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Note

Ganodermasides C and D, Two New Anti-Aging Ergosterols from Spores of the Medicinal Mushroom *Ganoderma lucidum*

Yufang WENG,[1] Jun LU,[1] Lan XIANG,[2] Akira MATSUURA,[3] Yang ZHANG,[4] Qianming HUANG,[4] and Jianhua QI[1,†]

[1]*College of Pharmaceutical Sciences, Zhejiang University, Yu Hang Tang Road 388, Hangzhou 310058, China*
[2]*College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310029, China*
[3]*Department of Biology, Faculty of Science, Chiba University, 1-33 Yayoi, Inage-ku, Chiba 263-8522, Japan*
[4]*College of Biology and Science, Sichuan Agricultural University, Yaan, Sichuan 625014, China*

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Two new anti-aging compounds, ganodermasides C and D, were isolated and their structures elucidated. They are novel ergosterols possessing a 4,6,8(14),22-tetraene-3-one unit with unique hydroxylation at C-9. Both of them significantly extended the replicative lifespan of the K6001 yeast strain. Ganodermasides C and D regulated the expression of the gene for *UTH1* to prolong the replicative lifespan of yeast.

Key words: spores of *Ganoderma lucidum*; ganodermaside; anti-aging; K6001 yeast strain; *UTH1* gene

Aging is an inevitable process of functional impairment which increases the susceptibility of an organism to a variety of age-dependent diseases. The precise molecular mechanisms that link aging to age-related diseases are not clearly understood, although, the close relationship between the process of aging and age-dependent neurodegenerative disorders has been extensively studied. Some small molecules, such as resveratrol, have been found not only to possess activity that extended the replicative lifespan of yeast, but also to produce positive effects in various animal models of age-related disorders.[1–4] Anti-aging substances could therefore be developed to serve as therapeutic drugs for treating age-related diseases.

The traditional yeast replicative-lifespan bioassay is one of the well-known methods for screening samples with anti-aging activity, in which the maximum number of daughter cells produced by an individual mother cell is counted by removing the daughters before self-replication can occur.[5] To establish a more convenient bioassay method, we used the *Saccharomyces cerevisiae* mutant, K6001, for evaluating the anti-aging activity toward yeast. Extracts of traditional Chinese medicines were screened by using the yeast K6001 bioassay method and compared with the result for the well-known anti-aging substance, resveratrol.[6,7] Our previous work, guided by this modified bioassay method, enabled two novel active ergosterols, ganodermasides A (**1**) and B (**2**) (Fig. 1A), to be isolated from a methanol extract of spores of *G. lucidum*.[7] Further examination of the first HPLC analysis to separate other active fractions led to the isolation of two more new active ergosterol derivatives, ganodermasides C (**3**) and D (**4**) (Fig. 1A). We report here the isolation, structures, and biological activities of these sterols.

The fruiting bodies of *G. lucidum* have been widely utilized, and their bioactive components have been intensively studied. However, the active components in the spores of *G. lucidum* have been rarely investigated due to the difficulty of gathering spores under natural conditions. The recent successful indoor cultivation of *G. lucidum* on a large scale in China enabled plentiful spores of *G. lucidum* to be easily collected.

The spores of *G. lucidum* were bought in Shanghai, China. Since the amounts of active compounds obtained from the first extract were not adequate for a structural determination and bioassay, we scaled up the extraction and purification processes. Another portion of the dried spores (0.85 kg) was extracted and purified by the procedures previously reported.[7] Two active fractions were obtained from the first HPLC purification in a Develosil ODS-HG-5 column $\varphi 10 \times 250$ mm (Nomura Chemicals) eluted at a flow rate of 3 mL/min by MeCN/H_2O (7:3, v/v). In addition, the sample having the same retention time as that previously obtained was combined. These two active samples were again purified by HPLC. The active fraction with the shorter retention time was purified to yield pure ganodermaside C (**3**; 9.5 mg, $t_R = 26.8$ min, 83% aqueous MeOH) and the latter to yield ganodermaside D (**4**; 6.8 mg, $t_R = 45.3$ min, 87% aqueous MeOH).

Ganodermaside C (**3**)[8] had the molecular formula of $C_{28}H_{38}O_3$, as determined by high-resolution electron-spray ionization mass spectral (HR-ESI-MS) measurements. Ganodermaside C (**3**) displayed fluorescence under ultraviolet (UV) light, as confirmed by the respective UV and infrared (IR) absorption at 336 nm and 1678 cm^{-1}. These data for UV and IR absorption corresponded to a long-range conjugated ketone group, while the IR absorption at 3500 cm^{-1} suggested the presence of a hydroxyl group. The ^1H- and ^{13}C-NMR spectra of **3**, together with the results of an HMQC experiment, showed the presence of four double bonds (δ_H 5.18, 5.31, 5.97, 6.39, and 7.57; δ_C 128.3, 128.7, 131.3, 133.6, 134.0, 138.3, 145.6, and 158.9), two

† To whom correspondence should be addressed. Tel/Fax: +86-571-88208627; E-mail: qijianhua@zju.edu.cn

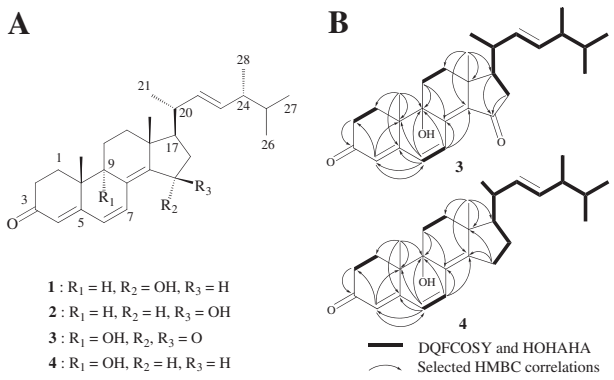

Fig. 1. Structures and Gross Structures of Ganodermasides.
A, structures of ganodermasides A (**1**), B (**2**), C (**3**), and D (**4**); B, gross structures of ganodermasides C (**3**) and D (**4**) with selected HMBC correlations.

ketones (δ_C 198.9 and 207.2), one hydroxyl-bearing quaternary sp^3 carbon (δ_C 72.6), and two other quaternary sp^3 carbons (δ_C 42.9 and 43.0; Table 1). The remaining proton and carbon signals were attributed to six methyl groups, including two tertiary methyls (C-18 and C-19), four secondary methyls (C-21, C-26, C-27, and C-28), five methylenes (C-1, C-2, C-11, C-12, and C-16), and four methines (C-17, C-20, C-24, and C-25). In addition, an analysis of the signals that appeared in the COSY and HOHAHA spectra led to the determination of the partial structures, C-1 to C-2, C-6 to C-7, C-11 to C-12, and C-16 to C-28, depicted by the bold bonds in Fig. 1B. These partial structures were connected by the long-range H–C correlations obtained by an HMBC experiment to yield the gross structure of **3** (Fig. 1B). The important HMBC correlations were as follows: methyl protons (H-19) to C-1, C-5, C-9, and C-10; methyl protons (H-18) to C-12, C-13, C-14, and C-17; H-2 to C-3; H-4 to C-2, C-6, and C-10; H-6 to C-4, C-5, C-9, and C-10; H-7 to C-8, C-9, and C-14; H-11 to C-8, and C-9; H-16 to C-15; and H-17 to C-15.

The stereochemical orientation of C-9 was presumed to be α, because all natural steroids have a *trans* B/C fusion.[9] Furthermore, the pyridine-induced deshielding effect was used to confirm the OH-9α configuration.[10] The proton signals at δ 2.54 (H-1α) and δ 1.77 (H-11α) ($CDCl_3$) were respectively shifted downfield to δ_H 2.90 and δ_H 2.00 (pyridine-d_5). The configuration of the chiral center at C-20 in the side chain was determined as 20*R* on the basis of the NOE correlations and a decoupling measurement, in which proton signal δ 1.14 (H-21) was irradiated. The coupling constant, $J_{17-20} =$ 9.0 Hz, obtained by the decoupling measurement suggested a converse relationship between H-17 and H-20. Furthermore, the 24*R* stereochemistry was assigned by considering the agreement of the ^{1}H- and ^{13}C-NMR data for the side chain of **3** with those of the known compound, gymnasterone D, whose stereostructure has been determined by an X-ray crystal-structure analysis.[11] Moreover, the *trans*-geometry was elucidated from the coupling constant, $J_{22-23} = 15.3$ Hz. The stereostructure of **3** was therefore established as 20*R*, 24*R*, 9α-hydroxy-$\Delta^{4,6,8(14),22}$-tetraene-3,15-dione-ergosterol.

Ganodermaside D (**4**),[8] having the molecular formula $C_{28}H_{40}O_2$, which contains one ketone group less than ganodermaside C (**3**), was deduced from HR-ESI-MS measurements. The ^{1}H- and ^{13}C-NMR spectra of **4** were superimposable on those of **3** (Table 1), except for the signals of a conjugated ketone (at C-15), which was replaced by that of a sp^3-methylene in **4**, and around that position. The analysis of the COSY and HOHAHA spectra led to the determination of the partial structures depicted by the bold bonds in Fig. 1B. The HMBC correlations are also summarized in Fig. 1B with arrows that connect these partial structures to give the planar structure of **4**.

The OH-9α configuration in **4** was determined to be the same as that in **3** by the pyridine-induced deshielding effect.[10] The proton signals at δ_H 2.50 (H-1α) and δ_H 1.70 (H-11α) in $CDCl_3$ were respectively shifted downfield to δ_H 2.91 and δ_H 1.85 in pyridine-d_5. The *R*-configuration at C-20 was determined from the NOE

Table 1. ^{1}H- and ^{13}C-NMR Data for Ganodermasides C (**3**) and D (**4**) in $CDCl_3$

Carbon	**3**		Carbon	**4**	
	^{1}H[a]	^{13}C[b]		^{1}H[a]	^{13}C[b]
1a	1.81 m	27.1	1a	1.78 m	27.4
1b	2.54 m		1b	2.50 m	
2	2.55 m	33.4	2	2.52 m	33.8
3		198.9	3		199.4
4	5.97 s	128.3	4	5.88 s	126.3
5		158.9	5		161.2
6	6.39 d (9.9)	131.3	6	6.11 d (9.6)	124.6
7	7.57 d (9.9)	128.7	7	6.49 d (9.6)	130.7
8		138.3	8		126.5
9		72.6	9		72.4
10		42.9	10		42.3
11a	1.77 m	25.0	11a	1.70 m	25.3
11b	1.95 m		11b	1.94 m	
12a	1.79 m	32.0	12a	1.64 m	31.9
12b	2.09 m		12b	1.96 m	
13		43.0	13		44.4
14		145.6	14		158.2
15		207.2	15a	2.38 m	25.5
			15b	2.48 m	
16a	2.14 dd (19.4, 12.2)	42.6	16a	1.52 m	27.7
16b	2.38 dd (19.4, 8.0)		16b	1.85 m	
17	1.75 m	50.7	17	1.35 m	55.5
18	1.08 s	18.7	18	0.96 s	17.7[c]
19	1.16 s	20.5	19	1.12 s	20.8
20	2.25 m	39.4	20	2.17 m	39.2
21	1.14 d (6.6)	21.3	21	1.08 d (6.6)	21.2
22	5.18 dd (15.3, 8.5)	133.6	22	5.21 dd (15.3, 8.1)	134.8
23	5.31 dd (15.3, 8.0)	134.0	23	5.28 dd (15.3, 7.4)	132.7
24	1.87 m	42.8	24	1.88 m	42.8
25	1.48 m	33.0	25	1.49 m	33.0
26	0.82 d (6.8)	19.6	26	0.84 d (7.0)	19.6
27	0.84 d (6.8)	20.0	27	0.85 d (7.0)	20.0
28	0.92 d (6.8)	17.7	28	0.94 d (6.8)	17.6[c]

[a] At 500 MHz; NMR chemical shifts in δ (ppm) were referenced to the solvent peaks of δ_C 77.0 and δ_H 7.26 for $CDCl_3$, and of δ_H 7.19 for pyridine-d_5; coupling constants (J in Hz) are in parentheses.
[b] At 125 MHz
[c] Interchangeable

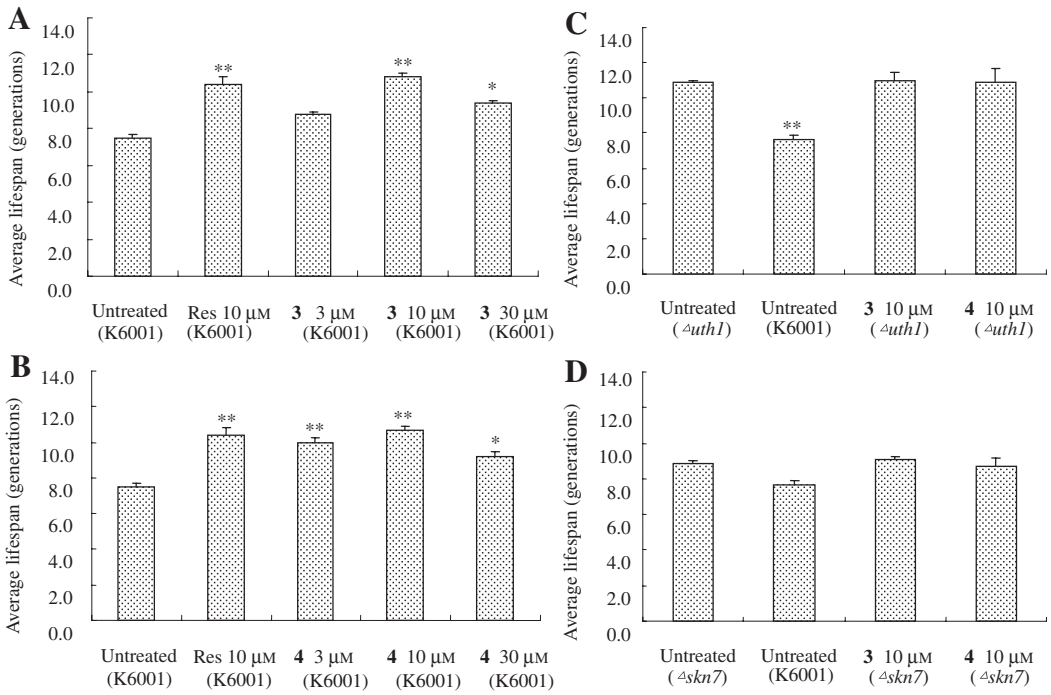

Fig. 2. Biological Activity of Ganodermasides C (**3**) and D (**4**), and Their Mechanism of Action on Yeast.

Each value is presented as the average lifespan ± SD (n = 3). The average lifespan is the mean number of generations for daughter cells produced by each of forty mother cells. * and ** indicate significant difference from the corresponding negative control; $^{*}p < 0.05$, $^{**}p < 0.01$. A and B respectively show the dose-response characteristics for the anti-aging effects of ganodermasides C (**3**) and D (**4**) on the replicative lifespan. The average lifespan untreated was 7.5, and 10.4** treated with resveratrol (Res) at 10 μM; A, **3** at 3 μM was 8.8, **3** at 10 μM was 10.8**, and **3** at 30 μM was 9.4*; B, **4** at 3 μM was 10.0**, **4** at 10 μM was 10.7**, and **4** at 30 μM was 9.2*. C and D respectively show the effects of ganodermasides C (**3**) and D (**4**) on the average replicative lifespan of *uth1* and *skn7* mutants. The *uth1* and *skn7* mutants with a K6001 background were constructed by using the kanamycin gene instead of the target gene.

correlations and a decoupling measurement, in which H-21 (δ_H 1.08) was irradiated. In addition, the 24*R* configuration was assigned by comparing the spectroscopic data with those already reported.[11] The *trans*-geometry was elucidated from the coupling constant of $J_{22-23} = 15.3$ Hz.

A lifespan assay was conducted according to the methods described in our previous paper.[7] The modified bioassay method used 10 μM resveratrol as a positive control to evaluate its reliability. The replicative lifespan of K6001 cells was significantly extended in the presence of resveratrol at a concentration of 10 μM.[6] Figure 2A and B shows the dose-response characteristics for the replicative lifespan with various concentrations of ganodermasides C (**3**) and D (**4**) in comparison with the positive control, resveratrol, and a negative control (EtOH). Compounds **3** (10 μM, Fig. 2A) and **4** (3 and 10 μM, Fig. 2B) significantly increased the average lifespan ($p < 0.01$) in comparison with the negative control. Each of these two compounds at a 10 μM concentration showed activity comparable to that at the best resveratrol concentration of 10 μM, being similar to the effects of ganodermasides A (**1**) and B (**2**).[7] Interestingly, considering the structures and the biological activities of ganodermasides, the 4,6,8(14),22-tetraene-3-one unit of the sterol, and probably the sterol skeleton itself, played an important role in their anti-aging effect on the yeast replicative lifespan, whereas the configuration of the hydroxyl or ketone group at C-15 and the hydroxyl group at C-9 were not similarly involved.

The structural similarity among the ganodermasides prompted the study on the mechanism of action of ganodermasides C (**3**) and D (**4**) on the replicative lifespan of yeast to be referenced against that of ganodermasides A (**1**) and B (**2**).[7] Figure 2C shows that **3** and **4**, each at a level of 10 μM (the best concentration for anti-aging activity), had no influence on the replicative lifespan of *uth1*-mutant cells, suggesting that they might regulate the expression of the gene for *UTH1*, an aging protein, and thereby extend the replicative lifespan of yeast. We examined the effects of 10 μM of the **3** and **4** on a *skn7* mutant with the K6001 yeast background and found that neither **3** nor **4** affected the lifespan of the *skn7*-mutant yeast (Fig. 2D). These results show that the extension of the replicative lifespan that was induced by the **3** and **4** was brought about by the same mechanism as that of **1** and **2**, in which the transcription factor, *SKN7*, regulated the effects of **1** and **2** on the expression of *UTH1*.

The significant differences between groups was determined by ANOVA, with a subsequent two-tailed multiple *t*-test according to Student-Newman-Keuls by SPSS biostatistics software. Values with $p < 0.05$ were considered significant.

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8) Ganodermaside C: yellowish powder; $[\alpha]_D^{25}$ +389 (*c* 0.46, $CHCl_3$); IR (KBr) ν cm^{-1}: 3500, 1678; Uv λ_{max} (MeOH) nm ($\log \varepsilon$): 336 (4.40); HR ESI-TOF-MS m/z $[M + H]^+$ Calcd. for $C_{28}H_{39}O_3$: 423.2894, Found: 423.2877. Ganodermaside D: yellowish powder; $[\alpha]_D^{25}$ +294 (*c* 0.27, $CHCl_3$); IR (KBr) ν cm^{-1}: 3455, 1635; Uv λ_{max} (MeOH) nm ($\log \varepsilon$): 342 (4.46); HR ESI-TOF-MS m/z $[M + H]^+$ Calcd. for $C_{28}H_{41}O_2$: 409.3101, Found: 409.3075.
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