STEROIDS 73 (2008) 1452–1464

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/steroids

Synthesis of ponasterone A derivatives with various steroid skeleton moieties and evaluation of their binding to the ecdysone receptor of Kc cells

Hirokazu Arai, Bunta Watanabe, Yoshiaki Nakagawa, Hisashi Miyagawa*

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

ARTICLE INFO

Article history:
Received 31 January 2008
Received in revised form
29 July 2008
Accepted 7 August 2008
Published on line 2 September 2008

Keywords:
Ecdysone agonists
Ponasterone A
Drosophila
Kc cells
Ecdysone receptor

ABSTRACT

A series of ponasterone A (PNA) derivatives with various steroid moieties were synthesized to measure their binding activity to the ecdysone receptors of *Drosophila* Kc cells. The activity of compounds was evaluated by determining the concentration required to give the 50% inhibition (IC_{50} in M) of the incorporation of [^{3}H]PNA to *Drosophila* Kc cells. Compounds with no functional groups such as OH and C=O group in the steroid skeleton moiety were inactive. By the introduction of functional groups such as the OH and the C=O group in the steroidal structure, these compounds became active. Some compounds containing the A/B-*trans* ring fusion, which is different from that (A/B-*cis*) of ecdysteroids were also active. The oxidation of CH_2 at 6-position to C=O, enhanced the activity 19 times, but the activity was erased by the reduction of oxo to OH group at 6-position. The activity was enhanced about 250 times by the conversion of A/B ring configuration from *trans* [(20R,22R)-2β,3β,20,22-tetrahydroxy-5α-cholestan-6-one: $pIC_{50} = 4.84$] to *cis* [(20R,22R)-2β,3β,20,22-tetrahydroxy-5β-cholestan-6-one: $pIC_{50} = 7.23$]. The latter *cis*-type compound which is the most potent among compounds synthesized in this study was equipotent to the natural molting hormone, 20-hydroxyecdysone, even though it is 1/50 of PNA.

1. Introduction

Insect molting is regulated by the molting hormone, 20-hydroxyecdysone (20-OH Ecd, **1** in Fig. 1). The biosynthesis of 20-OH Ecd has been studied intensively by Gilbert and co-workers [1,2]. It is well-known that insects have to intake cholesterol and other related steroidal compounds from their diet because they cannot construct the steroid skeleton. *In Drosophila*, ecdysone (Ecd; Fig. 1) is synthesized from cholesterol in the prothoracic gland and converted to 20-OH Ecd in other peripheral tissues, such as fat body and midgut, although 3-dehydroecdysone is secreted in *Bombyx mori* [3]. Recently, halloween genes coding the P450 hydroxylases that catalyze the final four steps of 20-OH Ecd biosynthesis have been identified in *Drosophila* [4–8].

Even though cholesterol has no receptor binding activity [9], Ecd and 2-deoxyecdysone have weak receptor binding and hormonal activities [10,11]. We also demonstrated that Ecd has a weak binding activity to *in vitro* translated ecdysone receptor proteins [12,13] and molting hormonal activity in tissue [14]. In addition, there are many steroidal compounds that have molting hormonal activity in plants [11] and a few animals, which are available in the web site EcdyBase (URL: http://ecdybase.org/). Among them, ponasterone A (PNA, **2** in Fig. 1), which is isolated from *Podocarpus Nakaii*, is well-known as the most potent ecdysteroid [15,16]. To date, many

Abbreviations: Ecd, ecdysone; 20-OH-E, 20-hydroxyecdysone; PNA, ponasterone A; CS, castasterone.

* *Corresponding author*. Tel.: +81 75 753 6117; fax: +81 75 753 6123.
E-mail address: naka@kais.kyoto-u.ac.jp (Y. Nakagawa).

0039-128X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2008.08.005

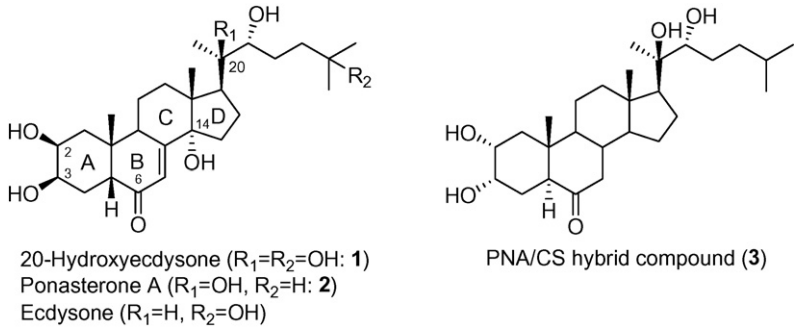


Fig. 1 – Structures of ecdysteroids and PNA/CS hybrid compound.

ecdysteroids have been identified and their hormonal activity has been evaluated in the cell-based assay [17,18]. The structure-activity relationship (SAR) of steroidal compounds is quantitatively analyzed using the three-dimensional quantitative SAR (3D QSAR) method [11,19].

In addition to ecdysteroids, non-steroidal compounds such as diacylhydrazines [20,21], acylaminoketones, [22,23] *N*-benzoyltetrahydroquinolines, [24] 3,5-di-*t*-butyl-4-hydroxybenzamides [25] and oxadiazolines [26] are reported to be ecdsyone agonists. We reported that the side chain moiety of ecdysteroids that is mimicked by the *t*-butylaminobenzoyl moiety of dibenzoylhydrazines is important to express the molting hormonal activity [27-29]. Moreover, crystal structure analyses of the ecdysone receptor proteins with ligand molecules demonstrated that steroidal and non-steroidal ligand molecules are only partially overlapping the ligand binding cavities of ecdysone receptor (EcR) of the tobacco budworm *Heliothis virescens* [30,31]. Thus, a steroid compound PNA and a nonsteroidal ecdysone agonist, a dibenzoylhydrazine-type compound (BYI06830) [30], are overlapped as shown in Fig. 2, which is constructed by fitting the conserved 4 amino acid residues in the ligand binding domain (LBD) of both the PNA- and the BYI06830-bound receptors (http://www.ncbi.nlm.nih.gov/) using the modeling software SYBYL 6.9 (Tripos, USA).

According to Voigt et al., few brassinosteroid/ecdysteroid hybrid compounds have weak ecdysone activity, while some other compounds are antagonists [32]. Previously we also synthesized the brassinosteroid/ecdysteroid hybrid compounds and found that the PNA/castasterone (PNA/CS: Fig. 3) hybrid compound **3** carrying the steroid moiety of a plant steroid hormone, CS, and the side chain of PNA, has a binding affinity to the EcRs and a hormonal activity against lepidopteran tissue [14,33]. Even though the steroid mother skeleton of ecdysteroids is replaceable with that of the brassinosteroids to show the ecdysone activity, the potency of the PNA/CS was 1/40 and 1/250 that of PNA against Diptera Kc and Lepidoptera Sf-9 cells, respectively. [33] This result indicates that the modification of the steroid mother skeleton may change the proper positioning of the side chain moiety of ecdysteroids in ligand binding pocket.

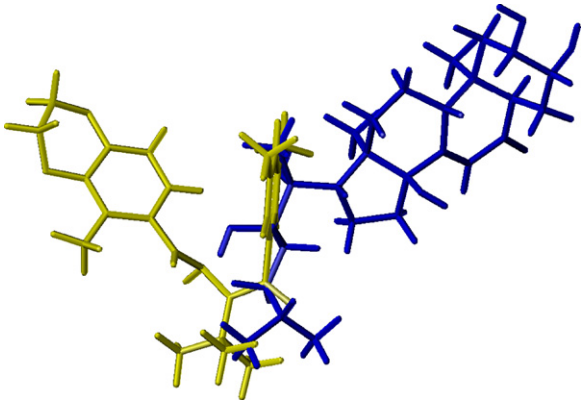


Fig. 2 – Superposition between PNA (black) and BYI06830 (gray).

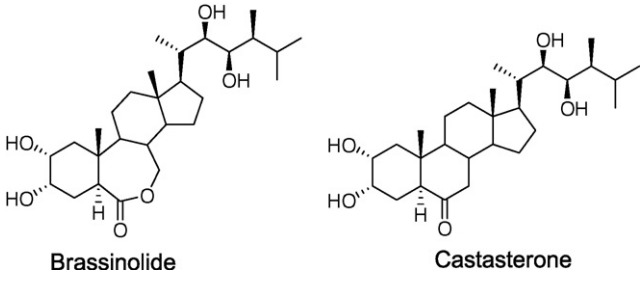


Fig. 3 – Structures of plant steroid hormones.

In this study, we synthesized a number of PNA analogs by modifying the mother skeleton moiety of PNA, and studied the SAR which would be fruitful to design new chemistry. The design of new compounds based on the structure of ecdysteroids will reach to the development of other ecdysone agonists possessing a broad insecticidal activity.

2. Experimental

2.1. *Synthesis*

Chemicals were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin, USA), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), and Nacalai Tesque Inc. (Kyoto, Japan). Dess–Martin periodinane was also prepared according to the conventional method [34,35]. Oven-dried glassware and positive Ar pres-

Table 1 – Binding activity of ecdysteroids against ecdysone receptor of Kc cells[a]

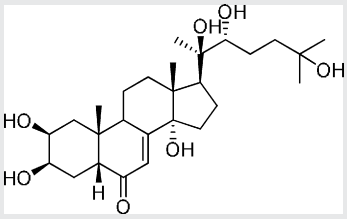
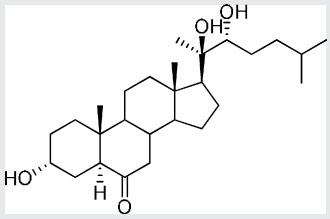
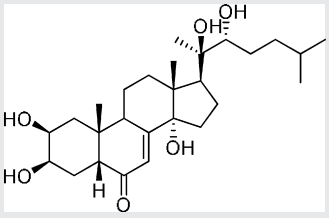
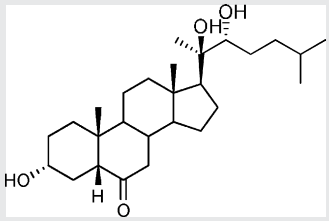
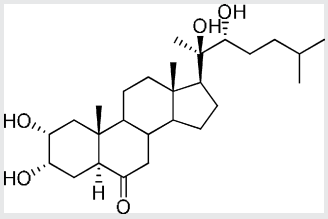
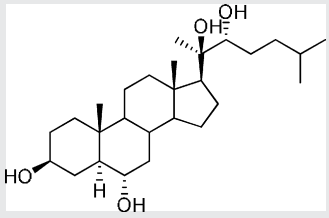
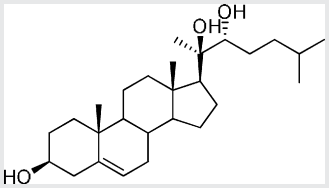
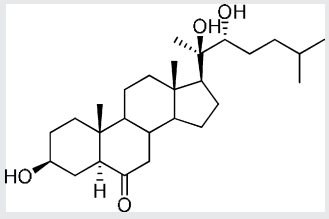
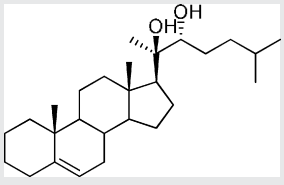
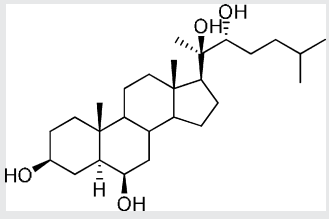
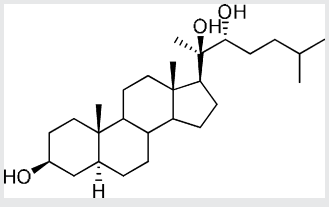
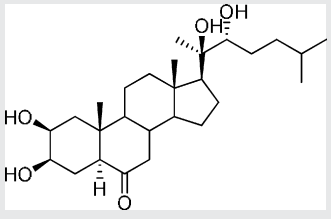
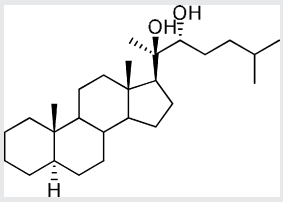
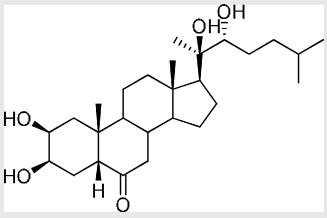
Compound No.[b]	Structure	pIC_{50} (M)	Compound No.	Structure	pIC_{50} (M)
1[c]		7.34[f]	20		6.10 ± 0.18
2[d]		8.89[f]	21		4.05 ± 0.27
3[e]		6.49[g]	24		<3.61 (42%)
4		4.38 ± 0.28	26		5.02 ± 0.45
8		<3.61 (23%)	28		<3.61 (19%)
9		4.38 ± 0.17	32		4.84 ± 0.30

Table 1 (*Continued*)

Compound No.[b]	Structure	pIC_{50} (M)	Compound No.	Structure	pIC_{50} (M)
12		<3.61 (8%)	34		7.23 ± 0.10

[a] Values are mean ± standard deviation for two experiments. The values in parentheses are the inhibition % at the corresponding concentration.
[b] Corresponds to the compound number in the text and schemes.
[c] 20-Hydroxyecdysone (20-OH Ecd).
[d] Ponasterone A (PNA).
[e] PNA/CS hybrid compound.
[f] Ref. [36].
[g] Ref. [33].

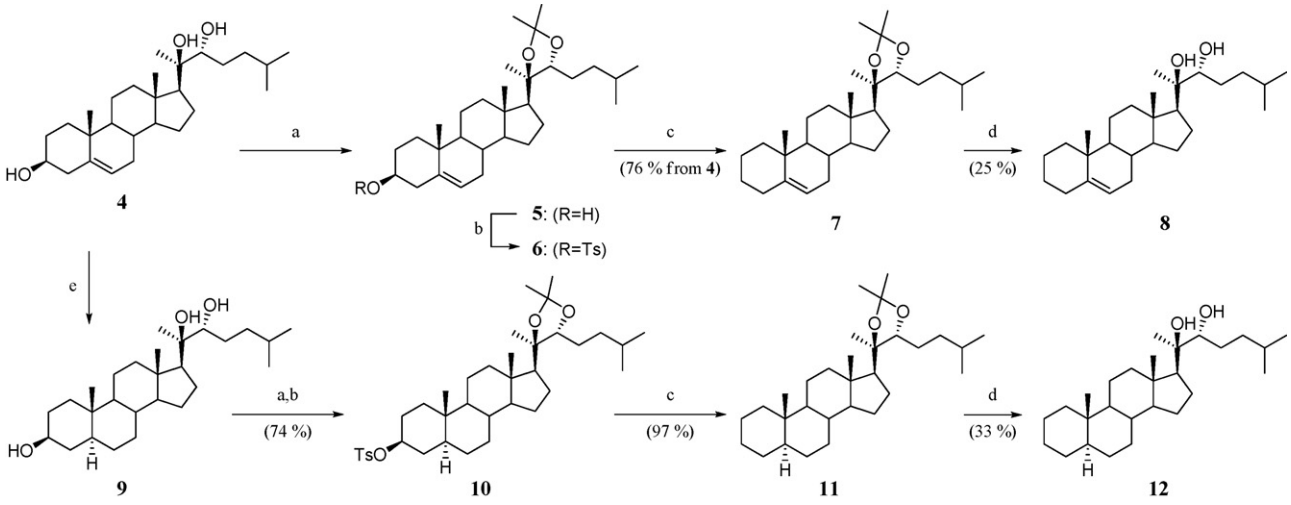
sure were used to keep anhydrous conditions. Anhydrous solvents were either commercially available or prepared conventionally in the laboratory. Flash column chromatography was conducted using Kieselgel 60 (Merck, Darmstadt, Germany) as the adsorbent. NMR spectra were recorded on a Bruker ARX-500 (500 MHz for ^1H and 125 MHz for ^{13}C) or Bruker AVANCE-400 (400 MHz for ^1H and 100 MHz for ^{13}C) or Bruker AC-300 (300 MHz for ^1H and 75 MHz for ^{13}C) in deuteriochloroform unless otherwise noted. Melting points (mp) were measured with a Yanagimoto melting point apparatus (Yanagimoto Seisakusho, Kyoto, Japan) and uncorrected. Optical rotations were measured on a JASCO P-1010 polarimeter (JASCO, Tokyo, Japan). Synthesized compounds listed in Table 1 were submitted to either elemental or high-resolution mass spectrum (HRMS) analysis. HRMS were recorded on a JEOL JMS 700 spectrometer (Tokyo, Japan). Elemental analyses were performed at the Microanalytical Center of Kyoto University. The synthetic methods are summarized in Schemes 1–4.

2.1.1. *(20R,22R)-20,22-Dihydroxycholest-5-ene* (4) (*Scheme 1*)

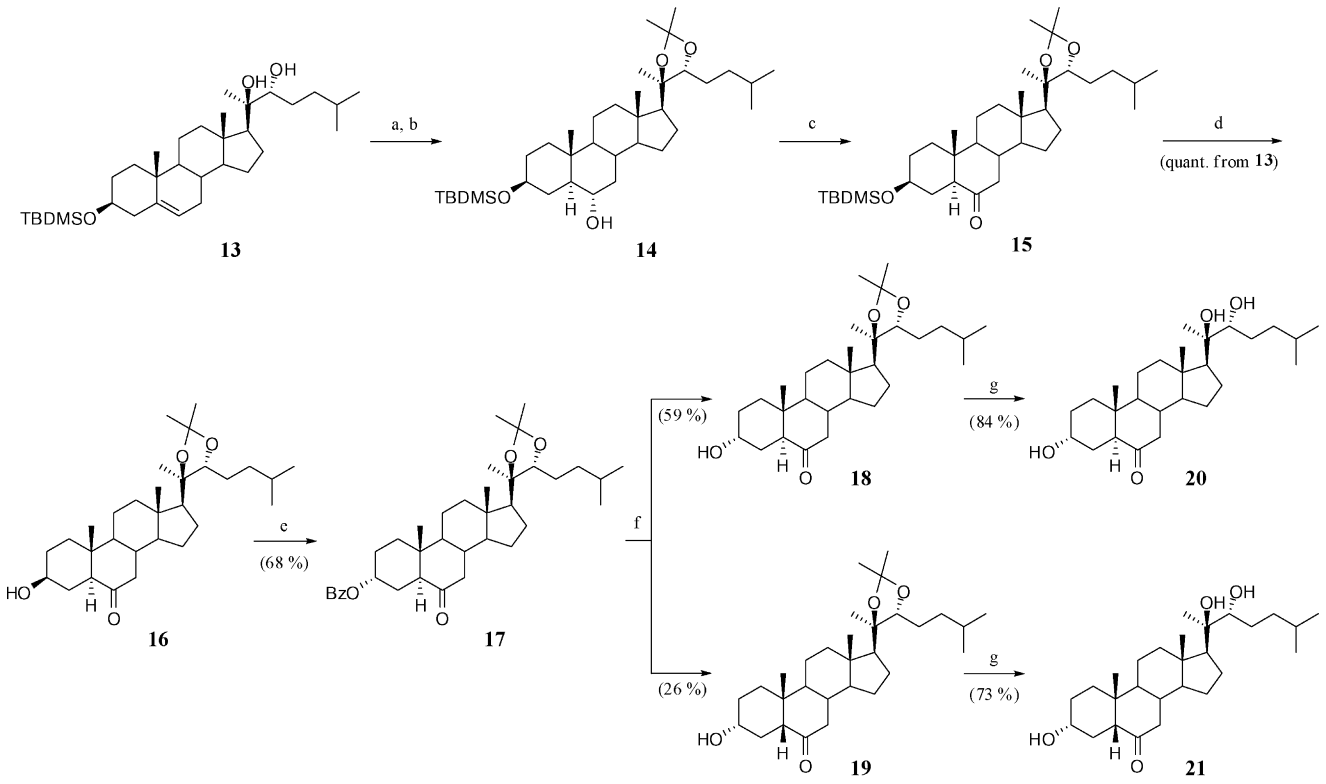
Compound **4** was synthesized from pregnenolone according to the procedure previously reported [33]. The NMR (500 MHz for ^1H and 125 MHz for ^{13}C) spectrum of compound **4** is follows: δ 0.89 (3H, s), 0.90 (3H, d, J = 6.3 Hz), 0.91 (3H, d, J = 6.3 Hz), 1.02 (3H, s), 1.22 (3H, s), 3.39 (1H, m), 3.53 (1H, m), 5.36 (1H, m); ^{13}C NMR (125 MHz) δ 13.57, 19.38, 20.38, 20.93, 21.92, 22.36, 22.94, 28.08, 29.15, 31.27, 31.61, 31.74, 36.32, 36.48, 37.23, 40.18, 42.25, 43.17, 50.04, 54.72, 56.67, 71.74, 76.39, 77.41, 121.54, 140.76; HRMS (FAB) m/z: $C_{27}H_{46}O_3Na$ $[M+Na]^+$, calcd 441.3345, found 441.3341.

2.1.2. *(20R,22R)-20,22-Isopropylidenedioxycholest-5-ene* (7) (*Scheme 1*)

A mixture of compound **4** (0.50 g, 1.19 mmol), 2,2-dimethoxypropane (0.29 mL, 2.38 mmol), and a catalytic amount of p-TsOH·H_2O in $CHCl_3$ (5 mL) was stirred for 1 min at room temperature. Since the reaction did not complete,

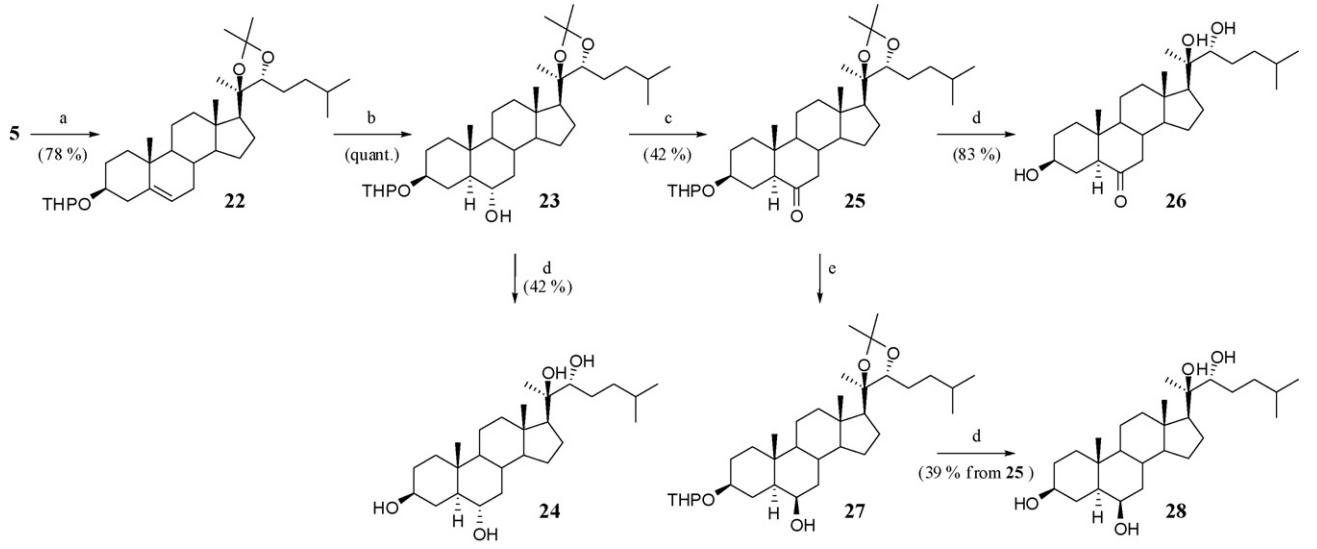


Scheme 1 – Reagents and conditions: (a) $(MeO)_2CMe_2$, p-TsOH·H_2O, $CHCl_3$, RT; (b) TsCl, pyridine, RT; (c) $LiEt_3BH$, THF, RT; (d) 60% AcOH/THF, 80 °C; (e) H_2, 10% Pd–C, EtOH, RT, 2 d.

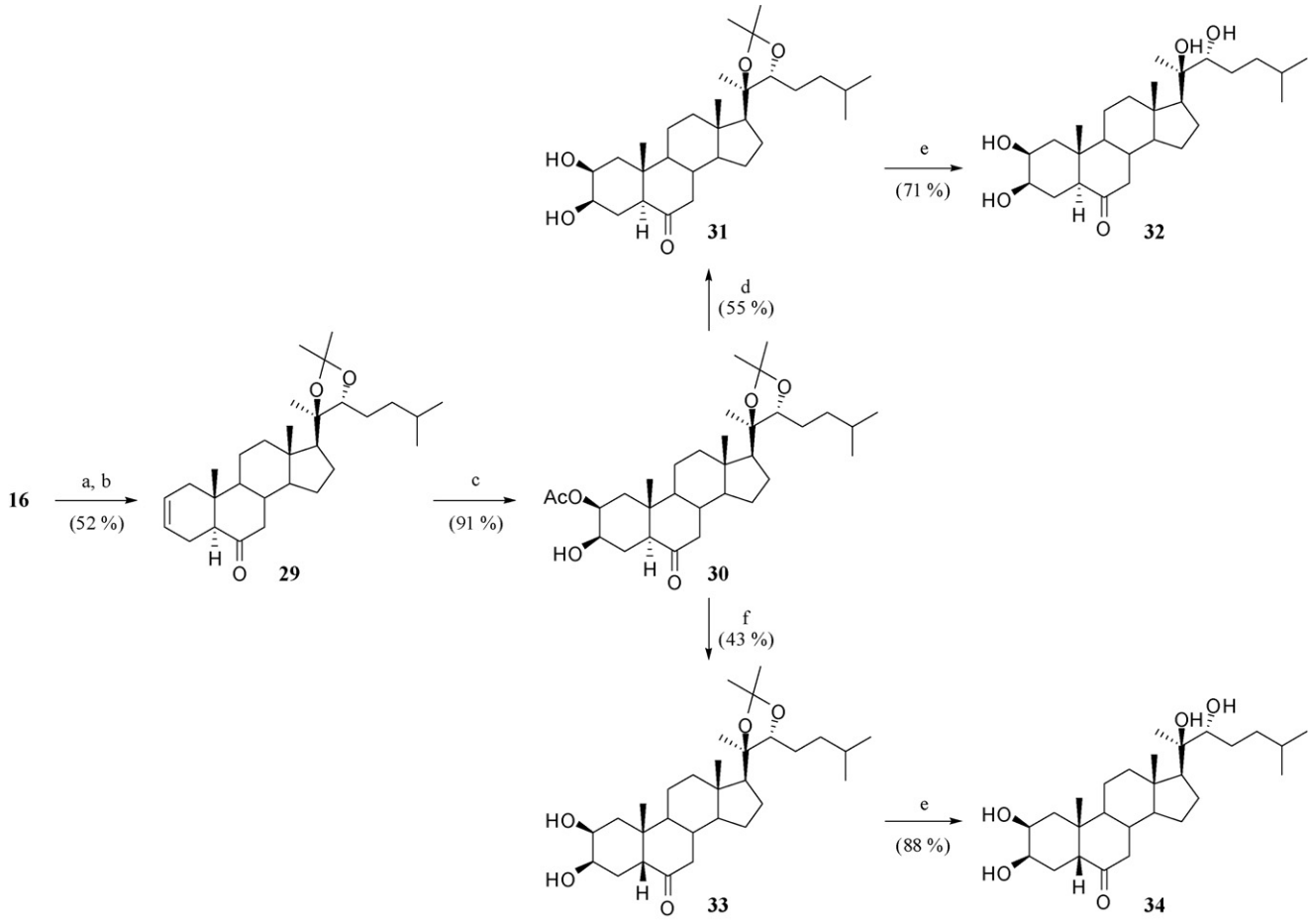


Scheme 2 – Reagents and conditions: (a) $(MeO)_2CMe_2$, *p*-TsOH•H_2O, $CHCl_3$, RT, 2 min; (b) BH_3•SMe_2, THF, RT, overnight; (c) Dess–Martin periodinane, CH_2Cl_2, RT, 2 h; (d) 80% AcOH, THF, 80 °C, 2 h; (e) benzoic acid, PPh_3, DEAD, THF, RT, overnight; (f) NaOH, MeOH, 60 °C, 30 min; (g) 60% AcOH, 80 °C.

additional 2,2-dimethoxypropane (0.29 mL, 2.38 mmol) was added and stirred for another 1 min. The mixture was diluted with $CHCl_3$ (50 mL), washed with saturated aqueous $NaHCO_3$ (20 mL) solution, and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to afford compound **5** (0.88 g) as a yellowish oil. This crude material was dissolved in anhydrous pyridine (8.8 mL). *p*-Toluenesulfonyl chloride was added to the solution (0.68 g, 3.57 mmol) and the reaction mixture was stirred overnight at room temperature. The mixture was added 3M HCl (88 mL) at 0 °C 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) solution, and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to give compound **6** (0.69 g) as a colorless



Scheme 3 – Reagents and conditions: (a) 2,3-Dihydro-2*H*-pyran, *p*-TsOH·H_2O, CH_2Cl_2, RT, 9 h; (b), BH_3·SMe_2, THF, RT, 12 h; (c) Dess–Martin periodinane, CH_2Cl_2, RT, 3 h; (d) 60% AcOH, EtOH, 80 °C; (e) $NaBH_4$, EtOH, 0 °C, 15 min then RT, 1 h.



Scheme 4 – Reagents and conditions: (a) MsCl, Et_3N, CH_2Cl_2, RT, 30 min; (b) $LiBr{\cdot}H_2O$, Li_2CO_3, DMF, reflux, 1.5 h; (c) (i) AcOAg, I_2, AcOH, RT, 1 h, (ii) H_2O, 90 °C, 1 h; (d) 1N NaOH, THF, RT, 1 h; (e) 60% AcOH, 80 °C, 4 h; (f) K_2CO_3, MeOH/H_2O, reflux, overnight.

solid. This crude material was dissolved in anhydrous THF (11.2 mL). $LiEt_3BH$ (1.05 M in THF, 3.20 mL, 3.36 mmol) was added to the solution at 0 °C under argon and the reaction mixture was stirred overnight at room temperature. Since the reaction did not complete, extra amount of $LiEt_3BH$ (1.05 M in THF, 5.33 mL, 5.60 mmol) was added at 0 °C and stirred for 1 day at room temperature. At this point, since **6** still remained, more $LiEt_3BH$ (1.05 M in THF, 5.33 mL, 5.60 mmol) was added at 0 °C. After stirring overnight at room temperature, 10% NaOH (8.0 mL) and 30% H_2O_2 (6.0 mL) were added dropwise successively at 0 °C, and stirred for 15 min at the same temperature. The mixture was diluted with water (40 mL) and extracted with EtOAc (4× 20 mL). The combined organic layer was washed successively with 20% $Na_2S_2O_3{\cdot}5H_2O$ (30 mL) and brine (15 mL), and dried over anhydrous $MgSO_4$. The solvent was evaporated and the crude product was purified by flash column chromatography (hexane/EtOAc = 300:1–200:1) to obtain compound **7** (0.40 g, 76% from **4**) as a colorless solid; ^{1}H NMR (500 MHz) δ 0.81 (3H, s), 0.90 (6H, d, J = 6.5 Hz), 1.00 (3H, s), 1.15 (3H, s), 1.30 (3H, s), 1.41 (3H, s), 3.63 (1H, dd, J = 9.3 and 2.7 Hz), 5.27 (1H, m).

2.1.3. *(20R,22R)-Cholest-5-ene-20,22-diol* (**8**) *(Scheme 1)*

A solution of compound **7** (0.35 g, 0.79 mmol) in 60% AcOH (7.5 mL) and THF (13 mL) was stirred 4 days at 80 °C. The solvent was removed *in vacuo*, and the residue was subjected to flash column chromatography (hexane/EtOAc = 9:1) to afford compound **8** (43 mg, 14%) as a colorless solid. Unreacted **7** was treated as above repeatedly and the total yield of **8** was 80 mg (25%), mp 136–137 °C (EtOH); $[\alpha]_D^{16}$ −20.9 (*c* 0.64, EtOH); ^{1}H NMR (400 MHz) δ 0.89 (3H, s), 0.90 (3H, d, J = 6.4 Hz), 0.91 (3H, d, J = 6.4 Hz), 1.00 (3H, s), 1.22 (3H, s), 3.39 (1H, m), 5.27 (1H, m); ^{13}C NMR (100 MHz) δ 13.59, 19.46, 20.37, 20.61, 21.92, 22.36, 22.51, 22.94, 23.91, 28.00, 28.08, 29.13, 31.20, 31.72, 32.84, 36.32, 37.51, 39.86, 40.25, 43.17, 50.49, 54.73, 56.77, 76.39, 77.47, 118.79, 143.74; HRMS (FAB) *m/z*: $C_{27}H_{46}O_2Na$ $[M+Na]^+$, calcd 425.3396, found 425.3398.

2.1.4. *(20R,22R)-5α-Cholestane-3β,20,22-triol* (**9**) *(Scheme 1)*

A mixture of compound **4** (0.94 g, 2.25 mmol) and 10% Pd–C (0.47 g) in EtOH (20 mL) was stirred for 2 days under hydrogen at room temperature. The catalyst was removed by filtration through Celite®. The filtrate was concentrated *in vacuo* and the residue was recrystallized from MeOH to yield compound **9** (0.57 g, 60%) as a colorless solid, mp 90–91 °C (MeOH); $[\alpha]_D^{27}$ +18.2 (*c* 0.50, $CHCl_3$); ^{1}H NMR (C_5D_5N, 500 MHz) δ 0.85 (3H, s), 0.95 (3H, d, J = 6.4 Hz), 0.96 (3H, d, J = 6.3 Hz), 1.19 (3H, s), 1.54 (3H, s), 3.77 (1H,

dd, J = 10.0 and 5.0 Hz), 3.88 (1H, m); ^{13}C NMR (C_5D_5N, 125 MHz) δ 12.58, 14.23, 21.26, 21.59, 22.61, 23.31, 24.46, 28.40, 29.24, 30.21, 32.40, 35.18, 35.84, 37.23, 37.61, 39.37, 41.02, 43.85, 45.31, 52.55, 54.73, 55.70, 56.91, 70.65, 76.58, 76.68; HRMS (FAB) m/z: $C_{27}H_{48}O_3Na$ $[M+Na]^+$, calcd 443.3501, found 443.3496.

2.1.5. *(20R,22R)-20,22-Isopropylidenedioxy-3β-(p-toluenesulfonyloxy)-5α-cholestane* (**10**) (*Scheme 1*)

A mixture of compound **9** (0.50 g, 1.19 mmol), 2,2-dimethoxypropane (0.72 mL, 5.95 mmol), and a catalytic amount of p-TsOH·H_2O in $CHCl_3$ (5 mL) was stirred for 1 min at room temperature. After addition of $CHCl_3$ (50 mL), the organic layer was washed with saturated aqueous $NaHCO_3$ solution (20 mL) and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to afford a yellowish oil (0.88 g). This crude product was dissolved in anhydrous pyridine (7.6 mL) and p-toluenesulfonyl chloride (0.68 g, 3.57 mmol) was added to the mixture. After stirring overnight at room temperature, the reaction mixture was added with 3M HCl (76 mL) at 0 °C and extracted with EtOAc (4× 30 mL). The combined organic layer was washed successively with brine (30 mL) and saturated aqueous $NaHCO_3$ solution (30 mL), and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to give compound **10** (0.54 g, 74% from **9**) as a colorless solid, which was used without further purification. ^{1}H NMR (500 MHz) δ 0.76 (3H, s), 0.78 (3H, s), 0.89 (6H, d, J = 6.6 Hz), 1.11 (3H, s), 1.29 (3H, s), 1.40 (3H, s), 2.44 (3H, s), 3.61 (1H, dd, J = 9.3 and 2.8 Hz), 4.42 (1H, m), 7.32 (2H, d, J = 8.2 Hz), 7.79 (2H, d, J = 8.2 Hz).

2.1.6. *(20R,22R)-20,22-Isopropylidenedioxy-5α-cholestane* (**11**) (*Scheme 1*)

$LiEt_3BH$ (1.05 M in THF, 2.51 mL, 2.64 mmol) was added at 0 °C to compound **10** (0.54 g, 0.88 mmol) in anhydrous THF (8.8 mL) under argon. The reaction mixture was stirred overnight at room temperature. Since the reaction did not complete, additional $LiEt_3BH$ (1.05 M in THF, 4.19 mL, 4.40 mmol) was added at 0 °C and the mixture was stirred overnight at room temperature. To the reaction mixture were added successively 10% NaOH (8.0 mL) and 30% H_2O_2 (6.0 mL) dropwise at 0 °C, and the mixture was stirred for 30 min at the same temperature. At this point, water (40 mL) was added and extracted with EtOAc (4× 20 mL). The combined organic layer was washed successively with 20% $Na_2S_2O_3$·$5H_2O$ (30 mL) and brine (15 mL), then dried over anhydrous $MgSO_4$. The solvent was evaporated and the crude product was purified by flash column chromatography (hexane/EtOAc = 500:1) to obtain the compound **11** (0.38 g, 97%) as a colorless solid. ^{1}H NMR (500MHz) δ 0.78 (6H, s), 0.90 (6H, d, J = 6.6 Hz), 1.13 (3H, s), 1.30 (3H, s), 1.41 (3H, s), 3.62 (1H, dd, J = 9.1 and 2.7 Hz).

2.1.7. *(20R,22R)-5α-Cholestane-20,22-diol* (**12**) (*Scheme 1*)

A solution of compound **11** (0.23 g, 0.52 mmol) in 60% AcOH (5.5 mL) and THF (11 mL) was stirred overnight at 80 °C. The solvent was evaporated and the residue was subjected to flash column chromatography (hexane/EtOAc = 9:1) to yield compound **12** (34 mg, 16%) as a colorless solid. Unreacted **11** was treated as above repeatedly and the total yield of **12** was 69 mg (33%), mp 122–123 °C (EtOH); $[\alpha]_D^{24}$ +19.5 (c 0.48, EtOH); ^{1}H NMR (400 MHz) δ 0.78 (3H, s), 0.86 (3H, s), 0.90 (3H, d, J = 6.4 Hz), 0.91 (3H, d, J = 6.4 Hz), 1.20 (3H, s), 3.38 (1H, m); ^{13}C NMR (100 MHz) δ 12.22, 13.78, 20.30, 20.67, 21.91, 22.15, 22.36, 22.92, 23.81, 26.80, 28.07, 29.00, 32.03, 34.81, 36.21, 36.31, 38.67, 40.53, 43.46, 47.02, 54.71, 54.82, 56.56, 76.38, 77.20, 77.49. Analysis calculated for $C_{27}H_{48}O_2$: C, 80.14; H, 11.96. Found: C, 79.91; H, 11.99.

2.1.8. *(20R,22R)-3β-Hydroxy-20,22-isopropylidenedioxy-5α-cholestan-6-one* (**16**) (*Scheme 2*)

A mixture of compound **13** (3.33 g, 6.25 mmol) that was derived from pregnenolone [33], 2,2-dimethoxypropane (1.53 mL, 12.5 mmol), and a catalytic amount of p-TsOH·H_2O in $CHCl_3$ (30 mL) was stirred for 1 min at room temperature. Since the reaction did not complete, additional 2,2-dimethoxypropane (0.77 mL, 6.24 mmol) was added and stirred for another 1 min. The reaction mixture was diluted with $CHCl_3$ (120 mL), washed with saturated aqueous $NaHCO_3$ solution (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to afford a colorless solid (4.78 g). This crude product was dissolved in anhydrous THF (50 mL). BH_3·SMe_2 solution (2.0 M in toluene, 12.5 mL, 25.0 mmol) was added to the solution at 0 °C under argon. The mixture was stirred overnight at room temperature. 10% NaOH (10 mL) and 30% H_2O_2 (10 mL) were added to the reaction mixture at 0 °C and this was stirred for 30 min at the same temperature. The reaction mixture was diluted with EtOAc (200 mL), washed successively with brine (50 mL) and 20% $Na_2S_2O_3$·$5H_2O$ (130 mL), and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to yield compound **14** (5.23 g) as a colorless solid. This crude material was dissolved in anhydrous CH_2Cl_2 (50 mL). Dess–Martin periodinane (4.76 g, 11.2 mmol) was added to the solution at 0 °C, and the mixture was stirred for 1 h at room temperature. Since the reaction was not completed, Dess–Martin periodinane (0.53 g, 1.25 mmol) was added again at 0 °C, and the mixture was stirred for another 1 h at room temperature. 20% $Na_2S_2O_3$·$5H_2O$/saturated aqueous $NaHCO_3$ (75 mL) solution was added to the mixture and this stirred for 30 min at room temperature. The mixture was diluted with CH_2Cl_2 (200 mL), and the separated organic layer was dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to give compound **15** (5.02 g) as a colorless solid. This crude material was dissolved in 80% AcOH (50 mL), and THF (10 mL), and the mixture was stirred for 2 h at 80 °C. The solvent was removed *in vacuo*, and the residue was subjected to flash column chromatography ($CHCl_3$/MeOH = 50:1) to yield compound **16** (2.96 g, quant. from **13**) as a colorless solid. ^{1}H NMR (400 MHz) δ 0.77 (3H, s), 0.80 (3H, s), 0.90 (6H, d, J = 6.6 Hz), 1.17 (3H, s), 1.30 (3H, s), 1.41 (3H, s), 2.21 (1H, dd, J = 12.4 and 2.6 Hz), 2.33 (1H, dd, J = 13.1 and 4.4 Hz) 3.58 (1H, m), 3.62 (1H, dd, J = 9.2 and 2.9 Hz).

2.1.9. *(20R,22R)-3α-Benzoyloxy-20,22-isopropylidenedioxy-5α-cholestan-6-one* (**17**) (*Scheme 2*)

Diethyl azodicarboxylate solution (40% in toluene, 1.44 mL, 3.16) mmol was added to a mixture of compound **16** (0.75 g, 1.58 mmol), triphenylphosphine (0.83 g, 3.16 mmol), and benzoic acid (0.39 g, 3.16 mmol) in anhydrous THF (10 mL) at 0 °C, and the mixture was stirred overnight at room temperature. After addition of silica gel (13 g), the solvent was removed *in vacuo* and residue was subjected to flash column chromatography (hexane/EtOAc = 10:1) to afford compound **16** (0.62 g, 68%)

as a colorless solid. ^{1}H NMR (400 MHz) δ 0.81 (6H, s), 0.91 (6H, d, J = 6.6 Hz), 1.15 (3H, s), 1.30 (3H, s), 1.41 (3H, s), 2.14 (1H, d, J = 12.0 Hz), 2.35 (1H, dd, J = 13.1 and 4.4 Hz), 2.67 (1H, dd, J = 12.4 and 3.0 Hz), 3.63 (1H, dd, J = 9.0 and 2.9 Hz), 5.38 (1H, t, J = 2.7 Hz), 7.45 (2H, m), 7.54 (1H, m), 8.02 (2H, m).

2.1.10. (20R,22R)-3α-Hydroxy-20,22-isopropylidenedioxy-5α-cholestan-6-one (18) (Scheme 2)

A mixture of compound **17** (0.62 g, 1.07 mmol) and NaOH (0.42 g, 10.5 mmol) in MeOH was stirred for 30 min at 60 °C. The solvent was removed *in vacuo*, and the residue was dissolved in $CHCl_3$ (50 mL). The organic layer was washed with saturated aqueous NH_4Cl solution (50 mL), and the separated aqueous layer was re-extracted with $CHCl_3$ (4× 20 mL). The combined organic layer was washed with brine (50 mL) and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* and the residue was subjected to flash column chromatography (hexane/EtOAc = 2:1) to obtain compound **18** (0.30 g, 59%) as a colorless solid. ^{1}H NMR (400 MHz) δ 0.74 (3H, s), 0.79 (3H, s), 0.90 (6H, d, J = 6.6 Hz), 1.15 (3H, s), 1.30 (3H, s), 1.41 (3H, s), 2.12 (1H, m), 2.31 (1H, dd, J = 13.0 and 4.5 Hz), 2.71 (1H, t, J = 7.9 Hz), 3.62 (1H, dd, J = 9.3 and 2.9 Hz), 4.17 (1H, br, s).

2.1.11. (20R,22R)-3α-Hydroxy-20,22-isopropylidenedioxy-5β-cholestan-6-one (19) (Scheme 2)

Compound **19** (0.13 g, 26%) was obtained in the course of preparation of compound **18** as described above as a more polar material than compound **18**. ^{1}H NMR (400 MHz) δ 0.79 (3H, s), 0.86 (3H, s), 0.91 (6H, d, J = 6.6 Hz), 1.15 (3H, s), 1.30 (3H, s), 1.41 (3H, s), 3.63 (1H, dd, J = 9.0 and 2.7 Hz), 3.64 (1H, m).

2.1.12. (20R,22R)-3α,20,22-Trihydroxy-5α-cholestan-6-one (20) (Scheme 2)

A solution of compound **18** (0.30 g, 0.63 mmol) in 60% AcOH (10 mL) was stirred for 7 h at 80 °C. The solvent was removed *in vacuo*, and the residue was subjected to flash column chromatography ($CHCl_3$/MeOH = 40:1) to obtain compound **20** (0.23 g, 84%) as a colorless oil. Recrystallization from EtOAc gave a colorless solid, mp 185–186 °C (EtOAc); $[\alpha]_D^{20}$ −5.3 (*c* 0.52, EtOH); ^{1}H NMR (400 MHz) δ 0.74 (3H, s), 0.88 (3H, s), 0.90 (3H, d, J = 6.5 Hz), 0.92 (3H, d, J = 6.5 Hz), 1.21 (3H, s), 2.31 (1H, dd, J = 13.0 and 4.5 Hz), 2.71 (1H, t, J = 7.9 Hz), 3.38 (1H, m), 4.17 (1H, br, s); ^{13}C NMR (100 MHz) δ 12.30, 13.66, 20.34, 20.93, 21.75, 22.34, 22.93, 23.59, 27.65, 28.05, 28.15, 29.20, 31.66, 36.28, 37.24, 39.95, 41.49, 43.76, 46.68, 51.69, 53.76, 54.65, 56.71, 65.41, 76.39, 77.14, 212.53; HRMS (FAB) *m/z*: $C_{27}H_{47}O_4$ $[M+H]^+$, calcd 435.3474, found 435.3472. Analysis calculated for $C_{27}H_{46}O_4$: C, 74.55; H, 10.74. Found: C, 74.61; H, 10.67.

2.1.13. (20R,22R)-3α,20,22-Trihydroxy-5β-cholestan-6-one (21) (Scheme 2)

A solution of compound **19** (0.13 g, 0.27 mmol) in 60% AcOH (10 mL) was stirred for 4 h at 80 °C. The solvent was removed *in vacuo*, and the residue was subjected to flash column chromatography ($CHCl_3$/MeOH = 40:1) to obtain compound **21** (85 mg, 73%) as a colorless oil. Recrystallization from EtOAc gave a colorless solid, mp 104–106 °C (EtOAc); $[\alpha]_D^{24}$ −24.8 (*c* 0.44, EtOH); ^{1}H NMR (400 MHz) δ 0.86 (3H, s), 0.87 (3H, s), 0.91 (3H, d, J = 6.5 Hz), 0.92 (3H, d, J = 6.4 Hz), 1.22 (3H, s), 3.38 (1H, m), 3.64 (1H, ddd, J = 15.4 and 10.4 and 4.9 Hz); ^{13}C NMR (100 MHz) δ 13.58, 20.33, 20.70, 21.83, 22.34, 22.90, 23.14, 23.63, 28.03, 29.21, 29.80, 34.30, 34.81, 36.26, 36.34, 37.90, 39.98, 40.05, 42.72, 43.83, 54.67, 56.79, 59.30, 70.09, 76.38, 77.08, 213.76; HRMS (FAB) *m/z*: $C_{27}H_{47}O_4$ $[M+H]^+$, calcd 435.3474, found 435.3483.

2.1.14. (20R,22R)-20,22-Isopropylidenedioxy-3β-(tetrahydropyran-2-yloxy)cholest-5-ene (22) (Scheme 3)

A mixture of crude compound **5** (2.27 g, 96%, 4.75 mmol), 2,3-dihydro-2*H*-pyrane (1.0 mL), and a catalytic amount of *p*-TsOH·H_2O in anhydrous CH_2Cl_2 (5 mL) was stirred for 9 h at room temperature. The reaction mixture was diluted with CH_2Cl_2 (25 mL), washed with saturated aqueous $NaHCO_3$ solution (10 mL), and dried over anhydrous Na_2SO_4. The solvent was evaporated, and the residue was subjected to flash column chromatography (hexane/EtOAc = 20:1) to afford compound **22** (2.00 g, 78%) as a colorless solid.

2.1.15. (20R,22R)-20,22-Isopropylidenedioxy-3β-(tetrahydropyran-2-yloxy)-5α-cholestan-6α-ol (23) (Scheme 3)

BH_3·SMe_2 solution (2.0 M in toluene, 7.2 mL, 14.4 mmol) was added to a solution of compound **22** (2.00 g, 3.68 mmol) in anhydrous THF (23 mL) at 0 °C under argon, and the mixture was stirred for 12 h at room temperature. 10% NaOH (7.2 mL) and 30% H_2O_2 (7.2 mL) were added to the reaction mixture at 0 °C and stirred for 15 min. The mixture was diluted with EtOAc (160 mL), successively washed with brine (50 mL) and 20% $Na_2S_2O_3$·$5H_2O$ (75 mL), and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to yield compound **23** (2.11 g, quant.) as a colorless solid, which was subsequently used without purification.

2.1.16. (20R,22R)-5α-Cholestane-3β,6α,20,22-tetraol (24) (Scheme 3)

A solution of compound **23** (1.05 g, 98%, 1.84 mmol) in 60% AcOH (12 mL) and EtOH (2 mL) was stirred for 6.5 h at 80 °C. The solvent was removed *in vacuo*, and the residue was subjected to flash column chromatography (2 times) to separate compound **24** from **23**. The latter was treated as above, and the combined **24** was further purified by recrystallization from EtOH to give a pure product (0.33 g, 42%) as a colorless solid, mp 132–134 °C (EtOH); $[\alpha]_D^{20}$ +31.9 (*c* 0.54, EtOH); ^{1}H NMR (C_5D_5N, 400 MHz) δ 0.93 (3H, s), 0.94 (3H, d, J = 6.5 Hz), 0.96 (3H, d, J = 6.4 Hz), 1.20 (3H, s), 1.56 (3H, s), 3.07 (1H, m), 3.76 (1H, m), 3.95 (1H, m), 5.09 (1H, d, J = 4.4 Hz), 5.91 (1H, s), 6.00 (1H, s), 6.13 (1H, s); ^{13}C NMR (C_5D_5N, 100MHz) δ 13.57, 13.78, 13.82, 14.22, 21.27, 21.58, 22.61, 22.69, 23.32, 24.51, 28.41, 30.22, 32.45, 33.82, 34.19, 36.62, 37.24, 38.16, 40.92, 43.83, 52.91, 54.44, 56.79, 68.78, 73.10, 76.57, 83.97; HRMS (FAB) *m/z*: $C_{27}H_{48}O_4Na$ $[M+Na]^+$, calcd 459.3450, found 459.3462.

2.1.17. (20R,22R)-20,22-Isopropylidenedioxy-3β-(tetrahydropyran-2-yloxy)-5α-cholestan-6-one (25) (Scheme 3)

Dess–Martin periodinane (0.86 g, 2.02 mmol) was added to a solution of compound **23** (1.05 g, 98%, 1.84 mmol) in anhydrous CH_2Cl_2 (16 mL) at 0 °C. After stirring for 2 h at room temperature, additional Dess–Martin periodinane (0.22 g, 0.50 mmol) was added at 0 °C, and the mixture was stirred for another 1 h

at room temperature. 20% $Na_2S_2O_3 \cdot 5H_2O$/saturated aqueous $NaHCO_3$ solution (10 mL) was added to the mixture and stirred for 5 min at room temperature. The mixture was diluted with CH_2Cl_2 (65 mL), and the separated organic layer was dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo*, and the residue was subjected to flash column chromatography (hexane/EtOAc = 7:1) to give compound **25** (0.43 g, 42%) as a colorless solid, mp 150-151 °C (EtOH); $[\alpha]_D^{27}$ −14.1 (*c* 0.50, EtOH); ^{1}H NMR (300 MHz) δ 0.77 (3H, s), 0.79 (3H, s), 0.90 (6H, d, *J* = 6.5 Hz), 1.14 (3H, s), 1.29 (3H, s), 1.41 (3H, s), 3.48 (1H, m), 3.56 (1H, m), 3.62 (1H, dd, *J* = 9.2 and 2.8 Hz), 3.90 (1H, m), 4.76 (1H, m).

2.1.18. *(20R,22R)-3β,20,22-Trihydroxy-5α-cholestan-6-one (26) (Scheme 3)*

A solution of compound **25** (1.73 g, 3.10 mmol) in 60% AcOH (24 mL) and EtOH (4 mL) was stirred for 8.5 h at 80 °C. After evaporation, the residue was subjected to flash column chromatography ($CHCl_3$/MeOH = 30:1) to separate compound **26** as a colorless solid and unreacted compound **25**. The latter was treated as above, and the total yield of compound **26** was 1.12 g (83%), mp 180–181 °C (EtOH); $[\alpha]_D^{20}$ −6.4 (*c* 0.52, EtOH); ^{1}H NMR (400 MHz) δ 0.76 (3H, s), 0.88 (3H, s), 0.90 (3H, d, *J* = 6.5 Hz), 0.90 (3H, d, *J* = 6.6 Hz), 1.21 (3H, s), 2.33 (1H, dd, *J* = 13.0 and 4.3 Hz), 3.38 (1H, m), 3.58 (1H, m); ^{13}C NMR (100 MHz) δ 13.65, 20.34, 21.38, 21.76, 22.34, 22.50, 22.90, 23.62, 28.05, 28.16, 29.21, 30.00, 30.66, 36.26, 36.64, 37.16, 39.92, 40.86, 43.77, 46.51, 53.86, 54.67, 56.78, 70.64, 76.36, 83.35, 210.66; HRMS (FAB) *m/z*: $C_{27}H_{47}O_4$ $[M+H]^+$, calcd 435.3474, found 435.3473.

2.1.19. *(20R,22R)-5α-Cholestan-3β,6β,20,22-tetraol (28) (Scheme 3)*

$NaBH_4$ (0.69 g, 18.3 mmol) was added to a solution of compound **25** (1.02 g, 1.83 mmol) in EtOH (20 mL) at 0 °C. After stirring for 15 min at the same temperature, the reaction mixture was stirred for 1 h at room temperature. 1M HCl (20 mL) was added to the mixture at 0 °C, and extracted with $CHCl_3$ (4× 10 mL). The combined organic layer was washed with saturated aqueous $NaHCO_3$ solution (20 mL) and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure to give compound **27** (1.10 g) as a colorless solid. This crude material was dissolved in 60% AcOH (12 mL) and EtOH (2 mL), and the mixture was stirred for 9 h at 80 °C. After evaporation, the residue was subjected to flash column chromatography ($CHCl_3$/MeOH = 15:1) to separate compound **28** and unreacted **27**. The latter was treated as above, and the total yield of **28** was 0.31 g (39%) as a colorless solid, mp 112-113 °C (EtOAc); $[\alpha]_D^{20}$ +12.1 (*c* 0.51, EtOH); ^{1}H NMR (300 MHz) δ 0.90 (3H, d, *J* = 6.3 Hz), 0.91 (3H, s), 0.91 (3H, d, *J* = 6.3 Hz), 1.04 (3H, s), 1.21 (3H, s), 3.38 (1H, m), 3.66 (1H, m), 3.82 (1H, d, *J* = 2.1 Hz); ^{13}C NMR (C_5D_5N, 100 MHz) δ 13.82, 14.23, 16.34, 21.28, 21.57, 22.61, 23.32, 24.61, 28.41, 30.23, 30.57, 36.13, 37.24, 39.29, 40.90, 43.95, 48.58, 53.16, 54.96, 55.75, 56.83, 71.22, 71.41, 76.58, 76.69; HRMS (FAB) *m/z*: $C_{27}H_{48}O_4Na$ $[M+Na]^+$, calcd 459.3450, found 459.3462.

2.1.20. *(20R,22R)-20,22-Isopropylidenedioxy-5α-cholest-2-en-6-one (29) (Scheme 4)*

Methanesulfonyl chloride (0.63 mL, 8.4 mmol) was added to a mixture of compound **16** (1.90 g, 4.0 mmol) and triethylamine (1.5 mL, 10.0 mmol) in anhydrous CH_2Cl_2 (15 mL) at 0 °C, and the reaction mixture was stirred for 20 min at room temperature. The mixture was diluted with CH_2Cl_2 (60 mL), washed with saturated aqueous $NaHCO_3$ solution (15 mL), and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to obtain a pale yellowish solid (2.26 g). This crude material was dissolved in anhydrous DMF (35 mL). $LiBr \cdot H_2O$ (4.24 g, 40.4 mmol) and Li_2CO_3 (2.99 g, 40.4 mmol) were added to the solution and the mixture was refluxed for 1.5 h. 1N HCl (50 mL) was added to the cooled mixture and this was extracted with $CHCl_3$ (4× 30 mL). The combined organic layer was successively washed with saturated aqueous $NaHCO_3$ solution (50 mL) and brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to obtain a pale yellowish oil, which was purified by flash column chromatography (hexane/EtOAc = 30:1) to yield compound **29** (0.95 g, 52%) as a colorless solid. ^{1}H NMR (400 MHz) δ 0.72 (3H, s), 0.81 (3H, s), 0.90 (6H, d, *J* = 6.6 Hz), 1.15 (3H, s), 1.31 (3H, s), 1.41 (3H, s), 3.62 (1H, dd, *J* = 9.2 and 3.0 Hz), 5.57 (1H, m), 5.68 (1H, m).

2.1.21. *(20R,22R)-2β-Acetoxy-3β-hydroxy-20,22-isopropylidenedioxy-5α-cholestan-6-one (30) (Scheme 4)*

Iodine (0.55 g, 2.18 mmol) was added to a mixture of compound **29** (0.95 g, 2.08 mmol) and silver(I) acetate (0.78 g, 4.68 mmol) in AcOH (35 mL) in portion under argon, and the reaction mixture was stirred for 1 h at room temperature. Water (37.4 μL, 2.08 mmol) was added to the mixture, and stirred for 1 h at 90 °C. After addition of NaCl (1.3 g), the mixture was cooled to room temperature. The insoluble materials were filtered off, and the filtrate was concentrated *in vacuo* to obtain a pale yellowish solid, which was purified by flash column chromatography ($CHCl_3$/MeOH = 50:1) to yield compound **30** (1.01 g, 91%) as a colorless solid. ^{1}H NMR (400 MHz) δ 0.79 (3H, s), 0.89 (3H, s), 0.90 (6H, d, *J* = 6.6 Hz), 1.14 (3H, s), 1.29 (3H, s), 1.41 (3H, s), 2.09 (3H, s), 2.17 (1H, dd, *J* = 12.1 and 2.8 Hz), 2.26 (1H, dd, *J* = 11.7 and 2.0 Hz), 2.33 (1H, dd, *J* = 13.2 and 4.5 Hz), 3.61 (1H, dd, *J* = 9.2 and 2.9 Hz), 3.67 (1H, m), 5.15 (1H, m).

2.1.22. *(20R,22R)-2β,3β-Dihydroxy-20,22-isopropylidenedioxy-5α-cholestan-6-one (31) (Scheme 4)*

A mixture of compound **30** (0.53 g, 1.0 mmol) in 1M NaOH (15 mL) and THF (8 mL) was stirred for 1 h at room temperature. After addition of saturated aqueous NH_4Cl solution (40 mL), the aqueous layer was extracted with $CHCl_3$ (4× 15 mL). The combined organic layer was washed successively with saturated aqueous NH_4Cl solution (30 mL) and brine (30 mL), and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to obtain a pale yellowish solid, which was purified by flash column chromatography ($CHCl_3$/MeOH = 40:1) to yield compound **31** (0.27 g, 55%) as a colorless solid. ^{1}H NMR (400 MHz) δ 0.80 (3H, s), 0.90 (6H, d, *J* = 6.6 Hz), 0.99 (3H, s), 1.14 (3H, s), 1.29 (3H, s), 1.41 (3H, s), 2.21 (1H, dd, *J* = 12.1 and 2.6 Hz), 2.33 (1H, dd, *J* = 13.1 and 4.5 Hz), 3.62 (1H, dd, *J* = 9.1 and 2.9 Hz), 3.63 (1H, m), 4.03 (1H, m).

2.1.23. *(20R,22R)-2β,3β,20,22-Tetrahydroxy-5α-cholestan-6-one (32) (Scheme 4)*

A solution of compound **31** (0.30 g, 0.61 mmol) in 60% AcOH was stirred for 4 h at 80 °C. The solvent was removed *in vacuo*, and the residue was purified by flash column chromatography ($CHCl_3$/MeOH = 20:1) to obtain a compound **32** (0.20 g, 71%)

as a colorless solid, mp 226–227 °C (hexane/EtOH); $[\alpha]_D^{21}$ +3.5 (*c* 0.52, EtOH); ^{1}H NMR (400 MHz) δ 0.88 (3H, s), 0.90 (3H, d, *J* = 6.5 Hz), 0.91 (3H, d, *J* = 6.5 Hz), 0.99 (3H, s), 1.21 (3H, s), 2.20 (1H, dd, *J* = 12.4 and 2.9 Hz), 2.32 (1H, dd, *J* = 13.1 and 4.2 Hz), 3.38 (1H, m), 3.64 (1H, m), 4.03 (1H, br, s); ^{13}C NMR (C_5D_5N, 100 MHz) δ 14.11, 15.81, 21.26, 21.89, 22.45, 22.62, 23.33, 24.13, 25.57, 28.41, 30.24, 36.85, 37.23, 40.55, 41.04, 43.59, 44.18, 46.70, 54.84, 55.49, 56.90, 57.93, 70.02, 72.15, 76.54, 210.53; HRMS (FAB) *m/z*: $C_{27}H_{47}O_5$ $[M+H]^+$, calcd 451.3423, found 451.3422.

2.1.24. *(20R,22R)-2β,3β-Dihydroxy-20,22-isopropylidenedioxy-5β-cholestan-6-one (33) (Scheme 4)*

A mixture of compound **30** (0.24 g, 0.45 mmol) and K_2CO_3 (2.0 g) in MeOH (60 mL) and water (10 mL) was refluxed overnight. The solvent was evaporated and water (50 mL) was added to the residue. The aqueous layer was extracted with $CHCl_3$ (1× 50 mL, 4× 20 mL), and the combined organic layer was washed with brine (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to obtain a pale yellowish solid, which was purified by flash column chromatography ($CHCl_3$/MeOH = 40:1) to yield compound **33** (95 mg, 43%) as a colorless solid· ^{1}H NMR (400MHz) δ 0.79 (3H, s), 0.90 (6H, d, *J* = 6.6 Hz), 0.91 (3H, s), 1.15 (3H, s), 1.29 (3H, s), 1.41 (3H, s), 2.43 (1H, dd, *J* = 12.9 and 4.7 Hz), 3.62 (1H, dd, *J* = 9.1 and 2.9 Hz), 3.78 (1H, m), 4.05 (1H, m).

2.1.25. *(20R,22R)-2β,3β,20,22-Tetrahydroxy-5β-cholestan-6-one (34) (Scheme 4)*

A mixture of compound **33** (0.14 g, 0.29 mmol) in 60% AcOH was stirred for 4 h at 80 °C. The solvent was removed *in vacuo*, and the residue was subjected to flash column chromatography ($CHCl_3$/MeOH = 20:1) to obtain compound **34** (0.12 g, 88%) as a colorless solid, mp 130–132 °C (EtOAc); $[\alpha]_D^{21}$ −46.1 (*c* 0.52, EtOH); ^{1}H NMR (400 MHz) δ 0.88 (3H, s), 0.90 (3H, d, *J* = 6.5 Hz), 0.91 (3H, d, *J* = 6.2 Hz), 0.92 (3H, s), 1.22 (3H, s), 2.44 (1H, dd, *J* = 12.9 and 4.5 Hz), 3.37 (1H, m), 3.78 (1H, m), 4.04 (1H, m); ^{13}C NMR (C_5D_5N, 100 MHz) δ 13.98, 21.18, 21.62, 22.46, 22.61, 23.21, 23.98, 28.40, 30.21, 33.31, 36.58, 37.15, 38.24, 40.54, 40.66, 40.75, 43.12, 44.10, 54.72, 55.42, 56.85, 67.52, 68.78, 76.49, 214.43; HRMS (FAB) *m/z*: $C_{27}H_{47}O_5$ $[M+H]^+$, calcd 451.3423, found 451.3422.

2.1.26. *Binding assay*

The inhibition of the binding of [^{3}H]PNA (85.2 Ci/mmol; ARC Inc., Carlsbad, CA, USA) to Kc cells was examined according to previously reported methods [9,36]. In brief, 400 μL of cell suspension (4×10^6 cells/ml) containing 1 μL of DMSO solution of the test compound and 2 μL of the 70% ethanol solution of [^{3}H] PNA (0.5 μM, ca. 60,000 dpm) was incubated for 30 min at 25 °C. The reaction mixture was immediately filtered through a glass filter (GF/F) and washed three times with water (1 mL) which was used to rinse the test tube. The filter was dried under infrared light and transferred to the vial for a liquid scintillation counter (LSC). The radioactivity collected in the filter was counted with LSC in 3 mL of Aquasol-2 (Packard Instrument Co., Meriden, CT, USA). The concentration-response curve for the inhibition of the [^{3}H]PNA binding was drawn for each compound. The concentration required to give 50% inhibition (IC_{50} in M) was determined by probit analysis [37] and the reciprocal logarithm of IC_{50}, pIC_{50}, was used as the index of the binding activity.

3. Results and discussion

3.1. *Chemistry*

The analogs with non-oxygenated steroidal mother skeleton moiety (**8** and **12**) were prepared according to the procedure of Scheme 1. Since there are three OH groups in the starting material (**4**), protections of OH are sometimes needed for certain reactions. The 20,22-diol moiety of compound **4** [33] was protected as acetonide and 3β-hydroxyl group was tosylated to give compound **6**. Even though $LiEt_3BH$ was used to remove the tosyl group of **6**, large amounts of $LiEt_3BH$ (13 equiv.) and a long reaction time (6 days) were required to obtain compound **7** in a favorable yield. The hydrolysis of compound **7** by 60% acetic acid/THF was quite sluggish, and the yield of compound **8** was low (25%) in spite of the repetition of the hydrolytic reaction. The double bond existing in the B-ring of compound **4** was hydrogenated to lead compound **9** using 10% Pd–C, which was then converted to **12** using a similar procedure to that used for the preparation of compound **8** from compound **5**. Other acids such as $BiCl_3$, trifluoroacetic acid, HBr, and HCl were used to hydrolyze acetonide, but the satisfactory result was not obtained.

The syntheses of 3α-OH analogs (**20** and **21**) are summarized in Scheme 2. The diol moiety of compound **13** was protected using dimethoxypropane, and the acetonide was submitted to hydroboration of the double bond at C-5 with $BH_3{\cdot}SMe_2$ obtaining the 6α-alcohol **14**. Since the addition of $BH_3{\cdot}SMe_2$ to double bond is sterically hindered by the presence of the methyl group (C19) at C-10 position, the stereochemistry of OH at C6 of **24** should be a alpha configuration having 6β-H[38,39]. Compound **28** with 6α-H is derived from the corresponding ketone **25** by reducing the carbonyl group carrying minor byproduct **24**.

The 6α-OH group of **14** was oxidized to a ketone with Dess–Martin periodinane, and the TBDMS group, which is used to protect the 3-OH group was selectively removed without hydrolyzing acetonide under the moderate acid condition (80% AcOH, THF, 80 °C). The yield of compound **16** from compound **13** was quantitative. The stereochemistry inversion of the 3β-OH group was achieved by a Mitsunobu reaction affording compound **17** in 68% yield. By hydrolyzing compound **17** under basic condition A/B *trans* (**18**) and A/B *cis* (**19**) were obtained in 59% and 26% yield, respectively. The stereochemistry of the A/B-ring fusion was determined by NMR. Even though the corresponding compounds have not been reported to date, the chemical shifts of H-5α of **32** and H-5β of **34** are thought to be similar to the analogous compounds such as brassinosteroids shown in Fig. 4. [40] The chemical shift of 5β-H of **35** (A/B *cis*) is 2.41 ppm (dd, *J* = 10 and 5 Hz), and those of 5α-H of **36** and **37** (A/B *trans*) are 2.21 ppm (dd, *J* = 11.7 and 3 Hz) and 2.22 ppm (dd, *J* = 11.7 and 3 Hz), respectively. Thus, the A/B ring fusion of **32** and **34** are assigned to be *trans* and *cis*, respectively. The similar shifts of H-NMR for H-5 are also reported for 22,23-epoxyecdysteroids (i.e. 2.42 ppm; *J* = 12 and 3.5 Hz for 5α-H; 3.02 ppm, *J* = 12 and 3 Hz for 5β-H). [41]

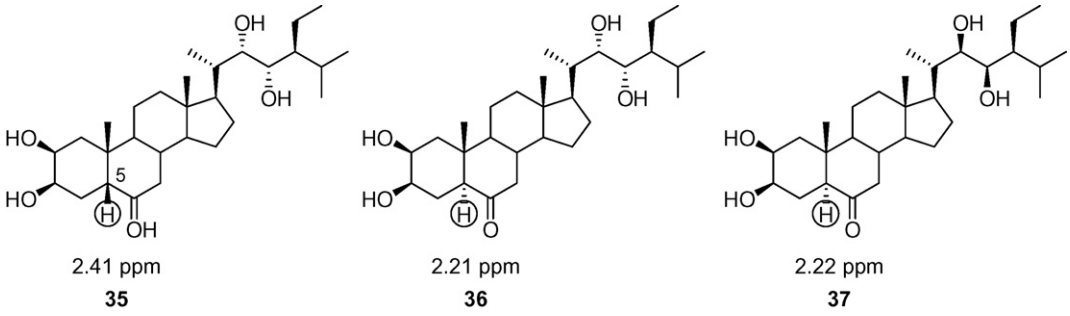


Fig. 4 – H NMR chemical shifts (δ) for Hα or Hβ at C5 for A/B *cis* and *trans* compounds. See Ref. [40].

The compounds **18** and **19** were hydrolyzed to compounds **20** and **21** in fairly good yields (84% and 73%) under the condition with 60% acetic acid in THF.

Compound **5** was used as starting material to synthesize 3β-OH analogs (**26** and **28**), as shown in Scheme 3. The 3β-OH group of compound **5** was protected by the THP group (compound **22**) with a 78% yield. Since the protection of OH by THP is not strong, both THP and isopropylidene groups compound **23** were easily removed using 60% acetic acid with a 42% yield. Compound **22** was converted to compound **23** quantitatively by the hydroboration of the double bond of the B-ring. Compound **23** was oxidized to compound **25** using Dess–Martin periodinane with a 42% yield, and compound **25** was hydrolyzed to compound **26** with a 83% yield. Compound **25** was also converted to compound **28**, in which the 6-keto group was reduced to the 6β-OH group with $NaBH_4$. The acid hydrolysis of compound **27** afforded compound **28**.

The analogs with the 2β,3β-diol moiety were prepared from compound **16**, as shown in Scheme 4. After mesylating the 3β-OH group of compound **16**, MsOH was eliminated to afford compound **29** with a 52% yield, by refluxing in DMF with a combination of $LiBr{\cdot}H_2O$ and Li_2CO_3. Woodward oxidation of compound **29** gave compound **30** with a 91% yield. By the brief treatment of compound **30** with 1 M NaOH in THF, the A/B *trans* steroid **31** was obtained in 55% yield, and hydrolyzed to compound **32** in 71% yield under acidic condition. On the other hand, by hydrolyzing compound **30** with K_2CO_3 in boiling aqueous MeOH overnight, the A/B *cis* compound **33** was obtained in 43% yield. This was then hydrolyzed to compound **34** under acidic condition.

3.2. *Receptor binding activity*

The binding activity of newly synthesized steroidal compounds is listed in Table 1. Compounds **8** and **12**, without functional groups such as OH and C=O group in the steroid skeleton were inactive. Introduction of the OH group at the 3-position of the steroidal skeleton elevated the activity to a measurable level ($pIC_{50} = 4.38$ for **4** and **9**), even though the configuration of the A/B ring fusion is different from that of the ecdysteroids. The additional modification of the B-ring moiety where CH_2 was oxidized to C=O at the C6 position increased the activity about 10 times (**26** *vs.* **9**). Interestingly, the activity was lost by the reduction of the oxo to the hydroxyl group (**26** *vs.* **24** or **28**). Further hydroxylation at 2-position had no effect on the activity (**32** *vs.* **26**). The conversion of A/B *trans* configuration to the *cis* enhanced the activity 250 times (**34** *vs.* **32**); the activity of **34** was equivalent or slightly higher than that of the insect molting hormone, 20-OH Ecd ($pIC_{50} = 7.34$).

In the above structure-activity relationship (SAR), the effect of the configuration of the 3-OH group on the activity was somewhat curious. Even though the configurations of the 3-OH group of 20-OH Ecd and PNA are both β, the activity of the compound **26** with 3β-OH group was 10 times lower than its 3α-epimer (compound **20**). This was also the case for the compounds carrying the *cis*-2,3-dihydroxy groups, where compound **32** carrying the 3β-OH group was about 50 times less potent than the (2α, 3α)-compound (**3**). The apparent inconsistency in these data is likely explained by the difference in the configuration of the A/B ring fusion between the series of tested compounds in this study and the natural ecdysteroids. As illustrated in Fig. 5, the 3-OH groups of compounds **20** and **3** are axially positioned to be located in a space close to that occupied by the 2,3-diOH groups of ecdysteroids, such as 20E and PNA. By contrast, the 3β-OH groups of compounds **26** and **32** are away from the 2,3-diOH groups of ecdysteroids. Conversely, it is suggested that the location of a hydrogen bond forming group in this space is very important for the molecular interaction between the steroid compound and the EcR.

As the compounds without functionality in the steroid skeleton were inactive, it is obvious that the properly located functional groups play a very important role in the binding of the steroidal ligand to its receptor. However, as indicated by the difference in the potency between the 3-OH epimers,

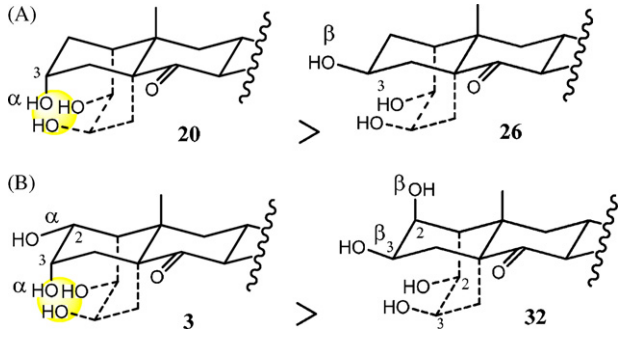


Fig. 5 – Superposition of A/B ring moieties of ecdysteroids on the corresponding moiety of 20-OH Ecd (broken line). The sign ">" indicates the difference of the activity. (A) 3-Hydroxy analogs (20 *vs.* 26) (B) 2,3-dihydroxyanalogs (3 *vs.* 32).

proper orientation of each functional group is also important. The positional relationship among the functional groups on the steroidal skeleton is considered to be highly fixed due to its rigid structure, and it is very likely that these fixed functionalities are important not only for the high affinity of the ligand-receptor interaction, but also for the capacity to cause some critical conformational change of the receptor molecule to exert its function, by anchoring the specific sites on the protein molecule to each other. In this regard, it is also interesting to note that the activity of PNA (25-dehydroxy 20-OH Ecd) and E (Fig. 1) are greatly different (about 2000 times), in spite of having the same number of functional groups (four OH and one C=O) at the same positions of the steroidal mother skeleton (A, B and C/D rings; Fig. 1). The structural difference between these molecules lies in the presence or absence of the OH group in the side chain, and therefore, it is suggested that the functionality in such a less rigid structure can also greatly affect the interaction between the firm steroidal moiety and the receptor, as a result of its own interaction with a specific moiety on the receptor.

4. Conclusion

It was shown that the receptor binding activity changes with the modification of the steroid mother skeleton. Based upon this SAR study, the number of OH and C=O groups that are able to form hydrogen bonds between ligand molecules and receptor proteins is important for the receptor-binding activity. The QSAR for ligand-receptor binding is currently in progress using the ligand-receptor binding model that was constructed for DmEcR. As stated above, even though diacylhydrazine-type compounds are specific to Lepidoptera due to the partial overlapping to ecdysteroid molecules (Fig. 2), the compounds which are superposable onto ecdsysteroids should evidence broad insecticidal specificity. Therefore, the QSAR study should be helpful in the design of new ecdysone agonists that have broad spectrum insecticidal.

Acknowledgements

This study was supported, in part, by the 21st Century COE program for Innovative Food and Environmental Studies Pioneered by Entomomimetic Sciences, from Ministry of Education, Culture, Sports, Science and Technology of Japan. A part of experiments was performed in the RI center of Kyoto University.

REFERENCES

[1] Gilbert LI, Warren JT. A molecular genetic approach to the biosynthesis of the insect steroid molting hormone. Vitam Horm 2005;73:31–57.
[2] Ono H, Rewitz KF, Shinoda T, Itoyama K, Petryk A, Rybczynski R, et al. Spook and Spookier code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. Dev Biol 2006;298:555–70.
[3] Kiriishi S, Rountree DB, Sakurai S, Gilbert LI. Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3 beta-reductase mediated conversion to ecdysone in representative insects. Experientia 1990;46:716–21.
[4] Chavez VM, Marques G, Delbecque JP, Kobayashi K, Hollingsworth M, Burr J, et al. The Drosophila disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. Development 2000;127: 4115–26.
[5] Niwa R, Matsuda T, Yoshiyama T, Namiki T, Mita K, Fujimoto Y, et al. CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of Bombyx and Drosophila. J Biol Chem 2004;279:35942–9.
[6] Petryk A, Warren JT, Marques G, Jarcho MP, Gilbert LI, Kahler J, et al. Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc Natl Acad Sci USA 2003;100:13773–8.
[7] Warren JT, Petryk A, Marques G, Parvy JP, Shinoda T, Itoyama K, et al. Phantom encodes the 25-hydroxylase of Drosophila melanogaster and Bombyx mori: a P450 enzyme critical in ecdysone biosynthesis. Insect Biochem Mol Biol 2004;34:991–1010.
[8] Warren JT, Petryk A, Marques G, Jarcho M, Parvy JP, Dauphin-Villemant C, et al. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of Drosophila melanogaster. Proc Natl Acad Sci USA 2002;99:11043–8.
[9] Nakagawa Y, Minakuchi C, Ueno T. Inhibition of [^{3}H]ponasterone A binding by ecdysone agonists in the intact Sf-9 cell line. Steroids 2000;65:537–42.
[10] Frisrom JW, Yund MA. Characteristics of the action of ecdysones on Drosophila imaginal discs cultured in vitro. In: Maramorosch K, editor. Invertebrate tissue culture research application. New York: Academic Press; 1976. p. 161–78.
[11] Dinan L, Hormann RE. Ecdysteroid agonists and antagonists. In: Gilbert LI, Iatrou K, Gill SS, editors. Comprehensive molecular insect science. Oxford; 2005. p. 197–242.
[12] Minakuchi C, Nakagawa Y, Kamimura M, Miyagawa H. Binding affinity of nonsteroidal ecdysone agonists against the ecdysone receptor complex determines the strength of their molting hormonal activity. Eur J Biochem 2003;270:4095–104.
[13] Ogura T, Minakuchi C, Nakagawa Y, Smagge G, Miyagawa H. Molecular cloning, expression analysis and functional confirmation of ecdysone receptor and ultraspiracle from the Colorado potato beetle *Leptinotarsa decemlineata*. FEBS J 2005;272:4114–28.
[14] Watanabe B, Nakagawa Y, Miyagawa H. Synthesis of a castasterone/ponasterone hybrid compound and evaluation of its molting hormone-like activity. J Pestic Sci 2003;28:188–93.
[15] Imai S, Fujioka S, Nakanishi K, Koreeda M, Kurokawa T. Extraction of ponasterone A and ecdysterone from podocarpaceae and related plants. Steroids 1967;10:557–65.
[16] Kobayashi M, Nakanishi K, Koreeda M. The moulting hormone activity of ponasterones on *Musca domestica* (Diptera) and *Bombyx mori* (Lepidoptera). Steroids 1967;9:529–36.
[17] Clement CY, Bradbrook DA, Lafont R, Dinan L. Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. Insect Biochem Mol Biol 1993;23:187–93.
[18] Swevers L, Kravariti L, Ciolfi S, Xenou-Kokoletsi M, Ragoussis N, Smagghe G, et al. A cell-based high-throughput screening system for detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic compounds. FASEB J 2004;18:134–6.
[19] Dinan L, Hormann RE, Fujimoto T. An extensive ecdysteroid CoMFA. J Comput Aided Mol Des 1999;13:185–207.

[20] Wing KD. RH: 5849: a nonsteroidal ecdysone agonist: effects on a *Drosophila* cell line. Science 1988;241:467–9.
[21] Wing KD, Slawecki RA, Carlson GR. RH-5849: a nonsteroidal ecdysone agonist: effects on larval lepidoptera. Science 1988;241:470–2.
[22] Tice CM, Hormann RE, Thompson CS, Friz JL, Cavanaugh CK, Michelotti EL, et al. Synthesis and SAR of α-acylaminoketone ligands for control of gene expression. Bioorg Med Chem Lett 2003;13:475–8.
[23] Tice CM, Hormann RE, Thompson CS, Friz JL, Cavanaugh CK, Saggers JA. Optimization of α-acylaminoketone ecdysone agonists for control of gene expression. Bioorg Med Chem Lett 2003;13:1883–6.
[24] Smith HC, Cavanaugh CK, Friz JL, Thompson CS, Saggers JA, Michelotti EL, et al. Synthesis and SAR of *cis*-1-benzoyl-1,2,3,4-tetrahydroquinoline ligands for control of gene expression in ecdysone responsive systems. Bioorg Med Chem Lett 2003;13:1943–6.
[25] Mikitani K. A new nonsteroidal chemical class of ligand for the ecdysteroid receptor 3,5-di-tert-butyl-4-hydroxy-N-isobutyl-benzamide shows apparent insect molting hormone activities at molecular and cellular levels. Biochem Biophys Res Commun 1996;227:427–32.
[26] Hormann RE, Chortyk O, Le DP. Oxadiazoline ligands for modulating the expression of exogenous genes via an ecdysone receptor complex. US 2004/017651 A1 USA; 2004.
[27] Nakagawa Y, Shimizu B, Oikawa N, Akamatsu M, Nishimura K, Kurihara N, et al. Three-dimensional quantitative structure-activity analysis of steroidal and dibenzoylhydrazine-type ecdysone agonists. In: Hansch C, Fujita T, editors. Classical and three-dimensional QSAR in agrochemistry. Washington, DC: American Chemical Society; 1995. p. 288–301.
[28] Shimizu B, Nakagawa Y, Hattori K, Nishimura K, Kurihara N, Ueno T. Molting hormonal and larvicidal activities of aliphatic acyl analogs of dibenzoylhydrazine insecticides. Steroids 1997;62:638–42.
[29] Nakagawa Y, Hattori K, Shimizu B, Akamatsu M, Miyagawa H, Ueno T. Quantitative structure-activity studies of insect growth regulators XIV. Three dimensional quantitative structure-activity relationship of ecdysone agonists including dibenzoylhydrazine analogs. Pestic Sci 1998;53:267–77.
[30] Billas IML, Iwema T, Garnier JM, Mitschler A, Rochel N, Moras D. Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. Nature 2003;426:91–6.
[31] Billas IM, Moras D. Ligand-binding pocket of the ecdysone receptor. Vitam Horm 2005;73:101–29.
[32] Voigt B, Whiting P, Dinan L. The ecdysteroid agonist/antagonist and brassinosteroid-like activities of synthetic brassinosteroid/ecdysteroid hybrid molecules. Cell Mol Life Sci 2001;58:1133–40.
[33] Watanabe B, Nakagawa Y, Ogura T, Miyagawa H. Stereoselective synthesis of (22*R*)- and (22*S*)-castasterone/ponasterone A hybrid compounds and evaluation of their molting hormone activity. Steroids 2004:483–93.
[34] Ireland RE, Liu L. An improved procedure for the preparation of the Dess–Martin periodinane. J Org Chem 1993;58:2899–999.
[35] Frigerio M, Santagostino M, Spotore S. A user-friendly entry to 2-iodoxybenzoic acid (IBX). J Org Chem 1999;64:4537–8.
[36] Nakagawa Y, Minakuchi C, Takahashi K, Ueno T. Inhibition of [^{3}H]ponasterone A binding by ecdysone agonists in the intact Kc cell line. Insect Biochem Mol Biol 2002;32:175–80.
[37] Sakuma M. Probit analysis of preference data. Appl Entmol Zool 1998;33:339–47.
[38] Thompson MJ, Robbins WE, Kaplanis JN, Cohen CF, Lancaster SM. Synthesis of analogs of alpha-ecdysone. A simplified synthesis of 2-beta, 3-beta, 14-alpha-trihydroxy-7-en-6-one-5-beta-steroids. Steroids 1970;16:85–104.
[39] Sung GC, Janzen L, Pharis RP, Back TG. Synthesis and bioactivity of 6alpha- and 6beta-hydroxy analogues of castasterone. Phytochemistry 2000;55:121–6.
[40] Brosa C, Capdevila JM, Zamora I. Brassinosteroids: a new way to define the structural requirements. Tetrahedron 1996;52:2435–48.
[41] Kovganko NV, Survilo VL. Synthesis of 22,23-epoxyecdysteroids from stigmasterol. Chem Nat Compd 2001;37:154–60.