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Stereoselective synthesis of (22*R*)- and (22*S*)-castasterone/ponasterone A hybrid compounds and evaluation of their molting hormone activity

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Abstract

Two stereoisomers of a castasterone/ponasterone A hybrid compound, the (20*R*,22*R*) and (20*R*,22*S*)-isomers of 2α,3α,20,22-tetrahydroxy-5α-cholestan-6-one, were synthesized stereoselectively and their binding activity to the ecdysteroid receptor was determined. From the concentration–response curve for the inhibition of the incorporation of tritiated ponasterone A into ecdysteroid receptor containing insect cells, the concentration (IC_{50}) required to inhibit 50% of the incorporation of radioactivity into cells was evaluated. The IC_{50} values of the (22*R*)- and (22*S*)-isomers were determined to be 0.30 and 38.9 μM against Kc cells, respectively, indicating that the (22*R*)-isomer is about 100 times more potent than the corresponding (22*S*)-isomer. IC_{50} values of these compounds against lepidopteran Sf-9 cells were determined to be 0.36 and 12.9 μM, respectively. The molting hormonal effect was examined in a *Chilo suppressalis* integument system and the 50% effective concentration for the stimulation of *N*-acetylglucosamine incorporation into the cultured integument was determined to be 2.7 μM for the (22*R*)-isomer, while the (22*S*)-isomer was inactive. On the other hand, both isomers did not show brassinolide-like activity in the rice lamina inclination assay.

Keywords: Castasterone; Ponasterone A; Molting hormone activity; *Chilo suppressalis*; Insect cells

1. Introduction

Insect molting is regulated by 20-hydroxyecdysone (20E; **1** in Fig. 1), and a number of ecdysone analogs, which are together termed ecdysteroids (ES), have been identified in plants, animals and microorganisms (http://ecdybase.org). In plants, steroid hormones which regulate growth and development are also present and are referred to as brassinosteroids (BR) [1–3]. The first identified BR was brassinolide (BL; **2** in Fig. 1) [4], which has a characteristic 7-membered B-ring containing a 7-oxa-6-keto system. To date, more than 50 BRs have been characterized [5]. Castasterone (CS; **3** in Fig. 1) identified from insect galls of the chestnut, has a six-membered ring structure instead of seven-membered lactone structure at the B-ring of BL [6].

Although ES-containing plants contain a wide variety of structural analogs, these endogenous ES do not have significant BR-like activity. On the other hand, two BR compounds, 24-epibrassinolide and 24-epicastasterone, displaced specifically-bound tritiated ponasterone A (PonA; **4**) in *S. littoralis* imaginal disc assays [7], whereas CS did not significantly inhibit tritiated PonA binding at the highest concentration tested (4% inhibition at 5 μM) against Sf-9 cell lines [8]. It is also reported that a few BR/ES hybrid compounds, such as compound **5** (Fig. 1), showed weak molting hormonal activity [9]. Previously, we reported that dibenzoylhydrazines and diacylhydrazines analogs have ES-like activity and the two carbonyl oxygens of diacylhydrazines correspond to the 20- and 22-hydroxy groups of 20E [10]. Recently, the crystal structure of EcR with bound ligand was solved and the superposition between 20E and one of diacylhydrazines was shown [11]. As we proposed, the steroid skeleton was not necessary for strong binding to Lepidoptera ecdysteroid receptors.

Recently, we synthesized a hybrid compound containing the steroidal moiety of CS and the side-chain of PonA, and showed that this hybrid compound is not BS-like but ES-like [12]. This hybrid compound was a mixture of

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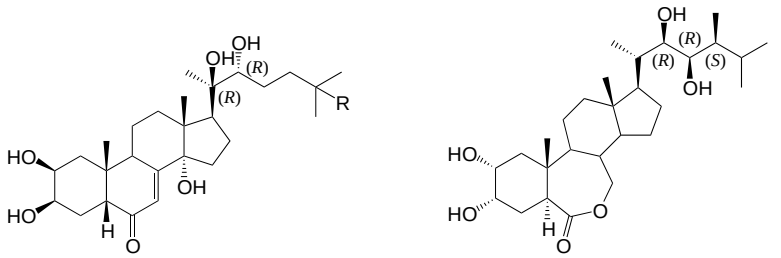
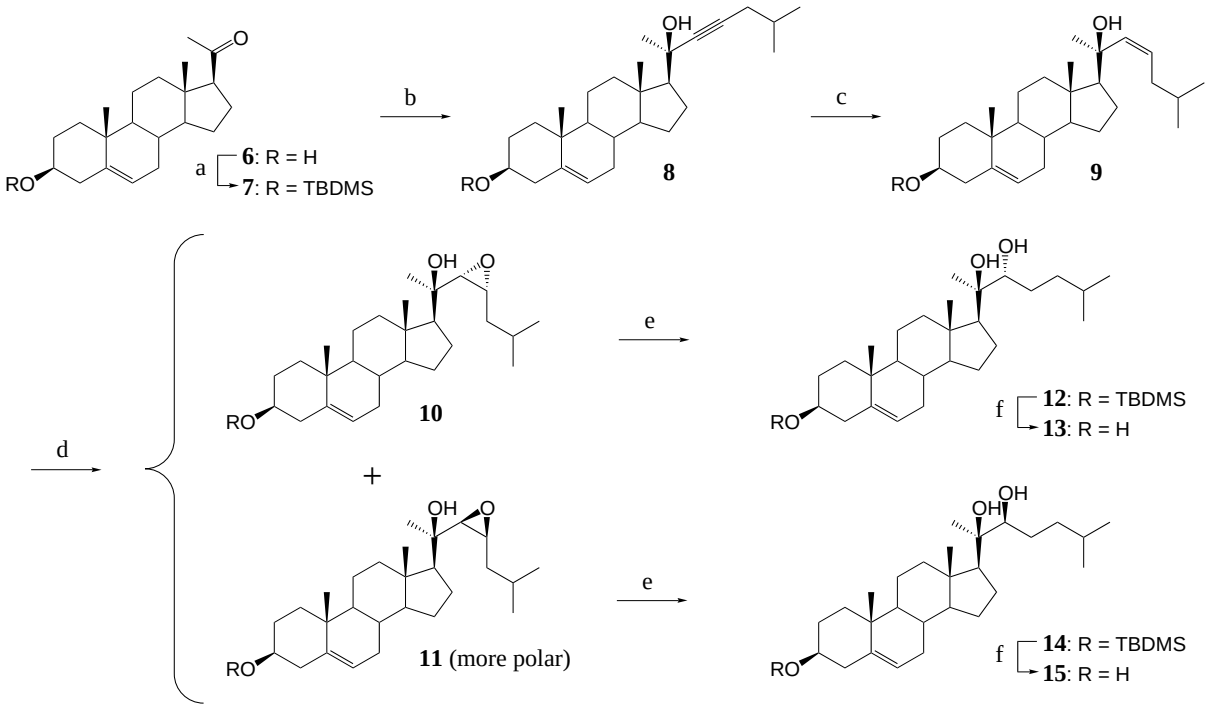
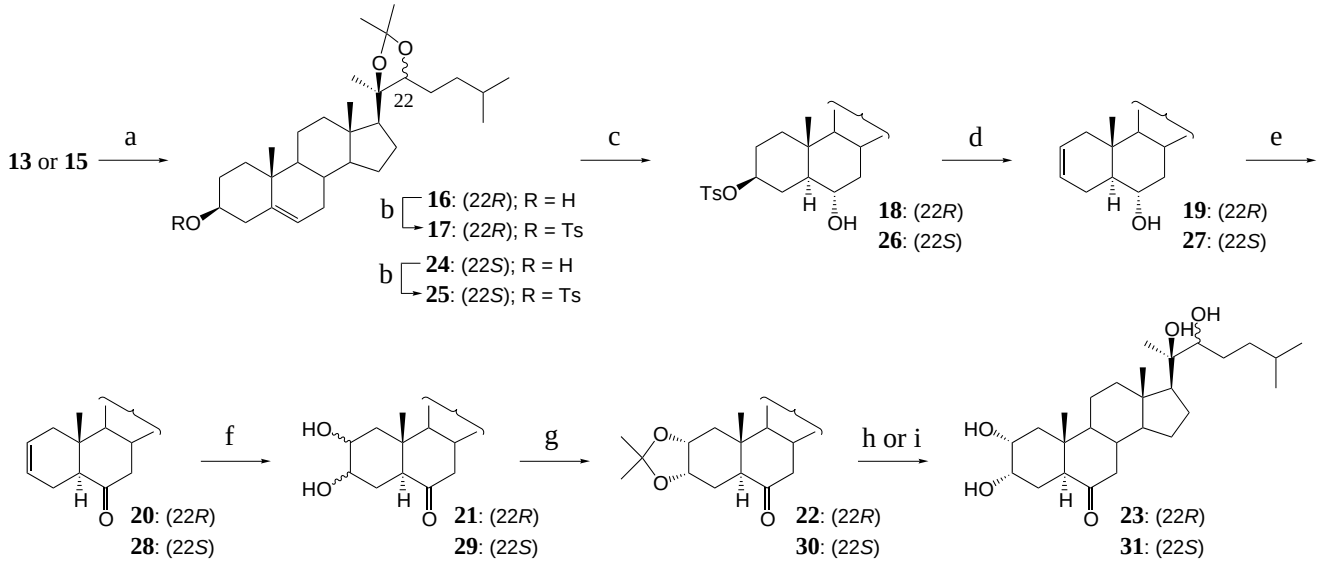


Fig. 1. Structures of ecdysteroids, brassinosteroids, and related compounds.

epimers with respect to C-22 of the side-chain. In this study, we stereoselectively synthesized both the (22*R*)- and (22*S*)-isomers (Schemes 1 and 2) and evaluated their ES-like activity. The receptor binding activity was evaluated by performing competitive binding assays with tritiated PonA with insect Kc and Sf-9 cells [8,13], and the molting hormonal activity was determined in cultured integument of *Chilo suppressalis* by observing the effect on chitin synthesis [14,15]. BR-like activity was also measured using the rice lamina inclination assay [16].



Scheme 1. Reagents and conditions: (a) TBDMSCl, imidazole, DMF, r.t., 5 days (b) 4-methyl-1-pentyne, *n*-BuLi, THF, −78 °C, 10 min (c) H_2 Lindlar catalyst, quinoline, EtOAc, r.t., 1.5 h, then H_2, Lindlar catalyst, EtOAc, r.t., 20 min (d) $VO(acac)_2$, TBHP, CH_2Cl_2, r.t., overnight, then chromatographic separation (e) $LiAlH_4$, THF r.t., overnight (f) TBAF, THF, r.t., 4 days.



Scheme 2. Reagents and conditions: (a) $(MeO)_2CMe_2$, *p*-TsOH, $CHCl_3$, r.t., 40 min, then MeOH, overnight (b) TsCl, pyridine, r.t., 2 days (c) $BH_3{\cdot}SMe_2$, THF, r.t., overnight then 10% NaOH and 30% H_2O_2, 0 °C, 20 min (d) LiBr, Li_2CO_3, DMF, 120 °C, 1 h (e) CrO_3, H_2SO_4, acetone, r.t., 50 min (f) OsO_4, NMO, acetone, r.t., overnight (g) $(MeO)_2CMe_2$, *p*-TsOH, r.t., 1 h, then chromatographic separation (h) for **22**, 60% AcOH, EtOH, 80 °C, 6 h (i) for **30**, 80% AcOH, 80 °C, 1 h.

2. Experimental

2.1. Synthesis

2.1.1. General

Melting points (Mp) were measured with a Yanagimoto melting point apparatus (Yanaco, Kyoto, Japan) and are uncorrected. Optical rotations were measured on a JASCO P-1010 polarimeter. HRMS was recorded on a JOEL JMS 700 spectrometer operating in the 70 eV EI mode. NMR spectra were recorded on a Bruker ARX-500 (500 MHz for ^1H and 125 MHz for ^{13}C) in $CDCl_3$ unless indicated otherwise. Complete assignment of ^{13}C signals were carried out using ^1H-^1H COSY, HMQC and HMBC spectra. Flash column chromatography was conducted using Kieselgel 60 (Merck, Darmstadt, Germany) as the adsorbent. Lindlar catalyst was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan and used without further treatment. Anhydrous *tert*-butyl hydroperoxide (TBHP)/CH_2Cl_2 solution was prepared as described in the literature [17]. Elemental analyses were performed at the Microanalytical Center of Kyoto University.

2.1.2. Chemical synthesis

*2.1.2.1. 3β-(tert-Butyldimethylsilyl)oxypregn-5-en-20-one (**7**).* Imidazole (6.13 g, 90.0 mmol) and *tert*-butyldimethylsilyl chloride (TBDMSCl; 6.78 g, 45.0 mmol) were added to pregnenolone (**6**; 9.49 g, 30.0 mmol) in anhydrous DMF (50 ml), successively. After stirring for 5 days at room temperature, toluene (200 ml) was added to the reaction mixture, and the organic layer was washed with water (3 × 75 ml) and brine (50 ml). After drying the organic layer over anhydrous Na_2SO_4, the solvent was evaporated in vacuo to yield compound **7** (13.20 g) as a white solid, which was subsequently used without purification.

*2.1.2.2. (20R)-3β-(tert-Butyldimethylsilyl)oxycholesta-5-en-22-yn-20-ol (**8**).* 4-Methyl-1-pentyne (9.0 ml, 76.7 mmol) in anhydrous THF (30 ml) was cooled to −78 °C under Ar, and 48 ml (75.8 mmol) of 1.58 M *n*-BuLi/hexane was added dropwise within 25 min. After stirring the mixture for 15 min, compound **7** (13.2 g, 30.0 mmol), dissolved in anhydrous THF (90 ml), was added dropwise within 10 min. Saturated aqueous NH_4Cl (5.0 ml) was added dropwise to the reaction mixture, which was then warmed to room temperature. After addition of the saturated aqueous NH_4Cl (100 ml), the mixture was extracted with EtOAc (4 × 30 ml). The combined organic layers were washed with brine (50 ml). After drying the organic layer over anhydrous Na_2SO_4, the solvent was evaporated in vacuo to yield compound **8** (15.72 g) as a white solid, which was used without further purification.

*2.1.2.3. (20R,22Z)-3β-(tert-Butyldimethylsilyl)oxycholesta-5,22-dien-20-ol (**9**).* Lindlar catalyst (2.50 g) was added to a mixture of compound **8** (15.72 g, 30 mmol), EtOAc (160 ml) and quinoline (0.13 ml). After stirring the mixture for 1.5 h under hydrogen at room temperature, the catalyst was filtered out and washed with EtOAc (140 ml). The combined EtOAc solutions were washed with 1N HCl (50 ml) and saturated aqueous $NaHCO_3$ (50 ml) successively. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo to yield a white solid (15.61 g). Since the

reaction was not complete, the products were dissolved in EtOAc (160 ml) and stirred for a further 20 min in the presence of the Lindlar catalyst (2.50 g) under hydrogen. The mixture was filtered through Celite®, and the solvent was evaporated to yield compound **9** (15.38 g) as a white solid, which was used without further purification. An analytical sample was purified by recrystallization from acetone. Mp 152–153 °C; $[\alpha]_D^{26}$ −48.4° (*c* 0.76, $CHCl_3$); ^{1}H NMR δ 0.06 (6H, s, TBDMS), 0.84 (^{3}H, s, 18-H_3), 0.89 (9H, s, TBDMS), 0.89–0.95 (1H, m, 9α-H), 0.91 (6H, d, *J* = 6.6 Hz, 26-H_3 and 27-H_3), 0.96–1.02 (1H, m, 14α-H), 1.00 (3H, s, 19-H_3), 1.04 (1H, td, *J* = 13.7 and 3.7 Hz, 1α-H), 1.15 (1H, m, 15-H), 1.24 (1H, td, *J* = 12.2 and 5.2 Hz, 12α-H), 1.40 (3H, s, 21-H_3), 1.43–1.73 (11H, m), 1.80 (1H, dt, *J* = 13.2 and 3.4 Hz, 1β-H), 1.97 (1H, dtd, *J* = 17.0, 4.9 and 2.7 Hz, 7β-H), 2.11 (1H, ddd, *J* = 12.4, 3.4 and 3.1 Hz, 12β-H), 2.16 (1H, ddd, *J* = 13.3, 4.9 and 2.2 Hz, 4α-H), 2.24 (2H, m, 24-H_2), 2.27 (1H, m, 4β-H), 3.48 (1H, tt, *J* = 10.9 and 4.7 Hz, 3α-H), 5.22 (1H, dt, *J* = 12.1 and 7.4 Hz, 23-H), 5.31 (1H, m, 6-H), 5.44 (1H, dt, *J* = 12.1 and 1.5 Hz, 22-H); ^{13}C NMR δ −4.59 (TBDMS), 13.58 (C-18), 18.26 (TBDMS), 19.43 (C-19), 20.90 (C-11), 22.40 (C-26/C-27), 22.52 (C-26/C-27), 23.24 (C-16), 23.89 (C-15), 25.94 (TBDMS), 29.10 (C-25), 29.56 (C-21), 31.30 (C-8), 31.81 (C-7), 32.08 (C-2), 36.60 (C-10), 37.04 (C-24), 37.38 (C-1), 40.27 (C-12), 42.81 (C-4), 42.81 (C-13), 50.16 (C-9), 56.78 (C-14), 60.64 (C-17), 72.61 (C-3), 76.92 (C-20), 121.02 (C-6), 128.29 (C-23), 137.39 (C-22), 141.61 (C-5); analysis calculated for $C_{33}H_{58}O_2Si$: C, 76.98; H, 11.35. Found: C, 76.74; H, 11.45.

*2.1.2.4. (20R,22R,23R)-22,23-Epoxy-3β-(tert-butyldimethylsilyl)oxycholest-5-en-20-ol (**10**).* Anhydrous CH_2Cl_2 (60 ml) was added to a mixture of compound **9** (15.38 g, 30 mmol) and vanadyl acetylacetonate (80 mg, 0.30 mmol) under Ar. After adding 4.43 M TBHP/CH_2Cl_2 (10 ml, 44.3 mmol), the mixture was stirred overnight at room temperature. Dimethyl sulfide (1.5 ml, 20.4 mmol) was added and the mixture was stirred for 30 min at room temperature followed by addition of silica gel (80 g). The solvent was removed in vacuo and the residue was subjected to repeated flash column chromatography (hexane/EtOAc = 25:1, three times) to afford the product **10** (5.13 g, 32% from **6**). Mp 203 °C (acetone/$CHCl_3$); $[\alpha]_D^{27}$ −24.8° (*c* 1.33, $CHCl_3$); ^{1}H NMR δ 0.06 (6H, s, TBDMS), 0.87 (3H, s, 18-H_3), 0.89 (9H, s, TBDMS), 0.92 (1H, td, *J* = 10.9 and 6.3 Hz, 9α-H), 0.97–1.07 (2H, m), 0.98 (3H, d, *J* = 6.4 Hz, 26-H_3/27-H_3), 0.99 (3H, d, *J* = 6.4 Hz, 26-H_3/27-H_3), 1.00 (3H, s, 19-H_3), 1.17 (1H, m, 15-H), 1.27 (1H, td, *J* = 11.9 and 6.1 Hz, 12α-H), 1.34 (3H, s, 21-H_3), 1.44–1.58 (6H, m), 1.64–1.74 (5H, m), 1.75–1.84 (3H, m), 1.97 (1H, m, 7β-H), 2.10 (1H, dt, *J* = 12.3 and 3.2 Hz, 12β-H), 2.17 (1H, ddd, *J* = 13.1, 5.0 and 2.1 Hz, 4α-H), 2.27 (1H, m, 4β-H), 2.83 (1H, d, *J* = 4.3 Hz, 22-H), 2.94 (1H, dt, *J* = 8.5 and 4.3 Hz, 23-H), 3.48 (1H, tt, *J* = 10.9 and 4.7 Hz, 3α-H), 5.32 (1H, m, 6-H); ^{13}C NMR δ -4.60 (TBDMS), 13.01 (C-18), 18.25 (TBDMS), 19.42 (C-19), 20.89 (C-11), 22.46 (C-16), 22.56 (C-26/C-27), 22.72 (C-26/C-27), 23.50 (C-21), 23.91 (C-15), 25.92 (TBDMS), 27.50 (C-25), 31.25 (C-8), 31.77 (C-7), 32.05 (C-2), 36.09 (C-24), 36.57 (C-10), 37.36 (C-1), 40.24 (C-12), 42.78 (C-4), 42.88 (C-13), 50.19 (C-9), 56.68 (C-14), 57.01 (C-23), 61.52 (C-17), 62.25 (C-22), 71.25 (C-20), 72.59 (C-3), 121.01 (C-6), 141.57 (C-5); analysis calculated for $C_{33}H_{58}O_3Si$: C, 74.66; H, 11.01. Found: C, 74.79; H, 10.73.

*2.1.2.5. (20R,22S,23S)-22,23-Epoxy-3β-(tert-butyldimethylsilyl)oxycholest-5-en-20-ol (**11**).* Compound **11** (7.18 g, 45% from **6**), derived from compound **9** as described above, was more polar than compound **10**. Mp 200–201 °C (acetone/$CHCl_3$); $[\alpha]_D^{29}$ −80.1° (*c* 1.55, $CHCl_3$); ^{1}H NMR δ 0.06 (6H, s, TBDMS), 0.85 (3H, s, 18-H_3), 0.89 (9H, s, TBDMS), 0.93 (1H, ddd, *J* = 11.5, 11.1 and 5.2 Hz, 9α-H), 0.99 (3H, d, *J* = 6.3 Hz, 26-H_3/27-H_3), 1.00 (3H, d, *J* = 6.6 Hz, 26-H_3/27-H_3), 1.01 (3H, s, 19-H_3), 1.04 (2H, m), 1.17 (1H, m, 15-H), 1.24 (1H, td, *J* = 12.6 and 4.7 Hz, 12α-H), 1.43 (3H, s, 21-H_3), 1.46–1.58 (5H, m), 1.60–1.73 (5H, m), 1.77–1.84 (4H, m), 1.98 (1H, dtd, *J* = 17.0, 4.8 and 2.6 Hz, 7β-H), 2.07 (1H, dt, *J* = 12.3 and 3.2 Hz, 12β-H), 2.17 (1H, ddd, *J* = 13.3, 4.8 and 2.1 Hz, 4α-H), 2.27 (1H, m, 4β-H), 2.75 (1H, d, *J* = 4.3 Hz, 22-H), 3.01 (1H, ddd, *J* = 9.4, 4.2 and 2.5 Hz, 23-H), 3.48 (1H, tt, *J* = 11.0 and 4.7 Hz, 3α-H), 5.32 (1H, m, 6-H); ^{13}C NMR δ -4.61 (TBDMS), 13.60 (C-18), 18.24 (TBDMS), 19.42 (C-19), 20.89 (C-11), 22.58 (C-26/C-27), 22.65 (C-16), 22.80 (C-26/C-27), 23.83 (C-15), 25.92 (TBDMS), 27.23 (C-21), 27.71 (C-25), 31.44 (C-8), 31.73 (C-7), 32.04 (C-2), 36.57 (C-10), 36.91 (C-24), 37.35 (C-1), 39.90 (C-12), 42.64 (C-13), 42.77 (C-4), 50.15 (C-9), 56.47 (C-17), 56.82 (C-14), 59.93 (C-23), 63.34 (C-22), 72.57 (C-3), 72.81 (C-20), 120.98 (C-6), 141.54 (C-5); analysis calculated for $C_{33}H_{58}O_3Si$: C, 74.66; H, 11.01. Found: C, 74.46; H, 10.71.

*2.1.2.6. (20R,22R)-3β-(tert-Butyldimethylsilyl)oxycholest-5-ene-20,22-diol (**12**).* Lithium aluminum hydride (1.98 g, 52.2 mmol) was added to an ice-cold solution of compound **10** (2.77 g, 5.22 mmol) in anhydrous THF (50 ml) and the mixture was stirred for 30 min at 0 °C, then further stirred overnight at room temperature. After cooling the mixture with an ice-water bath, water (2.0 ml) and 15% (w/v) NaOH (2.0 ml) were added dropwise, followed by the addition of water (5.9 ml). After stirring for 10 min at room temperature, the precipitate was removed by filtration and the organic layer was dried over anhydrous $MgSO_4$. The solvent was evaporated to yield compound **12** (2.49 g) as a white solid, which was subsequently used without purification.

*2.1.2.7. (20R,22R)-Cholest-5-ene-3β,20,22-triol (**13**).* A 1.0 M solution of tetra-*n*-butylammonium fluoride (TBAF)/THF (5.7 ml, 5.7 mmol) was added to the crude compound **12** (2.49 g) dissolved in anhydrous THF (11 ml) and the mixture was stirred for 3 days at room temperature. To

complete the reaction, additional TBAF solution (10.5 ml, 10.5 mmol) was added and the reaction mixture was further stirred overnight. After addition of $CHCl_3$ (150 ml), the mixture was washed with saturated aqueous NH_4Cl (50 ml) and dried over anhydrous $MgSO_4$. The solvent was evaporated to yield a yellowish oil (11.22 g), which was purified by flash column chromatography (hexane/EtOAc = 2:1−1:1) to obtain the compound **13** (2.13 g, 98%) as a white solid. Mp 183 °C (EtOAc) {literature [18] 178–180 °C (MeOH), literature [19] 176–178 °C (acetone/hexane)}; $[\alpha]_D^{28}$ −42.5° (*c* 0.76, $CHCl_3$); ^{1}H NMR δ 0.89 (3H, s, 18-H_3), 0.90 (3H, d, J = 6.1 Hz, 26-H_3/27-H_3), 0.91 (3H, d, J = 6.3 Hz, 26-H_3/27-H_3), 1.02 (3H, s, 19-H_3), 1.22 (3H, s, 21-H_3), 3.39 (1H, m, 22-H), 3.52 (1H, m, 3α-H), 5.35 (1H, m, 6-H); ^{13}C NMR δ 13.55, 19.37, 20.37, 20.93, 21.92, 22.35, 22.93, 23.92, 28.06, 29.15, 31.27, 31.60, 31.73, 36.32, 36.47, 37.23, 40.18, 42.24, 43.17, 50.04, 54.73, 56.67, 71.71, 76.39, 77.39, 121.52, 140.76; analysis calculated for $C_{27}H_{46}O_3$: C, 77.46; H, 10.07. Found: C, 77.24; H, 11.21.

*2.1.2.8. (20R,22S)-Cholest-5-ene-3β,20,22-triol (**15**).* Compound **11** (5.20 g, 9.80 mmol) was treated in the same way as compound **10** to yield compound **15** (3.43 g, 84%) as a white solid and recrystallized from acetone/$CHCl_3$. The melting point was not sharp (181–190 °C) {literature [20] 187–189 °C (MeOH)} and elemental analysis data was not acceptable. This may be due to the fact that the compound is hygroscopic. $[\alpha]_D^{27}$ −64.9° (*c* 0.89, $CHCl_3$); ^{1}H NMR δ 0.88 (3H, s, 18-H_3), 0.90 (3H, d, J = 6.6 Hz, 26-H_3/27-H_3), 0.91 (3H, d, J = 6.6 Hz, 26-H_3/27-H_3), 0.94 (1H, td, J = 11.2 and 5.6 Hz, 9α-H), 1.01 (3H, s, 19-H_3), 1.01 (1H, m, 14α-H), 1.08 (1H, m, 1α-H), 1.15 (1H, m, 15β-H), 1.20 (1H, m, 24-H), 1.24 (2H, m, 12α-H and 23-H), 1.27 (3H, s, 21-H_3), 1.42–1.62 (8H, m), 1.65–1.75 (2H, m, 15α-H and 16-H), 1.67 (1H, m, 17α-H), 1.78 (1H, m, 16-H), 1.84 (2H, m, 1β-H and 2α-H), 1.98 (1H, m, 7β-H), 2.10 (1H, dt, J = 12.4 and 3.2 Hz, 12β-H), 2.23 (1H m, 4β-H), 2.30 (1H, m, 4α-H), 3.25 (1H, dd, J = 9.3 and 5.3 Hz, 22-H), 3.53 (1H, m, 3α-H), 5.35 (1H, m, 6-H); ^{13}C NMR δ 13.49 (C-18), 19.37 (C-19), 20.97 (C-11), 22.47 (C-26 / C-27), 22.60 (C-16), 22.76 (C-26 / C-27), 23.48 (C-21), 24.12 (C-15), 28.14 (C-25), 29.38 (C-23), 31.33 (C-9), 31.61 (C-1), 31.75 (C-7), 36.44 (C-24), 36.47 (C-10), 37.23 (C-1), 40.10 (C-12), 42.25 (C-4), 43.11 (C-13), 50.04 (C-9), 54.04 (C-17), 56.35 (C-14), 71.74 (C-3), 76.47 (C-20), 80.53 (C-22), 121.59 (C-6), 140.74 (C-5); HRMS *m/z* calculated for $C_{27}H_{46}O_3$: 418.3447. Found: 418.3457.

*2.1.2.9. (20R,22R)-20,22-Isopropylidenedioxycholest-5-en-3β-ol (**16**).* Compound **13** (1.65 g, 3.94 mmol), 2,2-dimethoxypropane (4.5 ml) and catalytic amount of *p*-TsOH in $CHCl_3$ were stirred for 40 min at room temperature, then MeOH (3.0 ml) was added to hydrolyze undesired 3β-(1-methyl-1-methoxyethyl)ether resulting from excess 2,2-dimethoxypropane stirring overnight. After addition of $CHCl_3$ (50 ml), the organic layer was washed with saturated aqueous $NaHCO_3$ (20 ml) and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to afford compound **16** (1.76 g) as a white solid, which was used without further purification.

*2.1.2.10. (20R,22R)-20,22-Isopropylidenedioxy-5α-cholest-2-en-6-one (**20**).* *p*-Toluenesulfonyl chloride (3.00 g, 15.7 mmol) was added to compound **16** (1.76 g) in anhydrous pyridine (15 ml) and the mixture was stirred for 2 days at room temperature. The mixture was poured into ice-cold 3N HCl (75 ml) and extracted with EtOAc (4 × 30 ml). The combined organic layer was washed successively with brine (30 ml) and saturated aqueous $NaHCO_3$ (30 ml), then dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to give compound **17** (2.21 g) as a yellowish solid, which was dissolved in anhydrous THF (16 ml). To the mixture was added 2.0 M borane–methyl sulfide complex/toluene (7.9 ml, 15.8 mmol) at 0 °C. The mixture was stirred overnight under Ar at room temperature. After cooling in an ice-water bath, 10% NaOH (8.0 ml) and 30% H_2O_2 (8.0 ml) were added to the mixture and stirred for 20 min at 0 °C. After dilution with water (50 ml), the aqueous layer was extracted with EtOAc (4 × 30 ml). The combined organic layer was washed with 20% $Na_2S_2O_3$ (30 ml) and brine (20 ml), then dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to give compound **18** (2.29 g) as a white solid, which was dissolved in anhydrous DMF (35 ml) and stirred at 120 °C for 1 h in the presence of LiBr (2.07 g, 19.7 mmol) and Li_2CO_3 (2.19 g, 29.6 mmol) under Ar. After cooling to room temperature, insoluble materials were filtered out and the filtrate was diluted with EtOAc (200 ml). The organic layer was washed successively with water (2 × 50 ml) and brine (50 ml), and then dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to give compound **19** (2.13 g) as a brown solid, which was dissolved in acetone (40 ml). Jones reagent (2.0 ml) was slowly added to this solution at 0 °C, and the mixture was stirred for 50 min at the same temperature followed by addition of *i*-PrOH (1.0 ml). After the solvent was evaporated, water (60 ml) was added to the residue, and the aqueous layer was extracted with EtOAc (4 × 30 ml). The combined organic layer was washed successively with brine (30 ml) and saturated aqueous $NaHCO_3$ (30 ml), then dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to yield a pale yellowish solid (1.67 g), which was purified by flash column chromatography (hexane/EtOAc = 25:1) to obtain pure compound **20** (0.66 g, 37% from **13**). Mp 175 °C (EtOH); $[\alpha]_D^{20}$ +25.1° (*c* 0.25, $CHCl_3$); ^{1}H NMR δ 0.72 (3H, s, 19-H_3), 0.80 (3H, s, 18-H_3), 0.90 (6H, d, J = 6.6 Hz, 26-H_3 and 27-H_3), 1.11–1.19 (2H, m), 1.15 (3H, s, 21-H_3), 1.20–1.35 (4H, m), 1.30 (3H, s, acetonide), 1.40–1.49 (4H, m), 1.41 (3H, s, acetonide), 1.52–1.68 (4H, m), 1.75 (1H, dtd, J = 12.3, 10.7 and 4.1 Hz, 8β-H), 1.93 (1H, m, 16-H), 1.98 (1H, t, J = 13.3 Hz, 7α-H), 2.00 (2H, m, 1-H_2), 2.02 (1H, m, 4α-H), 2.14 (1H, dt, J = 12.5 and

3.2 Hz, 12β-H), 2.26 (1H, m, 4β-H), 2.35 (1H, dd, J = 10.0 and 4.7 Hz, 5α-H), 2.36 (1H, dd, J = 13.1 and 4.2 Hz, 7β-H), 3.62 (1H, dd, J = 9.2 and 2.8 Hz, 22-H), 5.57 (1H, m, 2-H), 5.69 (1H, ddd, J = 9.9, 4.6 and 2.3 Hz, 3-H); ^{13}C NMR δ 13.04 (C-18), 13.51 (C-19), 21.05 (C-11), 21.70 (C-4), 21.72 (C-21), 22.49 (C-26/C-27), 22.52 (C-26/C-27), 22.58 (C-16), 23.58 (C-15), 26.75 (acetonide), 26.85 (C-23), 28.22 (C-25), 29.03 (acetonide), 36.38 (C-24), 37.32 (C-8), 39.35 (C-1), 39.78 (C-12), 39.99 (C-10), 43.40 (C-13), 46.84 (C-7), 53.38 (C-9), 53.84 (C-5), 54.73 (C-17), 56.95 (C-14), 81.34 (C-22), 84.14 (C-20), 106.61 (acetonide), 124.48 (C-2), 124.96 (C-3), 211.89 (C-6); analysis calculated for $C_{30}H_{48}O_3$: C, 78.90; H, 10.59. Found: C, 78.67; H, 10.63.

*2.1.2.11. (20R,22R)-2,3-Dihydroxy-20,22-isopropylidenedioxy-5α-cholestan-6-one (**21**).* To compound **20** (700 mg, 1.52 mmol) in acetone (24 ml) was added 5 mg/ml OsO_4/*t*-BuOH solution (0.80 ml, 0.016 mmol), 50% aqueous *N*-methylmorpholine *N*-oxide (NMO; 0.90 ml, 3.84 mmol) and water (1.5 ml) successively. To dissolve the starting material completely, acetone (18 ml) was added and the reaction mixture was stirred overnight at room temperature, then 50% $Na_2S_2O_4$ (0.25 ml) was added with 5 min stirring. To the mixture were added Celite® (0.2 g) and activated charcoal (0.2 g) with 5 min stirring. Insoluble materials were filtered off and the filtrate was concentrated in vacuo. To the residue was added 1N HCl (60 ml) and the aqueous layer was extracted with $CHCl_3$ (4 × 30 ml). The combined organic layer was washed with saturated aqueous $NaHCO_3$ (30 ml), dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography ($CHCl_3$:MeOH = 30:1) to give **21** (723 mg, 97%), which contained both the 2α,3α- and 2β,3β-dihydroxy isomers in a ratio of 82/18 based on ^{1}H NMR analysis. For the 2α,3α-dihydroxyisomer: δ 0.76 (3H, s, 19-H_3), 0.79 (3H, s, 18-H_3), 0.90 (6H, d, J = 6.6 Hz, 26-H_3 and 27-H_3), 1.15 (3H, s, 21-H_3), 1.30 (3H, s, acetonide), 1.41 (3H, s, acetonide), 2.68 (1H, dd, J = 12.6 and 2.8 Hz, 5α-H), 3.62 (1H, m, 22-H), 3.78 (1H, m, 2β-H), 4.05 (1H, m, 3β-H). For the 2β,3β-dihydroxyisomer: δ 0.79 (3H, s, 18-H_3), 0.90 (6H, d, J = 6.6 Hz, 26-H_3 and 27-H_3), 0.99 (3H, s, 19-H_3), 1.15 (3H, s, 21-H_3), 1.30 (3H, s, acetonide), 1.41 (3H, s, acetonide), 3.62 (1H, m, 22-H), 3.63 (1H, m, 3α-H), 4.04 (1H, m, 2α-H). Since the two isomers could not be separated by recrystallization, the mixture was subjected to the next step.

*2.1.2.12. (20R,22R)-2α,3α,20,22-Bis(isopropylidenedioxy)-5α-cholestan-6-one (**22**).* To mixture **21** (600 mg, 1.22 mmol) was added 2,2-dimethoxypropane (10 ml) and a catalytic amount of *p*-TsOH, and the mixture was stirred for 1 h at room temperature. After addition of $CHCl_3$ (100 ml), the organic layer was washed with saturated aqueous $NaHCO_3$ (20 ml) and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo and the residue was purified by flash column chromatography (hexane/EtOAc = 7:1–5:1) to yield compound **22** (379 mg, 59%) as a white solid. Mp 92–95 °C; $[\alpha]_D^{27}$ +33.9° (*c* 0.38, $CHCl_3$); ^{1}H NMR δ 0.68 (3H, s, 19-H_3), 0.78 (3H, s, 18-H_3), 0.90 (6H, d, J = 6.6 Hz, 26-H_3 and 27-H_3), 1.10–1.18 (2H, m), 1.14 (3H, s, 21-H_3), 1.20–1.37 (6H, m), 1.29 (3H, s, 20,22-acetonide), 1.34 (3H, s, 2α,3α-acetonide), 1.40–1.50 (3H, m), 1.41 (3H, s, 20,22-acetonide), 1.50 (3H, s, 2α,3α-acetonide), 1.53–1.60 (2H, m), 1.62–1.69 (2H, m), 1.74 (1H, m, 8β-H), 1.92 (1H, m, 16-H), 1.97–2.05 (2H, m), 2.00 (1H, t, J = 13.3 Hz, 7α-H), 2.12 (2H, m), 2.33 (1H, dd, J = 13.0 and 4.3 Hz, 7β-H), 2.53 (1H, dd, J = 12.6 and 3.8 Hz, 5α-H), 3.62 (1H, dd, J = 9.1 and 2.7 Hz, 22-H), 4.11 (1H, m, 2β-H), 4.28 (1H, m, 3β-H); ^{13}C NMR δ 12.67 (C-19), 13.03 (C-18), 21.03 (C-11), 21.70 (C-21), 22.51 (C-26 and C-27), 22.55 (C-4), 22.55 (C-16), 23.59 (C-15), 26.51 (2α,3α-acetonide), 26.73 (20,22-acetonide), 26.83 (C-23), 28.21 (C-25), 28.59 (2α,3α-acetonide), 29.02 (20,22-acetonide), 36.35 (C-24), 37.15 (C-8), 39.60 (C-12), 41.12 (C-1), 42.45 (C-10), 43.40 (C-13), 46.73 (C-7), 51.46 (C-5), 53.31 (C-9), 54.65 (C-17), 56.85 (C-14), 72.12 (C-3), 72.29 (C-2), 81.31 (C-22), 84.08 (C-20), 106.61 (20,22-acetonide), 107.84 (2α,3α-acetonide), 211.40 (C-6); analysis calculated for $C_{33}H_{54}O_5$: C, 74.67; H, 10.25. Found: C, 74.44; H, 10.00.

*2.1.2.13. (20R,22R)-2α,3α,20,22-Tetrahydroxy-5α-cholestan-6-one (**23**).* A mixture of compound **22** (317 mg, 0.58 mmol), EtOH (1.7 ml) and 60% AcOH (10 ml) was stirred for 6 h at 80 °C. After cooling the mixture to room temperature, the solvent was evaporated and further concentrated azeotropically with toluene (2 × 5 ml). The residue was purified by column chromatography ($CHCl_3$/MeOH = 30:1) to give compound **23** (218 mg, 81%) as a white solid. Mp 227–230 °C (MeOH); $[\alpha]_D^{29}$ +1.4° (*c* 0.49, EtOH); ^{1}H NMR δ 0.76 (3H, s, 19-H_3), 0.87 (3H, s, 18-H_3), 0.90 (3H, d, J = 6.5 Hz, 26-H_3/27-H_3), 0.91 (3H, d, J = 6.5 Hz, 26-H_3/27-H_3), 1.12–1.34 (5H, m), 1.21 (3H, s, 21-H_3), 1.38 (2H, m), 1.45 (2H, m), 1.55 (4H, m), 1.67–1.77 (4H, m), 1.80–1.87 (2H, m), 1.92 (1H, m, 4α-H), 2.00 (1H, t, J = 12.7 Hz, 7α-H), 2.16 (1H, m, 12β-H), 2.30 (1H, dd, J = 13.1 and 4.4 Hz, 7β-H), 2.67 (1H, dd, J = 12.5 and 2.6 Hz, 5α-H), 3.38 (1H, br.d, J = 9.0 Hz, 22-H), 3.77 (1H, m, 2β-H), 4.05 (1H, br.s, 3β-H); ^{13}C NMR δ 13.55 (C-19), 13.65 (C-18), 20.35 (C-21), 21.05 (C-11), 21.75 (C-16), 22.34 (C-26/C-27), 22.92 (C-26/C-27), 23.61 (C-15), 26.25 (C-4), 28.04 (C-25), 29.22 (C-23), 36.27 (C-24), 36.95 (C-8), 39.83 (C-12), 40.15 (C-1), 42.51 (C-10), 43.74 (C-13), 46.56 (C-7), 50.72 (C-5), 53.66 (C-9), 54.62 (C-17), 56.58 (C-14), 68.24 (C-2), 68.35 (C-3), 76.41 (C-22), 77.09 (C-20), 211.90 (C-6); analysis calculated for $C_{27}H_{46}O_5$: C, 71.96; H, 10.29. Found: C, 71.69; H, 10.04.

*2.1.2.14. (20R,22S)-20,22-Isopropylidenedioxy-5α-cholest-2-en-6-one (**28**).* Compound **15** (2.56 g, 6.11 mmol) was treated in the same way as that for compound **13** *via* compounds **24**, **25**, **26**, and **27** to yield compound **28** (1.45 g, 52%) as a white solid. Melting point 138 °C (EtOH); $[\alpha]_D^{19}$

+31.7° (*c* 0.76, $CHCl_3$); ^{1}H NMR δ 0.71 (3H, s, 19-H_3), 0.81 (3H, s, 18-H_3), 0.92 (6H, d, *J* = 6.6 Hz, 26-H_3 and 27-H_3), 1.13 (1H, m, 15-H), 1.16 (1H, m, 14α-H), 1.19 (1H, m, 24-H), 1.22 (1H, m, 12α-H), 1.29 (1H, m, 9α-H), 1.38 (1H, m, 17α-H), 1.37 (3H, s, acetonide), 1.39 (3H, s, 21-H_3), 1.41–1.52 (3H, m), 1.49 (3H, s, acetonide), 1.53–1.67 (5H, m), 1.75 (1H, dtd, *J* = 12.4, 10.5 and 4.1 Hz, 8β-H), 1.89 (1H, m, 16-H), 1.98 (1H, t, *J* = 12.7 Hz, 7α-H), 1.99 (2H, m, 1-H_2), 2.02 (1H, m, 4α-H), 2.15 (1H, dt, *J* = 12.5 and 3.2 Hz, 12β-H), 2.26 (1H, m, 4β-H), 2.34 (1H, m, 5α-H), 2.37 (1H, dd, *J* = 13.2 and 4.1 Hz, 7β-H), 3.70 (1H, dd, *J* = 9.1 and 3.9 Hz, 22-H), 5.57 (1H, m, 2-H), 5.69 (1H, m, 3-H); ^{13}C NMR δ 12.92 (C-18), 13.50 (C-19), 21.11 (C-11), 21.71 (C-4), 22.51 (C-26/C-27), 22.58 (C-26/C-27), 24.00 (C-16), 24.11 (C-15), 26.33 (acetonide), 26.46 (acetonide), 27.49 (C-23), 27.79 (C-21), 28.19 (C-25), 36.81 (C-24), 37.15 (C-8), 39.36 (C-1), 39.94 (C-10), 40.19 (C-12), 43.91 (C-13), 46.88 (C-7), 53.43 (C-9), 53.83 (C-5), 54.96 (C-17), 56.45 (C-14), 83.85 (C-20), 87.88 (C-22), 106.71 (acetonide), 124.47 (C-2), 124.98 (C-3), 211.91 (C-6). Analysis calculated for $C_{30}H_{48}O_3$: C, 78.90; H, 10.59. Found: C, 78.61; H, 10.33.

*2.1.2.15. (20R,22S)-2,3-Dihydroxy-20,22-isopropylidenedioxy-5α-cholestan-6-one (**29**).* Compound **28** (1.80 g, 3.94 mmol) was treated as described for compound **20** to give compound **29** (1.80 g, 93%) as a white solid. The ratio of 2α,3α- and 2β,3β-isomer was determined to be 83/17 based on ^{1}H NMR analysis. For the 2α,3α-dihydroxy isomer: δ 0.76 (3H, s, 19-H_3), 0.80 (3H, s, 18-H_3), 0.92 (6H, m, 26-H_3 and 27-H_3), 1.36 (3H, s, acetonide), 1.39 (3H, s, 21-H_3), 1.48 (3H, s, acetonide), 2.67 (1H, dd, *J* = 12.5 and 2.8 Hz, 5α-H), 3.70 (1H, dd, *J* = 9.1 and 3.8 Hz, 22-H), 3.77 (1H, m, 2β-H), 4.05 (1H, m, 3β-H). For the 2β,3β-dihydroxy isomer: δ 0.80 (3H, s, 18-H_3), 0.92 (6H, m, 26-H_3 and 27-H_3), 0.98 (3H, s, 19-H_3), 1.36 (3H, s, acetonide), 1.39 (3H, s, 21-H_3), 1.48 (3H, s, acetonide), 3.62 (1H, m, 3α-H), 3.70 (1H, dd, *J* = 9.1and 3.8 Hz, 22-H), 4.03 (1H, m, 2α-H). Since the two isomers could not be separated by recrystallization, the mixture was subjected to the next step.

*2.1.2.16. (20R,22S)-2α,3α,20,22-Bis(isopropylidenedioxy)-5α-cholestan-6-one (**30**).* Compound **29** (1.60 g, 3.26 mmol) was treated as described for compound **21** to give compound **30** (0.98 g, 58%) as a white solid. Mp 229 °C (EtOH/$CHCl_3$); $[\alpha]_D^{27}$ +35.9° (*c* 0.79, $CHCl_3$); ^{1}H NMR δ 0.68 (3H, s, 19-H_3), 0.79 (3H, s, 18-H_3), 0.91 (6H, d, *J* = 6.6 Hz, 26-H_3 and 27-H_3), 1.08–1.20 (3H, m), 1.22–1.42 (5H, m), 1.34 (3H, s, 2α,3α-acetonide), 1.36 (3H, s, 20,22-acetonide), 1.39 (3H, s, 21-H_3), 1.43–1.67 (7H, m), 1.48 (3H, s, 20,22-acetonide), 1.50 (3H, s, 2α,3α-acetonide), 1.74 (1H, m, 8β-H), 1.90 (1H, m, 16-H), 1.96–2.03 (2H, m), 2.00 (1H, t, *J* = 12.7 Hz, 7α-H), 2.33 (1H, dd, *J* = 13.0 and 4.3 Hz, 7β-H), 2.53 (1H, dd, *J* = 12.6 and 3.8 Hz, 5α-H), 3.70 (1H, dd, *J* = 9.0 and 3.9 Hz, 22-H), 4.10 (1H, m, 2β-H), 4.28 (1H, m, 3β-H); ^{13}C NMR δ 12.66 (C-19), 12.91 (C-18), 21.09 (C-11), 22.49 (C-26/C-27), 22.49 (C-4), 22.58 (C-26/C-27), 23.96 (C-16), 24.12 (C-15), 26.30 (20,22-acetonide), 26.44 (20,22-acetonide), 26.50 (2α,3α-acetonide), 27.47 (C-23), 27.77 (C-20), 28.18 (C-25), 28.58 (2α,3α-acetonide), 36.77 (C-24), 36.97 (C-8), 40.01 (C-12), 41.13 (C-1), 42.40 (C-10), 43.89 (C-13), 46.77 (C-7), 51.45 (C-5), 53.35 (C-9), 54.89 (C-17), 56.34 (C-14), 72.13 (C-3), 72.30 (C-2), 83.79 (C-20), 87.82 (C-22), 106.70 (20,22-acetonide), 107.83 (2α,3α-acetonide), 211.42 (C-6); analysis calculated for $C_{33}H_{54}O_5$: C, 74.67; H, 10.25. Found: C, 74.40; H, 10.18.

*2.1.2.17. (20R,22S)-2α,3α,20,22-Tetrahydroxy-5α-cholestan-6-one (**31**).* A solution of compound **30** (580 mg, 1.09 mmol) in 80% AcOH (10 ml) was stirred for 1 h at 80 °C. After cooling to room temperature, the solvent was evaporated and further concentrated azeotropically with toluene (2 × 5 ml). The residue was purified by recrystallization from $CHCl_3$/MeOH to give compound **31** (284 mg, 58%) as a white solid. Mp 258 °C; $[\alpha]_D^{30}$ −24.6° (*c* 0.95, EtOH); ^{1}H NMR [C_5D_5N/$CDCl_3$ = 9:1 (approximately)]; δ 0.83 (3H, s, 19-H_3), 0.89 (3H, d, *J* = 6.6 Hz, 26-H_3/27-H_3), 0.90 (3H, d, *J* = 6.6 Hz, 26-H_3/27-H_3), 1.09 (3H, s, 18-H_3), 1.09–1.16 (1H, m, 15-H), 1.19 (1H, m, 14α-H), 1.27–1.37 (4H, m), 1.51 (1H, m, 15-H), 1.58 (3H, s, 21-H_3), 1.58–1.66 (3H, m), 1.76–1.86 (2H, m), 1.93–2.04 (5H, m), 2.09 (1H, td, *J* = 11.9 and 5.5 Hz, 23-H), 2.19–2.31 (4H, m), 2.36 (1H, dd, *J* = 12.9 and 4.5 Hz, 7β-H), 3.07 (1H, dd, *J* = 12.5 and 2.8 Hz, 5α-H), 3.68 (1H, br.d, *J* = 9.9 Hz, 22-H), 4.00 (1H, m, 2β-H), 4.36 (1H, m 3β-H); ^{13}C NMR [C_5D_5N/$CDCl_3$ = 9:1 (approximately)]; δ 13.78 (C-19), 13.92 (C-18), 21.44 (C-11), 21.93 (C-21), 22.31 (C-16), 22.76 (C-26/C-27), 23.04 (C-26/C-27), 24.00 (C-15), 27.78 (C-4), 28.58 (C-25), 29.24 (C-23), 37.18 (C-8), 37.27 (C-24), 40.37 (C-12), 41.15 (C-1), 42.60 (C-10), 43.65 (C-13), 46.90 (C-7), 51.44 (C-5), 54.00 (C-9), 54.84 (C-17), 56.97 (C-14), 68.33 (C-2), 68.84 (C-3), 76.38 (C-20), 78.33 (C-22), 211.80 (C-6); analysis calculated for $C_{27}H_{46}O_5$: C, 71.96; H, 10.29. Found: C, 71.76; H, 10.30.

2.2. Bioassay

2.2.1. Ecdysteroid receptor binding activity

The inhibition of the incorporation of [^{3}H]PonA (150 Ci/mmol; ARC Inc., St. Louis, MO, USA) into Kc or Sf-9 cells was examined according to previously reported methods [8,13]. In brief, 400 μl of cell suspension (4 × 10^6 cells/ml) was incubated with 1 μl of DMSO solution of the test compound and 2 μl of the 70% ethanol solution of [^{3}H]PonA (0.5 nM, ca. 60000 dpm) for 30 min at 25 °C. The reaction mixture was immediately filtered through a glass filter (GF/F) and washed three times with water. The radioactivity collected in the filter was counted with a liquid scintillation counter (LSC) in 3 ml of Aquasol-2 (Packard Instrument Co., Meriden, CT, USA). The concentration

curve for the inhibition of the incorporation of [^{3}H]PonA was derived, and the concentration required to give 50% inhibition (IC_{50}) was determined by probit analysis [21]. The reciprocal logarithm of IC_{50}, pIC_{50}, was used as the index of the binding activity.

2.2.2. Molting hormonal activity

Molting hormonal activity was measured using cultured integument of *C. suppressalis* according to previously reported methods [14,15]. Briefly, integument excised from diapause larvae of *C. suppressalis* was incubated in Grace's medium containing a test compound for 24 h, then transferred to fresh medium containing *N*-acetyl-[^{14}C]glucosamine ([^{14}C]GlcNAc: 56 mCi/mmol; Moravek Biochemical Inc., Brea, CA, USA) and incubated for 3 days. The radioactivity incorporated into the cultured integument was measured by LSC in Aquasol-2 as described above. The 50% effective concentration (EC_{50} in M) was determined from the concentration-response curve for the incorporation of [^{14}C]GlcNAc to the integuments by probit analysis [21]. The reciprocal logarithm of EC_{50}, pEC_{50}, was used as the index of the molting hormonal activity.

2.2.3. Brassinolide activity

The brassinolide activity was measured using the dwarf rice lamina inclination assay reported by Fujioka et al. [16] under synergistic condition with indole-3-acetic acid (IAA). Briefly, the seeds of dwarf rice (*Oryza sativa* cv. Tan-ginbozu, provided by Sankyo Agro, Ltd., Tokyo, Japan) were soaked in 0.25% aqueous Benlate-T solution for 2 days at 30 °C under light. Uniformly germinated seeds were selected by coleoptile length (ca. 1–2 mm) and planted in 20 ml of 1% agar medium (ca. 10 mm thickness) in a beaker (50 ml volume), then incubated for 3 days under the same conditions described above. A 0.5 μl (25 nmol/plant) of EtOH solution of IAA (50 mM) was applied by micro-syringe to the top portion of the lamina before the application of each test compound. Various concentrations of test samples (0.5 μl) in EtOH were applied to the same part of the plant. After 2 days incubation under identical growth condition, the external angle between the lamina and its leaf sheath was measured using a circular protractor. Seven plants were planted in each beaker, and three sets were used for each concentration. Thus, each point is the average of 21 observations. In each assay, both negative (EtOH) and positive control (brassinolide, 1 nmol/plant) were tested. To normalize the measured angle, the angles obtained for positive and negative controls were set as 0 and 100%, respectively.

3. Results and discussion

3.1. Synthesis

Two stereoisomers of a CS/PonA hybrid compound, (20*R*,22*R*)- and (20*R*,22*S*)-isomers of 2α,3α,20,22-tetrahydroxy-5α-cholestan-6-one, were synthesized stereoselectively from pregnenolone in good yield. In our previous study, the alkoxylithium reagent derived from (±)-1-(benzyloxymethyl)oxy-4-methyl-1-(tri-*n*-butylstannyl)pentane was added to pregnenolone to introduce the side-chain [12]. However, the product was a mixture of two diastereomers with respect to C-22 and the yield was low. We therefore, developed a new synthetic method to stereoselectively construct the side-chain (Scheme 1).

Pregnenolone **6** was converted to its TBDMS ether **7** followed by the stereoselective addition of 4-methylpentynyllithum to the C-20 carbonyl group to give the propargylic alcohol **8**. The triple bond of **8** was semihydrogenated to afford the *Z*-allylic alcohol **9** in the presence of the Lindlar catalyst, then the C-22 double bond was regioselectively epoxidized under $VO(acac)_2$/TBHP system [22] to give a mixture of two compounds. They were separated using silica gel column chromatography to give the product **10** and a more polar product **11** in 32% and 45% yield from **6**, respectively. The oxirane ring of **10** was cleaved by $LiAlH_4$ [23] to give **12**. Since a part of the TBDMS ether was also cleaved under these conditions, the crude product was treated directly with TBAF to give the triol **13** in 98% yield from **10**. ^{1}H and ^{13}C NMR spectra of **13** were identical with those reported for (20*R*,22*R*)-dihydroxycholesterol [19], thus the configuration of **10** was determined to be (20*R*,22*R*,23*R*). In the same way, the epimeric triol **15** was derived from **11** in 84% yield. In this case, the ^{1}H NMR spectrum for the side-chain of **15** was identical with that reported for (20*R*,22*S*)-3β-acetoxycholest-5-ene-20,22-diol, [24] and the configuration of the parent epoxide **11** was further confirmed to be (20*R*,22*S*,23*S*). This epoxide opening reaction with $LiAlH_4$ proved to be quite regioselective and to proceed in high yield. Application of other reagents such as (i-Bu)$_2$AlH [25], $LiEt_3BH$ [26], $LiBH_4$/Ti(O-i-Pr)$_4$ [27] and $LiBH_4$/Et_3B [28] were unsuccessful in this transformation.

The subsequent functionalization was executed in a conventional way [29] (Scheme 2). The diol moiety of **13** was selectively protected as an acetonide followed by tosylation of the 3β-hydroxyl group. Hydroboration of the double bond at C-5 with $BH_3 \cdot SMe_2$ followed by oxidative workup gave the 6α-alcohol **18**. Introduction of a new double bond at C-2 was accomplished by elimination of *p*-TsOH with LiBr/Li_2CO_3 in DMF. Oxidation of the 6α-hydroxy group with the Jones reagent gave the 6-keto compound **20** in 37% yield from **13**. Catalytic dihydroxylation of the C-2 double bond of **20** using NMO as co-oxidant [30] gave a mixture of 2α,3α- and the undesired 2β,3β-diol in a ratio of 82/18 as determined from ^{1}H NMR analysis. These isomers could not be separated by column chromatography or recrystallization. However, their acetonide derivatives could be separated by column chromatography to give pure 2α,3α-acetonide **22** in 57% yield from **20**. The two acetonide groups at the A-ring and the side-chain were removed by acidic hydrolysis (60% AcOH, EtOH, 80 °C, 6 h) to give the desired (22*R*)-CS/PonA hybrid compound **23**

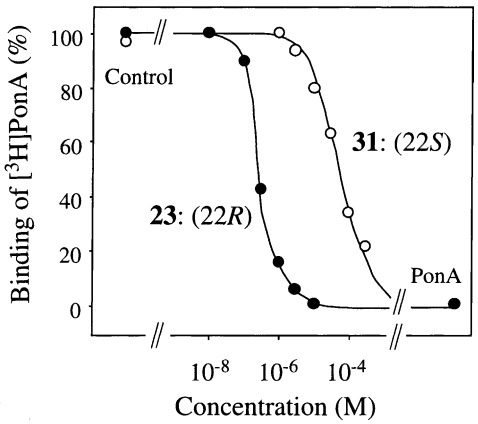


Fig. 2. Concentration–response curves of synthetic compounds (**23** and **31**) for the inhibition of [^{3}H]PonA binding in intact Kc cells. Data are shown as the average ± S.D. (n = 3).

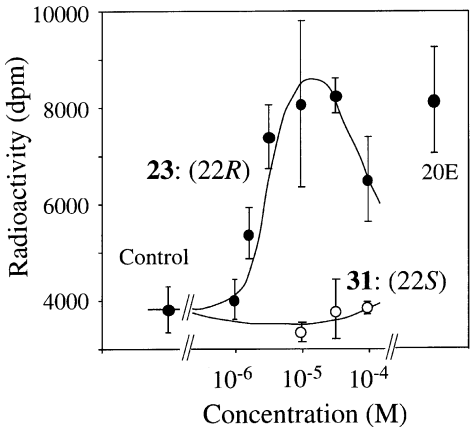


Fig. 3. Concentration-response curves of synthetic compounds (**23** and **31**) for the molting hormonal response in the cultured integument. Data are shown as the average ± S.D. (n = 3).

in 81% yield. The (20*R*,22*S*)-dihydroxycholesterol **15** was treated in the same way as **13** to give the (22*S*)-CS/PonA hybrid compound. The overall yields of the (22*R*)-isomer and the (22*S*)-isomer from the starting material **6** were 5.4% and 6.1% in 14 steps, respectively. Interestingly, the (20*R*,22*S*)-acetonide was hydrolyzed easier (80% AcOH, 80 °C, 1 h) than the (20*R*,22*R*)-isomer. Under these conditions, the (20*R*,22*R*)-acetonide of **22** was stable, while the 2α,3α-acetonide was completely removed. Similar differences in the susceptibility to hydrolysis between two epimeric acetonide compounds have been previously reported [31].

3.2. Inhibition of [^{3}H] PonA binding in Kc and Sf-9 cells

The concentration curves of the newly synthesized compounds for the competitive inhibition of [^{3}H]PonA incorporation to Kc cells are shown in Fig. 2. The experiment was repeated, and the average of the pIC_{50} values together with their standard deviations is listed in Table 1. A similar binding assay was performed using the Sf-9 cell line and the pIC_{50} was also determined. As shown in Table 1, compounds **23** and **31** were active in both cell lines, with pIC_{50} values ranging from 4.41 to 6.49. The (22*R*)-isomer **23** was 100 times more potent than (22*S*)-isomer with Kc cells, whereas the difference of the activity between the two isomers in Sf-9 cells was 35 times.

3.3. Molting hormonal activity

The concentration-response curves for the molting hormonal activity of these newly synthesized compounds are shown in Fig. 3, indicating that the (22*R*)-isomer evidenced ES-like activity, but the (22*S*)-isomer was not effective even at the highest concentration tested (100 μM). Molting hormonal activity in terms of pEC_{50} is listed in Table 1. Even though the activity of the (22*R*)-isomer is 1/100 that of PonA, it is three times higher than that of ecdysone (E). Voigt et al. [9] synthesized twenty-four BR/ES hybrid compounds and measured their ecdysteroid agonist activity and found that the compounds with (22*S*)-configuration positively responded as ecdysteroids. Among these ecdysteroid-active compounds, an EC_{50} value could only be determined for (22*S*,23*S*,24*R*)-3β,14α,22,23-tetrahydroxy-5α-ergost-7-en-6-one (**5**; Fig. 1), even though its potency was 1/13 that of E. Our CS/PonA compounds contain a 20-hydroxy group, but compounds synthesized by Voigt et al. lack this 20-hydroxy group [9]. According to Voigt et al., the

Table 1
Biological activity of newly synthesized CS/PonA hybrid compounds and ecdysteroids

Compounds	Binding activity (pIC_{50}; M)		Hormonal activity (pEC_{50}; M)
	Kc cells	Sf-9 cells	*C. suppressalis*
23 (20*R*,22*R*)	6.49 ± 0.11 (5)	6.44 ± 0.06 (2)	5.57 ± 0.08 (2)
31 (20*R*,22*S*)	4.41 ± 0.14 (2)	4.89 ± 0.24 (2)	< 4.00 (11.5%) (2)
Ecdysone	5.59[a]	5.63[b]	5.05[c]
20E	7.34[a]	6.78[b]	6.75[d]
PonA	8.89[a]	8.05[b]	7.53[d]

[a] Reference [13].
[b] Reference [8].
[c] Reference [12].
[d] Reference [15,32].

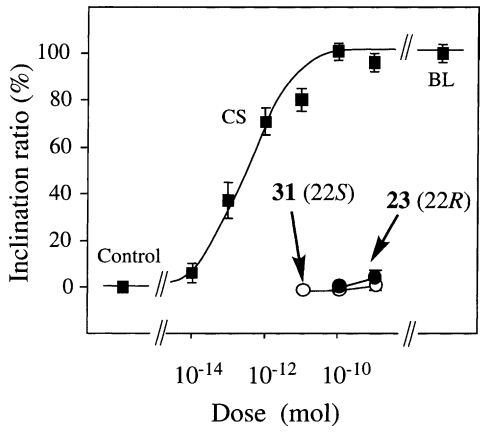


Fig. 4. Concentration-response curves of synthetic compounds (**23** and **31**) and CS for the bending of lamina in rice lamina inclination assay. Data are shown as the average ± S.D. (n = 21).

(22*S*)-configuration of the hybrid compound is essential for ecdysteroid activity, whereas the (22*R*)-isomer was the active component for CS/PonA hybrid compounds. Even though the absolute configurations of C-22 required for the molting hormonal activity are reversed between our compounds and Voigt's compounds, the stereostructure is essentially the same as depicted in Fig. 1: for Voigt's compounds, the hydroxyl group at C-23 reverses the configuration at C-22 by altering the priority sequence according to the Cahn–Ingold–Prelog rule.

3.4. Plant hormonal activity

The dose-response curve of CS against the inclination of rice lamina is shown in Fig. 4. The pEC_{50} value was 12.3 which is consistent with that previously determined in our laboratory [33]. We also examined the effect of CS/PonA hybrid compounds on the rice lamina inclination under identical conditions, however, both isomers were inactive even at very high doses as shown in Fig. 4. Thus, the presence of the hydroxyl group at C-20 was detrimental to activity.

In conclusion, we were able to synthesize both the (22*R*)- and (22*S*)-isomers of CS/PonA hybrid compounds stereoselectively, and measured their binding activity to ecdysteroid receptors and the molting hormonal activity in the cultured integument. The (22*R*)-isomer evidenced molting hormonal activity, but the (22*S*)-isomer was inactive, even though the (22*S*)-isomer showed the weak binding activity, being 1/35 to 1/100 that of the (22*R*)-isomer. Both isomers showed no plant hormonal activity at 1 nmol/plant.

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