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A Small-Scale Synthesis and Enantiomeric Resolution of *(RS)*-[1-^{14}C]-2-Phenylpropionic Acid and Biosynthesis of Its Diastereomeric Acyl Glucuronides

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SUMMARY

The 3 step synthesis for *(RS)*-[1-^{14}C]-2-phenylpropionic acid having overall yield of 30% is reported. The enantiomers were separated to an optical purity of 95 % (90 % ee) by semi-preparative chiral HPLC using an α_1-acid glycoprotein-based column (which has not previously been reported for this separation). Subsequently, the diastereomeric acyl glucuronides of (*R*)- and (*S*)-[1-^{14}C]-2-phenylpropionic acid were biosynthesised using a recirculating-mode isolated perfused rat liver preparation. The procedure for purification of the glucuronides by semi-preparative HPLC was optimised to ensure the biosynthetic products did not undergo degradation via acyl migration or hydrolysis.

Keywords: *(RS)*-[1-^{14}C]-2-phenylpropionic acid, acyl glucuronides, phase transfer catalyst, chiral HPLC

INTRODUCTION

2-Phenylpropionic acid (2-PPA) is the progenitive congener of the profens, a clinically important class of non-steroidal anti-inflammatory drugs (NSAIDs) used in the treatment of musculoskeletal and other inflammatory conditions. Both the (*R*)- and (*S*)-enantiomers of 2-PPA are metabolised predominantly by hepatic conjugation

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with D-(+)-glucuronic acid to form diastereomeric 1-*O*-β acyl glucuronides (2-PPA-G) (see Fig. 1) and it has been suggested that the step regulating the stereoselective elimination of (*R*)- and (*S*)-2-PPA-G in rats is their excretion from the liver in bile and blood (1). These excretory processes are therefore of particular clinical interest as exposure to reactive acyl glucuronides has been implicated in a number of immunological side effects of NSAIDs (2).

One experimental model that is well suited to studying the physiologic exchange of materials in the liver is the single-injection, impulse-response (3) isolated perfused rat liver. These experiments provide information on how the processes involved in hepatocellular uptake, efflux and biliary excretion affect a bolus input of drug to an isolated perfused liver. Given the large number of samples generated by these experiments, use of a radiolabelled substrate greatly reduces sample analysis times as simple radiometry (β-counting) can be used to quantify drug present in effluent perfusate.

As with the vast majority of profens, ^{14}C-labelled (*R*)- and (*S*)-2-PPA and their acyl glucuronides are not commercially available. Described here is a facile synthesis of labelled (*RS*)-[1-^{14}C]-2-PPA with subsequent resolution of the enantiomers and a procedure for biosynthesis of their labelled 1-*O*-β acyl-glucuronide conjugates, which could then be used in impulse-response experiments. The techniques should be equally amenable to other labelled 2-arylpropionate enantiomers and their respective major acyl glucuronide metabolites.

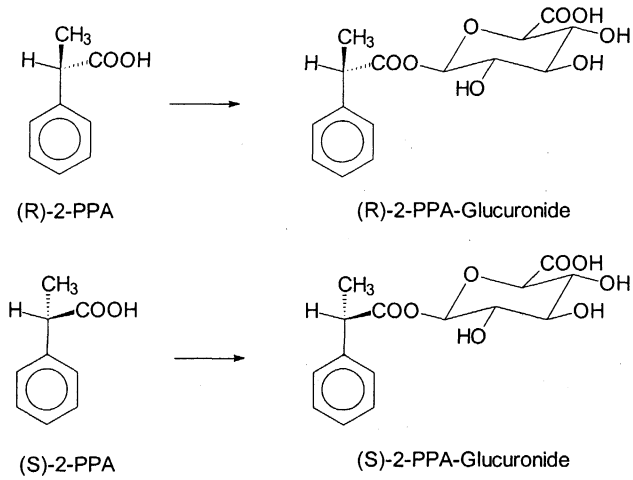
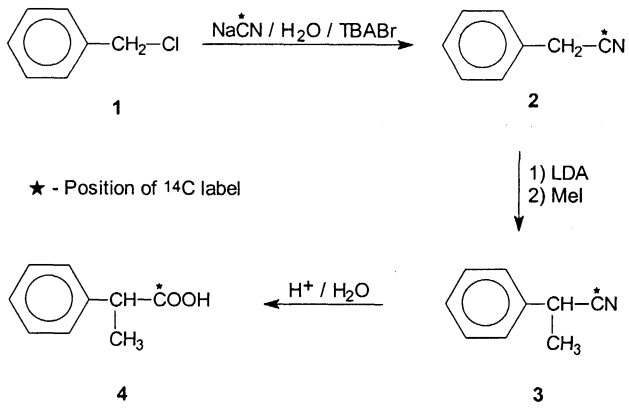


Figure 1: Structures of (*R*)- and (*S*)-2-PPA and their acyl glucuronide metabolites.

RESULTS AND DISCUSSION

The synthetic procedure outlined in Scheme 1 was based on that described for the synthesis of *(RS)*-[1-^{14}C]-ketoprofen (4).



Scheme 1: 3-Step synthesis of *(RS)*-[1-^{14}C]-2-phenylpropionic acid from benzyl chloride.

Conversion of unlabelled benzyl chloride **1** to benzyl cyanide **2** on a gram-scale according to the method of Vogel (5) achieved high yields. However, because the synthetic product had to be of sufficiently high specific activity for use in biological (impulse-response) experiments, it was necessary to perform the reaction incorporating the ^{14}C label on a milligram scale due to the high cost of ^{14}C-labelled sodium cyanide. When this reaction was conducted on a milligram scale, yields became highly variable (10-80%).

An NMR sample tube was found to be the ideal reaction vessel for this milligram-scale reaction as the length of tube above liquid acted as an air condenser. Furthermore, the reflux rate could be easily adjusted by varying the depth of the tube in the oil bath.

While the use of an NMR tube is elegant in its simplicity, the tube's small diameter gave rise to a very small interfacial area between the organic and aqueous phases. As cyanide ions are insoluble in the organic layer (6), contact between cyanide and benzyl chloride occurs only at this interface. The rate and extent of reaction is therefore (largely) determined by the interfacial area and degree of mixing about the 2-phase line. The progress of the reaction can be markedly improved by

using a phase transfer catalyst to facilitate movement of cyanide ions from the interface to the bulk of the organic phase (6).

A variety of catalysts have been used in cyanide displacement reactions involving aryl halides, however tetrabutylammonium bromide (TBABr in Scheme 1) is particularly convenient as it is easily removed from the final organic phase by washing with water (6). When TBABr was added to the aqueous phase such that it was present at a concentration of 1 % w/v, consistent (crude) yields of 72-76 % were achieved.

Methylation of [1-^{14}C]-benzyl cyanide **2**, followed by hydrolysis of (*RS*)-[1-^{14}C]-α-methylbenzyl cyanide **3** was then performed resulting in an overall yield of 30% for the synthesis of (*RS*)-[1-^{14}C]-2-PPA **4**, from benzyl chloride **1**. This compares well with the 23 % yield reported by Akira et al (7) for the synthesis of (*RS*)-[1-^{13}C]-ketoprofen and the 40% yield reported by Hayball et al. (4) for the synthesis of (*RS*)-[1-^{14}C]-ketoprofen.

(RS)-[methyl-^{14}C]-2-PPA has previously been optically resolved by recrystallisation of the diastereomeric amine salts formed upon reaction of *(RS)*-[methyl-^{14}C]-2-PPA with either (*R*)-(+)- or (*S*)-(-)-α-phenylethylamine (8). However, in our case, the mass of *(RS)*-[1-^{14}C]-2-PPA synthesised was small and, because of the substantial losses of (expensive) material that would be associated with resolution of milligram quantities by this procedure, it was deemed unsuitable. Separation of the enantiomers was therefore attempted by semi-preparative chiral HPLC. Radiometric detection was initially used to determine which peaks present in the UV_{212nm} chromatogram of the reaction product were associated with ^{14}C-labelled material.

A number of chiral columns are commercially available that claim to resolve (*R*)- and (*S*)-2-PPA, however the semi-preparative chiral column that was available in this laboratory was an α_1-acid glycoprotein-based column. This column has not previously been reported to facilitate this separation, so a mobile phase was developed that afforded the enantiomeric resolution evident in Figure 2A. Optical purity of the separated enantiomers of 2-PPA was assessed by chiral HPLC using an

analytical human serum albumin-based HPLC column with radiometric detection (Figure 2B and 2C).

The optical impurities shown in Figs. 2B and 2C were the result of the minor overlap that occurred when larger masses were applied to the ChiralAGP™ column. As a result, a 90 % ee (95 % of one enantiomer, 5 % of the other enantiomer) was achieved. Products of higher optical purity are easily obtained by injecting smaller amounts on the column.

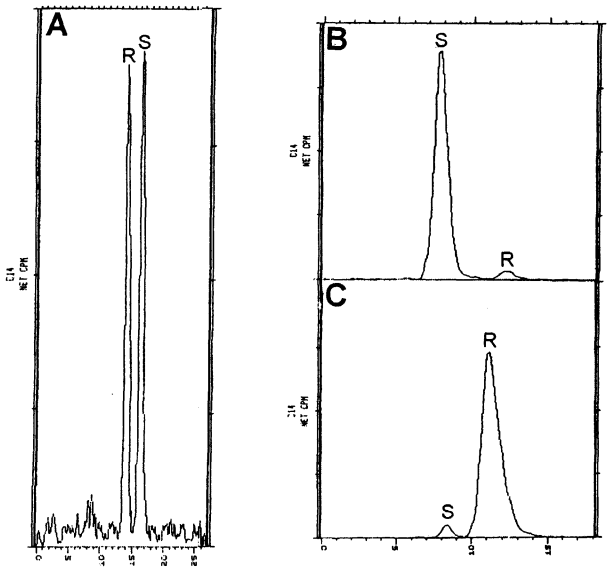


Figure 2 : Radiochromatograms showing (A) Separation of (*RS*)-[1-^{14}C]-2-PPA using a Chiral-AGP™ column (B and C) optical purity of separated (*S*)- and (*R*)-[1-^{14}C]-2-PPA (using a Chiral-HSA™ column.)

While the diastereomeric acyl glucuronides of (*R*)- and (*S*)-2-PPA can be chemically synthesised, the procedure is complex and has an overall yield of only 25 % when performed on a 0.75 mmol scale (9). As the isolated perfused rat liver (IPRL) has been used to investigate the hepatic disposition of (*R*)- and (*S*)-2-PPA (10), the recirculating-mode IPRL provided a convenient and high yield (efficient) method of biosynthesising (*R*)- and (*S*)-[1-^{14}C]-2-PPA-G.

1-*O*-β Acyl glucuronides are unstable, degrading via acyl migration or hydrolysis, giving rise to positional isomers and aglycone respectively (11). These degradation processes can be avoided by exposing samples containing 1-*O*-β acyl glucuronides to cold-acidic conditions (< 5°C, pH 3-5). As the 2-, 3- and 4-*O*-rearrangement isomers can exhibit markedly different pharmacokinetic profiles than

the biosynthetic 1-*O*-β form (12), it was of utmost importance that the purified product had not undergone isomerisation. Freeze-drying and an acidic HPLC mobile phase in the purification procedure ensures that the 1-*O*-β acyl glucuronides are maintained under these conditions until they are in a pure form. The doublets present at 5.57 and 5.55 in the ^{1}H-NMR spectra of (*S*)- and (*R*)-[1-^{14}C]-2-PPA-G and the fact that the spectra were comparable to the 500 MHz spectra reported previously for (*S*)- and (*R*)-2-PPA-G (9) indicated that the labelled glucuronides were indeed in the 1-*O*-β form and were therefore suitable for use in biological experiments.

Because of the propensity for acyl-glucuronides to degrade in buffer solutions under physiological conditions (11), the stability of (*S*)- and (*R*)-[1-^{14}C]-2-PPA-G in the isolated perfused liver perfusion medium (Kreb's bicarbonate buffer) at 37°C was assessed. Both diastereomers degraded with apparent first-order kinetics and degradation half-lives ($t_{1/2,deg}$) of 70 and 116 min for (*R*)- and (*S*)-[1-^{14}C]-2-PPA-G, respectively. The more rapid degradation of (*R*)-[1-^{14}C]-2-PPA-G was in accordance with the trend reported previously for the glucuronides of 2-PPA (9) and other profens (13, 14). The $t_{1/2,deg}$ values of (*S*)- and (*R*)-[1-^{14}C]-2-PPA-G in Kreb's bicarbonate buffer were, however, substantially smaller than those reported for the corresponding unlabelled compounds in 10 mM sodium phosphate buffer at the same temperature and pH (11). Degradation of unlabelled (*S*)- and (*R*)-2-PPA-G in Kreb's bicarbonate buffer (pH 7.4) at 37 °C was found to be comparable to that of (*S*)- and (*R*)-[1-^{14}C]-2-PPA-G (the $t_{1/2,deg}$ values being 55 and 110 min, respectively) suggesting that the more rapid degradation observed for (*S*)- and (*R*)-[1-^{14}C]-2-PPA-G compared with previously published data was due to a buffer effect, not the presence of the ^{14}C label.

In conclusion, this paper has described a simple method of synthesising (*R*)- and (*S*)-[1-^{14}C]-2-PPA on a small scale such that the product is of sufficiently high specific activity for use in biological experiments. Furthermore, a simple method of biosynthesising and purifying the diastereomeric 1-*O*-β acyl glucuronides of (*R*)- and (*S*)-[1-^{14}C]-2-PPA has also been described.

EXPERIMENTAL

All compounds were purchased from Sigma-Aldrich (Sydney, Australia) with the exception of ^{14}C-labelled sodium cyanide (40.19 mCi/mmol) which was from ICN (Irvine, USA) and unlabelled sodium cyanide which was from Merck (Darmstadt, Germany). H_2O was purified using the Waters™ Milli-Q Purification System. ^{1}H-NMR spectra were recorded with a Varian 600 MHz spectrometer using D_2O as solvent. Chemical shifts are reported as ppm downfield from the ^{1}H resonance of tetramethylsilane. All chromnatography was performed on a Hewlett-Packard Series 1100 system equipped with Series 1100 isocratic pump, Series 1100 autosampler and Series 1100 UV detector (detection wavelength - 212nm). Radiometric detection was by a Packard Radiomatic™ 150TR Flow Scintillation Analyser. All reactions were optimised using non-radiolabelled material.

Conversion of benzyl chloride **1** *to [1-^{14}C]-benzyl cyanide* **2**. ^{14}C-Labelled sodium cyanide (4 mCi, 4.6 mg, 0.12 mmol) and unlabelled sodium cyanide (10.5 mg, 0.27 mmol) were dissolved in 40 μL of a 0.30 M tetrabutylammonium bromide solution in a standard NMR sample tube which was then heated in a 90°C oil bath. Benzyl chloride (36 μL, 0.306 mmol) in ethanol (30 μL) was added slowly and the solution was allowed to reflux gently for 6.5 hr After cooling, water (500 μL) was added and the resultant mixture extracted with ether (5 x 600 μL). After removal of the ether under a nitrogen stream, the product was purified by preparative TLC (ethyl acetate : hexane, 1:4) to yield ^{14}C-benzyl cyanide (19.5 mg, 54 % yield) which was then reconstituted in dry THF (200 μL) and stored over Type 4Å molecular sieves until the next reaction.

Conversion of [1-^{14}C]-benzyl cyanide **2** *to (RS)-[1-^{14}C]-α-methylbenzyl cyanide* **3**. n-Butyllithium (90 μL, 1.69 M, 0.146 mmol) was added to diisopropylamine (20.5 μL, 0.146 mmol) and dry THF (100 μL) in a flamed, nitrogen-filled test-tube at -78°C. After the reaction mixture was stirred for 30 min, [1-^{14}C]-benzyl cyanide (16.5 mg, 0.148 mmol) in THF (200 μL) was added. A bright red colour evolved indicating the formation of the dianion after which the solution was stirred for a further 30 min (at -78°C). Iodomethane (100 μL, 0.66 mmol) was added and the

reaction mixture stirred at room temp for 1 hr. After adding 1M HCl (1 mL), the mixture was extracted with dichloromethane (4 x 2 mL). The latter was removed under vacuum to yield 13.9 mg (0.106 mmol, 72 %) of ^{14}C-*(RS)*-α-methylbenzyl cyanide yield). No further purification of the crude cyanide was performed prior to the next reaction.

Conversion of (RS)-[1-^{14}C]-α-methylbenzyl cyanide **3** *to (RS)-[1-^{14}C]-2-phenylpropionic acid* **4**. 9M H_2SO_4 (2 mL) was added to the flask containing the crude ^{14}C-*(RS)*-α-methylbenzyl cyanide from the previous reaction. The solution was then heated at 90-100°C for 8 hr after which it was extracted with dichloromethane. The dichloromethane solution was extracted with 1 M NaOH and the aqueous extract was then acidified with conc HCl and extracted with dichloromethane. The latter was evaporated off under a nitrogen stream and the product reconstituted in water prior to enantiomeric resolution by HPLC. HPLC analysis of the unresolved product revealed the presence of 12.5 mg (0.083 mmol) of ^{14}C-*(RS)*-2-phenylpropionic acid, i.e. 79 % yield.

Enantiomeric resolution of (RS)-[1-^{14}C]-2-phenylpropionic acid by semi-preparative chiral HPLC. (*R*)- and (*S*)-[1-^{14}C]-2-Phenylpropionic acid were separated using a 100 mm x 10.0 mm semi-preparative Chiral-AGP™ (Chromtech, Sweden) HPLC column. Mobile phase consisted of 0.1 % v/v THF in 0.1 M KH_2PO_4 (pH adj. to 4.0 with 85 % phosphoric acid) flowing at 3 mL/min. Effluent mobile phase fractions containing each enantiomer were pooled, and the resultant volume acidified (pH < 1) with conc HCl prior to extraction with dichloromethane (5 x equiv. vol.). The latter was removed under vacuum and the product taken up in 1mL of H_2O for storage. Optical purity was assessed by chiral HPLC using an analytical Chiral HSA™ column (Chromtech, Sweden) with a mobile phase containing 0.067 M Sorenson's phosphate buffer (pH 6.5) and 7.5% acetonitrile flowing at 0.8 mL/min.

Biosynthesis of (R)- and (S)-[1-^{14}C]-2-PPA acyl glucuronides.

The diastereomeric acyl glucuronide metabolites of 2-PPA were biosynthesised using the recirculating-mode isolated perfused rat liver preparation, details of which have been described previously (15).

The 100 mL volume of perfusion medium was spiked with 85 μCi of *(RS)*-[1-^{14}C]-2-PPA. Bile was collected directly into 2 mL of 1.55 M KH_2PO_4 solution (pH 4.2) throughout the 1 hr perfusion period; contents of the perfusate reservoir were collected and acidified to pH 4 with conc HCl at the end of this time. Approximately 31 % and 23 % of the dosed radioactivity was recovered as (unresolved) (*R*)- and (*S*)-[1-^{14}C]-2-PPA-glucuronide in perfusate and bile, respectively. The remainder of the dose was recovered unchanged as (unresolved) (*R*)- and (*S*)-[1-^{14}C]-2-PPA in perfusate. The *R*:*S* ratios of the [1-^{14}C]-2-PPA-glucuronides in perfusate and bile were approximately 1:1 and 1:3, respectively. The acidified perfusate and bile solutions were initially extracted at pH 4 with dichloromethane (3 x equiv. vol.) to selectively recover the residual unconjugated [1-^{14}C]-2-PPA. The solutions were then freeze-dried and the residue reconstituted in 2 mL of H_2O. Separation of the (*R*)- and (*S*)-[1-^{14}C]-2-PPA-glucuronides was achieved using a 300 mm x 7.8 mm semi-preparative reversed-phase HPLC column (SymmetryPrep® C18, Waters). Mobile phase consisted of H_2O/acetonitrile/trifluoroacetic acid (810/190/0.3) flowing at 3 mL/min and injection volume was 400 μL. Collected fractions were pooled and freeze-dried. The product was reconstituted in 0.1 mM KH_2PO_4 solution and stored at <5 °C.

^{1}H-NMR ((S)-[1-^{14}C]-2-PPA-G): δ 1.493 (3H, d, $J_{H3\text{-}H2}$=7.2 Hz, H3), 3.451-3.582 (3H,m, ring protons), 3.963 (1H, q, $J_{H2\text{-}H3}$=7.2 Hz, H2), 3.966 (1H, d, $J_{H'5\text{-}H'4}$=9.6 Hz, H'5), 5.571 (1H, d, $J_{H'1\text{-}H'2}$=8.1 Hz, H'1), 7.321-7.414 (5H, aromatic protons)
^{1}H-NMR ((R)-[1-^{14}C]-2-PPA-G): δ 1.49 (3H, d, $J_{H3\text{-}H2}$=7.2 Hz, H3), 3.417-3.455 (1H,m,ring protons), 3.521-3.544 (2H, m, ring protons) 3.958 (1H, q, $J_{H2\text{-}H3}$=7.2 Hz, H2), 5.55 (1H, d, $J_{H'1\text{-}H'2}$=8.4 Hz, H'1), 7.322-7.413 (5H, aromatic protons)

Time-dependent degradation of (R)- and (S)-[1-^{14}C]-2-PPA acyl glucuronides.

Modified Kreb's Bicarbonate buffer solution (4mL, 25 mM $NaHCO_3$, 118 mM NaCl, 5 mM KCl, 1.2 mM K_2HPO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 16.5 mM (D)-glucose, 0.00833 mM sodium taurocholate) containing either (*R*)- or (*S*)-[1-^{14}C]-2-PPA-G was incubated at 37 °C. Solutions were bubbled continuously with carbogen (95% CO_2 / 5% O_2) to ensure the pH did not change due to loss of CO_2. Aliquots (500 μL) were taken and immediately stabilised with 1M HCl (10 μL) prior to analysis by HPLC (3.9 x 150 mm Symmetry C8 column, 810/190/0.3 H_2O/acetonitrile/trifluoroacetic

acid, 0.8 mL/min, 200 μL injection vol.) using radiometric detection. Sample quantitation was based on radiochromatogram peak area.

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