Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1887–1893

BIOORGANIC & MEDICINAL CHEMISTRY

Inhibitors from Rhubarb on Lipopolysaccharide-Induced Nitric Oxide Production in Macrophages: Structural Requirements of Stilbenes for the Activity

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Received 11 January 2001; accepted 24 March 2001

Abstract—By bioassay-guided separation, three stilbenes (rhapontigenin, piceatannol, and resveratrol), two stilbene glucoside gallates (rhaponticin 2″-*O*-gallate and rhaponticin 6″-*O*-gallate), and a naphthalene glucoside (torachrysone 8-*O*-β-D-glucopyranoside) with inhibitory activity against nitric oxide (NO) production in lipopolysaccharide-activated macrophages were isolated (IC_{50} = 11–69 μM). The oxygen functions (–OH, $–OCH_3$) of stilbenes at the benzene ring were essential for the activity. The glucoside moiety reduced the activity, while the α,β-double bond had no effect. Furthermore, the active stilbenes (rhapontigenin, piceatannol, and resveratrol) did not inhibit inducible NO synthase activity, but they inhibited nuclear factor-κB activation following expression of inducible NO synthase.

Introduction

Korean rhubarb, the rhizome of *Rheum undulatum* L., is used as a remedy for the blood stagnation syndrome ('Oketsu syndrome' in Japanese traditional medicine) as well as a purgative agent. This rhubarb is considered to have a lesser purgative effect but more potent effect on Oketsu syndrome than other rhubarbs such as *R. palmatum*.[1] Previously, anti-allergic and anti-inflammatory effects of the hot water extract were reported to be responsible for its anti-Oketsu effect.[1] However, its pharmacological properties and bioactive constituents have not been studied in sufficient detail. Recently, we reported the isolation and structure elucidation of anti-oxidant constituents from the rhizome of *R. undulatum* and elucidated the structural requirements for anti-oxidant activity (Chart 1).[2]

Nitric oxide (NO) has been implicated in a number of diverse physiological or pathological processes, such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation. Among the NO synthase (NOS) family, inducible NOS (iNOS) has been shown to be involved in pathological processes via overproduction of NO, and is expressed in response to pro-inflammatory agents [interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and lipopolysaccharide (LPS), etc.] in various cell types including macrophages, endothelial cells and smooth muscle cells.[3] Nuclear factor (NF)-κB is a major transcription factor involved in iNOS, TNF-α, IL-1β, and IL-8 gene expression. NF-κB is present as an inactive form due to combination with an inhibitory subunit, IκB, which keeps NF-κB in the cytoplasm, thereby preventing activation of the target gene in the nucleus. Cellular signals lead to phosphorylation of IκB following elimination of IκB from NF-κB by proteolytic degradation. Then, the activated-NF-κB is released and translocated into the nucleus to activate transcription of its target genes.[4] Inhibition of iNOS enzyme activity or iNOS induction and inhibition of NF-κB activation may be of therapeutic benefit in various types of inflammation.[3–5]

In the course of our characterization studies on anti-Oketsu effect in traditional medicine, we reported various constituents of natural medicines that act as inhibitors of overproduction of NO.[6] In our continuing study, the methanolic extract from the dried rhizome of *R. undulatum* was found to show inhibitory activity against NO production in LPS-activated mouse peritoneal

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0968-0896/01/$ - see front matter
PII: S0968-0896(01)00093-1

macrophages. By bioassay-guided separation, we isolated 10 known stilbene constituents from the active fraction together with a naphthalene glucoside and four anthraquinone glucosides. This report describes the inhibitory effects of constituents from the rhizome of *R. undulatum*, and structural requirements of active constituents for the activity.[7] In addition, we examined the inhibitory effects of the principal active constituents on iNOS enzyme activity, iNOS induction and NF-κB activation to clarify their mechanisms of action.

Results and Discussion

Isolation of NO production inhibitors from the rhizome of *R. undulatum*

First, the effects of the methanolic extract on nitrite accumulation from LPS-activated macrophages were examined. Nitrite, an oxidative product of NO, was accumulated in the medium after 20 h of incubation with LPS. Nitrite concentration in the medium without inhibitors (control group) was 28.3 ± 5.7 μM, and that in

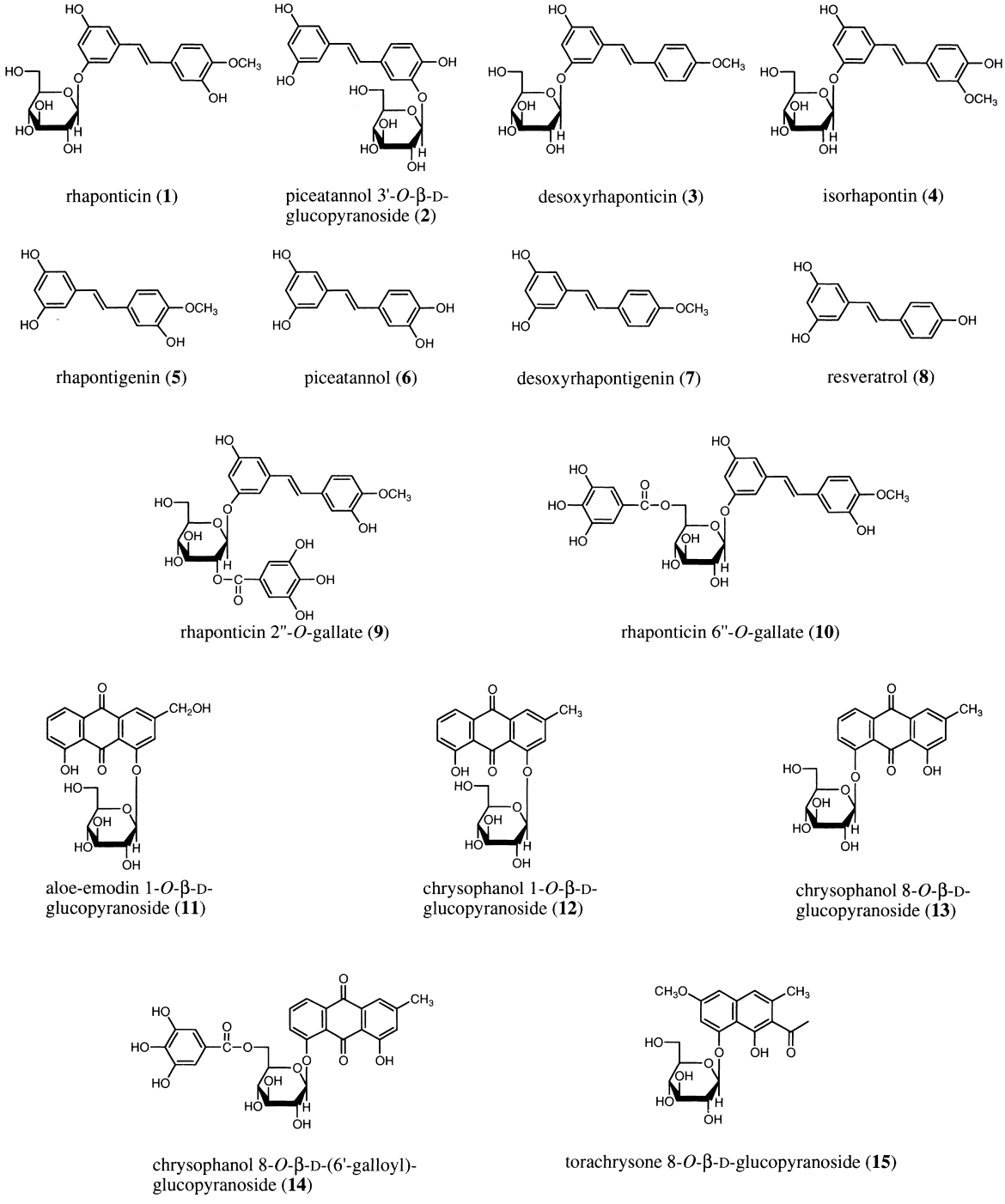


Chart 1. Chemical constituents from the rhizome of *R. undulatum*.

the medium without LPS (unstimulated group) was 2.9±2.1 μM (mean±SD of 38 experiments). Reference compounds, CAPE (an inhibitor of NF-κB activation),[5b] L-NMMA (a non-selective inhibitor of NOS),[8] and GED (an inhibitor of iNOS)[9] potently inhibited nitrite accumulation in the medium. The methanolic extract (33.8% from the natural medicine) of the dried rhizome of *R. undulatum* significantly inhibited the nitrite accumulation (Table 1).

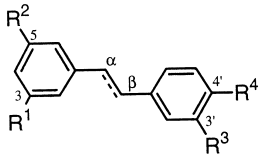
The methanolic extract was subjected to Diaion HP-20 column chromatography (H_2O→MeOH→acetone) to give the H_2O-eluted fraction (13.3%), MeOH-eluted fraction (18.7%), and acetone-eluted fraction (1.8%). Since the methanol (MeOH)-eluted fraction showed a stronger inhibitory effect on nitrite accumulation than the H_2O-eluted and acetone-eluted fractions (Table 1), the MeOH-eluated fraction was subjected to silica gel column chromatography ($CHCl_3$–MeOH→$CHCl_3$–MeOH–H_2O→MeOH) to give five fractions (fr. 1–fr. 5). Each fraction was subjected to ODS column chromatography (MeOH–H_2O) and finally HPLC (ODS, 250×20 mm i.d., MeOH–H_2O or CH_3CN–H_2O) to give rhaponticin (**1**,[10] 3.5%), piceatannol 3′-*O*-β-D-glucopyranoside (**2**,[10] 2.0%), desoxyrhaponticin (**3**,[10] 0.048%), isorhapontin (**4**,[11] 0.36%), rhapontigenin (**5**,[10] 0.58%), piceatannol (**6**,[10] 0.073%), desoxyrhapontigenin (**7**,[12] 0.015%), resveratrol (**8**,[13] 0.048%), rhaponticin 2″-*O*-gallate (**9**,[10] 0.12%), rhaponticin 6″-*O*-gallate (**10**,[10] 0.087%), aloe-emodin 1-*O*-β-D-glucopyranoside (**11**,[2,7]

Table 1. Inhibitory effects of MeOH extract, H_2O-eluted, MeOH-eluted, and acetone-eluted fractions from the rhizome of *R. undulatum* on NO production in LPS-activated mouse peritoneal macrophages[a]

	Concentration (μg/mL)	Inhibition[b] (%)		IC_{50} (μM)
Control	—	0.0±5.6	CAPE	4.0
MeOH extract	100	22.8±4.7**	L-NMMA	28
H_2O-eluted fraction	100	26.7±8.3**	GED	1.4
MeOH-eluted fraction	100	50.6±4.3**		
Acetone-eluted fraction	100	−3.5±4.4		

[a]Asterisks denote significant differences from control, $^{**}p<0.01$.
[b]Values indicate the means±SEM ($N=4$).

Table 2. Effects of stilbene constituents (**1**–**10**) from the rhizome of *R. undulatum* and related compounds (**16**–**23**, **1a**, **2a**, **5a**, **6a**, **8a**, **16a**) on NO production in LPS-activated mouse peritoneal macrophages[a,b]



	α—β	R^1	R^2	R^3	R^4	IC_{50} (μM) (%)
Rhaponticin (**1**)	C=C	O-Glc	OH	OH	OCH_3	>100 (25)
1a	C–C	O-Glc	OH	OH	OCH_3	>100 (8)
Piceatannol 3′-*O*-Glc (**2**)	C=C	OH	OH	O-Glc	OH	>100 (10)
2a	C–C	OH	OH	O-Glc	OH	>100 (6)
Desoxyrhaponticin (**3**)	C=C	O-Glc	OH	H	OCH_3	>100 (13)
Isorhapontin (**4**)	C=C	O-Glc	OH	OCH_3	OH	>100 (5)
Rhapontigenin (**5**)	C=C	OH	OH	OH	OCH_3	48
5a	C–C	OH	OH	OH	OCH_3	49
Piceatannol (**6**)	C=C	OH	OH	OH	OH	23
6a	C–C	OH	OH	OH	OH	32
Desoxyrhapontigenin (**7**)	C=C	OH	OH	H	OCH_3	>30 (13)[c]
Resveratrol (**8**)	C=C	OH	OH	H	OH	68
8a	C–C	OH	OH	H	OH	76
Rhaponticin 2″-*O*-gallate (**9**)	C=C	O-Glc(2-gallate)	OH	OH	OCH_3	13
Rhaponticin 6″-*O*-gallate (**10**)	C=C	O-Glc(6-gallate)	OH	OH	OCH_3	11
trans-Stilbene (**16**)	C=C	H	H	H	H	>100 (21)
16a	C–C	H	H	H	H	>100 (26)
17	C=C	OH	OH	OCH_3	OH	63
18	C=C	O-Glc$(CH_3)_4$	OCH_3	OCH_3	OCH_3	20
19	C=C	OCH_3	OCH_3	O-Glc$(CH_3)_4$	OCH_3	27
20	C=C	OCH_3	OCH_3	OCH_3	OCH_3	28
21	C=C	OCH_3	OCH_3	H	OCH_3	22
22	C=C	OH	OCH_3	OCH_3	OCH_3	19[c]
23	C=C	OCH_3	OCH_3	OH	OCH_3	23[c]

[a]Values in parentheses represent the inhibition (%) at 30 or 100 μM.
[b]Glc: β-D-glucopyranosyl.
[c]Cytotoxic effects were observed at 100 μM.

0.0065%), chrysophanol 1-*O*-β-D-glucopyranoside (**12**,[14] 0.25%), chrysophanol 8-*O*-β-D-glucopyranoside (**13**,[14] 0.16%), chrysophanol 8-*O*-β-D-(6′-galloyl)-glucopyranoside (**14**,[2,7] 0.092%), and torachrysone 8-*O*-β-D-glucopyranoside (**15**,[15] 0.12%).

As shown in Table 2, three stilbenes [rhapontigenin (**5**), piceatannol (**6**), and resveratrol (**8**)], two stilbene glucoside gallates [rhaponticin 2″-*O*-gallate (**9**) and rhaponticin 6″-*O*-gallate (**10**)], and a naphthalene glucoside [torachrysone 8-*O*-β-D-glucopyranoside (**15**)] inhibited LPS-induced NO production (IC_{50} = 11–69 μM). Other stilbene constituents (**1**–**4**) and anthraquinones (**11**–**14**) showed weak inhibitory effects (IC_{50} > 100 μM) (Table 3).

Strucutural requirements of stilbenes and anthraquinones for NO production inhibitory activity

To clarify structure–activity relationships of stilbenes and anthraquinones, related compounds **17**, **26**, and **27** were derived by enzymatic hydrolysis of **4**, **11**, and **12**; **18**–**21** by CH_3I-methylation of **1**, **2**, **5**, **8**; **22** and **23** by methanolysis of **18** and **19**; dihydrostilbenes (**1a**, **2a**, **5a**, **6a**, **8a**, **16a**) by hydrogenation of **1**, **2**, **5**, **6**, **8**, and **16** as reported previously.[2]

Related stilbene compounds (**5a**, **6a**, **8a**, **17**–**23**) showed inhibitory activity (IC_{50} = 19–76 μM), although *trans*-stilbene (**16**) and dihydrostilbene (**16a**) lacking the oxygen functions (−OCH_3 and −OH) showed little activity. Dihydrostilbene derivatives (**5a**, **6a**, **8a**) also showed equipotent activities as compared with their corresponding stilbenes (**5**, **6**, **8**), while the glucosides (**1a**, **2a**) did not. Permethylated stilbene glucosides (**18**, **19**) also showed activity (IC_{50} = 20, 27 μM). Furthermore, two gallates (**9**, **10**) showed more potent activity than **1** and gallic acid (**31**). With exception of desoxyrhapontigenin (**7**), **22**, and **23**, test compounds at 100 μM did not show cytotoxicity in MTT assay.

These results indicated the following structural requirements of stilbenes for the activity: (1) the oxygen functions (–OH, –OCH_3) at the benzene rings are essential for the activity; (2) the glucoside moiety reduced the activity but permethylation of the glucose moiety restored the activity; (3) the α,β-double bond did not affect the activity; and (4) the galloyl ester tended to potentiate the activity.

Sennosides A (**24**) and B (**25**) and anthraquinones (**26**–**29**) showed weak inhibitory effects or cytotoxic effects, although rhein (**30**) showed inhibitory effect (IC_{50} = 70 μM). Compounds **14** with a galloyl ester and **13** showed equipotent effect. This result indicated that the galloyl ester of anthraquinone glucoside did not potentiate the activity.

Effects of stilbenes (5, 6, 8) on iNOS enzyme activity, iNOS induction, and NF-κB activation

Next, the effects of three principal stilbenes [rhapontigenin (**5**), piceatannol (**6**), and resveratrol (**8**)] on iNOS enzyme activity and iNOS induction were examined. A reference compound, L-NMMA, inhibited iNOS enzyme activity with an IC_{50} of 13 μM, but compounds **5**, **6**, and **8** showed weak inhibitory effects on iNOS enzyme activity; their inhibitory effects at 100 μM were 9.3, 22, and 1.2%, respectively. iNOS protein was detected at 130 kDa after a 12-h incubation with LPS on SDS-PAGE-Western blotting analysis (Fig. 1). Three stilbenes (**5**, **6**, **8**) concentration-dependently inhibited iNOS induction, consistent with the above results.

Finally, the effects of **5**, **6**, and **8** on activation of NF-κB were analyzed by electrophoretic mobility shift assay. The cells were incubated with or without LPS and the test sample for 4 h, and proteins of the cell lysate were added to reaction mixtures containing NF-κB consensus oligonucleotide labeled with ^{32}P-ATP. The oligonucleotide–protein complex was separated electrophoretically and visualized by autoradiography. Recent studies demonstrated that compound **8**, a bioactive polyphenol found in grapes and wine, inhibited LPS-induced NO generation and LPS- and TNF-α-induced activation of NF-κB.[16] Similarly, compounds **5** and **6** also inhibited LPS-induced NF-κB activation (Fig. 2). These findings indicated that the active constituents (**5**, **6**, **8**), at least in part, inhibited the upstream signaling pathway of NF-κB activation following iNOS expression, thereby preventing NO production.

In conclusion, three stilbenes [rhapontigenin (**5**), piceatannol (**6**), and resveratrol (**8**)], two stilbene glucoside gallates [rhaponticin 2″-*O*-gallate (**9**) and rhaponticin 6″-*O*-gallate (**10**)], and a naphthalene glucoside [torachrysone 8-*O*-β-D-glucopyranoside (**15**)] with inhibitory activity against NO production in LPS-activated macrophages were isolated from the rhizome of *R. undulatum* (IC_{50} = 11–68 μM). The oxygen functions

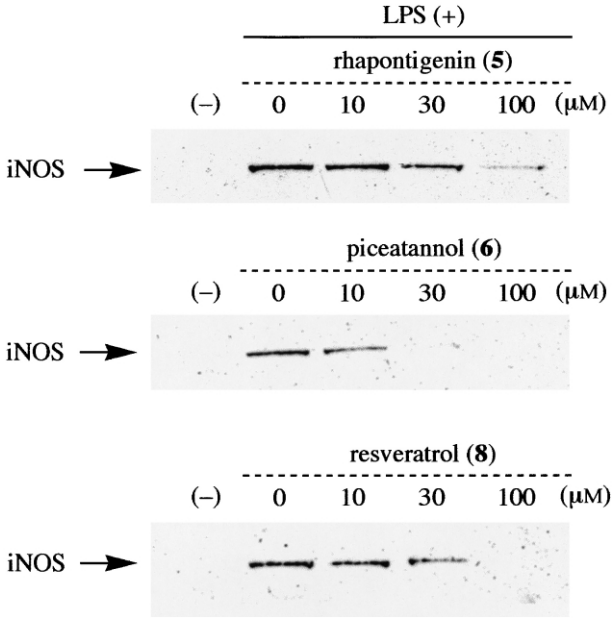


Figure 1. Effects of stilbenes (**5**, **6**, **8**) on LPS-induced induction of iNOS protein in macrophages. iNOS was detected at 130 kDa after 12-h incubation with 20 μg/mL LPS by SDS-PAGE-Western blotting analysis.

(–OH, $–OCH_3$) at the benzene ring of stilbenes were essential for the activity, and the glucoside moiety reduced the activity but permethylation of the glucose moiety restored the activity, while the α,β-double bond had no effect. Furthermore, the active stilbenes [rhapontigenin (**5**), piceatannol (**6**), and resveratrol (**8**)] inhibited iNOS induction and activation of NF-κB, but not iNOS enzyme activity.

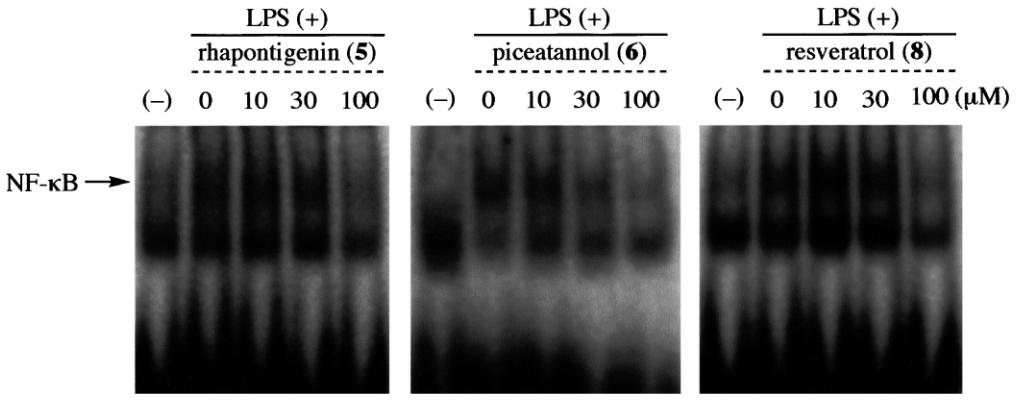
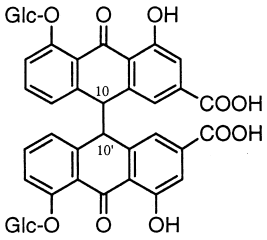
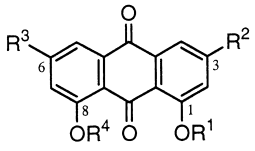


Figure 2. Effects of stibenes (**5**, **6**, **8**) on LPS-induced activation of NF-κB in macrophages. The cells were incubated with or without LPS and test sample for 4 h, and proteins of the cell lysate were added to reaction mixtures containing NF-κB consensus oligonucleotide labeled with ^{32}P-ATP. The oligonucleotide–protein complex was separated electrophoretically and visualized by autoradiography.

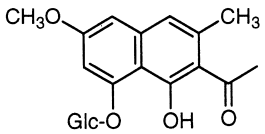
Table 3. Effects of anthraquinones (**11**–**14**), a naphthalene (**15**) and related compounds (**24**–**31**) on NO production in LPS-activated mouse peritoneal macrophages[a,b]



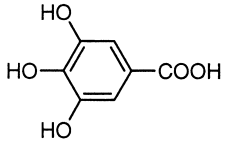
	10–10′	IC_{50} (μM) (%)
Sennoside A (**24**)	*threo*	>100 (31)
Sennoside B (**25**)	*erythro*	>100 (37)



	R^1	R^2	R^3	R^4	IC_{50} (μM) (%)
Aloe-emodin 1-*O*-Glc (**11**)	Glc	CH_2OH	H	H	>100 (46)
Chrysophanol 1-*O*-Glc (**12**)	Glc	CH_3	H	H	>100 (20)
Chrysophanol 8-*O*-Glc (**13**)	H	CH_3	H	Glc	>100 (31)
Chrysophanol 8-*O*-(6′-galloyl)-Glc (**14**)	H	CH_3	H	Glc(6-gallate)	>100 (31)
Aloe-emodin (**26**)	H	CH_2OH	H	H	>10 (38)[c]
Chrysophanol (**27**)	H	CH_3	H	H	>100 (2)
Emodin (**28**)	H	CH_3	OH	H	>30 (3)[d]
Physcion (**29**)	H	CH_3	OCH_3	H	>100 (−9)
Rhein (**30**)	H	COOH	H	H	70



torachrysone 8-*O*-Glc (**15**): IC_{50} = 69 μM



gallic acid (**31**): IC_{50} = 63 μM

[a]Glc: β-D-glucopyranosyl.
[b]Values in parentheses represent the inhibition (%) at 10, 30 or 100 μM.
[c]Cytotoxic effect was observed at 30 μM.
[d]Cytotoxic effect was observed at 100 μM.

Materials and Methods

Extraction and isolation

The dried rhizome of *R. undulatum* (5.8 kg, cultivated in Korea and purchased from MAE CHU Co. Ltd., Nara, Japan), which was botanically identified by comparison of the taxonomical features with authentic rhubarb samples, was crushed and then extracted three times with methanol at room temperature for 24 h. The methanolic extract (33.8% from the natural medicine) of the dried rhizome of *R. undulatum* (5.8 kg, cultivated in Korea) was subjected to Diaion HP-20 column chromatography ($H_2O \rightarrow$MeOH$\rightarrow$acetone) to give the H_2O-eluted fraction (13.3%), MeOH-eluted fraction (18.7%), and acetone-eluted fraction (1.8%). The active fraction (MeOH–eluted fraction) was subjected to silica gel column chromatography [$CHCl_3$–MeOH (10:1, v/v)$\rightarrow$(4:1)$\rightarrow$$CHCl_3$–MeOH–$H_2O$ (10:3:1, lower layer)$\rightarrow$MeOH] to give five fractions (fr. 1–fr. 5). Each fraction was subjected to ODS column chromatography (MeOH–H_2O) and finally HPLC [YMC-pack R&D ODS-5-A, 250×20 mm i.d., MeOH–H_2O or CH_3CN–H_2O] to give rhaponticin (**1**, 3.5%), piceatannol 3′-*O*-β-D-glucopyranoside (**2**, 2.0%), desoxyrhaponticin (**3**, 0.048%), isorhapontin (**4**, 0.36%), rhapontigenin (**5**, 0.58%), piceatannol (**6**, 0.073%), desoxyrhapontigenin (**7**, 0.015%), resveratrol (**8**, 0.048%), rhaponticin 2″-*O*-gallate (**9**, 0.12%), rhaponticin 6″-*O*-gallate (**10**, 0.087%), aloe-emodin 1-*O*-β-D-glucopyranoside (**11**, 0.0065%), chrysophanol 1-*O*-β-D-glucopyranoside (**12**, 0.25%), chrysophanol 8-*O*-β-D-glucopyranoside (**13**, 0.16%), chrysophanol 8-*O*-β-D-(6′-galloyl)-glucopyranoside (**14**, 0.092%) and torachrysone 8-*O*-β-D-glucopyranoside (**15**, 0.12%). Related compounds **17**, **26**, and **27** were derived by enzymatic hydrolysis of **4**, **11** and **12**; **18**–**21** by CH_3I-methylation of **1**, **2**, **5**, **8**; **22** and **23** by methanolysis of **18** and **19**; dihydrostilbenes (**1a**, **2a**, **5a**, **6a**, **8a**, **16a**) by hydrogenation of **1**, **2**, **5**, **6**, **8**, and **16** as reported previously.[2]

Reagents

Lipopolysaccharide (LPS, from *Salmonella enteritidis*) and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma (St. Louis, MO, USA); 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) was from Dojin (Kumamoto, Japan); caffeic acid phenethyl ester (CAPE) and guanidinoethyldisulfide (GED) were from Calbiochem (San Diego, CA, USA); RPMI 1640 was from Life Technologies (Rockville, MD, USA); protease inhibitor cocktail (Complete Mini) was from Boehringer Mannheim (Germany); fetal calf serum (FCS) was from Bio Whittaker (Walkersville, MD, USA); anti-mouse iNOS antibody (monoclonal) was from Transduction Laboratories (San Diego, CA, USA); anti-mouse IgG antibody conjugated to horseradish peroxidase and the enhanced chemiluminescense (ECL) kit, L-[U-^{14}C]- arginine, γ-[^{32}P]-ATP were from Amersham Pharmacia Biotech (Bucks, UK); thioglycolate (TGC) medium was from Nissui Seiyaku (Tokyo, Japan); iNOS was from OXIS International (Portland, OR, USA); NF-kB consensus oligonucleotide and T4 polynucleotide kinase (Gel Shift Assay Kit) were from Promega (Madison, WI, USA); Aquasol-2 was from Packard (Meriden, CT, USA); emodine (**28**), physcion (**29**), and rhein (**30**) were from Funakoshi (Tokyo, Japan); sennosides A (**24**) and B (**25**) and other chemicals were from Wako (Osaka, Japan). Nitrocellulose membranes (0.25 μm) were purchased from Bio Rad (Tokyo, Japan); 96-well microplates and culture dishes (6 cm) were from Nunc (Naperville, IL, USA); and spin columns (UFC30SV00) were from Millipore (Bedford, MA, USA).

Screening for NO production

Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice by washing with 6–7 mL of ice-cold PBS, and cells (5×10^5 cells/well) were suspended in 200 μL of RPMI 1640 supplemented with 5% fetal calf serum (FCS), penicillin (100 units/mL) and streptomycin (100 μg/mL), and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Non-adherent cells were removed by washing with PBS, and the adherent cells (more than 95% macrophages as determined by Giemsa staining) were cultured in fresh medium containing 10 μg/mL LPS and various concentrations of test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent.[17] Cytotoxicity was determined by MTT colorimetric assay. Briefly, after 20-h incubation with test compounds, MTT (10 μL, 5 mg/mL in PBS) solution was added to the wells. After a further 4 h in culture, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference: 655 nm). CAPE, L-NMMA and GED were used as reference compounds. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated by the following formula and IC_{50} was determined graphically ($N=4$).

$$\text{Inhibition (\%)} = \frac{A-B}{A-C}\times 100$$

A−C : NO_2^- concentration (μM)

[A : LPS (+), sample (−);
B : LPS (+), sample (+);
C : LPS (−), sample (−)]

Detection of iNOS

In this experiment, peritoneal exudate cells were obtained from the peritoneal cavities of male ddY mice that had been intraperitoneally injected with 4% TGC medium 4 days previously to ensure a large number of the cells. Cells (7.5×10^6 cells/3 mL/dish) were pre-cultured in culture dishes (6 cm i.d.) for 1 h, and the adherent cells (more than 95% macrophages) were collected as described above. After washing, the culture medium was exchanged for fresh medium containing 5% FCS, 20 μg/mL LPS and test compound for 12 h. Cells were collected in lysis buffer [100 mM NaCl,

10 mM Tris, Complete Mini (1 tab/10 mL), 0.1% Triton X-100, 2 mM EGTA] and sonicated. After determination of protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer.[18] For SDS-PAGE, aliquots of 50 μg of protein from each sample were subjected to electrophoresis in 10% polyacrylamide gels. Following electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membrane. The membrane were incubated with 5% nonfat dried milk in Tris-buffered saline (TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with mouse monoclonal IgG (dilution of 1:1000) against iNOS. The blots were washed in TBS and probed with secondary antibody, anti-mouse IgG antibody conjugated to horseradish peroxidase (dilution of 1:5000). Detection was performed using an ECL kit and X-ray film (Hyper Film, Amersham).

Electrophoretic mobility shift assay

TGC-induced peritoneal macrophages (7.5×10^6 cells/3 mL/dish) was prepared as described above. Cells were cultured in RPMI 1640 supplemented with 5% FCS, penicillin (100 units/mL) and streptomycin (100 μg/mL), 20 μg/mL LPS and test compound for 4 h. Cells were collected in ice-cold PBS and resuspended in four cell volumes of lysis buffer (420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1% Nonidet P40, 20 mM HEPES, pH 7.9). The cell lysate was incubated on ice for 1 h, then centrifuged at 13,000 rpm at 4 °C for 5 min. The protein content of each supernatant was determined, and equal amounts of protein (20 μg) were added to reaction mixtures containing 20 μg BSA and ^{32}P-labeled NF-κB consensus oligonucleotide. The oligonucleotide–protein complex was separated by non-denaturing polyacrylamide gel electrophoresis (Gel Shift Assay Kit, Promega), and autoradiography was performed using an imaging analyzer (BAS 5000, Fuji Film). ^{32}P-Labeled NF-κB consensus oligonucleotide was labeled using γ-[^{32}P]-ATP (3000 Ci/mmol) and T4 polynucleotide kinase.

iNOS Enzyme activity

NOS activity was measured by monitoring the conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline. Briefly, test sample solution (5 μL) and 40 μL of substrate and coenzyme solution [100 μM arginine (containing 50 nCi L-[U-^{14}C]-arginine), 1 mM NADPH, 3 μM tetrahydrobiopterin (BH_4), 1 μM flavin adenine dinucleotide (FAD), 1 μM flavin mononucleotide (FMN) in 25 mM Tris–HCl buffer (pH 7.4)] were pre-incubated at 37 °C for 10 min. iNOS (20 mU/5 μL) was then added to the reaction mixture. After incubation at 37 °C for 30 min, the reaction was terminated by addition of 400 μL of cold buffer containing 5 mM EDTA and 50 mM HEPES (pH 5.5). The substrate was adsorbed on AG 50W X-8 ion-exchange resin (Na^+ form, 60–70 mg) packed in spin columns. The L-citrulline, which is ionically neutral at pH 5.5, flowed through the column completely,[19] and was mixed with a scintillation cocktail (Aquasol-2) and radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman). Test compound was dissolved in DMSO and diluted with Tris–HCl buffer (pH 7.4) (final concentration of DMSO: 2%).

Statistical analysis

Values were expressed as means ± SD or SEM One-way analysis of variance following Dunnett's test for multiple comparison analysis were used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

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