Urinary Metabolites of Genistein Administered Orally to Rats

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In a study on the metabolism of flavonoids, the isoflavone genistein was administered orally to rats. Urine samples were collected and treated with β-glucuronidase and arylsulfatase. Genistein and its metabolites, 4′,5,7-trihydroxyisoflavanone (M1), 4′,7-dihydroxyisoflavan (M2), and *p*-ethylphenol (M3) were isolated from the urine following treatment with enzymes. The structures of M1, M2, and M3 were determined on the basis of chemical and spectral data.

Key words genistein; urinary metabolite; 4′,5,7-trihydroxyisoflavanone; 4′,7-dihydroxyisoflavan; equol; *p*-ethylphenol

Genistein is a type of phytoestrogen found as a glucoside in glycine, the genus Sophora[1] and soy products.[2] Genistein is known to have estrogenic activity,[3] a tyrosine kinase inhibitory action,[4] and a histidine kinase inhibitory action.[5] Accordingly, genistein appears to play a role in the prevention of hormone-dependent diseases such as breast and prostate cancers, osteoporosis, and coronary heart disease.[6—9] In addition to conjugation, genistein is metabolized to dihydrogenistein, equol, and *p*-ethylphenol.[10—14] Although many reports on the metabolites of genistein appear in the literature, there exist no systematic and detailed studies of their spectra or structure. To study the metabolism of genistein in greater detail, we used a three-dimensional (3D) HPLC equipped with a photodiode array detector as a new tool in the detection and structural identification of the metabolites.

The present paper reports the structures of the urinary metabolites of genistein administered orally to rats following treatment with β-glucuronidase and arylsulfatase.

Results and Discussion

The 3D HPLC profile of urine samples from rats after oral administration of genistein showed three distinct peaks tentatively designated as M1, M2, and M3 in decreasing order of polarity. In urine samples, the parent compound was also identified as genistein by direct comparison of the UV spectrum and retention time with those of an authentic sample in 3D HPLC. M1, M2, and M3 from urine treated with enzymes were isolated by chromatographic separation on a Sephadex LH-20 column and repeated preparative HPLC, as described in the Experimental section, followed by determination of their structures.

M1 was obtained as a white powder, mp 216—218 °C. Its molecular formula was $C_{15}H_{12}O_5$ based on high-resolution mass spectrum (HR-MS) analysis. The UV spectrum of M1 was typical of the isoflavanone skeleton. The ^{1}H-NMR spectrum also suggested an isoflavanone skeleton, with a signal at δ 4.48 (2H, d, J=6.4 Hz, H-2) and 3.92 (1H, t, J=6.4 Hz, H-3), and signal at δ 5.77 (2H, s) was assigned to H-6 and H-8, respectively, and another set of A_2B_2-type signals at δ 6.71 (2H, d, J=8.6 Hz) and 7.07 (2H, d, J=8.7 Hz) which were assigned to B-ring protons (H-3′, -5′, H-2′, -6′). The structure of M1 was thus concluded to be 4′,5,7-trihydroxyisoflavanone. As the yield of M1 was too small, the configuration at C-3 could not be determined.

M2 was obtained as a white powder, mp 156—159 °C, with a molecular formula determined to be $C_{15}H_{14}O_3$ from HR-electron impact (EI)-MS. The UV spectrum in methanol exhibited major absorbance at 281 nm, typical of the isoflavan skeleton. In the ^{1}H-NMR spectrum, signals assignable to H-2α, H-2β, H-3, and H-4 of the isoflavan skeleton appeared at δ 3.93 (1H, t, J =10.6 Hz), 4.26 (1H, dd, J =10.6, 2.8 Hz), 3.07—3.18 (1H, m), and 2.90 (2H, d, J =8.4 Hz), respectively. Observation of ABX-type aromatic proton signals at δ 6.37 (1H, d, J=2.3 Hz), 6.41 (1H, dd, J=8.2, 2.3 Hz), and 6.89 (1H, d, J =8.2 Hz) and A_2B_2-type aromatic proton signals at δ 6.84 (2H, d, J =8.7 Hz) and 7.09 (2H, d, J=8.7 Hz) in the ^{1}H-NMR spectrum indicated that M2 possibly has hydroxyl groups at C-7 and C-4′. These data indicate the structure to be the known isoflavan, 4′,7-dihydroxyisoflavan (equol). Direct comparison of M2 by MS and NMR spectra with an authentic sample synthesized from daidzein confirmed the planar structure of M2 to be identical with that of equol. As the yield of M2 was too small, the stereochemistry at C-3 could not be determined.

M3 was identified as *p*-ethylphenol by comparison with an authentic sample.

In previous papers,[12,15] we reported that genistin administered orally to rats was hydrolyzed to the aglycone genistein, which was then metabolized into the conjugated forms (sulfates or glucuronides) and finally excreted into the urine and bile *via* the bloodstream. Genistein was shown in this study to be metabolized to M1, M2, and M3. Chart 1 shows the metabolic pathway deduced from the present results; genistein is partially hydrogenated to an isoflavanone-type intermediate M1, and subsequently the carbonyl group at C-4 of M1 is reduced to an isoflavan-type M2. Since the most of the flavonoids occur in the intact plant as glucosides and are hydrolyzed in the rumen of livestock and further demethylated and reduced by the microorganisms,[16] it was thought possible that genistein could also be converted into the hydrogenated metabolite M1 by the gut flora. On the other hand, genistein is partially converted to M3 *via* ring fission of the C ring, as advocated by Griffiths.[17]

Batterham *et al.*[18] carried out studies in sheep and has identified *p*-ethylphenol as the major metabolite from genistein. They also reported that the compound was generated from the B ring and partially from the C ring of genistein and that no metabolite was detected that originated from the A ring. Moreover, Cayen *et al.*[19] conducted a study with intramuscular injection of ^{3}H-genistein into hens, and found equol as a metabolite in the urine. In our study using rats, both compounds were observed to be present.

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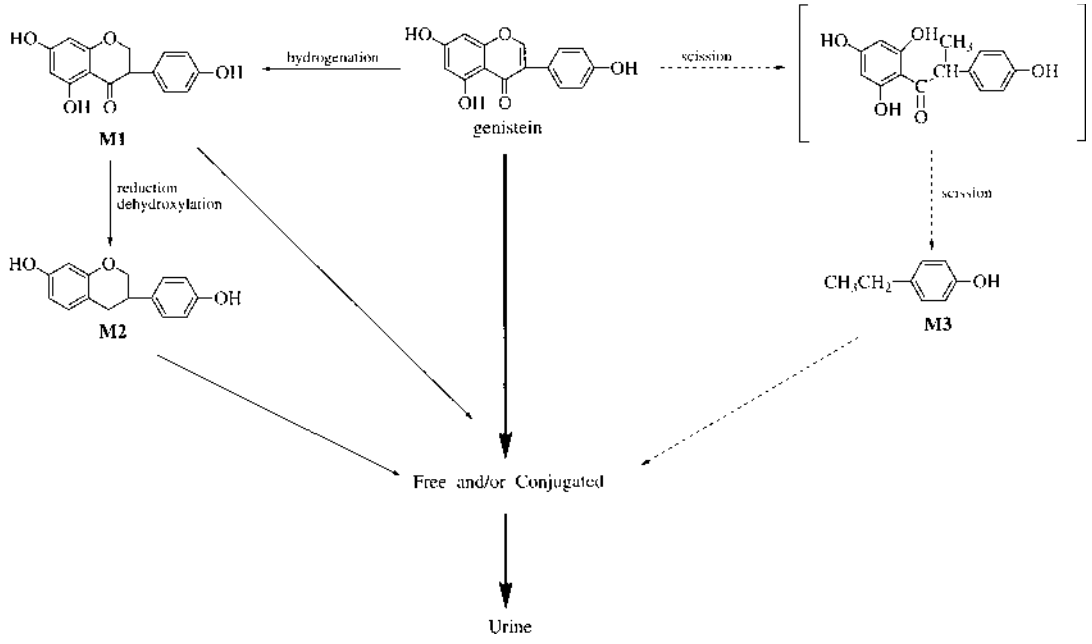

Chart 1. Proposed Metabolic Pathways of Genistein in Rats

The thickness of the arrows indicates the relative importance of the pathways. The dotted arrows indicate the metabolic pathways advocated by Griffiths.[17]

Studies on the effects of these metabolites on drug efficacy and mechanism of action will be very interesting, and will certainly provide useful information about isoflavonoid metabolism *in vivo*.

Experimental

Apparatus NMR spectra were recorded on a JEOL JNM-EX 270 with tetramethylsilane as the internal standard, and chemical shifts are given as δ values. MS were measured with a JEOL DX-303 mass spectrometer. The HPLC system consisted of a CCPM pump, CO-8010 column oven (Tosoh, Tokyo, Japan), and model M991J photodiode array detector (Waters Millipore, Milford, MA, U.S.A.).

Reagents Genistein was purchased from Fujicco (Kobe, Japan). β-Glucuronidase was from Sigma (St. Louis, MA, U.S.A.). All other reagents were of special grade.

Animals Male Sprangue-Dawley rats (Japan SLC Inc., Hamamatsu), 6 weeks old, were used. They were fasted but had free access to water for 18 h prior to the experiments.

HPLC Conditions A stainless steel column (250×4.6 mm i.d.), packed with reverse-phase TSKgel ODS-120T (5 μm, Tosoh) was used. The mobile phase was a linear gradient system comprised of 10 mM sodium phosphate buffer (pH 6.5) (solvent A) and 100% methanol (solvent B), A/B=95/5 (0 min)→60/40 (45 min)→60/40 (70 min). The flow rate was 1.0 ml/min at 40 °C.

Isolation of Urinary Metabolites For isolation of urinary metabolites, 0.8 g of genistein were administered orally in portions to each group of 12 rats at 100 mg/kg/d over a period of 30 d, and urine samples were collected using metabolic cages. The combined urine samples (350 ml) were dissolved in 100 ml of 0.2 M sodium acetate buffer (pH 5.5) to which 4.4 ml of β-glucuronidase/arylsulfatase solution was added and the solution was incubated at 37 °C for 24 h. The incubated solution was extracted with ethyl acetate (1000 ml) three times. The organic layer was evaporated to dryness at below 40 °C. The residue (230 mg) was dissolved in a small amount of methanol and chromatographed on a Sephadex LH-20 column with methanol as the eluant.

The fractions containing metabolites (M1—M3) were subjected to preparative HPLC under the following conditions: column, TSKgel ODS-120T (10 μm, 300×7.8 mm i.d., Tosoh); mobile phase, 20% methanol (solvent A) and 100% methanol (solvent B); linear gradient system, A/B=80/20 (0 min)→0/100 (90 min). The flow rate was 2.0 ml/min at room temperature. Each metabolite fraction was evaporated to dryness at 40 °C *in vacuo* to give M1 (1 mg), M2 (1 mg), and M3 (1 mg), respectively.

M1 (4′,5,7-Trihydroxyisoflavanone): A white powder, mp 216—218 °C. HR-EI-MS: Calcd for $C_{15}H_{12}O_5$: 272.0685. Found: 272.0693. ^{1}H-NMR (DMSO-d_6, 270 MHz) δ: 3.92 (1H, t, J=6.4 Hz, H-3), 4.48 (2H, d, J=6.4 Hz, H-2), 5.77 (2H, s, H-6, 8), 6.71 (2H, d, J=8.7 Hz, H-3′, 5′), 7.07 (2H, d, J=8.7 Hz, H-2′, 6′), 12.26 (1H, s, 5-OH).

M2 (4′,7-Dihydroxyisoflavan): A white powder, mp 155—157 °C. HR-EI-MS: Calcd for $C_{15}H_{14}O_3$: 242.0943. Found: 242.0940. ^{1}H-NMR (CD_3OD, 270 MHz) δ: 2.87 (2H, d, J=9.1 Hz, H-4), 2.94—3.11 (1H, m, H-3), 3.91 (1H, t, J=10.4 Hz, H-2α), 4.19 (1H, dd, J=10.5, 3.5 Hz, H-2β), 6.22 (1H, d, J=2.5 Hz, H-8), 6.31 (1H, dd, J=8.2, 2.5 Hz, H-6), 6.74 (2H, d, J=8.7 Hz, H-3′, 5′), 6.87 (1H, d, J=8.2 Hz, H-5), 7.09 (2H, d, J=8.4 Hz, H-2′, 6′).

M3 (*p*-Ethylphenol): Colorless oil. HR-EI-MS: Calcd for $C_8H_{10}O$: 122.0732. Found: 122.0662. ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.20 (3H, t, J=7.6 Hz, –CH_3), 2.58 (2H, q, J=7.6 Hz, –CH_2–), 6.75 (2H, d, J=8.6 Hz, H-2, 6), 7.06 (2H, d, J=8.7 Hz, H-3, 5).

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