Bioactive Constituents from Chinese Natural Medicines. XIV.[1] New Glycosides of β-Carboline-Type Alkaloid, Neolignan, and Phenylpropanoid from *Stellaria dichotoma* L. var. *lanceolata* and Their Antiallergic Activities

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A new β-carboline-type alkaloidal glycoside, glucodichotomine B, four new neolignan glycosides, dichotomosides A, B, C, and D, and a new phenylpropanoid glycoside, dichotomoside E, were isolated from a Chinese natural medicine, the roots of *Stellaria dichotoma* L. var. *lanceolata*. The structures of the new glycosides were determined on the basis of chemical and physicochemical evidence. Among them, dichotomoside D inhibited the release of β-hexosaminidase (IC_{50}=64 μM) as well as tumor necrosis factor-α and interleukin-4 (IC_{50}=16, 34 μM) in RBL-2H3 cells. These findings suggest that dichotomoside D is more effective against the late-phase reactions in type I allergy than in the immediate phase.

Key words *Stellaria dichotoma* L. var. *lanceolata*; glucodichotomine B; dichotomoside; degranulation inhibitor; antiallergy; neolignan

In the course of our studies on the bioactive constituents from Chinese natural medicines,[1—10] we previously reported the structures of six β-carboline-type alkaloids, dichotomines A (**7**), B (**8**), C (**9**), and D (**10**) and dichotomides I (**11**) and II (**12**) from the 95% aqueous ethanolic extract of the roots of *Stellaria dichotoma* L. var. *lanceolata* BGE. ("**銀柴胡**" in Chinese, Caryophyllaceae) together with 18 known constituents.[1] As a continuing study of this natural medicine, we additionally isolated a new β-carboline-type alkaloidal glycoside, glucodichotomine B (**1**), four new neolignan glycosides, dichotomosides A (**2**), B (**3**), C (**4**), and D (**5**), and a new phenylpropanoid glycoside, dichotomoside E (**6**). This paper deals with the isolation and structure elucidation of the new constituents (**1**—**6**). Furthermore, we describe the inhibitory effects of the new constituents on the release of β-hexosaminidase and also the inhibitory effects of dichotomoside D (**5**) on the releases of tumor necrosis factor (TNF)-α and interleukin (IL)-4 in RBL-2H3 cells.

The *n*-BuOH-soluble fraction obtained from the roots of *S. dichotoma* L. var. *lanceolata*, which was described previously,[1] was subjected to ordinary-phase [$CHCl_3$–MeOH–H_2O (50 : 3 : 1—10 : 3 : 1, lower layer—6 : 4 : 1, v/v/v)–MeOH] and reverse-phase column chromatography [MeOH–H_2O], and finally to HPLC [YMC-Pack ODS-5-A, 250×20 mm i.d., MeOH–H_2O] to give glucodichotomine B (**1**, 0.0014% from the natural medicine), and dichotomosides A (**2**, 0.0013%), B (**3**, 0.0004%), C (**4**, 0.0009%), D (**5**, 0.0002%), and E (**6**, 0.0009%).

Structure of Glucodichotomine B (1) Glucodichotomine B (**1**) was isolated as a yellow powder with negative optical rotation ($[\alpha]_D^{27}$ −28.2°) and was deduced to possess a nitrogen function based on TLC examination using Dragendorff's reagent. The IR spectrum of **1** showed an absorption band at 1761 cm^{-1} ascribable to the carbonyl function and strong absorption bands at 3420 and 1020 cm^{-1} suggestive of a glycosidic structure. In the positive-ion FAB-MS of **1**, a quasimolecular ion peak was observed at *m*/*z* 457 $(M+Na)^+$. The molecular formula $C_{20}H_{30}N_2O_9$ of **1** was determined from the quasimolecular ion peak observed in the FAB-MS and from high-resolution FAB-MS measurement. The ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (Table 1) spectra[11] of **1** showed signals assignable to a methylene and a methine bearing an oxygen function [δ 4.07 (m, 15-H_2), 5.42 (t, *J*=7.0 Hz, 14-H)], five aromatic protons [δ 7.32, 7.62 (both m, 6, 7-H), 7.70, 8.40 (both d, *J*=7.5 Hz, 8, 5-H), 8.88 (s, 4-H)], and an amino proton [δ 11.80 (br s, 9-NH)] together with a β-glucopyranosyl part [δ 4.46 (d, *J*=7.6 Hz, 1′-H)]. Acid hydrolysis of **1** with 1 M hydrochloric acid (HCl) liberated dichotomine B (**8**)[1] as its aglycon and D-glucose, which was identified in HPLC analysis using an optical rotation detector.[3,5—9] As shown in Fig. 1, the ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY) experiment on **1** indicated the presence of partial structures shown in bold and the heteronuclear multiple-bond correlations (HMBC) experiment were observed between the 1′-proton and 14-carbon. By comparison of the ^{13}C-NMR data for **1** with those for **8**,[1] a glycosylation shift[12] was observed around the 14-position. Thus the connectivity of the β-D-glucopyranosyl moiety in **1** was clarified. On the basis of the above-mentioned evidence, the structure of **1** was determined.

Structures of Dichotomosides A—E (2—6) Dichotomoside A (**2**) was obtained as a white powder with negative optical rotation ($[\alpha]_D^{27}$ −2.3°). The molecular formula of **2** was determined from the positive-ion FAB-MS and by high-resolution FAB-MS analysis to be $C_{26}H_{32}O_{13}$. In the UV spectrum of **2**, an absorption maxmum was observed at 284 (log ε 3.78) nm, while the IR spectrum of **2** showed absorption bands due to hydroxyl (3400 cm^{-1}), carboxyl (1718 cm^{-1}), and ether functions (1046 cm^{-1}) and aromatic rings (1585, 1458 cm^{-1}). The ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (Table 1) spectra[11] of **2** showed the presence of the following functions: four methylenes [δ 2.61 (m, 8, 8′-H_2), 2.86

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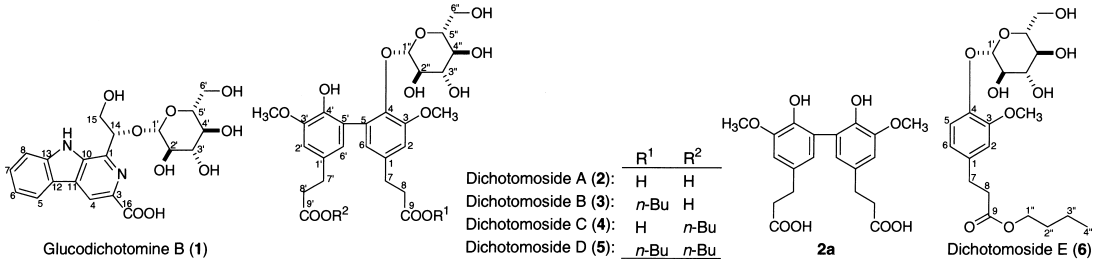

Chart 1

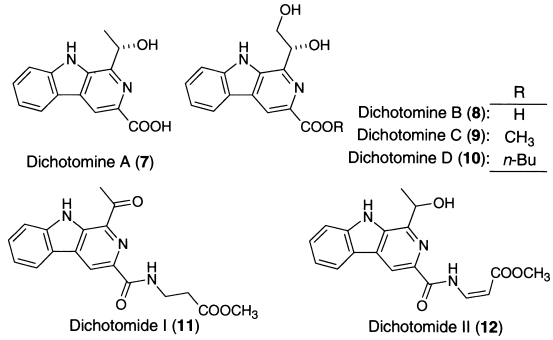

Chart 2

(m, 7, 7′-H_2)], two methoxyl [δ 3.86, 3.87 (both s, 3′, 3-OCH_3)], four aromatic protons [δ 6.68, 6.71 (both d, J=1.6 Hz, 6′, 6-H), 6.79, 6.90 (both d, J=1.6 Hz, 2′, 2-H)] together with an β-D-glucopyranosyl part [δ 4.74 (d, J=7.0 Hz, 1″-H)]. The acid hydrolysis of **2** liberated a new aglycon (**2a**) and D-glucose, which was identified by HPLC analysis using an optical rotation detector.[3,5—9] As shown in Fig. 1, the ^{1}H–^{1}H COSY experiment on **2** indicated the presence of the partial structures shown in bold and the HMBC experiment showed links between the following protons and carbons (2-H and 4, 6-C; 3-OCH_3 and 3-C; 6-H and 2, 4-C; 7-H_2 and 1, 2, 6, 9-C; 8-H_2 and 1, 9-C; 2′-H and 4′, 6′-C; 3′-OCH_3 and 3′-C; 6′-H and 2′, 4′-C; 7′-H_2 and 1′, 2′, 6′, 9′-C; 8′-H_2 and 1′, 9′-C; 1″-H and 4-C). Consequently, the positions of the methoxyl groups and the β-D-glucopyranoside linkage of **2** were clarified, and thus the structures of **2** and **2a** were determined as shown.

Dichotomosides B (**3**) and C (**4**) were also isolated as a white powder with positive optical rotation (**3**: $[\alpha]_D^{27}$ +8.4°, **4**: $[\alpha]_D^{27}$ +5.5°). In the positive-ion FAB-MS of **3** and **4**, a quasimolecular ion peak was observed at m/z 631 $(M+Na)^+$ and the molecular formula $C_{30}H_{40}O_{13}$ was determined by high-resolution MS measurement. The UV spectra of **3** and **4** showed absorption maxima at [275 (log ε 4.09) and 283 (3.89) nm], respectively, and the IR spectra showed absorption bands due to hydroxyl (**3**: 3468 cm^{-1}; **4**: 3400 cm^{-1}), ester carbonyl (**3**: 1734 cm^{-1}; **4**: 1734 cm^{-1}), carboxyl (**3**: 1718 cm^{-1}; **4**: 1718 cm^{-1}), and ether functions (**3**: 1040 cm^{-1}; **4**: 1040 cm^{-1}) and aromatic rings (**3**: 1560, 1458 cm^{-1}; **4**: 1561, 1474 cm^{-1}). The acid hydrolysis of **3** and **4** liberated D-glucose.[3,5—9] The ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 1) spectra[11] of **3** showed signals assignable to an n-butyl ester moiety [δ 0.90 (3H, t, J=7.1 Hz), 1.32, 1.56 (2H each, both m), 4.05 (2H, t, J=6.8 Hz), 9-OC_4H_9], four methylenes [δ 2.60, 2.63 (both m, 8′, 8-H_2), 2.83, 2.89 (both m, 7′, 7-H_2)], two methoxyl [δ 3.86, 3.87 (both s, 3′, 3-OCH_3)], an β-D-glucopyranosyl part [δ 4.74 (d, J=7.0 Hz, 1″-H)], and four aromatic protons [δ 6.67, 6.70 (both d, J=1.6 Hz, 6′, 6-H), 6.79, 6.88 (both d, J=1.6 Hz, 2′, 2-H)]. The proton and carbon signals of the ^{1}H- and ^{13}C-NMR spectra of **3** were superimposable on those of **2**, except for signals due to an n-butyl ester moiety. The structure of **3** was confirmed in ^{1}H–^{1}H COSY and HMBC experiments. Long-range correlations were observed between the 1‴-proton of the n-butyl ester moiety and the 9-carbon of the aglycon moiety, and between the 1″-proton of the β-D-glucopyranosyl moiety and the 4-carbon of the aglycon moiety. On the other hand, the proton and carbon signals in the ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 1) spectra[11] of **4** were found to be similar to those of **3** {**4**: an n-butyl ester [δ 0.91 (3H, t, J=7.3 Hz), 1.33, 1.58 (2H each, both m), 4.06 (2H, t, J=7.3 Hz), 9′-OC_4H_9], four methylenes [δ 2.59, 2.64 (both m, 8, 8′-H_2), 2.83, 2.88 (both m, 7′, 7-H_2)], two methoxyl [δ 3.87, 3.88 (both s, 3′, 3-OCH_3)], an β-D-glucopyranosyl part [δ 4.73 (d, J=7.0 Hz, 1″-H)], and four aromatic protons [δ 6.66, 6.71 (both d, J=1.6 Hz, 6′, 6-H), 6.78, 6.91 (both d, J=1.6 Hz, 2′, 2-H)]}. In the HMBC experiment on **4**, long-range correlations were observed between the 1‴-proton of the n-butyl ester moiety and the 9′-carbon of the aglycon moiety. On the basis of the above-mentioned evidence, the structures of **3** and **4** were elucidated as shown.[13]

Dichotomoside D (**5**) was isolated as a white powder with positive optical rotation ($[\alpha]_D^{27}$ +7.4°) and the molecular formulas of **5** was determined from the positive-ion FAB-MS and by high-resolution FAB-MS analysis to be $C_{34}H_{48}O_{13}$. The UV spectrum of **5** showed an absorption maximum at 283 (log ε 3.71) nm, and the IR spectrum of **5** showed an absorption band at 1732 cm^{-1} ascribable to the ester carbonyl function and strong absorption bands at 3350 and 1026 cm^{-1} suggestive of a glycosidic structure. The acid hydrolysis of **5** liberated D-glucose.[3,5—9] The ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (Table 1) spectra[11] of **5** showed signals assignable to two n-butyl ester moieties [δ 0.86 (6H, m), 1.31, 1.52 (4H each, both m), 4.00 (4H, t, J=6.8 Hz), 9, 9′-OC_4H_9], four methylenes [δ 2.62 (m, 8′, 8-H_2), 2.77 (m, 7′, 7-H_2)], two methoxyl [δ 3.78, 3.79 (both s, 3′, 3-OCH_3)], a β-D-glucopyranosyl part [δ 4.87 (d, J=7.0 Hz, 1″-H)], and four aromatic protons [δ 6.59, 6.77 (both d, J=1.6 Hz, 6, 6′-H), 6.76, 6.87 (both d, J=1.6 Hz, 2′, 2-H)]. In the HMBC experiment on **5**, long-range correlations were observed, as shown in Fig. 1. Consequently, the structure of **5** was determined as shown.[13]

Structure of Dichotomoside E (6) Dichotomoside E (**6**) was isolated as a white powder and its molecular formula $C_{20}H_{30}O_9$ was determined from a quasimolecular ion peak

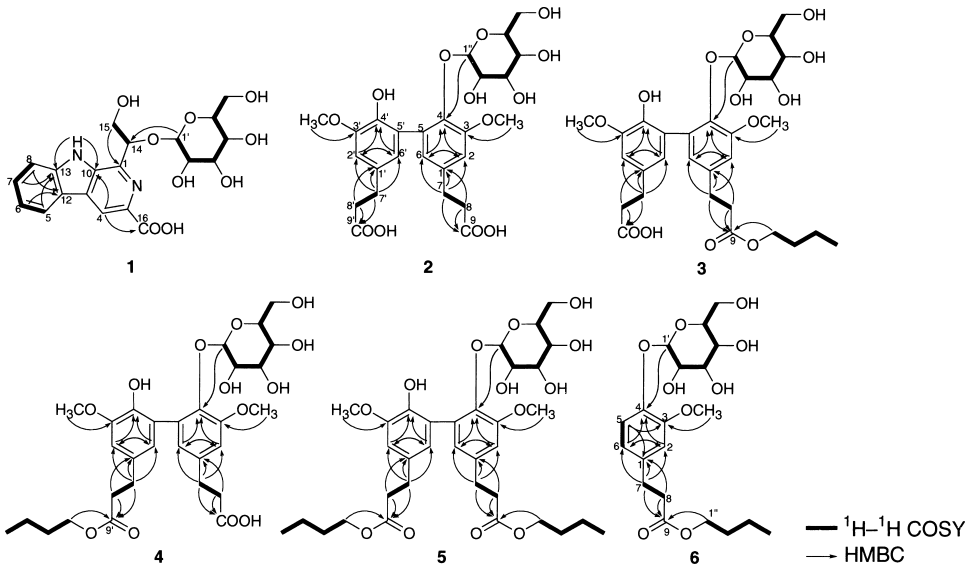

Fig. 1. ^{1}H–^{1}H COSY and HMBC Correlations of **1**—**6**

Table 1. ^{13}C-NMR Data on Glucodichotomine B (**1**), Dichotomosides A—E (**2**—**6**), and **2a**

	1[a]		**2**[b]	**2a**[b]	**3**[b]	**4**[b]	**5**[a]		**6**[b]
C-1	142.7	C-1	138.1	126.9	134.4	138.2	134.8	C-1	136.8
		C-2	113.1	123.9	113.1	113.2	112.2	C-2	113.8
C-3	136.2	C-3	153.1	149.2	153.1	153.1	151.3	C-3	150.5
C-4	116.4	C-4	142.4	142.7	142.4	142.4	140.5	C-4	146.2
C-5	121.8	C-5	134.4	133.0	134.5	134.4	132.1	C-5	118.1
C-6	120.0	C-6	124.5	124.2	124.5	124.5	123.2	C-6	121.6
C-7	128.5	C-7	31.7	31.7	31.8	31.7	30.0	C-7	31.6
C-8	112.4	C-8	36.6	37.0	36.7	36.8	34.8	C-8	36.9
		C-9	176.7	175.1	174.5	176.6	171.9	C-9	174.5
C-10	135.2	C-1′	132.6		132.6	132.4	129.7	3-$COOCH_3$	56.6
C-11	128.8	C-2′	111.6		111.6	111.6	110.4	9-$COOC_4H_9$	65.3
C-12	120.5	C-3′	149.0		149.0	149.1	147.1		31.8
C-13	140.9	C-4′	142.7		142.7	142.6	141.3		20.1
C-14	81.2	C-5′	127.4		127.3	127.4	125.5		14.0
C-15	64.4	C-6′	124.2		124.2	124.2	123.1	Glc	
C-16	166.6	C-7′	31.6		31.8	31.7	30.0	C-1′	102.9
Glc		C-8′	37.0		37.1	37.2	34.8	C-2′	74.8
C-1′	101.5	C-9′	176.5		176.7	174.8	172.0	C-3′	78.0
C-2′	73.8	3-$COOCH_3$	56.7	56.5	56.7	56.7	56.0	C-4′	71.2
C-3′	77.0	3′-$COOCH_3$	56.5		56.5	56.5	55.6	C-5′	77.7
C-4′	70.1	9-$COOC_4H_9$			65.3		63.3	C-6′	62.4
C-5′	76.3				31.7		30.1		
C-6′	61.0				20.1		18.5		
					14.0		13.4		
		9′-$COOC_4H_9$				65.3	63.2		
						31.8	30.1		
						20.2	18.5		
						14.1	13.3		
		Glc							
		C-1″	104.2		104.2	104.3	101.6		
		C-2″	75.4		75.4	75.5	73.7		
		C-3″	77.6		77.7	77.7	76.7		
		C-4″	71.1		71.2	71.2	69.7		
		C-5″	77.4		77.4	77.5	76.0		
		C-6″	62.5		62.5	62.5	60.8		

Measured in *a*) DMSO-d_6 and *b*) CD_3OD at 125 MHz.

observed in positive-ion FAB-MS *m/z* 437 $(M+Na)^+$ and by high-resolution MS measurement. The IR spectrum of **6** showed absorption bands at 3420, 1730, 1561, 1458, and 1040 cm^{-1}, suggesting the presence of hydroxyl, ester carbonyl, and ether functions and an aromatic ring. Acid hydrolysis of **6** liberated D-glucose.[3,5—9] The ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 1) spectra[11] of **6** showed signals assignable to a *n*-butyl ester moiety [δ 0.91 (3H, t, J=7.6 Hz),

1.35, 1.58 (2H each, both m), 4.05 (2H, t, J=6.5 Hz), 9-OC_4H_9], four methylenes [δ 2.60 (t, J=7.6 Hz, 8-H_2), 2.86 (t, J=7.6 Hz, 7-H_2)], a methoxyl [δ 3.83 (s, 3-OCH_3)], a β-D-glucopyranosyl part [δ 4.82 (d, J=7.0 Hz, 1′-H)], and three aromatic protons [δ 6.72 (dd, J=1.9, 8.4 Hz, 6-H), 6.86 (d, J=1.9 Hz, 2-H), 7.07 (d, J=8.4 Hz, 5-H)]. In the HMBC experiment of **6**, long-range correlations were observed between the following protons and carbons (2-H and 4, 6-C; 3-OCH_3 and 3-C; 5-H and 1, 3-C; 6-H and 2, 4-C; 7-H_2 and 1, 2, 6, 9-C; 8-H_2 and 1, 9-C; 1′-H and 4-C; 1″-H_2 and 9-H), and thus the stereostructure of **6** was determined as shown.

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.[14,15] 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 diarylheptanoids,[3,16] sesquiterpenes,[4] diterpenes,[17] flavonoids,[18] anthraquinones,[8] stilbenes,[10] phenanthrenes,[10] and phenylpropanoids.[19] In our continuous search for antiallergic principles from natural sources, we previously reported that the 95% aqueous ethanolic extract from the roots of *S. dichotoma* L. var. *lanceolata* showed an antiallergic effect on the ear passive cutaneous anaphylaxis (PCA) reactions in mice (*in vivo*) and inhibitory effects on the release of β-hexosaminidase induced by dinitrophenylated bovine serum albumin (DNP-BSA) in RBL-2H3 cells sensitized with anti-DNP IgE (*in vitro*).[1] Furthermore, a β-carboline-type alkaloid, dichotomine C (**9**), was isolated as one of the active constituents with an IC_{50} value of 62 μM for the release of β-hexosaminidase inhibitory activity. We examined the effects of additionally obtained constituents from this natural medicine on the release of β-hexosaminidase inhibitory activity (Table 2). As a result, a neolignan glycoside, dichotomoside D (**5**, IC_{50}=64 μM) showed inhibitory activity, and its activity was stronger than those of two antiallergic compounds, tranilast (492 μM) and ketotifen fumarate (216 μM).[1,18,19]

Recently, the biphasic allergic reaction mediated by antigen-IgE antibody has been reported. After challenge with an antigen, sensitized animals and atopic individuals exhibit early-phase responses, such as the appearance of wheals and flares on the skin and bronchoconstriction of the airways, and late-phase responses such as edema and erythema usually persist over a 6- to 24-h period at the site of challenge in the skin and airways.[20—23] The early-phase responses are mainly due to small-molecule chemical mediators (*e.g.*, histamine, serotonin) from mast cells. Mast cells also produce cytokines including TNF-α, IL-4, and IL-5, and these cytokines play an important role in the late-phase reactions.[20—23] From natural resources, several flavones[18] and phenylpropanoids[19] were reported to inhibit the release of TNF-α and IL-4. However, there have been no reports on the inhibitory effects of β-carboline-type alkaloids on the release of TNF-α and IL-4 from mast cells. In the present study, the effects of **5**, which exhibited inhibitory effects against the release of β-hexosaminidase, on the release of TNF-α and IL-4 in RBL-2H3 cells 4 h after challenge were examined. As a result, **5** inhibited the release of TNF-α and IL-4, with IC_{50} values of 16 and 34 μM, respectively. These findings suggest that dichotomoside D (**5**) is more effective against the late-phase reactions in type I allergy than in the early phase.

Table 2. Inhibitory Effects of Constituents from *S. dichotoma* L. var. *lanceolata* on the Release of β-Hexosaminidase from RBL-2H3 Cells

	Inhibition (%)	IC_{50} (μM)
	100 μM	
Glucodichotomine B (**1**)	3.2±1.1	
Dichotomoside A (**2**)	1.0±0.7	
Dichotomoside B (**3**)	8.2±2.3	
Dichotomoside D (**5**)	88.2±0.7**	64
Dichotomoside E (**6**)	5.7±3.1	
Tranilast	8.9±2.2	492
Ketotifen fumarate	19.1±1.3**	216

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 Dragendorff's reagent or 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Extraction and Isolation The 95% aqueous ethanolic extract (21.4%) from dried roots of *S. dichotoma* L. var. *lanceolata* (7.0 kg, purchased in Shenyang, Liaoning Province, China) was partitioned in an EtOAc–H_2O mixture and then the aqueous layer was extracted with *n*-BuOH to give the EtOAc-, *n*-BuOH-, and H_2O-soluble fractions (2.1, 6.7, 12.6%, respectively). Normal-phase silica gel column chromatography [3.0 kg, $CHCl_3$–MeOH–H_2O (50 : 3 : 1→10 : 3 : 1, lower layer→6 : 4 : 1, v/v/v)→MeOH] of the *n*-BuOH-soluble fraction (200 g) gave seven fractions [fr. 1 (30.3 g), 2 (28.7 g), 3 (30.8 g), 4 (29.5 g), 5 (24.8 g), 6 (23.1 g), and 7 (32.8 g)] as reported previously.[1] Fraction 3 (30.8 g) was subjected to reverse-phase silica gel column chromatography [Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh, 300 g), MeOH–H_2O (20 : 80→40 : 60→70 : 30, v/v)→MeOH] to furnish eight fractions [fr. 3-1 (3.2 g), 3-2 (3.4 g), 3-3 (4.2 g), 3-4 (4.2 g), 3-5 (4.4 g), 3-6 (5.5 g), 3-7 (3.7 g), 3-8 (2.2 g)]. Fraction 3-6 (5.5 g) was further purified by HPLC [YMC-Pack ODS-A (YMC Co., Ltd., Kyoto, Japan, 250×20 mm i.d.), MeOH–H_2O (50 : 50, v/v)] to give dichotomosides B (**3**, 12 mg, 0.0004%), C (**4**, 26 mg, 0.0009%), and E (**6**, 24 mg, 0.0009%). Fraction 5 (4.4 g) was subjected to reverse-phase silica gel column chromatography [300 g, MeOH–H_2O (20 : 80→40 : 60→70 : 30, v/v)→MeOH] to furnish nine fractions [fr. 5-1 (0.2 g), 5-2 (0.4 g), 5-3 (0.2 g), 5-4 (0.2 g), 5-5 (0.4 g), 5-6 (0.5 g), 5-7 (0.7 g), 5-8 (0.4 g), 5-9 (1.4 g)]. Fraction 5-4 (0.20 g) was further purified by HPLC [MeOH–H_2O (40 : 60, v/v)] to give glucodichotomine B (**1**, 39 mg, 0.0014%). Fraction 7 (32.8 g) was separated by reverse-phase silica gel column chromatography [1.0 kg, MeOH–H_2O (20 : 80→40 : 60→70 : 30, v/v)–MeOH] to furnish

eight fractions [fr. 7-1 (1.2 g), 7-2 (3.4 g), 7-3 (3.2 g), 7-4 (4.2 g), 7-5 (4.4 g), 7-6 (4.5 g), 7-7 (4.7 g), and 7-8 (7.2 g)]. Fraction 7-4 (4.2 g) was subjected to HPLC [MeOH–H_2O (30 : 70, v/v)] to furnish dichotomoside A (**2**, 36 mg, 0.0013%). Fraction 7-5 (4.4 g) was subjected to HPLC [MeOH–H_2O (30 : 70, v/v)] to furnish dichotomoside D (**5**, 6 mg, 0.0002%).

Glusodichotomine B (**1**): A yellow powder, Dragendorff's reagent positive, $[\alpha]_D^{27}$ −28.2° (c=0.20, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{20}H_{30}N_2O_9Na$ $(M+Na)^+$ 457.1223; Found 457.1231. UV [MeOH, nm (log ε)]: 218 (4.30), 239 (4.35), 270 (4.51). IR (KBr): 3420, 3300, 1716, 1635, 1508, 1020 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 4.07 (2H, m, 15-H_2), 4.46 (1H, d, J=7.6 Hz, 1′-H), 5.42 (1H, t, J=7.0 Hz, 14-H), 7.32, 7.62 (1H each, both m, 6, 7-H), 7.70, 8.40 (1H each, both d, J=7.5 Hz, 8, 5-H), 8.88 (1H, s, 4-H), 11.80 (1H, br s, 9-NH). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 457 $(M+Na)^+$.

Dichotomoside A (**2**): A white powder, $[\alpha]_D^{27}$ −2.3° (c=0.30, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{26}H_{32}O_{13}Na$ $(M+Na)^+$ 575.1741; Found 575.1747. UV [MeOH, nm (log ε)]: 284 (3.78). IR (KBr): 3400, 1718, 1585, 1458, 1046 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 2.61 (4H, m, 8, 8′-H_2), 2.86 (4H, m, 7, 7′-H_2), 3.86, 3.87 (3H each, both s, 3′, 3-OCH_3), 4.74 (1H, d, J=7.0 Hz, 1″-H), 6.68, 6.71 (1H each, both d, J=1.6 Hz, 6′, 6-H), 6.79, 6.90 (1H each, both d, J=1.6 Hz, 2′, 2-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 575 $(M+Na)^+$.

Dichotomoside B (**3**): A white powder, $[\alpha]_D^{27}$ +8.4° (c=0.50, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{30}H_{40}O_{13}Na$ $(M+Na)^+$ 631.2367; Found 631.2363. UV [MeOH, nm (log ε)]: 275 (4.09). IR (KBr): 3468, 1734, 1718, 1560, 1458, 1040 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: [0.90 (3H, t, J=7.1 Hz), 1.32, 1.56 (2H each, both m), 4.05 (2H, t, J=6.8 Hz), 9-OC_4H_9], 2.60, 2.63 (2H each, both m, 8′, 8-H_2), 2.83, 2.89 (2H each, both m, 7′, 7-H_2), 3.86, 3.87 (3H each, both s, 3′, 3-OCH_3), 4.74 (1H, d, J=7.0 Hz, 1″-H), 6.67, 6.70 (1H each, both d, J=1.6 Hz, 6′, 6-H), 6.79, 6.88 (1H each, both d, J=1.6 Hz, 2′, 2-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 631 $(M+Na)^+$.

Dichotomoside C (**4**): A white powder, $[\alpha]_D^{27}$ +5.5° (c=0.20, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{30}H_{40}O_{13}Na$ $(M+Na)^+$ 631.2367; Found 631.2363. UV [MeOH, nm (log ε)]: 283 (3.89). IR (KBr): 3400, 1734, 1718, 1561, 1474, 1040 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: [0.91 (3H, t, J=7.3 Hz), 1.33, 1.58 (2H each, both m), 4.06 (2H, t, J=7.3 Hz), 9′-OC_4H_9], 2.59, 2.64 (2H each, both m, 8, 8′-H_2), 2.83, 2.88 (2H each, both m, 7′, 7-H_2), 3.87, 3.88 (3H each, both s, 3′, 3-OCH_3), 4.73 (1H, d, J=7.0 Hz, 1″-H), 6.66, 6.71 (1H each, both d, J=1.6 Hz, 6′, 6-H), 6.78, 6.91 (1H each, both d, J=1.6 Hz, 2′, 2-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 631 $(M+Na)^+$.

Dichotomoside D (**5**): A white powder, $[\alpha]_D^{27}$ +7.4° (c=0.30, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{34}H_{48}O_{13}Na$ $(M+Na)^+$ 687.2993; Found 687.2996. UV [MeOH, nm (log ε)]: 283 (3.71). IR (KBr): 3350, 1732, 1587, 1464, 1026 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: [0.86 (6H, m), 1.31, 1.52 (4H each, both m), 4.00 (4H, t, J=6.8 Hz), 9, 9′-OC_4H_9], 2.62 (4H, m, 8′, 8-H_2), 2.77 (4H, m, 7′, 7-H_2), 3.78, 3.79 (3H each, both s, 3′, 3-OCH_3), 4.87 (1H, d, J=7.0 Hz, 1″-H), 6.59, 6.77 (1H each, both d, J=1.6 Hz, 6, 6′-H), 6.76, 6.87 (1H each, both d, J=1.6 Hz, 2′, 2-H). ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 687 $(M+Na)^+$.

Dichotomoside E (**6**): A white powder, $[\alpha]_D^{27}$ −29.5° (c=1.63, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{20}H_{30}O_9Na$ $(M+Na)^+$ 437.1788; Found 437.1794. UV [MeOH, nm (log ε)]: 275 (3.83). IR (KBr): 3420, 1730, 1561, 1458, 1040 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: [0.91 (3H, t, J=7.6 Hz), 1.35, 1.58 (2H each, both m), 4.05 (2H, t, J=6.5 Hz), 9-OC_4H_9], 2.60 (2H, t, J=7.6 Hz, 8-H_2), 2.86 (2H, t, J=7.6 Hz, 7-H_2), 3.83 (3H, s, 3-OCH_3), 4.82 (1H, d, J=7.0 Hz, 1′-H)], 6.72 (1H, dd, J=1.9, 8.4 Hz, 6-H), 6.86 (1H, d, J=1.9 Hz, 2-H), 7.07 (1H, d, J=8.4 Hz, 5-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: given in Table 1. Positive-ion FAB-MS: m/z 437 $(M+Na)^+$.

Acid Hydrolysis of 1—6 A solution of **1** (3.0 mg) or **2** (6.0 mg) in 1 M HCl (2.0 ml) was heated under reflux for 3 h. After cooling, the reaction mixture was extracted with EtOAc. The aqueous layer was subjected to HPLC analysis under the following conditions, respectively: HPLC column, Kaseisorb LC NH_2-60-5, 4.6 mm i.d.×250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; 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 an authentic sample. t_R: 12.3 min (D-glucose, positive optical rotation). The EtOAc layer was washed with 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, hexane–EtOAc (3 : 1, v/v)] to give dichotomine B (**8**, 1.5 mg, 84%) and **2a** (3.8 mg, 93%), respectively. Through a similar procedure, **3**—**6** (1.0 mg each) in 1 M HCl (0.5 ml) was heated under reflux for 3 h. After cooling, the reaction mixture was extracted with EtOAc and D-glucose was identified from the aqueous layer.

2a: A white powder. UV [MeOH, nm (log ε)]: 218 (4.55), 289 (3.80). IR (KBr): 3400, 1736, 1599, 1460 cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 2.63 (4H, m, 8, 8′-H_2), 2.87 (4H, m, 7, 7′-H_2), 3.87 (6H, s, 3, 3′-OCH_3), 6.66 (2H, d, J=1.6 Hz, 6, 6′-H), 6.79 (2H, d, J=1.6 Hz, 2, 2′-H). ^{13}C-NMR (125 MHz, CD_3OD) δ_C: given in Table 1. EI-MS (%): m/z 390 (M^+, 57), 154 (100).

Bioassay. 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 the Health Science Research Resources Bank, Osaka, Japan) were evaluated using a method reported previously.[3,4,8,10,16—19] 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% 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:

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

Where the control (C) is DNP-BSA (+), test sample (−); test (T), DNP-BSA (+), test sample (+); blank (B), DNP-BSA (−), test sample (+); and 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 based on the determination of the total β-hexosaminidase activity after sonication of the cell suspension.

Inhibitory Effect on Antigen-Induced TNF-α and IL-4 Release in RBL-2H3 Cells The inhibitory effects of test samples on the release of TNF-α and IL-4 in RBL-2H3 cells were evaluated using the method reported previously.[18,19] RBL-2H3 cells (2×10^5 cells/well) were sensitized with anti-DNP IgE as described above. The cells were washed twice with 500 μl of MEM containing 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml), and exchanged with 320 μl of fresh medium. Then 40 μl of test sample solution and 40 μl of antigen (DNP-BSA, final concentration was 10 μg/ml) were added to each well and incubated at 37 °C for 4 h. The supernatant (50 μl) was transferred into a 96-well ELISA plate, and TNF-α and IL-4 concentrations were determined using commercial kits (TNF-α, rat, ELISA system, code 3012, Biosource International Co., Ltd.; IL-4, rat, ELISA system, code 2737, Amersham Pharmacia Biotech Co., Ltd.). The test samples were dissolved in DMSO, and the solution was added to MEM (final DMSO concentration 0.1%). To estimate the release of TNF-α or IL-4 from cells, the same procedure was followed (normal), but without the addition of antigen. Thus the inhibition % of the release of TNF-α or IL-4 by the test sample was calculated using the following equation, and IC_{50} values were determined graphically:

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

Where the control (*C*) is DNP-BSA (+), test sample (−); test (*T*), DNP-BSA (+), test sample (+); and normal (*N*), DNP-BSA (−), test sample (−).

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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