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# Metabolism of (−)-Epigallocatechin Gallate by Rat Intestinal Flora

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Anaerobic metabolism of (−)-epigallocatechin gallate (EGCg) by rat intestinal bacteria was investigated in vitro. First, intestinal bacteria which are capable of hydrolyzing EGCg to (−)-epigallocatechin (EGC) and gallic acid (**2**) were screened with 169 strains of enteric bacteria. As a result, *Enterobacter aerogenes*, *Raoultella planticola*, *Klebsiella pneumoniae* susp. *pneumoniae*, and *Bifidobacterium longum* subsp. *infantis* were found to hydrolyze EGCg. Subsequent steps of EGCg metabolism are degradation of EGC (**1**) by intestinal bacteria. Then, EGC was incubated with rat intestinal bacteria in 0.1 M phosphate buffer (pH 7.1) and the degradation products were analyzed with time by HPLC or LC-MS. Further, the products formed from EGC were isolated and identified by LC-MS and NMR analyses. The results revealed that EGC was converted first to 1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (**3**) by reductive cleavage between 1 and 2 positions of EGC, and subsequently metabolite **3** was converted to 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (**4**) followed by the conversion to 5-(3,5-dihydroxyphenyl)-4-hydroxyvaleric acid (**5**) by decomposition of the phloroglucinol ring in metabolite **4**. This degradation pathway was considered to be the major route of EGCg metabolism in the in vitro study, but two minor routes were also found. In addition to the in vitro experiments, metabolites **3**, **4**, **5**, and **6** were detected as the metabolites after direct injection of EGC into rat cecum. When EGCg was administered orally to the rats, metabolites **4**, **5**, **6**, **11**, and **12** were found in the feces. Among the metabolites detected, metabolite **5** was dominant both in the cecal contents and feces. These findings suggested that the metabolic pathway of EGCg found in the in vitro study may be regarded as reflecting its metabolism in vivo.

**KEYWORDS: Metabolism; epigallocatechin gallate; intestinal flora**

## INTRODUCTION

It is well recognized that tea catechins have various physiological functions including antioxidative, blood cholesterol lowering, blood sugar level lowering, and cancer preventive activities (*1*−*4*). Many studies have shown that (−)-epigallocatechin gallate (EGCg), the most abundant catechin, has strong physiological activity. Accordingly, there has also been great interest in the metabolism of EGCg because of the importance of elucidating the mechanism responsible for its biological activity. Many studies to date have addressed the metabolism of EGCg in mammalia such as mouse, rat, and human. After oral administration of EGCg, it has been found in rat and human plasma (*5*−*9*). Distribution and excretion of EGCg (*10*−*12*) have been also reported in addition to its pharmacokinetics study (*13*, *14*). The metabolites of EGCg excreted in bile and urine were further identified (*10*, *15*−*18*). Among the metabolites identified, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone were found in human (*17*, *18*) and rat urine (*12*). These compounds are thought to be the ring-fission metabolites of EGCg and/or EGC by intestinal microflora. On the basis of the findings, the metabolic pathway of EGCg have been proposed in gut tract (*12*). Hattori et al. (*19*, *20*) has identified EGCg metabolites formed by human intestinal bacteria and then initial stage of EGCg metabolism was proposed. However, up until now there has been no detailed study on the whole metabolic pathway of EGCg by intestinal bacteria.

In this study, we investigated in detail the metabolic pathways of EGCg by rat intestinal bacteria in vitro. Further, we examine whether or not the in vitro metabolic pathway mirrors its metabolism in vivo.

## MATERIALS AND METHODS

**Chemicals.** (−)-Epigallocatechin-3-*O*-gallate (EGCg) and (−)-epigallocatechin (EGC) were obtained from Sigma-Aldrich Japan (Tokyo). General anaerobic medium (GAM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). All other chemicals were available products of analytical grade or HPLC grade.

**Animals.** Male Wistar rats were purchased from Charles Liver Laboratories Inc. (Yokohama, Japan) and housed in a room at 23 ± 3 °C and 50 ± 5% relative humidity, with a 12 h light-dark cycle. The rats were given normal pelleted food (Oriental Yeast Ltd., Tokyo, Japan)

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during experimental period. In this study, 15−21 weeks of age of rats were used. All experimental procedures were in accordance with the guidelines for animal experiments of Food Research Laboratories, Mitsui Norin Co., Ltd.

**Screening of Enteric Bacteria Capable of Hydrolyzing EGCg.** After sterilization of 9 mL of 0.1 M phosphate buffer (pH 7.1) by autoclaving at 121 °C for 15 min, EGCg solution (1 mL, 10 mg/mL) filtered through sterilized membrane filter DISMIC-25cs, 0.2 $\mu$m (Toyo Roshi Kaisha Ltd., Tokyo, Japan) was added to the buffer. Each bacterial strain (22 genera, 169 strains) was inoculated to the solution and then 2 mL of sterile mineral oil was layered over. The mixture was incubated at 37 °C for 24 h and then was centrifuged at 3500*g* for 10 min at room temperature. The supernatant was analyzed by HPLC to select the enterobacteria with the ability to hydrolyze EGCg. Analytical HPLC was carried out with a 250 × 4.6 mm i.d., 5 $\mu$m, Capcell Pak C18 UG120 (Shiseido Co. Ltd., Tokyo, Japan) in an Waters 2695 Separations Module (Nihon Waters, Tokyo, Japan) apparatus equipped with an Waters 2489 UV/VS Detector (270 nm). Elution was done with distilled water/methanol/acetonitrile/phosphoric acid (85/10/5/0.01, v/v/v/v) at a flow rate of 1 mL/min at 40 °C.

**Time-Course Analysis of the Metabolite Formation from EGC (1) by Rat Intestinal Flora.** Incubation method of EGC with rat enterobacteria was done according to that of Meselhy et al. (*22*). Fresh cecal contents (3.3 g) obtained from three male Wistar rats (16 weeks of age) were put into 300 mL of GAM broth and then incubated at 37 °C under anaerobic condition with an Anaeropack system (Mitsubishi Gas Chemical, Tokyo, Japan). After 3 days, the culture was centrifuged by Avanti J-25 (Beckman Coulter, Tokyo, Japan) at 15000*g* for 20 min at 4 °C, and the harvested cells were washed once with sterile water (200 mL). The cells were suspended in 150 mL of prefiltered 0.1 M phosphate buffer (pH 7.1), and 200 mg of EGC in 20 mL of 5% aqueous methanol prefiltrated with the sterilized membrane filter was added to the cell suspension. The mixture was incubated under anaerobic condition with the Anaeropack system. After incubation for 24, 48, 72, 96, and 168 h, aliquots (1 mL) of the incubation mixture were withdrawn in an anaerobic glovebox under $CO_2$ atmosphere. These samples were centrifuged by MX-301 centrifuge (TOMY Seiko, Tokyo, Japan) at 15000*g* for 10 min at 4 °C. The resulting supernatants were filtered with the membrane filter and then analyzed by Surveyor HPLC and LCQ Deca XPplus system (Thermo Fisher Scientific, USA). The above experiment was repeated more than 15 times with different cecal contents of different rats (15−21 weeks).

**Purification of EGC Metabolites.** The metabolite formation from EGC by rat intestinal flora was performed according to the procedure as described above. After incubation for 24, 48, and 96 h, 50 mL each samples of the incubation mixture were taken out in the anaerobic glovebox. Each sample was centrifuged at 15000*g* for 20 min at 4 °C to remove bacterial cells. The resulting supernatant was adjusted pH to about 3.5 with 5 M HCl and then extracted three times with equal volume of ethyl acetate. The ethyl acetate fraction was evaporated to dryness under reduced pressure. The residue obtained from the 24-h incubation mixture was used for the isolation of **3** and **8**, and the residue from the 48-h mixture for **4**. Similarly, the isolation of **5** to **7** was performed with the 96-h incubation mixture. Each residue obtained from the 24- and 48-h incubation mixtures were dissolved in 3 mL of 5% aqueous methanol and the solution was subjected to preparative HPLC for purification of **3**, **4**, and **8**. Preparative HPLC was performed with a 150 mm × 20 mm i.d., 5 $\mu$m, Capcellpak C18 MG column (Shiseido Co. Ltd., Tokyo, Japan) in a JASCO HPLC 800 series system (JASCO, Tokyo, Japan). The column was eluted with a linear gradient of solvent, starting with 10% (v/v) methanol aqueous solution containing 0.5% (v/v) acetic acid and ending with 60% (v/v) aqueous methanol containing 0.5% (v/v) acetic acid at a flow rate of 9.7 mL/min at 40 °C. The elution pattern was monitored by measuring the absorbance at 270 nm. Each fraction containing metabolite was collected and evaporated to dryness. The residue was dissolved in 5 mL of distilled water and then concentrated to dryness. This procedure was repeated two more times to remove acetic acid completely. The resultant residues were individually dissolved in a small amount of distilled water and freeze-dried to obtain metabolite **3** (17 mg), metabolite **4** (15 mg), and metabolite **8** (5 mg). For the isolation of **5** to **7**, the ethyl acetate fraction obtained from the 96-h incubation mixture was extracted with the one-fifth volume of 20 mM aqueous $Na_2CO_3$ solution. In this process, **6** remained in the organic layer, and **5** and **7** were distributed in the aqueous layer. Metabolite **6** in the ethyl acetate fraction was purified in the same manner as was metabolite **3** to obtain 5 mg of purified **6**. The aqueous solution containing **5** and **7** was evaporated to about 5 mL, and the concentrate was adjusted to around pH 5.0 with 5 M acetic acid. After removal of the precipitate (probably sodium acetate) by centrifugation, the supernatant was subjected to preparative HPLC. The HPLC were performed under the same conditions as described above except for using mobile phases without acetic acid. Metabolite **7** fraction was evaporated under reduced pressure and then freeze-dried to obtain purified metabolite **7** (3 mg). On the other hand, **5** fraction was concentrated to remove the organic solvents and the resulting concentrate was applied to an ion exchange column 10 × 65 mm, 5.1 mL of Diaion SK1B $Na^+$ form (Mitsubishi Chemical, Co., Tokyo, Japan) which had been equilibrated with distilled water. The column was eluted with 5 vol of distilled water. The eluate was concentrated to about 3 mL and then lyophilized to obtain 21 mg of metabolite **5** as sodium salt.

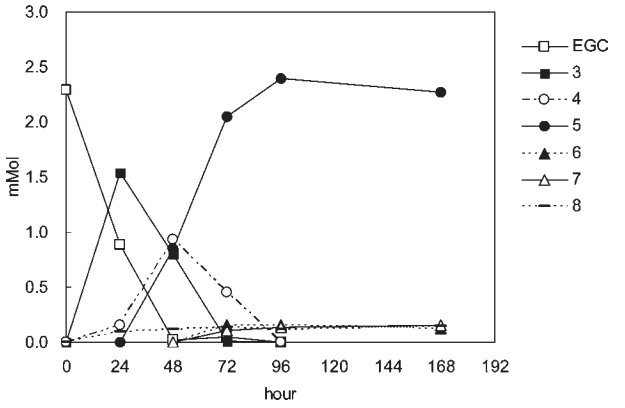


**Figure 1.** Time profile of EGC (metabolite **1**) metabolism by rat intestinal flora via the pathway of route I.

When different intestinal flora was used, metabolites **9**−**12** were formed from EGC in addition to **3**, **5**, and **6** (**Figure 2A**). Then, the 24-h incubation mixture for the isolation of **9** and **10** was adjusted pH to 1.5 with 5 M phosphoric acid and the metabolites were extracted three times with same volume of a mixture of ethyl acetate/*n*-butanol (1/1, v/v). The organic layer was then treated two times with half volume of 100 mM aqueous $Na_2CO_3$ containing 0.1% sodium ascorbate. In this procedure, **9** passed into the aqueous layer, while **10** remained in the organic layer. The aqueous layer was adjusted to around pH 7.0 with 5 M HCl and was concentrated to about 15 mL. To the concentrate 75 mL of ethanol was added and then it was centrifuged to remove insoluble material. The supernatant was concentrated to 1 mL and then the concentrate was adjusted to pH 2−3 with 2 M HCl and subjected to preparative HPLC. The HPLC conditions were the same as for metabolite **5** as mentioned above. Metabolite **9** fraction was collected and concentrated to remove organic solvent. The resultant solution was treated with the Diaion SK1B $Na^+$ form column in the same manner as for **5**. The eluate was concentrated and lyophilized to obtain 5 mg of purified **9**. The ethyl acetate-*n*-butanol layer containing **10** was concentrated to dryness. The resultant residue was treated in the same manner as was **3** to obtain 3 mg of purified **10**. Metabolites **11** and **12** were purified from 72-h and 168-h incubation mixtures, respectively, using the same procedure as for **3**. Finally, 10 mg of **11** and 19 mg of **12** were obtained. Metabolite **13** was also transformed from EGC by other rat intestinal flora (**Figure 2B**). This compound was also purified from 24-h incubation mixture using the same procedure as for **11** to obtain 8 mg of purified **13**.

**Interconversion between Metabolite 5 and Metabolite 6.** Interconversion between metabolites **5** and **6** was examined in the pH range of 1−10. Buffers used were 0.2 M potassium-hydroxy chroride buffer (pH 1.0 and 2.0), 0.2 M citrate-phosphate buffer (pH 3.0−8.0), 0.2 M borate-sodium hydroxide buffer (pH 9.0−10.0). Each buffer (0.9 mL) was placed in an individual tube, and then 0.1 mL each of 1 M **5** or **6** aqueous solution was added. The mixture was incubated under $CO_2$ gas at 37 °C. After 24 h, the incubation mixture was analyzed by HPLC.

**Analysis of Metabolites Formed from EGC (1) in Rat Cecum.** Male Wistar rats (21 weeks of age, $n = 6$) underwent laparotomy after intraperitoneal injection of 25 mg of thiopental sodium (Ravonal; Mitsuibishi

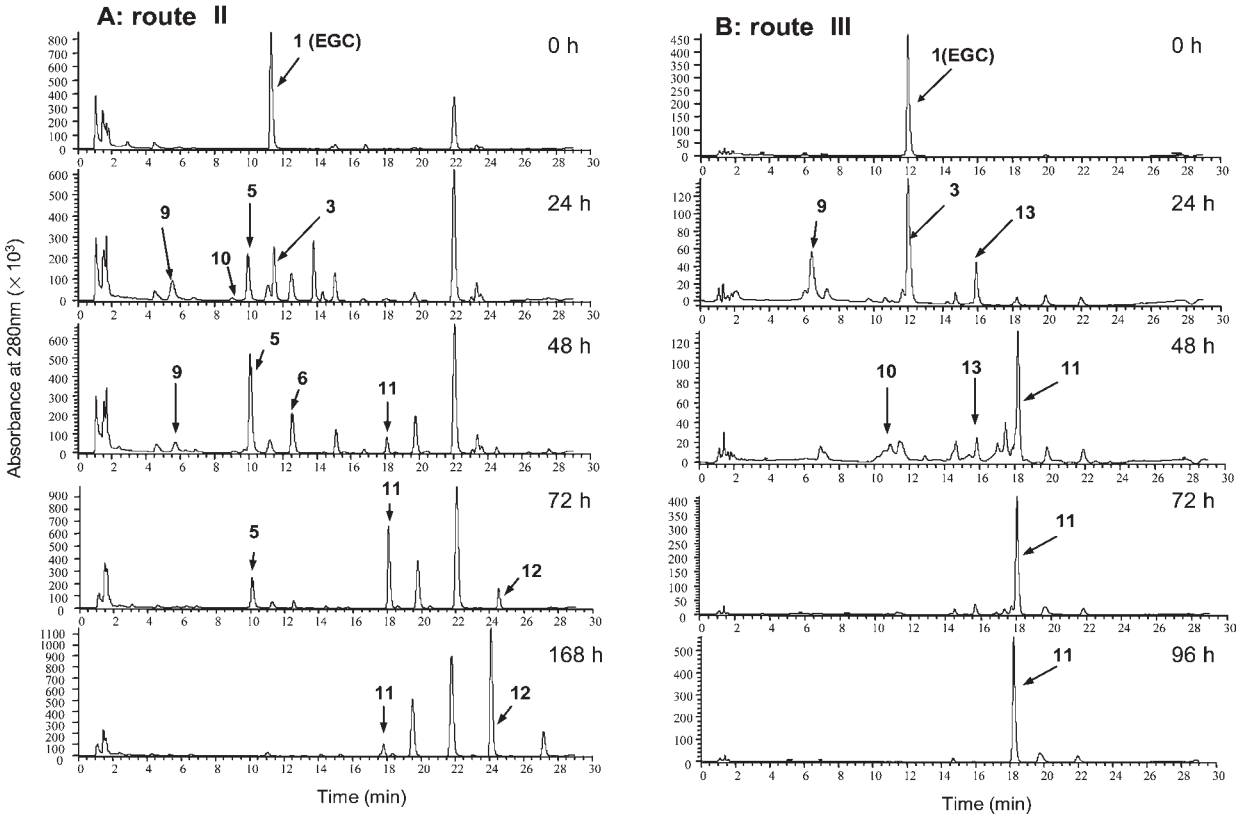


**Figure 2.** HPLC chromatograph profile of route II and route III.

Takeda Pharma Co., Ltd.). The segment of junction between cecum and large intestine was ligated. Then, EGC solution (3 mg/ 0.5 mL of saline) was directly injected into the cecum and the rats were maintained under the anesthesia gas. After 5 h, cecal contents were harvested. To the cecal contents (1 g) was added 0.8 mL of 10% aqueous methanol and 0.2 mL of 1 M sodium acetate buffer (pH 3.5) containing 1% ascorbic acid and 0.15 mM EDTA. After sonication for 30 min at 40 °C, the mixture was centrifuged at 25000*g* for 20 min at 10 °C. The supernatant was analyzed by the LC-MS system as described below.

**Analysis of Fecal Metabolites after Oral Administration of EGCg.** Fecal metabolites after oral administration of EGCg were determined. Male Wistar rats (15 weeks of age, $n = 4$) were orally administered EGCg (25 mg in 1 mL of saline). The rats were then placed in stainless steel metabolic cages. Fecal samples were separately collected at time intervals of 0−8, 8−24, 24−32, 32−48 h after dosing. The samples were frozen at −30 °C, and then lyophilized. The resulting fecal powder (1 g) was added to 0.8 mL of 10% aqueous methanol and 0.2 mL of 1 M sodium acetate buffer (pH 3.5) containing 1% ascorbic acid and 0.15 mM EDTA. The mixture was homogenized well with a vortex mixer (Scientific Industries, NY, USA) and ultrasonic bath for 1 h. The mixture was centrifuged at 25000*g* for 20 min at 10 °C. The resulting supernatant was subjected to the LC-MS system.

**LC-MS Analysis.** LC-MS analysis of EGC metabolites by rat intestinal flora in vitro was performed by a Finnigan Spectra System which consisted of a Surveyor HPLC system and an LCQ Deca XPplus system (Thermo Fisher Scientific, USA). HPLC was carried out with a 100 × 2.0 mm i.d., 5 $\mu$m, Capcell pack MG column (Shiseido Co.Ltd., Tokyo, Japan) at a flow rate of 0.2 mL/min at 40 °C. The column was eluted at a flow rate of 0.2 mL/min at 40 °C with 100% buffer A (distilled water/acetonitrile/acetic acid; 100/2.5/0.2, v/v/v) for 2 min, followed by linear increases in buffer B (distilled water/acetonitrile/methanol/acetic acid; 33/2.5/66/0.2, v/v/v) from 0 to 80% for 2 to 25 min. The mobile phase was then re-equilibrated with 100% buffer A for 5 min. The column elute was monitored by the diode array detector and subsequently was directed to the LCQ Deca XPplus ion trap mass spectrometer incorporated with ESI interface. The negative ion polarity mode was set for the ESI source. Analysis was initially carried out using full scan, data-dependent MS/MS scanning from $m/z$ 100 to 1000. LC-MS analysis for quantification of metabolites in cecum and feces was carried out with a 150 × 2.0 mm i.d., 4 $\mu$m, Synergi MAX RP-18 column (Phenomenex, Macclesfield, U.K.) (*21*). The column was eluted with buffer A (distilled water/acetonitrile/acetic acid; 98/2/0.3, v/v/v) and buffer B (distilled water/acetonitrile/acetic acid; 60/40/0.3, v/v/v) at a flow rate of 0.2 mL/min at 40 °C. The column was eluted initially with 100% buffer A for 2 min, followed by linear increases in buffer B to 100% from 2 to 20 min, and with 100% buffer B up to 24 min. The column was finally equilibrated with buffer A for 6 min. Quantification of the metabolites in cecum and feces was performed with the selected ion monitoring (SIM) mode. The separated $[M - H]^-$ ion chromatograms were selected at $m/z$ 305, 307, 291, 225, and 207 for the specific parent ions of metabolites **1**, **3**, **4**, **5**, and **6**, respectively. Metabolites in cecum and feces were determined based on the standard calibration curves obtained with the metabolites purified in this study.

**NMR and Optical Rotation Analyses.** NMR analysis was performed by a Bruker Ultrashield 400 plus system. ($^1$H, 400 MHz; $^{13}$C, 100 MHz: Bruker BioSpin, Ibaragi, Japan). All samples were dissolved in methanol-$d_4$ (Kanto Chemical, Tokyo, Japan). Chemical shifts were referenced with tetramethylsilane (TMS) as an internal standard. Specific rotation $[\alpha]_D^{20}$ of two metabolites (**5** and **6** ) was measured by a P-1020 polarimeter (JASCO, Tokyo, Japan). Metabolite **5** was dissolved in distilled water (c 0.225) and metabolite **6** in methanol (c 0.355).

## RESULTS AND DISCUSSION

**Screening of Enteric Bacteria Capable of Hydrolyzing EGCg.** It has been previously suggested that the metabolism of galloylated catechins such as ECg and EGCg by intestinal bacteria was initiated by releasing gallic acid from the catechins (*14*, *22*, *25*). In relation to this, Hackett and Griffiths (*23*, *24*) have also reported that 3-*O*-methyl-(+)-catechin, unlike (+)-catechin, did not undergo ring fission by intestinal flora. Thus, the free hydroxy group at the 3 position of catechins is important for their metabolism by intestinal flora. Accordingly, we first screened enteric bacteria capable of hydrolyzing EGCg. Among 169 stains of the bacteria

commercially available, four bacterial strains, *Enterobacter aerogenes*, *Raoultella planticola* (*Klebsiella planticola*), *K. pneumoniae* subsp. *pneumoniae*, and *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) were found to be capable of hydrolyzing EGCg. Since these bacteria are well recognized to exist commonly in the intestinal tract, EGCg is considered to be easily hydrolyzed to yield EGC (**1**) and gallic acid (**2**) in the gut.

**Time-Course Analysis of Metabolites by Rat Intestinal Bacteria.** After hydrolyzing EGCg to EGC (**1**) and gallic acid (**2**), subsequent metabolic processes of EGCg are degradation of EGC by intestinal bacteria. In this study, EGC was incubated with intestinal bacteria from rat cecal contents in 0.1 M sodium phosphate buffer (pH 7.1) and transformation of **1** was examined over time by LC-MS analysis. We performed the in vitro experiments more than 15 times, and finally found that EGC underwent degradation through at least three different metabolic pathways by rat intestinal flora. These three metabolic routes are referred to as route I, route II, and route III.

**Figure 1** shows time-course analysis of EGC (**1**) metabolites produced by rat intestinal flora through the pathway of route I. In this route, EGC was first converted to **3** within 24 h incubation, together with a small amount of **8**. Second metabolite (**4**) peaked at 48 h incubation accompanied by a decrease in **3**. Subsequently, **5** began to increase gradually after incubation for 48 h with decreasing **4**, reaching a plateau at about 96 h incubation and remained as the main product throughout the incubation periods (168 h). Two other small peaks (**6** and **7**) were also detected at the same incubation period when **5** was produced.

In route II (**Figure 2A**), the first metabolic step of EGC (**1**) was conversion into **3** just as in route I. However, **3** was converted to **9** at the second step in route II, and then **9** to **5**. Metabolite **5** was further transformed to **11** and finally **12** was produced from **11**. Thus, **5** was produced from **3** through **9** in route II, while this compound was transformed from **3** via **4** in the case of route I. Metabolite **5** was accumulated in route I, but was converted to **12** via **11** and hence **12** is regarded as the major product in route II. In addition, **6** and **10** were found to be formed.

In route III (**Figure 2B**), EGC (**1**) was first converted to **3** as well as in routes I and II. During incubation for 24 to 48 h, metabolite **3** was observed to be converted to **9**, and thereafter **9** was converted to **13** and **13** was converted to **11**, successively. Metabolite **10** was also produced after 48 h incubation. Metabolite **11** was hardly converted to any other metabolites even after extension of incubation time up to 168 h, and therefore the compound was regarded as the major product in route III. This metabolic route is very analogous to route II.

We have revealed three routes of EGC (**1**) metabolic pathway by rat intestinal flora in the in vitro experiments. Among the three routes, route I was shown to be more dominant pathway than routes II and III because the conversion of EGC into the compounds shown in route I occurred with high frequencies (probability with more than 90%) in the repeated experiments in vitro.

**Structural Analysis of Metabolites Produced in Routes I, II, and III.** EGC metabolites detected in each metabolic route as described above were then purified by preparative HPLC and their structures were determined by LC-MS and NMR analyses. Thus, six metabolites (**3**−**8**) in route I, four metabolites (**9**−**12**) in route II, and a metabolite (**13**) in route III were purified and identified. In the case of **5** and **9**, extraction and purification processes were somewhat cumbersome. As indicated in **Figure 3**, metabolite **5** could not be extracted from the incubation mixture with ethyl acetate until the pH of the mixture was adjusted to below pH 4.0. Furthermore, **5** was spontaneously converted into **6** during the concentration process, especially under acidic condition. Accordingly, purification of the compound was performed cautiously. Similar phenomena were observed in the isolation process of **9**, and hence this compound was also purified carefully.

Metabolite **3** showed a pseudomolecular ion peak at *m*/*z* 307 $[M - H]^-$ in its electoronspray ionization (ESI)-MS analysis, 2 mass units larger than that of EGC. $^1$H- and $^{13}$C NMR spectra of **3** were very similar to those of 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (*22*) and 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (*19*) except for their aromatic signal patterns. The substitution patterns of two aromatic rings in **3** were found to resemble those of EGC. Together with $^1$H−$^1$H shift correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments, metabolite **3** was identified to be 1-(3′,4′,5′-trihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol.

Metabolite **4** showed an $[M - H]^-$ ion peak at *m*/*z* 291 in negative ESI-MS, 16 mass units less than that of **3**. The $^1$H- and $^{13}$C NMR data of **4** could be superimposed with those of 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol reported by Wang et al. (*19*). Consequently, metabolite **4** was determined to be 1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol.

The $^1$H- and $^{13}$C NMR data of **5** were closely similar to those of 5-(3,4-dihydroxyphenyl)-4-hydroxyvaleric acid reported in our previous paper (*25*) except for the aromatic signal patterns. The aromatic pattern of **5** was in harmony with that of the 3′,5′-dihydroxyphenyl moiety in **4**. The negative ESI-MS data of **5** also showed $[M - H]^-$ ion peak at *m*/*z* 225, which is consistent with that of 5-(3,4-dihydroxyphenyl)-4-hydroxyvaleric acid. From these data together with COSY, HMQC, and HMBC experiments, the chemical structure of metabolite **5** was concluded to be 5-(3,5-dihydroxyphenyl)-4-hydroxyvaleric acid. The optical rotation value, $[\alpha]_D^{20}$ (c 0.225, $H_2O$) is −18.1°.

The MS, $^1$H NMR, and $^{13}$C NMR data of **6** agreed with those of 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone reported previously (*14*). Accordingly, metabolite **6** was concluded to be 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone. The optical rotation value, $[\alpha]_D^{20}$ −8.9° (c 0.355, $CH_3OH$), of **6** was very similar to that ($[\alpha]_D^{20}$ −12.9° (c 0.4, $CH_3OH$)) of the same compound identified previously (*14*). Thus, it is reasonable that **6** has 4*R* configuration.

Metabolite **7** showed a pseudomolecular ion peak at *m*/*z* 181 $[M - H]^-$ in negative ESI-MS. Molecular mass of this compound was 16 mass units larger than that of 3-(3-hydroxyphenyl) propionic acid (*25*). Aromatic signal patterns of **7** in $^1$H NMR and chemical shifts of the aromatic ring in $^{13}$C NMR were analogous to those of **6**. From these observations together with HMQC and HMBC experiments, metabolite **7** was determined to be 3-(3,5-dihydroxyphenyl) propionic acid.

Negative ESI-MS data of **8** exhibited an $[M - H]^-$ ion peak at *m*/*z* 289, 16 mass units less than that of EGC, suggesting the compound lacked one hydroxyl group relative to EGC. The $^1$H- and $^{13}$C NMR data were very similar to those of EGC, besides chemical shifts in the B ring. The chemical shifts of **8** were comparable to those in the 3′,5′-dihydroxyphenyl moiety of **4**. From further experiments with HMQC and HMBC, metabolite **8** was identified to be 4′-dehydroxy EGC.

A pseudomolecular ion peak at *m*/*z* 241 $[M - H]^-$ in negative ESI-MS was observed in **9**. The $^1$H- and $^{13}$C NMR data indicated good resemblance to those of **5**, except for its aromatic signal patterns. The aromatic chemical shifts of **9** were very similar to those in the 3′,4′,5′-trihydroxyphenyl group of **3**. Therefore, it is concluded that metabolite **9** is 5-(3,4,5-trihydroxyphenyl)-4-hydroxyvaleric

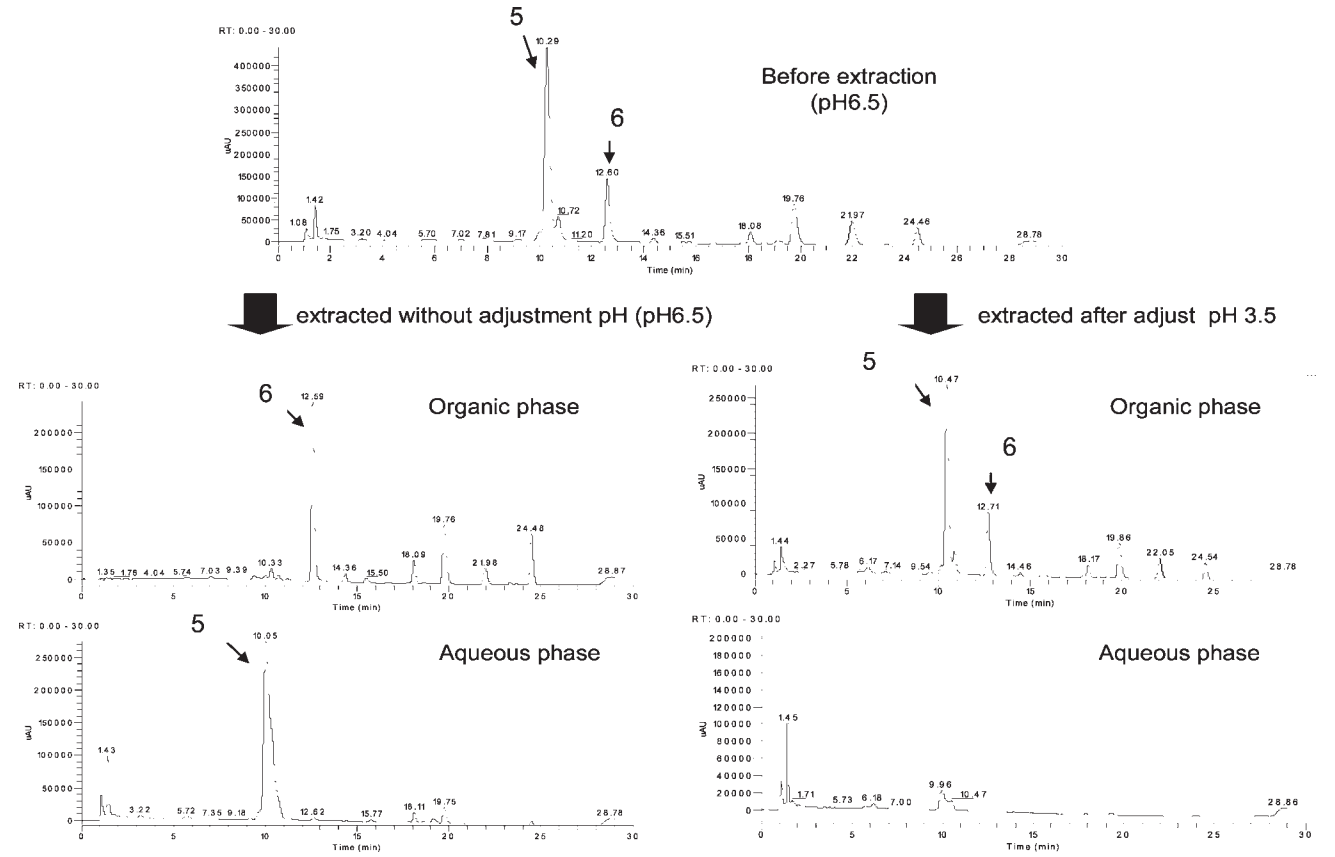


**Figure 3.** Influence of pH range on extraction process of metabolite **5**.

acid. This conclusion was further substantiated by COSY, HMQC, and HMBC experiments.

Negative ESI-MS data of metabolite **10** exhibited an $[M - H]^-$ ion peak at $m/z$ 223, 18 mass units less than that of **9**, suggesting the compound was dehydrated from **9**. The $^1$H- and $^{13}$C NMR spectra indicated that **10** has $\gamma$-valerolactone structure as well as **6**. The signal patterns and chemical shifts of the aromatic ring in **10** were found to be nearly identical with those in **9**. Together with COSY, HMQC, and HMBC experiments, metabolite **10** was determined to be 5-(3′,4′,5′-trihydroxyphenyl)-$\gamma$-valerolactone.

In the case of **11**, a pseudomolecular ion peak at $m/z$ 209 $[M - H]^-$ was observed, 16 mass units less than that of **5**, suggesting the compound lacked one hydroxyl group relative to metabolite **5**. The $^1$H- and $^{13}$C NMR data of **11** were compatible with those of 5-(3′-hydroxyphenyl)valeric acid previously reported by Meselhy et al. (*22*) except for the aromatic signal patterns of **11**. The aromatic patterns of **11** were observed to be closely similar to those of **7**. These observations suggested that this compound is 5-(3,5-dihydroxyphenyl)valeric acid. This suggestion was confirmed by further experiments with COSY, HMQC, and HMBC.

The $^1$H- and $^{13}$C NMR data of **12** and **13** were analogous to those of **11**, except that the signal patterns and chemical shifts of aromatic rings in three compounds were different from each other. The whole signal patterns and chemical shifts in **12** were superimposable on those of 5-(3′-hydroxyphenyl)valeric acid reported by Meselhy et al. (*22*). The ESI-MS data of **12** showed a pseudomolecular ion peak at $m/z$ 193 $[M - H]^-$, which was 16 mass units less than that of **11**. These observations clearly demonstrated that metabolite **12** is 5-(3-hydroxyphenyl)valeric acid. In metabolite **13**, the ESI-MS data exhibited a pseudomolecular ion peak at $m/z$ 225 $[M - H]^-$, 16 mass units larger than that of **11**. The $^1$H NMR and $^{13}$C NMR spectra of the aromatic signal patterns of **13** were very analogous to those of **3** and **9**. Accordingly, metabolite **13** was identified to be 5-(3,4,5-trihydroxyphenyl)valeric acid. This result was further supported by COSY, HMQC, and HMBC experiments.

The $^1$H NMR and $^{13}$C NMR spectroscopic data of the purified metabolites were summarized in **Tables 1** and **2**, respectively. The chemical structures of the metabolites are also shown in **Figure 6**.

**Interconversion between Metabolite 5 and Metabolite 6. Figure 4** shows the interconversion ratio of metabolites **5** and **6** in the pH range of 1.0−10.0. It was found that **5** is stable under alkaline conditions, but the conversion into **6** occurred under acidic conditions below pH 4. In particular, 90% or more of **5** was spontaneously converted to **6** at pH below 2. On the contrary, metabolite **6** was stable under acidic conditions and began to change to **5** at above pH 7. Similar phenomenon also occurred between **9** and **10**. Accordingly, much attention should be paid to handling these metabolites **5**, **6**, **9**, and **10**, especially in the purification process of these compounds.

**Metabolism of EGCg in Vivo.** In order to obtain further understanding of EGCg metabolism in the intestinal tract, we examined metabolites of EGC (**1**) after direct injection into rat cecum. The metabolites produced in the cecum 5 h after dosing of EGC (3 mg, 9.8 $\mu$mol) were determined by LC-MS system. As indicated in **Figure 5**, the amounts of each metabolite detected in the cecum were 0.026, 0.028, 0.857, 1.701, and 0.618 $\mu$mol in the cecal contents for metabolite **1**, **3**, **4**, **5**, and **6** respectively. These results demonstrated that EGC was transformed in vivo through the metabolic pathway of route I found in the in vitro experiments as described above. In addition, metabolite **5** was also the most dominant metabolite in rat cecum as well as in our in vitro experiments.

Further, fecal metabolites after oral administration of EGCg (25 mg, 54.5 $\mu$mol) were determined. Nine metabolites were detected in feces, but no EGCg was found. The amounts of the

**Table 1.** $^1$H-NMR Spectra Data of Catechin Metabolites[a]

| | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| H-1a | 2.601 dd (13.7, 7.6) | 2.942 dd (13.8, 6.5) | | | | | | | | | |
| H-1b | 2.432 dd (13.7, 8.3) | 2.719 dd (13.8, 6.9) | | | | | | | | | |
| H-2 (or 2a) | 3.938 dddd (8.3, 7.6, 4.3, 4.0) | 4.898 dddd (8.3, 7.5, 6.9, 6.5) | 2.329 ddd (7.5, 7.4, 7.3) | 2.532 ddd (17.9, 9.4, 9.0) | 2.510 t (6.6) | 4.620 d (6.8) | 2.441 ddd (15.7, 10.6, 7.4) | 2.485 ddd (17.9, 10.3, 8.2) | 2.286 m | 2.301t (6.8) | 2.286 t (7.0) |
| H-2b | | | 2.256 ddd (7.4, 7.4, 7.2) | 2.408 ddd (17.9, 8.9,4.7) | | | 2.326 ddd (15.7, 8.8, 6.8) | 2.341 ddd (17.9, 11.3, 4.9) | | | |
| H-3 (0r 3a) | 2.849 dd (13.8, 4.3) | 3.010 dd (13.1, 8.3) | 1.657 dddd (14.3, 7.5, 7.4, 7.4) | 2.262 dddd (12.2, 9.4, 6.9, 4.7) | 2.743t (7.6) | 4.000 m | 1.805 dddd (13.5, 10.6, 6.8, 3.3) | 1.945 dddd (15.0, 11.1, 8.2, 7.0) | 1.609 m | 1.620 m | 1.586 m |
| H-3b | 2.646 dd (13.8, 4.0) | 2.737 dd (13.1, 7.5) | 1.810 dddd (14.3, 7.3, 7.2, 3.7) | 1.964 dddd (12.2, 9.0, 8.9, 7.2) | | | 1.588 dddd (13.5, 8.8, 7.4,4.4) | 2.243 dddd (15.0, 10.3, 7.3, 4.9) | | | |
| H-4 (or 4a) | | | 3.784 m | 4.744 dddd (6.9,7.2,6.5,6.4) | | 2.522 dd (16.2,7.4) | 3.708 dddd (9.4,6.3,4.4,3.3) | 4.709 dddd (7.3,7.0,6.4,6.3) | 1.609 m | 1.620 m | 1.586 m |
| H-4b | | | | | | 2.761 dd (16.2,5.0) | | | | | |
| H-5a | | | 2.623 dd (13.5, 6.8) | 2.870 dd (13.9, 6.5) | | | | 2.715 dd (14.0, 6.0) | 2.464 m | 2.545 t (6.8) | 2.408 t (6.8) |
| H-5b | | | 2.562 dd (13.5, 6.2) | 2.771 dd (13.9,6.4) | | | | 2.816 dd (14.0,6.0) | | | |
| H-6 | | | | | | 5.883 or 5.926d (2.1) | | | | | |
| H-8 | | | | | | 5.883 or 5.926 d (2.1) | | | | | |
| H-2′ | 6.220 s | 6.240 d (2.1) | 6.195 d (2.4) | 6.193 d (2.1) | 6.165 d (1.9) | 6.333 d (2.1) | 6.217 s | 6.244 s | 6.133 d (2.0) | 6.637 t (2.4) | 6.180 s |
| H-4′ | | 6.151 t (2.1) | 6.109 t (2.2) | 6.141 t (2.1) | 6.085 d (1.9) | 6.202 t (2.1) | | | 6.078 t (2.0) | 6.597 dd (7.9, 2.0) | |
| H-5′ | | | | | | | | | | 7.064 dd (7.9, 8.1) | |
| H-6′ | 6.220 s | 6.240 d (2.1) | 6.195 d (2.4) | 6.193 d (2.1) | 6.165 d (1.9) | 6.333 d (2.1) | 6.217 s | 6.244 s | 6.133 d (2.0) | 6.650 d (8.1) | 6.180 s |
| H-3′′ | 5.863 s | 5.806 d (2.0) | | | | | | | | | |
| H-5′′ | 5.863 s | 5.773 d (2.0) | | | | | | | | | |
| temp (°C) | 25 | 25 | 40 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |

[a] Chemical shifts are expressed in ppm downfield from the signal of TMS in methanol-$d_4$. Coupling constants in Hz are in parentheses.

metabolites determined are summarized in **Table 3**. The metabolites were not detected at 0−8 h post dose and emerged mainly at 8−24 h and 24−31 h. In the feces, **5** (6.16 $\mu$mol/feces) was abundant, followed by **4** (3.45 $\mu$mol) and **6** (3.27 $\mu$mol). In this experiment, EGCg was considered to be converted via the mixed metabolic pathways of routes I and II found in the in vitro experiments.

The above in vivo experiments suggested that the metabolic pathway of EGCg found in the in vitro experiments probably reflected the pathway in vivo.

On the basis of the time-course analysis of the metabolites derived from EGCg and their structural identification, we proposed three metabolic pathways of EGCg by rat intestinal bacteria (route I to III) as illustrated in **Figure 6**. EGCg is hydrolyzed to EGC (**1**) and gallic acid (**2**) at the first step of metabolism. This step was common in the three pathways. In the most dominant metabolic pathway (route I) in this study, EGC formed from EGCg is converted to **3** by reductive opening between the 1 and 2 positions of EGC. The resulting **3** is transformed to **4** by dehydroxylation at 4′ position of **3**. Further, metabolite **4** undergoes ring-fission of the phloroglucinol moiety to yield **5** as the major metabolite in route I. At the same time, metabolite **6** may be formed by lactonization of **5** just after the ring-fission. Metabolite **5** is converted into **7**, but its conversion is only very slight. In addition, while the dominant pathway is **1** to **3**, a very small amount of **1** is converted to **8** by dehydroxylation at the 4′ position. This metabolite appeared not to be metabolized any more by the intestinal bacteria in this study. Wang et al. (*19*) have discussed the possibility that the presence of 4′-hydroxyl group of EGC may be necessary for the reductive opening of its heterocyclic ring, which is essential for further degradation of EGC. This consideration is seemed to be the case for **8**. In route II, one of the two minor pathways, metabolite **3** formed from EGC undergoes degradation of the phloroglucinol ring to produce **9**. This compound is converted into **5** by dehydroxylation at the 4′ position. Furthermore, metabolite **5** undergoes dehydroxylation at the 4 position to form **11**, followed by transformation to **12** by elimination of the 5′-hydroxyl group in **11** in route II. Metabolite **3** is transformed to **13** via **9** in route III. Finally, metabolite **13** is converted to **11** by undergoing dehydroxylation at the 4′ position of **13**. Metabolite **10** is also produced just after ring-fission of the phloroglucinol moiety of **3** in addition to **9** in routes II and III, as well as the formation of **5** and **6** in route I.

Thus, we determined the three metabolic pathways of EGCg by rat intestinal bacteria. The differences in the metabolic pattern of EGCg are considered to be a reflection of differences in the intestinal microflora of the different rats used. We further examined whether or not the metabolic pathways proposed in the in vitro experiments reflect the EGCg metabolism in gut tract in vivo. The metabolites derived from EGC were determined 5 h

**Table 2.** $^{13}$C-NMR Spectra Data of Catechin Metabolites[a]

| | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C-1 | 44.19 | 43.17 | 182.97 | 178.03 | 144.84 | | 178.4 | 180.42 | 178.19 | 177.56 | 177.83 |
| C-2 | 75.38 | 85.73 | 34.24 | 28.11 | 37.838 | 82.73 | 32.03 | 29.56 | 35.25 | 34.92 | 34.91 |
| C-3 | 31.64 | 32.76 | 35.91 | 29.53 | 32.63 | 68.7 | 32.87 | 27.93 | 25.89 | 25.70 | 25.68 |
| C-4 | | | 74.09 | 83.00 | | 27.82 | 73.26 | 83.31 | 31.91 | 31.95 | 32.17 |
| C-4a | | | | | | 100.65 | | | | | |
| C-5 | | | 45.13 | 42.25 | | 157.68 | 44.75 | 41.78 | 36.66 | 36.47 | 36.16 |
| C-6 | | | | | | 96.37 | | | | | |
| C-7 | | | | | | 157.91 | | | | | |
| C-8 | | | | | | 95.57 | | | | | |
| C-8a | | | | | | 156.71 | | | | | |
| C-1′′ | 105.75 | 104.52 | | | | | | | | | |
| C-2′′ | 158.29 | 159.55 | | | | | | | | | |
| C-3′′ | 95.92 | 96.06 | | | | | | | | | |
| C-4′′ | 157.76 | 162.87 | | | | | | | | | |
| C-5′′ | 95.92 | 90.56 | | | | | | | | | |
| C-6′′ | 158.29 | 159.55 | | | | | | | | | |
| C-1′ | 132.35 | 141.29 | 142.79 | 139.88 | | 143.17 | 131.11 | 128.46 | 145.88 | 145.07 | 134.67 |
| C-2′ | 109.47 | 108.92 | 109.16 | 108.93 | 107.78 | 106.49 | 109.42 | 109.55 | 107.97 | 116.23 | 108.36 |
| C-3′ | 146.74 | 159.68 | 159.40 | 159.49 | 159.52 | 159.55 | 146.9 | 147.11 | 159.38 | 158.23 | 146.87 |
| C-4′ | 132.01 | 101.78 | 101.65 | 102.10 | 101.44 | 103.13 | 132.55 | 133.11 | 101.13 | 113.69 | 132.03 |
| C-5′ | 146.74 | 155.38 | 159.40 | 159.49 | 159.52 | 159.55 | 146.9 | 147.11 | 159.38 | 130.29 | 146.87 |
| C-6′ | 109.47 | 108.92 | 109.16 | 108.93 | 107.78 | 106.49 | 109.42 | 109.55 | 107.97 | 120.81 | 108.36 |
| temp (°C) | 25 | 25 | 40 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |

[a] Chemical shifts are expressed in ppm downfield from the signal of TMS in methanol-$d_4$.

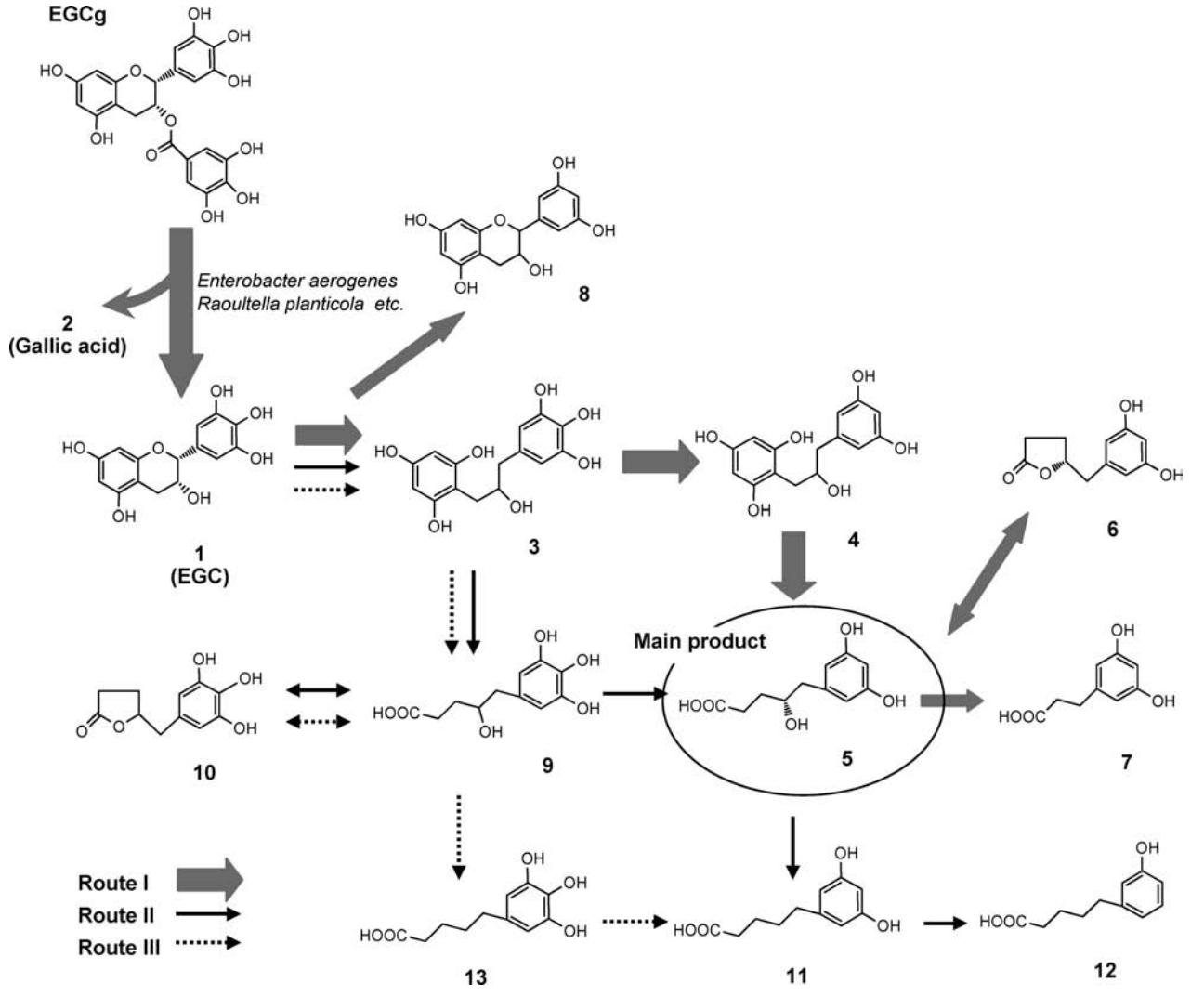


**Figure 6.** Putative metabolic pathway of EGCg by rat intestinal flora.

after direct injection of EGC into the cecum of the operating rats. As a result, metabolites **1**, **3**, **4**, **5**, and **6** were detected in the cecal contents and **5** was found to be the most dominant metabolite among them. We also determined the metabolites in rat feces after oral administration of EGCg. Metabolites **4**, **5**, and **6** were found as the major metabolites and metabolite **5** was dominant in the feces. Therefore, it is likely that the in vitro metabolic pathway proposed in this study reflects the in vivo metabolism of EGCg in the rats.

In this study, we have found that metabolite **5** (5-(3,5-dihydroxyphenyl)-4-hydroxyvaleric acid) is the major metabolite produced by rat intestinal bacteria. However, there have been no reports that 5-hydroxyphenyl-4-hydroxyvaleric acids were identified as the metabolites produced from catechins such as (+)-catechin (*23*) and (−)-epicatechin gallate (ECg) (*22*) by mammalian intestinal bacteria. Thus, there is an apparent discrepancy between our results and those obtained in previous reports. Although we have no sufficient explanation to this discrepancy at present, we have noticed that metabolite **5** (5-(3,5-dihydroxyphenyl)-4-hydroxyvaleric acid) was converted into metabolite **6** (5-(3′,5′-dihydroxyphenyl)-γ-valerolactone) during the isolation process of **5** in this study. The lactonization of **5** to **6** was found to take place easily during the concentration process, particularly under acidic conditions (data not shown). Such a lactonization reaction is well-known as γ-hydroxycarboxylic acid being converted rapidly to the corresponding γ-lactone under acidic conditions. Therefore, a possible explanation for no detection of 5-hydroxyphenyl-4-hydroxyvaleric acids in the previous papers is that the valeric acids were converted artificially, at least in part, to the corresponding γ-valerolactone during the isolation process. In addition, it was observed that 5-(3,5-dihydroxyphenyl)-4-hydroxyvaleric acid in aqueous solution could not be extracted with ethyl acetate when pH of the solution was at higher than 5, while the compound was shifted to the organic layer at the pH below 4 (**Figure 3**). This may be another reason that 5-hydroxyphenyl-4-hydroxyvaleric acids were not isolated as the metabolites of catechins.

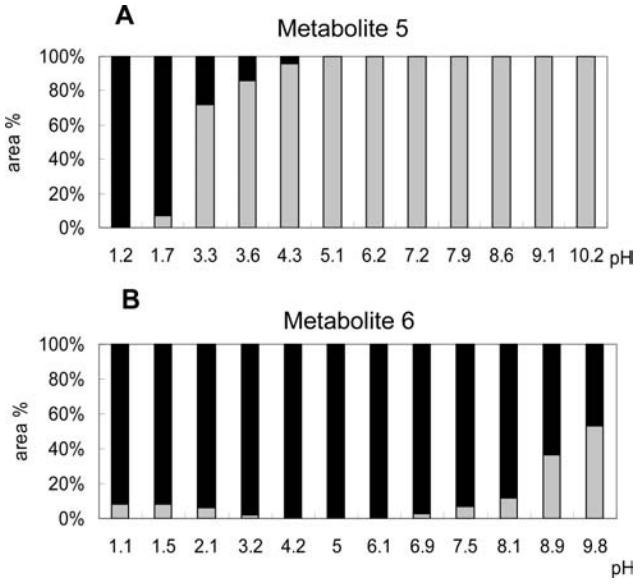


**Figure 4.** Interconversion of metabolite **5** to metabolite **6** (**A**) and metabolite **6** to metabolite **5** (**B**) in the pH range 1.2−10.2. These values are evaluated by peak area resulting HPLC analysis after 24 h incubation at 37 °C. Both metabolites have the maximum absorption of wavelength at 275 nm. (gray bars: metabolite **5**; black bars: metabolite **6**).

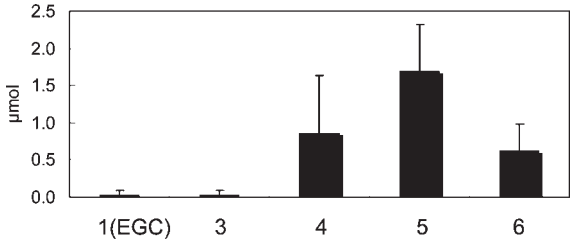


**Figure 5.** Cecal metabolites after direct injection of EGC (metabolite **1**) into rat cecum. Data expressed as mean ± SD values in μmol of six rats, five hours after injection.

Similarly, 5-hydroxyphenyl-4-hydroxyvaleric acids were still not found in urinary metabolites after EGCg dosing (*14*, *18*). We have reported that a kind of valeric acid, 5-(3,4-dihydroxyphenyl)-4-hydroxyvaleric acid, was found in rat urine after oral administration of ECg, but was a minor metabolite (*25*). To the contrary, lactonization products of 5-hydroxyphenyl-4-hydroxyvaleric acids, 5-hydroxyphenyl-γ-valerolactones such as 5-(3′, 4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,4′,5′-triihydroxyphenyl)-γ-valerolactone, and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone have been reported to be the major metabolites of (+)-catechin (*23*), ECg (*22*, *25*), and EGCg (*12*, *17*). Negligible detection of 5-hydroxyphenyl-4-hydroxyvaleric acids as the urinary metabolites of catechins in previous papers may be explained by lactonization of the compounds to the γ-valerolactones during their isolation process as discussed above. However, further work is needed to elucidate whether or not the conversion of valeric acids into the corresponding γ-valerolactones takes place in the body.

We also examined the effects of pH on interconversion of **5** into **6** to ascertain their stability in our in vitro experiments with intestinal bacteria. As shown in **Figure 4**, more than 90% of **5** was converted into **6** at pH values below 2.0 within 24 h at 37 °C, but the conversion rate decreased with increasing pH and no change was observed at pH 5.0 and above. On the other hand, although **6** was stable at about pH 6.9 and below, it changed to **5** in alkaline pH. Thus, the interconversion between **5** and **6** was caused by the change in the pH of the solution, but this conversion scarcely occurred in the pH range of about 5.0 to 6.9. Indeed, when EGC was incubated with intestinal bacteria in 0.1 M phosphate buffer (pH 7.0), pH of the incubation mixture was maintained between pH 6.0 and 7.0 throughout the experimental periods. Hence, the two compounds were considered unconverted in the in vitro

**Table 3.** Concentration of Metabolites Excreted in Feaces After Oral Administration of EGCg[a]

| | EGCg | 1 | 3 | 4 | 5 | 6 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|
| 0−8 h | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8−24 h | 0 | 0.38 ± 0.31 | 0.12 ± 0.17 | 3.44 ± 3.25 | 3.00 ± 1.47 | 1.60 ± 1.57 | 0.02 ± 0.03 | 0.01 ± 0.01 | 0.06 ± 0.09 | 0 |
| 24−32 h | 0 | 0.07 ± 0.05 | 0.02 ± 0.04 | 0.01 ± 0.01 | 2.52 ± 1.66 | 1.37 ± 0.87 | 0.01 ± 0.01 | 0.01 ± 0.01 | 0.40 ± 0.22 | 0.02 ± 0.03 |
| 32−48 h | 0 | 0.05 ± 0.02 | 0.01 ± 0.01 | 0 | 0.64 ± 0.29 | 0.30 ± 0.13 | 0 | 0 | 0.45 ± 0.26 | 0.07 ± 0.03 |
| total | 0 | 0.50 ± 0.38 | 0.15 ± 0.22 | 3.45 ± 3.26 | 6.16 ± 3.41 | 3.27 ± 2.57 | 0.03 ± 0.05 | 0.01 ± 0.02 | 0.09 ± 0.57 | 0.09 ± 0.06 |

[a] Data expressed as mean ± SD values in μmol of four rats.

study. The pH of the cecal contents and feces in our in vivo experiments were measured under the extraction conditions described. The pH was found to be in the range of 5.0−7.0 (data not shown), and therefore the interconversion between **5** and **6** was thought not to take place. These findings implied that both **5** and **6** detected in this study would be actually transformed from EGC and EGCg by rat intestinal bacteria. Similarly, **9** and **10** were also considered to be truly produced from EGC.

In this study, we revealed the whole metabolic pathway of EGCg by rat intestinal bacteria through both in vitro and in vivo experiments. It was also presumed that the metabolic pathway changed somewhat according to differences in the composition of rat intestinal bacteria. Substantial amounts of the metabolites from EGCg would be produced in the gut tract since intact EGCg is reported to be poorly absorbed in the body (*12*, *13*). Therefore, it will be important to examine the biological activities of the metabolites and their metabolic fate in the body.

## ABBREVIATIONS USED

EGCg, (−)-epigallocatechin gallate; EGC, (−)-epigallocatechin.

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