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## Synthesis of oxygenated oleanolic and ursolic acid derivatives with anti-inflammatory properties

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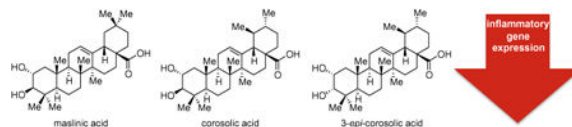
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### Abstract

The scalable syntheses of four oxygenated triterpenes have been implemented to access substantial quantities of maslinic acid, 3-*epi*-maslinic acid, corosolic acid, and 3-*epi*-corosolic acid. Semi-syntheses proceed starting from the natural products oleanolic acid and ursolic acid. Proceeding over five steps, each of the four compounds can be synthesized on gram scale. Divergent diastereoselective reductions of  $\alpha$ -hydroxy ketones provided access to the four targeted diol containing compounds from two precursors of the oleanane or ursane lineage. These compounds were subsequently evaluated for their ability to inhibit inflammatory gene expression in a mouse model of chemically induced skin inflammation. All compounds possessed the ability to inhibit the expression of one or more inflammatory genes induced by 12-*O*-tetradecanoylphorbol-13 acetate in mouse skin, however, three of the compounds, corosolic acid, 3-*epi*-corosolic acid and maslinic acid were more effective than the others. The availability of gram quantities will allow further testing of these compounds for potential anti-inflammatory activities as well as cancer chemopreventive activity.

### Graphical abstract



### Keywords

Triterpene; Cancer; Mouse; Skin inflammation; Maslinic acid; 3-*epi*-Maslinic acid; Corosolic acid; 3-*epi*-Corosolic acid; Synthesis; Natural product; Chemoprevention

Cancer continues to be a burden on mankind with 14.1 million new cases and 8.2 million cancer deaths in 2012.<sup>1</sup> These figures are projected to increase to 22.2 million new cases and 13.2 million deaths by the year 2030.<sup>2</sup> If melanoma and non-melanoma skin cancers (squamous cell carcinoma, basal cell carcinoma, etc.) are all placed under the umbrella of skin cancer, then this malignancy is the most common in the world,<sup>3</sup> particularly among fair populations.<sup>4</sup> According to the World Health Organization, skin cancer accounts for one third of all cancer diagnoses.<sup>5</sup> Furthermore, the incidence of skin cancer is on the rise.<sup>4</sup> Estimates indicate that from 1975 to 2006 there were more cases of skin cancer in the United States than all other cancers combined.<sup>6</sup> Thus, there is tremendous need to develop new agents for prevention and/or treatment of skin and other cancers.<sup>28,29</sup>

In light of previous studies showing the anti-tumor promoting activity of ursolic acid **4** (Fig. 1) in a murine two-stage skin carcinogenesis experiment<sup>30–32</sup>, the same mouse model was used to explore the efficacy of four pentacyclic triterpene natural products with ursolic acid-like structure and oxidative pattern: maslinic acid **5**, 3-*epi*-maslinic acid **6**, corosolic acid **7**, and 3-*epi*-corosolic acid **8** (Fig. 2). Maslinic acid and 3-*epi*-maslinic acid are oleanane triterpenes, while corosolic acid and 3-*epi*-corosolic acid are of the ursane variety. Oleanane **1** triterpenes differ from ursane **2** triterpenes in the position of a methyl group at C20 in lieu of C19. All four have been isolated from plant sources, including *Perilla frutescens*,<sup>7,8</sup> whose leaves are a common ingredient in Chinese, Japanese, and Korean cuisine.<sup>9</sup>

*P. frutescens* also finds use in traditional Chinese medicine as a treatment for gastrointestinal disturbances, fish and crab poisoning, cough, asthma, and the common cold.<sup>10</sup> In more modern work, all four of the above mentioned triterpenes have shown important biological activity, notably against human cancer cells. Maslinic acid inhibited the growth of adenoid,<sup>11</sup> astrocytoma,<sup>12</sup> breast,<sup>13,14</sup> cervical,<sup>12</sup> colon,<sup>15,16</sup> fibrosarcoma,<sup>17</sup> lung,<sup>15</sup> ovarian,<sup>15</sup> and melanoma<sup>13,15</sup> cancer cells. The compound 3-*epi*-maslinic acid demonstrated activity against gastric and uterine cancer cells.<sup>18</sup> Corosolic acid slowed the growth of breast,<sup>19–21</sup> cervical,<sup>22</sup> colon,<sup>15,23</sup> gastric,<sup>18,22</sup> liver,<sup>21,22</sup> leukemia,<sup>22,24</sup> lung,<sup>15,19</sup> melanoma,<sup>25</sup> nerve,<sup>15</sup> ovarian,<sup>19</sup> and uterine<sup>18</sup> cancer in vitro. The diastereomer of corosolic acid, 3-*epi*-corosolic acid, demonstrated cytotoxic activity against leukemia and breast cancer cell lines.<sup>26</sup> While these natural products have shown anti-cancer activity across many cell types, exploration of their effect on skin cancers like squamous cell carcinoma and basal cell carcinoma has been limited. Furthermore, there has been a shortage of studies employing these compounds in whole-organism, in vivo cancer trials as there are challenges in accessing the required quantities of natural product.

In order to evaluate the activity of maslinic acid, 3-*epi*-maslinic acid, corosolic acid, and 3-*epi*-corosolic acid in a murine model of skin cancer, each natural product was synthesized based on literature precedent on gram scale.<sup>27</sup> Maslinic acid and 3-*epi*-maslinic acid were synthesized starting from commercially available oleanolic acid **3** (Fig. 3). The carboxylic acid of oleanolic acid was protected with benzyl chloride in the presence of catalytic potassium iodide to afford the benzyl ester **9**. The crude ester was precipitated by combining the reaction mixture with cold water (0 °C) and, after filtration, used in the subsequent step without further purification. The secondary alcohol of **9** was oxidized cleanly with Jones reagent to the corresponding ketone **10**. Purification by recrystallization from 200 proof

ethanol gave pure **10** as a solid in 81% yield over two steps. Treatment of ketone **10** with *m*-chloroperbenzoic acid and catalytic sulfuric acid generated hydroxyketone **11** by diastereoselective oxidation of the transient enol on the less hindered  $\alpha$ -face. Reduction of  $\alpha$ -hydroxyketone **11** under different conditions afforded a variable mixture of two diastereomeric vicinal diols **12** and **13**. Sodium borohydride favored formation of diol **12** with hydride delivery to the *Si* face of the ketone. This selectivity was reversed under Meerwein–Ponndorf–Verley conditions, favoring diol **13**. Deprotection by cleavage of the benzyl ester of **12** was effected using an atmosphere of hydrogen gas and palladium on carbon, generating maslinic acid **5**. Subjecting diol **13** to the same conditions yielded 3-*epi*-maslinic acid **6**.

Corosolic acid **7** and 3-*epi*-corosolic acid **8** were synthesized in an analogous manner, starting from ursolic acid **4** (Fig. 4). The carboxylic acid of ursolic acid was protected as a benzyl ester using benzyl chloride in the presence of catalytic potassium iodide generating benzyl ester **14**. The crude ester was precipitated from the reaction by combining the reaction mixture with cold water (0 °C) following filtration the crude material was used without further purification. The secondary alcohol of **14** was oxidized to the ketone through the use of Dess–Martin periodinane to the yield ketone **15**. Purification of ketone **15** by recrystallization from 200 proof ethanol gave pure **15** in 92% yield over two steps. Treatment of **15** with *m*-chloroperbenzoic acid and catalytic sulfuric acid generated hydroxyketone **16** by diastereoselective oxidation of the enol from the less hindered face. Selective reduction of the  $\alpha$ -hydroxyketone **16** under two conditions afforded a different ratio of the vicinal diols, **17** and **18**. While sodium borohydride was selective for the formation of **17** with hydride delivery to the *Si* face of the ketone the selectivity was reversed using Meerwein–Ponndorf–Verley conditions to favor diol **18**. Reductive deprotection of diol **17** was achieved with hydrogen gas (1 atm) and palladium on carbon, generating corosolic acid **7**. In a similar transformation the diol **18** was debenzylated to yield 3-*epi*-corosolic acid **8**.

In preliminary experiments, the compounds were subsequently tested for their ability to inhibit inflammatory gene expression induced by topical treatment with the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse skin. Inflammation and changes in inflammatory gene expression are closely linked to the process of skin tumor promotion by TPA.<sup>33,34</sup> For these experiments, six-week old ICR mice (obtained from Harlan Laboratories Inc. Frederick, MD) were group housed and maintained on a chow diet for one week. All experiments were carried out according to institutional and the NIH guidelines. The dorsal skin of mice (at 7 weeks of age) was shaved, and two days later mice received topical treatments of acetone vehicle (200  $\mu$ l) or 2  $\mu$ mol of ursolic acid, oleanolic acid, corosolic acid, 3-*epi*-corosolic acid, maslinic acid, or 3-*epi*-maslinic acid 30 min before 6.8 nmol TPA treatment. All treatments were given twice-weekly over a two-week period. Epidermal tissue was then harvested 6 h after the last TPA treatment for RNA extraction as previously described.<sup>35,36</sup> For the analysis of inflammatory gene expression, cDNA was prepared from epidermal RNA samples using High Capacity cDNA Reverse Transcription Kits (AB Applied Biosystems). Briefly, mixtures were prepared in a total 20  $\mu$ l volume including 1  $\mu$ g of RNA, 2  $\mu$ l 10 $\times$  RT buffer, 2  $\mu$ l 10 $\times$  random primers, 0.8  $\mu$ l 25 $\times$  dNTP mix

(100 mM), 1  $\mu$ l reverse transcriptase and nuclease-free water. The mixtures were run under thermal cycler condition (25 °C for 10 min  $\rightarrow$  37 °C for 120 min  $\rightarrow$  85 °C for 5 min  $\rightarrow$  4 °C for 5 min). For qRT-PCR analysis, 150 ng of cDNA was mixed with 5  $\mu$ l of 2 $\times$  iTag™ universal SYBR® green supermix (Bio-rad), 0.5  $\mu$ l of 10  $\mu$ M forward primers, 0.5  $\mu$ l of 10  $\mu$ M reverse primers, and nuclease-free water. The mixtures were then subjected to qRT-PCR using ViiA™ 7 real time instrument and analysis software. The Mann–Whitney *U* test was used to evaluate significant decreases of TPA-induced gene expression by individual compounds when compared to TPA only treated group.

As shown in Figure 5, all of the compounds had some ability to inhibit TPA-induced mRNA expression of inflammatory cytokines in the epidermis following topical application. However, corosolic acid, 3-*epi*-corosolic acid and maslinic acid were the most effective at inhibition of inflammatory gene expression induced by TPA. In this regard, all three of these compounds inhibited expression of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-23 whereas the other compounds, including UA were less effective overall and in some cases did not significantly inhibit expression of specific genes. Thus, within this class of related compounds, corosolic acid, 3-*epi*-corosolic acid and maslinic acid, may be more effective anti-inflammatory agents. Because of our interest in cancer prevention, these compounds are now being tested in several mouse models of cancer including the two-stage skin carcinogenesis model for potential anti-carcinogenic activity.

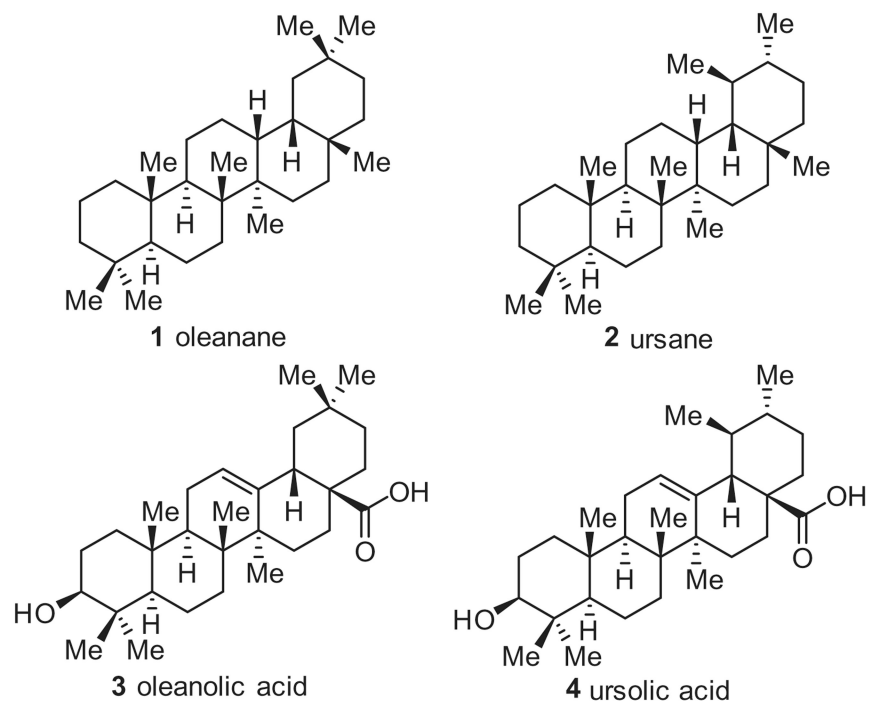
## Acknowledgments

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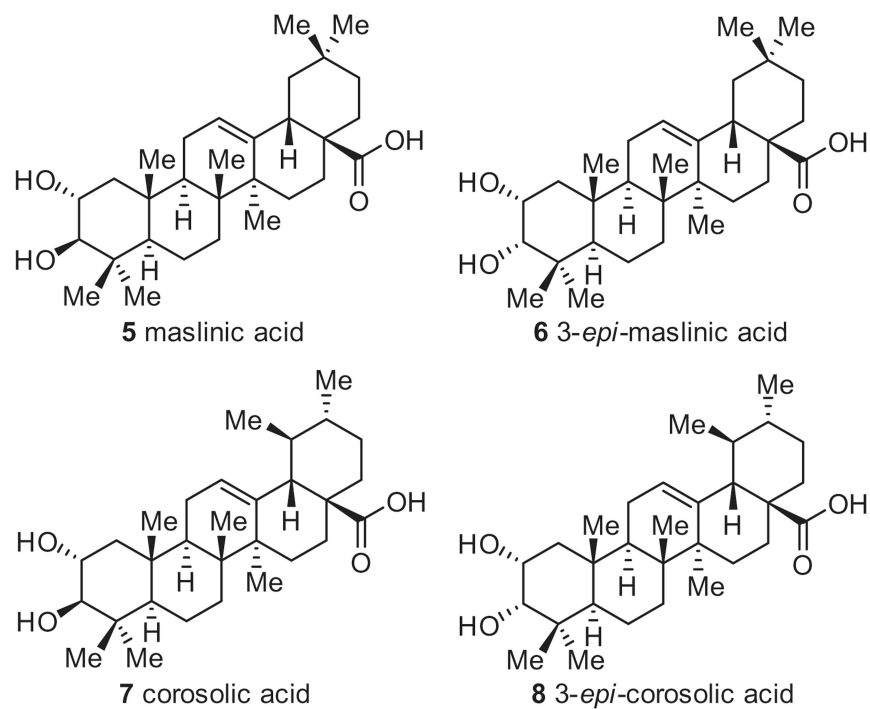
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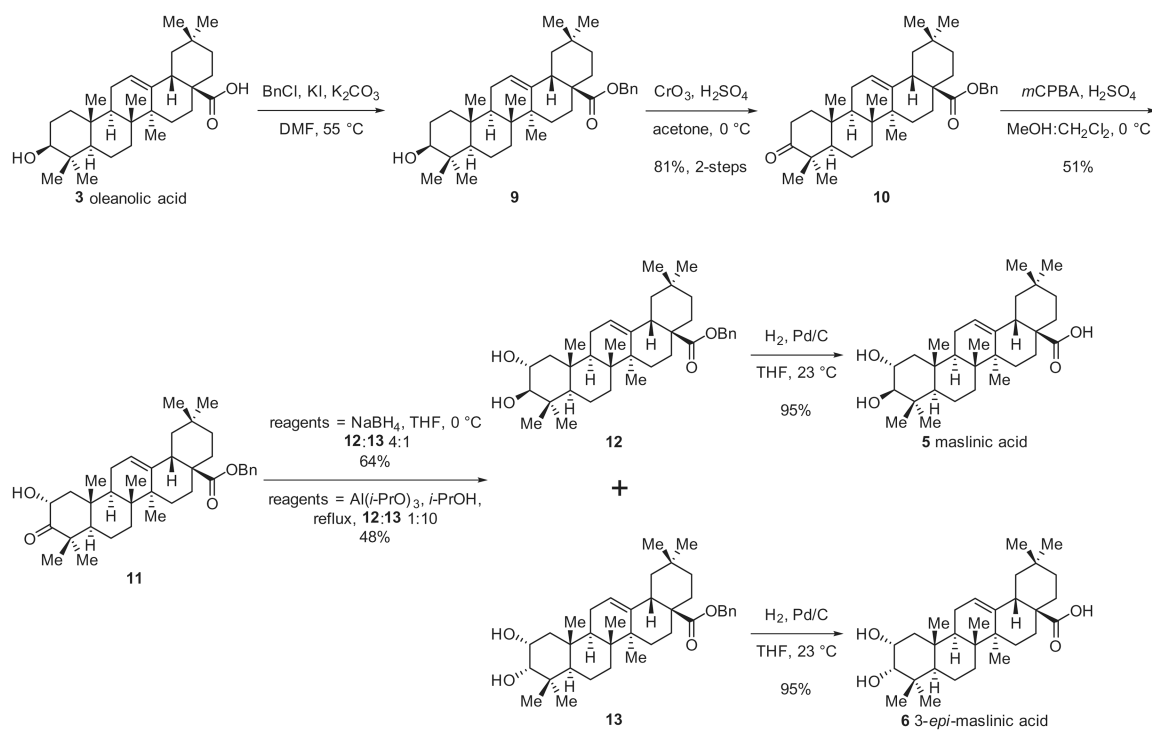
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**Figure 1.** Structures of oleanane and ursane carbocyclic cores and oleanolic acid, and ursolic acid.

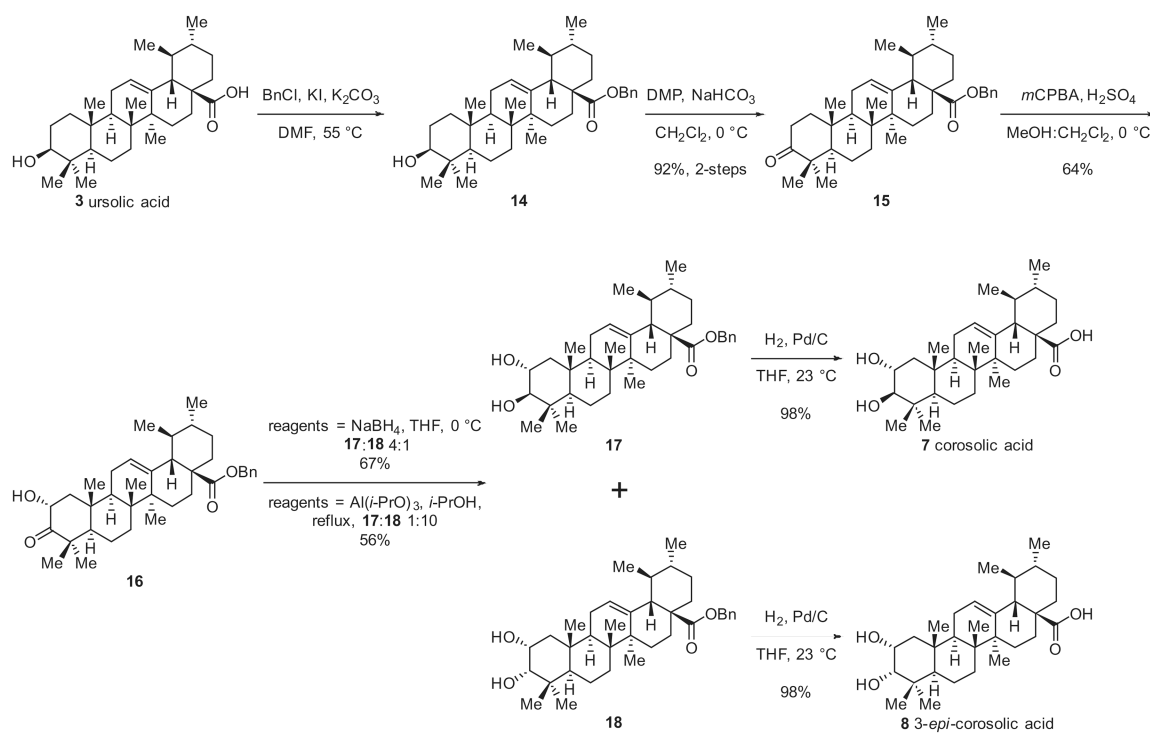


**Figure 2.**  
Structures of maslinic, 3-*epi*-maslinic, corosolic, and 3-*epi*-corosolic acids.

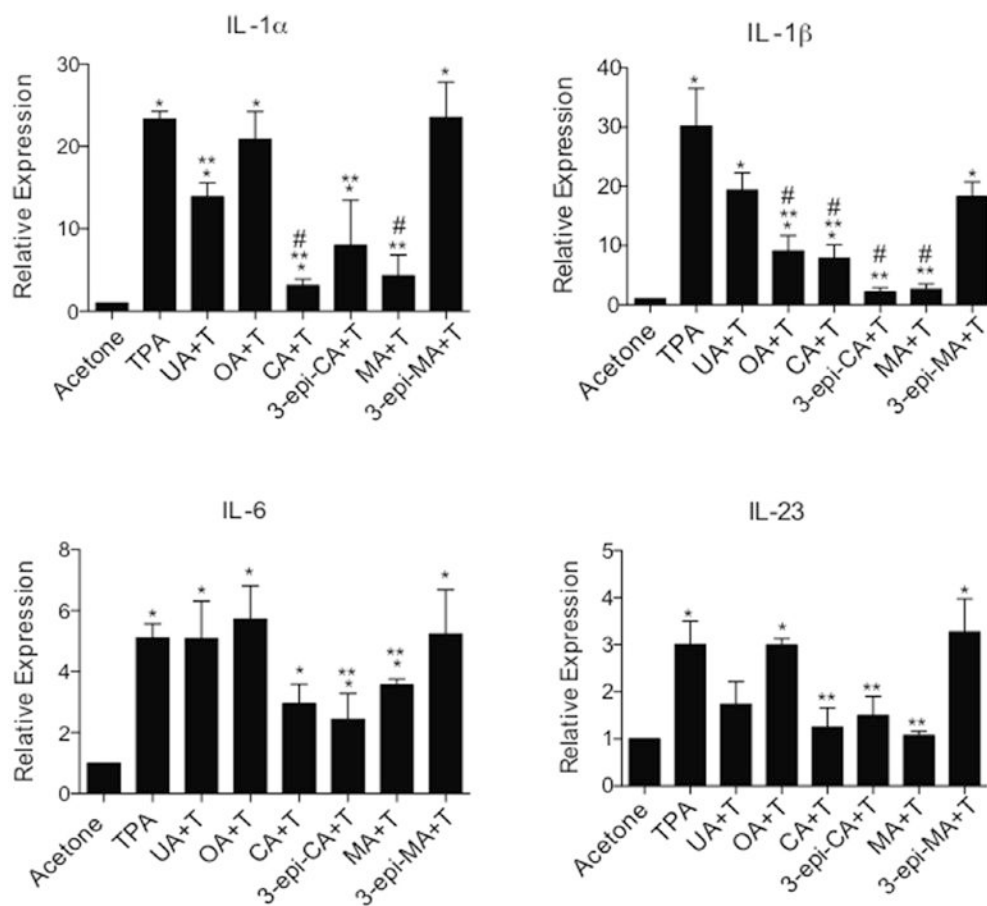


**Figure 3.** Syntheses of maslinic acid and 3-*epi*-maslinic acid proceeding from oleanolic acid.





**Figure 4.** Synthesis of corosolic acid and 3-*epi*-corosolic acid proceeding from ursolic acid.



**Figure 5.** Effect of triterpenes found in *P. frutescens* in TPA-induced inflammatory gene expression in mouse skin. Quantitative mRNA expression (assessed by qRT-PCR) of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and IL-23 was normalized to GADPH and to the value in acetone treated mice which was set to a value of 1. The graphs represent means  $\pm$ SEM. For statistical analysis, the Mann-Whitney *U* test was used. \*, significantly greater ( $p < 0.05$ ) than acetone-treated group; \*\*, significantly lower ( $p < 0.05$ ) than TPA-treated group; #, significantly lower ( $p < 0.05$ ) than UA + TPA group; †, significantly greater ( $p < 0.05$ ) than UA + TPA group. T; TPA, UA; ursolic acid, OA; oleanolic acid, CA; corosolic acid, 3-epi-CA; 3-*epi*-corosolic acid, MA; maslinic acid, and 3-*epi*-MA; 3-*epi*-maslinic acid.