A SYNTHESIS OF 25-HYDROXYCHOLECALCIFEROL-(26,27-^{3}H)

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SUMMARY

The synthesis of 25-hydroxycholecalciferol-(26,27-^{3}H) is described. 25-Oxo-27-norcholesta-5,dien-3β-yl acetate is converted photochemically to 25-oxo-27-norcholecalciferol-3-acetate, which is then reacted with tritiated methylmagnesium iodide and subsequently saponified to give 25-hydroxycholecalciferol-(26,27-^{3}H).

INTRODUCTION

Cholecalciferol* (Vitamin D_3) undergoes a sequence of metabolic conversions in vertebrates which leads to the formation of a compound active in the biochemical reactions necessary for the effective intestinal transport of calcium[1]. The first of these conversions occurs in the liver[2,3], to give 25-hydroxycholecalciferol[4] which is then released into the blood. Subsequently, 25-hydroxycholecalciferol is further hydroxylated in the kidney[5] to give 1,25-dihydroxycholecalciferol[6], the form of the vitamin most active in promoting calcium transport. In order to study the metabolic fate of 25-hydroxycholecalciferol in detail,

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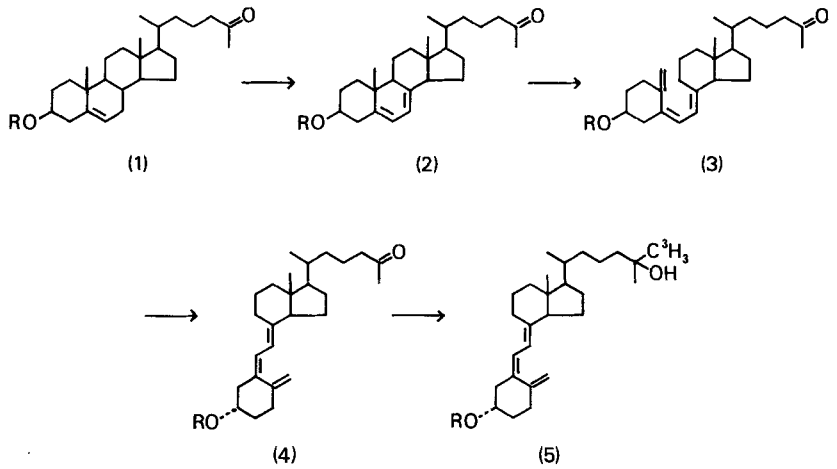
*Trivial names: Cholecalciferol, 9,10-Secocholesta-5,7,10(19)-trien-3β-ol

Tachysterol$_3$, 9,10-Secocholesta-5(10),6,8-trien-3β-ol

a highly radioactive preparation of the compound is required, and we report here the synthesis of 25-hydroxycholecalciferol tritiated at C-26(27), starting from 25-oxo-27-norcholesterol (1, R=H), a readily available oxidation product of cholesterol, as outlined in Figure 1 below.

Fig.1. Reaction Scheme



Syntheses of 25-hydroxycholecalciferol-[26,27-^{3}H] have been reported[7] which involve the reaction of a tritiated methyl grignard reagent with either 25-oxo-27-norcholesterol-3-acetate (1, R=Ac) or with the corresponding 5,7-diene (2, R=Ac). However, the critical step in all syntheses of vitamin D analogues from $\Delta^{5,7}$-steroids is the photochemical cleavage of the B ring to give the precholecalciferol analogue (3), which is then isomerised to the calciferol analogue (4) by heating. This photochemical reaction proceeds in low yield as a result of the presence of competing pathways, and since a cardinal principle of the design of radioactive syntheses is the minimisation of wastage and of the number and complexity of the synthetic procedures subsequent to the insertion of the radio-

active atoms, it is clearly of advantage to delay insertion of the radioactive atoms until a stage subsequent to the photochemical reaction.

This has been accomplished in the present synthesis by preparing 25-oxo-27-norcholecalciferol-3-acetate (4, R=Ac) from 25-oxo-27-norcholesta-5,7-dien-3β-yl acetate (2, R=Ac); preferential reaction of tritiated methyl-magnesium iodide with the 25-oxo group of 4(R=Ac) gave 25-hydroxycholecalciferol-[26,27-^{3}H] (5, R=H) after saponification. In the Grignard reaction it was necessary to exclude all traces of iodine, as a consequence of the extreme sensitivity of vitamin D analogues to reagents promoting cis-trans isomerisation[8,9].

EXPERIMENTAL

3β-Hydroxy-27-norcholesta-5,7-dien-25-one (2, R=H).

25-Oxo-27-norcholesta-5,7-dien-3β-yl acetate (2, R=Ac) (10 g), prepared by the method of Halkes and Van Vliet[10], was saponified by refluxing with 5% methanolic potassium hydroxide (200 ml) for 1 hr. The sterol crystallised on cooling and was filtered, washed with a little methanol, and recrystallised from methanol to give the free sterol (2, R=H) (8.2 g), m.p. 81-2°.

25-Oxo-27-norcholecalciferol-3-acetate (4, R=Ac).

3β-Hydroxy-27-norcholesta-5,7-dien-25-one (2, R=H) (6.0 g) was irradiated, in 300 mg-batches each in diethyl ether-methanol (9:1, 150 ml) for 20 min at 0° in the photochemical reactor described previously[11]. The batches were combined and evaporated to dryness below 20°, and the residue was suspended in diethyl ether (50 ml) and refrigerated. The crystalline residue, consisting of unchanged starting material, was filtered off and re-cycled through the same irradiation procedure. The combined product, after removal of solvent under reduced pressure, was refluxed in 90% ethanol (50 ml) for 45 min. 100 ml of 80% ethanol containing digitonin (5 g) was added, and refluxing was continued for a further 30 min. The cold solution was filtered 4 hr later, the residue being washed with ethanol.

Filtrate and washings were diluted with ether and successively washed with half-saturated NaCl solution and with water. After brief drying (Na_2SO_4) the ether extract was concentrated under reduced pressure to give an oily residue, whose ultraviolet absorption spectrum indicated that both 25-oxo-27-norcholecalciferol and 25-oxo-27-nortachysterol$_3$ were present.

This crude product was chromatographed on a column (50 x 1.5 cm) of silicic acid, eluting with a linear gradient from diethyl ether-light petroleum (1:1) to diethyl ether. Fractions (10 ml) were collected and assayed for ultraviolet absorbance at 265 or 281 nm. 25-Oxo-27-norcholecalciferol, still contaminated with some 25-oxo-27-nortachysterol$_3$, was recovered in fractions 15-33; fractions 34-40 contained pure 25-oxo-27-nortachysterol$_3$ which was later converted to 25-hydroxy-dihydrotachysterol$_3$ (D.E.M. Lawson, P.A. Bell & E. Kodicek, unpublished results).

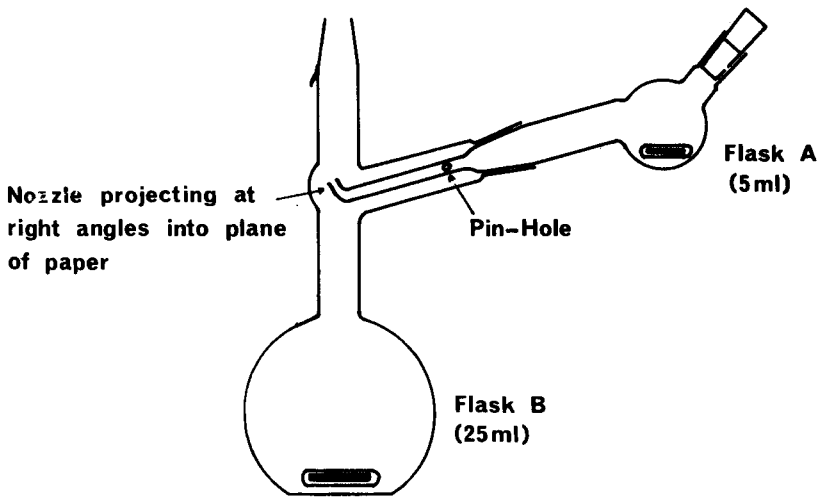
Fractions 15-33 were combined and evaporated to dryness. To the residue was added maleic anhydride (2 g) and dry benzene (40 ml) and the mixture was heated at 70° for 30 min. After removal of benzene under reduced pressure, methanolic potassium hydroxide (2 N, 40 ml) was added. 1 hr later, water and ether were added, and the ether layer was washed, separated, and dried (Na_2SO_4). Following removal of solvent, dry pyridine (20 ml) and acetic anhydride (5 ml) were added. After 12 hr at room temperature, the mixture was concentrated under reduced pressure and chromatographed on a column (50 x 1.5 cm) of silicic acid, eluting with a linear gradient (1000 ml) from light petroleum to light petroleum-diethyl ether (1:1) and collecting 10 ml fractions. The product was eluted between fractions 49-63. These fractions were combined and evaporated to dryness to give 25-oxo-27-norcholecalciferol-3-acetate as a colourless oil, λ_{max} 265 nm. The yield, calculated on the basis of ε_{265} = 18,300 was 0.83g. The compound gave a single spot on TLC on silica gel GF_{254} in chloroform, with R_F 0.58, and on silica gel GF_{254}: silver nitrate (4:1) in chloroform-acetone (4:1) gave two spots,

identified by spraying with sulphuric acid-methanol (1:1) and heating, with R_F values of 0.62 (major) and 0.50 (minor). Visual inspection indicated that the spots were in the ratio of approximately 10:1. On GLC, the compound gave two peaks, corresponding to pyro- and isopyro-derivatives, with retention times relative to 5α-cholestane of 2.80 and 3.67. The IR spectrum (in CS_2) showed absorption at 1730 ($CH_3.CO.O$-), 1710 (CH_3-$CO.CH_2$-), cm^{-1}.

25-Hydroxy-cholecalciferol-3-acetate-[26,27-^3H] (5, R=Ac).

The Grignard reaction was performed in an apparatus (Fig. 2) attached to a vacuum manifold to permit transfer of methyl iodide-[^3H] without escape to the atmosphere. 25-Oxo-27-norcholecalciferol-3-acetate (120 mg) was introduced in ethanol solution into bulb B, and the solvent was removed. The Grignard reagent was prepared in bulb A from magnesium powder (13.1 mg), dry deperoxidised ether (1 ml) and methyl iodide-[^3H] (95.4 mg) which was prepared immediately before use and distilled from mercury to prevent contamination with iodine.

Fig. 2. Vacuum Manifold Grignard Apparatus



Deperoxidised dry ether (5 ml) in the vapour phase was condensed into the apparatus by cooling bulbs A and B in liquid nitrogen. By turning bulb A, the Grignard solution was added drop by drop to the magnetically stirred steroid solution; addition of the first few drops of Grignard solution produced a yellow colour, but with further addition this disappeared, and a fine white precipitate was formed. The mixture was stirred for 1 hr.

The apparatus was flushed with nitrogen and an aqueous mixture containing 10% ammonium chloride and 10% sodium thiosulphate (5 ml) was added to the reaction mixture which was then well stirred. When the layers were clear, the mixture was transferred to a separating funnel, and washed in with benzene (10 ml) and ether (10 ml). The layers were separated, and the organic layer washed with water (2X) and 30% NaCl solution. The organic solution was dried (Na_2SO_4). In order to remove any traces of unreacted methyl iodide-[^{3}H] the solution was taken down to dryness at room temperature by means of a stream of nitrogen. The residue was redissolved in ether and the process repeated. The activity of the crude product was 4 curies.

The product was dissolved in a small volume of chloroform-acetone (1:1) and applied to 4 plates (20 x 20 x 0.1 cm) of silica gel PF_{254}. The plates were developed 3 successive times in chloroform in a cold room (+4°C). The main band identified by brief examination under an U.V. lamp was partly resolved into two components. The whole of this partly resolved band was removed from each plate by scraping into a Buchner funnel. The combined material was then washed with deperoxidised ether (200 ml). The resulting solution contained 1.4 curies.

A spot of the product on TLC, using chloroform-acetone (4:1) on silica gel GF_{254}, gave a single main peak on scanning the activity, with R_F 0.67, but on silica gel GF_{254}:silver nitrate (4:1) the peak was resolved into two components with R_Fs 0.42 and 0.52. The product was therefore purified by preparative TLC on 4 plates of silica gel PF_{254}:silver nitrate (4:1) (20 x 20 x 0.1 cm) using chloroform-acetone (4:1). The plates were developed once in the cold room.

Brief visual inspection under U.V. showed two well separated main bands, mean R_Fs approximately 0.4 (band I) and 0.6 (band II) respectively. The bands were scraped from the plates and eluted with ether as before. The eluates from band I (537 millicuries) and band II (601 Millicuries) were each found to consist of pure materials by TLC on silica gel:silver nitrate (4:1) using chloroform-acetone (4:1). The U.V. spectra (in ether) showed that band II contained 25-hydroxy-cholecalciferol-3-acetate-[26,27-^3H] (λ_{max} 266 nm; $\lambda_{max}/\lambda_{min}$ 1.8) and band I the trans isomer (λ_{max} 273 nm; $\lambda_{max}/\lambda_{min}$ 3.8).

25-Hydroxy-cholecalciferol-[26,27-^3H] (5, R=H).

601 millicuries of 25-hydroxy-cholecalciferol-3-acetate-[26,27-^3H] were dissolved in methanol (10 ml) and saponified with 10% aqueous potassium hydroxide (0.5 ml) under nitrogen at room temperature. The solution was stirred magnetically and samples of the reaction mixture were examined by TLC on silica gel GF_{254}:silver nitrate (4:1), developing with chloroform-acetone (4:1). 13.5% acetate remained after 90 min saponification, and 2.5% after 4 hr. Further saponification showed little change in the acetate content. After 6½ hr the solution was partitioned between ether (50 ml) and water (20 ml). The layers were separated, and the aqueous layer was back extracted twice with portions of ether (50 ml). The combined ethereal solutions were washed with water until the washings were neutral, then with 30% NaCl solution. The solution was dried over sodium sulphate. The solvent was removed at room temperature with a stream of nitrogen. The residue was redissolved in chloroform-acetone (1:1) (1 ml) and applied to two plates (20 x 20 x 0.1 cm) of silica gel PF_{254}:silver nitrate (4:1). The plates were given three successive developments with chloroform-acetone (9:1). The main band on each plate was identified under U.V., and scraped off into a Buchner funnel. The adsorbed material was eluted from the combined bands with ether (200 ml). In order to remove any dissolved silver nitrate and silicic acid, the ethereal solution was washed twice with water and once with sodium sulphate, the solvent was removed at room temperature using a

stream of nitrogen, and the residue redissolved in pure dry benzene (100 ml) to reduce radiation decomposition. The radiochemical purity of the product was 98% in three TLC systems and one paper chromatography system: cyclohexane-ether (4:1) on silica gel GF_{254}, chloroform-acetone (9:1) on silica gel GF_{254}: silver nitrate (4:1), acetone-water (4:1) (saturated with silicone oil) on silica gel GF_{254} containing 5% silicone oil, and on paper, 90% aqueous acetic acid (saturated with paraffin oil) on paper impregnated with paraffin oil.

The yield of product was 376 millicuries. The specific activity, calculated on the basis of E = 17,700(9) was 10.6 curies/millimole, λ_{max} = 266 nm.

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