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Bacterial Carotenoids

X. On the Constitution of the Minor Carotenoids of *Rhodopseudomonas*. 1. P518

SYNNØVE LIAAEN JENSEN

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

A description is given of the isolation and properties of a new bacterial carotenoid, provisionally termed P518, isolated from a tan mutant and the wild type of *Rhodopseudomonas spheroides*. This carotenoid exhibits absorption maxima in visible light at longer wavelengths than any other carotenoid hitherto described. The evidence obtained suggests the structure 2-keto-spirilloxanthin (III) for the new compound.

Whereas presumably correct structures have already been ascribed to the major carotenoids isolated from photosynthetic purple bacteria, some of the minor carotenoids occurring in the genus *Rhodopseudomonas*, such as hydroxy-Y, hydroxy-R and P512 are still lacking reliable structural formulae [1].

P512 has been isolated from *R. gelatinosa* [2], and its presence claimed in the tan mutant of *R. spheroides* [3] and probably also in the wild type of the latter bacterium [1]. In attempts to isolate this carotenoid from aerobically grown cultures of the wild type and tan mutant of *R. spheroides*, a new carotenoid has been detected. This carotenoid has provisionally been designated as P518, according to the manner of Goodwin [4,2], from the position of the main absorption band in petroleum ether.

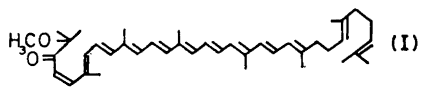
RESULTS AND DISCUSSION

P518 crystallized as the *trans* isomer as violet-black needles, m.p. 221°C, from acetone-petroleum ether; total amount 0.34 mg. Its absorption maxima in visible light, recorded in various solvents, are presented in Table 1. The spectra in CS_2, benzene and acetone were of the usual shape, with moderate fine-structure. The spectrum in petroleum ether showed somewhat better fine-structure and was "tilted" markedly towards longer wavelengths, whereas that in ethanol had a more rounded appearance. The shapes of the absorption

Table 1. Absorption maxima in visible light of P518, recorded in various solvents.

Solvent	Abs.max. in mμ		
Pet.ether	487.5	518	555
Ethanol		522	
Acetone	495	528	559
Benzene	510	539	575
CS_2	528	562	601

spectra in the two latter solvents were very similar to those of spheroidenone (I) [5], as seen from Fig. 1. This common spectral feature was strongly indicative



(I)

of the presence of a conjugated carbonyl group in P518. These groups are known to cause such changes in the absorption spectra of carotenoids when measured in petroleum ether and ethanol [6].

The presence of conjugated carbonyl in P518 was confirmed by the IR-spectrum (Fig. 2) which showed medium strength absorption at 1680 cm^{-1} at the same frequency and intensity as in spheroidenone (Fig. 3).

The IR-spectrum of P518, compared with that of spheroidenone (I), further revealed the absence of hydroxyl functions (in agreement with its

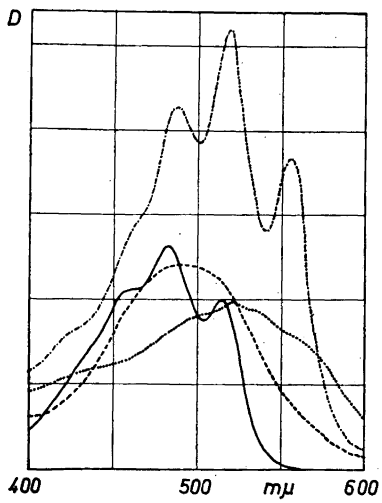


Fig. 1. Absorption spectra in visible light of spheroidenone and P518. ———— Spheroidenone in pet.ether; ------ Spheroidenone in ethanol; -·-·-·- P518 in pet.-ether; ····· P518 in ethanol

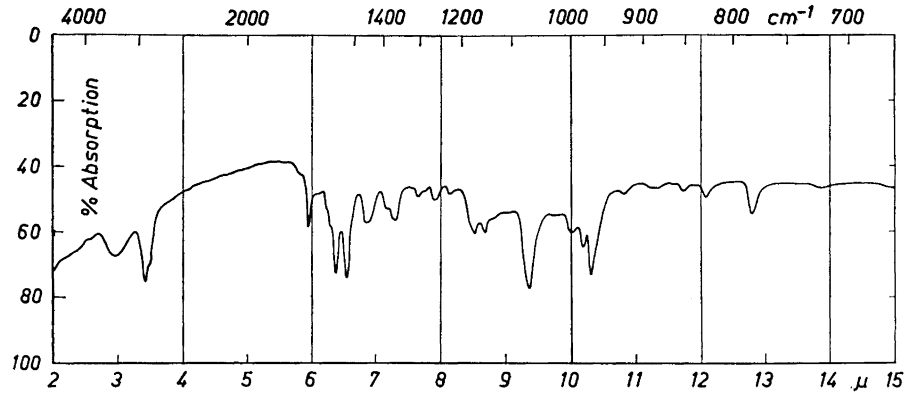


Fig. 2. IR-spectrum of 0.33 mg P518 in 0.14 g KBr.

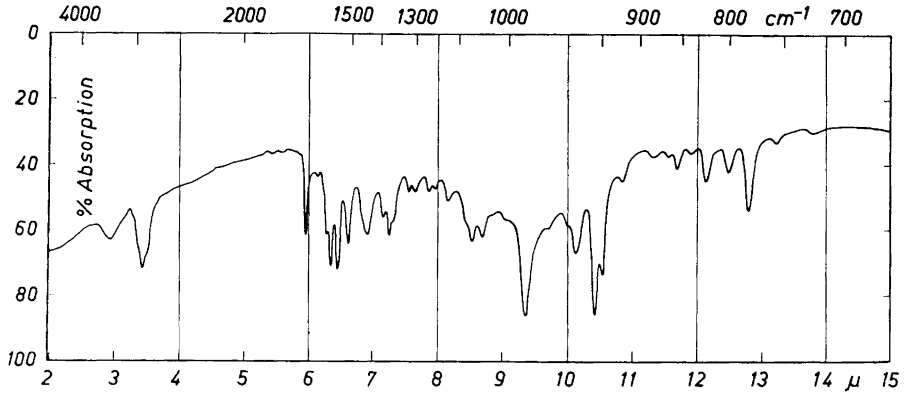


Fig. 3. IR-spectrum of 0.37 mg spheroidenone in 0.14 g KBr.

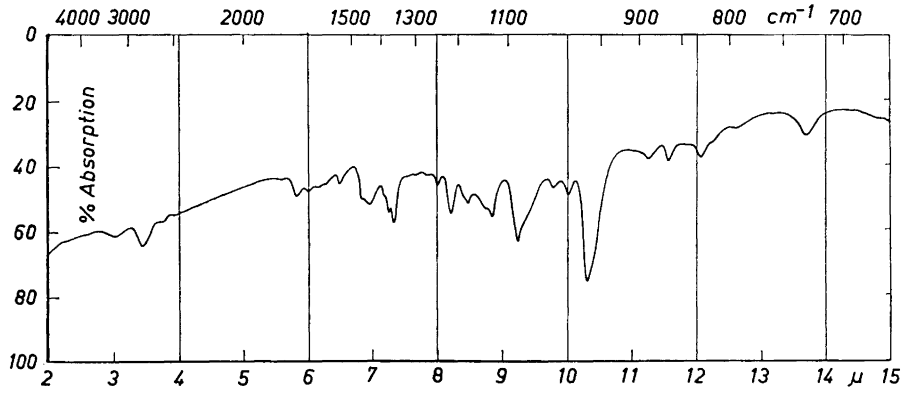
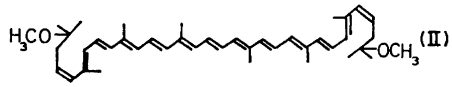


Fig. 4. IR-spectrum of 0.38 mg spirilloxanthin (synthetic) in 0.13 g KBr.

partition behaviour and chromatographic properties, *cf.* Table 2), and demonstrated the presence of at least one, presumably tertiary, methoxyl group (absorption at 1068 cm^{-1}). The absorption band at 3360 cm^{-1}, present in the IR-spectra of both spheroidenone and P518, probably represents a carbonyl overtone. The IR-spectra of P518 and spheroidenone showed great similarity, except for the absorption in the double bond regions.

On treatment of P518 with $HCl-CHCl_3$ no product with extended conjugated chain was produced. Allylic methoxyl groups are therefore presumably absent from P518.

Reduction of P518 with $LiAlH_4$ furnished a major product whose absorption spectrum in visible light was indistinguishable from that of spirilloxanthin (II) [7,8] and α-bacterioruberin [9], as seen from Fig. 5. The two latter compounds



(II)

contain 13 conjugated carbon-carbon double bonds in an aliphatic system, and reduced P518 should therefore contain the same number of conjugated double bonds in an aliphatic chain.

The partition ratios and chromatographic behaviour of $LiAlH_4$-reduced P518 are further evidence for the presence of one hydroxyl group in this compound, as seen from Table 2.

By comparison of the absorption spectra in visible light for P518 and the reduced compound, the contribution of the conjugated carbonyl group is found to be 24 mμ at the middle maximum in petroleum ether. A shift of 30 mμ is found for spheroidenone (I) and its $LiAlH_4$-reduction product [12,13].

$LiAlH_4$-reduced P518 was found to be stable to the $HCl-CHCl_3$ reagent employed.

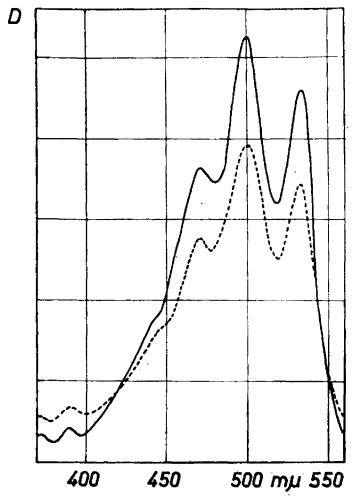


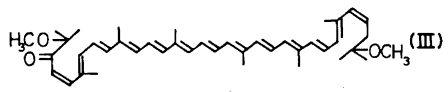
Fig. 5. The absorption spectra in visible light of *trans* spirilloxanthin ————, and *trans* reduced P518 ------, in acetone.

Table 2. Partition ratios and R_F-values of some structurally related carotenoids.

Carotenoid	No. of functional groups				Partition ratios [10]		R_F-value* 10 % acetone-pet.ether
	OCH_3	C=O	OH	Conj. double bonds	Pet.ether/ 95 % methanol	Pet.ether/ 85 % methanol	
Spheroidenone	1	1	0	10	98:2		0.86
Spirilloxanthin	2	0	0	13	88:12		0.76
P518	2?	1	0	13	82:18		0.46
Monodemethylated spirilloxanthin [7]	1	0	1	13	43:57	95:5	0.39
Reduced P518	2?	0	1	13	16:84	68:32	0.18
α-Bacterioruberin [9]	0	0	2	13	1:99	3:97	0.02

* On Scleicher and Schüll No. 287 paper,

Based on the evidence presented above the structure (III) as a 2-keto-spirilloxanthin is suggested for P518. This structure is in agreement with the



(III)

absorption spectra in visible light and the observed partition ratio and chromatographic properties. It is also compatible with the similarity in the IR-spectrum with spheroidenone and the properties of the $LiAlH_4$-reduction product, as well as the position and the intensity of the methoxyl band in the IR [14]. Data for comparison of the methoxyl band with that of other mono- and di-methoxylated carotenoids of related type are presented in Table 3. The tentative allocation of the two methoxyl groups to the 1,1′-positions is based on the IR-spectrum and the absence of allylic methoxyl in P518 and also on analogy with all other known methoxylated bacterial carotenoids [14].

Table 3. Relative intensities of the methoxyl band in the IR-spectra of some related carotenoids.

	Carotenoid	No. of OCH_3-groups	OCH_3:C=O *	OCH_3:*trans F* **
a	Anhydro-rhodovibrin (P481) [14]	1		0.32
	Synthetic spirilloxanthin	2		0.57
b	Spheroidenone	1	1.31	0.79
	P518	2?	1.58	1.10

* Relative intensity of the 1068 cm^{-1} band to that of the 1680 cm^{-1} band.

** Relative intensity of the 1068 − 1078 cm^{-1} band to that of the strongest band in the 950 cm^{-1} region. The figures for the group *a* and group *b* carotenoids in this column are not comparable, since the group *b* carotenoids have complex, split bands in the 950 cm^{-1} region, whilst the group *a* pigments exhibit only one band in this region.

A quantitative determination of methoxyl in P518 performed commercially on a small sample (0.7 mg) isolated from *R. gelatinosa* [13] gave a value corresponding to 1.36 OCH_3-groups based on a molecular formula $C_{40}H_{58}O(OCH_3)_2$.

The absorptive and chromatographic properties of the P518 stereoisomeric set is reported, and stereochemical stability studies have been carried out.

In an endeavour to confirm the suggested structure (III) for P518 a partial synthesis of P518 was attempted on a micro scale from spirilloxanthin by means of N-Br-succinimide according to the method of Petracek and Zechmeister [15]. This reagent was also used in an attempt to synthesize 2-OH-spirilloxanthin according to the method of Entschel and Karrer [18]. These experiments have so far only resulted in yellow decomposition products and recovered spirilloxanthin.

The structure of P518 as a 2-keto-spirilloxanthin, is, however, strongly supported by its biosynthetic formation in *R. gelatinosa* from spirilloxanthin [17].

Crystalline spheroidenone was isolated for comparison. A stereochemical study of this pigment revealed that neospheroidenone A had spectral and adsorptive properties similar to those of lycopene. Claims for the presence of lycopene in spheroidenone-producing bacteria [2], should therefore be re-examined.

EXPERIMENTAL

Cultures. Wild type *Rhodopseudomonas spheroides*, strain 2.4.9 C and a tan mutant 2H809 of the same bacterium, both organisms obtained from Prof. R. Y. Stanier, Department of Bacteriology, University of California, Berkeley, were used.

Medium and cultural conditions. All cultures were grown aerobically in darkness at 26—30°C in a medium consisting of:

0.5 % Difco yeast extract
0.01 M ammonium succinate
0.01 M K_2HPO_4—NaH_2PO_4 buffer pH 6.8
0.05 % $MgSO_4 \cdot 7\ H_2O$
tap water

Small cultures (2 l) were grown for about 30 h in Erlenmeyer flasks of 5 l capacity with aeration. Shaking cultures (200 ml) were used as inoculum after about 48 h of growth. The cells were harvested at *ca.* 5°C in a Servall refrigerated centrifuge (6000 × *g*).

Large cultures (170 l) were grown for 73 h in a fermentation tank of 190 l capacity as described elsewhere [14]. Aerobically grown cultures (total volume 7.5 l), prepared as

Table 4. Chromatographic separation of the carotenoids from aerobically grown wild type or tan mutant of *R. spheroides* on deactivated alumina.

Zones in order of increasing adsorption	Colour of zone	Required eluant	Abs.max. in mμ in pet.ether	Identification
a	red	3 % acetone-pet.ether	(460) 482.5 515	spheroidenone
b	blue-violet	8 % acetone-pet.ether	487.5 518 555	P518
c	red	10—15 % acetone-pet.ether	(460) 482 515	OH-R

Table 5. Carotenoid content of aerobically grown cultures of the tan mutant and wild type of *R. spheroides*.

	Tan mutant	Wild type		
		Batch 1	Batch 2	Batch 3
Volume of the culture (l)	2	2	170	170
Yield of acetone extracted residue (g/l)	0.39	0.46	0.56	0.73
Carotenoid as % of acetone extracted residue	0.039	0.196	0.411	0.031
Aeration rate	low *	low *	low **	high ***
Spheroidenone (As % of total carotenoid)	91.0	96.4	95.4	97.5
OH-R (As % of total carotenoid)	6.1	3.6	0.3	1.6
P518 (As % of total carotenoid)	3.0 (9.1)	trace (3.6)	4.3 (4.6)	0.7 (2.3)

* Air supply through a glass tube of 7 mm inner diameter at a low, unmeasured rate.
** *ca.* 0.4 cu. feet/min.
*** *ca.* 2 cu. feet/min.

described above, were used as inoculum. The cells from the large cultures were harvested on a continous flow Sharples centrifuge at a rate of about 10 l/h.

Extraction and separation of the carotenoids. The centrifuged bacteria were extracted at room temperature with successive portions of acetone until a beige-coloured residue remained. The acetone extract was filtered and the acetone removed *in vacuo*. The remaining aqueous suspension was saponified with a 10 % KOH-methanol solution (final alkali concentration 7 %), by allowing to stand at room temperature for 2 h. The unsaponifiable matter was transferred to pet.ether-benzene in a separatory funnel by gentle addition of concentrated aqueous NaCl-solution equivalent to 1/4 of the volume of the methanolic extract. The hypophase was re-extracted two times with pet.ether-benzene, and the combined epiphase was washed three times with water and dried over anhydrous Na_2SO_4.

The chromatographic separation of the carotenoid mixture was carried out on columns of Woelm neutral aluminium oxide, activity grade 2 [18], as described elsewhere [14]. Table 4 shows the development of a typical chromatogram.

The carotenoids were eluted and determined spectrophotometrically in the usual manner, using $E^{1\,\%}_{1\,cm} = 2100$ for spheroidenone [12], $E^{1\,\%}_{1\,cm} = 2000$ for OH-R and $E^{1\,\%}_{1\,cm} = 1900$ for P518, in each case at the middle main absorption maximum in pet.ether. The result is presented in Table 5.

As seen from Table 5 high aeration increased the cell yield, but greatly lowered the carotenoid content of the cells. The tan mutant gave a somewhat lower cell yield with a considerably lower carotenoid content than the wild type (Batch 1) when the two were grown under similar conditions, although the amount of the minor carotenoids was relatively higher in the mutant.

Carotenoid analysis by means of circular paper chromatography [11] of samples (0.5—2 l) withdrawn periodically from the growing culture of the wild type (Batches 2,3) revealed that P518 was formed relatively late in the growth period. Using the above analytical procedure carotenoids more saturated than spheroidenone could not be detected even early in the exponential growth stage.

P 5 1 8

Crystallization. P518 (from zone b) crystallized as single, short, violet-black, shiny needles from acetone-pet.ether. The crystals were filtered on a Pt-Gooch crucible of 1 cm diameter, washed with cold pet.ether and dried *in vacuo* over Dehydrite and paraffin wax; yield: 0.34 mg (from Batch 2).

The crystalline specimen melted sharply at 221°C (uncorr.) as measured in an evacuated tube on a Berl block.

Solubility. Crystalline P518 was readily soluble in CS_2, fairly soluble in acetone and tetrahydrofurane, slightly soluble in benzene, poorly soluble in ethanol and nearly insoluble in pet.ether (< 0.7 μg/ml at room temperature).

Absorption spectra in visible light. The solutions in CS_2, acetone, benzene and ethanol were prepared by direct dissolution of the crystalline sample, followed by rapid filtration and recording of the absorption spectrum on a Zeiss PMQ2 spectrophotometer. The pet.ether solution (pet.ether of boiling range 60—70°C was used throughout this work) was prepared from an acetone solution in a separatory funnel, by dilution with an excess of water. The pet.ether extract was washed twice with water and dried quickly over anhydrous Na_2SO_4 before the absorption spectrum was recorded. The result is presented in Table 1 and Fig. 1.

IR-spectrum. Crystalline P518 (0.33 mg) was ground with dry KBr (0.14 g) in an agate mortar, and transferred to a ring-like mould of aluminium foil (of the same shape as the light-opening of the Perkin-Elmer Model 21 spectrophotometer) placed symmetrically in the stainless steel ring of 14 mm inner diameter used for preparation of the ordinary KBr-discs. Dry KBr (0.47 g) was filled around the mould. The mould was removed and the disc pressed in the usual manner [14]. The IR-spectrum is presented in Fig. 2.

Partition ratio was determined in a quantitative manner according to the method of Petracek and Zechmeister [10]. The pet.ether solution was prepared from an acetone solution as described above, and the test was carried out immediately. Found: Pet.ether/95 % methanol, 82:18.

Test for allylic methoxyl. The test was performed according to the method of Entschel and Karrer [16,14]. The procedure was first tested with $LiAlH_4$-reduced spheroidenone, and found satisfactory[13]. Using 50 μg samples of P518 recovered from the KBr-disc, no products with extended chromophore could be demonstrated by paper-chromatographic [11] resolution of the reaction mixture in several independent experiments.

Stereochemical studies. P518 crystallized as the pure *trans* isomer, demonstrated by circular paper chromatography [11]. Negligible spontaneous isomerization occurred in darkness at 20°C over a period of 3 days. Iodine catalysis in acetone and determination of the quantitative composition of the iodine catalyzed equilibrium mixture was carried out as described elsewhere [14]. The result is presented in Table 6.

The neo C isomer exhibited the highest *cis*-peak. The true nature of the above *cis* isomers as members of the P518 stereoisomeric set was proved by reverse iodine catalyzed isomerization followed by paper-chromatographic resolution of the stereoisomeric mixtures obtained.

$LiAlH_4$-reduced P518

Reduction. To 156 μg of dry P518 (recovered from the KBr-disc), was added 4 ml of a dry, filtered tetrahydrofurane solution saturated with $LiAlH_4$ (containing approximately 72 μg of $LiAlH_4$[19]) under nitrogen with shaking at room temperature. After a reaction

Table 6. Quantitative composition of the iodine catalyzed equilibrium mixture of P518.

Member of the set	Abs.max. in mμ in acetone	R_F-value * 10 % acetone-pet.ether	% of total
Neo C	*ca.* 430 (485) 514 (555)	0.85	26
Neo B	*ca.* 430 (485) 518 (555)	0.73	17
Neo A	*ca.* 430 (490) 522 (557)	0.54	6
Trans	495 528 559	0.46	51
I_2-cat.eq.mixture	*ca.* 430 (495) 522 (555)		

* On Schleicher & Schüll No. 287 paper.

Table 7. Adsorptive and chromatographic properties of the reduced P518 stereoisomeric set.

Stereoisomer	R_F-value * 10 % acetone-pet.ether	Abs.max. in mμ in acetone
Neo B	0.48	371 389 (465) 491 523
Neo A	0.32	371 389 (465) 489 522
Trans	0.18	371 389 466 498 533

* On Schleicher and Schüll No. 287 paper.

time of 3 min the solution had turned from violet to red-orange, and was poured into diethyl ether in a separatory funnel. The carotenoids were transferred to diethyl ether in the usual manner and dried over anhydrous Na_2SO_4 before chromatographic resolution on deactivated alumina was carried out. Total pigment recovery: 59 %.

The reaction mixture contained 87 % of a major product (required eluant 26 % acetone-pet.ether), which is further described below, whereas the remaining 13 % consisted of less strongly adsorbed more yellow pigments.

Absorption spectra in visible light. The strongly *cis*-isomerized fraction from the deactivated alumina chromatogram had abs.max. at 369, 386, 462, 490, and 524 mμ in pet.ether. The paper-chromatographically purified *trans* isomer had abs.max. at 371, 389, 466, 498, and 533 mμ in acetone with pronounced fine-structure (*cf.* Fig. 5). The absorption spectrum in methanol also exhibited good fine-structure.

Partition test, carried out according to the method of Petracek and Zechmeister [10], gave as result: Pet.ether/95 % methanol, 16:84; Pet.ether/85 % methanol, 68:32.

Test for allylic hydroxyl or methoxyl groups was performed as above for P518 on 30 μg (spectrophotometrically estimated) samples. Reduced P518 was stable towards this reagent. Paper-chromatographic examination of the reaction mixture revealed that only *trans-cis* isomerization had occurred.

Stereochemical studies. Some properties of the main stereoisomers of reduced P518 are listed in Table 7.

Spheroidenone

Crystallization. Spheroidenone (from zone a) crystallized as small plates from acetone and as red, shiny needles forming clusters from 3% acetone-petroleum ether. After recrys-

Table 8. Composition of the iodine catalyzed equilibrium mixture of spheroidenone.

Stereo-isomer	Abs.max. in mμ in			R_F-value* Pet. ether	% of total
	Pet.ether	Acetone	Benzene		
Neo A	369 445 472 (505)	370 (455) 477 (502)		0.33	38
Trans	460 482.5 515	(455) 484 (505)	(475) 501 530	0.24	62
I_2-cat.eq. mixture			382 (470) 494 525		

* On Schleicher and Schüll No. 287 paper.

tallization from acetone-petroleum ether the dried crystals melted at 158—159°C in an evacuated tube.

Absorption spectra in visible light. The crystalline compound exhibited abs.max. in petroleum ether at (460), 482.5 and 515 mμ; in ethanol at 488 mμ (see Fig. 1); in benzene at (475), 501 and 530 mμ and in CS_2 at (490), 520 and 553 mμ.

IR-spectrum. The KBr-disc was prepared as for P518, and the spectrum is presented in Fig. 3.

Partition ratio, determined as above, gave as result: Pet.ether/95 % methanol, 98:2.

Stereochemical studies. Spheroidenone crystallized as the pure *trans* isomer as demonstrated by the paper-chromatographic purity test. Spontaneous isomerization in pet.ether during a period of 20 h i darkness at room temperature was not detectable by spectrophotometric measurements and paper-chromatographic examination. The result of iodine catalyzed stereoisomerization, carried out in benzene solution as described elsewhere [14], is presented in Table 8. Reversible iodine catalyzed isomerization of the neo A isomer to *trans* spheroidenone confirmed the nature of this isomer as a member of the spheroidenone stereoisomeric set.

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