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OCCURRENCE OF DEHYDROSQUALENE (C_{30} PHYTOENE) IN *STAPHYLOCOCCUS AUREUS*

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SUMMARY

1. A mutant of *Staphylococcus aureus* 209 P, which had lost the ability to synthesize colored carotenoids, accumulated a phytoene-like compound. By ultraviolet, infrared, nuclear magnetic resonance and mass spectra, together with chemical analyses, the structure of this compound was established to be 2,6,10,15,19,23-hexamethyl-2,6,10,12,14,18,22-tetracosaheptaene which has a 12,13-*cis* configuration (dehydrosqualene).

2. This compound was also found in the wild strain when the cells were grown in the presence of diphenylamine.

INTRODUCTION

It was reported that a mutant of *Staphylococcus aureus* 209 P lacked the ability to synthesize colored carotenoids and accumulated "bacterial phytoene"[1]. We further reported that the bacterial phytoene was synthesized from [2-^{14}C]mevalonate with cell-free extracts from this mutant[2] and was converted to colored carotenoids[3,4]. In a preliminary report it was shown that the bacterial phytoene is different from phytoene and has the same carbon skeleton as squalane, and further that its molecular formula is $C_{30}H_{48}$ (ref. 5). The present report is concerned with the confirmation of the structure of this compound (Compound X).

MATERIALS AND METHODS

Growth of Microorganisms

Staphylococcus aureus 209 P (IFO No. 3061) was provided by the Institute for Fermentation, Osaka. The isolation of mutant from the wild strain was previously reported[1]. The organisms were grown with continuous shaking for 48 h at 28° on a complex medium containing the following components: peptone, 10 g; beef broth, 10 g; glycerol, 10 g; NaCl, 2 g; Na_2HPO_4, 0.05 g; $MgSO_4$, 100 mg; $MnSO_4$, 100 mg; nicotinamide, 2 mg; thiamine, 0.03 mg; and ammonium molybdate, 1 mg in 1 l of

tap water (pH 7.5). For the growth of the wild strain, $6 \cdot 10^{-5}$M diphenylamine was added to the complex medium. The cells were harvested by centrifugation, washed with a 0.3% aqueous solution of NaCl and stored at −20°.

Solvents

Hexane was purified by successive shakings with conc. H_2SO_4, conc. H_2SO_4–conc. HNO_3(3:1, v/v), conc. H_2SO_4, water and 2% solution of NaOH, then dried on $CaCl_2$ and finally distilled. Ether was treated with *p*-hydroquinone and distilled immediately before use. Other solvents were freshly distilled before use.

Materials

Squalane was purchased from Nakarai Chemicals Ltd., Kyoto. It was treated with ozone in hexane to remove unsaturated compounds and washed with water, dried over anhydrous Na_2SO_4 and purified by passing through a column of alumina. Phytoene was prepared according to the procedure of RABOURN, QUACKENBUSH AND PORTER[6], and further purified by thin-layer chromatography (silica gel G, 1 mm thick) with hexane–ether (99:1, v/v) as developing solvent. The area of silica gel corresponding to phytoene was scraped off and extracted with ether. The ether solution was evaporated to dryness *in vacuo*.

Extraction and purification of Compound X

To the cells of mutant (wet wt. 100 g) were added 200 ml of 75% methanol, 20 g of NaOH and 10 g of pyrogallol. The mixture was heated for 30 min at 80–85°. After cooling, it was diluted with water and the unsaponifiable material was extracted 3 times with hexane. The yellow hexane extracts were washed 5 times with distilled water and dried over anhydrous Na_2SO_4. The solution was concentrated under reduced pressure. The concentrated crude extracts were chromatographed on a 1.6 cm × 15 cm column of permutit (60–80 mesh) with hexane as solvent. The elution of Compound X was monitored spectrophotometrically. The combined fractions of Compound X were concentrated and rechromatographed on a 1.6 cm × 10 cm column of activated alumina (200–300 mesh, activity grade I). It was eluted with 40 ml of hexane, 40 ml of hexane–ether (99:1, v/v) and finally with 60 ml of hexane–ether (98:2, v/v). Compound X was eluted with the last solvent. Since Compound X is liable to decomposition on exposure to air and light, all procedures were performed in subdued light and under N_2. The average yield was approx. 15 mg from 100 g of wet cells. The yield was reduced after the cells were stored for several months at −20°. The extraction procedure and the purification of the lipid from the wild strain were the same as mentioned above.

Preparation of perhydro Compound X

Compound X was dissolved in isopropanol–glacial acetic acid (1:1, v/v) and was completely hydrogenated using platinum black as catalyst. The perhydro Compound X thus obtained was passed through a column of activated alumina using hexane as solvent.

Measurement of spectra

The ultraviolet spectra were measured with a Hitachi recording spectrophoto-

meter, Model 124. The infrared spectra were recorded on a Perkin–Elmer recording infrared spectrophotometer, Model 621, with a KRS-5 window plate. The mass spectra were run with a Hitachi mass spectrometer, Model RMU-6. The nuclear magnetic resonance spectra were run in C^2HCl_3 with tetramethyl silane as the internal reference at 60 Mcycles/sec using a JEOL 3H60 spectrophotometer. Phytoene (approx. 20 mg) and Compound X (approx. 10 mg) were dissolved in 0.5 ml of C^2HCl_3, respectively. The number of protons was calculated with an electron integrator.

Dehydrogenation

Compound X (approx. 3 mg) was dehydrogenated with *N*-bromosuccinimide by the method of ZECHMEISTER AND KOE[7]. The products were chromatographed on a 2.2 cm × 15 cm column of $Ca(OH)_2$ using hexane as solvent and the elution was monitored spectrophotometrically.

Isomerization

Compound X was dissolved in hexane and was isomerized under ultraviolet light in the presence of iodine (2% of the weight of Compound X) for 5 min in a stoppered quartz cell. The ultraviolet spectra were measured before and after isomerization.

Thin-layer chromatography of the lipid from the mutant and the wild strain

After purification on a column of activated alumina, the fractions with phytoene-like absorption spectra were combined, concentrated and co-chromatographed on thin-layer plates. Samples and the authentic phytoene were spotted in hexane on 5% paraffin-impregnated thin-layer plates (silica gel G, 300 μ thick) and developed with acetone–water (95:5, v/v) saturated with paraffin. The developed chromatograms were sprayed with a 0.5% aqueous solution of $KMnO_4$ and the yellow spots appeared after a few minutes.

RESULTS AND DISCUSSION

Ultraviolet spectra

Compound X shows almost the same absorption spectrum as that of phytoene and has the following absorption maxima; 276, 286 ($E^{1\%}_{1\,cm}$, 1050 in hexane) and 298 mμ. Therefore, Compound X is not distinguished from phytoene by the ultraviolet spectral curves. However, the extinction coefficient of Compound X at 286 mμ is higher than that of phytoene (phytoene: $E^{1\%}_{1\,cm}$, 850).

NMR spectra

Compound X has seven ethenoid bonds, three of which proved to be conjugated bonds as judged from the ultraviolet spectrum, and perhydro Compound X is identical with squalane, as described in the next section. Therefore, the problem of the location of the seven ethenoid bonds in Compound X remained to be solved. NMR spectra of Compound X and phytoene are shown in Fig. 1. Both are quite similar to each other, and differences are observed only in the relative number of protons at each signal. These facts allow the assignments as listed in Table I. Although there was overlapping of the signals at 8.41 and 8.34 τ, careful estimation of the relative inten-

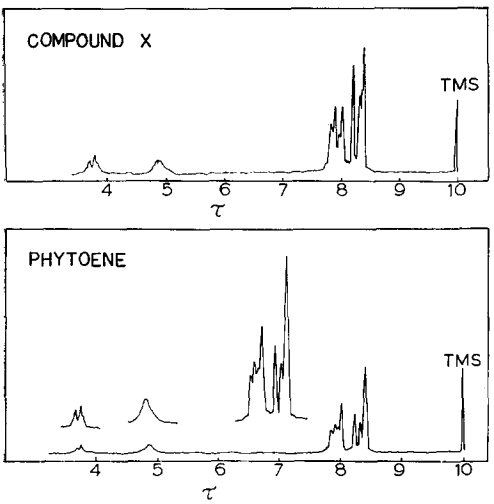

Fig. 1. NMR spectra of Compound X and phytoene in C^2HCl_3. TMS, trimethyl silane.

sities of each band showed that 6 protons (2 methyls) and 12 protons (4 methyls) are distributed to the peaks of 8.34 and 8.41 τ, respectively. Therefore, apart from the two methyls of isopropylidene end-groups, there is no methyl *cis* to the olefinic proton in the molecule of Compound X. The probability that the triene structure of

$$\begin{matrix} & CH_3 & & & CH_3 & \\ & | & & & | & \\ -C & = & CH-CH & = & C-C & = \end{matrix}$$

Compound X may be is ruled out from the result of mass spectrum.

TABLE I

ASSIGNMENT OF SIGNALS IN NMR SPECTRA OF COMPOUND X AND PHYTOENE

*Proton**	*τ-Value*	*Compound X*		*Phytoene*	
		Number of protons theory	*Number of protons observed*	*Number of protons theory*	*Number of protons observed*
$\begin{matrix} H \\ R \end{matrix}\!\!>\!C\!=\!C\!<\!\begin{matrix} CH_3 \\ CH_3 \end{matrix}$	8.41 (singlet)	12	18.2	18	24.2
$\begin{matrix} H \\ R \end{matrix}\!\!>\!C\!=\!C\!<\!\begin{matrix} CH_3 \\ CH_3 \end{matrix}$	8.34 (singlet)	6		6	
$-\overset{CH_3}{\overset{\vert}{C}}= CH-CH = CH-CH = \overset{CH_3}{\overset{\vert}{C}}-$	8.24 (singlet)	6	6.3	6	6.1
$=C-CH_2-CH_2-C=$	7.85–8.02 (multiplet)	16	16.2	24	24.2
$-CH=$	4.86 (broad)	4	3.7	6	5.7
$=CH-CH=CH-CH=$	3.70 (singlet) 3.76 (singlet)	4	3.7	4	3.8

* The protons under consideration are in italics.

Mass spectra

Mass spectra of Compound X and perhydro Compound X are shown in Fig. 2. The mass spectrum of perhydro Compound X is in complete agreement with that of squalane. In the case of Compound X, the peak at *m/e* 408 is the parent peak corresponding to $C_{30}H_{48}$. The peaks at *m/e* 339 (M-69) and *m/e* 271 (M-69-68) are explained as being derived from the successive loss of C_5H_9 and C_5H_8 units from the terminal of

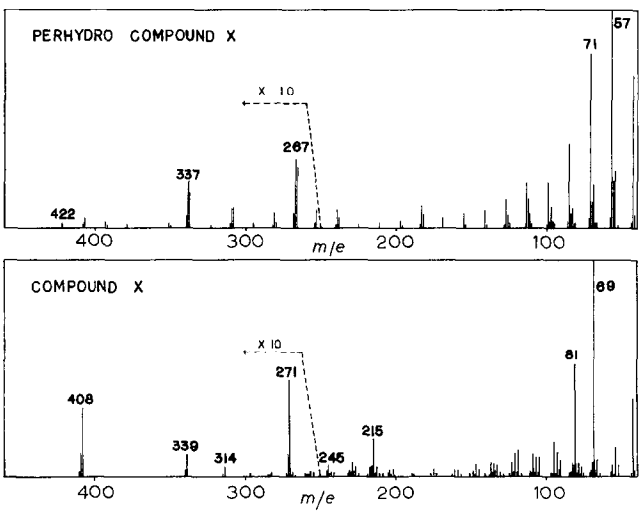

Fig. 2. Mass spectra of perhydro Compound X and Compound X.

the molecule. The peaks at *m/e* 314 (M-94) and *m/e* 245 (M-163) cannot be explained by a cleavage of the molecule from the terminal. In the case of more unsaturated carotenoids, M-92 and M-106 peaks are generally seen, due to the *cis*-oid rearrangement of the conjugated polyene chain[8]. It is clear that the M-94 peak of Compound X corresponds to the M-92 peak of the former compounds. Therefore, the peaks at *m/e* 314 (M-94) and *m/e* 245 (M-94-69) are probably caused by the fragmentation shown in Fig. 3.

Dehydrogenation with N-bromosuccinimide

When Compound X was treated with *N*-bromosuccinimide, two products, A and B, were obtained. Absorption maxima of A and B are as follows: A: 332, 348 and 376 mμ; B: 377, 398 and 423 mμ. These absorption spectra of A and B are very

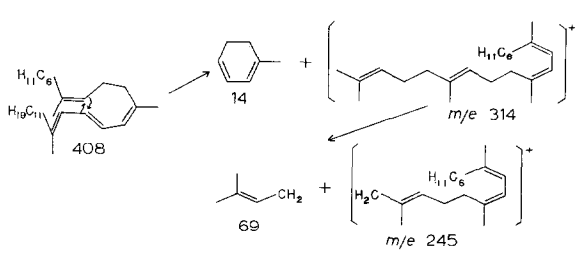

Fig. 3. Explanation of fragmentation of mass spectrum of Compound X.

similar to those of phytofluene (λ_{max}: 322, 348 and 378 mμ) and ζ-carotene ($\lambda_{max.}$ 378, 399 and 424 mμ), respectively.

Configuration of conjugated triene

As in the case of phytoene, the extinction value was increased and the shape of the absorption curve was caused by isomerization of Compound X. These facts suggest that the configuration of the conjugated triene changed from *cis* to *trans* by isomerization[9]. In addition, the presence of a strong band at 13.05 μ and the absence of a strong band at 10.2 μ in the infrared spectrum of Compound X indicate that the conjugated triene has a *cis* configuration[9].

The results mentioned above indicate that Compound X has the structure shown in Fig. 4, that is, this compound is dehydrosqualene (C_{30} phytoene).

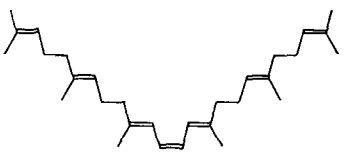

Fig. 4. The structure of Compound X.

Occurrence of dehydrosqualene in the wild strain

The lipid fraction extracted from the wild strain which had a phytoene-like absorption curve was co-chromatographed with C_{30} phytoene and phytoene by reverse-phase thin-layer chromatography. The typical R_F values were as follows: dehydrosqualene, 0.46; phytoene, 0.30; lipid fraction, 0.46. The lipid fraction gave a single spot and no spot was observed in the area corresponding to the R_F value of phytoene. It is certain that the amount of phytoene, even if present, is less than 1% of that of dehydrosqualene.

CHARGAFF AND DIERYCK[10] reported in 1932 the occurrence of zeaxanthin in *S. aureus*[10]. Later, SOBIN AND STAHLY[11] detected δ-carotene and rubixanthin in this organism. These reports may suggest that dehydrosqualene is a precursor of the C_{40} colored carotenoids. However, since the identifications of these colored carotenoids were based mainly on spectral data in the visible region, the evidence is not conclusive. Our recent investigation suggests the possibility that these colored carotenoids of *S. aureus* have a C_{30} skeleton. Further studies on their structure are now in progress.

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