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Xanthomonas Pigments

2.* The *Xanthomonas* "Carotenoids" — Non-carotenoid Brominated Aryl-polyene Esters

A. G. ANDREWES,[a] S. HERTZBERG,[a] S. LIAAEN-JENSEN[a] and M. P. STARR[b]

[a]*Organic Chemistry Laboratories, Norwegian Institute of Technology, University of Trondheim, Trondheim, Norway and* [b]*Department of Bacteriology, University of California, Davis, California 95616, USA*

The pigments of *Xanthomonas juglandis* strain XJ103, previously reported to be carotenoids, have been further investigated.

Two pigment complexes (I and II), each of estimated molecular weight *ca.* 2200, provided on saponification brominated aryl octaene methyl esters. Partial structures for the four components of the saponified pigment mixture are presented (*1a*, *1b*, *2a*, *2b*). The monobromides (*1a*, *2a*) could not be separated from the corresponding dibromides (*1b*, *2b*). The proposed structures are based on chemical data and physical properties including electronic, infrared and mass spectra, and comparison with dimethylcortisalin (*3b*) and model compounds (*4* – *7*) synthesized for this purpose.

Structures such as those proposed are unique and support the premise that the pigments may serve as a significant taxonomic marker for the genus *Xanthomonas*.

The genus *Xanthomonas* is a group of Gram-negative bacteria which cause diseases in plants.[1–4]

Although a few non-pigmented bacteria have been occasionally assigned to the genus *Xanthomonas*, and colourless mutants of *Xanthomonas* spp. are known, the yellow colour of their colonies has been of diagnostic importance for distinguishing *Xanthomonas* spp. from other bacteria occurring in the same habitats.

From previous studies, it appears that the same pigments occur in many nomenspecies of *Xanthomonas*,[5,6] that they are membrane-bound,[7] that they are different from other previously reported bacterial pigments,[5,6] and that they have some properties which led to their classification as carotenoids.[5,8]

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We now report partial structures for the pigments isolated from *Xanthomonas juglandis* strain XJ103 which, in fact, are not carotenoids but are unique aryl octaene methyl esters distinguished by carrying one or two bromine atoms.

The present work is restricted to *X. juglandis* strain XJ103. However, on the basis of previous work,[5,6] it seems likely that the results may be extended to other *Xanthomonas* nomenspecies.

RESULTS AND DISCUSSION

Members of the genus *Xanthomonas* form from glucose a complex polysaccharide [9–11] which interferes with pigment analysis. *X. juglandis* XJ103 was therefore cultivated in a sugar-free medium.

Pigments were extracted from lyophilized cells and isolated according to the scheme shown in Fig. 1. Pigments *1* and *2* obtained after mild saponification of the extracts correspond to the material isolated from the same strain of *X. juglandis* in previous investigations.[5–7]

Complexes I and II were obtained when the saponification procedure was omitted. Treatment of the complexes I and II with mild base afforded pigments *1* and *2*, respectively. Pigments *1* and *2* and the corresponding complexes exhibited virtually identical electronic spectra (*cf.* Fig. 2), but differed

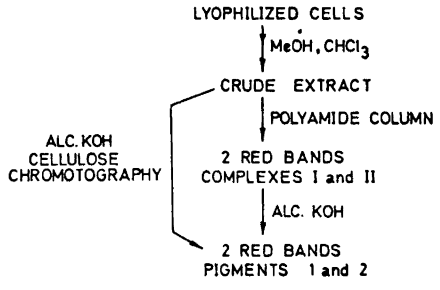


Fig. 1. Scheme for isolation of pigments from *X. juglandis* XJ103.

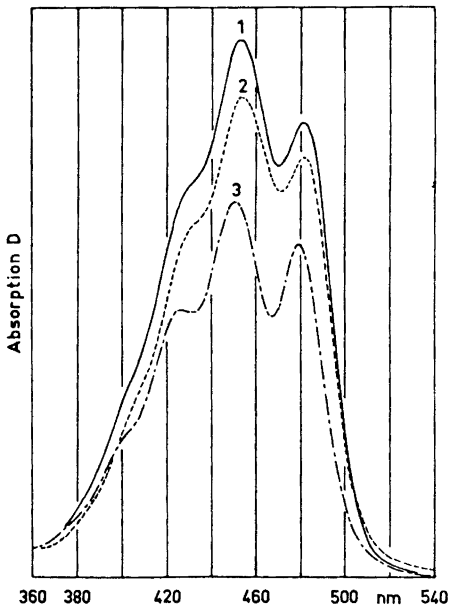


Fig. 2. Absorption spectra in visible light of *X. juglandis* XJ103 pigment *1* (curve 1), XJ103 pigment *2* (curve 2), and synthetic *4* (curve 3).

Table 1. Chromatographic properties of the *X. juglandis* XJ103 pigment complexes I and II, of the *trans* pigments *1* and *2* and of the acetate of *2*.

Chromatographic system	Required eluent or R_F-value				
	I	II	*1*	*2*	*2*-acetate
Polyamide 6-AC column	4 % MeOH-benzene	6–10 % MeOH-benzene			
Merck kieselgel column			benzene	$CHCl_3$ ether [a]	
$CaCO_3$ column	[b]			[b]	
Alumina activity grade 2 column					
Cellulose (Schleicher & Schüll No. 124) column			5 % acetone-pet.ether	20–100 % acetone-pet.ether	2–5 % acetone-pet.ether
Schleicher & Schüll No. 287 (kieselguhr) paper					
benzene	0.40	0.25	0.78	0.47	
20 % acetone in petroleum ether			0.80	0.50	0.80
Silicagel G plates: benzene			0.80	0.45	
2 % methanol in benzene	0.60	0.40			
chloroform			0.92	0.60	

[a] For development, zones extruded. [b] Not eluted with methanol.

in other physical properties. The complexes were readily soluble in most common organic solvents. Their mass and IR spectra were inconclusive, and the PMR spectra showed only a strong band characteristic of methylene groups in saturated environments. Pigments *1* and *2*, however, had minimum solubility in all common solvents to such an extent that the PMR spectra could not be obtained. A single attempt to obtain a time-averaged spectrum was unsuccessful because of degradation during the time required.

Considering now only the products obtained after mild saponification, Pigments *1* and *2* constituted 0.04 % of the lyophilized cells in the approximate ratio of 2:1. Pigment *1* crystallized as needles of m.p. 220°C, whereas *2* was obtained as an amorphous powder. The chromatographic behaviour is summarized in Table 1. The difference in polarity of *1* and *2* indicates that *2* has at least one polar group not present in *1*. Representative electronic absorption spectra of *1* and *2* are shown in Fig. 2. Absorption maxima for both compounds in various solvents are listed in Table 2. The general shape of

Table 2. Spectral properties of the *X. juglandis* XJ103 pigment complexes I and II and the *trans* pigment *1* and *2* in visible light.

Solvent	Absorption maxima in nm											
	I			II			*1*			*2*		
Methanol	420	442.5	469	420	442	469	(418)	442	470			
Acetone	(421)	444	472	(421)	443	471	(423)	445	473	420	444	472
Chloroform							(428)	453	482	(428)	453	480
Benzene	433	456	483	430	455	483						
Pyridine							(435)	460	489			
Dimethyl sulfoxide							(435)	462	490			

the absorption curves are typical for conjugated polyenes and resemble the spectra of aromatic polyene aldehydes characterized by Zechmeister's group.[12]

Mass spectrometric analysis of *1* showed the molecular ion as a triple peak at *m/e* 530, 532, and 534 with relative intensities 1 : 2 : 1, indicative of a dibromo-compound. Accurate mass measurement established the molecular formula $C_{25}H_{24}Br_2O_3$. Fragment ions centered at *m/e* 501 and *m/e* 473 were compatible with loss of methoxy and carbomethoxy radicals, respectively. Also noted were ions attributed to the loss of HBr and probably benzene. Pigment *2* gave a molecular ion centered at *m/e* 518 with isotopic distribution precisely that of a dibromo-compound. The molecular formula $C_{24}H_{22}Br_2O_3$ determined by high precision measurements indicated that *2* differs from *1* by an extra methyl or methylene group.

Bands in the IR spectrum of *1* (Fig. 3) at 1710 cm^{-1} and 1135 cm^{-1} are attributed to an α,β-unsaturated ester.[13] Absorption at 1010 cm^{-1} was assigned to out-of-plane C–H vibrations on *trans*-double bonds, the position of which is shifted to somewhat higher frequencies than that noted for carotenoids,[14] but consistent with the same assignment made here for cortisalin (*3a*)[15] which has no methyl substituents on the polyene chain. The IR spectra of dimethyl-

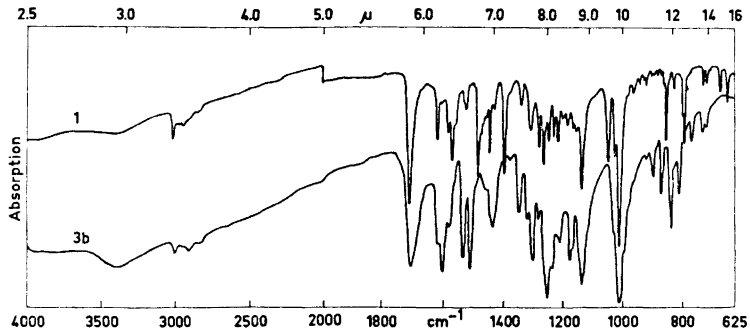
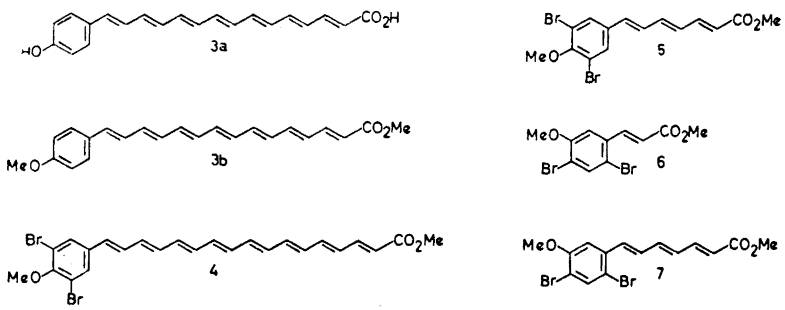


Fig. 3. Infrared spectra (KBr) of *Xanthomonas juglandis* XJ103 pigment *1* and dimethylcortisalin (*3b*).

cortisalin (*3b*) and *1* have many features in common (Fig. 3). Thus absorption at 1250 cm^{-1} in *3b* and *1* may be associated with an aromatic methoxy group.[13] The band at 855 cm^{-1} of *1* was originally attributed to the vibration of isolated C–H in an aromatic ring. We have been unable to assign the prevalent bands at 1396 cm^{-1} and 1050 cm^{-1}. The IR spectrum of *2* was less distinct. Strong absorption at 3410 cm^{-1} indicated a hydroxy group [13] and additional bands were present which suggested a conjugated ester and conjugated *trans*-disubstituted double bonds at the same frequencies noted in the spectrum of *1*.



The molecular formulas for *1* and *2* (13 double bond equivalents) in conjunction with the observed electronic spectrum dictate that in all probability the pigments contain one aromatic ring. The loss of methoxy and carbomethoxy fragments in the mass spectra [16] and the aforementioned IR data suggest that the chromophore terminates in a conjugated methyl ester function. Comparison of the electronic spectra with that of dimethylcortisalin (*3b*, λ_{max} 444 nm *vs.* 461 nm for *1* in pyridine) indicated that *1* and *2* have a polyene system longer than 7 conjugated double bonds. The difference in the absorption maxima of dimethylcortisalin (*3b*) and *1* or *2* suggested that the polyene system of *1* and *2* consists of 8 double bonds. Moreover, considering

the molecular formula and chromophore, *1* could contain at most one methyl group. Oxidation studies to be discussed below showed that this methyl group is connected with the aryl ring and not with the polyene chain. Thus the initial supposition was that *1* and *2* were aryl octaenes terminated by a conjugated methyl ester and further distinguished by having two bromine atoms incorporated in the structure, presumably on the phenyl ring. The presence of a phenolic hydroxy group in *2*, methylated in *1*, was consistent with the spectral data.

A model compound *4* was synthesized [17] and the properties compared with that of *1*. Compound *4* had solubility properties similar to and chromatographic properties identical with those of *1* in the systems tested. The electronic absorption spectrum of *4* closely resembled, but was not identical to, that of *1* (Fig. 2); the maximum of *4* was hypsochromically displaced by 3 nm in chloroform and the spectrum of *4* showed slightly more fine-structure. Comparison of the IR spectra (*cf.* Ref. 17) showed similarities in the bands assigned to the ester and methoxy groups and the *trans*-disubstituted double bonds. Differences were observed in the region 800 – 900 cm^{-1} and the bands at 1395 cm^{-1} and 1050 cm^{-1}, noted for, but not assigned for *1* were absent. With respect to the mass spectrum of *4* the expected molecular ion and isotopic peaks were present. Losses of methoxy and carbomethoxy fragments were noted along with a prominent loss of C_6H_6 (benzene from the polyene chain [17]), but no loss of HBr was detectable. Thus, *1* and *4* were not identical, but the similarities in the general characteristics of the synthetic pigment (*4*) and *1* are supporting evidence for the basic proposed structures of *1* and *2*.

Turning now to the chemical properties of *1* and *2:* Saponification of a mixture of *1* and *2* in methanol-chloroform (1:1) containing 10 % KOH gave a mixture of the corresponding carboxylic acids, confirmed by mass spectrometry and by the change in partition properties. Neither pigment *1* nor *2* reacted with diazomethane. This result does not disprove the presence of a phenolic hydroxy group in *2* since neither *3a* nor phenolic carotenoids [18] provide a methyl ether under the same conditions. Pigment *1* could not be acetylated or silylated. Pigment *2* gave a monoacetate, judged by chromatographic properties and mass spectrometry (M = *m/e* 558, 560, 562).

Reduction with complex metal hydrides were complicated by the low solubility of the pigments and low recoveries. As expected the ester group was not reduced with $NaBH_4$. A product obtained with $NaBH_4$ exhibited the same electronic spectrum as the starting material (*1*), but was chromatographically more strongly adsorbed. Its formation may involve displacement of bromine by hydride,[19] although mass spectrometric results were inconclusive.

When *1* was reduced with $LiAlH_4$, at least two products were obtained. One product could not be distinguished from the $NaBH_4$-product mentioned above. Reduction of the ester function can account for the second major acetylable product with λ_{max} 405, 422, and 448 nm in acetone. Other products, less consistently obtained, had shorter chromophores.

Oxidation of *1* with $KMnO_4 - CaCO_3$,[20] slow under reflux condition, gave an acid with molecular formula $C_8H_7BrO_3$ (high precision mass spectrometry), explicable by a benzoic acid substituted by a methoxy group and one bromine

atom. $KMnO_4-H_2SO_4$ oxidation was rapid and gave the same $C_8H_7BrO_3$ moiety. Ozonolysis of a mixture of *1* and *2* gave products with molecular ions corresponding to $C_8H_7BrO_3$ and $C_7H_5BrO_3$. The oxidation data thus support the idea that *2* is a phenol and *1* its methyl ether derivative.

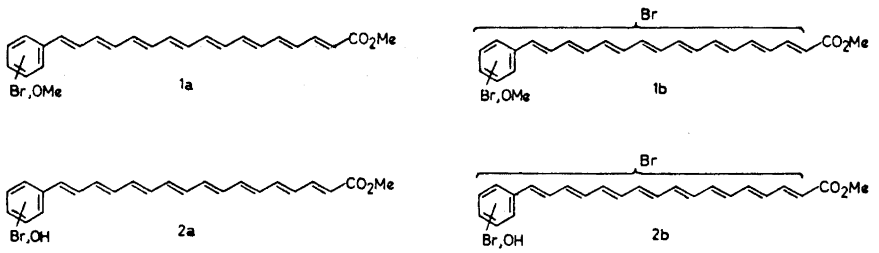
The absence of any dibromo acid in the oxidation mixture was unexpected, and may be explained in a number of ways:

First of all, the second bromine may be located on the polyene chain and not on the ring. However, vinylic bromides of the required type might be expected to suffer elimination under the alkaline conditions used for preparation of the free carboxylic acids of *1* and *2*.[21]

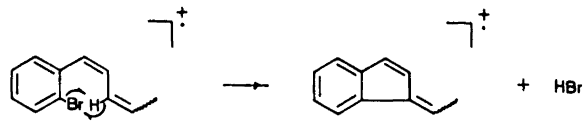
A second possibility would be that *1* and *2* each were mixtures of mono- and dibromo compounds and that (i) the dibromo compounds did not survive the oxidation conditions or (ii) that the dibromo compounds only represented a minor portion of the total pigment mixture. Alternative (i) is disregarded for *1* since $KMnO_4$ oxidation of the two model dibromomethoxy aryl compounds *5* and *6* gave the expected dibromo acids.

Careful re-examination of the mass spectra of *2* under different conditions and from different batches of cells indeed confirmed that *2* is, in fact, a mixture of the mono- and dibromo compounds: *2a*, $C_{24}H_{23}BrO_3$ *m/e* 438, 440, and *2b*, $C_{24}H_{22}Br_2O_3$ *m/e* 516, 518, 520. This conclusion was not reached earlier because the fragment ions at *m/e* 436, 438, caused by loss of HBr from the molecular ion of *2b*, give peaks in the same region. The relative quantities of *2a* and *2b* cannot be judged from the mass spectra and are not known, since we have been unable to separate the mixture although a number of chromatographic systems have been examined.

Consequently, it appears that *X. juglandis* strain XJ103 contains four pigments (*1a*, *1b*, *2a*, *2b*), two of which are monobromides (*1a*, *2a*) and two dibromides (*1b*, *2b*). In the case of the monobromides, the bromine atom must be located on the aromatic ring. With respect to the dibromides, one of the bromine atoms is on a phenyl ring while the position of the second bromine is not established.



The observed loss of HBr from *1b* and *2b* on electron impact may support the presence of bromine in *ortho* position to the polyene chain. The required hydrogen transfer for HBr elimination from an aryl bromide is best accommodated with *ortho* substitution, for example by the following mechanism:



Loss of a bromine radical from the model compounds *6* and *7* indicates that bromine substituted *ortho* to the polyene chain is labile on electron impact. Further support for an *ortho* bromine substituent in *1* and *2* follows from a comparison of the electronic spectra of *5* and *7*. Compounds *5* and *7* exhibit absorption at about the same wavelength. However, *7* displays less spectral fine-structure than *5*. The same situation is observed with respect to *1* and *2* *versus* the synthetic model *4*. Finally the IR-band at 1050 cm^{-1}, observed for *1* and *2* was also noted for synthetic *7*, and not for *4* and *5*.

The *Xanthomonas* complexes consist of the pigments *1a*, *1b*, *2a*, *2b*, or precursors thereof, bound in as yet an unknown fashion to a lipid-like moiety. Considering the extinction coefficient of the complexes, I and II and the corresponding pigments *1* and *2*, a rough calculation of the unit weight of the attached moiety can be made and gives a value of 1700 for complex I as well as II. It then appears that the same moiety is attached to each pigment. The PMR spectrum of the complexes indicates that the attached group is largely lipid in nature, and the fact that base is required to break the complex suggests that the bond between the chromophore moiety and the attached group is chemical in nature. Phospholipid/fatty alcohol esters of a coloured carboxylic acid, suffering re-esterification on alkali treatment in methanol to give the methyl esters *1* and *2*, have been considered. However, alkali treatment of the complexes I and II in ethanol did not result in the corresponding ethyl esters, thus disproving the above hypothesis.

One remaining possibility is that complexes I and II contain a hydroaromatic ring which gives rise to the aromatic structure on alkali treatment.

CHEMOTAXONOMICAL CONSIDERATIONS

Naturally occurring bromine compounds in living organisms are comparatively rare although several have been reported from marine sources, *e.g.* Refs. 22 – 33. Some of these marine compounds have bromine substituents on an aromatic ring,[22,23,29–33] but none have extended polyene chains. The instances of bromine compounds isolated from non-marine sources [34–37] are scarce and in each of the cases referred to the incorporation of bromine was accomplished by the addition of potassium bromide to a synthetic medium as a replacement for chloride ion.

In the present cultivation of *X. juglandis* no bromide was deliberately added to the complex culture medium, and the precise source of the bromine incorporated into the *Xanthomonas* pigments is so far not known. It is probably safe to assume that the concentration of chloride ion was higher than that of bromide in the beef extract-peptone nutrient broth used. If this is indeed the case, then these *Xanthomonas* bacteria must have a capacity for specific

enrichment of bromide over chloride. This in itself would constitute a novel feature of a non-marine organism.

With respect to the general features of the *Xanthomonas* pigments other than the presence of bromine in the molecule, we have found relatively few instances of naturally occurring compounds with related structures. Cortisalin (*3a*) isolated from the fungus *Corticium salicinum* [15] is a phenol *para* substituted by a conjugated polyene carboxylic acid, which has features common to the *Xanthomonas* pigments. An unbranched polyene dicarboxylic acid, corticrocin,[38] has been isolated from the related species *Corticium croceum*, but this lacks an aromatic ring. From *Aspergillus niger* two aromatic polyenes [39–41] have been reported, but both these pigments have substituents on the polyene chain and lack substituents on the aryl ring.

Thus, the *Xanthomonas* pigments, not yet encountered outside the bacterial genus *Xanthomonas*, represent unique structures of significant chemotaxonomic value and considerable chemo-ecological interest.

EXPERIMENTAL

Xanthomonas pigments

Biological material. Xanthomonas juglandis strain XJ103 [6,7] was obtained from the International Collection of Phytopathogenic Bacteria maintained at the Department of Bacteriology, University of California at Davis.

Cultivation. The cultures were grown in several 200 l batches of double-strength Difco nutrient broth (16 g/l) with aeration (7 l/min) and agitation. Initial pH of the broth was 6.9. During growth the pH was maintained at 7.2. The cells of one typical batch were harvested after 29 h of growth and the total yield was 1280 g wet weight; after lyophilization 150 g.

Materials and methods. Instrumentation and solvents were as generally used in the Norwegian laboratory.[17] Chromatographic properties are compiled in Table 1.

Pigment extraction. Lyophilized cells were exhaustively extracted with MeOH, then MeOH – $CHCl_3$ (1:1) and the extracts pooled and concentrated under vacuum. The resultant viscous oil was dispersed in benzene and filtered through Celite to eliminate insoluble material.

Chromatography of non-saponified extracts. Benzene solutions of the extracts were chromatographed on columns of polyamide 6-AC resin (Machery, Nagel & Co., Germany). The individual complexes of I and II were further purified by preparative thick layer chromatography on silica gel G plates developed with benzene.

Characterization of complexes I and II. I and II were isolated as deep red, amorphous powders from chilled MeOH solutions. Both were soluble in petroleum ether, ether, benzene, acetone, MeOH, $CHCl_3$, and CH_2Cl_2. The absorption maxima for I and II in visible light are listed in Table 2. In MeOH solution complexes I and II had E (1 %, 1 cm) = 515 and 505, respectively, at 443 nm. Assuming E (1 %, 1 cm) = 2200 and M = 530 and 516 for *1* and *2*, respectively, this corresponds to molecular weights of 2236 and 2212 for complexes I and II, respectively, provided only one chromophore unit is present in each complex.

The PMR spectra for both I and II ($CDCl_3$) showed only a single signal at τ 8.72; the mass spectra gave no identifiable molecular ion. The IR spectra (KBr) of I and II showed bands at 3320 (broad, s, OH), 2910 – 2840 (s, CH), 1715 (broad, vs., C=O), 1615 (m, C=C), 1580 (m), 1455 (m), 1130 (broad, s), 1010 (*trans*-disubstituted double bonds), 955 (s), 890 (m) and 850 (m) cm^{-1}.

Hydrolysis of complex I with ethanolic KOH. Complex I (0.2 mg) in ethanol (5 ml) containing 5 % KOH was stirred for 1 h at room temperature, whereupon the pigment precipitated. The pigment was extracted with $CHCl_3$ and, after drying and concentrating

the extract, was purified on silica gel plates. The mass spectrum of the product showed the molecular ion at *m/e* 534, 532, 530 (1:2:1, $C_{25}H_{24}Br_2O_3$).

Saponification of extracts. Extracts containing I and II were saponified in MeOH solution containing 5 % KOH at room temperature for 1 h. Under these conditions most of the pigments precipitated. Water was added and the pigments extracted with $CHCl_3$. Occasionally a small amount of NH_4Cl was necessary to transfer all pigments to the organic phase. The organic extracts were combined, dried over NaCl and concentrated under vacuum.

Chromatography of saponified extracts. Benzene solutions of pigments *1* and *2* were chromatographed on cellulose columns developed with benzene. The individual fractions were further purified by preparative thick layer chromatography on silica gel G plates developed with benzene. The handling and chromatography of *1* and *2* was continually hampered by the low solubility of these pigments. The approximate ratio of *1* and *2* was 2:1.

Characterization of 2. *2* was precipitated as an amorphous red powder from MeOH solution; total amount available from several isolations *ca.* 6 mg. *2* was chromatographically homogeneous in all systems tested. Upon chromatography on Al_2O_3 columns activity grade 3, *2* could not be eluted with pure MeOH.

Absorption characteristics in visible light are given in Fig. 2 and Table 2.

The IR-spectrum (KBr) exhibited ν_{max} 3410 (OH), 3020 – 2820 (C–H), 1710 (conj. C=O), 1010 (*trans*-disubstituted double bonds), 858 (aromatic C–H) and 800 cm^{-1}.

The mass spectrum showed molecular ions at *m/e* 516, 518, 520 (1:2:1, $M_{2a} = C_{24}H_{22}Br_2O_3$) and 438, 440 (1:1, $M_{2b} = C_{24}H_{23}BrO_3$).

Acetylation of 2. *2* (0.32 mg) was submitted to standard acetylation in acetic anhydride/pyridine; pigment recovery 82 %. The reaction mixture contained a single product less polar than *2* and slightly more polar than *1*. *2*-acetate was purified on cellulose columns and TLC; λ_{max} ($CHCl_3$) 430, 451, 479 nm; *m/e* 558 (M, 3 % of base), 530 (M – 28), 516 (M – 42), 470 (M′, 17 % of base) and 438 (M′ – 42).

Other attempted reactions of 2. *2* (0.26 mg) in 2.5 % methanolic KOH was resistant to ester hydrolysis overnight.

Careful $LiAlH_4$ reduction in ether of **2** (0.1 mg) for 2 min gave 43 % recovery. No new coloured products were formed.

2 (0.1 mg) in $CHCl_3$ was treated with diazomethane (10 ml, 1.5 % solution in ether) at room temperature for 2 h. Starting material (84 %) was recovered, and TLC analysis showed no methylation to have occurred. The methylation reaction was repeated and the reaction heated at 35°C for 4 h. Again the reaction was negative.

Characterization of 1. *1* crystallized from acetone-MeOH or $CHCl_3$-petroleum ether as red needles with metallic sheen, m.p. sharp 218°C. The needles were imperfect and not suitable for X-ray analysis. The total amount of crystalline *1* available from several isolations was *ca.* 15 mg.

Crystalline *1* was insoluble in petroleum ether, ether and MeOH; slightly soluble in DMSO, pyridine, $CHCl_3$, CH_2Cl_2, benzene, and acetone.

The visible light absorption spectrum of *1* is given in Fig. 2 and absorption maxima in various solvents in Table 2. In a mixed solvent system ($CHCl_3$-pyridine-DMSO, 4:4:92), *1* had *E* (1 %, 1 cm) = 2200 at the main maximum (462 nm). In the same solvent system crystalline β-carotene exhibited *E* (1 %, 1 cm) = 2190 at λ_{max}.

The IR-spectrum (KBr) of *1* (Fig. 3) had ν_{max} 3020 – 2820 (C – H), 1712 (conj. C=O), 1620 (C=C), 1395, 1260 (OCH_3), 1135 (C – O), 1050, 1010 (*trans*-disubstituted double bonds), 855 (aromatic C – H), and 792 cm^{-1}.

An attempt to record a time-averaged PMR spectrum failed due to decomposition of the sample over 24 h.

The mass spectrum of *1* showed peaks at *m/e* 530, 532, 534 (1:2:1, $M = C_{25}H_{24}{}^{79}Br^{81}BrO_3$: obs. 532.0086 calc. 532.0088). Fragment ions were observed at M – 31 (OCH_3), M – 59 ($COOCH_3$), M – 78 (C_6H_6), M – 80 (HBr), M – 111 (OCH_3 + HBr or $HOCH_3$ + Br), M – 139 (CH_3COO + HBr or CH_3COOH + Br).

1 (0.1 mg.) was stereoisomerized by stirring in benzene solution for 20 h in daylight with a catalytic amount of I_2. Paper chromatography on S&S 287 (benzene) showed the formation of a *neo* A isomer (15 %); λ_{max} (acetone) 420, 442 and 468 nm; $R_F = 0.89$.

Attempted acetylation of 1. *1* (0.2 mg) in dry pyridine was treated with acetic anhydride (0.2 ml) at room temperature; pigment recovery was 90 %. No new products were formed.

Lithium aluminium hydride reduction of 1. *1* (0.2 mg) partially dissolved in ether (3 ml) was reduced with $LiAlH_4$ for 5 min; pigment recovery 80 %. Traces of a new product were observed.

1 dissolved in tetrahydrofuran (THF) was in two experiments reacted with $LiAlH_4$ at room temperature with pigment recoveries 25 – 42 %.

1 (0.28 mg) in THF (10 ml) was reacted with $LiAlH_4$ for 2 h at −40°C; pigment recovery was 62 %. The reaction mixture contained unreacted *1*, a product inseparable from the $NaBH_4$-reduction product described below and a product which after purification on TLC had λ_{max} ($CHCl_3$) 415, 436, and 450 nm. The latter product was acetylated with acetic anhydride in pyridine to a less polar product with R_F-value near that of *1*.

Sodium borohydride reduction of 1. *1* (0.87 mg) in benzene (3 ml) and ethanol (3 ml) was treated with $NaBH_4$ for 12 h at 20°C; pigment recovery 40 %. After work-up in the usual manner, chromatography on a cellulose column gave *1* (30 %) and a product (70 %) more strongly adsorbed. The product was separated into two zones on kieselguhr paper (benzene); $R_F = 0.32$ (minor), λ_{max} (415), 437, and (465) nm in acetone and $R_F = 0.45$ (major), λ_{max} (420), 443, and (469) nm in acetone and (428), 453, and 480 nm in $CHCl_3$. The main product, further purified by TLC, appeared on mass spectrometry to have molecular ions at *m/e* 466, 468 (1:1).

Permanganate oxidation of 1. *1* (8 mg) in a benzene (5 ml) and water (5 ml) mixture containing $KMnO_4$ (90 mg) and $CaCO_3$ (140 mg) was refluxed for 10 h and the reaction mixture worked up according to the procedure described by Karrer *et al.*[20] About 60 % of starting material was recovered. The acid fraction was purified by GLC and the mass spectrum obtained; *m/e* 232, 230 (1:1, $M = C_8H_7BrO_3$, obs. 231.9557, calc. 231.9555 for ^{81}Br ion, 217, 215 (M − 15), 215, 213 (M − 17), 187, 185 (M − 45) and 161, 159 (M − 71).

1 (5 mg) was oxidized as described above in acid solution (0.1 N H_2SO_4). After 1 h of refluxing, the benzene layer was totally decolorized and the solution worked up. The organic acid was isolated, purified and analyzed by mass spectrometry. The spectrum was identical to that obtained for the product from the above oxidation.

Ozonization of 1 *and* 2. A mixture of *1* and *2* (10 mg) in acetic acid (2.5 ml) and $CHCl_3$ (2.5 ml) at 0°C was subjected to a 3 % stream of ozone for 4 min. The ozonides were oxidatively decomposed with H_2O_2 (30 %), water added and the organic acids extracted into ether. The ether solution was extracted with an aqueous sodium carbonate solution from which the organic acids were again extracted with ether after acidification with H_3PO_4. Mass spectrometry revealed ions at *m/e* 232, 230 (1:1, $M = C_8H_7BrO_3$), 217, 215 (M − 15), 215, 213 (M − 17), 187, 185 (1:1, M − 45); 218, 216 (1:1, $M' = C_7H_5BrO_3$) and 173, 171 (1:1, M′ − 45).

Hydrolysis of 1 *and* 2 *to the corresponding carboxylic acids.* A mixture of *1* and *2* (0.8 mg) in MeOH–$CHCl_3$ (1:1, 5 ml) containing 10 % KOH was stirred at room temperature for 5 h. After the addition of water and $CHCl_3$ the pigments were entirely in the aqueous phase. Dilute acetic acid was added until the aqueous layer was adjusted to pH 7.3 and the pigments extracted into $CHCl_3$. The organic extracts were dried with Na_2SO_4 and the solvent evaporated under vacuum. Mass spectrometry showed ions corresponding to $C_{24}H_{22}Br_2O_3$ and $C_{23}H_{20}Br_2O_3$, compatible with the expected carboxylic acids. The mixed carboxylic acids had λ_{max} (MeOH) 419, 443, and 472 nm.

In several other experiments where $CHCl_3$ was omitted, no hydrolysis of *1* or *2* was achieved.

Model compounds

Methyl 7-(3,5-dibromo-4-methoxyphenyl)-heptatrienoate (5). 3,5-Dibromo-4-methoxybenzaldehyde was prepared and characterized according to the procedure described by Andrewes.[17] Reaction with the phosphoran derived from methyl sorbate [17] gave *5*; m.p. 142 – 144°C; λ_{max} (MeOH) 325 shoulder, 337, 350 nm, (ether) 322, 338, 352 nm; ν_{max} (KBr) 3020 – 2840 (CH), 1708 (C=O), 1604 (C=C), 1270 (OCH_3), 1140 (C – O), 1000 (*trans*-disubstituted double bonds), 875 (aromatic C – H); τ ($CDCl_3$) 6.22 (3 H, OCH_3 on ring), 6.09 (3 H, OCH_3 in ester), 4.2 – 2.8 (6 H, olefinic protons), 2.43 (2 H, aromatic protons); *m/e* triple peaks centered at 402 (M), 387 (M − 15), 371 (M − 31), 343 (M − 59), no ions at M − 79, M − 80, M − 81 or M − 82.

Permanganate oxidation of 5. *5* (50 mg) in benzene (25 ml) and water (25 ml containing $KMnO_4$ (250 mg) and H_2SO_4 (0.1 N) was refluxed for 1 h. The cooled mixture was extracted with ether and the organic acid transferred into an aqueous solution of 5 % K_2CO_3. The aqueous solution was acidified with H_3PO_4 (0.5 N) and the organic acid transferred to ether. After drying the organic extracts with Na_2SO_4 and concentrating under vacuum, mass spectrometry of the residue showed a molecular ion as a triple peak at *m/e* 310, 312, 314 (M, $C_8H_6Br_2O_3$) and no ion corresponding to $C_8H_7BrO_3$.

Methyl 3-(2,4-dibromo-5-methoxyphenyl)-acrylate (6). 3-Hydroxybenzaldehyde was brominated according to the procedure described by Brink [42] and methylated using the procedure described by Lindemann.[43] The resulting 3,5-dibromo-4-methoxybenzaldehyde was added to a stirred methanolic solution of ethyl diethylphosphonoacetate [44] and NaOMe. After 20 h at 40°C, water was added and the mixture extracted with ether. The ether extracts were concentrated under vacuum and chromatographed on a deactivated Al_2O_3 column to give *6*; m.p. 162–164°C; λ_{max} (MeOH) 283, 327 nm; ν_{max} (KBr) 3095–2840 (C–H), 1710 (C=O), 1240 (OCH_3), 1170 (C–O), 870 (aromatic C–H); τ($CDCl_3$) 6.17 (3 H, OCH_3 on ring), 6.08 (3 H, OCH_3 in ester), 3.62 d (1 H, J = 16 Hz, H-1), 2.93 (1 H, aromatic, H-6), 2.23 (1 H, aromatic, H-3), 2.05 d (1 H, J = 16 Hz, H-3); *m/e* 348, 350, 352 (1:2:1, M), 319 (M–31) and 269, 271 (M–Br).

Permanganate oxidation of 6. *6* (50 mg) was oxidized with $KMnO_4$ as described above. Mass spectrometry of the product showed a molecular ion as a triple peak corresponding to $C_8H_6Br_2O_3$.

Methyl-7-(2,4-dibromo-5-methoxyphenyl)-heptatrienoate (7). 3,5-Dibromo-4-methoxybenzaldehyde was reacted with the phosphoran derived from methyl sorbate.[17] After work-up and purification by column chromatography on deactivated Al_2O_3, *6* crystallized from MeOH-$CHCl_3$ solution melted at 154–156°C; λ_{max} (MeOH) 352 nm, (broad band with inflection at 352 nm), (ether) 352 nm, (broad band with inflections at 322 and 337 nm); ν_{max} (KBr) 3075–2840 (C–H), 1710 (C=O), 1625 (C=C), 1135 (C–O), 1010, 1050 (*trans*-disubstituted double bonds), 880, 858, 820 (aromatic C–H) cm^{-1}; τ($CDCl_3$) 6.23 (3 H, OCH_3 on ring), 6.08 (3 H, OCH_3 in ester), 4.05 d (1 H, J = 15 Hz, H–2), 3.5–3.0 (5 H, olefinic protons), 2.95 (1 H, aromatic H-6), 2.17 (1 H, aromatic H-3); *m/e* triple peak centered at 402 (M), 385 (M–15), 371 (M–31), 329, 331 (1:1, M–79), 289, 291 (1:1, M–31–80 or M–32–79), 281 (M–123) and 265 (M–138).

Cortisalin (3a). Methylation of *3a* with diazomethane under the conditions used for *2* gave no product.

Dimethylcortisalin (3b). *3b* had λ_{max} (pyridine) at 418, 443, and 466 nm; ν_{max} see Fig. 3. Attempted hydrolysis of *3b* (0.3 mg) in 10 % methanolic KOH overnight at room temperature or at reflux temperature for 2 h failed.

Methyl 17-(3,5-dibromo-4-methoxyphenyl)-heptadeca-2,4,6,8,10,12,14,16-heptaenoate.[17] On co-chromatography with *1* on kieselguhr paper or silicagel plates (*cf.* Table 1), no separation was achieved.

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