Bioactive Constituents from Chinese Natural Medicines. XI.[1] Inhibitors on NO Production and Degranulation in RBL-2H3 from *Rubia yunnanensis*: Structures of Rubianosides II, III, and IV, Rubianol-g, and Rubianthraquinone

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Three new arborinane-type triterpene glycosides, rubianosides II, III, and IV, a new arborinane-type triterpene, rubianol-g, and a new anthraquinone, rubianthraquinone, were isolated from a Chinese natural medicine, the roots of *Rubia yunnanensis*. The structures of the new constituents including their absolute configurations were determined on the basis of chemical and physicochemical evidence. The inhibitory effects of the isolated constituents on nitric oxide production in lipopolysaccharide-activated macrophages were examined. Among them, a cyclic peptide constituent, RA-XII and its aglycon, RA-V (deoxybouvadin), potently inhibited overproduction of nitric oxide and induction of inducible nitric oxide synthase. In addition, an anthraquinone constituent, 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone, was found to show inhibitory effects on the release of β-hexosaminidase in RBL-2H3 cells.

Key words *Rubia yunnanensis*; rubianoside; rubianthraquinone; nitric oxide inhibitor; cyclic peptide; degranulation inhibitor

The roots of *Rubia yunnanensis* DIELS (Rubiaceae, "雲南茜草" in Chinese), which is cultivated in Yunnan Province, China are termed as "小紅参" in Chinese and are used in Chinese traditional medicine for the treatment of vertigo, insomnia, rheumatism, tuberculosis, hematemesis, menstrual disorder, and contusion.

During the course of our studies on bioactive constituents of Chinese natural medicines originating in Yunnan Province,[2—6] we previously reported the isolation and structure elucidation of five arborinane-type triterpenes named rubianols-a—e (**6**, **7**, **10**—**12**) and an arborinane-type triterpene glycoside, rubianoside I (**15**), from roots of *R. yunnanensis*.[1] As a continuation of the characterization studies on bioactive constituents of this natural medicine, we have isolated three new arborinane-type triterpene glycosides, rubianosides II (**1**), III (**2**), and IV (**3**), a new arborinane-type triterpene, rubianol-g (**4**), and a new anthraquinone, rubianthraquinone (**5**), together with seven known compounds (**16**—**18**, **20**, **24**, **25**, **28**). In this paper, we describe the isolation and structure elucidation of the new constituents (**1**—**5**) and the inhibitory effects of the isolated constituents on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages and the release of β-hexosaminidase in RBL-2H3 cells.

The aqueous acetone extract from the roots of *R. yunnanensis* (purchased in Kunming, Yunnan Province, China) was partitioned into an ethyl acetate (EtOAc)–water mixture to furnish the EtOAc-soluble and H_2O-soluble fractions as previously described.[1] The EtOAc-soluble fraction was subjected to normal-phase and reverse-phase silica gel column chromatography and repeated HPLC to give rubianosides II (**1**, 0.0006% from the natural medicine), III (**2**, 0.011%), and IV (**3**, 0.0035%), rubianol-g (**4**, 0.0021%), and rubianthraquinone (**5**, 0.0043%) together with **16**[7,8] (0.0016%), **17**[7,8] (0.22%), **18**[7,8] (0.062%), 5′-methoxylariciresinol (**20**,[9] 0.00048%), 2-methyl-1,6-dihydroxy-9,10-anthraquinone 3-*O*-(6′-acetyl)-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranoside (**24**,[10] 0.18%), rubinaphthin A (**25**,[11] 0.028%), and

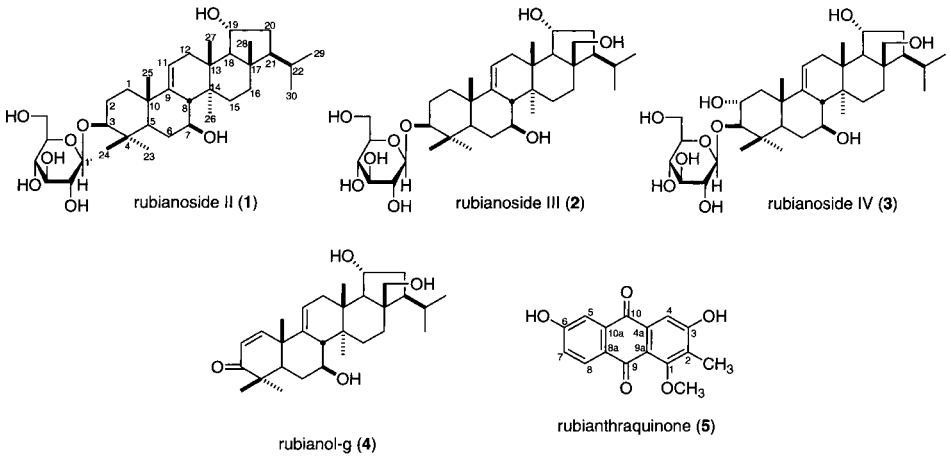

Chart 1

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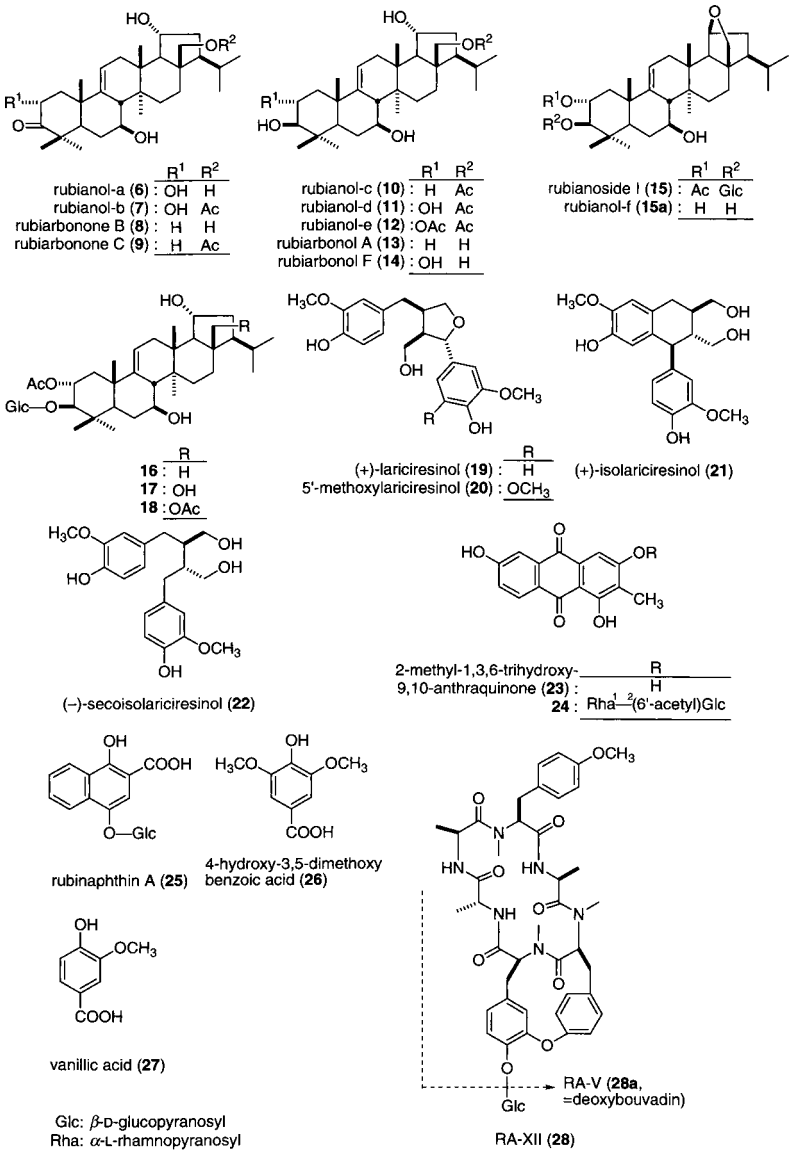

Chart 2

RA-XII (**28**,[12,13] 0.021%).

Absolute Stereostructures of Rubianosides II (1), III (2), and IV (3), and Rubianol-g (4) Rubianoside II (**1**) was isolated as a white powder with positive optical rotation ($[\alpha]_D^{25}$ +2.2°). The IR spectrum of **1** showed an absorption band at 1640 cm^{-1} ascribable to olefin function and strong absorption bands at 3431 and 1078 cm^{-1} suggestive of a glycosidic structure. In the negative-ion fast atom bombardment (FAB)-MS of **1**, a quasimolecular ion peak was observed at *m/z* 619 $(M-H)^-$, while the positive-ion FAB-MS showed a quasimolecular ion peak at *m/z* 643 $(M+Na)^+$. The molecular formula $C_{36}H_{60}O_8$ of **1** was determined from the quasimolecular ion peaks observed in the FAB-MS and by high-resolution FAB-MS measurement.

The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[14] of **1** showed signals assignable to eight methyls [δ 0.85, 0.89 (3H each, both d, J=6.1 Hz, 30, 29-H_3), 0.89, 1.08, 1.13, 1.13, 1.28, 1.35 (3H each, all s, 28, 24, 25, 27, 26, 23-H_3)], three methines bearing an oxygen function [δ 3.44 (1H, dd, J=4.0, 11.6 Hz, 3-H), 4.06 (1H, m, 7-H), 4.50 (1H, m, 19-H)], and an olefin function [δ 5.43 (1H, br d, J=*ca.* 6 Hz, 11-H)] together with a glucopyranosyl part [δ 4.98 (1H, d, J=7.6 Hz, 1′-H)]. Acid hydrolysis of **1** with 2 M hydrochloric acid (HCl) liberated a new triterpene termed rubianol-h (**1a**) as its aglycon and D-glucose, which was identified by HPLC analysis using an optical rotation detector.[1,15] The proton and carbon signals in the ^{1}H- and ^{13}C-NMR spectra of **1a** were similar to those of rubiarbonol A (**13**), except for the signals due to the 28-methyl group. The ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY) experiments on **1** and **1a** indicated the presence of five partial structures written in the bold lines (C-1–C-3, C-5–C-8, C-11–C-12, C-15–C-16, C-18–C-22–C-29, 30) (Fig. 1). The heteronuclear multiple-bond correlations (HMBC) were observed between the following proton and carbon pairs of **1** (H_3-23, 24 and C-3—5; H_3-25 and C-1, 5, 9, 10; H_3-26 and C-8, 13–15; H_3-27 and C-12–14, 18; H_3-28 and C-16–18, 21; H_3-29, 30 and C-21, 22; H-1′ and C-3). Thus, the connectivities of the quaternary carbons and the positions of the β-D-glucopyranosyl moiety in **1** were clarified. Next, the relative stereostructures of **1** and **1a** were elu-

cidated using nuclear Overhauser enhancement spectroscopy (NOESY), which showed the NOE correlations between the following proton pairs: H-3 and H-5, H_3-23; H-5 and H-7, H_3-23; H-7 and H_3-26; H-8 and H_3-25, 27; H-18 and H-21, H_3-26; H-19 and H_3-27, 28; H_3-24 and H_3-25; H_3-27 and H_3-28. Thus, the relative stereostructure of the aglycon part (**1a**) in **1** was determined to be 28-dehydroxyrubiarbonol A.

Finally, the absolute stereostructures of **1** and **1a** were characterized by the application of the modified Mosher's method.[16] Namely, treatment of **1a** with (*R*)- or (*S*)-2-methoxy-2-trifluoromethylphenylacetic acid [(*R*)- or (*S*)-MTPA] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC·HCl) and 4-dimethylaminopyridine (4-DMAP) selectively yielded the 19-mono-MTPA esters (**1b**, **c**), respectively. On the basis of the conformation analysis of rubianol-a (**6**),[1] the selectivity of the 19-esterization reaction with a bulky MTPA group seemed to be responsible for its less steric hinderance. As shown in Fig. 1, the signals due to protons attached to C-20, 21, 22, 29, and 30 in the 19-mono-(*S*)-MTPA ester (**1c**) were observed at lower

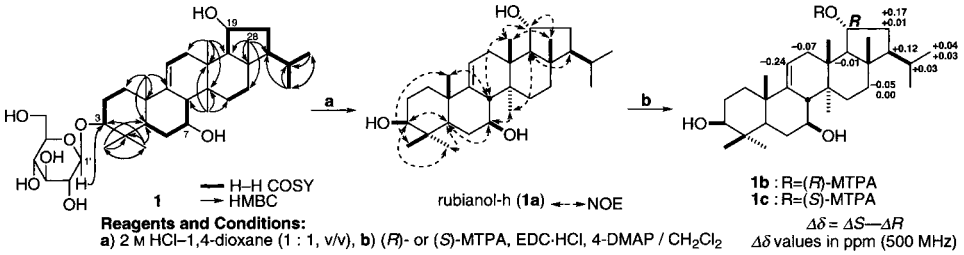

Fig. 1

Table 1. ^{13}C-NMR Data for Rubianosides II—IV (**1**—**3**), Rubianols-g (**4**) and -h (**1a**), and **16**—**18**

	1	**1a**	**2**	**3**	**4**	**16**	**17**	**18**
C-1	36.8	37.0	36.8	45.5	155.5	42.9	42.8	42.8
C-2	27.2	28.8	27.2	67.6	125.3	70.4	70.4	70.3
C-3	88.4	78.1	88.8	95.4	203.8	88.2	88.2	88.2
C-4	39.5	39.5	39.6	40.6	44.1	41.2	41.1	41.1
C-5	49.1	49.1	49.1	49.0	46.4	48.3	48.3	48.3
C-6	33.6	34.0	33.6	33.6	33.1	33.2	33.2	33.1
C-7	72.1	72.2	72.1	72.0	70.6	71.7	71.6	71.7
C-8	49.5	49.5	49.1	49.1	49.0	49.0	48.9	48.8
C-9	147.4	147.1	147.5	147.0	143.3	146.1	146.2	146.3
C-10	39.6	39.9	39.5	40.3	42.1	40.5	40.2	40.0
C-11	117.2	117.1	117.4	117.7	118.1	117.3	117.6	117.2
C-12	37.3	37.3	37.7	37.6	37.6	37.1	37.4	37.1
C-13	38.3	38.4	38.4	38.4	38.3	38.3	38.3	38.1
C-14	39.9	39.9	40.3	40.3	40.4	39.8	40.5	40.5
C-15	32.1	32.1	33.1	33.1	33.1	32.0	33.0	32.3
C-16	37.2	37.2	33.4	33.4	33.3	37.1	33.3	32.5
C-17	43.8	43.8	49.1	49.1	49.0	43.7	48.9	47.2
C-18	59.3	59.3	60.1	60.0	60.0	59.1	59.8	59.4
C-19	70.4	70.4	70.7	70.7	70.7	70.2	70.5	69.8
C-20	41.9	42.0	43.5	43.5	43.5	41.9	43.4	42.5
C-21	57.8	57.8	58.1	58.1	58.1	57.7	58.0	57.4
C-22	30.7	30.8	30.8	30.8	30.8	30.7	30.7	31.0
C-23	28.3	28.8	28.3	28.5	25.1	28.4	28.3	28.3
C-24	17.0	16.5	17.0	18.1	22.0	18.0	17.9	17.9
C-25	22.0	22.1	22.0	22.9	22.2	22.6	22.6	22.6
C-26	17.1	17.1	17.3	17.3	17.5	17.1	17.2	17.2
C-27	17.0	17.0	16.8	16.8	16.7	17.0	16.7	16.6
C-28	16.0	16.0	63.0	62.9	62.9	15.9	62.8	64.7
C-29	22.2*	22.2*	23.4*	23.4*	23.4*	22.2*	23.4*	22.9*
C-30	23.2*	23.2*	23.7*	23.7*	23.6*	23.2*	23.6*	23.4*
Glc-1′	107.0		107.0	106.6		106.0	106.0	106.0
Glc-2′	75.8		75.8	75.6		75.7	75.7	75.7
Glc-3′	78.8		78.8	78.7		78.6	78.5	78.5
Glc-4′	71.9		71.9	71.6		72.0	71.9	72.0
Glc-5′	78.4		78.4	78.6		78.1	78.1	78.1
Glc-6′	63.1		63.1	62.6		63.2	63.1	63.1
OCO<u>C</u>H$_3$						21.8	21.7	21.0
								21.7
O<u>C</u>OCH$_3$						170.7	170.7	170.4
								170.7

Measured in pyridine-d_5 at 125 MHz. * May be interchangeable within the same column.

fields compared with those of the 19-mono-(*R*)-MTPA ester (**1b**) [$\Delta\delta$: positive], while signals due to protons of C-11, 12, 16, and 18 in **1c** were observed at higher fields compared with those of **1b** [$\Delta\delta$: negative]. Consequently, the absolute configuration at the 19-position of **1a** was determined to be *R* configuration and the absolute stereostructures of **1** and **1a** were elucidated as shown.

Rubianosides III (**2**) and IV (**3**)[17,18] were also obtained as a white powder with positive optical rotation (**2**: $[\alpha]_D^{25}$ +3.5°; **3**: $[\alpha]_D^{25}$ +90.5°). The molecular formulas of **2** and **3** were determined from the negative and positive-ion FAB-MS and by high-resolution FAB-MS analysis to be $C_{36}H_{60}O_9$ and $C_{36}H_{60}O_{10}$, respectively. The IR spectrum of **2** and **3** showed absorption bands due to hydroxyl, olefin, and ether functions (**2**: 3431, 1640, 1085 cm^{-1}; **3**: 3400, 1655, 1076 cm^{-1}). The acid hydrolysis of **2** and **3** liberated D-glucose, which was identified by HPLC analysis using an optical rotation detector,[1,15] and rubiarbonols A (**13**)[1,19] and F (**14**)[1,19] as their aglycons, respectively. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[14] of **2** and **3** showed the presence of the following functions: a rubiarbonol A part in **2** {seven methyls [δ 0.97, 1.10 (3H each, both d, J=6.4 Hz, 30, 29-H_3), 1.07, 1.11, 1.35, 1.36, 1.43 (3H each, all s, 24, 25, 23, 26, 27-H_3)], a methylene and three methine bearing an oxygen function [δ 3.44 (1H, dd, J=4.0, 11.6 Hz, 3-H), 4.06 (1H, m, 7-H), 4.10, 4.22 (1H each, both d, J=9.5 Hz, 28-H_2), 5.06 (1H, m, 19-H)], and an olefin function [δ 5.47 (1H, br d, J=*ca.* 6 Hz, 11-H)]} and an β-D-glucopyranosyl part [δ 4.98 (1H, d, J=7.6 Hz, 1′-H)]; a rubiarbonol F part in **3** {seven methyls [δ 0.97, 1.10 (3H each, both d, J=6.4 Hz, 30, 29-H_3), 1.13, 1.20, 1.34, 1.42, 1.45 (3H each, all s, 24, 25, 26, 27, 23-H_3)], a methylene and four methine bearing an oxygen function [δ 3.35 (1H, d, J=9.2 Hz, 3-H), 4.05 (1H, m, 7-H), 4.13, 4.22 (1H each, both d, J=11.3 Hz, 28-H_2), 4.16 (1H, m, 2-H), 5.06 (1H, m, 19-H)], and an olefin function [δ 5.63 (1H, br d, J=*ca.* 5 Hz, 11-H)]} and an β-D-glucopyranosyl part [δ 4.99 (1H, d, J=7.9 Hz, 1′-H)]. Finally, the positions of the β-D-glucopyranoside linkage in **2** and **3** were also determined by HMBC, which showed long-range correlation was observed between the 1′-proton and 3-carbon (Fig. 2). Consequently, the absolute stereostructures of rubianosides III and IV were elucidated to be 3-*O*-β-D-glucopyranosyl rubiarbonols A (**2**) and F (**3**).

Rubianol-g (**4**)[17,18] was isolated as a white powder with positive optical rotation ($[\alpha]_D^{25}$ +206.1°). The positive-ion FAB-MS of **4** showed a quasimolecular ion peak at *m*/*z* 493 $(M+Na)^+$ and the molecular formula $C_{30}H_{46}O_4$ of **4** was determined by high-resolution FAB-MS measurement. The IR spectrum of **4** showed absorption bands at 3400, 1718, and 1670 cm^{-1} assignable to hydroxyl, carbonyl, and olefin functions, while the UV spectrum of **4** showed an absorption maximum at 230 nm ($\log\varepsilon$=4.03) due to an enone chromophore. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[14] of **4** showed signals assignable to seven methyls [δ 0.98, 1.10 (3H each, both d, J=6.5 Hz, 30, 29-H_3), 1.05, 1.24, 1.28, 1.28, 1.46 (3H each, all s, 24, 23, 25, 26, 27-H_3)], a methylene and two methines bearing an oxygen function [δ 4.11, 4.23 (1H each, both d, J=11.6 Hz, 28-H_2), 4.14 (1H, m, 7-H), 5.09 (1H, m, 19-H)], and three olefin protons [δ 5.62 (1H, br d, J=*ca.* 6 Hz, 11-H), 6.08 (1H, d, J=10.7 Hz, 2-H), 7.22 (1H, d, J=10.7 Hz, 1-H)] together with five methylenes (C-6, 12, 15, 16, 20), five methines (C-5, 8, 18, 21, 22), and seven quaternary carbons (C-3, 4, 9, 10, 13, 14, 17). The planar structure of **4** was confirmed by ^{1}H–^{1}H COSY and HMBC experiments. Furthermore, the relative stereostructure of **4** was clarified by NOESY, which showed nuclear Overhauser effect correlations were observed as shown in Fig. 2. On the basis of the above-mentioned evidence, the relative stereostructure of rubianol-g (**4**) was elucidated.

The absolute stereostructure of **4** was characterized by the application of the modified Mosher's method[16] as shown in Fig. 2. Consequently, the absolute configuration at the 19-position of **4** was determined to be the *R* configuration and the absolute stereostructures of **4** were elucidated as shown.

Structure of Rubianthraquinone (5) Rubianthraquinone (**5**) was isolated as a yellow powder and its molecular formula $C_{16}H_{12}O_5$ was determined from the molecular ion peak observed in the electron impact (EI)-MS [*m*/*z* 284 (M^+), 269 (M^+-CH_3)] and by high-resolution MS measurement. The IR spectrum showed absorption bands at 3500, 1718, 1670, 1601, 1566, and 1473 cm^{-1}, suggesting the presence of hydroxyl, carbonyl, and aromatic functions. The UV

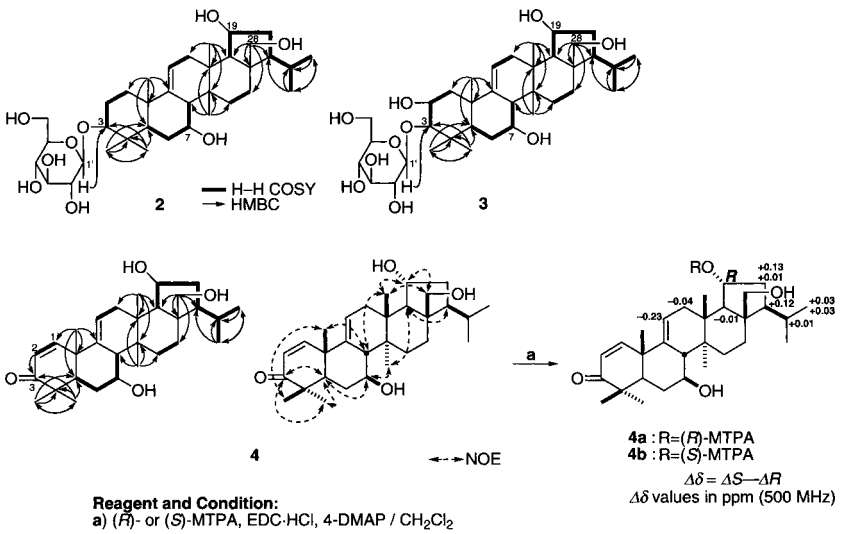

Fig. 2

spectrum of **5** indicated the presence of the anthraquinone moiety from the characteristic absorption maxima at 215 (log ε 4.36), 281 (4.59), 301 (4.20), 338 (3.84), and 411 (3.30) nm.[10] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 2) spectra[14] of **5** showed signals assignable to a methyl [δ 2.48 (3H, s, 2-CH_3)], a methoxyl [δ 4.07 (3H, s, 1-OCH_3)], and an anthraquinone structure [δ 7.46 (1H, dd, J=2.5, 8.3 Hz, 7-H), 7.95 (1H, s, 4-H), 7.97 (1H, d, J=2.5 Hz, 5-H), 8.46 (1H, d, J=8.3 Hz, 8-H)]. The proton and carbon signals in the ^{1}H- and ^{13}C-NMR spectra of **5** were superimposable on those of 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone (**23**),[1,10] except for the signals due to a methoxyl group. In the HMBC experiment of **5**, long-range correlations were observed as shown in Fig. 3 and the NOESY experiment on **5** showed a NOE correlation between the methoxyl proton and the 2-methyl proton. Consequently, the position of a methoxyl group of **5** was clarified as the 1-position and thus the structure of **5** was determined as shown.

Inhibitory Effects on NO Production and iNOS Induction in LPS-Activated Mouse Peritoneal Macrophages The inorganic free radical NO has been implicated in physiological and pathological processes, such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine by NO synthase (NOS). In the family of NOS, iNOS in particular is involved in pathological aspects with overproduction of NO, and can be expressed in response to pro-inflammatory agents such as interleukin-1β, tumor necrosis factor-α, and LPS in various cell types including macrophages, endothelial cells, and smooth muscle cells.

As a part of our studies to characterize the bioactive components of natural medicines, we have reported several NO production inhibitors; *i.e.* higher unsaturated fatty acids,[20] polyacetylenes,[21,22] coumarins,[21] flavonoids,[22] stilbenes,[23,24] lignans,[25] sesquiterpenes,[26—31] diterpenes,[32,33] triter-

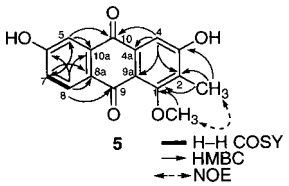

Fig. 3

Table 2. ^{13}C-NMR Data for Rubianthraquinone (**5**)

	5
C-1	161.4
C-2	127.2
C-3	162.3
C-4	109.8
C-4a	135.5
C-5	112.4
C-6	163.2
C-7	122.2
C-8	130.3
C-8a	127.8
C-9	180.3
C-9a	118.9
C-10	183.5
C-10a	135.2
2-CH_3	9.7
1-OCH_3	61.0

Measured in pyridine-d_5 at 125 MHz.

Table 3. Inhibitory Effects of Constituents from *R. yunnanensis* on NO Production in LPS–Activated Mouse Peritoneal Macrophages

	Inhibition (%)						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
Rubianoside III (**2**)	0.0±7.9	−2.1±3.5	10.3±9.4	10.4±9.8	−3.4±4.6	1.2±3.9	—
Rubianoside IV (**3**)	0.0±4.3	−1.2±5.3	10.2±5.3	−7.5±2.3	−0.6±0.7	13.6±2.3	—
Rubianol-g (**4**)	0.0±4.3	4.9±3.7	2.0±5.8	−4.3±3.6	−3.7±1.5	70.5±3.4**	70 (*ca.*)
Rubianthraquinone (**5**)	0.0±2.0	−3.9±1.3	−9.2±3.2	0.2±1.4	4.7±1.7	38.5±2.1**	—
16	0.0±4.9	3.8±2.0	−13.7±3.8	−3.4±1.7	−7.3±5.3	5.8±3.8	—
17	0.0±3.4	1.5±3.2	−1.2±5.3	−7.4±4.8	−1.9±1.9	−6.8±3.6	—
18	0.0±9.1	10.3±3.3	4.3±6.2	4.2±3.5	8.3±0.7	0.1±0.3	—
5′-Methoxylariciresinol (**20**)	0.0±3.7	4.8±5.8	−3.0±6.5	−13.1±3.8	−14.4±5.9	63.3±4.0	78 (*ca.*)
24	0.0±5.4	−6.5±2.0	−1.8±6.1	−12.5±4.5	0.1±6.5	7.2±4.1	—
Rubinaphthin A (**25**)	0.0±5.2	−5.2±4.6	−4.2±7.4	2.6±4.6	−4.5±6.3	4.5±5.2	—
L-NMMA	0.0±4.0	5.9±0.9	10.3±3.7	15.0±1.6**	34.1±3.2**	63.1±1.2**	57
CAPE	0.0±0.7	3.8±0.1	1.4±0.1	68.2±0.0**	93.7±0.2**	99.6±0.0**,#	15
GED	0.0±0.0	6.2±0.1	24.4±0.1**	57.9±0.1**	89.7±0.2**	97.9±0.0**	7.4

	Inhibition (%)						
	0 μM	0.3 μM	1 μM	3 μM	10 μM	30 μM	
RA-XII (**28**)	0.0±6.0	25.2±8.3	54.0±4.2**	71.4±4.1**,#	91.2±1.1**,#	98.5±1.4**,#	0.85

	Inhibition (%)						
	0 μM	0.003 μM	0.01 μM	0.03 μM	0.1 μM	0.3 μM	
RA-V (**28a**, =deoxybouvadin)	0.0±8.1	13.0±3.7	36.1±1.9**	77.1±1.9**	98.1±0.7**,#	100.6±2.0**,#	0.015
Herbimycin A	0.0±6.7	—	−3.8±6.4	14.9±5.1	48.0±2.0**	77.0±2.4**	0.094

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, ** p<0.01. # Cytotoxic effect was observed.

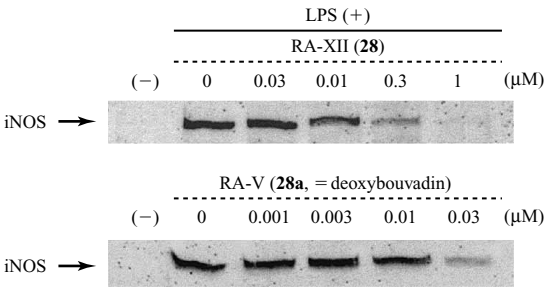

Fig. 4. Effects of RA-XII (**28**) and RA-V (**28a**, =Deoxybouvadin) on iNOS Induction in LPS-Activated Mouse Macrophages

penes,[1,34] and diarylheptanoids.[35,36] Previously, we examined the effects of 16 constituents from the roots of *R. yunnanensis* on NO production from LPS-activated macrophages and reported two arborinane-type triterpenes, rubianols-d (**11**) and -e (**12**), showed inhibitory activity.[1] In our continuing study of the antiinflammatory principles of this natural medicine, 11 isolated constituents were examined. Among them, a cyclic peptide glycoside, RA-XII (**28**) was found to show the most potent inhibitory activity (IC_{50}=0.85 μM). Moreover, the inhibitory activity of RA-V (**28a**,[37,38] IC_{50}=0.015 μM), which is an aglycon of **28**, was stronger than that of **28**. The inhibitory activities of **28** and **28a** were more potent than that of herbimycin A, an inhibitor of tyrosin kinase (IC_{50}=0.094 μM). Cyclic peptides were reported to show antitumor activity caused by their cyctotxic effect.[12,13,37,38] In the present study, **28** at 1 μM and **28a** at 0.03 μM showed the inhibitory activity without any cytotoxic effects in the MTT assay.

Next, the effects of two cyclic peptides (**28**, **28a**) on iNOS induction were examined. iNOS was detected at 130 kDa after a 12-h incubation with LPS by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-Western blotting analysis as shown in Fig. 4.[23—35] iNOS induction of LPS-activated macrophages was shown to be suppressed by two cyclic peptides (**28**, **28a**) in closely related to their inhibitions of NO. These results suggested that **28** and **28a** inhibited NO production mainly due to their inhibitory activities against iNOS induction in LPS-activated macrophages.

Inhibitory Effect on the Release of β-Hexosaminidase in RBL-2H3 Cells Histamine, which is released from mast cells stimulated by an antigen or a degranulation inducer, is usually determined as a degranulation marker in *in vitro* experiments on immediate allergic reactions. β-Hexosaminidase is also stored in the secretory granules of mast cells and is released concomitantly with histamine when mast cells are immunologically activated.[39,40] Therefore, it is generally accepted that β-hexosaminidase is a degranulation marker of mast cells.

As a part of our characterization studies on the bioactive components of natural medicines, we previously reported several inhibitors of the release of β-hexosaminidase such as diaylheptanoids,[41,42] diterpenes,[31,43] and flavonoids.[44] In our continuous search for antiallergic principles from natural sources, we examined the effects of constituents and their derivatives from the roots of *R. yunnanensis* on the release of β-hexosaminidase induced by dinitrophenylated bovine serum albumin (DNP-BSA) from RBL-2H3 cells sensitized with anti-DNP IgE (Table 4). As a result, an anthraquinone, 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone (**23**, IC_{50}=29 μM),[45] showed inhibitory activity, and their activities were stronger than those of two antiallergic compounds, tranilast (0.49 mM) and ketotifen fumarate (0.22 mM).[31]

Table 4. Inhibitory Effects of Constituents from *R. yunnanensis* on the Release of β-Hexosaminidase from RBL-2H3 Cells

	Inhibition (%)
	100 μM
Rubianoside III (**2**)	−2.9±3.5
Rubianoside IV (**3**)	−3.4±3.6
Rubianol-g (**4**)	21.4±3.4**
Rubianthraquinone (**5**)	4.4±1.5
Rubianol-a (**6**)	5.8±5.2
Rubianol-b (**7**)	1.9±7.3
Rubiarbonone B (**8**)	5.2±11.2
Rubiarbonone C (**9**)	15.1±4.5
Rubianol-c (**10**)	0.3±5.9
Rubianol-d (**11**)	39.7±2.4**
Rubianol-e (**12**)	17.5±4.7
Rubiarbonol A (**13**)	1.2±2.3
Rubiarbonol F (**14**)	42.5±2.9**
Rubianoside I (**15**)	4.4±1.6
16	−15.4±1.4
17	−2.1±4.2
18	1.2±2.4
(+)-Lariciresinol (**19**)	14.0±4.8
5′-Methoxylariciresinol (**20**)	15.4±1.7**
(+)-Isolariciresinol (**21**)	6.0±8.5
(−)-Secoisolariciresinol (**22**)	−2.8±5.3
2-Methyl-1,3,6-trihydroxy-9,10-anthraquinone (**23**)	82.3±0.7**
24	−9.9±1.6
Rubinaphthin A (**25**)	14.2±6.3
4-Hydroxy-3,5-dimethoxybenzoic acid (**26**)	7.6±5.4
Vanillic acid (**27**)	−6.6±6.0
RA-XII (**28**)	35.7±4.0**
RA-V (**28a**, =deoxybouvadin)	1.2±2.5

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, ** p<0.01.

Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; EI-MS and high-resolution MS, JEOL JMS-GCMATE mass spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JNM-LA500 (500 MHz) spectrometer; ^{13}C-NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; and HPLC detector, Shimadzu RID-6A refractive index detector.

The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh); reverse-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); TLC, precoated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reverse phase); reverse-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

Extraction and Isolation The 80% aqueous acetone extract (28.3%) from dried roots of *R. yunnanensis* (1.6 kg) was partitioned in an EtOAc–H_2O mixture to give the EtOAc-soluble fraction (4.3%). Normal-phase silica gel column chromatography [BW-200 (Fuji Silysia Co., Ltd., 750 g), *n*-hexane–EtOAc (5 : 1—1 : 1, v/v)–$CHCl_3$–MeOH (1 : 1, v/v)–MeOH] of the EtOAc-soluble portion (45 g) gave six fractions [Fr. 1 (11.0 g), 2 (16.7 g), 3 (2.8 g), 4 (3.9 g), 5 (8.9 g), 6 (1.7 g)] as reported previ-

ously.[1)]

Fraction 2-4 (822 mg) obtained from Fraction 2 (16.0 g) and previously isolated (+)-lariciresinol (**19**, 178 mg, 0.018%), (+)-isolariciresinol (**21**, 50 mg, 0.0050%), and (−)-secoisolariciresinol (**22**, 54 mg, 0.0054%),[1)] was further separated by HPLC [YMC-Pack ODS-A (YMC Co., Ltd., Kyoto, Japan, 250×20 mm i.d.), MeOH–H_2O (50 : 50, v/v)] to furnish (+)-5′-methoxylariciresinol (**20**, 22 mg, 0.00048%). Fraction 2-6 (783 mg), from which rubianol-a (**6**, 39 mg, 0.0039%) was previously isolated,[1)] was separated by HPLC [MeOH–H_2O (65 : 35, v/v)] to give rubianthraquinone (**5**, 43 mg, 0.0043%). Fraction 2-7 (1.10 g), from which rubianols-b (**7**, 12 mg, 0.0011%), -d (**11**, 41 mg, 0.0041%), and rubiarbonol A (**13**, 111 mg, 0.011%) and rubiarbonone B (**8**, 41 mg, 0.0041%) were previously isolated,[1)] was purified by HPLC [MeOH–H_2O (65 : 35, v/v)] to give rubianol-g (**4**, 21 mg, 0.0021%).

Fraction 4 (3.4 g) was subjected to reverse-phase silica gel column chromatography [Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh, 100 g), MeOH–H_2O (50 : 50→60 : 40→70 : 30, v/v)→MeOH] to furnish nine fractions [Fr. 4-1 (424 mg), 4-2 (345 mg), 4-3 (424 mg), 4-4 (1.10 g), 4-5 (161 mg), 4-6 (156 mg), 4-7 (95 mg), 4-8 (159 mg), 4-9 (536 mg)]. Fraction 4-2 (345 mg) was further purified by HPLC [MeOH–H_2O (50 : 50, v/v)] to give RA-XII (**28**, 191 mg, 0.021%). Fraction 4-4 (1.10 g) was further purified by HPLC [MeOH–H_2O (65 : 35, v/v)] to give **18** (522 mg, 0.062%). Fraction 4-6 (156 mg) was subjected to HPLC [MeOH–H_2O (70 : 30, v/v)] to furnish rubianoside II (**1**, 7 mg, 0.0006%) and **16** (19 mg, 0.0016%). Fraction 4-8 (159 mg) was further purified by HPLC [MeOH–H_2O (75 : 25, v/v)] to furnish rubianoside I (**15**, 17 mg, 0.0018%).

Fraction 5 (3.0 g) was subjected to reverse-phase silica gel column chromatography [90 g, MeOH–H_2O (50 : 50→60 : 40→70 : 30, v/v)→MeOH] to furnish seven fractions [Fr. 5-1 (327 mg), 5-2 (133 mg), 5-3 (71 mg), 5-4 (1.44 g), 5-5 (249 mg), 5-6 (=2-methyl-1,6-dihydroxy-9,10-antraquinone 3-*O*-(6′-acetyl)-α-L-rhamnosyl(1→2)-β-D-glucopyranoside, **24**, 649 mg, 0.18%), 5-7 (131 mg)]. Fraction 5-1 (327 mg) was further purified by normal-phase silica gel column chromatography [30 g, $CHCl_3$–MeOH (3 : 1→1 : 1, v/v)] to give rubinaphthin A (**25**, 97 mg, 0.028%). Fraction 5-3 (71 mg) was subjected to HPLC [MeOH–H_2O (65 : 35, v/v)] to furnish rubianoside IV (**3**, 12 mg, 0.0035%). Fraction 5-4 (1.44 g) was subjected to HPLC [MeOH–H_2O (65 : 35, v/v)] to furnish rubianoside III (**2**, 14 mg, 0.011%) and **17** (265 mg, 0.22%).

The known constituents were identified by comparison of their physical data ($[\alpha]_D$, IR, UV, and ^{1}H- and ^{13}C-NMR) with reported values.[7—13)]

Rubianoside II (**1**): A white powder, $[\alpha]_D^{25}$ +2.2° (c=0.20, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{36}H_{60}O_8Na$ $(M+Na)^+$ 643.4186; Found 643.4178. IR (KBr): 3431, 2940, 1640, 1465, 1375, 1166, 1078 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.85, 0.89 (3H each, both d, J=6.1 Hz, 30, 29-H_3), 0.89, 1.08, 1.13, 1.13, 1.28, 1.35 (3H each, all s, 28, 24, 25, 27, 26, 23-H_3), 1.05 (1H, m, 5-H), 1.38 (1H, m, 21-H), 1.40 (1H, m, 22-H), 1.40, 1.62 (1H each, both m, 1-H_2), 1.66 (2H, m, 16-H_2), 1.91, 2.39 (1H each, both m, 2-H_2), 1.91, 2.82 (1H each, both m, 15-H_2), 1.94, 2.24 (1H each, both m, 6-H_2), 1.97, 2.07 (1H, each, both m, 20-H_2), 2.04 (1H, d, J=9.8 Hz, 18-H), 2.42 (1H, m, 8-H), 2.43 (2H, m, 12-H_2), 3.44 (1H, dd, J=4.0, 11.6 Hz, 3-H), 4.06 (1H, m, 7-H), 4.50 (1H, m, 19-H), 4.98 (1H, d, J=7.6 Hz, 1′-H), 5.43 (1H, br d, J=*ca.* 6 Hz, 11-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 643 $(M+Na)^+$. Negative-ion FAB-MS: m/z 619 $(M-H)^-$.

Rubianoside III (**2**): A white powder, $[\alpha]_D^{25}$ +3.5° (c=0.10, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{36}H_{60}O_9Na$ $(M+Na)^+$ 659.4135; Found 659.4140. IR (KBr): 3431, 2961, 1640, 1470, 1375, 1166, 1085, 1034 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.97, 1.10 (3H each, both d, J=6.4 Hz, 30, 29-H_3), 1.07, 1.11, 1.35, 1.36, 1.43 (3H each, all s, 24, 25, 23, 26, 27-H_3), 1.05 (1H, m, 5-H), 1.40, 1.62 (1H each, both m, 1-H_2), 1.58 (1H, m, 21-H), 1.62, 2.03 (1H each, both m, 16-H_2), 1.92, 2.23 (1H each, both m, 6-H_2), 1.92, 2.40 (1H each, both m, 2-H_2), 2.03, 2.82 (1H each, both m, 15-H_2), 2.15 (1H, m, 22-H), 2.19, 2.65 (1H, each, both m, 20-H_2), 2.36 (1H, d, J=9.8 Hz, 18-H), 2.49 (1H, m, 8-H), 2.51, 2.60 (1H each, both m, 12-H_2), 3.44 (1H, dd, J=4.0, 11.6 Hz, 3-H), 4.06 (1H, m, 7-H), 4.10, 4.22 (1H each, both d, J=9.5 Hz, 28-H_2), 4.98 (1H, d, J=7.6 Hz, 1′-H), 5.06 (1H, m, 19-H), 5.47 (1H, br d, J=*ca.* 6 Hz, 11-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 659 $(M+Na)^+$. Negative-ion FAB-MS: m/z 635 $(M-H)^-$.

Rubianoside IV (**3**): A white powder, $[\alpha]_D^{25}$ +90.5° (c=0.10, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{36}H_{60}O_{10}Na$ $(M+Na)^+$ 675.4084; Found 675.4081. IR (KBr): 3400, 2960, 1655, 1458, 1387, 1259, 1076, 1023 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.97, 1.10 (3H each, both d, J=6.4 Hz, 30, 29-H_3), 1.13, 1.20, 1.34, 1.42, 1.45 (3H each, all s, 24, 25, 26, 27, 23-H_3), 1.17 (1H, m, 5-H), 1.60 (1H, m, 21-H), 1.61, 2.02 (1H each, both m, 16-H_2), 1.63, 2.39 (1H each, both m, 1-H_2), 1.93, 2.24 (1H each, both m, 6-H_2), 2.03, 2.81 (1H each, both m, 15-H_2), 2.14 (1H, m, 22-H), 2.16, 2.61 (1H, each, both m, 20-H_2), 2.34 (1H, d, J=10.0 Hz, 18-H), 2.47, 2.57 (1H each, both m, 12-H_2), 2.49 (1H, d, J=7.0 Hz, 8-H), 3.35 (1H, d, J=9.2 Hz, 3-H), 4.05 (1H, m, 7-H), 4.13, 4.22 (1H each, both d, J=11.3 Hz, 28-H_2), 4.16 (1H, m, 2-H), 4.99 (1H, d, J=7.9 Hz, 1′-H), 5.06 (1H, m, 19-H), 5.63 (1H, br d, J=*ca.* 5 Hz, 11-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 675 $(M+Na)^+$. Negative-ion FAB-MS: m/z 651 $(M-H)^-$.

Rubianol-g (**4**): A white powder, $[\alpha]_D^{25}$ +206.1° (c=0.10, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{30}H_{46}O_4Na$ $(M+Na)^+$ 493.3294; Found 493.3287. UV [MeOH, nm (log ε)]: 230 (4.03). IR (KBr): 3400, 2920, 1718, 1670, 1458, 1375, 1034 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.98, 1.10 (3H each, both d, J=6.5 Hz, 30, 29-H_3), 1.05, 1.24, 1.28, 1.28, 1.46 (3H each, all s, 24, 23, 25, 26, 27-H_3), 1.57 (1H, m, 21-H), 1.83 (1H, m, 5-H), 2.16 (1H, m, 22-H), 1.58, 2.02 (1H each, both m, 16-H_2), 2.07, 2.26 (1H each, both m, 6-H_2), 2.26, 2.68 (1H each, both m, 15-H_2), 2.16, 2.65 (1H, each, both m, 20-H_2), 2.33 (1H, d, J=9.8 Hz, 18-H), 2.57 (2H, m, 12-H_2), 2.61 (1H, d-like, 8-H), 4.11, 4.23 (1H each, both d, J=11.6 Hz, 28-H_2), 4.14 (1H, m, 7-H), 5.09 (1H, m, 19-H), 5.62 (1H, br d, J=*ca.* 6 Hz, 11-H), 6.08 (1H, d, J=10.7 Hz, 2-H), 7.22 (1H, d, J=10.7 Hz, 1-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 493 $(M+Na)^+$.

Rubianthraquinone (**5**): A yellow powder. High-resolution EI-MS: Calcd for $C_{16}H_{12}O_5$ $(M)^+$ 284.0687; Found 284.0685. UV [EtOH, nm (log ε)]: 215 (4.36), 281 (4.59), 301 (4.20), 338 (3.84), 411 (3.30). IR (KBr): 3500, 1718, 1670, 1601, 1566, 1473 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 2.48 (3H, s, 2-CH_3), 4.07 (3H, s, 1-OCH_3), 7.46 (1H, dd, J=2.5, 8.3 Hz, 7-H), 7.95 (1H, s, 4-H), 7.97 (1H, d, J=2.5 Hz, 5-H), 8.46 (1H, d, J=8.3 Hz, 8-H). ^{13}C-NMR (125 MHz pyridine-d_5) δ_C: given in Table 2. EI-MS: m/z 284 (M^+, 100), 269 (M^+-CH_3, 47).

Acid Hydrolysis of Rubianosides II (1), III (2), and IV (3) A solution of **1** (5.0 mg), **2** (3.0 mg), or **3** (3.0 mg) in 2 M HCl–1,4-dioxane (0.5 ml, 1 : 1, v/v) was heated under reflux for 2 h. After cooling, the reaction mixture was poured into ice-water and neutralized with Amberlite IRA-400 (OH^- form), and the resin was removed by filtration. The filtrate was then extracted with EtOAc. The aqueous layer was subjected to HPLC analysis under the following conditions, respectively: HPLC column, Shodex Asahipak NH-2P-50-4E, 4.6 mm i.d.×250 mm; detection, optical rotation; mobile phase, CH_3CN–H_2O (75 : 25, v/v); flow rate 0.8 ml/min; and column temperature, room temperature. Identification of D-glucose present in the aqueous layer was carried out by comparison of its retention time and optical rotation with those of authentic sample. t_R: 11.1 min (D-glucose, positive optical rotation).

The EtOAc layer was washed with saturated aqueous $NaHCO_3$ and brine, then dried over $MgSO_4$ powder and filtrated. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by silica gel column chromatography [0.5 g, *n*-hexane–EtOAc (3 : 1, v/v)] to give rubianol-h (**1a**, 2.6 mg, 71%, from **1**), rubiarbonol A (**13**, 2.1 mg, 93%, from **2**), and rubiarbonol F (**14**, 2.1 mg, 95%, from **3**), respectively.

Rubianol-h (**1a**): A white powder, $[\alpha]_D^{25}$ +12.6° (c=0.10, MeOH). High-resolution EI-MS: Calcd for $C_{30}H_{50}O_3$ (M^+) 458.3760; Found 458.3767. IR (KBr): 3389, 2940, 1655, 1465, 1375, 1264, 1093, 1038 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.86, 0.90 (3H each, both d, J=6.4 Hz, 30, 29-H_3), 0.90, 1.13, 1.17, 1.22, 1.26, 1.29 (3H each, all s, 28, 24, 27, 25, 23, 26-H_3), 1.15 (1H, m, 5-H), 1.37 (1H, m, 21-H), 1.42 (1H, m, 22-H), 1.51, 1.77 (1H each, both m, 1-H_2), 1.65 (2H, m, 16-H_2), 1.91, 2.85 (1H each, both m, 15-H_2), 1.98 (2H, m, 2-H_2), 2.01, 2.32 (1H each, both m, 6-H_2), 2.05 (1H, d, J=9.8 Hz, 18-H), 2.10 (2H, m, 20-H_2), 2.47 (1H, m, 8-H), 2.48 (2H, m, 12-H_2), 3.50 (1H, m, 3-H), 4.07 (1H, m, 7-H), 4.52 (1H, m, 19-H), 5.51 (1H, br d, J=*ca.* 6 Hz, 11-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. EI-MS: m/z 458 (M^+, 8), 440 (M^+-H_2O, 100).

Preparation of the (*R*)-MTPA Esters (1b, 4a) and (*S*)-MTPA Esters (1c, 4b) from Rubianols-h (1a) and -g (4) A solution of **1a** (1.0 mg) in CH_2Cl_2 (1.0 ml) was treated with (*R*)-2-methoxy-2-trifluoromethylphenylacetic acid [(*R*)-MTPA, 2.6 mg] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC·HCl, 2.1 mg) and 4-dimethylaminopyridine (4-DMAP, 0.8 mg), and the mixture was stirred under reflux for 8 h. After cooling, the reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was successively washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, and then dried over $MgSO_4$ powder and filtrated. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by silica gel col-

umn chromatography [0.5 g, *n*-hexane–EtOAc (3 : 1, v/v)] to give **1b** (0.7 mg, 50%). Using a similar procedure, (*S*)-MTPA ester (**1c**, 0.7 mg, 50%) was obtained from **1a** (1.0 mg) using (*S*)-MTPA (2.6 mg), EDC·HCl (2.1 mg), and 4-DMAP (0.8 mg).

1b: ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.77, 0.83 (3H each, both d, J=6.4 Hz, 30, 29-H_3), 0.83, 1.02, 1.14, 1.14, 1.19, 1.26 (3H each, all s, CH_3), 1.11 (1H, m, 5-H), 1.20 (1H, m, 21-H), 1.35 (1H, m, 22-H), 1.51, 1.89 (1H each, both m, 16-H_2), 1.52 (2H, m, 1-H_2), 1.82, 2.14 (1H each, both m, 20-H_2), 1.84, 2.81 (1H each, both m, 15-H_2), 2.00, 2.30 (1H each, both m, 6-H_2), 2.06 (2H, m, 2-H_2), 2.09 (2H, m, 12-H_2), 2.14 (1H, d, J=9.8 Hz, 18-H), 2.40 (1H, m, 8-H), 3.52 (1H, m, 3-H), 3.80 (3H, s, –OCH_3), 4.01 (1H, m, 7-H), 5.38 (1H, br d, J=*ca.* 6 Hz, 11-H), 5.54 (1H, m, 19-H), 7.50 (3H, m, Ph-H), 7.88 (2H, m, Ph-H).

1c: ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.81, 0.86 (3H each, both d, J=6.4 Hz, 30, 29-H_3), 0.83, 0.97, 1.08, 1.13, 1.20, 1.26 (3H each, all s, CH_3), 1.08 (1H, m, 5-H), 1.32 (1H, m, 21-H), 1.38 (1H, m, 22-H), 1.51, 1.84 (1H each, both m, 16-H_2), 1.52 (2H, m, 1-H_2), 1.82, 2.80 (1H each, both m, 15-H_2), 1.99, 2.15 (1H each, both m, 20-H_2), 1.99, 2.29 (1H each, both m, 6-H_2), 2.02 (2H, m, 12-H_2), 2.06 (2H, m, 2-H_2), 2.13 (1H, d, J=9.8 Hz, 18-H), 2.35 (1H, m, 8-H), 3.52 (1H, m, 3-H), 3.80 (3H, s, –OCH_3), 3.98 (1H, m, 7-H), 5.14 (1H, br d, J=*ca.* 6 Hz, 11-H), 5.54 (1H, m, 19-H), 7.50 (3H, m, Ph-H), 7.88 (2H, m, Ph-H).

A solution of **4** (5.0 mg) in CH_2Cl_2 (1.0 ml) was treated with (*R*)-MTPA (12.4 mg) in the presence of EDC·HCl (10.3 mg) and 4-DMAP (3.9 mg), and the mixture was stirred under reflux for 8 h. After cooling, the reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was successively washed with 5% aqueous HCl, saturated aqueous $NaHCO_3$, and brine, and then dried over $MgSO_4$ powder and filtrated. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was purified by silica gel column chromatography [0.5 g, *n*-hexane–EtOAc (2 : 1, v/v)] to give **4a** (1.5 mg, 20%). Using a similar procedure, (*S*)-MTPA ester (**4b**, 1.1 mg, 15%) was obtained from **4** (5.0 mg) using (*S*)-MTPA (12.4 mg), EDC·HCl (10.3 mg), and 4-DMAP (3.9 mg).

4a: ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.89, 0.96 (3H each, both d, J=6.4 Hz, 30, 29-H_3), 1.06, 1.14, 1.26, 1.28, 1.44 (3H each, all s, CH_3), 1.38 (1H, m, 21-H), 1.50, 1.92 (1H each, both m, 16-H_2), 1.85 (1H, m, 5-H), 1.93, 2.93 (1H each, both m, 20-H_2), 1.99, 2.64 (1H each, both m, 15-H_2), 2.01 (1H, m, 22-H), 2.09, 2.26 (1H each, both m, 6-H_2), 2.46 (1H, d, J=10.4 Hz, 18-H), 2.57 (1H, m, 8-H), 2.57 (2H, m, 12-H_2), 3.71 (3H, s, –OCH_3), 3.91, 4.29 (1H each, both d, J=11.6 Hz, 28-H_2), 4.12 (1H, m, 7-H), 5.54 (1H, br d, J=*ca.* 7 Hz, 11-H), 6.18 (1H, d, J=10.4 Hz, 2-H), 6.25 (1H, m, 19-H), 7.28 (1H, d, J=10.4 Hz, 1-H), 7.49 (3H, m, Ph-H), 7.87 (2H, m, Ph-H).

4b: ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.92, 0.99 (3H each, both d, J=6.7 Hz, 30, 29-H_3), 1.06, 1.10, 1.25, 1.26, 1.38 (3H each, all s, CH_3), 1.50 (1H, m, 21-H), 1.50, 1.92 (1H each, both m, 16-H_2), 1.80 (1H, m, 5-H), 1.97, 2.63 (1H each, both m, 15-H_2), 2.06, 2.94 (1H each, both m, 20-H_2), 2.02 (1H, m, 22-H), 2.04, 2.25 (1H each, both m, 6-H_2), 2.46 (1H, d, J=10.4 Hz, 18-H), 2.53 (1H, m, 8-H), 2.53 (2H, m, 12-H_2), 3.71 (3H, s, –OCH_3), 3.92, 4.29 (1H each, both d, J=11.6 Hz, 28-H_2), 4.07 (1H, m, 7-H), 5.31 (1H, br d, J=*ca.* 7 Hz, 11-H), 6.18 (1H, d, J=10.4 Hz, 2-H), 6.25 (1H, m, 19-H), 7.27 (1H, d, J=10.4 Hz, 1-H), 7.49 (3H, m, Ph-H), 7.87 (2H, m, Ph-H).

Enzymatic Hydrolysis of RA-XII (28) A solution of **28** (4.3 mg) in 0.2 M acetate buffer (pH 4.4, 2.0 ml) was treated with β-glucosidase (10 mg from almond, Oriental Yeast Co., Ltd., Tokyo, Japan), and the mixture was stirred at 38 °C for 7 d. After the addition of EtOH to the reaction mixture, the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography [1.0 g, $CHCl_3$–MeOH (10 : 1, v/v)] to give RA-V (**28a**, 3.5 mg, 99%), which was identified by comparison of the physical data ($[\alpha]_D$, IR, UV, and ^{13}C-NMR) with reported values.[37,38]

Bioassay

NO Production from Macrophages Stimulated by LPS The inhibitory effects of the test samples on NO production in LPS-activated mouse macrophages were evaluated by a method reported previously.[1] Briefly, peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice, which had been injected intraperitoneally with 4% thioglycolate medium 4 d previously, by washing with 6—7 ml of ice-cold phosphate-buffered saline (PBS). The cells (5×10^5 cells/well) were then suspended in 200 μl of RPMI 1640 supplemented with 5% fetal calf serum, 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. Nonadherent cells were removed by washing the cells with phosphate-buffered saline (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 test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent. Cytotoxicity was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay, after 20-h incubation with the test compounds. L-NMMA was used as a reference compound. Each test compound was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated using 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, TGC-induced peritoneal exudate cells (7.5×10^6 cells/3 ml/dish) from male ddY mice were pre-cultured in culture dishes (6 cm i.d.) for 1 h, and the adherent cells were obtained as described previously.[23—25,29,33,35] 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, protease inhibitor cocktail (1 tab/50 ml), 0.1% Triton X-100, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH 7.4] and sonicated. After determination of the protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer. For SDS-PAGE, aliquots of 40 μg of protein from each sample were subjected to electrophoresis in 7.5% polyacrylamide gels. Following electrophoresis, the proteins were transferred electrophoretically onto nitrocellulose membranes. The membranes were incubated with 5% nonfat dried milk in Tris-buffered saline (T-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 T-TBS and probed with the secondary antibody, anti-mouse IgG antibody conjugated with horseradish peroxidase (dilution of 1 : 5000). Detection was performed using an ECL™ and X-ray film (Hyperfilm-ECL™, Amersham).

Inhibitory Effect on the Release of β-Hexosaminidase in RBL-2H3 Cells The inhibitory effects of the test samples on the release of β-hexosaminidase from RBL-2H3 cells [Cell No. JCRB0023, obtained from Health Science Research Resources Bank (Osaka, Japan)] were evaluated by a method reported previously.[31,41—44] Briefly, RBL-2H3 cells were dispensed into 24-well plates at a concentration of 2×10^5 cells/well using Eagle's minimum essential medium (MEM, Sigma) containing fetal calf serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.45 μg/ml of anti-DNP IgE, and these were incubated overnight at 37 °C in 5% CO_2 for sensitization of the cells. Then the cells were washed twice with 500 μl of siraganian buffer [119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and 40 mM NaOH, pH 7.2], and incubated in 160 μl of siraganian buffer [5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albmin (BSA) were added] for an additional 10 min at 37 °C. Aliquots (20 μl) of test sample solution were added to each well and incubated for 10 min, followed by the addition of 20 μl of antigen (DNP-BSA, final concentration 10 μg/ml) at 37 °C for 10 min to stimulate the cells to evoke allergic reactions (degranulation). The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 μl) was transferred into a 96-well microplate and incubated with 50 μl of substrate (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 200 μl of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured using a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to siraganian buffer (final DMSO concentration 0.1%).

The percent inhibition of the release of β-hexosaminidase by the test material was calculated using the following equation, and IC_{50} values were determined graphically:

$$\text{inhibition (\%)} = \left(1-\frac{T-B-N}{C-N}\right)\times 100$$

Control (*C*): DNP-BSA (+), test sample (−); Test (*T*): DNP-BSA (+), test sample (+); Blank (*B*): DNP-BSA (−), test sample (+); Normal (*N*): DNP-BSA (−), test sample (−).

Under these conditions, it was calculated that 40—60% of β-hexosaminidase was released from the cells in the control groups by determination of the total β-hexosaminidase activity after sonication of the cell suspension.

Statistics Values are expressed as mean±S.E.M. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

References and Notes

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8) The ^{1}H- and ^{13}C-NMR data of **16**—**18** were not reported[7] and described follows.[14] **16**: ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.86, 0.91 (3H each, both d, J=6.1 Hz, 30, 29-H$_3$), 0.89, 1.13, 1.16, 1.24, 1.28, 1.39 (3H each, all s, 28, 27, 24, 25, 26, 23-H$_3$), 1.10 (1H, m, 5-H), 1.28, 2.09 (1H, each, both m, 20-H$_2$), 1.38 (1H, m, 21-H), 1.40 (1H, m, 22-H), 1.58, 2.15 (1H each, both m, 1-H$_2$), 1.64 (2H, m, 16-H$_2$), 1.67, 2.80 (1H each, both m, 15-H$_2$), 1.95, 2.20 (1H each, both m, 6-H$_2$), 2.04 (1H, d, J=9.7 Hz, 18-H), 2.41 (3H, s, –OAc), 2.41 (1H, m, 8-H), 2.45 (2H, m, 12-H$_2$), 3.63 (1H, d, J=9.7 Hz, 3-H), 4.01 (1H, m, 7-H), 4.50 (1H, m, 19-H), 5.00 (1H, d, J=7.6 Hz, 1′-H), 5.41 (1H, br d, J=*ca.* 6 Hz, 11-H), 5.65 (1H, m, 2-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1; **17**: ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.97, 1.10 (3H each, both d, J=6.5 Hz, 30, 29-H$_3$), 1.14, 1.21, 1.35, 1.38, 1.41 (3H each, all s, 24, 25, 26, 23, 27-H$_3$), 1.16 (1H, m, 5-H), H), 1.60 (1H, m, 21-H), 1.61, 2.12 (1H each, both m, 1-H$_2$), 1.62, 2.01 (1H each, both m, 16-H$_2$), 1.88, 2.21 (1H each, both m, 6-H$_2$), 1.99, 2.79 (1H each, both m, 15-H$_2$), 2.15 (1H, m, 22-H$_2$), 2.20, 2.63 (1H, each, both m, 20-H$_2$), 2.35 (1H, d, J=10.1 Hz, 18-H), 2.41 (3H, s, –OAc), 2.46 (1H, m, 8-H), 2.51, 2.61 (1H each, both m, 12-H$_2$), 3.63 (1H, d, J=10.0 Hz, 3-H), 4.02 (1H, m, 7-H), 4.11, 4.20 (1H each, both d, J=8.4 Hz, 28-H$_2$), 4.99 (1H, d, J=7.6 Hz, 1′-H), 5.06 (1H, m, 19-H), 5.44 (1H, br d, J=*ca.* 6 Hz, 11-H), 5.59 (1H, m, 2-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1; **18**: ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.88, 0.99 (3H each, both d, J=5.4 Hz, 30, 29-H$_3$), 1.11, 1.16, 1.21, 1.29, 1.39 (3H each, all s, 27, 24, 25, 26, 23-H$_3$), 1.10 (1H, m, 5-H), H), 1.57 (1H, m, 21-H), 1.61, 2.11 (1H each, both m, 1-H$_2$), 1.60 (1H, m, 22-H$_2$), 1.62, 1.95 (1H each, both m, 16-H$_2$), 1.89, 2.80 (1H each, both m, 15-H$_2$), 1.91, 2.21 (1H each, both m, 6-H$_2$), 2.16 (2H, m, 20-H$_2$), 2.09, 2.41 (3H each, both s, –OAc), 2.30 (1H, d, J=10.0 Hz, 18-H), 2.36 (1H, m, 8-H), 2.41, 2.50 (1H each, both m, 12-H$_2$), 3.62 (1H, d, J=10.0 Hz, 3-H), 4.00 (1H, m, 7-H), 4.32, 4.60 (1H each, both d, J=12.4 Hz, 28-H$_2$), 4.65 (1H, m, 19-H), 4.99 (1H, d, J=7.5 Hz, 1′-H), 5.40 (1H, br d, J=*ca.* 6 Hz, 11-H), 5.65 (1H, m, 2-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. The acid hydrolysis of **16**—**18** liberated D-glucose, which was identified by HPLC analysis using an optical rotation detector.[1,15]
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