Synthesis of Novel Mammalian Metabolites of the Isoflavonoid Phytoestrogens Daidzein and Genistein

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Abstract. **The synthesis of novel mammalian metabolites of dietary isoflavones, dihydrodaidzein (4′,7-dihydroxyisoflavanone) *7*,[2] dihydrogenistein (4′,5,7-trihydroxyisoflavanone) *9*, 6′-hydroxy-*O*-demethylangolensin [1-(2,4,6-trihydroxyphenyl)-2-(4-hydroxyphenyl)propan-1-one] *10*, and *cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ols *11*, *12* is described, and their characteristics by physical and chemical constants given for the first time.** [P.S.E.B.M. 1998, Vol 217]

Epidemiological studies reveal that foodstuffs containing isoflavonoid phytoestrogens may reduce the risk of certain hormone-dependent cancers such as breast, prostate and colon cancer (1–8). Vegetarian and semivegetarian diets, especially in some developing and Asian countries, are rich in plant isoflavonoids (9). Legumes (soy, lentils, various peas, beans, etc.), whole grains, vegetables, fruits, and berries are important ingredients responsible for the isoflavonoid content in those diets. Soy foods, in particular, associated with a reduced risk of breast and prostate cancers in Japan (3), provide significant amounts of isoflavones to the human diet. Various soy foods may contain approximately 0.1 mg–300 mg of isoflavones/100g. Soymilk and soynuts, examples of nonfermented soy foods, have two to three times more isoflavones than fermented soy foods such as tempeh, bean paste, miso, and fermented beancurd (10). The major isoflavones in soybeans are the acylated isoflavone glycosides genistin, daidzin, and glycitin and their aglycones, genistein (4′,5,7-trihydroxyisoflavone) *1* and daidzein (4′,7-dihydroxyisoflavone) *2* and glycitein (4′,7-dihydroxy-6-methoxyisoflavone). Other common isoflavones in edible plants are biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) and formononetin (7-hydroxy-4′-methoxyisoflavone) *3* (11, 12).

Daidzein, formononetin, genistein, and biochanin A possess estrogenic activity (12). This biological effect was first identified in sheep (13), later in cattle (14), and is now implicated in humans. These phytoestrogen isoflavones seem to affect hormone production and metabolism as well as cancer cell growth by a number of mechanisms that make them candidates for a protective role with regard to breast and prostate cancers particularly (1, 2, 5, 15–19). Since the detection and identification of isoflavonoid phytoestrogens in animals and humans, many studies on their biological role in health and disease have been carried out, and several reviews on the topic have appeared (1, 4, 12, 15–17, 20, 21).

The metabolism of the major plant isoflavones genistein *1*, daidzein *2*, biochanin A, and formononetin *3* has been studied in domestic animals such as sheep (13, 22–24), cattle (14, 24–26), horses (27), poultry (28–30) and dogs (31). There are also a few studies on subhuman primates (32–35), but the metabolism of these dietary isoflavones in man is not well known.

Most of the studies on the metabolism of phytoestrogen isoflavones have concerned sheep. Formononetin *3*, a major isoflavone in legume fodder (e.g., clover), is demethylated to daidzein *2* in the rumen. Daidzein *2* is reduced to equol *4*, which is resistant to further breakdown (23, 36, 37). In sheep, more than 80% of ingested formononetin *3* may ultimately be absorbed into the bloodstream as equol (4′,7-dihydroxyisoflavan) *4*. Much of this is rapidly transformed to a water soluble metabolite in the liver and excreted in the urine, but some invariably persists in the blood plasma either in "free" form or as a sulfoconjugate, exerting a harm-

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[2] The numbers in italics refer to code numbers assigned these compounds and used in the figures.

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ful and prolonged influence on the female reproductive cycle (23, 36). Alternative minor pathways of formononetin *3* degradation lead to 4′-*O*-methylequol (7-hydroxy-4′-methoxyisoflavan) *5* and *O*-demethylangolensin [1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)propan-1-one] *6* (5%–20%), both of which are estrogenic (13, 23), equol *4* and genistein *1* (20) being the most potent estrogens of the phytoestrogens. Biochanin A is demethylated to genistein *1*, which is degraded to the biologically inactive *para*-ethylphenol and 2,4,6-trihydroxybenzoic acid in the rumen (13).

The dietary isoflavones, genistein *1*, daidzein *2*, and the metabolites equol *4* and *O*-demethylangolensin *6* and their conjugates (38) have been isolated from the urine of macrobiotics, vegetarians, and also of omnivorous people consuming their everyday food (16, 20, 39). Formononetin *3* (40), and glycitein (4′,7-dihydroxy-6-methoxy-isoflavone) (41) have also been isolated from human urine. By a sensitive isotope dilution gas chromatography–mass spectrometric method (id-gc-ms) genistein *1*, daidzein *2*, equol *4*, and *O*-demethylangolensin *6* have been detected also in human feces (42) and plasma (43–45).

A metabolic pathway for dietary isoflavones in man has been proposed (1) on the basis of the isolation and identification of isoflavonoid phytoestrogens from human urine. The metabolism of isoflavones in humans seems to be similar to that in animals in general. In human (both male and female) ingestion, phytoestrogens are modified by intestinal bacteria, absorbed from the gastrointestinal tract, and then they undergo hepatic metabolism (1). Formononetin *3* undergoes demethylation, yielding daidzein *2*, which is reduced to the isoflavan equol *4*. Further degradation of equol appears not to occur, and most of the absorbed equol is conjugated in the liver with glucuronic acid before being excreted in the urine (1). Adlercreutz *et al.* have isolated and identified equol *4* (18), 4′-*O*-methylequol *5* (1, 17, 40), 3′,7-dihydroxyisoflavan (46), dihydrodaidzein (also called Intermediate *O*) *7* (1, 17, 40), and *O*-demethylangolensin *6* (47) as metabolites of daidzein *2* and/or formononetin *3* by using authentic synthetic reference compounds (Fig. 1). Four of these can now be quantitatively measured by id-gc-ms selected ion monitoring (sim) techniques and isotope dilution mass spectrometry using deuterated internal standards (17, 39, 48). Another potential metabolite of daidzein *2*, named Intermediate E *8*, has also been detected (1, 17, 40) and tentatively identified as 4′,7-dihydroxyisoflav-2-ene, but full verification awaits a chemical synthesis of the compound. Very recently five new potential metabolites of dietary isoflavones were reported by Kelly *et al.* in addition to genistein *1*, daidzein *2*, glycitein and the isoflavonoid metabolites equol *4*, dihydrodaidzein *7* and *O*-demethylangolensin *6*, from the urine of Caucasian people who were on a soy flour diet (41). The urine samples were analysed by gc-ms, suggesting two potential metabolites of genistein *1*, dihydrogenistein *9* and 6′-hydroxy-*O*-demethylangolensin *10* (Fig. 2). It was confirmed that daidzein *2* is reduced to dihydrodaidzein *7*, and suggested that the latter is further reduced to two tetrahydrodaidzeins (4′,7-dihydroxyisoflavan-4-ols) *11, 12*, which differ only in their stereochemistry, and to dehydro-*O*-demethylangolensin *7d*. The tentative identification of the five new potential metabolites was mainly based on mass spectral evidence owing to the lack of authentic reference compounds. In a subsequent paper by the same group, certain microscale syntheses of the potential metabolites were reported with very little experimental detail and no nuclear magnetic (nmr), infrared (ir), or ultraviolet (uv) spectroscopic characterization of the compounds in product mixtures (49). We now report unambiguous chemical synthesis of these human endogenous metabolites of the dietary isoflavones daidzein and genistein, and present their characterization by ^{1}H, ^{13}C nmr, ir, ms, and uv.

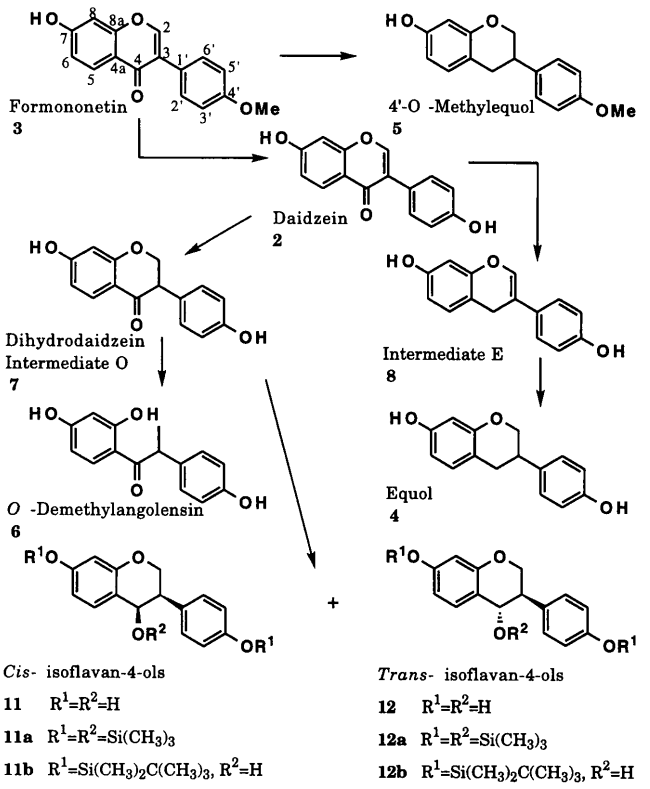

Figure 1. The proposed metabolic pathway of dietary plant isoflavonoids daidzein *2* and formononetin *3* in humans.

Materials and Methods

M.p.s were determined in open capillary tubes with an Electrothermal apparatus (Electrothermal Engineering Ltd., Essex, UK) and are uncorrected. Ir spectra were recorded on a FTIR Biorad FTS-7 instrument (Biorad Corp., Hercules, CA) on KBr disks. ^{1}H- and ^{13}C-nmr spectra were recorded on a Varian GEMINI-200 FT NMR spectrometer (Varian Instruments, Palo Alto, CA) using the standard $^{1}H/^{13}C$ dual probe (chemical shifts in ppm). *J* values are given in Hz. Lrms and hrms spectra were obtained with a JEOL JMS SX102 mass spectrometer operating at 70 eV. Samples were introduced at 120°–150°C by a direct inlet probe. The mass

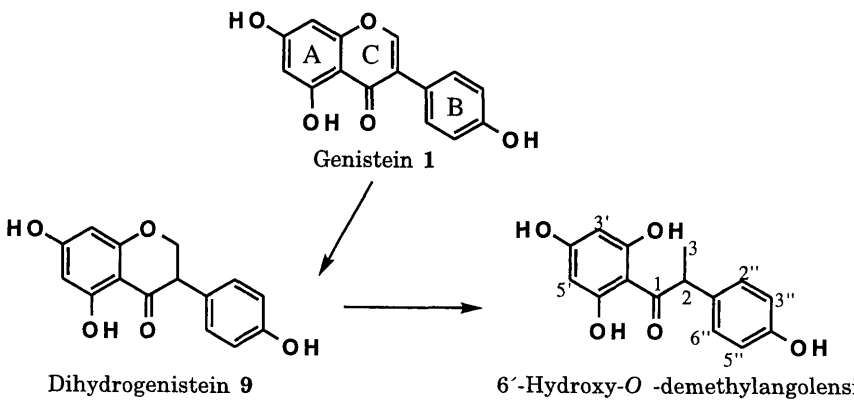

Figure 2. The proposed metabolic pathway for genistein *1* in humans.

spectrum of TMS-enolether of dihydrodaidzein *7b* was obtained with a Hewlett-Packard gc-ms (5890/5972) instrument (Palo Alto, CA) equipped with a HP 1 (12 m, 0.2 mm i.d.) silica capillary column with a film thickness of 0.33 μm. The carrier gas was helium at a flow rate of 1 ml/min. The column temperature was programmed from 197°C–280°C at 10°C/min scanning over the mass range 50–700. The injector temperature was 250°C and detector temperature 280°C. Injections were made in the splitless mode. *Cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ols *11, 12* were separated on an hplc instrument Isco Model 2350 (Lincoln, NE), equipped with a Shimadzu (Tokyo, Japan) SDP-6A spectrophotometric detector monitoring adsorption at 220 nm and a Shimadzu C-R6A integrator. The sample was filtered through a Millex LCR 0.45 μm cartridge (Millipore) and injected via a Rheodyne (Cotati, CA) Model 7125 sample injector with a 1-ml loop. A semipreparative column was a C_{18}-Silasorb 250 × 10 mm i.d., 15 μm. Mobile phase (MeOH-H_2O, 5: 6) was delivered at a flow rate of 9 ml/min. The methanol used was hplc grade and water filtered through a 0.45 μm membrane filter (Millipore). TMS ethers of *cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ols *11a, 12a* were separated by gc-ms analysis using a JEOL JMS SX102 mass spectrometer (Tokyo, Japan) and a Hewlett-Packard 5890A gas chromatograph equipped with SE 54 (15 m, 0.32 μm i.d.) capillary column. The column temperature was programmed from 100°C–240°C at 10°C/min. The injector temperature was 250°C, and the detector temperature was 300°C. The UV spectra were recorded with a CARY 5E UV-VIS-NIR spectrophotometer (Varian Australia Pty Ltd., Mulgrave, Victoria, Australia). Tlc was conducted on Merck (Merck KGaA, Darmstadt, Germany) silica gel 60 F_{254} plates, and Merck silica gel 60 (0.040 mm–0.063 mm, 230–400 mesh) was used for flash chromatography. Tetrahydrofuran (THF) was dried by distilling over CaH_2 before use.

Dihydrodaidzein *7.* Daidzein *1* (0.255 g, 1 mmol) was dissolved in methanol (20 ml) and 0.255 g 10% Pd/C and ammonium formate (0.252 g, 4 mmol) were added under argon. After refluxing for 2 hr, the reaction mixture was cooled, the Pd/C filtered off, the solvent evaporated, and the crude product was recrystallized from CH_2Cl_2-EtOAc to give *7* (0.211 g, 82%), m.p. 250°C (lit. (50) 250–1°C); ir ν_{max} 3262 (OH) 1658 cm^{-1} (CO); ^1H nmr (d_6-acetone); δ 3.86 (t, 1H, H-3), 4.62 (d, 2H, H-2, J = 6.7), 6.41 (d, 1H, H-8, J = 2.3), 6.57 (dd, 1H, H-6, J = 8.8 and 2.3), 6.80 (d, 2H, H-3′,5′, J = 8.2), 7.15 (d, 2H, H-2′, 6′, J = 8.2), 7.75 (d, 1H, H-5, J = 8.7); ^{13}C nmr (d_6-DMSO), δ 50.03 (C-3), 71.15 (C-2), 102.16 (C-8), 110.52 (C-6), 113.52 (C-4a), 115.09 (C-3′,5′), 126.08 (C-1′), 128.92 (C-5), 129.49 (C-2′,6′), 156.38 (C-4′), 162.97 (C-8a), 164.34 (C-7) and 190.50 (C-4); λ_{max} (94% EtOH)/nm 214 (ϵ 21900), 278 (13600) and 313 (7850); *m/z* 256 (M^+, 22%), 137 (100), 120 (35), 108 (5), and 91 (7); (Found: M^+, 256.0744. $C_{15}H_{12}O_4$ requires M, 256.0736).

Dihydrogenistein *9.* Genistein *1* was reduced as described above and purified by a flash filtration giving *9* as white crystals (0.154 g, 60%), m.p. 220°C (lit. (51) 217-18°C); ir υ_{max} 3440, 3152 (OH) 1645 cm^{-1} (CO);^1H nmr (d_6-acetone); δ 3.97 (t, 1H, H-3), 4.60 (d, 2H, H-2, J = 5.8), 5.95 (s, 2H, H-6,8), 6.82 (d, 2H, H-3′,5′, J = 6.6), 7.20 (d, 2H, H-2′,6′, J = 6.6); ^{13}C nmr (d_6-acetone); δ 50.91 (C-3), 72.15 (C-2), 95.59 (C-8), 96.94 (C-6), 103.21 (C-4a), 116.31 (C-3′,5′), 127.40 (C-1′), 130.61 (C-2′,6′), 157.78 (C-4′), 164.25 (C-5), 165.73 (C-8a), 167.35 (C-7), 197.86 (C-4); λ_{max} (94% EtOH)/nm 214 (ϵ 24900) and 292 (18300); *m/z* 272 (M^+, 41%), 252 (10), 235 (5), 153 (100), and 120 (35); (Found: M^+, 272.0691. $C_{15}H_{12}O_5$ requires M, 272.0685).

4,4′,7-tris-(trimethylsilyloxy)isoflav-3-ene *7b.* Dihydrodaidzein *7* (0.066 g, 0.26 mmol) was dissolved in pyridine (5 ml) and BSTFA (1 ml). The reaction mixture was stirred at 60°C for 1 hr, and LDA in dry THF (0.39 ml, 0.34 *M*) and BSTFA (1 ml) were added. After 2.5 hr the reaction mixture was poured into water, neutralized with 2*M* HCl and extracted with ether. The ether phase was washed with diluted HCl and dried with Na_2SO_4. After evaporation the crude product was crystallized from *n*-hexane to give *7b* (0.060 g, 50%), m.p. 120°C; ^1H nmr (d_6-acetone); δ 0.07 (s, 6H, 2 × Si-CH_3), 0.00 (s, 15H, 5 × Si-CH_3), 0.26 (s, 6H, 2 × Si-CH_3), 4.94 (s, 2H, H-2), 6.28 (d, 1H, H-8, J = 2.3), 6.42 (dd, 1H, H-6, J = 2.3 and 8.3), 6.84 (d, 2H, H-3′,5′, J = 8.7), 7.18 (d, 1H, H-5, J = 8.3), 7.35 (d, 2H, H-2′,6′, J = 8.7); ^{13}C nmr (d_6-acetone); δ 1.50 (Si-CH_3), 70.07

(C-2), 103.35 (C-3), 108.97 (C-8), 110.94 (C-6), 116.10 (C-4a), 120.79 (C-3′,5′), 124.96 (C-5), 128.54 (C-1′), 130.94 (C-2′,6′), 142.33 (C-4), 154.86 (C-4′), 157.65 (C-7) and 159.71 (C-8a); *m/z* 473 (41%), 472 (M^+, 97), 471 (100), 457 (31), 383 (21), 307 (11), 281 (22), 209 (9), 191 (10), and 73 (87). (Found: M^+, 472.1923. $C_{24}H_{36}O_4Si_3$ requires M, 472.1921).

6′-Hydroxy-*O*-demethylangolensin *10.* A solution of genistein *1* (0.2 g, 0.7 mmol) in THF (10 ml) was added over 30 min to a stirred slurry of lithium aluminium hydride (0.15 g, 4 mmol) in refluxing THF (5 ml). Refluxing was continued for 2.5 hr, the reaction mixture was cooled and poured into an icy NH_4Cl (sat.) solution. The mixture was neutralized with 2*M* HCl and some water added and extracted with EtOAc. The extract was washed with water, dried over Na_2SO_4 and evaporated. The residue (0.2 g, 100%) was purified by flash chromatography, eluent CH_2Cl_2-EtOAc 1:1 v/v. Crystallization from benzene gave reddish crystals of *10* (0.132 g, 66%), m.p. 99°C; ir υ_{max} 3379 (OH), 2978, 2936, 1632 cm^{-1} (CO); ^1H nmr (d_6-acetone); δ 1.39 (d, 3H, 3-H, J = 6.9), 5.25 (q, 1H, 2-H, J = 6.9), 5.89 (s, 2H, 3′,5′-H), 6.70 (d, 2H, 3″,5″-H, J = 8.6), 7.15 (d, 2H, 2″,6″-H, J = 8.6); ^{13}C nmr (d_6-acetone); δ 19.86 (C-3), 49.34 (C-2), 95.92 (C-3′,5′), 104.79 (C-1′), 115.85 (C-3″,5″), 129.95 (C-2″,6″), 134.11 (C-1″), 156.87 (C-4″), 165.65 (C-2′,6′,4′) and 207.32 (C-1); λ_{max} (94% EtOH)/nm 224 (ε 7990) and 294 (6440); *m/z* 274 (M^+, 8%), 272 (25), 257 (10), 153 (100), and 121 (22) (Found: M^+, 274.0835. $C_{15}H_{14}O_5$ requires M, 274.0841).

Cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ols *11, 12. Dihydrodaidzein *7* (0.150 g, 0.59 mmol) in THF (12 ml) was added slowly to a solution of $LiBH_4$ (0.089 g, 40 mmol) in THF (6 ml) in an ice bath under argon. The reaction was monitored by tlc and after 27 hr at r.t., the reaction was quenched by pouring the reaction mixture cautiously into a cold saturated NH_4Cl solution, extracted with EtOAc, dried with Na_2SO_4, and evaporated giving a mixture of two isomers in a 7:3 ratio (0.14 g, 94%). The isomers were separated by preparative HPLC.

Cis-4′,7-dihydroxyisoflavan-4-ol *11* m.p. 167°C (dec.); ^1H nmr (d_6-acetone); δ 3.12 (dt, 1H, H-3, J_d = 12.4, J_t = 3.2), 3.89 (d, 1H, 4-OH, J = 4.8), 4.13 (ddd, 1H, H-2_β equatorial, J = 10.3&3.7&1.3), 4.51 (dd, 1H, H-2_α axial, J = 10.3&12.1), 4.64 (br t, 1H, H-4, J = 4.0), 6.29 (d, 1H, H-8, J = 2.4), 6.39 (dd, 1H, H-6, J = 8.2&2.4), 6.79 (d, 2H, H-3′,5′, J = 8.4), 7.07 (d, 1H, H-5, J = 8.2), 7.19 (d, 2H, H-2′,6′, J = 8.4); ^{13}C nmr (d_6-acetone); δ 44.74 (C-3), 65.30 (C-2), 67.26 (C-4), 103.30 (C-8), 108.81 (C-6), 115.85 (C-3′,5′), 118.31 (C-4a), 130.58 (C-2′,6′), 130.87 (C-1′), 132.33 (C-5), 156.04 (C-4′), 157.12 (C-7), and 159.18 (C-8a); λ_{max} (94% EtOH)/nm 199 (ε 68200), 224s (19700) and 280 (4850); *m/z* 240 (M^+-H_2O, 90%), 239 (100), 223 (7), 210 (5), 181 (5), 165 (5), 147 (15), 120 (18), and 71 (10). 258 (M^+) was detected at 19 eV. (Found: M^+, 258.0874. $C_{15}H_{14}O_4$ requires M, 258.0892).

Trans-4′,7-dihydroxyisoflavan-4-ol *12* m.p. 124°C (dec.); ^1H nmr (d_6-acetone); δ 3.00 (dt, 1H, H-3, J_d = 4.0, J_t = 7.2), 4.18 (dd, 1H, H-2_α axial, J = 7.9 and 10.5), 4.25 (dd, 1H, H-2_β equatorial, J = 11.0 and 4.0) 4.77 (br t, 1H, H-4, J = 6.4), 6.25 (d, 1H, H-8, J = 2.4), 6.42 (dd, 1H, H-6, J = 8.4 and 2.4), 6.72 (d, 2H, H-3′,5′, J = 8.6), 7.16 (d, 2H, H-2′,6′, J = 8.6), 7.26 (d, 1H, H-5, J = 8.4); ^{13}C nmr (d_6-acetone); δ 47.22 (C-3), 68.87 (C-2), 69.26 (C-4), 103.06 (C-8), 109.13 (C-6), 116.08 (C-3′,5′), 118.47 (C-4a), 129.93 (C-2′,6′), 130.81 (C-5), 131.71 (C-1′), 156.33 (C-4′), 157.17 (C-7), and 158.71 (C-8a); λ_{max} (94% EtOH)/nm 199 (ε 44600), 225s (14100), and 280 (3550); *m/z* 258 (M^+, 2%), 254 (8), 240 (M^+-H_2O, 90), 239 (100), 238 (62), 226 (7), 210 (27), 197 (5), 181 (18), 165 (8), 147 (14), and 120 (15). (Found: M^+, 258.0901. $C_{15}H_{14}O_4$ requires M, 258.0892).

Cis-4′,7-di(trimethylsilyloxy)isoflavan-4-ol *11a*

Cis-4′,7-dihydroxyisoflavan-4-ol *11* was silylated as reported (41).

t_R 11′04″; *m/z* 474 (M^+, 2%), 385 (20), 384 (37), 309 (75), 307 (45), 294 (17), 282 (18), 268 (22), 267 (100), and 75 (55).

Trans-4′,7-di(trimethylsilyloxy)isoflavan-4-ol *12a*

Trans-4′,7-dihydroxyisoflavan-4-ol *12* was silylated as reported (41).

t_R 10′52″ *m/z* 474 (M^+, 2%), 385 (18), 384 (45), 383 (28), 309 (100), 307 (59), 294 (29), 268 (20), 267 (83), and 75 (66).

Cis- and *trans*-4′,7-bis-(dimethyl-*tert*-butylsilyloxy)isoflavan-4-ols *11b, 12b* Dihydrodaidzein *7* (0.92 g, 3.6 mmol) was silylated by stirring with *tert*-butyldimethylchlorosilane (1.35 g, 9 mmol) and imidazole (0.61g, 9 mmol) in dry *N,N*-dimethylformamide (10 ml). After 36 hr at r.t. the mixture was poured in water and extracted with ether. After evaporation 4′,7-bis-(dimethyl-*tert*-butylsilyloxy)isoflavan-4-one was obtained in quantitative yield (1.74 g). This compound (0.100 g, 0.2 mmol) in THF (6 ml) was added to a solution of $LiBH_4$ (0.009 g, 0.4 mmol) in THF (15 ml) under argon at 0°C. After 22 hr at r.t., the reaction mixture was poured into a cold saturated NH_4Cl solution, extracted with CH_2Cl_2, dried with Na_2SO_4, and evaporated to furnish a solid mixture of *cis*- and *trans*-4′,7-di(dimethyl-*tert*-butylsilyloxy)isoflavan-4-ols (in a ratio of 7:3) (0.096 g, 96%). Flash chromatography (eluting with hexane-acetone 7:4) gave pure *cis*-4′,7-bis-(dimethyl-*tert*-butylsilyloxy)isoflavan-4-ol *11b* (0.032 g), m.p. 110°C; R_f = 0.72 (hexane-acetone 7:4); ^1H nmr ($CDCl_3$); δ 0.21 (s, 12H, 4 × CH_3), 1.00 (s, 18H, 6 × CH_3), 3.23–3.30 (dt, 1H, H-3, J = 3.1 and 11.9), 4.23–4.31 (dd, 1H, H-2, J = 3.6 and 10.3), 4.49–4.60 (dd, 1H, H-2, J = 10.5), 4.70 (d, 1H, H-4, J = 2.3), 6.41 (d, 1H, H-8, J = 2.4), 6.47 (d, 1H, H-6, J = 2.5 and 8.2), 6.85 (d, 2H, H-3′,5′, J = 8.5), 7.17 (dd, 3H, H-2′,6′,5, J = 1.6 and 8.4); ^{13}C nmr ($CDCl_3$); δ -4.41 (Si-CH_3), 18.19 (C-$(CH_3)_3$), 25.66 (3 × CH_3), 43.52 (C-3), 64.10 (C-2), 66.82 (C-4), 107.84 (C-8), 113.28 (C-6), 116.89 (C-4a), 120.37 (C-3′,5′), 129.24 (C-2′,6′), 130.23 (C-1′), 131.19 (C-5), 154.99 (C-7,4′), and 157.02 (C-8a);

m/z 486 (M^+, 10%), 470 (18), 469 (25), 468 (60), 309 (35), 307 (20), 235 (23), 234 (100), 177 (45), and 75 (42), and pure *trans*-4′,7-bis-(dimethyl-*tert*-butylsilyloxy)isoflavan-4-ol *12b* (0.013 g), m.p. 88°C; R_f = 0.61 (hexane-acetone 7:4); ^1H nmr ($CDCl_3$); δ 0.19 (s, 12H, 4 × CH_3), 0.98 (s, 18H, 6 × CH_3), 3.00–3.14 (m, 1H, H-3), 4.14–4.38 (m, 2H, H-2), 4.86 (d, 1H, H-4, J = 7.7), 6.35 (d, 1H, H-8, J = 2.4), 6.48 (dd, 1H, H-6, J = 2.3 and 8.4), 6.82 (d, 2H, H-3′,5′, J = 8.6), 7.10 (d, 2H, H-2′,6′, J = 8.6), 7.30 (d, 1H, H-5, J = 8.4); ^{13}C nmr ($CDCl_3$); δ 4.42 (Si-CH_3), 18.20 (C-$(CH_3)_3$), 25.65 (3 × CH_3), 46.46 (C-3), 68.27 (C-2), 69.35 (C-4), 107.57 (C-8), 113.44 (C-6), 117.85 (C-4a), 120.38 (C-3′,5′), 128.88 (C-1′,2′,6′), 131.13 (C-5), 155.01 (C-7,4′) and 156.48 (C-8a); *m/z* 486 (M^+, 2%), 470 (18), 469 (41), 468 (100), 467 (17), 309 (32), 307 (18), 177 (14), 75 (27), 57 (30).

Results

Having developed an easy access to a number of isoflavones by a one-pot reaction starting from the appropriate phenol and an arylacetic acid (52, 53), our syntheses of the potential metabolites were designed on functional group changes in the isoflavone structures.

Since the hydrogenation of isoflavones with palladized charcoal (Pd/C) and hydrogen gave predominantly isoflavans, a milder hydrogenation method was used. The hydrogen transfer reduction (54) of daidzein *2* with ammonium formate in refluxing methanol over 10% Pd/C produced dihydrodaidzein *7* in 82% yield. The same method was applied successfully to genistein *1*, giving dihydrogenistein *9* in high purity.

6′-Hydroxy-*O*-demethylangolensin *10* was prepared from genistein *1* by the reduction and alkaline cleavage of the C ring with lithium aluminum hydride. The two stereoisomers of tetrahydrodaidzein *11, 12* were synthesized by reducing dihydrodaidzein *7* with excess lithium borohydride in THF to furnish a mixture of *cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ols *11, 12* in a 7:3 ratio. The slight difference in their R_f values made them detectable by tlc, but this was not enough to allow separation by plc or flash chromatography. Thus the *cis*- and *trans*-isoflavan-4-ols *11, 12* were isolated by preparative hplc using an RP 18 column and MeOH-H_2O elution. However, flash chromatography was used successfully in the separation of the *cis* and *trans* products *11b, 12b* after the $LiBH_4$ reduction of the bis-dimethyl-*tert*-butylsilyl protected dihydrodaidzein. We also derivatized *cis*- and *trans*-isoflavan-4-ols as their TMS-ethers *11a, 12a* and detected their mass spectra by gc-ms.

The chemical structures of *cis*- and *trans*-4′,7-dihydroxyisoflavan-4-ols are assigned by their 200 MHz ^1H nmr spectra. The chemical shift of H-3 in *cis*-4′,7-dihydroxyisoflavan-4-ol appears as a doublet of triplets at 3.12 ppm, the coupling constant of the doublet being 12.4 Hz and 3.2 Hz for the triplet. In the *trans* isomer H-3 is assigned at 3.00 ppm with J_d = 4.0 and J_t = 7.2 as expected. The geminal protons at C-2 in the *cis* isomer produce an AB quartet ($J_{A,B}$ = 10.3 Hz, $J_{H\text{-}2\beta,H\text{-}3}$ = 3.7 with long range coupling $J_{H\text{-}2\beta,H\text{-}4}$ = 1.3, confirmed by long-range HETCOR) at 4.13 and 4.51 ppm. In the *trans* isomer the long-range coupling is not seen, and the doublet of doublets for the axial H-2_α is observed at 4.18 with $J_{H\text{-}2\alpha,H\text{-}3}$ = 7.9 and $J_{H\text{-}2\alpha,H\text{-}2\beta}$ = 10.5 Hz, and a doublet of doublets for H-2_β equatorial at 4.25 with $J_{H\text{-}2\beta,H\text{-}3}$ = 4.0 and $J_{H\text{-}2\beta,H\text{-}2\alpha}$ = 11.0 Hz. The proton of C-4 in both isomers gives a broadish signal with poorly resolved peaks due to coupling with the geminal hydroxy group.

Discussion

Kelly *et al.* (41, 49) reported of three peaks in their gc-ms analysis, two at MU 26.65, MU 27.11 assigned as

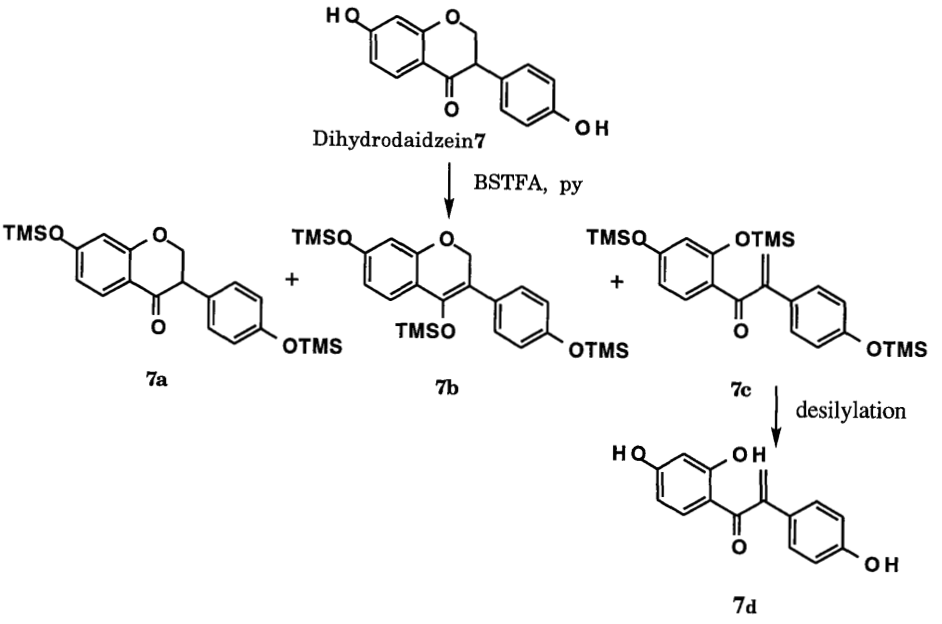

Figure 3. Silylation of dihydrodaidzein *7* by BSTFA in pyridine.

keto *7a* and enol *7b* forms of silylated dihydrodaidzein *7*, and the third at MU 25.92 (M^+ 472, base peak *m/z* 281) referred to as dehydro-*O*-demethylangolensin *7c* (Fig. 3). Also Woodward (55) has reported of more than one compound in analysing the bis-trimethylsilyltrifluoroacetamide (BSTFA) silylated dihydrodaidzein *7* in gc-ms. We have not previously observed keto-enol tautomerization of dihydrodaidzein *7*, but we have not used BSTFA and pyridine for derivatization like Kelly and co-workers (41, 49). We routinely silylate the isoflavonoid samples by 200 μl of pyridine/hexamethyldisilazane/trimethylchlorosilane (9:3:1 v/v/v) at room temperature overnight, and this procedure furnishes the desired silylated ketonic product *7a* in quantitative yield. When silylated with BSTFA and pyridine, dihydrodaidzein *7* indeed gave three products. Two of the products showed a molecular ion 72 mass unit heavier than the expected dihydrodaidzein bis-TMS ether *7a*, indicating the incorporation of an additional TMS group. This and the fragmentation patterns in ms spectra suggest that the three compounds are the bis-trimethylsilyl ether of isoflavanone (the keto form) *7a*, the tris-TMS ether of isoflav-3-en-4-ol (the enol form) *7b* and the tris-TMS-ether of 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)prop-2-ene-1-one *7c*, a ring cleavage product. The latter, always the minor product, is characterized by the two singlets of the methylene protons at 5.25 and 5.89 ppm in ^{1}H-nmr (d_6-DMSO). Kelly *et al.* (41, 49) suggested that this minor ring opened product that they called dehydro-*O*-demethylangolensin *7d* is a metabolite of daidzein *2*. However it seems to be, particularly in this conjunction, a silylation artefact (*7d*) and not a metabolite of dietary isoflavones.

To confirm the structure of 4,4′,7-tris-(trimethylsilyloxy)isoflav-3-ene (the enol form) *7b* it was synthesized by enolization of dihydrodaidzein *7* using lithium diisopropylamide (LDA) followed by silylation. All characteristics of this compound were identical to those of *7b* obtained by BSTFA silylation of dihydrodaidzein.

It has been shown that genistein *1* is metabolized to equol *4* in fowl (56). With the advent of highly specific and sensitive methods for the determination of the isoflavonoids, including the use of mass spectroscopy, it is inevitable that other phytoestrogens in the biosynthetic pathway to equol *4* will be identified in human biological fluids as well. It has been revealed that there are differences between individuals regarding the human metabolism of plant isoflavonoids. Studies of administration of soy protein have revealed that in some subjects equol *4* is not formed despite a challenge from a soy protein diet (36, 41). Also a diet with a high level of fermentative activity may well affect the isoflavonoid metabolism. The identification of genistein *1*, daidzein *2*, equol *4*, and *O*-demethylangolensin *6* in feces (42) has shown that ingested isoflavonoids are excreted not only through urine but also through the bile. Daidzein *2* and the isoflavonoid phytoestrogen metabolites equol *4*, 4′-*O*-methylequol *5*, 3,7′-dihydroxyisoflavan and *O*-demethylangolensin *6* are also present in cow's milk, and humans may be exposed to these mammalian metabolites also through the diet (39, 40). It is important to recognize that the overall consumption of isoflavonoid phytoestrogens in foods may be high, therefore a prolonged exposure may have notable biological effects. The clarification of the metabolism of isoflavonoid phytoestrogens in humans thus remains an important task.

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1. Setchell KDR, Adlercreutz H. Mammalian lignans and phytoestrogens: Recent studies on their formation, metabolism, and biological role in health and disease. In: Rowland IR, Ed. Role of the Gut Flora in Toxicity and Cancer. London: Academic Press, pp315–345, 1988.
2. Adlercreutz H. Diet, breast cancer, and sex hormone metabolism. Ann NY Acad Sci **595:**281–290, 1990.
3. Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hämäläinen E, Hasegawa T, Okada H. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Am J Clin Nutr **54:**1093–1100, 1991.
4. Barnes S, Grubbs C, Setchell KDR, Carlson J. Soybeans inhibit mammary tumors in models of breast cancer. In: Pariza MW, Aeschbachen H-U, Felton JS, Sato S, Eds. Mutagens and Carcinogens in the Diet. New York: Wiley-Liss Inc., pp239–253, 1990.
5. Adlercreutz H, Mousavi Y, Höckertstedt K. Diet and breast cancer. Acta Oncol **31:**175–181, 1992.
6. Rose DP. Dietary fiber, phytoestrogens, and breast cancer. Nutrition **8:**47–51, 1992.
7. Messina M, Barnes S. The role of soy products in reducing risk of cancer. J Nat Cancer Inst **83:**541–546, 1991.
8. Adlercreutz H, Goldin B, Gorbach SL, Höckerstedt K, Watanabe S, Hämäläinen E, Markkanen H, Mäkelä T, Wähälä K, Hase T, Fotsis T. Soybean phytoestrogen intake and cancer risk. J Nutr **125:**757S–770S, 1995.
9. Adlercreutz H, Mousavi Y, Loukovaara M, Hämäläinen E. Lignans, isoflavones, sex hormone metabolism, and breast cancer. In: Proceedings of the Ares-Serono International Symposium, The New Biology of Steroid Hormones, Budapest, Hungary, 1990.
10. Wang H-J, Murphy PA. Isoflavone content in the commercial soybean foods. J Agric Food Chem **42:**1666–1673, 1994.
11. Murphy PA, Farmakalidis E, Johnson LD. Isoflavone content of soya-based laboratory animal diets. Food Chem Toxicol **20:**315–317, 1982.
12. Price KR, Fenwick GR. Naturally occurring oestrogens in foods. Food Add Contam **2:**73–106, 1985.
13. Shutt DA. The effects of plant oestrogens on animal reproduction. Endeavour **35:**110–113, 1976.
14. Kallela K. The oestrogenic effect of red clover pasture on the ovariectomized heifer. Nord Veterinaermed **20:**185–192, 1968.
15. Adlercreutz H, Höckerstedt K, Bannwart C, Bloigu S, Hämäläinen E, Fotsis T, Ollus A. Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and sex hormone binding globulin (SHBG). J Steroid Biochem Mol Biol **27:**1135–1144, 1987.
16. Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Mäkelä T, Brunow G, Hase T. Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens and anticarcinogens, in urine of women on various habitual diets. J Steroid Biochem Mol Biol **25:**791–797, 1986.
17. Adlercreutz H. Lignans and phytoestrogens possible preventive role in cancer. Front Gastrointest Res **14:**165–176, 1988.
18. Adlercreutz H, Fotsis T, Heikkinen R, Dwyer R, Woods JT, Goldin B, Gorbach S. Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian postmenopausal women and in women with breast cancer. Lancet **2:**1295–1299, 1982.

19. Adlercreutz H, Mousavi Y, Loukovaara M, Hämäläinen E. Lignans, isoflavones, sex hormone metabolism, and breast cancer. In: Hochberg RB, Naftolin F, Eds. The New Biology of Steroid Hormones. New York: Serno Symposia Publications, Raven Press, Vol 74: pp145–154, 1991.
20. Adlercreutz H. Western diet and western diseases: Some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest **201**(Suppl 50):3–23, 1990.
21. Dewick PM. Isoflavonoids. In: Harborne JB, Mabry TJ; Eds. The Flavonoids: Advances in Research. Cambridge: Chapman and Hall, pp535–632, 1982.
22. Adams NR, Sanders MR, Ritar AJ. Oestrogenic damage and reduced fertility in ewe flocks in southwestern Australia. Aust J Agric Res **39:**71–77, 1988.
23. Davies HL, Hill JL. The effect of diet on the metabolism in sheep of the tritiated isoflavones formononetin and biochanin A. Aust J Agric Res **40:**157–163, 1989.
24. Lundh TJ-O, Pettersson H, Kiessling, K-H. Demethylation and conjugation of formononetin and daidzein in sheep and cow liver microsomes. J Agric Food Chem **36:**22–25, 1988.
25. Luk K-C, Stern L, Weigele M. Isolation and identification of diazepam-like compounds from bovine urine. J Nat Prod **46:**852–861, 1983.
26. Kallela K, Heinonen K, Saloniemi H. Plant oestrogens: The cause of decreased fertility in cows. Nord Vet-Med **36:**124–129, 1984.
27. Marrian GF, Haselwood GAD. Equol, a new inactive phenol isolated from the ketohydroxyestrin fraction of mare's urine. Biochem J **26:**1226–1232, 1932.
28. Chang HHS, Robinson AR, Common RH. Excretion of radioactive daidzein and equol as monosulfates and disulfates in the urine of the laying hen. Can J Biochem **53:**223–230, 1975.
29. Chang HHS, Robinson AR, Common RH. Lability of equol to acidic hydrolysis procedures. Anal Biochem **63:**290–292, 1975.
30. Tang G, Common RH. Urinary conversion products of certain orally administered isoflavones in the fowl. Biochim Biophys Acta **158:**402–413, 1968.
31. Juniewicz PE, Pallante Morell S, Moser A, Ewing LL. Identification of phytoestrogens in the urine of male dogs. J Steroid Biochem Mol Biol **31:**987–994, 1988.
32. Monfort J, Thompson MA, Czekala NM, Kasman LH, Shackleton CHL, Lasley BL. Identification of a nonsteroidal estrogen, equol, in the urine of pregnant maraques: Correlation with steroidal estrogen excretion. J Steroid Biochem Mol Biol **20:**869–876, 1984.
33. Thomson MA, Lasley BL, Rideout BA, Kasman LH. Characterization of the estrogenic properties of a nonsteroidal estrogen, equol, extracted from urine of pregnant macaques. Biol Reprod **31:**705–713, 1984.
34. Adlercreutz H, Musey P, Fotsis T, Bannwart C, Wähälä K, Mäkelä T, Brunow G, Hase T. Identification of lignans and phytoestrogens in urine of chimpanzees. Clin Chim Acta **158:**147–154, 1986.
35. Musey PI, Adlercreutz H, Gould KG, Collins DC, Fotsis T, Bannwart C, Mäkelä T, Wähälä K, Brunow G, Hase T. Effect of diet on lignans and isoflavonoid phytoestrogens in chimpanzees. Life Sciences **57:**655–664, 1995.
36. Ingham JL. Naturally occurring isoflavonoids. Prog Chem Org Nat Prod **43:**17–18, 1983.
37. Cox RI, Braden AWH. A new phyto-oestrogen metabolite in sheep. J Reprod Fertil **36:**492, 1974.
38. Adlercreutz H, van der Wildt J, Kinzel J, Attalla H, Wähälä K, Mäkelä T, Hase T, Fotsis T. Lignan and isoflavonoid conjugates in human urine. J Steroid Biochem Mol Biol **52:**97–103, 1995.
39. Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Brunow G, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. Clin Chim Acta **199:**263–278, 1991.
40. Bannwart C, Adlercreutz H, Wähälä K, Brunow G, Hase T. Isoflavonic phytoestrogens in humans, identification and metabolism. Eur J Cancer & Clin Oncol **23:**1732, 1987.
41. Kelly GE, Nelson C, Waring MA, Joannou GE, Reeder AY. Metabolites of dietary (soya) isoflavones in human urine. Clin Chim Acta **223:**9–22, 1993.
42. Adlercreutz H, Fotsis T, Kurzer MS, Wähälä K, Mäkelä T, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of unconjugated lignans and isoflavonoids in human feces, with preliminary results in omnivorous and vegetarian women. Anal Biochem **225:**101–108, 1995.
43. Adlercreutz H, Fotsis T, Lampe J, Wähälä K, Mäkelä T, Brunow G, Hase T. Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas chromatography. Scand J Clin Lab Invest **215**(Suppl 53):5–18, 1993.
44. Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phyto-oestrogens in Japanese men. Lancet **342:**1209–1210, 1993.
45. Adlercreutz H, Fotsis T, Watanabe S, Lampe J, Wähälä K, Mäkelä T, Hase T. Determination of lignans and isoflavonoids in plasma by isotope dilution gas chromatography-mass spectrometry. Cancer Detect Prev **18:**259–271, 1994.
46. Bannwart C, Adlercreutz H, Wähälä K, Kotiaho T, Hesso A, Brunow G, Hase T. Identification of the phyto-oestrogen 3′,7-dihydroxyisoflavan, an isomer of equol, in human urine and cow's milk. Biomed Environ Mass Spectrom **17:**1–6, 1988.
47. Bannwart C, Adlercreutz H, Fotsis T, Wähälä K, Hase T, Brunow G. Identification of *O*-desmethylangolensin, a metabolite of daidzein, and of matairesinol, one likely plant precursor of the animal lignan enterolactone, in human urine. Finn Chem Lett **11:**120–125, 1984.
48. Wähälä K, Mäkelä T, Bäcström R, Brunow G, Hase T. Synthesis of the [^{2}H]-labelled urinary lignans, enterolactone and enterodiol, and the phytoestrogen daidzein and its metabolites equol and *O*-desmethylangolensin. J Chem Soc Perkin Trans 1 95–98, 1986.
49. Joannou GE, Kelly GE, Reeder AY, Waring M, Nelson C. A urinary profile study of dietary phytoestrogens: The identification and mode of metabolism of new isoflavonoids. J Steroid Biochem Mol Biol **54:**167–184, 1995.
50. Jain AC, Naresh NK. Synthesis of (±)-sativanone and (±)-dihydrodaidzein. Indian J Chem **26B:**136–139, 1987.
51. Inoue N. Isoflavones and isoflavanones. VI. Dehydrogenation of isoflavanones with *N*-bromosuccinimide. Nippon Kagaku Zasshi **79:**1537–1540, 1958. Chem Abstr **54:**5635e, 1960.
52. Wähälä K, Hase T. Expedient synthesis of polyhydroxyisoflavones. J Chem Soc Perkin Trans 1 3005–3008, 1991.
53. Wähälä K, Hase T, Adlercreutz H. Synthesis and labeling of isoflavone phytoestrogens, including daidzein and genistein. Proc Soc Exp Biol Med **208:**27–32, 1995.
54. Wähälä K, Hase T. Hydrogen transfer reduction of isoflavones. Heterocycles **28:**183–186, 1989.
55. Woodward MD. Gas chromatography/mass spectroscopy of isoflavonones and related compounds. Phytochemistry **21:**1403–1407, 1982.
56. Cayen MN, Carter AL, Common RH. The conversion of genistein to equol in the fowl. Biochim Biophys Acta **86:**56–64, 1964.