



## Antiinflammatory triterpenoids and steroids from *Ganoderma lucidum* and *G. tsugae*

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Received 21 August 2006; received in revised form 4 April 2007

### Abstract

The antiinflammatory properties of triterpenoids and steroids from both *Ganoderma lucidum* and *Ganoderma tsugae* were studied. Twelve compounds, including ergosta-7,22-dien-3 $\beta$ -ol (**1**), ergosta-7,22-dien-3 $\beta$ -yl palmitate (**2**), ergosta-7,22-dien-3-one (**3**), ergosta-7,22-dien-2 $\beta$ ,3 $\alpha$ ,9 $\alpha$ -triol (**4**), 5 $\alpha$ ,8 $\alpha$ -epidioxyergosta-6,22-dien-3 $\beta$ -ol (**5**), ganoderal A (**6**), ganoderal B (**7**), ganoderic aldehyde A (**8**), tsugaric acid A (**9**), 3-oxo-5 $\alpha$ -lanosta-8,24-dien-21-oic acid (**10**), 3 $\alpha$ -acetoxy-5 $\alpha$ -lanosta-8,24-dien-21-oic acid ester  $\beta$ -D-glucoside (**11**), and tsugaric acid B (**12**), were assessed in vitro by determining their inhibitory effects on the chemical mediators released from mast cells, neutrophils, and macrophages. Compound **10** showed a significant inhibitory effect on the release of  $\beta$ -glucuronidase from rat neutrophils stimulated with formyl-Met-Leu-Phe (fMLP)/cytochalasin B (CB) whereas compound **9** significantly inhibited superoxide anion formation in fMLP/CB-stimulated rat neutrophils. Compound **10** also exhibited a potent inhibitory effect on NO production in lipopolysaccharide (LPS)/interferon- $\gamma$  (IFN- $\gamma$ )-stimulated N9 microglial cells. Moreover, compound **9** was also able to protect human keratinocytes against damage induced by ultraviolet B (UV B) light, which indicated **9** could protect keratinocytes from photodamage.

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**Keywords:** *Ganoderma*; *Lucidum*; *Ganoderma tsugae*; Fruiting bodies; Antiinflammatory; Triterpenoids; Steroids

### 1. Introduction

Crude extracts of *Ganoderma lucidum* and *G. tsugae* are used as a Chinese remedy for the treatment of hepatopathy (Aarisawa et al., 1986), enhancement of splenic natural killer cell activity and serum interferon production in mice (Won et al., 1992), respectively. In previous studies, analysis of constituents from *Ganoderma* genus afforded a series of triterpenoids and steroids (Lin and Shiao, 1989; Lin et al., 1990, 1993, 1997; Lin and Tome, 1991; Gan et al., 1998a,b; Su et al., 2000), some of which were cytotoxic against several human cancer cell lines (Lin et al., 1997;

Gan et al., 1998a). It is also believed that mast cells, neutrophils, and macrophages are important contributors to inflammatory disorders, and activation of microglial cells also play a crucial role in inflammatory diseases of CNS. Thus, inhibition of activation of these inflammatory cells appears to be an important therapeutic target for small molecular drugs for treatment of inflammatory disease. To continue our screening for bioactive compounds as inhibitors or stimulants of chemical mediators released from mast cells, neutrophils, macrophages, and microglial cells, the antiinflammatory and/or inflammatory effects of **1–12** (Fig. 1) were examined.

Photoaging of the skin and the development of skin cancer are both thought to result from exposure to ultraviolet (UV) radiation. Radiation from the UV spectrum encompasses ultraviolet A (UVA) (320–400 nm), UVB

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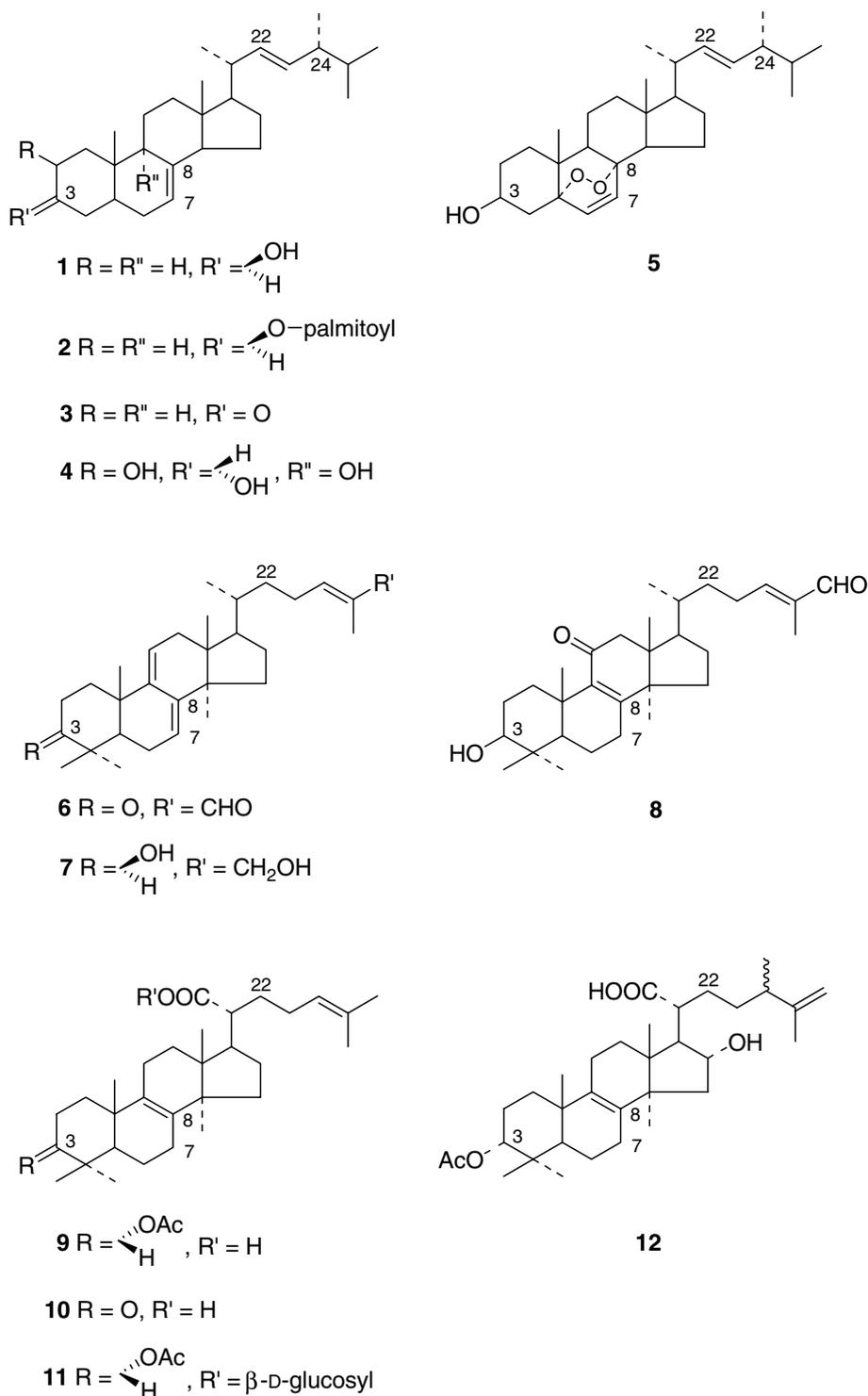


Fig. 1. Structures of triterpenoids and steroids isolated from *Ganoderma* genus.

(280–320 nm) and UVC (100–290 nm). However, as UVC is, for the most part, filtered out by atmospheric ozone, UVA and UVB radiation play a more significant role in the initiation of photocarcinogenesis (Lyons and O'Brien, 2002). A growing body of evidence suggests that reactive oxygen species (ROS) are generated by UV radiation, resulting in oxidative damage to cellular components such as the mitochondria as well as nuclear DNA, which in turn

accelerates aging and contributes to skin cancers (Miyachi, 1995). Therefore, either reducing the risk of UV-induced oxidative stress-mediated skin disease or protecting keratinocytes from photodamage, may be good for skin care. Hence, for a study on the properties of a bioactive compound with a protective effect on UVB-induced damage in keratinocytes, the protective effect of **9** on UVB-induced damage in keratinocytes was also examined.

## 2. Results and discussion

The antiinflammatory and/or inflammatory activities of known compounds **1–12** were studied *in vitro* on the basis of their effects on chemical mediators released from mast cells, neutrophils, and macrophage. Compounds **1–12** did not show significant inhibition of mast cell degranulation stimulated with *p*-methoxy-*N*-methylphenethylamine (compound 48/80) (Table 1), whereas formyl-Met-Leu-Phe (fMLP) or fMLP/cytochalasin B(CB) induced the release of  $\beta$ -glucuronidase and lysozyme from rat neutrophils. Compounds **1–7** did not display however, any significant inhibition of neutrophil degranulation stimulated with fMLP (Table 1). On the other hand, compound **10** had potent, concentration dependent, inhibitory effects on the release of  $\beta$ -glucuronidase stimulated with fMLP/CB, while the other compounds did not give any significant inhibition (Table 1). The above results thus indicated that oxidation of the C-3 $\alpha$ -OH moiety formed from saponification of **9** enhanced inhibitory effects on the release of  $\beta$ -glucuronidase stimulated with fMLP/CB. Compound **11** did not inhibit neutrophil degranulation, although it induced release of  $\beta$ -glucuronidase and lysozyme stimulated with fMLP/CB. Thus indicated that esterification of the carboxylic acid group at C-20 in **9** did not enhance any inhibitory effect on neutrophil degranulation. By contrast, compound **11** could induce inflammation through induction of granulation in rat neutrophils in response to fMLP/CB. fMLP, fMLP/CB or PMA stimulated superoxide anion formation in rat neutrophils. As shown in Table 2, compound **9** exhibited significant inhibitory effects on fMLP/CB-induced superoxide anion generation, while having no significant inhibition of the PMA-induced response. It is thus conceivable that fMLP/CB and PMA induce superoxide anion formation by activating the same oxidase in neutrophils, but

Table 2

Inhibitory effects of **1–12** on superoxide anion formation ( $n$  mol/( $10^6$  cells)/30 min) from rat neutrophils stimulated with fMLP (A) or fMLP/CB (B) and PMA (C)

Compound	IC <sub>50</sub> <sup>a</sup> ( $\mu$ M)		
	A	B	C
<b>1</b>	>30 (−19.0 $\pm$ 8.3)	ND	ND
<b>2</b>	>30 (−13.8 $\pm$ 14.5)	ND	ND
<b>3</b>	>30 (−17.6 $\pm$ 3.9)	ND	ND
<b>4</b>	>90 (−18.4 $\pm$ 8.8)	ND	ND
<b>5</b>	>90 (2.6 $\pm$ 21.3)	ND	ND
<b>6</b>	>90 (2.0 $\pm$ 7.3)	ND	ND
<b>7</b>	>30 (6.1 $\pm$ 6.2)	ND	ND
<b>8</b>	>90 (17.6 $\pm$ 8.7)	ND	ND
<b>9</b>	ND	14.7 $\pm$ 0.4	>30 (12.5 $\pm$ 0.1)
<b>10</b>	ND	>30 (−20.5 $\pm$ 2.9)	>30 (−9.2 $\pm$ 0.1)
<b>11</b>	ND	>30 (38.2 $\pm$ 0.5)	>30 (62.0 $\pm$ 2.2)
<b>12</b>	ND	>30 (36.4 $\pm$ 4.3)	>30 (−10.2 $\pm$ 1.3)
Positive control <sup>b</sup>	14.8 $\pm$ 1.7	9.0 $\pm$ 0.5	7.3 $\pm$ 0.6

<sup>a</sup> When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses. Data are presented as the means  $\pm$  SEM ( $n = 3–5$ ). ND: not determined.

<sup>b</sup> Trifluoperazine was used as positive control for A–C, respectively.

that they utilize different transduction mechanisms which are regulated differently (Segal and Abo, 1993). The observations that **9–12** had no appreciable effect on PMA-induced response and **1–8** and **10–12** had no appreciable effect on fMLP- and fMLP/CB-induced response, suggest the involvement of PMA-, fMLP- and fMLP/CB-independent signaling pathways, respectively.

Treatment of RAW 264.7 macrophages with either lipopolysaccharide (LPS) or N9 microglial cells with LPS/interferon- $\gamma$  (IFN- $\gamma$ ) for 24 h induced NO production as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in media based on the Griess reaction.

Table 1

Inhibitory effects of **1–12** on the release of  $\beta$ -glucuronidase and histamine from rat peritoneal mast cells stimulated with compound 48/80 (A) and the release of  $\beta$ -glucuronidase and lysozyme from rat neutrophils stimulated with fMLP (B) or fMLP/CB (C)

Compound	IC <sub>50</sub> <sup>a</sup> ( $\mu$ M)					
	A		B		C	
	$\beta$ Glucuronidase	Histamine	$\beta$ Glucuronidase	Lysozyme	$\beta$ Glucuronidase	Lysozyme
<b>1</b>	>30 (2.9 $\pm$ 8.4)	>30 (−11.6 $\pm$ 2.4)	>30 (−15.1 $\pm$ 10.9)	>30 (−10.1 $\pm$ 8.3)	ND	ND
<b>2</b>	>30 (7.6 $\pm$ 6.4)	>30 (−10.5 $\pm$ 4.2)	>30 (0.3 $\pm$ 10.1)	>30 (−27.0 $\pm$ 10.9)	ND	ND
<b>3</b>	>30 (2.2 $\pm$ 11.0)	>30 (−11.5 $\pm$ 1.4)	>30 (−1.6 $\pm$ 9.2)	>30 (−10.3 $\pm$ 10.0)	ND	ND
<b>4</b>	>90 (23.3 $\pm$ 7.1)	>90 (19.9 $\pm$ 2.6)	>90 (14.9 $\pm$ 8.2)	>90 (8.2 $\pm$ 11.1)	ND	ND
<b>5</b>	>90 (35.1 $\pm$ 10.8)	>90 (14.1 $\pm$ 7.4)	>90 (16.2 $\pm$ 6.7)	>90 (−5.9 $\pm$ 10.2)	ND	ND
<b>6</b>	>90 (34.4 $\pm$ 14.3)	>90 (32.1 $\pm$ 12.1)	>90 (33.8 $\pm$ 5.11)	>90 (3.2 $\pm$ 8.6)	ND	ND
<b>7</b>	>30 (6.0 $\pm$ 8.6)	>30 (−11.4 $\pm$ 2.5)	>30 (13.1 $\pm$ 11.2)	>30 (2.3 $\pm$ 12.1)	ND	ND
<b>8</b>	>90 (50.7 $\pm$ 5.3)	>90 (58.6 $\pm$ 10.4)	ND	>90 (0.7 $\pm$ 3.7)	ND	ND
<b>9</b>	>30 (−38.6 $\pm$ 21.2)	>30 (2.7 $\pm$ 3.1)	ND	ND	>30 (9.1 $\pm$ 2.9)	>30 (15.5 $\pm$ 3.0)
<b>10</b>	>30 (33.7 $\pm$ 3.8)	>30 (15.7 $\pm$ 3.5)	ND	ND	7.5 $\pm$ 0.4	>30 (−1.0 $\pm$ 5.4)
<b>11</b>	>30 (18.4 $\pm$ 6.4)	>30 (11.6 $\pm$ 1.3)	ND	ND	>30 (−131.3 $\pm$ 38.5)	>30 (−50.2 $\pm$ 8.7)
<b>12</b>	>30 (3.7 $\pm$ 7.2)	>30 (−3.9 $\pm$ 2.1)	ND	ND	>30 (51.6 $\pm$ 5.8)	>30 (16.7 $\pm$ 5.8)
Positive control <sup>b</sup>	12.4 $\pm$ 1.6	8.4 $\pm$ 1.8	22.2 $\pm$ 0.6	17.1 $\pm$ 1.0	14.2 $\pm$ 0.7	12.3 $\pm$ 1.2

<sup>a</sup> When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses. Data are presented as the means  $\pm$  SEM ( $n = 3–5$ ). ND: not determined.

<sup>b</sup> Mepacrine was used as positive control for A and trifluoperazine was used as positive control for B and C.

Table 3  
Inhibitory effects of **9–12** on the accumulation of  $\text{NO}_2^-$  (**A**) and  $\text{TNF-}\alpha$  formation (**B**) in the culture media of RAW 264.7 cells in response to LPS and N9 cells in response to LPS/IFN- $\gamma$

Compound	$\text{IC}_{50}^a$ ( $\mu\text{M}$ )			
	<b>A</b>		<b>B</b>	
	RAW 264.7	N9	RAW 264.7	N9
<b>9</b>	>30 (36.6 $\pm$ 0.7)	>30 (11.1 $\pm$ 2.8)	>30 (2.7 $\pm$ 6.5)	>30 (-68.6 $\pm$ 8.2)
<b>10</b>	>10 (9.0 $\pm$ 0.8)	17.3 $\pm$ 0.2	>10 (31.1 $\pm$ 2.9)	>30 (-81.3 $\pm$ 19.9)
<b>11</b>	>30 (19.3 $\pm$ 2.1)	>30 (-12.8 $\pm$ 2.0)	>30 (27.1 $\pm$ 5.5)	>30 (-61.2 $\pm$ 11.3)
<b>12</b>	>10 (-19.6 $\pm$ 3.7)	>10 (-0.7 $\pm$ 3.3)	>10 (29.4 $\pm$ 8.2)	>30 (-49.2 $\pm$ 8.3)
Positive control <sup>b</sup>	4.8 $\pm$ 0.2	4.6 $\pm$ 0.2	0.8 $\pm$ 0.3	0.05 $\pm$ 0.01

<sup>a</sup> When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses. Data are presented as the mean  $\pm$  SEM ( $n = 3-5$ ).

<sup>b</sup> *N*-(3-aminomethyl)benzylacetamide (1400 W) and dexamethasone were used as positive controls for **A** and **B**, respectively.

As shown in Table 3, NO production in N9 microglial cells was markedly suppressed by **10**, while compounds **9**, **11**, and **12** did not show any inhibitory effect on the NO accumulation in RAW 264.7 cells or N9 cells. This also clearly indicated that oxidation of the C-3 $\alpha$ -OH moiety formed from saponification of **9** enhanced the inhibitory effect on NO production stimulated with LPS/IFN- $\gamma$  in N9 cells. The above result showed that **10** can inhibit the production of proinflammatory mediator NO.

Treatment of RAW 264.7 macrophages with either LPS or N9 microglial cells with LPS/IFN- $\gamma$  induces formation of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Compounds **9–12** did not, however, inhibit TNF- $\alpha$  formation in RAW 264.7 and N9 cells in response to LPS and LPS/IFN- $\gamma$ , respectively, on the other hand compounds **9–10** induced

significant TNF- $\alpha$  formation in N9 cells in response to LPS/IFN- $\gamma$ . This indicates that these compounds can induce inflammation through release of TNF- $\alpha$  in N9 cells in response to LPS/IFN- $\gamma$ .

The present study verified that lanostanoids **9** and **10**, isolated from *G. tsugae*, exert inhibitory effects on the release of chemical mediators from inflammatory cells while steroids isolated from *Ganoderma* fungus did not show any measurable antiinflammatory activity. The present study also suggests that the inhibition of NO production by **10** in microglial cells may have value in either the therapeutic treatment or prevention of certain central inflammatory diseases associated with the increase of NO production. Further experiments are needed to elucidate in vivo their antiinflammatory activities.

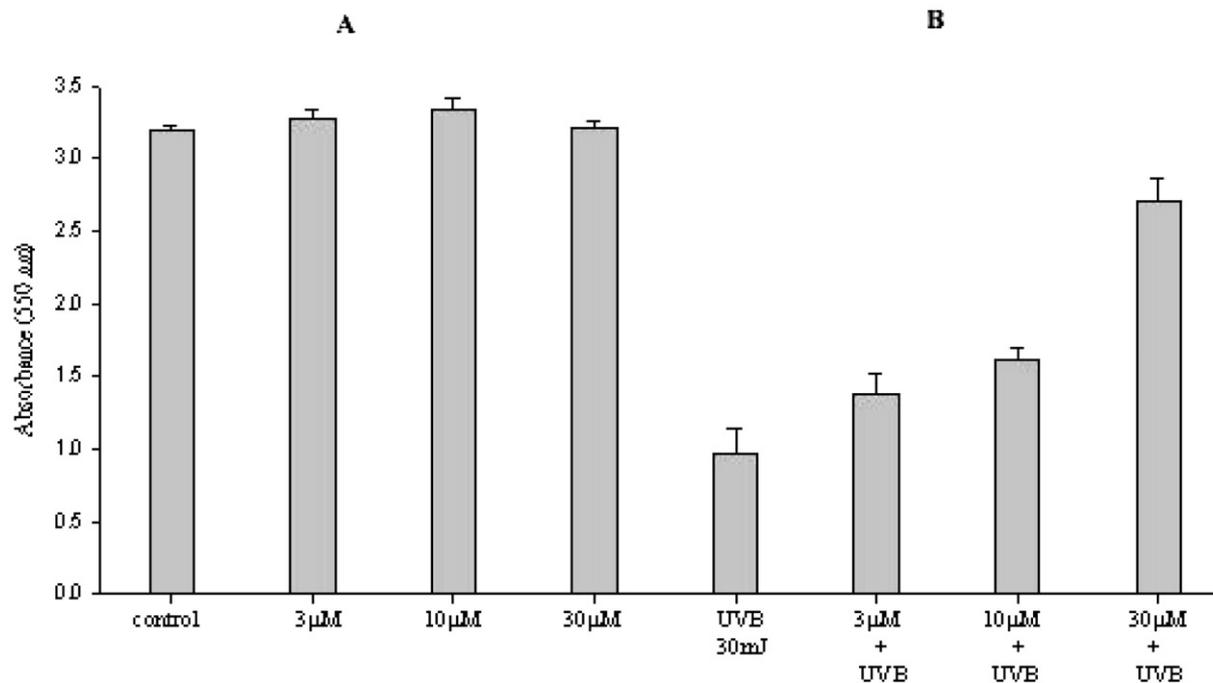


Fig. 2. Compound **9** is not cytotoxic to keratinocytes (**A**). The cells were treated with PBS (control) or the indicated concentrations of **9** for 24 h and cell viability was assessed by MTT assay. The results are expressed as absorbances of control and indicated concentrations of **9**, and data are presented as means  $\pm$  SE ( $n = 6$ ). Viability of keratinocytes following UVB treatment (**B**). The cells were treated with UVB at the indicated dose (30  $\text{mJ}/\text{cm}^2$ ), and then cell viability was assessed by MTT assay 24 h after UVB exposure. The results are expressed as absorbances of control and indicated concentrations of **9**, and data are presented as means  $\pm$  SE ( $n = 6$ ). \* $P < 0.05$ , vs control.

As shown in Fig. 2, compound **9** did not have any significant effect on cell viability when treated with various concentrations; thus indicates that **9** is not cytotoxic to keratinocytes. To clarify the protective effects of **9** on keratinocytes, cells were treated with **9** (3–30  $\mu$ M) and UVB-irradiated (30 mJ/cm<sup>2</sup>). The results obtained **9** inhibited UVB-induced cell death with a dose-dependent manner (Fig. 2). Reactive oxygen species (ROS), including singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical, are generated by UV radiation (Miyachi, 1995). Compound **9** had a significantly inhibitory effect on superoxide anion formation stimulated with fMLP/CB in rat neutrophils. The protective effect of **9** on UVB-induced damage in keratinocytes may be correlated with superoxide anion formation in keratinocytes. Our study provides the first evidence that tsugaric acid A (**9**) is a potent protective agent for keratinocytes after exposure to UVB. Further experiments are needed to evaluate the exact mechanism of action.

### 3. Conclusions

In the present study, we have found that triterpenoid **9** and **10**, isolated from *G. tsugae*, displayed significant inhibitory effects on fMLP/CB-induced superoxide anion generation, and release of  $\beta$ -glucuronidase stimulated with fMLP/CB from rat neutrophils and accumulation of NO in the culture media of N9 cells in response to LPS/IFN- $\gamma$ , respectively. It establishes that **9** and **10** may have value in the therapeutic treatment or prevention of peripheral or central inflammatory diseases associated with increase of superoxide anion formation and excess release of  $\beta$ -glucuronidase or excess of accumulation of NO in inflammatory cells. The data also indicated that **9** has a protective effect on keratinocytes against photodamage induced by ultraviolet B (UV B) light, and might find application as a agent for skin care.

### 4. Experimental

#### 4.1. General procedures

Melting points are reported uncorrected. The optical rotations were obtained on a JASCO model DIP-370 digital polarimeter. UV spectra were obtained on a JASCO model 7800 UV-Vis spectrophotometer, and IR spectra were recorded on a Hitachi model 260-30 spectrophotometer, <sup>1</sup>H (400 MHz) and <sup>13</sup>C (100 MHz) NMR spectra were recorded on a Varian Unity-400 spectrometer and MS were obtained on a JMS-HX 100 mass spectrometer.

#### 4.2. Chemicals

Compound 48/80, histamine, formyl-Met-Leu-Phe (fMLP), phorbol 12-myristate 13-acetate (PMA), mepa-

crine, trifluoperazine, heparin, bovine serum albumin, phenolphthalein- $\beta$ -D-glucuronidase, *O*-phthalaldehyde, cytochrome *c*, superoxide dismutase (type I, from bovine liver), bacterial LPS (*Escherichia coli*, serotype 0111: B4), *N*-(3-aminomethyl)benzylacetamide (1400 W), penicillin, streptomycin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were obtained from Sigma, St. Louis, MO. Hanks' balanced salt solution (HBSS) was obtained from Gibco Lab, Grand Island, NY. Dextran T 500 was purchased from Pharmacia LKB, Taipei, Taiwan. Dimethyl sulfoxide (DMSO) was obtained from Merck, Taiwan. All culture reagents were obtained from Gibco BRL.

#### 4.3. *Ganoderma* materials, extraction, and isolation

These items are briefly described as the following. *G. lucidum* and *G. tsugae* were collected at Liu-Kuei Shian, Kaohsiung Hsien, Taiwan, R.O.C., during June 1987 and July 1995, respectively. Air-dried fruit bodies were chipped and extracted with CHCl<sub>3</sub> for 1 week at room temperature. The CHCl<sub>3</sub> extracts of *G. lucidum* and *G. tsugae* were individually subjected to silica gel cc. Elution of the CHCl<sub>3</sub> extract of *G. lucidum* with cyclohexane-C<sub>6</sub>H<sub>6</sub> (4:1), C<sub>6</sub>H<sub>6</sub>, and C<sub>6</sub>H<sub>6</sub>-EtOAc (4:1) yielded **1–3**, **6–8** and **4–5**, respectively (Lin et al., 1990; Orcutt and Richardson, 1970; Gunatilaka et al., 1981; Jain and Gupta, 1984). Elution of CHCl<sub>3</sub> extract of *G. tsugae* with CHCl<sub>3</sub>-EtOAc (25:1) yielded **9** and **10**, CHCl<sub>3</sub>-MeOH (9:1) yielded **10** and cyclohexane-CHCl<sub>3</sub>-MeOH (2:40:1) yielded **12** (Lin et al., 1997; Gan et al., 1998a).

4.3.1. Compound identification of ergosta-7,22-dien-3 $\beta$ -yl palmitate (**2**), ergosta-7,22-dien-2 $\beta$ ,3 $\alpha$ ,9 $\alpha$ -triol (**4**), ganoderic aldehyde A (**8**), tsugaric acid A (**9**), 3 $\alpha$ -acetoxy-5 $\alpha$ -lanosta-8,24-dien-21-oic acid ester  $\beta$ -D-glucoside (**11**), and tsugaric acid B (**12**)

The physical and spectral data of these compounds have been described in previous reports (Lin et al., 1990, 1997; Lin and Tome, 1991; Gan et al., 1998a).

#### 4.4. Biological evaluation

The inhibitory assays for chemical mediator induced by various stimulants in mast cells, neutrophils, RAW 264.7 cells and N9 cells were performed by the methods previously described (Wei et al., 2005; Ko et al., 2003).

##### 4.4.1. Cell culture

Human keratinocytes (HaCaT cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (GibcoBRL, UK), 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich, UK). The cells were cultured in a humidified incubator at 37 °C under an atmosphere containing 5% CO<sub>2</sub>. Keratinocytes cultured on 1.5 cm culture dishes were pretreated with various concentrations of tsugaric acid A (**9**) for 2 h. After two washes

with DMEM, the cells were incubated with 0.5 mL phosphate-buffered saline (PBS). UVB irradiation was performed immediately as suggested by the manufacturer. Briefly, cells were irradiated in a Bio-Sun system illuminator (Vilber Lourmat, France) with a UV peak at 312 nm. UVB was supplied by a closed spaced array of four UVB lamps, which delivered uniform irradiation at a distance of 10 cm. After UVB exposure, cells were fed with fresh DMEM containing various concentrations of **9**, and then incubated for an additional 24 h (Huang et al., 2005). Cell viability was determined by MTT assay.

#### 4.4.2. Cell viability assay (MTT assay)

The viability of cells was determined by MTT assay as previously described (Wu and Huang, 2003) with minor modification. Briefly, MTT (0.5 mg/mL in DMEM) was used for the quantification of living metabolically active cells (Mosmann, 1983). Mitochondrial dehydrogenases metabolized MTT to a purple formazan dye, which is measured photometrically at 550 nm. Cell viability is proportional to the absorbance measured (Green et al., 1984). Cell viability was calculated as the percentage of control. For measurement of cytotoxicity of **9** on keratinocytes, cells were treated with various concentrations of **9** for 24 h and cell viability was measured as described above.

#### 4.5. Statistical analysis

Data are presented as the mean  $\pm$  SEM from four to six separated experiments. Statistical analyses were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the Student's *t*-test method for two-group comparison.  $P < 0.05$  was considered significant. Analysis of linear regression (at least three data within 20–80% inhibition) was used to calculate IC<sub>50</sub> values.

#### Acknowledgement

This work was partially supported by a grant from the National Science Council of the Republic of China (NSC 92-2320-B037-041).

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.phytochem.2007.06.008](https://doi.org/10.1016/j.phytochem.2007.06.008).

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