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Research Article

Synthesis of [$^2H_8$]-enterolactone and [$^2H_{10}$]-enterodiol

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Summary

Enterolactone and enterodiol are the main mammalian metabolites of dietary butyrolactone type lignans. The study of biological properties and potential health effects of these compounds requires isotopically labelled compounds as standards for quantitative measurements. An expedient deutero-labelling method for enterolactone is to use the $D_3PO_4 \cdot BF_3/D_2O$ complex at room temperature which will exchange all eight aromatic hydrogens, even from inactivated meta positions, to form [2,4,5,6,2′,4′,5′,6′-$^2H_8$]-enterolactone in 74% yield and 99% isotopic purity. [2,4,5,6,9,9,2′,4′,5′,6′-$^2H_{10}$]-Enterodiol was prepared from [2,4,5,6,2′,4′,5′,6′-$^2H_8$]-enterolactone by reduction with $LiAlD_4$ which introduces two more deuterium atoms into the molecule. Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: deuterium; enterodiol; enterolactone; labelling; lignan; phytoestrogen; metabolite

Introduction

In the human diet, good sources for plant lignans – precursors of the mammalian lignans enterolactone (3,3′-dihydroxylignano-9,9′-lactone) **1** and enterodiol (lignane-3,3′,9,9′-tetraol) – are flaxseed, wholegrain products, cereals especially rye, berries and vegetables.[1,2] The growing interest in these compounds is based on their possible health effects. Several epidemiological studies indicate that enterolignans, enterolactone **1** and enterodiol, may protect against breast, prostate and colon cancers and cardiovascular diseases.[3–5]

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Dietary plant lignans matairesinol (4,4′-dihydroxy-3,3′-dimethoxylignano-9,9′-lactone) and secoisolariciresinol (3,3′-dimethoxy-lignane-4,4′,9,9′-tetraol) are metabolized mainly to enterolactone **1** and enterodiol by human intestinal microflora.[6,7] These metabolites have been identified in human body fluids and tissues. Additional minor metabolites of plant lignans and new precursors for enterolactone **1** and enterodiol have been recently found *in vivo* and *in vitro* studies.[8,9]

Lignan concentrations are low in biological samples and therefore quantitation methods require good resolution and sensitivity. Commonly used analytical techniques for lignans are based on HPLC-MS and GC-MS.[10] In these chromatographic methods stable isotopically labelled enterolignans are needed as internal standards.

We now report that the $D_3PO_4 \cdot BF_3/D_2O$ complex, which we have used previously for the deuteration of flavonoids,[11–14] gives the [$^2H_8$]-enterolactone **2** in one step in excellent yield and isotopic purity. Remarkably all eight aromatic protons of enterolactone **1**, even from the non activated C-5 and C-5′ sites, are exchanged at room temperature. In the mass spectrum there is only an M^+ peak and no cluster of molecular ions with lesser amounts of deuterium and no unlabelled molecules. Thus the isotopic purity of [2,4,5,6,2′,4′,5′,6′-$^2H_8$]-enterolactone **2** is >99%.

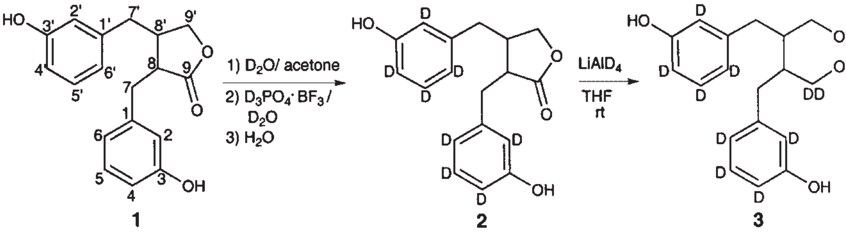
Previously, [9,9-$^2H_2$]-enterolactone has been prepared in 49% overall yield[15] by Kirk *et al.* by multistep synthesis, starting from dideuterated butenolide followed by a tandem Michael alkylation reaction, desulphurization and debenzylation with Raney nickel. Another route, only useful for preparing butyrolactone lignans such as enterolactone with identical aromatic substituents, was introduced by Bambagiotti-Alberti *et al.*[16] Dibenzylidene succinic acid, prepared by Stobbe condensation, was deuterated (D_2) over a $H_4Ru_4(CO)_8(PBu_3)_4$ catalyst to form the γ-lactone ring directly. [7,8,9,9,7′,8′-$^2H_6$]-enterolactone was obtained after debenzylation in 46% overall yield accompanied by 12% of the *cis* isomer. In our own earlier work,[17] enterolactone was refluxed with a mixture of PBr_3/D_2O to give [2,4,6,2′,4′,6′-$^2H_6$]-enterolactone directly in 35% yield in >90% isotopic purity. In this method aromatic hydrogens *ortho* or *para* to the phenolic hydroxy group are exchanged for deuterium.

Results and discussion

In the optimized procedure, hydrogens of the phenolic hydroxy groups are first removed from the equilibrium by exchanging them to deuteriums in an acetone–D_2O mixture and evaporating the solvents. The actual deuteration reagent $D_3PO_4 \cdot BF_3/D_2O$ is prepared by dissolving dry P_2O_5 (1.5 g) in D_2O (1.5 ml) at 0°C. The resulting deuterated phosphoric acid is then saturated with

BF_3 gas at room temperature. The reagent is added to the pre-deuterated enterolactone and the mixture stirred at room temperature.[12]

In previous methods only two to six hydrogens of enterolactone undergo exchange whereas the $D_3PO_4 \cdot BF_3/D_2O$ complex is acidic enough to exchange all eight aromatic hydrogens of enterolactone, even from the non-active aromatic sites C-5 and C-5′ (Scheme 1). [2,4,5,6,9,9,2′,4′,5′,6′-$^2H_{10}$]-Enterodiol **3** was obtained by the reduction of [2,4,5,6,2′,4′,5′,6′-$^2H_8$]-enterolactone **2** with $LiAlD_4$.



Scheme 1. Synthesis of [$^2H_8$]-enterolactone and [$^2H_{10}$]-enterodiol

Since the deuterated enterolactone will be used in analytical procedures as the TMS derivative, the deuterium content of [$^2H_8$]-enterolactone was determined by comparing the EI mass spectra of TMS-derivatised labelled and unlabelled compounds. Also, it was important to demonstrate that the newly generated D labels are stable under the silylation conditions. The ion patterns are practically superimposable in the M^+ region (Figure 1) and calculated isotopic purity for the TMS-derivatised [$^2H_8$]-enterolactone was >99%. EI mass spectroscopy cannot be used for the calculation of deuteration level of TMS-derivatised [$^2H_{10}$]-enterodiol as no molecular ion peak can be seen. However an APCI negative mode mass spectrum gives a strong M^+-1 peak, both for deuterated and undeuterated enterodiols, allowing the estimation of isotopic purity of [$^2H_{10}$]-enterodiol as >95%.

The ^1H and ^{13}C NMR data also confirm the exchange of all aromatic protons with excellent deuteration levels.

Experimental

All compounds, homogeneous by TLC, were characterized by ^1H NMR, ^{13}C NMR, LRMS and HRMS. Melting points were determined in open capillary tubes with a Büchi B-545 melting point apparatus and are uncorrected. NMR data were recorded on a Varian GEMINI 2000 or Bruker Avance 500 spectrometer. Chemical shifts are given in ppm and $SiMe_4$ was used as an

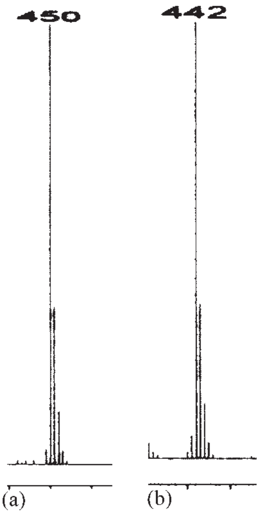


Figure 1. Part of the MS spectra for TMS derivatised [$^2H_8$]-enterolactone (a) and enterolactone (b)

internal standard. In the ^{13}C NMR spectra the shifts given for the C-D triplets or C-D_2 multiplet are those corresponding to the centre of the pattern and are marked 'D'. EIMS and HRMS were obtained using a JEOL JMS SX 102 spectrometer and the samples were introduced by a direct inlet probe. The deuterium content of TMS derivatised [$^2H_8$]-enterolactone was determined with a GC/MS Varian Saturn CP-3800. The deuterium content of [$^2H_{10}$]-enterodiol was determined with a Micromass Quattro II LC/MS using APCI in the negative mode. TLC was conducted with Merck silica gel 60 F_{254} plates and [$^2H_8$]-enterolactone was purified with Sephadex LH-20 (Sigma). D_2O (99.9%) was obtained from Aldrich and $LiAlD_4$ (99%) from Fluka. THF was dried by distilling over sodium and enterolactone was synthesized by a tandem Michael addition alkylation according to our published method.[18]

[2,4,5,6,2′,4′,5′,6′-$^2H_8$]-Enterolactone 2

A mixture of D_2O-treated enterolactone (0.10 g, 0.34 mmol) and $D_3PO_4 \cdot BF_3/D_2O$ (4 ml) reagent[12] was stirred at room temperature for 20 h. The reaction mixture was poured into ice water and the product extracted with EtOAc. The extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated. The crude product was re-treated with $D_3PO_4 \cdot BF_3/D_2O$ if the deuteration level as ascertained by NMR was unsatisfactory. The crude product was purified with Sephadex LH-20 (column: 0.5 cm × 17 cm, eluent: first 5 ml 20:1 (v/v) CH_2Cl_2–MeOH then 10 ml 40:3 (v/v) CH_2Cl_2–MeOH) and recrystallized from CH_2Cl_2–EtOAc as off white crystals (0.74 g, 74%), isotopic purity

$>99\%$, mp 145–146°C (Reference 19, 141–143°C D_0-enterolactone); ^{1}H NMR (200 MHz, D_6-acetone): δ 2.48–2.70 (4H, m, 7′, 8, 8′), 2.89 (1H, m, 7b), 2.96 (1H, m, 7a), 3.88 (1H, m, 9′a), 4.07 (1H, m, 9′b), 8.26 and 8.30 (s, $2\times$OH); ^{13}C-NMR (50 MHz, D_6-acetone): δ 35.1 (C-7), 38.6 (C-7′), 42.1(C-8′), 46.8 (C-8), 71.4 (C-9′), 114.1 (t, C-4, C-4′)^D, 116.2 (t, C-2, C-2′)^D, 120.6 (t, C-6, C-6′)^D, 129.8 (C-5, C-5′)^D, 140.6 (C-1), 141.1 (C-1′), 158.3 (C-3, C-3′), 178.7(C-9); EIMS (70 eV) *m*/*z*: M^+ 306 (39%), 196 (24%), 149 (16%), 137 (33%), 111 (100%), 80 (25%), 43 (46%); HRMS (*m*/*z*): M^+ calcd for $C_{18}H_{10}D_8O_4$, 306.1707, found, 306.1718.

[2,4,5,6,2′,4′,5′,6′-$^2H_8$]-3,3′-Di-O-TMS-enterolactone

[$^2H_8$]-Enterolactone (1 mg) was stirred in the pyridine/HDMS/TMCS 9:3:1 (v/v) reagent[8] for 2 h. Volatiles were evaporated under a flow of Ar and the product was dissolved in hexane. The filtered hexane solution was used as such for GC/MS analysis. EIMS (70 eV) *m*/*z*: M^+ 450 (100%), 185 (52%).

[2,4,5,6,9,9,2′,4′,5′,6′-$^2H_{10}$]-Enterodiol 3

[$^2H_8$]-Enterolactone (0.054 g, 0.18 mmol) was treated with $LiAlD_4$ (0.04 g, 0.95 mmol) in dry THF (6 ml) at room temperature. After 1h the reaction mixture was poured into a cold, saturated NH_4Cl (aq) solution. The solution was neutralised with 2M H_2SO_4 and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4 and evaporated. Crude [$^2H_{10}$]-enterodiol was recrystallized from $CHCl_3$–CH_3COCH_3 as off white crystals (0.041 g, 75%), isotopic purity $>95\%$, mp 174–175°C (Reference 20, 175–176°C for D_0-enterodiol).

^{1}H NMR (500 MHz, D_6-acetone): δ 1.95 (2H, m, 8, 8′), 2.70 (4H, m, 7,7′), 3.51 (1H, m, 9′a), 3.68 (1H, m, 9′b), 8.08 (s, arom. OH); ^{13}C-NMR (126 MHz, D_6-acetone): δ 36.1 (C-7, C-7′), 44.8 and 44.9 (C-8, C-8′), 61.1 (weak m, C-9^D and s, C-9′), 113.1 (C-4, C-4′)^D, 116.6 (C-2, C-2′)^D, 120.7 (C-6, C-6′)^D, 129.4 (C-5, C-5′)^D, 143.9 (C-1, C-1′), 158.1 (C-3, C-3′); EIMS (70 eV) *m*/*z*: M^+ 312 (3%), 294 (33%), 182 (36%), 164 (62%), 150 (50%), 137 (54%), 111 (100%), 80 (60%), 44 (59%).

Conclusion

All eight aromatic hydrogens, even at the nonactive C-5 and C-5′ sites, of enterolactone can be exchanged for deuterium under strongly acidic conditions using the $D_3PO_4\cdot BF_3/D_2O$ complex as deuteration reagent, in good yield and excellent isotopic purity. [2,4,5,6,2′,4′,5′,6′-$^2H_8$]-Enterolactone **2** and its reduction product [2,4,5,6,9,9,2′,4′,5′,6′ -$^2H_{10}$]-enterodiol **3** are stable molecules and can be used as reference compounds in quantitative analyses.

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