SYNTHESIS OF 4-HYDROXY[PHENYL-U-^{14}C]COUMARIN

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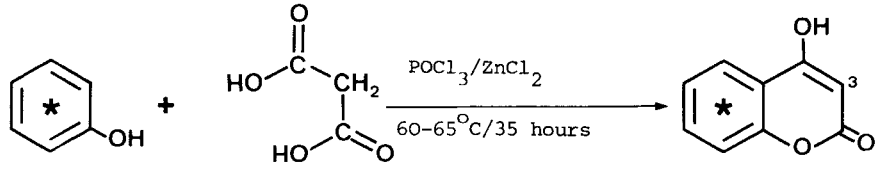
SUMMARY

Radiolabelled 4-hydroxycoumarin was synthesised on a microscale by condensing [U-^{14}C]phenol and malonic acid in the presence of anhydrous zinc chloride and phosphorus oxychloride. The yield was 14% on a 0.5 mmol scale at a specific activity of 107 μCi/mmol.

Key words : 4-Hydroxycoumarin, [U-^{14}C]phenol, microscale, synthesis.

INTRODUCTION

4-Hydroxycoumarin (I) is the fundamental moiety of a number of anticoagulant rodenticides, for example warfarin, coumachlor, coumatetralyl, difenacoum, brodifacoum and bromadiolone. Radiolabelled anticoagulants can be synthesised by linking the appropriate side chain to 4-hydroxy-[phenyl-U-^{14}C]coumarin at the 3-position for use in metabolic studies and the development of analytical methods for the determination of residues in post-mortem material. For *in vivo* studies of anticoagulant metabolism, it is necessary to produce radiolabelled 4-hydroxycoumarin of sufficiently high specific activity. Of the various methods[1-9] described for the synthesis of 4-hydroxycoumarin, the one-step method of Shah *et al.*[9] in which phenol and malonic acid are condensed in the presence of zinc chloride and phosphorus oxychloride was chosen for the present study.



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Other workers[10] have used this method on a smaller scale (24 mmol phenol) but obtained a low yield (25% by weight). To prepare radiolabelled 4-hydroxycoumarin of sufficient specific activity, while minimising the cost of [^{14}C]phenol to be purchased, it was necessary to scale the reaction down to 0.5 mmol phenol. The aim of the current investigation was to study the reaction conditions in detail to see whether this further reduction in the scale of the reaction could be achieved without concomitant loss in yield.

MATERIALS

Authentic 4-hydroxycoumarin (Ward, Blenkinsop & Co. Ltd.), [U-^{14}C]phenol (87 mCi/mmol; Amersham International plc), phenol, malonic acid, phosphorus oxychloride (Lab-reagent, BDH) and zinc chloride (anhydrous granular; Koch Light) were obtained commercially. All solvents used were of AnalaR grade except tetrahydrofuran which was HPLC grade (Rathburn) and devoid of stabiliser. Acetone and ether (which refers to diethy ether) were further purified by distillation and stored over molecular sieve (Type 4A; BDH).

EXPERIMENTAL

The micro-reaction apparatus was assembled from a Wheaton Micro Kit (Aldrich). Thin-layer (TLC) and preparative thin-layer chromatography (PTLC) plates, pre-coated with silica gel and 254 nm fluorescent indicator, were obtained commercially (Merck, Anachem or Whatman). All plates were pre-washed with methanol. The radioactivity on the chromatographic plates was located with a Berthold LB 2723 TLC radio-scanner and the radioactivity profile recorded on a Berthold LB 280 recorder. Reverse-phase high performance liquid chromatography (HPLC) was performed using two Altex 110A pumps and a 420 solvent programmer, by a method adapted from Walters *et al.*[11] Samples (25 µl) were chromatographed on a column (250 x 4.5 mm) of Zorbax-ODS (Du Pont) that was eluted with a multi-step gradient from water:formic acid (50:0.2, v/v) to tetrahydrofuran:water:formic acid (75:50:0.2, v/v/v). The eluant was monitored at 280 nm with a Pye LC3 variable-wavelength absorbance detector.

Infra-red (IR) spectra were recorded on a Pye Unicam SP200 spectrophotometer. Ultra-violet (UV) spectra were obtained using a Pye-Unicam SP8-100 recording spectrophotometer. Nuclear magnetic resonance (NMR proton) spectra were recorded on a Varian EM360 spectrometer (60 MHz) using tetramethylsilane as internal standard (δ = 0.00). Mass spectra were obtained on a Kratos MS30/DS50 mass spectrometer by direct insertion into the source at 200°C and at an ionising voltage of 70eV. Radioactivity measurements were made using a Packard Tricarb 460C liquid scintillation counter. Lipoluma or Luma gel (LKB Instruments) was used as the scintillant. All melting points (uncorrected) were determined on an Electro-thermal melting point apparatus.

Prior to microscale radiolabelled synthesis the yield of the reaction was examined in a series of experiments using unlabelled phenol. The initial syntheses were carried out on a large scale (24 mmol phenol) according to the method of Shah *et al.*[9] and the product was shown to be identical with authentic 4-hydroxycoumarin; R_F 0.32 [toluene:ethyl formate: formic acid (99:99:2, v/v/v)][12]; m.pt. 206-8°; ν max. (liquid paraffin mull) 1680, 1600, 1308, 950, 835, 740 cm^{-1}; λmax. [isopropanol:acetic acid (99:1, v/v)] 304, 279, 268 nm; δ (DMSO-d_6) 5.5 (s; 1-H), 7.7 (m; 5-H); m/e 162 (M^+, 2%) 161 (14%), 160 (95%), 120 (80%), 119 (100%), 92 (21%), 91 (92%). Microscale (0.5 mmol phenol) syntheses were performed by a method adapted from that of Shah *et al.* (see later). The results of both series of experiments are summarised in Table 1. On the microscale the highest yield obtained was 20%. In certain experiments moisture was suspected to be the prime cause of low yield, and since zinc chloride is highly deliquescent it was necessary to take stringent precautions to keep the reaction mixture anhydrous. Having ascertained the best conditions for the microscale reaction, a tracer experiment was undertaken with a small amount of radioactivity (1 μCi). For convenience in dispensing and to provide suitable aliquots for scintillation counting, samples of [U-^{14}C]phenol were dissolved

TABLE 1. Relationship between scale, reaction time and highest yield obtained.

Scale (mmol phenol)	Reaction time (h)	Yield (%)
2400[a]	35	64*
24[a]	35	16
24[b]	35	20
24[b]	26	25
24[c]	26	25
0.5[b]	26	10
0.5[b]	12	20

*Results of Shah *et al.*[9]

Molar ratio of phenol:malonic acid:phosphorus oxychloride

[a](1:1:2), [b](1:1:4), [c](1:2:4)

in ether. For the subsequent removal of the ether it was found necessary to use a vacuum manifold (-40°C, 0.1 mm Hg). The loss of phenol during this procedure was about 10%, which was considerably better than the losses suffered by using a rotavapor (25-90%).

Synthesis of radiolabelled 4-hydroxycoumarin on microscale (0.5 mmol phenol)

The contents of an ampoule of [U-^{14}C]phenol (0.28 mg, 250 µCi, specific activity 87 mCi/mmol) were rinsed into a 1 ml volumetric flask with ether. An aliquot (250 µl) of this solution was transferred to the micro-reaction vessel containing unlabelled phenol (47 mg, 0.5 mmol) and malonic acid (52 mg, 0.5 mmol). The reaction vessel was then attached to a vacuum manifold and the solvent removed (0.1 mm Hg, -40°C). Phosphorus oxychloride (0.3 ml, 3.3 mmol) was added to the contents of the reaction vessel, mixed thoroughly and two aliquots (15 µl) were withdrawn and the radioactivity measured by scintillation counting (total 52 µCi). Zinc chloride (205 mg, 1.5 mmol) was then added to the reaction vessel. The micro-reaction vessel was equipped with a reflux condenser fitted with a calcium chloride tube. The reaction

mixture was stirred at 60-65^{o} for 12 hours, then cooled on ice and crushed ice was added. The mixture was extracted with ethyl acetate (2 x 25 ml). The organic extracts were combined, dried (anhydrous sodium sulphate), filtered and concentrated on a rotavapor (40^{o}C/15 mm Hg). This solution was applied to a PTLC plate and developed three times in toluene:ethyl formate:formic acid (99:99:2, v/v/v).[12] Four bands, one of them at the origin, were detected when the plate was viewed under UV light (254 nm). The least mobile band above the origin was eluted with ether and found to be 4-hydroxycoumarin. Analytical TLC of the product showed it to be of high radiochemical purity (Berthold LB 2723 TLC radio-scanner/LB 280 recorder). There was good agreement in the yield (14%) measured by weight, HPLC, NMR, UV and radioactivity. Quantitation of yield by NMR and UV was carried out by comparison with standard solutions. Of these, the HPLC method was best for the simultaneous assessment of purity and yield. The UV spectrophotometric method needs to be treated with caution because although 4-hydroxycoumarin gives an easily recognised spectrum, it was found that other materials, presumably closely-related by-products also absorb at the wavelength (308 nm) used for quantifying 4-hydroxycoumarin. It would be unwise to rely on a single method to assess purity and yield.

In this study, at 0.5 mmol phenol scale, the yield of 4-hydroxycoumarin is only 14% and the optimum reaction time is 12 hours. It seems almost certain that the low yields obtained in the current work are an inherent property of undertaking the reaction on such a small scale. In view of the exhaustive nature of the investigation reported here it is unlikely that the yield could be improved. Despite this problem the reaction is still of great use because of its innate simplicity and it has facilitated the preparation of 4-hydroxycoumarin uniformly labelled with ^{14}C in the phenylene ring at a specific activity of 107 μCi/mmol, which is suitable for the synthesis of radiolabelled anticoagulants.

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