

Bacterial Carotenoids

XXIX.* The Carotenoids of two Yellow Halophilic Cocci — Including a New Glycosidic Methyl Apo-lycopenoate

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The carotenoid composition of two yellow, halophilic cocci is reported.

A crystalline apo-carotenoid isolated from strain SE20-4, has been characterized by spectral properties including mass spectra, formation of derivatives and glycoside hydrolysis. It is considered to be the tertiary glycoside methyl 1-hexosyl-1,2-dihydro-3,4-didehydro-apo-8'-lycopenoate (*I*). The R_F -value of the sugar component corresponded to that of mannose. Observations on the biosynthesis of *I* are discussed.

Whereas carotenoids of red halophilic bacteria have attracted considerable interest,¹⁻⁴ those of the yellow halophilic bacteria have been less closely examined.² Four years ago we investigated the carotenoids of two yellow halophilic cocci,⁵ first studied by Baxter.² A new apo-carotenoid was isolated. Further spectroscopic data and glycoside hydrolysis have now permitted the structural assignment of this compound.

RESULTS AND DISCUSSION

Strain H5B-2

The carotenoid content (0.018 % of total), consisted of neurosporene (81 %), a lycopene-like compound (4 %) and a dihydroxy- ζ -carotene-like compound (15 %) in agreement with earlier findings.² Neurosporene was identified by direct comparison with an authentic specimen including absorption spectrum in visible light, R_F -values, and stereomutation behaviour (five stereoisomers).

* Part XXVIII. *Acta Chem. Scand.* 23 (1969) 1057.

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Strain SE20-4

The carotenoid synthesis depended quantitatively and qualitatively on the aeration of the culture. When vigorous aeration was employed (maximum yield 0.007 % carotenoid of the dry weight) a new esterified apo-carotenoic acid (64 % of total) and its corresponding alcohol (13 %) were produced, in addition to some minor carotenoids. Neither of the main carotenoids were mentioned by Baxter.²

After mild alkali treatment of the crude extract the ester (*I*) was purified by repeated chromatography and fractional crystallization; total yield 1.5 mg, m.p. 182°C. Judged by R_F -values and partition ratios the natural ester (*I*) was a strongly polar compound. Kinetic acetylation experiments showed the presence of six intermediary acetates in the formation of the peracetate (*2*), hence revealing the presence of three or more primary or secondary hydroxyl groups in *I*. None of the hydroxyl groups appeared to be in allylic position to the polyene chain since oxidation with *p*-chloranil⁶ of the ester *I* and the free acid *3* (see below) failed.

Saponification of the natural ester (*I*) under strong conditions gave the corresponding acid (*3*) with a pK_a -value of *ca.* 4.2; yield 0.8 mg, m.p. 222–228°C. The acid (*3*) was chromatographically very strongly adsorbed, exhibited spectral fine-structure in methanol (Fig. 1) characteristic of conjugated car-

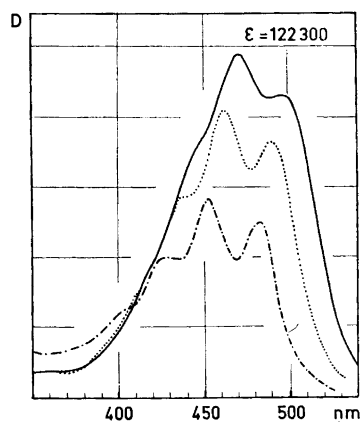


Fig. 1. Absorption spectra in visible light of — the natural ester (*I*) in acetone, the acid (*3*) in methanol, and - · - · - the corresponding alcohol (*5*) in ether.

boxylic acids⁷ and could not be reduced with sodium borohydride. Methylation with diazomethane gave a product indistinguishable from natural *I*. The peracetate (*4*) of the acid (*3*) was prepared.

The natural ester (*I*) was resistant to treatment with sodium borohydride, but treatment with lithium aluminium hydride caused a hypsochromic shift and gave an allylic alcohol (*5*). The same product (*5*) was obtained on similar reduction of *4* and *2*. Like other primary polyenols⁸ the allylic alcohol (*5*) provided no product with extended chromophore on treatment with acidified chloroform. The chemical reactions carried out are summarized below:

28 H, in-chain methyl and acetate methyl); 8.72 and 8.77 (strong, *gem.*-dimethyl at *tert.* oxygen + lipid contaminant). Signals in the regions τ 8.19 (end-of-chain methyl) and τ 8.3 and 8.9–9.1 (alicyclic rings) were absent.

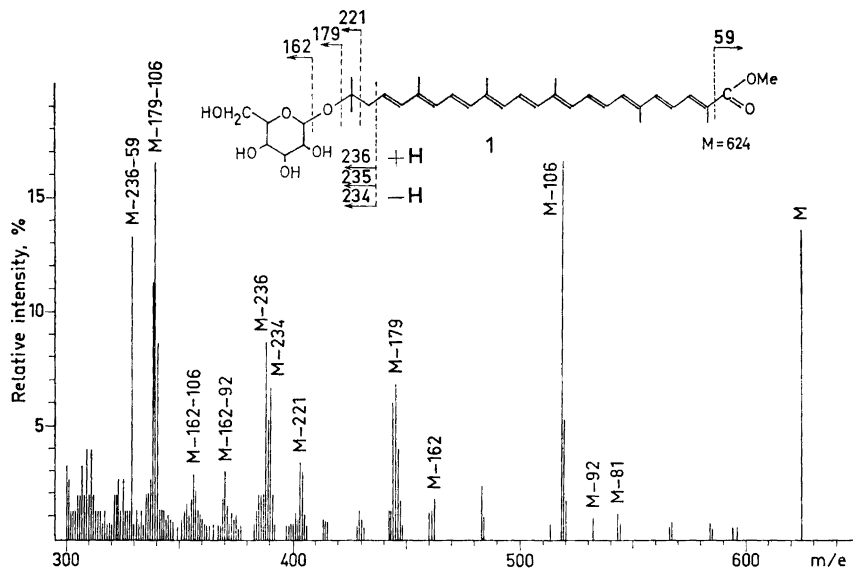


Fig. 3. Mass spectrum of the natural ester (1).

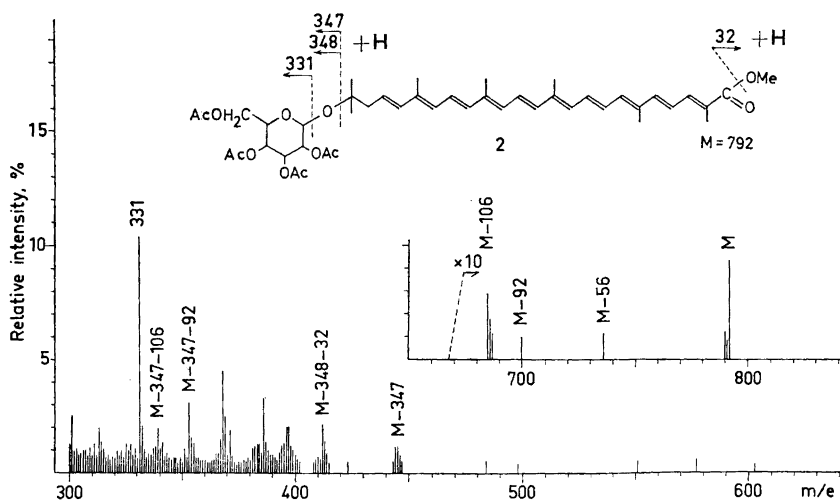
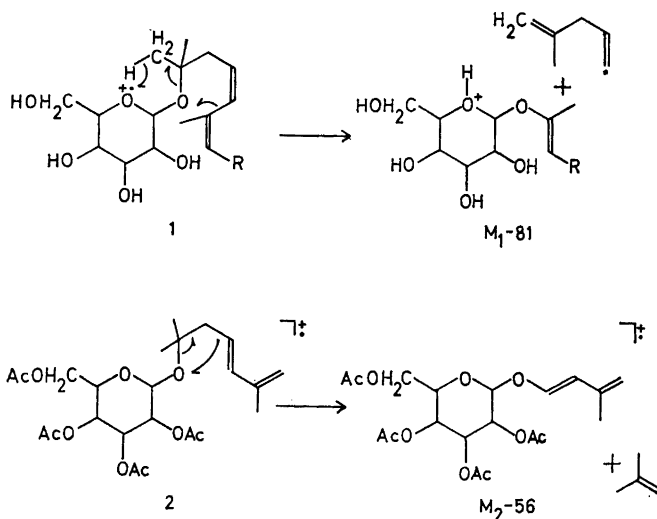


Fig. 4. Mass spectrum of the tetraacetate (2) of the natural ester.

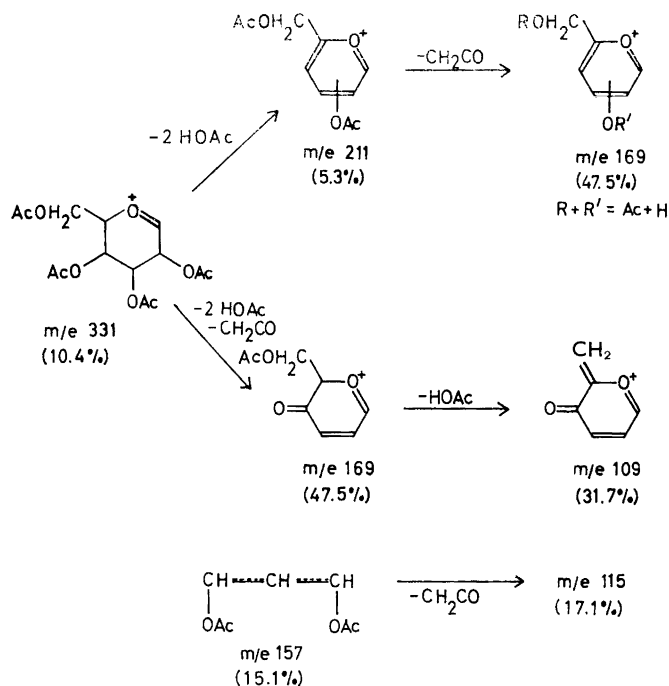
Further confirmation was obtained from the mass spectra of *1* and *2* (Figs. 3 and 4). The diagnostically important fragmentations are indicated on the respective figures. The assignment of the molecular ion of *1* at m/e 624 (corresponding to $C_{37}H_{52}O_8$) was confirmed by the characteristic losses of 92 and 106 mass units therefrom.¹³⁻¹⁵ In the upper part of the spectrum several peaks assigned to fragmentation of the end group containing the sugar residue are observed. Cleavage of the glycoside bond with hydrogen transfer from the charged aglycone gives rise to the $M-162$ peak. Prominent ions correspond to fracture of the carotenoid-oxygen ether bond ($M-179$), the C_1-C_2 bond ($M-221$) and the C_2-C_3 bond with charge retention on the carotenoid moiety. In the latter case cleavage without and with hydrogen transfer in both directions occurs, peaks at $M-234$, $M-235$, and $M-236$. The only evidence for the carbomethoxy group in the spectrum of *1* is an ion at m/e 329, explained as loss of 59 mass units from the $M-236$ ion. A low intensity ion at $M-81$ could be explained by elimination of the saturated end of the carotenoid molecule as indicated in Scheme 1.



Scheme 1.

The peracetate (*2*) of the natural ester showed only peaks of low intensity in the upper mass range. However, the molecular ion, now at m/e 792 (corresponding to $C_{45}H_{60}O_{12}$), is again confirmed by observed losses of 92 and 106 mass units. Hence the mass spectrum confirms the formulation of the peracetate (*2*) as a tetraacetate. Cleavage of the carotenoid-oxygen ether linkage gives rise to $M-347$ and $M-348$ ions. The loss of 32 mass units from the $M-348$ ion provides some support for the existence of the carbomethoxy group. A prominent ion at m/e 331 is ascribed to cleavage of the glycosidic linkage with charge retention on the sugar moiety. Such an ion was previously observed by Biemann, De Jongh and Schnoes¹⁶ in work on hexose acetates. They

showed that further fragmentation of this ion led to a series of characteristic ions formed by combined losses of ketene and acetic acid. Scheme 2 is that of Biemann *et al.*,¹⁶ and the values in brackets give the relative intensities of the corresponding peaks in our spectrum of 2. The high values for these intensities provide support for the formulation of 2 as a hexoside with four acetoxy groups in the sugar moiety.



Scheme 2.

The weak M—56 ion deserves comment since it normally occurs when α -ring structures are present.^{13,15} This ion may here be formed by a cyclic mechanism involving loss of the C₁—C₂ fragment, see Scheme 1.

At this stage only an air-bleached specimen of the peracetate (2) was available for glycoside hydrolysis, carried out in HCl-methanol.¹⁷ The resulting methyl glycoside gave the corresponding reducing sugar on further hydrolysis according to Painter.¹⁸ Our methyl glycoside and reducing sugar preparations both gave positive Molisch tests¹⁹ for carbohydrate. On paper chromatography the reducing sugar gave a single spot with R_F -value in two systems corresponding to that of mannose. The colour and fluorescence of the spot, developed with aniline phthalate,²⁰ deviated somewhat, and further sample would be required to establish the identity of the hexose unambiguously.

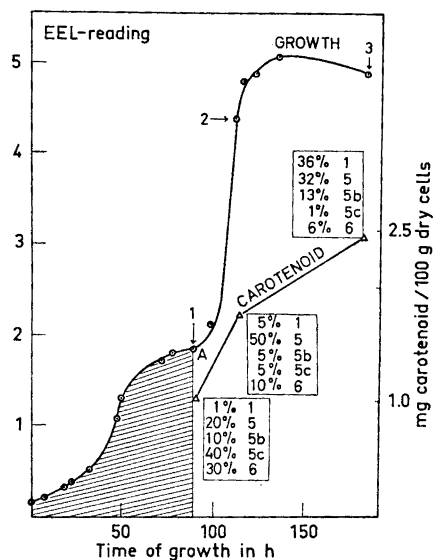
The allylic alcohol (5) obtained on hydride reduction of 2 had properties corresponding to a second carotenoid present in strain SE20—4, to which

structure 5 is tentatively ascribed. Additional minor carotenoids were not satisfactorily characterized.

Carotenoid glycosides hitherto known are crocin which is a digentiobiose ester,²¹ the tertiary β -D-glucosides phlei-xanthophyll and 4-keto-phlei-xanthophyll¹¹ and the rhamnosides myxoxanthophyll¹⁷ and oscillaxanthin.¹² Indications of other carotenoid glycosides also exist.^{22,23}

One culture (culture 1) was grown aiming at investigating the effect of aeration on the pigment synthesis. The growth curve and periodic carotenoid analysis of culture 1 (Fig. 5) revealed that carotenoid synthesis continued

Fig. 5. Growth curve of culture 1 strain SE20-4 measured by optical density (EEL-reading) and carotenoid content of dry cells. Carotenoid components in per cent of total carotenoid are included. Numbers denote withdrawal of samples 1, 2, 3 for carotenoid analyses. Shaded area: low aeration. Aeration increased at stage A.



after the late exponential phase of growth, during which period the glycosidic apo-ester (1) was predominantly produced. The synthesis of the natural decaenol, believed to be the glycoside 5, preceded that of 1, and is a tentative precursor of 1. Both glycosides (1 and 5) were only minor components in cells grown with limited oxygen supply. Whereas oxygen may play a more direct role in the synthesis of the carbomethoxy group, *cf.* Ref. 7, it presumably plays an indirect role in the glycosidation step. Pigments 5b and 5c encountered in cultures grown with limited oxygen supply appear to be non-glycosidic decaene carotenoids, probably chemically related to 5, and pigment 6 a hydroxy-neurosporene-like carotenoid different from chloroxanthin.

EXPERIMENTAL

Materials and methods. When not stated to the contrary these were as summarized elsewhere.²⁴

Cultures. Two yellow halophilic cocci strain H5B-2 and strain SE20-4,² obtained from Dr. R. M. Baxter, Division of Applied Biology, National Research Council, Ottawa, Canada, were used.

Medium and cultural conditions. The medium contained 15 % NaCl, 0.25 % trypton (Difco), 0.5 % $MgSO_4 \cdot 7H_2O$, 0.1 % KCl, 0.02 % $CaCl_2 \cdot 6H_2O$ and 0.5 % yeast extract (Difco) in tap water. Cultures were grown in diffuse light or darkness at 37°C. The subinoculum was grown in shaken flasks and the inoculum in aerated Erlenmeyer flasks. Mass cultures were grown in a fermentation tank of 190 l capacity. Cell growth was followed by optical density measurements and cultures harvested by centrifugation when the stationary growth phase was reached.

H5B-2. A 30 l aerated culture was harvested after 141 h growth, yield 9 g dry cells.

SE20-4. Culture 1 (67 l) was grown with low oxygen supply for 90 h (sample 1, 30 l, 8 g dry cells withdrawn), then with strong aeration (sample 2, 9 l, 13 g dry cells withdrawn after 114 h) and harvested after 186 h (sample 3, 28 l, 47 g dry cells). An apparently stationary phase of growth after 90 h was overcome by strong aeration; see Fig. 5.

Culture 2 (180 l) grown with strong aeration was harvested after 64 h, yield 222 g dry cells.

Isolation of the carotenoids. Centrifuged cells in water (1:1) were broken by treatment with ballotini beads in a cell homogenizer at 2°C and extracted with acetone at room temperature. The pigments were transferred to ether, saponified in 5 % methanolic KOH-solution for 2 h, chromatographed and crystallized in standard manner.²⁴ Adsorption properties pertaining to SE20-4 are compiled in Table 1.

Table 1. Adsorptive properties of the *trans* isomers of the carotenoids of strain SE20-4 and derivatives thereof.

| Compound | Required eluent from cellulose column | R_F -value | | |
|------------------------|---------------------------------------|---------------------------|---------------------------|---------------------------|
| | | Kieselguhr paper | | Aluminium oxide paper |
| | | 20 % acetone ^a | 30 % acetone ^b | 10 % acetone ^a |
| Natural ester (1) | 50 % acetone ^a | 0.01 | 0.34 | |
| Ester tetraacetate (2) | 15 % acetone | 0.75 | | |
| Acid (3) | 50-100 % acetone | | 0 | |
| Allylic alcohol (5) | 20 % acetone | | 0.22 | 0 |
| 5b | | | 0.99 | 0.52 |
| 5c | | | 0.99 | 0.83 |
| 6 | | | 0.99 | 0.95 |

^a In petroleum ether.

^b In benzene.

Strain H5B-2

A total of 0.97 mg carotenoids (10.8 mg/100 g dry cells) isolated after saponification, column (alumina) and paper chromatography, was estimated to be 81 % neurosporene, 4 % lycopene-like and 15 % di-hydroxy- ζ -carotene-like compounds. Neurosporene had abs.max. at 414, 438, and 467 nm in petroleum ether, $\% III/II=106$. Co-chromatography with authentic neurosporene gave a single zone ($R_F=0.72$ on kieselguhr paper in petroleum ether). Separate iodine catalyzed isomerization produced the same four *cis* isomers: $R_F=0.12$ (neo U ca. 10 %), 0.23 (*trans* ca. 70 %), 0.27 (neo A ca. 5 %), 0.30 (neo B ca. 5 %) and 0.35 (neo C ca. 10 %) on aluminium oxide paper (petroleum ether) in each case. Due to oily contaminants the lycopene-like compound was not satisfactorily characterized. The di-hydroxy- ζ -carotene-like compound had abs.max. 379, 396, and 422 nm in petroleum ether, $R_F=0.25$ on aluminium oxide paper (10 % acetone in petroleum ether) and partition ratio in petroleum ether/85 % methanol 4:6.

Strain SE20-4

Culture 1. The growth curve (measured by optical density in an EEL colorimeter, supplied with filter 608) and carotenoid synthesis are depicted in Fig. 5. The pigment extract of the three cell samples were saponified with 20 % methanolic KOH solution to facilitate the estimation of *I*.

Sample 1 contained 0.084 mg carotenoid (1.05 mg/100 g dry cells) consisting of *I* (trace), *5* (20 %), two less polar compounds with the same chromophore as *5*, *5b* (10 %) and *5c* (40 %), a hydroxy-neurosporene-like compound *6* (30 %), chromatographically different from chloroxanthin, and two minor compounds.

Sample 2 contained 0.23 mg carotenoid (1.77 mg/100 g dry cells) consisting of *I* (5 %), *5* (50 %), *5b* (5 %), *5c* (5 %), *6* (10 %) and three minor compounds.

Sample 3 contained 1.15 mg carotenoid (2.45 mg/100 g dry cells) consisting of *I* (36 %), *5* (32 %), *5b* (13 %), *5c* (trace), *6* (6 %) and four minor compounds.

Culture 2. The total carotenoid content of the culture, determined on aliquots, roughly followed the growth curve. The carotenoid content at harvest was 6.6 mg/100 g dry weight, yield 14.7 mg, consisting of *I* (64 % of total), *5* (13 %) and some minor components not further studied.

Methyl 1-hexosyl-1,2-dihydro-3,4-didehydro-*apo*-8'-lycopenoate (*I*)

Isolation. After mild alkali treatment²⁴ of the crude extract (resulting in partial saponification of *I* to give *3*) the ester (*I*) was purified by repeated chromatography on cellulose columns. Colourless contaminants were removed by precipitation from acetone and *I* was crystallized from acetone-petroleum ether; yield 1.5 mg, m.p. 182°C. The absorption spectrum in visible light had abs.max. at 469 ($\epsilon=122\ 300$, cf. 122 700 for natural methyl *apo*-6'-lycopenoate⁹) and 497 nm in acetone (Fig. 1). Its IR-spectrum (KBr) is given in Fig. 2 and the mass spectrum in Fig. 3. *I* had partition ratio in petroleum ether/95 % methanol 1:99 and in petroleum ether/60 % methanol 9:91.

Standard treatment of *I* (0.1 mg) with *p*-chloranil for 30 h gave no product with extended chromophore.

I (0.1 mg) gave no product with extended chromophore on standard treatment with HCl-chloroform; pigment recovery was 65 %.

I (0.1 mg) in ethanol was treated with sodium borohydride for 90 min, pigment recovery was 70 %. No reduction product was formed.

*Methyl 1-tetraacetylhexosyl-1,2-dihydro-3,4-didehydro-*apo*-8'-lycopenoate (2).* The course of acetylation of *I* (0.15 mg) in dry pyridine (1 ml) with acetic anhydride (0.2 ml) was followed by paper chromatography. Six intermediary acetates with $R_F=0.07, 0.13, 0.24, 0.38, 0.47,$ and 0.58 (kieselguhr paper, 20 % acetone in petroleum ether) were observed. In a larger scale experiment crystalline *I* (0.6 mg) was acetylated. The peracetate (*2*) exhibited the same absorption spectrum in visible light as *I*. Its mass spectrum, (Fig. 4), recorded with an LKB-9000 instrument and NMR-spectrum in CDCl_3 at 100 MC/sec were obtained. Signal positions are quoted under Results. *2* had partition ratio in petroleum ether/85 % methanol 18:82, in a parallel experiment found for *azafrin* methyl ester 15:85 in the same system.

An air-bleached sample of crystalline *2* (0.4 mg) was hydrolysed in 0.15 N HCl-methanol.¹⁷ The resulting methyl glycoside was hydrolyzed with polystyrene sulphonic acid.^{17,18} The Molisch test¹⁹ was positive for the methyl glycoside and reducing sugar preparations. The reducing sugar was examined by paper chromatography (Schleicher & Schüll No. 2043B) with mannose, glucose, galactose, and xylose as reference standards. The chromatograms were developed with aniline phthalate²⁰ giving brown spots with white fluorescence in UV-light for hexoses. The following R_{Gluc} -values were observed in system 5¹⁷ (benzene-butanol-pyridine-water 1:5:3:3, ascending method) galactose 0.90, glucose 1.00, mannose 1.06, hydrolysate 1.05, xylose 1.24 and in system 6²⁵ (pyridine-ethyl acetate-water 2:5:7, epiphase, descending method) galactose 0.84, glucose 1.00, mannose 1.23 and hydrolysate 1.23.

*1-Hexosyl-1,2-dihydro-3,4-didehydro-*apo*-8'-lycopenoic acid (3).* *3* was obtained by saponification of the natural ester (*I*) or its tetraacetate (*2*), using 10 % KOH in methanol-

ether over-night or 20 % KOH methanol-ether for 4 h at 35°C. Parallel experiments with azafurin methyl ester and torularhodin methyl ester resulted in quantitative hydrolysis under the latter condition, whereas saponification with the usual 5 % KOH solution in all cases only resulted in partial hydrolysis. **3** required acidification of the hypophase to pH 4.2 in order to be transferred to ether (*cf.* $pK_a=4.26$ for acrylic acid).²⁶ The free acid (**3**) was purified by chromatography on cellulose columns and crystallized from methanol-acetone-petroleum ether; total yield *ca.* 0.3 mg, m.p. 222–228°C. **3** had abs.max. (440), 466, and 491 nm in acetone and (435), 460, and 490 nm in methanol (Fig. 1).

3 (0.12 mg) in ethanol was not reduced by sodium borohydride, and **3** (0.38 mg) gave no oxidation products on treatment with *p*-chloranil.

3 (1.3 mg) in methanol (15 ml) was methylated with diazomethane in ether. The methylation product (**1**) had abs.max. 469 and 497 nm in acetone, partition ratio in petroleum ether/60 % methanol 9:91 and R_F -value identical with natural **1**. The methylation product (**1**) was submitted to standard acetylation. The peracetate (**2**) had partition ratio in petroleum ether/85 % methanol 20:80 and was not separated from **2**, prepared from natural **1**, when co-chromatographed.

1-Tetraacetylhexosyl-1,2-dihydro-3,4-didehydro-apo-8'-lycopenoic acid (**4**). **3** (0.1 mg) was acetylated in the usual manner in order to allow dissolution in ether for subsequent reduction with lithium aluminium hydride to **5**.

1-Hexosyl-1,2-dihydro-3,4-didehydro-apo-8'-lycopenol (**5**). **5** was prepared by reduction with lithium aluminium hydride in ether of **4**, **1**, and **2**, total yield 1.1 mg. Oily contaminants present in the mother liquors used for these experiments could not be removed by column chromatography (cellulose) and crystallization of **5** failed. The absorption spectrum in visible light had abs.max. at 427, 452, and 482 nm in ether (Fig. 1).

Standard treatment of **5** (0.1 mg) with HCl-chloroform gave 39 % pigment recovery and no less polar products.

Natural 1-hexosyl-1,2-dihydro-3,4-didehydro-apo-8'-lycopenol (**5**)

Isolation. This pigment was isolated by ether extraction of the alkaline hypophase after saponification under strong conditions of the crude extract of culture **2**, yield *ca.* 1 mg non-crystalline compound. The absorption spectrum in visible light was identical with that of synthetic **5** above (Fig. 1), and natural and synthetic **5** were inseparable in co-chromatography tests on kieselguhr paper.

Acknowledgements. The mass spectra were obtained by the courtesy of Dr. O. Isler Hoffmann-La Roche, Basel, and Dr. C. Enzell, Swedish Tobacco Co., Stockholm, and the NMR-spectrum by Dr. L. Phillips, Imperial College, London. S.L.J. acknowledges a grant for G.W.F. from *Norges Almenvitenskapelige Forskningsråd*.

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Received February 17, 1969.