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The Carotenoids of Flexibacteria

II.* A New Xanthophyll from *Saprospira grandis*

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The major pigment of *Saprospira grandis* Gross is a new xanthophyll, here designated saproxanthin. Saproxanthin exhibits physical and chemical properties in agreement with its being a 1′,2′-dihydro-3′,4′-dehydro-3,1′-dihydroxy-γ-carotene (III).

A partial synthesis of dehydrated saproxanthin acetate (V) has been carried out by N-bromosuccinimide dehydrogenation of rubixanthin acetate (VII). The two acetates (V) as well as the corresponding mono-ols (VIII) appeared to be identical.

The main diagnostic features of flexibacteria — aquatic, filamentous, non-sporulating, flagella-less, non-photosynthetic microorganisms — have been summarized by Soriano and Lewin.[1] The alternative classifications of *Saprospira grandis* Gross as a non-photosynthetic, blue-green alga or as a bacterium have been discussed in detail by Lewin.[2]

All flexibacteria so far examined produce carotenoids, and to provide comparative biochemical information Fox and Lewin [1b] initiated a study of their pigment composition. Dr. Lewin enthusiastically urged us to continue this work. The present paper deals with the carotenoids produced by *Saprospira grandis;* studies of other species are now in progress.

RESULT AND DISCUSSION

Lyophilized cells of *Saprospira grandis* contained 0.02 % carotenoids, 97 % of which consisted of a new xanthophyll, here designated saproxanthin. Crystalline saproxanthin, yield 1.2 mg from 46 g of cells, melted at 178–179°C.

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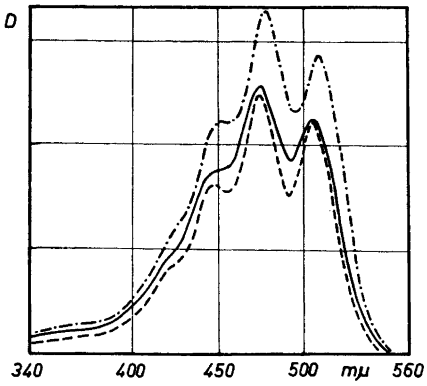
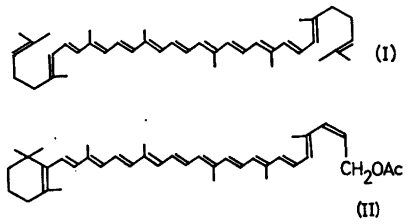


Fig. 1. Absorption spectra in visible light for acetone solution of:
— — — Lycopene (I);
——— β-Apo-carotenyl (C_{37}) acetate (II);
—·—·— Saproxanthin (III).

The absorption curve in visible light of saproxanthin recorded in acetone solution is presented in Fig. 1, together with those of lycopene (I) and β-apo-carotenyl (C_{37})-acetate (II). Absorption maxima and extinction values are given in Table 1. The wavelength position of the absorption maxima, the degree



of fine-structure in the spectrum, and the extinction coefficient, suggested for saproxanthin a chromophore analogous to β-apo-carotenyl (C_{37})-acetate (II). Absorption data for the three compounds in other solvents (petroleum ether, chloroform, pyridine and carbon disulphide) substantiated this assumption. Further support for this conclusion was derived from the spectral shape of the *cis*-peak in the *cis*-isomers of saproxanthin. According to Zechmeister,[6] double *cis*-peaks are characteristic of entirely aliphatic chromophores, whereas carotenoids in which the chromophore extends into an alicyclic group do not exhibit a pronounced splitting of the *cis*-peak. However, the remarkably small shift in absorption maxima for I and II should be pointed out.

Saproxanthin showed a partition ratio indicative of a di-ol, and gave upon acetylation a single reaction product with partition behaviour and adsorptive properties characteristic of a monoacetate. The infrared spectrum of saproxanthin (see Fig. 2) had absorption bands at 3320, *ca.* 1130, 1040, and 905 cm^{-1}, characteristic of one secondary, non-allylic hydroxyl group in a cyclohexene ring [7] and one tertiary hydroxyl group.[3] Support for the tertiary character of the second hydroxyl group was derived from the dehydration of saproxanthin acetate with phosphorus oxychloride in pyridine.[8] Neither the secondary, nor

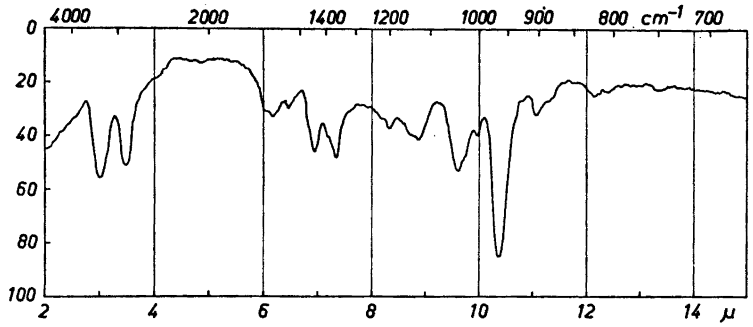


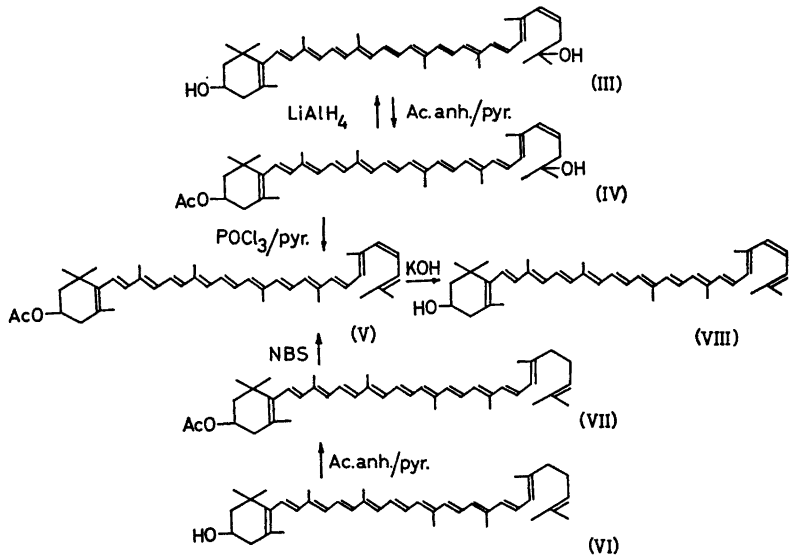
Fig. 2. Infrared spectrum of saproxanthin in KBr.

the tertiary hydroxyl group was in allyl-position to the polyene chain, as judged from its negative reaction when treated with acid chloroform according to the method of Karrer and Leumann [9] and its resistance towards allylic oxidation with *p*-chloranil.[10]

Hydride reduction of the monoacetate resulted in reversion to saproxanthin. Carbonyl groups are therefore absent from saproxanthin, as supported also by infrared data (see Fig. 2). Furthermore, the reduction test gave no evidence for epoxidic oxygen groups.

The oxygen functions were thus established as one secondary and one tertiary hydroxyl group, in non-allylic positions.

Based on these results the structure III was postulated for saproxanthin, the monoacetate being IV and the dehydration product of the latter V. The

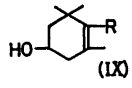


dehydration product indeed exhibited an extended chromophore with absorption spectrum identical with that of torulene (3′,4′-dehydro-γ-carotene).

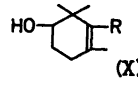
A partial synthesis of a compound indistinguishable from V was accomplished from rubixanthin, $C_{40}H_{56}O$, a carotenoid first isolated by Kuhn and Grundmann.[11] Evidence for its structure as a 3-hydroxy-γ-carotene was based on its absorption spectrum in visible light being identical with that of γ-carotene, its hydrogen uptake on catalytic hydrogenation, acetone formation on ozonolysis which secured the isopropylidene end-group, and determination of active hydrogen. Since rubixanthin exhibited no biological vitamin-A activity, the hydroxyl group was presumably located in the β-end group, and was by analogy placed in the 3-position. In later years, infrared and acetylation evidence as well as the resistance of the pigment towards allylic dehydration[12] support the structure VI for rubixanthin, although the position 2 for the hydroxyl group cannot be ruled out from the available evidence. However, it may be mentioned that no carotenoid carrying a hydroxyl group in the 2-position in a cyclohexene ring has yet been found in Nature.

Dehydrogenation of rubixanthin acetate (VII) with N-bromosuccinimide gave, in low yield, a product with an extended chromophore and with a visible light absorption spectrum identical with that of dehydrated saproxanthin acetate (V). Co-chromatography tests revealed identical R_F-values for the two main stereoisomers (*trans* and neo A). Furthermore the acetates (V), prepared according to different routes, on saponification yielded the same mono-ol (VIII), inseparable by co-chromatography on kieselguhr paper. This was true for the *trans* as well as the neo A isomer.

Provided rubixanthin has the formula VI and the chromatographic systems used[13,14] are able to separate on the single difference basis of end-groups IX



(IX)



(X)

and X (free or acetylated), our evidence supports the structure III for saproxanthin as a 1′,2′-dihydro-3′,4′-dehydro-3,1′-dihydroxy-γ-carotene.

Three minor carotenoids are described in the experimental section. These compounds were not identified with any known carotenoids, and were isolated in amounts insufficient for structural determination.

EXPERIMENTAL

Materials and methods. The reagents and solvents used, except for the acetone and petroleum ether (boiling range 40–70°C), were of analytical grade.

Column chromatography was carried out on Woelm neutral alumina, activity grade 2,[15] or magnesium silicate. Circular paper chromatography was performed on Schleicher & Schüll No. 287 paper (kieselguhr paper)[13] or Schleicher & Schüll No. 288 paper (aluminium oxide paper).[14] For co-chromatograms the 3-divided paper technique was used.[16]

Visible light absorption spectra were recorded on a Beckman DB recording spectrophotometer and infrared spectra on a Perkin Elmer Model 21 spectrophotometer.

Partition tests were carried out according to the procedure of Petracek and Zechmeister.[17]

Table 1. Absorption data in visible light for some related carotenoids in acetone solution.

Carotenoid	Abs.max. in mμ	$E^{1\%}_{1\,cm}$	ε	% III/II[3]
Lycopene (I)	448 474 505.5	3460*[4]	185 000	84
β-Apo-carotenyl (C_{37})-acetate (II)	450.5 476 505	2905*[5]	166 000	64
Saproxanthin (III)	451 478.5 509	2920	162 000	61

* For main maximum in petroleum ether.

Organism. Saprospira grandis Gross, Woods Hole strain, was obtained from the collection of Dr. R. A. Lewin.

Medium and cultural conditions. The cultivation was carried out at Karolinska Institutet by civ. ing. M. Fall through the courtesy of Dr. C. G. Hedén, Bakteriologisk Bioteknik, Karolinska Institutet, Stockholm, Sweden.

Cultures were grown in the nutrient medium recommended by Fox and Lewin [1b] in a fermentation tank of 200 l capacity at 30°C in very dim light. The pH was maintained at 6.8. The culture was aerated; frothing was reduced by use of a silicone-type antifoam. After 20 h of growth the temperature was lowered to 10°C, and the cells were harvested by centrifugation; yield, 400 g wet weight, giving 51.5 g freeze-dried cells.

Pigment extraction. Freeze-dried cells (46 g) were finely ground in a mortar. In order to facilitate the extraction, water (150 ml) was added, and the pigments were extracted at room temperature with successive portions of acetone (total 4.1 l) until the last acetone extract was colourless, followed by a final extraction with methanol overnight. No further pigment could then be extracted from the grey cell residue.

The extracts were pooled and concentrated, and the pigments were transferred to ether in a separatory funnel on mixing with aqueous salt solution. The total carotenoid content was estimated spectrophotometrically as 9.3 mg (using $E^{1\%}_{1\,cm} = 2700$ at 470 mμ in ether) or 0.02 % of the freeze-dried cells.

Partition behaviour. A preliminary partition test between pertoleum ether and 85 % methanol did not result in concentration of particular pigments in either of the two phases. Paper-chromatographic examination showed that the main pigment had a distribution ratio of *ca.* 1:2 between the epi- and hypophase, so that the diverse pigments in the raw extract could not be separated by this means.

Saponification. Preliminary experiments with an aliquot demonstrated that the carotenoid components were stable to alkali, since co-chromatography tests on kieselguhr paper of unsaponified and saponified aliquots of the pigment extract revealed no change in carotenoid composition.

Saponification for 1.5 h at room temperature in 7 % KOH-methanol was therefore included in the purification procedure.

Column chromatography. An aliquot was chromatographed on deactivated alumina. Since some pigment could not be eluted with pure methanol, a weaker adsorbent, magnesium silicate, was used for the first chromatographic separation. The main pigment, saproxanthin, which constituted more than 95 % of the total carotenoid, required 50 % ether-petroleum ether for elution. Upon concentration of the column fractions containing this carotenoid, a red oil was obtained. When re-chromatographed on deactivated alumina, saproxanthin required either 40 % acetone-petroleum ether or 3 % methanol-petroleum ether for elution.

Chromatographic data for the pigments of *Saprospira grandis* are compiled in Table 2.

Table 2. Chromatographic data for the carotenoids of *Saprospira grandis*.

Carotenoid	Required eluant from		R_F-value			
	Magnesium silicate	Deactivated alumina	S & S 288	S & S 287		
			1 %*	1 %	10 %	20 %
S.g. 434	10 % ether **	2 % acetone **	0.44			
S.g. 500		15–20 % acetone	0.25	0.45		
Saproxanthin	50 % ether	40 % acetone or 3 % methanol			0.47	
S.g. 460	50 % acetone or 5 % methanol	> 100 % methanol				0.15

* Acetone in petroleum ether.
** In petroleum ether.

Saproxanthin (III)

Crystallization. The main fractions from the alumina chromatogram containing *trans* saproxanthin was used for crystallization from ether-petroleum ether. The crystals were collected by centrifugation, washed with cold petroleum ether and dried under vacuum at room temperature in a small desiccator containing Dehydrite and paraffin wax; yield, 1.2 mg, m.p. 178–179°C (uncorr.) in evacuated capillary tube.

Solubility. Crystalline saproxanthin was sparingly soluble in petroleum ether, moderately soluble in ether, and readily soluble in acetone, chloroform, or pyridine.

Paper-chromatographic purity test. The crystalline specimen contained exclusively *trans* saproxanthin, as shown by a paper-chromatographic purity test; $R_F = 0.47$ on kieselguhr paper (10 % acetone-petroleum ether).

Absorption spectra in visible light. The extinction coefficient measured in acetone was $E_{1\,cm}^{1\,\%} = 2920$ at 478.5 mμ. The extinction curve is reproduced in Fig. 3; see also Table 1. Absorption spectra were recorded in other solvents: petroleum ether, abs. max. (360.5), 445, 470, and 500 mμ; ether, abs. max. 448, 472.5, and 503 mμ; chloroform, abs. max. (370), 460, 486, and 518 mμ; and pyridine, (372), 490, and 552 mμ. The position of the *cis*-peak is given in parentheses.

Infrared spectrum. A disc containing 0.31 mg saproxanthin in 0.2 g KBr was prepared as described elsewhere.[18] The spectrum is presented in Fig. 2.

Partition ratio. Found for petroleum ether/85 % methanol 24:76.

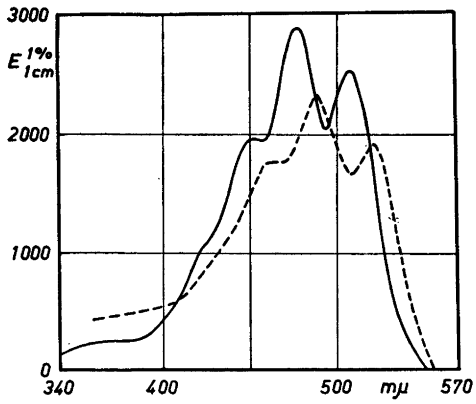


Fig. 3. Absorption spectra in visible light in acetone solution of:
——— Saproxanthin (III);
— — — Dehydrated saproxanthin (VIII). Ordinate value are on arbitrary basis for this curve.

Stereochemical studies. A neo U isomer of saproxanthin was more strongly adsorbed than the *trans* isomer on the alumina column. The neo U isomer had abs. max. at (349), 360.5, 443, 468, and 497 mμ, % D_B/D_{II} [3] = 40, % III/II = 38. This stereoisomer was less strongly adsorbed than *trans* saproxanthin on kieselguhr paper.

Co-chromatography tests with authentic carotenoids were carried out on kieselguhr paper (10 % acetone-petroleum ether). 1,1'-Dihydroxy-1,2,1',2'-tetrahydro-lycopene [19] and warmingol [20,21] were more strongly adsorbed than saproxanthin, and a similar pigment from *Corynebacterium poinsettiae* [22] was less strongly adsorbed than saproxanthin.

Acetylation of saproxanthin (III) to IV. Saproxanthin (70 μg) was dissolved in 2 ml dry pyridine, 0.5 ml acetic anhydride was added, and the mixture was kept at room temperature. Aliquots were withdrawn for periodic paper-chromatographic examination. No intermediate product was observed; the acetylation was complete after 290 min. Other experiments gave similar results.

The acetate (IV) exhibited the same absorption spectrum in visible light as saproxanthin, $R_F = 0.70$ on kieselguhr paper (10 % acetone-petroleum ether) and partition ratio in petroleum ether/95 % methanol 43:57.

Hydride reduction of saproxanthin acetate (IV) to III. An aliquot (15 μg) of the acetate was reduced with $LiAlH_4$ in dry ether, as described elsewhere.[18] The reduction product had an identical absorption spectrum in visible light to that of saproxanthin (III), and could not be separated from that pigment by co-chromatography tests on kieselguhr paper.

Dehydration of saproxanthin acetate (IV) to V with phosphorus oxychloride. The method of Surmatis and Ofner [8] was adapted. Preliminary experiments revealed that the free di-ol (III) could not be used in these experiments, but satisfactory results could be obtained with the monoacetate (IV). Various modifications of the method were tried; the best results were obtained by the following procedure:

Saproxanthin monoacetate (IV; 0.26 mg) was dissolved in 2 ml dry pyridine, $POCl_3$ (0.01 ml) was added, and the mixture was stirred mechanically for 30 min at 50°C. Paper-chromatographic examination showed that the reaction mixture contained *ca.* 50 % of a polar reaction product and *ca.* 50 % of a product more red and less polar than the starting material. The latter product was transferred to petroleum ether in a separatory funnel, and the epiphase was washed several times with aqueous sodium bicarbonate solution.

The petroleum ether extract was added directly to a cellulose column without prior concentration or drying with sodium sulphate. The red product was eluted with 10 % ether-petroleum ether, and was further purified on aluminium oxide paper ($R_F = 0.43$ in 2 % acetone-petroleum ether). In acetone this product (V) had abs. max. at 465, 489.5, and 520 mμ, its spectral curve being identical with that of torulene. It was finally obtained in 29 % yield from the initial saproxanthin monoacetate.

The main *cis* isomer (neo A) had $R_F = 0.54$ on kieselguhr paper (2 % acetone-petroleum ether).

Saponification of dehydrated saproxanthin acetate (V) to VIII. The red acetate (V) (31 μg) was saponified by treatment with 8 % KOH-methanol for 1 h at room temperature, and the reaction mixture was worked up in the usual manner. The free mono-ol obtained (VIII) had $R_F = 0.32$ on aluminium oxide paper (10 % acetone-petroleum ether), $R_F = 0.19$ on kieselguhr paper (2 % acetone-petroleum ether), abs. max. 458, 483, and 516 mμ in petroleum ether and abs. max. (465), 489, and 521 mμ, % III/II = 38 in acetone. Its spectrum in acetone is presented in Fig. 3 together with that of saproxanthin (III).

A presumed *cis* isomer had $R_F = 0.25$ on kieselguhr paper (2 % acetone-petroleum ether).

Attempt to obtain allylic oxidation of III.[10] To 0.1 mg saproxanthin (III) in 2 ml benzene was added 0.3 mg *p*-chloranil and 0.005 mg I_2 in 0.1 ml petroleum ether.[10] After 17.5 h in Na-light at room temperature no new products were formed, as shown by paper-chromatographic examination.

Attempt to obtain allylic dehydration of III with HCl/$CHCl_3$.[9] To 50 μg saproxanthin (III) in 5 ml of chloroform was added 7 capillary drops of saturated (0.32 N) HCl/$CHCl_3$. No colour change was observed during 30 min exposure to indirect daylight. The pigments were transferred to ether upon admixture of aqueous bicarbonate solution. Only polar decomposition products (*ca.* 20 %) and unchanged saproxanthin (*ca.* 80 %) were detected in the reaction mixture by paper-chromatographic examination.

Minor carotenoids

S.g. 434. By re-chromatography on deactivated alumina 10 μg of this carotenoid was obtained. Adsorption data for this pigment are given in Table 2. Abs. max. in visible light were located at 410, 434, and 462 mμ in petroleum ether; 411, 437, and 465 mμ in acetone, and 410, 433, and 460 mμ in methanol. The fine-structure of the spectrum was less pronounced than in that of neurosporene. S.g. 434 was more strongly adsorbed on aluminium oxide paper than neurosporene (isolated from *Rhodopseudomonas gelatinosa* [23] and γ-carotene, and less strongly adsorbed than chloroxanthin.[23] The pigment was resistant towards reduction with $LiAlH_4$ and gave no instant colour shift on treatment with 25 % formic acid in ether.

S.g. 500. By re-chromatography on deactivated alumina 40 μg of this pigment was obtained; for adsorption data see Table 2. S.g. 500 had a rounded absorption curve in visible light, with abs. max. around 490–505 mμ in acetone or in methanol. In partition test between petroleum ether and 95 % methanol, the pigment distributed approximately equally between the two phases. No new products were obtained on hydride reduction.

S.g. 460. By re-chromatography on magnesium silicate 67 μg of this pigment was obtained; for adsorption data see Table 2. The partition ratio between petroleum ether/ 75 % methanol was 17:83. No acetates could be detected after acetylation.

Authentic carotenoids

Lycopene (*I*) had abs. max. in petroleum ether at 445, 471, and 502 mμ; in acetone at 448, 474, and 505.5 mμ; in chloroform at 458, 484, and 518.5 mμ; in pyridine at 461, 490, and 526 mμ and in carbon disulphide at 477.5, 507.5, and 545 mμ.

β-Apo-carotenyl (C_{37}) acetate (II) had abs. max. in petroleum ether at 443, 471, and 501 mμ; in acetone at 450.5, 476, and 505 mμ; in chloroform at 487.5 and 519 mμ; in pyridine at (468), 490.5, and 525 mμ and in carbon disulphide at 506.5 and 540 mμ.

Torulene had abs. max. in petroleum ether at (379), 459, 484, and 517.5 mμ and in acetone at 490 and 522 mμ.

Rubixanthin acetate (VII). Rubixanthin (3.66 mg), isolated from *Rosa rubigenosa* as described elsewhere,[12] was acetylated in 5 ml dry pyridine with 0.5 ml acetic anhydride. The acetate was formed in quantitative yield after 18 h at room temperature, as shown by paper-chromatographic analysis. Rubixanthin acetate had abs. max. (440), 463, and 493 mμ in acetone and $R_F = 0.66$ on kieselguhr paper (2 % acetone-petroleum ether).

N-Bromosuccinimide dehydrogenation of rubixanthin acetate (VII) to V. Rubixanthin acetate (3.22 mg) was dissolved in 3 ml of carbon tetrachloride and 1.23 mg N-bromo-succinimide was added. After 22 h at room temperature the pigments were transferred to petroleum ether and chromatographed on a column of deactivated alumina. Rubixanthin acetate (1.2 mg) was recovered, and 50 μg of 3′,4′-dehydro-rubixanthin-acetate (V) was obtained in 1.6 % yield.

Trans V required 2 % acetone-petroleum ether for elution from deactivated alumina. It exhibited $R_F = 0.43$ on kieselguhr paper (2 % acetone-petroleum ether) and abs. max. (381), 462, 489, and 520 mμ in acetone. The assumed main *cis* isomer had $R_F = 0.54$ on kieselguhr paper (2 % acetone-petroleum ether).

Upon co-chromatography with dehydrated saproxanthin acetate (V) on kieselguhr paper (2 % acetone-petroleum ether), no separation of the corresponding *trans* and neo A isomers was observed.

Saponification of 3′,4′-dehydro-rubixanthin acetate (V) to VIII. Trans 3′,4′-dehydro-rubixanthin acetate (V; 20 μg) was saponified with 8 % KOH-methanol for 1.5 h at room temperature. The reaction mixture was worked up in the usual manner. VIII had $R_F = 0.32$ on aluminium oxide paper (10 % acetone-petroleum ether), on kieselguhr paper $R_F = 0.43$ (5 % acetone-petroleum ether) and $R_F = 0.19$ (2 % acetone-petroleum ether) and abs. max. (379), 458, 484, and 517.5 mμ in petroleum ether. The main *cis*-isomer had $R_F = 0.25$ on kieselguhr paper (2 % acetone-petroleum ether).

On co-chromatography with dehydrated saproxanthin (VIII) on kieselguhr paper, no separation of the corresponding *trans* and neo A isomers was obtained.

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