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Triterpenoids and Steroids from *Ganoderma mastoporum* and Their Inhibitory Effects on Superoxide Anion Generation and Elastase Release

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Abstract: The methanol extracts of the fruiting bodies of *Ganoderma mastoporum* collected in Vietnam was purified to afford eight compounds, including three triterpenoids and five steroids. The purified compounds were examined for their inhibitory effects against superoxide anion generation and elastase release. Among the tested compounds, ergosta-4,6,8(14),22-tetraen-3-one (**3**) exhibited the most significant inhibition towards superoxide anion generation and elastase release with IC₅₀ values of 2.30 ± 0.38 and 1.94 ± 0.50 µg/mL, respectively.

Keywords: *Ganoderma*; triterpenoid; steroid; superoxide anion generation; elastase release

1. Introduction

The genus *Ganoderma* (Polyporaceae) are extensively used as a Chinese remedy for the treatment of tumors [1,2], hepatopathy [3], enhancement of splenic natural killer cell activity, and serum interferon production in mice [4]. Species such as *G. lucidum* attract much attention because it is a well-known source of various therapeutic agents [5,6]. Previous phytochemical investigations of *Ganoderma* genus afforded a series of triterpenoids and steroids [7–13]. These principles displayed significant cytotoxicity and anti-inflammatory bioactivity [14,15]. *G. mastoporum* is a fungus widely distributed in Southern China, Northern Vietnam, Malaysia, Philippines, and reported to contain sesquiterpenoids [16]. Therefore, additional studies of the phytochemical constituents of *G. mastoporum* and their bioactivities are merited. In our continuous program aimed at the discovery of anti-inflammatory lead drugs from the natural sources, the chemical composition of the fruiting bodies of *G. mastoporum* collected in Vietnam was investigated to search for the bioactive constituents. In the present study, we wished to report the identification of eight compounds, as well as their inhibitory effects on superoxide anion generation and elastase release.

2. Results and Discussion

2.1. Isolation and Identification of Compounds 1–8

Air-dried and powdered fruiting bodies of *Ganoderma mastoporum* were extracted with methanol, and the combined extracts were concentrated under reduced pressure to give a deep brown syrup. The crude extract was suspended in water and partitioned with ethyl acetate to afford ethyl acetate and water soluble fractions, respectively. Purification of the ethyl acetate fraction by a conventional combination of column chromatographies yielded three triterpenes: Δ^1 -lupenone (**1**) [17], ganodermanondiol (**5**), lucidumol B (**6**) [18], and five steroids: ergosta-7,22-dien-3 β -ol (**2**) [19], ergosta-4,6,8(14),22-tetraen-3-one (**3**), ergosterol peroxide (**4**) [20], ergosta-7,22-dien-3-one (**7**) [19], and 3 β ,5 α -dihydroxy-(22*E*,24*R*)-ergosta-7,22-dien-6-one (**8**) [21], respectively. Their chemical structures (Figure 1) were identified by comparison of their physical and spectroscopic data with those reported in the literature.

2.2. The Inhibitory Effects of Isolated Compounds on Superoxide Anion Generation and Elastase Release

The purified compounds were subjected to the examination for inhibition of superoxide anion generation and elastase release by human neutrophils in response to *N*-formyl-L-methionyl-leucyl-phenylalanine/cytochalasin B (FMLP/CB) [22], and the data are displayed in Table 1. Only compounds **2** and **8** did not display significant inhibition of superoxide anion generation and elastase release. Other tested compounds **1** and **3–7** demonstrated inhibitory effects towards superoxide anion generation and elastase release in a concentration-dependent manner (data not shown). The IC₅₀ values were in the range between 2.30 ± 0.38 and 6.36 ± 0.36 µg/mL against superoxide anion generation, and between 1.94 ± 0.50 and 5.01 ± 0.82 µg/mL against elastase release, respectively, compared with the reference compound LY294002 (IC₅₀ of 0.40 ± 0.02 and 1.53 ± 0.25 µg/mL towards superoxide anion generation and elastase release). Among the tested compounds, ergosta-4,6,8(14),22-tetraen-3-one (**3**)

exhibited the most significant inhibition towards superoxide anion generation and elastase release with IC_{50} of 2.30 ± 0.38 and 1.94 ± 0.50 $\mu\text{g/mL}$. From the above results, the following structure-bioactivity relationships were deduced: triterpenoids or steroids possessing 3-hydroxy rather than 3-ketone functionalities, such as **2**, **4**, **6**, and **8**, displayed weaker superoxide anion generation inhibitory effects. Comparison of the bioactivity of **2** and **7** also exhibited this tendency. The 24,25-dihydroxy substitutions in **5** and **6**, and the 5-hydroxy group in **8** decreased the inhibition of superoxide anion generation. There is similar tendency in the inhibition of elastase release. However, lucidumol B (**6**) displayed more significant inhibition of elastase release than **5** and **7**.

Figure 1. Structures of purified compounds 1–8.

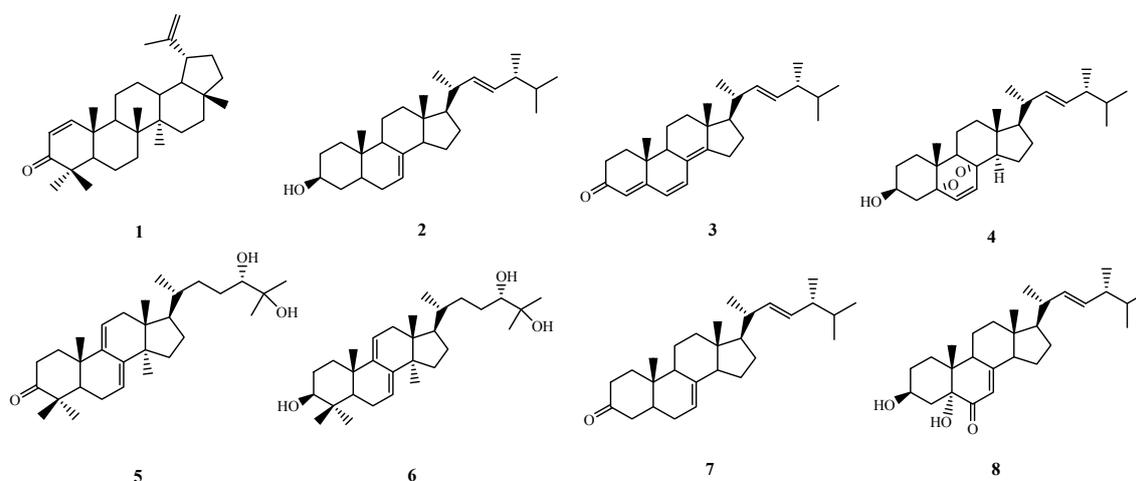


Table 1. Inhibitory effects of purified samples from *G. mastoporum* on superoxide anion generation and elastase release by human neutrophils in response to *N*-formyl-L-methionyl-leucyl-phenylalanine/cytochalasin B (FMLP/CB).

Compound	IC_{50} ($\mu\text{g/mL}$) ^a or (Inh %) ^b	
	Superoxide Anion Generation	Elastase Release
1	3.71 ± 0.79 ***	3.26 ± 0.07 ***
2	(34.46 ± 2.42) ***	(27.44 ± 4.90) **
3	2.30 ± 0.38 ***	1.94 ± 0.50 ***
4	5.28 ± 0.76 ***	(35.99 ± 3.42) ***
5	6.36 ± 0.36 ***	5.01 ± 0.82 **
6	5.33 ± 0.46 ***	3.32 ± 0.14 ***
7	5.02 ± 0.98 ***	4.41 ± 0.50 ***
8	(30.11 ± 3.17) ***	(19.46 ± 6.65) *
LY294002 ^c	0.40 ± 0.02 ***	1.53 ± 0.25 ***

^a Concentration necessary for 50% inhibition. ^b Percentage of inhibition (Inh %) at 10 $\mu\text{g/mL}$ concentration; results are presented as mean \pm S.E.M. (n = 3–4); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the control value. ^c A phosphatidylinositol-3-kinase inhibitor was used as a positive control for superoxide anion generation and elastase release.

The formation of superoxide anion in neutrophils can be inhibited by modulating cellular signaling pathways, but also by direct radical scavenging. Therefore the purified constituents **1–8** were

also subjected to assays of DPPH free radical scavenging activities and ferrous ion chelating capability [23]. None of the examined compounds exhibited significant scavenging of free radicals and capability of chelating ferrous ions at the tested concentration (data not shown). It indicated that the inhibition of superoxide anion generation and elastase release by compounds **1** and **3–7** is mediated by modulating cellular signaling pathways [24]. In the previous literature, Δ^1 -lupenone (**1**) and ergosterol peroxide (**4**) were reported to exhibit the inhibitory effects on LPS-induced *i*NOS-dependent NO production in RAW 264.7 cells [25–27]. Moreover, ergosta-7,22-dien-3 β -ol (**2**), ergosterol peroxide (**4**), and ergosta-7,22-dien-3-one (**7**) also inhibited superoxide anion generation release by rat neutrophils [15]. In the present study, the inhibitory effects of these isolated compounds on superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB were reported for the first time.

3. Experimental

3.1. General Procedure

Melting points were determined using Yanagimoto MP-S3 apparatus (Kyoto, Japan). Optical rotations were measured using a JASCO DIP-370 polarimeter (Oklahoma City, OK, USA). The UV spectra were obtained on a Hitachi UV-3210 spectrophotometer (Tokyo, Japan), and IR spectra were recorded on a Shimadzu FTIR-8501 spectrophotometer (Kyoto, Japan). ^1H - and ^{13}C -NMR, COSY, NOESY, HMQC, and HMBC spectra were obtained on the Bruker Avance III-400 NMR spectrometer (Fremont, CA, USA), with tetramethylsilane (TMS) as internal standard and the chemical shifts were reported in δ values (ppm). The electrospray ionization (ESI) mass spectrum was determined using an Agilent 1200 LC-MSD Trap spectrometer (Santa Clara, CA, USA). Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, E. Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was conducted on precoated Kieselgel 60 F 254 plates (Merck) and the compounds were visualized by spraying with 10% (v/v) H_2SO_4 followed by heating at 110 °C for 10 min.

3.2. Fungus Materials

The basidiomycete (*Ganoderma mastoporum* (Mont) Pat.) was collected at the Puhuong National Park of Nghean Province, Vietnam, in November 2010 and identified by Dr. Ngo Anh, Department of Biology, Hue University. A voucher specimen (Vinh-TSWu 20100805) was deposited at the herbarium of the Department of Chemistry, Vinh University.

3.3. Extraction and Isolation

The fruiting bodies of *Ganoderma mastoporum* (4.3 Kg) were air-dried, powdered, and extracted with methanol (10 L \times 3) at ambient temperature, and the combined extracts were concentrated under reduced pressure to give a deep brown syrup (125 g). The crude extract was suspended in water and partitioned with ethyl acetate to afford ethyl acetate (30 g) and water soluble (95 g) fractions, respectively.

The ethyl acetate soluble extracts were subjected to silica gel column chromatography (CC) with *n*-hexane and acetone gradients (15:1 to 1:1) to afford five fractions. Fractions 1–2 and 4–5 did not

display significant triterpenoid and steroid spots monitored by TLC so that they were not further purified. Fraction 3 was a triterpene-rich fraction visualized by spraying with 10% (v/v) H₂SO₄ followed by heating at 110 °C for 10 min. It was subjected to silica gel column chromatography eluted with chloroform and methanol gradients (100:1 to 1:1) to afford three subfractions. Subfraction 3-1 was purified by SiO₂ CC (eluted with chloroform/ethyl acetate 20:1) and further recrystallization of the resulted minor fractions with chloroform/methanol to yield Δ^1 -lupenone (**1**, 4 mg) and ergosta-7,22-dien-3 β -ol (**2**, 6 mg), respectively. Subfraction 3-2 was repeatedly purified by SiO₂ CC and preparative TLC (pTLC) (eluted with benzene/ethyl acetate 20:1) to afford ergosta-4,6,8(14),22-tetraen-3-one (**3**, 3 mg), ergosterol peroxide (**4**, 8 mg), and ganodermanondiol (**5**, 2 mg), respectively. Subfraction 3-3 was purified by SiO₂ CC (eluted with benzene/acetone 50:1) to yield three minor fractions (3-3-1~3-3-3). Fractions 3-3-1 and 3-3-2 were repeatedly purified by SiO₂ CC and pTLC (eluted with benzene/ethyl acetate 20:1) to afford lucidumol B (**6**, 3 mg), and ergosta-7,22-dien-3-one (**7**, 2 mg), respectively. Fractions 3-3-3 was further recrystallized with chloroform /methanol to result in 3 β ,5 α -dihydroxy-(22*E*,24*R*)-ergosta-7,22-dien-6-one (**8**, 2 mg).

3.4. Determination of Inhibitory Effects on Superoxide Anion Generation and Elastase Release

3.4.1. Preparation of Human Neutrophils

Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes. Blood was drawn from healthy human donors (20–30 years old) by venipuncture into heparin-coated vacutainer tubes, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital [22].

3.4.2. Measurement of Superoxide Anion Generation

The assay of the generation of superoxide anion was based on the SOD-inhibitable reduction of ferricytochrome c [22]. Calculations were based on differences in the reactions with and without SOD (100 U/mL) divided by the extinction coefficient for the reduction of ferricytochrome c ($\epsilon = 21.1/\text{mM}/10 \text{ mm}$).

3.4.3. Measurement of Elastase Release

Degranulation of azurophilic granules was determined by elastase release as described previously [22]. The results were expressed as the percent of elastase release in the FMLP/CB-activated, drug-free control system.

3.4.4. Assay of Scavenging Activity against Diphenyl Picrylhydrazyl (DPPH) Radical

The DPPH free radical scavenging assay was executed according to the reported method [23]. The DPPH radical scavenging activities of the sample solutions were expressed as inhibition percentage and calculated by using the following equation:

$$\text{Inhibition percentage (\%)} = [1 - (\text{absorbance of samples at 517 nm}) / (\text{absorbance of control at 517 nm})] \times 100 (\%).$$

3.4.5. Chelation of Ferrous Ions

The capability of chelating ferrous ions was determined by the reported method with some modifications [23]. The relative capability of chelating ferrous ions was derived from the formula:

$$[1 - (\text{sample absorbance at 562 nm})/(\text{control absorbance at 562 nm})] \times 100 (\%)$$

3.4.6. Statistical Analysis

Results were expressed as mean \pm S.E.M. Computation of 50% inhibitory concentration (IC₅₀) was computer-assisted (PHARM/PCS v.4.2). Statistical comparisons were made between groups using Student's *t* test. Values of *p* less than 0.05 were considered to be statistically significant.

4. Conclusion

In summary, three triterpenoids and five steroids from the fruiting bodies of *G. mastoporum* collected in Vietnam were characterized and their inhibition of superoxide anion generation and elastase release by human neutrophils in response to FMLP/cytochalasin B was examined. The results provide evidence for the use of the fruiting bodies of *G. mastoporum* as herbal medicines in the treatment of inflammatory diseases, and these purified principles may be potentially useful in developing new anti-inflammatory therapeutic agents.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Ruan, Z.; Su, J.; Dai, H.C.; Wu, M.C. Characterisation and immunomodulating activities of polysaccharides from *Lentinus edodes*. *Int. Immunopharmacol.* **2005**, *5*, 811–820.
2. Stanley, G.; Harvey, K.; Slivova, V.; Jiang, J.; Sliva, D. *Ganoderma lucidum* suppresses angiogenesis through the inhibition of secretion of VEGF and TGF- β 1 from prostate cancer cells. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 46–52.
3. Aarisawa, M.; Fujita, A.; Saga, M.; Fukumura, H.; Hayashi, T.; Shimizu, M.; Morita, N. Three new lanostanoids from *Ganoderma lucidum*. *J. Nat. Prod.* **1986**, *49*, 621–625.
4. Won, S.J.; Lin, M.T.; Wu, W.L. *Ganoderma tsugae* mycelium enhances splenic natural killer cell activity and serum interferon production in mice. *Japan J. Pharmacol.* **1992**, *59*, 171–176.
5. Paterson, R.R.M. *Ganoderma*—A therapeutic fungal biofactory. *Phytochemistry* **2004**, *67*, 1985–2001.

6. Wasser, S.P. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 258–274.
7. Lin, L.J.; Shiao, M.S. ¹³C-NMR correlation of stereochemistry in lanostanoids triterpenes. *J. Nat. Prod.* **1989**, *52*, 595–605.
8. Lin, C.N.; Tome, W.P.; Won, S.J. A lanostanoid of Formosan *Ganoderma lucidum*. *Phytochemistry* **1990**, *29*, 673–675.
9. Lin, C.N.; Kuo, S.H.; Won, S.J. Steroids of Formosan *Ganoderma amboinense*. *Phytochemistry* **1993**, *32*, 1549–1551.
10. Lin, C.N.; Fann, Y.F.; Chung, M.I. Steroids of Formosan *Ganoderma tsugae*. *Phytochemistry* **1997**, *46*, 1143–1146.
11. Gan, K.H.; Fann, Y.F.; Hsu, S.H.; Kuo, K.W.; Lin, C.N. Mediation of cytotoxicity of lanostanoids and steroids of *Ganoderma tsugae* through apoptosis and cell cycle. *J. Nat. Prod.* **1998**, *61*, 485–487.
12. Gan, K.H.; Kuo, S.S.; Lin, C.N. Steroidal constituents of *Ganoderma applanatum* and *Ganoderma neo-japonicum*. *J. Nat. Prod.* **1998**, *61*, 1421–1422.
13. Su, H.J.; Fann, Y.F.; Chung, M.I.; Won, S.J.; Lin, C.N. New lanostanoids of *Ganoderma tsugae*. *J. Nat. Prod.* **2000**, *63*, 514–516.
14. Giner-Larza, E.M.; Mánuez, S.; Giner-Pons, R.M.; Recio, M.C.; Ríos, J. On the anti-inflammatory and anti-phospholipase A2 activity of extracts from lanostane-rich species. *J. Ethnopharmacol.* **2000**, *73*, 61–69.
15. Ko, H.H.; Hung, C.F.; Wang, J.P.; Lin, C.N. Antiinflammatory triterpenoids and steroids from *Ganoderma lucidum* and *G. tsugae*. *Phytochemistry* **2008**, *69*, 234–239.
16. Hirotani, M.; Ino, C.; Hatano, A.; Takayanagi, H.; Furuya, T. Ganomastensols A, B, C and D, cadinene sesquiterpenes from *Ganoderma mastoporum*. *Phytochemistry* **1995**, *40*, 161–165.
17. Srivastava, R.; Kulshreshtha, D.K. Triterpenoids from *Glochidion heyneanum*. *Phytochemistry* **1988**, *27*, 3575–3578.
18. Fujita, A.; Arisawa, M.; Saga, M.; Hayashi, T.; Morita, N. Two new lanostanoids from *Ganoderma lucidum*. *J. Nat. Prod.* **1986**, *49*, 1122–1125.
19. Lu, W.; Adachi, I.; Kano, K.; Yasuta, A.; Toriizuka, K.; Ueno, M.; Horikoshi, I. Platelet aggregation potentiators from Cho-Rei. *Chem. Pharm. Bull.* **1985**, *33*, 5083–5087.
20. Jinming, G.; Lin, H.; Jikai, L. A novel sterol from chinese truffles *Tuber Indicum*. *Steroids* **2001**, *66*, 771–775.
21. Ishizuka, T.; Yaoita, Y.; Kikuchi, M. Sterol constituents from the fruit bodies of *Grifola frondosa* (Fr.) S. F. Gray. *Chem. Pharm. Bull.* **1997**, *45*, 1756–1760.
22. Hwang, T.L.; Li, G.L.; Lan, Y.H.; Chia, Y.C.; Hsieh, P.W.; Wu, Y.H.; Wu, Y.C. Potent inhibitors of superoxide anion production in activated human neutrophils by isopedicin, a bioactive component of the Chinese medicinal herb *Fissistigma oldhamii*. *Free Radic. Biol. Med.* **2009**, *46*, 520–528.
23. Kuo, P.C.; Lin, M.C.; Chen, G.F.; Yiu, T.J.; Tzen, J.T.C. Identification of methanol-soluble compounds in sesame and evaluation of antioxidant potential of its lignans. *J. Agric. Food Chem.* **2011**, *59*, 3214–3219.

24. Yang, M.L.; Kuo, P.C.; Hwang, T.L.; Chiou, W.F.; Qian, K.; Lai, C.Y.; Lee, K.H.; Wu, T.S. Synthesis, *in vitro* anti-inflammatory and cytotoxic evaluation, and mechanism of action studies of 1-benzoyl- β -carboline and 1-benzoyl-3-carboxy- β -carboline derivatives. *Bioorg. Med. Chem.* **2011**, *19*, 1674–1682.
25. Jin, S.E.; Son, Y.K.; Min, B.S.; Jung, H.A.; Choi, J.S. Anti-inflammatory and antioxidant activities of constituents isolated from *Pueraria lobata* roots. *Arch. Pharm. Res.* **2012**, *35*, 823–837.
26. Chao, W.W.; Kuo, Y.H.; Lin, B.F. Anti-inflammatory activity of new compounds from *Andrographis paniculata* by NF- κ B transactivation inhibition. *J. Agric. Food Chem.* **2010**, *58*, 2505–2512.
27. Ma, L.; Chen, H.; Dong, P.; Lu, X. Anti-inflammatory and anticancer activities of extracts and compounds from the mushroom *Inonotus obliquus*. *Food Chem.* **2013**, *139*, 503–508.

Sample Availability: Samples of the compounds **1–8** are available from the authors.

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