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NADPH AND PROTOPORPHYRIN IX DEPENDENT CONVERSION OF VIOLAXANTHIN TO A *RETRO*-CAROTENOID IN *NARCISSUS PSEUDONARCISSUS* CHROMOPLASTS *IN VITRO*

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Key Word Index—*Narcissus pseudonarcissus*; Liliaceae daffodil, flowers; carotenoid; protoporphyrin IX.

Abstract—In homogenates of chromoplasts isolated from petals of *Narcissus pseudonarcissus*, violaxanthin is efficiently converted into a novel *retro*-carotenoid [(3*S*,5*R*,3′*S*,5′*R*, all-*E*)-5,5′-dihydro-3,5,3′,5′-tetrahydroxy-*β*,*β*-carotene]. Here we have purified and characterized this compound by NMR spectroscopy. Its formation took place in chromoplast homogenates after supplementation with NAD(P)H in the presence of protoporphyrin IX. The tetrapyrrole could either be biosynthetically formed from added *δ*-aminolevulinate or supplemented externally. The reaction was greatly stimulated under anaerobic conditions, probably due to inhibition of a respiratory redox pathway known to operate in chromoplast membranes. Saturating behaviour with respect to substrates, high substrate specificities and proteinase susceptibility show the enzymic nature of the reaction. Neither chromoplast membranes alone nor the soluble stroma components deprived of membranes were capable of carrying out the reaction, indicative of a differentially localized multi-component system being involved in catalysis.

INTRODUCTION

Chlorophyll-free chromoplasts from the flowers of daffodil develop rapidly from chloroplast-like precursors within several hours. Isolated chromoplasts have served as a model system for studying carotenoid biosynthesis in past years, and increasingly also for studying the differentiation process. Many enzymes and reactions typical for chloroplasts persist functionally over the chloroplast-chromoplast transition e.g. enzymes of the chlorophyll biosynthetic pathway [1] translocators [2], CF_0CF_1 ATPase [3] and others, and it has proved advantageous to study a number of typical chloroplast reactions in chromoplasts, due to the absence of many competing reactions. For example, the investigation of carotenoid biosynthesis is greatly facilitated by the absence of the chlorophyll-synthase reaction which, in chloroplasts *in vitro*, channels geranylgeranyl-diphosphate almost completely into chlorophyll prenylation [4]. Membrane-associated redox-reactions such as respiratory activities [3] or carotene desaturation [5, 6], which are difficult to measure in chloroplasts because of competition by redox elements of the photosynthetic electron transport, are experimentally much more readily accessible in non-green chromoplasts. A new membrane-bound redox reaction in daffodil chromoplast membranes is described here, the NAD(P) H dependent reduction of violaxanthin into a hitherto undescribed xanthophyll with a *retro*-configuration. The reaction takes place in the presence of protoporphyrin IX under anaerobic conditions, which inhibit the NAD(P)H-dependent respiratory redox chain.

RESULTS

Chlorophyll-free chromoplast homogenates synthesize large amounts of protoporphyrin IX when incubated with *δ*-aminolevulinate, as described previously [1]. In the course of these investigations we also noticed a massive synthesis of a specific new polar carotenoid, when such assays were supplemented with NADPH and when anaerobic conditions were applied. As could be readily detected in TLC separations, the novel carotenoid was formed at the expense of violaxanthin. Two additional new carotenoids were also formed in trace amounts, but will not be considered here, since they represent degradation products. The main component synthesized was purified to homogeneity. It exhibited an absorption spectrum at slightly longer wavelengths than the violaxanthin precursor (423, 446, 474 nm versus 419, 441, 469 nm, in ethanol). Its increased polarity pointed to the possibility that the epoxy-groups of violaxanthin had been converted into hydroxy-groups. This was substantiated by acetylation and silylation reactions. By application of NMR techniques it could be unequivocally shown that

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the compound had shifted double bonds and hence must possess a symmetrical *retro*-C_{40} 3,5,3′,5′-tetrahydroxy carotenoid structure. Assuming that the absolute geometry at C-3 of violaxanthin is preserved, the structure [(3*S*,5*R*,3′*S*,5′*R*, all-*E*)-5,5′-dihydro-3,5,3′,5′-tetrahydroxy-β,β-carotene] is deduced (Fig. 1, II). This conclusion is based primarily on the observation of chemical shifts and coupling constants in the ^{1}H NMR that are characteristic for symmetrical *retro*-C_{40} carotenoids such as eschscholtzxanthin and others [7, 8]. The assignment of the olefinic in-chain ^{1}H signals was based on some 1D TOCSY experiments [9] that revealed the sub-sets of coupled protons H-7 (*d*, 6.76 ppm, $J_{7,8} = 12.5$ Hz), H-8 (*d*, 6.64 ppm) and H-19 (*s*, 1.95 ppm) with the H-8 doublet broadened by long-range coupling to H-19. A further 3-spin sub-set of protons H-10 (*d*, 6.41 ppm, $J_{10,11} = 14.8$ Hz), H-11 (*dd*, 6.65 ppm, $J_{11,12} \approx 15$ Hz) and H-12 (*d*, 6.22 ppm) was identified in the same way. The remaining olefinic protons (H-14, H-15, H-15′, H-14′) of the symmetrical compound were located as a narrow multiplet with almost identical chemical shifts near 6.40 ppm as in other *retro*-carotenoids [10].

The protons of the aliphatic end group were identified by a further 1D TOCSY experiment with magnetization transfer from the axially oriented H-3 at 4.25 ppm (*m*, half-width *ca* 20 Hz) to its neighbours H-4eq (*ddd*, 2.19 ppm, $J_{4,4} = 13.5$, $J_{3,4} = 6.5$, $J_{2,4} = J_W \approx 1$ Hz), H-2eq (*ddd*, 1.88 ppm, $J_{2,2} = 13$, $J_{2,3} = 5$, $J_{2,4} \approx 1$ Hz), H-4ax (*dd*, 1.65 ppm, $J_{3,4} = 8$ Hz), and H-2ax (*dd*, 1.56 ppm, $J_{2,3} = 9.5$ Hz). In the same experiment the doublet of 3-OH was assigned at 1.36 ppm ($J = 4.3$ Hz). The remaining singlet signals of H-20, H-18, H-16, H-17 and 5-OH were identified and assigned in agreement with ROESY experiments at 1.97, 1.54, 1.41, 1.37 and 1.22 ppm. A strong ROE between H-3ax and the methyl signal at 1.37 ppm revealed that the latter corresponded to the axially oriented 1β-methyl group. Moreover, through space connectivities between olefinic and 'in chain' methyl protons clearly identified the geometry of all double bonds (see Fig. 8 in 9).

It is known that ^{13}C chemical shifts are also very characteristic of *retro*-carotenoids [8]. Since it was impossible to acquire a normal ^{13}C NMR-spectrum from only 75 μg of sample, we recorded a proton-detected one-bond correlated ^{1}H^{13}C 2D COSY that revealed approximate chemical shifts for the different ^{13}C signals. As a main result, it was evident that protons H-14 and H-15 were correlated to carbons C-14 and C-15 absorbing at *ca* 137.7 (together with C-10) and 129.1 ppm (together with C-8), in full agreement with expectation [8].

The molecule was very acid labile. After four days in chloroform solution quantitative isomerization of the *retro*-compound into mutatoxanthin as a mixture of its two constitutive furanoid isomers (8R) and (8S) was observed in an ^{1}H NMR (Fig. 1, III). The UV/VIS and mass spectrum of this rearrangement product were in agreement with the proposed structure, and the former indicated a sample quantity of 75 μg, assuming $A^{1\%}_{1\,\mathrm{cm}} = 2000$.

For quantification, a HPLC-system was used (see Fig. 2) in which the peak area of lutein/zeaxanthin, which remained inert in the incubation system used, was treated as an internal standard to which changes in the violaxanthin and *retro*-carotenoid content could be related. With this system we were interested in investigating the enzymic nature of this reaction, as well as the cofactor requirements. Initial experiments had shown that boiling, as well as proteinase K-treatment prior to incubation,

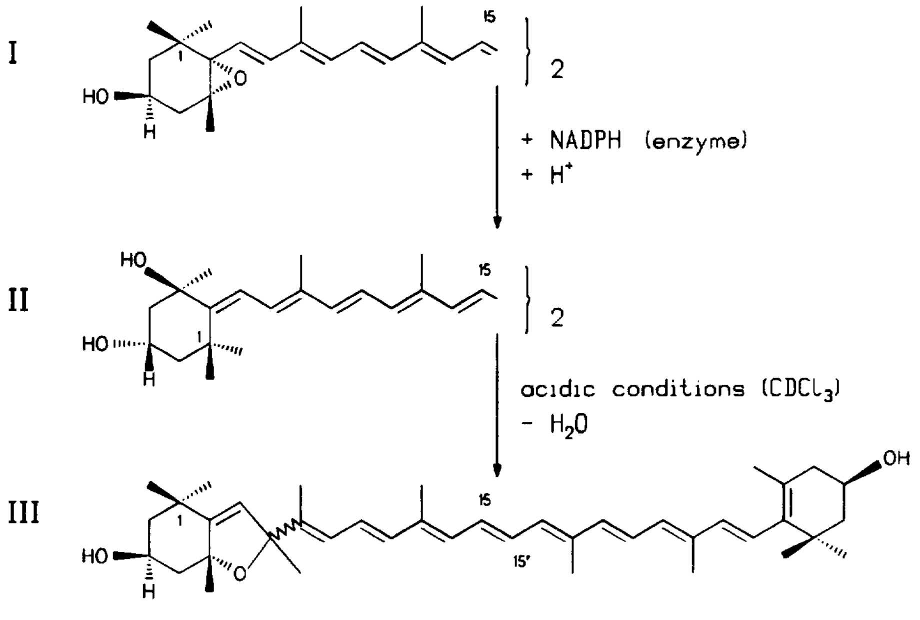

Fig. 1. Chemical structures. I, Violaxanthin; II, new *retro*-carotenoid, (3*S*,5*R*,6*E*,6′*E*)-5,6-dihydro-6,5′-*retro*-β,β-carotene-3,5,3′,5′-tetrol; III, mutatoxanthin.

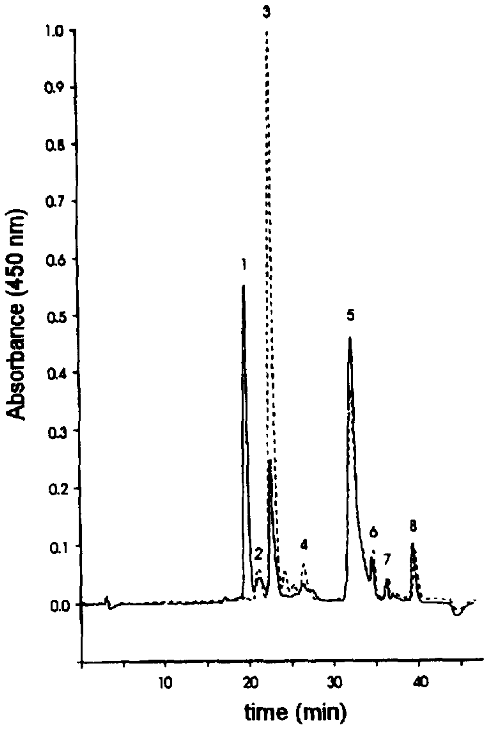

Fig. 2. HPLC analysis of *retro*-carotenoid formation. Dotted line, negative control – carotenoid pattern in an extract of non-incubated chromoplast homogenates; solid line, the same separation after 45 min incubation in the presence of NADPH, protoporphyrin IX and anearobic conditions as given in the Experimental section. 1, *retro*-carotenoid formed at the expense of violaxanthin; 2, neoxanthin; 3, violaxanthin; 4, antheraxanthin; 5, lutein; 6, zeaxanthin; 7, cryptoxanthin; 8, β-carotene.

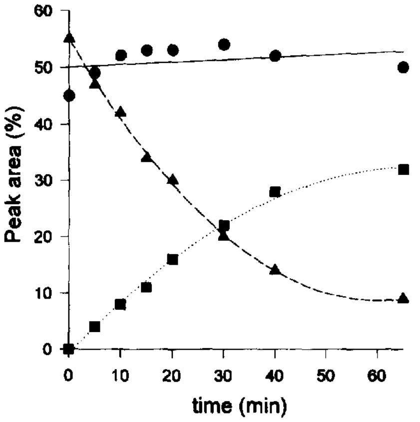

Fig. 3. Time course of the *retro*-carotenoid formation and of the decrease of the substrate, violaxanthin. ●, Lutein/zeaxanthin; ▲ violaxanthin; ■, *retro*-carotenoid. (Peak areas of lutein/zeaxanthin + violaxanthin + *retro*-carotenoid = 100%.)

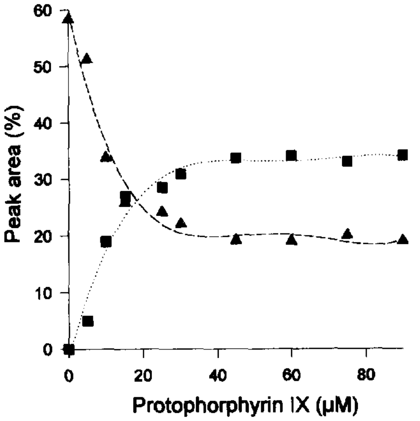

Fig. 4. Response of the enzymic activity towards increasing protoporphyrin IX concentrations. ▲ Violaxanthin; ■, *retro*-carotenoid. (Peak areas of lutein/zeaxanthin (not shown) + violaxanthin + *retro*-carotenoid = 100%.) NADPH was 1 mM, all other additives were as described in the Experimental section.

completely abolished *retro*-carotenoid formation, pointing to its enzymic basis. Under optimal conditions of all reactants, time linearity was observed for 25 min (see Fig. 3); a pronounced sigmoidal shape of the time curve could be avoided by a pre-incubation for 15 min with protoporphyrin IX, probably by allowing attainment of an equilibrium of this metabolite between the aqueous phase and membranes (see below). With protoporphyrin IX in saturating concentrations (90 μM) and using an incubation time of 30 min, NADPH was about twice as effective as NADH in *retro*-carotenoid formation. NADPH-saturation was observed at 0.1–0.15 mM concentrations.

To elucidate the role of protoporphyrin IX, we incubated chromoplast homogenates with equimolar (55 μM) concentrations of either protoporphyrin IX, coproporphyrinogen II, chlorophyll a or chlorophyll b in the presence of 1 mM NADPH under anaerobic conditions for 30 min. Only in the presence of protorphyrin IX was a (24%) conversion of violaxanthin observed; in all other cases no reaction occurred. Also chlorophyllide, prepared from spinach leaves using the chlorophyllase reaction according to Holden [11], when added in somewhat lower concentrations (30 μM), was ineffective. Thus, intermediates of the chlorophyll biosynthetic pathway ranging from steps before and after protoporphyrin IX are incapable of provoking *retro*-carotenoid formation.

In order to establish the effective protoporphyrin IX concentration, incubations were carried out with increasing amounts of this metabolite, maintaining saturating conditions for NADPH (Fig. 4). A saturating behaviour was found, as for NADPH, at a concentration of about 40–80 μM. However, assuming a catalytic participation of the tetrapyrrole (see below), this concentration range appeared to be somewhat high. Indeed, in the buffer alone without any membranes present, only a small fraction of protoporphyrin IX was actually dissolved, while the remainder was particulate and could be pelleted by centrifugation at 24 000 *g*. The graph in Fig. 5A indicates that under saturating conditions of 60–100 μM the concentration corresponded only to a 200–300 nM true solution. In order to establish which fraction was effective in *retro*-carotenoid formation we carried out incubations in which the tetrapyrrole was added to buffer and subsequently centrifuged. The supernatant with the dissolved portion was then supplemented with chromoplast membranes and all other additives. No conversion was achieved under these conditions. We conclude that protoporphyrin IX required the biphasic aqueous/membranous system for proper localization and participation in *retro*-carotenoid formation.

To test the role of membranes here, chromoplast homogenates were incubated with increasing amounts of protoporphyrin IX. By centrifugation, the dispersed particulate tetrapyrrole was pelleted, while membrane with any bound tetrapyrrole appeared on a 40% (w/v) sucrose cushion, leaving only soluble terapyrrole in the supernatant. Fig. 5B shows the protoporphyrin IX content of membranes after such a separation, bound either by adsorption to the membrane surface or by partition into the lipid phase. No saturation in the loading of the membrane was observed in the concentration range used; maximally, 26% of the applied amount of the tetrapyrrole was found here. However, since saturation of the *retro*-carotenoid formation was observed within the same concentration range, we conclude that this effect could indeed be due to substrate saturation of membrane-localized enzyme.

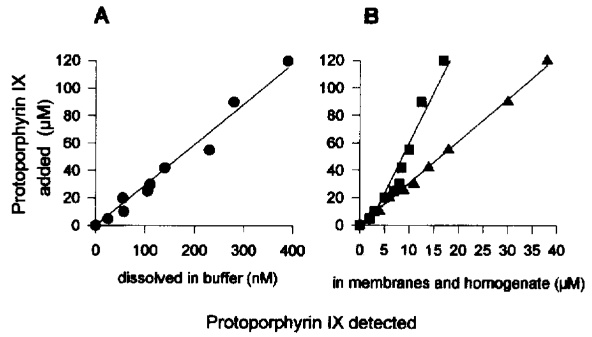

Fig. 5. Physical behaviour of protoporphyrin IX in a monophasic (A, buffer) and biphasic (B, buffer/membranes) system. The tetrapyrrole was added as indicated on the y-axis. The values in A represent the amount detected in buffered solution after centrifugation. The values in B represent the amount detected in membranes (▲) after centrifugation of the chromoplast homogenate onto a 40% sucrose cushion and of the amount detected in the membrane-band plus the supernatant (■). The remainder of the amount added to the system was particulate and appeared in the pellet.

In order to obtain preliminary information about the localization of the enzymes mediating *retro*-carotenoid formation, homogenates were subfractionated by high speed centrifugation into washed membrane and stroma fractions. These were incubated separately under optimal conditions for *retro*-carotenoid synthesis; the stroma fraction was also supplemented with protein-free liposomes made from chromoplast membrane lipids. The results (Table 1) show that membrane and stromal components are both required. Protein-free liposomes were unable to substitute for membranes, and membranes alone were inefficient. The stromal component seems to be rate limiting, as is indicated by the dose-response. Ultrafiltration of the stroma fraction showed the active factor to be larger than M_r 30000.

DISCUSSION

The conversion of violaxanthin into the novel *retro* carotenoid [(3*S*,5*R*,3′*S*,5′R, all-*E*)-5,5′-dihydro-3,5,3′,5′-tetrahydroxy-β,β-carotene] under anaerobic conditions in the presence of protoporphyrin IX and NADPH is apparently an enzymic reaction. This is indicated by the susceptibility towards proteinase treatment, the involvement of NADPH, and the saturating behaviour with respect to NAD(P)H, as well as the remarkable specificity for violaxanthin and protoporphyrin IX, the latter showing saturating behaviour as well. Two enzymic components mediating *retro*-carotenoid formation are involved, one soluble in the stroma, the other one being membrane-bound. This may reflect the different solubilities of substrates; NADPH is water soluble, whereas violaxanthin is localized in the hydrophobic region of the

Table 1. Localization of *retro*-carotenoid forming activities. Chromoplast homogenates and subfractions thereof were incubated as described. Membranes and stroma represent the pellet and supernatant of 200 000 *g* centrifugation (1.5 hr). A 1:1 ratio of stroma and membranes represents roughly the situation in homogenates. Neither stroma or membranes alone are capable of catalysing the *retro*-carotenoid formation. (Quantification in % from HPLC peak integrations relative to lutein–zeaxanthin as an internal standard = 100%)

Assay	*retro*-Carotenoid (area %)	Violaxanthin (area %)
Homogenate–NADPH (negative control)	0.4	133.1
Homogenate (positive control)	26.3	105.7
Membranes	0.8	143.3
Stroma	n.d.	n.d.
Stroma + Liposomes	1.2	107.5
Stroma + Membranes (1:2)	8.7	120.4
Stroma + Membranes (1:1)	23.7	100.0

lipid bilayer. The localization of protoporphyrin IX is not yet clear. Evidently, there are multiple equilibria, between aggregates and 'soluble' and between 'soluble' and 'adsorbed to membranes'. It seems likely that there is an additional equilibrium between 'surface-adsorbed' and partitioned into the lipophilic interior of membranes.

In fact, the *retro*-carotenoid is formed at the expense of violaxanthin by reduction. Thus, the participation of NADPH is explicable, since formally, two hydrogen atoms are necessary to carry out the reaction (for a possible reaction mechanism in eschscholtzxanthin formation, see [12]). The essential and specific participation of protoporphyrin IX is now established, but it remains to be ascertained whether it becomes chemically modified in the course of the reaction e.g. as a co-substrate, or whether its participation is catalytic.

The effect of oxygen on the reaction described here is paralleled in another carotenoid biosynthetic reaction, the NADPH-dependent cyclization of lycopene in chromoplast membranes, which takes place *in vitro* only under anaerobic conditions and requires NADPH [13]. This may be a common theme in chromoplast membranes when redox reactions are involved, since it has been determined recently that a NAD(P)H-dependent respiratory pathway is operating in just these membranes [3]. In one interpretation it is quite conceivable that the electron flow, in the absence of oxygen, is competitively shunted towards reactions employing redox centers at more positive redox potentials. Thus, redox reactions proceeding with relative slow velocities *in vitro* (and maybe also *in vivo*) can be significantly accelerated *in vitro* upon inhibition of the dominating process of 'chromorespiration' or other dominating redox phenomena. Thus, the situation becomes more complicated in chloroplasts. Similar to the problem with carotene desaturation (another membrane-bound redox reaction), we have been unable to perform this *retro*-carotenoid formation in chloroplasts isolated from spinach (*Spinacea oleracea*) or mustard seedings (*Sinapis alba*) under the same incubation conditions. However in the case of chloroplasts, not only a membrane-bound respiratory activity has to be taken into account in the design of the experiment (the so-called chlororespiration), but the (competing?) elements of the photosynthetic electron transport as well. These possible relationships are currently being investigated. As judged by chromatographic comparison, however, the *retro*-carotenoid is apparently present only in trace amounts in chloroplasts - insufficient material for a detailed analysis.

Only marginally related in our eyes, but mentioned here for the sake of completeness is that there is an additional reaction employing violaxanthin as a substrate: The reversible de-epoxidation to zeaxanthin via antheraxanthin, the so-called xanthophyll cycle (for reviews see [14, 15]). Ascorbate, which is reduced by NADPH, is an essential cofactor in the de-epoxidation of violaxanthin, whereas NADPH and oxygen are required in epoxidation of zeaxanthin. It cannot be fully excluded that the synthesis of the *retro*-carotenoid described here represents an alternative redox route acting on violaxanthin. However, there are obvious differences as well; oxygen plays a converse role and there is no requirement for light or for acidic pH values.

EXPERIMENTAL

Daffodil flowers were purchased from local markets and chromoplasts were isolated according to the procedure described in [21]. The isolation of pea chloroplasts was carried out according to [22]. Isolated chromoplasts in Tris–HCl buffer (100 mM, pH 7.4; $MgCl_2$, 10 mM) were frozen intact and sonicated prior to incubation experiments and/or subfractionation into membranes and stroma by centrifugation for 1.5 hr at 200 000 *g*. When necessary, membranes or washed membranes (by an additional centrifugation) were resuspended using a tight-fitting Potter-homogenizer. The stroma fr. was further subfractionated using a Microsep ultrafiltration cell (Filtron) equipped with a hydrophobic membrane (type omega) and a molecular weight cut-off of M_r 30 000. The retentate was diluted with buffer back to its original volume prior to incubation experiments.

For the preparation of liposomes, chromoplast membranes were subjected to $CHCl_3$–MeOH (2:1, v/v) extraction. The extract was washed with a 1% NaCl-solution. After centrifugation the organic phase was evapd to dryness in a rotatory evaporator and the dried lipids were sonicated in buffer at 0° until a homogeneous suspension was formed.

Protoporphyrin IX and coproporphyrinogen tetramethylester were from Sigma; the latter was saponified in ethanolic KOH prior to its use as a substrate. The free acid was partitioned against H_2O–Et_2O and the aqueous phase re-extracted with Et_2O under mild acidic conditions. The Et_2O was evapd under a stream of nitrogen and the tetrapyrrole was dissolved in DMSO. Concns of protoporphyrin IX and coproporphyrinogen II were determined photometrically using extinction coefficients given in [23]. Chlorophyll a and b concns were determined according to [24]. The tetrapyrroles were administered to the 1 ml incubation assays in a volume of 10 μl DMSO; control experiments had shown that the solvent did not affect the reaction under investigation.

The standard incubation assay for *retro*-carotenoid formation contained 1 ml homogenized chromoplast suspension (1 mg protein ml^{-1}) protoporphyrin IX, 70 μM; NADPH 1 mM and the components of the enzymic oxygen trap, catalase (45 μg/ml), glucose oxidase (150 μg ml^{-1}) and glucose at a final concn of 1.4 mg ml^{-1}. Incubation took place at 27° in the dark.

Carotenoids were extracted from incubation assays after addition of 100 μl of a 25% NH_4OH solution using $CHCl_3$–MeOH (2:1). The organic phase was dried under a stream of nitrogen and the extract dissolved in 100 μl $CHCl_3$ (stored over Al_2O_3). In order to prevent any possible acid-catalysed rearrangement reactions, 5 μl NH_4OH was added to each sample. The extracts were sepd on silica gel thin layer plates using the solvent system toluene–ethyl acetate–EtOH (40:10:10). Alternatively, the extracts were separated by HPLC using a

Nucleosil 10 C_{18} column (Macherey und Nagel) using a gradient with the solvents A, MeOH–H_2O (3:1, v/v) and B, acetonitrile–tetrahydrofuran (1:1). The sepn was performed using a linear gradient from 100% A to 60% A in 15 min, followed by an isocratic segment at 60% A for additional 15 min. From 30 min to 40 min the linear gradient was continued from 60% A to 80% A. The constant flow-rate was 1.5 ml min^{-1}.

For the determination of the functional groups, acetylation of secondary hydroxy-groups was done in pyridine using Ac_2O. Additional silylation of tertiary hydroxyl groups was achieved with chlorotrimethylsilane. The chemical conversion of the novel *retro*-carotenoid into the furanoid form of antheraxanthin was done by adding catalytical amounts of 0.2 N HCl to an ethanolic solution.

The preparative purification of the underivatized *retro*-carotenoid for NMR-spectroscopy was achieved by combining TLC as described above with an HPLC-system with the same column as above, employing acetonitrile as the only eluent at a flow-rate of 1.5 ml min^{-1}. NMR-spectra were acquired on a 400 MHz AM-400 Bruker-Spectrospin spectrometer with an ASPECT 3000 computer. Deuterated chloroform (so-called 100% D quality) was used with TMS as internal reference.

Through-bond and through-space connectivities between the different protons were determined by 1D TOCSY, 1D and 2D ROESY experiments. In the 1D experiments a 180° DANTE pulse sequence was used for selective inversion of a suitable proton signal. Magnetization transfer in the 1D TOCSY experiments was achieved by MLEV17 of *ca* 20 and 58 ms duration. In ROESY mixing was provided by a sequence of chopped 90° pulses with an interpulse delay corresponding to a duty cycle of 0.08. The 2D experiment was acquired in the phase-sensitive mode by adding the FIDs obtained with 0.5 and 0.8 s mixing times. A total of 400 experiments was acquired in 4K, zero-filled in F_1 to 2K and multiplied by cosine-square windows in both directions. A digital resolution of 1.95 and 3.91 Hz per address was achieved.

The phase-sensitive proton-detected HSQC $^1H^{13}C$ 2D COSY was acquired in reverse mode into 1K memory from 300 experiments of 224 scans each with total acquisition time of *ca* 36 h r. Zero-filling to 4K and 1K and multiplication by cosine-square windows gave a digital resolution of 3.9 and 39 Hz per address in F_2 and F_1, respectively. Further experimental details on the measuring techniques applied are given elsewhere [9, 10].

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