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Isolation, Identification, and Structure of a Potent Alkyl-Peroxyl Radical Scavenger in Crude Canola Oil, Canolol*

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Alkylhydroperoxides in oxidized oil are undesirable components because they become alkylperoxyl radicals (ROO•) in the presence of heme, hemoglobin, or myoglobin in red meat. ROO• are biochemically reactive and damage nucleic acids and proteins, thereby harming living cells. We isolated a component, a highly potent ROO• scavenger, from crude canola oil (rapeseed), which we designated canolol, and identified its chemical structure, 4-vinyl-2,6-dimethoxyphenol. The canolol content of crude canola oil greatly increased after the seed was roasted as compared with that from unroasted seed, but it decreased in highly purified oil. This anti-ROO• activity was highest in crude oil, deceased after each refining step, and was lowest in highly purified refined oil. Canolol was thus generated during roasting. As shown previously, canolol is one of the most potent anti-ROO• components in crude canola oil and its potency is much greater than that of well-known antioxidants, including α-tocopherol, vitamin C, β-carotene, rutin, and quercetin.

Key words: antioxidants; antimutagenic; seed oil; oil refining process; canolol

Polyunsaturated fatty acids are susceptible to oxidation, which results in formation of lipid hydroperoxides (LOOH). LOOH in oxidized oils are undesirable food components, because they give rise to reactive metabolites such as lipid-derived radicals and aldehydes (*e.g.*, malondialdehyde and 4-hydroxy-2-nonenal), which cause oxidative damage in vital molecules such as DNA and proteins.[3–5] Endogenous lipid peroxidation caused by oxidative stress is another important source of LOOH. We previously found that heme iron such as that in hemoglobin and myoglobin efficiently catalyzes the formation of lipid peroxyl radicals (LOO•) from LOOH, whereas nonheme iron catalyzes the formation of carbon centered radicals (R•) and alkoxy radicals (RO•).[6,7] The alkyl peroxyl radical ROO• is unique in that it is relatively stable, has a long half-life, and can travel long distances *in vivo* from the site of generation.[6,7] We also showed that intake of a fat-rich diet with hemoglobin, which may generate LOO• in the digestive tract, significantly promoted colon carcinogenesis in rats treated with low levels of a carcinogen.[8] LOO• can induce single-strand breakage of DNA[8] and can efficiently generate abasic sites in DNA.[9] These observations suggest that generation of LOO• *in vivo* contributes to carcinogenesis, and hence that compounds that scavenge LOO• might help to prevent cancer.[10–13]

Previously we reported that many natural edible oils contain high levels of anti-ROO• activity, with extra virgin olive oil exhibiting the highest ROO• scavenging activity, followed by crude sesame oil and then canola oil.[8] Such anti-ROO• activity might be attributed to various components such as sesaminol, lignans, tocopherols, flavonoids, and polyphenols. However, canola oil components have not been studied in detail. We coined canolol the most active component in canola oil, and identified its chemical structure after extensive purification to a single component, as described below.*

The methods we used for purification and identifica-

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Abbreviations: TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NMR, nuclear magnetic resonance; DTPA, diethylenetriaminepentaacetic acid; $IPOX_{50}$, inhibitory potential 50% of peroxyl radicals; LOO•, lipid peroxyl radicals; PMC, 2,2,5,7,8-pentamethy-6-couromanol

tion included differential solvent extraction, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), mass spectroscopy (MS), nuclear magnetic resonance (NMR), and infrared, visible, and ultraviolet spectroscopy. Purified canolol was found to have the structure 4-vinyl-2,6-dimethoxyphenol, a decarboxyl derivative of sinnapinic acid. Canolol exhibited the highest $ROO^{\bullet}$ scavenging activity among various known antioxidants such as α-tocopherol, vitamin C, β-carotene, rutin, and quercetin. Further, we found the steps used in the oil refining process of canola oil decreased the final canolol content, while roasting the canola seeds increased canolol content by as much as 100 times of that before roasting.

Materials and Methods

Materials. Canola seeds and degummed canola oil (expeller and hexane extraction after cooking at 80–90 °C) were obtained from Showa Sangyo (Tokyo). Diethylenetriaminepentaacetic acid (DTPA), *t*-butyl hydroperoxide (*t*-BuOOH), and hemoglobin were obtained from Sigma-Aldrich (St. Louis, MO). Tocopherols α-, β-, γ-, and δ-, and 2,2,5,7,8-pentamethyl-6-chromanol (PMC), were obtained from Eisai (Tokyo). Synthetic canolol was prepared by Junsei Chemical (Tokyo).

Assay of anti-$ROO^{\bullet}$ activity. As described previously.[6–8] $ROO^{\bullet}$ were generated from the reaction between *t*-BuOOH and hemoglobin. *t*-$BuOO^{\bullet}$ can oxidize luminol to form fluorescent products, and thus the amount of *t*-$BuOO^{\bullet}$ can be quantitated by measuring luminol-derived chemiluminescence. $ROO^{\bullet}$ scavenging activity can be determined by means of inhibition of chemiluminescence. In this assay, 125 µl of 0.01 M phosphate buffered saline, 25 µl of 10 mM DTPA, 25 µl of 0.3 M *t*-BuOOH, and 25 µl of ethanol solution of the test substance were added to a well of a 96-well microplate. The plate was shaken and preincubated at 37 °C for 210 s, after which it was placed in a chemiluminescence microplate reader (Labsystems Luminoskan RS, Dainippon Pharmaceuticals, Osaka). Chemiluminescence was initiated by the addition of 25 µl of 100 mg/l hemoglobin, using a dispenser supplied with the instrument. The intensity of maximum chemiluminescence was immediately recorded. The concentration of the test substance at which 50% of chemiluminescence was suppressed relative to the control (no addition of test substance) was measured, and this value was designated the 50% inhibitory concentration of peroxyl radicals ($IPOX_{50}$). Thus, the smaller the value of $IPOX_{50}$, the greater the radical scavenging potency.

TLC analyses. The samples, which were dissolved in chloroform, were applied to TLC plate silica gel, 0.25 mm thick (Kieselgel 60; Merck, Darmstad, Germany), and then developed in a solvent mixture of hexane, diethyl ether, and acetic acid (80:30:1, v/v/v). Neutral lipids were determined in the presence of iodine vapor, as used for detection of general organic compounds.

After the samples were sprayed with 50 µM β-carotene and 1 mM linoleic acid (Wako Pure Chemical Industries, Osaka) in chloroform solution, antioxidant substances were detected as yellow spots. Phenolic compounds were detected as dark blue spots, after samples were sprayed with phenol reagent (Nacalai Tesque, Kyoto) diluted two-fold with chloroform.

Isolation and purification.

Methanol extraction. In brief, methanol (90 ml) was added to 450 g of crude canola oil (that had been degummed by pressure after cooking at 80–90 °C), and the mixture was shaken for extraction for 10 min using a shaker to obtain a heavy dark biphasic solution. The mixture was then centrifuged at 1,500 × *g* for 30 min. The upper methanol layer was collected in an oval flask. Methanol (90 ml) was added again to the lower phase that contained the crude canola oil, and the mixture was shaken again for 10 min for extraction. This procedure was repeated three times.

Each methanol layer was combined and processed with a rotary evaporator *in vacuo* to remove methanol to dryness.

Liquid-liquid phase partition. The methanol extract was dissolved in 45 ml of hexane, the mixture was placed into a separating funnel (no. 1), and 15 ml of acetonitrile was added to the separating funnel. The funnel was shaken, and then the lower (acetonitrile) phase was transferred into another separating funnel (no. 2) containing 45 ml of new hexane, while 15 ml of fresh acetonitrile was introduced into separating funnel no. 1. Separating funnels no. 1 and no. 2 were shaken, and then the lower phase (acetonitrile) of separating funnel no. 2 was introduced into an oval flask (no. 3), and the lower phase of separating funnel no. 1 was combined with separating funnel no. 2. This procedure was repeated with 15 ml of acetonitrile two times. The acetonitrile layer and the remaining hexane layer were evaporated *in vacuo*, and the solvents were removed.

Fractionation by silica gel column chromatography. For column chromatography, 15 g of silica gel (Kanto Kagaku, Tokyo), with a particle size of <150 µm, was packed in a glass column $\phi 20 \times 300$ mm. The acetonitrile fraction (500 mg) was dissolved in 5 ml of chloroform and then applied to the column, which was developed with a mixture of solvents: (hexane/diethyl ether, 90:10 v/v), 175 ml; chloroform, 225 ml; acetone, 400 ml; and methanol, 200 ml. Elution proceeded at a rate of approximately 2 ml/min. The eluted fractions were collected and stored in an oval flask, and the

* A part of this work was described in the MS thesis of D. Wakamatsu in 2001,[1] and Koski *et al.* published the antioxidant activity of this component more recently, during preparation of this manuscript.[14]

solvents were evaporated with a rotary evaporator *in vacuo*.

Preparative TLC. The chloroform fraction (100 mg) was subjected to preparative TLC (Kieselgel 60, Merck, Darmstadt, Germany) with the solvent mixture hexane/diethyl ether (80:30 v/v). After confirmation of a yellow band on the plate near Rf = 0.15, the desired substance together with the silica gel was scraped off with a spatula. Chloroform (20–30 ml) was added to the collected silica gel powder. To extract the substance, it was removed from the silica gel *via* filtration with a glass filter, and the solvent was evaporated.

Purification by HPLC. The sample obtained of TLC was subjected to HPLC with a C18 column (Aquasil Keystone Scientific, Bellefonte, PA, $\phi 4.6 \times 250$ mm) used at room temperature. An acetonitrile/water mixture (90:10 v/v, eluent A) followed by acetonitrile (eluent B) were applied for gradient elution as follows: isocratic elution A, 0–20 min; linear gradient elution from 100% A to 100% B, 20–25 min; isocratic elution with 100% B, 25–40 min. The flow rate of the mobile phase was 1.0 ml/min, and the injection volumes were 100 µl of the sample solution (5 mg/ml). Eluate absorbance was measured at 300 nm.

Identification. The purified sample, a single identified spot by TLC and one peak in HPLC, was subjected to the following analyses for identification:

i) The elements C, H, and N were analyzed using a CHN corder (CHN Corder MT-3; Yanaco, Kyoto). The sulfur content was measured using a sulfur analyzer (Antec, 7000; Astech, Tokyo).

ii) For liquid chromatography/mass spectroscopy (LC/MS), canolol was dissolved in methanol and the mixture was applied to a reverse-phase HPLC column (Hewlett Packard Hypersil ODS, Palo Alto, CA; $\phi 40 \times 125$ mm) for separation; elution was with methanol. Mass analysis was performed using the JMS–LC mate mass spectrometer (JEOL, Tokyo) with positive-mode, atmospheric pressure chemical ionization. The vaporizer temperature was 500 °C.

iii) For NMR, a sample was dried in a nitrogen atomosphere, dissolved in chloroform-*d* ($CDCl_3$), and introduced into an ampule tube, after which measurement was taken at 400 Mz for ^{1}H NMR; JEOL (Tokyo) was measured.

iv) Ultraviolet and visible absorption was determined in water (V-550 UV/VIS spectrophotometer, Jasco, Tokyo) in the conventional manner.

Content of canolol and tocopherol. Canolol shows a fluorescence emission at 325 nm when excited at 298 nm. Hence canolol elution of content was monitored by means of an HPLC system with a fluorescence detector (FP-1520, Jasco, Tokyo). Separation was achieved *via* a column from Kanto Kagaku, LiChroCART 250-4 cartridge, LiChrospher 100 NH_2; column $\phi 4 \times 244$ mm, particle size of 5 µm; the internal standard for HPLC was PMC. HPLC was carried out at temperature below 25 °C. Elution was isocratic, with a 1.2% isopropanol/hexane (v/v) mixture. The flow rate of the mobile phase was 1.2 ml/min, and the injection volumes were 50 µl of the sample solution.

For detection of canolol in the eluate, the fluorescence detector was set at 298 nm for excitation and 325 nm for emission. PMC was detected at about 6 min, and canolol was detected at about 18 min. These conditions were also in effect for detection of the tocopherols: α-, β-, γ-, and δ-tocopherol.

Results

Reduced anti-ROO• activity in canola oil during processing

As shown in Table 1, anti-ROO• activity and canolol in canola oil decreased significantly as oil refining proceeded. Processes, such as degumming, alkaline-washing with 10% NaOH, decoloration with acid clay adsorption, and exposure to high-temperature (260 °C) steam led to anti-ROO• activity a significant decrease whereas the total content of tocopherol remained constant (Table 1, 2). This result indicates that the anti-ROO• activity in canola oil can be attributed in large part to compounds other than tocopherols.

Table 1. Change in Anti-ROO• Activity of Canola Oil during Industrial Processing

Oil	$IPOX_{50}$ (mg/ml)	Canolol conc (µg/g)	Total tocopherol conc (µg/g)
Degummed crude oil	1.46	220.0	785
Deacidified oil	21.6	38.0	754
Decolored oil	105	Not detectable	749
Deodorized (purified) oil	>300	Not detectable	409

Canolol as a potent anti-ROO• component in canola oil

To identify components with potent anti-ROO• activity in canola oil, degummed crude oil was subjected to methanol extraction. This procedure yielded 3,025 mg with an $IPOX_{50}$ value of 0.18, that is, activity that was 23.3 times higher than that of the starting material (Table 3). In the liquid-liquid partition experiment, strong anti-ROO• activity was recovered from the acetonitrile phase (Table 3). Further purification was carried out by means of silica gel column chromatography, and we observed the highest anti-ROO• activity in the chloroform fraction. TLC analysis showed that this chloroform fraction consisted of three spots having Rf values of 0.01, 0.15, and 0.3 (Fig. 1). A spot with an Rf value of 0.3 corresponded to free fatty acid. The spots with Rf = 0.15 exhibited potent antioxidant activity, as revealed by β-carotene. Use of the phenol reagent for detection of phenolic compounds indicated that fractions with Rf values of 0.01 and 0.15 were both phenolic compounds, the yield for each being 13 mg. The anti-ROO• activity of the fraction with an Rf value of 0.15

Table 2. Content of Canolol and Tocopherol in Canola Oil before and after Roasting of Seeds

Canola seed (lot)	Before roasting							After roasting							Change: after/before roasting	
	Canolol content (μg/g)	Tocopherol content (μg/g)					$IPOX_{50}$[a] (mg/ml)	**Canolol content (μg/g)**	Tocopherol content (μg/g)					$IPOX_{50}$ (mg/ml)	Canolol increase	Tocopherol increase
		α-Toc	β-Toc	γ-Toc	δ-Toc	Total Toc			α-Toc	β-Toc	γ-Toc	δ-Toc	Total Toc			
A	**48**	307	0	339	6	652	0.67	**549**	316	0	323	8	647	0.39	11	0.99
B	**20**	272	0	334	7	613	0.66	**616**	280	0	313	6	599	0.35	31	0.98
C	**58**	259	0	427	8	693	0.72	**758**	272	0	417	8	697	0.25	13	1.00
D	**10**	302	0	769	19	1090	0.62	**1088**	326	0	763	19	1108	0.22	109	1.01
E	**6**	171	0	460	12	643	0.72	**1536**	208	0	474	12	694	0.25	256	1.08

[a] 50% inhibitory concentration of peroxyl radical by canola oil.

Table 3. Anti-ROO• Activity in Canola Oil at Each Step during Canolol Purification[a]

Purification step	$IPOX_{50}$, mg/ml[b]	Recovery yield
Degummed crude oil	4.2 (1)[b]	450 g
↓		
Methanol extraction	0.18 (23.3)	3,025 mg
↓		
Liquid-liquid partition		
Hexane phase	2.5 (1.68)	1,533 mg
Acetonitrile phase	0.044 (95.4)	862 mg
↓		
Silicagel column chromatography		
Hexane/ether fraction	2.6 (1.6)	163 mg
Chloroform fraction	0.022 (190)	267 mg
Acetone fraction	0.12 (35)	379 mg
Methanol fraction	0.22 (19)	78 mg
↓ (100 mg of chloroform frn)		
TLC		
Upper layer (Rf = 0.15)	0.008 (530)	13 mg
Lower layer (Rf = 0.01)	0.016 (260)	13 mg
↓ (use frn of Rf 0.015 for HPLC)		
HPLC	0.004 (1,050)	1 mg

[a] Numbers in parentheses indicate relative anti-ROO• activity compared with degummed crude oil (starting material).

[b] Anti-ROO• activity relative to crude degummed oil is 1. Values in parentheses are ROO•-scavenging potential relative to the starting material.

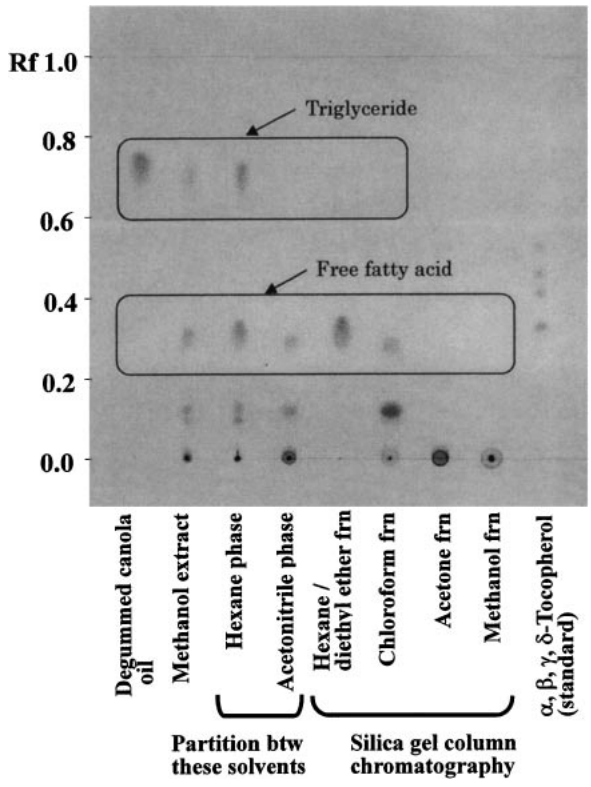
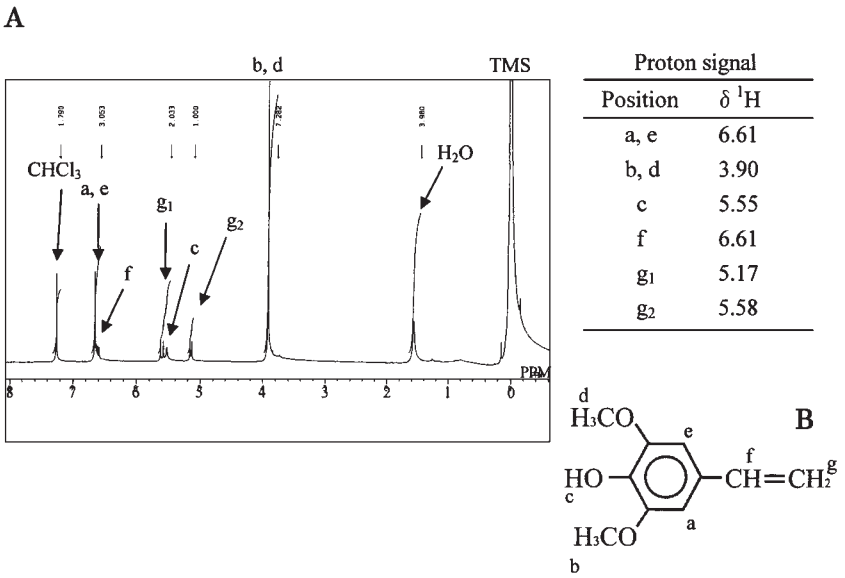


Fig. 1. TLC Analysis of Canola Oil Constituents Obtained from Different Purification Steps.

was two times higher than that of the Rf = 0.01 fraction. Finally, a TLC fraction with an Rf value of 0.15 was applied to reverse-phase HPLC purification. A single peak at a retention time of 12 min was collected and



Proton signal

Position	δ ^{1}H
a, e	6.61
b, d	3.90
c	5.55
f	6.61
g_1	5.17
g_2	5.58

Fig. 2. ^{1}H-NMR Spectrum of the Purified Component from Canola Oil in $CDCl_3$ (A) and Its Proposed Chemical Structure of Canolol (B). TMS, tetramethylsilanes were used as the standard.

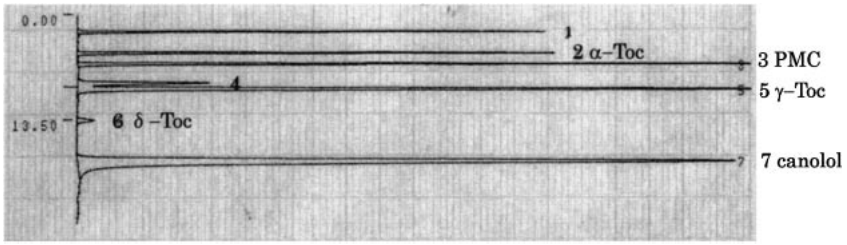


Fig. 3. HPLC Chromatogram of Crude Canola Oil, and References (Peak 1, BHT [antioxidant reagent]; peak 2, α-tocopherol; peak 3, PMC (internal standard); peak 4, unknown; peak 5, γ-tocopherol; peak 6, δ-tocopherol; peak 7, canolol). See text for details.

found to have an $IPOX_{50}$ value of 0.004. This compound had anti-ROO$^\bullet$ activity 1,050 times higher than that of the starting material (Table 3).

Identification of the chemical structure of canolol

We determined the chemical structure of this compound. Elemental analysis yielded neither nitrogen nor sulfur. High-resolution MS of atmospheric pressure chemical ionization (APCI) plus LC/MS showed a molecular mass of 180, and the structure $C_{10}H_{12}O_3$ was proposed. On the basis of these results plus the ^{1}H-NMR data, we concluded that this active component is 4-vinyl-2,6-dimethoxyphenol (Fig. 2B), and we designated it canolol. Synthesis *via* decarboxlation of sinnapinic acid yielded the same compound as canolol on TLC and HPLC; identity was also confirmed by NMR spectroscopy (data not shown). Therefore the assigned chemical formula is consistent with sinapinic acid.

Canolol had absorption peaks at 218 nm and 269 nm. The molecular extinction coefficient in water was 29,000 at 218 nm and 13,000 at 269 nm (ε_{mol}/l; 1 cm). We also found that canolol had a fluorescence emission maximum at 325 nm (data not shown) which was used for quantifying upon HPLC.

HPLC chromatography

Figure 3 shows a typical pattern of HPLC; the representative components are shown. The conditions are designated in "Methods". Canolol shows a distinct separation.

Increase of canolol after roasting

We examined the levels of canolol and tocopherols in five lots of crude oil from canola seeds before and after roasting (Table 2). The amounts of α, γ, and δ-tocopherol were unchanged during roasting (165 °C, 5 min), whereas β-tocopherol remained zero throughout. In contrast, the canolol content of each lot was increased by about 10–100 times after roasting, as shown in Table 2. This finding was consistent with antioxidant potential.

Discussion

In the plant seeds from which most edible oils are prepared, abundant antioxidants have been found. Highly potent oxygen radical scavengers play a crucial role in preventing radical-induced damage of DNA, RNA, and proteins in the seeds. These scavengers are thus vitally important not only for prevention of genetic damage but also for various nutritional and enzymatic functions needed by seeds for germination.

In the gut, where heme becomes available and accessible to alkyl hydroperoxides (or LOOH) in food, the peoxyl radical $ROO^{\bullet}$ is generated. Suppression of such hazardous radical species is of great practical value for human health and cancer prevention.

In the present study, we isolated a highly potent anti-$ROO^{\bullet}$ fraction from crude canola oil and identified its chemical structure as 4-vinyl-2,6-dimethoxyphenol, and coined this substance canolol. As shown in Fig. 2B, canolol is a decarboxylated analog of sinapinic acid. It was detected in five different lots of unroasted canola seeds (6–58 μg/g) (Table 2). Further, roasting the canola seeds markedly increased the canolol content in all five lots of seeds (to 549–1536 μg/g). Our data suggest that decarboxylation of sinapinic acid during roasting might produce canolol. This possibility, such as decarboxylation of sinapinic acid by heat, should be elucidated in a model experiment. The biosynthetic pathways of canolol also remain unclear.

Sinapinic acid and other 4-hydroxycinnamic acid derivatives such as ferulic acid possess radical scavenging activity.[15] It was proposed that these compounds can readily donate their phenoxyl hydrogen atom to neutralize radical species, with simultaneous formation of corresponding phenoxyl radicals.[16] Unpaired electrons of phenoxyl radicals, however, can be delocalized, and hence these phenoxyl radicals show weaker reactivity. In agreement with this finding, we discovered, *via* electron spin resonance spectroscopy, canolol-derived phenoxyl radicals in a reaction between canolol and *t*-$BuOO^{\bullet}$ (data not shown).

In previous studies, we showed that various sources such as nitroguanosine and heterocyclic amines, as well as inflammatory insults in hosts, generated superoxide anion radicals and nitric oxide.[17–20] Also, our data relating peroxyl radical formation by heme plus ROOH with enhanced colon carcinogenesis[8] have been validated subsequently.[21]

Canolol can scavenge not only $ROO^{\bullet}$ but also peroxynitrite ($ONOO^{-}$),[2] a highly potent oxidizing and nitrating agent formed during the reaction of superoxide radicals with nitric oxide.[22–24] We showed that both are formed endogenously *in vivo* at sites of inflammation and infection.[17,18,25] Because $ONOO^{-}$ can cause oxidative and nitrative damage to DNA, RNA, and proteins, overproduction of it might be associated with various pathogenic states or conditions such as inflammation, cancer, arteriosclerosis, and neurodegenerative diseases.[26–29] We found that $ONOO^{-}$ has, in addition, strong mutagenic effects on the *Salmonella typhimurium* TA102 strain[2] and the influenza and Sendai viruses.[18,30] We also found that canolol strongly suppressed bacterial mutations in *Salmonella* caused by $ONOO^{-}$.[2] The antimutagenic potency of canolol was almost equivalent to or more than that of ebselen, a potent $ONOO^{-}$ scavenger. All these data together indicate that canolol might have beneficial effects on both $ROO^{\bullet}$ and $ONOO^{-}$ mediated diseases. The presence of potent antioxidant components in the diet is thus clearly important.

The present results suggest that the oil refining processes used in the industry today should be revised so that such important scavengers are retained. It is also interesting that canolol levels appear to increase during roasting, which suggests that a precursor of canolol, such as sinnapinic acid, might exist, in which case decarboxylation of the precursor would result in formation of canolol. This possibility should be investigated in a model system.

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