Biohydrogenation of Unsaturated Compounds by *Saccharomyces cerevisiae*. Part 2†: (*S*)-(−)-Ethyl 4-Hydroxy-3-methylbutanoate as a Chiral Synthon for the Preparation of (25*S*)-26-Hydroxycholesterol

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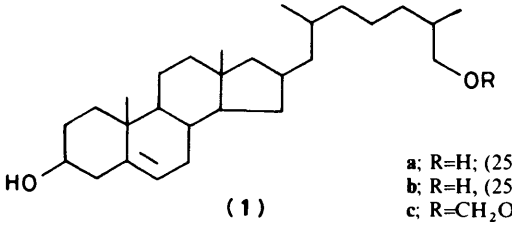
(25*S*)-26-Hydroxycholesterol (**1b**) has been synthesized from stigmasterol (**4a**) and (*S*)-(−)-ethyl 4-hydroxy-3-methylbutanoate (**2a**), the latter having the necessary chirality for the synthesis of the steroidal side-chain.

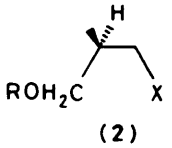
26-Hydroxycholesterol, a product of cholesterol metabolism, has been reported to be produced by stereospecific microsomal hydroxylation during bile acid biosynthesis.[1] As far as the synthesis of the single C-25 isomers is concerned, (25*R*)-26-hydroxycholesterol (**1a**) can be obtained from kryptogenin which is of limited availability. The other epimer, (25*S*)-26-hydroxycholesterol (**1b**) has been prepared by microbial hydroxylation of cholesterol with *Mycobacterium smegmatis*[2] or by two different chemical approaches. Hydroboration of 3β-tetrahydropyranyloxycholesta-5,25-diene gave samples of the (25*S*)-epimer (**1b**) of variable optical purity.[3] However, a stereospecific synthesis of (25*S*)-(**1b**) has been recently reported[4] from 3β-tetrahydropyranyloxy-23,24-dinorchol-5-en-22-ol and the chiral synthon (*S*)-(+)-3-hydroxy-2-methylpropanoic acid which is obtained from a microbial source.[5] In this case, the optical purity of the final product obviously depends on the purity of the chiral synthon used and the steroidal moiety has to be converted into a C-23 steroid in order to be connected to the C-4 chiral synthon.

We have realized an alternative approach to the synthesis of (25*S*)-26-hydroxycholesterol (**1b**) starting from a readily accessible steroid and using as chiral synthon (*S*)-ethyl 4-hydroxy-3-methylbutanoate (**2a**) or its synthetic equivalent, (*S*)-3-methyl-γ-butyrolactone (**3**). Either the hydroxy ester (**2a**) and γ-lactone (**3**) can be easily prepared by a biohydrogenation process, using baker's yeast as microbial source.[6] The steroidal moiety was prepared in a conventional manner from commercially available stigmasterol (**4a**) which was protected at C-3 as a 3,5-cyclo steroid, *via* its tosylate (**4b**), following a well established procedure.[7]



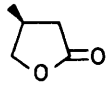
(**1**)

a; R=H; (25*R*)
b; R=H, (25*S*)
c; R=CH_2OEt; (25*S*)

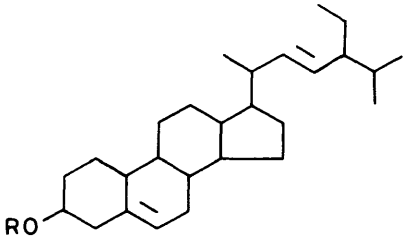


(**2**)

a; R=H, X=CO_2Et
b; R=CH_2OEt, X=CO_2Et
c; R=CH_2OEt, X=CH_2OH
d; R=CH_2OEt, X=CH_2OMes
e; R=CH_2OEt, X=CH_2SPh
f; R=CH_2OEt, X=CH_2SO_2Ph

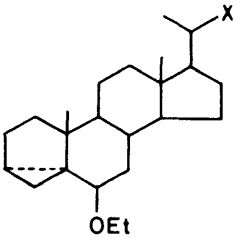


(**3**)



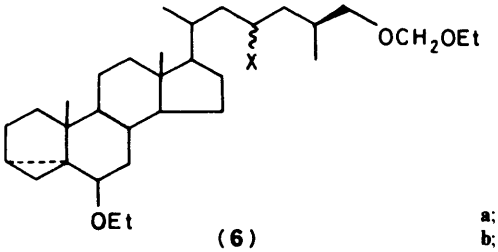
(**4**)

a; R=H
b; R=Tos



(**5**)

a; X=CH=CHCH(Et)Pr^i
b; X=CHO
c; X=CH_2OH
d; X=CH_2OTs
e; X=CH_2I
f; X=CH_2SO_2Ph
g; X=CH_2SPh



(**6**)

a; X=SO_2Ph
b; X=H

† Part 1, see preceding paper.

Subsequent treatment of the tosylate (**4b**) with ethanol and potassium acetate, afforded the ethoxy 3,5-cyclo steroid (**5a**) in good yields (80%). Ozonolysis and reduction of the intermediate ozonide could afford either the aldehyde (**5b**) or the alcohol (**5c**), according to the path followed. In a preliminary work on construction of the side chain, we chose the chiral lactone (**3**) as synthon, since it could be either prepared by baker's yeast biohydrogenation or was, at the time, commercially available (Fluka). Since a feature of this approach was the phenyl sulphone-induced nucleophilic opening of a lactone,[8] we prepared the phenyl sulphone (**5f**) from the 22-alcohol (**5c**) either *via* the tosylate (**5d**) or, better, the iodo derivative (**5e**). Nucleophilic displacement of iodine by sodium benzenesulphinate proceeded in methanol or PEG 400 in only modest yields as a result of cleavage of the 3,5-cyclo steroid moiety and compound (**5f**) was better prepared in two steps: substitution by sodium phenyl sulphide (90%) to give (**5g**) and then oxidation of this by *m*-chloroperbenzoic acid (90%). In the event, although the steroidal sulphone (**5f**) was accessible in good overall yields, its use for the desired nucleophilic opening of the lactone (**3**) using a variety of bases [BuLi, lithium di-isopropylamide (LDA), and EtMgBr[9] for the generation of the anion to the sulphone group] gave only indifferent yields of product. A report by DeLuca *et al.*[9] confirmed difficulties encountered in the same reaction and, therefore, the route was abandoned. As an alternative, ethyl 4-iodo-3-methylbutanoate was prepared by trimethylsilyl iodide-induced opening of the lactone,[10] but no reaction of the 22-sulphone (**5f**) with the iodo ester occurred: instead, ring closure of the iodo ester to the corresponding γ-lactone was observed. We, therefore, thought that an alkyl phenyl sulphone such as (**2f**) could be used for the construction of the steroidal side chain, using known sulphone chemistry.[11] For the preparation of sulphone (**2f**), the hydroxy ester (**2a**) is a more convenient starting material than the lactone (**3**), provided that the hydroxy group is suitably protected in order to avoid ring closure side-reactions. The ethoxymethoxy group was selected and the protected hydroxy ester (**2b**) was prepared in 75% yields with formaldehyde diethyl acetal in the presence of toluene-*p*-sulphonic acid.[12] Following $LiAlH_4$ reduction of the ester (**2b**) (see Part 1) to the protected diol (**2c**), mesylation to (**2d**), displacement with phenyl sulphide to (**2e**), and final *m*-chloroperbenzoic acid oxidation afforded (**2f**) (28% yield). The 22-iodo steroid (**5e**), prepared in 86% yield from the alcohol (**5c**), proved to be the best steroidal counterpart for coupling with the sulphone (**2f**), and the reaction was best effected with LDA in tetrahydrofuran. Following removal of the phenyl sulphone moiety with sodium amalgam and careful acidic hydrolysis (H_2SO_4) afforded the C-26 protected (**1c**). If the acidic hydrolysis was carried out with concentrated HCl, the ethoxymethoxy group at C-26 was also cleaved, but the main product was 3-chlorocholest-5-en-26-ol, along with 10% of the desired (**1b**). The final hydrolysis of C-26 protecting group required HCl in refluxing tetrahydrofuran and finally (25*S*)-(**1b**) was obtained in 45% starting from the 22-iodo derivative (**5e**). Since the optical purity of the final product depends only on the purity of the chiral synthon used, we established that the average purity of the hydroxy ester (**2a**) prepared by us by the fermentative route was generally 90—95%. This was easily achieved by converting the hydroxy ester (**2a**) into the lactone (**3**) of well defined configuration and optical purity (lit.,[6] $[\alpha]_D$ − 24.7°, 97% o.p.). For example, compound (**2a**) from a typical incubation according to our experimental protocol was converted into (**3**); $[\alpha]_D$ − 23° (*c* 4 in MeOH) (o.p. 90%).

The synthesis of (25*S*)-26-hydroxycholesterol (**1b**) from the chiral hydroxy ester (**2a**) provides another example of the synthetic utility of biohydrogenations of unsaturated compounds by fermenting baker's yeast. In our example, the bifunctional chiral synthon is constituted by an hydroxy ester which can be suitably protected and further chemically transformed. By this route several variations at C-26 are made possible by choosing the proper synthon and by manipulation of the hydroxy ester (**2a**); the synthesis of (25*R*)-(**1a**) would also be possible.

Experimental[13]

All steroids, except final product (**1b**), were oils which resisted all attempts at crystallization. All the above compounds were purified by column chromatography and gave satisfactory elemental analysis.

6β-*Ethoxy*,3α,5-*cyclo*-5α-23,24-*dinorcholan*-22-*ol* (**5c**).—Crude stigmasterol toluene-*p*-sulphonate (**4b**)[7] (2.5 g, 4.4 mmol) was refluxed in a solution of fused potassium acetate (2.5 g, 24.5 mmol) in absolute ethanol (125 ml) for 4 h. The mixture was evaporated and the residue taken up with ether. The solution was filtered and the filtrate was evaporated under reduced pressure to afford a mixture containing predominantly 3,5-cyclostigmasteryl ethyl ether (**5a**) (1.6 g), which was used as such for the next step: δ 2.90 (1 H, s, CHO), 3.25—3.75 (2 H, m, CH_2O), and 5.10 (2 H, m, CH=C).

Compound (**5a**) (1.6 g, 1.4 mmol) was dissolved in dry dichloromethane (20 ml). The solution, cooled to −78 °C, was ozonized until it turned blue (*ca.* 10 min at an ozone flux of 1 g h^{-1}) after which it was concentrated under nitrogen and diluted with methanol (25 ml). Sodium borohydride (1.1 g, 29 mmol) was added to the solution, cooled in an ice-bath, and the mixture was stirred and kept at room temperature for 2 h. The mixture was then evaporated under reduced pressure and the residue extracted with ethyl acetate (3 × 100 ml). Evaporation of the extract gave a crude reaction product which was purified by column chromatography on neutral alumina with hexane–ethyl acetate (6 : 4) as eluant to afford the product (**5c**) (0.46 g, 28%). The low yields are the result of the impure nature of the starting stigmasterol (**4a**), the commercial product containing variable amounts (up to 40—50%) of the saturated analogues of (**4a**), namely campesterol and sitosterol. The purified product gave a satisfactory elemental analysis (Found: C, 80.2; H, 11.3. $C_{24}H_{40}O_2$ requires C, 80.0; H, 11.1%); δ 2.90 (1 H, s, CHO), 3.20—3.90 (4 H, m, CH_2O); $[\alpha]_D$ + 68° (*c* 2.27 in $CHCl_3$).

6β-*Ethoxy*-21-*hydroxy*-3α,5-*cyclo*-5α-23,24-*dinorcholan*-21-*yl Tosylate* (**5d**).—Toluene-*p*-sulphonyl chloride (0.3 g, 1.57 mmol) was added to the alcohol (**5c**) (0.3 g, 0.83 mmol), dissolved in pyridine (2 ml), and cooled in an ice-bath. The mixture was kept at room temperature for 24 h after which it was diluted with water and extracted with dichloromethane. The organic phase was dried (Na_2SO_4) and evaporated to afford the oily tosylate (**5d**) (0.385 g, 90%), which was used for the next step without further purification: δ 2.50 (3 H, s, $MeC_6H_4SO_3$), 2.90 (1 H, m, CHO), 3.30—4.10 (4 H, m, CH_2O), 7.40 (2 H, d, *J* 7 Hz), and 7.90 (2 H, d, *J* 7 Hz).

6β-*Ethoxy*-22-*iodo*-3α,5-*cyclo*-5α-23,24-*dinorcholane* (**5e**).—Lithium iodide (0.5 g, 3.7 mmol) was added to the above tosylate (**5d**) (0.514 g, 1 mmol) dissolved in dry acetone (5 ml). The mixture was refluxed for 8 h and then evaporated under reduced pressue. The residue was extracted with dichloromethane and the organic phase washed with aqueous sodium thiosulphate, and dried (Na_2SO_4), and evaporated under reduced pressure to give the title compound (**5e**) (0.45 g, 96%) as an oil (Found: C, 61.5; H, 8.5%. $C_{24}H_{39}IO$ requires C, 61.3; H, 8.3% $[\alpha]_D$ + 47.7° (*c* 1.9 in $CHCl_3$); δ 2.90 (1 H, s, CHO) and 3.20—3.90 (4 H, m, CH_2O, CH_2I); *m/z* 470 (M^+), 425, and 343.

4-*Ethoxymethoxy*-3-*methylbutyl Methanesulphonate* (**2d**).—Triethylamine (1.92 g, 19 mmol) and methanesulphonyl

chloride (2.17 g, 19 mmol) were added to (*S*)-4-ethoxymethoxy-3-methylbutan-1-ol (**2c**) (prepared as described in Part 1) (1.37 g, 8.4 mmol) dissolved in dry dichloromethane (20 ml) and cooled in an ice-bath. The stirred mixture was kept at 0 °C for 3 h after which it was washed with water. The organic phase was then dried (Na_2SO_4) and evaporated under reduced pressure to afford the mesylate (**2d**) (2.17 g) which was used without further purification δ: 0.90 (3 H, d, CH_3CH), 1.30 (3 H, t, CH_3CH_2O), 1.50—2.20 (3 H, m, CH_2CH_2OMs, $CHCH_3$), 3.00 (3 H, m, CH_3SO_2), 3.60 (2 H, q, CH_3CH_2O), 4.30 (2 H, t, CH_2OMs), and 4.80 (2 H, s, OCH_2O).

(S)-1-*Ethoxymethoxy*-2-*methyl*-4-*phenylthiobutane* (**2e**).—A solution of thiophenol (1 g, 9.1 mmol) in dry tetrahydrofuran (3 ml) was added dropwise under nitrogen to a suspension of sodium hydride (81.8% suspension; 0.53 g, 18.1 mmol) in dry tetrahydrofuran (6 ml). After 10 min a few drops of ethanol were added until the precipitate was completely dissolved and this was followed by the above mesylate in dry tetrahydrofuran (6 ml). The stirred reaction mixture was kept at room temperature for 4 h after which it was diluted with water and extracted with diethyl ether (3 × 100 ml). The organic phase was dried (Na_2SO_4) and evaporated to leave a crude product that was purified by column chromatography on neutral alumina (hexane–ethyl acetate, 9:1) to afford the title compound (**2e**) (0.92 g, 43%), b.p. 220—225 °C (Found: C, 66.1; H, 8.6. $C_{14}H_{22}SO_2$ requires C, 66.1; H, 8.7%); δ 0.90 (3 H, d, CH_3CH), 1.30 (3 H, t, CH_3CH_2O), 3.00 (2 H, m, CH_2S), 3.30—3.80 (4 H, m, CH_3CH_2O and OCH_2CHMe), 4.70 (2 H, s, OCH_2O), and 7.40 (5 H, s, ArH); $[\alpha]_D$ − 3.25° (*c* 2 in $CHCl_3$); *m/z* 254 (M^+), 209, and 195.

(S)-1-*Ethoxymethoxy*-2-*methyl*-4-*phenylsulphonylbutane* (**2f**).—3-Chloroperbenzoic acid (0.63 g, 3.64 mmol) was added to the above compound (**2e**) (0.58 g, 2.3 mmol), dissolved in dry dichloromethane (11 ml), and cooled in an ice-bath. The stirred reaction mixture was kept at room temperature for 4 h after which the precipitate was filtered off and the filtrate neutralized with aqueous ammonium hydroxide (12%). The solution was extracted with dichloromethane (2 × 50 ml) and the organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to afford the sulphone (**2f**) (0.46 g, 70%), b.p. 235 °C (Found: C, 58.8; H, 7.8. $C_{14}H_{22}SO_4$ requires C, 58.7; H, 7.7%); δ 0.90 (3 H, d, CH_3CH), 1.30 (3 H, t, CH_3CH_2O), 3.00—3.80 (6 H, m, CH_3CH_2O, OCH_2CHMe, CH_2SO_2), 7.55—7.70 (3 H, m, ArH), and 7.90—8.10 (2 H, m, ArH); $\nu_{max.}$ 1 150 and 1 305 cm^{-1}; $[\alpha]_D$ − 3.4° (*c* 2 in $CHCl_3$); *m/z* 286 (M^+), 257, 241, 227, and 211.

(26S)-26-*Ethoxymethoxy*-6β-*ethyl*-23-*phenylsulphonyl*-3α,5-*cyclo*-5α-*cholestane* (**6a**).—Butyl-lithium (1.6M in hexane; 0.51 ml, 0.82 mmol) was added dropwise, at 0 °C under nitrogen, to a solution of di-isopropylamine (0.084 g, 0.83 mmol) in dry tetrahydrofuran (1 ml). The reaction was cooled to −70 °C and a solution of the sulphone (**2f**) (0.23 g, 0.82 mmol) in dry tetrahydrofuran (1 ml) was added. The reaction was kept at −70 °C and the stirring continued for 10 min. After this a solution of the 22-iodo compound (**5e**) (0.19 g, 0.4 mmol) and hexamethylphosphoric triamide (0.39 g, 2.2 mmol) in dry tetrahydrofuran (1 ml) was added dropwise. The temperature was then brought to 0 °C and the stirring continued for 5 h. After this the reaction mixture was neutralized with saturated aqueous ammonium chloride and extracted with dichloromethane (3 × 50 ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to give crude product reaction which was purified by column chromatography on neutral alumina (hexane–ethyl acetate, 95:5 as eluant) to afford the title compound (**6a**) (0.19 g, 75%) (Found: C, 72.8; H, 9.7. $C_{38}H_{60}O_5S$ requires C, 72.6; H, 9.55%); δ 2.90 (1 H, s, CHO), 3.10—3.80 (7 H, m, CH_2O, $CHSO_2Ph$), 4.60 (2 H, d, OCH_2O), 7.50—7.70 (3 H, m, ArH), and 7.90—8.10 (2 H, m, ArH); $[\alpha]_D$ + 39.4° (*c* 2.03 in $CHCl_3$); $\nu_{max.}$ 1 148 and 1 305 cm^{-1}; *m/z* 628 (M^+).

(25S)-26-*Ethoxymethoxy*-6β-*ethyl*-3α,5-*cyclo*-5α-*cholestane* (**6b**).—A 6% Na-Hg amalgam (freshly prepared, 1.18 g) was added to a solution of the sulphone (**6a**) (0.45 g, 0.72 mmol) in absolute ethanol (3 ml) under nitrogen at room temperature. The progress of the reaction was monitored by t.l.c. (benzene–ethyl acetate, 9:1) and portions of 6% Na–Hg were added until the reduction was complete. After 24 h the mixture was neutralized with saturated aqueous ammonium chloride and extracted with dichloromethane (3 × 10 ml). Work-up provided a crude product which was purified by column chromatography (neutral alumina, hexane–ethyl acetate, 9:1) to afford the title compound (**6b**) (0.34 g, 96%) (Found: C, 78.9; H, 11.65. $C_{32}H_{56}O_3$ requires C, 78.7; H, 11.5%); δ 0.80—1.90 (m), 2.90 (1 H, m, CHO), 3.20—3.80 (6 H, m, CH_2O), 4.70 (2 H, s, OCH_2O); $[\alpha]_D$ + 36.8° (*c* 2.45 in $CHCl_3$); *m/z* 487 (M^+ − 1), 472, 442, and 433.

(25S)-26-*Hydroxycholesterol* (**1b**).—A few drops of 3M sulphuric acid were added to a solution of compound (**6b**) (0.34 g, 0.7 mmol) in tetrahydrofuran (5 ml). After 12 h at room temperature the reaction was neutralized with saturated aqueous $NaHCO_3$ and extracted with ethyl acetate (3 × 10 ml) to afford (25*S*)-26-ethoxymethoxycholesterol (**1c**) (0.24 g, 75%); δ 0.80—2.20 (m), 3.20—3.80 (5 H, m, CH_2O, CHO), 4.70 (2 H, s, OCH_2O), and 5.30—5.50 (1 H, m, CH=C); *m/z* 460 (M^+), 442, 415, 401, and 385.

The 26-ethoxymethoxy group was removed by acidic hydrolysis with 1—2 drops of concentrated hydrochloric acid in tetrahydrofuran at reflux (3 h). After work-up and silica gel chromatography (hexane–ethyl acetate, 6:4) pure (**1b**) (0.192 g, 90%) was obtained. Its chemicophysical properties were in good accordance with the values reported in literature: $[\alpha]_D$ − 39.2° (*c* 0.9 in $CHCl_3$) [lit.[3], $[\alpha]_D$ − 38.0 + 1.16° (*c* 1.72 in $CHCl_3$)].

Hydrolysis of compound (**6b**) (0.16 g, 0.327 mmol) was also carried out directly with 1 drop of concentrated HCl in tetrahydrofuran (5 ml) at 40 °C (24 h). Silica gel chromatography of the crude products (0.15 g) gave by elution with hexane–ethyl acetate (9:1) 3β-chlorocholest-5-en-26-ol (0.06 g, 44%); δ 0.90—2.20 (m), 2.40—2.70 (2 H, m, CH_2CH=C), 3.40—4.20 (3 H, d + m, CH_2O and CHCl), and 5.25—5.55 (1 H, m, CH=C); *m/z* 421, 420 (M^+), 419, 406, 404, 384, and 369. Fractions eluted with the same eluant mixture (6:4) contained title compound (**1b**) (15 mg, 11%).

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References

1 (*a*) K. A. Mitropoulos and N. B. Myant, *Biochem. J.*, 1965, **97**, 26C—28C; (*b*) O. Berseus, *Acta Chem. Scand.*, 1965, **19**, 325.
2 (*a*) Schubert, G. Kaufman, and C. Horhold, *Biochem. Biophys. Acta*, 1969, **176**, 163, 170; (*b*) M. Galli Kienle, K. R. Varma, L. J. Mulheirn, B. Yagen, and E. Caspi, *J. Am. Chem. Soc.*, 1973, **95**, 1996.
3 R. K. Varma, M. Koreeda, B. Yagen, K. Nakanishi, and E. Caspi, *J. Org. Chem.*, 1975, **40**, 3680.
4 C. Y. Byon, M. Gut, and V. Toome, *J. Org. Chem.*, 1981, **46**, 3901.
5 N. Cohen, W. F. Eichel, R. J. Lopresti, C. Neukom, and C. Saucy, *J. Org. Chem.*, 1976, **41**, 3505.

6 H. G. W. Leuenberger, W. Boguth, R. Barner, M. Schmid, and R. Zell, *Helv. Chim. Acta*, 1979, **62**, 455.
7 E. Fernholz and W. L. Ruigh, *J. Am. Chem. Soc.*, 1940, **62**, 3346.
8 D. Savoia, C. Trombini, and A. Umani Ronchi, *J. Org. Chem.*, 1982, **47**, 564.
9 J. W. Morzycki, H. K. Schnoes, and H. F. DeLuca, *J. Org. Chem.*, 1984, **49**, 2148.
10 H. R. Kricheldorf, *Angew. Chem., Int. Ed. Engl.*, 1979, **18**, 689.
11 P. D. Magnus, *Tetrahedron*, 1977, **33**, 2019.
12 U. A. Schaper, *Synthesis*, 1981, 794. Our experimental conditions for ethoxymethylation were similar to the procedure described for methoxymethyl ethers: J. P. Yardley and H. Flechter, *Synthesis*, 1976, 244.
13 See Part 1.

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