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The synthesis and aqueous superoxide anion scavenging of water-dispersible lutein esters

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Abstract—Xanthophyll carotenoids of the C40 series, which includes commercially important compounds such as lutein, zeaxanthin, and astaxanthin, have poor aqueous solubility in the native state. Hawaii Biotech, Inc. (HBI) and others have shown that the aqueous dispersibility of derivatized carotenoids can be increased by varying the chemical structure of the esterified moieties. In the current study, the published series of novel, highly water-dispersible C40 carotenoid derivatives has been extended to include (3*R*,3′*R*,6′*R*)-lutein (β,ε-carotene-3,3′-diol) derivatives. Two novel derivatives were synthesized by esterification with inorganic phosphate and succinic acid, respectively, and subsequently converted to the sodium salts. Red-orange, clear, aqueous suspensions were obtained after addition of these novel derivatives to USP-purified water. Aqueous dispersibility of the disuccinate sodium salt of lutein was 2.85 mg/mL; the diphosphate salt demonstrated a >10-fold increase in dispersibility at 29.27 mg/mL. As reported previously, these aqueous suspensions were obtained without the addition of heat, detergents, co-solvents, or other additives. The direct aqueous superoxide scavenging abilities of these novel derivatives were subsequently evaluated by electron paramagnetic resonance (EPR) spectroscopy in a well-characterized in vitro isolated human neutrophil assay. The novel derivatives were nearly identical aqueous-phase scavengers, demonstrating dose-dependent suppression of the superoxide anion signal (as detected by spin-trap adducts of DEPMPO) in the millimolar range. These lutein-based soft drugs will likely find utility in those commercial and clinical applications for which aqueous-phase singlet oxygen quenching and direct radical scavenging may be required.

The synthesis of water-dispersible C40 carotenoids—as potential parenteral agents for clinical applications[1–6]—has proven to be an effective strategy to improve the injectability of these compounds as therapeutic agents, a result perhaps not achievable through other formulation methods.[7] The methodology has also been extended to non-symmetric carotenoids with fewer than 40 carbon atoms in the molecular skeleton and differing ionic character.[8,9] The aqueous dispersibility of these compounds also facilitates proof-of-concept studies in model systems (e.g., cell culture),[10,11] where the high lipophilicity of these compounds previously limited their bioavailability and hence proper evaluation of efficacy.[12] In addition, esterification has proven useful to increase oral bioavailability, a fortuitous side effect of the esterification process which can increase solubility in gastric mixed micelles.[13,14] The net overall effect is a vast improvement in potential clinical utility for the lipophilic carotenoid compounds as therapeutic agents.

In the current study, the principles of retrometabolic drug design were again utilized to produce novel soft drugs[15] from the asymmetric parent C40 carotenoid scaffold (3*R*,3′*R*,6′*R*)-lutein. Lutein was obtained commercially as purified natural source material (marigold extract), mainly the (3*R*,3′*R*,6′*R*)-stereoisomer (one of eight potential stereoisomers). Lutein (Scheme 1) possesses key characteristics—similar to those of HBI's previous starting material astaxanthin—which also made it an ideal starting platform for retrometabolic syntheses: one, synthetic handles (hydroxyl groups) for conjugation, and two, an excellent safety profile for the parent compound.[16] As stated previously, lutein is available commercially from multiple sources in bulk as predominantly the (3*R*,3′*R*,6′*R*)-stereoisomer, the primary isomer in the human diet and human retinal tissue.[17] Recent studies have demonstrated the utility of lutein-based supplementation for the clinical improvement of vision, reduction of ultraviolet (UV)-based

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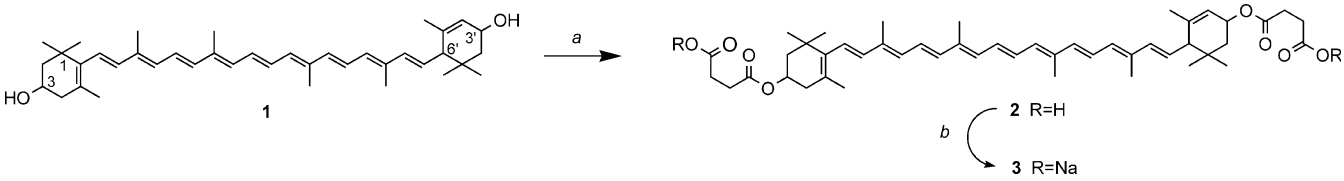


Scheme 1. Reagents and conditions: (a) succinic anhydride, *N*,*N*-diisopropylethylamine, CH_2Cl_2 (64%); (b) NaOMe, CH_2Cl_2 (91%).

inflammation, and potentially the inhibition and/or amelioration of age-related macular degeneration (ARMD).[16–19] The synthesis, characterization, and aqueous-phase superoxide anion scavenging abilities of the diphosphate and disuccinate sodium salts of natural source lutein are herein reported. The potential utility of lutein-based formulations has been extended for clinical application by providing compounds with sufficient aqueous dispersibility for parenteral administration, thereby capturing the significant human population of carotenoid oral non-responders as well as acute clinical application(s) requiring rapid loading of therapeutic doses. In addition, potent and nearly identical direct superoxide anion scavenging ability (at millimolar concentration) is documented for both novel compounds.

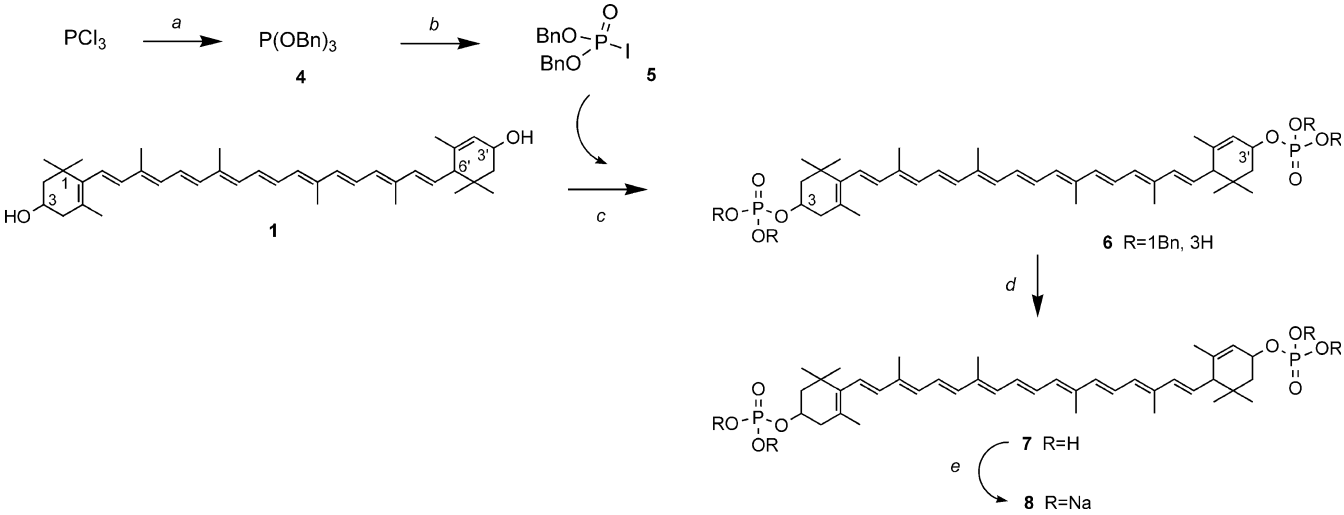
In HBI's continuing efforts to impart water dispersibility to clinically relevant hydrophobic carotenoids, the preparation of two water-dispersible lutein derivatives, the sodium salts of lutein disuccinate and lutein diphosphate, as seen in Schemes 1 and 2, is reported.[20]

The synthesis of disuccinate salt **3** was straightforward and began with succinylation of lutein using succinic anhydride and Hünig base (Scheme 1). Disuccinylation of lutein was optimized by running the reaction in a concentrated fashion and using modest excesses of anhydride and base. Aqueous acidic workup yielded disuccinate **2**; excess reagents were removed by washing with dilute HCl. The water-dispersible derivative (**3**) was generated by treatment of **2** with sodium methoxide. The resulting salt was diluted with water and lyophilized to provide sodium salt **3** in good yield.

In the pursuit of preparing a water-dispersible phosphate derivative of lutein, a three-step protocol was envisioned: one, phosphorylation of hydroxyls as protected phosphates; two, mild removal of protecting groups to yield free phosphates; and three, salt formation to provide the diphosphate sodium salt.

Successful diphosphorylation of lutein was first achieved using dimethyl phosphoroiodidate, formed in situ by reacting trimethyl phosphite with iodine.[25] Attempted deblocking of the phosphates using potassium cyanide,[26] phenyl thiolate,[26] *tert*-butylamine,[27] lithium hydroxide, or bromotrimethylsilane[28] in the presence of *N*,*O*-bis(trimethylsilyl)acetamide failed to provide target **7** (see Scheme 2). Such efforts resulted in the decomposition of the polyene, cleavage of phosphates, or the incomplete deprotection of methyl phosphate esters.

As seen in Scheme 2, sodium salt **8** was successfully prepared when using benzyl esters as phosphate-protecting groups. Lutein was phosphorylated using in situ generated dibenzyl phosphoroiodidate,[25] forming a mixture of benzyl-protected diphosphates. Workup of the phosphate mixture provided a crude red oil that was used in the deprotection step without further purification. Successful debenzylation of the protected phosphates was achieved using bromotrimethylsilane[28] in the



Scheme 2. Reagents and conditions: (a) benzyl alcohol, triethylamine, Et_2O (83%); (b) I_2, CH_2Cl_2; (c) **1**, pyridine, CH_2Cl_2 then **5**; (d) bromotrimethylsilane, pyridine, CH_2Cl_2; (e) NaOMe, CH_2Cl_2 (35% for three steps).

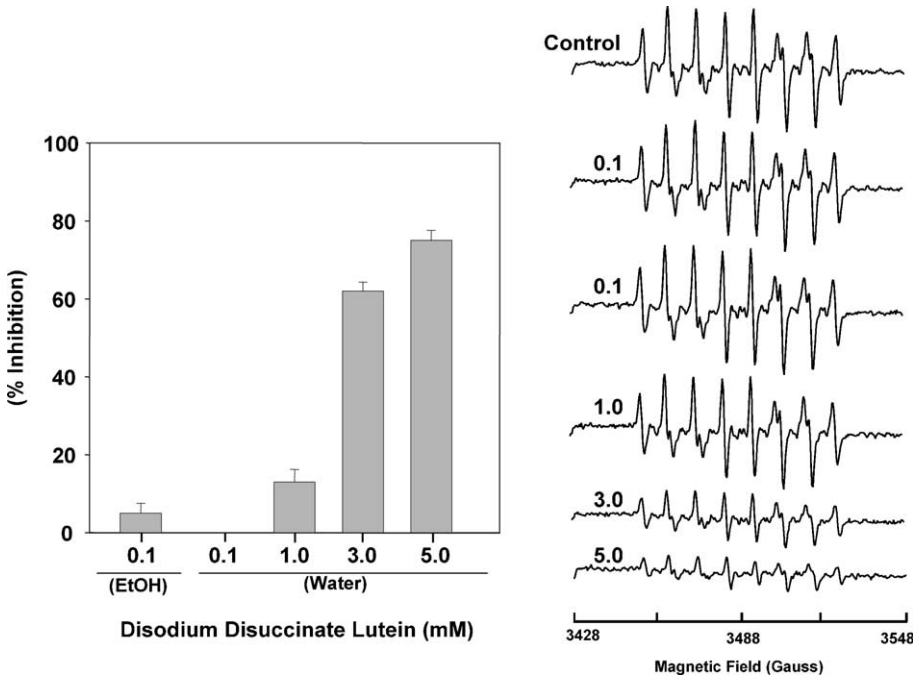


Figure 1. Mean percent inhibition (±SEM) of superoxide anion signal as detected by DEPMPO spin-trap by the sodium disuccinate derivative of lutein (tested in water). A 100 μM formulation (0.1 mM) was also tested in 40% EtOH, a concentration shown to produce a molecular (i.e., non-aggregated) solution (data not shown). As the concentration of the derivative increased, inhibition of the superoxide anion signal increased in a dose-dependent manner. At 5 mM, approximately 3/4 (75%) of the superoxide anion signal was inhibited. No apparent scavenging (0% inhibition) was observed at 0.1 mM in water, and scavenging was not significantly increased over background scavenging by the EtOH vehicle (5%)[1] by the addition of 40% EtOH. The millimolar concentration scavenging by the novel derivative was accomplished in water alone, without the addition of organic co-solvent (e.g., acetone, EtOH),[1] heat, detergents, or other additives.

presence of pyridine. As shown in Scheme 2, the sodium salt was generated by treating **8** with sodium methoxide. The resulting salt was washed with diethyl ether, then water, and resuspended in aqueous methanol. The solution was diluted with water and lyophilized to provide sodium salt **8** in good yield.

The mean percent inhibition of superoxide anion signal (±SEM) as detected by DEPMPO spin-trap by the sodium disuccinate derivative of lutein (tested in water) is shown in Figure 1. A 100 μM formulation (0.1 mM) was also tested in 40% EtOH, a concentration shown to produce a molecular (i.e., non-aggregated) solution. As the concentration of the derivative increased, inhibition of superoxide anion signal increased in a dose-dependent manner. At 5 mM, approximately 3/4 (75%) of the superoxide anion signal was inhibited. No significant scavenging (0% inhibition) was observed at 0.1 mM in water. Addition of 40% EtOH to the derivative solution at 0.1 mM did not significantly increase scavenging over that provided by the EtOH vehicle alone (5% inhibition).[1] These data suggested that card-pack ('H'-type) aggregation for this derivative was not occurring in aqueous solution (and thus limiting the interaction of the aggregated carotenoid derivative with aqueous superoxide anion) (see Tables 1 and 2).[29,30]

The mean percent inhibition of superoxide anion signal (± SEM) as detected by DEPMPO spin-trap by the sodium diphosphate derivative of lutein (tested in water) is shown in Figure 2. A 100 μM formulation (0.1 mM) was also tested in 40% EtOH, a concentration also shown to produce a molecular (i.e., non-aggregated)

Table 1. Descriptive statistics of mean % inhibition of superoxide anion signal for aqueous and ethanolic (40%) formulations of sodium disuccinate derivatives of lutein tested in the current study

Sample	Solvent	Concentration (mM)	*N*	Mean (% inhibition)	SD	SEM	Min	Max	Range
Lutein disuccinate sodium salt	40% EtOH	0.1	3	5.0	4.4	2.5	0	8	8
Lutein disuccinate sodium salt	Water	0.1	1	0.0	ND	ND	0	0	0
Lutein disuccinate sodium salt	Water	1.0	3	13.0	5.6	3.2	8	19	11
Lutein disuccinate sodium salt	Water	3.0	3	61.7	4.0	2.3	58	66	8
Lutein disuccinate sodium salt	Water	5.0	3	74.7	4.5	2.6	70	79	9

Sample sizes of three were evaluated for each formulation, with the exception of lutein in 40% EtOH stock solution ($N = 1$). Mean % inhibition did not increase over background[1] levels until sample concentration reached 1 mM in water; likewise, addition of 40% EtOH at the 0.1 mM concentration did not increase scavenging over background levels attributable to the EtOH vehicle (mean = 5% inhibition).[1]

Table 2. Descriptive statistics of mean % inhibition of superoxide anion signal for aqueous and ethanolic (40%) formulations of sodium diphosphate derivatives of lutein tested in the current study

Sample	Solvent	Concentration (mM)	*N*	Mean (% inhibition)	SD	SEM	Min	Max	Range
Lutein diphosphate sodium salt	40% EtOH	0.1	3	18.0	7.0	4.0	11	25	14
Lutein diphosphate sodium salt	Water	0.1	1	0.0	ND	ND	0	0	0
Lutein diphosphate sodium salt	Water	1.0	3	9.3	3.5	2.0	6	13	7
Lutein diphosphate sodium salt	Water	3.0	3	72.3	3.1	1.8	69	75	6
Lutein diphosphate sodium salt	Water	5.0	3	91.0	2.6	1.5	88	93	5

Sample sizes of three were evaluated for each formulation, with the exception of lutein diphosphate in water at 100 μM (0.1 mM) where $N = 1$. Mean % inhibition of superoxide anion signal increased in a dose-dependent manner as the concentration of lutein diphosphate was increased in the test assay. At 100 μM in water, no inhibition of scavenging was seen. The molecular solution in 40% EtOH (mean % inhibition = 18%) was increased above background scavenging (5%) by the ethanolic vehicle,[1] suggesting that disaggregation increased scavenging at that concentration. Slightly increased scavenging (on a molar basis) may have been obtained with the diphosphate derivative in comparison to disuccinate derivative (see Table 1 and Fig. 1).

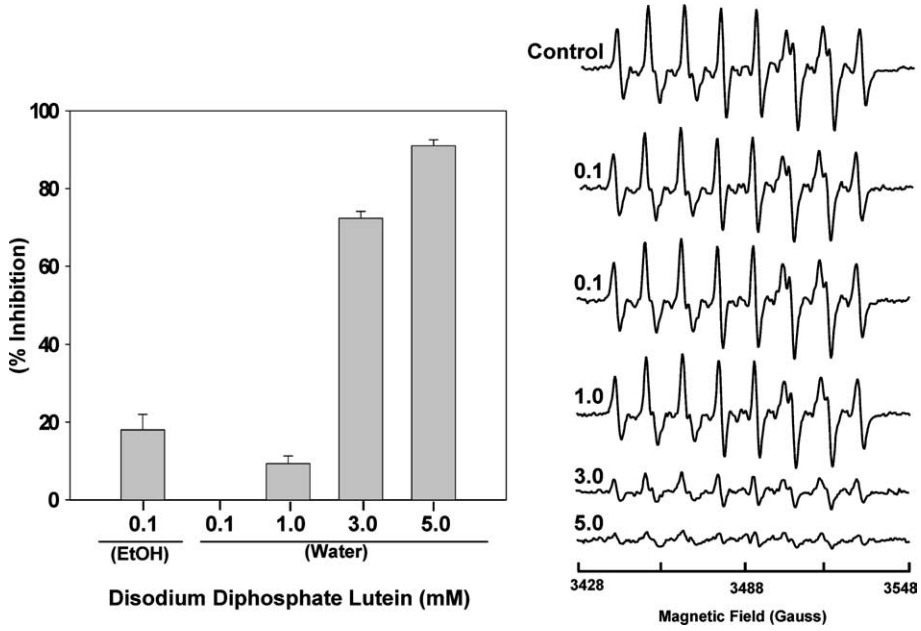


Figure 2. Mean percent inhibition (± SEM) of superoxide anion signal as detected by DEPMPO spin-trap by the sodium diphosphate derivative of lutein (tested in water). A 100 μM formulation (0.1 mM) was also tested in 40% EtOH, a concentration shown to produce a molecular (i.e., non-aggregated) solution (data not shown). As the concentration of the derivative increased, inhibition of the superoxide anion signal increased in a dose-dependent manner. At 5 mM, greater than 90% of the superoxide anion signal was inhibited. No significant scavenging (0% inhibition) was observed at 0.1 mM in water, however a significant increase over background scavenging by the EtOH vehicle (5%)[1] was observed after the addition of 40% EtOH (mean = 18% inhibition). This suggested that the molecular solution of the compound demonstrated increased scavenging over the aggregated solution, and that the diphosphate derivate may have increased scavenging potential in comparison to the disuccinate derivative. Again, the millimolar concentration scavenging by the novel derivative was accomplished in water alone, without the addition of organic co-solvent (e.g., acetone, EtOH),[29] heat, detergents, or other additives.

solution of this derivative. As the concentration of the derivative increased, inhibition of the superoxide anion signal increased in a dose-dependent manner. At 5 mM, slightly more than 90% of the superoxide anion signal was inhibited (versus 75% for the disuccinate lutein sodium salt). As for the disuccinate lutein sodium salt, no apparent scavenging (0% inhibition) was observed at 0.1 mM in water. However, a significant increase over background scavenging by the EtOH vehicle (5%) was observed after the addition of 40% EtOH, resulting in a mean 18% inhibition of superoxide anion signal. This suggested that disaggregation of the compound leads to an increase in scavenging ability by this novel derivative, pointing to a slightly increased scavenging ability of molecular solutions of the more water-dispersible diphosphate derivative relative to the disuccinate derivative.

In the current study, facile preparation of the sodium salts of (3*R*,3′*R*,6′*R*)-lutein disuccinate and diphosphate is described. These asymmetric C40 carotenoid derivatives exhibited aqueous dispersibility of 2.85 and 29.27 mg/mL, respectively. Increased dispersibility of the phosphate diesters of lutein versus the succinate diesters is believed to be primarily a function of the increased potential for hydrogen bonding by ionized phosphate groups; in aqueous solvent, the more polar phosphate moiety will impart greater water dispersibility to the parent carotenoid scaffold than a disuccinate functionality. Electronic paramagnetic resonance

spectroscopy of direct aqueous superoxide scavenging by these derivatives demonstrated nearly identical dose-dependent scavenging profiles, with slightly increased scavenging noted for the diphosphate derivative. In each case, scavenging in the millimolar range was observed. These results suggest that as parenteral soft drugs with aqueous radical scavenging activity, both compounds are potentially useful in those clinical applications in which rapid and/or intravenous delivery is desired for the desired therapeutic effect(s). In particular, parenteral administration of lutein esters should overcome the problems with dose-proportionality and carotenoid non-response observed with oral administration of lutein.[31]

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20. *Chemical syntheses (general).* Natural source lutein (90%) was obtained from ChemPacific, Inc. (Baltimore, MD) as a red-orange solid and was purified by dissolving in a minimal volume of CH_2Cl_2, passed through a 0.45 μm filter, and concentrated in vacuo. This process was repeated three times. All other reagents and solvents used were purchased from Acros (New Jersey, USA) and were used without further purification. All reactions were performed under N_2 atmosphere. All flash chromatographic purifications were performed on Natland International Corporation 230–400 mesh silica gel using the indicated solvents. LC/MS (APCI) was recorded on an Agilent 1100 LC/MSD VL system; column: Zorbax Eclipse XDB-C18 Rapid Resolution (4.6 × 75 mm, 3.5 μm); temperature: 25 °C; starting pressure: 105 bar; flow rate: 1.0 mL/ min; mobile phase (%A = 0.025% trifluoroacetic acid in H_2O, %B = 0.025% trifluoroacetic acid in acetonitrile). Gradient program: 70% A/30% B (start), step gradient to 50% B over 5 min, step gradient to 98% B over 8.30 min, hold at 98% B over 25.20 min, step gradient to 30% B over 25.40 min; PDA Detector: 470 nm. The presence of trifluoroacetic acid in the LC eluents acts to protonate lutein disuccinate and diphosphate salts to give the free diacid forms, yielding $M^+ = 768$ for the disuccinate salt and $M^+ = 728$ for the diphosphate salt. LRMS: + mode; APCI: atmospheric pressure chemical ionization, ion collection using quadrapole.
*Lutein (β,ε-carotene-3,3′-diol) (**1**).* LC/MS (APCI): 9.95 min (2.78%), λ_{max} 423 nm (70%), 446 nm (100%), 474 nm (80%); 10.58 min (3.03%), λ_{max} 423 nm (73%), 446 nm (100%), 474 nm (82%); 11.10 min (4.17%), λ_{max} 423 nm (68%), 447 nm (100%), 474 nm (79%); 12.41 min (90.02%), λ_{max} 423 nm (73%), 447 nm (100%), 474 nm (83%); *m/z* 568 M^+ (69%), 551 $[M-H_2O+H]^+$ (100%), 533 (8%).
*β,ε-Carotenyl 3,3′-disuccinate (**2**).* To a solution of lutein (**1**) (0.50 g, 0.879 mmol) in CH_2Cl_2 (8 mL) were added *N*,*N*-diisopropylethylamine (3.1 mL, 17.58 mmol) and succinic anhydride (0.88 g, 8.79 mmol). The solution was stirred at rt overnight and then diluted with CH_2Cl_2 and quenched with cold 5% aqueous HCl. The aqueous layer was extracted two times with CH_2Cl_2 and the combined organic layer was washed three times with 5% aqueous HCl, dried over Na_2SO_4, and concentrated to yield disuccinate **2** (0.433 g, 64%) as a red-orange solid; LC/MS (APCI):10.37 min (8.42%), λ_{max} 423 nm (74%), 446 nm (100%), 474 nm (83%); *m/z* 769 $[M+H]^+$ (8%), 668 $[M-C_4O_3H_4]^+$ (7%), 650 (100%), 532 (22%); 11.78 min (90.40%), λ_{max} 269 nm (18%), 423 nm (68%), 446 nm (100%), 474 nm (80%); *m/z* 769 $[M+H]^+$ (7%), 668 $[M-C_4O_3H_4]^+$ (9%), 650 (100%), 532 (23%).
*β,ε-Carotenyl 3,3′-disuccinate sodium salt (**3**).* To a solution of disuccinate **2** (0.32 g, 0.416 mmol) in CH_2Cl_2 (6 mL) at 0 °C was added dropwise sodium methoxide (25% wt in methanol; 0.20 mL, 0.874 mmol). The solution was diluted with water, and the clear, red-orange aqueous solution was lyophilized to yield **3** (0.278 g, 91%) as a red-orange, hygroscopic solid; LC/MS (APCI): 11.75 min (91.32%), λ_{max} 269 nm (16%), 423 nm (70%), 446 nm

(100%), 474 nm (82%); *m/z* 769 $[M+H]^+$ (7%), 668 $[M-C_4O_3H_4]^+$ (9%), 650 (100%), 532 (23%).

*Tribenzyl phosphite (**4**)*. To a well-stirred solution of phosphorus trichloride (1.7 mL, 19.4 mmol) in Et_2O (430 mL) at 0 °C was added dropwise a solution of triethylamine (8.4 mL, 60.3 mmol) in Et_2O (20 mL), followed by a solution of benzyl alcohol (8.1 mL, 77.8 mmol) in Et_2O (20 mL). The mixture was stirred at 0 °C for 30 min and then at rt overnight. The mixture was filtered and the filtrate concentrated to give a colorless oil. Silica chromatography (hexanes/Et_2O/triethylamine, 4/1/1%) of the crude product yielded **4** (5.68 g, 83%) as a clear, colorless oil that was stored under N_2 at −20 °C; ^1H NMR: δ 7.38 (15 H, m), 4.90 (6H, d).*Dibenzyl phosphoroiodidate (**5**)*. To a solution of tribenzyl phosphite (5.43 g, 15.4 mmol) in CH_2Cl_2 (8 mL) at 0 °C was added I_2 (3.76 g, 14.8 mmol). The mixture was stirred at 0 °C for 10 min or until the solution became clear and colorless. The solution was then stirred at rt for 10 min and used directly in the next step.

*(Monobenzyl-phosphoryloxy)-(phosphoryloxy)-β,ε-carotene (**6**)*. To a solution of lutein (**1**) (0.842 g, 1.48 mmol) in CH_2Cl_2 (8 mL) were added pyridine (4.8 mL, 59.2 mmol). The solution was stirred at 0 °C for 5 min and then freshly prepared **5** (14.8 mmol) in CH_2Cl_2 (8 mL) was added dropwise to the mixture at 0 °C. The solution was stirred at 0 °C for 1 h and then diluted with CH_2Cl_2 and quenched with brine. The aqueous layer was extracted twice with CH_2Cl_2 and the combined organic layer was washed once with $NaSSO_4$, once with brine, then dried over Na_2SO_4 and concentrated. Pyridine was removed by azeotropic distillation using toluene to yield mono benzyl-protected diphosphate **6**, used in the next step without further purification; LC/MS (APCI): 9.67 min (13.31%), λ_{max} 268 nm (26%), 423 nm (74%), 446 nm (100%), 476 nm (84%); *m/z* 850 (5%), 825 (4%), 810 (100%), 532 (96%); 10.02 min (86.69%), λ_{max} 268 nm (26%), 423 nm (72%), 446 nm (100%), 476 nm (89%); *m/z* 850 (5%), 825 (4%), 810 (100%), 532 (92%).

*3,3′-Diphosphoryloxy-β,ε-carotene (**7**)*. To a solution of **6** (1.48 mmol) in CH_2Cl_2 (10 mL) at 0 °C were added pyridine (1.2 mL, 14.8 mmol) and then bromotrimethylsilane (0.97 mL, 7.40 mmol). The solution was stirred at 0 °C for 30 min, quenched with triethylamine, diluted with CH_2Cl_2, and then concentrated to yield crude diphosphate **7** as a red-orange oil, used in the next step without further purification; LC/MS (APCI): 8.90 min (54.88%), λ_{max} 268 nm (20%), 423 nm (70%), 446 nm (100%), 476 nm (90%); *m/z* 693 (5%), 639 (48%), 555 (42%), 538 (100%); 9.18 min (43.33%), λ_{max} 423 nm (78%), 446 nm (100%), 476 nm (91%); *m/z* 693 (7%), 639 (45%), 555 (38%), 538 (100%).

*3,3′-Diphosphoryloxy-β,ε-carotene sodium salt (**8**)*. To a solution of crude **7** (1.48 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added dropwise sodium methoxide (25% wt in methanol; 6.77 mL, 29.6 mmol). The solution was stirred at rt overnight and then diethyl ether was added to the salt. The suspension was centrifuged and the supernatant discarded. Water was added to the salt and the suspension was centrifuged and the supernatant discarded. The salt was redissolved in methanol and diluted with water. Lyophilization of the clear, red-orange aqueous solution yielded **8** (0.38 g, 35% over three steps) as a red-orange, hygroscopic solid; LC/MS (APCI): 8.54 min (25.86%), λ_{max} 268 nm (25%), 423 nm (74%), 446 nm (100%), 474 nm (68%); 8.85 min (31.13%), λ_{max} 268 nm (20%), 423 nm (66%), 446 nm (100%), 474 nm (80%), *m/z* 912 (50%), 780 (18%), 692 (7%), 630 (100%), 550 (45%);9.15 min (30.62%), λ_{max} 268 nm (23%), 423 nm (75%), 446 nm (100%), 474 nm (86%), *m/z* 912 (41%), 780 (15%), 692 (5%), 630 (100%), 550 (43%); 9.45 min (12.40%), λ_{max} 268 nm (21%), 335 nm (16%), 423 nm (76%), 446 nm (100%), 474 nm (80%).

Determination of aqueous dispersibility. 30.13 mg of **3** was added to 1 mL USP-purified water. The sample was rotated for 2 h and then centrifuged for 5 min. After centrifuging, solid was visible in the bottom of the tube. A 125 μL aliquot of the solution was then diluted to 25 mL. The sample was analyzed by UV/Vis spectroscopy at 436 nm, and the absorbance was compared to a standard curve compiled from four standards of known concentration. The concentration of the original supernatant was calculated to be 2.85 mg/mL and the absorptivity was 36.94 AU mL/cm mg. Slight error may have been introduced by the small size of the original aliquot. Next, 30.80 mg of **8** was added to 1 mL USP-purified water. The sample was rotated for 2 h and then centrifuged for 5 min. After centrifuging, solid was visible in the bottom of the tube. A 125 μL aliquot of the solution was then diluted to 25 mL. The sample was analyzed by UV/Vis spectroscopy at 411 nm, and the absorbance was compared to a standard curve compiled from four standards of known concentration. The concentration of the original supernatant was calculated to be 29.27 mg/mL and the absorptivity was 2.90 AU mL/cm mg. Slight error may have been introduced by the small size of the original aliquot.

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.[1,21] Briefly, each 10 mL of whole blood was mixed with 0.8 mL of 0.1 M EDTA and 25 mL saline. The diluted blood was then layered over 9 mL 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 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 $MgCl_2$).

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.[1,22] The microwave frequency was measured with a Model 575 microwave counter (EIP Microwave, Inc., San Jose, CA). To measure superoxide anion (O_2^-) generation from phorbol-ester (PMA)-stimulated PMNs, EPR spin-trapping studies were performed using the spin-trap DEPMPO (Oxis, Portland, OR) at 10 mM. 1×10^6 PMNs were stimulated with PMA (1 ng/mL) and loaded into capillary tubes for EPR measurements. To determine the radical scavenging ability of **3** and **8** in aqueous and ethanolic formulations, PMNs were 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.[23,24]
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