Chemical Structures and Changes of Extracts during Growth of Reishi *(Ganoderma lucidum)*[*1]

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Compounds from the mushroom reishi *(Ganoderma lucidum* (Leys.)) were examined for chemical structure and over time. So were the extracts during the growth of fruiting bodies. Isolated compounds were identified as ganoderic acid A (I). triterpene alcohols (II) and (III), triterpenealdehyde (IV), possibly related to methylester of ganoderic acid Y(V), by carbon-13 nuclear magnetic resonance and other spectra. These triterpenes increased after the appearance of fruiting bodies, although they were not found in mycelia. Ergosterol and fatty acids were found in both mycelia and fruiting bodies but did not increase during growth of the latter.

Triterpenes were found more in the outer section than in the inner section of fruiting bodies. This infers that aging of a fruiting body increases its triterpenes because the outer section is older than the inner section.

1. INTRODUCTION

Reishi, *Ganoderma lucidum* (lays.) (Polyporaceae), grows naturally in Japan and China, and often is called mannentake. Reishi has been cultivated artificially on media of wood meal-rice bran and on wood blocks for the last ten years in Japan.The total annual production is reported to be 100 tons.

It also is reported that water extracts of reishi reduce blood pressure and platelet aggregation, (1,3) cholesterol in blood, (2,4) effect histamin-releasing inhibition (5) and promote antitumor activity. (6,7) The chemical structures of isolated compounds were clarified on branched glucan (8,9) and triterpenes. (10-15)

Medical effects are reported to depend on many factors, including the strains of reishi[5,16] and the sections of a fruiting body.[16] In addition it has been stated that the growth period of fruiting bodies has some effects on the medical value of reishi, and these writers have expected that the chemical constituents of a fruiting body might make some changes during its growth. In this paper, we survey the chemical constituents of extracts and examine their changes during the growth of reishi.

2. RESULT

2.1Chemical structures of compounds isolated from extracts

Fruiting bodies or sporophores of reishi were subjected to extraction with 70% aqueous methanol. The freeze-dried extract (yield, 7% of oven-dried sample) was extracted with hexane, chloroform, and ethylacetate. The chloroform extract (yield, 4% of oven-dried sample) was fractionated by silica-gel

Table 3-1. Methanol extracts from sections of a fruiting body (% of over dried weight)

Part	Section	Chloroform	
		Soluble	Insoluble
Pileus	Outer	7.55	3.63
	Inner	2.06	2.95
	Gill	2.57	4.14
Stipe	Outer	8.27	4.64
	Inner	1.72	3.60

3. EXPERIMENT

Six strains of commercial reishi were investigated for growth, and a strain from Nakano City, Nagano Prefecture, was selected as the best one for growing tests in this research. The fruiting bodies of this strain also were used for chemical analysis. Chemical analysis was made by ‘^{3}C-NMR, JEOL JNM-FX-lOO; high resolution MS, JEOL DX-300; UV, Shimazu UV-200; IR, Shimadzu IR-400; and ‘H-NMR, JEOL JNM-4H-l00. HPLC (high performance liquid chromatography analysis was by a Shimadzu LC3A.

3.1 Isolation and identification of compounds

One kg of air-dried fruiting bodies ~vas extracted with 8 1 of~70% aqueous methanol three times, and the extract was freeze-dried to become powder. The powder, suspended in 11 of water, was extracted successively with hexane (0.6 g), chloroform (43.9 g), and ethylacetate (6.3 g). (Yields are in parentheses.) The chloroform extract was chromatographed by silica gel with a hexane-ethylacetate-acetic acid system. Compounds A, B, C, D, and E were fractionated. Compounds A and E were identified as ganoderic acid A and

chromatography. Five compounds, A-E, were isolated.

Compound A was identified as ganoderic acid A (I), which had been isolated from this species [10,14,15] before, by comparison of carbon-l3 nuclear magnetic resonance (^{13}C-NMR) (Table 1) and mass spectra (MS) with those of an authentic sample.[t0~] Compound B had a ^{13}C-NMR pattern similar to that of methylester of ganoderic acid Y (V), which also had been isolated previously from reishi[11] (Table 1), and included a 26-alcohol structure (69.0 ppm) instead of the 26-acid structure (168.8 ppm) of (V). The structure was inferred to be 3p-hydroxy-Stx-lanost-7, 9-dien-26-ol (II), supported by high-MS, MS, ultraviolet and visible spectra. (UV), infra red spectra (IR), and proton nuclear magnetic resonance spectra (^{1}H-NMR) of the original compound and its acetate (Fig.l). ^{13}C-NMR of Compound C resembled that of Compound B. There was a 3-keto structure (216.8 ppm) in Compound C instead of the 3-alcohol structure (78.9 ppm) in Compound B. High-MS, MS, IR, and 'H-NMR supported this structure (III). Reduction of Compound C with $NaBH_4$ gave Compound

B. Oxidation of Compound C with $Mn0_2$ gave Compound D (IV) by High-MS, IR, and UV. Compound D also was found in the chloroform extract.

Twenty triterpenes already have been isolated from reishi. All were acids except for two C_{27} ketones. C_{30} ketones and C_{30} aldehyde were isolated for the first time. Residual Compound E was identified as ergosterol by UV, IR, and MS, but had been isolated previously from reishi.[5)]

2.2 Changes of extracts during growth of fruiting bodies

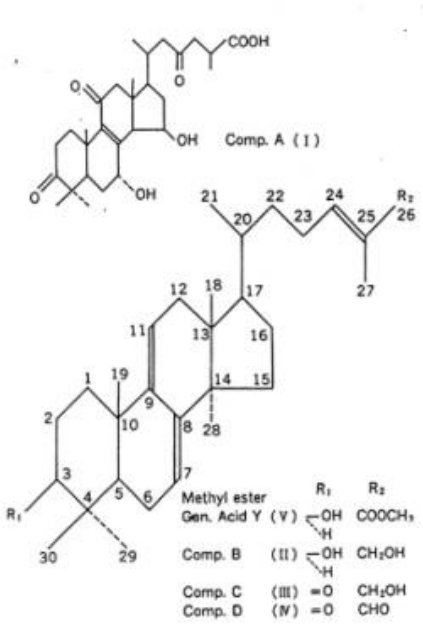

Fig. 1. Triterpenes from a fruiting body.

To obtain more information on chemical constituents, extracts from growing fruiting bodies were compared with those

ergosterol, respectively, as shown in the preceding section. Compound B: White crystals, mp l86-I8TC, High MS; Calculated for $C_{30}H_{48}O_2$: 440.3828, observed: 440.3662. MS *m/z:* 440 (M^+), 425 (M^+-CH_3), 422 (M^+-H_2O), and 407 (M^+-CH_3-H_2O). Uv? $^{MeOH}_{max}$ mm (log ?): 236 (4.12), 243 (4.17), and 252 (4.01). IR? $^{KBr}_{max}$ cm^{-1} 3370, 2930, 1450, 1375, 1040, and 990. ^{1}H-NMR (in $CDCI_3$) ?: 0.58 (5, 6H), 0.85 (S, 12H), 0.98 (S, 6H), 1.02 (S, 6H), 1.69 (S, 6H), 4.01 (S, 4H), and 5.40 **(**m**,** 6H).

Acetate of Compound B: MS *m/z:* 524 (M^+), 464 (M^+-AcOH), 449 (M^+-AcOH-CH_3), 408 (M^+-2 AcOH) and 393 (M^+-2 AcOH-CH_3). IR$_v$ $^{KBr}_{max}$$cm^{-1}$: 2960, 1740, 1495, 1375, 1260, 1040, and 990. ^{1}H-NMR (in $CDCI_3$) ?: 0.57 (S. 6H), 0.88 (S, 12H), 1.02 (S, 6H), 1.67 (S, 6H), 2.02 (S, 12H), 4.48 (S, 4H), and 5.40 **(**m, 6H).

Compound C: mp. 94-95ºC, High-MS; Calculated for $C_{30}H_{46}0_2$: 438.368, observed: 438.347. MS *m/z:* 438 (M^+), 420 (M^+-H_2O) and 405 (M^+-CH_3-H_20). IR? $^{KBr}_{max}$ cm^{-1} : 3400, 2940, 1710, 1450, 1375, and 1110. ^{1}H-NMR (in CDCJ3) ?: 0.59 (S, 6H), 0.89 (S. 6H), 1.09 (S, 6H), 1.13 (S, 6H), 1.20 (S, 6H), 1.65 (S, 6H), 3.97 (S, 4H), and 5.40 (M, 6H).

Compound D: mp. 143ºC, High-MS; Calculated for $C_{30}H_{44}0_2$: 436.352, observed: 436.334. MS *m/ z:* 436 (M^+) and 421 (M^+-CH_3). IR$_v$ $^{KBr}_{max}$ 2970, 1710, 1680, 1450, 1375, 1110, and 1000.Uv? $^{hexane}_{max}$ nm (log ?): 227 (4.15), 234 (4.22), 2.41 (4.19), and 2.51 (3.96).

Reduction of Compound C to Compound B by $NaBH_4$: Five mg of Compound C was dissolved in dioxane (1 ml) and $NaBH_4$ (29 rng in 70% aqueous EtOH, 1 ml) was added in a dioxane solution and kept for 1 hr at room temperature. Compound B was obtained after the decomposition of $NaBH_4$ with acetic acid.

Oxidation of Compound C to Compound D by activated MnO_2: $MnSO_4$ (1.2 g) was dissolved in 40% aqueous NaOH (15 ml). This solution was added slowly to a hot-water solution of $KMnO_4$ (60 ml: 9.6 g). The resulting precipitate, $Mn0_2$ was filtrated off, washed with water, dried at l2OC, and powdered. Forty-five mg of Compound C was dissolved in CH_2C1_2 (11.5 ml), added the above-mentioned activated $Mn0_2$ (46.0 mg), and vibrated for 1 hr at room temperature. This mixture was filtered, and the filtrate was evaporated to yield Compound D.

3.2Extracts from a fruiting body grown in an experimental chamber

Reishi was incubated in a sterilized medium, a *5* **X** 10 cm bottle having wood meal-rice bran (3: 1, v/v*)*, at 25ºC for 2 or 5weeks. When the mycelia prevailed in the medium,

of vegetative mycelia ("V.M.") and two other types of mycelia, one being extruded in air ("M.A.") and the other growing in a medium ("M.M."). Fruiting bodies, "M.A.", and "M.M." were sampled every five days after the appearance of the fruiting bodies, and extracts from these samples were quantified. The results are shown in Fig. 2-1. Fruiting bodies were found to have smaller amounts of extract than "M.A." and "M.M." after five days, and amounts decreased with time. On the contrary, triterpenes increased after fruiting body appearance. Results are shown in Fig. 2-2. No triterpenes were found in "V.M.", "M.A." and "M.M.". Thus triterpenes are very characteristic of fruiting bodies.

It has not been determined whether the increase of triterpenes depends on the increasing metabolism of old sections or on the accelerated metabolism of newly formed sections in a fruiting body, because the fruiting bodies analyzed contained both new and old sections as shown in Fig. 2-3. This is discussed in a later section.

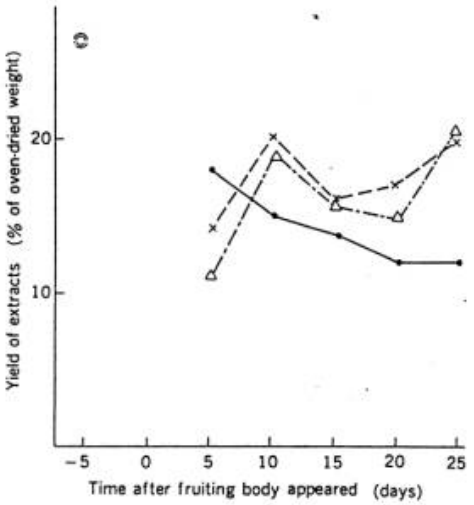

Fig. 2-1. Time course of amounts of extracts.
Legend: —●—: Fruiting bodies, —×—: "M. A.", —△—: "M.M.", ◎: "V.M.".

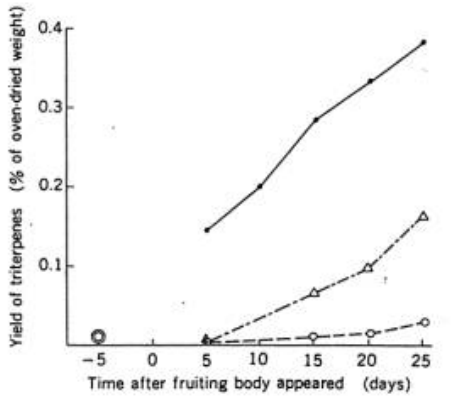

Fig. 2-2. Time course of amounts of triterpenes in fruiting bodies.
Legend: —●—: Ganoderic acid A, —△—: Compound B, —○—: Compound C, ◎: Ganoderic acid A in vegetative mycelium.

Amounts of extracts in sections of a fruiting body

vegetative mycelia ("V.M.") were collected from mycelia sticking on the inside wall of the bottle. Then the bottle was placed in a chamber at 27C. Thereafter, three samples were taken every five days, as follows: "M.A." was sampled from mycelia extending into the air over the medium. "M.M." was sampled from mycelia growing between the wall of the bottle and the medium because it was impossible for mycelia to be taken from the wood meal-rice bran medium. A fruiting body was taken from the surface of the medium in a bottle. Four samples were taken successively from separately incubated media.

Samples of fruiting bodies for investigations over time were taken every five days as a cluster of mycelia was produced on the surface of the medium and grew to become yellow on the 5th day when a new white cluster of mycelia was produced on top of the old yellow cluster (Fig. 2-3).

Each sample was extracted independently with MeOH: $CHC1_3$: H_2O (2: 1: 0.8, v/v/v). Each extract was freeze-dried, extracted with chloroform, and weighed.

Amounts of triterpenes, ergosterol, and free fatty-acids were measured by HPLC. Phenols were treated with Folin-Denis reagent.

The total fatty-acids were measured in methanolysis products of samples. That is, samples were hydrolysed by 1 N methanolic NaOH for 2 hrs at 90°C. After neutralization, the chloroform-soluble part was obtained. Fifty gram of the dried chloroform-part, containing ?-cholestan as an internal standard, was refluxed in 2.5% methanolic HCI (2 ml). The reaction mixture was fractionated by ethylacetate, and the ethylacetate layer was measured in methylester of fatty acids by gel-permeation chromatography.

3.3 Quantitative analysis of chemical constituents in sections of a fruiting body

The stipe and pileus of a fruiting body were separated. The stipe was divided into inner and outer sections, and the pileus was divided into gill and outer sections. The weights of each sections were shown in Fig. 4. The division was based on color. That is, only a thin layer of the surface was more dark-red than most of the brown outer layer which was more light in color than the inner layer.

A fruiting body was extracted with 70% aqueous methanol. The extract was divided into a chloroform-soluble part and an insoluble part.

The amounts of chemical constituents were determined by the methods previously described.

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Each section of a fruiting body has distinctive secondary metabolites. Both a stipe and a pileus were observed separately. in addition, we focused attention on the newly-produced mycelia of a fruiting body extended from the center of a stipe or between a gill and the upper side of a pileus, as shown in Fig. 3. Therefore, a stipe was divided into inner and outer sections, and a pileus was divided into a gill and inner and outer sections. This enabled us to obtain data for distinguishing extracts of newly produced mycelia from those of existing mycelia.

Amounts of 70% aqueous methanol extract (Table 3-1), triterpenes (Table 3-2), ergosterol, fatty acids, and phenols (Table 3-3) were measured. A gill was found to have less triterpenes and phenols but more fatty acids than other sections. A pileus had more ergosterol than a stipe. Further it is shown in Tables 3-1, 3-2, and 3-3 that old sections (outer parts) have more chloroform soluble substances, more triterpenes, and more phenols than new sections (inner parts), but all have similar amounts of ergosterol and fatty acids. These findings and the preceding discussion on increases of triterpenes during growth of a fruiting body. indicate some relationship between the age of a fruiting body and the content of triterpenes. That is. the content of triterpenes and phenols increased when a fruiting body was kept in a medium longer.

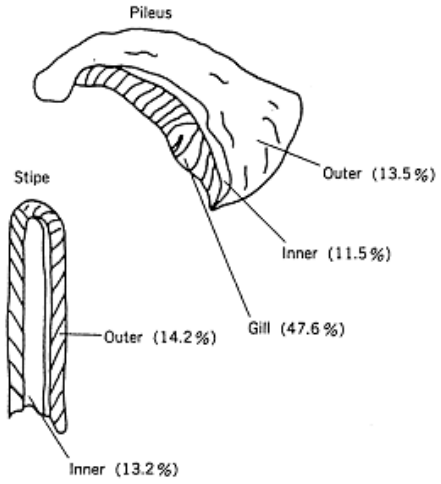

Fig.3. Cross-sections and weight distributions of a pileus

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and a stipe of a fruiting body.

Note: Parenthesizes figures are percents of the total

weight of the fruiting body.

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