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Synthesis and Biological Evaluation of Novel Furozan-Based Nitric Oxide-Releasing Derivatives of 23-Hydroxy Betulinic Acid and 3-oxo-23-hydroxybetulinic acid as Potential Anti-Tumor Agents

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Abstract

To search for novel nitric oxide (NO)-releasing anti-tumor agents, two series of furoxan-based NO-releasing derivatives of 23-Hydroxybetulinic acid and 3-oxo-23-hydroxybetulinic acid were designed and synthesized. The nitrate/nitrite levels in the cell lysates were assayed and the results showed that these derivatives could produce high levels of NO in vitro. Then the antiproliferative activity of these hybrids against four human cancer cell lines was further determined, among which, compound 20a was the most promising derivative with an IC_{50} under 10 μM on all tested cell lines. The preliminary structure-activity relationships were concluded based on present experimental data.

Keywords: 23-Hydroxy betulinic acid; NO-donor; Furozan; Anti-tumor activity

Introduction

Lupane triterpenoids such as betulinic acid (Figure 1) are prevalent in natural sources and have various biological activities. 23-Hydroxybetulinic acid (Figure 1) was isolated from the roots of a Chinese medicinal herb *Pulsatilla chinensis* (Bge) Regel, which has a very similar structure and pharmacological activity to betulinic acid [1-3]. A great deal of investigations on the structural modifications of betulinic acid and 23-hydroxybetulinic acid were carried out, and many derivatives with excellent anti-HIV and anti-tumor activities have been obtained [4-6]. Meanwhile, pharmacological studies suggested that 3-oxo-23-hydroxybetulinic acid (3) (Figure 1) had stronger cytotoxic activity on murine melanoma B16 cells (IC_{50}=22.5 μg/ml) than 23-hydroxybetulinic acid and betulinic acid (IC_{50}=32 and 76 μg/ml, respectively) [7]. On another hand, our previous study showed that the polarity and length of the chain in C-28 had an important impact on the anti-tumor activity. These results motivated us to undertake further modifications of the C-28 of 23-Hydroxybetulinic acid, and more intensive SARs have been obtained [8,9].

Nitric oxide (NO), a free radical gas, is a key mediator involved in many physiological and pathological processes. High levels of NO and its metabolic derivatives, the reactive nitrogen species (RNS) and reactive oxygen species (ROS), can modify functional proteins by S-nitrosylation, nitration, and disulfide formation, leading to bio-regulation, inactivation, and cytotoxicity, particularly in tumor cells [10-12]. Therefore, NO-releasing compounds as anti-cancer agents have been investigating for cancer therapy at clinic [13,14]. Furoxans are thermally stable compounds and represent one class of NO donors that can produce high levels of NO and exhibit strong anti-cancer activity [15,16].

Inspired by the obtained interesting results of our previous studies, in which an NO-donor moiety was connected to a 'native' molecule for the purpose of enhancing its therapeutic impact [17,18], in this study, two series of novel furozan-based nitric oxide-releasing derivatives of 23-hydroxybetulinic acid and its analogue 3-oxo-23-hydroxybetulinic acid were designed and synthesized.

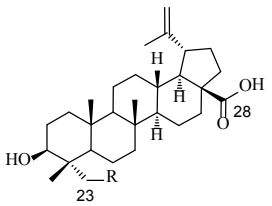
Methods and Materials

Synthesis

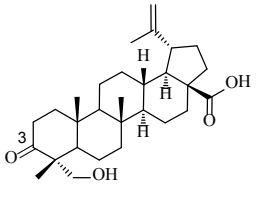
General: Commercially available reagents and solvents were used without further purification. Column chromatography was carried out on Merck silica gel 60 (200-300 mesh). ^{1}H NMR spectra were recorded with 300 MHz spectrometers in the indicated solvents (TMS as internal standard). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), double doublet (dd), multipet (m) and broad (br). Low-resolution mass spectra (LRMS) were measured on Agilent QTOF 6520.

Synthetic Procedures/Analytical Data of Compounds: The synthetic method and physicochemical date of the compounds **10a-i** were disclosed in our previous report [17].

1) Benzyl 3,23-dihydroxy-lup-20(29)-en-28-oate (11): To a mixture of 23-Hydroxybetulinic acid (2) (1.00 g, 2.1 mmol) and K_2CO_3(1.00 g, 7.2 mmol) in DMF (20 mL) was added benzyl chloride



Betulinic acid (**1**):R=H
23-Hydroxybetulinic acid (**2**):R=OH



3-oxo-23-hydroxybetulinic acid (**3**)

Figure 1: Structure of betulinic acid (**1**), 23-hydroxybetulinic acid (**2**) and 3-oxo-23-hydroxybetulinic acid (**3**).

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(0.3 ml, 2.5 mmol) at room temperature for 12 h. Then the reaction mixture was filtered, and washed with DMF (5 mL × 3). The filtrate was poured into ice-water to give a white precipitate. The precipitate was filtered, washed with water, and dried to give 11 (1.07g, 90%), which was almost a pure product, and was used for the next reaction without further purification. Pure product was obtained by recrystallization of the crude product from EtOH. ESI-MS *m/z*: 563.3 $[M + H]^+$, 585.3 $[M + Na]^+$, 601.4 $[M + K]^+$

2) Benzyl 3-hydroxy-23-t-butyldimethylsilyloxy-lup-20(29)-en-28-oate (12): To a solution of 11 (1.00g, 1.8mmol) in CH_2Cl_2(30 mL) was added TBSCl (0.36g, 2.4mmol), DMAP (0.3 g, 2.5 mmol) at room temperature for 4 h. After CH_2Cl_2 was removed by evaporation in vacuo, the residue was acidified with 10% HCl (20 mL) and extracted with EtOAc. The combined extract was washed with saturated brine (30 mL×3), dried over Na_2SO_4, filtered, and concentrated in vacuo to afford a yellow oil, which was purified by column chromatography (petroleum ether-EtOAc (20:1)) to give 12 as a white solid (1.11 g, 92%).^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.06 (6 H, s, Si-$(CH_3)_2$), 0.75, 0.84, 0.93, 1.67 (6 H for 0.84, each 3 H for others, s, 24, 25, 26, 27 and 30-CH_3), 0.90 (9 H, s, t-Bu), 2.13–2.20 (1 H, m), 2.25–2.28 (1 H, m), 3.01 (1 H, m, H-19), 3.33, 3.65 (each 1 H, d, *J*=9.3 Hz, H-23), 3.56 (1 H, m, H-3), 4.59, 4.72 (each 1 H, s, H-29), 5.09, 5.15 (each 1 H, d, *J*=12.2 Hz, CH_2-Ph), 7.32–7.35 (5 H, m, H-Ph).

3) Benzyl 3-oxo-23-t-butyldimethylsilyloxylup-20(29)-en-28-oate (13): To a solution of 12 (1.03 g, 1.5 mmol) in CH_2Cl_2 (30 mL) was added PCC (0.5 g, 2.3 mmol) at 0°C. After being stirred at 0°C for 4 h, the reaction mixture was warmed to room temperature and stirred overnight. The mixture was filtered and washed with CH_2Cl_2 (10 mL × 5). The filtrate was concentrated in vacuo to give a brown solid. Crystallization from ethanol gave 13 as a white solid (0.91g, 89%). mp 151-154°C. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.07 (6 H, s, Si-$(CH_3)_2$), 0.80, 0.83, 0.86, 0.96, 1.68 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 0.87 (9 H, s, t-Bu), 1.97 (3 H, s, Ac), 2.18–2.32 (2 H, m), 2.37–2.42 (2 H, m), 3.04 (1 H, m, H-19), 3.28, 3.56 (each 1 H, d, *J*=9.1 Hz, H-23), 4.61, 4.73 (each 1 H, d, *J*=1.2 Hz, H-29), 5.09, 5.16 (each 1 H, d, *J*=12.2 Hz, CH_2-Ar), 7.35–7.38 (5 H, m, H-Ar); ESI-MS *m/z*: 675.5 $[M + H]^+$, 697.5 $[M + Na]^+$, 714.5 $[M + K]^+$.

4) Benzyl 3-oxo-23-hydroxy-lup-20(29)-en-28-oate (14): To a solution of 13 (0.8 g, 1.2 mmol) in acetone (30 mL) were added 10% HCl (1 mL). The reaction mixture was stirred at room temperature for 2 h. At this point, the mixture was neutralized with $NaHCO_3$ saturated solution, and then extracted with CH_2Cl_2, the CH_2Cl_2 layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography (petroleum ether-EtOAc (5:1)) to give 14 as a white solid (0.6 g, 90%). mp 175-178°C. IR (film, cm^{-1}) 3444, 3067, 2950, 2863, 1732, 1705, 1640, 1459, 1381, 1257, 1121, 1087, 881, 835, 775, 701; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.74, 0.88, 0.92, 0.95, 1.61 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.48–2.60 (1 H, m), 2.95 (1 H, m, H-19), 3.33, 3.56 (each 1 H, d, *J*=11.3 Hz, H-23), 4.53, 4.66 (each 1 H, s, H-29), 5.02, 5.09 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.23–7.31 (5 H, m, H-Ar); ESI-MS *m/z*: 561.3 $[M + H]^+$, 583.3 $[M + Na]^+$, 599.0 $[M + K]^+$.

5) 3,23-(1-methylethylidene acetal)-lup-20(29)-en-28-oic acid (16): To a solution of 2 (1.00g, 2.1mmol) in anhydrous acetone (30 mL) was added TsOH (0.1g) and DMP (0.8ml, 7.6mmol). The reaction mixture was refluxed for 4 h. At this point, the mixture was evaporated and diluted with EtOAc (30mL), the EtOAc layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography (petroleum ether-EtOAc (4:1)) to give 16 as a white solid (0.94 g, 84%).^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.80, 0.86, 0.99, 1.06, 1.68 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.17~2.25 (2H, m), 2.99 (1H, m, H-19), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 4.61, 4.74 (each 1H, s, H-29); ESI-MS *m/z*: 511.4 $[M - H]^-$

6) Ethyl 3,23-(1-methylethylidene acetal)-lup-20(29)-en-28-oate (17): To a mixture of 16 (0.94 g, 1.8 mmol) and K_2CO_3(1.00 g, 7.2 mmol) in DMF (25 mL) was added ethyl bromide (0.27 ml, 3.6 mmol) at room temperature for 12 h. Then the mixture was diluted with EtOAc (30mL), the EtOAc layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography (petroleum ether-EtOAc (4:1)) to give 16 as a white solid (0.89g, 90%).IR (film, cm^{-1}) 3469, 2988, 2847, 2866, 1719, 1447, 1397, 1253, 1177, 1154, 1133, 1114, 1064, 880; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.86, 0.90, 0.95, 1.02, 1.68 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.17~2.25 (2H, m), 3.01 (1H, m, H-19), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 4.13 (2H, m, $COOCH_2CH_3$), 4.61, 4.74 (each 1H, s, H-29).

7) 3,23-(1-methylethylidene acetal)-lup-20(29)-en-28-ol (18): To a solution of 17 (0.89 g, 1.64 mmol) in dry THF (25 mL) was added $LiAlH_4$ (0.32 g, 8 mmol). The reaction mixture was refluxed for 4 h. At this point, the mixture was diluted with aqueous ether, and then extracted with CH_2Cl_2, the CH_2Cl_2 layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography (petroleum ether-EtOAc (2:1)) to give 18 as a white solid (0.62 g, 76%).IR (film, cm^{-1}) 3475, 2941, 2870, 1725, 1665, 1399, 1254, 1206, 1112, 1063, 1029, 853; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.87, 0.97, 1.02, 1.44, 1.68 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.38 (1H, m, H-19), 3.33, 3.79 (each 1H, d, *J*=10.5 Hz , H-28), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 4.58, 4.68 (each 1H, s, H-29).

8) General procedure for synthesis of compounds 15a-i and 19a-i: Compound 14 (0.1g, 0.18 mmol) or 18 (0.09g, 0.18 mmol)was mixed with corresponding Compounds 10a–i (0.22 mmol), EDCI (93 mg, 0.6 mmol) and DMAP (catalytic amount) in 15 mL of CH_2Cl_2 and stirred at room temperature for 8-16 h. The reaction mixture was washed with water and saturated NaCl solution sequentially, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude products were purified by column chromatography (petroleum ether-EtOAc (4:1)) to give the title compounds.

9) Benzyl 3-oxo-(23-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxyethyl)-lup-20(29)-en-28-oate (15a): White solid, yield 48.2%.mp. 50–53 °C; IR (KBr) υ_{max} 3435, 2951, 2869, 1737, 1455, 1375, 1334, 1160, 1074, 962, 736, 621 cm^{-1}; ^{1}H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.80, 0.88, 0.92, 0.95, 1.60 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.39–2.50 (1 H, m), 2.67 (4 H, m, $CO(CH_2)_2CO$), 3.02 (1 H, m, H-19), 4.05 (2 H, s, H-23), 4.18, 4.47 (each 2H, t, *J*=6.0 Hz, $O(CH_2)_2O$), 4.59, 4.72 (each 1H, s, H-29), 5.02, 5.09 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.23–7.31 (5 H, m, H-Ar), 7.59 (2H, t, *J*=7.8 Hz, H-Ar), 7.73 (2H, t, *J*=6.9 Hz, H-Ar), 8.06 (1H, d, *J*=7.5 Hz, H-Ar); ESI-MS *m/z*: 929.4 $[M + H]^+$.

10) Benzyl 3-oxo-(23-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxypropyl)-lup-20(29)-en-28-oate (15b): White solid, yield 47.6%. mp. 46–49 °C; IR (KBr) υ_{max} 3418, 2951, 2869, 1736, 1643, 1455, 1378, 1160, 736, 686 cm^{-1}; ^{1}H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.80, 0.88, 0.92, 0.95, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.67 (4H, m, $CO(CH_2)_2CO$), 3.02 (1H, m, H-19), 4.05, 4.11 (each 1H, s, H-23), 4.18 (4H, m, $O(CH_2)_3O$), 4.59, 4.71 (each 1H, s, H-29), 5.02, 5.09 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.26–7.31 (5 H, m, H-Ar), 7.53 (2H, t, *J*=7.8 Hz, H-Ar), 7.65 (2H, t, *J*=6.9 Hz, H-Ar), 8.07 (1H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 943.4 $[M + H]^+$

11) Benzyl 3-oxo-(23-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)- oxybutyl)-lup-20(29)-en-28-oate (15c): White solid, yield 47.2%. mp. 42–45 °C; IR (KBr) υ_{max} 3420, 2951, 2869, 1736, 1644, 1455, 1331, 1160, 736, 621 cm^{-1}; ^1H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.79, 0.88, 0.91, 0.96, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.39–2.50 (2 H, m), 2.65 (4H, m, $CO(CH_2)_2CO$), 3.01 (1H, m, H-19), 4.05 (2H, s, H-23), 4.18, 4.47 (each 2H, t, *J*=6.0 Hz, $O(CH_2)_4O$), 4.59, 4.71 (each 1H, s, H-29), 5.06, 5.09 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.27 –7.35 (5 H, m, H-Ar), 7.59 (2H, t, *J*=7.8 Hz, H-Ar), 7.65 (2H, t, *J*=6.9 Hz, H-Ar), 8.07 (1H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 957.4 $[M + H]^+$

12) Benzyl 3-oxo-(23-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxyethyl)-lup-20(29)-en-28-oate (15d): White solid, yield 48.1%. mp. 55–58 °C; IR (KBr) υ_{max} 3420, 2952, 2869, 1736, 1643, 1455, 1160, 736, 686 cm^{-1}; ^1H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.79, 0.88, 0.91, 0.96, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.38 (4H, m, $CO(CH_2)_3CO$), 3.00 (1H, m, H-19), 4.01 (2H, s, H-23), 4.38 (4H, t, *J*=5.4 Hz, $O(CH_2)_2O$), 4.60, 4.72 (each 1H, s, H-29), 5.07, 5.10 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.27–7.35 (5 H, m, H-Ar), 7.59 (2H, t, *J*=7.8 Hz, H-Ar), 7.65 (2H, t, *J*=6.9 Hz, H-Ar), 8.07 (1H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 942.4 $[M + H]^+$.

13) Benzyl 3-oxo-(23-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxypropyl)-lup-20(29)-en-28-oate (15e): White solid, yield 45.9%. mp. 60–63 °C; IR (KBr) υ_{max} 3417, 2951, 2869, 1736, 1643, 1455, 1331, 1160, 736, 686 cm^{-1}; ^1H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.79, 0.88, 0.91, 0.96, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.40 (4H, m, $CO(CH_2)_3CO$), 3.00 (1H, m, H-19), 4.02 (2H, s, H-23), 4.38 (4H, t, *J*=5.4 Hz, $O(CH_2)_3O$), 4.60, 4.72 (each 1H, s, H-29), 5.07, 5.10 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.27–7.35 (5 H, m, H-Ar), 7.59 (2H, t, *J*=7.8 Hz, H-Ar), 7.65 (2H, t, *J*=6.9 Hz, H-Ar), 8.07 (1H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 957.4 $[M + H]^+$

14) Benzyl 3-oxo-(23-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxybutyl)-lup-20(29)-en-28-oate (15f): White solid, yield 46.2 %. mp. 59–62 °C; IR (KBr) υ_{max} 3420, 2951, 2869, 1736, 1455, 1378, 1160, 1018, 736, 621 cm^{-1}; ^1H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.80, 0.88, 0.92, 0.95, 1.60 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.42 (4H, m, $CO(CH_2)_3CO$), 3.02 (1 H, m, H-19), 4.05 (2 H, s, H-23), 4.38 (4H, t, *J*=6.0 Hz, $O(CH_2)_4O$), 4.59, 4.72 (each 1H, s, H-29), 5.02, 5.09 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.23–7.31 (5 H, m, H-Ar), 7.59 (2H, t, *J*=7.8 Hz, H-Ar), 7.73 (2H, t, *J*=6.9 Hz, H-Ar), 8.06 (1H, d, *J*=7.5 Hz, H-Ar); ESI-MS *m/z*: 971.4 $[M + H]^+$

15) Benzyl 3-oxo-(23-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)- oxyethyl)-lup-20(29)-en-28-oate (15 g): White solid, yield 45.0 %. mp. 54–57 °C; IR (KBr) υ_{max} 3440, 2924, 2854, 1726, 1617, 1551, 1450, 1170, 740, 597 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.79, 0.88, 0.92, 0.95, 1.61 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.39–2.50 (1 H, m), 3.01 (1 H, m, H-19), 4.24 (2 H, m, H-23), 4.58, 4.71 (each 1H, s, H-29), 4.73 (4H, t, *J*=6.0 Hz, $O(CH_2)_2O$), 5.02, 5.09 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.23–7.31 (5 H, m, H-Ar), 7.55 (2H, t, *J*=5.0 Hz, H-Ar), 7.59 (2H, t, *J*=7.8 Hz, H-Ar), 7.71 (2H, t, *J*=5.0 Hz, H-Ar), 7.73 (2H, t, *J*=6.9 Hz, H-Ar), 8.06 (1H, d, *J*=7.5 Hz, H-Ar); ESI-MS *m/z*: 977.4 $[M + H]^+$

16) Benzyl 3-oxo-(23-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)- oxypropyl)-lup-20(29)-en-28-oate (15h): White solid, yield 44.9%. mp. 50–53 °C; IR (KBr) υ_{max} 3445, 2925, 2854, 1726, 1617, 1552, 1450, 1270, 1170, 740, 698, 597 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.79, 0.88, 0.92, 0.95, 1.61 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.39–2.50 (1 H, m), 3.01 (1 H, m, H-19), 4.15 (2 H, m, H-23), 4.52, 4.57 (each 1H, t, *J*=6.0 Hz, $O(CH_2)_3O$), 4.59, 4.71 (each 1H, s, H-29), 5.02, 5.09 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.23–7.31 (5 H, m, H-Ar), 7.55 (2H, t, *J*=7.5 Hz, H-Ar), 7.59 (2H, t, *J*=7.8 Hz, H-Ar), 7.71 (1H, t, *J*=5.0 Hz, H-Ar), 7.73 (2H, t, *J*=6.9 Hz, H-Ar), 8.06 (2H, d, *J*=7.5 Hz, H-Ar); ESI-MS *m/z*: 991.4 $[M + H]^+$

17) Benzyl 3-oxo-(23-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxybutyl)-lup-20(29)-en-28-oate (15i): White solid, yield 45.9%. mp. 49–52 °C; IR (KBr) υ_{max} 3445, 2925, 2853, 1726, 1617, 1551, 1450, 1270, 1170, 740, 698, 597, 556 cm^{-1}.^1H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.79, 0.88, 0.92, 0.95, 1.61 (each 3 H, s, 24, 25, 26, 27 and 30-CH_3), 2.39–2.50 (1 H, m), 3.01 (1 H, m, H-19), 4.27 (2 H, s, H-23), 4.52, 4.57 (each 1H, t, *J*=6.0 Hz, $O(CH_2)_3O$), 4.59, 4.71 (each 1H, s, H-29), 5.02, 5.09 (each 1 H, d, *J*=11.1 Hz, CH_2-Ar), 7.23–7.31 (5 H, m, H-Ar), 7.55 (2H, t, *J*=7.5 Hz, H-Ar), 7.59 (2H, t, *J*=7.8 Hz, H-Ar), 7.71 (1H, t, *J*=5.0 Hz, H-Ar), 7.73 (2H, t, *J*=6.9 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar); ESI-MS *m/z*: 1005.4 $[M + H]^+$

18) 3, 23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxyethyl)-ate (19a): White solid, yield 51.4%. ^1H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.81, 0.88, 0.91, 0.96, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.47 (1H, m, H-19), 2.69 (4H, m, $CO(CH_2)_2CO$), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 3.88, 4.30 (each 1H, d, *J*=10 Hz, H-28), 4.50, 4.68 (each 2H, d, *J*=7.5 Hz, $O(CH_2)_2O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, *J*=7.5 Hz, H-Ar), 7.74 (1H, t, *J*=7.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar)

19) 3, 23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxypropyl)-ate (19b): White solid, yield 48.9%. ^1H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.81, 0.88, 0.91, 0.96, 1.61 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.43 (1H, m, H-19), 2.65 (4H, m, $CO(CH_2)_2CO$), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 3.85, 4.30 (each 1H, d, *J*=10.5 Hz, H-28), 4.31, 4.51 (each 2H, t, *J*=6 Hz, $O(CH_2)_3O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, *J*=7.5 Hz, H-Ar), 7.74 (1H, t, *J*=7.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar)

20) 3, 23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxybutyl)-ate (19c): White solid, yield 50.5%. ^1H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.81, 0.88, 0.91, 0.96, 1.61 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.43 (1H, m, H-19), 2.66 (4H, m, $CO(CH_2)_2CO$), 3.43, 3.51 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 3.85, 4.30 (each 1H, d, J=11.1 Hz, H-28), 4.18, 4.47 (each 2H, t, J=6 Hz, $O(CH_2)_4O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, J=7.2 Hz, H-Ar), 7.74 (1H, t, J=6.3 Hz, H-Ar), 8.07 (2H, d, J=7.5 Hz, H-Ar)

21)3,23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxyethyl)-ate (19d): White solid, yield 53.2%. ^1H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.81, 0.88, 0.96, 1.02, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.41 (1H, m, H-19), 2.43 (4H, m, $CO(CH_2)_3CO$), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 3.86, 4.30 (each 1H, d, *J*=10 Hz, H-28), 4.51, 4.64 (each 2H, t, *J*=4.5 Hz, $O(CH_2)_2O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, *J*=7.5 Hz, H-Ar), 7.74 (1H, t, *J*=7.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar)

22) 3,23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxypropyl)-ate (19e): White solid, yield 49.7 %. ^1H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.81, 0.88, 0.96, 1.02, 1.61 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.41 (1H, m, H-19), 2.43 (4H, m, $CO(CH_2)_3CO$), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 3.85, 4.30 (each 1H, d, J=10 Hz, H-28), 4.51, 4.64 (each 2H, t, J=4.5 Hz, $O(CH_2)_3O$), 4.58,

4.69 (each 1H, s, H-29), 7.62 (2H, t, J=7.5 Hz, H-Ar), 7.74 (1H, t, J=7.5 Hz, H-Ar), 8.07 (2H, d, J=7.5 Hz, H-Ar)

23) 3,23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxybutyl)-ate (19f): White solid, yield 51.2 %. ^{1}H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.81, 0.88, 0.91, 0.96, 1.61 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.41 (1H, m, H-19), 2.43 (4H, m, $CO(CH_2)_3CO$), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 3.85, 4.30 (each 1H, d, J=11.1 Hz, H-28), 4.50, 4.64 (each 2H, t, J=4.5 Hz, $O(CH_2)_4O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, J=7.2 Hz, H-Ar), 7.74 (1H, t, J=6.3 Hz, H-Ar), 8.07 (2H, d, J=7.5 Hz, H-Ar)

24) 3,23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxyethyl)-ate (19 g): White solid, yield 53.8%. ^{1}H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.82, 0.87, 0.98, 1.02, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.50 (1H, m, H-19), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 4.06, 4.46 (each 1H, d, *J*=10.5 Hz, H-28), 4.59, 4.70 (each 1H, s, H-29), 4.74 (4H, m, $O(CH_2)_2O$), 7.46 (2H, t, *J*=7.5 Hz, H-Ar), 7.58 (2H, t, *J*=4.5 Hz, H-Ar), 7.64 (1H, t, *J*=7.5 Hz, H-Ar), 7.72 (1H, t, *J*=4.5 Hz, H-Ar), 7.82 (1H, t, *J*=4.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar)

25) 3, 23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxypropyl)-ate (19h): White solid, yield 48.7%.^{1}H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.82, 0.87, 0.98, 1.02, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.51 (1H, m, H-19), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 4.07, 4.47 (each 1H, d, *J*=10.5 Hz, H-28), 4.60, 4.70 (each 1H, s, H-29), 4.74 (4H, m, $O(CH_2)_3O$), 7.46 (2H, t, *J*=7.5 Hz, H-Ar), 7.58 (2H, t, *J*=4.5 Hz, H-Ar), 7.64 (1H, t, *J*=7.5 Hz, H-Ar), 7.72 (1H, t, *J*=4.5 Hz, H-Ar), 7.82 (1H, t, *J*=4.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar)

26) 3, 23-(1-methylethylidene acetal)-lup-20(29)-en-28-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxybutyl)-ate (19i): White solid, yield 46.5 %. ^{1}H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.82, 0.86, 0.98, 1.02, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.48 (1H, m, H-19), 3.43, 3.52 (each 1H, d, *J*=10.5 Hz , H-23), 3.49 (1H, m, H-3), 4.06, 4.46 (each 1H, d, *J*=10.8 Hz, H-28), 4.59, 4.70 (each 1H, s, H-29), 4.74 (4H, m, $O(CH_2)_4O$), 7.46 (2H, t, *J*=7.5 Hz, H-Ar), 7.58 (2H, t, *J*=4.5 Hz, H-Ar), 7.64 (1H, t, *J*=7.5 Hz, H-Ar), 7.71 (1H, t, *J*=4.5 Hz, H-Ar), 7.82 (1H, t, *J*=4.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar)

27) General procedure for synthesis of compounds 20a-i: To a solution of **19a-i** (2mmol) in THF (10 mL) were added 10% HCl (10 mL). The reaction mixture was stirred at room temperature for 2 h. At this point, the mixture was neutralized with $NaHCO_3$ saturated solution, and then extracted with CH_2Cl_2, the CH_2Cl_2 layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by column chromatography (dichloromethane-methanol, (30:1)) to give the title compounds.

28) 3,23-dihydroxy-lup-20(29)-en-28-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxyethyl)-ate (20a): White solid, yield 75.1 %. mp. 54–57 °C; IR (KBr) υ_{max} 3420, 2926, 2854, 1733, 1618, 1553, 1283, 739, 685, 598 cm^{-1}; ^{1}H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.81, 0.88, 0.91, 0.96, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.47 (1H, m, H-19), 2.69 (4H, m, $CO(CH_2)_2CO$), 3.41, 3.70 (each 1H, d, *J*=10 Hz, H-23), 3.60 (1H, m, H-3), 3.88, 4.30 (each 1H, d, *J*=10 Hz, H-28), 4.50, 4.68 (each 2H, d, *J*=7.5 Hz, $O(CH_2)_2O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, *J*=7.5 Hz, H-Ar), 7.74 (1H, t, *J*=7.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 844.4 $[M + NH_4]^+$.

29) 3,23-dihydroxy-lup-20(29)-en-28-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxypropyl)-ate (20b): White solid, yield 72.3 %. mp. 52–55 °C; IR (KBr) υ_{max} 3420, 2925, 2854, 1734, 1618, 1553, 1284, 740, 685, 598 cm^{-1}; ^{1}H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.81, 0.88, 0.91, 0.96, 1.61 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.43 (1H, m, H-19), 2.65 (4H, m, $CO(CH_2)_2CO$), 3.39, 3.69 (each 1H, d, *J*=8.4 Hz, H-23), 3.60 (1H, m, H-3), 3.85, 4.30 (each 1H, d, *J*=10.5 Hz, H-28), 4.31, 4.51 (each 2H, t, *J*=6 Hz, $O(CH_2)_3O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, *J*=7.5 Hz, H-Ar), 7.74 (1H, t, *J*=7.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 858.4 $[M + NH_4]^+$

30) 3,23-dihydroxy-lup-20(29)-en-28-O-(4-oxo-butyric acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxybutyl)-ate (20c): White solid, yield 70.3 %. mp. 48–51°C; IR (KBr) υ_{max} 3420, 2926, 2854, 1734, 1618, 1553, 1283, 740, 685, 598 cm^{-1}; ^{1}H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.81, 0.88, 0.91, 0.96, 1.61 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.43 (1H, m, H-19), 2.66 (4H, m, $CO(CH_2)_2CO$), 3.40, 3.69 (each 1H, d, J=8.4 Hz, H-23), 3.60 (1H, m, H-3), 3.85, 4.30 (each 1H, d, J=11.1 Hz, H-28), 4.18, 4.47 (each 2H, t, J=6 Hz, $O(CH_2)_4O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, J=7.2 Hz, H-Ar), 7.74 (1H, t, J=6.3 Hz, H-Ar), 8.07 (2H, d, J=7.5 Hz, H-Ar); MS(ESI) m/z: 872.4 $[M + NH_4]^+$

31) 3,23-dihydroxy-lup-20(29)-en-28-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxyethyl)-ate (20d): White solid, yield 74.9 %. mp. 73–76 °C; IR (KBr) υ_{max} 3420, 2925, 2854, 1733, 1618, 1553, 1285, 739, 685, 598 cm^{-1}; ^{1}H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.81, 0.88, 0.96, 1.02, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.41 (1H, m, H-19), 2.43 (4H, m, $CO(CH_2)_3CO$), 3.42, 3.71 (each 1H, d, *J*=10 Hz, H-23), 3.62 (1H, m, H-3), 3.86, 4.30 (each 1H, d, *J*=10 Hz, H-28), 4.51, 4.64 (each 2H, t, *J*=4.5 Hz, $O(CH_2)_2O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, *J*=7.5 Hz, H-Ar), 7.74 (1H, t, *J*=7.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 858.4 $[M + NH_4]^+$.

32) 3,23-dihydroxy-lup-20(29)-en-28-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxypropyl)-ate (20e): White solid, yield 70.3 %. mp. 63–66 °C; IR (KBr) υ_{max} 3420, 2926, 2854, 1734, 1618, 1553, 1283, 740, 685, 598 cm^{-1}; ^{1}H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.81, 0.88, 0.96, 1.02, 1.61 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.41 (1H, m, H-19), 2.43 (4H, m, $CO(CH_2)_3CO$), 3.40, 3.69 (each 1H, d, J=10 Hz, H-23), 3.60 (1H, m, H-3), 3.85, 4.30 (each 1H, d, J=10 Hz, H-28), 4.51, 4.64 (each 2H, t, J=4.5 Hz, $O(CH_2)_3O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, J=7.5 Hz, H-Ar), 7.74 (1H, t, J=7.5 Hz, H-Ar), 8.07 (2H, d, J=7.5 Hz, H-Ar); MS(ESI) m/z: 872.4 $[M + NH_4]^+$

33) 3,23-dihydroxy-lup-20(29)-en-28-O-(5-oxo-pentanoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxybutyl)-ate (20f): White solid, yield 67.8 %. mp. 61–64 °C; IR (KBr) υ_{max} 3420, 2926, 2854, 1734, 1618, 1553, 1283, 740, 685, 598 cm^{-1}; ^{1}H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.81, 0.88, 0.91, 0.96, 1.61 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.41 (1H, m, H-19), 2.43 (4H, m, $CO(CH_2)_3CO$), 3.40, 3.69 (each 1H, d, J=8.4 Hz, H-23), 3.60 (1H, m, H-3), 3.85, 4.30 (each 1H, d, J=11.1 Hz, H-28), 4.50, 4.64 (each 2H, t, J=4.5 Hz, $O(CH_2)_4O$), 4.58, 4.69 (each 1H, s, H-29), 7.62 (2H, t, J=7.2 Hz, H-Ar), 7.74 (1H, t, J=6.3 Hz, H-Ar), 8.07 (2H, d, J=7.5 Hz, H-Ar); MS(ESI) m/z: 886.4 $[M + NH_4]^+$

34) 3,23-dihydroxy-lup-20(29)-en-28-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxyethyl)-ate (20g): White solid, yield 65.1 %. mp. 95–98 °C; IR (KBr) υ_{max} 3440, 2924, 2854, 1726, 1617, 1551, 1450, 1170, 740, 597 cm^{-1}; ^{1}H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.82, 0.87, 0.98, 1.02, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.50 (1H, m, H-19), 3.42, 3.71 (each 1H, d, *J*=9.5 Hz, H-23), 3.62 (1H, m, H-3), 4.06, 4.46 (each 1H, d, *J*=10.5 Hz, H-28), 4.59, 4.70 (each 1H, s, H-29), 4.74 (4H, m, $O(CH_2)_2O$), 7.46 (2H, t, *J*=7.5 Hz, H-Ar), 7.58 (2H, t, *J*=4.5 Hz, H-Ar), 7.64 (1H, t, *J*=7.5 Hz, H-Ar), 7.72

(1H, t, *J*=4.5 Hz, H-Ar), 7.82 (1H, t, *J*=4.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 892.4 $[M + NH_4]^+$

35) 3,23-dihydroxy-lup-20(29)-en-28-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxypropyl)-ate (20h): White solid, yield 68.3%.mp. 78–81 °C; IR (KBr) υ_{max} 3439, 2924, 2854, 1726, 1618, 1551, 1450, 1170, 740, 597 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz), δ (ppm) 0.82, 0.87, 0.98, 1.02, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.51 (1H, m, H-19), 3.41, 3.70 (each 1H, d, *J*=9.5 Hz, H-23), 3.62 (1H, m, H-3), 4.07, 4.47 (each 1H, d, *J*=10.5 Hz, H-28), 4.60, 4.70 (each 1H, s, H-29), 4.74 (4H, m, $O(CH_2)_3O$), 7.46 (2H, t, *J*=7.5 Hz, H-Ar), 7.58 (2H, t, *J*=4.5 Hz, H-Ar), 7.64 (1H, t, *J*=7.5 Hz, H-Ar), 7.72 (1H, t, *J*=4.5 Hz, H-Ar), 7.82 (1H, t, *J*=4.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 906.4 $[M + NH_4]^+$

36) 3,23-dihydroxy-lup-20(29)-en-28-O-(2-formyl benzoic acid-(3-phenylsulfonyl-1,2,5-oxadiazole-2-oxide-4)-oxybutyl)-ate (20i): White solid, yield 64.5 %. mp. 75–78 °C; IR (KBr) υ_{max} 3440, 2925, 2854, 1726, 1618, 1551, 1450, 1170, 740, 597 cm^{-1}; ^1H NMR($CDCl_3$, 300 MHz), δ (ppm) 0.82, 0.86, 0.98, 1.02, 1.60 (each 3H, s, 24, 25, 26, 27 and 30-CH_3), 2.48 (1H, m, H-19), 3.40, 3.70 (each 1H, d, *J*=7.5 Hz, H-23), 3.61 (1H, m, H-3), 4.06, 4.46 (each 1H, d, *J*=10.8 Hz, H-28), 4.59, 4.70 (each 1H, s, H-29), 4.74 (4H, m, $O(CH_2)_4O$), 7.46 (2H, t, *J*=7.5 Hz, H-Ar), 7.58 (2H, t, *J*=4.5 Hz, H-Ar), 7.64 (1H, t, *J*=7.5 Hz, H-Ar), 7.71 (1H, t, *J*=4.5 Hz, H-Ar), 7.82 (1H, t, *J*=4.5 Hz, H-Ar), 8.07 (2H, d, *J*=7.5 Hz, H-Ar); MS(ESI) *m/z*: 920.4 $[M + NH_4]^+$

NO-releasing test

The levels of nitrate/nitrite formed from individual compounds were determined by the colorimetric assay using the nitrate/nitrite colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. 10μmol/L of each compound in phosphate buffer solution (PBS) containing 2% dimethyl sulfoxide and 5.0 mM L-cysteine at pH 7.4 was incubated at 37°C for 10–150 min and were sampled at 10 min, 30 min, 60 min, 80 min, 100 min, 120 min and 150min. The collected samples (2 mL) were mixed with 0.5 ml of Griess reagent and incubated at 37°C for 10 min, followed by measuring at 540 nm. The different concentrations of nitrite were used as standards to calculate the concentrations of NO formed by individual compounds.

MTT assay

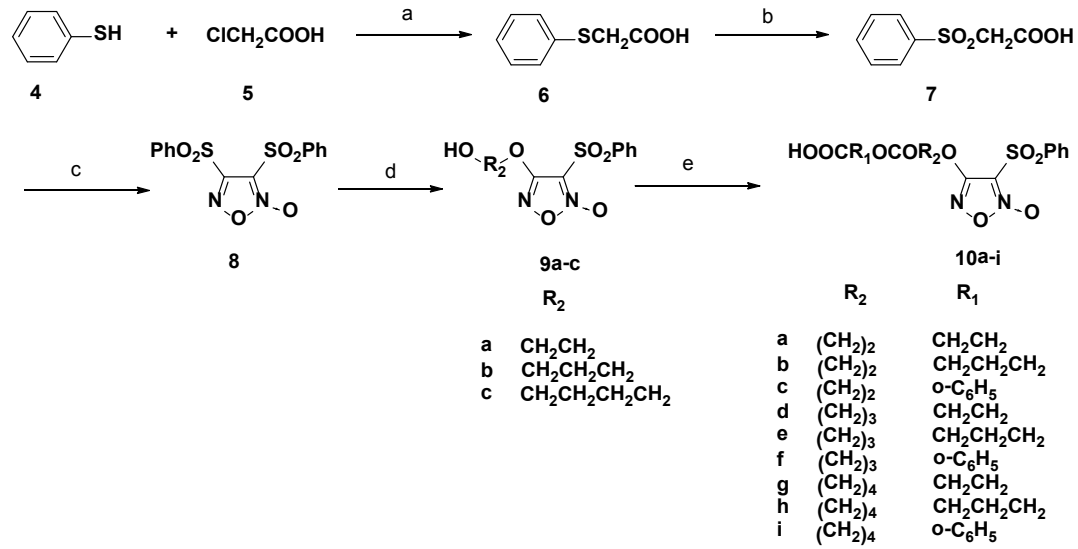
The MTT assay was employed *in vitro* for anti-proliferative activity assay, which was performed in 96-well plates. Four different cell lines were used: B16 (mice melanoma), A549 (human lung carcinoma), BEL-7402 (human hepatoma), K562 (human leukemic cell). Test cells at the log phase of their growth cycle (5×104 cell/mL) were added to each well (100 μL/well), then treated in four replicates at various concentrations of the samples (0.39-100 μg/mL), and incubated for 24 h at 37℃ in a humidified atmosphere of 5% CO_2. After 72 h, 20 μL of MTT solution (5 mg/mL) per well was added to each cultured medium, which was incubated for further 4 h. Then, DMSO was added to each well (150 μL/well). After 10 min at room temperature, the OD of each well was measured on a Microplate Reader (BIO-RAD instruments Inc NO.550) at a wavelength of 490 nm. In these experiments, the negative reference was 0.1% DMSO, and Taxol was used as the positive reference.

Results and Discussion

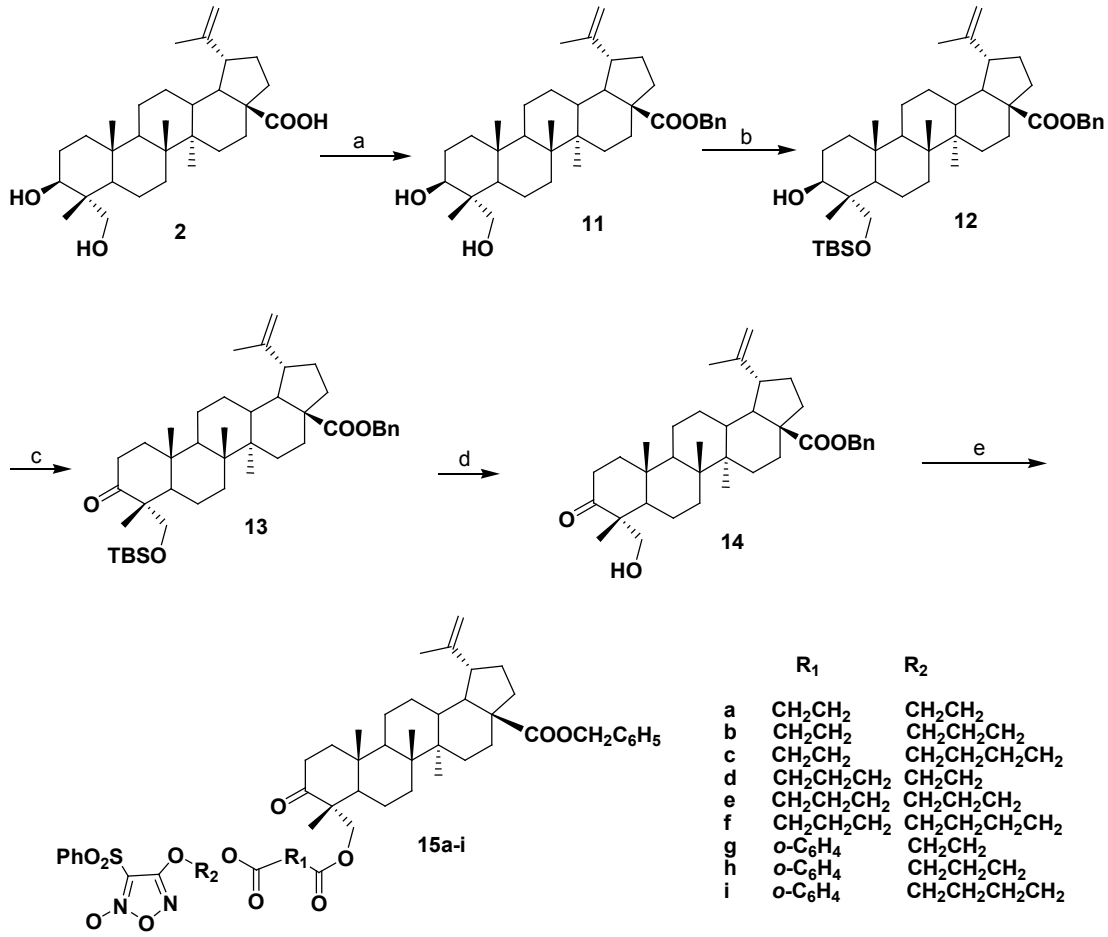
Chemistry

As shown in (Scheme 1), the substituted furoxans were prepared in five steps sequence. The starting material benzenethiol (4) was converted to 2-(phenylthio) acetic acid (6) by treatment with chloroacetic acid (5) in 97% yield. Then, compound 6 was oxidized by 30% H_2O_2 aqueous solution to generate 2-(phenylsulfonyl) acetic acid (7). Furthermore, fuming HNO_3 was added to obtain diphenylsulfonylfuroxan (8). Subsequently, 8 was then converted to various monophenylsulfonylfuroxans 9a–c by treatment with the corresponding diol. Finally, anhydrides were added and furoxan-based NO donors 10a–i were obtained.

The general procedure for the synthesis of derivatives 15a-i was described in (Scheme 2). For the synthetic experiments, the starting material 23-hydroxybetulinic acid (2) was isolated from the root of *Pulsatilla chinensis*. It was mixed with benzyl bromide and potassium carbonate in DMF at room temperature for 2h to give benzyl ester 11 in 92% yield. Silyl ether 12 was prepared in high yield using the regular method, and then an oxidation reaction was followed on C-3 position with PCC afforded ketone 13. Deprotection of 13 with 10%HCl in acetone at room temperature produced benzyl 3-oxo-23-



Scheme 1: General method for the synthesis of **10a–i**. Reagents and conditions: (a) NaOH (aq),140 °C, 2h; (b) 30% H_2O_2, AcOH, rt, 3h; (c) fuming HNO_3, 90 °C, 4h; (d) diol, THF, 30% NaOH, rt, 4–8h; (e) anhydrides, pyridine, rt, 6-12h.



	R_1	R_2
a	CH_2CH_2	CH_2CH_2
b	CH_2CH_2	$CH_2CH_2CH_2$
c	CH_2CH_2	$CH_2CH_2CH_2CH_2$
d	$CH_2CH_2CH_2$	CH_2CH_2
e	$CH_2CH_2CH_2$	$CH_2CH_2CH_2$
f	$CH_2CH_2CH_2$	$CH_2CH_2CH_2CH_2$
g	*o*-C_6H_4	CH_2CH_2
h	*o*-C_6H_4	$CH_2CH_2CH_2$
i	*o*-C_6H_4	$CH_2CH_2CH_2CH_2$

Scheme 2: General method for the synthesis of **15a–i**. Reagents and conditions: (a) benzyl bromide, DMF, rt, 2h; (b) TBSCl, DMAP, CH_2Cl_2, rt, 4h; (c) PCC, CH_2Cl_2, rt, 3h; (d) 10%HCl, acetone, rt, 2h; (e) **10**a-i, EDCI, DMAP, CH_2Cl_2, rt, 8-12h.

hydroxybetulinate 14. Treatment of 14 with intermediate furoxan-based NO donors 10a-i gave a series of hybrids 15a-i.

The synthesis of derivatives 20a-i was also started from isolated 23-hydroxybetulinic acid (2). Ketalization of 2 with 2, 2-dimethoxypropane in the presence of TsOH in anhydrous acetone gave cyclic ketal 16 in 84% yield. Treated with ethyl bromide and potassium carbonate in DMF at room temperature for 12h, esterification of 28-group was accomplished and 17 was obtained. The reduction of ester 17 with $LiAlH_4$ in THF afforded the alcohol 18, which was subsequently reacted with 10a-i to afford 19a-i in moderate yields. Deprotection of 19a-i with 10%HCl in acetone at room temperature produced derivatives 20a-i (Scheme 3).

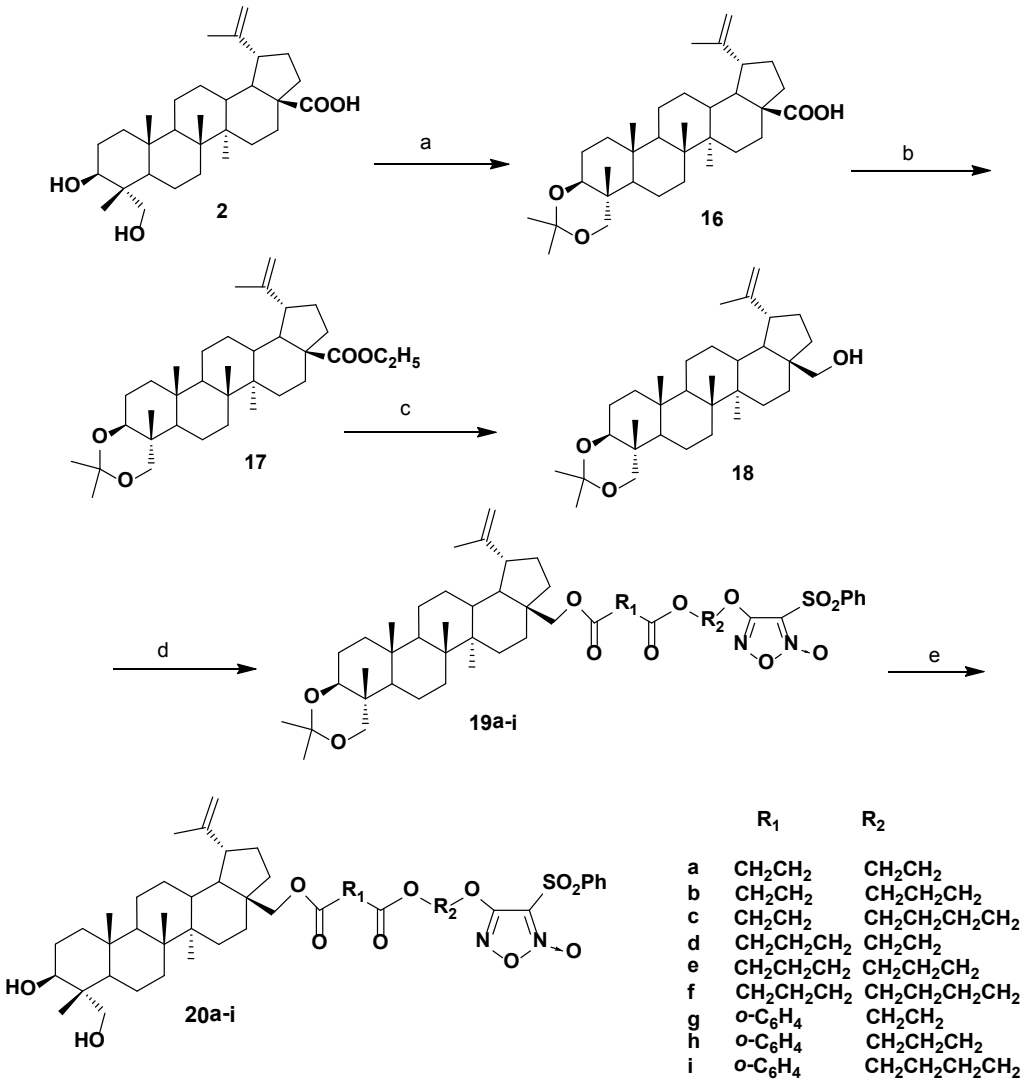
Biological evaluation

NO-releasing test: The levels of nitrate/nitrite in the lysates were determined of target compounds (15a-i and 20a-i) at 10 μmol/L by Griess assay through the duration of 0-150 min. As showed in Figure 2, all the target NO-donating derivatives were found to release different amounts of NO. In general, C-28 substituted NO-donating derivatives (20a-i) were found to release the more amount of NO than the 3-oxo-23-hydroxybetulinic acid derivatives (15a-i). Among them, compounds 20a and 20b showed the maximum releasing amount, with the highest level of 26.9 μmol/L and 25.48 μmol/L at the 150 min time point (Figures 2 and 3).

Cytotoxicity: To evaluate the anticancer potencies of these newly synthesized 23-hydroxybetulinic acid derivatives, the antiproliferative activities of compounds 15a-i and 20a-i were tested against four cancer cell lines (B16, A546, Bel-7402, K562). The present results demonstrated that nearly all synthesized NO-releasing 23-hydroxybetulinic acid derivatives can markedly inhibit the proliferation of cancer cells than their parent compounds 2 (23-hydroxybetulinic acid) and 3 (3-oxo-23-hydroxybetulinic acid) (Table 1). Among them, compound 20a was the most promising derivative with an IC_{50} under 10 μM on all tested cell lines. Noticeably, the antiproliferative activity evaluation also showed that C-28 substituted NO-donating derivatives of 23-hydroxybetulinic acid (20a-i) generally exhibited stronger activity than 3-oxo-23-hydroxybetulinic acid derivatives (15a-i). These results suggested that releasing of NO contributed to the antiproliferative activity and higher levels of NO releasing could produce stronger activity. Moreover, preliminary structure-activity relationships displayed that the variety and length of the linkers, which connected NO donor moiety to the 23- or 28-position of parent compounds, were important for compounds' activities. When R_1 were aliphatic linkers, the target compounds showed stronger cytotoxicity (20a-f) than those with aromatic linkers (20g-i), meanwhile, the order of substituent R_2 for the activities was as follows: Ethyl > Propyl> Butyl.

Conclusion

In summary, by coupling NO-donor moieties with natural products



	R_1	R_2
a	CH_2CH_2	CH_2CH_2
b	CH_2CH_2	$CH_2CH_2CH_2$
c	CH_2CH_2	$CH_2CH_2CH_2CH_2$
d	$CH_2CH_2CH_2$	CH_2CH_2
e	$CH_2CH_2CH_2$	$CH_2CH_2CH_2$
f	$CH_2CH_2CH_2$	$CH_2CH_2CH_2CH_2$
g	*o*-C_6H_4	CH_2CH_2
h	*o*-C_6H_4	$CH_2CH_2CH_2$
i	*o*-C_6H_4	$CH_2CH_2CH_2CH_2$

Scheme 3: General method for the synthesis of **20a–i**. Reagents and conditions: (a) 2, 2-dimethoxypropane, TsOH, anhydrous acetone, reflux, 4h; (b) ethyl bromide,K_2CO_3, DMF, rt, 12h; (c) $LiAlH_4$, THF, reflux, 4h; (d) **10**a-i, EDCI, DMAP, CH_2Cl_2, rt, 8-12h; (e) 10%HCl, acetone, rt, 2h.

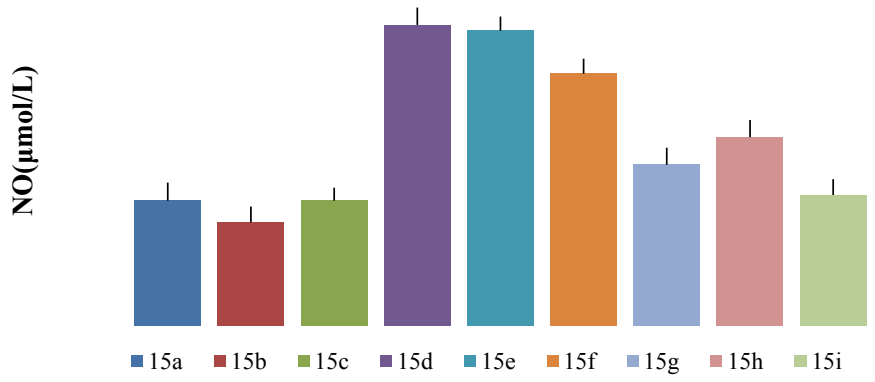


Figure 2: Variable levels of NO produced by the compounds **15a-i** (10μmol/L) at the time point of 150 min.

23-hydroxybetulinic acid and its analogue 3-oxo-23-hydroxybetulinic acid, two series of novel furozan-based nitric oxide-releasing derivatives were designed and synthesized. The NO-releasing assay indicated variable levels of NO have produced by the target compounds. Among them, compound 20a was found to release the maximum amount of NO, and furthermore 20a showed to have IC_{50} values under 10 μM

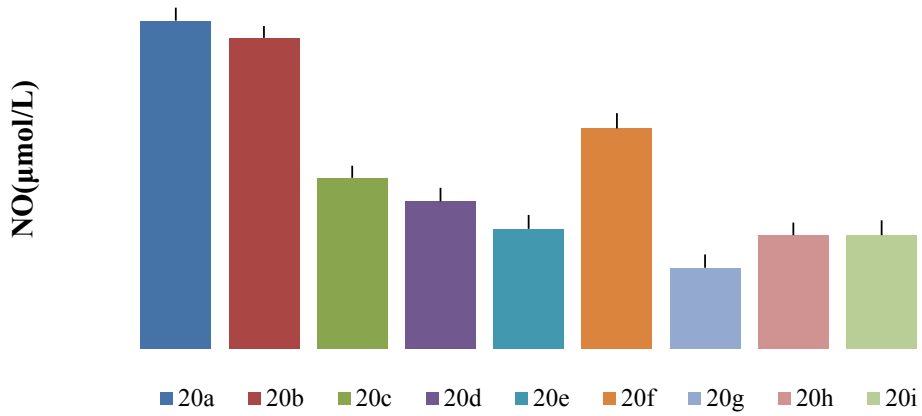


Figure 3: Variable levels of NO produced by the compounds **20a-i** (10μmol/L) at the time point of 150 min.

Compd.	IC_{50}(μM)			
	B16	A549	Bel-7402	K562
Taxol[b]	0.96 ± 0.01	0.72 ± 0.04	0.45 ± 0.10	0.91 ± 0.02
2	29.87 ± 3.64	33.08 ± 0.15	39.67 ± 4.22	42.03 ± 1.21
3	20.62 ± 1.02	29.70 ± 0.34	33.78 ± 2.12	38.33 ± 1.31
14	19.39 ± 0.18	20.92 ± 1.60	21.85 ± 0.73	22.69 ± 0.09
15a	7.98 ± 0.29	9.34 ± 0.54	11.97 ± 0.06	12.23 ± 0.12
15b	10.75 ± 0.14	10.09 ± 0.20	14.87 ± 0.24	15.56 ± 0.52
15c	11.92 ± 0.09	11.27 ± 0.33	15.01 ± 0.09	16.14 ± 0.43
15d	9.65 ± 0.31	9.60 ± 0.14	11.95 ± 0.19	12.48 ± 0.31
15e	9.71 ± 0.20	10.62 ± 0.11	14.70 ± 0.20	16.22 ± 0.14
15f	10.01 ± 0.13	10.33 ± 0.07	15.85 ± 0.12	16.01 ± 0.30
15g	11.89 ± 0.08	12.91 ± 0.05	15.54 ± 0.09	17.96 ± 0.11
15h	16.74 ± 0.12	17.74 ± 0.09	18.01 ± 0.04	19.41 ± 0.26
15i	20.81 ± 0.14	21.87 ± 0.28	22.70 ± 0.15	25.05 ± 0.14
20a	6.40 ± 0.42	8.05 ± 0.12	9.02 ± 0.16	8.46 ± 0.05
20b	6.93 ± 0.06	9.71 ± 0.30	10.17 ± 0.01	10.08 ± 0.06
20c	6.98 ± 0.04	10.03 ± 0.05	13.06 ± 0.03	12.10 ± 0.07
20d	6.29 ± 0.22	8.78 ± 0.19	9.08 ± 0.42	9.34 ± 0.15
20e	7.13 ± 0.16	9.85 ± 0.11	10.21 ± 0.08	11.72 ± 0.12
20f	7.97 ± 0.14	9.92 ± 0.16	13.13 ± 0.15	13.98 ± 0.08
20g	11.15 ± 0.31	10.94 ± 0.14	14.98 ± 0.13	15.81 ± 0.10
20h	15.16 ± 0.18	16.72 ± 0.17	17.81 ± 0.10	16.56 ± 0.09
20i	19.27 ± 0.15	20.60 ± 0.16	21.02 ± 0.11	23.14 ± 0.08

[a] Results are expressed as IC_{50} values in μM. [b] Taxol was used as a positive control.

Table 1: IC_{50} values of the target compounds against four human tumor cell lines[a].

on all tested human cancer cell lines, which was the most promising derivative. The present study demonstrates that introduction with NO-donor moieties at suitable positions of 23-hydroxybetulinic acid and its analogue could obtain the interesting derivatives with improved antiproliferative activity. Moreover, the assay data also revealed that the higher levels of NO-releasing could produce stronger activity. The present results may provide useful information for the subsequent design and synthesis of NO releasing derivatives of 23-hydroxybetulinic acid with improved biological response.

Acknowledgements

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