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Direct superoxide anion scavenging by a highly water-dispersible carotenoid phospholipid evaluated by electron paramagnetic resonance (EPR) spectroscopy

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Abstract—Synthetic carotenoid analogs, with increased utility for biological applications, are sparingly reported in the literature. Synthetic modification, which may increase the water solubility and/or water dispersibility of lipophilic carotenoids, allows their use in aqueous environments as potent antioxidants against potentially deleterious reactive oxygen species (ROS) that can be generated in vivo. Superoxide anion, produced by activated human neutrophils, can be a source of additional harmful ROS and nonradical species such as singlet oxygen in vivo. In the current study, direct scavenging of superoxide anion by a well-characterized C30 carotenoid phospholipid mixture was evaluated in a standard in vitro isolated human neutrophil assay by electron paramagnetic resonance (EPR) spectroscopy, employing the spin-trap DEPMPO. The carotenoid phospholipid was tested in aqueous formulation (aqueous dispersibility >60 mg/mL), in which supramolecular assembly takes place, as well as in ethanolic formulation as a monomeric solution of the carotenoid phospholipids. The carotenoid phospholipid (a highly unsaturated zwitterionic surfactant) was compared with a previously characterized rigid, long-chain, highly unsaturated dianionic bolaamphiphile, which contains an additional three conjugated double bonds in its extended conjugated system. As previously reported, direct scavenging by the carotenoid phospholipid derivatives in monomeric ethanolic formulation was superior at each tested concentration to aqueous, aggregated formulations of the compounds. Additionally, the percent inhibition of superoxide signal was related to the apparent or effective length of the conjugated chromophore, consistent with previous reports of radical inhibition and singlet oxygen quenching by polyene carotenoids of differing length.

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1. Introduction

The conjugated double bond structure of the polyene chain of carotenoids is responsible for the characteristic color of the chromophore for particular carotenoids. Antioxidant capacity is related to effective or apparent polyene chain length, and to the number of conjugated double bonds.[1] As scavengers of oxygen radicals, carotenoids are particularly potent. The reaction kinetics depend not only on the individual carotenoid, but also on the nature of the model used (solvent system), and the radical species involved.[2,3] As nearly all of the 700 natural carotenoids are lipophilic,[4] diverse strategies must be utilized to increase the water solubility of these compounds for introduction into aqueous test systems.[5,6] Synthetic carotenoids with increased water solubility and/or water dispersibility have been sparingly reported in the literature.[7,8] Supramolecular assembly in aqueous solution—which protects the individual molecules in the aggregated state from oxidation—limits scavenging of radical and nonradical species generated in or released into solution.[7,9,10] Therefore, comparisons of the scavenging ability of these compounds must take into consideration the behavior of the individual molecules while in aqueous solution.

In the current study, electron paramagnetic resonance (EPR) spectroscopy was used to characterize the ability

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Keywords: Carotenoid phospholipids; Zwitterionic surfactants; Astaxanthin derivatives; Carotenoid esters; Electron paramagnetic resonance; EPR; EPR spectroscopy; Spin traps; DEPMPO; Supramolecular assembly; Superoxide anion; Scavengers.

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of a well-characterized carotenoid phospholipid mixture [1-(β-apo-8′-carotenoyl)-glycero-3-phosphocholine, 1-(β-apo-8′-carotenoyl)-glycero-2-phosphocholine, and 2-(β-apo-8′-carotenoyl)-glycero-3-phosphocholine in a 49:43:8 ratio (aqueous dispersibility >60 mg/mL; Fig. 1)][11] to directly scavenge superoxide anion produced from maximally-activated isolated human neutrophils. The scavenging ability of this isomeric mixture of zwitterionic surfactant(s) was compared with that obtained both concurrently and previously with a rigid, long-chain, highly unsaturated bolaamphiphile (Cardax™).[7] The assay utilized here is a standard in vitro test system, in which the production of superoxide anion by stimulated human neutrophils is quantified by EPR in the presence of a spin trap (DEPMPO).[7] The results corroborate earlier results obtained with synthetic carotenoids, in which the scavenging activity of the carotenoid derivative in aqueous solution is improved by the addition of less polar solvent, thereby disintegrating the aggregates into monomers. Percent inhibition (% inhibition) of superoxide anion signal detected with the DEPMPO spin trap revealed that the effective chromophore length—related to both the number of conjugated double bonds in the compound and $\lambda_{\max}$—is one important determinant of the ultimate antioxidant potency of a particular carotenoid derivative.

2. Materials

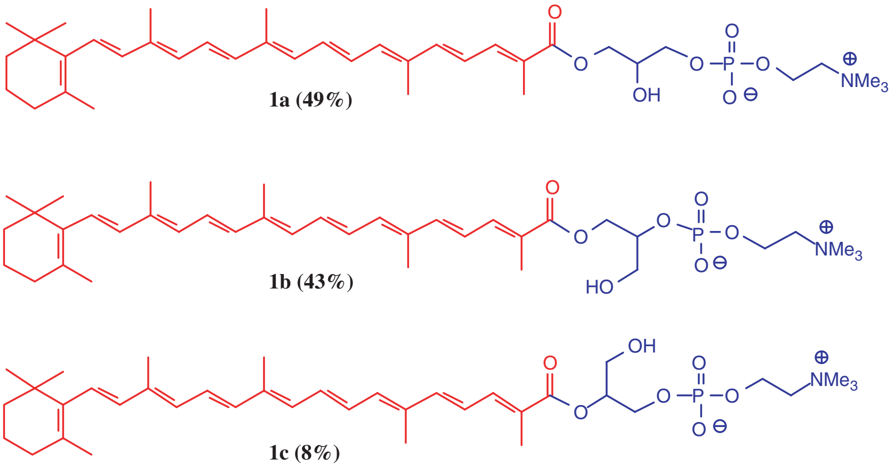
The positional isomers of the carotenoid lysophosphotidylcholines [1-(β-apo-8′-carotenoyl)-glycero-3-phosphocholine, 1-(β-apo-8′-carotenoyl)-glycero-2-phosphocholine, and 2-(β-apo-8′-carotenoyl)-glycero-3-phosphocholine] synthesized for the current study are shown in Figure 1a–c. An isomeric mixture was tested, containing compounds **1a**–**1c** in the ratio 49:43:8. The compounds were >95% pure by HPLC (as AUC). Clear red, evenly-colored aqueous suspensions were obtained without heat, detergents, or additives after addition of test compound to deionized (DI) water, as described previously.[11] Aqueous formulations of the compound mixture were evaluated at four concentrations: 0.5, 1.0, 3.0, and 10.0 mM. As these compounds demonstrate supramolecular assembly in aqueous formulation,[11] the compounds were also tested in ethanolic formulation (41.2% final EtOH concentration in stock solution), in which only monomers exist (UV/vis spectroscopic evidence; Fig. 2). The ethanolic formulations tested were 0.1, 0.3, 0.5, 1.0, and 3.0 mM (Table 1).

Cardax™ in 33% ethanol (EtOH) at 0.1 mM (100 μM), utilized as a reference standard, was tested as previously described.[7] Briefly, the disodium salt disuccinate derivatives of astaxanthin [statistical mixture of stereoisomers 3*S*,3′*S*, *meso* (3*R*,3′*S*), and 3*R*,3′*R* in a 1:2:1 ratio; Fig. 3] were introduced into the aqueous test system as the monomeric ethanolic solution. Cardax™ was synthesized at >97% purity by HPLC (as AUC). The results of inhibition of superoxide anion signal, as detected by the paramagnetic spin-trap DEPMPO-OOH adduct, are given in Table 1.

Ethanol vehicle negative controls (0.3% final EtOH concentration in isolated neutrophil assay) were also evaluated. Mean percent inhibition of the ethanol-only vehicle was within the coefficient of variation of the scavenging assay (<5%, data not shown).[7]

3. Leukocyte isolation and preparation

Human polymorphonuclear leukocytes (PMNs) were isolated from freshly sampled venous blood of a single volunteer (S.F.L.) by Percoll density gradient centrifugation as described previously.[7,12] Briefly, each 10 mL of whole blood was mixed with 0.8 mL of 0.1 M EDTA and 25 mL of saline. The diluted blood was then layered over

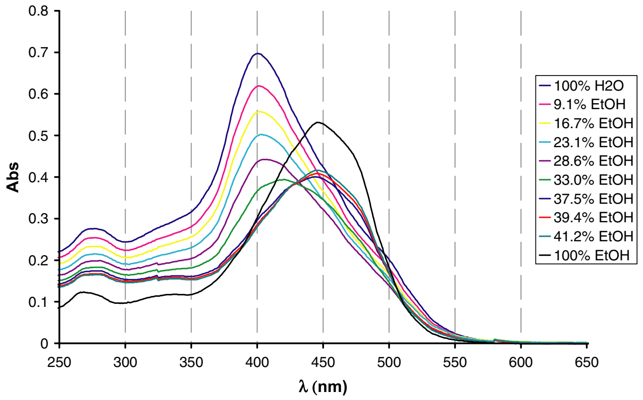


1a (49%)

1b (43%)

1c (8%)

Figure 1. The positional isomers of the carotenoid lysophosphotidylcholines [1-(β-apo-8′-carotenoyl)-glycero-3-phosphocholine (**1a**); 1-(β-apo-8′-carotenoyl)-glycero-2-phosphocholine (**1b**); 2-(β-apo-8′-carotenoyl)-glycero-3-phosphocholine (**1c**)] synthesized for the current study. The compounds were tested as a mixture of the positional isomers in the ratio of compounds **1a**–**1c** of 49:43:8. The compounds were >95% pure by HPLC (as AUC). The isomer mixture was tested in aqueous formulation, in which supramolecular assembly takes place, as a well as in ethanolic formulation (41.2% final EtOH concentration in stock solution), which maintains the compounds in monomeric solution.



UV_{max} (100% DI H_2O) = 400 nm
UV_{max} (9.1% EtOH) = 402 nm
UV_{max} (16.7% EtOH) = 402 nm
UV_{max} (23.1% EtOH) = 403 nm
UV_{max} (28.6% EtOH) = 406 nm
UV_{max} (33.0% EtOH) = 421 nm
UV_{max} (37.5% EtOH) = 444 nm
UV_{max} (39.4% EtOH) = 445 nm
UV_{max} (41.2% EtOH) = 446 nm
UV_{max} (100% EtOH) = 446 nm

Figure 2. UV/vis absorption spectra of **1a**–**c** aggregates in water (440 nm) with increasing amounts of EtOH (Fig. 1). The λ_{max} increases to 446 nm at an EtOH concentration of 41.2%, at which point no further shift of the absorption maximum occurs (i.e., a molecular solution is reached). The λ_{max} in EtOH of 446 nm reflects the effective chromophore length of this compound mixture; for comparison, the λ_{max} of Cardax™ in EtOH is 481.5 nm.[9] Blue-shifting of the absorption maximum in water versus the less polar solvent reflects supramolecular assembly (aggregation).

9 mL of Percoll at a specific density of 1.080 g/mL. After centrifugation at 400*g* for 20 min at 20 °C, the plasma, mononuclear cell, and Percoll layers were removed. Erythrocytes were subsequently lysed by addition of 18 mL of ice-cold water for 30 s, followed by 2 mL of 10× PIPES buffer (25 mM PIPES, 110 mM NaCl, and 5 mM KCl, titrated to pH 7.4 with NaOH). Cells were then pelleted at 4 °C, the supernatant was decanted, and the procedure was repeated. After the second hypotonic cell lysis, cells were washed twice with PAG buffer [PIPES buffer containing 0.003% human serum albumin (HSA) and 0.1% glucose]. Afterward, PMNs were counted by light microscopy on a hemocytometer. The isolation yielded PMNs with a purity of >95%. The final pellet was then suspended in PAG-CM buffer (PAG buffer with 1 mM $CaCl_2$ and 1 mM $MgCl_2$).

4. EPR measurements

All EPR measurements were performed using a Bruker ER 300 EPR spectrometer operating at X-band with a TM_{110} cavity as previously described.[7,13] The microwave frequency was measured with a Model 575 microwave counter (EIP Microwave, Inc., San Jose, CA). To measure superoxide anion ($O_2^{-}\cdot$) generation from phorbol-ester (PMA)-stimulated PMNs, EPR spin-trapping studies were performed using the spin-trap DEPMPO (Oxis, Portland, OR) at 10 mM. PMNs (1×10^6) were stimulated with PMA (1 ng/mL) and loaded into capillary tubes for EPR measurements. To determine the radical scavenging ability of Cardax™ in ethanolic formulation at 100 μM as a reference standard, as well as the aqueous and ethanolic formulations of the carotenoid phospholipid mixture, PMNs were

Table 1. Descriptive statistics for mean percent (%) inhibition of aqueous superoxide anion production for increasing concentrations of aqueous and ethanolic (concentration of EtOH in stock solution 41.2%) formulations of the C30 carotenoid phospholipid positional isomer mixture tested in the current study, as well as Cardax™ in 33% ethanol (EtOH) at 0.1 mM (100 μM)

Sample	Solvent	Concentration (mM)	*N*	Mean % inhibition	SD	SEM	Min	Max	Range
C30 carotenoid phospholipid mixture	Water	0.5	5	18.2	16.1	7.2	1	43	42
C30 carotenoid phospholipid mixture	Water	1.0	4	24.6	20.5	9.2	4	54	50
C30 carotenoid phospholipid mixture	Water	3.0	4	51.8	12.4	6.2	35	63	28
C30 carotenoid phospholipid mixture	Water	10.0	3	94.3	2.1	1.2	92	96	4
C30 carotenoid phospholipid mixture	EtOH	0.1	3	13.3	7.0	4.1	6	20	14
C30 carotenoid phospholipid mixture	EtOH	0.3	3	31.3	1.5	0.9	30	33	3
C30 carotenoid phospholipid mixture	EtOH	0.5	3	61.7	14.0	8.1	46	73	27
C30 carotenoid phospholipid mixture	EtOH	1.0	3	79.3	3.1	1.8	76	82	6
C30 carotenoid phospholipid mixture	EtOH	3.0	3	98.7	0.6	0.3	98	99	1
Cardax™	EtOH	0.1	3	38.0	8.7	5.0	32	48	16

Sample sizes of 3 or greater were evaluated for each formulation. Dose-dependent increases in superoxide anion scavenging were observed for the C30 isomer mixture in both formulations; the aggregated aqueous formulation was less efficacious, and more highly variable, than the monomeric solution in EtOH. Mean % inhibition of 13.3% by the C30 positional isomer mixture at 0.1 mM (with 10 conjugated double bonds in the chromophore) was less than that observed with Cardax™ (38.0%; 13 conjugated double bonds in the extended conjugated system); the result was statistically significant (unpaired Student's *t*-test, $t = 3.82$, $p = 0.019$).

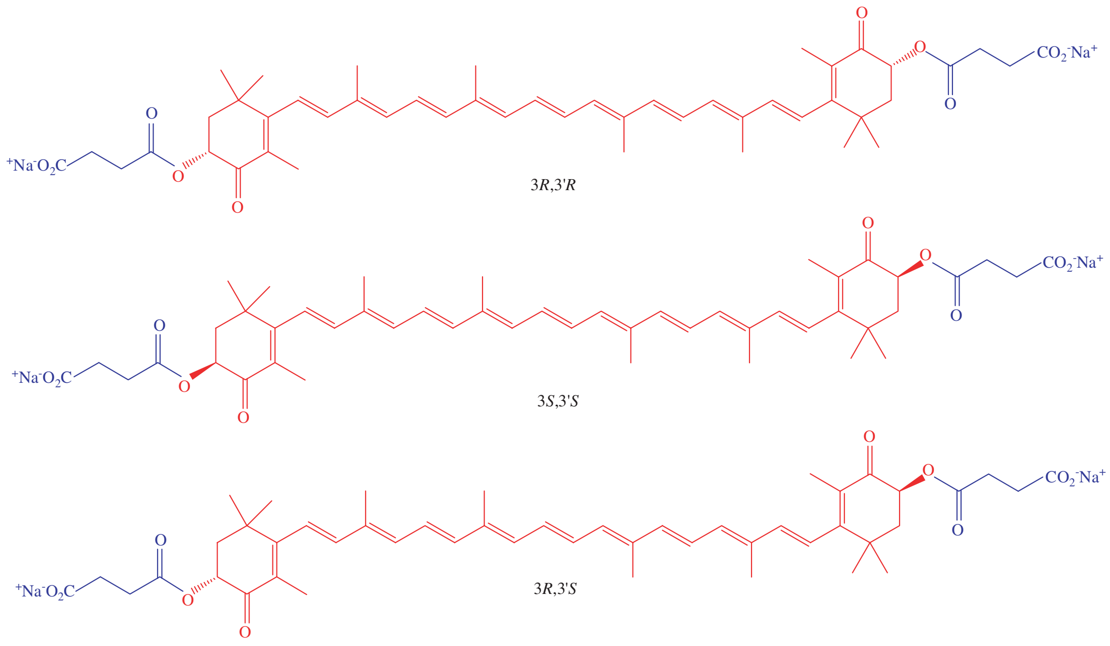


Figure 3. The three stereoisomers comprising the statistical mixture of stereoisomers of the disodium salt disuccinate diester of astaxanthin (Cardax^{TM}) synthesized for the current study (shown as the all-*E* geometric isomers). Cardax^{TM} was >97% pure by HPLC (as AUC). The mixture of stereoisomers contains 3*S*,3′*S*, *meso* (3*R*,3′*S*), and 3*R*,3′*R* in a 1:2:1 ratio. Cardax^{TM} was tested in ethanolic formulation (33% EtOH final concentration in stock solution) at 100 μM.

pre-incubated for 5 min with test compound, followed by PMA stimulation.

Instrument settings used in the spin-trapping experiments were as follows: modulation amplitude, 0.32 G; time constant, 0.16 s; scan time, 60 s; modulation frequency, 100 kHz; microwave power, 20 mW; and microwave frequency, 9.76 GHz. The samples were placed in a quartz EPR flat cell, and spectra were recorded. The component signals in the spectra were identified and quantified as reported previously.[14,15]

5. Statistical analysis

Statistical analyses were performed with the NCSS statistical software package (NCSS 2001 and PASS 2002, Kaysville, UT). All statistical tests were performed at a type I error rate (α) = 0.05.

Figure 4 and Table 1 show the mean relative scavenging abilities, with accompanying EPR spectra, of each of the four aqueous formulations of the positional isomer mixture (concentrations 0.5–10.0 mM). The mean %

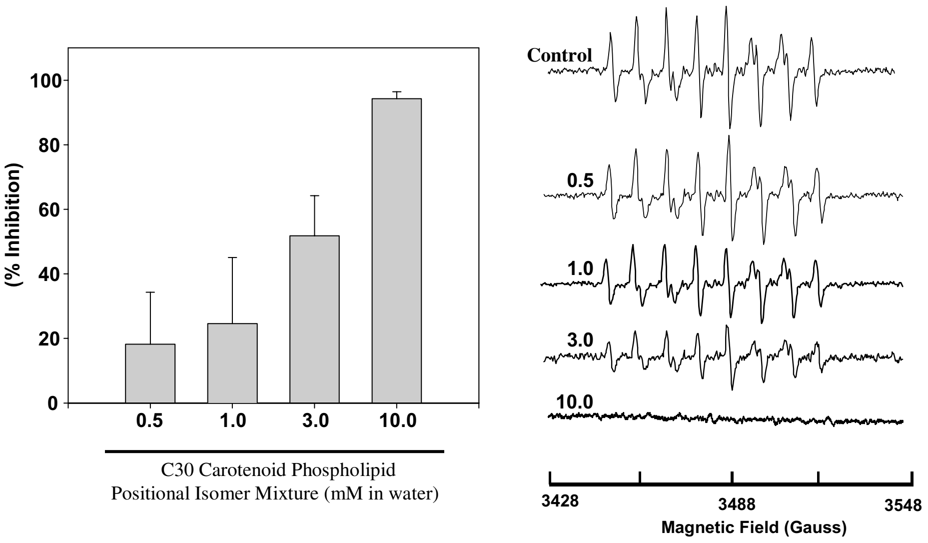


Figure 4. Mean percent inhibition of superoxide anion signal as detected by DEPMPO spin trap by the carotenoid phospholipid isomer mixture **1a**–**c** (49:43:8 ratio; Fig. 1) in aqueous formulation. Each derivative in aqueous formulation was standardized to control EPR signal detected without addition of compound (set at 0% inhibition by convention). Dose-dependent, nonlinear increase in scavenging efficiency is seen. In each case, the aqueous formulation is less efficacious than the corresponding formulation in EtOH (Fig. 5).

inhibition increased in a nonlinear, dose-dependent fashion (from 18.2% to 94.3%); an approximate 20-fold increase in concentration was necessary to effect slightly greater than a 5-fold increase in scavenging efficiency. This phenomenon was reported for the first time for Cardax™ in water, a statistical mixture of stereoisomers that also demonstrates supramolecular assembly in pure aqueous formulation.[7]

Figure 5 and Table 1 show the mean relative scavenging abilities, with accompanying EPR spectra, of each of the five ethanolic formulations of the positional isomer mixture. The scavenging efficiency was nearly linear below 0.5 mM. The concentrations tested increased from 0.1 to 3.0 mM, a 30-fold increase in absolute concentration. The mean % inhibition of superoxide anion signal increased from 13.3% at the lowest concentration tested, to 98.7% (near complete inhibition) at 3.0 mM, the highest concentration evaluated in ethanolic formulation. At each identical concentration (0.5, 1.0, and 3.0 mM), the ethanolic formulation demonstrated increased scavenging ability over the aqueous formulation, a phenomenon described previously.[7]

A comparison of the direct superoxide anion scavenging efficiency between Cardax™ at 100 μM in ethanolic formulation (38.0%) and the positional isomer mixture of the carotenoid phospholipid at 100 μM in ethanolic formulation (13.3%) demonstrates significantly increased scavenging by the C40 carotenoid derivative (Student's unpaired t-test, $t = 3.82$, $p = 0.019$), with 13 conjugated double bonds in the extended conjugated system, versus the 10 conjugated double bonds in the carotenoid phospholipid chromophores. The effective chromophore of the astaxanthin derivative can be estimated from the $\lambda_{\max}$ in ethanol of 481 nm;[9] similarly, the effective chromophore of the carotenoid phospholipids by the $\lambda_{\max}$ in EtOH of 446 nm (Fig. 2).

Carotenoids are typically potent lipophilic antioxidants. They have been traditionally evaluated as antioxidants in model systems operating with organic solvents due to their hydrophobic nature.[16] These compounds normally exert their antioxidant properties in cell membranes, lipoproteins, and the lipid compartments of other tissues in vivo.[1,17] The present study evaluated the utility of a highly water-dispersible, highly unsaturated carotenoid phospholipid mixture—with aqueous dispersibility >60 mg/mL—to directly scavenge aqueous-phase superoxide anion produced by maximally-stimulated isolated human neutrophils. The ability of this zwitterionic carotenoid phospholipid surfactant mixture to scavenge superoxide anion in the aqueous phase in vitro was compared with the scavenging ability of the rigid, long-chain, highly unsaturated carotenoid bolaamphiphile Cardax™ (maximum dispersibility 8.64 mg/mL). The characterization of the scavenging ability of Cardax™ in the identical test system, repeated here, has been described previously.[7]

In the current study, dose-dependent increases in mean scavenging ability were identified for both the aqueous and ethanolic formulations of the carotenoid phospholipid mixture (Table 1; Figs. 4 and 5). Nonlinear increases in scavenging ability were seen for both formulations, a phenomenon related to the supramolecular assembly which occurs in aqueous formulation.[7,9] At concentrations ⩽ 0.5 mM in ethanol, the mean scavenging ability of the carotenoid phospholipid mixture was nearly linear, suggesting a stoichiometric relationship between the scavenging ability of the carotenoid phospholipids while in monomeric solution, and the

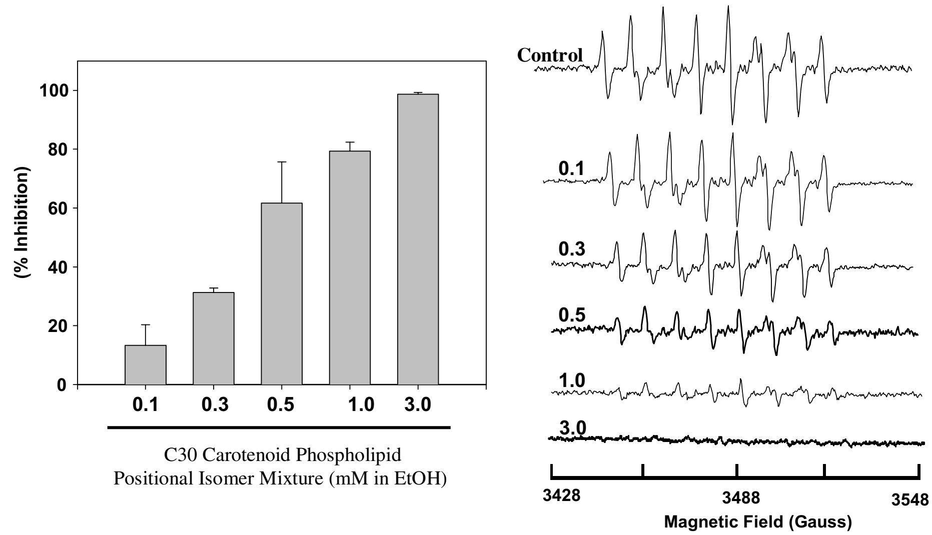


Figure 5. Mean percent inhibition of superoxide anion signal as detected by DEPMPO spin trap by the carotenoid phospholipid isomer mixture **1a–c** (49:43:8 ratio; Fig. 1) in ethanolic formulation (final EtOH concentration in isolated neutrophil assay 0.4%). Each derivative in ethanolic formulation was standardized to control EPR signal detected without addition of compound (set at 0% inhibition by convention). Dose-dependent, nearly linear increases in scavenging efficiency are seen at concentrations ⩽ 0.5 mM. At 3 mM, near-complete inhibition of superoxide anion signal was observed (98.7% inhibition). At each identical concentration tested, the ethanolic (i.e., monomeric) formulation is more efficacious than the corresponding formulation in deionized (DI) water (Fig. 4).

concentration of superoxide generated after maximal stimulation. The surfactant and aggregation properties of the positional isomers were expected to be virtually identical, as each contains the same polar head group and the same hydrophobic chain (Fig. 1a–c). The isomers in aqueous solution predominately form aggregates with an average hydrodynamic radius $r_H = 8$ nm; a few larger aggregates of sizes between $r_H = 40$–600 nm have also been detected by dynamic light scattering (DLS).[11] As has been suggested for Cardax™, aggregation in solution is dependent upon both the concentration of the target compound, and the ionic strength of the diluent (Foss et al., unpublished results). This may form one explanation for the loss of linearity at higher concentrations in ethanolic solution, after introduction into the buffered test assay.

Comparison of the mean scavenging efficiency of the positional isomer mixture in ethanol at 100 μM (13.3%) with the mean scavenging efficiency of Cardax™ in EtOH at 100 μM (38.0%) could reflect the contributions of the effective chromophores of each of these compounds to the overall antioxidant potential. As has been demonstrated previously for lycopene (λ_{max} in acetone 505 nm)[18] and nonesterified, free astaxanthin (λ_{max} in acetone 480 nm)[19] in model systems with regard to singlet oxygen quenching ability, it is the effective chromophore, and not the absolute number of conjugated double bonds, that determines quenching efficiency.[16] In this case, the number of conjugated double bonds parallels the mean scavenging efficiency (13 vs 10), and is also indicative of the effective chromophore (λ_{max} of the positional isomers in EtOH = 446 nm, Fig. 2; and λ_{max} of Cardax™ in EtOH = 481.5 nm).[9] The relative decrease in mean scavenging efficiency can be completely compensated for by the absolute differences in water dispersibility between the two compounds (>60 vs 8.64 mg/mL for the carotenoid phospholipids and Cardax™, respectively).

In summary, the current study corroborates earlier investigations on the utility of novel carotenoid derivatives with increased water dispersibility for aqueous-phase scavenging of biologically relevant reactive oxygen species (ROS). Dose-dependent increases in scavenging of superoxide anion produced by maximally-stimulated human neutrophils were detected by EPR spectroscopy with spin trapping. In every case, monomeric solutions of the test compounds in ethanol were more efficacious than the aggregated aqueous formulations of the test compound at the same concentration. Dose-ranging studies revealed that the concentration of the carotenoid phospholipid mixture could be increased to near-complete suppression of the induced superoxide anion signal. On a molar basis, the carotenoid phospholipids were slightly less potent direct radical scavengers than the previously evaluated astaxanthin derivative Cardax™, an observation attributed to the difference in effective chromophore length between the two compound mixtures. This difference may be overcome by increasing the dose of the carotenoid phospholipid mixture in vivo (Lockwood, unpublished results). These compounds may have significant potential as aqueous-phase direct scavengers of ROS and other nonradical species such as singlet oxygen, which can be particularly destructive in biological systems.

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