Inhibitors of Skin-Tumor Promotion. VIII.[1,2] Inhibitory Effects of Euglobals and Their Related Compounds on Epstein-Barr Virus Activation. (1)

Midori TAKASAKI,[a] Takao KONOSHIMA,[a] Kazuyoshi FUJITANI,[a] Shigeo YOSHIDA,[b] Hiroyuki NISHIMURA,[c] Harukuni TOKUDA,[d] Hoyoku NISHINO,[d] Akio IWASHIMA,[d] and Mutsuo KOZUKA*,[a]

Kyoto Pharmaceutical University,[a] 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607, Japan, Institute of Physical and Chemical Research,[b] 2-1 Hirosawa, Wako, Saitama 351-01, Japan, Department of Bioscience and Technology, School of Engineering, Hokkaido Tokai University,[c] Sapporo 005, Japan, and Department of Biochemistry, Kyoto Prefectural University of Medicine,[d] Kawaramachi-dori, Hirokoji, Kamigyo-ku, Kyoto 602, Japan.
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Twelve euglobals from *Eucalyptus globulus* and their twenty-six related compounds were examined for their inhibitory effects on Epstein-Barr virus activation by a short-term *in vitro* assay. The results showed that most of the euglobals having monoterpene structures, and euglobal-III (8) had strong inhibitory activity. Grandinol (18), homograndinols (19 and 20), and compounds 26, 27, 28, and 32 showed stronger inhibitory effects. Based on the results, the structural requirements for the activity of these compounds were discussed.

Keywords Epstein-Barr virus; euglobal; acylphloroglucinol; monoterpenoid; sesquiterpenoid; *Eucalyptus globulus*; TPA, anti-tumor-promoter; structural requirement

Twelve euglobals, compounds having novel acylphloroglucinol-monoterpene (or -sesquiterpene) structures, were isolated from leaves and flower buds of *Eucalyptus globulus* LABILL.[3] These compounds showed anti-inflammatory activity in a screening test using chick embryo,[4] as they have had strong inhibition of exuberant granulation.[5]

We have reported the inhibitory effects of several natural products including flavonoids,[6a] steroids,[1] triterpenoids,[6b] triterpenoid saponins,[6c] quinones,[6d] and crude drugs[6e] on 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced Epstein-Barr virus early antigen (EBV-EA) activation, as a result of screening test for anti-tumor-promoting activity.

To search for possible anti-tumor-promoters, we examined the inhibitory tendency of the euglobals and their related compounds on the EBV-EA activation, as most known anti-tumor-promoters also show anti-inflammatory activity on the skin of animals.[7]

Experimental

Materials Samples tested were obtained from the following sources: euglobal-Ia$_1$ (**1**),[3a] euglobal-Ia$_2$ (**2**),[3a] euglobal-Ib (**3**),[3a] euglobal-Ic (**4**),[3a] euglobal-IIa (**5**),[3a] euglobal-IIb (**6**),[3a] euglobal-IIc (**7**),[3a] euglobal-III (**8**),[3a] euglobal-IVa (**9**),[3b] euglobal-IVb (**10**),[3b] euglobal-V (**11**),[3b] and euglobal-VII (**12**)[3b] were isolated from the flower buds of *Eucalyptus globulus*. Aromadendrene (**15**),[8] and globulol (**16**),[8] were obtained from the essential oil of fruits of *Eucalyptus globulus*, and virdiflorol (**17**)[9] from the extract of *Mentha spicata*; their structures were confirmed by nuclear magnetic resonance (NMR) and mass spectra (MS). α-Phellandrene (**13**) and sabinene hydrate (**14**) were provided by Nippon Terpene Chemical Company, Ltd., Kobe, Japan. Grandinol (**18**),[10] (*S*)-homograndinol (**19**),[11] (±)homograndinol (**20**)[11] were synthesized according to the reported methods. Compounds **21**, **22**, **24**—**29**, **31**—**35**, **37**, and **38** were prepared by known methods.[12] Compound **30**[13a] was obtained by a Vilsmeier–Haack reaction on phloroglucinol (**23**). Compound **36**[13b] was synthesized by a Friedel–Crafts reaction on **23** with isovaleryl chloride/titanium (IV) chloride. Phloroglucinol (**23**) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Biological Activities The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer), the EBV genome-carrying human lymphoblastoid cells, which were cultivated in 8% FBS RPMI 1640 medium (Nissui). These indicator cells (Raji) (1×10^6/ml) were incubated at 37 °C for 48 h in 1 ml of a medium containing *n*-butyric acid (4 mM, inducer), TPA (20 ng = 32 pmol/ml in dimethyl sulfoxide (DMSO)) and a known amount of test compound in DMSO. Smears were made from the cell suspension. The activated cells were stained by high-titer EBV-EA positive sera from nasopharyngeal carcinoma (NPC) patients and detected by an indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the experiments were repeated three times. The average EA induction was compared with that of positive control experiments with *n*-butyric acid (4 mM) plus TPA (20 ng/ml). In the experiments, the EA induction values were ordinarily around 40%, and these values were taken as positive control (100%). In this screening method, the cell viability required for the judgment of inhibitory effects was more than 60%.[7]

Results and Discussion

As shown in the tables, euglobals, mono- and sesquiterpenoids related to euglobal structures, and acylphloroglucinols including non-natural synthetic compounds were tested for their inhibitory activity using short-term *in vitro* assay of EBV-EA activation in Raji cells induced by TPA. Their inhibitory effects on the activation and viabilities of Raji cells are shown in Tables I—III.

Among the compounds in Table I, euglobal-III (**8**) showed strong inhibitory activity, and euglobals-Ib (**3**) and -IIa (**5**) exhibited highly significant activities at 1000 mol/TPA ratios, respectively. Euglobal-Ic (**4**) was also noted for its activity at 1000 mol/TPA and 100 mol/TPA ratios. Euglobals-Ia$_1$ (**1**) and -Ia$_2$ (**2**) had considerable activity at 100 molar ratio per TPA but both indicated strong

TABLE I. Inhibitory Effects of Treatment with Euglobals on TPA Induced EBV-EA Activation

Sample	Concentration[a] 1000	100	10	1
Euglobal-Ia$_1$ (**1**)	0.0[b](0)[c]	31.5 (80)	100.0 (>80)	100.0 (>80)
Euglobal-Ia$_2$ (**2**)	0.0 (0)	38.9 (80)	89.5 (>80)	100.0 (>80)
Euglobal-Ib (**3**)	12.5 (70)	83.5 (80)	100.0 (>80)	100.0 (>80)
Euglobal-Ic (**4**)	21.1 (60)	51.3 (80)	95.1 (>80)	100.0 (>80)
Euglobal-IIa (**5**)	13.6 (60)	78.5 (80)	100.0 (>80)	100.0 (>80)
Euglobal-IIb (**6**)	0.0 (20)	73.6 (70)	92.3 (>80)	100.0 (>80)
Euglobal-IIc (**7**)	10.2 (30)	73.5 (80)	96.6 (>80)	100.0 (>80)
Euglobal-III (**8**)	0.0 (50)	2.7 (60)	100.0 (>80)	100.0 (>80)
Euglobal-IVa (**9**)	0.0 (20)	58.1 (80)	100.0 (>80)	100.0 (>80)
Euglobal-IVb (**10**)	0.0 (10)	68.7 (80)	91.3 (>80)	100.0 (>80)
Euglobal-V (**11**)	60.1 (70)	100.0 (>80)	100.0 (>80)	100.0 (>80)
Euglobal-VII (**12**)	0.0 (20)	70.2 (80)	84.3 (>80)	100.0 (>80)

a) Mol ratio/TPA (20 ng = 32 pmol/ml). *b*) Values represent relative percentages to the positive control value (100%). *c*) Values in parentheses are viability percentages of Raji cells.

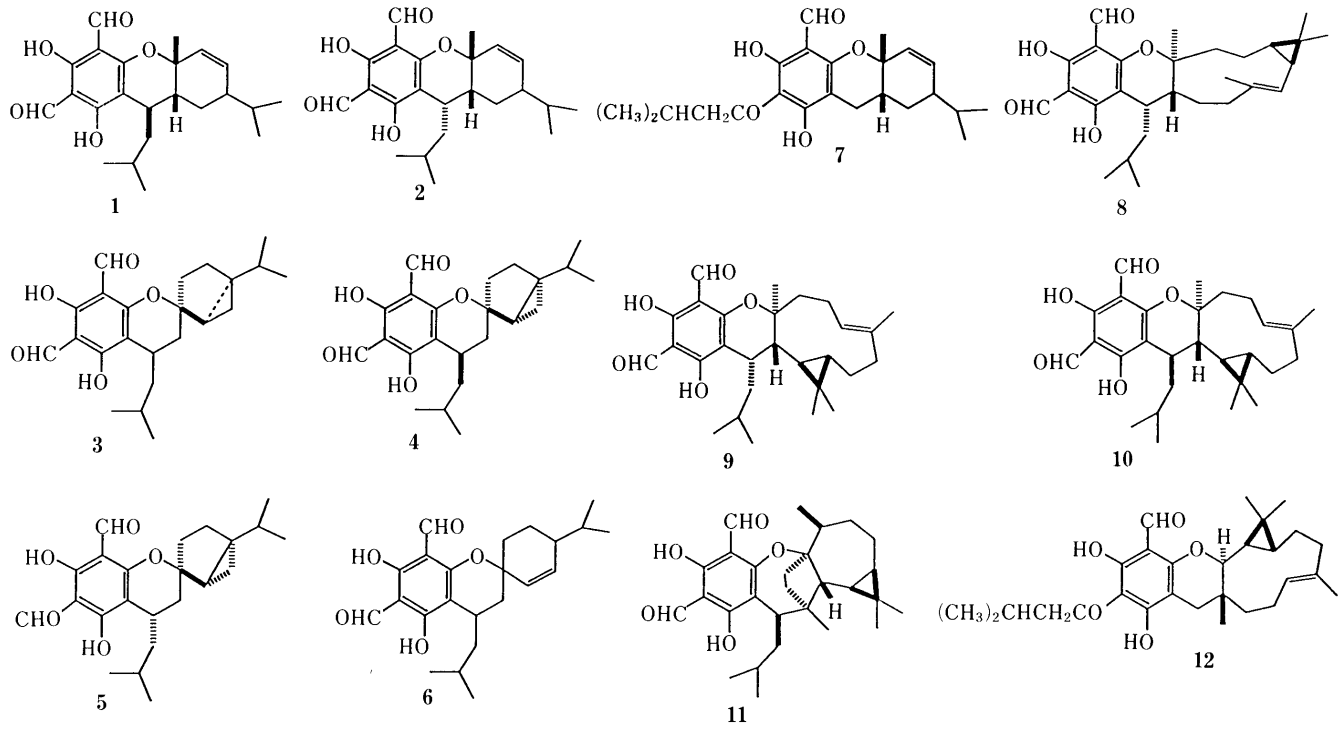

Chart 1

Table II. Inhibitory Effects of Treatment with Some Terpenoids on TPA Induced EBV-EA Activation

Sample	Concentration[a] 1000	100	10	1
α-Phellandrene (**13**)	69.6[b](80)[c]	76.4 (>80)	90.9 (>80)	100.0 (>80)
Sabinene hydrate (**14**)	42.4 (70)	56.3 (>80)	75.8 (>80)	100.0 (>80)
Aromadendrone (**15**)	33.3 (80)	63.6 (>80)	90.9 (>80)	100.0 (>80)
Globulol (**16**)	0.0 (0)	0.0 (0)	70.0 (10)	100.0 (>80)
Viridoflorol (**17**)	0.0 (0)	0.0 (0)	100.0 (70)	100.0 (>80)

a) Mol ratio/TPA (20 ng = 32 pmol/ml). *b*) Values represent relative percentages to the positive control value (100%). *c*) Values in parentheses are viability percentages of Raji cells.

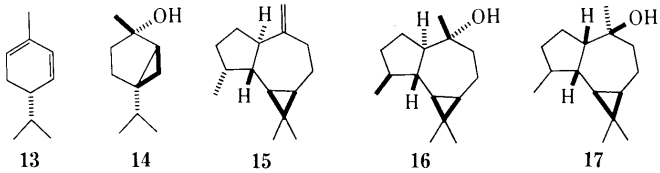

Chart 2

Table III. Inhibitory Effects of Treatment with Some Acylphloroglucinols on TPA Induced EBV-EA Activation

Sample	Concentration[a] 1000	100	10	1
Grandinol (**18**)	0.0[b](20)[c]	15.8 (60)	55.8 (>80)	89.3 (>80)
(*S*)-Homograndinol (**19**)	0.0 (20)	0.0 (70)	86.0 (70)	100.0 (>80)
(±)-Homograndinol (**20**)	0.0 (0)	0.0 (60)	78.5 (>80)	100.0 (>80)
21	0.0 (40)	60.2 (60)	100.0 (>80)	100.0 (>80)
22	0.0 (60)	78.8 (>80)	80.0 (>80)	100.0 (>80)
Phloroglucinol (**23**)	70.0 (60)	88.9 (>80)	100.0 (>80)	100.0 (>80)
24	36.8 (50)	100.0 (>80)	100.0 (>80)	100.0 (>80)
25	10.5 (40)	71.5 (60)	100.0 (>80)	100.0 (>80)
26	0.0 (0)	10.5 (70)	52.3 (>80)	100.0 (>80)
27	0.0 (60)	0.0 (>80)	62.6 (>80)	90.9 (>80)
28	0.0 (40)	5.3 (70)	81.2 (>80)	100.0 (>80)
29	0.0 (0)	55.2 (60)	83.5 (>80)	100.0 (>80)
30	0.0 (40)	41.6 (60)	85.3 (>80)	100.0 (>80)
31	0.0 (0)	26.8 (80)	62.5 (>80)	100.0 (>80)
32	0.0 (0)	0.0 (0)	10.0 (60)	52.6 (>80)
33	0.0 (40)	58.2 (80)	100.0 (>80)	100.0 (<80)
34	0.0 (0)	20.8 (70)	89.5 (>80)	100.0 (>80)
35	0.0 (50)	22.5 (60)	77.8 (>80)	100.0 (>80)
36	19.5 (50)	80.2 (>80)	100.0 (>80)	100.0 (>80)
37	0.0 (0)	63.6 (60)	83.8 (>80)	100.0 (>80)
38	0.0 (20)	69.6 (80)	100.0 (>80)	100.0 (>80)

a) Mol ratio/TPA (20 ng = 32 pmol/ml). *b*) Values represent relative percentages to the positive control value (100%). *c*) Values in parentheses are viability percentages of Raji cells.

cytotoxicity on Raji cells at 1000 mol/TPA ratio. Euglobal-V (**11**) was not effective at low concentration (under 100 mol/TPA ratio), while euglobals-IIb (**6**), -IVa (**9**), -IVb (**10**) and euglobals having isovaleryl group on the aromatic ring, *e.g.* euglobals-IIc (**7**) and -VII (**12**) showed weak activity and gave strong cytotoxicity on Raji cells at 100 and 1000 mol ratios, respectively. These results led us to investigate the structure requirements for the activity of euglobals.

As shown in Table II, two monoterpenoids (**13** and **14**) and three sesquiterpenoids (**15**—**17**) were tested and the results showed that their activities were insignificant. The essential moiety for the activity of euglobals was then considered to be the presence of the acylphloroglucinol structures. Thus, phloroglucinol (**23**) and nineteen acylphloroglucinols related to the euglobals were subjected to bioassay (Table III). Of the compounds having closely related structures with the acylphloroglucinol moieties of the euglobals, grandinol (**18**), (*S*)-homograndinol (**19**), and (±)-homograndinol (**20**), showed strong activities at 100 mol ratio, though at 1000 mol ratio strong cytotoxicities on Raji cells were observed. Compound **21** gave a similar result as the cases of euglobals-IIc (**7**) and -VII (**12**) with an isovaleryl group on the aromatic ring, whereas compound **22** was slightly less active.

It is clear from Table III that both formyl and ketone groups are required for activity as the removal of either (**24**

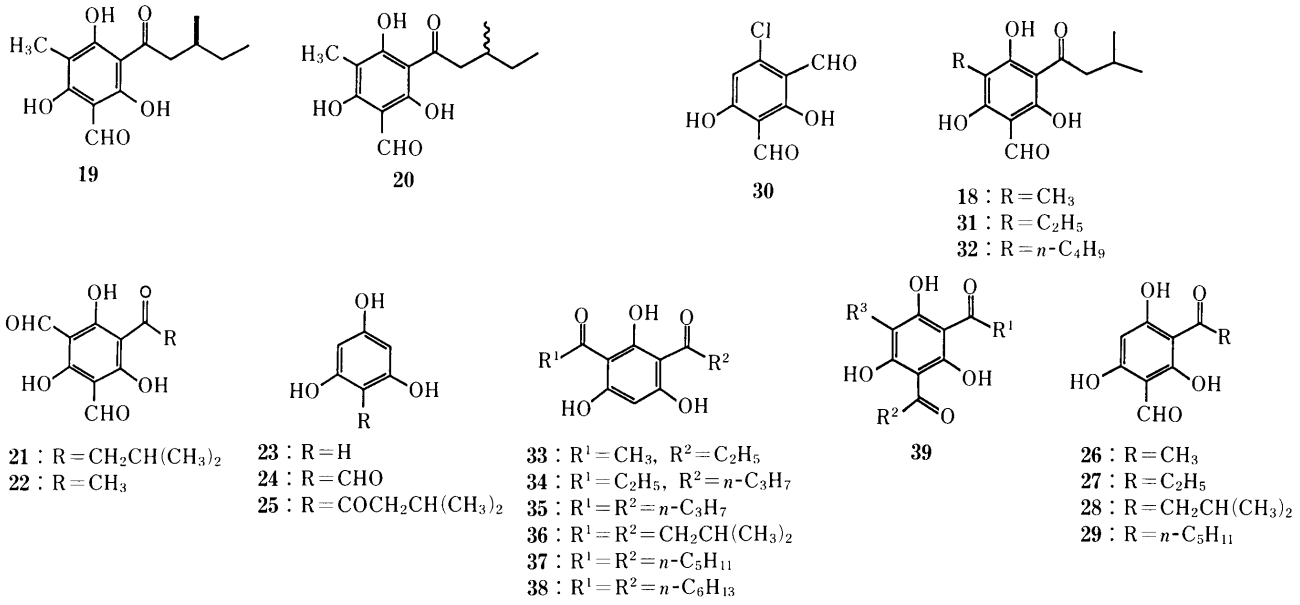

Chart 3

and **25**) or both (**23**) results in a remarkable decrease of activity and cytotoxicity. Compound **28** though lacking the aromatic methyl group that grandinol (**19**) has showed almost the same order of activity as grandinol (**19**). Compounds **26** and **27** also gave the same order of activity, whereas compound **29** demonstrated considerable decrease in the activity. It therefore seems that the alkyl substituent is not a prerequisite for activity. Compound **30**, which has a chlorine atom instead of a hydroxyl group, also gave moderate activity. As stated, the methyl group on the aromatic ring is not essential for activity though it is possible to enhance the activity by varying the alkyl group. Especially at low concentration (10 and 1 mol ratios), compounds **31** and **32** were more active than compound **28**. The results in Table III showed that the coexistence of formyl and keto-carbonyl groups is not always necessary for activities to be manifest. Compounds **34** and **35** both showed strong effects, while compound **33** having acetyl and propionyl groups, and compounds **36**—**38** with both carbonyl chains longer than five carbons showed a lack of activity, as was the case with compound **29**.

From the above results, the following conclusions can be drawn.

1. In general, the euglobals with monoterpene structures were more active than those whose molecules included sesquiterpene structures.
2. The essential moiety for the activity of euglobals was considered to be acylphloroglucinol structure (**39**).
3. In the acylphloroglucinol structure, two acyl groups are required for the activity.
4. The activity is affected by the steric factors of the chains, R^1 and R^2, in **39** while the presence or length of R^3 is insignificant.
5. It is noted that the activity pattern of the inhibitory effects of the compounds so far tested is very similar to that found in the germination inhibitory test.[12]

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References and Notes

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13) K. Fujitani and M. Kozuka, unpublished. Details of the syntheses will be reported elsewhere. Their structures were confirmed by spectral data (IR, ^{1}H-NMR, and MS). *a*) mp 106 °C. *b*) mp 120—122 °C. After this paper was completed, we learned that compound **36** had been independently synthesized by Professor M. Tada and his colleagues of Tokyo University of Agriculture and Technology [Abstracts of Papers, Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry, 1990. *Nippon Nogei Kagaku Kaishi*, **64**, 404 (1990)]. Their compound melts at 125—126 °C (Professor M. Tada, private communication).