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Bacterial Carotenoids

XI. On the Constitution of the Minor Carotenoids of *Rhodopseudomonas* 2. OH-R

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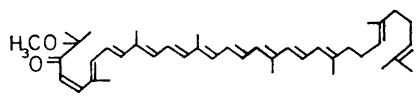
The present paper reports the isolation and characterization of crystalline OH-R fra *Rhodopseudomonas gelatinosa*. The evidence presented suggests the structure 1′,2′-dihydro-1′-OH-spheroidenone (V).

According to the survey of Goodwin [1] there occurs in aerobically grown cultures of *Rhodopseudomonas spheroides*, *R. gelatinosa* and *R. capsulata* a minor carotenoid referred to as hydroxy-R or OH-R [2], so called because of its spectral similarity to spheroidenone (R). The report of Nakayama [3] indicated its presence also in the tan mutant of *R. spheroides*. The presence of a hydroxyl group in this carotenoid has been obvious from its adsorptive properties and partition behaviour. This pigment has been only partly characterized [1,3], and on the basis of the few data available the structure as a 3′-OH-spheroidenone has been suggested by Goodwin [4]. Biosynthetic considerations have led us to propose that this pigment might be a demethylated spheroidenone. The structure of spheroidenone (I) has been unequivocally established by Davis, Jackman, Siddons and Weedon [6].



(I)

RESULTS AND DISCUSSION

In the present investigation the isolation of OH-R in amounts sufficient for work on its constitution has been attempted from the wild type and tan mutant of *R. spheroides*. In both these organisms OH-R is a minor constituent, not exceeding 5 % of the total carotenoid [7]. We therefore turned to *R. gelatinosa*, in which a content of up to 17 % of the total carotenoid had been reported [1]. We found that in contrast to the observations of Goodwin [1] aerobic cultures

Table 1. Absorption maxima (mμ) in visible light of crystalline *trans* OH-R and spheroidenone, recorded in various solvents.

Solvent	OH-R	Spheroidenone
Pet.ether b.p. 60—70°C	(460) 483 516	(460) 483 515
Ethanol	487	488
Acetone	484 (505)	484 (505)
$CHCl_3$	501	
Benzene	(475) 501 530	(475) 502 530
CS_2	(490) 520 555	(490) 520 553

of the same strain as used by him, when grown under our conditions invariably contained OH-R as the major carotenoid, *i.e.* generally 60—75 % of the total carotenoid, as shown in Table 6. This organism was therefore a very suitable source for the isolation and further characterization of OH-R.

OH-R from *R. gelatinosa* crystallized as needles from acetone-petroleum ether; m.p. 158.5—159.5°C in an evacuated tube. This pigment could not be distinguished from OH-R from the wild type or tan mutant of *R. spheroides* when the two were co-chromatographed on circular paper with a kieselguhr filler [8]. Its absorption maxima in visible light, recorded in various solvents, are given in Table 1, together with those of spheroidenone.

The position of the absorption maxima as well as the shapes of the spectra supported the previous assumption that the same chromophoric system occurred in the two pigments. The presence of a conjugated carbonyl group was evident from the fine-structure of the spectrum in petroleum ether as compared with the rather rounded spectrum in ethanol [9], and also from the IR-spectrum. The IR-spectra of OH-R and spheroidenone are presented in Fig. 1. These two spectra showed great similarity except for some differences in the 1000—900 cm^{-1} region, and a somewhat stronger absorption of OH-R at 3340 cm^{-1}

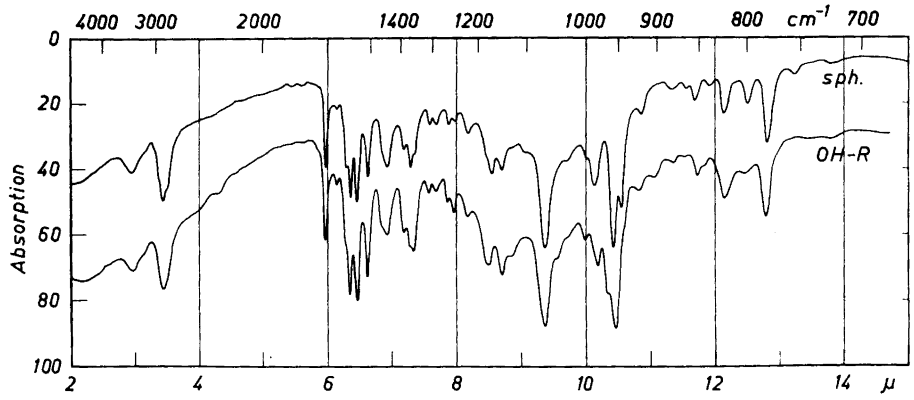


Fig. 1. IR-spectra of 0.37 mg spheroidenone in 0.14 g KBr [7] (upper curve) and of 0.34 mg OH-R in 0.15 g KBr (lower curve).

Table 2. Partition ratios and adsorptive properties of some carotenoids.

Carotenoid	No. of functional groups				Partition ratio [12] Pet.ether/ 95 % methanol	R_F-value [8]		Required eluant. Woelm alumina, activity grade 2
	OCH_3	C=O	OH	conj. double bonds		5 % acetone-pet.eth.	10 % acetone-pet.eth.	
Spheroidenone	1	1	0	10	98:2	0.86		3 % acetone*
Reduced spheroidenone	1	0	1	10	86:14	0.53		10–12% acetone
OH-R	1	1	1	10	63:37	0.45		10–12% acetone
Reduced OH-R	1	0	2	10	28:72	0.24	0.57	30–33% acetone
Zeaxanthin	0	0	2	11	12:88	0.30	0.59	

* In pet.ether.

and 1140 cm^{-1}. The absorption at these two wavelengths together with the rather weak band at 905 cm^{-1} are considered typical of carotenoids carrying tertiary hydroxyl groups [5]. Both compounds exhibited similar absorption at 1680 cm^{-1} (conjugated carbonyl) and at 1068 cm^{-1} (tertiary OCH_3 [10,5]). The IR-spectrum thus revealed the presence of a presumably tertiary methoxyl group and a tertiary hydroxyl group in OH-R. The tertiary character of the hydroxyl group was confirmed by a negative acetylation test, from which only unchanged OH-R was recovered. The presence of one hydroxyl group is also suggested by the partition coefficients given in Table 2. OH-R is presumably not an α-ketol, since the carbonyl absorption in the IR is at the same position as for spheroidenone. Hydrogen-bonded keto-groups are known to cause a frequency shift of the keto absorption [11].

Treatment of OH-R with HCl—$CHCl_3$ furnished no product with extended chromophore. Allylic hydroxyl or methoxyl groups therefore seem to be absent.

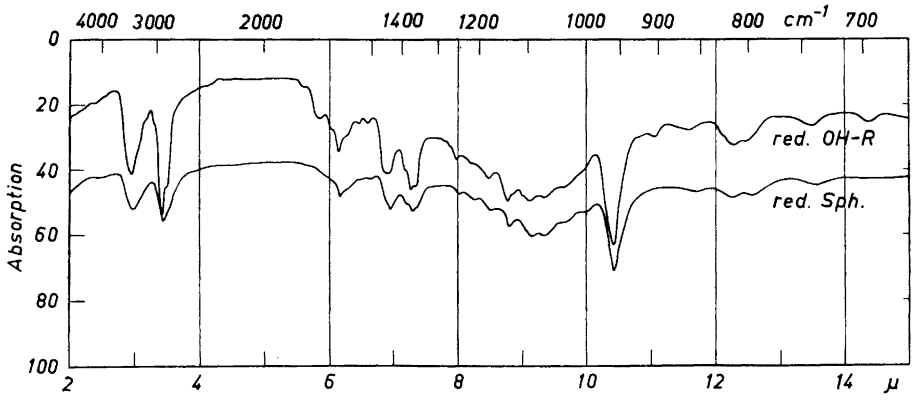


Fig. 2. IR-spectra of $LiAlH_4$-reduced OH-R (0.20 mg in 0.14 g KBr, upper curve) and of $LiAlH_4$-reduced spheroidenone (0.29 mg in 0.13 g KBr, lower curve).

Table 3. Properties of the products obtained on HCl—$CHCl_3$-treatment of reduced OH-R.

Product No.	Abs.max. in mμ in pet.ether	Req. eluant from Woelm alumina, activity grade 2	R_F-value [8]		Partition ratio [12] Pet.ether/ 95 % methanol	Assumed no. of	
			5 % acetone*	10 % acetone		conj. double bonds	OH groups
1	465 494 527.5	3—5 % acetone*	0.59	0.85	96:4	13	0
2	445 467 498	7 % acetone	0.47	0.80	83:17	11	1
3	466 493 527	10 % acetone	0.16	0.46	79:21	13	1

* In pet.ether.

Reduction of OH-R with $LiAlH_4$ gave a diol, m.p. 146°C, with abs.max. at 431, 454, and 487 mμ in petroleum ether. As seen from Table 2 reduced OH-R showed normal adsorptive properties and partition behaviour, and is therefore probably not an α-glycol. α-Glycols like oxy-β-carotene show abnormal chromatographic behaviour and partition coefficients [12].

The IR-spectra of reduced OH-R and reduced spheroidenone are presented in Fig. 2. The secondary hydroxyl group originating from the keto group exhibits a relatively weak absorption band at 1095 cm^{-1}.

Treatment of $LiAlH_4$-reduced OH-R with HCl—$CHCl_3$ [14] gave three products, the properties of which are listed in Table 3. This table also gives tentative conclusions as to the length of the chromophore and the number of hydroxyl groups in the molecule. The conclusions are based on considerations of the visible light absorption data, the partition ratios and the chromatographic behaviour of the products. Admittedly a more hypophasic behaviour would have been expected for a carotenoid containing 13 conjugated double bonds and one hydroxyl group than that found for Product 3, when compared with the partition ratios of monodemethylated spirilloxanthin [15].

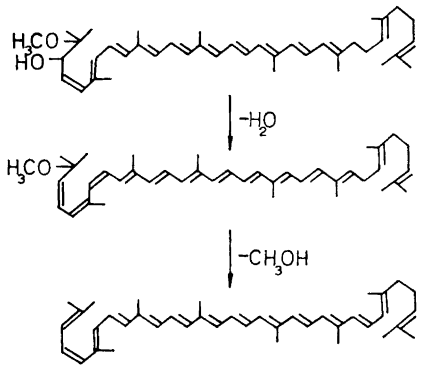
To obtain further evidence for the structural interpretation of the result, $LiAlH_4$-reduced spheroidenone (II) was prepared and treated with HCl—$CHCl_3$ in an analogous manner. The properties of the products obtained agree fairly

Table 4. Properties of the products obtained on HCl—$CHCl_3$-treatment of $LiAlH_4$-reduced spheroidenone.

Product No.	Abs.max. in mμ in pet.ether	Req.eluant from Woelm alumina, activity grade 2	R_F-value [8]		Partition ratio [12] Pet.ether/ 95 % methanol	Assumed no. of	
			Pet. ether	5 % acetone*		conj. double bonds	OH-groups
1	445 468 498	1 % acetone*	0.66		100:0	11	0
2	465 498 532	3 % acetone		0.64	98:2	13	0
3	475 503 538	5 % acetone		0.41	97:3		

* In pet.ether.

well with those previously described by Goodwin, Land and Sissins [16] and are given in Table 4. Product 2, the major product, could not be separated by circular paper chromatography [8] from 3,4-dehydrolycopene (IV) prepared by treatment of lycopene with N-Br-succinimide [17] and showed the same absorption properties in visible light. The expected reaction scheme for $HCl—CHCl_3$-treatment of reduced spheroidenone (II) can thus be formulated as follows:



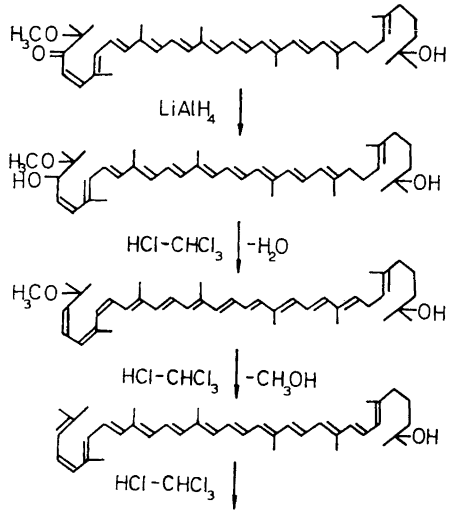
(II)

(III)

(IV)

The properties of Product 1 are as expected for compound (III). The third and minor product has a longer chromophore than 3,4-dehydrolycopene (IV).

Based on the assumption that OH-R exhibits structure (V), which is in agreement with the spectral data presented above, the analogous reaction scheme for treatment of $LiAlH_4$-reduced OH-R (VI) with $HCl—CHCl_3$ would be as depicted below:



(V)

(VI)

(VII)

(VIII)

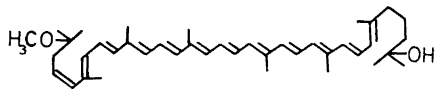
Product 1

The products actually obtained (*cf.* Table 3) exhibit properties compatible with this scheme. The predicted properties of structure (VII) seem to correspond to those of Product 2, and those of structure (VIII) to those found for Product 3. Product 1 must then be regarded as a secondary product arising by an unknown reaction (*e.g.* cyclization or chloronation) of Product 3. As has been discussed elsewhere [5], abnormal reactions are known to occur when carotenoids containing non-allylic, tertiary hydroxyl groups in the 1-positions are treated with $HCl-CHCl_3$. Product 1 was different from 3,4-dehydrolycopene (IV) as shown by a co-chromatography test.

The result of the $HCl-CHCl_3$ treatment of OH-R is thus in agreement with the arrangement of double bonds in structure (V) and rules out a possible extra double bond in the 3′,4′-position which would be difficult to detect in the visible light and IR-spectra. It also seems to support the localization of the presumably tertiary methoxyl group and the tertiary hydroxyl group of OH-R in the 1- and 1′-positions, respectively.

A rational name for OH-R directly based on the carbon skeleton would be very long and impractical. From the structure (V) ascribed to OH-R it is seen to be a 1′,2′-dihydro-1′-OH-derivative of spheroidenone (I). It can also be considered as a 2-keto-7′,8′-dihydro-derivative of rhodovibrin (IX) [18].

Demonstration of the biosynthetic formation of OH-R from 7′,8′-dihydrorhodovibrin is described elsewhere [19].



(IX)

It has been suggested [2,5] that OH-R is a demethylated spheroidenone. That this is not true implies that the enzyme responsible for the introduction of the keto-group is more specific than was previously assumed.

EXPERIMENTAL

Materials and methods. Reagents and solvents used, except for the acetone and the pet.ether (boiling range 60–70°C), were of analytical grade. Column chromatography was carried out on Woelm neutral aluminium oxide, activity grade 2 [20]. Circular paper chromatography was performed on Schleicher and Schüll No. 287 paper [8].

Visible light absorption spectra were recorded on a Zeiss PMQ2 Spectrophotometer; IR-spectra on a Perkin Elmer Model 21 Recording Spectrophotometer. KBr discs for determination of IR-spectra were prepared by the method described in an earlier paper of this series [7]. All melting points were determined in evacuated tubes on a Berl block and are uncorrected.

Culture. R. gelatinosa Strain 8290, obtained from the Department of Scientific and Industrial Research, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, was used. This strain had originally been obtained from Prof. C. B. van Niels collection (his number ATH 2.2.1) [1].

Medium and cultural conditions. The cultures were grown in a sterilized medium consisting of 0.3 % Difco yeast extract, 0.3 % Na-glutamate. The pH was 6.8.

Six cultures (each of 3 l) were grown for 4 days in flat bottommed flasks of 5 l capacity in a light cabinet at 30°C with sterile air bubbling through the cultures at a rate sufficient to provide presumably aerobic conditions. Two days old shaking cultures (200 ml) were used as inoculum for each 3 l culture.

Harvesting of the cells. Owing to the slimy consistency of the culture, the cells could not be harvested by centrifugation in a satisfactory manner. This difficulty was overcome

Table 5. Chromatographic resolution on deactivated alumina of the carotenoid mixture from aerobically grown *R. gelatinosa*.

Zone in order of increasing adsorbance	Colour of zone	Required eluant	Abs.max. in mμ in pet.ether	Identification
a	yellow	2 % acetone-pet.ether	416 439 469	neurosporene
b	red	3— 4 % acetone-pet.ether	(460) 483 515	spheroidenone
c	blue-violet	8 % acetone-pet.ether	487.5 518 555	P518
d	red	10—14 % acetone-pet.ether	(460) 483 515	OH-R

by adding 500 ml of a saturated, aqueous $MgSO_4$-solution with stirring to each 3 l culture, followed by 3 l of acetone. A red precipitate of cell material was hereby obtained. The transparent supernatant could easily be decanted and was discarded. The slimy, red suspension (*ca.* 1 l) of the cell material from each culture was further concentrated in a similar manner by addition of the same volume of acetone. Upon a third and final treatment of the bacterial concentrate with 0.5 l of acetone, a fairly dehydrated concentrate was obtained, from which the carotenoids were extracted on further addition of acetone. A total of 3 l of $MgSO_4$-solution and 27 l of acetone was used in this pretreatment of the cells.

Pigment extraction, saponification and chromatographic separation. The carotenoids were extracted from the cell mass by allowing it to stand at room temperature with 3 successive portions of acetone (about 10 h each time). A dark mauve acetone extract (total volume 8 l) and a decolorized beige cell residue was thus obtained. The saponification and chromatographic procedures were carried out as previously described [7,5]. Particulars of the deactivated alumina chromatogram are given in Table 5.

The carotenoids were eluted and the amounts determined spectrophotometrically in the usual manner, using $E^{1\%}_{1cm} = 2900$ for neurosporene [21] at the middle main maximum in pet.ether, and the extinction coefficients previously used for the three other carotenoids [7]. A total carotenoid yield of 29.4 mg (1.64 mg/l culture) was established spectrophotometrically. The composition of the carotenoid mixture is presented in Table 6.

P518

Crystallization. P518 (from zone c) crystallized as blackish-violet single needles from acetone-pet.ether. The crystals were collected by centrifugation, washed and dried as previously described [7]. After two recrystallizations a total yield of 1.66 mg, m.p. 222°C, was obtained.

Absorption spectra in visible light were determined as previously described [7], and found to be identical with those of P518 from *R. spheroides;* $E^{1\%}_{1cm} = 1745$ at 561 mμ in CS_2.

Table 6. Carotenoid composition of aerobically grown *R. gelatinosa*.

Carotenoid	% of total
Neurosporene	0.3
Spheroidenone	12.5
P518	18.1
OH-R	67.4
Decomposition products	1.7

Co-chromatography with P518 from R. spheroides. Trans P518 from *R. gelatinosa* could not be separated from *trans* P518 from *R. spheroides* when the two were co-chromatographed on the paper employed. Using 10 % acetone-pet.ether as developer a single violet zone (R_F = 0.46) was formed.

OH-R

Crystallization. OH-R (from zone d) crystallized as tiny, mauve needles, partly forming aggregates from acetone-pet. ether. After recrystallization from the same solvent system the crystals melted at 158.5 – 159.5°C; yield: 10.1 mg.

OH-R crystallized as the pure *trans* isomer as revealed by the paper-chromatographic purity test; R_F = 0.45, using 5 % acetone-pet.ether as developer.

Solubility. Crystalline OH-R was very readily soluble in CS_2, readily soluble in acetone, benzene and $CHCl_3$, fairly soluble in methanol and ethanol and slightly soluble in pet.ether.

Absorption spectra in visible light of the crystalline sample were recorded in various solvents, immediately after dissolution. The absorption maxima are given in Table 1. The shapes of the spectra were analogous to those of spheroidenone; $E^{1\%}_{1cm}$ = 2070 at 501 mμ in benzene.

IR-spectrum. The IR-spectrum is presented in Fig. 1.

Quantitative partition ratio was determined according to the method of Petracek and Zechmeister [13]. Found: Pet.ether/95 % methanol 63:37.

Acetylation test. 0.4 mg of OH-R was dissolved in 5 ml of dry pyridine and 0.15 ml of acetic anhydride was added under nitrogen. After standing overnight the reaction mixture was worked up in the usual manner. Chromatographic resolution on deactivated alumina revealed that no acetate had been formed.

Test for allylic hydroxyl and methoxyl groups was carried out on a 0.4 mg sample as described below for $LiAlH_4$-reduced OH-R. The red colour of the reaction mixture did not increase and neither were any products with prolonged chromophore detected paper-chromatographically.

Co-chromatography with OH-R from the wild type and tan mutant of R. spheroides [7] on the kieselguhr-containing paper gave a single zone, R_F = 0.45 using 5 % acetone-pet.ether as developer.

$LiAlH_4$-reduced OH-R

Preparation. A mother liquor containing 5.3 mg of OH-R (spectrophotometrically determined) was concentrated, and the residue dried at 0.1 mm Hg, room temperature. The carotenoid was dissolved in 10 ml of a dry tetrahydrofurane suspension of $LiAlH_4$. After 3 min the brown-yellow solution was poured into diethylether in a separatory funnel to destroy unchanged $LiAlH_4$. The carotenoids were transferred to ether in the usual manner on addition of water. The combined ether extract was washed with water and dried over anhydrous Na_2SO_4. A pigment recovery of 98 % was spectrophotometrically established using $E^{1\%}_{1cm}$ = 2600 at the middle main abs.max. in pet.ether) before chromatographic separation of the reaction mixture was carried out on deactivated alumina. The carotenoid mixture consisted of 2 % of indefinite decomposition products and 98 % of reduced OH-R (required eluant 25 – 30 % acetone-pet.ether).

Crystallization. Reduced OH-R crystallized in low yield as needles from acetone-pet. ether. The crystals were collected in a sintered glass funnel and recrystallization was carried out from the same solvent system; yield of dried crystals: 0.25 mg, m.p. 146°C.

Paper-chromatographic purity tests of the crystalline specimen gave one zone only, R_F = 0.24 in 5 % acetone-pet.ether, R_F = 0.57 % in 10 % acetone-pet.ether.

Solubility. Reduced OH-R was readily soluble in $CHCl_3$, moderately soluble in benzene and slightly soluble in pet.ether.

Absorption spectra in visible light were determined for the crystalline *trans* compound in various solvents, immediately after dissolution. The spectra exhibited pronounced fine-structure, and were analogous to those of crystalline Y [22]. The absorption maxima are given in Table 7.

Table 7. Spectral characteristics in visible light of $LiAlH_4$-reduced OH-R in various solvents.

Solvent	Abs.max. in mμ				% III/II[5]
Pet.ether	(408)	431	457.5	488.5	90
$CHCl_3$	(415)	440	466.5	500	80
Benzene	(418)	441.5	468	502.5	87

IR-spectrum. The KBr-disc was prepared as previously described [7], and the result is presented in Fig. 2.

Quantitative partition test carried out according to the method of Petracek and Zechmeister [13] gave as result: Pet.ether/95 % methanol 28:72.

Test of allylic hydroxyl and methoxyl groups. A mother liquor containing 1.34 mg (spectrophotometrically determined) reduced OH-R was concentrated and dried at 0.1 mm Hg, room temperature. To the residue dissolved in 3.5 ml of $CHCl_3$, was added under nitrogen 3 ml of 0.03 N HCl—$CHCl_3$, prepared according to the method of Entschel and Karrer [14]. The mixture was allowed to stand in diffuse daylight with occasional shaking for 8 min. The reaction mixture turned dark red during this period, and was worked up in the usual manner [14]. The pigments were transferred to pet.ether and the chromatographic separation was carried out on deactivated alumina. After the chromatographic resolution a pigment recovery of 86 % was spectrophotometrically established. The reaction mixture consisted of unchanged OH-R (49 %), Product 1 (8 %), Product 2 (7 %) and Product 3 (35 %). The properties of the products, determined as described above for OH-R, are listed in Table 3.

On treatment of Product 2 with the HCl—$CHCl_3$ reagent as described above, followed by paper-chromatographic resolution of the reaction mixture, the formation of Product 1 (*ca.* 20 %) and Product 3 (*ca.* 20 %) was demonstrated. When Product 3 was treated n a similar manner Product 1 (*ca.* 90 %) was formed.

Spheroidenone

A small amount of crystalline spheroidenone was obtained from zone b. It crystallized as the *trans* isomer, $R_F = 0.66$ in 2 % acetone-pet.ether and $R_F = 0.86$ in 5 % acetone-pet.ether on the usual paper. Its absorption maxima in visible light are presented in Table 1. The absorptive and chromatographic properties were identical with those of spheroidenone isolated from *R. spheroides* [7].

$LiAlH_4$-reduced spheroidenone

Preparation. To 15.3 mg spheroidenone isolated from *R. spheroides* [7] was added under nitrogen 10 ml of a dry tetrahydrofuran suspension of $LiAlH_4$. After 3 min the reaction mixture was worked up as described above for $LiAlH_4$-reduced OH-R. Prior to the chromatographic resolution a pigment recovery of 77 % was spectrophotometrically established. The reaction mixture consisted of 19 % of yellow decomposition products with ill-defined absorption spectra and 81 % of reduced spheroidenone (required eluant 10—12 % acetone from the usual alumina).

Crystallization. Reduced spheroidenone crystallized as tiny needles from acetone-pet.ether. After two recrystallizations a yield of 0.7 mg was obtained; mp. 145—147°C. The paper-chromatographic purity test gave one lemon yellow zone, $R_F = 0.53$ in 5 % acetone-pet.ether.

Solubility. The crystalline compound was readily soluble in SC_2 and $CHCl_3$, fairly soluble in benzene and slightly soluble in pet.ether.

Table 8. Spectral characteristics of $LiAlH_4$-reduced spheroidenone.

Solvent	Abs.max. in mμ				% III/II[5]
Pet.ether	(405)	429	455	486.5	88
95 % methanol	(405)	429	455	486.5	86
$CHCl_3$	(415)	441	466	500	78
Benzene	(415)	441	467.5	501	88
CS_2	(435)	457	487.5	522	92

Absorption spectra in visible light of the crystalline compound were determined in various solvents. The absorption spectra were analogous to those of crystalline Y[22]. Absorption data are given in Table 8.

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

Quantitative partition test carried out as described above gave as result: Pet.ether/95 % methanol 86:14.

Test for allylic hydroxyl and methoxyl groups was performed as for $LiAlH_4$-reduced OH-R on 2.02 mg (spectrophotometrically determined) reduced spheroidenone. After standing in diffuse daylight for 20 min, chromatographic separation on deactivated alumina gave a pigment recovery of 79 % (spectrophotometrically established). The reaction mixture contained 9 % of unreacted reduced spheroidenone, 2 % of decomposition products with ill-defined spectra and three additional products, 1, 2, and 3 in amounts of 15 %, 70 %, and 6 %, respectively. The properties of these products were examined according to the methods described above and are presented in Table 4. Product 2 could not be separated from 3,4-dehydrolycopene, prepared as described below, on the circular paper chromatogram. Upon co-chromatography one violet zone, $R_F = 0.64$ was obtained, using 5 % acetone-pet.ether as developer. Product 2 was different from Product 1 of $HCl-CHCl_3$-treated reduced OH-R. On co-chromatography on circular paper the formed exhibited $R_F = 0.66$ and the latter $R_F = 0.53$, using 5 % acetone-pet.ether as developer.

Preparation of 3,4-dehydrolycopene. 3,4-Dehydrolycopene was prepared from synthetic lycopene (Hoffmann-La Roche & Co.) using the procedure of Winterstein, Studer and Rüegg [17]. To 11.1 mg of lycopene in 5 ml of CCl_4 was added 3.1 mg of N-Br-succinimide. The mixture was refluxed for 1 h. After chromatographic separation on deactivated alumina a pigment recovery of 51 % was obtained. The recovered pigment consisted of 95 % of lycopene, 2 % of 3,4-dehydrolycopene and 3 % of bisdehydrolycopene. The latter two compounds required 3 % and 5 % acetone in pet. ether for elution. 3,4-Dehydrolycopene had abs.max. at (500), 534, and 574 mμ in CS_2.

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