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Bacterial Carotenoids

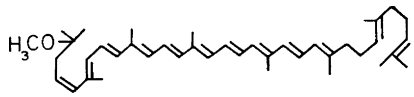
XII. The Constitution of the Minor Carotenoids of *Rhodopseudomonas* 3. OH-Y

SYNNØVE LIAAEN JENSEN

Institutt for Organisk Kjemi, Norges Tekniske Høgskole, Trondheim, Norway

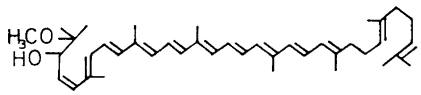
The chemical constitution of OH—Y from *Rhodopseudomonas gelatinosa* has been investigated, and the structure as a 7′,8′-dihydrorhodovibrin (IV) is suggested. The complex carotenoid composition of anaerobic cultures of *R. gelatinosa* has been analyzed, and the components have been identified.

According to the survey of Goodwin [1] a carotenoid designated as hydroxy-Y or OH—Y [2] is present in anaerobically grown cultures of the photosynthetic purple bacteria *R. gelatinosa*, *R. capsulata* and *R. spheroides*. This pigment exhibits the same absorption spectrum in visible light as Y (I), the structure of which has been established by Davis, Jackman, Siddons and Weedon [3].



(I)

The presence of a hydroxyl group in OH—Y has been assumed on the basis of its adsorptive properties and partition behaviour [4]. It can be differentiated from $LiAlH_4$-reduced spheroidenone (II) by a negative response to $HCl—CHCl_3$ treatment [5], which rules out the presence of allylic hydroxyl in

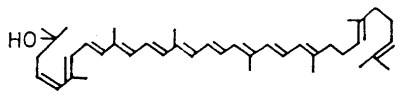


(II)

the molecule. On the basis of biosy thetic considerations we [2,6] have proposed that OH—Y might be a demethylated Y (III).

Table 1. Absorption maxima in visible light of crystalline *trans* Y and *trans* OH—Y, recorded in various solvents.

Solvent	Abs.max in mμ					
	Y			OH—Y		
Petroleum ether, b.p. 60—70°C	429	455	486.5	429	454	486
$CHCl_3$	440	465.5	500	439	465.5	498
Benzene	440.5	467.5	501.5	439	467	501
CS_2	457	486	522	457	486	522



(III)

A confirmation of this suggestion has been attempted in the present investigation.

RESULT AND DISCUSSION

In the present work OH—Y, which is the major carotenoid in photosynthetically grown cultures of *R. gelatinosa*, has been isolated in the crystalline state. OH—Y crystallized from acetone-petroleum ether as tiny needles, m.p. 157—157.5°C. The absorption spectra in visible light, recorded in various solvents, were conform as to shape and absorption maxima with those of Y (I), isolated from the same organism, and with those of $LiAlH_4$-reduced spheroidenone (II). The absorption maxima of Y and OH—Y are given in Table 1, and the absorption spectrum of OH—Y is presented in Fig. 1. The visible light absorption data thus confirm the presence of identical chromophores in Y and OH—Y.

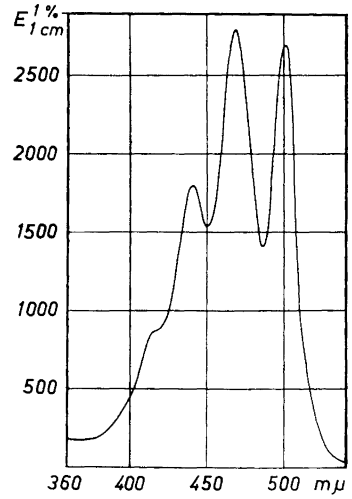


Fig. 1. Absorption spectrum in visible light of *trans* OH—Y in benzene.

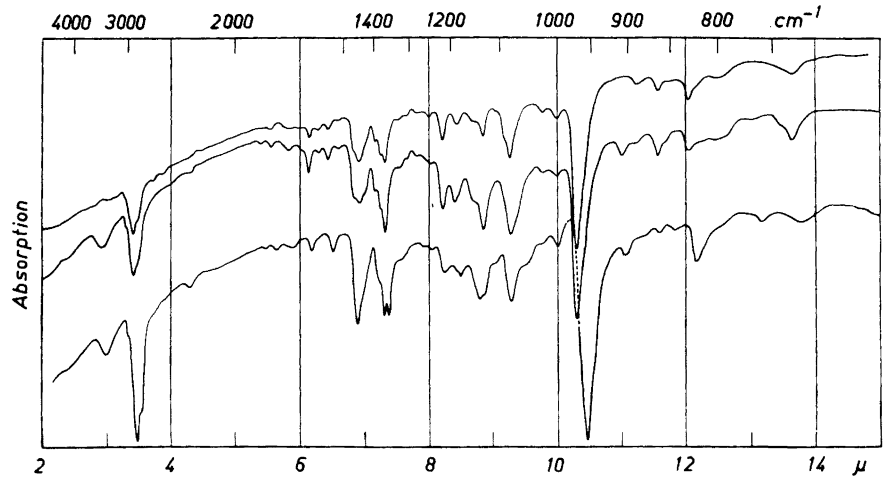
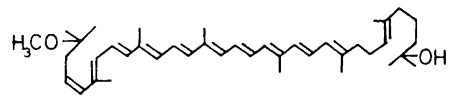


Fig. 2. IR-spectra of Y (0.30 mg in 0.14 g KBr, uppermost curve), OH—Y (0.30 mg in 0.13 g KBr, middle curve) and rhodovibrin [6] (1.2 mg in 0.6 g KBr, lowermost curve).

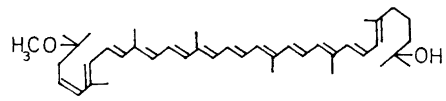
The IR-spectra of (I), OH—Y and $LiAlH_4$-reduced spheroidenone (II) are presented in Fig. 2. Examination of these spectra clearly reveals the presence of a methoxyl group (presumably tertiary, 1078 cm^{-1}) [6] and a tertiary hydroxyl group (3410, 1130, 905 cm^{-1}) [6] in OH—Y. The tertiary character of the hydroxyl group was confirmed by the resistance of OH—Y towards acetylating agents.

We have proposed [2,6] that there are five different types of reactions in the carotenoid transformations of the photosynthetic purple bacteria. On the basis of the spectral data presented above and the fact that Y and OH—Y occur together, it is plausible to assume that OH—Y is formed from Y (I) by a Type 3 reaction, *i.e.* by addition of H_2O to the isopropylidene double bond of Y with the formation of a tertiary alcohol (IV).



(IV)

Structure (IV) would be in good agreement with the role of OH—Y as the immediate biosynthetic precursor of OH—R (2-keto-7′,8′-dihydro-rhodovibrin) [7,8]. OH—Y should then be a 7′,8′-dihydro-derivative of rhodovibrin (V) [9].



(V)

The IR-spectra of OH—Y and rhodovibrin (V) are indeed very similar as can be seen from Fig. 1.

Table 2. Chromatographic resolution of the carotenoid mixture of anaerobically grown *R. gelatinosa* on Woelm neutral aluminium oxide, activity grade 2 [13].

Zone in order of increasing adsorbance	Colour of zone	Required eluant, % acetone **	Identification *
a	yellow	1	neurosporene
b	yellow	2	Y
c	red	3	R, anhydro-rhodovibrin
d	purple	5–7	spirilloxanthin
e	yellow	7	chloroxanthin
f	yellow	9	OH–Y
g	red	10–13	OH–R, rhodovibrin
h	purple	13	monodemethylated spirilloxanthin

* After paper-chromatographic[14] and spectrophotometric examination.
** In pet.ether.

The OH–Y present as a major carotenoid in photosynthetically growing cells of *R. gelatinosa* thus was found to be different from demethylated Y (III) — the hypothetical precursor of Y (I) [2,6]. We have also not been able to detect compound (III) in young anaerobic cultures of *R. gelatinosa*. There is, however, good reason to believe that a similar situation exists as in the case of the P481-group of bacterial carotenoids [9], and that compound (III) represents the third, so far missing, member of the Y-group of bacterial carotenoids.

The carotenoid composition of anaerobic cultures of *R. gelatinosa* has been reinvestigated and the result is presented in Table 2. The presence of small amounts of spheroidenone [3] and OH–R [7] is presumably caused by some exposure to oxygen during cultivation or harvesting. Crystalline Y (I) was obtained, and purity criteria are presented.

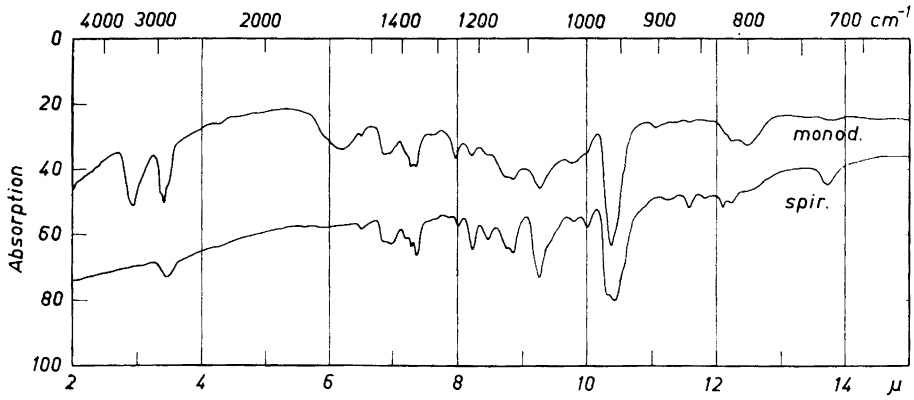
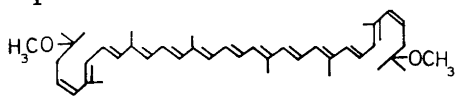


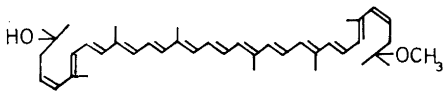
Fig. 3. IR-spectrum of monodemethylated spirilloxanthin (0.20 mg in 0.13 g KBr, upper curve) and of spirilloxanthin (0.40 mg in 0.13 g KBr, lower curve).

Sprilloxanthin (VI) [10,11,6] was isolated in the crystalline state and its identity proved by mixed melting point, co-chromatography tests and spectral analogy (visible light and IR) with synthetic spirilloxanthin.

Pure monodemethylated spirilloxanthin (VII) [10] was obtained crystalline for the first time, m.p. 208.5—209°C. The IR-spectrum is presented in Fig. 3, together with that of spirilloxanthin



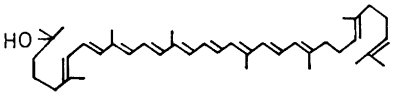
(VI)



(VII)

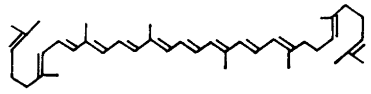
The presence of one methoxyl group (1078 cm^{-1}) and a tertiary hydroxyl group (3400, 1140, 905 cm^{-1}) in monodemethylated spirilloxanthin was evident from the IR-spectrum. On acetylation only about 8 % of monodemethylated spirilloxanthin was affected, confirming the tertiary character of the hydroxyl group.

Crystalline chloroxanthin (VIII) [12,3,6] which hitherto has been isolated only from the green mutant of *R. spheroides* [12], was in the present investigation obtained for the first time from another organism. Its spectra in visible light and IR were identical with those of authentic chloroxanthin, and the two pigments were inseparable when co-chromatographed.



(VIII)

The presence of neurosporene (IX) [3] was confirmed, and finally two carotenoids tentatively identified as anhydro-rhodovibrin (P481) (X) [6,9] and rhodovibrin (V) were shown to be present.



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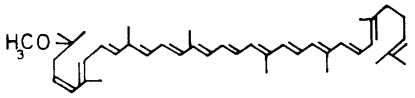

(X)

Provided that a sufficient quantity of cells is analyzed, the distribution pattern of carotenoids in anaerobically grown cultures of *R. gelatinosa* can thus be shown to be much more complex than that reported by Goodwin [1]. The biosynthetic implications of this distribution pattern will be discussed elsewhere [8].

EXPERIMENTAL

Materials and methods have been described in an earlier paper of this series [7].

Culture. The same strain of *R.gelatinosa* as was used for the aerobic cultures in the preceding investigation [7] was employed.

Medium and cultural conditions. The medium was that described previously [7]. Six cultures were grown in 4 glass-stoppered and 2 cotton-stoppered bottles of 5 l capacity, filled to the neck with sterilized medium. Illumination was provided by nine 150 watt lamps mounted about 20 cm from the bottles. The temperature was maintained at 25 – 30°C by water cooling [6]. A growth period of 4 days was allowed.

The inoculum (4 × 0.5 l) was grown in a light cabinet at 25°C for 2 days in glass-stoppered bottles completely filled with medium.

Harvesting of the cells. The cells were harvested as already described [7]. A total of 8 l of $MgSO_4$-solution and 45 l of acetone was used in the pre-treatment of the cells. Care was taken to avoid excess contact with oxygen prior to the acetone treatment.

Pigment extraction, saponification and chromatographic separation were carried out as has been described for the aerobically grown cells [7]. A total volume of 20 l of acetone was used for the pigment extraction. After saponification and prior to chromatographic separation, a carotenoid content of 49.2 mg was estimated spectrophotometrically (using $E^{1\,\%}_{1\,cm} = 2500$ at 456 mμ in pet.ether); *i.e.* 1.62 mg/l culture. A description of the deactivated alumina chromatogram is given in Table 2. The carotenoids were eluted and each mixed fraction was submitted to quantitative paper chromatography [14] prior to the spectrophotometric determination. The extinction coefficients previously employed [6,7] were used. For chloroxanthin the neurosporene value [6] and for OH—Y $E^{1\,\%}_{1\,cm} = 2500$ at 456 mμ in pet. ether were employed. The result of the quantitative determination of the carotenoid mixture is presented in Table 3.

Neurosporene

(from zone a) had abs.max. in pet.ether at 416, 439, and 469 mμ with pronounced fine-structure; $R_F = 0.88$ on the circular paper employed using pet.ether as developer.

Y

Crystallization. Clusters of tiny, ceris red needles were obtained from acetone-pet. ether. After recrystallization from the same solvent system the crystals were collected and dried as previously described [7]; yield 1.0 mg, m.p. sharp at 144.5°C.

In the paper-chromatographic purity test one lemon yellow zone, $R_F = 0.79$ was obtained, using 2 % acetone in pet.ether as developer.

Absorption spectra in visible light of the crystalline specimen were recorded in various solvents, immediately after dissolution. The spectra exhibited pronounced fine-structure. The abs.max. are given in Table 1; $E^{1\,\%}_{1\,cm} = 2840$ at 466.5 mμ in benzene.

IR-spectrum. The spectrum is presented in Fig. 2.

Quantitative partition test carried out according to the method of Petracek and Zechmeister [15] gave as result: Pet.ether/95 % methanol 99:1.

Table 3. Quantitative composition of the carotenoid mixture isolated from anaerobic cultures of *R. gelatinosa*.

Carotenoid	% of total
Neurosporene	1.4
Chloroxanthin	3.3
Y	27.0
R	1.8
OH—Y	41.6
OH—R	4.7
Anhydro-rhodovibrin (P481)	4.2
Rhodovibrin (OH—P481)	1.1
Monodemethylated spirilloxanthin	0.9
Spirilloxanthin	14.3

Spheroidenone

(from zone c) had after paper-chromatographic purification $R_F = 0.86$ in 5 % acetone-pet.ether; abs.max. at 488 mμ in methanol. The spectrum exhibited no fine-structure. Identity with spheroidenone from aerobic cultures of *R. spheroides* [15] was proved by co-chromatography on the usual paper.

Anhydro-rhodovibrin (P481)

(from zone c) had after paper-chromatographic purification $R_F = 0.65$ in 5 % acetone-pet.ether and abs.max. at 375, 455, 483, and 517 mμ in methanol, with pronounced fine-structure. The R_F-value obtained is close to that previously established for anhydro-rhodovibrin from *Rsp. rubrum* [14].

Spirilloxanthin

Crystallization. Spirilloxanthin (from zone d) crystallized from 5 % acetone-pet.ether as irregular violet plates. On further concentration of the mother liquor violet needles crystallized. The crystals were collected as previously described [15]; yield 2.54 mg; m.p. 212–214°C; abs.max. at 464, 493, and 528 mμ in pet.ether. Mixed m.p. determination with synthetic spirilloxanthin m.p. 215°C (kindly provided by Hoffmann-La Roche & Co.) gave m.p. 213–215°C.

Co-chromatography with synthetic spirilloxanthin. The crystalline specimen above gave one single zone on the circular paper chromatogram, and could not be separated from synthetic spirilloxanthin ($R_F = 0.44$ in 5 % acetone-pet.ether) on co-chromatography.

IR-spectrum. The spectrum is presented in Fig. 3.

Chloroxanthin

Crystallization. Chloroxanthin (from zone e) was rechromatographed on deactivated alumina to remove some spirilloxanthin present in the eluted fraction. A semi-crystalline orange precipitate was obtained from acetone-pet.ether. The precipitate was collected by centrifugation, washed and dried [15]; yield 0.25 mg, m.p. 130–132°C.

Co-chromatography with authentic chloroxanthin. In the paper chromatographic purity test the chloroxanthin sample above gave one lemon yellow zone $R_F = 0.48$ in 2 % acetone-pet.ether. No separation from chloroxanthin from the green mutant of *R. spheroides* [12] (pigment kindly provided by Dr. T. O. M. Nakayama, Dept. of Food Technology, University of California, Davis) was achieved on the circular paper chromatogram.

Absorption spectrum in visible light. Abs.max. in pet.ether were located at 417, 440, and 470 mμ, % III/II [6] = 89.

IR-spectrum. The spectrum agreed well with that of chloroxanthin from the green mutant of *R. spheroides* [6].

OH–Y

Crystallization. OH–Y (from zone f) crystallized as single, tiny red needles from acetone-pet.ether. After two recrystallizations from the same solvent system a yield of 9.5 mg was obtained, m.p. 157–157.5°C.

In the paper-chromatographic purity test a single yellow zone was obtained, $R_F = 0.63$ in 5 % acetone-pet.ether.

Solubility. Crystalline OH–Y was readily soluble in pyridine and CS_2, moderately soluble in acetone, $CHCl_3$ and benzene and only slightly soluble in pet.ether.

Absorption spectra in visible light. All spectra were determined as described previously [15] by direct dissolution of the crystalline sample in the various solvents. The result is presented in Table 1 and Fig. 1; $E\,{}^{1\,\%}_{1\,\mathrm{cm}} = 2785$ at 467.5 mμ in benzene.

IR-spectrum. The spectrum is presented in Fig. 2.

Quantitative partition test carried out as above gave as result: Pet.ether/95 % methanol 80:20.

Acetylation test. To 0.5 mg of OH—Y dissolved in 5 ml of dry pyridine was added under nitrogen 0.2 ml of acetic anhydride. After 21 h at room temperature the reaction mixture was worked up in the usual manner. A pigment recovery of 77 % was spectrophotometrically established after chromatography on deactivated alumina. The recovered carotenoid consisted of 99.5 % OH—Y and 0.5 % of a pigment with properties as predicted for the acetate. This had abs.max. at 430, 455, and 586 mμ in pet.ether. For elution from deactivated alumina 5 % acetone in pet.ether was required; R_F = 0.67 and R_F = 0.86 on the usual paper when 2 % acetone-pet.ether and 5 % acetone-pet.ether respectively were used for development of the chromatogram.

Paper-chromatographic examination of the original mother liquor revealed the absence of any other compounds with the same absorption spectrum in visible light as OH—Y.

OH—R

from zone g, also containing rhodovibrin, co-crystallized with the latter compound. OH—R exhibited R_F = 0.45 in 5 % acetone-pet.ether on the usual paper. The same R_F-value is found for OH—R from aerobically grown cultures of *R. gelatinosa* [7]. The abs. max. in methanol was at 486 mμ, and the spectrum showed no fine-structure.

Rhodovibrin (OH—P481)

from the mixed crystals above had R_F= 0.54 in 10 % acetone-pet.ether on the paper employed, as previously found for rhodovibrin from *Rsp. rubrum* [14]. Identity of the two compounds was proved by co-chromatography on the usual paper. The abs.max. in visible light were located at 458, 484, and 517 mμ in pet.ether, and the spectrum had pronounced fine-structure.

Monodemethylated spirilloxanthin

Crystallization. Monodemethylated spirilloxanthin (from zone h) crystallized as tiny, violet needles from acetone-pet.ether. The crystals were collected as previously described [7], yield 0.2 mg; m.p. 208.5—209°C, R_F= 0.39 in 10 % acetone-pet.ether on the usual paper.

Absorption spectrum in visible light recorded in pet.ether had abs.max. at 461, 493, and 528 mμ with pronounced finestructure.

IR-spectrum. The spectrum is presented in Fig. 3.

Acetylation. A fraction containing 0.3 mg (spectrophotometrically determined) of monodemethylated spirilloxanthin was used in the test. The dry pigment was dissolved in 5 ml of dry pyridine and 0.15 ml of acetic anhydride was added under nitrogen. After 46 h at room temperature the reaction mixture was worked up in the usual manner [6]. A pigment recovery of 68 % was spectrophotometrically established after chromatography on deactivated alumina. The recovered carotenoid contained 93 % of monodemethylated spirilloxanthin and 7 % of a pigment with properties as expected for the acetate. The acetate had abs.max. at 462, 492, and 527 mμ in pet.ether and R_F= 0.46 in 5 % acetone-pet.ether on the usual paper. Spirilloxanthin had R_F= 0.48 in the same solvent.

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