Novel Nitric Oxide and Superoxide Generation Inhibitors, Persenone A and B, from Avocado Fruit

Oe Kyung Kim,[†] Akira Murakami,[‡] Yoshimasa Nakamura,[†] Naohito Takeda,[§] Hideo Yoshizumi,[§] and Hajime Ohigashi*[,†]

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan; Department of Biotechnological Science, Faculty of Biology-Oriented Science and Technology, Kinki University, Iwade-Uchida, Wakayama 649-6493, Japan; and Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan

One known, (2*R*)-(12*Z*,15*Z*)-2-hydroxy-4-oxoheneicosa-12,15-dien-1-yl acetate (**1**), and two novel compounds, persenone A (**2**) and B (**3**), have been isolated from avocado fruit (*Persea americana* P. Mill), as inhibitors of superoxide (O_2^-) and nitric oxide (NO) generation in cell culture systems. They showed marked inhibitory activities toward NO generation induced by lipopolysaccharide in combination with interferon-γ in mouse macrophage RAW 264.7 cells. Their inhibitory potencies of NO generation (**1**, $IC_{50} = 3.6$; **2**, $IC_{50} = 1.2$; and **3**, $IC_{50} = 3.5$ μM) were comparable to or higher than that of a natural NO generation inhibitor, docosahexaenoic acid (DHA; $IC_{50} = 4.3$ μM). Furthermore, compounds **1**−**3** and DHA markedly suppressed tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate-induced O_2^- generation in differentiated human promyelocytic HL-60 cells (**1**, $IC_{50} = 33.7$; **2**, $IC_{50} = 1.4$; **3**, $IC_{50} = 1.8$; and DHA, $IC_{50} = 10.3$ μM). It is notable that they were found to be suppressors of both NO- and O_2^--generating biochemical pathways but not to be radical scavengers. The results indicate that these compounds are unique antioxidants, preferentially suppressing radical generation, and thus may be promising as effective chemopreventive agent candidates in inflammation-associated carcinogenesis.

Keywords: *Cancer chemoprevention; nitric oxide; superoxide; avocado; RAW 264.7 cells; HL-60 cells*

INTRODUCTION

One of the most effective strategies for cancer control is chemoprevention (Wattenberg, 1985; Tanaka, 1992). A variety of approaches directed toward biochemical phenomena in carcinogenesis have led to the discovery of several types of chemopreventive agents. Using screening tests for the inhibition of tumor-promoter-induced Epstein−Barr virus (EBV) activation (Koshimizu et al., 1988), we have so far isolated and identified several chemopreventive agents from edible plants, for example, 1′-acetoxychavicol acetate (ACA) (Kondo et al., 1993; Murakami et al., 1994, 1996; Ohnishi et al., 1996; Tanaka et al., 1997a,b), cardamonin (Murakami et al., 1993), glyceroglycolipids (Murakami et al., 1995), pheophorbides (Nakamura et al., 1996a,b), auraptene (Murakami et al., 1997; Tanaka et al., 1997c, 1998), and curcumin (Ohigashi et al., 1994), which was reported as a chemopreventive agent by Conney et al. (1997), Huang et al. (1991), and Lu et al. (1994). In our search for another line of effective chemopreventive agents, we have recently focused on a new bioassay system measuring the inhibition of nitric oxide (NO) generation.

Formation of NO from the guanidino nitrogen group of L-arginine is catalyzed by NO synthase (NOS). At least three distinct isoforms of NOSs have been identified (Ignarro et al., 1993). The first (eNOS) is a constitutive Ca^{2+}- and calmodulin-dependent membrane-bound isoform present in endothelial cells, which is known to play an important role in the dynamic control of vascular tone. The second (nNOS) is a constitutive Ca^{2+}- and calmodulin-dependent soluble isoform, present mainly in neural tissues. NO synthesized by this enzyme acts as a neurotransmitter. The third is an inducible Ca^{2+}-independent isoform (iNOS), which is induced by either bacterial lipopolysaccharide (LPS) or a number of cytokines including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) in macrophages, hepatocytes, and endothelial cells. iNOS induction is known to be involved in immune and inflammatory responses (Vane et al., 1994).

NO is a reactive radical that may react simultaneously with superoxide (O_2^-) to form highly cytotoxic peroxynitrite anion ($ONOO^-$) in inflammatory tissues, which may cause DNA damage (Szabo et al., 1997). It also reacts directly with a variety of enzymes and other proteins to either activate or inhibit their function by oxidizing SH groups, forming complexes with metal ions, or reacting with tyrosine residues (Szabo et al., 1997). Moreover, $ONOO^-$ activates cyclooxygenase and thus stimulates prostaglandin biosynthesis, in vivo, in perfused organs and macrophages (Salvemini et al., 1993; Landino et al., 1996). Collectively, generation of excess amounts of NO through iNOS functions may be closely associated with multistage processes of inflammatory carcinogenesis, such as those in the colon

* Corresponding author (telephone +81-75-753-6281; fax +81-75-753-6284; e-mail ohigashi@kais.kyoto-u.ac.jp).
[†] Kyoto University.
[‡] Kinki University.
[§] Meijo University.

10.1021/jf990977r CCC: $19.00
Published on Web 04/06/2000

(Takahashi et al., 1997). It is therefore widely considered that inhibition of excessive NO generation in inflammatory cells is beneficial for the prevention of carcinogenesis.

In a previous study, we examined the inhibitory properties of edible Japanese plants toward LPS/IFN-γ-induced NO generation in RAW 264.7 cells (Kim et al., 1998). In those tests, a methanol extract from avocado fruit (*Persea americana* P. Mill) showed notably higher NO generation inhibitory activity. Therefore, in the present study, we searched for the active principles and isolated one known and two novel NO generation inhibitors from avocado fruit in the RAW 264.7 murine macrophage cell line stimulated with LPS/IFN-γ. Furthermore, these compounds showed O_2^- generation inhibitory activity in differentiated human promyelocytic HL-60 cells.

MATERIALS AND METHODS

General Procedures. Fast atom bombardment mass spectra (FAB-MS) were obtained on a JEOL HX-100 (JEOL DATUM, Osaka, Japan). An infrared (IR) spectrum was taken on a Shimadzu model DR 8030 (Shimadzu Co. Ltd., Kyoto, Japan). An ultraviolet (UV) spectrum was taken on a Shimadzu model UV-2200AI (Shimadzu Co. Ltd.). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-300 using tetramethylsilane (TMS) as an internal standard (δ 0.00). Optical rotation was measured by a JASCO DIP-4 (JASCO Co. Ltd., Tokyo, Japan). Chromatographic materials used were as follows: Wako gel C-100, C-200, and C-300 (Wako Pure Chemical Industries Co. Ltd., Osaka, Japan), Keiselgel 60 F_{254} for TLC (Merck Co. Ltd., Darmstadt, Germany), and ODS gel $KC_{18}F$ for TLC (Whatman, Clifton, NJ).

Chemicals and Cells. L-Arginine and (6*R*)-tetrahydro-L-biopterin (BH_4), along with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO) and RPMI 1640 medium and fetal bovine serum (FBS) from Gibco BRL (Grand Island, NY). LPS (*Escherichia coli* serotype 0127, B8) was purchased from Difco Labs (Detroit, MI) and IFN-γ from Genzyme (Cambridge, MA). Other chemicals were purchased from Wako Pure Chemicals Co. Ltd.. RAW 264.7 cells were a gift from Dr. Tatsumi (Ohtsuka Pharmaceutical Co. Ltd.), and human promyelocytic leukemia HL-60 cells (Collins et al., 1977) were obtained from the Health Science Research Resources Bank (Osaka, Japan).

Isolation and Identification of Active Avocado Constituents. The fresh edible part of an avocado (*P. americana*, 350 g) was cut into small pieces (~1 cm^3) and extracted with 2 L of methanol (MeOH) at room temperature for 2 weeks. The active compounds were traced using the inhibitory assay of NO generation in RAW 264.7 cells stimulated by LPS in combination with IFN-γ. The active constituent was traced by testing each sample derived from the MeOH extract at a concentration of 100 μg/mL in the inhibitory assay of NO generation. The MeOH extract (9 g), obtained by filtration, showed an 83.4% inhibition of NO_2^- generation. Next, the extract was partitioned between water and *n*-hexane. The *n*-hexane layer (2.5 g, 85.4% inhibition) was then chromatographed on Wako gel C-100, eluting stepwise with *n*-hexane containing increasing amounts of ethyl acetate (EtOAc) to give an active 20% EtOAc eluate (1.2 g, 84.4% inhibition). This fraction was further chromatographed on Wako gel C-200, eluting stepwise with chloroform containing increasing amounts of acetone to give active 5% acetone eluate (644 mg, 88.1% inhibition). The active fraction was then purified by preparative HPLC on a YMC-Pack ODS column (150 × 20 mm, YMC Co. Ltd., Kyoto, Japan), eluted with 67% MeCN/H_2O (flow rate = 7 mL/min, detection = 224 nm) to give an active fraction. Final purification was performed by preparative TLC on silica gel ($CHCl_3$/acetone = 10:1) to yield **1** (19 mg), **2** (11.7 mg), and **3** (3.0 mg). To detect the bands on TLC, the edge of each TLC (~2 cm) was cut off and sprayed with 5% H_2SO_4 in EtOH followed by heating.

Table 1. ^{1}H and ^{13}C NMR Data of PA-3 (1)

	3		
position	δ_H	δ_C	lit.[a] δ_H
1	4.13, 2H, m	67.2	4.10, 2H, m
2	4.30, 1H, d	66.0	4.30, 1H, br m
3	2.67, 2H, d	27.22	2.60, 2H, d
4			
5	2.44, 2H, t, J = 7.4 Hz	27.1	2.45, 2H, t, J = 7.5 Hz
6	1.23−1.40, 16H, br s	45.2	1.26, 16H, br s
7	1.23−1.40, 16H, br s	29.2	1.26, 16H, br s
8	1.23−1.40, 16H, br s	29.3	1.26, 16H, br s
9	1.23−1.40, 16H, br s	29.6	1.26, 16H, br s
10	1.23−1.40, 16H, br s	31.5	1.26, 16H, br s
11	2.03, 4H, d		1.8−2.2, 4H, m
12	5.27−5.42, 4H, m	130.2	5.36, 4H, m
13	5.27−5.42, 4H, m	130.0	5.36, 4H, m
14	2.77, 2H, t	43.6	2.77, 2H, t
15	5.27−5.42, 4H, m	128.1	5.36, 4H, m
16	5.27−5.42, 4H, m	127.9	5.36, 4H, m
17	2.03, 4H, d	27.2	1.8−2.2, 4H, m
18	1.23−1.40, 16H, br s	25.6	1.26, 16H, br s
19	1.23−1.40, 16H, br s	23.5	1.26, 16H, br s
20	1.23−1.40, 16H, br s	22.5	1.26, 16H, br s
21	0/.89, 3H, t J = 6.7 Hz	14.0	0.88, 3H, t, J = 6.0 Hz
OH	3.20 1H, d		3.15, 1H, br s
$\underline{CH_3}CO$	2.09, 3H, s	20.8	2.09, 3H, s
$\overline{CH_3\underline{CO}}$		170.9	

[a] Chang et al., 1975.

Table 2. ^{1}H and ^{13}C NMR Data of Persenone (2)

position	δ_H	δ_C	m[a]	J (Hz)	DEPT	HMBC
1	4.13	67.8	s		CH_2	
2	4.34	66.7	m		CH	
3	2.78	26.1	m		CH_2	C-1, C-2, C-4
4		200.0			C	
5	6.12	130.8	d	16	CH	C-7
6	6.88	149.7	dt	16, 6.9	CH	C-4
7	2.24	32.0	m		CH_2	
8	1.27−1.37	29.86	m		CH_2	
9	1.27−1.37	29.82	m		CH_2	
10	1.27−1.37	29.3	m		CH_2	
11	2.04	33.0	m		CH_2	C-9, C-10
12	5.29−5.41	130.1	m		CH	
13	5.29−5.41	128.8	m		CH	
14	2.78	42.8	m		CH_2	C-12, C-13
15	5.29−5.41	128.2	m		CH	
16	5.29−5.41	126.4	m		CH	
17	2.04	27.5	m		CH_2	C-15, C-16
18	1.27−1.37	27.7	m		CH_2	
19	1.27−1.37	28.4	m		CH_2	
20	1.27−1.37	23.0	m		CH_2	
21	0.88	14.5	t	6.7		
OH	3.30		s		CH_3	C-19, C-20
$\underline{CH_3}CO$	2.09	2.13	s		CH_3	
$\overline{CH_3\underline{CO}}$		171.4			C	

[a] Multiplicity.

(2*R*)-(12*Z*,15*Z*)-2-Hydroxy-4-oxoheneicosa-12,15-dien-1-yl acetate (**1**) was obtained as a colorless oil: $[\alpha]^{22}_D$ +10.5° (*c* 0.26, $CHCl_3$); IR ν_{max} (KBr) 3300 (OH), 1740 (OCO), 1710 (CO), 1370, 1240, 1040 cm^{-1}; FAB-MS [*m*-nitrobenzyl alcohol (*m*NBA) as a matrix], *m*/*z* 403 $[M + Na]^+$, 381 $[M + H]^+$; HR-FAB-MS (*m*NBA as a matrix), *m*/*z* 381.3005 ($[C_{23}H_{40}O_4 + H]^+$, calcd for 381.3005); ^{1}H and ^{13}C NMR, see Table 1.

Persenone A (**2**) was obtained as a colorless oil: $[\alpha]^{22}_D$ +17.6° (*c* 0.48, $CHCl_3$); UV λ_{max} (MeCN) (ϵ) 224 (9000) nm; IR ν_{max} (KBr) 3300 (OH), 1740 (OCO), 1660, 1625, 1240, 1370, 1240, 1040, 980 cm^{-1}; FAB-MS (*m*NBA as a matrix), *m*/*z* 401 $[M + Na]^+$, 379 $[M + H]^+$; HR-FAB-MS (*m*NBA as a matrix), *m*/*z* 379.2848 ($[C_{23}H_{38}O_4 + H]^+$, calcd for 379.2848); ^{1}H and ^{13}C NMR, see Table 2.

Persenone B (**3**) was obtained as a colorless oil: $[\alpha]^{22}_D$ +8.3° (*c* 0.33, $CHCl_3$); UV λ_{max} (MeCN) (ϵ) 222 (3200) nm; IR ν_{max}

Table 3. ^{1}H and ^{13}C NMR Data of Persenone B (3)

position	δ_H	δ_C	m^a	J (Hz)	DEPT	HMBC
1	4.12	67.3	m		CH_2	
2	4.34	66.2	br s		CH	
3	2.76		m		CH_2	C-1, C-2, C-4
4		199.6			C	
5	6.11	130.3	d	16	CH	
6	6.88	149.5	dt	16, 6.9	CH	C-4, C-8
7	2.23	32.5	dd	14, 6.9	CH_2	C-5, C-6
8	1.47	31.9	s		CH_2	
9	1.26−1.29	29.6	m		CH_2	
10	1.26−1.29	29.5	m		CH_2	
11	1.26−1.29		m		CH_2	
12	1.26−1.29	125.9	m		CH_2	
13	1.26−1.29	42.3	m		CH_2	
14	1.26−1.29	28.0	m		CH_2	
15	1.26−1.29	29.3	m		CH_2	
16	1.26−1.29	29.2	m		CH_2	
17	1.26−1.29	22.6	m		CH_2	
18	1.26−1.29	20.8	m		CH_2	
19	0.88	14.1	t	6.7	CH_3	C-17, C-18
OH	3.30		s		CH_3	C-19, C-20
$\underline{CH_3}CO$	2.11		s		CH_3	
$\underline{CH_3CO}$		170.9			C	

*Multiplicity.

(KBr) 3300 (OH), 1740 (OCO), 1660, 1625, 1240, 1370, 1240, 1040, 980 cm^{-1}; FAB-MS (*m*NBA as a matrix), *m*/*z* 377 [M + Na]$^+$, 355 [M + H]$^+$; HR-FAB-MS (*m*NBA as a matrix), *m*/*z* 377.2668 ([$C_{21}H_{38}O_4$ + Na]$^+$, calcd for 377.2670), *m*/*z* 355.2848 ([$C_{21}H_{38}O_4$ + H]$^+$, calcd for 355.2848); ^{1}H and ^{13}C NMR, see Table 3.

LPS/IFN-γ-Induced NO Generation Test. The murine macrophage cell line RAW 264.7 was cultivated in RPMI medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 (Tayeh et al., 1989). The cells (2×10^5 cells/mL) were treated with LPS (100 ng/mL), BH_4 (10 μg/mL), IFN-γ (100 units/mL), L-arginine (2 mM), and the appropriate concentrations of test samples dissolved in 5 μL of DMSO. Cells treated without any test compounds but with LPS, BH_4, IFN-γ, L-arginine, and 5 μL of DMSO were used as a positive control. After 24 h, the levels of nitrite (NO_2^-), L-citrulline, and cell viability were measured as described below.

Measurement of NO_2^- Formation. NO_2^- production, an indicator of NO synthesis, was determined in the supernatant of the media by Griess reaction as reported previously (Green et al., 1982). After the 24 h incubation mentioned above, the supernatants (0.5 mL) were added to a solution of 0.5 mL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4) to form a purple azodye with an absorption maximum at 543 nm.

Measurement of L-Citrulline. L-Citrulline was formed from L-arginine by iNOS activity. Its level in the medium was determined colorimetrically by reaction of the medium supernatant with diacetyl monoxime as reported previously (Boyde et al., 1980). This method is based on the fact that carbamino compounds, such as urea and L-citrulline, react with diacetyl monoxime in the presence of both sulfuric and phosphoric acids to form a color complex. Briefly, to 0.4 mL of the supernatant was added 0.6 mL of a chromogenic reagent (5 mg of thiosemicarbazide in reagent 1/reagent 2 = 2:1), and the reaction mixture was heated at 100 °C for 5 min. The visible absorption of the mixture at 530 nm was then measured. Reagent 1 was 250 mL of concentrated sulfuric acid and 200 mL of concentrated phosphoric acid in 550 mL of distilled water. $FeCl_3$ (250 mg/L) was added to the solution. Reagent 2 was 500 mg of diacetyl monoxime in 100 mL of distilled water.

Cell Viability. Mitochondrial respiration, an indicator of cell viability, was assayed by the mitochondrial-dependent reduction of 3-(4,5-dimethiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan (Sladowski et al., 1992). Cells in 24-well plates were incubated with MTT (0.25 mg/mL) for 4 h. The cells were solubilized in 0.04 N HCl in 2-propanol. The extent of reduction of MTT to formazan within the cells was quantitated by the measurement of absorbance at 570 nm.

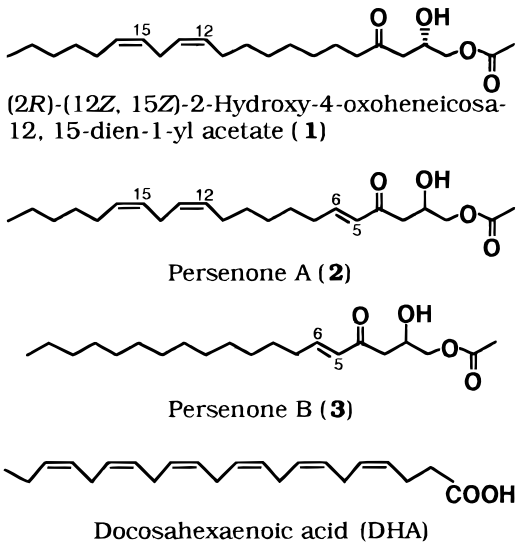

Figure 1. Structures of (2*R*)-(12*Z*,15*Z*)-2-hydroxy-4-oxoheneicosa-12,15-dien-1-yl acetate (**1**), persenone A (**2**), B (**3**), and DHA.

TPA-Induced O_2^- Generation Test. Inhibitory tests of TPA-induced superoxide generation were conducted as previously reported (Nakamura et al., 1998a). Human promyelocytic leukemia HL-60 cells were cultivated at 4×10^5 cells/mL in RPMI 1640 supplemented with 10% FBS. The cells were preincubated with 1.25% DMSO at 37 °C in a 5% CO_2 incubator for 6 days to induce their differentiation into granulocyte-like cells. The cells were then washed with Hank's buffer and suspended at a density of 1×10^6 cells/mL. Test compound dissolved in 5 μL of DMSO or 5 μL of DMSO (positive control) was added to the cell suspension, and the mixture thus obtained was incubated at 37 °C for 15 min. The cells were washed with Hank's buffer twice to remove extracellular test compounds. Next, 5 μL of TPA solution (20 μM) was added to the cell suspension. After 90 s, 50 μL of cytochrome *c* solution (20 mg/mL) was added to the reaction mixture, which was incubated for another 15 min. The reaction mixture was cooled on ice and centrifuged at 2000*g* for 1 min. The visible absorption of the supernatant at 550 nm, which is due to the formation of reduced cytochrome *c* by the reaction with O_2^-, was measured. Cell viability was measured by a trypan blue exclusion test.

RESULTS AND DISCUSSION

Isolation and Identification of NO Generation Inhibitors from Avocado. The active principles were traced by the inhibitory assay of NO generation in RAW 264.7 cells stimulated by LPS in combination with IFN-γ. Fresh *P. americana* fruits were extracted with MeOH, and the extract was partitioned between *n*-hexane and water. The active *n*-hexane layer was chromatographed on silica gel to afford an active fraction eluated with 20% EtOAc in *n*-hexane. This fraction was chromatographed on silica, ODS gel, preparative HPLC, and preparative TLC to yield compounds **1**−**3**. On the basis of the spectral data, compound **1** was identified as (2*R*)-(12*Z*,15*Z*)-2-hydroxy-4-oxoheneicosa-12,15-dien-1-yl acetate (Figure 1), which has been previously isolated from avocado leaves (Chang et al., 1975) and fruits as an antifungal compound (Prusky et al., 1982).

In the ^{1}H NMR spectrum of **2**, a marked difference from **1** was found only in the presence of two doublet signals [δ 6.12 (1H, d, J = 16 Hz) and δ 6.88 (1H, dt, J = 16, 6.9 Hz)], indicating the presence of a *trans*-olefin. The ^{1}H, ^{13}C, distortionless enhancement by polarization transfer (DEPT) and ^{1}H-detected multiple-bond hetero-

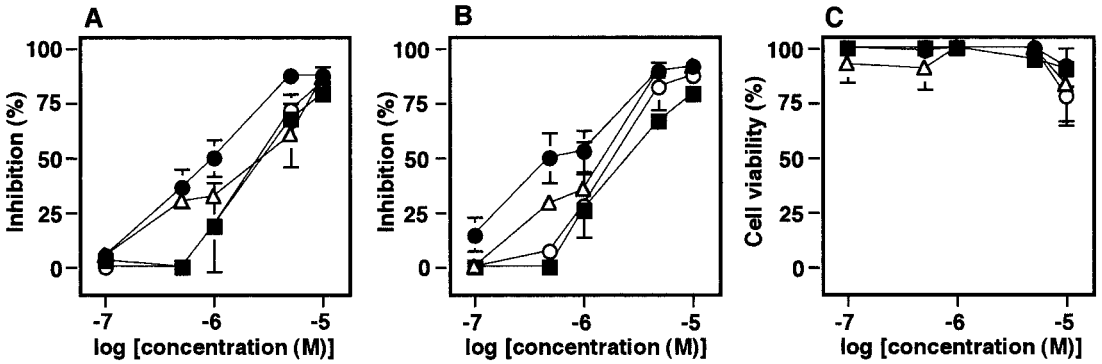

Figure 2. Inhibitory effects of test compounds against NO generation stimulated by LPS and IFN-γ in mouse macrophage RAW 264.7 cells: (A) NO_2^- inhibition; (B) L-citrulline inhibition; (C) cell viability; (■) **1**; (●) **2**; (△) **3**; (○) DHA. Data are expressed as means ± SE ($n = 4$). RAW 264.7 cells (2×10^5 cells/mL) were treated with LPS (100 ng/mL), BH_4 (10 μg/mL), IFN-γ (100 units/mL), L-arginine (2 mM), and the appropriate concentrations of test samples dissolved in 5 μL of DMSO. After 24 h, the levels of NO_2^-, L-citrulline, and cell viability were measured.

nuclear multiple bond connectivity (HMBC) NMR data of **2** are shown in Table 2. The HMBC spectrum showed the two- or three-bond couplings between C-4 and H-3, 6, and between C-7 and H-5. The presence of an *enone* group in **2** was supported by the UV absorption maximum at 224 nm ($\epsilon = 9000$). The IR spectrum of **2** revealed 980 cm^{-1}, which is characteristic of a trans double bond. The remaining spectral data of **2** were in good agreement with those of **1**. In the high-resolution FAB MS, a peak corresponding to $[M + H]^+$ was observed at *m/z*: 379.2848 ($[C_{23}H_{38}O_4 + H]^+$, calcd for 379.2848), showing the molecular formula of **2** to be $C_{23}H_{38}O_4$. Thus, the structure of **2** was determined as (12*Z*,15*Z*)-2-hydroxy-4-oxoheneicosa-5,12,15-trien-1-yl acetate (Figure 1).

In the ^1H NMR spectrum of **3**, the four *cis*-olefin signals present in both **1** and **2** disappeared. Compound **3** showed peaks at *m/z* 377.2668 ($[C_{21}H_{38}O_4 + Na]^+$, calcd for 377. 2670) and 355.2848 ($[C_{21}H_{38}O_4 + H]^+$, calcd for 355.2848) in the high-resolution FAB MS. Thus, **3** was identified to be a 2-hydroxy-4-oxononadeca-5-en-1-yl acetate. The absolute configuration at C-2 of **2** and **3** has not been determined yet. Compounds **2** and **3** were named persenone A and B, respectively.

Inhibitory Effects of 1—3 and DHA on LPS/IFN-γ-Induced NO Generation in RAW 264.7 Cells. NO generation was measured by the formation of both NO_2^- and L-citrulline. As noted above, L-citrulline is formed from L-arginine by iNOS, and the resultant NO is then converted to NO_2^-. RAW 264.7 cells were cultured in the presence of test compounds stimulated with LPS and IFN-γ. Cells incubated in the absence of the samples and treated only with LPS and IFN-γ were used as controls. Docosahexaenoic acid (DHA) (Figure 1), reported previously as a naturally occurring iNOS induction inhibitor (Ohata et al., 1997), was used as a positive control. As shown in Figure 2, compounds **1**−**3** and DHA inhibited NO generation concentration-dependently in stimulated macrophages with significant cell viability. The inhibitory potencies of NO_2^- generation for **1**−**3** (**1**, $IC_{50} = 3.6$; **2**, 1.2; and **3**, 3.5 μM, respectively) were comparable to or higher than those of DHA ($IC_{50} = 4.3$ μM), the same results as for the inhibitory potencies of L-citrulline generation (**1**, $IC_{50} = 3.3$; **2**, 0.5; **3**: 2.0; and DHA, 2.5 μM, respectively). The presence of both the *enone* and *cis*−*cis diene* may be increasing factors for NO generation inhibition because the activity of **2** was higher than those of **1** and **3** when compared with their IC_{50} values (although statistically not significant). It has been reported that nonsteroidal anti-inflammatory drugs (NSAIDS) such as sodium salicylate, aspirin, ibuprofen, and indomethacin inhibited the expression of the *iNOS* gene in NR8383 cells (Aeberhard et al., 1995). NSAIDS have been reported to suppress colorectal carcinogenesis (Giardiello et al., 1993; Labayle et al., 1991; Reddy et al., 1993) with which iNOS-mediated inflammation is closely associated. Also, dimethylhydrazine- and azoxymethane-induced aberrant crypt foci (ACF) formation and the incidence of carcinogenesis in rat colons were significantly reduced by diets containing *n*−3 type polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) and DHA (Ninoura et al., 1988; Reddy et al., 1988; Takahashi et al., 1993). Moreover, DHA was found to reduce the intestinal polyp numbers in $Apc^{\Delta 716}$ knockout mice (Oshima et al., 1995). Furthermore, ACA suppressed NO generation in RAW 264.7 cells concentration-dependently, which was shown to be parallel to the reduced expression of iNOS mRNA and protein (Ohata et al., 1998). ACA also inhibited azoxymethane-induced colonic ACF formation and tumorigenesis when fed to rats during the initiation or postinitiation phase (Tanaka et al., 1997a,b). Thus, we suggest that compounds **1**−**3** are promising chemopreventive agent candidates in digestive organs, including the colon.

Inhibitory Effects of 1—3 against O_2^- Generation. Numerous studies have thus far demonstrated that reactive oxygen intermediates, originated from O_2^-, interact with nucleosides and form oxidative DNA bases which induce a specific mutational spectrum considered to be a signature of oxidant-mediated DNA damage (Nguyen et al., 1992). In particular, TPA-type tumor promoters are reported to trigger O_2^- generation in epithelial cells and leukocytes, through the xanthine/xanthine oxidase (XA/XOD) and NADPH oxidase systems, respectively (Cross et al., 1991; Reiners et al., 1987). As shown in Figure 3, the inhibitory potencies of **1**−**3** and DHA with regard to O_2^- generation (**1**, $IC_{50} = 33.7$; **2**, 1.4; **3**, 1.8; and DHA, 10.3 μM) were remarkable, and they maintained significant cell viability at each concentration tested. Compounds **1**−**3** or DHA did not scavenge O_2^- at concentrations of up to 100 μM in the XA/XOD system (data not shown), indicating that they preferentially suppress the NADPH oxidase system responsible for O_2^- generation in differentiated HL-60 cells, similar to the NO assay. The *enone* group, but not the cis double bond, was indicated as an important

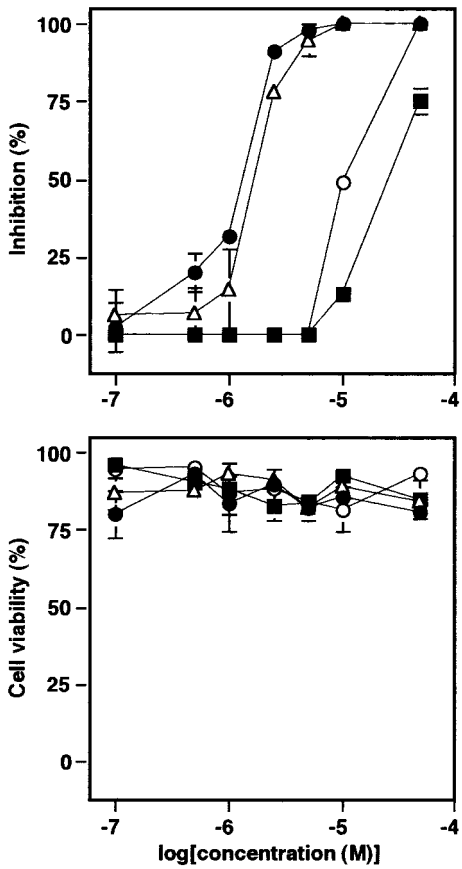

Figure 3. Inhibitory effects of test compounds against O_2^- generation in differentiated human promyelocytic HL-60 cells: (■) **1**; (●) **2**; (△) **3**; (○) DHA. Data are expressed as means ± SE (n = 4). HL-60 cells were preincubated with 1.25% DMSO at 37 °C for 6 days, differentiating them into granulocyte-like cells. Sample solution was added to the cell suspension, and the mixture was incubated at 37 °C for 15 min. Five microliters of TPA solution (20 μM) was added to the cell suspension (1 mL), which was incubated for 90 s to produce O_2^-. Then, 50 μL of cytochrome c solution (20 mg/mL) was added to the reaction mixture, which was incubated for another 15 min. The reaction mixture was centrifuged, and visible absorption at 550 nm was measured. Cell viability was measured by a trypan blue exclusion test.

structural factor for O_2^- generation inhibition, because the activities of **2** and **3** were equal to each other and exceeded those of **1** and DHA. ACA and aurcaptene [IC_{50} = 4.3 and 1.2 μM, respectively (Murakami et al., 1996, 1997)] have recently been reported as potent O_2^- generation inhibitors. They were originally identified as inhibitors of tumor promoter TPA-induced EBV activation in Raji cells and clarified to possess anti-tumor-promoting activity in a two-stage carcinogenesis experiment with 7,12-dimethylbenz[*a*]anthracene (DMBA) and TPA in ICR mouse skin (Murakami et al., 1996, 1997). Furthermore, they showed a marked reduction in the incidence of tumor marker ACF (Tanaka et al., 1997a,c) or colon adenocarcinomas (Tanaka et al., 1997b). It is important to note that colon carcinogenesis is associated with the generation of reactive oxygen species such as O_2^- on the surface of the intestinal lumen, and inflammatory cells in close proximity to the colon can produce reactive nitrogen species such as NO_2^- (Moghadasian et al., 1996; Nonaka et al., 1993; Stone et al., 1997). The treatments of mouse skin with TPA significantly increased H_2O_2 formation as well as myeloperoxidase (MPO) activity, a biomarker for infiltration of inflammatory cells into the target tissue, which led to chronic inflammation or tumor promotion (Wei et al., 1992). ACA completely inhibited H_2O_2 formation and reduced MPO activity (Nakamura et al., 1998b). These results suggested that inhibition of O_2^- generation in leukocytes, at least in part, might be important for the suppression of oxidative stress and hence tumor promotion in mouse skin.

The structures of compounds **1** and **2** are similar to those of $n-6$ PUFAs; **1** and **2** have a double bond at the $n-6$ position. Although $n-6$ PUFAs showed no appreciable effects on NO generation (Ohata et al., 1997), DHA, which is a 22-carbon fatty acid having double bonds in the $n-3$ position, suppressed NO generation by inhibiting *iNOS* gene transcription (Khair-EI-Din et al., 1996; Ohata et al., 1997). In addition, it has been reported that $n-6$ PUFAs enhance rat mammary tumor development, whereas $n-3$ PUFAs inhibit it (EI-Sohemy et al., 1997). Also, DHA has been reported to be a suppressor of carcinogenesis in rat colon (Ninoura et al., 1988; Reddy et al., 1988; Takahashi et al., 1993) and of intestinal polyp formation in $Apc^{\Delta 716}$ knockout mice (Oshima et al., 1995). Although compounds **1** and **2** are structurally similar to $n-6$ PUFAs, their profiles for the inhibition of NO generation and carcinogenesis may belong to $n-3$ PUFAs. Therefore, compounds **1**−**3** may act on colon cancer the same as DHA. However, the molecular mechanisms of DHA and compounds **1**−**3** remain to be elucidated.

Moreover, it is interesting to note that compounds **1**−**3** are diacylglycerol (DG) mimic compounds with a C−C bond between C-3 and C-4 replacing the ester group in DGs. Protein kinase C (PKC) activation was apparently obligatory for induction of the iNOS in RAW 264.7 cells stimulated by LPS and IFN-γ (Paul et al., 1997). Furthermore, a direct role of PKC or phospholipase A_2 in the activation of the assembled NADPH oxidase in neutrophils resulted in O_2^- generation (Cross et al., 1991; Curnutte et al., 1994). Therefore, the structural analogy of compounds **1**−**3** to DGs, an endogenous agonist of PKC activation (Nishizuka, 1995; Yamamoto et al., 1997), may be essential for their activity to be exhibited.

In conclusion, compound **1** and persenone A and B were evaluated as unique dual inhibitors of O_2^- and NO generation in inflammatory leukocytes and thus are expected to be notable cancer chemopreventive agents in inflammation-associated organs, including the stomach and colon.

LITERATURE CITED

Aeberhard, E. E.; Henderson, S. A.; Arabolos, N. S.; Griscavage, J. M.; Castro, F. E.; Barrett, C. T.; Ignarro, L. J. Nonsteroid anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. *Biochem. Biophy. Res. Commun.* **1995**, 1053−1059.

Boyde, T. R. C.; Rahmatullah, M. Optimization of conditions for the colorimetric determination of citrulline, using diacetyl monoxime. *Anal. Biochem.* **1980**, *107*, 424−431.

Chang, C. F.; Isoga, A.; Kamikado, T.; Murakoshi, S.; Sakura, A.; Tamura, S. Isolation and structure elucidation of growth inhibitors for silk worm larvae from avocado leaves. *Agric. Biol. Chem.* **1975**, *39*, 1167−1168.

Collins, S. J.; Gallo, R. C.; Gallagher, R. E. Continuous growth and differentiation of human myeloid leukemia cells in suspension culture. *Nature* **1977**, *270*, 347−349.

Conney, A. H.; Lou, Y. R.; Xie, J. G.; Osawa, T.; Newmark, H. L.; Liu, Y.; Chang, R. L.; Huang, M. T. Some perspectives

on dietary inhibition of carcinogenesis: Studies with curcumin and tea. *Proc. Soc. Exp. Biol. Med*. **1997**, *216*, 234−245.

Cross, A. R.; Jones, O. T. G. Enzymatic mechanisms of superoxide production. *Biochim. Biophys. Acta* **1991**, *1057*, 281−289.

Curnutte, J. T.; Erickson, R. W.; Ding, J.; Badwey, J. A. Reciprocal interactions between protein kinase C and components of the NADPH oxidase complex may regulate superoxide production by neutrophils stimulated with phorbol ester. *J. Biol. Chem*. **1994**, *269*, 10813−10819.

EI-Sohemy, A.; Archer, M. C. Regulation of mevalonate synthesis in rat mammary glands by dietary *n*-3 and *n*-6 polyunsaturated fatty acids. *Cancer Res*. **1997**, *57*, 3685−3687.

Giardiello, F. M.; Hamilton, S. R.; Krush, A. J.; Piantadosi, S.; Hylind, L. M.; Celano, P.; Booker, S. V.; Roninson, C. R.; Offerhaus, J. A. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *New Engl. J. Med*. **1993**, *328*, 1313−1316.

Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrate, nitrite and [^{15}N]nitrite in biological fluids. *Anal. Biochem*. **1982**, *126*, 131−138.

Huang, M. T.; Lysz, T.; Ferraro, T.; Abidi, T. F.; Laskin, J. D.; Conney, A. H. Inhibitory effects of curcumin on *in vitro* lipoxygenase and cyclooxygenase activities in mouse epidermis. *Cancer Res*. **1991**, *51*, 813−819.

Ignarro, L. J.; Fukuto, J. M.; Griscavage, J. M.; Rogers, N. E.; Byrns, R. E. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine. *Proc. Natl. Acad. Sci. U.S.A*. **1993**, *90*, 8103−8107.

Khair-EI-Din, T. A.; Sicher, S. C.; Vazquez, M. A.; Chung, G. W.; Stallworth, K. A.; Kitamura, K.; Miller, R. T.; Lu, C. Y. Transcription of the murine iNOS gene is inhibited by docosahexaenoic acid, a major constituent of fetal and neonatal sera as well as fish oils. *J. Exp. Med*. **1996**, *183*, 1241−1246.

Kim, O. K.; Murakami, A.; Nakamura, Y.; Ohigashi, H. Screening of edible Japanese plants for nitric oxide generation inhibitory activities in RAW 264.7 cells. *Cancer Lett*. **1998**, *125*, 199−207.

Kondo, A.; Ohigashi, H.; Murakami, A.; Suratwadee, J.; Koshimizu, K. Potent inhibitor of tumor-promoter-induced Epstein−Barr virus activation. 1′-acetoxychavicol acetate from *Languas galanga*, a traditional Thai condiment. *Biosci., Biotechnol., Biochem*. **1993**, *57*, 1344−1345.

Koshimizu, K.; Ohigashi, H.; Tokuda, H.; Kondo, A.; Yamaguchi, K. Screening of edible plants against possible antitumor promoting activity, *Cancer Lett*. **1988**, *39*, 247−257.

Labayle, D.; Fisher, D.; Vielh, P.; Drouhin, F.; Pariente, A.; Bories, C.; Duhamel, O.; Trousset, M.; Attali, P. Sulindac causes regression of rectal polyps in familial adenomatous polyposis. *Gastroenterology* **1991**, *101*, 635−639.

Landino, L. M.; Crews, B. C.; Timmons, M. D.; Morrow, J. D.; Marnett, L. J.Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A*. **1996**, *93*, 15069−15074.

Lu, Y. P.; Chang, R. L.; Lou, Y. R.; Huang, M. T.; Newmark, H. L.; Reuhl, K. R.; Conney, A. H. Effect of curcumin on 12-*O*-tetradecanoylphorbol-13-acetate- and ultraviolet B light-induced expression of c-Jun and c-Fos in JB6 cells and in mouse epidermis. *Carcinogenesis* **1994**, *15*, 2363−2370.

Moghadasian, M. H.; Freeman, H. T.; Godin, D. V. Endogenous antioxidant status in neoplastic and adjacent tissues in 1,2-dimethylhydrazine-induced colon cancer in rats: Effects of olsalazine. *Carcinogenesis* **1996**, *17*, 983−987.

Murakami, A.; Kondo, A.; Nakamura, Y.; Ohigashi, H.; Koshimizu, K. Possible antitumor promoting properties from edible plants of Thailand, and identification of an active constituent, cardamonin, of *Boesenbergia pandurata*. *Biosci., Biotechnol., Biochem*. **1993**, *57*, 1971−1973.

Murakami, A.; Ohigashi, H.; Koshimizu, K. Possible antitumor promoting properties of traditional Thai food items and some of their active constituents. *Asia Pacific J. Clin. Nutr*. **1994**, *3*, 185−191.

Murakami, A.; Nakamura, Y.; Koshimizu, K.; Ohigashi, H. Glyceroglycolipids from *Citrus hystrix*, a traditional herb in Thailand, potently inhibit the tumor-promoting activity of 12-*O*-tetradecanoylphorbol 13-acetate in mouse skin. *J. Agric. Food Chem*. **1995**, *43*, 2779−2783.

Murakami, A.; Ohura, S.; Nakamura, Y.; Koshimizu, K.; Ohigashi, H. 1′-acetoxychavicol acetate, a superoxide anion generation inhibitor, potently inhibits tumor promotion in ICR mouse skin. *Oncology* **1996**, *53*, 386-391.

Murakami, A.; Kuki, W.; Takahashi, Y.; Yonei, H.; Nakamura, Y.; Ohto, Y.; Ohigashi, H.; Koshimizu, K. Auraptene, a citrus coumarin, inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion in ICR mouse skin, possibly through suppression of superoxide generation in leukocytes. *Jpn. J. Cancer Res*. **1997**, *88*, 443−452.

Nakamura, Y.; Murakami, A.; Koshimizu, K.; Ohigashi, H. Identification of pheophorbide *a* and its related compounds as possible antitumor promoters in the leaves of *Neptunia oleracea*. *Biosci., Biotechnol., Biochem*. **1996a**, *60*, 1028−1030

Nakamura, Y.; Murakami, A.; Koshimizu, K.; Ohigashi, H. Inhibitory effect of pheophorbide *a*, a chlorophyll-related compound on skin tumor promotion in ICR mouse. *Cancer Lett*. **1996b**, *108*, 247−255.

Nakamura, Y.; Ohto, Y.; Murakami, A.; Osawa, T.; Ohigashi, H. Inhibitory effects of curcumin and tetrahydrocurcuminoids on the tumor promoter-induced reactive oxygen species generation in leukocytes *in vitro* and *in vivo*. *Jpn. J. Cancer Res*. **1998a**, *89*, 361−370.

Nakamura, Y.; Ohto, Murakami, Y., Ohto, Y., Torikai, K., Tanaka, T., Ohigashi, H. Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by a superoxide generation inhibitor 1′-acetoxychavicol acetate. *Cancer Res*. **1998b**, *58*, 4832−4839.

Nguyen, T.; Brunson, D.; Cresp, C. L.; Penman, B. W.; Wishnok, J. S.; Tannenbaum, S. R. DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *Proc. Natl. Acad. Sci. U.S.A*. **1992**, *89*, 3030−3034.

Ninoura, T.; Takata, T.; Sakaguchi, M.; Takada, H.; Yamamura, M.; Hioki, K.; Yamamoto, M. Effect of dietary eicosapentaenoic acid on azoxymethane-induced colon carcinogenesis in rats. *Cancer Res*. **1988**, *48*, 4790−4794.

Nishizuka, Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J*. **1995**, *9*, 484−496.

Nonaka, Y.; Iwagaki, H.; Kimura, T.; Fuchimoto, S.; Orita, K. Effect of reactive oxygen intermediates on the *in vitro* invasive capacity of tumor cells and liver metastasis in mice. *Int. J. Cancer* **1993**, *54*, 983−986.

Ohata, T.; Fukuda, K.; Takahashi, M.; Sugimura, T.; Wakabayashi, K. Suppression of nitric oxide production in lipopolysaccharide-stimulated macrophage cells by ω3 polyunsaturated fatty acids. *Jpn. J. Cancer Res*. **1997**, *88*, 234−237.

Ohata, T.; Fukuda, K.; Murakami, A.; Ohigashi, H.; Sugimura, T.; Wakabayashi, K. Inhibition by 1′-acetoxychavicol acetate of lipopolysaccharide- and interferon-γ-induced nitric oxide production in RAW264 cells. *Carcinogenesis* **1998**, *19*, 1007−1012.

Ohigashi, H.; Murakami, A.; Koshimizu, K. Antitumor promoters from edible plants. In *Food Phytochemicals for Cancer Chemoprevention*; Ho, C. T., Osawa, T., Huang, M.-T., Rosen, R. T., Eds.; ACS Symposium Series; American Chemical Society: Washington, DC, 1994; pp 251−261.

Ohnishi, M.; Tanaka, T.; Makita, H.; Kawamori, T.; Mori, H.; Satoh, K.; Hara, A.; Murakami, A.; Ohigashi, H.; Koshimizu, K. Chemopreventive effect of a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate, on rat oral carcinogenesis. *Jpn. J. Cancer Res*. **1996**, *87*, 349−356.

Oshima, M.; Takahashi, M.; Oshima, H.; Tsutsumi, M.; Yazawa, K.; Sugimura, T.; Nishimura, S.; Wakabayashi, K.; Taketo, M. M. Effects of docosahexaenoic acid (DHA) on intestinal polyp development in *Apc*$^{-716}$ knockout mice. *Carcinogenesis* **1995**, *16*, 2605−2607.

Paul, A.; Doherty, K.; Plevin, R. Differential regulation by protein Kinase C isoforms of nitric oxide synthase induction in RAW 264.7 macrophages and rat aortic smooth muscle cells. *Br. J. Pharm.* **1997**, *120*, 940−946.

Prusky, D.; Keen, N. T.; Sims, J. J.; Midland, S. L. Possible involvement of an antifungal diene in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. *Phytopathology* **1982**, *72*, 1578−1582.

Reddy, B. S.; Sugie, S. Effect of different levels of omega-3 and omega-6 fatty acids on azoxymethane-induced colon carcinogenesis in F344 rats. *Cancer Res.* **1988**, *48*, 6642−6647.

Reddy, B. S.; Rao, C. V.; Rivenson, A.; Kelloff, G. Inhibitory effect of aspirin on azoxymethane-induced colon carcinogenesis in F344 rats. *Carcinogenesis* **1993**, *14*, 1493−1497.

Reiners, Jr., J. J.; Pence, B. C.; Barcus, M. C. S.; Cantu, A. R. 12-*O*-Tetradecanoylphorbol-13-acetate-dependent induction of xanthine dehydrogenase and conversion to xanthine oxidase in murine epidermis. *Cancer Res.* **1987**, *47*, 1775−1779.

Salvemini, D.; Misko, T. P.; Masferrer, J. L.; Seibert, K.; Currie, M. G.; Needleman, P. Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7240−7244.

Sladowski, D.; Steer, S. J.; Clothier, R. H.; Balls, M. An improved MTT assay. *J. Immunol. Methods* **1992**, *157*, 203−207.

Stone, W. L.; Papas, A. M. Tocopherols and the etiology of colon cancer. *J. Natl. Cancer Inst.* **1997**, *89*, 1006−1014.

Szabo, C.; Ohshima, H. DNA damage induced by peroxynitrite: Subsequent biological effects. *Arch. Biochem. Biophys., Part B Nitric Oxide* **1997**, *1*, 373−385.

Takahashi, M.; Minamoto, T.; Yamashita, N.; Yazawa, K.; Sugimura, T.; Ezumi, H. Reduction in formation and growth of 1,2-dimethylhydrazine-induced aberrant crypt foci in rat colon by docosahexaenoic acid. *Cancer Res.* **1993**, *53*, 2786−2789.

Takahashi, M.; Fukuda, K.; Ohata, T.; Sugimura, T.; Wakabayashi, K. Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. *Cancer Res.* **1997**, *57*, 1233−1237.

Tanaka, T. Cancer chemoprevention. *Cancer J.* **1992**, *5*, 11−16.

Tanaka, T.; Makita, H.; Kawamori, T.; Kawabata, K.; Murakami, A.; Satoh, K.; Hara, A.; Ohigashi, H.; Koshimizu, K. A xanthine oxidase inhibitor 1′-acetoxychavicol acetate inhibits azoxymethane-induced colonic aberrant crypt foci in rats. *Carcinogenesis* **1997a**, *18*, 1113−1118.

Tanaka, T.; Kawabata, K.; Kakumoto, M.; Makita, H.; Matsunaga, K.; Mori, H.; Satoh, K.; Hara, A.; Murakami, A.; Koshimizu, K.; Ohigashi, H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate. *Jpn. J. Cancer Res.* **1997b**, *88*, 821−830.

Tanaka, T.; Kawabata, K.; Kakumoto, M.; Makita, H.; Akira, H.; Mori, H.; Satoh, K.; Hara, A.; Murakami, A.; Kuki, W.; Takahashi, Y.; Yonei, H.; Koshimizu, K.; Ohigashi, H. Citrus auraptene inhibits chemically induced colonic aberrant crypt foci in male F344 rats. *Carcinogenesis* **1997c**, *18*, 2155−2161.

Tanaka, T.; Kawabata, K.; Kakumoto, M.; Matsunaga, K.; Mori, H.; Akira, M.; Kuki, W.; Takahashi, Y.; Yonei, H.; Satoh, K.; Hara, A.; Maeda, M.; Ota, T.; Odashima, S.; Koshimizu, K.; Ohigashi, H. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by citrus auraptene in rats. *Carcinogenesis* **1998**, *19*, 425−431.

Tayeh, M. A.; Marletta, M. A. Macrophage oxidation of L-arginine to nitric oxide, nitrite and nitrate. *J. Biol. Chem.* **1989**, *264*, 19654−19658.

Vane, J. R.; Mitchell, J. A.; Appleton, I.; Tomlinson, A.; Bishop-Bailey, D.; Croxtall, J.; Willoughby, D. A. Inducible isoforms of cyclooxygenase and nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *81*, 2046−2050.

Wattenberg., L. W. Chemoprevention of cancer. *Cancer Res.* **1985**, *45*, 1−8.

Wei, H.; Frenkel, K. Sarcophytol a suppresses the oxidative events and DNA damage *in vivo*: A possible mechanism of antitumor promotion. *Cancer Res.* **1992**, *52*, 2298−2303.

Yamamoto, Y.; Kambayashi, Y.; Ito, T.; Watanabe, K.; Nakano, M. 1,2-Diacylglycerol hydroperoxides induce the generation and release of superoxide anion from human polymorphonuclear leukocytes. *FEBS Lett.* **1997**, *412*, 461−464.

Received for review September 23, 1999. Revised manuscript received February 10, 2000. Accepted February 23, 2000. This study was partly supported by a Grant-in-Aid for Scientific Research on Priority Areas—Cancer (No. 09253104, H.O.) and by JSPS Research Fellow (Y.N.) and Encouragement of Young Scientists (No. 09760118, A.M.) grants from the Ministry of Education, Science and Culture of Japan.

JF990977R