Steroids 75 (2010) 224–229

Contents lists available at ScienceDirect

Steroids

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Synthesis and anti-glioma activity of 25(R)-spirostan-3β,5α,6β,19-tetrol

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ARTICLE INFO

Article history:
Received 25 September 2009
Received in revised form 7 December 2009
Accepted 8 December 2009
Available online 21 December 2009

Keywords:
Polyhydroxy steroid
25(R)-Spirostan-3β,5α,6β,19-tetrol
Glioma

ABSTRACT

Malignant gliomas are common and aggressive brain tumours in adults. The rapid proliferation and diffuse brain migration are the main obstacles to successful treatment. Here, we show 25(R)-spirostan-3β,5α,6β,19-tetrol, a polyhydroxy steroid, is capable of suppressing proliferation and migration of C6 malignant glioma cells in a concentration-dependent manner. The compound 25(R)-spirostan-3β,5α,6β,19-tetrol was synthesised by seven steps starting from diosgenin in 8.55% overall yield. The structures of the synthetic compounds were characterised by infrared (IR), ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR spectra and EA.

1. Introduction

In the central nervous system (CNS), gliomas are the most common primary tumours with poor prognosis, which account for 60% of primary brain tumours [1,2]. Although systemic metastases are relatively rare, the highly infiltrative nature of glioma cells that migrate into surrounding brain parenchyma makes total surgical resection impossible [3,4]. The residual cancer cells peripheral to the excised lesion after surgery give rise to a recurrent tumour, which, in more than 90% of cases, develops immediately adjacent to the resection margin [4,5]. Therefore, it is needed to develop novel pharmacological agents to inhibit the proliferation and migration of the malignant gliomas, which could be used in combination with conventional systemic therapies.

Steroids are one of the most ancient molecules [6], which exhibit diverse biological activities, and are regarded as the key to life. In the past, much attention has been paid to the use of steroids in the treatment of some neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and cerebral ischaemia [7,8], partly due to their relatively high permeability across the blood–brain barrier. However, little attention has been paid to the use of steroids in the treatment of brain tumours, especially the malignant gliomas.

In this study, we synthesised the polyhydorxy steroid, 25(R)-spirostan-3β,5α,6β,19-tetrol starting from diosgenin (Scheme 1) in 8.55% overall yield. We found 25(R)-spirostan-3β,5α,6β,19-tetrol could significantly inhibit the proliferation and migration of glioma cells, implying the potential use of 25(R)-spirostan-3β,5α,6β,19-tetrol as a drug candidate for the treatment of malignant gliomas.

2. Experimental

2.1. Chemistry

All melting points were determined on an X_6 melting point apparatus and were uncorrected. IR spectra were recorded as KBr pellets on an EQUINOX 55 FT spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Unity INOVA 500 NB (500 MHz) spectrometer. Chemical shifts were reported in values relative to TMS as the internal standard. Flash column chromatography was carried out with silica gel (200–300 mesh). All chemicals and solvents were analytical grade and purchased from the Guangzhou Chemical Reagents Company China.

2.1.1. 25(R)-3β-Acetoxy-spirost-5-ene (**2**)

Diosgenin **1** (20 g, 0.48 mol) and pyridine (1 ml) were added to 150 ml acetic anhydride, and the mixture was heated and stirred at 90 °C for 1 h [9]. The solution was poured into ice water, The precipitate was filtered, washed with water and dried under vacuum to yield a white solid, which was recrystallised with CH_3OH to give compound **2** (20.9 g, 95%) as colourless needle crystals. mp 193.6–194.5 °C. IR (KBr) ν: 3047, 1723, 1451, 1378, 1051, 982, 901 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 0.78 (d, J = 5.2, 3H, 27-CH_3), 0.79 (s, 3H, 18-CH_3), 0.98 (d, 3H, J = 7.6, 21-CH_3), 1.04 (s, 3H, 19-CH_3), 2.03 (s, 3H, CH_3CO), 3.37 (t, 1H, J = 10.8 Hz, 26-Ha), 3.46 (m, 1H, 26-Hb),

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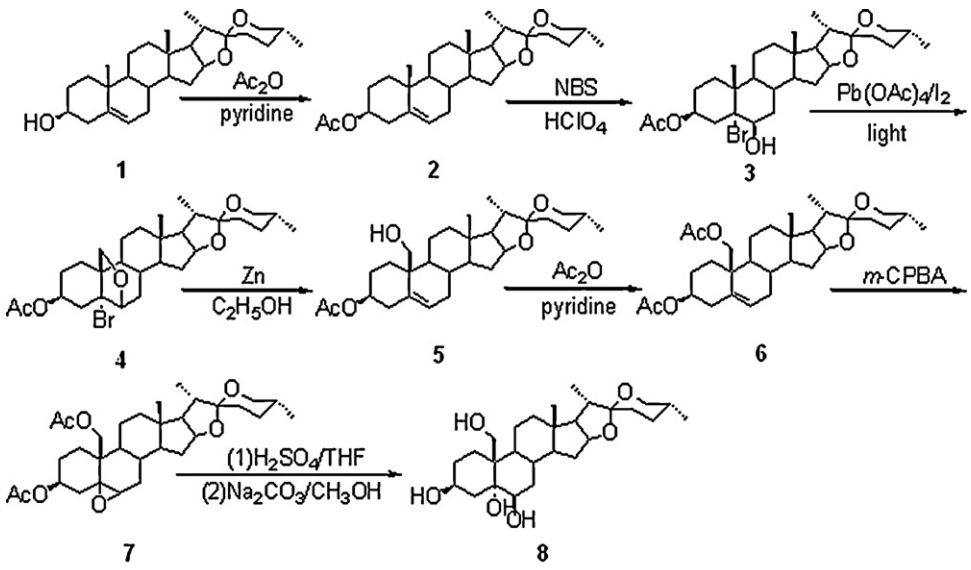
0039-128X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2009.12.005



Scheme 1.

Scheme 1.

4.41 (q, 1H, 16-H), 4.60 (m, 1H, 3-H), 5.37 (d, 1H, 6-H). ^{13}C NMR ($CDCl_3$) δ: 14.51 (CH_3), 16.27 (CH_3), 17.13 (CH_3), 19.92 (CH_3), 20.83 (CH_2), 21.39 (CH_3), 27.76 (CH_2), 28.83 (CH_2), 30.31 (CH), 31.43 (CH), 31.45 (CH_2), 31.45 (CH_2), 31.86 (CH), 32.06 (CH_2), 36.75 (CH_2), 36.98 (CH_2), 38.11 (CH_2), 39.75 (CH_2), 40.28 (C), 41.46 (CH), 49.99 (CH), 56.46 (CH), 62.15 (CH), 66.84 (CH_2), 73.89 (CH), 80.80 (CH), 109.25 (C), 122.36 (CH), 139.72 (C) and 170.46 (C). Analysis calculated for $C_{29}H_{44}O_4$: C, 76.27; H, 9.71; found: C, 76.53; H, 9.70.

2.1.2. 25(R)-3β-Acetoxy-5α-bromo-6β-hydroxy-spirostane (**3**)

Compound **3** was prepared as described previously with some modification [9]. A solution of compound **2** (19 g, 41.6 mmol) in dioxane (180 ml) containing 70% $HClO_4$ (1 ml) and water (10 ml) was stirred in the dark at 10 °C. Immediately, four portions of NBS (8 g, 45 mmol) were added stepwise at 10-min intervals to the solution. The mixture was stirred for 1 h, and then was poured into the cool saturated solution of sodium sulphite. The mixture was filtered and the residue was purified by flash column chromatography (petroleum ether/EtOAc 4:1) to give **3** as needle crystals (16.5 g, 72.10%). mp 135.5–143.1 °C. IR (KBr) ν: 3437, 1738, 1456, 1377, 1055, 690, 521 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 0.76 (d, J = 6.0, 3H, 27-CH_3), 0.77 (s, 3H, 18-CH_3), 0.94 (d, J = 7.2, 3H, 21-CH_3), 1.33 (s, 3H, 19-CH_3), 2.03 (s, 3H, CH_3CO), 3.35(t, 1H, J = 10.8 Hz, 26-Ha), 3.47 (m, 1H 26-Hb), 4.11 (m, 1H, 6-H), 4.17 (q, 1H, 16-H), 4.36 (m, 1H, 3-H). ^{13}C NMR ($CDCl_3$) δ:14.48 (CH_3), 16.59 (CH_3), 17.12 (CH_3), 17.98 (CH_3), 21.15 (CH_2), 21.32 (CH_3), 26.36 (CH_2), 28.83 (CH_2), 30.27 (CH_2), 30.30 (CH), 31.41 (CH_2), 31.66 (CH), 34.84 (CH_2), 35.10 (CH_2), 38.44 (CH_2), 39.72 (CH_2), 40.50 (C), 40.69 (C), 41.67 (CH), 47.54 (CH), 55.60 (CH), 62.13 (CH), 66.87 (CH_2), 72.04 (CH), 75.69 (CH), 80.73 (CH), 86.42 (C), 109.26 (C) and 170.36 (C). Analysis calculated for $C_{29}H_{45}BrO_4$: C, 62.92; H, 8.19; found: C, 62.58; H, 8.180.

2.1.3. 25(R)-3β-Acetoxy-5α-bromo-6β,19-epoxy-spirostane (**4**)

The synthesis of compound **4** was performed as described previously with some modification [9]. A mixture of anhydrous cyclohexane (1420 ml) and benzene (480 ml) containing lead tetraacetate (50.9 g, 112.76 mmol), calcium carbonate (20 g, 200 mmol) was stirred, then compound **3** (12 g, 21.68 mmol) and iodine (12 g, 47.28 mmol) were added. The solution was irradiated by a 250 W tungsten lamp for 6 h. The mixture was filtered, and the solvent was removed under reduced pressure. The residue was dissolved with ethyl acetate, and then the solution was sequentially washed with 10% solution of sodium sulphite and 5% $NaHCO_3$ and water, and then dried with Na_2SO_4. Evaporation of the solvent gave an oil which was purified by flash column chromatography (petroleum ether/EtOAc 9:1) and recrystallised from MeOH to afford colourless needles **4** (8.2 g, 70.18%). mp 196.6–197.9 °C. IR (KBr) ν: 1740, 1452, 1368, 1053, 694, 606, 544 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 0.74 (d, J = 6.4, 3H, 27-CH_3), 0.78 (s, 3H, 18-CH_3), 0.91 (d, J = 6.8, 21-CH_3), 1.99 (s, 3H, CH_3CO), 3.32 (t, 1H, J = 10.8Hz, 26-Ha), 3.35 (q, 1H, 26-Hb), 3.71 (d, J = 8.4, 1H, 19-Ha), 3.89 (d, J = 8.4, 1H, 19-Hb), 4.01 (d, J = 4.8, 1H, 16-H), 4.38 (m, 1H, 6-H), 5.15 (m, 1H, 3-H). ^{13}C NMR ($CDCl_3$) δ: 13.68 (CH_3), 15.99 (CH_3), 16.35 (CH_3), 20.48 (CH_2), 21.73 (CH_3), 22.52 (CH_2), 26.11 (CH_2), 28.03 (CH_2), 29.51 (CH_2), 30.54 (CH), 30.63 (CH_2), 32.23 (CH), 32.28 (CH_2), 38.95 (CH_2), 40.45 (CH_2), 40.57 (CH_2), 40.78 (C), 45.17 (C), 48.06 (CH), 53.42 (CH), 61.34 (CH), 66.07 (CH), 66.81 (CH_2), 69.12 (CH_2), 73.61 (CH), 80.04 (C), 81.44 (CH), 108.45 (C) and 169.44 (C). Analysis calculated for $C_{29}H_{43}BrO_4$: C, 63.15; H, 7.86; found: C, 63.14; H, 8.011.

2.1.4. 25(R)-3β-Acetoxy-19-hydroxy-spirost-5-ene (**5**)

Compound **5** was prepared according to the reported method [9], with some modification. Briefly, compound **4** (6 g, 10.88 mmol) in ethanol (500 ml) was treated with zinc dust (18 g, 276.92 mmol) under reflux with stirring for 5 h. The suspension was filtered, and the filtrate was evaporated under reduced pressure. The residue was dissolved in EtoAc, and washed with water and dried with Na_2SO_4. The solvent was removed under reduced pressure, and the resulting white solid was recrystallised from MeOH-H_2O to afford compound **5** (4.2 g, 78%) as needles; mp 223–225 °C. IR (KBr) ν: 3474, 3040, 2957, 1732, 1450, 1377, 1244, 1045 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 0.75 (d, J = 6.0, 3H, 27-CH_3), 0.82 (s, 3H, 18-CH_3), 0.93 (d, J = 6.8, 3H, 21-CH_3), 2.00 (s, 3H, CH_3CO), 3.34(t, 1H, J = 10.8 Hz, 26-Ha), 3.42–3.43 (m, 1H, 26-Hb), 3.59 (d, J = 11.2, 1H, 19-Ha), 3.89 (d, J = 11.6, 1H, 19-Hb), 4.37 (m, 1H, 16-H), 4.61 (m, 1H, 6-H) and 5.75 (m, 1H, 3-H). ^{13}C NMR ($CDCl_3$) δ: 14.48(CH_3), 16.62 (CH_3), 17.11 (CH_3), 21.35 (CH_2), 21.52 (CH_3), 28.08 (CH_2), 28.81 (CH_2), 30.30 (CH), 31.38 (CH_2), 31.72 (CH_2), 32.88 (CH), 33.16 (CH_2), 38.20 (C), 40.00 (CH_2), 40.50 (C), 41.62 (CH_2), 41.74 (CH), 50.26 (CH), 57.31 (CH), 62.06 (CH), 62.80 (CH_2), 66.85 (CH_2), 73.36 (CH), 80.84 (CH), 109.30 (CH), 127.99 (CH), 134.62 (C) and 170.46 (C). Analysis calculated for $C_{29}H_{44}O_5$: C, 73.69; H, 9.38; found: C, 73.94; H, 9.30.

*2.1.5. 25(R)-3β,19-Diacetoxy-spirost-5-ene (**6**)*

Compound **5** (4 g, 8.46 mmol) and pyridine (1 ml) were added to acetic anhydride (20 ml), and the mixture was heated and stirred at 90 °C for 1 h. The solution was poured into ice water, The precipitate was filtered, washed with water, dried under vacuum to yield compound **6** as colourless needle crystals (4.2 g, 95%). mp 156.8–157.3 °C. IR (KBr) ν: 3047, 1747, 1449, 1371, 1050 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.78 (d, *J* = 4.8, 3H, 27-CH_3), 0.81 (s, 3H, 18-CH_3), 0.96 (d, *J* = 5.6, 3H, 21-CH_3), 2.00 (s, 3H, 3-CH_3CO), 2.02 (s, 3H, 19-CH_3CO), 3.35 (t, 1H, *J* = 8.8 Hz, 26-Ha), 3.46–3.47 (m, 1H, 26-Hb), 3.96 (d, *J* = 9.2, 1H, 19-Ha), 4.39 (m, 1H, 16-H), 4.49 (d, *J* = 9.6, 1H, 19-Hb), 4.62 (m, 1H, 6-H), 5.63 (m, 1H, 3-H). ^{13}C NMR ($CDCl_3$) δ: 14.40 (CH_3), 16.27 (CH_3), 17.02 (CH_3), 20.92 (CH_3), 21.21 (CH_3), 21.29 (CH_2), 27.73 (CH_2), 28.67 (CH_2), 30.15 (CH), 31.29 (CH_2), 31.36 (CH_2), 31.66 (CH), 32.38 (CH), 33.41 (CH_2), 37.98 (CH_2), 39.71 (CH_2), 40.29 (C), 41.51 (CH), 49.90 (CH), 56.85 (CH), 61.94 (CH), 64.43 (CH_2), 66.70 (CH_2), 73.20 (CH), 80.66 (CH), 109.14 (C), 126.40 (CH), 134.47 (C), 170.42 (C) and 170.60 (C). Analysis calculated for $C_{31}H_{46}O_6$: C, 73.69; H, 9.38; found: C, 73.50; H, 9.60.

*2.1.6. 25(R)-3β,19-Diacetoxy-5α,6α-epoxy-spirostane (**7**)*

H_2O (80 ml), Na_2CO_3 (1.8 g, 21.68 mmol) and *m*-CPBA (2.2 g, 12.75 mmol) were added to the solution of compound **6** (2 g, 3.77 mmol) in 120 ml of CH_2Cl_2, and the mixture was stirred at room temperature for 6 h. The solvent was removed under reduced pressure, and extracted with ethyl acetate. The organic layer was washed with Na_2SO_3, $NaHCO_3$ and brine, and then dried with anhydrous Na_2SO_4. Evaporation of the solvent gave the crude product which was purified by flash column chromatography to give a white solid. The resulting product was recrystallised from MeOH to afford colourless needle crystals **7** (1 g, 55.4%). mp 214–216 °C. IR (KBr) ν: 2925, 2874, 1740, 1454, 1370, 1240, 1037 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.65 (s, 3H, 18-CH_3), 0.705 (d, *J* = 5.6 3H, 27-CH_3), 0.84 (m, 3H, 21-CH_3), 2.02 (s, 3H, 3-CH_3CO), 2.05 (s, 3H, 19-CH_3CO), 3.26–3.31 (m, 1H, 26-Ha), 3.38–3.40 (m, 1H, 26-Hb), 3.99 (d, *J* = 9.2, 1H, 19-Ha), 4.25–4.38 (m, 1H, 16-H), 4.41 (d, *J* = 9.2, 1H, 19-Hb), 4.74–4.78 (m, 1H, 6-H), 4.87–4.94 (m, 1H, 3-H). ^{13}C NMR ($CDCl_3$) δ: 14.38 (CH_3), 17.02 (CH_3), 20.89 (CH_2), 21.45 (CH_3), 27.31 (CH_2), 29.64 (CH_2), 30.57 (CH_2), 31.34 (CH), 31.64 (CH_2), 37.30 (CH_2), 38.34 (C), 39.78 (CH_2), 40.38 (C), 41.58 (CH), 42.63 (CH), 43.11 (CH), 56.98 (CH), 59.61 (CH), 61.82 (CH), 62.44 (C), 63.46 (CH_2), 66.78 (CH_2), 70.46 (CH), 80.49 (CH), 109.21 (C), 170.12 (C) and 170.67 (C). Analysis calculated for $C_{31}H_{46}O_7$: C, 70.16; H, 8.74; found: C, 70.35; H, 8.96.

*2.1.7. 25(R)-Spirostan-3β,5α,6β,19-tetrol (**8**)*

H_2SO_4 (1N, 5 ml) was added to the solution of compound **7** (0.5 g, 0.94 mmol) in THF (25 ml), and stirred at room temperature for 24 h. The mixture was neutralised with Na_2CO_3 and then evaporated. The residue was dissolved in methanol (100 ml), Na_2CO_3 (2 g, 24 mmol) was added and then refluxed for 2 h. The solution was poured into 200 ml ice water, and the precipitate was filtered and washed with water and purified by flash column chromatography to give white solid. The resulting solid was further purified by recrystallisation from MeOH-H_2O to afford colourless needles of 25(R)-spirostan-3β,5α,6β,19-tetrol (0.2 g, 43.31%). mp 153–155 °C. IR (KBr) ν: 3408, 2927, 2856, 1456, 1378, 1054 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.76 (s, 3H, 18-CH_3), 0.91 (d, *J* = 5.6 3H, 27-CH_3), 1.02 (m, 3H, 21-CH_3), 3.16–3.25 (m, 1H, 26-Ha), 3.32–3.42 (m, 1H, 26-Hb), 3.61–3.68 (m, 1H, 6-CH), 3.75–3.85 (m, 1H, 3-CH), 4.19 (d, *J* = 3.2, 1H, 19-Ha), 4.26–4.30 (m, 1H, 16-H) and 4.46 (d, *J* = 4.4, 1H, 19-Hb). ^{13}C NMR ($CDCl_3$) δ:14.52 (CH_3), 16.16 (CH_3), 16.99 (CH_3), 20.46 (CH_2), 21.62 (CH_2), 28.44 (CH_2), 28.92 (CH_2), 29.74 (CH), 30.82 (CH), 31.38 (CH_2), 32.92 (CH), 34.60 (CH), 39.40 (CH_2), 39.66 (CH_2), 39.92 (CH), 40.81 (C), 41.04 (CH_2), 43.77 (CH), 44.57 (C), 55.44 (CH), 61.85 (CH), 65.62 (CH), 65.82 (CH_2), 65.83 (CH_2), 73.98 (CH), 79.19 (C), 80.16 (CH) and 108.30 (C). Analysis calculated for. $C_{27}H_{44}O_6$: C, 69.79; H, 9.54; found: C, 70.19; H, 9.80.

2.2. Biological assays

25(R)-Spirostan-3β,5α,6β,19-tetrol was prepared by the above route described in Scheme 1. Dulbecco's modified eagle medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Hoechst 33258 was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Antibodies against cyclin D1, cyclin D3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Beverly, MA, USA), and cyclin-dependent kinases CDK2 and CDK4 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were of analytical grade unless otherwise indicated.

2.2.1. Cell culture and drug treatment

Rat C6 glioma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM (Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS (PAA Laboratories Inc., Etobicoke, ON, USA), 100 units ml^{-1} penicillin (PAA), and 100 μg ml^{-1} streptomycin (PAA) in a humidified atmosphere of 5% CO_2 at 37 °C. Before administration, the medium was changed to DMEM with 1% FBS, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. 25(R)-Spirostan-3β,5α,6β,19-tetrol was dissolved in DMSO and added to the cultures at indicated concentrations for 48 h. DMSO was used at a final concentration below 0.1% and the same volume of DMSO was added to the control group.

2.2.2. Proliferation and cytotoxicity assay

Cell proliferation was evaluated by MTT assay as we described previously [10]. Briefly, C6 cells were plated in 96-well plates and treated with 25(R)-spirostan-3β,5α,6β,19-tetrol for the 48 h, following which 10 μl MTT (5 mg ml^{-1}) was added and incubated for another 4 h at 37 °C. Finally, the MTT solution was removed and 100 μl DMSO was added to dissolve the crystal. Absorbance was read at 570 nm with an EXL800 microimmunoanalyser (BioTek, Burlington, VT, USA).

Cytotoxicity of 25(R)-spirostan-3β,5α,6β,19-tetrol was evaluated by lactate dehydrogenase (LDH) release. LDH release was quantified with a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Absorbance was measured at 490 nm with an EXL800 microimmunoanalyser.

2.2.3. Migration assay

Cell migration was evaluated by a wound healing assay as described previously with some modification [11]. Briefly, the cells were plated on a 6-well plate. The next day, scratches were made with a 200 μl sterile pipette tip, and then the cells were washed with phosphate-buffered saline (PBS) three times and incubated with 25(R)-spirostan-3β,5α,6β,19-tetrol for 48 h. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min. To make cell counting easier, the nucleus of C6 cells was stained with Hoechst 33258 (Invitrogen, 5 μg ml^{-1}, diluted in PBS) for 5 min after fixation. Finally, the cells were washed three times with PBS and photographed with an Olympus fluorescence microscope (Melville, NY, USA). The percentage of migration was calculated by cells that migrated into the scratched areas compared with cells that stayed in the peripheral areas.

2.2.4. Cell cycle analysis

A flow cytometry analysis of DNA content of cells was performed to assess the cell cycle phase distributions as described [2]. In brief, after treatment, the cells were collected by trypsinisation, washed

in PBS and fixed in 70% ethanol for 30 min at 4 °C. After washing with PBS, cells were incubated with the DNA-binding dye propidium iodide (50 μg ml^{-1}) and RNase (1.0 mg ml^{-1}) for 30 min at 37 °C in the dark. Finally, cells were washed and red fluorescence was analysed by a FACSCalibur flow cytometer (BD, Heidelberg, Germany) using a peak fluorescence gate to discriminate aggregates.

2.2.5. Western blot analysis

C6 cells were treated with 25(R)-spirostan-3β,5α,6β,19-tetrol for the indicated time and the total protein was extracted with M-PER (Mammalian Protein Extraction Reagent, Pierce, Rockford, IL, USA) according to the manufacturer's instructions. After measurement of the protein concentration with BCA Protein Assay Kit (Pierce, Rockford, IL, USA), equal amount of protein samples were combined with concentrated sodium dodecyl sulphate (SDS) loading buffer and heated to 95 °C for 5 min, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blots were performed. After incubation with horseradish peroxidase labelled secondary antibody (1:1000, Cell Signaling Technology, Beverly, MA, USA), the visualisation of protein was done with enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA) and a GeneGnome chemiluminescence imaging and analysis system (Syngene Bio Imaging, Cambridge, UK).

2.2.6. Data analysis

Data are expressed as mean ± SD from three independent experiments. Statistical evaluations were done by one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

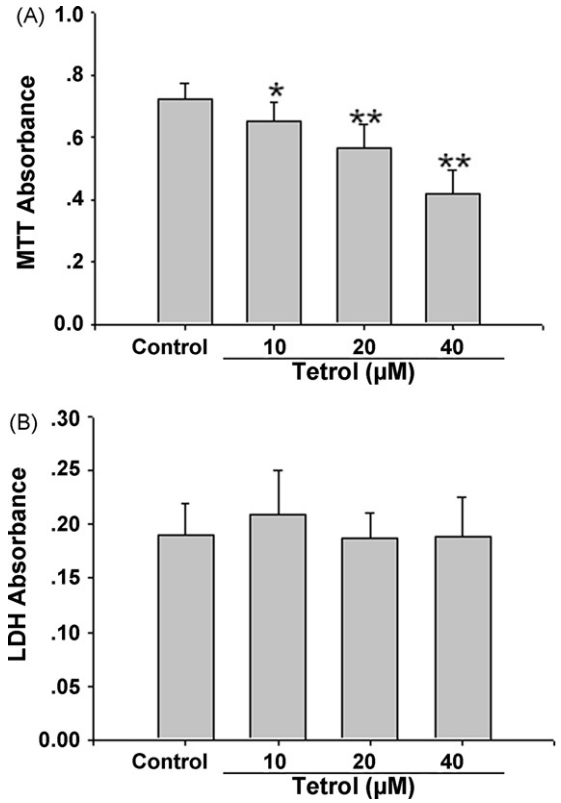


Fig. 1. 25(R)-Spirostan-3β,5α,6β,19-tetrol (Tetrol) induced proliferation inhibition but not cell death in C6 glioma cells. C6 cells were treated with different concentrations of Tetrol for 48 h. (A) MTT absorbance was measured for cell proliferation. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control. (B) LDH absorbance was measured for cytotoxicity.

3. Results and discussion

3.1. Chemistry

The planned synthetic route from diosgenin (**1**) to the target compound 25(R)-spirostan-3β,5α,6β,19-tetrol (**8**) is shown in Scheme 1. The 3β-hydroxy group of **1** was protected by acetylation to give 3β-acetoxydiosgenin (**2**). Compound **2** was converted to compound **3** with NBS containing a catalytic amount of 70% $HClO_4$. When the acidity was not suitable, it yielded a large amount of byproduct 25(R)-3β-acetoxy-5α-hydroxy-spirostan-6-one which was difficult to be separated from compound **3**. The 6β, 19-epoxide (**4**) was the product of remote oxidation when bromohydrin (**3**) was treated with Pb $(OAc)_4$ and I_2 under the irradiation of a tung-

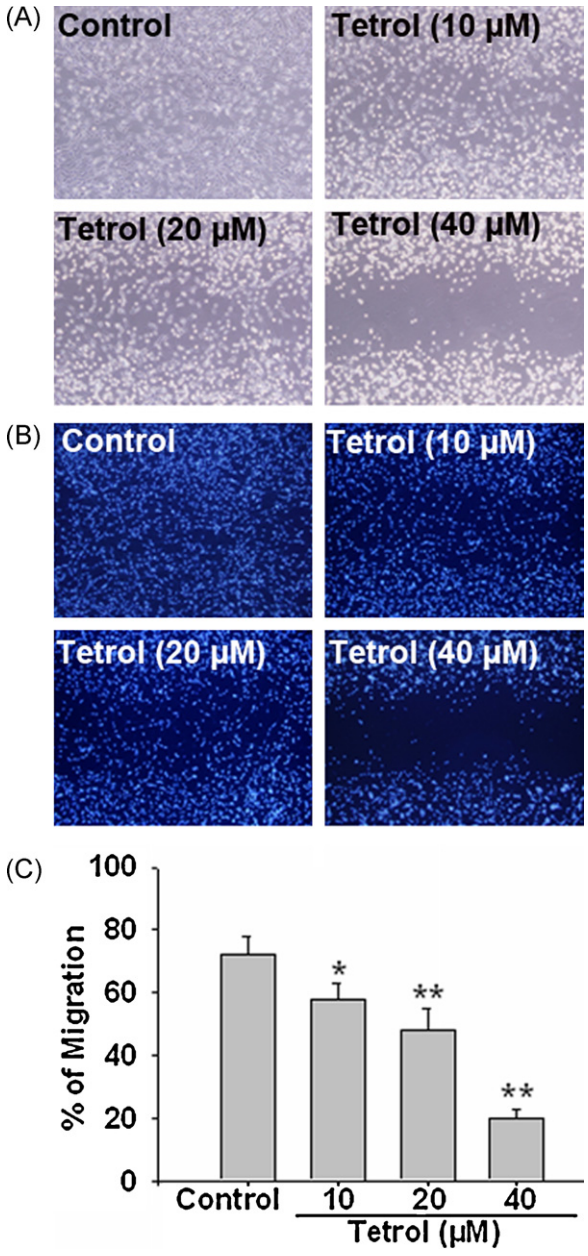


Fig. 2. 25(R)-Spirostan-3β,5α,6β,19-tetrol (Tetrol) inhibited migration of C6 glioma cells. C6 cells were plated on a 6-well plate and scraped with a 200 μl sterile pipette tip and then incubated with different concentrations of Tetrol for 48 h. (A) Phase contrast of migrated cells. Representatives of three independent experiments were shown. (B) Hochest 33258 staining of nucleus. (C) Statistical analysis of the percentage of migrated cells. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

sten lamp. Compound **5** was an important intermediate, which was synthesised via reduction of **4** with Zn powder.

The most characteristic features of 19-hydroxy steroid (**5**) involve the appearance of the ^{13}C NMR peak at 62.80 (19-CH_2OH), 134.62 (5-C), 127.99 (6-CH) and ^{1}H NMR peak at 3.59 (d, J = 8.4, 1H, 19-Ha), 3.89 (d, J = 8.4, 1H, 19-Hb) and 5.75 (t, 1H, 6-H), which are consistent with 19-OH and 5,6-double bonds. The C_{19}-OH of **5** was protected by acetylation to give **6**. Compound **6** was converted to 5,6-epoxide (**7**) with *m*-CPBA. The disappearance of ^{13}C NMR peak at 134.47 (5-C), 126.40 (6-CH), and the appearance of ^{13}C NMR peak at 59.61 (6-CH) and 61.98 (5-C) implied the formation of 5,6-epoxide (**7**). The target compound 25(R)-spirostan-3β,5α,6β,19-tetrol (**8**) was obtained when **7** was treated with H_2SO_4 and then hydrolysed using Na_2CO_3 in methanol. The appearance of ^{13}C NMR peak at 73.98 (3-CH), 79.19 (5-C), 65.62 (6-CH), 65.83 (19-CH_2) proved the formation of 3β, 5α, 6β, 19-tetrol (**8**). For compounds **2**–**7**, the peak still existed at 108–110 in the ^{13}C NMR spectrum. It was the chemical shift of spiral atom 22-C which showed that the spiral ring structure was kept unchanged.

3.2. Anti-glioma activity

Local migration and aggressive proliferation activity of the remaining tumour cells following operation are inherent features of malignant glioma s. Cell migration is a crucial event for tumour spreading, metastasis and invasiveness. An invasive nature of glioma cells leads to the local destruction of the adjacent tissue with an extensive cellular infiltration into the normal brain [12]. The infiltration of tumour cells and their high proliferative activity are key elements in turning gliomas into an unmanageable disease, which prevents complete tumour removal and leads to therapeutic failure and recurrence. Effective inhibition of the migration and proliferation activity of the malignant gliomas may provide help avoiding the spread and recurrence of the malignant gliomas, especially after surgical resection.

To evaluate the inhibiting activity of 25(R)-spirostan-3β,5α,6β,19-tetrol on glioma proliferation, we performed an MTT score assay. The conversion of MTT requires active mitochondria within cells; thus, MTT scores are used as a measure of cellular activity and cell number [13]. Compared with the control, 25(R)-spirostan-3β,5α,6β,19-tetrol could dose-dependently inhibit MTT scores of C6 cells (Fig. 1A). The reduction in MTT score in response to 25(R)-spirostan-3β,5α,6β,19-tetrol treatment could be explained either by cell injury or by reduced proliferation. LDH assay was used to investigate whether 25(R)-spirostan-3β,5α,6β,19-tetrol could cause cell injury. We found there was no statistically significant difference in the mean LDH absorbance between cells treated with 25(R)-spirostan-3β,5α,6β,19-tetrol and the control (Fig. 1B). Therefore, cytotoxicity was not the possible contributor to the reduction in MTT score. Taken together, the proliferation inhibiting activity of 25(R)-spirostan-3β,5α,6β,19-tetrol appears to be responsible for the reduction in MTT score.

Except for the infinite proliferation ability, migration is another characteristic feature of malignant gliomas. To determine the possible role of 25(R)-spirostan-3β,5α,6β,19-tetrol in migration, we performed a wound healing assay. As shown in Fig. 2A and B, untreated C6 cells were able to invade the scratched area that was fully re-colonised 48 h later. However, 25(R)-spirostan-3β,5α,6β,19-tetrol significantly inhibited this process in a concentration-dependent manner. In 25(R)-spirostan-3β,5α,6β,19-tetrol (40 μM) treated group, very few cells were in the scratched area and the distance between the borders of the wound was significantly different from that of the control 48 h after the scratch (Fig. 2A and B). Quantitative analysis clearly indicated significant decreases of the cell migration rate following 25(R)-spirostan-3β,5α,6β,19-tetrol treatment (Fig. 2C). These data suggest that 25(R)-spirostan-3β,5α,6β,19-tetrol has the ability to suppress the cellular migration of C6 malignant glioma cells.

To investigate the chemical basis of 25(R)-spirostan-3β,5α,6β,19-tetrol (**8**) as an anti-glioma agent, the precursors of **8**, together with **8**, were tested for their effect on the migration and proliferation ability of C6 glioma at the concentration of 20 μM (Table 1). We found, except for compound **8**, only compounds **3** and **4** have the inhibitory effect on C6 glioma migration and proliferation. However, compounds **3** and **4** could also cause significant cytotoxicity at the effective concentration, which may account for their inhibiting effect on the migration and proliferation of C6 glioma. By contrast, 25(R)-spirostan-3β,5α,6β,19-tetrol did not induce cytotoxicity as it significantly suppressed the C6 glioma migration and proliferation. Furthermore, other structurally related compounds (**1**–**7**) have no effect on C6 glioma migration and proliferation, which further confirm the polyhydroxy

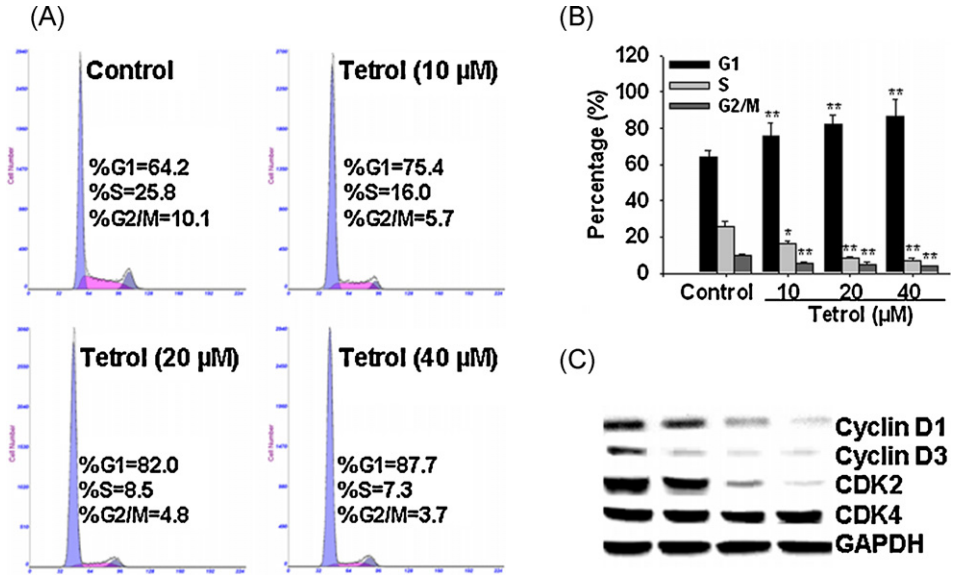


Fig. 3. Effects of 25(R)-spirostan-3β,5α,6β,19-tetrol (Tetrol) on the cell cycle and cell cycle regulatory proteins of C6 glioma cells. C6 cells were treated with 10, 20 and 40 μM of Tetrol for 48 h. (A) Cell cycle distributions. One of three independent experiments was shown. (B) Statistical analysis of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control. (C) Western blots analysis of cyclins and cyclin-dependent kinases.

Table 1
In Vitro anti-glioma activity of the tested compounds at 20 (M concentration.

Compound	% of Migration	MTT Absorbance	LDH Absorbance
1	69.32 ± 4.42	0.70 ± 0.08	0.19 ± 0.02
2	71.31 ± 5.34	0.71 ± 0.13	0.20 ± 0.03
3	58.35 ± 2.68*	0.62 ± 0.09*	0.41 ± 0.05**
4	62.38 ± 3.69*	0.64 ± 0.06*	0.35 ± 0.04*
5	65.44 ± 6.56	0.68 ± 0.15	0.18 ± 0.02
6	69.79 ± 5.76	0.71 ± 0.10	0.21 ± 0.03
7	65.97 ± 7.29	0.69 ± 0.08	0.20 ± 0.02
8	48.35 ± 3.29**	0.56 ± 0.07**	0.18 ± 0.02

Results are expressed as means ± SD (n = 3), *$p < 0.05$, **$p < 0.01$ compared with control.

structure is critical for the anti-glioma activity of this series of compounds.

Dysregulation of cell cycle progression is a fundamental reason that leads to aberrant proliferation of tumour cells. The G1/S-phase checkpoint is the first critical restriction point in the cell division cycle and the G1/S transition is closely linked to the activation of cell cycle regulatory proteins such as cyclins and CDKs [14]. To gain insight into the mechanism of growth inhibitory effects of 25(R)-spirostan-3β,5α,6β,19-tetrol, we assessed cell cycle distribution of C6 cells by flow cytometry. Analysis of cell cycle showed that 25(R)-spirostan-3β,5α,6β,19-tetrol led to a dose-dependent C6 accumulation in the G1 phase to reach approximately 75.4%, 82.0% and 87.7% of population at 10, 20 and 40 μM, respectively, while that in the control was 64.2%, indicating cell cycle arrest at this phase. Concomitantly, there was a striking decrease in the S and G2/M fractions over the basal level. Cells in the S phase decreased from 25.8% of control group to 7.3% of 40 μM 25(R)-spirostan-3β,5α,6β,19-tetrol group ($p < 0.01$) and G2/M phase cells from 10.1% to 3.7% ($p < 0.01$) (Fig. 3 A and B).

To further determine the molecular basis for the cell cycle arrest induced by 25(R)-spirostan-3β,5α,6β,19-tetrol, we examined the expression of multiple cell cycle regulatory proteins by Western blotting. Cyclins and CDKs associated with G1/S-phase checkpoint, such as cyclin D1, cyclin D3, CDK2 and CDK4, were investigated. Fig. 3C showed that 25(R)-spirostan-3β,5α,6β,19-tetrol downregulated the expression of cyclin D1, cyclin D3 and CDK2. However, the level of CDK4 was not altered (Fig. 3C). The inhibitory effect of 25(R)-spirostan-3β,5α,6β,19-tetrol on cyclin/CDK complex expression was consistent with the reduced S phase progression. Based on these findings, 25(R)-spirostan-3β,5α,6β,19-tetrol is likely to inhibit proliferation by blocking cell cycle progression through G1 to S phase by inactivation of cyclin D1, cyclin D3 and CDK2.

In conclusion, we demonstrated here that 25(R)-spirostan-3β,5α,6β,19-tetrol was able to effectively inhibit proliferation and migration of C6 glioma cells. Specially, it did not cause significant tumour cell cytotoxicity at the effective concentrations used in this study, which indicated 25(R)-spirostan-3β,5α,6β,19-tetrol could function as a novel and potential anti-glioma agent compared with conventional cytotoxic chemotherapies. At the same time, it may provide critical clues for the development of more potent anti-tumour agents.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Project No. 30472089), the National High Technology Development 863 Key Project (Project No. 2006AA090502).

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