The *C*-Glucosyl Bond of Puerarin Was Cleaved Hydrolytically by a Human Intestinal Bacterium Strain PUE to Yield Its Aglycone Daidzein and an Intact Glucose

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***C*-Glycosides are usually resistant against acidic hydrolysis and enzymatic treatments because C-1 of the sugar moiety is directly attached to the aglycone by C–C bonding. Nevertheless, a human intestinal bacterium, strain PUE, can cleave the *C*-glucosyl bond of puerarin to yield its aglycone daidzein.[1)] To clarify the mechanism of the cleaving reaction, we tried to identify the structure of the metabolite derived from the sugar moiety of puerarin. To detect it easily, deuterium labeled puerarin, [6″,6″-D_2]puerarin, was prepared in 7 steps. Sugars contained in a metabolite mixture from [6″,6″-D_2]puerarin was analyzed by an HPLC-electrospray ionization (ESI)-MS method after treatment of sugars with 1-phenyl-3-methyl-5-pyrazolone (PMP). Since deuterium labeled glucose was detected in the metabolite mixture of [6″,6″-D_2]puerarin, we concluded that puerarin was metabolized to daidzein and an intact glucose by strain PUE. As C-1 of the sugar was hydroxylated instead of hydrogenating, *C*-glucosyl bond-cleaving reaction is not reduction but hydrolysis. This is the first report of revealing the reaction manner and the exact products of *C*-glucosyl bond-cleaving reaction.**

Key words *C*-glucoside; puerarin; human intestinal bacterium

Many types of glycosides are widely distributed as major constituents in medicinal plants. However, due to their hydrophilic properties, they are poorly absorbed from gastro intestinal tract into body fluid, but their aglycones formed by intestinal bacterial hydrolysis are readily absorbed. Therefore, intestinal bacteria play an important role in increasing absorption and efficacy of drugs including glycosides. In nature, there is a special type of glycoside, *C*-glycosides, such as those of flavone, isoflavone, chromone, xanthone, anthrone, and gallic acid. Since C-1 of the sugar ring is directly connected to the aglycone by C–C bonding, *C*-glycosides are usually resistant against acidic and enzymatic hydrolysis in contrast with the corresponding *O*-glycosides. Thus, with the prospect of improving the stability, various *C*-glycoside analogues mimicking bioactive but labile *O*-glycosides have been prepared for developing the new drugs.[2)] In addition, a lot of publications describing in detail about *O*-glycosidase appeared, but that of *C*-glycosidase did not.

On the other aspects, in the last two decades, *C*-glucosyl bond-cleaving reactions by human intestinal bacteria were reported.[1,3—11)] Despite of the growing importance of *C*-glycosides in the field of pharmaceuticals, characteristic features of *C*-glucosyl bond-cleaving enzymes involved in its metabolism were still remain to be unclear.

Recently, we isolated a new bacterial species named strain PUE that transforms a *C*-glucoside puerarin to its aglycone daidzein.[1)] The aglycone moiety is normally detected easily because of UV active, however, the liberated sugar segment from *C*-glucoside has not been yet identified. The structural elucidation of the liberated sugar moiety could yield a valuable clue in revealing the reaction mechanism (Fig. 1). In the present paper, we describe the structural elucidation of the sugar moiety after cleavage of a *C*-glucosyl bond of puerarin by anaerobic incubation with a human intestinal bacterium, strain PUE, and a cell-free extract.

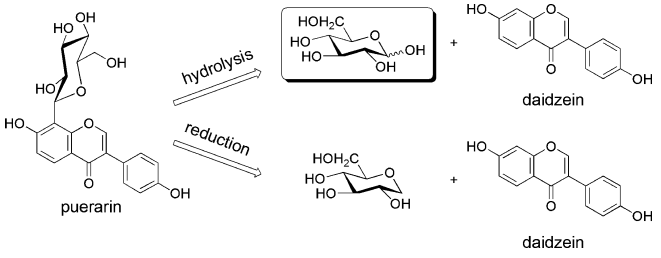

Fig. 1. Two Possible Processes for Cleaving *C*-Glucosyl Bond of Puerarin

Results

Synthesis of [6″,6″-D_2]puerarin (8) Since puerarin (**1**) has only one primary alcohol at C-6 of the sugar moiety, we targeted it for labeling with deuterium. First, **1** was treated with benzaldehyde and zinc chloride to give **2**. Other hydroxyl groups of **2** were acetylated with acetic anhydride in pyridine to obtain **3**. Benzylidene acetal of **3** was hydrolyzed under an acidic condition to afford **4**. The primary hydroxyl of **4** was oxidized to carboxylic acid with a Jones reagent to give **5**. The acetyl groups of **5** were removed by alkali to give **6**, then, the carboxyl group was esterified to yield **7** by a usual method. We failed the reduction of **7** to the corresponding alcohol with $LiAlD_4$ due to decomposition, but **8** was prepared from **7** by slow addition of CD_3OD into the reaction mixture containing $NaBD_4$.[12)] In this reaction, the addition of CH_3OH instead of CD_3OD gave a mixture of **8** and [6″-H,6″-D]puerarin (*ca.* 1 : 1 ratio), because hydrogen atoms originated from CH_3OH were also incorporated into the reduction products by any chance. In the ^{1}H-NMR spectrum of **8** obtained with the CD_3OD condition, the signals for H-6″ disappeared and in the high-resolution electrospray ionization mass spectrometric (HR-ESI-MS) spectrum, the [M−H] ion appeared at *m*/*z* 417.1154 (Calcd for $C_{21}H_{17}D_2O_9$: 417.1149). These results confirmed that the product was puerarin labeled with two deuterium at C-6″.

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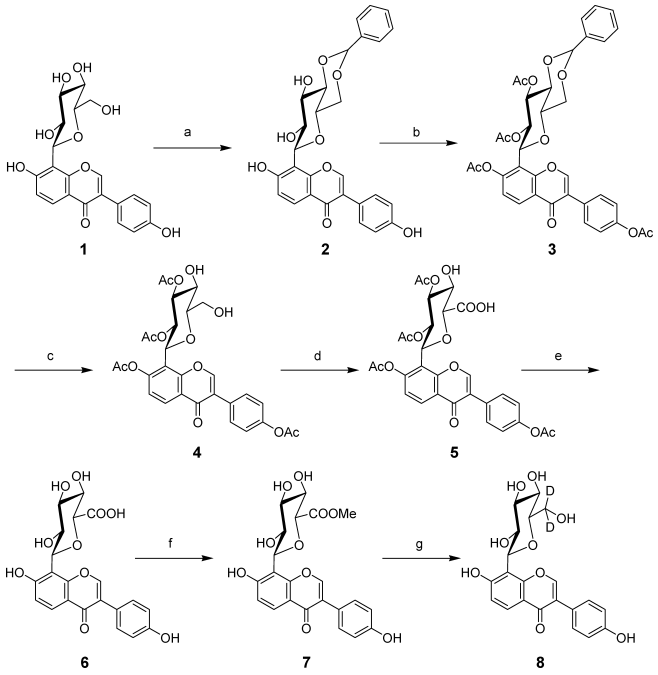

Reagents: (a) PhCHO, $ZnCl_2$, (b) Ac_2O, pyridine, (c) 80% AcOH, 50 °C, (d) CrO_3, H_2SO_4, H_2O, acetone, (e) K_2CO_3, MeOH, (f) MeOH, H_2SO_4, (g) (i) $NaBD_4$, THF, 60 °C, (ii) slow addition of CD_3OD.

Chart 1

HPLC-ESI-MS Analysis of a [6″,6″-D_2]Puerarin Metabolite Mixture In general, it is hard to analyze various sugars by an HPLC-ESI-MS method because of the lack of good chromatographic separation and the difficulty in ionization of sugars. To resolve these problems, many kinds of precolumn derivatization methods have been reported.[13] Of these, we adopted a method of PMP-labeling using 1-phenyl-3-methyl-5-pyrazolone (PMP).[14] A cell-free-system instead of living bacterial system was selected since the bacteria may consume liberated sugars quickly as a carbon source, and identification of the sugars seems to be difficult. A pilot study indicated that a cell-free extract (CFE) of strain PUE required for manganese ion on cleaving the *C*-glucosyl bond of puerarin, as observed in the case of mangiferin *C*-glucosyl cleaving enzyme.[11] Puerarin or [6″,6″-D_2]puerarin was added to a CFE in the presence of $MnCl_2$, and the reaction mixture was anaerobically incubated at 37 °C for 6 h. The mixture was filtered through a 10 kDa membrane, and a filtrate including low molecular weight compounds was treated with a PMP reagent before analyzing by HPLC-ESI-MS.

Figure 2 shows the LC-MS spectra of (a) standard PMP-glucose, (b) PMP-labeled sugar from the metabolite of puerarin, and (c) PMP-labeled sugar from the metabolite of [6″,6″-D_2]puerarin.

Standard PMP-glucose was eluted at 22.8 min and the [M+H] ion appeared at *m*/*z* 511.4. We could find a peak of the identical retention time and mass spectrum as PMP-glucose on (b), indicating the presence of glucose in the metabolite mixture of puerarin. On (c), a peak with the same retention time of PMP-glucose was also observed, however, the [M+H] ion appeared at *m*/*z* 513.3. The pseudo molecular ion for this PMP-labeled sugar was detected two mass units higher than that of PMP-glucose, which could be concluded that the sugar was originated from [6″,6″-D_2]puerarin. These

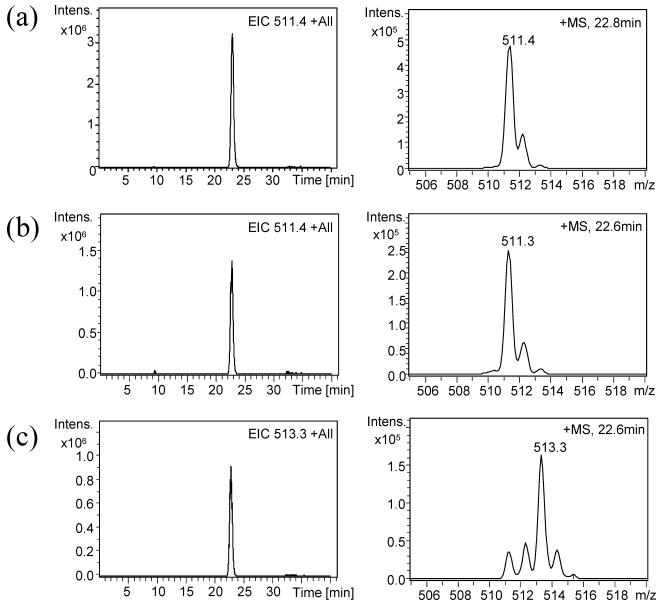

Fig. 2. LC-ESI-MS Spectra of PMP-Labeled Sugars

(a) Standard PMP-glucose, (b) PMP-labeled sugar from the metabolite of puerarin, (c) PMP-labeled sugar from the metabolite of [6″,6″-D_2]puerarin.

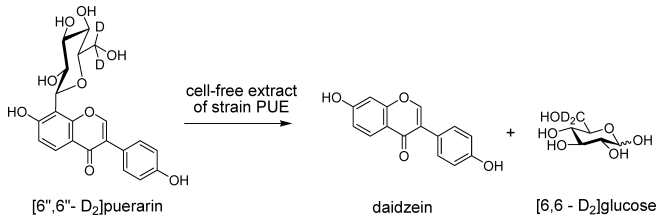

Fig. 3. Conversion of [6″,6″-D_2]Puerarin to Daidzein and [6,6-D_2]Glucose by a CFE of Strain PUE

results suggested that puerarin was metabolized to daidzein and an intact glucose by a human intestinal bacterium, strain PUE (Fig. 3).

Time Course of Conversion of Puerarin to Daidzein and Glucose To confirm further the fact that the metabolite derived from the sugar moiety of puerarin was D-glucose, an enzymatic method using a Glucose (GO) assay kit (SIGMA, U.S.A.) was performed to the CFE. The key enzyme of the kit was glucose oxidase which specifically oxidized β-D-glucose to gluconic acid and hydrogen peroxide. Generated hydrogen peroxide reacts with *o*-dianisidine in the presence of peroxidase to form a colored product.[15] L-Glucose, on the contrary, does not react with glucose oxidase at all.[16] A cell-free-system instead of living bacterial system was selected for the same reason as pointed out above. In the preparation of the CFE, strain PUE was cultured in general anaerobic medium (GAM) broth containing puerarin to induce the puerarin metabolizing enzyme. The collected bacterial cells were washed with buffer before sonication, but quite a little amount of daidzein was remained in CFE regrettably because of its hydrophobicity. To the CFE of strain PUE were added puerarin and $MnCl_2$, and the mixture was anaerobically incubated at 37 °C (Fig. 4). Almost whole puerarin was consumed before 8 h incubation, with the increase of daidzein and glucose. No increase of glucose was observed in the CFE containing neither puerarin nor $MnCl_2$. These observa-

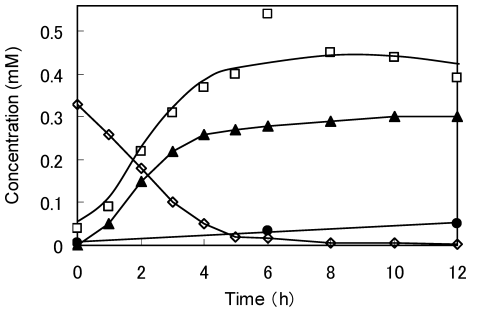

Fig. 4. Time Course of Conversion of Puerarin (◇) to Daidzein (□) and Glucose (▲) by a Cell-Free Extract of Strain PUE

0.3 mM puerarin and 1 mM $MnCl_2$ were added to the CFE of strain PUE. To the glucose of CFE (●) were added neither puerarin nor $MnCl_2$. A little amount of daidzein at 0 h is trace back to CFE preparation. The enzyme is induced only when strain PUE was cultured with puerarin.

tions combined with the spectral data of obtaining sugar product indicated the absolute configuration of the glucose liberated from puerarin to be confirmed as D-form.

Discussion

The C–C bonds of *C*-glucosides are difficult to disconnect by chemical and biological methods. However, a human intestinal bacterium, strain PUE, can cleave the *C*-glucosyl bond of puerarin.[1)] The reaction mechanism is unclear, and the structure of the metabolite derived from a sugar moiety of puerarin is not determined. For the purpose of elucidating the sugar metabolite, we synthesized [6″,6″-D_2]puerarin. After [6″,6″-D_2]puerarin was incubated with a cell-free extract of strain PUE, sugars contained in the metabolite mixture was prelabeled with PMP and analyzed by HPLC-ESI-MS. As a result, deuterium-labeled glucose was detected in the metabolite mixture of [6″,6″-D_2]puerarin. We consequently concluded that puerarin was converted to daidzein and intact glucose by strain PUE. Furthermore, liberation of glucose was also confirmed by an enzymatic glucose detection method using glucose oxidase. The reason why strain PUE cleaves the *C*-glucosyl bond of puerarin may be ascribed that this intestinal bacterium had ability to utilize liberated glucose as an energy source by cleaving *C*-glucosyls. A *C*-glucosyl bond-cleaving reaction is not hydrogenolysis but a new type of hydrolysis, because C-1 of the sugar moiety was hydroxylated. 1-Deoxyglucose possibly produced in the other reductively-cleaving mechanism (hydrogenolysis) did not detect in the metabolite mixture, thus means that the reaction with strain PUE does not include any reduction process. Until now, purification and characterization of the *C*-glucosyl bond-cleaving enzyme of puerarin has not been accomplished. Further study has to provide the new findings of novel glycoside hydrolase family catalyzing the hydrolysis of *C*-glycosides.

Experimental

General An anaerobic incubator, EAN-140 (Tabai Co., Osaka, Japan), was used for incubation with a bacterium, strain PUE or a cell-free extract (CFE). ^{1}H- and ^{13}C-NMR were taken on Varinan NMR system 600 (^{1}H, 600 MHz; ^{13}C, 125 MHz). Chemical shifts are given on a δ (ppm) scale with either CH_3OH (^{1}H, 3.31 ppm; ^{13}C, 49.0 ppm) for CD_3OD or dimethyl sulfoxide (DMSO) (^{1}H, 2.49 ppm; ^{13}C, 39.7 ppm) for DMSO-d_6 as the internal standard. HR-ESI-MS spectrum was taken with a micrOTOF-Q (Bruker Daltonics Inc., Billerica, U.S.A.). UV spectra were measured by a UV-2200 UV–VIS recording spectrophotometer (Shimadzu Co., Kyoto, Japan).

Chemicals Strain PUE was isolated from human feces as previously described.[1)] Puerarin was isolated from the roots of *Pueraria lobata* (Willd.) Ohwi (Tochimoto Tenkaido Co., Osaka, Japan). GAM broth was purchased from Nissui Seiyaku Co. (Tokyo, Japan). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was obtained from TCI (Tokyo, Japan). CD_3OD (99.8%) and $NaBD_4$ (99%) were purchased from Cambridge Isotope Laboratories, Inc. (U.S.A.). A glucose (GO) assay kit was obtained from Sigma (U.S.A.).

Synthesis of Deuterium-Labeled Puerarin. 4″,6″-*O*-Benzylidenepuerarin (2) **1** (433 mg, 1.04 mmol) and zinc chloride (140 mg, 1.03 mmol) were dissolved in benzaldehyde (3 ml) and the mixture was stirred for 3 h at room temperature. The reaction mixture was poured into water, and the precipitate was washed with water and hexane. The precipitate was purified by silica gel column chromatography [$CHCl_3$–MeOH (15 : 1)] to yield **2** (368 mg, 70%) as a white solid. ^{1}H-NMR (CD_3OD) δ: 3.64 (1H, m, H-5″), 3.73 (1H, dd, J=9.0, 9.5 Hz, H-4″), 3.78 (1H, dd, J=8.2, 9.5 Hz, H-3″), 3.83 (1H, br t, J=10.4 Hz, 6″a), 4.28 (1H, dd, J=5.0, 10.4 Hz, H-6″b), 4.39 (1H, dd, J=8.2, 10.0 Hz, H-2″), 5.20 (1H, d, J=10.0 Hz, H-1″), 5.65 (1H, s, Ph-C<u>H</u>), 6.86 (2H, d, J=8.7 Hz, H-3′, 5′), 7.01 (1H, d, J=8.9 Hz, H-6), 7.33—7.38 (3H, m, three protons of Ph), 7.38 (2H, d, J=8.7 Hz, H-2′, 6′), 7.52—7.56 (2H, m, two protons of Ph), 8.07 (1H, d, J=8.9 Hz, H-5), 8.21 (1H, s, H-2). ^{13}C-NMR δ: 69.8 (C-6″), 72.8 (C-5″), 73.2 (C-2″), 76.0 (C-1″), 76.7 (C-3″), 82.6 (C-4″), 103.0 (Ph-<u>C</u>H), 112.8 (C-8), 116.3 (three carbon signals of 3′, 5′, 6), 118.3 (C-4a), 124.1 (C-1′), 125.7 (C-3), 127.6, 129.1, 129.9 and 139.2 (six carbon signals of Ph), 128.3 (C-5), 131.4 (two carbon signals of C-2′, 6′), 154.4 (C-2), 158.7 (two carbon signals of C-4′, 8a), 163.2 (C-7), 178.2 (C-4). ESI-MS (positive) m/z: 505.4 $[M+H]^+$.

2″,3″,4′,7-Tetra-*O*-acetyl-4″,6″-*O*-benzylidenepuerarin (3) Acetic anhydride (1 ml) was added dropwise to a mixture of **2** (151 mg, 0.30 mmol) in pyridine (2 ml) and stirred for 2 h at room temperature. The reaction mixture was poured into water and extracted three times with ethyl acetate. Combined organic layer was washed with 1 M HCl and brine, dried over magnesium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography [hexane–acetone (8 : 3)] to yield **3** (170 mg, 85%) as a slightly yellow solid. According to the ^{1}H-NMR spectrum, inseparable two conformational isomers (*ca.* 3 : 4 ratio) were existed in the product, because free rotation of the *C*-glucosyl bond was restricted due to the steric hindrance between a phenolic hydroxyl group at C-7 and a bulky sugar moiety at C-8. ^{1}H-NMR data[17)] of one of the isomer (**A**) (DMSO-d_6) δ: 1.65—2.44 (12H, four singlet signals of acetates), 3.77 (1H, dd, J=9.8, 10.3 Hz, H-6″a), 3.99 (1H, m, H-5″), 4.04 (1H, t, J=9.4 Hz, H-4″), 4.26 (1H, dd, J=4.8, 10.3 Hz, H-6″b), 5.22 (1H, d, J=10.0 Hz, H-1″), 5.54 (1H, dd, J=9.2, 9.4 Hz, H-3″), 5.67 (1H, s, Ph-C<u>H</u>), 5.77 (1H, m, H-2″), 7.22 (2H, d, J=8.6 Hz, H-3′, 5′), 7.30 (1H, d, J=8.8 Hz, H-6), 7.35—7.42 (5H, m, five protons of Ph), 7.64 (2H, d, J=8.6 Hz, H-2′, 6′), 8.16 (1H, d, J=8.8 Hz, H-5), 8.70 (1H, s, H-2). ^{13}C-NMR δ: 20.3—21.5 (four carbon signals of acetates), 68.1 (C-6″), 70.2 (C-5″), 70.3 (C-2″), 71.8 (C-1″), 72.7 (C-3″), 78.3 (C-4″), 101.0 (Ph-<u>C</u>H), 118.2 (C-8), 121.5 (C-6), 122.3 (two carbon signals of C-3′, 5′), 122.4 (C-4a), 123.7 (C-3), 126.6, 128.6, 129.5 and 137.6 (six carbon signals of Ph), 127.7 (C-5), 129.2 (C-1′), 130.4 (two carbon signals of C-2′, 6′), 150.8 (C-4′), 153.4 (C-7), 154.8 (C-2), 155.9 (C-8a), 169.0—170.2 (four carbon signals of acetates), 175.0 (C-4). ^{1}H-NMR data of another isomer (**B**) (DMSO-d_6) δ: 1.65—2.44 (12H, four singlet signals of acetates), 3.67 (1H, dd, J=9.7, 10.3 Hz, H-6″a), 3.91 (1H, ddd, J=5.1, 9.6, 9.7 Hz, H-5″), 3.98 (1H, m, H-4″), 4.23 (1H, dd, J=5.1, 10.3 Hz, H-6″b), 5.46 (1H, m, H-3″), 5.52 (2H, m, H-1″, 2″), 5.78 (1H, s, Ph-C<u>H</u>), 7.20 (2H, d, J=8.6 Hz, H-3′, 5′), 7.32 (1H, d, J=8.8 Hz, H-6), 7.35—7.42 (5H, m, five protons of Ph), 7.63 (2H, d, J=8.6 Hz, H-2′, 6′), 8.18 (1H, d, J=8.8 Hz, H-5), 8.62 (1H, s, H-2). ^{13}C-NMR δ: 20.3—21.5 (four carbon signals of acetates), 67.9 (C-6″), 70.5 (C-5″), 71.1 (C-1″), 71.3 (C-2″), 72.8 (C-3″), 78.1 (C-4″), 100.8 (Ph-<u>C</u>H) 118.4 (C-8), 121.7 (C-4a), 122.2 (two carbon signals of C-3′, 5′), 122.9 (C-6), 123.6 (C-3), 126.6, 128.6, 129.5 and 137.6 (six carbon signals of Ph), 127.6 (C-5), 129.2 (C-1′), 130.6 (two carbon signals of C-2′, 6′), 150.8 (C-4′), 154.3 (two carbon signals of C-7, 8a), 154.9 (C-2), 169.0—170.2 (four carbon signals of acetates), 175.0 (C-4). ESI-MS (positive) m/z: 673.4 $[M+H]^+$.

2″,3″,4′,7-Tetra-*O*-acetylpuerarin (4) **3** (326 mg, 0.485 mmol) was dissolved in 80% acetic acid (6 ml) and the mixture was stirred for 3 h at 50 °C. After cooling, water was added to the reaction mixture and the mixture was extracted three times with ethyl acetate. Combined organic layer was washed with brine, dried over magnesium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography [$CHCl_3$–MeOH (50 : 1)] to yield **4** (230 mg, 81%) as a white solid. Two conformational isomers (*ca.* 1 : 1 ratio) were existed because of the steric interaction between an acetyl group at C-7 and a bulky sugar. ^{1}H-NMR data[17)] of

one of the isomer (**A**) (DMSO-d_6) δ: 1.61—2.43 (12H, four singlet signals of acetates), 3.48 (1H, m, H-6″a), 3.57 (2H, m, H-4″, 5″), 3.74 (1H, m, H-6″b), 4.95 (1H, d, J=10.1 Hz, H-1″), 5.17 (1H, m, H-3″), 5.60 (1H, m, H-2″), 7.20 (2H, d, J=8.4 Hz, H-3′, 5′), 7.30 (1H, d, J=8.7 Hz, H-6), 7.64 (2H, d, J=8.4 Hz, H-2′, 6′), 8.15 (1H, d, J=8.7 Hz, H-5), 8.69 (1H, s, H-2). ^{13}C-NMR δ: 20.3—21.2 (four carbon signals of acetates), 61.3 (C-6″), 68.4 (C-5″), 70.3 (C-2″), 71.3 (C-1″), 76.7 (C-3″), 81.4 (C-4″), 118.9 (C-8), 121.4 (C-6), 122.2 (two carbon signals of C-3′, 5′), 122.4 (C-4a), 123.4 (C-3), 127.3 (C-5), 129.3 (C-1′), 130.3 (two carbon signals of C-2′, 6′), 150.7 (C-4′), 153.3 (C-7), 155.0 (C-2), 155.9 (C-8a), 168.8—170.3 (four carbon signals of acetates), 174.9 (C-4). ^{1}H-NMR data of another isomer (**B**) (DMSO-d_6) δ: 1.61—2.43 (12H, four singlet signals of acetates), 3.48—3.51 (2H, m, H-4″, 6″a), 3.62 (1H, m, H-5″), 3.67 (1H, m, H-6″b), 5.10 (1H, dd, J=9.3, 9.5 Hz, H-3″), 5.22 (1H, d, J=10.0 Hz, H-1″), 5.40 (1H, dd, J=9.5, 10.0 Hz, H-2″), 7.20 (2H, d, J=8.5 Hz, H-3′, 5′), 7.30 (1H, d, J=8.7 Hz, H-6), 7.63 (2H, d, J=8.5 Hz, H-2′, 6′), 8.15 (1H, d, J=8.7 Hz, H-5), 8.63 (1H, s, H-2). ^{13}C-NMR δ: 20.3—21.2 (four carbon signals of acetates), 60.7 (C-6″), 67.8 (C-5″), 70.6 (C-1″), 71.1 (C-2″), 77.0 (C-3″), 81.6 (C-4″), 119.0 (C-8), 121.6 (C-4a), 122.2 (two carbon signals of C-3′, 5′), 122.9 (C-6), 123.4 (C-3), 127.2 (C-5), 129.3 (C-1′), 130.5 (two carbon signals of C-2′, 6′), 150.7 (C-4′), 154.3 (two carbon signals of C-7, 8a), 155.0 (C-2), 168.8—170.4 (four carbon signals of acetates), 175.0 (C-4). ESI-MS (positive) m/z: 585.5 $[M+H]^+$.

4′,7-Di-*O*-acetyldaidzein-8-*C*-(2,3-di-*O*-acetyl) Glucuronic Acid (5) A Jones reagent (2.17 mmol CrO_3, 180 μl H_2SO_4, 900 μl H_2O) was added to a solution of **4** (440 mg, 0.753 mmol) in acetone (5 ml). The mixture was stirred for 2 h at room temperature, and then quenched by addition of 2-propanol at 0 °C. Water was added to the reaction mixture and the mixture was extracted three times with ethyl acetate. Combined organic layer was washed with water, a 1% $NaHCO_3$ solution and brine. The organic layer was dried over magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography [$CHCl_3$–MeOH (50 : 1)] to afford **5** (220 mg, 49%) as a white solid. The products consisted of inseparable two conformational isomers, similar to compounds **3** and **4**. ^{1}H-NMR data[17] of one of the isomer (**A**) (DMSO-d_6) δ: 1.62—2.43 (12H, four singlet signals of acetates), 3.82 (1H, m, H-4″), 4.07 (1H, d, J=9.7 Hz, H-5″), 5.05 (1H, d, J=10.0 Hz, H-1″), 5.23 (1H, dd, J=9.1, 9.5 Hz, H-3″), 5.65 (1H, dd, J=9.5, 10.0 Hz, H-2″), 7.20 (2H, d, J=8.7 Hz, H-3′, 5′), 7.30 (1H, d, J=8.7 Hz, H-6), 7.62 (2H, d, J=8.7 Hz, H-2′, 6′), 8.16 (1H, d, J=8.7 Hz, H-5), 8.72 (1H, s, H-2). ^{13}C-NMR δ: 20.3—21.3 (four carbon signals of acetates), 69.9 (two carbon signals of C-2″, 4″), 71.5 (C-1″), 76.1 (C-3″), 79.0 (C-5″), 118.6 (C-8), 121.5 (C-6), 122.2 (two carbon signals of C-3′, 5′), 122.4 (C-4a), 123.7 (C-3), 127.6 (C-5), 129.3 (C-1′), 130.4 (two carbon signals of C-2′, 6′), 150.8 (C-4′), 153.5 (C-7), 154.9 (C-2), 155.9 (C-8a), 168.9—170.3 (four carbon signals of acetates), 170.0 (C-6″), 174.9 (C-4). ^{1}H-NMR data of another isomer (**B**) (DMSO-d_6) δ: 1.62—2.43 (12H, four singlet signals of acetates), 3.79 (1H, dd, J=9.5, 9.7 Hz, H-4″), 4.04 (1H, d, J=9.7 Hz, H-5″), 5.16 (1H, dd, J=9.1, 9.5 Hz, H-3″), 5.36 (1H, d, J=10.0 Hz, H-1″), 5.41 (1H, dd, J=9.1, 10.0 Hz, H-2″), 7.20 (2H, d, J=8.7 Hz, H-3′, 5′), 7.29 (1H, d, J=8.8 Hz, H-6), 7.62 (2H, d, J=8.7 Hz, H-2′,6′), 8.16 (1H, d, J=8.8 Hz, H-5), 8.62 (1H, s, H-2). ^{13}C-NMR δ: 20.3—21.3 (four carbon signals of acetates), 69.9 (C-4″), 70.7 (C-1″), 70.8 (C-2″), 76.0 (C-3″), 79.6 (C-5″), 118.6 (C-8), 121.7 (C-4a), 122.2 (two carbon signals of C-3′, 5′), 123.1 (C-6), 123.5 (C-3), 127.4 (C-5), 129.3 (C-1′), 130.5 (two carbon signals of C-2′, 6′), 150.8 (C-4′), 154.3 (two carbon signals of C-7, 8a), 154.9 (C-2), 168.9—170.3 (four carbon signals of acetate), 170.0 (C-6″), 175.0 (C-4). ESI-MS (positive) m/z: 599.4 $[M+H]^+$.

Daidzein-8-*C*-glucuronic Acid (6) K_2CO_3 (177 mg, 1.28 mmol) was added to a solution of **5** (156 mg, 0.261 mmol) in MeOH (5 ml) and the mixture was stirred for 2 h at room temperature. After acidified with 1 M HCl, the reaction mixture was extracted three times with BuOH, and then combined organic layer was concentrated under reduced pressure. The residue was purified by silica gel column chromatography [$CHCl_3$–MeOH–H_2O (8 : 3 : 0.5)] to yield **6** (60 mg, 53%) as a slightly yellow solid. ^{1}H-NMR (CD_3OD) δ: 3.55 (1H, dd, J=8.8, 9.1 Hz, H-3″), 3.73 (1H, dd, J=9.1, 9.7 Hz, H-4″), 3.97 (1H, d, J=9.7 Hz, H-5″), 4.22 (1H, m, H-2″), 5.13 (1H, d, J=10.0 Hz, H-1″), 6.85 (2H, dd, J=2.0, 8.7 Hz, H-3′, 5′), 7.00 (1H, d, J=9.0 Hz, H-6), 7.38 (2H, dd, J=2.0, 8.7 Hz, H-2′, 6′), 8.07 (1H, d, J=9.0 Hz, H-5), 8.21 (1H, s, H-2). ^{13}C-NMR δ: 72.5 (C-2″), 73.4 (C-4″), 75.8 (C-1″), 79.5 (C-3″), 81.0 (C-5″), 112.5 (C-8), 116.2 (three carbon signals of C-3′, 5′, 6), 118.5 (C-4a), 124.2 (C-1′), 125.6 (C-3), 128.3 (C-5), 131.4 (two carbon signals of C-2′, 6′), 154.5 (C-2), 158.7 (two carbon signals of C-4′, 8a), 163.2 (C-7), 172.9 (C-6″), 178.3 (C-4). ESI-MS (positive) m/z: 431.2 $[M+H]^+$.

Daidzein-8-*C*-glucuronic Acid Methyl Ester (7) A catalytic amount of H_2SO_4 was added to a solution of **6** (140 mg, 0.326 mmol) in MeOH (5 ml) and the mixture was stirred overnight at room temperature. Water was added to the mixture, which was then extracted three times with ethyl acetate. Combined organic layer was washed with brine, dried over magnesium sulfate, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography [$CHCl_3$–MeOH–H_2O (10 : 1.5 : 0.1)] to yield **7** (79 mg, 55%) as a slightly yellow solid. ^{1}H-NMR (CD_3OD) δ: 3.54 (1H, t, J=9.0 Hz, H-3″), 3.76 (1H, dd, J=9.0, 9.7 Hz, H-4″), 3.77 (3H, s, COO-Me), 4.00 (1H, d, J=9.7 Hz, H-5″), 4.28 (1H, m, H-2″), 5.12 (1H, d, J=9.8 Hz, H-1″), 6.84 (2H, dd, J=2.2, 8.7 Hz, H-3′, 5′), 6.99 (1H, d, J=8.9 Hz, H-6), 7.37 (2H, dd, J=2.2, 8.7 Hz, H-2′, 6′), 8.06 (1H, d, J=8.9 Hz, H-5), 8.19 (1H, s, H-2). ^{13}C-NMR δ: 52.8 (COO-Me), 72.3 (C-2″), 73.3 (C-4″), 75.9 (C-1″), 79.4 (C-3″), 81.2 (C-5″), 112.3 (C-8), 116.2 (three carbon signals of C-3′, 5′, 6), 118.5 (C-4a), 124.1 (C-1′), 125.6 (C-3), 128.4 (C-5), 131.4 (two carbon signals of C-2′, 6′), 154.5 (C-2), 158.7 (two carbon signals of C-4′, 8a), 163.2 (C-7), 171.4 (C-6″), 178.2 (C-4). ESI-MS (positive) m/z: 445.3 $[M+H]^+$.

[6″,6″-D$_2$]Puerarin (8) $NaBD_4$ (16 mg, 0.38 mmol) was added to a solution of **7** (34 mg, 0.077 mmol) in tetrahydrofuran (THF) (1 ml) and the mixture was stirred for 30 min at 60 °C. CD_3OD (400 ml) was slowly added to the reaction mixture and the mixture was stirred for 1 h at 60 °C. After cooling, the reaction was quenched with 0.1 M HCl (5 ml). The mixture was extracted three times with BuOH and combined organic layer was concentrated under reduced pressure. The residue was purified by silica gel column chromatography [$CHCl_3$–MeOH–H_2O (10 : 1.5 : 0.1)] to yield **8** (10 mg, 31%) as a slightly yellow solid. ^{1}H-NMR (CD_3OD) δ: 3.47 (1H, m, H-5″), 3.52—3.54 (2H, m, H-3″, 4″), 4.12 (1H, m, H-2″), 5.10 (1H, d, J=10.0 Hz, H-1″), 6.85 (2H, d, J=8.7 Hz, H-3′, 5′), 6.99 (1H, d, J=8.9 Hz, H-6), 7.38 (2H, d, J=8.7 Hz, H-2′, 6′), 8.06 (1H, d, J=8.9 Hz, H-5), 8.19 (1H, s, H-2). ^{13}C-NMR δ: 62.1 (C-6″), 71.7 (C-4″), 73.0 (C-2″), 75.6 (C-1″), 80.0 (C-3″), 82.7 (C-5″), 113.2 (C-8), 116.2 (three carbon signals of C-3′, 5′, C-6), 118.4 (C-4a), 124.2 (C-1′), 125.6 (C-3), 128.1 (C-5), 131.4 (two carbon signals of C-2′, 6′), 154.5 (C-2), 158.7 (two carbon signals of C-4′, C-8a), 163.2 (C-7), 178.3 (C-4). HR-ESI-MS (negative) m/z 417.1154 $[M-H]^-$ (Calcd for $C_{21}H_{17}D_2O_9$: 417.1149).

Preparation of a Cell-Free Extract Strain PUE was cultured under anaerobic conditions at 37 °C for 12 h in 1 l of GAM broth containing 0.3 mM puerarin. The bacterial cells were collected by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.5), and then suspended in 25 ml of same buffer. Cells were disrupted by sonication on ice and centrifuged at 100000×g for 60 min at 4 °C to obtain a supernatant as a cell-free extract.

Preparation of a Metabolite Mixture of Puerarin or [6″,6″-D$_2$]Puerarin A mixture of a cell-free extract (1 ml) , $MnCl_2$ (1 mM) and puerarin or [6″,6″-D_2]puerarin (each 0.5 mM) was anaerobically incubated for 6 h at 37 °C. The reaction mixture was filtrated through Centriplus YM-10 (Millipore, U.S.A.) with a 10 K molecular weight cut off by centrifugation at 3000×g for 30 min at 4 °C. The low molecular weight filtrate was labeled using a PMP reagent described follows.

PMP Labeling Standard PMP-glucose was prepared as previously reported.[14] PMP labeling of liberated sugars were as follows: A 0.5 M PMP solution (100 μl) in MeOH and 0.3 M aqueous sodium hydroxide (100 μl) were added to a solution (50 μl) of low molecular weight metabolites, and the mixture was incubated for 30 min at 70 °C. After cooling, the mixture was neutralized with 0.1 M HCl, diluted with water (400 μl), and washed three times with $CHCl_3$, then aqueous layer was concentrated. The residue was dissolved in MeOH (500 μl), and an aliquot was injected into a column of the HPLC-ESI-MS system.

HPLC-ESI-MS Analysis HPLC was performed on an Agilent 1100 system (Agilent Technologies, Germany) with a photodiode array detector and Agilent 1100 series binary pump, and an Esquire 3000 plus mass spectrometer (Bruker Daltonik GmbH, Germany) coupled with an ESI interface and an ion trap mass analyzer. Conditions for analyzing PMP-sugars are as follows: column, TSK-gel ODS-80Ts (Tosoh Co., Tokyo, Japan, 4.6 mm× 150 mm); mobile phase, 22% acetonitrile in 0.1% acetic acid for 30 min on isocratic mode; flow rate, 1.0 ml/min; temperature, 30 °C; monitoring, TIC (total ion chromatogram) and EIC (extracted ion chromatogram at 511.4 or 513.3) in the positive mode by ESI-MS.

Time Course A CFE (20 ml) containing $MnCl_2$ (1 mM) and puerarin (0.3 mM) was anaerobically incubated at 37 °C. The amount of puerain, daidzein, and glucose in the CFE were measured at 0, 1, 2, 3, 4, 5, 6, 8, 10, and 12 h.

Quantitative Analysis of Puerarin and Daidzein Puerarin and daidzein were extracted twice with BuOH (200 μl) from the reaction mixture (100 μl) mentioned above. The extract was evaporated to dryness, dissolved in 50% MeOH, and analyzed by HPLC. Concentrations of puerarin and daidzein were calculated from the calibration curves of the respective authentic samples. HPLC conditions were as follows: recorder, C-R6A Chromatopac; pump, LC-6A; system controller, SCL-6B; monitor, SPD-6A; injector, SIL-9A; column, TSK-gel ODS-80Ts (Tosoh Co., Tokyo, Japan, 4.6×150 mm); flow rate, 1 ml/min; detection, 250 nm; solvent system, 15—50% acetonitrile in 0.1% trifluoroacetic acid in a linear gradient for 20 min.

Quantitative Analysis of Glucose An aliquot of the reaction mixture (250 μl) was heated for 5 min at 90 °C. After centrifugation at 6000×$\boldsymbol{g}$, the amount of glucose in the supernatant was measured with a Glucose (GO) assay kit.

References and Notes

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17) ^{1}H- and ^{13}C-NMR were taken as a conformational mixture. The assignments of them were performed by gCOSY, gHSQC, gHMBC and gHSQC-TOCSY analysis.