Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 5432–5439
Bioorganic & Medicinal Chemistry

The synthesis of 18β-glycyrrhetinic acid derivatives which have increased antiproliferative and apoptotic effects in leukemia cells

Dan Liu,[a] Dandan Song,[a] Gang Guo,[a] Rui Wang,[a] Jinling Lv,[a] Yongkui Jing[b,*] and Linxiang Zhao[a,*]

[a]*Shenyang Pharmaceutical University, Shenyang 110016, PR China*
[b]*Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA*

Received 2 February 2007; revised 21 May 2007; accepted 23 May 2007
Available online 26 May 2007

Abstract—18β-Glycyrrhetinic acid (GA), 3β-hydroxyl-11-oxo-olean-12-ene-29-oic acid, has been found to inhibit growth and to induce apoptosis in cancer cells. Through structural modification, 16 GA derivatives (12 novel compounds) with modified structures at the C_3 and C_{29} positions were synthesized. The antiproliferative effects and apoptosis induction abilities of these compounds were determined in human leukemia HL-60 cells. The replacement of the hydroxyl group of GA with a carbonyl group or an oxime group at C_3 position does not influence the antiproliferative effect. However, the antiproliferative and apoptosis induction abilities of the compounds with a replaced alkoxyimino group at position C_3 and a free C_{29} carboxyl group are markedly increased.

1. Introduction

Glycyrrhizin and its metabolic product, aglycon 18β-glycyrrhetinic acid (GA, 3β-hydroxyl-11-oxo-olean-12-ene-29-oic acid, **1**), are the main active principles of the plant *Glycyrrhizae radix*. GA has been used as medicine to treat allergic and hepatic diseases.[1,2] GA suppresses the tumor promoting effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 7,12-dimethylbenz[*a*]anthracene on skin tumor formation in mice,[3,4] and has antitumor activities.[5,6] Recently, it has been shown that GA could induce apoptosis in human hepatoma, leukemia, and gastric cancer cells at high concentrations.[7] To improve the efficacy of GA in inhibiting cell growth and in inducing apoptosis in leukemia cells, oximino, acyloxyimino, and alkoxyimino groups were introduced into the C_3 position and/or an ester was formed at the C_{29} free carboxylic acid position in order to increase the lipophilic character of the compounds. Sixteen GA derivatives including 12 novel compounds (**4a**, **4b**, **5b**, **5c**, **6a–c**, **8a**, **8b**, **9a–c**) were synthesized and their antiproliferative and apoptotic effects were investigated in human HL-60 leukemia cells.

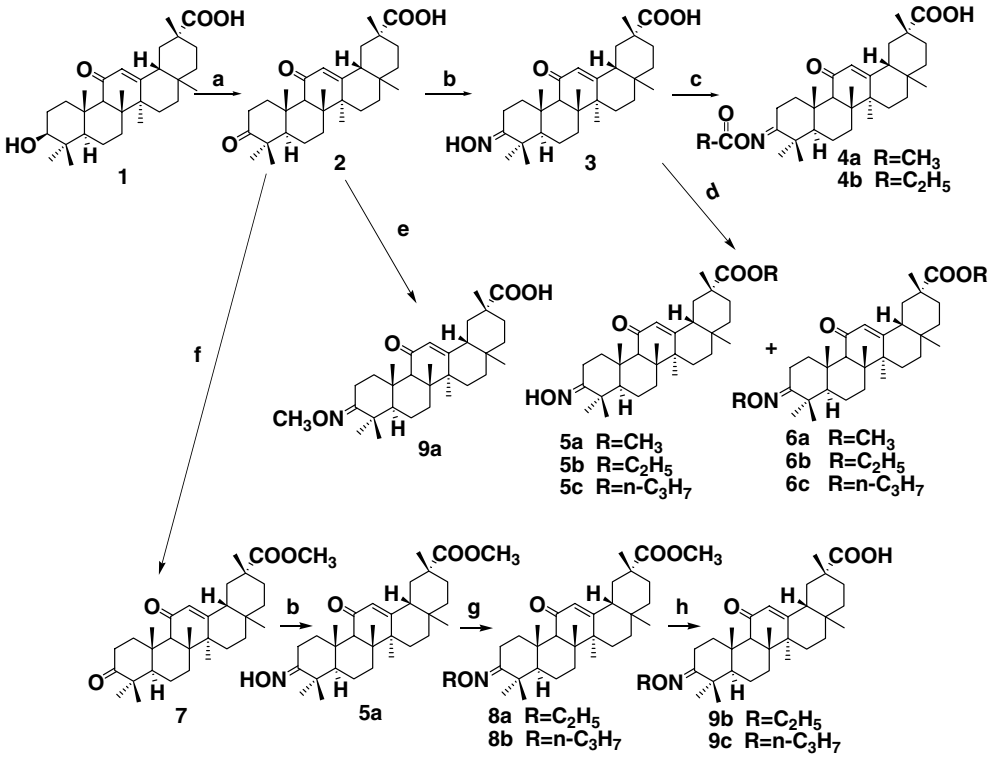
2. Results and discussion

2.1. Synthesis of GA derivatives

18β-Glycyrrhetinic acid (**1**) was used as the starting material and the structure modification was done at the positions of C_3 and C_{29} of 18β-glycyrrhetinic acid. The synthetic pathways are shown in Scheme 1. The target compounds **4–6** were synthesized from **1**, through the intermediate compounds **2** and **3** (Scheme 1). 3-Keto compound **2** was obtained in a high yield from **1** with the Jones' reagent,[8] which reacted with hydroxylamine hydrochloride in pyridine (Py) to produce an oxime compound **3**.[9] Refluxing compound **3** with the acetic anhydride or propionic anhydride in Py in the presence of 4-dimethylaminopyridine (DMAP)[10] afforded the esters of compounds **4a** and **4b** at position C_3, but had a free carboxyl group at position C_{29}. On the other hand, treatment of compound **3** with alkyl halide refluxed in tetrahydrofuran (THF) at a basic pH generated compounds **6a**, **6b**, and **6c**, which had an alkylated oxime at position C_3 and an esterified carboxyl group at position C_{29}.[11] However, under gentle conditions with a

Keywords: 18β-Glycyrrhetinic acid; Synthesis; Apoptosis; Structure–activity relationship.
* Corresponding authors. Fax: +86 24 2389 6576 (L.Z.); e-mail addresses: yongkui.jing@mssm.edu; linxiang.zhao@vip.sina.com

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.05.057



Scheme 1. The synthetic pathways of 18β-glycyrrhetinic acid derivatives. Reagents and conditions: (a) Jones' reagent, THF, ice-salt bath, 1 h; (b) $NH_2OH{\cdot}HCl$, Py, reflux, 4 h; (c) Ac_2O or $(CH_3CH_2CO)_2O$, Py, DMAP, reflux, 4–6 h; (d) CH_3I, or CH_3CH_2Br, or *n*-$CH_3CH_2CH_2Br$, KOH, THF, reflux, 1–24 h; (e) $CH_3ONH_2{\cdot}HCl$, Py, reflux, 2 h; (f) CH_3OH, concd H_2SO_4, reflux, 24 h; (g) CH_3CH_2Br or *n*-$CH_3CH_2CH_2Br$, KOH, THF, reflux, 10–20 h; (h) LiI, DMF, reflux, 3 h.

reduced amount of alkyl halide and a reduction in reaction time, compounds **5a**, **5b**, and **5c** were obtained, respectively, which were only esterified at the C_{29} carboxyl group without alkylation of the oxime at position C_3.

Compound **5a** was also obtained in a high yield from compound **7** through methylation of compound **2** condensed with hydroxylamine hydrochloride. Compound **5a** reacted with alkyl halide (C_2H_5Br, or *n*-C_3H_7Br) by refluxing in THF to obtain either **8a** or **8b**, which subsequently was reacted with lithium iodide in dry DMF to give the target acid **9b** and **9c**.[12] Acid **9a** was synthesized by using intermediate **2** in a reaction with methoxyl amine hydrochloride in Py directly. Target compounds **9a**–**c** had an alkylated C_3 oxime and a free carboxyl group at C_{29}.

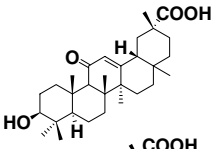
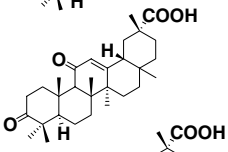
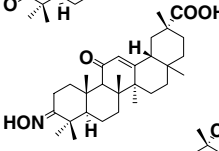
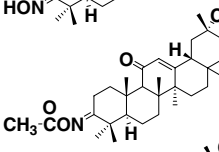
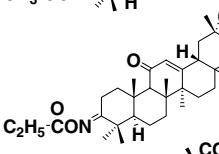
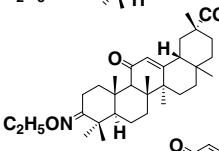
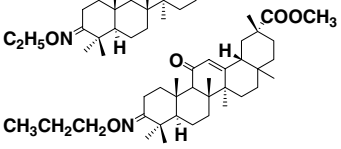
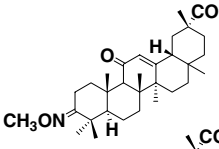
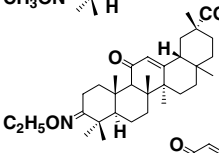
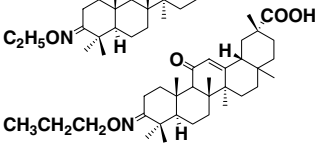
The structures of all the compounds were determined by application of mp, Infrared (IR), mass spectra (MS), high-resolution mass spectra (HR-MS), and NMR spectral data. The structure assignment for compounds **5**, **6**, and **9** was supported by comparing the ^1H NMR and ^{13}C NMR spectral data with each other. The ^1H NMR spectrum of compound **7**, the product of **2** refluxing in CH_3OH in the presence of concentrated H_2SO_4, displayed an added methyl group at δ 3.76 ppm (3H, s) while the chemical shift at C-29 was downfielded from 182.04 to 176.88 ppm in the ^{13}C NMR spectrum. The difference between oxime **5a** and acid **9a** was obvious in the NMR spectra, since there were $-OCH_3$ protons at δ 3.76 ppm (3H, s) and at δ 176.91 ppm at C_{29} in **5a**, while the corresponding data were δ 3.82 ppm (3H, s) and δ 181.52 ppm at C_{29} in **9a**. The same differences were seen in comparing the spectral data between compounds **5b** and **9b**, and between **5c** and **9c**.

2.2. Investigation of the abilities of these compounds to inhibit cell growth and to induce apoptosis in HL-60 cells

The antiproliferative effects of these GA derivatives in HL-60 cells were determined by direct cell counting. As shown in Table 1, compounds with a free hydroxyl group (**1**), a carbonyl group (**2**), or an oxime group (**3**) at the C_3 position were less effective than all of the other compounds tested with $GI_{50s} > 50$ μM. Esterification of the C_3 oxime group (**4a** and **4b**) did not improve the antiproliferative effects when compared to that of compound **3** which contains a free oxime group. Alkylation of the C_3 oxime (**9a**–**c**), which has a free carboxyl group at the C_{29} position, significantly increased antiproliferative effects. The activity order of these three compounds was **9a < 9b < 9c**. Of all the derivatives, **8a** and **8b** (which contained an alkoxyimino group at C_3 position and an esterified carboxyl group at position C_{29}) were the most potent inhibitors of cell growth with GI_{50s} of 19 μM.

To determine whether the antiproliferative effects of these GA derivatives were due to apoptosis induction, the percentage of apoptotic cells was determined after treatment. Since it has been reported that GA induced apoptosis at a concentration of approximately

Table 1. The antiproliferative and apoptotic effects of glycyrrhetinic acid derivatives in HL-60 cells

Code[a]	Structure	GI_{50}[b] (μM)	Apoptotic cells[c] (%)
1		63.2 ± 3.5	24.9 ± 3.7
2		59.6 ± 5.4	28.5 ± 4.1
3		63.0 ± 5.8	24.5 ± 4.5
4a		58.8 ± 3.0	19.1 ± 2.3
4b		57.7 ± 5.5	27.7 ± 3.3
8a		19.1 ± 0.9	8.6 ± 1.5
8b		19.0 ± 0.8	4.9 ± 1.3
9a		36.1 ± 3.7	60.2 ± 2.5
9b		24.4 ± 0.2	59.7 ± 2.9
9c		20.6 ± 2.5	87.3 ± 4.5

[a] The antiproliferative effects of compounds **5a–c**, **6a–c**, and **7** were not determined due to their poor solubility in H_2O, ethanol, and dimethylsulfoxide.

[b] GI_{50} is the concentration that inhibited 50% of cell growth. The cells were treated with various concentrations of the compounds for 72 h and their number was determined.

[c] Cells were treated with 80 μM of each compound for 6 h and the percentage of apoptotic cells were determined by morphological observation after staining with AO and EB as described in Section 3. Data shown are means ± SD of three independent experiments.

100 μM,[7] HL-60 cells were treated with 80 μM of each compound for 6 h, and the percentages of apoptotic cells were determined by morphological observation after staining with AO and EB. Table 1 shows that approximately 25% of HL-60 cells underwent apoptosis after treatment with compounds **1, 2, 3**, **4a** or **4b**. Compounds with a free C_{29} carboxyl group and a C_3 alkoxyimino group (**9a–c**) were more potent than the other compounds in apoptosis induction (Fig. 1). The apoptosis induction ability of compounds **9a–c** was dose-dependent. Compound **9c** was the most effective apoptosis inducer of these compounds (Fig. 2). To further confirm apoptosis induction, the percentage of cells in subG1 and the levels of DNA fragmentation after treatment with GA and compounds **9a–c** were determined. Cells treated with compounds **9a–c**, but not GA, had increased numbers of cells in the subG1 phase that counted as apoptotic cells and had a greater amount of DNA fragmentation (Fig. 3). Although compounds **8a** and **8b** were more potent in inhibiting cell growth, they did not induce apoptosis after 6 h of treatment at a concentration of 80 μM (Table 1). These data suggest

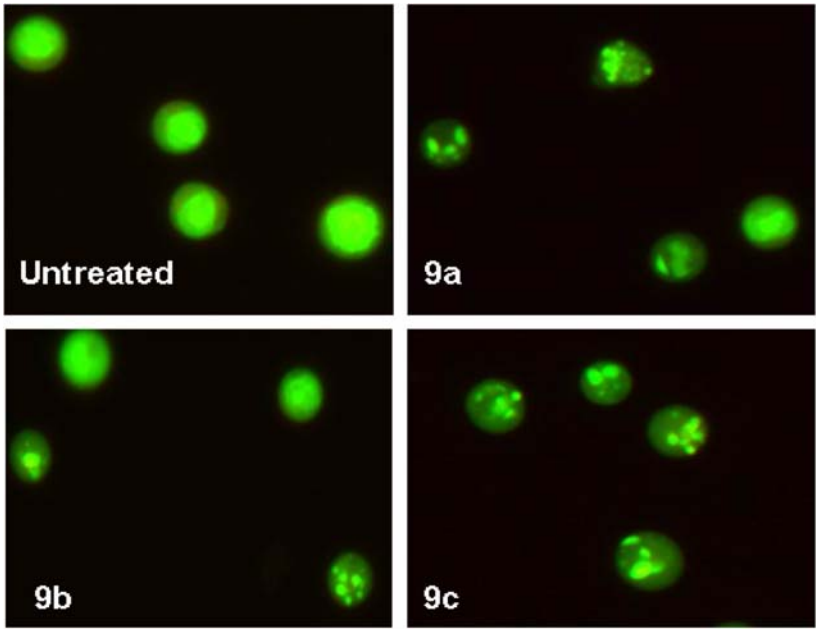


Figure 1. Apoptotic cells were determined based on morphological changes in HL-60 cells after treatment with compounds **9a**–**c**. HL-60 cells were treated with the indicated compounds at 80 μM for 6 h. Cells with nuclear apoptotic bodies were observed after staining with AO and EB with a fluorescence microscope.

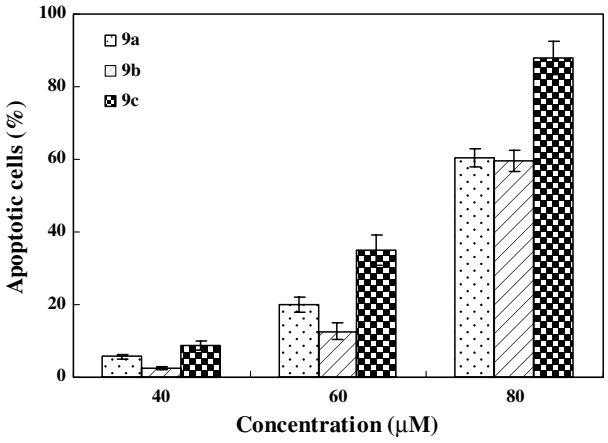


Figure 2. Dose-dependent apoptotic effects of compounds **9a**–**c**. HL-60 cells were treated with the indicated concentrations of compounds for 6 h and the percentages of apoptotic cells were determined by morphologic fluorescent observation after staining with AO and EB. Data shown are means ± SD of three independent experiments.

that the antiproliferative effects of compounds **8a** and **8b** might be through an apoptosis-induction independent pathway and that these compounds may inhibit cell growth and induce apoptosis via different pathways.

In summary, our data indicate that (1) replacement of the hydroxyl group of GA with a carbonyl group or an oxime group at the C_3 position does not influence the antiproliferative ability; (2) compounds with an alkoxyimino group at position C_3 and a free C_{29} carboxyl group have greater antiproliferative effect and apoptosis induction than other compounds tested; (3) compounds with an esterified C_{29} carboxyl group and an alkylated C_3 oxime group are potent cell growth inhibitors and lack apoptosis induction ability; (4) these compounds may inhibit cell growth and induce apoptosis through different pathways.

3. Experimental

GA was purchased from Shanghai Haokang Chemicals Co. Ltd, China, with a over 98% purity. Other reagents were bought from commercial suppliers in an analytic grade without further purification unless otherwise noted. Anhydrous LiI was obtained by drying purchased $LiI{\cdot}3H_2O$ for 10 h at 110 °C under vacuum. ^1H NMR, ^{13}C NMR spectra were recorded on a BRUKER ARX-300 instrument with tetramethylsilane as an internal standard. IR spectra were recorded on a BRUKER IR-27G spectrometer. MS were determined on either Finnigan MAT/USA spectrometer (LC–MS) or Shimadzu GCMS QP-1000 mass spectrometer. HR-MS were obtained on Finnigan MAT-711 mass spectrometer in EI mode. The purity was calculated in a N300 chromatographic working station with Hitachi UV detector L-2400 and Hitachi pump-L-2130. The melting points were determined on an electrically heated X4 digital visual melting point apparatus and were uncorrected. TLC board (Alugram silica gel G/UV_{254}) was obtained from Macherey–Nagel GmbH & Co., and the development system was cyclohexane:acetone (v/v) = 3:1.

3.1. 3,11-Dioxo-olean-12-ene-29-oic acid (2)

To a solution of GA (**1**) (10.0 g, 21.2 mmol) in THF (35 mL), Jones' reagent (15 mL) was added dropwise in an ice-salt bath. The solution was stirred for 1 h, added to H_2O (100 mL), filtered, and dried to afford compound **2** as a white solid (9.58 g, yield: 98%). R_f value 0.25, mp: 283–285 °C, purity: 98.7%. IR (KBr): 3313, 2965, 1728, 1683, 1645, 1457, 1386, 1207, 1144, 1087, 781, 676 cm^{-1};

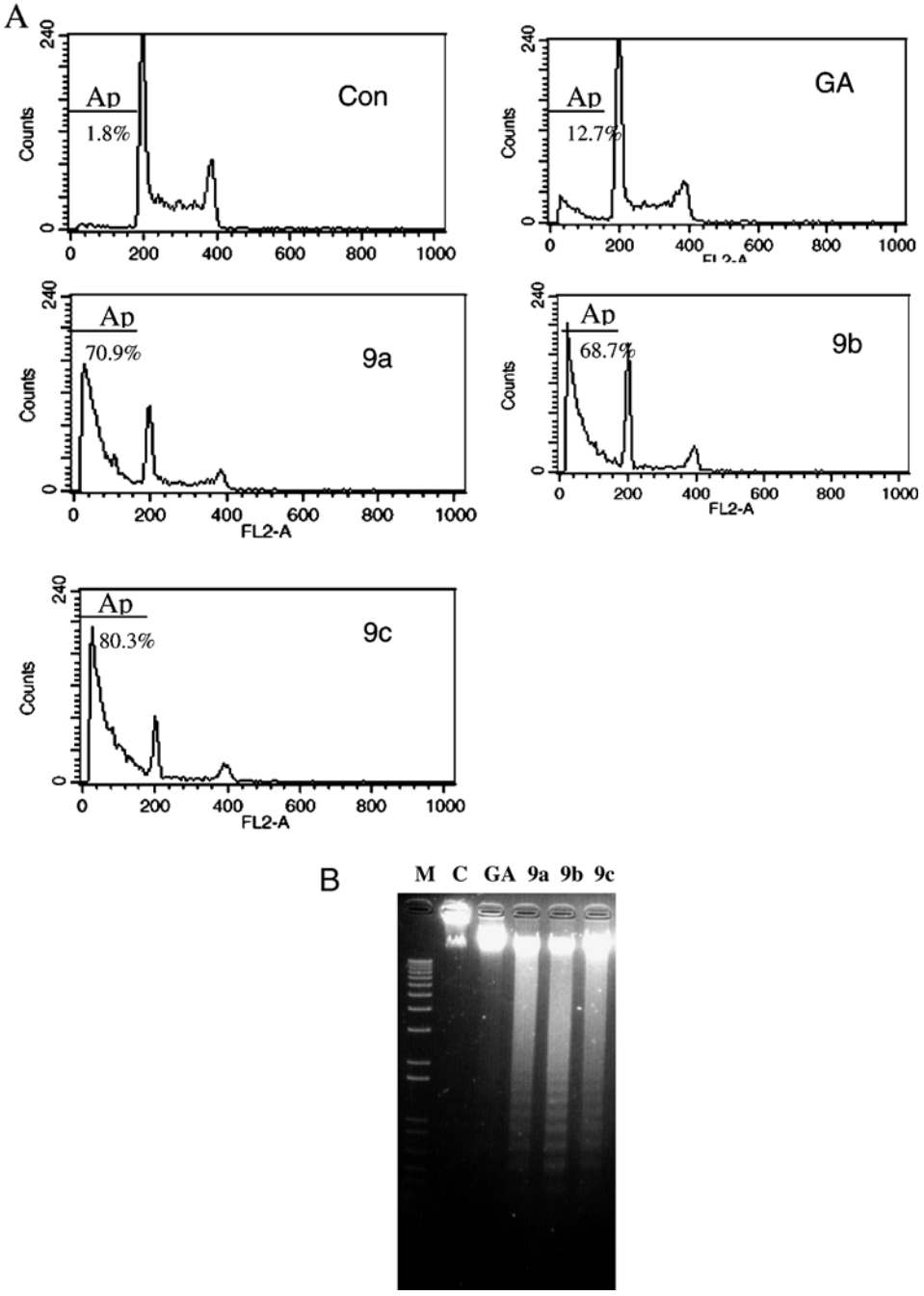


Figure 3. Apoptosis induction determined by FACS and DNA fragmentation. (A) FACS analysis of apoptotic cells. HL-60 cells were treated with the indicated compounds at 80 μM for 6 h. Apoptotic cells were determined after propidium iodide staining. (B) DNA fragmentation. HL-60 cells were treated with the indicated compounds at 80 μM for 6 h and the fragmented DNA was visualized as described in Section 3. Ap, apoptotic cells.

^{1}H NMR ($CDCl_3$): δ (ppm) 5.76 (1H, s, H-12), 3.74–3.72 (1H, m), 2.97–2.94 (1H, m), 2.61 (1H, s, H-9), 2.45–2.39 (1H, m, 18-H), 1.38, 1.32, 1.27, 1.23, 1.17, 1.11, 0.86 (s, $CH_3 \times 7$); ^{13}C NMR ($CDCl_3$): δ (ppm) 217.4 (C-3), 199.7 (C-11), 182.0 (C-29), 169.9 (C-13), 128.4 (C-12), 61.0 (C-9); LC–MS: 469.8 $(M+H)^+$, 491.6 $(M+Na)^+$.

3.2. 3-Hydroxyimino-11-oxo-olean-12-ene-29-oic acid (3)

A mixture of compound **2** (1.84 g, 3.9 mmol) with $NH_2OH{\cdot}HCl$ (0.81 g, 11.6 mol) and Py (10 mL) was refluxed for 4 h, cooled and poured into ice-water (10 mL), acidified by concentrated HCl to pH2-3, filtered, and dried. By purification on a silica gel column with cyclohexane: acetone (v/v) = 5:1, the oxime **3** was obtained as a white solid (1.69 g, yield: 89%). R_f value 0.18, mp: 290–293 °C, purity: 97.6%. IR (KBr): 3391, 2957, 1663, 1460, 1387, 1229, 955 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ (ppm) 12.19 (1H, s, COOH), 10.28 (1H, s, N–OH), 5.43 (1H, s, H-12), 2.88–2.83 (1H, m), 2.65–2.60 (1H, m), 2.40 (1H, s, H-9), 1.35, 1.14, 1.11, 1.10, 1.07, 0.99, 0.77 (s, $CH_3 \times 7$); ^{13}C NMR (DMSO-d_6): δ (ppm) 198.9 (C-11), 177.9 (C-29), 170.1 (C-13), 163.3 (C-3), 127.4 (C-12), 60.7 (C-9); LC–MS: 484.7 $(M+H)^+$, 506.7 $(M+Na)^+$.

3.3. General procedure for the preparation of 3-acyloxyimino-18β-glycyrrhetinic acid (4a, 4b)

To a solution of oxime **3** (0.4 mmol) in Py (2 mL), anhydride (0.6 mmol) was added dropwise in an ice-salt bath and then catalytic qualitative DMAP was added. The mixture was refluxed for 4–6 h, cooled and poured into ice-water, acidified to pH 2–3 with concentrated HCl, filtered, and dried. The crude was purified on a silica gel column with $CHCl_3$/MeOH (v/v) = 200:1 to yield a white solid.

3.3.1. 3-(*N*-Acetoxyimino)-11-oxo-olean-12-ene-29-oic acid (4a). Yield: 70%. R_f value 0.32, mp: 226-229 °C, purity: 95.1%. IR (KBr): 3284, 2964, 1736, 1656, 1464, 1388, 1366, 1209, 1153, 927, 870 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.73 (1H, s, H-12), 2.94–2.85 (2H, m), 2.40 (1H, s, H-9), 2.18 (3H, s, CH_3CO), 1.16 (6H, s, $CH_3 \times 2$) 1.36, 1.28, 1.25, 1.23, 0.85 (s, $CH_3 \times 5$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.9 (C-11), 181.3 (C-29), 174.6 (CH_3CO), 169.9 (C-13), 169.6 (C-3), 128.4 (C-12), 61.3 (C-9); LC–MS: 548.6 $(M+Na)^+$; HR-MS: *m/z*, calcd, $C_{32}H_{47}NO_5$ (M^+) 525.3454. Found: 525.3460.

3.3.2. 3-(*N*-Propionyloxyimino)-11-oxo-olean-12-ene-29-oic acid (4b). Yield: 73%. R_f value 0.31, mp: 145–146 °C, purity: 96.2%. IR (KBr): 3429, 2953, 1736, 1660, 1463, 1387, 1208, 1152, 922, 868 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.73 (1H, s, H-12), 2.94–2.89 (2H, m), 2.50–2.42 (2H, q, J = 22.7 Hz, CH_3CH_2CO–), 2.40 (1H, s, H-9), 1.36, 1.28, 1.25, 1.23, 1.21, 1.16, 0.85 (s, $CH_3 \times 7$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.9 (C-11), 181.6 (C-29), 174.7 (CH_3CH_2CO), 169.9 (C-13), 169.6 (C-3), 128.3 (C-12), 61.3 (C-9); LC–MS: 562.7 $(M+Na)^+$; HR-MS: *m/z*, calcd, $C_{33}H_{49}NO_5$ (M^+) 539.3611. Found: 539.3613.

3.4. General procedure for production of 3-oximino-18β-glycyrrhetinic acid ester (5a–c)

To a solution of compound **3** (0.48 mmol) in THF (2 mL), KOH (1.50 mmol) and CH_3I, CH_3CH_2Br, or $CH_3CH_2CH_2Br$ (6.7 mmol) were added and refluxed for 1–5 h. The reaction mixture was cooled and filtered. The filtrate was evaporated to dryness under reduced pressure. The crude was purified on a silica gel column with $CHCl_3$/MeOH (v/v) = 60:1 to yield a white solid.

3.4.1. Methyl 3-oximino-11-oxo-olean-12-ene-29-oate (5a). Yield: 82%. R_f value 0.43, mp: 270–272 °C, purity: 96.0%. IR (KBr): 3271, 2932, 1728, 1657, 1618, 1464, 1387, 1318, 1217, 1154, 1087, 941, 741 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.69 (1H, s, H-12), 3.69 (3H, s, –$COOCH_3$), 3.08–2.86 (2H, m), 2.38 (1H, s, H-9), 1.15 (6H, s, $CH_3 \times 2$), 1.35, 1.25, 1.18, 1.09, 0.81 (s, $CH_3 \times 5$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.8 (C-11), 176.9 (C-29), 169.4 (C-13), 167.0 (C-3), 128.4 (C-12), 61.3 (C-9); LC–MS: 498.6 $(M+H)^+$, 520.5 $(M+Na)^+$.

3.4.2. Ethyl 3-oximino-11-oxo-olean-12-ene-29-oate (5b). Yield: 86%. R_f value 0.43, mp: 259–261 °C, purity: 98.3%. IR (KBr): 3420, 2974, 1702, 1657, 1616, 1468, 1417, 1386, 1320, 1220, 1177, 1157, 1091, 922, 641 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.66 (1H, s, H-12), 4.18–4.14 (2H, q, J = 11.0 Hz, –OCH_2CH_3), 3.09–2.85 (2H, m), 2.38 (1H, s, H-9), 1.35, 1.26, 1.18, 1.15, 1.14, 1.08, 0.81 (s, $CH_3 \times 7$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.8 (C-11), 176.4 (C-29), 169.6 (C-13), 167.1 (C-3), 128.4 (C-12), 61.3 (CH_3CH_2O–), 60.3 (C-9); LC–MS: 512.6 $(M+H)^+$; HR-MS: *m/z*, calcd, $C_{32}H_{49}NO_4$ (M^+) 511.3662. Found: 511.3674.

3.4.3. *n*-Propyl 3-oximino-11-oxo-olean-12-ene-29-oate (5c). Yield: 85%. R_f value 0.46, mp: 252–253 °C, purity: 95.3%. IR (KBr): 3435, 2971, 1704, 1658, 1465, 1418, 1386, 1319, 1218, 1173, 1150, 1088, 921, 642 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.66 (1H, s, H-12), 4.09–4.03 (2H, q, J = 19.1 Hz, –OCH_2–), 3.09–2.87 (2H, m), 2.38 (1H, s, H-9), 1.15 (6H, s, $CH_3 \times 2$), 1.35, 1.26, 1.17, 1.08, 0.81 (s, $CH_3 \times 5$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.8 (C-11), 176.5 (C-29), 169.5 (C-13), 167.0 (C-3), 128.4 (C-12), 66.0 (–CH_2O–), 61.3 (C-9); LC–MS: 526.6 $(M+H)^+$; HR-MS: *m/z*, calcd, $C_{33}H_{51}NO_4$ (M^+) 525.3818. Found: 525.3813.

3.5. General procedure for the synthesis of 3-(alkoxyimino)-18β-glycyrrhetinic acid ester (6a–c)

To a solution of compound **3** (0.33 mmol) in THF (2 mL), KOH (1.35 mmol), CH_3I, CH_3CH_2Br, or $CH_3CH_2CH_2Br$ (1.2 mmol) were added and refluxed for 2–24 h. The mixture was cooled and filtered. The filtrate was evaporated to dryness under reduced pressure. The crude was purified on a silica gel column with cyclohexane–acetone to yield a white solid.

3.5.1. Methyl 3-(methoxyimino)-11-oxo-olean-12-ene-29-oate (6a). Yield: 83%. R_f value 0.66, mp: 257–260 °C, purity: 100.0%. IR (KBr): 3428, 2953, 1724, 1656, 1465, 1387, 1319, 1218, 1158, 1052, 882 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.68 (1H, s, H-12), 3.82 (3H, s, CH_3ON=), 3.69 (3H, s, –$COOCH_3$), 2.97–2.84 (2H, m), 2.38 (1H, s, H-9), 1.15 (6H, s, $CH_3 \times 2$), 1.34, 1.24, 1.17, 1.06, 0.81 (s, $CH_3 \times 5$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.9 (C-11), 176.9 (C-29), 169.4 (C-13), 165.8 (C-3), 128.5 (C-12), 61.3 (CH_3ON=), 61.0 (C-9); LC–MS: 512.3 $(M+H)^+$; HR-MS: *m/z*, calcd, $C_{32}H_{49}NO_4$ (M^+) 511.3662. Found: 511.3658.

3.5.2. Ethyl 3-(ethoxyimino)-11-oxo-olean-12-ene-29-oate (6b). Yield: 79%. R_f value 0.68, mp: 174-176 °C, purity: 100.0%. IR (KBr): 3420, 2982, 1722, 1656, 1463, 1386, 1317, 1217, 1154, 1090, 1056, 914, 879, 768 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.66 (1H, s, H-12), 4.15–4.10 (2H, q, J = 14.7 Hz, –$COOCH_2CH_3$), 4.09–4.03 (2H, q, J = 17.9 Hz, CH_3CH_2ON=), 2.97–2.78 (2H, m), 2.38 (1H, s, H-9), 1.15–1.14 (6H, $CH_3 \times 2$), 1.35, 1.26, 1.18, 1.07, 0.81 (s, $CH_3 \times 5$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.9 (C-11), 176.3 (C-29), 169.5 (C-13), 165.4 (C-3), 128.4 (C-12), 68.6 (CH_3CH_2ON=), 61.3 (–$COOCH_2CH_3$), 60.3 (C-9); LC–MS: 540.3 $(M+H)^+$, 562.4 $(M+Na)^+$; HR-MS: *m/z*, calcd, $C_{34}H_{53}NO_4$ (M^+) 539.3975. Found: 539.3968.

3.5.3. *n*-Propyl 3-(*n*-propoxyimino)-11-oxo-olean-12-ene-29-oate (6c). Yield: 75%. R_f value 0.72, mp: 113–114 °C, purity: 98.8%. IR (KBr): 3434, 2964, 2873, 1727, 1659,

1463, 1214, 1170, 1086, 1055, 985, 877 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.66 (1H, s, H-12), 4.09–4.03 (2H, dd, J = 19.0 Hz, –$COOCH_2$–), 3.99–3.94 (2H, t, J = 13.4 Hz, –CH_2ON=), 2.97–2.80 (2H, m), 2.38 (1H, s, H-9), 1.15 (6H, s, $CH_3 \times 2$), 1.35, 1.23, 1.17, 1.06, 0.75 (s, $CH_3 \times 5$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.8 (C-11), 176.4 (C-29), 169.4 (C-13), 165.2 (C-3), 128.4 (C-12), 74.7 (–CH_2ON=), 66.0 (–$COOCH_2$–), 61.3 (C-9); LC–MS: 568.8 $(M+H)^+$, 590.9 $(M+Na)^+$; HR-MS: m/z, calcd, $C_{36}H_{57}NO_4$ (M^+) 567.4288. Found: 567.4287.

3.5.4. Methyl 3,11-dioxo-olean-12-ene-29-oate (7). A solution of acid **3** in anhydrous methanol was added with concentrated H_2SO_4 in drops, refluxed 48 h, cooled, and filtered. Washed with H_2O to pH 5–6 and dried to obtain ester **7** as a white crystal. Yield: 79%. R_f value 0.52, mp: 245–246 °C, purity: 95.0%. IR (KBr): 3428, 2960, 1725, 1706, 1655, 1457, 1387, 1219, 997 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.71 (1H, s, H-12), 3.70 (3H, s, –$COOCH_3$), 2.99–2.94 (1H, m), 2.65–2.61 (1H, m), 2.44 (1H, s, H-9), 1.37, 1.27, 1.17, 1.15, 1.11, 1.07, 0.82 (s, $CH_3 \times 7$); ^{13}C NMR ($CDCl_3$): δ (ppm) 217.2 (C-3), 199.5 (C-11), 176.9 (C-29), 169.7 (C-13), 128.4 (C-12), 61.0 (C-9); LC–MS: 483.7 $(M+H)^+$, 505.6 $(M+Na)^+$.

3.6. General procedure for the synthesis of methyl 3-(alkoxyimino)-11-oxo-olean-12-ene-29-oate (8a–b)

The process was same as for the preparation of compound **6**, starting with oxime **5a** and CH_3CH_2Br or $CH_3CH_2CH_2Br$, to yield compound **8a** or **8b,** respectively. The crude material was purified on a silica gel column with $CHCl_3$/MeOH to produce the white solid.

3.6.1. Methyl 3-(ethoxyimino)-11-oxo-olean-12-ene-29-oate (8a). Yield: 89%. R_f value 0.66, mp: 222–224 °C, purity: 96.7%. IR (KBr): 3431, 1727, 1655, 1462, 1385, 1217, 1157, 1089, 1055, 918, 880 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.68 (1H, s, H-12), 4.10–4.03 (2H, dd, J = 21.0 Hz, CH_3CH_2ON=), 3.69 (3H, s, –$COOCH_3$), 2.99–2.91 (1H, m), 2.85–2.77 (1H, m), 2.38 (1H, s, H-9), 1.15 (6H, s, $CH_3 \times 2$), 1.35, 1.23, 1.18, 1.07, 0.81 (s, $CH_3 \times 5$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.9 (C-11), 176.9 (C-29), 169.3 (C-13), 165.3 (C-3), 128.5 (C-12), 68.6 (CH_3CH_2ON=), 61.3 (C-9); GC–MS (m/z): 525 (M^+, 13.91), 480 (100.00), 181 (32.17), 135 (60.08), 105 (27.42), 95 (35.13), 55 (41.04), 43 (37.37), 41 (45.56); HR-MS: m/z, calcd, $C_{33}H_{51}NO_4$ (M^+) 525.3818. Found: 525.3818.

3.6.2. Methyl 3-(propoxyimino)-11-oxo-olean-12-ene-29-oate (8b). Yield: 88%. R_f value 0.70, mp: 159-160 °C, purity: 97.0%. IR (KBr): 3437, 2964, 2872, 1731, 1658, 1461, 1386, 1217, 1155, 1055, 986 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.68 (1H, s, H-12), 4.01–3.96 (2H, t, J = 13.3 Hz, –CH_2ON=), 3.69 (3H, s, –$COOCH_3$), 2.98–2.93 (1H, m), 2.83–2.80 (1H, m), 2.38 (1H, s, H-9), 0.95–0.90 (3H, t, J = 14.8 Hz, $CH_3CH_2CH_2ON$=), 1.15 (6H, s, $CH_3 \times 2$), 1.35, 1.23, 1.18, 1.07, 0.81 (s, $CH_3 \times 5$); ^{13}C NMR ($CDCl_3$): δ (ppm) 199.9 (C-11), 176.9 (C-29), 169.4 (C-13), 165.3 (C-3), 128.5 (C-12), 76.6 (–CH_2ON=), 61.3 (C-9); GC–MS (m/z): 539 (M^+, 12.62), 480 (100.00), 135 (37.09), 95 (22.56), 55 (24.17), 43 (34.57), 41 (37.01); HR-MS: m/z, calcd, $C_{34}H_{53}NO_4$ (M^+) 539.3975. Found: 539.3956.

3.7. Synthesis of 3-(alkoxyimino)-18β-glycyrrhetinic acid (9a–c)

3.7.1. 3-(Methoxyimino)-11-oxo-olean-12-ene-29-oic acid (9a). The process was as same as for the preparation of compound **3** using compound **2** and NH_2OCH_3·HCl as starting materials. Yield: 85%. R_f value 0.35, mp: 259–261 °C, purity: 100.0%. IR (KBr): 3416, 2945, 1728, 1656, 1456, 1386, 1203, 1140, 1052, 889 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.72 (1H, s, H-12), 3.82 (3H, s, CH_3ON=), 2.99–2.93 (1H, m), 2.84–2.81 (1H, m), 2.38 (1H, s, H-9), 1.35, 1.24, 1.23, 1.18, 1.16, 1.07, 0.85 (s, $CH_3 \times 7$); ^{13}C NMR ($CDCl_3$): δ (ppm) 200.2 (C-11), 181.5 (C-29), 169.4 (C-13), 165.8 (C-3), 128.4 (C-12), 61.3 (CH_3ON=), 61.0 (C-9); GC–MS (m/z): 497 (M^+, 11.12), 466 (100.00), 262 (22.49), 135 (56.17), 119 (25.99), 95 (38.53), 55 (46.56), 43 (51.09), 41 (63.86); HR-MS: m/z, calcd, $C_{31}H_{47}NO_4$ (M^+) 497.3505. Found: 497.3515.

3.7.2. 3-(Ethoxyimino)-11-oxo-olean-12-ene-29-oic acid (9b). To a solution of compound **8a** (0.53 g, 1.0 mmol) in dry DMF (80 mL), dried LiI (3.52 g, 26.2 mmol) was added rapidly, then refluxed for 2 h under N_2 atmosphere. The reaction solution was cooled and poured into water (100 mL), acidified by 3% HCl to pH 3, filtered, and dried. The crude was purified on a silica gel column with $CHCl_3$/MeOH (v/v) = 200:1 to yield the acid **9b** as a white solid (0.15 g, yield: 30%). R_f value 0.61, mp: 221–223 °C, purity: 100.0%. IR (KBr): 3426, 2974, 1702, 1659, 1462, 1386, 1053, 947, 916, 879 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.72 (1H, s, H-12), 4.09–4.02 (2H, q, J = 21.0 Hz, CH_3CH_2ON=), 2.97–2.78 (2H, m), 2.39 (1H, s, H-9), 1.35, 1.24, 1.23, 1.18, 1.16, 1.07, 0.85 (s, $CH_3 \times 7$); ^{13}C NMR ($CDCl_3$): δ (ppm) 200.1 (C-11), 181.8 (C-29), 169.5 (C-13), 165.3 (C-3), 128.5 (C-12), 68.6 (CH_3CH_2ON=), 61.3 (C-9); GC–MS (m/z): 511 (M^+, 14.56), 466 (100.00), 262 (20.42), 135 (71.20), 55 (61.63), 43 (33.34), 41 (24.81); HR-MS: m/z, calcd, $C_{32}H_{49}NO_4$ (M^+) 511.3662. Found: 511.3663.

3.7.3. 3-(*n*-Propoxyimino)-11-oxo-olean-12-ene-29-oic acid (9c). The process was performed as done in the preparation of **9b** using **8b** instead of **8a** to afford acid **9c**. Yield: 28%. R_f value 0.41, mp: 205–207 °C, purity: 97.1%. IR (KBr): 3427, 2962, 2871, 1702, 1661, 1460, 1386, 1208, 1176, 1054, 984, 917, 877 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) 5.73 (1H, s, H-12), 3.99–3.95 (2H, t, J = 13.5 Hz, –CH_2ON=), 2.98–2.83 (2H, m), 2.39 (1H, s, H-9), 1.36, 1.23, 1.23, 1.18, 1.16, 1.07, 0.85 (s, $CH_3 \times 7$), 0.95–0.90 (3H, t, J = 14.8, Hz, $CH_3CH_2CH_2ON$=); ^{13}C NMR ($CDCl_3$): δ (ppm) 200.2 (C-11), 181.6 (C-29), 169.5 (C-13), 165.4 (C-3), 128.5 (C-12), 74.8 (–CH_2ON=), 61.3 (C-9); GC–MS (m/z): 525 (M^+, 9.85), 467 (35.18), 466 (100.00), 262 (18.39), 135 (62.53), 105 (37.58), 95 (48.18), 69 (52.32), 55 (49.04); HR-MS: m/z, calcd, $C_{33}H_{51}NO_4$ (M^+) 525.3818. Found: 525.3806.

3.8. Biological activity assays

3.8.1. Cells. HL-60 cells were cultured in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS).

3.8.2. Antiproliferative effect. Cells were seeded at a density of 1×10^5cells/mL and incubated with various concentrations of the tested compounds for 3 days. The total cell number in each group was determined. The antiproliferative ability was calculated and expressed as the ratio of the cell number in treated group to that of the untreated group. The concentration (GI_{50}) which inhibited half of the cell growth was calculated.

3.8.3. Apoptosis induction. Apoptotic cells were determined by morphology and fluorescence-activated cell sorting (FACS) analysis with propidium iodide (PI) as well as DNA fragmentation.[13] For morphologic evaluation, cells were stained with acridine orange (AO) and ethidium bromide (EB), and assessed by fluorescence microscopy. Briefly, 1 μL of stock solution containing 100 μg/mL AO and 100 μg/mL EB was added to 25 μL of cell suspension. EB-negative cells with nuclear shrinkage, blebbing, and apoptotic bodies were counted as apoptotic cells. For FACS analysis with PI staining, cells were fixed with ice-cold 70% ethanol at a cell density of 1×10^6/mL and treated with 1 mg/mL RNase for 30 min at 37 °C. PI was then added to the solution at a final concentration of 50 μg/mL and the DNA content was quantitated by a flow cytometry. Cells in subG1 phase were valuated as apoptotic cells. For DNA fragmentation assay, cells were harvested by centrifugation and cellular DNA was extracted with a PUREGENE DNA isolation kit (Gentra System, Minneapolis, MN). Electrophoresis of 5 μg DNA was performed in 1% agarose gel in 40 mM Tris–acetate buffer (pH 7.4), at 50 V. After electrophoresis, DNA was visualized by EB staining.

Acknowledgment

This work was partly supported by Joint Research Fund for Overseas Chinese Young Scholars of National Natural Science Foundation of China (30328030).

References and notes

1. Li, X. F.; Meng, F. M.; Pan, F. Sh.; Lin, W.; Lan, Zh. F.; Wang, F. L.; Luo, Q.; Wang, Y. *Chin. Pharmacol. Bull.* **1990**, *6*, 105–108.
2. Hayashi, H.; Fukui, H.; Tabata, M. *Plant Cell Rep.* **1988**, *7*, 508–511.
3. Haruo, T.; Sumie, K.; Hoyoku, N. *J. Kyoto Pref. Univ. Med.* **1985**, *94*, 999–1004.
4. Nishino, H.; Kitagawa, K.; Iwashima, A. *Carcinogenesis* **1984**, *5*, 1529–1530.
5. Huang, W.; Huang, J. Q.; Zhang, D. F.; Liao, Z. Q. *Chin. J. Integr. Tradit. West. Med. Liver Dis.* **2003**, *13*, 148–150.
6. Luo, H. L.; Zhang, Z. L.; Wu, Q. N.; Huang, M. S.; Huang, W.; Zhang, D. F.; Yang, F. Y. *Chin.—Ger. J. Clin. Oncol.* **2004**, *3*, 137–140.
7. Hibasami, H.; Iwase, H.; Yoshioka, K.; Takahashi, H. *Int. J. Mol. Med.* **2006**, *17*, 215–219.
8. Mulzer, J.; Angermann, A.; Schubert, B.; Seilz, C. *J. Org. Chem.* **1986**, *51*, 5294–5299.
9. Ma, Ch.; Nakamura, N.; Hattori, M. *Chem. Pharm. Bull.* **2000**, *48*, 1681–1688.
10. Hofle, G.; Sleglich, W. *Synthesis* **1972**, *11*, 619–621.
11. Welch, S.; Wong, P. *Tetrahedron Lett.* **1972**, *19*, 1853–1856.
12. Dean, P. D. G. *J. Chem. Soc.* **1965**, *11–12*, 6655.
13. Jing, Y. K.; Dai, J.; Chalmers-Redman, R. M. E.; Tatton, W. G.; Waxman, S. *Blood* **1999**, *94*, 2102–2111.