Investigation of the Biosynthesis of 3α-Hydroxy Triterpenoids, Ganoderic Acids T and S, by Application of a Feeding Experiment using [1,2-$^{13}C_2$]Acetate[1]

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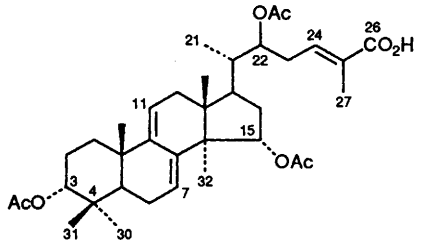
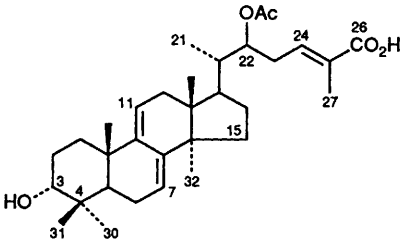
The biosynthesis of 3α-acetoxy and 3α-hydroxytriterpenoids, ganoderic acids T and S, in cultured mycelia of *Ganoderma lucidum* fed with [1,2-$^{13}C_2$]acetate, was investigated by ^{13}C NMR spectroscopy. The ^{13}C-labelling patterns of ganoderic acids T and S indicate that (3*S*)-squalene 2,3-epoxide is a precursor of 3α-hydroxy triterpenoids in higher fungi as well as of those in higher plants.

The fungus *Ganoderma lucidum* (Polyporaceae) has many pharmaceutical properties and has long been used as a home remedy for the alleviation of minor disorders, and to promote vitality and longevity. Recently, many lanostane-type triterpenoids have been isolated from both the fungus body and the cultured mycelia of *G. lucidum*.[2–19] Some of them are known to inhibit both histamine release in rat mast cells.[4] and angiotensin-converting enzyme,[12] and to have cytotoxic activity against hepatoma cells *in vitro*.[16,17] Ganoderic acid T, isolated as a major component from the cultured mycelia of *G. lucidum*, has a 3α-substituent.[18,19] In contrast, ganoderic acid derivatives from the fruit-bodies (the pileus and the stipe) of *G. lucidum* were 3β-substituted or 3-keto compounds.[5,10] However, ganoderic acid T was found in the underground part of the fruit-bodies, and characterized.[20] The difference in the C-3 substituents between ganoderic acid derivatives isolated from the cultured mycelia and the underground part of the fruit-bodies, and those from the fruiting bodies (the pileus stipe), greatly interested us with regard to the biosynthetic pathway of the triterpenoids of *G. lucidum*. In plant tissue cultures of *Isodon japonicus*, a 3α-hydroxy triterpenoid, 3-epimaslinic acid, is biosynthesized from its 3β-isomer *via* a 3-oxo compound derived from (*S*)-squalene 2,3-epoxide.[21] On the other hand, Rhomer *et al.*[22,23] reported that, in a cell-free system from the bacteria *Acetobacter pasteurianum* and *Methylococcus capsulatus*, 3α- and 3β-hydroxy triterpenoids were separately formed from (3*R*)- and (3*S*)-squalene 2,3-epoxides without the involvement of 3-oxo compounds. In order to clarify the biosynthetic origin of the 3α-hydroxy group of ganoderic acids T (**1**), and S (**2**) which were produced in the higher fungus *G. lucidum*, the pattern of incorporation of intact acetate units was investigated with [1,2-$^{13}C_2$]acetate.

In this paper, we report the ^{13}C-labelling patterns of compounds (**1**) and (**2**), which indicate that (3*S*)-squalene 2,3-epoxide is a precursor of 3α-hydroxy triterpenoids in higher fungi as well as of that of higher plants. To the best of our knowledge, this is the first study of the origin of 3α-hydroxy triterpenoids in higher fungi.



ganoderic acid T (**1**)



ganoderic acid S (**2**)

Results and Discussion

First, it was necessary to confirm the active time for the production of ganoderic acid derivatives in the cultured mycelia of *G. lucidum*. From an initial time–course experiment (Figure 1), the most active time for the production of ganoderic acid derivatives was shown to be after 2–3 weeks of culture.

After both 2 and 3 weeks of culture, a solution of sodium [1,2-$^{13}C_2$]acetate (67 mg) (1/4 dilution) in water (1 ml) was administered to each flask and the cultured mycelia were harvested after one more week of culture. The mycelia were extracted and the enriched acids (**1**) (92 mg) and (**2**) (27.7 mg) were isolated by use of silica gel and reversed-phase high-performance liquid chromatography (HPLC) according to the method outlined in a previous paper.[19] A full assignment of the natural-abundance ^{13}C NMR spectrum of compounds (**1**) and (**2**) was required as a prelude to the application of ^{13}C-labelling studies to the biosynthesis. The ^{13}C NMR assignments of

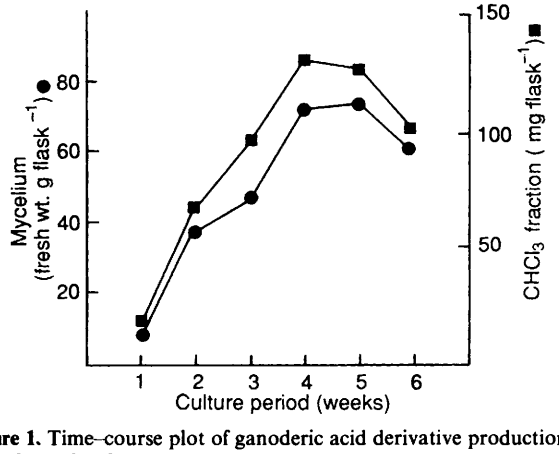
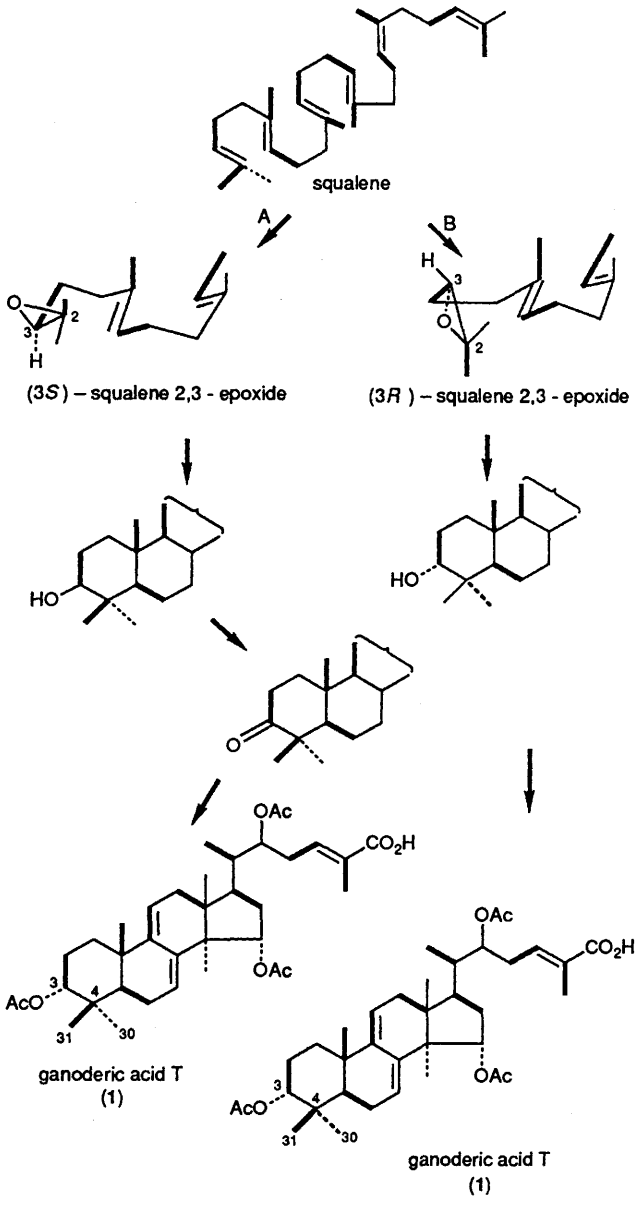


Figure 1. Time–course plot of ganoderic acid derivative production by *Ganoderma lucidum*.

Table 1. ^{1}H NOE difference spectral data for compound (1).

Irradiated proton (δ_H)	Enhanced proton (δ_H)
0.66 (18-H_3)	5.08 (5.1%, 15-H^β)
0.88 (30-H_3)	4.68 (5.5%, 3-H^β), 1.49 (17.5%, 5-H)
0.98 (31-H_3)	4.68 (5.5%, 3-H^β), 1.49 (5.0%, 5-H)
0.99 (19-H_3)	4.68 (3.8%, 3-H^β), 1.53 (5.0%, 20-H)
1.03 (32-H_3)	5.08 (1.7%, 15-H^β)



Scheme. Possible incorporation patterns of intact ^{13}C–^{13}C bonds derived from [1,2-$^{13}C_2$]acetate into ganoderic acid T (**1**) *via* (3*S*)- and (3*R*)-squalene 2,3-epoxides.

ganoderic acids T and S were made by comparison with the corresponding chemical-shift values observed for ganoderic acid X and methylganoderate X,[17] by consideration of the empirical substituent effect reported in previous papers.[5,10] However, there remained some uncertainly as to the C-30 and C-31 carbon-signal assignments. Since to distinguish the C-31 signal from the other methyl-carbon signals, especially from the C-30 signal, in the ^{13}C NMR spectrum was extremely important in our study, we provided direct evidence for the assignment of the C-31 carbon signal as follows: the C-31 methyl-proton signal of compound (**1**) was unambiguously assigned to the signal at δ 0.98 by means of nuclear Overhauser difference (NOE) spectroscopy. The NOE results are summarized in Table 1. A differential NOE spectrum obtained upon irradiation at the position of the methyl resonance (δ_H 0.88) showed significant enhancement of the 3-H^β (δ_H 4.68; 5.5%) and 5-H (δ_H 1.49; 17.5%) signals. Also, irradiation at the position of a second methyl resonance (δ_H 0.98) produced significant enhancement of the 3-H^β signal and enhancement of that of 5-H, as shown in Table 1, but to a much smaller extent than from irradiation at δ_H 0.88. These NOE experimental results show equal enhancements of 3-H^β but not of 5-H by irradiation at both C-30 and C-31. The signals at δ_H 0.88 and 0.98 were thus unambiguously assigned to those of the C-30 and C-31 methyl-proton resonances, respectively. It was very easy to identify the cross-peaks to the C-30 and C-31 carbon-atom bands and the proton signals in the proton–carbon shift-correlation diagram, and so the methyl-carbon bands at δ_C 27.7 and 22.4 were unambiguously assigned to C-30 and C-31.

The intact acetate bond from the [1,2-$^{13}C_2$]acetate should be incorporated into both compounds (**1**) and (**2**), as is observed in other triterpenoids, depending on the biosynthetic pathway.[24–26] The expected incorporation pattern of acetate *via* mevalonic acid, squalene, and lanosterol is shown in the Scheme. Thus the pairs of carbon atoms C-31 and -4 (or -30 and -4), -2 and -3, -5 and -6, -9 and -11, -10 and -19, -12 and -13, -16 and -17, -20 and - 21, -23 and -24, and -25 and -27 were expected to be derived from intact acetate units and to show ^{13}C–^{13}C spin couplings. The remaining carbon atoms, derived from acetate residues which have been cleaved at various stages along the biosynthetic pathway, should appear as uncoupled resonances.

The ^{13}C NMR data and the labelling patterns for compounds (**1**) and (**2**) are shown in the Table 2. As shown in Table 2, some of the expected ^{13}C–^{13}C spin couplings were not clearly observed, especially in the resonances of C-31, C-19, and C-6 of ganoderic acid T (**1**), since these signals at δ_C 22.4, 22.6, and 22.8 [for (**1**)] are unfortunately very close to each other. Thus, we decided to use the two-dimensional INADEQUATE method instead of the proton-noise-decoupled ^{13}C NMR spectrum to analyse the ^{13}C-labelling patterns of C-31, C-19, and C-6. The definitive assignment fo the ^{13}C–^{13}C coupling of ganoderic acid T (**1**) follows from the double quantum-coherence measurements performed at 100 MHz in $CDCl_3$ solution, shown in Figure 2.

As shown in Figure 2A, the carbon–carbon connectivity plots show directly the presence of 12 pairs of ^{13}C–^{13}C couplings. In the expanded spectrum of the high-field region (δ_C 10–45; Figure 2B), three carbon signals (C-6, C-19, and C-31) couple unambiguously with the anticipated carbon bands (C-5, C-10, and C-4). On the other hand, the connectivity plot for the C-30 carbon band (δ_C 27.7) is not observed in any coherence region. These results indicated that the intact acetate bond of [1,2-$^{13}C_2$]acetate was incorporated into the C-31–C-4 bond, but not into the C-30–C-4 bond, in ganoderic acid T. The results confirm that the biosynthetic pathway to ganoderic acid T (**1**) is route A in the Scheme. If compound (**1**) were to be biosynthesized *via* (3*R*)-squalene 2,3-epoxide, one of the ^{13}C–^{13}C couplings of ^{13}C-enriched acid (**1**) should correspond to the C-30–C-4.

In conclusion, 3α-acetoxy and 3α-hydroxy triterpenoids in fungi, such as ganoderic acids T and S, were shown to be biosynthesized *via* (3*S*)-squalene 2,3-epoxide as a precursor, a situation similar to that in higher plants.

Table 2. ^{13}C Chemical shifts and J_{CC} coupling constants observed for [1,2-$^{13}C_2$]acetate-enriched ganoderic acids T and S.

	Ganoderic acid T (1)		Ganoderic acid S (2)			Ganoderic acid T (1)		Ganoderic acid S (2)	
Carbon	Chemical shift	J_{CC}/Hz	Chemical shift	J_{CC}/Hz	Carbon	Chemical shift	J_{CC}/Hz	Chemical shift	J_{CC}/Hz
1	30.5	s	29.9	s	19	22.6		22.6	35
2	23.1	35	25.6		20	39.6	35	39.4	34
3	78.4	35	76.2	37	21	12.7	35	12.7	34
4	36.5	36	37.2	36	22	74.4	s	74.7	s
5	43.8	35	47.4	33	23	31.8	43	31.8	42
6	22.8		27.6	33	24	138.9	43	139.5	42
7	121.3	s	120.4	s	25	129.4	45	129.2	46
8	139.9	s	142.3	s	26	172.2	s	172.4	s
9	145.9	72	146.0	72	27	12.3	45	12.2	46
10	37.3	34	37.4	35	30	27.7	s	28.2	s
11	115.3	72	115.6	72	31	22.4		22.6	35
12	37.9	36	37.8	36	32	18.4	s	25.7	s
13	43.9	35	43.7	36	CO*Me*	21.0		21.0	60
14	51.4	s	50.4	s	CO*Me*	21.3			
15	76.9	s	31.4	s	CO*Me*	21.4			
16	36.6	34	23.0	32	C=O	170.6		170.7	60
17	45.4	34	43.2	35	C=O	170.9			
18	15.7	s	15.5	s	C=O	171.1			

s, Single label. Signals not reported are because one or two of the doublet signals overlapped with other signals.

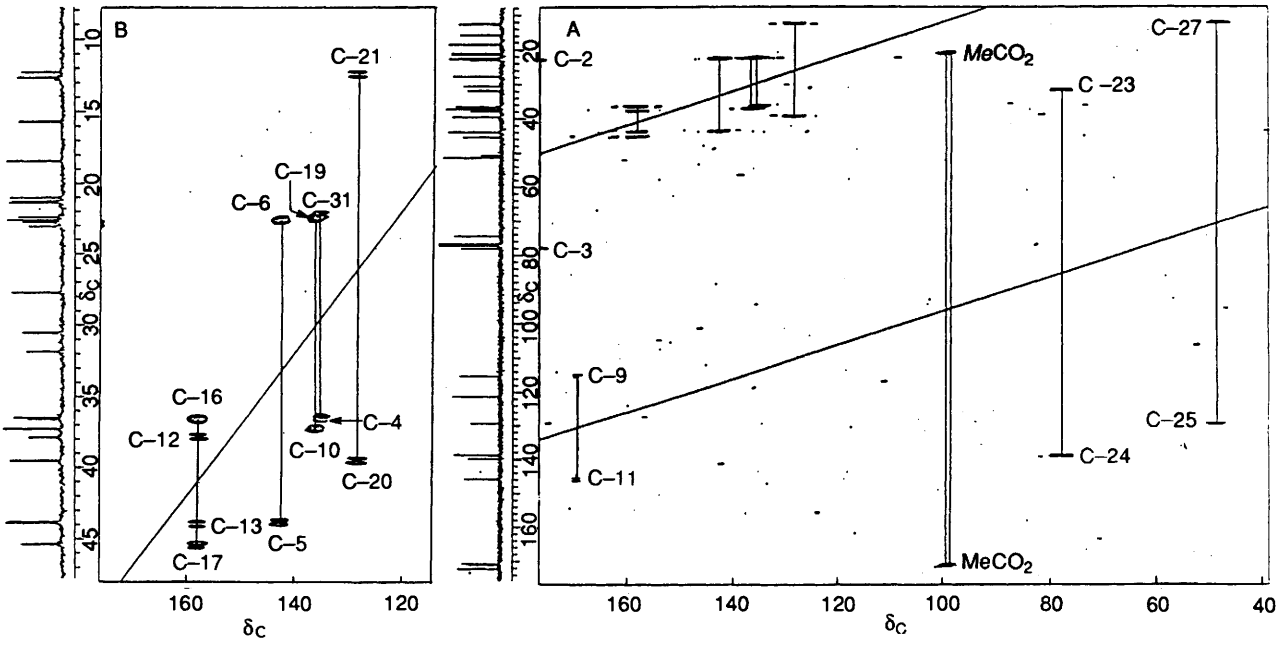


Figure 2. Carbon–carbon connectivity 2D plot for [1,2-$^{13}C_2$]acetate-enriched ganoderic acid T (1).

Experimental

M.p.s were determined on a Kofler hot-stage apparatus and are uncorrected. High-resolution MS was run on a direct-insertion probe with a JEOL JMS DX-300 spectrometer. ^1H NMR spectra were recorded on a Varian XL 400 spectrometer (400 MHz), and ^{13}C NMR spectra were recorded on the Varian XL 400 spectrometer (100.6 MHz). Sodium [1,2-$^{13}C_2$]acetate (90 atom%) was purchased from MSD (Canada).

Culture Conditions.—*G. lucidum* was grown on malt agar slopes and was transferred to malt agar in Petri dishes as a pre-culture. For the production of ganoderic acid derivatives, the medium was prepared as follows: glucose (45 g), soytone (3.0 g), yeast extract (1.5 g), KH_2PO_4 (1.5 g), NaCl (0.3 g), $CaCl_2 \cdot 2H_2O$ (0.33 g), $MgSO_4 \cdot 7H_2O$ (0.15 g), and distilled water (3 l). This medium was divided among fifteen 1-l Roux flasks and autoclaved at 121 °C for 20 min. The flasks were inoculated with 7 of 10-mm plugs cut from the malt agar pre-culture and were then maintained in an incubator at 25 °C in the dark. The time-course experiment was performed by harvesting the mycelia from three Roux flasks each week, and the amounts of the mycelia and the chloroform extract of the mycelia were measured as reported in a previous paper.[19]

Administration of Sodium [1,2-$^{13}C_2$]*Acetate and Extraction and Separation Procedures.*—After both 2 and 3 weeks of surface culture of *G. lucidum*, a solution of sodium [1,2-$^{13}C_2$]acetate (4-times dilution with sodium acetate) (67 mg) in water (1 ml) was administered to every flask under the sterile conditions and the cultured mycelia were harvested after a further week of culture. The mycelia (1.26 kg fresh wt) were homogenized with MeOH (2.5 l) in a Waring blender and the mixture was kept for 4 days

at room temperature. The homogenate was filtered and the residue was re-extracted with the same solvent (2.5 l). The filtrates were combined and MeOH was removed under reduced pressure. The residue was treated with water (500 ml) and extracted with $CHCl_3$ (1 l × 5); the extract was dried (Na_2SO_4), and evaporated to dryness. The $CHCl_3$ extract residue (1.645 g) was subjected to chromatography over silica gel (Wako-gel C-200) (300 g). Elution with benzene (0.5 l), 10% AcOEt in benzene (1 l), 20% AcOEt in benzene (0.7 l) (Fraction A), 20% AcOEt in benzene (1.3 l) (Fraction B), 30% AcOEt in benzene (1 l), and AcOEt (1 l) (Fraction C) yielded a crude mixture of acids (**1**) and (**2**) (from fraction B; 561.7 mg).

Isolation of Enriched Compounds (**1**) *and* (**2**).—Further separation and purification of compounds (**1**) and (**2**) was achieved by HPLC. Fraction B (561.7 mg) was run on a Nucleosil C_{18} packed column (10 × 300 mm) eluted with 95% MeOH (3 ml min^{-1}). Ganoderic acid T (**1**) was isolated from the fractions containing the peak eluted at 16.2 min. Compound (**1**) (92.9 mg) was obtained as needles, m.p. 210–212 °C (lit.,[19] 200–202 °C; Büchi apparatus) (Found: M^+, 612.3679. Calc. for $C_{36}H_{52}O_8$: M, 612.3662). Compound (**2**) was isolated from the peak eluted at 19.1 min, and was obtained as needles (27.7 mg), m.p. 206–208 °C (lit.,[19] 194–196 °C; Büchi apparatus) (Found: M^+, 512.3498. Calc. for $C_{32}H_{48}O_5$: M, 512.3500).

Conditions for NMR Spectral Measurements.—The heteronuclear, two-dimensional, ^1H–^{13}C chemical-shift correlation diagram was obtained using a 5-mm sample, spectral widths of 13 927.6 Hz (^{13}C, F_2) and +1 380 Hz (^1H, F_1), and a data acquisition of 192 scans, with 48 increments in t_1 to provide, after zero-filling, a matrix of 2 048 × 512 (t_2, t_1) which was transformed into (F_2, t_1) and then into (F_2, F_1) using shifted sine-bell functions for weighting in both dimensions. This provided digital resolution of 6.8 and 1.35 Hz per point in the F_2 and F_1 domain, respectively. The refocusing delay was 6.3 ms, the relaxation delay 1 s. For the carbon–carbon connectivity plots of ganoderic acid T (**1**) the pulse sequence CC2DQ was used under the following conditions: number of accumulations 1 000; J_{CC} 40 Hz; number of increments 64; delay time D_1 2.5 s; total acquisition time 60 h.

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