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Triterpenoid Carotenoids and Related Lipids

THE TRITERPENOID CAROTENES OF *STREPTOCOCCUS FAECIUM* UNH 564P

By RICHARD F. TAYLOR and BRIAN H. DAVIES
Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth SY23 3DD, U.K.

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1. The occurrence of a novel series of triterpenoid carotenes in *Streptococcus faecium* UNH 564P is reported. 2. This series, which comprises the C_{30} analogues of phytoene, phytofluene, ζ-carotene, 7,8,11,12-tetrahydrolycopene and neurosporene, appears to be analogous to the C_{40} Porter–Lincoln series and a pathway of triterpenoid carotene dehydrogenation is proposed to account for the formation of these compounds. 3. Two *cis* isomers of the C_{30} analogue of neurosporene are described. 4. An appropriate system of nomenclature for these novel compounds is proposed.

Carotenoids are known to occur in a number of non-photosynthetic bacteria. These carotenoids are of many structural varieties and include the C_{45} and C_{50} homocarotenoids, present in species of *Flavobacterium*, *Halobacterium*, *Sarcina* and *Corynebacterium* (Weeks & Andrewes, 1972), the common C_{40} carotenoids, which are allegedly present in all the carotenogenic non-photosynthetic bacteria examined so far, and the apocarotenoids. The last class of compounds includes the glycosidic apocarotenoid, methyl 1-mannosyloxy-3,4-didehydro-1,2-dihydro-8′-apo-ψ-caroten-8′-oate, found in a yellow halophilic coccus (Aasen *et al.*, 1969), and the triterpenoid 'bacterial phytoene' reported in *Staphylococcus aureus* (Suzue *et al.*, 1968) and in *Halobacterium cutirubrum* (Kushwaha *et al.*, 1972).

There is some confusion about the position of 'bacterial phytoene' in a general scheme of carotenoid biosynthesis. Suzue (1960) claimed that phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) was incorporated into δ-carotene (ε,ψ-carotene) by cell-free extracts of *Staph. aureus*. In a later report, however, Suzue *et al.* (1968) showed that the conjugated triene present in this bacterium is not phytoene but its C_{30} analogue, and suggested that either the C_{30} 'bacterial phytoene' is a precursor of C_{40} carotenes or the other carotenoids of *Staph. aureus* might also be triterpenoids. Clearly, further structural studies are required before the situation can be clarified in *Staph. aureus*. The carotenoids of *H. cutirubrum*, on the other hand, have been studied in rather more detail and include both C_{40} and C_{50} compounds in addition to the C_{30} 'bacterial phytoene'; these observations led to the suggestion that, in this organism, 'bacterial phytoene' may be a precursor of the C_{40} carotenoids (Kushwaha *et al.*, 1972).

The present studies on carotenogenic, non-photosynthetic bacteria are directed at species of Streptococci and, in particular, a strain of *Streptococcus faecium*. As a result of preliminary investigations, the occurrence of carotenoids, including a postulated apocarotenoid, has been reported in this organism (Taylor *et al.*, 1971). The present paper describes the identification of the carotenes of this bacterium not as tetraterpenoids but rather as a series of novel triterpenoid carotenes based on 'bacterial phytoene'. A brief report of some of the investigations included in this study has appeared (Taylor & Davies, 1973).

Materials and Methods

Organism

The organism used in these studies was isolated from soil and has been referred to as Enterococcus 564 (Taylor *et al.*, 1971; Taylor & Davies, 1973). In this and all subsequent reports, however, the organism will be referred to as *Streptococcus faecium* strain UNH 564P (personal communication, Dr. W. R. Chesbro, Department of Microbiology, University of New Hampshire, Durham, N.H., U.S.A.).

Culture conditions

The bacteria were grown in 45-litre batches in 10-litre flat-bottomed flasks at 37°C in the light (3200lx) for 24h. The aqueous medium was Trypticase Soy Broth (Difco Laboratories, Detroit, Mich., U.S.A.) supplemented with 0.1% yeast extract (Difco), 0.16% NaH_2PO_4 and 0.09% Na_2HPO_4 (all w/v). The pH was adjusted to 6.8 with 1.0M-NaOH before sterilization.

Determination of bacterial dry weight

After 24h growth, a sample (25ml) was withdrawn from any single culture flask. This sample was centrifuged at 8000*g* and the sedimented cells were washed twice with water. The washed cells were resuspended in 25ml of water and distributed as batches of 1, 2 and 5ml in triplicate into preweighed vessels. These were first oven-dried (90°C) for 12–18h and then transferred to a vacuum desiccator for 12–18h. The weights of the various samples were determined and used to determine the average weight of cells per litre of culture.

Solvents

Analytical-reagent-grade light petroleum (b.p. 40–60°C), hexane, diethyl ether, chloroform and acetone were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Methanol (B.P.) was obtained from James Burrough Ltd., London S.E.11, U.K. The light petroleum was dried over sodium wire, glass-redistilled, passed through a freshly activated silicic acid column and glass-redistilled once again before use. The diethyl ether was sodium-dried and then glass-redistilled from reduced iron powder, methanol was glass-redistilled from KOH and acetone was glass-redistilled twice. Chloroform was glass-redistilled twice, stored in the dark and used within 1 week of its purification. Hexane was redistilled once.

Standard compounds

Squalene was obtained from Eastman Kodak Ltd., Kirkby, Lancs., U.K., and lycopersene (7,8,11,12,15,-7′,8′,11′,12′,15′-decahydro-ψ,ψ-carotene) was a gift from F. Hoffman–La Roche and Co. Ltd., Basel, Switzerland; lycopene (ψ,ψ-carotene) and β-carotene (β,β-carotene) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Phytoene, phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene), ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene) and β-zeacarotene (7′,8′-dihydro-β,ψ-carotene) were all isolated from mutants of *Phycomyces blakesleeanus* by standard methods used in this laboratory (Davies, 1965, 1973*a*); a sample of neurosporene (7,8-dihydro-ψ,ψ-carotene), isolated from nicotine-inhibited cultures of *Rhodopseudomonas spheroides*, was kindly provided by Aung Than of this laboratory.

Extraction of carotenoids

The bacterial cells were collected from batch cultures by centrifugation with a Sharples continuous-flow centrifuge, washed once with 0.9% (w/v) NaCl and then once with water. The washed cells were extracted by homogenization with 4% (w/v) KOH in methanol and the resulting mixture was centrifuged (20000*g* for 10min) to sediment the cell residue. The cell residue was then repeatedly extracted with fresh 4% KOH in methanol until it was white. The methanolic extracts were combined and extracted in a separating funnel, water and diethyl ether being added to give a composition of methanolic extract–water–ether of 5:5:4 (by vol.). The aqueous methanolic hypophase was rendered colourless by repeated extractions with fresh diethyl ether and was discarded. The combined ether extracts were washed to neutrality with water, dried over anhydrous (ether-washed) Na_2SO_4 and concentrated *in vacuo* to dryness.

Purification of carotenoids

This dry material was weighed (extracted cell lipid, see Table 5) and then dissolved in 50ml of 95% (v/v) methanol. This solution was partitioned with 50ml of light petroleum in a separating funnel. After the phases had separated, the methanolic hypophase was extracted a further four times with 50ml volumes of fresh light petroleum before being set aside for later analyses which are not reported here. The light-petroleum extracts, containing the carotenes investigated in the present study as well as a number of xanthophylls, were combined, concentrated *in vacuo* to about 5ml and chromatographed on a column (24cm×2cm) of alumina (M. Woelm, Eschwege, Germany; neutral, Brockmann activity grade II). The column was developed with light petroleum followed by increasing concentrations of acetone in light petroleum. On elution, the fractions were examined spectrophotometrically and those having the same absorption spectrum and chromatographic behaviour were combined. The individual carotenoids from these fractions were purified by repeated chromatography on smaller columns (15cm×1cm) of alumina (neutral, grade II) developed with light petroleum or acetone in light petroleum (Table 1).

Absorption spectra

Electronic absorption spectra of the carotenoids were recorded from solutions in light petroleum contained in 1cm path-length quartz cuvettes in a Unicam SP.800 recording spectrophotometer. A holmium oxide filter was used to calibrate the wavelength scale of the instrument for each spectrum recorded. Quantitative determinations of the polyenes were carried out in solutions in light petroleum of known volumes by using values for the specific extinction coefficients ($E^{1\%}_{1cm}$) adapted from the molar extinction values of C_{40} carotenes reported by Davis *et al.* (1966). The $E^{1\%}_{1cm}$ values for the C_{40} carotenes are as follows: phytoene (15-*cis*), 757 at 286nm; phytofluene, 1577 at 347nm; 7,8,11,12-tetrahydrolycopene (7,8,11,12-tetrahydro-ψ,ψ-carotene), 2519 at 395nm; ζ-carotene,

2555 at 400nm; neurosporene, 2918 at 440nm. The corresponding values for the C_{30} carotenes are: 4,4'-diapophytoene (15-*cis*), 1009 at 285.5nm; 4,4'-diapophytofluene, 2105 at 346.5nm; 4,4'-diapo-7,8,11,12-tetrahydrolycopene, 3367 at 395nm; 4,4'-diapo-ζ-carotene, 3415 at 400nm; 4,4'-diaponeurosporene, 3905 at 435nm. The $E^{1\%}_{1cm}$ quoted for 4,4'-diaponeurosporene, was also used as a nominal value to determine *cis* isomers of diaponeurosporene as well as diaponeurosporene-based xanthophylls present in the organism.

Configuration of polyenes

The stereochemical configurations of the carotenes were investigated by following the changes in their absorption spectra on iodine-catalysed photoisomerization (Zechmeister, 1962). Isomerizations were carried out in light-petroleum solutions of the carotenes in stoppered 1cm quartz cells. Iodine (in light petroleum) was added (1–2% of the quantity of carotenoid) and absorption spectra were recorded before and after illumination for periods of up to 30min with either two parallel fluorescent lamps (Philips MCFE 65W, 4400°C, Warm White) at a distance of 40cm or a single 60W tungsten lamp at a distance of 30cm.

To define the components of the stereoisomeric equilibrium mixture of diaponeurosporene, a light-petroleum solution of all-*trans*-diaponeurosporene (500μg in 50ml) was treated with iodine and illuminated until stereochemical equilibrium, as indicated by the absence of further spectral changes, was attained. The equilibrium mixture was washed with 2% (w/v) $Na_2S_2O_3$ to remove the iodine, with water to remove $Na_2S_2O_3$, dried over anhydrous Na_2SO_4 and the solvent evaporated *in vacuo* to about 5ml. The stereoisomers were resolved by chromatography on a column (24cm×2cm) of a 1:1 (w/w) mixture of $Ca(OH)_2$ (British Drug Houses Ltd.) and Celite 545 (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.). The chromatogram was developed successively with 200ml volumes of light petroleum followed by 1,2 and 4% (v/v) acetone in light petroleum. Fractions eluted from the column were examined spectrophotometrically and the amount of each stereoisomer was determined by assuming an $E^{1\%}_{1cm}$ value of 3905 at its λ_{max}..

The stereochemistry of diapophytoene ('bacterial phytoene') was determined by n.m.r. spectroscopy at 100MHz on about 1.5mg of sample (in CCl_4) with a Varian HA-100 instrument at the Department of Chemistry, Queen Mary College, London.

Thin-layer chromatography

Comparisons of the t.l.c. behaviour of unknown and standard carotenoids and related terpenoids were made on thin layers (0.25mm) of silica gel G or alumina G (both from E. Merck, Darmstadt, Germany) by using a number of different solvent systems (Table 2). Chromatograms were run in closed glass tanks lined with chromatography paper (Whatman No. 1; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) which was pre-saturated with solvent for at least 1h before use. Compounds on chromatograms were located as appropriate by their colour in visible light, by fluorescence on illumination at 360nm or as brown zones on exposure to iodine vapour.

Hydrogenation and gas–liquid chromatography

Hydrogenations were carried out in a micro-hydrogenation apparatus supplied by A. Gallenkamp and Co. Ltd., London E.C.2, U.K. Samples (100–500μg) were dissolved in 25ml of chloroform containing 50mg of platinum oxide (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) and were shaken for 2–4h at room temperature under a positive hydrogen gas pressure of 1.33×10^4Pa. After this time, the catalyst was removed by filtering the reaction mixture through a sintered-glass funnel and the colourless filtrate was concentrated *in vacuo* to yield a sample concentration of approx. 10μg/μl of chloroform. Some 1–3μl of this solution was used for g.l.c. analysis.

G.l.c. analyses were performed on a Pye–Unicam Series 104 gas–liquid chromatograph equipped with a dual hydrogen-flame ionization detection system. Supports were prepared, packed into 1.52m×6.35mm (external diam.) glass columns and conditioned by methods described previously (Taylor & Ikawa, 1971). Three different liquid-phase and support combinations were used: 3% OV-17 on Universal B (85–100 mesh), 2% SE-52 on Gas-Chrom Q (80–100 mesh) and 2% Dow–Corning high-vacuum grease on acid-washed dimethylchlorosilane-treated Chromosorb W (85–100 mesh). Samples were run in a temperature programme proceeding from 225 to 300°C at 3°C/min rate of rise after an initial isothermal period of 3min and with a nitrogen-carrier-gas flow rate of 60ml/min.

Mass spectra

Purified carotenoids for mass spectral studies were stored in the dark under N_2 in sealed vials kept at −20°C until analyses were carried out. Mass spectra were recorded on an A.E.I. MS30 mass spectrometer by using direct probe sample insertion, a probe temperature of 100–150°C and an ionization potential of 12, 18 or 24eV. The mass-spectral data reported for each carotene are results of the analysis of material from at least two batches of bacteria. High-resolution measurements of ion masses were made relative to heptacosafluorotributylamine with an A.E.I. MS902

mass spectrometer at the Department of Chemistry, Queen Mary College, London.

Results

Repeated chromatography of the epiphasic pigments from *Strep. faecium* UNH 564P on columns of alumina yielded a number of chromatographically pure carotenoids. The electronic spectral and chromatographic adsorption properties of the first seven carotenoids eluted are listed in Table 1. It first appeared from these data that the seven pigments were members of a Porter–Lincoln carotene series (Porter & Lincoln, 1950) modified, as in some fungi, to include both isomeric conjugated heptaenes (ζ-carotene and 7,8,11,12-tetrahydrolycopene; Davies, 1973*a*; Davies *et al.*, 1974).

Further examination of the *Strep. faecium* carotenes by other methods, however, soon showed that they did not behave as C_{40} carotenes. When the *Strep. faecium* pigments were co-chromatographed on a number of t.l.c. systems with authentic C_{40} analogues, each bacterial pigment had a higher R_F value than, and separated from, the corresponding tetraterpene (Table 2).

The non-tetraterpenoid nature of the *Strep. faecium* carotenes was again demonstrated when they were hydrogenated and examined by g.l.c. in three systems. The hydrogenated bacterial carotenes chromatographed with a retention time less than that of the squalene standard. If any one of the hydrogenated *Strep. faecium* carotenes was injected in a mixture containing squalene, hydrogenated squalene (squalane) and the hydrogenated C_{40} analogue of the bacterial carotene, it always co-chromatographed with the squalane whereas the hydrogenated C_{40} carotene

Table 1. *Spectroscopic and chromatographic properties of the carotenes isolated from Streptococcus faecium UNH 564P*

For details see the text.

Fraction	Colour	Percentage of acetone in light petroleum required for elution from alumina (grade II)	Absorption maxima (nm) in light petroleum							Conjugated double bonds	Identification
I	Colourless	0				275	285.5	297.5		3	4,4′-Diapophytoene
II	Colourless and fluorescent	0.25			(315.5)	330	346.5	366		5	4,4′-Diapophytofluene
III	Pale yellow	0.5			(354)	374	395	419		7	4,4′-Diapo-7,8,11,12-tetrahydrolycopene
IV	Yellow	0.75	316	328.5	(382)	405	428	456		9	Neo-4,4′-diaponeurosporene C
V	Pale yellow	1.0			(358)	378	400	425		7	4,4′-Diapo-ζ-carotene
VI	Yellow	1.5	(316)	328.5	(384)	407	430	459		9	Neo-4,4′-diaponeurosporene B
VII	Yellow	2.0			(390)	412	435	465		9	All-*trans*-4,4′-diaponeurosporene

Table 2. *Behaviour of squalene, 4,4′-diapocarotenes and C_{40} carotenes on t.l.c.*

The t.l.c. systems were as follows: A, silica gel G developed with light petroleum (b.p. 40–60°C); B, silica gel G developed with 1% (v/v) diethyl ether in light petroleum; C, silica gel G developed with 5% (v/v) benzene in light petroleum; D, silica gel G developed with 10% (v/v) benzene in light petroleum; E, alumina G developed with 0.25% (v/v) diethyl ether in hexane; F, alumina G developed with 1% (v/v) diethyl ether in hexane; G, alumina G developed with 5% (v/v) benzene in hexane.

Compound	R_F value						
System ...	A	B	C	D	E	F	G
Squalene	0.16	0.53	0.45	0.65	0.58	0.68	0.73
4,4′-Diapophytoene	0.09	0.46	0.34	0.55	0.45	0.61	0.67
Phytoene	0.04	0.35	0.23	0.47	0.35	0.56	0.62
4,4′-Diapophytofluene	0.07	0.35	0.25	0.45	0.19	0.38	0.58
Phytofluene	0.01	0.28	0.17	0.34	0.11	0.32	0.51
4,4′-Diapo-7,8,11,12-tetrahydrolycopene	0.02	0.26	0.19	0.36	0.07	0.18	0.37
4,4′-Diapo-ζ-carotene	0.02	0.25	0.19	0.35	0.07	0.16	0.37
ζ-Carotene	0	0.23	0.11	0.28	0.03	0.10	0.35
4,4′-Diaponeurosporene	0	0.22	0.14	0.27	0	0.04	0.21
Neurosporene	0	0.18	0.09	0.21	0	0	0.18

Table 3. *Behaviour of squalene, lycopersene, their hydrogenation products and those of 4,4′-diapocarotenes and of C_{40} carotenes on g.l.c.*

The g.l.c. systems were as follows: 1, 3% OV-17 on Universal B (squalene-retention time = 11.15 min); 2, 2% SE-52 on Gas-Chrom Q (squalene-retention time = 5.50 min); 3, 2% Dow-Corning High-Vacuum Grease on Chromosorb W (squalene-retention time = 8.65 min).

Compound	System ...	Retention times relative to squalene 1	2	3
Squalene		1.00	1.00	1.00
Lycopersene		2.76	3.95	3.15
Hydrogenation products of:				
Squalene (i.e. squalane)		0.54	0.66	0.73
4,4′-Diapophytoene		0.53	0.67	0.73
4,4′-Diapophytofluene		0.54	0.67	0.74
4,4′-Diapo-ζ-carotene		0.53	0.67	0.73
4,4′-Diaponeurosporene		0.53	0.67	0.73
Hydrogenation products of:				
Lycopersene (i.e. lycopersane)		1.76	3.15	2.67
Phytoene		1.77	3.14	2.64
Phytofluene		1.75	3.15	2.66
ζ-Carotene		1.78	3.13	2.66
Neurosporene		1.77	3.14	2.68
Lycopene		1.75	3.15	2.66
β-Carotene		2.43	3.81	2.88

(i.e. lycopersane) always chromatographed with a much longer retention time. Table 3 lists the relative retention times of squalene, lycopersene, their hydrogenation products and those of the bacterial and C_{40} carotenes (including the bicyclic β-carotene). These g.l.c. data show that the *Strep. faecium* carotenes are all acyclic triterpenes.

Unambiguous proof of the structures of the *Strep. faecium* carotenes (shown in Scheme 1) was provided by mass-spectral analysis of each purified pigment.

4,4′-*Diapophytoene* (*I*)

The least polar member of the *Strep. faecium* carotene series had $\lambda_{max.}$ in light petroleum at 275, 285.5 and 297.5 nm and was therefore, like phytoene, a conjugated triene. On mass-spectral analysis, it gave a molecular ion (21%) of accurate mass 408.375995, which corresponded to a molecular formula of $C_{30}H_{48}$ (calc. 408.375610). Ions at *m/e* 69 (100%), 137 (78%), 339 (3.0%; *M*−69) and 271 (47%; *M*−137) indicated 'bis-allylic' fission of the 7,8- (or 7′,8′-) and 11,12- (or 11′,12′-) bonds. Losses of 69 and 137 mass units from the molecular ions were supported by the appearance of metastable ions at *m/e* 281.3 and 180.0 respectively ($339^2/408 = 281.3$; $271^2/408 = 180.0$). All these features of the mass spectrum were consistent with the assignment of structure (I) to this carotene.

The 100 MHz n.m.r. spectrum showed signals for the methyl groups at 1.58 (12H), 1.65 (6H) and 1.74 (6H) p.p.m. relative to tetramethylsilane. The allylic protons (16H, two each at carbon atoms 7, 7′, 8,8′, 11, 11′,12 and 12′) gave chemical shifts of 1.90–2.40 p.p.m., the vinylic protons (4H, at carbon atoms 6, 6′, 10 and 10′) gave an unresolved multiplet centred at 5.07 p.p.m. and the olefinic triene protons (4H, at carbon atoms 14, 14′, 15 and 15′) yielded a multiplet in the 5.9–6.4 p.p.m. region of the spectrum. These n.m.r. data are also consistent with structure (I).

4,4′-*Diapophytofluene*

The second of the bacterial carotenes was recognized as having a conjugated pentaene chromophore by its absorption spectrum, which had $\lambda_{max.}$ in light petroleum at 315.5 (infl.), 330, 346.5 and 366 nm. It gave a molecular ion at *m/e* 406 (24%) which corresponded to a molecular formula of $C_{30}H_{46}$. 'Bis-allylic' fragmentations led to the appearance of ions at *m/e* 69 (100%), 137 (68%), 337 (15%; *M*−69) and 269 (97%; *M*−137) and the losses of 69 and 137 mass units from the molecular ion were substantiated by metastable ions at *m/e* 280.2 ($337^2/406 = 280.2$) and 178.4 ($269^2/406 = 178.4$) respectively. A further rearrangement loss of 137 mass units from *m/e* 337 to yield an ion at *m/e* 200 (24%; *M*−69−137), supported by a metastable ion at *m/e* 118.7 ($200^2/337 = 118.7$), was also consistent with the proposed structure (II).

4,4′-*Diapo*-7,8,11,12-*tetrahydrolycopene* (*III*) *and* 4,4′-*diapo*-ζ-*carotene* (*V*)

As Tables 1 and 2 show, two conjugated heptaenes were present among the carotenoids of *Strep. faecium*. Their absorption spectra were consistent with the chromophore being placed symmetrically (in V) and unsymmetrically (in III) in the molecules, as in ζ-carotene and 7,8,11,12-tetrahydrolycopene respectively (Davies, 1970). Both carotenes had a molecular ion at *m/e* 404 (III, 17%; V, 73%), so that the molecular formula of each was $C_{30}H_{44}$. The spectra of both carotenes had ions at *m/e* 69 (100%) and at *m/e* 335 (*M*−69; III, 7.5%; V, 23%) with a metastable ion for compound (V) at *m/e* 277.9 ($335^2/404 = 277.9$), thus indicating that the 7,8- (or 7′,8′-) bond is single. The major difference between the mass spectra of the two carotenes was that whereas the symmetrical conjugated heptaene (V) showed no loss of 137 mass units, the other (III) yielded ions at both *m/e* 137 (41%) and 267 (7.3%; *M*−137). This 'bis-allylic' fission does not occur in the symmetrical compound because it does not possess a saturated 11′,12′-bond. This difference between the fission reactions of *Strep. faecium* conjugated heptaenes is analogous to

the mass-spectral differences which led to the differentiation of ζ-carotene and 7,8,11,12-tetrahydrolycopene (Davies *et al.*, 1969) and confirms the assignments of the symmetrical and unsymmetrical structures to compounds (V) and (III) respectively. Both of the conjugated heptaenes of *Strep. faecium* possess conjugated double-bond systems sufficiently extensive for their molecular ions to lose fragments corresponding to toluene (92 mass units) and *m*-xylene (106 mass units). Thus both mass spectra had ions at *m/e* 312 (*M*−92; III, 1.3%; V, 2.5%) and 298 (*M*−106; III, 1.0%; V, 2.0%).

4,4′-*Diaponeurosporene* (*VII*)

The most unsaturated component of the *Strep. faecium* carotene series, the conjugated nonaene, gave a molecular ion at *m/e* 402 (39%) which indicated a molecular formula of $C_{30}H_{42}$. The mass spectrum contained evidence not only of the 'bis-allylic' fission of the 7′,8′-bond, in ions at *m/e* 69 (89%) and 333 (4%; *M*−69), but also of the loss of a C_6H_{11} fragment (83 mass units) owing to fission of the 8′,9′-bond. An ion at *m/e* 83 was the base peak of the spectrum, and the fragmentation of the molecular ion to yield an ion at *m/e* 319 (3%; *M*−83) was supported by a metastable ion at 252.9 ($319^2/402 = 252.9$). Ions were also present at *m/e* 310 (3%; *M*−92), 296 (5%; *M*−106), 227 (7%; *M*−69−106 or *M*−83−92) and 213 (24%; *M*−83−106). All these mass-spectral features are consistent with the structure (VII) assigned to the conjugated nonaene.

Two further carotenes (IV and VI) isolated from *Strep. faecium* had electronic spectra and chromatographic properties consistent with their being *cis* isomers of 4,4′-diaponeurosporene (VII). Their mass spectra were characterized by the presence of molecular ions at *m/e* 402 (equivalent to $C_{30}H_{42}$) and fragmentation patterns which corresponded to that of 4,4′-diaponeurosporene. All three isomers (IV, VI and VII) were subjected to iodine-catalysed photoisomerization, whereupon they yielded identical stereoisomeric equilibrium mixtures with absorption maxima in light petroleum at 316 (infl.), 328.5, 386 (infl.), 410, 432 and 462nm. Whereas the isomeric diaponeurosporenes (IV) and (VI) showed hyperchromic and bathochromic shifts of their main absorption bands and decreases in extinction of their '*cis*-peaks' (at 328.5nm), photoisomerization of the other isomer (VII) resulted in the appearance of a '*cis*-peak' at 328.5nm and in hypochromic and hypsochromic changes of the other absorption bands. It was therefore concluded that the most polar diaponeurosporene (VII) is the all-*trans* isomer, whereas the less polar isomers (IV and VI) each have a different *cis* configuration.

To define further the relationship of the three diaponeurosporenes, 500μg of the all-*trans* isomer (VII) was photoisomerized and the stereoisomeric equilibrium mixture was resolved into its components by column chromatography. As Table 4 shows, six stereoisomeric forms of diaponeurosporene were separated and named according to the normal convention (Magoon & Zechmeister, 1957). The two natural *cis*-diaponeurosporenes, (IV) and (VI), corresponded in their electronic spectra and in their behaviour on co-chromatography, to the neo C and neo B isomers respectively.

The configurations of diapophytofluene and diapo-ζ-carotene (sufficient diapo-7,8,11,12-tetrahydrolycopene was not available) were also examined by iodine-catalysed photoisomerization. The absorption bands of diapophytofluene were shifted some 1.5nm to shorter wavelengths and underwent extinction losses; the spectrum of diapo-ζ-carotene showed hypsochromic shifts (2nm) and hypochromic changes of the main absorption bands and an increase in extinction in the '*cis*-peak' region (284 and 296nm). A tentative assignment of an all-*trans* configuration can therefore be made for each of these polyenes.

The predominant stereochemistry of the conjugated triene chromophore of diapophytoene was established as 13-*trans*,15-*cis*,13′-*trans* by its n.m.r. spectrum (100MHz). The signals for the methyl groups at the end of the central triene chromophore were both at 1.74p.p.m. relative to tetramethylsilane, thus showing

Table 4. *Spectroscopic and chromatographic properties of the components of a stereoisomeric equilibrium mixture obtained from 4,4′-diaponeurosporene by iodine-catalysed photoisomerization*

For details see the text.

Steric form	Percentage of acetone in light petroleum required for elution from $Ca(OH)_2$–Celite 545 (1:1, w/w)	Absorption maxima (nm) in light petroleum						Percentage of total isomers
All-*trans*	4			(390)	412	435	465	47
Neo A	4	(316)	(328.5)	(387)	410	433	463	4
Neo B	2	(316)	328.5	(384)	407	430	459	10
Neo C	2	316	328.5	(382)	405	428	456	33
Neo D	1	316	328.5	(379)	403	425	453	5
Neo E	1			(373)	(396)	417	(445)	1

that the triene unit is either *trans,trans,trans* or *trans,cis,trans* (Khatoon *et al.*, 1972; Aung Than *et al.*, 1972). The peaks of the multiplet (5.9–6.4p.p.m.) due to the triene protons had only a limited separation and were almost identical in appearance with those in the 100MHz spectrum of synthetic *trans,cis,trans*-C_{40}-phytoene; the corresponding synthetic all-*trans* isomer showed a much greater separation in this region, yielding two multiplets centred at 5.8 and 6.2p.p.m., the latter being particularly sharp (T. P. Toube, personal communication). The overall appearance of the olefinic region of the diapophytoene spectrum was consistent with the conjugated triene being predominantly *trans,cis,trans*, but with a small amount of the all-*trans* isomer also present; this reflects the situation for phytoene (C_{40}) in a number of other micro-organisms (Aung Than *et al.*, 1972). As the band for a methyl group (C-20 or C-20′ of a carotenoid) attached to a *cis* double bond moves downfield by 0.02 or 0.07p.p.m. depending on whether the neighbouring double bond is *trans* or *cis* (Khatoon *et al.*, 1972), the presence in the diapophytoene spectrum of a weak signal at 1.81p.p.m. and of an even weaker band at 1.77p.p.m. (as a slight shoulder on the 1.74p.p.m. peak) may be taken to indicate the presence of traces of the *trans,cis,cis* and *trans,trans,-cis* isomers respectively. For C_{40}-phytoene, the former configuration is known to result from the photoisomerization of the *trans,cis,trans* isomer (B. C. L. Weedon, personal communication).

The results of detailed quantitative analyses of the carotenoids of *Strep. faecium* are shown in Table 5.

Table 5. *Carotenoid composition of Streptococcus faecium UNH 564P*

These data are the averages from determinations carried out on four separate cell batches each of 45 litres cultured at 37°C in the light for 24h. The average yield of dry cells was 0.78g/litre of culture and the average weight of lipid extracted was 5.7mg/g dry wt. of cells.

Compound(s)	(μg/g dry wt. of cells)	(μg/mg of lipid)	Percentage of total carotenoid
4,4′-Diapophytoene	11.17	2.01	42.5
4,4′-Diapophytofluene	3.26	0.59	12.4
4,4′-Diapo-7,8,11,12-tetrahydrolycopene	0.27	0.05	1.0
Neo-4,4′-diaponeurosporene C	0.51	0.09	1.9
4,4′-Diapo-ζ-carotene	1.10	0.20	4.2
Neo-4,4′-diaponeurosporene B	0.45	0.08	1.7
4,4-Diaponeurosporene	3.39	0.61	12.9
Total carotenes	20.15	3.63	76.6
Total xanthophylls	6.15	1.11	23.4
Total carotenoids	26.30	4.74	100.0

Discussion

It is clear that the carotenes of *Strep. faecium* UNH 564P represent a new class of terpenoids, the triterpenoid carotenoids. The recognition of this class necessitates the introduction of a standardized system of nomenclature and it is proposed that these compounds should be named with both a trivial and a fully systematic nomenclature. The former is based on the 'apo-' nature of the C_{30} compounds with respect to the C_{40} carotenoids (Rule 10.1, IUPAC–IUB Tentative Rules for Carotenoid Nomenclature; Commission on Biochemical Nomenclature, 1972) rather than on their structural relationship to squalene. This approach is justified because (*a*) the C_{30} compounds have absorption spectra identical with and other properties similar to those of the corresponding C_{40} carotenoids, (*b*) considerations of their function and the later stages of their biosynthesis will be more akin to those of conventional carotenoids than to those of squalene, and (*c*) a defined set of nomenclature rules exists into which these novel compounds may be integrated (IUPAC–IUB Tentative Rules for Carotenoid Nomenclature; Commission on Biochemical Nomenclature, 1972). The additional requirement for a fully systematic nomenclature is also present since any trivial or semi-systematic nomenclature, irrespective of whether it is based on carotenoids or on squalene, could not readily be applied in the event that cyclized C_{30} carotenoids are found in nature. Thus the C_{30} analogue of phytoene, previously named 'bacterial phytoene' and 'dehydrosqualene' (Suzue *et al.*, 1968), becomes 4,4′-diapophytoene (I) or 2,6,10,15,19,23-hexamethyltetracosa-2,6,10,12,14,18,22-heptaene (Fig. 1). The names of the other members of the carotene series described in this paper are as follows: 4,4′-diapophytofluene (II; 2,6,10,15,19,23-hexamethyltetracosa-2,6,8,10,12,14,18,22-octaene), 4,4′-diapo-7,8,11,12-tetrahydrolycopene (III; 2,6,10,15,-19,23-hexamethyltetracosa-2,4,6,8,10,12,14,18,22-nonaene), 4,4′-diapo-ζ-carotene (V; 2,6,10,15,19,23-hexamethyltetracosa-2,6,8,10,12,14,16,18,22-nonaene) and 4,4′-diaponeurosporene (VII; 2,6,10,15,-19,23-hexamethyltetracosa-2,4,6,8,10,12,14,16,18,22-decaene).

The series of triterpenoid carotenes found in *Strep. faecium* UNH 564P probably represents a biosynthetic dehydrogenation sequence analogous to that of C_{40} carotene formation. The latter dehydrogenation sequence, proposed by Porter & Lincoln (1950) on the basis of their observations of the carotenoids of tomato fruit, was substantiated when further structural studies of the participating carotenes (Davis *et al.*, 1966) revealed that the phytoene molecule could yield, by successive losses each of two hydrogen atoms, phytofluene, ζ-carotene, neurosporene and then lycopene. This series of carotenes has since been

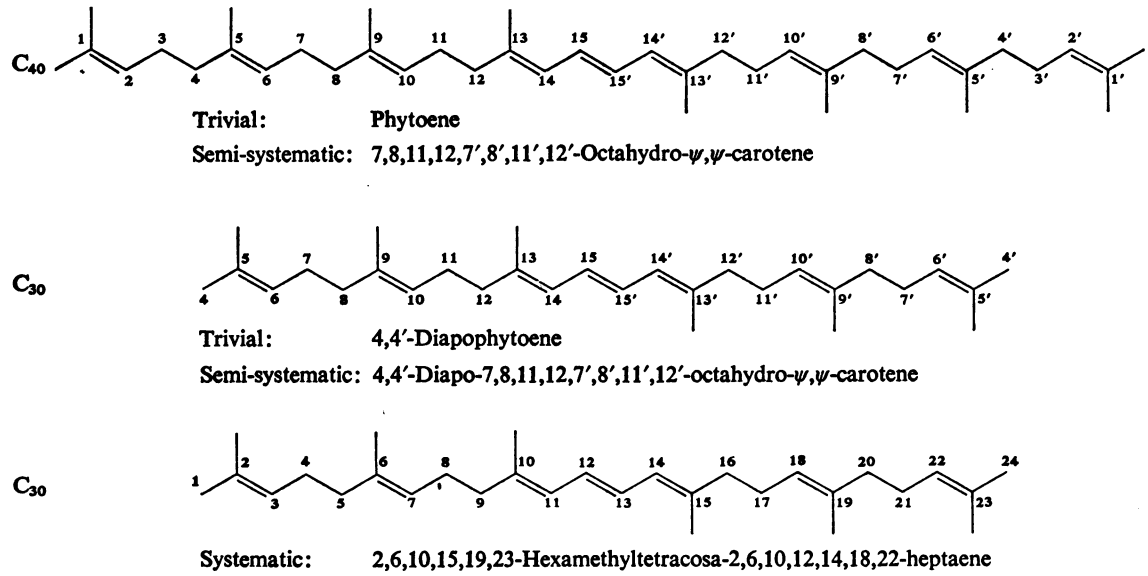
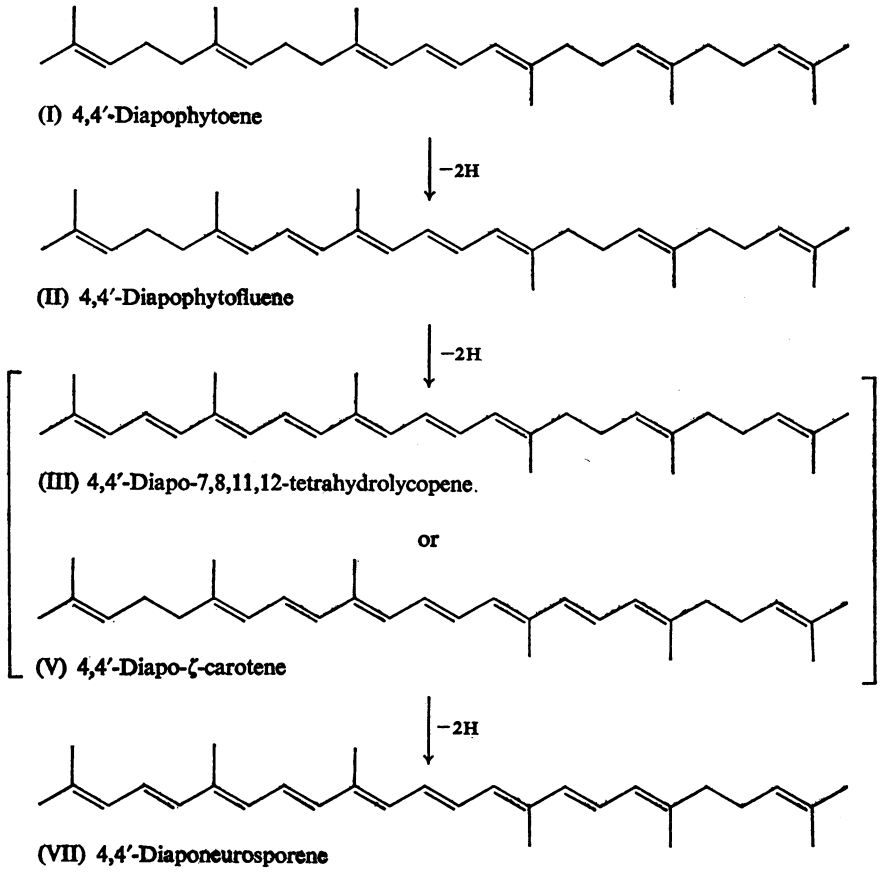


Fig. 1. *Structural relationships between C_{40} carotenes (e.g. phytoene) and C_{30} apocarotenes (e.g. 4,4′-diapophytoene) and the trivial, semi-systematic (CBN, 1972) and systematic nomenclature of triterpenoid carotenes*

shown to occur in most carotenogenic organisms, but with modifications in some instances at the conjugated heptaene level. One such modification is in the purple non-sulphur photosynthetic bacterium, *Rhodospirillum rubrum*, where the symmetrical ζ-carotene is apparently replaced by its unsymmetrical isomer, 7,8,11,12-tetrahydrolycopene (Davies, 1970). Studies on certain other micro-organisms, the photosynthetic bacterium *Rhodopseudomonas viridis* (Malhotra *et al.*, 1970), the non-photosynthetic *Flavobacterium dehydrogenans* (Weeks, 1971), the fungi *Neurospora crassa* (Davies *et al.*, 1974) and mutants of *Phycomyces blakesleeanus* (Davies, 1973*a*), indicate that both isomeric conjugated heptaenes must be involved as alternative intermediates in carotene dehydrogenation in the same organism.

It may be postulated that the triterpenoid carotenes of this strain of *Strep. faecium* participate in an analogous dehydrogenation sequence (Scheme 1). In this, the dehydrogenation of diapophytoene to yield diapophytofluene is followed by the conversion of the latter, again with the loss of two hydrogen atoms, into either diapo-ζ-carotene or diapo-7,8,11,12-tetrahydrolycopene, both of which can yield diaponeurosporene by further dehydrogenation. In contrast with the normal C_{40} carotene series, the *Strep. faecium* carotene dehydrogenation series terminates at this conjugated nonaene stage rather than continuing to the conjugated undecaene (e.g. 4,4′-diapolycopene), no diapolycopene has been detected in the organism; indeed, the structures of all the xanthophylls of the bacterium are based on that of diaponeurosporene. Whether the absence of diapolycopene reflects the absence of a specific dehydrogenase, or merely a high activity on the part of the enzymes responsible for converting diaponeurosporene into the xanthophylls, is not clear and must await further investigation. The nature of some of the xanthophylls of *Strep. faecium* UNH 564P is the subject of a subsequent report (Taylor & Davies, 1974).

Further studies on the significance of the above stereochemical data are also required. Although the diapophytoene is predominantly in the form of its *trans,cis,trans* isomer, with a small amount of the all-*trans* form, the other carotenes are all-*trans*, except for diaponeurosporene, the all-*trans* isomer of which is accompanied by smaller amounts of the neo B and neo C *cis* isomers (Table 5). The high concentration of diapophytoene (Table 5) may result from the simultaneous formation of the *trans,cis,trans* and all-*trans* isomers and the further metabolism only of the latter, in which case the inactive *trans,cis,trans* form would tend to accumulate. An alternative view, more likely by analogy with C_{40} phytoene metabolism (Davies, 1973*b*), is that the isomerization of *trans,cis,trans*-diapophytoene to the all-*trans* form, which precedes dehydrogenation of the latter to all-*trans*-diapophytofluene, may be rate-limited. The origin of the *cis*-diaponeurosporenes is also unclear; it is significant that the neo B and neo C *cis* isomers, with the all-*trans* form, predominate in the stereoisomeric equilibrium mixture formed from all-*trans* diaponeurosporene by photoisomerization (Table 4). They are present in too large a proportion, however,



Scheme 1. *Postulated sequence for the dehydrogenation of triterpenoid carotenes in Streptococcus faecium UNH 564P*

to have been formed in the course of chemical manipulation of the carotenes, but may have resulted from photoisomerization caused by illumination of the cultures. A third, but less likely, possibility is that they may be the final products of a dehydrogenation sequence which involves *cis* carotenes as its intermediates.

The recognition of a complete series of triterpenoid carotenoids in *Strep. faecium* UNH 564P may have some significance to bacterial taxonomy and may go some way towards explaining the role of 4,4'-diapophytoene in *Staph. aureus*. Correlations have been made between carotenoids and DNA base ratios in certain groups of non-photosynthetic bacteria; species of *Flavobacterium*, *Corynebacterium* and *Sarcina* all have high percentages of guanine and cytosine (52–80% G+C) and all contain C_{45} and C_{50} homocarotenoids in addition to C_{40} carotenoids (Weeks, 1971; Mamur *et al.*, 1963). It may be that species of *Streptococcus* and *Staphylococcus*, which have low percentages of guanine and cytosine (32–36% G+C; Mamur *et al.*, 1963), are members of another group in which triterpenoid carotenoids are formed. Thus, as suggested by Suzue *et al.* (1968), the occurrence of diapophytoene in *Staph. aureus* may indicate that this organism forms a series of C_{30} carotenoids similar to those of *Strep. faecium* UNH 564P. The occurrence of diapophytoene in *Halobacterium cutirubrum*, which has a high proportion of guanine and cytosine in its DNA (66–68% G+C; Mamur *et al.*, 1963) and contains both C_{40} and homocarotenoids (Kushwaha *et al.*, 1972), remains something of an anomaly.

In conclusion, it may be postulated that since no C_{40} carotenoids occur in *Strep. faecium* UNH 564P, the existence of this novel class of natural products, the triterpenoid carotenoids, indicates the operation of a new biosynthetic pathway. Although the C_{40} carotenoids, the C_{45} and C_{50} homocarotenoids and those apocarotenoids derived from C_{40} carotenoids appear to have a common origin in the dimerization of geranylgeranyl pyrophosphate (Goodwin, 1971), the C_{30} carotenoids from this pigmented streptococcus

may arise, like squalene, from the condensation of two molecules of farnesyl pyrophosphate. If this were so, the new biosynthetic pathway would have important implications for any artificial division of eukaryotes and prokaryotes on the basis of their terpenoid metabolism. A crucial question, to which our continuing biosynthetic studies may provide the answer, relates to the possible role of squalene, which is also present in the organism (R. F. Taylor & B. H. Davies, unpublished work), in the formation of the triterpenoid carotenoids. This problem is more intriguing even than that concerning lycopersene and C_{40} carotenoid formation.

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