Stereochemistry of Hydrogen Addition to C-25 of Desmosterol by Sterol-Δ^{24}-Reductase of Rat Liver Homogenate

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Chemically synthesized (26)- and (27)-methyl-^{13}C-labeled desmosterol (3, 5) were separately incubated with rat liver homogenate. ^{13}C-NMR analysis of the incubation products indicated that the C-25 hydrogen of cholesterol was introduced from the *si*-face of 3 and the *re*-face of 5.

Keywords [26-^{13}C]desmosterol; [27-^{13}C]desmosterol; sterol-Δ^{24}-reductase; cholesterol biosynthesis; [2-^{13}C][1-(methoxycarbonyl)ethyl]triphenylphosphonium iodide

Reduction of the 24,25-double bond of Δ^{24}-steroids such as desmosterol and lanosterol is catalyzed by sterol-Δ^{24}-reductase.[1] It has been demonstrated in a rat liver microsomal fraction that in the process of saturation of the 24,25-double bond of desmosterol, a hydrogen atom from the medium is added to C-24 and another from NADPH to C-25.[2] Investigation of the stereochemistry involved using (24*R*)-[$^{14}C_5$,17α,20,24-$^3H_3$]cholesterol (**2**) biosynthesized from (3*RS*,4*R*)-[2-^{14}C,4-^{3}H]mevalonic acid (**1**) revealed addition of the 24-*pro*-*S*-proton.[3] The addition of the hydrogen at C-25 was shown to occur from the *si*-face, resulting in cholesterol having the stereochemistry shown in **2** (Chart 1).[4] For this determination, [$^{14}C_5$]cholesterol biosynthesized from [2-^{14}C]mevalonic acid was mixed with [25-^{3}H]cholestrol and incubated with *Mycobacterium smegmatis* to yield (25*S*)-26-hydroxy [$^{14}C_5$,25-^{3}H]cholest-4-en-3-one. The microbial hydroxylation proceeded without loss (or epimerization) of the C-25 hydrogen, and it was shown that the C-26 methyl group (*pro*-*R*-methyl) of cholesterol derived from C-2 of mevalonic acid bore the oxygen function. Consequently the reduction of the 24,25-double bond proceeded by the *cis* acquisition of two hydrogens. These *in vitro* results were later confirmed by an *in vivo* experiment.[5] Although these previous investigations utilizing radio-labelled substrates were ingenious, they required extensive (and time-consuming) biological and chemical degradation of the metabolically produced cholesterol. It will be shown here that identical information can be obtained by a brief ^{13}C-NMR spectral analysis of crude incubation products from [26-^{13}C]- and [27-^{13}C]desmosterol (**3**, **5**). The advantage of this methodology has already been demonstrated by our recent investigations on the stereochemical course of desmosterol reduction in an insect.[6]

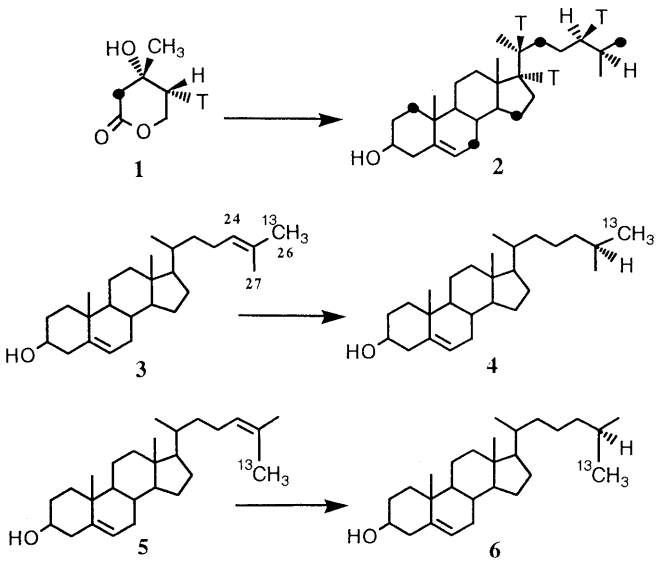

Chart 1

Preparation of the requisite [26-^{13}C]desmosterol (**3**) was previously described.[7] The ^{13}C-label is located 85% at C-26 and 15% at C-27. The corresponding [27-^{13}C] isomer (**5**) was prepared as depicted in Chart 2. Reaction of [^{13}C]methyl iodide with (carbomethoxymethylene) triphenylphosphorane according to the known method[8] gave the corresponding [2-^{13}C]ethyl phosphonium iodide

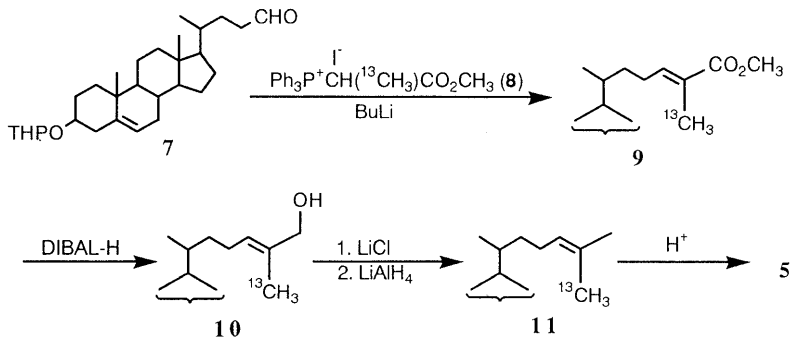

Chart 2

(**8**), which was then treated with *n*-butyllithium followed by 3β-tetrahydropyranyloxychol-5-en-24-al (**7**).[9] The resulting Wittig reaction product was (24*E*)-olefin (**9**, 57% yield), accompanied with a trace of the (24*Z*)-isomer. The ^{13}C-NMR spectrum of **9** showed an intense signal at δ 17.61 (27-C), while the (24*Z*)-isomer showed a prominent signal at δ 25.69 (26-C). The α,β-unsaturated ester (**9**) was reduced with diisobutyl aluminum hydride to afford an allylic alcohol (**10**) in 77% yield. Substitution of the hydroxyl with chlorine, followed by reduction with lithium aluminum hydride yielded [27-^{13}C]desmosterol tetrahydropyranyl ether (**11**, 31%). Final acidic treatment of **11** produced [27-^{13}C]desmosterol (**5**) in 90% yield. The ^{13}C-label is located 96% at C-27 (δ 17.59) and 4% at C-26 (δ 25.69).

The [26-^{13}C]- and [27-^{13}C]desmosterol (**3**, **5**) were separately incubated with the $10000 \times g$ supernatant fraction of rat liver homogenate in the presence of NADPH, ATP and $MgCl_2$. The ethyl acetate extract of the incubation product, after fractionation by thin layer chromatography, was analyzed by ^{13}C-NMR spectroscopy. It can be seen from Fig. 1 that the incubation product from [26-^{13}C]desmosterol (**3**) showed an intense signal at δ 22.55 assigned to *pro-R*-methyl (C-26) of cholesterol.[10] Other signals of lower intensity were thought to arise from the endogenous cholesterol. Further information was obtained from the ^{13}C-NMR spectrum of the incubation product from [27-^{13}C]desmosterol (**5**), wherein a prominent peak appeared at δ 22.79 due to *pro-S*-methyl (C-27) of cholesterol. These results strongly suggest that [26-^{13}C]desmosterol (**3**) was converted to *pro-R*-methyl[^{13}C]cholesterol (**4**), while [27-^{13}C]desmosterol (**5**) yielded *pro-S*-methyl[^{13}C]cholesterol (**6**). Therefore, it is concluded that the hydrogen atom at C-25 of cholesterol is introduced from the back side of the C-24,25 double bond of desmosterol, whose conformation is defined as depicted in Chart 1.

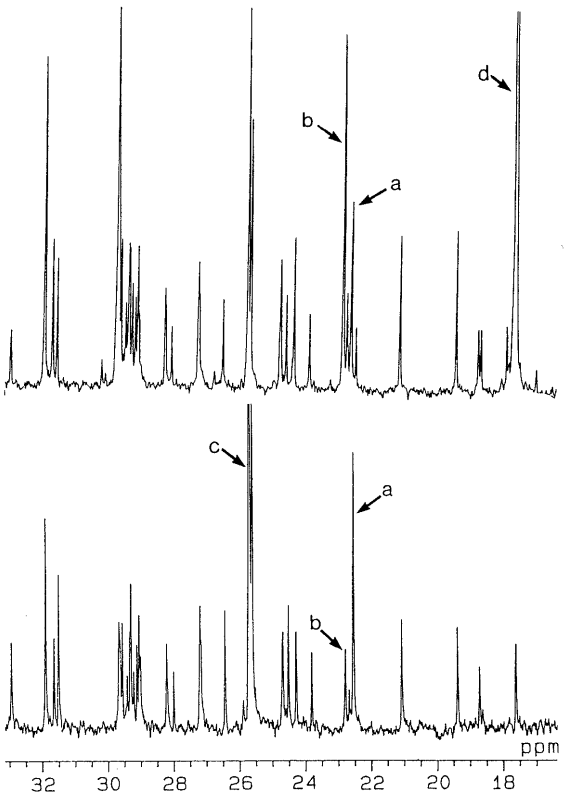

Fig. 1. ^{13}C-NMR Spectra of the Incubation Products (Cholesterol Fraction) from [26-^{13}C]Desmosterol (**3**) (Lower) and from [27-^{13}C]-Desmosterol (**5**) (Upper)

Methyl signals a, b, c and d are due to 26-C and 27-C of cholesterol and 26-C and 27-C of desmosterol, respectively.

This conclusion, obtained straightforward by ^{13}C-NMR analysis, is identical with that obtained circuitously by the use of ratio-labeled substrate.[4] It should also be mentioned that the same stereochemical course of desmosterol reduction has recently been demonstrated in an insect.[6]

Experimental

Incubation Procedure Male rats of the Wistar strain weighing *ca.* 200 g were used. Rat liver homogenate 50% (w/v) was prepared in 0.25 M sucrose solution with a loose-fitting glass homogenizer. The homogenate was centrifuged at $700 \times g$ for 15 min at 4 °C. The supernatant was recentrifuged at $10000 \times g$ for 20 min at 4 °C. To the resulting supernatant (2 ml) was added 0.1 M Tris–HCl buffer pH 8.5 solution (0.52 ml) containing ATP (12.5 mg), $MgCl_2$ (4 ml) and NADPH (1.5 mg). Incubation was conducted by adding acetone solution (70 μl) containing [26 or 27-^{13}C]desmosterol (300 μg) and by gently swirling at 37°C for 1 h. Incubation was terminated by addition of ethanol (2 ml) and 10% NaOH (1 ml). Water (30 ml) was added and the mixture was extracted with ethyl acetate (4×30 ml), washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated off and the residue was applied to a silica gel plate (20×20 cm) and developed with *n*-hexane–ethyl acetate (4 : 1). The band of cholesterol was eluted with ethyl acetate and subjected to ^{13}C-NMR spectroscopy.

Chemicals [26-^{13}C]Desmosterol (**3**) was prepared as described before.[7] NADPH and ATP were obtained from Boehringer Co. (Mannhemim, Germany).

(1-Methoxycarbonyl[2-^{13}C]ethyl)triphenylphosphonium Iodide (8) This labelled compound was prepared from [^{13}C]methyl iodide (99% ^{13}C enriched) and (carbomethoxymethylene)triphenylphosphorane according to the published method.[7] ^{1}H-NMR ($CDCl_3$) δ: 1.70 (3H, ddd, $J_{H-H}=7.3$ Hz, $J_{C-H}=133$ Hz, $J_{D-H}=18.3$ Hz), 3.59 (3H, s), 6.64 (1H, m), 7.64—8.01 (15H, m). ^{13}C-NMR ($CDCl_3$) δ: 13.29.

Methyl (24*E*)-3β-Tetrahydropyranyloxy[27-^{13}C]cholesta-5,24-dien-26-dien-26-oate (9) *n*-BuLi (1.3 M solution in hexane; 3.2 ml, 4.2 mmol) was added to a suspension of **8** (1.97 g, 4.13 mmol) in dry tetrahydrofuran (THF) (16 ml) at 0 °C under nitrogen, and the mixture was stirred for 5 min. A solution of 3β-tetrahydropyranyloxychol-5-en-24-al (**7**)[9] (2.20 g, 4.97 mmol) in dry THF (17 ml) was added, and stirring was continued at 0 °C for 5 min and then at room temperature for 3 h. Extractive (ether) work-up gave a crude product, which was chromatographed on silica gel using hexane–EtOAc as an eluent to afford **9** (1.21 g, 57%), mp 94—97 °C (softened at *ca.* 80°C) (from MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-H_3), 0.95 (3H, d, $J=6.4$ Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 1.85 (3H, d, $^1J_{C-H}=125.0$ Hz, 27-H_3), 3.43—3.97 (3H, m, 3-H, 6'-H_2 of THP), 3.73 (3H, s, OMe), 4.71 (1H, m, 2'-H of THP), 5.34 (1H, m, 6-H), 6.75 (1H, q, $J_{H-H}=6.0$ Hz, $^3J_{C-H}=6.0$ Hz, 24-H). ^{13}C-NMR ($CDCl_3$) δ: 17.61 (27-C). In addition, a weak signal was observed at δ 25.69 due to 26-C of the (24*Z*)-isomer. *Anal.* Calcd for $C_{31}{}^{13}CH_{52}O_4$: C, 77.34; H, 10.20. Found: C, 77.11; H, 10.05.

(24*E*)-26-Hydroxy-3β-tetrahydropyranyloxy[27-^{13}C]cholesta-5,24-diene (10) Diisobutylaluminum hydride (1 M solution in toluene; 4.8 ml, 4.8 mmol) was added to a solution of **9** (1.24 g, 2.41 mmol) in dry CH_2Cl_2 (10 ml) at 0 °C under nitrogen, and the mixture was stirred for 3 h. Extractive (CH_2Cl_2) work-up gave a crude product, which was chromatographed on silica gel using hexane–EtOAc as an eluent to afford **10** (908 mg, 77%), mp 123—124 °C (from MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-H_3), 0.95 (3H, d, $J=6.4$ Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 1.66 (3H, d, $^1J_{C-H}=125.0$ Hz, 27-H_3), 3.43—3.98 (3H, m, 3-H, 6'-H_2 of THP), 4.00 (2H, m, CH_2OH), 4.71 (1H, m, 2'-H of THP), 5.32—5.45 (2H, m, 6-H, 24-H). ^{13}C-NMR ($CDCl_3$) δ: 13.64 (27-C). A weak signal was observed at δ 21.22 due to 26-C of the (24*Z*)-isomer. *Anal.* Calcd for $C_{31}{}^{13}CH_{52}O_3$: C, 79.33; H, 10.79. Found: C, 79.31; H, 10.51.

3β-Tetrahydropyranyloxy[27-^{13}C]cholesta-5,24-diene (11) A mixture of lithium chloride (225 mg, 5.29 mmol), 2,6-lutidine (0.63 ml, 5.41 mmol) and **10** (825 mg, 1.70 mmol) in dry dimethylformamide (DMF) (15 ml) was stirred at 0 °C for 2 min. Methanesulfonyl chloride (MsCl) (0.43 ml, 5.55 mmol) was added dropwise and the mixture was stirred at room temperature for 6 h. The mixture was diluted with water and ether, and the separated organic layer was washed with saturated aqueous cupric nitrate solution, $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, and concentrated to afford a crude allylic chloride.

A solution of the chloride in dry ether (3 ml) was refluxed, after addition of $LiAlH_4$ (20 mg), under a nitrogen atmosphere for 2 h. Extractive (ether) work-up gave a crude product, which was chromatographed on silica gel using hexane–EtOAc as an eluent to afford **11** (241 mg, 31%), mp 122—124 °C (from MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-H_3), 0.94 (3H, d, J=6.4 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 1.60 (3H, d, $^1J_{C-H}$=125.0 Hz, 27-H_3), 1.68 (3H, d, $^3J_{C-H}$=3.9 Hz, 26-H_3), 3.44—3.96 (3H, m, 3-H, 6′-H_2 of THP), 4.71 (1H, m, 2′-H of THP), 5.08 (1H, q, J_{H-H}=6.0 Hz, $^3J_{C-H}$=6.0 Hz, 24-H), 5.34 (1H, m, 6-H). ^{13}C-NMR ($CDCl_3$) δ: 17.61 (27-C). A weak signal at δ 25.69 was observed due to 26-C of the side-chain isopropylidene group. *Anal.* Calcd for $C_{31}{}^{13}CH_{52}O_2$: C, 82.03; H 11.16. Found: C, 81.85; H, 10.86.

[27-^{13}C]Desmosterol (5) A mixture of **12** (231 mg, 0.49 mmol) in dry THF (3 ml), MeOH (0.5 ml) and 2 N HCl (0.3 ml) was stirred at room temperature for 8 h. Extractive (ether) work-up followed by purification on a silica gel column using hexane–EtOAc (5 : 1) as an eluent afforded **5** (171.2 mg, 90%), mp 121—122 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-H_3), 0.93 (3H, d, J=6.8 Hz, 21-H_3), 1.01 (3H, s, 19-H_3), 1.60 (3H, d, $^1J_{C-H}$=124.5 Hz, 27-H_3), 1.68 (3H, d, $^3J_{C-H}$=3.4 Hz, 27-H_3), 3.52 (1H, m, 3-H), 5.08 (1H, q, J_{H-H}=6.0 Hz, $^3J_{C-H}$=6.0 Hz, 24-H), 5.34 (1H, m, 6-H). *Anal.* Calcd for $C_{26}{}^{13}CH_{44}O$: C, 84.35; H, 11.50. Found: C, 84.14; H, 11.57. The ^{13}C-NMR spectrum exhibited signals at δ 17.59 (27-C) and at δ 25.69 (26-C) in the ratio of 20 : 1. The location of the ^{13}C label was calculated as 96% 27-C and 4% 26-C, because 26-C and 27-C signals for nonlabeled sample were observed in the ratio of 1.8 : 1 under our NMR conditions.

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