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Electron- and Energy-Transfer Properties of Hydrophilic Carotenoids

Hans-Richard Sliwka,*[a] Thor-Bernt Melø,*[b] Bente Jeanette Foss,[a] Shams H. Abdel-Hafez,[a] Vassilia Partali,[a] Geoff Nadolski,[c] Henry Jackson,[c] and Samuel F. Lockwood[c]

Abstract: The antioxidant activities—expressed as the electron-donating properties—of five hydrophilic carotenoids (carotenoid surfactants) and three related hydrophobic carotenoids were investigated by flash photolysis. The electron-transfer rates of the carotenoids to the triplet state of the sensitizer 2-nitronaphthalene and the energy transfer rates of triplet 2-nitronaphthalene to the carotenoids were determined. The results demonstrate that the electron-donating effects of the hydrophilic and hydrophobic carotenoids were comparable when evaluated in acetonitrile. In the presence of water, however, electron transfer (i.e., antioxidant efficiency) was enhanced by a factor of four for the hydrophilic carotenoids. The increased hydrophilicity of carotenoids, therefore, could expand their antioxidant properties, thus facilitating their use as aqueous-phase radical scavengers. At the same time, it was shown that supramolecular assembly ("aggregation") of the amphiphilic carotenoids prevented electron transfer, thus deactivating the antioxidant function. Modulation of the biophysical properties of carotenoids through synthetic modification is capable of increasing the biological and medical utility of this natural class of predominantly hydrophobic antioxidant compound.

Keywords: surfactants · aggregation · antioxidants · carotenoids · electron transfer · supramolecular assembly

Introduction

The in vivo antioxidant defense system relies on endogenous enzymatic and nonenzymatic components, as well as exogenously supplied dietary substrates, such as carotenoids.

Determination of the broadly defined "antioxidant activity" for a particular compound or set of compounds can be strongly dependent on 1) the test system used[1] (e.g., $^1O_2$ quenching,[2,3] radical[4–7] and superoxide anion scavenging,[8] voltammetry,[9] or epidemiological trials[10]); 2) the physicochemical structure of the antioxidant compounds;[11] 3) the interaction between the aqueous and lipid phases at the membrane bilayer,[12] amongst other variables. Consequently, attempts to uniformly characterize antioxidant activity with a single, inclusive method may be a futile undertaking.[13] Tailoring the test system to a specific and predictive purpose may provide more useful information for a particular application.

Antioxidants frequently possess conjugated double bonds (chromophores), and, accordingly, carotenoids (Car)—with their characteristic polyene chain—serve a multitude of functions for their producers (e.g., algae, yeast) and their consumers (e.g., crustaceans, fish, birds, and man).[14–16] Literally, the antioxidant activities of carotenoids—their reactions with oxygen $^3O_2$ and ozone—can be dependent on the intermediate formation of cation radicals ($Car^{\bullet+}$), whereas the reaction with singlet oxygen $^1O_2$ (physical quenching) is in contrast associated with the triplet energy level ^3Car.[17] In a broader sense, the antioxidant properties of carotenoids arise from their excellent ability to deactivate excited states and their strong reducing potential (electron donation). Although electron donation can immediately deactivate harmful radicals, the antioxidant mechanism can be inseparably

[a] H.-R. Sliwka, B. J. Foss, S. H. Abdel-Hafez, V. Partali
Department of Chemistry
Norwegian University of Science and Technology
7491 Trondheim (Norway)
Fax: (+47) 73-59-6155
E-mail: hrs@nvg.ntnu.no

[b] T.-B. Melø
Department of Physics
Norwegian University of Science and Technology
7491 Trondheim (Norway)
Fax: (+47) 73-59-7710
E-mail: thor.melo@phys.ntnu.no

[c] G. Nadolski, H. Jackson, S. F. Lockwood
Cardax Pharmaceuticals, Inc.
99–193 Aiea Heights Drive, Suite 400
Aiea, Hawaii 96701 (USA)

accompanied by the simultaneous formation of oxidized carotenoid molecules, for example, both neutral and charged radicals (Car$^{\bullet}$, Car$^{\bullet+}$). In some cases, the relatively long-lived carotenoid radicals could, in principle, act as deleterious pro-oxidants, if not scavenged ("regenerated or repaired") by other antioxidants, such as vitamin C, vitamin E, or other carotenoids. Appropriate redox pairing through diffusional encounter,[18] predefined proximity (exploited in micelles),[19,20] or covalent interactions, as in the case of several carotenoid/vitamin C, carotenoid/vitamin E, and carotenoid/carotenoid conjugates,[5,7,21–23] could regenerate the parent carotenoid, thus eliminating the pro-oxidant concerns related to carotenoid radical cations. Although, hydrogen-ion abstraction and adduct formation are also known mechanisms of antioxidant activity of carotenoids,[24] we will give attention to electron donation and energy transfer.

Herein, the antioxidant (i.e., electron-donating) mechanisms of carotenoids—particularly of hydrophilic carotenoids—were evaluated through results obtained from flash photolysis. In these experiments, an excited sensitizer donates and accepts energy or electrons to and from the carotenoid, respectively. The properties of the resulting transient species ^{3}Car and Car$^{\bullet+}$ allowed an estimation of the antioxidant activity of carotenoids.

From the outset, it was evident that such an in vitro assessment might not be directly applicable to in vivo systems, in which carotenoids can be preferentially bound to human serum albumin or other plasma proteins and solubilized in cellular membranes. Carotenoid/protein binding in particular may have a strong impact on biochemical activity.[25] However, a critical first step in the evaluation of hydrophilic antioxidants is the physicochemical assessment of radical-scavenging behavior in aqueous solutions, a key property in the formulation of parenteral therapeutics. The characterization of the antioxidant properties of carotenoid/protein associations would be a logical and subsequent measurement.[26]

Nearly all of the 732 registered natural carotenoids are lipophilic[27] and, for that reason, have been predominantly investigated in (halogenated) organic solvents.[2,28] In living systems, however, carotenoids are not likely to always react in either a pure lipid or water-based environment, but perhaps more often at the typical biological hydrophilic/hydrophobic interphases, such as emulsions or aggregate dispersions. Truly water-soluble carotenoids should have at least some exposure to the essential aqueous plasma phase before redistribution to other tissues, thus making the current evaluations directly relevant in vivo for such compounds.

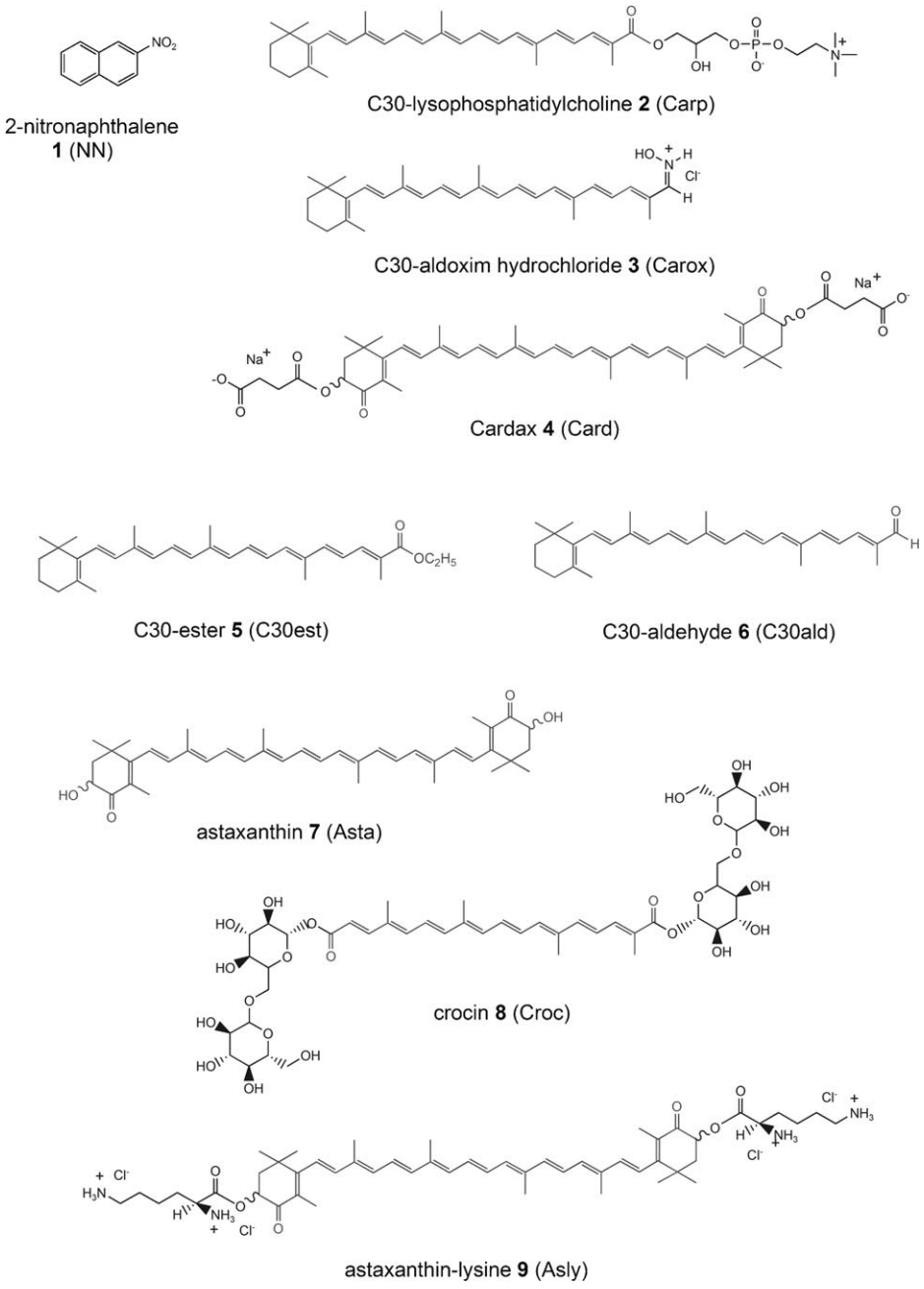
Carotenoid aggregation has been studied for many years.[29] Yet, most of the examined carotenoids have lacked covalently attached hydrophilic groups, which has considerably restrained evaluations in aqueous formulation. It has been shown that self-aggregation of carotenoids has an autoprotective effect: the photostability of carotenoid aggregate dispersions was significantly increased relative to carotenoid monomer solutions.[30] In contrast, photodegradation was observed to be enhanced in carotenoid/liposome aggregates.[31,32] Therefore, the physicochemical structure of the aggregated compound, the local biophysical environment, and the inciting stimulus can be important variables that govern reactivity.

In organic solvents and most likely also in lipid-rich environments, the radical scavenging of carotenoids depends principally on the length of the polyene chain and the presence of conjugated carbonyl groups.[11,33] Other groups directly attached to the carotenoid scaffold (e.g., thione, oxime, and Se ether functionalities) have limited or no impact on the antioxidant activity.[3,34] In water, antioxidant properties have occasionally been determined by incorporating hydrophobic carotenoids into micelles prepared with xenobiotic detergents. Extraneous surfactants, however, can obscure the experimental results.[23,31,35,36] To avoid this difficulty, carotenoids can be prepared synthetically with appropriate hydrophilic substituents: the carotenoids can subsequently become surfactants themselves.[37–40] Only under special circumstances can the properties of carotenoids in water be studied independently of the hydrophilicity of the compound.[41–43]

Recently, a wide range of novel water-soluble and water-dispersible[44] carotenoid derivatives were synthesized.[45] The physical properties (i.e., surface tension, critical micelle concentration (cmc), aggregate size) of some of these compounds have been measured: 1) monopolar zwitterionic carotenoid lysophosphatidylcholine "Carp" **2**;[38,39] 2) monopolar cationic oxime hydrochloride "Carox" **3**; 3) dianionic bolaamphiphile Cardax "Card" **4**;[40,46] 4) tetracationic bolaamphiphilic astaxanthin/lysine conjugate "Asly" **9**. [22] The surface and aggregate properties of the natural neutral bolaamphiphile crocin "Croc" **8** have also been determined[47] (Scheme 1).

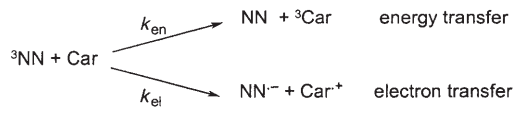
The antioxidant effects of C30 ester **5** ("C30est"), C30 aldehyde **6** ("C30ald"), and astaxanthin **7** ("Asta"; Scheme 1) are well established in organic solvents,[2,28] and it was assumed that antioxidant activity measured in vitro would be similar in vivo.[48] However, the question concerning the reactivity of conjugated polyenes in water-based environments remains open: Does the presence of hydrophilic groups modulate the antioxidant properties? Answering this question is not only of academic interest: compounds **2**, **4**, and **9** are excellent scavengers of biologically produced superoxide anions in ethanol and water,[8,49,50] and improved hydrophilicity is the basis for the clinical testing of **4**.[22,51]

The flash-photolysis behavior of the hydrophilic carotenoids **2**–**4** was investigated in acetonitrile (MeCN) and in a 1:1 mixture of MeCN/H_2O to evaluate the influence of conjugated polar groups on antioxidant activity. The MeCN portion of the aqueous solvent allowed the hydrophobic parent compounds **5**–**7** to be monitored. In pure water, only water-soluble **8** and **9** could be investigated (the results obtained are partially included herein for reference). A comprehensive account of the antioxidant activity in water (i.e., electron transfer, energy transfer, and $^1O_2$ quenching) of these particularly hydrophilic carotenoids, including bixin, will be presented elsewhere.



Scheme 1. Some of the water-soluble and water-dispersible carotenoid derivatives synthesized recently.

In this study, the sensitizer 2-nitronaphthalene (NN; **1**) was utilized, whose triplet state ^{3}NN has the characteristic property when encountering a carotenoid to either donate its triplet energy, thus forming ^{3}Car, or to accept an electron (resulting in the formation of $NN^{\bullet-}$ and $Car^{\bullet+}$). The ratio between the rate of energy or electron transfer (k_{en} and k_{el}, respectively) can be expressed by a bifurcation ratio:[52]



The probability of energy or electron transfer depends on the structure of the involved molecules (NN, Car) and on the polarity and viscosity of the solvent.

Results and Discussion

We first describe the kinetic and energetic aspects of energy and electron transfer that allowed us to calculate the rates and ratio of the transfer reaction. We then interpret the recorded transient spectra and focus on the consequences of the antioxidant effect.

Determination of transfer rates: The efficiency of energy and electron transfer is defined as the proportion of the ^{3}NN···Car quenching encounters that yield ^{3}Car and $Car^{\bullet+}$. When ^{3}NN is isoenergetic with the triplet level of an acceptor molecule, energy transfer prevails; otherwise, electron transfer occurs preferentially. Electron- or energy-transfer reactions follow a three-step process: 1) the formation of an encounter complex by diffusion, 2) exchange of electrons or energy, and 3) separation of the products from the encounter complex [Eq. (1)]. The transfer-reaction constants can be obtained by analysis of the decay of the triplet sensitizer Δ^3S. The decay constant k_t is found at a time interval $\Delta\tau$ to a fraction $1/e$ of the initial value divided by the concentrations of the two reactants [Eq. (2)].

$$^3\mathrm{NN} + \mathrm{Car} \overset{k_{-d},k_d}{\rightleftharpoons} {}^3\mathrm{NN}\cdots{}^0\mathrm{Car} \overset{k_{-e},k_e}{\rightleftharpoons} \mathrm{NN}^{\bullet-}\cdots\mathrm{Car}^{\bullet+} \xrightarrow{k_s} \mathrm{NN}^{\bullet-} + \mathrm{Car}^{\bullet+} \quad (1)$$

$$k_t = \frac{\Delta[^3\mathrm{NN}]/\Delta\tau}{[^3\mathrm{NN}][\mathrm{Car}]} = \frac{e^{-1}}{[\mathrm{Car}]\Delta\tau} = 0.37 \times 10^{10}\,\mathrm{M^{-1}\,s^{-1}} \quad (2)$$

The concentration of Car ([Car]) is approximately 10^{-5} M, and the lifetime of ^{3}NN is $\Delta\tau = 10^{-5}$ s (Figure 1). Hence, the encounter of Car with ^{3}NN is close to the diffusion limit.

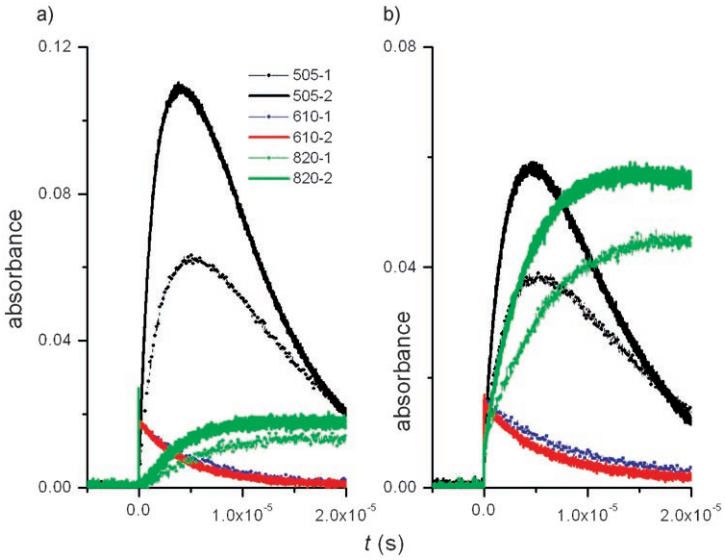


Figure 1. The kinetic traces of laser-flash-induced absorbance changes in a solutions of NN **1** ($c_1=2\times10^{-4}$, $c_2=2.8\times10^{-4}$ M) and C30est **5** ($c_1=0.55\times10^{-6}$, $c_2=1.05\times10^{-5}$ M) in a) MeCN and b) MeCN/H_2O (2:1). ^{3}Car was monitored at 505 nm (c_1: ——; c_2: ——), ^{3}NN at 610 nm (c_1: ——; c_2: ——), and Car$^{\bullet+}$ at 820 nm (c_1: ——; c_2: ——). The maximum signal strength was observed after 5 μs for ^{3}Car and after about 12 μs for Car$^{\bullet+}$. The concentrations were determined with $\varepsilon_5=115\,000$ ($E^{1\%}_{1cm}=2500$).[61]

The transfer rate constant of a diffusion-limited reaction is given by Equation (3):

$$k_D = 4\pi DR = 4\pi\frac{kT}{5\pi\eta}R = 0.66\times10^{10}\ \mathrm{M^{-1}\,s^{-1}} \qquad (3)$$

in which R denotes the distance between the molecules at collision ($R\approx10$ Å, sum of the radii r of the diffusing donor and acceptor molecules), and D is the diffusion coefficient, depending on the viscosity η of the solvent. As the viscosity of MeCN is 0.4 relative to H_2O, ($\eta^{H_2O}_{20°}=1.00$ cP, $\eta^{MeCN}_{20°}=0.44$ cP)[53] the lifetime of ^{3}NN in MeCN is expected to decrease by this factor relative to its lifetime in H_2O. The probability of a collision encounter of NN and Car is k_d, and the probability for the encounter complex to split is k_{-d}. Similarly, k_e and k_{-e} express electron or energy back transfer, and k_s is the probability for product separation. Taking into account that electron back transfer is energetically unfavorable ($k_s \gg k_{-e}$), transfer is usually much faster than back diffusion ($k_e \gg k_{-d}$), and the overall reaction is diffusion-limited, the rate of product formation under steady-state conditions can be expressed by Equations (4) or (5)

$$R_p = \frac{k_S}{k_S+k_{-e}}\cdot\frac{k_e}{k_e+k_{-d}}\cdot k_d[A][D] \qquad (4)$$

or

$$R_p = \frac{k_d[A][D]}{\left(1+\frac{k_{-d}}{k_e}\right)\left(1+\frac{k_{-e}}{k_s}\right)} \approx \frac{k_d[A][D]}{1+\frac{k_{-d}}{k_e}} \approx k_d[A][D] \qquad (5)$$

^{3}NN can either transfer energy or electrons; k_e could, therefore, mean electron k_{el} or energy k_{en} transfer [Eq. (6)]:

$$R_p(\mathrm{el}) = \frac{k_{el}}{k_e+k_{-d}}\cdot k_d[A][D] \quad \text{(electron transfer)} \qquad (6)$$

in which $k_e=k_{el}+k_{en}$. Thus, the ratio between the electron- and energy-transfer rates is identical to the ratio between the reaction rate constants for electron and energy transfer, even if the reactions are diffusion-limited.

The "golden rule" is applicable to nonadiabatic radiationless transitions, in which electronic interactions (V) are smaller than k_BT (weak coupling limit: $V \ll k_BT$). Therefore, when this rule is applied to electron and energy transfer [Eq. (7)]:[54]

$$k_e = \left(\frac{\pi}{\hbar\lambda_s k_b T}\right)^{1/2}\cdot V^2\sum_n\left(e_{-s}\left(\frac{S_n}{n!}\right)\right)\cdot\exp\left(-\frac{(\Delta G_0+\lambda_s+nh\nu)^2}{4\lambda_s k_B T}\right) \qquad (7)$$

in which λ_s is the solvent reorganization energy, λ_i is the inner reorganization energy, $S=\lambda_i/h\nu$, and ν is the averaged frequency for molecular vibrations. The free-energy change for electron transfer ΔG_0^{el} is calculated from Equation (8):

$$\Delta G_0^{el} = [E(\mathrm{Car}^{\bullet+})-E(\mathrm{NN}^{\bullet-})]-E(^3\mathrm{NN}) = E(\mathrm{Car}^{\bullet+}) -(-0.97\ \mathrm{eV})-2.39\ \mathrm{eV} = E(\mathrm{Car}^{\bullet+})-1.42\ \mathrm{eV} \qquad (8)$$

From reported potential values,[55] we estimate the value of $E(\mathrm{Car}^{\bullet+})$ to be approximately 1 eV and obtain $\Delta G_0^{el}\approx-0.4$ eV. The free-energy change for triplet–triplet energy transfer is derived from $\Delta G_0^{en}=E(^3\mathrm{Car})-E(^3\mathrm{NN})=E(^3\mathrm{Car})-2.39$ eV. The energy state for ^{3}Car has been approximately given as $E(^3\mathrm{Car})\approx1$ eV,[56] thus $\Delta G_0^{en}\approx-1.4$ eV (Figure 2). The energy levels $E(^3\mathrm{NN})$ and $E(\mathrm{NN}^{\bullet-})$ are considered to be constant, whereas $E(\mathrm{Car}^{\bullet+})$ depends on the particular carotenoid and the solvent-reorganization energy, that is, the electrical work for separating the charges at the two molecules in the encounter complex.

In the above expression [Eq. (8)] for energy–electron transfer, all the quantities are equivalent, except for the free energies of reaction ΔG_o. The transfer rates reach a maximum when the argument in the exponential is zero and ΔG_o is in opposition to the reorganization energies. A value of −1 eV was obtained for energy transfer and −0.7 and −0.4 eV were chosen for electron transfer. The calculated transfer constants as a function of reorganization energy λ_s are shown in Figure 2a. Concerning the bifurcation ratio, the two graphs in Figure 2b represent the groups of water-soluble and water-dispersible carotenoids (blue and red curves, respectively). Table 1 shows that the average peak and bifurcation ratio of carotenoids **2**–**7** in MeCN are 0.4 and 0.3, respectively. In MeCN/H_2O, the corresponding values increase to 2.0 and 1.5. The water-soluble carotenoids **8** and **9** show a low average value for the average peak and

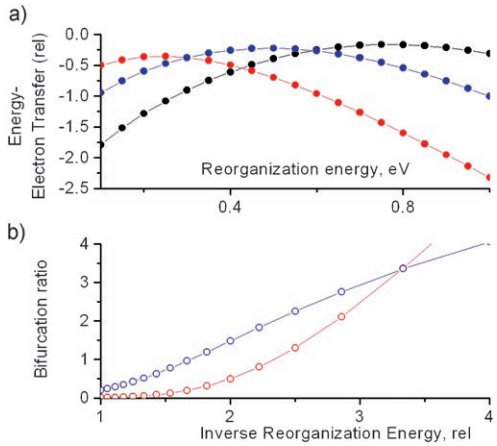


Figure 2. a) Calculated transfer rates according to the Mataga expression[54] for energy or electron transfer as a function of the solvent reorganization energy. The interaction energy was $V=0.024$ eV, inner reorganization energy $\lambda_i=0.3$ eV, and averaged frequency $\nu=0.15$ eV. The free-energy change is set to $\Delta G_0^{en}=-1.0$ eV (●) for energy transfer and $\Delta G_0^{el}=-0.7$ eV (●) and $\Delta G_0^{el}=-0.4$ eV (●), respectively, for electron transfer. b) The bifurcation ratio $\frac{\Delta G_0^{en}}{\Delta G_0^{el}}$ with $\Delta G_0^{el}=-0.7$ eV (○) (water-soluble Car) and $\Delta G_0^{el}=-0.4$ eV (○) (water-dispersible Car)

Table 1. Molar absorption coefficients ε and the peak and bifurcation ratios of the carotenoid transients.[a]

Car	$\varepsilon_T/\varepsilon_G$	$\varepsilon_{cat}/\varepsilon_G$	Peak ratio	Bifurcation ratio
		MeCN		
2 Carp	1.52	1.90	0.24	0.19
3 Carox	1.50	1.65	0.31	0.24
4 Card	1.50	1.74	0.44	0.34
5 C30est	1.54	1.95	0.51	0.40
6 C30ald	1.51	1.98	0.41	0.32
7 Asta	1.60	1.63	0.36	0.28
8 Croc	1.95	1.80	0.11	0.12
9 Asly	1.90	2.00	0.17	0.16
average **2**–**7**	1.5	1.8	0.4	
average **8** and **9**	1.9	1.9	0.14	0.14
		MeCN/H_2O		
2 Carp	1.56	1.87	1.84	1.43
3 Carox	1.69	1.78	1.67	1.30
4 Card	1.35	1.73	1.91	1.49
5 C30est	1.61	1.96	1.76	1.37
6 C30ald	1.77	2.02	2.31	1.80
7 Asta	1.75	1.90	1.67	1.30
8 Croc	2.00	2.20	2.17	2.00
9 Asly	1.80	1.90	2.50	2.40
average **2**–**7**	1.6	1.9	2.0	1.5
average **8** and **9**	1.9	1.6	2.3	2.2

[a] T = triplet, cat = cation radical $Car^{\bullet+}$, G = ground state.

bifurcation ratio in MeCN (both 0.14) and increase to 2.3 and 2.2, respectively, in MeCN/H_2O. The average relative molar absorption coefficient for carotenoids **2**–**7** for the triplet/ground state ($\varepsilon_T/\varepsilon_G$) and cation radical/ground state ($\varepsilon_{cat}/\varepsilon_G$) are quite similar in MeCN and MeCN/H_2O; the same corresponding values are seen with **8** and **9.** As the calculated bifurcation ratio is consistent with the peak ratio values

Table 2. Absorption of carotenoids and carotenoid transients in MeCN and MeCN/H_2O.[a]

	Car MeCN	Car H_2O/MeCN	^{3}Car MeCN	^{3}Car H_2O/MeCN	$Car^{\bullet+}$ MeCN	$Car^{\bullet+}$ H_2O/MeCN
8 Croc	430	443	478	492	681	677
5 C30 est	**440**	**435**	**504**	**511**	**822**	**822**
2 Carp	450	400	504	512	822	820
6 C30 ald	**450**	**460**	**521**	**532**	**820**	**820**
3 Carox	440	390	498	503	861	858
7 Asta	**475**	**488**	**555**	**565**	**845**	**840**
4 Card	475	484	557	584	850	847
9 Asly	480	486	554	585	856	843

[a] Parent compounds are indicated in bold for each group.

obtained from the transient absorption spectra, we consider these data to be fairly accurate.

Photophysical properties of NN and Car: The antioxidant properties of carotenoids are generally attributed to the conjugated polyene chain (Table 2). Carotenoids with greater conjugation should be better antioxidants, thus releasing an electron more easily than shorter-chain carotenoids. As expected, **2** and **5** had comparable λ_{max} values in MeCN concerning Car and ^{3}Car absorptions, whereas ^{3}**6** absorbed at longer wavelengths. In MeCN/H_2O, ^{3}**4** appears at significantly longer wavelengths than the related ^{3}**7**. Short-chain carotenoids $\mathbf{5}^{\bullet+}$, $\mathbf{2}^{\bullet+}$, and $\mathbf{6}^{\bullet+}$ on one hand and long-chain carotenoids $\mathbf{7}^{\bullet+}$, $\mathbf{4}^{\bullet+}$, and $\mathbf{9}^{\bullet+}$ on the other hand have similar absorption maxima in MeCN/H_2O. With the exception of $\mathbf{9}^{\bullet+}$, no significant differences were observed concerning the λ_{max} value of $Car^{\bullet+}$ in MeCN and MeCN/H_2O. The deviation of **3** will be addressed in the description of Figure 5.

Figure 1 displays kinetic traces of the transients ^{3}**5**, $\mathbf{5}^{\bullet+}$, and ^{3}NN at two different concentrations in H_2O and MeCN/H_2O. The increase of $\mathbf{5}^{\bullet+}$ in H_2O and its much longer lifetime relative to ^{3}**5** are well demonstrated. The lifetime of the triplet species ^{3}NN and ^{3}**5** are not affected by water.

Figure 3 a shows the transient absorption spectra of the hydrophobic parent compounds **5**–**7** in MeCN. The peak heights of ^{3}Car and $Car^{\bullet+}$ for **5**–**7**, respectively, are similar, thus demonstrating identical efficiencies for energy transfer. Figure 3 b illustrates that on addition of water to the solvent the intensity of the absorption of ^{3}Car decreases, followed by an approximate fourfold increase of the $Car^{\bullet+}$ peaks, which were stable in MeCN/H_2O during the investigated time scale. (This observation was confirmed by kinetic measurements; Figure 1.)

Figure 4 a presents the transient absorption spectra of hydrophilic **2**–**4** in MeCN. The intensity of the ^{3}Car bands shows that energy transfer is strongly favored. The hydrophilic carotenoids **2**–**4** show an increase of ^{3}Car formation relative to the parent compounds **5**–**7**. Figure 4 b confirms that energy transfer is decreased and electron transfer favored by a factor of about four in aqueous solution. The formation of $Car^{\bullet+}$ from **2**–**4** is similar to $Car^{\bullet+}$ from **5**–**7**; thus, the presence of hydrophilic groups on **2**–**4** does not affect the intensity of the $Car^{\bullet+}$ absorption.

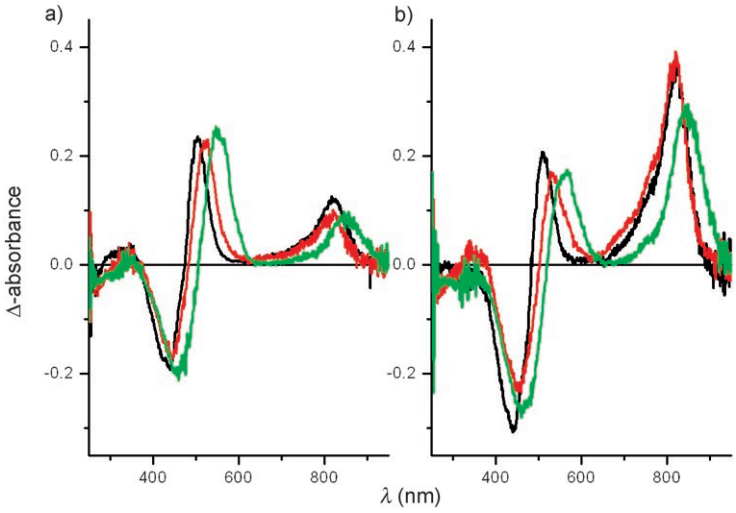


Figure 3. Flash-induced transient spectra of **5** (——), **6** (——), and **7** (——) in a) MeCN and b) MeCN/H_2O with NN **1** as a sensitizer. The equal heights of the ^{3}Car peaks indicate identical efficiencies for energy transfer. In the water-containing solvent, the energy transfer is slightly decreased, whereas the electron-transfer abilities are increased approximately fourfold. The negative absorbance is due to ground-state depletion. The delay between the pump and the probe pulse is 10 μs. The spectra have been divided by the peak ground-state absorbance of NN and Car to correct for differences in concentrations.

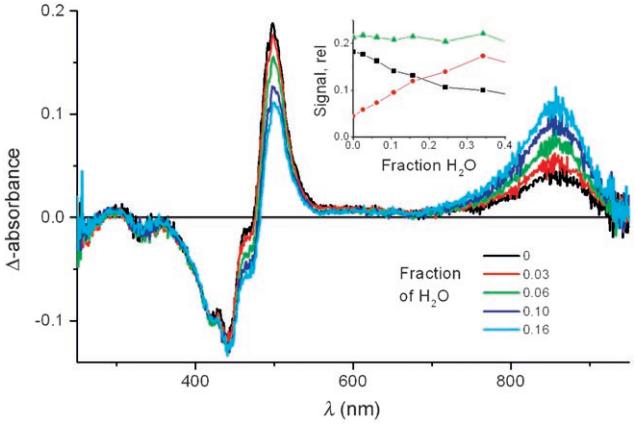


Figure 5. Transient absorption spectra of **3** at a delay of 10 μs for increasing amounts of water in MeCN. Higher water fractions favored **3**$^{\bullet+}$ formation. The insert indicates the peaks heights of ^{3}Car (■) and Car$^{\bullet+}$ (●) as a function of the proportion of water in the solvent and the sum of the peak heights, weighted by the extinction coefficients (▲). The weak absorption band around 600 nm was attributed to ^{3}NN.

Figure 5 demonstrates the subtle influence of water on the antioxidant activity of Car, exemplified by **3**. MeCN favors the formation of ^{3}**3**, whose energy level would be crucial for efficient $^1O_2$ quenching. On addition of water to the solvent, ^{3}**3** decreases simultaneously with the increase of **3**$^{\bullet+}$, thus indicating an enhanced ability of radical scavenging. At the appropriate water fraction, an optimum is achieved for both possible antioxidant actions (see insert of Figure 5). (The $^1O_2$ quenching rate at an optimal water fraction remains to be verified experimentally.)

The properties of **3** merit special attention. The oximium group shifts the λ_{max} values of **3** and ^{3}**3** to shorter wavelengths relative to **6** and ^{3}**6** (see Table 2 and Figure 4).

Decreasing λ_{max} value mimics a shortening of the polyene chain and, consequently, the antioxidant function should be negatively affected. However, the pragmatic rule "the higher the absorption the better the antioxidant" is not valid for polyenes with heteroatoms, as previously verified for $^1O_2$ quenching with carotenoid thiones.[34] Compound **3**$^{\bullet+}$ absorbs at a higher wavelength than **6**$^{\bullet+}$, and even higher than **7**$^{\bullet+}$ and **4**$^{\bullet+}$ (see Table 2 and Figure 4). Compound **3** went from a high ground-state energy level to a low energy level after electron release. The antioxidant effect of carotenoids might, therefore, be increased with other conjugated nitrogen substituents (hydrazones, semicarbazones, phenylimines).[57]

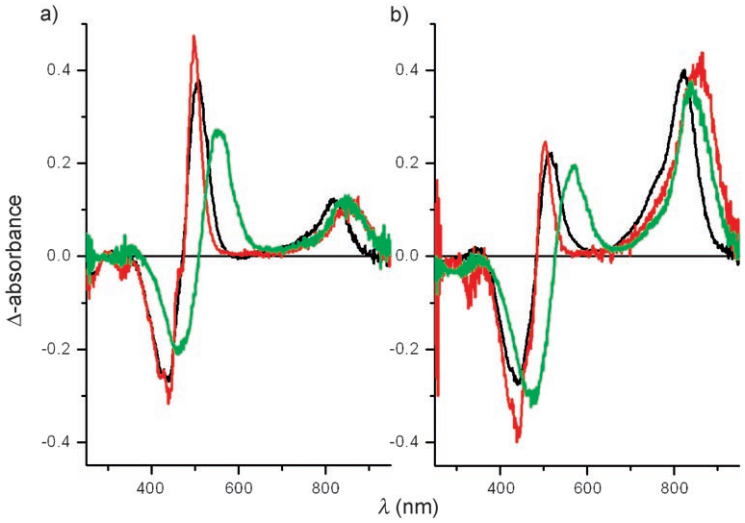


Figure 4. Flash-induced transient spectra of **2** (——), **3** (——), and **4** (——) in a) MeCN and b) MeCN/H_2O using NN as a sensitizer. In MeCN, **2** and **3** showed an increase in energy transfer relative to **5** and **6**; electron transfer was unaffected. In MeCN/H_2O, electron transfer was increased by a factor of four for all carotenoids.

Figure 6a presents the relative energy- and electron-transfer efficiencies of the carotenoids **3**, **5**, **6**, **8**, and **9** together with the bifurcation, taken as the ratio of the peak heights, which reflect the volume fraction of H_2O in MeCN. The formation of ^{3}**7** and ^{3}**3** was immediately blocked by water, whereas the formation of ^{3}**8** and ^{3}**9** increased at very low water fractions. Figure 6b reveals that **3** and **5**–**7** cease to transfer their electrons to the sensitizer at a water fraction of approximately

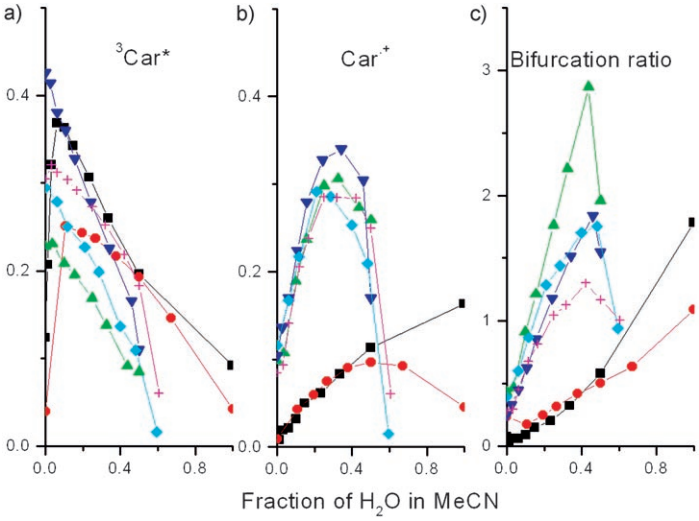


Figure 6. a) Changes in relative energy, b) electron-transfer efficiency, and c) bifurcation ratio for **5** (◆), **6** (▲), **7**(+), **3** (▼), **8** (■), and **9** (●) as a function of water added to MeCN. The peak heights were divided by the dilution factor and by the absorbance of NN and Car. The different behavior of the water-soluble carotenoids is apparent.

0.4, whereas electron transfer from **9** and especially from **8** remains possible, even in pure water. Figure 6c visualizes the bifurcation ratio, thus reflecting the influence of water on the formation of ^{3}Car and Car$^{\bullet+}$. The ratios derived from the transient spectra were compatible with the calculated transfer constants (Table 1). Figure 6c confirms the difference of water-dispersible and water-soluble carotenoids (previously illustrated in Figure 2b). As **9** only partially aggregated and **8** not at all within the investigated water contents (cmc, Vis spectroscopic evidence), encounter complex formation remained possible. The ease of electron transfer with these molecules was remarkable considering that a higher water content resulted in increased viscosity of the solvent, thus hindering diffusional collision of NN with carotenoids.

Most notably, we found that in pure aqueous dispersions of the amphiphilic carotenoids **2**–**4** energy and electron transfer is definitely obstructed by aggregation, as the addition of NN **1** to the aggregate dispersions with subsequent irradiation failed to form ^{3}Car and Car$^{\bullet+}$. The aggregation process located the sensitive polyene chain to the interior of the aggregate and the polar groups to the exterior of the aggregate. The polar-group membrane apparently prevents penetration of the sensitizer or close contact with the polyene chain; also, the hydrophilic sensitizers rose bengal and methylene blue failed to react (data not shown). Similarly, melittin, a natural membrane opener, did not assist the sensitizers in reaching the polyene chain.[58] Thus, aggregation of **2**–**4**, **8**, and **9** could prevent unwanted premature reactions in addition to the previously observed photostability.[30] Aggregate stability was further confirmed when **4** was heated with aqueous and methanolic HCl solutions (2%) at 65°C. Decomposition of the monomers in methanol–HCl was detected after 3 h and was completed after 32 h, whereas the aggregates in aqueous HCl resisted decomposition under these conditions. Autoprotection by aggregation, therefore, appeared to be more efficient than the protection conferred by encapsulating the carotenoids with gelatin/gum arabicum.[59] The aggregation of the appropriate hydrophilic carotenoid can, therefore, be regarded as a self-formulation process. The carotenoid molecules become reactive after the aggregate membrane is deconstructed by the addition of organic solvents or by lipophilic or protein-binding molecules.[60]

Figure 7 illustrates the determination of the relative molar absorption coefficients ε given in Table 1 (details are given in the Experimental Section).

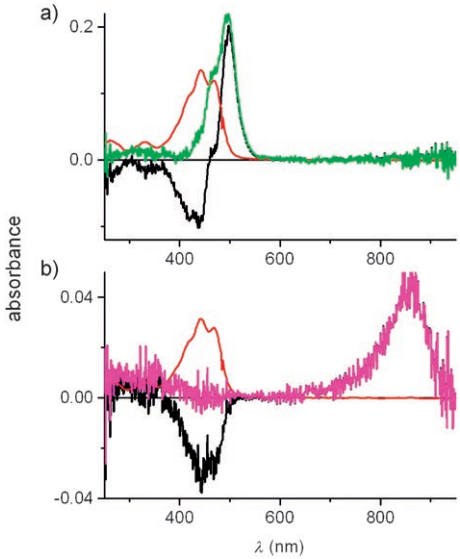


Figure 7. Spectra of a) ^{3}**3** (——) and b) **3**$^{\bullet+}$ (——) in MeCN, together with the spectra of **3** (——) and the corresponding ground-state depletion (——). The transient spectra were calculated by subtracting ^{3}**3** and **3**$^{\bullet+}$ from the spectra of **3**, respectively (by adding ground-state absorption in appropriate amounts to eliminate its contribution to the transient spectrum).

Conclusion

When measured in MeCN, the hydrophilic substituents did not significantly change the antioxidant (i.e., the electron-donating) properties of carotenoids **2**–**4** relative to the parent compounds **5**–**7**). By slightly increasing the proportion of water in the MeCN solvent, the electron-donating abilities were increased by a factor of approximately four. The investigated carotenoids were, for that reason, better antioxidants in water than in organic solvents, provided that the hydrophilicity was high enough to prevent aggregation, as was the case for **8** and **9**. Carotenoids whose substituents favor aggregation in water lose their antioxidant properties (**2**–**4**). The choice of the hydrophilic group covalently attached to a carotenoid is, therefore, of singular importance, particularly when designing novel chemical entities with improved pharmacokinetic and pharmacodynamic properties.

Hydrophilic substituents efficiently modulate the antioxidant properties of carotenoids with respect to aggregation tendency. Truly water-soluble carotenoids, such as **8** and **9**, which form monomolecular solutions, are immediately reactive in aqueous formulation; however, they are also prone to premature decomposition prior to the targeted use (leading to potentially reduced shelf life and susceptibility to light and heat). The investigated self-aggregating carotenoids are stable in a water-based environment and only become reactive when contacting a milieu (biological or otherwise) in which the aggregates are disrupted. It may be further possible to optimize the concerted action of singlet-oxygen quenching and radical-scavenging properties by the addition of a small amount of water to these hydrophilic, self-formulating carotenoids.

Experimental Section

Synthesis: The synthesis of Carp **2**, Card **4**, and Asly **9** and the purification of Croc **8** have been reported.[38,47,50] The synthesis and properties of Carox **3**, the surface and aggregation properties of **9**, and the antioxidant activity of **8** and **9** will be reported elsewhere. C30est **5**, C30ald **6**, and Asta **7** were kindly provided by H. Ernst, BASF AG (Ludwigshafen, Germany).

The stability of the molecular solutions and the aggregate dispersions for one of the aggregating carotenoids (**4**) were determined by heating it to reflux in methanolic HCl (2%) and aqueous HCl (2%) and following the decomposition by UV/Vis spectroscopic analysis. Anaerobic conditions for the flash-photolysis experiments were achieved by bubbling argon through the solutions and dispersions prior to the measurements. MeCN (Aldrich) was used as the solvent. The sensitizer NN (**1**) was given to the carotenoids after formation of the aggregate dispersion or monomolecular solution.

Transient absorption spectroscopy: Flash-induced changes in the absorbance of the samples were recorded with a homebuilt multichannel kinetic spectrometer. A linear flash lamp with a pulse width of approximately 5 μs served as the pump source for a sample placed in a standard fluorescence cuvette (cross section: 1×1 cm). The flash-induced change in the absorbance of the sample was monitored in right-angle geometry by using a miniature flash lamp with a pulse of 2 μs. The spectral analysis of the transmitted beam was carried out with the aid of a bifurcated fiber optic bundle and two spectrographs, one that covered the 200–620-nm range and the other the 600–1010-nm range. The light dispersed by the grating in each spectrograph was detected by a 512-element diode array. Time resolution was achieved by varying the delay t between the firing of the pump and the triggering of the probe source. The overall time resolution, determined by the width of the pump source, was close to 5 μs. The flash-induced changes in the absorbance of the sample are given as $\Delta A(\lambda,t) \equiv A(\lambda,t) - A(\lambda)$, in which $A(\lambda)$ is the absorbance of the sample before and $A(\lambda,t)$ the absorbance at time t after the instant at which the pump is fired. The contribution of ^{3}NN was removed from the absorption spectra, which were also corrected for small concentration differences by dividing the transient spectra by the ground-state absorbance.

Kinetic measurements: The time dependence of the signal $\Delta A(\lambda,t)$ at a fixed wavelength was recorded in a kinetic spectrometer with the third harmonic of a pulsed Nd:YAG laser (BM Industries) as the pump source. A pulsed xenon lamp (5 μs) provided the monitoring light, and a prism monochromator (Zeiss M4 QIII) placed after the sample compartment served as the wavelength selector. The pump and probe beams were set perpendicular to each other. A Hamamatsu photomultiplier (R928) with a 50-Ω terminating resistor was used for detecting the light emerging from the exit slit of the monochromator; the output of the detector was recorded by a 600-MHz digital storage oscilloscope (Agilent, Infinium 54830B). The transient absorption is given as the average of 32 individually recorded traces. Pump and probe flash lamps did not allow sufficient time resolution and complementary flash-laser experiments were therefore performed. A short laser pulse (6 ns) allowed the detection of the formation and decay of ^{3}Car. The Xe-flash spectra were recorded after 10 μs, at the maximum peak heights of the ^{3}Car and Car$^{\bullet+}$ absorption bands. The reaction constants were obtained from the recorded traces, thus expressing the bifurcation ratio between the electron- and energy-transfer rates. The bifurcation ratio determined from laser excitation is similar to Xe-lamp excitations. The peak heights of ^{3}**3** and **3**$^{\bullet+}$ were corrected by their molar absorption coefficients (Figure 5). **Excitation**: NN was excited at 355 nm using the laser. Excitation occurred at all emitted wavelengths with the Xe lamp.

Determination of molar absorption coefficients: The absorption coefficients of ^{3}Car were derived from the transient absorption spectrum $\Delta A(\lambda,t)$ [Eq. (9)]:

$$\Delta A(\lambda,t) = A(\lambda,t) - A(\lambda) = \varepsilon_P - c_P + \varepsilon_S - (c - c_P) - l - \varepsilon_S - c - l = (\varepsilon_P - \varepsilon_S) - c_P - l \quad (9)$$

in which $A(\lambda,t)$ and $A(\lambda)$ express the absorbance of the sample at time t before and after the flash, respectively; ε_P and ε_S are the extinction coefficients of the photoproducts with a concentration c_P and of ground-state molecules with concentration c. The pump flash triggers a certain fraction α of the ground-state molecules to be converted into photoproducts $c_P = a - c$. The absorption spectrum of the photoproduct is therefore: $\Delta A(\lambda,t) + \alpha A(\lambda)$, in which $\alpha A(\lambda)$ is the ground-state depletion. The factor α is found by adding a certain fraction of the ground-state absorption spectrum to the transient absorption spectrum, so that its ground-state character is vanishing. When the value of α is determined, the absorption coefficients for the photoproduct can be found by comparing the αA spectrum with that of $\Delta A + \alpha A$, as both originate from equal concentrations (Figure 7a). The spectrum of Car$^{\bullet+}$ was obtained by using the same procedure (Figure 7b). The bifurcation ratio was obtained by dividing the absorbance of ^{3}Car and Car$^{\bullet+}$ by the corresponding molar absorption coefficients (see Figure 6c and Table 1).

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