Triterpenoid Carotenoids and Related Lipids

TRITERPENOID MONOHYDROXY- AND MONOGLUCOSYLOXY-CAROTENOIDS FROM *STREPTOCOCCUS FAECIUM* UNH 564P

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1. The characterization of two novel triterpenoid xanthophylls occurring in *Streptococcus faecium* UNH 564P is described. 2. Both are derived formally, and probably biosynthetically, from the C₃₀ analogue of neurosporene and have been identified as 4-hydroxy-4,4'-diaponeurosporene and its glucoside, 4-D-glucopyranosyloxy-4,4'-diaponeurosporene. 3. Problems associated with the use of specific glucosidases in defining the anomerism of carotenoid glucosides are discussed.

In recent years a number of carotenoid glycosides have been found in non-photosynthetic bacteria. These glycosides are derived directly from the carotenoids of the organism and may contain aglycone moieties of different chain lengths. Glycosides with C₅₀ homocarotenoid aglycones include the monoglucoside of sarcinaxanthin [2-(4-hydroxy-3-methylbut-2-enyl)-2'-(3-methylbut-2-enyl)- ϵ,ϵ -caroten-18-ol] found in *Sarcina lutea* (Norgård *et al.*, 1970), corynexanthin, the monoglucoside of decaprenoxanthin [2,2'-bis-(4-hydroxy-3-methylbut-2-enyl)- ϵ,ϵ -carotene], found in a *Corynebacterium* sp. (Weeks & Andrewes, 1970) and, with the corresponding diglucoside, in an *Arthrobacter* sp. (Arpin *et al.*, 1972*b*), and the mono- and di-glucosides of bacterioruberin [2,2'-bis-(3-hydroxy-3-methylbutyl)-3,4,3',4'-tetrahydro-1,2,1',2'-tetrahydro- ψ,ψ -carotene-1,1'-diol] found in a halophilic bacterium (Arpin *et al.*, 1972*a*). Non-photosynthetic bacterial carotenoid glycosides with C₄₀ carotenoid aglycones include the glucosidic 'phleixanthophylls', such as phleixanthophyll [1'-(β -D-glucopyranosyloxy)-3',4'-didehydro-1',2'-dihydro- β,ψ -caroten-2'-ol], of *Mycobacterium phlei* (Hertzberg & Liaaen-Jensen, 1967) and a number of rhamnosidic and glucosidic carotenoids isolated from the gliding myxobacteria (e.g. Kleinig *et al.*, 1970, 1971). Only one carotenoid has been reported with an apocarotenoid aglycone, namely methyl 1-mannosyloxy-3,4-didehydro-1,2-dihydro-8'-apo- ψ -caroten-8'-oate, found in a halophilic coccus (Aasen *et al.*, 1969).

In the preceding paper (Taylor & Davies, 1974), a novel series of triterpenoid carotenes was reported to occur in the non-photosynthetic bacterium, *Streptococcus faecium* UNH 564P. This series proceeds from 4,4'-diapophytoene (4,4'-diapo-7,8,11,12,7',8',11',12'-octahydro- ψ,ψ -carotene) by dehydro-

genation through 4,4'-diapophytofluene (4,4'-diapo-7,8,11,12,7',8'-hexahydro- ψ,ψ -carotene) and the isomeric conjugated heptaenes (4,4'-diapo-7,8,7',8'-tetrahydro- ψ,ψ -carotene and 4,4'-diapo-7,8,11,12'-tetrahydro- ψ,ψ -carotene) to terminate at 4,4'-diaponeurosporene (4,4'-diapo-7,8-dihydro- ψ,ψ -carotene). In addition to these carotenes, the organism contains a number of xanthophylls; the present paper is concerned with the identification of two of these as the monohydroxy and monoglucosyloxy derivatives of 4,4'-diaponeurosporene.

Materials and Methods

Standard methods for the growth, harvesting and determination of bacterial dry weight of *Streptococcus faecium* UNH 564P, for the extraction of carotenoids from the bacterial cells, for the determination of electronic absorption spectra and their use in quantitative determination of carotenoids and the source and methods for purification of the solvents were described in the preceding paper (Taylor & Davies, 1974). Methods for the hydrogenation of carotenoids and g.l.c. of the products were described by Taylor & Ikawa (1971) and by Taylor & Davies (1974).

Purification of carotenoids

The lipid fraction from cultures of *S. faecium* UNH 564P was partitioned between light petroleum (b.p. 40–60°C) and aq. 95% (v/v) methanol (Taylor & Davies, 1974). Chromatography of the epiphasic fraction on a column (24cm×2cm) of alumina (M. Woelm, Eschwege, Germany; neutral, Brockmann activity grade II) with light petroleum containing increasing proportions of acetone (0–20%, v/v) led to the resolution of ten carotenoid fractions. The

components of fractions I to VII, eluted successively with 0–2% acetone in light petroleum, have been identified as 4,4'-diapocarotenenes (Taylor & Davies, 1974); fractions VIII and IX, eluted with 4 and 7.5% acetone in light petroleum respectively, have been partially characterized as carbonyl-containing 4,4'-diapocarotenoids (R. F. Taylor & B. H. Davies, unpublished work); and the identification of fraction X, eluted with 15–20% acetone in light petroleum, is described below. Fraction X was purified by repeated chromatography on columns (15cm×1cm) both of alumina (neutral, grade II), developed with 15–20% acetone in light petroleum, and silica gel (Woelm, grade I), developed with 5% acetone in light petroleum. The chromatographically pure carotenoid (X) had absorption maxima in light petroleum at 386 (infl.), 413, 435 and 465nm.

The hypophase from the above phase partition contained one major carotenoid component (fraction XI) which was recovered by rotary evaporation of the 95% methanol solvent (*in vacuo* at 35°C with the addition of benzene) and dissolving the residue in the minimum volume of diethyl ether. This residue was chromatographed on a column (25cm×2cm) of, CF-11 cellulose (Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) which had previously been washed and packed by using diethyl ether, and was developed with increasing concentrations of acetone in diethyl ether (0–30%). Those fractions that had the same absorption spectrum and chromatographic behaviour were combined and the fraction XI carotenoid was purified by further chromatography on smaller columns (15cm×1cm) of either CF-11 or Microgranular CT cellulose (Reeve Angel Scientific Ltd., London, E.C.4, U.K.), developed with 20–25% acetone in ether. The chromatographically pure carotenoid (XI) had absorption maxima in ethanol at 391 (infl.), 416, 439 and 468nm.

Chemical tests

Acetylation (Aasen & Liaaen-Jensen, 1966) was carried out by dissolving approx. 100–200µg of pigment in 1ml of dry pyridine (Hopkin and Williams Ltd., Romford, Essex, U.K.; refluxed for 1.5h over KOH and redistilled with the exclusion of moisture), adding 0.01ml of acetic anhydride (Hopkin and Williams Ltd.), and incubating the reaction mixture under N₂ in the dark at room temperature for 12–24h. Water (2ml) was added to the reaction mixture and the acetylated product was extracted with 3ml of diethyl ether. After three further extractions with fresh volumes of solvent, the ether extracts were combined, washed with water to remove pyridine and concentrated *in vacuo* to dryness. The residue was dissolved in light petroleum for further analyses as described below or purified for mass-spectrometric analysis by chromatography on a column (10cm×

0.8cm) of alumina (neutral grade II), developed with light petroleum followed by increasing concentrations of acetone in light petroleum.

Silylation of hydroxycarotenoids (McCormick & Liaaen-Jensen, 1966) was carried out by standard methods already described in detail (Davies, 1970b). The trimethylsilyl ethers were dissolved in light petroleum for further analyses.

Dehydration of allylic hydroxyl groups (Entschel & Karrer, 1958) was accomplished by treating a chloroform solution of the hydroxycarotenoid with 1–2 drops of chloroform saturated with conc. HCl. Standard analytical methods, described below, were applied directly to this solution.

A test for the presence of acid functions in xanthophylls was performed by first dissolving the pigment in 4ml of diethyl ether and, after determination of the extinction of this solution at its $\lambda_{\max}$, partitioning against either 50 (or 75)% aq. methanol or 50 (or 75)% aq. methanol containing 0.05% (w/v) KOH. Comparison of the two partition coefficients indicated the presence or absence of acidic functions.

Methylation of allylic secondary hydroxyl groups was attempted by the addition of 1–2 drops of 0.12M-HCl in 99% methanol (Bamji & Krinsky, 1966) to a solution of the pigment in methanol. The solution was then investigated as described below.

After each of the above chemical procedures, the properties of any products were compared with those of the starting material by t.l.c., electronic-absorption spectrophotometry, determination of partition coefficients and, where applicable, by g.l.c. Partition coefficients were determined between light petroleum (b.p. 40–60°C) and 95% methanol by the method of Petracek & Zechmeister (1956). They are quoted below as the percentages of the total carotenoid in each of the two phases (epiphase:hypophase) as determined by extinctions at the wavelength of maximal absorbance.

Thin-layer chromatography

T.l.c. was carried out on thin layers (0.25mm) of silica gel G (E. Merck, Darmstadt, Germany) in covered glass tanks which were lined with chromatography paper (Whatman no. 1), which was presaturated with solvent for at least 1h before use. The solvent systems used (Tables 1 and 2) were: 1, benzene-methanol-acetic acid (87:11:2, by vol.); 2, chloroform; 3, 15% (v/v) acetone in light petroleum. Carotenoids were detected on t.l.c. by their colour in visible light and as brown spots on exposure to iodine vapour.

Identification of sugar moiety of carotenoid glycoside

Approx. 100µg of the carotenoid (XI) was dissolved in 3ml of HCl-saturated chloroform. The solution

was heated in a boiling-water bath for 10 min, additional chloroform being added to maintain the volume. On completion of the reaction, the chloroform was evaporated *in vacuo*, the residue was dissolved in 5 ml of water and this aqueous solution was extracted with 5 ml of diethyl ether. The ether solution was washed once with a fresh 5 ml volume of water and was discarded. The aqueous solutions were combined, neutralized with 5M-NaOH and concentrated *in vacuo* to dryness. The dry residue was dissolved in 1 ml of water and the resulting solution, the 'water-soluble hydrolysate', was used for chromatographic and enzymic analysis of the sugar released on hydrolysis of the glycoside (XI).

The water-soluble hydrolysate was chromatographed in both t.l.c. and paper systems along with standard sugars including D-glucose, D-galactose, D-mannose, D-fructose and sucrose (all from British Drug Houses Ltd., Poole, Dorset, U.K.). Thin-layer chromatograms (0.25 mm) were run on a 1:1 (w/w) mixture of Silica gel G (Merck) and alumina G (Merck), i.e. the so-called 'alusi' system (Stahl, 1965), in closed glass tanks lined with Whatman no. 1 chromatography paper by using two solvent systems (Table 3): 4, butan-1-ol-acetic acid-water (6:3:1, by vol.) and 5, propan-1-ol-water-ethyl acetate-acetic acid (4:4:1:1, by vol.; Stahl, 1965). The resulting chromatograms were sprayed with an anisaldehyde-H₂SO₄ reagent (Stahl, 1965) and heated for 5 min at 110°C to detect the different carbohydrates, each of which yielded a characteristic colour (Table 3).

Paper chromatography was carried out on sheets (30 cm × 15 cm) of Whatman no. 1 chromatography paper. Unknown and authentic carbohydrates were applied to the paper, which was then rolled into a cylinder, and the chromatograms were run by ascending development in covered cylindrical glass tanks or large glass beakers. Four different solvent systems were used (Table 3): 6, benzene-*n*-butan-1-ol-pyridine-water (1:5:4:3, by vol.); 7, ethyl acetate-water-acetic acid (3:3:1, by vol.); 8, propan-1-ol-ethyl acetate-water (7:1:2, by vol.), and 9, ethyl acetate-water-pyridine (2:2:1, by vol.; Lederer & Lederer, 1957). The positions of the sugars on the chromatograms were determined by spraying with aniline phthalate (Partridge, 1949) and heating at 110°C for 5 min; the sugars appeared as brown spots on a white background.

The remainder of the water-soluble hydrolysate was analysed by an enzymic method. The glucose oxidase-peroxidase reaction, which was used for glucose determination, was monitored by the changes in extinction of *o*-dianisidine dihydrochloride [Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; enzymes were from Boehringer Corporation (London) Ltd., London, W.5, U.K.]. The reaction mixture (1 ml) in 0.1M-KH₂PO₄-Na₂HPO₄ buffer (pH 6.8) contained *o*-dianisidine

dihydrochloride (0.066 mg; 0.208 μmol), D-glucose oxidase (EC 1.1.3.4, grade I; 0.1 mg), peroxidase (EC 1.11.1.7, grade I; 0.1 mg) and the glucose substrate (25–100 nmol); it was incubated at 30°C for 5 min. Extinctions were measured at 436 and 500 nm in 0.1 cm path-length cuvettes by using a Pye-Unicam SP. 1800 spectrophotometer.

Enzymic hydrolysis of glycosides

Standard substrates included *p*-nitrophenol α-D-glucoside, *p*-nitrophenol β-D-glucoside, *N*-stearoyl-dihydroglucocerebroside, *N*-stearoyldihydrogalactocerebroside [all from Sigma (London) Chemical Co. Ltd.] and digitonin (Hopkin and Williams Ltd.); Tween 80, used as a solubilizing agent at concentrations described later, was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. The activities of glucosidases on these and other substrates were determined by coupling the production of glucose to the above glucose oxidase-peroxidase assay procedure. Initial incubations were carried out in 0.1M-KH₂PO₄-Na₂HPO₄ buffer (pH 6.8, 1.7 ml) containing α-glucosidase (EC 3.2.1.20; 0.02 mg) or β-glucosidase (EC 3.2.1.21; 0.05 mg) and the appropriate amount of substrate (to yield 25–100 nmol of glucose/ml) for 6 min at 30°C. Glucose oxidase (0.1 mg), peroxidase (0.1 mg) and *o*-dianisidine dihydrochloride (0.066 mg; 0.208 μmol) were added in the same buffer to give a final volume of 2 ml; the mixture was reincubated for a further 5 min and then the extinction was recorded at 500 nm. This high wavelength was chosen in preference to the customary 436 nm to avoid, as far as possible, the region of carotenoid absorption.

Mass spectra

Mass spectra of purified carotenoids were determined on an A.E.I. MS30 instrument by using a direct-insertion probe sampling technique. Analyses of hydroxycarotenoids, carotenoid glycosides and acetates were carried out with a probe temperature of 125–225°C and ionization potentials of 12 or 18 eV. Each mass spectrum reported was obtained on analysis of the carotenoid from at least two separate batches of *S. faecium* UNH 564P.

Infrared spectra

The i.r. spectrum of the carotenoid (X) was determined from a CCl₄ solution in a 0.2 ml NaCl cell of a Perkin-Elmer model 700 infrared spectrophotometer.

Results

Analysis of the carotenoids of *S. faecium* UNH 564P has already led to the recognition of a number

Table 1. Spectroscopic, partition and adsorption characteristics of the epiphasic carotenoid (X) and of a number of its reaction products

For details see the text.											
Sample	Absorption maxima (nm) in light petroleum				Partition coefficient in light petroleum- 95% methanol	R _F values on t.l.c. in system			Inference		
	(386)	413	435	465		1	2	3			
Carotenoid (X)	(386)	413	435	465	26:74	0.67	0.31	0.25	Conjugated nonaene xanthophyll		
Product obtained by:											
Acetylation	(386)	413	435	465	48:52	0.77	0.69	0.55	Primary or secondary OH		
Silylation	(386)	413	435	465	58:42	0.79	0.70	0.61	Primary, secondary or tertiary OH		
Acetylation followed by silylation	(386)	413	435	465	47:53	0.76	0.67	0.53	No tertiary OH		
Treatment with HCl-CHCl ₃	(397)	424	447	477	89:11	0.75	0.61	0.67	Allylic OH		

of triterpenoid carotenes (Taylor & Davies, 1973, 1974) and of two triterpenoid xanthophylls containing carbonyl functions (R. F. Taylor & B. H. Davies, unpublished work). The remaining carotenoids of the organism, the two most polar, one (X) epiphasic in a light petroleum-95% methanol partition and the other (XI) hypophasic, were obtained in a chromatographically pure form and were identified as described below.

Identification of carotenoid (X) as 4-hydroxy-4,4'-diaponeurosporene

The absorption maxima in light petroleum, at 386 (infl.), 413, 435 and 465nm, were characteristic of a conjugated nonaene chromophore (Davies, 1970a). The polarity of the carotenoid, which was eluted with 15-20% acetone in light petroleum from a column of neutral alumina (grade II), was consistent with its being a monohydroxycarotenoid (Davies & Aung Than, 1974).

Chemical tests were carried out to investigate further the nature of the functional group. The partition ratio, in distributions of the carotenoid between diethyl ether and 75% aq. methanol, was constant (95:5) irrespective of whether the hypophase was neutral or alkaline, thus indicating that no acidic function was present. Both acetylation and silylation could be accomplished to produce, in each case, a single reaction product (Table 1); no intermediates were detected by t.l.c. monitoring in the course of the reactions; thus only one hydroxyl function was indicated. That this hydroxyl function was not tertiary was shown by its acetylation and by the failure of silylation further to modify the acetylated product (Table 1). The possibility of its being secondary was eliminated by the failure of the carotenoid to undergo methylation (Liaaen-Jensen *et al.*, 1968); treatment with 0.12M-HCl in methanol resulted in no changes in the t.l.c. or partition behaviour. When the carotenoid was dissolved in chloroform and treated with HCl-saturated chloroform, its t.l.c. and partition behaviour and its absorption spectrum all underwent changes (Table 1) which indicated that dehydration had occurred to yield what was presumably a conjugated decaene with its double bonds in the *retro* configuration (Liaaen-Jensen, 1971). This behaviour was consistent with the primary hydroxyl function being allylic in nature.

The presence of a primary hydroxyl function in the carotenoid (X) was confirmed by its i.r. spectrum, which showed diagnostic absorption peaks at 3430 (hydroxyl) and 1020cm⁻¹ (primary hydroxyl) in addition to characteristic carotenoid absorption peaks at 965 (disubstituted *trans* carbon-carbon double bonds) and 850cm⁻¹ (trisubstituted carbon-carbon double bonds; Liaaen-Jensen & Jensen, 1965).

When the carotenoid (X) was hydrogenated and subjected to g.l.c. on 5% Dow-Corning high-vacuum grease on Chromosorb W (Taylor & Ikawa, 1971; Taylor & Davies, 1974), the product chromatographed with a retention time of 0.79 relative to a squalene standard. Acetylation and silylation of hydrogenated compound (X) each resulted in a single chromatographic peak; the retention times relative to squalene were 0.86 and 0.80 respectively. The volatilities of the derivatives indicated that the pigment has a triterpenoid carbon skeleton (Taylor & Davies, 1974) and confirmed the hydroxyl nature of the substituent.

The complete structure of the xanthophyll was established by examination of its mass spectrum. It had a molecular ion at m/e 418 (7%) corresponding to a molecular formula of $C_{30}H_{42}O$. Ions at m/e 402 (4%; $M-16$) and 400 (6%; $M-18$) indicated the presence of an allylic primary hydroxyl group (Enzell *et al.*, 1969). Ions at m/e 349 (13%; $M-69$) and 69 (base peak) were consistent with 'bis-allylic' fission of a saturated 7',8'-bond and cleavage of the 8',9'-single bond led to ions at m/e 83 (83%) and 335 (6%; $M-83$). Losses of toluene (92 mass units) and *m*-xylene (106 mass units), characteristic of carotenoids, also occurred, yielding ions at m/e 326 (15%; $M-92$) and 312 (25%; $M-106$) respectively. In addition, a number of ions were formed that represented combination losses from the molecular ion: m/e 331 (15%; $M-18-69$), 317 (9%; $M-18-83$), 308 (7%; $M-18-92$), 294 (6%; $M-18-106$), 257 (10%; $M-69-92$), 243 (9%; $M-83-92$), 239 (62%; $M-18-69-92$), 225 (16%; $M-18-69-106$ or $M-18-83-92$) and 211 (24%; $M-18-83-106$). The formation of a prominent ion at m/e 267 (26%; $M-151$) may be due to the cleavage of the 11,12-double bond (favoured by the presence of the hydroxyl substituent) accompanied by the transfer of a hydrogen atom to the smaller fragment; this would clearly be directly analogous to the formation of an $M-153$ species in the mass spectra of lycoxanthin (ψ,ψ -caroten-16-ol) and lycophyll (ψ,ψ -carotene-16,16'-diol; Enzell *et al.*, 1969). In contrast with lycoxanthin and lycophyll, however, the mass spectrum of this carotenoid (X) showed no loss of 85 mass units and was therefore consistent with the 7,8-bond being unsaturated.

All these analytical data are consistent with the carotenoid (X) being formulated as 4-hydroxy-4,4'-diaponeurosporene (4,4'-diapo-7',8'-dihydro- ψ,ψ -

caroten-4-ol) or 2,6,10,15,19,23-hexamethyltetracos-2,4,6,8,10,12,14,16,18,22-decaen-1-ol.

Identification of carotenoid (XI) as 4-glucopyranosyloxy-4,4'-diaponeurosporene

The most polar of the carotenoids from *S. faecium* UNH 564P was hypophasic (light petroleum-95% methanol) and was eluted from cellulose with 25% acetone in diethyl ether. Its absorption spectrum was characteristic of a conjugated nonaene chromophore and showed absorption maxima in ethanol at 391 (infl.), 416, 439 and 468nm, in diethyl ether at 390 (infl.), 414, 437 and 466nm and in chloroform at 400 (infl.), 424, 450 and 479nm.

A chromatographically pure sample of the carotenoid (XI) was subjected to a number of chemical tests. Partitioning between diethyl ether and either aq. 50% methanol or aq. 50% methanol containing 0.05% KOH revealed no evidence of any acidic functions; the partition coefficient was the same (94:6) in each solvent pair. Acetylation or silylation resulted in each case in the formation of a single final product (Table 2), although at least five intermediates could be detected by t.l.c. in the course of each reaction; there were therefore a number of hydroxyl groups in the molecule. The failure of the silylation procedure to alter the properties of the final acetylation product indicated that all the hydroxyl groups were of a primary or secondary nature. Treatment of the carotenoid with either HCl-saturated chloroform or 0.12M-HCl in methanol resulted in changes in the absorption spectrum and partition characteristics (Table 2); t.l.c. showed that the product was a mixture of the unreacted carotenoid (XI; major component) and a compound (minor component) which co-chromatographed in three t.l.c. systems with the product obtained by treating 4-hydroxy-4,4'-diaponeurosporene (X) with HCl-saturated chloroform (cf. Table 1). These data suggested that the polar carotenoid (XI) was a polyhydroxylated derivative, such as a glycoside, of 4-hydroxy-4,4'-diaponeurosporene.

The mass spectrum of the carotenoid (XI) showed a molecular ion at m/e 580 (14%) corresponding to a molecular formula of $C_{36}H_{52}O_6$. A weak ion at m/e 562 (2%; $M-18$) indicated the loss of the elements of water from the molecular ion. An ion at m/e 401 (8%; $M-179$) was consistent with α -fission at a hexoside oxygen (Aasen *et al.*, 1969) and ions

Table 2. Spectroscopic, partition and adsorption characteristics of the hypophasic carotenoid (XI) and a number of its reaction products

For details see the text.

Sample	Absorption maxima (nm) in the given solvent	Partition coefficient in light petroleum-95% methanol	R _F values on t.l.c. in system:			Inference
			1	2	3	
Carotenoid (XI)	(391) 416 439 468 Ethanol	6:94	0.19	0	0	Conjugated nonaene xanthophyll
Product obtained by: Acetylation	(391) 416 439 468 Ethanol	35:65	0.63	0.73	0.17	Primary or secondary OH
	(386) 413 435 465 Light petroleum	47:53	0.65	0.76	0.19	Primary, secondary or tertiary OH
Silylation	(391) 416 439 468 Ethanol	35:65	0.63	0.73	0.17	No tertiary OH
Acetylation followed by silylation	(386) 413 435 465 Light petroleum	18:82	0.75	0.61	0.65	Allylic OH possible
Mixture obtained by treatment of (XI) with HCl-CHCl ₃ or HCl-methanol	(395) 418 441 470 Ethanol		0.19	0	0	

at *m/e* 418 (16%; *M*-162), 402 (9%; *M*-178; both ions reported by Weeks & Andrewes, 1970) and 400 (11%; *M*-180) were all explicable in terms of simultaneous α -cleavage and hydrogen transfer at a hexoside linkage. The carotenoid (XI) was clearly the hexoside of a triterpenoid aglycone.

The mass spectrum, like that of 4-hydroxy-4,4'-diaponeurosporene (X), showed strong ions at *m/e* 83 (base peak) and at *m/e* 69 (75%), but the complementary ions at *m/e* 497 (1%; *M*-83) and at *m/e* 511 (2%; *M*-69) were relatively weak; these characteristics indicated saturation at the 7',8'- and 8',9'-bonds. The absence from the mass spectrum of any evidence of loss of either 83 or 69 mass units concomitant with that of the sugar moiety, other than those occurring (see below) with the rearrangement losses of toluene or *m*-xylene, suggested that the sugar residue must be at the other, less saturated, end of the molecule. Typical carotenoid losses of toluene and *m*-xylene gave ions at *m/e* 488 (2%; *M*-92) and 474 (11%; *M*-106) respectively and also contributed to the formation of ions at *m/e* 419 (9%; *M*-69-92), 257 (20%; *M*-162-69-92), 241 (40%; *M*-178-69-92) and 227 (50%; *M*-178-69-106 or *M*-178-83-92). These spectral data are consistent with the formulation of the carotenoid (XI) as a hexoside of 4-hydroxy-4,4'-diaponeurosporene.

Further mass-spectral data were obtained after acetylation of the carotenoid glycoside (XI) and purification of the fully acetylated product by chromatography on columns of alumina (neutral, grade II) with 20% acetone in light petroleum. The mass spectrum of the peracetate showed a molecular ion at *m/e* 748 (4%; C₄₄H₆₀O₁₀) which corresponded to that expected for a tetra-acetate of the hexoside of 4-hydroxy-4,4'-diaponeurosporene. Ions at *m/e* 417 (5%; *M*-331) and 401 (8%; *M*-347) corresponded to α -fissions on either side of the glycosidic oxygen, and a series of ions at *m/e* 331 (100%), 242 (14%), 211 (16%), 169 (73%), 157 (46%), 115 (39%), 109 (41%) and 43 (38%) represented the typical fragmentation pattern of a tetra-acetoxyoxonium ion derived from a hexapyranoside (Biemann *et al.*, 1963) observed in a number of acetylated carotenoid hexosides (Aasen *et al.*, 1969; Norgård *et al.*, 1970). Ions at *m/e* 69 (58%), 83 (70%), 679 (1%; *M*-69), 665 (3%; *M*-83), 656 (6%; *M*-92) and 642 (4%; *M*-106) indicated that the aglycone is 4-hydroxy-4,4'-diaponeurosporene. Combination losses led to ions at *m/e* 332 (52%; *M*-347-69), 318 (10%; *M*-347-83), 309 (12%; *M*-347-92) and 295 (14%; *M*-347-106). All the mass-spectral data are consistent with the formulation of the carotenoid (XI) as 4-hexopyranosyloxy-4,4'-diaponeurosporene.

To define the hexose moiety of the glycoside, the water-soluble hydrolysate from the natural pigment was examined by t.l.c., paper chromatography and enzymic oxidation. The carbohydrate component of

Table 3. Comparison of the properties of the sugar moiety of the carotenoid glycoside (XI) with those of a number of standard sugars

		For details see the text.					
Sample	Colour on t.l.c. plate with anisaldehyde-H ₂ SO ₄	R _F value on t.l.c. in system		R _F value on paper chromatography in system:			
		4	5	6	7	8	9
Duplicate samples of the water-soluble hydrolysate							
1	Blue-grey	0.64	0.63	0.50	0.52	0.32	0.64
2	Blue-grey	0.64	0.63	0.49	0.52	0.32	0.64
D-Glucose	Blue-grey	0.64	0.63	0.50	0.52	0.33	0.65
D-Mannose	Green	0.43	0.58	0.54	0.57	0.39	0.69
D-Galactose	Grey-green	0.36	0.56	0.43	0.50	0.28	0.60
D-Fructose	Violet	0.43	0.65	0.53	0.57	0.40	0.64
Sucrose	Violet	0.38	0.66	0.43	0.49	0.25	0.62

the hydrolysate co-chromatographed with D-glucose in all the chromatographic systems used and gave the colour reaction with anisaldehyde-H₂SO₄ characteristic of this sugar (Table 3). Further proof of identity was provided by the oxidation of the hydrolysis product by a D-glucose oxidase-peroxidase system with *o*-dianisidine acting as an indicator of D-glucose oxidation. Quantitative analyses by this method showed the recovery of 25–30% of the total carotenoid glycoside (XI) as glucose; this was in good agreement with the theoretical value of 31% for the yield of D-glucose from a monoglucoside of 4-hydroxy-4,4'-diaponeurosporene. The naturally occurring carotenoid can therefore be formulated as 4-D-glucopyranosyloxy-4,4'-diaponeurosporene (XI; 4-D-glucopyranosyloxy-4,4'-diapo-7',8'-dihydro- ψ,ψ -carotene) or 1-D-glucopyranosyloxy-2,6,10,15,19,23-hexamethyltetracos-2,4,6,8,10,12,14,16,18,22-decaene.

A number of attempts were made to distinguish whether the sugar in the carotenoid glycoside exists as the α or β anomer. As insufficient material was available to permit such an assignment to be made by n.m.r. spectroscopy (Liaaen-Jensen, 1973), the use of a linked enzyme method was attempted. This used either α -glucosidase or β -glucosidase in conjunction with the glucose assay system (D-glucose oxidase-peroxidase-*o*-dianisidine). This system worked well with such standards as the *p*-nitrophenyl glucosides, and it could be shown that Tween 80, necessary to

solubilize the lipid-soluble glycosides, did not interfere seriously with the detection of the simple water-soluble glycosides. Concentrations of Tween 80 as high as 5–10 mg/ml inhibited hydrolysis (e.g. of *p*-nitrophenyl β -D-glucoside by β -glucosidase) and/or enzymic glucose assay by only 10–20%. Water-insoluble standards with known glycosidic linkages, such as digitonin, *N*-stearoyldihydroglucocerebroside and *N*-stearoyldihydrogalactocerebroside, all dissolved in enzymic mixtures containing 5–10 mg of Tween 80/ml; similarly, some 50–70 μ g of the carotenoid glucoside/ml dissolved in the enzyme solution containing Tween 80 (5 mg/ml). None of these substrates, however, was amenable to hydrolysis by either glucosidase. The failure of this method to permit assignment of an anomeric form to the glucose moiety of the carotenoid glucoside is discussed below. By analogy with other carotenoid glycosides (Liaaen-Jensen, 1973), it might be expected that the glucose of the *S. faecium* carotenoid would be in the β form.

Quantitative determination of the xanthophylls, (X) and (XI)

4-D-Glucopyranosyloxy-4,4'-diaponeurosporene (XI) is the more abundant of these two xanthophylls when *S. faecium* UNH 564P is cultured under the standard conditions. It represents 13.7% of the total carotenoid (3.6 μ g/g dry cell wt.; 0.65 μ g/mg of ether-extractable lipid). The aglycone (X) constitutes

2.2% of the total carotenoid (0.59 µg/g dry cell wt.; 0.11 µg/mg of lipid).

Discussion

The above results have led to the characterization of two novel xanthophylls, isolated from cultures of *S. faecium* UNH 564P, as 4-hydroxy-4,4'-diaponeurosporene and 4-D-glucopyranosyloxy-4,4'-diaponeurosporene. These compounds are the first triterpenoid xanthophylls to be identified and the glucoside is only the fourth primary carotenoid glycoside to be reported, the others being sarcinaxanthin monoglucoside (Norgård *et al.*, 1970) and the mono- and di-glucosides of decaprenoxanthin (Weeks & Andrewes, 1970; Arpin *et al.*, 1972b).

In the course of the characterization of 4-D-glucopyranosyloxy-4,4'-diaponeurosporene, attempts were made to use specific glucosidases to define the anomerism of the glucose moiety. This method has also been attempted by other workers and previous failures have been ascribed to the insolubility of carotenoid glycosides in the aqueous reaction mixture (Hertzberg & Liaaen-Jensen, 1967). Our experiments have shown, however, that galactose and gluco-cerebrosides as well as the *S. faecium* carotenoid glucoside can be solubilized in a reaction mixture containing both a glucosidase and glucose oxidase by the utilization of Tween 80 and with little loss of enzyme activity owing to the detergent. It therefore appears that the problem involved in the assay is not a matter of solubility but is probably due to the specificity of the (β)-glucosidase used. Most, if not all, commercially available β-glucosidases are extracted from almonds and the activity of the almond enzyme has been shown to decrease markedly as the aglycone chain length of the substrate increases beyond seven carbon atoms; this is probably due to the slow dissociation of large aglycone moieties from the enzyme (Nisizawa & Hashimoto, 1970). This postulate is supported by a study of glucocerebroside hydrolysis, which indicated that two separate β-glucosidases may exist in animal tissues, one of which catalyses the hydrolysis of short-chain aglycone β-glucosides whereas the other hydrolyses more complex β-glucosides such as glucocerebrosides (Brady *et al.*, 1965). Thus the failure of the available β-glucosidases to hydrolyse the presumed β-glucosyloxy-carotenoids appears to be due to the specificity of the enzymes.

The recognition of the monohydroxy and monoglucosyloxy derivatives of 4,4'-diaponeurosporene in *S. faecium* extends the implications of the unique triterpenoid carotenoid series found in this organism. All carotenogenic non-photosynthetic bacteria examined to date appear to form complex oxygenated carotenoids and, in most cases, glycosidic carotenoids which appear to be derived directly from the hydroxy

compounds (and these from the carotenes) found in the same organism. Thus the occurrence of these novel carotenoids in *S. faecium*, which forms 4,4'-diaponeurosporene as its most highly unsaturated carotene, would seem to represent the logical conclusion of a scheme of carotenoid biosynthesis in which the terminal carotene is first hydroxylated (to yield compound X) and then glycosylated (to give compound XI).

The presence of carotenoid glycosides in non-photosynthetic bacteria raises important questions as to their function in these micro-organisms. The joining of a polar glycosidic moiety to a lipophilic carotenoid suggests that these compounds may be naturally integrated into the mixed-polarity environment encountered in cell membranes. Certainly the recent characterization of a carotenoid glycoside with a fatty acyl substituent as the major carotenoid from, and comprising 0.14% of the dry weight of, the membrane of *Myxococcus fulvus* (Kleinig, 1972) lends support to the postulate that mixed-polarity carotenoid glycosides may be important as bacterial cell-membrane components. Whether such an integration of carotenoid glycosides into bacterial cell membranes is the first step in their function as membrane-structure stabilizers, as photodynamic protection molecules (Krinsky, 1971) or as mediators of more complex enzyme-lipid interactions occurring in bacterial membranes must, for the time being, remain an intriguing problem.

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