Bioactive Constituents from Chinese Natural Medicines. XXVI.[1] Chemical Structures and Hepatoprotective Effects of Constituents from Roots of *Rhodiola sachalinensis*

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The methanolic extract from the roots of *Rhodiola sachalinensis* was found to show a protective effect on D-galactosamine-induced cytotoxicity in primary cultured mouse hepatocytes. From the methanolic extract, five new glycosides, two monoterpene glycosides, two flavonol bisdesmosides, and a cyanogenic glycoside, were isolated together with 34 known compounds. The structures of new constituents were elucidated on the basis of chemical and physicochemical evidence. In addition, the principal constituents, sachalosides III and IV, rhodiosin, and *trans*-caffeic acid, displayed hepatoprotective effects.

Key words Rhodiolae Radix; *Rhodiola sachalinensis*; sachaloside; flavonol bisdesmoside; cyanogenic glycoside; hepatoprotective effect

The roots of the alpine *Rhodiola* (Crassulaceae) plant, Rhodiolae Radix, have been widely used as a hemostatic, antibechic, tonic, and endermic liniment for burns and contusions in traditional Chinese medicine. Chemical and biological studies on several *Rhodiola* plants have been reported.[2—5] For example, the methanolic extract from the roots of *R. sachalinensis* was reported to show inhibitory activity on prolyl endopeptidase.[4] During the course of our serial studies on the bioactive constituents from Chinese natural medicines,[1,6—13] we have characterized the structures of several monoterpene oligoglycosides and cyanogenic glycosides from the roots of *R. quadrifida* (PALL.) FISCH. *et* MAY and *R. sacra* (PRAIN ex HAMET) S. H. FU.[14,15] Among the isolated constituents, monoterpene oligoglycosides, sacranosides A and B, were found to inhibit histamine release from rat exudate cells induced by an antigen–antibody reaction.[15] As a continuing study on the bioactive constituents of Rhodiolae Radix, the methanolic extract from the roots of *R. sachalinensis* A. BOR. was found to show a protective effect on D-galactosamine (D-GalN)-induced cytotoxicity in primary cultured mouse hepatocytes. From the methanolic extract, we have isolated two new monoterpene glycosides, sachalosides I and II, two new flavonol bisdesmosides, sachalosides III and IV, a new cyanogenic glycoside, sachaloside V, together with 34 known compounds. Furthermore, we examined the protective effects of principal constituents on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes. In this paper, we describe the isolation and structure elucidation of the new constituents (**1**—**5**) and the hepatoprotective effects of principal constituents from the roots of *R. sachalinensis*.

The roots of *R. sachalinensis* were extracted with methanol under reflux. The methanolic extract (14.6% from the roots) was partitioned into an EtOAc–H_2O mixture to furnish an EtOAc-soluble fraction (3.5%) and aqueous layer. The aqueous layer was further extracted with *n*-BuOH to give *n*-BuOH and H_2O-soluble fractions (4.4% and 6.5%, respectively). As shown in Table 1, the methanolic extract and the EtOAc- and *n*-BuOH-soluble fractions were found to show hepatoprotective effects, but the H_2O-soluble fractions showed weak effect. The EtOAc-soluble fraction was subjected to normal-phase and reversed-phase column chromatographies, and finally HPLC to give 13 known compounds, geranyl β-D-glucopyranoside (**6**, 0.0070%),[16] myrtenyl 10-*O*-β-D-glucopyranoside (**9**, 0.0054%),[15] *p*-tyrosol (**17**, 0.062%),[17] 3-phenylpropyl β-D-glucopyranoside (**21**, 0.052%),[18] *trans*-cinnamyl alcohol (**22**, 0.0065%),[19] *p*-coumaric acid (**26**, 0.017%),[20] *trans*-caffeic acid (**27**, 0.0011%),[21] *p*-hydroxyphenethyl anisate (**30**, 0.0003%),[22] eriodictyol (**32**, 0.019%),[23] luteolin (**33**, 0.0011%),[24] kaempferol (**34**, 0.023%),[25] tricin (**37**, 0.0069%),[26] 1-octyl-β-D-glucopyranoside (**38**, 0.0019%).[18] The *n*-BuOH-soluble fraction was also subjected to Diaion HP-20 column chromatography (H_2O→MeOH) to give the water- and methanol-eluted fractions (0.8% and 3.5%, respectively). The methanol-eluted fraction was subjected to normal- and reversed-phase column chromatographies, and finally HPLC to give sachalosides I (**1**, 0.0004%), II (**2**, 0.0005%), III (**3**, 0.0003%), IV (**4**, 0.0004%), and V (**5**, 0.0007%), together with 21 known compounds, geranyl 1-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**7**, 0.0029%),[27] kenposide A (**8**, 0.0028%),[28] sacranoside A (**10**, 0.0011%),[15] rhodiocyanoside A (**11**, 0.23%),[14] lotaustralin (**12**, 0.0014%),[29] hetorodendrin (**13**, 0.0002%),[30] benzyl β-D-glucopyranoside (**14**, 0.0022%),[31] phenylmethyl 6-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside (**15**, 0.0004%),[32] benzylalcohol 7-*O*-α-L-arabinopyranosyl(1→6)-β-D-glucopyranoside (**16**, 0.0002%),[33] 2-phenylethyl β-D-glucopyranoside (**18**, 0.0022%),[34] salidroside (**19**, 0.10%),[35] 2-(4-methoxyphenyl)-ethyl β-D-glucopyranoside (**20**, 0.0004%),[36] *trans*-cinnamyl β-D-glucopyranoside (**23**, 0.0046%),[37] rosarin (**24**, 0.0097%),[38] rosavin (**27**, 0.034%),[39] eugenyl *O*-β-D-apiofuranosyl(1″→6′)-β-D-glucopyranoside (**28**, 0.0001%),[40] gein (**29**, 0.0007%),[41] nodakenin (**31**, 0.0021%),[42] leucoside (**35**, 0.0028%),[32] rhodiosin (**36**, 0.014%),[43] 1-octyl-α-D-arabinofuranosyl(1→6)-β-D-glucopyranoside (**39**, 0.0009%).[44]

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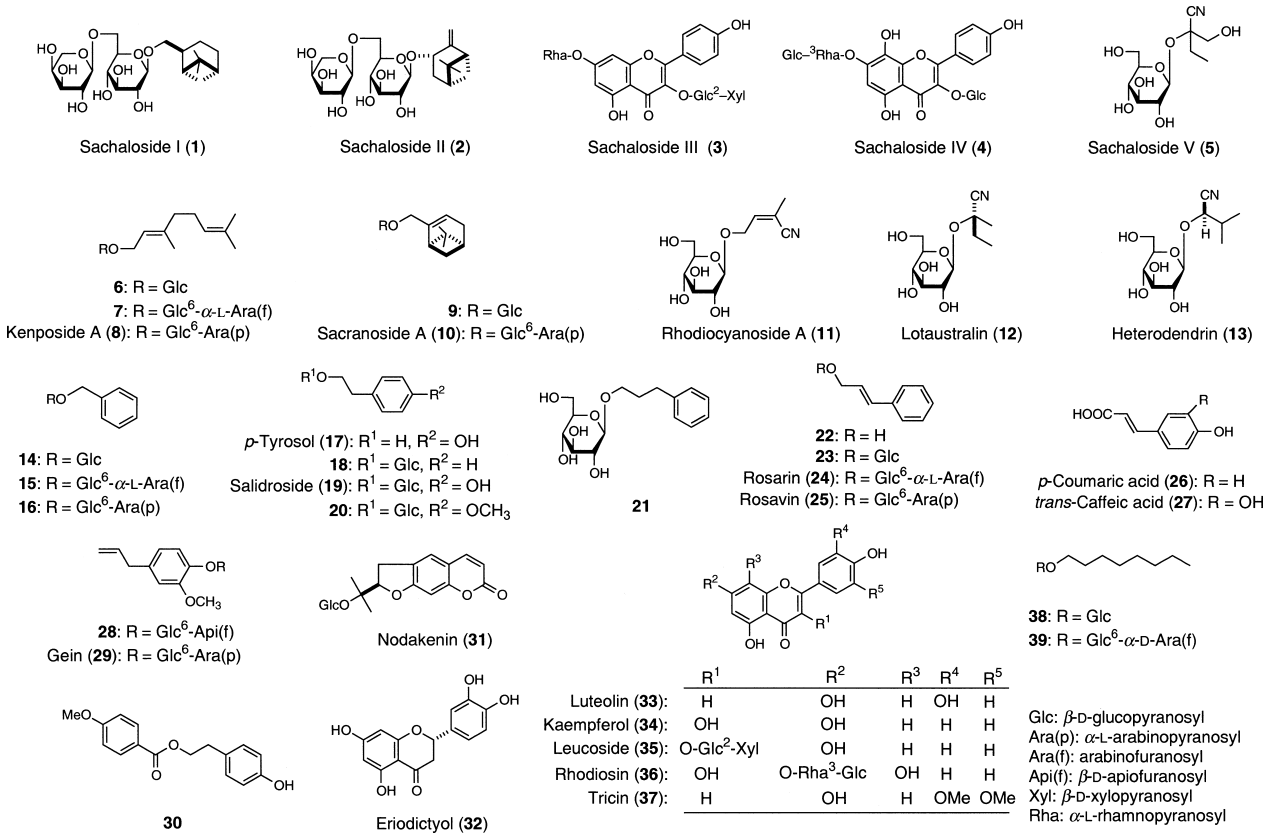

Chart 1

Table 1. Inhibitory Effects of Methanolic Extract and Its Fractions from *R. sachalinensis* on D-GalN-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes[a)]

	Inhibition (%)				
	0 μg/ml	3 μg/ml	10 μg/ml	30 μg/ml	100 μg/ml
MeOH ext.	0.0±3.2	16.7±3.9*	30.8±5.8**	51.1±2.0**	98.8±4.1**
EtOAc soluble fraction	0.0±5.2	49.2±4.1**	73.3±3.3**	89.4±1.6**	124.0±11.7**
n-BuOH-soluble fraction	0.0±6.9	10.3±5.0	22.8±8.1	69.7±12.1**	68.8±4.8**
H_2O-soluble fraction	0.0±5.2	7.3±2.2	10.2±7.7	20.8±5.0	34.7±3.4**

a) Each value represents the mean±S.E.M. (n=4). Significantly different from the control, * $p<0.05$, ** $p<0.01$.

Sachaloside I (**1**), obtained as colorless viscous oil with a negative optical rotation ($[\alpha]_D^{27}$ −33.6° in MeOH), showed absorption bands at 3503 and 1043 cm^{-1} assignable to hydroxyl and ether functions in the IR spectrum. In the positive-ion and negative-ion fast atom bombardment (FAB)-MS of **1**, quasimolecular ion peaks were observed at *m/z* 471 $(M+Na)^+$ and *m/z* 447 $(M-H)^-$, respectively. The high-resolution (HR)-MS analysis revealed the molecular formula of **1** to be $C_{21}H_{36}O_{10}$. The acid hydrolysis of **1** liberated L-arabinose and D-glucose, which was identified by HPLC analysis using an optical rotation detector.[1,9,10,45)] The ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 1) spectra[46)] of **1** showed signals assignable to a monoterpene moiety [δ 0.86, 2.29 (1H each, both m, H_2-7), 0.91, 1.10 (3H each, both s, H_3-8, 9), 1.43, 1.83 (1H each, both m, H_2-3), 1.79, 1.88 (1H each, both m, H_2-4), 1.80 (1H, m, H-5), 1.99 (1H, m, H-1), 2.30 (1H, m, H-2), 3.41, 3.71 (1H each, both m, H_2-10)], a β-D-glucopyranosyl moiety [δ 4.12 (1H, d, J=7.6 Hz, H-1′)], and a α-L-arabinopyranosyl moiety [δ 4.21 (1H, d, J=6.9 Hz, H-1″)]. The proton and carbon signals due to the 10-*O*-glycoside moiety in the ^{1}H- and ^{13}C-NMR spectra of **1** were similar to those of sacranoside A (**10**),[15)] whereas the proton and carbon signals due to the monoterpene part were superimposable on those of L-*cis*-myrtanol-β-D-glucopyranoside.[47)] The aglycon part of **1** was identified to be *cis*-myrtanol from the following physicochemical evidence. As shown in Fig. 1, the double quantum filter correlation spectroscopy (DQF COSY) experiment on **1** indicated the presence of partial structures written in bold lines, and in the heteronuclear multiple-bond correlations (HMBC) experiment, long-range correlations were observed between the following protons and carbons: H-1 and C-7, 10; H-2 and C-6, 7, 10; H_2-3 and C-4, 5, 10; H-5 and C-3, 4, 7; H_2-7 and C-1, 5, 6; H_3-8 and C-1, 5, 6, 9; H_3-9 and C-1, 5, 6, 8; H_2-10 and C-1, 2, 3, 1′; H-1′ and C-10.

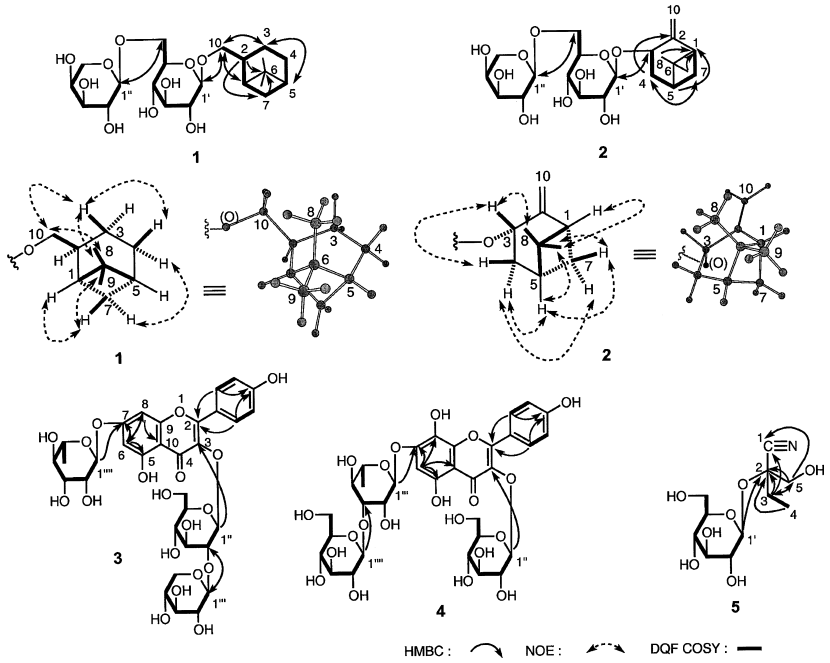

Fig. 1. Selected HMBC and NOE Correlations

Table 2. ^{13}C-NMR (125 MHz) Data for **1**—**2**, **5** (Methanol-d_4) and **3**, **4** (DMSO-d_6)

Carbon	**1**	**2**	**3**	**4**	**5**	Carbon	**1**	**2**	**3**	**4**
1	44.5	52.1			119.4	1″	104.5	102.3	97.8	100.7
2	42.8	152.8	155.7	156.6	81.6	2″	75.0	75.0	81.7	74.1
3	20.0	73.7	133.1	133.2	29.5	3″	77.9	78.2	77.5	76.3
4	27.0	31.3	177.5	177.9	8.5	4″	71.6	71.7	69.5	69.8
5	42.6	41.3	160.8	151.9	66.1	5″	76.8	77.0	76.7	77.4
6	39.6	41.6	99.2	98.9		6″	69.4	69.4	60.4	60.7
7	34.0	28.5	161.4	149.8		1‴	105.0	105.1	104.4	98.9
8	23.9	22.5	94.2	127.2		2‴	72.3	72.4	73.8	68.8
9	28.4	26.4	155.8	144.4		3‴	74.1	74.2	76.1	80.8
10	76.0	114.3	105.5	105.6		4‴	69.4	69.4	69.3	70.3
1′	104.5	102.3	120.6	120.9	101.5	5‴	66.6	66.7	65.7	69.3
2′	75.0	75.0	131.0	131.0	74.9	6‴				17.7
3′	77.9	78.2	115.1	115.0	77.9	1⁗			98.3	104.3
4′	71.6	71.7	160.5	160.0	71.2	2⁗			69.7	73.9
5′	76.8	77.0	115.1	131.0	78.0	3⁗			70.1	76.1
6′	69.4	69.4	131.0	115.0	62.4	4⁗			71.5	69.8
						5⁗			70.0	76.7
						6⁗			17.8	60.9

Those findings led us to confirm the planar structure of the monoterpene part and the diglycoside structure of **1**. The relative stereostructure of the monoterpene part in **1** was characterized by nuclear Overhauser enhancement spectroscopy (NOESY) experiment, which showed NOE correlations between the following proton pairs: H-3β and H-4β, H_3-8, H_2-10; H-4α and H-7α; H-7β and H-1, H_3-9; H_3-8 and H_2-10. On the basis of this evidence, the structure of sachaloside I was determined as shown.

Sachaloside II (**2**), obtained as colorless viscous oil with a negative optical rotation ($[\alpha]_D^{27}$ −47.6° in MeOH), showed absorption bands at 3524, 1635 and 1076 cm^{-1} assignable to hydroxyl, olefin, and ether functions in the IR spectrum. In the positive-ion and negative-ion FAB-MS of **2**, quasimolecular ion peaks were observed at *m*/*z* 469 $(M+Na)^+$ and *m*/*z* 445 $(M-H)^-$, respectively. The HR-MS analysis revealed the molecular formula of **2** to be $C_{21}H_{34}O_{10}$. The acid hydrolysis of **2** liberated L-arabinose and D-glucose, which were identified by HPLC analysis.[1,9,10,45] The ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 1) spectra[46] of **2** showed signals assignable to a monoterpene moiety [δ 0.67, 1.27 (3H each, both s, H_3-8, 9), 1.70, 2.37 (1H each, both m, H_2-7), 1.97 (1H, m, H-5), 2.04, 2.22 (1H each, both m, H_2-4), 2.46 (1H, m, H-1), 4.56 (1H, br d, J=7.6 Hz, H-3), 4.80, 5.11 (1H each, both br s, H_2-10)], a β-D-glucopyranosyl moiety [δ 4.41 (1H, d, J=7.5 Hz, H-1′)], and a α-L-arabinopyranosyl moiety [δ 4.37 (1H, d, J=7.0 Hz, H-1″)]. The proton and carbon signals due to the 10-*O*-glycoside moiety in the ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of sacranoside A (**10**)[15] and sachaloside I (**1**), while the signals due to the monoterpene part resembled those of *trans*-pinocarveol.[48] As shown in Fig. 1, long-range correlations in the HMBC experiment on **2** were

observed between the following proton and carbon: H-1 and C-3, 7, 10; H-3 and C-1, 10, 1′; H_2-4 and C-2, 3, 5, 7; H-5 and C-1, 6, 8, 9; H_2-7 and C-1, 5; H_3-8 and C-1, 6, 9; H_3-9 and C-1, 6, 8; H_2-10 and C-1, 3; H-1′ and C-3, so that the planar structure of the monoterpene part and the diglycoside structure were clarified. The relative stereostructure of the monoterpene part in **2** was characterized by NOESY experiment, which showed NOE correlation between the following proton pairs: H-1 and H-7β, H_3-9; H-3β and H-4β, H_3-8; H-5 and H-4α, H-7β, H_3-9; H-7β and H_3-9. On the basis of this evidence, the structure of sachaloside II was determined as shown.

Sachaloside III (**3**) was obtained as a yellow amorphous powder with a negative optical rotation ($[\alpha]_D^{26}$ −74.9° in MeOH). The IR spectrum of **3** showed absorption bands at 3400, 1655, 1072 cm^{-1} ascribable to hydroxyl, α,β-unsaturated carbonyl, and ether functions. The positive-ion and negative-ion FAB-MS of **3** exhibited quasimolecular ion peaks at *m/z* 749 $(M+Na)^+$ and *m/z* 725 $(M-H)^-$, respectively and the fragment ion peak was observed at *m/z* 593 $(M-C_5H_9O_4)^-$ in the negative-ion FAB-MS. The molecular formula $C_{32}H_{38}O_{19}$ of **3** was determined from the quasimolecular ion peaks and by HR-MS measurement. Acid hydrolysis of **3** with 1.0 M aqueous HCl liberated D-glucose, L-rhamnose and D-xylose, which were identified by HPLC analysis.[1,9,10,45] Enzymatic hydrolysis of **3** with narigninase gave a kaempferol (**34**)[25] as the aglycon. The ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (Table 1) spectra of **3**, which were assigned by various NMR experiments,[46] showed signals assignable to *meta*-coupled and *ortho*-coupled A_2B_2-type aromatic protons [δ 6.43, 6.83 (each 1H, both d, J=2.2 Hz, H-6, 8), 6.90, 8.14 (2H each, both d, J=8.9 Hz, H-3′,5′, 2′,6′)], a β-D-glucopyranosyl part [δ 5.71 (1H, d, J=7.3 Hz, H-1″)], an α-L-rhamnopyranosyl part [δ 1.12 (1H, d, J=6.1 Hz, H-6‴), 5.56 (1H, d, J=1.9 Hz, H-1⁗)], and a β-D-xylopyranosyl part [δ 4.60 (1H, d, J=7.4 Hz, H-1‴)]. The proton and carbon signals due to the kaempferol moiety including the 7-*O*-β-D-rhamnopyranosyl part in the ^{1}H- and ^{13}C-NMR spectra of **3** were similar to those of kaempferol 3,7-di-*O*-α-L-rhamnopyranoside,[49] while the proton and carbon signals due to the 3-*O*-glycoside moiety was similar to those of leucoside (**35**).[32] Furthermore, the structure of **3** was characterized by means of DQF COSY and HMBC experiments (Fig. 1). Long-range correlations in the HMBC experiment on **3** were observed between the following proton and carbon: H-1″ and C-3; H-1‴ and C-2″; H-1⁗ and C-7. On the basis of the above-mentioned evidence, the structure of sachaloside III was determined to be kaempferol 3-*O*-β-D-xylopyranosyl(1→2)-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**3**).

Sachaloside IV (**4**) was obtained as a yellow amorphous powder with a negative optical rotation ($[\alpha]_D^{24}$ −35.9° in MeOH). The IR spectrum of **4** showed absorption bands at 3400, 1655, 1034 cm^{-1} ascribable to hydroxyl, α,β-unsaturated carbonyl, and ether functions. The positive-ion and negative-ion FAB-MS of **4** exhibited quasimolecular ion peaks at *m/z* 795 $(M+Na)^+$ and *m/z* 771 $(M-H)^-$, respectively and the fragment ion peak was observed at m/*z* 608 $(M-C_6H_{11}O_5)^-$ in the negative-ion FAB-MS. The molecular formula $C_{33}H_{40}O_{21}$ of **4** was determined from the quasimolecular ion peaks and by HR-MS measurement. Acid hydrolysis of **4** with 1.0 M aqueous HCl liberated D-glucose and L-rhamnose, which were identified by HPLC analysis.[1,9,10,45] The ^{1}H-NMR (DMSO-d_6) and ^{13}C-NMR (Table 1) spectra of **4**, which were assigned by various NMR experiments,[46] showed signals assignable to a flavonol moiety [δ 6.63 (1H, s, H-6), 6.90, 8.13 (2H each, both d, J=8.9 Hz, H-3′,5′, 2′,6′)], together with two β-D-glucopyranosyl and an α-L-rhamnopyranosyl parts [δ 1.25 (1H, d, J=6.1 Hz, H-6‴), 4.99 (1H, d, J=7.9 Hz, H-1⁗), 5.56 (1H, d, J=1.9 Hz, H-1‴), 5.47 (1H, d, J=7.4 Hz, H-1″)]. The proton and carbon signals due to the flavonol moiety including the 7-*O*-glycoside moiety in the ^{1}H- and ^{13}C-NMR spectra of **4** were similar to those of 8-hydroxykaempferol glycoside, rhodiosin (**36**),[43] except for the signals due to the 3-*O*-β-D-glucopyranoside moiety of **4**, while the proton and carbon signals due to the 3-*O*-β-D-glucopyranoside moiety was similar to those of kaempferol 3-*O*-β-D-glucopyranoside.[50] The 8-hydroxykaempferol 3,7-diglycoside structure of **4** was characterized by means of DQF COSY and HMBC experiments, which showed long-range correlations between the following proton and carbon: H-1″ and C-3; H-1‴ and C-7; H-1⁗ and C-3‴ (Fig. 1). On the basis of the above-mentioned evidence, the structure of sachaloside IV was determined to be 8-hydroxykaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranoside (**4**).

Sachaloside V (**5**), obtained as a colorless amorphous powder with a negative optical rotation ($[\alpha]_D^{27}$ −27.4° in MeOH), showed absorption bands at 3450, 2250 and 1074 cm^{-1} assignable to hydroxyl, nitrile, and ether functions in the IR spectrum. The molecular formula $C_{11}H_{19}NO_7$ was determined from the positive-ion FAB-MS data [*m/z* 300 $(M+Na)^+$] and by HR-FAB-MS measurement. The acid hydrolysis of **5** liberated D-glucose, which were identified by HPLC analysis.[1,9,10,45] The ^{1}H-NMR (CD_3OD) and ^{13}C-NMR (Table 1) spectra[46] of **5** showed signals assignable to an aglycon moiety [δ 1.08 (3H, t, J=7.3 Hz, H_3-4), 1.95 (2H, q, J=7.3 Hz, H_2-3), 3.76 (2H, br s, H_2-5), 119.4 (C-1)] and a β-D-glucopyranosyl moiety [δ 4.71 (1H, d, J=7.7 Hz, H-1′)]. The proton and carbon signals of **5** in the ^{1}H- and ^{13}C-NMR spectra were similar to those of lotaustralin (**12**),[29] except for the signals due to the hydroxymethyl moiety in **5**. The structure of **5** was characterized by means of DQF COSY and HMBC experiments (Fig. 1). That is, long-range correlations in the HMBC experiment on **5** were observed between the following proton and carbon: H-3 and C-1, 2, 5; H-4 and C-2; H-5 and C-1, 2, 3; H-1′ and C-2. On the basis of this evidence, the structure of sachaloside V was determined as shown.[51]

Previously, we have reported the isolation and structure elucidation of several constituents with hepatoprotective effects from Chinese natural medicines, *Bupleurum scorzonerifolium*,[52,53] *Panax notoginseng*,[54] and *Sedum sarmentosum*.[1] Since the methanolic extract from the roots of *R. sachalinensis* was found to show protective effects on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes, the activities of the major isolated constituents were examined. As shown in Table 3, sachaloside III (**3**), sachaloside IV (**4**), *trans*-caffeic acid (**27**), and rhodiosin (**36**) were found to show the hepatoprotective effects. The effects of **3** and **4** were equivalent to that of the hepatoprotective agent silybin,[55] and the effect of **36** was equivalent to that of eriodictyol (**32**), which was already known to display hepatoprotective effect.[56] On the other hand, the effects of **27** were

Table 3. Inhibitory Effects of Constituents from *R. sachalinensis* on D-GalN-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes[a)]

	Inhibition (%)				
	0 μM	3 μM	10 μM	30 μM	100 μM
Sachaloside III (**3**)	0.0±1.3	9.4±2.6	25.0±4.9**	41.7±8.4**	68.1±3.5**
Sachaloside IV (**4**)	0.0±3.2	11.1±3.6	22.4±9.3	28.6±6.4*	84.1±4.7**
Geranyl β-D-glucoside (**6**)	0.0±1.0	1.6±1.4	1.7±0.9	2.0±0.4	4.1±1.3
Myrtenyl *O*-β-D-glucopyranoside (**9**)	0.0±1.8	−2.0±0.8	−1.9±1.3	4.8±1.0	10.1±0.7
Rhodiocyanoside A (**11**)	0.0±0.8	1.0±0.8	0.0±0.7	0.7±1.6	−1.8±0.4
Lotaustralin (**12**)	0.0±1.2	−1.3±0.7	−0.8±0.9	3.8±4.2	5.6±2.5
Heterodendrin (**13**)	0.0±0.7	2.2±0.8	1.4±0.9	0.7±0.8	0.2±2.4
p-Tyrosol (**17**)	0.0±0.3	3.9±2.8	2.8±7.0	6.2±0.8*	8.6±0.4**
Salidroside (**19**)	0.0±0.4	0.2±2.1	0.6±1.2	−0.9±0.5	−1.3±1.9
Rosarin (**24**)	0.0±1.5	−1.2±0.9	1.0±1.0	1.8±0.8	5.9±1.4**
p-Coumaric acid (**26**)	0.0±0.2	5.2±1.8	6.9±2.0	5.7±0.9	7.2±0.5*
trans-Caffeic acid (**27**)	0.0±1.2	3.3±0.7	9.8±1.5	14.5±1.3**	30.9±1.2**
Eriodictyol (**32**)	0.0±2.2	0.3±7.0	4.9±0.8	15.3±1.5**	52.2±2.2**
35	0.0±1.6	1.1±0.7	3.2±0.6	6.0±0.3	17.4±4.5**
Rhodiosin (**36**)	0.0±1.2	10.4±0.8**	17.4±0.4**	23.2±0.9**	49.6±0.9**
Silybin[b,55)]	0.0±0.3	4.8±1.1	7.7±0.7	45.2±8.8**	77.0±5.5**

a) Each value represents the mean±S.E.M. (n=4). Significantly different from the control, $*p<0.05$, $**p<0.01$. *b*) Commercial silybin (Funakoshi Co., Ltd., Tokyo, Japan) was used as a reference compound.

stronger than those of *p*-coumaric acid (**26**) and *p*-tyrosol (**17**). These results suggested that the pyrocatechol moiety of **27** was essential to exerting the effect. Monoterpenes, **6** and **9**, and cyanogenic glycosides, **12** and **13**, were found to lack the activities.

Experimental

General Experimental Procedures The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); IR spectra, Shimadzu FTIR-8100 spectrometer; EI-MS and HR-EI-MS, JEOL JMS-GCMATE mass spectrometer; FAB-MS and HR-FAB-MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JEOL EX-270 (270 MHz), JNM-LA500 (500 MHz), and JEOL ECA-600K (600 MHz) spectrometers; ^{13}C-NMR spectra, JEOL EX-270 (68 MHz) JNM-LA500 (125 MHz), and JEOL ECA-600K (150 MHz) spectrometers with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index detector; and HPLC column, YMC-Pack ODS-A (YMC, Inc., 250×4.6 mm i.d.) and (250×20 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following experimental materials were used for chromatography: normal-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); Diaion HP-20 column chromatography (Nippon Rensui); 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) (reversed phase); reversed-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.

Plant Material The dried roots of *R. sachalinensis* were collected at Jilin province of China in 2005 and identified by one of authors (M.Y.). A voucher of the plant is on file in our laboratory (2006. China-06).

Extraction and Isolation The dried roots of *R. sachalinensis* (10.0 kg) were powdered and extracted 3 times with methanol under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a methanolic extract (1457 g, 14.6% from the dried roots), and an aliquot (1395 g) was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish an EtOAc-soluble fraction (336 g, 3.5%) and an aqueous phase. The aqueous phase was further extracted with *n*-BuOH to give an *n*-BuOH-soluble fraction (418 g, 4.4%) and an H_2O-soluble fraction (620 g, 6.5%). The EtOAc fraction (139 g) was subjected to ordinary-phase silica gel column chromatography [3.0 kg, $CHCl_3$→$CHCl_3$–MeOH (20 : 1)→$CHCl_3$–MeOH–H_2O (15 : 3 : 1, lower layer→10 : 3 : 1, lower layer→7 : 3 : 1, lower layer)→MeOH] to give six fractions [Fr. 1 (18.0 g), Fr. 2 (38.7 g), Fr. 3 (19.8 g), Fr. 4 (13.1 g), Fr. 5 (15.1 g), Fr. 6 (17.4 g)]. Fraction 2 (16.8 g) was subjected to ordinary-phase silica gel column chromatography [450 g, *n*-hexane–EtOAc (20 : 1→10 : 1→5 : 1→2 : 1→1 : 1, v/v)→$CHCl_3$–MeOH–H_2O (20 : 3 : 1, lower layer)] to give eight fractions [Fr. 2-1 (117 mg), Fr. 2-2 (1681 mg), Fr. 2-3 (4256 mg), Fr. 2-4 (2233 mg), Fr. 2-5 (217 mg), Fr. 2-6 (2385 mg) , Fr. 2-7 (3549 mg) , Fr. 2-8 (1005 mg)]. Fr. 2-4 (2233 mg) was subjected to reversed-phase silica gel column chromatography [100 g, MeOH–H_2O (50 : 50→60 : 40→70 : 30→90 : 10, v/v)→MeOH] to give five fractions [Fr. 2-4-1 (904 mg), Fr. 2-4-2 (61 mg), Fr. 2-4-3 (27 mg), Fr. 2-4-4 (233 mg), Fr. 2-4-5 (524 mg)]. Fraction 2-4-1 (904 mg) was further purified by HPLC [MeOH–H_2O (60 : 40, v/v)] to give *trans*-cinnamyl alcohol (**22**, 187 mg, 0.0065%). Fraction 2-4-2 (61 mg) was further purified by HPLC [MeOH–H_2O (60 : 40, v/v)] to give *p*-hydroxyphenethyl anisate (**30**, 8.4 mg, 0.0003%). Fr. 2-7 (3549 mg) was subjected to reversed-phase silica gel column chromatography [180 g, MeOH–H_2O (50 : 50→60 : 40→70 : 30→90 : 10, v/v)→MeOH] to give seven fractions [Fr. 2-7-1 (866 mg), Fr. 2-7-2 (483 mg), Fr. 2-7-3 (37 mg), Fr. 2-7-4 (175 mg), Fr. 2-7-5 (107 mg), Fr. 2-7-6 (89 mg), Fr. 2-7-7 (166 mg). Fr. 2-7-7 (197.9 mg) was identified as tricin (**37**, 198 mg, 0.0069%). Fraction 3 (19.7 g) was subjected to reversed-phase silica gel column chromatography [600 g, MeOH–H_2O (30 : 70→40 : 60→50 : 50→60 : 40→70 : 30→90 : 10, v/v)→MeOH] to give ten fractions [Fr. 3-1 (267 mg), Fr. 3-2 (3085 mg), Fr. 3-3 (599 mg), Fr. 3-4 (1310 mg), Fr. 3-5 (4701 mg), Fr. 3-6 (889 mg), Fr. 3-7 (359 mg), Fr. 3-8 (807 mg), Fr. 3-9 (738 mg), Fr. 3-10 (4984 mg)]. Fr. 3-2 (277 mg) was further purified by HPLC [MeOH–H_2O (30 : 70, v/v)] to give *p*-tyrosol (**17**, 144 mg, 0.062%). Fr. 3-4 (165 mg) was further purified by HPLC [MeOH–H_2O (35 : 65, v/v)] to give *p*-coumaric acid (**26**, 77 mg, 0.017%). Fr. 3-6 (260 mg) was further purified by HPLC [MeOH–H_2O (50 : 50, v/v)] to give eriodictyol (**32**, 48 mg, 0.019%). Fr. 3-7 (294 mg) was further purified by HPLC [MeOH–H_2O (50 : 50, v/v)] to give luteolin (**33**, 21 mg, 0.0011%). Fr. 3-8 (166 mg) was further purified by HPLC [MeOH–H_2O (55 : 45, v/v)] to give kaempferol (**34**, 28 mg, 0.023%). Fraction 4 (13.0 g) was subjected to reversed-phase silica gel column chromatography [450 g, MeOH–H_2O (30 : 70→40 : 60→50 : 50→60 : 40→70 : 30→90 : 10, v/v)→MeOH] to give twelve fractions [Fr. 4-1 (1157 mg), Fr. 4-2 (241 mg), Fr. 4-3 (689 mg), Fr. 4-4 (538 mg), Fr. 4-5 (752 mg), Fr. 4-6 (2058 mg), Fr. 4-7 (540 mg), Fr. 4-8 (479 mg), Fr. 4-9 (1701 mg), Fr. 4-10 (976 mg), Fr. 4-11 (248 mg), Fr. 4-12 (2391 mg)]. Fr. 4-5 (234 mg) was further purified by HPLC [MeOH–H_2O (25 : 75, v/v)] to give *trans*-caffeic acid (**27**, 34 mg, 0.0011%). Fr. 4-6 (205 mg) was further purified by HPLC [MeOH–H_2O (35 : 65, v/v)] to give 3-phenylpropyl β-D-glucopyranoside (**21**, 21 mg, 0.052%). Fr. 4-9 (1771 mg) was further purified by HPLC [MeOH–H_2O (55 : 45, v/v)] to give myrtenyl 10-*O*-β-D-glucopyranoside (**9**, 155 mg, 0.0054 %), geranyl β-D-glucoside (**6**, 203 mg, 0.0070%), and 1-octyl-β-D-glucopyranoside (**38**, 56 mg, 0.0019%). The *n*-BuOH-soluble fraction (365.7 g) was subjected to Diaion HP-20 column chromatography (4.0 kg, H_2O→MeOH) to give H_2O-eluted fractions (66 g, 0.8%) and MeOH-eluted fractions (291 g, 3.5%), respectively. The MeOH-eluted fraction (189 g) was subjected to ordinary-phase silica gel column chromatography [3.0 kg, $CHCl_3$–MeOH–H_2O (15 : 3 : 1, lower layer→10 : 3 : 1, lower layer→7 : 3 : 1, lower layer→6 : 4 : 1,

v/v/v)→MeOH] to give seven fractions [Fr. 1 (428 mg), Fr. 2 (1.8 g), Fr. 3 (60.5 g), Fr. 4 (13.4 g), Fr. 5 (16.6 g), Fr. 6 (12.6 g), and Fr. 7 (64.1 g)]. Fraction 3 (60.5 g) was subjected to reversed-phase silica gel column chromatography [1.5 kg, MeOH–H_2O (10 : 90→20 : 80→30 : 70→40 : 60→50 : 50→60 : 40→70 : 30, v/v)→MeOH] to give 11 fractions [Fr. 3-1 (12.1 g), Fr. 3-2 (3.4 g), Fr. 3-3 (2.3 g), Fr. 3-4 (822 mg), Fr. 3-5 (572 mg), Fr. 3-6 (26.5 g), Fr. 3-7 (956 mg), Fr. 3-8 (221 mg), Fr.3-9 (347 mg), Fr. 3-10 (626 mg) and Fr. 3-11 (356 mg)]. Fr. 3-1 (12.1 g) was identified as rhodiocyanoside A (**11**, 12.2521 g, 0.23%). Fraction 3-2 (350 mg) was further purified by HPLC [MeOH–H_2O (10 : 90, v/v)] to give lotaustralin (**12**, 78 mg, 0.0014%). Fraction 3-3 (306 mg) was also purified by HPLC [MeOH–H_2O (25 : 75, v/v)] to give benzyl β-D-glucopyranoside (**14**, 121 mg, 0.0022%). Fr. 3-5 (572 mg) was separated by HPLC [MeOH–H_2O (35 : 65, v/v)] to give *trans*-cinnamyl β-D-glucopyranoside (**23**, 249 mg, 0.0046%), 2-phenylethyl β-D-glucopyranoside (**18**, 122 mg, 0.0022%), and 2-(4-methoxyphenyl)ethyl β-D-glucopyranoside (**20**, 22 mg, 0.0004%). Fr. 3-7 (956 mg) was separated by HPLC [MeOH–H_2O (45 : 55, v/v)] to give eugenyl *O*-β-D-apiofuranosyl(1″→6′)-β-D-glucopyranoside (**28**, 5.4 mg, 0.0001%) and nodakenin (**31**, 116 mg, 0.0021%). Fr. 3-10 (626 mg) was separated by HPLC [MeOH–H_2O (50 : 50, v/v)] to give geranyl-1-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**7**, 158 mg, 0.0029%), kenposide A (**8**, 154 mg, 0.0028%), and 1-octyl α-D-arabinofuranosyl(1→6)-β-D-glucopyranoside (**39**, 46 mg, 0.0009%). Fraction 4 (13.4 g) was subjected to reversed-phase silica gel column chromatography [450 g, MeOH–H_2O (10 : 90→20 : 80→30 : 70→40 : 60→50 : 50→60 : 40→70 : 30, v/v)→MeOH] to afford nine fractions [Fr. 4-1 (1.1 g), Fr. 4-2 (5.6 g), Fr. 4-3 (698 mg), Fr. 4-4 (2.8 g), Fr. 4-5 (903 mg), Fr. 4-6 (77 mg), Fr. 4-7 (439 mg), Fr. 4-8 (532 mg), Fr. 4-9 (127 mg)]. Fraction 4-1 (560 mg) was separated by HPLC [MeOH–H_2O (10 : 90, v/v)] to give sachaloside V (**5**, 38 mg, 0.0007%) and hetorodendrin (**13**, 11 mg, 0.0002%). Fraction 4-2 (5.6363 g) was identified as salidroside (**19**, 5.636 g, 0.10%). Fraction 4-4 (629 mg) was separated by HPLC [MeOH–H_2O (35 : 65, v/v)] to give rosarin (**24**, 526 mg, 0.0097%), gein (**29**, 35 mg, 0.0007%). Fraction 4-7 (439 mg) was separated by HPLC [MeOH–H_2O (50 : 50, v/v)] to give sachaloside I (**1**, 22 mg, 0.0004%), sachaloside II (**2**, 26 mg, 0.0005%), and sacranoside A (**10**, 58 mg, 0.0011%). Fraction 5 (15.1 g) was subjected to reversed-phase silica gel column chromatography [450 g, MeOH–H_2O (20 : 80→30 : 70→50 : 50→70 : 30→90 : 10, v/v)→MeOH] to afford nine fractions [Fr. 5-1 (142 mg), Fr. 5-2 (5021 mg), Fr. 5-3 (860 mg), Fr. 5-4 (761 mg), Fr. 5-5 (6560 mg), Fr. 5-6 (212 mg), Fr. 5-7 (381 mg), Fr. 5-8 (386 mg), Fr. 5-9 (143 mg)]. Fraction 5-3 (860 mg) was separated by HPLC [MeOH–H_2O (20 : 80, v/v)] to give benzylalcohol 7-*O*-α-L-arabinopyranosy(1→6)-β-D-glucopyranoside (**16**, 11 mg, 0.0002%) and phenylmethyl 6-*O*-α-L-arabinofuranosyl(1→6)-β-D-glucopyranoside (**15**, 19 mg, 0.0004%). Fraction 5-5 (446 mg) was separated by HPLC [MeOH–H_2O (35 : 65, v/v)] to give rosavin (**25**, 12 mg, 0.034%). Fraction 6 (11.6 g) was subjected to reversed-phase silica gel column chromatography [400 g, MeOH–H_2O (20 : 80→30 : 70→50 : 50→70 : 30→90 : 10, v/v)→MeOH] to afford ten fractions [Fr. 6-1 (1308 mg), Fr. 6-2 (813 mg), Fr. 6-3 (2004 mg), Fr. 6-4 (761 mg), Fr. 6-5 (586 mg), Fr. 6-6 (2039 mg), Fr. 6-7 (491 mg), Fr. 6-8 (2255 mg), Fr. 6-9 (787 mg) , Fr. 6-10 (218 mg)]. Fraction 6-7 (491 mg) was separated by HPLC [MeOH–H_2O (15 : 85, v/v)] to give sachaloside IV (**4**, 20 mg, 0.0004%). Fraction 6-8 (781 mg) was separated by HPLC [MeOH–H_2O (40 : 60, v/v)] to give sachaloside III (**3**, 16 mg, 0.0003%) and leucoside (**35**, 153 mg, 0.0028%). Fraction 6-9 (787 mg) was identified as rhodiosin (**36**, 787 mg, 0.014%).

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

Sachaloside I (**1**): Colorless viscous oil; $[\alpha]_D^{27}$ −33.6° (c=1.07, MeOH); IR (film) ν_{max} 3503, 1043 cm^{-1}; ^{1}H-NMR (CD_3OD, 600 MHz) δ: 0.86, 2.29 (1H each, both m, H_2-7), 0.91, 1.10 (3H each, both s, H_3-8, 9), 1.43, 1.83 (1H each, both m, H_2-3), 1.79, 1.88 (1H each, both m, H_2-4), 1.80 (1H, m, H-5), 1.99 (1H, m, H-1), 2.30 (1H, m, H-2), 3.41, 3.71 (1H each, both m, H_2-10), 4.12 (1H, d, J=7.6 Hz, H-1′), 4.21 (1H, d, J=6.9 Hz, H-1″); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z 471 $[M+Na]^+$; negative-ion FAB-MS m/z 447 $[M-H]^-$; HR-FAB-MS: m/z 471.2201 (Calcd for $C_{21}H_{36}O_{10}$ $[M+Na]^+$, 471.2206).

Sachaloside II (**2**): Colorless viscous oil; $[\alpha]_D^{27}$ −47.6° (c=0.77, MeOH); IR (film) ν_{max} 3524, 1635, 1076 cm^{-1}; ^{1}H-NMR (CD_3OD, 600 MHz) δ: 0.67, 1.27 (3H each, both s, H_3-8, 9), 1.70, 2.37 (1H each, both m, H_2-7), 1.97 (1H, m, H-5), 2.04, 2.22 (1H each, both m, H_2-4), 2.46 (1H, m, H-1), 4.37 (1H, d, J=7.0 Hz, H-1″), 4.41 (1H, d, J=7.5 Hz, H-1′), 4.56 (1H, br d, J=7.6 Hz, H-3), 4.80, 5.11 (1H each, both br s, H_2-10); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z 469 $[M+Na]^+$; negative-ion FAB-MS m/z 445 $[M-H]^-$; HR-FAB-MS: m/z 469.2055 (Calcd for $C_{21}H_{34}O_{10}$ $[M+Na]^+$, 469.2050).

Sachaloside III (**3**): A yellow amorphous powder; $[\alpha]_D^{26}$ −74.9° (c=0.57, MeOH); IR (KBr) ν_{max} 3400, 1655, 1072 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 1.12 (1H, d, J=6.1 Hz, H-6‴), 4.60 (1H, d, J=7.4 Hz, H-1‴), 5.56 (1H, d, J=1.9 Hz, H-1⁗), 5.71 (1H, d, J=7.3 Hz, H-1″), 6.43, 6.83 (each 1H, both d, J=2.2 Hz, H-6, 8), 6.90, 8.14 (2H each, both d, J=8.9 Hz, H-3′,5′, 2′,6′); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z 749 $[M+Na]^+$; negative-ion FAB-MS m/z 725 $[M-H]^-$, m/z 593 $[M-C_5H_9O_4]^-$; HR-FAB-MS: m/z 749.1909 (Calcd for $C_{32}H_{38}O_{19}$ $[M+Na]^-$, 749.1905).

Sachaloside IV (**4**): A yellow amorphous powder; $[\alpha]_D^{24}$ −35.9° (c=1.17, MeOH); IR (KBr) ν_{max} 3400, 1655, 1034 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 1.25 (1H, d, J=6.1 Hz, H-6‴), 4.99 (1H, d, J=7.9 Hz, H-1⁗), 5.56 (1H, d, J=1.9 Hz, H-1‴), 5.47 (1H, d, J=7.4 Hz, H-1″), 6.63 (1H, s, H-6), 6.90, 8.13 (2H each, both d, J=8.9 Hz, H-3′,5′, 2′,6′); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z 795 $[M+Na]^+$; negative-ion FAB-MS m/z 771 $[M-H]^-$, m/z 608 $[M-C_6H_{11}O_5]^-$; HR-FAB-MS: m/z 795.1967 (Calcd for $C_{33}H_{40}O_{21}$ $[M+Na]^+$, 795.1960).

Sachaloside V (**5**): A colorless amorphous powder; $[\alpha]_D^{27}$ −27.4° (c=1.81, MeOH); IR (KBr) ν_{max} 3450, 2250, 1074 cm^{-1}; ^{1}H-NMR (CD_3OD, 600 MHz) [δ: 1.08 (3H, t, J=7.3 6Hz, H_3-4), 1.95 (2H, q, J=7.3 Hz, H_2-3), 3.76 (2H, br s, H_2-5), 4.71 (1H, d, J=7.7 Hz, H-1′); ^{13}C-NMR data see Table 1; positive-ion FAB-MS m/z 300 $[M+Na]^+$; HR-FAB-MS: m/z 300.1053 (Calcd for $C_{11}H_{19}NO_7$ $[M+Na]^+$, 300.1059).

Acid Hydrolysis of 1—5 Solution of **1**—**5** (each 1.0 mg) in 1 M HCl (2.0 ml) were each heated under reflux for 3 h (in the case of hydrolysis of **5**, HCN gas produced by the reaction was trapped by KOH in a glass tube). After cooling, each reaction mixture was neutralized with Amberlite IRA-400 (OH^- form) and filtrated, and the solution was partitioned with EtOAc to give two layers. The aqueous layer was evaporated and then subjected to HPLC analysis using Kaseisorb LC NH_2-60-5 column (4.6 mm×250 mm i.d., Tokyo Kasei Co., Ltd., Tokyo, Japan) and an optical rotation detector (Shodex OR-2, Showa Denko Co., Ltd., Tokyo, Japan). D-glucose, L-arabinose, D-xylose, and L-rhamnose were confirmed by comparison of the retention times with the authentic samples (Wako Pure Chemicals Ltd., Osaka, Japan) [D-glucose and L-arabinose, mobile phase: CH_3CN–H_2O (75 : 25, v/v), flow rate: 0.5 ml/min, t_R: 13.8 min (D-glucose, positive optical rotation); t_R: 12.3 min (L-arabinose, positive optical rotation); D-xylose and L-rhamnose, mobile phase: CH_3CN–H_2O (80 : 20, v/v), flow rate: 0.8 ml/min, t_R: 8.0 min (D-xylose, positive optical rotation); t_R: 6.8 min (L-rhamnose, negative optical rotation)].

Enzymatic Hydrolysis of Sachaloside III (3) with Naringinase A solution of **3** (8.8 mg) in 0.1 M acetate buffer (PH 3.8, 2.0 ml) was treated with narigninase (Sigma Chemical Co., 2 units), and the solution was stirred at 40 °C for 24 h. After EtOH was added to the reaction mixture, the solvent was removed under reduced pressure and the residue was purified by HPLC [MeOH : H_2O (55 : 45, v/v)] to furnish kaempferol (**34**, 1.9 mg, 56%).

Protective Effect on Cytotoxicity Induced by D-GalN in Primary Cultured Mouse Hepatocytes The hepatoprotective effects of the constituents were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using primary cultured mouse hepatocytes. Hepatocytes were isolated from male ddY mice (30—35 g) by collagenase perfusion method. The cell suspension at 4×10^4 cells in 100 >μl William's medium E containing fetal calf serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml) was inoculated in a 96-well microplate, and precultured for 4 h at 37 °C under a 5% CO_2 atmosphere. The fresh medium (100 μl) containing D-GalN (2 mM) and a test sample were added and the hepatocytes were cultured for 44 h. The medium was exchanged with 100 μl of the fresh medium, and 10 μl of MTT (5 mg/ml in phosphate buffered saline) solution was added to the medium. After 4 h culture, the medium was removed, 100 μl of isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured by microplate reader at 562 nm (reference: 660 nm). Inhibition (%) was obtained by following formula.

$$\text{inhibition (\%)} = [(\text{O.D.}_{(\text{sample})} - \text{O.D.}_{(\text{control})})/(\text{O.D.}_{(\text{normal})} - \text{O.D.}_{(\text{control})})] \times 100$$

Statistics Values were expressed as means±S.E.M. For statistical analysis, one-way analysis of variance followed by Dunnett's test was used. Probability (p) values less than 0.05 were considered significant.

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