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Bacterial Carotenoids

XIX* The Carotenoids of *Mycobacterium phlei* strain Vera. 1. The Structures of the Minor Carotenoids

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Under the cultural conditions specified, *Mycobacterium phlei* strain Vera, produced as minor carotenoids the monocyclic compounds γ-carotene (I), 1′,2′-dihydro-1′-hydroxy-γ-carotene (II), 4-keto-γ-carotene (III), and 1′,2′-dihydro-1′-hydroxy-4-keto-γ-carotene (VI).

The structures of the three latter carotenoids were established by dehydration with phosphorus oxychloride of II to I, and of VI to III, by hydride reduction of VI to 1′,2′-dihydro-4,1′-dihydroxy-γ-carotene (VII) and of III to 4-hydroxy-γ-carotene (IV) and further allylic dehydration of IV to *retro*-dehydro-γ-carotene (V). Finally I, II, III, and VI were directly compared with the corresponding totally synthetic carotenoids, prepared by others; as were IV, V, and VII here prepared from the natural and totally synthetic samples. This is the first finding of II, III, and VI in Nature; only III was obtained in the crystalline state.

The structure of the major carotenoid, here called phlei-xanthophyll, was different from that of myxoxanthophyll, and will be discussed in a later paper.

A great number of carotenoid-producing strains of *Mycobacterium phlei* have been described in the literature, several of which synthesize aryl-carotenoids (*e.g.* Refs. 1, 2).

According to the investigations of Schlegel,[3,4] the bright red *M. phlei* strain Vera differs in carotenoid composition from the other strains examined. The main carotenoid synthesized by strain Vera was tentatively identified by Schlegel[3] as myxoxanthophyll, a poly-hydroxy carotenoid previously considered characteristic of blue-green algae.[5]

The structures of the majority of highly oxygenated carotenoids are unknown, and this project was undertaken primarily to examine the structures of such carotenoids in more detail. However, also three of the minor carotenoids synthesized by *M. phlei* strain Vera proved to be new compounds, and the present report is restricted to the structures of these epiphasic carotenoids.

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Table 1. Carotenoid composition of *M. phlei* strain Vera as established in the present investigation.

Group of carotenoids	Carotenoid	% Of total carotenoid
Epiphasic	γ-Carotene (I)	0.5
	1′,2′-Dihydro-1′-hydroxy-γ-carotene (II)	6
	4-Keto-γ-carotene (III)	5
	1′,2′-Dihydro-1′-hydroxy-4-keto-γ-carotene (VI)	4
Hypophasic	Phlei-xanthophyll	85

RESULTS AND DISCUSSION

Schlegel [3] claimed the occurrence of phytoene, phytofluene, ζ-carotene, neurosporene, lycopene, γ-carotene and β-carotene in the epiphasic fraction in extracts of cells cultivated in the presence of diphenylamine. He also found small amounts of the same compounds (*ca.* 5 % of total carotenoids) in normally grown cells.[4]

The medium recommended by Schlegel [3] was used in our experiments. However, the epiphasic fraction comprised a relatively higher proportion of the total carotenoid and exhibited a different composition, see Table 1. This may be caused by other differences in cultural conditions.

γ-Carotene (I) was identified as such on the basis of absorption spectra in visible light and R_F-values of the individual members of the stereoisomeric mixture produced on iodine catalysis, as directly compared with those of synthetic γ-carotene.

A second, more polar carotenoid with γ-carotene chromophore was also present. Adsorptive properties and partition ratio indicated the presence of one hydroxyl substituent. Because of failure to form an acetate, and of facile dehydration to γ-carotene (I) by treatment with phosphorus oxychloride, the structure II was ascribed to this carotenoid. This conclusion was confirmed by direct comparison with synthetic II, synthesized by Bonnett, Spark and Weedon [6] in another connection.

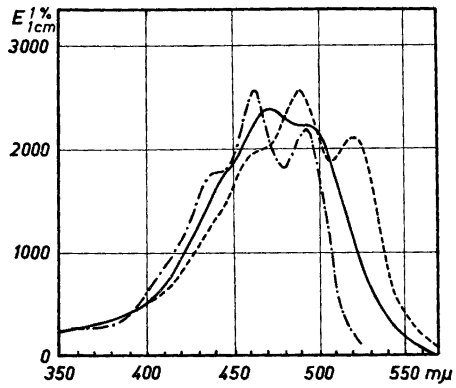


Fig. 1. Absorption spectrum in visible light in acetone solution of *trans* ——— natural 4-keto-γ-carotene (III), — · — 4-hydroxy--γ-carotene (IV), ------ *retro*-dehydro-γ--carotene (V). Ordinate values are on arbitrary basis for the spectra of IV and V.

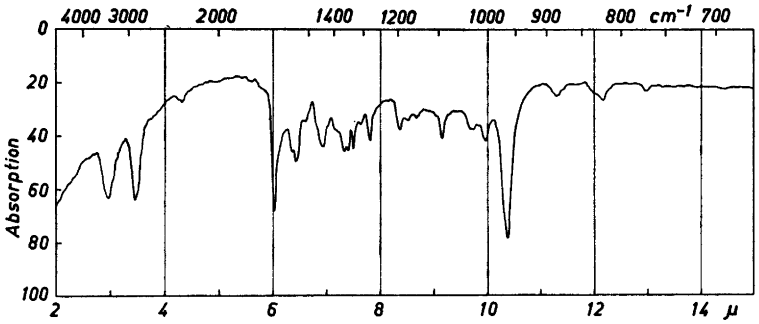
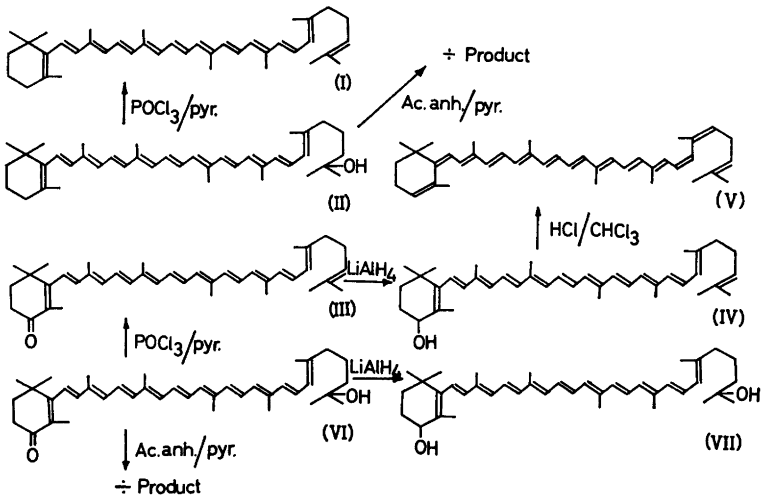


Fig. 2. Infrared absorption spectrum of natural 4-keto-γ-carotene (III) in KBr.

Furthermore, two carotenoids with identical, rather round-shaped absorption spectra in visible light (see Fig. 1, ———), were isolated. The less polar one crystallized as red needles, m.p. 146°C. The round-shaped absorption spectrum indicated the presence of conjugated carbonyl function(s), which was confirmed by the infrared spectrum, see Fig. 2. Upon hydride reduction a mono-ol with γ-carotene-type absorption spectrum was obtained (Fig. 1, —·—). This mono-ol was readily dehydrated with acid chloroform to a product (absorption spectrum Fig. 1, ---) hardly distinguishable from torulene (3′, 4′-dehydro-γ-carotene). The R_F-values for the four members of the stereoisomeric set produced on iodine catalysis were identical to those of torulene. However, the stereoisomers of the dehydration product exhibited absorption maxima in visible light at somewhat shorter wavelengths than those of torulene, see Table 3. The extent of the hypsochromic shift (5 mμ in petroleum ether) of the middle, main absorption maximum of the non-polar keto-carotenoid further suggested the 4-position of the carbonyl group in a cyclohexene ring.[7] On this basis the structure as a 4-keto-γ-carotene (III) was ascribed to the non-polar ketocarotenoid, the hydride reduction product being 4-hydroxy--γ-carotene (IV) and the dehydration product of the latter, *retro*-dehydro--γ-carotene (V). Further proof for structure III was sought in a partial synthesis of IV from γ-carotene (I) by treatment of the latter with N-bromosuccinimide in acetic acid-containing chloroform, according to the method of Karrer and Entschel.[8] β-Carotene was used as model substance and gave isocryptoxanthin (4-hydroxy-β-carotene) in satisfactory yield. However, IV could not be obtained from γ-carotene (I) under similar conditions. The structural assignment was subsequently confirmed by direct comparison with totally synthetic 4-keto-γ-carotene (III) synthesized by Leftwick and Weedon,[9] as well as by a comparative study of its derivatives (IV and V), prepared according to the route outlined below.

The absorptive and partition properties of the more polar derivative indicated the presence of one free hydroxyl group in this carotenoid. Again negative response towards acetylation, and facile dehydration with phosphorus oxychloride to III, secured the structure VI for this compound. In agreement with this inference, hydride reduction resulted in a di-ol (VII). A final verifica-



tion of structure 1′,2′-dihydro-1′-hydroxy-4-keto-γ-carotene (VI) for this carotenoid was obtained by direct comparison with synthetic VI, synthesized by Leftwick and Weedon,[9] as well as by a comparative study of the corresponding di-ols (VII).

The minor carotenoids synthesized by *M. phlei* strain Vera were thus established as the monocyclic C_{40}-carotenoids I, II, III, and VI. This is the first finding of II, III, and VI in Nature.

The major carotenoid synthesized by *M. phlei*, here called phlei-xanthophyll, was different from authentic myxoxanthophyll isolated from *Oscillatoria rubescens*.[10] The structure of phlei-xanthophyll will be discussed in a separate paper.[11]

EXPERIMENTAL

Materials and methods have been described in earlier papers of this series. This refers to reagents and solvents, deactivated alumina for column chromatography, procedure for hydride reduction, and instruments used (visible light absorption spectra were here recorded on a Beckman DB recording spectrophotometer),[12] paper-chromatographic methods and determination of partition ratios.[13] When not otherwise stated, paper chromatography was performed on circular kieselguhr paper, or alternatively on aluminium oxide paper. Iodine catalyzed stereoisomerization and acetylation experiments were carried out as described elsewhere.[14]

Culture. Mycobacterium phlei strain Vera, obtained from Prof. H. G. Schlegel, Institut für Mikrobiologie der Universität, Göttingen, was used.

Medium and cultural conditions. A mass culture (170 l) was grown at 34°C in a fermentation tank of 200 l capacity in the medium recommended by Schlegel.[3] The pH was maintained at 6.7—7.0. The culture was vigorously aerated, and illuminated from above. Light-grown cultures were used as inoculum. The cells were harvested by flotation after 43 h of growth; yield (dry weight) 281 g (determined by drying an aliquot at 105°C).

Carotenoid extraction. The cells were extracted at room temperature with successive portions of acetone/methanol 7:3. After concentration the pigments were transferred to peroxide-free ether-tetrahydrofurane in a separatory funnel by dilution with aqueous sodium chloride solution, followed by saponification for 24 h at room temperature in ether-methanol containing 5 % KOH. The unsaponifiable matter was again transferred

to ether-tetrahydrofurane in the usual manner; spectrophotometrically determined total carotenoid content *ca.* 200 mg or 0.7 % of the dried cells. Separation of the epiphasic and hypophasic pigments was carried out in a simple manner by column chromatography on Linters cellulose powder. The epiphasic carotenoids were washed through the column with petroleum ether while the hypophasic pigments were strongly retained.

Column chromatography. The above eluate was re-chromatographed on a column of deactivated alumina. The individual components are further described below in order of elution from the alumina column.

γ-Carotene (I)

This carotenoid required 2 % acetone-petroleum ether for elution from deactivated alumina. The *trans* isomer had abs. max. in petroleum ether at 362, 435, 460, and 490 mμ. After iodine catalysis the stereoisomers *trans* (0.35), neo A (0.39), and neo B (0.44) were present. R_F-values on aluminium oxide paper (petroleum ether) are given in parentheses.

Co-chromatography with a similarly prepared isomerization mixture of synthetic *trans* γ-carotene gave no separation of the corresponding stereoisomers.

4-Keto-γ-carotene (III)

Adsorptive properties. This pigment required 7 % acetone-petroleum ether for elution from deactivated alumina. The *trans* isomer had $R_F = 0.48$ on kieselguhr paper (2 % acetone-petroleum ether). It was more strongly adsorbed than synthetic echinenone and canthaxanthin in the latter chromatographic system.

Crystallization. After several months at −20°C III was obtained from acetone-petroleum ether as red needles, m.p. 146°C (uncorr.) in an evacuated tube; yield *ca.* 2 mg. The crystals were collected and dried as previously described.[15]

Absorption spectrum in visible light. Absorption maxima in petroleum ether were located at 465 and (490) mμ and in acetone at 471 ($E^{1\,\%}_{1\,cm} = 2380$) and (491) mμ, see Fig. 1.

Infrared spectrum. The spectrum of 0.45 mg natural III in 0.6 g KBr is presented in Fig. 2. Absorption bands were located at 3330 (overtone carbonyl); 2900 (CH), 1660 (conj. carbonyl); (1575), 1555 (conj. double bonds); 1365, 1350 (methyl, *gemini* methyl); 1332, 1272, 1198, 1178, 1155, 1092, 1030, 1005; 962 (*trans* disubst. double bonds), 885 and 823 cm^{-1} (*trans* trisubst. double bonds).

Partition ratio. Found for petroleum ether/95 % methanol, 82:18.

Stereochemical studies. The properties of the iodine-catalyzed equilibrium mixture are listed in Table 2.

Table 2. Properties of the iodine catalyzed equilibrium mixtures of natural and synthetic 4-keto-γ-carotene (III).

Carotenoid	Member of the set	R_F-value 2 % acetone-petroleum ether	In acetone			% Of total carotenoid
			Abs. max. in mμ	% III/II*	% D_B/D_{II}*	
Natural III	*trans*	0.47	471, (491)	0	17	45
	Neo A	0.60	466, (486)	0	18	27
	Neo B	0.69	360, 460, (480)	0	43	28
Synthetic III	*trans*	0.47	471, (491)	0	11	51
	Neo A	0.60	465, (486)	0	23	25
	Neo B	0.69	360, 460, 480	0	37	27

* For definition see Ref. 14.

Table 3. Composition of the iodine catalyzed equilibrium mixtures of *retro*-dehydro-γ-carotene (V) and torulene (3′,4′-dehydro-γ-carotene).

Carotenoid	Member of the set	R_F-value Al_2O_3-paper 2 % acetone-petroleum ether	In acetone					
			Abs. max. in mμ				% III/II	% D_B/D_{II}
Retro-dehydro-γ-carotene V	*trans*	0.53	(380,	(462),	487,	518	19	35
(prepared from natural III)	Neo A	0.62		(458),	481,	512	26	14
	Neo B	0.71						
	Neo C	0.90	380,		477,	(508)	41	0
Retro-dehydro-γ-carotene (V)	*trans*	0.53		463,	487,	519	17	30
(prepared from synthetic III)	Neo A	0.62		460,	482,	512	25	9
	Neo B	0.71						
	Neo C	0.90	380,		480,	510	26	0
Torulene	*trans*	0.53	(385),	465,	492,	525	17	59
	Neo A	0.62		463,	487,	519	28	45
	Neo B	0.71						
	Neo C	0.90	382,	(463),	485,	515	50	11

Hydride reduction of III to IV. Natural III (0.88 mg) in dry ether was reduced with $LiAlH_4$ in the usual manner; pigment recovery 60 %, all of which consisted of IV.

IV had abs. max. at (346), (438), 460, and 490 mμ in petroleum ether, $R_F = 0.62$ on aluminium oxide paper (10% acetone-petroleum ether and partition ratio in petroleum ether/95 % methanol, 74:26.

Allylic dehydration of IV to V with acid chloroform. The method of Karrer and Leumann [16] was used. To IV (0.34 mg) in 2 ml $CHCl_3$ was added 0.8 ml of a 0.07 N HCl/$CHCl_3$-solution. A bathochromic colour shift was immediately observed; pigment recovery 71 %. The de-acidified reaction mixture was chromatographed on a column of deactivated alumina. V required 2—5 % acetone-petroleum ether for elution. The composition of the iodine catalyzed equilibrium mixture of V and of synthetic torulene is given in Table 3.

Comparison between natural and totally synthetic III and between their derivatives IV and V. Absorption spectra of the *trans* isomers in visible light measured in acetone or petroleum ether solution were superimposable. A comparative study of the iodine catalyzed equilibrium mixtures was performed. The result is presented in Table 2. Synthetic III had partition ratio in petroleum ether/95 % methanol 83:17 and the infrared spectrum exhibited the same principal absorption bands as natural III.

1′,2′-Dihydroxy-1′-hydroxy-γ-carotene (II)

Adsorptive properties. This carotenoid required 13 % acetone-petroleum ether for elution from deactivated alumina and had $R_F = 0.61$ on kieselguhr paper (5 % acetone-petroleum ether). It was less strongly adsorbed than rubixanthin (3-hydroxy-γ-carotene) and OH-chlorobactene.[13]

Absorption spectrum in visible light. The *trans* isomer had abs. max. 350, (438), 459, and 488 mμ in petroleum ether with the same spectral shape as γ-carotene.

Partition test. Found for petroleum ether/95 % methanol, 75 :25.

Stereochemical studies. The R_F-values on aluminium oxide paper (10 % acetone-petroleum ether) of the zones obtained after iodine catalysis are listed below: *trans* (0.67), neo A (0.75), and neo B (0.95).

Acetylation test. Natural II (0.1 mg) in 3 ml dry pyridine was treated with 0.1 ml acetic anhydride for 20 h at room temperature. The reaction mixture was worked up in the usual manner; pigment recovery 93 %. Paper-chromatographic examination indicated that no acetylation product was formed.

Dehydration of II to I with phosphorus oxychloride. The method of Surmatis and Ofner [17] was adapted. To natural II (0.1 mg) in 5 ml dry pyridine was added 0.05 ml $POCl_3$. The reaction was carried out for 45 min at 50°C with magnetic stirring; pigment recovery 56 %. The *trans* and neo A isomers of the dehydration product (I) could not be distinguished from those of synthetic- γ-carotene, as shown by co-chromatography tests. The reaction product further exhibited the same absorption spectrum in visible light as synthetic γ-carotene.

Co-chromatography of natural and synthetic II. No separation of the three main stereosomers obtained after iodine catalysis was obtained on aluminium oxide paper.

1',2'-Dihydro-1'-hydroxy-4-keto-γ-carotene (VI)

Adsorptive properties. Natural VI required 15 % acetone-petroleum ether for elution from deactivated alumina. The *trans* isomer had $R_F = 0.54$ on kieselguhr paper (10 % acetone-petroleum ether).

Absorption spectrum in visible light. The spectra in petroleum ether and acetone solutions were identical with those of 4-keto-γ-carotene (III).

Partition ratio. Found for petroleum ether/95 % methanol, 27:73. The partition ratio remained unchanged in alkaline medium.

Stereochemical studies. The composition of the iodine catalyzed equilibrium mixture of natural VI is given in Table 4.

Acetylation test. Natural VI (0.12 mg) in 3 ml dry pyridine was treated with 0.1 ml acetic anhydride for 20 h at room temperature; pigment recovery 70 %. No acetylation products were formed.

Dehydration of VI to III with phosphorus oxychloride. The dehydration of natural VI (0.1 mg) was carried out in the manner described for II above; pigment recovery 64 %. The dehydration product (III) exhibited the same absorption spectrum in visible light as VI, but was less polar. Co-chromatography of the dehydration product and natural III supported identity of these compounds.

Hydride reduction of VI to VII. Natural VI (0.33 mg) in dry ether was reduced with $LiAlH_4$ in the usual manner; pigment recovery 50 %. The reduction product (VII) had a γ-carotene-like absorption spectrum. The *trans* isomer had $R_F = 0.45$ and the neo A isomer $R_F = 0.51$ on kieselguhr paper (10 % acetone-petroleum ether). In petroleum ether/85 % methanol the partition ratio 49:51 was found.

Comparison between natural and synthetic VI and their derivative VII. The composition of the iodine catalyzed equilibrium mixtures as determined by a direct, comparative study, is given in Table 4.

Synthetic VI had partition ratio in petroleum ether/95 % methanol, 25:75.

Hydride reduction of synthetic VI (0.22 mg) gave a pigment recovery of 46 %. The reaction mixture contained exclusively VII; abs. max. (438), 462, and 490 mμ in acetone; partition ratio in petroleum ether/85 % methanol, 51:49. A co-chromatography test on kieselguhr paper of the *trans* and neo A isomers of VII, prepared from the natural and synthetic samples, gave no separation of the corresponding stereoisomers.

Table 4. The composition of the iodine catalyzed equilibrium mixture of natural and synthetic 1',2'-dihydro-1'-hydroxy-4-keto-γ-carotene (VI).

Carotenoid	Member of the set	R_F-value 10 % acetone-petroleum ether	Abs.max. in mμ in acetone
Natural	*trans*	0.68	469, (489)
	Neo A	0.72	368, 460
Synthetic	*trans*	0.68	469, (490)
	Neo A	0.72	368, 460

Phlei-xanthophyll

Phlei-xanthophyll required 25—30 % acetone-petroleum ether for elution from Linters cellulose powder and had $R_F = 0.20$ on kieselguhr paper (30 % acetone-benzene). The crystalline *trans* isomer (m.p. 205°C) had abs. max. at (368), (428), 456, 478, and 509 mμ in acetone.

When co-chromatographed with *trans* myxoxanthophyll (m.p. 172°C) (abs. max. (370, (428), 456, 478, and 509 mμ in acetone) isolated from *Oscillatoria rubescens* [18] on kieselguhr paper (30 % acetone-benzene), phlei-xanthophyll was more strongly retained than myxoxanthophyll ($R_F = 0.40$). The result of iodine catalyzed stereoisomerization revealed that this difference was not caused by *cis-trans* isomerism. These carotenoids further proved to be chemically different.[11]

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