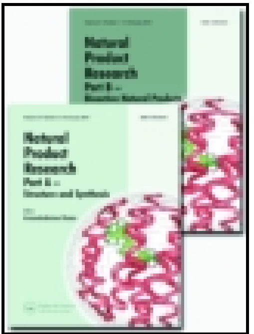

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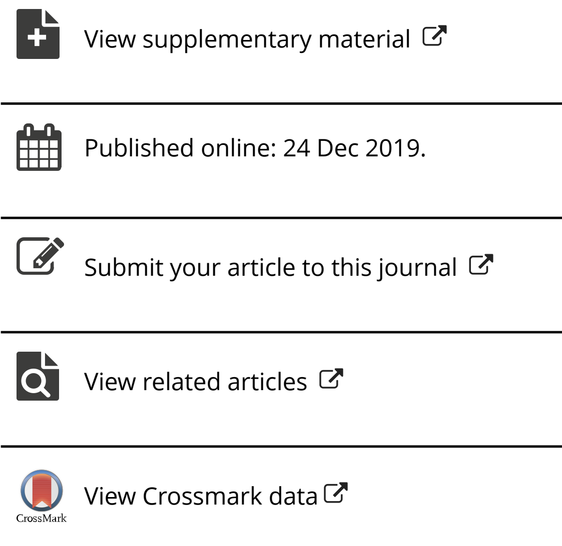

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Antiinflammatory lanostane triterpenoids from *Ganoderma lucidum*

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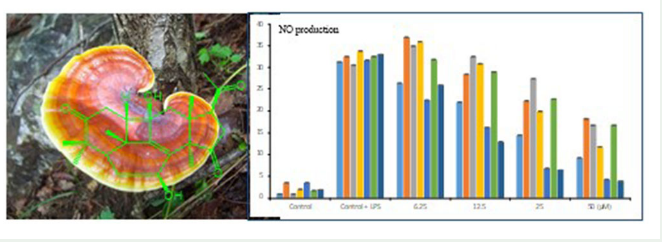
ABSTRACT

Phytochemical and biological studies of the methanolic extracts from *Ganoderma lucidum* (Polyporaceae) have led to the identification and isolation of a new lanostane triterpenoid, ganosidone A (**1**), and its eight known derivatives (**2**–**9**). The structure of new compound was determined by HREIMS, 1 D and 2 D NMR experiments and by comparing the acquired physicochemical data with the published values. All the compounds were evaluated for cancer chemopreventive potential based on their ability to inhibit nitric oxide (NO) production induced by lipopolysaccharides (LPS) in mouse macrophage RAW 264.7 cells in vitro. Notably, at a concentration of 50 μM, compounds **4** and **7** inhibited NO production by 86.5% and 88.2%, respectively.



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Ganoderma lucidum; polyporaceae; lanostane triterpenoid; inhibit nitric oxide production

1. Introduction

Ganoderma lucidum (Fr.) P. Karst. (Ganodermataceae) has been known as a traditional medicine in Korea, China, and Japan; and has been used for the treatment of chronic diseases, such as hepatopathy, nephritis, hypertension, arthritis, insomnia, bronchitis, asthma, diabetes, and cancer (Mizushina et al. 1998; Wasser and Weis 1999; Fatmawati et al. 2010). Analysis of chemical characteristics of the fruiting bodies of *G. lucidum* showed the presence of triterpenoids (Nishitoba et al. 1988; Hirotani et al. 1993;

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Figure 1. Structures of compounds **1**–**9** isolated from *G. lucidum*.

Li et al. 2005, 2006; Ruan et al. 2014), steroids (Zhang et al. 2008), and polysaccharides (Carrieri et al. 2017). Lanostane-type triterpenoids are the major constituents of *G. lucidum*, and they play a critical role in exhibiting its biological effects. Previous biological studies of *G. lucidum* have established its therapeutic potential as an anti-cancer agent (Muller et al. 2006; Rios et al. 2012). *G. lucidum* was also found to possess anti-viral effects, such as anti-HIV (Min et al. 1998) and anti-HSV (Eo et al. 1999), anti-inflammatory (Ko et al. 2008; Joseph et al. 2009), anti-androgenic (Fujita et al. 2005), cholesterol synthesis inhibitory (Hajjaj et al. 2005), hypoglycemic (Hikino et al. 1989), immunomodulating (Chen et al. 2006), lipid peroxidation/oxidative DNA damage inhibitory (Lee et al. 2001), antimicrobial (Yoon et al. 1994), and hepatic and renal protective (Shie et al. 2001) activities.

In our continuous search for cancer chemopreventive agents, the methanolic (MeOH)-soluble extracts of the fruiting body of *G. lucidum* exhibited inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in murine macrophage RAW 264.7 cells. This study reports the biological activity, isolation, and structural elucidation of a novel lanostane triterpene (**1**), and its eight known derivatives from the fruiting body of *G. lucidum* (Figure 1).

2. Results and discussion

2.1. Structure elucidation

Naturally occurring triterpenes in the genus *Ganoderma* have evolved from the intermediate lanosterol skeleton. Cyclization of squalene-2,3-epoxide yields a carbocation intermediate referred as protosterol, which on subsequent backbone rearrangement gives rise to tetracyclic lanosterol ($C_{30}H_{54}$) skeleton. Tetracyclic lanosterol acts as an intermediate molecule in the biosynthesis of various lanostane type triterpenoids. Nortriterpenoids, derived from lanostane type triterpenoids as a results of side chain degradation, are rare secondary metabolites found in the genus *Ganoderma*. The common nortriterpenoids possess carbon skeleton with 24 or 27 carbon atoms in *Ganoderma* genus.

Compound **1**, $[\alpha]_D^{25}+160$ (c 0.2, $CHCl_3$), was obtained as a white amorphous powder and its molecular formula was established as $C_{24}H_{32}O_5$ from a molecular ion of *m/z* 400.2248 $[M]^+$, (calcd for $C_{24}H_{32}O_5$, 400.2250) in the positive HREIMS. Inspection of the ^{13}C NMR spectrum, together with the molecular formula indicated that a nortriterpenoid contains 24 carbon atoms (Chen et al. 2017). The ^1H and ^{13}C NMR spectra showed signals similar to those of lucidone F (Peng et al. 2013): an acetyl group at [δ_H 2.24 (3 H, s)/δ_C 31.7 (C-21) and δ_C 209.1 (C-20)], five singlet methyl groups at [δ_H 1.46 (3 H, s, CH_3-19), δ_H 1.31 (3 H, s, CH_3-24), δ_H 1.05 (3 H, s, CH_3-23), δ_H 1.03 (3 H, s, CH_3-22), and δ_H 1.01 (3 H, s, CH_3-18)], two carbonyl carbons at [δ_C 219.0 (C-3) and δ_C 214.6 (C-15)], an oxygenated methine group at [δ_H 4.88 (2 H, dd, $J = 8.8$, 4.9 Hz)/δ_C 67.8 (C-7)], and a pair of olefinic quaternary carbons at [δ_C 155.8 (C-9) and δ_C 136.5 (C-8)]. However, compound **1** displayed a carbonyl group at δ_C 214.6 (C-15), an oxygenated quaternary carbon signal at δ_C 84.2 (C-11), and a downfield shifted methine carbon at δ_C 57.6 (C-1), in comparison to an oxygenated methine at δ_C 72.7 (C-15), a carbonyl group at δ_C 198.4 (C-11), and a methylene carbon at δ_C 35.6 (C-1) in lucidone F. Furthermore, a methylene carbon (C-12) signal in **1** seems to be shifted upfield by δ_C 37.0 ppm due to a lack of a neighbouring carbonyl group (C-11), as present in lucidone F. These results were confirmed by HMBC correlations of H-2 with C-3/C-11, H-12 with C-9/C-11/CH_3-18, and H-1 with C-9/CH_3-19, and with ^1H-^1H COSY analysis (Supplementary information Figure S1). Thus, HREIMS and 2 D NMR analyses established a unique nortriterpenoid skeleton, comprising a four-membered ring system (E-ring) across C-1 to C-11 (Wang et al. 2010). The C24-nortriterpenoid **1** could be biosynthesised through the pathways previously described: the degradation of C_{20}-C_{22} double bond by oxidation (Chen et al. 2017) and the nucleophilic addition at C-1 and C-11 positions (Wang et al. 2010) in ganoderenic acid D or 7β-hydroxy-3,11,15,23-tetraoxolanosta-8,20*E*(22)-dien-26-oic acid methyl ester (Supplementary information Scheme S1).

The orientation of a hydroxyl group at C-7 was determined to be a β-form based on NOESY spectrum that yielded correlations between H-7 and CH_3-24 and between CH_3-24 and H-17. The other hydroxyl group at C-11 was also confirmed be in β-orientation by NOESY spectrum that yielded correlation between C-11 and 7-OH (Supplementary information Figure S2). Further, NOESY spectrum exhibited correlation between H-1 and CH_3-19, indicating a cis junction for ring A/E. Consequently, **1** was determined as a new compound, ganosidone A.

The other isolates were identified as methyl ganoderate A (**2**) (Hirotani et al. 1985), methyl ganoderate H (**3**) (Kikuchi et al. 1986a), lucidumol A (**4**) (Min et al. 1998), lucidumol C (**5**) (Amen et al. 2016), ganoderic acid A (**6**) (Seo et al. 2009), ganodermanon triol (**7**) (Sato et al. 1986), ganolucidic acid A (**8**) (Kikuchi et al. 1986b) and ganolucidic acid E (**9**) (Komoda et al. 1985), by comparing their physical and spectroscopic data with those of the published values.

2.2. Antiinflammatory activity

Free radical nitric oxide (NO), synthesised by a family of enzymes termed NO-synthases (NOS), acts as a mediator of host defence mechanisms against pathogenic DNA and of homeostatic processes (Kuo and Schroeder 1995). However, excessive NO production has shown detrimental effects on many organ systems of the body, which can lead to

tissue damage with potentially fatal consequences, such as septic shock (Vincent et al. 2000). The inhibition of aberrantly active NF-κB activity has been correlated with decreased of NO production, inducible nitric oxide synthases has been associated with chronic inflammation. Therefore, inhibiting NO production may be of therapeutic benefit in various diseases that are caused by pathological levels of NO.

When treated with a concentration of 50 μM, compounds **1**–**4**, **6**, and **7** exhibited 44%–88.2% inhibitory activity against NO production (Supplementary information Figure S3). Out of these, compounds **4** and **7** inhibited NO production by 86.5% and 88.2%, respectively. However, these compounds exhibited cytotoxicity at 50 μM. Compounds **4** and **7** showed considerable inhibitory effects on NO production (78.6% and 80.7%, respectively) even when treated at a lower concentration of 25 μM, but without cytotoxicity. Thus, compounds **4** and **7** are more potent than the positive control L-NMMA (43.7% and 73.6% inhibition at 25 μM and 50 μM treatment concentrations, respectively), but with less cytotoxicity. Compounds **1**–**3** and **6** showed weak to moderate inhibitory effects (44.0%, 44.0%, 65.0%, and 48.5%, respectively) against NO production at 50 μM treatment. Compounds **5**, **8**, and **9** were inactive (data not shown).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Rudolph Research Autopol IV multi wavelength polarimeter. UV spectra were recorded on a Shimadzu PharmaSpec-1700 UV-visible spectrophotometer. IR spectra were measured on a Bruker Tensor-27 spectrophotometer. 1 D and 2 D NMR spectra were recorded on a Bruker AVANCE (600 MHz) spectrometer. High-resolution electrospray ionization mass spectra (HREIMS) were obtained with an Agilent 6530 LC-qTOF High Mass Accuracy mass spectrometer operated in the positive- and negative-ion modes. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (0.25 mm, Merck, Germany). Silica gel (230-400 mesh, Merck, Germany) and RP-18 (YMC·GEL ODS-A, 12 nm, S-150 μm) were used for column chromatography. Semi-preparative HPLC was conducted on YL9100 HPLC system (Young Lin, South Korea) equipped with a UV/Vis detector using an Alltech reversed-phase YMC-Pak C-18 column (10 μm, 20 × 250 mm) with a flow rate of 2 mL/min.

3.2. Plant material

The fruiting bodies of *G. lucidum* were purchased from in August 2018 from Kyong-dong Korean Traditional Market in Seoul. The mushroom was identified by Dr. K. Bae, Chung-Nam national University, Korea. A voucher specimen (no. GL001) was deposited at the Department of Life Sciences, Korea Polar Research Institute.

3.3. Extraction and isolation

The fruiting bodies of *G. lucidum* (1.5 kg) were extracted with methanol (MeOH, 3 × 2 L) at room temperature. The solvent was concentrated in vacuo to yield 27 g of a crude extract, which was then suspended in distilled water (0.3 L) and extracted

successively with *n*-hexane (1×0.3 L), ethyl acetate (EtOAc) (1×0.3 L), and *n*-butanol (1×0.3 L). The EtOAc extracts (7.0 g) were subjected to silica gel column chromatography (CC; ϕ 15 cm, 230-400 mesh, 2.5 kg), using a gradient solvent system of hexane-ethyl acetate (50:1 to 0:100), to afford 17 fractions (E1 – E17). Fraction E1 (3 g) was subjected to silica gel CC (ϕ 10 cm, 230-400 mesh, 500 g), using $CHCl_3$:MeOH (39:1 to 6:4) as the solvent system, yielding 9 subfractions (E1S1 - E1S9). Subfraction E1S4 (675 mg) was separated by a Sephadex LH-20 gel column and eluted with H_2O – MeOH (60:40 to 0:100), to afford 13 subfractions (E1S4L1 - E1S4L13). Subfraction E1S4L1 (174 mg) was subjected to semi-preparative HPLC (MeOH – H_2O = 30:70 to 100:0), to yield compounds **5** (11 mg, t_R 82 min) and **4** (2 mg, t_R 85 min). Compound **1** (1 mg) was obtained by repeated silica gel column chromatography and semi-preparative HPLC (MeOH – H_2O = 30:70 to 100:0) from the fraction E1S9. Subfraction E1S8 (150 mg) was subjected to semi-preparative HPLC (MeOH – H_2O = 60:40 to 100:0), to yield **6** (4 mg, t_R 75 min), **8** (10 mg, t_R 80 min), and **9** (6 mg, t_R 82 min). The combined subfractions E1S6 and E1S7 (100 mg) were subjected to separation on a semi-preparative RP-18 column by HPLC, using MeOH:H_2O mixtures (from 60:40 to 100:0) as the solvent system, to yield **2** (2.5 mg, t_R 85 min) and **3** (2 mg, t_R 90 min). Compound **7** (4 mg) was separated by a Sephadex LH-20 gel column; and was eluted with H_2O – MeOH (70:30 to 0:100) and semi-preparative HPLC (MeOH – H_2O = 30:70 to 100:0) from fraction E1S4L5.

3.3.1. Ganosidone A (1)

White amorphous powder. $[\alpha]_D^{25}+160$ (*c* 0.25, $CHCl_3$). UV (MeOH) λ_{max} (log ε) 230 (4.2) nm. ^{1}H NMR (CD_3OD, 600 MHz): δ_H 4.88 (dd, $J = 8.8$, 4.9 Hz, H-7), 3.55 (t, $J = 8.3$ Hz, H-17), 2.97 (t, $J = 12.7$ Hz, H-2a), 2.89 (dd, $J = 19.2$, 8.3 Hz, H-16a), 2.55 (dd, $J = 19.2$, 8.3 Hz, H-16b), 2.41 (d, $J = 13.7$ Hz, 12a), 2.38 (dd, $J = 11.7$, 7.7 Hz, H-1), 2.27 (m, H-6a), 2.24 (s, CH_3-21), 1.96 (d, $J = 13.7$ Hz, H-12b), 1.88 (dd, $J = 13.3$, 2.7 Hz, H-5), 1.73 (ddd, $J = 17.9$, 13.2, 4.9 Hz, H-6b), 1.46 (s, CH_3-19), 1.31 (s, CH_3-24), 1.05 (s, CH_3-23), 1.03 (s, CH_3-22), 1.01 (s, CH_3-18). ^{1}H NMR ($CDCl_3$, 600 MHz): δ_H 4.75 (dd, $J = 8.2$, 4.9 Hz, H-7), 3.36 (t, $J = 8.2$ Hz, H-17), 3.05 (dd, $J = 19.6$, 8.2 Hz, H-16a), 2.70 (t, $J = 12.3$ Hz, H-2a), 2.51 (dd, $J = 19.6$, 8.2 Hz, H-16b), 2.43 (m, H-1), 1.75 (m, H-6), 2.27 (d, $J = 13.7$ Hz, 12a), 2.24 (s, CH_3-21), 2.23 (dd, $J = 13.3$, 2.7 Hz, H-5), 1.98 (d, $J = 13.7$ Hz, H-12b), 1.51 (s, CH_3-19), 1.29 (s, CH_3-24), 1.06 (s, CH_3-23), 1.05 (s, CH_3-22), 1.01 (s, CH_3-18), 3.15 (s, 7-OH), 2.19 (s, 11-OH). ^{13}C NMR (CD_3OD, 150 MHz): δ_C 219.0 (C-3), 214.6 (C-15), 209.1 (C-20), 155.8 (C-9), 136.5 (C-8), 84.2 (C-11), 67.8 (C-7), 62.7 (C-14), 57.6 (C-1), 56.3 (C-5), 55.4 (C-17), 49.6 (C-10), 48.0 (C-4), 46.7 (C-13), 37.0 (C-12), 36.7 (C-2), 36.2 (C-16), 31.7 (C-21), 29.9 (C-6), 27.4 (C-22), 21.3 (C-24), 19.6 (C-18), 19.0 (C-23), 18.6 (C-19). HREIMS *m/z* 400.2248 $[M]^+$, (calcd for $C_{24}H_{32}O_5$, 400.2250).

3.3.2. Effects of compounds on LPS-induced NO production

The level of nitrite, the stable end product of NO, was estimated as described previously (Park et al. 2011). Stable constructed cells were seeded into 96-well plates at 2×10^5 cells per well. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Co.; Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine.

After 24 h incubation, the medium was replaced and the cells were treated with various concentrations of test compounds. LPS (Lipopolysaccharides from *Salmonella enterica* serotype typhimurium, sigma, NJ, USA) was used as an activator at a concentration of 0.5 ng/mL. Cellular proliferation of RAW 264.7 cells was not significantly affected in response to treatment with test compounds up to 25 μM compared with that of the cells without LPS or L-NMMA treatment (Supplementary information Figure S3A). The plate was incubated for 24 h. Nitric oxide (NO) concentration in the RAW264.7 cell culture supernatant was measured using the Griess reagent. Briefly, 100 μl of the collected each supernatant was mixed with equal amounts of Griess reagent (1% sulfanilamide in 5% phosphoric acid, 0.1% N-(1-naphthyl) ethylenediamine). The mixtures were incubated for 10 min at room temperature, and then the absorbance value of each well was determined at wavelength of 540 nm using a microplate reader. Nitrite concentration was determined using sodium nitrite calibration curve (0-100 μM). Data for NO constructs are expressed as inhibition ratio (i.e., the concentration required to inhibit NO production). The results showed that six of the total compounds decreased LPS-induced NO production (Supplementary information Figure S3B). L-N^G-monomethyl arginine citrate (L-NMMA), a positive control of this assay showed inhibition rates 43.7% and 73.6% in treatments of 25.1 and 50 μM concentration, respectively.

4. Conclusions

In this study, a novel nortriterpenoid, ganosidone A (**1**), possessing a four-membered ring system (E-ring) across C-1 to C-11, along with eight known derivatives (**2**–**9**) were isolated from the methanol extract from *G. lucidum* (Polyporaceae). Among the isolates, compounds **4** and **7** inhibited NO production by 86.5% and 88.2% at a concentration of 50 μM, respectively. These are more potent than the positive control, L-NMMA with less cytotoxicity. These results suggest that lanostane triterpenoids isolated from *G. lucidum* could possess inhibitory effect against NO production in vitro.

Supplementary material

The 1 D and 2 D NMR and HREIMS analyses of compound **1** are available as supporting information.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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