The Pigments of *Sarcina flava*: a new series of C₅₀ Carotenoids

By D. THIRKELL AND R. H. C. STRANG

Department of Biochemistry, Bute Medical Buildings, St Andrews, Fife

AND J. R. CHAPMAN

Consultant Laboratory, A.E.I., Manchester

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SUMMARY

The carotenoids of *Sarcina flava* were examined; four main fractions were separated. These were hydrocarbons, mono- and di-hydroxylated compounds and a very polar fraction. Further separation of the hydrocarbon fraction showed the presence of nine compounds and of the very polar fraction which showed an all-*trans* form and three *cis* isomers. Data for these fractions is presented; at least two of the fractions are C₅₀ carotenoids.

INTRODUCTION

After showing that *Sarcina flava* and *S. lutea* apparently synthesize identical pigments (Thirkell & Strang, 1967), the following work was undertaken to examine the chemical nature of these carotenoids. Most carotenoids which have been reported to occur in bacteria have 40 carbon atoms, but recently a novel C₅₀ carotenoid has been reported in *Flavobacterium dehydrogenans* (Jensen & Weeks, 1966). The work reported here shows the existence of C₅₀ carotenoids in another Gram-positive organism, *S. flava*.

METHODS

Bacteria. Large quantities of *Sarcina flava* (NTCC 7503) were grown on nutrient agar in large oblong aluminium dishes. The bacteria were harvested and stored at –20° until used.

Chemicals. All solvents used were Analar grade and were dried and redistilled before use. For spectrophotometric work, spectral quality solvents were used.

Pigment extraction and purification. Method (a) of Thirkell & Strang (1967) was used and the total pigment obtained was first purified by the lipid precipitation technique of Blessin (1962). The ether of the resultant supernatant fluid was removed on a rotary evaporator under reduced pressure at 35°. The pigment was dissolved in methanol, made 10% (w/v) with respect to KOH and saponified overnight at room temperature in the dark and in an atmosphere of nitrogen. The unsaponifiable material was extracted in the usual way.

Isolation of the individual pigment fractions. Preparative thin-layer chromatography on silica gel G (Merck) was used: 0.5 mm. layers on 200 × 200 mm. plates were strip-loaded by using the mechanical applicator produced by Desaga (Camlab (Glass) Ltd.). The plates were developed in chloroform + methanol (95 + 5, v/v); this separated four main fractions. The solvent used in the previous work (Thirkell & Strang, 1967) was not used in case the presence of a small concentration of acetic acid should induce the formation of isomers of certain fractions.

Tests used for characterization of fractions

Acetylation (Kuhn & Sorensen, 1938; Jensen, 1962). A sample of chromatographically pure material was taken to dryness on a rotary evaporator, and further dried under high vacuum for 30 min. The fraction was dissolved in 1 ml. pyridine which had been dried over NaOH pellets and redistilled; 0.1–0.2 ml. acetic anhydride was added and the reaction allowed to proceed at room temperature in the dark under an atmosphere of nitrogen. Samples were withdrawn at regular intervals in order to follow the course of the reaction chromatographically over a period of 24 hr, by which time it was complete. The chromatography was done on 18.5 cm. diameter kieselguhr-filled circular Schleicher & Schüll chromatography papers (Jensen & Jensen, 1959), using acetone and light petroleum (b.p. 60–80°) in various ratios as solvent. Thus the initial compound, the formation of intermediates during the reaction, and the formation of the final ester was followed. The ester formed was recovered into ether, and the ether thoroughly washed with water to remove pyridine. Spectrophotometric examination was then used to test that no degradation had occurred and to estimate the yield.

Test for tertiary hydroxyl groups after acetylation (S. L. Jensen, private communication). A sample of dry ester from acetylation was dissolved in 0.5 ml. dry pyridine and 0.2 ml. hexamethyl-disilane + 0.1 ml. trimethylchlorosilane added. The reaction proceeded at room temperature in an atmosphere of nitrogen. After 1 hr, carbon tetrachloride was added and the solution taken to dryness which removed the excess silane added. The product was dissolved in methanol and examined on kieselguhr-filled papers as before. The presence of a compound other than the substrate would be indicative of the formation of a silane from a tertiary hydroxyl group.

Reduction of esters with LiAlH_4 (Goodwin, 1956). A small quantity of LiAlH_4 was suspended in dry ether and the suspension filtered through glass wool to remove the larger particles. The filtered suspension was added to an ethereal solution of the ester in a separating funnel. After a short time, the reaction was terminated by adding wet ether. The products were removed, and excess LiAlH_4 destroyed by adding water. Chromatography on kieselguhr-filled papers was again used to follow the reaction. The appearance of products other than the ester or original fraction would indicate that reducible groups were present.

Oxidation with nickel peroxide (S. L. Jensen, private communication). The fraction was dissolved in dry ether and nickel peroxide added in the ratio 5 mg. nickel peroxide to 1 mg. sample. The reaction occurs spontaneously at room temperature under nitrogen. Samples were taken at intervals, and the course of the reaction followed chromatographically as before. The test is specific for the presence of allylic hydroxyl groups.

Iodine isomerization (Zechmeister & Polgár, 1943). To a solution of the fraction in acetone, a drop of iodine solution in hexane (10 $\mu\text{g./ml.}$) was added. The mixture was exposed to weak sunlight for 2–3 hr, samples were taken at intervals and the course of the reaction followed as before. When the pseudo-equilibrium was reached, the positions of the isomers were cut from the papers which were eluted with methanol, and the spectra and relative proportions determined.

Methylation of carboxyl group (Metcalf & Schmitz, 1961). A sample of the fraction under test was dried as for acetylation and dissolved in moisture-free methanol.

0.5 ml. methanolic boron trifluoride was then added and the reaction mixture refluxed for 2–3 min. The product was recovered into ether and examined chromatographically and spectrophotometrically.

Partition ratio (Petracek & Zechmeister, 1956). A mixture of methanol + water (95 + 5, v/v) and hexane was equilibrated and the sample was taken up in a known volume of one of them and transferred to a 10 ml. measuring cylinder. The sample was partitioned between equal volumes of both solvents and the two layers collected. The concentration of pigment in each layer was estimated spectrophotometrically.

Infrared spectroscopy. This was done with a Unicam SP 200 G instrument, by using either liquid cells and solutions in carbon tetrachloride or carbon disulphide, or KBr discs (approximately 50 µg. sample/90 mg. dry KBr).

Mass spectrometry. The sample was dissolved in the minimum volume of methylene dichloride and the solution spotted onto a ceramic direct insertion probe which was inserted through a vacuum lock into the ion chamber of an Associated Electrical Industries' MS 9 mass spectrometer. The sample was heated by contact with the walls of the ion chamber to achieve a reasonable rate of evaporation, and the mass spectrum scanned with the resolving power set at 1000. The temperature of the ion chamber was about 250°. Masses of the peaks were measured very accurately at a resolving power of 12,000.

RESULTS

The results of analysis of the four main fractions were as follows.

Fraction 1 (5% of the total pigments). With chloroform + methanol (95 + 5, v/v) as solvent, this fraction ran with the solvent front. It was rechromatographed on silica-loaded papers (Whatman SG 81) with light petroleum (b.p. 60–80°) as solvent. This resolved the fraction into nine bands as documented in Table 1.

Table 1. *The nature of the carotene fractions resolved from fraction 1 by circular paper chromatography on silica-loaded papers (Whatman SG 81) using light petroleum (b.p. 60–80°) as solvent*

The partition ratio of each fraction between methanol + water (95 + 5, v/v) and hexane, was 0/100.

Band	Type	R_F value	Colour	$\lambda_{\max}$ in methanol	Comment
F 1 (a)	Fluorescent	0.48	Colourless	—	Early carotene precursor?
(b)	Fluorescent	0.40	Colourless	—	Early carotene precursor?
(c)	Absorbent	0.28	Yellow	392, 424, 448	?
(d)	Fluorescent	0.17	Colourless	—	Very low concentration
(e)	Absorbent	0.12	Yellow	408, 431, 450	Unknown carotene
(f)	Absorbent	0.11	Yellow	406, 428, 450	Isomer of F 1 (e)
(g)*	Absorbent	0.07	Yellow	395, 415, 438	Unknown carotene
(h)*	Absorbent	0.06	Yellow	394, 415, 438	Isomer of F 1 (g)
(i)†	Absorbent	0.03	Yellow	414, 438, 469	Sarcinene?

* Subfractions (g) and (h) did not separate completely but gave an elongated egg-shaped spot.

† The $\lambda_{\max}$ of F 1 (i) are consistent with the presence of a chromophore containing nine conjugated double bonds. This is probably the parent hydrocarbon of the polar carotenoids in this bacterium.

Fraction 2 (8% of the total pigments). The results of the tests of this fraction are shown in Table 2. Some data obtained for this fraction and its derivatives from kieselguhr-filled circular chromatography papers are shown in Table 3.

Fraction 3 (26 % of the total pigments). The results of tests on this fraction are shown in Table 4. Some data obtained for this fraction and its derivatives from kieselguhr-filled circular chromatography papers are shown in Table 5.

Table 2. *The results of chemical tests carried out on fraction 2 in an attempt to gain information as to its nature*

Test	Result	Comment
Acetylation	Max. number of spots detected during reaction—2 (original compound + an ester) Final compound formed—monoester	Single hydroxyl group present
Tertiary hydroxyl group	Negative	No tertiary hydroxyl group present
Reduction with LiAlH_4	Negative	No other reducible group present
Oxidation with nickel peroxide	One α - β unsaturated aldehyde formed	One allylic hydroxyl group separated from the polyene chain
Iodine isomerization	One isomer induced (Neo U) (20 % of original converted to isomer)	—
Partition ratio	27:73	Polar compound, value consistent with monohydroxyl compound
Infrared spectroscopy	No <i>cis</i> peaks; peak at 1050 cm^{-1}	All <i>trans</i> form; primary hydroxyl group
Mass spectrometry	Parent ion at m/e 686; mass measurement gave the empirical formula as $\text{C}_{50}\text{H}_{70}\text{O}$	C_{50} carotenoid fraction

Table 3. *Data obtained for fraction 2 and its derivatives from separation on kieselguhr-filled circular chromatography papers*

The solvent used was acetone+light petroleum (b.p. $60\text{--}80^\circ$; 2+98, v/v).

Sample	R_F value	λ_{max} in methanol ($m\mu$)
All <i>trans</i> fraction 2	0.74	—, 415, 439, 469
Neo U isomer	0.61	331, 413, 437, 466
Monoacetate	0.95	—, 415, 439, 469

Fraction 4 (61 % of the total pigments). This polar fraction from the chloroform + methanol (95 + 5, v/v) separation was further separated on kieselguhr-filled circular chromatography papers into four fractions by using 40 % acetone in light petroleum (b.p. $60\text{--}80^\circ$) as solvent. The four subfractions were: fraction 4 (*a*), neo B isomer of fraction 4 (*c*) (6.5 % of fraction 4); fraction 4 (*b*), neo A isomer of fraction 4 (*c*) (10.5 % of fraction 4); fraction 4 (*c*), all *trans* compound (53.0 % of fraction 4); fraction 4 (*d*), neo U isomer of fraction 4 (*c*) (30.0 % of fraction 4). Fraction 4 (*c*) was examined in more detail and the results are given in Table 6. Some data obtained for this fraction and its derivatives from kieselguhr-filled circular chromatography papers are given in Table 7.

Table 4. *The results of chemical tests carried out on fraction 3 in an attempt to gain information as to its nature*

Test	Result	Comment
Acetylation	Max. number of spots detected during reaction—3 (original compound, mono- and di-ester). Final compound formed—di-ester	Two hydroxyl groups present
Tertiary hydroxyl group	Negative	No tertiary hydroxyl group present
Reduction with LiAlH_4	Negative	No other reducible group present
Oxidation with nickel peroxide	One mono and one di α - β unsaturated aldehyde formed	Two allylic hydroxyl groups separated from polyene chain
Iodine isomerization	One isomer induced (Neo U) (33 % original converted to the isomer)	—
Partition ratio	71:29	Polar compound, value consistent with the presence of two hydroxyl groups
Infrared spectroscopy	No <i>cis</i> peaks; peak at 1050 cm^{-1}	All <i>trans</i> form; primary hydroxyl groups
Mass spectrometry	Parent ion at $m/e\ 702$; mass measurement gave the empirical formula as $\text{C}_{50}\text{H}_{70}\text{O}_4$	C_{50} carotenoid fraction

Table 5. *Data obtained for fraction 3 and its derivatives from separation on kieselguhr-filled circular chromatography papers*

The solvent used was acetone + light petroleum (b.p. 60 – 80° ; $10+90$, v/v).

Sample	R_F value	λ_{max} in methanol (m μ)
All <i>trans</i> fraction 3	0.50	—, 415, 439, 469
Neo U isomer	0.42	331, 412, 436, 466
Monoacetate	0.60	—, 415, 439, 469
Diacetate	0.99	—, 415, 439, 469

Table 6. *The results of chemical tests carried out on fraction 4 (c) in an attempt to gain information as to its nature*

Test	Result	Comment
Acetylation	Max. number of spots detected during reaction—7. Final number of spots detected—1	Three hydroxyl groups present
Tertiary hydroxyl group	Negative	No tertiary hydroxyl group present
Reduction with LiAlH_4	Not carried out on this fraction	—
Iodine isomerization	Three isomers were induced: One Neo B isomer One Neo A isomer One Neo U isomer	Equivalent to fraction 4 (a) Equivalent to fraction 4 (b) Equivalent to fraction 4 (d)
Partition ratio	100:0	Very polar compound
Methylation of carboxyl group	Positive	Carboxyl group present?
Infrared spectroscopy	No <i>cis</i> peak. No absolute conclusion could be reached from the traces as to the nature of the hydroxyl groups or as to the presence or absence of a carboxyl group	All <i>trans</i> compound

Table 7. *Data obtained for fraction 4 and its derivatives from separation on kieselguhr-filled circular chromatography papers*

The solvents used were *acetone+light petroleum (b.p. 60–80°; 40+60, v/v) and †acetone+light petroleum (b.p. 60–80°; 10+90, v/v).

Sample	R_F value	$\lambda_{\max}$, in methanol (m μ)
Neo B isomer	0.99*	331, 414, 437, 468
Neo A isomer	0.83*	331, 414, 438, 468
All <i>trans</i> fraction 4 (c)	0.77*	—, 416, 439.5, 469.5
Neo U isomer	0.69*	331, 414, 436, 467
Triacetate	0.67†	—, 416, 439.5, 469.5

DISCUSSION

This present work revealed the presence of a new series of C_{50} carotenoids in *Sarcina flava* (and presumably also in *S. lutea*; Thirkell & Strang, 1967). We suggest that a C_{50} carotene (sarcinene ?; Chargaff & Dieryck, 1932) would be the logical parent compound of the series. As fraction 1 (i) has the same chromophore as the other fractions, it is likely that this is the parent hydrocarbon in question. The addition of a further ten carbon atoms to the normal C_{40} skeleton may make the hydrocarbon relatively more polar as suggested by its low R_F value. Examination of the other compounds in fraction 1 by co-chromatography showed that the normal precursors of the C_{40} carotenoids, e.g. phytoene, phytofluene and neurosporene, were absent. This may suggest an alternative biosynthetic pathway for this series of compounds. On the basis of the evidence presented, it would seem that the parent hydrocarbon is first converted to a monohydroxyl compound (fraction 2), then to a dihydroxyl compound (fraction 3), and finally to a more polar compound (fraction 4).

Aged chloroform has a tendency to form a trace of HCl which could dehydrate allylic alcohols or induce isomerism in carotenoids. The chloroform used in this work was redistilled immediately before use, stored in the dark, and the chromatographic separations, which were reproducible, were done in an inert atmosphere. If *cis* isomers are present, appropriate $\lambda_{\max}$ are detected on a spectrophotometer. Further separation attempted on the fractions revealed the presence of *cis* isomers in fractions 1 and 4. Two subfractions of fraction 1 appeared to be *cis* isomers, but the concentration of these fractions was very low. Fraction 4 was resolved into four subfractions on kieselguhr-filled chromatography papers using the solvent system as reported. Three of these subfractions were *cis* isomers and constituted 47% of fraction 4. Using a different solvent system, Thirkell & Strang (1967) also found *cis* isomers present in the pigments of *Sarcina flava*, and whether or not these *cis* isomers are naturally occurring remains to be elucidated. It is extremely difficult to show that a *cis* isomer is naturally occurring and not induced by a preparative procedure.

Under Dr S. L. Jensen's guidance, fraction 3 was compared with compound P439 from *Flavobacterium dehydrogenans* (Jensen & Weeks, 1966), the only previously reported C_{50} carotenoid. The two compounds gave identical results on chemical examination and neither the original compounds nor their derivatives could be separated by co-chromatography. Whereas compound P439 has an empirical formula of $C_{50}H_{72}O_2$, the mono- and dihydroxyl fractions reported here have their most intense peak in mass spectral analysis corresponding to compounds with 70 hydrogen atoms.

Evidence was also obtained which indicates the co-existence of both an H_{72} and an H_{68} series. Other than mass spectral data, no evidence was found which suggested the presence of more than one carotenoid in fractions 2 and 3.

The nature of fraction 4 is obscure. It forms a triple acetate, indicating three hydroxyl groups, but the R_F value of this ester was still relatively polar and much more so than the esters produced from fractions 2 and 3. This polarity was not due to a tertiary hydroxyl group, since no silane derivative was produced. Some other polar group must be present; the methylation reaction indicated the possibility of a carboxyl group. This was not proved or disproved by infrared analysis. If the hydroxyl groups in fractions 2 and 3 are allylic and primary, hydroxylation of carbons 5 and 5' would be the most likely explanation. It is conceivable that one of these groups could be oxidized to carboxyl and that the three hydroxyl groups of fraction 4 are such that one primary hydroxyl group remains on carbon 5 or 5', and that two others, presumably secondary hydroxyl groups, are substituted elsewhere on a ring.

It is not possible to arrive at the exact structure of any of the fractions but the following may be said. The work of Jensen & Weeks (1966) has suggested the presence of two α -ionone rings in compound P439. Since P439 gave identical chemical results and co-chromatographs with fraction 3, it is reasonable to assume the presence of two α -ionone rings in this series. Fractions 2 and 3 have primary hydroxyl groups (again similar to compound P439). The absorption spectra indicate that the main chromophore has nine conjugated double bonds and that the conjugated system does not extend into the rings. However, hydroxylation of methyl groups substituted on carbons 5 and 5' of the rings may cause a spectral shift and thus definite proof of this proposed chromophore is still lacking. One would assume that the ten extra carbon atoms are derived from the inclusion of two extra isoprene units into the skeleton of these molecules, as compared with C_{40} carotenoids. This inclusion would be at some stage during biosynthesis and since many C_{40} carotenoids are mirror images about the central C 15-15' bond, it is most likely that one C_5 unit is added on each side of this bond. The mass spectra of these compounds are not consistent with the C_5 units being substituted either on the rings or on the central chain of carbon atoms. It may be that the central chain is elongated in some way so that the chromophore of nine conjugated double bonds is not altered.

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