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Bacterial Carotenoids

XXXI.* C_{50}-Carotenoids 5.** Carotenoids of *Halobacterium salinarium*, Especially Bacterioruberin

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Data including silylation, dehydration, NMR and mass spectra leading to revision of the structure of bacterioruberin, the principal carotenoid of red halophilic bacteria, are reported. A C_{50}-tetraol structure (*4*) is ascribed to bacterioruberin. In addition three minor carotenoids with C_{50}-skeletons were isolated from *Halobacterium salinarium*. The monoanhydro-bacterioruberin (*9*) and bisanhydro-bacterioruberin (*10*) structures were assigned to two of these. Dehydration of tertiary carbinols with phosphorus oxychloride in the carotenoid series and polarity data for C_{50}-carotenoids are discussed. Biosynthetic considerations are briefly made.

Early studies on the carotenoid composition of red, obligate and extremely halophilic bacteria by Petter,[1] Lederer,[2] Spruit and Pijper,[3] and Baxter [4] have been reviewed elsewhere.[5,6]

On the basis of previous work in this laboratory the principal carotenoid, α-bacterioruberin, was assigned structure *1* [5–7] at a time when NMR and mass spectrometry were not yet available. The apparent identity of a methylation product with spirilloxanthin (*2*) was of decisive importance.[6,7] However, when the C_{40}-diol (*1*) became synthetically available [8] it was found to differ from α-bacterioruberin. In a re-investigation α-bacterioruberin was found to be a C_{50}-tetraol for which particularly two structures (*3* or *4*) were considered.[9] The prefix α has since been deleted deliberately [9] since this prefix is used to denote α-cyclogeranylidene end groups. In the present paper details of the preliminary communication [9] and further data [10] in favour of structure *4* are reported.

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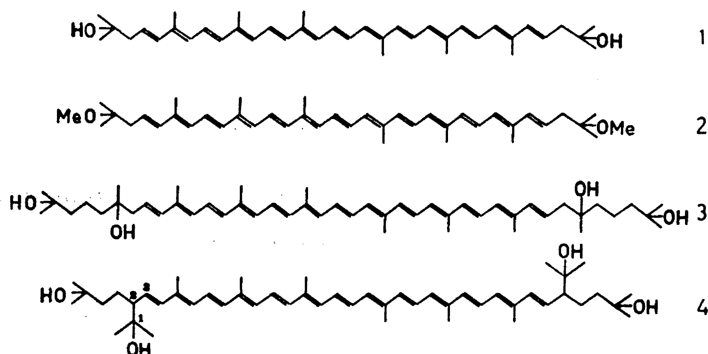


Various minor carotenoids [1,3,4,6] were previously isolated in quantities not permitting structural studies. Data presented here lead to structural assignments of two of these as a C_{50}-triol (*9*) and a C_{50}-diol (*10*, *cf.* Ref. 11).

RESULTS AND DISCUSSION

The same strain of *Halobacterium salinarium* (Harrison et Kennedy) Volcani as previously studied [5,6] was used. For a period during the mass cultivation the temperature rose unintendedly above optimum. This resulted in slow growth and increased yield of minor carotenoids.

Bacterioruberin. The main carotenoid (85 % of total) was identical with bacterioruberin, produced under normal growth conditions.[6] The previously reported physical data for bacterioruberin were confirmed, as was the absence of functional groups susceptible to alkali treatment or forcing hydride reduction – including epoxy groups. In agreement with previous acetylation and infrared evidence all hydroxy groups were found to be tertiary. The detailed course of the silylation [12] and dehydration (with phosphorus oxychloride [13]) reactions showed that four such groups were present, see Fig. 1. Thus the low-temperature silylation reaction was found to comprise four consecutive steps by isolation of three intermediates (or inseparable groups of intermediates) of different degree of silylation. If there are four hydroxy groups in a symmetrical molecule theoretically the two monoethers should give sequentially a mixture of four diethers and a mixture of two triethers. Although isolation of the individual isomers at the same silylation level was not achieved, the second intermediate (diethers) represented a broader chromatographic zone than the first (monoether) and third (triether) intermediate in accordance with the predicted isomer distribution. The three intermediates of different degree of silylation gave the expected products on further silylation or alkaline hydrolysis. Complete hydrolysis of the mono (*5*), di (*6*), tri (*7*), and tetra (*8*) trimethylsilyl ethers gave bacterioruberin (*4*). The dehydration of bacterioruberin likewise produced mono (*9*), bis (*10*), tris (*11*), and tetra (*12*) anhydroderivatives. The extent of dehydration of each derivative was verified by silylation (Fig. 1); for mass spectrometry, see below. No change in the visible spec-

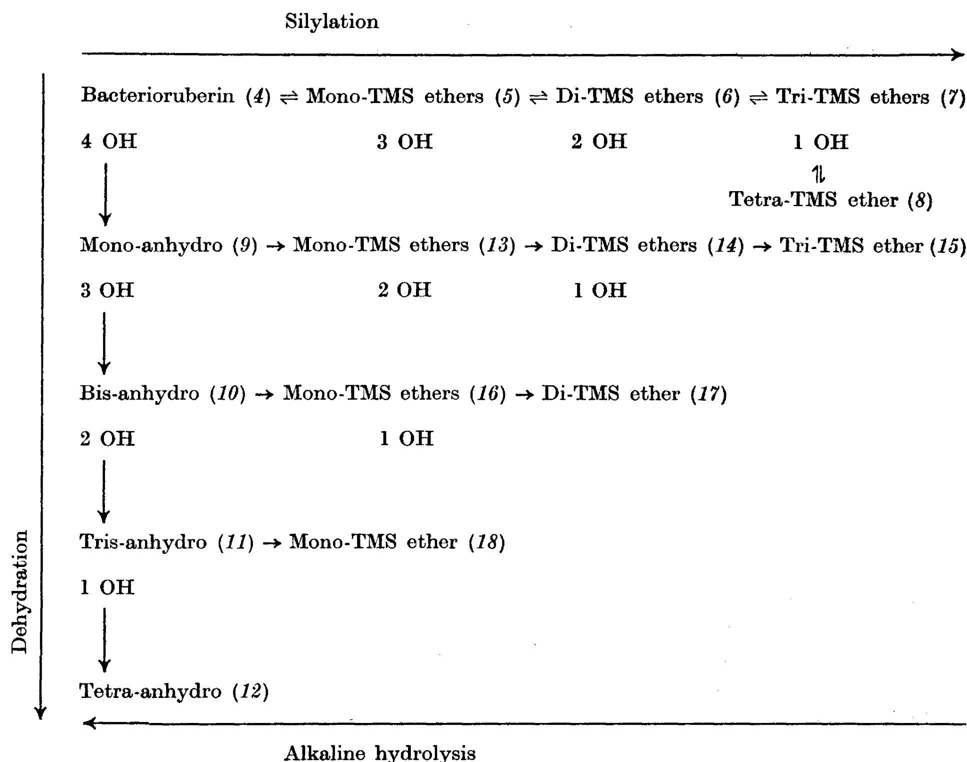


Fig. 1. Reaction scheme for bacterioruberin (*4*), natural bisanhydrobacterioruberin (*10*), and natural trisanhydro-bacterioruberin (*11*).

trum occurred during dehydration confirming the absence of allylic hydroxy groups.

The molecular formula $C_{50}H_{76}O_4$ ($C_{50}H_{72}(OH)_4$) was established by high precision mass spectrometry. Combined with the tridecaene chromophore of the visible spectrum [5,6] this demonstrated an aliphatic C_{50}-skeleton. Symmetrical location of the tridecaene chromophore in bacterioruberin (*4*) was apparent from the NMR spectrum (four in-chain and two in-chain/end-of-chain methyl groups). The degree of branching followed from NMR data, Fig. 2. The presence of altogether fourteen methyl groups, eight of which were attached to tertiary oxygen in very similar magnetic environments, rules out a linearly extended isoprenoid structure like *3*. For structure *4* the four *gem.* methyl groups of the extra C_5-units are expected to be magnetically equivalent, *cf.* rhodopin.[14] The chemical shift position of the two other pairs of *gem.* dimethyl groups (16, 17 and 16′, 17′) may, because of the closer proximity to the polyene chain be slightly different to those in the extra C_5-units, but should because of symmetry be identical in pairs. Slight magnetic non-

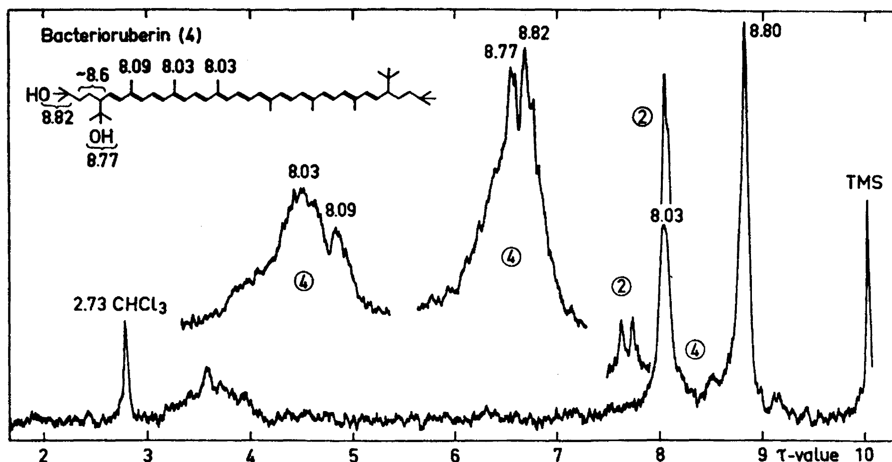


Fig. 2. NMR-spectra in $CDCl_3$ of bacterioruberin (*4*) and spirilloxanthin (*2*).

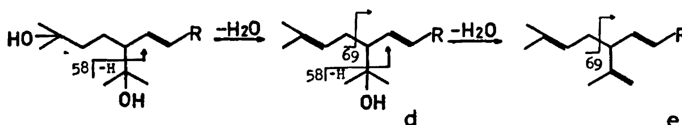
equivalence because of the asymmetric carbon atoms in the 2(2′)-position may be expected within the 16, 17 and 16′, 17′-pairs. The expanded spectrum showed signals at τ 8.77 and 8.82 in intensity ratio 1:1. Further resolution could not be observed because of the noise level. By comparison with the NMR spectrum of spirilloxanthin (*2*, doublet τ 7.70, $J=7$ cps) [15] the absence of allylic methylene groups in bacterioruberin was clearly established, see Fig. 2. The NMR spectrum of the tetra(trimethylsilyl) ether exhibited signals compatible with silylated *4*. Two pairs of magnetically equivalent trimethylsilyl ether groupings were evident (singlets at τ 9.88 and 9.92).

The mass spectrum of bacterioruberin showed only the usual losses of 92, 106, and 158 mass units from the polyene chain,[16,17] demonstrating the absence of extra in-chain substituents.[18] Losses of water up to four times and acetone up to twice (but the total of such losses never exceeding four) from the molecular ion and other major fragment ions are readily accomodated by structure *4; cf.* Ref. 17. Loss of acetone is not expected from structure *3*. Moreover, no fragments caused by cleavage of the doubly activated allylic bonds of *3* were observed. The data presented are considered strong support of structure *4* for bacterioruberin.

The C_{50}-skeleton of bacterioruberin (*4*) was not revealed by the extinction coefficient in visible light. The highest value measured in acetone in the previous study [E(1 cm, 1 %) = 2620][6] was confirmed for the highest melting sample in the present investigation. However, most samples in the present and previous work [6] had E(1 cm, 1 %) around 2250 corresponding to $\varepsilon = 167\ 000$. This is still a high value compared with $\varepsilon = 157\ 000$ in acetone for the synthetic C_{40}-diol (*1*)[8] and $\varepsilon = 155\ 000$ in petroleum ether for spirilloxanthin (*2*).[19]

The course of dehydration will now be considered. Dehydration of 1,1′-dihydroxy-1,2,1′,2′-tetrahydrolycopene by the above method yields, as con-

firmed in the present study and in accordance with Saytzeff's rule, nearly exclusively lycopene.[20] Less than 10 % of a lycopene-like hydrocarbon with one terminal methylene group, chromatographically inseparable from lycopene, was estimated to be present from spectroscopic evidence on dehydration of the synthetic C_{40}-undecaene-diol in our experiments. Likewise dehydration of rhodopin,[21] chlorobactene,[21] OH-spheroidene,[22] and OH-spheroidenone[23] (see Fig. 2) are reported to give the isopropylidene products. However, in order to satisfy the observed chromophore of the anhydro products of bacterioruberin (*4*) the dehydration of the 1-hydroxy group in this case cannot proceed according to Saytzeff's rule. An analogous result has been reported for the dehydration of 5-hydroxy-5,6-dihydro-β-carotene which furnished mainly α-carotene.[24] A still better analogy is found in the dehydration[20] of the C_{40}-diol (*1*)[8] with end groups similar to bacterioruberin (*4*). No extention of the chromophore was observed in this case either and the dehydration products were considered to possess terminal methylene groups.[20] A corresponding result was obtained on dehydration of 3,4-dehydro-rhodopin.[25] The reason why saproxanthin acetate[26] (1′-hydroxy-3-acetoxy-1,1′-dihydro-3′,4′-didehydro-γ-carotene) with one end group in common with the C_{40}-diol (*1*) yielded 3-acetoxy-torulene with extended chromophore is not apparent. Dehydration of the C_{40}-diol (*1*)[20] with end group *b* (*cf.* Fig. 2) appeared to proceed less smoothly than in the cases of end group *a*. Hence the expected sequence and course of the dehydration of bacterioruberin (*4*) is:



the end group *d* being present once in the monoanhydro (*9*) and twice in the bisanhydro (*10*) products, *e* once in the trisanhydro (*11*) and twice in the tetraanhydro (*12*) products. Mass spectra of the trisanhydro (*11*) and tetraanhydro (*12*) compounds were obtained. In support of the above sequence loss of 69 mass units ascribed to cleavage of the triply allylic bond of *e* and doubly allylic bond of *d* were observed in the spectra of both the tris (*11*) and tetraanhydro (*12*) derivatives; loss of 58 (acetone) mass units from the trisanhydro compound only.

In comparison with the previous examination[5–7] only the apparent identity of the methylation product of bacterioruberin (*4*) with spirilloxanthin (*2*) requires explanation. Whereas the products previously considered to be the mono- and dimethyl ethers of bacterioruberin were not available, the major product X of the methylation reaction[6] could be submitted to chromatographic re-examination and silylation. Correlation of polarity data (Table 1) together with the silylation and dehydration results discussed above enables the following explanation to be made.

Product X is a bacterioruberin monomethyl ether or an inseparable mixture of two monomethyl ethers. The product considered to be a C_{40}-monohydroxy-monomethyl ether is a C_{50}-dihydroxy-dimethyl ether or an

Table 1. R_F-values and partition ratios of the carotenoids studied.

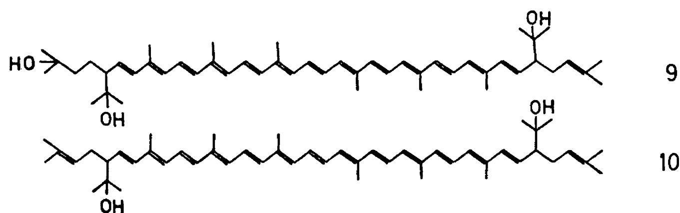
Trans carotenoid	R_F-value								Partition ratio		No. OH-groups	
	Kieselguhr paper					Alumina paper			Petroleum ether 95 % methanol	Petroleum ether 85 % methanol	C_{40}	C_{50}
	20 %[a]	10 %	5 %	2 %	0 %	30 %	20 %	5 %				
C_{40}-diol (*1*)	0.65	0.11				0.73	0.44		18:82		2	
di-TMS-ether				0.44							0	
Spirilloxanthin (*2*)			0.40	0.18					88:12		0	
Bacterioruberin (*4*)	0.43					0.18				3:97		4
mono-TMS ether (*5*)	0.71	0.29	0.07						16:84	60:40		3
di-TMS ether (*6*)		0.44	0.16	0.10					68:32			2
tri-TMS ether (*7*)		0.91	0.66	0.24					100:0			1
tetra-TMS ether (*8*)				0.96					100:0			0
Monoanhydro-bacterioruberin (*9*)	0.70	0.22				0.14			3:97	29:71		3
mono-TMS ether (*13*)		0.46	0.12									2
di-TMS ether (*14*)		0.90	0.61									1
tri-TMS ether (*15*)					0.82							0
Bisanhydro-bacterioruberin (*10*)	0.84	0.45	0.19	0.05			0.73		34:66	85:15		2
mono-TMS ether (*16*)			0.65	0.23								1
di-TMS ether (17)			0.99	0.99	0.71							0
Trisanhydro-bacterioruberin (*11*)		0.83	0.50	0.23			0.66	0.88	84:16			1
mono-TMS ether (*18*)			0.96	0.70								0
Tetraanhydro-bacterioruberin (*12*)			0.92	0.70				0.94				0
C_{50}-diol (*19*)		0.48				0.48	0.25		18:82	60:40		2
Product X[6]	0.59	0.20				0.08			2:8	4:6		(3)
mono-TMS ether		0.46	0.16	0.10								(2)
di-TMS ether		0.94	0.64									(1)
tri-TMS ether			1.00	0.95								(0)
Bacterioruberin "mono" methyl ether [6]		0.39							42:58	95:5		(2)
Bacterioruberin "di" methyl ether [6]		0.76	0.40						22:18			(1)

TMS = trimethylsilyl. [a]Acetone in petroleum ether.

inseparable mixture of four dimethyl ethers and the product previously considered to be the C_{40}-dimethyl ether spirilloxanthin (*2*) is a C_{50}-monohydroxy trimethyl ether or an inseparable mixture of two trimethyl ethers. Since the last compound was produced in at most 2 % yield [6,7] it is not surprising that no tetramethyl ether was isolated.

Since the previous examination of bacterioruberin other cases of apparent identity in partition and chromatographic behaviour, even of stereoisomeric sets, have been experienced, and identity proofs based on these criteria are now considered with scepticism, see below.

Minor carotenoids. The C_{50}-triol to which structure *9* is ascribed comprised 10 % of the total carotenoid. According to the absorption spectrum in visible light the C_{50}-triol possessed the same tridecaene chromophore as bacterioruberin (*4*), but it was less polar (Table 1). Acetylation furnished no acetate. In the silylation reaction three trimethylsilyl ethers were isolated, demonstrating the presence of two (or more) tertiary hydroxyl groups. The mass spectrum of the pertrimethylsilyl ether (*15*), $M = 938$ corresponding to $C_{50}H_{73}(Si(CH_3)_3)_3$ was in agreement with the C_{50}-triol structure (*9*). Dehydration with phosphorus oxychloride gave the peranhydro compound which exhibited a tridecaene chromophore in its visible absorption. Co-chromatography of the stereoisomeric set of this product with that of semi-synthetic tetraanhydro-bacterioruberin (*12*) suggested identity, as did partition ratios (Table 1). Moreover, the natural triol co-chromatographed with semi-synthetic monoanhydro-bacterioruberin (*9*), and in a co-silylation experiment semi-synthetic monoanhydro-bacterioruberin (*9*) a mixture with the natural triol and the natural triol alone behaved identically, giving indistinguishable intermediates and products in the same proportions at any time, see Fig. 1. In spite of repeated chromatography and recrystallization non-carotenoid contaminants in the C_{50}-triol were evident from the NMR spectrum, which also exhibited the signals required by structure *9*, monoanhydro-bacterioruberin. The structure of the hydroxylated end groups derived further support from peaks in the mass spectrum of the tri(trimethylsilyl) ether (*15*) at $M - 131$ and base peak at *m/e* 131 ($(CH_3)_2C = \overset{+}{O} - Si(CH_3)_3$), ascribed to α-cleavage of the trimethylsilyl ether groupings.



The C_{50}-diol, comprising 2 % of the total carotenoid could not be obtained in a pure, crystalline state either. The natural diol was chromatographically identical with semi-synthetic bisanhydro-bacterioruberin (*10*) and possessed an aliphatic tridecaene chromophore. Acetylation gave no acetate and only one intermediate was observed in the silylation reaction leading to the per-

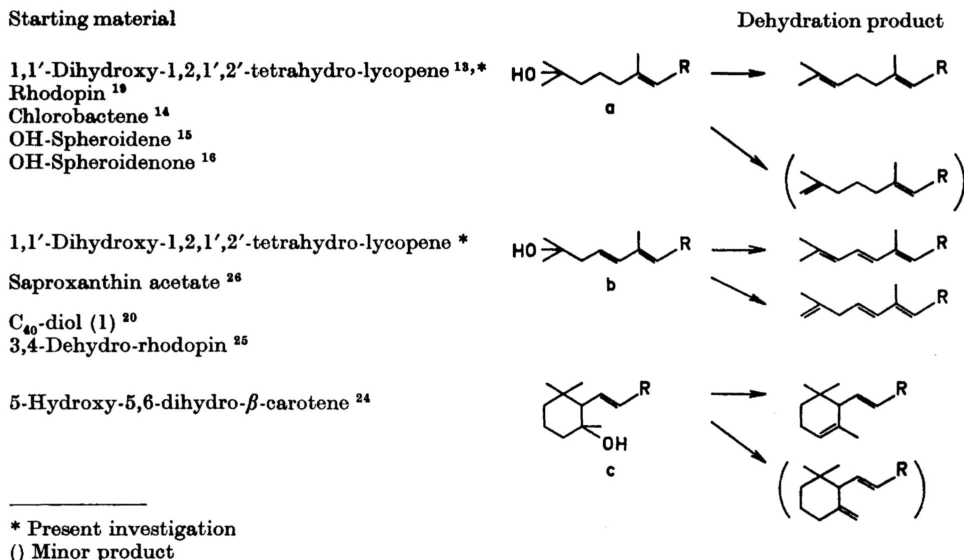


Fig. 3. End group modifications in the carotenoid series effected by dehydration of tertiary carbinols with phosphorus oxychloride in pyridine.

(trimethylsilyl) ether. Likewise only one intermediate was formed on dehydration with phosphorus oxychloride, Fig. 3. Single intermediates in the silylation and dehydration reactions favour a symmetrical diol. The peranhydro compound was chromatographically identical with semi-synthetic tetra-anhydro-bacterioruberin (*12*). Finally direct comparison with natural bis-anhydro-bacterioruberin isolated in the pure state from *Corynebacterium poinsettiae* and assigned structure *10* on the basis of further evidence, including NMR and mass spectra,[11] supported identity.

The structure of another C_{50}-diol (*19*) with aliphatic dodecaene chromophore, comprising 3 % of the total carotenoid, is receiving further attention. This last C_{50}-diol was more polar than Pigment VI of Baxter [4] or OH-482,[6] but less polar than β-bacterioruberin.[1] Identity with Pigment VII of Baxter [4] is questionable.

The mono (*9*) and bisanhydro-bacterioruberin (*10*) do not appear to have been isolated from halophilic bacteria previously.

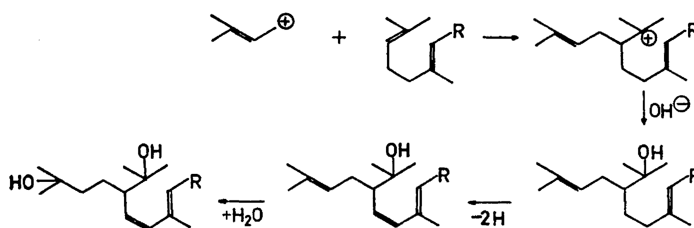
Of the minor carotenoids "390", "450", "OH-450", "OH-472", and "OH-482", previously isolated from cultures of *Halobacterium salinarium* grown with low oxygen supply,[6] only "450" was left in a quantity permitting further spectrometric data to be obtained. Pigment "450" was unexpectedly identified as β-carotene ($C_{40}H_{56}$).

Polarity of C_{50}-carotenoids. In recent years there has been a tendency to draw very detailed conclusions concerning the number and type of functional groups in carotenoids from the partition ratio.[27] Partititon ratios are primarily determined by the functional groups.[28] However, the influence of the chro-

mophore has already been pointed out,[6] and it is of course to be expected that variations of the carbon skeleton should have further influence. The partition data compiled in Table 1 serve to demonstrate the considerably lower polarity of C_{40}-carotenoids than of structurally related C_{50}-carotenoids with the same functional groups. Great caution must therefore be shown in considering partition data without paying attention to the polyene chain and the carbon skeleton.

Likewise chromatographic comparison of C_{40}- and C_{50}-carotenoids may lead to erroneous results, as evident from the discussion above and data compiled in Table 1. Whereas *cis-trans* isomerism and chromophore length are major factors for chromatographic separation on kieselguhr papers, separation on aluminium oxide papers appears to be mainly determined by the number of hydroxyl groups present. The two systems should therefore be used in combination.

Biosynthetic considerations. Although not regularly isoprenoid the new structure (*4*) of bacterioruberin may be considered biosynthetically sound. The skeleton corresponds in principle to those of previously established C_{50}-structures [29–31] and may be considered to be formed by addition of isoprenoid C_5-units to a traditional C_{40}-skeleton with isopropylidene end group:



The isolation of the monoanhydro (*9*) and bisanhydro (*10*) derivatives may indicate that the hydroxy group in the extra C_5-unit is introduced at a late biosynthetic stage.

So far the halophilic bacteria have appeared unique in their carotenoid composition. The isolation of bisanhydro-bacterioruberin (*10*) also from *Corynebacterium poinsettiae* [11] is interesting from a chemotaxonomic point of view.

The isolation of *β*-carotene from cells grown with low oxygen supply reveals the co-occurrence of C_{40}- and C_{50}-carotenoids and demonstrates a limited capability of *Halobacterium salinarium* to synthesize cyclic carotenoids.

EXPERIMENTAL

Methods. When not specified these were as summarized elsewhere.[32] Column chromatography was carried out on Spence type H alumina, activity grade 3. R_F-values and partition ratios of the compounds studied are compiled in Table 1. Values are only tabulated for the *trans* isomers. Carotenoids with the aliphatic tridecaene chromophore are sterically very labile and *cis*-isomerization cannot be prevented. Properties of the bacterioruberin (*4*) stereoisomeric set have been reported previously.[6] Two to four *cis* isomers of each *trans* derivative studied were generally observed on kieselguhr paper. The *cis*-isomers are readily recognized from the double *cis*-peak at 369, 385 nm in petroleum ether and

373, 389 nm in acetone and the parent *trans* compound easily determined by reversible isomerization.[33] Description of the stereoisomeric set of each derivative is not included here.

Biological material. Halobacterium salinarium, strain 1, obtained from the Department of Biochemistry, this University, was cultivated in mass culture (170 l) with strong aeration as described elsewhere.[6] After 1 day the temperature rose by accident to 60°C for a few hours, whereby the growth rate was reduced. The cells were harvested after 7 days (yield 7 g wet weight/l culture; previously found *ca.* 6 g/l [6]), and deepfrozen.

Extraction. Two separate isolations were carried out from 600 g portions. For one portion petroleum ether was completely omitted in the isolation procedure in order to avoid co-precipitation of bacterioruberin with high-molecular hydrocarbons derived from this solvent.[26,9]

The frozen cells were thawed and lysed [35] for 12 h in water suspension. The carotenoids were repeatedly extracted with acetone in a Waring blendor and by standing; yield 1.4 ml/l culture or 0.04 % of the acetone-extracted residue (previously found 0.4–1.5 mg/l).

Saponification. Separate experiments confirmed that the carotenoids present were stable towards alkali. According to previous experience [6] saponification was necessary in order to obtain pure bacterioruberin, and was carried out by the standard method;[32] pigment recovery was 97 %.

Column chromatography. After removal of white contaminants by precipitation with acetone column chromatography of a carbon disulphide concentrate was carried out on alumina. Components present in quantity above 1 % of total carotenoid were eluted as follows: Bisanhydro-bacterioruberin (10, 2 % of total) 10 % ether in benzene, unidentified C_{50}-diol (19, 3 % of total) 1 % methanol in benzene, monoanhydro-bacterioruberin (9, 10 % of total) 2 % methanol in benzene and bacterioruberin (4, 85 % of total) 3–4 % methanol in benzene.

Bacterioruberin (*4*)

Characterization. 4, crystallized from methanol-benzene or acetone-petroleum ether, had m.p. 183°C (previously found 183.5°C [6]), abs. max. in acetone 372, 388, 468, 498 [E(1 cm, 1 %)=2540] and 532 nm. This spectrum corresponds to that previously reported.[6] The IR spectrum (KBr) was also in agreement.[6] Co-chromatography tests (kieselguhr paper and aluminium oxide paper) confirmed identity with bacterioruberin produced under normal growth conditions.[6]

The NMR spectrum had methyl singlets at τ 8.04 (in-chain methyl) and 8.09 (in-chain/end-of-chain methyl) corresponding altogether to 6 methyl groups with intensity ratio *ca.* 2:1, singlet at τ 8.77 and 8.82 (ratio 1:1) corresponing to 8 methyl groups interpreted as 2+2 pairs of *gem.* methyl groups attached to tertiary oxygen.

The mass spectrum had peaks in the upper region at *m/e* 740.5743±0.0040 (M, $C_{50}H_{74}O_4$ required 740.5748), M–18, M–18–18, M–18–18–18, M–18–18–18–18, M–58–18, M–58–18–18, M–58–18–18–18, M–58–58, M–58–58–18, M–58–58–18–18, M–92, M–92–18, M–92–18–18, M–92–58, M–92–58–58, M–92–58–18, M–106, M–106–18, M–106–18–18, M–106–18–18–18, M–106–58, M–106–58–18, M–106–58–18–18, M–106–58–18–18–18, M–106–58–58, M–106–58–58–18, M–158.

Hydride reduction. *4* (0.5 mg) was treated with an excess of lithium aluminium hydride (10 mg) in tetrahydrofuran (1 ml). The reaction was followed by paper chromatography. Isomerization occurred rapidly, but no carotenoid other than *4* was found; pigment recovery was 44 % after 20 h.

Silylation. When carried out at room temperature the reaction was so fast that the mono(trimethylsilyl) ether intermediate (*5*) was overlooked and the tetraether (*8*) was quantitatively formed after 5 min. Silylations were carried out in acetone-CO_2-ice bath at –35°C according to the following procedure: To the carotenoid (1 mg) in pyridine (1 ml) with mechanical stirring was added the silylation reagent (0.1 ml hexamethyldisilazane and 0.05 ml trimethylsilyl chloride in 0.5 ml pyridine). Samples were removed by glass capillaries at intervals and submitted to circular paper chromatography. The coloured zones were eluted with acetone and the carotenoid content determined spectrophotometrically or visually. R_F-values and partition ratios of the products are given in Table 1. Each product contained a set of *trans* and *cis* isomers. The diether (*6*) set

appeared to contain more components, presumably isomers of the position of etherification.

Bacterioruberin (*4*) gave three intermediates and a final pertrimethylsilyl ether (*8*), quantitatively produced after *ca.* 25 min. The time/concentration plots suggested that the intermediates were derived from a consecutive sequence rather than parallel reactions. The first intermediate (monoether *5*) reached its maximum concentration after *ca.* 15 sec at −35°C and was completely reacted after 60 sec. The second intermediate (diether *6*) was at maximum concentration after 10 sec at 20°C and gave on separate silylation the final tetraether (*8*) with the third intermediate (triether *7*) as the only intermediate. The third intermediate (triether *7*) was at maximum after *ca.* 45 sec at 20°C and gave on separate silylation the final tetraether (*8*) and no detectable intermediate.

The tertiary trimethylsilyl ethers were hydrolysed with 5 % KOH in ether-methanol (1:1) at room temperature.[12] The tetraether (*8*) gave bacterioruberin (*4*) and the intermediates described above on hydrolysis. After 18 h only the monoether (*5*) and bacterioruberin (*4*) were left. The triether (*7*) gave after 18 h the diether (*6*, 20 % of total), the monoether (*5*, 40 % of total) and bacterioruberin (*4*, 35 % of total). The diether (*6*) gave the monoether (*5*) and bacterioruberin (*4*), whereas the monoether (*5*) gave only bacterioruberin (*4*).

The tetra(trimethylsilyl) ether (*8*) was synthesised on a preparative scale. Useful conclusions could only be drawn from the part of the NMR spectrum ($CDCl_3$ without internal standard) not influenced by a hydrocarbon contaminant (τ 8.72 and 9.05). *8* exhibited well resolved singlets at τ 8.02 (in-chain methyl), 8.09 (in-chain/end-of-chain methyl), 8.80 (*gem.* methyl at *tert.* OH), 9.88 and 9.92 (trimethylsilyl). No doublet at τ 7.70 was observed. The singlets at (τ 8.02 and 8.09):(τ 9.88 and 9.92) were in intensity ratio 1:2, calc. for *8* 1:2, and the singlets at τ 9.88:9.92 in intensity ratio 1:1 (each signal corresponding to two trimethylsilyl ether groups). For comparison the di(trimethylsilyl) ether groups). For comparison the di(trimethylsilyl) ether of 1,1′-dihydroxy-1,2,1′,2′-tetrahydro-lycopene, prepared [13] from the corresponding diol, obtained from Dr. J. D. Surmatis, exhibited one signal only at τ 9.88 for the trimethylsilyl ether groups. In the upper region of the mass spectrum of *8* significant peaks were observed at *m/e* 1028 (M, corresponding to $C_{50}H_{72}(OSi(CH_3)_3)_4$, M−92, M−106 and M−158 and peaks derived from the silylated end groups at M−15, M−131, M−131−131, M−106−131, M−106−131−131 and *m/e* 131.

Dehydration. Preliminary experiments aiming at demonstrating the total number of intermediates and reaction rates were conducted at room temperature on aliquots (*ca.* 0.5 mg) in dry pyridine (5 ml) with phosphorus oxychloride (0.05 ml) and magnetic stirring at room temperature. A larger scale experiment with *4* (9.2 mg) in pyridine (15 ml) and phosphorus oxychloride (0.4 ml) at room temperature for 45 min, worked up in the usual manner [21] gave after column chromatography 36 % carotenoid recovery, consisting of monoanhydro-bacterioruberin (*9*, 16 % of total), bisanhydro-bacterioruberin (*10*, 31 %), trisanhydro-bacterioruberin (*11*, 35 %), tetraanhydro-bacterioruberin (*12*, 8 %), and unidentified components (7 %). Another experiment with *4* (4.8 mg) in pyridine (10 ml) and phosphorus oxychloride (0.05 ml) for 5 h at 50°C gave after column chromatography 49 % pigment recovery; the tetraanhydro product (*12*) comprised 39 % of the recovered carotenoid.

Monoanhydro-bacterioruberin (*9*) was isolated in least quantity. It was identical in chromatographic behaviour and partition ratio with natural *9*, for which silylation and dehydration studies are described below.

Bisanhydro-bacterioruberin (*10*) was silylated as described above for *4* and gave a mono- (*16*) and di(trimethylsilyl) ether (*17*). The diether (*17*) was formed in quantitative yield after 30 min. Hydrolysis of *12* was effected as described above. After 17 h the reaction mixture contained the diether (*17*, 5 % of total), the monoether (*16*, 10 %), and *10* (85 %).

Trisanhydro-bacterioruberin (*11*) was silylated. Formation of a monoether (*18*) was just detectable after 10 sec and complete in 3 min. No intermediate was detected. The monoether (*18*) was hydrolysed to *11* to an extent of 95 % after 18 h. The mass spectrum of *11* had peaks in the upper mass region at *m/e* 686 (M), M−69, M−106, and M−106−58.

Tetraanhydro-bacterioruberin (*12*) was the ultimate dehydration product. It gave no silyl ether on silylation. The mass spectrum of *12* had peaks in the upper mass region

at *m/e* 668 (M), M−69, M−92, M−106, and M−202 (ascribed to cleavage of the 7,8-bond with hydrogen transfer).

For comparison 1,1′-dihydroxy-1,2,1′,2′-tetrahydro-lycopene (300 mg) in pyridine (50 ml) and phosphorus oxychloride (5 ml) was completely dehydrated at 50°C after 40 min. The product, crystallized from acetone-petroleum ether had melting point 174−175°C, abs. max. (360, 444, 471 and 503 nm, % III/II=76 in petroleum ether, ν_{max} (KBr), τ-values ($CDCl_3$), chromatographic properties before and after iodine catalysed isomerization on kieselguhr paper in addition to abs. max, and abundance of each stereoisomer [6] in agreement with lycopene. However, in the IR spectrum ν_{max} at 880 cm^{-1} was somewhat enhanced relative to lycopene and resonance at τ 5.45 further indicated presence of some terminal methylene, estimated from NMR data to correspond to *ca.* 6 % of a lycopene-type hydrocarbon with one terminal methylene group.

Methylation product X of bacterioruberin (*4*), from the previous investigation,[6] was examined chromatographically. Low-temperature silylation revealed two intermediates in formation of the ultimate silylation product.

A sample of the synthetic C_{40}-diol (*1*), obtained from Professor B.C.L. Weedon,[8] and the di(trimethylsilyl) ether prepared therefrom, were examined chromatographically.

Natural monoanhydro-bacterioruberin (*9*)

The natural triol (*9*), repeatedly chromatographed on alumina and precipitated from methanol-benzene (yield *ca.* 4 mg) exhibited absorption in visible light corresponding to *4*, but contained non-carotenoid impurities. In the NMR spectrum ($CDCl_3$) methyl signals ascribed to the triol were: τ 8.05 (in-chain methyl), 8.34, 8.39 (isopropylidene methyl) and 8.80 (*gem.* methyl at *tert.* hydroxyl). R_F-values and partition ratio corresponded to those of synthetic *9*.

Standard acetylation gave no acetate. Silylation of *9* (0.4 mg) gave after 65 min 88 % pigment recovery. Two intermediates were detected in the formation of the ultimate product (*13*). Co-silylation with synthetic monoanhydro-bacterioruberin (*9*) gave indistinguishable intermediates and products at any time. The ultimate product (*13*) was purified by column chromatography and subjected to mass spectrometry. Significant peaks in the upper mass region were at *m/e* 938 (M), M−15, M−92, M−106, M−120, M−131, M−158, M−120−92, M−106−106; base peak at *m/e* 131.

9 (0.4 mg) in pyridine (0.5 ml) was dehydrated with phosphorus oxychloride (0.05 ml) for 85 min; pigment recovery was 40 %. Two (possibly three) intermediates in addition to the starting material (*9*) and final product were observed. The ultimate dehydration product corresponded in partition behaviour and co-chromatography tests (stereoisomeric set) on aluminium oxide paper to tetraanhydro-bacterioruberin (*12*).

Natural bisanhydro-bacterioruberin (*10*)

The natural diol (*10*), yield 0.5 mg after rechromatography, was not obtained in the crystalline state. Absorption data in visible light corresponded to those of *4*, and R_F-values and partititon ratios to those of synthetic *10* (Table 1) as well as to those of a C_{50}-diol isolated from *Corynebacterium poinsettiae*.[11] The natural C_{50}-diol (*10*) was less polar than the synthetic C_{40}-diol.[6]

The natural diol (*10*) was resistant towards acetylation under standard conditions. Low-temperature silylation gave one intermediate (monosilyl ether, *16*) and a final product (disilyl ether, *15*). The di(trimethylsilyl) ether (*15*) was hydrolysed, giving the diol (*10*) as the only product after 40 h.

Dehydration of *10* gave one reaction intermediate in addition to the ultimate product; pigment recovery was 62 %. The final dehydration product had partition ratio and its stereoisomeric set R_F-values (determined by co-chromatography) identical with those of synthetic tetraanhydrobacterioruberin (*12*), derived from *4* or natural *9* on kieselguhr and aluminium oxide papers.

C_{50}-diol (*19*)

Polarity data (Table 1) only are reported. Further data will be published.

β-Carotene

A sample of "450", left from a previous investigation,[8] exhibited after further purification on alumina columns an absorption spectrum in visible light characteristic of β-carotene, and could not be separated from authentic β-carotene on aluminium oxide paper. The mass spectrum had peaks in the upper mass region at m/e 536 (M), M−92, M−106.

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