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Stereospecific Hydrogen Transfer from C-4 to C-6 during Enzymatic Transformation of Cholesterol into Cholestenone[1]

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In order to clarify the steric mechanism for biotransformation of Δ^5-3β-hydroxysteroid into Δ^4-3-ketosteroid the metabolic fate of hydrogen at C-4 during conversion of cholesterol into cholestenone by 3β-hydroxysteroid oxidase has been investigated. Of three substrates required for this purpose 4α-d_1-cholesterol was prepared through 4-d_1-cholest-4-en-3β-ol *tert*-butyldimethylsilyl ether as a key intermediate. The substrate was incubated with the enzyme, and the content and locality of deuterium in resulting cholestenone were determined by inspection of the mass, nuclear magnetic resonance, and infrared spectra. The transformation products formed from 4α- and 6- deuterated cholesterols retained the label almost intact, while that derived from 4β-d_1-cholesterol showed *ca.* 50% retention of the heavy isotope at 6β. Incubation studies using 6β-d_1-cholestenone as a substrate implied that a 50% loss of the label would be ascribable in part to the exchange with the incubation medium under the enzymatic control. These results led to a definite conclusion that 4β-hydrogen was transferred stereospecifically to the 6β-position during biotransformation of cholesterol into cholestenone.

Keywords——3β-hydroxysteroid oxidase; $\Delta^5\rightarrow\Delta^4$ isomerization; steric mechanism; stereospecific labeling; lithium aluminum deuteride; 4β-d_1-cholesterol; 4α-d_1-cholesterol; 6β-d_1-cholestenone; deuterium retention; hydrogen exchange

The microbial transformation of Δ^5-3β-hydroxysteroid into Δ^4-3-ketosteroid is of particular interest in connection with the biosynthesis of steroid hormones in the living animals. The steric mechanism for the migration of the double bond has been extensively investigated with several enzyme systems.[3-9] Ringold and his co-worker have previously demonstrated that during the isomerization of androst-5-ene-3,17-dione into androst-4-ene-3,17-dione by Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni* 4β-hydrogen is transferred to the 6β-position.[10] This explanation, however, is not fully acceptable because the only 4β-deuterated substrate was used for the incubation study. As a series of our studies on the microbial transformation of steroids,[11] elucidation of the steric mechasinm for the hydrogen transfer from C-4 to C-6 during conversion of cholesterol (cholest-5-en-3β-ol) into cholestenone (cholest-

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4-en-3-one) by 3β-hydroxysteroid oxidase [E.C. 1.1.3.6], derivable from *Brevibacterium sterolicum*,[12] has been undertaken.

Of the substrates required for this purpose, 4β-d_1-cholesterol (**1b**) was prepared by the route previously established in these laboratories,[13] and 6-d_1-cholesterol (**1c**) was obtained from 6-d_1-cholest-5-en-3β-ol acetate[14] by alkaline hydrolysis under the mild conditions. The remaining substrate, 4α-d_1-cholesterol (**1a**), was conveniently synthesized as shown in Chart 1.

An initial effort was directed to the preparation of 4-d_1-cholest-4-en-3β-ol *tert*-butyldimethylsilyl ether as a key intermediate. Hydroboration of cholest-4-en-3β-ol *tert*-butyldimethylsilyl ether (**2a**) and subsequent oxidation of the organoborane with alkaline hydrogen peroxide provided solely a *cis*-addition product, 5α-cholestane-3β,4α-diol 3-monosilyl ether (**3b**). The configurational assignment of the *cis*-adduct was justified by leading to the known 3β,4α-diol (**3a**) by acid hydrolysis. Being treated with pyridinium chlorochromate, **3b** underwent oxidation to yield the 4-ketone (**4**) as a single product. It is sufficiently substantiated

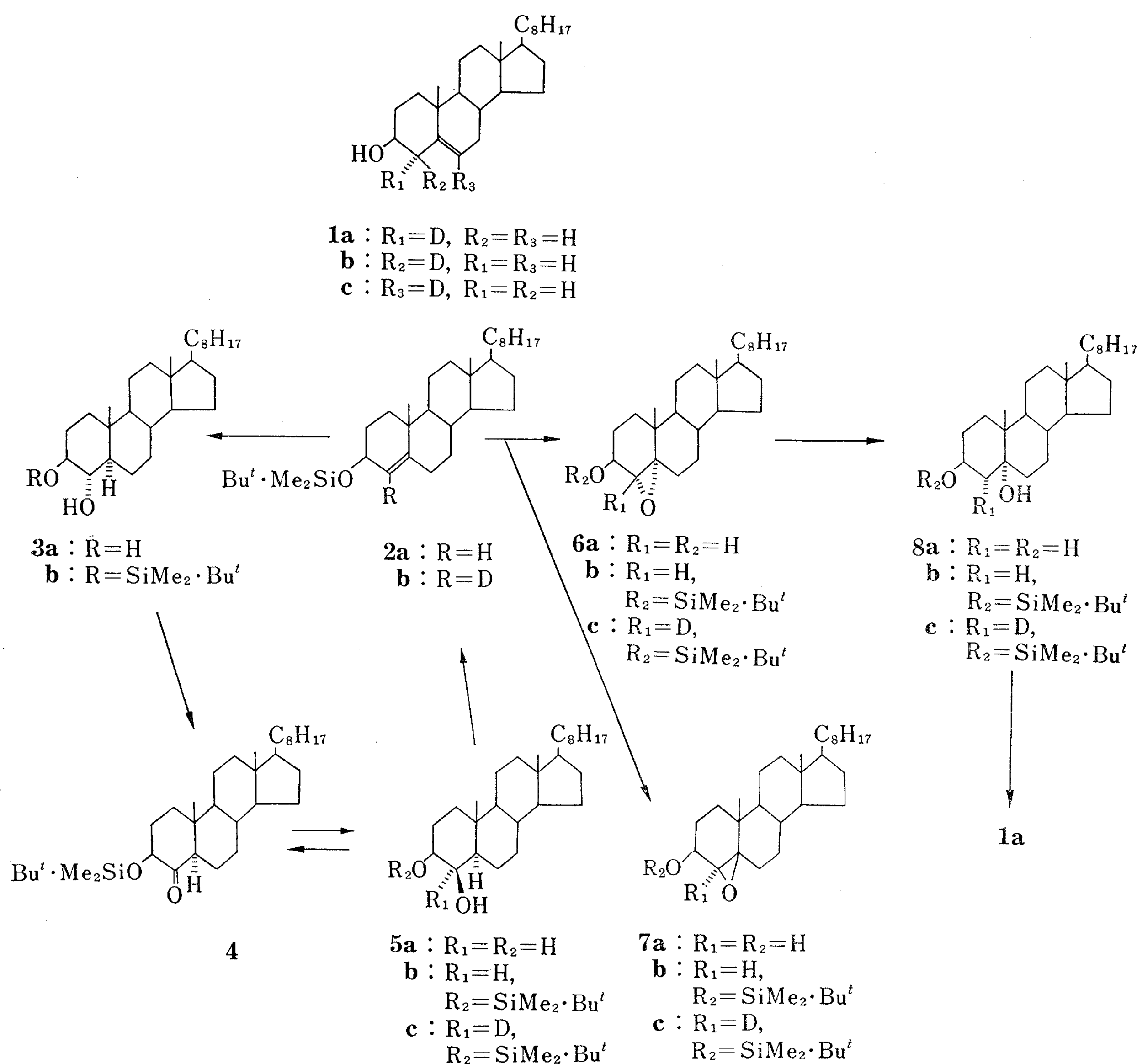

Chart 1

12) T. Uwajima, H. Yagi, and O. Terada, *Agr. Biol. Chem.* (Tokyo), **37**, 2345 (1973).
13) T. Nambara, S. Ikegawa, T. Ishizuka, and J. Goto, *Chem. Pharm. Bull.* (Tokyo), **22**, 2656 (1974).
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that the A/B-*trans* fusion is thermodynamically more stable than the A/B-*cis* juncture. In actuality the stereochemistry at C-5 retained as judged from the nuclear magnetic resonance (NMR) spectral data. The signal of 3α-proton appeared as a doublet of doublets ($J=7$, 10.5 Hz) at 4.15 ppm and that of 19-methyl proton at 0.17 ppm. Reduction of the oxo group with lithium aluminium hydride did take place along the stereoselective course to form the 4β-hydroxyl compound (**5b**) in a satisfactory yield. The product was obviously differentiated from the epimeric 4α-hydroxyl compound (**3b**) and was oxidized back to the 4-ketone (**4**). The β-*cis*-glycol structure was confirmed by leading to the known $3\beta,4\beta$-diol (**5a**) by acid hydrolysis. The configuration of the hydroxyl function newly introduced into the C-4 position was thus unambiguously characterized. When **5b** was treated with phosphorous oxychloride in pyridine, dehydration reaction proceeded to the desired direction to yield the Δ^4-olefine (**2a**).

The synthetic route thus established proved to be promising to introduce a deuterium label into the C-4 position of the Δ^4-olefine. Reduction of **4** with lithium aluminum deuteride under the conditions described above, followed by resilylation yielded 4α-d_1-5α-cholestane-$3\beta,4\beta$-diol 3-monosilyl ether (**5c**). Subsequent dehydration with phosphorus oxychloride in pyridine provided the desired 4-d_1-cholest-4-en-3β-ol *tert*-butyldimethylsilyl ether (**2b**). The locality of the heavy isotope at C-4 was justified by the fact that the signal due to C-4 proton disappeared in the NMR spectra.

Treatment of **2b** with *m*-chloroperbenzoic acid in the usual manner yielded the $4\alpha,5\alpha$-epoxide (**6c**) together with a trace amount of the $4\beta,5\beta$-epoxide (**7c**). Reductive cleavage of the oxido ring in **6c** with lithium aluminum hydride furnished the 4α-deuterated 5α-hydroxyl derivative (**8c**) as a sole product. Dehydration of **8c** was effected on brief exposure to thionyl chloride in pyridine resulting in formation of the Δ^4- and Δ^5-olefines. The mixture was treated with hydrochloric acid and chromatographed on silica gel to provide the desired 4α-d_1-cholesterol (**1a**). The mass spectra (MS) and NMR spectra using a shift reagent revealed that these substrates were stereospecifically labeled with deuterium at C-4 or C-6.

The deuterated substrates (**1a**, **1b** and **1c**) were incubated with 3β-hydroxysteroid oxidase under the conditions similar to those described by Uwajima, *et al.*[15] The incubation mixture was extracted with ethyl acetate and the extract was purified by preparative thin–layer chromatography (TLC) to afford cholestenone in *ca.* 95% yield. The deuterium content of the biotransformation product was determined by means of mass spectrometry. As listed in Table I the products formed from the 4α- and 6-deuterated substrates (**1a** and **1c**) retained the label almost intact, while that derived from the 4β-epimer (**1b**) showed a 51% retention of the isotope.

Inspection of the NMR spectra of the biotransformation products was then performed. The C-4 proton signal of the product from the 4α-deuterated substrate almost disappeared, while those of the products from the 4β- and 6-deuterated compounds were observed as a sharpened singlet and as a doublet ($J=2$ Hz), respectively and each signal was equivalent to one proton. Furthermore in the mass spectra of these products two important fragment ion peaks appeared at m/e 343 and 125[16] (see Fig. 1). It seemed likely that the heavy isotope of the products formed from the 4β- and 6-deuterated substrates would be located at the C-6 position. It is generally accepted that the infrared (IR) spectrum of a deuterated compound is distinguishable from those of its epimer and the non-deuterated compound. Determination of the isotopic purity by IR spectroscopy was undertaken employing epimeric 6-deuteriocholestenones as authentic specimens.[14] Unfortunately the C-D stretching band could not be observed with 6β-d_1-cholestenone. Accordingly, the characteristic bands at 660 and 845 cm^{-1} for 6β-d_1-cholestenone, at 800 cm^{-1} for 6α-d_1-cholestenone, and at 675 and 865 cm^{-1}

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Table I. Results of Deuterium Retention

Product	Substrate (%)		
	4α-D (**1a**)	4β-D (**1b**)	6-D (**1c**)
Cholestenone	98	51	98

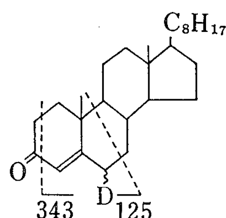

Fig. 1. Mass Fragmentation of 6-Deuterated Cholestenone

for non-labeled cholestenone were used for this purpose. The IR spectrum of the product from 6-deuterated substrate was entirely identical with that of 6α-d_1-cholestenone. Comparison of the IR spectrum of the product from the 4β-deuterated substrate with those of the synthetic mixtures of 6β-deuterated and non-labeled cholestenones in a variety of ratios was carried out. The spectra in the finger-print region of the biotransformation product and a synthetic mixture of the 6β-labeled and non-labeled cholestenones (1:1) were superimposable (Fig. 2). In consequence, it was clarified that the labels retained in the products formed from the 4β- and 6-deuterated substrates were located exclusively at the 6β- and 6α- position, respectively. The present result is fairly consistent with the previous finding that 4β-hydrogen was transferred directly to the 6β-position during isomerization of androst-5-ene-3,17-dione into androst-4-ene-3,17-dione catalyzed by the pure enzyme from *Pseudomonas testosteroni*.[10]

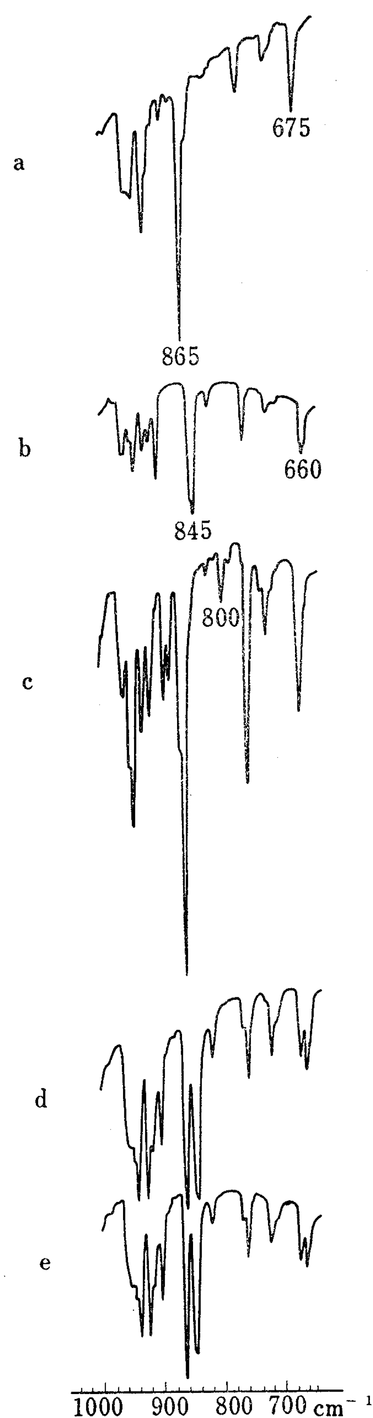

Fig. 2. Infrared Spectra of 6-Deuterated and Non-deuterated Cholestenones

(a) cholestenone; (b) 6β-d_1-cholestenone; (c) 6α-d_1-cholestenone; (d) mixture of a and b (1:1); (e) biotransformation product from 4β-deuterated substrate.

It should be pointed out that *ca.* 50% of deuterium labeled at the 4β-position was lost during the course of this biotransformation. A problem whether the loss of the label would proceed under the enzymatic control or non-enzymatic control remained unclear. In order to clarify this point non-labeled and 6β-deuterated cholestenones were incubated in distilled water or heavy water at pH 7.2 with or without the enzyme. The transformation products were isolated in the manner as described above, and the content and locality of deuterium were determined by inspection of the mass, NMR, and IR spectra. The results obtained are collected in Table II.

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Table II. Results of Deuterium Exchange between Cholestenone and Incubation Medium

Substrate	Condition	Deuterium content (%)
6β-D	Enzyme control in H_2O	72
Non-D	Enzyme control in D_2O	41
6β-D	Nonenzyme control in H_2O	98
Non-D	Nonenzyme control in D_2O	0

When 6β-d_1-cholestenone was conducted in water in the presence of the enzyme, 28% of labeled deuterium was lost. On the other hand, when the unlabeled substrate was treated in heavy water with the enzyme, deuterium was incorporated into the 6β-position at a rate of 41%. In the absence of the enzyme, however, any deuterium exchange was not observed with the two substrates irrespective of the incubation medium. This experiment provided an explanation in part for the *ca.* 50% loss of deuterium in cholestenone enzymatically produced from 4β-d_1-cholesterol.

These results were led to a definite conclusion that 4β-hydrogen was transferred stereospecifically to the 6β-position during transformation of cholesterol into cholestenone by 3β-hydroxysteroid oxidase. It is generally accepted that conversion of Δ^5-3β-hydroxysteroid into Δ^4-3-ketosteroid by 3β-hydroxysteroid oxidase proceeds *via* the Δ^5-3-ketone as an intermediate. The $\Delta^5 \rightarrow \Delta^4$ isomerization may probably involve the stereospecific elimination of 4β-hydrogen in the Δ^5-3-ketosteroid resulting in formation of the $\Delta^{3,5}$-dienol intermediate, followed by protonation at the 6β-position. Further work in progress in these laboratories will provide the more precise knowledge on the biotransformation mechanism.

Experimental[17)]

4β-d_1-Cholesterol (1b), 6α-d_1-Cholest-4-en-3-one, 6β-d_1-Cholest-4-en-3-one——Prepared by the methods previously established in these laboratories.[13,14)]

6-d_1-Cholesterol (6-d_1-Cholest-5-en-3β-ol) (1c)——To a solution of 6-d_1-cholest-5-en-3β-ol acetate (310 mg) in tetrahydrofuran (3 ml)–MeOH (6 ml) was added 30% KOH (1 ml) and allowed to stand at room temperature for 10 min. The resulting solution was extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. Recrystallization of the crude product from MeOH gave **1c** (226 mg) as colorless leaflets. mp 147.5—148°. IR ν_{max}^{KBr} cm^{-1}: 2240 (C–D). Mixed melting point on admixture with the non-labeled authentic sample showed no depression. MS *m/e*: 387 (M^+) (98% d_1).

5α-Cholestane-3β,4α-diol 3-*tert*-Butyldimethylsilyl Ether (3b)——To a stirred solution of cholest-4-en-3β-ol *tert*-butyldimethylsilyl ether (**2a**)[18)] (300 mg) and $LiAlH_4$ (360 mg) in anhydrous ether (18 ml) was added BF_3-etherate (3 g) in anhydrous ether (18 ml) at 0° over a period of 15 min under a stream of N_2 gas and stirred at room temperature for 1 hr. After addition of moist ether the resulting solution was extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. To a solution of this residue in tetrahydrofuran (10 ml) were added dropwise 10% NaOH (4 ml) and 30% H_2O_2 (3 ml) under ice-cooling and stirred at 0° for 1 hr. The resulting solution was diluted with H_2O and extracted with ether. The organic layer was washed with 5% $NaHSO_3$, 5% $NaHCO_3$, and H_2O, successively, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified by preparative TLC using hexane–AcOEt (100: 1) as developing solvent. Recrystallization of the eluate from ether–MeOH gave **3b** (210 mg) as colorless leaflets. mp 197—198°. $[\alpha]_D^{22}$ +20.0° (c=0.20). *Anal.* Calcd. for $C_{33}H_{62}O_2Si$: C, 76.38; H, 12.04. Found: C, 76.28; H, 12.08. NMR ($CDCl_3$) δ: 0.08 (6H, s, $Si(CH_3)_2$), 0.64 (3H, s, 18-CH_3), 0.90 (9H, s, *t*-C_4H_9), 3.1—3.5 (2H, m, 3α- and 4β-H).

17) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured in $CHCl_3$. Infrared (IR) spectral measurements were run on a JASCO Model IRA-1 spectrometer. Mass spectra were obtained on a Hitachi Model RMU-7 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL Model PS-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet, dd=doublet of doublets, and m=multiplet. The shift reagent was stored *in vacuo* over P_2O_5 until the use. Abbreviation used Eu(fod)$_3$=tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionate)europium (III). For preparative TLC silica gel H and silica gel HF_{254} (E. Merck AG, Darmstadt) were used as adsorbents.

18) H. Hosoda, K. Yamashita, H. Sagae, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **23**, 2118 (1975).

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Hydrolysis of 3b——To a solution of **3b** (5 mg) in acetone (5 ml) was added 5 N HCl (0.05 ml) and allowed to stand at 60° for 30 min. The resulting solution was neutralized with 5% $NaHCO_3$, concentrated to its half volume under the reduced pressure, and extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was purified by preparative TLC using hexane–AcOEt (4:1) as developing solvent. Recrystallization of the eluate from MeOH gave 5α-cholestane-3β,4α-diol (**3a**) (3 mg) as colorless leaflets. mp 232—234° (reported mp 236—238°).[19]

3β-Hydroxy-5α-cholestan-4-one *tert*-Butyldimethylsilyl Ether (4)——To a solution of AcONa (75 mg) and pyridinium chlorochromate (1 g) in anhydrous CH_2Cl_2 (5 ml) was added **3b** (500 mg) in anhydrous CH_2Cl_2 (5 ml) and stirred at room temperature for 14 hr. After addition of anhydrous ether the resulting solution was passed through a short pad of alumina. The organic layer was combined and evaporated. Recrystallization of the crude product from ether–MeOH gave **4** (400 mg) as colorless leaflets. mp 135—138°. $[\alpha]_D^{21}$ +1.9° (c=0.27). *Anal.* Calcd. for $C_{33}H_{60}O_2Si$: C, 76.68; H, 11.70. Found: C, 76.48; H, 11.89. NMR ($CDCl_3$) δ: 0.12 (6H, s, $Si(CH_3)_2$), 0.64 (3H, s, 18-CH_3), 0.71 (3H, s, 19-CH_3), 0.89 (9H, s, t-C_4H_9), 4.15 (1H, dd, J=7, 10.5 Hz, 3α-H).

5α-Cholestane-3β,4β-diol 3-*tert*-Butyldimethylsilyl Ether (5b)——To a solution of **4** (70 mg) in anhydrous ether (5 ml) was added $LiAlH_4$ (30 mg) and stirred at room temperature for 40 min. After addition of moist ether and 20% Rochelle salt solution the resulting solution was extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue was treated with *tert*-butyldimethylsilyl chloride (200 mg) and imidazole (400 mg) in dimethylformamide (DMF) (2 ml)–pyridine (1 ml) at room temperature for 3 hr. The reaction mixture was diluted with H_2O and extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified by preparative TLC using hexane–benzene (2:1) as developing solvent. Recrystallization of the eluate from ether–MeOH gave **5b** (52 mg) as colorless leaflets. mp 185—187°. $[\alpha]_D^{21}$ +14.0° (c=0.58). *Anal.* Calcd. for $C_{33}H_{62}O_2Si\cdot 1/2H_2O$: C, 75.08; H, 12.03. Found: C, 74.65; H, 11.65. NMR ($CDCl_3$) δ: 0.06 (6H, s, $Si(CH_3)_2$), 0.64 (3H, s, 18-CH_3), 0.89 (9H, s, t-C_4H_9), 1.03 (3H, s, 19-CH_3), 3.40—3.72 (2H, m, 3α- and 4α-H).

Oxidation of 5b with CrO_3-pyridine Complex——To a solution of **5b** (30 mg) in pyridine (1 ml) was added CrO_3-pyridine complex (1:10 w/v) (1 ml) and stirred at room temperature for 5 hr. The reaction mixture was diluted with ether, washed with 10% AcOH, 5% $NaHCO_3$, and H_2O, successively, and dried over anhydrous Na_2SO_4. After usual work-up the crude product was purified by preparative TLC using hexane–benzene (2:1) as developing solvent. Recrystallization of the eluate from ether–MeOH gave **4** (22 mg) as colorless leaflets. mp 128.5—130°. Mixed melting point on admixture with the authentic sample showed no depression.

Hydrolysis of 5b——Treatment of **5b** (10 mg) in MeOH with 5 N HCl was carried out in the manner as described with **3b**. Recrystallization of the crude product from MeOH gave 5α-cholestane-3β,4β-diol (**5a**) (7 mg) as colorless needles. mp 198—201° (reported mp 199—201°).[20]

Dehydration of 5b——To a solution of **5b** (10 mg) in pyridine (1 ml) was added $POCl_3$ (0.2 ml) under ice-cooling and stirred at room temperature overnight. After addition of moist ether the resulting solution was extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified by preparative TLC using hexane as developing solvent. Recrystallization of the eluate from ether–MeOH gave **2a** (6 mg) as colorless leaflets. mp 111—112°. Mixed melting point on admixture with the authentic sample showed no depression.

4α-d_1-5α-Cholestane-3β,4β-diol 3-*tert*-Butyldimethylsilyl Ether (5c)——To a solution of **4** (1.4 g) in anhydrous ether (30 ml) was added $LiAlD_4$ (600 mg) and stirred at room temperature for 30 min. After addition of moist ether and 20% Rochelle salt solution the resulting solution was extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was treated with *tert*-butyldimethylsilyl chloride (2.2 g) and imidazole (4.5 g) in DMF (8 ml)–pyridine (4 ml) at room temperature for 3 hr. The reaction mixture was diluted with H_2O and extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product was submitted to column chromatography on silica gel. Elution with hexane–benzene (2:1) gave a crystalline product (1.25 g) which was submitted to further elaboration without purification. A portion of the eluate was recrystallized from ether–MeOH to give **5c** as colorless leaflets. mp 193—194°. Mixed melting point on admixture with the non-labeled authentic sample (**5b**) showed no depression.

4-d_1-Cholest-4-en-3β-ol *tert*-Butyldimethylsilyl Ether (2b)——Dehydration of **5c** (1.25 g) with $POCl_3$ in pyridine was carried out in the manner as described with **5b**. The crude product was submitted to column chromatography on silica gel. Elution with hexane–AcOEt (50:1) gave a crystalline product (1 g) which was submitted to further elaboration without purification. A portion of the eluate was recrystallized from ether–MeOH to give **2b** as colorless leaflets. mp 111—114°. Mixed melting point on admixture with the non-labeled authentic sample (**2a**) showed no depression. NMR ($CDCl_3$) δ: 0.08 (6H, s, $Si(CH_3)_2$), 0.67 (3H, s, 18-CH_3), 0.89 (9H, s, t-C_4H_9), 1.03 (3H, s, 19-CH_3), 4.20 (1H, m, 3α-H).

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4α,5-Epoxy-5α-cholestan-3β-ol *tert*-Butyldimethylsilyl Ether (6b)——To a solution of 4α,5-epoxy-5α-cholestan-3β-ol (**6a**)[13] (40 mg) in DMF (0.5 ml)–pyridine (0.2 ml) were added imidazole (200 mg) and *tert*-butyldimethylsilyl chloride (100 mg) and allowed to stand at room temperature for 10 min. The resulting solution was diluted with ether, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. Recrystallization of the crude product from ether–MeOH gave **6b** (45 mg) as colorless leaflets. mp 176.5—178°. $[\alpha]_D^{13}$ +50.0° (c=0.38). *Anal.* Calcd. for $C_{33}H_{60}O_2Si$: C, 76.68; H, 11.70. Found: C, 76.62; H, 11.84. NMR ($CDCl_3$) δ: 0.08 (6H, s, $Si(CH_3)_2$), 0.67 (3H, s, 18-CH_3), 0.89 (9H, s, t-C_4H_9), 1.08 (3H, s, 19-CH_3), 2.82 (1H, s, 4β-H), 3.90 (1H, m, 3α-H).

4β,5-Epoxy-5β-cholestan-3β-ol *tert*-Butyldimethylsilyl Ether (7b)——Silylation of 4β,5-epoxy-5β-cholestan-3β-ol (**7a**)[21] (30 mg) with *tert*-butyldimethylsilyl chloride and imidazole was carried out in the manner as described with **6a**. Recrystallization from MeOH gave **7b** (33 mg) as colorless needles. mp 89—89.5°. $[\alpha]_D^{18}$ +1.5° (c=0.49). *Anal.* Calcd. for $C_{33}H_{60}O_2Si$: C, 76.68; H, 11.70. Found: C, 76.46; H, 11.89. NMR ($CDCl_3$) δ: 0.05, 0.06 (each 3H, s, $Si(CH_3)_2$), 0.70 (3H, s, 18-CH_3), 0.96 (9H, s, t-C_4H_9), 1.07 (3H, s, 19-CH_3), 3.01 (1H, d, J=3 Hz, 4α-H), 4.08 (1H, m, 3α-H).

5α-Cholestane-3β,5-diol 3-*tert*-Butyldimethylsilyl Ether (8b)——Silylation of 5α-cholestane-3β,5-diol (**8a**)[13] (100 mg) with *tert*-butyldimethylsilyl chloride and imidazole was carried out in the manner as described with **6a**. Recrystallization from ether–MeOH gave **8b** (114 mg) as colorless needles. mp 186—188°. $[\alpha]_D^{21}$ −11.9° (c=0.21). *Anal.* Calcd. for $C_{33}H_{62}O_2Si$: C, 76.68; H, 11.70. Found: C, 76.35; H, 12.10. NMR ($CDCl_3$) δ: 0.04 (6H, s, $Si(CH_3)_2$), 0.64 (3H, s, 18-CH_3), 0.89 (9H, s, t-C_4H_9), 0.99 (3H, s, 19-CH_3), 4.08 (1H, m, 3α-H).

Epoxidation of 2b——To a solution of **2b** (1 g) in benzene (20 ml) was added m-chloroperbenzoic acid (500 mg) and stirred at room temperature for 1.5 hr. The reaction mixture was diluted with ether, washed with 5% $NaHSO_3$, 5% $NaHCO_3$, and H_2O, successively, dried over anhydrous Na_2SO_4, and evaporated. The crude product was submitted to column chromatography on silica gel. Elution with hexane–benzene (3:1) and recrystallization of the less polar eluate from ether–MeOH gave 4β-d_1-4α,5-epoxy-5α-cholestan-3β-ol *tert*-butyldimethylsilyl ether (**6c**) (845 mg) as colorless leaflets. mp 176—177°. Mixed melting point on admixture with the non-labeled authentic sample (**6b**) showed no depression. Recrystallization of the more polar eluate from MeOH gave 4α-d_1-4β,5-epoxy-5β-cholestan-3β-ol *tert*-butyldimethylsilyl ether (**7c**) (52 mg) as colorless leaflets. mp 88—89°. Mixed melting point on admixture with the non-labeled authentic sample (**7b**) showed no depression.

4α-d_1-5α-Cholestane-3β,5-diol 3-*tert*-Butyldimethylsilyl Ether (8c)——To a solution of **6c** (900 mg) in anhydrous ether (30 ml) was added $LiAlH_4$ (700 mg) and refluxed for 30 hr. After addition of moist ether and 20% Rochelle salt solution the resulting solution was extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product (820 mg) was submitted to further elaboration without purification. A portion of the crude product was recrystallized from MeOH to give **8c** as colorless needles. mp 186—188°. Mixed melting point on admixture with the non-labeled authentic sample (**8b**) showed no depression.

4α-d_1-Cholesterol (1a)——To a solution of **8c** (820 mg) in pyridine (10 ml) was added $SOCl_2$ (0.8 ml) in $CHCl_3$ (0.8 ml) under ice-cooling and stirred at 0° for 5 min. After addition of moist ether the resulting solution was extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue obtained was treated with 6 N HCl (2 ml) in acetone (10 ml)–ether (10 ml) at 60° for 30 min. The resulting solution was neutralized with 5% $NaHCO_3$, concentraed to its half volume under the reduced pressure, and extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified by column chromatography on silica gel. Elution with hexane–AcOEt (10:1) and recrystallization of the eluate from MeOH gave **1a** (234 mg) as colorless leaflets. mp 145—146.5°. IR ν_{max}^{KBr} cm^{-1}: 2175. Mixed melting point on admixture with the non-labeled authentic sample showed no depression. MS m/e: 387 (M^+) (98% d_1).

NMR Spectra of Substrates——NMR spectra of non-labeled and deuterated cholesterols (**1a**, **1b** and **1c**) with 0.21 mol equivalent each of $Eu(fod)_3$ in CCl_4 were measured at 28°. The C-4 and C-6 proton signals in the NMR spectra were assigned as follows:

Compound	Chemical shift (δ) ppm		
	4α-H	4β-H	6-H
Cholesterol	5.24, m	5.48, m	5.90, broad d J=4 Hz
4α-D (**1a**)		5.74, m	5.96, broad s
4β-D (**1b**)	5.14, m		5.92, broad d J=4 Hz
6-D (**1c**)	5.38, m	5.68, m	

21) D.J. Collins, *Aust. J. Chem.*, 16, 658 (1963).

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Incubation Study——To a solution of deuterated cholesterol (**1a, 1b** or **1c**) (*ca.* 20 mg) in 95% EtOH (0.2 ml) was added cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.) (4 mg) in 0.03 M phosphate buffer (pH 7.2) (60 ml) and incubated at 37° for 21 hr. The incubation mixture was extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product was purified by preparative TLC using hexane–AcOEt (5: 1) as developing solvent. Recrystallization of the eluate from MeOH gave cholestenone as colorless plates in *ca.* 95% yield. The deuterium content of cholestenone formed from each substrate was determined by inspection of the molecular ion peak in the mass spectra (Table I). The C-4 proton signals of cholestenone in the NMR spectra were assigned as follows:

Product	Substrate		
	4α-D	4β-D	6-D
Cholestenone	Disappeared	5.76 s, W 1/2=1.5 Hz	5.76 ppm d, J=2 Hz

Deuterium Exchange between Cholestenone and Incubation Medium——Cholestenone and 6β-d_1-cholestenone were incubated at 37° for 21 hr under the following conditions:

Cholestenone	mg	EtOH ml	Enzyme mg	Medium	ml
6β-D	4.4	0.2[a]	2	H_2O	5
Non-D	5.6	0.2[b]	2	D_2O	5
6β-D	5.1	0.2[a]	None	H_2O	5
Non-D	5.7	0.2[b]	None	D_2O	5

Enzyme: cholesterol oxidase, H_2O: 0.03 M phosphate buffer (pH 7.2), D_2O: 0.03 M phosphate buffer in D_2O (pH 7.2).
a) 99% EtOH *b*) 95% EtOH

Isolation of cholestenone from the incubation mixture was carried out in the manner as described above. The deuterium content was determined by inspection of the molecular ion peak in the mass spectra (Table II).

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