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Bacterial Carotenoids

XX.* The Carotenoids of *Mycobacterium phlei* strain Vera. 2. The Structures of the Phlei-xanthophylls — two Novel Tertiary Glucosides

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The strongly hypophasic carotenoid fraction of *Mycobacterium phlei* strain Vera was shown to consist of two carotenoid components (V and VI), separable only as their acetates (VII and VIII).

The structures of phlei-xanthophyll (V) and 4-keto-phlei-xanthophyll (VI) were established by chemical and spectroscopic (electronic, infrared, proton magnetic resonance, and mass spectrometry) methods and shown to be tertiary D-glucosides of carotenoid aglucones, the first to be found in Nature.

The preparation of eleven derivatives of V and thirteen derivatives of VI is described. A partial synthesis of 2′-keto-phlei-xanthophyll tetraacetate (XI), a derivative of V, was achieved from synthetic XXI according to the Koenigs-Knorr procedure.

Finally the biosynthetic interrelationship between V, VI, and the previously isolated minor carotenoids I, II, III, and IV is considered.

In a recent paper [1] we have described the chemical structures of the minor carotenoids produced by *Mycobacterium phlei* strain Vera, namely the monocyclic C_{40}-carotenoids γ-carotene (I), 1′,2′-dihydro-1′-hydroxy-γ-carotene (II), 4-keto-γ-carotene (III), and 1′,2′-dihydro-1′-hydroxy-4-keto-γ-carotene (IV). The structures of II, III, and IV were confirmed by total synthesis by Bonnett, Spark and Weedon [2] and Leftwick and Weedon.[3]

The main carotenoid synthesized by this bacterium was tentatively identified by Schlegel [4] as myxoxanthophyll, a poly-hydroxy carotenoid of unknown structure, previously considered characteristic of blue-green algae.[5]

We have lately shown that the major xanthophyll, designated phlei-xanthophyll,[1,6] was different from myxoxanthophyll. Subsequent studies have revealed that phlei-xanthophyll consisted of two carotenoid components, and the present paper reports on the structural determination of the two hypophasic phlei-xanthophylls.

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RESULTS AND DISCUSSION

Under the condition of culture employed, *M. phlei* strain Vera produced a relatively high content of carotenoids (*ca.* 0.7 % of the dried cells), 85 % of which consisted of strongly hypophasic carotenoids, *cf.* Ref. 1.

The major problem in the isolation of the phlei-xanthophylls turned out to be one of purification rather than the usual limitation of material available. Owing to its strongly polar character, the hypophasic carotenoid fraction could only be chromatographed on a restricted number of adsorbents. The phlei-xanthophylls were barely soluble in the most commonly used organic solvents, and always crystallized with a large content of ash (containing alkali metals and sulphur). The crystalline specimens exhibited relatively low extinction values in visible light and had blurred infrared spectra.

On cellulose columns as well as on kieselguhr paper the hypophasic carotenoid fraction seemed to comprise a single carotenoid. However, upon acetylation, two final acetates, chromatographically separable and with somewhat different absorption spectra in visible light, were obtained. Individual saponification of each fully acetylated product furnished single xanthophylls with different absorption spectra in visible light, but with identical adsorptive properties. Separate re-acetylation of each xanthophyll resulted in a single peracetate in each case. Separation of the two phlei-xanthophylls was consequently carried out *via* the corresponding peracetates according to the scheme outlined in Fig. 1.

The major xanthophyll, phlei-xanthophyll (V), comprised 75 % of the hypophasic carotenoid fraction, whereas the second hypophasic xanthophyll, subsequently shown to be a 4-keto-phlei-xanthophyll (VI), accounted for the remaining 25 %.

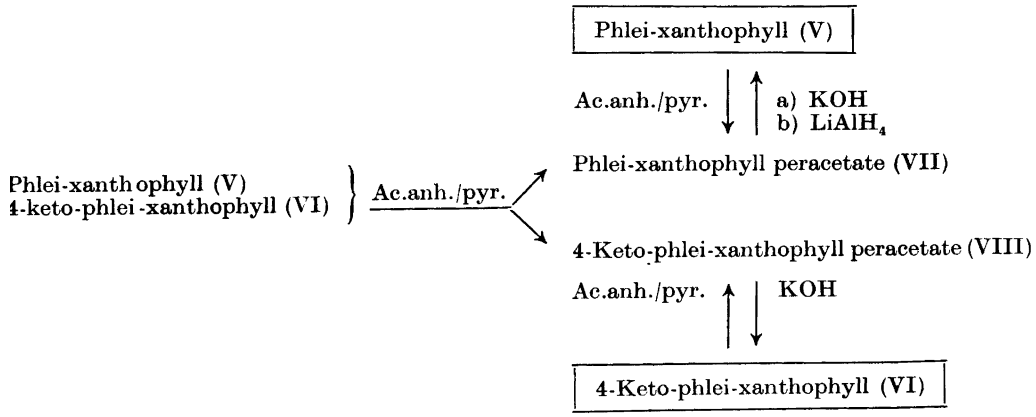


Fig. 1. Separation scheme for the two phlei-xanthophylls.

1. Phlei-xanthophyll (V)

Phlei-xanthophyll, m.p. 209°C, crystallized from pyridine-petroleum ether. The absorption spectrum in visible light, recorded in chloroform solution, is presented in Fig. 2 together with that of lycopene and β-apo-2′-carotenyl (C_{37})

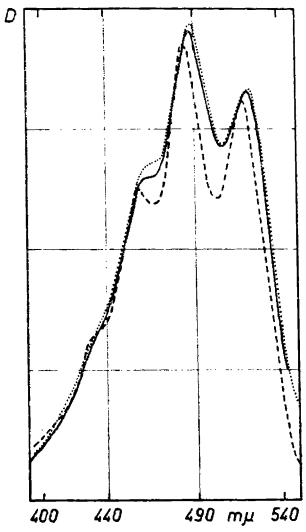


Fig. 2. Absorption spectra is visible light recorded in chloroform solution of *trans*. ····· β-apo-2′-carotenyl (C_{37}) acetate (IX), ——— phlei-xanthophyll (V), — — — lycopene.

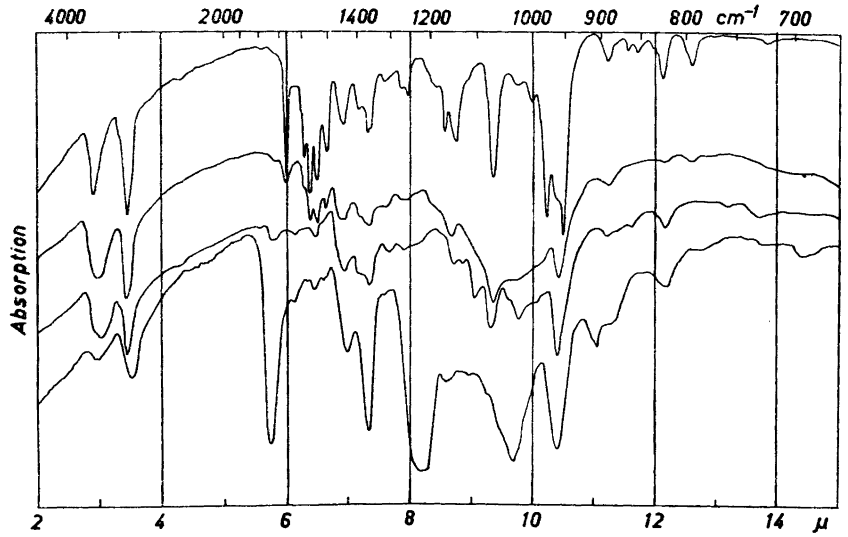


Fig. 3. Infrared absorption spectra measured in KBr of synthetic 1′,2′-dihydro-1′-hydroxy-2′-keto-torulene (XXI, uppermost curve), 2′-keto-phlei-xanthophyll (X, curve No. 2 from above), phlei-xanthophyll (V, curve No. 3 from above) and phlei-xanthophyll pentaacetate (VII, lowest curve).

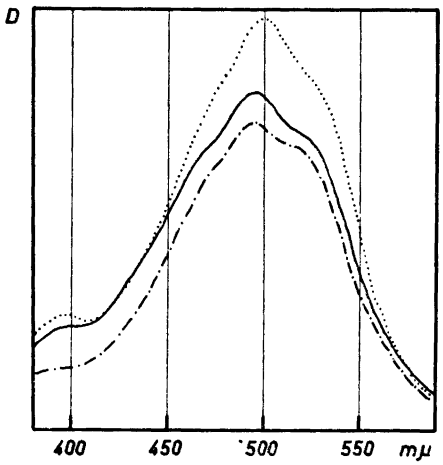
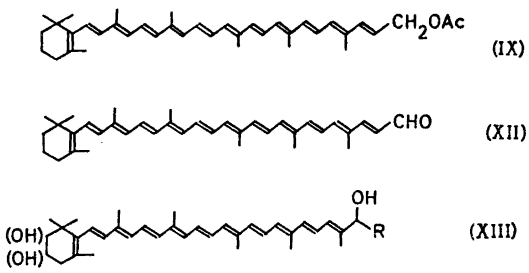


Fig. 4. Absorption spectra in visible light recorded in acetone solution of *trans*. — · — β-apo-2′-carotenal (C_{37}) (XII), ——— acetate of *p*-chloranil oxidized phlei-xanthophyll (XI) · · · · *p*-chloranil oxidized 4-keto-phlei-xanthophyll (XXVIII).

acetate (IX). Good spectral agreement was observed for phlei-xanthophyll and IX. The absence of conjugated (or isolated) carbonyl functions in phlei-xanthophyll was further inferred from the infrared spectrum (Fig. 3).

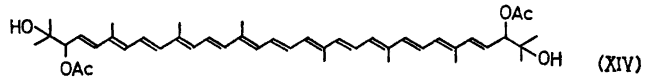
Allylic oxidation of phlei-xanthophyll with *p*-chloranil [7] gave an oxidation product (X), the *trans* peracetate (XI) of which exhibited an absorption spectrum in visible light indistinguishable from that of β-apo-2′-carotenal (C_{37}) (XII), (see Fig. 4). The presence of a conjugated keto-group in the allylic oxidation product (X) was also indicated by absorption at 1675 cm^{-1} in the infrared spectrum (see Fig. 3). Such a low intensity absorption band has previously been found to be characteristic of aliphatic carotenoids with conjugated carbonyl groups in the 2-position, *e.g.* spheroidenone, OH-spheroidenone,[8] and 2,2′-diketo-spirilloxanthin (P518),[9] and it was later found to be typical also of synthetic 1′,2′-dihydro-1′-hydroxy-2′-keto-torulene (XXI), see Fig. 3. Treatment of the allylic oxidation product (X) with the stronger oxidizing reagent nickel peroxide, [10,11] resulted in no further allylic oxidation. Since no dihydro product was obtained on treatment with zinc and acetic acid in pyridine according to the method of Kuhn and Winterstein, [12] under conditions where canthaxanthin (4,4′-diketo-β-carotene) and rhodoxanthin did yield dihydro-derivatives, the formulation of the allylic oxidation product (X) as an ω, ω′-diketone appeared unlikely. On this basis the partial structure XIII was indicated for phlei-xanthophyll. A hydroxyl group in 4-position seemed unlikely because of the above-mentioned lack of further allylic oxidation, as well as failure of the allylic oxidation product (X) or its peracetate (XI) to give elimination products with extended chromophore on standard treatment with acid chloroform.[13] However, at this stage hydroxyl substituents in the 2- or 3-positions or hydroxyl substituents in the *gem* methyl groups could not be disregarded.

The strong adsorptive properties and the polar partition ratio indicated a large number of hydroxyl groups in phlei-xanthophyll. The course of acetylation was followed by periodic analysis (see Fig. 10), and the number (five) and



properties of the transitory acetates formed, which were separable by circular chromatography, revealed the presence of at least three hydroxyl groups in positions accessible for acetylation. The correct number of acetoxy groups in the peracetate (VII) was later established as five (see below). The infrared spectrum of the pentaacetate (VII) is included in Fig. 3.

Although no absorption bands characteristic of tertiary hydroxyl groups were present in the infrared spectrum of phlei-xanthophyll (Fig. 3), the partition ratio, R_F-value and infrared spectrum of phlei-xanthophyll pentaacetate suggested the presence of such a group. However, the pentaacetate (VII) furnished no dehydration product when treated with phosphorus oxychloride in pyridine,[14] nor a trimethylsilyl ether on silylation. Whereas carotenoids with tertiary hydroxyl groups generally give trimethylsilyl ethers, very hindered hydroxyl groups like the one in the 6-position in azafrin methyl ester do not form such derivatives.[15] However, the diacetate (XIV) of hydride reduced, synthetic 1,2,1′,2′-tetrahydro-3,4,3′,4′-dehydro-1,1′-dihydroxy-2,2′-diketo-lycopene, did form a di-trimethylsilyl ether. The presence of tertiary hydroxy groups in phlei-xanthophyll was therefore disregarded.



On the other hand a tertiary oxygen substituent in the 1′-position appeared likely, because of the presence of a signal at 8.81 τ (*ca.* 6 protons) in the proton

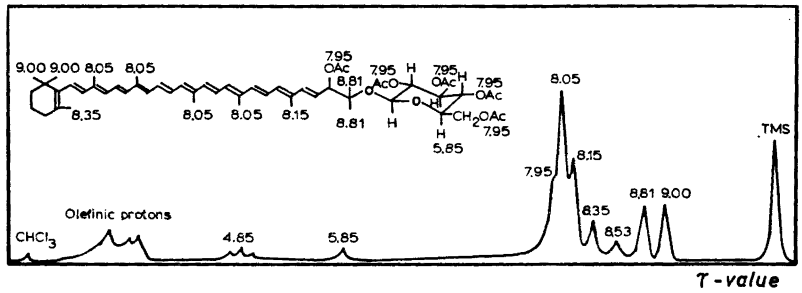


Fig. 5. Proton magnetic resonance spectrum of phlei-xanthophyll pentaacetate (VII) in deuterochloroform.

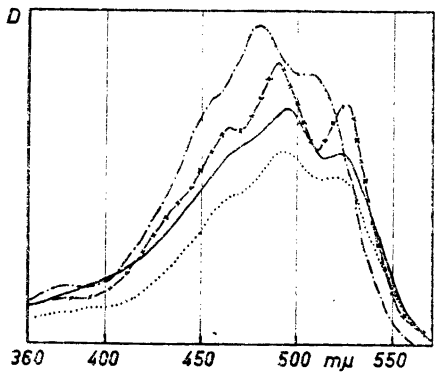
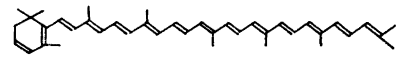
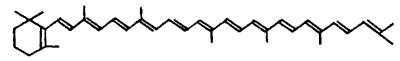


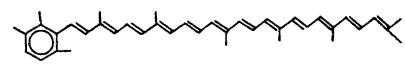
Fig. 6. Absorption spectra in visible light recorded in acetone solution of *trans*. — · — 4-keto-phlei-xanthophyll pentaacetate (VIII), — × — 4-hydroxy-torulene (XXXII), ——— 3,4-dehydro-torulene (XV), ····· 4-keto-torulene (XXXI).

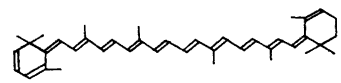
magnetic resonance spectrum of phlei-xanthophyll pentaacetate (VII); see Fig 5.[16] The latter spectrum also suggested the presence of unhydroxylated *gem* methyl groups in a cyclohexene ring (signal at 9.00 τ, *ca.* 6 protons).

A striking reaction occurred on treatment of phlei-xanthophyll with acid chloroform.[13] This strongly hydroxylated carotenoid was efficiently converted to a product with extended chromophore and properties compatible with a carotene. The absorption spectrum in visible light of this product (XV) is presented in Fig. 6. Efforts were made toward identification of this elimination product. The following compounds with related absorption spectra were used for direct comparison: torulene (XVI), 3',4'-dehydro-chlorobactene (XVII, synthesized by N-bromosuccinimide treatment of chlorobactene [17]), 2,3-dehydro-*retro*-carotene (XVIII, prepared by acid chloroform treatment of isozeaxanthin [18]) and finally 3,4-dehydro-torulene (XV, obtained by others[19] *via* defined routes from anhydro-deoxy-flexixanthin = 2-keto-torulene or by N-bromosuccinimide treatment of torulene).


(XV)

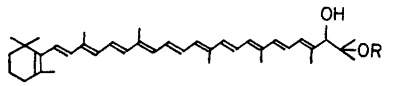

(XVI)


(XVII)


(XVIII)

Identity with XV was established by direct, qualitative and quantitative comparison of the stereoisomers present in the iodine catalyzed equilibrium mixtures,[20] see Table 3. On this basis the C_{40}-carotenoid skeleton of phlei-xanthophyll was established. Moreover, a hydroxyl group in the 2-position in phlei-xanthophyll (V, XIII) could also be disregarded.

Since most carotenoid pigments are C_{40}-compounds, it is tempting to make structural considerations of new carotenoids on this basis. However, at this stage a mass-spectrometric molecular weight determination offered helpful advice. By high precision mass spectrometry the molecular weight of phlei-xanthophyll was determined as 730.449 ± 30 ppm ($C_{45}H_{62}O_8$ or $C_{41}H_{62}O_{11}$). A pronounced peak was also observed at *m/e* 624.4021 ± 20 ppm ($C_{38}H_{56}O_7$) or M − *ca.* 106. Since no reference compound with sufficiently high molecular weight was available for matching any of these peaks, the measurements were not considered fully reliable. However, from the work of Schwieter *et al.*[21] carotenoids are known to lose xylene (*m/e* 106.160, C_8H_{10}) in the mass spectrometer, and the (M − 106) peak was used for calculation of the molecular formula of phlei-xanthophyll: $C_{38}H_{56}O_7$ (624.4026) + C_8H_{10} (106.160) = $C_{46}H_{64}O_7$ (730.5626). When incorporating the established structural features of phlei-xanthophyll ($C_{46}H_{64}O_7$) into formula XIX, the tertiary oxygen substituent R in the 1′-position turned out to be $C_6H_{11}O_5$ − strongly indicative that phlei-xanthophyll was a glycoside of a hexose sugar. Mass spectrometric molecular weight determination of the peracetate (XII) gave M = 940, thus revealing the presence of five acetate units in the peracetate, in fair agreement with the proton magnetic resonance spectrum of phlei-xanthophyll peracetate (VII) and compatible with the above assumption that R was a hexose residue.



(XIX)

R = $C_6H_{11}O_5$

The presumed sugar residue was sought for and found in the aqueous hypophase following acid chloroform treatment of phlei-xanthophyll (V) to yield 3,4-dehydro-torulene (XV). The aqueous hydrolysis concentrate gave a positive Molisch test [22] for carbohydrate, and the hexose present was established as glucose by paper chromatography according to the method of Fischer and Percival.[23] Since the glucose in question was oxidized by the D-glucose oxidase notatin,[24] it could be inferred that phlei-xanthophyll was a tertiary glycoside of D-glucose. Attempts were further made to establish by enzymic means the stereochemistry of the glycosidic linkage. *t*-Trimethylcarbinol-*β*-D-glucoside [25] was used as test substance for treatment with the *β*-glycosidase almond emulsin.[26] No enzymic hydrolysis of phlei-xanthophyll (V) was observed. Nor was any enzymic hydrolysis achieved using *α*-glucosidase, the activity of which was tested with maltose. These results may probably be explained from the unsatisfactory solubility properties of phlei-xanthophyll (V). However, since the vast majority of naturally occurring glycosides are *β*-glycosides,[27] it seems reasonable to assume that this is the case with phlei-xanthophyll too. Some support for this assumption came from the observation that the Koenigs-Knorr synthesis of the phlei-xanthophyll derivative XI described below, gave rise to a product inseparable from XI derived from natural phlei-xanthophyll. Under the conditions used the Koenigs-Knorr synthesis is known to yield *β*-glycosides.[27,49]

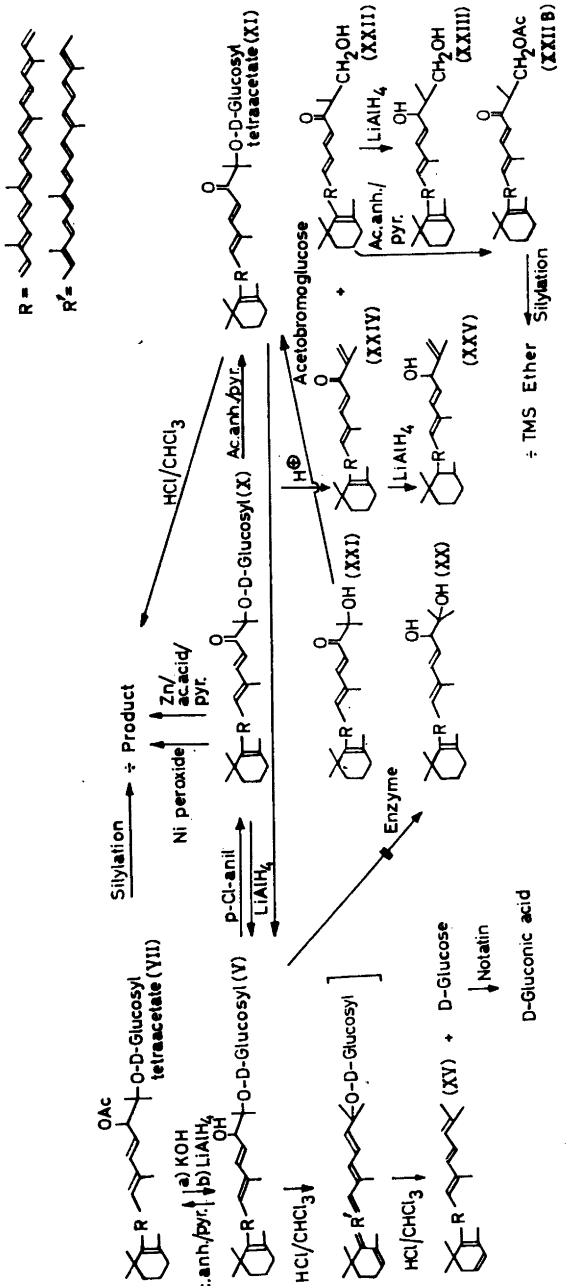
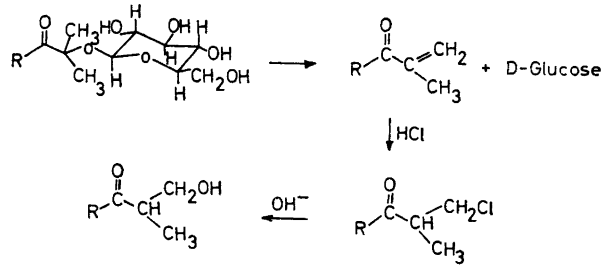


Fig. 7. Reaction scheme for phlei-xanthophyll (V).

The structure of phlei-xanthophyll was thus established as a tertiary D-glucoside. Tertiary glycosides in general appear to be very rare in Nature. Phlei-xanthophyll (V) represents the first tertiary glycoside of a carotenoid, and, apart from crocin (the digentiobiose ester of the carotenoid dicarboxylic acid crocetin [28]), this is the first finding of a crystalline glycoside with a carotenoid aglycone.*

The chemical transformations of phlei-xanthophyll so far discussed are summarized in Fig. 7. As already mentioned, the allylic oxidation product (X) and its tetraacetate (XI) were found to be stable towards standard treatment with hydrochloric acid in chloroform.[13] Hydrolysis of the glucosidic linkage, however, was expected under stronger acidic conditions Whereas X decomposed on treatment with 0.06 N hydrochloric acid in methanol,[25] two carotenoid products resulted on similar treatment in dry or ethanol-free, moist chloroform solution. These products exhibited absorption spectra in visible light indistinguishable from those of the allylic oxidation product (X, see Fig. 4) and synthetic 1′,2′-dihydro-1′-hydroxy-2′-keto-torulene (XXI). The most polar product, considered as XXII, showed a partition ratio indicative of a mono-ol, was somewhat more strongly adsorbed than XXI and afforded a monoacetate (considered as XXIIB) on acetylation and a di-ol (considered as XXIII) on hydride reduction. The monoacetate (XXIIB) failed to give a trimethylsilyl ether on silylation, thus demonstrating the absence of tertiary hydroxyl groups in XXII. The absorption spectra in visible light and the R_F-values and partition ratios of the less polar product and its hydride reduction product, as well as the alkali stability of the non-polar product were compatible with structures XXIV and XXV, respectively, for these compounds.

The formation of XXIV from X could be explained by a β-elimination. XXII might possibly be the result of an anti-Markownikoff addition of hydrogen chloride to XXIV followed by hydrolysis, although admittedly the reaction conditions employed were not expected to promote such hydrolysis. An attempted transformation of XXIV to XXII on acid (0.06 N) chloroform treatment failed.



According to Veibel,[25] tertiary glucosides are far more readily hydrolyzed than the corresponding primary or secondary glucosides. This explains in part the easy hydrolysis of phlei-xanthophyll (V) by acid chloroform treatment. The

* On the basis of insufficient evidence Smith [50] has a few years ago claimed the presence of carotenoid glucosides in *Mycoplasma* sp.

observed need for stronger conditions of hydrolysis for X, must probably be explained by differences in the mechanisms involved, since hydrolysis of V may be facilitated by the coupled elimination sequence form V to XV.

Finally a partial synthesis of XI was achieved by the Koenigs-Knorr procedure [29] using tetraacetoxy-bromoglucose and synthetic XXI. The latter compound was made available by Schwieter *et al.*[30] The glucoside synthesis, carried out on a micro scale gave a 5 % yield, and synthetic XI was identified with XI derived from 4-keto-phlei-xanthophyll (VI).

2. 4-Keto-phlei-xanthophyll (VI)

The absorption spectrum in visible light of 4-keto-phlei-xanthophyll was located at somewhat longer wavelengths than that of phlei-xanthophyll (V) and exhibited less fine-structure (*cf.* Fig. 6). Hydride reduction resulted in a hypsochromic shift of 4 mμ in acetone solution. The size of this shift indicated the presence of a conjugated carbonyl group in the 4-position in a cyclohexene ring [31] in 4-keto-phlei-xanthophyll. Moreover, the absorption spectra in visible light of its hydride reduction product (XXVI) and its peracetate (XXVII) were superimposable on that of phlei-xanthophyll (V, *cf.* Fig. 2). The presence of a conjugated keto group in 4-keto-phlei-xanthophyll was further inferred from infrared absorption at 1660 cm^{-1} (*cf.* Fig. 8 for spectrum of 4-keto-phlei-xanthophyll peracetate, VIII). The location of this keto-group in the 4-position in a so-called β-ring was also suggested by the observed methyl signals in the proton magnetic resonance spectra of 4-keto-phlei-xanthophyll (VI) and its hydride reduction product (XXVI). As with canthaxanthin (4,4'-diketo-β-carotene) and β-carotene, the carbonyl group in the 4-position in the cyclohexene ring causes a de-shielding of the gemini methyl groups as well as of the methyl group in 5-position.

Provided that 4-keto-phlei-xanthophyll had a hydroxyl group in 3-position, alkali treatment should have resulted in the corresponding diosphenol:[32]

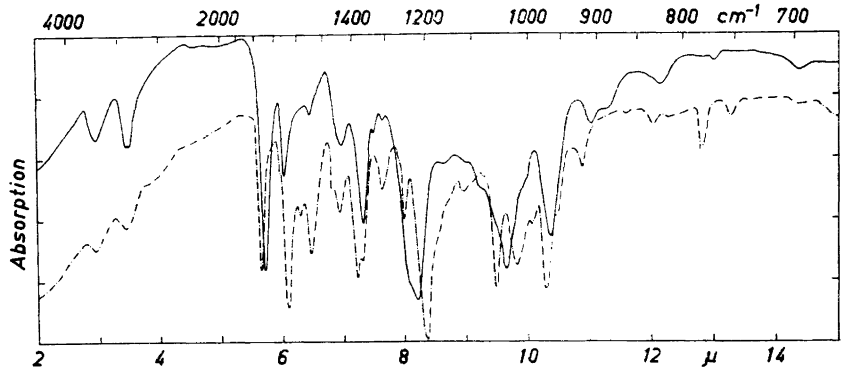
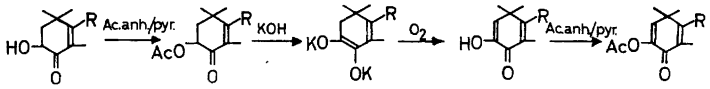


Fig. 8. Infrared spectra recorded in KBr of ——— 4-keto-phlei-xanthophyll pentaacetate (VIII), — · — astacene diacetate.



No blue-coloured enol salt was observed on alkali treatment of 4-keto-phlei-xanthophyll peracetate (VIII) in vacuum, nor were any absorption bands at 1620, 1540, 1245, and 1065 cm^{-1}, characteristic of the diosphenol groups in astacene, present in the infrared spectrum of 4-keto-phlei-xanthophyll. Furthermore, the peracetate (VIII) of alkali-treated, acetylated 4-keto-phlei-xanthophyll exhibited no enolacetate absorption at 1770 cm^{-1} as found in astacene diacetate (see Fig. 8). A hydroxyl function in the 3-position in 4-keto-phlei-xanthophyll was consequently disregarded.

Allylic oxidation of 4-keto-phlei-xanthophyll gave an oxidation product with extended chromophore which was considered to be a ω,ω'-diketone (XXVIII). This assumption was supported by zinc-acetic anhydride-pyridine reduction [12] to the unstable XXIX which rapidly reverted to XXVIII in the presence of alkali and oxygen. Although this allylic oxidation product (XXVIII) had an absorption spectrum in visible light hardly distinguishable from the allylic oxidation product (X) of phlei-xanthophyll (V), see Fig. 4, the adsorptive properties of XXVIII and X, as well as of the corresponding peracetates (XXX and XI), presented in Table 1, revealed the non-identity of these compounds.

Judging by their polarity properties (adsorption and partition behaviour), a similar number of hydroxyl groups was predicted for phlei-xanthophyll (V) and 4-keto-phlei-xanthophyll. The peracetate of 4-keto-phlei-xanthophyll gave no trimethylsilyl ether on silylation, thus arguing against the presence of tertiary hydroxyl groups. However, the proton magnetic resonance spectrum of the peracetate (VIII) exhibited a signal at 8.82 τ (*ca.* 6 protons) just as the phlei-xanthophyll pentaacetate (VII), thus indicating a tertiary oxygen substituent in the 1′-position.

Again, acid treatment of 4-keto-phlei-xanthophyll resulted in the formation of a non-polar elimination product, the main stereoisomers of which showed identical absorption spectra in visible light and corresponding R_F-values with those of anhydro-deoxy-flexixanthin = 4-keto-torulene (XXXI),[19] (see Fig. 6 and Table 4). Hydride reduction of the elimination product (XXXI) yielded a mono-ol (XXXII) with torulene-like absorption spectrum. On treatment with acid chloroform this mono-ol (XXXII) was converted to 3,4-dehydro-torulene (XV), identical with the allylic elimination product obtained from phlei-xanthophyll (V), see Table 3. The spectral changes occurring during these transformations from VI (with the same absorption spectrum as XIII) to XXXI to XXXII to XV are shown in Fig. 6.

On the basis of the identification of the allylic elimination product (XXXI) of 4-keto-phlei-xanthophyll, the C_{40}-carotenoid skeleton of the latter carotenoid was established, and hydroxyl groups in the 2 or the 3-position of 4-keto-phlei-xanthophyll could be disregarded. By inference, its hydroxyl groups were located in the 2′-position and in the assumed tertiary oxygen substituent in the 1′-position.

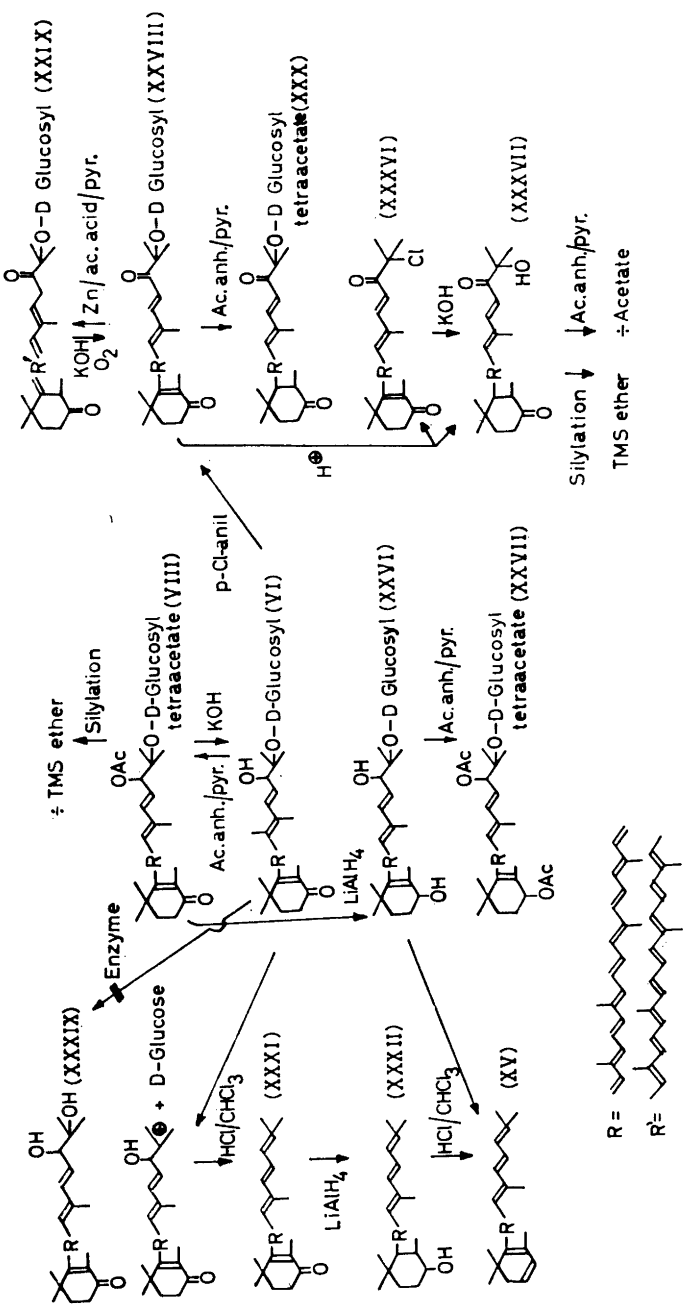
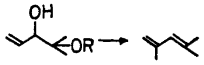


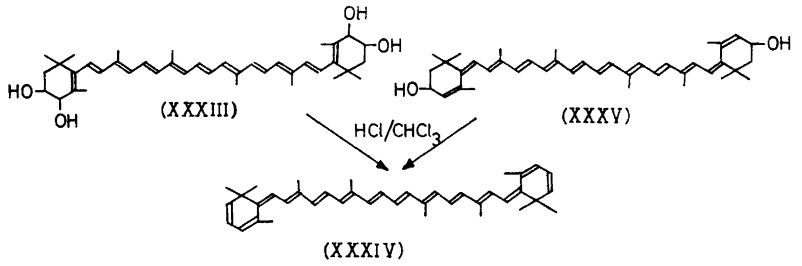
Fig. 9. Reaction scheme for 4-keto-phlei-xanthophyll (VI).

As with phlei-xanthophyll (V), glucose was isolated from the aqueous hypophase after acid chloroform treatment of 4-keto-phlei-xanthophyll; in this case in insufficient amount for subsequent notatin treatment. However, it seems reasonable to assume that 4-keto-phlei-xanthophyll is a D-glucoside like phlei-xanthophyll (V).

The reactions discussed above for 4-keto-phlei-xanthophyll are compatible with the structure VI for this carotenoid, see Fig. 9. In accordance with this scheme treatment with hydrochloric acid-chloroform of hydride reduced 4-keto-phlei-xanthophyll peracetate (XXVI) resulted in the formation of 3,4-dehydro-torulene (XV). Since this reaction proceeded less readily than the corresponding elimination from phlei-xanthophyll (V), it may be inferred that different mechanisms are involved. The reaction from phlei-xanthophyll (V) to XV on Fig. 7 may be formulated as a normal dehydration, but the reactions of 4-keto-phlei-xanthophyll (VI) and hydride reduced 4-keto-phlei-xanthophyll pentaacetate (XXVI) apparently also comprise eliminations of the following type:



As a model for the latter type of elimination the influence of acid chloroform on 3,4,3′,4′-tetrahydroxy-β-carotene (XXXIII, prepared by borohydride reduction of astacene) was studied. One of the two main elimination products was identified as anhydro-eschscholtzxanthin (XXXIV; for comparison prepared by acid chloroform treatment of hydride reduced rhodoxanthin = eschscholtzxanthin (XXXV)).



Also in this case a somewhat related type of elimination occurred. However, a comparison with the reaction from 3,4-dihydroxy-torulene to 3,4-dehydro-torulene (XV) on similar treatment,[19] might be more relevant.

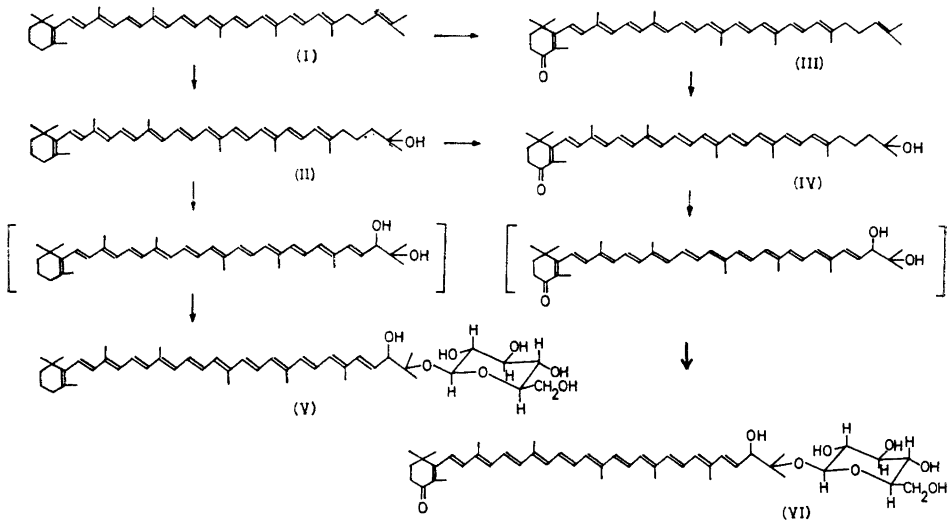
As for the allylic oxidation product (X) of phlei-xanthophyll (V) standard treatment [13] of the corresponding ω,ω′-diketone (XXVIII) with acid chloroform resulted in no reaction. However, stronger acid conditions promoted the hydrolysis of XXVIII.

Prolonged treatment with 0.13 N dry hydrogen chloride in chloroform resulted in complete conversion to two products, considered as XXXVI and XXXVII. The non-polar product (XXXVI) was converted to the more polar one (XXXVII) on alkali treatment, and the tertiary character of the hydroxyl

group in the latter was made evident by failure of XXXVII to form an acetate and by a successful transformation to a trimethylsilyl ether. There seems to be no obvious explanation of the different course of the reaction by acid treatment of the monoketone X to yield XXIV and XXII.

Finally enzymic hydrolysis of 4-keto-phlei-xanthophyll (VI), expected to yield the aglucone XXXIX, failed; presumably owing to solubility difficulties.

In summary we consider the structures V and VI established for the two strongly hypophasic phlei-xanthophylls. Their biosynthetic formation may possibly occur *via* the minor carotenoids previously studied (I, II, III, and IV) [1] as intermediates:



EXPERIMENTAL

Materials and methods used have been described in earlier papers of this series and have recently been summarized by Aasen and Liaaen Jensen.[19] This paper refers to the solvents and instruments used, as well as to the general isolation procedure for crystalline carotenoids. Peroxide-free tetrahydrofurane was prepared by distillation over lithium aluminium hydride. Pigment recoveries were determined spectrophotometrically. Elementary analysis was carried out by Fa. A. Bernhardt, Mülheim/Ruhr, Germany. Melting points were measured in evacuated capillary tubes on a Berl block and are uncorrected.

Partition tests were measured according to the method of Petracek and Zechmeister.[33] Iodine catalyzed stereoisomerization was carried out as previously described; this refers also to the terms in which spectral fine-structures are explained.[20]

Unless otherwise stated, reactions were carried out at room temperature.

Acetylations were effected in the usual way by acetic anhydride in pyridine.[20] Silylations were carried out according to the procedure of Golding, Rickards and Barber.[34a] Reduction with lithium aluminium hydride was performed in dry ether or tetrahydrofurane as described elsewhere.[9] Reduction tests with sodium borohydride required longer reaction periods. The reaction mixture was shaken mechanically and the solvents employed are specified in each case. Oxidation with *p*-chloranil was performed as described elsewhere.[7] The procedure of Entschel and Karrer was used for standard treatment with acid chloroform.[35]

Table 1. Adsorptive properties of various derivatives of phlei-xanthophyll (V).

Carotenoid	Required eluant from		R_F-value on S. & S. No. 287 paper			
	Cellulose column	Neutral alumina Activity grade 2	30 % acetone[a]	10 % acetone[b]	5 % acetone[b]	.2 % acetone[b]
4-Hydroxy-phlei-xanthophyll (XXVI)	25–30 % acetone[b]		0.34			
4-Keto-phlei-xanthophyll (VI)	25–30 % acetone		0.34			
Phlei-xanthophyll (V)	25–30 % acetone		0.34			
4,2′-Diketo-phlei-xanthophyll (XXVIII)	25 % acetone		0.39			
2′-Keto-phlei-xanthophyll (X)	20 % acetone		0.50			
4,2′-Diketo-phlei-xanthophyll tetraacetate (XXX)				0.14		
4-Keto-phlei-xanthophyll pentaacetate (VIII)		30 % acetone[b]		0.25		
1′,2′-Dihydro-1′-hydroxy-4,2′-diketo-torulene (XXXVII)				0.25		
1′,2′-Dihydro-2′,16′-dihydroxy-torulene (XXIII)				0.29		
4-Acetoxy-phlei-xanthophyll pentaacetate (XXVII)				0.34		
2′-Keto-phlei-xanthophyll tetraacetate (XI)				0.44		
1′,2′-Dihydro-1′,2′-dihydroxy-torulene (XX)				0.46		
1′,2′-Dihydro-2′-keto-16′-hydroxy-torulene (XXII)				0.57	0.12	
Phlei-xanthophyll penta-acetate (VII)		20–25 % acetone		0.59		
1′,2′-Dihydro-4,2′-diketo-1′-chloro-torulene (XXXVI)				0.68		
4-Hydroxy-torulene (XXXII)					0.30	
1′,2′-Dihydro-1′,16′-dehydro-2′-hydroxy-torulene (XXV)					0.39	
1′,2′-Dihydro-1′-hydroxy-2′-keto-torulene (XXI)					0.40	
1′,2′-Dihydro-2′-keto-16′-acetoxy-torulene (XXII B)					0.49	
4-Keto-torulene (XXXI)		5 % acetone			0.53	
1′,2′-Dihydro-1′,16′-dehydro-2′-keto-torulene (XXIV)		2–5 % acetone				0.42
3,4-Dehydro-torulene (XV)		2 % acetone				0.36[c]

[a] In benzene. [b] In petroleum ether. [c] 1 % acetone in petroleum ether.

Culture, medium, cultural conditions, extraction and saponification procedures have been described before.[1] Since alkali treatment of aliquots of the hypophasic carotenoids revealed no influence on their adsorptive properties or visible light absorption s ectra, saponification was included in the purification procedure, unless otherwise stated.

Chromatography. Column chromatography was performed on Schleicher and Schüll No. 124 cellulose powder or Woelm, neutral aluminium oxide, activity grade 2.[36] Circular paper chromatography was carried out on Schleicher and Schüll No. 597 paper, Schleicher

and Schüll No. 287 paper (kieselguhr paper)[37] or Schleicher and Schüll No. 288 paper (aluminium oxide paper).[38] For co-chromatography tests, the 3-divided paper technique was used.[39]

Adsorptive properties for the derivatives of phlei-xanthophyll studied are compiled in Table 1.

Separation of phlei-xanthophyll (V) and 4-keto-phlei-xanthophyll (VI) via *their acetates (VII and VIII).* V and VI could not be separated on a cellulose column, and the eluate containing the two phlei-xanthophylls was subjected to acetylation with acetic anhydride in dry pyridine. The resulting pentaacetates (VII and VIII) were separated by column chromatography on deactivated alumina. The properties of VII and VIII are further described below.

Phlei-xanthophyll (V)

Isolation. Chromatographically pure pentaacetate (VII) dissolved in dry tetrahydrofurane was reduced with lithium aluminium hydride in the usual manner. V was transferred to ether-tetrahydrofurane in a separatory funnel on admixture with aqueous sodium chloride solution and finally chromatographed on a cellulose column.

Alternatively the pentaacetate (VII) was saponified in ether-methanol containing 5 % potassium hydroxide, and treated as above. However, phlei-xanthophyll (V) of higher purity was obtained using the first procedure.

Crystallization. V crystallized from pyridine-petroleum ether as mauve-black needles forming aggregates; m.p. 209°C after repeated recrystallization; yield *ca.* 70 mg.

Solubility properties. Crystalline V was insoluble in petroleum ether and exhibited increasing solubility in the following solvents: carbon disulphide, ether, acetone, benzene, methanol, chloroform, tetrahydrofurane, and pyridine. The two latter were the only satisfactory solvents for pure V.

Adsorptive properties are given in Table 1.

Absorption spectrum in visible light. Absorption maxima for V in various solvents are presented in Table 2. The spectrum in chloroform solution is presented in Fig. 2. At 493 mμ in pyridine $E^{1\,\%}_{1\,cm} = 1740$ ($\varepsilon = 127\,000$).

For direct comparison the absorption spectra of synthetic lycopene, β-apo-2′-carotenyl (C_{37}) acetate (IX) as well as of eschscholtzxanthin [13] were recorded (see Table 2 and Fig. 2).

Table 2. Absorption maxima in visible light of *trans* eschscholtzxanthin, lycopene, β-apo-2′-carotenyl (C_{37}) acetate, and phlei-xanthophyll (V).

	Abs. max. in mμ in		
Carotenoid	Acetone	Chloroform	Pyridine
Eschscholtzxanthin		457 481 511	464 489 521
Lycopene	448 474 505	458 484 518	462 490 525
β-Apo-2′-carotenyl (C_{37}) acetate	451 474 505	(465) 489 522	(465) 493 526
Phlei-xanthophyll (V)	454 478 509	(465) 489 522	(465) 493 526

Eschscholtzxanthin was prepared by lithium aluminium hydride reduction of natural rhodoxanthin (9 mg) in dry tetrahydrofurane; pigment recovery was 80 %. Crystallization was effected from acetone-petroleum ether; m.p. 185 – 187°C, $R_F = 0.76$ on kieselguhr paper (20 % acetone in petroleum ether). The IR-spectrum exhibited characteristic splitting of the absorption band caused by *trans* di-substituted double bonds (950 and 975 cm^{-1}).

Infrared spectrum of 0.3 mg V in 0.2 g KBr is shown in Fig. 3.

Proton magnetic resonance (NMR) spectrum of V was recorded at 100 MC/sec in deuteropyridine. In the methyl region the following signals were observed: 8.95 (*gem.* methyl in β-ring). 8.52(?), 8.28 (methyl in 5-position in β-ring), 8.09 (methyl in 5′-position) and 8.04 τ (in-chain-methyl).

The spectrum of γ-carotene at 60 Mc/sec in the same solvent was recorded for comparison: 8.98 (*gem.* methyl in β-ring), 8.39 and 8.33 (isopropylidene), 8.24 (methyl in 5-position in β-ring), 8.17 (end-of-chain methyl in 5′-position) and 7.98 τ (in-chain-methyl).

The absence of methoxyl (6.70 τ) and aryl methyl (*ca.* 7.70—7.90 τ) in V was inferred from the NMR-spectrum.

Mass spectrum. The mass spectrum of V showed peaks at 730 (M) and 624.4021 ± 20 ppm (M − 106.160, $C_{38}H_{56}O_7$ theor. 624.4026).

Elementary analysis for the purest sample analyzed was found to be C 61.47, H. 7.81, ash 15.81, O (by difference) 14.91; re-calculated to ash-free specimen 73.0 C, 9.25 H, 17.75 O; calc. for $C_{46}H_{66}O_7$ 75.56 C, 9.12 H, 15.32 O. In addition the following was found: S 1.56; calc. for one S atom/mole, S 4.38, and OCH_3 0.77; calc. for one OCH_3/mole, OCH_3 4.23. One particular sample ($E^{1\,\%}_{1\,cm}$ = 500 at 494 mμ in pyridine) contained 41 % ash, comprising the elements K, Na, and also Ca, Mg, Al, Si, and Fe (determined by flame photometry).

Partition test. V was completely hypophasic when partitioned between petroleum ether and 60 % aqueous methanol.

Stereoisomerization studies. Iodine catalyzed isomerization in benzene solution resulted in a drop in the extinction coefficient and in the appearance of a *cis*-peak at 366 mμ (in acetone). A neo A isomer (abs.max. 366, (450), 473, and 504 mμ, % III/II = 25, % D_B/D_{II} = 33) was isolated by chromatography on kieselguhr paper (R_F = 0.42, compared with R_F = 0.34 for *trans* V; 30 % acetone in benzene). This isomer was shown to be a true member of the phlei-xanthophyll (V) stereoisomeric set by reversible isomerization in light. Boiling with reflux in benzene solution resulted in the formation of the same *cis*-isomer.

Epoxide test.[40] No blue colour was produced on treament of an ether solution of V with conc. hydrochloric acid.

Lead tetraacetate oxidation.[41] V (2.5 mg) in glacial acetic acid (5 ml) was treated with 0.1 N lead tetraacetate in glacial acetic acid (0.2 ml) for 3 days; pigment recovery 35 %. The main non-polar product (12 % of recovered pigment) had abs.max. at 465, 486, and 515 mμ in acetone and R_F = 0.70 on aluminium oxide paper (5 % acetone in petroleum ether), in the same region as β-apo-2′-carotenal (C_{37}) (XII) (R_F = 0.50 in a similar system). However, no co-chromatography tests were carried out.

Silylation. To V (0.5 mg) in dry pyridine (1 ml) was added hexamethyldisilazane (0.4 ml) and trimethylchlorosilane (0.2 ml). The reaction mixture was treated according to the method of Golding, Rickards and Barber [34a] and shown to contain a single carotenoid product. The pertrimethylsilyl ether had the same absorption spectrum in visible light as V and its R_F = 0.51 on kieselguhr paper (petroleum ether).

Acetylation. The course of acetylation of V (0.3 mg) in dry pyridine (3 ml) with acetic anhydride (0.2 ml) was followed by paper chromatography. In addition to phlei-xanthophyll (V) itself and the final acetate (VII), five transitory products (a,b,c,d,e) appeared on the circular paper chromatogram, the amounts of which were determined spectrophotometrically (see Fig. 10). Their R_F-values on kieselguhr paper (20 % acetone in petroleum ether) were as follows: a (0.12), b (0.30), c (0.50), d (0.71), and e (0.90).

When V (0.35 mg, derived from hydride-reduced, chromatographically pure pentaacetate, VII) in dry pyridine (0.2 ml) was re-acetylated with acetic anhydride (0.2 ml) for 18 h, a pigment recovery of 55 % was obtained. The reaction mixture contained exclusively VII. A similar result was obtained on re-acetylation of saponified VII.

Phlei-xanthophyll pentaacetate (VII). Adsorptive properties are presented in Table 1. Crystallization of VII (m.p. 124°C) was effected from acetone-petroleum ether. The absorption spectrum in visible light in acetone solution conformed with that of V; $E^{1\,\%}_{1\,cm}$ = 1850 (ε = 173 500, compared with ε = 174 900[42] for β-apo-2′-carotenyl (C_{37}) acetate (IX) at 478 mμ. The IR-spectrum (see Fig. 3) exhibited absorption at 3420, 2860 (CH_2), 1740 (acetate), 1630, 1540 (conj. double bonds), 1435 (CH_2), 1365 (CH_3), 1220 (acetate), (1165), 1035 (acetate), 962 (*trans* disubst. double bonds), 908 and 820 cm^{-1} (*trans* trisubst.

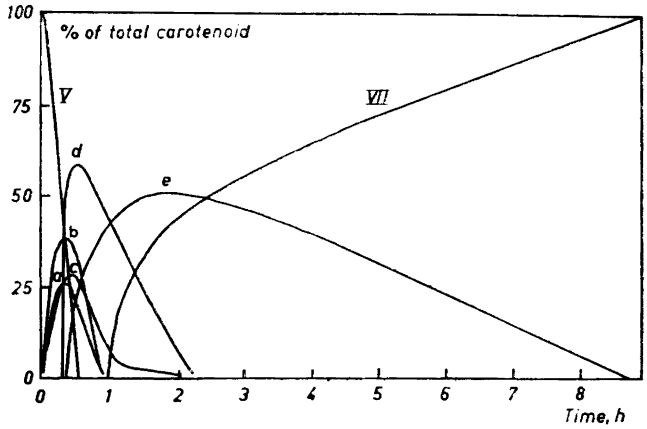


Fig. 10. Course of acetylation of phlei-xanthophyll (V); for explanation of transitory acetates (a, b, c, d, e) see text.

double bonds). The NMR-spectrum, recorded at 60 MC/sec in deuterochloroform is presented in Fig. 5. Methyl signals were located at 9.00 (*ca.* 6 protons, *gem.* methyl in β-ring), 8.81 (*ca.* 6 protons, *gem.* methyl in 1′-position), 8.53 (*ca.* 4 protons, non-allylic methylene), 8.35 (*ca.* 4 protons, methyl in 5-position in β-ring), 8.15, 8.05, and 7.95 (*ca.* 36 protons, in-chain/end-of-chain methyl, in-chain methyl and acetate methyl) and for CH_2-O at 5.95 τ (*ca.* 2 protons). The NMR-spectrum of *t*-trimethylcarbinol-β-D-glucoside tetraacetate at 60 Mc/sec in deuterochloroform was recorded for comparison. It exhibited signals at 8.77 (*t*-trimethyl), 8.00 (acetate methyl) and 5.94 τ (CH_2-O). This tetraacetate (m.p. 144—145°C) was prepared by acetylation of *t*-trimethylcarbinol-β-D-glucoside by acetylation with acetic anhydride in pyridine.

The mass spectrum of VII had peaks at 940 (M), 880 (M − 60, acetic acid), 834 (M − 106, xylene), 742 (M − 106 (xylene) − 92 (toluene)), 682 (M − 106 (xylene) − 92 (toluene) − 60 (acetic acid)). VII had the partition ratio 30:70 in petroleum ether/95 % methanol.

Further acetylation of VII was attempted by treatment with acetyl chloride in pyridine for 24 h. No less polar acetates were formed.

Attempted silylation of VII (2.4 mg) in dry pyridine (1 ml) was carried out by treatment with hexamethyldisilazane (0.4 ml) and trimethylchlorsilane (0.2 ml) for 1 h. The reaction mixture was treated in the usual manner. No trimethylsilyl ether was detected by spectrophotometric and paper-chromatographic examination. In a parallel experiment XIV (1 mg, prepared by hydride reduction in tetrahydrofurane, followed by acetylation of synthetic 1,2,1′,2′-tetrahydro-3,4,3′,4′-dehydro-1,1′-dihydroxy-2,2′-diketo-lycopene [34b] was submitted to the same treatment. The di-trimethylsilyl ether of XIV was formed in quantitative yield, had the same absorption spectrum in visible light as XIV and showed R_F = 0.88 (10 % acetone in petroleum ether) compared with R_F = 0.64 (20 % acetone in petroleum ether) for XIV on kieselguhr paper. 1,2,1′,2′-Tetrahydro-3,4,3′,4′-dehydro-1,1′-dihydroxy-2,2′-diketo-lycopene itself also gave a di-trimethylsilyl ether in quantitative yield (R_F = 0.68 on kieselguhr paper; 10 % acetone in petroleum ether, compared with R_F = 0.14 for 1,2,1′,2′-tetrahydro-3,4,3′,4′-dehydro-1,1′-dihydroxy-2,2′-diketo-lycopene on the same type of paper; 20 % acetone in petroleum ether).

Treatment of VII (0.9 mg) in dry pyridine (5 ml) with phosphorus oxychloride (0.05 ml) for 30 min at 50°C was performed according to the method of Surmatis and Ofner.[14,17] The reaction mixture, treated in the standard manner, contained unreacted VII in addition to some decomposition products.

Saponification of VII (9.8 mg) was carried out in ether-methanol (40 ml, 1:7) containing 5 % KOH for 1 h. The unsaponifiable matter was transferred to ether-tetrahydro-

furane in the usual manner; pigment recovery was 83 %. Crystallization of the product, chromatographically and spectrophotometrically (visible light) inseparable from phleixanthophyll (V), was effected from ether. However, following alkali treatment, V always had a low extinction coefficient and high ash content.

Hydride reduction of VII (8.33 mg) was carried out with lithium aluminium hydride in dry tetrahydrofurane in the usual manner; pigment recovery, 86 %. Some unreduced and partly reduced carotenoid was removed by partition between petroleum ether and 90 % aqueous methanol. The hypophasic pigment was isolated and identified as phleixanthophyll (V) by absorption characteristics in visible light and R_F-values. Other experiments gave a similar result.

Allylic oxidation of phlei-xanthophyll (V) to 2'-keto-phlei-xanthophyll (X). V (4.69 mg) in benzene (14 ml) and ethanol (6 ml) was treated with *p*-chloranil (10.65 mg) and iodine (30 μg in 3 ml hexane) for 18 h in artificial Na-light; pigment recovery, 98 %. The reaction mixture contained X (90 %) and unreacted V (10 %). Other experiments gave a similar result.

Further allylic oxidation of X (0.3 mg) was attempted by treatment with nickel peroxide [10,11] (2.8 mg, available oxygen 2.87×10^{-3} g atom/g nickel peroxide determined by titration; *ca.* 40 times molar excess). No oxidation product with the expected properties of an ω,ω'-diketone was obtained.

2'-Keto-phlei-xanthophyll (X). X was purified by chromatography on a cellulose column; for adsorptive properties see Table 1.

Crystallization was carried out from acetone-petroleum ether. X exhibited absorption maxima in visible light at 490 and (525) mμ in petroleum ether, at 493 mμ in methanol and at 495 and (522) mμ in acetone, see Fig. 4. The IR-spectrum of X (0.3 mg in 0.2 g KBr), presented in Fig. 3, had abs.max. at 3350, 2930 (CH), 1675 (conj. carbonyl), 1575, 1535, 1505 (conj. double bonds), 1445 (CH_2), 1370 (CH_3), 1155, 1080, 960 (*trans* disubst. double bonds), 890, 825 (*trans* trisubst. double bonds) and 795 cm^{-1}. X was entirely hypophasic when partitioned between petroleum ether and 60 % methanol.

Reduction of X (0.2 mg) in dry pyridine (3 ml) was attempted, involving treatment with glacial acetic acid (0.1 ml) and zinc powder (15 mg) for 2 min.[12] The pigments were transferred to ether in a separating funnel; pigment recovery was 45 %. The reaction mixture exhibited an unchanged absorption spectrum in visible light, and paper-chromatographic examination demonstrated the presence of X only. In a parallel experiment canthaxanthin (1.08 mg) was treated in like manner; pigment recovery was 27 %. The resulting impure 5,5'-dihydro-canthaxanthin had abs.max. in petroleum ether at 420, 443, and 474 mμ and was readily autoxidized by air in the presence of alkali to canthaxanthin. Also, rhodoxanthin (1.3 mg) was easily reduced in the same way; pigment recovery, 65 %. The reaction mixture, chromatographed on a sucrose column, contained 3,3'-diketo-β-carotene (75 % of total). The latter product had abs.max. at (425), 449, and 476 mμ in petroleum ether, $R_F = 0.95$ on kieselguhr paper (10 % acetone in petroleum ether) and was readily autoxidized to rhodoxanthin in alkaline medium.

Acid hydrolysis of 2'-keto-phlei-xanthophyll (X) to XXI and XXIV. Standard treatment of X with 0.02 N hydrogen chloride in chloroform [35] did not result in the isolation of allylic elimination products.

Whereas attempted hydrolysis of X (*ca.* 2 mg) in 0.1 N or 0.03 N hydrogen chloride in methanol resulted in decomposition of the pigment, treatment of X (0.5 mg) with 0.06 N hydrogen chloride in moist chloroform (washed free of ethanol with water) proceeded with 75 % pigment recovery and resulted in hydrolysis to two non-polar products in about equal amounts (XXI and XXIV). Similar treatment of X (1.1 mg) with dry hydrogen chloride in chloroform gave the same products and 45 % pigment recovery. These products were separated by chromatography on a column of deactivated alumina.

1',2'-Dihydro-2'-keto-16'-hydroxy-torulene (XXII). Adsorptive properties for the most polar product (XXII) are given in Table 1. Co-chromatography tests revealed that XXII was somewhat more strongly adsorbed than synthetic 1',2'-dihydro-2'-keto-1'-hydroxy-torulene (XXI). However, partition ratios in petroleum ether/95% methanol (55:45 for XXII and 60:40 for XXI) and absorption spectra in visible light (abs.max. in acetone at 500 mμ) were quite similar.

Acetylation of XXII (0.3 mg) in dry pyridine with acetic anhydride gave 70 % pigment recovery and complete conversion to a monoacetate (XXIIB); for R_F-value

consult Table 1. The monoacetate (XXIIB) exhibited the same absorption spectrum in visible light as XXII, and was resistant towards silylation [34] in the usual manner.

Hydride reduction of XXII (0.05 mg) proceeded with 69 % pigment recovery, and resulted in a di-ol (XXIII) with abs.max. at 452, 478, and 509 mμ in acetone, as found for phlei-xanthophyll (V). The partition ratio was 14:86 in petroleum ether/95 % methanol and 51:49 in petroleum ether/85 % methanol; for R_F-value see Table 1.

1',2'-Dihydro-1',16'-dehydro-2'-keto-torulene (XXIV). The least polar product (XXIV) obtained on acid treatment of X, had the same absorption spectrum in visible light as the more polar product (XXII) above and the partition ratio was 93:7 in petroleum ether/95 % methanol; for R_F-value see Table 1. This product (XXIV) was resistant towards acetylation in the usual manner. Treatment with 5 % methanolic KOH-solution for 1 h did not result in the formation of new products.

Hydride reduction of XXIV (0.07 mg) gave 86 % pigment recovery and complete conversion to a mono-ol (XXV) with abs.max. at 455, 479, and 510 mμ in acetone; for R_F-value see Table 1.

Further treatment of XXIV with 0.03 — 0.06 N hydrogen chloride in chloroform (dry or moist) did not result in conversion to XXII.

2'-Keto-phlei-xanthophyll tetraacetate (XI). X was readily acetylated with acetic anhydride in dry pyridine in the usual manner. Adsorptive properties for the fully acetylated *trans* acetate are given in Table 1. *Trans* XI exhibited abs.max. in acetone at (470), 497, and (523) mμ. The spectrum is presented in Fig. 4, together with that of synthetic β-apo-2'-carotenal (C_{37}) (XII), recorded for comparison. The IR-spectrum of XI (in KBr) had abs.max. at 2900 (CH), 1740 (acetate), 1670 (conj. carbonyl), 1615, 1520 (conj. double bonds), 1448 (CH_2), 1368 (CH_3), 1227 (acetate), 1157, 1075, 1032 (acetate), and 962 cm^{-1} (*trans* disubst. double bonds). In petroleum ether/95 % methanol the partition ratio was 38:62.

Standard treatment of XI (0.21 mg) with acid chloroform did not result in the formation of allylic elimination products with extended chromophore.

Reduction of XI (0.05 mg) with lithium aluminium hydride in dry tetrahydrofurane, yielded phlei-xanthophyll (V), characterized by its absorption spectrum in visible light and by co-chromatography tests with authentic V.

Allylic elimination of phlei-xanthophyll (V) to 3,4-dehydro-torulene (XV). To V (2.2 mg) in chloroform (5 ml) was added 7 drops of a 0.32 N dry hydrogen chloride-chloroform solution (final acid concentration 0.02 N). A bathochromic colour shift was immediately observed, and after 5 min the reaction mixture was treated as usual; pigment recovery 65 %. The reaction invariably proceeded with the same ease. The reaction mixture was chromatographed on a column of deactivated alumina (for adsorptive properties of the main non-polar elimination product (XV), see Table 1).

3,4-Dehydro-torulene (XV). The *trans* isomer of the above product (XV) exhibited abs.max. in acetone at (470), 492, and 523 mμ (see Fig. 6), and had a partition ratio of 90:10 in petroleum ether/95 % methanol. It was unaffected by hydride reduction, and was further characterized by a determination of the composition of the iodine catalyzed equilibrium mixture, see Table 3.

The composition of the corresponding equilibrium mixture of XV, obtained by Aasen and Liaaen Jensen [19] by acid chloroform treatment of hydride reduced anhydro-deoxy-flexixanthin = 4-hydroxy-torulene, was determined for comparison. The identity of the corresponding stereoisomers was supported by direct co-chromatography tests, see Table 3.

XV was also synthesized in low yield from torulene (3.1 mg) by dehydrogenation with N-bromosuccinimide (1.7 mg) in chloroform (3 ml). The reaction was interrupted after 2 days; pigment recovery 44 %. XV comprised *ca.* 5 % of the recovered carotenoid.

For comparison with the allylic elimination product (XV) the following compounds with related properties were also studied:

Synthetic torulene (XVI) had abs.max. in petroleum ether at 460, 485, and 517 mμ and exhibited a more pronounced fine-structure in its spectrum than XV. Moreover, *trans* XVI was less strongly adsorbed (R_F = 0.50 on kieselguhr paper; petroleum ether) than the allylic elimination product (XV) of V. Also the stereoisomeric set produced on iodine catalysis of XVI turned out to be different from that of XV.

3,4-Dehydro-chlorobactene (XVII) was prepared by dehydrogenation of chlorobactene [17] (5.1 mg) with N-bromosuccinimide (1.4 mg) in CCl_4 (4 ml) for 44 h;

Table 3. Composition of the iodine catalyzed equilibrium mixtures of the main allylic elimination product (XV) obtained from phlei-xanthophyll (V), hydride reduced anhydro-deoxy-flexixanthin = 4-hydroxy-torulene (XXXII) and hydride reduced 4-keto-phlei-xanthophyll (XXVI).

Product (XV) obtained from	Member of the set	R_F-value S. & S. No. 288 paper 2 % acetone-petroleum ether	Abs.max. in mμ in acetone				Approximate % of total carotenoid
Phlei-xanthophyll (V)	neo D	0.67	385	(460)	483	(510)	42
	neo C	0.57					
	neo B	0.51	390	(465)	489	(509)	33
	neo A	0.47					
	trans	0.40		(470)	492	(523)	25
Hydride reduced anhydro-deoxy-flexixanthin= 4-hydroxy-torulene (XXXII)	neo D	0.67	390		480		34
	neo C	0.57					
	neo B	0.51	390		487	(512)	31
	neo A	0.47					
	trans	0.40			492	(521)	34
4-Hydroxy-phlei-xanthophyll (XXVI)	neo D	0.67	390		484	(510)	32
	neo C	0.57					
	neo B	0.51	390	(465)	488	(512)	36
	neo A	0.47					
	trans	0.40		(470)	492	(522)	32

pigment recovery 35 %. XVII, purified by chromatography on a column of deactivated alumina, comprised 4 % of the recovered pigments, exhibited abs.max. in petroleum ether at 464, 486, and 517 mμ and had R_F = 0.70 on aluminium oxide paper (5 % acetone in petroleum ether compared with R_F = 0.62 for XV in the same system.

2,3-Dehydro-*retro*-carotene (XVIII) was prepared by treatment of synthetic, chromatographically purified isozeaxanthin (1.2 mg) with acid chloroform;[18] pigment recovery 66 %. The main product (XVIII) had abs.max. at 470, 489, and 518 mμ in acetone and R_F = 0.66 on aluminium oxide paper (2 % acetone in petroleum ether), compared with R_F = 0.35 for XV determined by a co-chromatography test.

Isolation of glucose after hydrolysis of phlei-xanthophyll (V) by acid chloroform. Phlei-xanthophyll (V, 9.5 mg) was treated with 0.02 N hydrogen chloride in chloroform for 3 min in the standard manner. The pigments were transferred to ether in a separatory funnel on admixture with water (750 ml). The weakly acidic hypophase was neutralized with 0.1 N NaOH-solution to pH 7.0 and concentrated to dryness under vacuum. The residue was treated with warm ethanol, and the ethanolic extract filtered and concentrated to 1 ml. An aliquot gave a positive Molisch test for carbohydrates with α-naphthol-ethanol-sulphuric acid.[22] Other aliquots were submitted to ascending paper chromatography in the benzene-butanol-pyridine-water (1:5:3:3) system of Fisher and Percival [23] overnight with galactose, xylose, and glucose as reference substances. The chromatogram was dried at room temperature and developed with aniline-trichloroacetate (2.5 %) in glacial acetic acid reagent at 100°C. The sample showed a single spot with $R_{glucose}$ = 1.0. Schleicher and Schüll No. 2043 B paper was used.

Other aliquots of the above ethanolic concentrate were treated with D-glucose oxidase (notatin).[24,43] D-Glucose (with and without enzyme) and a glucose-free enzyme solution was used for reference. Samples with 0 %, 0.4 %, and 4 % enzyme in 0.2 M phosphate buffer (pH 5.7), containing 25 % ethanol, were kept at 37°C for 1½ h and then chromatographed in the above descending system overnight. The activity of the enzyme was evident from the result of the D-glucose reference tests. Also the test extract did not

show a glucose spot following notatin treatment, whereas the untreated extract gave a clear glucose spot.

Enzymic hydrolysis of phlei-xanthophyll (V). As a model test the enzymic hydrolysis of *t*-trimethyl-carbinol-β-D-glucoside by β-glucosidase (almond emulsin) [26] was studied. Satisfactory hydrolysis was effected using 1 % glucoside and 1 % enzyme in 0.03 M acetate buffer (pH 5.05) at 30°C for 24 h. The presence of 20 % ethanol in the system had insignificant inhibitory effect, whereas the presence of 80 % ethanol partly inhibited the hydrolysis.

Phlei-xanthophyll (V, 0.5 mg) was dissolved in ethanol (0.5 ml) and vigorously stirred. Distilled water (0.4 ml) and acetate buffer (0.2 ml) was added dropwise to make a colloidal suspension of V. However, on addition of the β-glucosidase (9.7 mg) the carotenoid seemed to precipitate. The mixture was stirred for 24 h at 30°C, and the reaction interrupted by transfer of the pigment to ether in a separatory funnel; pigment recovery 30 %. The recovered carotenoid consisted exclusively of unreacted V. Other experiments gave similar results.

The activity of the α-glucosidase (Koch and Light) used was tested with pure maltose.[44] Maltose (100 mg) was treated with α-glucosidase (10 mg) in 0.2 M phosphate buffer (1 ml, pH 6.9) at room temperature for 6 h. The reaction mixture, submitted to paper chromatography according to Fisher and Percival [23] as described above, contained mainly glucose besides some unreacted maltose.

To a stirred solution of phlei-xanthophyll (V, 0.8 mg) in ethanol (0.5 ml) was added phosphate buffer (0.6 ml) and α-glucosidase (10 mg) as described above. The reaction was interrupted after 20 h by transfer of the pigment to ether. No hydrolysis was achieved, judged by circular chromatography of the recovered carotenoid.

Partial synthesis of 2'-keto-phlei-xanthophyll tetraacetate (XI). The glucoside synthesis was performed according to the method of Koenigs-Knorr following the procedure used by others.[45,46] Acetobromoglucose, m.p. 81°C, was prepared from glucose pentaacetate and hydrogen bromide according to the method of Fischer.[47] Silver carbonate was freshly prepared from silver nitrate and sodium bicarbonate.[48]

To synthetic XXI (8.4 mg) dissolved in dry ether (5 ml) was added acetobromoglucose (6.1 mg) and silver carbonate (8.3 mg). The mixture was stirred mechanically for 20 h at room temperature. A quantitative pigment recovery and a 5 % conversion to XI, was recorded. XI, thus obtained, exhibited the same absorption spectrum in visible light as XI derived from phlei-xanthophyll. It had a partition ratio in petroleum ether/95 % methanol of 34:66 and the *trans* (R_F = 0.44) and neo A (R_F = 0.50) isomer (produced on iodine catalysis in light) co-chromatographed on circular kieselguhr paper (10 % acetone in petroleum ether) with those of XI derived from natural phlei-xanthophyll.

4-Keto-phlei-xanthophyll (VI)

Isolation. Chromatographically pure pentaacetate (VIII) was saponified for 1 h in 5 % methanolic KOH-solution. The unsaponifiable matter was transferred to ether-tetrahydrofurane and finally chromatographed on a cellulose column.

Crystallization from pyridine-petroleum ether furnished dark mauve needles: yield *ca.* 25 mg.

Adsorptive properties are given in Table 1. Chromatographic separation from phlei-xanthophyll (V) was not achieved in any of the systems used.

Absorption spectrum in visible light. The absorption spectrum in visible light of VI was identical with that of its pentaacetate (VIII), see below. In a mixed acetone-tetrahydrofurane solvent the $E^{1\,\%}_{1\,cm}$ = 1160 (ε = 86 000) at the main abs.max.

Infrared spectrum of VI (0.3 mg in 0.2 g KBr) showed absorption at 3200 (OH), 2850 (CH), 1660 (conj. carbonyl), 1570, 1400, 1070, 1015, 960, (*trans* double bonds), 880, and 820 cm^{-1} (*trans* disubst. double bonds). The IR-spectrum of astacene, which exhibited absorption bands at 1620, 1540, 1245, and 1065 cm^{-1}, characteristic of diosphenol groupings [19] was recorded for comparison.

Mass spectrometric determination of molecular weight of VI as well as of the pentaacetate (VIII) was unsuccessful.

Partition test. VI was completely hypophasic when partitioned between petroleum ether and 60 % methanol.

Acetylation of VI (3.2 mg) in pyridine (4 ml) was carried out with acetic anhydride (0.4 ml) for 18 h; pigment recovery was 92 %. During this period complete transformation to VIII was achieved.

4-Keto-phlei-xanthophyll pentaacetate (VIII). *Trans* VIII was purified by column chromatography on deactivated alumina. Adsorptive properties are given in Table 1. VIII was more strongly adsorbed than phlei-xanthophyll pentaacetate (VII). Crystallization from acetone-petroleum ether furnished mauve-black needles. In visible light abs. max. were located at 480 ($E^{1\,\%}_{1\,cm}$ = 1490, ε = 142 000) and (507) mμ in acetone solution, see Fig. 6. The absorption spectrum of VIII was analogous to that of deoxy-flexixanthin = 1′,2′-dihydro-1′-hydroxy-4-keto-torulene ($\varepsilon \geqq 150\,000$ at 480 mμ).[19] The IR-spectrum (Fig. 8) of VIII (0.3 mg) in KBr (0.2 g) had abs.max. at 3360 (overtone C=O), 2880 (CH), 1740 (acetate), 1660 (conj. C=O), 1550 (conj. double bonds), 1440 (CH_2), 1370 (CH_3), 1220 (acetate), 1036 (acetate), 965 (*trans* disubst. double bonds), 908, and 827 cm^{-1} (*trans* trisubst. double bonds). The spectrum of astacene diacetate in KBr, recorded for comparison (see Fig. 8), exhibited enol-acetate absorption at 1770 cm^{-1}. Astacene diacetate was prepared by acetylation of synthetic astacene in the usual manner.

The NMR-spectrum of VIII, recorded in deuterochloroform at 100 Mc/sec exhibited signals at 7.97 (acetate methyl), 8.05 (in-chain methyl), 8.18 (methyl in 5-position in β-ring), 8.43 (CH_2), 8.82 (*gem.* methyl at *tert.* ether), and 8.85 τ (*gem.* methyl in 4-carbonyl substituted β-ring). The spectrum of canthaxanthin also recorded in deuterochloroform at 100 Mc/sec for comparison, had signals at 8.80 (*gem.* methyl), 8.12 (methyl in 5-position in β-ring), and 8.00 τ (in-chain methyl).

Attempts to form a trimethylsilyl ether of VIII were negative, as for VII above.

Alkali treatment of VIII (originating from previously non-saponified VI) in dry pyridine with a saturated solution of KOH in butanol in a two-finger device at room temperature under vacuum (0.1 mm Hg),[32] did not result in the formation of any blue-coloured enol salt of VI.

Saponification of VIII (13.6 mg) in 5 % methanolic KOH-solution for 1 h gave 100 % pigment recovery and resulted in quantitative conversion to VI.

4-Hydroxy-phlei-xanthophyll (XXVI). Hydride reduction of 4-keto-phlei-xanthophyll pentaacetate (VIII, 16.5 mg) was carried out in dry tetrahydrofurane for 1 min in the usual manner. Unreduced acetate was removed by partition between petroleum ether and 85 % methanol. The hypophase contained XXVI.

Adsorptive properties for XXVI are given in Table 1. XXVI could not be chromatographically separated from phlei-xanthophyll (V). Moreover, the absorption spectrum in visible light (abs.max. at 453, 478, and 508 mμ in acetone) could not be distinguished from that of phlei-xanthophyll (V, *cf.* Fig. 2).

Crystallization of XXVI from pyridine-petroleum ether gave dark red needles; $E^{1\,\%}_{1\,cm}$ = 1320, ε = 98 500, at the main abs.max. recorded in a solution of 14 % pyridine in acetone. The IR-spectrum had abs.max. at 3200 (OH), 2860 (CH), 1435 (CH_2), 1360 (CH_3), 1100, 1068 (OH), 1020 (*sec.* allylic OH) and 965 cm^{-1} (*trans* disubst. double bonds) and much resembled that of phlei-xanthophyll (V). Its NMR-spectrum, recorded at 100 Mc/sec in deuteropyridine, had characteristic signals at 8.96 (*gem.* methyl in β-ring) and 8.32 τ (methyl in 5-position in β-ring).

4-Acetoxy-phlei-xanthophyll pentaacetate (XXVII). Acetylation of 4-hydroxy-phlei-xanthophyll (XXVI, 0.4 mg) was effected by treatment with acetic anhydride (0.3 ml) in dry pyridine (3 ml) for 22 h; pigment recovery 100 %. *Trans* XXVII had abs.max. at 450, 478, and 508 mμ in acetone and was more strongly adsorbed than phlei-xanthophyll pentaacetate (VII), see Table 1.

Allylic elimination of 4-hydroxy-phlei-xanthophyll (XXVI) to 3,4-dehydro-torulene (XV). Treatment of XXVI (1.58 mg) with acid chloroform (0.02 N) was carried out for 20 min in the usual manner; pigment recovery 88 %. The reaction mixture was submitted to column chromatography on deactivated alumina, and the main product (XV) required from 20 % ether in petroleum ether to 2 % acetone in petroleum ether for elution. The *trans* product (XV) was further characterized by its absorption spectrum in visible light, R_F-value and composition of the iodine catalyzed equilibrium mixture (see Table 3). The stereoisomeric set of XV thus obtained appeared to be identical with that of the allylic dehydration product (XV) of V, as judged by direct comparison of the iodine catalyzed equilibrium mixtures.

Allylic oxidation of 4-keto-phlei-xanthophyll (VI) to 4,2'-diketo-phlei-xanthophyll (XXVIII). VI (0.81 mg) in ethanol-benzene (5 ml, 2:3) was oxidized with *p*-chloranil (2 mg) in the presence of iodine (5 μg) in the usual manner. The reaction was followed by paper chromatography, and a 95 % conversion to XXVIII was observed after 46 h; pigment recovery was 85 %. Other experiments gave a similar result.

4,2'-Diketo-phlei-xanthophyll (XXVIII). Adsorptive properties are given in Table 1. XXVIII had abs.max. in acetone at (473), 497, and (526) mμ, see Fig. 4. Semicrystalline XXVIII was obtained from acetone-petroleum ether. The IR-spectrum of XXVIII (0.1 mg) in KBr (0.2 g) exhibited abs.max. at 3250 (OH), 2870 (CH), 1645 (conj. C=O), 1535 (conj. double bonds), 1440 (CH_2), 1352 (CH_3), 1255, 1150, 1065 (OH), 962 (*trans* disubst. double bonds), and 885 cm^{-1}. XXVIII was completely hypophasic when partitioned between petroleum ether and 85 % methanol.

Reduction of 4,2'-diketo-phlei-xanthophyll (XXVIII) to 4,2'-diketo-5,3'-dihydro-phlei-xanthophyll (XXIX). XXVIII (0.7 mg) in pyridine (3 ml) was treated with glacial acetic acid (0.1 ml) and zinc powder (15 mg) for 2 min,[12] whereupon a hypsochromic colour shift was observed. The reaction was interrupted by transfer to ether on admixture with aqueous sodium chloride solution in a separatory funnel. The epiphasic pigment mixture, transferred to methanol, exhibited abs.max. at 460 mμ. When the methanolic extract was shaken with alkali and air a bathochromic colour shift to 475 mμ occurred. Pure XXIX could not be isolated by chromatography, and appeared to be very easily autoxidisable.

4,2'-Diketo-phlei-xanthophyll tetraacetate (XXX). XXX was synthesized in quantitative yield by acetylation of XXVIII with acetic anhydride in pyridine in the usual manner. *Trans* XXX exhibited the same absorption spectrum in visible light as XXVIII. Co-chromatography tests revealed that XXX was more strongly adsorbed than 2'-keto-phlei-xanthophyll tetraacetate (XI); for R_F-values see Table 1.

Acid treatment of 4,2'-diketo-phlei-xanthophyll (XXVIII). XXVIII (0.1 mg) was resistant towards standard treatment with 0.02 N hydrogen chloride in chloroform for 15 min.[13]

On treatment of XXVIII (1 mg) with 0.12 N dry hydrogen chloride in chloroform, complete conversion to two red, non-polar products (about equal amounts) occurred in 1 h; pigment recovery 49 %.

1',2'-Dihydro-4,2'-diketo-1'-chloro-torulene (XXXVI). The least polar product (considered as XXXVI) had abs.max. at 505 mμ in acetone and partition ratios of 41:59 in petroleum ether/95 % methanol and 71:29 in petroleum ether/85 % methanol; for R_F-value see Table 1. Attempts at silylation were unsuccessful. Saponification in 5 % methanolic KOH-solution for 1 h resulted in conversion to a product, inseparable from XXXVII, which did give a trimethylsilyl ether (R_F = 0.45 on kieselguhr paper; 5 % acetone in petroleum ether).

1',2'-Dihydro-4,2'-diketo-16'-hydroxy-torulene (XXXVII). The more polar product (XXXVII), exhibited the same absorption spectrum in visible light as XXXVI and had a partition ratio of 54:46 in petroleum ether/85 % methanol; for R_F-value see Table 1. This product (XXXVII) gave no acetate on repeated acetylation, but yielded a trimethylsilyl ether (R_F = 0.45 on kieselguhr paper; 5 % acetone in petroleum ether.

Allylic elimination of 4-keto-phlei-xanthophyll (VI) to 4-keto-torulene (XXXI). VI (0.5 mg) was treated with 0.02 N hydrogen chloride in chloroform for 15 min in diffuse daylight in the usual manner; pigment recovery 95 %. The reaction mixture, submitted to column chromatography on deactivated alumina, contained 23 % of a more red, non-polar product (XXXI); further characterized below. In another larger scale experiment XXXI (1.24 mg) was isolated.

4-Keto-torulene (XXXI). Adsorptive properties of the above product (XXXI) are given in Table 1. Its IR-spectrum in KBr exhibited characteristic absorption at 1660 cm^{-1} (conj. C=O) and showed much resemblance to that of 1',2'-dihydro-1'-hydroxy-4-keto-torulene = deoxy-flexixanthin.[19] XXXI was further characterized by determination of the composition of the iodine catalyzed equilibrium mixture, and directly compared with the corresponding equilibrium mixture made from paper-chromatographically purified *trans* anhydro-deoxy-flexixanthin = 4-keto-torulene [19] (see Table 4). Co-chromatography tests supported identity of the corresponding stereoisomers.

4-Hydroxy-torulene (XXXII). XXXI (0.68 mg derived from VI) was reduced with lithium aluminium hydride in dry ether in the usual manner; pigment recovery 71 %.

Table 4. Composition of the iodine catalyzed equilibrium mixtures of anhydro-flexixanthin = 4-keto-torulene (XXXI) and the main allylic elimination product obtained from 4-keto-phlei-xanthophyll (VI).

Carotenoid	Member of the set	R_F-value S. & S. No. 287 paper 5 % acetone-petroleum ether	Abs. max. in mμ in acetone
Anhydro-deoxy-flexixanthin	neo B	0.84	480
(4-Keto-torulene; XXXI)	neo A	0.71	486 (510)
	trans	0.57	491 (520)
Product obtained from VI	neo B	0.84	480
	neo A	0.71	488 (508)
	trans	0.57	491 (520)

XXXI exhibited abs.max. in petroleum ether at 456, 483, and 518 mμ and in acetone at 465, 490, and 523 mμ (see Fig. 6); for R_F-value see Table 1. The IR-spectrum of XXXII (0.2 mg) in KBr (0.2 g) had abs.max. at 3280 (OH), 2850 (CH), 1440 (CH_2), 1370 (CH_3), 1018 (allylic *sec.* hydroxyl), and 962 cm^{-1} (*trans* disubst. double bonds).

Allylic dehydration of 4-hydroxy-torulene (XXXII) to 3,4-dehydro-torulene (XV). XXXII (0.25 mg originally derived from VI) was treated with 0.02 N hydrogen chloride in chloroform in the usual manner; pigment recovery was 64 %. The resulting XV was purified by chromatography on a column of deactivated alumina. Its absorption spectrum in visible light and adsorptive properties (co-chromatography tests) supported identity with 3,4-dehydro-torulene, isolated above *via* a different route.

Allylic elimination of 3,4,3',4'-tetrahydroxy-β-carotene (XXXIII). XXXIII was prepared from synthetic astacene (10.7 mg) in ethanol-benzene (10 ml, 2:1) by sodium borohydride reduction at 70°C for 2 h; pigment recovery was 65 %. The recovered carotenoid consisted exclusively of XXXIII (m.p. 174°C, R_F = 0.31 on kieselguhr paper with 20 % acetone in petroleum ether), the IR-spectrum of which exhibited no carbonyl absorption.

XXXIII (1.3 mg) was treated with 0.02 N hydrogen chloride in chloroform for 15 min in the usual manner; pigment recovery, 62 %. Paper-chromatographic examination revealed the formation of two products with extended chromophore.

The product absorbing at longest wavelengths was identified as anhydro-eschscholtzxanthin (XXXIV), abs.max. (480), 500, and (531) mμ in acetone and 474, 496, and 528 mμ in petroleum ether, R_F = 0.77 on kieselguhr paper (2 % acetone in petroleum ether) and partition ratio 82:18 in petroleum ether/95 % methanol. Authentic anhydro-eschscholtzxanthin (XXXIV) for direct comparison was prepared from eschscholtzxanthin (1 mg, derived from hydride-reduced natural rhodoxanthin) by acid chloroform treament;[13] pigment recovery 40 %.

A second, unidentified product, had a round-shaped spectrum with abs.max. at 475 mμ in acetone and 472 mμ in petroleum ether. The partition ratio was 66:34 in petroleum ether/95 % methanol and it had an R_F = 0.44 on kieselguhr paper (2 % acetone in petroleum ether). It was slightly less strongly adsorbed than canthaxanthin and considerably less strongly adsorbed than synthetic 3,4,3',4'-tetradehydro-β-carotene.

Isolation of glucose after hydrolysis of 4-keto-phlei-xanthophyll (VI) by acid chloroform. 4-Keto-phlei-xanthophyll (VI, 5.7 mg) was treated with 0.02 N hydrogen chloride in chloroform for 15 min. After transfer of the pigments to ether on admixture with water, the aqueous hypophase was treated as described under isolation of glucose from phlei-xanthophyll (V).

An aliquot of the ethanolic concentrate gave a positive Molisch test,[22] and the presence of a single hexose, glucose, was again established by paper chromatography as described for phlei-xanthophyll (V) above.

Attempt at enzymic hydrolysis of 4-keto-phlei-xanthophyll (VI). The enzymic hydrolysis of VI (0.3 mg) was attempted using almond emulsin according to the procedure used for V above; final ethanol concentration 32 %. A pigment recovery of 95 % was recorded after 24 h. No aglucone (XXXIX) formation was observed judging from paper-chromatographic examination. Treatment of VI (0.8 mg) with α-glucosidase was carried out as described for V above. A 69 % pigment recovery, but no hydrolysis, was obtained after 24 h.

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