Reaction of 3-Dehydroshikimic Acid with Molecular Oxygen and Hydrogen Peroxide: Products, Mechanism, and Associated Antioxidant Activity

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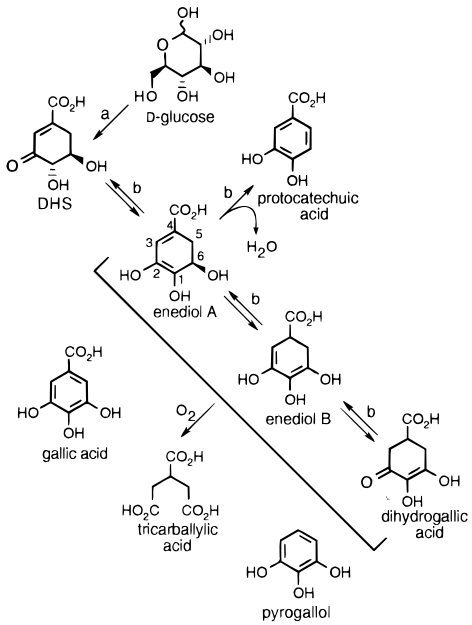
Abstract: In the presence of molecular oxygen or hydrogen peroxide, inorganic phosphate catalyzes the conversion of 3-dehydroshikimic acid (DHS) into gallic acid. Other products formed in the reaction of DHS with oxygen include protocatechuic acid, tricarballylic acid, and pyrogallol. With hydrogen peroxide as oxidant, pyrogallol formation is not observed, and smaller amounts of tricarballylic acid are produced. Evidence favoring a mechanism involving phosphate-catalyzed tautomerization of DHS to a reactive enediol intermediate follows from the successful isolation of dihydrogallic acid when oxidant is excluded from phosphate-buffered solutions of DHS. The reductone-like solution chemistry of DHS and the radical quenching reactivity of the phenolics formed from DHS prompted an appraisal of DHS antioxidant activity. Based on two different analyses, DHS was discovered to possess significant antioxidant activity relative to α-tocopherol, gallic acid, propyl gallate, and *tert*-butylhydroquinone.

3-Dehydroshikimic acid (DHS) is a hydroaromatic metabolite situated midway through the common pathway of aromatic amino acid biosynthesis.[1] During ^{1}H NMR analysis of the culture supernatants of DHS-synthesizing[2] *E. coli* AB2834/pKD136, a singlet at δ 7.1 was routinely observed. Subsequent characterization of this byproduct revealed that the resonance was associated with the aromatic protons of gallic acid. To determine whether gallic acid formation was due to enzymatic or chemical catalysis, DHS was incubated at 37 °C in minimal salts medium[3] in the absence of *E. coli* AB2834/pKD136. Gallic acid formation was still observed. Ultimately, inorganic phosphate was identified as the only solution component required for conversion of DHS into gallic acid in aqueous solutions exposed to O_2 or containing H_2O_2.

During investigation of the chemistry responsible for gallic acid formation, dihydrogallic acid (Scheme 1) was isolated from anaerobic, phosphate-buffered solutions of DHS. Formation of dihydrogallic acid suggests that observed DHS reactivity can be attributed to intermediacy of reactive enediol tautomers (Scheme 1). This reductone-like[4] chemistry along with the phenolics formed from dehydration and oxidation of DHS and its enediol tautomers led to an evaluation of DHS antioxidant activity. Use of a sensitive fluorescence assay to detect oxidation of a liposome and a separate assay based on quantitation of peroxide and hydroperoxide formation in lard revealed that DHS is an antioxidant comparable in its activity to a number of commercially employed antioxidants.

Scheme 1



Results

Product Analysis and Mechanism. Air at 1 atm reacted with aqueous solutions containing DHS and 1 M $Na_{1.5}H_{1.5}PO_4$ at pH 6.6. After 50 h at 40 °C, 10% of the DHS remained and a product mixture was formed consisting of gallic acid (14%), protocatechuic acid (12%), tricarballylic acid (14%), and pyrogallol (3%) from which gallic acid was purified in 13% isolated yield. Reaction of DHS with O_2 was a general base-catalyzed process (Figure 1A) first order in both DHS and inorganic phosphate with a 1.4×10^{-5} M^{-1} s^{-1} overall second order rate constant. DHS reacted with H_2O_2 under nearly identical conditions to those employed for reaction of DHS with O_2. However, oxidation of DHS with stoichiometric amounts of

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(1) For entry points into this extensively reviewed area, see: (a) Dewick, P. M. *Nat. Prod. Rep.* **1995**, *12*, 100. (b) Haslam, E. *Shikimic Acid Metabolism and Metabolites*; Wiley: New York, 1993.
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(3) M9 salts: Na_2HPO_4 (42 mM), KH_2PO_4 (22 mM), NaCl (8.5 mM), NH_4Cl (19 mM).
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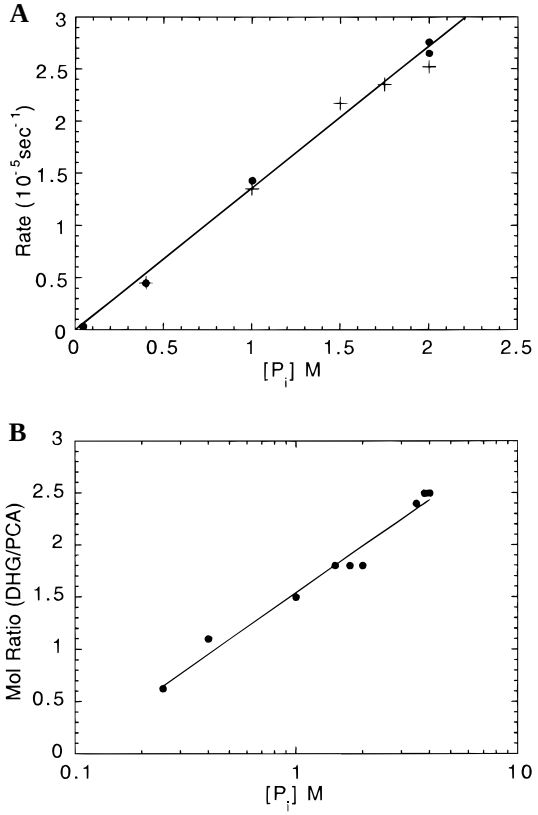


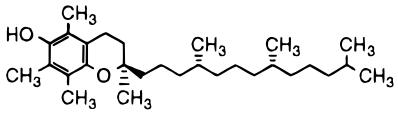
Figure 1. The effect of inorganic phosphate concentration on DHS reactivity. (**A**) Rate dependence of the aerobic (●) and anaerobic (+) reaction of aqueous DHS at 37 °C in the presence of varying concentrations of $Na_{1.5}H_{1.5}PO_4$. (**B**) The impact of varying concentrations of $Na_{1.5}H_{1.5}PO_4$ on the mol ratio of dihydrogallic acid (DHG)/protocatechuic acid (PCA) in the absence of O_2.

H_2O_2 afforded product mixtures that lacked pyrogallol and gave higher yields of gallic acid (22%) relative to protocatechuic acid (10%) and tricarballylic acid (2%). The only literature precedent for derivation of gallic acid from DHS employed Fehling solution for oxidation of DHS.[5]

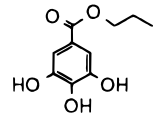
A plausible mechanism (Scheme 1) for DHS oxidation involves initial tautomerization of DHS to enediol A that is catalyzed by inorganic phosphate. Enediol A could then partition between elimination of water to form the protocatechuic acid which is observed in all DHS oxidations and reaction with either O_2 or H_2O_2 to yield gallic acid, tricarballylic acid, and pyrogallol. Formation of oxidation products need not be restricted to the reaction of a single enediol given the possibility that enediol A may be in equilibrium with enediol B and dihydrogallic acid. Although more than one mechanism can account for conversion of enediol A into enediol B (Scheme 1), a sigmatropic 1,5-hydride shift is an intriguing possibility. Subsequent tautomerization of enediol B would afford dihydrogallic acid.

Evidence for enediol intermediacy followed from isolation of oxygen-sensitive dihydrogallic acid (Scheme 1) along with protocatechuic acid in respective yields of 54% and 36% from an anaerobic, K_2HPO_4 (1 M) solution of DHS stirred for two days at rt. These reaction conditions differ markedly from the high pressure, Raney nickel hydrogenation of sodium gallate previously used to synthesize dihydrogallic acid.[6] Based on the observation (Figure 1B) that the dihydrogallic acid/protocatechuic acid product ratio was displaced in favor of dihydrogallic acid by increasing the concentration of inorganic phosphate, DHS was reacted with 4 M $Na_{1.5}H_{1.5}PO_4$. Anaerobic acidification and EtOAc extraction to remove the protocatechuic acid was followed by addition of NaOH and dilution to afford a $Na_{1.5}H_{1.5}PO_4$ (1 M) solution. Reaction of this dihydrogallic acid-containing solution with air at 37 °C for 12 h produced gallic acid (3%), pyrogallol (6%), and tricarballylic acid (35%).

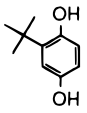
The rate dependence of DHS loss on the concentration of inorganic phosphate (Figure 1A) is nearly the same in the presence and absence of O_2. Although this indicates that phosphate-catalyzed oxidation of DHS and conversion of DHS to dihydrogallic acid share the same rate-determining step, intermediacy of dihydrogallic acid during DHS oxidation is a different matter. The product ratios determined for dihydrogallic acid oxidation and DHS oxidation differ markedly. In addition, the dihydrogallic acid/protocatechuic acid product ratio displayed a pronounced dependence on inorganic phosphate concentration (Figure 1B) in contrast to the gallic acid/protocatechuic acid product ratio that was nearly unaffected by inorganic phosphate concentration during oxidation of DHS. Therefore, while isolation of dihydrogallic acid establishes that phosphate-catalyzed isomerization of DHS to enediol tautomers is occurring (Scheme 1), the contribution of dihydrogallic acid relative to enediol A and enediol B to the products formed from DHS reaction with O_2 and H_2O_2 is uncertain.



α-tocopherol



propyl gallate



TBHQ

Appraisal of Antioxidant Activity. A convenient, rapid and sensitive model system consisting of liposomes impregnated with a fluorophore was used as one method for appraising DHS antioxidant activity. This method utilizes an extrusion technique[7] to produce large, unilamellar lipid vesicles (liposomes) formed from 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine containing the fluorophore, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid.[8] Upon initiation of peroxidation, the fluorescence decreases as peroxidation products react with the fluorophore localized within the artificial membrane. Therefore, the rate of the fluorescence decay correlates with the rate of peroxidation of the membrane lipids. The efficacy of test compounds as antioxidants is measured as the degree of inhibition of fluorescence decay. Using this technique (Figure 2), DHS displayed more potent antioxidative activity relative to α-tocopherol and weaker antioxidative activity relative to propyl gallate, gallic acid, and *tert*-butylhydroquinone (TBHQ).

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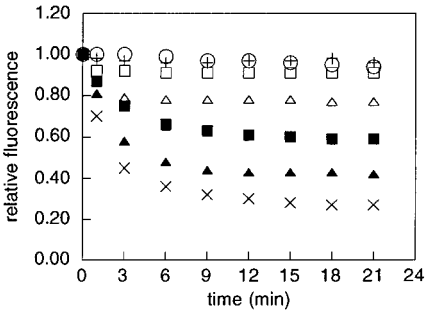

Figure 2. Oxidation of a 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine liposome in 50 mM phosphate buffer at pH 7.0 at 23 °C in the absence of added Fe^{2+} (○); in the presence of 20 μM Fe^{2+} (×); in the presence of 20 μM Fe^{2+} and 25 μM of either α-tocopherol (▲), DHS (■), propyl gallate (△), gallic acid (□), or TBHQ (+). Relative fluorescence (F_t/F_o) is the ratio of the fluorescence after a given time of oxidation (F_t) over the fluorescence (F_o) at $t = 0$ min.

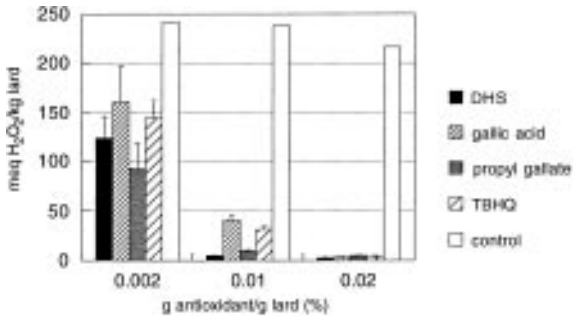

Figure 3. Peroxide values determined for lard after exposure in darkness to atmospheric oxygen at 60 °C for 28 days in the absence (control) of antioxidants and in the presence of varying concentrations of DHS, gallic acid, propyl gallate, and TBHQ.

DHS was also tested for its ability to inhibit the formation of peroxides and hydroperoxides in lard which are the initial products of lipid oxidation (Figure 3). Lard samples, with or without antioxidants, were kept at 60 °C in darkness for 28 days.[9] Peroxide values in units of milliequivalents per kg of lard were determined every 7 days over a 28 day period by standard titration methods.[10] At 0.02 wt%, DHS provided virtually complete protection for 28 days against oxidative decomposition of the lard (Figure 3). At all concentrations examined, the antioxidant activity of DHS was equal to or superior to the antioxidant activities of propyl gallate, gallic acid, and TBHQ. Even at concentrations as low as 0.002 wt%, DHS provided significant protection against oxidative decomposition. Peroxide values determined at 28 days for lard samples containing α-tocopherol at 0.02 wt% were almost indistinguishable from control experiments where no antioxidant was added to the lard.

Discussion

The products formed during DHS oxidation have intriguing ramifications relative to the genesis of gallic acid in nature. Gallic acid (Scheme 1) biosynthesis has been proposed to proceed via enzymes that catalyze the oxidation of 3-dehydroshikimic acid (DHS, Scheme 1), hydroxylate protocatechuic acid (Scheme 1), or degrade phenylpropanoids.[11] Curiously, not a single enzyme proposed for gallic acid biosynthesis has yet been isolated or even detected in biological extracts by enzyme assay. The facile reactivity of DHS with O_2 and H_2O_2 suggests that any report[12] of gallic acid biosynthesis by an intact organism needs to be viewed with caution. In lieu of assayable enzyme activity in cell lysate, observed gallic acid formation may not result from formal biosynthesis of the molecule but rather may reflect nonenzymatic oxidation of biosynthesized DHS. Plants, fungi, and other organisms may actually exploit DHS as an antioxidant whereby detected concentrations of gallic acid are the signature of DHS removal of O_2 or H_2O_2 from subcellular environments. Generation of tricarballylic acid during oxidation of DHS suggests that nonenzymatic processes might also contribute to the formation of this widely distributed plant metabolite.

Beyond its biosynthetic relationship to gallic acid, the antioxidant activity observed for DHS is particularly compelling. Use of higher temperatures to accelerate antioxidant measurements has been criticized for not providing an accurate parallel of the conditions typically encountered during storage of food and plant oils.[13] The 23 °C temperatures used during the fluorescent assay of synthetic liposome oxidation are thus advantageous. Nonetheless, the determination of peroxide values for lard maintained at 60 °C is a method for appraising antioxidant activity that is widely used in the food industry. The effectiveness of an antioxidant will vary as a function of the concentration of radical initiators, endogenous antioxidants, and the lipid composition of a given food matrix. Therefore, more than one assay needs to be employed in evaluating antioxidant activity. Both assays used in this report indicated that DHS possessed significant antioxidant activity.

Commercial antioxidants employed in foods, materials, medicinals, and cosmetics are designed to interfere with either individual or multiple steps in oxidative decomposition processes.[14] One of the larger antioxidant classes quenches radical intermediates formed during initiation and propagation of radical chain reactions involving organic compounds and reactive oxygen species. Radical scavengers include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), TBHQ, Irganox 1010, ethoxyquin, propyl gallate and the tocopherols. Ascorbic acid is a widely used O_2 scavenger. Thiodipropionic acid functions by degrading peroxides and hydroperoxides by nonradical routes. Ethylenediaminetetraacetic acid (EDTA) and citric acid deactivate metals towards redox reactions with peroxides by complexation and stabilization of the oxidized states of metal ions.

DHS is unique in that all of the reactivities associated with antioxidant activity are present either in DHS, DHS enediol tautomers, or the products formed from DHS (Scheme 1). How much of the observed antioxidant activity can be attributed to DHS and its enediol tautomers relative to the phenolic products formed from DHS is unclear. As reductones, DHS enediols should be able to quench radicals by donation of a hydrogen atom or electron and be capable of metal complexation.[4]

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Clearly, DHS and its enediol tautomers are quite reactive with O_2 and H_2O_2. As for the phenolic products formed from DHS, gallic acid is a phenolic radical scavenger and is known to form complexes with metals.[14c] Protocatechuic acid is also a phenolic radical scavenger precedented to possess antioxidant activity.[15]

Ultimately, the ease of synthesizing DHS may prove to be as important as its antioxidant activity. Propyl gallate is derived from esterification of gallic acid which is isolated from gall nuts and tara powder whose supply has historically been unreliable.[16] α-Tocopherol is obtained by an expensive isolation process from deodorized soybean oil[17] which is in short supply.[18] By contrast, suitably engineered microbes can synthesize DHS in a single step from D-glucose.[2] This starting material, in turn, is derived from inexpensive plant starch whose supplies are reliable and abundant. Depending on its toxicological profile in mammals and the particular antioxidant application, DHS could supplant expensive antioxidants such as propyl gallate[19a] and TBHQ.[19b] The natural biogenesis of DHS may also allow it to be used in applications previously restricted to natural antioxidants such as α-tocopherol.

Experimental Section

General. Isomerization, dehydration and oxidation reactions of 1−2% aqueous solutions of DHS were monitored by ^{1}H NMR using a Varian VXR-300 FT-NMR Spectrometer (300 MHz) and solvent suppression techniques.[20] Presaturation used the program "Presat" supplied with the instrumentation. For a lock signal, solutions were diluted by addition of a weighed amount of D_2O (5−12% of initial sample on a v/wt basis) containing 0.5−1.0 wt% of maleic acid, pivalic acid or disodium fumarate as internal integration and shift references. The lock signal for deuterium was quite broad, usually 3−4 Hz, as indicated by observing the dominant proton exchange peak prior to presaturation. Standard shimming and auto shimming procedures were inadequate under these conditions. For each sample, shimming was optimized to produce a symmetrical exchange peak with minimized peak width by repetitive shimming and observation of the spectrum obtained after one transient. Presat spectra were obtained from sixteen transients at relatively high saturation power and gain. This technique typically produced spectra where normally sharp lines were resolved to widths of less than 1 Hz. In these presaturated spectra, reliable integrations were obtained excluding the region from δ 3.0−5.8. Inorganic phosphate buffer was prepared by combining equimolar amounts of dibasic (HPO_4^{2-}) and monobasic ($H_2PO_4^{1-}$) sodium or potassium phosphate. Octadecyl-functionalized silica gel (Aldrich) was activated by initial elution with CH_3OH followed by elution with CH_3OH/H_2O (1:1, v/v).

Reaction of DHS with O_2. DHS (1 g, 5.81 mmol) was dissolved in 30 mL of 1.0 M $Na_{1.5}H_{1.5}PO_4$ and the solution adjusted to pH 6.7. The reaction was then stirred for 50 h exposed to the atmosphere at 40 °C. At the end of the reaction, 10% of the DHS remained unreacted. Products consisted of gallic acid (14%), protocatechuic acid (12%), tricarballylic acid (14%), and pyrogallol (3%). Extraction (2×) with EtOAc to remove pyrogallol was followed by acidification of the solution to pH 3.6 and extraction (2×) with EtOAc. Drying of the organic layer and concentration gave a solid (0.26 g). Chromatography using octadecyl-functionalized silica gel (86 mL) eluted with CH_3OH/H_2O (1:9, v/v, adjusted to pH 2.7) afforded pure gallic acid (0.13 g, 13%).

Reaction of DHS with Hydrogen Peroxide. DHS (1 g, 5.81 mmol) and H_2O_2 (2 wt%, 10 mL) were dissolved in 20 mL of 1.0 M $Na_{1.5}H_{1.5}PO_4$ and the solution adjusted to pH 6.7. After 36 h reaction at 40 °C, 5% of the DHS starting material remained unreacted. The crude reaction product consisted of gallic acid (22%), protocatechuic acid (10%) and tricarballylic acid (2%). After acidification to pH 3, the reaction solution was extracted (3×) with EtOAc. Drying of the organic layer and concentration gave a solid (0.33 g). Chromatography using octadecyl-functionalized silica gel (86 mL) eluted with CH_3OH/H_2O (3:7, v/v, adjusted to pH 2.8) afforded pure gallic acid (0.18 g, 18%).

Isolation of Dihydrogallic Acid. A warm filtered solution of DHS (0.18 g, 1.06 mmol) in 4 M $Na_{l.5}H_{1.5}PO_4$ (0.91 mL) was sparged with N_2 for about 3 min, capped, and then heated 44 h at 40−42 °C. After adding 0.11 g of D_2O containing 1.0 wt% maleic acid and 0.59 wt% pivalic acid, NMR analysis indicated a 69% yield of dihydrogallic acid and 31% yield of protocatechuic acid. Crystals of dihydrogallic acid or, more likely, its sodium or disodium salt, separated during the NMR acquisition. Unlike crystals of Na_2HPO_4 which also can separate from this saturated solution, the crystals of dihydrogallic acid did not readily dissolve on gentle warming but required brief heating in boiling water to redissolve. The resulting solution was acidified by addition of 4 M H_3PO_4 (0.46 mL). Extraction with EtOAc removed protocatechuic acid. On standing, the resulting aqueous phase deposited crystals of dihydrogallic acid which were collected and washed once with cold water. The hydrate of dihydrogallic acid melted 181−2 °C (dec). After vacuum drying these crystals melted 191.5−192.5 °C (lit.[6a] mp 192−3 °C, dec); ^{1}H NMR (1.2M P_i, TSP, $\delta = 0$ ppm) 2.70 (dd, $J = 17$, 8 Hz, 2H), 2.65 (dd, $J = 17$, 6 Hz, 2H), 2.94 (m, lH); ^{13}C NMR (H_2O/D_2O, 1,4-dioxane, $\delta = 70.0$ ppm) 180.5, 179.7, 133.6, 40.8, 36.8; IR (mineral oil mull, cm^{-1}) 3610 (w), 3425 (m), 1706 (m), 1665 (m), 1551 (s), 1319 (s), 1238 (s), 1210 (m), 1158 (s), 1020 (m) 962 (m); HRMS (EI) calcd. for $C_7H_8O_5$ (M^+) 172.0372, found 172.0368.

Oxygenation of Dihydrogallic Acid in Phosphate Buffer. A 5−6% solution of DHS in 0.75 mL of 4 M $Na_{1.5}H_{1.5}PO_4$ was isomerized to dihydrogallic acid, acidified and extracted (3×) to remove protocatechuic acid as described above. Two final extractions with cyclohexane removed EtOAc. The aqueous solution was neutralized with dilute NaOH to make l M $Na_{1.3}H_{1.7}PO_4$, stoppered under air in a 10 mL flask, and then incubated at 37 °C in a rotary shaker oven. After 12 h, the solution was concentrated under vacuum, transferred to NMR tubes, diluted with D_2O and reference, and sparged with N_2. Yields obtained from integration of these NMR spectra are 3% gallic acid, 6% pyrogallol and 35% tricarballylic acid.

Analysis of Aerobic Reactions of DHS in Aqueous Phosphate Solutions. Three aqueous solutions of DHS (0.12 g, 0.70 mmol) and 8 mL of l.0 M $Na_{l.5}H_{l.5}PO_4$ were made in 50 mL Erlenmeyer flasks. The reaction mixtures were briefly sparged with O_2, stoppered, and then incubated at 37 °C in a rotary shaker oven. Aliquots (0.70 mL) were taken at t_0 and at subsequent timed intervals from each of the three parallel reactions. After weighing, 10 vol/wt% D_2O containing 1 wt% of maleic acid was added to each aliquot followed by thorough mixing and sparging with N_2. ^{1}H NMR with solvent suppression were acquired for each aliquot and analyzed by integrating the aromatic signals for protocatechuic acid (3H), gallic acid (2H) and pyrogallol (3H); the vinyl signals for DHS (lH) and maleic acid (2H); and the highest field multiplet for tricarballylic acid (2H). In cases where dihydrogallic acid or other high field products were detected, the integrations of the overlapping signals of DHS and tricarballylic acid were subtracted from the integrals. Analysis for each component in each spectrum was reduced to a mol percentage of the initial DHS. Averages and standard deviations (σ_{n-1}) were based on aliquots taken at the same timed intervals from the three parallel reactions.

Analysis of Anaerobic Reactions of DHS in Aqueous Phosphate Solutions. A solution of DHS (0.011 g, 0.064 mmol) and D_2O (0.96 g containing 1 wt% maleic acid and 0.59 wt% pivalic acid) in 0.75 mL of l M K_2HPO_4 was deoxygenated directly in an NMR tube by sparging 1−3 min with N_2 gas introduced through a fine capillary. The initial NMR spectrum gave molar integration ratios of DHS (82.8%), maleic acid (10.2%) and pivalic acid (7.0%). After standing at room temperature for two days, the molar composition of this solution was protocatechuic acid (32.3%), dihydrogallic acid (49.4%), maleic acid (10.6%) and pivalic acid (7.7%). These data indicate that the yields

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of protocatechuic acid and dihydrogallic acid are 36−38% and 54−57%, respectively. Note however, the total yield of 90−95% reflects inherent error expected for NMR integrations (±5%) and also the exchange of deuterium into the products (and to a much lesser extent, the integration standards).

Analysis of Model Liposome Oxidation by Fluorescence Spectroscopy. A mixture containing 5 μmol of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine (Avanti Polar Lipids, Inc., Alabaster, AL) and 0.015 μmol of the fluorescence probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (Molecular Probes, Inc., Eugene, OR) were dried under vacuum using a rotary evaporator. The resulting lipid film was suspended in 500 μL of a solution containing NaCl (0.15 M), EDTA (0.1 mM), and MOPS (0.01 M) and subjected to ten freeze-thaw cycles using a dry ice/ethanol bath. The buffer solution was previously treated with chelating resin Chelex 100 (Sigma, St. Louis, MO) (5 g/100 mL buffer) to remove trace metal ions. The lipid-buffer suspension was then extruded twenty-nine times through a LiposoFast extruder (Avestin, Inc., Ottawa, Canada) containing a polycarbonate membrane with a pore size of 100 nm to produce unilamellar liposomes.[7c] A 20 μL aliquot of this liposome suspension was diluted to 2 mL in Chelex 100-treated buffer containing 100 mM NaCl, 50 mM sodium phosphate buffer at pH 7.0, incubated 5 min at room temperature, followed by incubation for 5 min in the thermostatted cuvette holder (23 °C) of the spectrofluorometer. Peroxidation was then initiated by the addition of 20 μL of 2 mM stock $FeCl_2$ solution to achieve a final concentration of 20 μM of Fe^{2+} in the absence or presence of test compounds. The control sample did not contain either Fe^{2+} or test compounds. Fluorescence intensity of these liposome solutions at an excitation wavelength of 384 nm were recorded every 3 min on a SLM4800 spectrofluorometer (SLM Instruments, Urbana, IL) over a period of 21 min. The decrease in relative fluorescence intensity with time indicates the rate of peroxidation.

Analysis of Lard Oxidation. Antioxidative activity of DHS and other commercially available antioxidants were tested for their ability to inhibit peroxide and hydroperoxide formation in lard. Lard samples (prime steam lard obtained from Monfort of Colorado, Inc.), with or without antioxidants, were kept at 60 °C in darkness for 28 days.[9] Aliquots (5 g) of the lard samples were collected for peroxide value measurements every seven days. A saturated KI solution (500 μL) was added to a solution containing $CHCl_3$/HOAc (3:2, v/v, 30 mL) and 5 g of lard sample. The solution was allowed to stand for 1 min and followed by the addition of 30 mL of distilled H_2O. The resulting acidic lard solution was titrated against $Na_2S_2O_3$ solution (0.1 N or 0.01 N) using 1% starch solution as indicator with continuous, vigorous shaking of the sample. The end point was determined by the disappearance of blue color from the $CHCl_3$ layer.[10]

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