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Synthesis of a Highly Unsaturated, Stable Hydroxy Peroxide: A Yellow ⇆ Blue Color-Changing Carotenoid Oxidation Product with Leuco Dye Properties

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A stable, highly unsaturated hydroxy peroxide was synthesized by reacting a common food color carotenoid with $FeCl_3$ in air. The obtained pale-yellow carotenoid hydroxy peroxide is easily converted into a blue, cationic polyene – then changes back to the yellow compound, showing the same characteristically dichromic properties like polycyclic, aromatic leuco dyes.

Introduction

Electron transfer to reactive radicals characterizes one of the antioxidant roles of carotenoids.[1] This process generates carotenoid radical cations, which are detectable by their distinctive NIR spectra.[2] We have previously generated the carotenoid radical cations of the food color ethyl β-apo-8′-carotenoate (Car) by photophysical means;[3] we now wanted to verify whether chemically initiated electron-transfer reactions would give the same transient. The electron release of Car to $FeCl_3$ has been described and we based our experiments on the published protocol.[4] Instead of employing $FeCl_3$ in the prescribed amount we added the iron salt abundantly.

Results and Discussion

Highly concentrated $FeCl_3$. We observed the earlier outlined reaction pattern (Figure 1): the peak at 850 nm represents the initially formed cation radical transient $Car^{\cdot+}$, the broad band at 665 nm has been attributed to $CarO_2^{\cdot+}$, unreacted Car was seen at 455 nm and the band at 425 nm was assigned to 5,8-endoperoxide $CarO_2$ (notation according to ref.[4]). We found that chemically (850 nm CH_2Cl_2) and photophysically (822 nm MeCN)[3] generated $Car^{\cdot+}$ absorbed at similar NIR-wavelengths (solvent effects taken into consideration) and with this result the reaction mixture was set aside. Four weeks later, a quick glance to the solution revealed a more brilliant coloration and, indeed, the Vis spectra showed an increase of the blue expressing absorption band. Opening the flask exposing the solution to air had no apparent effect. The stability of the blue color motivated us to purify the compound, at this point provisionally denoted Car*. We anticipated that the expected cation species would not survive general work-up procedures. Therefore, Car* was purified by fractional diffusion. However, impurities – mostly $FeCl_4^-$[4] specified by the band around 350 nm (Figure 2) – could not be removed completely by this delicate procedure, i.e. iron was still detected by reaction with 1,10-phenanthroline and utilization of ICP-MS. The color of the solvent layer faded to yellow by adding water to the blue solution. The yellow product was applied to analytical TLC silica plates, where, to our surprise, a polar, deep blue band was eluted with the same retention time as co-chromatographed Car* obtained from fractional diffusion. The blue band was extracted from the silica gel TLC plate with acetone and, astonishingly, turned yellow. When this yellow product was again subjected to TLC, it turned blue and then another time to yellow when extracted from the silica plate with acetone. Car* was likewise purified by precipitation of contaminations in toluene and hexane. Purification of the yellow compound by preparative HPLC was inefficient. EI-MS of the yellow product detected a molecular ion corresponding to the uptake of three oxygen atoms: $CarO_3$.

Low concentrated $FeCl_3$. The reaction of Car with $FeCl_3$ was repeated changing concentration and time: the reactants were diluted 10 times and the chromatographic work-up procedure started after 1 h. Again, a yellow compound was isolated with the same λ_{max} as the product obtained from the high concentrated solution. Nonetheless, the molecular mass was now compatible with previously characterized $CarO_2$.[4]

Comparison of products and properties. The precise assignment of protons and carbons in $CarO_2$ and $CarO_3$ was

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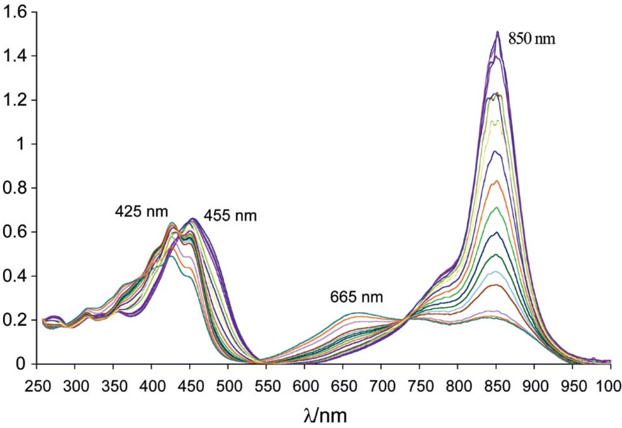


Figure 1. Vis spectra of reactions in CH_2Cl_2. Reaction a) Car with 0.5 mM $FeCl_3$, short reaction time: 850 nm $Car^{\cdot+}$, 665 nm $CarO_2^{\cdot+}$, 455 nm Car, 425 nm $CarO_2$ (compound assignments according to ref.[4]; reaction b) Car with 1 mM $FeCl_3$, long reaction time: 850 nm $Car^{\cdot+}$, 665 nm $CarO_2^+$, 455 nm Car, 425 nm $CarO_3$. Spectrum taken from reaction b), first scan recorded after 1 min, intermediate scans with increased time intervals.

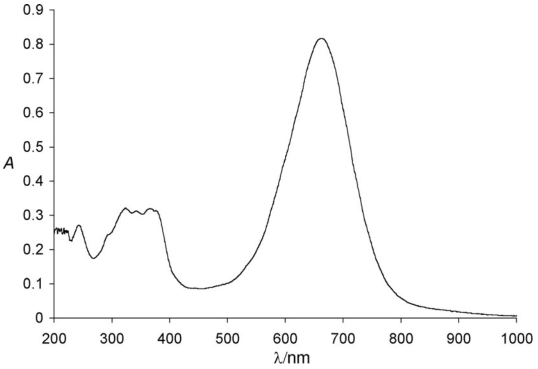


Figure 2. Vis spectrum of Car* ($CarO_2^+$) in CH_2Cl_2 after purification by fractional diffusion or precipitation of impurities. The band around 350 nm indicates $FeCl_4^-$ (Figure taken from the diffusion experiment).

achieved by iterative interpretation of spectra from different NMR techniques (^{1}H, ^{13}C, DEPT, homo and hetero nuclear COSY, HMBC). The NMR spectra of $CarO_3$ were nearly identical to those of $CarO_2$. The major differences in the spectra of $CarO_3$ were a new signal for quaternary C8 at δ = 111.74 ppm, a non-coupled one proton signal at δ = 5.29 ppm for H-C7; H8 was missing and C9 appeared at higher field, see Exp. Section.

Isoabsorbing $CarO_2$ and $CarO_3$ had quite different reaction behavior: yellow $CarO_2$ did not change to blue when applied on silica gel and it decomposed in the presence of $FeCl_3$. Recording the UV/Vis spectra of a) Car* adding H_2O, b) $CarO_3$ adding small amounts of $FeCl_3$ or c) $CarO_3$ in CH_2Cl_2 over time, blue Car* and yellow $CarO_3$ interchanged quantitatively (isosbestic point, Figure 3).

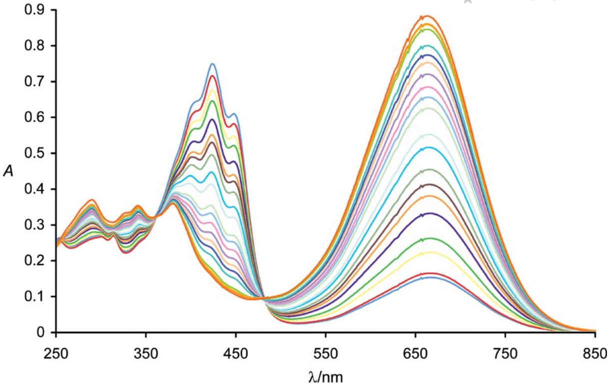
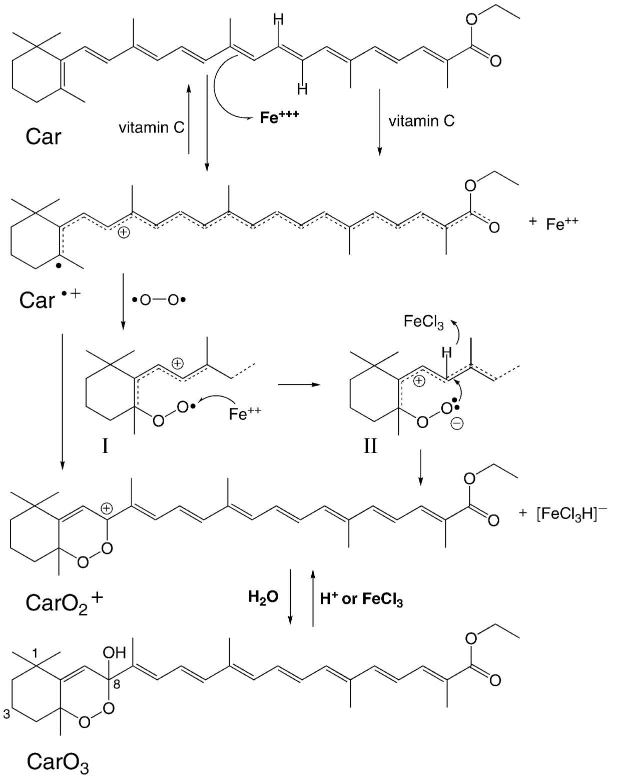


Figure 3. UV/Vis spectra of the blue and yellow compound: a) conversion of yellow (425 nm) $CarO_3$ to blue (665 nm) $CarO_2^+$ in CH_2Cl_2 b) formation of blue $CarO_2^+$ from yellow $CarO_3$ with $FeCl_3$ c) formation of yellow $CarO_3$ from blue $CarO_2^+$ after addition of H_2O. Figure taken from a), scans recorded every minute.

Since color conversion was not observed in acetone, MeOH and MeCN, traces of HCl in CH_2Cl_2 or $CDCl_3$ – initially present or formed and increased during spectra recording[5] – are likely to initiate the reaction. Incomplete conversion gave green solutions resulting from mixing yellow $CarO_3$ and blue Car*.

It has been demonstrated that rainbow-colored carotenoids originate from carotenoid cations.[6] Car* was therefore considered a positively charged species. Indeed, capillary electrophoresis of Car* showed a significantly shorter migration time than a neutral reference. Combination of spectral information, electrophoretic mobility and chemical reactions directed to $CarO_2^+$ as structure for blue Car* and to stereoisomers of $CarO_3$ for the yellow counterpart (see



Scheme 1. Reaction of Car with equimolar amounts of $FeCl_3$ (“long reaction time”).

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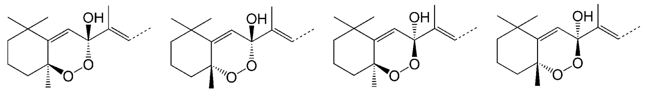
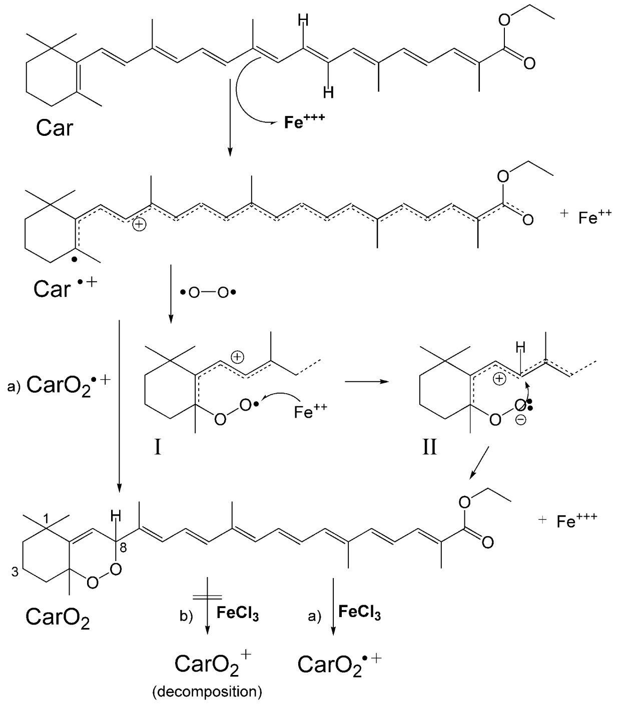
Schemes 1 and 2). HRMS of $CarO_3$ indicated the presence of an easily cleavable OH group and confirmed the structure of $CarO_2^+$.



Scheme 2. Stereoisomers of $CarO_3$.

Stoichiometry and related compounds. Repeating the experiments varying stoichiometry and reaction time we found that 1 mM Car reacted with 0.5 mM $FeCl_3$ overnight to a grey bluish solution from which $CarO_2$ was isolated. Equimolar amounts of Car and $FeCl_3$ gave after 12 h a deep blue solution containing $CarO_2^+$. Exposure of $CarO_2$ to air resulted in a carotenoid trioxide with an epoxy group.[7] Vis-spectroscopy of $CarO_3$ did not corroborate the formation of an epoxy ring, which would have shortened the double bond system below the λ_{max} of $CarO_3$.

Reaction mechanisms. It seems appropriate to describe the formation of $CarO_2^+$ and $CarO_3$ by adhering to the published results. Unfortunately, the presented reactions sequences only concentrated on electron balance and omits to consider the determinant structural transformations.[4] For structural information the previously used acronyms were interpreted and transcribed (Scheme 3). In a fast reaction, Car transfers an electron to Fe^{3+}, this electron is released from the C13-C14 double bond as suggested from a HOMO density plot. The unpaired electron and charge of the formed $Car^{\cdot+}$ are delocalized over the polyene chain, creating a large bathochromic shift into the NIR. Charge and electrons are in fact slightly localized at the 5,6,7,8-double bonds, increasing the reactivity at this side.[8] The reaction was inhibited in the presence of vitamin C and Car was regenerated when vitamin C was given to the solution shortly after the reaction was started (Scheme 1). These observations confirmed the radical character of the primary product. $Car^{\cdot+}$ could react with ground state (triplet) oxygen $^3O_2$ to intermediate cation radical I ($CarO_2^{\cdot+}$), it accepts an electron from byproduct $FeCl_2$ and forms peroxidate II, which then reacts to $CarO_2$ (Scheme 3). No reaction was reported in the absence of $^3O_2$. The reaction to $CarO_2$ runs independently of high or low $FeCl_3$-concentration as long as the reaction time is kept short.[4] Endoperoxides such as $CarO_2$ are usually obtained from dienes and excited (singlet) oxygen $^1O_2$,[9] but endoperoxides are also formed with dienes and $^3O_2$ in the presence of $FeCl_3$.[10]



Scheme 3. Reaction of Car with low amounts of $FeCl_3$ or "short reaction time"; a) ref.[4], b) this work. Structures transcribed from a summarizing notation in ref.[4] The structure of $CarO_2^{\cdot+}$ (intermediate I) was not specified in ref.[4]

The color inverting species $CarO_3 \leftrightarrows CarO_2^+$ from the reaction with higher amounts of $FeCl_3$ spontaneously reminds of leuco dyes, of which one form is deeply colored, the other one is colorless or weakly colored. In analogy, $CarO_2^+$ could be formed from $CarO_2$ by a formal bisallylic hydride abstraction from C8, similar to the benzylic oxidation of leucodye hydrocarbons with $^3O_2$ and $FeCl_3$ or other metal salts.[11,12] Nevertheless, the decomposition of $CarO_2$ with $FeCl_3$ stands in the way of such an analogous reaction (Scheme 3), thus indicating a concerted addition of $^3O_2$ and H-abstraction at $Car^{\cdot+}$ via intermediates such as I and II (Scheme 1). The isosbestic point at 740 nm between the absorption of $Car^{\cdot+}$ and $CarO_2^+$ could be taken as support for a direct process $Car^{\cdot+} \rightarrow CarO_2^+$ (Figure 1). Meanwhile, we observed a first order decay of $Car^{\cdot+}$ with no apparent relation to Car-consumption and $CarO_2^+$-formation (Figure 4). The driving force from $CarO_3$ to $CarO_2^+$ is probably found in the linearization of the ring double bond with the polyene chain resulting in extended charge delocalization; in $CarO_3$ the distorted conformation of the dioxane ring prevents conjugation (Figure 5). Elucidating the mechanism of $^3O_2$-addition to $Car^{\cdot+}$ may also clarify H-abstraction (postulated Lewis acid-base reaction with $FeCl_3$ and electron transfer from Fe^{2+} to $^3O_2$ or to R–O–O$^\cdot$).[4,13–16] A precise mechanistic discussion for endoperoxidation and hydride abstraction would be speculative at this juncture; Scheme 1 and Scheme 3 merely reconstruct a tentative train of thought to stimulate forthcoming verification studies. A possible not yet identified transient behind the so far overlooked small shoulder at 790 nm in Figure 1 may be significant in resolving mechanism and kinetic measurements. This shoulder is not seen in the spectra of photophysically generated $Car^{\cdot+}$ (in MeCN).[3]

$Car^{\cdot+}$ could as well transfer an electron to $FeCl_3$ yielding Car^{2+}, whose λ_{max} appears around 670 nm, just as observed for $CarO_2^{\cdot+}$ and $CarO_2^+$. However, Car^{2+} is unstable at room temperature,[17] has been excluded as precursor for $CarO_2$[4] and is hardly conceivable with the observed color inversion properties. As demonstrated earlier with carotenoid cations, $CarO_2^+$ could be the target for nucleophiles other than H_2O.[18]

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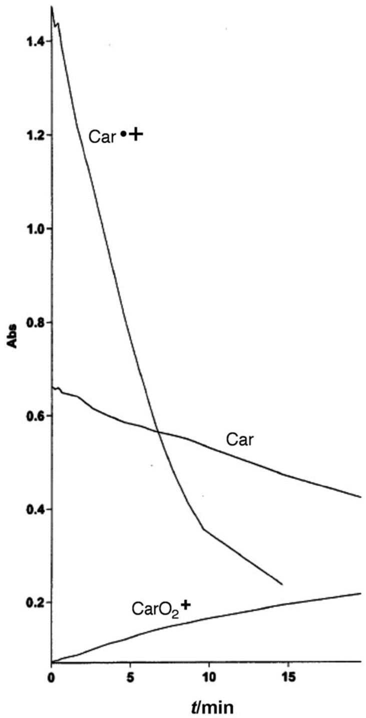


Figure 4. Kinetic traces of Vis/NIR absorbance changes in CH_2Cl_2 in the reaction of Car with $FeCl_3$: decay of $Car^{\cdot+}$ and Car and formation of product.

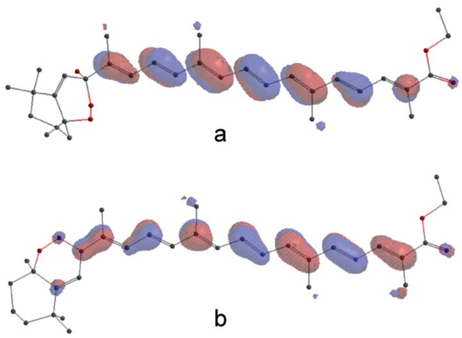


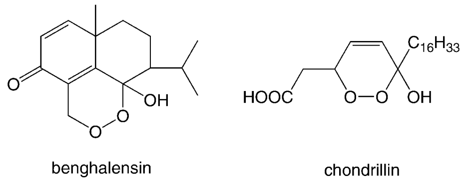
Figure 5. Topology of HOMOs in $CarO_3$ (a) and $CarO_2^+$ (b). (HF/6-31G*/CH_2Cl_2 in Spartan 08, Wavefunction Irvine CA 92612).

Leuco dye properties of $CarO_3$. Leuco dyes are normally associated with polymethines, particularly with di- or triarylmethines.[12] Those dyes have been defined to possess an odd number and polyenes an even number of methine groups.[19] Regardless of the electronic distribution and structural features the term leuco dye is now applied phenomenologically to describe the reversible color change of any class of colorants.[20] The crucial feature of leuco dyes is found in the stabilization of a chromatic carbocation by inductive effects and charge delocalization.

Yellow $CarO_3$ reacted analogous to polymethine carbinols, allylic and benzylic alcohols: the *tert*-OH groups in these derivatives are removed by silica gel, traces of HCl (in chlorinated solvents), hydrolysis, Lewis acids such as $FeCl_3$ or light.[5,11,12,21–27] Irradiation of $CarO_3$, however, led to bleaching. $FeCl_3$ removed the hydroxy group in $CarO_3$ exclusively as Lewis acid and occurred not as electron acceptor, since variation in spectra and reactions were not noticed. (Figure 3). Lewis acids other than $FeCl_3$ are expected to react with $CarO_3$ to $CarO_2^+$.[22,25,28]

Molecular modeling. It would have been desirable to sustain the structure of the new molecule by simulating the absorption spectra. However, reliable calculations of charged carotenoids are quite complex and need a lot of CPU time on supercomputers.[29] The spectra of Car, $CarO_2$, $Car^{\cdot+}$ and $CarO_2^{\cdot+}$ have been generated and did not match the experimental values.[4] Our own calculation of neutral carotenoids had previously pointed to important variations.[30] Cationic triphenylmethanes have only recently been studied by computational methods; the results were not convergent with the experimental data.[31] Consequently, molecular modeling of charged polyenes such as $CarO_2^+$ should be the subject of comprehensive forthcoming investigations.

Tertiary peroxidols. It is generally accepted that "two hydroxys at one carbon is not a stable entity". Consequently, most chemists will perceive intuitively – or by experience – that compounds with hydroxy and peroxyl groups attached to one carbon should be highly reactive or hazardously unstable. Nevertheless, the allylic 5,8-endoperoxide alcohol substructure of $CarO_3$ is found in several stable natural products, e.g., benghalensin and chondrillin, where the peroxidol fragment is indeed hazardous: against *Plasmodium* parasites causing malaria (Scheme 4).[32,33] The potential leuco dye properties of cochloxanthin,[34] a natural occurring antiplasmodial diallylic tertiary carotenoid carbinol, has not yet been described.



Scheme 4.

Conclusions

We have repeated the reported oxidation of Car with $FeCl_3$[4] introducing an important modification: by extending the reaction time we could identify a new yellow endoperoxide compound $CarO_3$. We have recently combined the structural characteristics of carotenoids and anthocyanidins;[35] the yellow ⇆ blue conversion $CarO_3$ ⇆ $CarO_2^+$ now connects carotenoids with leuco dyes. Depending on the point of view, $CarO_3$ can either be considered a new type of leuco dye carbinol or a new carotenoid with potential antimalaria properties. At least, $CarO_3$ can be added to the short list of stable carotenoid oxidation products with intact chain length.[36–38] The observed conversion $CarO_2^+$ ⇆ $CarO_3$ exemplarily illustrates the statement in the conclusion of ref.[39], that reactions of carotenoids with $FeCl_3$ are quite complex. Since iron and oxygen are constant companions of carotenoids[40] possible biochemical effects could be imagined. Further efforts in elucidation the intricate mechanistic aspects of chemically generated carotenoid ion radicals and by-products are warranted in line with similar photochemical,[3,41] radiolytical[2] and electrochemical[39,42] studies. Preliminary experiments with C20–C35 ester (5–11 double bonds) show all immediate electron transfer to $FeCl_3$ whatever the oxidation potential of the carotenoids ($E° = 1.05–0.63$ V),[43] though the decay pattern to final products is more ambiguous than with Car (C30-ester, 9 double bonds).

Carotenoid radical cations have also been detected by EMR after electron transfer from carotenoids to Cu^{2+}-silica compounds. However, the typical NIR-absorption was not seen and carotenoid oxidation products were not separated.[44]

We conclude by mentioning other reported reactions of carotenoids with iron. Addition of Fe^{3+} to (hydroxy) carotenones gave stable carotenoid-Fe-chelate complexes with only a slight increase in absorption.[45] Crocin and crocetin are bleached in the presence of Fe^{3+} without formation of highly colored products.[46] $Fe(CO)_4$ reacts with β,β-carotene to give yellow, stable iron carbonyl complexes[47] and ferrocenylcarotenoids have been used in molecular-wire studies.[48]

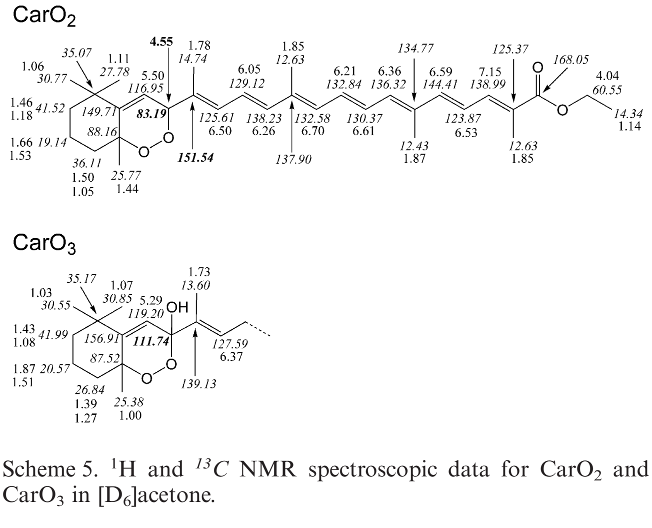
Experimental Section

General: Eluent mixture ratios are given in vol.-% units. ^1H and ^{13}C NMR spectra were recorded on Bruker Avance 400 MHz and 600 MHz instruments in [D$_6$]acetone with calibration against CHD_2COCD_3 for ^1H ($\delta = 2.04$ ppm) and ^{13}C ($\delta = 206$ ppm). UV/Vis spectra were obtained on a single beam Thermo Spectronic Helios γ, Varian 50 UV/Vis-IR spectrophotometer (decay of $Car^{\cdot+}$) or HP 8453 UV/Vis Spectrometer (see Figures 3, 4). MS spectra were recorded on MAT 95XL (TermoQuest Finigan). Solvents of spectroscopic quality were used for spectra recording; for reaction and elution solvents were of p.a. quality. Ethyl β-Apo-8′-carotenoate (C30-ester, Car) was used as delivered.

$CarO_3/CarO_2^+$: To a vigorously stirred solution of Car (800 mg, 1.7 mm) in CH_2Cl_2 (50 mL) was added $FeCl_3$ (275 mg, 1.7 mm) in a mixture of CH_2Cl_2 and acetone (50:1, 18 mL) at 20 °C. The solution turned immediately black, was stirred overnight (or longer) and became green and finally blue. Vanishing of the Car band in the Vis-spectra indicated complete reaction. No reaction was observed in the presence of vitamin C and the current reaction was stopped when vitamin C was added: the $Car^{\cdot+}$ peak at 850 nm disappeared and Car was regenerated. After solvent evaporation the blue residue was washed with hexane until hexane became colorless. After removing the solvent the remaining solid was dissolved in CH_2Cl_2 and carefully applied to the top of a CH_2Cl_2 solvent column and cautiously overlaid with CH_2Cl_2. The narrow blue band broadened slowly overnight by diffusion. The blue central layer was removed with a pipette and the procedure repeated. H_2O (20 mL) was added whilst stirring to the blue solution during which the organic layer turned greenish-yellow. After solvent evaporation, the yellow compound was applied to analytical TLC plates. Elution first with hexane/acetone (7:3), then CH_2Cl_2/acetone separated a polar blue band with the same R_f value as co-chromatographed $CarO_2^+$ from diffusional purification. Blue $CarO_2^+$ turned to yellow $CarO_3$ after extraction from the TLC plate with aqueous acetone. TLC-purified $CarO_3/CarO_2^+$ was used for spectra recording (Figure 3) Purification of higher amounts of $CarO_3/CarO_2^+$ with TLC or column chromatography (actone-hexane 70:30) and HPLC (first methyl *tert*-butyl ether, then MeOH) on silica, alumina and reversed-phase adsorbents was inefficient. In another purification attempt the residue after reaction of Car with $FeCl_3$ was dissolved in CH_2Cl_2 (3 mL) and toluene (47 mL) in a centrifugation tube. After cooling in liquid N_2 for 1 min a gray precipitation appeared after centrifugation. The supernatant green solution was removed and the solvents evaporated. This procedure was repeated. The obtained residue was then dissolved in CH_2Cl_2 (3 mL) and hexane (47 mL). Cooling, precipitation, centrifugation afforded a greenish solid, which in CH_2Cl_2 turned blue, containing roughly the same amount of iron impurities as obtained with fractional diffusion (Figure 2). The precipitation method allowed separating 40 mg of $CarO_3$ (from 800 mg of Car). Prepurifying $CarO_3$ by precipitation and subjecting the sample to preparative HPLC (cyano column 25 × 1 cm, Techsphere 5 µm) afforded trace amounts of pure product while requiring an excessive number of injections and a disproportionate amount of eluents. Attempted crystallization of $CarO_2^+$ failed.

$CarO_2$: The protocol of the $CarO_3/CarO_2^+$ experiment was modified by reacting Car (800 mg, 1.7 mm) with $FeCl_3$ (112 mg, 0.68 mm) overnight or by diluting the primary reaction mixture 10 times with CH_2Cl_2 and beginning the work-up procedure after 1 h. The solution did not turn to deep blue. Application of the product on analytic TLC plates (Silica 60 F254, Merck) and elution with hexane/acetone (7:3) separated a predominant yellow band which, after extraction, was purified on analytical TLC with CH_2Cl_2/acetone (6:3). The analytical data (λ_{max}, MS, NMR) were in accordance with previously identified $CarO_2$.[4]

Structural information was provided by the comparison of the products. UV/Vis: Figures 1 and 3. FT-IR (film on KBr): The two detected bands at 850 cm^{-1} and 880 cm^{-1}, appearing in the region of –O–O– vibrations around 800, 900 and 1100 cm^{-1} were too small for diagnostic use.[49–51] $CarO_2$: MS (EI, 70 eV): 492 M$^+$, 474 M$^+$ – H_2O. $CarO_3$: MS (EI, 70 eV): 508 M$^+$, 476 M$^+$ – 2O, 460 M$^+$ – 3O. HRMS: calcd. for $C_{32}H_{44}O_5OH$ [M + H – H_2O] 491.3156; found 491.3141; calcd. for $C_{32}H_{43}O_3$ 475.3207; found 475.3205. NMR ^1H and 13 C NMR ([D$_6$]acetone), see Scheme 5.



Scheme 5. ^1H and *^{13}C* NMR spectroscopic data for $CarO_2$ and $CarO_3$ in [D$_6$]acetone.

Interconversion of $CarO_3/CarO_2^+$: a) An occasionally stirred solution of $CarO_3$ in a quartz cell of 3 mL was UV/Vis scanned every minute at room temperature. Spectra recording in CH_2Cl_2 gave $CarO_2^+$; in acetone, MeOH and MeCN no reaction was observed, b) to a solution of $CarO_3$ in CH_2Cl_2 small amounts of $FeCl_3$ in CH_2Cl_2 were added with a micro syringe and the reaction mixture regularly scanned with UV/Vis for formation of $CarO_2^+$; c) To a solution of $CarO_2^+$ in CH_2Cl_2 were added low amounts of H_2O with a microsyringe; the formation of $CarO_3$ was followed by UV/Vis scans. Irradiation of $CarO_3$ with intensive light sources (sunlight or 125 W UV lamp) resulted in bleaching.

Reaction of $CarO_2$: Continuous spectra recording of yellow $CarO_2$ in CH_2Cl_2 over time did not show a blue expressing absorption band. $CarO_2$, dissolved in CH_2Cl_2, decomposed when reacted with $FeCl_3$ under high concentration conditions.

Electrophoresis: Capillary electrophoresis was carried out on a Beckman P/ACE™ MDQ 2000 (75 µm × 60 cm fused silica capillary, diode array detector) with 20 mM tetrabutylammonium tetrafluoroborate in $CH_3CN/CH_3OH/CH_3COOH$ (49:50:1). Samples were injected pneumatically and the electropherogram recorded at 15 kV over 30 min at 25 °C. Acetophenone was used as a neutral electroosmotic flow marker.

Determination of Residual Iron: The blue solution of $CarO_2^+$ purified by fractionated diffusion in CH_2Cl_2 was reduced with hydroxylamine to Fe^{2+}. Addition of 1,10-phenanthroline yielded a red solution with λ_{max} = 510 nm.[52] Fe was likewise detected by ICP-MS.

Estimation of the Decay Rate for $Car^{\cdot+}$ in Air: First-order fits resulted by calculating the decay of $Car^{\cdot+}$ with the Cary WinUV software over the first 10 min. The initial absorption values and rates were estimated as described[53] and used as the first approximation for the Marquardt non-linear regression analysis (Figure 4).

Acknowledgments

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