The Heterocyclic Ring Fission and Dehydroxylation of Catechins and Related Compounds by *Eubacterium* sp. Strain SDG-2, a Human Intestinal Bacterium

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A human intestinal bacterium, *Eubacterium* (*E.*) sp. strain SDG-2, was tested for its ability to metabolize various (3*R*)- and (3*S*)-flavan-3-ols and their 3-*O*-gallates. This bacterium cleaved the C-ring of (3*R*)- and (3*S*)-flavan-3-ols to give 1,3-diphenylpropan-2-ol derivatives, but not their 3-*O*-gallates. Furthermore, *E.* sp. strain SDG-2 had the ability of *p*-dehydroxylation in the B-ring of (3*R*)-flavan-3-ols, such as (−)-catechin, (−)-epicatechin, (−)-gallocatechin and (−)-epigallocatechin, but not of (3*S*)-flavan-3-ols, such as (+)-catechin and (+)-epicatechin.

Key words biotransformation; heterocyclic ring fission; dehydroxylation; flavan-3-ol; *Eubacterium* sp. strain SDG-2; human intestinal bacterium

Catechins are a group of polyphenolic flavan-3-ols, which are distributed in most medicinal plants and are major ingredients in green tea infusion. Catechins have been recognized as efficient free-radical scavengers, and green tea catechins have been reported to induce apoptosis in human lymphoblastic leukemia Molt 4B cells,[1] stomach cancer KATO III cells,[2] and promyelocytic leukemia HL-60 cells.[3] It is noteworthy that orally administered catechins as ingredients of crude drugs used in traditional medicine inevitably come into contact with human intestinal bacteria in the gastrointestinal tract and are transformed to various metabolites. The initial metabolic studies with (+)-catechin showed that it was converted to several simple phenolic acids and a series of phenylvalerolactones when given orally to rabbits.[4] Recently, we reported the biotransformation of catechins and their gallates by human or rat fecal bacteria *in vitro*; catechins were subjected to heterocyclic ring fission, followed by *para*-dehydroxylation.[5]

During the course of our study on the metabolism of a lignan glycoside, secoisolariciresinol diglucoside from flaxseed, to mammalian lignans, enterolactone and enterodiol, we isolated a human intestinal bacterium, *Eubacterium* (*E.*) sp. strain SDG-2, capable of *p*-dehydroxylation of the aromatic ring in the metabolic intermediates.[6] It is of interest to investigate whether this bacterium is able to catalyze *p*-dehydroxylation of the catechol moiety of catechins and related compounds.

In this paper, we report heterocyclic C-ring fission and the *p*-dehydroxylation of the B-ring in catechins and related compounds by *E.* sp. strain SDG-2.

Results

Transformation of (3*S*)-Flavan-3-ols by *E.* sp. Strain SDG-2 After anaerobic incubation of a (3*S*)-flavan-3-ol, either (+)-catechin or (+)-epicatechin, with *E.* sp. strain SDG-2, the metabolite in the fermentation broth was extracted with an organic solvent and then purified by repeated column chromatography to afford compound **1** (Fig. 1). This compound was identified as (2*R*)-1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol, which was previously isolated by anaerobic incubation of (+)-catechin with a bacterial mixture from human feces.[5]

Transformation of (3*R*)-Flavan-3-ols by *E.* sp. Strain SDG-2 Anaerobic incubation of (−)-catechin and (−)-epicatechin with *E.* sp. strain SDG-2 resulted in the formation of two compounds, **2** and **3**, which were identified as (2*S*)-1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol and (2*S*)-1-(3′-hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol, respectively, by direct comparison of the spectral data with those of authentic samples obtained from (−)-epicatechin 3-*O*-gallate after anaerobic incubation with a human intestinal bacterial mixture.[5] Similarly, (−)-gallocatechin and (−)-epigallocatechin were transformed to compound **4** by *E.* sp. strain SDG-2.

Compound **4** showed a molecular ion peak at *m*/*z* 292 $[M]^+$ in its electron impact (EI)-MS spectrum, 14 mass units less than that of (−)-gallocatechin and (−)-epigallocatechin. The ^{1}H-NMR spectrum showed protons at δ 2.48 (1H, dd,

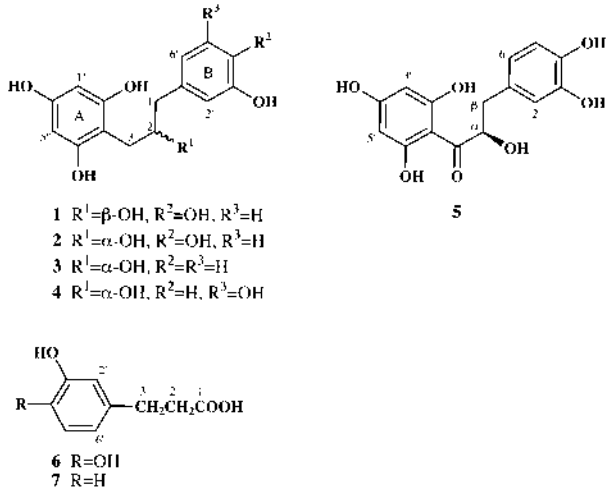

Fig. 1. Metabolites of Catechins and Related Compounds by *E.* sp. Strain SDG-2

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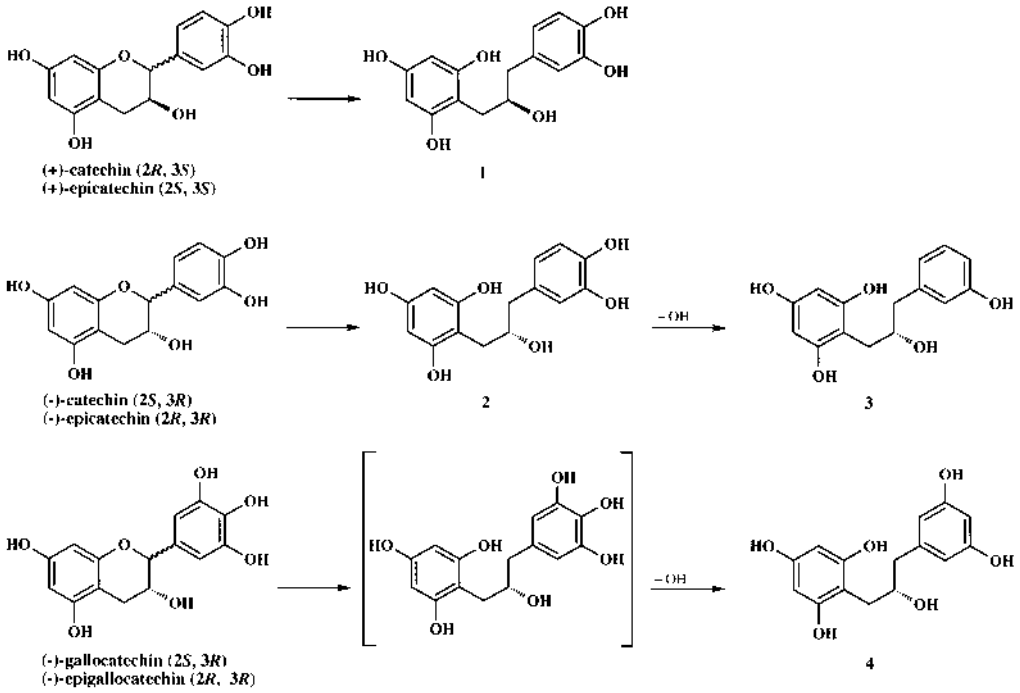

Fig. 2. Possible Metabolic Pathway of Catechins by *E.* sp. Strain SDG-2

J=13.8, 8.7 Hz, H_a-1), 2.57 (1H, dd, J=13.8, 3.6 Hz, H_b-1), 2.58 (1H, dd, J=13.8, 7.3 Hz, H_a-3), 2.84 (1H, dd, J=13.8, 4.6 Hz, H_b-3) and 3.90 (1H, dddd, J=8.7, 7.3, 4.6, 3.6 Hz, H-2), which suggested the heterocyclic ring was cleaved to produce a 1,3-diarylpropan-2-ol skeleton. This speculation was further confirmed by the presence of a methylene carbon at δ 44.5 (C-1) in compound **4**, instead of an oxygenated methylene carbon in (−)-gallocatechin and (−)-epigallocatechin in the ^{13}C-NMR spectrum. In the aromatic region of the ^{1}H-NMR spectrum, five protons were integrated into two spin systems; the signal at δ 5.78 (2H, br s) was assigned to H-3″ and H-5″ in the A-ring (as usual in compounds **1**—**3**), while the signals at δ 6.00 (1H, t, J=2.2 Hz) and 6.09 (2H, d, J=2.2 Hz) were assigned to the three protons H-4′, H-2′ and H-6′ in the B-ring, respectively. The structure of compound **4** was consequently established as (2*S*)-1-(3′,5′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol.

Transformation of Related Compounds by *E.* sp. Strain SDG-2 *E.* sp. strain SDG-2 transformed (+)-taxifolin to α, 2′,3,4,4′,6′-hexahydroxydihydrochalcone (**5**), and caffeic acid to dihydrocaffeic acid (**6**) and *m*-hydroxyphenylpropionic acid (**7**).

Discussion

Most of the previous metabolic studies on catechins have been undertaken with fecal flora of humans or animals. Although Schneider and Blaut[7] have tried to transform (+)-catechin and (−)-epicatechin using *Eubacterium ramulus*, a quercetin-3-glucoside-degrading anaerobic microorganism, these compounds were not metabolized. As shown in Fig. 2, our present experiments showed that *E.* sp. strain SDG-2 was capable of degrading catechins by ring fission and/or *p*-dehydroxylation. (+)-Catechin and (+)-epicatechin were degraded only by ring fission to **1**, which was not further converted to any other metabolite (confirmed by independent incubation of **1** with *E.* sp. strain SDG-2). In contrast, (−)-catechin and (−)-epicatechin were transformed to **2** by ring fission, and subsequently to **3** (confirmed by incubation of **2**) by *p*-dehydroxylation in the B-ring. In the case of (−)-gallocatechin and (−)-epigallocatechin, they were converted either by ring fission or *p*-dehydroxylation to give **4**, though no metabolic intermediate was detected as shown in Fig. 2.

E. sp. strain SDG-2 was first isolated as a bacterial strain capable of eliminating a *p*-hydroxy group in the aromatic ring of didemethylsecoisolariciresinol (a metabolic intermediate of enterolactone from secoisolariciresinol diglucoside from flaxseed).[6] It is quite interesting that *E.* sp. strain SDG-2 is capable of cleaving the heterocyclic ring of catechins except for their gallates, such as (−)-catechin 3-*O*-gallate, (−)-epicatechin 3-*O*-gallate, (−)-gallocatechin 3-*O*-gallate, (−)-epigallocatechin 3-*O*-gallate (data not shown). Similar results were also previously obtained for (−)-epicatechin 3-*O*-gallate,[5] (−)-epigallocatechin 3-*O*-gallate[5] and 3-*O*-methyl-(+)-catechin[7] when incubated anaerobically with rat fecal flora *in vitro.* These findings suggested the possible role of a free hydroxy group at C-3 in the degradation by *E.* sp. strain SDG-2 and rat fecal microorganisms. In addition, as with the ring fission, the presence of a free hydroxy group at C-4′ in the B-ring seems to be necessary, since 4′-(*O*)-methyl-(−)-epigallocatechin was not transformed even after a prolonged period of incubation (3 d) with *E.* sp. strain SDG-2 (data not shown).

E. sp. strain SDG-2 showed *p*-dehydroxylation activity in the B-ring for (3*R*)-flavan-3-ols: (−)-catechin, (−)-epicatechin, (−)-gallocatechin and (−)-epigallocatechin, but not for (3*S*)-flavan-3-ols: (+)-catechin and (+)-epicatechin. Regarding the structural differences, (−)-catechin and (−)-epicatechin are only different from (+)-catechin and (+)-epicatechin in the configuration at C-2 and C-3. The C-2 configuration has no significant influence, since the chiral center no

longer exists after the ring fission. The *R* configuration at C-3 seems to be essential for *p*-dehydroxylation, for (−)-catechin (3*R*) and (−)-epicatechin (3*R*) underwent *p*-dehydroxylation to give **3**. Similarly, *E.* sp. strain SDG-2 showed the ability to eliminate a hydroxy group in the B-ring of (−)-gallocatechin and (−)-epigallocatechin (both with 3*R* configurations). However, (+)-catechin (3*S*) and (+)-epicatechin (3*S*) were only transformed to their ring fission products, even after 4 d of incubation. Previous studies in our laboratory showed that (+)-catechin and their gallates were transformed to their ring fission and *p*-dehydroxylation products after incubation with a mixture of human intestinal bacteria.[5] This finding suggested the presence of different intestinal bacterial species responsible for the *p*-dehydroxylation of compound **1** derived from (+)-catechin. In the case of (+)-taxifolin, although the configuration at C-3 was *R*, it shared the same 3β-OH as (+)-catechin and (+)-epicatechin, and was accordingly transformed to **5** only by ring fission.

In the case of (−)-gallocatechin and (−)-epigallocatechin, the presence of three vicinal hydroxy groups at 3′, 4′ and 5′ in the B-ring seems to enhance the removal of the 4′-OH. Since no intermediate was detected in the samples taken out at 1 h intervals, *p*-dehydroxylation may have taken place quickly after the formation of a possible intermediate (see Fig. 2). Similarly, the *p*-dehydroxylation of **6** to **7** was demonstrated by *E.* sp. strain SDG-2, as well by a strain of *Pseudomonas* sp. isolated from rat feces.[8] In comparison with dihydrocaffeic acid, (4-hydroxyphenyl)propionic acid, a compound lacking a *meta*-OH, and (−)-secoisolarici-resinol, a compound with a *meta*-OMe group, were not degraded by *E.* sp. strain SDG-2. These findings indicated the importance of a vicinal hydroxy group for *p*-dehydroxylation. Furthermore, this bacterium showed no ability to transform 3,4-dihydroxyphenylacetic acid and gallic acid by dehydroxylation (data not shown), suggesting the necessity of at least three carbon atoms in the side chain for dehydroxylation. This is in contrast to the previous report that 3,4-dihydroxyphenylacetic acid was converted to *m*-hydroxyphenyl derivatives after its oral administration to rabbits.[9,10]

In fact, catechins are easily transformed into various metabolites when incubated with a human or rat intestinal bacteria mixture, and it is difficult to obtain some metabolites only with ring fission or *p*-dehydroxylation. The present study made it possible to obtain metabolites through ring fission or *p*-dehydroxylation of catechins in high yield by using a single bacterium isolated from human feces. The observation that *E.* sp. strain SDG-2 can selectively eliminate a *p*-hydroxy group in the B-ring of catechins with a 3-OH group in the *R* configuration, suggests the possible involvement of enzyme(s) in these reactions and deserves further investigation.

Experimental

General Optical rotations were measured in MeOH solution using a Jasco DIP-360 automatic polarimeter at 25 °C. ^{1}H- and ^{13}C-NMR spectra were taken on a Jeol JNM GX-400 with tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were measured with a Jeol JMS DX-300 mass spectrometer at an ionization voltage of 70 eV. For anaerobic cultivation, an anaerobic incubator, EAN-140 (Tabai Co. Osaka, Japan), was used. Wacogel C-400 (Wako Pure Chemical Co., Osaka, Japan) and Sephadex LH-20 (Pharmacia, Sweden) were used for column chromatography. Merck Kieselgel 60 F_{254} plates (layer thickness 0.25 mm) were used with a solvent system of $CHCl_3$–MeOH (4 : 1), and RP-18 F_{254}S plates (layer thickness 0.25 mm) with a solvent system of MeOH–H_2O (3 : 7). Spots were detected under a UV lamp or after spraying with anisaldehyde/H_2SO_4 reagent, followed by heating.

Chemicals and Media Catechins were provided by Mitsui Norin Co., Ltd. (Fujieda, Japan). (+)-Taxifolin was isolated in our laboratory. Caffeic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxylphenylpropionic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). General anaerobic medium (GAM) was purchased from Nissui Co. (Tokyo, Japan).

Organism *E.* sp. strain SDG-2 was isolated from human feces in our laboratory,[6] and maintained in a semisolid GAM.

Preparation of a Bacterial Suspension A 2 ml portion of precultured *E.* sp. strain SDG-2 was inoculated in 500 ml of GAM broth and anaerobically incubated overnight. The culture was used as a bacterial suspension in this experiment.

Incubation of (+)-Catechin with *E.* sp. Strain SDG-2 (+)-Catechin (30 mg in 2 ml MeOH) was added to a bacterial suspension (50 ml) of *E.* sp. strain SDG-2, and incubated at 37 °C in an anaerobic incubator for 36 h. The reaction mixture was then extracted with water sat. BuOH (3×50 ml). The organic layer was evaporated under reduced pressure to give a residue. The residue was applied to a column of silica gel. The column was thoroughly washed with $CHCl_3$ and then eluted with $CHCl_3$–MeOH (10 : 1). Fractions were pooled to give fractions A and B. Repeated column chromatography of fraction B on Sephadex LH-20 (aq. 95% MeOH) and RP-18 (MeOH–H_2O, 4 : 6) gave **1** (16 mg).

Incubation of (+)-Epicatechin with *E.* sp. Strain SDG-2 Similarly, (+)-epicatechin (10 mg in 1 ml MeOH) was anaerobically incubated with a bacterial suspension (20 ml) for 36 h. The reaction mixture was then treated as mentioned above to give **1** (4 mg).

Incubation of (−)-Catechin with *E.* sp. Strain SDG-2 (−)-Catechin (10 mg in 1 ml MeOH) was anaerobically incubated with a bacterial suspension (20 ml) at 37 °C for 36 h. After extraction and evaporation, the residue was applied to a column of silica gel, which was washed thoroughly with $CHCl_3$, followed by $CHCl_3$–MeOH (20 : 1, 10 : 1) to give fractions A—C. After repeated column chromatography on Sephadex LH-20 (aq. 95% MeOH) and RP-18 (MeOH–H_2O, 4 : 6), fractions B and C gave **3** (2.0 mg) and **2** (1.5 mg), respectively.

Incubation of (−)-Epicatechin with *E.* sp. Strain SDG-2 Similarly, (−)-epicatechin (30 mg in 1 ml MeOH) was anaerobically incubated with a bacterial suspension for 36 h. The reaction mixture was then treated as mentioned above to give **2** (10 mg) and **3** (8 mg), respectively.

Incubation of (−)-Gallocatechin and (−)-Epigallocatechin with *E.* sp. Strain SDG-2 (−)-Gallocatechin or (−)-epigallocatechin (30 mg each in 2 ml MeOH) was added to a bacterial suspension and incubated anaerobically for 36 h, separately. The respective reaction mixtures were then treated as mentioned above to give **4** (11 and 12 mg, respectively).

Incubation of (+)-Taxifolin with *E.* sp. Strain SDG-2 (+)-Taxifolin (25 mg in 1 ml MeOH) was incubated with a bacterial suspension (50 ml) for 3 d. The reaction mixture was then treated as mentioned above to give **5** (12 mg).

Incubation of Caffeic Acid with *E.* sp. Strain SDG-2 Caffeic acid (50 mg in 1 ml MeOH) was incubated with a bacterial suspension (100 ml) for 2 d. The reaction mixture was then treated as usual to give **6** (8 mg) and **7** (10 mg).

Compound **1**: Amorphous powder, $[\alpha]_D^{25}$ +19.8° (c=0.83, MeOH). EI-MS *m*/*z*: 292 $[M]^+$. This compound was identified as (2*R*)-1-(3′,4′-dihydroxyphenyl-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol by direct comparison of its spectral data with that published.[5]

Compound **2**: Amorphous powder, $[\alpha]_D^{25}$ −16.8° (c=1.14, MeOH). EI-MS *m*/*z*: 292 $[M]^+$. This compound was identified by comparing the ^{1}H- and ^{13}C-NMR spectra with those reported for (2*S*)-1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol.[5]

Compound **3**: Amorphous powder, $[\alpha]_D^{25}$ −13.5° (c=0.78, MeOH). EI-MS *m*/*z*: 276 $[M]^+$. The ^{1}H- and ^{13}C-NMR spectra of this compound were superimposed with those reported for (2*S*)-1-(3′-hydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol.[5]

Compound **4**: Amorphous powder, $[\alpha]_D^{25}$ −20.0° (c=0.1, MeOH). EI-MS *m*/*z*: 292 $[M]^+$. ^{1}H-NMR (MeOH-d_4) δ: 2.48 (1H, dd, J=13.8, 8.7 Hz, H_a-1), 2.57 (1H, dd, J=13.7, 3.6 Hz, H_b-1), 2.58 (1H, dd, J=14.2, 7.3 Hz, H_a-3), 2.84 (1H, dd, J=14.2, 4.6 Hz, H_b-3), 3.90 (1H, dddd, J=8.7, 7.3, 4.6, 3.6 Hz, H-2), 5.78 (2H, br s, H-3″, 5″), 6.00 (1H, t, J=2.2 Hz, H-4_), 6.09 (2H, d, J=2.2, 1.0 Hz, H-2′, 6′). ^{13}C-NMR (MeOH-d_4) δ: 31.7 (C-3), 44.5 (C-1), 75.0 (C-2), 95.9 (C-3″, 5″), 101.3 (C-4′), 109.1 (C-2′, 6′), 143.3 (C-1′), 157.7 (C-2″, 6″), 158.2 (C-4″), 159.1 (C-3′, 5′).

Compound **5**: Amorphous powder, $[\alpha]_D^{25}$ +5.2° (c=0.27, MeOH). EI-MS *m*/*z*: 306 $[M]^+$. ^{1}H-NMR (MeOH-d_4) δ: 2.79 (1H, dd, J=14.6, 7.4 Hz, H_a-

β), 3.10 (1H, dd, J=14.6, 3.7 Hz, H_b-β), 4.67 (1H, dd, J=7.4, 3.7 Hz, H-α), 5.82 (1H, d, J=1.4 Hz, H-2′ or H-4′), 5.88 (1H, d, J=1.4 Hz, H-4′ or H-2′), 6.56 (1H, dd, J=8.1, 2.1 Hz, H-6), 6.63 (1H, d, J=8.1 Hz, H-5), 6.70 (1H, d, J=2.1 Hz, H-2). ^{13}C-NMR (MeOH-d_4) δ: 38.0 (C-β), 88.0 (C-α), 91.4 (C-3′), 97.5 (C-5′), 104.1 (C-1′), 116.2 (C-5), 117.7 (C-2), 122.0 (C-6), 129.1 (C-1), 145.1 (C-4), 146.0 (C-3), 159.6 (C-4′), 171.3 (C-2′), 176.3 (C-6′), 198.9 (C=O).

Compound **6**: Amorphous powder, EI-MS m/z: 182 $[M]^+$. This compound was identified as (3′,4′-dihydroxyphenyl)propionic acid.[5)]

Compound **7**: Amorphous powder, EI-MS m/z: 166 $[M]^+$. The ^{1}H- and ^{13}C-NMR spectra were comparable with those reported for (3′-hydroxyphenyl)propionic acid.[5)]

Acknowledgements Part of this study was financially supported by Suntory, Ltd., Shimamotocho, Mishimagun, Osaka, and by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan. The authors express their thanks to Dr. G. M. E. Hussein for providing 4′-methyl-(−)-epigallocatechin. Catechins were kindly provided by Mitsui Norin Co., Ltd. (Fujieda, Japan).

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