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Potent Antioxidant Activity of a Dithiocarbamate-Related Compound from a Marine Hydroid

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ABSTRACT. Recently, we discovered a novel class of natural products, named the tridentatols, in a marine hydroid. Close examination of their molecular structures suggested that they may have antioxidant activity. This observation prompted us to evaluate *in vitro* the capacity of one of these tridentatols, viz. tridentatol A, to inhibit lipid peroxidation using human low density lipoprotein (LDL) as an experimental model. LDL was incubated with 5 μM cupric chloride (Cu^{2+}) in the absence and presence of tridentatol A or a reference antioxidant standard, i.e. vitamin E. The onset of rapid formation of conjugated lipid hydroperoxides was delayed in a concentration-dependent manner by tridentatol A. More specifically, LDL incubated with Cu^{2+} had a lag-phase time (the elapsed time before the onset of rapid formation of conjugated lipid hydroperoxides) of 150 min. However, when 0.5 μM tridentatol A was present during incubation, the lag phase time was extended to 225 min. With 1 μM tridentatol A, the lag phase time was 300 min. The same concentrations of vitamin E produced noticeably lower lag phase times. Thus, compared with vitamin E, tridentatol A better protected against the formation of conjugated lipid hydroperoxides in LDL. Direct colorimetric measurements of both lipid hydroperoxides and thiobarbituric acid-reactive substances confirmed the greater potency of tridentatol A relative to vitamin E. Furthermore, tridentatol A negated the Cu^{2+}-induced increase in electrophoretic mobility of LDL to a greater extent than vitamin E. In conclusion, tridentatol A is a powerful antioxidant against lipid peroxidation of LDL and is significantly more potent than vitamin E in this regard. BIOCHEM PHARMACOL **58**;8: 1313–1319, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. antioxidants; lipid peroxidation; low density lipoprotein; tridentatol; vitamin E

Identification of new antioxidants remains a highly active research area because antioxidants may reduce the risk of various chronic diseases believed to be caused by free radicals [1]. Many important antioxidants from terrestrial plant sources are already well known. For example, ascorbic acid, vitamin E, carotenoids, and flavonoids are present in many fruits and vegetables. Ascorbic acid and vitamin E are essential nutrients. Carotenoids and flavonoids are considered non-essential dietary components, but they may promote optimum health. Additionally, phytochemical antioxidants are found in other edible plant products such as herbs and spices, which include *Ginkgo biloba* [2] and rosemary [3].

Natural antioxidants are not limited to terrestrial plant species. They have also been discovered in marine plant species such as seaweeds. For example, carotenoid antioxidants having antimutagenic properties were identified in the red alga *Porphyra tenera* [4]. The possibility that marine antioxidants exist in non-plant sources has not been investigated widely. Recently, mycosporine-glycine, which is found in many marine invertebrates, was reported to have antioxidant activity [5]. We previously isolated three novel dithiocarbamate-related compounds (Fig. 1) from the marine hydroid *Tridentata marginata* [6]. This general class of natural products was named the tridentatols. *T. marginata* may produce the tridentatols as secondary metabolites that protect the hydroid against predators and also damaging solar UV radiation [7]. However, in addition to these two ecologically important activities, it is conceivable that the tridentatols have other chemical properties that benefit the hydroid.

Phenolic substances typically possess antioxidant activity that enables them to scavenge free radicals [8]. Thus, the presence of a phenolic hydroxyl group on the structures of the tridentatols suggests that the tridentatols each can furnish a hydrogen atom to scavenge free radicals as well. Accordingly, we tested a representative tridentatol, viz. tridentatol A, for antioxidant activity by evaluating its capacity to inhibit lipid peroxidation of human LDL§, a widely accepted model for evaluating antioxidants [2, 3, 9].

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§ *Abbreviations*: AAPH, 2,2′-azo-bis(2-amidinopropane)dihydrochloride; DDC, diethyldithiocarbamate; LDL, low density lipoprotein; and TBARS, thiobarbituric acid-reactive substances.

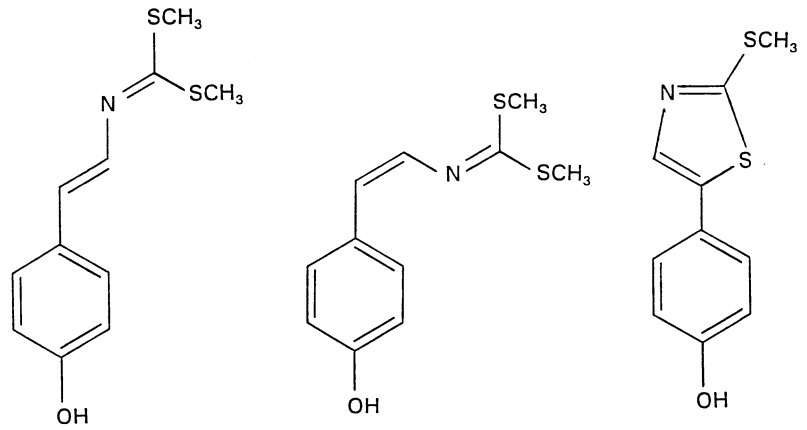

FIG. 1. Structures of the tridentatols.

MATERIALS AND METHODS

Materials

Tridentatol A was extracted and purified from *T. marginata* as reported previously [6]. Vitamin E and all other reagents were obtained from the Sigma Chemical Co.

Isolation of Human LDL

Blood was collected by venipuncture on two different occasions from a healthy female human subject after fasting overnight. EDTA was used as an anticoagulant (1.5 mg/mL of blood). After low-speed centrifugation of the whole blood to obtain plasma, LDL was isolated from the plasma by discontinuous density gradient ultracentrifugation as described previously [9, 10]. Subsequently, LDL was desalted by passing it through a gel filtration column packed with Bio-Gel P-6DG (Bio-Rad) and equilibrated with 10 mM PBS, pH 7.4. After filtration through a 0.45-μm filter, LDL in PBS was stored at 4° under nitrogen gas and used within 2 weeks. Protein content of the LDL was determined using BSA as the standard [11].

Initial Testing of Tridentatol A for Antioxidant Activity

LDL (0.05 mg protein/mL) was incubated with either 5 μM cupric chloride (Cu^{2+}) or 1 mM AAPH, in the absence and presence of tridentatol A or vitamin E (0–50 μM) dissolved in ethanol. The concentration of ethanol in the PBS-based incubation mixtures, including the controls, was 5% (v/v). Incubation was performed at 25° for various lengths of time (either 6, 6.5, or 10 hr), depending on the experiment. The formation of fatty acid conjugated dienes (conjugated lipid hydroperoxides) during LDL oxidation was monitored continuously [12] with a Beckman DU 640 spectrophotometer by measuring the increase in absorbance at 234 nm over time. After plotting the data, the lag-phase times were determined [12].

Complementary Testing of Tridentatol A for Antioxidant Activity Using Other Measurements of LDL Oxidation

LDL (0.4 mg protein/mL) was incubated with 5 μM Cu^{2+} in the absence and presence of tridentatol A or vitamin E (0–100 μM) dissolved in ethanol. The concentration of ethanol in the PBS-based incubation mixtures, including the controls, was 5% (v/v). Incubation was performed at 37° for 2–6 hr. Three complementary methods then were used to measure the extent of LDL oxidation, as detailed below.

Levels of lipid hydroperoxides in LDL were determined spectrophotometrically using FOX reagent [13]. Briefly, 0.9 mL of FOX reagent was added to 0.1 mL of post-incubation mixture, and also to hydrogen peroxide standards (0–10 μmol). After mixing and allowing the mixture to stand for 30 min, absorbance was measured at 560 nm against a reagent blank.

Levels of TBARS in LDL also were determined spectrophotometrically [14]. To 0.1-mL aliquots of post-incubation mixture and also to tetramethoxypropane standards (0–4 nmol) was added 1 mL of 20% (w/v) trichloroacetic acid containing EDTA (79 mg/100 mL). After adding 1 mL of 1% (w/v) thiobarbituric acid and mixing, tubes were placed in a boiling water bath for 15 min. After cooling, tubes were centrifuged at 1500 *g* for 15 min. Absorbance of the supernatant was measured at 532 nm.

Changes in electrophoretic mobility of LDL were evaluated as before [9] using a Ciba-Corning electrophoresis system/lipoprotein gel kit [1% (w/v) agarose gels with Fat Red 7B stain]. Instructions furnished by the manufacturer (Ciba-Corning) were followed.

RESULTS

Initially, the capacity of tridentatol A to inhibit either Cu^{2+}-induced or AAPH-induced lipid peroxidation of LDL was evaluated by kinetic measurement. In a concentration-dependent manner, tridentatol A delayed the onset of rapid

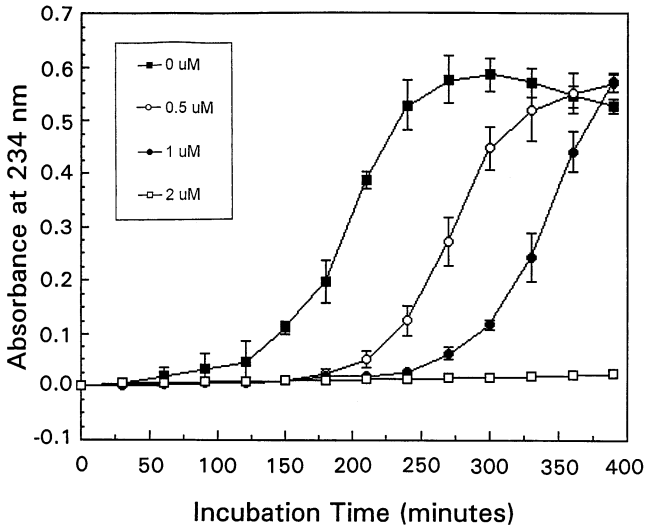

FIG. 2. Effect of tridentatol A on the onset of rapid formation of conjugated lipid hydroperoxides in LDL incubated with cupric ions. LDL (0.05 mg protein/mL) was incubated with 5 μM Cu^{2+} and 0–2 μM tridentatol A. Absorbance at 234 nm by the conjugated lipid hydroperoxides was monitored continuously for 390 min with recordings taken at 30-min intervals. Points are the means ± SD of 3 different experiments.

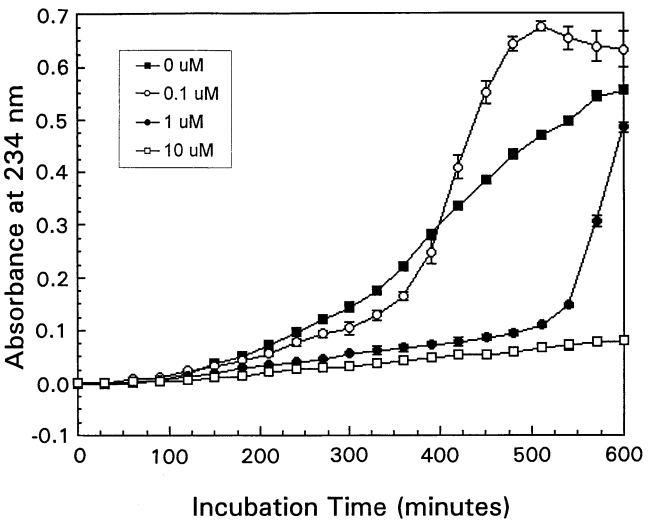

FIG. 3. Inhibitory effect of tridentatol A on formation of conjugated lipid hydroperoxides in LDL incubated with AAPH. LDL (0.05 mg protein/mL) was incubated with 1 mM AAPH and 0–10 μM tridentatol A. Absorbance at 234 nm by the conjugated lipid hydroperoxides was monitored continuously for 600 min with recordings taken at 30-min intervals. Points are the means ± SD of 3 different experiments.

formation of conjugated lipid hydroperoxides, which absorb light at 234 nm, in LDL incubated with 5 μM Cu^{2+} (Fig. 2). LDL incubated with Cu^{2+} alone had a lag-phase time (the elapsed time before the onset of rapid formation of conjugated lipid hydroperoxides) of 150 min. However, when 0.5 μM tridentatol A was present during incubation of LDL with Cu^{2+}, the lag-phase time was extended to 225 min. With 1 μM tridentatol A, the lag-phase time was extended to 300 min. Formation of conjugated lipid hydroperoxides was essentially prevented with 2 μM tridentatol A.

When 1 mM AAPH was used as the prooxidant to induce LDL oxidation, tridentatol A delayed the onset of rapid formation of conjugated lipid hydroperoxides in a similar manner as Cu^{2+} (Fig. 3). A marked effect was seen at 1 μM tridentatol A. A concentration of 10 μM tridentatol A delayed the onset of rapid formation of conjugated lipid hydroperoxide for at least 600 min. On the other hand, 0.1 μM tridentatol A, oddly, increased the net production of conjugated lipid hydroperoxides (indicated by the higher absorbance at 510 min of incubation). Whether this reflects a true pro-oxidant effect is equivocal.

As shown in Fig. 4, compared with an antioxidant standard (vitamin E), tridentatol A better delayed the onset of rapid formation of conjugated lipid hydroperoxides in LDL incubated with Cu^{2+}. The lag phase time for LDL incubated with Cu^{2+} alone was 155 min. In the presence of 1 μM vitamin E, the lag phase time was extended to 185 min, but the same concentration of tridentatol A extended the lag phase time to 280 min. This notable effect by 1 μM tridentatol A was greater than that of vitamin E even at 2 μM, which produced a lag phase time of only 215 min.

Direct colorimetric measurements of lipid hydroperoxides and TBARS confirmed the superior antioxidant potential of tridentatol A relative to vitamin E. As shown in Fig. 5, when LDL was incubated with Cu^{2+} for either 2 or 3 hr, tridentatol A inhibited production of lipid hydroperoxides better than vitamin E, with especially obvious results at concentrations of 3.125, 6.25, 12.5, and 25 μM. Consistent with these results, tridentatol A inhibited the produc-

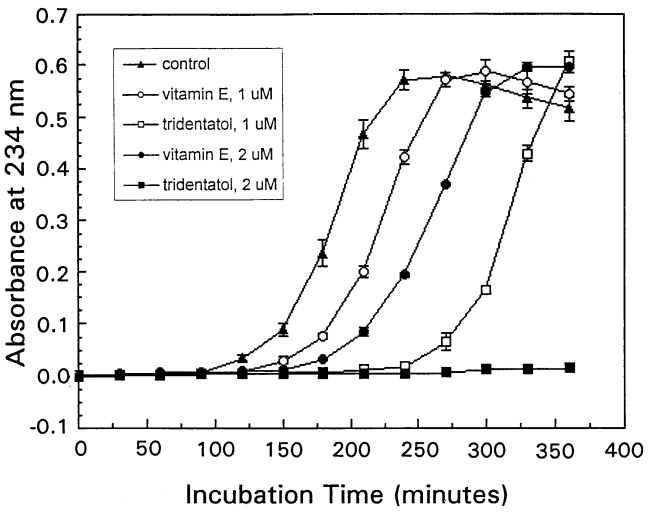

FIG. 4. Comparison between tridentatol A and vitamin E in delaying onset of the rapid formation of conjugated lipid hydroperoxides in LDL incubated with cupric ions. LDL (0.05 mg protein/mL) was incubated with 5 μM Cu^{2+} and 0–2 μM tridentatol A or vitamin E. Absorbance at 234 nm by the conjugated dienes was monitored continuously for 360 min with recordings taken at 30-min intervals. Points are the means ± SD of 3 different experiments.

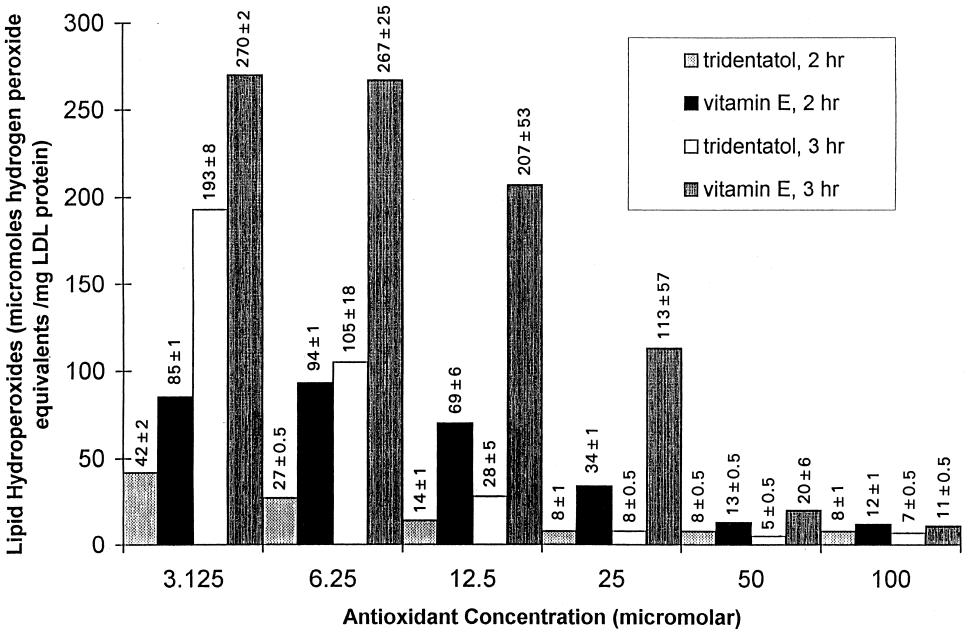

FIG. 5. Comparison between tridentatol A and vitamin E in preventing formation of lipid hydroperoxides in LDL incubated with cupric ions. LDL (0.4 mg protein/mL) was incubated at 37° for either 2 or 3 hr with 5 μM Cu^{2+} and 0–100 μM tridentatol A or vitamin E. Levels of lipid hydroperoxides in the control samples incubated for 2 and 3 hr were 6 ± 0.5 and 5 ± 1 μmol hydrogen peroxide equivalents/mg of LDL protein, respectively. Levels of lipid hydroperoxides in samples incubated with cupric ions in the absence of tridentatol A or vitamin E for 2 and 3 hr were 77 ± 4 and 291 ± 13 μmol hydrogen peroxide equivalents/mg of LDL protein, respectively. All values are the means ± SEM of data from two different experiments, performed in duplicate.

tion of TBARS better than vitamin E at 12.5, 25, and 50 μM concentrations of the antioxidants when LDL was incubated with Cu^{2+} for 4 hr (Fig. 6). With 6 hr of incubation, it required 50 μM tridentatol to inhibit TBARS production, while vitamin E was without effect at 50 or even 100 μM in this particular experiment. Thus, vitamin E at 25, 50, and 100 μM was able to inhibit TBARS formation for 4 hr, but not for 6 hr, perhaps due to exhaustion of vitamin E in further scavenging of lipid radicals during the additional 2 hr of incubation. A similar situation existed in examining the effects of 12.5 and 25 μM tridentatol. There were inhibitory effects on TBARS formation at 4 but not at 6 hr.

Finally, electrophoretic analyses (Figs. 7 and 8) corroborated the spectrophotometric analyses (Figs. 4–6) in terms of the capability of tridentatol A to inhibit Cu^{2+}-induced LDL oxidation better than vitamin E. Compared with control LDL (lane 1), LDL incubated with Cu^{2+} (lane 2) had enhanced mobility toward the anode upon electrophoresis in agarose gel, i.e. a greater extent of oxidative modification of the LDL (Fig. 7). However, the presence of incremental levels of tridentatol A during incubation of LDL with Cu^{2+} negated the increase in electrophoretic mobility of LDL in a concentration-dependent manner (lanes 3–8). In a comparative experiment where LDL was incubated with Cu^{2+} in the absence or presence of tridentatol A or vitamin E (Fig. 8), it is most notable that 25 μM tridentatol A (lane 3) versus 25 μM vitamin E (lane 4) better inhibited the increase in electrophoretic mobility of LDL caused by Cu^{2+} (lane 2). Moreover, it required 100 μM vitamin E (lane 8) to match the effect of 25 μM tridentatol A (lane 3).

DISCUSSION

Four experimental methods, which are recognized as standard procedures to measure LDL oxidation [15] and to test substances for antioxidant activity [2, 3, 9], were used to critically evaluate the capacity of tridentatol A to inhibit oxidative modification of LDL. The results from monitoring the onset of rapid formation of conjugated lipid hydroperoxides (Figs. 2–4), measuring levels of lipid hydroperoxides (Fig. 5) and TBARS (Fig. 6), and also determining changes in electrophoretic mobility of LDL (Figs. 7 and 8) all demonstrated that tridentatol A had antioxidant activity, and more so than vitamin E.

The antioxidant effects of tridentatol A were similar to those of other phenolic substances. For example, phenolic drugs such as acetaminophen and 5-aminosalicylate, which each contain a single phenolic hydroxyl group as does tridentatol A, scavenge AAPH-derived peroxyl radicals and inhibit membrane lipid peroxidation induced by iron/ascorbate [16]. Phenolic substances from an exotic plant drug inhibit membrane lipid peroxidation as well [8]. Having more than one phenolic hydroxyl group or phenolic structural units, natural phenolic substances such as flavonoids [17, 18] and polyphenols [19] present in many fruits and vegetables also have free radical-scavenging abilities.

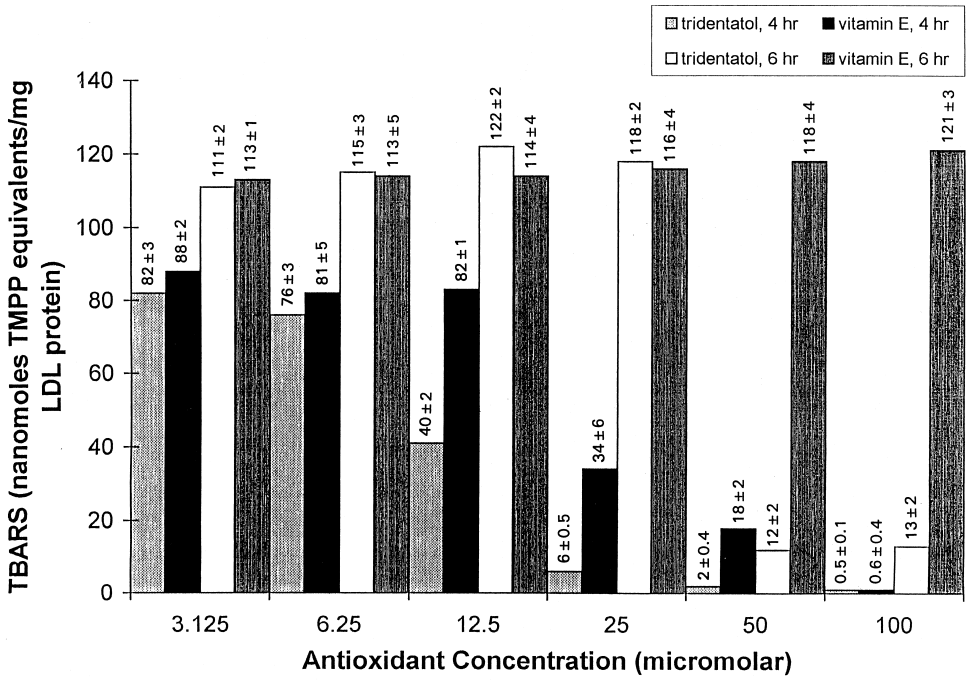

FIG. 6. Comparison between tridentatol A and vitamin E in preventing formation of TBARS in LDL incubated with cupric ions. LDL (0.4 mg protein/mL) was incubated at 37° for either 4 or 6 hr with 5 μM Cu^{2+} and 0–100 μM tridentatol A or vitamin E. Levels of TBARS in the control samples incubated for 4 and 6 hr were below 1 nmol of tetramethoxypropane (TMPP) equivalents/mg of LDL protein. Levels of TBARS in samples incubated with cupric ions in the absence of tridentatol A or vitamin E for 4 and 6 hr were 85 ± 1 and 109 ± 4 nmol of TMPP equivalents/mg of LDL protein. All values are the means ± SEM of data from two different experiments, performed in duplicate.

Moreover, flavonoids [20] and polyphenols [21] can prevent oxidative modification of LDL, as tridentatol A did in the present work.

The mechanism by which tridentatol A exerts its antioxidant action was not examined, but may be similar to how other phenolic substances function as antioxidants. Specifically, phenolic hydroxyl groups of phenolic antioxidants can provide hydrogen atoms to scavenge free radicals [22]. Accordingly, it can be inferred that the single phenolic hydroxyl group of tridentatol A furnishes a hydrogen atom to scavenge free radicals in inhibiting either copper- or AAPH-induced LDL oxidation. Upon treatment of LDL with cupric ions (Cu^{2+}) to induce oxidation, it is believed [23] that pre-existing lipid hydroperoxides in LDL reduce the Cu^{2+} to the cuprous (Cu^{+}) state, the ionic form of copper that actually initiates formation of lipid peroxyl radicals. It is logical to say that the phenolic hydroxyl group of tridentatol A releases its hydrogen atom to scavenge such lipid peroxyl radicals. Consequently, the propagation phase of lipid peroxidation [24] is blocked, and hence, LDL oxidation is inhibited.

Additional lines of thinking support the view that the phenolic hydroxyl group of tridentatol A furnishes a hydrogen atom in scavenging free radicals and inhibiting LDL oxidation. Tridentatol A delayed the onset of rapid formation of conjugated lipid hydroperoxides in LDL treated with AAPH (Fig. 3). Being thermolabile, AAPH decomposes upon incubation to produce water-soluble peroxyl radicals [25]. These peroxyl radicals are scavenged by acceptance of hydrogen atoms, such as would be available from the phenolic hydroxyl group of tridentatol A. Also, we found that tridentatol A reacted readily with the stable free radical DPPH (data not shown), which accepts a hydrogen atom as it is scavenged [26].

Because copper ions induce LDL oxidation, decreasing the availability of copper ions would be expected to decrease the extent of LDL oxidation. It is well established that other phenolic substances, although not necessarily all of them, can chelate transition metals [27, 28] such as copper. Thus, another possible mechanism for the inhibitory effect of tridentatol A on copper-induced LDL oxidation may be that the phenolic hydroxyl group of tridentatol A chelates Cu^{+}, believed to be generated upon reduction of Cu^{2+} by LDL lipid hydroperoxides [23].

It appears that the single phenolic hydroxyl group of tridentatol A is critical for its antioxidant activity. When the hydroxyl group was methylated to form methoxytridentatol A, this product was unable to inhibit LDL oxidation induced by copper ions (data not shown). Hence, these data support the mechanistic concept that the phenolic hydroxyl group of tridentatol A either releases a hydrogen atom to scavenge free radicals or perhaps is involved in chelating copper ions. Furthermore, the fact that LDL oxidation induced by copper ions was not inhibited by methoxytridentatol A indicates that the *S*-methyl-dithiocarbamic moiety of tridentatol A is not involved in inhib-

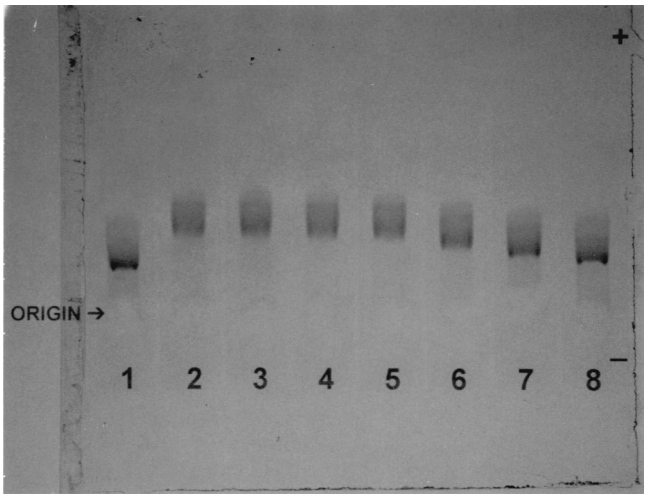

FIG. 7. Representative electrophoretic analysis of LDL after incubation with cupric ions in the absence and presence of tridentatol A. LDL (0.4 mg protein/mL) was incubated for 4 hr at 37° with 5 μM Cu^{2+} and 0–25 μM tridentatol A. After incubation, 0.8 μg LDL protein was loaded onto a 1% (w/v) agarose gel for electrophoresis. Subsequently, the gel was dried and then stained with Fat Red 7B. Lane 1: control; lane 2: Cu^{2+}; lane 3: Cu^{2+}/0.1 μM tridentatol A; lane 4: Cu^{2+}/0.5 μM tridentatol A; lane 5: Cu^{2+}/1 μM tridentatol A; lane 6: Cu^{2+}/5 μM tridentatol A; lane 7: Cu^{2+}/10 μM tridentatol A; and lane 8: Cu^{2+}/25 μM tridentatol A.

iting LDL oxidation by possibly interacting with copper ions.

A most noteworthy finding is that tridentatol A proved to be more potent than vitamin E (α-tocopherol) in inhibiting copper-induced LDL oxidation (Figs. 4–6 and 8). The greater potency of tridentatol A relative to vitamin E is not easy to explain fully, but is likely attributable to structural differences between the two substances. Both substances have one hydroxyl group capable of releasing a hydrogen atom to scavenge free radicals or perhaps chelate copper ions. Apparently, the hydroxyl group attached to the basic benzene ring of tridentatol A releases its hydrogen atom more readily than the single hydroxyl group attached to the chromanol ring system of vitamin E in scavenging free radicals. Other structural components attached to the respective ring systems of tridentatol A and vitamin E influence electron density of the hydroxyl group and ultimate release of its hydrogen atom [22]. This suggests that the electron density of the hydroxyl group of tridentatol A is greater than that of the hydroxyl group of vitamin E, thus favoring easier release of the hydrogen atom to react with LDL lipid free radicals.

The existence of such a potent antioxidant as tridentatol A in *T. marginata* may help survival of the hydroid in a harsh environment. *T. marginata* is commonly found attached to the Sargasso seaweed, which floats at the sea surface. Hence, *T. marginata* is under exposure to intense solar UV radiation, which is known to generate free radicals [29] that can damage important biomolecules such as DNA [30]. Therefore, the antioxidant and UV-absorbing capabilities of the tridentatols [7], which make up 10% of the hydroid dry mass [7], may be pivotal in protecting *T. marginata* against potentially lethal UV radiation.

Because the tridentatols are basically dithiocarbamate derivatives, this raises the question whether they can function like other kinds of dithiocarbamates that have been studied. For example, DDC has antioxidant activity [31], as demonstrated by its ability to chelate transition metal ions and also to scavenge hypochlorous acid, hydroxyl radical, and peroxynitrite. DDC also inhibited oxidative modification of LDL [32], an effect consistent with that of tridentatol A in our work. However, the mechanism of DDC is different from that of tridentatol A. DDC has two free sulfur atoms, one being highly active since DDC exists as a monothiolate anion under experimental conditions. In contrast, the two sulfur atoms of tridentatol A are methylated, likely rendering them inactive. DDC has been used in the treatment of acquired immunodeficiency syndrome [32]. Obviously, we cannot say that tridentatol A has such a clinical application.

In conclusion, tridentatol A is a powerful antioxidant against lipid peroxidation of LDL and is significantly more potent than vitamin E in this regard. Additional studies will be needed to explore the potential of tridentatol A and the other tridentatols as novel reagents for basic biochemical research. If shown to be safe in biological studies, these marine products might be useful in the prevention or treatment of various chronic diseases associated with oxidative stress.

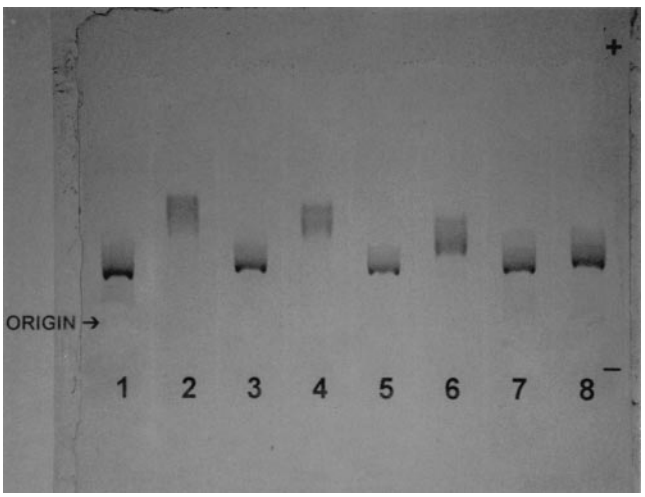

FIG. 8. Comparison between tridentatol A and vitamin E in preventing changes in electrophoretic mobility of LDL incubated with cupric ions. LDL (0.4 mg protein/mL) was incubated for 6 hr at 37° with 5 μM Cu^{2+} and 0–100 μM tridentatol A or vitamin E. Lane 1: control; lane 2: Cu^{2+}; lane 3: Cu^{2+}/25 μM tridentatol A; lane 4: Cu^{2+}/25 μM vitamin E; lane 5: Cu^{2+}/50 μM tridentatol A; lane 6: Cu^{2+}/50 μM vitamin E; lane 7: Cu^{2+}/100 μM tridentatol A; and lane 8: Cu^{2+}/100 μM vitamin E.

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