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Short communication

Efficient construction of novel D-ring modified steroidal dienamides and their cytotoxic activities

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ABSTRACT

Two series of steroidal dienamides **4a**–**q** and **5a**–**f** were designed, synthesized and evaluated for cytotoxic activities against five human cancer cell lines (MGC-803, EC109, PC-3, SMMC-7721 and MCF-7). The protocol developed efficiently achieved the construction of carbon–carbon double bond and selective conversion of nitrile group into carboxamide in one-pot procedure. Besides, compounds **4a**–**q** and **5a**–**f** showed moderate to excellent cytotoxic activities with the IC_{50} values ranging from 0.1 to 40 μM and most of them were more potent than 5-fluorouracil. Particularly, four compounds **4d**, **4e**, **4q** and **5a** showed excellent selectivity against MGC-803 with the IC_{50} values less than 1 μM. Flow cytometry analysis demonstrated that compound **4c** caused the cellular early apoptosis and cell cycle arrest in G2/M phase in a concentration-independent manner.

1. Introduction

Steroids are a class of important multi-cyclic compounds that exhibit diverse biological activities in living organisms. It is proved that a number of biologically important properties of modified steroids are dependent upon structural features of the steroid ring system [1] and side chain [2]. Chemical modification of the steroid ring system and side chain provides a way to alter the functional groups, and numerous structure–activity relationships have been established by such synthetic alterations [3]. These compounds could be used for development of new potential therapeutics, as well as tools for probing the spatial orientation of important binding responsive features in target macromolecules.

Dienamides are also recognized as key reactive intermediates due to their great diversities, potential synthetic values and commonly existence in nature [4–6]. Examples of these include Apicularen A [7,8], Salicylihalamide A [9] (Fig. 1). Among them, Zampanolide represents potent cytotoxicity against P388, A549, HT29 and MEL28 cell lines (IC_{50} = 1–5 ng/mL) [10]. Besides, dienamides could be used as electron-rich or electron-deficient dienes in Diels–Alder reactions effectively [11], which have already been applied to the synthesis of natural alkaloids [12] and some interesting heterocycles [13–15]. Dienamides are also key constituents in a number of pharmaceutically relevant units [16] and have been reported to have antioxidant, cytotoxic [17] and insecticidal activities [18]. For example, Saku et al. reported that 5,5-diarylpentadienamides (Fig. 1) as the transient receptor potential vanilloid I (TRPVI) antagonists were under further evaluation for clinical treatment of neuropathic pain [19]. Recently, Chen and co-workers reported that (*E*, *E*)-2-(benzylaminocarbonyl)-3-styrylacrylonitrile (Fig. 1) as the Mcl-1 protein inhibitor represented a 6-fold enhancement compared to its parent structure (K_d = 0.16 μM) [20].

Recently, we achieved the synthesis of steroidal dienamides from dehydroepiandrosterone via rearrangement of 2*H*-pyrans [21]. In view of the therapeutic importance of dienamides and being involved in finding new biologically active modified steroids [22–28], we are interested in the design, synthesis and biological evaluation of novel steroidal dienamides. Here, we further determine the scope of the steroidal dienamides synthesis and evaluate their cytotoxic activities against human cancer cell lines *in vitro* and the effects toward the cell cycle and apoptosis.

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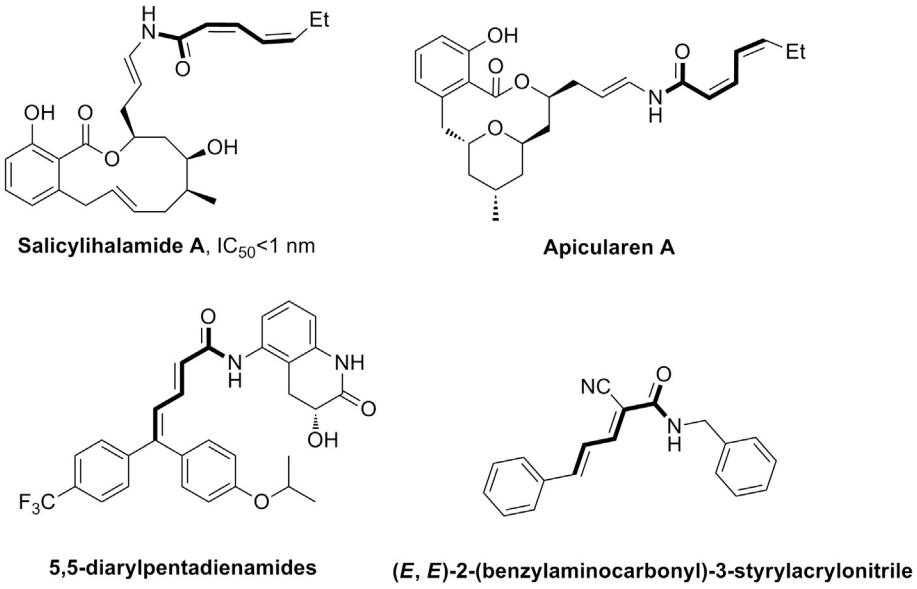


Fig. 1. Some natural occurring and synthetic pharmaceutically active dienamides.

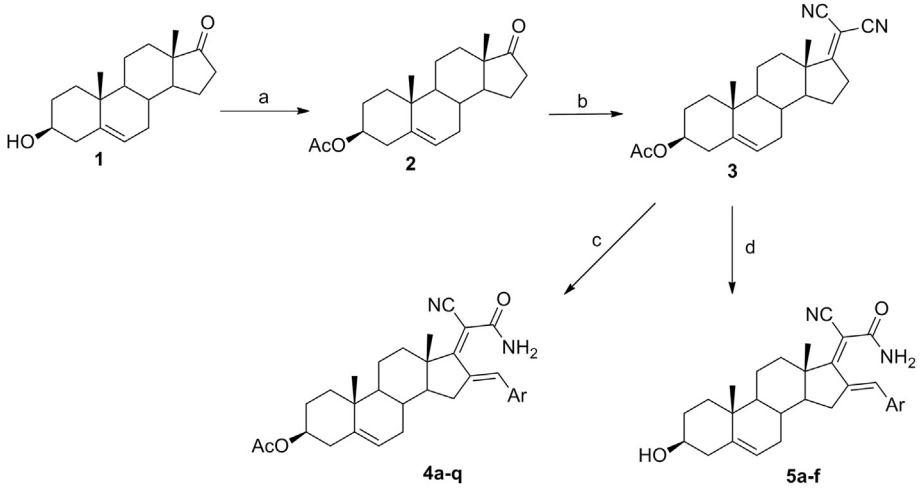
2. Results and discussion

2.1. Chemistry

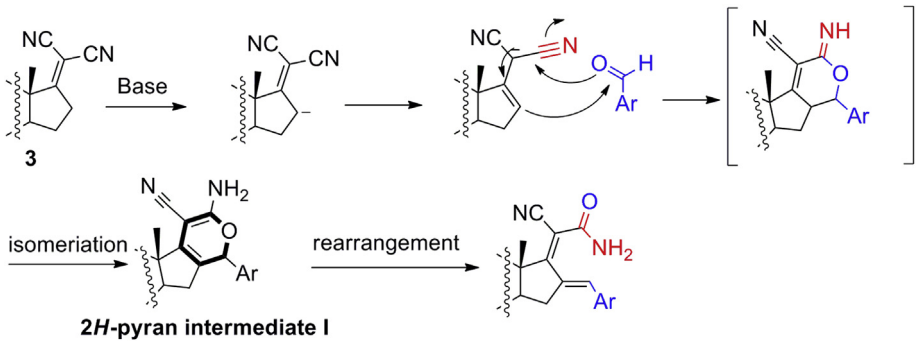
The protocol for the synthesis of D-ring modified steroidal dienamides (**4a**–**q** and **5a**–**f**) was very simple and straightforward involving the vinylogous aldol reaction of steroidal α,α-dicyanoalkene **3** and aldehydes, and then followed by rearrangement of the 2*H*-pyran intermediate (Scheme 1). The intermediate **3** was prepared in high yield via Aldol condensation of 3β-acetyl dehydroepiandrosterone **2** with malononitrile in ethanol catalyzed by ammonium acetate. According to our previously reported method [21], compounds **4a**–**q** were synthesized in moderate to high yields using NaOAc/EtOH system. 3β-Hydroxyl steroids, as the most potent LXRs activators, would function as a hydrogen bond acceptor and serve as anchor for binding of either oxysterol or its analogs to receptor [29,30]. In light of these, 3β-hydroxyl counterparts **5a**–**f** were also designed and synthesized in high yields without further deprotection of 3β-acetyl group by treatment compound **3** with Na_2CO_3 in boiling methanol. The direct condensation reaction of dehydroepiandrosterone **1** with malononitrile was also performed in ethanol catalyzed by ammonium acetate, affording the corresponding product in low yield (about 50%) even with prolonged reaction time (>12 h).

All the synthesized compounds were fully characterized by ^1H, ^{13}C NMR and high resolution mass spectra as described for **4a**. In the ^1H NMR spectra, the olefinic proton of compound **4a** appeared at $\delta = 7.25$ ppm as a sharp singlet, where the NH_2 protons resonated at $\delta = 6.02$ and 5.77 ppm as two broad singlets. The amide carbon in ^{13}C NMR signaled at $\delta = 164.92$ ppm. In the HSQC spectra, the proton of the olefinic proton ($\delta = 7.25$ ppm) had a direct correlation with the carbon ($\delta = 98.51$ ppm), revealing that the olefinic carbon of **4a** resonated at 98.51 ppm. The presence of a molecular ion peak at $m/z = 485.2802$ ($[M + H]^+$) in the mass spectra (calcd. 485.2804) further confirmed the structure of **4a**.

A possible mechanism for the formation of compounds **4a**–**q** and **5a**–**f** was shown in Scheme 2. Under basic conditions, the steroidal α,α-dicyanoalkene **3** was first deprotonated to form a nucleophile that then attacked the aldehyde via vinylogous aldol



Scheme 1. Synthesis of steroidal dienamides (**4a**–**q** and **5a**–**f**). Reagents and conditions: (a) $(Ac)_2O$, DMAP, Et_3N, DCM, r.t; (b) malononitrile, NH_4OAc, EtOH, reflux; (c) aldehydes, NaOAc, EtOH, reflux; (d) aldehydes, Na_2CO_3, MeOH, reflux.



Scheme 2. Proposed mechanism for the formation of steroidal dienamides.

reaction. Subsequent intramolecular nucleophilic addition and isomerization afforded the 2*H*-pyran intermediate I, which further rearranged to the steroidal dienamides. Interestingly enough, this protocol efficiently achieved the construction of carbon–carbon double bond and selective conversion of nitrile group into carboxamide with another nitrile group intact in one-pot under mild condition, which demonstrated that the formation of carboxamide did not attribute to the conventional hydrolysis of nitrile group. It should be noted that our final products with a conjugated dienamide group could be allowed to further modification into other novel steroidal derivatives [13–15].

However, different from our results, Elmegeed and coauthors reported that they obtained A-ring fused steroidal 2*H*-pyrans, not the steroidal dienamides via the intramolecular cyclization under the same condition [31].

2.2. Biological evaluation

2.2.1. Cytotoxic activity

The IC_{50} values (concentration required to inhibit tumor cell proliferation by 50%) for the synthesized compounds against five 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), human prostate cancer cell line (PC-3) and human esophageal cancer cell line (EC-109) were determined using the MTT assay. The results were listed in Table 1 and the well-known anticancer drug 5-fluorouracil was used as positive control.

As shown in Table 1, all the synthesized compounds showed moderate to excellent cytotoxic activities against five human cancer cell lines with the IC_{50} values ranging from 0.1 to 40 μM and most of them were more potent than 5-fluorouracil. 3β-Hydroxyl steroidal dienamides **5a**–**f** and their 3β-acetyl counterparts (compounds **4e**, **4m**, **4f**, **4o**, **4p** and **4q**) had the similar cytotoxic activities against EC109, MCF-7 and PC-3. However, compound **4e** was about 9-fold more potent than **5a** against MGC-803 with the IC_{50} values of 0.58 and 5.13 μM, respectively. Compound **4q** was about 3.5-fold less potent than **5f** (6.34 μM versus 1.83 μM). Compound **5b** showed excellent inhibitory effect against SMMC-7721 (IC_{50} = 1.90 μM), which was about 8- and 5-fold more potent than **4m** (IC_{50} = 15.44 μM) and 5-fluorouracil (IC_{50} = 9.78 μM), respectively. Compounds **4j** and **4k** with heteroaryl groups (furyl

Table 1
Primary *in vitro* cytotoxic activities of the steroidal dienamides against five human cancer cell lines.

No	Ar	IC_{50} (μM)[a]				
		EC109	MGC-803	MCF-7	PC-3	SMMC-7721
4a	Phenyl	11.72 ± 1.40	3.10 ± 0.36	7.47 ± 1.15	12.75 ± 1.63	5.03 ± 0.80
4b	2,4-Dichlorophenyl	5.24 ± 0.84	7.38 ± 1.10	3.98 ± 0.55	24.26 ± 2.04	5.04 ± 0.59
4c	4-Chlorophenyl	5.56 ± 0.09	1.54	3.85 ± 0.58	11.53 ± 1.49	3.90 ± 0.59
4d	3-Chlorophenyl	8.97 ± 0.84	0.11	8.01 ± 1.19	11.27 ± 1.48	7.12 ± 1.09
4e	2-Chlorophenyl	11.86 ± 1.88	0.58	6.27 ± 1.01	24.31 ± 2.12	18.38 ± 1.89
4f	3-Methoxylphenyl	7.04 ± 0.80	2.19 ± 0.10	8.36 ± 1.23	12.95 ± 1.60	11.53 ± 1.50
4g	2-Cyanophenyl	nd[b]	nd[b]	22.40 ± 2.08	nd[b]	nd[b]
4h	3,4-Difluorophenyl	3.05 ± 1.73	1.56	4.77 ± 0.76	8.48 ± 0.16	5.66 ± 0.90
4i	4-Isoproplphenyl	10.03 ± 1.37	1.09	15.35 ± 1.72	13.46 ± 0.25	9.30 ± 1.31
4j	Furyl	39.35 ± 0.57	0.12	39.37 ± 2.68	23.44 ± 2.20	22.85 ± 2.18
4k	Thienyl	20.33 ± 0.09	0.15	22.79 ± 2.14	20.45 ± 2.04	20.39 ± 2.04
4l	4-Bromophenyl	5.30 ± 0.70	2.71 ± 0.33	2.95 ± 0.39	26.78 ± 2.09	16.48 ± 1.18
4m	2-Fluorophenyl	15.57 ± 1.78	2.99 ± 0.35	4.66 ± 0.73	11.36 ± 1.51	15.44 ± 1.77
4n	4-Methylphenyl	12.03 ± 1.56	8.10 ± 1.22	6.35 ± 1.01	22.93 ± 2.12	18.55 ± 1.94
40	3,4-Dichlorophenyl	4.37 ± 0.69	3.20 ± 0.45	2.21 ± 0.16	8.18 ± 1.19	2.55 ± 0.27
4p	4-Fluorophenyl	4.78 ± 0.25	3.11 ± 1.38	3.67 ± 0.51	9.17 ± 1.32	5.90 ± 0.94
4q	3,4,5-Trimethoxylphenyl	5.28 ± 1.04	6.34 ± 0.98	5.23 ± 0.83	11.74 ± 1.44	2.82 ± 0.37
5a	2-Chlorophenyl	13.16 ± 1.67	5.13 ± 0.82	5.34 ± 0.85	16.94 ± 1.90	3.63 ± 0.54
5b	2-Fluorophenyl	14.59 ± 1.80	2.69 ± 0.20	6.58 ± 1.13	13.29 ± 1.71	1.90
5c	3-Methoxylphenyl	9.27 ± 1.01	3.96 ± 0.58	5.57 ± 0.20	13.95 ± 1.73	6.09 ± 0.97
5d	3,4-Dichlorophenyl	8.61 ± 1.26	5.11 ± 0.82	2.41 ± 0.18	8.84 ± 1.28	3.34 ± 0.46
5e	4-Fluorophenyl	8.69 ± 0.93	5.67 ± 0.90	5.92 ± 0.07	14.64 ± 1.80	3.34 ± 0.42
5f	3,4,5-Trimethoxylphenyl	8.72 ± 1.25	1.83	6.54 ± 0.01	12.23 ± 1.53	7.94 ± 1.18
5-Fu	–	10.61 ± 1.08	6.92 ± 0.35	7.54 ± 0.70	24.76 ± 3.44	9.78 ± 0.99

[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 determined.

and thienyl) at 16-position of steroidal skeleton showed weak antiproliferative activity against EC109, MCF-7, PC-3 and SMMC-7721 ($IC_{50} > 20$ μM) but excellent inhibitory effect against MGC-803 with the IC_{50} values of 0.12 and 0.15 μM, respectively (57- and 45-fold more potent than 5-Fu). This excellent and selective inhibition against MGC-803 promoted us to perform further investigation and the results will be reported in due course. By contrast, when substituted phenyl groups were introduced into 16-position of the steroidal skeleton, most of these compounds (**4a**–**i** and **5a**–**f**) represented moderate to excellent inhibitory activity, the electronic effect and the position of substituent on the phenyl group had a remarkable effect on their cytotoxic activity. To EC109, Compounds **4i** and **4n** with electron-donating groups such as 4-isopropyl and 4-methyl on the phenyl group had the moderate inhibitory effect (10.03 and 12.03 μM, respectively), while compound **4p** with the electron-withdrawing fluorine atom on the phenyl group showed excellent inhibition (4.78 μM). Compounds **4c**, **4d** and **4e** with a chlorine atom at the 4-, 3-and 2-position on the phenyl group represented excellent inhibitory effect against MGC-803 with the IC_{50} values of 1.54, 0.11 and 0.58 μM, respectively. A similar trend was also observed to other cancer cell lines.

2.2.2. Apoptosis assay

Considering the excellent cytotoxic activities of these compounds against all tested human cancer cell lines, compound **4c** was chosen to further explore its mechanism of action. In order to better characterize the mode of cell death induced by compound **4c**, we performed a biparametric cytofluorimetric analysis using propidium iodide (PI) and annexin-V-FITC in MGC-803 cells. After treatment with compound **4c** for 24 h at different concentrations (0, 2.0, 4.0, 8.0 μM), MGC-803 cells were labeled with the two dyes, and the resulting red (PI) and green (FITC) fluorescence was monitored by flow cytometry. As shown in Fig. 2, after treatment for 24 h, compound **4c** caused the extent of early apoptosis to increase significantly from 8.9% (DMSO control) to 80.9%. The results showed that compound **4c** markedly increased the cellular apoptosis in a concentration-independent manner.

2.2.3. Cell cycle analysis

Molecules that inhibit the growth of cancer cells invariably cause alteration of cell cycle distribution, with preferential G2/M blockade [32]. We therefore examined the effect of different concentrations of compound **4c** on cell cycle progression with MGC-803 cell line (Fig. 3). A cell-cycle cytotoxicity assay was performed by treating MGC-803 cells at various concentrations of compound **4c** (0, 2.0, 4.0, 8.0 μM) for 24 h. Compound **4c** caused an increase in the proportion of cells in G2/M phase in a concentration-dependent manner with a concomitant decrease of cells in other phases of the cell cycle. Specifically, the percentage of cells in G2/M phase at different concentrations was 20.41%, 33.68%, 49.43% and 56.91%, respectively.

3. Conclusions

In summary, two series of steroidal dienamides **4a**–**q** and **5a**–**f** were designed and synthesized from dehydroepiandrosterone in

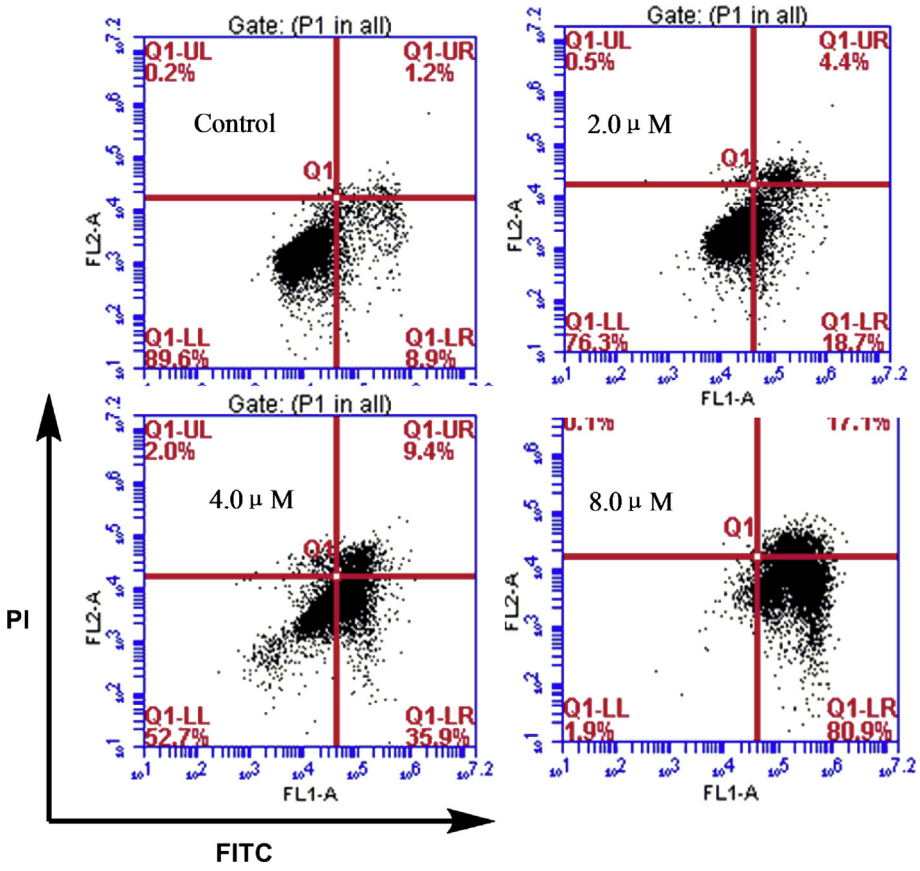


Fig. 2. Apoptosis effect on human MGC-803 cell line induced by compound **4c**. Apoptotic cells were detected with Annexin V/PI double staining after incubation with compound **4c** (0, 2.0, 4.0, 8.0 μM) for 24 h. The lower left quadrants represent live cells, the lower right quadrants are for early/primary 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.

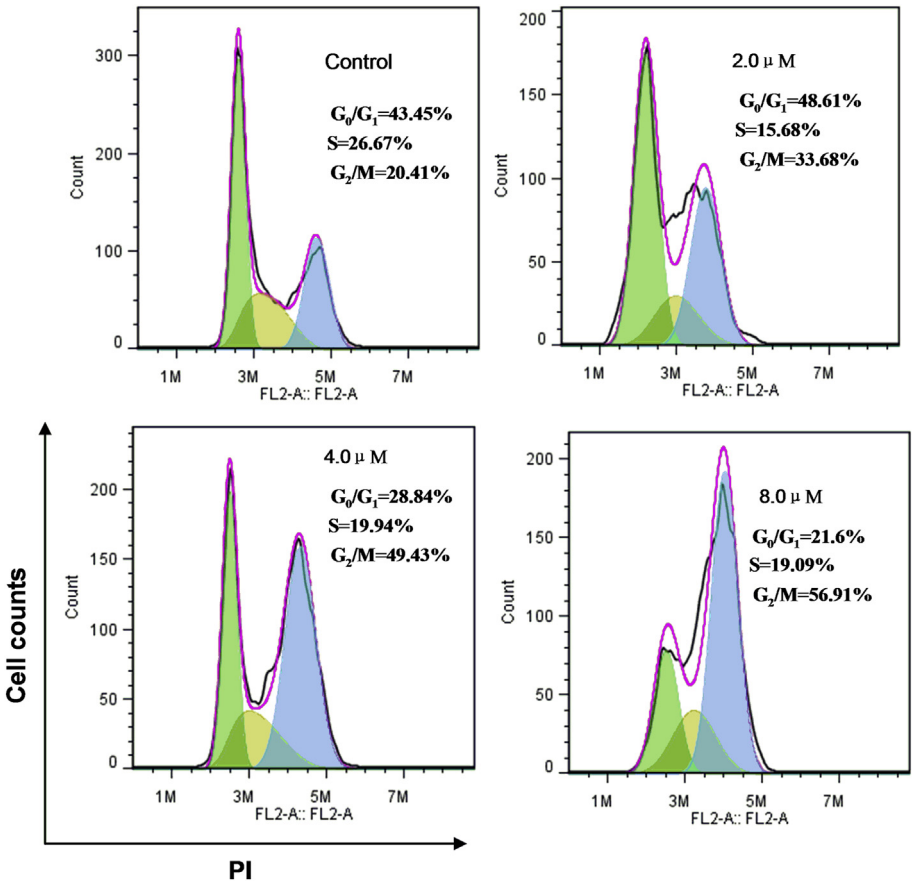


Fig. 3. Effect of compound **4c** on the cell cycle distribution of MGC-803 cells. Cells were treated with different concentrations (0, 2.0, 4.0, 8.0 μM) for 24 h. Then the cells were fixed and stained with PI to analyze DNA content by flow cytometry. The experiments were performed three times, and a representative experiment is shown.

high yields under mild conditions. This protocol efficiently achieved the construction of carbon–carbon double bond and selective conversion of nitrile group into carboxamide in one-pot. Besides, compounds **4a**–**q** and **5a**–**f** were also screened for cytotoxic activities against five human cancer cell lines, revealing that these compounds showed moderate to excellent cytotoxic activities with the IC_{50} values ranging from 0.1 to 40 μM and most of them were more potent than 5-fluorouracil. It is worth mentioning that four compounds **4d**, **4e**, **4q** and **5a** showed excellent inhibitory activity against MGC-803 with the IC_{50} values less than 1 μM. Further investigation showed that compound **4c** caused the cellular early apoptosis and an increase in the proportion of cells in G2/M phase in a concentration-independent manner.

4. Experimental section

4.1. 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 (Most of the peaks due to the steroidal skeleton are merged and could not be differentiated. Thus δ values of only those peaks that distinguish the product and could easily be differentiated are reported). High-resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-T of Micromass spectrometer by electrospray ionization (ESI).

4.2. Synthesis of 3-acetyl dehydroepiandrosterone *2*

A mixture of dehydroepiandrosterone (4.0 mmol), acetic anhydride (4.4 mmol), 4-dimethylaminopyridine (DMAP, 0.02 mmol) and Et_3N (8.0 mmol) in dichloromethane (50 mL) was stirred for about 5 h at room temperature. After completion of the reaction as evident from TLC, the organic phase was washed with water and brine, dried over Na_2SO_4. Removal of solvent afforded compound **2** quantitatively without further purification. White solid, mp 169.4–170.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 5.43 (d, J = 5.1 Hz, 1H), 4.63 (m, 1H), 2.06 (s, 3H), 1.07 (s, 3H), 0.91 (s, 3H). HRMS (ESI): m/z calcd for $C_{21}H_{30}NaO_3$ $(M + Na)^+$, 353.2093; found, 353.2094.

4.3. Synthesis of the D-ring modified steroidal dicyanoalkene *3*

To a solution of compound **2** (3.0 mmol) in ethanol (10 mL) containing ammonium acetate (0.5 g), malononitrile (4.5 mmol) was added. The reaction mixture was heated under reflux for about 3 h until compound **2** had disappeared as indicated by TLC. The solid product formed upon cooling at room temperature was washed with ethanol, collected by filtration to yield white solid of compound **3**, yield: 95%, mp 187.9–188.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 5.38 (d, J = 5.1 Hz, 1H), 4.74–4.45 (m, 1H), 3.04–

2.86 (m, 1H), 2.74 (m, 1H), 2.04 (s, 3H), 1.05 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 196.08, 170.50, 139.93, 121.58, 112.26, 111.16, 79.73, 73.58, 55.22, 49.34, 48.91, 37.99, 36.83, 36.56, 34.73, 33.61, 31.39, 31.37, 27.63, 23.56, 21.40, 20.78, 19.27, 16.24. HRMS (ESI): *m*/*z* calcd for $C_{24}H_{30}N_2NaO_2$ $(M + Na)^+$, 401.2205; found, 401.2229.

4.4. General procedure for the synthesis of the steroidal dienamides **4a–q**

To a solution of compound **3** (1.0 mmol) in ethanol, aldehydes (1.0 mmol) and sodium acetate (2.0 mmol) were added. The reaction mixture was heated under reflux for about 3–7 h. The solvent was removed and CH_2Cl_2 was added, the organic phase was washed with water and brine, dried over Na_2SO_4. After removal of the solvent, the residue was purified by silica gel chromatography with ethyl acetate/petroleum (1/2) as the eluent to give the corresponding steroidal dienamides.

4.4.1. 3β-Acetoxyl-5-en-16-benzylidene-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4a**)

Yellow solid, yield: 76%, mp 134.1–135.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.55–7.29 (m, 5H), 7.25 (s, 1H), 6.02 (s, 1H), 5.77 (s, 1H), 5.40 (d, *J* = 4.7 Hz, 1H), 4.61–4.50 (m, 1H), 2.04 (s, 3H), 1.06 (s, 3H), 1.05 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 175.44, 170.54, 164.92, 140.09, 137.42, 136.19, 135.78, 129.90, 128.90, 128.67, 121.61, 116.36, 98.51, 73.70, 52.54, 49.51, 47.13, 38.05, 36.79, 36.61, 34.73, 31.48, 31.42, 31.29, 29.70, 27.66, 21.42, 21.10, 19.31, 16.51. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{37}N_2O_3$ $(M + H)^+$, 485.2804; found, 485.2802.

4.4.2. 3β-Acetoxyl-5-en-16-(2,4-dichlorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxo-ethylidene) (**4b**)

Yellow solid, yield: 87%, mp 146.5–147.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.47 (s, 1H), 7.38 (dd, 2H), 7.25 (dd, 1H), 6.55 (s, 1H), 6.38 (s, 1H), 5.35 (d, *J* = 4.7 Hz, 1H), 4.68–4.52 (m, 1H), 2.03 (s, 3H), 1.08 (s, 3H), 1.04 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 172.51, 170.60, 165.08, 140.04, 135.38, 134.70, 133.19, 130.36, 130.16, 129.64, 127.01, 121.55, 116.08, 100.11, 73.70, 52.33, 49.41, 47.31, 38.00, 36.75, 36.57, 34.67, 31.34, 31.24, 31.03, 27.63, 21.41, 21.22, 19.27, 16.59. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{35}Cl_2N_2O_3$ $(M + H)^+$, 553.2025; found, 553.2007.

4.4.3. 3β-Acetoxyl-5-en-16-(4-chlorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4c**)

Yellow solid, yield: 86%, mp 161.0–162.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.34 (d, 4H), 7.19 (s, 1H), 6.32–5.96 (m, 2H), 5.39 (d, *J* = 3.9 Hz, 1H), 4.63–4.58 (m, 1H), 2.04 (s, 3H), 1.05 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 174.96, 170.58, 164.98, 140.07, 138.03, 134.69, 134.21, 130.99, 128.89, 121.56, 116.31, 98.83, 73.69, 52.44, 49.46, 47.14, 38.02, 36.68, 34.70, 31.58, 31.07, 27.64, 21.42, 21.07, 19.29, 16.51, 15.27. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{36}ClN_2O_3$ $(M + H)^+$, 519.2414; found, 519.2411.

4.4.4. 3β-Acetoxyl-5-en-16-(3-chlorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4d**)

Yellow solid, yield: 84%, mp 147.0–148.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.38 (s, 1H), 7.36–7.24 (m, 3H), 7.18 (s, 1H), 6.04 (m, 2H), 5.40 (d, *J* = 4.7 Hz, 1H), 4.71–4.49 (m, 1H), 2.04 (s, 3H), 1.05 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 174.60, 170.51, 164.71, 140.02, 138.89, 137.91, 134.55, 133.83, 129.80, 129.43, 128.65, 127.81, 121.52, 116.16, 99.18, 73.64, 54.81, 52.39, 49.42, 47.13, 37.99, 36.65, 34.67, 31.51, 31.05, 29.66, 27.60, 21.37, 21.03, 19.25, 16.48. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{35}ClN_2NaO_3$ $(M + Na)^+$, 541.2234; found, 541.2230.

4.4.5. 3β-Acetoxyl-5-en-16-(2-chlorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4e**)

Yellow solid, yield: 81%, mp 227.4–228.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.55 (s, 1H), 7.45 (d, *J* = 7.2 Hz, 1H), 7.38 (t, *J* = 6.2 Hz, 1H), 7.33–7.17 (m, 2H), 6.14 (m, 2H), 5.35 (s, 1H), 4.70–4.50 (m, 1H), 2.03 (s, 3H), 1.10 (s, 3H), 1.05 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 173.27, 170.54, 164.76, 140.02, 139.50, 134.67, 131.61, 129.84, 129.70, 129.61, 126.60, 121.59, 116.17, 99.69, 73.69, 52.37, 49.44, 47.33, 38.02, 36.77, 36.59, 34.68, 31.37, 31.25, 30.96, 29.69, 27.64, 21.41, 21.06, 19.28, 16.60. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{35}ClN_2NaO_3$ $(M + Na)^+$, 541.2234; found, 541.2232.

4.4.6. 3β-Acetoxyl-5-en-16-(3-methoxylbenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4f**)

Yellow solid, yield: 90%, mp 146.1–147.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.28 (m, 1H), 7.20 (s, 1H), 7.01 (d, *J* = 7.6 Hz, 1H), 6.93 (s, 1H), 6.86 (d, *J* = 8.2 Hz, 1H), 6.37 (s, 2H), 5.39 (d, *J* = 4.3 Hz, 1H), 4.69–4.51 (m, 1H), 3.81 (s, 3H), 2.03 (s, 3H), 1.04 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 174.67, 170.63, 165.60, 159.59, 140.00, 137.85, 137.55, 135.31, 129.60, 122.40, 121.66, 116.36, 115.29, 114.38, 98.80, 73.75, 55.32, 52.40, 49.44, 47.02, 38.02, 36.67, 34.69, 31.33, 29.68, 27.63, 21.41, 21.06, 19.28, 16.48. HRMS (ESI): *m*/*z* calcd for $C_{32}H_{38}N_2NaO_4$ $(M + Na)^+$, 537.2729; found, 537.2740.

4.4.7. 3β-Acetoxyl-5-en-16-(2-cyanobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4g**)

Brown solid, yield: 55%, mp 211.7–212.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.77–7.52 (m, 4H), 7.47–7.36 (m, 1H), 6.27 (s, 1H), 6.06 (s, 1H), 5.38 (d, *J* = 5.1 Hz, 1H), 4.73–4.50 (m, 1H), 2.05 (s, 3H), 1.12 (s, 3H), 1.07 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 172.42, 170.55, 164.21, 142.24, 140.12, 139.78, 133.21, 132.66, 129.67, 128.77, 128.49, 121.46, 117.68, 115.83, 112.96, 100.84, 73.67, 52.28, 49.42, 47.31, 38.03, 36.77, 36.60, 34.68, 31.41, 31.26, 29.70, 27.64, 21.41, 21.03, 19.30, 16.63. HRMS (ESI): *m*/*z* calcd for $C_{32}H_{35}N_3NaO_3$ $(M + Na)^+$, 532.2576; found, 532.2578.

4.4.8. 3β-Acetoxyl-5-en-16-(3,4-difluorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxo-ethylidene) (**4h**)

Yellow solid, yield: 85%, mp 143.2–145.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.20 (m, 4H, ArH and Ar–CH═, overlapped), 6.38 (s, 2H), 5.40 (d, *J* = 4.6 Hz, 1H), 4.60 (m, 1H), 2.04 (s, 3H), 1.05 (s, 3H), 1.04 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 174.28, 170.64, 165.23, 151.45, 148.96, 140.04, 138.41, 133.56, 132.96, 126.54, 121.55, 118.12, 117.94, 117.62, 117.45, 116.21, 99.23, 77.40, 77.08, 76.77, 52.36, 49.43, 47.10, 38.00, 36.76, 36.57, 34.69, 31.42, 31.24, 31.21, 27.62, 21.40, 21.05, 19.27, 16.49. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{35}F_2N_2O_3$ $(M + H)^+$, 521.2616; found, 521.2614.

4.4.9. 3β-Acetoxyl-5-en-16-(4-isopropylbenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4i**)

Yellow solid, yield: 70%, mp 142.2–143.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.38 (d, *J* = 8.2 Hz, 2H), 7.29–7.23 (m, 3H, ArH and Ar–CH═, overlapped), 6.37 (m, 2H), 5.41 (d, *J* = 4.4 Hz, 1H), 4.60 (m, 1H), 2.93 (m 1H), 2.04 (s, 3H), 1.26 (s, 3H), 1.25 (s, 3H), 1.06 (s, 3H), 1.04 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 175.07, 170.63, 165.84, 150.03, 140.01, 136.52, 135.59, 133.88, 130.08, 126.80, 121.70, 116.51, 98.26, 73.76, 52.50, 49.49, 47.00, 38.03, 36.68, 34.70, 33.98, 31.36, 29.69, 27.64, 23.79, 21.42, 21.09, 19.29, 16.48. HRMS (ESI): *m*/*z* calcd for $C_{34}H_{43}N_2O_3$ $(M + H)^+$, 527.3274; found, 527.3271.

4.4.10. 3β-Acetoxyl-5-en-16-furylmethylene-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4j**)

Yellow solid, yield: 86%, mp 156.8–157.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.51 (s, 1H), 7.11 (s, 1H), 6.50 (m, 4H), 5.40 (d, *J* = 3.9 Hz, 1H), 4.77–4.44 (m, 1H), 2.02 (s, 3H), 1.04 (s, 3H), 1.00 (s,

3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 174.01, 170.64, 165.58, 152.49, 144.44, 139.95, 134.60, 122.23, 121.77, 116.61, 114.60, 112.49, 97.92, 77.47, 77.24, 76.99, 73.78, 52.00, 49.45, 47.80, 38.01, 36.76, 36.56, 34.73, 31.64, 31.41, 31.26, 27.63, 21.40, 21.11, 19.28, 16.40, 14.18. HRMS (ESI): *m*/*z* calcd for $C_{29}H_{35}N_2O_4$ $(M + H)^+$, 475.2597; found, 475.2599.

4.4.11. 3β-Acetoxyl-5-en-16-thienylmethylene-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4k**)

Yellow solid, yield: 84%, mp 153.3–154.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): δ 7.53 (s, 1H), 7.49 (d, *J* = 4.8 Hz, 1H), 7.20 (d, *J* = 3.2 Hz, 1H), 7.10 (dd, *J* = 4.8, 3.2 Hz, 1H), 6.46 (s, 2H), 5.42 (d, *J* = 3.8 Hz, 1H), 4.61 (m, 1H), 2.04 (s, 3H), 1.05 (s, 3H), 1.02 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): δ 174.43, 170.65, 165.62, 140.50, 140.01, 134.64, 131.50, 129.58, 128.67, 127.79, 121.70, 116.61, 97.87, 77.48, 77.17, 76.85, 73.77, 52.13, 49.44, 47.98, 38.02, 36.76, 36.58, 34.76, 31.65, 31.44, 31.27, 27.64, 21.42, 21.10, 19.30, 16.43. HRMS (ESI): *m*/*z* calcd for $C_{29}H_{35}N_2O_3S$ $(M + H)^+$, 491.2368; found, 491.2365.

4.4.12. 3β-Acetoxyl-5-en-16-(4-bromobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4l**)

Yellow solid, yield: 80%, mp 163.9–164.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.50 (d, *J* = 8.5 Hz, 2H), 7.33–7.22 (d, *J* = 8.5 Hz, 2H), 7.18 (s, 1H), 6.16 (s, 1H), 6.08 (s, 1H), 5.39 (d, *J* = 4.5 Hz, 1H), 4.60 (m, 1H), 2.76 (dd, *J* = 16.8, 6.5 Hz, 1H), 2.73–2.62 (m, 1H), 2.04 (s, 3H), 1.06 (s, 3H), 1.05 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) δ 174.88, 170.59, 165.04, 140.06, 138.19, 135.07, 134.24, 131.86, 131.22, 123.08, 121.57, 116.32, 98.88, 73.69, 52.42, 49.44, 47.14, 38.02, 36.77, 36.58, 34.69, 31.42, 31.26, 27.64, 21.44, 21.07, 19.30, 16.53. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{35}BrN_2NaO_3$ $(M + Na)^+$, 585.1729; found, 585.1725.

4.4.13. 3β-Acetoxyl-5-en-16-(2-fluorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4m**)

Yellow solid, yield: 65%, mp 140.1–140.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.53–7.39 (m, 2H), 7.29 (m, 1H), 7.15 (t, *J* = 7.5 Hz, 1H), 7.12–6.98 (m, 1H), 6.27 (s, 2H), 5.37 (d, *J* = 4.7 Hz, 1H), 4.68–4.50 (m, 1H), 2.80–2.61 (m, 2H), 2.04 (s, 3H), 1.07 (s, 3H), 1.05 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) δ 173.85, 170.60, 165.07, 161.93, 159.43, 140.00, 139.47, 130.38, 129.59, 126.92, 124.44, 124.31, 124.00, 121.63, 116.28, 115.86, 115.64, 99.47, 73.71, 52.33, 49.44, 47.21, 38.02, 36.75, 36.58, 34.65, 31.37, 31.25, 31.20, 27.64, 21.42, 21.05, 19.29, 16.58. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{35}FN_2NaO_3$ $(M + Na)^+$, 525.2529; found, 525.2531.

4.4.14. 3β-Acetoxyl-5-en-16-(4-methylbenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4n**)

Yellow solid, yield: 61%, mp 149.6–149.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.32 (d, *J* = 8.0 Hz, 1H), 7.22 (s, 1H), 7.19 (d, *J* = 8.0 Hz, 1H), 6.15 (s, 1H), 6.11 (s, 1H), 5.40 (d, *J* = 4.5 Hz, 1H), 4.60 (m, 1H), 2.81 (dd, *J* = 15.9, 6.1 Hz, 1H), 2.76–2.63 (m, 1H), 2.36 (s, 3H), 2.04 (s, 3H), 1.05 (s, 3H), 1.04 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) δ 175.47, 170.58, 165.45, 140.04, 139.24, 136.40, 135.80, 133.46, 129.95, 129.43, 121.67, 116.51, 98.12, 73.73, 52.52, 49.49, 47.08, 38.04, 36.78, 36.59, 34.70, 31.44, 31.28, 29.70, 27.65, 21.43, 21.42, 21.09, 19.30, 16.48. HRMS (ESI): *m*/*z* calcd for $C_{32}H_{38}N_2NaO_3$ $(M + Na)^+$, 521.2780; found, 521.2782.

4.4.15. 3β-Acetoxyl-5-en-16-(3,4-dichlorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4o**)

Yellow solid, yield: 84%, mp 159.8–162.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.47 (d, *J* = 1.7 Hz, 1H), 7.44 (d, *J* = 8.4 Hz, 1H), 7.24 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.14 (s, 1H), 6.20 (s, 1H), 6.10 (s, 1H), 5.39 (d, *J* = 4.6 Hz, 1H), 4.60 (m, 1H), 2.75 (dd, *J* = 16.1, 5.8 Hz, 1H), 2.72–2.63 (m, 1H), 2.04 (s, 3H), 1.05 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) δ 174.35, 170.60, 164.82, 140.06, 139.36, 136.17, 132.84, 132.70, 131.22, 130.56, 128.74, 121.52, 116.16, 99.43, 73.68, 52.37, 49.42, 47.19, 38.01, 36.76, 36.58, 34.69, 31.42, 31.32, 31.27, 27.63, 21.43, 21.05, 19.30, 16.53. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{35}Cl_2N_2O_3$ $(M + H)^+$, 553.2025; found, 553.2023.

4.4.16. 3β-Acetoxyl-5-en-16-(4-fluorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4p**)

Yellow solid, yield: 72%, mp 138.5–138.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.41 (dd, *J* = 8.7, 5.5 Hz, 2H), 7.22 (s, 1H), 7.08 (t, *J* = 8.7 Hz, 2H), 6.02 (s, 1H), 5.72 (s, 1H), 5.40 (d, *J* = 4.9 Hz, 1H), 4.60 (m, 1H), 2.79 (dd, *J* = 15.9, 7.4 Hz, 1H), 2.75–2.64 (m, 1H), 2.04 (s, 3H), 1.06 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) δ 175.20, 170.59, 165.20, 163.96, 161.46, 140.07, 137.00, 134.39, 132.45, 131.72, 121.58, 116.39, 115.89, 115.68, 98.51, 73.69, 52.45, 49.46, 47.13, 38.02, 36.77, 36.59, 34.69, 31.43, 31.32, 31.26, 27.64, 21.43, 21.07, 19.30, 16.51. HRMS (ESI): *m*/*z* calcd for $C_{31}H_{35}FN_2NaO_3$ $(M + Na)^+$, 525.2529; found, 525.2529.

4.4.17. 3β-Acetoxyl-5-en-16-(3,4,5-trimethoxylbenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**4q**)

Yellow solid, yield: 89%, mp 150.1–150.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.17 (s, 1H), 6.65 (s, 2H), 6.34 (s, 1H), 6.23 (s, 1H), 5.39 (d, *J* = 4.5 Hz, 1H), 4.74–4.51 (m, 1H), 3.86 (s, 9H), 2.82 (dd, *J* = 15.8, 6.2 Hz, 1H), 2.75–2.60 (m, 1H), 2.04 (s, 3H), 1.05 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) δ 174.88, 170.62, 165.52, 153.06, 140.15, 138.90, 136.68, 135.64, 131.82, 121.49, 116.43, 107.30, 98.41, 73.70, 60.95, 56.20, 52.39, 49.42, 46.99, 38.01, 36.75, 36.59, 34.66, 31.39, 31.22, 27.62, 21.42, 21.04, 19.28, 16.50. HRMS (ESI): *m*/*z* calcd for $C_{34}H_{42}N_2NaO_6$ $(M + Na)^+$, 597.2941; found, 597.2939.

4.5. General procedure for the synthesis of the steroidal dienamides **5a–f**

To a solution of compound **3** (1.0 mmol) in methanol, aldehydes (1.0 mmol) and sodium carbonate (2.0 mmol) were added. The reaction mixture was heated under reflux for about 3–6 h. The solvent was removed and CH_2Cl_2 was added, the organic phase was washed with water and brine, dried over Na_2SO_4. After removal of the solvent, the residue was purified by silica gel chromatography with ethyl acetate/petroleum (1/2) as the eluent to give the corresponding steroidal dienamides.

4.5.1. 3β-Hydroxyl-5-en-16-(2-chlorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxo-ethylidene) (**5a**)

White solid, yield: 75%, mp 228.1–228.3 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) δ 8.20 (s, 1H), 7.99 (s, 1H), 7.89–7.57 (m, 2H), 7.52 (dd, *J* = 13.9, 7.9 Hz, 1H), 7.49–7.31 (m, 2H), 5.26 (s, 1H), 4.63 (d, *J* = 4.2 Hz, 1H), 3.26 (d, *J* = 4.7 Hz, 1H), 1.03 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm) δ 168.80, 164.11, 141.74, 141.52, 134.54, 133.82, 130.67, 130.38, 130.07, 127.77, 127.02, 120.44, 118.08, 100.57, 70.39, 52.29, 49.40, 47.77, 42.59, 36.48, 31.19, 19.57, 16.70. HRMS (ESI): *m*/*z* calcd for $C_{29}H_{33}ClN_2NaO_2$ $(M + Na)^+$, 499.2128; found, 499.2125.

4.5.2. 3β-Hydroxyl-5-en-16-(2-fluorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxo-ethylidene) (**5b**)

Yellow solid, yield: 82%, mp 173.0–173.2 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) δ 8.19 (d, *J* = 22.8 Hz, 1H), 7.93–7.57 (m, 3H), 7.51–7.37 (m, 1H), 7.37–7.15 (m, 2H), 5.28 (s, 1H), 4.63 (dd, *J* = 4.3, 2.3 Hz, 1H), 3.33–3.16 (m, 1H), 0.99 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm) δ 169.37, 168.32, 165.02, 164.06, 161.70, 159.23, 159.14, 141.86, 141.77, 141.43, 140.94, 130.94, 130.26, 130.15, 125.15, 125.08, 124.58, 124.46, 124.31, 124.20, 123.45, 122.27,

120.43, 118.11, 116.95, 116.14, 116.10, 115.93, 115.89, 101.64, 100.31, 70.40, 52.27, 49.58, 49.46, 47.60, 46.59, 42.60, 37.16, 36.57, 36.50, 34.83, 33.59, 31.82, 31.49, 31.35, 31.23, 31.07, 19.57, 17.01, 16.68. HRMS (ESI): *m/z* calcd for $C_{29}H_{33}FN_2NaO_2$ $(M + Na)^+$, 483.2424; found, 483.2426.

4.5.3. 3β-Hydroxyl-5-en-16-(3-methoxylbenzylidene)-androstano-17-(2-amino-1-cyano-2-oxo-ethylidene) (**5c**)

Yellow solid, yield: 82%, mp 206.4–206.6 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) δ 8.13 (d, J = 21.9 Hz, 1H), 7.73 (s, 1H), 7.62 (s, 1H), 7.36 (dd, J = 16.4, 8.3 Hz, 1H), 7.11 (d, J = 8.0 Hz, 1H), 7.07–6.87 (m, 2H), 5.30 (d, J = 2.8 Hz, 1H), 4.63 (dd, J = 4.5, 2.3 Hz, 1H), 3.79 (s, 3H, δ, ppm), 3.27 (d, J = 4.3 Hz, 1H), 2.62–2.40 (m, 3H), 0.99 (s, 3H), 0.98 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm) δ 170.78, 169.18, 165.26, 164.19, 159.79, 141.83, 139.69, 139.30, 138.09, 137.81, 132.25, 131.30, 130.27, 122.33, 120.49, 118.29, 117.03, 115.08, 114.92, 114.63, 100.84, 99.70, 70.41, 55.58, 52.36, 49.57, 47.38, 46.50, 42.62, 37.14, 36.54, 34.90, 33.55, 31.83, 31.32, 21.03, 19.57, 16.96, 16.61. HRMS (ESI): *m/z* calcd for $C_{30}H_{36}N_2NaO_3$ $(M + Na)^+$, 495.2624; found, 495.2627.

4.5.4. 3β-Hydroxyl-5-en-16-(3,4-dichlorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxo-ethylidene) (**5d**)

White solid, yield: 79%, mp 185.5–185.7 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm) δ 8.14 (s, 1H), 7.76 (d, J = 3.2 Hz, 2H), 7.69 (dd, J = 8.3, 5.8 Hz, 1H), 7.56 (dd, J = 15.9, 12.8 Hz, 2H), 5.30 (s, 1H), 4.65 (s, 1H), 3.27 (s, 1H), 2.77 (dd, J = 16.3, 6.5 Hz, 1H), 2.73–2.57 (m, 1H), 0.97 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm) δ 170.22, 168.67, 164.99, 163.95, 141.87, 141.81, 141.47, 137.37, 137.15, 131.96, 131.89, 131.37, 131.34, 131.23, 131.10, 129.77, 129.57, 128.71, 120.41, 118.05, 101.66, 100.54, 70.38, 52.29, 52.14, 49.51, 47.39, 46.55, 42.61, 37.12, 36.57, 36.51, 33.51, 31.81, 31.27, 31.09, 20.93, 19.59, 16.93, 16.56. HRMS (ESI): *m/z* calcd for $C_{29}H_{32}Cl_2N_2NaO_2$ $(M + Na)^+$, 533.1739; found, 533.1735.

4.5.5. 3β-Hydroxyl-5-en-16-(4-fluorobenzylidene)-androstano-17-(2-amino-1-cyano-2-oxoethylidene) (**5e**)

Yellow solid, yield: 73%, mp 165.5–165.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.50 (dd, J = 8.5, 5.5 Hz, 1H), 7.41 (dd, J = 8.5, 5.5 Hz, 1H), 7.21 (s, 1H), 7.09 (q, J = 9.1 Hz, 2H), 6.14 (s, 1H), 6.03 (s, 1H), 5.37 (d, J = 3.3 Hz, 1H), 3.71–3.40 (m, 1H), 2.78 (dd, J = 15.7, 5.7 Hz, 1H), 2.74–2.57 (m, 1H), 1.06 (s, 3H), 1.05 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) δ 175.20, 165.41, 164.38, 163.94, 161.44, 141.18, 141.14, 138.19, 137.03, 134.29, 132.79, 132.48, 132.45, 131.77, 131.73, 131.68, 120.66, 120.61, 118.29, 116.45, 115.89, 115.68, 98.49, 96.93, 71.51, 52.89, 52.49, 49.55, 48.24, 47.15, 42.09, 42.00, 37.03, 36.49, 34.73, 33.48, 31.46, 31.33, 31.27, 29.70, 27.02, 24.98, 21.12, 19.39, 16.53, 16.16. HRMS (ESI): *m/z* calcd for $C_{29}H_{34}FN_2O_2$ $(M + H)^+$, 461.2604; found, 461.2602.

4.5.6. 3β-Hydroxyl-5-en-16-(3,4,5-trimethoxyl benzylidene)-andros tano-17-(2-amino-1-cyano-2-oxoethylidene) (**5f**)

Yellow solid, yield: 86%, mp 166.1–166.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm) δ 7.72 and 7.19 (1H), 6.76 (s, 1H), 6.67 (s, 1H), 6.17 (d, J = 12.1 Hz, 1H), 5.97 (d, J = 25.6 Hz, 1H), 5.38 (s, 1H), 3.91 (s, 3H), 3.88 (s, 6H), 3.55 (s, 1H), 2.84 (dd, J = 15.6, 6.9 Hz, 1H), 2.72 (d, J = 6.6 Hz, 1H), 1.12 (s, 3H), 1.07 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm) δ 177.84, 175.25, 165.40, 164.33, 153.14, 153.10, 141.31, 141.28, 138.99, 137.85, 136.67, 135.75, 134.11, 131.80, 120.55, 120.49, 118.35, 116.48, 107.38, 98.28, 96.62, 71.51, 60.98, 56.23, 52.85, 52.49, 49.54, 48.22, 47.07, 42.09, 41.98, 37.03, 36.51, 34.72, 33.49, 31.45, 31.31, 31.26, 29.69, 27.02, 24.98, 21.11, 19.37, 16.53, 16.17. HRMS (ESI): *m/z* calcd for $C_{32}H_{40}N_2NaO_5$ $(M + Na)^+$, 555.2835; found, 555.2837.

4.6. Effect of compounds on cell viability

Exponentially growing cells were seeded at 5 × 103 cells per well into 96-well plates. 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. The medium containing MTT was discarded, 150 μL of dimethyl sulfoxide (DMSO) was added to each well and the plates agitated until the dark blue crystals (formazan) had completely dissolved; the absorbance was measured using a microplate reader at a wavelength of 570 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 concentration–response curves according to the inhibition ratio for each concentration.

4.7. Analysis of cellular apoptosis

MGC-803 cells were plated in 6-well plates (1.0×10^6 cells/well) and incubated at 37 °C for 24 h. Exponentially growing cells were then incubated for 24 h with complete medium (blank) or with the compound **4c**. Cells were then harvested and the 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 flow cytometer.

4.8. Flow cytometric analysis of cell cycle distribution

For flow cytometric analysis of DNA content, 5×10^5 MGC-803 cells in exponential growth were treated with different concentrations of the test compounds for 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).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi:10.1016/j.ejmech.2013.05.035.

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