Synthesis of the [^{2}H]-Labelled Urinary Lignans, Enterolactone and Enterodiol, and the Phytoestrogen Daidzein and its Metabolites Equol and *O*-Demethylangolensin

Kristiina Wähälä, Taru Mäkelä, Reijo Bäckström, Gösta Brunow and Tapio Hase *
Department of Chemistry, University of Helsinki, Vuorikatu 20, SF-00100 Helsinki, Finland

Methods are described for the synthesis of [$^2H_6$]enterolactone, {[$^2H_6$]-*trans*-2,3-bis(3-hydroxybenzyl)-γ-butyrolactone}, [$^2H_6$]enterodiol, {[$^2H_6$]-2,3-bis(3-hydroxybenzyl)butane-1,4-diol}, [$^2H_4$]daidzein, {[$^2H_4$]-7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one}, [$^2H_4$]equol, {[$^2H_4$]-3(4-hydroxyphenyl)-2*H*-1-benzopyran-7-ol}, and [$^2H_5$]-*O*-demethylangolensin, {[$^2H_5$]-1-(2′,4′-dihydroxy-2-(*p*-hydroxyphenyl)propiophenone} by hydrogen–deuterium exchange at aromatic rings using PBr_3 or NaOD in deuterium oxide or labelled trifluoroacetic acid. The structures, isotopic purities and positions of uptake of deuterium were determined by n.m.r. and mass spectrometry (m.s.)

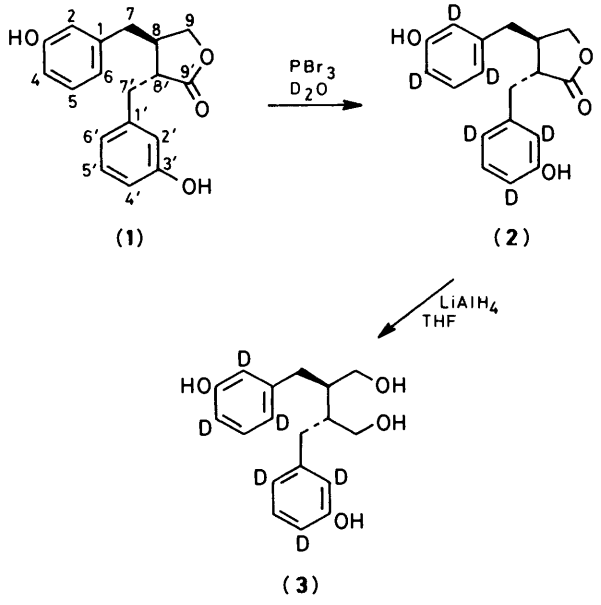
The detection of lignans and phytoestrogens in man and animals has opened a new field of research pertinent to both hormone and cancer research.[1] Lignans of various structures have been found as constituents of some higher plants,[2] and several lignans of unusual structure have been identified in man and in several animals.[3–5]

Investigation of the estrogenic isoflavones began in the 1940s as an attempt to explain the causes of 'clover disease' which resulted in infertility in sheep grazing on certain forages.[6] Daidzein is known to degrade in the rumen of the sheep to give equol (70%),[7] *O*-demethylangolensin (5—20%),[8] and *O*-methylequol.[9] Very little is known about the metabolism in non-ruminants in general and in man in particular.

So far six diphenolic compounds of lignan or phytoestrogen origin have been identified in human urine: enterolactone, enterodiol,[3,4] matairesinol,[10] daidzein,[11] equol,[12,13] and *O*-demethylangolensin.[10] Animal lignans and isoflavonic phytoestrogens produced in the gut by bacterial metabolism of plant, especially grain, lignan and isoflavone precursors have been discussed with regard to their possible role as antiestrogens and anticarcinogens.[1,10]

To study the metabolism, biological and physiological effects, sensitive and specific methods were required for the quantification of the enterolignans and phytoestrogens from samples of human origin. Thus to facilitate the quantification of these compounds, a method was developed[14] based on ion exchange chromatography and g.c.–m.s. (SIM technique) using deuteriated internal standards of enterolactone, enterodiol, equol, and daidzein. For this technique it was desirable that the internal standard be labelled by at least three deuterium atoms in order to obtain a straight line calibration curve free of interference from the compound to be measured.[15]

Kirk *et al.*[16] have very recently reported the synthesis of the isotopically labelled urinary lignan [9′,9′-$^2H_2$] enterolactone and of the derived [1,1,4,4-$^2H_4$]enterodiol, employing a route where the label is carried along from a deuteriated starting material over a multistep synthetic sequence into the deuteriated product. We report here the successful application of another approach, namely the H/D exchange within the finished molecular framework. Our route, based on the exchange of aromatic protons that are *ortho*- or *para*- to a phenolic OH group is of course rather attractive for diphenolic compounds in particular. Thus, we have access to the two [$^2H_6$]enterolignans (Scheme 1) having highly satisfactory m.s. quantification characteristics. Similarly, certain [$^2H_5$]- or [$^2H_4$]-isoflavonic phytoestrogens (Scheme 2) are readily obtainable from the protio compounds.



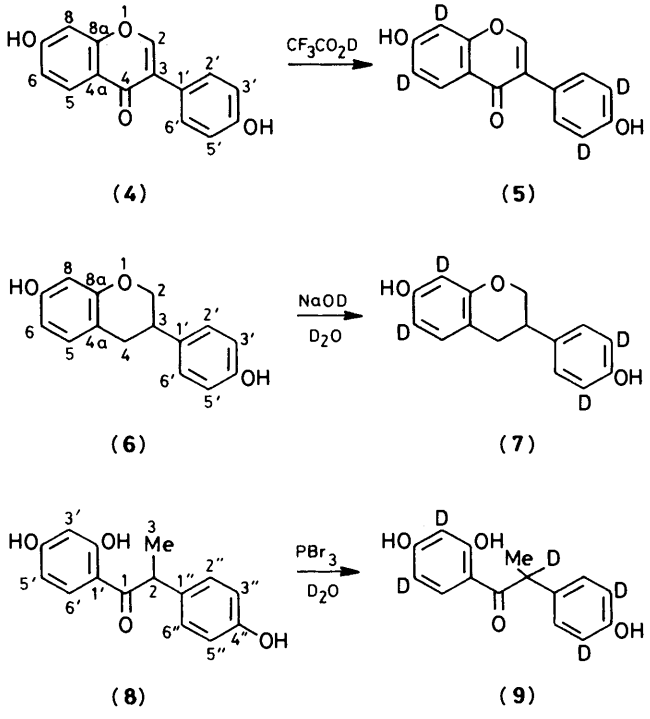
Scheme 1.

Enterolactone (**1**), daidzein (**4**), and *O*-demethylangolensin (**8**) were labelled with deuterium by exchange in DBr–D_2O or CF_3CO_2D. Labelled [$^2H_6$]enterolactone (**2**) was reduced by $LiAlH_4$ to give hexadeuterioenterodiol (**3**). Equol (**6**) is known to be decomposed under acidic conditions[17] but a base-catalysed exchange of hydrogen with deuterium works very well in this case. In most cases, the deuteriated products are available in an isotopic purity of 90% or better.

The position of the remaining unexchanged protons in aromatic rings of enterolactone (**2**) was determined from a non-

decoupled ^{13}C n.m.r. spectrum (Figure), which revealed that the only proton-carrying carbon atoms in the aromatic rings were C-5 and C-5′ (which give an overlapping signal at 130.1 p.p.m. in [$^2H_6$]acetone).

N.m.r. spectroscopy was used as a qualitative and mass spectroscopy as a quantitative method for following deuteriation in the case of isoflavonic phytoestrogens. The distribution of the label into [$^2H_4$]-, [$^2H_5$]-, *etc.* species was deduced from the molecular ion pattern in the mass spectra. The deuteriation sites were determined by ^{13}C n.m.r. spectroscopy. Deuteriated methine carbons appear as triplets in the proton noise decoupled spectra as compared to the singlets in the spectra of undeuteriated compounds. The ^{13}C n.m.r. spectrum of daidzein (**4**) is on the record,[18] while that of *O*-demethyl angolensin (**8**) was assigned by comparing the ^{13}C chemical shifts with literature data available for deoxybenzoins, especially 2,4,4′-trihydroxyphenylbenzoin.[19] Equol (**6**) assignments are based on comparison with spectra of simpler model compounds and consideration of substituent effects.[20] The ^{13}C n.m.r. spectrum of deuteriated *O*-demethylangolensin (**9**) showed evidence that the substitution occurred more rapidly at positions 2, 3″, 5′, and 5″ than at position 3′. Measurement by m.s. confirmed the isotopic composition as [2,. 3″, 5′, 5″-$^2H_4$] (20%) and [2,3′,3″,5′,5″-$^2H_5$] (80%) in *O*-demethylangolensin (**9**).



Scheme 2.

The protons of phenolic hydroxy groups were replaced by deuterons by D_2O treatment before the deuteriation procedure proper. In this way, the theoretical deuterium yields per DBr exchange step are much improved, for example, from 67% D to 78% D for *O*-demethyl angolensin.

In each procedure the H/D exchange treatment was repeated to ascertain complete deuteriation. After the final exchange, the reaction products were treated with a large excess of H_2O, C_2H_5OH, or CH_3OH to reinstate the protic hydroxy groups, so as to avoid ambiguities resulting from uncontrolled OD/OH exchange.

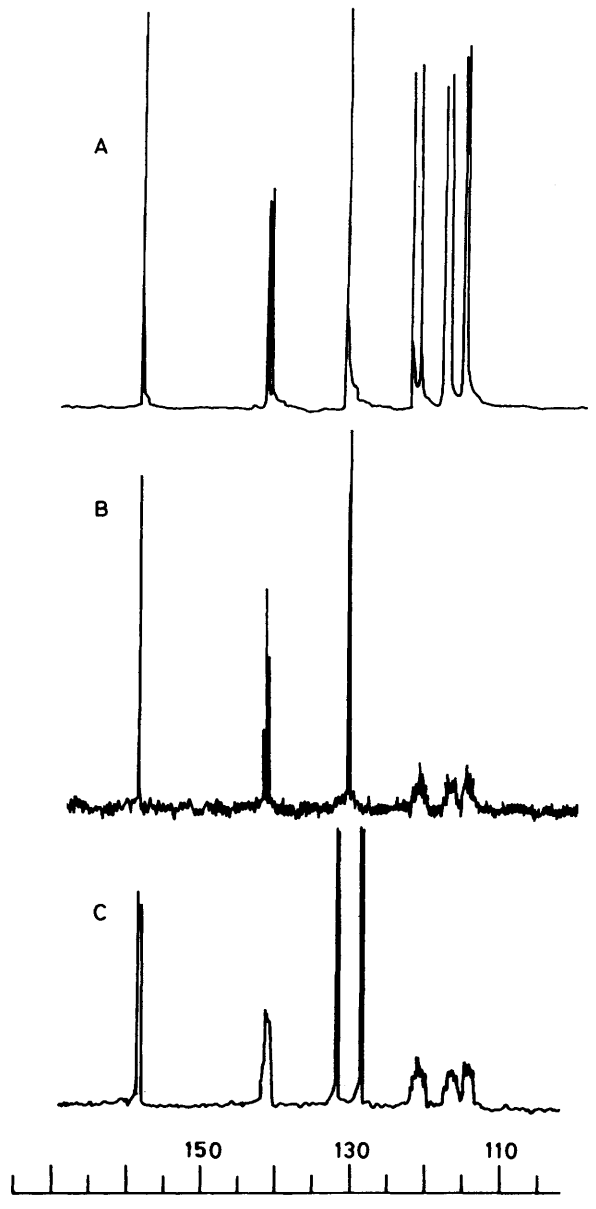


Figure. ^{13}C N.m.r. signals from aromatic ring carbons in (*a*) enterolactone, (*b*) hexadeuteriated enterolactone, and (*c*) deuteriated enterolactone recorded without proton noise decoupling. (The spectra were run in [$^2H_6$]acetone on a Jeol JNM FX 200 instrument).

Experimental

Improved methods for the preparation of the compounds for deuteriation will be published elsewhere.[10,21] ^{13}C N.m.r. spectra were recorded on a Jeol JNM PET 100 or a Jeol JNM FX 200 spectrometer for solutions in [$^2H_6$]acetone or [$^2H_6$]dimethyl sulphoxide with $SiMe_4$ as an internal reference. In numerical listings of the ^{13}C n.m.r. spectra, the shifts given for the C-D triplets are those corresponding to the central peaks, and are marked by a D. Mass spectra were obtained with a Jeol JMS 01SG 2 instrument. The samples were introduced by direct inlet probe with electron energy 40—75 eV, or 10—20 eV when determining the deuterium content; the sample temperature was in the range 60—155 °C. Ether refers to diethyl ether.

[2,2′,4,4′,6,6′-$^2H_6$]*Enterolactone* (**2**).—Deuterium oxide (2.2 g), PBr_3 (0.5 g), and D_2O-treated enterolactone (**1**) (0.66 g) were refluxed for 5 h.[22] After cooling, the reaction mixture was extracted with ether, the ether extracts were washed with D_2O (× 3), then dried (Na_2SO_4) and evaporated. The crude product was treated a second time with PBr_3 and D_2O as above and then purified by preparative t.l.c. acetone–hexane (1:2) to yield the deuteriated amorphic enterolactone (**2**) (0.23 g). The mass spectrum of the product (M^+, 304) indicated an isotopic purity of >90%; *m/z* 304 (10%), 194 (15), 148 (10), 137 (14), 136 (23), 111 (100), 110 (81), 94 (11), 93 (10), 80 (30), and 79 (41) (lit.,[23] for [$^2H_0$]enterolactone); δ_H (200 MHz; [$^2H_6$]acetone) signals *ca.* 6.58, 6.74 (2-, 2′-H), 6.73, 6.76 (4-, 4′-H), and 6.53, 6.70 (6-, 6′-H) were absent and the only aromatic proton signals were 7.09, 7.13 (5-, 5′-H, *J* 8.3 Hz) for the deuteriated sample. For the ^{13}C n.m.r. spectrum, see the Figure.

[2,2′,4,4′,6,6′-$^2H_6$]*Enterodiol* (**3**).—[$^2H_6$]Enterodiol (**3**) was prepared from [$^2H_6$]enterolactone (**2**) by $LiAlH_4$ reduction,[24] m.p. 165—169 °C (from $CHCl_3$). The mass spectrum of the product (M^+, 308) indicated the presence of 6 D atoms in the molecule and an isotopic purity of >90%; *m/z* 308 (0.1%), 290 (0.9), 289 (0.9), 288 (0.7), 181 (1.0), 180 (1.7), 179 (1.5), 178 (1.0), 163 (1.4), 162 (2.7), 161 (3.1), 150 (2.3), 147 (2.8), 136 (3), 111 (97), 110 (100), 94 (3), 93 (6), 80 (12), and 79 (19) (lit.,[23] for [$^2H_0$]enterodiol).

[6,8,3′,5′-$^2H_4$]*Daidzein* (**5**).—A mixture of D_2O-treated daidzein (**4**) (0.30 g) and labelled trifluoroacetic acid (19 ml) was heated under reflux for 27 h. CF_3CO_2D was prepared by adding trifluoroacetic anhydride (15 ml) to deuterium oxide (4 ml). After cooling, the solvent was removed and a fresh portion of CF_3CO_2D was added and the mixture was refluxed for 8 days. The reaction mixture was allowed to cool and the solvent was evaporated. The crude product was dissolved twice in boiling ethanol (95%; 50 ml) and evaporated. [$^2H_4$]Daidzein (**5**) was recrystallized from aqueous ethanol (0.2 g, 65%), m.p. 335 °C (lit.,[25] 325 °C for non-deuteriated material); *m/z* 258 (19.0%), 257 (21.0), 139 (96.0), 138 (100.0), 121 (33.9), 120 (84.1), 109 (16.7), 92 (15.0), and 91 (25.3). [$^2H_0$]Daidzein *m/z* 254 (45.8%), 253 (24.0), 137 (100), 118 (60.5), 108 (18.1), 89 (42.3), 81 (10.7), and 80 (16.7). In undeuteriated daidzein, the base peak is at *m/z* 137 whilst *m/z* 136 is very weak.[21] We are at present unable to explain why *m/z* 138, and not *m/z* 139, is the base peak in the [$^2H_4$]-derivative. The tetradeuterio isotopic purity of [6,8,3′,5′-$^2H_4$]daidzein (**5**) was estimated from a low-voltage mass spectrum (to minimize the $M^+ - 1$ peak); as 91%; δ_C (100 MHz; [$^2H_6$]-DMSO) 174.56 (C-4), 162.30 (C-7), 157.30 (C-8a), 156.96 (C-4′), 152.59 (C-2), 129.84 (C-2′, 6′), 127.11 (C-5), 123.41 (C-1′), 122.43 (C-3), 116.49 (C-4a), 114.97 (C-6, 3′, 5′),[D] and 101.75 (C-8).[D]

[6,8,3′,5′-$^2H_4$]*Equol* (**7**).—D_2O-Treated equol (**6**) (0.5 g), deuterium oxide (2.5 mol), and NaOD in D_2O (40%; 0.09 ml) were heated for 20 h at 100 °C in a Teflon-lined stainless steel autoclave.[26] Before sealing the autoclave was flushed with argon. After cooling, the reaction mixture was acidified with conc. H_2SO_4 (0.3 ml) in D_2O (0.7 ml). The resulting precipitate was collected and washed three times with D_2O. The entire process was repeated after adding a fresh sample of deuterium oxide and 40% NaOD in D_2O. After acidification of the reaction mixture with H_2SO_4–H_2O, the pale crystalline residue was washed with water and dissolved twice in methanol (30 ml) and evaporated. [6,8,3′5′-$^2H_4$]Equol (**7**) was recrystallized from aqueous ethanol (0.31 g, 63%), m.p. 158 °C (lit.,[27] 158 °C for non-deuteriated material). The isotopic purity was 90% [$^2H_4$] as determined by mass spectrometry; *m/z* 246 (3.2%), 137 (24.3), 136 (18.1), 125 (85.1), 123 (14.5), 122 (100.0), 121 (13.1), 109 (23.1), 93 (7.8), 79 (2.0), and 67 (0.7) (lit.,[28] for undeuteriated equol); δ_C (100 MHz; [$^2H_6$]acetone) 157.32 and 156.96 (C-7 and C-4′), 155.87 (C-8a), 133.36 (C-4a), 130.75 (C-5), 128.99 (C-2′, -6′), 116.00 (C-3′, -5′),[D] 114.00 (C-1′), 108.60 (C-6),[D] 103.90 (C-8),[D] 71.53 (C-2), 38.77 (C-3), and 32.58 (C-4).

[2,3′,3″,5′,5″-$^2H_5$]-O-*Demethylangolensin* (**9**).—Deuterium oxide (1.94 g), PBr_3 (0.16 ml), and D_2O-treated *O*-demethyl angolensin (**8**) (0.5 g) were refluxed for 5 h.[22] After cooling, the crystallized product was filtered off and washed with two portions of D_2O. The exchange process was repeated and the labelled *O*-demethylangolensin was isolated and washed with water and extracted with ether. The organic layer was dried (Na_2SO_4) and evaporated. The crude product was dissolved twice in methanol, evaporated and then recrystallized from benzene (0.342 g, 65%), m.p. 110 °C (lit.,[29] 103 °C for non-deuteriated material); *m/z* 263 (M^+), and 139 (base peak). A low-voltage mass spectrum established the isotopic purity of [$^2H_5$]-*O*-demethylangolensin (**9**) as [$^2H_4$], 20%; [$^2H_5$], 80%. δ_C (100 MHz; [$^2H_6$]acetone) 199.49 (C-1), 166.91 (C-4′), 165.15 (C-2′), 157.02 (C-4″), 133.72 (C-6′, -1″), 129.23 (C-2″, -6″), 116.19 (C-3″, -5″),[D] 113.09 (C-1′), 107.99 (C-5′),[D] 103.75 (C-3′),[D] 45.85 (C-2),[D] and 19.42 (C-3).

Acknowledgements

We thank Mrs. Eila Bernström and Miss Liisa Heino for recording the n.m.r. spectra and Mr. Seppo Kaltia for running the mass spectra.

References

1 H. Adlercreutz, *Gastroenterology*, 1984, **86**, 761.
2 C. B. S. Rao, 'Chemistry of Lignans,' Andhra University Press, 1978.
3 K. D. R. Setchell, A. M. Lawson, F. L. Mitchell, H. Adlercreutz, D. N. Kirk, and M. Axelson, *Nature (London)*, 1980, **287**, 740.
4 S. R. Stitch, J. K. Toumba, M. B. Groen, C. W. Funke, J. Leemhuis, J. Vink, and G. F. Woods, *Nature (London)*, 1980, **287**, 738.
5 K. D. R. Setchell, A. M. Lawson, S. P. Borriello, R. Harkness, H. Gordon, D. L. M. Morgan, D. N. Kirk, H. Adlercreutz, L. C. Anderson, and M. Axelson, *Lancet*, 1981, 4.
6 D. A. Shutt, *Endeavour*, 1976, **35**, 110.
7 D. A. Shutt, R. H. Weston, and J. P. Hogan, *Aust. J. Agric. Res.*, 1970, **21**, 713.
8 T. J. Batterham, D. A. Shutt, N. K. Hart, A. W. H. Braden, and H. J. Tweeddale, *Aust. J. Agric. Res.*, 1971, **22**, 131.
9 M. C. Nottle and A. B. Beck, *Aust. J. Agric. Res.*, 1974, **25**, 509.
10 C. Bannwart, H. Adlercreutz, T. Fotsis, K. Wähälä, T. Hase, and G. Bronow, *Finn. Chem. Lett.*, 1984, 120.
11 C. Bannwart, T. Fotsis, R. Heikkinen, and H. Adlercreutz, *Clin. Chim. Acta*, 1984, **136**, 165.
12 M. Axelson, D. N. Kirk, R. D. Farrant, G. Cooley, A. M. Lawson, and K. D. R. Setchell, *Biochem. J.*, 1982, **210**, 353.
13 H. Adlercreutz, T. Fotsis, R. Heikkinen, J. T. Dwyer, M. Woods, B. R. Goldin, and S. L. Gorbach, *Lancet*, 1982, 1295.
14 H. Adlercreutz, T. Fotsis, C. Bannwart, T. Mäkelä, K. Wähälä, G. Brunow, and T. Hase in 'Second Symposium on Estrogens in the Environment Influences on Development,' Raleigh, 1985.
15 A. P. De Leenheer and A. A. Cruyl in 'Biomedical Applications of Mass Spectrometry,' eds. G. R. Waller and O. C. Dermer, John Wiley & Sons, New York, 1980, 1169.
16 D. N. Kirk, L. M. McLaughlin, A. M. Lawson, K. D. R. Setchell, and S. K. Patel, *J. Chem. Soc., Perkin Trans. 1*, 1985, 35.
17 H. S. Chang, A. R. Robinson, and R. H. Common, *Anal. Biochem.*, 1975, **63**, 290.
18 H. C. Jha, F. Zilliken, and E. Breitmaier, *Can. J. Chem.*, 1980, **58**, 1211.
19 A. Pelter, R. S. Ward, and R. J. Bass, *J. Chem. Soc., Perkin Trans. 1*, 1978, 666.
20 M. Hesse, H. Meier, and B. Zeeh, 'Spektroskopische Methoden in der organischen Chemie,' Georg Thieme Verlag, Stuttgart, 1979, 236.
21 H. Adlercreutz, T. Mäkelä, K. Wähälä, G. Brunow, and T. Hase, unpublished work.

22 A. A. Bothner-By and E. Moser, *J. Am. Chem. Soc.*, 1968, **90**, 2347.
23 K. D. R. Setchell, A. M. Lawson, E. Conway, N. F. Taylor, D. N. Kirk, G. Cooley, R. D. Farrant, S. Wynn, and M. Axelson, *Biochem. J.*, 1981, **197**, 447.
24 M. B. Groen and J. Leemhuis, *Tetrahedron Lett.*, 1980, **21**, 5043.
25 R. B. Bradbury and D. E. White, *J. Chem. Soc.*, 1951, 3447.
26 D. H. Williams, S. W. Tam, and R. G. Cooks, *J. Am. Chem. Soc.*, 1968, **90**, 2150.
27 F. Wessely and F. Prillinger, *Chem. Ber.*, 1939, **72**, 629.
28 Y. Hayashi, T. Shirato, K. Sakurai, and T. Takahashi, *Mokuzai Gakkaishi*, 1978, **24**, 898.
29 R. A. Micheli, A. N. Booth, A. L. Livingston, and E. M. Bickoff, *J. Med. Pharm. Chem.*, 1962, **5**, 321.

Received 3rd *May* 1985; *Paper* 5/733