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18β-Glycyrrhetinic Acid Derivatives Possessing a Trihydroxylated A Ring Are Potent Gram-Positive Antibacterial Agents

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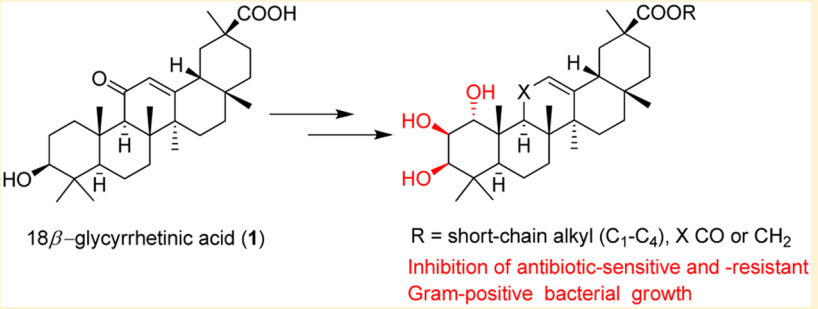
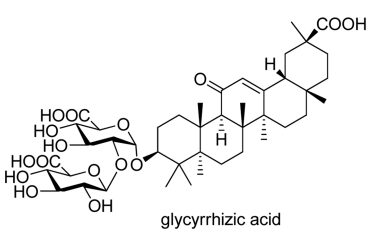
Supporting Information

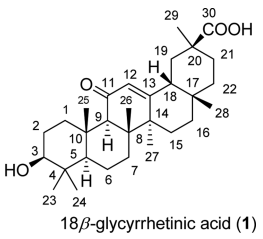


ABSTRACT: The oleanane-type triterpene 18β-glycyrrhetinic acid (**1**) was modified chemically through the introduction of a trihydroxylated A ring and an ester moiety at C-20 to enhance its antibacterial activity. Compounds **22**, **23**, **25**, **28**, **29**, **31**, and **32** showed more potent inhibitory activity against *Streptomyces scabies* than the positive control, streptomycin. Additionally, the inhibitory activity of the most potent compound, **29**, against *Bacillus subtilis*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* was greater than that of the positive controls. The antibacterial mode of action of the active derivatives involved the regulation of the expression of genes associated with peptidoglycans, the respiratory metabolism, and the inherent virulence factors found in bacteria, as determined through a quantitative real-time reverse transcriptase PCR assay.

18β-Glycyrrhetinic acid (**1**), an oleanane-type triterpene, is found as a minor component of licorice (the roots of the leguminous plants *Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fisch., and *Glycyrrhiza inflata* Batalin).[1] However, it can also be obtained from the hydrolysis of glycyrrhizic acid, the major active component of licorice.[1,2] 18β-Glycyrrhetinic acid (**1**) and its analogues have demonstrated a variety of biological activities, such as cytotoxic, hepatoprotective, antiviral, and anti-inflammatory effects.[3] Furthermore, these compounds exhibit broad-spectrum antibacterial activity by inhibiting the growth of *Bacillus subtilis*,[4] *Mycobacterium tuberculosis*,[5] *Staphylococcus aureus*,[6] and *Actinobacillus actinomycetemcomitans*.[6] Structure−activity relationship studies of **1** have indicated that its A ring and the carboxylic acid group at C-20 not only are involved in various biological activities but also act as important sites for structural modification.[7] Other researchers have also reported that the introduction of oxygen functionalities into ring A of **1** and the esterification of the carboxylic acid group confer improved biological activities.[8]

The increasing morbidity and mortality caused by infections with pathogenic bacteria, particularly multi-drug-resistant pathogens, have highlighted an urgent requirement for developing novel antibacterial agents to protect the health





and integrity of human and plant life. A significant proportion of the currently approved drugs that have been designed as new antibacterial agents are natural products or are derived from a natural product scaffold.[9] Natural products and their derivatives with unique and diverse chemical structures usually exhibit broad-spectrum bactericidal activity, particularly potent inhibitory activity against antibiotic-resistant bacterial strains, which provides a platform for the rational design and development of novel antimicrobial therapeutics.[9]

1α-Hydroxy-12-olean-30-oic acid shows weak inhibitory activity against *Escherichia coli*, with a minimum inhibitory

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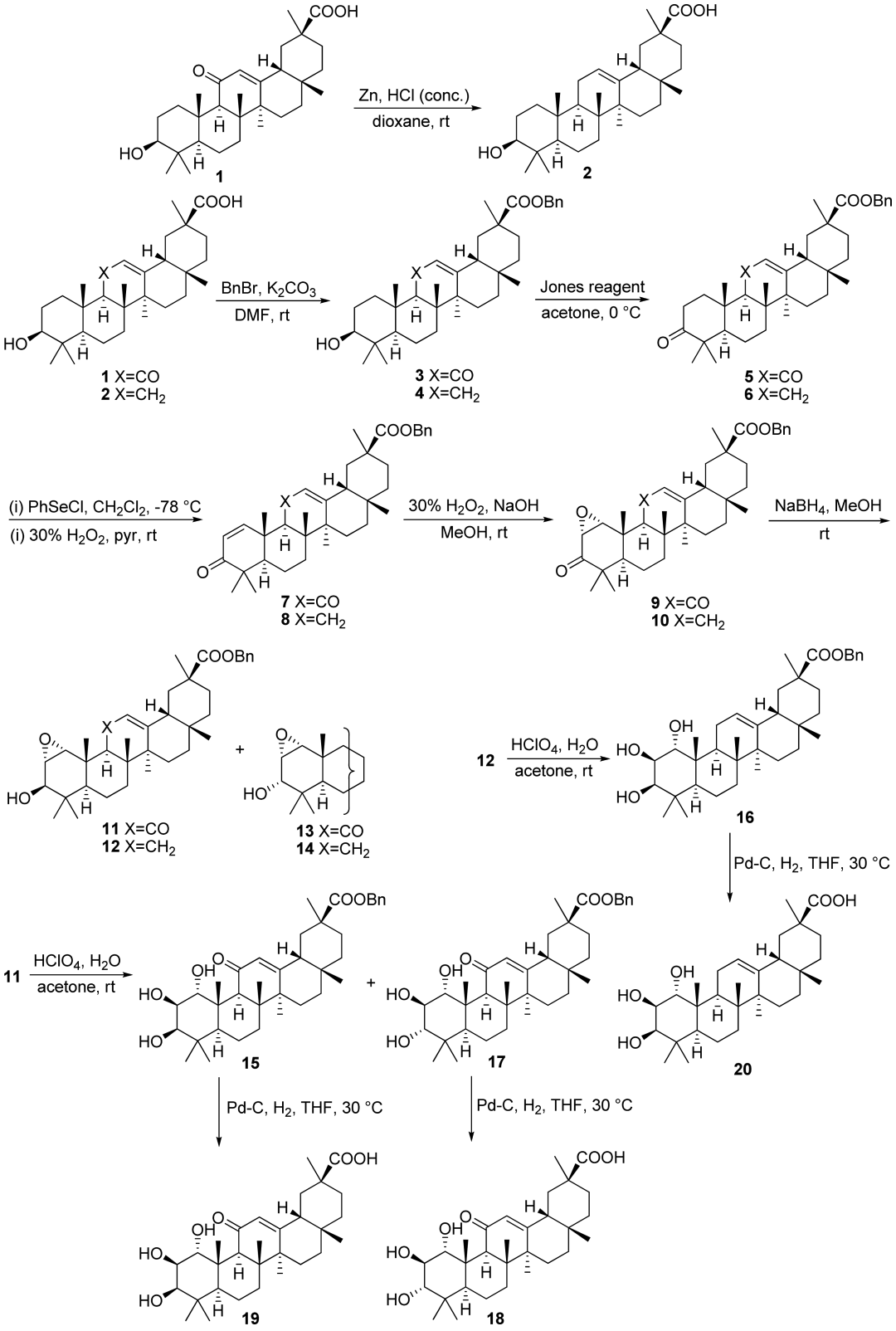
Scheme 1. Synthesis of Derivatives of 18β-Glycyrrhetinic Acid



concentration (MIC) greater than 250 μg/mL.[10] However, 1α,3β-dihydroxy-12-oleanen-30-oic acid, which possesses one more hydroxy group in ring A than the above-mentioned compound, displays better antibacterial activity against *Escherichia coli*, with a MIC of 16 μg/mL.[10] Similarly, oleanolic acid exhibits MIC values of 1.9 and 7.8 μg/mL against *Bacillus thuringiensis* and *Escherichia coli*, respectively, whereas its analogue, maslinic acid, which bears an additional hydroxy group at position C-2 in ring A, exhibits MIC values of 0.9 and 3.9 μg/mL, respectively, against these bacterial strains.[11] It thus appears that the addition of a hydroxy group to ring A of oleanane-type triterpenes increases their antibacterial proper-

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ties. On the basis of these observations, modifying 18β-glycyrrhetinic acid (**1**) through the addition of two hydroxy groups at positions C-1 and C-2 may be expected to enhance the resultant antibacterial activity. To evaluate this hypothesis, a series of trihydroxylated derivatives was synthesized in the present study using 18β-glycyrrhetinic acid (**1**) as the starting scaffold. The antibacterial properties of the parent compound and the synthesized derivatives were assayed against three Gram-negative bacteria, namely, *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and multi-drug-resistant *Pseudomonas aeruginosa* (MDRPA), and four Gram-positive bacteria, namely, *Streptomyces scabies* (*S. scabies*), *Bacillus subtilis* (*B. subtilis*), *Staphylococcus aureus* (*S. aureus*), and methicillin-resistant *Staphylococcus aureus* (MRSA), to identify the structure−activity relationships of the derivatives. It was found that some of the trihydroxylated compounds obtained showed markedly enhanced inhibitory activity against Gram-positive bacterial strains when compared with the parent compound **1** and were even more potent than the positive controls. Furthermore, the most potent derivatives were selected and assayed to investigate the potential mechanisms underlying their activity. These findings may provide new insights for the design of new pentacyclic triterpene derivatives as promising antibiotics.

RESULTS AND DISCUSSION

Chemistry. It was hypothesized that the antibacterial activities of 18β-glycyrrhetinic acid (**1**) and its derivatives can be changed significantly by the reduction of the carbonyl group at position C-11 to a methylene group. Thus, to determine the influence of the carbonyl group on the antibacterial activity, 11-deoxo-18β-glycyrrhetinic acid (**2**) was prepared from **1**.[12] Similar modifications were then made to compounds **1** and **2**.

On the basis of a previous study, triterpenoid acids bearing a trihydroxylated A ring were prepared as detailed in Scheme 1.[13] Enones **7** and **8** were obtained through a two-step process with moderate yields (approximately 70%). Ketones **5** and **6** were first treated with phenylselenenyl chloride (PhSeCl) in CH_2Cl_2 at −78 °C for 15 min, and the mixture was further oxidized with 30% hydrogen peroxide in the presence of pyridine.[14] Additionally, the enones (**7** and **8**) could be synthesized directly by the treatment of the ketones (**5** and **6**) with selenium dioxide in acetic anhydride.[13] However, this method is undesirable because it generates many byproducts in the process, lowering the yield (<50%). The epoxy ketone intermediates (**9** and **10**) were prepared with high selectivity through the epoxidation of the enones (**7** and **8**) using hydrogen peroxide, and yields higher than 90% were obtained after purification by silica gel column chromatography.[15] The relative configuration of the epoxy ketones was proposed based on the results of 1D NMR (^{1}H, ^{13}C, and DEPT NMR) and 2D NMR (HSQC, HMBC, and ROESY) analysis. For example, for compound **10**, oxygenated methines δ_H 3.51 (d, J = 4.4 Hz) and 3.38 (d, J = 4.4 Hz) were assigned to H-1 and H-2 based on the HSQC and HMBC spectra, respectively. The ROESY spectra were used to confirm the relative configuration. NOE correlations were observed between H_3-25β, H-1, and H-2, indicating that these protons are in the β-orientation and therefore suggesting that the epoxy ring is in the α-orientation. Additionally, the formation of the epoxy ring in the α-orientation is in agreement with the reaction mechanism, which involves the nucleophilic attack of the hydrogen peroxide anion at the less-hindered C-1 from the α-side opposite the β-methyl group at the C-10 position. Similar signals were also observed for compound **10**.

Epoxy ketones **9** and **10** were reacted with $NaBH_4$ to obtain the 1α,2α-epoxy-3β-ols **11** and **12** as the major products (yields more than 60%) and the 1α,2α-epoxy-3α-ols **13** and **14** as the minor products (yields less than 25%), after purification by silica gel column chromatography.[16] Based on 1D NMR analysis, the protons at C-1, C-2, and C-3 in compounds **11** and **13** were assigned according to their HSQC, HMBC, and COSY spectra. The OH-3 relative configuration was deduced from the ROESY spectrum. Strong NOE correlations for compound **11** were observed between H-3 and H-5α and between H-1 and H-25β, and no NOE correlation was observed between H-3 and H-25α, implying that H-3 is in the β-orientation and that the hydroxy group is α-oriented. However, the distinct NOE correlations between H-25β, H-1, H-2, and H-3 for compound **13** indicated β-orientations for these protons, thereby suggesting an α-orientation for OH-3. Similarly, the OH-3 configuration of compounds **12** and **14** was confirmed in each case from their ROESY data.

The treatment of the major product **11** with perchloric acid in acetone produced the trihydroxylated compounds **15** and **17** with yields of 66% and 16%, respectively, after purification by silica gel column chromatography.[13] These compounds were crystallized from EtOH, and the structures were confirmed by X-ray crystallography (Figures 1 and 2). The same reaction

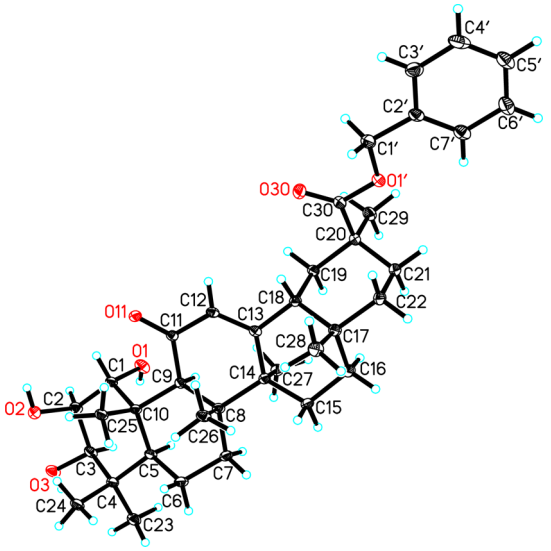

Figure 1. X-ray crystallographic structure of compound **15**. Displacement ellipsoids are shown at the 30% probability level.

with compound **12** generated compound **16** with a yield of 78%. The relative configuration of the hydroxy groups in compound **16** was determined by comparison of its NMR spectra with the data for compounds **15** and **17**.

The protected benzyl ester groups of compounds **15**−**17** were then hydrogenated with 10% palladium over carbon in the presence of hydrogen gas in tetrahydrofuran (THF) to produce the target compounds **18**−**20** in high yields (>85%).[16]

The acid-catalyzed ring-opening reaction of the epoxy compound **11** produced compound **15** and its 3α-hydroxy epimer **17**. In general, this reaction follows mechanism **a** (Scheme 2): the weak nucleophile H_2O attacks the C-2 position from the β-side in the presence of acid to obtain a *trans*-hydroxy group at positions C-1 and C-2 and thereby

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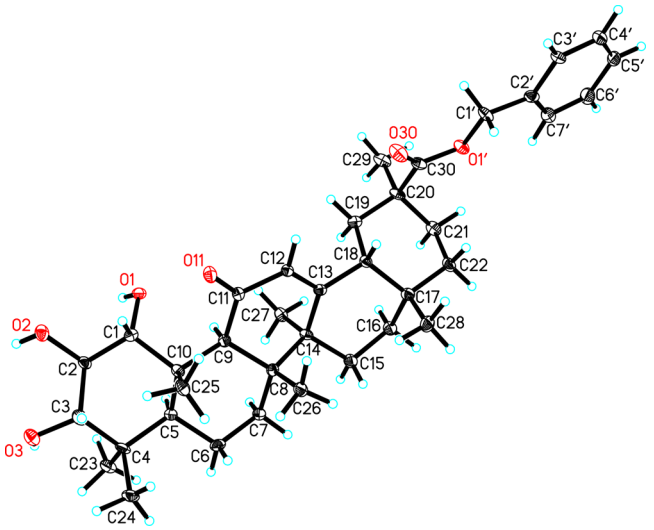


Figure 2. X-ray crystallographic structure of compound **17**. Displacement ellipsoids are shown at the 30% probability level.

generate compound **15**.[17] The formation of compound **17** is explained by mechanism **b** (Scheme 2): the position of the oxirane ring moves in the presence of the neighboring hydroxy group, and the nucleophile H_2O then attacks C-3 from the α-side to produce compound **17**.[18] However, the same reaction with **12** did not yield the 3α-hydroxy epimer of **16**, perhaps due to high stereospecificity or its removal during the purification process.

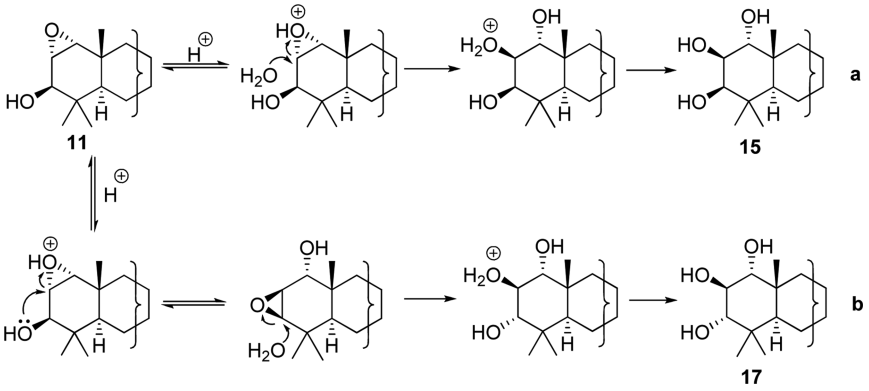
Additional modifications were focused on the esterification of the carboxylic acid group at C-20 of compounds **19** and **20** (Scheme 3). Due to the insufficient quantities obtained, compound **18** could not be further modified. Compounds **19** and **20** were reacted with various alkyl halides to obtain ester derivatives (compounds **21**−**42**),[16] including alkyl esters (linear-chain alkyl ester and branched-chain alkyl ester derivatives) and benzyl esters (nitro-substituted and chloro-substituted benzyl ester derivatives), in high yields (>95%).

In Vitro Antibacterial Activity. The parent compound **1** and all of its prepared derivatives were subjected to turbidimetric assays to assess their in vitro inhibitory activities against the bacterial growth of seven bacterial strains, namely, *E. coli*, *P. aeruginosa*, MDRPA, *S. scabies*, *B. subtilis*, *S. aureus*, and MRSA.[19] Ampicillin, streptomycin, and vancomycin were used as positive controls. The compounds were tested initially using a single dose of 100 μM to determine whether they completely inhibited bacterial growth. After identification of the active compounds, the MIC values of these compounds, determined through broth microdilution assays and representing the lowest concentration of the antibacterial agent at which bacterial growth was not apparent when compared with the negative control, which consisted of vehicle without microorganisms, were determined to assess the potency of the compounds.

The results indicated that none of the derivatives showed antibacterial activity against the Gram-negative bacterial strains, namely, *E. coli*, *P. aeruginosa*, and MDRPA. In contrast, some of the compounds exhibited an improvement in potency against the Gram-positive bacterial strains, namely, *S. scabies*, *B. subtilis*, *S. aureus*, and MRSA, as presented in Table 1. Similar to the findings obtained in previous studies, these results confirmed that the oleanane-type triterpenes preferentially inhibit the growth of Gram-positive bacterial growth, although the mechanism of action has not been fully elucidated.[20]

The intermediates (**7**−**14**) possessing an α,β-unsaturated ketone or an α-epoxy group in ring A showed negligible inhibitory activity against all of the bacterial strains tested. Notably, the derivatives with the highest activity against Gram-positive bacteria were compounds **22**, **23**, **25**, **28**, **29**, **31**, and **32**, which possess three hydroxy groups in ring A and short-chain alkyl (C_1−C_4) ester functionalities at C-20 (Table 1). However, both the trihydroxylated derivatives (**26**, **27**, **33**−**42**), which contain long-train alkyl ester groups or substituted benzyl ester groups, and compounds **18**, **19**, and **20**, which have a carboxylic acid group at C-20, were inactive or exhibited weak activity, suggesting that the short-chain alkyl ester moiety is essential for the antibacterial activity of the trihydroxylated compounds. Among the compounds tested, compound **29**, which has an ethyl ester group at C-20, showed the highest activity against *B. subtilis*, *S. aureus*, and MRSA, with MIC values lower than those of the positive controls. Additionally, compounds **22**, **23**, **25**, **28**, **29**, **31**, and **32** exhibited potent inhibitory activity against *S. scabies*, with MIC values ranging from 1.0 to 4.4 μg/mL. Of these compounds, **28**, which possesses a methyl ester group, was the most active, with a MIC value that was 5-fold lower than that of the positive control. Csuk et al. found no direct relationship between the C-11 ketone group and the apoptotic activity of compound **1** derivatives.[21] However, in the present study, a significant difference in bacterial inhibition activity was observed between the 11-oxo and 11-deoxo compounds. Of these two compound types, the 11-oxo compounds, such as **13** and **23**, showed higher activity levels than their corresponding 11-deoxo

Scheme 2. Mechanism for the Acid-Catalyzed Epoxide Ring-Opening Reaction



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Scheme 3. General Synthesis of Compounds 21−42

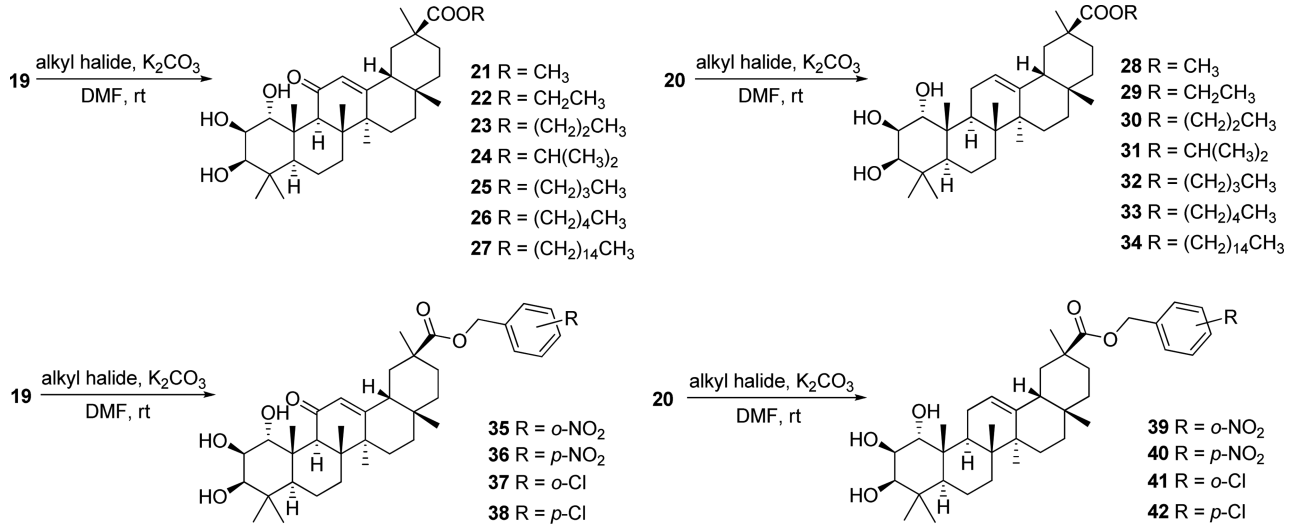

Table 1. In Vitro Antibacterial Activity of Selected Derivatives[a]

	MIC (μg/mL)			
compound	*B. subtilis*	*S. scabies*	*S. aureus*	MRSA
8	>34.6	34.6	>34.6	>32.0
13	>36.7	36.7	>36.7	>32.0
15	>37.8	>37.8	>37.8	4.0
16	>36.9	>36.0	>36.9	8.0
17	>37.8	>37.8	>37.8	4.0
20	>31.2	31.2	15.6	>32.0
22	16.9	2.1	4.2	4.0
23	>34.8	4.3	4.3	2.0
24	>34.8	>34.8	>34.8	8.0
25	>35.7	4.4	>35.7	4.0
28	4.0	1.0	2.0	>32.0
29	2.0	4.1	1.0	1.0
30	>33.9	>33.9	8.4	8.0
31	>33.9	4.2	8.4	2.0
32	>34.8	4.3	>34.8	>32.0
ampicillin	nt[b]	nt	1.4	nt
streptomycin	2.9	5.8	nt	nt
vancomycin	nt	nt	nt	2.0

[a]The derivatives not listed in this table were inactive (MIC > 50 μg/mL). [b]Not tested.

compounds, **14** and **30**, respectively (Table 1). Conversely, the 11-deoxo compounds, such as **8**, **20**, and **28**, displayed higher activity levels than their corresponding 11-oxo compounds, **7**, **19**, and **21**, respectively (Table 1). Thus, these results support the initial hypothesis that the antibacterial activity of compound **1** and its derivatives can be altered significantly by the reduction of the carbonyl group at position C-11 to a methylene group, although the structure−activity relationships are not entirely clear.

The results indicated that the introduction of two hydroxy groups at the C-1 and C-2 positions of compound **1** can improve its antibacterial activity against Gram-positive bacteria. Moreover, although no studies conducted to date have concentrated on the role of the hydroxy groups of pentacyclic triterpenes as a possible contributor to the inhibitory activity against antibiotic-resistant bacterial strains, this study showed that trihydroxylated derivatives (compounds **23**, **29**, and **31**) exhibited potent inhibitory activity against the antibiotic-resistant strain MRSA, implying that this type of compound exhibits the potential to be developed as agents against antibiotic-resistant bacteria.

Time-Kill Assays. The inhibitory activities of derivatives **28** and **29**, which had the lowest MIC values, were studied using time-kill assays.[19] As shown in Figure 3, compounds **28** and **29** displayed dose-dependent inhibitory activity against the growth of three Gram-positive bacterial strains. At concentrations equal to 0.5 times their MIC values, the compounds showed a limited inhibitory effect before regrowth, whereas at the other concentrations tested (1× MIC, 2× MIC, and 4× MIC), the survival initially decreased at a rapid rate and then gradually slowed. Similar growth inhibition patterns were observed for both *B. subtilis* and *S. aureus* using different concentrations of the compounds. The killing time of compound **28** was longer than 24 h for *S. scabies* due to the slow growth of this bacterium.

Effects of the Compounds on the Expression of Bacterial Genes Related to Metabolism and Virulence. Compound **1** represses the synthesis of DNA, RNA, and protein in *B. subtilis* and reduces the expression of virulence genes in MRSA.[22] To investigate the antibacterial mechanism of the active derivatives at the molecular level, the parent compound **1** and selected derivatives (**28** and **29**) were subjected to a Q-RT-PCR assay to determine whether these compounds (at a concentration equal to 0.5× MIC) could regulate the expression of bacterial genes related to metabolism and virulence.[22b,23] The genes selected for this experiment are associated with peptidoglycans (*ykuD* and *lytF* in *B. subtilis*, *lytM* and *fmhB* in *S. aureus* and MRSA, and *murX* in *S. scabies*), respiratory metabolism (*mmgD* in *B. subtilis*, *narG* in *S. aureus* and MRSA, and *p450* in *S. scabies*), and virulence regulation (*yhdT* and *yqhB* in *B. subtilis*, *seaR* and *hla* in *S. aureus*, *seaR*, *hla*, and *mecA* in MRSA, and *txtA*, *txtC*, *nec1*, and *tomA* in *S. scabies*).

Peptidoglycans, which are essential cell-wall components in bacteria, play an important role in adhesion, morphological control, and signal transduction and have been the most important target for therapeutic intervention in bacterial infections, particularly antibiotic treatment.[24] The *ykuD* gene

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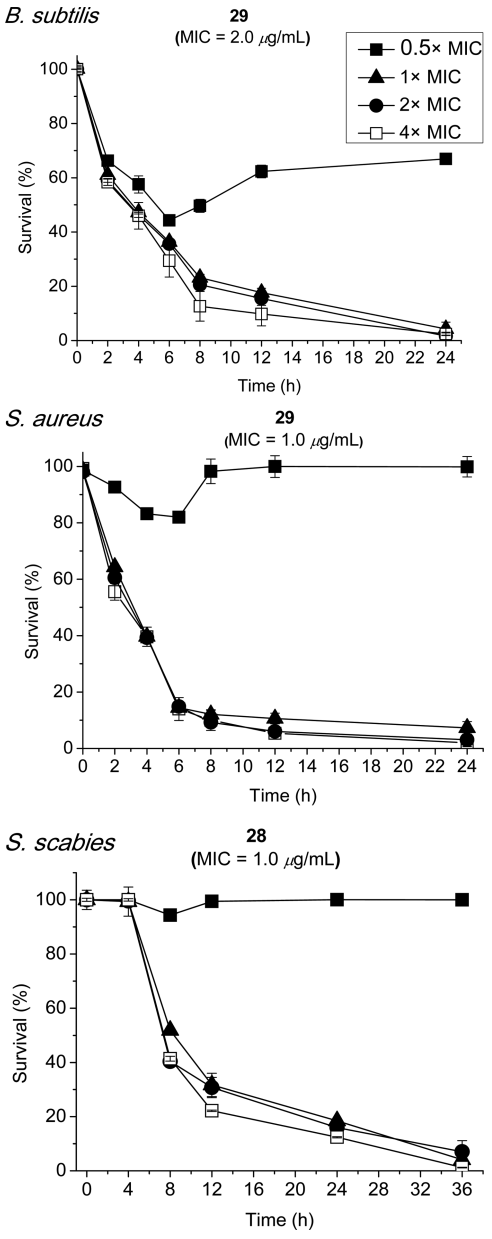

Figure 3. Time-kill analysis of selected compounds **28** and **29** against three Gram-positive bacterial strains. The curves represent the survival rates of the bacteria treated with different concentrations of the compounds. Each data point represents the results from three experiments, and the error bars show the standard errors of the means (SEM).

encodes a peptidoglycan transglycosylase that participates in peptidoglycan degradation during bacterial autolysis,[25] and *lytF* encodes a peptidoglycan endopeptidase that plays essential roles in peptidoglycan turnover, cell separation, and bacterial autolysis.[26] These two genes were significantly up-regulated by compound **29** in *B. subtilis* (Figure 4A), suggesting that the inhibitory activity of this compound may induce bacterial autolysis. Another autolytic gene, *lytM*, was not altered in *S. aureus* in response to treatment with compounds **1** and **29** (Figure 4B).[23] However, compound **29** inhibited the expression of this gene in MRSA (Figure 4C). The *fmhB* and *murX* genes encode the peptidoglycan synthases FmhB and phospho-*N*-acetylmuramoyl-pentapeptide-transferase, respectively.[27] Notably, the FmhB protein, which catalyzes the synthesis of the

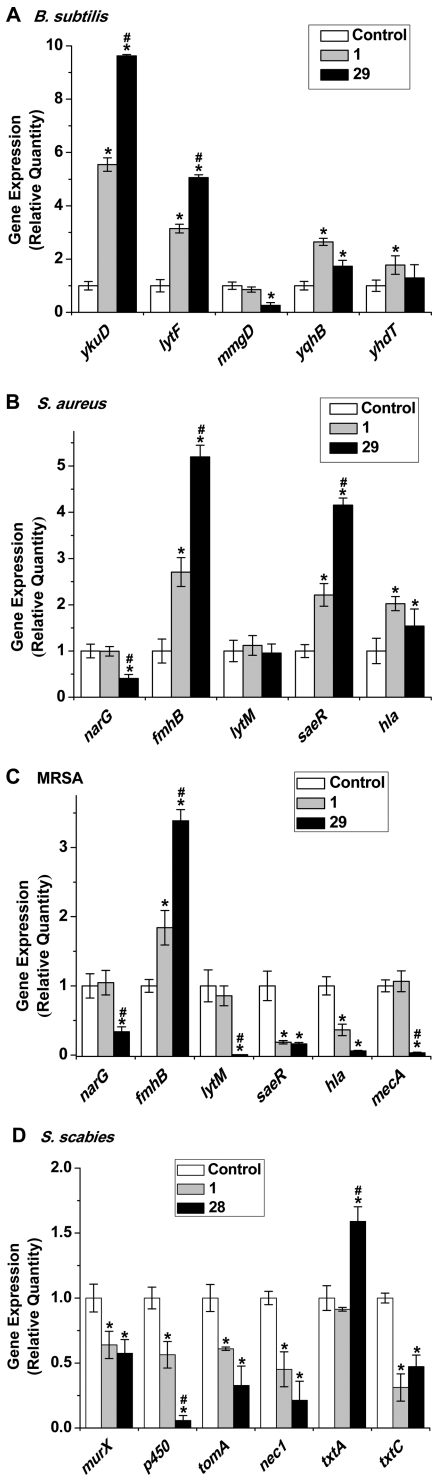

Figure 4. Relative gene expression in three bacterial strains treated with the test compounds in vitro. The data were analyzed by Q-RT-PCR and are expressed as the means ± SEM (n = 3). The asterisks indicate statistically significant differences between the derivative-treated group and the control group ($^{*}p < 0.05$), and the pound signs indicate statistically significant differences between the derivative-treated group and the compound **1**-treated group ($^{\#}p < 0.05$).

pentaglycine interpeptide in *S. aureus* peptidoglycans, is important for cell-wall cross-linking and stability and acts as an anchor for cell-wall-associated proteins and pathogenicity determinants.[27] The expression of *fmhB* in *S. aureus* and MRSA was increased after treatment with compound **29** compared

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with the control and the parent compound **1**, whereas *murX* expression in *S. scabies* was reduced by treatment with compound **28** as compared with the control (Figure 4B, C, and D). These results provide the first evidence that the active derivatives of compound **1** may affect the enzymes participating in peptidoglycan metabolism, resulting in cell-wall instability or disruption. A similar finding was also observed in previous studies of oleanolic acid, which belongs to the family of oleanane-type triterpenes, similar to compound **1**.[28] After this alteration in the peptidoglycan structure, the Gram-positive bacteria are perhaps more susceptible to the compounds, which can now enter the cell more easily.

The levels of the *mmgD* and *narG* genes, which are related to respiratory metabolism, were reduced in the bacterial strains when treated with the test compounds when compared with the control (Figure 4A, B, and C). However, these genes were not significantly reduced in compound **1**-treated bacteria, indicating that compound **1** and its derivatives have different antibacterial mechanisms. Interestingly, *p450* expression was decreased in *S. scabies* after treatment with compounds **1** and **28** (Figure 4D). The *mmgD* gene contributes to the methylcitric acid cycle by converting propionyl-CoA to pyruvate, which is a key intersection in the metabolic network.[29] The inhibition of *mmgD* expression would block the methylcitric acid cycle and affect propionyl-CoA metabolism and bacterial growth.[29] The *narG* gene in *S. aureus* encodes a membrane-bound nitrate reductase that participates in the nitrogen metabolic pathway.[30] In turn, *p450* monooxygenase gene encodes the key functional enzyme in the respiratory chain, which transforms a wide variety of secondary metabolite and xenobiotic substrates and thus plays an important role in detoxification.[31] These critical respiratory metabolism genes were down-regulated by the selected compounds, which may indicate that the derivatives lower metabolic efficiency and detoxification and inhibit bacterial energy mechanisms, resulting in the observed antibacterial activity.

The virulence genes *yhdT* and *yqhB* in *B. subtilis* and *seaR* and *hla* in *S. aureus*, which are related to hemolytic activity, were up-regulated by compounds **1** and **29** (Figure 4A and B). However, the expression of *seaR* and *hla* in MRSA was inhibited by these two compounds (Figure 4C), suggesting that the test compounds may decrease the pathogenicity of MRSA. The *mecA* gene is the main determinant for methicillin resistance and encodes a specific penicillin-binding protein (PBP), PBP2a, in MRSA to confer resistance to β-lactam antibiotics.[22b] This gene was down-regulated by compound **29** when compared with the control and compound **1** (Figure 4C). This observation indicates that compound **29** has the potential to restore the inhibitory activity of β-lactam antibiotics against MRSA when administered together with these antibiotics.The *txtA*, *txtC*, *nec1*, and *tomA* genes in *S. scabies*, which are responsible for potato scab disease, were significantly down-regulated by the test compounds, with the exception of *txtA* (Figure 4D).[32] The altered expression of the virulence genes demonstrates the influence of the derivatives produced an in vitro investigation on bacterial pathogenicity.

In summary, 28 new compounds possessing a trihydroxylated A ring were synthesized from the natural product 18β-glycyrrhetinic acid (**1**), and the absolute structure of this novel compound type was confirmed by X-ray crystallography as compounds **15** and **17**. The derivatives exhibited preferential growth-inhibiting activity against Gram-positive bacteria in vitro. Among the compounds prepared, compounds **28** and **29** displayed the most potent activity, with MIC values that were lower than those of the positive controls. Structure−activity relationship studies showed that the substitution of positions C-1 and C-2 of 18β-glycyrrhetinic acid (**1**) with α- and β-hydroxy groups, respectively, and the replacement of the carboxylic acid group at C-20 with a short-chain alkyl ester group led to increased antibacterial activity against antibiotic-sensitive and -resistant Gram-positive bacteria. Furthermore, Q-RT-PCR studies revealed that the antibacterial activity of the active derivatives involved the regulation of several genes associated with peptidoglycans, respiratory metabolism, and virulence factors. Therefore, the modifications, structure−activity relationships, and mechanistic studies reported herein provide insights that should enable the further development of 18β-glycyrrhetinic acid (**1**) or its analogues as viable and highly effective antibacterial agents, particularly as inhibitors of antibiotic-resistant bacteria.

EXPERIMENTAL SECTION

General Experimental Procedures. Ampicillin, streptomycin, and vancomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and the commercial reagents used were of analytical reagent grade. The melting points are uncorrected (Leica hot-stage microscope). The optical rotations were measured using a PerkinElmer 241 polarimeter, and the $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. The IR spectra were recorded using KBr disks on a PerkinElmer FT-IR spectrometer. The 1D NMR and 2D NMR spectroscopic data were collected on a Varian INOVA 400 MHz or a Varian Nova 500 MHz spectrometer using tetramethylsilane as the internal standard. EIMS was performed using an HP1100-MSD spectrometer, and ESIMS was performed using an Agilent 1100/MS-G1946 spectrometer. TLC was performed on silica gel 60 F_{254} plates purchased from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, People's Republic of China) and visualized by exposure to UV light and the use of an iodine chamber or by spraying with a 10% solution of sulfuric acid in ethanol followed by heating. All of the compounds produced were subjected to HPLC analysis on a Waters Sunfire CH, C_{18} (5 μm, 250 × 4.6 mm) column, with two solvent systems (acetonitrile−water and methanol−water). The X-ray crystal structure data were obtained using a Bruker APEX-II CCD diffractometer at 100(2) K with Cu Kα radiation (λ = 1.541 78 Å).

Microorganisms and Culture Media. The *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *B. subtilis* ATCC 6051, *S. aureus* ATCC 25923, and *S. scabies* CGMCC4.7610246 bacterial strains were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, People's Republic of China). MDRPA and MRSA were isolated from clinical specimens obtained from Kunming General Hospital of Chengdu Military Area Command, People's Republic of China, from 2009 to 2010 and were stored frozen at −80 °C. Specimen collection procedures were approved by the Institutional Review Board (human studies review committee), and the protocol number was 96−-049. *E. coli*, *S. aureus*, and *B. subtilis* were cultured in Luria−Bertani medium (LB, Oxoid, Basingstoke, UK) on a shaker at 37 °C, MDRPA and MRSA were cultured in Mueller-Hinton broth (Oxoid, Basingstoke, UK) supplemented with 25.0 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+} at 35 °C, and *S. scabies* was cultured in oat bran broth prepared according to the instructions provided by Goyer et al. at 28 °C.[33] The microorganisms were collected at the log phase of growth by centrifugation, washed once, and suspended in the broth for turbidimetry assays.

Plant Material. The roots of *Glycyrrhiza uralensis* were produced in the People's Republic of China and were purchased from the Sanqiao Crude Drug Market, Guiyang, People's Republic of China, in October 2011. The plants were identified by Prof. Lu-Tai Pan at the School of Pharmacy, Guiyang College of Traditional Chinese Medicine. A voucher specimen (No. 2010-0916) has been deposited at the Herbarium of the Guiyang College of Traditional Chinese Medicine.

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Extraction and Isolation of 18β-Glycyrrhetinic Acid (1).[2] Air-dried and powdered roots of *G. uralensis* (20 kg) were extracted three times with 75% ethanol for 2 h under reflux. The extract was concentrated under a vacuum to yield a residue, which was partitioned successively three times between dichloromethane, ethyl acetate, *n*-butanol, and water. After evaporation, the *n*-butanol fraction (530 g) was chromatographed over a silica gel column to obtain eight fractions, which were eluted with a gradient of MeOH in $CHCl_3$ (0 to 50%). Fraction 5 was subjected to silica gel H (average particle size of 10 μM) column chromatography, with an eluent of $CHCl_3$–MeOH (8:2). This was purified further using Sephadex LH-20 (MeOH) to afford a glycoside (5.6 g), which was identified as glycyrrhizic acid based on a comparison of its NMR spectroscopic data with literature values.[2] Glycyrrhizic acid (650 mg) was then dissolved in 200 mL of MeOH containing 10% H_2SO_4 for hydrolysis under reflux for 4 h. The reaction mixture was diluted with water, neutralized with a 10% NaOH solution, and extracted three times with $CHCl_3$, and the organic layer was evaporated under vacuum. The crude aglycone was purified by silica gel H column chromatography and then recrystallized from acetic acid to yield compound **1** as white needle crystals (purity >95% by HPLC; mp 293–295 °C, lit.[34] mp 295 °C); its spectroscopic data were consistent with the published literature data.[34]

Synthesis of α,β-Unsaturated Ketones (Compounds 7 and 8). The 3-ketones **5** and **6** were prepared with good yields (>90%) as described in the literature.[12,16] PhSeCl (1.84 g, 9.6 mmol, 1.2 equiv) was added to a solution of the 3-ketone compound (**5** or **6**, 8.0 mmol, 1.0 equiv) in dry dichloromethane (200 mL). The mixture was stirred at −78 °C for 15 min, and 30% hydrogen peroxide (0.29 mL, 9.6 mmol, 1.2 equiv) and pyridine (20 mL) were then added to the mixture. The reaction mixture was stirred at room temperature for 1.5 h, and most of the solvent was then distilled off under vacuum. The residue was extracted with EtOAc (3 × 100 mL), and the organic phase was washed with saturated aqueous $NaHCO_3$ and brine, dried with anhydrous $MgSO_4$, filtered, and concentrated under vacuum. Each crude product was purified by silica gel column chromatography with petroleum ether–ethyl acetate as the eluent to yield the enone products.

*Benzyl 3,11-dioxo-18β-olean-1,12-dien-30-oate (**7**):* 3.14 g, 71% yield; yellow, amorphous powder; $[\alpha]^{30}_D$ +190.6 (*c* 0.67, $CHCl_3$); IR (KBr) ν_{max} 3062, 3033, 2968, 2867, 1727, 1652, 1615, 1462, 1386, 1212, 1162, 766, 750, 716 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.73 (1H, d, *J* = 10.0 Hz, H-1), 7.38–7.34 (5H, m, H-Ar), 5.79 (1H, d, *J* = 10.0 Hz, H-2), 5.64 (1H, s, H-12), 5.22 (1H, d, *J* = 12.4 Hz, Bn-CH_{2a}), 5.09 (1H, d, *J* = 12.4 Hz, Bn-CH_{2b}), 2.65 (1H, s, H-9), 1.41, 1.37, 1.17, 1.16, 1.16, 1.11, 0.75 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.6, 198.9, 176.1, 170.5, 161.6, 136.0, 128.5, 128.5, 128.3, 128.2, 128.2, 128.0, 124.5, 66.2, 52.5, 52.7, 48.2, 45.4, 44.7, 43.9, 43.4, 41.0, 38.7, 37.5, 31.9, 31.7, 31.0, 28.4, 28.2, 27.5, 26.4, 26.2, 23.3, 21.5, 20.0, 18.8, 18.1; EIMS *m/z* 556 $[M]^+$ (50), 541 (10), 468 (24), 91 (100).

*Benzyl 3-oxo-18β-olean-1,12-dien-30-oate (**8**):* 3.13 g, 72% yield; yellow, amorphous powder; $[\alpha]^{30}_D$ +115.2 (*c* 0.58, $CHCl_3$); IR (KBr) ν_{max} 3442, 2929, 1728, 1668, 1455, 1382, 1214, 1155, 1083, 824, 733, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.34 (5H, m, H-Ar), 7.04 (1H, d, *J* = 10.4 Hz, H-1), 5.81 (1H, d, *J* = 10.4 Hz, H-2), 5.22 (1H, br s, H-12), 5.20 (1H, d, *J* = 12.4 Hz, Bn-CH_{2a}), 5.09 (1H, d, *J* = 12.4 Hz, Bn-CH_{2b}), 1.18, 1.15, 1.14, 1.13, 1.10, 1.04, 0.75 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.3, 176.8, 159.2, 144.8, 136.3, 128.4, 128.4, 128.0, 128.0, 128.0, 124.9, 121.7, 65.9, 53.3, 48.2, 44.5, 44.2, 42.5, 41.8, 41.6, 40.5, 39.3, 38.1, 32.3, 31.9, 31.2, 28.5, 28.1, 27.8, 26.8, 26.0, 25.8, 23.4, 21.6, 18.9, 18.8, 17.3; EIMS *m/z* 542 $[M]^+$ (6), 451 (64), 405 (35), 247 (62), 91 (100).

Synthesis of Epoxy Ketones (Compounds 9 and 10). A 10% NaOH aqueous solution (7.7 mmol, 1.4 equiv) was added dropwise to a solution of ketone (**7** or **8**, 5.5 mmol, 1.0 equiv) in MeOH (200 mL) with continuous stirring in an ice bath for 30 min. After a 30% H_2O_2 solution (1.7 mL, 55.0 mmol, 10.0 equiv) was added to the mixture, the reaction mixture was stirred at room temperature until the starting material was not observed by TLC. The resulting reaction mixture was quenched with a 1 M HCl solution, and most of the solvent was then distilled off under vacuum. The residue was extracted with EtOAc (3 × 100 mL). The organic phase was washed with saturated aqueous $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. Each crude product was then chromatographed on a silica gel column with a petroleum ether–acetone solution as the eluent to obtain the pure compound.

*Benzyl 1α,2α-epoxy-3,11-dioxo-18β-olean-12-en-30-oate (**9**):* 2.9 g, 93% yield; white, amorphous powder; $[\alpha]^{30}_D$ +243.8 (*c* 0.26, $CHCl_3$); IR (KBr) ν_{max} 2955, 2866, 1731, 1698, 1649, 1466, 1389, 1216, 1160, 1085, 865, 750, 696 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.41–7.33 (5H, m, H-Ar), 5.64 (1H, s, H-12), 5.21 (1H, d, *J* = 12.4 Hz, Bn-CH_{2a}), 5.10 (1H, d, *J* = 12.4 Hz, Bn-CH_{2b}), 4.49 (1H, d, *J* = 4.4 Hz, H-2), 3.38 (1H, d, *J* = 4.4 Hz, H-1), 2.90 (1H, s, H-9), 1.41, 1.21, 1.17, 1.13, 1.11, 1.02, 0.75 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.4, 198.9, 176.1, 170.7, 136.0, 128.5, 128.5, 128.3, 128.2, 128.2, 127.9, 66.2, 64.6, 57.2, 54.5, 48.2, 45.5, 45.1, 44.8, 43.9, 43.4, 41.0, 38.3, 37.5, 31.7, 31.6, 31.0, 28.4, 28.2, 27.9, 26.5, 26.2, 23.2, 20.9, 18.6, 18.1, 15.8; EIMS *m/z* 572 $[M]^+$ (40), 557 (6), 481 (35), 463 (22), 91 (100).

*Benzyl 1α,2α-epoxy-3-oxo-18β-olean-12-en-30-oate (**10**):* 2.8 g, 92% yield; white, amorphous powder; $[\alpha]^{30}_D$ +160.0 (*c* 0.40, $CHCl_3$); IR (KBr) ν_{max} 2956, 1727, 1699, 1455, 1383, 1212, 1155, 1084, 732, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.37–7.26 (5H, m, H-Ar), 5.22 (1H, br s, H-12), 5.21 (1H, d, *J* = 12.4 Hz, Bn-CH_{2a}), 5.08 (1H, d, *J* = 12.4 Hz, Bn-CH_{2b}), 3.51 (1H, d, *J* = 4.4 Hz, H-1), 3.38 (1H, d, *J* = 4.4 Hz, H-2), 1.20, 1.16, 1.11, 1.01, 1.01, 0.98, 0.75 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 212.9, 198.9, 176.9, 144.8, 136.3, 128.4, 128.4, 128.0, 128.0, 128.0, 121.7, 65.9, 63.9, 56.9, 48.2, 45.9, 44.8, 44.2, 42.6, 41.7, 40.5, 39.9, 38.4, 38.2, 32.0, 31.9, 31.2, 28.5, 28.1, 27.9, 26.8, 26.1, 25.7, 23.9, 20.9, 18.8, 17.1, 15.1; EIMS *m/z* 558 $[M]^+$ (3), 467 (38), 421 (24), 338 (16), 247 (63), 91 (100).

General Procedure for the Synthesis of Compounds 11–14. The epoxy ketone (**9** or **10**, 4.3 mmol, 1.0 equiv) was dissolved in MeOH (100 mL), and $NaBH_4$ (817.0 mg, 21.5 mmol, 5.0 equiv) was added slowly to the solution while stirring in an ice bath for 2 h. After quenching with water (200 mL), the mixture was extracted with EtOAc (3 × 200 mL), and the organic phase was washed with saturated aqueous $NaHCO_3$ and brine. The combined organic phases were dried over anhydrous $MgSO_4$, filtered, and concentrated. Each residue was then isolated by silica gel column chromatography using petroleum ether–acetone as the eluent to yield a pure compound.

*Benzyl 1α,2α-epoxy-3β-hydroxy-11-oxo-18β-olean-12-en-30-oate (**11**):* 1.49 g, 61% yield; white, amorphous powder; $[\alpha]^{30}_D$ +173.9 (*c* 0.29, $CHCl_3$); IR (KBr) ν_{max} 3446, 2957, 2868, 1728, 1658, 1455, 1386, 1215, 1160, 826, 751, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.40–7.33 (5H, m, H-Ar), 5.59 (1H, s, H-12), 5.21 (1H, d, *J* = 12.4 Hz, Bn-CH_{2a}), 5.09 (1H, d, *J* = 12.4 Hz, Bn-CH_{2b}), 4.10 (1H, d, *J* = 2.8 Hz, H-1), 3.55 (1H, d, *J* = 5.6 Hz, H-3), 3.05 (1H, d, *J* = 3.6 Hz, H-2), 2.79 (1H, s, H-9), 1.36, 1.30, 1.16, 1.12, 0.94, 0.81, 0.73 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.9, 176.2, 169.8, 136.0, 128.6, 128. 6, 128.2, 128.1, 128.1, 128.0, 75.3, 66.2, 61.2, 57.9, 56.1, 48.2, 45.2, 44.5, 43.9, 43.3, 40.9, 37.6, 36.9, 36.3, 32.4, 31.7, 31.1, 28.3, 28.2, 28.2, 26.4, 26.3, 23.2, 18.9, 17.9, 17.8, 16.0; EIMS *m/z* 574 $[M]^+$ (12), 559 (13), 491 (27), 91 (100).

*Benzyl 1α,2α-epoxy-3β-hydroxy-18β-olean-12-en-30-oate (**12**):* 1.67 g, 69% yield; white, amorphous powder; $[\alpha]^{30}_D$ +109.1 (*c* 0.33, $CHCl_3$); IR (KBr) ν_{max} 3442, 2955, 1729, 1658, 1455, 1381, 1214, 1148, 1082, 1049, 1027, 752, 697 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.37–7.32 (5H, m, H-Ar), 5.20 (1H, d, *J* = 12.0 Hz, Bn-CH_{2a}), 5.18 (1H, br s, H-12), 5.08 (1H, d, *J* = 12.8 Hz, Bn-CH_{2b}), 3.57 (1H, s, H-3), 3.07 (1H, d, *J* = 4.0 Hz, H-2), 2.99 (1H, d, *J* = 4.0 Hz, H-1), 1.15, 1.14, 1.13, 0.97, 0.95, 0.79, 0.74 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.9, 144.4, 136.4, 128.4, 128.4, 128.0, 127.9, 127.9, 121.9, 75.8, 65.9, 60.3, 57.3, 48.1, 45.5, 44.2, 42.7, 41.5, 41.1, 39.7, 38.2, 36.9, 36.4, 32.3, 31.8, 31.3, 28.5, 28.4, 28.1, 26.8, 26.1, 25.8, 23.6, 18.0, 16.9, 16.8, 16.6; EIMS *m/z* 558 $[M - 2H]^+$ (6), 451 (22), 405 (22), 338 (82), 247 (100), 91 (65).

*Benzyl 1α,2α-epoxy-3α-hydroxy-11-oxo-18β-olean-12-en-30-oate (**13**):* 575.1 mg, 23% yield; white, amorphous powder; $[\alpha]^{30}_D$ +122.4 (*c* 0.49, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.40–7.33

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(5H, m, H-Ar), 5.59 (1H, s, H-12), 5.21 (1H, d, J = 12.4 Hz, Bn-CH_{2a}), 5.09 (1H, d, J = 12.0 Hz, Bn-CH_{2b}), 4.29 (1H, d, J = 4.4 Hz, H-1), 3.54 (1H, d, J = 4.8 Hz, H-3), 3.42 (1H, dd, J = 4.4, 4.8 Hz, H-2), 2.80 (1H, s, H-9), 1.39, 1.28, 1.17, 1.11, 0.89, 0.82, 0.74 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.8, 176.2, 169.9, 136.1, 128.6, 128. 6, 128.2, 128.1, 128.1, 128.0, 72.5, 66.2, 63.1, 56.2, 55.9, 48.3, 45.3, 43.9, 43.3, 41.7, 40.9, 37.6, 37.3, 36.4, 32.4, 31.7, 31.1, 28.4, 28.2, 26.4, 26.3, 25.8, 23.7, 23.3, 18.9, 17.8, 16.6; EIMS m/z 575 $[M + H]^+$ (10), 560 (27), 491 (17), 393 (31), 352 (21), 135 (53), 91 (100).

*Benzyl 1α,2α-epoxy-3α-hydroxy-18β-olean-12-en-30-oate (**14**):* 484.0 mg, 20% yield; white, amorphous powder; $[\alpha]^{30}_D$ +120.6 (c 0.49, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35−7.29 (5H, m, H-Ar), 5.18 (1H, d, J = 12.4 Hz, Bn-CH_{2a}), 5.16 (1H, br s, H-12), 5.06 (1H, d, J = 12.4 Hz, Bn-CH_{2b}), 3.51 (1H, d, J = 4.0 Hz, H-3), 3.42 (1H, t, J = 4.4 Hz, H-2), 3.16 (1H, d, J = 4.0 Hz, H-1), 1.14, 1.13, 1.07, 0.94, 0.87, 0.78, 0.72 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.9, 144.6, 136.4, 128.4, 128.4, 127.9, 127.9, 127.9, 121.8, 72.7, 65.9, 62.3, 55.4, 48.1, 44.2, 42.7, 42.6, 41.5, 41.4, 39.8, 38.2, 37.2, 36.4, 32.3, 31.8, 31.3, 28.5, 28.1, 26.8, 26.0, 25.9, 25.8, 23.8, 23.6, 17.3, 16.9, 16.4; EIMS m/z 558 $[M - 2H]^+$ (9), 405 (6), 338 (41), 247 (82), 201 (12), 91 (100).

General Procedure for the Synthesis of Target Compounds 15−17 Using the Ring-Opening Reaction. The major compound (**11** or **12**, 2.5 mmol, 1 equiv) obtained from the above-described reaction was dissolved in acetone (100 mL), and perchloric acid (5.0 mmol, 2 equiv) was added dropwise while stirring in an ice bath. Stirring was continued for an additional 24 h. The mixture was neutralized with 10% NaOH solution and then extracted with EtOAc (3 × 200 mL). The organic phase was washed with saturated aqueous $NaHCO_3$ and brine, and the combined organic phases were dried with anhydrous $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel column chromatography using petroleum ether−acetone as the eluent to yield the target product.

*Benzyl 1α,2β,3β-trihydroxy-11-oxo-18β-olean-12-en-30-oate (**15**):* 982.7 mg, 66% yield; colorless, monoclinic crystals; mp 89−91 °C, $[\alpha]^{30}_D$ +178.5 (c 0.29, $CHCl_3$); IR (KBr) ν_{max} 3441, 2948, 2863, 1731, 1655, 1455, 1384, 1214, 1159, 1045, 999, 732, 695 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41−7.33 (5H, m, H-Ar), 5.61 (1H, s, H-12), 5.20 (1H, d, J = 12.4 Hz, Bn-CH_{2a}), 5.09 (1H, d, J = 12.0 Hz, Bn-CH_{2b}), 4.69 (1H, br s, H-1), 4.00 (1H, br s, H-2), 3.57 (1H, br s, H-3), 3.28 (1H, br s, OH), 3.24 (1H, s, H-9), 3.00 (1H, br s, OH), 2.68 (1H, br s, OH), 1.39 (3H, s, H-25), 1.36 (3H, s, H-27), 1.16 (3H, s, H-29), 1.15 (3H, s, H-26), 1.02 (3H, s, H-23), 0.99 (3H, s, H-24), 0.74 (3H, s, H-28); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.7, 176.3, 171.4, 136.1, 128.6, 128. 6, 128.4, 128.3, 128.2, 128.2, 76.0, 75.2, 74.5, 66.2, 54.7, 48.0, 47.5, 44.9, 43.9, 43.6, 41.2, 41.1, 38.0, 37.7, 31.8, 31.7, 31.1, 29.8, 28.5, 28.2, 26.5, 26.4, 23.5, 19.2, 17.7, 17.7, 16.9; EIMS m/z 592 $[M]^+$ (1), 574 (25), 491 (21), 393 (14), 225 (30), 135 (31), 91 (100).

*Benzyl 1α,2β,3α-trihydroxy-11-oxo-18β-olean-12-en-30-oate (**17**):* 232.4 mg, 16% yield, colorless monoclinic crystal; mp 114−116 °C, $[\alpha]^{30}_D$ +185.6 (c 0.37, $CHCl_3$); IR (KBr) ν_{max} 3424, 2959, 2869, 1728, 1650, 1456, 1385, 1214, 1152, 1007, 876, 751, 698 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41−7.32 (5H, m, H-Ar), 5.70 (1H, s, H-12), 5.21 (1H, d, J = 12.0 Hz, Bn-CH_{2a}), 5.10 (1H, d, J = 12.0 Hz, Bn-CH_{2b}), 3.90 (1H, d, J = 8.0 Hz, H-1), 3.72 (1H, d, J = 11.6 Hz, H-3), 3.54 (1H, dd, J = 8.0, 11.6 Hz, H-2), 3.14 (1H, s, H-9), 2.89 (1H, br s, OH), 2.77 (1H, br s, OH), 2.48 (1H, br s, OH), 1.35 (3H, s, H-27), 1.17 (3H, s, H-29), 1.16 (3H, s, H-25), 1.15 (3H, s, H-26), 1.02 (3H, s, H-23), 0.94 (3H, s, H-24), 0.76 (3H, s, H-28); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.6, 176.1, 172.5, 136.0, 128.5, 128.5, 128.3, 128.2, 128.1, 128.1, 82.6, 74.6, 74.3, 66.2, 54.5, 48.1, 46.8, 44.5, 43.9, 43.7, 42.0, 41.1, 37.6, 37.5, 31.6, 31.0, 30.9, 28.5, 28.1, 26.5, 26.3, 23.4, 23.3, 23.3, 21.3, 19.3, 18.8; EIMS m/z 574 $[M - H_2O]^+$ (82), 557 (32), 491 (54), 393 (52), 225 (35), 135 (30), 91 (100).

Crystallization and X-ray Crystallographic Analysis of Compounds 15 and 17. Compounds **15** and **17** were crystallized through the slow evaporation of an EtOH solution. The cell parameter measurements and data collection were performed at T = 100(2) K with a Bruker APEX II CCD diffractometer using Cu Kα radiation (λ = 1.541 78 Å).[35] The structure was resolved by direct methods (SHELXS-97) and refined with full-matrix least-squares on F^2. All of the non-hydrogen atoms were refined anisotropically, and all of the hydrogen atoms were placed in idealized positions and refined as riding atoms with relative isotropic parameters. The absolute structures were determined by refining the Flack parameter and computing the Hooft parameter. The absorption correction was performed by SADABS using multiscan parameters. The crystallographic data for compounds **15** (deposition number CCDC 1042471) and **17** (deposition number CCDC 1042473) have been deposited in the Cambridge Crystallographic Data Centre. Copies of the data can be obtained free of charge by applying to the Director of the Cambridge Crystallographic Data Centre (12 Union Road, Cambridge CB2 1EZ, UK; fax: + 44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

*Crystal Data for Compound **15**:* $C_{37}H_{52}O_6{\cdot}2(H_2O)$, M = 628.82, colorless prism, size 1.21 × 0.37 × 0.27 mm^3, monoclinic, a = 34.1981(12) Å, b = 6.6186(2) Å, c = 15.6744(6) Å, α = 90.00°, β = 108.2830(10)°, γ = 90.00°, V = 3368.7(2) Å^3, space group $C2$, Z = 4, D_c = 1.240 g/cm^3, μ(Cu Kα) = 0.688 mm^{-1}, F(000) = 1368 mm^{-1}. A total of 27 880 reflections and 5872 independent reflections (R_{int} = 0.0499) were collected in the θ range from 2.72° to 69.55°, with index ranges of h(−41/41), k(−8/7), l(−18/18), and completeness θ_{max} = 97.8%. The final R indices [$I > 2\sigma(I)$] were R_1 = 0.0548 and $wR(F^2)$ = 0.1530. The final R indices (all data) were R_1 = 0.0549 and $wR(F^2)$ = 0.1532 (all data). The goodness of fit on F^2 was 1.083. The largest difference peak and hole were 0.673 and −0.385 e·Å^{-3}, respectively. The Flack parameter was 0.0(2), and the Hooft parameter was 0.11(5) for 2490 Bijvoet pairs.

*Crystal Data for Compound **17**:* $C_{37}H_{52}O_6{\cdot}2(H_2O)$, M = 628.82, colorless prism, size 0.95 × 0.46 × 0.42 mm^3, monoclinic, a = 15.7402(6) Å, b = 6.5876(2) Å, c = 17.5417(6) Å, α = 90.00°, β = 93.2380(10)°, γ = 90.00°, V = 1816.00(11) Å^3, space group $P2_1$, Z = 2, D_c = 1.150 g/cm^3, μ(Cu Kα) = 0.638 mm^{-1}, F(000) = 684 mm^{-1}. A total of 13 439 reflections and 5404 independent reflections (R_{int} = 0.0432) were collected in the θ range from 2.52° to 69.31°, with index ranges of h(−16/19), k(−7/7), l(−20/21), and completeness θ_{max} = 95.0%. The final R indices [$I > 2\sigma(I)$] were R_1 = 0.0996 and $wR(F^2)$ = 0.2866. The final R indices (all data) were R_1 = 0.1011 and $wR(F^2)$ = 0.2944 (all data). The goodness of fit on F^2 was 1.504. The largest difference peak and hole were 1.204 and −0.914 e·Å^{-3}, respectively. The Flack parameter was 0.0(3), and the Hooft parameter was 0.04(7) for 1976 Bijvoet pairs.

*Benzyl 1α,2β,3β-trihydroxy-18β-olean-12-en-30-oate (**16**):* 1.13 g, 78% yield; white, amorphous powder; $[\alpha]^{30}_D$ +121.6 (c 0.33, $CHCl_3$); IR (KBr) ν_{max} 3425, 2949, 1729, 1455, 1382, 1214, 1155, 1029, 697 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.39−7.32 (5H, m, H-Ar), 5.19 (1H, d, J = 12.0 Hz, Bn-CH_{2a}), 5.13 (1H, br s, H-12), 5.07 (1H, d, J = 12.0 Hz, Bn-CH_{2b}), 3.93 (1H, t, J = 4.0 Hz, H-2), 3.61 (1H, d, J = 2.4 Hz, H-1), 3.45 (1H, d, J = 4.0 Hz, H-3), 1.24 (3H, s, H-25), 1.17 (3H, s, H-27), 1.11 (3H, s, H-29), 1.00 (3H, s, H-23), 0.99 (3H, s, H-26), 0.97 (3H, s, H-24), 0.71 (3H, s, H-28); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.1, 144.5, 136.3 128.4, 128.4, 128.0, 128.0, 128.0, 122.2, 75.9, 74.5, 73.9, 65.9, 48.1, 47.9, 44.2, 42.7, 42.0, 40.2, 39.6, 38.5, 38.3, 38.2, 32.0, 31.8, 31.2, 29.5, 28.5, 28.1, 26.9, 26.1, 26.0, 23.4, 17.9, 17.2, 17.0, 14.3; EIMS m/z 578 $[M]^+$ (1), 560 (10), 338 (64), 247 (100), 91 (63).

General Procedure for the Synthesis of Target Compounds 18−20 Using a Hydrogenolysis Reaction. After 10% palladium/carbon was added to a solution of trihydroxylated compound (**15**, **16**, or **17**) in THF (50 mL), the resulting mixture was stirred at 30 °C under H_2 at atmospheric pressure for 24 h and then filtered through Celite. The residue was washed with THF (10 mL × 3), and the filtrate was evaporated to yield a crude product, which was subjected to silica gel column chromatography using $CHCl_3$−MeOH as the eluent to produce a pure compound.

*1α,2β,3α-Trihydroxy-11-oxo-18β-olean-12-en-30-oic acid (**18**):* 72.5 mg, 85% yield, colorless needles; mp 114−116 °C; $[\alpha]^{30}_D$ +153.0 (c 0.29, $CHCl_3$); IR (KBr) ν_{max} 3424, 2959, 2869, 1728, 1650, 1456, 1385, 1214, 1152, 1007, 876, 751, 698 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.85 (1H, s, H-12), 3.92 (1H, d, J = 7.2 Hz, H-

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1), 3.73 (1H, d, J = 11.6 Hz, H-3), 3.55 (1H, dd, J = 7.2, 11.6 Hz, H-2), 3.16 (1H, s, H-9), 2.89 (1H, br s, OH), 1.35 (3H, s, H-27), 1.23 (3H, s, H-29), 1.17 (3H, s, H-25), 1.16 (3H, s, H-26), 1.03 (3H, s, H-23), 0.95 (3H, s, H-24), 0.87 (3H, s, H-28); ^{13}C NMR (100 MHz, $CDCl_3$ and CD_3OD) δ 203.9, 179.2, 173.5, 127.9, 81.9, 74.6, 74.5, 54.4, 48.2, 46.5, 44.5, 43.7, 43.5, 41.8, 41.1, 37.6, 37.3, 31.6, 30.9, 30.8, 28.5, 28.2, 26.5, 26.2, 23.4, 23.2, 23.0, 20.7, 19.1, 18.6; EIMS m/z 502 $[M]^+$ (2), 484 (22), 303 (18), 135 (60), 43 (100).

*1α,2β,3β-Trihydroxy-11-oxo-18β-olean-12-en-30-oic acid (**19**):* 520.4 mg, 86% yield, colorless needles; mp 167−169 °C; $[\alpha]^{30}_D$ +155.3 (c 0.21, $CHCl_3$); IR (KBr) ν_{max} 3431, 2949, 2867, 1704, 1646, 1464, 1387, 1219, 1117, 1049, 975, 819 cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 6.06 (1H, s, H-12), 5.74 (1H, br s, H-1), 4.81 (1H, t, J = 3.6 Hz, H-2), 4.28 (1H, d, J = 4.0 Hz, H-3), 3.63 (1H, s, H-9), 2.56 (1H, d, J = 10.4 Hz, H-18), 2.29 (1H, d, J = 11.2 Hz, H-21), 2.02 (3H, s, H-25), 1.51 (3H, s, H-24), 1.46 (3H, s, H-27), 1.43 (3H, s, H-23), 1.32 (3H, s, H-29), 1.29 (3H, s, H-26), 0.83 (3H, s, H-28); ^{13}C NMR (100 MHz, C_5D_5N) δ 201.4, 182.9, 169.7, 129.0, 77.4, 74.8, 74.4, 54.9, 48.7, 48.4, 45.4), 44.2, 44.1, 41.9, 41.7, 39.6, 38.5, 32.8, 32.2, 31.6, 30.4, 28.8, 28.7, 26.8, 26.7, 23.7, 19.5, 18.1, 17.8, 16.6; EIMS m/z 503 $[M + H]^+$ (1), 484 (38), 469 (18), 401 (32), 207 (30), 135 (77), 43 (100).

*1α,2β,3β-Trihydroxy-18β-olean-12-en-30-oic acid (**20**):* 498.9 mg, 85% yield, colorless needles; mp 255−257 °C; $[\alpha]^{30}_D$ +140.6 (c 0.17, $CHCl_3$); IR (KBr) ν_{max} 3428, 2948, 1701, 1455, 1383, 1227, 1177, 1029, 969 cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.59 (1H, br s, H-12), 4.80 (1H, t, J = 3.6 Hz, H-2), 4.29 (1H, br s, H-1), 4.27 (1H, d, J = 4.0 Hz, H-3), 1.69 (3H, s, H-25), 1.49 (3H, s, H-24), 1.41 (3H, s, H-27), 1.40 (3H, s, H-23), 1.40 (3H, s, H-29), 1.18 (3H, s, H-26), 0.95 (3H, s, H-28); ^{13}C NMR (100 MHz, C_5D_5N) δ 179.8, 145.1, 123.5, 77.6, 74.7, 74.3, 49.1, 48.8, 44.4, 43.6, 42.7, 41.2, 40.1, 39.3, 39.2, 38.9, 32.9, 32.5, 31.9, 30.5, 29.2, 28.7, 27.4, 26.7, 26.4, 24.0, 18.7, 18.3, 17.6, 15.0; EIMS m/z 488 $[M]^+$ (2), 470 (8), 248 (100).

General Procedure for the Preparation of Target Compounds (21−42) from Compound 19 or 20. K_2CO_3 (0.1 mmol, 2.0 equiv) was added to a solution of compound **19** or **20** (0.05 mmol, 1.0 equiv) in dry DMF (20 mL). After 30 min of stirring at room temperature, an alkyl halide (0.06 mmol, 1.2 equiv) was added, and the mixture was stirred for an additional 4 h. The mixture was poured into water (50 mL), neutralized with 1.0 M HCl solution, and then extracted with EtOAc (3 × 100 mL). The organic phase was washed with saturated aqueous $NaHCO_3$ and brine, and the combined organic phases were dried over anhydrous $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel column chromatography using petroleum ether−acetone as the eluent to yield a pure compound. The NMR spectra and MS data for compounds **22−42** are presented in the Supporting Information.

*Methyl 1α,2β,3β-trihydroxy-11-oxo-18β-olean-12-en-30-oate (**21**):* 24.6 mg, 95% yield, colorless needles; mp 128−130 °C; $[\alpha]^{30}_D$ +169.4 (c 0.43, $CHCl_3$); IR (KBr) ν_{max} 3438, 2945, 2864, 1733, 1655, 1464, 1386, 1216, 1155, 1116, 1044, 998, 866 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.72 (1H, s, H-12), 4.68 (1H, br s, H-1), 4.00 (1H, br s, H-2), 3.70 (3H, s, $COOCH_3$), 3.57 (1H, br s, H-3), 3.29 (1H, br s, OH), 3.24 (s, 1H, H-9), 3.02 (br s, 1H, OH), 2.69 (br s, 1H, OH), 1.39, 1.37, 1.18, 1.15, 1.02, 0.99, 0.83 (each 3H, s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.9, 177.0, 171.4, 128.4, 75.4, 75.2, 74.3, 54.6, 51.8, 48.2, 47.5, 44.9, 44.0, 43.7, 41.1, 41.1, 38.1, 37.7, 31.8, 31.7, 31.1, 29.7, 28.6, 28.3, 26.5, 26.4, 23.5, 19.2, 17.6, 17.6, 16.7; EIMS m/z 516 $[M]^+$ (3), 498 (100), 483 (35), 317 (21), 276 (50).

Evaluation of the Antibacterial Activity in Vitro. Turbidimetric assays were performed to determine the MIC values.[19] In brief, the bacterial cultures were seeded in 96-well microculture plates at a concentration of 1 × 10^5 CFU/mL. Twofold serial dilutions of the test compounds were prepared with broth in microplates using DMSO as the cosolvent, and the DMSO concentration never exceeded 1% (v/v). Negative (medium containing vehicle without microorganisms) and normal controls (medium containing vehicle and microorganisms) were also designed. After sedimentation and measurement of the first absorbance at 620 nm using an ELISA plate reader, the bacteria were incubated for an appropriate time with vigorous shaking on a vibrating platform at the appropriate temperature. The second optical density of the bacterial suspension was then measured. The MIC values were determined as the minimum concentration of the test compounds that caused no apparent bacterial growth compared with the negative control. The assay was performed in triplicate to verify the MIC values.

Time-kill assays were performed according to previously published methods.[19] Briefly, each bacterial suspension was adjusted to a concentration of 5 × 10^5 CFU/mL and divided into three groups: negative control, positive control (as described above), and the test compounds at concentrations of 0.5 × , 1 × , 2 ×, and 4 × the MIC. Under the appropriate culture conditions, the *S. aureus* and *B. subtilis* cultures were incubated for 0, 2, 4, 6, 8, 12, and 24 h, and the *S. scabies* cultures were incubated for 0, 4, 8, 16, 24, and 36 h. At the indicated time points, 1 mL samples of the treated bacteria and controls were removed, serially diluted, and plated onto agar plates to enumerate the viable colonies, and the survival was then calculated. The tests were repeated in triplicate, and the killing kinetics curves were constructed.

Quantitative Real-Time Reverse-Transcriptase PCR (Q-RT-PCR) Assay. The test bacterial strains were cultured in broth supplemented with either the test compounds at a concentration of 0.5 × MIC or vehicle alone. The bacteria were allowed to grow to the exponential phase (OD_{620} = 0.4) and were then harvested by centrifugation.[22b,23] The total RNA was isolated according to a previously described procedure.[22b] The mRNAs were reverse-transcribed into cDNAs using the PrimeScript reverse transcriptase kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. Q-RT-PCR was performed with an UltraSYBR mixture (with ROX) kit (CWBIO, Beijing, People's Republic of China) using a StepOnePlus real-time PCR system (Applied Biosystems, Grand Island, NY, USA); the sequences of the primers used are presented in the Supporting Information. The relative expression levels for each sample compared with the control were determined by the $2^{-\Delta\Delta CT}$ method.[36] The *yqxC*, *16s rRNA*, and *rpoB* genes were used as housekeeping genes for the *B. subtilis*, *S. aureus*, MRSA, and *S. scabies* strains, respectively. Each experiment was performed in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00641.

Spectroscopic data for the new compounds (compounds **22−42**), copies of the NMR spectra of all of the new compounds (compounds **7−42**), and the sequences of the primers used in the Q-RT-PCR assay (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) (a) Lin, G.; Nnane, I. P.; Cheng, T. Y. *Toxicon* **1999**, *37*, 1259−1270. (b) Kao, T. C.; Wu, C. H.; Yen, G. C. *J. Agric. Food Chem.* **2014**, *62*, 542−553.
(2) Kalani, K.; Agarwal, J.; Alam, S.; Khan, F.; Pal, A.; Srivastava, S. K. *PLoS One* **2013**, *8*, e74761.
(3) (a) Yu, D.; Sakurai, Y.; Chen, C. H.; Chang, F. R.; Huang, L.; Kashiwada, Y.; Lee, K. H. *J. Med. Chem.* **2006**, *49*, 5462−5469. (b) Maitraie, D.; Hung, C. F.; Tu, H. Y.; Liou, Y. T.; Wei, B. L.; Yang, S. C.; Wang, J. P.; Lin, C. N. *Bioorg. Med. Chem.* **2009**, *17*, 2785−2792. (c) Csuk, R.; Schwarz, S.; Kluge, R.; Ströhl, D. *Eur. J. Med. Chem.* **2010**, *45*, 5718−5723. (d) Chen, S.; Zou, L.; Li, L.; Wu, T. *PLoS One* **2013**, *8*, e53662.
(4) Nick, A.; Wright, A. D.; Sticher, O.; Rali, T. *J. Nat. Prod.* **1994**, *57*, 1245−1250.
(5) Zhou, X.; Zhao, L.; Liu, X.; Li, X.; Jia, F.; Zhang, Y.; Wang, Y. *Phytother. Res.* **2012**, *26*, 253−258.
(6) Salari, M. H.; Eshraghi, S.; Noroozi, M. *Daru* **2001**, *9*, 37−39.
(7) Graebin, C. S.; Verli, H.; Guimarães, J. A. *J. Braz. Chem. Soc.* **2010**, *21*, 1595−1615.
(8) Sun, H.; Fang, W. S.; Wang, W. Z.; Hu, C. *Bot. Bull. Acad. Sin.* **2006**, *47*, 339−368.
(9) Brown, D. G.; Lister, T.; May-Dracka, T. L. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 413−418.
(10) Angeh, J. E.; Huang, X.; Sattler, I.; Swan, G. E.; Dahse, H.; Härtl, A.; Eloff, J. N. *J. Ethnopharmacol.* **2007**, *110*, 56−60.
(11) Wang, J.; Ren, H.; Xu, Q. L.; Zhou, Z. Y.; Wu, P.; Wei, X. Y.; Cao, Y.; Chen, X. X.; Tan, J. W. *Food Chem.* **2015**, *168*, 623−629.
(12) Beseda, I.; Czollner, L.; Shah, P. S.; Khunt, R.; Gaware, R.; Kosma, P.; Jordis, U. *Bioorg. Med. Chem.* **2010**, *18*, 433−454.
(13) Huang, L. R.; Luo, H.; Zhang, J. X.; Wang, D. P.; Li, Q. J.; Hao, X. J.; Yang, X. S. *Eur. J. Med. Chem.* **2015**, *95*, 64−75.
(14) Brown, R. S.; Eyley, S. C.; Parsons, P. J. *J. Chem. Soc., Chem. Commun.* **1984**, 438−439.
(15) Yamaguchi, K.; Mori, K.; Mizugaki, T.; Ebitani, K.; Kaneda, K. *J. Org. Chem.* **2000**, *65*, 6897−6903.
(16) Wen, X.; Sun, H.; Liu, J.; Cheng, K.; Zhang, P.; Zhang, L.; Ni, P.; Zographos, S. E.; Leonidas, D. D.; Alexacou, K. M.; Gimisis, T.; Hayes, J. M.; Oikonomakos, N. G. *J. Med. Chem.* **2008**, *51*, 3540−3554.
(17) Eddingsaas, N. C.; VanderVelde, D. G.; Wennberg, P. O. *J. Phys. Chem. A* **2010**, *114*, 8106−8113.
(18) Roush, W. R.; Brown, R. J.; DiMare, M. *J. Org. Chem.* **1983**, *48*, 5083−5093.
(19) Fischer, C. L.; Drake, D. R.; Dawson, D. V.; Blanchette, D. R.; Brogden, K. A.; Wertz, P. W. *Antimicrob. Agents Chemother.* **2012**, *56*, 1157−1161.
(20) Fontanay, S.; Grare, M.; Mayer, J. *J. Ethnopharmacol.* **2008**, *120*, 272−276.
(21) Csuk, R.; Schwarz, S.; Kluge, R.; Ströhl, D. *Arch. Pharm.* **2012**, *345*, 28−32.
(22) (a) Kim, H. K.; Park, Y.; Kim, H. N.; Choi, B. H.; Jeong, H. G.; Lee, D. G.; Hahm, K. S. *Biotechnol. Lett.* **2002**, *24*, 1899−1902. (b) Long, D. R.; Mead, J.; Hendricks, J. M.; Hardy, M. E.; Voyich, J. M. *Antimicrob. Agents Chemother.* **2013**, *57*, 241−247.
(23) Singh, V. K.; Carlos, M. R.; Singh, K. *FEMS Microbiol. Lett.* **2010**, *311*, 167−175.
(24) (a) Wang, J. E.; Dahle, M. K.; McDonald, M.; Foster, S. J.; Aasen, A. O.; Thiemermann, C. *Shock* **2003**, *20*, 402−414. (b) Schneider, T.; Sahl, H. G. *Int. J. Med. Microbiol.* **2010**, *300*, 161−169.
(25) Kitano, K.; Tuomanen, E.; Tomasz, A. *J. Bacteriol.* **1986**, *167*, 759−765.
(26) Margot, P.; Pagni, M.; Karamata, D. *Microbiology* **1999**, *145*, 57−65.
(27) Rohrer, S.; Ehlert, K.; Tschierske, M.; Labischinski, H.; Berger-Bachi, B. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 9351−9356.
(28) Wolska, K. I.; Grudniak, A. M.; Fiecek, B.; Kraczkiewicz-Dowjat, A.; Kurek, A. *Cent. Eur. J. Biol.* **2010**, *5*, 543−553.
(29) Reddick, J. J.; Williams, J. K. *Biotechnol. Lett.* **2008**, *30*, 1045−1050.
(30) Muthaiyan, A.; Silverman, J. A.; Jayaswal, R. K.; Wilkinson, B. J. *Antimicrob. Agents Chemother.* **2008**, *52*, 980−990.
(31) Tian, Z.; Cheng, Q.; Yoshimoto, F. K.; Lei, L.; Lamb, D. C.; Guengerich, F. P. *Arch. Biochem. Biophys.* **2013**, *530*, 101−107.
(32) Bignell, D. R. D.; Huguet-Tapia, J. C.; Joshi, M. V.; Pettis, G. S.; Loria, R. *Antonie van Leeuwenhoek* **2010**, *98*, 179−194.
(33) Goyer, C.; Vachon, J.; Beaulieu, C. *Phytopathology* **1998**, *88*, 442−445.
(34) Subba Rao, G. S. R.; Kondaiah, P.; Singh, S. K.; Ravanan, P.; Sporn, M. B. *Tetrahedron* **2008**, *64*, 11541−11548.
(35) *APEX2*; Bruker AXS Inc.: Madison, WI, 2010.
(36) Livak, K. J.; Schmittgen, T. D. *Methods* **2001**, *25*, 402−408.

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J. Nat. Prod. XXXX, XXX, XXX−XXX