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Stereoselective synthesis of novel antiproliferative steroidal (*E*, *E*) dienamides through a cascade aldol/cyclization process

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ABSTRACT

The stereoselective and metal-free protocol involving a cascade aldol/cyclization process for the synthesis of steroidal (*E*, *E*) dienamides from steroidal α, α-dicyanoalkene was reported. This protocol efficiently achieved the construction of C=C bond and selective conversion of cyano group into carboxamide in one-pot procedure under mild condition. Further biological evaluation showed that some of these compounds had moderate to excellent cytotoxic activities against all the tested cancer cell lines and were more potent than well-known drug 5-fluorouracil. Particularly, compound **3c** represented excellent inhibitory effect against MCF-7 (IC_{50} = 0.76 μM), which was about 10-fold more potent than 5-fluorouracil.

1. Introduction

Dienamides have long been recognized as key reactive intermediates due to their great diversities, potential synthetic values and commonly existence in nature [1–3] and have been reported to have antioxidant, cytotoxic [4] and insecticidal activities [5]. Acyclic dienamides are also key constituents in a number of biologically active natural products and pharmaceutically relevant units. Examples of these include Apicularen A [6,7], Salicylihalamide A [8], 5,5-diarylpentadienamides [9] and (*E*, *E*)-2-(benzylaminocarbonyl)-3-styrylacrylonitrile [10] (Fig. 1). For example, Saku *et al.* reported that 5,5-diarylpentadiena-mides as the transient receptor potential vanilloid I (TRPVI) antagonists are under further evaluation for clinical treatment of neuropathic pain [9]. Recently, Chen and co-workers reported that (*E*, *E*)-2-(benzylaminocarbonyl)-3-styrylacrylonitrile as the Mcl-1 protein inhibitor represented a 6-fold enhancement compared to its parent structure (K_d = 0.16 μM) [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 total synthesis of natural alkaloids [12] and some interesting heterocycles [13–15].

Despite their synthetic utility and biological potential, the synthetic routes available for the synthesis of dienamides are relatively limited [16,17]. A number of recent approaches for the synthesis of dienamides are mainly transition metal-based protocols, which require rather severe catalytic reaction conditions [18]. This can make these preparative methods environmentally unfriendly and less cost effective. Moreover, heavy metals may contaminate the final products, a problem which is frequently encountered in the pharmaceutical industry [19]. The development of efficient, mild and metal-free methods for the synthesis of more versatile and new dienamides is also desirable. Recently, Zhao [20] and Perumal [21] reported a cascade one-pot approach to a variety of dienamides from α,α-dicyanoalkenes and aldehydes via vinylogous aldol reaction by the electrocyclic ring opening of the initially formed pyran derivatives under mild basic condition with good diastereoselectivity, respectively. In view of the therapeutic importance of dienamides and being involved in finding new biologically active modified steroids [22–25], we are interested in the design, synthesis and biological evaluation of novel steroidal dienamides. Our group recently reported that dehydroepiandrosterone-based steroidal dienamides showed excellent cytotoxic activities with the IC_{50} values ranging from 0.1 to 40 μM and caused the cellular early apoptosis and cell cycle arrest in G2/M phase in a concentration-independent manner (Scheme 1) [26]. To better understand the structure-activity relationship of steroidal dienamides, herein we report the stereoselective and metal-free synthesis of steroidal (*E*, *E*) dienamides from steroidal α,α-dicyanoalkene through a cascade aldol/cyclization process, affording a series of conjugated

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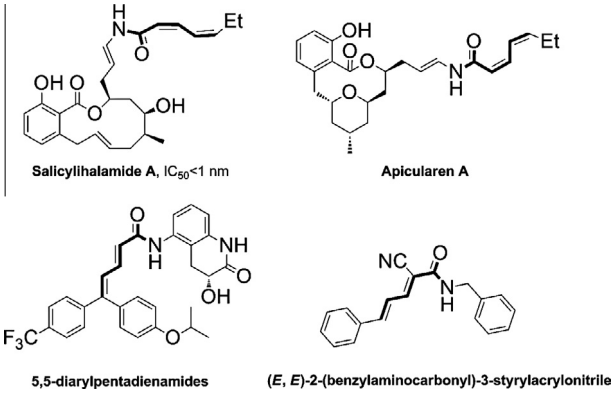


Fig. 1. Some natural occurring and synthetic pharmaceutically active dienamides.

steroidal dienamides in moderate yields. Besides, we also evaluated their cytotoxic activities against MGC-803, MCF-7, EC109 and SMMC-7721 cancer cell lines.

2. Experimental

2.1. General remarks

Reagents and solvents were purchased from commercial sources and were used without further purification. 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-*d6*. 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). Signal patterns are indicated as s, singlet; d, doublet; dd, double doublet; *t*, triplet; m, multiplet; bs, broad singlet. Coupling constant (*J*) is given in Hertz (Hz). High-resolution mass spectra (HRMS) were recorded on a Waters Micromass Q–T of Micromass spectrometer by electrospray ionization (ESI).

2.2. 20-Dicyanometylidene-5-pregnen-3β-ol acetate (**2**)

To a solution of compound **1** (1.076 g, 3.0 mmol) in ethanol (10 mL) containing ammonium acetate (0.5 g, 6.49 mmol), malononitrile (297 mg, 4.5 mmol) was added. The reaction mixture was heated under reflux for about 3 h until compound **1** disappeared as indicated by TLC (petroleum ether/ethyl acetate = 9/1). The solid product formed upon cooling at room temperature was washed with ethanol, collected by filtration to yield compound **2**. White solid; mp 205.2–205.3 °C; Yield: 95%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38 (d, *J* = 5.0 Hz, 1H), 4.70-4.53 (m, 1H), 3.03 (*t*, *J* = 8.9 Hz, 1H), 2.28 (s, 3H), 2.04 (s, 3H), 1.02 (s, 3H), 0.73 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.47, 170.53, 139.81, 122.05, 112.74, 112.39, 86.79, 73.74, 57.38, 56.20, 50.22, 49.74, 38.04, 37.65, 36.92, 36.63, 31.88, 31.75, 27.68, 25.45, 25.09, 21.86, 21.45, 20.72, 19.33, 14.25. HRMS (ESI): *m/z* calcd for $C_{26}H_{34}N_2NaO_2$ $(M + Na)^+$, 429.2518; found, 429.2516.

2.3. General procedure for the synthesis of compounds ***3a–k***

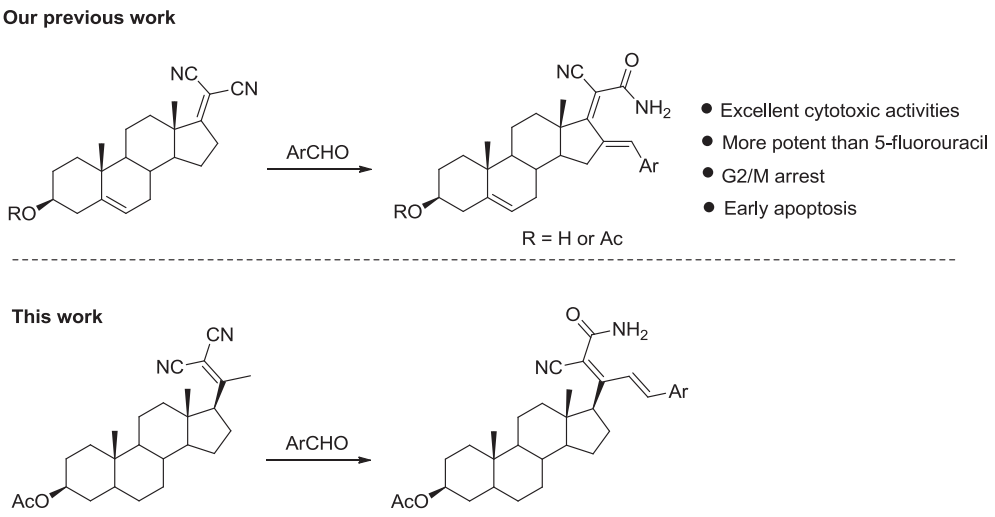
To a solution of compound **2** (407 mg, 1.0 mmol) in ethanol, aldehyde (1.2 mmol) and sodium acetate (405 mg, 2.0 mmol) were added. The reaction mixture was heated under reflux for about 3–7 h. The solvent was removed and CH_2Cl_2 was added, the organic phase was washed with water and brine, dried over Na_2SO_4. After removal of the solvent, the residue was purified by silica gel chromatography using ethyl acetate/petroleum ether (1/2) as the eluent to give the corresponding steroidal dienamides.

2.3.1. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-phenyl-(2E, 4E)-penta-2,4-dienoic acid amide (***3a***)

White solid; mp 109.9–110.0 °C; Yield: 75%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51-7.40 (m, 2H), 7.41-7.29 (m, 3H), 7.20 (d, *J* = 16.3 Hz, 1H), 6.86 (d, *J* = 16.3 Hz, 1H), 6.03 (s, 1H), 5.67 (s, 1H), 5.39 (d, *J* = 5.0 Hz, 1H), 4.94-4.27 (m, 1H), 3.13 (*t*, *J* = 9.2 Hz, 1H), 2.03 (s, 3H), 1.02 (s, 3H), 0.81 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.55, 166.53, 164.01, 139.80, 137.48, 135.78, 129.38, 128.91, 127.44, 125.42, 122.24, 117.99, 106.98, 73.83, 56.56, 56.38, 49.94, 48.65, 38.14, 38.07, 36.96, 36.68, 31.99, 31.85, 27.71, 25.58, 25.12, 21.43, 20.84, 19.32, 14.44. HRMS (ESI): *m/z* calcd for $C_{33}H_{41}N_2O_3$ $(M + H)^+$, 513.3117; found, 513.3118.

2.3.2. 2-Ccyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(4″-chlorophenyl)-(2E, 4E)-penta-2,4-dienoic acid amide (***3b***)

White solid; mp 199.0–199.1 °C; Yield: 82%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (d, *J* = 8.6 Hz, 2H), 7.32 (d, *J* = 8.6 Hz, 2H), 7.18 (d, *J* = 16.3 Hz, 1H), 6.79 (d, *J* = 16.3 Hz, 1H), 6.13 (s, 1H), 5.99 (s, 1H), 5.39 (d, *J* = 4.6 Hz, 1H), 4.70-4.53 (m, 1H), 3.12 (*t*, *J* = 9.1 Hz, 1H), 2.04 (s, 3H), 1.02 (s, 3H), 0.79 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.52, 166.31, 164.00, 139.71, 135.74, 135.06, 134.24, 129.06,



Scheme 1. Design of pregnenolone-based dienamides.

128.50, 126.05, 122.17, 117.89, 107.09, 73.75, 56.50, 56.29, 49.84, 48.64, 38.06, 37.99, 36.89, 36.60, 31.91, 31.77, 27.64, 25.48, 25.05, 21.40, 20.77, 19.26, 14.37. HRMS (ESI): *m/z* calcd for $C_{33}H_{40}ClN_2O_3$ $(M+H)^+$, 547.2727; found, 547.2729.

*2.3.3. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(2″-chlorophenyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3c**)*

Yellow solid; mp 121.2–121.5 °C; Yield: 90%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69-7.54 (m, 1H), 7.38 (dd, *J* = 7.2, 1.9 Hz, 1H), 7.32-7.23 (m, 2H), 7.20 (d, *J* = 2.7 Hz, 2H), 6.16 (s, 1H), 5.88 (s, 1H), 5.39 (d, *J* = 4.3 Hz, 1H), 4.60 (m, 1H), 3.15 (*t*, *J* = 9.2 Hz, 1H), 2.04 (s, 3H), 1.02 (s, 3H), 0.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.56, 167.20, 163.73, 139.78, 134.32, 134.15, 132.72, 130.01, 129.87, 128.31, 127.39, 127.21, 122.25, 117.93, 107.58, 73.83, 56.84, 56.34, 49.89, 48.79, 38.23, 38.06, 36.95, 36.68, 31.99, 31.85, 27.71, 25.54, 25.17, 21.45, 20.87, 19.33, 14.59. HRMS (ESI): *m/z* calcd for $C_{33}H_{40}ClN_2O_3$ $(M+H)^+$, 547.2727; found, 547.2726.

*2.3.4. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(3″-chlorophenyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3d**)*

Yellow solid; mp 197.7–197.8 °C; Yield: 84%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (s, 1H), 7.39-7.22 (m, 3H), 7.18 (d, *J* = 16.3 Hz, 1H), 6.73 (d, *J* = 16.3 Hz, 1H), 6.06 (s, 1H), 5.69 (s, 1H), 5.39 (d, *J* = 4.5 Hz, 1H), 4.73-4.48 (m, 1H), 3.11 (*t*, *J* = 9.1 Hz, 1H), 2.04 (s, 3H), 1.02 (s, 3H), 0.79 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.57, 166.68, 163.63, 139.79, 137.65, 135.29, 134.89, 130.12, 129.12, 127.10, 126.95, 125.57, 122.23, 117.82, 107.45, 73.81, 56.78, 56.34, 49.91, 48.78, 38.11, 38.06, 36.95, 36.67, 31.98, 31.83, 27.71, 25.48, 25.10, 21.45, 20.83, 19.33, 14.47. HRMS (ESI): *m/z* calcd for $C_{33}H_{40}ClN_2O_3$ $(M+H)^+$, 547.2727; found, 547.2725.

*2.3.5. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(4″-bromophenyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3e**)*

White solid; mp 176.8–177.0 °C; Yield: 79%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J* = 8.4 Hz, 2H), 7.31 (d, *J* = 8.4 Hz, 2H), 7.22 (d, *J* = 16.3 Hz, 1H), 6.77 (d, *J* = 16.3 Hz, 1H), 6.06 (s, 1H), 5.71 (s, 1H), 5.39 (d, *J* = 4.7 Hz, 1H), 4.75-4.50 (m, 1H), 3.12 (*t*, *J* = 9.2 Hz, 1H), 2.04 (s, 3H), 1.02 (s, 3H), 0.79 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.79, 166.88, 164.03, 140.03, 136.05, 135.00, 132.31, 129.06, 126.50, 123.64, 122.46, 118.21, 107.29, 74.04, 56.85, 56.61, 50.16, 48.97, 38.39, 38.30, 37.19, 36.91, 32.22, 32.08, 29.95, 28.35, 27.94, 26.64, 25.76, 25.35, 21.69, 21.08, 14.68. HRMS (ESI): *m/z* calcd for $C_{33}H_{39}BrN_2NaO_3$ $(M+Na)^+$, 613.2042; found, 613.2044.

*2.3.6. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(4″-nitrophenyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3f**)*

Brown solid; mp 138.9–139.0 °C; Yield: 86%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, *J* = 8.8 Hz, 2H), 8.21 (d, *J* = 8.8 Hz, 2H), 7.62 (d, *J* = 8.8 Hz, 2H), 7.59 (d, *J* = 8.8 Hz, 2H), 7.35 (d, *J* = 16.4 Hz, 1H), 7.15 (d, *J* = 16.2 Hz, 1H), 6.97 (d, *J* = 16.2 Hz, 1H), 6.76 (d, *J* = 16.4 Hz, 1H), 6.19 (d, *J* = 25.9 Hz, 2H), 5.73 (d, *J* = 9.0 Hz, 2H), 5.38 (s, 2H), 4.61 (d, *J* = 5.9 Hz, 2H), 4.06 (*t*, *J* = 9.4 Hz, 1H), 3.13 (*t*, *J* = 9.1 Hz, 1H), 2.04 (s, 6H), 1.02 (s, 6H), 0.79 (s,6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.57, 170.37, 168.55, 167.15, 165.65, 163.28, 163.21, 163.12, 150.95, 147.97, 147.69, 144.94, 142.16, 141.56, 139.90, 139.80, 139.74, 136.60, 135.70, 133.25, 130.72, 130.27, 127.86, 127.79, 124.24, 124.20, 122.26, 122.17, 120.01, 118.18, 117.61, 117.52, 115.30, 108.02, 106.04, 73.79, 57.20, 56.31, 56.24, 50.97, 49.90, 49.85, 49.01, 48.74, 38.05, 36.97, 36.89, 36.66, 32.02, 31.94, 31.81, 29.71, 27.70, 26.18, 25.33, 25.14, 25.08, 21.44, 20.84, 20.71, 19.32, 14.56, 14.51. HRMS (ESI): *m/z* calcd for $C_{33}H_{39}N_3NaO_5$ $(M+Na)^+$, 580.2787; found, 580.2787.

*2.3.7. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(4″-fluorophenyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3g**)*

White solid; mp 226.1–226.3 °C; Yield: 83%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51-7.37 (m, 2H), 7.14 (d, *J* = 16.3 Hz, 1H), 7.05 (*t*, *J* = 8.6 Hz, 2H), 6.82 (d, *J* = 16.3 Hz, 1H), 6.08 (s, 1H), 5.78 (s, 1H), 5.39 (d, *J* = 4.9 Hz, 1H), 4.69-4.54 (m, 1H), 3.12 (*t*, *J* = 9.2 Hz, 1H), 2.04 (s, 3H), 1.02 (s, 3H), 0.80 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.80, 166.80, 164.80, 164.27, 162.31, 140.03, 136.32, 132.31, 132.28, 129.45, 129.36, 125.55, 122.47, 118.28, 116.33, 116.11, 107.08, 74.05, 56.76, 56.62, 50.16, 48.91, 38.39, 38.29, 37.19, 36.90, 32.21, 32.08, 29.94, 27.94, 25.81, 25.36, 21.68, 21.08, 19.56, 14.67. HRMS (ESI): *m/z* calcd for $C_{33}H_{40}FN_2O_3$ $(M+H)^+$, 531.3023; found, 531.3023.

*2.3.8. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(3″,4″-difluorophenyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3h**)*

Yellow solid; mp 122.7–122.8 °C; Yield: 85%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32-7.23 (m, 1H), 7.15 (d, *J* = 7.3 Hz, 2H), 7.12 (d, *J* = 10.3 Hz, 1H), 6.71 (d, *J* = 16.3 Hz, 1H), 6.09 (s, 1H), 5.72 (s, 1H), 5.39 (d, *J* = 4.9 Hz, 1H), 4.61 (m, 1H), 3.11 (*t*, *J* = 9.2 Hz, 1H), 2.04 (s, 3H), 1.02 (s, 3H), 0.79 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.57, 166.70, 163.60, 139.79, 134.49, 126.70, 123.88, 122.21, 117.88, 117.83, 117.65, 115.69, 115.51, 107.26, 73.80, 56.76, 56.35, 49.91, 48.79, 38.12, 38.06, 36.96, 36.67, 31.98, 31.83, 27.70, 25.47, 25.10, 21.45, 20.83, 19.33, 14.45. HRMS (ESI): *m/z* calcd for $C_{33}H_{39}F_2N_2O_3$ $(M+H)^+$, 549.2929; found, 549.2926.

*2.3.9. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-3″, 4″,5″-trimethoxylphenyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3i**)*

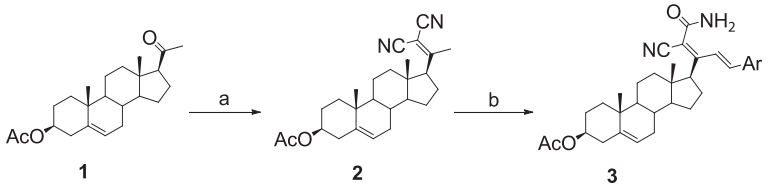
White solid; mp 201.2–201.4 °C; Yield: 80%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17 (d, *J* = 16.2 Hz, 1H), 6.80 (d, *J* = 16.2 Hz, 1H), 6.67 (s, 2H), 6.09 (s, 1H), 5.74 (s, 1H), 5.39 (d, *J* = 4.6 Hz, 1H), 4.70-4.52 (m, 1H), 3.89 (s, 6H), 3.86 (s, 3H), 3.14 (*t*, *J* = 9.1 Hz, 1H), 2.04 (s, 3H), 1.02 (s, 3H), 0.81 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.50, 166.43, 164.09, 153.45, 139.73, 139.43, 137.53, 131.37, 124.76, 122.18, 118.12, 106.51, 104.60, 73.75, 56.18, 49.87, 48.58, 38.11, 38.00, 36.90, 36.61, 31.92, 31.80, 29.65, 27.65, 25.56, 25.22, 25.09, 24.70, 21.39, 20.78, 19.26, 14.45. HRMS (ESI): *m/z* calcd for $C_{36}H_{47}N_2O_6$ $(M+H)^+$, 603.3434; found, 603.3433.

*2.3.10. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(4″-furyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3j**)*

Yellow solid; mp 145.3–145.6 °C; Yield: 86%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 1.2 Hz, 1H), 7.12 (d, *J* = 16.1 Hz, 1H), 6.75 (d, *J* = 16.1 Hz, 1H), 6.48 (d, *J* = 3.3 Hz, 1H), 6.46-6.35 (m, 1H), 6.03 (s, 1H), 5.82 (s, 1H), 5.39 (d, *J* = 4.7 Hz, 1H), 4.61 m, 1H), 3.08 (*t*, *J* = 9.2 Hz, 1H), 2.04 (s, 3H), 1.02 (s,3H), 0.81 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.80, 165.33, 164.64, 152.01, 144.45, 140.02, 125.50, 123.36, 122.49, 118.42, 113.34, 112.50, 106.55, 74.07, 56.64, 56.30, 50.17, 48.77, 38.39, 38.30, 37.19, 36.91, 32.19, 32.10, 29.94, 27.95, 25.96, 25.38, 21.68, 21.07, 19.56, 14.60. HRMS (ESI): *m/z* calcd for $C_{31}H_{39}N_2O_4$ $(M+H)^+$, 503.2910; found, 503.2912.

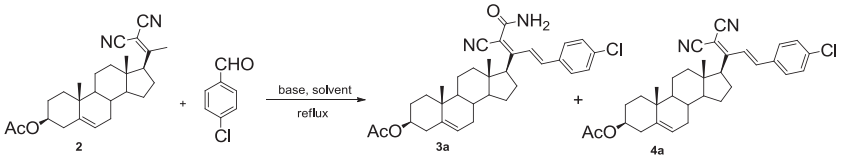
*2.3.11. 2-Cyano-3-[(3′β,17′β)-3′-(acetyloxy) androst-5′-en-17′-yl]-5-(4″-thienyl)-(2E, 4E)-penta-2,4-dienoic acid amide (**3k**)*

Yellow solid; mp 124.0–124.3 °C; Yield: 85%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, *J* = 4.9 Hz, 1H), 7.18-7.11 (m, 1H), 7.09 (s, 2H), 7.02 (dd, *J* = 5.0, 3.7 Hz, 1H), 6.05 (s, 1H), 5.72 (s, 1H), 5.39 (d, *J* = 4.9 Hz, 1H), 4.71-4.50 (m, 1H), 3.10 (*t*, *J* = 9.2 Hz, 1H), 2.03 (s, 3H), 1.02 (s, 3H), 0.81 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.56, 165.29, 164.19, 141.36, 139.80, 131.06, 129.32, 128.09, 127.53, 124.52, 122.25, 118.26, 106.20, 73.83, 56.42, 56.18, 49.94, 48.55, 38.19, 38.07, 36.96, 36.68, 31.97, 31.87,



Scheme 2. Synthesis of steroidal dienamides (**3a–k**). Reagents and conditions: (a) malononitrile, NH_4OAc, EtOH, reflux; (b) aldehydes, NaOAc, EtOH, reflux.

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



Entry	Solvent	Base	Yield[b] (%)
1	EtOH	Et_3N (1.0 eq)	Trace
2	EtOH	Piperidine (1.0 eq)	24
3	EtOH	Na_2CO_3 (1.0 eq)	67
4	$HOCH_2CH_2OH$	Et_3N (1.0 eq)	No reaction
5	DCM	Et_3N (1.0 eq)	No reaction
6	EtOH	NaOAc (1.0 eq)	30
7	EtOH	NaOAc (2.0 eq)	56
8	EtOH	NaOAc (5.0 eq)	82

[a] Unless otherwise noted, the reaction was carried out with **2** (0.5 mmol) and 4-chlorobenzaldehyde (0.6 mmol) under reflux in solvent (5 mL) for 12 h.
[b] Isolated yields.

27.71, 25.71, 25.16, 21.44, 20.85, 19.32, 14.40. HRMS (ESI): *m/z* calcd for $C_{31}H_{39}N_2O_3S$ $(M+H)^+$, 519.2681; found, 519.2679.

2.4. Cytotoxic activity 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 a 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.

3. Results and discussion

3.1. Chemistry

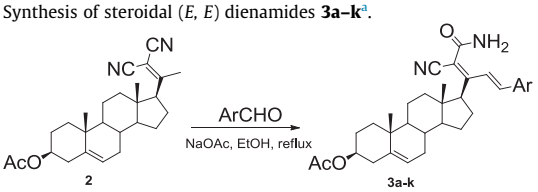
The protocol for the synthesis of steroidal (*E*, *E*) dienamides **3a–k** was very simple and straightforward involving the tandem aldol/cyclization process of steroidal α,α-dicyanoalkene **2** and aldehydes (Scheme 2). The intermediate **2** was prepared in high yield via aldol condensation of 3β-acetyl pregnenolone **1** with malononitrile in ethanol catalyzed by ammonium acetate according to our previously reported method [25].

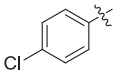
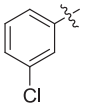
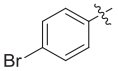
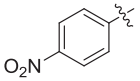
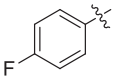
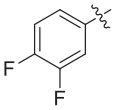
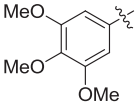
In order to improve the yields of final products, we evaluated the key step of this protocol. Initially, the reaction of compound **2** and 4-chlorobenzaldehyde was selected as a model reaction for catalyst and solvent evaluation (Table 1). While using Et_3N as the organic base, the yield was very low or no reaction was observed regardless of the solvent used (Table 1, entries 1, 4 and 5). When piperidine was used, the yield increased to 24% (Table 1, entry 2). As shown in Table 1, the inorganic bases were beneficial to this reaction. Specifically, one equivalent of NaOAc was used, the yield was about 30% (Table 1, entry 6), when the amount of NaOAc increased, the yield increased correspondingly (Table 1, entries 7 and 8). However, when Na_2CO_3 was used, the yield decreased to 67% probably due to the formation of the competitive dehydration product **4a** (Table 1, entry 3). The present reaction was best performed with 5.0 equivalents of NaOAc in EtOH under reflux for about 12 h.

Having established the optimal condition, the scope and reproducibility of this methodology was explored by applying the same reaction condition to other aldehydes and the representative results were listed in Table 2. Excellent yields were obtained irrespective of the position of the substituents on the phenyl ring (**3a–k**). Besides, when heteroaromatic aldehydes such as furfural and 2-thiophenecarboxaldehyde were used, the corresponding products (**3j** and **3k**) were also obtained in high yields. It should be noted that the electronic nature of the substituents on the phenyl ring had a remarkable effect on the yield. When 4-morpholinyl benzaldehyde, 4-dimethylamino benzaldehyde, pyrrole-2-carboxaldehyde, indole-3-formaldehyde and aliphatic aldehydes were used, the reaction gave a complex reaction mixture. Besides, no reaction was observed with compound **2** recovered quantitatively when ketones such as acetone and acetophenone were used due to their low reactivity.

All the synthesized compounds were fully characterized by ^1H, ^{13}C NMR and high-resolution mass spectra as described for **3a**. In the ^1H NMR spectra of **3a** (Fig. 2), the NH_2 protons resonated at δ 6.03 and 5.67 ppm as two singlets. The two olefinic protons of acyclic dienamide gave two doublets at δ 7.20 (J = 16.3 Hz, 1H) and δ

Table 2
Synthesis of steroidal (*E*, *E*) dienamides **3a–k**[a].



Compounds	Ar	Yield[b] (%)
3a		75
3b		82
3c		90
3d		84
3e		79
3f		86
3g		83
3h		85
3i		80
3j		86
3k		85

[a] Unless otherwise noted, the reaction was carried out with **2** (1.0 mmol), aldehyde (1.2 mmol) and NaOAc (5.0 mmol) under reflux in EtOH for 12 h.
[b] Isolated yields.

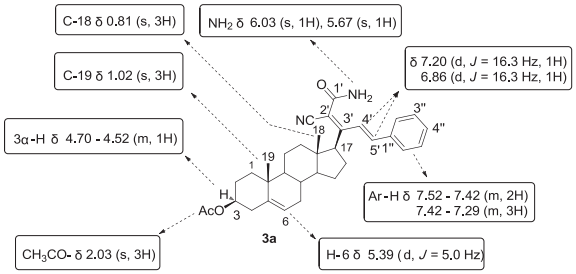


Fig. 2. Selected ^{1}H NMR chemical shifts of compound **3a**.

6.86 (J = 16.3 Hz, 1H), showing that the newly formed double bond had the *E*-configuration. The aromatic protons appeared at δ 7.52-7.42 (m, 2H) and δ 7.42-7.29 (m, 3H), respectively. The proton of 3α-H appeared as a multiplet in the region of δ 4.70-4.52 ppm. The protons attached to C-18, C-19 and acetyl group occurred at δ 0.81 (s, 3H), 1.02 (s, 3H) and 2.03 (s, 3H), respectively. The presence of a molecular ion peak at m/z = 513.3118 ($[M + H]^+$) in the mass spectrum (calcd. 513.3117) further confirmed the structure of **3a**.

Table 3
Preliminary *in vitro* cytotoxic activities of compounds **1**, **2** and **3a–k** against four human cancer cell lines.

Compounds	IC_{50} (μM)[a]			
	SMMC-7721	MCF-7	EC109	MGC-803
1	124.05 ± 1.62	73.48 ± 1.87	32.03 ± 1.51	24.80 ± 1.40
2	>128	15.30 ± 1.19	>128	61.67 ± 1.79
3a	36.90 ± 1.57	91.05 ± 1.96	50.66 ± 1.71	80.04 ± 1.90
3b	>128	9.11 ± 0.96	38.19 ± 1.58	34.76 ± 1.54
3c	11.08 ± 1.05	0.76 ± 0.12	5.57 ± 0.75	20.71 ± 1.32
3d	>128	9.58 ± 0.98	38.54 ± 1.59	25.69 ± 1.41
3e	>128	13.21 ± 1.12	54.94 ± 1.74	68.75 ± 1.84
3f	72.80 ± 1.86	5.23 ± 0.72	49.14 ± 1.69	31.01 ± 1.49
3 g	14.40 ± 1.16	2.82 ± 0.45	9.66 ± 0.99	26.19 ± 1.42
3 h	10.15 ± 1.01	2.59 ± 0.41	9.78 ± 0.99	19.33 ± 1.29
3i	>128	6.05 ± 0.78	63.13 ± 1.8	32.54 ± 1.51
3j	55.80 ± 1.67	4.59 ± 0.66	26.16 ± 1.42	27.64 ± 1.44
3k	21.30 ± 1.33	2.18 ± 0.34	4.78 ± 0.68	24.17 ± 1.38
5-Fu	9.78 ± 0.99	7.54 ± 0.70	10.61 ± 1.08	6.92 ± 0.35

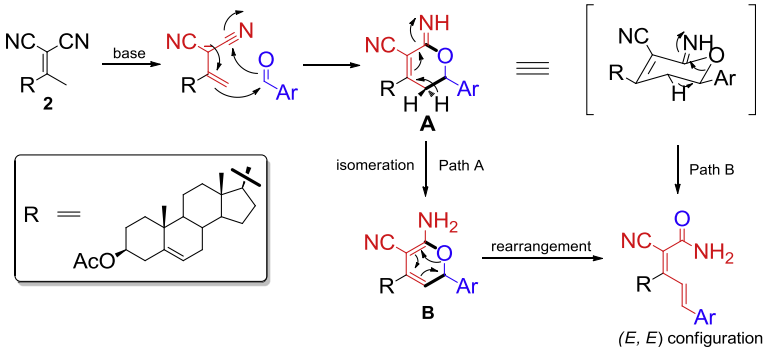
[a] Inhibitory activity was assayed by exposure for 72 h to substances and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are presented as the means ± SDs of three independent experiments.

A possible mechanism for the formation of compounds **3a-k** was proposed in Scheme 3. In the presence of base, the first step involved facile deprotonation of vinyl malononitrile **2** to furnish a nucleophile that attacked the aldehyde via vinylogous aldol reaction and subsequent intramolecular nucleophilic addition, affording the intermediate A. Then, there were two possible pathways to form the final products. (A) Isomerization of intermediate A afforded intermediate B, which underwent electrolytic ring opening to give the final products (path A); (B) A second deprotonation of active hydrogen of the intermediate A catalyzed by NaOAc occurred via a stable chair conformation and generated the products (path B). The process forming the intermediate A involved the tandem construction of the C–C and C–O bonds (highlighted in bold in scheme 3). Interestingly enough, this protocol efficiently achieved the construction of carbon–carbon double bond and selective conversion of cyano group into carboxamide with another cyano group intact in one-pot procedure under mild condition, which demonstrated that the formation of carboxamide did not attribute to the conventional hydrolysis of cyano group. The final products were isolated as single diastereomers with (*E*, *E*) configuration. It should be noted that our final products with a conjugated dienamide group could be allowed to convert into other novel steroidal derivatives [13–15].

3.2. Bioactivity

With these compounds in hand, we next performed tests of their cytotoxic activities against human cancer cell lines and preliminary structure-activity relationship (SAR) study. 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 results were listed in Table 3 and the well-known cytotoxic drug 5-fluorouracil was used as positive control.

As represented in Table 3, compounds **1** and **2** showed weak inhibitory effect against all the tested cancer cell lines with the IC_{50} values ranging from 15 to 128 μM. Besides, all the compounds were less potent than 5-Fu against MGC-803 and SMMC-7721. Among them, compound **3h** was the most potent one with the IC_{50} values of 10.05 and 19.33 μM, respectively. Compared with



Scheme 3. Possible mechanism for the synthesis of steroidal (*E*, *E*)-dienamides.

compounds **3b**–**k**, compound **3a** had the relatively weak inhibitory effect against all the tested cancer cell lines with the IC_{50} values more than 36 μM, indicating that aromatic ring and the substituents on the phenyl ring had the remarkable effect on their cytotoxic activities. Specifically, compounds **3b**–**d** with chlorine atom on the 4-, 2- and 3-position on the phenyl ring had different inhibition against the tested cell lines, compound **3c** was relatively more potent than compounds **3b** and **3d** and showed excellent inhibitory effect against MCF-7 and EC109 cells with the IC_{50} values of 0.76 and 5.57 μM, respectively (about 10- and 2-fold potent than 5-Fu). Compound **3e** had the moderate inhibition against MCF-7 (IC_{50} = 13.21 μM) and showed weak inhibitory effect against the remaining three cancer cell lines. Compounds **3f**–**h** with electron withdrawing groups such as nitro and fluorine atom on the phenyl ring had the moderate inhibitory effect against SMMC-7721 and MGC-803. However, to MCF-7 and EC109 cell lines, these compounds represented excellent inhibition with the IC_{50} values ranging from 2.5 to 10 μM, which were also more potent than 5-Fu. Compound **3i** with electron donating group demonstrated excellent cytotoxic activities against MCF-7 (IC_{50} = 6.05 μM) but weak inhibition against MGC-803, EC109 and SMMC-7721. Compounds **3j** and **3k** with heteroaryl groups (furyl and thienyl) had the similar inhibitory effect with compounds **3f**–**h** against the tested cell lines and were more potent than 5-Fu. This excellent inhibition of these compounds, especially compound **3c**, against MCF-7 promoted us to perform further investigation and the results will be reported in due course.

4. Conclusion

In conclusion, we have reported the stereoselective and metal-free synthesis of steroidal (*E*, *E*) dienamides from steroidal α, α-dicyanoalkene through a cascade aldol/cyclization process. This protocol efficiently achieved the construction of C=C bond and selective conversion of cyano group into carboxamide with another cyano group intact in one-pot procedure under mild condition. Further biological evaluation showed that some of these compounds had moderate to excellent cytotoxic activities against all the tested cancer cell lines and were more potent than well-known drug 5-Fu. Particularly, compound **3c** represented excellent inhibitory effect against MCF-7 (IC_{50} = 0.76 μM), which was about 10-fold more potent than 5-Fu. Preliminary SAR analysis showed that the cytotoxic activities varied greatly depended on the position and electronic nature of substituents on the phenyl ring. Further investigation of mechanism of action is under way and will be reported in due course.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2013.08.001.

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