Synthesis of (24*R*)-11α-(4-Carboxybutyryloxy)-24,25-dihydroxyvitamin D_3: A Novel Haptenic Derivative Producing Antibodies of High Affinity for (24*R*)-24,25-Dihydroxyvitamin D_3

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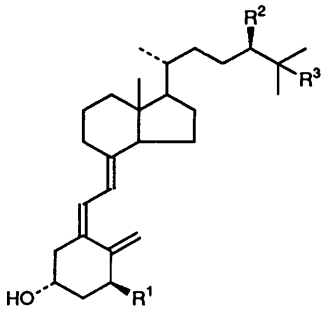
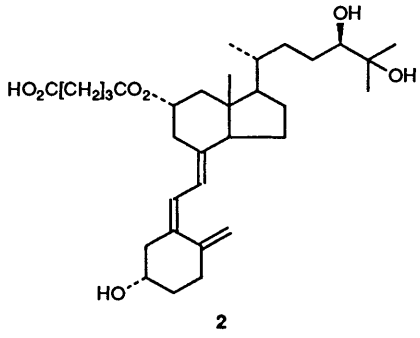
The measurement of serum/plasma (24*R*)-24,25-dihydroxyvitamin D_3 **1a** levels, which is one of the major metabolites of vitamin D_3 **1b**, is important to clarify its physiological significance. To obtain highly specific antibodies to compound **1a** which are useful for development of immunoassays, a novel haptenic derivative, (24*R*)-11α-(4-carboxybutyryloxy)-24,25-dihydroxyvitamin D_3 **2** was synthesized in 21 steps from a [17(20)*Z*]-ethylidene derivative **3** of 11α-hydroxydehydroepiandrosterone. The preparation of an immunogenic conjugate of the hapten **2** with bovine serum albumin and the properties of the resulting antibodies are also described briefly.

(24*R*)-24,25-Dihydroxyvitamin D_3 **1a** is one of the major metabolites of vitamin D_3 **1b**, produced *via* 25-hydroxyvitamin D_3 **1c** by further stereoselective hydroxylation on its side chain at the vitamin D-supplemented state. Since the discovery of



1a R^1 = H, R^2 = R^3 = OH
1b R^1 = R^2 = R^3 = H
1c R^1 = R^2 = H, R^3 = OH
1d R^1 = R^3 = OH, R^2 = H



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dihydroxyvitamin D_3 **1a**, it has been a matter of controversy as to whether or not this metabolite has a physiological role. However, in the last decade, evidence suggesting that dihydroxyvitamin D_3 **1a** has its own specific biological activity has been accumulated.[1–6] Among these findings, the actions on bone are especially noteworthy. Recently, it has been demonstrated by Nakamura *et al.* that a massive dose of dihydroxyvitamin D_3 **1a** causes a marked increase in bone volume[5] and mechanical strength[6] in rats without inducing hypercalcemia. Thus, the metabolite has received much attention as a novel agent for the diseases that cause bone atrophy.

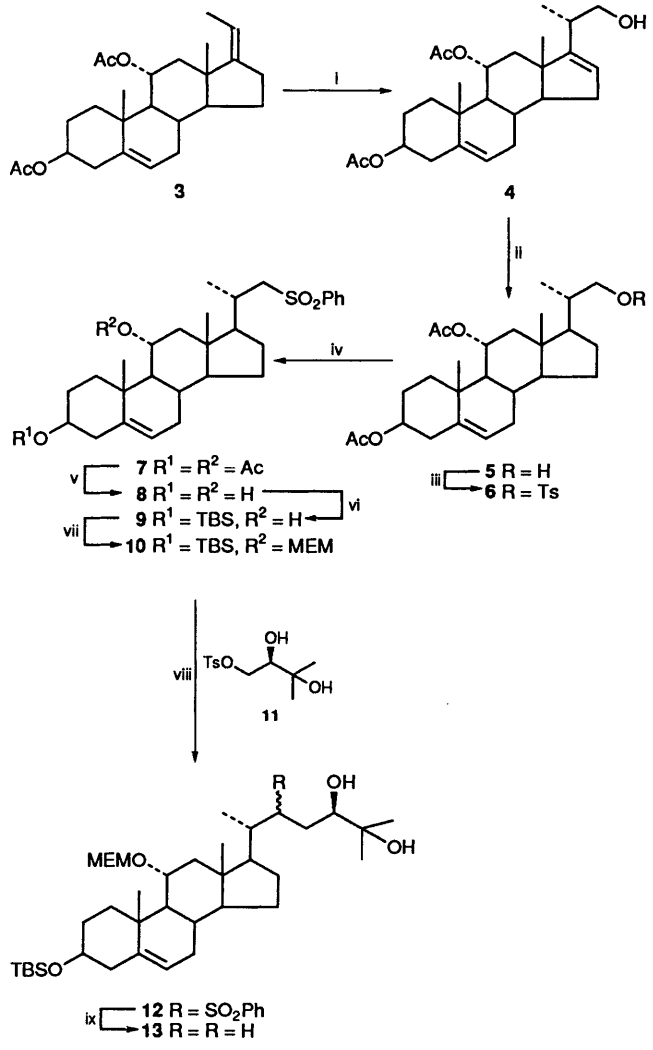
A reliable method for the determination of the serum/plasma dihydroxyvitamin D_3 **1a** levels is needed to support studies on the metabolite in order to develop a new drug and clarify its physiological significance. The determination of dihydroxyvitamin D_3 **1a** levels is now performed mainly by competitive protein binding assay (CPBA) using vitamin D binding protein (DBP).[7] However, CPBA requires a large amount of serum/plasma specimens and their careful pretreatment to remove interfering substances, because the method lacks sufficient sensitivity and specificity for this metabolite to be measured in biological fluids.

Immunoassays based on an antibody having high specificity and affinity for dihydroxyvitamin D_3 **1a** are expected to be an alternative methodology which is simpler, more feasible and suitable for routine use. However, few attempts have been made to produce specific antibodies to the metabolite. Previously, Hummer and Christiansen prepared two kinds of antisera using (24*R*)-24,25-dihydroxyvitamin D_3 3-hemisuccinate as a haptenic derivative and developed a radioimmunoassay (RIA) using one of them.[8] These antisera, however, did not show enough specificity, and thus the RIA required pretreatment of the serum sample by Sephadex® LH-20 and high-performance liquid chromatography (HPLC) to separate dihydroxyvitamin D_3 **1a** from some cross-reacting metabolites.

The use of the hapten-carrier conjugate exposing both the A-ring and side chain is expected to provide antibodies having much higher specificity, the C-11α position of the vitamin D_3 metabolites being an attractive coupling site with the carrier protein. For this reason, we have synthesized the haptenic derivatives 25-hydroxyvitamin D_3 **1c**[9,10] and 1α,25-dihydroxyvitamin D_3 **1d** (the active form of vitamin D_3)[11] having C-11α bridges, which produced antibodies having useful properties. The present paper reports the synthesis of a novel (24*R*)-24,25-dihydroxyvitamin D_3 hapten, (24*R*)-11α-(4-carboxybutyryloxy)-24,25-dihydroxyvitamin D_3 **2**. The preparation of an immunogenic conjugate of the hapten **2** with bovine serum albumin (BSA); the properties of the resulting antibodies are also described briefly.

Results

The [17(20)*Z*]-ethylidene derivative[9,10] **3** of 11α-hydroxydehydroepiandrosterone[12] previously synthesized in our laboratory seemed to be a reasonable starting material. Initially, the 24,25-dihydroxylated side chain with the correct stereochemistry (17β, 20*R* and 24*R*) was constructed by reported



Scheme 1 *Reagents:* i, paraformaldehyde, $BF_3{\cdot}OEt_2$, CH_2Cl_2; ii, H_2, Pt/C, AcOEt; iii, TsCl, pyridine; iv, LiBr, Li_2CO_3, DMF; then $PhSO_2Na$, DMF; v, KOH, MeOH–THF–H_2O; vi, TBSCl, imidazole, DMF; vii, MEMCl, Pr^i_2NEt, CH_2Cl_2; viii, BuLi, THF; ix, Na–Hg, Na_2HPO_4, THF–MeOH; TBS = Bu^tMe_2Si; MEM = $MeO[CH_2]_2OCH_2$

methods[13–16] (Scheme 1). Thus, the ene reaction of compound **3** with paraformaldehyde in the presence of boron trifluoride–diethyl ether[13] stereoselectively afforded the (20*S*)-alcohol **4** (83% yield) the configuration at C-20 of which was suitable for the construction of the natural steroid side chain. Formation of the corresponding (20*R*)-isomer was not observed by TLC or ^1H NMR. Subsequent catalytic hydrogenation of compound **4** with Pt/C[13] proceeded at the C-16 double bond selectively from the less hindered α-face. Thus, the 5-ene **5** having the desired 17β-configuration could be obtained in 98% yield (these assignments for stereochemistry are described below), which was then converted into its toluene-*p*-sulfonate **6** with toluene-*p*-sulfonyl chloride (TsCl) in 93% yield. Nucleophilic substitution of the tosyloxy group in compound **6** by treatment with lithium bromide and lithium carbonate followed by reaction with sodium benzenesulfinate in a one-pot procedure[14,16] produced the phenyl sulfone **7** in 84% yield. A single crystal X-ray analysis was performed on compound **7**. The perspective view plotted using ORTEP clearly demonstrated that the compound has the desired configuration (17β, 20*S*; Fig. 1), from which the compounds **4**–**6** were also proved to possess the above-mentioned stereochemistry. Saponification of compound **7** gave the diol **8** (98% yield), whose 3β-hydroxy group was then selectively silylated using 1.2 mol equiv. of *tert*-butyldimethylsilyl chloride (TBSCl)[9,10] to give the 3-monosilyl ether **9** in 87% yield. Subsequently, the 11α-hydroxy group of compound **9** was protected by a 2-methoxyethoxymethyl (MEM) group[17] to distinguish it from the 24-hydroxy group which was introduced later. Thus, the reaction of the silyl ether **9** with 2-methoxyethoxymethyl chloride (MEMCl) and *N*,*N*-diisopropylethylamine gave the MEM ether **10** in 97% yield. The C-(23–27) unit having a (24*R*)-configuration was then introduced using a chiral side chain synthon **11**.[14–16] The reaction of compound **10** with the synthon **11** in the presence of butyllithium at −20 °C afforded the coupling product **12** as an epimeric mixture at C-22, whose phenylsulfonyl group was then eliminated by treatment with sodium amalgam.[14,16] The MEM group was stable enough under these reactions, and the desired intermediate **13** could be obtained in 77% yield from compound **10**.

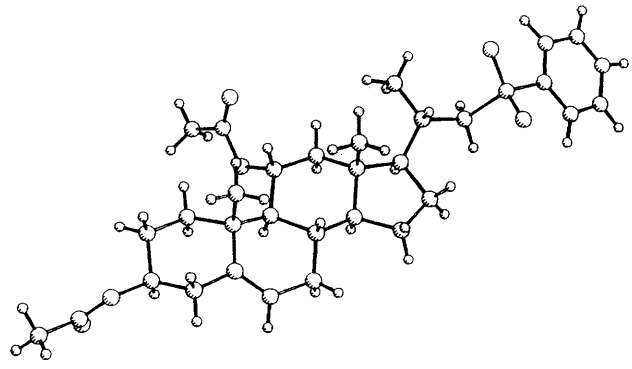
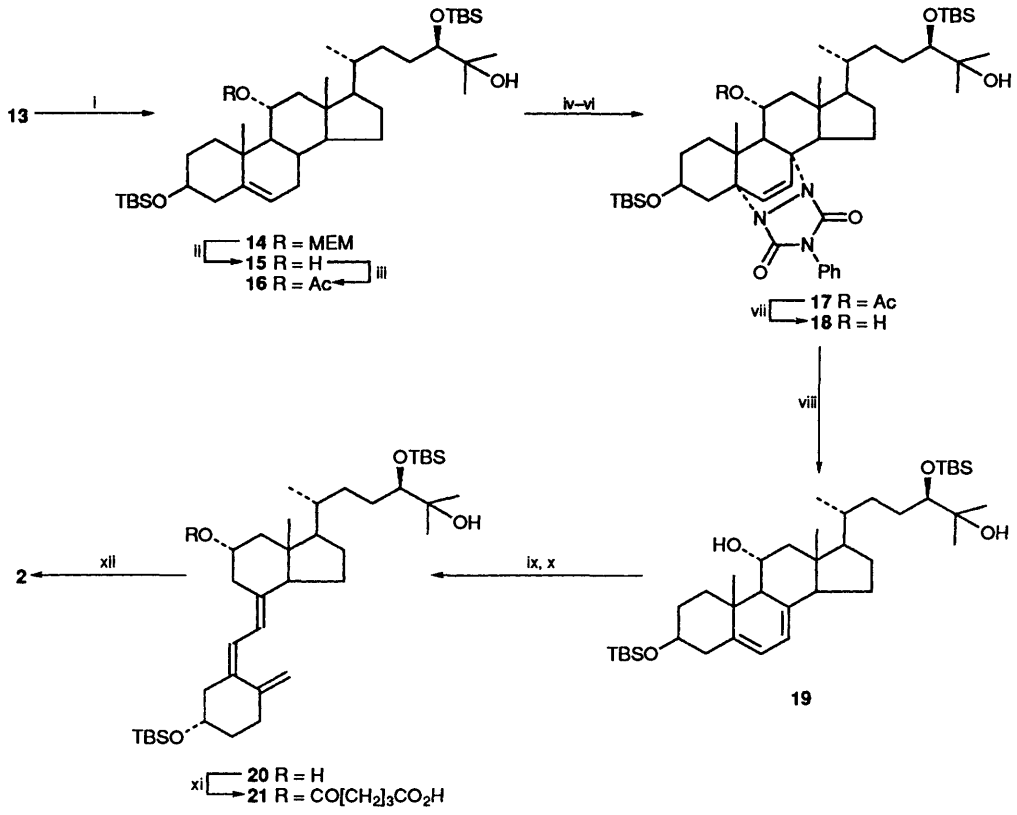


Fig. 1 Perspective view of compound **7** plotted by ORTEP

In the next sequence of reactions, compound **13** was converted into the 5,7-diene derivative **19** whose 3β- and 24-hydroxy groups were selectively protected (Scheme 2). Firstly, the 24-hydroxy group of the diol **13** was silylated with TBSCl to give the bis-TBS ether **14** (87% yield), whose MEM group was then converted into an acetyl group which is stable under the subsequent 7-dehydrogenation. Thus, selective cleavage of the MEM ether of compound **14** in the presence of the TBS group was achieved using bromodimethylborane in dichloromethane at −70 °C[18] to give the 11α-alcohol **15** in 93% yield. The usual acetylation of compound **15** provided its 11-acetate **16** quantitatively (98% yield). Next, the 5,7-diene structure was introduced into compound **16**. Allylic bromination of the acetate **16** with *N*-bromosuccinimide (NBS) and a catalytic amount of 2,2′-azoisobutyronitrile (AIBN), and subsequent dehydrobromination by successive treatment with tetrabutylammonium bromide (TBAB) and tetrabutylammonium fluoride (TBAF)[19] provided a mixture of several components containing the 5,7-diene derivative of the acetate **16**. To facilitate its purification, the mixture was treated with 4-phenyl-4*H*-1,2,4-triazole-3,5-dione (PTAD)[20] to convert the 5,7-diene into the Diels–Alder adduct **17**, which was easily isolated by flash column chromatography (44% yield). Deacetylation of compound **17** with potassium hydroxide followed by removal of the PTAD group from the diol **18** by refluxing in 1,1,3,3-tetramethylguanidine[21] gave the suitably protected intermediate **19** in 52% yield from compound **17**.

Finally, compound **19** was transformed into the hapten **2**. Irradiation of the diene **19** with a high-pressure mercury lamp (400 W) through a Vycor filter and subsequent thermal isomerization at room temperature afforded a reaction mixture from which vitamin D_3 derivative **20** was separated in 33% yield by preparative TLC (PLC). Acylation of the 11α-hydroxy



Scheme 2 *Reagents and conditions:* i, TBSCl, imidazole, DMF; ii, Me_2BBr, CH_2Cl_2; iii, Ac_2O, pyridine; iv, NBS, AIBN, hexane; v, TBAB, THF; then TBAF, THF; vi, PTAD, CH_2Cl_2; vii, KOH, MeOH; viii, 1,1,3,3-tetramethylguanidine; ix, *hv*, Et_2O; x, room temp., hexane–THF; xi, glutaric anhydride, DMAP, pyridine; xii, TBAF, THF

group of compound **20** with glutaric anhydride in the presence of 4-(dimethylamino)pyridine (DMAP) proceeded smoothly and gave the hemiglutarate **21**, which was then subjected to desilylation with TBAF to provide the desired hapten **2** in 83% yield from compound **20**.

All the novel compounds (**2** and **4**–**21**) exhibited satisfactory spectral data and/or elemental analyses. In the ^1H NMR spectra of the vitamin D_3 derivatives **20**, **21** and **2**, the twin singlet-like signals due to the exocyclic methylene protons were assigned based on the nuclear Overhauser effect experiments performed on compound **2** (data not shown), as in the previous case of the 11α-derivative of 25-hydroxyvitamin D_3 (in the reverse order to the conventional assignment of vitamin D_3 and D_2 derivatives).[10]

The hapten **2** was then coupled with BSA by the *N*-succinimidyl ester method[22] (Scheme 3). Repeated immunization of rabbits with the resulting hapten-carrier conjugate afforded three kinds of polyclonal antibodies whose properties were then examined in an RIA procedure using [23,24(N)-^3H](24*R*)-24,25-dihydroxyvitamin D_3 (N indicates nominal labelling; in this case the 23- and 24-positions are expected to be labelled with ^3H, based on the synthetic method, however, it is uncertain) as a labelled antigen. All the antibodies exhibited extremely high titres (optimum final dilution 1:25 000–1:330 000) and affinity constants[23] (K_a = 0.2–1.0 × 10^{10} dm^3 mol^{-1}). The RIA systems employing these antibodies possessed much higher sensitivity (detection limit 2–4 pg/tube) than that of conventional CPBA (larger than 50 pg/tube).[7]

Discussion

We have succeeded in the synthesis of the novel hapten **2** in 21 steps and in a 2.0% overall yield from compound **3**.

The construction of the 24,25-dihydroxylated side chain having the necessary absolute configuration (17β, 20*R* and 24*R*) was a major problem in synthesizing the hapten **2** starting with the steroid precursor **3**. The stereochemistry at C-17 and C-20 was constructed by the stereoselective ene reaction and the following regio- and stereo-selective hydrogenation.[13] The introduction of (24*R*)-configuration was performed utilizing the chiral synthon **11**,[14–16] though another approach based on the asymmetric reduction of an enone system[24] might also be possible. To introduce the bridge structure into the 11α-position selectively, it was also necessary to protect the 11α-hydroxy group adequately during the reaction coupling the steroid synthon having a phenylsulfonyl group and the side chain synthon **11** and subsequent treatment with sodium amalgam. This protective group can be removed selectively in the presence of TBS groups which were considered to be optimum for the protection of the 3β- and 24-hydroxy groups. We chose the MEM group from a range of acetal groups which seemed to fulfil the requirement and obtained a satisfactory result. It should be noted, however, that the cleavage of the MEM group of a 5,7-diene analogue of compound **14** (diene **22**; Scheme 4) with bromodimethylborane gave the intermediate **19** only in poor yield (maximum 18%), even when triethylamine was added as an acid scavenger. Thus, the reaction sequence in which the introduction of the 5,7-diene system into compound **9** precedes the coupling reaction with the chiral synthon **11** was unsuccessful (Scheme 4).

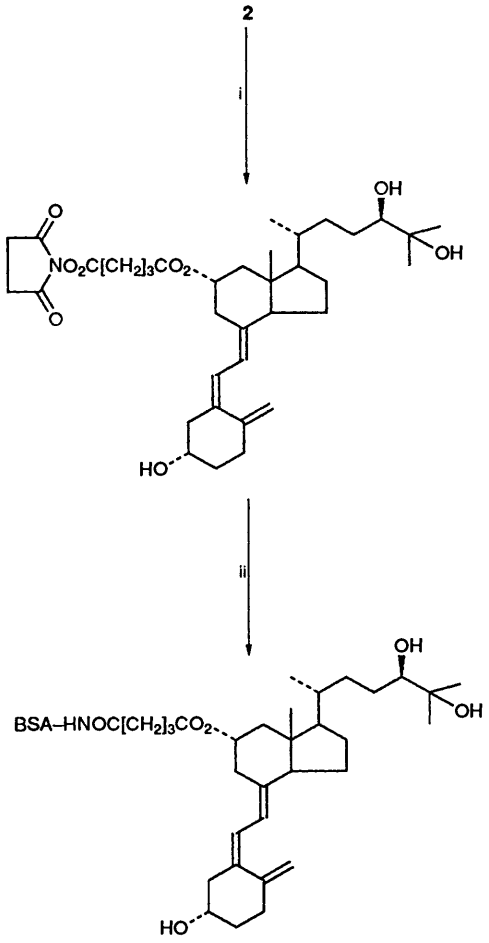
In the two-step reaction converting the adduct **17** into the 5,7-diene **19**, both steps of which were done under basic conditions, we observed by-products having a TBS group at their 25-position which migrated from the 24-position. Therefore, appropriate protection of the 25-hydroxy group prior to these reactions may improve the overall yield of the hapten **2**.

To our knowledge, this is the first report dealing with the synthesis of a dihydroxyvitamin D_3 **1a** derivative having a functional group at its C-ring. The antibodies raised against the

hapten **2** conjugated with BSA showed much higher affinity for dihydroxyvitamin D_3 **1a** than that of DBP (in the range of 10^8 dm^3 mol^{-1})[25] and gave highly sensitive dose–response curves in an RIA procedure. The specificity of these RIA systems and their application to the measurement of serum/plasma dihydroxyvitamin D_3 **1a** are now under investigation in our laboratory. Details of these results will be reported in the future.

Experimental

M.p.s were recorded with a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-181 digital polarimeter for solutions in $CHCl_3$, and $[\alpha]_D$ values are given in units of 10^3 deg cm^2 g^{-1}. UV Spectra were taken on a Hitachi U-2000 spectrophotometer for solutions in ethanol. The low- and high-resolution mass spectra [electron impact (EI) or fast-atom bombardment (FAB) ionization] were determined with Hitachi M-80 and JEOL JMS-DX-303 spectrometers, respectively. ^1H NMR spectra were obtained with a JEOL JNM-FX-100 (100 MHz) or a JNM-EX-270 (270 MHz) spectrometer. $CDCl_3$ was used as the solvent with tetramethylsilane as internal standard. *J* Values are given in Hz. HPLC was performed on a JASCO TRI ROTAR or TOSOH CCPD chromatograph equipped with a JASCO UVIDEC-100-II UV detector (265 nm). A WAKOSIL 5SIL or a Develosil ODS-5 column (each 5 μm; 15 × 0.4 cm i.d.) was used at a flow rate of 1 cm^3 min^{-1} under ambient temperature. Column and flash chromatography were carried out with Merck silica gel 60 (60–200 μm) and Wakogel FC-40 (20–40 μm), respectively. PLC was carried out with Merck silica gel 60 F_{254} (0.5 mm). [17(20)*Z*]-Pregna-5,17(20)-diene-3β,11α-diyl diacetate (the starting substance **3**) was synthesized from 11α-hydroxydehydroepiandrosterone[12] as described in the previous report.[9,10] (*R*)-2,3-Dihydroxy-3-methylbutyl toluene-*p*-sulfonate (chiral side chain synthon **11**) was synthesized from commercially available methyl (*R*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate according to the reported method.[15] All air-sensitive reactions were carried out under argon or nitrogen. The phrase 'dried and evaporated' indicates drying with Na_2SO_4 followed by evaporation of the solvents under reduced pressure.



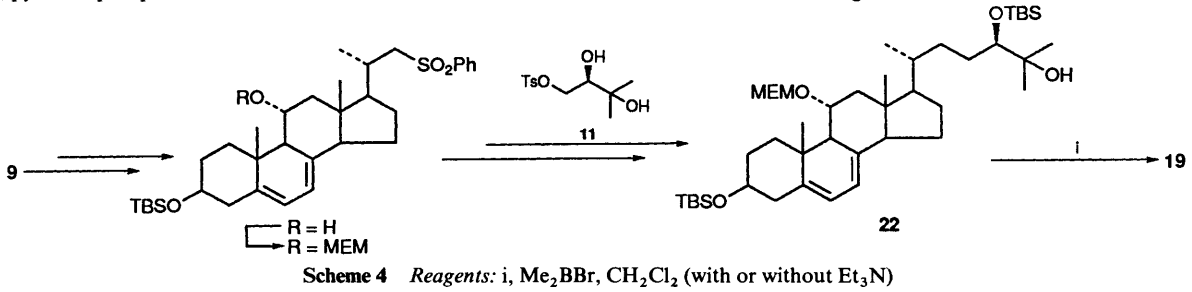
Scheme 3 *Reagents:* i, *N*-Hydroxysuccinimide, EDC, dioxane–H_2O; ii, BSA, pyridine–phosphate buffer

(20S)-22-*Hydroxy*-23,24-*dinorchola*-5,16-*diene*-3β,11α-*diyl Diacetate* * **4**.—BF_3·OEt_2 (0.04 mol dm^{-3} solution in CH_2Cl_2; 7.6 cm^3) was added dropwise to a stirred solution of the ethylidene diacetate **3** (3.04 g, 7.59 mmol) and paraformaldehyde (295 mg, 1.15 equiv.) in CH_2Cl_2 (20 cm^3). The mixture was stirred at 0 °C for 6 h, and the resulting solution was poured into 5% aq. $NaHCO_3$. The mixture was extracted with Et_2O, and the organic layer was washed (brine) and then dried and evaporated. The crude product was purified by flash column chromatography (hexane–AcOEt, 2:1) to give *compound* **4** (2.72 g, 83%) as needles, m.p. 183.5–184.5 °C (from hexane–AcOEt); $[\alpha]_D^{17}$ −101.5 (*c* 0.20) (Found: C, 72.5; H, 8.8. $C_{26}H_{38}O_5$ requires C, 72.5; H, 8.9%); δ(270 MHz) 0.87 (3 H, s, 18-H_3), 1.02 (3 H, d, *J* 6.9, 21-H_3), 1.16 (3 H, s, 19-H_3), 2.02 and 2.03 (each 3 H, s, OAc), 3.56 (2 H, m, 22-H_2), 4.59 (1 H, m, 3α-H), 5.36 (1 H, m, 11β-H) and 5.46–5.48 (2 H, m, 6- and 16-H).

(20S)-22-*Hydroxy*-23,24-*dinorchol*-5-*ene*-3β,11α-*diyl Diacetate* **5**.—A solution of the diene **4** (2.69 g, 6.25 mmol) in AcOEt (105 cm^3) was stirred with 5% Pt/C (270 mg) at room temperature under hydrogen for 13 min. After removal of the catalyst by filtration, the solvent was evaporated off. The crude product was purified by column chromatography (hexane–AcOEt, 3:2) to give *compound* **5** (2.65 g, 98%) as needles, m.p. 167.5–169 °C (from hexane–AcOEt); $[\alpha]_D^{17}$ −76.4 (*c* 0.20) (Found: C, 72.1; H, 9.2. $C_{26}H_{40}O_5$ requires C, 72.2; H, 9.3%); δ(270 MHz) 0.78 (3 H, s, 18-H_3), 1.03 (3 H, d, *J* 6.6, 21-H_3), 1.11 (3 H, s, 19-H_3), 2.01 and 2.03 (each 3 H, s, OAc), 3.36 and 3.61 (each 1 H, m, 22-H), 4.58 (1 H, m, 3α-H), 5.27 (1 H, m, 11β-H) and 5.44 (1 H, d, *J* 5.3, 6-H).

(20S)-22-(*Tolyl*-p-*sulfonyloxy*)-23,24-*dinorchol*-5-*ene*-3β,11α-

* Nomenclature according to ref. 26.



Scheme 4 *Reagents:* i, Me_2BBr, CH_2Cl_2 (with or without Et_3N)

diyl Diacetate **6**.—Toluene-*p*-sulfonyl chloride (TsCl) (11.5 g, 60.3 mmol) was added to a solution of 5-ene **5** (1.51 g, 3.49 mmol) in pyridine (34 cm^3), and the mixture was stirred at 4 °C for 16 h. A small amount of water was added to the mixture, which was then stirred for a further 10 min. The mixture was then extracted with AcOEt, and the organic layer was washed (5% aq. HCl; brine; 5% aq. $NaHCO_3$ and then brine) and then dried and evaporated. The crude product was purified by flash column chromatography (hexane–AcOEt, 3:1) to give *compound* **6** (1.91 g, 93%) as needles, m.p. 157–158 °C (from hexane–AcOEt); $[\alpha]_D^{17}$ −62.2 (*c* 0.20) (Found: C, 67.35; H, 8.0. $C_{33}H_{46}O_7S$ requires C, 67.55; H, 7.9%); δ(100 MHz) 0.72 (3 H, s, 18-H_3), 0.96 (3 H, d, *J* 7, 21-H_3), 1.09 (3 H, s, 19-H_3), 2.00 and 2.02 (each 3 H, s, OAc), 2.45 (3 H, s, Ts-Me), 3.66–4.00 (2 H, m, 22-H_2), 4.58 (1 H, m, 3α-H), 5.03–5.51 (2 H, m, 6- and 11β-H) and 7.26–7.82 (4 H, m, Ph).

(20S)-22-*Phenylsulfonyl*-23,24-*dinorchol*-5-*ene*-3β,11α-*diyl Diacetate* **7**.—LiBr (1.40 g, 13.4 mmol) and Li_2CO_3 (980 mg, 13.3 mmol) were added to a solution of toluene-*p*-sulfonate **6** (2.56 g, 4.36 mmol) in *N*,*N*-dimethylformamide (DMF; 90 cm^3), and the mixture was stirred at 85 °C (bath temperature) for 95 min. Sodium benzenesulfinate (5.76 g, 35.1 mmol) was then added to the resulting mixture, and the mixture was stirred at 85 °C (bath temperature) for a further 3 h. The resulting solution was diluted with AcOEt, washed (brine), dried and evaporated. The crude product was purified by flash column chromatography (hexane–AcOEt, 4:1) to give *compound* **7** (2.05 g, 84%) as prisms, m.p. 210.5–211.5 °C (from hexane–AcOEt); $[\alpha]_D^{17}$ −40.8 (*c* 0.20) (Found: C, 68.9; H, 8.35. $C_{32}H_{44}O_6S$ requires C, 69.0; H, 8.0%); δ(100 MHz) 0.73 (3 H, s, 18-H_3), 1.08 (3 H, s, 19-H_3), 1.16 (3 H, d, *J* 7, 21-H_3), 2.00 and 2.02 (each 3 H, s, OAc), 2.66–3.20 (2 H, m, 22-H_2), 4.55 (1 H, m, 3α-H), 5.23 (1 H, m, 11β-H), 5.41 (1 H, m, 6-H) and 7.51–7.94 (5 H, m, Ph).

(20S)-22-*Phenylsulfonyl*-23,24-*dinorchol*-5-*ene*-3β,11α-*diol* **8**.—A solution of the phenyl sulfone **7** (2.05 g, 3.68 mmol) in a mixture of MeOH (50 cm^3), tetrahydrofuran (THF; 20 cm^3) and 20% aq. KOH (84 cm^3) was stirred at 60 °C (bath temperature) for 2 h. After neutralization with 50% aq. AcOH, the mixture was extracted with AcOEt. The organic layer was washed (brine; 5% aq. $NaHCO_3$ and then brine) and then dried and evaporated. The crude product was purified by flash column chromatography (toluene–AcOEt, 2:3) to give *compound* **8** (1.70 g, 98%) as needles, m.p. 122–124 °C (from hexane–AcOEt); $[\alpha]_D^{17}$ −19.5 (*c* 0.20); δ(100 MHz) 0.69 (3 H, s, 18-H_3), 1.15 (3 H, s, 19-H_3), 1.22 (3 H, d, *J* 6, 21-H_3), 2.40–3.20 (2 H, m, 22-H_2), 3.48 (1 H, m, 3α-H), 4.02 (1 H, m, 11β-H), 5.30 (1 H, m, 6-H) and 7.55–7.96 (5 H, m, Ph); *m*/*z* (EI) 472 (M^+, 19%), 454 ($M^+ - H_2O$, 17), 436 ($M^+ - 2H_2O$, 4.1) and 77 (100).

(20S)-3β-(tert-*Butyldimethylsiloxy*)-22-*phenylsulfonyl*-23,24-*dinorchol*-5-*en*-11α-*ol* **9**.—A mixture of diol **8** (1.70 g, 3.60 mmol), TBSCl (630 mg, 4.18 mmol) and imidazole (645 mg, 9.47 mmol) in DMF (20 cm^3) was stirred at room temperature for 3.5 h. The mixture was diluted with AcOEt, washed (brine) and then dried and evaporated. The crude product was purified by flash column chromatography (hexane–AcOEt, 3:1) to give *compound* **9** (1.83 g, 87%) as prisms, m.p. 198.5–200 °C (from hexane–AcOEt); $[\alpha]_D^{17}$ −15.7 (*c* 0.20) (Found: C, 69.3; H, 9.1. $C_{34}H_{54}O_4SSi$ requires C, 69.6; H, 9.3%); δ(270 MHz) 0.05 (6 H, s, $SiMe_2$), 0.68 (3 H, s, 18-H_3), 0.88 (9 H, s, SiBu^t), 1.14 (3 H, s, 19-H_3), 1.22 (3 H, d, *J* 6.6, 21-H_3), 2.79–3.13 (2 H, m, 22-H_2), 3.47 (1 H, m, 3α-H), 3.96 (1 H, m, 11β-H), 5.34 (1 H, d, *J* 5.6, 6-H) and 7.54–7.93 (5 H, m, Ph).

(20S)-3β-(tert-*Butyldimethylsiloxy*)-11α-(2-*methoxyethoxymethoxy*)-22-*phenylsulfonyl*-23,24-*dinorchol*-5-*ene* **10**.—Pr^i_2NEt (1.18 cm^3, 6.94 mmol) and MEMCl (0.80 cm^3, 7.01 mmol) were added to a solution of ether **9** (505 mg, 0.860 mmol) in CH_2Cl_2 (15 cm^3), and the mixture was stirred at room temperature for 3 h. The resulting solution was poured into 5% aq. $NaHCO_3$, and the mixture was extracted with AcOEt. The organic layer was washed (brine) and then dried and evaporated. The crude product was purified by column chromatography (hexane–AcOEt, 3:1) to give *compound* **10** (561 mg, 97%) as a powder, m.p. 122.5–123.5 °C (from MeOH); $[\alpha]_D^{17}$ −6.79 (*c* 0.20) (Found: C, 67.5; H, 9.1. $C_{38}H_{62}O_6SSi$ requires C, 67.6; H, 9.3%); δ(270 MHz) 0.05 (6 H, s, $SiMe_2$), 0.65 (3 H, s, 18-H_3), 0.88 (9 H, s, SiBu^t), 1.10 (3 H, s, 19-H_3), 1.22 (3 H, d, *J* 6.3, 21-H_3), 2.79–3.13 (2 H, m, 22-H_2), 3.37 (3 H, s, OMe), 3.41–3.78 (5 H, m, 3α-H and OCH_2CH_2O), 3.89 (1 H, m, 11β-H), 4.73 and 4.80 (each 1 H, d, *J* 7.3, OCH_2O), 5.34 (1 H, d, *J* 5.1, 6-H) and 7.54–7.92 (5 H, m, Ph).

(20S,22ζ,24R)-3β-(tert-*Butyldimethylsiloxy*)-11α-(2-*methoxyethoxymethoxy*)-22-*phenylsulfonylcholest*-5-*ene*-24,25-*diol* **12**.—BuLi (1.6 mol dm^{-3} solution in hexane; 4.15 cm^3) was added dropwise to a solution of MEM ether **10** (560 mg, 0.829 mmol) and chiral synthon **11** (455 mg, 1.66 mmol) in THF (22 cm^3) at −20 °C. The resulting mixture was stirred at −20 °C for 2 h and quenched with saturated aq. NH_4Cl. The mixture was extracted with AcOEt, and the organic layer was washed (brine) and then dried and evaporated. The crude product was purified by flash column chromatography (hexane–AcOEt, 5:6) to give *compounds* **12a** and **12b** (less polar and more polar epimers at C-22; total 593 mg, 92%) each as a foam. Spectral data for **12a**, δ(270 MHz) 0.04 (6 H, s, $SiMe_2$), 0.43 (3 H, s, 18-H_3), 0.88 (9 H, s, SiBu^t), 1.02 (3 H, d, *J* 6.6, 21-H_3), 1.06 (3 H, s, 19-H_3), 1.21 and 1.27 (each 3 H, s, 26- and 27-H_3), 3.46 (3 H, s, OMe), 3.40–3.89 (7 H, m, 3α-, 11β-, 24-H and OCH_2CH_2O), 4.70 and 4.78 (each 1 H, d, *J* 7.3, OCH_2O), 5.31 (1 H, d, *J* 5.1, 6-H) and 7.54–7.89 (5 H, m, Ph); *m*/*z* (EI) 776 (M^+, 0.71%), 644 (M^+ − TBSOH, 5.1), 397 (644 − MEMOH − SO_2Ph, 15), 379 (397 − H_2O, 59), 253 (M^+ − side chain − TBSOH − MEMOH, 32), 133 (100) and 59 [$CH_3C(OH)CH_3^+$, 24]. Spectral data for **12b**, δ(270 MHz) 0.06 (6 H, s, $SiMe_2$), 0.67 (3 H, s, 18-H_3), 0.89 (9 H, s, SiBu^t), 1.04, 1.12 and 1.19 (each 3 H, s, 19-, 26- and 27-H_3), 1.37 (3 H, d, *J* 6.9, 21-H_3), 3.27 (1 H, m, 24-H), 3.39 (3 H, s, OMe), 3.42–3.95 (6 H, m, 3α-, 11β-H and OCH_2CH_2O), 4.75 and 4.83 (each 1 H, d, *J* 7.3, OCH_2O), 5.36 (1 H, d, *J* 5.1, 6-H) and 7.52–7.90 (5 H, m, Ph); *m*/*z* (EI) 776 (M^+, 0.65%), 644 (M^+ − TBSOH, 6.1), 397 (644-MEMOH-SO_2Ph, 12), 379 (397-H_2O, 55), 253 (M^+-side chain-TBSOH-MEMOH, 28), 133 (100) and 59 [$CH_3C(OH)CH_3^+$, 23].

(24R)-3β-(tert-*Butyldimethylsiloxy*)-11α-(2-*methoxyethoxymethoxy*)*cholest*-5-*ene*-24,25-*diol* **13**.—5.0% Na–Hg (6.64 g) and Na_2HPO_4 (2.81 g) were added to a solution of diol **12** (mixture; 590 mg, 0.759 mmol) in a mixture of THF (28 cm^3) and MeOH (1.76 cm^3) and then the reaction mixture was stirred at room temperature for 30 min. The resulting suspension was poured into chilled sodium phosphate buffer (0.1 mol dm^{-3}; pH 6.8) and then the insoluble material was removed by filtration. The filtrate was extracted with AcOEt, and the organic layer was washed (brine) and then dried and evaporated. The crude product was purified by flash column chromatography (hexane–AcOEt, 6:5) to give *compound* **13** (405 mg, 84%) as a solid, δ(270 MHz) 0.05 (6 H, s, $SiMe_2$), 0.69 (3 H, s, 18-H_3), 0.89 (9 H, s, SiBu^t), 0.96 (3 H, d, *J* 6.2, 21-H_3), 1.12, 1.16 and 1.22 (each 3 H, s, 19-, 26- and 27-H_3), 3.29 (1 H, m, 24-H), 3.38 (3 H, s, OMe), 3.42–3.78 (5 H, m, 3α-H and OCH_2CH_2O), 3.95 (1 H, m, 11β-H), 4.74 and 4.83 (each 1 H, d, *J* 7.3, OCH_2O) and 5.36 (1 H, d, *J* 5.6, 6-H).

(24R)-3β,24-*Bis*(tert-*butyldimethylsiloxy*)-11α-(2-*methoxyethoxymethoxy*)*cholest*-5-*en*-25-*ol* **14**.—A mixture of diol **13** (358 mg, 0.562 mmol), TBSCl (1.23 g, 8.16 mmol) and imidazole (1.15 g, 16.9 mmol) in DMF (1.2 cm^3) was stirred at room temperature for 19 h. The resulting solution was poured into chilled water and extracted with AcOEt. The organic layer was washed (brine) and then dried and evaporated. The crude product was purified by flash column chromatography (hexane–AcOEt, 8:1) to give *compound* **14** (361 mg, 86%) as a solid, δ(270 MHz) 0.05, 0.08 and 0.09 (6 H, 3 H and 3 H, each s, 4 × SiMe), 0.67 (3 H, s, 18-H_3), 0.89 and 0.92 (each 9 H, s, SiBu^t), 1.11, 1.13 and 1.14 (each 3 H, s, 19-, 26- and 27-H_3), 3.38 (3 H, s, OMe), 3.40–3.73 (6 H, m, 3α-, 24-H and OCH_2CH_2O), 3.93 (1 H, m, 11β-H), 4.74 and 4.83 (each 1 H, d, *J* 6.9, OCH_2O) and 5.36 (1 H, d, *J* 5.3, 6-H); *m*/*z* (EI) 750 (M^+, 0.24%), 253 (8.5), 75 (100) and 59 [$CH_3C(OH)CH_3^+$, 61].

(24R)-3β,24-*Bis*(tert-*butyldimethylsiloxy*)*cholest*-5-*ene*-11α,25-*diol* **15**.—Bromodimethylborane (0.37 mol dm^{-3} solution in CH_2Cl_2; 0.62 cm^3) was added dropwise to a solution of the tris-ether **14** (234 mg, 0.311 mmol) in CH_2Cl_2 (12 cm^3) at −70 °C, and then the mixture was stirred at −70 °C for 80 min. The resulting solution was poured into a chilled mixture of THF (20 cm^3) and saturated aq. $NaHCO_3$ (40 cm^3) and stirred for 10 min at 0 °C. The mixture was extracted with AcOEt, and the organic layer was washed (10% aq. $NaHSO_3$; 5% aq. $NaHCO_3$ and then brine) and then dried and evaporated. The crude product was purified by flash column chromatography (toluene–AcOEt, 12:1) to give *compound* **15** (191 mg, 93%) as a foam [Found: $(M + Na)^+$ (FAB), 685.5007. $C_{39}H_{74}NaO_4Si_2$ requires *M*, 685.5023]; δ(270 MHz) 0.06, 0.08 and 0.09 (6 H, 3 H and 3 H, each s, 4 × SiMe), 0.69 (3 H, s, 18-H_3), 0.89 and 0.92 (each 9 H, s, SiBu^t), 0.93 (3 H, d, *J* 7.9, 21-H_3), 1.13, 1.14 and 1.16 (each 3 H, s, 19-, 26- and 27-H_3), 3.39 (1 H, m, 24-H), 3.46 (1 H, m, 3α-H), 4.03 (1 H, m, 11β-H) and 5.37 (1 H, d, *J* 5.3, 6-H); *m*/*z* (EI) 662 (M^+, 2.1%), 253 (M^+ − side chain − TBSOH − H_2O, 26), 75 (100) and 59 [$CH_3C(OH)CH_3^+$, 31].

(24R)-3β,24-*Bis*(tert-*butyldimethylsiloxy*)-25-*hydroxycholest*-5-*en*-11α-*yl Acetate* **16**.—A solution of diol **15** (191 mg, 0.288 mmol) in pyridine (3 cm^3)–Ac_2O (1.5 cm^3) was stirred at room temperature for 5 h. The resulting solution was poured into chilled H_2O and extracted with AcOEt. The organic layer was washed (H_2O; chilled 5% aq. HCl; brine; 5% aq. $NaHCO_3$ and then brine) and then dried and evaporated. The crude product was purified by column chromatography (hexane–AcOEt, 10:1) to give *compound* **16** (198 mg, 98%) as a foam, δ(270 MHz) 0.05 and 0.09 (each 6 H, s, $SiMe_2$), 0.75 (3 H, s, 18-H_3), 0.89 and 0.92 (each 9 H, s, SiBu^t), 1.08 (3 H, s, 19-H_3), 1.13 and 1.14 (each 3 H, s, 26- and 27-H_3), 2.01 (3 H, s, OAc), 3.36–3.53 (2 H, m, 3α- and 24-H), 5.27 (1 H, m, 11β-H) and 5.38 (1 H, d, *J* 5.3, 6-H); *m*/*z* (EI) 704 (M^+, 0.45%), 253 (M^+ − side chain − TBSOH − AcOH, 13), 75 (100) and 59 [$CH_3C(OH)CH_3^+$, 23].

(24R)-3β,24-*Bis*(tert-*butyldimethylsiloxy*)-25-*hydroxy*-3′,5′-*dioxo*-4′-*phenyl*-5,8-[1,2]*epi*[1,2,4]*triazolo*-5α,8α-*cholest*-6-*en*-11α-*yl Acetate* **17**.—A mixture of 5-ene **16** (197 mg, 0.279 mmol), NBS (74.6 mg, 0.419 mmol) and AIBN (4.6 mg, 28.0 μmol) in hexane (9.0 cm^3) was refluxed for 12 min. The mixture was then cooled in an ice bath and the resulting precipitate was filtered off. The filtrate was concentrated under reduced pressure, and the residue thus obtained was dissolved in THF (8.0 cm^3). TBAB (9.1 mg, 28.2 μmol) was added to the solution, which was then stirred at 0 °C for 30 min. TBAF (1 mol dm^{-3} solution in THF; 1.2 cm^3) was then added to the resulting solution, which was stirred at 0 °C for a further 2 h. The resulting mixture was diluted with AcOEt, washed (H_2O; 5% aq. $NaHCO_3$ and then brine) and then dried and evaporated. The residue was dissolved in CH_2Cl_2 (15 cm^3), and a solution of PTAD (0.15 mol dm^{-3} in CH_2Cl_2) was added dropwise to the solution until a faint red colour due to PTAD persisted. After addition of PTAD, the mixture was stirred at room temperature for 1 h. The resulting solution was diluted with hexane–AcOEt (1:1) and submitted to a short column of silica gel 60 (25 g). The eluate was concentrated under reduced pressure, and the crude product thus obtained was further purified by flash column chromatography (hexane–AcOEt, 5:1) to give *compound* **17** (108 mg, 44%) as a pale yellow solid, δ(270 MHz) 0.07, 0.08 and 0.10 (3 H, 6 H and 3 H, each s, 4 × SiMe), 0.88 (9 H, s, SiBu^t), 0.89 (3 H, s, 18-H_3), 0.90 (9 H, s, SiBu^t), 0.93 (3 H, s, 19-H_3), 1.12 and 1.13 (each 3 H, s, 26- and 27-H_3), 2.04 (3 H, s, OAc), 3.14 (1 H, m, 9α-H), 3.39 (1 H, m, 24-H), 4.37 (1 H, m, 3α-H), 4.89 (1 H, m, 11β-H), 6.24 and 6.35 (each 1 H, d, *J* 8.3, 6-and 7-H) and 7.29–7.47 (5 H, m, Ph); *m*/*z* (EI) 702 (M^+ − PTAD, 0.51%), 378 (702 − 2 × TBSOH − AcOH, 15), 251 (702 − side chain − TBSOH − AcOH, 24), 73 (100) and 59 [$CH_3C(OH)CH_3^+$, 28].

(24R)-3β,24-*Bis*(tert-*butyldimethylsiloxy*)-11α,25-*dihydroxy*-4′-*phenyl*-5,8-[1,2]*epi*[1,2,4]*triazolo*-5α,8α-*cholest*-6-*ene*-3′,5′-*dione* **18**.—A solution of acetate **17** (108 mg, 0.123 mmol) and KOH (150 mg, 2.67 mmol) in MeOH (6.0 cm^3) was stirred at room temperature for 1.5 h. The resulting solution was diluted with AcOEt, washed (brine) and then dried and evaporated. The crude product was submitted to flash column chromatography (hexane–AcOEt, 4:1) to give a mixture containing *compound* **18** and the by-product having the 25-TBS group migrated from the 24-position (*ca.* 25%; estimated by ^1H NMR) (96.5 mg). The mixture was used without further purification, δ(270 MHz) 0.08, 0.10 and 0.11 (6 H, 3 H and 3 H, each s, 4 × SiMe), 0.81 (3 H, s, 18-H_3), 0.88 and 0.91 (each 9 H, s, SiBu^t), 1.13, 1.14 and 1.17 (each 3 H, s, 19-, 26- and 27-H_3), 3.12 (1 H, m, 9α-H), 3.39 (1 H, m, 24-H), 3.76 (1 H, m, 11β-H), 4.38 (1 H, m, 3α-H), 6.23 and 6.32 (each 1 H, d, *J* 8.3, 6- and 7-H) and 7.31–7.47 (5 H, m, Ph).

(24R)-3β,24-*Bis*(tert-*butyldimethylsiloxy*)*cholesta*-5,7-*diene*-11α,25-*diol* **19**.—A solution of adduct **18** (mixture; 95.0 mg) in 1,1,3,3,-tetramethylguanidine (2.0 cm^3) was refluxed for 70 min. The resulting solution was diluted with AcOEt, washed (H_2O; chilled 5% aq. HCl; H_2O; 5% aq. $NaHCO_3$ and then brine) and then dried and evaporated. The crude product was purified by flash column chromatography (hexane–AcOEt, 8:1) to give *compound* **19** (42.6 mg, 52% from compound **17**) as a solid, λ_{max}/nm 273, 284 and 296; δ(270 MHz) 0.06, 0.09 and 0.10 (6 H, 3 H and 3 H, each s, 4 × SiMe), 0.61 (3 H, s, 18-H_3), 0.89 and 0.92 (each 9 H, s, SiBu^t), 0.96 (3 H, d, *J* 5.6, 21-H_3), 1.10 (3 H, s, 19-H_3), 1.14 and 1.15 (each 3 H, s, 26- and 27-H_3), 3.38 (1 H, m, 24-H), 3.60 (1 H, m, 3α-H), 4.21 (1 H, m, 11β-H), 5.38 (1 H, m, 7-H) and 5.52 (1 H, m, 6-H); *m*/*z* (EI) 660 (M^+, 2.4%), 378 (M^+ − 2 × TBSOH − H_2O, 3.5), 251 (M^+ − side chain − TBSOH − H_2O, 5.9), 75 (100) and 59 [$CH_3C(OH)CH_3^+$, 12].

(5Z,7E)-(3S,24R)-3,24-*Bis*(tert-*butyldimethylsiloxy*)-9,10-*secocholesta*-5,7,10(19)-*triene*-11α,25-*diol* **20**.—A solution of diene **19** (72.5 mg, 0.110 mmol) in Et_2O (400 cm^3) was irradiated intermittently (for 5 s, 20 s, 30 s and 15 s) with a 400 W high-pressure mercury lamp through a Vycor filter at 0 °C with argon bubbling through the solution. After removal of the solvent under reduced pressure, the residue was dissolved in a mixture of hexane (20 cm^3) and THF (5 cm^3) and stored in the dark at room temperature under argon for 5 days. The solvent was evaporated off and the crude product thus obtained was

purified by PLC (hexane–Pr^{i}OH, 20:1, developed three times) to give *compound* **20** (23.8 mg, 33%) as an oil [Found: $(M + Na)^+$ (FAB), 683.4851. $C_{39}H_{72}NaO_4Si_2$ requires *M*, 683.4867]; λ_{max}/nm 265; λ_{min}/nm 230; δ(270 MHz) 0.06, 0.07, 0.09 and 0.10 (each 3 H, s, SiMe), 0.56 (3 H, s, 18-H_3), 0.89 and 0.92 (each 9 H, s, SiBu^t), 1.14 and 1.15 (each 3 H, s, 26- and 27-H_3), 3.39 (1 H, m, 24-H), 3.78–3.95 (2 H, m, 3- and 11β-H), 4.76 [1 H, br s, 19(*Z*)-H], 5.01 [1 H, br s, 19(*E*)-H], 6.07 and 6.17 (2 H, ABq, *J* 11.2, 7- and 6-H); *m*/*z* (EI) 660 (M^+, 0.94%), 251 (M^+ – side chain – TBSOH – H_2O, 4.8), 193 (6.9), 76 (6.1), 75 (100) and 59 [$CH_3C(OH)CH_3^+$, 14]. The purity of compound **20** was also confirmed by HPLC [column, WAKOSIL 5SIL; mobile phase, hexane–Pr^{i}OH (50:1); t_r 6.9 min].

(5Z,7E)-(3S,24R)-4-{[3,24-*Bis*(tert-*butyldimethylsiloxy*)-25-*hydroxy*-9,10-*secocholesta*-5,7,10(19)-trien-11α-*yl*]*oxycarbonyl*}*butyric Acid* **21**.—A mixture of triene **20** (23.8 mg, 36.0 μmol), glutaric anhydride (515 mg, 4.51 mmol) and DMAP (0.660 mg) in pyridine (0.6 cm^3) was stirred at room temperature for 4.5 h. Pyridine (4.0 cm^3) and H_2O (0.8 cm^3) were added to the mixture, which was then stirred for a further 30 min. The mixture was then extracted with AcOEt, and the organic layer was washed (H_2O) and then dried and evaporated. The crude product was purified by PLC (hexane–AcOEt, 2:3) to give *compound* **21** (25.6 mg, 92%) as an oil, λ_{max}/nm 265; λ_{min}/nm 230; δ(270 MHz) 0.07, 0.08, 0.09 and 0.10 (each 3 H, s, SiMe), 0.60 (3 H, s, 18-H_3), 0.88 (9 H, s, SiBu^t), 0.92 (9 H, s, SiBu^t), 1.14 and 1.15 (each 3 H, s, 26- and 27-H_3), 3.40 (1 H, m, 24-H), 3.83 (1 H, m, 3-H), 4.74 [1 H, br s, 19(*Z*)-H], 5.00 [2 H, m + br s, 11β- and 19(*E*)-H] and 6.11 (2 H, m, 6- and 7-H); *m*/*z* (FAB; positive ions) 797 [$(M + Na)^+$, 2.9%] and 175 (100); *m*/*z* (FAB; negative ions) 773 [$(M - H)^-$, 7.3%] and 131 (100).

(5Z,7E)-(3S,24R)-4-{[3,24,25-*Trihydroxy*-9,10-*secocholesta*-5,7,10(19)-*trien*-11α-*yl*]*oxycarbonyl*}*butyric Acid* **2**.—A solution of hemiglutarate **21** (6.33 mg, 8.16 μmol) and TBAF (0.12 mmol) in THF (0.62 cm^3) was stirred at room temperature for 6 h. The resulting solution was diluted with AcOEt, washed (H_2O) and then dried and evaporated. The crude product was purified by PLC ($CHCl_3$–MeOH, 5:1) to give *compound* **2** (4.03 mg, 90%) as an oil [Found: $(M + Na)^+$ (FAB), 569.3455. $C_{32}H_{50}NaO_7$ requires *M*, 569.3454]; λ_{max}/nm 264; λ_{min}/nm 229; δ(270 MHz) 0.62 (3 H, s, 18-H_3), 0.95 (3 H, d, *J* 5.6, 21-H_3), 1.17 and 1.22 (each 3 H, s, 26- and 27-H_3), 3.33 (1 H, m, 24-H), 3.96 (1 H, m, 3-H), 4.79 [1 H, d, *J* 2.3, 19(*Z*)-H], 4.99 (1 H, m, 11β-H), 5.04 [1 H, br s, 19(*E*)-H], 6.11 and 6.20 (2 H, ABq, *J* 11.2, 7- and 6-H); *m*/*z* (FAB; positive ions) 569 [$(M + Na)^+$, 9.4%] and 153 (100); *m*/*z* (FAB; negative ions) 545 [$(M - H)^-$, 4.8%] and 176 (100). The purity of compound **2** was also confirmed by HPLC [column, Develosil ODS-5; mobile phase, MeCN–0.5% aq. $(NH_4)_2CO_3$ (1:2); t_r 5.6 min].

Preparation of Hapten–BSA Conjugate.—1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (EDC) (27.1 mg, 0.141 mmol) and *N*-hydroxysuccinimide (14.5 mg, 0.126 mmol) were added to a solution of hapten **2** (8.47 mg, 15.5 μmol) in 95% aq. dioxane (0.2 cm^3), and the mixture was stirred at room temperature for 4.5 h. The resulting solution was diluted with AcOEt, washed (H_2O) and then dried and evaporated. The crude *N*-succinimidyl ester of hapten **2** (8.34 mg; determined by UV spectra at 265 nm on the assumption that the molar extinction coefficient is 17 000[27]) thus obtained was dissolved in pyridine (0.4 cm^3). Then, a solution of BSA (20.0 mg, 0.303 μmol) in a sodium phosphate buffer (0.05 mol dm^{-3}; pH 7.4) (0.4 cm^3) was added to the solution, and the mixture was stirred at 4 °C for 1 day. The resulting solution was dialysed against H_2O at 4 °C for 1 day. After addition of acetone and a small amount of NaCl, the resulting suspension was centrifuged at 4 °C (1000 *g* for 15 min) and the supernatant was discarded. The procedure was repeated until free secosterols were removed. The precipitate was dissolved in 20% aq. pyridine (*ca.* 4 cm^3) and dialysed against 0.9% aq. NaCl at 4 °C for 1 day to give the desired conjugate with a coupling ratio of hapten : BSA of 23 (11.4 mg) as a solution in 0.9% aq. NaCl.

Acknowledgements

Part of this work was supported by grants from the Ministry of Education, Science and Culture of Japan, which are gratefully acknowledged. The authors thank Dr. S. Hosoi of this Faculty for the X-ray analysis and Dr. N. Kubodera of Chugai Pharmaceutical Co. (Tokyo, Japan) for his helpful suggestions. Our thanks are also due to Taiho Pharmaceutical Co. (Tokyo, Japan) for measuring the FABMS spectra.

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Paper 3105623F
Received 17*th September* 1993
Accepted 8*th October* 1993