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Design, synthesis and biological evaluation of novel steroidal spiro-oxindoles as potent antiproliferative agents

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ABSTRACT

Two series of novel steroidal spiro-pyrrolidinyl oxindoles **3a–t** and **6a–c** were designed and synthesized from dehydroepiandrosterone using the 1,3-dipolar cycloaddition as the key step and further evaluated for their antiproliferative activities for four human cancer cell lines (MGC-803, EC109, SMMC-7721 and MCF-7). This protocol achieved the formation of two C—C bonds, one C—N bond and the creation of one new five-membered pyrrolidine ring and three contiguous stereocenters in a single operation. Biological evaluation showed that these synthesized steroidal spiro-pyrrolidinyl oxindoles possessed moderate to good antiproliferative activities against the tested cell lines and some of them were more potent than 5-Fu. Particularly, compound **3g** showed good antiproliferative activity against SMMC-7721 (IC_{50} = 0.71 μM). Steroid dimer **6b** showed improved antiproliferative activities against SMMC-7721 and MCF-7 with the IC_{50} values of 4.30 and 2.06 μM, respectively. Flow cytometry analysis demonstrated that compound **3n** caused the cellular early apoptosis and cell cycle arrest at G2/M phase in a concentration- and time-independent manner.

1. Introduction

Steroids are a family of molecules that play a crucial role in a wide range of biological processes and in human physiology. Their hormonal action *via* binding to a specific receptor is well known and has led to the development of antagonists to treat certain hormone-dependent diseases [1]. It is proved that a number of biologically important properties of modified steroids depend upon structural features of the steroid ring system [2] and side chain [3]. Chemical modifications of the steroid ring system and side chain provide a way to alter the functional groups, and numerous structure activity relationships (SARs) have been established by such synthetic alterations [4]. Among them, dimeric steroids are a special group of compounds, which have recently received significant attention [5,6]. There is evidence that dimerization of steroid skeleton offers some unique characteristics that are applicable in different areas, especially in pharmacology [7–9].

The specificity of biological activities found in natural compounds is generally in connection with the characteristic structural complexity and well-defined stereo-architecture [10,11]. The biological activity and structural complexity found in nature has stimulated generations of synthetic chemists to design strategies for assembling challenging structures found in natural products [12,13]. Particularly intriguing are the spirocyclic oxindole scaffolds, which feature in a large number of natural and unnatural compounds with important biological activities [14–18] and can also serve as key intermediates for the synthesis of alkaloids and many kinds of pharmaceuticals or drug precursors [19]. Among them, the spiro-pyrrolidinyl oxindoles possess a myriad of biological activities. For example, coerulescine [13], the simplest spirooxindole-pyrrolidine hybrid found in nature, displays local anesthetic effect. Pteropodine modulates the function of muscarinic serotonin receptors [20]. The spirotryprostatins have antibiotic properties and are of interest as anticancer lead compounds [21], and the recently discovered small-molecule MDM2 inhibitor MI-219 and its analogs are in advanced preclinical development for cancer therapeutics [14,16] (Fig. 1).

Recently, we have achieved the synthesis and antiproliferative evaluation of steroidal dienamides from dehydroepiandrosterone [22,23]. Inspired by the varied and significant biological activities of spiro-oxindole skeletons [24–29] and being involved in finding new biologically active modified steroids [30,31], we are interested in the design, synthesis and biological evaluation of novel steroidal spiro-pyrrolidinyl oxindoles. In this paper, we report the efficient and catalyst-free construction of steroidal spiro-oxindoles with spirotricyclic skeleton *via* a one-pot three-component protocol and evaluate their antiproliferative activities against human cancer cell lines *in vitro* and the effects toward the cell cycle and apoptosis.

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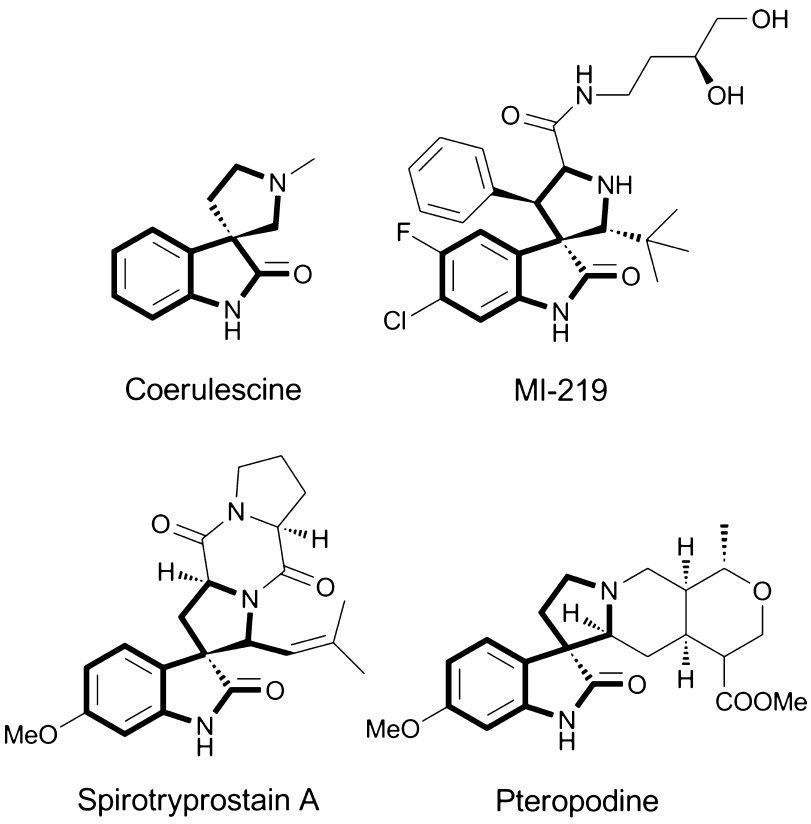


Fig. 1. Some naturally occurring and synthetic spiro-pyrrolidinyl oxindoles.

2. Experimental

2.1. General

Reagents and solvents were purchased from commercial sources and were used without further purification. Thin-layer chromatography (TLC) was carried out on glass plates coated with silica gel (Qingdao Haiyang Chemical Co., G60F-254) and visualized by UV light (254 nm). The products were purified by column chromatography over silica gel (Qingdao Haiyang Chemical Co., 200–300 mesh). Melting points were determined on a X-5 micromelting apparatus and are uncorrected. All the NMR spectra were recorded with a Bruker DPX 400 MHz spectrometer with TMS as internal standard in $CDCl_3$ or DMSO-d_6. Chemical shifts are given as δ ppm values relative to TMS. High-resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-T of Micromass spectrometer by electrospray ionization (ESI).

2.2. General procedure for the synthesis of 16-arylidene-17-ketosteroids (**2**)

A mixture of dehydroepiandrosterone (DHEA, **1**) (2.0 mmol), aromatic aldehydes (2.1 mmol) and KF/Al_2O_3 (2.0 mmol) in EtOH (20 mL) was heated under reflux for about 1 h. After completion of the reaction as evident from TLC, the slurry was filtered and the residue was washed thoroughly with CH_2Cl_2. The filtrate was condensed under reduced pressure, and the solid obtained was crystallized from EtOH or MeOH to yield the corresponding 16-arylidene-17-ketosteroids **2**. All the intermediates **2** were synthesized following the procedure previously reported by our group [30]. All the intermediates were reported before by our group [30] and Kumar [41], so spectral data for a representative compound **2a** was given below. White solid, yield 93%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (d, J = 8.3 Hz, 2H, ArH), 7.71 (d, J = 8.4 Hz, 2H, ArH), 7.46 (s, 1H, Ar-C<u>H</u>=), 5.45–5.36 (m, 1H, 6-H), 3.60–3.50 (m, 1H, 3α-H), 3.09 (s, 3H, Ar-SO_2C<u>H_3</u>), 2.94–2.82 (m, 1H), 2.50 (ddd, J = 16.0, 12.8, 3.0 Hz, 1H), 2.40–2.32 (m, 1H), 2.32–2.26 (m, 1H), 2.26–2.15 (m, 1H), 2.01 (dd, J = 9.6, 3.1 Hz, 1H), 1.86 (ddd, J = 15.8, 12.0, 4.3 Hz, 2H), 1.80–1.63 (m, 4H), 1.57 (ddd, J = 14.5, 11.9, 3.3 Hz, 2H), 1.47–1.33 (m, 2H), 1.19–1.11 (m, 1H), 1.09 (s, 3H, 19-CH_3), 1.01 (s, 3H, 18-CH_3). HRMS (ESI): m/z calcd for $C_{27}H_{34}O_4SNa$ $(M+Na)^+$, 477.2075; found, 477.2075.

2.3. General procedure for the synthesis of steroidal spiro-pyrrolidinyl oxindoles (**3a**–**t**)

A mixture of 16-arylidene-17-ketosteroids (**2a**–**t**, 1 mmol) (substituted) isatin (1.5 mmol) and sarcosine (2.0 mmol) in methanol/1,4-dioxane mixture (v/v = 1/1, 20 mL) was refluxed for about 5 h. After completion of the reaction as evident from TLC (petroleum ether/ethyl acetate = 2/1), the mixture was evaporated and purified by column chromatography on silica gel using petroleum ether/ethyl acetate = 2/1(v/v) as the eluent to obtain the pure products **3a**–**t**.

2.3.1. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-methylsulfonyl phenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3a**)

White solid, yield: 89%, m.p. 262.0–264.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (s, 1H, NH), 7.92 (d, J = 8.2 Hz, 2H, ArH), 7.70 (d, J = 6.7 Hz, 2H, ArH), 7.28–7.15 (m, 1H, ArH), 7.23 (t, J = 5.9 Hz, 1H, ArH), 7.07–6.96 (m, 1H, ArH), 6.85 (d, J = 7.7 Hz, 1H, ArH), 5.10 (d, J = 4.0 Hz, 1H, 6-H), 4.11 (t, J = 8.7 Hz, 1H, 12′-H), 3.92 (t, J = 9.4 Hz, 1H, 11′-H), 3.54 (t, J = 8.1 Hz, 1H, 11′-H), 3.51–3.39 (m, 1H, 3α-H), 3.09

(s, 3H, CH_3SO_2—), 2.17 (s, 3H, *N*-CH_3), 2.28–2.14 (m, 1H), 2.14–1.89 (m, 3H), 1.79 (dd, *J* = 10.7, 6.3 Hz, 2H), 1.63 (dd, *J* = 29.1, 12.9 Hz, 2H), 1.44 (dd, *J* = 18.6, 8.1 Hz, 1H), 1.38–1.11 (m, 3H), 0.98–0.86 (m, 1H), 0.82 (s, 3H, 19-CH_3),0.81 (dd, *J* = 18.4, 6.6 Hz, 1H), 0.75–0.64 (m, 1H), 0.52 (s, 3H, 18-CH_3), 0.41 (td, *J* = 11.6, 4.9 Hz, 1H), 0.30 (dt, *J* = 12.7, 5.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.64, 146.62, 142.11, 141.16, 139.00, 131.34, 129.66, 128.67, 127.53, 126.95, 122.80, 120.44, 109.58, 78.08, 71.47, 67.44, 60.56, 51.25, 50.20, 47.79, 47.02, 44.55, 42.16, 36.94, 36.51, 34.81, 33.36, 31.32, 31.04, 30.76, 30.20, 19.79, 19.26, 14.82. HRMS (ESI): *m*/*z* calcd for $C_{35}H_{45}N_2O_5S$ $(M+H)^+$, 629.3049; found, 629.3043.

2.3.2. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-nitrophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3b**)

Yellow solid, yield: 91%, m.p. 202.3–204.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (d, *J* = 7.8 Hz, 2H, ArH), 7.68 (d, *J* = 5.9 Hz, 2H, ArH), 7.25 (d, *J* = 7.6 Hz, 1H, ArH), 7.12 (d, *J* = 7.6 Hz, 1H, ArH), 7.05 (t, *J* = 7.5 Hz, 1H, ArH), 6.85 (d, *J* = 7.7 Hz, 1H, ArH), 5.22 (s, 1H, 6-H), 3.93 (t, *J* = 9.4 Hz, 1H, 12′-H), 3.59 (t, *J* = 8.4 Hz, 1H, 11′-H), 3.55–3.37 (m, 2H, 11′-H and 3α-H), 2.19 (s, 3H, *N*-CH_3), 2.30–2.07 (m, 2H), 1.88 (d, *J* = 16.2 Hz, 2H), 1.79 (d, *J* = 12.1 Hz, 2H), 1.66 (dd, *J* = 23.1, 13.0 Hz, 2H), 1.46–1.31 (m, 3H), 1.31–1.25 (m, 1H), 1.01–0.88 (m, 1H), 0.85 (s, 3H, 19-CH_3), 0.73 (m, 2H), 0.57 (s, 3H, 18-CH_3), 0.43 (td, *J* = 11.6, 4.9 Hz, 1H), 0.39–0.28 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.51, 147.71, 147.11, 141.82, 141.16, 131.16, 129.72, 128.75, 126.92, 123.70, 122.98, 120.47, 109.48, 78.02, 71.52, 67.45, 65.88, 60.44, 51.19, 50.20, 47.88, 47.07, 42.14, 36.92, 36.50, 34.79, 33.36, 31.42, 31.09, 30.74, 30.23, 19.21, 14.82. HRMS (ESI): *m*/*z* calcd for $C_{36}H_{42}N_3O_5$ $(M+H)^+$, 596.3124; found, 596.3126.

2.3.3. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(3″-nitrophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3c**)

Yellow solid, yield: 93%, m.p. 189.5–193.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H, NH), 8.59 (s, 1H, ArH), 8.11 (dd, *J* = 8.2, 1.3 Hz, 1H, ArH), 7.75 (s, 1H, ArH), 7.50 (t, *J* = 7.9 Hz, 1H, ArH), 7.34–7.27 (m, 1H, ArH), 7.11 (d, *J* = 7.3 Hz, 1H, ArH), 7.04 (t, *J* = 7.5 Hz, 1H, ArH), 6.96 (d, *J* = 7.7 Hz, 1H, ArH), 4.93 (s, 1H, 6-H), 4.12 (m, 1H, 12′-H), 3.95 (t, *J* = 9.3 Hz, 1H, 11′-H), 3.64 (t, *J* = 8.5 Hz, 1H, 11′-H), 3.60–3.45 (m, 1H, 3α-H), 2.22 (s, 3H, *N*-CH_3), 2.20–1.99 (m, 2H), 1.76 (m, 3H), 1.65 (t, *J* = 13.5 Hz, 2H), 1.48–1.31 (m, 2H), 1.31–1.21 (m, 2H), 1.16 (ddd, *J* = 15.5, 9.3, 4.4 Hz, 1H), 0.96 (dd, *J* = 19.6, 7.6 Hz, 1H), 0.82 (s, 3H, 19-CH_3), 0.89–0.67 (m, 2H), 0.58 (s, 3H, 18-CH_3), 0.50–0.30 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.33, 148.66, 142.30, 141.99, 141.06, 136.50, 129.84, 129.34, 128.43, 126.75, 125.11, 122.91, 122.15, 120.27, 109.99, 78.22, 71.31, 67.30, 60.77, 51.05, 49.94, 47.67, 47.06, 42.01, 36.75, 36.50, 34.85, 33.31, 31.53, 31.01, 30.68, 30.28, 19.21, 14.87. HRMS (ESI): *m*/*z* calcd for $C_{36}H_{42}N_3O_5$ $(M+H)^+$, 596.3124; found, 596.3126.

2.3.4. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(3″-methoxylphenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3d**)

Yellow solid, yield: 86%, m.p. 174.0–175.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (s, 1H, NH), 7.23 (t, *J* = 7.8 Hz, 2H, ArH), 7.13 (d, *J* = 7.5 Hz, 2H, ArH), 7.02 (t, *J* = 7.6 Hz, 2H, ArH), 6.81 (dd, *J* = 11.0, 5.1 Hz, 2H, ArH), 5.19 (d, *J* = 4.9 Hz, 1H, 6-H), 4.09–3.89 (m, 2H, 12′-H and 11′-H), 3.82 (s, 3H, CH_3O-), 3.55 (t, *J* = 7.4 Hz, 1H, 11′-H), 3.48 (m, 1H, 3α-H), 2.19 (s, 3H, *N*-CH_3), 2.28–2.10 (m, 2H), 2.03 (m, 1H), 1.95–1.74 (m, 3H), 1.74–1.57 (m, 2H), 1.46 (dd, *J* = 18.7, 8.0 Hz, 1H), 1.40–1.31 (m, 2H), 1.24–1.16 (m, 1H), 0.92 (dt, *J* = 12.4, 9.0 Hz, 2H), 0.86 (s, 3H, 19-CH_3), 0.75–0.63 (m, 1H), 0.6 (s, 3H, 18-CH_3), 0.43 (td, *J* = 11.6, 5.0 Hz, 1H), 0.34 (td, *J* = 12.7, 4.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.69, 159.70, 141.92, 141.22, 141.02, 129.41, 129.31, 128.78, 127.40, 122.74, 122.62, 120.69, 115.38, 112.84, 109.26, 78.17, 71.54, 67.77, 60.43, 60.35, 55.20, 51.59, 50.27, 47.96, 47.09, 42.13, 36.96, 36.51, 34.90, 33.19, 31.52, 31.14, 30.83, 30.27, 19.23, 14.61. HRMS (ESI): *m*/*z* calcd for $C_{37}H_{45}N_2O_4$ $(M+H)^+$, 581.3379; found, 581.3376.

2.3.5. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-fluorophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3e**)

Yellow solid, yield: 90%, m.p. 178.4–180.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (s, 1H, NH), 7.43 (s, 2H, ArH), 7.22 (t, *J* = 7.6 Hz, 1H, ArH), 7.11 (d, *J* = 7.5 Hz, 1H, ArH), 7.00 (dd, *J* = 15.7, 7.9 Hz, 3H, ArH), 6.85 (d, *J* = 7.7 Hz, 1H, ArH), 5.17 (s, 1H, 6-H), 4.06–3.93 (m, 1H, 12′-H), 3.87 (t, *J* = 9.4 Hz, 1H, 11′-H), 3.62–3.39 (m, 2H, 11′-H and 3α-H), 2.15 (s, 3H, *N*-CH_3), 2.59–2.17 (m, 3H), 2.01 (d, *J* = 4.1 Hz, 1H), 1.83 (dd, *J* = 20.4, 16.3 Hz, 2H), 1.65 (dd, *J* = 28.5, 12.8 Hz, 2H), 1.52–1.39 (m, 1H), 1.36 (d, *J* = 20.7 Hz, 2H), 1.23–1.14 (m, 1H), 0.92 (dd, *J* = 16.7, 12.1 Hz, 2H), 0.85 (s, 3H, 19-CH_3), 0.74–0.62 (m, 1H), 0.55 (s, 3H, 18-CH_3), 0.43 (td, *J* = 11.6, 5.0 Hz, 1H), 0.38–0.26 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.10, 163.11, 160.67, 142.15, 141.07, 135.34, 135.31, 131.77, 131.69, 129.52, 128.65, 127.23, 122.76, 120.65, 115.39, 115.18, 109.46, 78.21, 71.50, 67.57, 60.68, 50.86, 50.21, 47.89, 47.06, 42.14, 36.93, 36.51, 34.84, 33.21, 31.46, 31.08, 30.76, 30.27, 19.24, 14.56. HRMS (ESI): *m*/*z* calcd for $C_{36}H_{42}FN_2O_3$ $(M+H)^+$, 569.3179; found, 569.3176.

2.3.6. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(2″-fluorophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3f**)

White solid, yield: 79%, m.p. 189.4–192.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97–7.82 (m, 2H, ArH and NH), 7.26–7.09 (m, 4H, ArH), 7.07–6.93 (m, 2H, ArH), 6.80 (d, *J* = 7.7 Hz, 1H, ArH), 5.21 (d, *J* = 5.0 Hz, 1H, 6-H), 4.52 (dd, *J* = 9.7, 8.3 Hz, 1H, 12′-H), 3.91 (t, *J* = 9.4 Hz, 1H, 11′-H), 3.50 (t, *J* = 8.5 Hz, 1H, 11′-H), 3.47–3.39 (m, 1H, 3α-H), 2.18 (s, 3H, *N*-CH_3), 2.28–2.08 (m, 2H), 2.07–1.96 (m, 1H), 1.93–1.82 (m, 1H), 1.78 (d, *J* = 11.2 Hz, 2H), 1.73–1.56 (m, 2H), 1.48–1.38 (m, 1H), 1.38–1.17 (m, 3H), 0.98–0.87 (m, 2H), 0.85 (s, 3H, 19-CH_3), 0.74–0.63 (m, 1H), 0.55 (s, 3H, 18-CH_3), 0.42 (td, *J* = 11.2, 5.4 Hz, 1H), 0.31 (td, *J* = 12.6, 4.9 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.10, 178.49, 162.47, 160.03, 141.85, 141.11, 132.03, 129.46, 128.96, 128.40, 128.32, 127.30, 126.50, 126.37, 124.40, 124.36, 122.81, 120.66, 115.15, 114.92, 109.21, 78.05, 71.56, 67.05, 59.56, 50.27, 47.95, 47.20, 42.14, 36.94, 36.52, 34.88, 33.20, 31.52, 31.15, 30.95, 30.90, 30.29, 19.81, 19.23, 14.78. HRMS (ESI): *m*/*z* calcd for $C_{36}H_{42}FN_2O_3$ $(M+H)^+$, 569.3179; found, 569.3178.

2.3.7. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-chlorophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3g**)

Yellow solid, yield: 86%, m.p. 197.3–199.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H, NH), 7.39 (s, 2H, ArH), 7.27 (d, *J* = 8.4 Hz, 2H, ArH), 7.21 (t, *J* = 7.6 Hz, 1H, ArH), 7.09 (d, *J* = 7.5 Hz, 1H, ArH), 7.00 (t, *J* = 7.6 Hz, 1H, ArH), 6.83 (d, *J* = 7.7 Hz, 1H, ArH), 5.19 (d, *J* = 3.7 Hz, 1H, 6-H), 3.97 (t, *J* = 8.8 Hz, 1H, 12′-H), 3.86 (t, *J* = 9.4 Hz, 1H, 11′-H), 3.49 (m, 2H, 11′-H and 3α-H), 2.13 (s, 3H, *N*-CH_3), 2.52 (s, 1H), 2.31–2.16 (m, 2H), 2.02 (d, *J* = 4.1 Hz, 1H), 1.84 (dd, *J* = 29.2, 14.0 Hz, 2H), 1.65 (dd, *J* = 29.8, 12.8 Hz, 2H), 1.45 (dd, *J* = 23.0, 10.2 Hz, 1H), 1.33 (t, *J* = 13.3 Hz, 2H), 1.21 (d, *J* = 4.8 Hz, 1H), 1.00–0.87 (m, 2H), 0.85 (s, 3H, 19-CH_3), 0.76–0.61 (m, 1H), 0.55 (s, 3H, 18-CH_3), 0.43 (td, *J* = 11.5, 4.9 Hz, 1H), 0.34 (td, *J* = 12.5, 3.9 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.97, 142.16, 141.11, 138.18, 132.80, 131.61, 129.51, 128.67, 128.61, 127.20, 122.74, 120.61, 109.45, 78.16, 71.50, 67.50, 50.99, 50.21, 47.89, 47.07, 42.17, 36.94, 36.53, 34.80, 33.22, 31.49, 31.09, 30.77, 30.28, 21.07, 19.82, 19.23, 14.63. HRMS (ESI): *m*/*z* calcd for $C_{36}H_{42}ClN_2O_3$ $(M+H)^+$, 585.2884; found, 585.2882.

*2.3.8. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(3″-chlorophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3h**)*

White solid, yield: 83%, m.p. 227.4–229.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H, NH), 7.56 (s, 1H, ArH), 7.31 (s, 1H, ArH), 7.28–7.17 (m, 3H, ArH), 7.10 (d, J = 7.6 Hz, 1H, ArH), 7.01 (t, J = 7.5 Hz, 1H, ArH), 6.87 (d, J = 7.6 Hz, 1H, ArH), 5.11 (s, 1H, 6-H), 4.03–3.94 (m, 1H, 12′-H), 3.94–3.82 (m, 1H, 11′-H), 3.54 (t, J = 8.1 Hz, 1H, 11′-H), 3.48 (m, 1H, 3α-H), 2.17 (s, 3H, N-CH_3), 2.28–2.09 (m, 2H), 2.09–2.01 (m, 1H), 1.99 (s, 1H), 1.83 (dd, J = 29.1, 14.6 Hz, 2H), 1.65 (dd, J = 27.4, 13.0 Hz, 2H), 1.50–1.39 (m, 1H), 1.35 (d, J = 10.8 Hz, 2H), 1.20 (dd, J = 12.8, 8.1 Hz, 1H), 0.94 (dd, J = 18.4, 8.5 Hz, 2H), 0.85 (s, 3H, 19-CH_3), 0.75–0.63 (m, 1H), 0.55 (s, 3H, 18-CH_3), 0.43 (td, J = 11.6, 5.0 Hz, 1H), 0.35 (td, J = 13.0, 4.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.96, 142.07, 141.91, 140.90, 134.36, 130.18, 129.66, 129.53, 128.63, 128.51, 127.26, 127.16, 122.77, 120.66, 109.50, 78.14, 77.37, 77.06, 76.74, 71.52, 67.52, 51.23, 50.20, 47.86, 47.04, 42.13, 36.94, 36.51, 34.84, 33.17, 31.54, 31.13, 30.77, 30.29, 19.82, 19.24, 14.20. HRMS (ESI): m/z calcd for $C_{36}H_{42}ClN_2O_3$ $(M+H)^+$, 585.2884; found, 585.2886.

*2.3.9. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(2″-chlorophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3i**)*

White solid, yield: 81%, m.p. 208.7–210.7 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1H, NH), 7.94 (d, J = 7.7 Hz, 1H, ArH), 7.42 (dd, J = 7.3, 3.5 Hz, 2H, ArH), 7.35–7.17 (m, 2H, ArH), 6.95 (t, J = 7.5 Hz, 1H, ArH), 6.84 (dd, J = 16.0, 7.6 Hz, 2H, ArH), 5.16 (s, 1H, 6-H), 4.57 (dd, J = 12.8, 6.8 Hz, 2H, 12′-H and 11′-H), 3.70 (t, J = 9.2 Hz, 1H, 11′-H), 3.17 (dd, J = 10.0, 5.3 Hz, 1H, 3α-H), 2.09 (s, 3H, N-CH_3), 2.08–2.04 (m, 1H), 1.98–1.92 (m, 1H), 1.73 (d, J = 16.4 Hz, 1H), 1.57 (d, J = 10.0 Hz, 2H), 1.41 (d, J = 12.2 Hz, 1H), 1.31–1.07 (m, 5H), 0.77 (s, 3H, 19-CH_3), 0.73–0.59 (m, 3H), 0.55 (s, 3H, 18-CH_3), 0.24 (td, J = 11.2, 5.1 Hz, 1H), 0.18–0.07 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.40, 143.94, 141.90, 137.64, 134.32, 132.61, 130.02, 129.61, 129.05, 128.30, 127.87, 127.35, 121.94, 119.99, 109.85, 77.71, 70.22, 66.79, 60.58, 50.69, 47.61, 46.99, 45.29, 42.47, 37.14, 36.49, 34.63, 33.63, 31.71, 31.17, 31.07, 30.96, 30.08, 19.37, 15.51. HRMS (ESI): m/z calcd for $C_{36}H_{42}ClN_2O_3$ $(M+H)^+$, 585.2884; found, 585.2881.

*2.3.10. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(3″, 4″-dichlorophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3j**)*

Yellow solid, yield: 88%, solid, m.p. 201.4–203.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H, NH), 7.64 (s, 1H, ArH), 7.37 (d, J = 8.0 Hz, 1H, ArH), 7.32 (d, J = 6.2 Hz, 1H, ArH), 7.23 (t, J = 7.5 Hz, 1H, ArH), 7.09 (d, J = 7.4 Hz, 1H, ArH), 7.01 (t, J = 7.5 Hz, 1H, ArH), 6.86 (d, J = 7.7 Hz, 1H, ArH), 5.15 (s, 1H, 6-H), 3.94 (t, J = 8.7 Hz, 1H, 12′-H), 3.84 (t, J = 9.3 Hz, 1H, 11′-H), 3.63–3.43 (m, 2H, 11′-H and 3α-H), 2.14 (s, 3H, N-CH_3), 2.53–2.18 (m, 3H), 1.84 (dd, J = 33.8, 13.9 Hz, 2H), 1.65 (dd, J = 25.2, 12.9 Hz, 2H), 1.55–1.39 (m, 1H), 1.35 (d, J = 10.6 Hz, 2H), 1.31–1.16 (m, 1H), 1.00–0.87 (m, 2H), 0.85 (s, 3H, 19-CH_3), 0.75–0.62 (m, 1H), 0.58 (s, 3H, 18-CH_3), 0.49–0.30 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.01, 142.14, 141.05, 140.24, 132.46, 132.03, 131.03, 130.38, 129.71, 129.64, 128.56, 126.98, 122.82, 120.55, 109.61, 78.10, 71.48, 67.28, 50.73, 50.18, 47.85, 47.09, 42.16, 36.93, 36.52, 34.77, 33.21, 31.49, 31.09, 30.74, 30.29, 26.90, 21.08, 19.23, 14.78. HRMS (ESI): m/z calcd for $C_{36}H_{41}Cl_2N_2O_3$ $(M+H)^+$, 619.2494; found, 619.2493.

*2.3.11. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-phenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3k**)*

White solid, yield: 85%, m.p. 246.9–248.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (s, 1H, NH), 7.34 (dd, J = 12.8, 5.5 Hz, 4H, ArH), 7.29–7.18 (m, 2H, ArH), 6.93 (t, J = 7.5 Hz, 1H, ArH), 6.87 (d, J = 7.4 Hz, 1H, ArH), 6.80 (d, J = 7.7 Hz, 1H, ArH), 5.16 (d, J = 4.2 Hz, 1H, 6-H), 4.57 (d, J = 4.6 Hz, 1H, 12′-H), 3.80 (m, 2H, 12′-H and 11′-H), 3.18 (m, 1H, 3α-H), 2.09 (s, 3H, N-CH_3), 2.09–2.06 (m, 2H), 2.01–1.93 (m, 1H), 1.77 (d, J = 16.3 Hz, 1H), 1.58 (d, J = 10.2 Hz, 2H), 1.42 (d, J = 12.4 Hz, 1H), 1.33–1.19 (m, 4H), 1.14–1.08 (m, 2H), .0.77 (s, 3H, 19-CH_3), 0.71–0.63 (m, 2H), 0.44 (s, 3H, 18-CH_3), 0.26 (td, J = 11.5, 4.8 Hz, 1H), 0.17 (td, J = 12.5, 4.0 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.38, 143.92, 141.91, 140.04, 130.26, 129.88, 128.85, 128.20, 127.55, 127.42, 121.82, 119.99, 109.73, 77.74, 70.24, 67.66, 60.37, 51.17, 50.70, 47.67, 46.74, 42.48, 37.15, 36.49, 34.72, 33.24, 31.73, 31.16, 31.04, 30.96, 30.12, 19.36, 14.77. HRMS (ESI): m/z calcd for $C_{36}H_{43}N_2O_3$ $(M+H)^+$, 551.3274; found, 551.3268.

*2.3.12. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-methylphenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3l**)*

Yellow solid, yield: 82%, m.p. 184.8–186.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (s, 1H, NH), 7.33 (d, J = 5.5 Hz, 2H, ArH), 7.21 (t, J = 7.6 Hz, 1H, ArH), 7.12 (d, J = 7.6 Hz, 3H, ArH), 7.00 (t, J = 7.5 Hz, 1H, ArH), 6.83 (d, J = 7.5 Hz, 1H, ArH), 5.20 (s, 1H, 6-H), 4.06–3.95 (m, 1H, 12′-H), 3.96–3.80 (m, 1H, 11′-H), 3.60–3.36 (m, 2H, 11′-H and 3α-H), 2.33 (s, 3H, CH_3Ph-), 2.15 (s, 3H, N-CH_3), 2.28–2.17 (m, 2H), 2.10–2.01 (m, 1H), 1.84 (dd, J = 33.5, 14.3 Hz, 2H), 1.64 (dd, J = 30.2, 12.9 Hz, 2H), 1.51–1.29 (m, 4H), 1.24–1.12 (m, 1H), 0.96–0.87 (m, 2H), .0.86 (s, 3H, 19-CH_3), 0.74–0.62 (m, 1H), 0.44 (s, 3H, 18-CH_3), 0.43 (td, J = 11.6, 5.0 Hz, 1H), 0.33 (td, J = 12.6, 4.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.00, 178.91, 142.17, 142.09, 141.04, 136.55, 136.40, 130.13, 129.37, 129.16, 128.75, 127.47, 122.65, 120.75, 109.38, 78.23, 71.53, 67.77, 51.30, 50.23, 47.96, 47.06, 42.17, 36.95, 36.53, 34.87, 33.15, 31.53, 31.11, 30.83, 30.29, 21.14, 19.25, 14.53. HRMS (ESI): m/z calcd for $C_{37}H_{45}N_2O_3$ $(M+H)^+$, 565.3430; found, 565.3426.

*2.3.13. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″isopropylphenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3m**)*

Yellow solid, yield: 73%, m.p. 186.4–188.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61 (s, 1H, NH), 7.36 (d, J = 6.4 Hz, 2H, ArH), 7.27–7.06 (m, 4H, ArH), 7.01 (t, J = 7.6 Hz, 1H, ArH), 6.83 (d, J = 7.6 Hz, 1H, ArH), 5.23 (d, J = 3.5 Hz, 1H, 6-H), 4.04–3.96 (m, 1H, 12′-H), 3.96–3.87 (m, 1H, 11′-H), 3.49 (m, 2H, 11′-H and 3α-H), 2.87 (heptet, J = 6.9 Hz, 1H, —C<u>H</u>(CH_3)$_2$), 2.46–2.18 (m, 4H), 2.15 (s, 3H, N-CH_3), 2.10–2.00 (m, 1H), 1.91 (d, J = 16.8 Hz, 1H), 1.81 (d, J = 11.4 Hz, 1H), 1.69 (d, J = 13.3 Hz, 1H), 1.62 (d, J = 12.3 Hz, 1H), 1.53–1.29 (m, 3H), 1.25 (d, J = 6.9 Hz, 6H, —CH(C<u>H</u>$_3$)$_2$), 1.00–0.88 (m, 2H), 0.86 (s, 3H, 19-CH_3), 0.75–0.63 (m, 1H), 0.57 (s, 3H, 18-CH_3), 0.44 (td, J = 11.3, 4.9 Hz, 1H), 0.34 (td, J = 12.6, 4.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.89, 147.44, 142.12, 141.10, 136.68, 130.11, 129.36, 128.75, 127.49, 126.47, 122.66, 120.77, 109.34, 78.22, 71.54, 67.82, 51.28, 50.24, 47.98, 47.07, 42.15, 36.97, 36.56, 34.86, 33.66, 33.11, 31.52, 31.13, 30.82, 30.32, 23.98, 19.86, 19.28, 14.57. HRMS (ESI): m/z calcd for $C_{39}H_{49}N_2O_3$ $(M+H)^+$, 593.3743; found, 593.3742.

*2.3.14. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-tert-buthylphenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3n**)*

Yellow solid, yield: 83%, m.p. 205.4–207.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (s, 1H, NH), 7.43–7.28 (m, 4H, ArH), 7.21 (m, 1H, ArH), 7.12 (d, J = 7.5 Hz, 1H, ArH), 7.00 (t, J = 7.7 Hz, 1H, ArH), 6.81 (d, J = 7.6 Hz, 1H, ArH), 5.23 (d, J = 4.7 Hz, 1H, 6-H), 3.99 (dd, J = 9.8, 7.6 Hz, 1H, 12′-H), 3.96–3.86 (m, 1H, 11′-H), 3.48 (m, 2H, 11′-H and 3α-H), 2.14 (s, 3H, N-CH_3), 2.30–2.07 (m, 4H), 2.08–1.99 (m, 1H), 1.96–1.85 (m, 1H), 1.79 (d, J = 11.6 Hz, 1H), 1.69 (d, J = 13.4 Hz, 1H), 1.61 (d, J = 12.6 Hz, 1H), 1.47 (dd, J = 19.1, 8.6 Hz, 1H), 1.31 (s, 9H, —C(C<u>H</u>$_3$)$_3$), 1.25–1.21 (m, 1H), 0.99–0.87 (m, 2H), 0.86 (s, 3H,

19-CH_3), 0.74–0.63 (m, 1H), 0.57 (s, 3H, 18-CH_3), 0.43 (td, J = 11.5, 5.0 Hz, 1H), 0.33 (td, J = 12.9, 4.7 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.76, 149.73, 142.03, 141.12, 136.30, 129.82, 129.35, 128.78, 127.50, 125.31, 122.67, 120.77, 109.28, 78.19, 71.55, 67.82, 51.14, 50.25, 47.99, 47.07, 42.14, 36.97, 36.56, 34.88, 34.45, 33.10, 31.51, 31.39, 31.15, 30.82, 30.31, 19.28, 14.59. HRMS (ESI): m/z calcd for $C_{40}H_{51}N_2O_3$ $(M+H)^+$, 607.3900; found, 607.3884.

*2.3.15. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-naphthyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3o**)*

White solid, yield: 79%, m.p. 262.0–264.2 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1H, NH), 7.94 (t, J = 6.9 Hz, 2H, ArH), 7.83 (dd, J = 16.4, 8.0 Hz, 2H, ArH), 7.61 (t, J = 7.6 Hz, 1H, ArH), 7.49 (d, J = 3.7 Hz, 2H, ArH), 7.25 (t, J = 7.4 Hz, 1H, ArH), 7.11–6.88 (m, 2H, ArH), 6.82 (d, J = 7.6 Hz, 1H, ArH), 5.11 (s, 1H, 6-H), 4.88 (t, J = 8.6 Hz, 1H, 12′-H), 4.55 (d, J = 4.3 Hz, 1H, —OH), 4.13–3.90 (m, 1H, 11′-H), 3.42 (t, J = 7.7 Hz, 1H, 11′-H), 3.16 (d, J = 3.7 Hz, 1H, 3α-H), 2.05 (s, 3H, N-CH_3), 1.99–1.91 (m, 2H), 1.68 (d, J = 16.5 Hz, 1H), 1.53 (d, J = 13.3 Hz, 2H), 1.37 (d, J = 11.8 Hz, 1H), 1.31–1.11 (m, 4H), 1.11–0.90 (m, 2H), 0.78–0.58 (m, 2H), 0.67 (s, 3H, 19-CH_3), 0.51 (t, J = 13.9 Hz, 1H), 0.18 (t, J = 11.1 Hz, 1H), 0.17 (s, 3H, 18-CH_3), 0.09–0.04 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.19, 143.98, 141.82, 136.00, 133.91, 132.62, 129.99, 129.34, 128.32, 128.10, 127.64, 127.55, 126.67, 126.17, 126.06, 123.45, 121.93, 120.02, 109.82, 99.99, 77.81, 70.21, 67.98, 60.71, 50.69, 47.50, 47.19, 42.91, 42.45, 37.13, 36.44, 34.76, 33.48, 31.70, 31.03, 29.95, 19.83, 19.23, 15.81. HRMS (ESI): m/z calcd for $C_{40}H_{45}N_2O_3$ $(M+H)^+$, 601.3430; found, 601.3428.

*2.3.16. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-pyridyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3p**)*

White solid, yield: 87%, m.p. 189.9–191.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (dd, J = 4.8, 0.9 Hz, 1H, ArH), 7.98–7.82 (m, 2H, ArH and NH), 7.69 (m, 1H, ArH), 7.22 (m, 1H, ArH), 7.19–7.11 (m, 2H, ArH), 7.08–6.92 (m, 1H, ArH), 6.80 (d, J = 7.6 Hz, 1H, ArH), 5.20 (d, J = 5.1 Hz, 1H, 6-H), 4.29 (dd, J = 9.8, 8.3 Hz, 1H, 12′-H), 4.03 (t, J = 9.5 Hz, 1H, 11′-H), 3.64 (t, J = 8.6 Hz, 1H, 11′-H), 3.52–3.38 (m, 1H, 3α-H), 2.18 (s, 3H, N-CH_3), 1.97 (dd, J = 13.5, 4.3 Hz, 1H), 1.89 (d, J = 21.5 Hz, 1H), 1.84–1.72 (m, 2H), 1.72–1.56 (m, 2H), 1.49–1.38 (m, 1H), 1.38–1.14 (m, 5H), 0.96–0.81 (m, 1H), 0.85 (s, 3H, 19-CH_3),0.78 (d, J = 13.7 Hz, 1H), 0.70 (s, 3H, 18-CH_3), 0.67–0.63 (m, 1H), 0.47–0.27 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.55, 160.28, 148.87, 141.85, 141.15, 136.56, 129.46, 128.93, 127.22, 124.22, 122.78, 121.93, 120.63, 109.23, 78.10, 71.54, 67.21, 58.90, 53.39, 50.24, 47.99, 47.24, 42.13, 36.94, 36.52, 34.97, 32.83, 31.53, 31.13, 30.95, 30.92, 30.33, 19.24, 14.90. HRMS (ESI): m/z calcd for $C_{35}H_{42}N_3O_3$ $(M+H)^+$, 552.3226; found, 552.3223.

*2.3.17. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-furyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3q**)*

Yellow solid, yield: 88%, m.p. 180.0–181.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (s, 1H, NH), 7.34 (d, J = 1.0 Hz, 1H, ArH), 7.21 (dd, J = 11.0, 4.2 Hz, 1H, ArH), 7.08 (d, J = 7.4 Hz, 1H, ArH), 6.99 (t, J = 7.5 Hz, 1H, ArH), 6.79 (d, J = 7.7 Hz, 1H, ArH), 6.41 (d, J = 3.1 Hz, 1H, ArH), 6.38–6.31 (m, 1H, ArH), 5.23 (d, J = 4.7 Hz, 1H, 6-H), 4.12 (m, 1H, 12′-H), 3.96 (t, J = 9.3 Hz, 1H, 11′-H), 3.51 (t, J = 8.5 Hz, 1H, 11′-H), 3.48–3.41 (m, 1H, 3α-H), 2.16 (s, 3H, N-CH_3), 2.28–2.09 (m, 2H), 2.01 (d, J = 4.6 Hz, 1H), 1.97–1.74 (m, 3H), 1.74–1.58 (m, 1H), 1.50–1.30 (m, 2H), 1.04 (t, J = 14.1 Hz, 1H), 0.98–0.84 (m, 1H), 0.89 (s, 3H, 19-CH_3), 0.79 (s, 3H, 18-CH_3), 0.66–0.61 (m, 1H), 0.49–0.32 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.19, 154.35, 141.96, 141.52, 141.05, 129.49, 128.50, 126.97, 122.70, 120.77, 110.59, 109.27, 108.07, 77.83, 71.56, 67.02, 57.20, 50.25, 47.93, 47.62, 44.10, 42.15, 36.97, 36.54, 34.95, 32.09, 31.53, 31.12, 30.91, 30.40, 21.07, 19.28, 15.10. HRMS (ESI): m/z calcd for $C_{34}H_{41}N_2O_4$ $(M+H)^+$, 541.3066; found, 541.3063.

*2.3.18. Spiro [2′.16]-1′-methyl-2′-(indolin-2-one)-4′-(4″-thienyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3r**)*

Yellow solid, yield: 89% m.p. 192.6–193.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (s, 1H, NH), 7.27–7.18 (m, 2H, ArH), 7.09 (dd, J = 13.9, 5.2 Hz, 2H, ArH), 7.05–6.94 (m, 2H, ArH), 6.84 (d, J = 7.7 Hz, 1H, ArH), 5.22 (t, J = 12.9 Hz, 1H, 6-H), 4.31 (t, J = 8.8 Hz, 1H, 12′-H), 3.97 (t, J = 9.4 Hz, 1H, 11′-H), 3.64 (t, J = 8.6 Hz, 1H, 11′-H), 3.55–3.41 (m, 1H, 3α-H), 2.18 (s, 3H, N-CH_3), 2.30–2.10 (m, 2H), 2.05–1.98 (m, 1H), 1.95–1.74 (m, 4H), 1.67 (dd, J = 25.4, 13.0 Hz, 2H), 1.49–1.35 (m, 3H), 1.10 (t, J = 14.1 Hz, 1H), 1.00–0.81 (m, 1H), 0.88 (s, 3H, 19-CH_3), 0.76–0.61 (m, 1H), 0.70 (s, 3H, 18-CH_3), 0.47–0.36 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.52, 142.96, 142.08, 140.98, 129.53, 128.48, 127.17, 127.04, 126.92, 124.78, 122.75, 120.76, 109.40, 77.98, 71.55, 67.51, 61.12, 50.24, 47.83, 47.27, 45.75, 42.15, 36.96, 36.52, 34.87, 32.60, 31.52, 31.12, 30.81, 30.32, 21.07, 19.26, 14.21. HRMS (ESI): m/z calcd for $C_{34}H_{41}N_2O_3S$ $(M+H)^+$, 557.2838; found, 557.2836.

*2.3.19. Spiro [2′.16]-1′-methyl-2′-(5‴-fluoro-indolin-2-one)-4′-(4″-chlorophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3s**)*

Yellow solid, yield: 79%, m.p. 187.5–189.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (Brs, 1H, NH), 7.39 (d, J = 6.8 Hz, 2H, ArH), 7.30 (s, 1H, ArH), 7.27 (d, J = 2.6 Hz, 1H, ArH), 7.03–6.82 (m, 2H, ArH), 6.76 (dd, J = 8.4, 4.2 Hz, 1H, ArH), 5.21 (d, J = 4.0 Hz, 1H, 6-H), 3.96 (dd, J = 9.7, 8.0 Hz, 1H, 12′-H), 3.84 (t, J = 9.4 Hz, 1H, 11′-H), 3.51 (t, J = 8.4 Hz, 1H, 11′-H), 3.48–3.39 (m, 1H, 3α-H), 2.16 (s, 3H, N-CH_3), 2.33–2.09 (m, 2H), 2.02 (dd, J = 14.5, 5.1 Hz, 1H), 1.89 (d, J = 15.2 Hz, 1H), 1.79 (d, J = 11.9 Hz, 2H), 1.70 (dd, J = 13.2, 3.2 Hz, 3H), 1.45–1.38 (m, 2H), 1.36–1.19 (m, 1H), 1.00–0.90 (m, 1H), 0.86 (s, 3H, 19-CH_3), 0.81–0.74 (m, 1H), 0.72–0.59 (m, 1H), 0.57 (s, 3H, 18-CH_3), 0.51–0.31 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.58, 160.14, 157.74, 141.16, 137.92, 137.72, 132.95, 131.58, 129.34, 129.26, 128.67, 120.52, 116.97, 116.71, 116.07, 115.83, 109.86, 109.78, 78.26, 71.57, 67.78, 60.53, 51.06, 50.32, 48.29, 47.16, 42.14, 36.95, 36.53, 34.83, 33.14, 31.50, 31.11, 30.95, 30.28, 22.66, 19.84, 19.22, 14.57. HRMS (ESI): m/z calcd for $C_{36}H_{41}ClFN_2O_3$ $(M+H)^+$, 603.2790; found, 603.2783.

*2.3.20. Spiro [2′.16]-1′-methyl-2′-(5‴-bromo-indolin-2-one)-4′-(4″-chlorophenyl)-tetrahydro-1H-pyrrolo-dehydroandrosterone (**3t**)*

White solid, yield: 76%, m.p. 203.4–207.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.95 (s, 1H, NH), 7.46–7.32 (m, 3H, ArH), 7.28 (d, J = 8.1 Hz, 2H, ArH), 7.24 (d, J = 1.9 Hz, 1H, ArH), 6.75 (d, J = 8.2 Hz, 1H, ArH), 5.19 (d, J = 4.6 Hz, 1H, 6-H), 4.04–3.89 (m, 1H, 12′-H), 3.83 (t, J = 9.4 Hz, 1H, 11′-H), 3.62–3.41 (m, 2H, 11′-H and 3α-H), 2.15 (s, 3H, N-CH_3), 2.42 (brs, 1H), 2.30–2.09 (m, 2H), 2.01 (dd, J = 13.9, 4.3 Hz, 1H), 1.94–1.78 (m, 2H), 1.78–1.67 (m, 2H), 1.47 (dd, J = 26.0, 12.5 Hz, 2H), 1.34 (d, J = 9.9 Hz, 2H), 1.02–0.89 (m, 2H), 0.87 (s, 3H, 19-CH_3), 0.71–0.60 (m, 1H), 0.58 (s, 3H, 18-CH_3), 0.51–0.38 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.42, 141.17, 141.12, 137.89, 132.94, 132.37, 131.78, 131.57, 129.49, 128.68, 120.54, 115.58, 110.90, 78.05, 71.57, 67.74, 51.01, 50.26, 48.27, 47.17, 42.17, 36.95, 36.56, 34.89, 33.12, 31.51, 31.11, 30.65, 30.29, 19.26, 14.64. HRMS (ESI): m/z calcd for $C_{36}H_{41}BrClN_2O_3$ $(M+H)^+$, 663.1989; found, 663.1990.

*2.4. General procedure for the synthesis of phenyl-linked 16-methylene-17-ketosteroid dimer (**5**)*

A mixture of dehydroepiandrosterone (DHEA, **1**) (2.0 mmol), isophthalaldehyde (1.1 mmol) and KF/Al_2O_3 (1.0 mmol) in EtOH

(20 mL) was heated under reflux for about 1 h. After completion of the reaction as evident from TLC (petroleum ether/ethyl acetate = 3/1), the slurry was filtered and the residue was washed thoroughly with CH_2Cl_2. The filtrate was condensed under reduced pressure, and the solid obtained was crystallized from EtOH to yield the phenyl linked 16-methylene-17-ketosteroid dimer **5**. White solid, yield 90%, mp 222.9–224.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (s, 1H, Ar–H), 7.55 (d, J = 7.8 Hz, 2H, Ar–H), 7.52–7.48 (m, 1H, Ar–H), 7.46 (s, 2H, Ar–CH=), 5.41 (d, J = 4.8 Hz, 2H, 6-H), 3.61–3.50 (m, 2H, 3α-H), 2.92 (dd, J = 16.0, 5.6 Hz, 2H), 2.55–2.42 (m, 2H), 2.40–2.26 (m, 4H), 2.27–2.16 (m, 2H), 2.01 (d, J = 12.5 Hz, 2H), 1.95–1.79 (m, 6H), 1.79–1.64 (m, 4H), 1.64–1.48 (m, 4H), 1.49–1.34 (m, 4H), 1.18–1.04 (m, 4H), 1.10 (s, 6H, 19-CH_3), 1.01 (s, 6H, 18-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 209.53, 141.19, 136.72, 136.08, 132.43, 131.90, 130.84, 129.08, 120.75, 71.59, 50.32, 49.78, 47.39, 42.22, 37.14, 36.76, 31.59, 31.56, 31.27, 31.04, 29.51, 20.40, 19.46, 14.24; HRMS (ESI): m/z cacld for $C_{46}H_{58}O_4Na$ (M+Na)$^+$, 697.4233; found, 697.4235.

2.5. General procedure for the synthesis of dimeric steroidal spiro-oxindoles (**6a**–**c**)

A mixture of 16-methylene-17-ketosteroid dimer (**5**, 0.5 mmol) (substituted) isatin (2.5 mmol) and sarcosine (2.5 mmol) in methanol/1, 4-dioxane mixture (v/v = 1/1, 20 mL) was refluxed for about 5 h. After completion of the reaction as evident from TLC (petroleum ether/ethyl acetate = 2/1), the mixture was evaporated and purified by column chromatography on silica gel using petroleum ether/ethyl acetate = 2/1(v/v) as the eluent to obtain the pure products **6a**–**c**.

2.5.1. Steroid dimer **6a**

White solid, yield: 73%, m.p. 233.2–235.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, 1H, NH), 7.88–7.59 (m, 1H, ArH), 7.46 (s, 1H, ArC<u>H</u>=), 7.36 (m, 3H, ArH), 7.23 (t, J = 7.6 Hz, 1H, ArH), 7.12 (d, J = 7.4 Hz, 1H, ArH), 7.02 (t, J = 7.5 Hz, 1H, ArH), 6.83 (d, J = 7.7 Hz, 1H, ArH), 5.40 (s, 1H, 6-H), 5.20 (d, J = 4.1 Hz, 1H, 6-H), 4.11–4.00 (m, 1H, 12′-H), 3.93 (t, J = 9.5 Hz, 1H, 11′-H), 3.51 (m, 3H, 11′-H and 3α-H), 2.17 (s, 3H, N-CH_3), 2.86 (dd, J = 16.1, 6.0 Hz, 1H), 2.63 (d, J = 6.9 Hz, 1H), 2.37–2.19 (m, 4H), 2.06–1.94 (m, 3H), 1.87–1.83 (m, 5H), 1.73–1.47 (m, 7H), 1.47–1.30 (m, 7H), 1.08 (s, 3H, 19-CH_3), 1.00 (s, 3H, 19-CH_3), 0.99–0.91 (m, 2H), 0.83 (s, 3H, 18-CH_3), 0.81–0.75 (m, 1H), 0.76–0.64 (m, 1H), 0.54 (s, 3H, 18-CH_3), 0.48–0.37 (m, 1H), 0.32 (td, J = 12.7, 4.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.88, 178.46, 141.98, 141.38, 141.25, 140.11, 136.32, 135.88, 133.13, 131.42, 129.48, 128.84, 128.75, 127.33, 122.75, 120.83, 120.52, 109.34, 78.04, 71.57, 71.48, 67.74, 60.51, 51.61, 50.36, 50.21, 50.06, 47.98, 47.45, 47.04, 42.25, 42.13, 38.97, 37.13, 36.93, 36.77, 36.53, 34.84, 33.40, 31.59, 31.44, 31.22, 30.81, 30.26, 29.69, 29.66, 29.52, 22.66, 20.44, 19.84, 19.47, 19.22, 19.18, 14.64, 14.34. HRMS (ESI): m/z calcd for $C_{56}H_{69}N_2O_5$ (M+H)$^+$, 849.5206; found, 849.5208.

2.5.2. Steroid dimer **6b**

White solid, yield: 71%, m.p. 240.3–242.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (s, 1H, NH), 7.73 (s, 1H, ArC<u>H</u>=), 7.52–7.29 (m, 4H, ArH), 7.04–6.86 (m, 2H, ArH), 6.78 (dd, J = 8.4, 4.2 Hz, 1H, ArH), 5.39 (s, 1H, 6-H), 5.20 (d, J = 3.9 Hz, 1H, 6-H), 4.08–3.98 (m, 1H, 12′-H), 3.90 (t, J = 9.4 Hz, 1H, 11′-H), 3.65–3.37 (m, 3H, 11′-H and 3α-H), 2.18 (s, 3H, N-CH_3), 2.86 (dd, J = 16.0, 5.8 Hz, 1H), 2.68–2.48 (m, 1H), 2.40–2.20 (m, 5H), 2.15–2.07 (m, 2H), 2.00–1.83 (m, 8H), 1.70 (d, J = 13.0 Hz, 4H), 1.64–1.48 (m, 3H), 1.47–1.31 (m, 6H), 1.08 (s, 3H, 19-CH_3), 1.00 (s, 3H, 19-CH_3), 0.84 (s, 3H, 18-CH_3), 0.81–0.75 (m, 1H), 0.75–0.62 (m, 1H), 0.56 (s, 3H, 18-CH_3), 0.44 (ddd, J = 24.8, 12.1, 6.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.90, 178.37, 160.10, 157.70, 141.39, 141.32, 139.89, 137.91, 136.33, 135.92, 133.11, 131.41, 129.51, 129.38, 129.30, 128.79, 120.82, 120.43, 116.98, 116.72, 116.05, 115.82, 109.88, 109.80, 78.18, 71.58, 71.50, 67.90, 51.69, 50.34, 50.28, 50.03, 48.30, 47.45, 47.14, 42.25, 42.11, 38.97, 38.74, 37.12, 36.93, 36.76, 36.54, 34.85, 34.12, 33.31, 31.59, 31.46, 31.22, 30.96, 30.27, 29.66, 29.49, 27.95, 22.96, 22.66, 20.44, 20.15, 19.85, 19.48, 19.23, 19.17, 14.58, 14.41, 14.33, 14.16, 14.11, 11.40. HRMS (ESI): m/z calcd for $C_{56}H_{68}FN_2O_5$ (M+H)$^+$, 867.5112; found, 867.5114.

2.5.3. Steroid dimer **6c**

White solid, yield: 68%, m.p. 233.5–235.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 (d, J = 48.3 Hz, 1H, NH), 7.69 (s, 1H, ArC<u>H</u>=), 7.55–7.32 (m, 4H, ArH), 7.32–7.20 (m, 1H, ArH), 7.02 (dt, J = 15.6, 7.6 Hz, 2H, ArH), 5.42 (s, 1H, 6-H), 5.22 (s, 1H, 6-H), 4.11–3.98 (m, 1H, 12′-H), 3.92 (t, J = 9.5 Hz, 1H, 11′-H), 3.66–3.42 (m, 3H, 11′-H and 3α-H), 2.19 (s, 3H, N-CH_3), 2.87 (dd, J = 16.1, 6.0 Hz, 1H), 2.60 (dd, J = 27.3, 13.8 Hz, 1H), 2.42–2.21 (m, 5H), 2.12 (dd, J = 14.2, 4.3 Hz, 2H), 1.95 (dd, J = 41.1, 11.4 Hz, 9H), 1.75–1.52 (m, 6H), 1.40–1.30 (m, 4H), 1.10 (s, 3H, 19-CH_3), 1.02 (s, 3H, 19-CH_3), 1.00–0.91 (m, 3H), 0.85 (s, 3H, 18-CH_3), 0.71–0.57 (m, 1H), 0.55 (s, 3H, 18-CH_3), 0.48 (td, J = 11.7, 5.0 Hz, 1H), 0.33 (td, J = 12.6, 4.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 209.82, 177.63, 141.41, 141.06, 139.78, 139.54, 136.36, 135.94, 133.02, 131.35, 129.51, 129.33, 128.93, 128.84, 127.07, 123.56, 120.75, 120.47, 114.43, 79.07, 71.58, 71.38, 67.95, 60.48, 51.78, 50.34, 50.17, 49.99, 48.29, 47.43, 47.09, 42.25, 42.08, 37.14, 36.89, 36.79, 36.59, 34.91, 33.36, 31.61, 31.56, 31.39, 31.29, 31.23, 31.17, 30.89, 30.34, 29.50, 26.91, 22.66, 20.44, 19.79, 19.50, 19.22, 14.57, 14.31, 14.12. HRMS (ESI): m/z calcd for $C_{56}H_{68}ClN_2O_5$ (M+H)$^+$, 883.4817; found, 883.4816.

2.6. Cell culturing

Human cancer cell lines were maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cancer cells were maintained in RPMI1640 medium. All cell lines were purchased from the China Center for Type Culture Collection (CCTCC, Shanghai, China). For pharmacological investigations, 10 mM stock solutions of the tested compounds were prepared with dimethyl sulfoxide (DMSO). The highest DMSO concentration of the medium (0.1%) did not have any substantial effect on the determined cellular functions.

2.7. Antiproliferative assays

Exponentially growing cells were seeded into 96-well plates at a concentration of 5×10^3 cells per well. After 24 h incubation at 37 °C, the culture medium was removed and replaced with fresh medium containing the candidate compounds in different concentrations. The cells were incubated for another 72 h. Then, 20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL) was added to all wells and incubated for 4 h at 37 °C. Discarded the suspension and added 150 μL of dimethyl sulfoxide (DMSO) to each well and shook the plates to dissolve the dark blue crystals (formazan); the absorbance was measured using a microplate reader at the wavelength of 490 nm. Each concentration was analyzed in triplicate and the experiment was repeated three times. The average 50% inhibitory concentration (IC_{50}) was determined from the dose-response curves according to the inhibition ratio for each concentration.

2.8. Analysis of cellular apoptosis

MGC-803 cells were plated in 6-well plates (5.0×10^6 cells/mL) and incubated at 37 °C for 12 or 24 h. Exponentially growing cells were then incubated for 12 or 24 h with complete medium (blank) or with the compound **3n**. Cells were then harvested and the

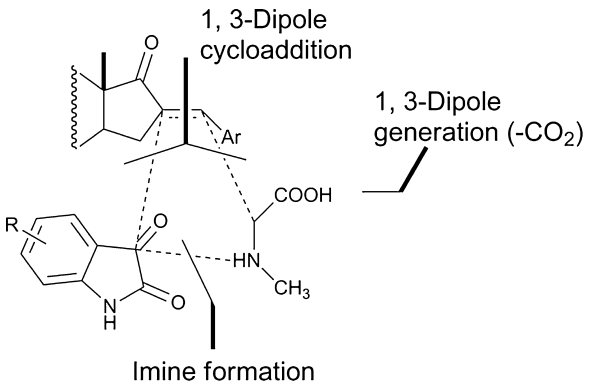


Fig. 2. The strategy for the synthesis of steroidal spiro-pyrrolidinyl oxindoles.

Annexin-V-FITC/PI apoptosis kit (Biovision) was used according to the manufacturer's instructions to detect apoptotic cells. Ten thousand events were collected for each sample and analyzed by Accuri C6 flowcytometer.

2.9. Flow cytometric analysis of cell cycle distribution

For flow cytometric analysis of DNA content, 5.0×10^6 MGC-803 cells in exponential growth were treated with different concentrations of the test compounds for 12 or 24 h. After an incubation period, the cells were collected, centrifuged and fixed with ice-cold ethanol (70%). The cells were then treated with buffer containing RNAse A and 0.1% Triton X-100 and then stained with PI. Samples were analyzed on Accuri C6 flow cytometer (Becton, Dickinson). Data obtained from the flow cytometer was analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

3. Results and discussion

3.1. Chemistry

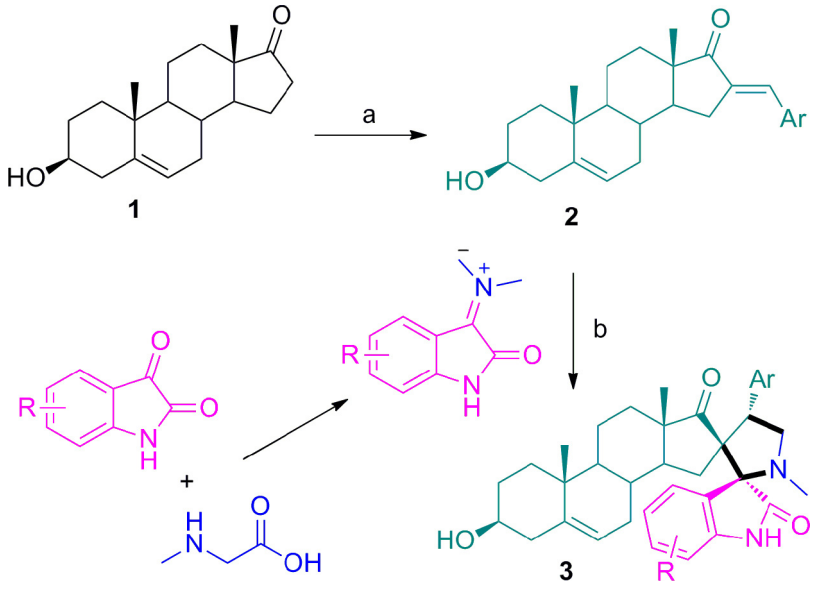
As shown in Fig. 2, Our approach involved the 1, 3-dipolar cycloaddition between (*E*)-3β-hydroxy-5-en-16-arylidene-17-ketosteroids (**2**) and azomethine ylides generated *in situ* from substituted isatins and sarcosine. Initially, (*E*)-3β-hydroxy-5-en-16-arylidene-17-ketosteroids were prepared by the KF/Al_2O_3-catalyzed Claisen-Schmidt condensation of dehydroepiandrosterone (DHEA, **1**) with aromatic aldehydes in excellent yields following the procedure previously reported by our group [30]. Subsequent 1,3-dipolar cycloaddition of azomethine ylides, which were generated from the decarboxlative condensation of isatins and sarcosine, with **2** afforded novel steroidal spiro-pyrrolidinyl oxindoles **3** (Scheme 1). Apart from the expected products, some uncharacterisable impurities were also generated in this reaction.

Table 1
Optimization for the synthesis of **3a**.

Entry	Solvent	Yield [a] (%)
1	Methanol	13
2	Methanol/H_2O (1:1)	0
3	Toluene	44
4	Acetonitrile	60
5	1,4-Dioxane	63
6	Methanol/1,4-dioxane (1:1)	75
7[b]	Methanol/1,4-dioxane (1:1)	89

[a] Isolated yield after purification by column chromatography.
[b] The ratio of **2a**, isatin and sarcosine was 1:1.5:2.0.

Solvent-optimization for this cycloaddition was investigated by the choosing the model three-component reaction between 16-(4-methylsulfonylbenzylidene)-17-ketosteroid (**2a**, 1 mmol), isatin (1 mmol) and sarcosine (1 mmol) in toluene, methanol, methanol/H_2O mixture (v/v = 1/1), acetonitrile, 1, 4-dioxane, methanol/1, 4-dioxane mixture (v/v = 1/1) under reflux. As can be seen from Table 1, the best result was obtained by refluxing the reaction mixture in methanol/1, 4-dioxane for 5 h to furnish steroidal spiro-pyrrolidinyl oxindole **3a** in 75% yield (Table 1, entry 6), 12%, 31% and 15% higher than those in 1, 4-dioxane, toluene and acetonitrile, respectively (Table 1, entries 3–5). This could be ascribed to the diminished stabilization of the polar transition states involved in this reaction in 1, 4-dioxane, toluene and acetonitrile. The yields were very low when this reaction was performed in methanol or methanol/H_2O mixture due to the poor solubility of 17-ketosteroid **2a** (Table 1, entries 1–2). The reaction when performed with 2 moles of sarcosine and 1.5 moles isatin per 1 mole of 17-ketosteroid **2a** afforded a remarkably enhanced 89% yield (Table 1, entry 7). The optimum yield was obtained in methanol/1, 4-dioxane when the ratio of 17-ketosteroid, isatin and sarcosine



Scheme 1. Synthesis of steroidal spiro-pyrrolidinyl oxindoles (**3**). Reagents and conditions: (a) Aldehydes, KF/Al_2O_3, EtOH, reflux; (b) 1, 4-Dioxane/MeOH (v/v = 1/1), 100 °C.

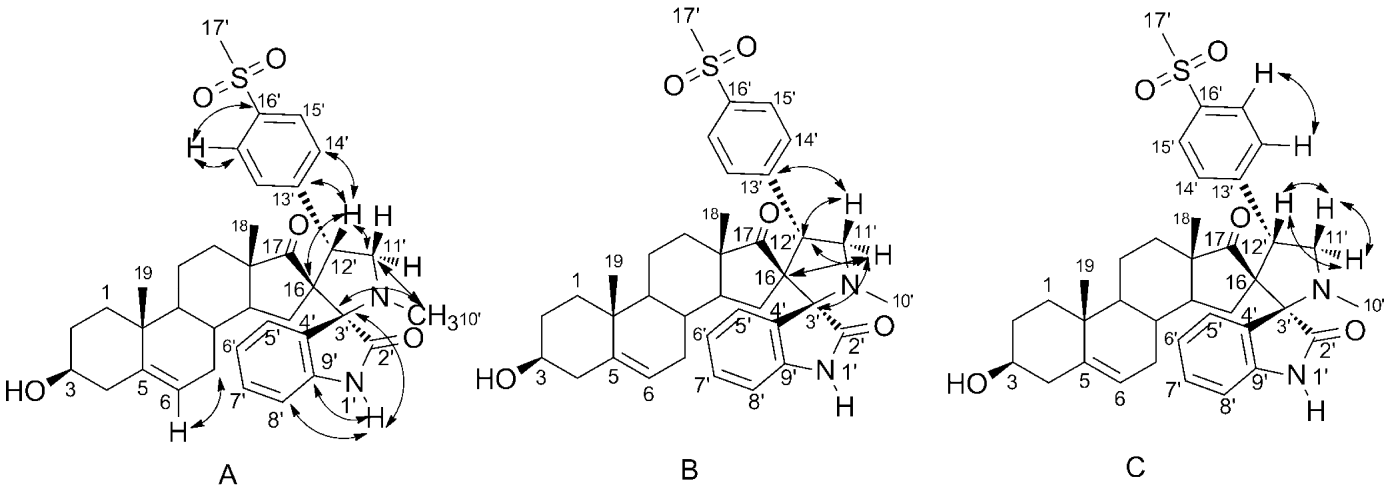


Fig. 3. Selected HMBC (A and B) and H–H COSY (C) correlations of **3a** (arbitrary numbering).

was 1:1.5:2.0. Having established the optimal condition, we further investigated the scope and reproducibility of this methodology by applying the same reaction condition to other substrates.

All the synthesized compounds were fully characterized by ^{1}H, ^{13}C NMR and high-resolution mass spectra as described for **3a**. The ^{1}H NMR spectrum of **3a** has four singlets at 3.10, 2.18, 0.83 and 0.53 ppm for 17′-CH_3, *N*-CH_3 of the pyrrolidine ring, 19-CH_3 and 18-CH_3, respectively. The multiplet at 3.43–3.51 ppm is assigned to the 3α-H of steroid A-ring. The hydrogens of the *N*-CH_3 of the pyrrolidine ring have the HMBC correlations with a 11′-CH_2 methylenic carbon and the quaternary carbon at 3′-position (78.08 ppm), which also shows the HMBC correlation with the NH proton of the oxindole ring appears at 8.59 ppm (Fig. 3). From the HMBC and HSQC correlations of 11′-CH_2, the triplets at 3.90–3.95 ppm (J = 9.4 Hz) and 3.53–3.57 ppm (J = 8.4 Hz) are assigned to 11′-CH_2 with its carbon signal at 60.56 ppm. The multiplicity of H-12′ and its H-H COSY correlation with the 11′-CH_2 hydrogens and its HMBCs with C-16, C-13′ and C-12′ enables the assignment of the former to the multiplet at 4.09–4.15 ppm. The hydrogen attached to C-6 appears as a singlet at 5.11 ppm and the aromatic hydrogens appears as doublets and multiplets at 6.85–6.94 ppm. Associated with DEPT135 spectrum, the quaternary carbon signals of C-16 and C-3′ appears at 67.45 and 78.08 ppm. These signal assignments are consistent with the HMBCs and H-H COSY correlations depicted in Fig. 3. No trace of the other possible regioisomer **4** was observed (Scheme 2). The benzylic proton (H-12′) appears as a multiplet at 4.09–4.15 ppm, which clearly confirms the regiochemistry of the cycloaddition reaction. If other regioisomer **4** was formed, the benzylic proton would appear as a singlet in the ^{1}H NMR spectrum (Scheme 2). The presence of a molecular ion peak at m/z = 629.3049 ([M+H]$^+$) in the mass spectrum (calcd. 629.3043) further confirms the structure of **3a**.

The mechanism proposed to rationalize the formation of the novel steroidal spiro-pyrrolidinyl oxindoles is summarized in Scheme 2. Decarboxylative condensation of the isatin with sarcosine gives the azomethine ylide, which then undergoes 1, 3-dipolar cycloaddition reaction with the dipolarophile **2** in a regioselective manner. The formation of other possible regioisomer was not observed. The regioselectivity of the reaction is explicable by the preference of the electron-rich carbon of the dipole adding to the electron-deficient β-carbon of the α, β-unsaturated moiety of **2**. The steroidal spiro-pyrrolidinyl oxindoles were also formed with complete stereoselectivity, affording one diastereomer exclusively, despite the presence of three newly formed stereocenters in the product. The carbonyl group linked to the steroid ring system and the amide carbonyl are in trans-relationship with respect to C_2—C_3 bond of the pyrrolidine ring system, this is presumably ascribable to the preferred spatial arrangement of the dipolarophile and the azomethine ylide in the cycloaddition step, which would minimize the repulsion between the carbonyl groups. The aryl ring attached to the pyrrolidine ring adjacent to the spiro carbon of **3** is also the trans-relationship to the carbonyl of the steroid skelton with respect to C_3—C_4 bond of the pyrrolidine ring system due to the *E*-configuration of exocyclic double bond at the 16-position of 17-ketosteroids **2**. It should be noted that three stereocenters (two quaternary carbon centers), two C—C bonds and one C—N bond were formed in one-pot procedure under catalyst-free condition. This complete regioselectivity and stereoselectivity was also observed by other groups [32–36].

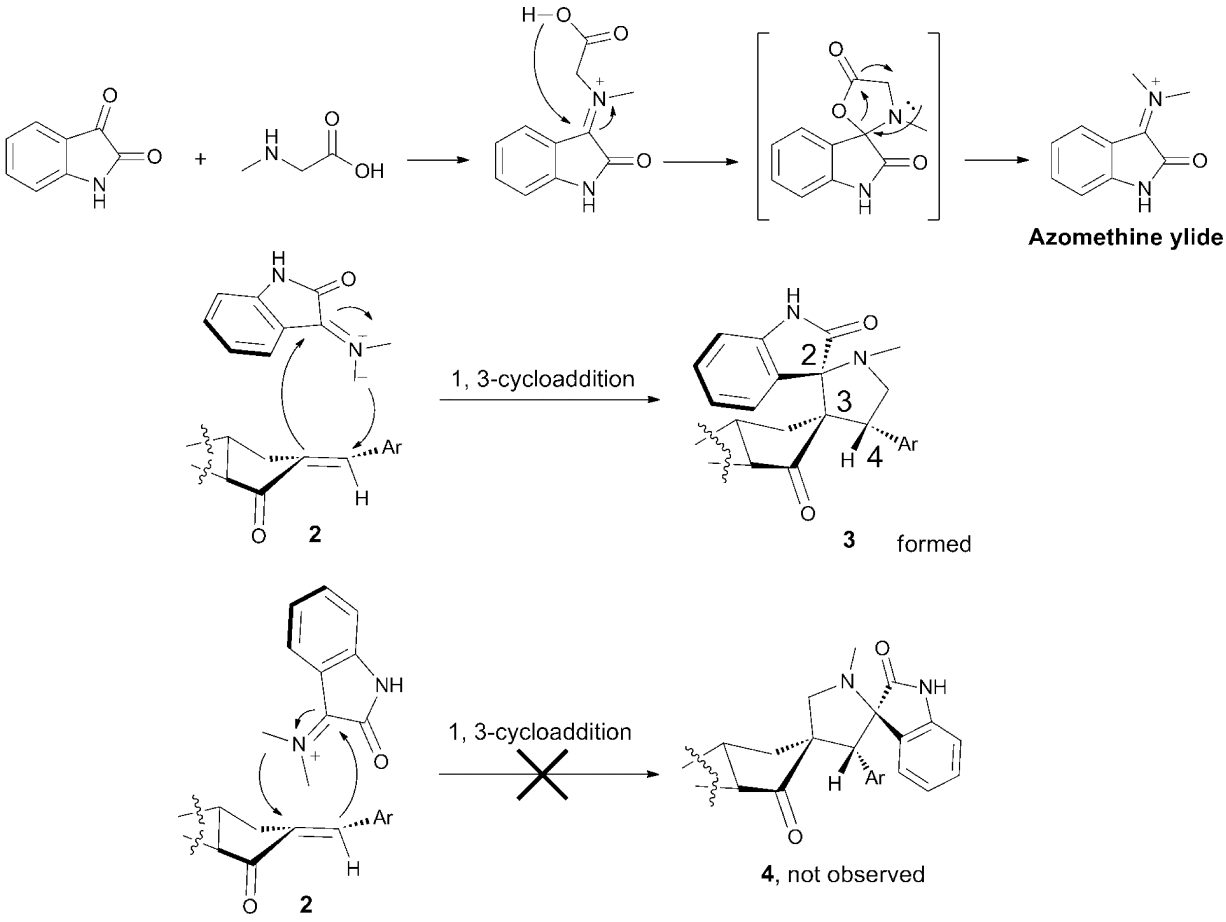
Natural products such as cephalostatins, ritterazines and Crellastatins are classical dimeric steroids with potent cytotoxicity [37,38]. However, the natural supply of dimeric steroids with any direct pharmacological action is extremely limited, the synthesis of natural steroid dimers and their analogs has drawn wide attention [39,40]. Considering this, we also synthesized three steroid dimers with dispiropyrrolidine skeleton (Scheme 3). The protocol involved the condensation reaction of dehydroepiandrosterone (DHEA, **1**) with isophthalaldehyde *via* Claisen-Schmidt condensation catalyzed by KF/Al_2O_3 in ethanol, affording a novel phenyl-linked ketosteroid dimer **5** in 90% yield, followed by the [3 + 2] cycloaddition with substituted isatin and sarcosine and finally gave compounds **6a–c**. Unexpectedly, compounds **7** were not obtained even 5 equivalents of isatin and sarcosine were used probably due to the steric hindrance.

3.2. Biological evaluation

3.2.1. Antiproliferative activity

The IC_{50} values (concentration required to inhibit tumor cell proliferation by 50%) for the synthesized compounds against four human cancer cell lines including human gastric cancer cell line (MGC-803), human breast cancer cell line (MCF-7), human liver cancer cell line (SMMC-7721) and human esophageal cancer cell line (EC-109) were determined using the MTT assay. The well-known anticancer drug 5-fluorouracil was evaluated in parallel as a positive control and the results were listed in Table 2.

As shown in Table 2, most of the synthesized compounds showed moderate to good antiproliferative activities against four human cancer cell lines with the IC_{50} values ranging from 0.7 to 43 μM and some of them were more potent than 5-Fu. The structure–activity relationships were probed through altering

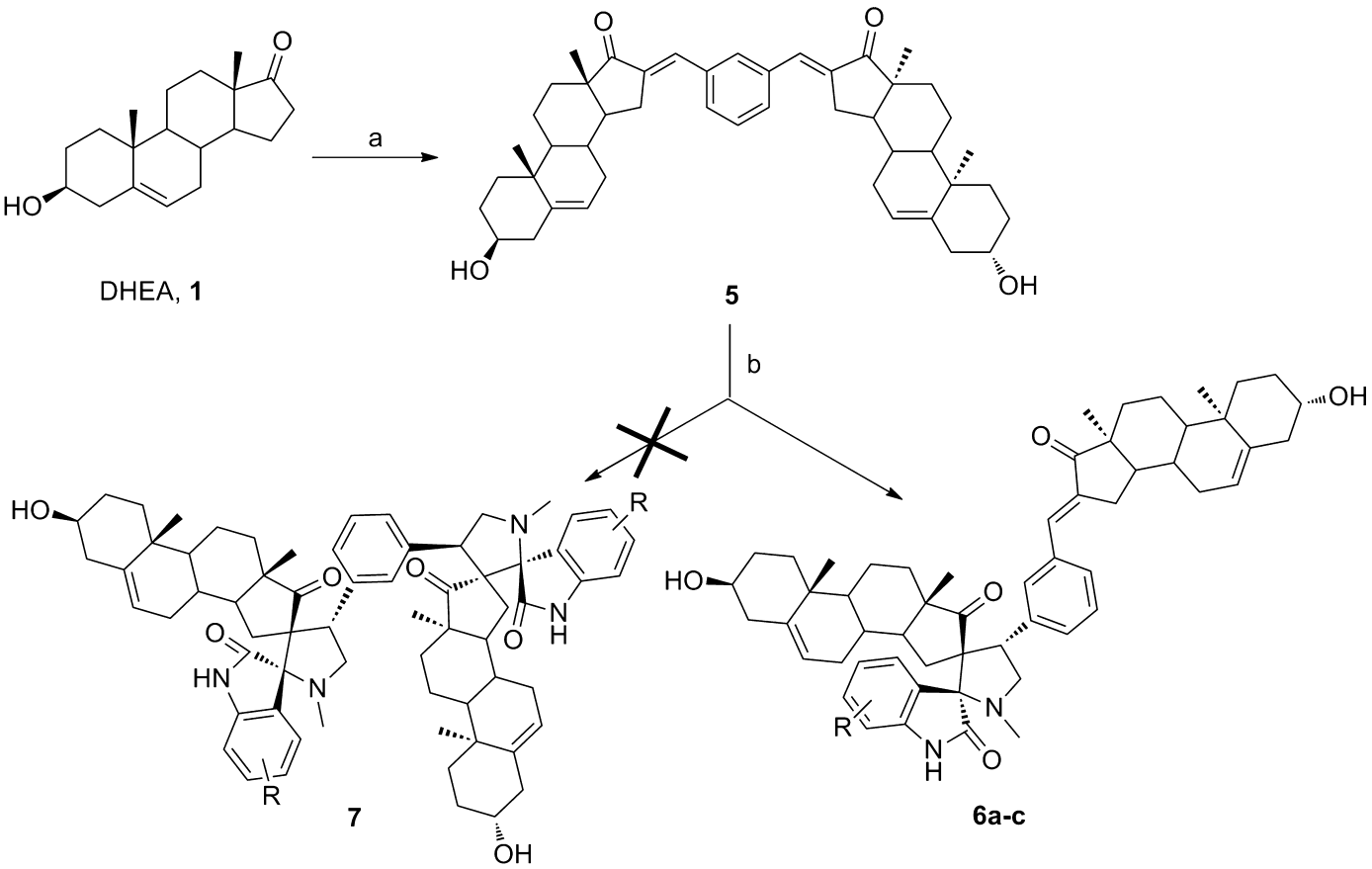


Scheme 2. Plausible mechanism for the formation of steroidal spiro-pyrrolidinyl oxindole **3a**.

Table 2
Preliminary *in vitro* antiproliferative activities of steroidal spiro-oxindoles against four human cancer cell lines.

Compounds	Ar	R	IC_{50} (μM)[a]			
			EC109	MGC-803	SMMC-7721	MCF-7
3a	4-Methylsulfonylphenyl	H	42.02 ± 1.62	17.82 ± 1.13	101.51 ± 2.00	18.15 ± 1.26
3b	4-Nitrophenyl	H	5.06 ± 0.40	5.09 ± 0.71	14.14 ± 0.85	20.49 ± 1.31
3c	3-Nitrophenyl	H	12.91 ± 0.81	9.64 ± 0.98	13.08 ± 0.82	25.26 ± 1.40
3d	3-Methoxylphenyl	H	24.21 ± 1.08	12.99 ± 1.00	16.66 ± 0.92	27.71 ± 1.43
3e	4-Fluorophenyl	H	14.68 ± 0.87	6.62 ± 0.82	18.77 ± 0.97	18.31 ± 1.27
3f	2-Fluorophenyl	H	22.16 ± 1.04	10.02 ± 1.06	6.94 ± 0.54	11.41 ± 1.06
3g	4-Chlorophenyl	H	10.45 ± 0.72	7.26 ± 0.86	0.71 ± 0.11	14.27 ± 1.15
3h	3-Chlorophenyl	H	11.77 ± 0.77	5.21 ± 0.72	14.13 ± 0.85	14.37 ± 1.16
3i	2-Chlorophenyl	H	9.16 ± 0.66	6.12 ± 0.79	12.36 ± 0.79	17.87 ± 1.25
3j	3, 4-Dichlorophenyl	H	9.11 ± 0.66	5.93 ± 0.77	9.79 ± 0.69	18.00 ± 1.26
3k	Phenyl	H	23.44 ± 1.07	14.46 ± 1.06	4.33 ± 0.34	23.35 ± 1.37
3l	4-Methylphenyl	H	12.79 ± 0.81	11.87 ± 1.07	10.12 ± 0.70	14.65 ± 1.17
3m	4-Isopropylphenyl	H	3.51 ± 0.24	4.22 ± 0.63	14.13 ± 0.85	37.97 ± 1.58
3n	4-*t*-butylphenyl	H	13.57 ± 0.83	5.79 ± 0.76	6.05 ± 0.48	10.25 ± 1.01
3o	1-Naphthyl	H	13.72 ± 1.14	8.40 ± 0.92	19.86 ± 1.30	11.82 ± 1.07
3p	2-Pyridyl	H	ns[b]	ns	ns	ns
3q	Furyl	H	8.95 ± 0.65	11.10 ± 1.05	14.30 ± 0.85	34.11 ± 1.53
3r	Thienyl	H	16.49 ± 0.92	8.00 ± 0.90	12.46 ± 0.79	28.02 ± 1.45
3s	4-Chlorophenyl	5-F	6.78 ± 0.83	9.79 ± 0.99	13.24 ± 1.12	8.09 ± 0.91
3t	4-Chlorophenyl	5-Br	10.32 ± 1.01	7.58 ± 0.88	10.62 ± 1.03	5.54 ± 0.74
6a	–	H	29.76 ± 1.47	17.03 ± 1.23	27.73 ± 1.44	15.28 ± 1.18
6b	–	5-F	7.01 ± 0.85	15.38 ± 1.19	4.30 ± 0.63	2.06 ± 0.31
6c	–	7-Cl	13.74 ± 1.14	16.81 ± 1.23	26.56 ± 1.42	6.86 ± 0.84
5-Fu	–	–	10.61 ± 1.08	6.92 ± 0.35	9.78 ± 0.99	7.54 ± 0.70

[a] Inhibitory activity was assayed by exposure for 72 h to substances and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are presented as the means ± SDs of three independent experiments.
[b] Not soluble in DMSO.



Scheme 3. Protocol for the synthesis of steroid dimers (**6a–c**). Reagents and conditions: (a) Isophthalaldehyde, KF/Al_2O_3, EtOH, reflux; (b) substituted isatin, sarcosine, MeOH/dioxane (v/v = 1/1), 100 °C.

substituents in the isatin nucleus and those attached to the 4-position of pyrrolidine. Compounds **3a–d** with electron-withdrawn groups had moderate inhibitory effect toward most of the tested cancer cell lines with the IC_{50} values less than 28 μM. However, compound **3a** showed weak inhibition against EC109 and SMMC-7721 (IC_{50} > 42 μM). It should be noted that compound **3b** having a nitro substituent at the 4-position of phenyl group was more potent than 5-Fu against EC109 and MGC803 with the IC_{50} values of 5.06 and 5.09 μM, respectively. Compared to compounds **3a–d**, halogenated compounds **3e–j** represented similar antiproliferative activities against EC109, SMMC-7721 and MCF-7. To MGC-803, compounds **3e–j** demonstrated improved antiproliferative activities with the IC_{50} values ranging from 5.21 to 10.02 μM. Among them, compounds **3e** and **3h–j** were more potent than 5-Fu. Particularly, compound **3g** showed the most potent inhibitory effect against SMMC-7721 (IC_{50} = 0.71 μM). Compared to **3k**, compounds **3l–n** with electron-donating groups displayed enhanced antiproliferative activities against EC109, MGC-803 and MCF-7, but to SMMC-7721, compound **3k** was about 2.3-fold more potent than 5-Fu (IC_{50} = 4.33 μM). Compounds **3q–r** with heteroaryl groups (furyl and thienyl) exhibited similar antiproliferative activities against the tested cancer cell lines with those having substituted phenyl group. Substituents on the isatin nucleus had remarkable effect on their antiproliferative activities toward SMMC-7721 cells. Incorporation of a fluoro or bromo substituent at the 5-position of isatin nucleus was found to be negative for activity. Compounds **3s** and **3t** were about 19- and 15-fold less potent than **3g**. Steroid dimers **6a–c** did not represent significant improvement on their antiproliferative activities toward the cancer cell lines. Among them, compound **6b** had the moderate inhibition against EC109, SMMC-7721 and MCF-7 with the IC_{50} values of 7.01, 4.30 and 2.06 μM, respectively.

3.2.2. Apoptosis assay

Considering the moderate to good antiproliferative activities of these compounds against all tested human cancer cell lines, compound **3n** was chosen to further explore its mechanism of action. In order to better characterize the mode of cell death induced by compound **3n**, we performed a biparametric cytofluorimetric analysis using propidium iodide (PI) and annexin-V-FITC in MGC-803 cells. After treatment MGC-803 cells with compound **3n** for 12 or 24 h at different concentrations (0, 2.5, 5.0, 10.0 μM), MGC-803 cells were labeled with the two dyes, and the resulting red (PI) and green fluorescence (FITC) was monitored by flow cytometry. As shown in Fig. 4, compound **3n** caused the remarkable early apoptosis. Specifically, after treatment for 12 h, the early apoptosis rate was 8.7% for the control group, the early apoptosis rate increased gradually with the increase of the concentration of Compound **3n** and finally amounted to 32.1% for the high concentration group (10.0 μM) (Fig. 4A). A similar trend was also observed after treatment for 24 h and the early apoptosis rate increased significantly and stood at 65.7% for the high concentration group (10.0 μM) (Fig. 4B). Besides, the late apoptosis rate also witnessed a slight increase. The results showed that compound **3n** markedly increased the cellular apoptosis in a concentration-and time-dependent manner.

3.2.3. Cell cycle analysis

Molecules that inhibit the growth of cancer cells invariably cause alteration of cell cycle distribution, with preferential G2/M blockade. To better understand the antiproliferative activity of compound **3n**, a cell cycle progression was performed by treating MGC-803 cells at different concentrations of compound **3n** (0, 2.5, 5.0, 10 μM). Treatment MGC-803 cells for 12 h, the percentage of cells at G2/M phase increased to 35.87% for the high concentration

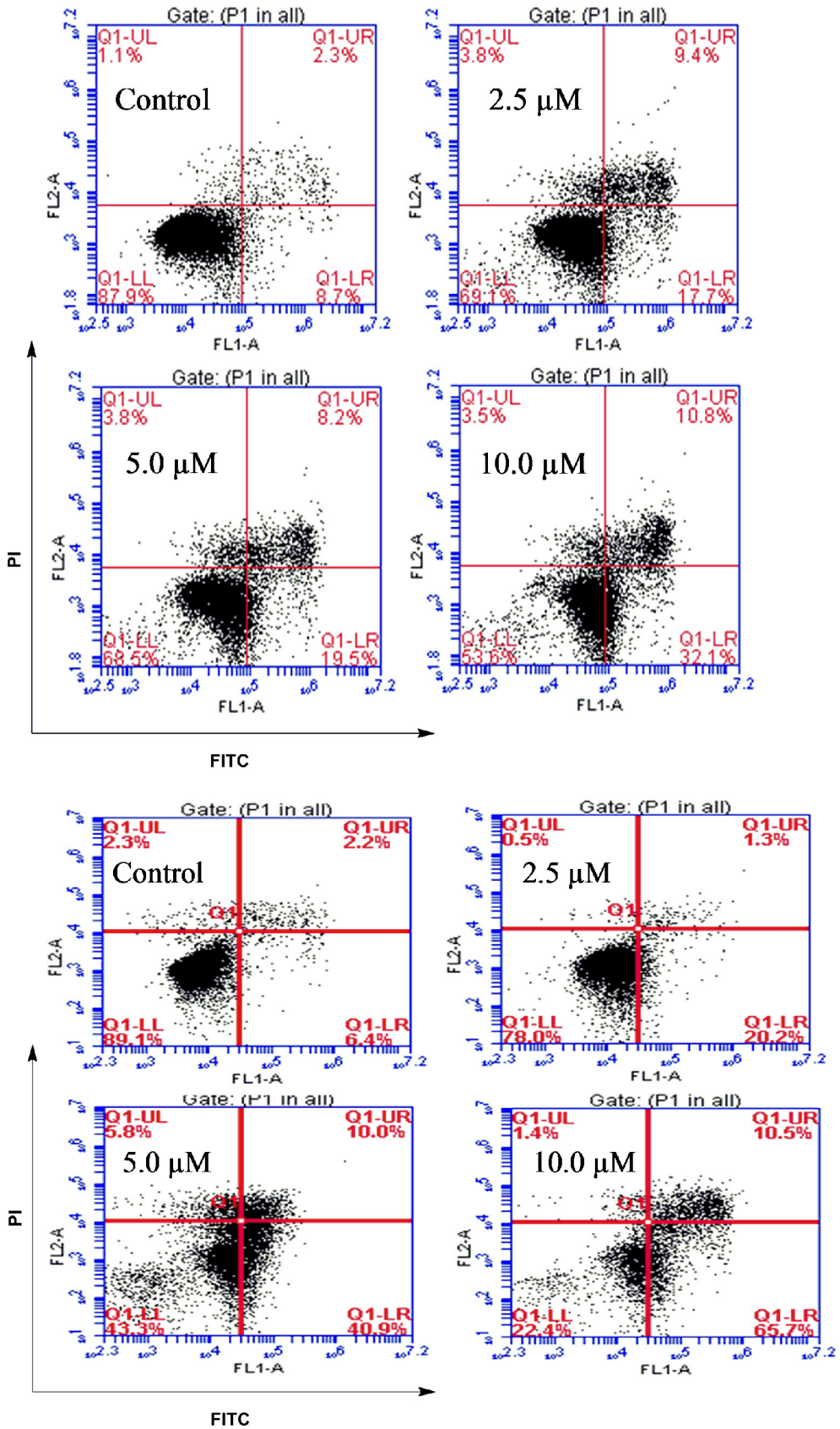


Fig. 4. Apoptosis effect on human MGC-803 cell line induced by compound **3n**. Apoptotic cells were detected with Annexin V-FITC/PI double staining after incubation with compound **3n** (0, 2.5, 5.0, 10.0 μM) for 12 h or 24 h. (A) Incubated for 12 h; (B) incubated for 24 h. The lower left quadrants represent live cells, the lower right quadrants are for early/primary apoptotic cells, upper right quadrants are for late/secondary apoptotic cells, while the upper left quadrants represent cells damaged during the procedure. The experiments were performed three times, and a representative experiment is shown.

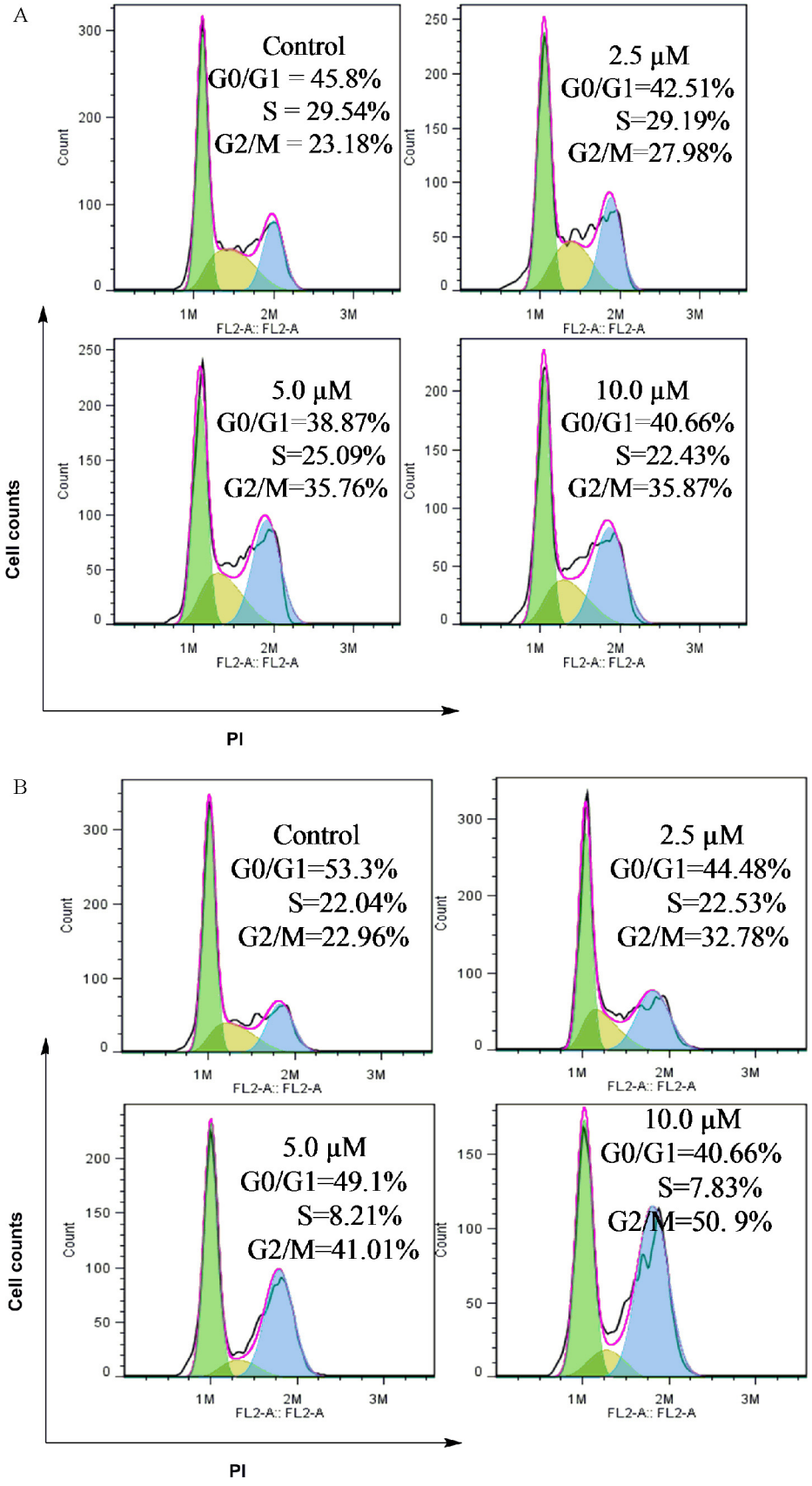


Fig. 5. Effect of compound **3n** on the cell cycle distribution of MGC-803 cells. Cells were treated with different concentrations (0, 2.5, 5.0, 10.0 μM) for 12 h or 24 h. Then the cells were fixed and stained with PI to analyze DNA content by flow cytometry. (A) Incubated for 12 h; (B) incubated for 24 h. The experiments were performed three times, and a representative experiment is shown.

group (23.18% for the control group) (Fig. 5A), whereas when treatment for 24 h, the percentage of cells at G2/M phase increased remarkably from 22.96% to 50.9% (Fig. 5B). The results revealed that **3n** caused an obvious G2/M arrest pattern in a concentration- and time-dependent manner with a concomitant decrease of cells in other phases of the cell cycle.

4. Conclusions

In summary, we have developed an efficient and catalyst-free protocol for the synthesis of novel steroidal spiro-pyrrolidinyl oxindoles using the (*E*)-3β-hydroxy-5-en-16-arylidene-17-ketosteroids as the dipolarophiles. The method involved the one-pot, three-component 1, 3-dipolar cycloaddition of azomethine ylides generated *in situ* from decarboxylative condensation of isatin with sarcosine and afforded the final products with high regio and stereoselectivity. This protocol achieved the formation of two C—C bonds, one C—N bond and the creation of one new five-membered ring and three contiguous stereocenters, three of which are quaternary. Biological evaluation showed that these synthesized steroidal spiro-pyrrolidinyl oxindoles possessed moderate to good antiproliferative activities against a panel of cell lines and some of them were more potent than 5-Fu. Particularly, compound **3g** showed good antiproliferative activity against SMMC-7721 (IC_{50} = 0.71 μM). Steroid dimer **6b** showed significantly improved antiproliferative activities against SMMC-7721 and MCF-7 with the IC_{50} values of 4.30 and 2.06 μM, respectively. Further investigation showed that compound **3n** caused the cellular early apoptosis and an increase in the proportion of cells at G2/M phase in a concentration-and time-independent manner.

Acknowledgment

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