Pergamon

Tetrahedron 56 (2000) 913–916

TETRAHEDRON

Synthesis of Deuterium Labeled Polyhydroxy Flavones and 3-Flavonols

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Received 22 October 1999; revised 22 November 1999; accepted 9 December 1999

Abstract—Syntheses are described for seven new deuterium labeled flavones, [3,6,8,3′,5′-D_5]-apigenin **5**, [3,3′,5′-D_3]-apigenin **6**, [3,6,8,2′,5′,6′-D_6]-luteolin **7**, [3,2′,5′,6′-D_4]-luteolin **8**, [6,8,2′,5′,6′-D_5]-quercetin **9**, [2′,5′,6′-D_3]-quercetin **10** and [8,2′,5′,6′-D_4]-fisetin **11**, using $D_3PO_4 \cdot BF_3/D_2O$ as a deuteration reagent. The products were characterized by NMR and mass spectrometry.

Introduction

We have recently described[1] the synthesis and structural characterization of polyphenolic polydeuterated isoflavonoids by H/D exchange, also discussing in depth the mechanics and relative ease of exchange at the various ring sites. It was of interest to extend these studies to the isomeric flavonoid group of compounds, as the change of aryl substitution from C-3 to C-2 would be expected to cause subtle changes in the H/D exchange patterns at the phenolic ring sites. Perhaps more obviously, the C-3 hydrogen would now appear to be easily exchanged compared to the low exchange rate at C-2 in the isoflavones. We report here the synthesis by H/D exchange and structural characterization of a number of polydeuterated polyhydroxyflavones, using the $D_3PO_4 \cdot BF_3/D_2O$ reagent system that proved very satisfactory with the polyhydroxyisoflavones.[1,2] Obviously, sites that are activated for electrophilic aromatic substitution will be preferentially exchanged, but we will also discuss the order in which the exchanges occur in the flavone ring system.

The synthetic polydeuterated flavonoids and isoflavonoids have a very significant application potential in quantitation of the silylated natural compounds by the isotope dilution GC/MS method in the selected ion monitoring mode (ID/GC/MS/SIM).[3–7] As far as the flavonoids are concerned, they are found in fruits, vegetables, nuts, seeds, stems and flowers and in many food products such as tea and wine, and are thus important constituents of animal and human diet.[8,9] They have also been identified as active constituents in many medicinal plants[10] and have been reported to possess certain beneficial effects in mammalian biology, including antiallergic, antiviral, antiproliferative and anticarcinogenic activities.[11] For the clarification of these effects, it is clearly desirable to be able to carry out epidemiological studies on the effects of food constituents in large populations. For use as internal standards in the ID/GC/MS/SIM method, it is important that the polydeuterated analogs are isomerically and isotopically pure, contain no unlabeled species, and that the D labels are stable under the analytical conditions employed.[6] Reference compounds for polyhydroxy aromatics must contain three to five stable D atoms since the TMS-derivatized unlabeled compound 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.[12] Finally, it is obvious that derivatization using D-containing reagents such as $(CD_3)_3SiCl$ is not applicable.

There is very little previous work on the controlled synthesis of polydeuterated polyhydroxyflavones, and the only report involving an exchange within the intact target flavone deals with the deuterium labeling of rutin, a 3-flavonol glycoside.[13–15] [6,8,2′,5′,6′-D_5]-Rutin was obtained by treating rutin twice with NaOH in D_2O at 95° for 8 h, but incorporation of D atoms at C-2′ and C-6′ was only 70%. Furthermore, owing to the lability of the 6- and 8-D substituents these were back-exchanged using NaOH in H_2O at 25° to give [2′,5′,6′-D_3]-rutin in 60% yield. However, the D_1:D_2:D_3 ratio in the final product was 1:6:3 by ^1H NMR[13–15] which is not satisfactory for accurate ID/GC/MS/SIM work. Also, just three D labels are not sufficient for the quantitation of a compound that contains five hydroxy groups.

Keywords: flavones; deuterium; labeling; 3-flavonols.
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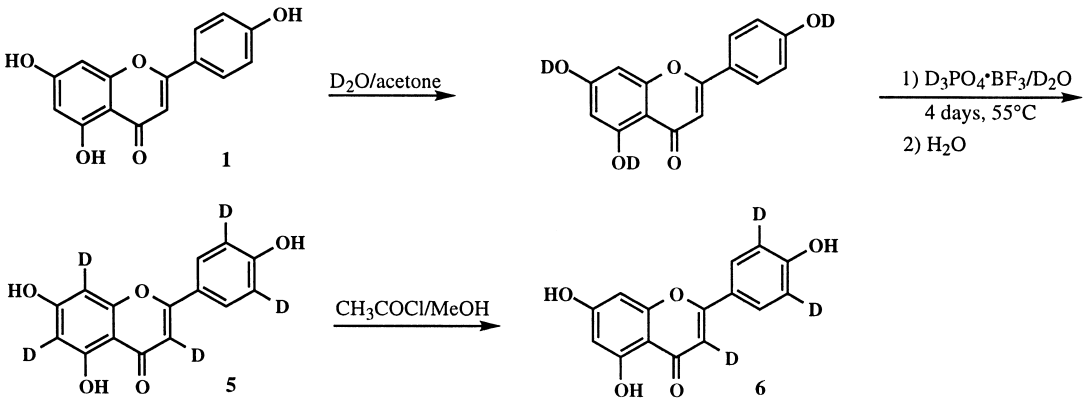
PII: S0040-4020(99)01085-6



Scheme 1. Synthesis of deuterium labeled apigenin.

Results and Discussion

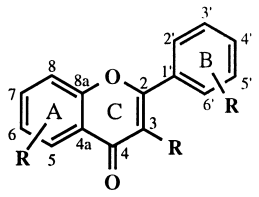
Before the actual C–H exchange we prefer to exchange the phenolic protons for deuterons by D_2O treatment to minimize the amount of available protons in the equilibrium mixture. After the completion of C–H exchange the protic phenol groups are reinstated on aqueous work-up. The deuteration reagent, $D_3PO_4{\cdot}BF_3/D_2O$,[1] prepared just before the reaction is added to the predeuterated flavone and the reaction mixture is stirred under the exclusion of atmospheric moisture (Scheme 1).

Apigenin **1** is deuterated to the D_5-product **5** in 4 days at 55°C (Scheme 1). The C-2′ and C-6′ positions of **1** remain unchanged even after many repetitions presumably due to the inability of the ring oxygen to activate those sites towards electrophilic aromatic substitution. It is interesting that in the corresponding isoflavone genistein (5,7,4′-trihydroxyisoflavone), under same conditions and with two repetitions, also the less activated C-2′ and C-6′ positions take up deuteriums,[2] undoubtedly owing to the now activating effect of the ether oxygen.

In deuterated apigenin **5**, luteolin **7** and quercetin **9**, each having two hydroxyls in a *meta* relationship, the deuteriums at C-6 and C-8 are quite labile, and would probably not survive the isolation, purification and derivatization steps used in ID/GC/MS/SIM. Actually the 8-D in luteolin **7** and in quercetin **9** is so labile that it is partially back-exchanged already during the aqueous work-up. Thus the isotopic purity for D_6-luteolin **7** and D_5-quercetin **9** is not very good (Table 1). As with the analogous isoflavonoids,[1,2] the labile deuteriums can be selectively back-exchanged to hydrogens. This time the dedeuteration conditions can be even milder; 0.5% of CH_3COCl in MeOH is enough to replace the C-6 and C-8 deuteriums with hydrogens. The deuteriums on B and C rings are not affected at all by this dedeuteration method. Thus our deuteration method appears more effective and the dedeuteration method more selective for the preparation of stable, isotopically pure flavonoids than that used previously for rutin.[13–15] Nevertheless, although our D_3-quercetin **10** is 95% isotopically pure, only three deuterium atoms may not be sufficient for accurate ID/GC/MS/SIM work for a compound having five hydroxyl groups as mentioned above. We will report shortly on a completely different approach to overcome this problem.

The sites of deuteration were determined from the ^1H and ^{13}C NMR spectra by comparison with those of undeuterated compounds. Deuterium carrying carbon atoms appear as low intensity triplets in the proton noise decoupled spectra

Table 1. Deuteration and dedeuteration of polyhydroxyflavonoids



Flavonoid	Method[a,b]	Reaction time	Substituents in product	Yield/isotopic purity (%)
1 5,7,4′-$(OH)_3$	A	4 d	**5** 5,7,4′-$(OH)_3$, 3,6,8,3′,5′-D_5	83/88
2 5,7,3′,4′-$(OH)_4$	A	2 d	**7** 5,7,3′,4′-$(OH)_4$, 3,6,8,2′,5′,6′-D_6	78/82
3 3,5,7,3′,4′-$(OH)_5$	A	2 d	**9** 3,5,7,3′,4′-$(OH)_5$, 6,8,2′,5′,6′-D_5	86/76
4 3,7,3′,4′-$(OH)_4$	A	1 d	**11** 3,7,3′,4′-$(OH)_4$, 8,2′,5′,6′-D_4	78/93
5	B	20 min	**6** 5,7,4′-$(OH)_3$, 3,3′,5′-D_3	94/94
7	B	”	**8** 5,7,3′,4′-$(OH)_4$, 3,2′,5′,6′-D_4	71/86
9	B	”	**10** 3,5,7,3′,4′-$(OH)_5$, 2′,5′,6′-D_3	72/95

[a] Method A: deuteration with $D_3PO_4{\cdot}BF_3/D_2O$.
[b] Method B: dedeuteration with $CH_3COCl/MeOH$.

as compared to the intense singlets in the undeuterated compounds. Proton and carbon assignments were also confirmed by GHMBC (gradient selected heteronuclear multiple bond correlation) techniques. The order of exchange of hydrogens was assigned by monitoring the progress of different deuteration and dedeuteration experiments by NMR. Thus, the reactivity order for apigenin **1** is 8,6>3>3′,5′⩾2′,6′, for luteolin **2** 8,6>3>2′,5′,6′, for quercetin **3** 8>6>2′,5′>6′ and for fisetin **4** 8>2′,5′,6′⩾6,5.

The isotopic purity of the products (Table 1) was determined from the mass spectra operating in the negative ion electrospray mode since normal electron impact ionization mass spectra did not give reliable results for all deuterated flavones. It appears that some kind of deuterium scrambling between two molecules can take place in EI/MS before the ionization.

The new polydeuterated flavonoids are stable, isotopically and chemically pure and thus can be reliably used as reference compounds in quantification or in metabolic studies.

Experimental

General

All compounds were characterized by ^{1}H, ^{13}C and 2D (GHMBC) NMR, LRMS and HRMS and 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 Inova 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 peaks 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 negative ion electrospray LC-MS obtained with Micromass Quattro II, using 20 μl Rheodyne loop and MeOH–H_2O (1:1) eluent. The UV spectra were recorded with a CARY 5E UV-VIS-NIR spectophotometer. TLC was conducted with Merck silica gel 60 F_{254} plates. D_2O (99.9 at.%) and quercetin was obtained from Sigma, apigenin (mp 345°C) and luteolin (mp 325–327°C) from Indofine and fisetin (mp >330°C) from Aldrich.

General procedure for deuteration of flavonoids (Method A)[1]

Temperature for all flavone deuterations was 55°C. Reaction times are in the Table 1.

General procedure for dedeuteration of labile deuteriums (Method B)

Flavonoid (0.1 g) containing labile deuterium atoms was refluxed in 0.5% CH_3COCl/MeOH (10 ml) for 20 min. The mixture was then poured into ice water, the product filtered and washed with water or extracted with EtOAc, the extracts washed with water (until neutral), dried (Na_2SO_4) and evaporated.

[3,6,8,3′,5′-D_5]-Apigenin 5 {5,7-dihydroxy-2-(4-hydroxyphenyl-3,5-D_2)-4H-1-benzopyran-4-one-3,6,8-D_3}. Recrystallization from aqueous EtOH gave yellow crystals, mp 345–346°C (345°C for D_0-apigenin); λ_{max} (EtOH)/nm 269 (ϵ 27 500), 335 (30 200); δ_H [$(CD_3)_2SO$] 7.93 (2H, s, 2′- and 6′-H), 10.4 and 10.8 (1H, br, -OH), −2.0 (1H, br, 5-OH); δ_C [$(CD_3)_2SO$] 93.9 (C-8)^D, 98.8 (C-6)^D, 102.5 (C-3)^D, 103.7 (C-4a), 115.6 (C-3′, -5′)^D, 121.1 (C-1′), 128.3 (C-2′, -6′), 157.2 (C-8a), 161.1 (C-4′), 161.4 (C-5), 163.7 (C-2), 164.0 (C-7), 181.7 (C-4); *m/z* (EI) 275 (M^+, 100%), 247 (13), 155 (20), 123 (19), 121 (13), (Found: M^+, 275.0834. $C_{15}H_5D_5O_5$ requires M, 275.0842).

[3,3′,5′-D_3]-Apigenin 6 {5,7-dihydroxy-2-(4-hydroxyphenyl-3,5-D_2)-4H-1-benzopyran-4-one-3-D}. Recrystallization from aqueous EtOH gave yellow crystals, mp 345–346°C (345°C for D_0-apigenin); λ_{max} (EtOH)/nm 269 (ϵ 27 000), 335 (31 800); δ_H [$(CD_3)_2SO$] 6.21 (1H, s, 6-H), 6.50 (1H, s, 8-H), 7.93 (2H, s, 2′- and 6′-H), 9.3 and 9.7 (1H, br, -OH), −2.0 (1H, br, 5-OH); δ_C [$(CD_3)_2SO$] 93.9 (C-8), 98.8 (C-6), 102.8 (C-3)^D, 103.7 (C-4a), 115.7 (C-3′, -5′)^D, 121.1 (C-1′), 128.3 (C-2′, -6′), 157.3 (C-8a), 161.0 (C-4′), 161.4 (C-5), 163.7 (C-2), 164.1 (C-7), 181.7 (C-4); *m/z* (EI) 273 (M^+, 100%), 245 (12), 153 (20), 123 (10), 121 (8), (Found: M^+, 273.0716. $C_{15}H_7D_3O_5$ requires M, 273.0717).

[3,6,8,2′,5′,6′-D_6]-Luteolin 7 {5,7-dihydroxy-2-(3,4-dihydroxyphenyl-2,5,6-D_3)-4H-1-benzopyran-4-one-3,6,8-D_3}. Recrystallization from aqueous *i*-propanol gave yellow crystals, mp 322°C (325°C for D_0-luteolin); λ_{max} (EtOH)/nm 256 (ϵ 28 600), 352 (18 300); δ_C [D_6-acetone] 94.7 (C-8)^D, 99.5 (C-6)^D, 104.5 (C-3)^D, 105.4 (C-4a), 113.9 (C-2′)^D, 116.3 (C-5′)^D, 120.1 (C-6′)^D, 123.7 (C-1′), 146.5 (C-3′), 150.1 (C-4′), 158.8 (C-8a), 163.4 (C-5), 164.9 (C-7), 165.2 (C-2), 183.1 (C-4); *m/z* (EI) 292 (M^+, 100%), 264 (20), 155 (28), 138 (17), (Found: M^+, 292.0844. $C_{15}H_4D_6O_6$requires M, 292.0854).

[3,2′,5′,6′-D_4]-Luteolin 8 {5,7-dihydroxy-2-(3,4-dihydroxyphenyl-2,5,6-D_3)-4H-1-benzopyran-4-one-3-D}. Recrystallization from aqueous *i*-propanol gave yellow crystals, mp 321°C (325°C for D_0-luteolin); λ_{max} (EtOH)/nm 255 (ϵ 20 600), 352 (18 800); δ_H [D_6-acetone] 6.26 (1H, s, 6-H), 6.53 (1H, s, 8-H); δ_C [D_6-acetone] 94.6 (C-8), 99.6 (C-6), 103.9 (C-3)^D, 105.3 (C-4a), 113.8 (C-2′)^D, 116.3 (C-5′)^D, 120.1 (C-6′)^D, 123.1 (C-1′), 146.3 (C-3′), 149.9 (C-4′), 158.6 (C-8a), 163.2 (C-5), 164.7 (C-7), 165.0 (C-2), 182.9 (C-4); *m/z* (EI) 290 (M^+, 100%), 262 (13), 153 (20), 138 (10), (Found: M^+, 290.0732. $C_{15}H_6D_4O_6$ requires M, 290.0728).

[6,8,2′,5′,6′-D_5]-Quercetin 9 {3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl-2,5,6-D_3)-4H-1-benzopyran-4-one-6,8-D_2}. Recrystallization from aqueous EtOH gave yellow crystals, mp 310°C (311–313°C for D_0-quercetin)[16]; λ_{max} (EtOH)/nm 256 (ϵ 20 900), 374 (22 800); δ_C [D_6-acetone] 94.5 (C-8)^D, 99.0 (C-6)^D, 104.2 (C-4a), 115.9 (C-2′, -5′)^D, 121.4 (C-6′)^D,123.6 (C-1′) 136.8 (C-3), 145.8 (C-3′), 147.0 (C-2), 148.4 (C-4′), 157.8 (C-8a), 162.3 (C-5), 165.0 (C-7),

176.6 (C-4); *m*/*z* (EI) 307 (M^+, 100%), 279 (10), 155 (13), 140 (15), (Found: M^+, 307.0745. $C_{15}H_5D_5O_7$ requires M, 307.0740).

[2′,5′,6′-D_3]-Quercetin 10 {3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl-2,5,6-D_3)-4H-1-benzopyran-4-one}. Recrystallization from aqueous EtOH gave yellow crystals, mp 312–313°C (311–313°C for D_0-quercetin)[16]; λ_{max} (EtOH)/nm 256 (ϵ 17 500), 374 (19 000); δ_H [D_6-acetone] 6.28 (1H, s, 6-H), 6.53 (1H, s, 8-H); δ_C [D_6-acetone] 94.5 (C-8), 99.2 (C-6), 104.2 (C-4a), 115.9 (C-2′, -5′)^D, 121.6 (C-6′)^D, 123.6 (C-1′), 136.8 (C-3), 145.8 (C-3′), 147.0 (C-2), 148.4 (C-4′), 157.8 (C-8a), 162.4 (C-5), 165.0 (C-7), 176.6 (C-4); *m*/*z* (EI) 305 (M^+, 100%), 276 (12), 153 (15), 140 (10), (Found: M^+, 305.0608. $C_{15}H_7D_3O_7$ requires M, 305.0615).

[8,2′,5′,6′-D_4]-Fisetin 11 {3,7-dihydroxy-2-(3,4-dihydroxyphenyl-2,5,6-D_3)-4H-1-benzopyran-4-one-8-D}. Recrystallization from aqueous EtOH gave yellow crystals, mp 335–337°C (>330°C for D_0-fisetin); λ_{max} (EtOH)/nm 250 (ϵ 14 400), 319 (10 500), 365 (22 900); δ_H [$(CD_3)_2SO$] 6.92 (1H, d, 6-H, *J* 8.7), 7.94 (1H, d, 5-H, *J* 8.7), 9.06, 9.30, 9.55, 10.77 (1H, br s, -OH); δ_C [$(CD_3)_2SO$] 101.4 (C-8)^D, 114.0 (C-6), 114.5 (C-4a), 114.9 (C-2′, -5′)^D, 119.1 (C-6′)^D, 122.2 (C-1′), 126.3 (C-5), 137.0 (C-3), 144.8 (C-3′, -2), 147.1 (C-4′), 156.1 (C-8a), 162.1 (C-7), 171.8 (C-4); *m*/*z* (EI) 290 (M^+, 100%), 261 (21), 138 (39), (Found: M^+, 290.0733. $C_{15}H_6D_4O_6$ requires M, 290.0728).

Acknowledgements

We thank Dr Jorma Matikainen and Mr Heikki Björk for running the mass spectra and Mr Seppo Kaltia for running the 2D-NMR spectra.

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