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# Anti-inflammatory Triterpenes from the Leaves of *Rosa laevigata*

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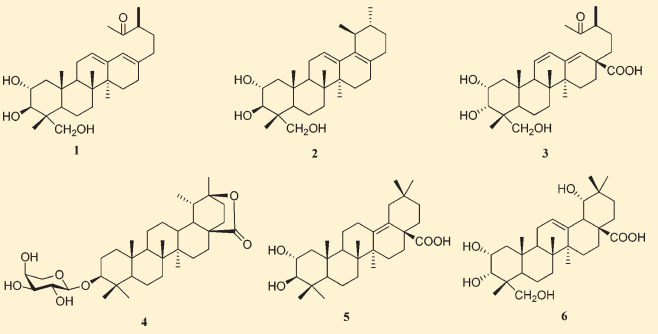
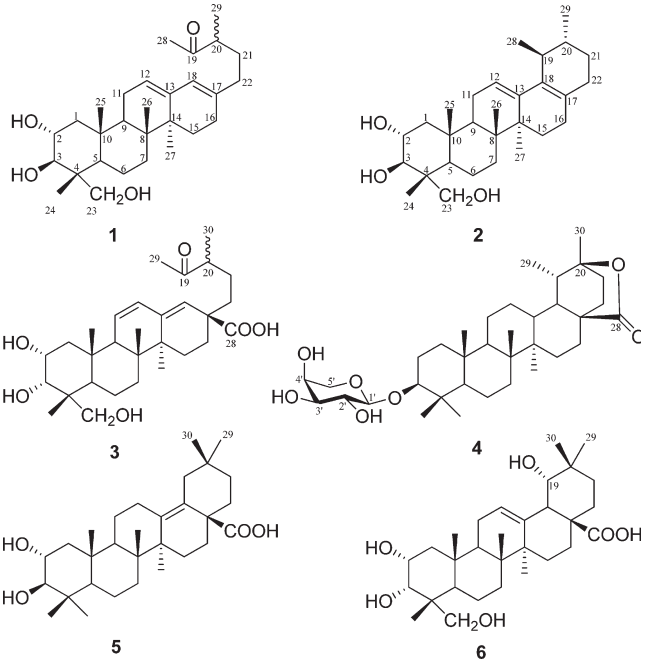
Supporting Information

**ABSTRACT:** Bioassay-guided fractionation of an EtOAc extract of the leaves of *Rosa laevigata* afforded two new 19-oxo-18,19-seco-ursane-type triterpenes (**1** and **3**), a new ursane-type nortriterpene (**2**), a new ursane-type triterpene lactone saponin (**4**), and two new oleanane-type triterpenoids (**5** and **6**), together with eight known triterpenoids (**7**−**14**). Compound **1**, a 19-oxo-18,19-seco-28-norursane, possesses a conjugated diene between C-12 and C-17. Several of the isolated compounds (**1**, **5**, **7**, **11**, and **13**) exhibited moderate activities in anti-inflammatory assays in vitro.



Severe burns cause a pronounced hypermetabolic response characterized by catabolism and extensive protein wasting. Studies have shown that this hypermetabolic state is driven by a severe inflammatory response.[1] Immediately after trauma via burning, cytokines and pro-inflammatory mediators show marked changes in the cytokine expression profile.[2] Therefore, anti-inflammatory responses are associated with the process of healing burn injuries.

*Rosa laevigata* Michx. (Rosaceae) is an evergreen climbing shrub widely distributed throughout southern China.[3] The leaves of *R. laevigata* are widely used as a traditional Chinese folk medicine for the treatment of skin tumors, burns, and ulcers.[4,5] Previous investigations on the chemical constituents of *R. laevigata* have led to the characterization of several compound classes including polyphenols, steroids, triterpenoids, and triterpene glucosides.[5−9] However, bioactivity studies have not been previously reported for this plant, and there are no prior reports on the chemical and biological activities of metabolites from the leaves of *R. laevigata*. As part of our ongoing search for bioactive natural products, we found that a 70% ethanol extract of the leaves of *R. laevigata* showed potent effects on curing burn injuries in a wound-healing test. Chemical and biological investigations on the leaves of *R. laevigata* were carried out, and the EtOAc extract showed significant anti-inflammatory activity in in vitro assays. Bioassay-guided fractionation led to the isolation of 14 triterpenes, including six new (**1**−**6**) and eight known compounds (**7**−**14**). Here, we report the isolation and structure elucidation of these triterpenes and their anti-inflammatory activities.



## RESULTS AND DISCUSSION

A 70% EtOH extract of the leaves of *R. laevigata* was suspended in $H_2O$ and successively extracted with petroleum

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Table 1. $^1$H NMR Data of Compounds 1−6 in Pyridine-$d_5$ (500 MHz, $J$ in Hz)

| no. | 1 | 2 | 3 | 4[a] | 5 | 6 |
|---|---|---|---|---|---|---|
| 1 | 1.49 (m) | 1.50 (m) | 1.95 (t, 12.0) | 0.90, 1.71 (m) | 1.42 m | 1.91 (t, 12) |
| | 2.45 (dd, 4.0, 12.5) | 2.50 (dd, 4.0, 12.5) | 2.31 (dd, 3.5, 11.5) | | 2.51 (dd, 4.5, 13.0) | 2.04 (m) |
| 2 | 4.36 (m) | 4.36 (m) | 4.42 (d, 11.0) | 1.71, 1.85 (m) | 4.23 (m) | 4.37 (d, 8.5) |
| 3 | 4.31 (m) | 4.28 (d, 10) | 4.25 (s) | 3.13 (m) | 3.51 (d, 9.0) | 4.23 (s) |
| 5 | 1.97 (m) | 1.95 (m) | 2.18 (m) | 0.69 (d, 10) | 1.12 (br s) | 2.18 (m) |
| 6 | 1.56, 1.88 (m) | 1.58, 1.88 (m) | 1.50, 1.72 (m) | 1.36, 1.50 (m) | 1.44, 1.65 (m) | 1.50, 1.71 (m) |
| 7 | 1.56, 1.78 (m) | 1.58, 1.78 (m) | 1.43, 1.61 (m) | 1.34, 1.42 (m) | 1.50, 1.54 (m) | 2.10, 2.27 (m) |
| 9 | 1.97 (m) | 1.97 (m) | 2.44 (br s) | 1.28 (m) | 1.74 (m) | 2.25 (m) |
| 11 | 2.10, 2.21 (m) | 2.22, 2.33 (m) | 5.89 (d, 10.5) | 1.26, 1.51 (m) | 1.50, 1.71 (m) | 2.22, 2.27 (m) |
| 12 | 5.47 (br s) | 5.71 (br s) | 6.25 (dd, 2.0, 10.0) | 1.00, 1.63 (m) | 1.99 (m) | 5.64 (s) |
| | | | | | 2.90 (d, 14.0) | |
| 13 | | | | 1.22 (m) | | |
| 15 | 1.35 (dd, 4.0, 12.5) | 1.33, 1.69 (m) | 1.28, 2.21 (m) | 1.09, 2.03 (m) | 1.27 (m) | 1.31, 2.22 (m) |
| | 1.65 (m) | | | | 2.15 (t, 12.0) | |
| 16 | 1.99, 2.13 (m) | 1.95, 2.04 (m) | 1.63, 2.61 (m) | 1.23, 1.92 (m) | 1.73 (m) | 2.21, 2.87 (m) |
| | | | | | 2.35 (d, 13.5) | |
| 18 | 5.91 (s) | | 5.92 (s) | 1.08 (m) | | 3.70 (d, 9.5) |
| 19 | | 1.78 (m) | | 1.56 (m) | 2.27 (d, 14.0) | 3.68 (d, 7.0) |
| | | | | | 2.68 (d, 14.0) | |
| 20 | 2.56 (m) | 2.65 (m) | 2.62 (m) | | | |
| 21 | 1.51, 1.93 (m) | 1.42, 1.48 (m) | 1.70, 2.07 (m) | 1.63, 1.92 (m) | 1.40, 1.71 (m) | 1.22, 2.22 (m) |
| 22 | 2.10 (2H, m) | 2.07, 2.14 (m) | 1.76, 2.07 (m) | 1.54, 1.63 (m) | 1.54 (m) | 1.44, 1.81 (m) |
| | | | | | 2.65 (d, 16.5) | |
| 23 | 3.82 (d, 10.5) | 3.82 (d, 10.0) | 3.84 (d, 10.5) | 0.97 (s) | 1.36 (s) | 3.83 (d, 10.5) |
| | 4.31 (m) | 4.28 (d, 10.0) | 3.99 (d, 10.5) | | | 4.00 (d, 11.0) |
| 24 | 1.18 (s) | 1.19 (s) | 0.93 (s) | 0.80 (s) | 1.16 (s) | 0.95 (s) |
| 25 | 1.25 (s) | 1.27 (s) | 1.09 (s) | 0.83 (s) | 1.08 (s) | 1.13 (s) |
| 26 | 1.02 (s) | 1.05 (s) | 0.99 (s) | 0.93 (s) | 1.24 (s) | 1.17 (s) |
| 27 | 1.06 (s) | 1.05 (s) | 1.05 (s) | 0.90 (s) | 1.31 (s) | 1.66 (s) |
| 28 | 2.18 (s) | 0.90 (d, 10.0) | | | | |
| 29 | 1.13 (d, 7.0) | 1.03 (d, 9.0) | 2.19 (s) | 0.99 (d, 5.0) | 1.04 (s) | 1.26 (s) |
| 30 | | | 1.14 (d, 7.0) | 1.31 (s) | 0.93 (s) | 1.19 (s) |
| 1′ | | | | 4.38 (d, 5.0) | | |
| 2′ | | | | 3.76 (br s) | | |
| 3′ | | | | 3.71 (br s) | | |
| 4′ | | | | 3.92 (br s) | | |
| 5′ | | | | 3.55 (d, 10.0) | | |
| | | | | 3.90 (d, 10.0) | | |

[a] Measured in $CDCl_3$.

ether, EtOAc, and n-BuOH. Separation of the EtOAc extract using a combination of silica gel, Sephadex LH-20, and semi-preparative HPLC yielded compounds **1**−**14**. Comparison of their NMR and MS data with reported values confirmed the structures of the known compounds **7**−**14** as 2α,3α,23-trihydroxyolean-12-en-28-oic acid (**7**),[10] euscaphic acid (**8**),[11] ursolic acid (**9**),[12] maslinic acid (**10**),[13] 19α-hydroxyasiatic acid (**11**),[11] 2α,3β,23-trihydroxylup-20 (29)-en-28-oic acid (**12**),[14] 19α-hydroxyasiatic acid-28-*O*-β-D-glucopyrannoside (**13**),[11] and 3β,23α-dihydroxyursan-28-oic acid δ-lactone (**14**).[15]

Compound **1** was obtained as white crystals in MeOH. The positive HRESIMS exhibited a pseudomolecular ion peak at $m/z$ 481.3291 $[M + Na]^+$ (calcd 481.3294), consistent with a molecular formula of $C_{29}H_{46}O_4$, implying seven degrees of unsaturation. The UV spectrum showed an absorption maximum at 241 nm, indicating the presence of a conjugated system. IR absorption bands indicated the existence of hydroxy (3420 cm$^{-1}$), carbonyl (1712 cm$^{-1}$), and olefinic (1634 cm$^{-1}$) functional groups. The $^1$H NMR spectrum (Table 1) revealed four methyl singlets ($\delta_H$ 1.02, 1.06, 1.18, and 1.25), one methyl doublet ($\delta_H$ 1.13, $J$ = 7.0 Hz), one acetyl singlet ($\delta_H$ 2.18), two olefinic methine signals ($\delta_H$ 5.47 and 5.91), two oxygen-bearing methine signals ($\delta_H$ 4.31 and 4.36), and one oxygen-bearing methylene signal ($\delta_H$ 3.82 and 4.31). The $^{13}$C NMR (Table 2) displayed 29 carbon signals, which were identified by the DEPT and HMBC spectra as one ketocarbonyl ($\delta_C$ 211.0), two olefinic methines ($\delta_C$ 122.2 and 125.9), two olefinic quaternary carbons ($\delta_C$ 136.3 and 139.7), two oxymethines ($\delta_C$ 69.1 and 78.3), one oxymethylene ($\delta_C$ 66.5), four aliphatic quaternary carbons ($\delta_C$ 38.6, 38.7, 40.9, and 43.8), three aliphatic methines ($\delta_C$ 46.8, 48.3, and


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Table 2. $^{13}$C NMR Data of Compounds 1−6 in Pyridine-$d_5$ (125 MHz)

| no. | 1 | 2 | 3 | 4[a] | 5 | 6 |
|---|---|---|---|---|---|---|
| 1 | 48.3 | 48.3 | 42.5 | 38.8 | 48.3 | 42.6 |
| 2 | 69.1 | 69.0 | 66.3 | 25.9 | 69.0 | 66.4 |
| 3 | 78.3 | 78.3 | 79.1 | 89.8 | 84.0 | 79.1 |
| 4 | 43.8 | 43.8 | 42.2 | 39.1 | 40.0 | 42.1 |
| 5 | 48.4 | 48.3 | 43.6 | 55.7 | 56.1 | 43.8 |
| 6 | 18.6 | 18.7 | 18.3 | 18.1 | 19.0 | 18.6 |
| 7 | 33.8 | 34.1 | 32.4 | 33.9 | 35.6 | 33.8 |
| 8 | 38.7 | 39.3 | 41.2 | 40.5 | 42.0 | 40.3 |
| 9 | 48.3 | 47.9 | 54.9 | 50.6 | 51.3 | 48.5 |
| 10 | 38.6 | 38.5 | 38.3 | 36.9 | 39.0 | 38.8 |
| 11 | 24.0 | 24.2 | 127.7 | 21.0 | 22.4 | 24.4 |
| 12 | 122.2 | 117.5 | 130.7 | 25.2 | 25.6 | 123.9 |
| 13 | 139.7 | 137.7 | 142.7 | 42.8 | 138.1 | 145.1 |
| 14 | 40.9 | 41.3 | 41.7 | 41.0 | 45.0 | 42.4 |
| 15 | 27.9 | 27.5 | 26.7 | 27.3 | 27.9 | 29.3 |
| 16 | 26.6 | 28.7 | 27.6 | 27.6 | 33.8 | 28.5 |
| 17 | 136.3 | 128.9 | 47.7 | 42.0 | 49.0 | 46.2 |
| 18 | 125.9 | 133.6 | 129.0 | 48.4 | 129.5 | 45.0 |
| 19 | 211.0 | 33.1 | 211.8 | 41.9 | 41.7 | 81.4 |
| 20 | 46.8 | 32.5 | 47.6 | 84.1 | 33.1 | 35.8 |
| 21 | 31.2 | 25.0 | 28.3 | 27.1 | 37.6 | 29.4 |
| 22 | 35.4 | 32.4 | 39.2 | 32.2 | 36.6 | 33.2 |
| 23 | 66.5 | 66.5 | 71.4 | 28.1 | 29.4 | 71.4 |
| 24 | 14.5 | 14.5 | 17.3 | 16.4 | 17.7 | 17.8 |
| 25 | 18.2 | 18.2 | 19.7 | 16.3 | 18.1 | 17.1 |
| 26 | 17.1 | 17.4 | 17.1 | 15.7 | 18.3 | 17.8 |
| 27 | 21.2 | 21.0 | 20.3 | 14.2 | 21.4 | 25.0 |
| 28 | 28.2 | 13.5 | 178.1 | 177.2 | 179.3 | 181.2 |
| 29 | 16.4 | 20.0 | 28.2 | 18.7 | 32.5 | 29.0 |
| 30 | | | 16.5 | 24.0 | 24.5 | 25.0 |
| 1′ | | | | 104.5 | | |
| 2′ | | | | 71.6 | | |
| 3′ | | | | 72.4 | | |
| 4′ | | | | 67.0 | | |
| 5′ | | | | 64.1 | | |

[a] Measured in $CDCl_3$.

48.4), eight aliphatic methylenes ($\delta_C$ 18.6, 24.0, 26.6, 27.9, 31.2, 33.8, 35.4, and 48.3), and six methyl carbons ($\delta_C$ 14.5, 16.4, 17.1, 18.2, 21.2, and 28.2). The above findings accounted for three of the seven degrees of unsaturation, suggesting that **1** is a tetracyclic nortriterpene. Analysis of the $^{13}$C NMR spectrum indicated that **1** closely resembled swinhoeic acid,[16] with the main difference in the position of a conjugated diene. The double bonds in **1** were located at $\Delta^{12,13}$ and $\Delta^{17,18}$, as determined by the HMBC correlations from $\delta_H$ 5.91 (H-18) to $\delta_C$ 122.2 (C-12), 139.7 (C-13), 40.9 (C-14), 26.6 (C-16), and 35.4 (C-22), from $\delta_H$ 5.47 (H-12) to $\delta_C$ 48.3 (C-9), and from $\delta_H$ 1.06 ($H_3$-27) to $\delta_C$ 139.7 (C-13) and 27.9 (C-15) (Figure 1). The HMBC correlations of both $H_2$-23 ($\delta_H$ 3.82 and 4.31) and $H_3$-24 ($\delta_H$ 1.18) with C-3 ($\delta_C$ 78.3) and C-4 ($\delta_C$ 43.8) indicated the hydroxymethylene group was attached at C-4. Moreover, the HMBC correlations from $\delta_H$ 4.36 (H-2) to $\delta_C$ 48.3 (C-1) and from $\delta_H$ 4.31 (H-3) to $\delta_C$ 48.3 (C-1), 69.1 (C-2), and 43.8 (C-4) suggested that the hydroxy groups were attached to C-2 and C-3,

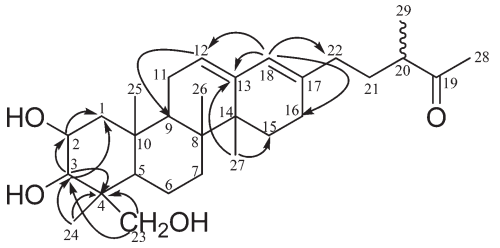


**Figure 1.** Key HMBC correlations of compound **1**.

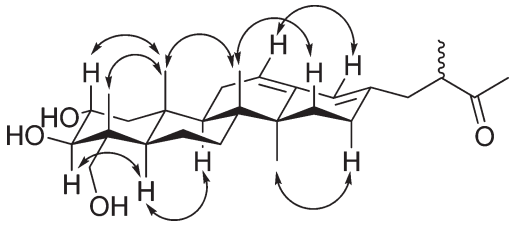


**Figure 2.** Key NOESY correlations of compound **1**.

repectively. The relative configuration of **1** was determined through inspection of the NOESY spectrum (Figure 2). NOE correlations between the methyl protons at $\delta_H$ 1.18 ($H_3$-24) and the methyl protons at $\delta_H$ 1.25 ($H_3$-25) indicated the α-orientation of the hydroxymethylene group (23-$CH_2$OH). Additionally, the NOESY cross-peaks of H-2/$H_3$-25 and H-3/H-5 established the *β*-orientation of H-2 and α-orientation of H-3. Thus, **1** was elucidated as 2α,3*β*,23-trihydroxy-19-oxo-18,19-seco-12,17-dien-28-norursane.

Compound **2** was isolated as a white, amorphous powder. Positive mode HRESIMS indicated a molecular formula of $C_{29}H_{46}O_3$ ($[M + Na]^+$ $m/z$ 465.3343, calcd 465.3345), containing seven degrees of unsaturation. The IR absorption bands suggested the presence of hydroxy (3446 cm$^{-1}$) and olefinic (1636 cm$^{-1}$) functional groups. The $^1$H and $^{13}$C NMR data showed that **2** was an ursane-type triterpene based on proton signals at $\delta_H$ 0.90 (d, $J$ = 10.0 Hz, $H_3$-28), 1.03 (d, $J$ = 9.0 Hz, $H_3$-29), 1.05 (s, $H_3$-26 and $H_3$-27), 1.19 (s, $H_3$-24), and 1.27 (s, $H_3$-25), as well as the corresponding $^{13}$C NMR signals at $\delta_C$ 13.5 (C-28), 20.0 (C-29), 17.4 (C-26), 21.0 (C-27), 14.5 (C-24), and 18.2 (C-25). The proton signals at $\delta_H$ 3.82 (d, $J$ = 10.0 Hz) and 4.28 (d, $J$ = 10.0 Hz) and corresponding carbon resonance at $\delta_C$ 66.5 were ascribed to one hydroxymethylene group. Comparison of the NMR data of **2** with those of **1** indicated that they were structurally closely related. Significant differences included the replacement of the ketocarbonyl group ($\delta_C$ 211.0, C-19) in **1** with a methine ($\delta_C$ 33.1, C-19) in **2** and an olefinic methine ($\delta_C$ 125.9, C-18) in **1** was replaced by an olefinic quaternary carbon ($\delta_C$ 133.6, C-18) in **2**. These assignments were in accord with the observed changes of the chemical shifts for C-20 and C-28, from $\delta_C$ 46.8 and 28.2 in **1** to $\delta_C$ 32.5 and 13.5 in **2**, and confirmed by the HMBC correlations from $H_3$-28 ($\delta_H$ 0.90) to C-18 ($\delta_C$ 133.6) and C-19 ($\delta_C$ 33.1). The relative configuration of **2** was determined to be the same as that of **1** by analysis of the NOESY spectrum. Consequently, the structure of **2** was established as 2α,3*β*,23-trihydroxy-12,17-dien-28-norursane.

Compound **3** was assigned a molecular formula of $C_{30}H_{46}O_6$ and eight degrees of unsaturation, as deduced from the HRESIMS ($m/z$ 525.3188 $[M + Na]^+$, calcd 525.3192) and $^{13}$C NMR spectra. The $^1$H, $^{13}$C, and HSQC NMR data revealed the


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existence of six methyl, eight methylene, eight methine, and eight quaternary carbons, including two cyclic olefinic bonds ($\delta_C$ 127.7, 129.0, 130.7, and 142.7) and two carbonyl carbons ($\delta_C$ 178.1 and 211.8). The $^{13}$C NMR spectrum was similar to that of swinhoeic acid,[16] except for the signals at C-23 and C-24. The HMBC correlations of both $H_2$-23 ($\delta_H$ 3.84 and 3.99) and $H_3$-24 ($\delta_H$ 0.93) with C-3 ($\delta_C$ 79.1) and C-4 ($\delta_C$ 42.2) suggested the hydroxymethylene moiety was attached at C-4. The relative configuration of **3** was established by analysis of the NOESY spectrum. NOE correlations from $H_3$-24 to $H_3$-25 suggested that the hydroxymethylene group (23-$CH_2$OH) was in the α-orientation. Strong NOE correlations between H-2 and $H_3$-24 and between H-3 and $H_3$-24 indicated that these protons were cofacial and were assigned a β-orientation. Therefore, compound **3** was designated as 2α,3α,23-trihydroxy-19-oxo-18,19-seco-urs-11,13(18)-dien-28-oic acid.

Compound **4** was obtained as a white, amorphous powder. Its molecular formula was determined as $C_{35}H_{56}O_7$ by observation of an ion at $m/z$ 611.3925 $[M + Na]^+$ (calcd 611.3924) in the HRESIMS, which indicated eight degrees of unsaturation. The IR spectrum of **4** showed a prominent ester carbonyl absorption band at 1740 cm$^{-1}$. The $^1$H NMR spectrum exhibited six methyl singlets ($\delta_H$ 1.31, 0.97, 0.93, 0.90, 0.83, and 0.80), one methyl doublet ($\delta_H$ 0.99, $J$ = 5.0 Hz), and one oxygen-bearing methine signal ($\delta_H$ 3.13). An anomeric proton signal was also observed at $\delta_H$ 4.38 (d, $J$ = 5.0 Hz). The $^{13}$C NMR data in combination with analysis of the DEPT and HSQC spectra revealed 35 carbon signals due to seven quaternary, 10 methine, 11 methylene, and seven methyl carbons, of which 30 carbons were assigned to the aglycone part including a lactone carbonyl at $\delta_C$ 177.2, and the remaining carbon signals were ascribable to an α-arabinopyranosyl moiety ($\delta_C$ 104.5, 71.6, 72.4, 67.0, and 64.1) attached at C-3, as evidenced by HMBC correlations from $\delta_H$ 4.38 (H-1′) to $\delta_C$ 89.8 (C-3). The observed oxygen-bearing quaternary carbon signal at $\delta_C$ 84.1 (C-20) as well as the HMBC correlations from $\delta_H$ 1.23 (H-16), 1.08 (H-18), and 1.54 (H-22) to $\delta_C$ 177.2 (C-28) indicated a six-membered lactone ring between C-20 and C-28. The $^{13}$C NMR signals of **4** were similar to 3β-[(*O*-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]-20β-hydroxyursan-28-oic acid δ-lactone,[17] with the marked difference being the absence of a glucosyl moiety. Acid hydrolysis of **4** yielded L-Arabinose (see Experimental Section). NOESY correlations were observed from $H_3$-23 to H-1′ and H-3 and from $H_3$-30 to $H_3$-29 and $H_3$-27. These data are consistent with the structure 3β-[(α-L-arabinopyranosyl)oxy]-20β-hydroxyursan-28-oic acid δ-lactone, which was assigned to **4**.

Compound **5** was isolated as a white, amorphous powder. Its HRESIMS displayed a quasi-molecular ion peak at $m/z$ 495.3451 $[M + Na]^+$ (calcd 495.3450) consistent with a molecular formula of $C_{30}H_{48}O_4$. The $^1$H, $^{13}$C, and HSQC NMR data demonstrated the presence of seven methyl, 10 methylene, four methine, and nine quaternary carbons, including two olefinic quaternary carbons ($\delta_C$ 129.5 and 138.1), and a carboxylic acid carbon ($\delta_C$ 179.3). Attachment of a double bond to C-13 and C-18 was established by HMBC correlations from $H_3$-27 ($\delta_H$ 1.31) to C-13 ($\delta_C$ 138.1) and from H-19 ($\delta_H$ 2.27) to C-18 ($\delta_C$ 129.5). Analysis of the $^{13}$C NMR data of **5** established its close structural resemblance to centellasapogenol A,[18] differing only in the absence of a hydroxymethylene group ($\delta_C$ 66.8, C-23), which was replaced by a methyl carbon ($\delta_C$ 29.4, C-23) in **5**. HMBC correlations from $H_3$-23 ($\delta_H$ 1.36) to C-4 ($\delta_C$ 40.0), C-5 ($\delta_C$ 56.1), and C-24 ($\delta_C$ 17.7) were observed. The NOESY correlations, from H-2 to $H_3$-25 and from H-3 to $H_3$-23, indicated a β-orientation for H-2 and an α-orientation for H-3. Thus, the structure of **5** was determined to be 2α,3β-dihydroxyolean-13(18)-en-28-oic acid.

Compound **6** exhibited the molecular formula $C_{30}H_{48}O_6$, as deduced from HRESIMS ($m/z$ 527.3344 $[M + Na]^+$, calcd 527.3349). The $^{13}$C NMR spectrum showed 30 carbon signals including six methyls [$\delta_C$ 17.1, 17.8 (×2), 25.0 (×2), and 29.0)] and two olefinic carbons ($\delta_C$ 123.9 and 145.1), which were typical of the double bond at C-12(13) of oleanane-type triterpenes. The overall appearance of the $^{13}$C NMR spectrum showed the same planar structure as that of 2α,3α,19α,24-tetrahydroxyolean-12-en-28-oic acid.[19] The relative configuration of **6**, assigned on the basis of NOESY data, differed from 2α,3α,19α,24-tetrahydroxyolean-12-en-28-oic acid at one stereogenic center. NOESY correlations from $H_3$-24 to $H_3$-25 indicated that the hydroxymethylene group (23-$CH_2$OH) was in an α-orientation. Thus, the structure of **6** was established as 2α,3α,19α,23-tetrahydroxyolean-12-en-28-oic acid.

Numerous triterpenoids have been isolated from *R. laevigata*.[7−9] However, there are no reports on the 18,19-seco-ursane triterpenes. The co-occurrence of 19-oxo-18,19-seco-ursane-type triterpenes (**1** and **3**) within the plant raises interesting questions about their biogenesis. The possible biosynthetic pathways to compounds **1**−**3** are postulated in Scheme 1. The precursor of **1**−**3** was proposed to be compound **11**, which was transformed into a key intermediate **1a** by an oxidative cleavage of the C-18−C-19 bond. Decarboxylation of **1a** would afford **1**. Rearrangement and dehydrogenation of **1a** could lead to the production of **3**. In an alternative route, precursor **11** would undergo protonation to give intermediate **2a**, whose decarboxylation would result in the formation of **2**.

Compounds **1**−**14** were evaluated for their anti-inflammatory activities in a luciferase assay, induced by LPS macrophages. Compounds **1**, **5**, **7**, **11**, and **13** showed moderate activities in this assay (see Supporting Information). To explain the observed anti-inflammatory effects, the in vitro effects of these compounds on the inflammatory response were investigated further. The anti-inflammatory effects were evaluated by investigating the inhibitory activity of the compounds on the production of nitric oxide, TNF-α, IL-1β, IL-6, and IL-10 in LPS-stimulated mouse monocyte macrophage RAW 264.7 cells (Table 3). Initially, the cell viability in the presence of the test compounds was examined using the MTT method. Concentrations of up to 50 μg/mL (with LPS 0.01, 0.1, and 1 μg/mL) of the test compounds did not result in significant changes in the viability of the cells, although a concentration of 100 μg/mL of the test compounds (along with 10 μg/mL of LPS) decreased cell viability significantly. For all assays, hydrocortisone was used as a positive control. At concentrations in the range 0.5−50 μg/mL, the test compounds exhibited significant inhibitory activity on the production of all inflammation factors tested in vitro.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** Melting points were measured with an SCW X-4 melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer model 341 polarimeter with a 1 dm cell. UV spectra were obtained on a U-3010 spectrophotometer. IR spectra were recorded on a Bruker vector 22 spectrometer with KBr pellets. 1D and 2D NMR experiments were performed on a Bruker AMX-500 MHz instrument in pyridine-$d_5$ or

**Scheme 1. Possible Biogenetic Pathway for Compounds 1−3**

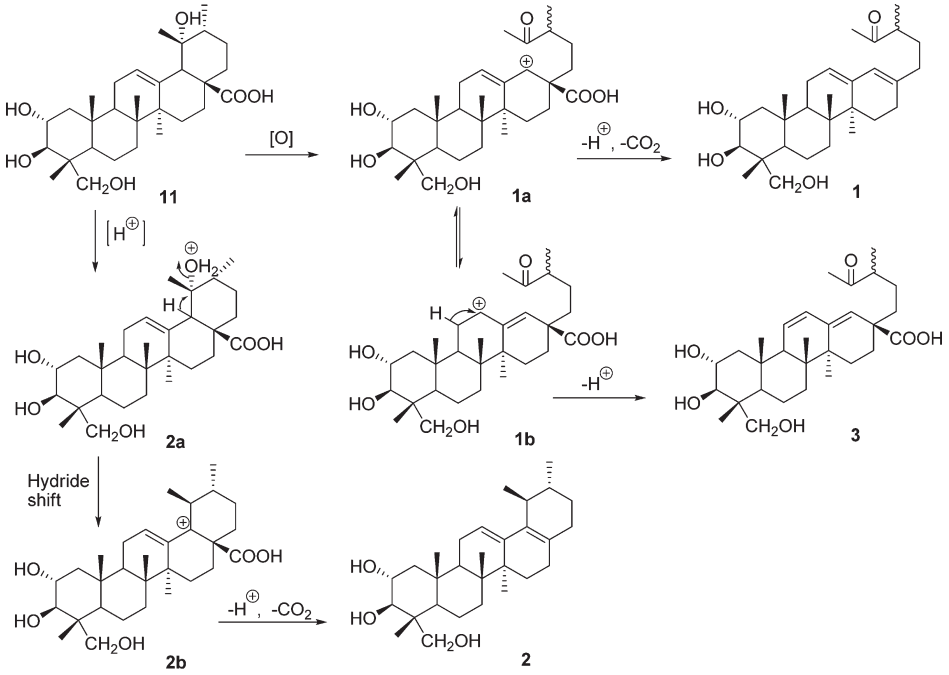


**Table 3. Inhibitory Effects of Compounds 1, 5, 7, 11, and 13 (50 $\mu$g/mL) on NO, TNF-α, IL-1$\beta$, IL-6, and IL-10 Production Stimulated by LPS (1 $\mu$g/mL) in RAW 264.7 Cells (mouse leukemic monocyte macrophage cell line)**

| | anti-inflammatory activity (means ± SDs, $n$ = 3) | | | | |
|---|---|---|---|---|---|
| compound | NO | TNF-α | IL-1$\beta$ | IL-6 | IL-10 |
| LPS | 11.7 ± 1.4 | 686 ± 54 | 264 ± 34 | 108 ± 10 | 484 ± 35 |
| **1** | 3.6 ± 0.5[b] | 188 ± 50[b] | 68.7 ± 14.0[b] | 35.9 ± 11.5[b] | 181 ± 12[b] |
| **5** | 3.5 ± 0.5[b] | 170 ± 51[b] | 73.2 ± 36.3[b] | 31.8 ± 9.1[b] | 135 ± 15[b] |
| **7** | 3.4 ± 0.6[b] | 226 ± 48[b] | 76.3 ± 33.3[b] | 44.9 ± 6.4[b] | 172 ± 14[b] |
| **11** | 4.1 ± 0.8[b] | 189 ± 41[b] | 39.3 ± 14.8[b] | 30.4 ± 12.8[b] | 129 ± 29[b] |
| **13** | 4.8 ± 0.5[b] | 325 ± 45[b] | 77.8 ± 40.1[b] | 43.8 ± 18.2[b] | 199 ± 20[b] |
| hydrocortisone[a] | 4.0 ± 1.1[b] | 221 ± 43[b] | 53.8 ± 30.8[b] | 30.6 ± 12.8[b] | 136 ± 17[b] |

[a] Positive control. [b] $p < 0.05$ vs control.

$CDCl_3$ with TMS as an internal standard. High-resolution TOF-ESIMS spectra were acquired with a Waters Q-Tof micro YA019 mass spectrometer. A Waters 1525 HPLC (using a PDA 2998 detector) was used for isolation and purification with a Sunfire ODS-18 semipreparative HPLC column (250 × 10 mm, 5 $\mu$m). GC was carried out using an Agilent 6890N gas chromatograph. Vacuum liquid chromatography (VLC) was performed on silica gel (200−300 mesh, Qingdao Ocean Chemical Company). Column chromatography (CC) was performed on Sephadex LH-20 (Pharmacia). The fractions were monitored by TLC (HSGF 254, Yantai, China), and spots were visualized by heating silica gel plates sprayed with 10% $H_2SO_4$ in $H_2O$.

**Plant Material.** Leaves of *R. laevigata* were collected at Huangshan, Anhui Province, China, in November 2008 and identified by Professor Hong-Fang Li (School of Pharmacy, Second Military Medical University). A voucher specimen (No. JYY2008368) was deposited in Laboratory of Marine Drugs, Department of Pharmacy, Changzheng Hospital, Second Military Medical University, China.

**Extraction and Isolation.** Dried leaves of *R. laevigata* (5 kg) were percolated with 70% (v/v) EtOH at room temperature three times, filtered, and concentrated to give an EtOH extract (450 g). The extract was suspended in $H_2O$ and successively extracted with petroleum ether, EtOAc, and n-BuOH. The EtOAc fraction was subjected to VLC on silica gel using a stepwise gradient elution of $CH_2Cl_2$−MeOH (50:1, 30:1, 20:1, 15:1, 10:1, 5:1, and 2:1) to afford five subfractions (A−E). Fraction B was passed through a silica gel column using a petroleum ether−acetone mixture as eluent, with a stepwise gradient (20:1 to 1:1) to give 10 subfractions (B1−B10). Fraction B4 was recrystallized from MeOH to yield **1** (58 mg). Repeated silica gel column chromatography of fraction B6 using $CH_2Cl_2$−MeOH (30:1−10:1), followed by purification using semipreparative HPLC with a gradient elution mixture of MeOH−$H_2O$ (85:15−95:5), yielded **8** (23 mg), **2** (8 mg), **14** (7 mg), and **7** (18 mg). Fraction B8 was subjected to chromatography on a Sephadex LH-20 column to provide seven subfractions (B8.1−B8.7). Fraction B8.2 was separated on silica gel eluted with petroleum ether−acetone (3:1) to yield **4** (15 mg). Fraction B8.3 was passed through a silica gel eluted with petroleum ether−acetone (5:1 and 3:1) to afford **9** (20 mg), **10** (25 mg), and **11** (20 mg). Fraction B8.4 was purified by semipreparative HPLC with MeOH−$H_2O$ (80:20−85:15) to afford **3**


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(15 mg) and **6** (20 mg). Fraction B8.5 was purified by silica gel CC, using petroleum ether−acetone as eluent (3:1 and 2:1), followed by purification using semipreparative HPLC with MeOH−$H_2O$ (75:25−80:20), to yield **12** (30 mg) and **5** (12 mg). Fraction B8.6 was recrystallized from MeOH to yield **13** (100 mg).

**Luciferase Assay.** The NF-$\kappa$B 293 cells were cultured in a DMEM medium supplemented with 10% FBS (fetal bovine serum), 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin. The cells were pretreated with the 14 compounds at concentrations of 12.5, 25, 50, 100, and 200 $\mu$g/mL for 4 h and then stimulated with 1 $\mu$g/mL LPS for 48 h. The cells were rinsed twice with phosphate-buffered saline (PBS, pH 7.4) and lysed with passive lysis buffer (Promega). Then luciferase activity was analyzed using the luciferase assay system (Promega) according to the manufacturer's instructions.[20]

**Measurement of Nitric Oxide Content and Cell Viability.** The cells were cultured in serum-free medium for 8 h and then incubated in medium containing 0.5, 5, and 50 $\mu$g/mL of test compounds for 4 h. The cells were then treated with 1 $\mu$g/mL of LPS for 24 h. Hydrocortisone (50 $\mu$g/mL) was used as a positive control. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using Griess reagent. Briefly, 100 $\mu$L of the supernatant from incubates was mixed with an equal volume of Griess reagent (0.1% *N*-[1-naphthyl]ethylenediamine and 1% sulfanilamide in 5% $H_3PO_4$).[21] Cytotoxicity was determined using the MTT colorimetric assay, after 24 h incubation with the test compounds.

**Measurement of TNF-α, IL-1$\beta$, IL-6, and IL-10.** The test groups, LPS groups, and positive control groups were treated in the same way as the NO analysis experiments. The supernatants of cell culture were harvested and centrifuged at 3000*g* at 4 °C for 2 min for the analysis of TNF-α, IL-1$\beta$, IL-6, and IL-10. Enzyme-linked immunosorbent assays for detecting the cytokines in the supernatants were carried out according to the instructions provided by the manufacturer. Finally, the standard provided with the kits was used to quantify each cytokine in the supernatants.[22]

*Compound* ***1***: white crystals from MeOH; mp 145 °C; $[\alpha]^{20}_D$ +25 (*c* 0.16, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 241 (4.40), 202 (4.08) nm; IR (KBr) $\nu_{max}$ 3420, 2937, 2837, 1712, 1634, 1384, 1096 cm$^{-1}$; $^1$H NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C NMR (pyridine-$d_5$, 125 MHz), see Tables 1 and 2; HRESIMS *m*/*z* 481.3291 $[M + Na]^+$ (calcd for $C_{29}H_{46}O_4Na$, 481.3294).

*Compound* ***2***: white, amorphous powder; $[\alpha]^{20}_D$ +10 (*c* 0.07, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 244 (4.46), 202 (4.28) nm; IR (KBr) $\nu_{max}$ 3446, 2925, 2873, 1636, 1384, 1096 cm$^{-1}$; $^1$H NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C NMR (pyridine-$d_5$, 125 MHz), see Tables 1 and 2; HRESIMS *m*/*z* 465.3343 $[M + Na]^+$ (calcd for $C_{29}H_{46}O_3Na$, 465.3345).

*Compound* ***3***: white, amorphous powder; $[\alpha]^{20}_D$ −13 (*c* 0.173, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 243 (4.70), 202 (4.55) nm; IR (KBr) $\nu_{max}$ 3406, 2935, 2868, 1701, 1638, 1384, 1088 cm$^{-1}$; $^1$H NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C NMR (pyridine-$d_5$, 125 MHz), see Tables 1 and 2; HRESIMS *m*/*z* 525.3188 $[M + Na]^+$ (calcd for $C_{30}H_{46}O_6Na$, 525.3192).

*Compound* ***4***: white, amorphous powder; $[\alpha]^{20}_D$ +3 (*c* 0.173, $CH_2Cl_2$); IR (KBr) $\nu_{max}$ 3425, 2939, 2872, 1740, 1384, 1075 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 500 MHz) and $^{13}$C NMR ($CDCl_3$, 125 MHz), see Tables 1 and 2; HRESIMS *m*/*z* 611.3925 $[M + Na]^+$ (calcd for $C_{35}H_{56}O_7Na$, 611.3924).

**Acid Hydrolysis of 4 and GC Analysis.** A solution of compound **4** (2.0 mg) was heated in 1 N HCl (1 mL) at 90 °C for 2 h in a sealed ampule. The reaction mixture was concentrated to dryness under $N_2$, the residue was dissolved in 0.1 mL of Tri-Sil Z (*N*-trimethylsilylimidazole−pyridine, 1:4, Pierce Biotechnology, Rockford, IL), and the solution was stirred at 60 °C for 1 h. After drying the solution, the residue was partitioned between cyclohexane and $H_2O$ (1 mL, 1:1 v/v). The combined organic phase was filtered through a 0.45 $\mu$m membrane to remove the precipitate and analyzed by GC.[23] Gas chromatography was performed on a HP-5 column (28 m × 0.32 mm) using a FID detector with He as carrier gas (1.0 mL/min flow rate). The FID detector operated at 260 °C (column temp 180 °C). L-Arabinose was identified by comparison with the retention time of authentic L-Ara ($t_R$ = 4.92 min) after treatment in the same manner with Tri-Sil Z.

*Compound* ***5***: white, amorphous powder; $[\alpha]^{18}_D$ +0 (*c* 0.08, MeOH); IR (KBr) $\nu_{max}$ 3421, 2942, 2869, 1699, 1384, 1093 cm$^{-1}$; $^1$H NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C NMR (pyridine-$d_5$, 125 MHz), see Tables 1 and 2; HRESIMS *m*/*z* 495.3451 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_4Na$, 495.3450).

*Compound* ***6***: white, amorphous powder; $[\alpha]^{20}_D$ +24 (*c* 0.22, MeOH); IR (KBr) $\nu_{max}$ 3421, 2946, 1700, 1384, 1091 cm$^{-1}$; $^1$H NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C NMR (pyridine-$d_5$, 125 MHz), see Tables 1 and 2; HRESIMS *m*/*z* 527.3344 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_6Na$, 527.3349).

## ■ ASSOCIATED CONTENT

**Supporting Information.** This information is available free of charge via the Internet at http://pubs.acs.org.

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## ■ REFERENCES

(1) Gauglitz, G. G.; Song, J.; Herndon, D. N.; Finnerty, C. C.; Boehning, D.; Barral, J. M.; Jeschke, M. G. *Shock* **2008**, *30*, 503–507.

(2) Jeschke, M. G.; Chinkes, D. L.; Finnerty, C. C.; Kulp, G.; Suman, O. E.; Norbury, W. B.; Branski, L. K.; Gauglitz, G. G.; Mlcak, R. P.; Herndon, D. N. *Ann. Surg.* **2008**, *248*, 387–401.

(3) Xu, G. J.; He, H. X.; Xu, L. S.; Jin, R. Y. *Chinese Materia Medical*; Chinese Medicinal Science & Technology Publishing House: Beijing, 1998; Vol. 7, p 223.

(4) Editorial Committee of Chinese Pharmacopeia. *Chinese Pharmacopeia* (2005 ed.); Chemical & Industry Press: Beijing, 2005; p 153.

(5) Yoshida, T.; Tanaka, K.; Chen, X. M.; Okuda, T. *Chem. Pharm. Bull.* **1989**, *37*, 920–924.

(6) Yoshida, T.; Tanaka, K.; Chen, X. M.; Okuda, T. *Phytochemistry* **1989**, *28*, 2451–2454.

(7) Fang, J. M.; Wang, K. C.; Cheng, Y. S. *Phytochemistry* **1991**, *30*, 3383–3387.

(8) Yuan, J. Q.; Yang, X. Z.; Miao, J. H.; Tang, C. P.; Ke, C. Q.; Zhang, J. B.; Ma, X. J.; Ye, Y. *Molecules* **2008**, *13*, 2229–2237.

(9) Gao, P. Y.; Li, L. Z.; Peng, Y.; Piao, S. J.; Zeng, N.; Lin, H. W.; Song, S. J. *Biochem. Syst. Ecol.* **2010**, *38*, 457–459.

(10) UIIah, F.; Hussain, H.; Hussain, J.; Bukhari, I. A.; Gilani, A. H.; Ahmad, V. U. *Phytother. Res.* **2007**, *21*, 1076–1081.

(11) Seto, T.; Tanaka, T.; Tanaka, O.; Naruhashi, N. *Phytochemistry* **1984**, *23*, 2829.

(12) Tundis, R.; Deguin, B.; Menichini, F.; Tillequin, F. *Biochem. Syst. Ecol.* **2002**, *30*, 689–691.

(13) Taniguchi, S.; Imayoshi, Y.; Kobayashi, E.; Takamatsu, Y.; Shimura, S.; Yoshidda, T. *Phytochemistry* **2002**, *59*, 315–323.

(14) Yoshikawa, K.; Kimura, Y. K. E.; Arihara, S. *Phytochemistry* **1998**, *49*, 2057.
(15) Rahman, A.; Ansari, A. A.; Drexler, S. A.; Clardy, J. *Heterocycles* **1982**, *19*, 217.
(16) Zhao, W. Q.; Ding, L. S.; Wang, M. K. *Chin. Chem. Lett.* **2001**, *12*, 245.
(17) Matsuo, Y.; Watanabe, K.; Mimaki, Y. *J. Nat. Prod.* **2009**, *72*, 1155–1160.
(18) Matsuda, H.; Morikawa, T.; Ueda, H.; Yoshikawa, M. *Heterocycles* **2001**, *55*, 1499–1504.
(19) Liang, X.; Song, S. J.; Dai, B. D.; Ma, Z. Q. *Chin. J. Med. Chem.* **2008**, *18*, 381.
(20) Lu, W. Q.; Qiu, Y.; Li, T. J.; Tao, X.; Sun, L. N.; Chen, W. S. *Arch. Pharm. Res.* **2009**, *32*, 1301–1308.
(21) Qiu, L.; Zhao, F.; Jiang, Z. H.; Chen, L. X.; Zhao, Q.; Liu, H. X.; Yao, X. S.; Qiu, F. *J. Nat. Prod.* **2008**, *71*, 642–646.
(22) Gao, H.; Zhao, F.; Chen, G. D.; Chen, S. D.; Yu, Y.; Yao, Z. H.; Wang, Z.; Li, J.; Yao, X. S. *Phytochemistry* **2009**, *70*, 795–806.
(23) Bai, N. S.; He, K.; Ibarra, A.; Bily, A.; Roller, M.; Chen, X. Z.; Rühl, R. *J. Nat. Prod.* **2010**, *73*, 2–6.