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Synthesis of [3α-^{3}H]7-dehydrocholesterol via stable tritiated 4-phenyl-1,2,4-triazoline-3,5-dione derivative

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Synthesis of [3α-^{3}H]7-dehydrocholesterol is described via protection of the 5,7-diene system in 7-dehydrocholesterol as the Diels–Alder adduct with 4-phenyl-1,2,4-triazoline-3,5-dione followed by oxidation of the hydroxyl group to give the 3-oxo adduct. Reduction of the keto adduct with [^{3}H]sodium borohydride produced the adduct of [3α-^{3}H]7-dehydrocholesterol from which the radiolabeled sterol was obtained via treatment with lithium aluminum hydride. The advantage of the method is that highly labeled [3α-^{3}H]7-dehydrocholesterol can be prepared. Further, unlike 7-dehydrocholesterol, its adduct with 4-phenyl-1,2,4-triazoline-3,5-dione is stable and can be stored. This allows the preparation of small batches of [3α-^{3}H]7-dehydrocholesterol for immediate use in biological experiments, and losses due to decomposition of excess radiolabeled 7-dehydrocholesterol are minimized. (*Steroids* **62**:700–702, 1997) © *1997 by Elsevier Science Inc.*

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Introduction

Patients with Smith–Lemli–Opitz syndrome, a devastating, often fatal autosomal recessive disorder characterized by a large number of birth defects, e.g., dysmorphic facial features, limb and genital abnormalities, and severe mental retardation, have extremely low plasma cholesterol.[1–5] We have recently demonstrated a severe abnormality in cholesterol biosynthesis in these patients and have shown that the very low plasma cholesterol levels are associated with the accumulation of large amounts of the cholesterol precursor 7-dehydrocholesterol and isomer 5,8-cholestadien-3β-ol.[6–9] We demonstrated that deficient 7-dehydrocholesterol-Δ^7-reductase activity, which catalyzes the conversion of 7-dehydrocholesterol to cholesterol, is inherited in homozygotes with Smith–Lemli–Opitz syndrome and is responsible for the accumulation of 7-dehydrocholesterol and the clinical abnormalities.[7] To further characterize 7-dehydrocholesterol-Δ^7-reductase, we needed radiolabeled 7-dehydrocholesterol with high specific activity. This paper describes a convenient method for the synthesis of tritium-labeled 7-dehydrocholesterol.

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Experimental

Reference standards of cholesterol and 7-dehydrocholesterol were purchased from Steraloids, Inc. (Wilton, New Hampshire, USA). 7-Dehydrocholesterol was crystallized from methanol before use. Sodium borotritiide was purchased from New England Nuclear (Boston, Massachusetts, USA). All other reagents and solvents were reagent grade and were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA).

Methods

Melting points were determined on a Thermolyne apparatus (Thermolyne Corp., Dubuque, Iowa, USA) model 12,600 and are uncorrected. Optical rotations were obtained on a Perkin-Elmer (Norwalk, Connecticut, USA) model 141 polarimeter with ethanol as the solvent. IR spectra were obtained on a Perkin-Elmer model 421 spectrophotometer as KBr discs. Ultraviolet Spectra were obtained in chloroform solution with a Hewlett-Packard (Palo Alto, California, USA) model HP8450 spectrophotometer. Thin-layer chromatography (TLC) was carried out on silica gel O plates (Analabs, New Haven, Connecticut, USA) in a solvent system of chloroform/acetone (94:6, v/v), and the spots were visualized by spraying the plate with phosphomolybdic acid (3.5%, in isopropanol) and sulfuric acid (20%) and heating at 110°C for 2 min.[10]

Gas-liquid chromatography (GLC). GLC was performed on a Hewlett-Packard model 5890A gas chromatograph equipped with

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a flame ionization detector and an injector with a split/splitless device for capillary columns. A chemically bonded fused silica CP-Sil-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25 m × 0.22 mm internal diameter) (Chrompack, Inc., Raritan, New Jersey, USA) was used with helium as the carrier gas. The chromatographic conditions employed were as described previously.[11] The trimethylsilyl ether derivatives of the sterols [5–10 μg of sterol plus 100 μL of Sil-Prep (hexamethyldisilazane: trimethylchlorosilane: pyridine, 3:1:9; Alltech Associates, Inc., Deerfield, Illinois, USA) at 55°C for 20 min] were dissolved in hexane, and aliquots were injected into the GLC column simultaneously with 5α-cholestane as the internal standard.

Gas chromatography-mass spectrometry. Mass spectra of the sterols when needed were carried out on a Hewlett-Packard Model 5988 gas chromatograph-mass spectrometer using a 25-m CP-Sil-5 CB capillary column.

4-Phenyl-1,2,4-triazoline-3,5-dione adduct of 7-dehydrocholesterol (Figure 1, I). 7-Dehydrocholesterol (1 g) was dissolved in acetone (100 mL), and 4-phenyl-1,2,4-triazoline-3,5-dione (0.55 g, in 10 mL of acetone) was added.[12] The reaction was complete when a slight excess of the triazoline solution resulted in a persistent light pink-colored solution. The product was kept overnight at 0°C, and the colorless long needles of the adduct *I* (1.1 g) were filtered (melting point, 152–154°C). Concentration of the mother liquor yielded another 300 mg of the product (total yield, 95%). The product showed a single spot on TLC-R_f, 0.20. Analysis calculated for $C_{35}H_{49}O_3N_3$: C, 75.13; H, 8.77; N, 7.51. Found: C, 74.99; H, 8.65; N, 7.61. The physical characteristics of the compound were identical with those reported in the literature.[12]

Oxidation of adduct I to 3-oxo adduct (II). The 4-phenyl-1,2,4-triazoline-3,5-dione adduct *I* (1 g) was dissolved in acetone (20 mL) and cooled to 5°C in ice water. Sodium bicarbonate (500 mg) was added, and 1 mL of a cold solution of Jones' reagent (prepared by the addition of 2.2 g of CrO_3 in 2.8 mL of concentrated sulfuric acid and dilution with water to a final volume of 10 mL) was added with vigorous stirring of the reaction mixture. The reaction was worked up after 5 min, and the light brown-colored product obtained was passed through a small bed of silica gel. The compound was eluted with chloroform (100 mL), and the eluate was evaporated to dryness under reduced pressure at 45°C. The product (0.75 g) showed only one spot on TLC-R_f, 0.56, melting point 170–172°C (colorless plates from acetone-hexane), $[\alpha]_D^{20}$, −53.2°C (concentration, 1.6).[13] Analysis calculated for $C_{35}H_{47}O_3N_3$: C, 75.40; H, 8.44; N, 7.54. Found: C, 75.13; H, 8.59; N, 7.39.

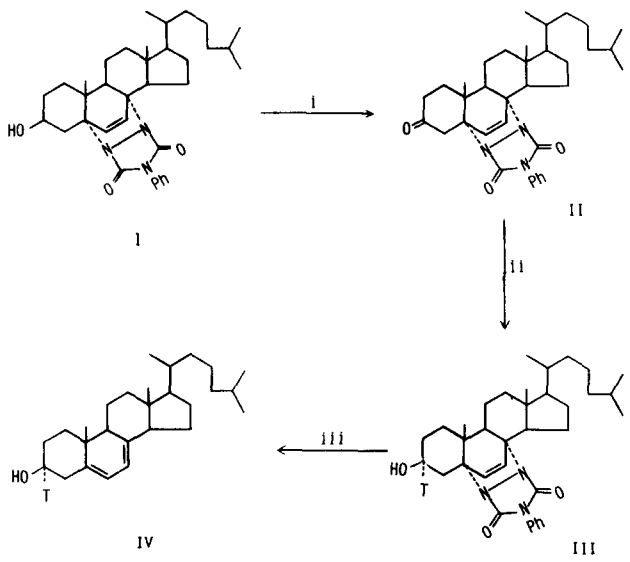

Figure 1 Synthesis of [3α-^{3}H]7-dehydrocholesterol. I, 4-Phenyl-1,2,4-triazoline-3,5-dione adduct of 7-dehydrocholesterol; II, 3-oxo derivative of 4-phenyl-1,2,4-triazoline-3,5-dione adduct of 7-dehydrocholesterol; IV, 4-phenyl-1,2,4-triazoline-3,5-dione adduct of [3α-^{3}H]7-dehydrocholesterol; and IV, [3α-^{3}H]7-dehydrocholesterol. i, Jones' oxidation; ii, NaB^3H_4, iii, $LiA1H_4$.

Sodium borohydride reduction of II. The keto adduct *II* (90 mg) was treated with sodium borohydride (20 mg) in methanol (5 mL) at 0–5°C for 20 min and then was poured into excess cold water. The white precipitate obtained was collected, washed with water, and dried (82 mg). On TLC, the product showed a major spot, R_f, 0.20. The pure compound corresponding to this R_f value was obtained by preparative TLC (42 mg) and was found to be identical with the 4-phenyl-1,2,4-triazoline-3,5-dione adduct of 7-dehydrocholesterol (*I*) (TLC, melting point, and IR).

Lithium aluminum hydride reduction of the 4-phenyl-1,2,4-triazoline-3,5-dione adduct of 7-dehydrocholesterol. The pure 4-phenyl-1,2,4-triazoline-3,5-dione adduct of 7-dehydrocholesterol obtained above after sodium borohydride reduction of the 3-oxo adduct (5 mg) was dissolved in anhydrous tetrahydrofuran (0.2 mL) in a dry screw cap tube and was treated with lithium aluminum hydride at 55°C for 2 h.[12] After the usual work-up, the product (3.6 mg) was crystallized from chloroform-methanol as colorless plates (melting point 142–143°C). This compound was found to show only one spot on TLC (R_f, 0.62) and was confirmed to be 7-dehydrocholesterol (melting point, TLC, ultraviolet, IR, GLC, and mass spectra).

Synthesis of [3α-^{3}H]7-dehydrocholesterol. Sodium borotritiide (25 mCi) was added to 5 mg of the keto adduct *II* dissolved in 1 mL of methanol at 5°C. The products were diluted with 4 mL of cold water, and sterols were extracted with ethyl ether (5 mL × 3). Ether was washed with water (5 mL) and then was evaporated under a stream of nitrogen at 40°C. The residue was subjected to preparative TLC. The spot due to a 4-phenyl-1,2,4-triazoline-3,5-dione adduct of 7-dehydrocholesterol was applied at one end of the plate, and the band with the R_f value corresponding to this compound (Figure 1, *III*) was scraped and the product was eluted with 25 mL of chloroform/methanol (75:25, v/v). After the evaporation of solvents, the residue was taken in 0.5 mL of tetrahydrofuran and was reacted with lithium aluminum hydride as described above. The free sterol (*IV*) that was liberated was extracted with ethyl acetate, and the solvent was evaporated. The residue was dissolved in 100 μL of chloroform and was applied on a TLC plate. The spot due to 7-dehydrocholesterol was applied as reference standard, and the plate was allowed to develop to a height of approximately 15 cm in the solvent system. The plate was then sprayed with water to visualize the spot according to reference standard, the band corresponding to 7-dehydrocholesterol was scraped from the plate, and the compound was eluted from the silica gel with chloroform/methanol (75:25, v/v; 50 mL). After the evaporation of solvents under reduced pressure, the residue was dissolved in methanol and an aliquot was used to check its purity. Thin-layer chromatography followed by zonal radioactivity counting with a scintillation counter showed that the [^{3}H]7-dehydrocholesterol obtained was over 97% pure (specific activity of 150 × 10^6 dpm). The compound was stored at −20°C in tubes that were sealed under argon. Stored in this way, up to 25% of the [3α-^{3}H]-7-dehydrocholesterol decomposed in 3 months. Stored in the same way, compound *III* was less than 10% decomposed in that time.

Results and discussion

A major limitation of the use of radiolabeled 7-dehydrocholesterol is the relative instability of the compound. Unlabeled 7-dehydrocholesterol is stable when kept in the cold and away from light. We found that a solution of 7-dehydrocholesterol prepared for thin-layer chromatographic purposes was more than 50% decomposed when left on the shelf unprotected from light for 1 month, and the problem is further aggravated in the case of radiolabeled 7-dehydrocholesterol. Thus, a solution of [1,2-^{3}H]7-dehydrocholesterol with a specific activity of approximately 100×10^6 dpm was 96% decomposed after 3 months at 0°C in the dark. Even leaving the compound as a dilute solution (specific activity, 5×10^6 dpm) in benzene-methanol (1:1) in the dark at −20°C resulted in up to a 20% loss of 7-dehydrocholesterol in just 2 weeks. It is therefore imperative that radiolabeled 7-dehydrocholesterol be purified immediately before use. This also makes it more practical to repeatedly synthesize small batches of the radiolabeled compound.

The homoannular 5,7-diene system in 7-dehydrocholesterol is very susceptible to cleavage by even very mild oxidation of 7-dehydrocholesterol and it results in highly complex mixtures. This precluded the direct oxidation of 7-dehydrocholesterol to the corresponding 3-oxo compound, which could then be reduced with tritiated sodium borohydride to tritiated 7-dehydrocholesterol. The diene system can, however, be protected as a Diels–Alder adduct with 4-phenyl-1,2,4-triazoline-3,5-dione,[14] and the sterol can be regenerated by reduction with lithium aluminum hydride.[12] The hydroxyl group in the adduct could be oxidized to the 3-oxo compound.[13] We found that whereas lithium aluminum hydride reduced the 3-oxo group as well as regenerated the sterol from the adduct, sodium borohydride only reduced the 3-oxo group, while the adduct remained intact. Taking advantage of the stability of the adduct toward sodium borohydride, we could prepare the tritium-labeled adduct of 7-dehydrocholesterol from which the radiolabeled 7-dehydrocholesterol could then be obtained with lithium aluminum hydride. The advantage of this method for preparation of the radiolabeled 7-dehydrocholesterol is that the radiolabeled adduct of 7-dehydrocholesterol (III) is stable, and, unlike radiolabeled 7-dehydrocholesterol, it can be stored without appreciable decomposition. Thus, whenever needed, only the required amount of the ^{3}H-labeled adduct can be reduced to radiolabeled 7-dehydrocholesterol, which can be immediately used for the biological reactions. Furthermore, [3α-^{3}H]7-dehydrocholesterol can be prepared with very high specific activity by this method, an important factor for enzymatic assays where very little mass of high specific activity is desirable.

7-Dehydrocholesterol is synthesized from cholesterol via dehydrobromination of 7-bromocholesterol benzoate followed by chromatographic purification.[15] This method can be adopted for preparation of radiolabeled 7-dehydrocholesterol from radiolabeled cholesterol. However, substantial amounts of 4,6-cholestadien-3β-ol are formed during the reaction that not only lower the yield of 7-dehydrocholesterol but necessitate chromatographic removal of 4,6-cholestadien-3β-ol.[15] Although 4,6-cholestadien-3β-ol is not formed in the modified method developed by Confalone et al.,[16] the method involves extra steps and, in our hands, was inconvenient to adopt due to the small mass of radiolabeled cholesterol to be used in these reactions, and less than 15% overall yield was obtained.

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