A New Vitamin E (α-Tocomonoenol) from Eggs of the Pacific Salmon *Oncorhynchus keta*

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A novel α-tocomonoenol [3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-12-tridecenyl)-2H-1-benzopyran-6-ol] having an unusual methylene unsaturation at the isoprenoid-chain terminus of α-tocopherol was isolated from the lipophilic fraction of chum salmon eggs. The structure of this marine-derived tocopherol (MDT) was established by spectral analyses. The peroxyl radical-trapping activities of MDT and α-tocopherol were compared in aqueous phosphatidylcholine liposomal suspension and in methanolic solution at 37 °C. The antioxidant activity of MDT was found to be identical to that of α-tocopherol under the experimental conditions of measurement.

Oxidative stress in human pathophysiology has been implicated to be a causative factor in aging and in the etiology of degenerative diseases such as heart attack, diabetes, and cancer.[1] Intrinsic antioxidants, such as vitamins E and C, are the first defense against oxidative damage. Human cells, other than in tissues exposed to the atmosphere, are generally present to low oxygen tension ($pO_2 \sim 30$ mmHg) and are additionally protected from chronic exposure to environmental solar radiation. In our quest to find novel antioxidants with potential to outperform endogenous free-radical scavengers, we are searching leads from biodiverse tissues typically exposed to elevated levels of oxidative stress. One such potential source for novel antioxidants is the eggs of fish, as they are adapted to higher levels of oxygen ($pO_2 = 150$ mmHg at air saturation) and are chronically exposed to environmental challenge. Additionally, genetic material within the cytosol must also be protected against potential damage arising from the diffusion of H_2O_2 produced at the cellular envelope during the respiratory burst of fertilization.[2]

Salmon eggs are a popular item in the Japanese diet, and a ready source of fresh material is available from local markets. We extracted soluble components from the eggs of chum salmon (*Oncorhynchus keta*) into 2-propanol and analyzed the supernatant for constituent antioxidants using reversed-phase HPLC separation with electrochemical detection (ECD). ECD is a useful technique for the selective detection of antioxidants because such molecules can be oxidized at a low potential with high molar sensitivity. Figure 1 presents a typical ECD chromatogram showing the presence of α-tocopherol (α-Toc) and an unknown antioxidant (compound **1**) from the 2-propanol extract of salmon eggs. Additionally, compound **1** proved to be chromatographically distinct from all other known tocopherols, including tocotrienols and a known plant α-tocomonoenol **2**[3] (data not shown).

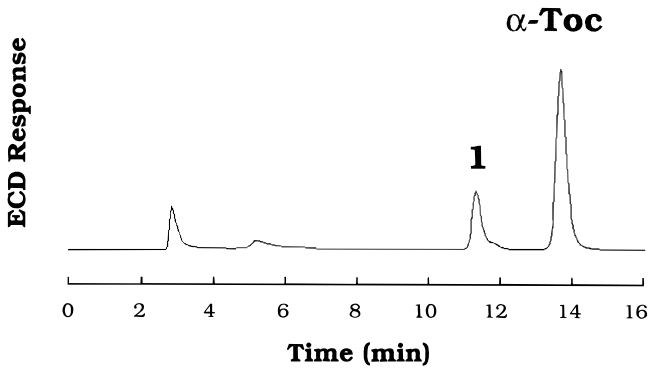
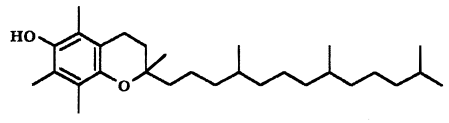
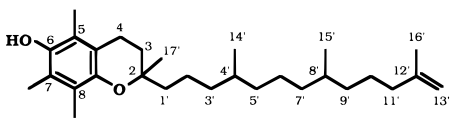


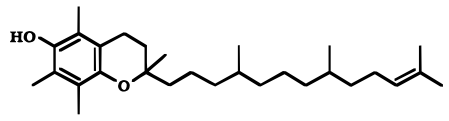
Figure 1. Reversed-phase HPLC-ECD chromatogram in the analysis of tocopherols extracted from chum salmon eggs.



α-Tocopherol (α-Toc)



1 (MDT)



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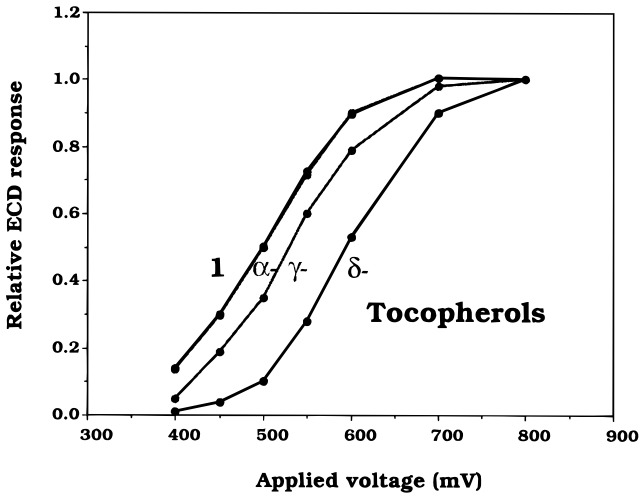

Figure 2. Hydrodynamic voltammograms of compound **1** (MDT) and α-, γ-, and δ-tocopherols prepared by HPLC-ECD measurements. The voltammograms of α-Toc and MDT (**1**) are overlapping.

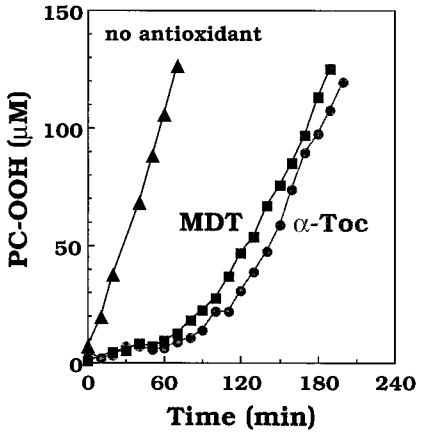

Figure 3. Formation of phosphatidylcholine hydroperoxides (PC−OOH) in the oxidation of 5.2 mM soybean phosphatidylcholine liposomal dispersion in 10 mM Tris buffer (pH 7.4) initiated with 1.0 mM AMVN and with added MDT (1.9 μM) or α-Toc (2.2 μM) under aerobic conditions at 37 °C.

We compared the electrochemical properties of both antioxidants in the salmon egg extract by comparing the ECD response of the chromatographic signals obtained on varying the applied oxidation potential at the working ECD electrode. The combined hydrodynamic voltammogram prepared from these measurements (Figure 2) revealed that the electrochemical properties of α-Toc and compound **1** are identical, offering an expectation that compound **1** and α-Toc have similar antioxidant activities. Figure 2 also shows that γ- and δ-tocopherols have greater oxidation potentials than α-Toc (and compound **1**), and this behavior is consistent with having reduced antioxidant activities.[4]

We isolated compound **1** by extracting chum eggs with hexane−2-propanol (1:1) and purifying the product by sequential chromatographic fractionation on Si gel and reversed-phase, semipreparative separation, as described in the Experimental Section. That compound **1** eluted with α-Toc on Si gel fractionation and had similar retention characteristics by reversed-phase separation suggests that both compounds have closely related structures.

GC−MS, ^{1}H NMR, and ^{13}C NMR analyses provided evidence for the structure of compound **1** as 3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-12-tridecenyl)-2H-1-benzopyran-6-ol. GC−MS analysis gave the molecular weight of compound **1** as 428, being 2 mass units less than α-Toc (M_r = 430). Fragments at *m*/*z* 205 and 165 are attributed to the benzopyranol ion $(C_{13}O_2H_{17})^+$ of α-Toc without its isoprenoid side chain and that of its characteristic tropylium ion $(C_{10}O_2H_{13})^+$,[5] respectively. These data suggest that compound **1** has the identical benzopyranol (chromanol) structure of α-Toc and differs only by having a double-bond unsaturation in the isoprenoid side chain. The ^{1}H NMR spectra of compound **1** were very similar to the spectra of α-Toc except for the appearance of the terminal methylene protons at 4.67 and 4.69 ppm in the ^{1}H NMR spectrum. The DEPT experiment also showed that the signal at 109.5 ppm in the ^{13}C NMR spectrum was attributed to a methylene carbon. Compound **1** differs from the isomeric α-tocomonoenol **2** found in palm and rice oils[3] and, given its distinctive origin, we have assigned the trivial name "marine-derived tocopherol" (MDT) to compound **1**.

The ratio of MDT to α-Toc was 1:4 in chum salmon eggs (*Oncorhynchus keta*). We also found MDT:α-Toc ratios of 1:5.5 and 1:6.5 in the eggs of sockeye salmon (*Oncorhynchus nerka*) and walleye pollack (Alaska pollack; *Theragra chalcogramma*), respectively. These coldwater species are caught from the northern Sea of Japan and the Okhotsk Sea. In contrast, only small quantities of MDT were found in the eggs of flyingfish (*Cypselurus hiraii*) and Pacific herring *(Clupea pallasii*) captured from southern regions, indicating that MDT may be functionally associated with adaption to the coldwater environment. We are currently investigating this hypothesis.

The antioxidant activity of compound **1** (MDT) was expected to be identical to that of α-Toc because the antioxidant properties of these tocopherols are characteristic of their benzopyranol (chromanol) electrophore,[4] with MDT and α-Toc having identical chromanol structures. We compared their antioxidant properties using the *in vitro* phosphatidylcholine (PC) oxidation assay[6,7] whereby oxidation of soybean PC liposomes with a lipid-soluble, radical initiator proceeds by a free-radical chain mechanism at a near constant rate (Figure 3). Addition of MDT (1.9 μM) or α-Toc (2.2 μM) significantly inhibits the control rate of oxidation, as shown in Figure 3, by scavenging peroxyl radicals at similar rates. The duration of the inhibition period is directly dependent on the numbers of peroxyl radicals trapped by the antioxidant[4] and, because we used a slightly greater concentration of α-Toc than MDT in the PC oxidation assay, α-Toc as expected showed a longer period of inhibition (Figure 3). When MDT and α-Toc were present in methanol at identical concentrations with an added peroxyl radical generator, both substrates were oxidized at identical rates (Figure 4). The latter data confirms that the rate constants for MDT and α-Toc to scavenge peroxyl radicals are equivalent in homogeneous solution under experimental conditions conducted at 37 °C.

Experimental Section

Extraction and Isolation. We homogenized 380 g of chum salmon eggs (purchased from the local market) in a domestic food blender and stirred the paste with 1000 mL of hexane−2-propanol (1:1) for 10 min at room temperature. The extraction phase was decanted, and volatile solvents were removed on a rotary evaporator at reduced pressure. Hexane (500 mL) was added to the residue and a clear reddish-orange solution was obtained after the addition of anhydrous magnesium sulfate to dry the organic phase. After the removal of hexane, the precipitate was charged to an open column (70 mm i.d.) containing a bed of chromatographic grade Si gel (600 mL volume; 75−150 μm) equilibrated to hexane. Compound **1** (MDT) and α-Toc eluted with hexane−2-propanol (25:1) were

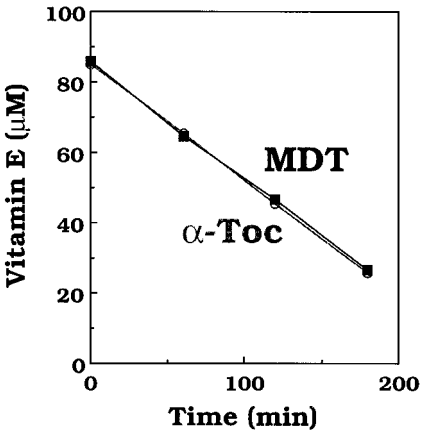

Figure 4. Competitive oxidation of MDT and α-Toc in equimolar quantities (83 μM, each) by peroxyl radicals produced by 5.5 mM AMVN in methanol under aerobic conditions at 37 °C.

collected. Organic solvents were removed from the tocopherol-containing fraction, and the tocopherols were re-extracted from the triglyceride-rich residue with 3 volumes (ca. 300 mL) of methanol. The methanol was removed and the extraction procedure was repeated three times. The remaining triglycerides were removed by passing the methanolic extract through a solid-phase extraction cartridge (C_{18}, 100 mL, Supelco, Tokyo). Finally, the component tocopherols were separated on a semipreparative ODS column (20 × 250 mm, YMC, Kyoto) with methanol at a flow rate of 8 mL/min. Elution of MDT and α-Toc was monitored by UV detection at 290 nm, and they were separately collected at retention times of 26−30 and 33−40 min, respectively. The final yield of MDT after the removal of solvent was 7.6 mg.

Analytical Measurements. MDT and α-Toc concentrations in tissue extracts were determined by HPLC separation on an analytical column (Supelcosil LC-18, 5 μm, 250 × 4.6 mm, Supelco) and measured by amperometric electrochemical detection (model Σ985, Irica, Kyoto). The ECD oxidation potential was maintained at +600 mV (vs Ag/AgCl) on a glassy carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol delivered at a flow rate of 1.0 mL/min. A typical chromatogram is presented in Figure 1.

GC−EIMS Analysis of Compound 1 (MDT). The GC−MS spectrum of compound **1** was recorded on a Hitachi (Tokyo) M-80B spectrometer. Separation was achieved on an OV-1 column (3 mm$^{\phi}$ × 1 m) coated with 1% Chromosorb W-HP at an initial column temperature at 160 °C with a linear gradient (10 °C/min) to 300 °C with He as the carrier gas (35 mL/min). Ionization voltage was fixed at 70 eV producing major ions at *m*/*z* (%) = 165 (100), 205 (11), and 428 (82).

NMR Spectra of Compound 1 (MDT). ^{1}H and ^{13}C NMR spectra were measured on a Bruker AMX-500 spectrometer ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.72 (s, 3H, 14′-H), 1.99 (br t, J = 7.2 Hz, 2H, 11′-H), 4.67 (br s, 1H, 13′-H), 4.69 (br s, 1H, 13′-H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.26, 11.76, 12.19 (q, $ArMe_3$); 19.64, 19.71 (q, 15′, 16′-C); 22.38, 23.79 (q, 14′, 17′-C); 20.75, 21.03, 24.44, 25.07, 29.69, 31.56, 36.65, 37.39, 37.47, 38.14, 39.81 (t, 3, 4, 1′, 2′, 3′, 5′, 6′, 7′, 9′, 10′, 11′-C); 32.70, 32.70 (d, 4′, 8′-C); 74.52 (s, 2-C); 109.53 (t, 13′-C); 117.35, 118.44, 120.99, 122.61, 144.52,145.55, 146.35 (s, 4a, 5, 6, 7, 8, 8a, 12′-C).

PC Oxidation Inhibition Assay. Soybean PC liposomes were prepared containing test tocopherols with the lipid-soluble, radical initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) in 10 mM Tris buffer (pH 7.4), as previously described.[6,7] The assay was conducted by oxidation of a 5.2 mM dispersion of PC liposomal membranes on initiation with 1.0 mM AMVN under aerobic conditions at 37 °C. PC hydroperoxide (PC-OOH) concentrations for rate measurements were determined by HPLC analyses[6] on a Si gel column (5 μm, 4.6 × 250 mm, Supelco) using methanol−40 mM monobasic sodium phosphate (9:1) as the mobile phase (1 mL/min) with UV detection at 234 nm.

Antioxidant Activities Measured in Homogeneous Solution. Both MDT (compound **1**) and α-Toc (83 μM each) were oxidized with 5.5 mM AMVN in methanol at 37 °C under aerobic conditions. Consumption of the two compounds was measured by HPLC-ECD as described above.

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