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Synthesis, anti-inflammatory, and antioxidant activities of 18β-glycyrrhetinic acid derivatives as chemical mediators and xanthine oxidase inhibitors

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ABSTRACT

Twenty 18β-glycyrrhetic acid (18β-GA) derivatives **2**–**21** including 13 new 18β-GA derivatives were synthesized and evaluated as anti-inflammatory and antioxidant agents. Compounds **7** and **20** with a 3,4-*seco*-structure and compound **6** with a lactone moiety showed potent inhibitory effect on superoxide anion generation in rat neutrophils response to fMLP/CB and PMA, respectively. Compound **6** with a lactone moiety revealed stronger inhibitory effect on XO activity than those of compounds **13** and **14** with a 3,4-*seco*-struture. Compound **14**, a 30-isoproylcarbamoyl *seco*-compound exhibited potent inhibitory effect on NO accumulation and iNOS protein expression while compounds **3**, **10**, **13**, **15**, **17**, and **21** revealed potent inhibitory effect on tumor necrosis factor-α (TNF-α) formation in RAW 264.7 cells in response to lipopolysaccharide (LPS). The cleavage of ring A of **3** attenuated the inhibitory effect on TNF-α formation in RAW 264.7 cells in response to LPS except for **17**. The present results suggested these compounds were potential to be served as anti-inflammatory and antioxidant agents.

1. Introduction

Mast cells and neutrophils, stimulated with various inducers may contribute to inflammatory disorders. Macrophages are important in nonspecific host resistant to microbial pathogens and serve as central regulators of the specific immune response.[1] Following the activation of macrophage, TNF-α and NO were generated in response to LPS.[2,3] NO plays a central role in macrophage-induced cytotoxicity and excess NO may contribute to the pathophysiology of septic shock.[4] TNF-α is an important pro-inflammatory cytokine with immune and inflammatory functions and generally considered as principal mediator of septic shock. The overexpression of TNF-α is associated with autoimmune diseases such as rheumatoid arthritis and Crohn's disease.[5]

Reactive oxygen species (ROS) is associated in pathological events including inflammation, metabolic disorders, cellular aging, reperfusion damages, artherosclerosis, and carcinogenesis.[6] ROS induce programmed cell death or necrosis, induce or suppress the expression of many genes, and activate cell signaling cascades.[7] The oxidative damage of DNA in the development of certain cancers and lipid oxidative damage in the occurrence and progression of vascular disease are also associated with ROS.[6]

XO is a key enzyme that catalyzes the oxidation of xanthine and hypoxanthine into uric acid and plays a vital role in causing hyperuricemia and gaut.[8]

Synthetic oleanane and ursane with various enone functionalities in ring A were reported to have anti-inflammatory effect.[9] A several of natural triterpenoids, such as 18β-GA, an oleanane triterpenoid, showed remarkably active against the edema produced by 12-*O*-tetradecanoylphorbol acetate (TPA).[10] Natural oleanolic acid and ursolic acid possessed nonenzymatic antioxidative and antiglycative properties.[11] Here, we report the preparation of a series of 18β-GA derivatives from 18β-GA (**1**). The structure modification was focused on cleavage of ring A and introduction of various ester groups at C-3 and C-30 for biological evaluation. The structure and biological activity of 18β-GA derivatives relationship also reported in the present paper.

2. Results and discussion

2.1. Chemistry

Compounds **2**–**21** were synthesized as depicted in Schemes 1–3. Starting material, 18β-GA (**1**) was oxidized to 3-keto compound (**2**)

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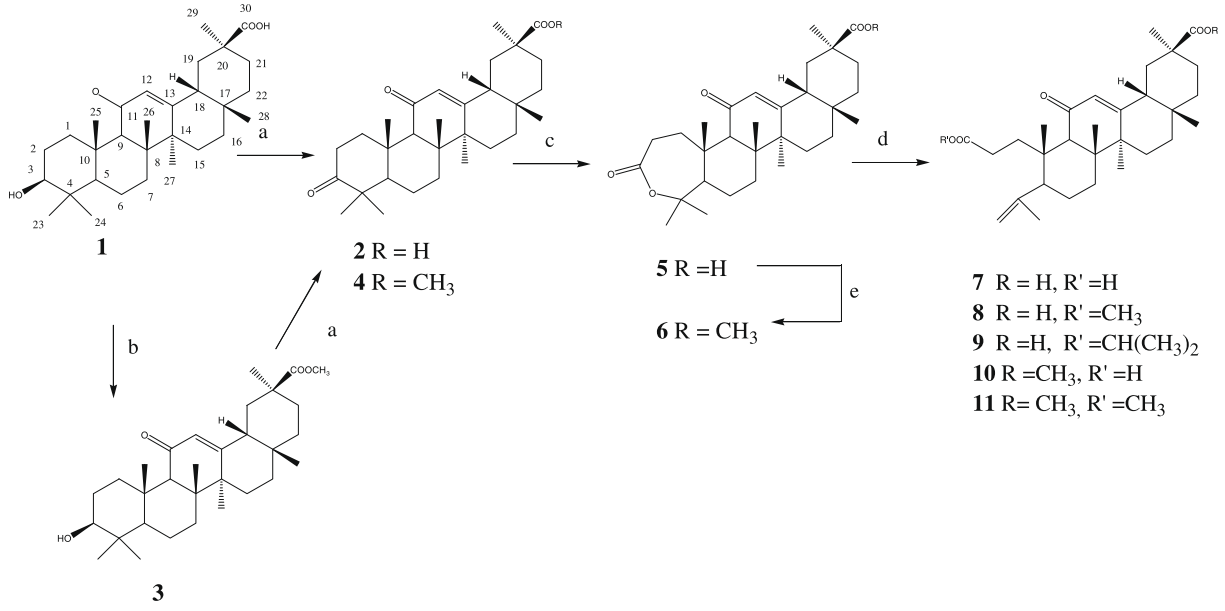
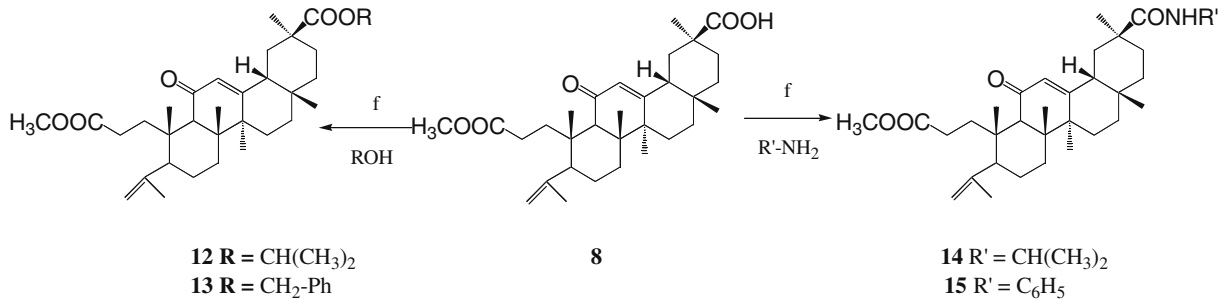


Scheme 1. Reagents and conditions: (a) CrO_3, DMF, rt, 12 h; (b) CH_3OH, H_2SO_4, reflux, 48 h; (c) *m*-CPBA, CH_2Cl_2, rt, 12 h; (d) appropriate alcohol, *p*-TSA, rt, 6–8 h; (e) EDCI, 4-dimethylaminopyridine (DMAP), CH_2Cl_2, MeOH, rt, 6–8 h.



Scheme 2. Reagents and conditions: (f) EDC, DMAP, CH_2Cl_2, ROH or R′-NH_2, rt, 24 h.

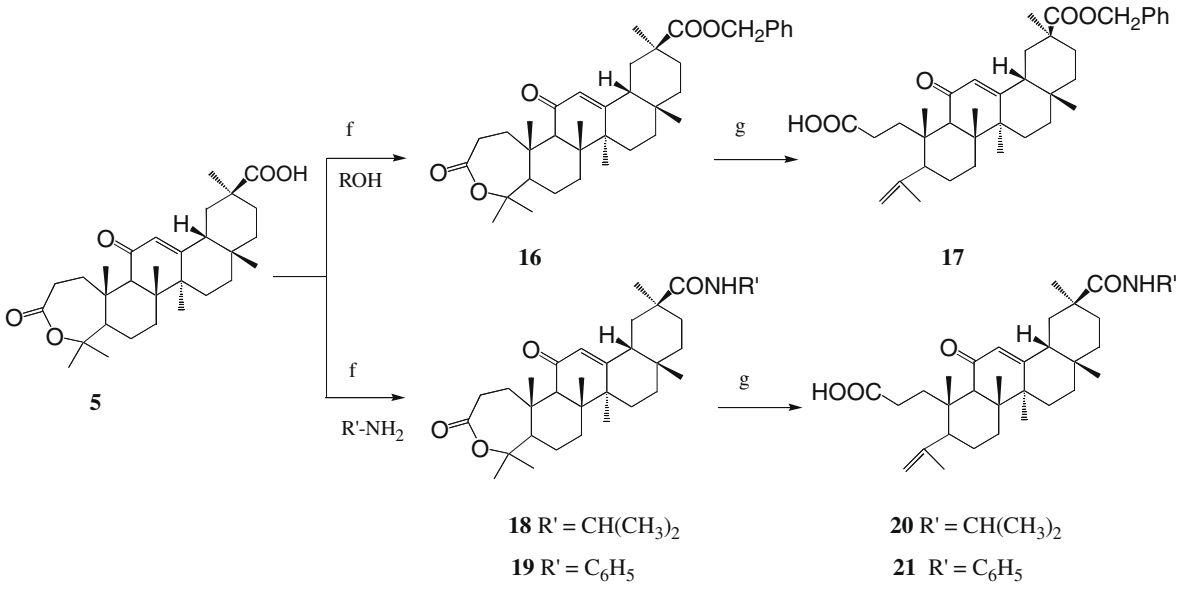
using CrO_3 in DMF.[12] The treatment of 3-oxo-derivative **2** with *m*-chloroperbenzoic acid (*m*-CPBA) afforded lactone **5**.[13] Methylation of 18β-GA with excess of MeOH in the presence of H_2SO_4 as catalyst to yield **3**. The oxidation of C-3–OH of **3** to give **4**. Compound **4** was further treated with *m*-CPBA to give 30-methyl ester lactone (**6**). This compound was synthesized in a different synthetic route, by the esterification of lactone (**5**) used 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) as the activating agent in methanol.[14] The lactone ring of **5** or **6** was cleaved by treatment of *p*-toluenesulfonic acid (*p*-TSA) in appropriate solvent to yield products **7**–**11**. Treatment of *seco*-methyl ester **8** with isopropyl alcohol and benzyl alcohol in the presence of EDCI and 4-dimethylaminopyridine (DMAP) provided the corresponding 30-ester compounds **12** and **13**, respectively. Similar treatment of **8** with amines, isopropylamine, and aniline afforded 30-amide *seco*-compounds **14** and **15**, respectively (Scheme 2). Compounds **16**, **18**, and **19** were prepared from lactone (**5**) which was the common starting material for synthesis of these compounds. Acid group at C-30 was modified to ester and amide, respectively, followed by ring cleavage in the presence of *p*-TSA in methylene chloride yielded C-30 substituted *seco*-acids **17**, **20**, and **21** (Scheme 3).

The known 18β-GA derivatives **2**–**4**, **5**, **7**, **8**, **11**, and **22** were identified by spectroscopic data and compared with those of data reported in literatures.[14–17] The new 18β-GA derivatives were characterized by various spectroscopic methods (Table 1 and Section 4) and compared with data reported in literatures.[14–17]

2.2. Biological results and discussion

The degranulation of mast cells and neutrophils, and macrophages contribute to inflammatory disorder. Combining potent inhibition of chemical mediators released from mast cells, neutrophils, and macrophages would suggest a promising anti-inflammatory agents.[18] The anti-inflammatory effects of **1**–**20** were studied in vitro for their inhibitory effects on chemical mediators released from mast cells, neutrophils, and macrophages. Synthetic compounds **1**–**20** did not show significant inhibitory effects on mast cell and neutrophil degranulation.

FMLP (0.3 μM)/CB (5 μg/mL) or PMA (3 nM) stimulated superoxide anion generation in rat neutrophils. As shown in Figures 1 and 2, positive control (diphenylene iodonium chloride, DPI), **2**, **7**, and **20** and DPI, **6**, **13**, and **14** had potent and concentration-dependent inhibitory effects on fMLP/CB and PMA induced superoxide anion generation with IC_{50} values of 2.7 ± 2.0, 10.3 ± 5.2, 7.0 ± 2.0, and 9.8 ± 5.2 and 1.8 ± 0.4, 12.9 ± 1.8, 17.0 ± 1.5, and 15.6 ± 1.7 μM (Table 2), respectively while the other compounds had no significant inhibitory effects (data not shown). These results indicate that reduction of the carbonyl group at C-3 of **2** to **1** or introduction of a lipophilic alkyl group at C-30 of 18β-GA derivatives might attenuate their inhibitory effects on fMLP/CB-induced responses while introduction of a lipophilic group at C-30 of **5** or a benzylester moiety at C-30 such as **13** or an isopropylcarbamoyl group at C-30 such as **14** significantly enhance the inhibitory ef-



Scheme 3. Reagents and conditions: (f) EDC, DMAP, CH_2Cl_2, ROH or R′-NH_2, rt, 24 h; (g) CH_2Cl_2, *p*-TSA, rt, 24 h.

fects on PMA-induced responses. As shown in Figure 1 also reveals that cleavage of ring A of **2** to afford compounds with 3,4-*seco*-structure, such as **7**, enhances its inhibitory effect on fMLP/CB-induced responses. Introduction of a lipophilic moiety at C-30 or C-3 such as **14**, **15**, or **21** weakened the inhibitory activity on fMLP/CB-induced response but introduction of an isopropylcarbamoyl moiety at C-30 such as **20** enhances the inhibitory activity on response induced by same inducer (Fig. 1). As shown in Figure 2, lactone derivative from 18β-GA such as **6** displays stronger and concentration-dependent effect on inhibitory effect on PMA-induced response than those of **13** and **14**. The result indicated that cleavage of ring A of **6** and introduction of a side chain greater than methyl group at C-30 attenuate the inhibitory effect on PMA-induced response. Because fMLP and PMA activate NADPH oxidase to produce superoxide anion through different cellular signaling mechanism.[19] These 18β-GA derivatives indicated different inhibitory effect on fMLP/CB- and PMA-induced responses. These results supported that above observations.

Treatment of RAW 264.7 macrophage-like cells with LPS (1 μg/mL) for 24 h induced NO production as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the media based on Griess reaction.[20] As shown in Figure 3, LPS induced a significant increase of NO production and this effect was concentration dependently suppressed by positive control (*N*-(3-aminometyl)benzylacetamidine, 1400 W) and **14** with IC_{50} values of 1.5 ± 0.2 and 13.1 ± 5.0 μM (Table 2), respectively while compounds **2**, **11**, **12**, and **13** each at 30 μM indicated about 40% (Table 2) of inhibitory effect on NO accumulation in the media induced by LPS. The above observation shows that a lipophilic group substituted at C-3 or C-30 of 3,4-*seco*-type derivatives from 18β-GA might enhance the suppression of NO accumulations in the media induced by LPS except for compound **15**. To determine whether the inhibition of NO production in RAW 264.7 cells is attributed to the decrease of iNOS protein expression, Western blotting analysis was performed. Unstimulated cells expressed very low level of iNOS protein, whereas LPS 50 ng/mL induced a large amount of iNOS protein expression (Fig. 4). Compound **14** significantly inhibited the iNOS protein expression. Thus, the blockade of iNOS transcription has a critical role as evidenced from the parallelism of the inhibition of NO production and iNOS protein expression by **14**.

Effect on the generation of TNF-α was determined in RAW 264.7 cells stimulated with LPS.[2,3,21] As shown in Figure 5, positive control (genistein), **3**, **10**, **13**, **15**, **17**, and **21** strongly and concentration dependently inhibited TNF-α generation in LPS-stimulated RAW 264.7 cells with IC_{50} values of 26.5 ± 9.1, 1.3 ± 0.4, 26.1 ± 14.7, 13.7 ± 4.3, 15.5 ± 3.2, 2.3 ± 0.5, 27.7 ± 7.2 μM (Table 2), respectively. Among them the suppression of TNF-α generation in RAW 264.7 cells stimulated with LPS by these compounds except for **21** were stronger than that of positive control. The esterification of C-30 at 18β-GA enhances the suppressed effect on TNF-α generation while reduction of C-3–OH of 18β-GA or cleavage of ring A attenuates the inhibition of TNF-α generation on LPS-stimulated response. The esterification of C-30 at 3,4-*seco*-type derivatives from 18β-GA with benzyl alcohol strongly enhances the inhibitory effect on TNF-α generation while amidation with isopropylamine attenuate the inhibition of TNF-α generation on LPS-stimulated response.

ROS have been known to damage many biological macromolecules, with DNA being a significant target. The ability of 18β-GA derivatives to inhibit the DNA damage caused by $O_2^{-\cdot}$ [generated by xanthine (XA)/XO] was studied in vitro by agarose gel electrophoresis.[22] As shown in Figure 6, compounds **2**, **6**, **7**, **13**, **14**, and **20** revealed significant inhibitory effects on superoxide anion formation released from rat neutrophils activated by fMLP/CB or PMA and showed significant protective effects on oxidative DNA damage caused by $O_2^{-\cdot}$. The above results clearly revealed that these compounds may possess 1,1-diphenyl-2-picrylhydrazl (DPPH), a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecules,[22] scavenging activity or inhibitory effect on XO, an enzyme that catalyzed the oxidation of XA and hypoxanthine into uric acid,[8] activity. For determined the antioxidant activities of these compounds, the radical scavenging activities and XO inhibitory activities of these compounds were analyzed. As shown in Figure 7, compounds **6**, **13**, **14**, **22**, and allopurinol (positive control) significantly inhibit the XO activity in a concentration-dependent manner with IC_{50} values of 131.5 ± 2.7, 175.0 ± 0.9, 192.4 ± 2.7, 186.1 ± 1.0, and 2.0 ± 0.7 μM, respectively. Compounds **2**, **7**, and **20** weakly inhibit the XO activity (data not shown). All the compounds used for study on inhibitory effects on XO activity did not show DPPH scavenging activity.

The XO inhibitors **6**, **13**, and **14** also significantly suppressed the superoxide anion formation in rat neutrophils stimulated with PMA. This result showed the suppression of superoxide anion formation by these compound in rat neutrophils stimulated with PMA

Table 1
^{13}C NMR spectral data for compounds **6**, **9**, **10**, and **12–21**

Position	6	9	10	12	13	14	15	16	17	18	19	20	21
C-1	38.7	23.5	23.7	23.7	23.7	23.7	23.8	38.6	23.8	38.7	38.6	23.7	23.5
C-2	32.1	31.8	31.0	31.4	31.3	31.4	31.4	32.0	31.4	32.1	32.0	31.4	31.4
C-3	175.4	173.5	179.6	175.7	176.1	174.6[a]	173.9[a]	176.1	179.0	175.5	175.6	179.0	180.6
C-4	85.5	146.6	146.4	146.5	146.4	146.5	146.5	85.6	146.5	85.6	85.6	146.4	146.3
C-5	54.2	38.7	38.6	38.7	38.6	38.7	38.8	54.5	38.7	54.2	54.5	38.7	39.1
C-6	22.0	29.7	29.0	28.6	28.4	28.6	28.4	22.0	28.4	22.0	22.0	28.6	28.8
C-7	32.0	34.3	34.1	34.4	34.3	34.4	34.4	32.4	34.2	32.2	32.4	34.1	34.1
C-8	43.3	43.6	40.3	43.6	43.9	43.3	43.7	43.4	43.9	43.3	43.4	43.3	44.0
C-9	61.2	52.7	50.9	52.8	52.6	52.9	52.8	61.2	52.9	61.3	61.3	52.8	52.5
C-10	39.4	40.9	38.5	41.1	41.1	42.1	42.0	39.6	41.1	39.4	39.6	41.9	41.7
C-11	198.7	199.8	199.6	199.6	199.4	199.6	199.5	198.8	199.5	198.7	198.7	199.8	200.6
C-12	128.4	128.5	128.3	128.3	128.2	128.3	128.5	128.6	128.3	128.3	128.7	128.3	128.5
C-13	169.3	169.4	169.5	169.7	169.2	169.6	169.3	169.2	169.4	169.6	169.1	170.0	170.7
C-14	45.2	45.1	45.0	45.0	44.9	45.0	45.1	45.3	45.0	45.2	45.3	45.1	45.3
C-15	26.2	26.4	26.3	26.3	26.3	26.4	26.4	26.3	26.4	26.2	26.4	26.4	26.4
C-16	26.2	26.5	26.4	26.5	26.4	26.5	26.5	26.4	26.5	26.3	26.4	26.5	26.7
C-17	31.7	31.4	31.6	31.7	31.6	31.8	32.0	31.8	31.7	31.4	31.7	31.8	31.8
C-18	48.2	48.2	48.3	48.3	48.0	48.1	48.1	48.1	48.1	48.0	48.0	48.2	48.4
C-19	41.0	40.9	43.6	43.7	43.5	41.1	44.5	41.0	43.6	41.7	41.8	41.1	44.6
C-20	43.8	43.8	43.9	50.8	50.7	43.7	50.8	43.9	51.1	43.2	44.5	43.7	50.4
C-21	30.9	30.8	31.1	31.0	31.0	31.4	31.6	31.1	31.2	31.8	32.2	31.4	31.6
C-22	37.6	37.7	37.7	37.6	37.5	37.4	37.4	37.6	37.6	37.3	37.4	37.4	37.5
C-23	31.7	114.2	114.2	114.1	114.1	114.2	114.2	32.2	114.3	31.8	32.2	114.3	114.2
C-24	25.9	23.4	23.3	23.4	23.4	23.4	23.4	25.9	23.4	25.9	25.9	23.3	23.2
										S15			
C-25	17.4	19.5	19.4	19.5	19.4	19.5	19.5	17.4	19.4	17.4	17.4	19.5	19.4
C-26	18.0	18.6	18.6	18.6	18.5	18.6	18.6	18.2	18.6	18.0	18.2	18.6	18.5
C-27	23.0	23.8	23.4	23.3	23.2	23.2	23.3	23.1	23.3	23.0	23.1	23.2	23.1
C-28	28.2	28.5	28.5	29.2	29.1	29.5	29.4	28.4	28.9	29.3	29.3	29.4	29.7
C-29	28.1	28.4	28.2	28.2	28.1	29.2	29.2	28.2	28.3	28.5	28.4	29.1	29.1
C-30	176.7	181.8	176.9	174.2	174.2	174.3[a]	174.3[a]	175.6	176.2	174.5	173.9	174.8	174.5
OCH_3	51.6		51.7	51.5	51.4	51.5	51.5						
$OCH(CH_3)_2$		50.6		67.3									
$OCH(CH_3)_2$		21.8, 21.8		21.6, 21.8									
OCH_2					66.1			66.2	66.2				
1′					136.0		137.8	136.0	136.1		137.8		137.9
2′					128.3		120.1	128.2	128.2		120.2		121.4
3′					128.5		129.0	128.5	128.6		128.7		128.7
4′					128.3		124.4	128.3	128.4		124.4		124.4
5′					128.5		129.0	128.5	128.6		128.7		128.7
6′					128.3		120.1	128.2	128.2		120.2		121.4
$NH–CH(CH_3)_2$						50.8				41.0		50.8	
$NH–CH(CH_3)_2$						22.7				22.6		22.6	
						22.9				22.7		22.9	

[a] Signals may be interchanged in the same column.

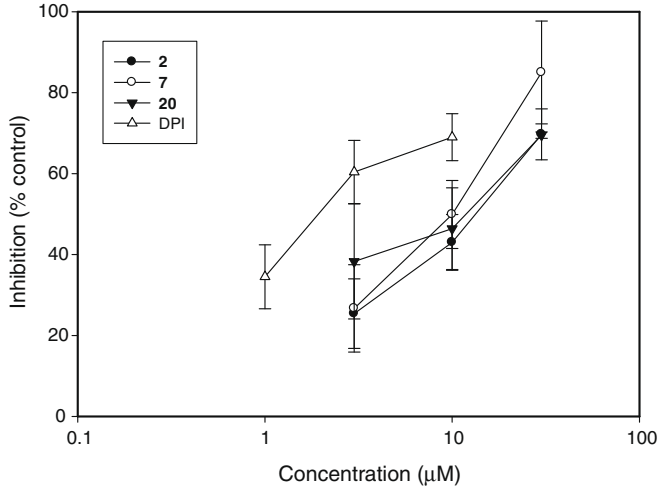


Figure 1. The inhibitory effects of **2**, **7**, **20**, and DPI on superoxide anion generation in rat neutrophils stimulated with fMLP/CB. Data are presented as means ± SD (n = 3–6). Diphenylene iodonium chloride (DPI) was used as positive control.

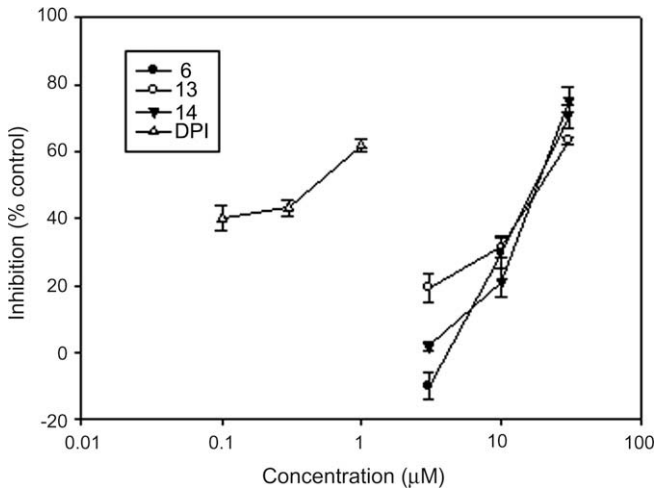


Figure 2. The inhibitory effects of **6**, **13**, **14**, and DPI on superoxide anion generation in rat neutrophils stimulated with PMA. Data are presented as means ± SD (n = 3–6). DPI was used as positive control.

Table 2
The inhibitory effect of 18β-GA derivatives on the superoxide anion formation from rat neutrophils stimulated with fMLP/CB or PMA (**A**), the accumulation of NO_2^- in RAW 264.7 cells stimulated with LPS (**B**), and TNF-α formation from RAW 264.7 cells stimulated with LPS (**C**)

Compound	IC_{50}[a] (μM) (**A**)		IC_{50}[a] (μM) (**B**)	IC_{50}[a] (μM) (**C**)
	fMLP/CB	PMA	RAW 264.7	RAW 264.7
2	10.3 ± 5.2		>30 (45.6 ± 9.1)	
3				1.3 ± 0.4
6		12.9 ± 1.8		
7	7.0 ± 2.0			
10				26.1 ± 14.7
11			>30 (44.3 ± 10.5)	
12			>30 (43.0 ± 4.3)	
13		17.0 ± 1.5	>30 (44.5 ± 4.2)	13.7 ± 4.3
14		15.6 ± 1.7	13.1 ± 5.0	
15				15.5 ± 3.2
17				2.3 ± 5.0
20	9.8 ± 5.2			
21				27.7 ± 7.2
DPI	2.7 ± 2.0	1.8 ± 0.4		
1400 W			1.5 ± 0.2	
Genistein				26.5 ± 9.1

Diphenylene iodonium chloride (DPI), *N*-(3-aminomethyl)benzylacetamidine (1400 W), and genistein were used as positive controls for **A**, **B**, and **C**, respectively.

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. Data are presented as means ± SD (n = 3–6).

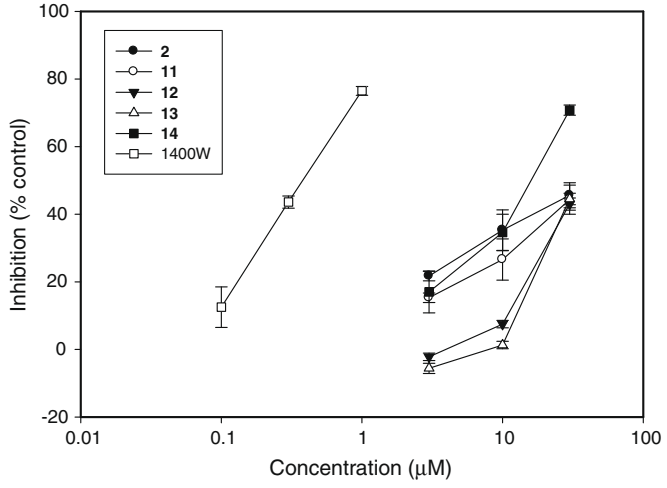


Figure 3. The inhibitory effects of **2**, **11**–**14**, and 1400 W on the accumulation of NO_2^- in the culture media of RAW 264.7 cells in response to LPS. Data are presented as means ± SD (n = 3–6). *N*-(3-Aminomethyl)benzylacetamidine) (1400 W) was used as positive control.

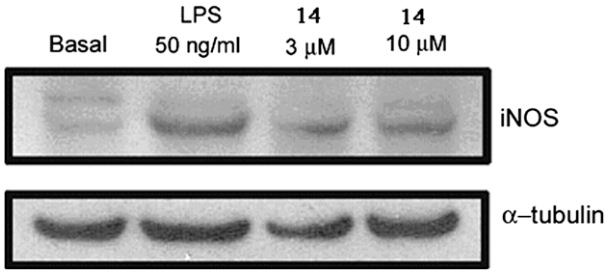


Figure 4. Effect of **14** on the expression of iNOS protein. RAW 264.7 cells were pretreated with **14** at 3 μM or 10 μM for 1 h, followed by stimulation with 50 ng/mL of LPS. After 24 h, the expression of iNOS protein was analyzed by Western blotting. Similar results were obtained from three independent experiments.

may correlate with XA/XO system while the suppression of superoxide anion formation by compounds **2**, **7**, and **20** in rat neutrophils stimulated with fMLP/CB did not correlate with XA/XO system. The above results also indicated that the cleavage of lactone ring of **6** attenuates the inhibitory effect on XO activity.

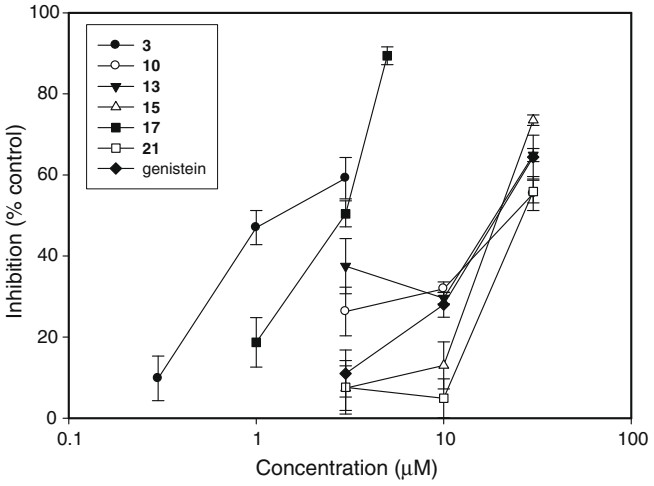


Figure 5. The inhibitory effects of **3**, **10**, **13**, **15**, **17**, **21**, and genistein on the formation of TNF-α in the culture media of RAW 264.7 cells in response to LPS. Data are presented as means ± SD (n = 3–6). Genistein was used as positive control.

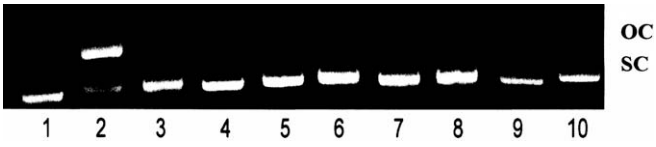


Figure 6. Inhibition of DNA strand breaks induced by $O_2^{-\cdot}$ (generated by XA/XO) in the presence of **2**, **6**, **7**, **13**, **14**, and **20** studied by gel electrophoresis. Supercoiled plasmid pBR322 DNA (500 ng) in phosphate buffer (pH 7.4) solution was incubated for 20 min with XA/XO acting as the control. Lane 1, DNA (without XA/XO); lane 2, control; lane 3, control + SOD (300 μM); lane 4, control + quercetin (300 μM) serving as positive control; lane 5, control + **2** (300 μM); lane 6, control + **6** (300 μM); lane 7, control + **7** (300 μM); lane 8, control + **13** (300 μM); lane 9, control + **14** (300 μM); lane 10, control + **20** (300 μM).

3. Conclusion

Twenty 18β-GA derivatives were synthesized and biologically evaluated as agents with antioxidant and anti-inflammatory activities. Compounds **2**, **3**, **6**, **7**, **10**, **13**, **14**, **15**, **17**, **20**, **21**, and **22** exert potent inhibitory effects on the release of chemical mediators from inflammatory cells and compounds **6**, **13**, **14**, and **22** display antioxidant and anti-inflammatory activities.

NO plays a central role in macrophage induced cytotoxicity and has been demonstrated to implicate in the pathology of central neurologic disease and in the peripheral tissue damage associated with acute and chronic inflammation[18] and septic shock.[4] The present study suggests that the inhibition of NO generation and suppression of superoxide anion formation by **14** in RAW 264.7 cells and rat neutrophils, respectively, and also the inhibition of XO activity and protective effect on oxidative DNA damage by **14** may have value in the therapeutical treatment or prevention of certain central as well as peripheral inflammatory diseases associated with the increase of NO production. The suppression of TNF-α production by compounds **3**, **10**, **13**, **17**, and **21** in macrophage may also have value in the treatment or prevention of certain inflammatory diseases or septic shock associated with increase of TNF-α production.

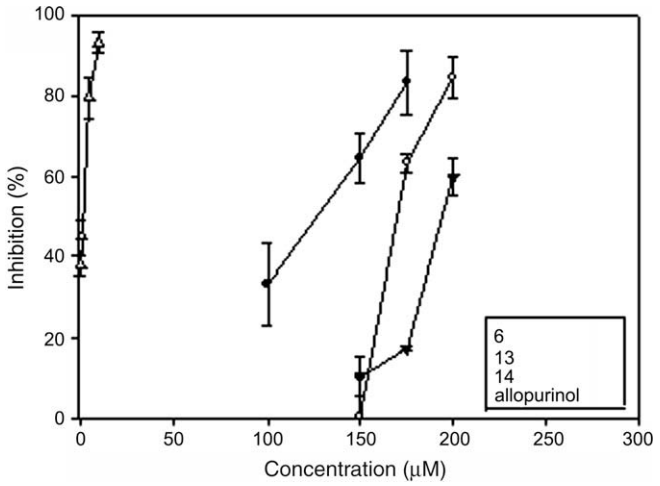


Figure 7. Dose-dependent inhibition of XO by **6**, **13**, **14**, and allopurinol. Data are presented as means ± s.e.m., $n = 6$.

4. Experimental

4.1. Chemistry

Melting points (uncorrected) were determined with a Yanco Micro-Melting point apparatus. IR spectra were determined with a Perkin–Elmer system 2000 FTIR spectrophotometer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR were recorded on a Varian UNITY-400 spectrometer. Low-resolution mass spectra and high-resolution mass spectra data were obtained on a JMX-HX 100 mass spectrometer. Chromatography was performed using a flash-column technique on Silica Gel 60 supplied by E. Merck.

4.2. Synthesis of 18β-glycyrrhetinic acid derivatives

4.2.1. 4-Hydroxy-3,4-*seco*-11-oxo-18β-olean-12-en-3,30-dioic acid 3,4 lactone 30 methyl ester (6)

Compound **4** (1 g, 2.1 mmol) in CH_2Cl_2 (30 mL) was added 3-chloroperoxybenzoic acid (3.6 g, 21.3 mmol). The mixture was allowed to stand at room temperature in dark for 12 h. The solution was diluted with $CHCl_3$, washed with 5% KI solution and 5% sodium sulfite solution, dried over Na_2SO_4, and concentrated to give **6**, as white solid (0.82 g, 1.6 mmol, 78%): mp 166–171 °C; $[\alpha]_D^{25}$ 189 (*c* 0.1, $CHCl_3$). IR (KBr): 1715, 1648 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.67 (3H, s, Me-28), 1.10 (3H, s, Me-29), 1.11 (3H, s, Me-26), 1.33 (6H, s, Me-25 and Me-27), 1.40 (3H, s, Me-23), 1.43 (3H, s, Me-24), 3.64 (3H, s, –OCH_3), 5.65 (1H, s, H-12). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 498 $[M]^+$ (3). HRESIMS: Calcd for $C_{31}H_{46}O_5Na$: 521.3243. Found: 521.3241.

4.2.2. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30-dioic acid 3-isopropyl ester (9)

Compound **5** (0.1 g, 0.2 mmol) in isopropyl alcohol (5 mL), CH_2Cl_2 (2 mL) was added 0.3 g of *p*-toluenesulfonic acid. The mixture was stirred at room temperature for 6–8 h, concentrated in vacuo, poured into water, extracted with $CHCl_3$. The $CHCl_3$ layer was washed with 5% sodium bicarbonate and brine, dried over Na_2SO_4, and concentrated. The residue was purified by a column to yield **9**, as light yellow solid (0.06 g, 0.12 mmol, 60%): mp 94–99 °C; $[\alpha]_D^{25}$ 154 (*c* 0.1, $CHCl_3$). IR (KBr): 2976, 1727, 1658, 1459, 1382, 1175 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.85 (3H, s, Me-28), 1.16 (3H, s, Me-29), 1.17 (3H, s, Me-25), 1.19 (3H, s, Me-26), 1.21 (6H, d, $J = 6.4$ Hz, $CH(CH_3)_2$), 1.39 (3H, s, Me-27), 1.76 (3H, s, Me-24), 4.69 (1H, br s, H-23), 4.89 (1H, br s, H-23), 4.95 (1H, m, –OCH), 5.72 (1H, s, H-12). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 526 $[M]^+$ (2). HRESIMS: Calcd for $C_{33}H_{50}O_5Na$: 549.3556. Found: 549.3554.

4.2.3. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30-dioic acid 30-methyl ester (10)

Compound **6** (0.1 g, 0.2 mmol) in CH_2Cl_2 (10 mL) was added 0.3 g of *p*-toluenesulfonic acid. The mixture was stirred at room temperature for 6–8 h. The mixture was diluted with water and extracted with $CHCl_3$. The organic layer was washed with 5% sodium bicarbonate solution, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by a column to give **10** as white solid (0.08 g, 0.16 mmol, 78%): mp 89–94 °C; $[\alpha]_D^{25}$ 171 (*c* 0.1, $CHCl_3$). IR (KBr): 1729, 1658 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.78 (3H, s, Me-28), 1.12 (3H, s, Me-29), 1.13 (6H, s, Me-25 and Me-26), 1.35 (3H, s, Me-27), 1.72 (3H, s, Me-24), 3.66 (3H, s, –$COOCH_3$), 4.66 (1H, br s, H-23), 4.86 (1H, br s, H-23), 5.65 (1H, s, H-12). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 498 $[M]^+$ (10). HRESIMS: Calcd for $C_{31}H_{46}O_5Na$: 521.3243. Found: 521.3245.

4.2.4. General procedure for esterification and amidation of lactone and *seco*-compounds

To a solution of lactone or *seco*-compound (1 mmol) in dry CH_2Cl_2 (10 mL) were added EDCI (2 mmol) and DMAP (catalytic amount) followed by alcohol or amine (2 mmol). The reaction mixture was stirred at room temperature overnight. After the reaction was finished (monitored by TLC) the mixture was diluted with water and extracted with chloroform. The organic solution was washed with 3% hydrochloric acid solution, brine, dried over $MgSO_4$, and filtered. The solvent was removed in vacuo and purified by column chromatography.

4.2.5. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30-dioic acid 3-methyl,30-isopropyl ester (12)

Compound **12** was prepared from **8** following the general procedure described for esterification. Compound **12** was obtained as light yellow solid (0.28 g, 0.52 mmol, 52%): mp 116–119 °C; $[\alpha]_D^{25}$ 126 (*c* 0.1, $CHCl_3$). IR (KBr): 1724, 1658 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.79 (3H, s, Me-28), 1.11 (3H, s, Me-29), 1.14 (3H, s, Me-25), 1.15 (3H, s, Me-26), 1.21 (3H, d, $J = 6.4$ Hz, –$CHCH_3$), 1.24 (3H, d, $J = 6.4$ Hz, –$CHCH_3$), 1.37 (3H, s, Me-27), 1.74 (3H, s, Me-24), 3.60 (3H, s, OCH_3), 4.67 (1H, br s, H-23), 4.87 (1H, br s, H-23), 5.02 (1H, septet, $J = 6.4$ Hz, OCH), 5.63 (1H, s, H-12). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 540 $[M]^+$ (18). HRESIMS: Calcd for $C_{34}H_{52}O_5Na$: 563.3712. Found: 563.3711.

4.2.6. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30-dioic acid 3-methyl,30-benzyl ester (13)

Compound **13** was prepared from **8** following the general procedure described for esterification using benzyl alcohol as alcohol moiety. Compound **13** was obtained as light yellow solid (0.35 g, 0.6 mmol, 60%): mp 32–38 °C; $[\alpha]_D^{25}$ 120 (*c* 0.1, $CHCl_3$). IR (KBr): 1730, 1657 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.72 (3H, s, Me-28), 1.12 (3H, s, Me-29), 1.14 (3H, s, Me-25), 1.15 (3H, s, Me-26), 1.35 (3H, s, Me-27), 1.74 (3H, s, Me-24), 3.59 (3H, s, –OCH_3), 4.67 (1H, br s, H-23), 4.87 (1H, br s, H-23), 5.07 (1H, d, $J = 12$ Hz, –CHH–C_6H_5), 5.19 (1H, d, $J = 12$ Hz, CHH–C_6H_5), 5.55 (1H, s, H-12), 7.35 (5H, m, C_6H_5). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 588 $[M]^+$ (3). HRESIMS: Calcd for $C_{38}H_{52}O_5Na$: 611.3712. Found: 611.3709.

4.2.7. Methyl 30-isopropylcarbamoyl-11-oxo-18β-3,4-*seco*-olean-4(23),12-dien-3-oate (14)

Compound **14** was prepared from **8** following the general procedure described for amidation using *iso*-propyl amine as amine moiety. Compound **14** was obtained as light yellow solid (0.34 g,

0.63 mmol, 63%): mp 80–85 °C; $[\alpha]_D^{25}$ 116 (*c* 0.1, $CHCl_3$). IR (KBr): 3379, 1720, 1645 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.82 (3H, s, Me-28), 1.11 (3H, s, Me-29), 1.13, 1.15 (6H, each d, J = 6.4 Hz, –CH(CH_3)$_2$), 1.15 (3H, s, Me-26), 1.16 (3H, s, Me-25), 1.39 (3H, s, Me-27), 1.75 (3H, s, Me-24), 3.62 (3H, s, OCH_3), 4.11 (1H, m, NCH), 4.68 (1H, br s, H-23), 4.89 (1H, br s, H-23), 5.37 (1H, d, J = 8.4, –NH), 5.64 (1H, s, H-12). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 539 $[M]^+$ (34). HRESIMS: Calcd for $C_{34}H_{53}NO_4Na$: 562.3872. Found: 562.3874.

4.2.8. Methyl 30-phenylcarbamoyl-11-oxo-18β-olean-3,4-*seco*-olean-4(23),12-dien-3-oate (15)

Compound **15** was prepared from **8** with the general procedure described for amidation using aniline as amine moiety. Compound **15** was obtained as light yellow solid (0.38 g, 0.66 mmol, 66%): mp 149–154 °C; $[\alpha]_D^{25}$ 192 (*c* 0.1, $CHCl_3$). IR (KBr): 3375, 1728, 1655, 1598 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.83 (3H, s, Me-28), 1.15 (3H, s, Me-25), 1.16 (3H, s, Me-26), 1.26 (3H, s, Me-29), 1.41 (3H, s, Me-27), 1.75 (3H, s, Me-24), 3.63 (3H, s, OCH_3), 4.69 (1H, br s, H-23), 4.89 (1H, br s, H-23), 5.72 (1H, s, H-12), 7.11 (1H, brt, J = 8.4 Hz, aromatic H), 7.33 (2H, br t, J = 8.4 Hz, aromatic H), 7.37 (1H, br s, NH), 7.51 (1H, dd, J = 8.4 Hz, 1.2 Hz, aromatic H). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 573 $[M]^+$ (32). HRESIMS: Calcd for $C_{37}H_{51}NO_4Na$: 596.3716. Found: 596.3718.

4.2.9. 4-Hydroxy-3,4-*seco*-11-oxo-18β-olean-12-en-3,30-dioic 3,4-lactone 30-benzylester (16)

Compound **16** was synthesized from **5** following the general procedure described for esterification using benzyl alcohol as one of the reactant. Compound **16** was obtained as white solid (0.36 g, 0.62 mmol, 62%): mp 83–89 °C; $[\alpha]_D^{25}$ 254 (*c* 0.1, $CHCl_3$). IR (KBr): 1726, 1654 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.73 (3H, s, Me-28), 1.14 (3H, s, Me-29), 1.16 (3H, s, Me-26), 1.36 (3H, s, Me-25), 1.37 (3H, s, Me-27), 1.44 (3H, s, Me-23), 1.47 (3H, s, Me-24), 5.08 (1H, d, J = 12.4 Hz, –OC*H*H–), 5.21 (1H, d, J = 12.4 Hz, –OCH*H*–), 5.58 (1H, s, H-12), 7.36 (5H, m, aromatic proton). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): $[M]^+$ 574 (15). HRESIMS: Calcd for $C_{37}H_{50}O_5Na$: 597.3556. Found: 597.3553.

4.2.10. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30-dioic acid 30-benzylester (17)

Compound **17** was prepared from **16** following the procedure described for synthesis of **10**. Compound **17** was obtained as white solid (0.08 g, 0.14 mmol, 70%): mp 87–92 °C; $[\alpha]_D^{25}$ 145 (*c* 0.1, $CHCl_3$). IR (KBr): 1726, 1657 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.73 (3H, s, Me-28), 1.15 (3H, s, Me-29), 1.16 (6H, s, Me-25 and 26), 1.36 (3H, s, Me-27), 1.75 (3H, s, Me-24), 4.69 (1H, br s, H-23), 4.89 (1H, br s, H-23), 5.09 (1H, d, J = 12.4 Hz, –OC*H*H–), 5.21 (1H, d, J = 12.4 Hz, –OCH*H*–), 5.57 (1H, s, H-12), 7.37 (5H, m, C_6H_5). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): $[M]^+$ 574 (25). HRESIMS: Calcd for $C_{37}H_{50}O_5Na$: 597.3556. Found: 597.3558.

4.2.11. 4-Hydroxy-3,4-*seco*-11-oxo-18β-olean-12-en-3,30-dioic 3,4-lactone 30-isopropylcarbamate (18)

Compound **18** was synthesized from **5** following the general procedure described for amidation using *iso*-propylamine as one of the reactant. Compound **18** was obtained as light yellow solid (0.32 g, 0.6 mmol, 60%): mp 94–99 °C; $[\alpha]_D^{25}$ 154 (*c* 0.1, $CHCl_3$). IR (KBr): 3382, 1720, 1653 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.77 (3H, s, Me-28), 1.07 (3H, s, Me-29), 1.11 (3H, s, Me-26), 1.33 (3H, s, Me-27), 1.34 (3H, s, Me-25), 1.40 (3H, s, Me-23), 1.44 (3H, s, Me-24), 4.07 (1H, m, C*H*(CH_3)$_2$), 5.52 (1H, d, J = 8.0 Hz, NH), 5.64 (1H, s, H-12). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 525 $[M]^+$ (27). HRESIMS: Calcd for $C_{33}H_{51}NO_4Na$: 548.3716. Found: 548.3717.

4.2.12. 4-Hydroxy-3,4-*seco*-11-oxo-18β-olean-12-en-3,30-dioic 3,4-lactone 30-phenylcarbamate (19)

Compound **19** was synthesized from **5** following the general procedure described for amidation using aniline as one of the reactant. Compound **19** was obtained as white solid (0.37 g, 0.66 mmol, 66%): mp 123–128 °C; $[\alpha]_D^{25}$ 268 (*c* 0.1, $CHCl_3$). IR (KBr): 3359, 1717, 1655 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.81 (3H, s, Me-28), 1.16 (3H, s, Me-29), 1.26 (3H, s, Me-26), 1.37 (3H, s, Me-27), 1.41 (3H, s, Me-25), 1.44 (3H, s, Me-23), 1.48 (3H, s, Me-24), 5.75 (1H, s, H-12), 7.11 (1H, m, aromatic proton), 7.34 (3H, m, NH, and aromatic proton), 7.50 (2H, m, aromatic proton). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 559 $[M]^+$ (20). HRESIMS: Calcd for $C_{36}H_{49}NO_4Na$: 582.3559. Found: 582.3557.

4.2.13. 30-Isopropylcarbamoyl-11-oxo-18β-3,4-*seco*-olean-4(23),12-dien-3-oic acid (20)

Compound **20** was prepared from **18** following the procedure described for synthesis of **10**. Compound **20** was obtained as light yellow solid (0.06 g, 0.12 mmol, 62%): mp 142–146 °C; $[\alpha]_D^{25}$ 126 (*c* 0.1, $CHCl_3$). IR (KBr): 3348, 1738, 1651 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.81 (3H, s, Me-28), 1.11 (6H, d, J = 6.8 Hz, –CH(CH_3)$_2$), 1.12 (3H, s, Me-29), 1.15 (6H, s, Me-25, and Me-26), 1.37 (3H, s, Me-27), 1.74 (3H, s, Me-24), 4.11 (1H, m, –C*H*(CH_3)$_2$), 4.68 (1H, br s, H-23), 4.88 (1H, br s, H-23), 5.64 (1H, s, H-12), 5.71 (1H, d, J = 8.0 Hz, NH). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 525 $[M]^+$ (32). HRESIMS: Calcd for $C_{33}H_{51}NO_4Na$: 548.3716. Found: 548.3718.

4.2.14. 30-Phenylcabamoyl-11-oxo-18β-3,4-*seco*-olean-4(23),12-dien-3-oic acid (21)

Compound **21** was prepared from **19** following the procedure described for synthesis of **10**. Compound **21** was obtained as white solid (0.07 g, 0.13 mmol, 65%): mp 162–166 °C; $[\alpha]_D^{25}$ 262 (*c* 0.1, $CHCl_3$). IR (KBr): 3365, 1652, 1526 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84 (3H, s, Me-28), 0.97 (3H, s, Me-25), 1.00 (3H, s, Me-26), 1.31 (3H, s, Me-29), 1.40 (3H, s, Me-27), 1.69 (3H, s, Me-24), 4.62 (1H, br s, H-23), 4.86 (1H, br s, H-23), 5.84 (1H, s, H-12), 7.07 (1H, m, aromatic proton), 7.28 (2H, m, aromatic proton), 7.45 (2H, m, aromatic proton), 8.27 (1H, br s, NH). ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV) *m/z* (% rel. int.): 559 $[M]^+$ (64). HRESIMS: Calcd for $C_{36}H_{49}NO_4Na$: 582.3559. Found: 582.3558.

4.3. Mast cell degranulation, neutrophil degranulation, superoxide anion generation, macrophage culture and drug treatment, NO determination, and Western blotting analysis

Compound stock solution (30 mM in DMSO) was prepared and stored at −25 °C, and was diluted with DMSO to 1–20 mM range at room temperature before experiment. The final percentage of DMSO in the reaction mixture was less than 0.5% (v/v). Rat (Sprague Dawley) peritoneal mast cells[23] and peripheral blood neutrophils[24] were isolated and incubated with test compounds for 5 min at 37 °C before stimulation with 10 μg/mL of compound 48/80 for another 15 min or with 1 μM formyl-Met-Leu-Phe (fMLP) for another 45 min, respectively. The degranulation of mast cells and neutrophils was assessed by the determination of histamine and β-glucuronidase, and β-glucuronidase and lysozyme, respectively, in the supernatant.[23,25] The total content of lysozyme and β-glucuronidase was measured from the Triton X-100-treated cells. In the superoxide anion generation experiments, neutrophils were stimulated with fMLP (0.3 μM)/CB (5 μg/mL) for 30 min in the presence of cytochrome *c*, and the superoxide anion generation was measured in terms of superoxide dismutase-inhibitable cytochrome *c* reduction.[26,27] Murine macrophage-like cell line RAW 264.7 cells were plated in 96-well plate, and incubated with test compounds for 1 h at 37 °C before stimulation with 1 μg/mL of

lipopolysaccharide (LPS) for 24 h. Nitric oxide (NO) in the cell medium was determined by the Griess reaction.[28] In Western blot analysis, cells were washed with PBS twice and harvested in Laemmli sodium dodecyl sulfate (SDS) sample buffer. Cell lysates were separated by 10% SDS–PAGE, and electrophoretically transferred to poly(vinylidene difluoride) membranes. Membranes were blocked for 1 h at room temperature in TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.1% Tween 20) containing 5% nonfat milk. Membranes were washed with TBST buffer and then incubated for 1 h with a monoclonal anti-iNOS antibody (1:1000 dilution). Following washed with TBST buffer, horseradish peroxidase-labeled anti-mouse IgG (1:10,000 dilution) was added at room temperature for 1 h. The blots were developed using ECL Western blotting reagents.[22] Secretory levels of TNF-α in culture supernatants were determined by the EIA kit according to the procedure of manusfacturer.

4.4. Inhibition of oxidative DNA damage

A mixture of supercoiled plasmid pBR322 DNA (1 μg/μL) and xanthine (2 mM)/xanthine oxidase (0.7 U/mL) in 10 mM phosphate buffer (pH 7.4) was incubated for 20 min with 500 μM of superoxide dismutase, quercetin, or **2**, **6**, **7**, **13**, **14**, and **20** in a total volume of 20 μL in a 1.5 mL microfuge tube at 37 °C, respectively. Quercetin was used as positive control. After incubating for 20 min, a 15 μL aliquot of mixture was loaded into 1.0% agarose gel containing ethidium bromide (0.05 μg/mL) in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer. The electrophoresis was carried out for 30 min at 100 V. Then the gels were illuminated with UV light and photographed. Plasmid DNA subjected to electrophoresis without superoxide dismutase, quercetin, or **2**, **6**, **7**, **13**, **14**, and **20** served as the control. The gel electrophoretic motility of the various forms of DNA was compared with the control.[6]

4.5. Assay of xanthine oxidase activity

The xanthine oxidase activity with xanthine as the substrate was measured at 25 °C, according to the protocol of Kong and associates[29] with modification. The assay mixture consisting of 50 μL of test solution, 60 μL of 70 mM phosphate buffer (pH 7.5), and 30 μL of enzyme solution [0.1 U/mL in 70 mM phosphate buffer (pH 7.5)] was prepared immediately before use. After preincubation at 25 °C for 15 min, the reaction was initiated by addition of 60 μL of substrate solution (150 μM xanthine in the same buffer). The reaction was monitored for 5 min at 295 nm. The xanthine oxidase activity was expressed as micromoles of uric acid per minute.

4.6. Statistical analysis

Data were expressed as means ± SD. Statistical analysis were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the student's *t*-test method for two group comparison, with $p < 0.05$ was considered to be statistically significant.

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