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Synthesis of Isoflavonoid Deuterium Labeled Polyphenolic Phytoestrogens

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Dedicated to the memory of Professor Sir Derek Barton

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Abstract: Deuteration methods are described for the synthesis of new stable deuterium labeled isoflavonoids, [8,3',5'-D_3]-daidzein **4a**, [8,3',5'-D_3]-formononetin **4b**, [2',3',5',6'-D_4]-biochanin A **4c**, [6,8,3',5'-D_4]dihydrodaidzein **5a**, [3,6,8,3',5'-D_5]-dihydrodaidzein **5b**, [3,2',3',5',6'-D_5]-dihydrogenistein **5c** and [2,4,8,10-D_4]-coumestrol **6**, using D_3PO_4•BF_3/D_2O as a deuteration reagent. Positions of deuterium labels and isotopic purities were determined by NMR and mass spectrometry. The relative ease of H/D exchange at the various aromatic sites is discussed.

INTRODUCTION

The interest in stable isotope labeled standards has arisen from the widespread application of mass spectrometry as a specific detection tool in biomedical, pharmacological and environmental analysis. An expedient access to these compounds is of critical importance as they are only rarely commercially available. ^{13}C would be an optimal label to be used in an internal standard since it cannot be exchanged to ^{12}C under ordinary circumstances, and total or partial synthesis using highly expensive reagents is required. ^{15}N labels are seldom used since total synthesis is also needed and with the exception of alkaloids, the compounds of interest rarely contain nitrogen. $^{16}O/^{18}O$ -exchange is limited to compounds such as carboxylic acids that contain functional groups prone to facile oxygen exchange reactions.[1] Reactive phenolic compounds may undergo $^{16}O/^{18}O$ - exchange, but the conditions are quite harsh, yields are low and protection is needed.[2] Deuteration remains the most important exchange reaction to obtain stable isotope labeled compounds. Since in many applications several labels are necessary (*vide infra*) no other stable isotope can compete with deuterium in terms of expediency and reagent cost. On the negative side however certain C-D labels may be easily exchanged back to C-H.

Many early articles concerning hydrogen isotope exchange reactions deal with hydrogen-tritium exchange. Preparatively the requirements for deuteration compared to tritiation differ significantly. Since the radioactivity of T can be measured precisely using only small proportion of the isotopic label, exchange conditions that introduce labels in low yield are sufficient. In deuterium labeled compounds the incorporation of labels is monitored by less sensitive analytical techniques so the introduction of multiple labels is generally desired.[3]

Ideally the chemical and physicochemical behaviour should be identical for both the labeled standard and analyte to ensure low variability and high precision in quantitative mass spectrometry. For use as an internal

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standard in quantitative MS, no unlabeled species must be present and the isotope labels must remain stable under the analytical conditions employed.[1] Working with polyhydroxy aromatics, it is desirable that the reference compound contains three to five stable D atoms since the unlabeled compound, TMS-derivatized for GC, will show fairly intense m+1 and m+2 ions in its mass spectrum owing to the high number of carbon and silicon atoms in the molecule. Thus several D atoms are required to shift the peaks of the reference compound to higher m/z values free of interference from the peaks of the analyte.[4] Derivatization using $(CD_3)_3Si$ ethers is not applicable since silyloxy groups will not survive the various extraction and purification procedures often under moderately acidic or basic conditions.[5] Finally, the standard must be isomerically and isotopically pure.

The precursors of polyphenolic isoflavonoids are found in legumes, particularly soy, various seeds, bean sprouts, mushrooms, vegetables, fruits and berries.[6] Epidemiological studies reveal that foodstuffs containing isoflavonoids may reduce the risk of certain hormone-dependent cancers.[7] After the intake of isoflavonoids in various foods they are modified by intestinal bacteria in humans to biologically active, hormone-like substances. The weakly estrogenic polyphenols formed influence sex hormone production, metabolism and biological activity, intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation, cell differentiation, cell adhesion, and angiogenesis in such a way as to make them strong candidates for natural cancer protective compounds.[6]

To study the metabolism and biological and physiological effects, sensitive methods were required for the quantification of the isoflavonoids in plant extracts and biological fluids. In early studies, owing to the lack of suitable isoflavonoid reference compounds, certain steroids such as androstane-3,17-diol[8]were used as GC/MS standards for the quantitation of isoflavonoid phytoestrogens. However, an isotope dilution technique using deuterated analogs of the compounds to be measured would be much preferred because the solubility, chemical behaviour and GC characteristics would then remain unaltered. To this end, an isotope dilution gas chromatographic mass spectrometric method in the selected ion monitoring mode (ID/GC/MS/SIM) was developed,[6,9] using synthetic deuterated internal standards for the correction of losses during the analytical procedure. Each compound to be measured has a corresponding stable deuterated internal reference in this method. This allows a very specific, sensitive, accurate and reproducible determination of isoflavonoids.

Previously, a total synthesis of 3',5'-D_2-formononetin (7-hydroxy-4'-methoxyisoflavone) from deuterated benzaldehyde has been reported.[10] We have reported the synthesis of [6,8,3',5'-D_4]-equol (7,4'dihydroxyisoflavan) under basic conditions ($NaOH/D_2O$),[11] and [6,8,3',5'-D_4]-daidzein (7,4'-dihydroxyisoflavone) and [6,8,3',5'-D_4]-genistein (5,7,4'-trihydroxyisoflavone) using CF_3COOD as the reagent[9,12] . We also found that [6,8,3',5'-D_4]-genistein may rather easily lose the deuterium labels at the highly activated 6- and 8-positions.[9,13] In a preliminary communication, we described a procedure involving perdeuteration using D_3PO_4•BF_3/D_2O followed by selective dedeuteration for the synthesis of the isotopically stable [2',3',5',6'-D_4]-genistein **4d**.[14] The other reagents we have tested for the deuteration of isoflavonoids, D_3PO_4,[9] D_2O-BF_3•Et_2O,[15] D_2O•BF_3[16] and $CH_3COOD/D_2O/K_2PtCl_4$[17] are much less effective and do not completely deuterate even the most activated protons in genistein **1d**. CF_3COOD is good reagent for activated isoflavonoids[13] but the 2'- and 6'-protons of 4'-hydroxyisoflavonoids resist exchange.

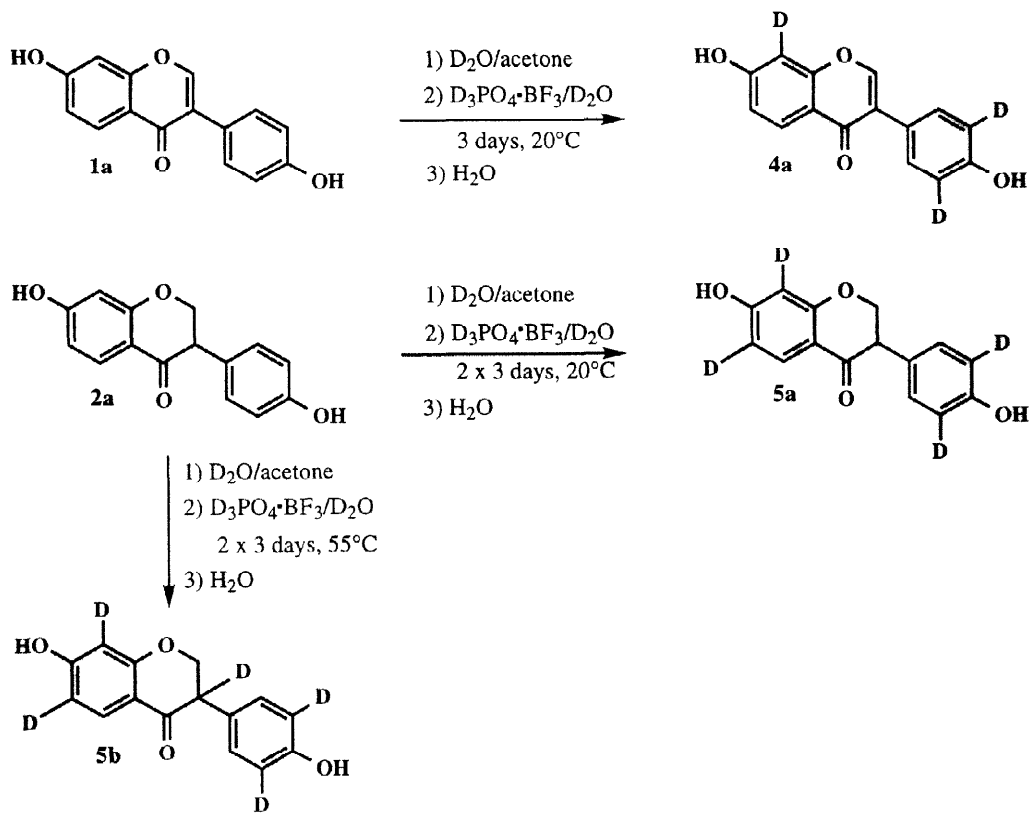
We now present full details of our deuteration method (Scheme), based the use of deuterated phosphoric acid — boron trifluoride complex[18-20] for the synthesis of isotopically pure polydeutero isoflavonoids. As the method involves H-D exchanges within the target isoflavonoid framework, problems inherent in multi-step total synthesis using deuterated starting materials are avoided. We also comment on the relative ease of H-D exchange

at the various aromatic sites, and present results of AM1 calculations that are in accord with the experimental results.

RESULTS AND DISCUSSION

In the deuteration procedure the protons of phenolic hydroxy groups are first replaced by deuterons by mixing the isoflavone with acetone – D_2O and evaporating the solvents. This serves to remove the phenolic protons from the equilibrium mixture prior to the exchange reaction proper. In the final aqueous work-up, protic phenol groups are reinstated. The deuteration reagent is prepared by adding D_2O (8 eq) to dry P_2O_5 (1 eq) at 0 °C and saturating the resulting deuterated phosphoric acid with BF_3 gas. The D_3PO_4•BF_3/D_2O reagent is added to the predeuterated isoflavonoid and the reaction mixture is stirred under exclusion of atmospheric moisture.

The anhydrous 1:1 trideuterophosphoric acid – boron trifluoride complex has been proposed to have the structure $[P(OH)_4]^+[(OH)_2P(OBF_3)_2]^-$.[21] As we use about 8 equivalents of D_2O, to obtain a less viscous and easier to handle reagent, the exact structure of our complex is uncertain.



Scheme. Deuteration of daidzein **1a** and dihydrodaidzein **2a**.

Daidzein **1a** and formononetin **1b** are deuterated chemo- and regioselectively to D_3-products (**4a** and **4b**) at room temperature (Table). The 6-position remains unchanged under these conditions even though the deuteration of dihydrodaidzein **2a** gives the D_4-product **5a** where also the 6-position has been exchanged. Inspection of the heats of formation calculated with AM1 for the respective benzenonium cation intermediates agree with this

observation (Figure 1). In daidzein the intermediate leading to 6-substitution (**A**) is 4.6 kcal/mol higher in energy than the 8-substitution intermediate (**B**). In contrast, the 6-substitution intermediate (**C**) in dihydrodaidzein is lower by 8.8 kcal/mol compared to **D**. In daidzein the 8-substitution intermediate (**B**) has aromatic stabilization which is absent in the corresponding structure of dihydrodaidzein.

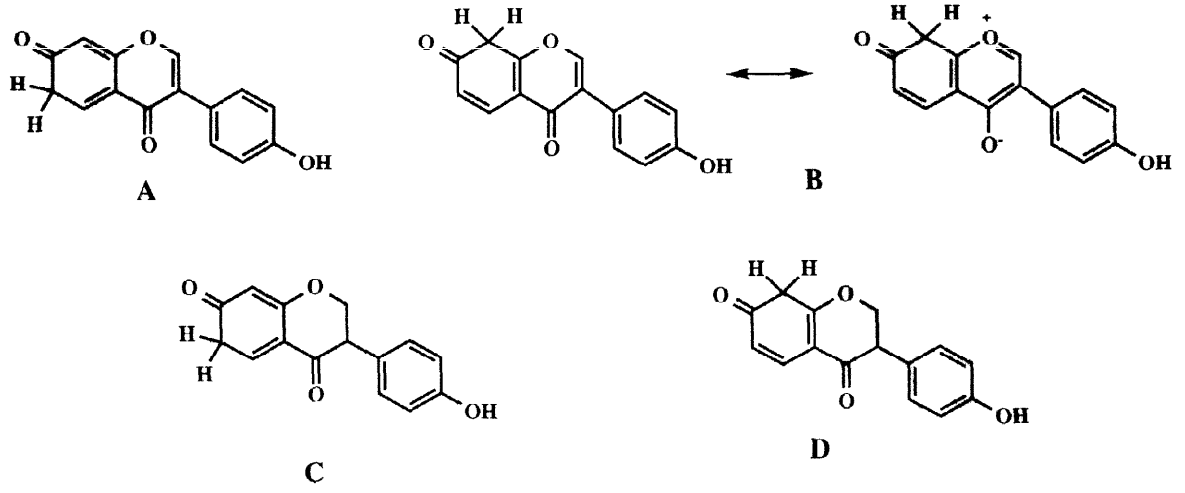


Figure 1. Proton exchange intermediates of daidzein and dihydrodaidzein.

At 55°C also the less activated C-2' and C-6' sites of biochanin A **1c**, genistein **1d** and dihydrogenistein **2c** can be exchanged to deuteriums. After complete deuteration the labile deuterium atoms at the highly activated positions D-6 and D-8 can be selectively removed by acid treatment[14] to give the stable deutero compounds. The 2'- and 6'-positions in daidzein **1a** and formononetin **1b** are not exchanged at 55 °C, even with many repetitions. Thus a hydroxyl group at position 5 promotes the exchange at 2'- and 6'-positions (Figure 2). Under more severe conditions (7 days at 100 °C in an autoclave) the 6,8,2',3',5',6'-protons of daidzein can be exchanged to deuterons.[22]

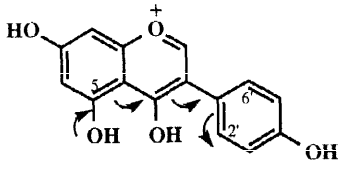
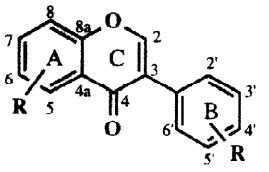
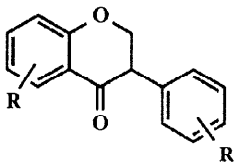


Figure 2.

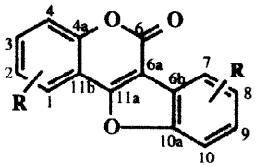
Table



1 a-d
4 a-d



2 a,c
5 a-c



3
6

Isoflavonoid	Reaction time days/no. of runs	T/°C	Substituents in product	Yield / isotopic purity (%)
1a 7,4'-OH	3/1	20	**4a** 7,4'-OH, 8,3',5'-D	98 / 93[a]
1b 7-OH, 4'-OMe	3/1	20	**4b** 7-OH, 4'-OMe, 8,3',5'-D	96 / 91
1c 5,7-OH, 4'-OMe	3 / 4	40	**4c** 5,7-OH, 4'-OMe, 2',3',5',6'-D	10 / 70[b]
1d 5,7,4'-OH	3 / 3	55	**4d** 5,7,4'-OH, 2',3',5',6'-D	91 / 94[c]
2a 7,4'-OH	3 / 2	20	**5a** 7,4'-OH, 6,8,3',5'-D	74 / 94
2a 7,4'-OH	3 / 2	55	**5b** 7,4'-OH, 3,6,8,3',5'-D	82 / 93
2c 5,7,4'-OH	4 / 3	55	**5c** 5,7,4'-OH, 3,2',3',5',6'-D	60 / 66[d]
3 3,9-OH	4/1	55	**6** 3,9-OH, 2,4,8,10-D	95 / 92

[a] less than 1% exchange at C-2'(C-6') was observed by MS even after 4 runs.
[b] biochanin A **1c/4c** is demethylated during the reaction to genistein **1d/4d**. At 90 % conversion, deuteration at C-2' and C-6' of **1c/1d** is only 70 % complete.
[c] synthesis of stable D4-genistein.[14]
[d] product is a mixture of D5/D4-dihydrogenistein (66/33; partial loss of the C-3 label on ring A dedeuteration).

The sites of deuteration were determined from the ^{1}H- and ^{13}C-NMR spectra by comparison with those of undeuterated compounds. D-carrying carbon atoms appear as low intensity triplets in the proton noise decoupled spectra as compared to the intensive singlets in spectra of undeuterated compounds. Previous studies do not agree in assigning the 3- and 1'-carbon atoms of daidzein.[23] Using GHMBC (gradient selected heteronuclear multiple bond correlation) we have ascertained that C-1' appears at δ 122.5 and C-3 at δ 123.4, based on the observed couplings between C-1' and H-3'(5'), and between C-3 and H-2'(6'). The ^{13}C-NMR spectrum of coumestrol has not been assigned previously. We report here the correlation of the carbon atoms of coumestrol with the ^{13}C δ values with the exception of the the three pairs C-7/C-1, C-10a/C-9 and C-3/C-11a which cannot be resolved even with GHMBC.

The isotopic purity of the products were determined from the mass spectra of trimethylsilylated products to avoid M-1 losses from phenolic hydroxyls. Since TMS-derivatized isoflavones usually have M-15 (M-CH_3) as the base peak and M-16 is very weak, isotopic purities are easily and reliably calculated from this region. Thus the fragmentation involving the loss of 2'-H from isoflavones[24] will not interfere either.

The order of exchange of hydrogens has been determined from different deuteration and dedeuteration experiments by following the progress of the reaction by NMR. The reactivity order for daidzein **1a** and formononetin **1b** is 8>3'(5')>6>2'(6')>>2,5, and for biochanin A **1c** and genistein **1d** 8>6>3'(5')>2'(6')>2. Biochanin A **1c/4c** is partly demethylated during the deuteration by the very acidic D_3PO_4•BF_3 to genistein **1d/4d** and thus the yield is poor. For dihydrodaidzein **2a** the reactivity order is 6>8>3'(5')>3>2'(6')>5>2, for dihydrogenistein **2c** 6,8>3>3'(5')>2'(6')>2 and for coumestrol **3** 4,10>2,8>1,7. The Mulliken charges calculated with the semiempirical AM1 method (see Figure 3) reveal that the order of reactivity in electrophilic substitutions for daidzein is 8>3'(5')>6>2'(6') and for genistein 8>6>3'(5')>2'(6') which is exactly the same as observed experimentally. Also the relative unreactivity of 6-position of daidzein compared to 8-position (see above) is manifested in the charges. Thus it may be concluded that the different reactivities in electrophilic hydrogen exchange are mainly caused by the electron density at the exchange site.

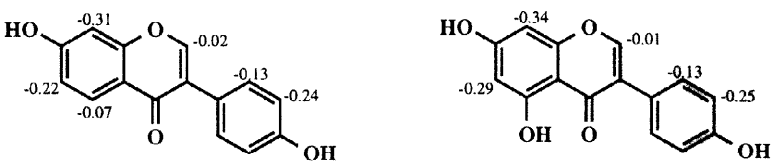


Figure 3.

The new deuterium labeled isoflavonoids can be reliably used as reference compounds and introduced at the beginning of the analytical procedure, since the deuterium labels are securely bound and will survive the various isolation, purification and derivatization steps. For example, the isolation procedure[6] involves a hydrolysis step with 2 M HCl at 100° for 2.5 h followed by neutralisation with 10 M NaOH. The ID/GC/MS/SIM method has now been used for the quantitation of isoflavonoids in human urine,[25] plasma,[26] feces,[27] saliva, breast aspirate, prostatic fluid[28] and foodstuffs such as legumes,[29] seeds,[4] tea and coffee.[30]

EXPERIMENTAL

General

All compounds, characterized by ^1H, ^{13}C and 2D (GHMBC) NMR, LRMS and HRMS, were homogenous by TLC. Melting points were determined in open capillary tubes with an Electrothermal apparatus, and are uncorrected. NMR spectra were recorded on a Varian GEMINI 2000 and Varian Innova 300 WB spectrometers. $SiMe_4$ was used as an internal standard. Chemical shifts are given in δ and *J* values in Hz. In the ^{13}C-NMR spectra the shifts given for the C-D triplets are those corresponding to the central peak and are marked by "D". Mass spectra were obtained with a JEOL JMS SX102 mass spectrometer operating at 70 eV. Samples were introduced by a direct inlet probe. Isotopic purities are calculated from the mass spectra of silylated compounds. The mass spectrometry samples were silylated using *N,O*-bis(trimethylsilyl)trifluoroacetamide and pyridine. The UV spectra were recorded with a CARY 5E UV-VIS-NIR spectophotometer. TLC was conducted with Merck silica gel 60 F_{254} plates; Merck silica gel 60 F_{254} PLC plates were used for preparative thick-layer chromatography (PLC). Coumestrol was from Kodak and D_2O (99.9 atom %) from Sigma. Daidzein and

formononetin were prepared by a one pot procedure,[31] and biochanin A was prepared from the corresponding deoxybenzoin, obtained by the Hoesch condensation[32] and cyclization.[33] Genistein was demethylated from biochanin A,[34] and dihydrodaidzein and dihydrogenistein were obtained by the reduction of the corresponding isoflavone.[35]

General Procedure for Deuteration of Isoflavonoids

An OH/OD exchange of phenolic groups in an isoflavonoid was first conducted by adding the corresponding isoflavonoid (0.1 g) to acetone (1 ml) and deuterium oxide (1 ml). The mixture was then heated for 5 minutes at 50°C and the volatiles evaporated. The deuteration reagent (D_3PO_4•BF_3 in D_2O) was prepared by slowly adding D_2O (1.5 ml, 83 mmol) to dry P_2O_5 (1.5 g, 11mmol) with stirring at 0 °C. This mixture was saturated with BF_3 gas at room temperature. The resulting complex (4 ml) was added to the predeuterated isoflavonoid and the reaction mixture was stirred under anhydrous conditions (reaction time and temperature, Table). After deuteration the cooled mixture was poured into ice water (200 ml) and the product was extracted with EtOAc, the extracts were washed with water (until neutral), dried (Na_2SO_4) and evaporated. The deuteration procedure was repeated if necessary. Unstable hexadeutero biochanin A and heptadeutero dihydrogenistein were directly dedeuterated to the stable derivatives.

[8,3',5'-D_3]-Daidzein {7-hydroxy-3-(4-hydroxyphenyl-3,5-D_2)-4H-1-benzopyran-4-one-8-D} **4a**.
Recrystallization from EtOH gave white crystals, mp 332 °C (325 °C for D_0-daidzein)[36]; λ_{max} (EtOH)/nm 249 (ε 36 000), 302 (14 800); δ_H [$(CD_3)_2SO$] 6.94 (1H, d, *J* 8.7, 6-H), 7.39 (2H, s, 2'- and 6'-H), 7.97 (1H, d, *J* 8.7, 5-H), 8.29 (1H, s, 2-H), 9.54 (1H, br s, 4'-OH), 10.78 (1H, br s, 7-OH); δ_C [$(CD_3)_2SO$] 101.8 (C-8)^D, 114.6 (C-3', -5')^D, 115.0 (C-6), 116.6 (C-4a), 122.5 (C-1'), 123.4 (C-3), 127.2 (C-5), 129.9 (C-2', -6'), 152.8 (C-2), 157.0 (C-4'), 157.4 (C-8a), 162.4 (C-7), 174.7 (C-4); *m/z* (EI) 258 (36%), 257 (M^+, 100), 256 (53), 228 (4), 200 (4), 138 (37), 128 (6), 120 (18), 109 (4), 91(4) (Found: M^+, 257.0765. $C_{15}H_7O_4D_3$ requires M, 257.0767).

[8,3',5'-D_3]-Formononetin {7-hydroxy-3-(4-methoxyphenyl-3,5-D_2)-4H-1-benzopyran-4-one-8-D} **4b**.
Recrystallization from EtOH gave white crystals, mp 254 °C (258 °C for D_0-formononetin)[36]; λ_{max} (EtOH)/nm 250 (ε 27 500), 302 (11 100); δ_H [$(CD_3)_2SO$] 6.95 (1H, d, *J* 8.8, 6-H), 7.51 (2H, s, 2'- and 6'-H), 7.98 (1H, d, *J* 8.8, 5-H), 8.35 (1H, s, 2-H), 10.81 (1H, br s, 7-OH); δ_C [$(CD_3)_2SO$] 55.1 (OMe), 101.8 (C-8)^D, 113.3 (C-3', -5')^D, 115.1 (C-6), 116.6 (C-4a), 123.1 (C-1'), 124.2 (C-3), 127.2 (C-5), 129.9 (C-2', -6'), 153.1 (C-2), 157.4 (C-8a), 158.8 (C-4'), 162.5 (C-7), 174.6 (C-4); *m/z* (EI) 272 (32%), 271 (M^+, 100), 270 (35), 256 (12), 228 (6), 134 (36), 128 (3), 119 (8), 109 (3), 91(8) (Found: M^+, 271.0919. $C_{16}H_9O_4D_3$ requires M, 271.0923).

[2',3',5',6'-D_4]-Biochanin A {5,7-dihydroxy-3-(4-methoxyphenyl-2,3,5,6-D_4)-4H-1-benzopyran-4-one} **4c**. The crude product, [6,8,2',3',5',6'-D_6]-biochanin A, was refluxed in 1% CH_3COCl/MeOH (20 ml) for 30 min and poured into ice water. The D_4-product was filtered, purified with PLC ($CHCl_3$-MeOH, 9:1) and recrystallized from aqueous EtOH giving white crystals, mp 211-213 °C (214.5-215 °C for D_0-biochanin A)[37]; λ_{max} (EtOH)/nm 211 (ε 22 600), 262 (39 200); δ_H (D_6-acetone) 3.84 (3H, s, 4'-OMe), 6.30 (1H, s, 6-H), 6.43

(1H, s, 8-H), 7.56 (0.6 H, s, 2'- and 6'-H), 8.21 (1H, s, 2-H); δ_C (D_6-acetone) 55.7 (OMe), 94.6 (C-8), 99.9 (C-6), 106.2 (C-4a), 114.2 (C-3', -5')^D, 123.8 (C-1'), 124.1 (C-3), 130.7 (C-2', -6')^D, 154.6 (C-2), 159.1 (C-8a), 160.6 (C-4'), 164.0 (C-5), 165.1 (C-7), 181.6 (C-4); *m/z* (EI) 289 (36%), 288 (M^+, 100), 287 (49), 286 (14), 273 (10), 259 (5), 152 (10), 136 (22), 135 (16) (Found: M^+, 288.0945. $C_{16}H_8O_5D_4$ requires M, 288.0936).

[6,8,3',5'-D_4]-Dihydrodaidzein {2,3-dihydro-7-hydroxy-3-(4-hydroxyphenyl-3,5-D_2)-4H-1-benzopyran-4-one-6,8-D_2} **5a**. The crude product was purified by refluxing 5 min with activated carbon in EtOH, then by PLC (CH_2Cl_2-EtOAc, 7:2) and recrystallized from Pr^iOH/H_2O giving white crystals, mp 254 °C (250 °C for D_0-dihydrodaidzein)[35]; λ_{max} (EtOH)/nm 278 (ε 31 500), 312 (17 200); δ_H (D_6-acetone) 3.86 (1H, t, *J* 6.6, 3-H), 4.62 (2H, d, *J* 6.6, 2-H), 7.14 (2H, s, 2'- and 6'-H), 7.75 (1H, s, 5-H); δ_C (D_6-acetone) 51.7 (C-3), 72.6 (C-2), 103.0 (C-8)^D, 111.5 (C-6)^D, 115.3 (C-4a), 116.0 (C-3' -5')^D, 127.9 (C-1'), 130.0 (C-5), 130.4 (C-2', -6'), 157.4 (C-4'), 164.3 (C-8a), 165.0 (C-7), 191.1 (C-4); *m/z* (EI) 261 (12%), 260 (M^+, 28), 259 (10), 140 (27), 139 (100), 138 (33), 123(11), 122 (42), 121(10), 110 (4), 93 (7), (Found: M^+, 260.0991. $C_{15}H_8O_4D_4$ requires M, 260.0986).

[3,6,8,3',5'-D_5]-Dihydrodaidzein {2,3-dihydro-3-D-7-hydroxy-3-(4-hydroxyphenyl-3,5-D_2)-4H-1-benzopyran-4-one-6,8-D_2} **5b**. The crude product was purified with PLC (CH_2Cl_2-EtOAc, 7:2) and recrystallized from Pr^iOH/H_2O giving white crystals, mp 252-253 °C (250 °C for D_0-dihydrodaidzein)[35] ; λ_{max} (EtOH)/nm 277 (ε 30 700), 313 (16 900); δ_H (D_6-acetone) 4.62 (2H, s, 2-H), 7.14 (2H, s, 2'- and 6'-H), 7.75 (1H, s, 5-H); δ_C (D_6-acetone) 51.2 (C-3)^D, 72.6 (C-2), 102.9 (C-8)^D, 111.2 (C-6)^D, 115.4 (C-4a), 116.4 (C-3' -5')^D, 127.9 (C-1'), 130.1 (C-5), 130.5 (C-2', -6'), 157.5 (C-4'), 164.6 (C-8a), 165.0 (C-7), 191.2 (C-4); *m/z* (EI) 262 (15%), 261 (M^+, 20), 260 (8), 140 (28), 139 (100), 138 (17), 124 (17), 123 (24), 122 (9), 110 (5), 94 (5), (Found: M^+, 261.1058. $C_{15}H_7O_4D_5$ requires M, 261.1049).

[3,2',3',5',6'-D_5]-Dihydrogenistein {2,3-dihydro-3-D-5,7-dihydroxy-3-(4-hydroxyphenyl-2,3,5,6-D_4)-4H-1-benzopyran-4-one} **5c**. The crude product, [3,6,8,2',3',5',6'-D_7]-dihydrogenistein, was refluxed in 1% CH_3COCl/MeOH (10 ml) for 30 min and poured into ice water. The D_5-product was filtered, purified with PLC (CH_2Cl_2-EtOAc, 7:2) and recrystallized from hexane/acetone giving white crystals, isotopic purity 66%, mp 217 °C (220 °C for D_0-dihydrogenistein)[35] ; λ_{max} (EtOH)/nm 214 (ε 28 400), 225 (25 500), 292 (23 000); δ_H (D_6-acetone) 4.00 (0.68H, t, 3-H), 4.60 (2H, s, 2-H), 5.96 (2H, s, 6- and 8-H); δ_C (D_6-acetone) 50.4 (C-3)^D, 72.0 (C-2), 95.7 (C-8), 97.1 (C-6), 103.0 (C-4a), 115.9 (C-3' -5')^D, 127.3 (C-1'), 130.2 (C-2', -6')^D, 157.7 (C-4'), 164.2 (C-5), 165.8 (C-8a), 168.0 (C-7), 197.7 (C-4); *m/z* (EI) 278 (13%), 277 (M^+, 35), 276 (21), 154 (32), 153 (100), 152 (8), 125 (22), 124 (17), (Found: M^+, 277.0994. $C_{15}H_7O_5D_5$ requires M, 277.0998).

[2,4,8,10-D_4]-Coumestrol {3,9-dihydroxy-2,4,8,10-D_4-6H-benzofuro[3,2-c][1]benzopyran-6-one} **6**. Recrystallization from EtOH gave pale brown crystals, mp 385-386 °C (decomp.) (385 °C for D_0-coumestrol)[38]; λ_{max} (EtOH)/nm 237 (ε 33 100), 345 (26 000); δ_H [$(CD_3)_2SO$] 7.70 (1H, s, 7-H), 7.85 (1H, s, 1-H), 10.05 (1H, s, OH), 10.72 (1H, s, OH); δ_C [$(CD_3)_2SO$] 98.3 (C-10)^D, 102.0 (C-6a), 102.8 (C-4)^D, 104.1 (C-11b), 113.7 (C-2, -8)^D, 114.5 (C-6b), 120.5 and 122.5 (C-7 and C-1), 154.6 (C-4a), 155.9 and 156.9 (C-10a and C-

9), 157.6 and 159.5 (C-3 and C-11a), 161.1 (C-6); *m/z* (EI) 273 (42%), 272 (M^+, 100), 271 (18), 244 (9), 215 (4), 136 (7), 122 (8) (Found: M^+, 272.0634. $C_{15}H_4O_5D_4$ requires M, 272.0623).

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