Novel Antioxidative Metabolites in Rat Liver with Ingested Sesamin

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Sesamin, a major lignan in sesame oil, is known to have many biological activities, especially protective effects against oxidative damage in the liver. As sesamin itself has no antioxidative properties in vitro, to elucidate the mechanism of its antioxidative effects, the reaction products of sesamin in rat liver homogenate were analyzed. The methylenedioxyphenyl moiety in the structure of sesamin was shown to be changed into a dihydrophenyl (catechol) moiety. The enzymatic reaction products in vitro were identified as (1*R*,2*S*,5*R*,6*S*)-6-(3,4-dihydroxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo[3,3,0]octane and (1*R*,2*S*,5*R*,6*S*)-2,6-bis(3,4-dihydroxyphenyl)-3,7-dioxabicyclo[3,3,0]-octane, which showed strong radical scavenging activities; the latter was a novel compound. The same metabolites were found as glucuronic acid and/or sulfic acid conjugates in substantial amounts in rat bile after oral administration of sesamin. It is suggested that sesamin is a prodrug and the metabolites containing the catechol moieties in their structures are responsible for the protective effects of sesamin against oxidative damage in the liver.

KEYWORDS: Sesamin; antioxidant; metabolite

INTRODUCTION

Sesamin is a major lignan in sesame oil, and its biological effects have been extensively studied by many researchers. It was previously reported that sesamin is a specific inhibitor of $\Delta 5$ desaturase (*1*), which catalyzes the conversion of dihomo-γ-linolenic acid to arachidonic acid, in both microorganisms and animals, and exerts hypocholesterolemic activity through the inhibition of cholesterol absorption and synthesis (*2*). It was also reported that sesamin prevents the damage to the liver caused by alcohol or carbon tetrachloride (*3*) and shows a suppressive effect against 7,12-dimethylbenz[*a*]anthracene-induced rat mammary carcinogenesis (*4*) and antihypertensive effects (*5–7*), although the mechanisms of action of this lignan remain unclear. The proposed mechanisms of hepatotoxicity of alcohol or carbon tetrachloride and of carcinogens involve a free radical-mediated process leading to lipid peroxidation and DNA damage. In sesame oil, lignans carrying a hydroxy group, that is, sesaminol, episesaminol, and sesamolinol, exhibit antioxidant activity (*8, 9*); however, sesamin as an antioxidant has not been evaluated clearly. As the physiological effects of sesamin are most manifest in the liver, we hypothesized that the metabolized products of sesamin in the liver after oral administration to rats are responsible for the observed antioxidative properties. In this study the antioxidative metabolites of

sesamin were isolated from the reaction mixture with rat liver homogenate in vitro and with rat bile in vivo and structurally identified.

MATERIALS AND METHODS

Chemicals. Sesamin (a mixture of sesamin and episesamin, 51.1: 48.2, w/w) was prepared from refined sesame oil and purified as described previously (*10*). Sesamin was purified from a sesamin–episesamin mixture by high-performance liquid chromatography (HPLC), on a 250 mm × 20 mm i.d. Develosil ODS-UG-5 reversed-phase HPLC column (Nomura Chemical Co., Aichi, Japan) with UV detection at 280 nm. The column was eluted with a linear gradient from solvent A [water/acetonitrile (80:20, v/v)] to solvent B [water/acetonitrile (20: 80, v/v)] in 100 min at a flow rate of 5 mL/min. Rat S9 and cofactor I were purchased from Oriental Yeast Co. (Tokyo, Japan). The enzyme mixture of glucuronidase and sulfatase was from Sigma Chemical Co. (St. Louis, MO). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from Labotec Co. (Tokyo, Japan). The other reagents were of analytical grade, and all solvents used were of HPLC grade (Nacalai Tesque Co., Kyoto, Japan).

Animals. Male Wistar rats, weighing ∼280–320 g, were obtained from Oriental BioService (Kyoto, Japan). The animals were maintained in an air-conditioned room at 22 °C with a 12-h light/dark cycle. Drinking water and regular feed were supplied ad libitum. The experiments were started after the animals had acclimatized for at least 1 week.

Enzymatic Reaction in Vitro. To determine the reaction products of sesamin by microsomal enzymes in rats, sesamin was reacted with rat S9 mix. S9 mix contained rat S9 and cofactor I in a ratio of 1:9. Cofactor I was composed of $MgCl_2 \cdot 6H_2O$ (8 μmol), KCl (33 μmol),

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D-glucose-6-phosphate (5 μmol), NADPH (4 μmol), NADH (4 μmol), Na$_2$HPO$_4$ (100 μmol), and NaH$_2$PO$_4$ (100 μmol) in one vial. The reaction mixture, containing 10 mL of S9 mix and 1 μmol of sesamin dissolved in DMSO, was incubated at 37 °C for 1 h. Then sesamin and the enzymatic reaction products in the reaction mixture were extracted with ethyl acetate. After concentration in vacuo, the resultant extract was dissolved in methanol and then subjected to reversed-phase HPLC on a 150 mm × 4.6 mm i.d. Develosil ODS-UG-5 with UV detection at 280 nm. The column was eluted with a linear gradient from solvent A [water/acetonitrile (80:20, v/v)] to solvent B [water/acetonitrile (20:80, v/v)] in 30 min at a flow rate of 1 mL/min.

Spectroscopy. To determine the products of sesamin reacted with S9 mix and the metabolites of sesamin in rat bile, all NMR spectra were obtained on a Bruker DMX-500 spectrometer with ^{1}H NMR at 500 MHz and with ^{13}C NMR at 125 MHz. Chemical shifts are given in parts per million, using DMSO-d_6 as the solvent and internal standard. The mass spectra were recorded with an LCQ LC/MS system (Thermo Finnigan, San Jose, CA), equipped with an ESI ion source, which was used in the negative ion mode. The ESI parameters were as follows: spray voltage, 4.5 kV; sheath gas flow rate, 20 (arbitrary units); auxiliary gas flow rate, 0 (arbitrary units); capillary voltage, −14 V; capillary temperature, 240 °C; tube lens offset, 5 V; octapole 1 offset, 1 V; lens voltage, 14 V; octapole 2 offset, 6 V; and octapole radio frequency amplitude, 400 V$_{p-p}$. High-resolution FAB-MS spectra were recorded on a JMX-HX/HX110A system (JEOL Ltd., Tokyo, Japan) in the negative mode.

Analysis of Metabolites of Sesamin in Rat Bile. The animals were fasted for 15 h prior to administration of sesamin. Under diethyl ether anesthesia, a cannula was placed into the biliary duct. After surgery, the animals were placed in restraining cages and allowed free access to drinking water. Sesamin suspended in carboxymethyl cellulose was administered by oral intubation at a dosage of 500 mg/kg of body weight after the animals regained consciousness. In the second experiment for assessment of the excretion rate, sesamin dissolved in olive oil was orally administered at a dosage of 1 mg/kg of body weight. Bile was collected in a tube over 24 h, and bile samples were stored at −80 °C until needed. The bile samples were added to the enzyme mixture of glucuronidase and sulfatase and incubated at 37 °C for 3 h. After the addition of 1 N HCl to a final concentration of 0.05 N to the reaction mixtures, they were extracted with ethyl acetate. After concentration in vacuo, the resultant extract was dissolved in methanol/ethyl acetate (1:1, v/v) and then subjected to reversed-phase HPLC under the same conditions as the enzymatic reaction.

Superoxide Anion Radical Scavenging Activity. Superoxide anion radical (O$_2$$^-$) scavenging activity was analyzed by electron spin resonance (ESR) spectrometry, using the DMPO spin adduct generated by the hypoxanthine and xanthine oxidase reaction. ESR spectra were recorded with a JEOL JES-FR30 spectrometer, using an aqueous quartz flat cell (JEOL LC-12 ESR cuvette, internal size of 60 mm × 10 mm × 0.31 mm, effective volume of 160 μL). The sampling procedure used a 100 mM sodium phosphate buffer solution (pH 7.4) as the solvent. Fifty microliters of 2.0 mM hypoxanthine solution, 35 μL of 5.5 mM diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid, 50 μL of the sample solution, 15 μL of DMPO, and 50 μL of xanthine oxidase (0.4 unit/mL) were put into a test tube. After rapid stirring, 200 μL of the mixture was taken into the flat cell. ESR spectrum recording started 60 s after the addition of xanthine oxidase, with a recording rate of 5 mT/min. After recording, the signal intensity of the lowest field peak of the spectrum was normalized as the relative height against the standard signal intensity of the manganese oxide marker. The absolute concentration of DMPO−O$_2$$^-$ was finally determined by double-integration of the ESR spectrum.

Hydroxy Radical Scavenging Activity. Hydroxy radical (•OH) scavenging activity was analyzed in the same way as superoxide anion radical scavenging activity, using the DMPO spin adduct generated by the Fenton reaction. The sampling procedure used 100 mM sodium phosphate buffer solution (pH 7.4) as the solvent. Seventy-five microliters of 200 μM FeSO$_4$ and 200 μM DTPA solution, 50 μL of the sample solution, and 20 μL of 0.879 M DMPO were put into a test tube. Then, 20 μL of 10 mM H$_2$O$_2$ was added to the mixture. After 10 s of stirring, the reaction mixture was put into the flat cell. ESR
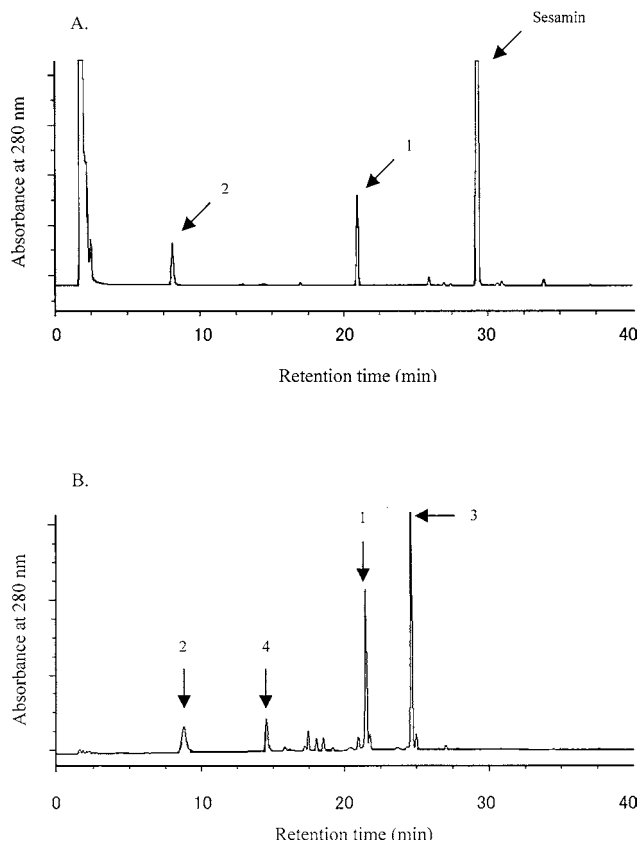
Figure 1. Reversed-phase HPLC chromatograms for isolating (A) the reaction products of sesamin with rat S9 mix and (B) the metabolites of sesamin in rat bile.

spectrum recording started 60 s after the addition of H$_2$O$_2$, with a recording rate of 5 mT/min. After recording, the signal intensity of the lowest field peak of the spectrum was normalized as the relative height against the standard signal intensity of the manganese oxide marker. The absolute concentration of DMPO−OH was finally determined by double-integration of the ESR spectrum.

1,1-Diphenyl-2-picrylhydradyl (DPPH) Scavenging Activity. DPPH scavenging activity was analyzed by ESR spectrometry. One hundred microliters of 60 μM DPPH dissolved in 50% CH$_3$CN and 100 μL of the sample solution dissolved in 50% CH$_3$CN were put into a test tube. After 10 s of stirring, the reaction mixture was put into the flat cell. ESR spectrum recording started 60 s after the addition of DPPH, with a recording rate of 5 mT/min. After recording, the signal intensity of the lowest field peak of the spectrum was normalized as the relative height against the standard signal intensity of the manganese oxide marker.
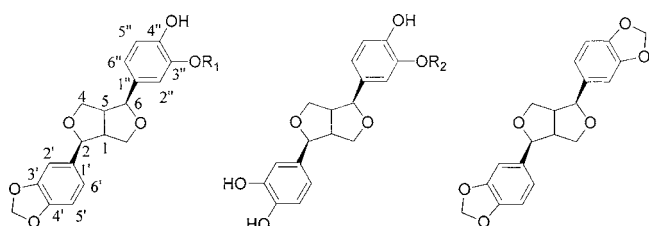
Inhibitory Activity of Lipid Peroxidation. Inhibitory activity of lipid peroxidation was measured by the thiobarbituric acid-reactive substance (TBARS) method (*11*−*13*). The reaction mixture, comprising 100 μL of phosphatidylcholine (10 mg/mL) in 0.1 M potassium phosphate buffer, pH 7.4, 50 μL of sample solution in CH$_3$CN, 20 μL of 2,2′-azobis(2-methylpropionamidine)·2HCl (510 mM) in H$_2$O, was incubated at 37 °C for 1 h. One hundred microliters of the reaction mixture, 50 μL of 1,3-diphenyl-2-thiobarbituric acid in CH$_3$CN, and 250 μL of HCl−CH$_3$COOH buffer (pH 2.0) were put into a test tube and reacted at 100 °C for 30 min. TBARS was extracted with *n*-BuOH, and its concentration was determined by fluorescence spectroscopy with excitation at 532 nm and emission at 553 nm.

RESULTS

Structural Determination of the Enzymatic Reaction Products. Two enzymatic reaction products were shown to be generated from sesamin by HPLC analysis (**Figure 1**). These products had molecular weights of 342 (compound **1**) and 330

Table 1. ¹H and ¹³C NMR Spectral Data for the Metabolites of Sesamin

| | chemical shifts of compounds 1–4 | | | | | | | |
| | 1 | | 2 | | 3 | | 4 | |
position	δ_C	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C	δ_H (J = Hz)
1	53.9	2.95 m	53.6	2.92 m	53.8	3.00 m	53.6	2.69 m
2	85.0	4.61 d (4.3)	84.9	4.51 d (4.2)	84.9	4.37 d (4.9)	85.0	4.53 d (4.9)
4a	71.1	4.10 dd (6.8, 8.9)	70.7	4.06 dd (6.8, 8.9)	71.0	4.10 dd (7.8, 8.8)	70.7	4.06 dd (6.8, 8.8)
4b		3.69 dd (3.6, 9.1)	70.7	3.67 dd (3.4, 8.9)		3.73 dd (3.9, 8.8)		3.70 dd (4.0, 8.8)
5	53.6	2.95 m			53.5	3.00 m	53.6	2.69 m
6	84.8	4.52 d (4.3)			85.1	4.59 d (4.9)	85.2	4.57 d (4.9)
8a	70.8	4.06 dd (6.8, 8.9)			70.8	4.10 dd (7.8, 8.8)	71.0	4.10 dd (6.8, 8.8)
8b		3.71 dd (3.6, 9.1)				3.73 dd (3.9, 8.8)		3.68 dd (4.0, 8.8)
1′	135.6		132.3		135.5		132.3	
2′	106.6	6.90 d (1.4)	113.5	6.72 d (1.9)	106.5	6.91 d (1.0)	113.6	6.72 d (1.8)
3′	147.5		144.5		146.5		145.1	
4′	146.5		145		147.4		144.6	
5′	108.0	6.85 d (8.0)	115.2	6.66 d (8.1)	107.9	6.86 d (8.3)	115.3	6.66 d (8.1)
6′	119.4	6.82 dd (1.4, 8.0)	116.9	6.58 dd (1.9, 8.1)	119.3	6.82 dd (1.0, 8.3)	117.0	6.58 dd (1.8, 8.1)
1″	132.3				132.2		132.2	
2″	113.6	6.71 d (2.0)			110.4	6.87 d (1.5)	110.4	6.87 d (1.6)
3″	145.1				147.5		147.5	
4″	144.7				145.9		145.9	
5″	115.3	6.66 d (8.0)			115.1	6.71 d (8.3)	115.1	6.71 d (8.1)
6″	117.1	6.57 dd (2.0, 8.0)			118.6	6.74 dd (1.5, 8.3)	118.6	6.73 dd (1.6, 8.1)
−O−CH₂−O−	100.9	5.97 s			100.9	5.98 s		
−OH		8.84 s		8.83 br s		8.88 s		
−CH₃					55.6	3.75 s	55.6	3.48 s

Figure 2. Structures of sesamin and its metabolites (compounds **1**–**4**).

Compound **1**; R₁ = H   Compound **2**; R₂ = H
Compound **3**; R₁ = CH₃   Compound **4**; R₂ = CH₃   sesamin

(compound **2**) on ESI-MS. The chemical shift of the proton of the methylenedioxy moiety (−O−CH₂−O−) in sesamin was 5.97 ppm with a signal intensity of four protons, but the proton signal intensity of the methylenedioxy moiety in compound **1** was only two protons by ¹H NMR analysis. No proton signal of the methylenedioxy moiety in compound **2** was observed. The chemical shifts of other proton signals in compounds **1** and **2** were similar to those of sesamin. The structure of compound **1** was therefore identified as (1R,2S,5R,6S)-6-(3,4-dihydrox-yphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo[3,3,0]-octane, which was reported as demethylpiperitol (*14*). Compound **2** was obtained as a white powder. The molecular formula was determined to be C₁₈H₁₈O₆ ([M]⁻ at *m/z* 329.1031) by HRFABMS. The structure of compound **2** was identified as (1R,2S,5R,6S)-2,6-bis(3,4-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]octane. ¹H and ¹³C NMR spectral data of these compounds are shown in **Table 1**.

Structural Determination of the Metabolites of Sesamin in Rat Bile. The metabolites of sesamin in rat bile after oral administration were analyzed using reversed-phase HPLC. Sesamin was mainly metabolized to four products as shown in **Figure 2**. The metabolites were obtained after treatment with glucuronidase and sulfatase, so they existed as substances conjugated by glucuronic acid and/or sulfuric acid.

Two of the four metabolites were determined as compounds **1** and **2**, the same products obtained as in the case of S9 mix

reaction. The molecular weights of the other metabolites (compounds **3** and **4**) were determined as 356 and 344 by ESI-MS. The ¹H and ¹³C NMR spectra of compounds **3** and **4** showed that the structures contained one methoxy moiety. The heteronuclear multiple-bond correlation (HMBC) spectrum displayed ¹H−¹³C long-range couplings from −OCH₃ to C-3″, from −OH to C-3″, C-4″, and C-5″, from H-2″ to C-1″, C-3″, C-4″, and C-6″, from H-5″ to C-3″ and C-4″, and from H-6″ to C-1″, C-2″, and C-3″. These ¹H−¹³C long-range correlations indicated a 3-methoxy-4-hydroxyphenyl moiety. The struc-tures of compounds **3** and **4** were confirmed as (1R,2S,5R,6S)-6-(4−hydroxy-3-methoxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo[3,3,0]octane and (1R,2S,5R,6S)-6-(4-hydroxy-3-methoxyphenyl)-2-(3,4--dihydroxyphenyl)-3,7-dioxabicyclo[3,3,0]octane by COSY, HSQC, HMBC, and HSQC-TOQCY experiments (*15*). Com-pound **3** was previously reported as piperitol (*16*), and com-pound **4** had a similar structure of 3′-O-demethylepipinoresinol (*17*). ¹H and ¹³C NMR spectra of compounds **3** and **4** are shown in **Table 1**.

Radical Scavenging Activity in Vitro. The methylenediox-yphenyl moiety of sesamin was changed to the 3,4-dihydrox-yphenyl (catechol) moiety in compound **1** and to the bis(3,4-dihydroxyphenyl) (dicatechol) moiety in compound **2** both in vitro and in vivo. The 4-hydroxy-3-methoxyphenyl moiety in compounds **3** and **4** was found only in vivo. We examined whether the metabolites had antioxidative activities in vitro in comparison with catechin, which is a typical compound with a catechol moiety. As shown in **Table 2**, the superoxide anion radical scavenging activities of compounds **1**, **2**, and **4** were 55.5, 73.7, and 53.6%, respectively, at 50 μM. The DPPH radical scavenging activities of compounds **1**, **2**, and **4** were 11.4, 70.5, and 43.2%, respectively, at 5 μM. The inhibitory activities of compounds **1**, **2**, and **4** on lipid peroxidation were 37.9, 71.3, and 42.0%, respectively, at 60 μM. Compounds **1** and **4** showed weak hydroxy radical scavenging activity (5.4 and 11.9%), but compound **2** showed strong activity (59.2%) at 250 μM. On the other hand, sesamin and compound **3** did not show

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Table 2. Antioxidative Activities of Sesamin, Its Metabolites, and Catechin against O_2^-, $\cdot OH$, DPPH, and Lipid Peroxidation Estimated by the Inhibition of the Production of TBARS

compound	antioxidative activity (%)			
	$O_2^{-\ a}$	$\cdot OH^b$	DPPH^c	TBARS^d
1	55.5	5.4	11.4	37.9
2	73.7	59.2	70.5	71.3
3	2.5	5.7	1.2	6.7
4	53.6	11.9	43.2	42.0
sesamin	3.0	2.3	0.0	5.8
catechin	60.5	14.3	40.9	76.3

^{a} Cocentration $= 50\ \mu M$. ^{b} Concentration $= 250\ \mu M$. ^{c} Concentration $= 5\ \mu M$. ^{d} Concentration $= 50\ \mu M$. (The head is arranged).

antioxidative activities. The antioxidative activities of catechin against O_2^-, DPPH, and lipid peroxidation were 60.5, 40.9, and 76.3%, respectively. However, catechin showed weak $\cdot OH$ scavenging activity (14.3%) in our experimental method.

DISCUSSION

It was previously reported that sesamin has antioxidative activity in the liver, but the mechanism of this effect was unclear. Flavonoids such as catechin, epicatechin, quercetin, kaemferol, and cyanidin generally show antioxidative effects (*18*, *19*), because they have a catechol moiety in their structures. The *o*-dihydroxyphenyl structure at the 3′- and 4′-positions in the B ring of flavonoids is necessary for high radical scavenging activity (*20*). However, sesamin does not have a hydroxyphenyl moiety, so we hypothesized that sesamin should be metabolized to biologically active compounds after absorption into the body.

Methylenedioxyphenyl compounds such as methylenedioxybenzene, methylenedioxyamphetamine, and methylenedioxymethamphetamine are demethylated by cytochrome P-450-catalyzed oxidation (*21*, *22*). Numerous studies have established the metabolism of a variety of methylenedioxyphenyl compounds both in vivo and in vitro. Although many of the metabolic pathways involve the action of microsomal mixed function oxidases or conjugating enzymes, or both, on specific functional groups or substituents in the aromatic ring, the major pathway common to all methylenedioxyphenyl compounds results from oxidative demethylenation of the methylenedioxy ring to yield the corresponding catechol.

In the present study, sesamin was demonstrated to be enzymatically demethylated by rat liver homogenate in vitro. Two enzymatic reaction products, compounds **1** and **2**, showed radical scavenging effects on O_2^- and DPPH radicals and inhibitory effects against lipid peroxidation. Compound **2**, a novel product, showed especially strong $\cdot OH$ scavenging activity, although sesamin, compound **1**, and catechin did not show scavenging activity. It might therefore be necessary for the structure to contain more than two catechol (dicatechol) moieties for the scavenging effect against the hydroxy radical.

Sesamin was also metabolized to at least four compounds in vivo after oral administration to rats. Two of the metabolites were identified as compounds **1** and **2**. Another metabolite was established as compounds **3** and **4**, containing the 4-hydroxy-3-methoxyphenyl moiety. The methylating reaction on the catechol moiety was considered to be catalyzed by catechol-*O*-methyl transferase (COMT). In view of the observation that compounds **1**, **2**, and **4** showed O_2^- and $\cdot OH$ scavenging activities, but compound **3** did not show any radical scavenging activities, the antioxidative effects of sesamin in vivo were ascribed to the three metabolites, compounds **1**, **2**, and **4**, generated in the liver.

Much attention has been focused on natural products, including phenolic acids, tannins, lignans, flavonoids, and carotenoids, for prevention and treatment of many diseases. The in vitro effectiveness of antioxidants against oxidative stress has already been demonstrated in many studies. In view of the metabolism of flavonoids, such as catechin and epicatechin (*23*), β-glucuronidation of the hydroxyphenyl moiety in the structure was rapid and the major metabolic pathway in vivo. UDP-β-glucuronosyl transferase catalyzes β-glucuronidation of flavonoids in both the intestine and liver. Some of the metabolites with free catechol moieties retained the antioxidative properties. The methylation of the catechol moiety is generally followed by the action of COMT in both the liver and kidney before excretion of the metabolites into the bile and urine. Previously, we reported that $\sim 10-15\%$ of the orally ingested catechin and epicatechin was excreted in rat bile (*23*). In the present study, $>40\%$ of the orally ingested sesamin was excreted in bile through the liver as metabolites. The difference of the excretion rates in bile could be explained by the higher hydrophobicity of sesamin as compared with that of flavonoids. It is possible that sesamin absorbed through the intestine was selectively captured in the liver due to its hydrophobicity, where the antioxidative metabolites were generated by demethylation of the methylenedioxyphenyl moieties. Therefore, sesamin might effectively exert antioxidative actions in the liver. One of the metabolites of sesamin, compound **2** with two catechol (dicatechol) moieties, is a novel antioxidant and shows much higher radical scavenging activities than any flavonoid reported to date.

In conclusion, sesamin itself has no antioxidative properties, but it is converted to prominently antioxidative metabolites in the liver, where they exert protective actions against oxidative damage in the liver. Sesamin should be regarded as a prodrug, and the three antioxidative metabolites may be responsible for the protective effects of sesamin against oxidative stress in vivo.

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