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GANODERIC ACID DERIVATIVES AND ERGOSTA-4,7,22-TRIENE-3,6-DIONE FROM *GANODERMA LUCIDUM**

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Key Word Index—*Ganoderma lucidum*; Basidiomycetes; cultured mycelia; triterpenoids; ganoderic acids T; S; R; Q; P; O; ergosta-4,7,22-triene-3,6-dione.

Abstract—Seven new triterpenoids, ganoderic acid T, ganoderic acid S, ganoderic acid R, ganoderic acid P, ganoderic acid Q, ganoderic acid 0, 7-*O*-methyl-ganoderic acid 0 and a known ergosterol derivative, ergosta-4,7,22-triene-3,6-dione were isolated from the cultured mycelium of *Ganoderma lucidum*. The structure of the first compound was determined using spectroscopic and X-ray analysis, and the structures of the other compounds were elucidated by spectroscopic data.

INTRODUCTION

Ganoderic acid derivatives, highly oxygenated and pharmaceutical active lanostane type triterpenoids, were recently isolated from the fungus *Ganoderma lucidum* (Polyporaceae) [1–15]. Toth *et al.* [16, 17] isolated ganoderic acids Z, Y, X, W, V and U, which were cytotoxic to hepatoma cells *in vitro*, from the cultured mycelium of *G. lucidum*. In a preliminary paper [18], we have reported the isolation and structure elucidation of three new ganoderic acid derivatives, ganoderic acid T(**2a**), S(**2b**) and R(**2c**) as the major triterpene constituents of the cultured mycelium. In this paper we wish to deal with the isolation and structure elucidation of these ganoderic acid derivatives as major triterpenoids and further new ganoderic acid derivatives and a new fungal metabolite, ergosta-4,7,22-triene-3,6-dione as a minor component of the cultured mycelium of *G. lucidum*.

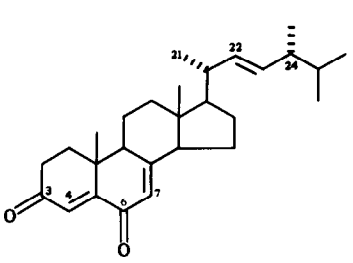
RESULTS AND DISCUSSION

The chloroform extract (16.78 g) of the cultured mycelia was chromatographed on silica gel and separated into fractions A–E. Further purification by reversed-phase HPLC afforded compounds **1**, **2a**, **2b**, **2c**, **2d**, **2e** and **3b** from fractions B, C and E.

Compound **1**, ergosta-4,7,22-triene-3,6-dione, analysed for $C_{28}H_{40}O_2$ and showed in the UV spectrum an absorption at 275 (log ε 3.8) characteristic of an α,β,α′,β′-unsaturated carbonyl group. The IR spectrum of **1** showed the presence of an α,β-unsaturated carbonyl group (1665, 1620 cm^{-1}) and an α,β,α′,β′-unsaturated carbonyl group(1640, 1600 cm^{-1}). The ^1H NMR spectrum of **1**(Table 1) clearly showed the presence of two tertiary methyl and four secondary methyl groups, thus suggesting an ergostane skeleton. This was supported by the base peak ion in the mass spectrum at *m/z* 283 (100%) corresponding to the fragment obtained by the loss of the side chain resulting from cleavage between C-17 and C-20. Furthermore, in the ^1H NMR spectrum of **1**, the chemical shift values of this protons of four secondary methyl groups were completely consistent with those of ergosterol [19]. Thus, compound **1** was suggested to have the same side chain as ergosterol. This suggestion was supported by the ^1H NMR spectrum, which showed two coupled olefinic proton signals (δ5.14 and 5.24). On the other hand, two olefinic protons (δ5.98 *br* s and 6.47 *s*) were observed in the ^1H NMR spectrum of **1**. Also four tertiary carbon signals (δ123.9, 126.0, 132.8, 134.8), two quarternary carbon signals (δ158.2 and 167.7) and two carbonyl carbon signals (δ187.0 and 199.3) were observed in the ^{13}C NMR spectrum of **1**. All these data indicated that **1** was an ergostane derivative with two trisubstituted olefins, an α,β-unsaturated carbonyl group and an α,β,α′,β′-unsaturated carbonyl group in the ergostane skeleton. By comparison of the ^1H NMR spectral data of compound **1**, ergosterol and 3β,5α,14α-trihydroxyergosta-7,22-dien-6-one, the C-18 methyl proton signals were observed at δ0.68, 0.63 and 0.68, respectively, but the chemical shift values of the C-19 methyl proton signals were very different (Table 1). These data led to the conclusion that the functional groups described above were present in the A and B ring portion of the molecule. Furthermore, the olefinic proton (δ6.47) was not coupled with any other proton. Therefore, the structure of 1 was elucidated as ergosta-4,7,22-triene-3,6-dione. Malorini *et al.* [21] reported the occurrence of ergosta-4,7,22-triene-3,6-dione in a mixture of sterioidal $\Delta^{4,7}$-3,6-diketones from the extract of the marine sponge *Raphidostila incisa*. The isolation of ergosta-4,7,22-triene-3,6-dione from the cultured fungi has been demonstrated for the first time [22].

Compound **2a**, ganoderic acid T, analysed for $C_{36}H_{52}O_8$ and showed mp 200–202°, and absorptions in

*Part 7 in the series 'Studies on the Metabolites of Higher Fungi'. For Part 6 see ref. [18].



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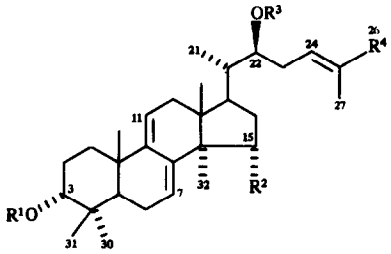
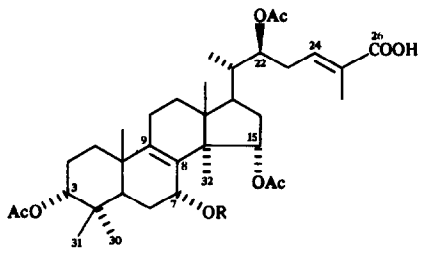


2a $R^1 = R^3 = Ac$, $R^2 = OAc$, $R^4 = COOH$
2b $R^1 = R^2 = H$, $R^3 = Ac$, $R^4 = COOH$
2c $R^1 = R^3 = Ac$, $R^2 = H$, $R^4 = COOH$
2d $R^1 = R^3 = Ac$, $R^2 = OH$, $R^4 = COOH$
2e $R^1 = H$, $R^2 = OAc$, $R^3 = Ac$, $R^4 = COOH$
2f $R^1 = R^3 = H$, $R^2 = OH$, $R^4 = CH_2OH$



3a R = H
3b R = Me

Table 1. ^{1}H NMR spectral data of compound **1**, ergosterol and 3β,5α,14α-trihydroxyergosta-7,22-dien-6-one

Proton	Compound 1	Ergosterol	3β,5α,14α-Trihydroxy-ergosta-7,22-dien-6-one
18-H_3	0.68	0.63	0.68
19-H_3	1.30	0.95	0.94
21-H_3	1.04 (*d*, *J* = 6.6Hz)	1.04 (*d*, *J* = 6Hz)	1.02 (*d*, *J* = 6.2Hz)
26 and 27-H_3	0.82 (*d*, *J* = 6.6Hz)	0.82 (*d*, *J* = 6Hz)	0.82 (*d*, *J* = 6.6Hz)
	0.84 (*d*, *J* = 6.6Hz)	0.84 (*d*, *J* = 6Hz)	0.84 (*d*, *J* = 6.6Hz)
28-H_3	0.92 (*d*, *J* = 6.6Hz)	0.92 (*d*, *J* = 6Hz)	0.92 (*d*, *J* = 6.6Hz)

The data for ergosterol and 3β,5α,14α-trihydroxyergosta-7,22-dien-6-one are cited from refs [19, 20].

the UV spectrum at 234, 242 and 250 nm, suggesting the presence of a heteroannular diene moiety. The IR spectrum of **2a** showed the presence of a hydroxyl group (3420 cm^{-1}) and ester carbonyl group (1720 cm^{-1}). The ^{1}H NMR spectrum of **2a** showed the presence of five tertiary methyl (δ0.66, 0.88, 0.98, 0.99 and 1.03), one allyl methyl (δ1.86) and one secondary methyl (δ0.97, *d*, *J* = 6.7 Hz) signals. Furthermore, the signals due to three secondary acetoxyl groups and three olefinic proton signals were observed (Table 2). These spectral data suggested **2a** to be a lanostane type triterpenoid having a heteroannular diene moiety and three acetoxyl groups. The ^{13}C NMR spectral data of **2a** supported this suggestion and revealed the presence of one carboxyl group (Table 3). Comparison of the ^{1}H NMR and ^{13}C NMR spectral data of **2a** with those of methyl ganoderate X and 3-*O*-acetyl methyl ganoderate X[17] led to the conclusion that **2a** is a tri-acetoxy-ganoderic acid of the 7,9(11)-24-triene type. Two of the three acetoxyl were proved to be at C-3α and C-15α on the basis of the chemical shift values and the coupling patterns of the methine proton signals which corresponded to those due to the 3β-H and 15β-H of 3-*O*-acetyl ganoderate X[17]. The third acetyl group was located at C-22 by reference to the ^{1}H–^{1}H shift correlation NMR spectrum, in which correlation between 23-H[δ2.33(1H, *ddd*, *J* = 15.0, 7.5, 7.0 Hz) and 2.57(1H, *ddd*, *J* = 15.0, 7.6, 7.1 Hz)] and the third acetoxyl methine proton signal at δ5.03 was observed. The relative stereostructure of **2a** was determined by X-ray analysis. Crystal data: M_r = 612.8, orthorhombic, space group $P2_12_12_1$, *a* = 16.740(1), *b* = 29.115(3), *c* = 7.213(1) Å, *V* = 3515.6(6) Å^3, *z* = 4, *d*(calcd) = 1.157 g cm^3, *d*(obsd) = 1.13 g cm^3 (flotation). The structure was solved by MULTAN 84 and refined by a block-diagonal least-squares technique to *R* = 0.057 for 2130 reflections [$|FC| > (Fo)$ and $|\Delta F| < 3\sigma(Fo)$] out of 2994 unique ones obtained in the range of $\theta \leqslant 60°$ for CuKα radiation. The structure represented Fig. 1 was drawn using the PLUTO program [23].

The absolute stereostructure was determined by the CD spectrum of compound **2f** prepared from **2a** by reduction, which showed a positive Cotton effect ($[\theta]_{239}$ +12689, *c* = 0.012 EtOH) like that of (26*S*)-26-*O*-methyl-perenniporiol ($[\theta]_{240}$ +29824, *c* = 0.012 EtOH) [24]. Thus, the structure of **2a** was determined as (22*S*, 24*E*)-3α,15α,22-triacetoxy-5α-lanosta-7,9(11),24-trien-26-oic acid.

Compound **2c**, ganoderic acid R, $C_{34}H_{50}O_6$, showed mp 201–202° and it had UV absorptions at 234, 242, 250 nm and hydroxyl (3400 cm^{-1}), ester carbonyl (1720 cm^{-1}) and α,β-unsaturated carbonyl (1675 cm^{-1}) absorptions in the IR spectrum. The ^{1}H NMR spectrum of **2c** was very similar to that of **2a** except for the loss of the methine proton signal at δ5.08 (15α-H) and of one acetoxyl methyl proton signal. The ^{13}C NMR spectrum of **2c** was also closely similar to that of **2a** except for the appearance of a methylene carbon signal (δ31.3) instead

Table 2. ^{1}H NMR spectral data of compounds **2a-2e**, **3a** and **3b** (400 MHz, $CDCl_3$)*

Proton	2a	2b	2c	2d	2e	3b	3a†
18-H_3	0.66 *s*	0.57 *s*	0.57 *s*	0.60 *s*	0.65 *s*	0.70 *s*	0.68 *s*
19-H_3	0.99 *s*	1.00 *s*	1.05 *s*	0.99 *s*	0.98 *s*	0.94 *s*	0.92 *s*
21-H_3	0.97 *d*	0.98 *d*	0.98 *d*	0.96 *d*	0.96 *d*	0.96 *d*	1.00 *d*
	(6.7)	(6.7)	(6.7)	(6.7)	(6.7)	(6.7)	(6.7)
30-H_3	0.88 *s*	0.98 *s*	0.87 *s*	0.87 *s*	0.98 *s*	0.89 *s*	1.17 *s*
31-H_3	0.98 *s*	0.94 *s*	0.99 *s*	0.98 *s*	0.93 *s*	0.98 *s*	0.89 *s*
32-H_3	1.03 *s*	0.87 *s*	0.90 *s*	1.00 *s*	0.99 *s*	1.18 *s*	1.32 *s*
27-H_3	1.86 *d*	1.86 *d*	1.87 *d*	1.86 *d*	1.86 *d*	1.86 *s*	1.94 *s*
	(1.6)	(1.6)	(1.6)	(1.2)	(1.4)		
22-OAc	2.05 *s*	2.05 *s*	2.05 *s*	2.05 *s*	2.06 *s*	2.05 *s*	1.76 *s*
3-OAc	2.07 *s*	—	2.06 *s*	2.07 *s*	2.09 *s*	2.07 *s*	1.77 *s*
15-OAc	2.08 *s*	—	—	—	—	2.09 *s*	1.85 *s*
H-3β	4.68 *dd*	3.45 *dd*	4.67 *dd*	4.67 *dd*	3.45 *dd*	4.68 *dd*	5.03 *dd*
	(3.0, 3.0)	(3.0, 3.0)	(3.0, 3.0)	(2.5, 2.5)	(2.7, 2.7)	(2.3, 2.3)	(2.5, 2.5)
7-H	5.48 *brs*	5.47 *brs*	5.47 *br dd*	5.88 *brd*	5.48 *dd*	3.56 *brs*	4.30 *brd*
			(4.6, 4.6)	(5.7)	(3.3, 3.3)		(3.1)
11-H	5.32 *d*	5.33 *d*	5.32 *d*	5.31 *brd*	5.33 *br d*	—	—
	(6.4)	(6.4)	(6.4)	(5.9)	(6.2)		
15-H	5.08 *dd*	—	—	4.27 *dd*	5.07 *dd*	5.11 *dd*	5.53 *dd*
	(8.0, 4.0)			(9.6, 4.9)	(8.8, 5.0)	(7.6, 3.5)	(9.5, 6.0)
22-H	5.03 *ddd*	5.10 *ddd*	5.11 *ddd*	5.04 *dd*	5.02 *dd*	5.02 *dd*	5.30 *dd*
	(7.1, 7.0, 1.4)	(6.8, 6.0, 1.7)	(7.1, 7.0, 1.4)	(7.2, 7.2)	(7.6, 7.6)	(7.5, 7.5)	(8.0, 5.5)
23-H_a	2.33 *ddd*	2.36 *ddd*	2.37 *ddd*	2.36 *ddd*	2.33 *ddd*	2.33 *ddd*	2.08 *ddd*
	(15.0, 7.5, 7.0)	(15.0, 7.5, 6.0)	(15.0, 7.5, 7.0)	(15.0, 7.3, 7.2)	(15.0, 7.6, 7.3)	(15.2, 7.5, 7.5)	(15.0, 7.5, 5.5)
23-H_b	2.57 *ddd*	2.56 *ddd*	2.57 *ddd*	2.56 *ddd*	2.57 *ddd*	2.57 *ddd*	2.46 *ddd*
	(15.0, 7.6, 7.1)	(15.0, 7.6, 7.0)	(15.0, 7.6, 7.1)	(15.0, 7.3, 7.2)	(15.0, 7.6, 7.3)	(15.2, 7.5, 7.5)	(15.0, 8.0, 7.5)
24-H	6.78 *ddq*	6.82 *ddq*	6.84 *ddq*	6.80 *ddq*	6.78 *ddq*	6.78 *dd*	7.11 *ddq*
	(7.6, 7.5, 1.6)	(7.6, 7.5, 1.6)	(7.6, 7.5, 1.6)	(7.3, 7.3, 1.2)	(7.3, 7.3, 1.4)	(7.5, 7.5)	(7.5, 7.5, 1.3)
O-Me						3.16 *s*	

*Values in parentheses are coupling constants in Hz.
†^{1}H NMR spectrum of **3a** was taken in C_6D_6.

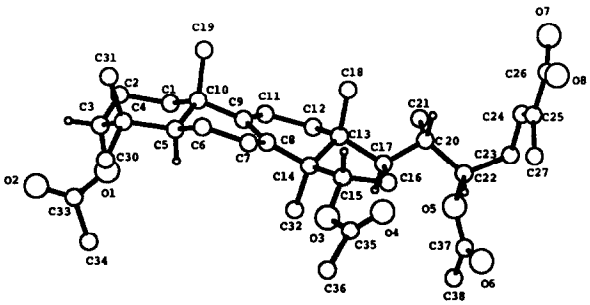


Fig. 1. A computer-generated perspective drawing of **2a**.

of the methine carbon signal (δ77.4) seen in the spectrum of **2a**. These spectral data showed the structure of **2c** to be (22*S*, 24*E*)-3α,22-diacetoxy-5α-lanosta-7,9(11),24-trien-26-oic acid.

Compound **2b**, ganoderic acid S, $C_{32}H_{48}O_5$, showed mp 194–196° and it had UV absorptions at 231, 240, 248 nm and hydroxyl (3410 cm^{-1}), ester carbonyl (1720 cm^{-1}) and α,β-unsaturated carbonyl (1675 cm^{-1}) absorptions in the IR spectrum. The ^{13}C NMR spectrum of **2b** was very similar to that of **2c**, except for the upfield shift of the C-3 carbon signal (δ 76.0) relative to that of **2c** (δ78.0) and the loss of one acetoxyl group. In the ^{1}H NMR spectrum of **2b**, a methine proton signal at δ5.10 (*ddd*, *J* = 6.8, 6.0, 1.7 Hz) due to 22-H was observed and a proton signal at δ3.45 (*dd*, *J* = 3.0, 3.0, 3β-H) which was absent in those of **2a** and **2c** appeared. Based on the spectral evidence, **2b** was deduced to be (22*S*, 24*E*)-22-acetoxy-3α-hydroxy-5α-lanosta-7,9(11),24-trien-26-oic acid. Fujimoto *et al.* [13] reported the isolation of a new ganoderic acid derivative from the same fungus which was designated ganoderic acid S. After discussion with Dr. Fujimoto, their new compound has been designated as ganoderic acid S_1.

Compound **2d**, ganoderic acid Q, mp 131–132°, analysed for $C_{34}H_{50}O_7$ and showed absorptions in the UV spectrum at 235, 243, 251 nm (log ε4.3, 4.3, 4.1), suggesting the presence of a heteroannular diene moiety similar to that in compounds **2a**, **2b** and **2c**. The IR spectrum of **2d** showed the presence of absorptions for hydroxyl

Table 3. ^{13}C NMR spectral data of compounds **2a–2e**, **3a** and **3b**(100.6 MHz, $CDCl_3$)*

Carbon	2a	2b	2c	2d	2e	3b	3a†
1	30.8	29.8	30.5	30.6	29.9	30.0	31.3
2	23.3	25.5	23.0	23.1	25.5	23.3	24.3
3	78.2	76.0	78.0	78.1	76.1	77.2	77.7
4	36.8	37.1[a]	36.4	36.5	37.0	36.3	37.2
5	44.1	43.1	44.0	45.0	42.9	39.6	40.8
6	22.7	27.5	27.5	22.8	23.0	21.3	28.5
7	121.6	120.3	120.2	121.6	121.5	75.9	67.1
8	140.2	142.2	142.2	140.5	140.0	142.9	141.7
9	146.2	145.9	145.8	146.2	146.0	132.7	135.1
10	37.5	37.3[a]	37.1	37.3	37.3	38.7	39.2
11	115.5	115.5	115.5	115.3	115.3	21.1	21.5
12	38.1	37.6	37.6	38.4	37.9	30.7	32.1
13	44.1	43.6	43.6	44.2	43.9	45.6	46.0
14	51.6	50.3	50.3	52.0	51.4	51.7	52.4
15	77.4	31.2	31.3	74.5	76.9	75.2	76.0
16	36.7	22.9	22.8	39.8	36.7	37.1	37.3
17	45.6	47.3	47.3	45.0	45.4	45.2	46.9
18	15.9	15.4	15.4	15.8	15.8	16.1	17.0
19	23.0[a]	22.7[b]	22.4[a]	22.6[a]	22.7[a]	17.6	18.0
20	39.8	39.3	39.3	39.3	39.6	39.9	41.0
21	12.8	12.6	12.6	12.8	12.7	12.7	13.6
22	74.6	74.6	74.6	74.6	74.4	74.3	74.8
23	32.1	31.7	31.8	31.7	31.9	31.8	32.8
24	139.2	139.5	139.6	139.2	139.1	139.1	140.0
25	129.4	129.0	129.0	129.4	129.2	129.3	130.2
26	172.0	172.2	172.3	172.0	171.6	172.2	172.9
27	12.4	12.2	12.2	12.3	12.3	12.3	13.1
30	27.9	28.1	27.7	27.7	28.2	27.3	28.2
31	22.6[a]	22.5[b]	22.5[a]	22.5[a]	22.8[a]	22.2	22.6
32	18.6	25.7	25.6	17.3	18.5	19.0	21.1
AcMe	21.2	21.0	21.0	21.6	21.0	21.0	21.1
AcMe	21.4		21.3	21.7	21.5	21.6	21.3
AcMe	21.5					21.7	21.3
C=O	170.7	170.6	170.6	170.7	170.6	170.6	170.3
C=O	170.9		170.6	170.9	171.2	170.1	170.3
C=O	170.2					170.2	170.5
O–Me						55.2	

*The number of directly attached protons to each individual carbon was verified with the DEPT pulse sequence[25].

†^{13}C NMR spectrum of **3a** was taken in C_6D_6.

[a,b]Assignments may be interchanged in each compound.

(3450 cm^{-1}), ester carbonyl (1740 cm^{-1}) and α-β-unsaturated carboxylic acid (1690 cm^{-1}). In the mass spectrum of **2d**, the molecular ion peak was observed at *m/z* 570[M]$^+$, suggesting **2d** was a deacetyl ganoderic acid T. This suggestion was consistent with the ^{13}C NMR spectrum of **2d**, which did not contain signals for an acetoxyl methyl and a carbonyl carbon. In the ^{13}C NMR spectrum of **2d** the C-15 carbon signal was shifted upfield to δ74.5 and C-16 carbon signal was shifted downfield to δ39.8. From these spectral investigations, compound **2d** was established to be (22*S*, 24*E*)-3α,22-diacetoxy-15α-hydroxy-5α-lanosta-7,9(11),24-trien-26-oic acid.

Compound **2e**, ganoderic acid P, mp 211–212.5°, showed in the UV spectrum absorptions at 235, 243, 251 nm, suggesting the presence of a heteroannular diene moiety. The IR spectrum of **2e** showed the presence of hydroxyl (3450 cm^{-1}), ester carbonyl (1735 cm^{-1}) and α,β-unsaturated carbonyl (1720 cm^{-1}). In the mass spectrum the molecular ion peak was observed at *m/z* 570[M]$^+$ suggesting that **2e** was a deacetyl ganoderic acid T. In the ^{13}C NMR spectrum of **2e**, the C-3 carbon signal was shifted upfield to δ76.1 and the C-2 carbon signal was shifted downfield to δ25.5. From these data, compound **2e** was established as (22*S*, 24*E*)-15α,22-diacetoxy-3α-hydroxy-5α-lanosta-7,9(11),24-trien-26-oic acid.

Compound **3b**, *O*-methyl ganoderic acid O, $C_{37}H_{56}O_9$, mp 228–229.5°, showed absorption only at 208 nm (log ε 4.1) in the UV spectrum and hydroxyl (3450 cm^{-1}), ester carbonyl (1740 cm^{-1}) and α,β-unsaturated carbonyl (1720 cm^{-1}) absorptions were seen in the IR spectrum. The ^1H NMR spectrum of **3b** (Table 2) clearly showed the presence of one secondary and five tertiary methyl groups, thus suggesting a lanostane skeleton. This was supported by a positive Liebermann–Burchard colour reaction

(brown-yellow). The ^{1}H NMR and ^{13}C NMR spectral patterns of **3b** were very similar to those of **2a** except for the olefinic region and the appearance of an *O*-methyl signal (δ55.2). In the ^{13}C NMR spectrum of **3b**, four olefinic carbon signals were observed at δ129.3 (*s*, C-25), 132.7 (*s*, C-9), 139.1 (*d*, C-24) and 142.9 (*s*, C-8). On the other hand, six olefinic carbon signals were observed at 115.5 (*d*, C-11), 121.6 (*d*, C-7), 129.4 (*s*, C-25), 139.2 (*d*, C-24), 140.2 (*s*, C-8) and 146.2 (*s*, C-9) in the spectrum of **2a**. The loss of two olefinic carbon signals in the spectrum of **3b** relative to that of **2a** indicated that the diene moiety of **2a** had been replaced by a single double bond. This was supported by the ^{1}H NMR spectrum, which showed only one olefinic proton signal at δ6.78 (24-H). These data indicated that **3b** was 7,11-dihydroganoderic acid T but with an extra methoxyl group. The position and configuration of the additional methoxyl group of **3b** were elucidated from its ^{1}H NMR and ^{1}H–^{1}H shift correlation NMR spectral data. In the ^{1}H NMR spectrum of **3b** a new signal at δ3.56, which was absent in **2a**, appeared as a broad singlet. The cross peaks between the new signal and the C-6 methylene protons (δ1.36 and 1.89) were clearly observed.

One of the methylene protons (δ1.36) coupled with three different protons (J = 13.5, 13.5, 3.2 Hz) and another methylene proton only coupled with the geminal methylene proton (J = 13.5 Hz). The arrangement from this evidence can only correspond to the H_5-$H_{6\alpha}$-H_{6b}-$H_{7\beta}$ linkages and it permitted the conclusion that the additional methoxyl group was attached at C-7 with an α-configuration. Therefore, the structure of **3b** was elucidated as 7α-methoxy-7,11-dihydroganoderic acid T, (22*S*, 24*E*)-3α,15α,22-triacetoxy-7α-methoxy-5α-lanosta-8,24-dien-26-oic acid.

In the second experiment the benzene extract of the dried cultured mycelia was separated by silica gel column chromatography (see Experimental). After repeating the HPLC, compound **3a**, ganoderic acid O, was obtained as colourless needles, mp 156–158°. The molecular formula was established as $C_{36}H_{54}O_9$ by elementary analysis. The UV spectrum of **3a** showed only the presence of an α,β-unsaturated carbonyl group [206 nm(log ε4.1)]. Compound **3a** exhibited hydroxyl (3420 cm^{-1}) and ester carbonyl (1720 cm^{-1}) absorptions in its IR spectrum. The ^{1}H NMR spectrum of **3a** was very similar to that of **3b** except for the loss of the methoxy methyl proton signal at δ3.16. ^{13}C NMR spectrum of **3a** was also closely similar to that of **3b** except for the loss of the methoxy methyl carbon signal at δ55.2 and the up-field shift (δ67.1) of the methoxy methine carbon signal in the spectrum of **3b**. These data suggested that **3a** had a hydroxyl group instead of a methoxyl group attached to C-7. In order to verify the proposed structure, a mixture of **3a** and methanol was left at room temperature for 7 days. Compound **3b** was detected by HPLC (R_t 16.1 min. Nucleosil C18 85% CH_3CN 3 ml/min.) in the mixture. From these results, the structure of compound **3a**, named ganoderic acid O, was established as (22*S*, 24*E*)-3α,15α,22-triacetoxy-7α-hydroxy-5α-lanosta-7,24-dien-26-oic acid. The fact that **3b**, containing a methoxyl group at C-7, was obtained by methanol extraction and was not present in the benzene extract suggests that it is an artifact.

The six new ganoderic acid derivatives identified as major or minor triterpenoid components from the cultured mycelia of *G. lucidum* have 3α-substituents. In contrast, the ganoderic acid derivatives from the fruit bodies of *G. lucidum* are 3β-substituted or 3-keto compounds. In plant tissue cultures of *Isodon japonicus*, a 3α-hydroxytriterpenoid, 3-epimaslinic acid, is biosynthesized from its 3β-isomer maslinic acid via a 3-oxo-compound [26]. On the other hand, Rohmer *et al.* [27, 28] reported that in a cell free system from the bacteria *Acetobacter pasteurianum* and *Methylococus capsulantus*, 3α- and 3β-hydroxytriterpenoids were separately formed from (3*R*)- and (3*S*)-2,3-oxidosqualenes, respectively, without involvement of 3-oxo-compounds. The difference in the C-3 substituents between ganoderic acid derivatives isolated from the cultured mycelia and those from the fruiting bodies of *G. lucidum* biosynthetically interesting. We recently observed, using [1,2-$^{13}C_2$]actate, that the incorporation pattern of intact bonds of enriched acetate units suggest ganoderic acid T to be biosynthesized from (3*S*)-oxidosqualene via a 3-oxo-compound by a process similar to that of in higher plant.

EXPERIMENTAL

Mps were uncorr. Mass spectra were run on a direct insertion probe. NMR spectra were taken in $CDCl_3$ at 23°; ^{13}C NMR at 75.0 and 100.6 MHz and ^{1}NMR at 300 and 400 MHz. HPLC of ganoderic acid derivatives was performed using a Nucleosil C18(10 × 300 mm) column, coupled to a UV detector and a differential refractometer.

Isolation and culture conditions of Ganoderma lucidum. After sterilizing the cultivated fruiting body of *G. lucidum*, a small piece was inoculated onto a malt agar medium. Developing colonies of the new hyphae from the fruiting body were transferred to malt agar in Petri dishes. The formation of the clamp connection of the pure cultured hyphae was observed under a microscope. The pure mycelium was subcultured for 2 weeks and grown in Roux flasks in 200 ml of medium containing 15 g glucose, 1.0 g soytone, 0.5 g yeast extract, 0.5 g KH_2PO_4, 0.1 g NaCl, 0.05 g $MgSO_4.7H_2O$ and 0.11 g $CaCl_2.2H_2O$ in 1 l of distilled H_2O. The pH of the medium was adjusted to 5.5 with 1 N HCl. Usually, each flask was seeded with 5 of the 10 mm plugs cut from the malt agar culture. The culture was maintained in the dark at 25°.

Extraction procedure and separation of MeOH *extract of the mycelia.* After 6 weeks culture (100 Roux flasks), the mycelia (5.32 kg, fr. wt) were harvested with Nylon cloth, homogenized with MeOH(10 l) in a Waring blender and allowed to stand for 1 week at room temp. The homogenate was filtered and the residue was re-extracted with the same solvent (10 l). The filtrates were combined and organic solvent was removed under red. pres. The residue was extracted with $CHCl_3$(1000 ml × 2), dried and evapd to dryness. The $CHCl_3$, extract(16.78 g) was subjected to chromatography over silica gel (800 g Wako gel C-200). Elution with 2 l C_6H_6 and 2 l C_6H_6–EtOAc (9:1) (fraction A), 1 l (fraction B), 4.5 l C_6H_6–EtOAc (8:2) (fraction C), 0.5 l C_6H_6–EtOAc (8:2) and 1 l C_6H_6–EtOAc (6:4) (fraction D) 2 l C_6H_6–EtOAc (6:4) and 2 l EtOAc (fraction E) yielded the crude mixture of compound 1 (fraction B 0.314 g), compounds **2a**, **2b**, **2c** (fraction C 9.332 g) and compounds **2d**, **2e**, **3b** (fraction E 4.35 g).

*Isolation and structure elucidation of ergosta-*4,7,22-*triene*-3,6-*dione* (1). After repeated HPLC (Nucleosil C18, MeOH 3.0 ml/min flow rate, R_t 12.2 min) of fraction **B**, compound 1 (27.7 mg) was obtained as pale yellow leaflets, mp 156–159° (decomp), $C_{28}H_{40}O_2$ (required 408.3028, $[M]^+$ at m/z 408.3034); IR ν_{max}^{KBr} cm^{-1}: 2950, 2870 (CH), 1665(CO), 1640(CO), 1620(C=C), 1600 (C=C); UV λ_{max}^{EtOH} nm (log ε): 275 (3.8). ^{1}H NMR: 0.68 (3H, *s*), 0.82(3H, *d*, J = 6.6 Hz), 0.84(3H, *d*, J = 6.6 Hz), 0.92 (3H, *d*, J = 6.6 Hz), 1.04 (3H, *d*, J = 6.6 Hz), 1.30 (3H, *s*), 5.14 (1H, *dd*, J = 15.0, 6.7 Hz), 5.24 (1 H, *dd*, J = 15.0, 7.5), 5.98 (1 H, *t*, J

= 1.8), 6.47 (1H, *s*). ^{13}C NMR 75.2 MHz ($CDCl_3$): 12.8(3), 17.6(3), 19.5(3), 19.7 (3), 20.0(3), 21.1(3), 21.9(2), 22.6(2), 27.7(2), 33.1(1), 34.3(2), 35.4(2), 38.5(2), 39.1(0), 40.2(1), 42.9(1), 44.6(0), 47.1(1), 56.1(1), 56.3(1), 123.9(1), 126.0(1), 132.8(1), 134.8(1), 158.2(0), 167.7(0), 187.0(0), 199.3(0). EIMS (direct inlet) 20 eV, *m/z* (rel. int.): 408[M]$^+$ (80), 283[M–side chain]$^+$ (100), 229[M–$C_{13}H_{23}$]$^+$ (33), 125[M]$^+$ (13).

Isolation and elucidation of ganoderic acid T(**2a**), *ganoderic acid S*(**2b**) *and ganoderic acid R*(**2c**). Further separation of fraction C was achieved by HPLC. A small part of fraction C(1.32 g) was run on a Nucleosil C18 packed column(10 mm × 300 mm) eluted with 95% MeOH (3 ml/min). Compound **2a** was isolated from the fraction containing the peak at 16.2 min. Compound **2a** (175.4 mg), colourless needles, mp 200–202°, $C_{36}H_{52}O_8$ (required 612.3662, [M]$^+$ at *m/z* 612.3666), $[\alpha]_D^{24}$ +23° ($CHCl_3$, *c* 0.13); IR ν_{max}^{KBr} cm^{-1}: 3420(OH), 2940(CH), 1720(COO), 1700(COO), 1240(C-O); UV λ_{max}^{EtOH} nm (log ε): 214(4.0), 225(4.0), 234(4.0), 242(4.0), 250(3.8). ^{1}H NMR and ^{13}C NMR: see Tables 2 and 3. EIMS(direct inlet) 20 eV, *m/z* (rel. int.): 612[M]$^+$ (100), 552[M – HOAc]$^+$ (20), 537[M – HOAc – Me]$^+$ (15), 492[M – 2 × HOAc]$^+$ (18), 477[M – 2 × HOAc – Me]$^+$ (26), 417[M – 3 × HOAc – Me]$^+$ (42), 293[M – side chain – HOAc – OAc]$^+$ (22), 277[M – side chain – 2 × HOAc – Me]$^+$ (20), 251(20), 239(46), 223(18), 197(16), 132(16). Compound **2b** was also isolated from the fraction containing the peak at 19.1 min. compound **2b**(33.5 mg), colourless needles, mp 194–196°, $C_{32}H_{48}O_5$ (required 512.3502, [M]$^+$ at *m/z* 512.3511), $[\alpha]_D^{24°}$ +19.8°($CHCl_3$, *c* 0.085); IR ν_{max}^{KBr} cm^{-1}: 3410(OH), 2870(CH), 1720(COO), 1675(COO), 1230(C–O); UV λ_{max}^{EtOH} (log ε): 214(4.3), 231(4.2), 240(4.2), 248(4.0). ^{1}H NMR and ^{13}C NMR: see Tables 2 and 3. EIMS(direct inlet) 20 eV, *m/z*(rel. int.): 512[M]$^+$ (100), 479(14), 452[M – HOAc]$^+$ (14), 419(17), 312[M – side chain]$^+$ (30), 311(27), 271(34), 253(31), 171(20), 159(27), 133(16), 119(14), 107(16). Compound **2c** was isolated from the last fraction containing the peak at 26.6 min. Compound **2c** (22.6 mg), colourless needles, mp 201–202°, $C_{34}H_{50}O_6$ (required 554.3606, [M]$^+$ at *m/z* 554.3595), $[\alpha]_D^{24°}$ +8.7($CHCl_3$, *c* 0.092), IR ν_{max}^{KBr} cm^{-1}: 3400(OH), 2870(CH), 1720(COO), 1675(COO), 1230(C – O); UV λ_{max}^{EtOH} nm(log ε): 224(4.0), 234(4.0), 242(4.0), 250(3.8). ^{1}H NMR and ^{13}C NMR: see Tables 2 and 3. EIMS(direct inlet) 20 eV, *m/z*(rel. int.): 554[M]$^+$(100), 494[M – HOAc]$^+$ (25), 479[M – HOAc – Me]$^+$ (28), 419[M – 2 × HOAc – Me]$^+$ (30), 354[M – side chain]$^+$ (24), 313[$C_{21}H_{29}O_2$]$^+$ (21), 295(17), 253(53), 239(23), 159(20), 145(16), 133(11), 107(14).

Isolation and elucidation of ganoderic acid Q(**2d**), *ganoderic acid P*(**2e**) *and 7-O-methyl ganoderic acid O*(**3b**). Further separation of Fraction E was achieved by repeated HPLC(Nucleosil C18, solvent 85% CH_3CN, flow rate 3.0 ml/min.) and compounds **2d**, **2e** and **3b** were isolated from the fraction *b* (21.3 mg) containing the peak at 15.9 min, the fraction *a* (8.9 mg) containing the peak at 15.0 min and the fraction *c*(277.9 mg) containing the peak at 17.6 min respectively. Compound **2d**, mp 131–132°, $C_{34}H_{50}O_7$(required 570.3555, [M]$^+$ at *m/z* 570.3568); IR ν_{max}^{KBr} cm^{-1}: 3450(OH), 2950(CH), 1740(COO), 1690(CO), 1250; UV λ_{max}^{EtOH} nm (log ε): 216(4.3), 225(4.2), 235(4.3), 243(4.3), 251(4.1), ^{1}H NMR and ^{13}NMR: see Tables 2 and 3. EIMS(direct inlet) 20 eV, *m/z* (rel. int.): 570[M]$^+$ (100), 510 [M – HOAc]$^+$ (35), 495[M – HOAc-Me]$^+$ (44), 435[M – HOAc × Me]$^+$ (19), 417[M – HOAc × 2-H_2O – Me]$^+$ (25). Compound **2e**, mp 211–212.5, $C_{34}H_{50}O_7$(required 570.3555, [M]$^+$ at *m/z* 570.3552), IR ν_{max}^{KBr} cm^{-1}: 3450(OH), 2950(CH), 1735(COO), 1720(COO), 1250(C – O), UV λ_{max}^{EtOH} nm (log ε): 214(4.3), 225(4.2), 235(4.2), 243(4.3), 251(4.1). ^{1}H NMR and ^{13}C NMR: see Tables 2 and 3. EIMS(direct inlet) 20 eV, *m/z* (rel. int.): 570[M]$^+$(100), 510 [M – HOAc]$^+$ (23), 495[M – HOAc – Me]$^+$ (39), 435[M – 2 × HOAc – Me]$^+$(12), 417[M – 2 × HOAc – H_2O – Me]$^+$(12). Compound **3b**, mp 228–229.5°, $C_{37}H_{56}O_9$(required 644.3924, [M]$^+$ at *m/z* 644.3895); IR ν_{max}^{KBr} cm^{-1} 3450(OH), 2970, 2950(CH), 1730(COO), 1720(COO), 1250(C – O); UV λ_{max}^{EtOH} nm(log ε): 208(4.1), ^{1}H NMR and ^{13}C NMR: see Tables 2 and 3, EIMS(direct inlet) 20 eV, *m/z* (rel. int.): 644[M]$^+$(3), 612 [M – HOMe]$^+$(17), 584[M – HOAc]$^+$(79), 537[M – HOAc – Me]$^+$ (17), 419[M – 3 × HOAc – HCHO – Me]$^+$(100).

Synthesis of compound **2f**. After methylation of compound **2a** (9.8 mg) with CH_2N_2, methylated compound **2a** was reacted with $LiAlH_4$ in 1.5 ml THF for 5 min at room temp. The reaction was stopped with 5 drops of 10% NH_4Cl. After removing of THF, the residue was partitioned with H_2O and $CHCl_3$. The $CHCl_3$ fraction was recrystallized from H_2O–MeOH to give colourless plates (1.78 mg), mp 201–202°, $C_{30}H_{48}O_4$(required 472.3552, [M]$^+$ at *m/z* 472.3545); CD curve $[\theta]_{239}$ +12689 EtOH; *c* = 0.012). ^{1}H NMR (400 MHz, $CDCl_3$): δ0.63 (3H, *s*), 0.89(3H, *d*, *J* = 6.5 Hz), 0.98(6H, *s*), 1.00(3H, *s*), 1.40(1H, *m*), 1.51(1H, *dd*, *J* = 10.5, 5.0 Hz), 1.69(3H, *s*), 3.45(1H, *br s*), 3.66(1H, *brdd*, *J* = 9.0, 5.0 Hz), 4.04(2H, *s*), 4.28(1H, *br t*, *J* = 7 Hz), 5.34(1H, *brd*, *J* = 6.2 Hz), 5.46(1H, *brt*, *J* = 7 Hz), 5.85(1H, *brd*, *J* = 6 Hz). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ11.8(3), 14.0(3), 15.9(3), 17.4(3), 22.7(3), 22.8(3), 22.9(2), 25.6(2), 28.2(3), 29.9(2), 33.9(2), 37.3(0), 37.4(0), 38.6(2), 39.5(2), 40.5(1), 43.1(1), 44.3(0), 45.3(1), 52.1(0), 68.7(2), 73.3(1), 74.8(1), 76.1(1), 115.7(1), 121.4(1), 122.1(1), 137.6(0), 140.8(0), 146.2(0).

Extraction procedure and separation of the C_6H_6 *extract*. After 42 days culture (97 Roux flasks), the mycelia(fr. wt 5.64 kg) were lyophilized and the dried mycelia(147 g) were extracted with ×2 C_6H_6(total 12 l) at room temp. After filtration, the C_6H_6 extract (10.5 g) was obtained. A part (6.1 g) of the benzene extract was subjected to chromatography over silica gel(500 g Wako gel C-200) and eluted as follows: fraction 1, 11 C_6H_6 – Me_2CO (9:1) and 2.1 l C_6H_6–Me_2CO (1:3); fraction 2, 1.9 l C_6H_6–Me_2CO 0.2 l C_6H_6–Me_2CO (8:2); fraction 3, 0.6 l C_6H_6–Me_2CO (8:2); fraction 4, 1.55 l C_6H_6–Me_2CO (8:2); fraction 5, 3.05 l C_6H_6–Me_2CO(8:2) and 1 l C_6H_6–Me_2CO (50:50). Fractions 1 and 2 contained the mixture of compounds **2a**, **2b**, **2c**(2.07 g) and compound **3a**(1.65 g), respectively.

Isolation and structure elucidation of compound **3a**. Further purification of fraction 2 was achieved by HPLC. A part(951.5 mg) of fraction 2 was run on Nucleosil C18 packed column(10 × 300 mm) eluted with CH_3CN–H_2O 17:3 3 ml/min). Compound **3a** was obtained from the fraction (214.0 mg) containing the peak at 12.7 min. but it was still impure and further purification was carried out with different HPLC condition(Nucleosil C18, solvent CH_3CN–H_2O (3:1 3.0 ml/min.) The pure compound **3a** (128.6 mg) colourless needles, had mp 156–158° (Found: C, 68.45; H, 8.68, $C_{36}H_{54}O_9$ requires: C, 68.54; H, 8.63). IR ν_{max}^{KBr} cm^{-1}: 3450(OH), 2980(CH), 1735(COO), 1260(C–O); UV λ_{max}^{EtOH} nm (log ε): 206(4.1). ^{1}H NMR and ^{13}C NMR: see Tables 2 and 3. EIMS(direct inlet) 20 eV, *m/z* (rel. int.): 612[M – H_2O]$^+$ (17), 570[M – HOAc]$^+$(36), 537 [M – HOAc – H_2O – Me]$^+$ (31), 495[M – HOAc × 2 – Me]$^+$(100), 435[M – HOAc × 3 – Me]$^+$ (10), 417 – M – HOAc × 3 – Me – H_2O]$^+$ (12), 239 (18), 171(9).

X-ray data of compound **2a**. Atomic coordinates have been deposited at the Cambridge Crystallographic Data Centre.

The conditions for the NMR measurements. The homo and hetero nuclear two-dimensional chemical shift correlation diagram was obtained according to the method described in a previous paper [4].

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