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Carotenoid Pigments of *Stigmatella aurantiaca (Myxobacterales)*

II. Acylated Carotenoid Glucosides

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Summary. The two main carotenoids of *Stigmatella aurantiaca* were identified as 1′,2′-dihydro-1′-hydroxy-3,4-dehydro-torulene glucoside (myxobactin) and 1′,2′-dihydro-1′-hydroxy-4-keto-torulene glucoside (myxobacton). Both pigments occur as monoesters of various fatty acids. The structural formulas were established by chemical and chromatographical analysis and by visible, infrared, and mass spectroscopy.

In a recent communication we reported on 9 minor carotenoids of the myxobacterium *Stigmatella aurantiaca*, namely the acyclic compounds phytoene, phytofluene, lycopene, 1,2,1′,2′,-tetrahydro-1,1′-dihydroxy-lycopene, and the monocyclic structures γ-carotene, 4-keto-γ-carotene, 1′,2′-dihydro-1′-hydroxy-γ-carotene, 1′,2′-dihydro-1′-hydroxy-4-keto-γ-carotene, 1′,2′-dihydro-1′-hydroxy-4-keto-torulene (Kleinig and Reichenbach, 1969). The present paper deals with the structural determination of the two major carotenoids which were tentatively identified as inositol ethers (Kleinig and Reichenbach, 1970). Both pigments, however, occur as tertiary glucosides acylated in the glucose moiety with fatty acids, as will be shown in this communication. The fatty acid free compounds have been named myxobactin (formula XI) and myxobacton (formula III).

In the past few years carotenoid glycosides have been described from a variety of bacteria (Smith, 1963; Hertzberg and Liaaen Jensen, 1967; Prebble, 1968; Aasen *et al.*, 1969; Weeks, 1970) and blue green algae (Hertzberg and Liaaen Jensen, 1969a, b; Francis *et al.*, 1970). The structures of myxobactin and myxobacton, however, have not been previously found in nature. To our knowledge this is also the first description of acylated carotenoid glycosides.

Material and Methods

Part of the studies was done on myxobacton isolated from *Myxococcus fulvus*, strain *Mx f2*, which could be cultivated with better yields than *S. aurantiaca*.

Culture conditions, pigment extraction, thin layer chromatography (TLC), and chemical reactions have been described before (Kleinig and Reichenbach, 1969). In addition the following methods were employed.

Infrared Spectroscopy. Spectra were measured in chloroform using NaCl cells with a Perkin Elmer double beam infrared spectrophotometer.

Gas-Liquid Chromatography. Fatty acids were determined by gas-liquid chromatography after hydrolysis and methylation with diazomethane as reported elsewhere (Schröder and Reichenbach, 1970).

Identification of Glucose. The saponified carotenoid glucosides were hydrolyzed with methanolic HCl (5 ml methanol plus 1 ml concentrated HCl) for 30 min at 60° C (Prebble, 1968). Glucose was identified by TLC on cellulose with the solvent system *n*-butanol-pyridine-water (6:4:2, v/v/v) by comparison with reference substances. For detection an anisidine phthalate spray was used. Glucose was also proved by a D-glucose oxidase test.

Enzymatic Hydrolysis of Myxobacton. To an ethanolic solution of myxobacton 0.1 M acetate buffer (pH 6.0) and α- or β-glucosidase were added (final ethanol concentration about 30%). Incubation was performed at 30° C for 30 min. No hydrolysis was achieved.

Production of Glucose Free Carotenoids. By treatment with strong alkali (in a solution of sodium in 96% ethanol) about 5% of the employed material was obtained as glucose free carotenoid. From the remainder only the fatty acids were removed. The yields of free carotenoid could be increased to about 40% by heating the reaction mixture for several hours at 60° C. Also treatment with HCl-methanol as described above resulted in free carotenoids.

Allylic Oxidation with p-Chloranil. The oxidation of allylic hydroxyl functions to the corresponding ketones was performed with *p*-chloranil, iodine, and artificial sodium light according to Liaaen Jensen (1963).

Acetylation with Acetic-1-C14 Anhydride. To a few hundred micrograms of myxobacton in 1 ml pyridine about 5 μCi of acetic-1-C14 anhydride (29.7 mCi/mM, The Radiochemical Center Amersham) were added. The mixture was incubated for various lengths of time. Activity was counted in a nonpolar toluene scintillant in a Packard Tri Carb.

Methylation. Myxobacton was methylated according to the method of Wallenfels *et al.* (1963). The dry pigment was dissolved in dimethyl formamide—dimethyl sulfoxide (1:1, v/v) and a small amount of $Ba(OH)_2$ was added. The solution was cooled on an ice bath and $^1/_3$ of its volume of dimethyl sulfate was added. The mixture was allowed to warm up to about 40—50° C and was then kept for 2—3 hrs at room temperature in the dark. The methylated products were extracted with ether and washed with $NaHCO_3$ solution. The different methyl ethers were separated and purified on a silica gel column.

Phospholipids. Phospholipids of *S. aurantiaca* were analyzed according to methods described elsewhere (Kleinig, 1970).

Incorporation of D-Glucose-C14 and Myo-Inositol-T in vivo. To 100 ml batches of logarithmically growing light cultures of *S. aurantiaca* was added either 10—25 μC of myo-inositol-T (3460 mCi/mM, New England Nuclear) + 130 μg of cold *meso*-inositol or 5—10 μCi of D-glucose-C14(U) (288 mCi/mM, The Radiochemical Center Amersham). In the latter case the bacteria were grown either with 1% glucose or without glucose in the medium. After addition of the labelled compounds the cul-

tures were incubated for 2 hrs. The cells were harvested, washed twice with cold water, and extracted as usually.

Mass Spectra. Mass spectra were taken on a Varian-MAT mass spectrometer SM1B using the direct inlet system. The ionizing energy was 70 eV. The temperature of the substances ranged from 170 to 190° C.

Results

The two major pigments of *S. aurantiaca* were eluted from a silica gel column after the minor carotenoids with acetone-diethyl ether mixtures. These two pigments accounted for at least 80% of the total carotenoids. The absorption spectra in visible light and the infrared spectra are shown in Figs. 1 and 2, respectively.

The Chromophore System

The light absorption of the orange pigment eluted first from the column did not change after reduction with $NaBH_4$, nor was there any change in polarity after reduction as determined by TLC. This meant that there was no keto group either in our out of conjugation with the carbon-carbon double bond system. The second pigment which was red in color had a slightly higher polarity and showed a typical ketocarotenoid spectrum (Fig. 1). After reduction a spectrum resulted which was identical with the spectrum of apo-2′-carotinol (Fig. 1). There was a hypsochromic

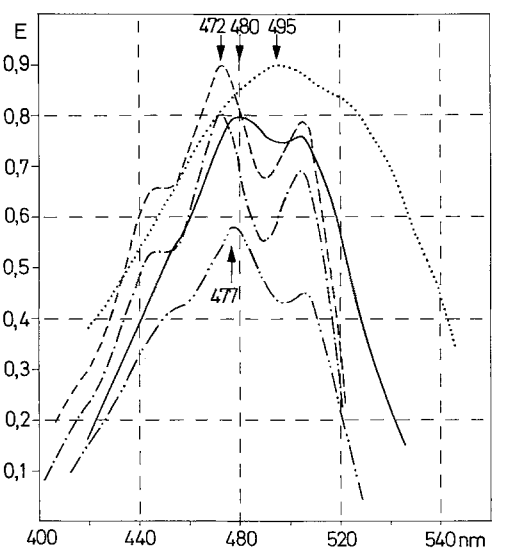


Fig. 1. Absorption spectra of myxobactin ester (orange pigment) — · · — · ·, myxobacton ester (red pigment) ——, reduced myxobacton ester — · — · —, apo-2′-carotinal, · · · · · ·, apo-2′-carotinol – – – –. Solvent ethanol

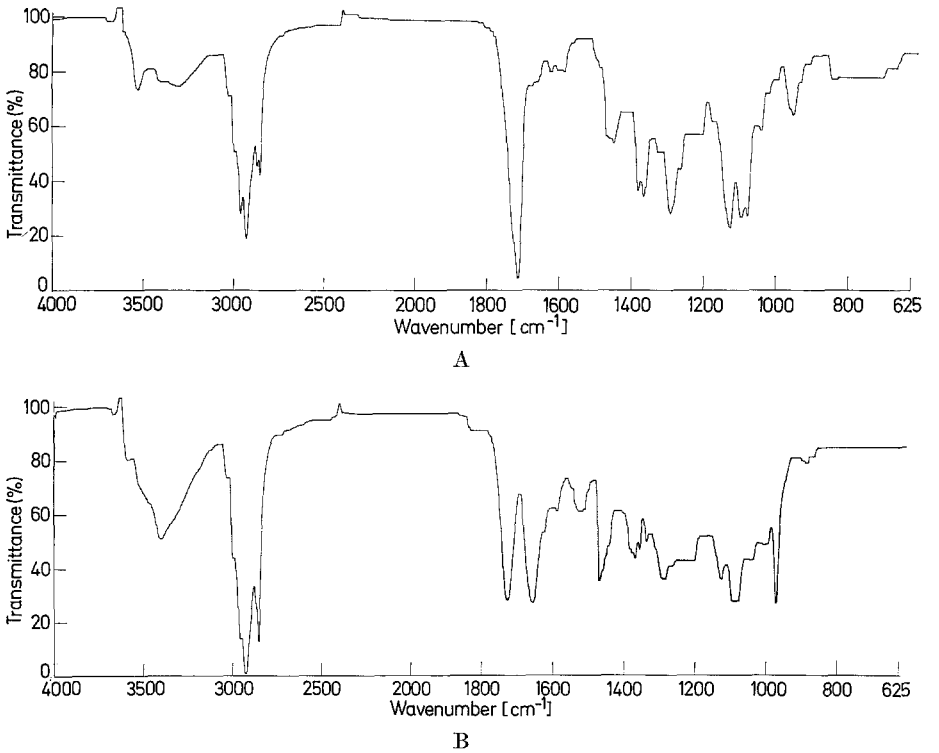


Fig. 2. Infrared spectra of myxobactin ester (orange pigment), A, and myxobacton ester (red pigment), B

shift of about 8 nm from the natural to the reduced pigment (Table), which indicated that the keto function was located in an ionone ring. Reduction of a conjugated keto function located in the chain would result in a shift of about 20 nm (see Fig. 1: apo-2′-carotinal and apo-2′-carotinol). Overtly the chromophore of the red pigment consisted of a carbonyl group in a β-ionone ring in conjugation with 11 carbon-carbon double bonds in the chain. The difference of about 5 nm in the spectra of the orange pigment and the reduced red pigment suggested that the orange pigment possesses an additional double bond in the ionone ring instead of the keto function in the 4 position. An additional conjugated double bond in the chain would exhibit a bathochromic shift of about 12 nm in this wavelength range.

The absence of a conjugated carbonyl function in the orange pigment and the presence of such a function in the red pigment were further inferred from the infrared spectra (Fig. 2: band at 1660 cm^{-1}). In addi-

tion, the infrared absorption of both pigments at about 1720 cm^{-1} indicated a carboxyl function, which will be discussed below.

Functional Groups

Both, the orange and the red pigment, migrated as uniform bands in adsorption TLC on silica gel, but split into at least 3 components in partition TLC on oil impregnated cellulose. This indicated differences in the lipophilic parts of individual molecules. After partial saponification, a single more polar derivative of each pigment appeared in partition TLC in addition to the native pigment groups. After prolonged saponification only the polar derivatives could be found. This suggested that in both pigments there is an acylated hydroxyl function as was also inferred from the infrared absorption at 1720 cm^{-1}.

Partial and total acetylation tests of the native orange and red pigments with acetic anhydride as well as partial methylation revealed the presence of 3 free hydroxyls. 4 hydroxyl groups could be demonstrated with the saponified pigments. This is depicted in Fig. 3 for the red pigment in adsorption TLC. As can be seen from the figure, the interpretation of the different acetates in adsorption TLC is quite difficult due to the appearance of isomers and could only be resolved by comparison with partition TLC and by acetylation with acetic-C14 anhydride. After acetylation with the radioactive agent and TLC the individual spots were scraped from the plate and counted in a non-polar scintillant. The ratios of relative specific activities of the mono-, di-, tri- and tetra-acetates were found to be about 1:2:3:4.

There were no free tertiary hydroxyls present in either pigment, since silylation of the peracetates gave no silyl ether. Secondary hydroxyls could be expected in position 2 and 2′ for the orange pigment and 2, 3, and 2′ for the red pigment. The 2′ position was excluded because of the negative results of the allylic oxidation test with chloranil (reference substance: reduced canthaxanthin) and because no allylic ether could be formed with 0.01 N ethanolic HCl. These two tests also excluded the presence of a hydroxyl function in position 2 of the orange pigment. A hydroxyl in position 3 of the red pigment was also excluded because alkali treatment did not result in an astacene-like diosphenol (Kuhn and Sörensen, 1938). Neither could a hydroxyl function in the above mentioned positions be demonstrated after saponification of the pigments.

The large number of functional groups (3 hydroxyls, 1 ester group) and the negative tests for special hydroxyls within the carotenoid molecules suggested that another polar molecule might be linked to the carotenoid skeleton. Therefore attempts were made to detect polar compounds after appropriate hydrolytic procedures. Glucose could be

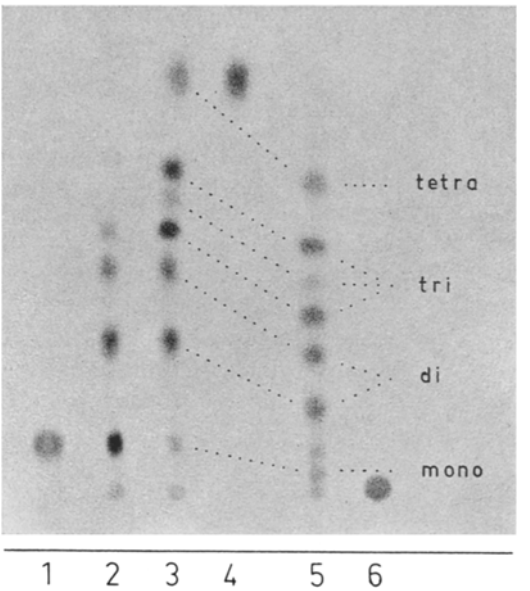


Fig. 3. Adsorption chromatogram on silica gel thin layer. *1* Native myxobacton monoester. *2* Native myxobacton monoester, after mild acetylation; *3* Native myxobacton monoester, after prolonged acetylation; *4* Native myxobacton monoester, after exhaustive acetylation, resulting in the triacetate exclusively; *5* Myxobacton, after acetylation; *6* Myxobacton. The different acetates (n) of myxobacton are connected by dotted lines with the corresponding myxobacton monoester acetates ($n-1$). The acetates of myxobacton are more strongly adsorbed than the corresponding myxobacton monoester acetates, because an acetate grouping is more polar than a long chain fatty acid grouping

demonstrated after treatment of the pigments with methanolic HCl by subsequent TLC (Fig. 4) or by a D-glucose oxidase test.

We first thought that the pigments in question were inositol ethers (Kleinig and Reichenbach, 1970), due to a misinterpretation of isomeric acetates, and because our pigments could not be split by treatment with $CHCl_3$/HCl (according to Hertzberg and Liaaen Jensen, 1967). The interpretation of the isomeric derivatives became unequivocal, however, when methyl ethers rather than acetates were prepared. The poor reactivity of the pigments can be explained by the fact that we have tertiary glucosides (as will be shown below). In the cases mentioned above the cleavage with $CHCl_3$/HCl was possible only because a free interacting hydroxyl was present in C2′ (mechanism according to Arpin and Liaaen Jensen, 1967).

These analyses indicate that the two pigments are glucosides with one of the glucose hydroxyls esterified with an acid. The orange caro-

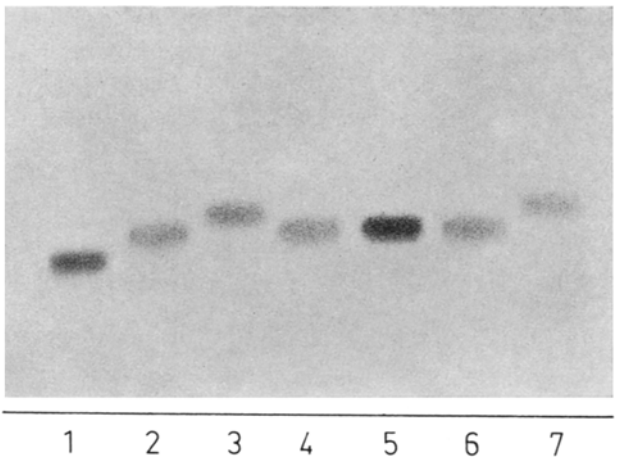


Fig. 4. Thin layer chromatogram of myxobacton hydrolysate and reference sugars: *1* Galactose; *2* Myxobacton hydrolysate; *3* Mannose; *4* Myxobacton hydrolysate; *5* Glucose; *6* Myxobacton hydrolysate; *7* Fructose

Table

Absorption maxima of myxobactin and myxobacton and derivatives (in ethanol)

Carotenoid	nm		
Myxobactin (XI) and its esters	454i	477	506
Myxobacton (III) and its esters	460i	480	504s
Reduced myxobacton	449s	472	504
1′,2′-Dihydro-1′-hydroxy-4-keto-torulene (V)	460i	480	504s
4-Keto-torulene (VI)	470i	490	516s
4-Hydroxy-torulene (VII)	460s	485	519
3,4-Dehydro-torulene (VIII)	467s	491	524
1′,2′-Dihydro-1′-hydroxy-3,4-dehydro-torulene (IX)	454i	477	506

i = inflexion; s = shoulder.

tenoid glucoside we call myxobactin, the red one myxobacton. The native pigments are then myxobactin ester and myxobacton ester. The position of the glucosidic bond in the carotenoid molecule and the nature of the acids will be discussed below.

An enzymatic cleavage of the free glucosides with α- or β-glucosidase could not be obtained. The same observation was made by Hertzberg and Liaaen Jensen (1967) with their carotenoid glucosides.

In vivo incorporation studies with glucose-C14 and *myo*-inositol-T gave negative results. After application of glucose-C14 for 30 min no activity could be detected in the pigments, although glucose is used by *S. aurantiaca* (Reichenbach and Dworkin, 1969). This phenomenon will

be considered in detail in a subsequent paper on the biosynthesis of these pigments. As expected from the outset, inositol was not incorporated into the pigments. The specific activity of phosphatidyl inositol, however, became rather high (about 120,000 cpm/μM).

Glucose Free Carotenoids

Besides the HCl treatment mentioned above the glucose moiety could also be split off from the carotenoids by harsh alkali treatment. With both methods, two main derivatives were obtained from either pigment, as shown in the reaction scheme. Myxobacton ester (I) yielded 1′,2′-dihydro-1′-hydroxy-4-keto-torulene (V), already known from *S. aurantiaca*, and 4-keto-torulene (VI) (for absorption maxima of the derivatives see Table), (V) could be easily converted into (VI) by $POCl_3$ treatment. From keto-torulene (VI) 3,4-dehydro-torulene (VIII) could be formed by $CHCl_3$/HCl treatment via the reduced keto-torulene (VII). Under identical conditions myxobactin ester (II) yielded a pigment with one tertiary hydroxyl (IX) and also 3,4-dehydro-torulene (VIII).

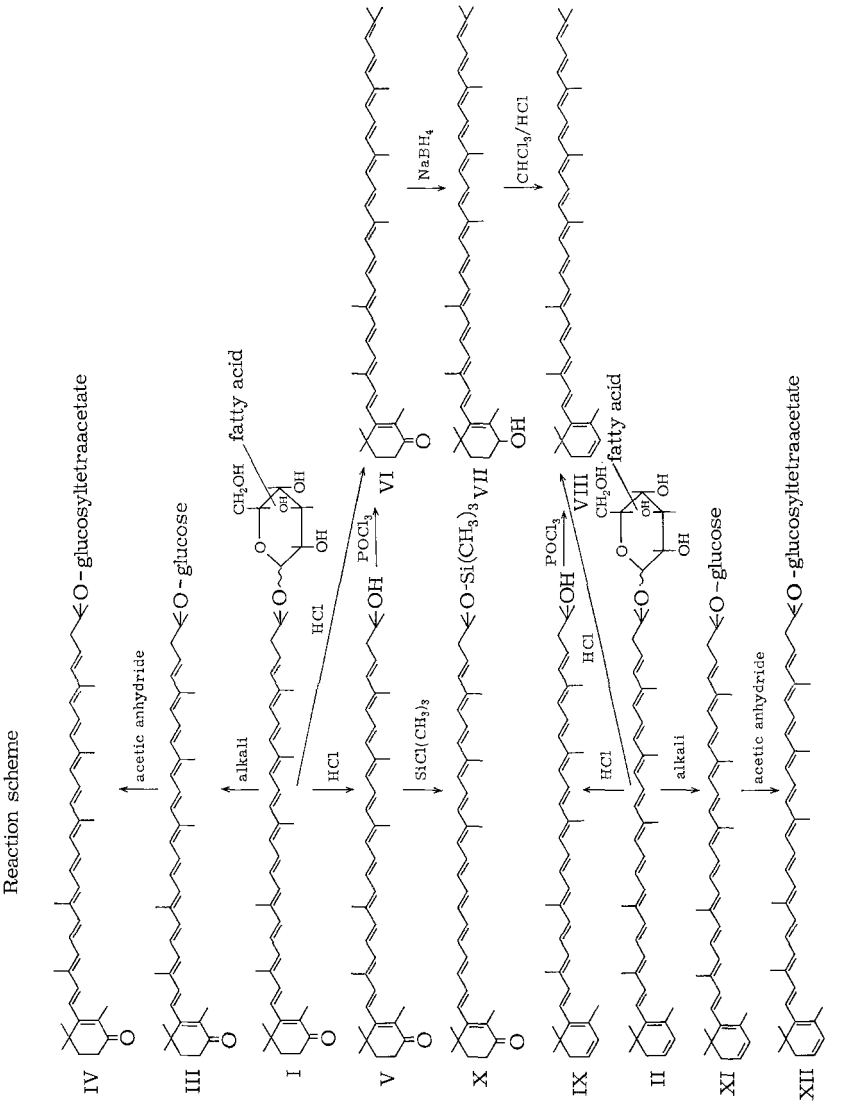
From these data it becomes evident that in myxobactin and myxobacton the glucose moiety must be linked to the tertiary hydroxyl group in C1′, the only hydroxyl available within the carotenoid molecule. This shows that the basic carotenoid structures of both pigments are related to the minor carotenoids of *S. aurantiaca* which also have hydroxyls only in the 1 or 1′ positions.

Fatty Acids. While in whole cells of *S. aurantiaca* the main fatty acids were found to be br15:0, 16:1, and br17:0 (together 75%, Schröder and Reichenbach, 1970), the fatty acids bound to myxobactin and myxobacton were mainly 14:0, 16:0, and 18:0 (together 68%). Three main esters were suggested also by partition TLC. Obviously, in whole cells prevail fatty acids with odd carbon numbers, branched chains, or with a double bond, whereas the pigment esters contain primarily even-numbered unbranched saturated fatty acids.

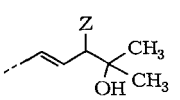
Mass Spectra

The mass spectrum of peracetylated myxobacton (IV) exhibited the molecular ion at m/e 896 (Fig. 5). In the mass spectrum of the permethylated compound the molecular ion appeared at m/e 784. Thus 4 hydroxyl groups originally present had been acetylated and methylated, respectively. These results are in agreement with the elemental composition $C_{46}H_{60}O_3(OH)_4$ for (III).

In the mass spectrum of (IV) peaks at m/e 804 (M−92) and m/e 790 (M−106) were caused by loss of xylene or toluene from the polyene chain (Schwieter *et al.*, 1965). The lack of a significant fragment at m/e 838



(M—58) excluded an endgrouping (XIII) in (IV) (Enzell *et al.*, 1969; Francis *et al.*, 1970):



XIII(Z=H, alkyl or oxygen function)

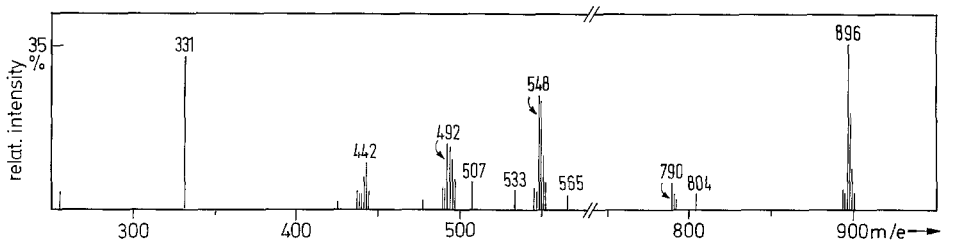
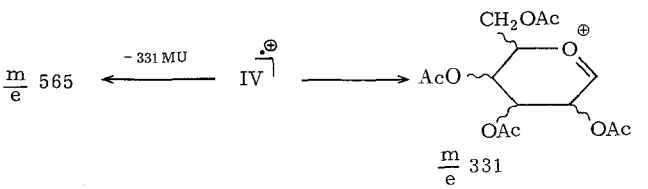
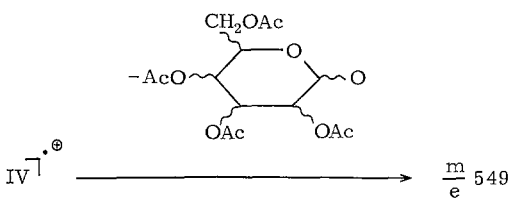


Fig. 5. Mass spectrum of myxobacton (base peak at m/e 43 = 100%)

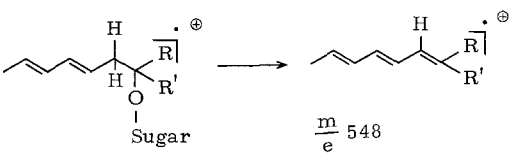
The ions at m/e 565 (M−331) and m/e 331 correspond: They are indicative for the presence of a tetraacetyl-hexose moiety:



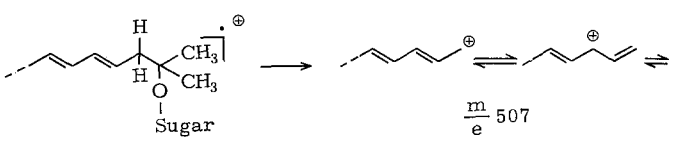
The fragment at m/e 549 originates, if the sugar is lost with the glycosidic oxygen atom included:



The elimination of the sugar can be accompanied by loss of hydrogen as is shown from m/e 548. The intensity of this peak is in good agreement with a glycosidic linkage in β-position to the polyene chain:



A fragment at m/e 507 is explained easily if $R = R' = CH_3$:



Further breakdown of m/e 548 and m/e 549, respectively, may lead to fragments at m/e 533, 493, and 492 (breakdown of the β-ionone ring) or m/e 442 (expulsion of xylene from the polyene chain).

In summary, from the experiments described above it is evident that myxobactin is 1′,2′-dihydro-1′-glucosyl-3,4-dehydro-torulene (XI) and myxobacton is 1′,2′-dihydro-1′-glucosyl-4-keto-torulene (III). Both pigments occur in *S. aurantiaca* as monoesters of various fatty acids. It could not be determined which hydroxyl of the glucose moiety is esterified or whether there is any preference at all. Also the nature of the glucosidic bond (α or β) is not known.

Discussion

Within the wide structural variety among bacterial carotenoides only few complex structures are known. These are the methylated spirilloxanthins and related pigments from purple bacteria (see e.g. Liaaen Jensen, 1963; Aasen and Liaaen Jensen, 1967) and the tertiary glycosides (Hertzberg and Liaaen Jensen, 1967; Prebble, 1968; Aasen *et al.*, 1969; Weeks, 1970). The new glucosides myxobactin and myxobacton occur as monoesters of fatty acids. The acyl function is located at the glucose moiety. Real carotenoid fatty acid esters, which are so common in the secondary carotenoids of green algae and higher plants, have not been detected so far in bacteria.

Myxobactin and myxobacton are monocyclic C_{40} carotenoids like most of the minor compounds of *S. aurantiaca* (Kleinig and Reichenbach, 1969). To our knowledge the carotenoid moiety of myxobactin (1′,2′-dihydro-1′-hydroxy-3,4-dehydro-torulene) is not described as yet, whereas the carotenoid moiety of myxobacton (1′,2′-dihydro-1′-hydroxy-4-keto-torulene) is present in *S. aurantiaca* as free pigment in very low concentration, and is further known as deoxiflexixanthin from *Flexibacter spec.* (Aasen and Liaaen Jensen, 1966).

We have found myxobactin and myxobacton also in some other myxobacteria. The natural distribution of these pigments will be discussed later from a chemotaxonomical point of view.

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References

Aasen, A. J., Liaaen Jensen, S.: Carotenoids of *Flexibacteria*. III. The structure of flexixanthin and deoxiflexixanthin. Acta chem. scand. **20**, 1970—1988 (1966).

— — Bacterial carotenoids. XXI. Isolation and synthesis of 3,4,3′,4′-tetrahydrospirilloxanthin. Acta chem. scand. **21**, 371—377 (1967).

Aasen, A. J., Francis, G. W., Liaaen Jensen, S.: Bacterial carotenoids. XXIX. The carotenoids of two yellow halophilic cocci—including a new glycosidic methyl apolycopenoate. Acta chem. scand. **23**, 2605—2615 (1969).

Arpin, N., Liaaen Jensen, S.: Recherches chimiotaxonomiques sur les champignons. Sur la présence de l'estre méthylique de la torularhodine chez *Cookeina sulcipes* (Berk.) Kuntze (Ascomycètes). C.R. Acad. Sci. Paris, Sér. D **265**, 1083—1085 (1967).

Enzell, C. R., Francis, G. W., Liaaen Jensen, S.: Mass spectrometric studies of carotenoids. II. A survey of fragmentation reactions. Acta chem. scand. **23**, 727—750 (1969).

Francis, G. W., Hertzberg, S., Andersen, K., Liaaen Jensen, S.: New carotenoid glycosides from *Oscillatoria limosa*. Phytochem. **9**, 629—635 (1970).

Hertzberg, S., Liaaen Jensen, S.: Bacterial carotenoids. XX. The carotenoids of *Mycobacterium phlei* strain *Vera* 2. The structures of the phleixanthophylls—two novel tertiary glucosides. Acta chem. scand. **21**, 15—41 (1967).

— — The structure of myxoxanthophyll. Phytochem. **8**, 1259—1280 (1969).

— — The structure of oscillaxanthin. Phytochem. **8**, 1281—1292 (1969).

Kleinig, H.: Nuclear membranes from mammalian liver. II. Lipid composition. J. Cell Biol. **46**, 396—402 (1970).

— Reichenbach, H.: Carotenoid pigments of *Stigmatella aurantiaca (Myxobacterales)*. I. The minor carotenoids. Arch. Mikrobiol. **68**, 210—217 (1969).

— — A new type of carotenoid pigment isolated from myxobacteria. Naturwissenschaften **57**, 93—94 (1970).

Kuhn, R., Sörensen, N. A.: Über Astaxanthin und Ovoverdin. Ber. dtsch. chem. Ges. **71**, 1879—1888 (1938).

Liaaen Jensen, S.: Carotenoids of photosynthetic bacteria—distribution, structure, and biosynthesis. In: H. Gest, A. San Pietro, and L. P. Vernon: Bacterial photosynthesis, pp. 19—34. Yellow Springs: Ohio, The Antioch Press 1963.

Prebble, J.: The carotenoids of *Corynebacterium fascians* strain 2 *Y*. J. gen. Microbiol. **52**, 15—24 (1968).

Reichenbach, H., Dworkin, M.: Studies on *Stigmatella aurantiaca (Myxobacterales)*. J. gen. Microbiol. **58**, 3—14 (1969).

Schröder, J., Reichenbach, H.: The fatty acid composition of vegetative cells and myxospores of *Stigmatella aurantiaca (Myxobacterales)*. Arch. Mikrobiol. **71**, 384—390 (1970).

Schwieter, U., Bollinger, H. R., Chopard-dit-Jean, L. H., Englert, G., Kofler, M., König, A., Planta, C. v., Rüegg, R., Vetter, W., Isler, O.: Synthesen in der Carotenoid Reihe. 19. Mitteilung: Physikalische Eigenschaften der Carotine. Chimia **19**, 294—302 (1965).

Smith, P. F.: The carotenoid pigments of Mycoplasma. J. gen. Microbiol. **32**, 307—319 (1963).

Wallenfelds, K., Bechtler, G., Kuhn, R., Trischmann, K., Egge, H.: Permethylierung von oligomeren und polymeren Kohlenhydraten und quantitative Analyse der Spaltprodukte. Angew. Chem. **75**, 1014—1022 (1963).

Weeks, O. B., Andrewes, A. G.: Structure of the glycosidic carotenoid corynexanthin. Arch. Biochem. **137**, 284—286 (1970).

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