THE SYNTHESIS OF CHOLESTEROL-2,2,4,4,6-d_5

Larry D. Gruenke and J. Cymerman Craig*
Department of Pharmaceutical Chemistry, School of Pharmacy,
University of California, San Francisco, California 94143.

SUMMARY

Cholesterol-3,4-d_2 and -2,2,4,4,6-d_5 have been synthesized from Δ^4-cholestene-3-one for use as mass spectrometric stable isotope internal standards. Conversion of Δ^4-cholesten-3-one to the enol acetate followed by reduction with sodium borodeuteride in a deuterated solvent yielded cholesterol-3,4-d_2. Similarly Δ^4-cholesten-3-one-2,2,4,6,6-d_5 obtained by base-catalyzed exchange of Δ^4-cholesten-3-one in ethanol-O-d_1 was converted to the enol acetate, followed by reduction with sodium borohydride in a deuterated medium to give cholesterol-2,2,4,4,6-d_5.

Key Words: Cholesterol, Deuterium

INTRODUCTION

The accurate quantitation of cholesterol ($\underset{\sim}{1}$-d_0) by the sensitive mass spectrometric technique of selected ion recording (SIR) requires the synthesis of cholesterol labeled with a stable isotope for use as an internal standard. The labeled cholesterol should be at least two mass units heavier than cholesterol itself and should be specificially labeled with a high degree of incorporation in order to insure lack of mutual interference between the ion currents for the labeled and unlabeled species in the mass spectrum. Although a synthesis of cholesterol-2,2,4-d_3 and cholesterol-2,2,4,4-d_4 has been described (1), isotopic distributions reported were (%) 8, d_1; 29, d_2; 55, d_3; 6, d_4; 2, d_5 for the d_3-compound and 3, d_0; 3, d_1; 8, d_2; 29, d_3; 54, d_4; 3, d_5 for the d_4-compound. Deuteriocholesterol prepared by exchange with D_2O and acetic acid-O-d_1 in the presence of active platinum (2,3) contained approximately one d-atom at C-6 and one d-atom distributed among C-24 to C-27, as well as a trace at C-3. A recent

0362-4803/79/0316-0495$01.00

Received September 6, 1978

preparation of cholesterol-d_{14} (4) while of high isotopic purity and specificity (deuterium at C-20 and C-22 to C-27) is relatively difficult.

We now report a convenient method for the synthesis of specifically deuterium labeled cholesterol-3,4-d_2 ($\underset{\sim}{1}$-d_2) and cholesterol-2,2,4,4,6-d_5 ($\underset{\sim}{1}$-d_5) from the readily available starting material Δ^4-cholesten-3-one ($\underset{\sim}{2}$-d_0) via Δ^4-cholesten-3-one enol acetate ($\underset{\sim}{3}$-d_0). The introduction of deuterium is accomplished by a base catalyzed exchange into the starting material and by the use of a deuterated medium or deuterated reducing agent in the reduction of the enol acetate.

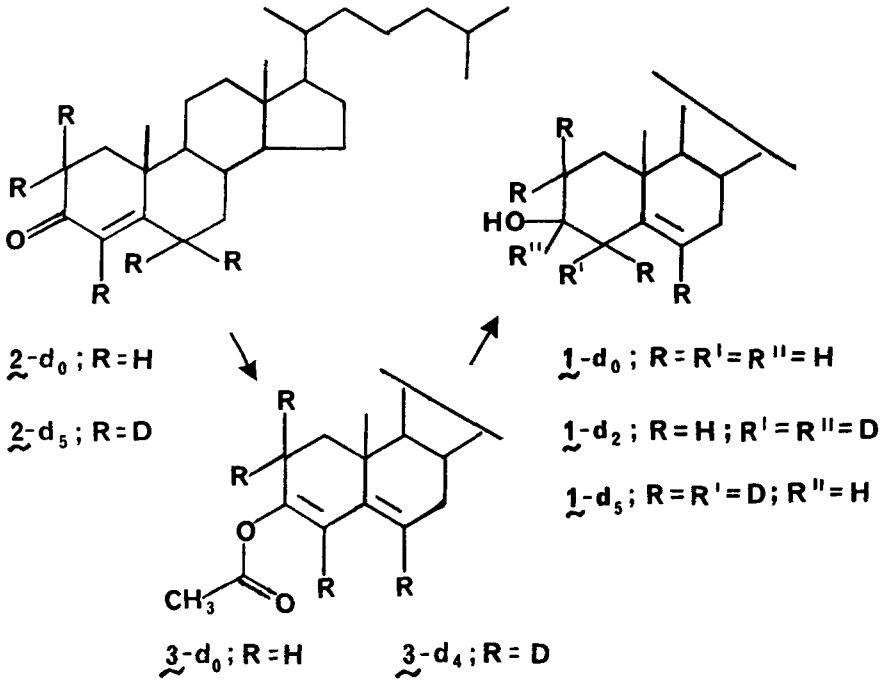
RESULTS AND DISCUSSION

The reduction of Δ^4-cholesten-3-one enol acetate (5) by sodium borohydride (6) probably proceeds in two steps (7): alkaline hydrolysis of the ester to yield the unconjugated enone, followed by reduction of the ketone function by borohydride. Thus the simultaneous use of a deuterated medium and of borodeuteride gave cholesterol-3,4-d_2 as expected. However, mass spectral analysis showed an isotopic composition of 3%-d_1, 87%-d_2, and 10%-d_3. The unexpected excess incorporation is presumably due to base catalyzed exchange at the highly activated allylic C-4 position of the unconjugated enone intermediate (1).

The mutual interference caused in the mass spectrum at m/e 386 by proton loss from the labelled standard and at m/e 388 from isotopic abundance in the natural compound could be minimized by using, as labelled standard, a variant containing a larger number of deuterium atoms. It has been shown in other Δ^4-3-one steroids (8,9) that five deuterium atoms can be introduced into the 2,2,4,6,6 positions by exchange using sodium in a mixture of methanol-O-d_1 and D_2O. Although attempts to exchange Δ^4-cholesten-3-one in a D_2O-tetrahydrofuran medium using KOD as the basic catalyst failed, the exchange proceeded smoothly with sodium methoxide in ethanol-O-d_1. After three such exchanges Δ^4-cholesten-3-one with an isotopic composition of 10%-d_4 and 90%-d_5 was obtained. If necessary further exchanges could be carried out in order to obtain even higher incorporation. However, this would have only a limited effect in improving the isotopic purity of the final cholesterol-d_5 since conversion of Δ^4-cholesten-3-one-d_5 to the enol acetate -d_4 proceeds with some scrambling of the label; a

mass spectrometric analysis at the molecular ion of the enol acetate -d_4 showed an isotopic composition of 25%-d_5, 58%-d_4 and 17%-d_3. Since only a catalytic amount (2%) of D_2SO_4 is present, the source of the additional (25%) deuterium found in the enol acetate-d_4 must therefore be the C-6 deuterium of the Δ^4-en-3-one precursor (lost in the formation (10) of the enol acetate) which becomes incorporated into the methyl group of the enol acetate by an acid-catalyzed exchange. Sodium borohydride reduction in a deuterated medium of this cholestenone-d_4 enol acetate gave cholesterol-d_5 without any excess incorporation of deuterium, so that the additional deuterium in the enol acetate-d_4 is confirmed as having been situated entirely in the acetate moiety.

The isotopic composition of the cholesterol-2,2,4,4,6-d_5 as determined by mass spectrometry was 68% d_5, 27% d_4, 4% d_3 and 1% d_2. This variant is therefore a suitable internal standard for mass spectrometric assays involving natural cholesterol since it possesses neither of the deficiencies noted above for the d_2-variant.



EXPERIMENTAL

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Literature melting points refer to the undeuterated compounds. Nmr spectra were measured on a Varian A-60A spectrometer using tetramethylsilane as the internal reference. Electron impact mass spectra were recorded on an AEI MS-12 spectrometer by direct insertion. Deuterium analysis by mass spectrometry was performed on the molecular ions.

Δ^4-Cholesten-3-one enol acetate ($\underset{\sim}{3}$-d_0). The following procedure was adapted from the method of Dauben and Eastham (5): One drop of conc sulfuric acid (98%) was added to a mixture of Δ^4-cholesten-3-one (2.0 g, 5.2 mmoles) and isopropenyl acetate (2.0 ml, 20 mmoles). The mixture was heated at 100° under a direct stream of nitrogen for two hr., anhydrous sodium acetate (0.1 g) was added, and the reaction mixture extracted with 10 ml of chloroform and filtered. Removal of the solvent and crystallization from methanol-chloroform gave 1.5 g (58%) of white crystals of $\underset{\sim}{3}$-d_0, mp 77-79° (lit. (5) mp 76-78°); eims m/e (relative intensity) M^+ 426 (3.9%), 384 (100%).

Cholesterol-3,4-d_2 ($\underset{\sim}{1}$-d_2). To a solution at 0° of sodium borodeuteride (0.25 g, 6.0 mmoles, Merck) in a mixture of 10 ml of ethanol-O-d_1 and 1 ml of deuterium oxide was added dropwise over a period of one hour a solution of Δ^4-cholesten-3-one enol acetate (0.30 g, 0.70 mmoles) in 15 ml of tetrahydrofuran. The solution was refluxed for one hr, cooled, and 3 ml of conc HCl added dropwise. The resulting mixture was poured into 50 ml of water and extracted with dichloromethane (3 x 30 ml). The extracts were dried (anhydrous sodium sulfate), filtered, and the solvent removed on the rotary evaporator. The residue was dissolved in 20 ml of hot ethanol, 10 drops of conc HCl added, and the solution was refluxed for 2 hr. Removal of solvent and crystallization from ethanol gave 0.16 g (59%) of $\underset{\sim}{1}$-d_2, mp 146-147.5°; eims m/e (relative density) 389 (41.4%), M^+ 388 (100%), 387 (8.2%), 386 (1.1%), 373 (25.8%), 370 (35.7%), 355 (23.8%), 301 (35.9%), 275 (67.7%).

Δ^4-Cholesten-3-one-2,2,4,6,6-d_5 ($\underset{\sim}{2}$-d_5). A solution of Δ^4-cholesten-3-one (2.0 g, 5.2 mmoles) in 10 ml of ethanol-O-d_1 (170 mmoles, 99.5% d) was treated

with 0.1 g of sodium methoxide and refluxed under dry nitrogen overnight. This exchange was repeated two more times, each time by removing the solvent under a direct stream of nitrogen, adding a fresh 10 ml aliquot of ethanol-O-d_1 and refluxing overnight. After the third exchange the reaction was quenched by adding 1 ml of deuterium oxide followed by anhydrous sodium sulfate (3 g) and 20 ml of hexane. The resulting mixture was filtered, the solvent removed on the rotary evaporator, and the residue recrystallized from hexane to give 1.4 g (70%) of yellowish crystals, mp 78.5-79.5°; a mixed m with authentic Δ^4-cholesten-3-one-d_0 was not depressed; eims m/e (relative intensity) M^+ 389 (53.7%), 388 (7.0%), 387 (0.8%), 345 (18.1%), 261 (29.9%), 260 (20.4%), 232 (29.9%), 129 (100%).

A comparison of the proton nmr spectrum of the deuterated compound with that of the undeuterated compound shows the expected loss of the signals in the 2.25 to 2.6 δ region (C-2 protons) and at 5.57 δ (vinylic hydrogen at C-4).

Δ^4-Cholesten-3-one-2,2,4,6-d_4 enol acetate ($\underset{\sim}{3}$-d_4). To a mixture of Δ^4-cholesten-3-one-2,2,4,6,6-d_5 (1.0 g, 2.6 mmoles) and isopropenyl acetate (1.0 ml, 10 mmoles) was added 1 µl of conc D_2SO_4 (96-98% in D_2O). The resulting mixture was heated to 100° under a direct stream of nitrogen for 1.5 hr and was then worked up as for the undeuterated compound to yield 0.6 g (54%) of white crystals, mp 78-79°; a mixed up with the undeuterated compound was not depressed; eims m/e (relative intensity) 431 (4.0%), M^+ 430 (5.3%), 429 (1.6%), 389 (42.8%), 388 (100%), 387 (44.5%).

Cholesterol-2,2,4,4,6-d_5 ($\underset{\sim}{1}$-d_5). To a solution at 0° of sodium borohydride (0.5 g, 13.2 mmoles) in a mixture of 10 ml of ethanol-O-d_1 (99.5% d) and 1 ml of deuterium oxide (99.8% d) was added dropwise over a period of one hr a solution of Δ^4-cholesten-3-one-2,2,4,6-d_4 enol acetate (0.5 g, 1.16 mmoles) in 15 ml of tetrahydrofuran. The resulting solution was refluxed for one hr under a nitrogen atmosphere and was then worked up as described for cholesterol-d_2 to give 0.22 g (49%) of $\underset{\sim}{1}$-d_5, mp 145-146.5°; eims m/e (relative intensity) 392 (20.7%), M^+ 391 (66.2%), 390 (28.3%), 389 (7.4%), 376 (20.6%), 373 (22.8%), 372 (15.2%), 358 (17.6), 302 (15.2%), 301 (15.9%), 275 (48.1%).

ACKNOWLEDGMENT

This work was supported by the American Cancer Society.

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