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Identification of Metabolites of (−)-Epicatechin Gallate and Their Metabolic Fate in the Rat

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After intravenous administration of (−)-epicatechin gallate to Wistar male rats, its biliary metabolites were examined. Deconjugated forms of (−)-epicatechin gallate metabolites were prepared by β-glucuronidase/sulfatase treatment and purified by HPLC. Five compounds were subjected to FAB-MS and NMR analyses. These metabolites were shown to be (−)-epicatechin gallate, 3′-*O*-methyl-(−)-epicatechin gallate, 4′-*O*-methyl-(−)-epicatechin gallate, 4″-*O*-methyl-(−)-epicatechin gallate, and 3′,4″-di-*O*-methyl-(−)-epicatechin gallate. After oral administration, five major metabolites excreted in rat urine were purified in their deconjugated forms and their chemical structures identified. They were degradation products from (−)-epicatechin gallate, pyrogallol, 5-(3,4-dihydroxyphenyl)-γ-valerolactone, 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid, 3-(3-hydroxyphenyl)propionic acid, and *m*-coumaric acid. Time course analysis of the identified (−)-epicatechin gallate metabolites showed that (−)-epicatechin gallate and its conjugate appeared in the plasma with their highest levels 0.5 h after oral administration; their levels rapidly decreased, and then they disappeared by 6 h. The degradation products, mainly in their conjugated forms, emerged at 6 h, peaked at 24 h, and disappeared by 48 h. In urine samples, (−)-epicatechin gallate and its methylated metabolites were hardly detected and the degradation products began to be excreted in the 6−24 h period, peaked in the 24−48 h period, and then began to disappear. The most abundant metabolite in both the plasma and the urine was found to be the conjugated form of pyrogallol. On the basis of these results, a possible metabolic route of (−)-epicatechin gallate orally administered to the rat is proposed.

KEYWORDS: (−)-Epicatechin gallate; catechin; tea; metabolism

INTRODUCTION

Recent epidemiological studies have revealed that the intake of flavonoids is inversely associated with the risk of coronary heart disease and stroke (*1*−*3*). These studies have suggested that tea catechins, as major ingestible flavonoids particularly in Japan, may be responsible for this inverse association (*1*, *2*). Imai *et al*. (*4*) have reported that an inverse association between green tea consumption and cancer incidence was found in a prospective cohort study of a Japanese population.

With the potentially beneficial role of tea catechins in human health becoming increasingly significant, the metabolic fate of tea catechins in the body has recently become a subject of considerable interest and is beginning to be elucidated to some extent with respect to (−)-epicatechin (*5*, *6*), (−)-epigallocatechin (*7*), and (−)-epigallocatechin gallate (*8*−*11*). However, the metabolic fate of (−)-epicatechin gallate, one of the major tea catechins, still remains to be clarified. We describe here identification of (−)-epicatechin gallate metabolites in the rat bile and urine, and their metabolic fate in the plasma and urine.

MATERIALS AND METHODS

Chemicals and Reagents. (−)-Epicatechin gallate was purchased from Kurita Chemical Co. (Tokyo, Japan). β-Glucuronidase type H-1 from *Helix pomatia* and D-saccharic acid 1,4-lactone were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were available products that were analytical grade or HPLC grade.

MS and NMR Analyses. MS (FAB ionization method) analysis was carried out with a JEOL JMS DX-300 mass spectrometer. NMR spectra were recorded on a JEOL Lambda-500 system. Samples (1−5 mg) were dissolved in 0.5 mL of deuterated methanol (methanol-d_4) (Merck). Chemical shifts are expressed in parts per million relative to tetramethylsilane (TMS) as an internal standard. Specific rotation data were determined by using a Jasco DIP-1000 polarimeter. CD measurements were performed with a Jasco J-600 apparatus.

Animals and Animal Experiments. Wistar male rats were maintained on a special diet free from naturally occurring polyphenols for 1 week (*12*). Bile duct cannulation was carried out on the animals according to the method of Hackett *et al*. (*13*). (−)-Epicatechin gallate (10 mg) in 1 mL of 10% aqueous ethanol was administered intravenously to the rats (300−400 g). Bile samples from 10 rats were collected

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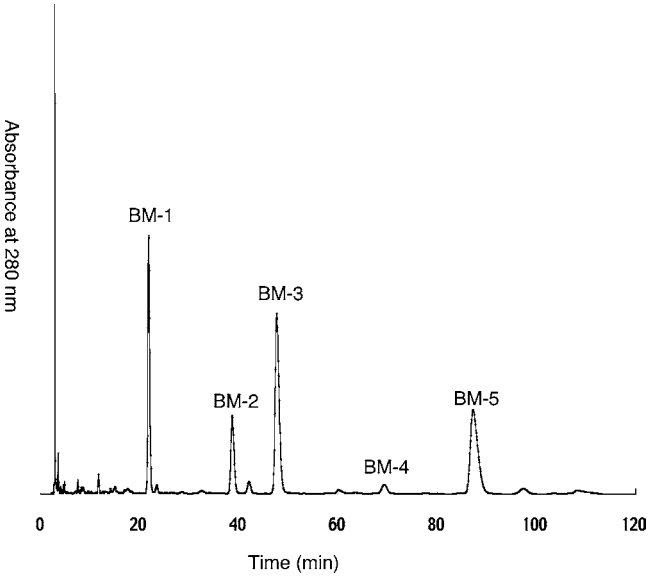

Figure 1. HPLC chromatogram of (–)-epicatechin gallate metabolites from a rat bile sample.

in test tubes containing 3 mL of 0.2 M sodium acetate buffer [containing 0.15 mM EDTA and 1% ascorbic acid (pH 4.8)] over a 4 h period after administration. All samples were frozen immediately and stored at −20 °C until use. To collect urinary samples, (−)-epicatechin gallate (50 mg/rat) was administered orally to the rats (400−500 g). Urine was collected for 48 h after administration in a tube containing 0.2 M sodium acetate buffer (pH 4.8).

In time course analysis of (−)-epicatechin gallate metabolites, (−)-epicatechin gallate was administered to rats (6 weeks of age, 130−150 g) by intragastric gavage at a dose of 100 mg/kg of body weight. Samples of the blood were taken into a tube containing heparin 0.5, 1, 3, 6, 24, and 48 h after administration. Plasma samples were separated from blood cells by centrifugation (3000*g*). Urine samples were collected 0−6, 6−24, 24−48, and 48−72 h after administration in chilled vessels. Plasma and urine samples were stored at −20 °C until use.

Purification of (−)-Epicatechin Gallate Metabolites from Bile. To the bile sample (∼60 mL) was added β-glucuronidase type H-1 (80 mg) which is a mixture of β-glucuronidase (28 000 units) and sulfatase (1400 units). The reaction mixture was incubated at 37 °C for 6 h with gentle shaking. The reaction mixture was extracted three times with the same volume of ethyl acetate. The organic phase was concentrated to dryness. The residue was dissolved in an acetonitrile/ethyl acetate/0.1% acetic acid mixture (18:0.6:90, v/v/v) and subjected to preparative HPLC. Preparative HPLC was performed with a 250 mm × 20 mm (inside diameter) Capcell Pak C18 AG120 column (Shiseido Co., Ltd., Tokyo, Japan) in a Jasco liquid chromatograph apparatus equipped with a Jasco UV-970 detector. Elution was done with an acetonitrile/ethyl acetate/0.1% acetic acid mixture (18:0.6:90, v/v/v) at a flow rate of 10 mL/min at room temperature. The elution pattern was monitored by measuring the absorbance at 280 nm. Five fractions (**BM-1**−**BM-5**) were collected (**Figure 1**), concentrated under reduced pressure to remove the organic solvents, and then freeze-dried. Since the **BM-2** fraction contained a contaminant compound, this fraction was further purified by preparative HPLC under the same conditions as described above except using 20% (v/v) aqueous methanol containing 0.1% acetic acid as a mobile phase. Finally, five purified compounds were obtained (yields of 10 mg for **BM-1**, 4.8 mg for **BM-2**, 6.4 mg for **BM-3**, 0.8 mg for **BM-4**, and 12.1 mg for **BM-5**) and were analyzed by analytical HPLC, MS, and NMR. Analytical HPLC was carried out with a 250 mm × 4.6 mm (inside diameter) Capcell Pak C18 AG120 column (Shiseido Co., Ltd.) in a Waters liquid chromatograph apparatus equipped with a Waters model M996 photodiode array detector. The column was eluted with an acetonitrile/ethyl acetate/0.05% phosphoric acid mixture (12:0.6:90, v/v/v) at a flow rate of 1.0 mL/min at 40 °C. The elution pattern was monitored by measuring the absorbance at 280 nm, and UV spectra of the compounds were also recorded by using wavelengths in the range of 200−400 nm.

BM-1: negative FAB-MS *m*/*z* 441 ([M − H]$^-$). The compound was identified as (−)-epicatechin gallate by direct comparison of ^{1}H and ^{13}C NMR spectral data with those of an authentic sample.

BM-2: negative FAB-MS *m*/*z* 455 ([M − H]$^-$); ^{1}H NMR (500 MHz, methanol-d_4) δ 2.86 (1H, dd, J = 1.9, 17.7 Hz, H-4a), 3.00 (1H, dd, J = 4.9, 17.7 Hz, H-4b), 3.82 (3H, s, OCH_3), 5.04 (1H, s, H-2), 5.53 (1H, m, H-3), 5.96 (2H, s, H-6,8), 6.70 (1H, d, J = 8.2 Hz, H-5′), 6.80 (1H, dd, J = 1.8, 8.2 Hz, H-6′), 6.92 (2H, s, H-2″,6″), 6.93 (1H, d, J = 1.8 Hz, H-2′). The ^{1}H NMR spectrum was in good agreement with that of an authentic 4″-*O*-methyl-(−)-epicatechin gallate reported previously (*14*).

BM-3: negative FAB-MS *m*/*z* 455 ([M − H]$^-$); ^{1}H NMR (500 MHz, methanol-d_4) δ 2.86 (1H, dd, J = 1.3, 17.6 Hz, H-4a), 3.02 (1H, dd, J = 4.6, 17.6 Hz, H-4b), 3.60 (3H, s, OCH_3), 5.06 (1H, s, H-2), 5.53 (1H, m, H-3), 5.95 (1H, d, J = 2.3 Hz, H-8), 5.97 (1H, d, J = 2.3 Hz, H-6), 6.73 (1H, d, J = 8.3 Hz, H-5′), 6.86 (1H, dd, J = 1.5, 8.3 Hz, H-6′), 6.99 (2H, s, H-2″,6″), 7.07 (1H, d, J = 1.5 Hz, H-2′); ^{13}C NMR (methanol-d_4, 125 MHz) δ 27.0 (C-4), 56.2 (methoxy carbon), 70.0 (C-3), 79.0 (C-2), 96.0 (C-8), 96.7 (C-6), 99.4 (C-4a), 110.2 (C-2″,6″), 111.6 (C-2′), 115.8 (C-5′), 120.9 (C-6′), 121.5 (C-1″), 131.5 (C-1′), 139.9 (C-4″), 146.5 (C-3″,5″), 147.4 (C-3′), 148.7 (C-4′), 157.4 (C-8a), 157.9 (C-5,7), 167.4 (C═O).

BM-4: negative FAB-MS *m*/*z* 455 ([M − H]$^-$); ^{1}H NMR (500 MHz, methanol-d_4) δ 2.85 (1H, dd, J = 2.1, 17.1 Hz, H-4a), 3.00 (1H, dd, J = 4.4, 17.1 Hz, H-4b), 3.80 (3H, s, OCH_3), 5.06 (1H, s, H-2), 5.53 (1H, m, H-3), 5.96 (2H, s, H-6,8), 6.85 (1H, d, J = 8.3 Hz, H-5′), 6.93 (1H, dd, J = 2.5, 8.3 Hz, H-6′), 6.94 (2H, s, H-2″,6″), 6.96 (1H, d, J = 2.5 Hz, H-2′).

BM-5: negative FAB-MS *m*/*z* 469 ([M − H]$^-$); ^{1}H NMR (500 MHz, methanol-d_4) δ 2.88 (1H, d, J = 16.0 Hz, H-4a), 3.04 (1H, d, J = 16.0 Hz, H-4b), 3.61 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 5.07 (1H, s, H-2), 5.54 (1H, m, H-3), 5.97 (1H, s, H-8), 5.98 (1H, s, H-6), 6.73 (1H, d, J = 8.4 Hz, H-5′), 6.87 (1H, d, J = 8.4 Hz, H-6′), 6.96 (2H, s, H-2″,6″), 7.06 (1H, s, H-2′); ^{13}C NMR (methanol-d_4, 125 MHz) δ 26.9 (C-4), 56.2 (methoxy carbon), 60.8 (methoxy carbon), 70.4 (C-3), 78.9 (C-2), 96.0 (C-8), 96.7 (C-6), 99.3 (C-4a), 110.3 (C-2″,6″), 111.6 (C-2′), 115.8 (C-5′), 120.8 (C-6′), 126.6 (C-1″), 131.4 (C-1′), 141.3 (C-4″), 147.3 (C-3′), 148.7 (C-4′), 151.7 (C-3″,5″), 157.3 (C-8a), 157.9 (C-7), 158.0 (C-5), 166.9 (C═O).

Purification of (−)-Epicatechin Gallate Metabolites from Urine. The urine sample (∼200 mL) was treated with β-glucuronidase type H-1 (80 mg). The reaction mixture was incubated at 37 °C for 6 h with gentle shaking. The reaction mixture was extracted three times with the same volume of ethyl acetate. The organic phase was concentrated to dryness. The residue was dissolved in an acetonitrile/ethyl acetate/0.1% acetic acid mixture (15:0.6:90, v/v/v) and subjected to preparative HPLC. Preparative HPLC was performed in the same way as for the bile metabolites, except using an acetonitrile/ethyl acetate/0.1% acetic acid mixture (15:0.6:90, v/v/v) as a mobile phase. Five fractions (**UM-1**−**UM-5**) were collected (**Figure 2**), concentrated under vacuum to remove the organic solvents, and then freeze-dried. Two fractions, **UM-2** and **UM-4**, were found to contain impurities by analytical HPLC, and hence, the fractions were further purified by preparative HPLC under the same conditions described above, except that 18% (v/v) methanol containing 0.1% acetic acid was used as a solvent. Finally, five purified compounds were obtained (yields of 2.9 mg for **UM-1**, 1.2 mg for **UM-2**, 7.4 mg for **UM-3**, 1.1 mg for **UM-4**, and 3.3 mg for **UM-5**) and were analyzed by analytical HPLC, MS, and NMR. Analytical HPLC was carried out under the same conditions as described for purification of (−)-epicatechin gallate metabolites from bile.

UM-1. See the Results.

UM-2: negative FAB-MS *m*/*z* 225 ([M − H]$^-$); $[\alpha]_D$ 9.3° (*c* 0.05%, in methanol, 30 °C); ^{1}H NMR (500 MHz, methanol-d_4) δ 1.60 (1H, m, H-3b), 1.80 (1H, m, H-3a), 2.31 (1H, ddd, J = 7.1, 8.6, 15.7 Hz, H-2b), 2.42 (1H, ddd, J = 6.3, 9.3, 15.7 Hz, H-2a), 2.56 (1H, dd, J = 6.5, 13.6 Hz, H-5b), 2.62 (1H, dd, J = 6.6, 13.6 Hz, H-5a), 3.72 (1H, m, H-4), 6.52 (1H, dd, J = 1.8, 7.9 Hz, H-6′), 6.65 (1H, d, J = 1.8 Hz,

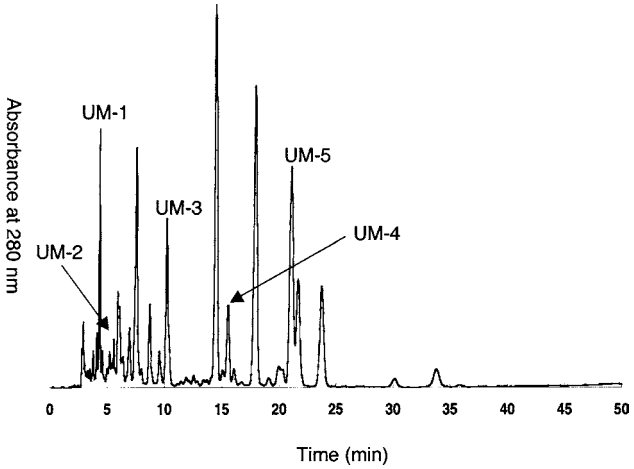

Figure 2. HPLC chromatogram of (–)-epicatechin gallate metabolites from a rat urine sample.

H-2′), 6.66 (1H, d, $J = 7.9$ Hz, H-5′); ^{13}C NMR (125 MHz, methanol-d_4) δ 32.7 (C-2), 33.1 (C-3), 44.5 (C-5), 73.5 (C-4), 116.3 (C-2′,5′), 121.8 (C-6′), 131.8 (C-1′), 144.7 (C-4′), 146.1 (C-3′), 171.3 (C-1, C=O).

UM-3: negative FAB-MS *m*/*z* 207 ([M − H]$^-$); $[\alpha]_D$ −12.0° (*c* 0.1%, in methanol, 30 °C); ^{1}H NMR (500 MHz, methanol-d_4) δ 1.95 (1H, dddd, $J = 6.9$, 9.7, 10.0, 12.9 Hz, H-3b), 2.24 (1H, dddd, $J = 4.9$, 6.9, 9.6, 12.9 Hz, H-3a), 2.33 (1H, ddd, $J = 4.9$, 10.0, 17.7 Hz, H-2b), 2.48 (1H, ddd, $J = 9.6$, 9.7, 17.7 Hz, H-2a), 2.79 (1H, dd, $J = 6.2$, 14.1 Hz, H-5b), 2.87 (1H, dd, $J = 6.3$, 14.1 Hz, H-5a), 4.72 (1H, dddd, $J = 6.2$, 6.3, 6.9, 6.9 Hz, H-4), 6.56 (1H, dd, $J = 2.0$, 8.1 Hz, H-6′), 6.68 (1H, d, $J = 2.0$ Hz, H-2′), 6.68 (1H, d, $J = 8.1$ Hz, H-5′); ^{13}C NMR (125 MHz, methanol-d_4) δ 27.9 (C-3), 29.5 (C-2), 41.5 (C-5), 83.3 (C-4), 116.4 (C-5′), 117.7 (C-2′), 122.0 (C-6′), 129.1 (C-1′), 145.3 (C-4′), 146.3 (C-3′), 180.4 (C-1, C=O).

UM-4: positive FAB-MS *m*/*z* 167 ([M + H]$^+$); ^{1}H NMR (500 MHz, methanol-d_4) δ 2.56 (2H, t, $J = 7.7$ Hz, H_2-2), 2.83 (2H, t, $J = 7.7$ Hz, H_2-3), 6.60 (1H, dd, $J = 2.2$, 7.8 Hz, H-6′), 6.65 (1H, dd $J = 2.2$, 2.2 Hz, H-2′), 6.68 (1H, d, $J = 7.8$ Hz, H-4′), 7.07 (1H, dd, $J = 7.8$, 7.8 Hz, H-5′); ^{13}C NMR (125 MHz, methanol-d_4) δ 32.1 (C-3), 36.8 (C-2), 114.1 (C-4′), 116.2 (C-2′), 120.5 (C-6′), 130.5 (C-5′), 143.7 (C-1′), 158.5 (C-3′), 176.9 (C-1, C=O).

UM-5: positive FAB-MS *m*/*z* 165 ([M + H]$^+$); ^{1}H NMR (500 MHz, methanol-d_4) δ 6.40 (1H, d, $J = 16.0$ Hz, H-2), 6.82 (1H, ddd, $J = 1.2$, 2.3, 7.9 Hz, H-6′), 6.99 (1H, dd, $J = 1.2$, 2.3 Hz, H-2′), 7.04 (1H, d, $J = 7.9$ Hz, H-4′), 7.21 (1H, dd, $J = 7.9$, 7.9 Hz, H-5′), 7.56 (1H, d, $J = 16.0$ Hz, H-3); ^{13}C NMR (125 MHz, methanol-d_4) δ 115.2 (C-2′), 118.5 (C-6′), 119.8 (C-2), 120.8 (C-4′), 131.0 (C-5′), 137.3 (C-3), 146.1 (C-1′), 159.1 (C-3′), 170.8 (C-1, C=O).

HPLC Analysis of (−)-Epicatechin Gallate Metabolites in Plasma and Urine. Each plasma (0.5 mL) and urinary (5 mL) sample was adjusted to pH 5.0 with 0.2 M sodium acetate buffer (pH 3.0) containing 1% ascorbic acid and 0.15 mM EDTA. The samples were then extracted three times with ethyl acetate. The ethyl acetate fractions were concentrated to dryness and dissolved in an acetonitrile/ethyl acetate/0.05% phosphoric acid mixture (12:0.6:90, v/v/v) for analytical HPLC. The aqueous phases were treated with β-glucuronidase type H-1 (700 units) for 6 h at 37 °C. The reaction mixtures were extracted three times with ethyl acetate. The organic fractions were concentrated and redissolved in the HPLC solvent. Urine samples were treated with the same procedure as mentioned above for plasma samples. HPLC analysis was carried out under the same conditions for analytical HPLC as described for purification of (−)-epicatechin gallate metabolites from urine, except that a Coulochem II model 5011 electrochemical detector was used for the plasma sample.

RESULTS

Structural Analysis of (−)-Epicatechin Gallate Metabolites from Bile. In this study, we succeeded in determining structures of (−)-epicatechin gallate metabolites in rat bile after intravenous administration. Deconjugated forms of five (−)-epicatechin gallate metabolites (**BM-1**−**BM-5**) were purified after enzymatic treatment with β-glucuronidase type H-1, which has both β-glucuronidase and sulfatase activities. Structural analysis of each compound was performed by HPLC, MS, and NMR analyses. Compound **BM-1** exhibited the same retention time and UV spectrum as authentic (−)-epicatechin gallate, and its ^{1}H NMR data could be superimposed with those of (−)-epicatechin gallate. Also, it was found that the retention time, UV spectrum, and mass spectrum of **BM-2** were the same as those of authentic 4″-*O*-methyl-(−)-epicatechin gallate reported in our previous paper (*14*). The ^{1}H NMR spectrum of **BM-2** was shown to be in accord with that of 4″-*O*-methyl-(−)-epicatechin gallate. As a result, compounds **BM-1** and **BM-2** were identified as (−)-epicatechin gallate and 4″-*O*-methyl-(−)-epicatechin gallate, respectively.

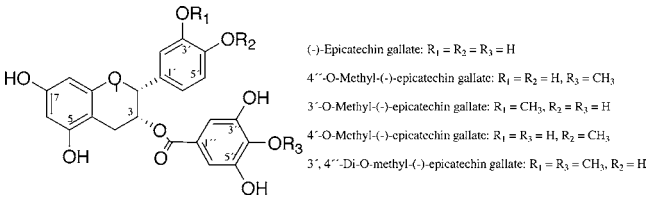

Figure 3. Structures of (–)-epicatechin gallate metabolites from rat bile.

Negative FAB-MS data of **BM-3** exhibited an [M − H]$^-$ ion peak at *m*/*z* 455, corresponding to monomethylated (−)-epicatechin gallate. The ^{1}H NMR spectrum of **BM-3** was similar to that of (−)-epicatechin gallate, except for a signal derived from methoxy protons (δ 3.60) and the downfield shift of H-2′ [from δ 6.92 for H-2′ of (−)-epicatechin gallate to δ 7.07] that were observed. The position of the methyl group was confirmed by the observation of the nuclear Overhauser effect between methyl protons and H-2′. Thus, compound **BM-3** was identified as 3′-*O*-methyl-(−)-epicatechin gallate.

A pseudomolecular ion peak of **BM-4** at *m*/*z* 455 ([M − H]$^-$) in negative FAB-MS was in agreement with that of monomethylated (−)-epicatechin gallate. Although the ^{1}H NMR spectrum of **BM-4** was similar to that of 4″-*O*-methyl-(−)-epicatechin gallate, the chemical shifts of H-2′ (δ 6.96), H-5′ (δ 6.85), and H-6′ (δ 6.93) of **BM-4** were observed to be more downfield than those of H-2′ (δ 6.93), H-5′ (δ 6.69), and H-6′ (δ 6.80) of 4″-*O*-methyl-(−)-epicatechin gallate. These downfield shifts of B ring protons were also found between (−)-epicatechin and 4′-*O*-methyl-(−)-epicatechin in our previous study (*6*). From these observations, **BM-4** was identified as 4′-*O*-methyl-(−)-epicatechin gallate.

In the case of **BM-5**, a pseudomolecular ion peak at *m*/*z* 469 ([M − H]$^-$) was observed by negative FAB-MS, suggesting that **BM-5** is dimethylated (−)-epicatechin gallate. The ^{1}H NMR spectrum of **BM-5** was very similar to that of 3′-*O*-methyl-(−)-epicatechin gallate, except for the appearance of two methoxy signals. The chemical shift of one methoxy signal (δ 3.61) was in accord with that (δ 3.60) of 3′-*O*-methyl-(−)-epicatechin gallate, and the shift of another one (δ 3.83) agreed with that (δ 3.82) of 4″-*O*-methyl-(−)-epicatechin gallate. Two cross-peaks between methyl protons (δ 3.61) and C-3′ and between methyl protons (δ 3.83) and C-4″ were observed by a heteronuclear multiple-bond connectivity (HMBC) experiment. These results demonstrated that compound **BM-5** is 3′,4″-di-*O*-methyl-(−)-epicatechin gallate. The structures of the five metabolites of (−)-epicatechin gallate identified are illustrated in **Figure 3**.

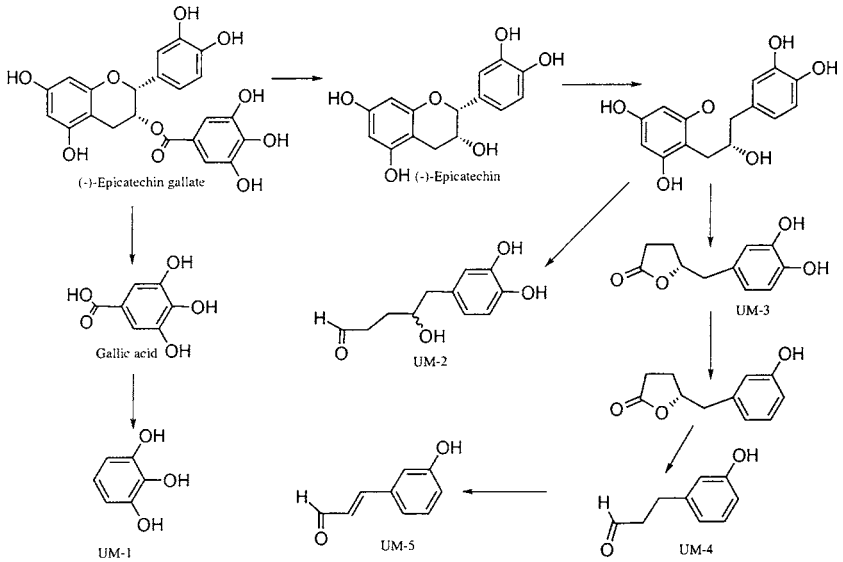

Figure 4. Proposed metabolic pathways of (–)-epicatechin gallate by rat intestinal bacteria.

Structural Analysis of Metabolites Derived from (−)-Epicatechin Gallate in Urine. In this study, Urinary metabolites derived from (−)-epicatechin gallate were the degradation products, and neither (−)-epicatechin gallate, methylated (−)-epicatechin gallate, nor their conjugates were detected. We isolated five compounds after treatment with a glucuronidase/sulfatase mixture to remove conjugates, and their chemical structures were determined by HPLC, MS, and NMR analyses.

Compound **UM-1** exhibited the same retention time and UV spectrum as authentic pyrogallol, and its ^{1}H and ^{13}C NMR data could be superimposed with those of pyrogallol reported by Meselhy *et al*. (*15*). Thus, **UM-1** was identified as pyrogallol.

The MS, ^{1}H NMR, and ^{13}C NMR data of **UM-3** agreed with those of 5-(3,4-dihydroxyphenyl)-γ-valerolactone (*15*). The optical rotation value ($[\alpha]_D$ −12.0°) was similar to those of 5-(3,4-dihydroxyphenyl)-γ-valerolactone ($[\alpha]_D$ −8.6°) (*15*) and 5-(3,5-dihydroxyphenyl)-γ-valerolactone ($[\alpha]_D$ −12.9°) (*10*). In addition, **UM-3** exhibited a (+) Cotton effect at ∼280 nm on CD measurement. In our previous report (*10*), 5-(3,5-dihydroxyphenyl)-γ-valerolactone, which exhibited a (+) Cotton effect at ∼280 nm, was reported to have an *R* configuration. It was therefore concluded that **UM-3** is 5-(3,4-dihydroxyphenyl)-γ-valerolactone with a 4*R* configuration.

The ^{1}H and ^{13}C NMR data of **UM-2** were comparable with those of 5-(3,4-dihydroxyphenyl)-γ-valerolactone (**UM-3**). However, in the ^{1}H NMR data, chemical shifts of H-3b (δ 1.60), H-3a (δ 1.80), H-5b (δ 2.56), H-5a (δ 2.62), and H-4 (δ 3.72) were observed to be significantly more upfield than those of **UM-3** [δ 1.95 (H-3b), 2.24 (H-3a), 2.79 (H-5b), 2.87 (H-5a), and 4.72 (H-4)]. In particular, there was a large upfield shift (1 ppm) of H-4 of **UM-2** as compared with that of the γ-valerolactone. In the ^{13}C NMR spectrum, C-4 of **UM-2** (δ 73.5) was also observed to be remarkably upfield compared with that of the γ-valerolactone (δ 83.3). The negative FAB-MS data of **UM-2** showed a pseudomolecular ion peak at *m*/*z* 225 ($[M - H]^-$), which was 18 mass units greater than that of **UM-3**. These observations suggested that 4-hydroxyvaleric acid was a part of the **UM-2** structure. From further experiments with H−H correlation spectroscopy, heteronuclear multiple-quantum coherence (HMQC), and HMBC, the chemical structure of **UM-2** was finally concluded to be 4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid. This compound was optically active ($[\alpha]_D$ 9.3°).

The ^{1}H and ^{13}C NMR data of **UM-4** were nearly identical with those of 3-(3-hydroxyphenyl)propionic acid as reported by Meselhy *et al*. (*15*). The positive FAB-MS data of **UM-4** [*m*/*z* 167 ($[M + H]^+$)] were in good agreement with those of the propionic acid. As a result, **UM-4** was determined to be 3-(3-hydroxyphenyl)propionic acid.

The positive FAB-MS data of **UM-5** exhibited a protonated molecular ion peak at *m*/*z* 165 ($[M + H]^+$), which was 2 mass units smaller than that of **UM-4**. The ^{1}H NMR data of **UM-5** showed ABCD-type aromatic protons at δ 6.82 (ddd, H-6′), 6.99 (dd, H-2′), 7.04 (d, H-4′), and 7.21 (dd, H-5′) and two proton signals derived from a *trans* olefinic (CH=CH) at δ 6.40 (1H, d, J = 16.0 Hz, H-2) and 7.56 (1H, d, J = 16.0 Hz, H-3). In the ^{13}C NMR spectrum of **UM-5**, a carbon signal derived from a carbonyl group at δ 170.8 was observed in addition to eight carbon signals. From these observations and further HMQC and HMBC experiments, **UM-5** was identified as 3-(3-hydroxyphenyl)propenoic acid (*m*-coumaric acid).

Structures of five urinary metabolites that have been identified are shown in **Figure 4**.

Time Course Analysis of Metabolites Derived from (−)-Epicatechin Gallate in Plasma and Urine. Using the metabolites identified above as standards, we determined by HPLC analysis the amount of each (−)-epicatechin gallate metabolite both present in the plasma and excreted in the urine over a time course. **Table 1** shows the concentration of metabolites in the plasma over time. (−)-Epicatechin gallate and its conjugated form were detected in the plasma, peaking at 0.5 h postdose and having completely disappeared at 6 h. The concentration ratio of (−)-epicatechin gallate and the (−)-epicatechin gallate conjugate at their peak was ∼8:1. The degradation metabolites from (−)-epicatechin gallate were also detected in their conjugated forms but not in their free forms except for **UM-4** [3-(3-hydroxyphenyl)propionic acid]. They emerged in the plasma 6 h after oral administration; their plasma concentrations peaked at 24 h and then gradually decreased, and almost all of the compounds finally disappeared after 48 h. The most abundant metabolite in the plasma was the **UM-1** (pyrogallol) conjugate followed by the **UM-3** [5-(3,4-dihydroxyphenyl)-γ-valerolactone] conjugate, and the **UM-4** [3-(3-hydroxyphenyl)propionic acid] conjugate. Plasma concentrations at their peak levels were ∼3.7 (**UM-1**), ∼2.4 (**UM-3**), and ∼1.9 times (**UM-4**)

Table 1. Time Course Analysis of the Concentration of ECg Metabolites in Rat Plasma

	plasma concentration (pmol/mL)[a]						
time (h)	ECg	ECg conjugate	**UM-1** conjugate	**UM-2** conjugate	**UM-3** conjugate	**UM-4**	**UM-4** conjugate
0.5	233 ± 232	29 ± 33	0	0	0	0	0
1	73 ± 69	4 ± 3	0	0	0	0	0
3	2 ± 2	5 ± 2	0	0	0	0	0
6	0	0	745 ± 375	136 ± 55	214 ± 82	323 ± 135	144 ± 29
24	0	0	868 ± 629	157 ± 138	562 ± 307	36 ± 62	439 ± 36
48	0	0	0	0	0	0	154 ± 16

[a] Values are the mean ± the standard deviation of three rats.

Table 2. Excretion of ECg Metabolites in Rat Urine

	urinary excretion amounts of metabolites (nmol)[a]						
period (h)	**UM-1** conjugate	**UM-2** conjugate	**UM-3** conjugate	**UM-4**	**UM-4** conjugate	**UM-5**	**UM-5** conjugate
0–6	0	0	0	0	0	0	0
6–24	832 ± 382	99 ± 5	25 ± 23	372 ± 68	58 ± 39	43 ± 60	48 ± 66
24–48	1224 ± 218	162 ± 4	201 ± 113	698 ± 154	711 ± 168	667 ± 216	753 ± 367
48–72	0	0	0	0	69 ± 60	220 ± 20	100 ± 57

[a] Values are the mean ± the standard deviation of three rats.

higher than those of intact (−)-epicatechin gallate. These results suggested that after oral administration of (−)-epicatechin gallate the metabolites that existed in the rat plasma were mainly degradation products, not intact (−)-epicatechin gallate, its methylated derivatives, or their conjugates.

Excretion of the (−)-epicatechin gallate metabolites in urine is shown in **Table 2**. None of the (−)-epicatechin gallate metabolites were detected during the first 6 h postdose. The degradation metabolites emerged between 6 and 24 h, peaked between 24 and 48 h, and greatly decreased between 48 and 72 h. Neither intact (−)-epicatechin gallate, methylated (−)-epicatechin gallate, nor their conjugates were found in the urine throughout the entire period. The most abundant metabolite excreted in urine was the **UM-1** (pyrogallol) conjugate, and the total amounts of this metabolite were 2 μmol (∼6% of the dose). The cumulative amounts of all metabolites excreted in urine by 72 h were 6.25 μmol [∼20% of the administered (−)-epicatechin gallate].

DISCUSSION

To examine the metabolic fate of (−)-epicatechin gallate in the body, we first determined the chemical structures of (−)-epicatechin gallate metabolites excreted in rat bile and urine. Subsequently, plasma concentrations and urinary excretion amounts of (−)-epicatechin gallate metabolites were examined using the identified metabolites as standards by HPLC.

After intravenous administration of (−)-epicatechin gallate, metabolites excreted in rat bile were isolated and identified as intact (−)-epicatechin gallate, three monomethylated (−)-epicatechin gallates, and a dimethylated (−)-epicatechin gallate. Most of these metabolites were found to exist in their conjugated forms (data not shown). Subsequently, five metabolites excreted in rat urine were isolated after oral administration of (−)-epicatechin gallate and their chemical structures determined. All of them were the degradation products derived from (−)-epicatechin gallate by intestinal bacteria. Three metabolites (**UM-1**, **UM-3**, and **UM-4**) were previously reported as the metabolites of (+)-catechin (*16*) and/or the degradation products of (−)-epicatechin gallate by human intestinal bacteria (*15*). **UM-5** (*m*-coumaric acid) and **UM-2** [4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid] are the new metabolites with respect to flavan-3-ols. On the basis of our results, and previous research on the biotransformation of (−)-epicatechin gallate by human intestinal bacteria (*15*) and on the metabolism of (+)-catechin (*16*, *18*), we outlined a proposal for the metabolic pathway of (−)-epicatechin gallate by intestinal bacteria as shown in **Figure 4**.

We also examined (−)-epicatechin gallate metabolites in rat plasma and urine over time. As a result, (−)-epicatechin gallate and its conjugate were found to exist in plasma during an earlier period (0.5−1 h) after oral administration, whereas the metabolites degraded from (−)-epicatechin gallate by intestinal bacteria were detected at high levels in the plasma 24 h postdose and were excreted in the urine at peak levels during the period from 24 to 48 h. These degradation metabolites were detected mainly in their conjugated forms and hence may be conjugated in lower parts of the small intestine, cecum, and large intestine and/or liver (*5*). On the other hand, since neither (−)-epicatechin gallate nor its conjugate was detected in the urine at any period, it is thought that almost all of these compounds were excreted into the bile as is the case with (−)-epigallocatechin gallate (*9*, *10*). Although it has been shown that (−)-epicatechin gallate hydrolyzes to form (−)-epicatechin and gallic acid in rat intestine prior to undergoing further degradation (**Figure 4**), they were hardly detected in either the plasma or the urine. In previous reports concerning the metabolism of (−)-epicatechin (*5*, *6*) and gallic acid (*17*), (−)-epicatechin or gallic acid in addition to their methylated and conjugated derivatives was found to be in the urine and plasma after oral administration to rats. Accordingly, it may be reasonable to surmise that (−)-epicatechin and gallic acid are absorbed into the body from upper parts but not lower parts of the intestine in the rat.

In a pharmacological study on the metabolites [5-(3-hydroxyphenyl)-γ-valerolactone, 5-(3,4-dihydroxyphenyl)-γ-valerolactone, 5-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone, and 3-(3-hydroxyphenyl)propionic acid] derived from (+)-catechin (*18*), these ring scission metabolites were reported to have weak or very slight toxicity. Further study on the physiological functions of degradation products from catechins is still required to clarify the beneficial effects on the body after oral administration of catechins.

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