# Stereoselective Oxidation at C-4 of Flavans by the Endophytic Fungus *Diaporthe* sp. Isolated from a Tea Plant[1)]

Andria AGUSTA,[a] Shoji MAEHARA,[a] Kazuyoshi OHASHI,[a] Partomuan SIMANJUNTAK,[b] and Hirotaka SHIBUYA*,[a]

[a] *Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University; Sanzo, 1 Gakuen-cho, Fukuyama, Hiroshima 729–0292, Japan: and* [b] *Research Centre for Biotechnology, Indonesian Institute of Sciences; Jalan Raya Bogor Km 46, Cibinong, Bogor 16911, Indonesia.* Received July 25, 2005; accepted August 19, 2005

**The microbial transformation of five flavans (1—5) by endophytic fungi isolated from the tea plant *Camellia sinensis* was investigated. It was found that the endophytic filamentous fungus *Diaporthe* sp. oxidized stereoselectively at C-4 position of (+)-catechin (1) and (−)-epicatechin (2) to give the correspondent 3,4-*cis*-dihydroxyflavan derivatives (6, 10), respectively. (−)-Epicatechin 3-*O*-gallate (3) and (−)-epigallocatechin 3-*O*-gallate (4) were also oxidized by the fungus into 3,4-dihydroxyflavan derivatives (10, 12) *via* (−)-epicatechin (2) and (−)-epigallocatechin (11), respectively. Meanwhile, (−)-gallocatechin 3-*O*-gallate (5), (−)-catechin (ent-1) and (+)-epicatechin (ent-2), which possess a 2*S*-phenyl substitution, resisted the biotransformation.**

**Key words** microbial transformation; flavan; endophyte; *Diaporthe* sp.; tea plant

Endophytic microbes living inside plants[2)] may have a capacity to transform the chemical constituents of the plants to yield their chemical derivatives. Flavans, [*e.g.* (+)-catechin (**1**), (−)-epicatechin (**2**), (−)-epicatechin 3-*O*-gallate (**3**), (−)-epigallocatechin 3-*O*-gallate (**4**) and (−)-gallocatechin 3-*O*-gallate (**5**)], have been isolated from tea leaves[3)] and are well known as free-radical scavengers.[4)] In order to shed light on the availability of endophytic microbes, we attempted the microbial transformation of five typical flavans (**1**—**5**, shown in Fig. 1) by endophytic fungi isolated from the tea plant *Camellia sinensis* (L.) O.K. (Theaceae), which is parasitized with *Scurrula atropurpurea* (BL.) DANS. (Loranthaceae).[5,6)]

A total of 35 filamentous endophytic fungi was obtained from the young stems of *Camellia sinensis* collected in the Puncak area, West Java, Indonesia, through the same procedure as described in our previous paper.[7)] The fungi obtained were classified into 6 species by morphological considerations and RAPD analyses.

By screening tests in several liquid cultivation mediums, it was found that one filamentous fungus among them transformed (+)-catechin (**1**) into the chemical derivative.[1)] The fungus was inoculated in glucose–yeast extract–peptone medium (glucose 20 g, yeast extract 1.0 g, peptone 5.0 g, $K_2HPO_4$ 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $CaCO_3$ 1.0 g and tap water 1 l, at pH 6.44) and then cultivated for 5 d under shaking at 90 rpm at 27 °C. A MeOH solution of (+)-catechin (**1**) was then added to the medium and shaken for one more day. The whole medium was extracted with EtOAc and evaporated to give a product. The product was purified by $SiO_2$ column chromatography (eluted with $CHCl_3 : MeOH : H_2O = 65 : 35 : 10$ lower phase) and subsequent reverse-phase HPLC (with $H_2O$) to afford a biotransformed product (**6**, 45%) in addition to the recovered (+)-catechin (**1**, 8.5%).

The IR spectrum of the product (**6**), $C_{15}H_{14}O_7$, $[\alpha]_D$ +5.5° (EtOH), showed absorption bands due to hydroxyl and aromatic groups. The $^1$H-NMR spectrum led us to belive that **6** was the known substance (+)-2,3-*trans*-3,4-*cis*-3,4,5,7,3′,4′-hexahydroxyflavan (leucocyanidin). However, since the physicochemical data in the literature[8,9)] was insufficient for comparison with **6**, we attempted the following chemical derivatization according to a previously reported procedure,[10)] as shown in Fig. 2.

(+)-(2*R*,3*R*)-3,5,7,3′,4′-Pentahydroxyflavanone (taxifolin, **7**) was converted by $CH_2N_2$ methylation followed by $NaBH_4$ reduction into two 3,4-dihydroxyflavan derivatives (**8**, **9**), whose stereostructures were clarified by the NOE observation between 2$\beta$-H and 4$\beta$-H in **9**. The methylated product of **6** was identical with **8**, including the optical rotation. From the above findings, the biotransformed product (**6**) was determined as (+)-(2*R*,3*S*,4*S*)-3,4,5,7,3′,4′-hexahydroxyflavan.

Next, we conducted rDNA analysis for the 18S, ITS1, 5.8S and ITS2 regions of the fungus that exhibited biotransformation activity against (+)-catechin (**1**). After consideration of the phylogenetic relationships for the ITS1, 5.8S, and ITS2 rDNA regions using the databases,[11)] we directly compared the rDNA base sequences of the endophytic fungus and *Diaporthe phaseolorum* var. *sojae* (IFO 6709). The results

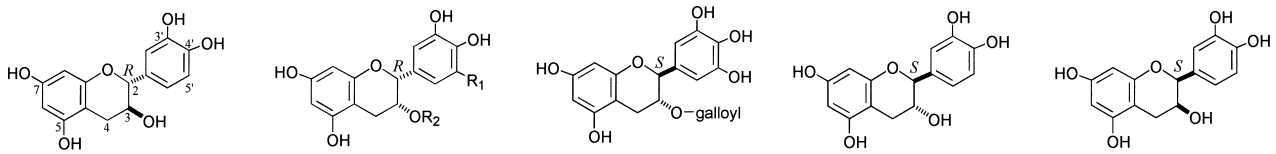


Fig. 1

* To whom correspondence should be addressed. e-mail: shibuya@fupharm.fukuyama-u.ac.jp

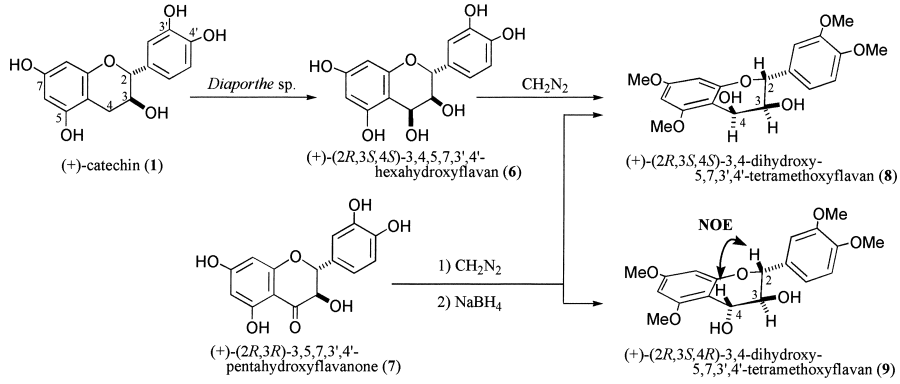


Fig. 2

Table 1. Comparison 18S-ITS1-5.8S-ITS2 rDNA Sequence of the Endophytic Fungus with *Diaporthe phaseolorum* var. *sojae*

| | 18S rDNA (bp) | Similarity (%) | ITS1 (bp) | Similarity (%) | 5.8S rDNA (bp) | Similarity (%) | ITS2 (bp) | Similarity (%) | Total similarity (%) |
|---|---|---|---|---|---|---|---|---|---|
| The endophytic fungus | 1734 | | 177 | | 153 | | 156 | | |
| *Diaporthe phaseolorum* | 1736 | 99.5 | 179 | 93.2 | 153 | 100.0 | 158 | 90.4 | 98.3 |
| Insert | 2 | | 2 | | 0 | | 2 | | 6 |
| Difference | 4 | | 7 | | 0 | | 7 | | 18 |
| Gap | 0 | | 0 | | 0 | | 0 | | 0 |

showed that the similarity for the 18S region is 99.5% with *D. phaseolorum*, 93.8% for the ITS1, 100% for the 5.8S, and 93.0% for the ITS2, shown in Table 1. These findings indicated that the endophytic fungus may be a species closely related to *Diaporthe phaseolorum* var. *sojae*.

The transformation of (−)-epicatechin (**2**) by the abovementioned endophytic fungus *Diaporthe* sp. gave a product (**10**, 39%) together with unchanged **2** (2.4%). The IR and UV spectra of **10**, $C_{15}H_{14}O_7$, $[\alpha]_D$ −8.4° (EtOH), showed similar absorptions to those of **6** transformed from (+)-catechin (**1**). The $^1$H- and $^{13}$C-NMR spectra both showed a characteristic three consecutive methine-proton system ($\delta$ 5.00, br s, 2-H; $\delta$ 3.88, dd, $J$=1.2, 2.2 Hz, 3-H; $\delta$ 4.75, d, $J$=2.2 Hz, 4-H). Furthermore, the NOE was observed between 2$\beta$-H and 4$\beta$-H in the $^1$H-NMR spectrum. From these findings, the chemical structure of **10** was elucidated as a new compound, (−)-(2*R*,3*R*,4*R*)-3,4,5,7,3′,4′-hexahydroxyflavan.

(−)-Epicatechin 3-*O*-gallate (**3**) was also biotransformed into the same product **10** (53%) *via* (−)-epicatechin (**2**, 13%), which was obtained as another product.

The biotransformation of (−)-epigallocatechin 3-*O*-gallate (**4**), a major chemical constituent of tea leaves, by the fungus gave a product (**12**, 43%) together with (−)-epigallocatechin (**11**, 19%). The IR and UV spectra of **12**, $C_{15}H_{14}O_8$, $[\alpha]_D$ −16.9°, showed similar absorption patterns to those of **10**, the product from (−)-epicatechin (**2**) and (−)-epicatechin 3-*O*-gallate (**3**). The $^1$H- and $^{13}$C-NMR spectra of **12** also showed similar signal patterns to those of **10** except for a signal pattern due to a phenyl moiety; $\delta$ 6.61 (2H, s, 2′-, 6′-H) in $^1$H-NMR, $\delta_C$ 131.1 (1′-C), 106.9 (2′-, 6′-C), 146.2 (3′-, 5′-C), 132.9 (4′-C) in the $^{13}$C-NMR. Furthermore, the NOE was observed between 2$\beta$-H and 4$\beta$-H. These findings prove that the chemical structure of **12** is a new compound, (−)-(2*R*,3*R*,4*R*)-3,4,5,7,3′,4′,5′-heptahydroxyflavan (leucodelphinidin) (Fig. 3).

In contrast, exposure of the endophytic fungus to (−)-gallocatechin 3-*O*-gallate (**5**) did not yield a product oxidized at C-4, but only afforded hydrolyzed (−)-gallocatechin (**13**, 75%). Since the resistance to biooxidation at the C-4 position was thought to be due to a 2*S*-configuration function, we next examined the biotransformation of (−)-catechin (ent-**1**) and (+)-epicatechin (ent-**2**), which also have a 2*S*-phenyl substitution. As expected, ent-**1** and -**2** strongly resisted the biooxidation (Fig. 4).

In conclusion, the endophytic fungus *Diaporthe* sp. isolated from the tea plant *Camellia sinensis*, stereoselectively oxidizes the C-4 carbon of flavans possessing a 2*R*-substitution from the same direction to the configuration of 3-hydroxyl function. This evidence agrees with the hypothesis that endophytic microbes exhibit the ability to transform the chemical constituents of the plants.

Finally, to determine the factors for this microbial oxidation, the following cultivations were examined. When the cultivation reaction was carried out under an environment of nitrogen gas, the oxidation did not proceed, but restarted to afford the oxidized products (**6**, **10**, **12**) after the rebubbling of oxygen gas. The filtrate of the fungus cultivation medium did not transform the flavans (**1**—**4**). These findings show that the biooxidation occurs in an endoenzyme manner using molecular oxygen.

**Experimental**

Optical rotations were measured using a JASCO DIP-360 digital polarimeter and a cell length of 50 mm. FAB-MS and high resolution FAB-MS were taken on a JMS SX-102 A mass spectrometer. IR spectra were run on a Shimadzu FT-IR 8500 spectrophotometer and UV spectra on a Hitachi U-3500 spectrometer. $^1$H- and $^{13}$C-NMR spectra were recorded on a JEOL JNM-Lambda 500 spectrometer operating at 500 MHz and 125 MHz for $^1$H and $^{13}$C nuclei, respectively. Chemical shifts are given in $\delta$ scale (ppm) relative to tetramethylsilane ($\delta$=0) as an internal standard, and coupling constants are given in hertz. PCR amplification was carried out with an ABI GeneAmp PCR System 9700 amplifier. DNA sequencing was performed

Table 2. Primers Used for PCR and rDNA Sequencing

| Primer | Sequence (5′–3′) | Application | Location |
|---|---|---|---|
| 18STscFw | TAA GCC ATG CAA GTC TAA GT | 18S amplification<br>18S sequencing | 18S rDNA |
| 18SFt1 | ACG GGT AAC GGA GGG TTA GG | 18S sequencing | 18S rDNA |
| 18SFt2 | CTG GCT GGC CGG TCT GCC TC | 18S sequencing | 18S rDNA |
| 18SFt3 | GCA TTC GCC AAG GAT GTT TT | 18S sequencing | 18S rDNA |
| 18SFt5 | ATC CCT AGT AAG CGC AAG TC | 18S sequencing | 18S rDNA |
| 18SFt6 | TCC AAA CTC GAT CAT TTA GAG | 18S sequencing | 18S rDNA |
| 18SFt7 | CAG ACA CAA CTA GGA TTG AVA G | 18S sequencing | 18S rDNA |
| 18SFt8 | GGA ATC CCT AGT AAG CGC AAG T | 18S sequencing | 18S rDNA |
| 18SRt2 | AGA ACA TCT AAG GGC ATC AC | 18S sequencing | 18S rDNA |
| 18SRt4 | TCC CTC CGC TGG TTC ACC AAC GG | 18S sequencing | 18S rDNA |
| 18SRt5 | CAA TTG TTC CTC GTT AGG GG | 18S sequencing | 18S rDNA |
| 18SRt6 | CAT CGC CGG CAC AAG GCC AT | 18S sequencing | 18S rDNA |
| 18SRt7 | CCT TGG ATG TAG TAG CCG TTT C | 18S sequencing | 18S rDNA |
| 18SRt8 | ACG GGC TAT TTA GCA GGT TA | 18S sequencing | 18S rDNA |
| 18STscRev | TCC CTC CGC TGG TTC ACC AAC GG | 18S amplification<br>18S sequencing | 18S rDNA |
| FG18SF10 | TCT GTG ATG CCC TTA GAT GTT TG | ITS1-5.8S-ITS2 amplification<br>ITS1-5.8S-ITS2 sequencing | 18S rDNA |
| 5.8SFt1 | CAC GGT AGT GGT TCG GTC CG | ITS1-5.8S-ITS2 sequencing | 5.8S rDNA |
| 5.8SRt1 | GCC TGG CTT GGT GAT GGC AC | ITS1-5.8S-ITS2 sequencing | 5.8S rDNA |
| ITS-TscRev | CAC ACA GGC TTG GTG TCC GAC C | ITS1-5.8S-ITS2 sequencing<br>ITS1-5.8S-ITS2 sequencing | 28S rDNA |

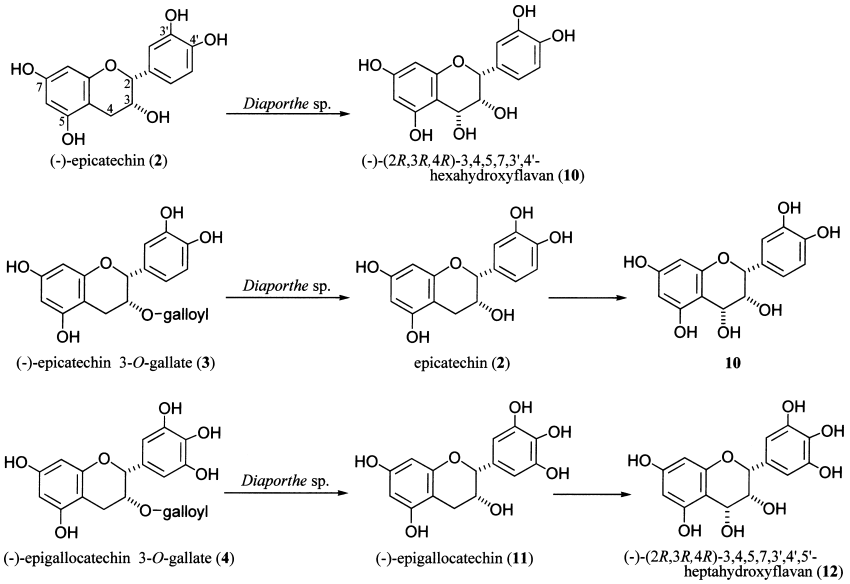


Fig. 3

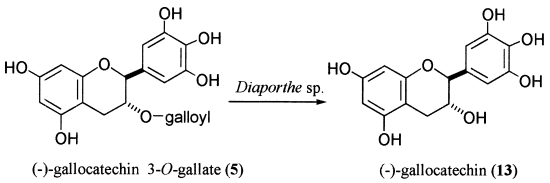


Fig. 4

with an ABI PRISM 3100 Genetic Analyzer. HPLC was carried out with a TOSOH PD-8020. Column chromatography was carried out using Kieselgel 60 (230—400 mesh, Merck). TLC on Kieselgel 60 $F_{254}$ (Merck) was used to ascertain the purity of the compounds. The spots were visualized by spraying with 5% vanillin in concentrated $H_2SO_4$. All microbial transformations were carried out in glucose–yeast extract–peptone medium containing 20 g of glucose, 1.0 g of yeast extract, 5.0 g of peptone, 0.5 g of $K_2HPO_4$, 0.5 g of $MgSO_4$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 1.0 g of $CaCO_3$ and 1 l of tap-water, at pH 6.44.

DNA extraction, PCR amplification, DNA sequencing, and phylogenetic analysis were carried out using the primers shown in Table 2, through the same procedures as described in our previous paper.[7)]

**Plant Material** The young stems of the tea plant *Camellia sinensis* (L.) O.K. (Theaceae) were collected in the Puncak area, West Java, Indonesia, in August 2003 and identified at the Herbarium Bogoriense, the Research Centre for Biology, Indonesian Institute of Sciences, Indonesia.

**Isolation of the Endophytic Fungi from the Tea Plant** The young stems were cut into pieces (*ca.* 1 cm in length) and washed with tap water for 10 min. The pieces were treated with 70% aq. EtOH for 1 min, 5.3% aq. sodium hypochlorite for 5 min, and 75% aq. EtOH for 0.5 min, and then cut into two pieces. The cut stems were placed on corn malt meal agar containing chloramphenicol (0.05 mg/ml) in a Petri dish and incubated at 27 °C. After 3 d, individual colonies were transferred onto PDA in a Petri dish and then incubated again at 27 °C for several days with periodic checks for the purity to obtain the 35 endophytic filamentous fungi and 9 yeasts.

**RAPD Analysis of the Endophytic Fungi** Random amplified polymorphic DNA (RAPD) analyses of the extracted DNA from 35 endophytic filamentous fungi were performed in a mixture of 50 ng of the DNA template, 5 $\mu$l of a primer [either primer-1 (5′-GTAGACCCGT-3′) or primer-2 (5′-CCCGTCAGCA-3′)], Ready-To-Go RAPD Analysis Beads (Amersham Biosciences Corp.) and sterile distilled water to a final volume of 25 $\mu$l. The whole mixture was subjected to thermal cycling programmed for 5 min at 95 °C followed by 45 cycles of 1 min at 95 °C, 1 min at 36 °C, and 2 min at 72 °C. The RAPD products were then loaded on 2.3% agarose gel at 150 volts and 140 mA for 240 min and then stained in ethidium bromide for 20 min. The electrophoresis patterns of the amplified products were visualized under UV light.

**Microbial Transformation of (+)-Catechin (1) by the Endophytic Fungus *Diaporthe* sp.** The endophytic fungus *Diaporthe* sp. was inoculated into the glucose–yeast extract–peptone medium (200 ml) and cultivated for 5 d under shaking at 90 rpm at 27 °C. A solution of (+)-catechin (**1**, 20 mg) in MeOH (20 ml) was added to the cultivation medium and shaking continued for 1 d. The reaction mixture was filtrated to remove the fungus bodies. The filtrate was extracted with EtOAc and concentrated under reduced pressure to give a product (45 mg), which was purified by silica gel column chromatography ($SiO_2$ 10 g, $CHCl_3$ : MeOH : $H_2O$=65 : 35 : 10 lower phase) and HPLC (Capcell Pak Phenyl SG-120, $H_2O$) to afford (+)-(2*R*,3*S*,4*S*)-3,4,5,7,3′,4′-hexahydroxyflavan (**6**, 9.5 mg, 45%)[8,9] and the recovered (+)-catechin (**1**, 1.7 mg, 8.5%).

The physicochemical properties of (+)-(2*R*,3*S*,4*S*)-3,4,5,7,3′,4′-hexahydroxyflavan (**6**) are given here, since Kristiansen[8,9] reported only $^1$H-NMR and UV spectra for **6**.

(+)-(2*R*,3*S*,4*S*)-3,4,5,7,3′,4′-Hexahydroxyflavan (**6**): A white powder, $[\alpha]_D$ +5.5° (*c*=0.55, in EtOH at 25 °C). IR (KBr) cm$^{-1}$: 3300, 1612. UV (EtOH) nm ($\varepsilon$): 280 (4,000). $^1$H-NMR (acetone-$d_6$: 3.85 (1H, dd, *J*=3.7, 9.5 Hz, 3-H), 4.84 (1H, d, *J*=9.5 Hz, 2-H), 4.87 (1H, d, *J*=3.7 Hz, 4-H), 5.65 (1H, d, *J*=2.1 Hz, 8-H), 6.02 (1H, d, *J*=2.1 Hz, 6-H), 6.80 (1H, dd, *J*=2.2, 7.9 Hz, 6′-H), 6.81 (1H, d, *J*=7.9 Hz, 5′-H), 6.94 (1H, d, *J*=2.2 Hz, 2′-H). $^{13}$C-NMR (acetone-$d_6$) $\delta$: 62.8 (4-C), 71.4 (3-C), 77.7 (2-C), 95.2 (8-C), 96.4 (6-C), 103.9 (4a-C), 115.7 (2′-C, 5′-C), 120.5 (6′-C), 131.8 (1′-C), 145.6 (4′-C), 145.8 (3′-C), 157.1 (8a-C), 158.9 (5-C), 159.8 (7-C). FAB-MS *m/z*: 307 $[M+H]^+$. High-resolution FAB-MS *m/z*: Calcd for $C_{15}H_{15}O_7$: 307.0818 $[M+H]^+$. Found 307.0810.

**Chemical Transformation of (+)-(2*R*,3*S*)-3,5,7,3′,4′-Pentahydroxyflavanone (7) into 8 and 9** A solution of (+)-(2*R*,3*S*)-3,5,7,3′,4′-pentahydroxyflavanone (**7**, 80 mg, Wako Pure Chemical Industries Ltd.) in MeOH (5.0 ml) was treated with ethereal diazomethane for 8 h at 0 °C. The reaction mixture was worked up in a usual manner to give a product (105 mg), which was purified by silica gel column chromatography ($SiO_2$ 15 g, benzene : acetone=8 : 1) to afford (+)-3-hydroxy-5,7,3′,4′-tetramethoxyflavanone (44 mg, 46%). $NaBH_4$ (3.1 mg) was added to a solution of (+)-3-hydroxy-5,7,3′,4′-tetramethoxyflavanone (10 mg) in MeOH (3.0 ml) at −20 °C and the mixture was stirred for 1.5 h at −20 °C. The reaction mixture was poured into $H_2O$ and extracted with $CHCl_3$. The $CHCl_3$ extract was washed with brine and dried over $Na_2SO_4$. Removal of the solvent gave a product (12 mg), which was separated by silica gel column chromatography ($SiO_2$ 1 g, benzene : acetone=5 : 1) to afford (+)-(2*R*,3*S*,4*S*)-3,4-dihydroxy-5,7,3′,4′-tetramethoxyflavan[10] (**8**, 2.9 mg, 29%) and (+)-(2*R*,3*S*,4*R*)-3,4-dihydroxy-5,7,3′,4′-tetramethoxyflavan[10] (**9**, 7.0 mg, 68%).

**Methylation of (+)-(2*R*,3*S*,4*S*)-3,4,5,7,3′,4′-Hexahydroxyflavan (6)** (+)-(2*R*,3*S*,4*S*)-3,4,5,7,3′,4′-Hexahydroxyflavan (**6**, 24 mg) was dissolved in MeOH (1.0 ml) and treated with ethereal diazomethane for 8 h at −20 °C. The reaction mixture was worked up in a usual manner to give a product (35 mg), which was purified by silica gel column chromatography ($SiO_2$ 10 g, benzene : acetone=5 : 1) to afford (2*R*,3*S*,4*S*)-3,4-dihydroxy-5,7,3′,4′-tetramethoxyflavan (**8**, 12 mg, 42%), which was identified with **8** prepared from **7**.

**Microbial Transformation of (−)-Epicatechin (2) by the Endophytic Fungus *Diaporthe* sp.** After cultivation of the endophytic fungus *Diaporthe* sp. in glucose–yeast extract–peptone medium (200 ml) for 5 d under shaking at 90 rpm at 27 °C, a solution of (−)-epicatechin (**2**, 20 mg) in MeOH (20 ml) was added to the cultivation medium and shaken for one more day. The reaction mixture was filtrated and the filtrate was extracted with EtOAc. The EtOAc layer was concentrated under reduced pressure to give a product (48 mg), which was purified by silica gel column chromatography ($SiO_2$ 10 g, $CHCl_3$ : MeOH : $H_2O$=65 : 35 : 10 lower phase) and HPLC (Capcell Pak Phenyl SG-120, $H_2O$) to afford **10** (8.2 mg, 39%) and the recovered (−)-epicatechin (**2**, 0.48 mg, 2.4%).

(−)-(2*R*,3*R*,4*R*)-3,4,5,7,3′,4′-Hexahydroxyflavan (**10**): A white powder, $[\alpha]_D$ −8.4° (*c*=0.77, in EtOH at 26 °C). IR (KBr) cm$^{-1}$: 3250, 1628. UV (EtOH) nm ($\varepsilon$): 279 (4200). $^1$H-NMR (acetone-$d_6$) $\delta$: 3.88 (1H, dd, *J*=1.2, 2.2 Hz, 3-H), 4.75 (1H, d, *J*=2.2 Hz, 4-H), 5.00 (1H, br s, 2-H), 5.92 (1H, d, *J*=2.1 Hz, 8-H), 6.03 (1H, d, *J*=2.1 Hz, 6-H), 6.80 (1H, d, *J*=7.9 Hz, 5′-H), 6.85 (1H, dd, *J*=1.8, 7.9 Hz, 6′-H), 7.08 (1H, d, *J*=1.8 Hz, 2′-H). $^{13}$C-NMR (acetone-$d_6$) $\delta$: 64.6 (4-C), 72.3 (3-C), 75.8 (2-C), 95.4 (8-C), 96.3 (6-C), 103.6 (4a-C), 115.5, 115.6 (2′-C, 5′-C), 119.4 (6′-C), 131.9 (1′-C), 145.4, 145.5 (3′-C, 4′-C), 157.6 (8a-C), 159.2 (5-C), 159.3 (7-C). FAB-MS *m/z*: 307 $[M+H]^+$. High-resolution FAB-MS *m/z*: Calcd for $C_{15}H_{15}O_7$: 307.0818 $[M+H]^+$. Found 307.0819.

**Microbial Transformation of (−)-Epicatechin 3-*O*-Gallate (3) by the Endophytic Fungus *Diaporthe* sp.** The endophytic fungus *Diaporthe* sp. was inoculated into glucose–yeast extract–peptone medium (200 ml) and shaken at 90 rpm at 27 °C for 5 d. A solution of (−)-epicatechin 3-*O*-gallate (**3**, 20 mg) in MeOH (20 ml) was added to the medium and shaking continued for 1 d. After filtration to remove the fungus bodies, the filtrate was extracted with EtOAc. The EtOAc extract was concentrated under reduced pressure to give a product (51 mg), which was purified by silica gel column chromatography ($SiO_2$ 10 g, $CHCl_3$ : MeOH : $H_2O$=65 : 35 : 10 lower phase) followed by HPLC (Capcell Pak Phenyl SG-120, $H_2O$) to afford (−)-(2*R*,3*R*,4*R*)-3,4,5,7,3′,4′-hexahydroxyflavan (**10**, 7.2 mg, 53%) and (−)-epicatechin (**2**, 1.7 mg, 13%).

**Microbial Transformation of (−)-Epigallocatechin 3-*O*-Gallate (4) by the Endophytic Fungus *Diaporthe* sp.** The endophytic fungus *Diaporthe* sp. was inoculated into glucose–yeast extract–peptone medium (200 ml) and cultivated for 5 d under shaking at 90 rpm at 27 °C. A solution of (−)-epigallocatechin 3-*O*-gallate (**4**, 20 mg) in MeOH (20 ml) was added to the medium and then shaken for one more day. The reaction mixture was filtrated and the filtrate was extracted with EtOAc. The EtOAc layer was concentrated under reduced pressure to give a product (48 mg), which was purified by silica gel column chromatography ($SiO_2$ 10 g, $CHCl_3$ : MeOH : $H_2O$= 65 : 35 : 10 lower phase) and HPLC (Capcell Pak Phenyl SG-120, $H_2O$) to afford (−)-(2*R*,3*R*,4*R*)-3,4,5,7,3′,4′,5′-heptahydroxyflavan (**12**, 6.2 mg, 43%) and (−)-epigallocatechin (**11**, 2.6 mg, 19%).

(−)-(2*R*,3*R*,4*R*)-3,4,5,7,3′,4′,5′-Heptahydroxyflavan (**12**): A white powder, $[\alpha]_D$ −16.9° (*c*=0.69, in EtOH at 24 °C). IR (KBr) cm$^{-1}$: 3300, 1628. UV (EtOH) nm ($\varepsilon$): 279 (4200). $^1$H-NMR (acetone-$d_6$) $\delta$: 3.90 (1H, dd, *J*=1.2, 3.1 Hz, 3-H), 4.76 (1H, d, *J*=3.1 Hz, 4-H), 4.95 (1H, br s, 2-H), 5.93 (1H, d, *J*=2.4 Hz, 8-H), 6.03 (1H, d, *J*=2.4 Hz, 6-H), 6.61 (2H, s, 2′-, 6′-H). $^{13}$C-NMR (acetone-$d_6$) $\delta$: 64.5 (4-C), 72.3 (3-C), 75.7 (2-C), 95.4 (8-C), 96.3 (6-C), 103.6 (4a-C), 106.9 (2′-C, 6′-C), 131.1 (1′-C), 132.9 (4′-C), 146.2 (3′-C, 5′-C), 157.5 (8a-C), 159.2 (5-C, 7-C). FAB-MS *m/z*: 323 $[M+H]^+$. High-resolution FAB-MS *m/z*: Calcd for $C_{15}H_{15}O_8$: 323.0767 $[M+H]^+$. Found 323.0737.

**Microbial Transformation of (−)-Gallocatechin 3-*O*-Gallate (5) by the Endophytic Fungus *Diaporthe* sp.** The endophytic fungus *Diaporthe* sp. was inoculated into glucose–yeast extract–peptone medium (200 ml). After cultivation for 5 d under shaking at 90 rpm at 27 °C, a solution of (−)-gallocatechin 3-*O*-gallate (**5**, 20 mg) in MeOH (20 ml) was added to the medium and shaking continued for 1 d. The reaction mixture was filtrated and the filtrate was extracted with EtOAc. The EtOAc layer was evaporated *in vacuo* to give a product (60 mg), which was purified by silica gel column chromatography ($SiO_2$ 10 g, $CHCl_3$ : MeOH : $H_2O$=65 : 35 : 10 lower phase) and HPLC (Capcell Pak Phenyl SG-120, $H_2O$) to give (−)-gallocatechin (**13**, 10.1 mg, 75%).

**Treatment of (−)-Catechin (ent-1) and (+)-Epicatechin (ent-2) by the Endophytic Fungus *Diaporthe* sp.** After cultivation of the endophytic fungus *Diaporthe* sp. in glucose–yeast extract–peptone medium (200 ml) for 5 d under shaking at 90 rpm at 27 °C, each solution of (−)-catechin (ent-**1**, 20 mg) and (+)-epicatechin (ent-**2**, 20 mg) in MeOH (20 ml) was individually added to the medium and shaking continued for 1 d. After filtration of each reaction mixture, the filtrates were extracted with EtOAc and concentrated under reduced pressure to give products (43 mg from ent-**1**, 48 mg from ent-**2**), which were purified by silica gel column chromatography ($SiO_2$ 10 g, $CHCl_3$ : MeOH : $H_2O$=65 : 35 : 10 lower phase) to recover (−)-catechin (ent-**1**, 16 mg, 80%) and (+)-epicatechin (ent-**2**, 17 mg, 85%), respectively.

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**References and Notes**


1) The preliminary report: Shibuya H., Agusta A., Ohashi K., Maehara S., Simanjuntak P., *Chem. Pharm. Bull.*, **53**, 866—867 (2005).
2) Bacon C. W., White J. F., Jr., "Microbial Endophytes," Marcel Dekker, New York, 2000.
3) Nonaka G., Kawahara O., Nishioka I., *Chem. Pharm. Bull.*, **31**, 3906—3014 (1983).
4) Nanjo F., *New Food Industry*, **40**, 61—67 (1998).
5) Ohashi K., Winarno H., Mukai M., Inoue M., Prana M. S., Simanjuntak P., Shibuya H., *Chem. Pharm. Bull.*, **51**, 343—345 (2003).
6) Ohashi K., Winarno H., Mukai M., Shibuya H., *Chem. Pharm. Bull.*, **51**, 463—466 (2003).
7) Shibuya H., Kitamura C., Maehara S., Nagahata M., Winarno H., Simanjuntak P., Kim H. S., Wataya Y., Ohashi K., *Chem. Pharm. Bull.*, **51**, 71—74 (2003).
8) Kristiansen K. N., *Carlsberg Res. Commun.*, **49**, 503—524 (1984).
9) Kristiansen K. N., *Carlsberg Res. Commun.*, **51**, 51—60 (1986).
10) Takahashi H., Li S., Harigaya Y., Onda M., *J. Nat. Prod.*, **51**, 730—735 (1988).
11) The international nucleotide sequence databases of EBI/EMBL, NIG/DDBJ, and NCBI/GenBank were used for the phylogenetic analysis.