Isolation of Furocoumarins from Bergamot Fruits as HL-60 Differentiation-Inducing Compounds

Satoru Kawaii, Yasuhiko Tomono, Eriko Katase, Kazunori Ogawa, and Masamichi Yano*

National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka 424-0204, Japan

The HL-60 differentiation-inducing compounds in bergamot fruits were isolated with column chromatography and identified as bergamottin, bergapten, and citropten by ^{1}H and ^{13}C NMR. Their HL-60 differentiation-inducing activity was measured by examining nitro blue tetrazolium (NBT) reducing, nonspecific acid esterase (NSE), specific esterase (SE), and phagocytic activities, and bergamottin showed the strongest activity among the coumarins isolated from bergamot fruits. The structure−activity relationship obtained from HL-60 differentiation assay suggests that hydrophobicity of furocoumarins is correlated with their activity.

Keywords: *Bergamot; furocoumarin; coumarin; differentiation; HL-60*

INTRODUCTION

Citrus plants are rich sources of various physiologically active substances, including flavonoids (Attaway, 1994; Benavente-García et al., 1997; Kawaii et al., 1999b,c), acridones (Chou et al., 1989; Takemura et al., 1995; Kawaii et al., 1999d), and limonoids (Lam et al., 1989a,b, 1993). It is commonly accepted that cancer formation can be prevented by the consumption of certain foods (Wattenberg, 1985, 1990; Stavric, 1994), and *Citrus* fruits and juices are one of the most prominent cancer-preventing foods (Stinson et al., 1996; So et al., 1996; Higashimoto et al., 1998). From a viewpoint of health promotion by dietary habits, we have surveyed various *Citrus* juices for differentiation-inducing activity toward HL-60 leukemia cells.

Since the measurable activity of the edible part tended to be hindered by the more abundant substances, which had no activity, we have prepared a readily extractable fraction from various *Citrus* juices, including adsorption on a porous polymer resin and successive elution from the resin with ethanol and acetone (Kawaii et al., 1999a). This method led us to a substantial decrease in flavonoid glycosides and sugars. During the course of examining biological activity in *Citrus* juices, we found strong activity in juices of king (*C. nobilis*) and bergamot (*C. bergamia*). The active principles in king juice have been identified as four polymethoxylated flavones (Kawaii et al., 1999a). However, only small amounts of polymethoxylated flavones were detected in bergamot juice. In the present study, we have isolated two furocoumarins and one coumarin from bergamot fruits as HL-60 differentiation-inducing compounds. Furocoumarins and coumarins are generally distributed throughout the *Citrus* species (Gray and Waterman, 1978), and the highest amounts are found in *Citrus* peel oils (Stanley and Jurd, 1971). These compounds are reported to have a broad spectrum of biological activities, including antimicrobial (Nakatani et al., 1978), anti-platelet-aggregation (Chen et al., 1995), and antimutagenic (Edenharder et al., 1995) activities. Structure−activity relationships obtained from HL-60 differentiation assay are also discussed.

* To whom correspondence should be addressed. Fax: +81-543-69-2115. E-mail: ym6082@okt.affrc.go.jp.

MATERIALS AND METHODS

Nuclear Magnetic Resonance Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a DPX 400 spectrometer (Bruker, Tsukuba, Japan) in $CDCl_3$. Chemical shifts were reported using residual C*H*Cl_3 (δ 7.24) and *C*DCl_3 (δ 77.0) as internal standards.

Isolation of HL-60 Differentiation-Inducing Compounds. Bergamot fruits were harvested from trees at the National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka, Japan in February 1998. The edible part (445 g), which consisted of the juice sac and segment epidermis, was homogenized in ethanol. The supernatant of the homogenate was concentrated in vacuo and absorbed on 250 g of polystyrene resin (Diaion HP-20, Mitsubishi Chemical, Tokyo, Japan) that had been preconditioned by thorough washing with deionized water. The resin was eluted with 750 mL of ethanol and then 750 mL of acetone. The eluents were concentrated in vacuo, and the thus obtained aqueous solution (approximately 250 mL) was partitioned between diethyl ether (200 mL × 3) and then *n*-butanol (200 mL × 3) to obtain the diethyl-ether-soluble extract (261.4 mg) and the *n*-butanol-soluble extract (2.48 g). The bioactive ether extract was subjected to silica gel column chromatography [Wako Gel C-200, Wako Pure Chemicals, Osaka, Japan; column size 200 mm × 30 mm (i.d.)] eluted with 20% ethyl acetate in hexane, chloroform, 20% chloroform in methanol, and then methanol. The eluate with 20% ethyl acetate in hexane was further purified by HPLC [mobile phase; 20% ethyl acetate in hexane, column; LiChrospher Si60, particle size; 5 μm, column size; 250 mm × 10 mm (i.d.), Kanto Chemicals, Tokyo, Japan], giving bergamottin (**1**, isolation yield; 4.0 mg) and bergapten (**2**, isolation yield; 0.5 mg) as active compounds. Ethanolic extracts of lyophilized bergamot peel (60.6 g) were similarly purified as mentioned above, giving bergamottin (**1**, isolation yield; 5.1 mg), citropten (**4**, isolation yield; 13.2 mg), and bergapten (**2**, isolation yield; 34.8 mg) as active compounds. Identification of these compounds was done by comparison of NMR spectral data with those of the authentic compounds.

Bergamottin: ^{1}H NMR data ($CDCl_3$) δ 8.14 (1H, d, J = 9.8 Hz, H-4), 7.57 (1H, d, J = 2.5 Hz, H-2′), 7.13 (1H, s, H-8), 6.94 (1H, dd, J = 0.9, 2.5 Hz, H-3′), 6.25 (1H, d, J = 9.8 Hz, H-3), 5.51 (1H, m, H-2″), 5.05 (1H, m, H-6″), 4.93 (2H, d, J = 6.8 Hz, H-1″), 2.08 (4H, m, H-4″, -5″), 1.67 (3H, s, H-10″), 1.66 (3H, s, H-8″), 1.58 (3H, s, H-9″); ^{13}C NMR data ($CDCl_3$) δ 161.3

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(s, C-2), 158.2 (s, C-7), 152.7 (s, C-8a), 149.0 (s, C-5), 144.9 (d, C-2′), 143.0 (s, C-3″), 139.6 (d, C-4), 132.0 (s, C-7″), 123.5 (d, C-6″), 118.9 (d, C-2″), 114.3 (s, C-6), 112.6 (d, C-3), 107.6 (s, C-4a), 105.0 (d, C-3′), 94.3 (d, C-8), 69.8 (t, C-1″), 39.5 (t, C-4″), 26.2 (t, C-5″), 25.7 (q, C-8″), 17.7 (q, C-9″), 16.7 (q, C-10″).

Bergapten: ^{1}H NMR data ($CDCl_3$) δ 8.14 (1H, d, J = 9.8 Hz, H-4), 7.58 (1H, d, J = 2.5 Hz, H-2′), 7.12 (1H, s, H-8), 7.00 (1H, dd, J = 0.9, 2.5 Hz, H-3′), 6.26 (1H, d, J = 9.8 Hz, H-3), 4.25 (3H, s, 5-OMe); ^{13}C NMR data ($CDCl_3$) δ 161.2 (s, C-2), 158.4 (s, C-7), 152.7 (s, C-8a), 149.6 (s, C-5), 144.8 (d, C-2′), 139.2 (d, C-4), 112.7 (s, C-6), 112.6 (d, C-3), 106. 4 (s, C-4a), 105. 0 (d, C-3′), 93.9 (d, C-8), 60. 1 (q, 5-OMe).

Citropten: ^{1}H NMR data ($CDCl_3$) δ 7.95 (1H, d, J = 9.7 Hz, H-4), 6.40 (1H, d, J = 2.1 Hz, H-6 or 8), 6.26 (1H, d, J = 2.1 Hz, H-6 or 8), 6.14 (1H, d, J = 9.7 Hz, H-3), 3.87 (3H, s, 5-OMe), 3.84 (3H, s, 7-OMe); ^{13}C NMR data ($CDCl_3$) δ 163.7 (s, C-7), 161.5 (s, C-2), 157.0 (s, C-5), 156.9 (s, C-8a), 138.7 (d, C-4), 111.0 (d, C-3), 104. 0 (s, C-4a), 94.9 (d, C-6 or 8), 92.8 (d, C-6 or 8), 55.9 (q, 5-OMe), 55.8 (q, 7-OMe).

Cell Differentiation Assay. The HL-60 cell line was obtained from the Riken Gene Bank (Tsukuba, Japan) and was maintained in RPMI1640 medium (Iwaki, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker Inc., Walkersville, MD). HL-60 cells in log phase ($\sim 10^6$ cells/mL) were diluted to 1.2×10^5 cells/mL and preincubated for 18 h in a 24-well plate ($\sim 2 \times 10^5$ cells/mL). Samples dissolved in DMSO were then added, keeping the final DMSO concentrations < 0.4% (v/v). In a blank experiment, the cells were treated with the same concentration of DMSO. Neither retardation of proliferation nor induction of differentiation was observed in the blank experiment. After 4 days of incubation, the cells were analyzed to determine the percentage exhibiting morphological and biochemical differentiation. For nitro blue tetrazolium reducing assay, a 1:1 (v/v) mixture of a cell suspension (10^6 cells/500 μL) and freshly prepared 12-*O*-tetradecanoylphorbol 13-acetate (TPA)/NBT solution (phosphate buffered saline containing 1 mg/mL of NBT and 1 μg/mL of TPA) was incubated for 15 min at 37 °C. Cells were then smeared on a slide glass and counter-stained by 0.25% (w/v) safranin O in 10% ethanol. Differentiated cells, which gave intracellular black-blue formazan deposit, were examined by counting minimum of 200 cells in triplicate for each experiment. For determination of nonspecific and specific esterase activities, assays by α-naphthyl acetate esterase (nonspecific acid esterase, NSE) and by naphthyl AS-D chloroacetate esterase (specific acid esterase, SE) were done using cytochemical kits from Sigma-Aldrich (91-A and 91-C, Tokyo, Japan). Differentiated cells were examined by counting a minimum of 200 cells in triplicates for each experiment. Phagocytic activity was assayed using polystyrene latex particles (average diameter = 0.81 μm, Difco Lab, Detroit, MI), which were suspended in RPMI1640 medium at a concentration of 10^9 particles/mL. Sample-treated HL-60 cells were washed and suspended in RPMI1640 containing 20% AB serum (BioWhittaker Inc., Walkersville, MD) at a final concentration of 2×10^6 cells/mL. A 1:1 mixture of the latex particle and HL-60 cell suspension was incubated for 4 h at 37 °C. Phagocytic activity was determined by counting cells ingesting the latex particles with a hemacytometer on a minimum of 200 cells.

Cell Proliferation Assay. Proliferation of HL-60 grown in 96-well microtiter plates was measured by using alamar Blue (Biosource International, Lewisville, TX), an oxidation−reduction colorimetric indicator based on detection of metabolic activity. The indicator changes color in response to chemical reduction of growth medium resulting from cellular growth (White et al., 1996; Zhi-Jun et al., 1997). Staining with alamar Blue appears to be well suited for the HL-60 cell line since the alamar Blue absorbance correlates with cell number (r = 0.995) (Kawaii et al., 1999d). Triplicate plates were prepared. To each well 10^4 cells/100 μL of HL-60 cell suspension was added, grown for 24 h, and then mixed with 100 μL of medium containing serial dilution of samples to be assayed. After 3 days of incubation, 20 μL of alamar Blue was asceptically added to each well and the mixture incubated for 24 h. Cellular proliferation (% of untreated control) was calculated with the following equation:

$$\text{inhibition (\%)} = 100 - \frac{[(A_{570} - A_{595})_{\text{test agent dilution}}] - [(A_{570} - A_{595})_{\text{blank}}]}{[(A_{570} - A_{595})_{\text{positive growth control}}] - [(A_{570} - A_{595})_{\text{blank}}]} \times 100 \quad (1)$$

where A_{570} and A_{595} are the absorbance at 570 and 595 nm, respectively.

HPLC Analysis of Furocoumarins and Coumarin. The freeze-dried fruits were divided into peel and edible part, which consisted of the juice sac and segment epidermis. One hundred milligrams of ground sample was extracted with 1 mL of MeOH−DMSO (1:1) three times. The extracts were combined, and MeOH was added to reach 5 mL. Twenty microliters of the combined extracts were injected to HPLC. Analytical conditions were as follows: column; Hypersil RP-18, particle size; 5 μm, 12.5 cm × 4.0 mm (i.d.) (Hewlett-Packard, Wilmington, DE), mobile phase; 70% MeOH/H_2O was used for bergamottin and 20% MeOH/H_2O was for bergapten and citropten. Detection was done by UV absorption at 330 nm. Concentrations of the compounds were calculated from peak areas integration of the sample and corresponding standards.

Statistical Analysis. One-way ANOVA, according to the Tukey−Kramer honestly significant difference test, was applied to the variables that could contribute to find significant effects on HL-60 cell growth and differentiation. The statistical significance at P = 0.05 was estimated by the actual absolute difference in the means minus the least significant difference, which is the difference that would be significant.

Table 1. Induction of NBT Reducing Activity by Bergamot Fruit Extracts

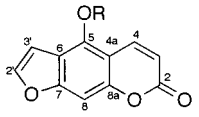
	edible part		peel	
	NBT[a] (%)	yield[b] (mg)	NBT[a] (%)	yield[c] (mg)
diethyl ether fraction	63	261	71	280
n-butanol fraction	32	2480	62	2330
aqueous fraction	25	498	9	410

[a] Percent of NBT-positive cells determined at 400 μg/mL. [b] Yield from 445 g of fresh edible part. [c] Yield from 60.6 g of lyophilized peel.

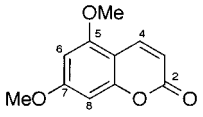
RESULTS AND DISCUSSION

Isolation and Identification of HL-60 Differentiation-Inducing Compounds from Bergamot Fruits. The edible part (445 g) of bergamot fruit was homogenized in ethanol. After removal of ethanol in vacuo, the homogenate was absorbed on HP-20 resin, which was then eluted with ethanol and acetone. The eluent was partitioned between diethyl ether and water and then between *n*-butanol and water. Table 1 shows the percent of NBT reducing activity and yield of the bergamot extracts. The most bioactive ether extract was chromatographed on a silica gel column. The fraction eluted with 20% ethyl acetate in hexane was further purified by reversed-phase HPLC to afford 4.0 mg of bergamottin (**1**) and 0.5 mg of bergapten (**2**) (Figure 1).

Expecting the existence of active compounds in bergamot peel, we also examined the ethanolic extract of lyophilized bergamot peels. The ethanolic extract of lyophilized bergamot peel (60.6 g) was similarly purified as mentioned above, giving 5.1 mg of bergamottin (**1**), 13.2 mg of citropten (**4**), and 34.8 mg of bergapten (**2**) as active compounds. Identification of citropten was done by comparison of NMR spectral data with those of the authentic compound.



	R
bergamottin (**1**)	$CH_2CH=C(Me)CH_2CH_2CH=C(Me)_2$
bergapten (**2**)	Me
bergaptol (**3**)	H



citropten (**4**)

Figure 1. Structures of furocoumarins and coumarin studied.

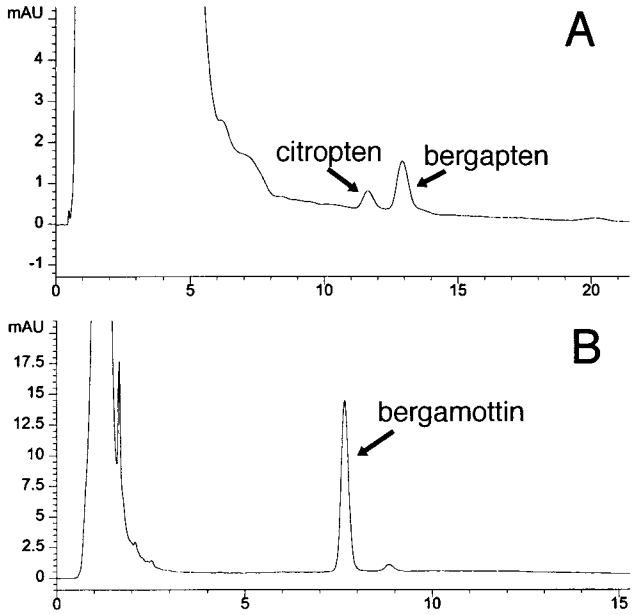


Figure 2. HPLC profiles of (A) citropten and bergapten and (B) bergamottin in the edible part of bergamot fruits.

Table 2. Coumarin and Furocoumarin Contents in Bergamot Fruits

	juice sac and segment epidermis (μg/100 mg of dry sample)	peel (μg/100 mg of dry sample)	readily extractable fraction (mg/100 mL of juice)
bergamottin	7.52	96.6	2.1
bergapten	1.07	152.5	0.4
citropten	0.17	21.7	n.d.

Table 2 shows contents of coumarin and furocoumarins in bergamot fruits, examined by HPLC analysis (Figure 2). The concentration of bergamottin in peel was approximately 10 times higher than that in the edible part, while the peel contained more than 100 times higher concentration of citropten and bergapten than the edible part.

HPLC analysis indicated that the readily extractable fraction of bergamot juice contained 2.1 mg/100 mL of juice of bergamottin, and thus the test medium of the readily extractable fraction of bergamot contained approximately 1.1 μM of bergamottin. The concentration was similar to the minimum effective concentration of bergamottin (Figure 3). In contrast to bergamot, these furocoumarins and coumarin were not detected in king juice, which contained four polymethoxylated flavones as active principles. We, therefore, considered that bergamottin mainly contributed the differentiation-inducing activity of the readily extractable fraction of bergamot juice.

HL-60 Differentiation Activity. The HL-60 cell line (Collins et al., 1978), established from an acute myeloid leukemia patient, provides a useful model system for studying differentiation of leukemic cells. Terminal differentiation of HL-60 can be monitored by changes of morphological, biochemical, and immunological properties (Yam et al., 1971). Certain compounds, known to be efficacious cancer-preventative agents, including interferon (Degos, 1990; Harris et al., 1985), retinoids (Breitman et al., 1980; Honma et al., 1980), and 1α,25-dihydroxyvitamin D_3 (Tanaka et al., 1982; Koeffler et al., 1984), are potent inducers of HL-60 cell differentiation and appear to be clinically effective against myeloproliferative disorders and human colon, mammary, and lung xenografts and melanoma.

Bergamottin, citropten, and bergapten were examined for their HL-60 differentiation-inducing activity as well as their effect on HL-60 proliferation. The concetration−response effect of these compounds is shown in Figure 3. To obtain information on structure−activity relationship in furocoumarins, we have also assayed bergaptol (**3**) (Funakoshi, Tokyo, Japan). A precise comparison of the differentiation-inducing activity of furocoumarins was made by the percentage of cells that had been induced to have NBT-reducing activity at a concentration of 10 μM, since the NBT test tended to give results with lower variance than those of other three tests performed, providing more reliable information for understanding the structure requirements for the activity. The decreasing sequence of the differentiation-inducing activity in furocoumarins is given as follows: bergamottin > bergapten > bergaptol. The percentages of NBT-reducing cells were 51 $\pm$ 9, 37 $\pm$ 3, and 14 $\pm$ 2, respectively. A decreasing HL-60 differentiation-inducing activity was observed when the longer alkyl side chain, that is a geranyloxy group, was replaced by shorter one, that is a methoxyl group, and by a hydroxyl group.

Hydrophobicity of furocoumarins seems to be correlated with their HL-60 differentiation-inducing activity. The logarithms of the partition coefficient between *n*-octanol and water (log *P*) were predicted by calculations (ChemDraw Pro 4.5, Cambridge Soft Co., Cambridge, MA). Log *P* values of bergamottin, bergapten, and bergaptol were calculated as 4.07, 1.31, and 1.05, respectively, supporting the relationship between hydrophobicity and HL-60 differentiation-inducing activity of furocoumarins.

The primary site of the furocoumarins is probably the cytoplasmic membrane. The correlation between hydrophobicity and the ability to membrane is demonstrated in several types of compounds, including short-chain alcohols (MaKarns et al., 1997) and indole alkaloids (Irie et al., 1987), and the quantitative relationship between chemical structure and biological activity reveals the role of the hydrophobicity in the loss of membrane integrity. The impairment of the biological membrane by hydrophobic xenobiotics most likely results from hydrophobic interaction with the membrane, which

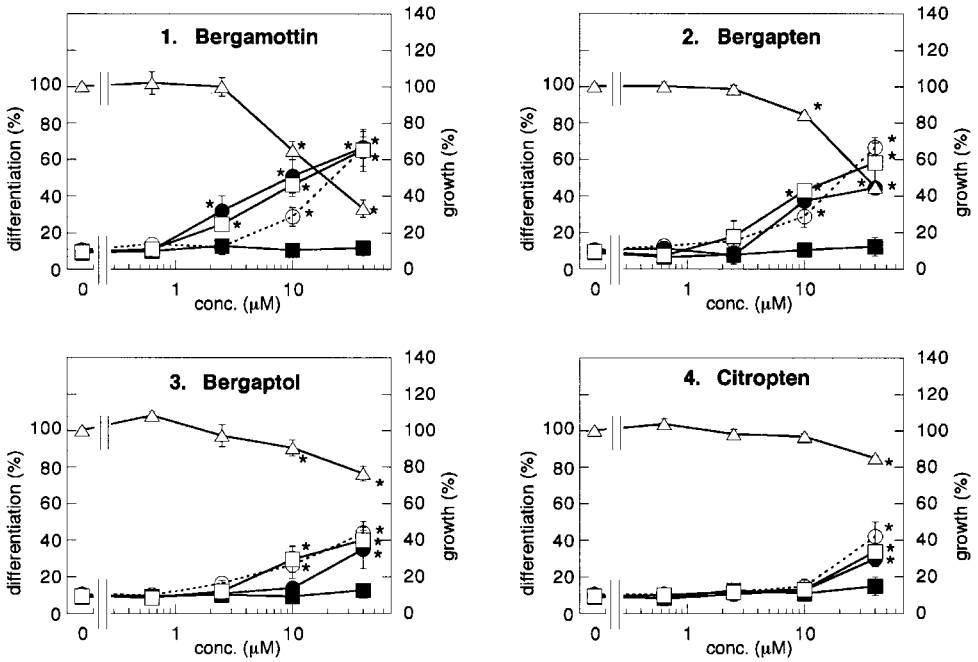


Figure 3. Concentration−response of furocoumarins and coumarin on differentiation and clonal proliferation of HL-60 cells: bergamottin (1); bergapten (2); bergaptol (3); citropten (4). Nitro blue tetrazolium reducing activity (●), nonspecific esterase activity (○), specific esterase activity (■), phagocytic activity (□), and cellular proliferation (△). Each point represents the mean of triplicates of experiments. Vertical bars indicate standard deviations. Asterisks indicate significant difference ($P < 0.05$) after assessment by one-way ANOVA (Tukey−Kramer honestly significant difference test).

affects the functioning of the membrane and membrane-embedded proteins (Sikkema et al., 1994; Cascorbi and Ahlers, 1981).

HL-60 cells treated with these compounds showed NBT reducing, nonspecific esterase, and phagocytic activities in a concentration-dependent manner. Monocyte/macrophage cells can be distinguished from granulocytes by the substrate specificity of their cytosolic esterases; cellular esterases of monocyte/macrophage cell hydrolyze α-naphthyl acetate, whereas granulocyte esterases cleave naphthyl AS-D chloroacetate. HL-60 cells treated with these furocoumarins and coumarin did not express significant elevation of naphthyl AS-D chloroacetate esterase activities, whereas they demonstrated marked increases in α-naphthyl acetate esterase activity (Figure 3). The results of NBT reduction, NSE, and phagocytic assays correlated well each other: these compounds induced similar percentages of monocyte/macrophage cells by these assays. Following treatment with bergamottin at a concentration of 40 μM, more than 60% of HL-60 cells were induced differentiation. There was a concomitant decrease in cellular proliferation, indicating that these compounds suppressed HL-60 cell growth. After a 4-day treatment, bergamottin, bergapten, bergaptol, and citropten at 40 μM inhibited HL-60 proliferation by 66, 55, 23, and 15, respectively.

Relevant with the above discussion, HL-60 differentiation-inducing activity of these furocoumarins is noteworthy as potential cancer-preventative agents. Bergamottin inhibits formation of epidermal DNA adducts and skin tumors induced by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene in SENCAR mice (Cai et al., 1997). Auraptene, which is a coumarin isolated from *Citrus* fruits, possesses dose-dependent chemopreventive activity in rat large bowel tumorigenesis (Tanaka et al., 1998a,b). The inhibitory activity is explained to be correlated with suppression of cell proliferation and lipid peroxidation and with induction of phase-II enzymes.

Furocoumarins are found in a wide variety of plants and have been indicated as phytoalexins in celery (Beier and Oertil, 1983). It is well-known that celery field workers and handlers are frequently affected with photosensitization caused by celery furocoumarins, namely psoralen, bergapten, xanthotoxin, and isopimpinellin (Berkley et al., 1986). In contrast to most furocoumarins, bergamottin does not strongly interact with DNA (Aubin et al., 1994; Morliere et al., 1991).

In conclusion, our results suggest that bergamottin and bergapten are responsible for HL-60 differentiation-inducing activity of the bergamot juices and that hydrophobicity of furocoumarins is correlated with the biological activity. The feasibility of physiological survey on the readily extractable fraction of *Citrus* juices is also demonstrated.

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