Metabolism of epicatechin 3-*O*-gallate and epigallocatechin 3-*O*-gallate by human intestinal flora.

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Summary

The biotransformation of (-)-epicatechin 3-*O*-gallate (**1**) and related compounds was undertaken using a human fecal suspension. Of fifteen metabolites isolated, four compounds were new, namely, two epimers of 1-(3-hydroxyphenyl)-3-(2, 4, 6-trihydroxyphenyl)propan-2-ols; 2,3-dihydroxyphenoxyl 3-(3,4-dihydroxyphenyl)propionate (**14**) and 1-(3,4-dihydroxyphenyl)-3-(2, 4, 6-trihydroxyphenyl)propan-2-ol (**15**). Similarly, (-)-epigallocatechin 3-*O*-gallate (**15**) was transformed to five metabolites including 1-(3,5-dihydroxy)-3-(2,4,6-trihydroxyphenyl)propan-2-ol (**18**) and 5-(3,5-dihydroxyphenyl)pentanoic acid (**19**).

(-)-Epicatechin (**2**), (-)-epigallocatechin (**16**) and their 3-O-gallates (**1** and **15**) were extensively metabolized by a human fecal suspension after incubation for 24 h, whereas both gallates (**1** and **15**) resisted any degradation by a rat fecal suspension, even after a prolonged incubation time (48 h), suggesting a difference in metabolic ability between two intestinal bacterial mixtures from different species.

Furthermore, a bacterial strain, *Eubacterium* sp. SDG-2, responsible for the cleavage of C-ring and 4-dehydroxylation of B-ring in flavan-3-ols was isolated from a human feces.

Keywords Biotransformation; (-)-epicatechin 3-*O*-gallate; (-)-epigallocatechin 3-*O*-gallate; human intestinal bacteria; *Eubacterium* sp.

Introduction

(-)-Epicatechin 3-*O*-gallates (**1**) and (-)-epigallocatechin 3-*O*-gallates (**15**) were found to be the most relevant antioxidant constituents of green tea, which protects us against lipid peroxidation,[1] inflammatory dermatoses and immunosuppression.[2] (-)-Epicatechin (**2**) was found to inhibit lung metastasis in mice.[3] However, studies dealing with the biotransformation of the flavan-3-ols by intestinal bacteria have been carried out only in a few instances. The metabolism of (-)-epicatechin (**2**) and its epimer (+)-catechin in rats and guinea pig have long been studied.[4] The present report deals with the biotransformation of (-)-epicatechin 3-*O*-gallate (**1**), (-)-epigallocatechin 3-*O*-gallate (**15**) and related compounds *in vitro*, using human and rat fecal suspensions.

Results and Discussion

After anaerobic incubation with a mixture of human intestinal bacteria (HIB), the fermentation broth of **1**, **2** and **15** was separately extracted with a BuOH-Et_2O mixture (1:1) and then purified by Sephadex LH-20 and medium pressure liquid chromatography (MPLC) to afford fifteen metabolites (**2-14**) (Chart 1). Compounds **2-4** were obtained from **1** after incubation for 12 h, **5-8** were mainly obtained from **1** and **2** after incubation for 24 h, and **9-14** could be mainly obtained after incubation for 48 h. Compounds **3**, and **16-19** were obtained from **15** after incubation for 24 h. The structures of these metabolites were determined by chemical and spectroscopic methods.

(-)-Epicatechin (**2**), (-)-epigallocatechin (**16**) and their gallates (**1** and **15**) were completely metabolized within 12 h by an HIB mixture when the reaction was monitored by thin-layer chromatography densitometry. On the other hand, 75-60% of **2** and **16** were metabolized by a rat intestinal bacterial (RIB) mixture for 12 h, and most of them within 24 h, indicating a slower conversion rate compared to that of HIB. In contrast to the case of HIB, the gallates **1** and **15** were hardly converted to any metabolites, even after prolonged incubation time up to 48 h. These findings revealed that the HIB mixture had appreciable activity of hydrolyzing 3-*O*-galloyl esters of flavan-3-ols, but the RIB mixture had little activity.

A number of metabolites obtained after incubation of **1** with HIB indicates massive ring fission catalyzed by intestinal bacteria. Except for **2**, **5** and **6**, a phloroglucinol part of the original substrate (**1**) was missing in all metabolites. In this connection, reincubation of some intermediate metabolites provided evidence of their sequential relationships, which supports the pathway of **1** by HIB, as shown in Chart 1. Following the incubation of **2**, there was conclusive evidence that **5-9** were degradation products of **1**. When **7** was incubated with HIB, **9** and **12** were also obtained, together with **8**. Without any exception, **12** was found to be the main metabolite in all incubates when the incubation time was extended for 48 h.

The proposed metabolic processes for the formation of these metabolites involve the initial hydrolysis

of a galloyl ester group in **1** to give **2** and **3**, where **2** underwent reductive cleavage to give **5**, while **3** was decarboxylated to give **4**. It seems evident that the reductive cleavage of **2** is due to the presence of a free *p*-hydroxyl group (C4'-OH) in **2**, since a 3',4',5,7-tetra-*O*-methyl ether of **2** was unchanged after incubation with HIB for 60 h.

Another pathway which has been prominent in most studies of flavan-3-ols to date involves the destruction of the phloroglucinol part (ring A) leading to CO_2, with the remainder of the molecule as phenolic lactone and sometimes phenolic acids.[5,6] In connection to this study, the possible lactonization of C2-OH in **5** with a carbonyl carbon left after the degradation of A-ring can be considered to give **7**, while *p*-dehydroxylation produced **6**, which similarly gave **8**.

Massive ring fission of **7** and **8** produced phenolic acids (**9-13**) which were mainly detected in all incubates after prolonged incubation of **2** with either HIB or RIB. Mono-methylation of an aromatic hydroxy group in **10** gave **13**. Condensation of **4** and **11** may also lead to the formation of **14**.

As regards the structural requirements for flavan-3-ol degradation, it is clear that the *p*-hydroxyl group (C4'-OH) is a must. On the other hand, studies with 3-*O*-methyl-(+)-catechin indicated that this substrate was resistant to ring fission by RIB.[7,8] This experiment suggested a possible contribution of C3-OH in the degradation of flavan-3-ols. Taking into account this conclusion and by comparing the degradation profiles for each pair, (**1** and **2**) and (**15** and **16**), after anaerobic incubation with HIB or RIB, we can conclude that methylation or acylation of the hydroxyl group (C3-OH) in **2** and **16** protects them from further degradation with RIB. This could be accounted for on the basis of species difference, which in tern reflects a difference in the types and numbers of potential bacteria in the intestinal flora of particular species.

The present findings indicate that **1** undergoes several metabolic alterations when incubated with HIB, which was not demonstrated with RIB. This difference was also seen in the *in vivo* experiments, as a substantial amount of **1** could be detected in rat plasma following its oral administration,[9] but when fed to humans, **1** was completely absent in plasma or urine samples.[10] Therefore, it is not surprising that the 3-*O*-gallates (**1** and **15**) could be absorbed unchanged through the intestinal wall of rats, thus demonstrating their antioxidant activity. However, the human consumption of green tea rich in these compounds may lead to metabolites of diverse structures. Against this background, the question is unanswered whether the remaining substrate (*i.e.* **1**) or, more likely, its metabolites are mainly responsible for antioxidant properties. This point will be a subject of particular interest to be discussed in the near future.

In order to clarify the transformation of **1** and **15** by intestinal bacteria, we tried to isolate bacterial strains capable of transforming **1** and **15** to a variety of metabolites from human feces and found that *Eubacterium* sp. strain SDG-2 was capable of ring fission followed by *p*-dehydroxylation of (+)-catechin, (+)-epicatechin, (-)-catechin, (-)-epicatechin (**2**), (-)-gallocatechin, and (-)-epigallocatechin (**16**). *i. e.,* After anaerobic incubation with *E.* sp. strain SDG-2, the fermentation broths with two (3*S*)-flavan-3-ols: (+)-catechin and (+)-epicatechin were separately extracted and then purified to afford the same product. It was identified as 1-(3, 4-dihydroxyphenyl)-3-(2, 4, 6-trihydroxyphenyl)propan-2β-ol. Four (3*R*)-flavan-3-ols: (-)-catechin, (-)-epicatechin (**2**), (-)-gallocatechin and (-)-epigallocatechin (**16**) (Chart 3), were transformed by *E.* sp. strain SDG-2. Compounds **5** was obtained from incubation of (-)-catechin and (-)-epicatechin (**2**) by ring fission, and then transformed to **6** by *p*-dehydroxylation.

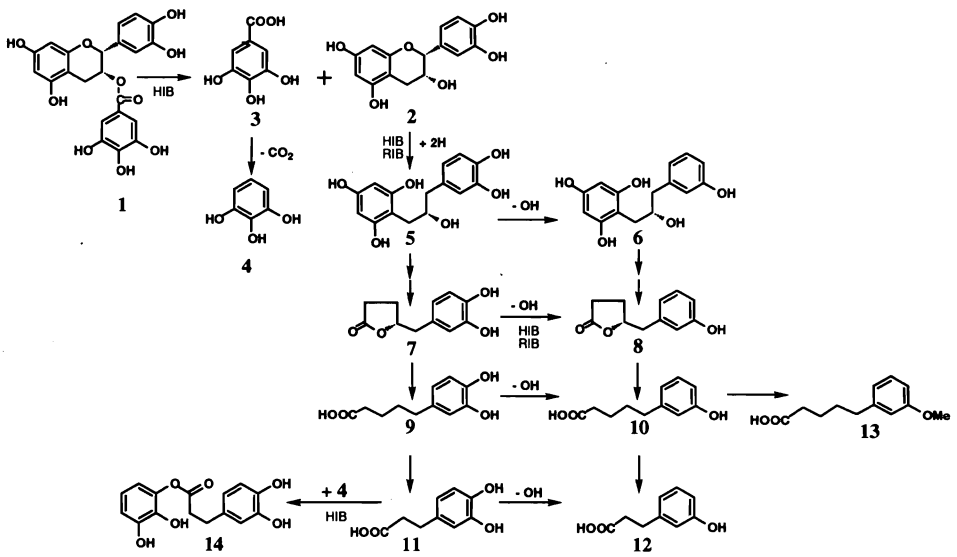

Chart 1 Metabolic process of epicatechin 3-*O*-gallate by human intestinal flora

The previous metabolic studies on catechins were undertaken with intestinal microorganisms from humans or animals. Schneider *et al.* have ever tried to transform (+)-catechin and (-)-epicatechin (**2**) using *Eubacterium ramulus*, a quercetin-3-glucoside-degrading anaerobic microorganism, unfortunately, they were not degraded. It is the first time, to the best of our knowledge, to report herein using a single bacterium isolated from human feces for the transformation of catechins and related compounds. As shown in Chart 3, *E.* sp. strain SDG-2 is capable of degrading catechins by ring fission and / or *p*-dehydroxylation. For (+)-catechin and (+)-epicatechin, they were only degraded by ring fission to **20**, which were not converted further. (-)-Catechin and (-)-epicatechin (**2**) were transformed to their degradation products by ring fission to **5**, which was subsequently followed by *p*-dehydroxylation in B-ring to give **6**. In the case of (-)-epigallocatechin (**16**) and (-)-gallocatechin, they were undergone either by ring fission or *p*-dehydroxylation to give **21**.

For most flavonoids including catechins, it seems that ring fission of heterocyclic ring is a very common reaction, catalyzed by intestinal bacteria. *E.* sp. strain SDG-2 was firstly isolated capable of dehydroxylating *p*-hydroxyl group of didemethylsecoisolariciresinol in the aromatic ring. It is very interesting that *E.* sp. strain SDG-2 is capable of cleaving the heterocyclic ring of catechins except for catechin gallates, such as (-)-catechin 3-*O*-gallate, (-)-epicatechin 3-*O*-gallate (**1**), (-)-gallocatechin 3-*O*-gallate, (-)-epigallocatechin 3-*O*-gallate (**15**). This finding suggested a possible contribution of a free C3-OH in the degradation by *E.* sp. strain SDG-2. In addition, as to the ring fission, the presence of 4'-OH in B-ring seems necessary since 4'-methyl-(-)-epigallocatechin was not transformed even after long time incubation with *E.* sp. strain SDG-2 for three days.

E. sp. strain SDG-2 showed activity of *p*-dehydroxylation in B-ring for (3*R*)-flavan-3-ols: (-)-catechin, (-)-epicatechin (**2**), (-)-epigallocatechin (**16**) and (-)-gallocatechin, while not for (3*S*)-flavan-3-ols: (+)-catechin and (+)-epicatechin. As regards the structural differences, (-)-catechin and (-)-epicatechin (**2**) 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 after ring fission, the chiral center will not exist. The *R* configuration at C-3 seems essential for *p*-dehydroxylation since (-)-catechin (3*R*) and (-)-epicatechin (**2**) (3*R*) were finally transformed into **6** by *p*-dehydroxylation, contrarily, (+)-catechin (3*S*) and (+)-epicatechin (3*S*) were only transformed to their ring fission products even after 4 days incubation. However, in the case of transformation by HIB, (+)-catechin was degraded either by ring fission or *p*-dehydroxylation, suggesting that other kinds of bacteria are responsible for the *p*-dehydroxylation of (+)-catechin. Similarly, *E.* sp. strain SDG-2 showed activity for (-)-gallocatechin and (-)-epigallocatechin by *p*-dehydroxylation, because of *R* configuration at C-3.

As indicated in our previous paper, the vicinal hydroxyl group in the aromatic rings was necessary for the *p*-dehydroxylation since (-)-secoisolariciresinol was not reacted with *E.* sp. strain SDG-2.8 To compounds (-)-epigallocatechin (**16**) and (-)-gallocatechin, the each two vicinal hydroxyls of 4'-OH in B-ring enhanced, to some extent, the removal of 4'-OH. The fact that it was not detected any intermediate(s) even picking samples at 1hr intervals led to assume that the reaction of dehydroxylation took place very quickly after ring fission. *E.* sp. strain SDG-2 transformed caffeic acid to dihydrocaffeic acid by hydrogenation, and then by *p*-dehydroxylation to give 3-(3-hydroxyphenyl)propionic acid. Similar study was also demonstrated by a strain of *Pseudomonas* sp. isolated from rat feces. In addition, 3-(4-hydroxyphenyl)propionic acid, lacking a meta-OH in comparison with dihydrocaffeic acid, was not degraded by *E.* sp. strain SDG-2, which also indicated the importance of a vicinal hydroxyl group. Furthermore, this bacterium cannot transform 3, 4-

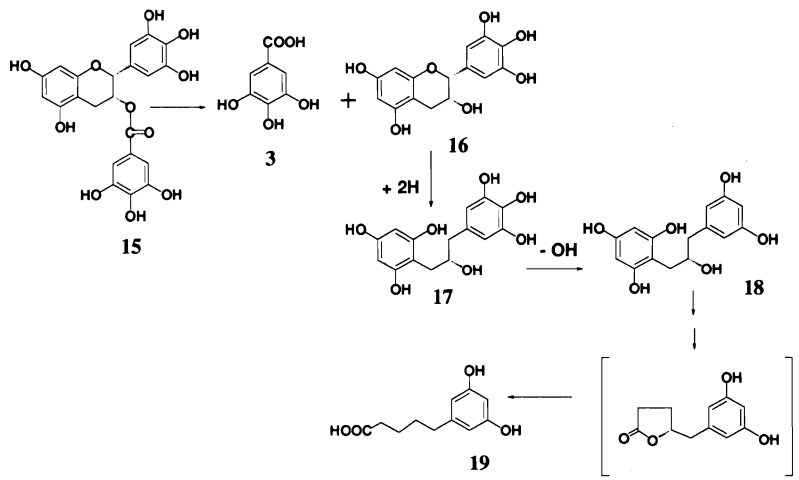

Chart 2 Metabolic process of epigallocatechin 3-*O*-gallate by human intestrinal flora

dihydroxyphenylacetic acid and gallic acid by dehydroxylation, indicated the necessity of at least three carbons in side chain for dehyroxylation.

As is known, catechins are easily transformed into various metabolites when incubated with human or rat intestinal bacteria mixture, and it is very difficult to get some metabolites only with ring fission or *p*-dehydroxylation. The present study made it possible to get metabolites through ring fission or *p*-dehydroxylation of catechins easily and with high yield. It is noteworthy that *E.* sp. strain SDG-2 can selectively dehydroxylate the *p*-hydroxyl in B-ring of catechins when 3-OH takes *R* configuration, which suggests the possible enzymatic action.

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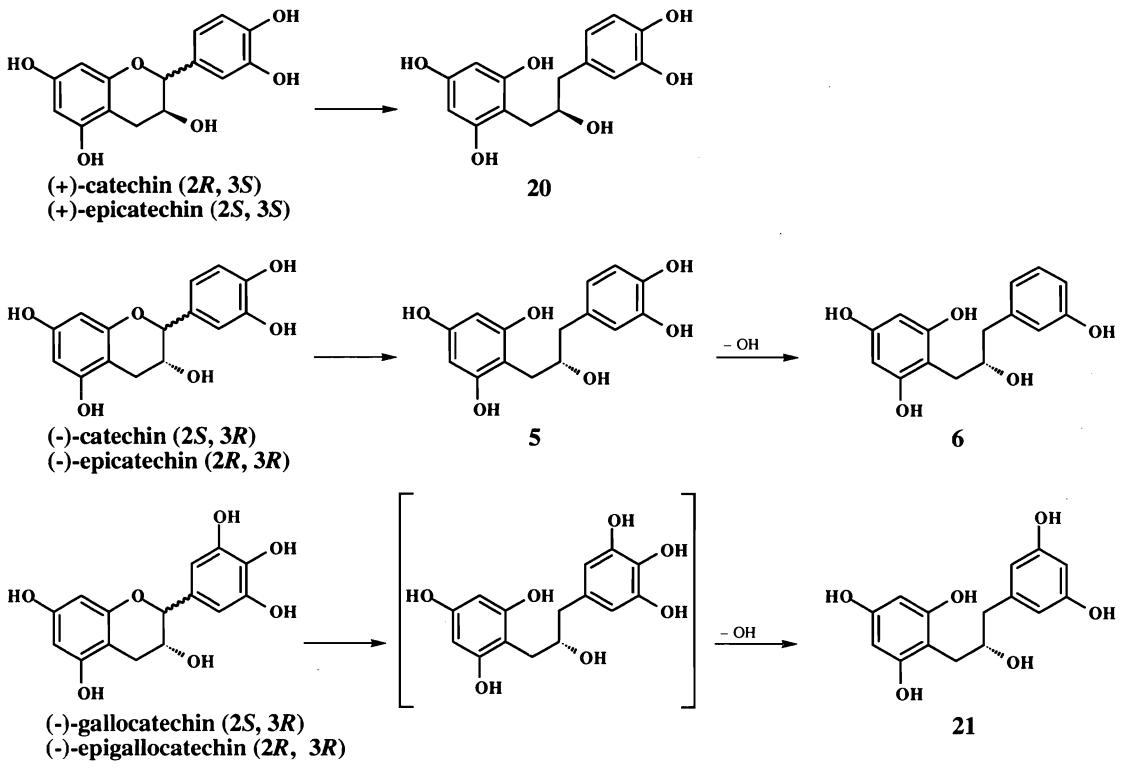

Chart 3 Transformation of catechins by *Eubacterium* sp. SDG-2