

Research Article

Synthesis of [²H₈]-enterolactone and [²H₁₀]-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 deuterio-labelling method for enterolactone is to use the D₃PO₄·BF₃/D₂O 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'-²H₈]-enterolactone in 74% yield and 99% isotopic purity. [2,4,5,6,9,9,2',4',5',6'-²H₁₀]-Enterodiol was prepared from [2,4,5,6,2',4',5',6'-²H₈]-enterolactone by reduction with LiAlD₄ 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'-dimethoxy-lignano-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%.

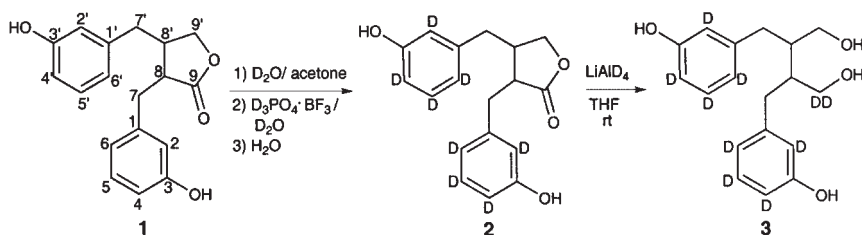
Previously, [9,9- 2H_2]-enterolactone has been prepared in 49% overall yield¹⁵ by Kirk *et al.* by multistep synthesis, starting from dideuterated butenolide followed by a tandem Michael alkylation reaction, desulphurization and debenzoylation with Raney nickel. Another route, only useful for preparing 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 debenzoylation 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

BF₃ 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 D₃PO₄ · BF₃/D₂O 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'-²H₁₀]-Enterodiol **3** was obtained by the reduction of [2,4,5,6,2',4',5',6'-²H₈]-enterolactone **2** with LiAlD₄.



Scheme 1. Synthesis of [²H₈]-enterolactone and [²H₁₀]-enterodiol

Since the deuterated enterolactone will be used in analytical procedures as the TMS derivative, the deuterium content of [²H₈]-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 [²H₈]-enterolactone was >99%. EI mass spectroscopy cannot be used for the calculation of deuteration level of TMS-derivatised [²H₁₀]-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 [²H₁₀]-enterodiol as >95%.

The ¹H and ¹³C 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, ¹³C 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 SiMe₄ was used as an

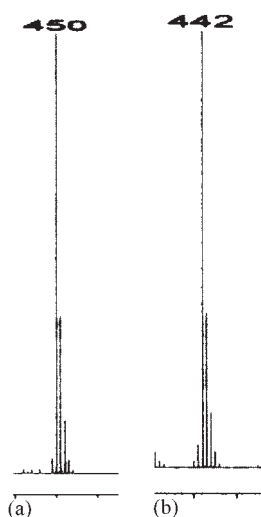


Figure 1. Part of the MS spectra for TMS derivatised $[^2\text{H}_8]$ -enterolactone (a) and enterolactone (b)

internal standard. In the ^{13}C NMR spectra the shifts given for the C-D triplets or C-D₂ 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 $[^2\text{H}_8]$ -enterolactone was determined with a GC/MS Varian Saturn CP-3800. The deuterium content of $[^2\text{H}_{10}]$ -enterodiol was determined with a Micromass Quattro II LC/MS using APCI in the negative mode. TLC was conducted with Merck silica gel 60 F₂₅₄ plates and $[^2\text{H}_8]$ -enterolactone was purified with Sephadex LH-20 (Sigma). D₂O (99.9%) was obtained from Aldrich and LiAlD₄ (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'-²H₈]-Enterolactone 2

A mixture of D₂O-treated enterolactone (0.10 g, 0.34 mmol) and D₃PO₄·BF₃/D₂O (4 ml) reagent¹² 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 Na₂SO₄ and evaporated. The crude product was re-treated with D₃PO₄·BF₃/D₂O 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) CH₂Cl₂-MeOH then 10 ml 40:3 (v/v) CH₂Cl₂-MeOH) and recrystallized from CH₂Cl₂-EtOAc as off white crystals (0.74 g, 74%), isotopic purity

> 99%, mp 145–146°C (Reference 19, 141–143°C D₀-enterolactone); ¹H NMR (200 MHz, D₆-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 × OH); ¹³C-NMR (50 MHz, D₆-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 C₁₈H₁₀D₈O₄, 306.1707, found, 306.1718.

[2,4,5,6,2',4',5',6'-²H₈]-3,3'-Di-O-TMS-enterolactone

[²H₈]-Enterolactone (1 mg) was stirred in the pyridine/HDMS/TMCS 9:3:1 (v/v) reagent⁸ 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'-²H₁₀]-Enterodiol 3

[²H₈]-Enterolactone (0.054 g, 0.18 mmol) was treated with LiAlD₄ (0.04 g, 0.95 mmol) in dry THF (6 ml) at room temperature. After 1 h the reaction mixture was poured into a cold, saturated NH₄Cl (aq) solution. The solution was neutralised with 2M H₂SO₄ and extracted with EtOAc. The extract was washed with brine, dried over Na₂SO₄ and evaporated. Crude [²H₁₀]-enterodiol was recrystallized from CHCl₃–CH₃COCH₃ as off white crystals (0.041 g, 75%), isotopic purity > 95%, mp 174–175°C (Reference 20, 175–176°C for D₀-enterodiol).

¹H NMR (500 MHz, D₆-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); ¹³C-NMR (126 MHz, D₆-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 D₃PO₄·BF₃/D₂O complex as deuteration reagent, in good yield and excellent isotopic purity. [2,4,5,6,2',4',5',6'-²H₈]-Enterolactone **2** and its reduction product [2,4,5,6,9,9,2',4',5',6'-²H₁₀]-enterodiol **3** are stable molecules and can be used as reference compounds in quantitative analyses.

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