Antitubercular Activity and Inhibitory Effect on Epstein-Barr Virus Activation of Sterols and Polyisoprenepolyols from an Edible Mushroom, *Hypsizigus marmoreus*

Toshihiro AKIHISA,^{*,a} Scott Gary FRANZBLAU,^b Harukuni TOKUDA,^c Masaaki TAGATA,^a Motohiko UKIYA,^a Tsunetomo MATSUZAWA,^d Koichi METORI,^e Yumiko KIMURA,^e Takashi SUZUKI,^e and Ken YASUKAWA^e

^a College of Science and Technology, Nihon University; 1–8 Kanda Surugadai, Chiyoda-ku, Tokyo 101–8308, Japan:

^b Institute for Tuberculosis Research, College of Pharmacy (M/C 964), University of Illinois at Chicago; 833 S. Wood St., Chicago, IL 60612, U.S.A.; ^c Department of Biochemistry and Molecular Biology, Kyoto Prefectural University of Medicine; Kyoto 602–0841, Japan; ^d Agricultural Technology Institute of Nagano Farmers' Federation; 787–1 Suzaka, Sazaka, Nagano 382–0084, Japan; and ^e College of Pharmacy, Nihon University; 7–7–1 Narashinodai, Funabashi, Chiba 274–8555, Japan. Received January 18, 2005; accepted February 28, 2005

Seven sterols (1–7) and eight polyisoprenepolyols (8–15), isolated from the non-saponifiable lipid fraction of the dichloromethane extract of an edible mushroom, *Hypsizigus marmoreus* (Buna-shimeji), were tested for their antitubercular activity against *Mycobacterium tuberculosis* strain H₃₇Rv using the Microplate Alamar Blue Assay (MABA). Six sterols (2–7) and two polyisoprenepolyols (8, 12) showed a minimum inhibitory concentration (MIC) in the range of 1–51 µg/ml, while the others (1, 9–11, 13–15) were inactive (MIC > 128 µg/ml). The seven sterols (1–7) and three polyisoprenepolyols (8, 10, 12) were further evaluated for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells. Sterols 6 and 7 showed potent inhibitory effects while preserving the high viability of Raji cells.

Key words *Hypsizigus marmoreus*; sterol; polyisoprenepolyol; antituberculosis; Epstein-Barr virus early antigen

Hypsizigus marmoreus (Buna-shimeji) has recently become a popular edible mushroom in Asia, and has been cultivated by culture methods such as bottle culture and bag-culture. It has been reported to exhibit many biological activities such as anti-tumor,^{1,2)} antioxidant,¹⁾ and antiproliferative activity.³⁾ Several homologous polyisoprenepolyols named hypsiziprenols have recently been characterized in this mushroom.⁴⁾ Hypsiziprenols are the possible biosynthetic precursors of gymnopilins, bitter principles of *H. marmoreus*.⁴⁾ In the course of our studies on the bioactive principles of natural medicines and foodstuffs,^{5,6)} we evaluated the constituents of *H. marmoreus* for their antitubercular activity⁷⁾ as well as their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA).^{5,6)} In this paper, we describe the antitubercular activity against *Mycobacterium tuberculosis* of seven sterols (1–7) and eight polyisoprenepolyols, hypsiziprenols (8–15), and inhibitory effects on the induction of EBV-EA by the TPA of seven sterols (1–7) and three hypsiziprenols (8, 10, 12) isolated from the non-saponifiable lipid (NSL) fraction of the extract of *H. marmoreus*.

MATERIALS AND METHODS

Extraction, Isolation, and Identification Sliced fresh bodies of *Hypsizigus marmoreus* (11 kg), cultivated in Nagano Prefecture in 2001, were air-dried. The dried mushroom (833 g) was then extracted with hot CH₂Cl₂ to give an extract (86 g). Alkaline hydrolysis (5% KOH in methanol (MeOH), reflux, 3 h) of the extract yielded a neutral non-saponifiable lipid (NSL) fraction (9.0 g). Crystallization of the NSL fraction from MeOH yielded a precipitate (2.8 g), which was identified as ergosterol (1),²⁾ and a filtrate (4.9 g).

The filtrate was subjected to column chromatography on silica gel (150 g) using an *n*-hexane–ethyl acetate (EtOAc) gradient of 1 : 0–1 : 1 as an eluent, which yielded fractions containing monohydroxy sterols (fraction A; *R*_f 0.83 on TLC developed with EtOAc–MeOH, 4 : 1), polyhydroxy sterols (fraction B; *R*_f 0.48), and polyisoprenepolyols (fraction C; *R*_f 0.24). Fractions A–C were purified by further chromatography on silica gel. Reversed-phase preparative HPLC (HPLC I; 25 cm × 10 mm i.d., Pegasil ODS II (Senshu Scientific Co., Ltd., Tokyo, Japan) column; mobile phase: MeOH–H₂O–

Chart 1. Structures of Compounds 1–15

* To whom correspondence should be addressed. e-mail: akihisa@chem.cst.nihon-u.ac.jp

acetic acid (AcOH), 19 : 1 : 1, 2.0 ml/min) of purified fraction A (0.39 g) gave compound **1** (60 mg; retention time (t_R) 28.3 min) and ergosterol peroxide (**2**)²⁾ (12 mg; t_R 27.8 min). HPLC (HPLC II; column used was the same as that of HPLC I; mobile phase: MeOH–H₂O–AcOH, 9 : 1 : 1, 2.0 ml/min) of purified fraction B (0.38 g) gave five compounds, cerevisterol (**3**)⁸⁾ (40 mg; t_R 23.6 min), 6-epicerevisterol (**4**)⁹⁾ (1.7 mg; t_R 38.6 min), 22,23-dihydrocerevisterol (**5**)¹⁰⁾ (6.7 mg; t_R 28.6 min), 6-*O*-methylcerevisterol (**6**)¹¹⁾ (2.4 mg; t_R 37.1 min), and (22*E*,24*R*)-5 α ,6 α -epoxyergosta-8,22-diene-3 β ,7 β -diol (**7**)¹²⁾ (5.0 mg; t_R 21.7 min). HPLC (HPLC III; 25 cm $\times$ 10 mm i.d., Hypersil ODS-5 (Senshu Scientific Co., Ltd., Tokyo, Japan) column; mobile phase: MeOH–H₂O–AcOH, 75 : 25 : 1, 0.8 ml/min) of purified fraction C (1.28 g) gave eight hypsiziprenols,⁴⁾ hypsiziprenol A₉ (**8**) (77 mg; t_R 24.8 min; mp 30–31 °C after crystallization from MeOH; $[\alpha]_D^{25} + 5.3^\circ$ ($c=0.10$, acetone); positive FAB-MS m/z 739 $[M+H]^+$), hypsiziprenol B₉ (**9**) (1.1 mg; t_R 23.3 min; mp 34–35 °C; $[\alpha]_D^{25} + 6.2^\circ$ ($c=0.10$, acetone); positive FAB-MS m/z 721 $[M+H]^+$), hypsiziprenol AA₉ (**10**) (2.3 mg; t_R 20.6 min; mp 65–67 °C; $[\alpha]_D^{25} + 2.8^\circ$ ($c=0.11$, acetone); negative FAB-MS m/z 753 $[M-H]^-$), hypsiziprenol B₁₀ (**11**) (2.0 mg; t_R 18.8 min; mp 45–47 °C; $[\alpha]_D^{25} + 8.5^\circ$ ($c=0.10$, acetone); positive FAB-MS m/z 807 $[M+H]^+$), hypsiziprenol BA₁₀ (**12**) (4.3 mg; t_R 21.8 min; mp 41–43 °C; $[\alpha]_D^{25} + 5.0^\circ$ ($c=0.11$, acetone); negative FAB-MS m/z 821 $[M-H]^-$), hypsiziprenol A₁₁ (**13**) (1.6 mg; t_R 29.0 min; mp 41–43 °C; $[\alpha]_D^{25} + 1.7^\circ$ ($c=0.20$, acetone); negative FAB-MS m/z 823 $[M-H]^-$), hypsiziprenol A₁₂ (**14**) (14.8 mg; t_R 26.0 min; mp 51–53 °C; $[\alpha]_D^{25} + 4.5^\circ$ ($c=0.10$, acetone); negative FAB-MS m/z 995 $[M-H]^-$), and hypsiziprenol AA₁₂ (**15**) (2.5 mg; t_R 33.2 min; mp 65–67 °C; $[\alpha]_D^{25} + 2.8^\circ$ ($c=0.11$, acetone); negative FAB-MS m/z 1011 $[M-H]^-$).

Identification of sterols **1**–**7** was done by ¹H-NMR (400 MHz) and EI-MS spectral comparison with the corresponding compounds in the literature,^{2,8–12)} and identifica-

tion of hypsiziprenols **8**–**15** by ¹H-NMR and FAB-MS spectral comparison with the corresponding or relevant compounds in the literature.⁴⁾

Determination of Antitubercular Activity The antitubercular activity of compounds in DMSO was determined against *Mycobacterium tuberculosis* H₃₇Rv (ATCC 27294) in Middlebrook 7H12 medium using the Microplate Alamar Blue Assay (MABA), as previously described.¹³⁾ The minimum inhibitory concentration (MIC) is defined as the lowest concentration effecting a reduction in fluorescence of 90% relative to controls.

EBV-EA Induction Tests The EBV genome-carrying lymphoblastoid cells, Raji cells, derived from Burkitt's lymphoma, were cultured in RPMI-1640 medium. The Raji cells were incubated for 48 h at 37 °C in a medium containing *n*-butyric acid (4 mM), TPA (32 pM), and various amounts of test compounds. Smears were made from the cell suspension, and the EBV-EA-inducing cells were stained by means of an indirect immunofluorescence technique. The details of the *in vitro* assay on EBV-EA induction have been reported previously.¹⁴⁾

RESULTS AND DISCUSSION

Although a homologous series of hypsiziprenols have previously been characterized in *Hypsizigus marmoreus*, mainly with the aid of FAB-MS spectroscopy,⁴⁾ this study constitutes the first instance of their isolation and full characterization.

The MIC values of seven sterols (**1**–**7**) and eight hypsiziprenols (**8**–**15**) against *M. tuberculosis* are shown in Table 1. Among the sterols tested, compounds **2**–**7** exhibited activity with MIC values of 1–51 μ g/ml, among which ergosterol peroxide (**2**; MIC 1 μ g/ml)¹⁵⁾ followed by cerevisterol (**3**; 6 μ g/ml) and 6-epicerevisterol (**4**; 11 μ g/ml) showed the most potent activity, although still being an order of magnitude less potent than the first-line antitubercular drug, ri-

Table 1. Minimum Inhibitory Concentrations (MIC Values) against *Mycobacterium tuberculosis* H₃₇Rv and Percentage of Epstein-Barr Virus Early Antigen (EBV-EA) Induction of Compounds **1**–**15**

Compound		Antitubercular activity	Percentage of EBV-EA induction ^{a)}				IC ₅₀ ^{b)} (mol ratio/32 pmol TPA)
			Concentration (mol ratio/32 pmol TPA)				
			MIC (μg/ml)	1000	500	100	10
1	Ergosterol	>128 ¹⁵⁾	12.2 (70)	49.6	82.3	100	520
2	Ergosterol peroxide	1 ¹⁵⁾	14.5 (70)	51.8	83.6	100	525
3	Cerevisterol	6	11.6 (70)	49.0	80.9	100	518
4	6-Epicerevisterol	11	8.5 (70)	45.9	80.1	100	512
5	22,23-Dihydrocerevisterol	51	10.3 (70)	48.0	80.2	100	515
6	6- <i>O</i> -Methylcerevisterol	36	0 (70)	26.4	72.0	91.2	298
7	(22 <i>E</i> ,24 <i>R</i>)-5α,6α-Epoxyergosta-8,22-diene-3β,7β-diol	45	0 (70)	23.1	70.0	89.3	192
8	Hypsiziprenol A ₉	15	48.6 (70)	72.3	92.7	100	926
9	Hypsiziprenol B ₉	>128					
10	Hypsiziprenol AA ₉	>128	13.5 (70)	51.0	75.1	100	510
11	Hypsiziprenol B ₁₀	>128					
12	Hypsiziprenol BA ₁₀	44	12.1 (70)	50.0	74.2	100	508
13	Hypsiziprenol A ₁₁	>128					
14	Hypsiziprenol A ₁₂	>128					
15	Hypsiziprenol AA ₁₂	>128					
	Rifampin ^{c)}	0.25					
	β-Carotene ^{c)}		8.6 (70)	34.2	82.1	100	397

a) Values represent percentages relative to the positive control value. TPA (32 pmol, 20 ng)=100%. Values in parentheses are viability percentages of Raji cells. b) IC₅₀ represents the mol ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol TPA. c) Reference compound.

fampin (Table 1). From the limited data set, it appears that C-6 α ,8 α -epidioxidation (**2**), hydroxylation at both C-5 α and C-6 α / β (**3**—**6**), or hydroxylation at C-7 β accompanied with C-5 α ,6 α -epoxidation (**7**) gives rise to higher activity for the 3 β -hydroxy sterols. On the other hand, two compounds, hypsiziprenol A₉ (**8**; MIC 15 μ g/ml) and hypsiziprenol BA₁₀ (**12**; 44 μ g/ml), showed potent antitubercular activity among the eight hypsiziprenols (**8**—**15**) tested. There was no apparent correlation between the structures and antitubercular activity for the hypsiziprenols.

The inhibitory effects of the seven sterols (**1**—**7**) and three hypsiziprenols (**8**, **10**, **12**) on EBV-EA activation induced by TPA were examined for the primary screening of anti-tumor promoting activities, and the results are shown in Table 1. Sterols **6** (IC₅₀ value 298 mol ratio/32 pmol TPA) and **7** (192 mol ratio/32 pmol TPA) showed potent inhibitory effects while preserving the high viability of Raji cells. The inhibitory effects of these sterols were more potent than β -carotene (397 mol ratio/32 pmol TPA), a vitamin A precursor that has been studied extensively in cancer chemoprevention using animal models.¹⁶ The inhibitory effects against EBV-EA activation have been demonstrated to be closely parallel to those against tumor promotion *in vivo*,^{5,6} and the sterols **6** and **7** from *H. marmoreus* were therefore suggested to be valuable anti-tumor promoters (potential cancer chemopreventive agents). Although ergosterol (**2**) exhibited only a moderate inhibitory effect against EBV-EA activation (Table 1), this still might be of importance as a chemopreventive constituent of *H. marmoreus* since this constitutes the most predominant lipid constituent of *H. marmoreus* and has been demonstrated to inhibit the promotion of skin papillomas by TPA in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mice.²⁾

In summary, concerning with the lipid constituents of *H. marmoreus*, the sterols might be of importance from the points of view of antituberculosis and cancer chemoprevention, whereas the hypsiziprenols are considered to be of less importance. No hypsiziprenols from *H. marmoreus* tested

have been reported to show anti-tumor activity on NCI-H292 or EL-4 cell lines.⁴⁾

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