TRITIATION OF 1,2,3-BENZENETRIOL (PYROGALLOL)

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SUMMARY

The synthesis of 4,6-$^{2}H_2$-1,2,3-benzenetriol from 1,2,3-benzenetriol was possible under both acid and base catalyzed conditions. However, under simulated physiological conditions it was observed that the label underwent exchange and was lost. Using a three step synthesis, 5-^{2}H-1,2,3-benzenetriol was synthesized and the label was found to be stable to exchange under the same conditions. Using this synthesis, 5-^{3}H-1,2,3-benzenetriol was synthesized with a specific activity of 7.6 mCi/mmole and of suitable purity for use in metabolic studies.

Key Words: 1,2,3-Benzenetriol (Pyrogallol), 5-^{3}H-1,2,3-Benzenetriol, deuterium, label exchange.

INTRODUCTION

Pyrogallol (pyrogallic acid, 1,2,3-trihydroxybenzene, 1,2,3-benzenetriol) (I) is a polyphenol which has found extensive use in industrial processes as an antioxidant (1) and as a synthetic intermediate (2). It exhibits a wide range of toxic effects including renal and hepatic damage, methemoglobinemia, azotemia (3), and at high doses circulatory collapse and death (1). It has also been employed in pharmacological studies as a specific inhibitor of catechol-O-methyltransferase (4,5). To date, however, there have been only limited studies on its metabolism.

To facilitate the initial metabolism studies it was necessary to synthesize radiolabeled pyrogallol. This paper reports a simple synthesis of a tritium-labelled pyrogallol suitable for use in metabolic studies in which the label is placed at a chemically stable position.

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EXPERIMENTAL

All chemicals used were reagent grade or better. The tritium oxide, 1 Ci/gm, was obtained from New England Nuclear. The deuterium oxide (99.8 atom%) and 30% sodium deuteroxide were obtained from Aldrich. Thin layer chromatography (TLC) was performed on Eastman TLC precoated silica gel sheets with fluorescent indicator. The TLC solvent systems were: System A, benzene-methanol-acetic acid (45-8-4); System B, toluene-ethyl acetate (8:2); System C, methanol, drop of acetic acid. High performance liquid chromatography (HPLC) was performed on an Altex Model 334 MP Liquid Chromatograph with a Rheodyne Model 7125 Syringe Loading Injector and a Whatman Partisil M9 ODS-3, 50 cm column. Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra were obtained with a Beckman Acculab 8 spectrophotometer. Nmr spectra were obtained on a Perkin-Elmer R-24 spectrophotometer and chemical shifts are reported relative to tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Uv spectra were obtained with a Beckman Model 25 Ultraviolet Spectrophotometer. Radioactivity was determined using a Beckman LS7500 Liquid Scintillation Counter using automatic quench control and external standardization. Gas chromatography/mass spectrometry was carried out using a Hewlett-Packard Model 5993 GC/MS system. Label exchange studies were carried out using a GCA Corporation Shaking Constant Temperature Bath Model 25.

4,6-$^{2}H_2$-1,2,3-benzenetriol(4,6-$^{2}H_2$-I) Pyrogallol (1.5 g, 12 mmoles) was placed in a flame-dried round-bottom flask and dissolved in 3.0 ml D_2O, then evaporated to dryness under reduced pressure. To the remaining white solid was added 1.5 g (79 mmoles) $Na_2S_2O_5$, 3.75 ml D_2O and 1.25 ml of a 30% solution of NaOD in D_2O under nitrogen. The approximate pH of the solution was 8-9. After 24 hours the solution was acidified to pH 5-6 with dropwise addition of 38% DCl, then extracted with 5x5 ml diethyl ether. The ether extracts were combined, dried over anhydrous Na_2SO_4, and evaporated to give 1.38 g (92% theoretical yield) of a white powder; m.p. 130-131°C (lit 131-133°C); the product co-chromatographed with I in

system A, rf=0.37; B, rf=0.10; C, rf=0.83 nmr (D_2O), 6.61 (S,1H,H-5). The mass spectrum of the trisilylated trimethylsilyl derivative contained the parent peak at m/z 344 (20.8%) with base peak at m/z 241, which indicated 62.6 atom% deuterium for the aromatic ring protons. The trisilylated trimethylsilyl derivative of I gave a parent peak at m/z 342 (17.6%) with a base peak at m/z 239.

Under acidic conditions at room temperature using 3.0 g (23.7 mmoles) I, 4.0 ml D_2O and 1.0 ml CF_3CO_2D, two deuteriums were again incorporated at the 4- and 6-positions as determined by nmr and GC/MS.

3,4,5-trimethoxybromobenzene. 3,4,5-trimethoxyaniline (0.25 g, 1.4 mmole), was dissolved in 5 ml of 4% H_2SO_4 solution, cooled to 0°C, and diazotized with 0.9 g (1.43 mmole) $NaNO_2$. The diazonium salt was then added dropwise to a 5 ml aqueous solution containing 0.20 g (1.40 mmole) CuBr and 1.0 ml of 50% HBr at 0°C. The mixture was stirred for 2 hours at 0-5°C after addition was complete. The precipitate was extracted with ether and the ether extract was filtered, dried over anhydrous Na_2SO_4, evaporated under reduced pressure and sublimed to give 0.183 g (53% theoretical yield) of the desired product: m.p. 79-80°C, nmr ($CDCl_3$) 3.84 (s,6H,3- and 5-CH_3), 3.87 (s,3H,2-CH_3) 6.65 (s,2H,Ar-H); Calcd for $C_9H_{11}BrO_3$: C, 43.74, H, 4.50; Found: C, 43.79, H, 4.50.

5-^{2}H-1,2,3-trimethoxybenzene. In a flame-dried, 50 ml 3-necked round bottom flask was added 100 mg (0.41 mmole) 3,4,5-trimethoxybromobenzene and 7 ml of freshly distilled dry hexanes. The reaction mixture was cooled to -73°C and 6.0 equivalents of freshly prepared n-butyllithium (0.7 - 1.2 M) in hexanes was added. The reaction was allowed to stir and warm from -73°C to -30°C until starting material was no longer detected by TLC (solvent system A), usually 1.5 - 2.0 hours. The reaction was quenched with 1.0 ml of D_2O and the mixture was allowed to warm to room temperature with stirring for 16 hours. The reaction was then quenched with 5.0 ml 0.1 M H_3PO_4 and stirred 10-15 min. The aqueous fraction was extracted with 5x2 ml diethylether and the hexane and ether fractions were combined, dried over anhydrous sodium sulfate, and evaporated under reduced

pressure to give a 50.0 mg (73.5% theoretical yield) of a white solid, which co-chromatographed as a single spot with authentic trimethoxybenzene in systems A (rf=0.68) and B (rf=0.59). Nmr ($CDCl_3$): 3.82 (S,6H,1- and 3-CH_3), 3.80 (S,3H, 2-CH_3), 6.70 (S,2H,Ar-H). The mass spectrum contained the parent peak and base at m/z 169 (100%). This was calculated to be 33.3 atom% deuterium for the aromatic ring protons. Trimethoxybenzene gave a parent peak and base peak at m/z 168.

5-^{2}H-1,2,3-benzenetriol(5-^{2}H-I). The deuterated trimethoxybenzene (27.4 mg, 16.2 mmoles) was dissolved in 3.0 ml of methylene chloride and cooled to -73°C. To this solution was added 9 molar equivalents of 1.0 M BBr_3 in methylene chloride and the reaction progress was monitored by TLC in solvent system A. After 2 hours, TLC showed evidence of both I and 3-methoxycatechol; at this time, 1 molar equivalent HBr in HOAc (37µl) was added to assist the final demethylation. When the demethylation was complete, it was quenched with 5.0 ml of 1M K_2HPO_4 (pH 8.0). The aqueous solution was saturated with NaCl and extracted with 5x1 ml ether; the ether extracts were combined, dried over anhydrous Na_2SO_4 and evaporated to give 6.2 mg (30% theoretical yield) of a white solid. The material co-chromatographed with pyrogallol in solvent systems A, B and C as a single spot. Nmr (D_2O): 6.53 (S,2H,Ar-H). The mass spectrum of the trisilyltrimethylsilyl derivative gave a parent peak at m/z 343. The deuterium incorporation was 33.4 atom% for the aromatic positions. As additional evidence that there was exclusive labeling at the 5 position, 0.1 g (0.4 moles) 3,4,5-trimethoxybromobenzene was lithiated at -73°C as previously outlined. To the mixture was added 5 g of finely ground solid carbon dioxide. The reaction was stopped by addition of 5 ml of 0.1M H_3PO_4 followed by diethylether extraction to give a solid white powder. Comparison of this material to commercial 2,3,4- and 3,4,5-trimethoxybenzoic acid by TLC, infrared, gas chromatography and GC/MS indicated that the sole product formed was 3,4,5-trimethoxybenzoic acid.

5-^{3}H-1,2,3-benzenetriol(5-^{3}H- I). The radiolabelled material was prepared using the same procedure as the deuterated compounds except for the following modifica-

tions and additions: the 5-lithio-3,4,5-trimethoxybenzene was quenched with 25 µl of 1 Ci/gm tritiated water (maximum amount allowed due to institutional restrictions) and allowed to warm to room temperature with stirring overnight. The demethylation with BBr_3 was done as previously outlined; however, the 5-^{3}H-I was further purified using semipreparative reverse phase high performance liquid chromatography (89% H_2O:1% HOAc:10% methanol with a flow rate of 3.0 ml/minute). Fractions were collected every minute, assayed for radioactivity and the retention volume from 48 ml to 60 ml was pooled and quantitated by uv using the maxima at 267 nm (ε = 628). Evaporation to dryness under reduced pressure gave 7.0 mg (34% overall yield) of 5-^{3}H-I with specific activity of 7.6 mCi/mmole. Radiochemical purity was determined to be greater than 95% based on the uv spectrum and TLC analysis in systems A, B and C. The material was dissolved in 1.0 ml of water containing 1 mg ascorbic acid and stored at -25°C.

Stability of Deuterium Label to Chemical Exchange:

Buffer solutions for the pH range 2 through 12 were prepared by combination of the appropriate 0.1M solutions of H_3PO_4, KH_2PO_4, K_2HPO_4 and K_3PO_4. The buffer solutions (10 ml) were placed in 125x15 mm screw-capped test tubes with Teflon-lined screw caps. The test tubes were placed in a 37°C constant temperature shaking water bath and allowed to equilibrate for 1 hour. To the buffer was then added 10.0 mg $Na_2S_2O_5$, and pH of the solution was checked. Each tube was then flushed with helium and 2-2.5 mg of either 4,6-$^2H_2$- or 5-^{2}H-I was added to each tube and the capped tubes were allowed to equilibrate for 30 minutes at which time the pH was again checked. The solutions were incubated at 37° for 24 hours after which the pH of the solutions was measured. Test tubes containing buffers of pH greater than 7 were acidified to pH 3-4 with concentrated HCl, then all buffers were saturated with sodium chloride and extracted with 3 xl ml diethyl-ether. The ether extracts were combined, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.1 ml acetonitrile, transferred to a 0.3 ml Reacti-vial, derivatized with

Sylon BFT (Supelco, Inc.) and analyzed by GC/MS. The gas chromatography conditions used a coiled glass column (4 ft x 2 mm i.d.) packed with 3% SE-30 on 100/-120 mesh Gas Chrom Q, injection port 270°C; the column temperature was temperature-programmed after 120°C → 160°C at 20°C per minute. The trisilylated trimethysilylderivative of I eluted at approximately 2 minutes. The mass spectrometer scanned from m/z 235 to 245 with 8 analog/digital measurements per datum point.

RESULTS AND DISCUSSION

In metabolic studies it would be preferred to synthesize pyrogallol containing a ^{14}C label. The synthesis of (U-^{14}C) 2,6-dimethoxyphenol from (U-^{14}C) phenol has been reported by Miller et al. (6); however, using the same methodology to obtain (U-^{14}C) pyrogallol would have required a five step synthesis (7). The only reported route (8) from aliphatics utilizes dihydroxymalonic acid and dimethylglutarate and also does not lend itself to a facile synthesis for labelling pyrogallol. Therefore, our initial investigations were directed toward incorporating a tritium label by simple acid or base exchange.

Approximation of π-orbital charge densities of 1.1704 for C-4 and C-6, and 1.0694 for C-5 as predicted by Schug and Deck (9) would predict that acid-catalyzed proton exchange on the aromatic ring of I should take place at positions 4 and 6. The studies of Olah and Mo (10) on the site of protonation of I in superacids corroborates these predictions. Hydrogen exchange under basic conditions would also be predicted to occur primarily at C-4 and C-6, since 2,3,4-trihydroxybenzoic acid is synthesized by reaction of I with sodium bicarbonate (11). It was unexpected, however, that under both acid- and base-catalyzed conditions exchange took place almost exclusively at these two positions with very little exchange (<5%) at C-5. The fact that two deuteriums could be incorporated under such facile conditions made this an appealing compound to synthesize; however, labels which are easy to chemically incorporate under acidic and basic conditions can also potentially undergo exchange in the physiological system in which the label-

Figure. Synthesis of 1,2,3-Benzenetriol Specifically Labelled in the 5-Position: a, n-BuLi, hexanes, -73°C, quenched with D_2O or T_2O; b, BBr_3, CH_2Cl_2,-73°C, then HBr/HOAc.

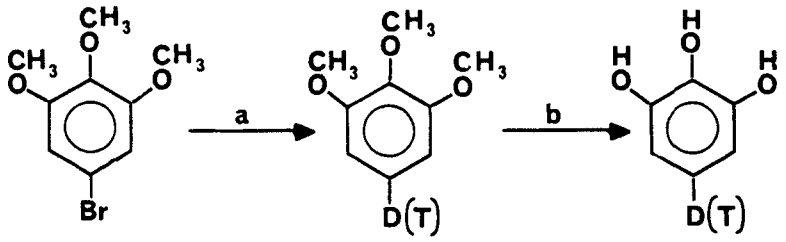

Table Percent Total Loss of Label from Deuterated Pyrogallol at Various pH's after 24 Hr Incubation at 37°C

pH	[4,6-$^2H_2$] PY	[5-^2H] PY
1.9	34.6	NED
2.9	3.7	NED
4.3	1.6	NED
5.7	1.7	NED
6.1	2.3	NED
7.1	6.7	NED
7.7	4.3	NED
8.7	18.3	NED
10.1	26.0	NED
11.1	99.3	NED
11.7	100.0	NED

NED = No exchange Detected

ed substrate is used. To evaluate the extent of this potential problem, the incubation of this compound at various pHs and at physiological temperature (37°C) for 24 hours was carried out. As depicted in the Table, loss of 5-7% of the label occurs at physiological pH; at acidic pH (i.e. resembling stomach pH), a significant amount of label was lost (35%). Also, under basic conditions (pH-10.1) 26% of the label was lost with virtually 100% label exchange at pH 11. Therefore, based on this information it was questionable if pyrogallol labeled in the 4- and 6-position would be suitable for further use in our studies.

The observation that position 5 was quite resistant to exchange suggested that a label incorporated in this position would be stable to relatively mild acid and base exchange. The synthetic approach used is outlined in the Figure. The 3,4,5-trimethoxybromobenzene was synthesized from commercially available 3,4,5-trimethoxyaniline using the conventional Sandmeyer reaction conditions. Some difficulty was encountered in lithiation of 3,4,5-trimethoxybromobenzene, however, using a 6-fold excess and maintaining the temperature below -30°C afforded reasonable yields of labeled trimethoxybenzene (50-73%). Quenching of the lithiated 3,4,5-trimethoxybromobenzene with only 25 µl of D_2O or T_2O required that the reaction be allowed to warm to room temperature overnight. If the workup was initiated 30 minutes after the addition of T_2O, pyrogallol with 0.42 mCi/mmole was obtained instead of 7.6 mCi/mmole. Demethylation of the trimethoxybenzene with BBr_3 led primarily to 3-methoxycatechol, and addition of one equivalent of HBr in glacial acetic acid was required to convert the intermediate catechol to I (13). Purification using HPLC provided labeled pyrogallol of adequate specific activity in an overall yield of 34%. To determine if a label incorporated in this position would be stable under simulated physiological conditions, the monodeuterated compound, was incubated in buffers as done with the other labeled material and no loss of deuterium was detected using GC/MS in the buffer range of pH 1.9 to 11.7 (see Table).

In conclusion, it would appear that pyrogallol in which the label is incor-

porated into the 5 position can be prepared in reasonable yield, of reasonably high specific activity and suitable for use in metabolic studies.

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