Chem. Pharm. Bull. 43(8) 1388—1390 (1995) Vol. 43, No. 8

Screening of Tissue Cultures and Thalli of Lichens and Some of Their Active Constituents for Inhibition of Tumor Promoter-Induced Epstein–Barr Virus Activation

Yoshikazu YAMAMOTO,*[,a] Yasutaka MIURA,[a] Yasuhiro KINOSHITA,[a] Masako HIGUCHI,[a] Yasuyuki YAMADA,[b] Akira MURAKAMI,[d] Hajime OHIGASHI,[c] and Koichi KOSHIMIZU[d]

Central Research Institute, Nippon Paint Co., Ltd.,[a] Ikeda Nakamachi, Neyagawa, Osaka 572, Japan, Graduate School of Science and Technology, Nara Institute of Science and Technology,[b] Takayama-cho, Ikoma, Nara 630–01, Japan, Food Science and Technology, Faculty of Agriculture,[c] Kyoto University, Kyoto 606–01, Japan, and Department of Biotechnological Science, Faculty of Biology-Oriented Science and Technology, Kinki University,[d] Iwade-Uchita, Wakayama 649–64, Japan. Received January 27, 1995; accepted April 13, 1995

Inhibition of tumor promoter-induced Epstein–Barr virus (EBV) activation was screened using tissue culture and thallus extracts of lichens. *Usnea longissima* ACH. thallus and *Cetraria ornata* MÜLL. ARG. tissue culture showed strong inhibitory activity. We identified (+)-usnic acid (1), barbatic acid (2), diffractaic acid (3), 4-*O*-demethylbarbatic acid (4), and evernic acid (5) as inhibitors of EBV activation from the *U. longissima* thallus. Of these compounds, (+)-usnic acid exhibited the highest inhibitory activity (IC_{50} = 1.0 μM).

Key words Epstein–Barr virus activation inhibitor; lichen tissue culture; lichen secondary metabolite

Lichens produce characteristic secondary metabolites[1] and they have been used as crude drugs all over the world. Recently, a variety of natural products isolated from lichens have been found to exhibit a wide range of potentially useful biological activities.[2] An anthraquinone, norsolorinic acid, inhibits monoamine oxidase.[3] New phenolics from *Protousnea* species have been isolated as tyrosinase inhibitors.[4] Metadepsides inhibit prostaglandin biosynthesis[5] and a dibenzofuran derivative, usnic acid, inhibits cancer growth,[6] while lichen polysaccharides exhibit antitumor and antiviral activities.[7] Usnic acid and the depside diffractaic acid have anti-inflammatory, analgesic, and antipyretic effects[8] and other depsides inhibit plant and insect growth.[9] Orsellinic acid derivatives exhibit nematocidal and anti-cholesterol activities.[10]

Most types of carcinogenesis are known to be caused by environmental factors. Chemically-induced carcinogenesis is known to pass through two successive stages, initiation (changing normal cells into dormant tumor cells) and promotion (changing these dormant cells into tumor cells). Compounds acting at these stages are called initiators or promoters, respectively. Recently, an assay for inhibitors of tumor promotion using Epstein–Barr virus (EBV) in Raji cells[11] has been established. Consequently, some natural inhibitors have been isolated; triterpenoids[12] (oleanolic, ursolic, and glycyrrhetinic acids) and flavonoids[13] (quercetin and kaempferol).

We established lichen tissue cultures in 1985[14] and have now accumulated cultures from about 400 species. These cultures may be promising sources of novel biologically active compounds. We previously reported the inhibitory activity of tyrosinase in some of these cultures.[15] In this paper, we describe the results of screening tests to detect inhibition of tumor promoter-induced EBV activation in tissue cultures and thalli of lichens as well as the isolation of some of these inhibitors.

Results and Discussion

Screening Tests for the Inhibition of Tumor Promoter-Induced EBV Activation The inhibitory effect of each acetone extract from tissue cultures and thalli of the 29 species was tested at 10 μg/ml on EBV activation induced by a potent tumor promoter, teleocidin B-4 at 20 ng/ml. The activity was expressed by a relative index (RI) with reference to the inhibitory activity of a positive control, 3-oxoursolic acid, at 2 μg/ml. As shown in Table 1, more than half of the acetone extracts showed a greater inhibitory activity than that of 3-oxoursolic acid. The extracts from *Cetraria ornata* MÜLL. ARG. tissue culture and *Usnea longissima* ACH. thallus showed marked RI values of 2.4 and 4.0, respectively. Cladoniaceae (*Cladonia* and *Gymnoderma*) tissue cultures markedly inhibited EBV activation.

Isolation of the Major Constituents of *Usnea longissima* Thallus The acetone and methanol extract from *U. longissima* thallus gave five major constituents (**1**—**5**). These compounds had been previously isolated as plant growth inhibitors from *U. longissima* thallus collected in Hokkaido Prefecture.[9]

Activities of Lichen-Derived Constituents on EBV Activation The dose producing EBV activation inhibition was measured in lichen-derived substances (**1**—**9**) (Table 2). (+)-Usnic acid exhibited a marked activity (IC_{50} 1.0 μM). We are currently testing the *in vivo* antitumor promoting activity of (+)-usnic acid. The inhibitory activity of (−)-usnic acid appeared to be somewhat lower than that of (+)-usnic acid. Evernic acid is the strongest active depside tested so far, suggesting that the balance between hydrophobicity and hydrophilicity in the depsides is significant for activity. Lichesterinic acid also exhibited inhibitory activity, equal to that of evernic acid, and to that of known antitumor promoters, quercetin (IC_{50} 23 μM)[13] and oleanolic acid (IC_{50} 20 μM).[12]

In conclusion, we have performed screening tests on lichens to investigate inhibition of EBV activation and found that usnic acid exhibits strong inhibitory activity.

* To whom correspondence should be addressed.

Table 1. Inhibitory Effects of Acetone Extracts of Tissue Cultures and Thalli of Lichens on Epstein–Barr Virus Activation Induced by Teleocidin B-4

Family	Species	Culture		Thallus	
		RI[a]	CV[b]	RI	CV
Baeomycetaceae	*Baeomyces placophyllus* (Lam.) Ach.	0.0	83	1.0	94
Cladoniaceae	*Cladia aggregata* (Sw.) Nyl.	2.9	0.1>	1.6	94
	Cladonia amaurocraea (Flörke) Schaer.	1.3	95	2.1	75
	Cladonia cristatella Tuck.	1.7	96	1.5	93
	Cladonia furcata (Huds.) Schaer.	1.7	94	1.8	97
	Cladonia gracilis (L.) Willd. var. *dilatata* (Hoffm.)	1.9	97	2.1	94
	Cladonia nigripes (Nyl.) Trass.	1.9	98	2.1	99
	Cladonia pleurota (Flörke) Schaer.	2.1	97	2.5	85
	Cladonia rangiferina (L.) Web.	1.6	96	2.1	98
	Cladonia vulcani Savicz	1.9	84	2.8	87
	Gymnoderma coccocarpum Nyl.	1.8	90	1.7	88
Graphidaceae	*Graphis scripta* (L.) Ach.	1.8	71	NT[c]	NT
Parmeliaceae	*Asahinea chrysantha* (Tuck.) W. Culb. *et* C. Culb.	0.4	96	0.8	95
	Cetraria ornata Müll. Arg.	2.4	85	3.1	78
	Cetrelia japonica (Zahlbr.) W. Culb. *et* C. Culb.	0.6	97	1.0	94
	Hypogymnia physodes (L.) Nyl.	0.6	86	0.1	82
	Parmelia saccatiloba Tyl.	0.3	90	NT	NT
	Pseudevernia furfuracea (L.) Zopf	0.5	92	1.9	92
Pertusariaceae	*Pertusaria flavicans* Lamy	0.4	86	NT	NT
Stereocaulaceae	*Stereocaulon intermedium* (Sav.) Magn.	0.5	92	1.4	88
Teloschistaceae	*Caloplaca scopularis* (Nyl.) Lett.	0.0	89	0.2	91
	Xanthoria fallax (Hepp) Arn.	1.7	91	1.3	91
Umbilicariaceae	*Umbilicaria kisovana* (Zahlbr.) Kurok.	1.2	87	2.3	80
Usneaceae	*Alectoria sulcata* (Lev.) Nyl.	0.2	94	2.5	89
	Evernia prunastri (L.) Ach.	1.2	92	0.0	93
	Letharia vulpina (L.) Hue	1.1	91	1.2	89
	Ramalina farinacea (L.) Ach.	1.4	88	NT	NT
	Usnea longissima Ach.	0.7	88	4.0	75
No family	*Thamnolia vermicularis* Ach.	1.5	90	1.0	93

a) RI, relative index = %I for the tested extract/%I for 3-oxoursolic acid. %I = (number of total cells − number of labelled cells) × 100/number of total cells. *b*) CV, cell viability (%) after 48 h. *c*) NT, no test.

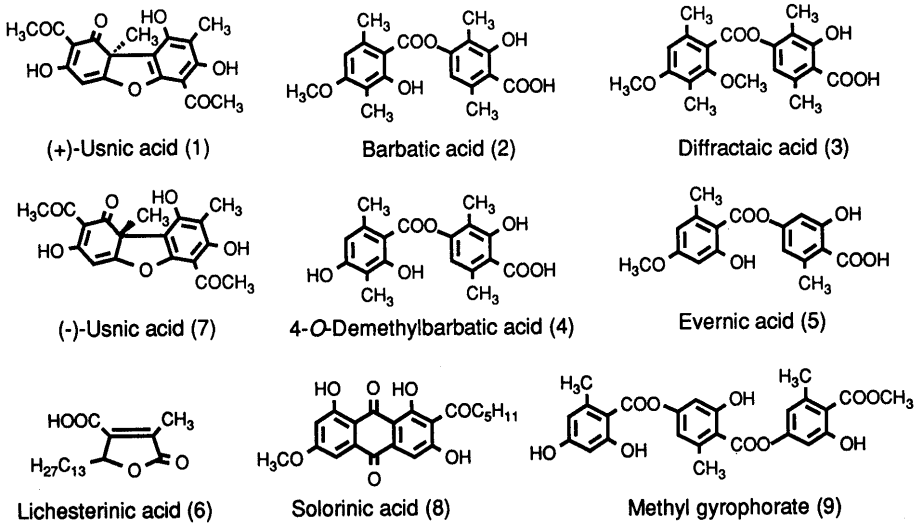

Fig. 1. Lichen-Derived Inhibitors of Epstein–Barr Virus Activation

Natural and cultured lichens are likely to be promising sources for potential anti-tumor promoters.

Experimental

Preparative HPLC was carried out using a Waters model Delta Prep 3000 at 25 °C under the following conditions: column, 19 × 150 mm packed with μBondasphere 5μ C_{18}-100 Å; solvent system, a linear gradient of 0.1% formic acid in methanol–0.1% formic acid in H_2O, 70:30 to 100:0 over 30 min at a flow rate of 10 ml/min; detector, spectrophotometer (254 nm). Preparative TLC was performed on Merck Kieselgel 60 F_{254} (layer thickness, 0.25 or 1 mm) with hexane–ether–formic acid (13:8:2, v/v). Spots were detected under UV light.

Materials Lichens were collected in Canada, England, Finland and Japan. They were deposited in the herbarium of the Research Center, Nippon Paint Co., Ltd. The lichen tissue cultures shown in Table 1, which we have established[14] from thallus segments, were maintained on agar-plates of malt-yeast extract (MY) medium[16] at 15 °C in the dark in our laboratory. Cultures were transferred to fresh medium every 8 weeks. *Usnea longissima* thalli for the isolation of inhibitors of EBV

Table 2. Inhibitory Effect of Lichen-Derived Compounds on Epstein–Barr Virus Activation Induced Teleocidin B-4

Compound	%I[a] (CV[b])								IC_{50} (μM)
	50 μM		5 μM		0.5 μM		0.05 μM		
(+)-Usnic acid (**1**)	>99	(50)	77.8	(83)	36.7	(95)	0.1>	(94)	1.0
Barbatic acid (**2**)	0.1	(92)	NT[c]		NT		NT		>100
4-*O*-Demethylbarbatic acid (**3**)	44.7	(95)	0.1>	(99)	NT		NT		72
Diffractaic acid (**4**)	30.0	(92)	0.1>	(99)	NT		NT		>100
Evernic acid (**5**)	64.6	(96)	13.3	(96)	0.1>	(99)	NT		42
Lichesterinic acid (**6**)	ND[d]	(0.1)	33.7	(96)	0.1>	(91)	NT		22
(−)-Usnic acid (**7**)	>99	(94)	49.6	(99)	28.5	(87)	NT		5.0
Solorinic acid (**8**)	17	(73)	NT		NT		NT		>100
Methyl gyrophorate (**9**)	24	(72)	NT		NT		NT		>100

a) %I, (number of total cells − number of labelled cells) × 100/number of total cells. *b*) CV, cell viability (%) after 48 h. *c*) NT, no test. *d*) ND, not determined.

activation were collected on Mt. Yatsugatake in Nagano Prefecture in 1990 and stored at −25 °C for 2 years.

Chemicals Authentic 4-*O*-demethylbarbatic, barbatic, diffractaic, and evernic acids were gifts from Dr. Huneck. Lichesterinic acid from *Cetraria islandica*, solorinic acid, and methyl gyrophorate from *Solorina crocea* for EBV assay were donated by Profs. Takahashi and Yamazaki. (−)-Usnic acid was purchased from Extrasynthèse (Genay, France).

Screening Tests of Lichen Extracts and Determination of the Activity of Epstein–Barr Virus Activation Inhibition Lyophilized thalli or tissue cultures (each 200 mg) were powdered in a mortar and then submerged overnight at 4 °C in 10 ml acetone. The acetone solution was then filtered and evaporated to dryness *in vacuo*. The Raji cells carrying the EBV genome were prepared at a cell density of 5×10^5 cells/ml, and sodium *n*-butyrate (3 mM), teleocidin B-4 (20 ng/ml), and the test extract (10 μg/ml) dissolved in 5 μl dimethyl sulfoxide (DMSO) were added to the medium. The Raji cells were incubated at 37 °C for 48 h in a 5% CO_2 atmosphere. After harvesting the cells, smears were prepared from cell suspensions. Early antigens induced by teleocidin B-4 were detected by treatment of the smears with nasopharyngeal carcinoma serum, then with FITC-labelled antihuman IgG. The percentage inhibition of EBV activation (%I) was calculated from cell counts and normalized to give a RI by comparison with the %I determined for a positive control (3-oxoursolic acid). In the screening test, each inhibitor activity was measured in a single experiment and the average inhibitory activity was measured in duplicate experiments for the inhibitors isolated.

Isolation and Identification of Major Constituents of *Usnea longissima* Thallus Dry *Usnea longissima* thalli (10 g) were cut into pieces and submerged three times in fresh acetone (200 ml) overnight, then filtered and submerged in the same way in methanol (200 ml). Combined filtrates were evaporated to dryness *in vacuo* to obtain a dry extract (1.1 g) This extract was subjected to silica-gel column chromatography to give 2 fractions (stepwise elution with hexane to ethyl acetate). Compound **1** was obtained from the first elute. Its physical data were consistent with that of authentic (+)-usnic acid. The second elute was subjected to preparative TLC and HPLC to give compounds **2** (51.7 mg), **3** (5.9 mg), **4** (9.0 mg), and **5** (6.2 mg). Their physical data were consistent with those of authentic barbatic, diffractaic, 4-*O*-demethylbarbatic and evernic acids, respectively.

Acknowledgements We are very grateful to Prof. I. Yoshimura for identification of lichens and valuable advice. We thank Prof. M. R. D. Seaward of Bradford University, Prof. Schofield of British Columbia University, and Prof. T. Ahti of Helsinki University for their help in collecting the lichens. We also thank Dr. S. Huneck of the Institute for Plant Biochemistry, Prof. K. Takahashi of Meiji College of Pharmacy, and Prof. M. Yamazaki of Chiba University for their gifts of lichen substances.

References

1) Asahina Y., Shibata S., "Chemistry of Lichen Substances," Kawade Syobo, Tokyo, 1954.
2) Yamamoto Y., "Plant Cell Culture in Japan," ed. by Komamine A., Misawa M., DiCosmo F., CMC Co., Ltd., Tokyo, 1991.
3) Okuyama E.,, Hossain C. F., Yamazaki M., *Shoyakugaku Zasshi*, **45**, 159 (1991).
4) Kinoshita K., Matsubara H., Koyama K., Takahashi K., Yoshimura I., Yamamoto Y., Higuchi M., Miura Y., Kinoshita K., Kawai K., *J. Hattori Bot. Lab.*, **75**, 359 (1994).
5) Sankawa U., Shibuya M., Ebizuka Y., Noguchi H., Kinoshita T., Iitaka Y., *Prostaglandin*, **24**, 21 (1982).
6) Kupchan S. M., Kopperman H. L., *Experientia*, **31**, 625 (1975); Takai M., Uehara Y., Beisler J. A., *J. Med. Chem.*, **22**, 1380 (1979).
7) Fukuoka F., Nakanishi M., Shibata S., Nishikawa Y., Takeda T., Tanaka M., *Gann*, **59**, 421 (1968); Hirabayashi L., Iehata S., Ito M., Shigeta S., Narui T., Shibata S., *Chem. Pharm. Bull.*, **37**, 2410 (1989).
8) Otsuka H., Komiya T., Tsukumi M., Toyosato T., Fujimura H., *J. Takeda Res. Lab.*, **31**, 247 (1972); Okuyama E., Umeyama K., Yamazaki M., Kinoshita Y., Yamamoto Y., *Planta Medica*, **61**, 113 (1995).
9) Nishitoba Y., Nishimura H., Nishiyama T., Mizutani J., *Phytochemistry*, **26**, 3181 (1987); Slansky F., Jr., *Environ. Entomol.*, **8**, 865 (1979).
10) Ahad A. M., Goto Y., Kiuchi F., Tsuda Y., Sato T., *Chem. Pharm. Bull.*, **39**, 1043 (1991); Kim C. H., *J. Iwate Med. Ass.*, **34**, 669 (1982).
11) Zur Hausen H., Bornkamm G. W., Schmidt R., Hecker E., *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 782 (1979); Ito Y., Kawanishi M., Harayama T., Takebayashi S., *Cancer Lett.*, **12**, 175 (1981).
12) Nishio H., Kitagawa K., Iwashima A., *Carcinogenesis*, **5**, 1529 (1984); Ohigashi H., Takamura H., Koshimizu K., Tokuda H., Ito Y., *Cancer Lett.*, **30**, 143 (1986).
13) Konoshima T., Takasaki M., Kozuka M., Lin Y.-M., Chen F.-C., Tokuda H., Matsumoto T., *Syoyakugaku Zasshi*, **42**, 343 (1988).
14) Yamamoto Y., Mizuguchi R., Yamada Y., *Agric. Biol. Chem.*, **49**, 3347 (1985).
15) Higuchi M., Miura Y., Boohene J., Kinoshita Y., Yamamoto Y., Yoshimura I., Yamada Y., *Planta Medica*, **59**, 253 (1993).
16) Ahmadjian V., *Bryologist*, **64**, 168 (1961).