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Dental caries prevention by traditional medicines. XII. Effect of components of *Ganoderma lucidum* on glucosyltransferase from *Streptococcus mutans*

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Abstract

By a bioassay-directed fractionation of an extract of the fruiting bodies of *Ganoderma lucidum*, which was previously shown to have *in vitro* anti-plaque action, ganoderic acids S1 and C2 were identified as inhibitory substances against glucosyltransferase (GTF) from a primary cariogenic bacterium, *Streptococcus mutans*. In addition, effect of some triterpenes and saponins on GTF was investigated.

Key words *Ganoderma lucidum, Streptococcus mutans*, glucosyltransferase, dental caries prevention, ganoderic acid S1, ganoderic acid C2.

Abbreviations GTF, glucosyltransferase ; IC_{50}, 50%-inhibitory concentration ; ISG, insoluble glucan ; SG, soluble glucan ; MS, mass spectrum ; ^{1}H-NMR, proton nuclear magnetic resonance ; ^{13}C-NMR, carbon-13 nuclear magnetic resonance.

Introduction

Streptococcus mutans is a primary cariogenic bacterium which causes dental caries in animals and humans.[1,2] It produces water-soluble and water-insoluble glucans from sucrose, which is catalyzed by membrane-bound or extracellular glucosyltransferase (GTF ; EC, 2, 4, 1, 4).[1,2] The glucans, especially the insoluble glucan, facilitate the accumulation of the microorganisms on smooth tooth surfaces (a dental plaque) and the subsequent development of dental caries.[1,2]

In the course of our basic studies on prevention of dental caries by traditional medicines, we have screened various Chinese[3] and Ayurvedic medicines[4] for *in vitro* anti-plaque action and have identified some antibacterial[5-8] and anti-GTF[3,4,9,10] substances hitherto.

In the present paper, we describe additional anti-GTF substances from the fruiting bodies of *Ganoderma lucidum* (FR.) KARST. (霊芝 ; Reishi in Japanese), whose extract was previously shown to have significant inhibition in the *in vitro* plaque formation mediated by GTF of *S. mutans*.[3]

Materials and Methods

Plant material : Cultivated fruiting bodies of *G. lucidum* were products of Daiichi Sangyo Co., Takayama, Gifu Prefecture, Japan.

Chemicals : Oleanolic acid was isolated from *Swertia japonica* in our laboratory. Soyasapogenols A and B, ginsenosides Rg_1 and Rb_1, saikosaponin c, glycyrrhetic acid and glycyrrhizin were purchased from Wako Pure Chemical Industry Co. Echinocystic acid, ursolic acid were obtained from Funakoshi Co. Saponins, β-D-glc^{-2}α-L-ara^{-3}hederagenin^{28}—β-D-glc^{6}—β-D-glc^{4}—α-L-rham (cauloside F), α-L-rham^{-2}α-L-ara^{-3}hederagenin^{28}—β-D-glc^{6}—β-D-glc^{4}—α-L-rham (kizutasaponin K_{12}), α-L-ara^{-3}hederagenin^{28}—β-D-glc^{6}—β-D-glc^{4}—α-L-rham (kizutasaponin K_{10}) and α-L-ara^{-3}hederagenin (kizutasaponin K_3) were provided by Professor T. Tomimori, Hokuriku University, Kanazawa.

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Bacto brain heart infusion (BHI) broth was a product of Difco Laboratories, Detroit, U.S.A. Uniformly labeled ^{14}C-sucrose was purchased from New England Nuclear, Boston, U.S.A. ACS II (Amersham) was used as scintillation fluid.

Microorganism : *S. mutans* OMZ 176 (serotype d) was provided from Professor S. Kotani of Osaka University Dental School.

Crude GTF preparation : Crude GTF from *S. mutans* OMZ 176 was prepared by the modified procedure of Mukasa and Slade.[11]

Chromatography : Silica gel, wako gel C-200, was used for column chromatography. Merck Kiselgel 60 F_{254} plates were used for thin-layer chromatography (TLC) and Merck PSC 60 F_{254} plates for preparative TLC. Solvent systems uesd were as follows : A, $CHCl_3$-EtOH (9 : 1, v/v) ; B, benzene-EtOAc (9 : 1, v/v). Spots on the plate were detected under UV light or by spraying anisaldehyde-H_2SO_4 reagent followed by heating. Preparative HPLC was carried out with a column of Chemosorb 5 Si (10 × 500 mm, Chemco Co.) using a solvent system, hexane-dichloroethane-EtOH (19 : 4 : 2) or $CHCl_3$-MeOH (9 : 1).

GTF-Inhibitory activity : A mixture (20 μl) consisting of crude GTF (0.34 mg protein/ml), 0.1 mM (^{14}C) sucrose (0.1 μCi/μl), 50 mM phosphate buffer, pH 6.8, and test sample dissolved in 50% MeOH (The final concentration of MeOH was approximately 10%) was incubated for 60 min at 37℃. An aliquot (5 μl) of the reaction mixture (containing both soluble and insoluble glucans ; SG and ISG) was applied to filter paper (Toyo No. 51A). The rest of the reaction mixture was centrifuged at 5,500 × *g* for 3 min, and an aliquot (5 μl) of the supernatant (containing SG) was also applied to the paper. The paper was developed with a mixture of BuOH-pyridine-H_2O (6 : 4 : 3) to the top. The radioactivity of glucans which remained at the origin of the paper was measured in a liquid scintillation counter. The amount of insoluble glucan (ISG) was calculated by the difference in radioactivity of the above two experiments. GTF-inhibition was represented by the percentage of insoluble glucan (ISG) formed in the presence of a test sample against a control (without the test sample ; 3977±102 dpm).

Extraction and fractionation : Powder of the fruiting bodies of *G. lucidum* (270 g) was extracted successively with boiling hexane (500 ml × 3), MeOH (500 ml × 3) and H_2O (500 ml × 3) to give the respective extracts in yields of 2.18, 15.9 and 12.42 g. The hexane-soluble was partitioned between hexane and 5% $NaHCO_3$ phases. The hexane phase was evaporated to give fraction H-1 and the $NaHCO_3$ phase was acidified with dilute HCl. The aqueous solution was extracted again with hexane (fraction H-2). The MeOH extract was suspended in water and extracted successively with hexane and EtOAc (fractions M-1 and M-2, respectively). EtOH was added to the H_2O extract dissolved in water to give precipitates, which were filtered off. The filtrate was applied to a Diaion column and the column was eluted with water and then MeOH to give a water-eluate (W-1) and a MeOH-eluate (W-2). Fractions H-1 and M-2 which showed potent GTF-inhibitory activity were separately chromatographed on silica gel columns, eluting with stepwise gradients of EtOAc in hexane and of MeOH in $CHCl_3$, respectively. Compounds **1**–**3** and fatty acids were isolated from fraction H-1, and compounds **4**–**9** from fraction M-2.

Ganoderic acid S1 (**1**) : Amorphous powder, MS *m/z* : 452 (M^+, 100%). The ^1H- and ^{13}C-NMR spectra agreed with those reported.[12]

Ganoderal A (**2**) : MS *m/z* : 436 (M^+, 100%). The ^1H-NMR spectrum was in agreement with that reported by Morigiwa *et al.*[12]

5α, 8α-Epidioxy-24ξ-methylcholesta-6, 22-diene-3β-ol (**3**) : Amorphous powder, $[\alpha]_D^{25} = -113.6$ (MeOH, c = 0.088), MS *m/z* : 428 (M^+, 50%). The ^1H-NMR spectrum agreed with that reported.[13]

Physcion (**4**) : MS *m/z* : 284 (M^+, 100%). The IR, ^1H-NMR and ^{13}C-NMR spectra were identical with those of an authentic sample.

Chrysophanol (**5**) : MS *m/z* : 254 (M^+, 100%). The IR, ^1H-NMR and ^{13}C-NMR spectra were identical with those of an authentic sample.

Chrysophanol glucoside (**6**) : On acid hydrolysis, the compound gave chrysophanol and D-glucose. However, this amorphous powder was a

mixture of 1-*O*-glucosylchrysophanol and 8-*O*-glucosylchrysophanol.[14]

Portensterol (**7**) : High-resolution MS *m/z* : 412.3378 (M^+), Calcd for $C_{28}H_{44}O_2$: 412.3342 ; ^{1}H-NMR (270 MHz, $CDCl_3$-MeOH) : δ 0.60 (3H, s, H_3-18), 0.82 (3H, d, J=6.8 Hz, H_3-26), 0.84 (3H, d, J = 6.6 Hz, H_3-27), 0.91 (3H, d, J = 6.8 Hz, H_3-21), 1.02 (3H, d, J=6.6 Hz, H_3-28), 1.07 (3H, s, H_3-19), *ca.* 3.59 (1H, br d-like, J = *ca.* 5.5 Hz, W/2=10.6 Hz, H-11α), *ca.* 4.1 (1H, m. H-3), 5.15 (1H, dd, J=14.9, 7.08 Hz, H-23), 5.23 (1H, dd, J = 14.9, 6.8 Hz, H-22), 5.32 (1H, br dd-like, H-6). The configuration of 3- and 11-hydroxy groups in portensterol was not determined in the literature[15,16] but the compound isolated in this experiment was assigned to be 3β, 11β-portensterol on the basis of the ^{1}H-NMR spectrum.

Ganodermanontriol (**8**) : MS *m/z* : 472 (M^+, 97%), The ^{1}H- and ^{13}C-NMR spectra agreed with those reported by Fujita *et al.*[17]

Ganoderic acid C2 (**9**) : Amorphous powder, $[\alpha]_D^{25}$ = +60° (MeOH, c=0.51), MS *m/z* : 518 (M^+, 100%). On methylation with diazomethane, it gave a methyl ester (MS *m/z* : 532 (M^+, 100%), mp. 188−190℃). The ^{13}C-NMR spectra of the acid and its methyl ester were in good agreement with those reported by Kohda *et al.*[18]

Analysis of fatty acid fraction : The fatty acid fraction was treated with diazomethane and analyzed by gas chromatography/mass spectrometry (GC/MS) under the conditions : column, 3% OV-1 (2.5 mm i.d.×2 m) ; column temperature, 150−280℃ (8℃/min) ; injector temperature, 250℃ ; flow rate, 50 ml He/min ; ionization voltage, 70 eV. The relative composition was as follows : pentadecanoic (1.4), hexadecanoic (24.0), heptadecanoic (1.5), octadecenoic (48.4), eicosanoic (2.3), docosanoic (5.3), tricosanoic (3.4), tetracosanoic (11.4) and pentacosanoic (2.3) acids. Parentheses represent their relative peak ratios. The fatty acid fraction had anti-bacterial action with a minimal inhibitory concentration of 50 μg/ml but showed weak GTF-inhibition at concentrations of 0.1−1.0 mg/ml.

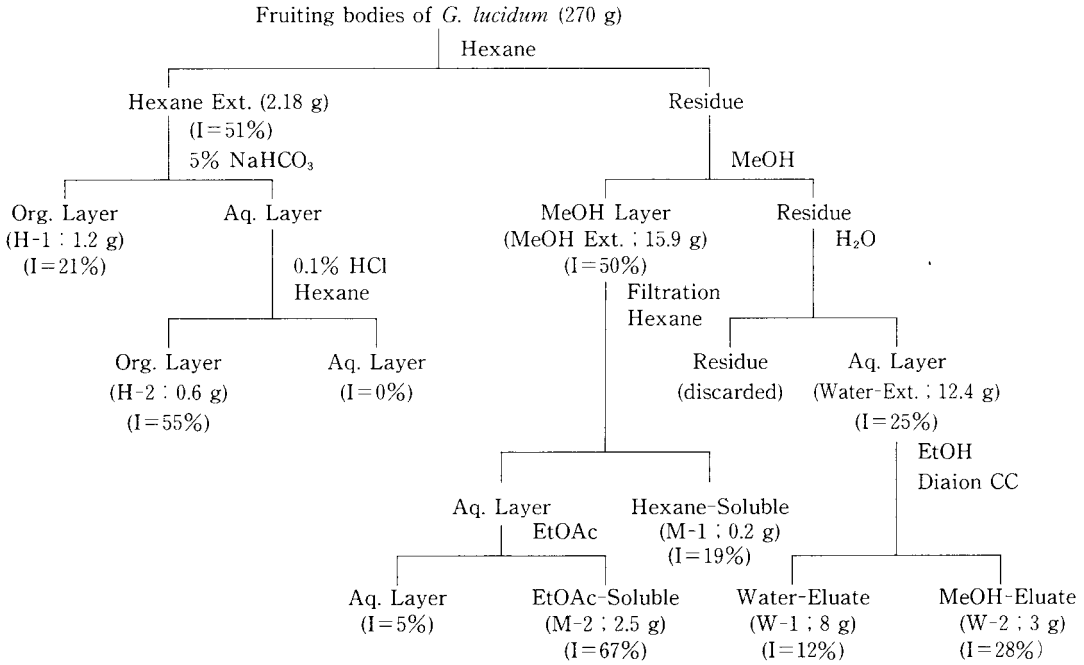

Fig. 1 Extraction and fractionation processes of the fruiting bodies of *G. lucidum*.
Yields of extracts and fractions, and percentages of GTF-inhibition by respective extracts and fractions at a concentration of 1 mg/ml are indicated in parentheses.

Results and Discussion

Bioassay-directed fractionation of an extract of G. lucidum

As reported in a previous paper,[3] an extract of the fruiting bodies of *G. lucidum* (Polyporaceae) showed potent inhibition in the adherence of heat-killed cells of *S. mutans* to smooth surfaces in the presence of sucrose and GTF, suggesting inhibitory action of the extract against GTF. For the purpose of isolating the inhibitory principles from the extract, the powder of the fruiting bodies of *G. lucidum* was extracted successively with hexane, MeOH and water as shown in Fig. 1. Table I shows inhibitory activities of these extracts in the insoluble glucan synthesis catalyzed by GTF in the presence of ^{14}C-sucrose. Both hexane and MeOH extracts showed appreciably inhibitory activities (51 and 50% inhibition, respectively, at 1.0 mg/ml), while the water extract showed less activity. The hexane extract was further fractionated into acidic and basic-neutral fractions. The former showed

Table I Inhibitory activities of various extracts and fractions obtained from the fruiting bodies of *G. lucidum*.

Extract and fraction	Percentage of GTF-inhibition at 1.0 mg/ml
Hexane extract	51
Basic-neutral fraction (H-1)	21
Acidic fraction (H-2)	55
MeOH extract	50
Hexane-soluble (M-1)	19
EtOAc-soluble (M-2)	67
Water extract	25
Water-soluble (W-1)	12
MeOH-soluble (W-2)	28

Assay was carried out in duplicate under the conditions described in "Materials and Methods." The percentage of GTF-inhibition was calculated as follows:

$$\left(1-\frac{\text{ISG formed in the presence of a sample (dpm)}}{\text{ISG formed without a sample (control ; dpm)}}\right)\times 100$$

The average variation in the GTF-inhibition in the presence of each sample was approximately ±6%.

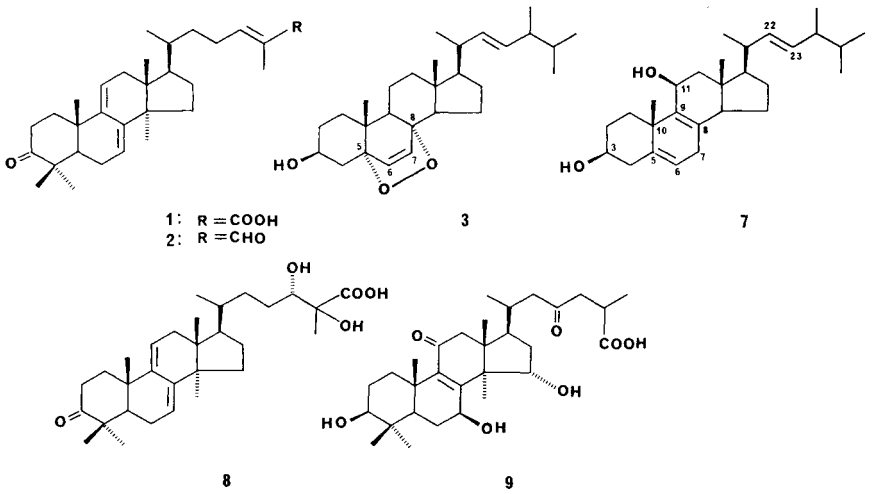

Fig. 2 Triterpenes and sterols isolated from the fruiting bodies of *G. lucidum*.

Table II Inhibitory activities of the components isolated from the fruiting bodies of *G. lucidum* against GTF.

Compound	Percentage of GTF-inhibition[a]	
	Concentration (mg/ml)	
	1.0	0.1
Ganoderic acid S1 (**1**)	—[b]	62
Ganoderal A (**2**)	0	0
Steroidal peroxide[c] (**3**)	31	0
Physcion (**4**)	0	0
Chrysophanol (**5**)	0	0
Chrysophanol glucoside[d] (**6**)	0	0
Portensterol (**7**)	34	32
Ganodermanontriol (**8**)	50	11
Ganoderic acid C2 (**9**)	83	55

Standard assay conditions are described in "Materials and Methods."
a) average of two experiments ; b) not determined ; c) 5α, 8α-epidioxy-24-methylcholesta-6, 22-dien-3β-ol ; d) a mixture of 1-*O*-glucosylchrysophanol and 8-*O*-glucosylchrysophanol (*ca.* 1 : 1) The average percent variation in the individual reading was *ca.*±5%.

stronger in inhibitory activity than the latter. Repeated column-chromatography of the acidic fraction led to the isolation of triterpenes (ganoderic acid S1 (**1**)[12] and ganoderal A (**2**)[12]), a steroid (5α, 8α-epidioxy-24-methyl-cholesta-6, 22-diene-3β-ol (**3**)[13]) and fatty acids. The composition of fatty acids were determined by means of GC/MS and the structures of other compounds were identified by spectroscopic methods (Fig. 2).

Similarly, the MeOH extract was fractionated and the active subfraction (the EtOAc-soluble fraction) afforded anthraquinones (physcion (**4**), chrysophanol (**5**) and chrysophanol glucoside (**6**)[14]), a steroid (portensterol (**7**)[15,16]) and triterpenes (ganodermanontriol (**8**)[17] and ganoderic acid C2 (**9**)[18,19]). Three anthraquinones (**4**−**6**) and two steroids (**7** and **3**) were isolated for the first time from the fruiting bodies of *G. lucidum*.

GTF-inhibitory activities of the components of G. lucidum extract

Table II shows GTF-inhibitory activities of various compounds isolated from the acidic fraction of the hexane extract and the EtOAc-soluble fraction of the MeOH extract. Among these compounds, ganoderic acids C2 and S1 showed potent GTF-inhibition with 50% inhibitory concentrations (IC_{50}) of 1.3 and 1.0×10^{-4} M, respectively. Other triterpens, sterols, fatty acids and anthraquinones showed less or no inhibitory activities at a concentration of 0.1 mg/ml. Under the same conditions, pentagalloyl glucose used as a positive control showed more potent inhibitory activity with an $IC_{50}=0.21\times10^{-4}$ M, thus indicating moderate inhibitory potency of ganoderic acids C2 and S1 against GTF.

GTF-inhibitory activities of triterpenes and triterpene glycosides

For a comparison of GTF-inhibitory activities, some other triterpenes and triterpene glycosides were investigated under the same assay conditions. Oleanolic acid, glycyrrhetic acid and glycyrrhizin showed appreciable anti-GTF action at a concentration of 0.1 mg/ml. The inhibitory activities of these compounds were slightly stronger than those of ganoderic acids C2 and S1. Kozai *et al.*[20] and Kohda *et al.*[21] have reported GTF-inhibitory action of ursolic acid and oleanolic acid isolated from a crude drug Zizyphi Fructus but we could not confirm the inhibitory action of the former compound in this experiment. Except for glycyrrhizin, triterpene glycosides had no inhibitory action against GTF. The inhibitory properties of glycyrrhetic acid and glycyrrhizin were already reported elsewhere.[4]

The fruiting bodies of *G. lucidum* have been used for the treatment of neurasthenia, insomnia,

Table III Inhibitory activities of some triterpenes and triterpene glycosides against GTF.

Compound	Percentage of GTF-inhibition[a]			
	Concentration (mg/ml)			
	10	1.0	0.1	0.01
Soyasapogenol A	23	49	11	0
Soyasapogenol B	77	68	0	0
Glycyrrhetic acid	100	79	72	12
Glycyrrhizin	100	81	64	26
Echinocystic acid	100	68	0	0
Ursolic acid	0	0	0	0
Oleanolic acid	100	84	84	50
Ginsenoside Rg_1	16	22	38	24
Ginsenoside Rb_1	0	0	0	4
Saikosaponin c	68	0	0	0
Cauloside F[b]	0	0	0	0
Kizutasaponin K_{12}[c]	0	0	0	0
Kizutasaponin K_{10}[d]	0	0	0	0
Kizutasaponin K_3[e]	0	0	0	0

Standard assay conditions are described in "Materials and Methods." The average percent variation in the individual reading was *ca.* 6%. a) average of two experiments ; b) β-D-glc^{2}-α-L-ara^{3}-hederagenin^{28}-β-D-glc^{6}-β-D-glc^{4}-α-L-rham ; c) α-L-rham^{2}-α-L-ara^{3}-hederagenin^{28}-β-D-glc^{6}-β-D-glc^{4}-α-L-rham ; d) α-L-ara^{3}-hederagenin^{28}-β-D-glc^{6}-β-D-glc^{4}-α-L-rham ; e) α-L-ara^{3}-hederagenin.

indigestion, bronchitis, cancer, *etc.* in Japanese folk medicine and traditional Chinese medicine. There are many reports on the pharmacological and biological activities of the crude extract and the components.[18,22-28] The inhibitory actions of *G. lucidum* triterpenes have been investigated in the *in vitro* assay systems of angiotensin-converting enzyme,[12] histamine release from rat mast cells[18] and cytotoxicity on hepatoma cells (HTC).[28] Our present results show an additional example for the biological activities of triterpenes from *G. lucidum*.

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和文抄録

In vitro で抗プラーク（歯苔形成の抑制）作用を示す霊芝（*Ganoderma lucidum*）エキスからその活性成分を単離，同定する目的で，プラーク形成に必須なグルカン合成酵素（glucosyltransferase ; GTF）の阻害活性を指標に同エキスの分画を行なった。その結果，ganoderic acid S1, ganoderic acid C などのトリテルペンが齲蝕原性菌（*Streptococcus mutans*）由来の GTF を強く阻害することを見いだした。また同時に，他のいくつかのトリテルペン，サポニンの同酵素に対する阻害効果も調べた。

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