Studies on the Antitumor-Promoting Activity of Naturally Occurring Substances. II.[1] Inhibition of Tumor-Promoter-Enhanced Phospholipid Metabolism by Umbelliferous Materials

Toru OKUYAMA,*,[a] Mitsumasa TAKATA,[a] Hoyoku NISHINO,[b] Atsuko NISHINO,[b] Junko TAKAYASU[b] and Akio IWASHIMA[b]

Department of Pharmacognosy and Phytochemistry, Meiji College of Pharmacy,[a] Nozawa 1-35-23, Setagaya-ku, Tokyo 154, Japan and Department of Biochemistry, Kyoto Prefectural University of Medicine,[b] Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602, Japan. *Received September 14, 1989*

Ninety-five extracts prepared from 14 kinds of Umbelliferous materials were studied to determine their effects on tumor-promoter-induced phenomena *in vitro*. Of the materials, 5 Chinese crude drugs, two Bai-Hua Qian-Hu classified as Q-I and Q-II types, the root of *Peucedanum praeruptorum* DUNN., Zi-Hua Qian-Hu, the root of *P. decursivum* MAXIM., Tang-Bai-Zhi, the root of *Angelica dahurica* BENTH. *et* HOOK. var. *pai-chi* KIMURA, HATA *et* YEN., Dang-Gui, the root of *A. acutiloba* KITAGAWA and 2 Umbelliferous plants, ashita-ba, *A. keiskei* KOIDZ., and ama-nyuu, *A. edulis* MIYABE, showed potent inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated ^{32}Pi incorporation into phospholipids of cultured cells.

From the active fraction of the crude drug "Tang-Bai-Zhi," imperatoin (1), isoimperatoin (2), oxypeucedanin (3), pabulenol (4), neobyakangelicol (5) and byakangelicin (6) were identified as active or inactive principles. Compound 4 had not previously been isolated from Tang-Bai-Zhi, *A. dahurica* var. *pai-chi*.

We also discuss the structure–activity relationship among the above 6 kinds of linear-type furanocoumarins, together with 3 kinds of antitumor-promoter coumarins having the same skeleton, psoralen (7), bergapten (8) and xanthotoxin (9), obtained from "ashita-ba" (eaten as a vegetable in Japan). Among the compounds in the present experiment, compounds 1 and 2 showed potent inhibitory activity at the concentration of 50 μg/ml and 3—9 were found to have less or no activity.

Keywords Umbelliferous plant; *Angelica dahurica*; *A. keiskei*; 12-*O*-tetradecanoylphorbol-13-acetate; phospholipid metabolism; radioactive inorganic phosphate incorporation; imperatoin; isoimperatoin; tumor promoter; antitumor promoter

Umbellifers were recognized long ago as natural aromatic and important medicinal plants or Chinese crude drugs. During research on the active components of the Chinese crude drug "Qian-Hu" belonging to Umbelliferae, Okuyama *et al.* performed structural analyses[2] and biochemical investigations[3] on a number of coumarins isolated from Qian-Hu preparations classified as Q-I, -II, -III and -IV types. As described previously, Pd-II, a seselin type coumarin, was isolated from the Chinese drug "Bai-Hua Qian-Hu," the root of *Peucedanum praeruptorum* DUNN.,[2a] and found to suppress the action of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) both *in vitro* and *in vivo*.[1]

The present paper describes the antitumor-promoter activity of 95 kinds of extracts derived from 14 Umbelliferous plants, and identification of the active constituents contained in them. Among the samples in Table I, 5 kinds of Chinese crude drugs, two types of "Bai-Hua Qian-Hu," the root of *Peucedanum praeruptorum* DUNN., "Zi-Hua Qian-Hu," the root of *P. decursivum* MAXIM., "Tang-Bai-Zhi," the root of *Angelica dahurica* BENTH *et* HOOK. var. *pai-chi* KIMURA, HATA *et* YEN., and "Dang-Gui," *A. acutiloba* KITAGAWA, and two plants, "ashita-ba," *Angelica keiskei* KOIDZ., and "ama-nyuu," *A. edulis* MIYABE, showed an excellent inhibitory effect, whereas 6 Chinese crude drugs and 1 plant were less active or showed no activity.

To find possible antitumor-promoters, extraction and separation of the most active Chinese crude drug "Tang-Bai-Zhi," *A. dahurica* BENTH. *et* HOOK. var. *pai-chi*, used in the treatment of headache, toothache, rhinorrhea, hemorrhoids, *etc.* and "ashita-ba," *A. keiskei* KOIDZ. (eaten as a vegetable in Japan), were carried out, and separated samples were monitored for activity on TPA-enhanced radioactive inorganic phosphate (^{32}Pi) incorporation into phospholipids of HeLa cells. From Tang-Bai-Zhi, six kinds of linear-type furanocoumarins, imperatoin (**1**), isoimperatoin (**2**), oxypeucedanin (**3**), pabulenol (**4**), neobyakangelicol (**5**) and byakangelicin (**6**), were identified, among which compounds **1** and **2** were found to have significant inhibitory effects. Moreover, from the root of ashita-ba 3 kinds of linear-type furanocoumarins, psoralen (**7**), bergapten (**8**) and xanthotoxin (**9**), were identified as shown in Chart 1, although these compounds were less active than compounds **1** and **2**. Based on these results, the structure–activity relationship is discussed.

Materials and Methods

Chemicals and Materials 7,12-Dimethylbenz[*a*]anthracene (DMBA) was purchased from Wako Pure Chemical Industries. TPA was obtained from PL Biochemicals, Inc. ^{32}Pi (carrier-free) was purchased from Japan Radioisotope Association. The Chinese crude drugs were supplied by Mikuni Co. (Osaka, Japan) and plants of Umbelliferae for the test were collected by ourselves as shown in the footnote of Table I.

Each sample (100 g) was successively extracted with *n*-hexane, Et_2O, AcOEt, MeOH and water for 3 h (each 1 l × 3) under reflux. The organic solvent was evaporated off *in vacuo* and the water solution was lyophilized to yield the corresponding *n*-hexane (He), Et_2O (Et), AcOEt (Ac), MeOH (Me) and water (Wa) extracts, respectively.

Isolation of Coumarins Chopped Chinese crude drug Tang-Bai-Zhi (400 g), the root of *Angelica dahurica* BENTH. *et* HOOK. var. *pai-chi* KIMURA, HATA *et* YEN (Umbelliferae), was extracted with *n*-hexane, Et_2O and AcOEt. The combined organic solutions were concentrated *in vacuo* to afford a corresponding extract (6.4 g). The extract was separated by chromatography on silica gel using a solvent gradient system (*n*-hexane–AcOEt = 10 : 1—1 : 2) to afford eight fractions. The fraction with potent inhibitory activity was purified by high performance liquid chromatography (HPLC) followed by recrystallization from *n*-hexane–AcOEt to yield six linear type furanocoumarins, 400 mg of imperatoin (**1**); colorless needles; mp 99.0—100.0 °C, 300 mg of isoimperatoin (**2**); colorless needles; mp 107.0—108.0 °C, 110 mg of oxypeucedanin (**3**); colorless leaflets; mp 141.0—142.0 °C, 35 mg of pabulenol (**4**); white powder; mp 123.0—124.0 °C, 10 mg of neobyakangelicol (**5**); yellow needles; mp 104.5—106.0 °C, 20 mg of byakangelicin (**6**); yellow needles; mp 124.0—125.0 °C. The ashita-ba (100 g), *A. keiskei* KOIDZ., was extracted and worked up in the same way to give three linear-type furanocoumarins, 500 mg of psoralen (**7**); colorless needles; mp 99.0—100.0 °C, 10 mg of bergapten

(**8**); colorless needles; mp 190—191.5 °C, 100 mg of xanthotoxin (**9**); colorless needles; mp 143.0—144.0 °C.

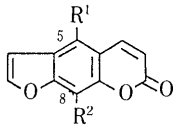
Each compound was identified by comparison of melting point and mass (MS), proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectral data with those of authentic samples.[4]

^{32}Pi Incorporation into Phospholipids of Cultured Cells Incorporation of ^{32}Pi into phospholipids of HeLa cells was assayed by the method described previously.[5]

Results and Discussion

Effect of Each Extract on ^{32}Pi Incorporation into Phospholipids of Cultured Cells It has been reported that the exposure of mammalian cells to tumor promoters causes a rapid increase in the incorporation of ^{32}Pi into phospholipids. This alteration in phospholipid metabolism is known to be one of the earliest phenomena caused by tumor promoters. Interestingly, it was found that various kinds of chemicals which inhibited this effect *in vitro* also suppressed *in vivo* carcinogenesis at the stage of promotion.

During our survey of antitumor-promoting agents among naturally occurring substances, we have undertaken bioassay of the extracts of Umbelliferous plants for activity on tumor-promoter-enhanced phospholipid metabolism of cultured cells. Most of the *n*-hexane, Et_2O and AcOEt extracts of the samples markedly inhibited TPA-stimulated ^{32}Pi incorporation into phospholipids of HeLa cells, whereas the MeOH and water extracts did not (Table I). The nonpolar extracts of two Chinese crude drugs, Zi-Hua Qian-Hu, the root of *Peucedanum decursivium*, and Tang-Bai-Zhi, the root of *Angelica dahurica*, and the root of ashita-ba, *A. keiskei*, (eaten as a vegetable in Japan), showed a strong inhibitory activity of about 100%. The nonpolar extract of Chinese crude drug Bai-Hua Qian-Hu, the root of *P. praeruptorum* classified as Q-I and Q-II type Qian-Hu, and Dang-Gui, the root of *A. acutiloba*, and a plant, the root, stem, leaf and seed of ama-nyuu, *A. edulis*, showed quite strong inhibitory effects of 80—50%. Among the parts of active plants used in this experiment as shown in Table I, the root of "ashita-ba" showed remarkable activity while the leaf was less active, whereas all parts of "ama-nyuu" showed strong inhibition. These results justify further studies to identify the active constituents of the extracts. Studies on Umbelliferous plants seem to be worthwhile for public health reasons, since the plants are widely used in Chinese and Japanese traditional medicine.



1: $R^1=H$, $R^2=OCH_2CH=C(CH_3)_2$
2: $R^1=OCH_2CH=C(CH_3)_2$, $R^2=H$
3: $R^1=OCH_2CH(O)C(CH_3)_2$, $R^2=H$
4: $R^1=OCH_2CH(OH)C(CH_3)=CH_2$, $R^2=H$
5: $R^1=OCH_3$, $R^2=OCH_2CH(OH)C(CH_3)=CH_2$
6: $R^1=OCH_3$, $R^2=OCH_2CH(OH)C(CH_3)_2(OH)$
7: $R^1=R^2=H$
8: $R^1=OCH_3$, $R^2=H$
9: $R^1=H$, $R^2=OCH_3$

Chart 1

TABLE I. Inhibitory Effect of Extracts Obtained from Umbelliferous Plants on TPA-Enhanced ^{32}Pi Incorporation into Phospholipids of HeLa Cells

Sample name	Original plant name	Extract	Inhibition (%)
Chinese drugs			
1. Bai-Hua Qian-Hu Q-I type (root)	*Peucedanum praeruptorum* DUNN.	He	63.1
		Et	49.7
		Ac	38.5
		Me	3.9
		Wa	14.9
2. Bai-Hua Qian-Hu Q-II type (root)	*P. praeruptorum* DUNN.	He	42.4
		Et	60.7
		Ac	56.4
		Me	37.2
		Wa	1.7
3. Zi-Hua Qian-Hu Q-III type (root)	*P. decursivum* MAXIM.	He	100
		Et	100
		Ac	50.3
		Me	36.4
		Wa	5.4
4. Tang-Bai-Zhi (root)	*Angelica dahurica* BENTH. *et* HOOK. var. *pai-chi* KIMURA, HATA *et* YEN.	He	100
		Et	100
		Ac	100
		Me	1.5
		Wa	0
5. Du-Huo (root)	*A. pubescene* MAXIM.	He	0
		Et	8.0
		Ac	0
		Me	0
		Wa	0
6. Qiang-Huo (root)	*A. sylvestris* L.	He	0
		Et	0
		Ac	1.8
		Me	0
		Wa	0
7. Chuan-Xiong (root)	*Cnidium officinale* MAKINO	He	1.1
		Et	1.6
		Ac	5.7
		Me	0
		Wa	0
8. Chai-Hu (root)	*Bupleurum falcatum* L.	He	19.7
		Et	27.9
		Ac	0.3
		Me	15.7
		Wa	0
9. Dang-Cui (root)	*A. acutiloba* KITAGAWA	He	79.8
		Et	72.9
		Ac	29.1
		Me	1.0
		Wa	0
10. Fang-Feng (root)	*Saposhnikovia divaricata* SCHISCHKIN	He	34.3
		Et	26.0
		Ac	7.7
		Me	5.2
		Wa	0
11. Bang-Fang-Feng (root)	*Glehnia littoralis* FR. SCHMIDT *et* MIQUEL	He	40.3
		Et	21.1
		Ac	13.3
		Me	0.5
		Wa	0
Plants			
12. Ashita-ba (root)	*A. keiskei* KOIDZ.	He	100
		Et	a)
		Ac	91.9
		Me	18.9
		Wa	1.9
Ashita-ba (stem)	*A. keiskei* KOIDZ.	He	23.9
		Et	74.8
		Ac	51.3
		Me	10.9
		Wa	5.5

TABLE I. (continued)

	Sample name	Original plant name	Extract	Inhibition (%)
	Ashita-ba (leaf)	*A. keiskei* KOIDZ.	He	9.2
			Et	26.9
			Ac	18.9
			Me	11.3
			Wa	8.0
13.	Ama-nyuu (root)	*A. edulis* MIYABE	He	72.7
			Et	66.8
			Ac	57.1
			Me	24.4
			Wa	0
	Ama-nyuu (stem)	*A. edulis* MIYABE	He	64.0
			Et	72.5
			Ac	43.1
			Me	14.0
			Wa	15.0
	Ama-nyuu (leaf)	*A. edulis* MIYABE	He	47.8
			Et	52.5
			Ac	38.7
			Me	11.0
			Wa	11.5
	Ama-nyuu (seed)	*A. edulis* MIYABE	He	75.5
			Et	83.1
			Ac	39.5
			Me	25.0
			Wa	6.9
14.	Du-Qin (whole plant)	*Cicuta virosa* L.	He	25.2
			Et	8.4
			Ac	6.7
			Me	0
			Wa	4.6

Extracts: He, *n*-hexane; Et, Et_2O; Ac, AcOEt; Me, MeOH; Wa, water. *a*) This extract could not be evaluated, because it showed strong toxicity to HeLa cells. Ashita-ba was collected at Miyake island, Tokyo. Ama-nyuu was collected at Nagai City in Yamagata Prefecture. Du-Qin was cultivated in the Medicinal Plant Garden at Meiji College of Pharmacy. HeLa cells cultured in Petri dishes were incubated with one of the test compounds (final concentration: 50 μg/ml). After 1 h, ^{32}Pi (10 μCi/culture) was added with or without TPA (50 nM). Incubation was continued for 4 h, and then radioactivity incorporated in the phospholipid fraction was assayed. Data are mean values of duplicate experiments and are expressed as % inhibition.

Effect of Coumarins Isolated from "Tang-Bai-Zhi" and "Ashita-ba" on ^{32}Pi Incorporation into Phospholipids of Cultured Cells As the *n*-hexane, Et_2O and AcOEt extracts of Chinese crude drug "Tang-Bai-Zhi," the root of *A. dahurica*, showed the highest activity, the active constituents in these samples were surveyed, and we identified 6 kinds of linear-type furanocoumarins, **1**—**6**, having a dimethylallyl or a related group linked through an ether bond at the C_5 and/or C_8 positions. On the other hand, 3 kinds of linear-type furanocoumarins without dimethyallyl moieties, **7**—**9**, were obtained from the extract of the edible plant "ashita-ba," *A. keiskei*, as shown in Chart 1. All coumarins in this experiment were screened for anti-tumor-promoter activity at concentration of 50 μg/ml. Some structure–activity relationships can be deduced.

Of the coumarins in Table II, compound **7**, which was the fundamental skeleton lacking substituents on the benzene ring, showed a relatively low inhibitory effect (12.9%) on TPA-enhanced ^{32}Pi incorporation into phospholipids of HeLa cells. The activity of compounds **8** and **9** with a methoxy group at the C_5 or C_8 position was similar to that of **7**. In compounds **4** and **5**, the former without a methoxyl group showed 36.7% inhibition, whereas the latter with a methoxyl group exhibited considerably less inhibitory effect. Compounds **1** and **2** having a dimethylallyl group showed excellent inhibitory effects of 86.5% and 94.1%, respectively, but **3** and **4**, in which the double bond of the dimethylallyl group was replaced by a hydroxyl or epoxyl group, were found to be significantly less active than **1** and **2**. Compounds **1** and **2** were about 7- to 8-fold more potent than the corresponding fundamental skeleton **7**. On the other hand, compound **6** with the dimethylallyl group saturated by two hydroxyls showed no activity (Table II). On the basis of this evidence, a dimethylallyl moiety on a linear furanocoumarin skeleton seem to be very important for activity, and addition of methoxyl group decreases the activity.

TABLE II. Inhibitory Effect of Coumarins Obtained from "Tang-Bai-Zai" and "Ashita-ba" on TPA-Enhanced ^{32}Pi Incorporation into Phospholipids of HeLa Cells

Coumarin (50 μg/ml)	Inhibition (%)
Imperatoin (**1**)	86.5
Isoimperatoin (**2**)	94.1
Oxypeucedanin (**3**)	31.9
Pabulenol (**4**)	36.7
Neobyakangelicol (**5**)	2.9
Byakangelicin (**6**)	0
Psoralen (**7**)	12.9
Bergapten (**8**)	15.7
Xanthotoxin (**9**)	23.8

TPA: 50 nM.

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