Identification of Coumarins from the Fruit of *Citrus hystrix* DC as Inhibitors of Nitric Oxide Generation in Mouse Macrophage RAW 264.7 Cells

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Three known coumarins have been isolated from *Citrus hystrix* DC as inhibitors of both lipopolysaccharide (LPS) and interferon-γ (IFN-γ)-induced nitric oxide (NO) generation in RAW 264.7 cells. The inhibitory activity of bergamottin ($IC_{50} = 14\ \mu M$) was comparable to that of *N*-(iminoethyl)-L-ornithine (L-NIO) ($IC_{50} = 7.9\ \mu M$), whereas oxypeucedanin and 5-[(6′,7′-dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen, structurally different from bergamottin only in their side-chain moieties, were notably less active. Using 21 coumarins, we structurally classified various types of coumarins into groups A−C: (A) bearing an isoprenyl (IP) or a geranyl (GR) group, highly active; (B) bearing an IP group cyclized to a coumarin ring, moderately active; (C) bearing an IP group modified with hydroxyl group(s) and/or having other functional groups except for the IP, completely inactive. Cellular uptake studies suggested that coumarins in group C are inactive because of poor permeability to the cell membrane.

Keywords: *Citrus hystrix DC; psoralen; coumarin; nitric oxide; RAW 264.7 cells; structure−activity relationships*

INTRODUCTION

Nitric oxide (NO), a gaseous free radical, is synthesized in biological systems by a family of enzymes, constitutive NO synthase (cNOS) and an inducible one (iNOS) (Vanvaskas and Schmidt, 1997). In the latter, NO is readily released by the iNOS function, which is induced by stimulation of bacterial lipopolysaccharide (LPS) or interferon-γ (IFN-γ) to form stoichiometric amounts of L-citrulline from L-arginine in some cell lines such as macrophages (Nathan and Xie, 1994). Although NO was primarily reported as an endothelium-derived relaxing factor and is thought to act as an intra- and intercellular messenger, excess generation of NO by iNOS has attracted attention because of its relevance to carcinogenesis (Ohshima and Bartsch, 1994; Xie et al., 1997). NO has been reported to cause mutagenesis (Arroyo et al., 1992) and deamination of DNA bases (Wink et al., 1991) and, more importantly, to form carcinogenic *N*-nitroso compounds through its simultaneous conversion to nitrite (NO_2^-), which covalently binds to primary and secondary amines in acidic conditions (Miwa et al., 1987). Moreover, an important chemical property of NO is that it reacts rapidly and spontaneously with a superoxide anion (O_2^-) to form a peroxynitrite anion ($ONOO^-$) (Ischiropoulos et al., 1992), which is more toxic than O_2^- or NO to biological systems by causing modification of proteins (e.g., 3-nitrotyrosine) (van der Vliet et al., 1995) or nucleic acid (e.g., 8-nitroguanine) (Yermilov et al., 1995). Collectively, suppression of the iNOS-induced NO generation in excess amounts is now accepted widely as a new paradigm for the chemoprevention of cancer.

We have recently reported NO generation suppressive properties of the methanol extracts from edible Japanese plants (Kim et al., 1998) and presumed that their inhibitory effects on NO generation may, in part, rationalize lower cancer risks by ingestion of vegetal plants. On the contrary, Miwa et al. (1997) reported that the water extracts from meats and offal may contain inducer(s) of NO generation using a murine macrophage cell line, RAW 264.7 cells, supporting the implications of the relevance of a “Western-style diet” to human carcinogenesis.

We have focused on edible Southeast Asian plants as promising sources of effective chemopreventive agents. By using an in vitro antitumor-promoting bioassay, we found greater cancer preventive potential from edible Thai and Indonesian plants than from common Japanese plants (Murakami et al., 1995a, 1998a). On this basis, 1′-acetoxychavicol acetate (Murakami et al., 1996), cardamonin (Murakami et al., 1993), pheophor-

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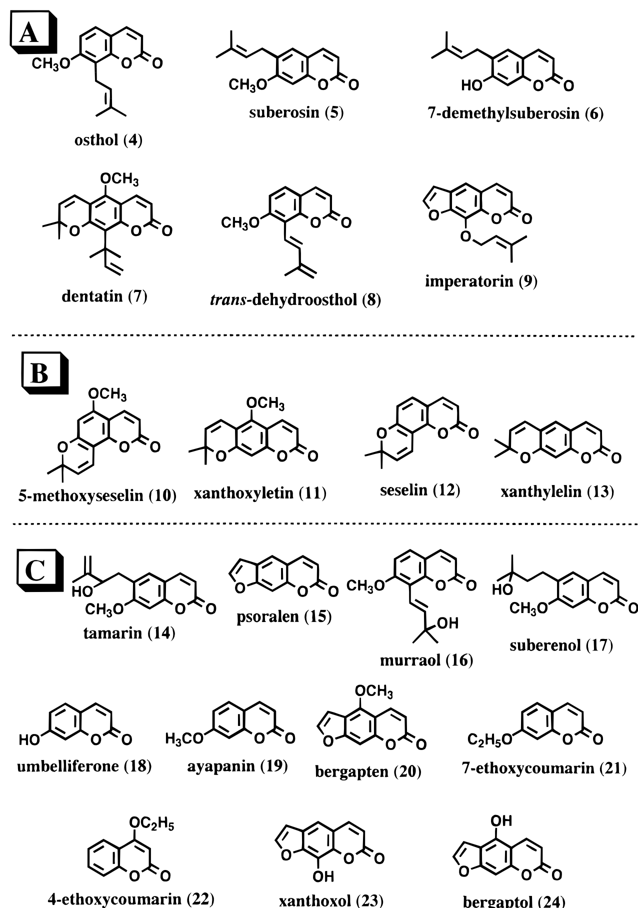

Figure 1. Structures of coumarins tested for structure−activity relationship studies: (A) compounds bearing IP or GR groups; (B) those in which IP groups are cyclized to coumarin rings; (C) those possessing IP or GR groups attached without hydroxyl group(s) or other functional groups.

bide *a* (Nakamura et al., 1996), niaziminin (Murakami et al., 1998b), and others have been isolated as the active principles. They showed notable chemopreventive effects when tested in rodent models. In 1995, we reported that glyceroglycolipids from the leaves of *Citrus hystrix* DC (Rutaceae), a herbal flavor in Southeast Asia, strongly inhibited the tumor-promoting activity of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in vitro and in vivo (Murakami et al., 1995b). The above led us in turn to search for such inhibitors from the fruit of *C. hystrix* of LPS/IFN-γ-induced NO generation in RAW 264.7 cells. In the present study, we identified three known coumarins as NO generation inhibitors, and then we examined the structure−activity relationships of the 21 related coumarins. In addition, the influence of the cellular uptake efficiency of coumarins on their NO inhibitory potential is discussed.

MATERIALS AND METHODS

General Procedure. UV (UV-2200, Shimadzu, Kyoto, Japan), IR (FTIR-8200, Shimadzu), $[\alpha]_D$ DIP-1000 (JASCO, Tokyo, Japan), ^{1}H NMR (DRX-300, Brucker, TMS as an internal standard), and APCI-MS (Hitachi MH-2000), HPLC (Hitachi, Japan), YMC I-40/64 gel (octadecylsilane, ODS) (Yamamura Chemical Laboratory, Kyoto, Japan), and Kieselgel 60 F254 for TLC (Merck Co. Ltd., Darmstadt, Germany) analyses were performed.

Chemicals and Cells. L-Arginine and (6*R*)-tetrahydro-L-biopterin (BH_4) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). RPMI 1640 medium was from Gibco BRL

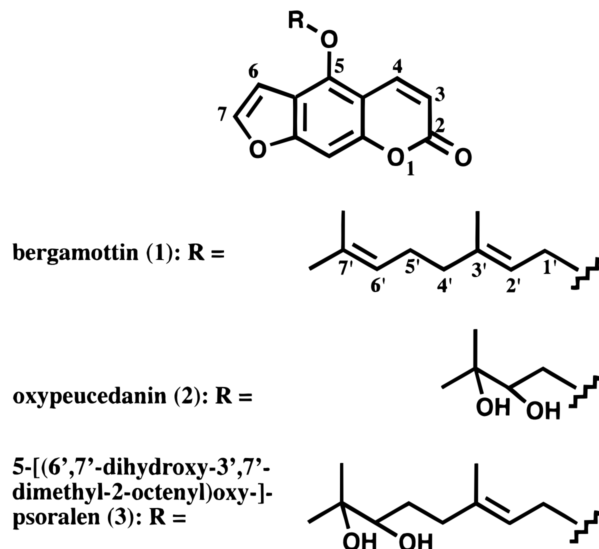

Figure 2. Structures of bergamottin, oxypeucedanin, and 9-[(6′,7′-dihydroxy-3′, 7′-dimethyl-2-octenyl)oxy]psoralen isolated from the fruit of *C. hystrix*.

(Grand Island, NY); LPS (*Escherichia coli* serotype 0127, B8) from Difco Labs (Detroit, MI);, and IFN-γ from Genzyme (Cambridge, MA). Coumarins (Figure 1), osthol (**4**), suberosin (**5**), 7-demethylsuberosin (**6**), dentatin (**7**), *trans*-dehydroosthol (**8**), 5-methoxyseselin (**10**), xanthylelin (**11**), seselin (**12**), xanthyletin (**11**), tamarin (**14**), murraol (**16**), suberenol (**17**), and umbelliferone (**18**) were isolated as previously reported (Wu et al., 1983; Ito et al., 1987, 1990, 1991); imperatorin (**9**), psoralen (**15**), ayapanin (**19**), bergapten (**20**), 7-ethoxycoumarin (**21**), 4-ethoxycoumarin (**22**), xanthoxol (**23**), and bergaptol (**24**) (Figure 1) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Other chemicals were obtained from Wako Pure Chemical Co. Ltd. (Osaka, Japan). RAW 264.7 cells were a kind gift from Ohtsuka Pharmaceutical Co. Ltd. (Ohtsu, Japan).

Isolation and Identification of Active Constituents. One kilogram of fresh fruits of *C. hystrix*, purchased from a market in Thailand, were extracted with methanol at room temperature. After filtration, each extract was concentrated in vacuo. After partition between ethyl acetate (EtOAc) and deionized water, the EtOAc layer (5.3 g) was subjected to silica gel column chromatography (Wakogel C-200, EtOAc/*n*-hexane, stepwise) to give 20% EtOAc eluate (444 mg), which was finally purified by preparative TLC (EtOAc/*n*-hexane, 1:10) to give bergamottin (**1**, 66.2 mg). Oxypeucedanin (**2**, 475 mg) and 5-[(6′,7′-dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen (**3**, 98.5 mg) (Figure 2) were isolated from 80% EtOAc eluate (984 mg) by ODS column chromatography (50% MeOH/H_2O) and then preparative TLC (EtOAc/*n*-hexane, 4:1). Bergamottin: UV λ_{max} (EtOH) nm (ϵ) 220 (31 200), 250 (22 500), 258 (20 000), 267 (19 200), 309 (17 100); IR ν_{max} ($CHCl_3$) cm^{-1} 1724, 1628, 1458;^{1}H NMR ($CDCl_3$) δ 1.60 (3H, s, 3′-Me), 1.68 (3H, s, 7′-Me), 1.69 (3H, s, 7′-Me), 2.10 (4H, m, H-4′,5′), 4.94 (2H, d, J = 6.8 Hz, H-1′), 5.07 (1H, m, H-6′), 5.54 (1H, t, J = 6.8 Hz, H-2′), 6.27 (1H, d, J = 9.8 Hz, H-3), 6.95 (1H, d, J = 2.2 Hz, H-6), 7.16 (1H, s, H-8), 7.59 (1H, J = 2.2 Hz, H-7), 8.16 (1H, d, J = 9.8 Hz, H-4); APCI-MS *m/z* (rel int, %) 339 ([M + H]$^+$, 63), 203 (100), 85 (89). Oxypeucedanin: $[\alpha]^{20}_D$ +16.5° (*c* 1.1, $CHCl_3$); UV λ_{max} (EtOH) nm (ϵ) 221 (21 200), 250 (16 200), 259 (14 400), 267 (14 400), 309 (12 900); IR ν_{max} ($CHCl_3$) cm^{-1} 3300−3500 (br) 1725, 1628, 1458; ^{1}H NMR ($CDCl_3$) δ 1.62 (3H, s, 3′-Me), 1.67 (3H, s, 3′-Me), 4.36 (1H, dd, J = 8.2, 2.3 Hz, H-2), 4.81 (1H, t, J = 8.2 Hz, H-3a), 5.20 (1H, dd, J = 8.2, 2.3 Hz, H-3b), 6.26 (1H, d, J = 9.8 Hz, H-3), 7.32 (1H, d, J = 2.3 Hz, H-6), 7.78 (1H, d, J = 2.3 Hz, H-7), 8.31 (1H, d, J = 9.8 Hz, H-4); APCI-MS *m/z* (rel int, %) 305 [(M + H)$^+$, 76], 287 [M − H_2O + H]$^+$, 100], 203 (84). 5-[(6′,7′-Dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen: $[\alpha]^{20}_D$ +11.0° (*c* 0.99, $CHCl_3$); UV λ_{max} (EtOH) nm (ϵ) 221 (25 700), 250 (16 200), 258 (17 200), 267 (16 500),

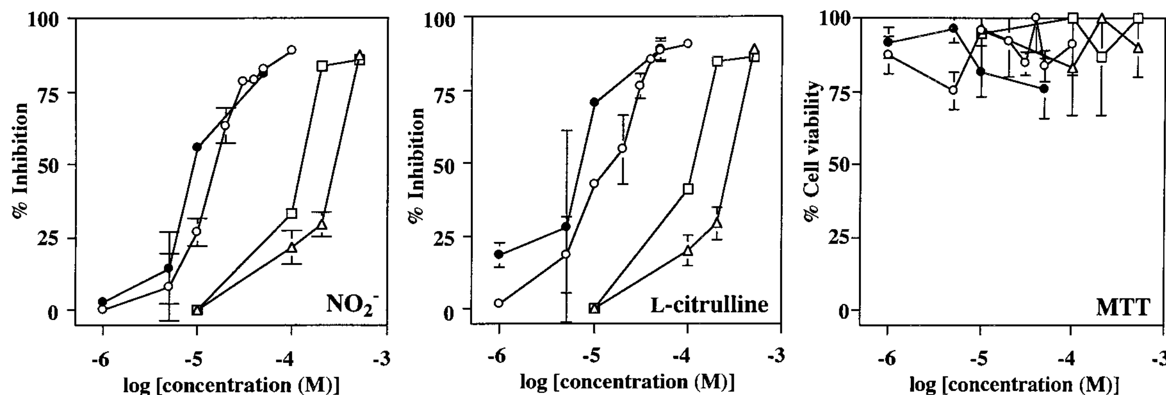

Figure 3. Inhibitory activities of bergamottin (○), oxypeucedanin (△), 5-[(6′, 7′-dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen (□), and L-NIO (●) toward LPS/IFN-γ-induced NO generation in RAW 264.7 cells. NO generation was induced by LPS (100 ng/mL) and IFN-γ (100 U/mL) in RAW 264.7 cells at 37 °C for 24 h (left, inhibition of NO_2^-; middle, inhibition of L-citulline; right, MTT assay).

309 (14 900); IR ν_{max} ($CHCl_3$) cm^{-1} 3300−3500 (br) 1725, 1628, 1458; ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, s, 7′-Me), 1.20 (3H, s, 7′-Me), 1.71 (3H, s, 3′-Me), 1.22−2.41 (4H, m), 3.32 (1H, dd, J = 10.3, 1.5 Hz, H-6′), 4.95 (2H, d, J = 6.8 Hz, H-1′), 5.61 (1H, t, J = 6.8 Hz, H-2′), 6.27 (1H, d, J = 9.8 Hz, H-3), 6.95 (1H, d, J = 2.3 Hz, H-6), 7.59 (1H, d, J = 2.3 Hz, H-7), 8.15 (1H, d, J = 9.8 Hz, H-4); APCI-MS m/z (rel int, %) 373 ([M + H]$^+$, 83), 355 ([M − H_2O + H]$^+$, 100), 203 (63), 153 (58).

NO Generation Test in RAW 264.7 Cells. Inhibitory tests of LPS/IFN-γ-induced NO generation were done as previously reported (Kim et al., 1998). Briefly, murine macrophage cell line RAW 264.7 cells were cultivated in RPMI medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2 atmosphere. The cells (2 × 10^5 cells/mL) grown in 1 mL of RPMI medium on a 24-well plate were treated with LPS (100 ng/mL), tetrahydrobiopterin (BH_4, 10 mg/mL), IFN-γ (100 U/mL), L-arginine (2 mM), and a test compound dissolved in DMSO at appropriate concentrations. The final DMSO concentration in the medium was 0.5%.

After 24 h, the levels of both NO_2^- and L-citrulline were measured by Griess (Green et al., 1982) and diacetyl monoxime (Boyde and Rahmatullah, 1980) assays, respectively. Cytotoxicity was measured by a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sladowaski et al., 1992). Each experiment was done in duplicate, and the data are shown as mean ± standard deviation values.

Cellular Uptake Experiment. RAW 264.7 cells (5 × 10^5 cells/mL) were grown in 2 mL of RPMI medium on a 35 mm dish overnight, and 10 μL of a test compound solution (10 mM in DMSO) was added to the cell culture. After an appropriate time, the medium was removed and cells were washed with PBS (1.5 mL) twice. The PBS used for washing (3.0 mL) was combined with the removed medium, and the mixture thus obtained was extracted with 5 mL of $CHCl_3$ for measurement of extracellular compounds. The $CHCl_3$ layer thus obtained was dried in vacuo and then dissolved in 50 μL of $CHCl_3$ for HPLC analysis. HPLC conditions: YMC pack ODS-A (Yamamura Chemical Laboratory, Kyoto, Japan, 6.0 mm × 150 mm); mobile phase, 80% (bergamottin) or 45% (bergapten) acetonitrile in water; flow rate, 1.0 mL/min; detection, 320 nm. In the above HPLC conditions, bergamottin and bergapten were detected at retention times of 13.5 and 11.0 min, respectively. For intracellular measurement, cells were trypsinized and washed with PBS twice followed by centrifugation. After being partitioned between 5 mL of PBS and 5 mL of $CHCl_3$, cells were sonicated for 10 s twice on ice. The $CHCl_3$ layer thus obtained was analyzed on HPLC as described above.

RESULTS

Isolation and Identification of NO Generation Inhibitors. Fresh fruits of *C. hystrix* were extracted with methanol. After partitioning between EtOAc and water, the EtOAc layer was subjected to silica gel column chromatography (EtOAc/n-hexane, stepwise) to give 20% EtOAc eluate, which was further purified by preparative TLC (EtOAc/n-hexane, 1:10) to give an active compound (**1**). Compounds **2** and **3** were isolated from the 80% EtOAc eluate by ODS column chromatography and then by preparative TLC. Their spectral data were in good agreement with those previously reported [**1**, bergamottin (Dreyer and Huey, 1973); **2**, oxypeucedanin (Harkar et al., 1984); **3**, 5-[(6′,7′-dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen (Dreyer and Huey, 1973] (Figure 2). The structural differences among the psoralens (furanocoumarins) were found only in the side-chain structures.

NO Generation Inhibitory Activities of Active Constituents. The concentration of nitrite (NO_2^-) in the medium of positive control only with LPS/IFN-γ-stimulation after 24 h was 15.8 ± 4.9 μM. As shown in Figure 3, bergamottin (**1**) showed marked inhibition of both NO_2^- and L-citrulline formation in LPS/IFN-γ-stimulated RAW 264.7 cells in a concentration-dependent manner after 24 h. It should be noted that its inhibitory potency (IC_{50} = 14 μM) is comparable to that of N-(iminoethyl)-L-ornithine (L-NIO, IC_{50} = 7.9 μM), a synthetic L-arginine analogue inhibitor of iNOS. Interestingly, **2** (IC_{50} = 310 μM) and **3** (IC_{50} = 130 μM), both closely related coumarins to bergamottin, were found to be notably less active than bergamottin. The isolated compounds and L-NIO showed no detectable cytotoxicity by MTT assay at every concentration tested.

Structure−Activity Relationships of Coumarins. As shown above, it is interesting to note that the characteristics of side chains between bergamottin and oxypeucedanin, 5-[(6′,7′-dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen, all of which have psoralen (furocoumarin) rings, clearly differentiate their inhibitory potencies toward NO generation. Namely, psoralen possessing a geranyl (GR) group (bergamottin) is highly active (IC_{50} = 14 μM), whereas psoralens bearing isoprenyl (IP) or GR chains with hydroxyl groups were drastically less active, that is, oxypeucedanin, 310 μM, and 5-[(6′,7′-dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen, 130 μM. These results led us to examine the coumarins modified with various functional groups for studies of their structure−activity relationships. A total of 21 naturally occurring coumarin- or psoralen-related compounds from *Murraya* or *Citrus* plants (Rutaceae each) or commercially available compounds were tested at concentrations of 10 and 50 μM (Figure 1). No notable cytotoxicity was observed in each experiment (data not

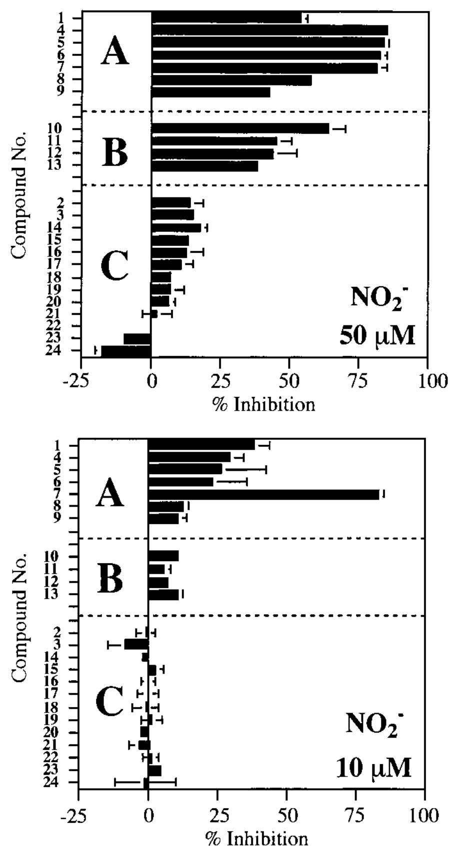

Figure 4. Inhibitory activities of coumarins at concentrations of 50 μM (above) and 10 μM (below) toward LPS/IFN-γ-induced NO_2^- production in RAW 264.7 cells. See captions of Figures 2 and 3 for assay conditions and classifications of groups A–C. The names of numbered compounds are shown in Figure 3.

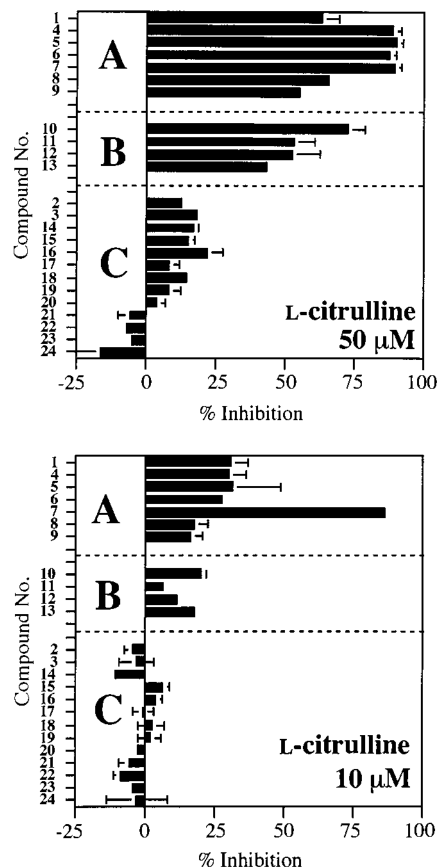

Figure 5. Inhibitory activities of coumarins are concentrations of 50 (above) and 10 μM (below) toward LPS/IFN-γ-induced L-citrulline production in RAW 264.7 cells. See captions of Figures 2 and 3 for assay conditions and classifications of groups A–C. The names of numbered compounds are shown in Figure 3.

shown). Including the isolated compounds, they were classified into three groups A–C on the basis of their structural status in the side chain: (A) IP or GR group on which no hydroxyl groups are substituted; (B) IP group cyclized to form dimethylchromene rings; (C) IP or GR group modified with hydroxyl group(s) and/or other functional groups except for the IP group. As clearly shown in Figures 4 and 5, groups A–C were highly active, moderately active, and inactive, respectively, in the inhibition of the NO_2^- (Figure 4) and L-citrulline (Figure 5) formation. The mean ± SD values of inhibition rates at 10 or 50 μM in group A are statistically different from those in groups B and C (Figure 6). Of all the coumarins tested, the inhibitory potency of dentanin (**7**) is conspicuous because it inhibited NO_2^- and L-citrulline formation by ~80% at 10 μM, whereas others in group A showed only moderate or weak inhibition (<50%) (Figures 4 and 5).

Cellular Uptake of Coumarins. For understanding the distinct differences in the activity of coumarins between groups A and C, we estimated the uptake efficiency of test compounds into RAW cells using HPLC for monitoring the intra- and extracellular compound levels. The uptake rates of bergamottin (**1**, IC_{50} = 14 μM) and bergapten (**20**, IC_{50} > 50 μM) were compared due to their significant activity contrast. As shown in Figure 7, although significant cellular uptake was observed for bergamottin (8.9–18.6% of the total during at 10 min–7.2 h), bergapten was found to be hardly incorporated into RAW 264.7 cells (0–1.1% of the total) at anytime tested.

DISCUSSION

The Rutaceae plants are known as one of the natural sources for structurally diverse coumarins (Gray and Waterman, 1978). Their biological functions have been reported to be anti-platelet-aggregating (Chen et al., 1995), antimicrobial (Nakatani et al., 1987), antimutagenic (Edenharder et al., 1995), and anti-tumor-promoting (Mizuno et al., 1994; Murakami et al., 1997) activities. Moreover, inhibitory activities of coumarins toward O_2^- generation in leukocytes have recently been demonstrated (Paya et al., 1993; Murakami et al., 1997). To our knowledge, however, this is the first report regarding the inhibition of NO generation by coumarin-related compounds in macrophages. The inhibitory potency of bergamottin (**1**, IC_{50} = 14 μM) was comparable to that of L-NIO (IC_{50} = 7.9 μM), a synthetic iNOS inhibitor. The mechanism of action of L-NIO is the competent inhibition of iNOS enzyme with an iNOS substrate, L-arginine, which is structurally analogous to L-NIO. On the other hand, the action mechanisms of bergamottin by which it reduces extracellular NO levels remain to be elucidated in detail, and some discussion is possible. As noted above, NO is readily released by LPS/IFN-γ stimulation to form stoichiometric amounts of L-citrulline from L-arginine in RAW 264.7 cells and is then converted simultaneously to NO_2^- in physiological conditions. The data showing that bergamottin inhibited the formation of L-citrulline and NO_2^- to a similar extent (Figure 3) clearly indicate that bergamottin has no NO scavenging potential and probably

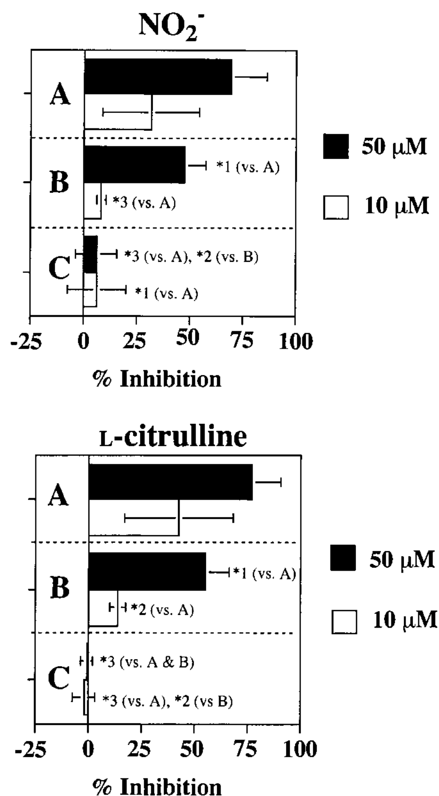

Figure 6. Average inhibitory percentages for NO_2^- (above) and L-citrulline (below) production in groups A−C. See caption of Figure 3 for classification of groups A−C. Solid and open bars indicate inhibitory percent at 50 and 10 μM, respectively. Statistical analysis was done by Student *t* test. *1, $P < 0.05$; *2, $P < 0.01$; *3, $P < 0.001$.

inhibits the LPS/IFN-γ-triggered iNOS expression pathways and/or iNOS enzyme activity. A similar inhibitory tendency is shown in other coumarin derivatives tested in the present study (Figures 4 and 5). It is of interest that their inhibitory manner, that is, generation inhibition rather than scavenging, has also been observed in the case of the inhibition of the O_2^- generating system by coumarins (Paya et al., 1993; Murakami et al., 1997). LPS-induced iNOS gene expression is considered to occur partly through the activation of mitogen-activated protein kinases (MAPK), subsequently activating kinase bound to microtubules (Ding et al., 1996), and the nuclear translocation of the transcription factor nuclear factor κ B (NF-κB) triggered by proteolysis of inhibitors κB-α (IκB-α) and IκB-β (Milligan et al., 1996). The effects of bergamottin on the above biochemical pathways should be examined in the future.

Interestingly, bergamottin ($IC_{50} = 14$ μM) was exclusively active for NO generation inhibition as compared with oxypeucedanin ($IC_{50} = 310$ μM) and 5-[(6′,7′-dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen ($IC_{50} =$ 130 μM), although they are structurally different only in their side-chain moieties. As noted above, because there are structurally diverse coumarins in the plant kingdom (Gray and Waterman, 1978), categorization of their various potentials for NO generation inhibition based on chemical structures is important and useful to arrange and predict the inhibitory activities of various coumarins. As presented in Figures 1 and 4−6, we could structurally classify various types of coumarins into groups A−C by their side-chain structures and inhibitory potencies toward NO generation: (A) compounds bearing an IP or a GR group are highly active; (B) compounds bearing an IP group cyclized to form dimethylchromene rings are moderately active; (C) compounds bearing an IP group modified with hydroxyl group(s) and/or having other functional groups except for an IP are totally inactive. According to this formula, auraptene (7-genranyloxycoumarin), which we have recently isolated from *Citrus natsudaidai* as a powerful chemopreventer in mouse skin (Murakami et al., 1997), rat colon (Tanaka et al., 1997), and tongue (Tanaka et al., 1998), is deduced to be effective for NO inhibition. In fact, auraptene showed marked inhibition toward NO generation in RAW 264.7 cells (Murakami et al., unpublished results). The carbon chain length or location of the terpene side chain does not regulate activity because bergamottin and osthol (**4**), for example, are similarly active. Yet, the presence or absence of the ether oxygen atom between the IP group and coumarin ring is unimportant (e.g., osthol versus imperatorin). We cannot explain clearly why only dentatin (**7**) shows a notably higher activity at a concentration of 10 μM than other coumarins in group A (Figures 4 and 5). However, it is tempting to speculate that the presence of another IP group cyclized to the coumarin ring and/or the presence of a structurally unique 1,1-dimethylallyl group may act as increasing factors for activity. We have no explanations why several coumarins such as bergaptol slightly enhanced NO_2^- and L-citrulline production. Additional experiments are necessary to address the mechanisms.

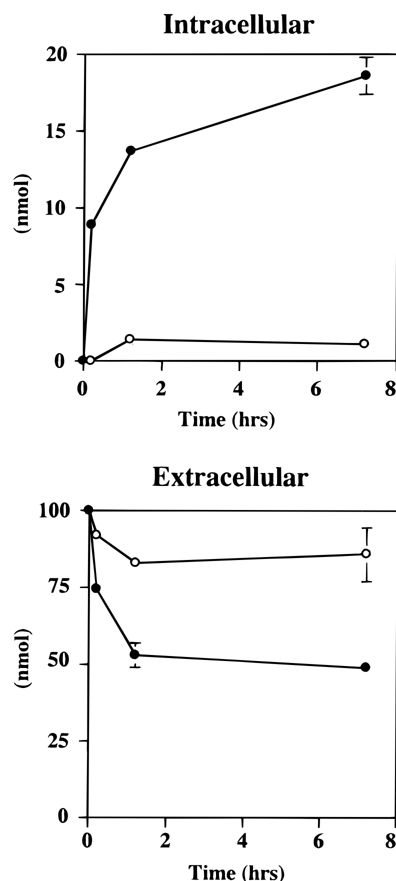

Figure 7. Cellular uptake of bergamottin (●) and bergapten (○) into RAW 264.7 cells. RAW 264.7 cells (5×10^5 cells/mL) were grown on a 35 mm dish overnight, and final concentration of test compounds was 50 μM. Their intra- and extracellular levels were determined by using HPLC (see details under Materials and Methods).

Cellular uptake rate is one of the crucial factors for activity exhibition because more hydrophilicity, in general, is a decreasing factor of test compounds for cellular incorporation (e.g., hydroxyl group in group C). Cellular uptake studies (Figure 7) clearly demonstrated that inactivity of bergapten (**20**) is attributable to its poor permeability to the cell membrane in contrast to bergamottin, which is structurally analogous to bergapten and has both significant activity (Figure 3) and high uptake rate (Figure 7), suggesting that the IP group plays an important role in cellular uptake to exert activity. It is uncertain, however, if the IP or GR group also contributes to activity exhibition within cells. In general, a hydroxyl group decreases molecular hydrophobicity. Therefore, the inactivity of tamarin, murraol, suberenol, xanthoxol, and bergaptol, all of which have hydoxyl group(s) and are thus supposed to be more hydrophilic than bergapten, may similarly be due to low efficiency for cellular incorporation. However, it should be noted that the presence of an IP group compensates the activity decrease due to the presence of a phenolic hydroxyl group because 7-demethylsuberosin, the only coumarin in this study bearing both an unmodified IP and a phenolic hydroxyl group, is significantly active in contrast to the inactive umbelliferone.

In conclusion, the present study shows that IP or GR coumarins are potent inhibitors of NO generation in macrophages. In particular, it is notable that the side chain is not allowed to be modified with any hydroxyl groups or replaced by other functional groups (methoxyl, ethoxyl, etc.) for activity exhibition. The inactive types of coumarins were suggested to have no inhibitory potential, resulting from their poor permeability to the cell membrane. Because NO is closely associated with various types of cancer, inhibition of NO generation through iNOS by coumarins may, in part, explain their chemopreventive activity in rodents and humans.

ABBREVIATIONS USED

LPS, lipopolysaccharide; IFN-γ, interferon-γ; NO, nitric oxide; iNOS, inducible NO synthase; L-NIO, *N*-(iminoethyl)-L-ornithine; IP, isoprenyl; GR, geranyl; cNOS, constitutive NO synthase; NO_2^-, nitrite; O_2^- superoxide anion; $ONOO^-$, peroxynitrite anion; COX, cylooxygenease; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ODS, octadecylsilane; EtOAc, ethyl acetate; BH_4, tetrahydrobiopterin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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