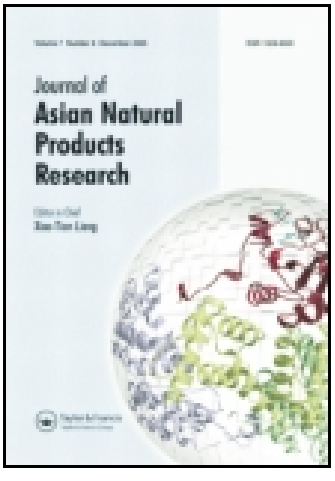

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Design, synthesis, and anti-tumor activity of novel betulinic acid derivatives

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Design, synthesis, and anti-tumor activity of novel betulinic acid derivatives

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Seventeen new derivatives of betulinic acid (BA) with potential anti-tumor activity have been synthesized. In order to improve the bioactivity of BA, we connected BA and nitric oxide donors together via different linkers. The results of the biological activity of these derivatives showed that four compounds exhibited obvious cytotoxicity against human hepatocellular carcinoma cells *in vitro*.

Keywords: betulinic acid; anti-cancer; NO donors; structural modification; NO-releasing

1. Introduction

Betulinic acid (BA) is a pentacyclic lupine triterpene with multiple biological activities, such as anti-bacterial [1,2], anti-inflammatory [3], anti-plasmodium [4,5], anti-cancer [6], and anti-HIV [7,8] activities. Particularly, BA was proved to possess selective cytotoxicity to melanoma cells without affecting the normal cells [9]. Then, the research aiming to improve the anti-cancer activity of BA surged for a long time. By analyzing the scaffold of BA, the OH group at C-3 position, the COOH group at C-28 position, the isopropenyl group at C-19 position, and A-ring can be modified and may afford many compounds with high activity (Figure 1).

Currently, nitric oxide (NO) is considered to promote tumor angiogenesis at low concentrations and inhibit tumor cells via different methods at high concentrations [10]. NO donors refer to compounds that can release a certain amount of NO by the action of enzymes and non-enzymes, including organic nitrates, furoxans, metal–NO complexes, nitrosothiols, NONOates, etc. With the development of NO donors, NO-donating drugs gradually emerged and developed rapidly. Among these, the most successful drug is NO-nonsteroidal anti-inflammatory drugs (NO-NSAIDs), which reduces the side effect of NSAIDs on the one hand and increases anti-cancer effect on the other hand [11].

Considering the selective inhibition of BA on tumor cells and the synergy on anti-tumor effect of NO donors, we connected NO donors to BA at the C-3 and C-28 positions to improve the anti-tumor activity of BA. Seventeen NO-BA hybrids have been designed and synthesized with the determination of these derivatives by IR, ^{1}H NMR and MS. *In vitro* cytotoxic activity of these compounds was evaluated against human hepatocellular carcinoma (HepG2) cells by MTT assay, and several of these derivatives exhibited considerable activity compared with sorafenib (positive control). We will also discuss the structure and activity relationships of these derivatives.

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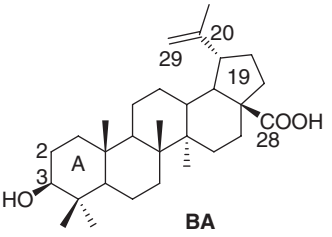


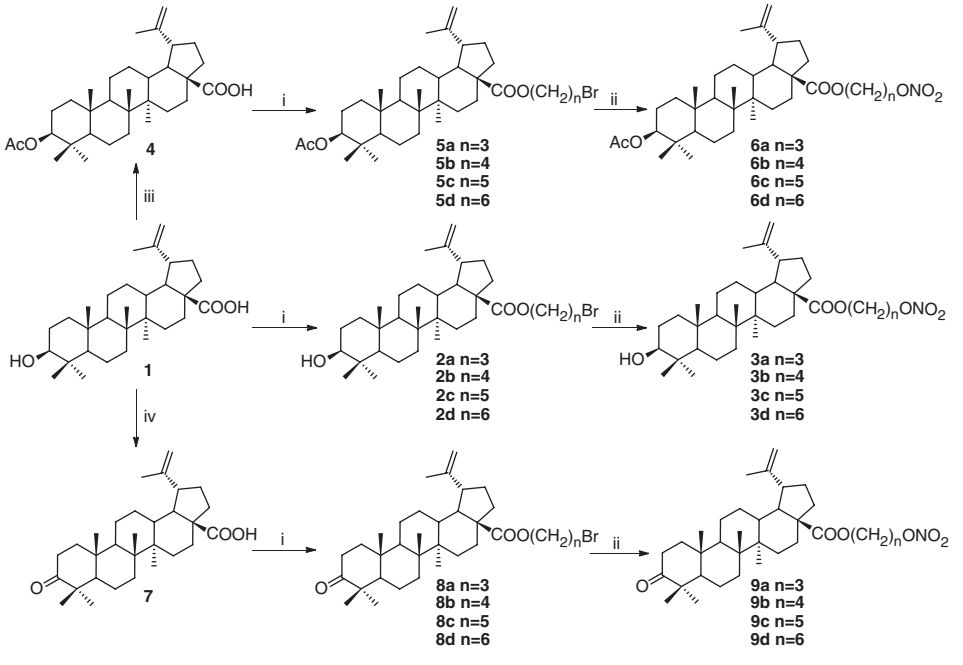
Figure 1. Structure of BA.

2. Results and discussion

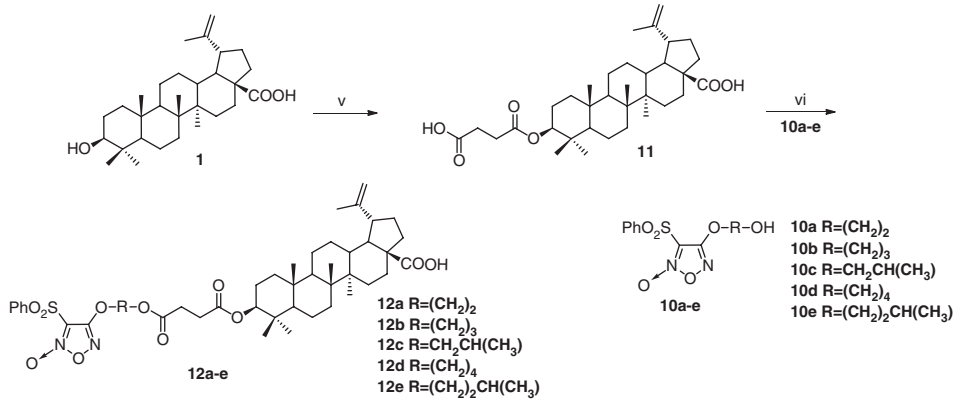
2.1 Chemistry

This paper describes two groups of BA derivatives coupled with nitrates and furoxans. Compounds **3a–d**, **6a–d**, and **9a–d** belong to the first group coupled with nitrates. The general routes for these derivatives are outlined in Scheme 1. BA was first modified with bromoalkane in the presence of K_2CO_3 in DMF to give the corresponding C-28 esters (**2a–d**) in 63%–70% yields. Subsequently, **2a–d** reacted with $AgNO_3$ in the wholly dark condition to afford compounds **3a–d**. 3-*O*-acyl betulinic acid (**4**) and betulonic acid (**7**) were prepared using BA with acetic anhydride and Jones' reagent, respectively. According to the same method above, compounds **6a–d** and **9a–d** were obtained from **4** and **7**. Thus, 12 target compounds were generated by coupling nitrates to the C-28 position of BA.

Compounds **12a–e** belong to the second group of BA derivatives coupled with furoxans, and the general route is outlined in Scheme 2. BA was treated with succinic anhydride to prepare compound **11**, then reacted with compounds **10a–e** in the presence of 4-dimethylamino pyridine (DMAP) and 1-ethyl-(3-dimethylamino-propyl)-carbodiimide hydrochloride (EDCI) to generate target compounds **12a–e**. In this route, important intermediates **10a–e** were prepared as described previously by Fang *et al.* [12].



Scheme 1. Reagents and conditions: (i) $Br(CH_2)_nBr$, K_2CO_3, DMF, r.t., 6 h; (ii) $AgNO_3$, THF/CH_3CN, reflux, 6 h; (iii) Ac_2O, DMAP, pyridine, r.t., 2 h; (iv) Jones' reagent, 0–5°C, 30 min.



Scheme 2. Reagents and conditions: (v) succinic anhydride, pyridine, reflux, 10 h; (vi) **10a–e**, DMAP, EDCI, dry CH_2Cl_2, reflux, 24 h.

2.2 *Cytotoxicity on HepG2*

All the derivatives of BA synthesized in this study were screened for cytotoxicity against HepG2 cells *in vitro* by MTT assay. Sorafenib was selected to be the positive control drug as it has been approved in the EU for the treatment of hepatocellular carcinoma.

As shown in Table 1, all the derivatives coupled with nitrates at the C-28 position of BA resulted in poor cytotoxicity against HepG2 cells. While the derivatives coupled with furoxans showed higher cytotoxicity, among them, compounds **12a–c** and **12e** showed obvious cytotoxicity against HepG2 cells with the IC_{50} values in the same order of magnitude as sorafenib. All of the furoxan derivatives except compound **12d** showed higher cytotoxicity than the lead compound BA. Compound **12e** ($IC_{50} = 9.24\,\mu M$), with iso-butanediol as the linker (group R in Scheme 2), exhibited the highest cytotoxicity, while compound **12d**, with butanediol as the linker, displayed lower cytotoxicity. Meanwhile, the inhibition rate of compound **12e** (69.76% at the concentration of 1×10^{-5} mol/l) was almost threefold higher than compound **12d** (24.09% at the concentration of 1×10^{-5} mol/l), which indicates that branched methyl in the linker with four carbon atoms can slightly improve the potency against HepG2 cells. By analyz-

Table 1. Inhibitory effects of the target compounds against HepG2 cell lines.

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
3a	NA[a]	**9c**	NA
3b	NA	**9d**	NA
3c	NA	**12a**	9.91
3d	NA	**12b**	9.81
6a	NA	**12c**	9.95
6b	NA	**12d**	NA
6c	NA	**12e**	9.24
6d	NA	BA	NA
9a	NA	Sorafenib[b]	4.29
9b	NA		

[a] NA: not active. Inhibition rate of the tested cells is less than 50% at the highest concentration of 1×10^{-5} mol/l.
[b] Positive control.

ing compounds **12a–e**, we speculate that lengthening the linker may not improve the activity of these derivatives.

The fact that nitrate derivatives displayed lower activity than furoxan derivatives can be explained by the theory that nitrates can only release equimolar NO, which may not be high enough to inhibit the tumor growth, while furoxans can release more NO under physiological conditions, which can synergistically increase antitumor activity [13]. Further work is required to summarize more definite structure and activity relationships.

3. Experimental

3.1 General experimental procedures

Melting points were determined using a capillary apparatus (RDCSY-I, Drug Research Institute of Chinese Academy of Science, Shanghai, China) without correction. The IR spectra were measured on a fourier transform infrared spectroscopy Nicolet Impact 410 (Nicolet Instrument Company, Madison, WI, USA). ^{1}H NMR spectra were recorded on a Bruker AVANCE 300-MHz instrument in $CDCl_3$ (Bruker Corporation, Zurich, Switzerland). The molecular weights were detected on HP 1100LC/MSD spectrometer (Agilent Technologies Corporation, Santa Clara, CA, USA). All the materials and solvents were used directly unless otherwise stated. CH_2Cl_2 was refluxed over P_2O_5 for an hour and distilled. All the compounds synthesized were purified by recrystallization or column chromatography on silica gel (200–300 mesh, Qingdao Ocean Chemical Company, Qingdao, China).

3.2 Synthesis of specific compounds by Scheme 1

3.2.1 Betulinic acid-3-bromo-propyl ester (2a)

To a solution of BA (500 mg, 1.09 mmol) in DMF (20 ml), 1,3-dibromo-propane (0.5 ml, 4.3 mmol) and K_2CO_3 (593 mg, 4.3 mmol) were added. The reaction mixture was stirred for 6 h at room temperature, then poured into water (100 ml) and extracted with EtOAc (3 × 40 ml). The combined organic layer was washed with water and saturated NaCl solution sequentially. The dried (Na_2SO_4) organic layer was evaporated *in vacuo*. The product was purified by column chromatography (petroleum ether (PE)–EtOAc; 15:1) to give compound **2a** (440 mg, 70%).

Compounds **2b–d** were prepared as the same method of **2a** in 72%, 63%, and 65% yields, respectively.

3.2.2 Betulinic acid-3-nitrooxy-propyl ester (3a)

A solution of **2a** (300 mg, 0.52 mmol) and $AgNO_3$ (128 mg, 0.75 mmol) in THF (4 ml) and CH_3CN (4 ml) was covered by black shielding layer to avoid illumination and was heated under reflux for 6 h. After cooling to room temperature and filtration, the filtrate was evaporated *in vacuo*. The product was purified by column chromatography (PE–EtOAc; 6:1) to give compound **3a** (218 mg, 75%). m.p. 76–78°C. IR ν_{max} (KBr, cm^{-1}): 3420, 3085, 1727, 1705, 1636; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.76 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.97 (s, 6H, 2 × CH_3), 1.68 (s, 3H, 30-CH_3), 2.98 (m, 1H, 19-H), 3.18 (dd, 1H, J = 10.8, 5.1 Hz, 3α-H), 4.18 (m, 2H, $COOCH_2$), 4.55 (t, 2H, J = 6.3 Hz, OCH_2), 4.61 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 577 $[M + NH_4]^+$.

3.2.3 Betulinic acid-4-nitrooxy-butyl ester (3b)

Compound **3b** was prepared from **2b** and $AgNO_3$ as described for **3a** to afford a white solid in 80% yield. m.p. 73–75°C. IR ν_{max} (KBr, cm^{-1}): 3454, 2941, 1723, 1701, 1632; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.76 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.97 (s, 6H, 2 × CH_3),

1.68 (s, 3H, 30-CH_3), 3.00 (m, 1H, 19-H), 3.18 (dd, 1H, $J = 11.1$, 5.1 Hz, 3α-H), 4.11 (m, 2H, $COOCH_2$), 4.50 (t, 2H, $J = 6.3$ Hz, OCH_2), 4.60 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 591 $[M + NH_4]^+$.

3.2.4 *Betulinic acid-5-nitrooxy-pentyl ester (**3c**)*

Compound **3c** was prepared from **2c** and $AgNO_3$ as described for **3a** to afford a white solid in 70% yield. m.p. 84–86°C. IR ν_{max} (KBr, cm^{-1}): 3409, 2943, 1722, 1699, 1632; ^1H NMR (300 MHz, $CDCl_3$): δ 0.76 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.97 (s, 6H, 2 × CH_3), 1.68 (s, 3H, 30-CH_3), 3.00 (m, 1H, 19-H), 3.18 (dd, 1H, $J = 10.8$, 5.1 Hz, 3α-H), 4.09 (m, 2H, $COOCH_2$), 4.46 (t, 2H, $J = 6.6$ Hz, OCH_2), 4.60 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 588 $[M + H]^+$.

3.2.5 *Betulinic acid-6-nitrooxy-hexyl ester (**3d**)*

Compound **3d** was prepared from **2d** and $AgNO_3$ as described for **3a** to afford a white solid in 72% yield. m.p. 72–74°C. IR ν_{max} (KBr, cm^{-1}): 3414, 2946, 1719, 1631; ^1H NMR (300 MHz, $CDCl_3$): δ 0.76 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.96 (s, 6H, 2 × CH_3), 1.69 (s, 3H, 30-CH_3), 3.00 (m, 1H, 19-H), 3.18 (dd, 1H, $J = 10.8$, 5.1 Hz, 3α-H), 4.07 (m, 2H, $COOCH_2$), 4.45 (t, 2H, $J = 6.6$ Hz, OCH_2), 4.60 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 602 $[M + H]^+$.

3.2.6 *3-O-Acetyl betulinic acid (**4**)*

A solution of BA (2.28 g, 5 mmol) and DMAP (122 mg, 1 mmol) in anhydrous pyridine (10 ml) was cooled and acetic anhydride was slowly added. The reaction mixture was stirred for 2 h at room temperature, then poured into cold water (100 ml), and extracted with EtOAc (3 × 50 ml). The combined organic layer was washed with water and saturated NaCl solution sequentially, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The product was recrystallized in MeOH (for three times) as a white solid (2.92 g, 92%).

Compounds **5a–d** were prepared from **4** and the corresponding dibromopropane as described for **2a** in 90%, 79%, 80%, and 86% yields, respectively.

3.2.7 *3-O-Acetyl betulinic acid-3-nitrooxy-propyl ester (**6a**)*

Compound **6a** was prepared from **5a** and $AgNO_3$ as described for **3a** to afford a white solid in 92% yield. m.p. 65–67°C. IR ν_{max} (KBr, cm^{-1}): 3453, 2950, 1728, 1630; ^1H NMR (300 MHz, $CDCl_3$): δ 0.83 (s, 9H, 3 × CH_3), 0.90 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 2.04 (s, 3H, CH_3CO), 2.98 (m, 1H, 19-H), 4.18 (m, 2H, $COOCH_2$), 4.46 (dd, 1H, $J = 9.6$, 6.3 Hz, 3α-H), 4.55 (t, 2H, $J = 6.3$ Hz, OCH_2), 4.60 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 619 $[M + NH_4]^+$.

3.2.8 *3-O-Acetyl betulinic acid-4-nitrooxy-butyl ester (**6b**)*

Compound **6b** was prepared from **5b** and $AgNO_3$ as described for **3a** to afford a white solid in 91% yield. m.p. 63–65°C. IR ν_{max} (KBr, cm^{-1}): 3424, 2950, 1735, 1721, 1626; ^1H NMR (300 MHz, $CDCl_3$): δ 0.83 (s, 9H, 3 × CH_3), 0.90 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 1.68 (s, 3H, 30-CH_3), 2.04 (s, 3H, CH_3CO), 2.99 (m, 1H, 19-H), 4.11 (m, 2H, $COOCH_2$), 4.47 (dd, 1H, $J = 9.9$, 6.3 Hz, 3α-H), 4.49 (t, 2H, $J = 5.1$ Hz, OCH_2), 4.61 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 633 $[M + NH_4]^+$.

3.2.9 *3-O-Acetyl betulinic acid-5-nitrooxy-pentyl ester (**6c**)*

Compound **6c** was prepared from **5c** and $AgNO_3$ as described for **3a** to afford a white solid in 85% yield. m.p. 64–66°C. IR ν_{max} (KBr, cm^{-1}): 3452, 2949, 1734,

1712, 1625; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.84 (s, 9H, 3 × CH_3), 0.91 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 2.04 (s, 3H, CH_3CO), 2.99 (m, 1H, 19-H), 4.10 (m, 2H, $COOCH_2$), 4.44 (dd, 1H, J = 12.9, 6.6 Hz, 3α-H), 4.46 (t, 2H, J = 6.3 Hz, OCH_2), 4.60 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 647 $[M + NH_4]^+$.

3.2.10 3-O-Acetyl betulinic acid-6-nitrooxy-hexyl ester (**6d**)

Compound **6d** was prepared from **5d** and $AgNO_3$ as described for **3a** to afford a white solid in 88% yield. m.p. 66–68°C. IR ν_{max} (KBr, cm^{-1}): 3449, 2947, 1732, 1712, 1625; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.83 (s, 6H, 2 × CH_3), 0.84 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 2.03 (s, 3H, CH_3CO), 3.00 (m, 1H, 19-H), 4.09 (m, 2H, $COOCH_2$), 4.43 (dd, 1H, J = 12.9, 6.3 Hz, 3α-H), 4.45 (t, 2H, J = 6.3 Hz, OCH_2), 4.60 (s, 1H, 29-H), 4.72 (s, 1H, 29-H); ESI-MS: *m/z* 644 $[M + H]^+$.

3.2.11 Betulonic acid (**7**)

Jones' reagent: CrO_3 (26.72 g) was dissolved in H_2SO_4 (23 ml); then, the solution was diluted to 100 ml with water. A solution of BA (500 mg, 1.09 mmol) in CH_2Cl_2 (12 ml) and acetone (12 ml) was cooled, to which Jones' reagent (1 ml) was slowly added. The mixture was stirred at room temperature for 15 min; then, MeOH (5 ml) was added. After another stirring for 10 min, CH_2Cl_2 (20 ml) was added and the organic layer was washed with water and saturated NaCl solution sequentially, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The product was recrystallized in MeOH for three times as a white solid (462 mg, 93%).

Compounds **8a**–**d** were prepared from **7** and the corresponding dibromopropane as described for **2a** in 81%, 79%, 70%, and 75% yields, respectively.

3.2.12 Betulonic acid-3-nitrooxy-propyl ester (**9a**)

Compound **9a** was prepared from **8a** and $AgNO_3$ as described for **3a** to afford a white solid in 85% yield. m.p. 67–69°C. IR ν_{max} (KBr, cm^{-1}): 3441, 2954, 1728, 1702, 1627; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.93 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 1.02 (s, 6H, 2 × CH_3), 1.07 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 3.00 (m, 1H, 19-H), 4.12 (m, 2H, $COOCH_2$), 4.50 (t, 2H, J = 6.3 Hz, OCH_2), 4.61 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 558 $[M + H]^+$.

3.2.13 Betulonic acid-4-nitrooxy-butyl ester (**9b**)

Compound **9b** was prepared from **8b** and $AgNO_3$ as described for **3a** to afford a white solid in 80% yield. m.p. 71–73°C. IR ν_{max} (KBr, cm^{-1}): 3454, 2949, 1724, 1705, 1630; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.93 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 2.99 (m, 1H, 19-H), 4.13 (m, 2H, $COOCH_2$), 4.50 (t, 2H, J = 6.0 Hz, OCH_2), 4.61 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 572 $[M + H]^+$.

3.2.14 Betulonic acid-5-nitrooxy-pentyl ester (**9c**)

Compound **9c** was prepared from **8c** and $AgNO_3$ as described for **3a** to afford a white solid in 74% yield. m.p. 65–67°C. IR ν_{max} (KBr, cm^{-1}): 3455, 2947, 1720, 1705, 1625; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.93 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 2.99 (m, 1H, 19-H), 4.13 (m, 2H, $COOCH_2$), 4.50 (t, 2H, J = 6.6 Hz, OCH_2), 4.61 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 586 $[M + H]^+$.

*3.2.15 Betulinic acid-6-nitrooxy-hexyl ester (**9d**)*

Compound **9d** was prepared from **8d** and $AgNO_3$ as described for **3a** to afford a white solid in 78% yield. m.p. 69–71°C. IR ν_{max} (KBr, cm^{-1}): 3454, 2962, 1723, 1694, 1628; ^1H NMR (300 MHz, $CDCl_3$): δ 0.92 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.68 (s, 3H, 30-CH_3), 2.99 (m, 1H, 19-H), 4.13 (m, 2H, $COOCH_2$), 4.46 (t, 2H, $J = 6.6$ Hz, OCH_2), 4.61 (s, 1H, 29-H), 4.73 (s, 1H, 29-H); ESI-MS: *m/z* 617 $[M + NH_4]^+$.

3.3 Synthesis of compounds 12a–d

*3.3.1 3-O-Succinyl-betulinic acid (**11**)*

A solution of BA (2.74 g, 6 mmol) and succinic anhydride (2.4 g, 24 mmol) in anhydrous pyridine (20 ml) was kept under reflux for 10 h. Then, the reaction mixture was poured into boiled water (100 ml), cooled and stirred for 10 min, and then acidized with 10% HCl to a pH of 5. The mixture was extracted with EtOAc (3 × 30 ml). The combined organic layer was washed with water and saturated NaCl solution sequentially, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product was recrystallized in MeOH for three times as a white solid (2.90 g, 87%).

*3.3.2 3-O-{4-[2-(2-Oxy-3-phenylsulfonyl-1,2,5-oxadiazol-4-oxy)ethoxy]}-succinyl-betulinic acid (**12a**)*

A solution of **10a** (61.7 mg, 0.22 mmol), **11** (100 mg, 0.18 mmol), DMAP (22 mg, 0.18 mmol), and EDCI (35 mg, 0.18 mmol) in anhydrous CH_2Cl_2 (10 ml) was stirred at reflux for 24 h. After filtration, the filtrate was evaporated to dryness *in vacuo*, and the product was purified by column chromatography ($CHCl_3$–MeOH; 100:1) to give compound **12a** (59 mg, 40%). m. p. 165–167°C. IR ν_{max} (KBr, cm^{-1}): 3453, 2930, 1728, 1627, 1372, 1170; ^1H NMR (300 MHz, $CDCl_3$): δ 0.82 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 2.63 (t, 2H, $J = 6.3$ Hz, $COCH_2$), 2.66 (t, 2H, $J = 6.3$ Hz, $COCH_2$), 3.45 (brs, 1H, 3α-H), 4.24 (t, 2H, $J = 6.0$ Hz, OCH_2), 4.50 (t, 2H, $J = 4.2$ Hz, OCH_2), 4.60 (s, 1H, 29-H), 4.73 (s, 1H, 29-H), 7.60–7.65 (m, 2H, ArH), 7.73–7.79 (m, 2H, ArH), 8.06 (d, 2H, $J = 7.8$ Hz, ArH); ESI-MS: *m/z* 823 $[M - H]^-$.

*3.3.3 3-O-{4-[2-(2-Oxy-3-phenylsulfonyl-1,2,5-oxadiazol-4-oxy) propoxy]}-succinyl-betulinic acid (**12b**)*

Compound **12b** was prepared from **10b** and **11** as described for **12a** to afford a white solid in 45% yield. m.p. 180–182°C. IR ν_{max} (KBr, cm^{-1}): 3449, 2929, 1726, 1627, 1375, 1166; ^1H NMR (300 MHz, $CDCl_3$): δ 0.80 (s, 3H, CH_3), 0.81 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 2.62 (t, 2H, $J = 5.4$ Hz, $COCH_2$), 3.47 (brs, 1H, 3α-H), 4.30 (t, 2H, $J = 6.3$ Hz, OCH_2), 4.49 (t, 2H, $J = 6.0$ Hz, OCH_2), 4.61 (s, 1H, 29-H), 4.73 (s, 1H, 29-H), 7.60–7.65 (m, 2H, ArH), 7.73–7.79 (m, 2H, ArH), 8.06 (d, 2H, $J = 7.5$ Hz, ArH); ESI-MS: *m/z* 837 $[M - H]^-$.

*3.3.4 3-O-{4-[2-(2-Oxy-3-phenylsulfonyl-1,2,5-oxadiazol-4-oxy)-1-methyl-ethoxy]}-succinyl-betulinic acid (**12c**)*

Compound **12c** was prepared from **10c** and **11** as described for **12a** to afford a white solid in 42% yield. m.p. 160–162°C. IR ν_{max} (KBr, cm^{-1}): 3452, 2944, 1732, 1625, 1384, 1170; ^1H NMR (300 MHz, $CDCl_3$): δ 0.77 (s, 3H, CH_3), 0.80 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.33 (d, 3H, $J = 6.2$ Hz, OCH*CH_3*), 1.69 (s, 3H, 30-CH_3), 2.60 (s, 4H, 2 × $COCH_2$), 3.00 (m, 1H, 19-H), 3.56 (brs, 1H, 3α-H), 4.45–4.48 (m, 3H, OCH_2,

OCH), 4.61 (s, 1H, 29-H), 4.74 (s, 1H, 29-H), 7.60–7.65 (m, 2H, ArH), 7.72–7.77 (m, 2H, ArH), 8.06 (d, 2H, $J = 7.5$ Hz, ArH); ESI-MS: *m/z* 837 $[M - H]^-$.

*3.3.5 3-O-{4-[2-(2-Oxy-3-phenylsulfonyl-1,2,5-oxadiazol-4-oxy) butoxy]-succinyl-betulinic acid (**12d**)*

Compound **12d** was prepared from **10d** and **11** as described for **12a** to afford a white solid in 48% yield. m.p. 185–187°C. IR ν_{max} (KBr, cm^{-1}): 3452, 2944, 1731, 1621, 1384, 1170; ^1H NMR (300 MHz, $CDCl_3$): δ 0.82 (s, 9H, $3 \times CH_3$), 0.93 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.69 (s, 3H, 30-CH_3), 2.64 (s, 4H, $2 \times COCH_2$), 3.00 (m, 1H, 19-H), 3.46 (brs, 1H, 3α-H), 4.18 (t, 2H, $J = 6.0$ Hz, OCH_2), 4.45 (t, 2H, $J = 6.3$ Hz, OCH_2), 4.61 (s, 1H, 29-H), 4.74 (s, 1H, 29-H), 7.60–7.65 (m, 2H, ArH), 7.73–7.79 (m, 2H, ArH), 8.06 (d, 2H, $J = 7.8$ Hz, ArH); ESI-MS: *m/z* 851 $[M - H]^-$.

*3.3.6 3-O-{4-[2-(2-Oxy-3-phenylsulfonyl-1,2,5-oxadiazol-4-oxy)-1-methyl-propoxy]}-succinyl-betulinic acid (**12e**)*

Compound **12e** was prepared from **10e** and **11** as described for **12a** to afford a white solid in 44% yield. m.p. 164–166°C. IR ν_{max} (KBr, cm^{-1}): 3447, 2947, 1732, 1619, 1384, 1170; ^1H NMR (300 MHz, $CDCl_3$): δ 0.77 (s, 3H, CH_3), 0.81 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.33 (d, 3H, $J = 6.3$ Hz, OCHCH_3), 1.69 (s, 3H, 30-CH_3), 2.60 (s, 4H, $2 \times COCH_2$), 3.00 (m, 1H, 19-H), 3.66 (brs, 1H, 3α-H), 4.45–4.49 (m, 3H, OCH_2, OCH), 4.61 (s, 1H, 29-H), 4.74 (s, 1H, 29-H), 7.60–7.65 (m, 2H, ArH), 7.72–7.77 (m, 2H, ArH), 8.06 (d, 2H, $J = 7.8$ Hz, ArH); ESI-MS: *m/z* 870 $[M + NH_4]^+$.

4. Conclusion

In summary, two groups of novel NO-releasing derivatives of BA have been designed and synthesized. Derivatives coupled with nitrates have been successfully prepared with the corresponding bromoalkanes, instead of alcohols, to overcome the steric hindrance at the C-28 position. However, the exhibited cytotoxicity of these derivatives was inferior to that of furoxan derivatives, which may be caused by the different NO-releasing amount under physiological conditions. Compound **12e** (IC_{50} of 9.24 μM) displayed approximative cytotoxicity against HepG2 cells compared to the positive control drug. Our findings may lead to further researches on the NO-releasing derivatives of BA for the therapeutic interventions of some human cancers.

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