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Pigments of *Staphylococcus aureus*, a Series of Triterpenoid Carotenoids

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The pigments of *Staphylococcus aureus* were isolated and purified, and their chemical structures were determined. All of the 17 compounds identified were triterpenoid carotenoids possessing a C_{30} chain instead of the C_{40} carotenoid structure found in most other organisms. The main pigment, staphyloxanthin, was shown to be α-D-glucopyranosyl 1-*O*-(4,4′-diaponeurosporen-4-oate) 6-*O*-(12-methyltetradecanoate), in which glucose is esterified with both a triterpenoid carotenoid carboxylic acid and a C_{15} fatty acid. It is accompanied by isomers containing other hexoses and homologs containing C_{17} fatty acids. The carotenes 4,4′-diapophytoene, 4,4′-diapophytofluene, 4-4′-diapo-ζ-carotene, 4,4′-diapo-7,8,11,12-tetrahydrolycopene, and 4,4′-diaponeurosporene and the xanthophylls 4,4′-diaponeurosporenal, 4,4′-diaponeurosporenoic acid, and glucosyl diaponeurosporenoate were also identified, together with some of their isomers or breakdown products. The symmetrical 4,4′-diapo- structure was adopted for these triterpenoid carotenoids, but an alternative unsymmetrical 8′-apo- structure could not be excluded.

Rosenbach (26), in one of the first descriptions of pyogenic cocci, distinguished between the catalase-negative streptococci and the catalase-positive staphylococci and further subdivided the staphylococci into those which produced orange-yellow or "golden" colonies on appropriate culture media (*Staphylococcus aureus*) and those which produced white colonies (*Staphylococcus albus*). The division of the genus into species is now based on properties considered more reliable than pigment formation (4, 28), but the specific epithet *aureus* remains as a reminder of one of the most readily observed features of *S. aureus*, the characteristic color of its colonies. When freshly isolated from natural sources, most strains produce colonies which are orange in color (4); some, particularly those of bovine origin or those showing multiple resistance to antibiotics, may be yellow (45), whereas others, although being in other respects typical strains of *S. aureus*, produce no pigment ("white" strains).

There is general agreement that these orange and yellow pigments are carotenoids but, in spite of studies by a number of workers, there is considerable disagreement about their precise chemical structure. Chargaff (6) claimed, mainly on the basis of the electronic absorption spectra of extracted pigments, to have identified zeaxanthin (β,β-caroten-3,3′-diol) and zeaxanthin esters, and Allegra et al. (3) and Steuer (30) also considered the main pigment to be zeaxanthin. Other workers have reported the main components to be not zeaxanthin but other carotenoids, singly or in mixtures, such as δ-carotene (ε, ψ-carotene), rubixanthin (β,ψ-caroten-3-ol), and rubixanthin esters (10, 29, 31) or δ-carotene and sarcinaxanthin [2,2′-bis(4-hydroxy-3-methyl-2-butenyl) γ,γ-carotene; 9, 27]. In addition, various amounts of more reduced compounds such as phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene), ζ-carotene (7,8,7′,8′-tetrahydro-ψ,ψ-carotene), and phytofluenol-like compounds have also been reported (10) and are presumed to be biosynthetic precursors of the main pigments.

Marshall and Rodwell have already presented evidence (J. H. Marshall and E. S. Rodwell, 3rd Int. Symp. Carotenoids Abstr. Commun., p. 56–57, 1972) that the major pigment of orange-pigmented strains of *S. aureus* cannot be any of these compounds, but must be a previously undescribed structure, which was named staphyloxanthin. More recently, Taylor and Davies (R. F. Taylor and B. H. Davies, 4th Int. Symp. Carotenoids Abstr. Commun., p. 66–67, 1975) have reported briefly that the major xanthophyll of *S. aureus* (strain 209P) does not possess the common tetraterpenoid (C_{40}) carotenoid structure, but is a triterpenoid (C_{30}) carotenoid with the structure 4,4′-diaponeurosporen-4-oic acid (4,4′-diapo-7′,8′-dihydro-ψ,ψ-caroten-4-oic acid); there is also evidence that it may form esters and glycosides (7). This major xanthophyll is

accompanied by triterpenoid carotenes which, it has been suggested (8), may be intermediates of a pathway for triterpenoid carotenoid biosynthesis which is analogous to the Porter-Lincoln pathway for tetraterpenoid carotenoid biosynthesis (24, 25). "Bacterial phytoene," isolated by Suzue from a white mutant of *S. aureus* 209P, had already been identified as a C_{30} homolog of phytoene, and it was proposed that it is a precursor of C_{40} carotenoids in this organism, although the possibility that it is the precursor of other C_{30} carotenoids was not excluded (32, 33).

Triterpenoid carotenoids have been reported in certain other bacteria. Aasen et al. (1) obtained the triterpenoid carotenoid glycoside methyl 1-mannosyloxy-3,4-didehydro-1,2-dihydro-8'-apo-ψ-caroten-8'-oate from two organisms described as yellow halophilic cocci. Taylor and Davies (34, 35, 37) isolated and characterized a series of triterpenoid carotenes and xanthophylls from *Streptococcus faecium* UNH564P, a yellow-pigmented strain (recent work [40] argues in favor of classifying these pigmented streptococci as a separate species, *Streptococcus casseliflavus*). The main pigments produced by this organism in unaerated cultures were carotenes, whereas in aerated cultures it produced mainly the glucoside 4-D-glucopyranosyloxy-4,4'-diapo-7,8-dihydro-ψ,ψ-carotene (glucosyl-diaponeurosporenol; 38). *Halobacterium cutirubrum*, an organism which produces both C_{40} and C_{50} carotenoids, also produces a C_{30} phytoene (17).

We reexamined the nature of the pigments produced by *S. aureus*, and from the results reported here, we conclude that they are all triterpenoid carotenoids or derivatives of them, some possessing novel structures. In the accompanying paper (19), we present evidence for the pathway by which these pigments are produced biosynthetically. A preliminary report of this work has been given previously (J. H. Marshall and G. J. Wilmoth, 5th Int. Symp. Carotenoids Abstr. Commun., p. 36, 1978).

MATERIALS AND METHODS

Organisms and growth conditions. The organism used for most of this work was *S. aureus* S41, isolated originally in 1965 in Melbourne, Australia, and chosen initially because of its ability to produce strongly pigmented colonies. Its phage typing pattern is 52/52A/42E/83A/81/95.

A number of mutants with altered pigment patterns were derived from this strain and, in some cases, were more convenient sources of certain carotenoids than the wild-type strain; details of their isolation and properties are given in the accompanying paper (19). *S. aureus* strain FDA209P (NCTC 7447) and the neotype strain NCTC 8532 were obtained from the National Collection of Type Cultures, Colindale, England.

Organisms were normally maintained as freeze-dried cultures, except for certain mutants (19). Working stocks were maintained on nutrient agar or glycerol monoacetate agar at 4°C after overnight growth at 37°C. The use of glycerol monoacetate as a substrate supporting good pigment production was described by Willis and Turner (46). Glycerol monoacetate broth contained (per liter): tryptone (Oxoid Ltd., London, England), 10.0 g; yeast extract (Difco Laboratories, Detroit, Mich.), 2.5 g; glycerol monoacetate (Koch-Light), 6.0 ml; Tris (Sigma Chemical Co., St. Louis, Mo.), 12.0 g; nicotinic acid, 1.2 mg; thiamine hydrochloride, 0.4 mg; biotin, 0.002 mg; the final pH was adjusted to 7.0 to 7.2 before autoclaving at 121°C for 10 min. In some experiments, the Tris buffer in the medium was replaced by phosphate (Na_2HPO_4, 2.1 g; KH_2PO_4, 0.7 g). Solid medium was prepared by incorporating 1% agar in the medium. Cells for pigment studies were normally grown in glycerol monoacetate broth in wide-mouthed Erlenmeyer flasks, the medium occupying one-fifth of the flask volume. Flasks were inoculated from an 18-h broth culture (0.1 ml per 100 ml) and incubated on a rotary shaker (eccentric radius, 3.5 cm) at 160 rpm and 37°C for 24 to 40 h (conditions which ensure good aeration) or under similar conditions in an orbital incubator (Gallenkamp, model IH-400).

Dry weight determination. The dry weight of cells was determined by measurement of the absorbance of cell suspensions at 580 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). The relation between dry weight and absorbance was linear up to an absorbance of 0.7; dry weight (milligrams per milliliter) = 0.27 × absorbance reading.

Chemicals. Analytical reagent grade chemicals were used where possible, failing which the purest commercially available grade was used. Light petroleum was Petroleumbenzin (b.p., 40 to 60°C; E. Merck, Darmstadt, W. Germany) unless otherwise specified. Most organic solvents were obtained from E. Merck or British Drug Houses (Poole, England) and used without further purification; where further purification was necessary, the methods described by Taylor and Davies (34) were used.

Extraction of carotenoids. Cells were harvested by centrifugation (5,000 × *g*, 10 min) and washed twice with water. The packed cells could be extracted immediately or stored at −20°C for up to 3 months without their carotenoid content being affected. They were suspended in methanol (40 ml per g [dry weight] of cells), heated in a water bath at 55°C for a few minutes while being stirred with a gentle stream of nitrogen, cooled, and centrifuged. The extraction was repeated if necessary until all pigment had been extracted. To avoid alteration of the carotenoids, manipulations were performed under nitrogen, exposure to bright light was avoided, and material was never stored in polar solvents. The combined methanol extracts were shaken with 1 volume of ethyl acetate and 3 volumes of 1.7 M aqueous NaCl, the ethyl acetate layer was removed, the aqueous layer if still colored was extracted with more ethyl acetate, and the combined ethyl acetate extracts were dried with anhydrous Na_2SO_4. The solvent was then removed in vacuo, the residue was dissolved in light petroleum-acetone (1:1,

vol/vol), and the solution was cooled to −20°C to precipitate phospholipids. For storage, it was preferable to replace this solvent mixture with light petroleum even though some of the polar carotenoids were not soluble in it.

Chromatographic methods. (i) Sephadex LH-20 columns. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was swollen before use by suspension in chloroform and then packed in a column (20 by 1 cm). Carotenoid extracts were transferred from light petroleum to chloroform solution. The chloroform solution was applied to the column, and the carotenoids were eluted with chloroform or, for more-polar components, a chloroform-methanol mixture.

(ii) Alumina columns. Alumina (Merck or Calbiochem; neutral, deactivated to Brockmann grade II) was suitable for separation of carotenes and low-polarity xanthophylls, but higher-polarity xanthophylls were difficult or impossible to elute. Some carotenoids, when left in contact with alumina, are degraded, and delays or interruption in the flow of solvent during separations should be avoided. Elution started with light petroleum, followed by light petroleum-acetone mixtures of increasing polarity, acetone, and in some cases acetone-methanol mixtures.

(iii) TLC. Thin-layer chromatography (TLC) was used as an analytical method and a preparative method. Plates were prepared by spreading silica gel (Kieselgel 60HR; Merck) on glass plates in 0.25-mm layers for analytical work or in 0.5-mm layers for preparative work and were activated at 110°C for 30 min. Silver nitrate-impregnated silica gel plates were prepared by incorporating 2.5% $AgNO_3$ and 5% concentrated NH_4OH in the suspending fluid for slurrying the silica gel (47). The following solvent systems were used: (i) light petroleum-acetone, 99:1 (vol/vol); (ii) light petroleum-acetone, 4:1 (vol/vol); (iii) light petroleum-acetone, 13:7 (vol/vol); (iv) benzene-methanol-acetic acid, 87:11:2 (vol/vol/vol). Spots or bands from thin-layer plates were recovered by scraping the appropriate portion of silica gel into a tube and eluting with light petroleum-acetone or acetone. Most carotenoids are readily detected as colored spots or bands; diapophytofluene, although colorless, fluoresces strongly in UV light. Colorless components were detected by exposure to iodine vapor, by spraying with sulfuric acid and heating, or by eluting and determining electronic absorption spectra.

(iv) GLC. Analyses by gas-liquid chromatography (GLC) were performed on a Perkin-Elmer model F-11 gas chromatograph (Perkin-Elmer, Beaconsfield, England). Glass columns (1.5 m by 4 mm) packed with 2% SE-52 on Gas-Chrom Q (80 to 100 mesh) were run isothermally at temperatures up to 330°C, using nitrogen as the carrier gas and a flame ionization detector. Many carotenoids are not stable under these conditions but can be converted into stable derivatives by hydrogenation over platinum oxide (36).

Spectroscopy. Electronic absorption spectra were determined with a Hitachi–Perkin-Elmer double-beam recording spectrophotometer (model 124; Perkin-Elmer, Norwalk, Conn.) calibrated against the 651.1-nm band of deuterium. Infrared spectra were determined in carbon tetrachloride solution with a Perkin-Elmer infrared recorder console (model 180); the "waxy" consistency of solid carotenoids made it difficult to compress them into a KBr pellet. Some initial determinations of mass spectra were made by G. P. Moss, Department of Chemistry, Queen Mary College, London, England; later determinations were made by S. Middleton, Department of Chemistry, Monash University, on a VG-Micromass 70/70F mass spectrometer, using a direct-probe insertion technique, a probe temperature of 200 to 220°C, and an ionizing potential of 70 eV.

Quantitative determination of carotenoids. Carotenoids were estimated quantitatively by measuring absorbance of solutions in light petroleum at appropriate wavelengths. Values for the specific extinction coefficients ($E^{1\%}_{1cm}$) of individual carotenoids were adapted from the molar extinction values of corresponding C_{40} carotenoids as described by Taylor and Davies (34). Values used for C_{30} carotenes are: 4,4′-diapophytoene (15-*cis*), 1,009 at 286 nm; 4,4′-diapophytofluene, 2,105 at 347 nm; 4,4′-diapo-7,8,11,12-tetrahydrolycopene, 3,367 at 395 nm; 4,4′-diapo-ζ-carotene, 3,415 at 400 nm; 4,4′-diaponeurosporene, 3,905 at 435 nm; 4,4′-diapolycopene, 4,450 at 466 nm. The $E^{1\%}_{1cm}$ value for 4,4′-diaponeurosporene was also used as a nominal value for its *cis* isomers and for the xanthophylls 4,4′-diaponeurosporenal (3,905 at 466 nm) and 4,4′-diaponeurosporenoic acid (3,905 at 455 nm). Corresponding values for the glycosides were: glucosyl-diaponeurosporenoate, 2,860 at 462 nm; staphyloxanthin, 1,920 at 462 nm.

Chemical characterization reactions. (i) Acetylation was used to determine the number and nature of hydroxyl functions (2). The carotenoid (50 to 500 μg) was dissolved in 1 ml of dry pyridine, 0.1 ml acetic anhydride was added, and the mixture was placed in the dark under nitrogen at room temperature for 12 to 24 h. Sodium chloride solution (5 ml, 1.7 M) was then added, the product was extracted into diethyl ether (two 5-ml volumes), and the combined extracts were washed with 1.7 M NaCl to remove pyridine; the solvent was then removed in vacuo. The acetylated product was dissolved in light petroleum and purified by TLC on silica gel with solvent iii. Primary, secondary, and tertiary alcohols are distinguished by their ease of acetylation (18); primary alcohols are fully acetylated within 10 to 30 min, secondary alcohols require 3 to 6 h for acetylation, and tertiary alcohols do not react. To determine whether tertiary hydroxyls were present, the fully acetylated product was further treated with dry pyridine and bis(trimethylsilyl)-acetamide (Pierce Chemical Co., Rockford, Ill.), and the product was recovered and purified as for acetyl derivatives. Tertiary hydroxyls are silylated under these conditions (13).

(ii). Sodium borohydride reduces aldehydes and ketones but not carboxylic acids or their esters (2, 18), but some carotenol esters may be saponified (12). An ethanolic solution of carotenoid was treated with a few crystals of $NaBH_4$ and placed in the dark at room temperature for 60 min; 1.7 M NaCl was then added, and the product was extracted into diethyl ether and purified.

(iii) Lithium aluminum hydride reduces aldehydes, ketones, and esters to hydroxyl derivatives (2). To the carotenoid dissolved in dry diethyl either, $LiAlH_4$ (1

to 2 mg) was added and allowed to react at room temperature for a few minutes. The reaction was terminated by the addition of a few milliliters of wet ether followed by 5 ml of 1.7 M NaCl, and the product in the ether phase was concentrated and purified. In some cases, difficulties were encountered in isolating products from strongly polar xanthophylls owing to their adsorption on the alumina precipitate.

(iv) Saponification with methanolic KOH is a standard procedure for preliminary purification of carotenoids in which it is assumed that not only glycerol esters but also any carotenol- or carotenoic acid-containing esters will be hydrolyzed to free acids. Taylor and Davies, however, have shown that 4% KOH at room temperature produces rapid transesterification of carotenoic esters or esterification of free carotenoic acids to the corresponding methyl esters (7; Taylor and Davies, 4th Int. Symp. Carotenoids Abstr. Commun.). More concentrated alkali (10% KOH) acting for 12 to 24 h is needed to ensure hydrolysis of esters (including methyl esters) to free acids. Esters can also be hydrolyzed to free acids with no possibility of methyl ester formation by treatment with 5% KOH in acetone at room temperature for 60 min; the alkaline acetone solution should be freshly prepared to keep to a minimum the formation of alkali-catalyzed polymerization products of acetone, which do not then interfere.

(v) Carotenoid isomerization was performed by the Zechmeister method, employing UV irradiation in the presence of iodine (34).

(vi) Partition coefficients between light petroleum and 95% methanol were determined by the method of Petracek and Zechmeister (21).

Identification of sugar moiety of carotenoid glycosides. The carotenoid glycoside (100 to 500 μg) was hydrolyzed in HCl-saturated chloroform as described by Taylor and Davies (35), and the water-soluble fraction was used for chromatographic and enzymatic analyses for carbohydrate. Chromatographic analysis was by TLC on Kieselguhr G plates buffered with phosphate at pH 5 and eluted with butan-1-ol–acetone–0.05 M phosphate (pH 5; 4:5:1, vol/vol/vol) (41). Sugars were detected by spraying the plates with $AgNO_3$ solution followed by NaOH solution and heating (16). Enzymatic analyses for glucose employed Glucostat reagent (glucose oxidase-peroxidase reagent; Worthington Diagnostics, Freehold, N.J.).

Identification and estimation of fatty acids. Material containing fatty acids was treated with methanol-boron trifluoride; the resulting fatty acid methyl esters were extracted into diethyl ether and analyzed by GLC in the system described previously, using a column temperature of 175°C. Quantitative estimation was by comparison of peak areas with those of known standards. Reference compounds included the saturated *n* acids C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, and C_{18} as well as the branched-chain anteiso- acids C_{15} and C_{17} prepared from *S. aureus* lipids.

Glycerol determination. Glycerol was measured enzymatically by using glycerol kinase and NAD-dependent glycerol 3-phosphate dehydrogenase (Boehringer Mannheim).

Radioactivity measurements. Activity of ^{14}C-containing material was measured with a liquid scintillation spectrometer (model 2002; Packard Instrument Co., Rockville, Md.). The scintillation fluid was prepared by dissolving 5 g of 2,5-diphenyloxazole [PPO] and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene in 1 liter of toluene and adding 500 ml of Triton X-100. Carotenoid samples were bleached with benzoyl peroxide before being counted (42). [^{14}C]acetic anhydride was obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Extraction and purification methods. Brief treatment of wet-packed cells with warm methanol (43) proved the most satisfactory of the methods tried for extracting pigments from *S. aureus*. Such extracts usually contain, in addition to carotenoids, other polyisoprene compounds such as squalene, menaquinones, and bactoprenol (undecaprenol and homologs) as well as phospholipids and glycolipids. It is customary to remove saponifiable lipids either by incorporating alkali in the extracting solvent or by a subsequent saponification step with methanolic KOH (18). With *S. aureus* extracts, saponification must be avoided; otherwise some xanthophylls, which are rapidly and irreversibly changed by dilute alkali, will be lost (Marshall and Rodwell, 3rd Int. Symp. Carotenoids Abstr. Commun.). Phospholipids can be removed by an alternative method involving precipitation by cold acetone (see above).

For purification and isolation of individual carotenoids, a combination of chromatographic methods was used. Chromatography on Sephadex LH-20 columns was the most useful system for initial fractionation of crude extracts; elution with chloroform followed by chloroform-methanol separated four main fractions and permitted complete recovery of all material from the column (Table 1). By suitable adjustment of the column size and flow rate of the solvent, fraction 3 could be resolved into two separate bands (diaponeurosporenoic acid and staphyloxanthin), but it was usually preferable to use other chromatographic systems to resolve each of fractions 1 to 3 into individual components. Individual carotenoids isolated from *S. aureus*, together with their spectral characteristics and order of elution from alumina (grade II) columns by light petroleum and light petroleum-acetone, are listed in Table 2. Alumina gave good resolution of fraction 1 but was less satisfactory for chromatography of more polar components, some of which became irreversibly bound to alumina and could not be eluted. There was also evidence of breakdown of some components, the extent being proportional to the time they were in contact with alumina.

TLC on silica gel with different solvent systems was capable of separating most of the carotenoids (Table 3), although it did not separate carotenes as well as did alumina columns. It was particularly useful when only limited amounts of material were available, for rapid qualitative analysis of crude extracts or fractions, and as a final purification step for some components. For example, up to 350 μg of staphyloxanthin could be separated as a narrow band without trailing on a 20-cm by 10-cm plate, 0.5 mm thick.

TABLE 1. *Chromatography of carotenoids and related compounds of S. aureus on Sephadex LH-20*

Fraction	Eluted by:	Composition
1	Chloroform	Squalene Carotenes Menaquinone Carotenals
2	Chloroform	Bactoprenol Isostaphyloxanthin Hydroxy-400 compounds
3	Chloroform-methanol (99:1)[a]	Diaponeurosporenoic acid Staphyloxanthin
4	Chloroform-methanol (19:1)[b]	Glucosyl-diaponeurosporenoate

[a] Chloroform alone will slowly elute fraction 3.
[b] Increasing methanol concentration causes swelling of Sephadex, and the flow rate decreases considerably.

Carotenes. The hydrocarbon fraction contained, in addition to squalene (20 μg/g [dry weight]), several carotenes which appeared to correspond to those first isolated by Taylor and Davies (34) from *Streptococcus faecium* and later reported by them to be also present in *S. aureus* 209P (4th Int. Symp. Carotenoids Abstr. Commun.). The chromatographic properties, epiphasic behavior (partition ratio, 100:0), and absorption maxima are in good agreement with those reported here (Table 2). Upon hydrogenation and analysis by GLC (Table 4), the derivatives all ran with the same retention time as squalane, indicating that they were all C_{30} compounds; mass spectral determinations on the staphylococcal diapophytoene (*m/e*, 408, corresponding to a molecular formula of $C_{30}H_{48}$) and

TABLE 2. *Chromatography on alumina and identity of carotenoids isolated from S. aureus*

Chromatography[a]		Color	λ_{max} (nm) in light petroleum[b]					Identification	Carotenoid[c] content (μg/g [dry wt])
Band	Solvent								
1	0	Colorless			—			Squalene	20
2	0	Colorless		275	286	298		15-*cis*-4,4′-Diapophytoene	40
3	0	Colorless (fluorescent)		330	347	366		4,4′-Diapophytofluene	3
4	0	Pale yellow		374	395	419		4,4′-Diapo-7,8,11,12-tetrahydrolycopene	2
5	0.5	Yellow		405	428	456		Neo-4,4′-diaponeurosporene C	1
6	0.5	Pale yellow		378	400	425		4,4′-Diapo-ζ-carotene	6
7	0.75	Yellow		407	430	459		Neo-4,4′-diaponeurosporene B	1
8	1	Yellow		412	435	465		4,4′-Diaponeurosporene (all-*trans*)	10
9	1	Red		440	466	498		4,4′-Diapolycopene	ND[d]
10	1.5	Colorless	243	248	260	269	325	Menaquinone	
11	3	Red	346	(441)	463	492		*cis*-4,4′-Diaponeurosporenal	ND
12	3	Red		(444)	466	496		4,4′-Diaponeurosporenal	1
13	3	Red		(454)	476	508		4,4′-Diapolycopenal	ND
14[e]	20	Pale yellow			—			Bactoprenol	
		Pale yellow		378	400	422	(445)	Hydroxy-400 compounds	10
15	30	Orange		—	460	(489)		Isostaphyloxanthin	10
16	40	Orange		—	462	(491)		Staphyloxanthin	360
NE[f]		Yellow		432	455	483		4,4′-Diaponeurosporenoic acid	38
NE		Yellow		(430)	453	481		*cis*-4,4′-Diaponeurosporenoic acid	2
NE		Orange		—	462	(491)		Glucosyl-4,4′-diaponeurosporenoate	10

[a] Chromatography on alumina (grade II) column. Bands eluted successively by light petroleum-acetone mixtures of increasing polarity; percent acetone indicated in solvent column. Relative positions of three noncarotenoid polyisoprenes also shown (bands 1, 10, and 14).
[b] Dashes indicate no peak; parentheses indicate point of inflection.
[c] Average figures for several experiments; cells grown with good aeration (see text) for 40 h at 37°C, yielding 2.5 to 3.0 mg (dry weight) of cells per ml of culture; total carotenoid content, ca. 500 μg/g (dry weight).
[d] ND, Not detected in the wild-type strain but found in some mutants (19).
[e] 4,4′-Diaponeurosporenol (λ_{max}, 412, 435, and 465) was never detected in *S. aureus*, but authentic material eluted in band 14.
[f] Not eluted even by polar solvents.

TABLE 3. *TLC of carotenoids, squalene, menaquinone, and bactoprenol from S. aureus*[a]

Compound	R_f value			
	i	ii	iii	iv
Squalene	0.87			
4,4'-Diapophytoene	0.80			
4,4'-Diapophytofluene	0.62			
4,4'-Diapo-7,8,11,12-tetrahydrolycopene	0.49			
Neo-4,4'-diaponeurosporene C	0.47	>0.85	>0.85	>0.90
4,4'-Diapo-ζ-carotene	0.47			
Neo-4,4'-diaponeurosporene B	0.45			
4,4'-Diaponeurosporene	0.39			
4,4'-Diapolycopene	0.36			
Menaquinone	0.25	0.71	0.81	0.82
cis-4,4'-Diaponeurosporenal	0.10	0.64	0.72	0.75
4,4'-Diaponeurosporenal	0.10	0.60	0.68	0.75
4,4'-Diapolycopenal	0.10	0.57	0.65	0.75
Bactoprenol		0.55	0.65	0.60
4,4'-Diaponeurosporenol		0.54	0.63	0.60
cis-4,4'-Diaponeurosporenoic acid		0.12	0.52	0.47
4,4'-Diaponeurosporenoic acid	0	0.12	0.49	0.45
Isostaphyloxanthin		0.07	0.43	0.40
Staphyloxanthin		0.05	0.38	0.36
Glucosyl-diaponeurosporenoate		0	0.05	0.25

[a] TLC on silica gel. For composition of solvents i through iv, see the text.

diaponeurosporene (*m/e*, 402, corresponding to a molecular formula of $C_{30}H_{42}$) provided conclusive evidence of their size. Under the growth conditions used, the total carotene content of the cells was about 60 μg/g (dry weight) (10 to 15% of the total carotenoids); however, some of the mutant strains (19) produced much higher proportions of carotenes and were a more convenient source for their isolation.

The main members of this carotene series are 4,4'-diapophytoene (4,4'-diapo-7,8,11,12,7',8',11',12'-octahydro-ψ,ψ-carotene), 4,4'-diapophytofluene (4,4'-diapo-7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene), 4,4'-diapo-ζ-carotene (4,4'-diapo-7,8,7',8'-tetrahydro-ψ,ψ-carotene) 4,4'-diapo-7,8,11,12-tetrahydrolycopene (4,4'-diapo-7,8,11,12-tetrahydro-ψ,ψ-carotene), and 4,4'-diaponeurosporene (4,4'-diapo-7,8-dihydro-ψ,ψ-carotene), possessing systems of 3,5,7,7- and 9-conjugated double bonds, respectively (Fig. 1). Only one isomer of 4,4'-diapophytoene was isolated; its properties correspond to those of the 15-*cis* isomer (7), in contrast to the other members of the series, which were all-*trans* isomers. For 4,4'-diaponeurosporene, in addition to the *trans* isomer, which constituted 80 to 90% of the total, two *cis* isomers were also isolated, the neo B and neo C isomers. Each of the three isomers, when subjected to iodine-catalyzed photoisomerization, gave an equilibrium mixture of the

TABLE 4. *GLC of carotenoids of S. aureus*

Compound	Retention[a] time (min)	Retention time/retention time of squalane
Reference compound		
Squalene	1.50	1.43
Hydrogenation products of:		
Squalene (i.e., squalane)	1.05	1.00
Lycopene[b] (i.e., lycopersane)	5.00	4.76
β-Carotene	3.40	3.26
Compound from *S. aureus*		
4,4'-Diapophytoene	2.10	2.00
Hydrogenation products of:		
Squalene	1.05	1.00
4,4'-Diapophytoene	1.05	1.00
4,4'-Diapophytofluene	1.05	1.00
4,4'-Diapo-ζ-carotene	1.05	1.00
4,4'-Diaponeurosporene	1.05	1.00
4,4'-Diaponeurosporenol[c]	1.50	1.43
4,4'-Diaponeurosporenal	1.55	1.47
4,4'-Diaponeurosporenoic acid	1.55	1.47
4,4'-Diaponeurosporenoic acid methyl ester	1.70	1.61
Staphyloxanthin	No clear peaks	

[a] Column: 2 % SE-52 on Gas-Chrom Q run isothermally at 250°C.

[b] Prepared from tomatoes.

[c] Prepared by reduction of staphyloxanthin with $LiAlH_4$.

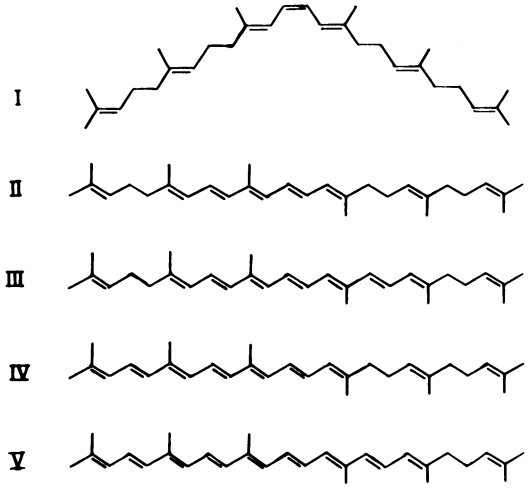

FIG. 1. *Triterpenoid carotenes of S. aureus. (I) 15-cis-4,4'-Diapophytoene; (II) 4,4'-diapophytofluene; (III) 4,4'-diapo-ζ-carotene; (IV) 4,4'-diapo-7,8,11,12-tetrahydrolycopene; (V) 4,4'-diaponeurosporene.*

trans (55%) and five *cis* isomers, neo A (2%), neo B (10%), neo C (30%), neo D (2%), and neo E (1%); the amounts found and positions of absorption maxima were in good agreement with reported values (34).

One novel carotene found in small quantities in certain mutants but not in the wild-type strain (21) was identified as 4,4'-diapolycopene (4,4'-diapo-ψ,ψ-carotene) on the basis of its absorption spectrum (λ_{max}, 440, 466, and 498 nm), which is identical to that reported for synthetic material prepared by dehydrogenation of squalene with *N*-bromosuccinimide (37). It has not previously been reported to occur naturally.

Carotenals. Carotenoid extracts from the wild-type organism contained a fraction present in small amounts (0.2% of the total) which could be separated as a red band (band 12; Table 2), but not in sufficient quantity for identification. Material sufficient for characterization was obtained from type IV mutants (21), for which it was the major carotenoid. Two isomers were found, separable by TLC with solvent iii, each having a partition ratio of 78:22 and being present in a ratio of approximately 4:1. The main all-*trans* isomer had λ_{max} values of (444), 466, and 496 nm in light petroleum, 467 nm in methanol, and 466 nm in acetone, whereas the values for the *cis* isomer in light petroleum were (441), 463, and 492 nm, with a prominent *cis* peak at 346 nm. Iodine-catalyzed photoisomerization of either gave an equilibrium mixture containing 67% *trans* isomer. The compound did not react with 4% methanolic KOH, could not be acetylated, and showed no acidic properties but was reduced by $NaBH_4$ or $LiAlH_4$ to a product with λ_{max} values (in light petroleum) at 413, 435, and 465 nm, which was identified as 4,4'-diaponeurosporen-4-ol. Hydrogenation gave a product, the retention time of which on GLC indicated that it had a C_{30} chain (Table 4). These properties are consistent with the identification of the compound as the aldehyde 4,4'-diaponeurosporen-4-al (4,4'-diapo-7',8'-dihydro-ψ,ψ-caroten-4-al) described by Taylor and Davies (37) (Fig. 2, VI).

In extracts from type IV mutants, small amounts of a second aldehyde were present which ran as a faint purple band (band 13) closely following band 12 on alumina columns and was separable by repeated chromatography. Its properties were very similar to those of diaponeurosporenal but with λ_{max} values of (454) 476 and 508 nm, a partition ratio of 80:20, and a $NaBH_4$ reduction product having λ_{max} values of (444), 465, and 496 nm. Its properties suggest that it contains 11 conjugated double bonds, and it would appear to correspond to the compound identified by Taylor and Davies (37) as 4,4'-diapolycopen-4-al (4,4'-diapo-ψ,ψ-caroten-4-al).

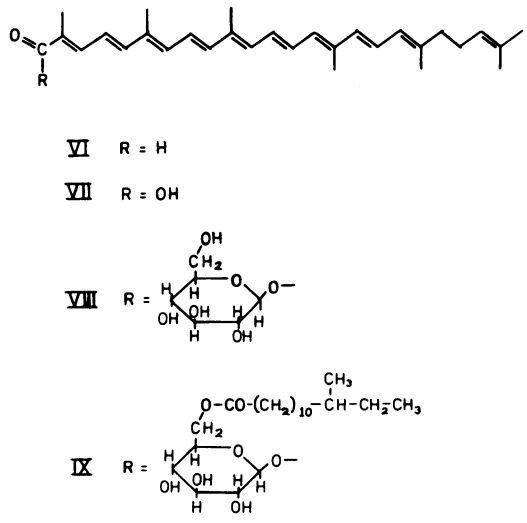

FIG. 2. *Triterpenoid xanthophylls of S. aureus. (VI) 4,4'-Diaponeurosporen-4-al; (VII) 4,4'-diaponeurosporen-4-oic acid; (VIII) glucosyl-diaponeurosporenoate; (IX) staphyloxanthin (α-D-glucopyranosyl 1-O-(4,4'-diaponeurosporen-4-oate) 6-O-(12-methyltetradecanoate).*

4,4'-Diaponeurosporen-4-oic acid. 4,4'-Diaponeurosporen-4-oic acid was obtained from crude extracts by preliminary fractionation on Sephadex LH-20 followed by TLC of fraction 3 with solvent iii; alumina columns could not be used, since the compound could not be eluted even by polar solvents. Some of its properties are listed in Table 5. The variation in the partition ratio with pH and its reduction to diaponeurosporenol by $LiAlH_4$ but not by $NaBH_4$ indicated that it was the carboxylic acid 4,4'-diaponeurosporen-4-oic acid (4,4'-diapo-7',8'-dihydro-ψ,ψ-caroten-4-oic acid), which Taylor and Davies have reported to be the major xanthophyll of *S. aureus* 209P (Taylor and Davies, 4th, Int. Symp. Carotenoids Abstr. Commun.) (Fig. 2, VII). The behavior with methanolic KOH was unusual; 4% KOH at 20°C caused no detectable change over short periods (20 min), but longer exposure led to slow esterification (50% in 12 h); 10% KOH, however, favored hydrolysis, the ester being 95% hydrolyzed within 24 h. Esterification to the same product was also effected by methanol-BF_3. Acetylation with acetic anhydride-pyridine was slow, 40% of the acid being unchanged after 24 h, and a single product was formed which had the properties of a mixed anhydride; silylation also yielded a single product. The retention time of the hydrogenation product (Table 4), which, as with other xanthophylls, consisted of a mixture of hydrogenated xanthophyll and the corresponding hy-

TABLE 5. *Comparison of some properties of staphyloxanthin and 4,4'-diaponeurosporen-4-oic acid*

Compound	λ_{max}[a]			Partition ratio (light petroleum/95% methanol)	Reaction product
4,4'-Diaponeurosporenoic acid	432	455	483	10:90[b]	
	(422)	448	475[c]		
	(430)	455	(483)[d]		
Reaction with:					
4% Methanolic KOH	432	455	485	85:15	Methyl diaponeurosporenoate
10% Methanolic KOH	432	455	483	10:90[b]	No reaction
$NaBH_4$	432	455	483	10:90[b]	No reaction
$LiAlH_4$	413	435	465	50:50	Diaponeurosporenol
Acetylating agent		460		55:45	Acetyl anhydride
Silylating agent		460		55:45	Silyl derivative
cis Isomer	(430)	453	481	10:90[b]	
Staphyloxanthin		462	(491)	18:82	
		460	[c]		
		460	[d]		
Reaction with:					
4% Methanolic KOH	432	455	485	85:15	Methyl diaponeurosporenoate
10% Methanolic KOH	432	455	483	10:90[b]	Diaponeurosporenoic acid
4% KOH in acetone	432	455	483	10:90[b]	Diaponeurosporenoic acid
$NaBH_4$	432	455	483	10:90[b]	Diaponeurosporenoic acid
$LiAlH_4$	413	435	465	50:50	Diaponeurosporenol
Acetylating agent	(440)	463	492	55:45	Triacetate (see text)
Silylation of fully acetylated product	(440)	463	492	55:45	No silylation
cis Isomer 350		460		20:80	

[a] Spectra measured in light petroleum except where otherwise indicated. Parentheses indicate point of inflection.

[b] Partition ratio increased in dilute acid to 45:55 and decreased in dilute alkali to 0:100.

[c] Measured in methanol.

[d] Measured in acetone.

drogenated hydrocarbon, indicated a C_{30} chain.

The infrared spectrum showed a prominent carbonyl peak at 1,715 cm^{-1}, which disappeared after $LiAlH_4$ reduction, and a broad peak at 3,350 cm^{-1}, corresponding to a carboxylic hydroxyl which became more intense, moved to 3,400 cm^{-1} after $LiAlH_4$ reduction, and was not present in the methyl ester. Mass spectral data for the acid and its methyl ester were in close agreement with those reported by Davies (7), indicating a molecular weight for the acid of 432 and a molecular formula of $C_{30}H_{40}O_2$. These analytical data confirm the identification of the compound as 4,4'-diaponeurosporen-4-oic acid.

Staphyloxanthin. Under the conditions of growth used in this work, 70 to 80% of the total carotenoid produced by *S. aureus* S41 was staphyloxanthin. A comparison of some properties of staphyloxanthin and diaponeurosporenoic acid is made in Table 5. Their spectra differed considerably, the acid showing a three-peak spectrum, whereas staphyloxanthin showed a single broad peak at 460 nm in methanol or acetone and a broad peak at 462, with an inflection at 491 in light petroleum (Fig. 3). Treatment

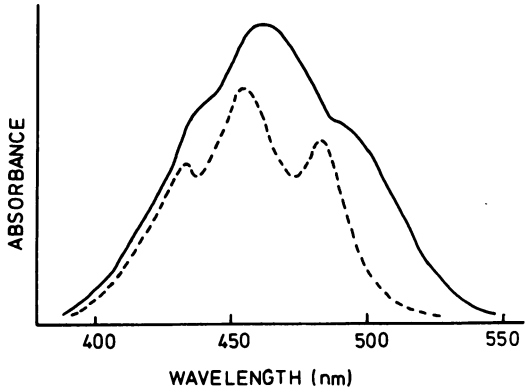

FIG. 3. *Electronic absorption spectra of xanthophylls. (——) Staphyloxanthin; (---) 4,4'-diaponeurosporenoic acid. Solvent, Light petroleum.*

of staphyloxanthin with dilute alkali caused its spectrum to change rapidly to a three-peak spectrum, and the change could not be reversed by acidification; when 4% methanolic KOH was used, transesterification occurred, and the product was diaponeurosporenoic acid methyl ester; when 10% methanolic KOH, 4% KOH in acetone,

or $NaBH_4$ was used, the product was the free acid.

Acetylation of staphyloxanthin with acetic anhydride-pyridine produced three separate products, the initial product reaching its maximum level within 40 to 50 min and then decreasing, the second reaching a maximum after about 120 min, and the third being the sole product after about 6 h. That each of these steps was sequential could be shown by isolating the first acetyl derivative by TLC (solvent iii; R_f, 0.51), subjecting it to further acetylation to produce the second derivative, isolating it (R_f, 0.62), and again acetylating to produce the fully acetylated product (R_f, 0.72). The presence of three acetylatable hydroxyl groups was confirmed by a quantitative measurement of the incorporation of ^{14}C into the products after acetylation of staphyloxanthin with [1-^{14}C]acetic anhydride, when the ^{14}C content of the three products corresponded to mono-, di-, and triacetyl derivatives, respectively (Table 6).

Earlier attempts to determine the structure of staphyloxanthin suggested that it was an ester of diaponeurosporenoic acid containing carbohydrate, fatty acid, and glycerol (Marshall and Wilmoth, 5th Int. Symp. Carotenoids Abstr. Commun.). Further work showed that these samples of staphyloxanthin were contaminated with glycolipid, which could not be separated by repeated TLC on silica gel with solvent iii or by TLC of acetylated derivatives in the same system. The presence of the glycolipid was shown by the blue color obtained after spraying with diphenylamine (16). Separation was achieved by TLC on silver nitrate-impregnated silica gel with solvent iii, in which system the R_f of staphyloxanthin was appreciably less than that of the glycolipid. Purified in this way it still contained diaponeurosporenoic acid, carbohydrate, and fatty acid, but no glycerol.

TABLE 6. *Determination of free hydroxyl groups with [1-^{14}C]acetic anhydride*

Carotenoid	Acetylated product[a] (cpm)	CH_3CO content[b] (nmol)	Carotenoid (nmol)	Acetyl/carotenoid ratio
Diaponeurosporenol	12,700	177	170	1.04
Staphyloxanthin				
First acetyl derivative	7,600	106	96	1.1
Second acetyl derivative	8,600	120	57	2.1
Third acetyl derivative	11,200	155	48	3.2
Isostaphyloxanthin	5,300	74	22	3.35
Glucosyl-diaponeurosporenoate	9,200	129	30	4.3

[a] Carotenoids were acetylated with [1-^{14}C]acetic anhydride-pyridine; values are for fully acetylated products, except for the first and second acetyl derivatives of staphyloxanthin.

[b] Specific activity of [1-^{14}C]acetic anhydride, 20.6 μCi/mmol.

The carbohydrate moiety was obtained in the water-soluble fraction after acid hydrolysis, and the main component had an R_f of 0.36 in the TLC system of Waldi (41), corresponding to glucose; about 5% each of two other carbohydrates was present with R_f values of 0.44 and 0.25, corresponding to mannose and galactose. The presence of glucose as the main component was confirmed by enzymatic assay with the Glucostat reagent.

Identification and measurement of fatty acids were carried out by GLC of their methyl esters after hydrolysis of the carotenoid with methanolic KOH. The main component was identified as the C_{15} anteiso- acid, 12-methyl-tetradecanoic acid; some of its C_{17} homolog 14-methyl-hexadecanoic acid was also present. Quantitative determinations of carotenoid, glucose, and fatty acid in staphyloxanthin yielded a ratio near 1:1:1, although the figure for glucose was always slightly less than one.

The infrared spectrum differs from that of methyl diaponeurosporenoate, mainly in showing, in addition to the carbonyl peak at 1,715 cm^{-1}, a second strong carbonyl band at 1,740 cm^{-1}, attributable to the fatty acid carboxyl, and a broad peak at 3,400 cm^{-1}, attributable to the glucose hydroxyls.

Mass spectra determinations on staphyloxanthin gave a pattern very similar to that of diaponeurosporenoic acid but with some additional peaks. The largest fragment detected was at *m/e* 432, corresponding to diaponeurosporenoic acid, with other peaks at *m/e* 415 (M-17) and 387 (M-45), indicating loss of —OH and —COOH. A prominent peak at *m/e* 242 accompanied by peaks at *m/e* 225 (M-17) and 197 (M-45) corresponded to a C_{15} monocarboxylic acid, whereas peaks at *m/e* 185 (M-57) and 57 were consistent with methylation at the third carbon,

$$CH_3-CH_2-\underset{\displaystyle CH_3}{\overset{|}{CH}}-$$

giving the fragment $CH_3—CH_2—\underset{\displaystyle CH_3}{\overset{|}{CH}}—$ (5). A small peak at *m/e* 269 indicated the presence of the homologous C_{17} fatty acid. Triacetylstaphyloxanthin gave a parent ion at *m/e* 944, corresponding to a molecular formula of $C_{57}H_{84}O_{11}$ (Fig. 4). Fragments at *m/e* 513 and 432 corresponded to splitting of diaponeurosporenoic acid from the rest of the molecule, whereas *m/e* 288 was the triacetylhexose moiety (M-432-225) and showed a typical fragmentation pattern of triacetylhexose (23), giving successive loss of acetyl groups producing peaks at *m/e* 229, 169, and 109; a small peak at *m/e* 703 corresponded to loss of fatty acid (M-242). A small peak was also de-

tected at *m/e* 972, attributable to the homolog containing a C_{17} fatty acid.

Staphyloxanthin thus appeared to be a derivative of diaponeurosporenoic acid linked by an ester linkage to glucose, which was also esterified with a C_{15} fatty acid. G. P. Moss (Queen Mary College, London, England) very kindly examined a sample of this material by mass and nuclear magnetic resonance spectrometry. He suggested that the carotenoid chain may have the unsymmetrical 8'-apo- structure, rather than the 4,4'-diapo- structure, and proposes for staphyloxanthin the structure α-D-glucopyranosyl 1-*O*-(8'-apo-ψ-carotenoate) 6-*O*-(12-methyl-tetradecanoate) (20), although we prefer the symmetrical 4,4'-diaponeurosporenoate for the carotenoid moiety (see Discussion). This structure is shown in Fig. 2 (IX). The relationship between the two alternative structures for the C_{30} chain, the 4,4'-diapo- and the 8'-apo-, and the C_{40} carotenoid chain are shown in Fig. 5. The analytical results indicate that staphyloxanthin is accompanied by small amounts of two isomeric forms in which glucose is replaced by mannose or galactose and by homologous forms containing the C_{17} fatty acid in place of the C_{15} fatty acid.

Isostaphyloxanthin. Material which ran just ahead of staphyloxanthin on Sephadex LH-20 or alumina could be separated as a distinct fraction by TLC (solvent iii). When subjected to the tests listed in Table 5, it behaved like staphyloxanthin and differed from it only in having a slightly higher partition ratio (23:77) and λ_{max} values of 461 and (489) nm, the 489-nm inflection being slightly less prominent. It is not a *cis* isomer and, when subjected to iodine-catalyzed photoisomerization, showed hypochromic and hypsochromic changes in its spectrum to a λ_{max} of 459 nm and a *cis* peak at 357 nm. Stepwise acetylation and acetylation with [1-^{14}C]acetic anhydride (Table 6) showed the presence of three free hydroxyl groups. By the same methods as were used for staphyloxanthin, it was shown to contain diaponeurosporenoic acid, glucose, and a C_{15} fatty acid, although there was not sufficient material for a quantitative enzymatic assay for glucose. Its properties suggest that it is an isomer of staphyloxanthin which may differ only in the position of the ester linkages between glucose and the C_{15} and C_{30} chains.

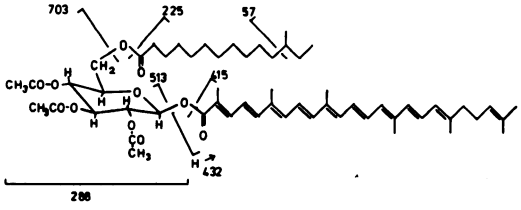

FIG. 4. *Mass spectrometry of triacetyl staphyloxanthin; derivation of main fragments.*

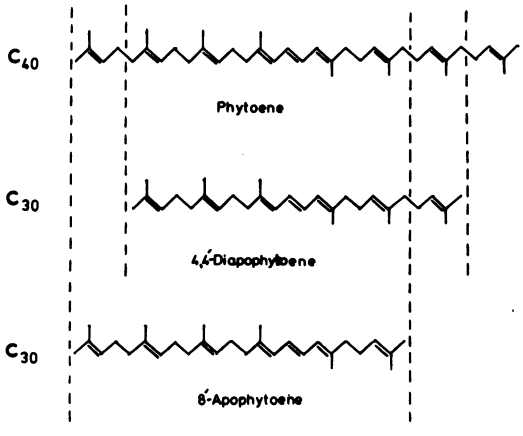

FIG. 5. *Relationship between alternative structures for "C_{30} phytoene" and phytoene.*

Glucosyl-diaponeurosporenoate. After the structure of staphyloxanthin became clear, it seemed likely that its biosynthesis would proceed via an intermediate involving two of its three constituents. Such a compound was detected in the most polar fraction from the Sephadex LH-20 column (Table 1, fraction 4). This fraction also contained glycolipid and phospholipid, and purification required several separations by TLC on silica gel (solvent iv) followed by TLC of acetylated material on silver nitrate-impregnated silica gel (solvent iii). Hydrolysis of the pure material yielded diaponeurosporenoic acid and glucose (as shown by TLC and enzymatically). By the chemical tests used to characterize staphyloxanthin (Table 5), it was indistinguishable from it. Its spectrum was also indistinguishable from staphyloxanthin [λ_{max}, 462 and (491) nm], but it was more hypophasic (partition ratio, 2:98), and its more polar nature was apparent on chromatography. A total of five acetyl derivatives were detected during acetylation; determination of free hydroxyl groups by exhaustive acetylation with [1-^{14}C]acetic anhydride showed that there were four (Table 6). Only small amounts of material were available for analysis, which limited the accuracy of determination of the carotenoid/glucose ratio; the value obtained was 1:0.6. Its properties are consistent with it being a glucosyl ester of diaponeurosporenoic acid.

"Hydroxy-400" compounds. Trace amounts of a group of xanthophylls were isolated which all gave spectra similar to that of diapo-ζ-carotene but with an additional shoulder at 445 nm [λ_{max}, 378, 400, 425, and (445) nm]. More of this material was obtained from certain mu-

tant strains (19), which permitted limited investigation of this group. TLC (solvent iii) separated bands at R_f 0.50 and 0.55, with two fainter bands at R_f 0.38 and 0.25. Their polarity and absorption spectra were unaffected by dilute acid or alkali. There is evidence that they are breakdown products of diaponeurosporene and possess hydroxyl or carbonyl groups (19).

4,4'-Diaponeurosporenol. 4,4'-Diaponeurosporenol is a major xanthophyll in *Streptococcus faecium* (35, 38), and it was anticipated that it would be present in *S. aureus* also, as an intermediate if not a major component. Several specific searches of appropriate fractions from both the wild-type organism and several mutant strains failed to detect it. Authentic material for comparison was readily prepared by reduction of staphyloxanthin or diaponeurosporenoic acid with $LiAlH_4$.

Carotenoids in other strains of *S. aureus*. Pigment formation in *S. aureus* is known to vary in different strains, and it is also dependent on growth conditions. The results presented here were obtained with strain S41 grown under conditions which gave good yields of pigment. A survey of a number of orange-pigmented strains showed that they possessed the same carotenoid pattern as strain S41, with staphyloxanthin as the major component. A more detailed analysis of two strains, the neotype strain of *S. aureus* (NCTC 8532) and strain 209P (NCTC 7447), the strain used by Suzue (31) and by Taylor and Davies (Taylor and Davies, 4th Int. Symp. Carotenoids Abstr. Commun.) is presented in Table 7. An investigation of other pigment types and of the influence of growth conditions on pigmentation will be reported elsewhere.

TABLE 7. *Comparison of carotenoid content of three strains of S. aureus*

Carotenoid	Amt[a] (μg/g [dry wt])		
	S41[b]	8532	209P
4,4'-Diapophytoene	40	60	55
4,4'-Diapophytofluene	3	2	2
4,4'-Diapo-ζ-carotene	6	4	4
4,4'-Diaponeurosporene	12	8	8
4,4'-Diaponeurosporenal	tr		tr
4,4'-Diaponeurosporenoic acid	40	45	65
Glucosyl-diaponeurosporenoate	10	20	25
Staphyloxanthin	360	220	320
Hydroxy-400 compounds	10	5	8

[a] For growth conditions, see Table 2, footnote *c*.
[b] Strain.

DISCUSSION

Much of the earlier work on the chemical nature of the pigments of *S. aureus* is confused and contradictory. Some of the reasons for this are now clear. While it has been generally accepted that the pigments were carotenoids, it was assumed that, like most naturally occurring carotenoids, they possessed a C_{40} chain and that identification was only a matter of matching their electronic absorption spectra with the spectra of known carotenoids. Suzue et al. (33), however, presented clear evidence that the "bacterial phytoene" which they isolated from a strain of *S. aureus* possessed a C_{30} and not a C_{40} structure; our results now show that the whole series of carotenoid-like pigments in *S. aureus* belong to a C_{30} series, none having a C_{40} structure. This triterpenoid carotenoid series was first described and studied by Taylor and Davies (35, 37; Taylor and Davies, 4th Int. Symp. Carotenoids Abstr. Commun.). The compounds were isolated from a yellow-pigmented group D streptococcus (*Streptococcus faecium* UNH564P), and a review of C_{30} carotenoids by Davies (7) also includes information about some members of the series obtained from *S. aureus* 209P (partly based on unpublished data). Triterpenoid carotenoids cannot be distinguished from corresponding tetraterpenoids on the basis of their electronic absorption spectra, the spectra being very similar or identical, and conclusive evidence as to their molecular size must be obtained in other ways, such as measurement of retention times by GLC, measurement of R_f values in appropriate TLC systems, and determination of mass spectra.

A second unusual feature of staphylococcal pigments which has misled other workers is the instability of staphyloxanthin towards acid or alkali. The ester linkage between diaponeurosporenoic acid and glucose is readily split by dilute alkali, yielding the free acid or, in alkaline methanol, its methyl ester. Standard methods for extraction of carotenoids frequently employ alkaline methanol or, alternatively, employ a saponification step early in the purification procedure. Such treatment will rapidly convert staphyloxanthin to either diaponeurosporenoic acid or its methyl ester, which will then be isolated as apparently the main pigment component. Hammond and White in their study of *S. aureus* pigments (10) did consider the possibility that saponification may affect their product but concluded that it did not and that no esters or glycosides were present, since the products obtained had the same mobilities on alumina paper chromatography whether a saponification step was included or not.

Staphyloxanthin proved difficult to purify, being accompanied by glycolipid material in several chromatographic systems, and analyses of earlier samples of what was thought to be pure

material suggested that it contained glycerol as well as hexose and fatty acid. The most successful method of freeing it from glycolipid proved to be TLC on $AgNO_3$-impregnated silica gel.

Carotenoid glycosides have been found in several procaryotes, including *Streptococcus faecium* (35), cyanobacteria (11), and myxobacteria (14, 15), but staphyloxanthin is unusual among natural products in possessing not a glycosidic bond linking glucose to a hydroxy-carotenoid but a glucosyl ester bond linking glucose to a carotenoic acid. There is also evidence that, in addition to the main structure containing glucose, small amounts of isomers containing galactose or mannose are also present. A second glucosyl ester bond links glucose to a fatty acid, predominantly the C_{15} anteiso- acid 12-methyltetradecanoic acid, which is the major fatty acid found in other lipids of *S. aureus* (44); it is also accompanied by some of its C_{17} homolog, 14-methylhexadecanoic acid. A similar fatty acyl glucose linkage was found in the acylated carotenoid glycosides of *Nocardia kirovani* (39) and of some gliding bacteria, including *Stigmatella aurantiaca* (15) and *Herpetosiphon giganteus* (14), the acyl moieties here also consisting of mixtures of fatty acids. The position of attachment to glucose of the carotenoic acid (1-*O*-) and fatty acid (6-*O*-) were suggested by Moss (20) on the basis of nuclear magnetic resonance and other studies. Isostaphyloxanthin was never isolated in sufficient quantity for detailed structural studies, but its properties suggest that it is an isomer of staphyloxanthin, differing from it only in the position of attachment of the two acyl groups. Final confirmation of these structures may have to await the application of appropriate synthetic methods (22).

Triterpenoid carotenoids in several types of gram-positive cocci have been reported and may provide a useful taxonomic feature. However, there are several differences between those found in *S. aureus* and those found in *Streptococcus faecium*. The carotenes are identical and include both 4,4′-diapo-ζ-carotene and its unsymmetrical isomer 4,4′-diapo-7,8,11,12-tetrahydrolycopene and *cis* isomers of 4,4′-diaponeurosporene, which may be artifacts produced during isolation; Davies, however, reported only the first of these in *S. aureus* (7). In *Streptotoccus faecium*, the main xanthophylls are the 4-hydroxy- and 4-glucosyloxy- derivatives of 4,4′-diaponeurosporene, with a trace of the corresponding aldehyde; in *S. aureus*, 4,4′-diaponeurosporenoic acid, its glucosyl ester, staphyloxanthin, and a trace of aldehyde were all identified, but no hydroxy compound was found, nor have we been able to find evidence for a 4′-hydroxy- or a 4′-glucosyloxy- derivative of 4,4′-diaponeurosporenoic acid as postulated by Davies (7). The strain of *S. aureus* used for much of this work (S41) was different from that used by Taylor and Davies (209P), but we also examined their strain, the neotype strain NCTC 8532, and other orange-pigmented strains, and essentially the same carotenoid pattern was found for each. Other strains readily distinguished from the predominant orange type by the different color of their colonies also produced triterpenoid carotenoids but in different proportions or they lacked one or more members of the series. The carotenoid glycoside isolated from two yellow halophilic cocci of uncertain taxonomic position by Aasen et al. (1) appears to be a closely related triterpenoid carotenoid possessing a mannosyloxy- group at one end of the molecule and a carboxymethyl- group at the other, although the methyl ester may be an artifact of the saponification procedure used.

The evidence available at present is not sufficient to permit an unequivocal decision between two possible arrangements of the carbon chain of triterpenoid carotenoids (Fig. 5): the symmetrical 4,4′-diapo- structure proposed by Taylor and Davies (34) or the unsymmetrical 8′-apo- structure proposed by Aasen et al. (1). It is possible, but we consider it unlikely, that both forms may occur naturally. Evidence based on a comparison of samples of our staphylococcal carotenes with synthetic models led Moss to favor the unsymmetrical structure (20), but consideration of their biosynthetic origin favors the symmetrical one (7). 4,4′-Diapophytoene could be formed directly by condensation of two molecules of farnesyl pyrophosphate (C_{15} + C_{15}) analogous to phytoene formation (C_{20} + C_{20}). Formation of 8′-apophytoene, however, would require either the loss of C_{10} from a C_{40} chain or the condensation of two unequal units (C_{20} + C_{10}). The first mechanism would require a C_{40} intermediate, whereas in the second, unless the enzyme possessed very high specificity, some condensation products involving C_{15} would be expected, leading to C_{25} or C_{35} products. Neither in the work described here nor in the work with mutant strains (19) was there any evidence for C_{25}, C_{35}, or C_{40} products and, consequently, until more conclusive evidence is available, we propose to use the symmetrical structure.

The triterpenoid carotenoids of *S. aureus* described here possess structures which suggest they may constitute sequential steps in a pathway for the biosynthesis of staphyloxanthin. Details of this pathway and further evidence in support of it will be presented in the accompanying paper (19).

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