Microbial Transformation of Flavonoids in Cultures of *Mucor hiemalis*

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Abstract

Flavonoids are plant secondary metabolites that are well known for their health-promoting properties as nutraceuticals in diets. Bioavailability and biological activities of flavonoids vary among the individual subclasses with different patterns of substitution, inclusive of glycosylation, to their basic structures. Many flavonoids exist as glycosides in plants. This study investigated the possibility of glycosylation of flavonoids through biotransformation using filamentous fungi as whole-cell biocatalysts. Microbial transformations of ten flavonoids (four flavones, four flavonols, a flavanone, and an aurone) were performed in cultures of *Mucor hiemalis* KCTC 26779. As a result, a flavonoid glycoside was obtained which has not been described previously. The chemical structure of this product was elucidated as 6,2′-dimethoxyflavonol-3-O-β-D-glucopyranoside by analyzing 1-dimensional and 2-dimensional-nuclear magnetic resonance spectral and high-resolution electrospray ionization mass spectral data. This compound could be useful for further biological and bioavailability studies, as well as expanding the library of flavonoid derivatives.

Keywords

microbial transformation, flavonoid, *Mucor hiemalis*, glycosylation, 6,2′-dimethoxyflavonol-3-O-β-D-glucopyranoside

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Flavonoids, found in fruits, vegetables, grains, roots, flowers, tea, and wine, have been shown to possess biological activities that vary due to the diversity of their phenolic structures.\(^{1,3}\) Reported pharmacological activities of flavonoids include antioxidant,\(^{4,5}\) anti-inflammatory,\(^{6}\) cardioprotective,\(^{7}\) anticancer,\(^{8,9}\) antiallergic,\(^{10}\) and antidiabetic effects.\(^{11,12}\) Because of the health-promoting and pharmacological effects of flavonoids, these compounds are utilized in food technology, as supplements, and by the pharmaceutical industries.\(^{13}\) However, most flavonoids are unsatisfactory in terms of water solubility and stability in certain media, resulting in low absorption.\(^{14}\) To overcome these problems, the glycosylation or hydroxylation of flavonoids is a well-known method that can improve solubility, intracellular and intercellular transport, chemical stability, and bioactivity.\(^{7,14,17}\) The solubility and stability of flavonoids can be improved by structural modification using chemical methods, biocatalysts, and biotransformation.\(^{8,19}\)

Microbial biotransformation is extensively used to transform many compounds, including hydrocarbons and pharmaceutical substances.\(^{20}\) It has the potential to generate chemoselective, regioselective, and stereoselective compounds under milder conditions than those used in chemical synthesis.\(^{21}\) Microbial biotransformation has been used for hydroxylation, dehydroxylation, O-methylation, O-demethylation, glycosylation, deglycosylation, dehydrogenation, and hydrogenation.\(^{22,23}\) Therefore, it is able to produce novel bioactive compounds and enhance physicochemical and pharmacological properties.\(^{24}\) Microbial biotransformation also has the advantage of being cost-effective, environmentally friendly, producing high yields and significant amounts of metabolites.\(^{25}\)

In our previous study on Coreopsis lanceolata, several types of flavonoids were isolated as flavone, flavonol, flavanone, chalcones, and aurone.\(^{26}\) As part of our search for new derivatives and/or enhancers of bioactivity and bioavailability through microbial transformation, we performed biotransformations of isolates 1 and 2 from *C. lanceolata* and commercially available compounds (3-10) with similar structures to those isolated, in cultures of *Mucor hiemalis* KCTC 26779, which has been an efficient biocatalyst for biotransformations of isolates 1 and 2 from *C. lanceolata* and commercially available compounds (3-10) with similar structures to those isolated, in cultures of *Mucor hiemalis* KCTC 26779, which has been an efficient biocatalyst for...
 Results and Discussion

Compound 11 was obtained as a brown solid and gave a molecular ion peak at \( m/z \) 483.1448 [M + Na]^+ in its high-resolution electrospray ionization mass spectrum (HR-ESI-MS), corresponding to an elemental formula of C_{23}H_{24}O_{10}Na. The \(^1\)H and \(^{13}\)C-nuclear magnetic resonance (NMR) spectra of 11 were similar to those of 6,3′-dimethoxyflavonol and peterin, except for the difference in signals of the sugar moiety. The differences between the NMR spectroscopic data of the aglycone of 11 and 6,3′-dimethoxyflavonol were in the signals of the B-ring. On the other hand, the C-ring pattern was different between the aglycone of 11 and peterin. In the \(^1\)H-NMR spectrum of 11, the signals at \( \delta_{\text{H}} \) 7.59 (1H, d, \( J = 9.2 \) Hz, H-8), 7.45 (1H, d, \( J = 3.0 \) Hz, H-5), and 7.38 (1H, dd, \( J = 9.2, 3.0 \) Hz, H-7) showed a 1,3,4-trisubstituted B ring system in the flavonol moiety. In addition, a 1,2-disubstituted C-ring was evident with signals at 7.70 (1H, dd, \( J = 7.6, 1.7 \) Hz, H-6'), 7.48 (1H, br t, \( J = 8.3 \) Hz, H-4'), 7.12 (1H, dd, \( J = 8.3, 1.0 \) Hz, H-3'), and 7.00 (1H, td, \( J = 7.6, 1.0 \) Hz, H-5). The position of two methoxyl groups were assigned to C-6 and C-2' by the correlations of two methyl proton signals at \( \delta_{\text{H}} \) 3.86 (3H, s, OCH\(_3\)) and 3.74 (3H, s, OCH\(_3\)) with two quaternary carbon signals at \( \delta_{\text{C}} \) 156.9 (C-6) and 157.7 (C-2'), respectively, in the \(^1\)H-\(^{13}\)C heteronuclear multiple bond correlation (HMBC) spectrum of 11. In the \(^{13}\)C-NMR spectrum of 11, signals for a carbonyl carbon at \( \delta_{\text{C}} \) 173.7 (C-4), three oxygenated aromatic carbons at \( \delta_{\text{C}} \) 157.3 (C-2), 150.5 (C-9), and 137.2 (C-3), and a quaternary carbon at \( \delta_{\text{C}} \) 124.8 (C-10) were observed. The \(^1\)H-\(^{13}\)C HMBC correlations of H-1'/C-9, H-1'/C-4, H-6'/C-2 indicated that the B ring was linked to C-9 and C-10, and the C-ring to C-2, in a 3-hydroxypryan-4-one ring. Accordingly, the aglycone moiety of 11 was identified as 6,2′-dimethoxyflavonol, for which there has been no report on its NMR data so far. The sugar moiety was identified by the \(^1\)H and \(^{13}\)C signals at \( \delta_{\text{H}} \) 2.98 (1H, m, H-5’)/\( \delta_{\text{C}} \) 77.8 (C-5’), 3.12 (1H, m, H-3’)/76.9 (C-3’), 2.86 (1H, m, H-2’)/74.5 (C-2’), 2.98 (1H, m, H-4’)/70.2 (C-4’), and 3.35 (1H, d, \( J = 11.3 \) Hz, H-6’a) and 3.53 (1H, d, \( J = 11.3 \) Hz, H-6’b)/61.4 (C-6’), and the \(^1\)H-\(^1\)H COSY correlations of H-2’/H-1’, H-3’, H-3’’/H-2’, H-4’, and H-5’/H-6’a. The sugar moiety was determined as β-d-glucopyranose by the anomeric proton at \( \delta_{\text{H}} \) 5.22, with a large coupling constant, \( J = 7.8 \) Hz, and by the \(^1\)H-\(^1\)H nuclear Overhauser effect spectroscopy correlations of H-3’/H-1’, and H-5’ and H-6’/H-4’. The \(^1\)H-\(^{13}\)C HMBC NMR correlations of H-1’/C-3 indicated that the β-d-glucopyranose was positioned at C-3 in the structure of 11 (Figure 2). Therefore, metabolite 11 was elucidated as 6,2′-dimethoxyflavonol-3-O-β-d-glucopyranoside, which has not been previously described.

In this study, the microbial transformation was attempted for a number of compounds with hydroxyl and/or methoxyl groups at diverse positions in flavone and flavonol structures. Among them, only compound 8, in which a methoxyl group was substituted for each of the A-ring and B-ring of flavonol was successfully glucosylated by \( \text{Mucor hiemalis} \) KCTC 26779. Other compounds (1-7, 9, and 10), with structures in which only hydroxyl groups are substituted, thus differing from compound 8, did not undergo biotransformation in this study.
However, in the previous reports on microbial transformation using this microorganism, flavonoids such as quercetin, apigenin, naringenin, and prenylated flavonoids have been glycosylated.

Many natural compounds are discovered in nature in the form of glycosides and they show a broad spectrum of benefits for human health. Almost all natural flavonoids are present in plants as either their O-glycosides or C-glycosides. These glycosylated flavonoids have been reported to have greater bioactivity, solubility, stability, and/or bioavailability than their aglycones. For example, the solubility of 5-O-a-D-glucopyranosyl(+)-catechin and 4’-O-a-D-glucopyranosyl(+)-catechin were increased by more than 40 times and had better thermal stability than their aglycone, catechin. In the 2,2-diphenyl-1-picyrylhydrazyl radical scavenging test, xanthohumol had a half-maximal inhibitory concentration value of 1.98 µM, while the value for xanthohumol-4’-O-ß-D-glucopyranoside was 0.77 µM, indicating that the glycosylated compound had more than twice the antioxidant effect of its aglycone. Puerarin-7-O-fructoside had increased antioxidant activity and enhanced water solubility than puerarin. It has also been reported that compared with puerarin, puerarin-7-O-glucoside has higher concentrations in plasma and a longer residence time in blood. Based on these studies, it is speculated that the new metabolite could also have enhanced bioactivity, solubility, and bioavailability. However, additional studies of this compound are required, such as biological evaluation and water solubility test.

Conclusions

In conclusion, microbial transformation studies of 10 compounds with flavonoid structures with different substituents were carried out with M. biennalis, and only 6,2’-dimethoxy-flavonol (8) was transformed into a novel metabolite, 6,2’-dimethoxyflavonol-3-O-ß-D-glucopyranoside (11). Biotransformation using M. biennalis KCTC 26779 is a relatively easy, economic, and eco-friendly method to produce glycosylated flavonoids, which allows the products to be obtained in quantities required for further studies.

Experimental

General

The measurement of optical rotations was conducted on a JASCO DIP-1000 polarimeter (JASCO Co., Tokyo, Japan). One-dimensional (1D) and 2-dimensional (2D)-NMR spectra were obtained on a JEOL-ECA 500 MHz NMR instrument (JEOL Ltd., Tokyo, Japan) with tetramethylsilane used as the internal standard. HR-ESI-MS were obtained on a Waters SYNAPT G2 mass spectrometer (Waters, Milford, MA, USA). Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F254 (Merck, Darmstadt, Germany) and Kieselgel 60 RP-18-F254S (Merck, Darmstadt, Germany). Analytical plates were visualized under ultraviolet light (254 and 365 nm) and sprayed with 10% (v/v) sulfuric acid, followed by heating at 180 ºC for 2 minutes. Column chromatography (CC) was conducted using silica gel (70, 230 mesh, Merck, Darmstadt, Germany), RP-18 (YMC gel ODS-A, 12 nm, S-75 µm, YMC Co., Tokyo, Japan), and Sephadex LH-20 (GE Healthcare Biosciences, Uppsala, Sweden).

Materials and Microorganism

Luteolin (1) and quercetin (2) were isolated from the dried flowers of Coreopsis lanceolata, as described in a previous study. 7,3’-Dihydroxyflavone (3), 6,4’-dihydroxyflavone (4), 3,5’-dihydroxyflavone (5), and 6,2’-dimethoxyflavonol (8) were purchased from Sigma-Aldrich (St Louis, MO, USA); galangin (6), (2S)-isoookanin (9), and sulfuretin (10) from Wuhan ChemFaces Biochemical Co., Ltd. (Hubei, China); and resokamplatin (7) from Extrasynthese (Genay, France). Mucor biennalis 26779 was obtained from the Korean Collection for Type Cultures (KCTC). The ingredients for microbial media, including dextrose, peptone, and malt extract, were purchased from Becton, Dickinson and Company (Sparks, MD, USA). Mucor biennalis was cultured on malt medium (malt extract 20 g/L, dextrose 20 g/L, peptone 1 g/L).

Screening Procedures

Microbial biotransformation studies were performed according to the standard two-stage procedure. The actively growing microbial cultures were inoculated in 250 mL flasks containing 50 mL of malt medium and incubated with gentle agitation (200 rpm) at 25 ºC in a temperature-controlled shaking incubator. After inoculation for 24 hours, the dimethylsulfoxide (DMSO) solutions (1.5 mg/200 µL) of 1-10 were added to each flask, and the incubation was continued under the same conditions for another 7 days. Sampling and TLC monitoring were performed at an interval of 24 hours. Culture controls consisted of fermentation cultures in which the microorganisms were grown without the addition of 1-10.

Scale-Up Fermentations of 8

Preparative scale-up fermentations were carried out in three 500 mL flasks each containing 150 mL of malt medium, and each 15 mg of 8, dissolved in DMSO, distributed evenly among the flasks. After incubation for 7 days, the microbial culture broth was extracted with ethyl acetate (EtOAc, 400 mL × 3), and the organic layers were combined and concentrated in vacuo. The EtOAc extract (263 mg) of 8 from M. biennalis culture broth was subjected to silica gel CC with a solvent system of dichloromethane-methanol (9:1 to 0:1, v/v) to produce metabolite 11 (12 mg).

6,2’-Dimethoxyflavonol-3-O-ß-D-Glucopyranoside (11)

Brown solid.

$[\alpha]_D^{25} +28.5^{\circ}$ (c 0.01, CH$_3$OH).

$^1$H NMR (DMSO-d$_6$, 500 MHz) δ H 7.70 (1H, dd, $J = 7.6, 1.7$ Hz, H-6’), 7.59 (1H, dd, $J = 9.2, 7.6$ Hz, H-8), 7.48 (1H, br t, $J = 8.3$ Hz, H-4’), 7.45 (1H, d, $J = 3.0$ Hz, H-5), 7.38 (1H, dd, $J = 9.2, 3.0$ Hz, H-7), 7.12 (1H, dd, $J = 8.3, 1.0$ Hz, H-3’), 7.00 (1H,
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Declarations of Conflicting Interests
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