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Synthesis and structure–activity relationships study of cytotoxic bufalin 3-nitrogen-containing-ester derivatives

Biao Ma, Zhi-Yong Xiao, Yi-Jia Chen, Min Lei, Yu-Hui Meng, De-An Guo, Xuan Liu *, Li-Hong Hu *

Shanghai Research Center for Modernization of Traditional Chinese Medicine, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, PR China

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ABSTRACT

A series of bufalin 3-nitrogen-containing-ester derivatives (**2–6**) were designed, synthesized, and evaluated for their proliferation inhibition activities against human cervical epithelial adenocarcinoma (HeLa) and non-small-cell lung cancer (A549) cell lines. The structure–activity relationships (SARs) of this new series were described in this paper. Cytotoxicity data revealed that C3 moiety had important influence on cytotoxic activity. On two cell lines, the bufalin-3-piperidinyl-4-carboxylate compound **2** (IC_{50} values on HeLa and A549 cell lines were 0.76 nM and 0.34 nM, respectively) displayed a significant cytotoxic potency compared to the parent compound bufalin.

1. Introduction

Bufadienolides are a type of natural cardiac steroids with potent antitumor activities, originally isolated from the Traditional Chinese Medicine Chan'Su. Bufalin (Fig. 1) is one of major components of Chan'Su, and its content in the crude drug can be as high as 1–5% of the dry weight. It also is one of the most potent bufadienolides against cancer cells, such as human prostate carcinoma cells (PC3, DU145) [1], human leukemia cells (U937, HL60) [2,3] and human cervical carcinoma cells (HeLa) [4], with IC_{50} values of 10^{-9} to 10^{-8} mol/L. Furthermore, bufalin can inhibit the growth of human HepG2 cell-transplanted tumor in nude mice and prolong the survival of host significantly [5]. The mechanism of the anti-cancer effects of bufalin is still subject to study. While, similar to other cardiac glycosides, bufalin could also bind to Na^+/K^+-ATPase and inhibit the Na^+/K^+ pump [6,7]. Therefore, present knowledge about the anti-cancer mechanisms of other cardiac glycosides such as ouabain or digitoxin might provide useful information for the understanding of the mechanism of bufalin. By inhibiting the Na^+/K^+ pump, cardiac glycosides could increase the intracellular concentration of Ca^{2+} [(Ca^{2+}) intra], which would lead to cardiotonic effect in cardiomyocytes but cause endoplasmic reticulum stress that eventually become lethal in cancer cells expressing particular Na^+/K^+-ATPase subunits [8]. Furthermore, at lower dose that would not exhibit inhibiting effect on Na^+/K^+ pump, cardiac glycosides could activate Na^+/K^+-ATPase signalosome and thus activate associated downstream signaling pathways such as phospholipase C (PLC) signaling, mitogen-activated protein kinase (MAPK) signaling, phosphatidyl-inositol-3-kinase (PI3 K) signaling, and Src kinase signaling. The activation of Na^+/K^+-ATPase signalosome would influence cellular activities including apoptosis, cell proliferation, cell motility, and tight junctions [9]. Similar results were also found in the study of bufalin. For example, the cytotoxicity and anti-invasion effects of bufalin on cancer cells were also found to be related to its inhibition on MAPK pathway [10,11].

From the 1990s, a lot of bufalin analogues have been prepared by isolation, and chemical or biological transformation, and these compounds are evaluated in vitro and in vivo for their cytotoxicities [12–16]. The essential structural requirements of bufalin for increasing cytotoxicities have been indentified: a steroidal C/D cis ring junctures with a C14β-hydroxyl group and a C17β-2-pyrone ring [17]. Unfortunately, these efforts have provided few new more active bufalin derivatives. Therefore, new ideas and approaches are needed to extend investigations of the use of these bufadienolides as anticancer agents. Recently, O'Doherty et al found that introduction of mono-sugars at C3 site of digitoxigenin (Fig. 1) enhanced its cytotoxicity [18–20]. Henrik group reported that ethylene glycol linked digitoxin mimics were comparable with digitoxigenin for cytotoxicity [21]. However, both synthesis of the sugar and sugar-mimics coupling with bufalin are laborious, which hinder further analogue development.

Acknowledging the limited SARs for bufalin 3-O-glycosides and its cumbersome preparation, it is obvious that structurally simpler polar substituents could be advantageously grafted on C3 of bufalin to increase activity while simultaneously increasing solubility. N-Heterocycles are found in a variety of biologically active

* Corresponding authors. Tel.: +86 21 20231965.
E-mail addresses: xuanliu@mail.shcnc.ac.cn (X. Liu), simmhulh@mail.shcnc.ac.cn (L.-H. Hu).

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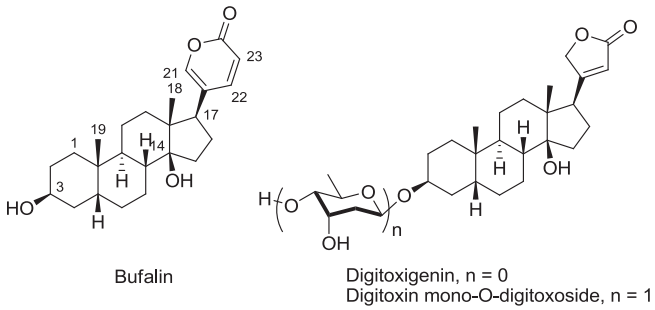
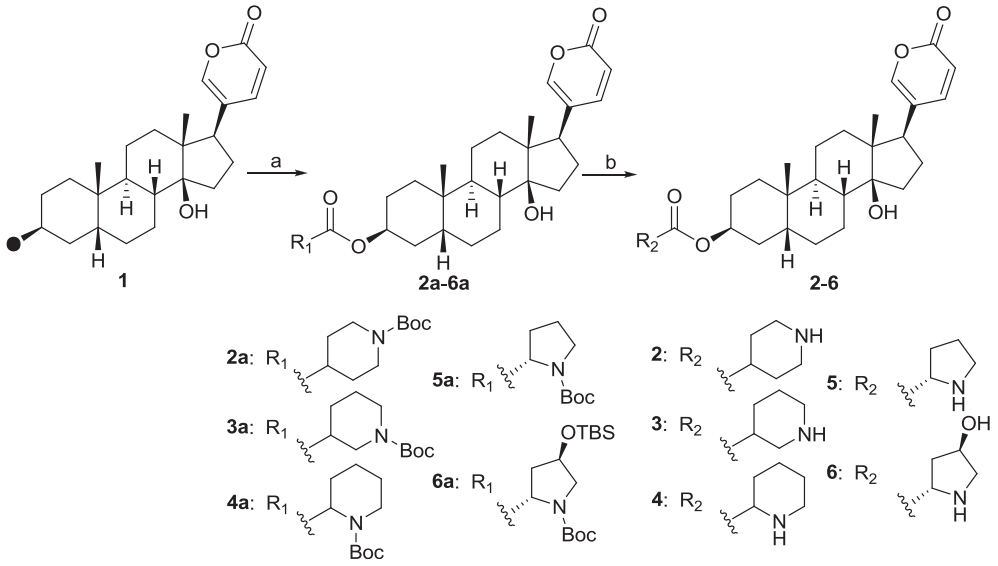


Fig. 1. Structures of bufalin, digitoxigenin and digitoxin mono-O-digitoxoside.

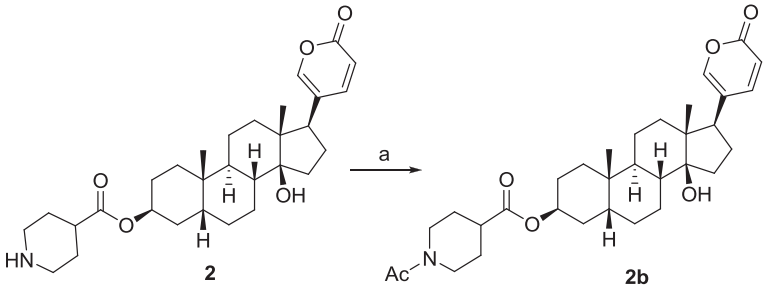
compounds and are easily to form water-soluble salts with organic or inorganic acids [22]. Therefore, we designed and synthesized a series of nitrogen-contained carbocycle esters, and the structures of these compounds were identified using MS, ^{1}H NMR and ^{13}C NMR spectroscopy. Meanwhile, their biological activities (IC_{50} values) were compared using a MTT assay with two human cancer cell lines including human lung cancer cells A549 and human cervical carcinoma cells HeLa. Bufalin was used as reference.

2. Experimental

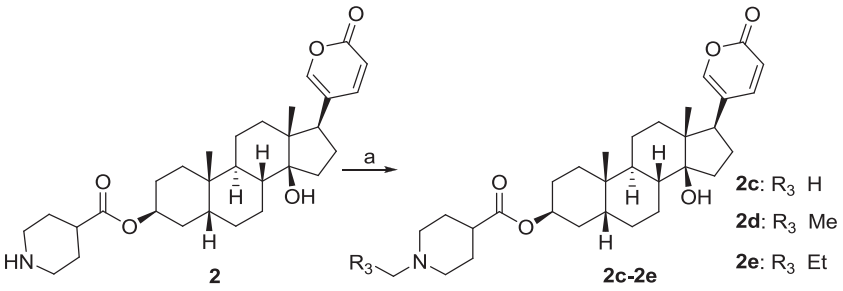
The derivatives **2**–**6** were synthesized from bufalin by the method as shown in Scheme 1. Boc-masked amino acids were reacted with bufalin to give nitrogen-contained bufalin esters, then Boc protecting group was removed with *p*-toluenesulfonic acid in methanol to give desired products **2**–**5**. Of note, TBS protecting group was also hydrolyzed in the same condition to afford compound **6**. For a further insight into the impact of C4′-nitrogen atom in piperidine ring on their cytotoxicities, we synthesized different substituents at C4′-nitrogen atom. The N-acetylated derivative **2b** was synthesized from **2** via acylation reaction with acetic anhydride (Scheme 2). N-alkylated derivatives **2c**–**e** was achieved via reductive amination of **2** and the corresponding aldehyde (Scheme 3). All reactions were performed under a nitrogen atmosphere with dry solvents in oven-baked or flame-dried glassware, unless otherwise noted. All reagents were commercially available, and were used without further purification unless otherwise specified. All solvents were redistilled under argon atmosphere. The progress of reactions were monitored by thin layer chromatography (TLC) plates (silica gel 60GF, Yantai jiangyou company) visualized with 254-nm UV light and/or by staining with Vanillin solution (2.7 g Vanillin + 100 mL H_2O + 35 mL concentrated H_2SO_4 diluted to 300 mL with 95% ethanol). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury-VX300 Fourier transform spectrometer or a Bruker AM-400 spectrometer, and chemical shifts (δ) were reported in ppm; the hydrogenated residues of deuterated solvent were used as internal standard $CDCl_3$: 7.26 ppm for 1H NMR, 77.00 ppm for ^{13}C NMR; CD_3OD: 3.31 ppm for 1H NMR; DMSO-d_6: 2.50 ppm for ^{1}H NMR, 39.52 ppm for ^{13}C NMR. ESIMS was run on a Bruker Esquire 3000 plus spectrometer in MeOH. HRESIMS were determined on a Micromass Q-Tof Global mass spectrometer.



Scheme 1. Reagents and conditions: (a) R_1COOH, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, DCM, 12 h, rt and (b) TsOH, MeOH, 2 d, rt.



Scheme 2. Reagents and conditions: (a) AcCl, 4-Dimethylaminopyridine, DCM, 6 h, rt.



Scheme 3. Reagents and conditions: (a) R_3CHO, $NaBH_3CN$, AcOH, MeOH, 12 h, rt.

2.1. General synthesis of bufalin 3-nitrogen-containing-ester derivatives

Method A: To a stirred solution of bufalin (200 mg, 0.52 mmol), 4-dimethylaminopyridine (63 mg, 0.52 mmol, 1 eq), and N-protected acid (1.56 mmol, 3 eq) in anhydrous DCM (2 mL) at room temperature under nitrogen gas was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (300 mg, 1.56 mmol, 3 eq), and stirring continued for 12 h. After completion (by TLC), DCM (10 mL), and H_2O (10 mL) were added. The organic phase was separated, washed with H_2O and brine, dried over Na_2SO_4, and concentrated in vacuo to afford yellow residue. To a solution of the residue in MeOH (2.5 mL) was added a solution of TsOH in MeOH (2.5 mL, 4%), and stirring continued at room temperature for 2d. After completion (by TLC), DCM (15 mL) and H_2O (15 mL) were added. The organic phase was separated, washed with aq. Na_2CO_3 and brine, dried over Na_2SO_4, and concentrated in vacuo to give yellow residue. Dissolved in minimal DCM, the crude product was subjected to purify on silica gel.

Method B: To a stirred solution of bufalin-3-piperidinyl-4-carboxylate compound **2** (200 mg, 0.52 mmol) and 4-dimethylaminopyridine (32 mg, 0.26 mmol, 0.5 eq) in anhydrous DCM (2 mL) at room temperature under nitrogen gas was added acyl chloride (0.78 mmol, 1.5 eq) or anhydride (0.78 mmol, 1.5 eq) dropwise, and stirring continued for 6 h. After completion (by TLC), the mixture solution was extracted with DCM (twice). The organic phase was combined, washed with aq. Na_2CO_3 and brine, dried over Na_2SO_4, and concentrated in vacuo to give yellow residue. Dissolved in minimal DCM, the crude product was subjected to purify on silica gel.

Method C: To a solution of compound bufalin-3-piperidinyl-4-carboxylate compound **2** (200 mg, 0.40 mmol) in anhydrous MeOH (2 mL) was added with aldehyde (0.60 mmol, 1.5 eq), AcOH (0.24 mmol, 0.6 eq) and $NaBH_3CN$ (1.20 mmol, 3 eq), then the resulting solution was stirred at room temperature overnight. After completion (by TLC), DCM (15 mL) and H_2O (15 mL) were added. The organic phase was separated, washed with H_2O and brine, dried over Na_2SO_4, and concentrated in vacuo to give yellow residue. Dissolved in minimal DCM, the crude product was subjected to purify on silica gel.

2.1.1. Compound **2**

Synthesis of compound **2** with method A to yield a white, amorphous powder. Yield: 57%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (dd, J = 9.7, 2.5 Hz, 1H, H22), 7.23 (d, J = 2.5 Hz, 1H, H21), 6.26 (d, J = 9.7 Hz, 1H, H23), 5.13 (s, 1H, H3), 3.40–3.38 (m, 2H, H3′a, H5′a), 3.03 (t, J = 9.8 Hz, 2H, H3′b, 5′b), 2.60–2.58 (m, 1H, H1′), 2.48–2.44 (m, 1H), 2.13–1.21 (m, 24H), 0.95 (s, 3H, H19), 0.69 (s, 3H, H18); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.8, 162.4, 148.5, 146.7, 122.6, 115.3, 85.3, 71.3, 51.2, 48.3, 42.8, 42.3, 40.8, 38.4, 37.1, 35.8, 35.2, 32.7, 30.6, 30.4, 28.7, 26.4, 25.0, 24.8, 24.7, 23.8, 21.4, 21.3, 16.5; MS (ESI) m/z: 498 $[M+H]^+$; HRMS (ESI): calcd for $C_{29}H_{42}NO_6$ $[M+H]^+$ 498.3214, found 498.3228.

2.1.2. Compound **3**

Synthesis of compound **3** with method A to yield a white, amorphous powder. Yield: 53%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (d, J = 9.0 Hz, 1H, H22), 7.22 (s, 1H, H21), 6.24 (d, J = 9.0 Hz, 1H, H23), 5.08 (s, 1H, H3), 3.66 (s, 1H, NH), 3.19–3.15 (m, 1H, H2′a), 2.93–2.91 (m, 1H, H2′b), 2.79–2.75 (m, 1H, H4′a), 2.63–2.61 (m, 1H, H4′b), 2.47–2.42 (m, 2H, H1′), 2.23–1.16 (m, 26H), 0.95 (s, 3H, H19), 0.70 (s, 3H, H18); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.6, 162.4, 148.5, 146.8, 122.6, 115.3, 85.3, 70.2, 51.2, 48.4, 46.2, 42.6, 42.3, 40.8, 36.9, 35.8, 35.1, 32.7, 30.5, 30.4, 28.7, 27.4, 26.4, 25.4, 25.0, 23.8, 23.7, 21.4, 21.3, 16.5; MS (ESI) m/z: 498 $[M+H]^+$; HRMS (ESI): calcd for $C_{30}H_{44}NO_5$ $[M+H]^+$ 498.3214, found 498.3198.

2.1.3. Compound **4**

Synthesis of compound **4** with method A to yield a white, amorphous powder. Yield: 55%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (d, J = 9.0 Hz, 1H, H22), 7.22 (s, 1H, H21), 6.25 (d, J = 9.0 Hz, 1H, H23), 5.12 (s, 1H, H3), 3.34–3.31 (m, 1H, H1′), 3.10–3.07 (m, 1H, H3′a), 2.68–2.64 (m, 1H, H3′b), 2.48–2.43 (m, 1H), 2.24–1.15 (m, 28H), 0.97 (s, 3H, H19), 0.70 (s, 3H, H18); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.8, 162.4, 148.4, 146.9, 122.7, 115.0, 85.0, 70.7, 58.5, 51.0, 48.2, 45.6, 42.1, 40.7, 36.8, 35.7, 35.0, 32.6, 30.4, 30.2, 29.2, 28.6, 26.3, 25.7, 24.9, 24.0, 23.7, 21.3, 21.1, 16.4; MS (ESI) m/z: 498 $[M+H]^+$; HRMS (ESI): calcd for $C_{30}H_{44}NO_5$ $[M+H]^+$ 498.3214, found 498.3223.

2.1.4. Compound **5**

Synthesis of compound **5** with method A to yield a white, amorphous powder. Yield: 61%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (d, J = 9.0 Hz, 1H, H22), 7.23 (s, 1H, H21), 6.26 (d, J = 9.0 Hz, 1H, H23), 5.13 (s, 1H, H3), 3.76 (dd, J = 9.0, 6.0 Hz, 1H, H1′), 3.12–3.08 (m, 1H, H3′a), 2.93–2.90 (m, 1H, H3′b), 2.49–2.44 (m, 1H), 2.25–1.18 (m, 26H), 0.96 (s, 3H, H19), 0.70 (s, 3H, H18); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.8, 162.4, 148.5, 146.8, 122.6, 115.3, 85.2, 77.2, 71.1, 59.9, 51.2, 48.3, 76.0, 42.3, 40.8, 36.8, 35.8, 35.1, 32.7, 30.5, 30.3, 28.7, 26.3, 25.5, 25.0, 23.8, 21.4, 21.3, 16.5; MS (ESI) m/z: 484 $[M+H]^+$; HRMS (ESI): calcd for $C_{29}H_{42}NO_5$ $[M+H]^+$ 484.3057, found 484.3042.

2.1.5. Compound **6**

Synthesis of compound **6** with method A to yield a white, amorphous powder. Yield: 62%; ^{1}H NMR (300 MHz, CD_3OD): δ 7.96 (dd, J = 9.7, 2.5 Hz, 1H, H22), 7.47 (s, 1H), 7.31 (dd, J = 2.5, 1.0 Hz, 1H, H21), 6.27 (dd, J = 9.7, 1.0 Hz, 1H, H23), 5.12 (s, 1H, H3), 4.40–4.36 (m, 1H, H4'), 3.96 (t, J = 8.0 Hz, 1H, H1'), 3.16 (dd, J = 11.8, 4.6 Hz, 1H, H3′a), 2.86 (d, J = 11.8 Hz, 1H, H3'b), 2.52–2.47 (m, 1H), 2.24–1.17 (m, 24H), 0.96 (s, 3H, H19), 0.70 (s, 3H, H18); 13C NMR (75 MHz, DMSO-d_6): δ 174.0, 161.4, 149.2, 147.4, 122.8, 114.2, 83.4, 70.9, 70.2, 58.7, 55.1, 50.1, 48.1, 41.2, 39.9, 39.6, 36.7, 34.9, 34.8, 32.0, 30.3, 29.9, 28.5, 26.3, 24.4, 23.7, 21.1, 21.0, 16.7; MS (ESI) m/z: 500 $[M+H]^+$; HRMS (ESI): calcd for $C_{29}H_{42}NO_6$ $[M+H]^+$ 500.3007, found 500.3001.

*2.1.6. Compound **2a***

To a stirred solution of bufalin (200 mg, 0.52 mmol), 4-dimethylaminopyridine (63 mg, 0.52 mmol, 1 eq) and 1-Boc-4-piperidinecarboxylic acid (357 mg, 1.56 mmol, 3 eq) in anhydrous DCM (2 mL) at room temperature under nitrogen gas was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (300 mg, 1.56 mmol, 3 eq), and stirring continued for 12 h. After completion (by TLC), DCM (10 mL), and H_2O (10 mL) were added. The organic phase was separated, washed with H_2O and brine, dried over Na_2SO_4, and concentrated in vacuo to afford yellow residue (250 mg). Dissolved in minimal DCM, the crude product was subjected to purify on silica gel (4:1, petroleum ether–acetone) to yield a white, amorphous powder (215 mg, 69%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (dd, J = 9.7, 2.6 Hz, 1H, H22), 7.23 (d, J = 2.6 Hz, 1H, H21), 6.26 (d, J = 9.7 Hz, 1H, H23), 5.10 (s, 1H, H3), 4.03 (m, 2H, H3′a, 5′a), 2.84 (t, J = 9.0 Hz, 2H, H3′b, 5′b), 2.46–2.42 (m, 2H), 2.17–1.21 (m, 26H), 1.45 (s, 9H, $C(CH_3)_3$), 0.96 (s, 3H, H19), 0.70 (s, 3H, H18); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.9, 162.4, 154.7, 148.5, 146.8, 122.7, 115.2, 85.2, 79.6, 70.4, 51.2, 48.3, 42.3, 41.4, 40.8, 37.0, 35.8, 35.1, 32.7, 30.5, 30.4, 28.7, 28.4, 28.1, 28.0, 26.4, 25.0, 23.8, 21.4, 21.3, 16.5; MS (ESI) m/z: 598 $[M + H]^+$; HRMS (ESI): calcd for $C_{35}H_{52}NO_7$ $[M + H]^+$ 598.3738, found 598.3733.

*2.1.7. Compound **2b***

Synthesis of compound **2b** with method B to yield a white, amorphous powder. Yield: 91%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (d, J = 9.0 Hz, 1H, H22), 7.23 (s, 1H, H21), 6.26 (d, J = 9.0 Hz, 1H, H23), 5.11 (s, 1H, H3), 4.42 (d, J = 12.0 Hz, 1H, H3′a), 3.78 (d, J = 12.0 Hz, 1H, H5′a), 3.14 (t, J = 9.0 Hz, 1H, H3′b), 2.80 (t, J = 9.0 Hz, 1H, H5′b), 2.54–2.43 (m, 2H), 2.09 (s, 3H, CH_3COO), 2.24–1.21 (m, 26H), 0.96 (s, 3H, H19), 0.70 (s, 3H, H18); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.5, 168.9, 162.4, 148.5, 146.7, 122.6, 115.3, 85.3, 70.6, 51.2, 48.3, 45.7, 42.3, 41.3, 40.9, 40.8, 37.0, 35.8, 35.2, 32.7, 30.5, 30.4, 28.7, 28.5, 27.9, 26.4, 25.0, 23.8, 21.5, 21.4, 21.3, 16.5; MS (ESI) m/z: 540 $[M + H]^+$; HRMS (ESI): calcd for $C_{32}H_{46}NO_6$ $[M + H]^+$ 540.3320, found 540.3314.

*2.1.8. Compound **2c***

Synthesis of compound **2c** with method C to yield a white, amorphous powder. Yield: 77%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (d, J = 9.0 Hz, 1H, H22), 7.22 (s, 1H,H21), 6.25 (d, J = 9.0 Hz, 1H, H23), 5.08 (s, 1H, H3), 2.83–2.79 (m, 2H, H3′a, 5′a), 2.46–2.44 (m, 1H), 2.26 (s, 3H, NCH_3), 2.20–1.15 (m, 29H), 0.94 (s, 3H, H19), 0.69 (s, 3H, H18); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.4, 162.4, 148.5, 146.8, 122.7, 115.2, 85.2, 70.1, 54.9, 51.1, 48.4, 48.3, 46.3, 42.2, 41.0, 40.8, 36.9, 35.7, 35.1, 32.7, 30.5, 30.4, 28.7, 28.3, 28.2, 26.4, 25.0, 23.7, 21.4, 21.2, 16.5; MS (ESI) m/z: 512 $[M + H]^+$; HRMS (ESI): calcd for $C_{31}H_{46}NO_5$ $[M + H]^+$ 512.3371, found 512.3357.

*2.1.9. Compound **2d***

Synthesis of compound **2d** with method C to yield a white, amorphous powder. Yield: 71%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (d, J = 9.0 Hz, 1H, H22), 7.22 (s, 1H, H21), 6.25 (d, J = 9.0 Hz, 1H, H23), 5.08 (s, 1H, H3), 2.92–2.87 (m, 2H, H3′a, 5′a), 2.46–2.44 (m, 1H), 2.38 (q, J = 6.0 Hz, 2H, NCH_2CH_3), 2.24–1.12 (m, 29H), 1.07 (t, J = 6.0 Hz, 3H, NCH_2CH_3), 0.94 (s, 3H, H19), 0.69 (s, 3H, H18); 13C NMR (75 MHz, $CDCl_3$): δ 174.4, 162.4, 148.5, 146.8, 122.6, 115.3, 85.3, 70.1, 52.6, 52.5, 51.2, 48.3, 42.3, 41.7, 40.8, 36.9, 35.8, 35.1, 32.7, 30.5, 30.4, 29.7, 28.7, 28.3, 28.2, 26.4, 25.0, 23.8, 21.4, 21.3, 16.5, 12.0; MS (ESI) m/z: 526 $[M + H]^+$; HRMS (ESI): calcd for $C_{32}H_{48}NO_5$ $[M + H]^+$ 526.3527, found 526.3534.

*2.1.10. Compound **2e***

Synthesis of compound **2e** with method C to yield a white, amorphous powder. Yield: 73%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (dd, J = 9.0, 3.0 Hz, 1H, H22), 7.22 (s, 1H, H21), 6.25 (d, J = 9.0 Hz, 1H, H23), 5.08 (s, 1H, H3), 2.90–2.86 (m, 2H, H3′a, 5′a), 2.46–2.44 (m, 1H), 2.28–2.24 (q, J = 6.0 Hz, 2H, CH_2CH_3), 2.02–1.20 (m, 31H), 0.93 (s, 3H, H19), 0.88 (t, J = 6.0 Hz, 3H,CH_2CH_3), 0.69 (s, 3H, H18); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.5, 162.4, 148.5, 146.8, 122.7, 115.3, 85.3, 70.1, 61.0, 53.0, 51.2, 48.3, 42.3, 41.7, 40.8, 36.9, 35.8, 35.2, 32.8, 30.6, 30.5, 28.7, 28.4, 28.3, 26.4, 25.1, 23.8, 21.4, 21.3, 20.1, 16.5, 12.0; MS (ESI) m/z: 540 $[M + H]^+$; HRMS (ESI): calcd for $C_{33}H_{50}NO_5$ $[M + H]^+$ 540.3684, found 540.3669.

2.2. In vitro cytotoxic assay

Human cervix epithelial adenocarcinoma cell line (HeLa) and non-small-cell lung cancer (A549) cell lines, obtained from American Type Culture Collection (Rockville, MD), were used for the cytotoxicity assay in vitro by MTT assay as reported before [23]. Briefly, cells were plated in 96-well flat-bottomed plates at density of 1×103 cells/well in complete medium and incubated overnight. Then, the media were changed into fresh media containing various amounts of compounds for 72 h. At the end of the incubation, 20 μL of the dye (3,[4,5-dimethylthiazol-2-yl-] diphenyltetrazolium bromide, 5 mg/mL), MTT, was added to each well and the plates were incubated for 3 h at 37 °C. Then, 100 μL of lysis buffer (20% sodium dodecyl sulfate [SDS] in 50% N,N-dimethylformamide, containing 0.5% [v:v] 80% acetic acid and 0.4% [v:v] 1 N HCl) was added to each well and incubated overnight (16 h). Cell viability was evaluated by measuring the mitochondrial-dependent conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The optical density (proportional to the number of live cells) was assessed with a Microplate Reader Bio-Rad 550 at 570 nm. Each experiment was performed in triplicate. Results of three independent experiments were used for statistical analysis. IC_{50} value (half-maximal inhibitory concentration) was calculated by the Logit method.

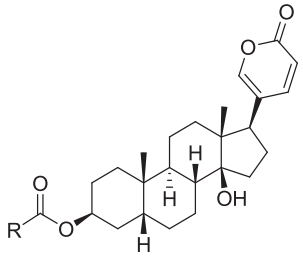
3. Results and discussion

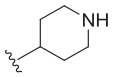
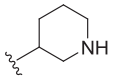
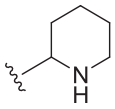
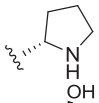
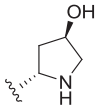
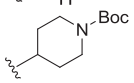
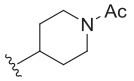
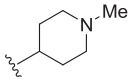
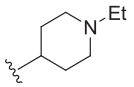
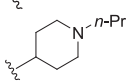
All the bufalin 3-nitrogen-containing-ester derivatives (**2**–**6**) were evaluated in vitro for their cytotoxicities against human cervical epithelial adenocarcinoma (HeLa) and non-small-cell lung cancer (A549) cell lines, and the results are presented in Table 1.

Among three bufalin-3-piperidinyl-ester derivatives (**2**–**4**), the piperidinyl-4-carboxylate (**2**) exhibited greater potency (IC_{50} values on HeLa and A549 cell lines are 0.76 and 0.34 nM, respectively) than 3-carboxylate (**3**) or 2-carboxylate (**4**) against both cell lines. For example, against the HeLa cell line, compound **2** was 32-time more active than **3** (IC_{50} = 24.8 nM), and 101-time more active than compound **4** (IC_{50} = 77.1 nM), respectively. These results indicated that the location of the nitrogen was important for cytotoxicity. In the five-membered ester series (**5** and **6**), L-prolinyl ester **5** showed less activity (IC_{50} = 42.7 nM) than bufalin against HeLa cell line, whereas L-hydroxyprolinyl ester **6** with one additional C4′-hydroxyl group demonstrated higher activity (IC_{50} = 9.9 nM) than bufalin (IC_{50} = 26.3 nM). Similar SARs results of these N-heterocycles analogues (**2**–**6**) also could be found against the A549 cell line, and in general, all these compounds were more sensitive against A549 than HeLa cell line.

We further investigated the impact of different substituents at C4′-nitrogen atom in piperidine ring on cytotoxicities. In Table 1, we observed that incorporation of the Boc (**2a**) and Ac (**2b**) groups resulted in a significant loss of potency against two cell lines,

Table 1
Cytotoxicity of compounds **2**–**6**.



No.	R	IC_{50} (nM)[a]	
		HeLa	A549
1		26.3 ± 3.7	6.2 ± 0.4
2		0.76 ± 0.06	0.34 ± 0.02
3		24.8 ± 3.0	2.2 ± 0.3
4		77.1 ± 6.4	7.9 ± 0.6
5		42.7 ± 5.1	21.3 ± 3.8
6		9.9 ± 1.0	7.7 ± 0.8
2a		>100	>100
2b		>100	39.5 ± 5.9
2c		28.0 ± 3.1	4.4 ± 0.3
2d		>100	7.7 ± 0.6
2e		>100	81.0 ± 7.9

[a] IC_{50}: 50% inhibitory concentration. Values are an average of three separate experiments.

whereas introduction of the methyl (**2c**) group displayed moderate potency (IC_{50} values on HeLa and A549 cell lines are 28.0 and 4.4 nM, respectively) compared to bufalin. Among three alkyl piperidinyl-4-carboxylates (**2c**–**e**), we observed a significant loss of cytotoxicity against HeLa and A549 cell lines with increasing the size alkyl groups compared to **2**. These results clearly indicated that C4′-NH was favorable to its cytotoxic activity. In conclusion, a series of bufalin derivatives were investigated in their cytotoxicities against human lung tumor cells A549 and human cervical carcinoma cells HeLa in the current study. Some derivatives demonstrated good cytotoxic activities, in some cases are better than bufalin. The most promising compounds in this series of synthetic compounds were **2**, **3**, **6** and **2c**, which showed strong cytotoxic activities.

In summary, we synthesize a new series of bufalin 3-nitrogen-containing-ester derivatives via a two-step simple and highly efficient method, and find that C3 moiety has important influence on cytotoxic activity. The bufalin-3-piperidinyl-4-carboxylate compound **2** (IC_{50} values on HeLa and A549 cell lines are 0.76 and 0.34 nM, respectively) displays a significant cytotoxic potency compared to the parent bufalin. Moreover, these bufalin 3-nitrogen-containing-ester derivatives will provide better insight into the effect of the C3 site on cytotoxic activity for designing potential cardiac glycoside antitumor agents. For the compounds found in the present study to possess stronger cytotoxicity than bufalin, further study checking their effects on Na^+/K^+-ATPase signalosome and downstream signaling pathways would be conducted.

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.2013.02.007.

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