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METABOLITES OF DAIDZEIN AND GENISTEIN AND THEIR BIOLOGICAL ACTIVITIES

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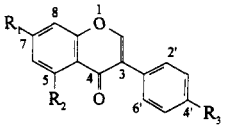
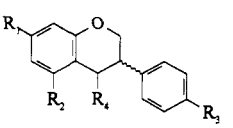
ABSTRACT.—A number of metabolites of daidzein and genestein have been synthesized and their biological activities determined. Equol [**3**], 5,7,4′-trihydroxyisoflavan [**5**], 4,7,4′-trihydroxyisoflavan [**6**], dihydrodaidzein [**8**], and dihydrogenistein [**9**] were synthesized either from daidzein [**1**] or genistein [**2**] by hydrogenation. Similarly, the derivatives **4**, **7**, and **11** were synthesized from **3**, **6**, and **10**, respectively. During acetylation and nmr experiments, **9** was converted to a novel enol intermediate [**10**]. Antifungal, antibacterial, mosquitocidal, nematocidal, and topoisomerase inhibition activities of these compounds were evaluated, with equol [**3**] being the most active of the compounds tested against topoisomerase I.

Several epidemiological studies have shown that soybean products have reduced the incidence of breast cancer in women (1,2). Soybean diets were shown to lower mammary tumor rates in rats induced by radiation (3) or by the carcinogen, *N*-methyl-*N*-nitrosourea (MNU) (4). Isoflavonoids present in soybean metabolize in the digestive tract (5), and are considered to reduce the incidence of breast cancer (1). Animal studies also revealed that the soybean isoflavonoids daidzein [**1**] and genistein [**2**] are responsible for this protective effect (1,3,4). Genistein has been shown to inhibit H_2O_2 formation in vivo and in vitro (6,7) and also to act as an inhibitor of tyrosine kinase and DNA synthesis (8,9).

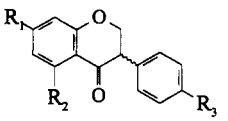
Several metabolites are detected in urine from human subjects on soybean diets (10). The major metabolites of **1** and **2** are their reduction products, equol [**3**], 7,4′-dihydroxyisoflavanone [**8**], tetrahydrodaidzein [**6**], and *O*-desmethylangolensin and 5,7,4′-trihydroxyisoflavanone [**9**]. The synthesis of isoflavanones and isoflavans from **1** and **2** was reported previously (11–13). Hydrogenation reactions of isoflavones were non-selective and often resulted in complex mixtures (11–13). A selective catalytic hydrogenation of several isoflavones to their corresponding isoflavanones has been published (14), but **1** and **2** were not used to produce isoflavanones by previous researchers (14). The biological activities of these metabolites, isolated from physiological samples in trace quantities, have not yet been evaluated. We have now synthesized compounds **3**, **5**, **6**, **8**, and **9** and the derivatives **4**, **7**, and **11**



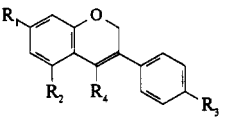
	R_1	R_2	R_3
1	OH	H	OH
2	OH	OH	OH



	R_1	R_2	R_3	R_4
3	OH	H	OH	H
4	OMe	H	OMe	H
5	OH	OH	OH	H
6	OH	H	OH	OH
7	OAc	H	OAc	OAc



	R_1	R_2	R_3
8	OH	H	OH
9	OH	OH	OH



	R_1	R_2	R_3	R_4
10	OH	OH	OH	OH
11	OAc	OAc	OAc	OAc

from **1** and **2**, respectively, in sufficient quantities to evaluate their biological activities.

Equol [**3**] was previously synthesized by the reduction of *0,0*-diacetyldaidzein followed by the hydrolysis of the resulting product in ethanolic NaOH (13). However, we have synthesized **3** by the hydrogenation of **1** synthesized previously in our laboratory (15) in glacial HOAc with Pd/C as catalyst, and confirmed its structure by comparison with ^{1}H- and ^{13}C-nmr data (13). Methylation of **3** afforded compound **4**, as confirmed by ^{1}H-nmr data.

Aldercreutz *et al.* (16) reported the synthesis of equol [**3**] through the hydrogenation of daidzein [**1**] in EtOH. In our laboratory, this procedure yielded only 4,7,4′-trihydroxyisoflavan [**6**] (10). The ^{13}C-nmr spectral data of **6** gave a signal at δ 67.70 assigned to C-4, which confirmed that the C=O group in **1** was partially reduced. Acetylation of **6** gave a triacetate, **7**, and provided additional evidence for the existence of a C-4 hydroxy group in **6**.

The hydrogenation of **1** over Pd/$BaSO_4$ in EtOH yielded **8** as the major product. The ^{1}H-nmr data of **8** were identical to published values (10,14). The ^{13}C-nmr spectrum of compound **8** showed a signal at δ 194.07 which indicated that the C=O group at C-4 was not reduced. Also, the ^{1}H-nmr spectrum of **8** did not exhibit an olefinic proton signal at δ 8.02. Therefore, the nmr data confirmed the existence of a C=O group at C-4 and the reduction of the olefinic bond between C-2 and C-3 in compound **8**.

Compound **9** was produced by the hydrogenation of **2** in EtOH over Pd/C. This compound gave a similar R_f value to **2** on Si gel tlc and its structure was confirmed by ^{1}H- and ^{13}C-nmr spectra. However, hydrogenation of **2** in glacial HOAc over Pd/C yielded a mixture of compounds **5** and **9** due to partial hydrogenation. Compound **5** had a lower R_f value on tlc than did **9**. The structure of compound **5** was identified as 5,7,4′-trihydroxyisoflavan by comparison of its nmr spectra with literature values (14).

Acetylation of compound **9** in pyridine and Ac_2O gave interesting results. Spectral characterization of the acetylated product **11** showed the presence of four acetate groups, even though the parent compound [**9**] had only three hydroxy groups. The H-2 proton of **11** in the ^{1}H-nmr spectrum appeared as a 2H singlet at δ 5.03. The signals at 136.14 and 111.29 ppm were assigned to the olefinic carbons C-3 and C-4, formed by the enolization of the C-4 carbonyl group.

The formation of **11** must involve the enol **10**, which means that during the acetylation process the keto form [**9**] was converted to the enol. The enolization of **9** was also observed during an nmr experiment with pyridine-d_5 as the solvent. The addition of 100 μl of HOAc-d_4 to a pyridine-d_5 solution of **9** caused an instant enolization of **9** to **10**. The H-2a and H-2b signals of **10** appeared as doublets at 4.48 and 4.43 ppm, respectively. However, the enol form was unstable and reverted completely to the keto form during isolation and purification. The same was true when attempts were made to hydrolyze the tetraacetate, **11**, to **10**. The enolization of **9** did not occur in CD_3OD/HOAc-d_4. The enolization of **9** in pyridine/HOAc may be induced by the OH-5 group in the A-ring complexing with pyridine. It is possible that **10** can

exist in biological systems as a metabolite of **2**. The related isoflavanone **8** did not enolize in pyridine-d_5 and HOAc-d_4.

Equol [**3**] showed growth inhibition for *Fusarium oxysporum, Fusarium moniliforme, Gleosporum* spp., *Rhizoctonia* spp., and *Aspergillus flavus* (all fungi), *Candida albicans* (yeast), and *Staphylococcus epidermidis, Streptococcus aureus,* and *Escherichia coli* (all bacteria) at 250 μg in plate assays. Mosquitocidal (*Aedes aegyptii* larvae) and nematocidal (*Panagrellus redivivus* and *Caenorhabditis elegans*) assays with **3** showed 100% mortality at 250 ppm within 24 h. Compound **9** inhibited the growth of all microorganisms tested at 250 μg concentration excluding *A. flavus* and *E. coli*. Neither mosquitocidal nor nematocidal activities were observed for **9**. Compounds **1**, **2**, **5**, **6**, **8**, and **11** were not active against these bacteria and fungi, the yeast, the mosquito larvae, or the nematodes, at the doses tested.

Genistein [**2**] is reported to have topoisomerase II inhibitory activity (17,18). Therefore, we have evaluated compounds **1–9** for both topoisomerase I and II activities using the mutant yeast strains JN394, JN394 t_1, and JN394 $t_{2\text{-}5}$ (19,20). In our experiments, the inhibitory concentration (IC_{50} value) for **2** was 250 ppm for JN394 and inhibited the growth of JN394 t_1 and JN394 $t_{2\text{-}5}$ by 30%. Compounds **1** and **4–7** did not show topoisomerase inhibition in preliminary plate assays. Camptothecin, a topo-I poison, was used as the positive control against JN394 and JN394 $t_{2\text{-}5}$ (97% inhibition of growth) at 10 ppm. Camptothecin has no effect on the growth of JN394 t_1 (20). Compounds **8** and **9** inhibited the growth of JN394 $t_{2\text{-}5}$, but had no effect on JN394. Compound **3**, the most active among all the compounds tested, inhibited the growth of these yeast strains and showed an IC_{50} value of 50 ppm.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H- and ^{13}C-nmr spectra were recorded on Varian VXR 300 and 500 MHz spectrometers, respectively, at room temperature. The mps were recorded on a Thomas model 40 micro hot-stage apparatus and were not corrected. Mass spectra were acquired on a JEOL HX-110 double-focusing mass spectrometer. Prep. tlc plates were purchased from Analtech Inc. (Newark, DE). Prep. recycle-hplc was used with a LC-20 and a C_{18} reversed-phase column (Jaigel, S-343-15; 15 μm, 250×20 mm), with the latter purchased from Dychrom (Santa Clara, CA).

CHEMICALS AND CELL CULTURE MEDIA.—Daidzein [**1**] and genistein [**2**] were synthesized in our laboratory (15). Pd/C and Pd/$BaSO_4$ (5%) were purchased from Aldrich Chemical Company (Milwaukee, WI). YMG (yeast extract 4 g/liter, maltose 10 g/liter, glucose 4 g/liter and agar 12 g/liter), PDA (potato dextrose agar) and Emmons (neopeptone 10 g/liter, glucose 20 g/liter, and agar 15 g/liter) media were prepared as described previously (21) and the ingredients were purchased from Difco Labs (Detroit, MI). NG medium (NaCl 3.0 g/liter, bacto peptone 2.5 g/liter, cholesterol 1 ml/liter from 5 mg/ml stock solution, $CaCl_2$ 1 ml/liter from 1 M stock solution, $MgSO_4$ 1 ml from 1 M stock solution, and potassium phosphate buffer 25 ml/liter of stock solution containing KH_2PO_4 11.97 g/100 ml and K_2HPO_4 2.09 g/100 ml) was used. YPDA medium (yeast extract 20 g/liter, peptone 10 g/liter and dextrose 20 g/liter, and adenine sulfate 2 ml/liter from 0.5% stock solution) was purchased from Difco Labs. Adenine sulfate and camptothecin were purchased from Aldrich Chemical Company (Milwaukee, WI).

HYDROGENATION OF ISOFLAVONES.—A solution of **1** or **2** (50 mg) in EtOH or glacial HOAc (100 ml) was bubbled with H_2 for 15 min and then added to a pre-reduced EtOH or glacial HOAc solution containing 5% Pd/C. The reaction mixture was stirred at room temperature under a H_2 atmosphere until the isoflavone was not detectable by tlc. The reaction mixture was filtered through a Celite bed and the resulting solution was dried *in vacuo*.

Equol [**3**].—The product from the hydrogenation of **1** (49 mg) in glacial HOAc was recrystallized from MeOH/H_2O to yield **3** (22.9 mg, 46.7%); mp 150–152°; its structure was confirmed by comparison of published spectral data (14).

7,4′-Dimethoxyequol [**4**].—A solution of compound **3** (20.4 mg) in Me_2CO (15 ml) was stirred with K_2CO_3 (5 g) for 15 min and refluxed with dimethyl sulfate (50 μl) for 8 h. The reaction mixture was cooled to room temperature, filtered, and the resulting solution dried *in vacuo*. The product was dissolved in $CHCl_3$ (50 ml) and washed with H_2O (20 ml×2) followed by washing with saturated $NaHCO_3$ solution (25 ml×1) and

H_2O (25 ml×2). The resulting $CHCl_3$ solution was evaporated *in vacuo* and the white precipitate was recrystallized from MeOH to give needle-like crystals (16 mg); mp 112–113°; its structure was confirmed by ^{1}H- and ^{13}C nmr spectroscopy.

4,7,4′-Trihydroxyisoflavan [**6**].—Amorphous powder (30 mg), produced from the hydrogenation of daidzein [**1**] (50 mg) using EtOH as the solvent (60% yield); mp 204–208°; nmr spectral data were identical to published values (10).

4,7,4′-Triacetylisoflavan [**7**].—To a solution of compound **6** (9.8 mg) in pyridine (1 ml), Ac_2O (200 μl) was added and left in the dark at room temperature for 24 h. The reaction mixture was dried *in vacuo* and purified by prep. tlc using $CHCl_3$-MeOH (12:1) as the mobile phase to yield an amorphous white solid (14.6 mg); mp 115–117°; its structure was confirmed by ^{1}H-nmr spectroscopy.

Dihydrodaidzein [**8**].—The reaction mixture from the hydrogenation of **1** in EtOH and Pd/$BaSO_4$ was purified by prep. recycle-hplc (C_{18} column, mobile phase H_2O-MeOH, 30:70, at a flow rate of 2 ml/min, detected at 210 nm) and yielded compound **8** (41.4%) as the major product; mp 198–200°; identification was made by comparison with published nmr spectral data (14).

Dihydrogenistein [**9**].—The reaction product from the hydrogenation of **2** in EtOH and Pd/C was purified by prep. tlc using a solvent system of $CHCl_3$-MeOH (10:1). The major component was recrystallized from MeOH/H_2O and gave colorless needle-like crystals, compound **9** (yield, 67%); mp 196–198°; confirmed by comparison with published spectral data (14).

5,7,4′-Trihydroxyisoflavan [**5**].—The hydrogenation of **2** with Pd/C in glacial HOAc yielded a mixture of two compounds. The reaction mixture was purified by prep. tlc developed with $CHCl_3$-MeOH (10:1). The compound with a R_f value similar to **2** was identified as compound **9** (yield, 17.8%). The second compound, with a lower R_f value, was recrystallized from MeOH/H_2O and gave needle-like crystals of compound **5** (yield, 27.0%); mp 209–211°; ^{1}H-nmr chemical shifts were identical to published values (14).

4,5,7,4′-Tetrahydroxyisoflavanone [**10**].—Compound **9** (12 mg) in pyridine-d_5 (0.75 ml) was treated with HOAc-d_4 (100 μl) in an nmr tube and its ^{1}H-nmr spectrum was recorded; ^{1}H nmr (pyridine-d_5 and 100 μl of HOAc-d_4) δ 7.20 (2H, d, J=8.7 Hz, H-2′, H-6′), 7.05 (2H, d, J=8.7 Hz, H-3′, H-5′), 6.36 (1H, d, J=1.8 Hz, H-6, H-8), 4.48 (1H, d, J=12.0 Hz, H-2a), 4.43 (1H d, J=12.0 Hz, H-2b).

4,5,7,4′-Tetraacetateisoflavanone [**11**].—Ac_2O (200 μl) was added to a solution of compound **9** (20 mg) in pyridine (1 ml) and stored in the dark at room temperature for 24 h. Crushed ice was added into the reaction mixture and the white precipitate formed was isolated by centrifugation. This precipitate was then recrystallized from MeOH to give colorless needle-like crystals of the novel compound **11** (22 mg); mp 191–192°; ^{1}H nmr ($CDCl_3$) δ 7.36 (2H, d, J=8.7 Hz, H-2′, H-6′), 7.11 (2H, d, J=8.7 Hz, H-3′, H-5′), 6.66 (1H, d, J=2.1 Hz, H-6), 6.47 (1H, d, J=2.4 Hz, H-8), 5.03 (2H, s, H-2), 2.31 (3H, s, Me), 2.29 (3H, s, Me), 2.27 (3H, s, Me), 2.10 (3H, s, Me); ^{13}C nmr ($CDCl_3$) δ 167.92 (C=O), 168.77 (C=O), 167.97 (C=O), 167.29 (C=O), 156.13 (C-8a), 150.72 (C-4′), 149.83 (C-5), 145.38 (C-7), 136.14 (C-4), 130.74 (C-1′), 128.58 (C-2′, C-6′), 121.31 (C-3′, C-5′), 111.29 (C-3), 109.88 (C-8), 107.54 (C-6), 68.55 (C-2), 20.62 (2×Me), 20.49 (Me), 20.08 (Me).

ANTIMICROBIAL ASSAYS.—Antifungal and antibacterial assays of compounds **1–3**, **5**, **6**, **8**, **9**, and **11** were carried out according to procedures reported earlier (21). Cultures of *Fusarium oxysporum* (MSU-SM-1322), *Fusarium moniliforme* (MSU-SM-1323), *Gleosporum* spp., and *Rhizoctonia* spp. were grown on potato dextrose agar (PDA) medium. Cultures of *Candida albicans* and *Aspergillus flavus* (MSU strains) were grown on YMG medium, and cultures of *Staphylococcus epidermidis* (ATCC 25923), *Streptococcus aureus* (MSU strain), and *Escherichia coli* (ATCC 25922) were grown on Emmons medium.

MOSQUITOCIDAL AND NEMATOCIDAL ASSAYS.—Mosquito larvae, *Aedes aegyptii* (Michigan State University, courtesy of Dr. A. Raikhel), were used to test the insecticidal properties of compounds **1–3**, **5**, **6**, **8**, **9**, and **11** using the procedure reported earlier (21). Nematocidal activity was carried out on *Panagrellus redivivus* and *Caenorhabditis elegans* using the procedure reported earlier (22).

TOPOISOMERASE ASSAYS.—*Saccharomyces cerevisiae* mutant cell cultures, JN394, JN394 t_1, and JN394 t_{2-5}, were supplied by one of us (J.L.N.) (19,20). The organisms were cultured in Petri dishes containing YPDA medium (20 ml). The cells from a fully grown plate were suspended in saline solution (10 ml), which was diluted to obtain 5×10^6 CFU/ml. YPDA liquid medium (1.95 ml) was inoculated with 25 μl of the cell suspensions (5×10^6 CFU/ml) from JN394, JN394 t_1, and JN394 t_{2-5}, respectively. The test compounds, daidzein [**1**], genistein [**2**], dihydrodaidzein [**8**], and dihydrogenistein [**9**] were dissolved in DMSO and were added to the test tubes (25 μl) to give the final concentration at 250 ppm. Each treatment was repeated in triplicate (data not shown). The positive control, a topo-I poison, camptothecin, was tested at 10 ppm. Because equol [**3**] showed an excellent activity in the preliminary plate assay, it was tested at con-

centrations of 100, 50, 25, and 10 ppm. The test tubes containing cell cultures and compounds were incubated at 27° for 24 h. At the end of the incubation period, a serial dilution of each cell suspension was prepared. An aliquot (100 μl) from each dilution was spread evenly on a Petri dish containing YPDA media and incubated at 27° for 72 h. The number of colonies were counted at the end of the incubation period and evaluated for the activity of test compounds.

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