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Synthesis and cytotoxicity of some novel 21*E*-benzylidene steroidal derivatives

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ABSTRACT

A series of novel derivatives of 21*E*-benzylidene-pregn-1,4-diene-3,20-dione **7a–g** and 21*E*-benzylidene-4-chloro-pregn-1,4-diene-3,20-dione **8a–g** was synthesized from the commercially available progesterone. These title compounds were evaluated for their cytotoxic activity against brine shrimp (*Artemia salina*) and murine Lewis lung carcinoma cells (LLC). It was found that compounds **7a–g** exhibited stronger activities than **8a–g** against the brine shrimps, and some of the tested compounds possessed weak inhibition of LLC cells.

1. Introduction

Steroids have always attracted considerable interest because of their biological signaling molecules. They can regulate a variety of biological processes and have a potential of being drugs for the treatment of a large number of diseases including cardiovascular [1], autoimmune diseases [2], breast cancer, prostate cancer, osteoarthritis [3,4], etc. Recent studies have shown that benzylidene derivatives are a range of versatile physiologically active compounds and are used as substrates for various organic syntheses. These compounds also possess a variety of biological activities such as cytotoxic [5–8], antimalarial [9,10], antileishmanial [11,12], anti-inflammatory [13,14], anti-HIV [15], antifungal [16], and tyrosine kinase inhibition [17]. More recently, some steroidal benzylidene derivatives have been shown to serve as dehydrogenase inhibitors [18], antimicrobial agents [19] and antineoplastic agents [20–22].

In order to obtain biologically potent compounds with diverse structures, the present studies prepared a series of new 21*E*-benzylidene derivatives from the commercially available progesterone **1** by structural modifications. Δ^1-Dehydrogenation of **1** gave a pivotal intermediate pregn-1,4-diene-3,20-dione **2**, and introduction of a chlorine atom at C-4 of **1** and then Δ^1-dehydrogenation gave another intermediate, 4-chloro-pregn-1,4-diene-3,20-dione **5**. The benzylidene derivatives **7a–g** and **8a–g** were prepared from the two intermediates **2** and **5**, respectively, with various aromatic aldehydes by aldol condensation reactions, and their cytotoxic activities against brine shrimp (*Artemia salina*) and murine Lewis lung carcinoma cells (LLC) were also evaluated, which we wish to report herein.

2. Experimental

2.1. General methods

Melting points were measured on an X-4 micromelting point apparatus. NMR spectra were recorded on a Bruker Advance III 500 instrument in $CDCl_3$ with TMS as internal standard for protons and solvent signals as internal standard for carbon spectra. Chemical shift values are mentioned in δ (ppm) and coupling constants are given in Hz. Mass spectra were obtained on either a ESI-esquire 3000 or maXis 4G Bruker Daltonics instrument.

Column chromatography (CC) was performed over silica gel (200–300 mesh, Qingdao Marine Chemical Ltd.). All solvents were distilled prior to use. The progress of all reactions was monitored by TLC on 2 cm × 5 cm precoated silica gel 60 F_{254} plates of thickness of 0.25 mm (Qingdao Marine Chemical Group, Co.). The chromatograms were visualized under UV 254–366 nm and iodine.

2.2. Chemical synthesis

2.2.1. 4α,5α- and 4β,5β-Epoxypregnan-3,20-dione 3 (3a and 3b)

A mixture of 10% NaOH (2.8 mL) and 30% H_2O_2 (5.6 mL) was added to a solution of progesterone **1** (3.14 g, 10 mmol) in CH_2Cl_2

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(30 mL) and methanol (40 mL). The reaction mixture was stirred at room temperature for 24 h, at which time TLC indicated reaction completion. The solvent was evaporated in vacuum, and then CH_2Cl_2 (150 mL) was added. The organic layer was washed with 1 mol/L HCl solution, H_2O and brine, and dried over anhydrous Na_2SO_4. Upon evaporation, 4,5-epoxypregnan-3,20-dione **3** was obtained (2.58 g, yield 78%) as a mixture of 4α,5α and 4β,5β stereomeric epoxides (**3a** and **3b**) (ratio α:β = ca.1:3). mp 119–122 °C (Ref. [23]: mp 122–124 °C); MS (ESI): *m*/*z* 331 $[M+H]^+$. The diastereomeric mixture ratio was determined by proton integration of the 4-H of the product.

4α,5α-Epoxypregnan-3,20-dione (**3a**): ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.67 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 2.13 (3H, s, 21-CH_3), 3.05 (1H, s, 4β-H); ^{13}C NMR (500 MHz, $CDCl_3$) δ ppm 13.34, 16.48, 21.39, 24.39, 28.87, 29.12, 29.62, 29.68, 31.48, 33.07, 35.40, 36.73, 38.75, 44.02, 50.57, 55.82, 62.84, 63.61, 69.98, 206.72 (C-3), 209.27 (C-20).

4β,5β-Epoxypregnan-3,20-dione (**3b**): ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.66 (3H, s, 18-CH_3), 1.16 (3H, s, 19-CH_3), 2.12 (3H, s, 21-CH_3), 2.99 (1H, s, 4α-H); ^{13}C NMR (500 MHz, $CDCl_3$) δ ppm 13.36, 18.92, 21.53, 22.83, 24.37, 26.20, 29.77, 30.31, 31.44, 32.53, 35.02, 37.23, 38.49, 44.09, 46.43, 56.00, 62.68, 63.45, 70.20, 206.68 (C-3), 209.16 (C-20).

2.2.2. 4-Chloro-pregn-4-ene-3,20-dione (4)

According to the procedure described in Ref. [24], to a solution of the diastereomeric mixture **3** (2.57 g, 7.8 mmol) in acetone (100 mL) was added concentrated HCl (2 mL). The solution was allowed to reflux for 1 h. The organic solvent was evaporated in vaccum and the desired crude product **4** was precipitated upon addition of cold water (200 mL). Recrystallization from methanol afforded pure compound **4** as white solid (1.68 g, 62%). mp 216–218 °C (Ref. [23]: mp 214–217 °C). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 1.24 (3H, s, 19-CH_3), 2.13 (3H, s, 21-CH_3), 3.26 (1H, dq, *J* = 3.0, 4.0, 15.5, 6-H); ^{13}C NMR (500 MHz, $CDCl_3$) δ ppm 13.32, 17.76, 21.10, 22.91, 24.29, 28.92, 31.05, 31.44, 33.98, 34.46, 35.09, 38.60, 41.34, 43.86, 53.84, 55.93, 63.41, 127.37 (C-4), 164.38 (C-5), 190.59 (C-3), 209.14 (C-20). MS (ESI): *m*/*z* 349 $[M+H]^+$.

2.2.3. Preparation of pregn-1,4-diene-3,20-dione (2) and 4-chloro-pregn-1,4-diene-3,20-dione (5)

According to a procedure described by Chen et al. [25]. To a solution of steroid **1** or **4** (4 mmol) and *tert*-butyldimethylchlorosilane (TBDMSCl, 30.4 mg, 0.2 mmol) in dioxane (20 mL) was added 2,3-dichloro-5,6-dicyano-benzoquinone (DDQ, 1.18 g, 5.2 mmol) in two portions at 0 °C. The reaction mixture was warmed to room temperature and then refluxed for 2 h with stirring. After the solution cooled down to room temperature, the reaction mixture was diluted with chloroform (100 mL) and was subjected to vacuum filtration over Al_2O_3 layer, which was washed with chloroform (100 mL). The filtrate was washed with saturated Na_2CO_3 solution (2 × 50 mL) and brine (50 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to yield a crude product as yellow oil. Recrystallization from petroleum ether/ethyl acetate afforded compounds **2** and **5**, respectively.

2.2.3.1. Pregn-1,4-diene-3,20-dione (2). White solid (810 mg, 65%): mp 149–150 °C (Ref. [25]: 149–151 °C); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 1.24 (3H, s, 19-CH_3), 2.12 (3H, s, 21-CH_3), 6.08 (1H, s, 4-H), 6.24 (1H, dd, *J* = 2.0, 10.0 Hz, 2-H), 7.05 (1H d, *J* = 10.0 Hz, 1-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.42 (C-18), 18.69 (C-19), 22.81, 22.84, 24.55, 31.43, 32.77, 33.52 35.48, 38.52, 43.48, 44.07; 52.21, 55.61, 63.38, 123.95 (C-4), 127.61 (C-2), 155.57 (C-1), 168.83 (C-5), 186.27 (C-3), 209.09 (C-20). MS (ESI): *m*/*z* 313 $[M+H]^+$.

2.2.3.2. 4-Chloro-pregn-1,4-diene-3,20-dione (5). White crystals (624 mg, 45%): mp 191–193 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 0.72 (3H, s, 18-CH_3), 1.31 (3H, s, 19-CH_3), 2.13 (3H, s, 21-CH_3), 3.33 (1H, dq, *J* = 3.5, 4.5, 16.0, 6-H), 6.38 (1H, d, *J* = 10.0 Hz, 2-H), 7.09 (1H, d, *J* = 10.5 Hz, 1-H); ^{13}C NMR (500 MHz, $CDCl_3$) δ ppm 13.42, 19.14, 22.89, 23.16, 24.50, 28.88, 31.41, 32.40, 35.41, 38.46, 44.01, 46.18, 52.90, 55.47, 63.29, 126.34 (C-2), 128.32 (C-4), 155.18 (C-1), 162.33 (C-5), 178.27 (C-3), 209.02 (C-20). MS (ESI): *m*/*z* 347 $[M+H]^+$. HR-MS (ESI): *m*/*z* 347.1775 $[M+H]^+$ (calcd. for $C_{21}H_{28}ClO_2$, 347.1772).

2.2.4. General procedure for the synthesis of benzylidene derivatives (7a–g and 8a–g)

To a solution of **2** or **5** (0.25 mmol) in ethanol (2.5 mL) was added 0.06 mL solution of 50% KOH. Then benzaldehyde **6a–g** (0.3 mmol) was charged into the reaction mixture and the solution was stirred at room temperature for 12 h. The reaction mixture was neutralized with 1 mol/L HCl solution, and then ethyl acetate (60 mL) was added. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the corresponding compounds **7a–g** and **8a–g** were obtained as solid powder by crystallization from ethyl acetate/petroleum ether.

*2.2.4.1. 21E-Benzylidene-pregn-1,4-diene-3,20-dione (**7a**).* White powder (82 mg, 82%), mp 80–81 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.71 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 6.09 (1H, s, 4-H), 6.24 (1H, dd, *J* = 1.5, 10.0 Hz, 2-H), 6.76 (1H, d, *J* = 15.5 Hz, 21-H), 7.04 (1H, d, *J* = 10.0 Hz, 1-H), 7.39–7.40 (3H, m, 3H of Ar–H), 7.54–7.57 (3H, m, 2H of Ar–H and 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.67 (C-18), 18.69 (C-19), 22.72, 22.87, 24.77, 32.82, 33.62, 35.63, 38.81, 43.57, 45.04, 52.33, 55.87, 61.72, 123.89 (C-4), 126.52 (C-21), 127.54 (C-2), 128.31, 128.96, 130.44, 134.67, 141.84 (C-22), 155.82 (C-1), 169.16 (C-5), 186.37 (C-3), 199.94 (C-20). MS (ESI): *m*/*z* 401 $[M+H]^+$. HR-MS (ESI): *m*/*z* 401.2476 $[M+H]^+$ (calcd. for $C_{28}H_{33}O_2$, 401.2475).

*2.2.4.2. 21E-(2-Chlorobenzylidene) pregn-1,4-diene-3,20-dione (**7b**).* White powder (93 mg, 86%), mp 98–99 °C(ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.71 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 6.09 (1H, s, 4-H), 6.24 (1H, dd, *J* = 1.5, 10.0 Hz, 2-H), 6.73 (1H, d, *J* = 16.0 Hz, 21-H), 7.05 (1H, d, *J* = 10.0 Hz, 1-H), 7.29–7.33 (2H, m, Ar–H), 7.42 (1H, d, *J* = 8.0 Hz, Ar–H), 7.63 (1H d, *J* = 7.5 Hz, Ar–H), 7.93 (1H d, *J* = 16.0 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.75 (C-18), 18.69 (C-19), 22.88, 22.88, 24.78, 32.81, 33.60, 35.62, 38.92, 43.56, 45.03, 52.24, 55.86, 61.02, 123.89 (C-4), 127.14 (C-21), 127.55 (C-2), 127.55, 129.31, 130.27, 131.16, 132.91, 135.40, 137.83 (C-22), 155.82 (C-1), 169.14 (C-5), 186.41 (C-3), 200.02 (C-20). MS (ESI): *m*/*z* 435 $[M+H]^+$. HR-MS (ESI): *m*/*z* 435.2087 $[M+H]^+$ (calcd. for $C_{28}H_{32}ClO_2$, 435.2085).

*2.2.4.3. 21E-(4-Chlorobenzylidene) pregn-1,4-diene-3,20-dione (**7c**).* White powder (93 mg, 86%), mp 76–77 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.71 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 6.09 (1H, s, 4-H), 6.24 (1H, dd, *J* = 1.5, 10.0 Hz, 2-H), 6.72 (1H, d, *J* = 16.0 Hz, 21-H), 7.04 (1H, d, *J* = 10.5 Hz, 1-H), 7.36 (2H, d, *J* = 8.5 Hz, Ar–H), 7.48 (2H, d, *J* = 8.5 Hz, Ar–H), 7.50 (1H d, *J* = 16.0 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.67 (C-18), 18.70 (C-19), 22.73, 22.86, 24.75, 32.80, 33.61, 35.65, 38.84, 43.52, 45.09, 52.33, 55.89, 61.91, 123.97 (C-4), 126.85 (C-21), 127.61 (C-2), 129.24, 129.45, 133.18, 136.31, 140.36 (C-22), 155.62 (C-1), 168.92 (C-5), 186.32

(C-3), 199.71 (C-20). MS (ESI): *m/z* 435 $[M + H]^+$. HR-MS (ESI): *m/z* 435.2088 $[M + H]^+$ (calcd. for $C_{28}H_{32}ClO_2$, 435.2085).

*2.2.4.4. 21E-(2-Methoxybenzylidene) pregn-1,4-diene-3,20-dione (**7d**).* White powder (90 mg, 84%), mp 66–67 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 3.89 (3H, s, —OCH_3), 6.09 (1H, s, 4-H), 6.25 (1H, dd, *J* = 1.5, 10.0 Hz, 2-H), 6.83(1H, d, *J* = 16.0 Hz, 21-H), 6.91–6.95 (2H, m, Ar–H), 7.05 (1H, d, *J* = 10.5 Hz, 1-H), 7.36(1H, d, *J* = 7.5 Hz, Ar–H), 7.54 (1H, d, *J* = 7.5 Hz, Ar–H), 7.87 (1H, d, *J* = 16.0 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.67 (C-18), 18.69 (C-19), 22.79, 22.93, 24.80, 32.85, 33.66, 35.65, 38.75, 43.63, 44.91, 52.37, 55.55, 55.86, 61.22, 111.21, 120.75, 123.64, 123.85 (C-4), 127.41 (C-21), 127.50 (C-2), 128.74, 131.63, 137.27 (C-22), 155.94 (C-1), 158.63, 169.32 (C-5), 186.40 (C-3), 200.47 (C-20). MS (ESI): *m/z* 431 $[M + H]^+$. HR-MS (ESI): *m/z* 431.2586 $[M + H]^+$ (calcd. for $C_{29}H_{35}O_3$, 431.2581).

*2.2.4.5. 21E-(4-Methoxybenzylidene) pregn-1,4-diene-3,20-dione (**7e**).* White powder (88 mg, 82%), mp 87–88 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 3.84 (3H, s, —OCH_3), 6.09 (1H, s, 4-H), 6.24 (1H, dd, *J* = 1.5, 10.0 Hz, 2-H), 6.65 (1H, d, *J* = 15.5 Hz, 21-H), 6.91 (2H, d, *J* = 8.0 Hz, Ar–H), 7.05 (1H, d, *J* = 10.5 Hz, 1-H), 7.49 (2H, d, *J* = 7.5 Hz, Ar–H), 7.52 (1H, d, *J* = 15.5 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.63 (C-18), 18.68 (C-19), 22.75, 22.87, 24.77, 32.88, 33.63, 35.62, 38.79, 43.60, 44.99, 52.36, 55.42, 55.85, 61.60, 114.41, 123.88 (C-4), 124.37, 127.31 (C-21), 127.54 (C-2), 130.02, 141.62 (C-22), 155.90 (C-1), 161.55, 169.28 (C-5), 186.38 (C-3), 199.84 (C-20). MS (ESI): *m/z* 431 $[M + H]^+$. HR-MS (ESI): *m/z* 431.2575 $[M + H]^+$ (calcd. for $C_{29}H_{35}O_3$, 431.2581).

*2.2.4.6. 21E-(2-Nitrobenzylidene) pregn-1,4-diene-3,20-dione (**7f**).* Pale yellow powder (94 mg, 84%), mp 150–152 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.73 (3H, s, 18-CH_3), 1.24 (3H, s, 19-CH_3), 6.08 (1H, s, 4-H), 6.23 (1H, dd, *J* = 1.5, 10.0 Hz, 2-H), 6.64 (1H, d, *J* = 16.0 Hz, 21-H), 7.05 (1H, d, *J* = 10.0 Hz, 1-H), 7.57 (1H, d, *J* = 7.0 Hz, Ar–H), 7.64–7.69 (2H, m, Ar–H), 7.94 (1H, d, *J* = 16.0 Hz, 22-H), 8.05 (1H, d, *J* = 8.0 Hz, Ar–H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.78 (C-18), 18.71 (C-19), 22.85, 22.96, 24.77, 32.80, 33.57, 35.61, 38.83, 43.52, 45.17, 52.19, 55.82, 60.92, 123.91 (C-4), 125.00, 127.55 (C-2), 129.11 (C-21), 130.35, 131.02, 131.61, 133.58, 137.22 (C-22), 148.57, 155.74 (C-1), 169.01 (C-5), 186.33 (C-3), 199.76 (C-20). MS (ESI): *m/z* 446 $[M + H]^+$. HR-MS (ESI): *m/z* 446.2321 $[M + H]^+$ (calcd. for $C_{28}H_{32}NO_4$, 446.2326).

*2.2.4.7. 21E-(4-Methylbenzylidene) pregn-1,4-diene-3,20-dione (**7g**).* White powder (86 mg, 83%), mp 92–93 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 1.22 (3H, s, 19-CH_3), 2.38 (3H, s, CH_3-Ph), 6.09 (1H, s, 4-H), 6.23 (1H, dd, *J* = 1.5, 10.0 Hz, 2-H), 6.72 (1H, d, *J* = 16.0 Hz, 21-H), 7.04 (1H, d, *J* = 10.0 Hz, 1-H), 7.20 (2H, d, *J* = 7.5 Hz, Ar–H), 7.45 (2H, d, *J* = 7.5 Hz, Ar–H), 7.53 (1H d, *J* = 16.0 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.66 (C-18), 18.70 (C-19), 21.53, 22.73, 22.87, 24.78, 32.83, 33.63, 35.64, 38.81, 43.55, 45.03, 52.35, 55.88, 61.65, 123.93 (C-4), 125.62 (C-21), 127.58 (C-2), 128.33, 129.70, 131.91, 140.96, 141.91 (C-22), 155.75 (C-1), 169.07 (C-5), 186.38 (C-3), 200.00 (C-20). MS (ESI): *m/z* 415 $[M + H]^+$. HR-MS (ESI): *m/z* 415.2629 $[M + H]^+$ (calcd. for $C_{29}H_{35}O_2$, 415.2632).

*2.2.4.8. 21E-Benzylidene-4-chloro-pregn-1,4-diene-3,20-dione (**8a**).* White powder (90 mg, 83%), mp 91–92 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.71 (3H, s, 18-CH_3), 1.29 (3H, s, 19-CH_3), 6.36 (1H, d, *J* = 10.0 Hz, 2-H), 6.76 (1H, d, *J* = 16.0 Hz, 21-H), 7.07 (1H, d, *J* = 10.0 Hz, 1-H), 7.39–7.40 (3H, m, 3H of Ar–H), 7.54–7.57 (3H, m, 2H of Ar–H and 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.67 (C-18), 19.14 (C-19), 22.75, 23.21, 24.72, 28.93, 32.51, 35.55, 38.75, 44.99, 46.24, 53.01, 55.74, 61.65, 126.30 (C-2), 126.46 (C-21), 128.29, 128.32 (C-4), 128.98, 130.48, 134.64, 141.93 (C-22), 155.33 (C-1), 162.52 (C-5), 178.35 (C-3), 199.89 (C-20). MS (ESI): *m/z* 435 $[M + H]^+$. HR-MS (ESI): *m/z* 435.2089 $[M + H]^+$ (calcd. for $C_{28}H_{32}ClO_2$, 435.2085).

*2.2.4.9. 21E-(2-Chlorobenzylidene) -4-chloro-pregn-1,4-diene-3,20-dione (**8b**).* White powder (103 mg, 88%), mp 200–202 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.72 (3H, s, 18-CH_3), 1.29 (3H, s, 19-CH_3), 6.37 (1H, d, *J* = 9.5 Hz, 2-H), 6.73 (1H, d, *J* = 16.0 Hz, 21-H), 7.07 (1H, d, *J* = 10.0 Hz, 1-H), 7.29–7.33 (2H, m, Ar–H), 7.43 (1H, d, *J* = 8.0 Hz, Ar–H), 7.63 (1H d, *J* = 8.0 Hz, Ar–H), 7.94 (1H d, *J* = 16.0 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.77 (C-18), 19.16 (C-19), 22.93, 23.24, 24.75, 28.93, 32.50, 35.56, 38.88, 44.98, 46.24, 52.92, 55.74, 60.88, 126.34 (C-2), 127.14 (C-21), 127.56, 128.31 (C-4), 129.30, 130.31, 131.19, 132.91, 135.44, 137.95 (C-22), 155.33 (C-1), 162.50 (C-5), 178.38 (C-3), 199.96 (C-20). MS (ESI): *m/z* 469 $[M + H]^+$. HR-MS (ESI): *m/z* 469.1697 $[M + H]^+$ (calcd. for $C_{28}H_{31}Cl_2O_2$, 469.1696).

*2.2.4.10. 21E-(4-Chlorobenzylidene)-4-chloro-pregn-1,4-diene-3,20-dione (**8c**).* White powder (103 mg, 88%), mp 198–200 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 1.29 (3H, s, 19-CH_3), 6.36 (1H, d, *J* = 10.0 Hz, 2-H), 6.71 (1H, d, *J* = 16.5 Hz, 21-H), 7.07 (1H, d, *J* = 10.0 Hz, 1-H), 7.36 (2H, d, *J* = 8.5 Hz, Ar–H), 7.47 (2H, d, *J* = 8.0 Hz, Ar–H), 7.52 (1H, d, *J* = 16.5 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.68 (C-18), 19.14 (C-19), 22.75, 23.20, 24.69, 28.91, 32.49, 35.53, 38.75, 45.03, 46.22, 52.98, 55.72, 61.80, 126.30 (C-2), 126.78 (C-21) 128.27 (C-4), 129.24, 129.47, 133.14, 136.33, 140.43 (C-22), 155.30 (C-1), 162.49 (C-5), 178.33 (C-3), 199.68 (C-20). MS (ESI): *m/z* 469 $[M + H]^+$. HR-MS (ESI): *m/z* 469.1690 $[M + H]^+$ (calcd. for $C_{28}H_{31}Cl_2O_2$, 469.1696).

*2.2.4.11. 21E-(2-Methoxybenzylidene)-4-chloro-pregn-1,4-diene-3,20-dione (**8d**).* White powder (97 mg, 84%), mp 152–154 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 1.28 (3H, s, 19-CH_3), 3.88 (3H, s, -OCH_3), 6.36 (1H, d, *J* = 8.5 Hz, 2-H), 6.83(1H, d, *J* = 16.0 Hz, 21-H)), 6.92–6.96 (2H, m, Ar–H), 7.07 (1H, d, *J* = 10.0 Hz, 1-H), 7.36(1H, d, *J* = 8.0 Hz, Ar–H), 7.53 (1H, d, *J* = 7.5 Hz, Ar–H), 7.88 (1H, d, *J* = 16.0 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.68 (C-18), 19.14 (C-19), 22.80, 23.26, 24.75, 28.96, 32.54, 35.54, 38.67, 44.87, 46.30, 53.01, 55.56, 55.69, 61.12, 111.21, 120.76, 123.55, 126.25 (C-2), 127.32 (C-21), 128.74 (C-4), 128.97, 131.71, 137.35 (C-22), 155.51 (C-1), 158.63, 162.72 (C-5), 178.41 (C-3), 200.45 (C-20). MS (ESI): *m/z* 465 $[M + H]^+$. HR-MS (ESI): *m/z* 465.2193 $[M + H]^+$ (calcd. for $C_{29}H_{34}ClO_3$, 465.2191).

*2.2.4.12. 21E-(4-Methoxybenzylidene)-4-chloro- pregn-1,4-diene-3,20-dione (**8e**).* White powder (95 mg, 82%), mp 124–125 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 1.29 (3H, s, 19-CH_3), 3.84 (3H, s, -OCH_3), 6.36 (1H, d, *J* = 9.5 Hz, 2-H), 6.64 (1H, d, *J* = 15.0 Hz, 21-H), 6.91 (2H, d, *J* = 7.5 Hz, Ar–H), 7.07 (1H, d, *J* = 9.5 Hz, 1-H), 7.49 (2H, d, *J* = 8.0 Hz, Ar–H), 7.52 (1H, d, *J* = 15.0 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.65 (C-18), 19.14 (C-19), 22.76, 23.21, 24.73, 28.94, 32.52, 35.53, 38.73, 44.96, 46.27, 53.01, 55.45, 55.71, 61.53, 114.42, 124.29, 126.28 (C-2), 127.27 (C-21), 128.67 (C-4), 130.06, 141.73 (C-22), 155.45 (C-1), 161.57, 162.66 (C-5), 178.41 (C-3), 199.85 (C-20). MS (ESI): *m/z* 465$[M + H]^+$. HR-MS (ESI): *m/z* 465.2189 $[M + H]^+$ (calcd. for $C_{29}H_{34}ClO_3$, 465.2191).

*2.2.4.13. 21E-(2-Nitrobenzylidene)-4-chloro-pregn-1,4-diene-3,20-dione (**8f**).* White powder (99 mg, 83%), mp 205–207 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.74 (3H, s, 18-CH_3), 1.30 (3H, s, 19-CH_3), 6.36 (1H, d, *J* = 10.0 Hz, 2-H), 6.63 (1H, d, *J* = 16.0 Hz, 21-H), 7.06 (1H, d, *J* = 9.5 Hz, 1-H), 7.57 (1H, d, *J* = 7.0 Hz, Ar–H), 7.63–7.68 (2H, m, Ar–H), 7.94 (1H, d, *J* = 16.0 Hz, 22-H), 8.05 (1H, d, *J* = 8.0 Hz, Ar–H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.81 (C-18), 19.17 (C-19), 23.03, 23.21, 24.75, 28.92, 32.48, 35.54, 38.77, 45.14, 46.23, 52.86, 55.70, 60.74, 125.04, 126.31 (C-2), 128.28 (C-4), 129.14 (C-21), 130.38, 131.05, 131.64, 133.61, 137.36 (C-22), 148.54, 155.35 (C-1), 162.49 (C-5), 178.35 (C-3), 199.74 (C-20). MS (ESI): *m*/*z* 480 $[M + H]^+$. HR-MS (ESI): *m*/*z* 480.1936 $[M + H]^+$ (calcd. for $C_{28}H_{31}ClNO_4$, 480.1936).

*2.2.4.14. 21E-(4-Methylbenzylidene)-4-chloro-pregn-1,4-diene-3,20-dione (**8g**).* White powder (93 mg, 83%), mp 184–186 °C (ethyl acetate-petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 1.28 (3H, s, 19-CH_3), 2.38 (3H, s, CH_3-Ph), 6.35 (1H, d, *J* = 10.0 Hz, 2-H), 6.72 (1H, d, *J* = 15.5 Hz, 21-H), 7.07 (1H, d, *J* = 10.0 Hz, 1-H), 7.20 (2H, d, *J* = 8.0 Hz, Ar–H), 7.44 (2H, d, *J* = 7.5 Hz, Ar–H), 7.53 (1H, d, *J* = 15.5 Hz, 22-H); ^{13}C NMR (500 MHz, $CDCl_3$): δ ppm 13.66 (C-18), 19.15 (C-19), 21.54, 22.76, 23.21, 24.73, 28.93, 32.51, 35.55, 38.74, 44.96, 46.25, 53.02, 55.73, 61.56, 125.55, 126.28 (C-2), 128.34 (C-4), 128.34, 129.22, 129.71, 131.88, 140.99, 141.97 (C-22), 155.36 (C-1), 162.56 (C-5), 178.34 (C-3), 199.91 (C-20). MS (ESI): *m*/*z* 449 $[M + H]^+$. HR-MS (ESI): *m*/*z* 449.2241 $[M + H]^+$ (calcd. for $C_{29}H_{34}ClO_2$, 449.2242).

2.3. Biology

2.3.1. Brine shrimp cytotoxicity assay

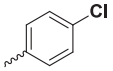
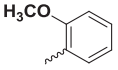
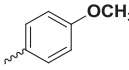
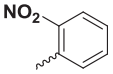
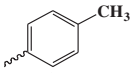
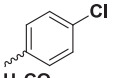
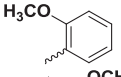
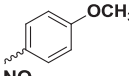
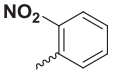
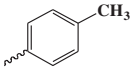
The procedure for brine shrimp lethality assay was modified from the assay previously described by our group [26]. Brine shrimp larvae (*A. salina*) were hatched in a small partitioned tank in artificial seawater. Illumination was provided on one side to attract newly hatched larvae. Brine shrimp larvae with second instar stage were used in this assay. Two milligrams of compounds and **5**, **7a–g** and **8a–g** were made up to 2 mg/mL in DMSO. Through analysis and filtration, five different concentrations (50, 40, 30, 20 and 10 μg/mL) of the compounds were used in this assay. Serial dilutions were made in the wells of 96-well microplates (Nunc, Denmark), to each microplates twenty to thirty brine shrimp larvae were added and some more artificial seawater were put to make exact 200 μL. Negative control was prepared with corresponding concentrations of DMSO in artificial seawater. Following 24 h of incubation at room temperature, casualties were counted under microscope. One hundred microliters of methanol were then added to each well to immobilize the brine shrimp larvae and after 15 min the total numbers of brine shrimp in each well were counted. The experiment was repeated three times, lethality data were transformed by probit analysis in SPSS 16.0 computer program to estimate LC_{50} values. Table 1 gives LC_{50} values of different compounds.

The lethality rate was calculated using the formula: $L = [(A - B - N)/(G - N)] \times 100$, where *L* = percent of the dead larvae after 24 h; *A* = number of the dead larvae after 24 h; *B* = average number of the dead larvae in the blind samples after 24 h; *N* = number of the dead larvae before starting the test; *G* = number of selected larvae for test.

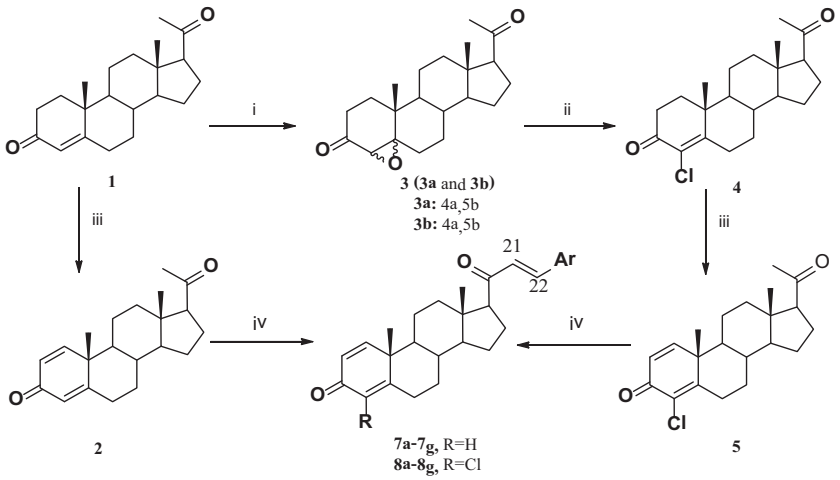
2.3.2. Cell cytotoxicity assay

Murine Lewis lung carcinoma cells (LLC) were grown in 100 mm culture flask at 37 °C under 5% CO_2 in high glucose Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat inactivated fetal bovine serum, 10 μg/mL penicillin, and 10 μg/mL streptomycin. Exponentially growing cells were used throughout. The cell cytotoxicity was assessed by Solarbio MTT kit (Beijing Solarbio Science & Technology Co., LTD) based on the reduction of MTT by the mitochondrial succinate dehydrogenase of intact cells to a purple formazan product [27]. LLC cells were plated in 96-well flat-microtiter plates at a density of 3000–10,000 cells/well for adherence before 6–24 h, then the media was replaced by fresh media and cells were incubated with various concentrations of 21*E*-benzylidene steroidal derivatives for 48 h. After the indicated time, the media was aspirated, and 100 μL media containing 10 μL MTT solution (5 mg mL^{-1} in PBS) stored at −20 °C in the dark was added to each well and plates were incubated for 4 h at 37 °C. Then, the media was moved and 110 μL of DMSO was added to each well to solubilize the formant crystals. The absorbance was measured at 490 nm using an ELISA microplate reader. Each sample was performed in four replicate wells, and the entire experiment was repeated two or three times.

Table 1
Cytotoxic activity of the tested compounds against brine shrimps and murine Lewis lung carcinoma cells (LLC) at 30 μg/mL.

Entry	Ar	LC50[a] (μg/mL)	Inhibitory rate[b]
2	–	>100	0
4	–	>100	0
5	–	>100	0
7a		22.6	31.29
7b		26.5	27.44
7c		24.0	24.36
7d		17.7	22.23
7e		19.8	14.04
7f		28.3	66.18
7g		21.1	17.08
8a		>100	51.12
8b		>100	12.02
8c		>100	12.96
8d		>100	8.34
8e		>100	5.96
8f		>100	25.63
8g		>100	14.22
Toosendanin		<1	

[a] The LC_{50} represents the concentration for the compound to cause 50% lethality of brine shrimps.
[b] All data are the average of four determinations, which were reproducible with deviation less than ±10%.



Scheme 1. Synthesis of 21*E*-benzylidene derivatives. Reagents: (i) H_2O_2, NaOH, MeOH–CH_2Cl_2; (ii) conc. HCl, acetone, reflux; (iii) DDQ, TBDMSCl, Dioxane, reflux; (iv) benzaldehydes **6a–g**, EtOH–KOH.

3. Results and discussion

3.1. Chemistry

The synthetic pathways of a series of new progesterone-derived 21*E*-benzylidene steroids **7a–g** and **8a–g** are shown in Scheme 1. Synthesis of intermediate 4-chloro-pregn-1,4-diene-3,20-dione **5** started with the commercially available progesterone **1**. According to the procedure previously described by Kirk and Hartshorn [28], treatment of **1** with alkaline hydrogen peroxide afforded a 1:3 mixture of epoxide **3**, i.e., 4α,5α-epoxypregnan-3,20-dione **3a** and 4β,5β-epoxypregnan-3,20-dione **3b**, favoring the desired isomer **3b**, as the ratio of α to β isomers was determined based on the proton integration of the H-4 (Hα and Hβ) in the ^{1}H NMR spectra of **3** (see Supporting Information). This result is significantly different from that of the reaction of **1** with dimethyldioxirane, which gave a mixture of the epoxide favoring α-epoxide **3a** [29]. The resulting epoxides (**3a** and **3b**) were directly utilized without further purification for the next step. As a result, treatment of the resulting 4,5-epoxy-steroid **3** with concentrated HCl at reflux afforded 4-chloro-pregn-4-ene-3,20-dione **4** in good yield. Subsequently, Δ^1-dehydrogenation of **4** with 2,3-dichloro-5,6-dicyano-benzoquinone (DDQ) in dioxane in the presence of *tert*-butyldimethylchlorosilane (TBDMSCl) [25] afforded the desired compound **5** in 45% yield.

Similarly, the other intermediate, pregn-1,4-diene-3,20-dione **2**, can be directly obtained through Δ^1-dehydrogenation of **1** using DDQ in 65% yield. Finally, aldol condensation reactions of the intermediates **2** and **5** with various benzaldehydes **6a–g**, followed by crystallization from ethyl acetate-petroleum ether without the need of laborious chromatography, afforded the corresponding target compounds **7a–g** and **8a–g** (Scheme 1), respectively. The structures of these newly synthesized steroidal derivatives were characterized by NMR and HR-ESIMS analyses. Specifically, from the ^{13}C NMR spectra of **3** (see Supporting Information), we can observe that each carbon signal appears in pairs, and the ^{13}C NMR spectrum of the minor 4α,5α-epoxypregnan-3,20-dione (**3a**) presented in this study is well matched to that of α-epoxide recorded by Carvalho et al. [30], accordingly, we assigned the epoxy configuration of the major isomer **3b** to the β- isomer. On the other hand, a new double bond formed was placed at the position 21 based on the ^{1}H NMR spectroscopic data. The ^{1}H NMR spectroscopic data of compounds **7a–g** and **8a–g** showed two characteristic signals in the range of 6.6–6.8 and 7.5–7.9 ppm, which can be assigned to the coupling protons of the double bond at C-21 and C-22. Their coupling constant was about 16 Hz, thereby indicating the geometry of the double bond is *E*, which was in agreement with that of 21*E*-benzylidene steroidal derivatives reported [22,31].

3.2. Biology

In order to evaluate cytotoxic effects of the compounds **2**, **4**, **5**, **7a–g** and **8a–g**, their median lethal concentration (LC_{50}) values were determined against brine shrimp (*Artemia salina* L.) larvae as reported previously [26]. As summarized in Table 1, LC_{50} values of all the tested compounds were found to be quite higher than those of the positive control toosendanin, a commercial botanical insecticide derived from *Melia azedarach*. Compounds **7a–g**, which lacked a chlorine atom at C-4 position, significantly inhibited the growth of the brine shrimp larvae in a dose-depentent manner. It is clear that **7a–g** showed similar cytotoxicity with LC_{50} values of 17.7–28.3 μg/mL, which were stronger than the positive control **2** (LC_{50} > 100 μg/mL). In contrast, compounds **8a–g**, with the same counterpart of aromatic aldehydes as **7a–g**, caused a loss of activity. Among the compounds tested, compound **7d**, having an *ortho*-OCH_3 in the aromatic ring, was found to be the most active (LC_{50} = 17.7 μg/mL). It should be noteworthy that the electron-donating group on the benzene ring was favorable for the cytotoxic activity (**7a** vs. **7d**, **7e**, **7g**), while the electron-withdrawing group could weaken the cytotoxicity (**7a** vs. **7b**, **7c**, **7f**), as compared to **7a** (Table 1). The results indicate that introducing benzylidene groups into **2** was capable of enhancing the cytotoxicity against the brine shrimp and that introducing a chlorine atom into C-4 position resulted in a loss of activity.

Furthermore, these synthetic compounds were tested by the MTT assay for their *in vitro* cytotoxicity against the murine Lewis lung carcinoma cells (LLC) [27]. From the results of Table 1, we found that compounds **7a–g** and **8a–g** showed some inhibitive effects (inhibition rate of 5% to 66%) to LLC cells at concentrations of 30 μg/mL, of which **7f** and **8a** displayed a weak cytotoxic activity (66.2% and 51.1%, respectively) at this concentration.

4. Conclusions

In summary, we have described the synthesis of a series of novel 21*E*-benzylidene derivatives of progesterone and their cytotoxicity to the brine shrimps and LLC cells. Compounds **7a–g** were found to have significant cytotoxic effects against brine shrimps,

while compounds **8a–g** were almost inactive. Moreover, all the synthetic compounds exhibited weak cytotoxicity toward LLC cells.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2013.04.016.

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