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Catabolism of (+)-Catechin and (−)-Epicatechin by Rat Intestinal Microbiota

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ABSTRACT: Catabolism of (+)-catechin (+C) and (−)-epicatechin (EC) by rat intestinal microbiota was examined in vitro. +C and EC metabolites isolated were identified by LC-MS and NMR analyses. As a result, 4-hydroxy-5-(3-hydroxyphenyl)valeric acid (**C-5** and **EC-5**), 4-oxo-5-(3,4-dihydorxyphenyl)valeric acid (**EC-7**), 4-oxo-5-(3-hydorxyphenyl)valeric acid (**EC-8**), and 1-(4-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (**EC-13**) were identified as new metabolites of +C or EC. From the measurement of optical rotation, each of the +C and EC metabolites possessing the same chemical structure and chiral carbon was inferred to have an enantiomeric relationship to each other and to maintain the configurations at the 3-position of the original catechins. On the basis of these findings together with previous information, the proposed metabolic pathway of +C and EC by rat intestinal microbiota was updated.

KEYWORDS: *catechin, intestinal microbiota, metabolite, valeric acid, valerolactone*

INTRODUCTION

There are many studies on preventive effects of tea catechins against cancer, heart disease, diabetes, and neurodegenerative diseases.[1−4] Over the past decade, study on the metabolism of tea catechins has been recognized to be important in clarifying the mechanisms of their physiological activities. Recently, catabolism of tea catechins by intestinal microbiota was also considered to be important in the explanation of their physiological activities because the intact catechins have been shown to be poorly absorbed in the body.[5−7] We have already investigated the biotransformation of (−)-epigallocatechin gallate (EGCg) by rat intestinal microbiota in both in vitro and in vivo experiments.[8] This study revealed that EGCg underwent ring fission by rat intestinal microbiota to produce 4-hydroxy-5-(3,5-dihydroxyphenyl)valeric acid as a main metabolite in the gut tract. Although the ring fission metabolites of catechol type catechins such as (+)-catechin (+C),[9] (−)-epicatechin (EC),[10,11] and (−)-epicatechin gallate (ECg)[7,12] have been reported, there is a little information on the biotransformation of +C and EC to 4-hydroxy-5-phenylvaleric acids by intestinal microbiota.[10,11,13] In addition, some +C and EC metabolites including the 4-hydroxy-5-phenylvaleric acids were still not definitively determined.

In this paper, we identify ring fission metabolites formed from +C and EC by rat intestinal microbiota and update the proposed metabolic pathway of these catechins.

MATERIALS AND METHODS

Chemicals. (−)-Epicatechin (EC) and (+)-catechin (+C) were obtained from Sigma-Aldrich Japan (Tokyo), methanol-d_4 was from Kanto Chemical Co., Inc. (Tokyo, Japan), and tetramethylsilane (TMS) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). General anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were available products of analytical or HPLC grade.

Animals. Male Wistar rats were purchased from Charles Liver Laboratories Japan, Inc. (Yokohama, Japan) and housed in a room at 23 ± 3 °C and 60 ± 5% relative humidity, with a 12 h light−dark cycle. The rats were given normal pelleted diet (Oriental Yeast Co., Ltd., Tokyo, Japan) throughout the experimental period. In this study, rats of 15−21 weeks age were used. All experimental procedures were in accordance with the guidelines for animal experiments of Food Research Laboratories, Mitsui Norin Co., Ltd.

In Vitro Incubation of +C and EC with Rat Intestinal Microbiota. Incubation of +C and EC with rat intestinal microbiota was carried out according to our method previously reported.[8] Fresh feces (12g) obtained from three or four rats of the same batch were preincubated with GAM broth (400 mL) at 37 °C for 60 h under anaerobic condition. The incubated culture broth was centrifuged (4500*g* for 30 min at 10 °C) to collect crude bacterial cells. The bacterial cells were washed once with sterile water and were then collected by centrifugation. The resultant cells were suspended with 0.1 M sodium phosphate buffer (pH 7.1), and the cell suspension turbidity was adjusted from 1.4 to 1.8 with the same buffer by measuring absorbance at 660 nm (DU 640 spectrophotometer, Beckman Coulter, Inc., Tokyo, Japan). To the bacterial suspension (210 mL) was added 5% aqueous methanol (25 mL) containing 200 mg of +C or EC previously filtered with a sterilized membrane filter (DISMIC-25cs, cellulose acetate 0.2 μm, Advantec Toyo Kaisha, Ltd., Tokyo, Japan), and the mixture was incubated at 37 °C under anaerobic condition. Every 24 h during the incubation period, samples (1 mL each) were withdrawn in an anaerobic glovebox under CO_2 atmosphere. After centrifugation to remove bacterial cells, the resulting supernatants were filtered and analyzed by a Surveyor HPLC and an LCQ Deca XPplus system (LC-MS; Thermo Fisher Scientific K.K., Yokohama, Japan) under the conditions as described below under LC-MS Analysis. Metabolites were monitored by absorbance at 230 nm and were confirmed by MS analysis.

Purification and Identification of Metabolites from +C and EC. Purification procedures were also performed according to our previous paper.[8] Samples (100 mL) of the incubation mixture were withdrawn after adequate incubation time. The samples were centrifuged at 10000*g* for 20 min at 4 °C to remove bacterial cells. The resultant supernatants were adjusted to about pH 3.5 with 5 M

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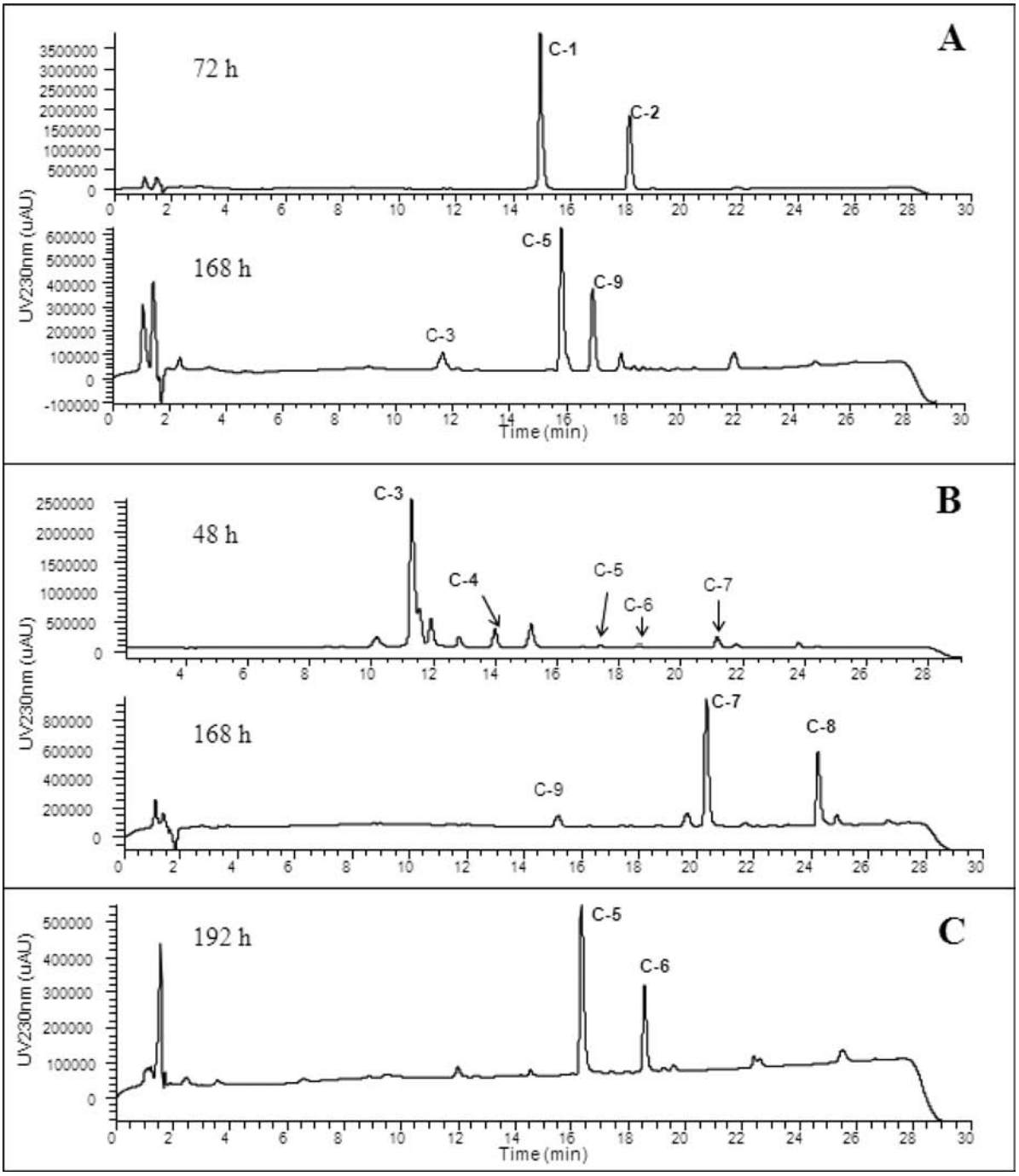

Figure 1. HPLC profiles of metabolite formation from +C by rat intestinal microbiota. Panels A–C show HPLC profiles of metabolite formation with different microbiota prepared from rats of different batches. Peaks C-1–C-9 show metabolites from +C. The metabolites presented in bold type were isolated from each incubation mixture.

HCl and then extracted three times with equal volumes of ethyl acetate. The ethyl acetate fractions were evaporated to dryness. Each residue thus obtained was dissolved in 5 mL of 5% aqueous methanol, and the solution was subjected to preparative HPLC. The HPLC was performed with a 150 mm × 20 mm i.d., 5 μm, CAPCELL PAK C_{18} MG column (Shiseido Co. Ltd., Tokyo, Japan) in a JASCO HPLC 800 series system (JASCO Corp., 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 patterns were monitored by measuring the absorbance at 270 nm. Each fraction containing a metabolite was collected and evaporated to dryness. Each residue was dissolved in distilled water and concentrated to dryness. This procedure was repeated twice or more to remove acetic acid. Finally, each residue was dissolved in distilled water and then lyophilized. Exceptionally, the ethyl acetate fraction containing **C-3** and **C-5** or **EC-3** and **EC-5** was extracted with 100 mL of 10 mM Na_2CO_3 solution. The above compounds moved from the organic fraction to the Na_2CO_3 solution. The Na_2CO_3 solution containing **C-3** and **C-5** or **EC-3** and **EC-5** was concentrated, and the pH of the concentrate was adjusted to 3.0–5.0 with 5 M HCl. After centrifugation to remove precipitate if necessary, the supernatant was subjected to the preparative HPLC under the same conditions as mentioned above except for using 10% (v/v) aqueous methanol for starting solvent and 60% (v/v) aqueous methanol for ending solvent. Each metabolite fraction was collected and concentrated to

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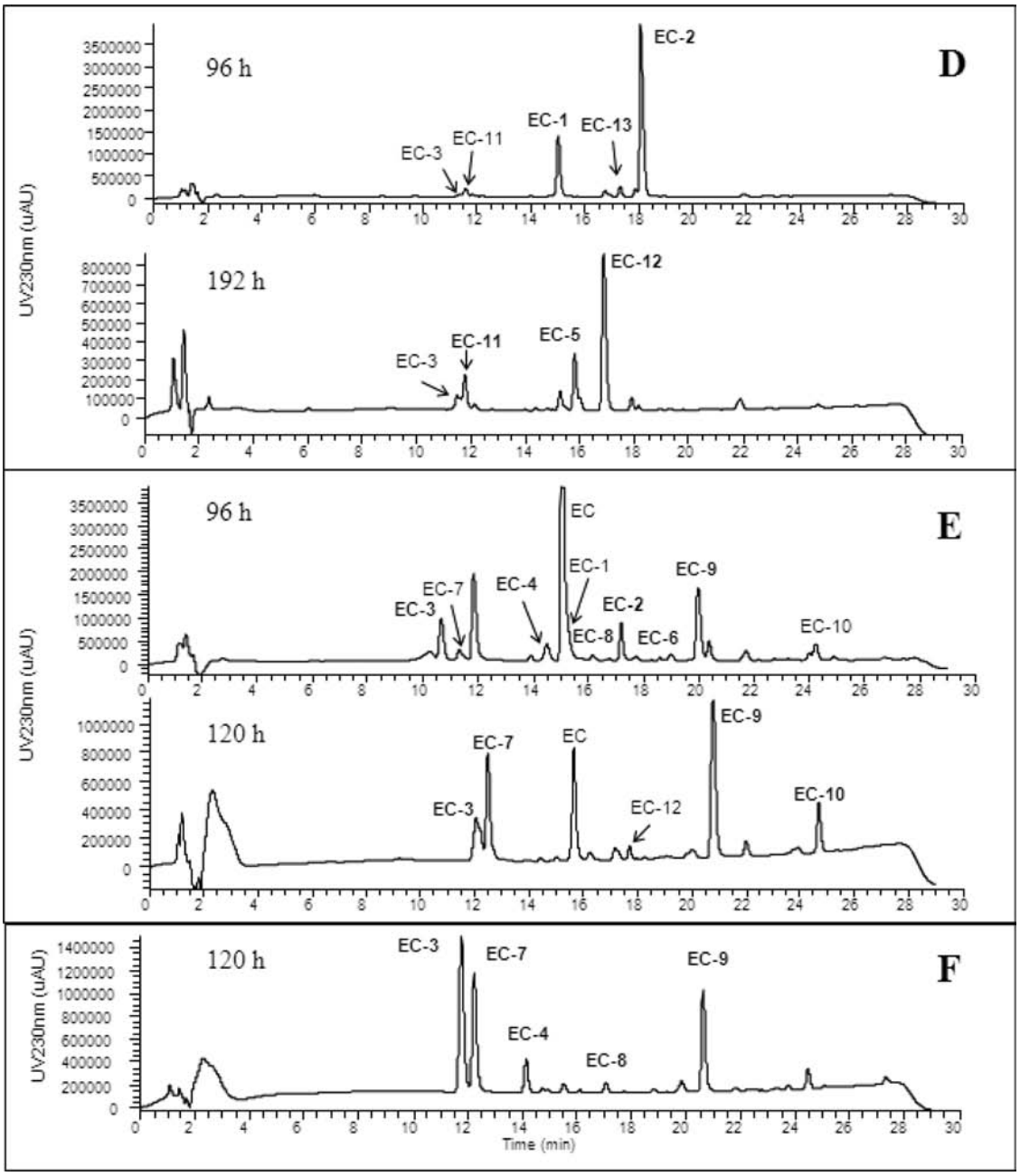

Figure 2. HPLC profiles of metabolite formation from EC by rat intestinal microbiota. Panels D–F show HPLC profiles of metabolite formation with different microbiota prepared from rats of different batches. Peaks EC-1–EC-13 show metabolites from EC. The metabolites presented in bold type were isolated from each incubation mixture.

remove methanol. The resultant **C-3**, **C-5**, **EC-3**, and **EC-5** fractions were individually treated with a cation exchange column with a Diaion SK1B Na^+ form (Mitsubishi Chemical Co., Tokyo, Japan) according to our previous paper[8] to obtain their sodium salt. Finally, purified metabolites **C-1** (15 mg), **C-2** (11 mg), **C-3** (22 mg), **C-4** (7 mg), **C-5** (15 mg), **C-6** (18 mg), **C-7** (20 mg), **C-8** (15 mg), and **C-9** (13 mg) were obtained as +C metabolites. Similarly, purified **EC-1** (10 mg), **EC-2** (14 mg), **EC-3** (18 mg), **EC-4** (7 mg), **EC-5** (6 mg), **EC-6** (2.5 mg), **EC-7** (12 mg), **EC-8** (2 mg), **EC-9** (31 mg), **EC-10** (17 mg), **EC-11** (7 mg), **EC-12** (21 mg), and **EC-13** (3 mg) were obtained as EC metabolites.

LC-MS Analysis. LC-MS/MS analysis was performed by a Surveyor HPLC and an LCQ Deca XPplus system (Thermo Fisher Scientific K.K.). HPLC was carried out with a 100 mm × 2 mm i.d., 5 μm, CAPCELL PAK C_{18} MG column (Shiseido Co. Ltd.) at a flow rate of 0.2 mL/min at 40 °C. The column was eluted 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/v) from 0 to 80% for 2 to 25 min. The mobile phase was re-equilibrated with 100% buffer A for 5 min. The elution patterns were monitored by the diode array detector and then directed to the 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.

NMR and Optical Rotation Analyses. NMR analysis was taken on a Bruker Ultrashield 400 plus system (^{1}H, 400 MHz; ^{13}C, 100 MHz: Bruker BioSpin K.K., Yokohama, Japan). All samples were dissolved in methanol-d_4 (Kanto Chemical). Chemical shifts were referenced to tetramethylsilane (TMS) at 0 ppm. ^{1}H–^{1}H shift correlation spectroscopy (COSY), heteronuclear multiple-bond correlation spectroscopy (HMBC), and heteronuclear single-quantum correlation spectroscopy

(HSQC) experiments were conducted on all metabolites purified in this study. Specific rotations ($[\alpha]_D^{20}$) of metabolites were measured by a P-1020 polarimeter (JASCO Corp.), and the solvents and sample concentrations used were described in the text.

RESULTS

Isolation of Metabolites Formed from both +C and EC. HPLC profiles of the metabolite formation from +C by rat intestinal microbiota prepared from three different batches are shown in Figure 1. **C-1** and **C-2** were purified from the 72 h and **C-5** and **C-9** from the 168 h incubation mixture A. Metabolites **C-3** and **C-4** were isolated from the 48 h and **C-7** and **C-8** from the 168 h incubation mixture B. **C-5** and **C-6** were isolated from the 192 h incubation mixture C. Similarly, metabolites **EC-1**, **EC-2**, and **EC-13** were isolated from the 96 h and **EC-5**, **EC-11**, and **EC-12** from the 192 h incubation mixture D as indicated in Figure 2. **EC-2**, **EC-3**, **EC-4**, **EC-6**, **EC-8**, and **EC-9** were isolated from the 96 h and **EC-3**, **EC-7**, **EC-9**, and **EC-10** from the 120 h incubation mixture E. **EC-3**, **EC-4**, **EC-7**, **EC-8**, and **EC-9** were isolated from the 120 h incubation mixture F. Finally, we isolated 9 different kinds of metabolites (**C-1**−**C-9**) from +C and 13 different metabolites (**EC-1**−**EC-13**) from EC. Structure determination of the +C and EC metabolites was performed by LC-MS, NMR, and optical rotation analyses. Table 1 shows the LC-MS data of all the metabolites isolated. NMR spectral data of novel metabolites (**C-5**, **EC-5**, **EC-7**, **EC-8**, and **EC-13**) are indicated in Tables 2 and 3, with **C-3** and **EC-3** as their analogous metabolites. Tables 4 and 5 show NMR spectral data of known +C metabolites (**C-1**, **C-2**, **C-4**, **C-6**, **C-7**, and **C-9**) and **EC-11** because their NMR data remain insufficient until now. NMR spectral data of EC metabolites were not shown because their NMR data were observed to be almost the same as those of the corresponding +C metabolites listed in Tables 4 and 5.

Table 1. LC-MS/MS Data of +C and EC Metabolites

metabolite	$[M - H]^-$ (m/z)	MS^2 ions (m/z)
C-1	291	247, 233, 205, 167, 123
C-2	275	231, 217, 191
C-3	225	207, 163, 123, 101
C-4	207	163
C-5	209	191, 147, 101
C-6	191	173, 147, 107
C-7	209	191, 135
C-8	193	175, 149
C-9	165	121
EC-1	291	247, 233, 205, 167, 123
EC-2	275	231, 217, 191
EC-3	225	207, 163, 123, 101
EC-4	207	163
EC-5	209	191, 147, 101
EC-6	191	173, 147, 107
EC-7	223	179, 123
EC-8	207	189, 163, 107, 99
EC-9	209	191, 135
EC-10	193	175, 149
EC-11	181	137
EC-12	165	121
EC-13	275	231, 189, 173, 119

Structural Analysis of Metabolites Formed from both +C and EC. **C-1** and **EC-1** were identified as 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol based on LC-MS and NMR data (Tables 1, 4, and 5). The compound was previously reported by Wang et al.[14] The optical rotation value of **C-1** ($[\alpha]_D^{20}$ = +19.5°, *c* 0.44 in methanol) was closely similar to that of (2*R*)-1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol ($[\alpha]_D^{25}$ = +19.8°, *c* 0.83 in methanol),[14] indicating **C-1** has 2*R* configuration. The value of **EC-1** ($[\alpha]_D^{20}$ = −16.2°, *c* 0.28 in methanol) was found to be opposite in sign to that of **C-1**. The **EC-1** value was closely analogous to that of (2*S*)-1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol ($[\alpha]_D^{25}$ = −16.8°, *c* 1.14 in methanol),[14] showing that **EC-1** has 2*S* configuration. Consequently, **C-1** and **EC-1** were considered to be enantiomers of each other.

C-2 and **EC-2** were found to have the same chemical structure and were identified to be 1-(3-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (Tables 1, 4, and 5). This metabolite derived from EC was reported[12,14] but not from +C. The optical rotation value of **C-2** was found to be $[\alpha]_D^{20}$ = +18.9° (*c* 0.36 in methanol). On the other hand, the value of **EC-2** was observed to be $[\alpha]_D^{20}$ = −14.4° (*c* 0.28 in methanol) and was close to that of (2*S*)-1-(3-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol ($[\alpha]_D^{25}$ = −13.5°, *c* 0.78 in methanol) from EC.[14] The optical rotation of **C-2** was thus opposite in sign to that of **EC-2**. Accordingly, it was presumed that **C-2** and **EC-2** were each other's enantiomer and had 2*R* and 2*S* configurations, respectively.

On the basis of NMR analysis (Tables 2 and 3) together with LC-MS data (Table 1), **C-3** and **EC-3** were determined to be 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid. This compound was already identified as a urinary metabolite of (−)-epicatechin gallate (ECg) in our previous paper.[7] Recently, Kutschera et al.[13] reported that this metabolite was biotransformed from +C and EC by human intestinal bacteria (using a combination of *Eggerthella lenta* rK3 and *Flavonifractor plautii* aK2), but their NMR data were not shown. The optical rotation values were observed to be $[\alpha]_D^{20}$ = +3.0° (*c* 0.31 in methanol) for **C-3** and $[\alpha]_D^{20}$ = −3.9° (*c* 0.39 in methanol) for **EC-3**. Accordingly, **C-3** and **EC-3** were considered to bear an enantiomeric relationship to each other, but their absolute configurations at the 4-position are still not determined.

C-4 and **EC-4** were found to be the same compound and were identified as 5-(3,4-dihydroxyphenyl)-γ-valerolactone by LC-MS and NMR analyses (Tables 1, 4, and 5). The above γ-valerolactone was already reported.[12,15,16] The optical rotation value of **C-4** ($[\alpha]_D^{20}$ = +10.5°, *c* 0.37 in methanol) was found to be the same sign as that of the γ-valerolactone having 4*S* configuration ($[\alpha]_D^{26}$ = +39.6°, *c* = 0.1 in methanol) as previously reported.[15] The value of **EC-4** ($[\alpha]_D^{20}$ = −25.0°, *c* 0.12 in methanol) was similar to that of the γ-valerolactone having 4*R*-configuration ($[\alpha]_D^{26}$ = −29.2°, *c* 1.00 in methanol) reported by Hamada et al.[16] and was shown to be opposite in sign to that of both **C-4** and the γ-valerolactone having 4*S* configuration. These observations suggested that **C-4** and **EC-4** were each other's enantiomer and had 4*S* and 4*R* configurations, respectively.

Metabolites **C-5** and **EC-5** exhibited a deprotonated molecular ion peak at m/z 209 $[M - H]^-$ in negative ESI-MS, 16 mass units less than that of **C-3** and **EC-3** (Table 1). The ^1H and ^{13}C NNR spectra of **C-5** were superimposed with those of **EC-5** and were similar to those of 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid (**C-3** and **EC-3**), except for the aromatic signal patterns (Tables 2 and 3). The aromatic signal patterns in **C-5** and **EC-5** were similar to those of the B ring

Table 2. ^{1}H NMR Spectral Data of Unidentified +C and EC Metabolites[a]

	C-3[b]	EC-3[b]	C-5[b]	EC-5[b]	EC-7	EC-8	EC-13
H-1a							2.72 dd (14.0, 4.2)
H-1b							2.56 dd (14.0, 8.0)
H-2					2.44 t (6.6)	2.48 t (6.3)	4.02 dddd (8.0, 7.6, 4.2, 4.2)
H-2a	2.33 ddd (15.0, 8.0, 7.2)	2.36 ddd (15.2, 8.3, 6.8)	2.35 ddd (15.1, 7.7, 7.6)	2.35 ddd (15.3, 7.6, 7.5)			
H-2b	2.26 ddd (15.0, 7.6, 6.9)	2.28 ddd (15.2, 8.1, 6.9)	2.27 ddd (15.1, 7.5, 7.5)	2.27 ddd (15.3, 7.6, 7.5)			
H-3					2.72 t (6.6)	2.75 t (6.3)	
H-3a	1.79 m	1.79 m	1.80 m	1.80 m			2.89 dd (14.0, 4.2)
H-3b	1.63 dddd (14.4, 7.6, 7.3, 7.2)	1.62 dddd (12.5, 8.3, 8.1, 6.3)	1.65 dddd (14.6, 7.6, 7.5, 7.4)	1.65 dddd (14.7, 7.5, 7.5, 7.4)			2.66 dd (14.0, 7.6)
H-4	3.73 m	3.72 m	3.78 m	3.78 m			
H-5					3.59 s	3.69 s	
H-5a	2.59 dd (13.8, 7.0)	2.62 dd (13.6, 6.8)	2.69 dd (13.5, 6.6)	2.68 dd (13.3, 6.8)			
H-5b	2.56 dd (13.8, 6.6)	2.56 dd (13.6, 6.0)	2.64 dd (13.5, 6.0)	2.64 dd (13.3, 5.8)			
H-3″							5.92 s
H-5″							5.92 s
H-2′	6.66 d (2.0)	6.66 d (1.9)	6.67 s	6.67 s	6.64 d (2.0)	6.66 s	7.02 d (8.4)
H-3′							6.68 d (8.4)
H-4′			6.60 d (8.0)	6.60 d (8.4)		6.67 d (1.2, 7.7)	
H-5′	6.66 d (7.8)	6.66 d (8.1)	7.06 t (8.0)	7.06 t (8.4)	6.70 d (8.0)	7.12 t (7.7)	6.68 d (8.4)
H-6′	6.52 dd (7.8, 2.0)	6.52 dd (8.1, 1.9)	6.68 d (8.0)	6.68 d (8.4)	6.53 dd (8.0, 2.0)	6.68 d (1.2, 7.7)	7.02 d (8.4)

[a]Chemical shifts are expressed in ppm downfield from the signal for TMS in methanol-d_4, and coupling constants in hertz are in parentheses. Proton numbering is illustrated in Figure 4. [b]**C-3**, **EC-3**, **C-5**, and **EC-5** are of their sodium salt forms.

in **C-2** (Tables 4 and 5). From the observations together with ^{1}H−^{1}H shift correlation spectroscopy (COSY), heteronuclear multiple-bond correlation spectroscopy (HMBC), and heteronuclear single-quantum correlation spectroscopy (HSQC) experiments, the chemical structures of **C-5** and **EC-5** were the same and were determined to be 4-hydroxy-5-(3-hydroxyphenyl)valeric acid. Their optical rotations were observed to be $[\alpha]_D^{20}$ = +10.1° (*c* 0.16 in methanol) for **C-5** and $[\alpha]_D^{20}$ = −9.3° (*c* 0.31 in methanol) for **EC-5**. It was suggested that the relationship between **C-5** and **EC-5** was enantiomeric. However, their absolute configurations are still not determined.

C-6 and **EC-6** were identified to be 5-(3-hydroxyphenyl)-γ-valerolactone on the basis of LC-MS and NMR data (Tables 1, 4, and 5). This compound was already synthesized as an EC metabolite.[16] The optical rotation of **EC-6** ($[\alpha]_D^{20}$ = −25.0°, *c* 0.12 in methanol) was analogous to the γ-valerolactone having 4*R* configuration ($[\alpha]_D^{26}$ = −29.2°, *c* 1.00 in methanol).[16] On the other hand, the optical rotation of **C-6** ($[\alpha]_D^{20}$ = +28.5°, *c* 0.48 in methanol) showed an opposite sign against **EC-6**. From the observations, it was presumed that **C-6** and **EC-6** were each other's enantiomer and possessed 4*S* and 4*R* configurations, respectively.

Table 3. ^{13}C NMR Spectral Data of Unidentified +C and EC Metabolites[a]

	C-3[b]	EC-3[b]	C-5[b]	EC-5[b]	EC-7	EC-8	EC-13
C-1	182.9	181.9	182.6	182.5	178.6	176.4	43.6
C-2	34.1	33.8	34.2	34.2	30.3	33.6	75.4
C-3	35.8	35.0	35.5	35.5	38.0	38.1	31.6
C-4	74.3	74.1	74.0	74.0	211.1	210.4	
C-5	44.4	44.4	45.0	45.0	50.2	50.6	
C-1″							105.8
C-2″							158.3
C-3″							96.0
C-4″							157.8
C-5″							96.0
C-6″							158.3
C-1′	132.1	132.1	142.0	142.0	127.6	137.3	131.9
C-2′	116.2	116.2	117.4	117.4	116.5	117.5	131.5
C-3′	146.0	146.0	158.3	158.3	146.5	158.7	116.0
C-4′	144.5	144.6	114.0	114.0	145.4	114.9	156.5
C-5′	117.6	117.6	130.2	130.2	117.6	130.7	116.0
C-6′	121.9	121.8	121.8	121.8	122.0	121.9	131.5

[a]Chemical shifts are expressed in ppm downfield from the signal for TMS in methanol-d_4. Carbon numbering is shown in Figure 4. [b]**C-3**, **EC-3**, **C-5**, and **EC-5** are of their sodium salt forms.

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Table 4. ^{1}H NMR Spectral Data of Known +C and EC Metabolites[a]

	C-1	C-2	C-4	C-6	C-7	C-9	EC-11
H-1a	2.64 dd (13.9, 7.6)	2.74 dd (13.9, 4.1)					
H-1b	2.52 dd (13.9, 7.8)	2.58 dd (13.9, 8.3)					
H-2	3.95 dddd (7.8, 7.6, 4.3, 4.1)	4.02 m				2.56 t (7.6)	2.51 t (7.8)
H-2a			2.48 ddd (15.3, 9.3, 9.2)	2.50 ddd (17.6, 9.1, 9.1)	2.42 ddd (15.7, 9.3, 6.3)		
H-2b			2.33 ddd (15.3, 9.2, 4.8)	2.37 ddd (17.6, 9.4, 4.6)	2.31 ddd (15.7, 8.6, 7.1)		
H-3						2.83 t (7.6)	2.74 t (7.8)
H-3a	2.86 dd (14.0, 4.3)	2.88 dd (14.9, 4.4)	2.23 dddd (12.2, 9.2, 6.8, 4.8)	2.26 m	1.81 m		
H-3b	2.67 dd (14.0, 4.1)	2.70 dd (14.9, 7.7)	1.95 dddd (12.2, 9.3, 9.2, 7.0)	1.97 m	1.60 m		
H-4	3.73 m	3.72 m	4.72 m	4.76 m	3.73 m		
H-5							
H-5a		2.62 dd (13.6, 6.8)	2.87 dd (14.0, 6.3)	2.95 dd (14.0, 6.6)	2.63 dd (13.6, 6.5)		
H-5b		2.56 dd (13.6, 6.0)	2.78 dd (14.0, 6.4)	2.86 dd (14.0, 6.4)	2.56 dd (13.6, 6.5)		
H-3″	5.87 s	5.89 s					
H-5″	5.87 s	5.89 s					
H-2′	6.66 d (2.0)	6.67 d (1.9)	6.68 d (2.0)	6.70 d (2.4)	6.65 dd (2.2, 2.0)	6.66 d (2.0)	6.65 d (2.0)
H-3′							
H-4′		6.60 dd (7.8, 1.9)		6.66 d (7.8)	6.68 d (7.8)	6.61 dd (7.9, 2.0)	
H-5′	6.66 d (8.0)	7.06 dd (8.1, 7.8)	6.69 d (8.0)	7.10 dd (8.4, 7.8)	6.66 d (7.8)	7.07 t (7.9)	6.67 d (8.0)
H-6′	6.53 dd (8.0, 2.0)	6.69 d (8.1)	6.56 dd (8.0, 2.0)	6.71 d (8.4)	6.52 dd (7.8, 2.0)	6.68 d (7.9)	6.53 dd (8.0, 2.0)

[a]Chemical shifts are expressed in ppm downfield from the signal for TMS in methanol-d_4, and coupling constants in hertz are in parentheses. Proton numbering is shown in Figure 4. Pairs **C-1** and **EC-1**, **C-2** and **EC-2**, **C-4** and **EC-4**, **C-6** and **EC-6**, **C-7** and **EC-9**, and **C-9** and **EC-12** gave almost the same chemical shifts.

EC-7 exhibited a deprotonated molecular ion peak at m/z 223 $[M - H]^-$ in negative ESI-MS, 2 mass units less than that of **EC-3** (Table 1). In ^{1}H and ^{13}C NMR, the aromatic signal patterns of **EC-7** were observed to be similar to those of **C-3** (Tables 2 and 3). In ^{1}H NMR, three methylene signals at δ 2.44 (t), 2.72 (t), and 3.59 (s) were observed. HSQC and HMBC experiments showed that these signals were assigned to be H-2 (2.44), H-3 (2.72), and H-5 (3.59). In ^{13}C NMR, a characteristic carbon signal at δ 211.1 was observed and was assigned to be C-4 derived from a keto group of **EC-7**. The presence of the keto group at the 4-position was confirmed by observing each of the cross peaks between H-3 and C-4 and between H-5 and C-4 in the HMBC experiment. From these observations, **EC-7** was determined to be 4-oxo-5-(3,4-dihydroxyphenyl)valeric acid.

EC-8 exhibited a deprotonated molecular ion peak at m/z 207 $[M - H]^-$ in negative ESI-MS, 2 mass units less than that of **EC-5** and 16 mass units less than that of **EC-7** (Table 1). The ^{1}H and ^{13}C NMR signal patterns were similar to those of **EC-7**, except for the aromatic patterns (Tables 2 and 3). The aromatic patterns of **EC-8** were similar to those of **EC-5**. The cross peaks between H-3 and C-4 and between H-5 and C-4 were also observed as well as **EC-7** in the HMBC experiment (Figure 3). Consequently, the metabolite **EC-8** was identified as 4-oxo-5-(3-hydroxyphenyl)valeric acid.

C-7 and **EC-9** were the same compound and were determined to be 5-(3,4-dihydroxyphenyl)valeric acid (Tables 4 and 5), which was found to be produced from ECg by human intestinal bacteria.[12] LC-MS and NMR data of **C-8** and **EC-10** showed these are the same compound (NMR data not shown) and were superimposed with 5-(3-hydroxyphenyl)valeric acid reported in our previous paper.[8] From LC-MS and NMR data (Tables 1, 4, and 5), **EC-11** was determined to be 3-(3,4-dihydroxyphenyl)propionic acid, which was reported as an ECg metabolite.[12] **C-9** and **EC-12** were the same compound and were identified to be 3-(3-hydroxyphenyl)propionic acid as reported in our previous paper.[7]

EC-13 exhibited a deprotonated molecular ion peak at m/z 275 $[M - H]^-$ in negative ESI-MS, which was the same as **EC-2**. The ^{1}H and ^{13}C NMR data (Tables 2 and 3) indicated that **EC-13** was analogous to **C-2** (Tables 4 and 5) except for its aromatic signal patterns. The two signals derived from the B ring protons of **EC-13** were observed at δ 6.68 (d, 2H, J = 8.4, H-3′ and 5′) and 7.02 (d, 2H, J = 8.4, H-2′ and 6′). The carbon signals from the B ring were also observed at δ 119.0 (C-3′ and 5′),

Table 5. ^{13}C NMR Spectral Data of Known +C and EC Metabolites[a]

	C-1	C-2	C-4	C-6	C-7	C-9	EC-11
C-1	43.8	44.4	180.4	180.2	178.0	176.9	177.3
C-2	75.4	75.1	29.5	29.5	35.0	36.8	37.3
C-3	31.6	31.8	27.9	28.1	25.7	32.1	31.6
C-4			83.3	83.0	32.3		
C-5			41.5	42.1	35.9		
C-1″	105.7	105.6					
C-2″	158.3	158.3					
C-3″	95.9	96.0					
C-4″	157.7	157.7					
C-5″	95.9	96.0					
C-6″	158.3	158.3					
C-1′	132.6	142.6	129.1	139.3	135.3	143.6	133.9
C-2′	117.7	117.4	117.7	117.4	116.5	116.3	116.5
C-3′	145.9	158.1	146.4	158.7	146.0	158.5	146.2
C-4′	144.4	113.9	145.3	114.8	144.1	114.1	144.6
C-5′	116.1	130.1	116.4	130.6	116.3	130.5	116.4
C-6′	121.9	121.9	122.0.	121.7	120.7	120.5	120.5

[a]Chemical shifts are expressed in ppm downfield from the signal for TMS in methanol-d_4. Carbon numbering is indicated in Figure 4. Pairs **C-1** and **EC-1**, **C-2** and **EC-2**, **C-4** and **EC-4**, **C-6** and **EC-6**, **C-7** and **EC-9**, and **C-9** and **EC-12** gave almost the same chemical shifts.

131.5 (C-2′ and 6′), 131.9 (C-1′), and 156.5 (C-4′), which were confirmed by HSQC and HMBC experiments. As a result, **EC-13** was concluded to be 1-(4-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol.

Figure 4 illustrates the structures of the metabolites identified in this study (**C-1**−**C-9** and **EC-1**−**EC-13**).

DISCUSSION

Meselhy et al.[12] have previously reported the identification of metabolites of (−)-epicatechin gallate (ECg) by human intestinal bacteria and proposed its metabolic pathway. We have recently reported the metabolic pathway of (−)-epigallocatechin gallate (EGCg) by rat intestinal microbiota.[8] In the present paper, we isolated and identified five different kinds of EC metabolites (**EC-3**, **EC-5**, **EC-7**, **EC-8**, and **EC-13**) other than the metabolites reported by Meselhy et al.[12] A search of their chemical structures with Scifinder suggested that **EC-5**, **EC-7**, **EC-8**, and **EC-13** are novel compounds. With regard to +C catabolism, **C-5** was also suggested to be a new compound. **C-3** and **EC-3** were recently reported to be biotransformed from +C and EC, respectively, by the combination of two human intestinal bacteria, *Eg. lenta* rK3 and *F. plautii* aK2,[13] but their chemical structures have not been definitively determined. In this study, we clearly demonstrated their structures by NMR analysis (Tables 1 and 2) together with LC-MS analysis. Furthermore, we presumed the steric configuration of some metabolites on the basis of their optical rotation values with reference to the values reported previously.[14−16] The configurations of +C and EC metabolites were presumed as follows; 2*R* for **C-1** and **C-2**, 2*S* for **EC-1** and **EC-2**, 4*S* for **C-4** and **C-6**, and 4*R* for **EC-4** and **EC-6**. The presumptive results implied that the +C and EC metabolites possessing the same chemical structure had enantiomeric relationships to each other and maintained the configurations at the 3-position in +C (3*S*) and EC (3*R*). Although the configurations of **C-3**, **EC-3**, **C-5**, and **EC-5** could not be determined in this study, the enantiomeric relationships were presumed between **C-3** and **EC-3** and between **C-5** and **EC-5**. With this information and the discussion above taken into account, it is expected that the +C (**C-3** and **C-5**) and EC metabolites (**EC-3** and **EC-5**) have 4*S* and 4*R* configurations, respectively.

On the basis of the present results and previous studies,[7,10−14] we updated the proposed metabolic pathways of +C and EC by rat intestinal microbiota (Figure 4). +C and EC are each converted to (2*R*)- (**C-1**) and (2*S*)- 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ols (**EC-1**) at the first

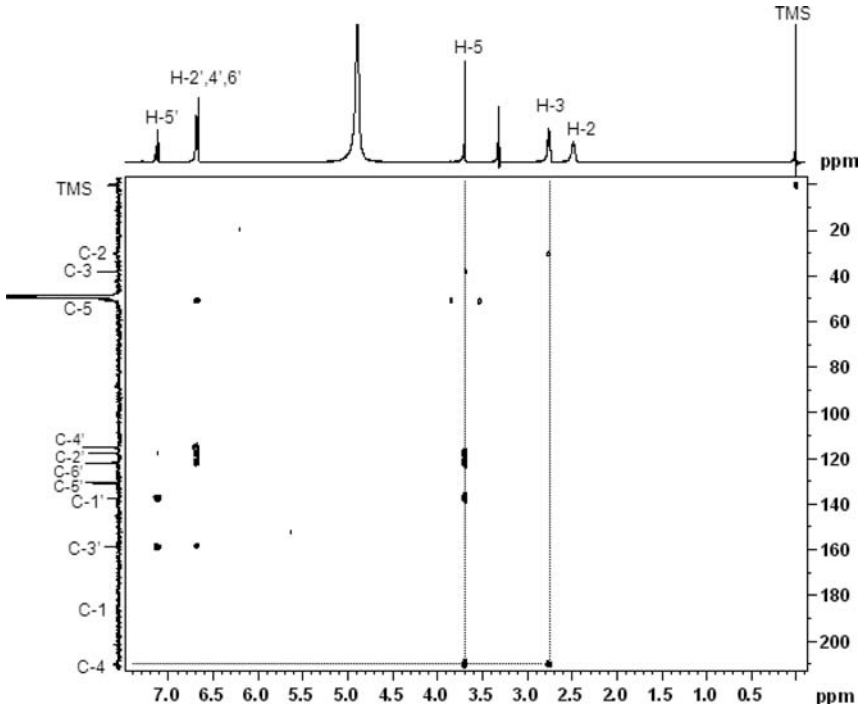

Figure 3. 2D NMR spectra of EC-8 in HMBC experiment. Chemical shifts are expressed in ppm downfield from the signal for TMS in methanol-d_4.

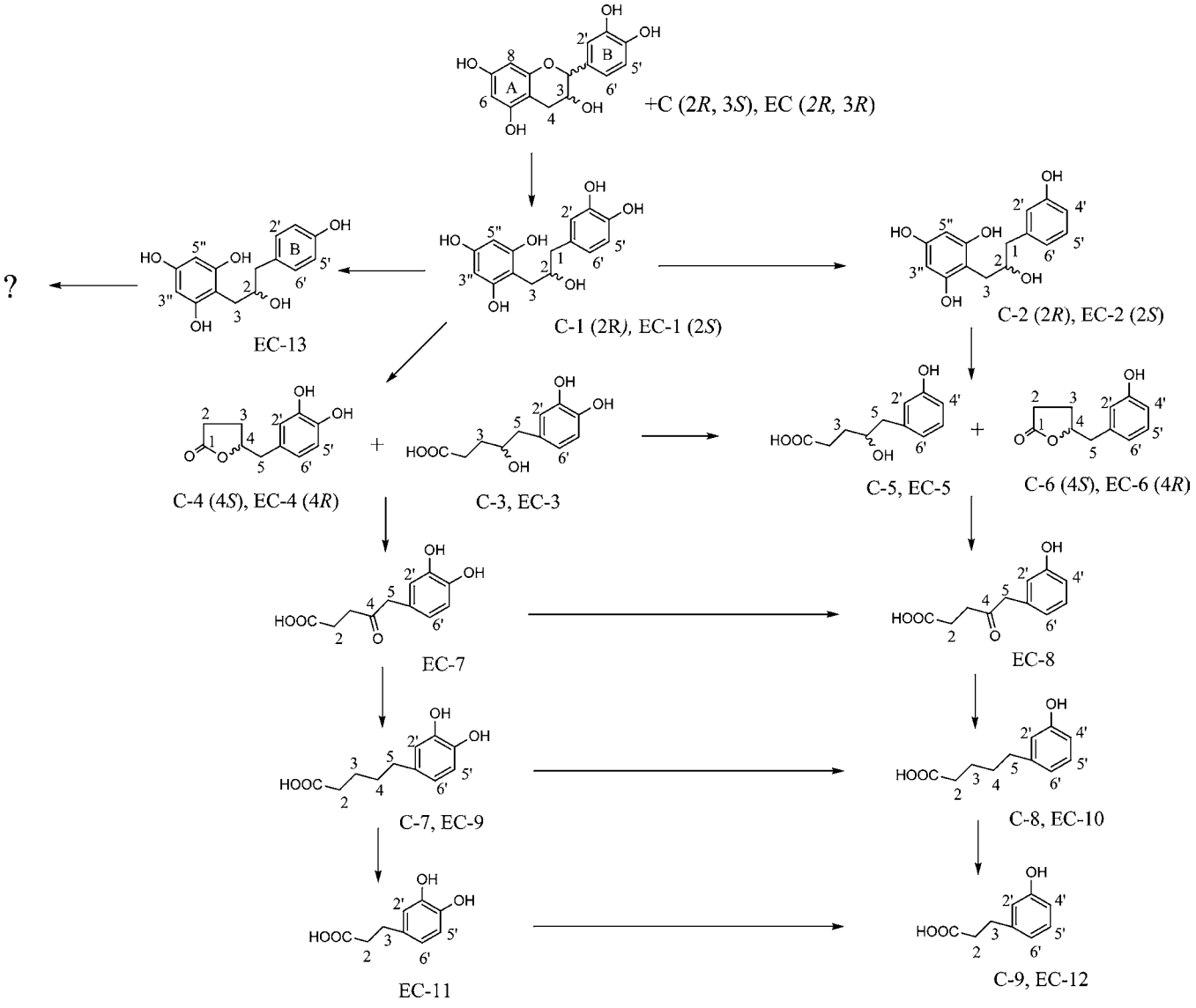

Figure 4. Proposed metabolic pathway of +C and EC by rat intestinal microbiota. *R* and *S* show configuration.

step. Subsequent dehydroxylation of **C-1** and **EC-1** at the 4′-position takes place at least in part to form (2*R*)- (**C-2**) and (2*S*)-1-(3-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ols (**EC-2**). In addition, 1-(4-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (**EC-13**) is produced as a minor metabolite in EC catabolism. The major metabolites (**C-1**, **C-2**, **EC-1**, and **EC-2**) undergo ring fission of the phloroglucinol ring to form mainly their corresponding 4-hydroxy-5-phenylvaleric acids (**C-3**, **C-5**, **EC-3**, and **EC-5**) with the formation of (4*S*)- (**C-4** and **C-6**) and (4*R*)-5-phenyl-γ-valerolactones (**EC-4** and **EC-6**). The biotransformation to **C-3** and **EC-3** from **C-1** and **EC-1** by *F. plautii* aK2 was reported to take place with accompanying formation of **C-4** and **EC-4**.[13] In the same way, it seems likely that the biotransformation to **C-5** and **EC-5** from **C-2** and **EC-2** occurs with **C-6** and **EC-6** production, respectively. **C-3**, **C-5**, **EC-3**, and **EC-5** are each then biotransformed to the corresponding valeric acids (**C-7**, **C-8**, **EC-9**, and **EC-10**) by 4-dehydroxylation. We revealed that the dehydroxylation proceeded in a two-step reaction; that is, the 4-hydroxy-5-phenylvaleric acids (**EC-3** and **EC-5**) were converted to their corresponding 4-oxo-5-phenylvaleric acids (**EC-7** and **EC-8**) by oxidation, followed by reductive elimination of oxygen at the 4-oxo group to produce 5-phenylvaleric acids (**EC-9** and **EC-10**). We have no explanation why the 4-oxo-5-phenylvaleric acids have not been detected during +C catabolism. However, it seems reasonable that the 4-hydroxy-5-phenylvaleric acids from +C are also converted to the corresponding 5-phenylvaleric acids via their 4-oxo-5-phenylvaleric acids as well as EC catabolism because the degradation pathway of +C is very similar to that of EC as illustrated in Figure 4. The 5-phenylvaleric acids, **C-7**, **C-8**, **EC-9**, and **EC-10**, are further degraded to their corresponding 3-phenylpropionic acids. This process may proceed by β-oxidation as proposed by Stoupi et al.[10]

In this study, we revealed more detailed metabolic pathways of +C and EC by rat intestinal microbiota. Wang et al.[14] have already reported that *Eubacterium* sp. strain SDG-2 converted +C and (+)-epicatechin to **C-1** (2*R* configuration), EC and (−)-catechin (−C) to **EC-2** (2*S*), and (−)-gallocatechin (GC) and (−)-epigallocatechin (EGC) to (2*S*)-1-(3,5-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol. Strain SDG-2 was also found to catalyze 4′-dehydroxylation of −C, EC, EGC, and GC. Recently, Kutschera et al.[13] revealed that +C and EC were each biotransformed by *Eg. lenta* rK3 to produce **C-1** and **EC-1** and that then these metabolites were converted into **C-3**

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and **EC-3** by *F. plautii* aK2. These findings are very important for elucidating the metabolic pathway of not only +C and EC but also other tea catechins such as EGC, ECg, and EGCg. More detailed study on the identification of intestinal bacteria involved in the degradation of the tea catechins is desired.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

+C, (+)-catechin; EC, (−)-epicatechin; −C, (−)-catechin; GC, (−)-gallocatechin; EGC, (−)-epigallocatechin; ECg, (−)-epicatechin gallate; EGCg, (−)-epigallocatechin gallate

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