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STUDIES ON INHIBITORS OF SKIN TUMOR PROMOTION, XII.[1] ROTENOIDS FROM *AMORPHA FRUTICOSA*

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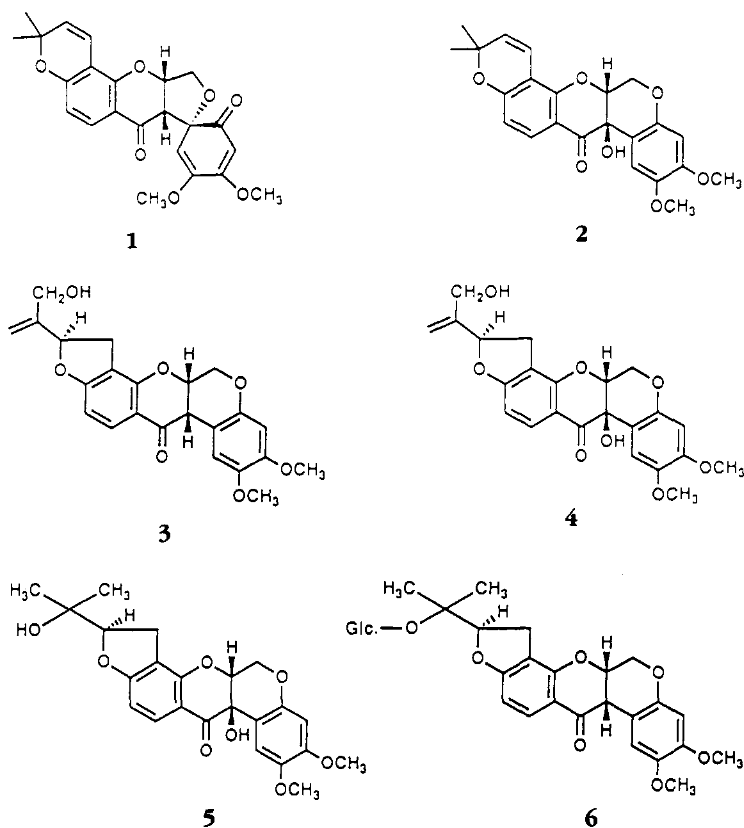
ABSTRACT.—As a part of screening studies for chemopreventive agents (anti-tumor-promoters), six North American plants belonging to the *Amorpha* genus were tested using an in vitro assay system. Of these plants, *Amorpha fruticosa* exhibited strong inhibitory effects on Epstein-Barr virus early antigen (EBA-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Also six rotenoids, amorphispironone [**1**], tephrosin [**2**], amorphigenin [**3**], 12a-hydroxyamorphigenin [**4**], 12a-hydroxydalpanol [**5**], and 6′-*O*-D-glucopyranosyldalpanol [**6**], were isolated from the leaves of *A. fruticosa*. Among these rotenoids, **1** and **2** exhibited remarkable inhibitory effects on EBV-EA activation induced by TPA. Further, **1** and **2** exhibited significant anti-tumor-promotion effects on mouse skin tumor promotion in an in vivo two-stage carcinogenesis test. These investigations suggested that these rotenoids might be valuable anti-tumor-promoters.

The insect feeding deterrence, antiparasitic, and hypotensive activities of the crude extract of *Amorpha fruticosa* L. (Leguminosae) have appeared in the literature (1). On the other hand, we have reported that several natural products showed strong inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as a primary screening test for chemopreventive agents (anti-tumor promoting agents) (2–4). Many compounds that inhibit EBV-EA induction by tumor promoters have been shown to act as inhibitors of tumor promotion in vivo (5–8).

As a result of our continuing search among North American plants (*Amorpha canescens* Pursh, *A. fruticosa*, *Amorpha georgiana* Wilb., *Amorpha glabra* Poir., *Amorpha herbacea* Walter, and *Amorpha schwerinii* C.K. Schneid.) for naturally occurring potential antitumor promoters, an MeOH extract of the leaves of *A. fruticosa* was found to show significant inhibitory effect on EBV-EA activation. Bioassay-directed fractionation of the active extract led to the isolation and characterization of six rotenoids **1–6** as inhibitory principles of EBV-EA activation. Previously, we have reported the structure elucidation of these rotenoids and an attempt on the chemical conversion of **1** into known rotenoids (9,10).

This paper describes the results of the biological assay on inhibitory effects of the extracts and these rotenoids **1–6** from the leaves of *A. fruticosa* on EBV-EA activation, the effects of **1** and **2** on the Raji cell cycle using a flow cytometer, and the results of in

[1]For Part XI, see Konoshima *et al.* (8).



vivo two-stage carcinogenesis test of **1** and **2** on mouse skin tumor (8,11). Several rotenoids have been isolated from the fruits and the root bark of the plant (12–14), but studies on their inhibitory effects on EBV-EA activation and skin-tumor-promotion have not been published thus far.

RESULTS AND DISCUSSION

As shown in Table 1, the *n*-hexane extract of the leaves of *A. fruticosa* showed the most significant inhibitory effects on EBV-EA activation (100% inhibition of activation at 10 μg/ml and 33.3% inhibition of activation even at 1 μg/ml) among these *Amorpha* plants. These inhibitory effects are almost at the same level as those of the Et_2O extract of *Magnolia officinalis* reported by Konoshima *et al.* (7).

We carried out bioassay-directed fractionation and purification of the most active fraction of the *n*-hexane extract. A novel rotenoid, amorphispironone [**1**], and tephrosin [**2**] were isolated by repeated cc and flash chromatography as active principles (10). Amorphigenin [**3**], 12a-hydroxyamorphigenin [**4**], and 12a-hydroxydalpanol [**5**] were isolated together with amorphispironone [**1**] from the CH_2Cl_2 extract, and 6′-*O*-D-glucopyranosyldalpanol [**6**] was isolated from the EtOAc extract. Of these rotenoids, amorphispironone [**1**] (1.1 g, 0.094%) and tephrosin [**2**] (0.4 g, 0.034%) were the major rotenoids of the leaves of these plants.

These six rotenoids **1–6** were tested utilizing the short-term in vitro assay of EBV-EA activation. Their inhibitory effects on activation induced by TPA and viabilities of Raji cells are shown in Table 2.

The rotenoids **1**, **2**, and **6** exhibited remarkable inhibitory effects on EBV-EA acti-

TABLE 1. Relative Ratio of EBV-EA Activation with Respect to Positive Control (100%) in Presence of Extracts of *Amorpha* Plants.[a]

Sample	Concentration[b]		
	100	10	1
Hexane extract			
Amorpha canescens	0.0 (30)	100.0 (50)	100.0 (80)
Amorpha fruticosa	0.0 (20)	0.0 (40)	66.7 (80)
Amorpha georgiana	14.9 (60)	39.2 (80)	83.7 (80)
Amorpha glabra	0.0 (70)	43.8 (80)	85.1 (80)
Amorpha herbacea	11.6 (10)	55.8 (80)	88.9 (80)
Amorpha schwerinii	0.0 (70)	93.6 (80)	100.0 (80)
MeOH extract:			
A. canescens	12.5 (70)	100.0 (80)	100.0 (80)
A. fruticosa	0.0 (80)	53.5 (80)	93.3 (80)
A. georgiana	15.4 (70)	64.3 (80)	92.1 (80)
A. glabra	18.4 (60)	92.9 (80)	100.0 (80)
A. herbacea	0.0 (60)	53.8 (80)	94.7 (80)
A. schwerinii	27.7 (60)	89.5 (80)	100.0 (80)

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

vation (more than 60% inhibition at 5×10^2 mol ratio of inhibitor/TPA). Compounds **1** and **2** exhibited more remarkable inhibitory effects (about 30% inhibition even at a 1×10^2 mol ratio) than other compounds. In our experiments, the inhibitory activities of **1**, **2**, and **6** were similar to those of retinoic acid and glycyrrhetinic acid, which are known as strong anti-tumor-promoters (6,16). The present investigation using an in vitro assay suggested that these rotenoids and the extract of *A. fruticosa* may be valuable anti-tumor-promoters and also suggested that the inhibitory effects of the extract are due to the combined effect of several of the constituents.

The effects of **1** and **2** on the cell cycle of Raji cells were then examined by flow cytometry. As shown in Table 3, the promoter TPA increased the percentage of G_2 and M phase of Raji cells and decreased the percentage of G_1 phase in comparison with negative control. When treated with compounds **1** and **2**, the percentage of the S phase was decreased and the percentage of the G_1 phase was increased as compared with the positive control. From these facts, it was deduced that compounds **1** and **2** arrested Raji

TABLE 2. Relative Ratio of EBV-EA Activation with Respect to Positive Control (100%) in Presence of Rotenoids from *Amorpha fruticosa*.

Compound	Concentration[a]			
	1000	500	100	10
1	0.0[b] (20)[c]	31.8 (80)	70.4 (80)	100.0 (80)
2	8.0 (40)	26.1 (60)	60.4 (70)	89.3 (80)
3	31.7 (80)	64.2 (80)	100.0 (80)	100.0 (80)
4	24.2 (80)	89.7 (80)	100.0 (80)	100.0 (80)
5	22.6 (80)	43.9 (80)	82.1 (80)	100.0 (80)
6	0.0 (80)	36.3 (80)	87.3 (80)	100.0 (80)

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

TABLE 3. Flow Cytometric Analysis of Raji Cell Cycle Treated with Rotenoids **1** and **2**[a].

Phase	Positive control[b]	Medium only[c]	Treated with **1**[d]	Treated with **2**[d]
G_1	42.6	67.2	59.1	57.8
S	26.4	21.2	10.3	13.4
G_2+M	31.0	11.6	30.6	28.8
Total	100.0	100.0	100.0	100.0

[a]Percentages of Raji cells in each phase.
[b]Treated with TPA (32 pmol) and *n*-butyric acid.
[c]Raji cells cultivated in RPMI-1640 medium containing 10% FCS.
[d]Treated with TPA (32 pmol), *n*-butyric acid, and **1** (32 nmol) or **2** (32 nmol).

cells in the G_1 phase, and consequently the percentage of G_1 phase in Raji cells was restored to normal value.

On the basis of the results of in vitro assays (inhibitory effects on EBV-EA activation and effects on cell cycle), the effects of rotenoids **1** and **2** on a two-stage carcinogenesis test in vivo using dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter were investigated. The activities, evaluated by both rate (%) of papilloma-bearing mice and average number of papillomas per mouse, were compared with those of a positive control. As shown in Figure 1, in the positive control, 100% of mice bore more than ten papillomas after twenty weeks of promotion. On the other hand, in the group treated with amorphispironone [**1**], only 20% of mice bore only one papilloma. Consequently, both rotenoids **1** and **2**, when applied continuously before each TPA treatment, delayed the formation of papillomas in mouse skin and reduced the rate of papilloma-bearing mice (A: about 80% and 60% reduction even at 20 weeks, respectively) as compared with the control experiments with TPA alone. Furthermore, these two compounds reduced the number of papillomas per mouse (B: about 90% and 70% reduction even at 20 weeks).

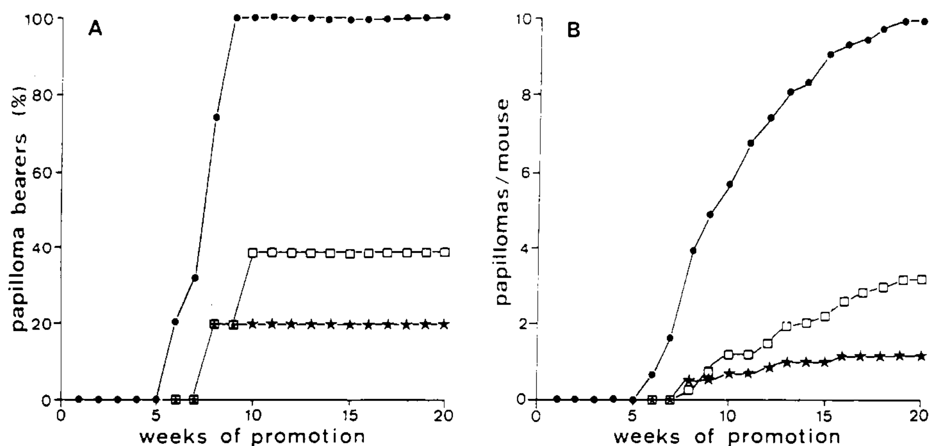


FIGURE 1. Inhibition of TPA-induced tumor promotion by multiple application of amorphispironone (85 nmol) and tephrosin (85 nmol). All mice were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) given twice weekly starting 1 week after initiation. A: Percentage of mice with papilloma. B: Average number of papillomas per mouse. ●, control TPA alone; ★, TPA + 85 nmol of amorphispironone [**1**]; □, TPA + 85 nmol of tephrosin [**2**].

These anti-tumor-promoting activities are extremely high in our experiments (7,8), and these results strongly suggested that amorphispironone [**1**] and tephrosin [**2**] might be valuable anti-tumor promoters in carcinogenesis. Studies on the details of the inhibitory mechanisms of rotenoids and on the details of the combined effects by plural constituents are now in progress.

EXPERIMENTAL

PLANT MATERIAL, EXTRACTION AND ISOLATION.—The leaves of *A. fruticosa* and *A. canescens* were collected in Oklahoma, and *A. georgiana*, *A. glabra*, *A. herbacea*, and *A. schwerinii* were collected in North Carolina, in 1990 and 1992. Voucher specimens are deposited in the herbarium of Kyoto Pharmaceutical University.

The cut leaves (1157 g) of *A. fruticosa* were extracted with *n*-hexane at room temperature and then extracted with hot MeOH exhaustively. After the solvent was removed in vacuo, a dark green residue was suspended in H_2O and extracted with CH_2Cl_2, EtOAc, and *n*-BuOH saturated with H_2O, successively. Each organic layer was evaporated in vacuo to give a residue (*n*-hexane 34.0 g, CH_2Cl_2 45.5 g, EtOAc 15.6 g, and *n*-BuOH 35.0 g). The details of isolation and structure elucidation of active rotenoids **1–6** have been described in our preliminary report (10).

CHEMICALS.—Dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma Chemical Co. (St. Louis, MO).

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

BIOLOGICAL ACTIVITIES IN VITRO.—The inhibition of EBV-EA activation induced by TPA was assayed according to literature methods (2,3).

FLOW CYTOMETRIC ANALYSIS.—Cellular DNA content of Raji cells was measured by flow cytometry. Fluorescence spectra were obtained on a commercially available FAC Scan (Becton Dickinson Co., Mountain View, CA). The cultured cells (1×10^6 per ml) in plastic tubes were stained with propidium iodide by a rapid staining technique (15). The nonionic detergent Triton 100 (Nacalai Tesque Co., Kyoto, Japan) (0.1%) was added to the tubes for the purpose of lysis of the cell membrane. Treated Raji cells were filtered through a 37 μm-pore nylon filter before staining. Treatment of RNase (Sigma) in phosphate-buffered saline (PBS, final 0.1%) decreased the fluorescence intensities of RNA. Finally, propidium iodide (final 50 μg/ml) was used for viable DNA staining. The flow cytometric analysis was carried out with FAC Scan cell fit DNA system, and the cell cycle pattern was analyzed by its program.

TWO-STAGE CARCINOGENESIS TEST IN VIVO.—The two-stage carcinogenesis test in vivo on mouse skin tumor was carried out according to literature methods (7).

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