5α-Reductase Inhibitory Effect of Triterpenoids Isolated from *Ganoderma lucidum*

Jie Liu,[a] Kenji Kurashiki,[b] Kuniyoshi Shimizu,[a] and Ryuichiro Kondo*,[a]

[a] *Department of Forest and Forest Products Science, Faculty of Agriculture, Kyushu University; Fukuoka 812–8581, Japan: and* [b] *Kurume Research Park Co. Ltd.; Fukuoka 839–0864, Japan.*

Received October 25, 2005; accepted November 22, 2005; published online November 25, 2005

5α-Reductase inhibitory activity-guided fractionation of the EtOH extract of the fruiting body of *Ganoderma lucidum* (Leyss.:Fr.) Karst. (Ganodermataceae), which is called Reishi, or Mannentake in Japan and Lingzhi in China, led to the isolation of two active compounds which were ganoderic acid DM and 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one with an IC_{50} of 10.6 μM and 41.9 μM respectively. A carboxyl group of side chain of ganoderic acid DM is essential to elicit the inhibitory activity because of much less activity of its methyl ester.

Key words 5α-reductase; *Ganoderma lucidum*; anti-androgen activity; benign prostatic hyperplasia (BPH)

The microsomal enzyme steroid 5α-reductase [EC 1.3.99.5] catalyzes the NADPH-dependent reduction of $\Delta^{4,5}$ double bond of a variety of 3-oxo-Δ^4 steroids.[1] The principal circulating androgen is testosterone. In several androgen target tissues, like the prostate, testosterone is converted to 5α-dihydrotestosterone (DHT), which is the most potent natural androgen. This process amplifies of the androgenic response, perhaps because of the higher affinity of the androgen receptor for DHT than for testosterone.[2] Both 5α-reductase and DHT perform critical roles physiologically and pathologically in man. The plasma level of DHT has been reported to be elevated in patients with either benign prostatic hyperplasia (BPH) or prostatic cancer. As a result, the inhibition of 5α-reductase has become a pharmacological strategy for the treatment of BPH as well as other DHT-related disorders such as acne and male pattern baldness.[3]

The study of the inhibition of 5α-reductase with organic molecules has lasted more than two decades; consequently, numerous nonsteroidal and steroidal compounds have been designed and synthesized as competitive, noncompetitive, and uncompetitive inhibitors of 5α-reductase. However, it should be noted that these inhibitors have the potential to cause adverse effects such as those reported for finasteride[4]—*i.e.*, gynecomastia, impairment of muscle growth, and severe myopathy. Hence, the emergence of therapeutic materials having fewer side effects—preferably, edible natural products—would be highly desirable if their safety could be guaranteed.

For thousands of years, mushrooms have been known as a source of medicine. In our previous screening of mushrooms, we discovered that the EtOH extract of *Ganoderma lucidum* (Leyss.:Fr.) Karst. (Ganodermataceae) (Fig. 1) showed the strongest 5α-reductase inhibitory activity. Also, the treatment of *G. lucidum* itself or the EtOH extract prepared from it significantly inhibited the growth of the ventral prostate induced by testosterone in rat.[5,6]

In this paper, we report the isolation of the oxygenated lanostane-type triterpenoids with 5α-reductase inhibition, ganoderic acid DM (**1**) and 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (**2**) from *G. lucidum* and their inhibitory effects on 5α-reductase.

MATERIALS AND METHODS

Materials *G. lucidum* (BMC9049) was obtained from Bisoken Inc. (Fukuoka, Japan). The mushroom was identified by Mr. S. Kaneko, Fukuoka Prefecture Forest Research and Extension Center. The voucher specimen (BMC9049) is preserved at the herbarium of the Department of Forest and Forest Products Sciences, Kyushu University in Japan. The fruiting body was dried and ground to powder before use. Unless otherwise specified, chemicals were obtained from Sigma Aldrich Japan Co., Ltd. (Tokyo, Japan). Organic solvents were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). [4-^{14}C] Testosterone was obtained from PerkinElmer Japan Co., Ltd. (Kanagawa, Japan).

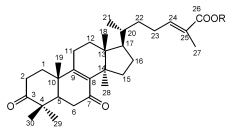
EtOH Eextracts of *Ganoderma lucidum* Dried and chipped *G. lucidum* (15 kg) was extracted with 95% EtOH (126 l) at room temperature for 24 h by using blender. The extracts were filtered through ADVANTEC No. 2 filter paper, concentrated under vacuum, and then freeze-dried. The extracts (571.1 g) were stored in −20 °C before assay.

Dried and chipped *G. lucidum* (200 g) was extracted with 30% EtOH at room temperature for 24 h by using blender. The extracts were filtered through ADVANTEC No. 2 filter paper, concentrated under vacuum, and then freeze-dried. The extracts (10 g) were stored in −20 °C before assay.

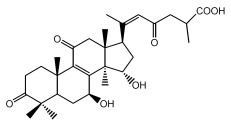
The 95% EtOH extracts (571 g) was fractionated into three fractions [Fr. A (240 g), Fr. B (35 g), Fr. C (269 g)] (Fr. A: TLC, silica gel, I_2 detection, EtOAc/*n*-hexane, 7 : 3, *Rf* 0.48—0.97, Fr. B: *Rf* 0.03—0.67, Fr. C: *Rf* 0—0.04.) by column chromatography on silica gel. Repeated column chro-

Fig. 1. The Photograph of *G. lucidum*

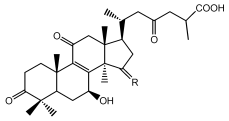
* To whom correspondence should be addressed. e-mail: kondo@agr.kyushu-u.ac.jp



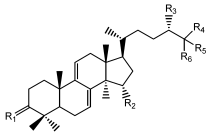
constituent	R
ganoderic acid DM (**1**)	H
methyl ester of **1**(**10**)	CH_3



ganoderenic acid A (**6**)



constituent	R
ganoderic acid D (**7**)	O
ganoderic acid A (**9**)	



constituent	R_1	R_2	R_3	R_4	R_5	R_6
5α-lanosta- 7,9(11),24- triene-15α,26- dihydroxy-3-one (**2**)	O	OH	$\Delta^{24(25)}$		CH_2OH	CH_3
ganodermadiol (**3**)		H	$\Delta^{24(25)}$		CH_2OH	CH_3
lucidumol B (**4**)		H	OH	CH_2OH	CH_3	CH_3
ganodermanontriol (**5**)	O	H	OH	CH_2OH	OH	CH_3
ganoderiol A (**8**)		H	OH	CH_2OH	OH	CH_3

Fig. 2. The Chemical Structure of Triterpenoids Isolated from *G. lucidum*

matography of Fr. B led to the isolation of four compounds (**1**—**4**). They are identified as ganoderic acid DM (**1**),[7] 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (**2**),[8] ganodermadiol (**3**),[9] lucidumol B (**4**),[10] by comparison of the MS, NMR and optical rotation matched with published data (Fig. 2).

The 30% EtOH extracts (10 g) were suspended in water (0.3 l) and extracted with $CHCl_3$ (1 l×5), water-saturated BuOH (2 l×5) successively. Repeated column chromatography of $CHCl_3$-soluble fraction and BuOH-soluble fraction led to the isolation of five compounds (**5**—**9**). They are identified as ganodermanontriol (**5**),[11] ganoderenic acid A (**6**),[12] ganoderic acid D (**7**),[13] ganoderiol A (**8**),[11] ganoderic acid A (**9**)[14]: MS, NMR and optical rotation matched with published data (Fig. 2).

Preparation of Rat Microsomes Rat liver from female SD rats (7 weeks age) was prepared by a method previously reported by Shimizu *et al.* with some modifications.[15] From two mature SD female rats, the liver was removed and minced tissue was homogenized in 4 tissue volumes of medium A (0.32 M sucrose, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 6.5). The resulting supernatant from the centrifugations was further centrifuged at 105000×***g*** for 1 h twice. The washed microsomes were suspended in 1 pellet volume of medium A, and the dispersion of microsomes was achieved using a syringe with 18 G, 23 G, and 26 G needles in succession. The microsome suspension was stored at −80 °C just before use.

Measurement of 5α-Reductase Inhibitory Activity A complete reaction mixture included 1 mM dithiothreitol, 20 mM phosphate buffer (pH 6.5), 1.9 nCi [4-^{14}C] testosterone, 150 μM testosterone, 167 μM NADPH, and the microsomes (0.6 mg of protein) in a final volume of 0.3 ml. The concentration of testosterone contributed by [4-^{14}C] testosterone was negligible. The sample was added to the solution at each concentration. The incubation was carried out for 10 min at 37 °C. It was started by the addition of 10 μl microsomes to the pre-heated reaction solution in a tube. After 10 min, the incubation was terminated by adding 10 μl of 3 M NaOH. To extract metabolites, 1 ml of diethyl ether was added, and the tubes were capped and shaken. The organic phase was applied to a silica plate (Kieselgel 60 F_{254}), and the plate was developed in EtOAc–*n*-hexane (7 : 3) at room temperature. The radioactivity profile was determined with an imaging analyzer (FLA-5000 RF, Fuji Film Co., Ltd., Tokyo, Japan). The 5α-reductase activity was calculated from the percentage of the extent of the conversion of [4-^{14}C] testosterone to [4-^{14}C] dihydrotestosterone.

Preparation of Methyl Ester (10) *N*-Methyl-*N*-nitroso-*p*-toluenesulfonamide (1.25 g, 5.83 mmol) was dissolved in diethyl ether (60 ml), and was dropped into the solvent that included KOH (0.7 g, 12.5 mmol), EtOH (1.6 ml) and water (0.5 ml). The water bath was not higher than 60 °C. The solution of CH_2N_2 was collected into a flask, and put into **1** until the bubble was not produced. The diethyl ether was dried, and the white powder was obtained. The methyl ester (**10**) was confirmed by GC-MS ($[M]^+$ 482) (Fig. 2).

RESULTS AND DISCUSSION

In our previous screening of 19 edible and medicinal mushrooms, we discovered that the EtOH extract of the fruiting body of *G. lucidum* showed the strongest 5α-reductase inhibitory activity.[5,6] In addition, the treatment of the EtOH

extract prepared from *G. lucidum* at 1.5 and 15 mg/kg/d significantly inhibited the growth of the ventral prostate induced by testosterone in rat.[6] To clarify the active principles of the EtOH extract of *G. lucidum*, 5α-reductase inhibitory activity-guided fractionation was carried out. The inhibitory concentration leading to 50% activity loss (IC_{50}) of the EtOH extract was estimated to be 93.6 μg/ml. The EtOH extracts were roughly separated into three fractions (Fr. A, B, C). Fr. B showed 5α-reductase inhibitory activity of more than 90% at 200 μg/ml.

G. lucidum has been reported to produce many bioactive oxygenated triterpenoids. Up to now, over 120 species of triterpenoids have been isolated from *G. lucidum* and the genus *Ganoderma*.[16] Considering the results of our TLC analysis (data not shown), it is likely that most of triterpenoids are present in Fr. B. Therefore, we focused on the triterpenoids in the EtOH extract of *G. lucidum* and investigated the 5α-reductase inhibitory effects of nine triterpenoids isolated from *G. lucidum*.

The results are shown in Table 1. α-Linolenic acid, known as a naturally occurring potent inhibitor, was used as a positive control. It should be noted that finasteride,[17] which is known as a potent steroidal inhibitor, showed an IC_{50} of 0.73 μM in our assay system. Ganoderic acid DM (**1**) and 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (**2**) showed stronger inhibitory activity than did α-linolenic acid.

The IC_{50} of **1** and **2** was estimated to be 10.6 μM and 41.9 μM, respectively. In contrast to **1**, its methyl ester (**10**) showed much less inhibitory activity on 5α-reductase as shown in Fig. 3. The inhibitory activities of 5α-reductase at 20 μM were 55% and 3% for **1** and **10**, respectively. These results suggested that a carboxyl group of side chain of **1** is essential to elicit the inhibitory activity, but the reason for this difference of inhibition between **1** and **10**, and for the potent inhibitory activity of **2** is still largely unknown. These still seems to be a lack of important knowledge for designing effective 5α-reductase inhibitors.

Table 1. Comparison of IC_{50} of Each Compound (**1**—**9**) from *G. lucidum* and α-Linolenic Acid on 5α-Reductase Activity

Compound	IC_{50} (μM)
Ganoderic acid DM (**1**)	10.6
5α-Lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (**2**)	41.9
Ganodermadiol (**3**)	>453
Lucidumol B (**4**)	>436
Ganodermanontriol (**5**)	>423
Ganoderenic acid A (**6**)	>389
Ganoderic acid D (**7**)	>194
Ganoderiol A (**8**)	>421
Ganoderic acid A (**9**)	>387
α-Linolenic acid (positive control)	116

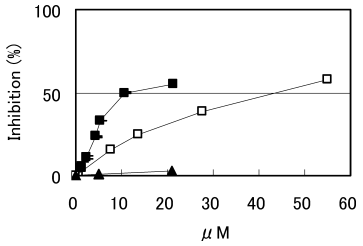


Fig. 3. Inhibitory Effects of **1**, **2** and **10** on 5α-Reductase
■: **1**, □: **2**, ▲: **10**.

The fungi *G. lucidum* (Reishi, Mannentake, or Lingzhi) has been used for centuries in East Asia to cure various human diseases such as hepatitis, hepatopathy, hypertension, nephritis, bronchitis, and cancers.[18] In the last few years, the use of herbal therapies in alternative medicine has been increasing, and although the number of cancer patients using herbal dietary supplements is not exactly known, there is evidence of the increasing use of dietary supplements in cancer treatment. *G. lucidum* is one of the herbs in the herbal mixture PC-SPES, which showed activity against hormone-refractory disease in two prostate cancer patients.[19] Extracts of PC-SPES demonstrated estrogenic effects and decreased growth of hormone-sensitive as well hormone-insensitive prostate cancer cells. These effects might be related to not only anti-cancer effects of *G. lucidum* but also anti-androgen effects by considering our results. Since excessive 5α-reductase activity has been proposed to be a possible contributing factor in prostate cancer development and progression, the development and progression of prostate cancer may also be affected by diets containing inhibitors of 5α-reductase. For example, the use of finasteride, the 5α-reductase inhibitor, can lower the androgen levels in the prostate and reduce the risk of prostate cancer.[20] Although the inhibitory effects on proliferation and migration of prostate cancer cells by *G. lucidum*[21] had been reported, 5α-reductase inhibitory activity of triterpenoids isolated from *G. lucidum* has never been reported.

Acknowledgments The authors are grateful to Mr. S. Kaneko, Mr. Y. Suimi, Dr. S. Kumamoto, Dr. F. Konishi and Dr. K. Noda, for providing mushroom samples and critical advices, and Ms. M. Sato for her experimental assistance.

REFERENCES

1) Russell D. W., Wilson J. D., *Annu. Rev. Biochem.*, **63**, 25—61 (1994).
2) Liao S., Liang T., Fang S., Castaned E., Shao T. C., *J. Biol. Chem.*, **248**, 6154—6162 (1973).
3) Metcalf B. W., Levy M. A., Holt D. A., *Trends Pharmacol. Sci.*, **10**, 491—495 (1989).
4) Uygur M. C., Gur E., Arik A. I., Altug U., Erol D., *Andrologia*, **30**, 5—10 (1998).
5) Liu J., Fujita R., Sato M., Shimizu K., Konishi F., Noda K., Kumamoto S., Ueda C., Tajiri H., Kaneko S., Suimi Y., Kondo R., *J. Wood. Sci.*, **51**, 189—192 (2005).
6) Fujita R., Liu J., Shimizu K., Konishi F., Noda K., Kumamoto S., Ueda C., Tajiri H., Kaneko S., Suimi Y., Kondo R., *J. Ethnopharmacol.*, **102**, 107—112 (2005).
7) Wang F., Cai H., Yang J., Zhang Y., Hou C., Liu J., Zhao M., *Yao Hsueh Hsueh Pao*, **32**, 447—450 (1997).
8) Gonzalez A. G., Leon F., Rivera A., Padron J. I., Gonzalez-Plata J., Zuluaga J. C., Quintana J., Estevez F., Bermejo J., *J. Nat. Prod.*, **65**, 417—421 (2002).
9) Arisawa M., Fujita A., Saga M., Fukumura H., Hayashi T., Shimizu M., Morita N., *J. Nat. Prod.*, **49**, 621—625 (1986).
10) Fujita A., Arisawa M., Saga M., Hayashi T., Morita N., *J. Nat. Prod.*, **49**, 1122—1125 (1986).
11) Sato H., Nishitoba T., Shirasu S., Oda K., Sakamura S., *Agric. Biol. Chem.*, **50**, 2887—2890 (1986).
12) Nishitoba T., Goto S., Sato H., Sakamura S., *Phytochemistry*, **28**, 193—197 (1988).
13) Komoda Y., *Tokyo Ika Shika Daigaku Iyo Kizai Kenkyusho Hokoku*, **19**, 45—54 (1985).
14) Kubota T., Asaka Y., Miura I., Mori H., *Helv*, *Chim. Acta*, **65**, 611—

619 (1982).
15) Shimizu K., Fukuda M., Kondo R., Sakai K., *Planta Med.*, **66**, 16—19 (2000).
16) Shiao M. S., *Chem. Rec.*, **3**, 172—180 (2003).
17) Metcalf D. A., Levy M. A., Holt D. A., "Advances in Medicinal Chemistry," Vol. 2, ed. by Maryanoff B. E., Maryanoff C. A., Elsevier, Oxford, UK, 1993.
18) Wasser S. P., Weis A. L., *Crit. Rev. Immunol.*, **19**, 65—96 (1999).
19) A de la Taille A., Hayek O. R., Burchardt M., Burchardt T., Katz A. E., *J. Altern. Complement Med.*, **6**, 449—451 (2000).
20) Thompson I. M., Goodman P. J., Tangen C. M., Lucia M. S., Miller G. J., Ford L. G., Lieber M. M., Cespedes R. D., Atkins J. N., Lippman S. M., Carlin S. M., Ryan A., Szczepanek C. M., Crowley J. J., Coltman C. A., Jr., *N. Engl. J. Med.*, **349**, 215—224 (2003).
21) Jiang J. H., Slivova V., Valachovicova T., Harvey K., Sliva D., *Int. J. Oncol.*, **24**, 1093—1099 (2004).