Synthesis and Labeling of Isoflavone Phytoestrogens, Including Daidzein and Genistein (43827)

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Abstract. **The synthesis of the important diphenolic isoflavone type phytoestrogens starting from the corresponding unprotected phenols and arylacetic acids is discussed. The aryl rings may carry additional alkyl, methoxy, and/or halogeno groups. Intermediate polyhydroxy deoxybenzoins can also be isolated in good yield. Isotopically labeled isoflavone phytoestrogens were prepared for use as internal standards in ion exchange chromatography and GC-MS selected ion monitoring (SIM technique). Traditional methods rely on total synthesis using deuterated starting materials for the preparation of labeled isoflavonoid structures. We have used successfully an application where the H/D exchange is performed within the finished molecular framework, based on the exchange of aromatic protons that are *ortho* or *para* to a phenolic OH group. By this method the deuterated products are available in an isotopic purity of 90% or higher.** [P.S.E.B.M. 1995, Vol 208]

In 1979, unknown diphenolic compounds were isolated from urine of the green monkey and human subjects. These compounds were identified as lignans and later similar diphenolic phytoestrogens possessing an isoflavonoid structure (1–4). Isoflavonoid phytoestrogens are known to cause a serious infertility syndrome in sheep (5) and cattle (6) grazing in certain clover fields. It became apparent that the disease syndrome was triggered not only by plant isoflavonoids but also their intestinal metabolites (5). These compounds bind to estrogen receptors having both antiestrogenic and estrogenic properties. Some plants in the human diet are rich in these isoflavonoid phytoestrogens (7), and their possible biological effects in humans are of considerable interest.

Our aim was to identify these compounds in human fluids and tissues and to study their biological properties and intestinal metabolism in mammals and humans, and their mechanism of action and anticancer properties. For the purpose of developing qualitative and quantitative analytical methods, authentic isoflavonoid phytoestrogens were needed. In addition to known and potential metabolites of the isoflavonoids, synthetic deuterated internal standards were required for the quantitative analysis.

The chemical structures of these biologically interesting isoflavonoid phytoestrogens are not particularly complex, yet their simplicity creates opportunities for state-of-art synthesis. This is the case particularly when the goal is to develop an efficient macroscale preparation method for isoflavonoids.

In the past, synthetic isoflavonoids have been mainly needed as comparison samples to confirm the new isoflavonoid structures isolated from nature. A more important incentive for synthetic work is the emerging biological activity of the isoflavonoids. Relatively large quantities of variously substituted isoflavonoids are frequently required both structure-activity studies and pharmacological applications.

The various isoflavonoid classes are often very interconvertible to some degree. It was reasoned that a short, efficient method of synthesizing one of these classes might provide ready access to other classes through functionality changing reactions within the finished molecular framework. For that reason we fo-

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cused on isoflavones, themselves a group of biologically active compounds of edible plants. In many cases, deoxybenzoins and chalcones can serve as starting materials for natural isoflavones, which typically have polyhydroxy and/or methoxy substitution.

Variously substituted hydroxy/methoxy isoflavones (3-phenyl-4*H*-1-benzopyran-4-ones) were required for clinical chemistry studies. The demand for ready availability meant that short routes would be preferred. Traditional synthesis of isoflavones involve several steps, including protection-deprotection sequences for the free hydroxy groups, and the overall yields are quite often very low.

The classical methods (8, 9) (Figs. 1 and 2) are still occasionally used to confirm the structure of a new natural product by total or partial synthesis. Conformation is particularly important if biogenetically unusual structures are proposed. A wide variety of C_1 reagents are now available, and many of these are still routinely used. Ethoxalyl chloride (10, 11), triethyl orthoformate (12), ethyl formate (13, 14), and dimethyl formamide (DMF) (15–19) under various conditions all make quite efficient C_1 sources (Fig. 1), although the protection of functional groups other than the hydroxyl required to make the pyrone ring (ring C) may be necessary. More frequently, isoflavones are synthesized by an oxidative conversion of chalcones (Fig. 2). Chalcones are readily obtained by the condensation of acetophenones and aromatic aldehydes and are thus more accessible than deoxybenzoins, particularly if complex substitution patterns are required. However, the availability of a particular polyhydroxy/methoxy substituted starting material for chalcone (or deoxybenzoin) synthesis may be a problem. Ethoxymethyl chloride has proved to be a suitable protecting agent for polyhydroxy/methoxychalcones and isoflavones since the ethoxymethoxy group is stable under alkaline conditions but easily cleaved by acids (20).

The rearrangement of chalcone epoxides catalyzed by boron trifluoride (Fig. 2) is still used (21), but product yields tend to be poor. The alternative thallium (III) nitrate (TTN) oxidation of 2′-hydroxychalcones (Fig. 2) in methanol (20–24) or trimethyl orthoformate (TMOF) (18, 19, 25) is now widely used. The intermediate acetal arising *via* an aryl migration mechanism may be transformed into the isoflavone by either acid or base treatment, thereby allowing consid-

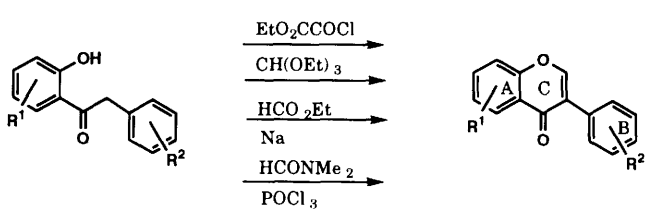


Figure 1. Synthesis of isoflavones from deoxybenzoins.

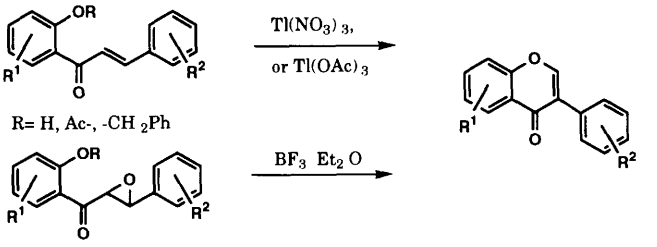


Figure 2. Syntheses of isoflavones from chalcones.

erable flexibility when acid sensitive groups are present. Problems may arise, however, if a chromene ring is present in the starting chalcone.

In the last few years, improved biogenetic-type oxidative aryl migrations have been developed. These are mediated by reagents such as thallium (III) acetate (TTA) with thallium (III) *p*-tolylsulfonate (*p*-TSA) in propionitrile (26), thallium (III) nitrate in methanol-chloroform containing 70% perchloric acid (27), or [hydroxy(tosyloxy)iodo]benzene in acetonitrile (28) (Fig. 3).

Isoflavones can also be synthesized by a direct arylation of 3-bromo (29) or 3-iodochromones (30) in a cross-coupling reaction with arylboronic acids or their esters catalyzed by tetrakis(triphenylphosphine)palladium (Fig. 4).

Another new route to isoflavones starts from salicylaldoximes (31). These are oxidized to nitrile oxides, which react with ω-oxy-substituted styrenes or the enamine (Fig. 5) to give 4-phenyl-substituted isoxazoles. The isoxazoles are acetylated, reduced, and cyclized in the presence of acid to isoflavones. The acylation is essential since Raney nickel reduction (RaNi) in methanol leads to partial overreduction giving, among other products, isoflavanones in 10%–15% yield (31).

Material and Methods

Dimethyl formamide and boron trifluoride etherate were dried and vacuum distilled over CaH_2 before use. Thin layer chromatography was conducted on Merck silica gel 60 F_{254} plates using dichloromethane-

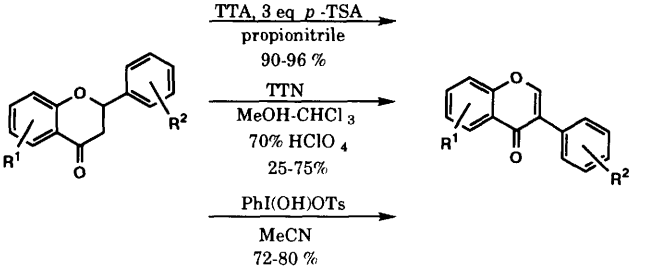


Figure 3. Biomimetic synthesis of halo/methoxy isoflavones from flavanones by an oxidative 1,2-aryl rearrangement (26–28).

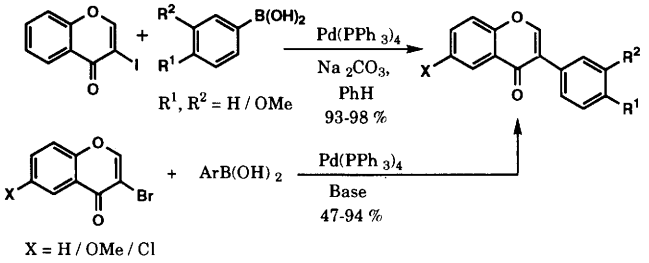


Figure 4. Synthesis of isoflavones by the palladium catalyzed crosscoupling reaction of 3-halochromones with arylboronic acids (29, 30).

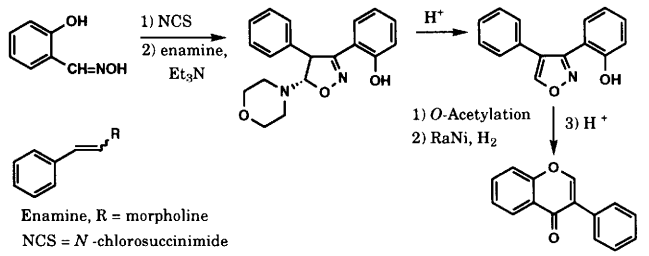


Figure 5. The use of nitrile oxides in isoflavone synthesis (31).

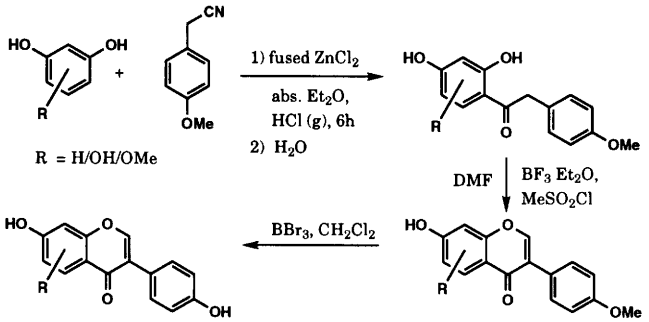


Figure 6. Synthesis of isoflavones from deoxybenzoins (33).

ethyl acetate 7:2 as eluent and detection under UV light.

General One-Pot Procedure for Isoflavones and Deoxybenzoins. A phenol (Fig. 7, Table I, Panel 1) (0.050 mol) and an arylacetic acid (0.050 mol) (Fig. 7, Table I, Panel 2) were dissolved into freshly distilled boron trifluoride etherate (20 mol eq) under argon. The mixture was stirred and heated at 65°–70°C. The reaction was monitored by thin layer chromatography. For the cyclization the reaction mixture was cooled to room temperature and dry dimethylformamide (77 ml) was added. The mixture was again heated to 50°C, and a solution of methanesulphonyl chloride (12 ml) in dry dimethylformamide (20 ml) was added slowly. After reaction at 60°–70°C the reaction mixture was cooled to room temperature and poured into a large volume of ice cold water or aqueous sodium acetate (12 g/100 ml). The crude product (Fig. 7, Table I, Panel 4) was filtered off and recrystallized from a suitable solvent.

The intermediate deoxybenzoins (Fig. 7, Table I, Panel 3) in many cases crystallized from the reaction mixture. They can be isolated by washing the collected material thoroughly with cold water or aq. NaOAc (12 g/100 ml) and recrystallized. If the reaction mixture is homogeneous, deoxybenzoins can be isolated by pouring the mixture into a large volume of water or aq. NaOAc, extracting with diethyl ether, drying with Na_2SO_4, evaporation, and recrystallization from an appropriate solvent.

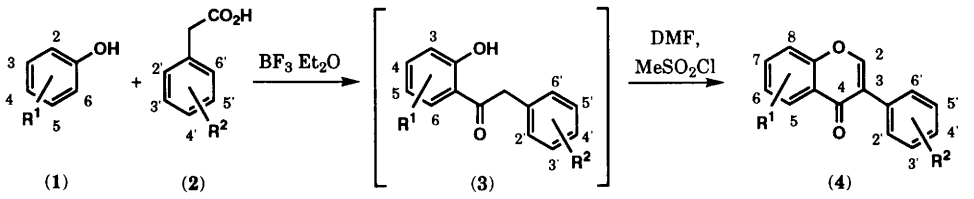
Labeling of Isoflavones. An OH/OD exchange of phenolic groups in isoflavone (0.9 mmol) was first conducted by dissolving the corresponding isoflavone in acetone (1 ml) and adding deuterium oxide (1 ml) (Fig. 8). The mixture was then heated for 5 min and evaporated. Labeled trifluoroacetic acid (CF_3COOD) was prepared by adding trifluoroacetic anhydride (1.25 ml) to deuterium oxide (3.5 ml) and refluxing the mixture for 10 min. This reagent was added to the D_2O-treated isoflavone and the mixture was refluxed for 2 days. The cooled mixture was then evaporated and fresh CF_3COOD was added and the reaction mixture was again heated under reflux for 2 days. The treatment was repeated for a third time. The reaction product was then treated twice with boiling aqueous ethanol to reinstate the protic hydroxy groups. d_4-Daidzein was recrystallized from aqueous ethanol (mp. 335°C) and d_4-genistein was recrystallized from ethanol (mp. 290°–292°C, decomp.).

Results and Discussion

In our laboratory, an expedient one-pot procedure was developed for the synthesis of polyhydroxy isoflavones from the corresponding free polyhydroxy phenols and phenylacetic acids. The intermediate deoxybenzoins are directly cyclized with dimethylformamide catalyzed by boron trifluoride etherate and mesyl chloride, to give the isoflavones in 50%–98% yields (Table I) (Fig. 7) (32).

In our early synthetic work (33), estrogenic isoflavones such as daidzein, formononetin, and genistein were synthesized from the corresponding methoxy substituted deoxybenzoins, prepared by the Hoesch condensation (34) in 40%–50% yields (Fig. 6) (33, 35). The Bass cyclization method (36) in which only the phenolic hydroxy groups in the A-ring exist unprotected was found to be useful for various hydroxymethoxy substituted isoflavones (33, 35). De-*O*-methylation at the B-ring with commonly employed HI or HBr was found to be unsatisfactory, and yields of the polyhydroxyisoflavones were poor. In contrast, deprotection with more selective reagent boron tribromide in methylene chloride (37) furnished the required end products in good yields and purity (33, 35).

The cyclization reaction of deoxybenzoins with dimethylformamide as a C_1 source and catalyzed by



(1) (2) (3) (4)

Figure 7. The one pot synthesis of polyhydroxyisoflavones (32).

boron trifluoride etherate and mesyl chloride was tested with unprotected 2,4,4′-trihydroxybenzoin. This high-yielding cyclization proved to be suitable for a number of polyhydroxy and/or methoxy isoflavones. However, the synthesis of deoxybenzoins in low yields by the time-consuming Hoesch condensation reaction from protected starting materials meant that the overall yield of isoflavones was low. The search for another approach to the deoxybenzoins was therefore initiated.

In 1983, Luk (38) and coworkers reported the Friedel-Crafts acylation of resorcinol with methoxy substituted phenylacetic acids catalyzed by gaseous boron trifluoride or boron trifluoride etherate. An application of this method (32) to unprotected starting materials, using the more easily handled boron trifluoride etherate as the catalyst and *as the solvent,* proved highly successful.

Thus 2,4,4′-trihydroxydeoxybenzoin, the precursor for daidzein, was isolated in highly pure state in 98% yield and could be directly cyclized, without resort to any of the often employed protection-deprotection sequences. Since the cyclization could now be done in unprotected form, the two new methods could also be combined in a one-pot procedure (Fig. 7) (32). To demonstrate the scope of the new route, nineteen hydroxyisoflavones (three of them new compounds and nine known natural products) and 16 hydroxydeoxybenzoins (five of them new) were synthesized (Table I) (32).

A valuable aspect of the new procedural modification is that, since free hydroxy groups are compatible with the reaction conditions, numerous mixed hydroxy/methoxy substituted isoflavones, which are commonly encountered as natural products, are directly accessible. Benzyl or silyl ethers or acyl pro-

Table I. The Reaction Conditions and Yields of the One-Pot Synthesis of Isoflavones

Substituents in phenol	Substituents in $ArCH_2COOH$	Product, yield %			
		Reaction time[a]	Deoxybenzoin	Reaction time[a]	Isoflavone
H	H	90 min	2-OH; 23%	1 hr	H; 98%[b]
H	H	90 min	4-OH; 75%		
2-OH	4′-OH	2 hr	3,4,4′-OH; 78%[c]	1 hr	8,4′-OH; 15%
4-Me	4′-OH	5 hr		5 hr	6-Me, 4′-OH; 64%[c]
3-OH	H	1 hr	2,4-OH; 89%	1 hr	7-OH; 93%
3-OH	3′-OH	1 hr	2,4,3′-OH; 93%	3 hr	7,3′-OH; 91%
3-OH	4′-OH	1 hr	2,4,4′-OH; 98%	1 hr	7,4′-OH; 98%
3-OH	2′-OMe	1 hr	2,4-OH, 2′-OMe; 98%	1 hr	7-OH, 2′-OMe; 98%
3-OH	3′-OMe	1 hr	2,4-OH, 3′-OMe; 96%	1 hr	7-OH, 3′-OMe; 84%
3-OH	4′-OMe	1.5 hr	2,4-OH, 4′-OMe; 98%	90 min	7-OH, 4′-OMe; 96%
3-OH	3′-OMe, 4′-OH	1 hr	2,4,4′-OH, 3′-OMe; 99%	90 min	7,4′-OH, 3′-OMe; 94%
3-OH	2′-OH	1 hr		1 hr	7,2′-OH; 72%
3-OH, 4-Cl	4′-OH	1 hr	2,4,4′-OH, 5-Cl; 67%[c]	1 hr	7,4′-OH, 6-Cl; 94%[c]
3-OH, 2-Me	4′-OH	5 hr	2,4,4′-OH, 3-Me; 97%[c]	5 hr	7,4′-OH, 8-Me; 91%
3-OH, 5-Me	4′-OH	2 hr	2,4,4′-OH, 6-Me; 86%[c]	2 hr	7,4′-OH, 5-Me; 84%
3,5-OH	H	1 hr		1 hr	5,7-OH; 90%
3,5-OH	4′-OMe	1 hr		1 hr	5,7-OH, 4′-OMe; 90%
3,5-OH	4′-OH	0°C/5 hr	2,4,4′,6-OH; 83%	1 hr	5,7,4′-OH; 53%
3,4-OH	4′-OH		Intractable complex mixture		Intractable complex mixture
2,3-OH	4′-OH	1 hr	2,3,4,4′-OH; 92%[c]	1 hr	7,8,4′-OH; 83%

[a] The reaction mixture was heated on a water bath at 65–70°C.
[b] The cyclization product of the isolated 2-hydroxydeoxybenzoin.
[c] New compound.

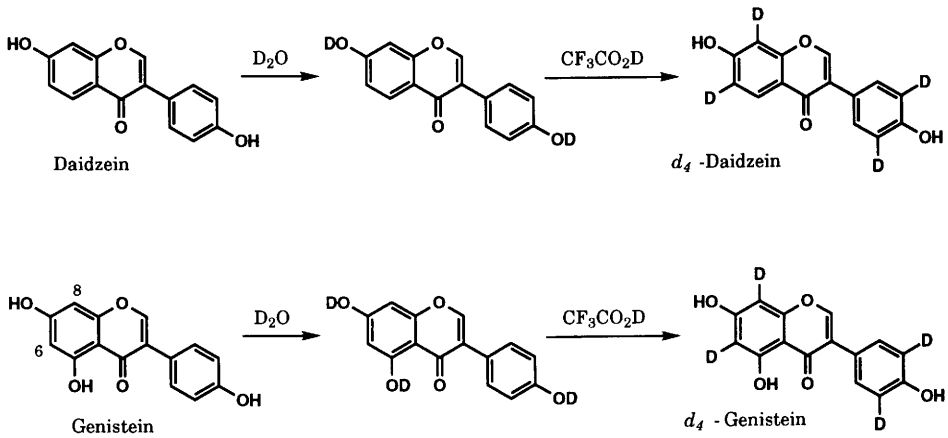


Figure 8. Deuterium labelling of phytoestrogen isoflavones daidzein and genistein (40, 41).

tecting groups would not be stable under these reaction conditions, and protection as the methyl ethers, one of the very few phenol masks stable under such reaction conditions, is clearly not applicable for any mixed hydroxy/methoxy substituted target. The same applies to the synthesis of polyoxygenated deoxybenzoins, although these compounds occur much less frequently in nature.

Synthesis of Labeled Isoflavones. Sensitive and specific methods were required for quantification of the isoflavonoid phytoestrogens in samples of human origin. A method was developed based on ion exchange chromatography and GC-MS selecting ion monitoring (39) (SIM technique), using deuterated internal standards of phytoestrogen isoflavonoids such as daidzein and genistein. It was desirable for this technique that the internal standard be labeled with at least three deuterium atoms in order to obtain a straight line calibration curve free of interference from the compound to be measured. For use as standards, the deuterated isoflavonoids must also be compatible with ion exchange chromatographic conditions (i.e., any reexchange of C-D to C-H cannot be tolerated).

Isotopically labeled natural products are often synthesized starting from labeled synthetic precursors, but we used another approach, namely the H/D exchange of aromatic protons that are *ortho* or *para* to a phenolic OH group.

Daidzein was d_4-labeled in 91% isotopic purity with deuterium by exchange in deuterated trifuoroacetic acid (40), and genistein afforded d_4-genistein in 90% isotopic purity determined by low voltage mass spectrum (41). To enhance the C-H to C-D exchange efficiencies, the protons of phenolic hydroxy groups were replaced by deuterons through D_2O treatment before the deuteration procedure proper. In addition, in each procedure the H/D exchange treatment was performed twice to ascertain complete deuteration. After the final exchange, the reaction products were treated with a large excess of H_2O, EtOH, or MeOH to reinstate the protic hydroxy groups, so as to avoid ambiguities resulting from uncontrolled OD/OH exchange under subsequent ion exchange chromatographic conditions.

However, it was found that d_4-genistein may rather easily lose one or two of its deuterium labels making it unreliable as an analytical standard. This is due to the hydroxy substitution pattern in the ring A of genistein. The activating effects of phenolic hydroxy groups combine to promote the easy electrophilic exchange reaction at Position 6 and 8 under protic conditions.

Current studies involve labeling methods in which nonactivated aromatic protons can also be exchanged to deuterons. Preliminary results indicate that we now have access to a new, *isotopically stable* d_4-genistein derivative. We are actively pursuing our studies on the selective polydeuteration of isoflavonoids, lignans, and steroids.

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