Anti-Tumor-Promoting Activities of Euglobals from *Eucalyptus* Plants

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To search for possible anti-tumor-promoters (chemopreventive agents), we carried out a primary screening of 21 euglobals (acylphloroglucinol-monoterpene or -sesquiterpene structures) isolated from the juvenile leaves of five species of *Eucalyptus* plants using an *in vitro* synergistic assay system. Of these compounds, euglobal-G1—-G5 (1—5), -Am-2 (15) and -III (16) exhibited significant inhibitory effects on Epstein-Barr virus (EBV) activation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Furthermore, the effects of compounds 1 and 16 on the cell cycle of Raji cells were also examined by a flow cytometer, and both compounds 1 and 16 exhibited strong inhibition on the effect of the cell cycle induced by TPA. These two euglobals (1 and 16) exhibited remarkable anti-tumor-promoting effects on mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis test.

Key words euglobal; anti-tumor-promoter; cell cycle; *Eucalyptus* plant; euglobal-G1; euglobal-III

The mechanism of chemical carcinogenesis has been explained by a two-stage theory or multi-stage theory,[1] and the development of anti-tumor-promoters has been regarded as the most effective method for the chemoprevention of cancer.[2] As a continuation of our chemical and biological studies on potential anti-tumor-promoters (chemopreventive agents), we carried out a primary screening of many kinds of natural products (triterpenoids,[3] flavonoids,[4] quinones,[5] crude drugs[6] and kampo prescriptions[7]) using their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Further, many compounds, which inhibit EBV-EA induction by tumor promoters and also exhibit anti-inflammatory activities, have been shown to act as inhibitors of tumor-promotion *in vivo*.[8]

On the other hand, we reported the isolation and structural elucidation of many kinds of euglobals (**1**—**21**) which have unique structures (acylphloroglucinol-monoterpene or -sesquiterpene structures) from some species of *Eucalyptus* genus (Myrtaceae).[9] The anti-inflammatory activities of several euglobals, indicated by the inhibition of exuberant granulation using chick embryo, have been also reported.[10]

In this paper, we report the results of the primary screening test on the inhibitory effects of these 21 new euglobals on EBV-EA activation. Furtheremore, the results of the cell cycle analysis on Raji cells using flow cytometry, and an *in vivo* two-stage carcinogenesis test on mouse skin tumor promotion of **1** and **16**, which exhibited strong inhibitory effects on EBV-EA activation, will be reported.

MATERIALS AND METHODS

Cells The EBV genome-carrying lymphoblastoid cells (Raji cells derived from Barkitt's lymphoma) were cultured in RPMI-1640 medium (Nissui) under the conditions described before.[4] Spontaneous activation of EBV-EA in our subline Raji cells was less than 0.1%.

Chemicals The tissue culture reagents, *n*-butyric acid and other reagents, were purchased from Nakalai Tesque (Kyoto, Japan). TPA, 7,12-dimethylbenz[*a*]anthracene (DMBA), and ribonuclease (RNase) were obtained from Sigma Chemical Co. (U.S.A.). EBV-EA positive serum, from a patient with nasopharyngeal carcinoma and used for an immunofluorescence test, was a gift from the Department of Otorhinolaryngology, Kobe University.

Animals Specific pathogen-free female ICR mice (6 weeks old) were obtained from Nippon SLC Co., Ltd. (Shizuoka, Japan), and housed in polycarbonate cages in a temperature-controlled room.

Isolation of Euglobals Euglobal-G1—-G5 (**1**—**5**) were isolated from the juvenile leaves of *Eucalyptus grandis* W. HILL,[9a] and euglobal-T1 (**6**) and -IIc (**7**) were isolated from the juvenile leaves of *E. tereticornis* SMITH,[9b] and euglobal-Am-2 (**15**), -IVb (**18**) and -VII (**20**) were isolated from *E. amplifolia* NAUDIN,[9c] and these plants were collected at Higashiyama Zoological and Botanical Garden, in January, 1988, in Nagoya, Japan. Euglobal-Bl-1—-IIa (**10**—**13**) were isolated from the juvenile leaves of *E. blakelyi* MAIDEN,[9c] which was collected in April, 1990, in Australia, and euglobal-III (**16**), -V (**19**) and -In-1 (**21**) were isolated from *E. incrassata* LABILL,[9d] collected in May 1990, in Australia. Euglobal-Ia$_1$ (**8**), -Ia$_2$ (**9**), -IIb (**14**) and -IVa (**17**) were isolated from *E. globulus* LABILL collected in Oct., 1979, in Kyoto, Japan.[9e] The details of the extraction, purification and chemical structural elucidation of these euglobals have been reported in previous literature.[9a-e]

***In Vitro* EBV-EA Activation Experiments** The inhibition of EBV-EA activation was assayed using the same method described previously.[3] The cells were incubated for 48 h at 37 °C in a medium (1 ml) containing *n*-butyric acid (4 mmol), TPA (32 pmol) and various amounts of the test compounds in dimethylsulfoxide (5 μl). Smears were made from the cell suspension and the EBV-EA inducing cells were stained by means of an indirect immunofluorescence technique.[11] In each assay, at least 500 cells were counted and the number of stained cells (positive cells) among them was recorded. Triplicate assays were performed for each data point. The EBV-EA inhibitory activity of the test compound was compared with that of the control experiment (100%) with *n*-butyric acid plus

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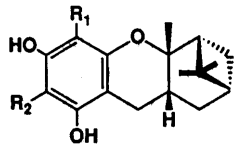
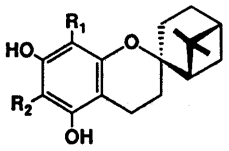
TPA. In the experiments, the EBV-EA activities were ordinarily around 40%, and these values were taken as the positive control (100%). The viability of Raji cells was assayed against treated cells by the trypan-blue staining method.

Cell Cycle Analysis by Flow Cytometry The cellular deoxyribonucleic acid (DNA) content of Raji cells was measured by flow cytometry. Fluorescence spectra were obtained and accomplished on a commercially available FAC Scan (Becton and Dikinson). The cells (1×10^6/ml), cultured using the same method as in the EBV-EA inhibitory assay, in plastic tubes were stained with propidium iodide by a rapid staining technique.[12] The nonionic detergent Triton × 100 (Nacalai Tesque Co., Ltd.) 0.1% was added to the tubes for the purpose of lysis of the cell membrane. Treated Raji cells were filtered through a 37 μ-pore nylon filter before staining. Treatment of RNase in phosphate-buffered saline (PBS) (final 0.1%) decreased the fluorescence intensities of RNA. Finally, we used propidium iodide (final: 50 μg/ml) for viable DNA staining. The flow cytometric analysis was carried out with an FAC Scan cell fit DNA system and the cell cycle pattern was analyzed by its program.

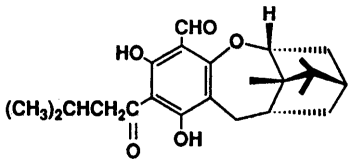
***In Vivo* Two-Stage Carcinogenesis Test on Mouse Skin Papillomas** Each group was composed of 15 mice housed five per cage and given water *ad libitum*. The back of each mouse was shaved with surgical clippers. The mice were carcinogenically initiated with DMBA (100 μg, 390 nmol) in acetone (0.1 ml). One week after initiation, carcinogenic growth was promoted twice a week by the application of TPA (1 μg, 1.7 nmol) in acetone (0.1 ml). At the same time, the mice were classified into the following three groups. Group I, the positive control group, was given TPA (1.7 nmol) in acetone (0.1 ml) alone. Group II mice,



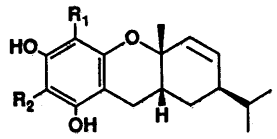
euglobal-G1 (1) R_1=isovaleroyl, R_2=aldehyde
euglobal-G2 (2) R_1=aldehyde, R_2=isovaleroyl



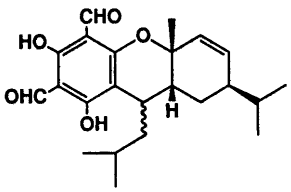
euglobal-G3 (3) R_1=aldehyde, R_2=isovaleroyl
euglobal-G4 (4) R_1=isovaleroyl, R_2=aldehyde



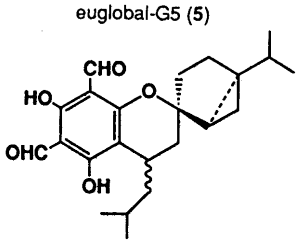
euglobal-G5 (5)



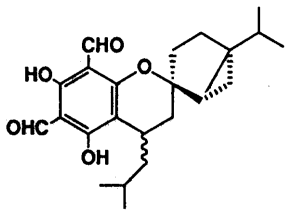
euglobal-T1 (6) R_1=isovaleroyl, R_2=aldehyde
euglobal-IIc (7) R_1=aldehyde, R_2=isovaleroyl



7β-isobutyl euglobal-Ia$_1$ (8) 7α-isobutyl euglobal-Ia$_2$ (9)



7β-isobutyl euglobal-BI-1 (10) 7α-isobutyl euglobal-Ib (11)



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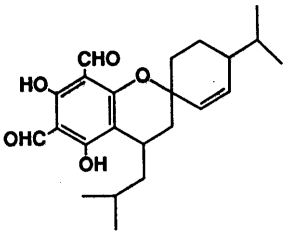
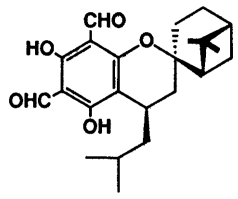
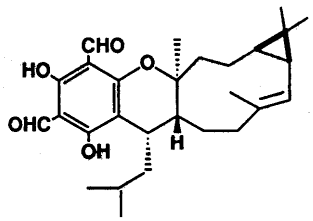


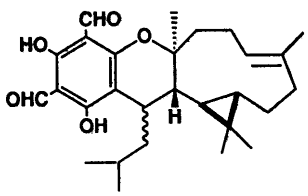
euglobal-IIb (14)



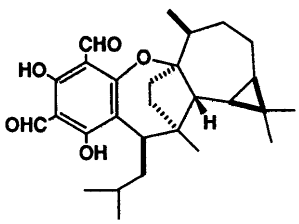
euglobal-Am-2 (15)



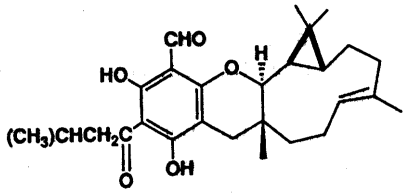
euglobal-III (16)



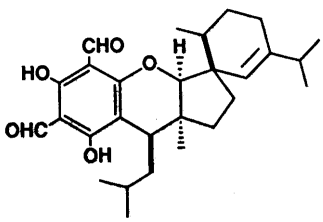
7α-isobutyl euglobal-IVa (17) 7β-isobutyl euglobal-IVb (18)



euglobal-V (19)



euglobal-VII (20)



euglobal-In-1 (21)

Chart 1

the treated group, were treated with each test compound (85 nmol) in acetone (0.1 ml) 1 h before each TPA treatment. Group III, the control group, was treated with glycyrrhetic acid (85 nmol) in acetone (0.1 ml) 1 h before each TPA treatment. The incidence of papillomas was observed weekly for 20 weeks, on the percentages of mice bearing papillomas and the average number of papillomas per mouse.[2,13]

RESULTS AND DISCUSSION

The primary screening test of euglobals (**1**—**21**) was carried out utilizing a short-term *in vitro* synergistic assay on EBV-EA activation. Their inhibitory effects on EBV-EA activation induced by TPA and the viability of Raji cells are shown in Table I. In these euglobals, euglobal-G1 (**1**), -G2 (**2**), -G4 (**4**), -G5 (**5**) and -Am-2 (**15**), which have acylphloroglucinol-monoterpene structures, exhibited significant inhibitory effects on EBV-EA activation (100% inhibition of activation at 1×10^3 mol ratio/TPA, more than 70% inhibition of activation at 5×10^2 mol ratio/TPA and 25—55% inhibition of activation even at 1×10^2 mol ratio/TPA) and preserved the high viability of Raji cells even at a high concentration. On the other hand, euglobal-Ia_1 (**8**), -Ia_2 (**9**) and -IIb (**14**) showed strong cytotoxicity on Raji cells (exhibiting less than 40% viability of Raji cells at 1×10^3 and 5×10^2 mol ratio/TPA).[14] Furthermore, in euglobals which have an acylphloroglucinol-sesquiterpene structure (**16**—**21**), euglobal-III (**16**) exhibited significant inhibitory effects (100% and 70% inhibition of activation at 1×10^3 mol ratio and 5×10^2 mol ratio/TPA, respectively) and preserved the high viability of Raji cells. And euglobal-IVa (**17**), -IVb (**18**) and -VII (**20**) showed strong cytotoxicity on Raji cells (exhibiting less than 30% viability of Raji cells even at 5×10^2 mol ratio/TPA).[14] In our experiments, these inhibitory activities of **1**, **2**, **4**, **5**, **15** and **16** were stronger than those of glycyrrhetic acid, which is known as the one of stronger anti-tumor-promoters.[15] Furthermore, many natural products which strongly inhibit EBV-EA activation induced by the tumor promoter, TPA, have been shown to act as inhibitors of tumor promotion *in vivo*.[3,4,8]

The effects of **1** and **16** on the cell cycle of Raji cells treated with TPA were also examined by flow cytometry. As shown in Table II, the promoter TPA increased the percentage of the G_2 and M phase of Raji cells and decreased the percentages of both the G_1 and S phase in comparison with those of the negative control cultivated without TPA. When treated with euglobal-G1 (**1**), the percentages of both G_1 and S phase were on the increase and the percentage of the G_2 and M phase was on the decrease (57.2% of G_1, 24.0% of S, and 18.8% of G_2 and M at 3.2 nmol of **1**, and 60.8% of G_1, 28.8% of S, and 10.4% of G_2 and M at 32 nmol of **1**) as compared with the positive control treated with TPA. When treated with euglobal-III (**16**), the same tendency was observed as in the cells treated with compound **1**. From these results, it was deduced that compounds **1** and **16** accumulated in Raji cells in the S phase dependent on the concentration of **1** and **16**, and that consequently, the percentage of the G_2 and M phase was restored to a normal value. Therefore, compounds **1** and **16** also strongly inhibited the one of the biological activities, by influencing the cell cycle, of TPA.

On the basis of the results of the *in vitro* assays described above (inhibitory effects on EBV-EA activation and effects on the cell cycle induced by the promoter, TPA), the inhibitory effects of euglobal-G1 (**1**) and -III (**16**) on the two-stage carcinogenesis test *in vivo* using DMBA as an initiator and TPA as a promoter would be expected.[16] The inhibitory activities, evaluated by both the rate (%) of papilloma-bearing mice (Fig. 1A) and the average number of papillomas per mouse (Fig. 1B), were compared with those of the positive control group and those of the control group which was treated with glycyrrhetic acid.

In the positive control, more than 80% and 100% of mice bore papillomas even at 7 and 9 weeks of promotion,

TABLE I. Relative Ratio[a,b] of EBV-EA Activation with Respect to Positive Control (100%) in Presence of Euglobals (**1**—**21**) from *Eucalyptus* Plants

Samples	Concentration[c]			
	1000	500	100	10
Euglobal-G1 (**1**)	0.0 (50)	15.6 (>80)	70.3 (>80)	100.0 (>80)
Euglobal-G2 (**2**)	0.0 (60)	25.4 (>80)	73.3 (>80)	100.0 (>80)
Euglobal-G3 (**3**)	10.5 (>80)	23.6 (>80)	65.7 (>80)	100.0 (>80)
Euglobal-G4 (**4**)	0.0 (70)	26.2 (>80)	68.4 (>80)	100.0 (>80)
Euglobal-G5 (**5**)	0.0 (70)	0.0 (>80)	55.7 (>80)	93.8 (>80)
Euglobal-T1 (**6**)	15.6 (>80)	66.9 (>80)	91.3 (>80)	100.0 (>80)
Euglobal-IIc (**7**)	7.8 (>80)	62.2 (>80)	89.5 (>80)	100.0 (>80)
Euglobal-Ia_1 (**8**)	—[d] (0)	0.0 (20)	31.5 (>80)	100.0 (>80)
Euglobal-Ia_2 (**9**)	—[d] (0)	0.0 (20)	38.9 (>80)	89.5 (>80)
Euglobal-BI-1 (**10**)	27.3 (70)	38.1 (>80)	54.5 (>80)	100.0 (>80)
Euglobal-Ib (**11**)	18.2 (70)	27.3 (>80)	51.5 (>80)	85.1 (>80)
Euglobal-Ic (**12**)	12.1 (60)	30.3 (>80)	69.7 (>80)	100.0 (>80)
Euglobal-IIa (**13**)	13.6 (60)	43.7 (>80)	78.5 (>80)	100.0 (>80)
Euglobal-IIb (**14**)	0.0 (20)	0.0 (40)	73.6 (70)	100.0 (>80)
Euglobal-Am-2 (**15**)	0.0 (70)	15.3 (>80)	45.2 (>80)	79.6 (>80)
Euglobal-III (**16**)	0.0 (60)	28.9 (>80)	80.5 (>80)	100.0 (>80)
Euglobal-IVa (**17**)	0.0 (20)	0.0 (30)	59.1 (>80)	100.0 (>80)
Euglobal-IVb (**18**)	0.0 (10)	0.0 (20)	68.7 (>80)	91.3 (>80)
Euglobal-V (**19**)	14.7 (70)	56.4 (>80)	87.1 (>80)	100.0 (>80)
Euglobal-VII (**20**)	0.0 (20)	11.4 (30)	70.2 (>80)	100.0 (>80)
Euglobal-In-1 (**21**)	16.3 (70)	68.3 (>80)	90.5 (>80)	100.0 (>80)

a) Values represent relative percentages to the positive control value (100%). *b*) Values in parentheses are viability percentages of Raji cells. *c*) Mol ratio/TPA (20 ng = 32 pmol/ml). *d*) Not detected.

TABLE II. Flow Cytometric Analysis of Raji Cell Cycle Treated with Compounds **1** and **16**[a]

		Phase			Total
		G_1	S	G_2+M	
Medium only[b]		61.7	27.9	10.4	100.0
Positive control[c]		53.6	8.4	38.0	100.0
Treated with	32.0 nmol[f]	60.8	28.8	10.4	100.0
euglobal-G1 (**1**)[d]	3.2 nmol	57.2	24.0	18.8	100.0
	0.32 nmol	52.7	8.2	39.1	100.0
Treated with	32.0 nmol	62.0	29.1	8.9	100.0
euglobal-III (**16**)[e]	3.2 nmol	60.5	25.6	13.9	100.0
	0.32 nmol	54.5	8.0	37.5	100.0

a) Percentages of Raji cells in each phase. *b*) Raji cells cultivated in RPMI-1640 medium (1 ml) containing 10% fetal calf serum. *c*) Treated with TPA (32 pmol) and *n*-butyric acid. *d*) Treated with TPA (32 pmol), *n*-butyric acid and compound **1**. *e*) Treated with TPA (32 pmol), *n*-butyric acid and compound **16**. *f*) 32, 3.2 and 0.32 nmol = 1000, 100 and 10 mol ratio/TPA.

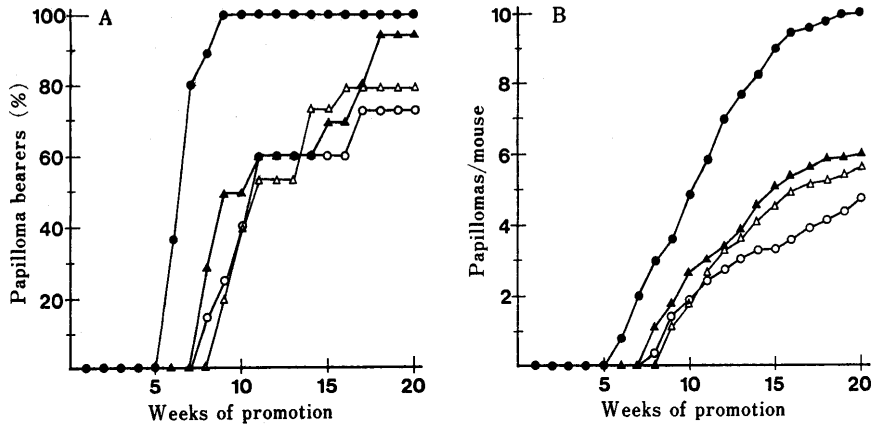


Fig. 1. Inhibition of TPA-Induced Tumor Promotion by Multiple Application of Euglobal-G1 (**1**), -III (**16**) and Glycyrrhetic Acid

All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA given twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas. B: average numbers of papillomas per mouse. ●, control TPA alone; ▲, TPA + 85 nmol of glycyrrhetic acid; ○, TPA + 85 nmol of euglobal-G1 (**1**); △, TPA + 85 nmol of euglobal-III (**16**).

respectively, as shown in Fig. 1A. Further, more than 5 and 10 papillomas were formed per mouse even at 10 and 20 weeks of promotion, respectively, as shown in Fig 1B. On the other hand, when euglobal-G1 (**1**) and -III (**16**) were applied continuously before each TPA treatment, they delayed the formation of papillomas in mouse skin and reduced the number of papillomas per mouse as follows. In the group treated with **1**, only about 40% and 70% of mice bore papillomas at 10 and 20 weeks of promotion, respectively. And 2 papillomas were formed at 10 weeks and only 4.5—5 papillomas were formed per mouse even at 20 weeks of promotion. Further, in the group treated with **16**, only about 40% and 80% of mice bore papillomas at 10 weeks and 20 weeks of promotion, respectively. And 2 papillomas were formed at 10 weeks and 5.5—6 papillomas were formed per mouse even at 20 weeks of promotion. Consequently, these euglobals (**1**, **16**) exhibited about 55% and 44% inhibition, respectively, even at 20 weeks of promotion, as shown in Fig. 1B. These results suggested that the inhibitory effects of **1** were stronger than those of glycyrrhetic acid, and the effects of **16** were similar to those of glycyrrhetic acid. These results from the two-stage carcinogenesis tests suggest that euglobal-G1 (**1**) and -III (**16**) might be valuable as anti-tumor-promoters in chemical carcinogenesis. The inhibitory mechanism of these compounds on tumor promotion and the inhibitory effects on other forms of carcinogenesis (pulmonary tumor or liver carcinoma) are now being studied.

REFERENCES AND NOTES

1) I. Berenblum, *Cancer Res.*, **1**, 807 (1941).
2) N. Yamamoto, K. Bister, H. Zur Hausen, *Nature* (London), **278**, 553 (1797); A. K. Verma, T. J. Slaga, P. W. Werz, G. C. Mueller, R. K. Boutwell, *Cancer Res.*, **40**, 2367 (1980); T. Kakizoe, K. Takai, K. Tobisu, M. Ohtani, S. Sato, *Jpn. J. Cancer Res.* (*Gann*), **79**, 235 (1988); Y. Takaishi, K. Ujita, H. Tokuda, H. Nishino, A. Iwashima, T. Fujita, *Cancer Lett.*, **1992**, 19, and references cited therein.
3) T. Konoshima, M. Takasaki, M. Kozuka, H. Tokuda, *J. Nat. Prod.*, **50**, 1167 (1987); H. Tokuda, T. Konoshima, M. Kozuka, T. Kimura, *Cancer Lett.*, **1988**, 309; T. Konoshima, M. Takasaki, T. Tatsumoto, M. Kozuka, R. Kasai, O. Tanaka, R. L. Nie, H. Tokuda, H. Nishino, A. Iwashima, *Biol. Pharm. Bull.*, **17**, 668 (1994); T. Konoshima, M. Takasaki, M. Kozuka, T. Nagao, H. Okabe, N. Irino, T. Nakasumi, H. Tokuda, H. Nishino, *ibid.*, **18**, 284 (1995).
4) T. Konoshima, E. Okamoto, M. Kozuka, H. Nishino, H. Tokuda, M. Tanabe, *J. Nat. Prod.*, **51**, 1266 (1988); T. Konoshima, M. Takasaki, M. Kozuka, A. Inada, T. Nakanishi, H. Tokuda, T. Matsumoto, *Shouyakugaku Zasshi*, **43**, 135 (1989); T. Konoshima, M. Kokumai, M. Kozuka, M. Iinuma, M. Mizuno, T. Tanaka, H. Tokuda, H. Nishino, A. Iwashima, *Chem. Pharm. Bull.*, **40**, 531 (1992).
5) T. Konoshima, M. Kozuka, J. Koyama, T. Okatani, K. Tagahara, H. Tokuda, *J. Nat. Prod.*, **52**, 987 (1989).
6) T. Konoshima, H. Tokuda, M. Kozuka, E. Okamoto, M. Tanabe, *Shouyakugaku Zasshi*, **41**, 344 (1987).
7) T. Konoshima, M. Takasaki, M. Kozuka, H. Tokuda, *Yakugaku Zasshi*, **114**, 248 (1994), and references cited therein.
8) H. Tokuda, T. Konoshima, M. Kozuka, T. Kimura, *Oncology*, **48**, 77 (1991); K. Ujita, Y. Takaishi, H. Tokuda, H. Nishino, A. Iwashima, T. Fujita, *Cancer Lett.*, **1993**, 129.
9) *a*) M. Takasaki, T. Konoshima, T. Shingu, H. Tokuda, H. Nishino, A. Iwashima, M. Kozuka, *Chem. Pharm. Bull.*, **38**, 1444 (1990); M. Takasaki, T. Konoshima, M. Kozuka, M. Haruna, K. Ito, T. Shingu, *ibid.*, **42**, 2591 (1994); *b*) M. Kokumai, T. Konoshima, M. Kozuka, M. Haruna, K. Ito, *J. Nat. Prod.*, **54**, 830 (1991); *c*) M. Takasaki, T. Konoshima, M. Kozuka, M. Haruna, K. Ito, S. Yoshida, *Chem. Pharm. Bull.*, **42**, 2177 (1994); *d*) M. Takasaki, T. Konoshima, M. Kozuka, M. Haruna, K. Ito, W. D. Crow, D. M. Paton, *ibid.*, **42**, 2113 (1994); *e*) M. Kozuka, T. Sawada, F. Kasahara, E. Mizuta, T. Amano, T. Komiya, M. Goto, *ibid.*, **30**, 1952 (1982).
10) M. Kozuka, T. Sawada, F. Kasahara, E. Mizuta, T. Amano, T. Komiya, M. Goto, *Chem Pharm. Bull.*, **30**, 1964 (1982).
11) G. Henle, W. Henle, *J. Bacteriol.*, **91**, 1248 (1966).
12) I. W. Taylor, *J. Histochem. Cytochem.*, **28**, 102 (1980); M. Takahashi, *Rinshou Kensa*, **30**, 1258 (1986); T. Shimomatsuya, N. Tanigawa, R. Muraoka, *Jpn. J. Cancer Res.*, **82**, 357 (1991).
13) C. E. Weeks, T. J. Slaga, H. Hennings, G. L. Gleason, W. M. Bracken, *J. Natl. Cancer Inst.*, **63**, 401 (1979); H. Tokuda, H. Ohigashi, K. Koshimizu, Y. Ito, *Cancer Lett.*, **1986**, 279.
14) A high viability of Raji cells is needed for our *in vitro* assay using indirect immunofluorescens technique by antigen–antibody reaction and is beneficial for the following *in vivo* assay.
15) K. Mizutani, "Food Phytochemicals for Cancer Prevention II, Teas, Spices and Herbs," ed. by C.-T. Ho, T. Osawa, M.-T. Huang, R. T. Rosen, American Chemical Society, Washington, DC, 1994, Chapter 32, p.322, and in our *in vitro* assay, the relative ratios of EBV-EA activation with respect to the positive control (100%) in presence of glycyrrhetic acid were 15.6, 54.3, 100 and 100% at 1×10^3, 5×10^2, 1×10^2 and 1×10 mol ratio/TPA, respectively, and the viability percentage of Raji cells was more than 80% at each concentration.
16) The inhibitory effects of **5** and **15** on EBV-EA activation were stronger than those of **1** and **16**, and the inhibitory effects of **2** and **4** were similar to those of **1** and **16**, but the yields of **2**, **4**, **5** and **15** from the plant were deficient for *in vivo* biological assay.