Anti-tumor-Promoting Activity of the Diterpene from *Excoecaria agallocha*. II

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Eight new diterpenoids (1—8) have been isolated from the wood of *Excoecaria agallocha* (Euphorbiaceae) and their inhibitory effects on the induction of Epstein–Barr virus early antigen (EBV-EA) in Raji cells were examined to search for potent anti-tumor-promoters from natural resources. Of these compounds, the secolabdane-type diterpenoid, compound 7 exhibited a remarkable inhibitory effect on EBV-EA induction, and a significant anti-tumor-promoting effect in the mouse two-stage carcinogenesis test using 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoyl-phorbol-13-acetate.

Key words anti-tumor-promoter; secolabdane-type diterpene; *Excoecaria agallocha*; two-stage carcinogenesis; Euphorbiaceae

The latex and leaves of *Excoecaria agallocha* (Euphorbiaceae) have been used as a dart poison and fish poison in New Caledonia,2) India3) and Malaysia,4) and used in traditional medicine in Thailand. 5) Further, the resinous wood including latex of the so-called “Okinawa-Jinko” (in Japanese) has been used as a substitute for the incense of agarwood (Jinko) in Okinawa, Japan.6) In the course of our studies on constituents of *E. agallocha*, we reported the isolation and structural elucidation of many labdane- and beyerane-type diterpenoids and their inhibitory effects on the induction of Epstein–Barr virus early antigen (EBV-EA) by the tumor promoter.1,7) In this paper, we describe the results of further screening tests [in vitro: EBV-EA induction, in vivo: two-stage carcinogenesis test using 12-O-tetradecanoylphorbol-13-acetate (TPA) as a promoter and 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator] for the anti-tumor-promoting activity of the novel diterpenoid isolated from the resinous wood of this plant.

MATERIALS AND METHODS

Isolation of Diterpenes Compounds 1—8 were isolated from the acetone extract of resinous wood of *Excoecaria agallocha* L. (Euphorbiaceae) collected in Okinawa. Details of the isolation and structural elucidation of these compounds were reported previously.8)

Chemicals The cell culture reagents, *n*-butyric acid and other reagents, were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). TPA and DMBA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Wako Pure Chemical Industries (Osaka, Japan). EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medical University.

Cells The EBV genome-carrying lymphoblastoid cells (Raji cells derived from Birkitt’s lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Hamamatsu, Japan) under previously described conditions.9) Spontaneous activation of EBV-EA in our subline Raji cells was less than 0.1%.

In Vitro EBV-EA Activation Experiments The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type) as described.9) The indicator cells (Raji, 1×10⁶/ml) were incubated at 37°C for 48 h in 1 ml of medium containing *n*-butyric acid (4 mM), TPA [32 pmol= 20 ng in dimethyl sulfoxide (DMSO), 2 μl] as inducer and various amounts of test compounds in 5 μl of DMSO. Smears were made from the cell suspension, and the activated cells which were stained by EBV-EA positive serum from NPC patients were detected by an indirect immunofluorescence technique. The EBV-EA induction of the test compounds was expressed as a relative ratio to the control experiment (100%) which was carried out only with *n*-butyric acid

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In Vivo Two-Stage Mouse Skin Carcinogenesis Test
The animals (specific pathogen-free female ICR mice, 6-weeks old) were divided into three experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and each mouse was topically treated with DMBA (100 μg, 390 nmol) in acetone (0.1 ml) as an initiation treatment. One week after initiation with DMBA, mice were promoted by the application with TPA (1 μg, 1.7 nmol) in acetone (0.1 ml) twice a week. Groups II and III received a topical application of compound 7 (85 nmol) and glycyrrhetic acid (85 nmol) in acetone (0.1 ml) 1 h before each promotion treatment, respectively. The incidence of papillomas was observed weekly for 20 weeks, and the percentage of mice bearing papillomas and the average number of papillomas per mouse were recorded. In our experiment, only typical papillomas larger than about 1 mm in diameter were counted in each case. During this experiment, the increase in body weight of the treated mice was not affected by any of the tested compounds.

RESULTS AND DISCUSSION

As shown in Table 1, the primary screening test of diterpenes 1—8 was carried out using a short-term synergistic assay on EBV-EA induction with TPA. Of these diterpenes, 4, 5, 7 and 8 exhibited remarkable inhibitory effects on EBV-EA induction (100%, more than 75% and more than 25% inhibitions at 1×10⁻⁴, 5×10⁻² and 1×10⁻⁴ mol ratio/TPA, respectively), and preserved a high viability of Raji cells. In our experiments, the inhibitory effects of these compounds were stronger than those of glycyrrhetic acid which has been known as an anti-tumor-promoting reagent. Especially, the secolabdane-type diterpenoid, compound 7, exhibited the strongest inhibitory effects of all these diterpenes. Based on our experiments in which many natural compounds which strongly inhibited EBV-EA induced by the tumor promoter exhibited a remarkable anti-tumor-promoting effect on the two-stage carcinogenesis, the effect of compound 7 was expected and was investigated by a two-stage carcinogenesis test of mouse skin tumors induced by DMBA as an initiator and TPA as a promoter. The inhibitory effect determined by both the rate (%) of papilloma-bearing mice and the average number of papillomas per mouse were compared with those of the positive control group (I) and the group treated with glycyrrhetic acid (III).

As shown in Fig. 1, the diterpene 7 exhibited a significant inhibitory effect on the tumor promotion induced by TPA. In the positive control group, which received treatment with DMBA and TPA, there was 100% incidence of papillomas within 10 weeks of promotion (Fig. 1A: % of papilloma bearers). In the group treated with DMBA, TPA and glycyrrhetic acid, about 43, 70 and 95% of mice bore papillomas at 10, 15 and 20 weeks of promotion, respectively. The animals treated with DMBA, TPA and compound 7 for 10 weeks showed papilloma formation of less than 20%, by 15 weeks showed 60% and by 20 weeks showed 95%. These tumor inhibitory effects were also seen as a reduction in the multiplicity of papillomas (Fig. 1B: the number of papillomas per mouse) over a ten week period. In the positive control group, 4.8, 8.1 and 9.1 papillomas were formed per mouse after 10, 15 and 20 weeks of promotion, respectively. In the group treated with DMBA, TPA and glycyrrhetic acid, average number of papillomas per mouse was significantly different from the positive control group (p<0.01, using Student’s t-test) in terms of papilloma bearers (%) (n=15), and at 10, 15 and 20 weeks of promotion, the group treated with compound 7 was different from the control group (p<0.01, using Student’s t-test) in terms of papillomas per mouse (n=15, at 10, 15 and 20 weeks promotion, positive control group: 4.8±0.2, 8.1±0.6 and 9.1±0.6, glycyrrhetic acid treated group: 2.6±0.1, 5.0±0.3, 5.8±0.3 and the group treated with 7: 1.0±0.1, 3.1±0.2 and 4.7±0.3).

Table 1. Percentages of EBV-EA Induction in the Presence of Diterpenes (1—8) with Respect to Positive Control (100%)

<table>
<thead>
<tr>
<th>Sample</th>
<th>1×10⁻⁴</th>
<th>5×10⁻²</th>
<th>1×10⁻⁴</th>
<th>1×10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.1⁰⁰ (70)¹</td>
<td>36.2 (&gt;80)</td>
<td>79.5 (&gt;80)</td>
<td>96.4 (&gt;80)</td>
</tr>
<tr>
<td>2</td>
<td>0.0 (70)</td>
<td>23.8 (&gt;80)</td>
<td>76.8 (&gt;80)</td>
<td>92.3 (&gt;80)</td>
</tr>
<tr>
<td>3</td>
<td>5.3 (70)</td>
<td>39.9 (&gt;80)</td>
<td>81.3 (&gt;80)</td>
<td>100 (&gt;80)</td>
</tr>
<tr>
<td>4</td>
<td>0.0 (70)</td>
<td>21.2 (&gt;80)</td>
<td>73.7 (&gt;80)</td>
<td>90.3 (&gt;80)</td>
</tr>
<tr>
<td>5</td>
<td>0.0 (70)</td>
<td>20.8 (&gt;80)</td>
<td>74.5 (&gt;80)</td>
<td>91.5 (&gt;80)</td>
</tr>
<tr>
<td>6</td>
<td>0.0 (70)</td>
<td>25.7 (&gt;80)</td>
<td>75.3 (&gt;80)</td>
<td>92.6 (&gt;80)</td>
</tr>
<tr>
<td>7</td>
<td>0.0 (70)</td>
<td>22.6 (&gt;80)</td>
<td>72.4 (&gt;80)</td>
<td>89.2 (&gt;80)</td>
</tr>
<tr>
<td>8</td>
<td>0.0 (70)</td>
<td>24.4 (&gt;80)</td>
<td>74.8 (&gt;80)</td>
<td>91.0 (&gt;80)</td>
</tr>
</tbody>
</table>

a) TPA (32 pmol/ml=20 ng/ml). b) Values represent relative percentages to the positive control value (at least 500 cells were counted, n=3, and all S.D. were less than ±2.5 at each concentration). c) Values in parentheses are viability percentages of Raji cells.

Fig. 1. Inhibitory Effect of 7 on Mouse Skin Carcinogenesis Induced by DMBA and TPA
All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly starting 1 week after initiation. A: percentage of mice bearing papillomas, B: average number of papillomas per mouse. Positive control, TPA alone; 6. TP A+85 nmol of glycyrrhetic acid; 7. TPA+85 nmol of 7. At 10 and 15 weeks of promotion, the group treated with compound 7 was significantly different from the positive control group (p<0.01, using Student’s t-test) in terms of papilloma bearers (%) (n=15), and at 10, 15 and 20 weeks of promotion, the group treated with compound 7 was different from the control group (p<0.01, using Student’s t-test) in terms of papillomas per mouse (n=15, at 10, 15 and 20 weeks promotion, positive control group: 4.8±0.2, 8.1±0.6 and 9.1±0.6, glycyrrhetic acid treated group: 2.6±0.1, 5.0±0.3, 5.8±0.3 and the group treated with 7: 1.0±0.1, 3.1±0.2 and 4.7±0.3).
2.6, 5.0 and 5.8 papillomas were formed per mouse after 10, 15 and 20 weeks of promotion, respectively. On the other hand, in the group treated with DMBA, TPA and compound 7, less than 1.0, 3.1 and 4.7 papillomas were formed per mouse after the same respective periods. These results showed that when 7 was applied before each TPA treatment, the formation of papillomas on mouse skin was remarkably delayed and the number of papillomas was significantly reduced. As shown in Figs. 1A and B, the inhibitory effect of 7 on two-stage carcinogenesis of mouse skin tumor was apparently more potent than that of glycyrrhetic acid.

A secolabdane-type diterpenoid like compound 7 is the first example of an anti-tumor-promoter of chemical carcinogenesis, although several abietane- or rearranged labdane-type diterpenoids were isolated from Pinaceous plants and their potential anti-tumor-promoting effects were reported.13) Further studies on details of the mechanism of the anti-tumor-promoting effect are now underway.

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REFERENCES AND NOTES
11) A high viability (more than 60%) of Raji cells is necessary for in vitro assay using an indirect immunofluorescence technique by antigen-antibody reaction and is beneficial for the following in vivo assay.
12) Percentages of EBV-EA induction in the presence of glycyrrhetic acid were 15.6, 54.3, 100 and 100% at 1×103, 5×102, 1×102 and 1×10 mol ratio/TPA, respectively. Tokuda H., Ohigashi H., Koshimizu K., Ito Y., *Cancer Lett.*, 33, 279—282 (1986).