Antitumor-Promoting Constituents from *Chaenomeles sinensis* Koehne and Their Activities in JB6 Mouse Epidermal Cells

Huiyuan Gao,[a] Lijun Wu,[a] Masanori Kuroyanagi,*[,b] Kazunori Harada,[b] Nobuo Kawahara,[c] Takahisa Nakane,[d] Kaoru Umehara,[e] Ayako Hirasawa,[e] and Yoshiyuki Nakamura[e]

[a] *Department of Traditional Chinese Medicines, Shenyang Pharmaceutical University; Shenyang 110016 China:* [b] *School of Bioresources, Hiroshima Prefectural University; 562 Nanatsukacho, Shobara, Hiroshima 727–0023, Japan:* [c] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan:* [d] *Tsukuba Medicinal Plant Station, National Institute of Health Sciences; 1 Hachimandai, Tsukuba, Ibaraki 305–0843, Japan: and* [e] *School of Pharmaceutical Sciences, University of Shizuoka; 52–1 Yada, Shizuoka 422–8526, Japan.* Received June 5, 2003; accepted July 22, 2003

Primary screening of antitumor-promoting activity using soft agar colony assays with JB6 cells was employed to isolate 22 compounds from *Chaenomeles sinensis* Koehne. These compounds were lyoniresinol-2a-*O*-α-L-rhamnopyranoside (1), lyoniresinol-2a-*O*-β-D-glucopyranoside (2), aviculin (3), betulinic acid (4), betulin (5), 3-*O*-(*E*)-*p*-coumaroylbetulin (6), 3-*O*-(*E*)-caffeoylbetulin (7), 3-*O*-(*Z*)-*p*-coumaroylbetulin (8), 3-*O*-(*E*)-caffeoyllupeol (9), alphitolic acid (10), sorbikortal II (11), tormentic acid (12), euscaphic acid (13), corosolic acid (14), maslinic acid (15), erythrodiol (16), 1-β-D-glucopyranosyloxy-3,4,5-trimethoxybenzene (17), avicularin (18), 7-*O*-β-D-glucopyranosylkaempferol (19), 5-*O*-β-D-glucopyranosylgenistein (20), 7-*O*-β-D-glucopyranosylgenistein (21), epicatechin (22), and β-sitosterol (23) and were identified using spectral data such as MS, ^{1}H- and ^{13}C-NMR. Compound 1, having a rhamnosyl group, showed greater activity than 2, having a glucosyl group, and 3, which was a bis-demethoxy derivative of 1. Betulinic acid (4), having a C-28 carboxyl group, 3-*O*-(*E*)-caffeoylbetulin (7), and tormentic acid (12) showed more potent activity than betulin (5), which has a C-28 hydroxymethyl group.

Key words antitumor-promoting constituent; JB6 mouse epidermal cell; soft agar colony assay; *Chaenomeles sinensis*; lupane-type triterpene; lyoniresinol-rhamnoside

Substantial attention has been given to primary cancer prevention in daily life.[1)] Inhibition of the tumor promotion step by various plant constituents is thought to be able to prevent cancer development. In the course of our studies on the isolation and structural elucidation of biologically active constituents from plant sources, we have reported antibacterial,[2,3)] antiandrogenic,[4,5)] cell differentiating,[6,7)] and antitumor-promoting compounds.[8)] To take advantage of the woody plant resources of Japan, and in particular to find novel antitumor-promoting constituents, 100 woody plants were screened using a soft agar colony assay with JB6 mouse epidermal cells.[9)]

Anchorage-dependent preneoplastic cells (JB6 cells) are transformed into anchorage independent neoplastic cells by treatment with a promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and are then able to grow in soft agar.[10)] We observed that a MeOH extract of the woody parts of *Chaenomeles sinensis* Koehne (Japanese name Karin) had an inhibitory effect on soft agar colony induction by TPA. *C. sinensis* is a woody plant found in eastern Asia, and is widely distributed as an ornamental tree in Japan. The fruit of this plant is known as Mu Gua in China and is used to treat throat diseases in traditional Chinese medicine and has been utilized in numerous Chinese health beverages. In Japan, the juice of this fruit is usually added to lozenges to treat throat swelling and coughing.

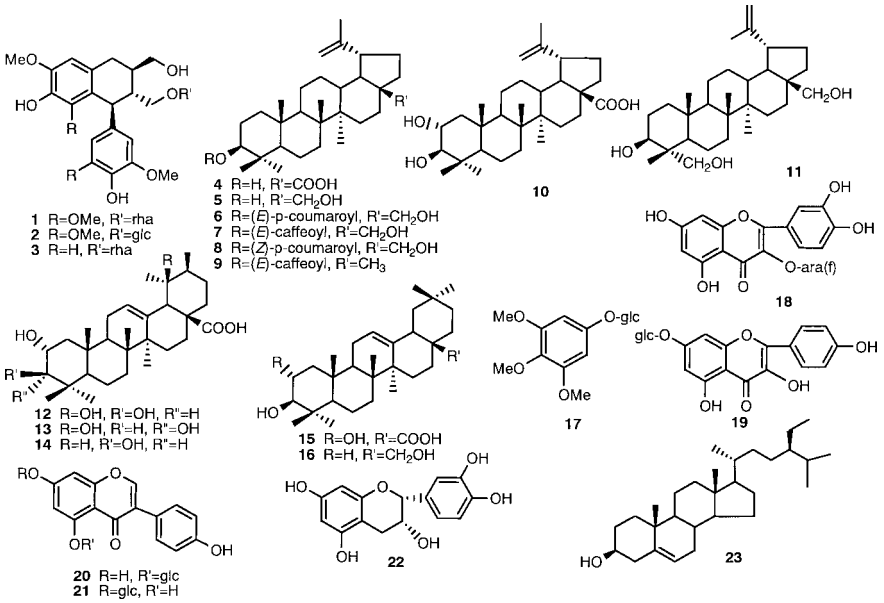
The EtOAc soluble fraction of the MeOH extract showed potent activity, and thus the fraction was purified using repeated silica gel column chromatography and HPLC on an ODS column, giving numerous triterpene derivatives along with lignan, flavone, and isoflavone derivatives. The MeOH eluate from a Diaion HP20 column purification of a hot water extract of the MeOH extraction residue, which was also purified by silica gel column chromatography and HPLC, gave lignan glycosides at relatively high yields. Some of these compounds were tested for antitumor-promoting activity.

This report describes the isolation and structural elucidation of constituents isolated from *C. sinensis* twigs and their antitumor-promoting activities.

Results and Discussion

Constituents and Their Structures More than a hundred MeOH extracts of woody plants were tested and several active extracts were identified. MeOH extracts of *C. sinensis* twigs showed potent antitumor-promoting activity. Three fractions were tested and the EtOAc soluble fraction (CSMA) showed the most potent activity, although the *n*-BuOH soluble fraction (CSMB) also showed activity (Table 1). The MeOH eluate (CSWM) showed a similar TLC pattern as the CSMB. Therefore, CSMA and CSWM were purified by means of repeated silica gel column chromatography and HPLC using an ODS column to give 22 compounds. Compounds **1**—**3** were identified as lyoniresinol-2a-α-L-rhamnopyranoside (**1**),[11)] lyoniresinol-2a-β-D-glucopyranoside (**2**),[12)] and aviculin (**3**),[13)] respectively, based on ^{1}H-, ^{13}C-NMR and MS data. Some of the ^{1}H- and ^{13}C-NMR data for **1** showed overlapping signals. This suggested that **1** could be a diastereomeric mixture containing enantiomeric aglycone parts that were not separated. The acetylation of **1** gave two hexaacetates **1a** and **1b** (ratio; **1a** : **1b**=3 : 2) and these were easily separated. Compound **1** gave an aglycone (**1′**), lyoniresinol, and methyl rhamnoside. The optical rotation of **1′** showed a positive value ($[\alpha]_D$ +5.0°), which was lower than reported data ($[\alpha]_D$ +13.3°).[12)] These data indicated that **1** was a mixture of (+)-lyoniresinol- and (−)-lyoniresinol-α-L-rhamnopyranosides, with (+)-lyoniresinol-α-L-rhamno-pyranoside being predominant. Compounds **4**, **5**, **7**, **9**—**11** were identi-

* To whom correspondence should be addressed. e-mail: kuroyang@bio.hiroshima-pu.ac.jp
© 2003 Pharmaceutical Society of Japan



fied as the lupane-type triterpenes betulinic acid (**4**),[14] betulin (**5**),[14] 3-*O*-(*E*)-*p*-coumaroylbetulin (**6**),[15] 3-*O*-(*E*)-cafffeoylbetulin (**7**),[16] 3-*O*-(*Z*)-*p*-coumaroylbetulin (**8**),[15] 3-*O*-(*E*)-caffeoyllupeol (**9**),[16] alphitolic acid (**10**),[17] and ilekudinol C (**11**).[18] Compounds **12**—**14** were identified as the ursane-type triterpenes tormentic acid (**12**),[17] euscaphic acid (**13**),[17] and corosolic acid (**14**).[17] Compounds **15** and **16** were identified as the oleanane-type triterpenes maslinic acid (**15**)[17] and erythrodiol (**16**).[19] Compounds **17**—**22** were identified as aromatic and flavonoid derivatives; 1-*β*-D-glucopyranosyloxy-3,4,5-trimethoxybenzene (**17**),[20] avicularin (**18**),[21] kaempferol-7-*O*-*β*-D-glucopyranoside (**19**),[22] 5-*O*-*β*-D-glucopyranosylgenistein (**20**),[23] 7-*O*-*β*-D-glucopyranosylgenistein (**21**)[24] and epicatechin (**22**). Compound **23** was identified as *β*-sitosterol.

Antitumor-Promoting Activity Eleven compounds, **1**—**7**, **12**, **17**, **22** and **23**, which were isolated in relatively high yields, were tested for antitumor-promoting activity. The results are shown in Table 2. Betulinic acid (**4**), having a lupane skeleton and a 28-carboxyl group, showed the most potent inhibitory activity (IC_{50} 5.5 μM) against soft agar colony induction by TPA in JB6 cells. Betulin (**5**), with a 28-hydroxyl group, showed weaker activity (IC_{50} 20.9 μM). This indicated that converting the C-28 carboxyl group to a hydroxymethyl group decreased the activity by 1/4. The carboxyl group at C-28 in lupane-type compounds was therefore important for inhibitory activity. 3-*O*-(*E*)-*p*-Coumaroylbetulin (**6**) and 3-*O*-(*E*)-caffeoylbetuline (**7**) exhibited more potent activity than **5**. Caffeoylation and *p*-cinnamoylation at C-3 of betuline (**5**) increased the activity to a level comparable to that of **4**. This indicated that esterfication of lupane-type alcohols with phenylpropanoid-type carboxylic acid enhances inhibitory activity. Betulinic acid (**4**) is a pentacyclic lupane-type triterpene and is one of most common lupan-type triterpenes in plants. It has several botanical sources, but was found in abundance in the bark of white birch trees (*Betula alba*). Betulinic acid (**4**) is known to exert several biological activities, such as anti-tumor activity,[25,26] apoptosis inducing activity,[27,28] inhibitory activity against HIV,[29,30] and anti-malarial activity.[31] Betulinic acid (**4**) is not poisonous and is relatively inexpensive because it is abundantly available from white birch bark. Tormentic acid (**12**), which has an ursane-skeleton, also showed potent suppressing activity (IC_{50} 7.8 μM) against soft agar colony formation. This indicated that the carboxyl group of the triterpenes was important for this activity.

Table 1. Inhibitory Effects of CSM, CSMA, CSMB, and CSMW on Soft Agar Colony Induction by TPA in JB6 Cells (TPA 1 ng/ml)

Fraction	Max. dose tested[a] (μg/ml)	Cell survival % at max. conc.	Soft agar colony induction	
			% of TPA control[b]	IC_{50} (μg/ml)
CSM	30.0	97.8	3.2	6.3
CSMA	10.0	89.0	8.4	3.3
CSMB	30.0	101.2	23.7	9.7
CSMW	30.0	114.9	55.1	50.1

a) Maximum dose of samples was determined from the cytotoxicity test results. *b*) Percentage is the average of two independent experiments in Cl 22 and Cl 41 cell lines of JB6 cells.

Table 2. Inhibitory Effects of Compounds from *C. sinensis* on Soft Agar Colony Induction by TPA in JB6 Cells (TPA 1 ng/ml)

Compound	Max. dose tested[a] (μg/ml)	Cell survival % at max. conc.	Soft agar colony induction	
			% of TPA-control[b]	IC_{50} (μM)
1	30.0	94.9	25.6	8.9
2	30.0	94.1	73.3	>17.2
3	10.0	105.0	71.4	>20.1
4	10.0	99.4	−4.1	5.5
5	30.0	97.6	39.3	20.9
6	10.0	58.6	12.8	7.0
7	30.0	72.7	−6.5	5.6
12	10.0	90.2	0	7.8
17	10.0	86.0	82.0	>28.9
22	10.0	81.1	68.2	>34.5
23	30.0	114.0	40.4	39.6

a) Maximum doses of samples were determined from the cytotoxicity test results. *b*) Percentage is the average of two independent experiments in JB6 Cl 22 and Cl 41 cell lines.

Lyoniresinol-rhamnoside (**1**) also showed potent suppressing activity (IC_{50} 8.9 μM) towards neoplastic transformation of JB6 cells treated with TPA. Lyoniresinol-glucoside (**2**) and aviculin (**3**) demonstrated lower inhibitory activity on soft agar colony induction. The aglycon (**1′**) exhibited greatly reduced inhibitory activity (data not shown). This indicates that sugars attached to lyoniresinol were important and that the methoxyl groups on the lignan part were also important for the inhibitory activity.

Compound **22** [(−)-epicatechin] exerted poor preventive activity against soft colony induction in JB6 cells. This result was different from that of (+)-catechin, which showed more potent activity (IC_{50} 45.1 μM).[8)]

We are interested in the contributions of the functional groups of triterpenoids and lignans to their antitumor-promoting activity. We isolated numerous lupane-type, ursane-type, and oleanane-type triterpenes, but we were unable to carry out antitumor-promoting testing for all of them because of the low yields of these samples. In the future, we hope to investigate the antitumor-promoting activity of a wide range of triterpene derivatives that are more abundant in plant sources.

Experimental

General Experimental Methods NMR measurements were carried out on a JEOL α-500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) with tetramethylsilane as an internal standard. FAB-MS data were recorded on a JEOL HX 110 mass spectrometer. HPLC was carried out using a reverse-phase column (YMC R-ODS-5A) with a CH_3CN–H_2O and MeOH–H_2O solvent system and was monitored at 210 nm. Silica gel 60 (Merck) was used for column chromatography. TLC was carried out using precoated silica gel $60F_{254}$ plates (Merck). Cell incubation assays were performed in a CO_2 gas incubator.

Plant Material *C. sinensis* twigs were collected from a farm in Hiroshima Prefecture, Japan, in June 2000 and after collection, were air-dried.

Extraction and Isolation of Plant Constituents Chipped twigs of *C. sinensis* (2.5 kg) were extracted with MeOH under reflux to give a methanol extract (CSM), which was suspended in water and then extracted with AcOEt to give an AcOEt fraction (CSMA) (62 g) and an aqueous layer. The aqueous layer was extracted with *n*-BuOH to give an *n*-BuOH fraction (CSMB) (89 g) and an aqueous fraction (CSMW). The MeOH extraction residue was extracted with water under reflux to give an aqueous extract, which was subjected to chromatography on a Diaion HP20 column, washed with water, and eluted with MeOH to give a MeOH eluate (CSWM) (50 g). The most active AcOEt soluble fraction (CSMA) (60 g) was subjected to chromatography on a silica gel column with a $CHCl_3$–MeOH gradient system, and afforded 9 fractions. Fraction 5 (12.1 g) was run through a silica gel column using a hexane–AcOEt gradient system and the resultant subfractions were further purified by HPLC using a reverse-phase (ODS) column to give **4** (103 mg), **5** (174 mg), **6** (18 mg), **8** (6 mg), **16** (20 mg), and **23** (75 mg). Fraction 7 (12.1 g) was subjected to chromatography on a silica gel column using a $CHCl_3$–MeOH gradient system. The given subfractions were further purified by HPLC to give **7** (157 mg), **9** (5 mg), **10** (8 mg), **11** (12 mg), **12** (18 mg), **13** (3 mg), **14** (5 mg), and **15** (6 mg). Fraction 9 (19.3 g) gave **1** (6 mg), **2** (3 mg), **3** (3 mg), **18** (5 mg), **19** (6 mg), **20** (16 mg) and **22** (8 mg) after silica gel column and HPLC purification. The MeOH eluate (CSWM) (50 g) was first subjected to chromatography on a silica gel column using a $CHCl_3$–MeOH gradient system, followed by HPLC to give **1** (1.27 g), **2** (31 mg), **3** (13 mg), **17** (144 mg), and **22** (150 mg). The structures of known constituents were identified from ^{1}H-, ^{13}C-NMR and MS data. Some of these compounds were confirmed by two dimensional (2D) NMR experiments such as heteronuclear multiple bond connectivity (HMBC) and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC).

Chemicals TPA (Eden Prairie, MN, U.S.A.), MEM (Nissui Pharmaceutical Co. Ltd., Tokyo), fetal bovine serum (FBS) (Biofluids, Rockville, MD, U.S.A.), Bacto Agar (Difco laboratories, U.S.A.).

Cells JB6 (Cl 22, Cl 41) cells, kindly provided by N. H. Colburn (NCI, Frederick, MD, U.S.A.), were grown at 36.5 °C in 8% FBS (fetal bovine serum)-MEM medium in a CO_2 incubator.

Cell Survival In order to determine the maximal doses of test materials for antitumor-promoting activity assays, cytotoxicity experiments were performed. JB6 Cl 22 and Cl 41 cells (density; 1×10^4 cells/well) cultured overnight in 48-well dishes were treated with samples at six concentrations (0, 1, 3, 10, 30, 100 μg/ml) with or without TPA (1 ng/ml) and were incubated for three additional days. Cell growth was compared with that in the solvent control using the crystal violet staining method. The maximal concentration used in a soft agar experiment was usually the concentration that resulted in 80% or greater cell survival.

Soft Agar Colony Assay Antitumor-promoting activity was estimated based on inhibition of soft agar colony induction by TPA in the JB6 Cl 22 and Cl 41 cell lines, as previously described (10). Cells growing logarithmically in a monolayer culture were trypsinized and suspended in 0.33% agar medium containing 10% FBS and 1 ng/ml TPA (1.6×10^{-9} M) with or without samples at the concentrations indicated in Tables 1 and 2. In duplicate 60-mm Petri dishes, 1.5 ml of the suspension (1×10^4 cells) was poured onto an agar layer containing the same concentration of TPA and/or sample. Soft agar colonies of 8 or more cells were counted after 2 weeks of incubation. The inhibitory activities were expressed as a percentage of that of the TPA control, and were the average of two independent experiments using Cl 22 and Cl 41.

Acknowledgements We thank Dr. K. Masuda and Mr. Y. Takase of Showa Pharmaceutical University for performing the FAB-MS and HR-FAB-MS measurements.

References

1) World Cancer Research Fund/American Institute for Cancer Resarch, "Food, Nutrition and Prevention of Cancer: a Global Perspective," American Institute for Cancer Research, Washington, 1997.
2) Kuroyanagi M., Sugiyama K., Kanazawa M., Kawahara N., *Chem. Pharm. Bull.*, **48**, 1917—1929 (2000).
3) Xiao D., Kuroyanagi M., Itani T., Matsuura H., Udayama M., Murakami M., Umehara K., Kawahara N., *Chem. Pharm. Bull.*, **49**, 1479—1481 (2001).
4) Kuroyanagi M., Arakawa T., Hirayama Y., Hayashi T., *J. Nat. Prod.*, **62**, 1595—1599 (1999).
5) Kuroyanagi M., Seki T., Hayashi T., Nagashima Y., Kawahara N., Sekita S., Satake M., *Chem. Pharm. Bull.*, **49**, 954—957 (2001).
6) Matsuda T., Kuroyanagi M., Sugiyama S., Umehara K., Ueno A., Nishi K., *Chem. Pharm. Bull.*, **42**, 1216—1225 (1994).
7) Kuroyanagi M., Shibata K., Umehara K., *Chem. Pharm. Bull.*, **47**, 1646—1649 (1999).
8) Gao H., Kuroyanagi K., Wu L., Kawahara N., Yasuno T., Nakamura Y., *Biol. Pharm. Bull.*, **25**, 1241—1243 (2002).
9) Gindhart T. D., Nakamura Y., Stevens L. A., Hagameyer G. A., West M. W., Smith B. M., Colburn N. H., "Carcinogenesis," Vol. 8, Raven Press, New York, 1985, pp. 341—367.
10) Nakamura Y., Colburn N. H., Gindhart T. D., *Carcinogenesis*, **6**, 229—235 (1985).
11) Kaneda N., Kinghorn A. D., Farnsworth N. R., Tuchinda P., Udchachon J., Satisuk T., Reutrakul V., *Phytochemistry*, **29**, 3366—3368 (1990).
12) Dada G., Corbani A., Manitto P., Speranza G., Lunazzzi L., *J. Nat. Prod.*, **52**, 1327—1330 (1989).
13) Kim H. J., Woo E.-R., Park H., *J. Nat. Prod.*, **57**, 581—586 (1994).
14) Siddiqui S., Hafeez F., Begum S., Siddiqui B. S., *J. Nat. Prod.*, **51**, 229—233 (1998).
15) Rashid M. A., Gray A. I., Waterman P. G., *J. Nat. Prod.*, **55**, 851—858 (1992).
16) Fuchino H., Satho T., Tanaka N., *Chem. Pharm. Bull.*, **43**, 1937—1942 (1995).
17) Kuang H.-X., Kasai R., Ohtani K., Liu Z.-S., Yuan C.-S., Tanaka O., *Chem. Pharm. Bulll.*, **37**, 2232—2233 (1989).
18) Nishimura K., Fukuda T., Miyase T., Noguchi H., Chen X.-N., *J. Nat. Prod.*, **62**, 1061—1064 (1999).
19) Xue H. Z., Lu Z. Z., Konno C., Soejarto D. D., Cordell G. A., Fong H. H. S., Hodgson W., *Phytochemistry*, **27**, 233—235 (1988).
20) Shimomura H., Sashida Y., Oohara M., Tenma H., *Phytochemistry*, **27**, 644—646 (1988).
21) Fukunaga T., Nishiya K., Kajikawa I., Takeya K., Itokawa H., *Chem. Pharm. Bull.*, **37**, 1543—1546 (1989).
22) Markham K. R., Ternai B., Stanley R., Geiger H., Mabry T. J., *Tetrahedron*, **34**, 1389—1397 (1978).

23) Geibel M., Geiger H., Treutter D., *Phytochemistry*, **29**, 1351—1353 (1990).
24) Hosny M., Rosazza J. P. N., *J. Nat. Prod.*, **62**, 853—858 (1999).
25) Fulda S., Jeremis I., Pietsch T., Debatin K. M., *Klin. Padiatr.*, **211**, 319—322 (1999).
26) Zuco V., Supino R., Righetti S. C., Cleris L., Marchesi E., Gambacorti-Passerini C., Formelli F., *Cancer Lett.*, **10**, 17—25 (2002).
27) Fulda S., Freisen C., Los M., Scaffidi C., Mier W., Benedict M., Nunez G., Karmmmet P. H., Peter M. E., Debatin K. M., *Cancer Res.*, **57**, 4956—4964 (1997).
28) Fulda S., Debatin K. M., *Med. Pediatr. Oncol.*, **35**, 616—618 (2000).
29) Soler F., Poujade C., Evers M., Carry J. C., Henin Y., Bousseau A., Heut T., Pauwels R., De Clercq E., Mayaux J. F., Le Pecq J. B., Dereu N., *J. Med. Chem.*, **39**, 1069—1083 (1996).
30) Vlietinck A. J., De Gruyne T., Apers S., Pieters L. A., *Planta Med.*, **64**, 97—109 (1998).
31) Steele J. C., Warhurst D. C., Kibry G. C., Simmonds M. S., *Phytother. Res.*, **13**, 115—119 (1999).