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Graphic abstract

Synthesis and biological evaluation of novel ocotillol-type triterpenoid derivatives as antibacterial agents

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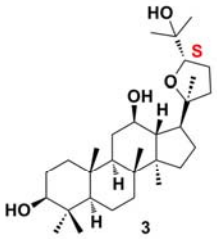

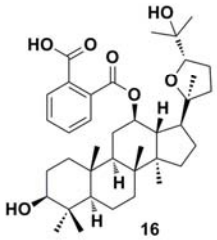

Compounds	MIC against *S. aureus* RN4220
3	8 μg/mL
16	4 μg/mL

Copounds	MIC (μg/mL)	
	CA-MRSA USA 300	*B. subtilis* 168
Kanamycin	1	0.25
3 + Kanamycin	0.125	0.2
16 + Kanamycin	0.0078	< 0.0020
Chloramphenicol	4	2
3 + Chloramphenicol	4	2
16 + Chloramphenicol	0.016	< 0.0078

Compounds **3** and **16** combined with kanamycin and chloramphenicol showed strong synergistic inhibitory effects at their sub-MIC concentrations against *S.aureus* USA 300 and *B.subtilis* 168.

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Abstract

A novel class of ocotillol-type triterpenoid derivatives have been synthesized and evaluated for their *in vitro* antibacterial activity against several representative pathogenic bacterial strains. Compounds 20(*S*)-protopanaxadiol (**PPD**), **3**, **5**, **16** and **24** exhibited potent antibacterial activity against Gram-positive bacteria. Compounds **3** and **5** also displayed promising antibacterial activity against a community-associated methicillin-resistant *S. aureus* (CA-MRSA; strain USA300). Furthermore, compounds **PPD**, **3** and **16** combined with two commercially available antibiotics kanamycin and chloramphenicol showed strong synergistic inhibitory effects at their sub-MIC concentrations against *S.aureus* USA 300 and *B.subtilis* 168. Additionally, cytotoxic activity assay showed that the compounds tested did not affect cell viability of the human epithelial kidney (HEK-293) and human cervical (HeLa) cells at their MICs.

Keywords

Triterpenoid; Ocotillol; Antibacterial activity; synergistic effect; cytotoxicity

1. Introduction

Since the discovery of Penicillin, the development of antibiotics has been carried out for over 80 years. In addition to the β-lactam family antibiotics, that have many structural

variants, other antibiotics or synthetic antimicrobial agents have also been developed, including the macrolides, tetracyclines, quinolones and glycopeptides which have different mechanisms of antibacterial action [1]. However, due to horizontal gene transfer and unlinked point mutations, more and more bacteria with resistance to antibiotics were isolated, with methicillin-resistant *Staphylococcus aureus* (MRSA) infections representing a particularly serious health problem worldwide [2]. More recently, infections due to community-associated (CA) MRSA, which is responsible for diseases such as necrotizing pneumonia, severe sepsis and necrotizing fasciitis, have risen rapidly, sparking fears of an epidemic [3]. In recent decades, vancomycin-resistant *Staphylococcus aureus* (VRSA) strains were also identified [4]. Consequently, novel antimicrobial agents with distinct structures are urgently needed to help battle emerging antibiotic-resistant bacterial infections.

Natural products have been the single most productive source of leads for the development of drugs particularly as anti-cancer agents and anti-infectives [5]. The triterpenoids, including sterols, steroids, and saponins, are a large and structurally diverse group of natural products with well-characterized biological activities [6]. Many of them such as ursolic acid (UA), oleanolic acid (OA), betulinic acid (BA) (Figure 1) show antimicrobial activities [7]. Previous reports indicated that the weak antimicrobial activity of butulinic acid as compared to its analogues ursolic acid and oleanolic acid is probably due to lack of the E ring that is present oleanene [8]. However, surprisingly, 23-hydroxybetulinic acid (HBA) (Figure 1) demonstrates moderate antimicrobial activity against Gram-positive bacteria *Bacillus subtilis* (closely related to *Bacillus anthracis*) strain 168 and *Staphylococcus aureus* strain RN4220 in our preliminary testing (Table 1), which suggests the E ring of oleanene/oleanane in triterpenoid natural products may not be the unique critical factor for antimicrobial activity.

Although the antibacterial activity of some natural products may be less than that of certain marketed drugs, their lower cytotoxicity compared to the traditional antibiotics was preferred. In addition, natural products usually have an element of structural complexity which is required for the inhibition of many antibacterial protein targets [9]. Ocotillol (Figure 1), a triterpene isolated from *Fouquieria splendens Engelm*, is characterized by bearing a five-membered epoxy ring at C-20 [10]. Pseudo-ginsenoside-

F11 (Figure 1) which is an ocotillol-type ginsenoside found in *Panax quinquefolium* (American ginseng) [11] has been proven to possess a protective effect on myocardial ischemia injury [12]. Recent research showed plant derived triterpenoid saponins commonly involve in plant defense system by perturbating membranes of bacteria or virus [13], as well as increasing membrane permeability of mammalian cells [14]. Thus, triterpenoid derivatives are potential to be developed as antimicrobial agents by affecting bacterial cell viability through membrane destruction. So far few studies have been devoted to the antimicrobial effect of ocotillol-type triterpenoid derivatives; furthermore, compared to oleanane, tetracyclic triterpenoid compounds are more potent in drug discovery for their low cytotoxicity [15] and diverse functional groups as well as their prospects for modification. Therefore, the present study described the synthesis of the tetracyclic triterpenoid derivatives from 20(*S*)-protopanaxadiol (PPD) and evaluated for their *in vitro* antibacterial, synergistic antibacterial activities and cytotoxicity [16].

2. Synthetic chemistry

We chose **PPD**, one of the main ginsengenin components of *Panax ginseng*, as our starting material (Scheme 1), as it can also be isolated from other natural sources [17]. As previously described [16], the 3, 12-diol of PPD was protected using acetic anhydride in the presence of DMAP; epoxidation of the resulting acetate **1** followed by nucleophilic addition *in situ* furnished a mixture of tetrahydrofuran **2**. After base treatment, two stereoisomeric triols **3** and **4** were obtained with desired yields. Triol **3** was then oxidized by 3 molar equivalents of pyridinium chlorochromate to provide dione **5**, whereas 1 molar equivalent of PCC only oxidized 3-hydroxyl of triol **3** to furnish ketone **6**; the same procedure applied to triol **4** formed dione **7** and ketone **8**, respectively (Scheme 2).

Regioselective protection of 3-hydroxyl group of triol **3** as mono acetate, followed by oxidation and/or esterification of the 12-ol and deprotection of the acetic group gave the derivatives **15-17**. Additionally, treatment of triol **3** with excess acetic anhydride provided diacetate, which was hydrolyzed in basic media to produce **14**. Target compounds **22-24** were also obtained with the same method (Scheme 2, Scheme 3).

3. Results and Discussion

We have designed and synthesized a series of ocotillol-type triterpenoid derivatives based on PPD. The minimum inhibitory concentration (MIC) of the synthesized compounds was determined against several bacterial strains using a standard LB medium dilution technique. Initial MIC screening results are presented in Table 1. The data demonstrated that PPD and the ocotillol-type triterpenoid derivatives **3**, **5**, **6, 16** and **24** inhibited the growth of Gram-positive bacteria *in vitro* with MIC values about 4-32 μg/mL. Among the synthesized compounds, **16** was found to be the most active with MIC value of 4 μg/mL against *S. aureus*. Meanwhile, stereoisomers **3** and **4** displayed dramatically dissimilar antibacterial activity against Gram-positive bacteria, which suggest the stereochemistry of the isopropanol group at C-24 may play a key role for the binding of the pharmacologically active compounds with the target receptor in bacteria. However, the activity of compound **5** illustrated that the reverse stereochemistry of C-24 is preferred when the hydroxyl groups at C-3 and C-12 are both oxidized, and compound **6** could only mildly inhibit the growth of *B. subtilis* when the hydroxyl group at C-3 is oxidized. With a phthalic acid mono ester at C-12 position, both compounds **16** and **24** displayed good activity against Gram-positive bacteria, *B. subtilis* with MIC values of 16 μg/mL, Again, their activity against *S. aureus* confirmed the significance of stereochemistry, with MICs of 4 and 64 μg/mL, respectively. Surprisingly, **15** and **22** exhibited mild activity against both Gram-positive and Gram-negative bacteria, *S. aureus* and *E. coli*, while **14** and **17** showed activity only against Gram-negative bacteria, which illustrated ocotillol-type derivatives' potential to somehow selectively inhibit Gram positive or negative bacteria.

As shown in Table 2, the bioactive compounds against Gram-(+) bacteria were chosen for testing against a significant highly pathogenic community-associated methicillin resistant strain *S. aureus* USA300 [18]. The results showed compounds **3** and **5** also possess good antibacterial activity against MRSA USA300. However, compound **16** could only exhibited moderate activity against this pathogen. The testing of minimum bactericidal concentration (MBC) was also carried out. Only **16** and **24** showed direct bactericidal activity against Gram positive bacteria, including CA-MRSA strain for **16** (**16**: 64 μg/mL against *S. aureus* RN 4220 and *B. subtilis* 168; 128 μg/mL against *S. aureus* USA300; **24**: 128 μg/mL against *B. subtilis* 168). The bactericidal effects revealed

the ocotillol-type triterpenoid derivatives not only can affect bacterial cell viability, but also cause cell death, which is a common effect by increasing cell membrane permeability.

Mechanistically, kanamycin is known as a bactericidal agent targeting the 30S of bacterial ribosome to cause mistranslation and inhibiting translocation during protein synthesis, while as a bacteriostatic drug, chloramphenicol inhibits the peptidyl transferase activity of bacterial ribosome, and then stop the bacterial growth by inhibiting protein synthesis. Like all of the other antibiotics, both of these two drugs have to cross the cell membrance and enter the cytoplasm for their antibacterial functions. As a result, reducing membrane permeability is one of the three mechanisms that bacteria generate resistance to antibiotics including chloramphenicol [19]. Conversely, an antibacterial agent targeting cell membrane could synergistically strengthen the sensitivity of bacteria to these antibiotics. The synergistic effects of compounds **PPD**, **3** and **16** were then investigated at their sub-MIC concentrations with above two conventional antibiotics against CA-MRSA *S. aureus* USA300 and *B. subtilis* 168 to verify our hypothesis on the effect of membrane perturbation as the mechanism of action, and the effects were evaluated by calculating the Fractional Inhibitory Concentration Index (FICI) using fractional inhibitory concentration (FIC) [20]. The results of the combination assay are listed in Table 3. As shown in Figure 2, at a specified concentration, most of the combinations showed effectiveness. Compounds **PPD** and **3** could reduce the MICs of kanamycin against *S. aureus* USA300 from 1 μg/mL to 0.25 and 0.125 μg/mL, respectively (FICI=0.26, 0.14). Synergistic activity against *B.subtilis* 168 were also observed when combination of **PPD** with kanamycin (FICI=0.25). Furthermore, the MICs of kanamycin and chloramphenicol were dramatically reduced in combination with compound **16**. The FICIs of **16** and kanamycin or chloramphenicol were calculated to be between 0.004 and 0.008, which was significant smaller than 0.5, suggesting that they acted synergistically to inhibit the growth of both strains. An additive effect (FICI=0.53) was observed when **PPD** was combined with chloramphenicol against *B.subtilis* 168 and the others' combinational activities were exhibited as indifferent interactions (1<FICI<2).

In contrast, when **PPD**, **3** and **16** were combined with kanamycin, the bactericidal activities of kanamycin against *S. aureus* USA300 were significantly enhanced from 4

μg/mL to 2, 1, and 2 μg/mL, respectively. For *B.subtilis* 168, potent bactericidal effects were also observed when combination of kanamycin with **PPD** and **16.** Surprisingly, chloramphenicol alone was bacteriostatic against *B.subtilis* 168, but exhibited significant bactericidal avtivity when combined with **16** with MBC value of less than 0.0078.

Reports shown that kanamycin crosses bacterial membrane through lipid-mediated transportation [21], and chloramphenicol can use both lipid and porin pathways to enter the bacterial cytoplasm [22]. Compounds **PPD**, **3** and **16** synergistically enhanced above two drugs' antibacterial activity against Gram positive bacteria, which suggest ocotillol-type derivatives target the phospholipid-mediated pathway, as affecting specifically porin transportation will not enhance the activity of drugs such as kanamycin. Furthermore, the outer membrane of Gram negative bacteria consisting lipopolysaccharides (LPS), which are the essential difference from the membrane structure of Gram positive bacteria, may explain the ineffectiveness of **PPD**, **3** and **16** against Gram negative bacteria. However, as shown in table 1, compounds **14** and **17** displayed specific activity against Gram-negative bacteria, which could provide us some information for further investigation.

Additionally, as described above, in the presence of **16** at sub-MIC concentration, chloramphenicol turned from bacteriostatic to bactericidal agent, this interesting result revealed ocotillol-type derivatives are suitable for combination with other antibiotics, inculding those bacteriostatics to cause bacterial cell death. This discovery is very significant and highly potential for the development of new drugs in clinic to reduce the toxicity of antibiotics, specially the ones like kanamycin and chloramphenicol, which are currently second-line drugs limited to last resort because of their toxicity.

The cytotoxicity of compounds **3**, **5** and **16** were then evaluated by MTT assay. The IC_{50} and selectivity index values (SI, rate between IC_{50} against human cervical (HeLa) or human epithelial kidney (HEK 293) cells and MIC against *S. aureus* RN4220 and CA-MRSA USA 300) are presented in Table 4 to determine if their observed antibacterial activity was caused by selective toxicity towards the bacterial cells. The high SI values indicate low toxicity of the compounds. As shown in Table 4, compounds **3**, **5** and **16** showed much higher IC_{50} values against both HeLa (~50 μg/mL) and HEK-293 (>150 μg/mL) cells than that of the positive control 5-fluorouracil (~0.8 μg/mL), and thus they will not affect cell viability at their antibacterial MICs (8, 16, 4 μg/mL). The tested

compounds **3**, **5** and **16** provided a better selectivity index on HEK-293 cell with high values of 19.3, 10.6 and 43.8 against *S. aureus* RN4220 and 19.3, 10.6 and 5.5 against CA-MRSA USA 300, respectively. And for HeLa cell, compounds **3** and **16** have also exhibited low toxicity (compound **3**, SI: 6 against *S. aureus* RN4220 and CA-MRSA USA 300; compound **16**, SI: 13.6 against *S. aureus* RN4220 and 1.7 against CA-MRSA USA 300). These results suggested that compounds **3**, **5** and **16** could exhibit *in vitro* antibacterial activity at non-cytotoxic concentrations.

Based on the antibacterial activity of ocotillol-type triterpenoid derivatives, a plausible structure-activity relationships (SARs) can be reasoned out as shown in Figure 3, hydrogen donors at C-3 and C-12 are preferred to maintain the activity against Gram-positive bacteria; on the contrary, decreased activity was observed when the functional groups at C-3 and C-12 turned to ketone as hydrogen accepter; However, substitution at C-12 as non-hydrogen donor ester resulted in mild activity against Gram-negative bacteria, especially compounds **14** and **17** with 24(*S*)-configuration displayed specific activity against Gram-negative bacteria; Furthermore, the stereochemistry at C-24 dramatically affects the antibacterial activity with *S*-configuration preferred, probably due to the diferent interaction between the substitution of C-12 and the tetrafuran ring.

4. Conclusions

In this paper we have designed and synthesized a series of ocotillol-type triterpenoid derivatives based on **PPD**, in which compounds **3**, **5**, **16** and **24** showed good antibacterial activity particularly against Gram-positive bacteria, and compounds **14** and **17** exhibited notably activity against Gram negative bacteria. Additional testing against CA-MRSA strain USA300 demonstrated compounds **3** and **5** also possess potent antibacterial activity. The subsequent synergistic antibacterial assay revealed that the ocotillol-type compounds enhanced the susceptibility of *S.aureus* USA300 and *B.subtilis* 168 to kanamycin and chloramphenicol (FICI<0.5). Compounds **3**, **5** and **16** were also evaluated for their cytotoxicity and exhibited no significant influence on cell viability on both HEK-293 and Hela cells at their MICs. These results suggested that ocotillol-type triterpenoid derivatives represent as potential leads for the development of antibacterial agents against antibiotic-resistant superbugs. Further investigation is required to

determine how those organisms can be targeted with our compounds, and the related experiments are being carried on in our laboratories and will be reported in due course.

5. Experimental

5.1 Chemistry

Most chemicals and solvents were analytical grade and, when necessary, were purified and dried with standard methods. Melting points were taken on an XT-4 micro melting point apparatus and uncorrected. ^{1}H NMR spectra were recorded with a Bruker AV-300 or ACF 500 spectrometer in the indicated solvents (TMS as internal standard): the values of the chemical shifts are expressed in δ values (ppm) and the coupling constants (J) in Hz. High-resolution mass spectra were recorded using an Agilent QTOF 6520.

5.1.1 20(*S*)-Protopanaxadiol (PPD)

PPD were prepared from total ginsenosides by alkaline hydrolysis according to the literature [23]**.** mp. 197–199℃; ESI-MS *m/z* 461.5 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.16 (t, J = 7.8 Hz, 1H), 3.58 (m, 1H), 3.20 (m, 1H), 1.70 (s, 3H), 1.63 (s, 3H),1.18 (s, 3H), 0.98 (s, 3H), 0.97 (s, 3H), 0.88 (s, 6H), 0.78 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 131.7, 125.0, 77.8, 74.2, 70.8, 55.8, 53.4, 51.5, 50.0, 47.7, 39.7, 38.9, 38.9, 37.0, 34.7, 34.5, 31.7, 31.2, 28.0, 27.3, 26.9, 26.4, 25.7, 22.3, 18.2, 17.7, 16.8, 16.1, 15.7, 15.3. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{52}NaO_3[M + Na]^+$: 483.3809, found: 483.3818.

5.1.2 (20*S*,24*S*)-Epoxy-dammarane-3β,12β,25-triol (3) and (20*S*,24*R*)-Epoxy-dammarane-3β,12β,25-triol (4)

To a solution of **PPD** (500 mg, 1.08 mmol) and DMAP (20 mg, 0.16 mmol) in dry pyridine (4 mL), acetic anhydride (0.42 mL, 4.43 mmol) was slowly added, and the mixture stirred for 24 h at room temperature.The reaction mixture was then diluted with ethyl acetate, washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated and the residue was purified by column chromatography, eluting with petroleum ether :ethyl acetate 10:1 mixture. 3β, 12β-diacetoxy-20 (*S*)-protopanaxadiol (**1**) was obtained as a white solid (515mg, 86%).

A solution of **1** (208mg, 0.38mmol) in dichloromethane (10 ml) was cooled to -3°C. Then a solution of *m*-CPBA (185mg, 75%, 0.76mmol) in dichloromethane (10 ml) was

added slowly and stirred for 2 h. The organic solution was washed with saturated. aq. $NaHCO_3$, saturated. aq. $Na_2S_2O_3$, water and brine successively, and dried over Na_2SO_4. The dichloromethane was evaporated in vacuo and the residue was purified over silica gel (8:1 petroleum ether-ethyl acetate) to get (20*S*, 24)-epoxy-3β, 12β-diacetoxy-dammarane-25-ol (**2**) as a white solid (148mg, 69%).

Potassium hydroxide (255 mg, 4.55 mmol) was added to a solution of **2** (405 mg, 0.72 mmol) in methanol (10 mL). The resulting mixture was refluxed for 2 h. The solvent was removed under reduced pressure, ethyl acetate was added,then washed with water and bine, dried over anhydrous sodium sulfate, concentrated in vacuo and the residue was purified over silica gel (2:1-1:1 petroleum ether-ethyl acetate), and then crystallized from ethyl acetate yielded (20*S*, 24*S*)-Epoxy-dammarane-3β, 12β, 25-triol (**3**) as white pellet-like crystal (166 mg, 48%) and from acetone yielded (20*S*, 24*R*)-Epoxy-dammarane-3β, 12β, 25-triol **(4)** as white lump-like crystal (155 mg, 45%).

(20*S*,24*S*)-Epoxy-dammarane-3β,12β,25-triol (3). mp. 224–225℃; ESI-MS *m*/*z* 477.3 $[M + H]^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.87 (dd, J = 10.2Hz, 5.1 Hz, 1H), 3.52 (td, J = 10.2Hz, 4.8 Hz, 1H), 3.19 (dd, J = 10.8Hz, 5.4 Hz, 1H), 2.25 (td, J = 10.5Hz, 4.2 Hz, 1H), 1.27 (s, 3H), 1.23 (s, 3H), 1.10 (s, 3H), 1.01 (s, 3H), 0.97(s, 3H) , 0.91 (s, 3H), 0.88 (s, 3H), 0.78 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 87.3, 87.0, 78.7, 70.4, 69.9, 55.8, 52.0, 50.1, 48.8, 48.7, 39.6, 38.8 (overlapping signal), 37.0, 34.6, 32.1, 31.5, 31.5, 28.7, 28.4, 27.9, 27.8, 27.3, 24.9, 24.1, 18.2, 17.6, 16.1, 15.3, 15.2. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{53}O_4[M + H]^+$: 477.3938, found: 477.3946.

(20*S*,24*R*)-Epoxy-dammarane-3β,12β,25-triol (4). mp. 167–169℃; ESI-MS *m*/*z* 477.3 $[M + H]^+$; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.84 (dd, J = 8.4Hz, 6.6 Hz, 1H), 3.52 (td, J = 10.5Hz, 4.8 Hz, 1H), 3.19 (dd, J = 10.8Hz, 5.1 Hz, 1H), 2.19 (td, J = 10.8Hz, 3.6 Hz, 1H), 1.28 (s, 3H), 1.27 (s, 3H), 1.10 (s, 3H), 0.99 (s, 3H), 0.97(s, 3H) , 0.90 (s, 3H), 0.86 (s, 3H), 0.78 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 86.4, 85.3, 78.7, 70.9, 70.0, 55.9, 51.9, 50.4, 49.3, 47.9, 39.7, 38.9, 38.8, 37.1, 34.7, 32.5, 31.2, 31.1, 28.5, 27.9, 27.8, 27.5, 27.39, 26.0, 24.9, 18.2, 18.1, 16.2, 15.3, 15.2. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{53}O_4$ $[M + H]^+$: 477.3938, found: 477.3934.

5.1.3 (20*S*,24*S*)-Epoxy-25-hydroxy-dammarane-3,12-dione (5)

To a solution of **3** (100 mg, 0.21 mmol) in dry dichloromethane (6 mL) was added pyridinium chlorochromate (131 mg, 0.60 mmol), the mixture was stirred at room temperature for 8 h, then filtrated, concentrated in vacuo and the residue was purified over silica gel (4:1 petroleum ether-ethyl acetate) to give the product as a white solid (75 mg, 75%). mp. 169–171℃; ESI-MS *m/z* 473.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.69-3.73 (m, 1H), 3.01 (d, J = 9.8Hz, 1H), 2.49-2.58 (m, 1H), 2.45-2.47 (m, 1H), 2.24-2.29 (m, 1H), 2.20-2.30 (m, 2H), 1.40 (s, 3H), 1.25 (s, 6H),1.20 (s, 3H), 1.10 (s, 3H), 1.04 (s, 3H), 0.88 (s, 3H), 0.87 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 216.6, 216.6, 87.6, 85.3, 70.2, 57.3, 56.9, 55.1, 53.7, 47.3, 40.2, 39.8, 39.1, 37.3, 36.5, 33.8, 33.6, 31.9, 29.3, 27.7, 26.7, 26.6, 26.2, 24.8, 23.9, 21.0, 19.7, 16.5, 15.7, 15.2. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{48}NaO_4$ $[M + Na]^+$: 495.3445, found: 495.3453.

5.1.4 (20*S*,24*S*)-Epoxy-12β,25-dihydroxy-dammarane-3-one (6)

To a solution of **3** (86 mg, 0.18mmol) in dry dichloromethane (8 mL) was added pyridinium chlorochromate (87 mg, 0.40 mmol), the mixture was stirred at room temperature for 8 h,then filtered, concentrated in vacuo, and the residue was purified over silica gel (1:1 petroleum ether-ethyl acetate) to give the product as a white solid (55 mg, 65%). mp. 168–170℃; ESI-MS *m/z* 475.3 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.89 (dd, *J* = 10.5Hz, 5.0 Hz, 1H), 3.54 (td, *J* = 10.0Hz, 4.5Hz, 1H), 2.49-2.54 (m, 1H), 2.45 (ddd, *J* = 11.0Hz, 8.0Hz, 3.5Hz, 1H), 2.27 (td, *J* = 10.5Hz, 4.5Hz, 1H), 1.28 (s, 3H), 1.23 (s, 3H), 1.11 (s, 3H), 1.08 (s, 3H), 1.053 (s, 3H), 1.046 (s, 3H), 0.96 (s, 3H), 0.93 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 217.5, 87.3, 87.0, 70.2, 69.9, 55.2, 52.1, 49.4, 48.8, 48.8, 47.2, 39.6, 39.5, 36.7, 33.9, 33.9, 32.1, 32.0, 31.5, 28.7, 28.4, 27.8, 26.6, 24.9, 24.2, 20.8, 19.6, 17.6, 15.9, 15.1. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{51}O_4$ $[M + H]^+$: 475.3782, found: 475.3780.

5.1.5 (20*S*,24*R*)-Epoxy-25-hydroxy-dammarane-3,12-dione (7)

To a solution of **4** (160 mg, 0.33 mmol) in dry dichloromethane (10 mL) was added pyridiniumchlorochromate (217 mg, 1.00 mmol), the mixture was stirred at room temperature for 8 h, then filtered, concentrated in vacuo and the residue was purified over silica gel (3:1 petroleum ether-ethyl acetate) to give the product as a white solid (119 mg, 76%). mp. 157–159℃; ESI-MS *m/z* 473.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.69

(dd, *J* = 10.0 Hz, 6.5 Hz, 1H), 2.93 (d, *J* = 9.5 Hz, 1H), 2.56 (td, *J* = 10.5 Hz, 4.5 Hz, 1H), 2.48-2.50 (m, 1H), 2.42 (ddd, *J* = 11.5 Hz, 7.5 Hz, 4.0 Hz, 1H), 2.20-2.30 (m, 2H), 1.26 (s, 3H), 1.24 (s, 3H),1.21 (s, 3H), 1.11 (s, 3H), 1.10 (s, 3H), 1.06 (s, 3H), 1.03 (s, 3H), 0.78 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 216.7, 216.7, 85.0, 83.5, 71.1, 57.1, 55.7, 55.1, 53.5, 47.3, 40.3, 39.8, 39.1, 37.2, 35.0, 33.7, 33.5, 32.0, 29.6, 27.6, 26.6, 26.5, 25.6, 24.9, 24.4, 20.9, 19.7, 16.5, 15.7, 15.2. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{49}O_4$ $[M + H]^+$: 473.3625, found: 473.3639.

5.1.6 (20*S*,24*R*)-Epoxy-12β,25-dihydroxy-dammarane-3-one (8)

To a solution of **4** (100mg, 0.21mmol) in dry dichloromethane (8 ml) was added pyridinium chlorochromate (87mg, 0.40mmol), the mixture was stirred at room temperature for 3 h. Then filtrated, concentrated in vacuo and the residue was purified over silica gel (5:1 petroleum ether-ethyl acetate) to give the product as a white solid (65 mg, 66%). mp. 162–164℃; ESI-MS *m/z* 475.3 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.85 (dd, *J* = 8.6 Hz, 6.0 Hz, 1H), 3.53 (td, *J* = 10.5 Hz, 4.5 Hz, 1H), 2.48-2.54 (m, 1H), 2.42 (ddd, *J* = 11.5 Hz, 7.5 Hz, 4.0 Hz, 1H), 2.21 (td, *J* = 11.5 Hz, 3.5 Hz, 1H), 1.28 (s, 6H), 1.10 (s, 3H), 1.07 (s, 3H), 1.04 (s, 3H), 1.02 (s, 3H), 0.96 (s, 3H), 0.91 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 217.4, 86.4, 85.3, 70.7, 70.0, 55.3, 51.9, 49.7, 49.4, 47.8, 47.2, 39.7, 39.5, 36.7, 34.0, 33.9, 32.5, 31.6, 31.1, 28.5, 27.8, 27.4, 26.6, 26.0, 24.9, 20.8, 19.6, 17.9, 15.9, 15.1. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{51}O_4$ $[M + H]^+$: 475.3782, found: 475.3790.

5.1.7 (20*S*,24*S*)-Epoxy-12β-*O*-acetyl-dammarane-3β,25-diol (14)

To a solution of **3** (500 mg, 1.05 mmol) and DMAP (20 mg, 0.16 mmol) in dry pyridine (4 ml), acetic anhydride (0.30 ml, 3.15 mmol) was slowly added. The reaction mixture was stirred at room temperature for 5 h, then extracted with ethyl acetate, and the organic layer was washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated, and purified by column chromatography (petroleum ether: ethyl acetate = 8:1) to yield a white solid (500mg, 85%): (20*S*, 24*S*)-Epoxy-3β, 12β-diacetoxy-dammarane -25-ol (**10**).

To a solution of **10** (100 mg, 0.18 mmol) in methanol (10 ml), Potassium hydroxide (21 mg, 0.36 mmol) was added. The reaction mixture was stirred at room temperature for 6 h. The methanol was evaporated in vacuo, ethyl acetate was added. The organic layer

was washed with water and brine, dried over anhydrous sodium sulfate, concentrated, and purified over silica gel (petroleum ether: ethyl acetate=4:1) to get (20*S*, 24*S*)-Epoxy-12β-*O*-acetyl-dammarane-3β, 25-diol (**14**) as a white solid (60 mg, 65%). mp. 200–203℃; ESI-MS *m*/*z* 519.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.83 (td, *J*=10.6 Hz, 5.4 Hz, 1H), 3.63 (t, *J*=8.6 Hz, 6.3Hz, 1H), 3.20 (dd, *J*=10.9 Hz, 4.1Hz, 1H), 2.13-2.28 (m, 1H), 2.01 (s, 3H), 1.26 (s, 3H), 1.18 (s, 3H), 1.09 (s, 3H), 0.99 (s, 3H), 0.98 (s, 3H), 0.93 (s, 3H), 0.85 (s, 3H), 0.78 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.8, 85.7, 84.7, 78.9, 75.8, 70.7, 56.0, 52.6, 50.4, 49.9, 46.6, 40.0, 39.8, 39.1, 39.0, 37.3, 34.7, 31.2, 28.6, 28.2, 27.8, 27.4, 27.1, 25.9, 24.2, 23.0, 22.1, 18.4, 17.6, 16.2, 15.7, 15.6. HR-MS (ESI) *m/z*: calculated for $C_{32}H_{54}NaO_5$ $[M + Na]^+$: 541.3863, found: 541.3863.

5.1.8 (20*S*,24*S*)-Epoxy-3β,25-dihydroxy-dammarane-12-one (15)

To a solution of **3** (100 mg, 0.21 mmol) and DMAP (10 mg, 0.08 mmol) in dry pyridine (4 ml) was slowly added acetic anhydride (0.03 ml, 0.32 mmol), the mixture was stirred for 12 h at room temperature, then diluted with ethyl acetate, washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated and the residue was purified by column chromatography, eluting with petroleum ether : ethyl acetate 3:1 mixture. (20*S*, 24*S*)-Epoxy-3β-acetoxy-dammarane-12β, 25-diol (**9**) was obtained as a white solid (94 mg, 86%).

To a solution of **9** (120 mg, 0.23 mmol) in dry dichloromethane (5 mL) was added pyridiniumchlorochromate (120 mg, 0.54 mmol), the mixture was stirred at room temperature for 10 h, then filtrated, concentrated in vacuo and the residue was purified over silica gel (4:1 petroleum ether-ethyl acetate) to give (20*S*, 24*S*)-Epoxy-3β-acetoxy-25-hydroxy-dammarane-12-one (**11**) as a white solid (85 mg, 72%).

To a solution of **11** (85 mg, 0.16 mmol) in methanol (5 mL) was added potassium hydroxide (40 mg, 0.71 mmol), the mixture was refluxed for 5 h. The solvent was removed in vacuo and ethyl acetate was added, then washed with water and brine, dried over anhydrous Na_2SO_4. The residue was purified by column chromatography over silica gel (2:1 petroleum ether-ethyl acetate) to afford (20*S*, 24*S*)-Epoxy-3β, 25-dihydroxy-dammarane-12-one (**15**) as white powder (68 mg, 87%). mp. 162–164℃; ESI-MS *m*/*z* 475.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.71 (dd, *J* = 10.5 Hz, 6.0 Hz, 1H), 3.20

(dd, *J* = 11.0 Hz, 4.5 Hz, 1H), 2.96 (d, *J* = 9.5 Hz, 1H), 2.53 (td, *J* = 10.5 Hz, 4.5 Hz, 1H), 2.20-2.22 (m, 2H), 1.20 (s, 3H), 1.19(s, 3H), 1.09 (s, 3H), 1.04 (s, 3H), 0.99 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 211.0, 87.5, 85.2, 78.5, 70.2, 57.2, 55.9, 55.7, 54.3, 40.3, 39.6, 38.8, 38.5, 37.6, 36.5, 34.2, 31.9, 29.6, 27.9, 27.6, 27.1, 26.5, 26.1, 24.7, 23.9, 18.3, 16.5, 16.0, 15.5, 15.2. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{51}O_4$ $[M + H]^+$: 475.3782, found: 475.3791.

5.1.9 (20*S*,24*S*)-Epoxy-12β-*O*-(2-carboxybenzoyl)-dammarane-3β,25-diol (16)

To a solution of **3** (200 mg, 0.42 mmol) and DMAP (20 mg, 0.16 mmol) in dry pyridine (4 ml), acetic anhydride (0.06 ml, 0.63 mmol) was slowly added. The reaction mixture was stirred at room temperature for 5 h, then extracted with ethyl acetate, and the organic layer was washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated, and purified by column chromatography (petroleum ether: ethyl acetate = 4:1) to yield a white solid (178mg, 82%): (20*S*, 24*S*)-Epoxy-3β-*O*-acetyl-dammarane-12β, 25-diol (**9**).

To a solution of **9** (178 mg, 0.34 mmol) and DMAP (12 mg, 0.10 mmol) in dry pyridine (4 ml), phthalic anhydride (164mg, 1.11 mmol) was added. The reaction mixture was stirred at 115^{o}C for 20 h, then extracted with ethyl acetate, and the organic layer was washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated, and purified by column chromatography (petroleum ether: ethyl acetate = 1:1) to yield the product as a white solid (124mg, 55%): (20*S*, 24*S*)-Epoxy-3β-*O*-acetyl-12β-*O*-(2-carboxy benzoyl)-dammarane-25-ol (**12**).

To a solution of **12** (100 mg, 0.15 mmol) in methanol (10 ml), Potassium hydroxide (17 mg, 0.30 mmol) was added. The reaction mixture was stirred at room temperature for 6 h. The methanol was evaporated in vacuo, ethyl acetate was added. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, concentrated, and purified over silica gel (dichloromethane : methanol=80:1) to get (20*S*, 24*R*)-Epoxy-12β-*O*-(2-carboxy benzoyl)-dammarane-3β, 25-diol (**16**) as a white solid (56 mg, 60%). mp. 192–195□; ESI-MS *m/z* 625.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.71-7.75 (m, 2H), 7.44-7.52 (m, 2H), 5.11 (td, *J*=10.5 Hz, 5.5 Hz, 1H), 3.55 (dd, *J*=7.0 Hz, 6.2 Hz, 1H), 3.18 (dd, *J*=11.0 Hz, 4.9 Hz, 1H), 2.04-2.18 (m, 2H), 1.27 (s, 3H),1.11 (s, 3H), 1.05 (s, 3H),1.01 (s, 3H), 0.98 (s, 3H),0.97 (s, 3H),0.86 (s, 3H),0.75 (s, 3H); ^{13}C NMR ($CDCl_3$,

75 MHz) δ172.8, 167.7, 131.6, 130.2, 129.5, 129.2, 86.0, 85.0, 79.0, 76.6, 71.1, 56.1, 52.9, 50.2, 50.2, 46.9, 39.8, 39.2, 39.1, 39.0, 37.3, 34.8, 31.4, 28.2, 27.6, 27.4, 27.3, 27.2, 26.4, 24.0, 23.7, 18.4, 18.0, 16.3, 15.8, 15.6. HR-MS (ESI) *m/z*: calculated for $C_{38}H_{56}NaO_7$ $[M + Na]^+$: 647.3918, found: 647.3922.

5.1.11 (20*S*,24*S*)-Epoxy-12β-*O*-cinnamoyl-dammarane-3β,25-diol (**17**)

To a solution of **3** (200 mg, 0.42 mmol) and DMAP (20 mg, 0.16 mmol) in dry pyridine (4 ml), acetic anhydride (0.06 ml, 0.63 mmol) was slowly added. After stirring at room temperature for 5 h. The reaction mixture was extracted with ethyl acetate, and the organic layer was washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated, and purified by column chromatography (petroleum ether: ethyl acetate = 2:1) to yield a white solid (163mg, 75%): (20*S*, 24*S*)-Epoxy-3β-*O*-acetyl-dammarane-12β, 25-diol (**9**).

To a solution of **9** (163 mg, 0.31 mmol) and DMAP (20 mg, 0.16 mmol) in dry pyridine (4 mL), cinnamoyl chloride (154 mg, 0.93 mmol) was added. The reaction mixture was stirred at 120^{o}C for 25 h, then diluted with ethyl acetate, and the organic layer was washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated, and purified by column chromatography (petroleum ether: ethyl acetate = 4:1) to yield the product as a white solid (104mg, 52%): (20*S*, 24*S*)-Epoxy-3β-*O*-acety1-12β-*O*-benzene acryloyl-dammarane-25-ol (**13**).

To a solution of **13** (104 mg, 0.16 mmol) in methanol (10 mL), Potassium hydroxide (18 mg, 0.32 mmol) was added. The reaction mixture was stirred at room temperature for 6 h. The methanol was evaporated in vacuo, ethyl acetate was added. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, concentrated, and purified over silica gel (petroleum ether: ethyl acetate = 2:1) to get (20*S*, 24*S*)-Epoxy-12β-*O*-benzene acryloyl-dammarane-3β, 25-diol (**17**) as a white solid (63 mg, 66%). mp. 188–192℃; ESI-MS *m/z* 607.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.68 (d, *J*=16.0 Hz, 1H), 7.52-7.55 (m, 2H), 7.41 (t, *J*=3.4 Hz, 3.1Hz, 3H), 6.38 (d, *J*=16.0 Hz, 1H), 5.02 (td, *J*=10.5 Hz, 5.5 Hz, 1H), 3.63 (dd, *J*=7.0 Hz, 6.2 Hz, 1H), 3.23 (dd, *J*=11.0 Hz, 4.9 Hz, 1H), 2.11-2.29 (m, 2H), 1.26 (s, 3H), 1.18 (s, 3H), 1.08 (s, 3H), 1.04 (s, 3H), 1.00(s, 3H), 0.99 (s, 3H), 0.87 (s, 3H), 0.79 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.7, 145.1, 134.6, 130.6, 129.1, 128.3, 118.9, 85.7, 84.7, 79.0, 75.8, 70.7, 56.0, 52.6, 50.6, 50.0, 46.8, 40.2,

39.8, 39.1, 39.0, 37.4, 34.7, 31.1, 28.7, 28.2, 27.8, 27.5, 27.2, 25.9, 24.2, 23.1, 18.5, 17.7, 16.2, 15.8, 15.6. HR-MS (ESI) *m/z*: calculated for $C_{39}H_{58}NaO_5$ $[M + Na]^+$: 629.4190, found: 629.4192.

5.1.12 (20*S*,24*R*)-Epoxy-12β-*O*-acetyl-dammarane-3β,25-diol (22)

To a solution of **4** (500 mg, 1.05 mmol) and DMAP (20 mg, 0.16 mmol) in dry pyridine (4 mL), acetic anhydride (0.30 mL, 3.15 mmol) was slowly added. The reaction mixture was stirred at room temperature for 5 h, then extracted with ethyl acetate, and the organic layer was washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated, and purified by column chromatography (petroleum ether: ethyl acetate = 8:1) to yield a white solid (500mg, 85%): (20*S*, 24*R*)-Epoxy-3β, 12β-diacetoxy-dammarane-25-ol (**19**).

To a solution of **19** (100 mg, 0.18 mmol) in methanol (10 mL), Potassium hydroxide (21 mg, 0.36 mmol) was added. The reaction mixture was stirred at room temperature for 6 h. The methanol was evaporated in vacuo, ethyl acetate was added. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, concentrated, and purified over silica gel (petroleum ether: ethyl acetate=4:1) to get (20*S*, 24*R*)-Epoxy-12β-*O*-acetyl-dammarane-3β, 25-diol (**22**) as a white solid (60 mg, 65%). mp. 205–208℃; ESI-MS *m*/*z* 519.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.83 (td, *J*=10.6 Hz, 5.3 Hz, 1H), 3.65 (t, *J*=7.1 Hz, 6.7 Hz, 1H), 3.19 (dd, *J*=11.2 Hz, 4.2 Hz, 1H), 2.14-2.33 (m, 1H), 2.01 (s, 3H), 1.28 (s, 3H), 1.26 (s, 3H), 1.10 (s, 3H), 1.00 (s, 3H), 0.98 (s, 3H), 0.94 (s, 3H), 0.85 (s, 3H), 0.77 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.8, 85.9, 83.5, 78.8, 75.8, 71.2, 56.2, 56.0, 52.4, 50.7, 50.0, 46.5, 39.8, 39.1, 39.0, 37.3, 34.7, 31.4, 28.6, 28.2, 27.7, 27.4, 27.0, 26.3, 24.4, 22.4, 22.0, 18.4, 17.8, 16.2, 15.7, 15.6. HR-MS (ESI) *m/z*: calculated for $C_{32}H_{54}NaO_5$ $[M + Na]^+$: 541.3863, found: 541.3865.

5.1.13 (20*S*,24*R*)-Epoxy-3β,25-dihydroxy-dammarane-12-one (23)

To a solution of **4** (100 mg, 0.21 mmol) and DMAP (10 mg, 0.08 mmol) in dry pyridine (4 ml) was slowly added acetic anhydride (0.03 ml, 0.32 mmol), the mixture was stirred for 12 h at room temperature,then diluted with ethyl acetate, washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated and the residue was purified by column chromatography,eluting with petroleum

ether :ethyl acetate 10:1 mixture. (20*S*, 24*R*)-Epoxy-3β-acetoxy-dammarane-12β, 25-diol (**18**) was obtained as a white solid (94 mg, 86%).

To a solution of **18** (120 mg, 0.23 mmol) in dry dichloromethane (5 mL) was added pyridinium chlorochromate (120 mg, 0.54 mmol), the mixture was stirred at room temperature for 10 h, then filtered, concentrated in vacuo and the residue was purified over silica gel (10:1 petroleum ether-ethyl acetate) to give (20*S*, 24*R*)-Epoxy-3β-acetoxy -25-hydroxy-dammarane-12-one (**20**) as a white solid (85 mg, 72%).

To a solution of **20** (85 mg, 0.16 mmol) in methanol (5 mL) was added potassium hydroxide (40 mg, 0.71 mmol), and the mixture was refluxed for 5 h. The solvent was removed in vacuo and ethyl acetate was added, and then washed with water and brine, dried over anhydrous Na_2SO_4. The residue was purified by column chromatography over silica gel (2:1 petroleum ether-ethyl acetate) to afford (20*S*, 24*R*)-Epoxy-3β, 25-dihydroxy-dammarane-12-one (**23**) as white powder (68 mg, 87%). mp. 193–195℃; ESI-MS *m/z* 475.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.69 (dd, J = 8.5 Hz, 6.0 Hz, 1H), 3.19 (dd, J = 11.5 Hz, 5.0 Hz, 1H), 2.90 (d, J = 10.0 Hz, 1H), 2.57 (td, J = 10.5 Hz, 4.5 Hz, 1H), 2.19-2.21 (m, 2H), 1.207(s, 3H), 1.198 (s, 3H), 1.11 (s, 3H), 1.10 (s, 3H), 0.99 (s, 3H), 0.93 (s, 3H), 0.80 (s, 3H), 0.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 211.4, 85.1, 83.5, 78.5, 71.1, 57.0, 55.7, 55.7, 54.2, 40.3, 39.7, 38.8, 38.5, 37.6, 35.1, 34.2, 32.0, 29.6, 27.9, 27.5, 27.1, 26.5, 25.5, 24.9, 24.3, 18.3, 16.6, 16.0, 15.5, 15.2. HR-MS (ESI) *m/z*: calculated for $C_{30}H_{51}O_4$ $[M + H]^+$: 475.3782, found: 475.3791.

5.1.14 (20*S*,24*R*)-Epoxy-12β-*O*-(2-carboxy benzoyl)-dammarane-3β,25-diol (24)

To a solution of **4** (200 mg, 0.42 mmol) and DMAP (20 mg, 0.16 mmol) in dry pyridine (5 mL), acetic anhydride (0.06 mL, 0.63 mmol) was slowly added. The reaction mixture was stirred at room temperature for 5 h, then extracted with ethyl acetate, and the organic layer was washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated, and purified by column chromatography (petroleum ether: ethyl acetate = 4:1) to yield a white solid (178 mg, 82%): (20*S*, 24*R*)-Epoxy-3β-*O*-acetyl-dammarane-12β, 25-diol (**18**).

To a solution of **18** (178 mg, 0.34 mmol) and DMAP (12 mg, 0.10 mmol) in dry pyridine (4 mL), phthalic anhydride (164 mg, 1.11 mmol) was added. The reaction mixture was stirred at 120^{o}C for 25 h, then extracted with ethyl acetate, and the organic

layer was washed with 10% HCl, water and brine successively, dried over anhydrous sodium sulfate, concentrated, and purified by column chromatography (petroleum ether: ethyl acetate = 1:1) to yield the product as a white solid (124 mg, 55%): (20*S*, 24*R*)-Epoxy-3β-*O*-acetyl-12β-*O*-(2-carboxy benzoyl)-dammarane-25-ol (**21**).

To a solution of **21** (100 mg, 0.15 mmol) in methanol (10 mL), Potassium hydroxide (17 mg, 0.30 mmol) was added. The reaction mixture was stirred at room temperature for 6 h. The methanol was evaporated in vacuo, ethyl acetate was added. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, concentrated, and purified over silica gel (dichloromethane: methanol=80:1) to get (20*S*, 24*R*)-Epoxy-12β-*O*-(2-carboxy benzoyl)-dammarane-3β, 25-diol (**24**) as a white solid (56 mg, 60%). mp. 196–198℃; ESI-MS *m*/*z* 625.4 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.84-7.86 (m, 1H), 7.68-7.70 (m, 1H), 7.56-7.59 (m, 2H), 5.12 (td, *J*=10.5 Hz, 5.0 Hz, 1H), 3.56 (t, *J*=7.5 Hz, 6.5 Hz, 1H), 3.26 (dd, *J*=10.5 Hz, 4.5 Hz, 1H), 2.11-2.19 (m, 2H), 1.28 (s, 3H), 1.09 (s, 3H), 1.08 (s, 3H), 1.07 (s, 3H), 1.03(s, 3H) , 1.00 (s, 3H), 0.87 (s, 3H), 0.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.4, 167.4, 133.1, 131.4, 131,2, 130.8, 129.5, 128.9, 85.9, 83.1, 78.8, 76.7, 71.3, 55.8, 52.4, 50.2, 50.0, 46.5, 39.6, 38.8, 38.7, 38.6, 37.1, 34.5, 31.5, 27.9, 27.7, 27.1, 27.0, 26.5, 26.2, 23.9, 21.9, 18.2, 17.8, 16.0, 15.5, 15.3. HR-MS (ESI) *m/z*: calculated for $C_{38}H_{56}NaO_7$ $[M + Na]^+$: 647.3918, found: 647.3916.

5.2 Pharmacology

Antibacterial activity assay

The minimum inhibitory concentration (MIC) of the synthesized compounds was determined against several bacterial strains using a standard LB medium dilution technique as described below. The compounds were dissolved to 50 mM in DMSO and transferred into 100 µl of Luria-Bertani medium as a serial dilution from 128 µg/ml to 2 µg/ml in 96-well NUNC Microwell™ plates. Bacteria were grown at 37°C in 5 ml LB with shaking until the optical density reached 0.6-0.7, and 5 µl of the culture was added to each well. The plate was incubated in a FLUOstar Optima plate reader (BMG Labtech) at 37°C with 600 rpm shaking. The OD_{600} of the culturewas taken every 10 min using LB

as the blank. The samples were tested in triplicate and the growth pattern of each sample was compared to cells exposed to equal amounts of DMSO.

Synergistic antibacterial activity assay

The synergistic antibacterial activity of the synthesized compounds was determined against *S. aureus* strain USA300 and *B. subtilis* strain 168 together with kanamycin or chloramphenicol. Kanamycin was dissolved in MilliQ water to 50 mM and chloramphenicol was dissolved in DMSO. Using a standard LB medium dilution technique, kanamycin or chloramphenicol was transferred into 100 µl of Luria-Bertani medium as a serial dilution from 1 µg/ml to 0.0020 µg/ml or from 4 µg/ml to 0.0078 µg/ml, respectively (minimum bactericidal or bacteriostatic concentrations) in 96-well NUNC Microwell™ plates. Bacteria were grown at 37°C in 5 ml LB with shaking until the optical density reached 0.6-0.7, 5 µl of the culture and the compounds to be tested at half of their minimum inhibitory concentrations were added to each well. The plate was incubated in a FLUOstar Optima plate reader (BMG Labtech) at 37°C with 600 rpm shaking. The OD_{600} of the culture was taken every 10 min using LB as the blank. The samples were tested in triplicate and the growth pattern of each sample was compared to cells exposed to equal amounts of DMSO. The FICI was calculated using fractional inhibitory concentration (FIC) according to the formulae from the literature [19] (Table 3).

MTT cytotoxicity assay

The MTT assay was employed in cytotoxicity assay in vitro, which was performed in 96-well plates. Human cervical (HeLa) and human epithelial kidney (HEK-293) cells at the log phase of their growth cycle (5×10^4 cell/mL) were added to each well (100 µL/well), then treated in three replicates at various concentrations of the samples (0.39-100 µg/mL), and incubated for 24 h at 37 □ in a humidified atmosphere of 5% CO_2. After 72 h, 20 µL of MTT solution (5 mg/mL) per well was added to each cultured medium, which was incubated for another 4 h. Then DMSO was added to each well (150 µL/well). After 10 min at room temperature, the OD of each well was measured on a Microplate Reader (BIO-RAD Instruments Inc NO.550) at the wavelength of 490 nm. In these experiments, the negative reference agent was 0.1% DMSO, and 5-fluorouracil was used as the positive reference with the concentration of 10 µg/mL.

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Figure captions

Table 1.

In vitro antibacterial activity of ocotillol-type derivatives. (MIC: μg/mL)

Strain	*S. aures*	*B. sub*	*E. coli*	*P. aer*	*A. baum*
HBA	16	8	>128	>128	>128
PPD	16	32	64	>128	64
3	8	8	>128	>128	>128
4	64	128	>128	>128	128
5	16	64	>128	>128	>128
6	>128	32	>128	>128	>128
7	128	128	>128	>128	>128
8	128	128	>128	>128	>128
14	>128	>128	64	64	64
15	64	>128	64	>128	>128
16	4	16	>128	128	>128
17	>128	>128	>128	128	32
22	64	>128	64	128	128
23	>128	128	>128	>128	>128
24	64	16	>128	>128	>128
KAN[a]	1	0.25	1	8	1

[a] KAN: kanamycin

Table 2.

Antibacterial activity of compounds **PPD, 3, 5, 16** and **24** against MRSA[a].

(MIC: μg/mL)

compound	MRSA USA 300
HBA	16
PPD	32
3	8
5	16
16	32
24	64
KAN[b]	1

[a] MRSA: methicillin-resistant *S. aureus*; [b] KAN: kanamycin

Table 3 .

Synergistic effect of different antibiotics with compounds **PPD**, **3** and **16** against *S. aureus* USA 300 and *B. subtilis* 168

Compound	MIC (μg/mL)		MBC (μg/mL)		FICI (FIC index)[d]	
	S. aureus USA300	*B.sub* 168	*S. aureus* USA300	*B.sub* 168	*S. aureus* USA300	*B.sub* 168
KAN[a]	1	0.25	4	1	-	-
CHL[b]	4	2	N/A[c]	N/A	-	-
PPD+KAN	0.25	0.0625	2	0.25	0.26	0.25
3+KAN	0.125	0.2	1	1	0.14	1.03
16+KAN	0.0078	<0.0020	2	<0.0020	0.008	<0.008
PPD+CHL	4	1	N/A	N/A	1.13	0.53
3+CHL	4	2	N/A	N/A	1.5	1.25
16+CHL	0.016	<0.0078	N/A	<0.0078	0.005	<0.004

[a] KAN: kanamycin; [b] CHL: chloramphenicol; [c] N/A: not applicable.

[d] FICI: according to the literature [19]: FIC of drug A (FIC A) = MIC of drug A in combination/MIC of drug A alone; FIC of drug B (FIC B) = MIC of drug B in combination/MIC of drug B alone; hence FICI = FIC A + FIC B. “Synergy” was defined when FICI was less than or equal to 0.5; while “additive” in which the FICI was greater than 0.5 and less than or equal to 1.0; whereas “indifferent” when the FICI was greater than 1.0 and less than or equal to 2.0; and “antagonistic” in cases which the FICI was greater than 2.0.

Table 4.

Cytotoxic activity of compounds **3**, **5** and **16** against HeLa and HEK-293 cells

Compound	IC_{50}[a] (μg/mL)		SI[b] (HeLa / HEK-293)		SI (HeLa / HEK-293)	
	HeLa	HEK-293	*S.aureus* RN4220		CA-MRSA USA300	
3	47.64±3.46	154.26±10.53	6.0	19.3	6.0	19.3
5	46.98±4.12	170.19±12.34	2.9	10.6	2.9	10.6
16	54.39±5.70	175.32±16.09	13.6	43.8	1.7	5.5
5-FU[c]	0.80±0.13	-	-	-	-	-

[a] IC_{50} is the concentrations required to inhibit 50% of cell growth and the results are expressed as the mean ± S.D. of three independent experiments.

[b] SI: Selectivity index (IC_{50} / MIC).

[c] 5-FU: 5-fluorouracil.

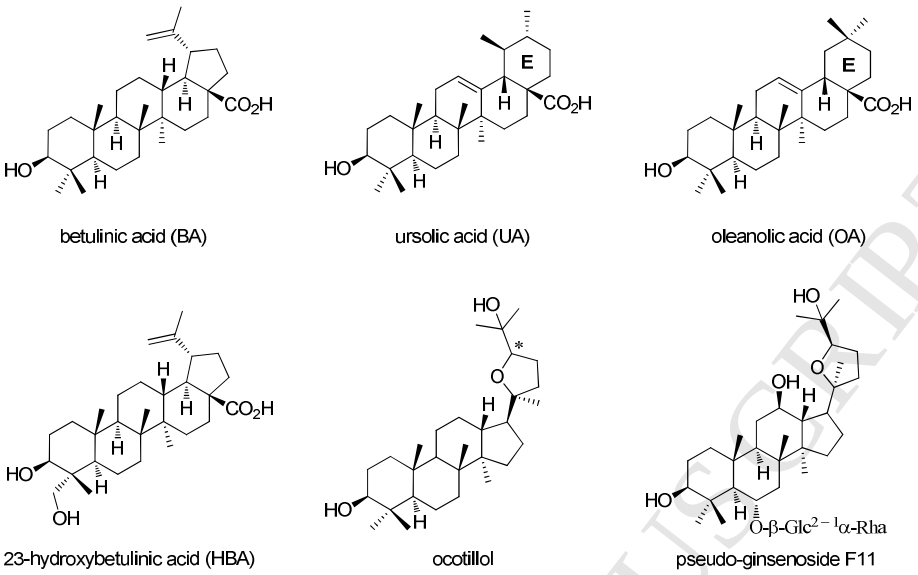


Figure 1. Natural triterpenoid products

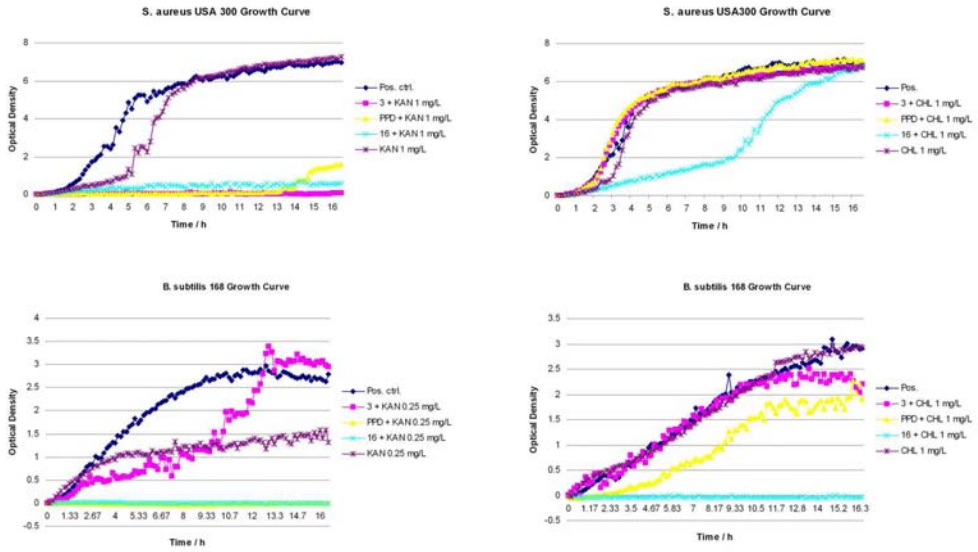


Figure 2. The synergistic effects of compounds **PPD**, **3** and **16** with two conventional antibiotics against CA- MRSA USA300 and *B. subtilis* 168.

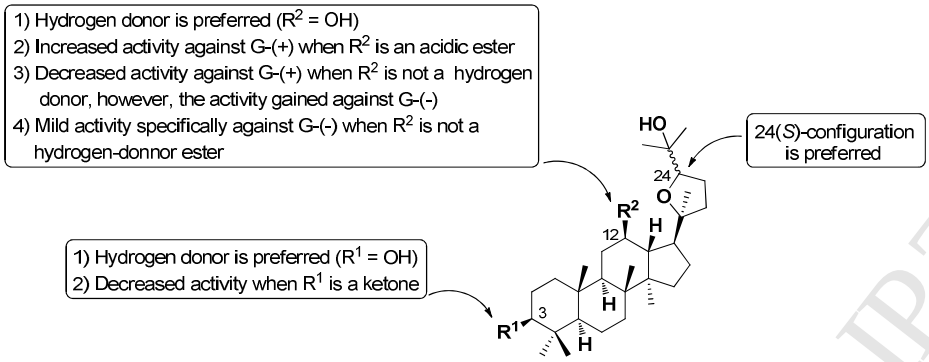
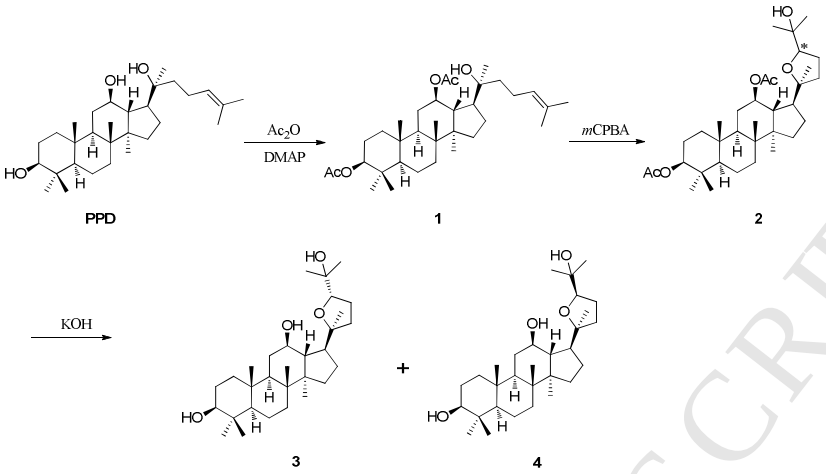
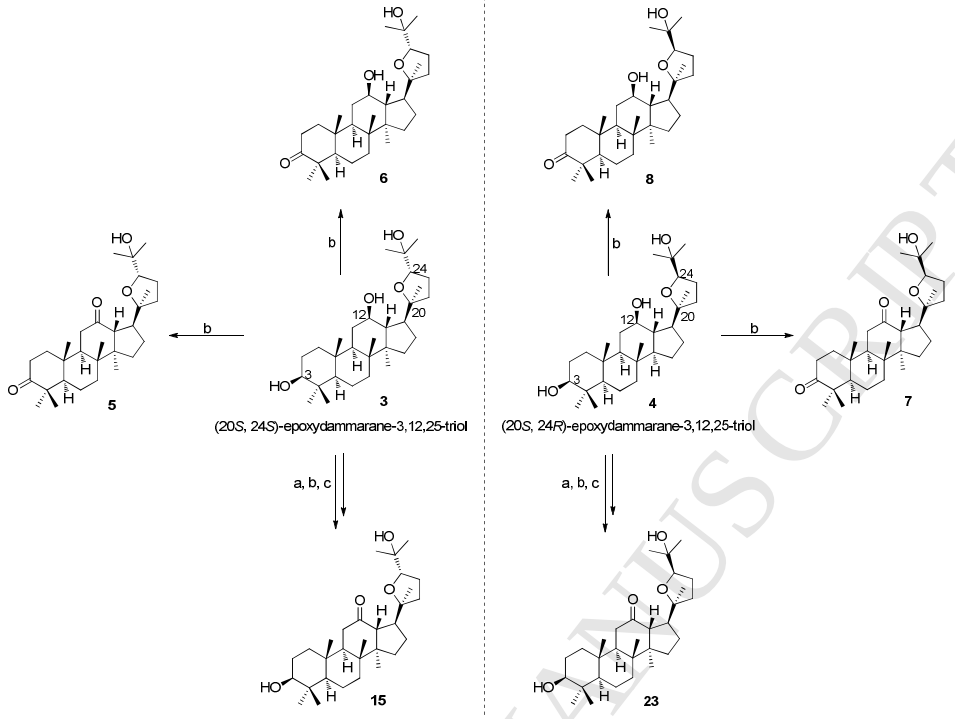


Figure 3. The preliminary SARs of ocotillol-type triterpenoid derivatives.

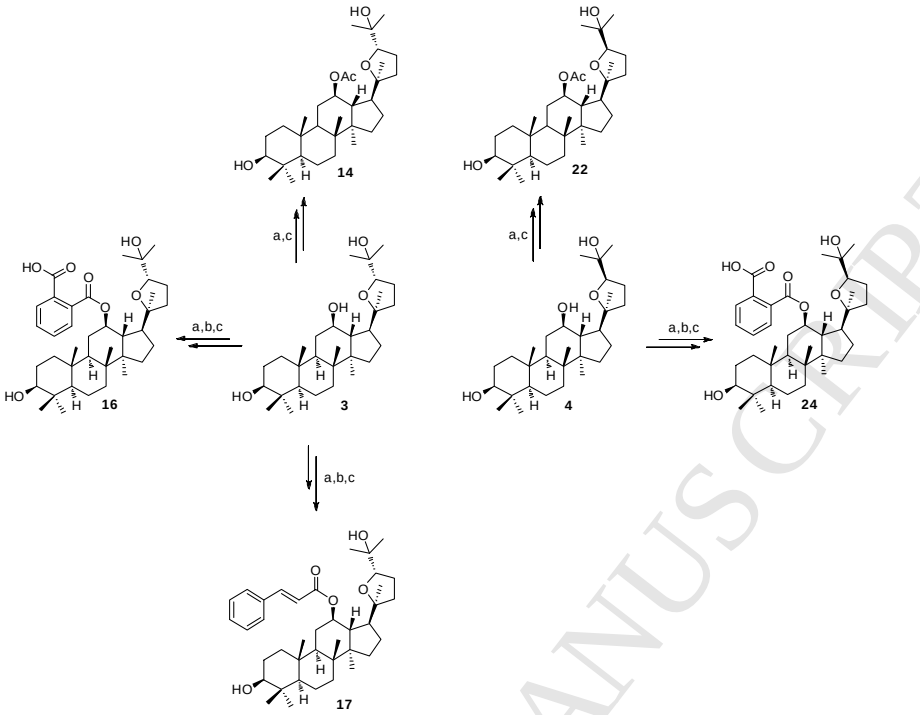


Scheme 1. Synthesis of triols **3** and **4**.



Reagents and conditions: (a) anhydrous pyridine, Ac_2O, DMAP, rt. (b) anhydrous CH_2Cl_2, PCC, rt. (c) CH_3OH, KOH, ref.

Scheme 2. Synthesis of oxidation derivatives (**5**-**8**, **15**, **23**) of **3** and **4**.



Reagents and conditions: (a) anhydrous pyridine, Ac_2O, DMAP, rt. (b) anhydrous pyridine, phthalic anhydride, DMAP, ref. (c) CH_3OH, KOH, rt. (d) anhydrous pyridine, cinnamoyl chloride, DMAP, Et_3N, ref.

Scheme 3. Synthesis of ester derivatives (**14**, **16**, **17**, **22**, **24**).

Highlights:

- A series of ocotillol-type derivatives derived from natural PPD were obtained.
- Compounds **3**, **5**, **16** and **24** showed potent activities against Gram-(+) bacteria.
- Compounds **3** and **16** highly sensitized Gram-(+) bacteria to antibiotics KAN and CHL.
- Compounds **3**, **5** and **16** exhibited no cytotoxicity at their MICs.