Peroxynitrite Scavenging and Cytoprotective Activity of 2,3,6-Tribromo-4,5-dihydroxybenzyl Methyl Ether from the Marine Alga *Symphyocladia latiuscula*

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Peroxynitrite ($ONOO^-$), formed from the reaction of superoxide ($O_2^{\bullet -}$) and nitric oxide ($^\bullet NO$), is a cytotoxic species that can oxidize several cellular components such as proteins, lipids, and DNA. It has been implicated in diseases such as Alzheimer's disease, rheumatoid arthritis, cancer, and atherosclerosis. Due to the lack of endogenous enzymes responsible for $ONOO^-$ inactivation, developing a specific $ONOO^-$ scavenger is of considerable importance. The aim of this study was to evaluate the ability of marine natural products to scavenge $ONOO^-$ and to protect cells against $ONOO^-$. Methanolic extracts of 17 marine alga were tested for their $ONOO^-$ scavenging activity. Among them, *Symphyocladia latiuscula* showed the potent scavenging activity. CH_2CH_2 fraction was partitioned with CH_2CH_2 following *n*-hexanal extraction from the methanol extract of *S. latiuscula*. It was highly effective for $ONOO^-$ scavenging activity. Further analysis of the active fractionated extract identified 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (TDB) as a potent $ONOO^-$ scavenger. The data demonstrated that TDB led to decreased $ONOO^-$-mediated nitration of tyrosine through electron donation. TDB showed significant inhibition on nitration of bovine serum albumin and low-density lipoprotein by $ONOO^-$ in a dose-dependent manner. It also provided cytoprotection from cell damage induced by $ONOO^-$. TDB can be developed as an effective peroxynitrite scavenger for the prevention of the involved diseases.

Keywords: *Marine alga; $ONOO^-$ scavenging activity; 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether; nitrotyrosine; cytoprotection*

INTRODUCTION

Peroxynitrite ($ONOO^-$) is formed by the reaction of nitric oxide ($^\bullet NO$) and superoxide ($O_2^{\bullet -}$). $ONOO^-$ is a cytotoxic reactive species that can be generated by endothelial cells, Kupffer cells, neutrophils, and macrophages (*1*). Compared to free radicals, $ONOO^-$ is a relatively stable species but once protonated, it gives highly reactive peroxynitrous acid (ONOOH), which yields various cytotoxicants (*1*, *2*). $ONOO^-$ is a relatively long-lived cytotoxicant with strong oxidizing properties toward various cellular constituents, including sulfhydryls, lipids, amino acids, and nucleotides (*3*). A number of publications report that $ONOO^-$ can induce oxidation of thiol (−SH) groups on proteins, nitration of tyrosine, nitrosation (e.g., formation of *S*-nitrosoglutathione), and lipid peroxidation that affect cell metabolism and signal transduction (*3*−*5*). Furthermore, $ONOO^-$ directly inhibits mitochondrial respiratory enzymes, reduces cellular oxygen consumption, and inhibits membrane sodium transport (*6*). It can ultimately contribute to cellular and tissue injury with DNA strand breakage and apoptotic cell death, for example, in thymocytes, cortical cells, and HL-60 leukemia cells (*4*).

Excessive formation of $ONOO^-$ may also be involved in several human diseases such as Alzheimer's disease, rheumatoid arthritis, cancer, and atherosclerosis (*7*). $ONOO^-$ was demonstrated to cause vasoconstriction at relatively low concentrations, and at higher concentrations, vessel injury was observed in vitro (*8*). $ONOO^-$ is considered to be a major reactive species in vivo in many forms of tissue injury, including ischemia−reperfusion injury of heart, lung, and neurons, inflammatory cell-mediated lung injuries, and cold-induced brain edema (*9*). Moreover, modification of low-density lipoproteins (LDL) by $ONOO^-$ may initiate cellular signals that lead to inflammation, mitosis, or cholesterol accumulation, consequently resulting in atherosclerosis (*10*).

Due to the lack of endogenous enzymes responsible for $ONOO^-$ inactivation, developing specific $ONOO^-$ scavengers is considerably important. The naturally occurring and synthetic $ONOO^-$ scavengers such as ergothioneine (*11*), deferoxamine, urate, glutathione (*12*), melatonin (*13*), mercaptoethylguanidine (*14*), Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin (*15*), and propofol (*16*) have been recently reported. The selenium-containing compounds D-(−)penicillamine (*17*) and ebselen (*18*) have been found to be powerful $ONOO^-$ scavengers in vitro. Ascorbic acid, γ-tocopherol, flavonoids, and polyhydroxyphenols, which are constituents

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of fruits, wines, teas, and green vegetables, were demonstrated to be effective antioxidants against $ONOO^-$ (*1*). To prevent the formation of $ONOO^-$, the $O_2^{\bullet -}$ level can be regulated by a number of enzymes, such as superoxide dismutase (SOD), catalase, and peroxidase (*1*), whereas the $^{\bullet}NO$ level can be controlled by oxyhemoglobin and $^{\bullet}NO$ synthase (*3*).

There is little described in the medical literature of $ONOO^-$ scavenging activity of alga and their isolated compounds. In previous studies, the natural products of *Eriobotrya japonica* (*19*), green tea (*20*, *21*), and *Ginkgo biloba* (*22*) have been shown to be potent $ONOO^-$ scavengers. In the present study, the extracts of various algae were screened for $ONOO^-$ scavenging activity. The fractions and the isolated active components of the most active alga, *Symphyocladia latiuscula*, were further tested for $ONOO^-$ scavenging activity. The protective effect of the active component 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (TDB) from *S. latiuscula* on nitration of tyrosine, bovine serum albumin (BSA), and low-density lipoprotein (LDL) by $ONOO^-$ and on $ONOO^-$-mediated cell damage was examined.

MATERIALS AND METHODS

Materials. 3-Morpholinosydnonimine (SIN-1) and DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid) were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123 (DHR 123) and $ONOO^-$ were from Molecular Probes (Eugene, OR) and Cayman Chemical Co. (Ann Arbor, MI), respectively. 4,5-Diaminofluorescein (DAF-2) was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). LDL and BSA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Poly(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA), and the chemiluminescence detection system was from Amersham Life Sciences, Inc. (Arlington Heights, IL). Anti-nitrotyrosine antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep were purchased from Upstate Biotechnology (Lake Placid, NY) and Amersham (Buckinghamshire, U.K.), respectively. All other chemicals were of the highest purity available from either Sigma Chemical Co. (St. Louis, MO) or Junsei Chemical Co. (Tokyo, Japan).

Alga Extracts. Leafy thalli of alga were collected at Cungsapo, Pusan, Korea, in January 1998 and authenticated by an algologist, Dr. K. W. Nam of the Department of Marine Biology, Pukyoung National University. TDB was isolated as described previously (*23*).

Measurement of $ONOO^-$ Scavenging Activity. $ONOO^-$ scavenging was measured by monitoring the oxidation of DHR 123 according to a modification of the method of Kooy et al. (*5*). A stock solution of DHR 123 (5 mM) in dimethylformamide was purged with nitrogen and stored at −80 °C. A working solution with DHR 123 (final concentration, f.c., 5 μM) diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride with 100 μM (f.c.) diethylenetriaminepentaacetic acid (DTPA) was purged with nitrogen and placed on ice before use. $ONOO^-$ scavenging by the oxidation of DHR 123 was measured with a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments) with excitation and emission wavelengths of 485 and 530 nm, respectively, at room temperature. The background and final fluorescent intensities were measured 5 min after treatment with or without SIN-1 (f.c. 10 μM) or authentic $ONOO^-$ (f.c. 10 μM) in 0.3 N sodium hydroxide. Oxidation of DHR 123 by decomposition of SIN-1 gradually increased, whereas authentic $ONOO^-$ rapidly oxidized DHR 123 with its final fluorescent intensity being stable over time.

Measurement of $O_2^{\bullet -}$ Scavenging Activity. 2,7-Dichlorodihydrofluorescein diacetate (H_2DCFDA, f.c. 2.5 μM) mixed with esterase (f.c. 1.5 units/mL) was incubated at 22 °C for 20 min and placed on ice in the dark until immediately prior to the study. Phosphate buffer (50 mM) at pH 7.4 was used. H_2DCFDA was deacetylated to nonfluorescent 2,7-dichlorodihydrofluorescein (DCFH) by esterase and subsequently oxidized to highly fluorescent 2,7-dichlorofluorescein (DCF) by $O_2^{\bullet -}$. The conversion of DCFH into DCF was gradually increased by $O_2^{\bullet -}$. The fluorescence intensity of oxidized DCFH was measured by using a microplate fluorescence reader (FL 500, Bio-Tek Instruments) at the excitation and emission wavelengths of 485 and 530 nm, respectively, for 1 h with or without the addition of menadione (f.c. 50 μM) as an $O_2^{\bullet -}$ source (*24*).

Measurement of $^{\bullet}NO$ Scavenging Activity. 4,5-Diaminofluorescein (DAF-2) as a specific $^{\bullet}NO$ indicator selectively traps $^{\bullet}NO$ between two amino groups in its molecule and yields triazolofluorescein, which emits green fluorescence when excited at 490−495 nm (*25*). One milligram of DAF-2 in 0.55 mL of dimethyl sulfoxide was diluted with 50 mM phosphate buffer (pH 7.4) to 1/400-fold. An $^{\bullet}NO$ donor, sodium nitroprusside (f.c. 2 mM), and DAF-2 (f.c. 3.14 μM) were added to a 96-well microplate. The fluorescence intensity was dependent on the amount of $^{\bullet}NO$ trapped by DAF-2. The fluorescence signal caused by the reaction of DAF-2 with $^{\bullet}NO$ was measured using a fluorescence spectrometer (FL 500, Bio-Tek Instruments) at the excitation and emission wavelengths of 485 and 530 nm after 10 min.

Interaction of TDB with $ONOO^-$. To identify the reaction mechanism of TDB, $ONOO^-$ was measured by a spectrophotometric analysis, as described by Pannala et al. (*26*). $ONOO^-$ (500 μM) in 0.3 N NaOH was added to a solution containing 100 μM TDB in 50 mM phosphate buffer (pH 7.4), making a final volume of 1 mL. Each mixed solution was incubated at 37 °C with shaking for 1 h and scanned between 190 and 600 nm on an Ultraspec 2000 UV−visible spectrophotometer (Pharmacia-Biotech). The spectral change in the visible region of TDB in the presence of $ONOO^-$ was monitored at 430 nm to determine the existence of nitration. The sample containing no $ONOO^-$ was also included for comparative purposes.

Determination of 3-Nitrotyrosine. The ability of TDB to suppress $ONOO^-$-mediated tyrosine nitration was determined according to the following procedure as described previously (*23*). $ONOO^-$ (500 μM) in 0.3 N NaOH was added to a solution containing 100 μM tyrosine in the presence of 100 μM TDB in 50 mM phosphate buffer (pH 7.4), making a final volume of 1 mL. The formation of 3-nitrotyrosine by the reaction of tyrosine with $ONOO^-$ was also included for comparative purposes. The spectrum of the peak displayed at 430 nm indicates the formation of 3-nitrotyrosine.

Sample Preparation for the Detection of Nitrated Proteins. A 2.5 μL aliquot of TDB dissolved in 10% EtOH was added to 95 μL of BSA (0.5 mg of protein/mL) or LDL (1 mg of protein/mL). The mixed samples were incubated with shaking at 25 °C for 1 h. After the 1 h incubation, 2.5 μL of $ONOO^-$ (f.c. 100 μM for BSA, f.c. 100 μM for LDL) in 0.3 N NaOH was added. The samples were further incubated with shaking for 30 min at 25 °C.

Determination of Nitrated Proteins. The prepared samples in gel loading buffer, pH 6.8 (0.125 M Tris[hydroxymethyl]aminomethane, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue) in a ratio of 1:1 were boiled for 5 min. 20 μL of each sample was separated on an SDS−polyacrylamide minigel (10% for BSA, 6% for LDL) at 100 V and transferred to a poly(vinylidene fluoride) membrane at 100 V for 1.5 h in a wet transfer system (Bio-Rad, Hercules, CA). The membrane was immediately placed into a blocking solution (10% nonfat dry milk in TBS-T buffer containing 10 mM Tris, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) at 4 °C overnight. The membrane was washed in TBS-T buffer for 30 min and then incubated with a monoclonal anti-nitrotyrosine antibody (diluted 1:2000 in TBS-T buffer) at room temperature for 2 h. After two 15-min washings in TBS-T buffer, the membrane was reacted with a horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep (0.1% nonfat milk, diluted 1:2000 in TBS-T buffer) at

room temperature for 2 h. After three 15-min washings in TBS-T buffer, antibody labeling was detected using enhanced chemiluminescence and exposed to radiographic film. Prestained blue protein markers were used for molecular weight determination.

Cell Culture. Bovine smooth muscle cells (SMC) were cultured with DMEM medium (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated (56 °C for 30 min) fetal serum (Gibco, Grand Island, NY), 233.6 mg/mL glutamine, 0.25 g/mL amphotericin, and 100 units/mL penicillin−streptomycin solution (Gibco) and adjusted to pH 7.4−7.6 by $NaHCO_3$ in an atmosphere of 5% CO_2. The fresh medium was replaced after 1 day to remove nonadherent cells or cell debris.

Cell Viability. Trypan blue dye exclusion was assessed as described by Sandoval et al. (*27*). SMCs in a 48-well plate were preincubated overnight. Cells were pretreated with SIN-1 (f.c. 1 mM) for 6 h and then treated with either phloroglucinol or penicillamine as the standard for comparison and incubated for ~14 h. Briefly, cells were detached with trypsin-EDTA (Gibco, BRL, Gaithersburg, MD), and 0.4% trypan blue dye was added. Within a 10-min incubation, the number of cells excluding dye was expressed as a percentage of total cells counted from four chambers of the hemocytometer.

Fluorescence Microscopy. SMCs were cultured for adherence on the slide for 12 h and pretreated with 500 μM SIN-1 for 6 h. Then, 100 μM phloroglucinol or 100 μM penicillamine was used to treat the cells for 12 or 24 h. Each cell group was stained with a 2 μL mixture of 270 μM acridine orange and 254 μM ethidium bromide (1:1). Apoptotic cells and viable cells were observed by reflected fluorescence microscopy (Olympus). Dead cells fluoresced red, and live cells fluoresced green.

Laser Cytometry ACAS 570. To observe the morphological change of smooth muscle cells, laser cytometry was used. Cells were cultured in 35 mm Acas dishes (Mat-Tek) and observed with a fluorescence microscope. The treated cells were stained with a 5 μL mixture of 270 μM acridine orange and 254 μM ethidium bromide (1:1) for each dish. At this time, the image of the cells displayed on the monitor was photographed with the technique of a dual image program at the resolution of 1024 × 1024 pixels. Laser cytometry was established with the parameter of 10% transmission, 100 μm pinhole, 589 nm emission filter, 600 V photomultiplier voltage, and 488 nm excitation wavelength. The cell images were measured exactly at single optical sites and analyzed by a color video copy processor (Mitsubishi, CP2000). Dead cells fluoresced red, and live cells fluoresced green.

Statistical Analysis. Data are expressed as means ± standard errors (SE) of three determinations. IC_{50} values (micromolar concentration required to inhibit $ONOO^-$ formation by 50%) were calculated using the dose inhibition curve with Microsoft Excel 97.

RESULTS

$ONOO^-$ Scavenging Activity of Methanolic Alga Extracts. The oxidation of DHR 123 to fluorescent rhodamine 123 mediated by authentic $ONOO^-$ or SIN-1 was determined in the presence of the alga extracts at the same concentration of 5.0 μg/mL noted in Table 1. The potency of alga extracts in the inhibition of DHR 123 oxidation by authentic $ONOO^-$ was in the order of *Symphyocldia latiuscula* (66.6 ± 3.6%) > *Sargassum miyabei* (48.0 ± 1.7%) > *Ecklonia stolonifera* (38.2 ± 2.7%). Moreover, the ability of the alga extracts to scavenge $ONOO^-$ formed from the decomposition of SIN-1, which simultaneously generates $^{\bullet}NO$ and $O_2^{\bullet-}$, was overall similar to their inhibitory effects on scavenging authentic $ONOO^-$. The ability of alga extracts to inhibit $ONOO^-$ from SIN-1 by >50% was *Acrosorium flabellatum* (72.6 ± 1.9%) > *E. stolonifera* (72.5 ± 4.2%) > *Gymnogongrus flabellifornis* (59.6 ± 2.9%) > *S. miyabei* (58.1 ± 2.4%) > *Gelidium amansii* (53.2 ± 2.6%) > *S. latiuscula* (52.9 ± 3.0%).

Table 1. $ONOO^-$ Scavenging Activity of Marine Alga Extracts (5.0 μg/mL)

alga MeOH extract	authentic $ONOO^-$ (%)	$ONOO^-$ from decomposition of SIN-1 (%)
Gigartina tenella	0.6 ± 6.8	48.8 ± 3.0
Gelidium amansii	0.4 ± 1.8	53.2 ± 2.6
Gartelopia elliptica	1.3 ± 4.0	49.1 ± 3.9
Chondrus ocellatus	−8.8 ± 1.3	47.6 ± 1.3
Gymnogongrus flabellifornis	0.9 ± 6.1	59.6 ± 2.9
Galasaura fastigiata Decaisne	−4.0 ± 3.3	42.9 ± 2.3
Lomentaria catenate	−5.6 ± 9.6	11.1 ± 6.2
Sargassum cinereum	−16.1 ± 7.2	39.4 ± 3.3
Sargassum miyabei	48.0 ± 1.7	58.1 ± 2.4
Ulva pertusa	−7.6 ± 9.7	48.7 ± 2.1
Enteropmorphacrinita	−8.7 ± 4.3	47.1 ± 1.9
Rhodymenia intricata	3.8 ± 5.9	44.4 ± 4.7
Sargassum thunbergii	12.2 ± 2.0	42.8 ± 3.1
Acrosorium flabellatum	14.9 ± 5.5	72.6 ± 1.9
Chondrus crispus	3.9 ± 6.8	43.7 ± 1.7
Ecklonia stolonifera	38.2 ± 2.7	72.5 ± 4.2
Symphyocladia latiuscula	66.6 ± 3.6	52.9 ± 3.0
penicillamine	90.9 ± 1.0	85.4 ± 0.2

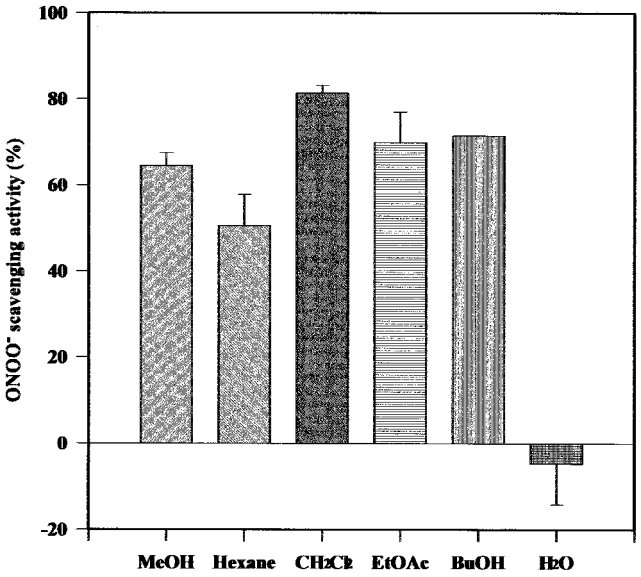

Figure 1. $ONOO^-$ scavenging activity of fractionated *S. latiuscula* (final concentrations: MeOH, 5 μg/mL; others, 2.5 μg/mL).

The results indicate that *S. latiuscula* ($ONOO^-$, 66.6 ± 3.6%; SIN-1, 52.9 ± 3.0%) scavenged both authentic $ONOO^-$ and $ONOO^-$ from SIN-1 most efficiently compared to a well-known $ONOO^-$ scavenger, penicillamine ($ONOO^-$, 90.9 ± 1.0%; SIN-1, 85.4 ± 0.2%).

$ONOO^-$ Scavenging Activity of Fractions from *S. latiuscula*. For further investigation, the $ONOO^-$ scavening activity of each fraction from *S. latiuscula* was also determined. The CH_2Cl_2 fraction of *S. latiuscula* (81.4 ± 1.7%) exhibited the highest $ONOO^-$ scavenging activity at 2.5 μg/mL concentration (Figure 1).

$ONOO^-$ Scavenging Activity of TDB from *S. latiuscula*. TDB from the active fraction of *S. latiuscula* was identified, and its structure is given in Figure 2. Table 2 presents IC_{50} values of the active component for $ONOO^-$, $O_2^{\bullet-}$, and $^{\bullet}NO$ scavenging activities. For $ONOO^-$ scavenging activity, TDB (0.013 ± 0.010 μM) showed a more potent scavenging activity than penicillamine (2.585 ± 0.133 μM). Compared to the antioxidant Trolox (109.950 ± 19.315 μM), TDB (16.432 ± 0.116 μM) was shown to scavenge $O_2^{\bullet-}$ much effectively. TDB

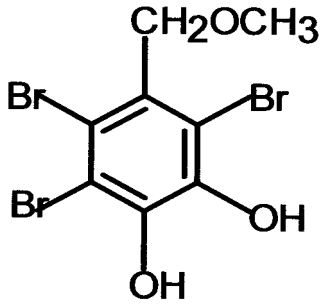

Figure 2. Structure of *S. latiuscula*.

(13.830 ± 0.164 μM) showed a relatively high •NO scavenging activity like carboxy-PTIO (6.433 ± 0.694 μM) as a standard.

Interaction of TDB with ONOO⁻. To establish whether TDB can undergo a nitration reaction after the addition of ONOO⁻, a spectrophotometric analysis to reveal a change in absorbance at 430 nm was used. The interaction of TDB with ONOO⁻ gave no specific change at 430 nm (Figure 3), suggesting that no nitration formation occurred. The absence of peak formation in the reaction of TDB with ONOO⁻ can be explained by an electron donation reaction for its ONOO⁻ scavenging activity.

Table 2. IC_{50} (Micromolar) of TDB on Scavenging Activities[a]

	ONOO⁻	$O_2^{\bullet -}$	•NO
TDB	0.013 ± 0.010	16.432 ± 0.116	13.830 ± 0.164
penicillamine[b]	2.585 ± 0.133		
Trolox[b]		109.950 ± 19.315	
carboxy-PTIO[b]			6.433 ± 0.694

[a] Data are means ± SE for triplicate measurements. [b] Used as a positive control.

Effect of TDB on ONOO⁻-Mediated 3-Nitrotyrosine. A peak at 430 nm was observed in the reaction of tyrosine with ONOO⁻, which results in 3-nitrotyrosine (Figure 4A). Nitration of tyrosine was easily detectable by color change from the colorless to the characteristic yellow color when tyrosine and ONOO⁻ were mixed. Once nitrotyrosine was formed at pH 7.4, the absorbance was maximal at the 420−440 nm range. Incubation of TDB with tyrosine prior to the addition of ONOO⁻ resulted in the disappearance of the nitrotyrosine peak at 430 nm, implying that TDB inhibited the formation of 3-nitrotyrosine.

Effect of TDB on Nitration of BSA and LDL by ONOO⁻. A major product of the reaction of proteins with ONOO⁻ is nitrotyrosine formation (*9*). Monoclonal antibodies specific for nitrotyrosine were used to detect

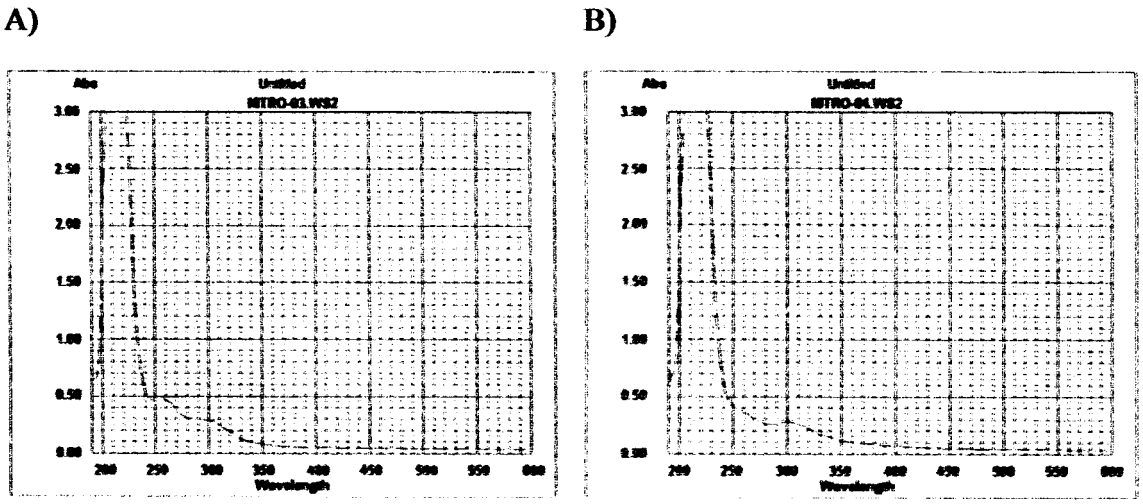

Figure 3. Interaction of TDB with ONOO⁻. TDB (100 μM) was incubated without (A) or with ONOO⁻ (500 μM) (B).

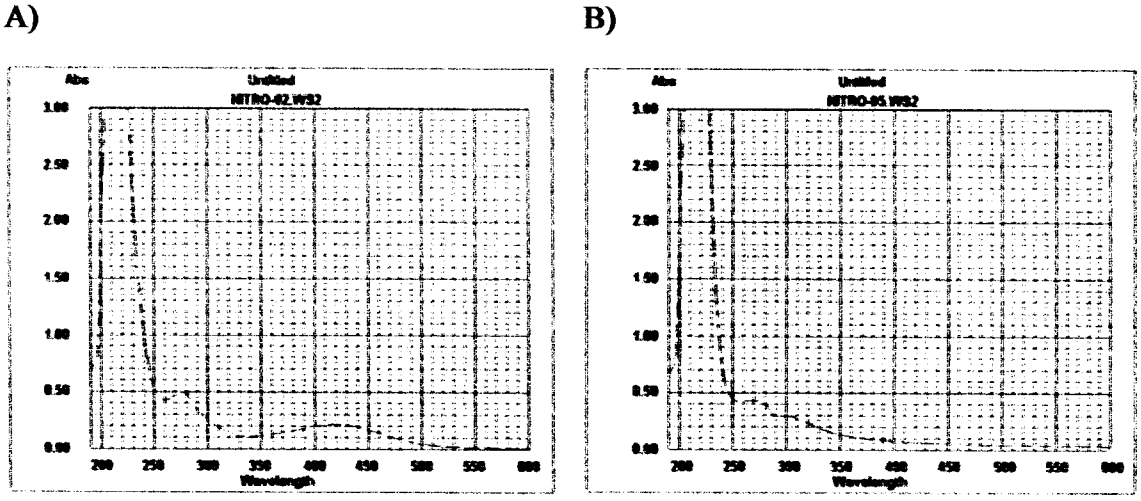

Figure 4. Effect of TDB with ONOO⁻-mediated 3-nitrotyrosine. Tyrosine (100 μM) was incubated without (A) or with TDB (B) prior to the addition of ONOO⁻. Each mixed solution was incubated at 37 °C with shaking for 1 h and scanned between 190 and 600 nm with spectrophotometric analysis. The spectrum of the peak displayed at 430 nm reflects the formation of 3-nitrotyrosine.

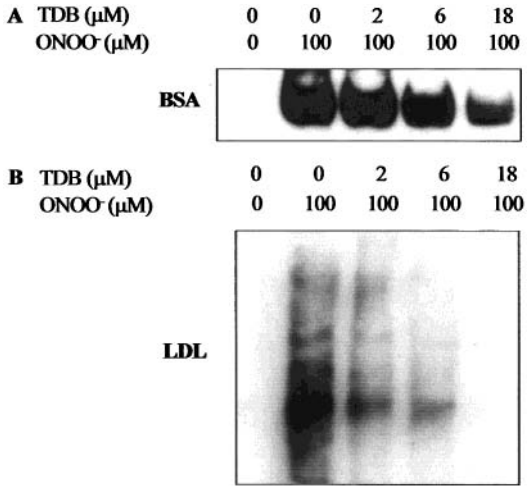

Figure 5. Effect of TDB on the nitration of BSA and LDL by $ONOO^-$. TDB was added to BSA (A) or LDL (B). The reaction samples were incubated with shaking at 25 °C for 1 h. After $ONOO^-$ was added, all samples were further incubated with shaking at 25 °C for 30 min.

Table 3. Effect of TDB on Cell Viability (Percent) of Cultured Bovine Smooth Muscle Cells Exposed to SIN-1 (1 mM) for 6 h[a]

		concn of TDB or PA		
sample		4 μM	20 μM	100 μM
control	100.0 ± 4.1			
SIN-1	24.7 ± 4.6			
SIN-1 + TDB		45.5 ± 1.7	51.1 ± 3.4	57.9 ± 2.4
SIN-1 + PA		46.6 ± 3.0	55.6 ± 1.0	64.6 ± 1.5

[a] Values show the relative percent of cell viability by trypan blue assay. Values are means ± SE for triplicate measurements. SIN-1,3-morpholinosydnonimine; TDB, 2,3,6-tribromo-4,5-dihydroxy benzyl methyl ether; PA, DL-penicillamine.

nitrotyrosine in proteins BSA (0.5 mg of protein/mL) and LDL (1 mg of protein/mL) after treatment with $ONOO^-$ (100 μM). The preincubation of TDB at concentrations of 2, 6, and 18 μM attenuated the nitration of BSA or LDL in a dose-dependent manner as shown in Figure 5.

Effect of TDB on Cell Viability. Cell viability was quantified by trypan blue dye exclusion (Table 3). SMC treated with only SIN-1 showed a marked decrease (24.7 ± 4.6%) in viability compared to the control group (100.0 ± 4.1%). Incubation of TDB at three different concentrations (4, 20, and 100 μM) with SIN-1-treated cells led to increases in the number of viable cells dose dependently.

Effect of TDB on Cell Damage Mediated by $ONOO^-$. The cytotoxicity of $ONOO^-$ was determined by exposing SMC to 500 μM SIN-1 for 6 h. The results indicated that the control group (with only serum-free medium) appeared bright green (Figure 6A), whereas exposure to SIN-1 led to marked cell damage, appearing as an orange color (Figure 6B), and, eventually, cell death after long-term (≥24 h) incubation (data not shown). The SIN-1-pretreated SMCs with 100 μM TDB all retained green color as they enhanced cell protection against $ONOO^-$, compared to the cells treated with SIN-1 only. The SMCs incubated for 24 h with TDB (Figure 6D) showed greater protection than the 12-h-incubated cells (Figure 6C). Penicillamine as a standard showed protection similar to that of TDB (Figure 6E,F).

Effect of TDB on Cell Damage Mediated by $ONOO^-$. In addition to fluorescence microscopy, laser cytometric analysis was used to scan a single cell. The fluorescence distribution within a cell was displayed in different colors. The untreated control SMCs (Figure 7A) gave a bright blue color, whereas a red-orange circularity at the center, probably the nucleus, was observed in the SIN-1-treated cell (Figure 7B). Like penicillamine (Figure 7E,F), TDB (Figure 7C,D) showed protection against cell damage by $ONOO^-$ mediated by SIN-1, which appears green/blue compared to the SIN-1-treated cell.

DISCUSSION

$ONOO^-$ is a mediator of toxicity in inflammatory processes and atherogenesis with strong oxidizing properties toward biological molecules (*28*). The toxicity of $ONOO^-$ can be attributed to nitration of tyrosine and tryptophan residues and alteration of protein function (*8*). Plant-food-derived antioxidants and active principles such as flavonoids, hydroxycinnamates (ferulic acid, chlorogenic acids, vanilin, etc.), β-carotene and other carotenoids, vitamin E, vitamin C, rosemary, sage, tea, and numerous extracts are proposed as important dietary antioxidant substances (*29*). A red alga, *S. latiuscula*, and its component TDB were shown to have strong scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and to inhibit peroxidation of linoleic acid (*30, 31*). The results obtained from the present study show that *S. latiuscula* could effectively inhibit $ONOO^-$ formation either from SIN-1 or with authentic $ONOO^-$ (Table 1). It would seem to be reasonable to use known $ONOO^-$, $^{\bullet}NO$, and $O_2^{\bullet -}$ scavenging agents penicillamine (*26*), carboxy-PTIO (*32*), and Trolox (*33*), respectively, for comparative purposes in this study. Indirectly, the level of $ONOO^-$ can also be controlled by regulation of the levels of $ONOO^-$ precursors, $^{\bullet}NO$ and $O_2^{\bullet -}$. The active component TDB from *S. latiuscula* not only directly enabled $ONOO^-$ scavenging but also involved in inhibition of $O_2^{\bullet -}$ and $^{\bullet}NO$ radical formation (Table 2). Hydroxyl groups in the molecule are responsible for the $ONOO^-$ scavenging activity of the phenolic compound TDB.

The detailed mechanism of the $ONOO^-$ scavenging action of each component is not yet clearly known, but two possible pathways (nitration or electron donation) in the reaction of a phenolic $ONOO^-$ scavenger with $ONOO^-$ were proposed (*26*). The phenolic compound, especially with a monohydroxyl group such as the phenolic amino acid, tyrosine, is preferentially nitrated by $ONOO^-$ (*26*). The nitration has not clarified whether it is derived from the breakdown of $ONOO^-$ to nitrogen dioxide radical ($^{\bullet}NO_2$) or the nitronium ion (NO_2^+) (*26*). As seen in Figure 4A, the conversion of tyrosine into 3-nitrotyrosine by $ONOO^-$ occurred and gave a peak at 430 nm. The increase in absorbance at 430 nm by a $ONOO^-$ scavenger strongly implies nitration. However, unlike tyrosine, nitration was not seen with TDB as the reaction of TDB with $ONOO^-$ produced no peak at 430 nm. In this respect, the electron transfer reaction of TDB for its $ONOO^-$ scavenging activity was believed to occur. TDB was also examined for its involvement with the reaction of tyrosine and $ONOO^-$. After the addition of TDB, the peak of 3-nitrotyrosine was no longer observed. This result suggests that TDB directly blocked the formation of 3-nitrotyrosine by $ONOO^-$ or possibly reduced the availability of $ONOO^-$.

BSA, the most abundant protein in plasma for the maintenance of colloid osmotic pressure and the trans-

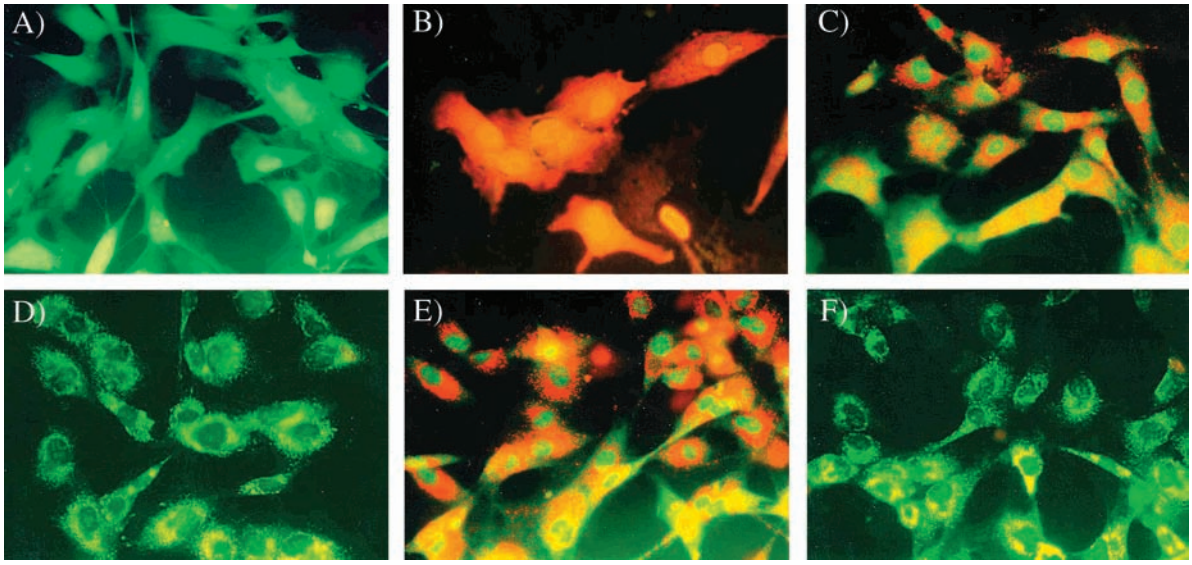

Figure 6. Protection of SIN-1-treated smooth muscle cell by TDB using laser cytometry: no treatment (serum-free media only) (A); SIN-1 (B); SIN-1 + TDB for 12 h (C); SIN-1 + TDB for 24 h (D); SIN-1 + penicillamine for 12 h (E); SIN-1 + penicillamine for 24 h (F).

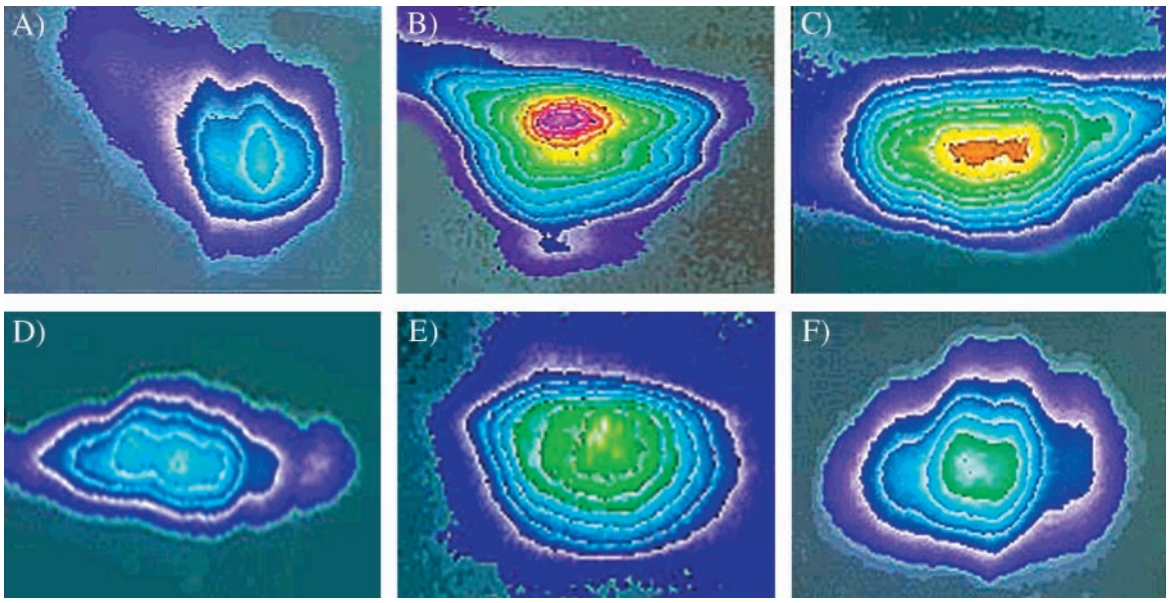

Figure 7. Protection of SIN-1-treated smooth muscle cell by TDB using fluorescence microscopy: no treatment (serum-free media only) (A); SIN-1 (B); SIN-1 + TDB for 12 h (C); SIN-1 + TDB for 24 h (D); SIN-1 + penicillamine for 12 h (E); SIN-1 + penicillamine for 24 h (F).

port of different ligands, and LDL of human plasma were used as models for $ONOO^-$ reactivity toward proteins. Protein tyrosine nitration by $ONOO^-$ may interfere with phosphorylation/dephosphorylation signaling pathways and alter enzyme functions (*34*−*36*).

It was demonstrated by Beckman and Koppenol (*37*) that nitrotyrosine was formed in atherosclerotic lesions of human coronary arteries. High plasma total cholesterol and LDL values showed significant correlation with development of atherosclerosis and cardiovascular diseases (*38*). $ONOO^-$, formed in the arterial wall from the reaction of $O_2^{\bullet -}$ with $^{\bullet}NO$, can initiate lipid peroxidation and oxidative damage to LDL and leave arteries susceptible to chronic inflammation, causing modification of arterial gene expression and promotion of lesion development (*38*). In vitro nitrated and oxidized LDLs were known to be taken up and degraded by monocyte-macrophages and smooth muscle cells, leading to excessive accumulation of cholesterol and foam cell formations that develop atherosclerotic lesions (*39*−*41*). Several reports demonstrated that catechin polyphenols, salicylate, and aminoethylcysteine ketimine decarboxylated dimer could protect against $ONOO^-$-induced modification of LDL (*26*, *42*, *43*). In the present study, evidence from Western blot analysis indicated that TDB even at concentrations as low as 2 μM could significantly reduce nitrotyrosine present in BSA and LDL.

Treatment with TDB enhanced SMC survival and gave cytoprotection against $ONOO^-$ similar to that shown by penicillamine. $ONOO^-$ plays a role in cytotoxicity in that a low dose of $ONOO^-$ has been implicated in apoptosis, whereas its high levels cause necrosis (*44*). Morphological changes during the apoptotic process include plasma membrane blebbing, membrane integrity, chromatin condensation, formation of apoptotic bodies, cell shrinkage, cytoplasmic vacuolization, and DNA fragmentation (*45*−*47*). Cell death appeared to

proceed gradually from the outer part of the cytoplasm toward the nucleus, accompanied by cytoplasmic vacuolization. The results of laser cytometric analysis were consistent with those of the fluorescence microscopic analysis. SIN-1 induced the morphological changes of the cells due to the cytotoxic effect of $ONOO^-$ compared with the untreated control group. Cellular injury by SIN-1 was attenuated by TDB. TDB provided cytoprotection from damage induced by $ONOO^-$ as significant changes from red-orange fluorescing cells to viable, green fluorescing cells were observed.

In conclusion, TDB, containing a number of hydroxyl groups, scavenged $ONOO^-$ efficiently through electron donation. The significant $ONOO^-$ scavenging properties of TDB can be useful in the prevention and treatment of $ONOO^-$-related diseases such as Alzheimer's disease, rheumatoid arthritis, cancer, inflammation, and atherosclerosis.

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