Note

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Synthesis of highly pure ^{14}C-labelled *DL*-allantoin and ^{13}C NMR analysis of labelling integrity

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A number of synthetic approaches are assessed to prepare allantoin labelled with ^{14}C given certain requirements and technical limitations. A method that fulfils these criteria is described to achieve the synthesis of highly pure ^{14}C-labelled allantoin with the label introduced to the ureido carbonyl group in the final step by reaction of 5-chlorohydantoin with [^{14}C]urea. The chosen method favours high purity at the expense of radiochemical yield, which is achieved at a level of 8%. The integrity of the label is then investigated by performing an NMR analysis of ^{13}C-labelled allantoin synthesized by the same method. The ^{13}C NMR spectrum confirms partial scrambling of the label to the C-2 position by equilibration of the product via a putative bicyclic intermediate, which had been suggested by other workers. The ^{14}C-labelled allantoin synthesized by this method is therefore assigned as *DL*-[$H_2N^{14}CO/^{14}$C-2]allantoin. This study also includes the first full characterization of a side product, 5-hydroxy-5-methoxyhydantoin, obtained by the reaction of a 5-hydroxyhydantoin intermediate with the methanol solvent.

Keywords: 5-substituted hydantoins; ^{14}C-labelling; allantoin; ^{13}C NMR analysis

Introduction

DL-5-monosubstituted hydantoins are important precursors to optically pure D- or L-amino acids, which are intermediates in the production of a number of drugs and pharmaceuticals via their enzyme-catalysed hydrolyses.[1,2] As part of our studies on the structure–function relationships of hydantoin transporters in pathogenic bacteria,[3–6] we required *DL*-allantoin (*DL*-5-ureidohydantoin) labelled with ^{14}C, which is not commercially available. Here we describe our approach to the synthesis of ^{14}C-labelled *DL*-allantoin **5**, where the target site for the ^{14}C label was the ureido carbonyl group. A number of important considerations in our choice of synthetic route were the costs of starting compounds and ^{14}C-labelled reagent, avoidance of overcomplicated or technically demanding reactions (due to limited facilities for radiolabelled work), introduction of the ^{14}C label at the final step (to minimize handling and to optimize radiolabelled yield), production of the final compound as a solid with >99% purity from a relatively small-scale (~100 mg) reaction. Avoiding chromatography was also desirable to minimize further handling. We investigated a number of routes for the synthesis of **5** under these limitations.

Results and discussion

An attractive one-step synthesis of *DL*-allantoin is the reaction of glyoxylic acid (OHC-COOH) with urea in the presence of an acid, for which the use of heterogeneous catalysts has been studied,[7] especially as the relatively cheap and available [^{14}C]urea could be used as the source of the radiolabel. We investigated this reaction catalysed by both HCl[8] and by Dowex 50W × 8 resin (pre-treated with 10% H_2SO_4)[7] on a 100 mg scale. In both cases allantoin could not be obtained as a crystalline solid, without purification by chromatography, and the product contained some unreacted urea (^{1}H NMR 5.41 ppm, ^{13}C NMR 160.0 ppm); therefore, this route was considered not suitable for the synthesis of **5**.

We also investigated the reaction of *DL*-5-aminohydantoin with potassium cyanate[9] for the synthesis of **5**, which would use [^{14}C]KCNO as the source of radiolabel. For this, *DL*-5-aminohydantoin was prepared as described by Sarges *et al*.[10] with the final removal of the benzyloxycarbonyl protecting group using HBr-AcOH cleavage. However, on a small scale, the reaction with potassium cyanate did not produce allantoin as a pure solid.

We then investigated a route to **5**, which was devised independently in this study, but has been described earlier by Abblard and Meynaud[11] and is shown in Scheme 1, where the radiolabel is introduced in the last step by reaction of *DL*-5-chlorohydantoin **4** with [^{14}C]urea.

To produce **4**, we began with the commercially available parabanic acid **1**, which was reduced with KBH_4 in a similar manner to that described by Abblard and Meynaud[11] to give *DL*-5-hydroxyhydantoin **2** in 77% yield. In this reaction, some of the starting parabanic acid (molar equivalent to KBH_4) was lost as the potassium salt, which was removed as a solid. Some of the

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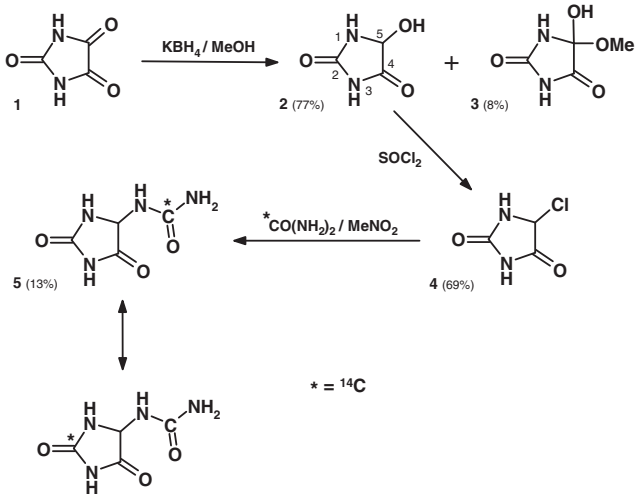

Scheme 1. Synthesis of ^{14}C-labelled *DL*-allantoin.

required product was lost by reaction of **2** with the methanol solvent to give 5-hydroxy-5-methoxyhydantoin **3**, which was also removed as a solid, in 8% yield. The loss of material to these side products had already been suggested by Abblard and Meynaud,[11] but isolation of **3** was surprising; therefore, a full NMR and physical analysis of this compound was performed, which had not been reported previously. Compound **3** was isolated as highly pure colourless crystals with melting point 222–225°C and with elemental analysis and high-resolution mass spectrometry results entirely consistent with the 5-methoxy structure shown in Scheme 1. The NMR spectra for this compound obtained in DMSO-d_6 were also consistent with the given structure, except that C-5 was not observed in the ^{13}C spectrum; this may be due to the absence of directly attached protons at C-5. Observation of NMR spectra also demonstrated that compound **3** was not stable for long in the DMSO solution. Compound **3** was not sufficiently soluble to allow ^{13}C NMR analysis in alternative solvents, including methanol-d_4; this was not surprising as methanol is the solvent from which compound **3** cleanly crystallizes from the reaction mixture.

Substitution of **2** to produce *DL*-5-chlorohydantoin **4** was achieved by reflux in thionyl chloride. As described by Sarges *et al*. in their preparation of *DL*-5-[(benzyloxy-carbonyl)amino]-hydantoin,[10] **4** was not isolated as it is unstable; instead the reaction mixture was evaporated and the residue dissolved in nitromethane for direct use in the next reaction. Importantly, any unreacted **2** is insoluble in nitromethane and thus can be removed by filtration, and the recovered **2** provided an approximate and indirect measure for the yield of **4**. This approach avoids isolating the unstable compound **4** as a solid, which had to be kept covered with hexane and used contaminated with ~10% 5-hydroxyhydantoin **2** in the method described by Abblard and Meynaud.[11]

DL-allantoin **5** was produced by reflux of **4** with urea in nitromethane in a similar manner to that described by Abblard and Meynaud,[11] except that a solution of **4** was used directly from the previous reaction. The product was obtained as a pure solid, with no contaminating urea, by its precipitation from the reaction mixture and washing with water. The yield of *DL*-allantoin **5** using this method was 15% from urea. The yield of **4** is only approximated; therefore, the quantity of **4** in the reaction mixture is likely to be significantly less than the molar

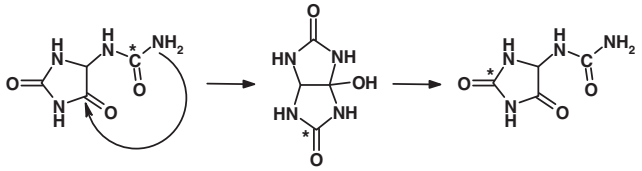

Figure 1. Putative mechanism for the scrambling of a label at the ureido carbonyl group of allantoin to the C-2 position. This type of mechanism had been suggested earlier by Abblard and Meynaud.[11]

equivalents of urea, which will affect the theoretical maximum yield possible. The relatively low yield was tolerated in order to obtain highly pure *DL*-allantoin, important when performing biological assays with radiolabelled compounds, and to fulfil all of the limitations described above. The reaction was then performed in the presence of [^{14}C]urea (0.5% at 56 mCi/mmol) to produce 34.4 mg (0.22 mmol) of ^{14}C-labelled *DL*-allantoin **5**, with a ^{14}C specific activity of 166 μCi/mmol. This corresponds to a chemical yield of 13% from urea and a radiochemical yield of 8%.

The work performed by Abblard and Meynaud[11] suggested that a ^{14}C label incorporated at the ureido carbonyl group of allantoin undergoes scrambling to C-2, possibly by the attack of the ureido primary amino group on the C-4 carbonyl function and therefore via a bicyclic symmetrical hydroxyglycouril intermediate (Figure 1). To investigate potential scrambling through this mechanism the synthesis was performed on the same scale as for the radiolabelled synthesis, except using 100% [^{13}C]urea to give ^{13}C-labelled *DL*-allantoin **5** in 17% yield. The ^{13}C NMR spectrum of ^{13}C-labelled **5** (Figure 2(A)) confirmed that a label at the ureido carbonyl group does undergo partial scrambling to C-2. Both the ^{13}C and ^{1}H NMR spectra of the ^{13}C-labelled allantoin (Figure 2(A) and (B)) also confirm the high purity of *DL*-allantoin produced by this method, especially with regard to any residual urea, which would have been easily detected (at 160.0 ppm) in the ^{13}C spectrum, with it being ^{13}C-labelled, and at 5.41 ppm in the ^{1}H spectrum.

The ^{14}C-labelled allantoin **5** produced using the method chosen here therefore contains molecules with ^{14}C either at the ureido carbonyl group or at C-2 and so can be assigned as *DL*-[$H_2N^{14}CO/^{14}C$-2]allantoin. Having the label at a mixture of the two positions does not cause any problems with its use in our intended biological assays. This study has highlighted a simple and reliable method to synthesize ^{14}C-labelled *DL*-allantoin under certain experimental limitations where high purity and consistency was favoured over yield.

Experimental

General

Except where stated otherwise, chemicals, ion-exchange resins and NMR solvents were from Sigma-Aldrich and reaction solvents used were of analytical grade, and all used without further purification. Column chromatography was performed on silica gel 60 using general-purpose solvents. Melting points were measured using a STUART Melting Point Apparatus. NMR spectra were recorded on a Bruker Avance 300 spectrometer and chemical shifts (δ) are given in ppm relative to the internal standard TMS. High-resolution mass spectra were obtained using a Waters GCT Premier instrument. Liquid scintillation analysis was performed on a Packard Tri-Carb 2100TR instrument and using an emulsifier-safe scintillation cocktail from Perkin-Elmer.

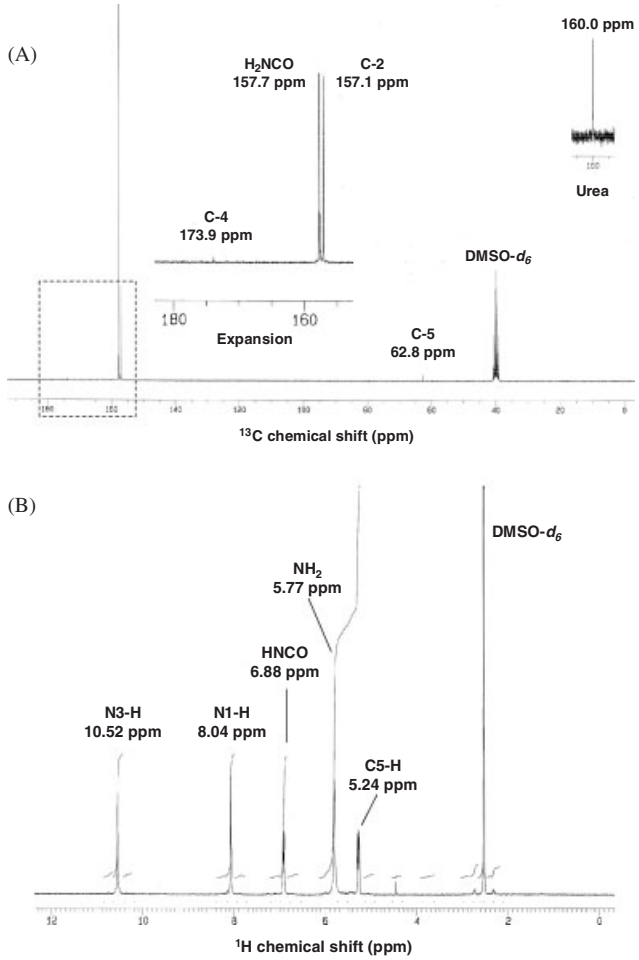

Figure 2. NMR analysis of ^{13}C-labelled *DL*-allantoin. ^{13}C (A) and ^{1}H (B) NMR spectra of the synthesized ^{13}C-labelled *DL*-allantoin **5** obtained in DMSO-d_6 and using a 300 MHz magnet. The ^{13}C NMR signal for urea, obtained for a separate sample of unlabelled urea in DMSO-d_6 and using the same magnet, is also shown in (A).

DL-5-hydroxyhydantoin (2)[11]

Potassium borohydride (273 mg, 5.1 mmol) was added in portions over ~30 min to a stirred solution of parabanic acid **1** (2 g, 17.5 mmol) in anhydrous methanol (44 ml) and the mixture was stirred for a further 1 h at room temperature. During this time, the potassium salt of **1** formed as a colourless solid, which was removed by filtration. The filtrate was stood at room temperature for ~24 h, during which 5-hydroxy-5-methoxyhydantoin **3** formed as colourless crystals (208 mg, 1.42 mmol, 8%). [M.p. 222–225°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.46 (s, 1 H, N3-H), 7.35 (s, 1 H, N1-H), 7.10 (s, 1 H, C5-OH), 3.77 (s, 3 H, C5-OCH$_3$). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 160.2 (C-2), 152.7 (C-4), no C-5 detected, 53.3 OCH$_3$. HRMS (EI$^+$/TOF): *m/z* calcd. for $C_4H_6N_2O_4$ = 146.0328; found: 146.0323. Anal. calcd. for $C_4H_6N_2O_4$: C, 32.88; H, 4.14; N, 19.17. Found: C, 32.95; H, 4.1; N, 19.4. Unstable to storage in DMSO]; these were removed by filtration. The filtrate was evaporated to leave a colourless solid, which was washed with methanol and dried under vacuum to give **2** (1.1 g, 9.48 mmol, 77%) as a colourless solid. The yield is corrected for the loss of **1** to the potassium salt (5.1 mmol). M.p. 141°C (lit. 140–142°C[11]). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.58 (s, 1 H, N3-H), 8.31 (s, 1 H, N1-H), 6.67 (s, 1 H, C5-OH), 5.08 (d, 1 H, *J* = 3.0 Hz, C5-H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 174.6 (C-2), 156.9 (C-4), 77.2 (C-5). HRMS (EI$^+$/TOF): *m/z* calcd. for $C_3H_4N_2O_3$ = 116.0222; found: 116.0217.

DL-5-chlorohydantoin (4)[10]

5-Hydroxyhydantoin **2** (500 mg, 4.31 mmol) was refluxed with vigorous stirring in thionyl chloride (8.7 ml) for 2 h. After cooling, the mixture was evaporated and the residue dissolved in nitromethane (10 ml) (5-chlorohydantoin dissolves to give a yellow solution and unreacted 5-hydroxyhydantoin remains insoluble). The solution was filtered to remove the contaminating 5-hydroxyhydantoin and 5 ml of the filtrate was used in the next reaction. The yield of **4** was ~401 mg, 2.98 mmol, 69% based on the amount of recovered 5-hydroxyhydantoin.

DL-allantoin (5)[7,11]

A solution of **4** in nitromethane (5 ml containing ~200 mg, 1.49 mmol, prepared as above) was added in portions over ~30 min to a solution of urea (100 mg, 1.66 mmol) in boiling nitromethane (5 ml) and the mixture was refluxed for 1 h with vigorous stirring. After allowing to cool, water (3 ml) was added and the mixture was stood at 4°C for ~5 days. A pale precipitate formed at the interface with the aqueous layer, which was collected under vacuum, washed thoroughly with water and dried under vacuum over phosphorus pentoxide for ~2 days to give **5** (39.3 mg, 0.25 mmol, 15% from urea) as a pale buff powder. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.53 (s, 1 H, N3-H), 8.05 (s, 1-H, N1-H), 6.88 (d, 8.4 Hz, 1 H, HNCO), 5.78 (s, 2 H, NH$_2$), 5.24 (dd, 1 H, *J* = 1.1 and 8.4 Hz, C5-H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 173.2 (C-4), 156.9 (H$_2$NCO), 156.3 (C-2), 61.99 (C-5). HRMS (EI$^+$/TOF): *m/z* calcd. for $C_4H_6N_4O_3$ = 158.0440; found: 158.0444. Anal. calcd. for $C_4H_6N_4O_3$: C, 30.39; H, 3.82; N, 35.43. Found: C, 30.55; H, 3.7; N, 35.15.

DL-[H$_2$N^{14}CO/^{14}C-2]allantoin (5)

The procedure was as above for the unlabelled **5**, except that 0.5 mg of the unlabelled urea was replaced by 0.5 mg [^{14}C]urea (American Radiolabelled Chemicals) with ^{14}C specific activity 56 mCi/mmol or 0.93 mCi/mg. The chemical yield of the reaction was 34.4 mg (0.22 mmol, 13% from urea). A total of 1.0 mg of the solid **5** gave ^{14}C counts (Packard Tri-Carb 2100TR) with an average of 2 360 945 dpm = 39.35 kBq = 1.0635 μCi; therefore, 34.4 mg of the solid has 36.58 μCi and a ^{14}C specific activity of 166 μCi/mmol or 0.166 mCi/mmol. The [^{14}C]urea (0.5 mg) used in the reaction had 465 μCi; the radiochemical yield of the reaction was therefore 36.58/465 μCi × 100 = 8%.

DL-[H$_2$N^{13}CO/^{13}C-2]allantoin (5)

The procedure was as above for the unlabelled **5**, except that 101.3 mg (1.66 mmol) of [^{13}C]urea (Cambridge Isotope Laboratories) was used in the reaction. The yield of **5** was 44.9 mg (0.28 mmol, 17% from urea). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.52 (s, 1 H, N3-H), 8.04 (s, 1-H, N1-H), 6.88 (d, 8.4 Hz, 1 H, HNCO), 5.77 (s, 2 H, NH$_2$), 5.24 (dd, 1 H, *J* = 1.1 and 8.4 Hz, C5-H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 173.9 (C-4), 157.7 (H$_2$NCO), 157.1 (C-2), 62.8 (C-5). HRMS (ESI$^+$): *m/z* calcd. for $C_3^{13}CH_6N_4O_3$+H = 160.0546; found: 160.0541.

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