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Carotenoids of Flexibacteria

III.* The Structures of Flexixanthin and Deoxy-flexixanthin

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Two new monocyclic xanthophylls, flexixanthin (XI) and deoxy-flexixanthin (XXIV), have been isolated from *Flexibacter* sp. Their structures have been established by chemical and physical methods. The properties of carotenoid α-ketols in relation to their dehydro-derivatives have been studied in particular.

Our understanding of the autoxidation of carotenoid α-ketols in alkaline medium is due to the fundamental investigations by Kuhn and Sørensen.[1] These workers demonstrated that on alkali treatment the red lobster pigment astaxanthin (I) gave a blue di-enol salt (II), which in the presence of oxygen was oxidized to the tetraketone astacene (IIIa). They further proposed a structure analogous to II for the blue-green chromoprotein ovoverdin (IV) of lobster eggs.

The structure suggested by Karrer, Loewe and Hübner [2] for astacene, *viz.* 3,4,3′,4′-tetra-keto-β-carotene (IIIa), was assigned the tautomeric diosphenol structure IIIb in order to account for its acidic partition behaviour in neutral versus alkaline medium [3] and for the formation of a dioxime with four active hydrogens (two arising from the oxime residues and two from the enolic protons).[4] Judging by the negative response of astacene towards etherification to VI,[3] by diazomethane, Karrer and Jucker [5] concluded that free astacene was only slightly enolized. However, infrared evidence [6] (*cf.* Fig. 1) has revealed that crystalline astacene or neutral solutions thereof exhibit predominantly the tautomeric structure IIIb.

The elucidation of the structure of astacene (IIIa) [2] was partly based on the formation of a bis-phenazine derivative (V) [4] with *o*-phenylenediamine. Its structure (III) has since been confirmed by total synthesis performed by Davis and Weedon.[7]

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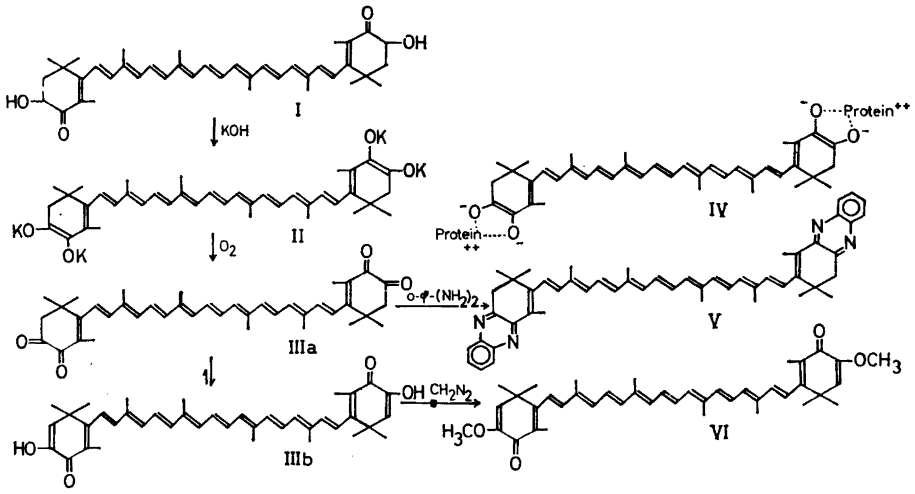
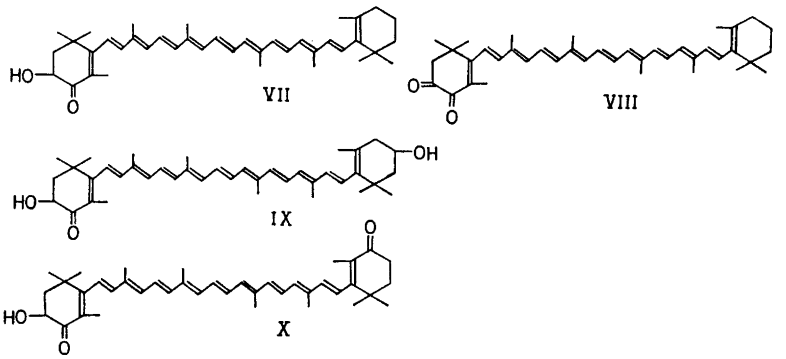


In recent years the natural occurrence of further carotenoid α-ketols has been claimed [8–10] without due regard to the reaction behaviour characteristic of these compounds.[1–5]

Thus, Krinsky and Goldsmith [8] reported the isolation of hydroxy-echinenone (VII) and euglenanone (VIII) from the alga *Euglena gracilis*. Weedon [11] later disproved the latter identification. Hydroxy-asteroidenone isolated from sea-stars has been assigned the structure IX by Nicola.[9] More recently Egger [10] ascribed the same structure (IX) to adonixanthin and structure X to adonirubin isolated from flowers of the angiosperm *Adonis annua* L., which, he claimed, also contained hydroxy-echinenone (VII).



RESULTS AND DISCUSSION

In the present investigation, a new monocyclic carotenoid α-ketol, here designated flexixanthin, has been isolated from a species of *Flexibacter*. Flexixanthin (XI) represented *ca.* 80 % of the total carotenoid of the organism

studied. The remaining *ca.* 20 % was a second carotenoid, deoxy-flexixanthin (XXIV).

1. Flexixanthin (XI)

Flexixanthin possesses one substituted cyclohexene ring with an α-ketol grouping, analogous to that of astaxanthin (I), and one aliphatic, hydrated end-group as found in many bacterial carotenoids.[12,13] Unequivocal proof for the structure XI for flexixanthin has been obtained by chemical and physical methods.

Upon alkali treatment, flexixanthin (XI) was autoxidized to dehydro-flexixanthin (XII). This reaction apparently proceeded less readily than the corresponding transformation of astaxanthin (I) to astacene (III).

Dehydro-flexixanthin (XIIb) exhibited infrared absorption characteristic of diosphenols like astacene (IIIb), as shown in Fig. 1. Of particular diagnostic value were absorption bands at 1612 and *ca.* 1540 cm^{-1}, as found for various tropolones,[6] as well as absorption bands at 1245 and 1060 cm^{-1}.

Dehydro-flexixanthin (XIIb) furnished an enol acetate (XIII) different from flexixanthin acetate (XIV). It should, however, be pointed out that the properties of XI and XII, like those of XIII and XIV, are confusingly similar, as evident from the data compiled in Tables 1 and 2 and Fig. 2. The absorption maxima in visible light of the dehydro-derivatives (XII and XIII) are at slightly longer wavelengths and are somewhat less distinct than those of the corresponding α-ketol compounds (XI and XIV). On circular kieselguhr paper flexixanthin (XI) could be separated from dehydro-flexixanthin (XII) only by very prolonged development, and no distinct separation could be obtained on a cellulose column. However, on the alkaline aluminium oxide

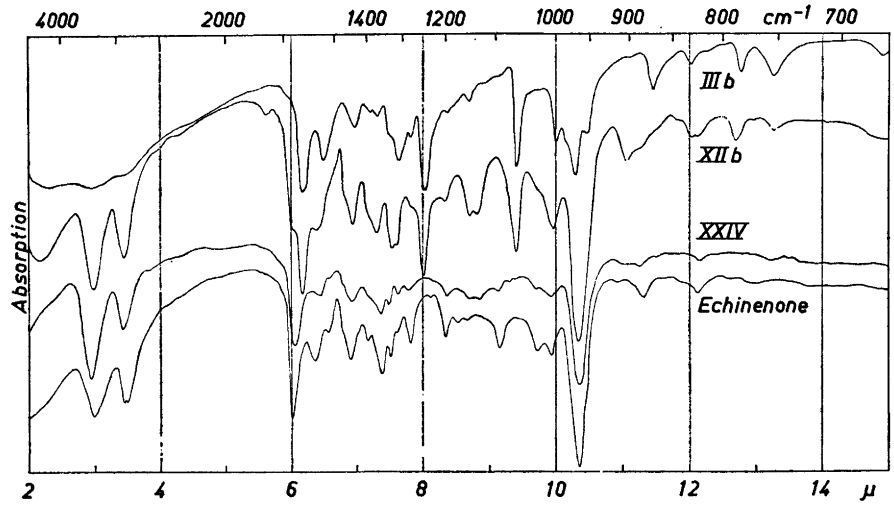


Fig. 1. Infrared spectra recorded in KBr of astacene (IIIb), dehydro-flexixanthin (XIIb), deoxy-flexixanthin (XXIV) and echinenone (4-keto-β-carotene).

Table 1. Properties of flexixanthin (XI), dehydro-flexixanthin (XII), flexixanthin acetate (XIV) and dehydro-flexixanthin acetate (XIII).

Properties	Flexixanthin (XI)	Dehydro-flexixanthin (XII)	Flexixanthin acetate (XIV)	Dehydro-flexixanthin acetate (XIII)
Abs.max. in acetone in mμ	483 510	485 (505)	483 510	485 (505)
R_F-value, kieselguhr paper (10 % acetone-petroleum ether)	0.44	0.42	0.47	0.41
R_F-value, aluminium oxide paper (20 % acetone-petroleum ether)	0.18	0	0.47	0.43
Partition ratio in petroleum ether/ 85 % methanol				
Neutral	39:61	47:53	46:54	52:48
Alkaline		29:71		

paper, the diosphenol compound (XIIb) was, as might be predicted, much more strongly adsorbed. Compounds XI and XII behaved rather similarly on partition in a neutral system. However, as expected, a much more hypophasic behaviour was observed for dehydro-flexixanthin (XIIb) when partitioned in an alkaline medium. Furthermore, flexixanthin acetate (XIV) could not be chromatographically separated from dehydro-flexixanthin acetate (XIII) on a cellulose column, whereas this succeeded either on kieselguhr paper or on aluminium oxide paper. Again the partition ratios in a neutral system was rather similar.

On the basis of preliminary saponification experiments and chromatographic considerations, we first drew the wrong conclusion that flexixanthin was unaltered upon alkali treatment. Consequently saponification was included in the isolation procedure. Most experiments were therefore performed with dehydro-flexixanthin (XII). Under our standard saponification conditions, flexixanthin (XI) gave only a 85—90 % conversion to dehydro-flexixanthin

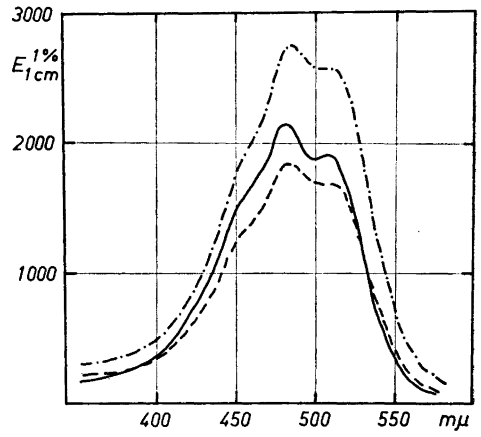
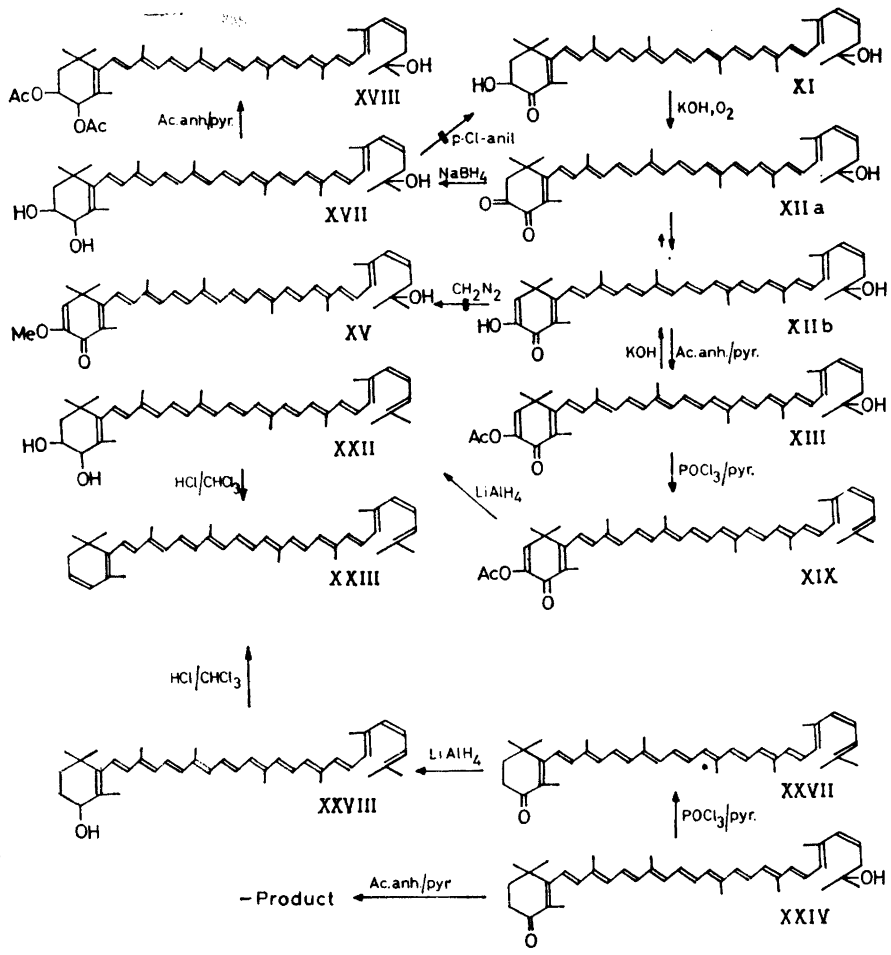
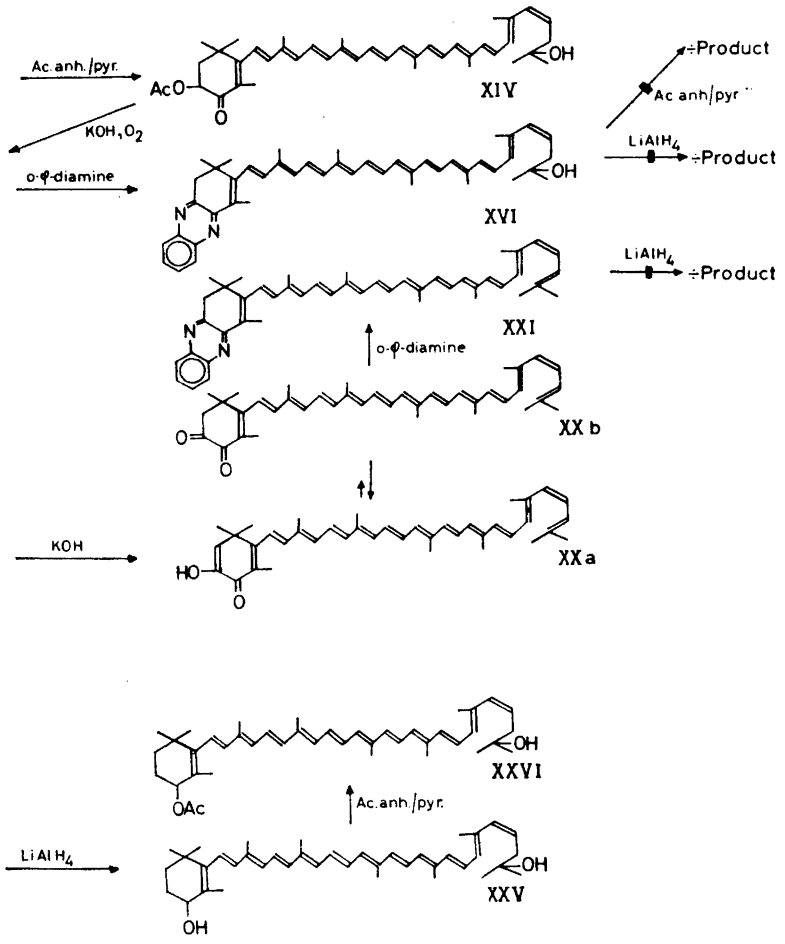


Fig. 2. Absorption spectra in visible light measured in acetone solution of — · — dehydro-flexixanthin (XII), ——— flexixanthin (XI), — — — deoxy-flexixanthin (XXIV). Extinction values are only valid for XII.



(XII). The inflexion at 1660 cm^{-1} in the infrared spectrum of dehydro-flexixanthin (XII) in Fig. 1 is thus due to a *ca.* 10 % contamination of flexixanthin (XI). The contamination of flexixanthin acetate (XIV) in our crystalline specimen of dehydro-flexixanthin acetate (XIII) had a more marked effect on the infrared spectrum, as seen from Fig. 3. In the carbonyl region, astaxanthin (I) diacetate exhibited absorption at 1745 cm^{-1}, astacene (IIIb) diacetate at 1768 cm^{-1} (enol acetate) and the dehydro-flexixanthin acetate (XIII) specimen at 1740 cm^{-1} with an inflexion at 1780 cm^{-1}. However, the absorption for the latter compound at 1540, 1200, and 1052 cm^{-1} was in agreement with that of the enol acetate of astacene.

Like astacene (IIIb), which failed to give the corresponding dimethyl ether (VI) with diazomethane, dehydro-flexixanthin (XIIb) yielded no methyl ether (XV) with the same reagent. Since infrared evidence favours the predominance of the tautomeric diosphenol structures (IIIb and XIIb), we consider



the acidity of the enolic protons to be insufficient to promote a reaction with diazomethane.

The existence of the tautomeric structure (XIIa) of dehydro-flexixanthin could be demonstrated by the smooth formation of the phenazine derivative (XVI) upon condensation with *o*-phenylenediamine The phenazine derivative (XVI) exhibited in visible light an absorption spectrum analogous to that of dehydro-flexixanthin (XII), was less strongly adsorbed on kieselguhr paper than the latter, and gave no new product when treated with lithium aluminium hydride. A corresponding change in properties was observed on formation of the bis-phenazine derivative (V) of astacene (IIIa). Furthermore, XVI furnished no new product on acetylation, proving the absence of hydroxyl groups accessible for acetylation. As a check was tested the reaction of echinenone (4-keto-β-carotene) with *o*-phenylenediamine. Echinenone gave under similar conditions, in very low yield, a more polar reaction product with an echinenone-

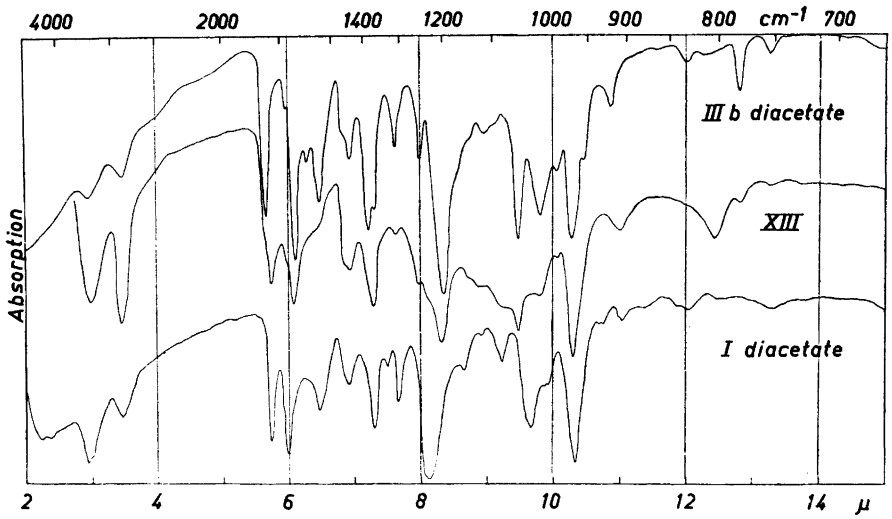


Fig. 3. Infrared spectra recorded in KBr of astacene (IIIb) diacetate, dehydro-flexixanthin acetate (XIII) and astaxanthin (I) diacetate.

like absorption spectrum, presumably the corresponding imine (Schiffs' base). The latter product, too, was resistant towards hydride reduction.

The location of the α-ketol grouping of flexixanthin (XI) was proved by hydride reduction of dehydro-flexixanthin (XIIa) to XVII. On this reduction was observed a hypsochromic shift of 7.5 mμ in acetone solution, accompanied by a gain in fine-structure of the absorption spectrum. Shifts of this size are characteristic of carbonyl groups in 4-position in a cyclohexene ring.[13] Acetylation tests revealed that the reduction product (XVII) contained two hydroxyl groups in positions available for acetylation. The diacetate so formed is considered to have the formula XVIII.

Allylic oxidation with *p*-chloranil of the reduction product (XVII) to flexixanthin (XI) was expected in view of the smooth formation of keto-products from isozeaxanthin (4,4'-dihydroxy-β-carotene) by treatment with this reagent.[14] However, neither XVII nor hydride-reduced astacene (3,4,3',4'-tetrahydroxy-β-carotene) yielded allylic oxidation products by this method or by oxidation with nickel peroxide.[15] Seemingly the α-glycol grouping prevented this reaction.

Further evidence for the cyclic end-group of flexixanthin (XI) was obtained by comparing the proton magnetic resonance data of dehydro-flexixanthin (XII) (see Fig. 4) with previously reported NMR data for astacene (III)[11] and similar data for the latter compound obtained in the present work. The diosphenol grouping in the cyclohexene ring causes a paramagnetic shift of the gemini methyl groups to 8.72 τ (6 protons) and a similar shift of the methyl group in 5-position to 7.94 τ (3 protons). The latter signal thus appears at lower fields than that of the in-chain methyl groups.

For establishment of the entire chromophore of flexixanthin (XI), the absorption spectrum in visible light of hydride-reduced dehydro-flexixanthin

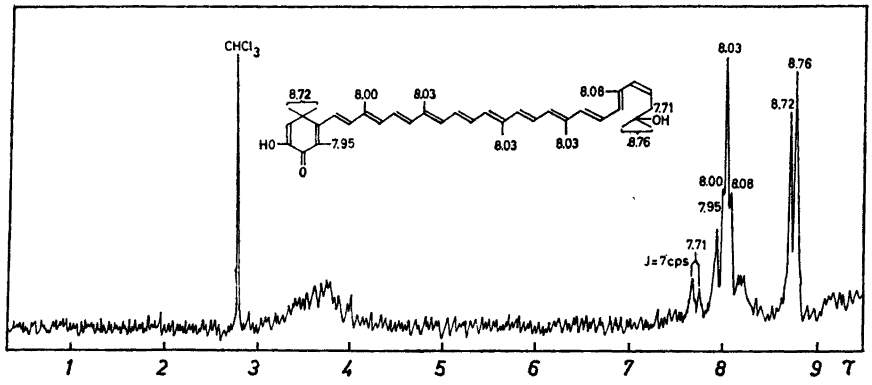


Fig. 4. Proton magnetic resonance spectrum of dehydro-flexixanthin (XII) measured in $CDCl_3$ at 100 Mc/sec.

(XVII) was of importance. As seen from Fig. 5, its spectrum was identical with that of β-apo-2′-carotenyl (C_{37}) acetate,[16] the chromophore of which comprises eleven aliphatic carbon-carbon double bonds in conjugation with a trimethyl-substituted cyclohexene ring.

We shall now turn to a consideration of the second end-group. The infrared absorption of dehydro-flexixanthin (XI) at 1140 and 905 cm^{-1} (see Fig. 1) indicated the presence of a tertiary hydroxyl group.[13] This was confirmed by proton magnetic resonance data (Fig. 4) from signals at 8.76 τ (6 protons) characteristic of gemini methyl groups adjacent to a tertiary hydroxyl group.[17] Additional evidence for such a grouping was derived from the partition ratios of flexixanthin (XI) and dehydro-flexixanthin (XII) presented in Table 1, as well as those of the tri-ol (XVII) and the mono-ol (XVIII). A final proof was obtained from the dehydration of dehydro-flexixanthin acetate (XIII) with phosphorus oxychloride to anhydro-dehydro-flexixanthin acetate (XIX) with an extended chromophoric system (see Figs. 2 and 6). In the proton magnetic resonance spectrum the methylene group in the 2′-position of dehydro-flexixanthin (XII) appeared, as predicted, as a doublet at 7.71 τ (2 protons) with $J = 7$ cps.[17]

In accordance with the structural scheme suggested, anhydro-dehydro-flexixanthin acetate (XIX) was saponified to XX, the diketo-form of which (XXb) again underwent smooth condensation with *o*-phenylenediamine to a phenazine derivative (XXI). The properties of this derivative (XXI) bore the same relationship to XX as XVI to XII.

Finally, XIX on hydride reduction yielded the α-glycol XXII (see Fig. 6), identical with the hydride reduction product of XXb. On treatment with acid chloroform [18] the α-glycol (XXII) gave a product with properties as expected for 3,4-dehydro-torulene (XXIII). The absorption spectrum of the last mentioned product (XXIII) is also reproduced in Fig. 6.

The data so far presented favoured structure XI for flexixanthin. A final verification was obtained from the mass-spectrometric molecular weight determination of a mixture of flexixanthin (XI; MW 582) and dehydro-flexixanthin

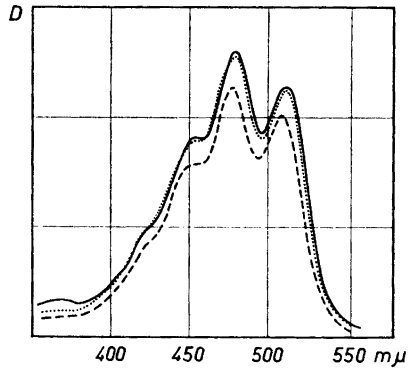


Fig. 5. Absorption spectra in visible light measured in acetone solution of ——— hydride reduced dehydro-flexixanthin (XVII), ... acetate (XXVI) of hydride reduced deoxy-flexixanthin, — — — β-apo-2′-carotenyl (C_{37}) acetate.

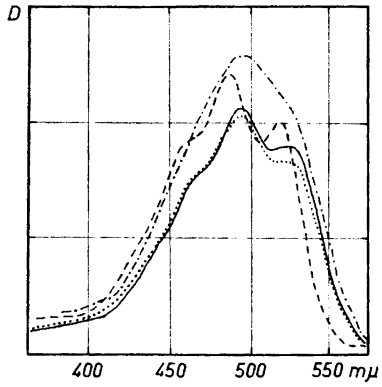


Fig. 6. Absorption spectra in visible light measured in acetone solution of — · — anhydro-dehydro-flexixanthin acetate (XIX), — — — 3,4-dihydroxy-torulene (XXII), ——— anhydro-deoxy-flexixanthin (XXVII), ····· 3,′4′-dehydro-torulene (XXIII).

(XII; MW 580) and from the complete proton magnetic resonance spectrum of dehydro-flexixanthin (XII). The assignments of the various methyl signals are given on the formula in Fig. 4.

Since there was no obvious colour change when flexixanthin (XI) was released from the cells by an organic solvent, it seems unlikely that flexixanthin would be bound in a protein complex, as is astaxanthin in ovoverdin (IV).

2. Deoxy-flexixanthin (XXIV)

The absorption spectrum in visible light of deoxy-flexixanthin was indistinguishable from that of flexixanthin (XI); see Fig. 2. However, judged by chromatographic properties and partition behaviour, deoxy-flexixanthin (XXIV) was less polar, and furnished no acetate on acetylation. Its infrared spectrum showed a characteristic absorption band at 1650 cm^{-1} for a conjugated carbonyl group (see Fig. 1, which includes for comparison the spectrum of echinenone (4-keto-β-carotene)). Hydride reduction resulted in a di-ol (XXV), which on acetylation gave a monoacetate (XXVI). The size of the hypsochromic shift observed on hydride reduction of deoxy-flexixanthin suggested the 4-position for the carbonyl group in XXIV. Furthermore, the absorption spectrum in visible light of the acetate of the reduction product (XXV) was nearly superimposable upon that of β-apo-2′-carotenyl (C_{37})-acetate or hydride reduced dehydro-flexixanthin (XVII) (see Fig. 5), suggesting the same chromophoric system for flexixanthin (XI) and deoxy-flexixanthin (XXIV).

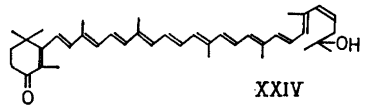
The partition ratio of deoxy-flexixanthin (XXIV), hydride reduced deoxy-flexixanthin (XXV), and its acetate (XXVI) suggested the presence in all these compounds of one tertiary hydroxyl group (inaccessible for acetylation). In the infrared spectrum of deoxy-flexixanthin (XXIV) (see Fig. 1), weak absorp-

tion at *ca.* 1140 cm^{-1} pointed in the same direction. In line with these observations, treatment with phosphorus oxychloride resulted in an epiphasic dehydration product (XXVII) with absorption in visible light in the same spectral region, but with more pronounced maxima than for anhydro-dehydro-flexixanthin acetate (XIX) (see Fig. 6).

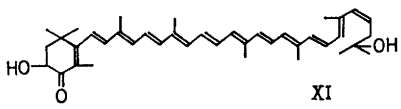
Hydride reduction of anhydro-deoxy-flexixanthin (XXVII) resulted in a mono-ol (XXVIII) with an absorption spectrum identical to that of torulene (3′,4′-dehydro-γ-carotene). Acid chloroform treatment of this mono-ol (XXVIII) resulted in normal allylic dehydration to 3,4-dehydro-torulene (XXIII). This compound (XXIII) proved to be identical with the allylic elimination product obtained from hydride reduced anhydro-dehydro-flexixanthin acetate (XXII). Allylic elimination of related type has also been observed on acid chloroform treatment of 3,4,3′,4′-tetrahydroxy-β-carotene.[19]

The data presented so far are in agreement with structure XXIV (MW 566) for deoxy-flexixanthin. Further support was derived from a mass-spectrometric determination of its molecular weight. Admittedly the determination was carried out on a specimen of dehydro-flexixanthin (XII) containing *ca.* 10 % flexixanthin (XI) and *ca.* 2 % deoxy-flexixanthin (XXIV). However, all three carotenoids gave molecular ions as well as (M — 106) peaks. According to the work of Schwieter *et al.*[20] carotenoids are known to lose xylene (*m/e* 106) in the mass spectrometer by a *cis*oid rearrangement of the polyene chain.

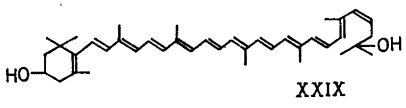
Accordingly we consider the carotenoids produced by this *Flexibacter* sp. to be unequivocally established as the new monocyclic xanthophylls flexixanthin (XI) and deoxy-flexixanthin (XXIV). These structures bear a striking resemblance to saproxanthin (XXIX), previously isolated from the related flexibacterium *Saprospira grandis*.[21] The difference in substitution on the cyclohexene ring serves to show how a simple substituent may greatly alter the chemical and physical behaviour of a compound.



XXIV



XI



XXIX

EXPERIMENTAL

Materials and methods. The reagents and solvents used, except for the acetone and petroleum ether (boiling range 40—65°C), were of analytical grade. Peroxides were removed from the diethyl ether by column chromatography on highly activated alumina (Spence).

Column chromatography was carried out on Woelm neutral alumina, activity grade 2,[22] or on Schleicher & Schüll cellulose powder No. 124. Circular paper chromatography

was performed on Schleicher & Schüll No. 287 paper (kieselguhr paper) [23] or Schleicher & Schüll No. 288 paper (aluminium oxide paper).[24] For co-chromatograms the 3-divided paper technique was used.[25]

Visible light absorption spectra were recorded on a Beckman DB recording spectrophotometer, and infrared spectra of samples in semimicro KBr discs were recorded with a Perkin Elmer Model 21 spectrophotometer, as previously described.[26] Nuclear magnetic resonance (NMR) spectra were recorded in deuterochloroform with tetramethylsilane as internal standard on an AEI type RS2 spectrometer (60 Mc/sec) or a Varian HA-100 (100 Mc/sec) spectrometer. Mass spectra were recorded on an AEI Type MS 9 mass spectrometer with direct inlet system.

When not stated to the contrary, extractions and chemical reactions were carried out at room temperature in darkness or subdued light. Carotenoid solutions were always flushed with pure nitrogen. Concentrations were carried out under reduced pressure on a Büchi rotating evaporator at temperatures not exceeding 45°C. Crystals were collected by centrifugation, or filtration on a platinum filter, washed with an appropriate, chilled solvent, dried in a small desiccator at 0.1 mm Hg at room temperature, and stored under nitrogen in sealed ampoules. Melting points were measured in evacuated capillary tubes on a Berl Block, and are uncorrected.

Partition tests were carried out according to the procedure of Petracek and Zechmeister.[27] Iodine-catalyzed stereoisomerization was carried out in petroleum ether or benzene solutions as previously described.[28]

Acetylation was effected in the usual way by acetic anhydride in dry pyridine.[28] Reduction with lithium aluminium hydride was performed in dry ether as described elsewhere.[26] For reduction tests with sodium borohydride, various solvents were used as specified in each case, and the reaction mixture was shaken mechanically. Dehydration with phosphorus oxychloride in dry pyridine was carried out at 40—50°C according to the method of Surmatis and Ofner.[29] Oxidation with *p*-chloranil was performed as described elsewhere.[14] Nickel peroxide was prepared according to the procedure of Nakagava, Konaka and Nakata,[15] and their method for allylic oxidation was adapted. The procedure of Entschel and Karrer [30] was employed for treatment with acid chloroform. A saturated solution of diazomethane, prepared from N-nitroso-N-methyl-urea and collected in ether,[31] was used for etherification.

Culture. The strain of *Flexibacter* studied here, 14—1, was isolated by Dr. R. A. Lewin in 1963 from a hot-spring at Agua Caliente, Costa Rica, and has since been maintained by weekly transfers in the medium described below. A sub-culture sent to Karolinska Institutet in 1965 served as inoculum for subsequent mass cultures used for the present work.

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

Cultures were grown in a nutrient medium developed by Lewin.[32] Each liter, prepared with distilled water contained $MgSO_4 \cdot 7H_2O$ (0.1 g), KNO_3 (0.1 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), sodium glycerophosphate (0.1 g), "tris" buffer (1 g), thiamine (1 g), cobalamine (1 μg), "casamino acids" (Difco) (1 g), glucose (1 g), and trace mineral solution (1 ml). The pH was adjusted to 7.5. The final culture was grown in a fermentation tank of 200 l capacity at 30°C in very dim light. The culture was aerated, but no antifoam was required. After 17 h of growth the temperature was lowered to 10°C and the cells were harvested by centrifugation and finally freeze-dried; yield 89 g.

Pigment extraction. Freeze-dried cells (76 g) were ground and extracted with acetone as previously described.[21] The acetone extracts were pooled and concentrated, and the pigments were transferred to petroleum ether in a separatory funnel on admixture with aqueous salt solution. The total carotenoid content was estimated spectrophotometrically to 21.8 mg (using $E\,\frac{1\,\%}{1\,cm} = 2500$ at the main maximum in petroleum ether), representing 0.03 % of the lyophilized cells.

Saponification was performed in 5 % methanolic KOH-solution (200 ml) for 1 h at room temperature. The pigments were transferred to ether in a separatory funnel in the usual manner; pigment recovery, 95 %.

The extract from another 3 g cells, containing *ca.* 1 mg carotenoids, was not submitted to saponification.

Column chromatography. The unsaponifiable matter was chromatographed on a column (1) of cellulose powder (18 × 5 cm). Distinct separation of the pigmented zones was not

Table 2. Chromatographic separation of the *Flexibacter* carotenoids on columns of cellulose and deactivated alumina.

Carotenoid	Required eluent in petroleum ether from			
	cellulose			neutral alumina
	Column 1	Column 2	Column 3	activity grade 2 [22]
Deoxy-flexixanthin (XXIV)	0–20 % ether	0–30 % ether	10–50 % ether	100 % ether-1 % methanol
Flexixanthin (XI)	0–100 % ether	30–50 % ether	50–75 % ether	irreversibly adsorbed
Dehydro-flexixanthin (XII)				

obtained. Pigments requiring more than 10 % ether-petroleumn ether for elution (14.3 mg) were combined and re-chromatographed on a similar column (2). The petroleum ether eluates (7.0 mg) were likewise pooled and re-chromatographed on a cellulose column (3), thereby effecting fairly satisfactory separation of deoxy-flexixanthin from the more strongly adsorbed carotenoids. Deoxy-flexixanthin was finally purified by chromatography on columns of deactivated alumina. The eluants required are compiled in Table 2. The composition of each column fraction was checked spectrophotometrically and by paper chromatography (*cf.* Table 1).

Carotenoia composition. The estimated carotenoid composition of the saponified pigment extract, spectrophotometrically determined after column and paper chromatography (in per cent of total carotenoid), was as follows: Deoxy-flexixanthin 13 %, flexixanthin 10 % and dehydro-flexixanthin (derived from native flexixanthin) 77 %. Other carotenoids were not detected.

Flexixanthin (XI)

Purification. Chromatographically pure flexixanthin was isolated from a non-saponified pigment extract after chromatography on a cellulose column and kieselguhr paper.

Adsorptive properties on various adsorbents are given in Tables 1 and 2.

Absorption spectrum in visible light. The absorption maxima in acetone solution are cited in Table 1 and the spectral curve is presented in Fig. 2.

Mass spectrum. For mass-spectrometric determination of molecular weight, see under dehydro-flexixanthin below.

Partition ratios are given in Table 1.

Flexixanthin acetate (XIV). To flexixanthin (15 μg) dissolved in 1 ml dry pyridine was added 0.1 ml acetic anhydride. After 2 ½ h 80 % had been converted to the acetate (XIV), as indicated by paper chromatography. The reaction mixture was worked up in the usual manner; pigment recovery, 81 %.

Flexixanthin acetate (XIV) exhibited the same absorption spectrum in visible light as flexixanthin (XI). Its partition ratio is given in Table 1. The R_F-values, also cited in that table, were determined in co-chromatography tests with dehydro-flexixanthin acetate (XIII).

Saponification of flexixanthin acetate (XIV) to dehydro-flexixanthin (XII). Flexixanthin acetate (10 μg) was treated with 10 % KOH-methanol (0.5 ml). After 2 min the R_F-value in 20 % acetone-petroleum ether decreased to 0.20 on aluminium oxide paper, a characteristic of flexixanthin (XI). The reaction mixture was flushed with air a few times during the following 2 ½ h reaction period. The final reaction product had the same absorption spectrum in visible light as dehydro-flexixanthin (XII) and co-chromatographed with that pigment (*cf.* Table 1).

Acetylation of the reaction product (*ca.* 8 μg) with 0.5 ml acetic anhydride in 0.5 ml dry pyridine at 5°C overnight gave dehydro-flexixanthin acetate (XIII), as judged by co-chromatography tests with that pigment.

Dehydro-flexixanthin (XII)

Purification. Dehydro-flexixanthin, twice chromatographed on a cellulose column, was crystallized from ether-petroleum ether; yield 5.1 mg, m.p. 183.5—185°C.

Purity test. The carotenoid composition of this specimen was estimated by paper-chromatographic examination on kieselguhr paper and on aluminium oxide paper, and by a consideration of its NMR-spectrum. The mass spectrum gave additional qualitative information about the three carotenoids present. The crystalline specimen (sample 1) contained *ca.* 88 % dehydro-flexixanthin (XII), *ca.* 10 % flexixanthin (XI) and *ca.* 2 % deoxy-flexixanthin (XXIV). At the crystallization stage we were not aware of the mixed composition, and further purification by alkali treatment was not attempted.

Absorption spectrum in visible light. Sample 1 exhibited abs.max. at the same positions as paper-chromatographically pure XII, namely in petroleum ether at 479.5 and (500) mμ and in acetone at 485 ($E\ {}^{1\%}_{1\ cm} = 2765$) and (505) mμ. The spectral curve in acetone is presented in Fig. 2.

Infrared spectrum. The IR-spectrum of sample 1 (0.3 mg) in 0.2 g KBr is presented in Fig. 1, together with that of 0.3 mg synthetic astacene (III b) in 0.2 g KBr, recorded for comparison.

NMR-spectrum. The proton magnetic resonance spectrum of sample 1 (2.1 mg) recorded in deuterochloroform at 100 Mc/sec is given in Fig. 4. The spectrum at 60 Mc/sec of 2.13 mg astacene (III) in deuterochloroform was recorded for comparison. Methyl signals were observed at 7.91 (*ca.* 6 protons), 8.03 (*ca.* 12 protons) and 8.71 τ (*ca.* 12 protons).

Mass spectrum. The mass spectrum of sample 1 (0.15 mg) gave the following peaks in the high-mass-unit end of the spectrum: $M_1 = 582$, $M_2 = 580$, $M_3 = 566$, $M_1 - 106 = 476$, $M_2 - 106 = 474$ and $M_3 - 106 = 460$.

Partition tests. The partition ratios of dehydro-flexixanthin (XII) (sample 1) in neutral and alkaline medium are reported in Table 1.

Acetylation of dehydro-flexixanthin (XII) to dehydro-flexixanthin acetate (XIII). To sample 1 of dehydro-flexixanthin (2.3 mg) dissolved in 10 ml dry pyridine was added 1 ml acetic anhydride. The reaction mixture was left for 4 h and then worked up in the usual manner; spectrophotometrically determined pigment recovery, 79 %. Paper-chromatographic examination on aluminium oxide paper of aliquots sampled during the reaction period revealed no formation of transitory products. The final reaction mixture contained exclusively dehydro-flexixanthin acetate (XIII). XIII could not be separated from the non-acetylated carotenoid (XII) on kieselguhr paper, but a clear separation was effected by co-chromatography tests on aluminium oxide paper (for R_F-values see Table 1). The reaction mixture was submitted to preparative chromatography on a cellulose column, from which the acetate (XIII) required 40—50 % ether-petroleum ether for elution.

Crystallization of the acetate (XIII) was carried out from ether-petroleum ether; yield 0.18 mg. *Paper-chromatographic purity test* of this crystalline specimen (sample 1) revealed the presence of 90 % dehydro-flexixanthin acetate (XIII) and 10 % flexixanthin acetate (XIV). *Absorption spectrum in visible light* of the acetate (XIII) was identical with that of dehydro-flexixanthin (XII) above. *Infrared spectrum.* The IR-spectrum of 0.18 mg dehydro-flexixanthin acetate (sample 1 above) in 0.2 g KBr is presented in Fig. 3. For comparison the spectra of astaxanthin (I) diacetate (0.3 mg in 0.2 g KBr) and astacene (III b) diacetate (0.3 mg in 0.2 g KBr) were also recorded.

Astaxanthin (I) diacetate, m.p. 200°C, was prepared by acetylation of astaxanthin (I) in the usual manner, followed by quick chromatographic purification on a column of neutral deactivated alumina. Astacene (III b) diacetate was prepared in a similar manner, excluding chromatography. *Partition ratio* of dehydro-flexixanthin acetate (XIII) is given in Table 1.

Saponification of dehydro-flexixanthin acetate (XIII) to XII. Dehydro-flexixanthin acetate (40 μg) was treated with 3 ml 10 % KOH-methanol for 2 h. The reaction product, worked up in the usual manner, could not be separated chromatographically from dehydro-flexixanthin (XII) either on kieselguhr paper or aluminium oxide paper.

Etherification of dehydro-flexixanthin (XIIb) with diazomethane. An aliquot of sample 1 of dehydro-flexixanthin (56 μg) was dissolved in 50 ml of a saturated solution of diazo-

methane in ether. No methylation product (XV) could be detected after 1 1/2 day at 5°C by chromatography either on kieselguhr paper or on a deactivated alumina column.

In a parallel experiment, 0.1 mg astacene (III) was treated in like manner; spectrophotometrically determined pigment recovery, 84 %. Again no methylation products were observed by chromatography on circular aluminium oxide paper.

Preparation of the phenazine derivative (XVI) of dehydro-flexixanthin (XIIa). To sample 1 (0.11 mg) of dehydro-flexixanthin (XII) dissolved in 1 ml glacial acetic acid was added 11.2 mg *o*-phenylenediamine. The mixture was kept at 100°C on a water bath, and the reaction was followed chromatographically on kieselguhr paper. After 45 min the mixture was cooled, the pigments were transferred to ether, and the ether extract was washed several times with water; spectrophotometrically determined carotenoid recovery, 64 %. The reaction mixture was chromatographed on a column of deactivated alumina. *Adsorptive properties.* The phenazine derivative (XVI) required 10—20 % acetone-petroleum ether for elution from the alumina column, and had $R_F = 0.64$ on kieselguhr paper (10 % acetone-pet.ether) compared with $R_F = 0.42$ for XII in the same system. *Absorption spectrum in visible light.* Paper-chromatographically pure XVI had abs. max. in petroleum ether at 480 mμ and in acetone at 486 mμ. *Attempt at hydride reduction.* XVI (50 μg) was treated with lithium aluminium hydride in dry ether in the usual manner; spectrophotometrically determined pigment recovery, 60 %. Judged by chromatographic examination on the two papers commonly used, the recovered pigment consisted exclusively of unaltered XVI. Its absorption spectrum in visible light remained unchanged. *Attempt at acetylation.* To XVI (20 μg) dissolved in 0.5 ml dry pyridine was added 0.3 ml acetic anhydride. No acetylation product was obtained during 24 h, as indicated by frequent chromatographic examination of aliquots on kieselguhr paper.

Preparation of the phenazine derivative (V) of astacene (IIIa). For comparative purposes the phenazine derivative (V) of astacene (IIIa) was prepared in a parallel experiment. To astacene (0.12 mg) dissolved in 2 ml glacial acetic was added 0.2 mg *o*-phenylenediamine. The condensation was followed by paper chromatography until all astacene had reacted, after 3 1/2 h at 100°C. The reaction mixture contained 70 % of the bis-phenazine derivative ($R_F = 0.70$ on kieselguhr paper: 5 % acetone-petroleum ether) and 15 % of the mono-phenazine derivative ($R_F = 0.55$ in the same system), compared with $R_F = 0.40$ for astacene.

The bis-phenazine derivative (V), purified by chromatography on a deactivated alumina column, required 5 % acetone-petroleum ether for elution, and exhibited in acetone a rounded absorption spectrum with abs. max. at 488 mμ.

Treatment of V (0.12 mg) with lithium aluminium hydride in dry ether in the usual manner gave no reduction products as revealed by paper chromatography.

Treatment of echinenone with o-phenylenediamine. Echinenone (2.8 mg) dissolved in 1.5 ml glacial acetic acid was treated with 28.4 mg *o*-phenylenediamine for 60 min at 100°C followed by 30 min at room temperature, and was then worked up in the usual manner; spectrophotometrically determined pigment recovery was only 11 %.

In addition to echinenone ($R_F = 0.58$ on kieselguhr paper; petroleum ether) the reaction mixture contained 35 % of a more polar compound ($R_F = 0.25$ in the same system) with abs. max. at 457 mμ in petroleum ether and 461 mμ acetone, values similar to those of echinenone.

The product (30 μg) was treated with lithium aluminium hydride in the usual manner; spectrophotometrically determined pigment recovery, 70 %. Paper-chromatographic examination revealed no change.

Hydride reduction of dehydro-flexixanthin (XII) to the triol XVII. Dehydro-flexixanthin (0.14 mg) was reduced with lithium aluminium hydride in the usual manner; spectrophotometrically determined pigment recovery, 91 %. XII was only slightly soluble in ether, and paper-chromatographic examination revealed that 10 % remained unreduced.

In another experiment, XII (0.46 mg) dissolved in 10 ml ethanol was reduced with sodium borohydride for 5 h; spectrophotometrically determined pigment recovery, 89 %. The reaction mixture contained 95 % of the triol (XVII) and 5 % unreacted XII. *Adsorptive properties.* The *trans* triol (XVII) had $R_F = 0.44$ and a presumed neo A isomer $R_F = 0.56$ on kieselguhr paper (20 % acetone-petroleum ether). *Absorption spectrum in visible light.* In acetone, *trans* XVII had abs. max. at (365), 450.5, 477, and 508 mμ (see Fig. 5) and the presumed neo A isomer at (365), 450, 475.5, and 507.5 mμ.

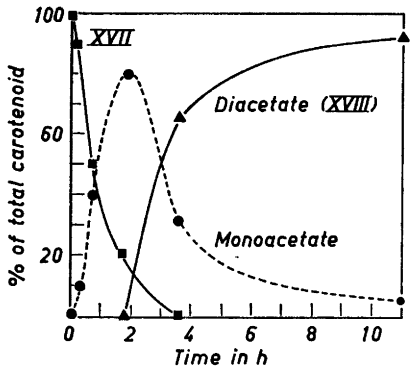


Fig. 7. Course of acetylation of the triol XVII to the diacetate XVIII. For conditions see text.

The spectrum of *trans* β-apo-2′-carotenyl (C_{37}) acetate, recorded for comparison in the same solvent, had abs. max. at 450.5, 476, and 505 mμ (see Fig. 5). *Partition test.* In petroleum ether/85 % methanol, the partition ratio of XVII was 12:88.

Acetylation of the triol XVII to the diacetate XVIII. To XVII (53 μg) dissolved in 2 ml dry pyridine was added 0.2 ml acetic anhydride. The course of acetylation was followed paper-chromatographically, and the results are presented in Fig. 7. The reaction was interrupted after 20 h; spectrophotometrically determined pigment recovery 79 %.

Chromatographed on kieselguhr paper (10 % acetone-petroleum ether), the monoacetate(s) appeared as two zones with R_F = 0.29 and 0.37. Their *cis-trans* relationship was not checked.

The following properties were found for the diacetate (XVIII): *Adsorptive properties.* On kieselguhr paper (10 % acetone-petroleum ether), the *trans* isomer had R_F = 0.50, and a neo A isomer R_F = 0.61. *Absorption spectrum in visible light* was similar to that of the triol (XVII). *Partition test.* In petroleum ether/95 % methanol, the partition ratio was 31:69.

Attempted allylic oxidation of the triol XVII. To XVII (0.31 mg) dissolved in 2 ml benzene was added 15 μg I_2 (in a small volume of petroleum ether) and 1 mg *p*-chloranil. After 40 h in Na-light no oxidation products were detected by paper-chromatographic or spectrophotometric analysis. The reaction mixture was worked up in the usual manner; spectrophotometrically determined pigment recovery, 60 %.

Similar oxidation attempts with 3,4,3′,4′-tetrahydroxy-β-carotene. 3,4,3′,4′-Tetrahydroxy-β-carotene (0.13 mg) dissolved in 8 ml ethanol-benzene (3:5) was mixed with 1.8 mg *p*-chloranil and 10 μg I_2 and illuminated with Na-light for 44 h. Spectrophotometric (pigment recovery 81 %) and paper-chromatographic examination showed that no allylic oxidation had occurred.

In another experiment, 3,4,3′,4′-tetrahydroxy-β-carotene (0.10 mg) dissolved in 1 ml dry ether-ethanol (1:1) was treated with nickel peroxide (0.98 mg; 15 times calculated excess based on titration data) for 4 h. There was no evidence for any allylic oxidation. In other experiments, though as much as 150 times excess of nickel peroxide was used, no keto-products could be detected by paper chromatography.

3,4,3′,4′-Tetrahydroxy-β-carotene was produced from astacene (0.15 mg) by sodium borohydride reduction in ethanol; spectrophotometrically determined pigment recovery, 92 %.

Dehydration of dehydro-flexixanthin acetate (XIII) to XIX. To XIII (1.7 mg) dissolved in 10 ml dry pyridine was added 0.05 ml phosphorus oxychloride. The reaction was followed paper-chromatographically, and after 30 min another 0.05 ml of the latter reagent was added. After 40 min the reaction was interrupted in the usual manner; spectrophotometrically determined recovery of epiphasic pigments, 42 %. The epiphasic carotenoids were chromatographed on a cellulose column. XIX comprised 59 %, and the rest was unreacted XIII. The following properties were established for XIX: *Adsorptive properties.* XIX required 40–50 % ether-petroleum ether for elution from

the cellulose column. The *trans* isomer exhibited $R_F = 0.57$ on kieselguhr paper (10 % acetone-petroleum ether). *Absorption spectrum in visible light. Trans* XIX had abs. max. in petroleum ether at 488 mμ and in acetone at 491 mμ (see Fig. 6).

Saponification of XIX to XX. A sample of XIX (0.20 mg) was saponified for 1 h in 15 ml ether-methanol (1:7) containing 5 % KOH. The reaction mixture was worked up in the usual manner; spectrophotometrically determined pigment recovery was 73 %. The reaction mixture contained exclusively XX. *Adsorptive properties. Trans* XX had $R_F = 0$ on aluminium oxide paper (20 % acetone-petroleum ether) and $R_F = 0.57$ on kieselguhr paper (10 % acetone-petroleum ether). *Absorption spectrum in visible light* was analogous to that of XIX. *Partition tests.* In the neutral system petroleum ether/85 % methanol, the partition ratio was 80:20. In the presence of alkali the partition ratio changed to 54:46.

Preparation of the phenazine derivative (XXI) of XX. To XX (0.15 mg) dissolved in 1.5 ml glacial acetic acid was added 6.2 mg *o*-phenylenediamine. The mixture was heated at 100°C and the reaction, followed paper-chromatographically, was interrupted after 3 h when XX had been completely converted to XXI; spectrophotometrically determined pigment recovery, 66 %. XXI was purified by chromatography on a column of deactivated alumina. *Adsorptive properties.* XXI required 5 % acetone-petroleum ether for elution from deactivated alumina. The *trans* isomer had $R_F = 0.15$ on kieselguhr paper (2 % acetone-petroleum ether). *Absorption spectrum in visible light.* XXI had abs.max. in petroleum ether at 488 mμ and in acetone at 493 mμ.

Attempted hydride reduction. XXI (29 μg) was treated with lithium aluminium hydride in the usual manner; spectrophotometrically determined pigment recovery, 82 %. As judged by paper-chromatographic examination no reduction product was formed.

Hydride reduction of XIX to the diol XXII. XIX (30 μg) was reduced with lithium aluminium hydride in dry ether in the usual manner; spectrophotometrically determined pigment recovery, 53 %. The reaction mixture contained 70 % XXII; for properties of the diol, see below.

Hydride reduction of XX to the diol XXII. XX (0.11 mg) was most conveniently reduced with sodium borohydride in ethanol. The reaction, followed paper-chromatographically, was completed after 3 h; spectrophotometrically determined pigment recovery, 45 %. *Adsorptive properties. Trans XXII* had $R_F = 0.40$ on kieselguhr paper (10 % acetone-petroleum ether). *Absorption spectrum in visible light.* In acetone, abs. max. were located at (460), 487, and 519 mμ (see Fig. 6).

Allylic elimination of XXII to XXIII. XXII (45 μg) was treated with a 0.03 N HCl—$CHCl_3$-solution (3 ml) for 18 min. The reaction was interrupted in the usual manner; spectrophotometrically determined pigment recovery, 75 %. Paper-chromatographic examination demonstrated complete conversion to XXIII. This product was further purified by chromatography on a column of deactivated alumina. *Adsorptive properties.* 3,4-Dehydro-torulene (XXIII) required 5 % acetone-petroleum ether for elution from deactivated alumina. The *trans* isomer had $R_F = 0.25$ on aluminium oxide paper (1 % acetone-petroleum ether). *Absorption spectrum in visible light.* The *trans* isomer had abs.max. in petroleum ether at 491 mμ and in acetone at 493 mμ. *Partition test.* In the system petroleum ether/95 % methanol the partition ratio was 100:0.

Deoxy-flexixanthin (XXIV)

Purification. Deoxy-flexixanthin was chromatographed 2 to 3 times on cellulose columns, and then once more on a column of deactivated alumina (see Table 2). A final re-chromatography on deactivated alumina was required.

Adsorptive properties on cellulose and deactivated alumina columns are given in Table 2. The following R_F-values were found: on kieselguhr paper, $R_F = 0.53$ (10 % acetone-petroleum ether) and on aluminium oxide paper, $R_F = 0.21$ (10 % acetone-petroleum ether) and $R_F = 0.55$ (20 % acetone-petroleum ether).

Crystallization was effected from ether-petroleum ether; yield *ca.* 0.3 mg with indistinct m.p. 150—160°C.

Purity test. The crystals were paper-chromatographically pure.

Absorption spectrum in visible light. Abs.max. in petroleum ether were located at 476.5 ($E\ ^{1\%}_{1\ cm} \geq 2650$) and 503 mμ, in acetone at 480.5 and 508 mμ (see Fig. 2), and in methanol at 477.5 and (500) mμ.

Infrared spectrum. The spectrum of XXIV (0.1 mg) in 0.2 g KBr is presented in Fig. 1. The spectrum of echinenone (0.3 mg) in 0.2 g KBr was recorded for comparison. Echinenone was isolated from *Aphanizomenon flos-aque.*[33]

Mass spectrum. For mass-spectrometric molecular weight determination, see under dehydro-flexixanthin above.

Partition tests. In the system petroleum ether/95 % methanol, the partition ratio was 36:64, and in petroleum ether/85 % methanol, 74:26. Addition of alkali did not alter the partition ratios.

Attempted acetylation. To XXIV (48 μg) dissolved in 1 ml dry pyridine was added 0.3 ml acetic anhydride. The reaction was interrupted after 19 h; spectrophotometrically determined pigment recovery, 83 %. Judged by paper chromatography no acetylated product was formed.

Hydride reduction of XXIV to XXV. XXIV (63 μg) was treated with lithium aluminium hydride in the usual manner; spectrophotometrically determined pigment recovery, 92 %. The reaction mixture consisted exclusively of XXV. *Adsorptive properties. Trans* XXV had $R_F = 0.31$ on kieselguhr paper (10 % acetone petroleum ether). The neo A and neo B isomer had $R_F = 0.37$ and 0.40, respectively, in the same system. Their true nature as members of the XXV stereoisomeric set was established by reversible iodine-catalyzed isomerization in light, followed by paper-chromatographic examination. XXV was less strongly adsorbed than XVII, as shown by a co-chromatography test. *Absorption spectrum in visible light. Trans* XXV had abs.max. in petroleum ether at (361), 444, 471, and 500 mμ, and in acetone at (450), 476, and 506 mμ. *Partition test.* In petroleum ether/85 % methanol the partition ratio was 48:52.

Acetylation of XXV to XXVI. To XXV (44 μg) dissolved in 1 ml dry pyridine was added 0.2 ml acetic anhydride, and the reaction was followed paper-chromatographically. No intermediates were observed, the conversion to XXVI was complete after 6 1/2 h; pigment recovery, 82 %. *Adsorptive properties.* On kieselguhr paper, *trans* XXVI had $R_F = 0.18$ (2 % acetone-petroleum ether) and $R_F = 0.67$ (10 % acetone-petroleum ether). *Absorption spectrum in visible light. Trans* XXVI had abs.max. in petroleum ether at (361), 447, 472, and 502.5 mμ, and in acetone at 451, 477.5, and 508.5 mμ (see Fig. 5). *Partition test.* In petroleum ether/95 % methanol, XXVI exhibited a partition ratio of 55:45.

Dehydration of deoxy-flexixanthin (XXIV) to XXVII. To XXIV (0.55 mg) dissolved in 2 ml dry pyridine was added 0.02 ml phosphorus oxychloride, and the reaction, followed paper-chromatographically, was interrupted after 48 min. Spectrophotometrically determined recovery was 66 %. The reaction mixture, which contained XXVII (95 % of total) and some unreacted XXIV, was chromatographed on a column of deactivated alumina. *Adsorptive properties.* XXVII required 5 % acetone-petroleum ether for elution from deactivated alumina. On kieselguhr paper the *trans* isomer had $R_F = 0.48$ (5 % acetone-petroleum ether) and $R_F = 0.76$ (10 % acetone-petroleum ether). *Absorption spectrum in visible light. Trans* XXVII had abs.max. in petroleum ether at 489.5 and 519.5 mμ, in acetone at 493 and 522.5 mμ (see Fig. 6), and in methanol at 490 and (516) mμ. *Partition test.* In petroleum ether/95 % methanol the partition ratio was 92:8.

Hydride reduction of XXVII to XXVIII. XXVII (0.17 mg) was reduced with lithium aluminium hydride in dry ether in the usual manner; spectrophotometrically determined pigment recovery, 91%. The reaction mixture consisted exclusively of XXVIII. *Adsorptive properties. Trans* XXVIII had $R_F = 0.32$ on kieselguhr paper (5 % acetone-petroleum ether). *Absorption spectrum in visible light.* The *trans* isomer had abs.max. at (378), 457.5, 483, and 516 mμ in petroleum ether, and at (380), (463), 488.5, and 520 mμ in acetone. The spectrum was identical with those of torulene (3′,4′-dehydro-γ-carotene) and XXII. *Partition ratio.* In petroleum ether/95 % methanol the partition ratio was 68:32.

Allylic dehydration of XXVIII to XXIII. XXVIII (0.15 mg) was treated with 2 ml 0.03 N HCl—$CHCl_3$-solution according to the standard method. The reaction was followed paper-chromatographically, and was interrupted after 11 min; spectrophotometrically determined pigment recovery, 81 %. XXIII, comprising 95 % of the reaction

mixture, was purified by chromatography on a column of deactivated alumina. *Adsorptive properties.* XXIII required 2 % acetone-petroleum ether for elution from deactivated alumina. The *trans* isomer had $R_F = 0.24$ on aluminium oxide paper (1 % acetone-petroleum ether) and $R_F = 0.36$ on kieselguhr paper in the same solvent system.

Trans 3,4-dehydro-torulene (XXIII) thus obtained was inseparable by chromatography from XXIII obtained by acid-chloroform treatment of XXII above. *Absorption spectrum in visible light. Trans* XXIII exhibited abs.max. in petroleum ether at 489.5 and 519.5 mμ, and in acetone at 494 and (523) mμ. The spectrum in acetone is presented in Fig. 6. *Partition test.* In petroleum ether/95 % methanol the partition ratio for XXIII was 100:0.

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