SYNTHESIS OF STEREOSPECIFICALLY LABELLED [4-^{3}H]CHOLESTEROL

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SUMMARY

[4α-^{3}H]Cholesterol was prepared in three steps from 4-oxo-cholest-5-en-3β-yl acetate, which was conveniently prepared from cholesterol. Introduction of the 4α ^{3}H label utilised NaB^3H_4 as the source of tritium. Details of this synthesis and also the synthesis of [4β-^{3}H]cholesterol are given. Procedures for quantitative estimation of the tritium label at the 4α and 4β positions are also described.

Key words: [4α-^{3}H]cholesterol, [4β-^{3}H]cholesterol, 4-oxo-cholest-5-en-3β-yl acetate.

INTRODUCTION

Metabolic modification of the A and B rings of steroids frequently involves stereospecific removal of a hydrogen from C-4 of cholesterol with formation of a Δ^5 bond. Investigation of such metabolic transformations often necessitates the availability of both [4α-^{3}H] and [4β-^{3}H]cholesterol substrates. The present paper reports the synthesis of [4α-^{3}H]cholesterol; synthesis of [4β-^{3}H]cholesterol is only outlined briefly, since the reactions for the introduction of isotope at the 4β-position have been described previously [1,2].

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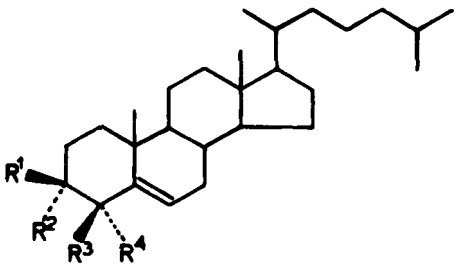
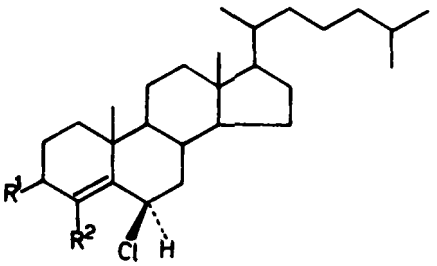


1 R^1=OBz, R^2=R^3=R^4=H
2 R^1=OBz, R^2=R^4=H, R^3=OH
4 R^1=OH, R^2=R^4=H, R^3=^{3}H
5 R^1=OH, R^2=R^3=R^4=H
6 R^1,R^2=O, R^3=R^4=H
7 R^1,R^2=O, R^3=H, R^4=OAc
8 R^1=OAc, R^2=H, R^3,R^4=O
9 R^1=OAc, R^2=H, R^3=OH, R^4=^{3}H
10 R^1=OAc, R^2=H, R^3=^{3}H, R^4=OH
12 R^1=OH, R^2=H, R^3=H, R^4=^{3}H



3 R^1=OBz, R^2=H
11 R^1=OAc, R^2=^{3}H

RESULTS AND DISCUSSION

[4β-^{3}H]Cholesterol

[4β-^{3}H]Cholesterol was prepared by the following procedure. 4β-Hydroxy-cholest-5-en-3β-yl benzoate 2 was synthesized from cholesteryl benzoate 1 by the method of Rosenheim and Starling[3] and was transformed into the chloro compound 3 by treatment with thionyl chloride using a modification of the method of Ireland, Wrigley and Young[1]. [4β-^{3}H]Cholesterol 4 was obtained in good yield by an $SN2'$ displacement of chlorine from 3 using lithium aluminium tritide[1].

[4α-^{3}H]Cholesterol

[4α-^{3}H]Cholesterol was prepared in three stages from 4-oxo-cholest-5-en-3β-yl acetate 8. This material itself was readily available via a modification of the method of Fieser and Stevenson[4]. Reduction of 8 with tritiated borohydride enabled the introduction of ^{3}H label at the 4 position in the resulting 4β-hydroxy compound 9. This material after careful separation from the 4α-hydroxy epimer 10, also produced by the reduction, was chlorinated to yield the rather unstable 6-chloro derivative 11 which was immediately reduced with lithium aluminium hydride to give the required [4α-^{3}H]cholesterol.

Location of ^{3}H in [4β-^{3}H]cholesterol

An aliquot of the [4β-^{3}H]cholesterol was mixed with [4-^{14}C]cholesterol and the mixture benzoylated to yield [4β-^{3}H,4-^{14}C]cholesteryl benzoate. Oxidation of this material with selenium dioxide[3] in acetic acid-water resulted in the loss of 91% of the tritium label (Table 1). This result indicates that the tritium is present at C-4, but since the

Table 1. ^{3}H/^{14}C Ratios of derivatives of [4β-^{3}H]cholesterol mixed with [4-^{14}C]cholesterol

	^{3}H/^{14}C Atomic ratio
Cholesterol	1:1
Cholesteryl benzoate	1.04:1
4β-Hydroxy-cholest-5-en-3β-yl benzoate	0.09:1

mechanism of this selenium dioxide reaction has not been investigated fully, it is not known with certainty which of the C-4

hydrogens is removed during the reaction. However, from the work of Ireland et al.[1], it is known that reduction of the compound 3 introduces tritium into the 4β-position of the sterol. Thus, the selenium dioxide oxidation reaction removes the 4β-hydrogen of cholesterol.

Location of ^{3}H in [4α-^{3}H]cholesterol

The $^{3}H/^{14}C$ ratios in the various transformation products formed from [4α-^{3}H]cholesterol in admixture with [4-^{14}C]cholesterol during location of the ^{3}H label are shown in Table 2. Practically all the tritium is retained in the 4β-hydroxy-cholest-5-en-3β-yl

Table 2. $^{3}H/^{14}C$ Ratios of derivatives of [4α-^{3}H]cholesterol mixed with [4-^{14}C]cholesterol

	$^{3}H/^{14}C$ Atomic ratio
Cholesteryl benzoate	1:1
4β-Hydroxy-cholest-5-en-3β-yl benzoate	0.98:1
4-Oxo-5,6-oxido-cholest-5-en-3β-yl benzoate	0.02:1

benzoate. Taken in conjunction with the preceding demonstraion that during introduction of the 4β-hydroxyl group, the 4β-hydrogen is stereospecifically removed, this result indicates that only 2% of the tritium in [4α-^{3}H]cholesterol is in the 4β-position. The $^{3}H/^{14}C$ ratio of 4-oxo-5,6-oxido-cholest-5-en-3β-yl benzoate indicates that a further 2% of the tritium in cholesteryl benzoate is not at C-4.

EXPERIMENTAL [5]

Synthesis of [4β-^{3}H]cholesterol

6β-Chloro-cholest-4-en-3β-yl benzoate 3 -4β-Hydroxy-cholest-5-en-3β-yl benzoate 2[1] (100 mg) was dissolved in dry pyridine (3 ml) and cooled in ice. To the ice-cold solution, thionyl chloride (0.1ml) was added and the mixture allowed to stand for 90 sec.. The reaction mixture was poured into ice water, extracted with ether and the ethereal extract washed successively with 4N HCl, saturated $NaHCO_3$ solution and water. The ether extract was dried over anhydrous sodium sulphate and after removal of the ether under reduced pressure the oily residue was dissolved in hexane (20ml), filtered through cotton wool, and evaporated to dryness to yield 6β-chloro-cholest-4-en-3β-yl benzoate 3 as a semi-crystalline gum (87mg). This compound was used as soon as possible after preparation and was pure by t.l.c. (silica gel G/benzene) and n.m.r. : δ ($CDCl_3$, 60MHz) 0.71 (C-18 methyl); 1.33 (C-19 methyl); 4.65 (C-6H); ca. 5.5 (3α-H), 5.64 (C-4H) 7.2-8.2 (aromatic protons).

[4β-^{3}H]Cholesterol 4 - 6β-Chloro-cholest-4-en-3β-yl benzoate 3 (18mg) dissolved in dry ether (1.5ml) was added to lithium aluminium tritide (5mCi, 0.32mg.) and the resulting suspension was allowed to stand for 15 min. and then refluxed for 3 hr.. After cooling, saturated sodium potassium tartrate solution (2 ml.) was added dropwise and the mixture allowed to stand for 30 min.. The resulting white suspension was extracted well with ether, the ethereal layer dried over anhydrous sodium sulphate and the ether removed under reduced pressure. The crude [4β-^{3}H]cholesterol was purified by t.l.c. (silica gel G/chloroform) followed by two recrystallizations from chloroform-methanol, washing well with ice-cold

methanol after each crystallization to yield pure [4β-^{3}H]cholesterol (600μCi).

Synthesis of [4α-^{3}H]cholesterol

Cholest-5-en-3-one 6 prepared from cholesterol 5 by the method of Pfitzner and Moffatt[6] was transformed into 3-oxo-cholest-5-en-4α-yl acetate 7 by the procedure of Fieser and Stevenson[4].

4-Oxo-cholest-5-en-3β-yl acetate 8 - 3-Oxo-cholest-5-en-4α-yl acetate 7 (360 mg) dissolved in light petroleum (b.p. 40-60°; 60ml) was added to deactivated alumina (10g), which had been prepared by addition of 1.3 ml water to 10g. of Brockmann Grade I neutral alumina (Woelm) and left to stand for 1 hr. prior to use. The suspension was stirred at room temperature for 5 min. and allowed to stand (at room temperature) with occasional shaking. The suspension was filtered, the alumina washed twice with diethyl ether (2 x 20ml) and the ether and petrol filtrates combined and evaporated to dryness. Preparative t.l.c. on silica gel GF_{254} with 10% ethyl acetate in petrol for development yielded 4-oxo-cholest-5-en-3β-yl acetate 8[4] (130 mg after recrystallization from chloroform/methanol). The mass spectrum showed a molecular ion at ^{m}/e 442 and expected prominent ions at ^{m}/e 414, 400, 382, 372 and 354. N.m.r. : δ($CDCl_3$, 60MHz), 0.70 (C-18 methyl), 1.00 (C-19 methyl), 2.14 (acetate protons), 5.16 (dd, J = 9, 10.5 Hz, 3α H), 6.32 (C-6H).

[4α-^{3}H]4β-Hydroxy-cholest-5-en-3β-yl acetate 9- 4-Oxo-cholest-5-en-3β-yl acetate (160mg) dissolved in dry methanol/tetrahydrofuran (1:1, 1.5ml) was added to sodium borotritide (100mCi, 18.4mCi/mg). The solution was allowed to stand for

45 min. at room temperature, water was added and the resulting suspension extracted into ether. The ethereal layer was washed with water, dried over anhydrous magnesium sulphate, and evaporated to dryness. T.l.c. indicated that the residue (33mCi) consisted of the 4β- and 4α-hydroxy compounds 9 and 10 (ca. 9:1) together with about 30% of an unidentified less polar impurity.

The crude mixture was separated by preparative t.l.c. on silica gel GF_{254} with 20% ethyl acetate in petrol for development, and the 4β- and 4α-alcohols 9 and 10 removed from the plates together as a broad band (30mCi). The mixture of alcohols 9 and 10 was subjected to further careful preparative t.l.c. on silica gel GF_{254} (0.5mm) and the plates developed twice in 17% ethyl acetate in petrol. The band (3.8mCi) co-chromatographing with the authentic 4β-hydroxy compound 9 was removed and re-chromatographed on the same system to yield pure [4α-^{3}H]4β-hydroxy-cholest-5-en-3β-yl acetate 9 (3mCi). The mass spectrum showed a molecular ion at m/e 444 and expected prominent ions at m/e 426, 402, 384, 287. N.m.r.: δ ($CDCl_3$, 60MHz) 0.69 (C-18 methyl), 1.17 (C-19 methyl), 2.01 (acetate methyl); 4.10 (4αH, broad), 4.53 (3α-H, broad), 5.52 (C-6H, broad).

[4-^{3}H]6β-Chloro-cholest-4-en-3β-yl acetate 11 - [4α-^{3}H]4β-Hydroxy-cholest-5-en-3β-yl acetate 9 (3m Ci) was dissolved in dry pyridine (2ml) and cooled in ice. Thionyl chloride (200μl.) was added and the mixture allowed to stand for 90 sec. at 0°C. The mixture was poured onto ice, extracted with ether, washed successively with 4N H_2SO_4, saturated $NaHCO_3$ solution, water and dried over anhydrous sodium sulphate. The solvent was removed under a stream of dry nitrogen to yield [4-^{3}H]6β-chloro-cholest-4-en-3β-

yl acetate 11, which was used immediately without further purification

[4α-^{3}H]Cholesterol 12 - The [4-^{3}H]6β-chloro-cholest-4-en-3β-yl acetate 11 was dissolved in dry ether (3ml) and lithium aluminium hydride (40 mg) added. The suspension was allowed to stand at room temperature for 1-1/2 hr. and then ethyl acetate (0.4ml) was added dropwise to destroy excess lithium aluminium hydride. Saturated sodium potassium tartrate solution (3ml) was added slowly and the resulting mixture allowed to stand for 15 min.. After dilution with water, the mixture was extracted with ether, the ether extract washed with water, dried over anhydrous sodium sulphate, and the ether removed under a stream of dry nitrogen to yield crude [4α-^{3}H]cholesterol.This was purified by preparative t.l.c. on silica gel G (0.5mm.) with 25% ethyl acetate in petrol for development to give [4α-^{3}H] cholesterol (1.01 mCi).

Purity of [4β-^{3}H] and [4α-^{3}H]cholesterol samples

The purity of the [4β-^{3}H] and [4α-^{3}H]cholesterol samples was established by micro-preparative g.l.c. with collection of fractions every 2 min. for radioassay. For this, a Pye 104 gas chromatograph was employed fitted with a metal splitter between the end of the column (4 mm. i.d. glass) and the flame ionization detector, so that approximately one part in ten passed to the detector, whereas the remainder was collected in glass capillary tubes at ambient temperature. With both [4β-^{3}H] and [4α-^{3}H]cholesterol, only one radioactive peak, corresponding to cholesterol was observed.

Location of ^{3}H label

Cholesteryl benzoate - The respective [^{3}H]cholesterol samples (ca. 0.7μCi) mixed with [4-^{14}C]cholesterol (ca. 0.1μCi) were diluted with non-radioactive cholesterol (160mg). Pyridine (600μl.) and benzoyl chloride (200μl.) were added and the mixture kept at room temperature overnight. The mixture was poured into ether, which was washed successively with water, 4N H_2SO_4, saturated $NaHCO_3$ solution, and water. The ether layer was dried over anhydrous magnesium sulphate and evaporated to dryness. The resulting cholesteryl benzoate was recrystallized from chloroform-methanol to constant $^3H/^{14}C$ ratio.

4β-Hydroxy-cholest-5-en-3β-yl benzoate - A solution of selenium dioxide (20mg) in water (10μl) and glacial acetic acid (1ml) was added to cholesteryl benzoate (103mg) dissolved in boiling acetic acid (1ml.). The resulting solution was boiled for 2 min. and anhydrous sodium acetate (25mg.) added. The mixture was allowed to cool, filtered, extracted with ether and the ether extract washed successively with saturated $NaHCO_3$ solution and water, and dried over anhydrous magnesium sulphate. After evaporation of the ether under vacuum the product was purified by preparative t.l.c. on silica gel GF_{254} with 5% ethyl acetate in benzene for development, to yield pure 4β-hydroxy-cholest-5-en-3β-yl benzoate (3) (30mg.). Particular care was taken to ensure that the plates were not overloaded in order to obtain a pure product. The 4β-hydroxy-cholest-5-en-3β-yl benzoate 2 was recrystallized from chloroform-methanol to constant $^3H/^{14}C$ ratio. The mass spectrum showed a molecular ion at m/e 506.3751 ($C_{34}H_{50}O_3$ requires 506.3760). N.m.r.: δ ($CDCl_3$, 60 MHz) 0.70 (s, 3H, C-18 protons), 0.76 (d, J = 6.5

Hz, 6H, C-26/27 protons), 0.90 (d, J = 6.5 Hz, 3H, C-21 protons), 1.26 (s, 3H, C-19 protons), 4.36 (d, J = 4Hz, 1H, C-4 proton), 4.94 (m, 1H, C-3 proton), 5.70 (1H, C-6 proton), 7.3-8.2 (5H, aromatic protons).

4-Oxo-5,6-oxido-cholest-5-en-3β-yl benzoate. - 4β-Hydroxy-cholest-5-en-3β-yl benzoate (22mg.) in benzene (0.5ml.) and acetic acid (0.05ml.) was stirred with chromium trioxide (12mg.) in water (0.02ml.) and acetic acid (0.2ml) for 6 hr.. The solution was poured into water, extracted with ether and the ethereal layer washed successively with $NaHCO_3$ solution and water, dried over anhydrous magnesium sulphate and evaporated to dryness. Preparative t.l.c. (Kieselgel GF_{254}, 5% ethyl acetate in benzene) of the product yielded the 5,6 α and β epoxides of 4-oxo-cholest-5-en-3β-yl benzoate in approximately equal yield (8mg. each). The least polar epoxide had m.s. m/e 520.3558 ($C_{34}H_{48}O_4$ requires 520.3553), 398.3188 ($C_{27}H_{42}O_2$ requires 398.3185); n.m.r.:δ ($CDCl_3$, 220MHz) 0.77 (s, 3H, C-18 protons), 0.88 (d, J = 6.5Hz, 6H, C-26/C-27 protons), 0.90 (d, J = 6.5Hz, 3H, C-21 protons), 1.07 (s, 3H, C-19 protons), 3.28 (d, J = ca. 2.5Hz, 1H, C-6 proton), 5.5 (m, 1H, 3α proton), 7.4-8.2 (5H, aromatic protons), whilst the more polar material had m.s. m/e 520.3558 ($C_{34}H_{48}O_4$ requires 520.3553), 398.3195 ($C_{27}H_{42}O_2$ requires 398.3185); n.m.r.: δ ($CDCl_3$, 220 MHz) 0.72 (s, 3H, C-18 protons), 0.87 (d, J = 6.5 Hz, 6H, C-26/27 protons), 0.93(d, J = 6.5 Hz, 3H, C-21 protons), 1.09 (s, 3H, C-19 protons), 3.44(d, J = ca. 2.5Hz, 1H, C-6 proton), 5.35 (m,1H, C-3 proton), 7.4-8.2 (5H, aromatic protons). The epoxides were accompanied by a smaller amount (5 mg.) of 4-oxo-

cholest-5-en-3β-yl benzoate, which had m.s. m/e 504.3618 ($C_{34}H_{48}O_3$ requires 504.3604), 476.3654 ($C_{33}H_{48}O_2$ requires 476.3654), 382.3242 ($C_{27}H_{42}O$ requires 382.3236); n.m.r.: δ ($CDCl_3$, 220MHz) 0.71 (s, 3H, C-18 protons), 0.87 (d, J = 6.5Hz, 6H, C-26/27 protons), 0.94 (d, J = 6.5Hz, 3H, C-21 protons), 5.64 (m, 1H, C-3 proton), 6.23 (1H, C-6 proton), 7.40-8.20 (5H, aromatic protons).

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5. Reaction sequences were carried out initially without introduction of tritium and the individual reaction products were characterised. The radiochemical syntheses were carried out in exactly the same manner and the products were characterised by co-chromatography on t.l.c. with authentic samples. N.m.r. spectra were recorded in $CDCl_3$ at 60MHz or 220MHz. Low resolution mass spectra were recorded on an AEI MS 12 spectrometer by direct inlet probe at 160-175°C. Accurate Mass measurements were carried out on an AEI MS 902 spectrometer by direct inlet probe at 150-200°C.

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