Bioactive Constituents of Chinese Natural Medicines. VII.[1] Inhibitors of Degranulation in RBL-2H3 Cells and Absolute Stereostructures of Three New Diarylheptanoid Glycosides from the Bark of *Myrica rubra*

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Three new diarylheptanoid glycosides, named (+)-*S*-myricanol 5-*O*-β-D-glucopyranoside, myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside, and myricanene B 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside, were isolated from the bark of Chinese *Myrica rubra*, together with twenty known compounds. The absolute stereostructures of the new diarylheptanoid glycosides were elucidated on the basis of chemical and physicochemical evidence, including the application of the modified Mosher's method. The inhibitory effects of isolated constituents on the release of β-hexosaminidase from RBL-2H3 cells were examined, and several diarylheptanoids, myricanol, (+)-*S*-myricanol, myricanone, and myricanenes A and B, and a flavonol, myricetin, were found to show the inhibitory activity.

Key words *Myrica rubra*; degranulation inhibitor; RBL-2H3 cell; (+)-*S*-myricanol 5-*O*-β-D-glucopyranoside; myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside; myricanene B 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside

The Myricaceae plant *Myrica rubra* SIEB. et ZUCC. is widely distributed in China, Taiwan, Japan and Korea. The bark of *M. rubra* (Myricae cortex) has been used locally as an astringent, antidote, and antidiarrheic in Japanese folk medicine, and has also been used externally for burns and skin diseases in Chinese traditional medicine. Previously, several flavonoids,[2] tannins,[2] triterpenes,[3] and diarylheptanoids[4—8] were isolated from the bark of *M. rubra*. Pharmacological studies of this natural medicine have reported that its methanolic extract showed protective effects on CCl_4^- and α-naphthylisothiocyanate-induced liver injury,[9] whereas the 50% aqueous ethanolic extract and some constituents inhibited melanin biosynthesis[10] and showed anti-androgenic activity.[11]

In the course of characterization studies on the bioactive constituents of Chinese natural medicines,[1,12] we have isolated three new diarylheptanoid glycosides, termed (+)-*S*-myricanol 5-*O*-β-D-glucopyranoside (**1**), myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**2**), and myricanene B 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**3**), together with twenty known compounds from the bark of *M. rubra*. This paper deals with the absolute

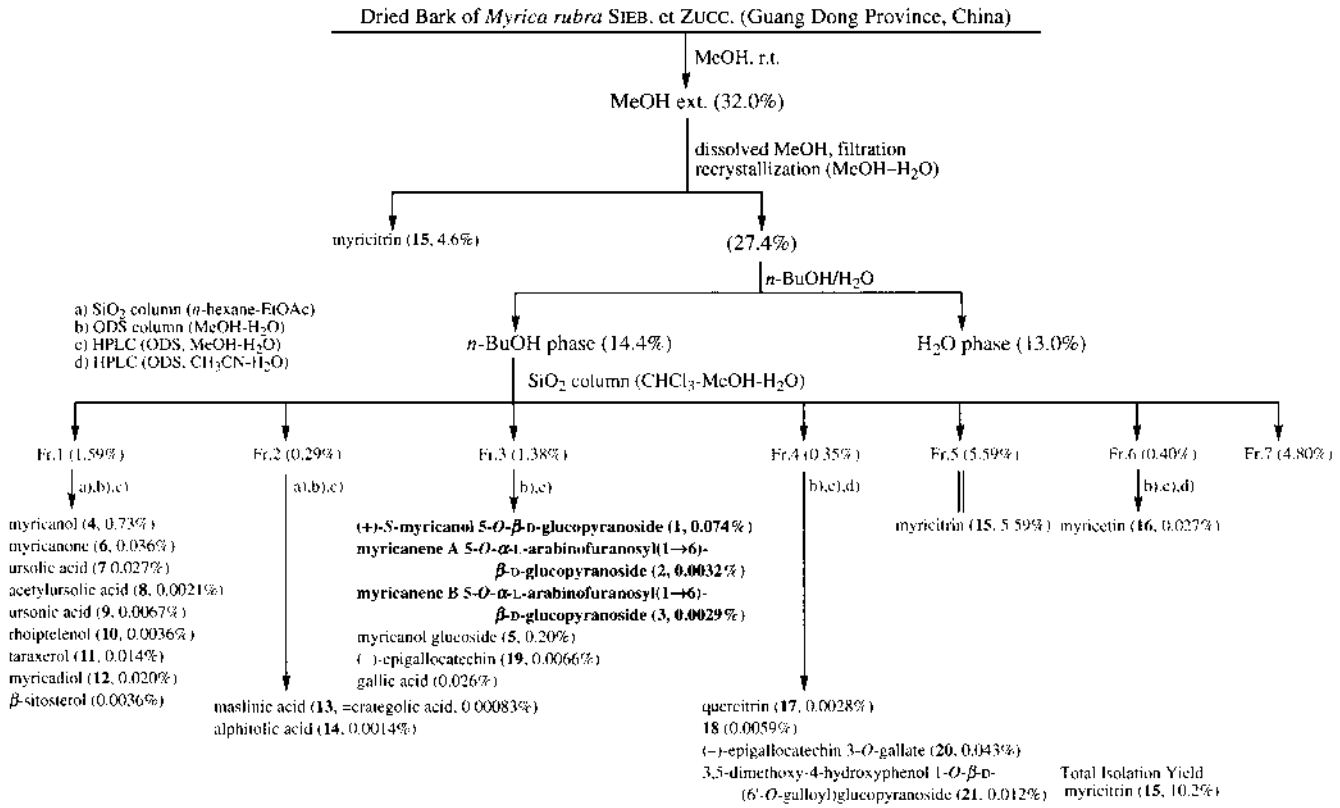

Chart 1

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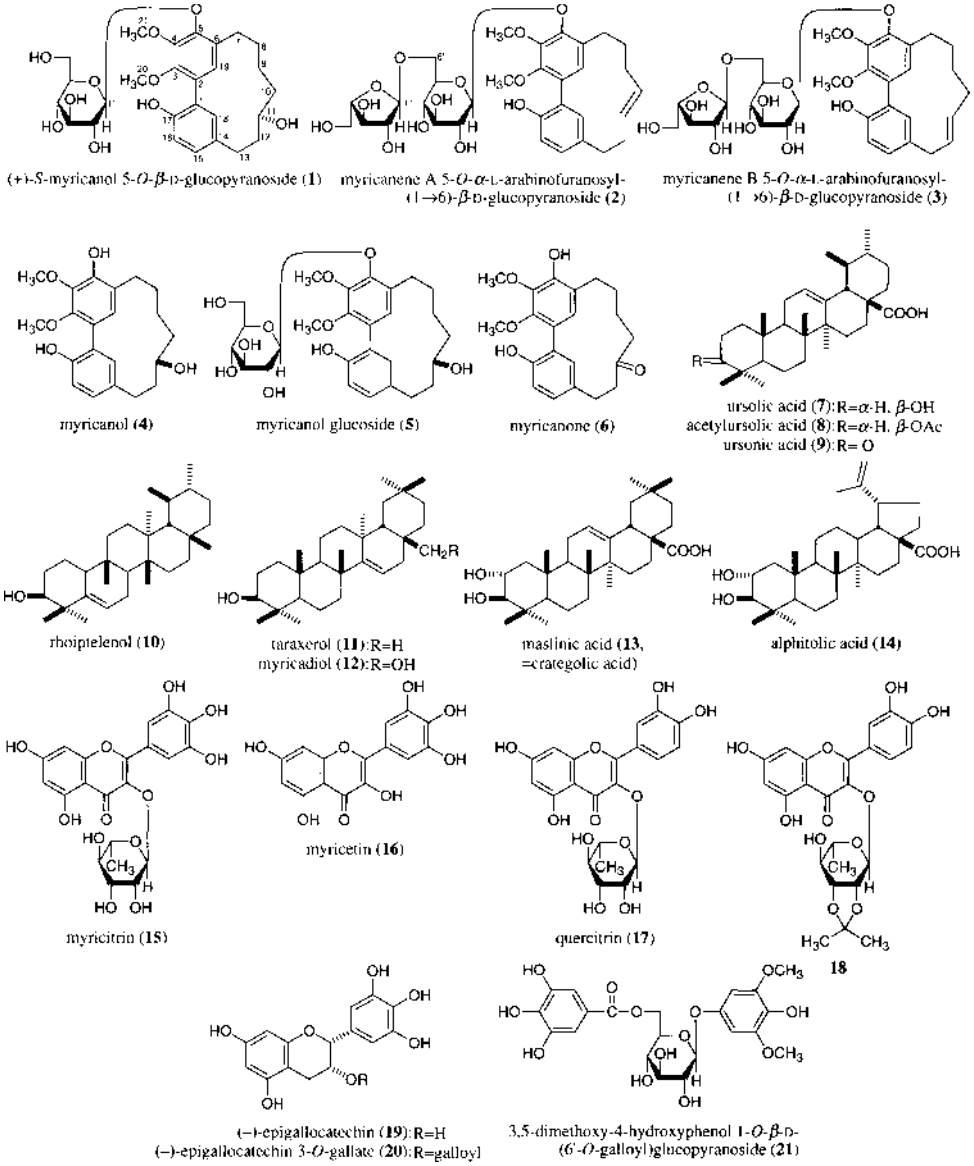

Chart 2

stereostructure elucidation of three new diarylheptanoid glycosides (**1**—**3**). In addition, we describe the inhibitory effect of components isolated from the bark of *M. rubra* on an immediate allergic reaction by monitoring the release of β-hexosaminidase from rat basophilic leukemia (RBL-2H3) cells.

Isolation of Chemical Constituents from the Dried Bark of *M. rubra* The methanolic extract of the bark of *M. rubra* (cultivated in Guang Dong province, China) was suspended in MeOH and filtered by Kiriyama funnel to give a soluble fraction and insoluble residue, which was recrystallized with aqueous MeOH to furnish myricitrin (**15**, 4.6% from natural medicine). The soluble fraction was partitioned in an *n*-BuOH–H_2O (1 : 1) mixture to give an *n*-BuOH-soluble fraction and H_2O-soluble fraction. The *n*-BuOH-soluble fraction was subjected to ordinary- and reversed-phase silica gel column chromatography, and finally HPLC, to furnish (+)-*S*-myricanol 5-*O*-β-D-glucopyranoside (**1**, 0.074%), myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**2**, 0.0032%), and myricanene B 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**3**, 0.0029%), together with twenty known compounds, myricanol (**4**,[4,5] 0.73%), myricanol glucoside (**5**,[4] 0.20%), myricanone (**6**,[4,5] 0.036%), ursolic acid (**7**,[13] 0.027%), acetylursolic acid (**8**,[13] 0.0021%), ursonic acid (**9**,[13] 0.0067%), rhoiptelenol (**10**,[14] 0.0036%), taraxerol (**11**,[3] 0.014%), myricadiol (**12**,[3] 0.020%), maslinic acid (**13**,[6,15] =crategolic acid, 0.00083%), alphitolic acid (**14**,[6] 0.0014%), myricitrin (**15**,[2,10] 5.59%), myricetin (**16**,[2,10] 0.027%), quercitrin (**17**,[16] 0.0028%), **18**[17] (0.0059%), (−)-epigallocatechin (**19**,[18] 0.0066%), (−)-epigallocatechin 3-*O*-gallate (**20**,[18] 0.043%), 3,5-dimethoxy-4-hydroxyphenol 1-*O*-β-D-(6′-*O*-galloyl)glucopyranoside (**21**,[19] 0.012%), β-sitosterol[20] (0.0036%), and gallic acid[20] (0.026%).

Absolute Stereostructure of (+)-*S*-Myricanol 5-*O*-β-D-Glucopyranoside (1) (+)-*S*-Myricanol 5-*O*-β-D-glucopyranoside (**1**) was isolated as a white powder with positive optical rotation ($[\alpha]_D^{22}$ +81.3°, EtOH). The positive- and negative-ion fast atom bombardment (FAB)-MS of **1** showed quasimolecular ion peaks at *m/z* 543 $(M+Na)^+$ and 519 $(M-H)^-$ respectively, in addition to a fragment ion peak at *m/z* 357 $(M-C_6H_{11}O_5)^-$. The molecular formula $C_{27}H_{36}O_{10}$ of **1**

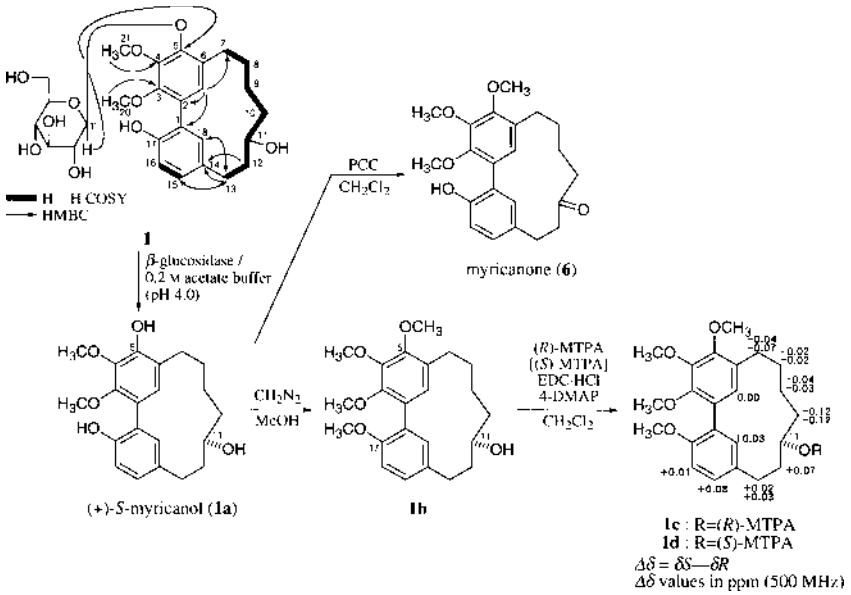

Fig. 1. Absolute Stereostructure of (+)-*S*-Myricanol 5-*O*-*β*-D-Glucopyranoside (**1**)

was determined from quasimolecular ion peaks and by high-resolution MS measurement. In the UV spectrum of **1**, absorption maxima were observed at 216 (log ε 4.5), 256 (4.2), and 299 (3.7) nm, which were suggestive of a biphenyl type diarylheptanoid structure.[8] The IR spectrum of **1** showed absorption bands at 3568, 1598, 1560, 1508, and 1073 cm^{-1}, ascribable to hydroxyl, benzene ring, and ether functions. On acid hydrolysis with 5% aqueous sulfuric acid (H_2SO_4)–1,4-dioxane (1 : 1, v/v), **1** liberated D-glucose, which was identified by GLC analysis of the trimethylsilyl thiazolidine derivative.[21] The ^{1}H-NMR (C_5D_5N) and ^{13}C-NMR (Table 1) spectra of **1** showed signals assignable to two methoxyl groups [δ 3.96, 4.10 (both s, 20, 21-H_3)], a methine bearing a hydroxyl group [δ 4.34 (m, 11-H)], a D-glucopyranosyl moiety [δ 5.75 (d, J=7.0 Hz, 1′-H)], and four aromatic protons [δ 7.12 (s, 19-H), 7.18 (d, J=8.0 Hz, 16-H), 7.19 (dd, J=2.0, 8.0 Hz, 15-H), 7.44 (br s, 18-H)], together with six methylenes (7, 8, 9, 10, 12, 13-H_2), which were very similar to those of myricanol glucoside (**5**).

The planar structure of **1**, including the positions of two methoxyl groups and a glycosidic linkage, which was the same as that **5**, was constructed on the basis of ^{1}H–^{1}H correlation spectroscopy (H–H COSY) and heteronuclear multiple bond correlation (HMBC) experiments. And the H–H COSY experiment on **1** indicated the presence of two partial structures written in the bold line, whereas, in the HMBC experiment, long-range correlations were observed between the protons and carbons, as shown in Fig. 1. Enzymatic hydrolysis of **1** with *β*-glucosidase liberated (+)-*S*-myricanol (**1a**). The proton and carbon signals in the ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR (Table 1) spectra of **1a** were superimpersable on those of myricanol (**4**), while the optical rotation of **1a** ($[\alpha]_D^{22}$ +37.3°, $CHCl_3$) was the opposite to that of **4** ($[\alpha]_D^{24}$ −48.3°, $CHCl_3$) [lit.[4] ($[\alpha]_D^{30}$ −62.9°, $CHCl_3$)]. Finally, pyridinium chlorochromate (PCC) oxidation of **1a** furnished myricanone (**6**). The above-mentioned evidence suggests that **1a** is an enantiomer of **4**.

In order to confirm the absolute stereostructure of **1a**, we carried out an application of the modified Mosher's method,[22] as shown in Fig. 1. Thus, diazomethane methylation of **1a** furnished the dimethylated-derivative (**1b**), which was subjected to the modified Mosher's method for the 11-mono-(*R*)-

Table 1. ^{13}C-NMR Data for (+)-*S*-Myricanol 5-*O*-*β*-D-Glucopyranoside (**1**), Myricanene A 5-*O*-*α*-L-Arabinofuranosyl(1→6)-*β*-D-glucopyranoside (**2**), and Myricanene B 5-*O*-*α*-L-Arabinofuranosyl(1→6)-*β*-D-glucopyranoside (**3**) and Their Aglycons (**1a**, **2a**, **3a**)

	1[a]	**2**[a]	**3**[a]	**1a**[b]	**2a**[b]	**3a**[b]
C-1	126.4	126.2	126.1	123.1	124.7	123.8
C-2	129.6	129.0	129.3	122.7	123.9	123.3
C-3	149.0	148.6	148.6	147.8	145.5	145.5
C-4	146.3	145.7	146.3	138.7	138.3	138.5
C-5	149.6	149.5	150.3	145.9	146.9	147.5
C-6	130.5	132.1	131.6	124.7	124.6	124.3
C-7	27.1	30.8	27.1	34.8	29.3	25.6
C-8	26.6	25.2	25.4	23.0	25.0	24.7
C-9	23.4	31.4	30.1	25.5	31.4	29.9
C-10	40.5	134.5	32.6	25.8	134.2	32.3
C-11	67.7	132.5	135.9	68.7	132.8	135.3
C-12	35.7	35.6	130.2	27.0	35.2	129.9
C-13	27.8	33.6	34.6	39.5	33.5	34.3
C-14	131.0	132.7	132.1	130.7	133.2	132.0
C-15	130.5	128.9	129.4	129.9	128.7	128.7
C-16	117.1	116.1	116.4	116.9	116.0	116.0
C-17	153.1	153.2	153.0	151.5	151.5	151.6
C-18	135.2	137.0	138.4	133.1	135.5	136.4
C-19	130.2	130.2	130.0	129.4	130.0	129.4
C-20	61.2	61.2	61.3	61.4	61.7	61.5
C-21	61.7	61.5	61.8	61.4	61.5	61.4
Glc-1	105.7	105.6	105.8			
2	75.7	75.5	75.7			
3	78.3	78.2	78.4			
4	71.5	71.8	72.0			
5	78.4	76.8	76.9			
6	62.5	68.1	68.3			
Ara(f)-1		109.8	110.1			
2		83.1	83.2			
3		78.7	78.7			
4		85.7	86.0			
5		62.5	62.6			

a) Measured in pyridine-d_5; *b*) $CDCl_3$ at 125 MHz. Glc: *β*-D-glucopyranosyl; Ara(f): *α*-L-arabinofuranosyl.

and (*S*)-2-methoxy-2-trifluorophenylacetate (MTPA esters, **1c**, **d**). As shown in Fig. 1, the signals due to protons attached to 12, 13, 15, 16, and 18-carbons in the 11-mono-(*S*)-MTPA ester (**1d**) were observed at lower field fields compared with those of the 11-mono-(*R*)-MTPA ester (**1c**) [$\Delta\delta$: positive], while signals due to protons of 7, 8, 9, 10-carbons in **1d** were observed at higher fields compared with those of **1c** [$\Delta\delta$: negative]. Consequently, the absolute stereostructure at the 11-positions of **1** and **1a** have been determined to be *S*. Previously, a (±)-myricanol mixture was isolated from the bark of *Myrica cerifera*, and its structure was established by crystal X-ray analysis.[23] This paper is the first report for the (+)-form of myricanol.

Stereostructures of Myricanene A 5-*O*-α-L-Arabinofuranosyl(1→6)-β-D-glucopyranoside (2) and Myricanene B 5-*O*-α-L-Arabinofuranosyl(1→6)-β-D-glucopyranoside (3) Myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**2**) and myricanene B 5-*O*-α-L-arabinofuranosyl (1→6)-β-D-glucopyranoside (**3**) were isolated as white powders with negative optical rotations (**2**: $[\alpha]_D^{22}$ −0.6°; **3**: $[\alpha]_D^{22}$ −10.8° in EtOH). In the positive- and negative-ion FAB-MS of **2** and **3**, common quasimolecular ion peaks were observed at *m/z* 657 $(M+Na)^+$ and *m/z* 633 $(M-H)^-$, together with a fragment ion peak at *m/z* 339 $(M-C_{11}H_{19}O_9)^-$, and the molecular formula $C_{32}H_{42}O_{13}$ was determined by high-resolution MS measurement. The UV spectra (EtOH) of **2** and **3** showed absorption maxima [nm (log ε): **2**, 215 (4.2), 257 (3.9), 300 (3.6); **3**, 214 (4.5), 253 (4.1), 297 (3.7)] suggestive of a biphenyl type diarylheptanoid moiety. The IR spectra of **2** and **3** showed absorption bands due to hydroxyl, olefin, benzene ring, and ether functions (**2**: 3651, 1659, 1610, 1502, 1059 cm^{-1}; **3**: 3657, 1651, 1593, 1504, 1059 cm^{-1}). On acid hydrolysis with 5% aqueous H_2SO_4–1,4-dioxane (1 : 1, v/v), **2** and **3** liberated D-glucose and L-arabinose, which were identified by GLC analysis of their trimethylsilyl thiazolizine derivatives.[21] The ^{1}H-NMR (C_5D_5N) and ^{13}C-NMR (Table 1) spectra of **2** showed signals assignable to two methoxyl groups [δ 3.97, 4.12 (both s, 20, 21-H_3)], an *E*-conformed olefin function [δ 5.22 (dt, *J*=15.5, 6.0 Hz, 10-H), 5.62 (dt, *J*=15.5, 8.5 Hz, 11-H)], a L-arabinofuranosyl moiety [δ 5.58 (br s, 1″-H)], a D-glucopyranosyl moiety [δ 5.69 (d, *J*=7.3 Hz, 1′-H)], and four aromatic protons [δ 7.13 (dd, *J*=2.0, 8.0 Hz, 15-H), 7.14 (d, *J*=8.0 Hz, 16-H), 7.21 (s, 19-H), 7.41 (br s, 18-H)], together with five methylenes (7, 8, 9, 12, 13-H_2). The proton and carbon signals in the ^{1}H-NMR (C_5D_5N) and ^{13}C-NMR (Table 1) spectra of **3** were found to be similar to those of **2**, except for signals due to an olefin function. New aglycons, myricanenes A (**2a**) and B (**3a**), were obtained by enzymatic hydrolysis of **2** and **3** with naringinase, respectively. On the other hand, dehydration of myricanol (**4**) with thionyl chloride ($SOCl_2$) yielded **2a** and **3a**, so that the structures of **2a** and **3a** were determined to be as shown in Fig. 2. The structure of the disaccharide moiety and the position of the glycosidic linkage in **2** and **3** were elucidated by HMBC experiment. Namely, long-range correlations were observed between the 1′-proton of the glucopyranosyl moiety and the 5-carbon of the aglycon moiety, and between the 1″-proton of the arabinofuranosyl moiety and the 6′-carbon of the glucopyranosyl moiety. Consequently, the stereostructures of **2** and **3** were elucidated as shown.

Inhibitory Effect of Constituents from *M. rubra* on the Release of β-Hexosaminidase from 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 secretory granules of mast cells, and is also released concomitantly with histamine when mast cells are immunologically activated.[24] Therefore, it is generally accepted that β-hexosaminidase is a degranulation marker of mast cells. Previously, we reported the isolation and structural elucidation of various antiallergic constituents from natural medicines, such as *Hydrangea macrophylla* var. *thunbergii*,[25] *Hovenia dulcis*,[26] *Rhodiola quadrifida*,[27] *R. sacra*,[28] *Alisma orientale*,[29] *Kochia scoparia*,[30] *Corchorus olitorius*,[31] *Phaseolus vulgaris*,[32] *Zizyphus jujuba* var. *spinosa*,[33] and *Benincasa hispida*.[34] In our continuous search for antiallergic principles from natural sources, we examined the effects of constituents from the bark of *M. rubra* on the release of β-hexosaminidase induced by dinitrophenylated bovine serum albumine (DNP-BSA) from RBL-2H3 cells sensitized with anti-DNP IgE. As shown in Table 2, biphenyl type diarylheptanoids, (+)-*S*-myricanol (**1a**, IC_{50}=28 μM), myricanenes A (**2a**, 98 μM) and B (**3a**, *ca.* 100 μM), myricanol (**4**, 63 μM), myricanone (**6**, 46 μM), and a flavonoid, myricetin (**16**, 23 μM), showed strong activity. On the other hand, biphenyl

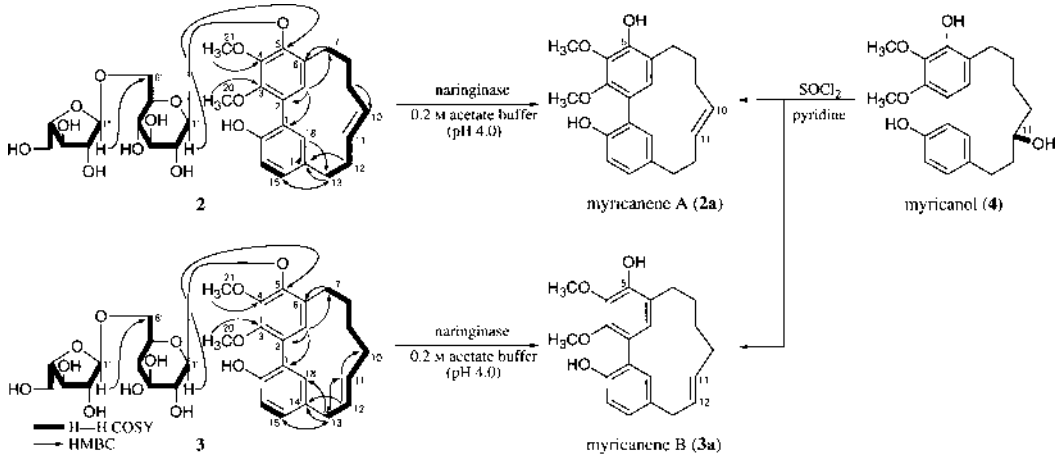

Fig. 2. Chemical Correlations of **2** and **3** with Myricanol (**4**)

Table 2. Inhibitory Effects of Constituents from the Bark of *M. rubra* on the Release of β-Hexosaminidase from RBL-2H3 Cells

Sample	Inhibition (%)				IC_{50} (μM)
	0 μM	10 μM	30 μM	100 μM	
(+)-*S*-Myricanol 5-*O*-β-D-glucopyranoside (**1**)	0.0±3.7	−3.6±5.9	−5.8±4.0	−13.1±3.6	—
(+)-*S*-Myricanol (**1a**)	0.0±2.0	17.0±1.6** (−1.1)	54.0±1.9** (4.5)	96.1±0.5** (7.4)	28
Myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**2**)	0.0±3.5	0.1±3.4	−0.4±3.3	7.9±1.4	—
Myricanene A (**2a**)	0.0±3.4	16.4±5.1 (−1.5)	29.5±4.0** (2.5)	50.6±3.9** (3.5)	98
Myricanene B 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**3**)	0.0±2.7	−0.2±4.5	4.4±2.8	−5.2±2.8	—
Myricanene B (**3a**)	0.0±2.4	13.6±2.1 (1.2)	25.2±3.5** (4.2)	44.1±2.9** (5.1)	—
Myricanol (**4**)	0.0±3.0	13.4±2.7 (−1.1)	28.6±3.1** (4.9)	73.5±1.3** (4.1)	63
Myricanol glucoside (**5**)	0.0±3.4	1.6±3.2	6.3±4.9	6.7±5.5	—
Myricanone (**6**)	0.0±3.3	10.4±2.7 (0.2)	39.4±3.4** (4.3)	85.3±0.7** (7.3)	46
Ursonic acid (**9**)	0.0±2.5	4.4±2.4	13.5±2.7**	20.2±1.3**	—
Rhoiptelenol (**10**)	0.0±2.6	−1.6±4.8	−3.5±4.0	−8.0±1.1	—
Taraxerol (**11**)	0.0±1.7	−3.3±3.4	−0.5±3.1	−3.0±0.9	—
Myricadiol (**12**)	0.0±1.4	−1.1±3.7	−3.7±1.1	−12.0±1.9	—
Myricitrin (**15**)	0.0±1.9	3.7±1.4	12.1±1.4	12.6±4.2	—
Myricetin (**16**)	0.0±5.6	3.9±5.1 (−3.2)	74.2±0.7** (1.2)	99.6±0.2** (−4.8)	23
18	0.0±3.8	2.5±3.4	−3.3±1.9	39.8±1.4**	—
(−)-Epigallocatechin (**19**)	0.0±2.9	2.1±5.0	12.1±1.4	12.6±4.2	—
(−)-Epigallocatechin 3-*O*-gallate (**20**)	0.0±4.1	−3.7±3.9	1.6±3.3	0.8±1.6	—
3,5-Dimethoxy-4-hydroxy-phenol 1-*O*-β-D-(6′-*O*-galloyl)-glucopyranoside (**21**)	0.0±2.4	7.0±3.0	6.1±3.6	−4.7±2.3	—
Gallic acid	0.0±2.1	−4.5±2.2	−3.3±3.5	−6.5±1.3	—
Curcumin	0.0±1.8	2.2±2.0 (2.6)	14.2±1.6** (4.1)	62.6±1.0** (6.9)	82

Each value represents the mean±S.E.M. (n=4). Significantly different from the control, $^{**}p<0.01$. Values in parentheses indicate enzyme inhibition (%) against β-hexosaminidase.

type diarylheptanoid glycosides (**1**—**3**, **5**), flavonoid glycosides (**15**, **18**), and catechins (**19**, **20**) exhibited weak inhibition. In addition, the effects of test compounds on β-hexosaminidase activity were examined to clarify whether their effects were due to the inhibition of enzyme activity or of degranulation. As a result, these active constituents (**1a**, **2a**, **3a**, **4**, **6**, **16**) did not affect the enzyme activity of β-hexosaminidase. Previously, a diarylheptanoid, curcumin, and a few flavonoids such as myricetin were reported to inhibit the degranulation,[35] but this is the first report of the degranulation inhibitory activity of biphenyl type diarylheptanoids.

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; electron impact (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; HPLC detector, Shimadzu RID-6A refractive index detector and Shimadzu SPD-10A UV-VIS 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); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); TLC, pre-coated TLC plates with Silica gel $60F_{254}$ (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed phase); reversed-phase high performance thin-layer chromatography (HPTLC), pre-coated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Extraction and Isolation The dried bark of *Myrica rubra* (5.0 kg, cultivated in Guang Dong, China and purchased from MAE CHU Co., Ltd., Nara, Japan) was finely cut and extracted three times with MeOH at room temperature for 1 d. Evaporation of the solvent under reduced pressure provided a MeOH extract (1600 g). The MeOH extract (1400 g) was suspended in a small amount of MeOH, and the suspension was filtered to give a soluble fraction (1200 g) and an insoluble fraction, which was recrystallized with aqueous MeOH to furnish myricitrin (**15**, 200 g). The soluble fraction (722 g) was partitioned in an n-BuOH–H_2O (1 : 1) mixture to give n-BuOH- and H_2O-soluble fractions (380 g and 342 g), respectively. The n-BuOH-soluble fraction (50 g) was subjected to ordinary-phase silica gel column chromatography [1.5 kg, $CHCl_3$–MeOH (10 : 1→5 : 1)→$CHCl_3$–MeOH–H_2O (6 : 4 : 1)→MeOH] to give seven fractions {Fr. 1 (5.5 g), Fr. 2 (1.0 g), Fr. 3 (4.8 g), Fr. 4 (1.2 g), Fr. 5 [=myricitrin (**15**, 19.4 g)], Fr. 6 (1.4 g), Fr. 7 (16.7 g)}. Fraction 1 (4.4 g) was subjected to reversed-phase silica gel column chromatography [130 g, MeOH–H_2O (60 : 40)→MeOH] to furnish two fractions [Fr. 1-1 (2.4 g), Fr. 1-2 (2.0 g)]. Fraction 1-1 (2.4 g) was purified by ordinary-phase silica gel column chromatography [70 g, n-hexane–AcOEt (4 : 1→3 : 1)] to give myricanol (**4**, 2.0 g) and myricanone (**6**, 100 mg). Fraction 1-2 (2.0 g) was subjected to reversed-phase silica gel column chromatography [60 g, MeOH–H_2O (70 : 30→80 : 20→90 : 10)→MeOH] to furnish seven fractions [Fr. 1-2-1 (145 mg), Fr. 1-2-2 (93 mg), Fr. 1-2-3 (388 mg), Fr. 1-2-4 (424 mg), Fr. 1-2-5 (100 mg), Fr. 1-2-6 (234 mg), Fr. 1-2-7 (616 mg)]. Fraction 1-2-3 (388 mg) was purified by HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (90 : 10)] to give ursolic acid (**7**, 76 mg) and ursonic acid (**9**, 19 mg). Fraction 1-2-4 (424 mg) was recrystallized with MeOH to furnish myricadiol (**12**, 57 mg) and a triterpene mixture, which was further purified by HPLC [detection RI, YMC-Pack

ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (90 : 10)] to give acetylursolic acid (**8**, 6 mg). Fraction 1-2-6 (334 mg) was subjected to ordinary-phase silica gel column chromatography [10 g, *n*-hexane–AcOEt (25 : 1→5 : 1)→$CHCl_3$] to give rhoiptelenol (**10**, 10 mg), taraxerol (**11**, 46 mg), and β-sitosterol (10 mg). Fraction 2 (1.0 g) was subjected to reversed-phase silica gel column chromatography [30 g, MeOH–H_2O (25 : 75→40 : 60→60 : 40→90 : 10→95 : 5)→MeOH] to give six fractions [Fr. 2-1 (141 mg), Fr. 2-2 (114 mg), Fr. 2-3 (113 mg), Fr. 2-4 (56 mg), Fr. 2-5 (150 mg), Fr. 2-6 (426 mg)]. Fraction 2-5 (150 mg) was subjected to ordinary-phase silica gel column chromatography [5 g, *n*-hexane–AcOEt (2 : 1)] and finally to HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (85 : 15)] to give maslinic acid (**13**, =crategolic acid, 3 mg) and alphitolic acid (**14**, 5 mg). Fraction 3 (4.8 g) was subjected to reversed-phase silica gel column chromatography [140 g, MeOH–H_2O (50 : 50→70 : 30)→MeOH] to give six fractions [Fr. 3-1 (371 mg), Fr. 3-2 (184 mg), Fr. 3-3 (1.4 g), Fr. 3-4 (2.5 g), Fr. 3-5 (105 mg), Fr. 3-6 (240 mg)]. Fraction 3-1 (371 mg) was purified by HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (25 : 75 and 5 : 95)] to give (−)-epigallocatechin (**19**, 23 mg) and gallic acid (90 mg). Fraction 3-3 (400 mg) was purified by HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (50 : 50)] to give (+)-*S*-myricanol 5-*O*-β-D-glucopyranoside (**1**, 74 mg) and myricanol glucoside (**5**, 201 mg). Fraction 3-5 (105 mg) was purified by HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (50 : 50)] to give myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**2**, 11 mg) and myricanene B 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**3**, 10 mg). Fraction 4 (1.2 g) was subjected to reversed-phase silica gel column chromatography [36 g, MeOH–H_2O (20 : 80→50 : 50→70 : 30)→MeOH] to furnish five fractions [Fr. 4-1 (65 mg), Fr. 4-2 (507 mg), Fr. 4-3 (100 mg), Fr. 4-4 (253 mg), Fr. 4-5 (275 mg)]. Fraction 4-2 (114 mg) was purified by HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (35 : 65)] to give (−)-epigallocatechin 3-*O*-gallate (**20**, 30 mg) and 3,5-dimethoxy-4-hydroxyphenol 1-*O*-β-D-(6′-*O*-galloyl)glucopyranoside (**21**, 9 mg). Fraction 4-3 (100 mg) was purified by HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (30 : 70)] to give (−)-epigallocatechin 3-*O*-gallate (**20**, 15 mg). Fraction 4-4 (253 mg) was purified by HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), CH_3CN–H_2O (35 : 65)] to give quercitrin (**17**, 9 mg) and **18** (20 mg). Fraction 6 (1.4 g) was subjected to reversed-phase silica gel column chromatography [45 g, MeOH–H_2O (30 : 70→50 : 50→70 : 30)→MeOH] and finally to HPLC [detection RI, YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), CH_3CN–H_2O (25 : 75)] to give myricetin (**16**, 98 mg). The known compounds (**4**—**21**) were identified by comparison of their physical data ($[\alpha]_D$, IR, ^{1}H-NMR, ^{13}C-NMR) with reported values.[2—6,10,13—19]

(+)-*S*-Myricanol 5-*O*-β-D-glucopyranoside (**1**): A white powder, $[\alpha]_D^{22}$ +81.3° (c=0.2, EtOH). High-resolution positive-ion FAB-MS: Calcd for $C_{27}H_{36}O_{10}Na$ $(M+Na)^+$: 543.2206. Found: 543.2218. UV [EtOH, nm, (log ε)]: 216 (4.5), 256 (4.2), 299 (3.7). IR (KBr): 3568, 2932, 1598, 1560, 1508, 1450, 1073 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 1.52, 1.79 (1H each, both m, 9-H_2), 1.82, 1.91 (1H each, both m, 8-H_2), 1.88, 1.93 (1H each, both m, 10-H_2), 1.97, 2.44 (1H each, both m, 12-H_2), 2.99, 3.40 (1H each, both m, 7-H_2), 3.01, 3.33 (1H each, both m, 13-H_2), 3.96, 4.10 (each 3H, both s, 20, 21-H_3), 4.34 (1H, m, 11-H), 5.75 (1H, d, J=7.0 Hz, 1′-H), 7.12 (1H, s, 19-H), 7.18 (1H, d, J=8.0 Hz, 16-H), 7.19 (1H, dd, J=2.0, 8.0 Hz, 15-H), 7.44 (1H, br s, 18-H). ^{13}C-NMR (C_5D_5N) δ_C: given in Table 1. Positive-ion FAB-MS m/z: 543 $(M+Na)^+$. Negative-ion FAB-MS m/z: 519 $(M-H)^-$, 357 $(M-C_6H_{11}O_5)^-$.

Myricanene A 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**2**): A white powder, $[\alpha]_D^{22}$ −0.6° (c=0.1, EtOH). High-resolution positive-ion FAB-MS: Calcd for $C_{32}H_{42}O_{13}Na$ $(M+Na)^+$: 657.2523. Found: 657.2531. UV [EtOH, nm, (log ε)]: 215 (4.2), 257 (3.9), 300 (3.6). IR (KBr): 3651, 2907, 1659, 1610, 1502, 1495, 1059 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 1.93 (2H, m, 8-H_2), 2.27 (2H, m, 12-H_2), 2.44 (2H, m, 9-H_2), 2.65 (2H, m, 13-H_2), 3.15, 3.45 (1H each, both m, 7-H_2), 3.97, 4.12 (each 3H, both s, 20, 21-H_3), 5.22 (1H, dt, J=15.5, 6.0 Hz, 10-H), 5.58 (1H, br s, 1″-H), 5.62 (1H, dt, J=15.5, 8.5 Hz, 11-H), 5.69 (1H, d, J=7.3 Hz, 1′-H), 7.13 (1H, dd, J=2.0, 8.0 Hz, 15-H), 7.14 (1H, d, J=8.0 Hz, 16-H), 7.21 (1H, s, 19-H), 7.41 (1H, br s, 18-H). ^{13}C-NMR (C_5D_5N) δ_C: given in Table 1. Positive-ion FAB-MS m/z: 1291 $(2M+Na)^+$, 657 $(M+Na)^+$. Negative-ion FAB-MS m/z: 633 $(M-H)^-$, 339 $(M-C_{11}H_{19}O_9)^-$.

Myricanene B 5-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**3**): A white powder, $[\alpha]_D^{22}$ −10.8° (c=0.1, EtOH). High-resolution positive-ion FAB-MS: Calcd for $C_{32}H_{42}O_{13}Na$ $(M+Na)^+$: 657.2523. Found: 657.2518. UV [EtOH, nm, (log ε)]: 214 (4.5), 253 (4.1), 297 (3.7). IR (KBr): 3657, 2910, 1651, 1593, 1504 1495, 1059 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 1.65, 1.75 (1H each, both m, 9-H_2), 1.91 (2H, m, 8-H_2), 2.09, 2.18 (1H each, both m, 10-H_2), 3.00, 3.39 (1H each, both m, 7-H_2), 3.30 (2H, m, 13-H_2), 3.84, 4.22 (each 3H, both s, 20 and 21-H_3), 5.59 (1H, br s, 1″-H), 5.65 (1H, d, J=6.5 Hz, 1′-H), 5.69 (1H, dt, J=15.5, 7.5 Hz, 12-H), 5.89 (1H, dt, J=15.5, 7.5 Hz, 11-H), 7.10 (1H, s, 19-H), 7.15 (1H, d, J=8.5 Hz, 16-H), 7.19 (1H, dd, J=1.5, 8.5 Hz, 15-H), 7.75 (1H, br s, 18-H). ^{13}C-NMR (C_5D_5N) δ_C: given in Table 1. Positive-ion FAB-MS m/z: 1291 $(2M+Na)^+$, 657 $(M+Na)^+$. Negative-ion FAB-MS m/z: 633 $(M-H)^-$, 339 $(M-C_{11}H_{19}O_9)^-$.

Enzymatic Hydrolysis of 1—3 A solution of **1** (21.0 mg, 0.040 mmol) in 0.2 M acetate buffer (pH 4.4, 3.0 ml) was treated with β-glucosidase (40 mg, from almond, Oriental Yeast Co., Ltd.), and the solution was stirred at 38 °C for 2 d. After treatment of the reaction mixture with EtOH, the mixture was evaporated to dryness under reduced pressure and the residue was purified by ordinary-phase silica gel column chromatography [1.0 g, $CHCl_3$–MeOH–H_2O (7 : 3 : 1, lower layer)] to give (+)-*S*-myricanol (**1a**, 14.4 mg, 99%).

Through a similar procedure, a solution of **2** or **3** (3.0 mg each, 0.005 mmol) in 0.2 M acetate buffer (pH 4.0, 2.0 ml) was treated with naringinase (10 mg, from *Penicillium decumbens*, Sigma Co., Ltd.), and the solution was stirred at 40 °C for 4 d, respectively. After treatment of the reaction mixture with EtOH, the mixture was evaporated to dryness under reduced pressure, and the residue was purified by ordinary-phase silica gel column chromatography [500 mg, *n*-hexane–AcOEt (3 : 1)] to give myricanenes A (**2a**, 1.0 mg, 62%) or B (**3a**, 1.0 mg, 62%).

(+)-*S*-Myricanol (**1a**): A white powder, $[\alpha]_D^{22}$ +37.3° (c=0.2, $CHCl_3$). High-resolution EI-MS: Calcd for $C_{21}H_{26}O_5$ (M^+): 358.1780. Found: 358.1774. UV [EtOH, nm, (log ε)]: 214 (4.2), 261 (3.7), 298 (3.5). IR (KBr): 3400, 2932, 1598, 1560, 1500, 1450 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.54, 1.91 (1H each, both m, 10-H_2), 1.56, 1.70 (1H each, both m, 9-H_2), 1.68, 2.34 (1H each, both m, 12-H_2), 1.92 (2H, m, 8-H_2), 2.55, 2.79 (1H each, both m, 7-H_2), 2.92 (2H, m, 13-H_2), 3.88, 4.00 (each 3H, both s, 20, 21-H_3), 4.09 (1H, m, 11-H), 6.89 (1H, s, 19-H), 6.91 (1H, d, J=8.5 Hz, 16-H), 7.09 (1H, dd, J=2.5, 8.5 Hz, 15-H), 7.18 (1H, br s, 18-H). ^{13}C-NMR ($CDCl_3$) δ_C: given in Table 1. EI-MS m/z (%): 358 (M^+, 100), 340 (M^+-H_2O, 14).

Myricanene A (**2a**): A white powder. High-resolution EI-MS: Calcd for $C_{21}H_{24}O_4$ (M^+): 340.1674. Found: 340.1668. UV [EtOH, nm, (log ε)]: 214 (4.0), 259 (3.4), 297 (3.3). IR (KBr): 3362, 2926, 1655, 1610, 1506, 1456 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.93 (2H, m, 8-H_2), 2.37 (2H, m, 12-H_2), 2.42 (2H, m, 9-H_2), 2.72 (2H, m, 13-H_2), 2.77 (2H, m, 7-H_2), 3.86, 4.00 (each 3H, both s, 20, 21-H_3), 5.39 (1H, dt, J=15.5, 5.5 Hz, 10-H), 5.59 (1H, dt, J=15.5, 6.5 Hz, 11-H), 6.83 (1H, d, J=8.0 Hz, 16-H), 7.02 (1H, dd, J=2.5, 8.0 Hz, 15-H), 7.07 (1H, s, 19-H), 7.36 (1H, d, J=2.5 Hz, 18-H). ^{13}C-NMR ($CDCl_3$) δ_C: given in Table 1. EI-MS m/z (%): 340 (M^+, 100), 325 (M^+-CH_3, 14).

Myricanene B (**3a**): A white powder. High-resolution EI-MS: Calcd for $C_{21}H_{24}O_4$ (M^+): 340.1674. Found: 340.1683. UV [EtOH, nm, (log ε)]: 215 (4.1), 253 (3.7), 301 (3.4). IR (KBr): 3566, 2926, 1655, 1633, 1506, 1456 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.72 (2H, m, 9-H_2), 1.94 (2H, m, 8-H_2), 2.21 (2H, m, 10-H_2), 2.65 (2H, m, 7-H_2), 3.31 (2H, m, 13-H_2), 3.89, 4.00 (each 3H, both s, 20, 21-H_3), 5.73 (1H, dt, J=15.5, 6.8 Hz, 12-H), 5.90 (1H, dt, J=15.5, 7.0 Hz, 11-H), 6.84 (1H, d, J=8.0 Hz, 16-H), 6.96 (1H, s, 19-H), 7.09 (1H, dd, J=2.5, 8.0 Hz, 15-H), 7.63 (1H, d, J=2.5 Hz, 18-H). ^{13}C-NMR ($CDCl_3$) δ_C: given in Table 1. EI-MS m/z (%): 340 (M^+, 100), 325 (M^+-CH_3, 9).

PCC Oxidation of 1a A solution of **1a** (4.0 mg, 0.011 mmol) in CH_2Cl_2 (1.0 ml) was treated with pyridinium chlorochromate (PCC, 5.0 mg, 0.023 mmol), and the whole mixture was stirred at room temperature for 30 min under N_2 atmosphere. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract was washed with saturated aqueous $NaHCO_3$ and brine, then dried over $MgSO_4$ powder and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a residue, which was then purified by silica gel column chromatography [500 mg, *n*-hexane–AcOEt (4 : 1)] to give **6** (3.0 mg, 75%).

Diazomethane Methylation of 1a A solution of **1a** (4.0 mg, 0.011 mmol) in MeOH (1.0 ml) was treated with $CH_2N_2 \cdot Et_2O$ (*ca.* 5 ml), and the whole mixture was stirred at room temperature for 30 min. Removal of the solvent under reduced pressure gave **1b** (4.3 mg, quant.).

1b: A white powder. High-resolution EI-MS: Calcd for $C_{23}H_{30}O_5$ (M^+): 386.2093. Found: 386.2104. ^{1}H-NMR ($CDCl_3$) δ: 1.54, 1.72 (1H each, both m, 9-H_2), 1.56, 1.92 (1H each, both m, 10-H_2), 1.67, 2.32 (1H each, both m, 12-H_2), 1.92 (2H, m, 8-H_2), 2.54, 2.81 (1H each, both m, 7-H_2), 2.92 (2H, m, 13-H_2), 3.89, 3.92, 3.97, 4.00 (all 3H, s, –OCH_3), 4.10 (1H, m, 11-H), 6.92

(1H, d, J=8.5 Hz, 16-H), 6.89 (1H, s, 19-H), 7.09 (1H, dd, J=2.5, 8.0 Hz, 15-H), 7.19 (1H, br s, 18-H). EI-MS m/z (%): 386 (M^+, 19), 372 (M^+-CH_3, 100), 358 ($M^+-C_2H_6$, 44).

Preparation of the (*R*)-MTPA Ester (1c) and (*S*)-MTPA Ester (1d) from 1b A solution of **1b** (1.0 mg, 0.029 mmol) in CH_2Cl_2 (1.0 ml) was treated with (*R*)-2-methoxy-2-trifluoromethylphenylacetic acid [(*R*)-MTPA] (6.6 mg, 0.028 mmol) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC·HCl, 5.5 mg, 0.028 mmol) and 4-dimethylaminopyridine (4-DMAP, 2.0 mg, 0.016 mmol), and the mixture was heated under reflux for 6 h. After cooling, it 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, then dried over $MgSO_4$ powder and filtered. Removal of the solvent from the filtrate under reduced pressure furnished a residue which was purified by silica gel column chromatography [500 mg, *n*-hexane–AcOEt (2 : 1)] to give **1c** (1.0 mg, 69%). Through a similar procedure, **1d** (1.0 mg, 69%) was prepared from **1b** (1.0 mg, 0.029 mmol) using (*S*)-2-methoxy-2-trifluoromethylphenylacetic acid [(*S*)-MTPA] (6.6 mg, 0.028 mmol), EDC·HCl (5.5 mg, 0.028 mmol) and 4-DMAP (2.0 mg, 0.016 mmol).

1c: ^{1}H-NMR ($CDCl_3$) δ: 1.62, 1.74 (1H each, both m, 9-H_2), 1.98 (2H, m, 12-H_2), 2.01 (2H, m, 10-H_2), 2.30 (2H, m, 8-H_2), 2.36, 2.75 (1H each, both m, 7-H_2), 2.58, 2.86 (1H each, both m, 13-H_2), 3.49, 3.57, 3.90, 3.92, 3.98 (all 3H, s, –OCH_3), 5.35 (1H, m, 11-H), 6.79 (1H, dd, J=1.8, 8.3 Hz, 15-H), 6.92 (1H, d, J=8.3 Hz, 16-H), 7.00 (1H, s, 19-H), 7.13 (1H, br s, 18-H).

1d: ^{1}H-NMR ($CDCl_3$) δ: 1.58, 1.71 (1H each, both m, 9-H_2), 1.89 (2H, m, 10-H_2), 2.05 (2H, m, 12-H_2), 2.28 (2H, m, 8-H_2), 2.32, 2.68 (1H each, both m, 7-H_2), 2.60, 2.89 (1H each, both m, 13-H_2), 3.49, 3.51, 3.91, 3.92, 3.98 (all 3H, s, –OCH_3), 5.35 (1H, m, 11-H), 6.93 (1H, d, J=8.3 Hz, 16-H), 7.00 (1H, s, 19-H), 7.05 (1H, dd, J=2.1, 8.3 Hz, 15-H), 7.16 (1H, br s, 18-H).

Dehydration of 4 A solution of **4** (50.0 mg, 0.014 mmol) in pryidine (2.0 ml) was treated with $SOCl_2$ (500 μl), and the mixture was stirred at 0 °C for 1 h. The reaction mixture was poured into ice-water and the whole was extracted with AcOEt. The AcOEt extract 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 gave a residue. The residue was purified by HPLC [detection UV (254 nm), YMC-Pack ODS-A (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (80 : 20)] to give myricanenes A (**2a**, 13.2 mg, 28%) and B (**3a**, 9.5 mg, 20%).

Acid Hydrolysis of 1—3 A solution of **1**—**3** (2 mg each) in 5% aqueous H_2SO_4–1,4-dioxane (0.5 ml, 1 : 1, v/v) was heated under reflux for 1 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form), and the residue was removed by filtration. After removal of the solvent from the filtrate under reduced pressure, the residue was transferred to a Sep-Pak C18 cartridge with H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (4 mg) in pyridine (0.5 ml) at 60 °C for 1 h. After reaction, the solution was treated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (0.2 ml) at 60 °C for 1 h. The supernatant was then subjected to GLC analysis to identify the derivatives of D-glucose (i) from **1**—**3**; and of L-arabinose (ii) from **2** and **3**. GLC conditions: column: Supeluco STBTM-1, 30 m×0.25 mm (i.d.) capillary column; injector temperature: 230 °C; detector temperature: 230 °C; column temperature: 230 °C; He flow rate: 15 ml/min; t_R: i: 24.2 min, ii: 15.1 min.

Bioassay

Inhibitory Effect on the Release of β-Hexosaminidase from RBL-2H3 Cells Inhibitory effects of test samples on the release of β-hexosaminidase from RBL-2H3 cells were evaluated by the following procedure.[36] RBL-2H3 cells were grown in Minimum Essential Medium Eagle (MEM, Shigma Co., Ltd.) containing fetal calf serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Before the experiment, cells were dispensed into 24-well plates at the concentration of 2×10^5 cells/well using a medium containing 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, 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), 40 mM NaOH, pH 7.2] and incubated in 160 μl of siraganian buffer (5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA were added) for an additional 10 min at 37 °C. Then, 20 μl of test sample solution was added to each well and incubated for 10 min, followed by the addition of 20 μl of antigen (DNP-BSA, final concentration was 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. 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 by 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 was 0.1%).

For estimating the spontaneous release of β-hexosaminidase from cells, exactly the same procedure was followed (Normal), but without adding antigen and IgE. Blank absorbance of the test material was measured to eliminate interference caused by the color of the test material itself. For this, only test material and substrate were added without adding cell extract (Blank). Thus, the inhibition % of the release of β-hexosaminidase by the test material was calculated by the following equation.

$$\text{inhibition (\%)}=\left(1-\frac{T-B-N}{C-N}\right)\times100$$

Control (C): antigen-IgE response was evoked without test sample; Test (T): antigen-IgE response was evoked in the presence of test sample; Blank (B): only test sample and substrate were added; Normal (N): antigen-IgE response was not evoked, test sample was not added.

In this condition, it was calculated that 40—50% 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.

β-Hexosaminidase Inhibitory Activity The cell suspension (5×10^7 cells) in 6 ml of PBS was sonicated. The solution was then centrifuged and the supernatant was diluted with siraganian buffer and adjusted to equal the enzyme activity of the degranulation test described above. The enzyme solution (45 μl) and test sample solution (5 μl) were transferred into a 96-well microplate and incubated with 50 μl of the substrate solution at 37 °C for 1 h. The reaction was stopped by adding 200 μl of the stop solution. The absorbance was measured by a microplate reader at 405 nm.

Statistics Values were expressed as means±S.E.M. One-way analysis of variance following Dunnett's test was used for statistical analysis.

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