An unusual vitamin E constituent (α-tocomonoenol) provides enhanced antioxidant protection in marine organisms adapted to cold-water environments

Yorihiro Yamamoto*†, Akio Fujisawa*, Akihiko Hara‡, and Walter C. Dunlap§

*Department of Chemistry and Biotechnology, School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan; ‡Department of Marine Biological Sciences, Faculty of Fisheries, University of Hokkaido, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-0821, Japan; and §Australian Institute of Marine Science, PMB No. 3 Townsville MC, Queensland 4810, Australia

Edited by Bruce N. Ames, University of California at Berkeley, Oakland, CA, and approved September 17, 2001 (received for review January 16, 2001)

A new vitamin E constituent having an unusual methylene unsaturation at the isoprenoid-chain terminus of α-tocopherol (α-Toc) was isolated from chum salmon eggs and was found to have identical antioxidant activity as does α-Toc in methanol or liposomal suspension at 37°C. Here we report that this marine-derived tocopherol (MDT) is broadly distributed with α-Toc in the tissue of marine fish, and that the MDT composition of total vitamin E is greater in the flesh of cold-water salmon (12–20%) than in that of tropical fish (≤2.5%). Vitamin E analysis of cultured masu salmon maintained on a MDT-deplete diet showed substantially less MDT content than native masu salmon, suggesting a trophic origin of MDT. This contention is supported by the finding of MDT in marine plankton from the cold waters of Hokkaido. We found that MDT inhibited peroxidation of cholesterol-containing phosphatidylcholine liposomes to a greater extent than did α-Toc at 0°C. Furthermore, the ratios of the rate constants for MDT and α-Toc to scavenge peroxyl radicals increased with decreasing rates of radical flux in liposomes and fish oil at 0°C, indicating that the enhanced activity of MDT at low temperature is attributed to its greater rate of diffusion in viscous lipids. These results suggest that MDT production, or its trophic accumulation, may reduce lipid peroxidation in marine organisms functionally adapted to cold-water environments.

Vitamin E is one of the most important lipid-soluble antioxidants to occur in plants and animals, specifically for protection against lipid peroxidation in biological membranes. Four homologue pairs (α-, β-, γ-, δ-tocopherols and -tocotrienols) have been described, of which the α forms (Fig. 1) have the greatest activities (1). Matsumoto *et al.* (2) discovered α-tocomonoenol (Fig. 1) in palm oil, attributed as a biosynthetic intermediate along the reductive pathway to α-tocopherol (α-Toc) from α-tocotrienol in higher plants (3). Recently, we isolated an isomeric and chemically distinct α-tocomonoenol [3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-12-tridecenyl)-2H-1-benzopyran-6-ol] from the lipophilic fraction of chum salmon eggs (4) having an unusual methylene unsaturation at the isoprenoid-chain terminus (Fig. 1). This 10th known vitamin E constituent was given the name "marine-derived tocopherol" (MDT) to differentiate its structure in designation of its unique origin. MDT was found to have identical antioxidant activity as does α-Toc in methanol or liposomal suspension at 37°C (4).

In this report, we examine the occurrence of MDT in tissues of a variety of fish and compare the antioxidant activity of MDT with that of α-Toc at low temperature. The occurrence of greater MDT concentrations in species inhabiting cold-water environments and its enhanced antioxidant activity at low temperature suggest a selective antioxidant function of MDT in cold-water adaptation.

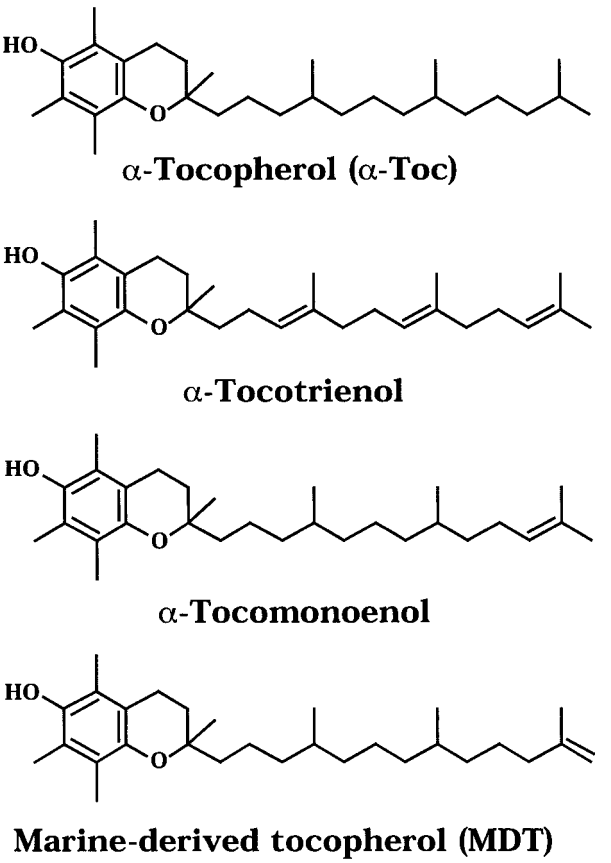

Fig. 1. Chemical structures for the α forms of vitamin E.

Materials and Methods

Materials. Fish eggs and tropical fish fillets were obtained at local markets in Tokyo and Townsville (Australia), respectively. Native chum and masu salmon were obtained from the Faculty of Fisheries, Hokkaido University. Farmed masu salmon were reared at the Nanae Fish Culture Experimental Station (Faculty of Fisheries, Hokkaido University) in outdoor concrete ponds supplied with a continuous flow of river water at ambient temperature. The fish were fed ad libitum with commercial trout food once a day. Plankton samples were collected during the cruise of T/S Oshoro Maru, Hokkaido University, at the mouth of Funka (Uchiura) Bay on the

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: α-Toc, α-tocopherol; MDT, marine-derived tocopherol; soyPC, soy phosphatidylcholine; LH, lipids; LOO·, lipid peroxyl radicals.

†To whom reprint requests should be addressed. E-mail: junkan@oxygen.t.u-tokyo.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

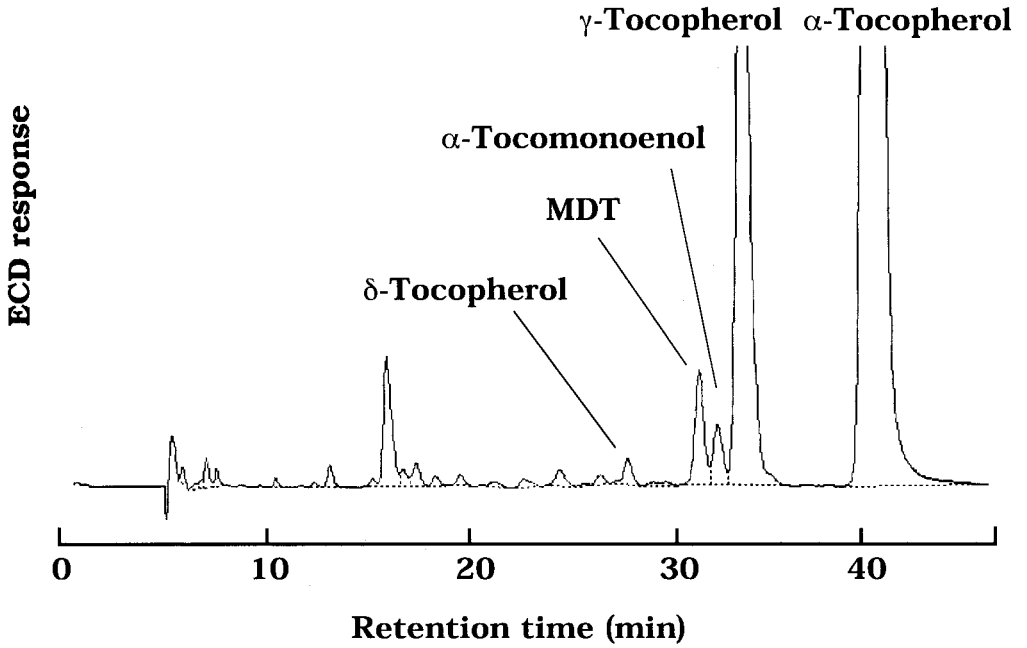

Fig. 2. RP-HPLC with electrochemical detection chromatogram for the separation of δ-tocopherol, MDT, α-tocomonoenol, γ-tocopherol, and α-Toc in a hexane extract of human plasma.

southeastern shore of Hokkaido on March 8, 1998, when the Oyashio (cold-water currents) prevailed in the upper layer. Vertical hauls from 100-m depth to the surface were made with a twin-type NORPAC (Rigosha, Tokyo) net (0.33- and 0.10-mm mesh), and samples were stored at −20°C until analyzed. Soy phosphatidylcholine (soyPC) was purified as described (5). Fish oil and cholesterol were purchased from Sigma. Fish oil was purified by silica gel column chromatography, loaded on with hexane and eluted with 5% 2-propanol in hexane. α-Toc was removed from fish oil by repetitive washing with methanol. After removal of solvents under high vacuum, residual α-Toc was determined by HPLC with electrochemical detection as described below. MDT was purified from salmon eggs as described (4). α-Tocomonoenol (from palm oil), α-tocotrienol, and other tocopherols were gifts from Eisai (Tokyo). Cholesterol was recrystallized from hot ethanol.

Vitamin E Analysis. Tocopherols in fish eggs and tissues were extracted with 5 equivalents (vol/wt) of 2-propanol with vigorous grinding. We previously described an RP-HPLC procedure for the analysis of MDT and α-Toc extracted from the eggs of chum salmon (4). An improved separation was achieved by applying two analytical columns (Supelcosil LC-18, 5 μm, 250 × 4.6 mm, Supelco) in tandem. Fig. 2 shows the separation of δ-tocopherol, MDT, α-tocomonoenol, γ-tocopherol, and α-Toc in the hexane extract of human plasma obtained from a healthy young Japanese donor. Tocopherols were measured by amperometric electrochemical detection (Model Σ985; Irica, Kyoto) with an oxidation potential at +600 mV (vs. Ag/AgCl) on a glassy carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/water (50:1 vol/vol) delivered at a flow rate of 1.0 ml/min.

Inhibition of soyPC Liposome Oxidation. We measured the effect of 5 μM MDT or α-Toc on the photooxidation of 5 mM multilamellar soyPC liposome containing 5 mM cholesterol and 0.5 μM benzophenone under aerobic conditions at 0°C. Liposomes were dispersed in 50 mM Tris buffer containing 0.15 M NaCl, pH 7.6. Oxidations were initiated by irradiation with a UV (λ_{max} = 350 nm) light source (BLE-270; Spectronic, Westbury, NY) at a dose of 1 J/m^{2}s. Formation of soyPC hydroperoxide was measured by HPLC separation with UV detection at 234 nm (6).

Cooxidation of MDT and α-Toc. Antioxidant activities of MDT and α-Toc were compared at 0°C by measuring vitamin E consumption during the aerobic oxidation of 5 mM multilamellar soyPC liposomes containing 5 μM each of MDT and α-Toc, with and without 5 mM cholesterol and 5 μM (or less) benzophenone. MDT and α-Toc concentrations were determined as described previously. Liposomes were dispersed in Tris buffer, and oxidations were initiated with UV light. Antioxidant activities of MDT and α-Toc were similarly determined in purified neat fish oil, but the oxidation proceeded with substantially less benzophenone (≤0.65 μM). This difference is attributed to fish oil having a greater polyunsaturated fatty acid concentration than soyPC liposomal dispersion, thereby enhancing the rate of peroxyl radical formation. Irradiation of 10 μM each of MDT and α-Toc in methanol without benzophenone did not affect the oxidation of either tocopherol (data not shown), thus eliminating the possibility for direct photochemical oxidation of MDT and α-Toc.

Results and Discussion

Vitamin E in Fish Tissues. The vitamin E concentration and the MDT composition [%MDT = 100 × MDT/(MDT + α-Toc)] in eggs and tissues from a selection of fish are given in Table 1. The eggs of chum salmon, sockeye salmon, and walleye pollack showed a higher %MDT composition (19.9, 14.2, and 13.2%, respectively) than eggs of warmer water fish. Similarly, native chum and masu salmon (sakura-masu) had greater %MDT values in their muscle (20.2 and 12.3%, respectively) than did tropical fish (≤2.5%) such as coral trout, mangrove jack, redthroat sweetlip, and blue-tailed cod. This comparison provides that greater concentrations of MDT generally occur in species of cold-water fish.

Table 1. Vitamin E concentrations in fish eggs and muscle tissue

	α-Toc*	MDT*	%MDT†	γ-Toc*	δ-Toc*
Egg					
Chum salmon ($n = 4$)	144	35.8	19.9	1.4	2.1
Masu salmon ($n = 2$)	352	35.1	9.1	0.0	2.8
Cultured masu salmon ($n = 3$)	280	0.9	0.3	4.5	1.0
Sockeye salmon ($n = 1$)	227	37.7	14.2	4.0	0.0
Walleye pollack ($n = 1$)	40.6	6.2	13.2	0.1	0.0
Pacific herring ($n = 1$)	2.5	0.2	7.4	0.0	0.0
Pacific cod ($n = 1$)	41.0	3.1	7.0	0.1	0.0
Flyingfish ($n = 1$)	3.3	0.2	5.0	0.1	0.0
Muscle					
Chum salmon ($n = 4$)	6.7	1.7	20.2	0.1	0.1
Masu salmon ($n = 4$)	39.3	5.2	12.3	0.0	0.1
Cultured masu salmon ($n = 4$)	60.4	0.3	0.4	1.5	0.6
Coral trout ($n = 1$)	5.02	0.13	2.5	0.00	0.08
Mangrove jack ($n = 1$)	3.45	0.04	1.0	0.00	0.00
Red-throat sweetlip ($n = 1$)	3.01	0.01	0.5	0.03	0.00
Blue-tailed cod ($n = 1$)	4.30	0.01	0.3	0.00	0.00
Gonad					
Chum salmon ($n = 2$)	91.7	20.6	18.3	0.0	0.5
Masu salmon ($n = 3$)	207	16.5	7.5	0.0	0.3
Cultured masu salmon ($n = 2$)	508	0.5	0.1	1.7	0.2
Spleen					
Chum salmon ($n = 3$)	26.7	3.3	11.0	0.0	0.2
Masu salmon ($n = 2$)	349	31.1	8.0	0.0	0.7
Cultured masu salmon ($n = 3$)	240	0.5	0.2	2.5	0.0
Liver					
Chum salmon ($n = 1$)	402	33.3	7.6	0.0	0.0
Masu salmon ($n = 1$)	555	38.3	6.4	0.0	0.7
Cultured masu salmon ($n = 1$)	1040	1.2	0.1	13.5	0.8
Phytoplankton	0.64	0.17	21.0	0.00	0.00
Zooplankton	4.60	0.54	10.5	0.00	0.00
Commercial feed for masu salmon	16.2‡	Trace‡	<0.1	14.9‡	2.6‡

*Nmol/g of wet tissue.
†%MDT = 100 × MDT/(MDT + α-Toc).
‡Nmol/g of dry feed.

The MDT concentration and %MDT composition in tissues of native masu salmon were much greater than those in corresponding tissues of masu salmon reared by aquaculture on a low MDT diet (Table 1). This comparison provides evidence that MDT is not synthesized *de novo* or produced by modification of consumed α-Toc, but is acquired directly from the diet. Vitamin E is known to be synthesized only by photosynthetic organisms (7), and our evidence suggests that this is also the case for MDT. In fact, a high %MDT composition (21.0%) was observed in a mixed phytoplankton sample collected from the Oyashio (cold waters) of Hokkaido, although mixed zooplankton separated from the same trawl had a lower %MDT composition (10.5%) (Table 1). This finding may indicate a reduced metabolic preference for accumulating MDT at the next trophic level. However, the selectivity for acquiring MDT across the food chain warrants greater examination. It is clear that humans can also absorb MDT from the diet (Fig. 2).

The biosynthesis of α-Toc is known to occur by methylation of γ-tocopherol in plants (7, 8) and algae (9) or by the reduction of α-tocotrienol in higher plants such as the eucalyptus tree (3). As expected, tocotrienols were not detected in our marine samples. It is therefore possible that MDT is derived from α-Toc in marine algae by desaturation in a manner similar to the introduction of double bonds in fatty acids (10). However, this conjecture requires further examination. It is well known that psychrophilic organisms maintain membrane order and functional integrity by increasing cellular proportions of long-chain polyunsaturated fatty acids (PUFAs; ref. 11). Yet by increasing PUFA, this homeoviscous (homeophasic) adaptation occurs at the expense of enhancing membrane oxidative vulnerability. Accordingly, the high dietary vitamin E requirement of cold-water fish is attributed to having a greater metabolic need for providing enhanced lipid-phase antioxidant protection (12). Our finding of greater MDT/total vitamin E ratios in cold-water fish suggests that MDT may enhance antioxidant protection at low temperature.

Antioxidant Properties of MDT and α-Toc at Low Temperature. Oxidation of unsaturated cellular lipids (LH) proceeds by a free radical chain mechanism whereby lipid peroxyl radicals (LOO·) function as the radical-chain carrier (Scheme 1, Eqs. **1** and **2**). α-Toc (or MDT) serves as an antioxidant by scavenging two peroxyl radicals to inhibit lipid peroxidation (Scheme 1, Eqs. **3** and **4**) (1). Given that α-Toc and MDT share identical chromanol structures in their reactive electrophores, it was not surprising to find identical antioxidant activities when measured in homogeneous methanolic solution or in soybean phosphatidylcholine liposomal dispersion at 37°C (4).

To test the hypothesis that MDT may provide enhanced antioxidant protection at low temperature, the antioxidant properties of MDT and α-Toc were compared in multilamellar soyPC liposomal membranes at 0°C. By using UV-irradiation of benzophenone (0.5 μM) to initiate lipid peroxidation (13), oxidation

$$\text{LOO}\bullet + \text{LH} \longrightarrow \text{LOOH} + \text{L}\bullet \quad (1)$$

$$\text{L}\bullet + \text{O}_2 \longrightarrow \text{LOO}\bullet \quad (2)$$

$$\alpha\text{-Toc (MDT)} + \text{LOO}\bullet \xrightarrow{k\ (k')} \alpha\text{-Toc}\bullet\ (\text{MDT}\bullet) + \text{LOOH} \quad (3)$$

$$\alpha\text{-Toc}\bullet\ (\text{MDT}\bullet) + \text{LOO}\bullet \longrightarrow \alpha\text{-Toc-OOL (MDT-OOL)} \quad (4)$$

$$k'/k = \ln([\text{MDT}]/[\text{MDT}]_0)/\ln([\alpha\text{-Toc}]/[\alpha\text{-Toc}]_0) \quad (5)$$

Scheme 1.

of 5 mM soyPC liposome containing 5 mM cholesterol under aerobic conditions formed soyPC hydroperoxide (PC-OOH) at a rate of 4630 pM/s (Fig. 3). Addition of 5 μM MDT or α-Toc reduced the control rate of soyPC oxidation to 91.4 $\pm$ 35.2 pM/s (n = 3) and 230 $\pm$ 26.4 pM/s (n = 3), respectively (Fig. 3). Because MDT or α-Toc combines with two peroxyl radicals (Scheme 1 Eqs. **3** and **4**), the rate of peroxyl radical formation was 86.3 $\pm$ 25.4 pM/s (n = 6) as calculated from the rate of vitamin E consumption (43.2 $\pm$ 12.7 pM/s). Kinetic chain length, determined as the ratio of the rate of PC-OOH formation to the rate of peroxyl radical formation, provides a measure of antioxidant efficiency to inhibit free-radical chain oxidation. Kinetic chain lengths for MDT- and α-Toc-inhibited liposomal oxidation were 1.28 $\pm$ 0.24 (n = 3) and 3.74 $\pm$ 0.25 (n = 3), respectively.

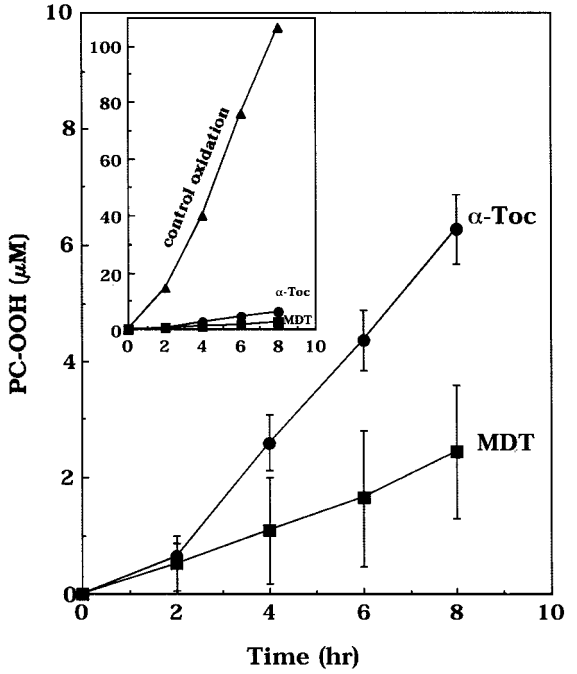

Fig. 3. Formation of soyPC hydroperoxide (PC-OOH) during 0.5 μM benzophenone-sensitized photooxidation of 5 mM soyPC multilamellar liposomes containing 5 mM cholesterol at 0°C with 5 μM MDT or α-Toc. Vertical bars indicate SD (n = 3). The average rate of LOO· formation was calculated as 86.3 pM/s from the rate of MDT or α-Toc consumption. (*Inset*) Data scaled to show the control rate of oxidation without the addition of vitamin E antioxidants.

Thus, MDT has a significant 2.9-fold advantage over equimolar α-Toc ($P < 0.001$) in its efficiency to inhibit liposomal oxidation 0°C.

We also evaluated the antioxidant activities of MDT and α-Toc by comparing the rate constants for MDT (k') and α-Toc (k) to scavenge LOO· (Scheme 1 Eq. **3**) during soyPC oxidation. The ratio of k'/k can be calculated from Eq. **5** by measuring the competitive time-dependent changes in MDT and α-Toc concentrations during lipid cooxidation. In a cholesterol-free soyPC liposomal membrane, MDT and α-Toc decreased at identical rates (k'/k = 1) during photosensitized oxidation at 0°C (data not shown). However, MDT consumption exceeded α-Toc oxidation (i.e., $k'/k > 1$; Fig. 4) when equimolar cholesterol was incorporated to increase liposomal membrane microviscosity (14). This rate difference was more pronounced at 0°C when reactivities were measured at the higher viscosity of neat fish oil (Fig. 4).

Data presented in Fig. 4 show that the k'/k ratio also increased significantly on lowering the photoproduction rate of LOO· (by reducing the amount of photosensitizer) as calculated by the total vitamin E (MDT + α-Toc) consumption rate. The greatest k'/k ratios observed were 1.8 in cholesterol-containing soyPC liposome and 3.9 in fish oil (Fig. 4). It should be emphasized, however, that the measured rate constants (Scheme 1 Eq. **3**) do not account for differences in the reactivities of MDT and α-Toc radicals to combine with LOO· (Scheme 1, Eq. **4**). Hence, the k'/k ratio may not reflect the true difference in the antioxidant activities of MDT and α-Toc. As discussed above, the efficiency of MDT to inhibit the oxidation of cholesterol-containing soyPC liposome at 0°C was 2.9-fold greater than equimolar α-Toc, although the k'/k ratio was only 1.1 when determined at an equivalent rate (86.3 pM/s) of peroxyl radical formation (Fig. 4).

The observed viscosity- and radical concentration-dependent changes in the antioxidant activities of MDT and α-Toc should reflect differences in their physical characteristics within cold viscous lipids. Given that α-tocotrienol with three olefinic bonds in its isoprenoid chain was shown to have a higher degree of intramembrane mobility than α-Toc (15), MDT is likely to have greater diffusion than α-Toc in cold viscous lipids. It is noteworthy that we also found α-tocotrienol to have a greater antioxidant activity in cholesterol-containing soyPC liposomes at 0°C than both MDT and α-Toc (data not shown). However, α-tocotrienol does not occur in marine organisms, including those adapted to cold environments.

Our data (Fig. 4) show that MDT and α-Toc have almost identical antioxidant activities (k'/k = 1) at high concentrations of peroxyl radicals. As concentrations of peroxyl radicals are

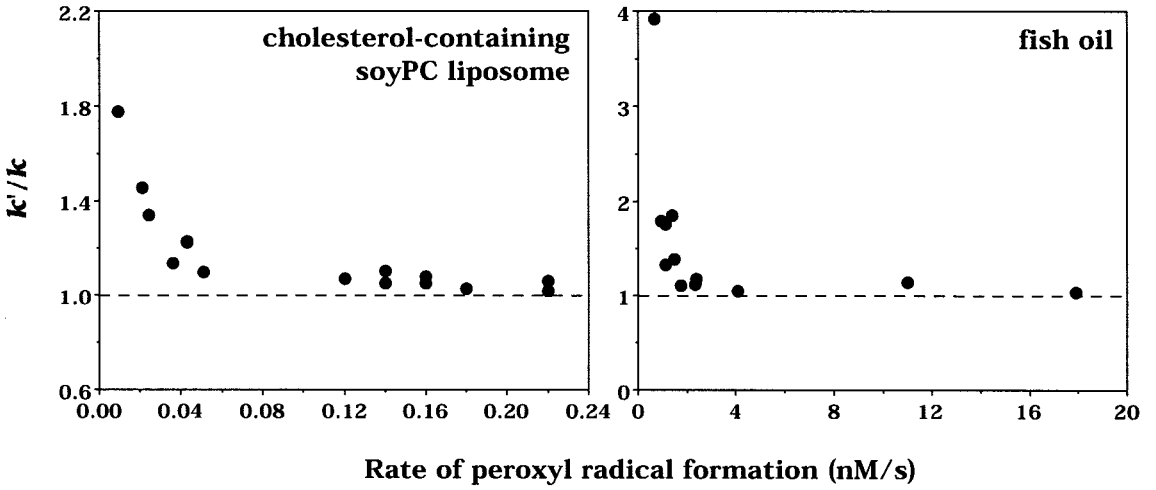

Fig. 4. Dependence in the reactivities of MDT and α-Toc expressed as k'/k ratios on LOO· flux during the benzophenone-sensitized photooxidation of 5 mM soyPC multilamellar liposomes containing 5 mM cholesterol, and of neat fish oil, at 0°C. Tocopherol oxidation rates decreased with decreasing concentrations of benzophenone; maximum concentrations were 5 μM and 0.65 μM for the oxidation of soyPC liposome and fish oil, respectively. The rate of LOO· formation was calculated by the rate of the total vitamin E (MDT and α-Toc) consumption.

reduced, a threshold is attained where diffusion of vitamin E becomes rate limiting, thus favoring the greater mobility of MDT ($k'/k > 1$). Although our experimental design did not permit the measurement of k'/k ratios at very low rates of radical flux, the competitive advantage of MDT to inhibit lipid peroxidation (Fig. 3) may be significantly greater at low physiological rates of cellular oxidation.

In summary, we demonstrate that biosynthetic production of MDT, or its accumulation at higher trophic levels, may reduce lipid peroxidation in marine organisms adapted to cold-water environments.

We thank Mr. Nobuo Maita and Dr. Tetsuya Suzuki for prior contributions to this study, and Dr. Naonobu Shiga for providing plankton samples. We also appreciate Dr. David Barnes and Ms. Elizabeth Tynan for editorial advice. We are also indebted to reviewers for their valuable comments. This work was supported by Grants-in-Aid for Scientific Research from the Ministries of Education, Science, and Culture, and Health and Welfare of Japan.

1. Burton, G. W. & Ingold, K. U. (1981) *J. Am. Chem. Soc.* **103,** 6472–6477.
2. Matsumoto, A., Takahashi, S., Nakano, K. & Kijima, S. (1995) *J. Jpn. Oil Chem. Soc.* **44,** 593–597.
3. Wellburn, A. R. (1970) *Phytochemistry* **9,** 743–748.
4. Yamamoto, Y., Maita, N., Fujisawa, A., Takashima, J., Ishii, Y. & Dunlap, W. C. (1999) *J. Nat. Prod.* **62,** 1685–1687.
5. Singleton, W. S., Gray, M. S., Brown, M. L. & White, J. L. (1965) *J. Am. Oil Chem. Soc.* **42,** 53–56.
6. Kohen, R., Yamamoto, Y., Cundy, K. C. & Ames, B. N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3175–3179.
7. Shintani, D. & DellaPenna, D. (1998) *Science* **282,** 2098–2100.
8. d'Harlingue, A. & Camara, B. (1985) *J. Biol. Chem.* **260,** 15200–15203.
9. Shigeoka, S., Ishiko, H., Nakano, Y. & Mitsunaga, T. (1992) *Biochim. Biophys. Acta* **1128,** 220–226.
10. McConn, M., Hugly, S., Browse, J. & Summerville, C. (1994) *Plant Physiol.* **106,** 1609–1614.
11. Hazel, J. R. (1995) *Annu. Rev. Physiol.* **57,** 19–42.
12. Woodward, B. (1994) *Aquaculture* **124,** 133–168.
13. Barclay, L. R. C., Baskin, K. A., Locke, S. J. & Schafer, T. D. (1987) *Can. J. Chem.* **65,** 2529–2540.
14. Wu, E., Jacobson, K. & Papahadjopoulos, D. (1977) *Biochemistry* **16,** 3936–3941.
15. Serbinova, E., Kagan, V., Han, D. & Packer, L. (1991) *Free Radical Biol. Med.* **10,** 263–275.