Three Cycloartane Glycosides from Cimicifuga Rhizome and Their Immunosuppressive Activities in Mouse Allogeneic Mixed Lymphocyte Reaction

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One known (1) and two new cycloartane triglycosides, 20*S*,22*R*,23*S*,24*R*-16β,23;22,25-diepoxy-cycloartane-3β,23,24-triol 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-xylopyranoside (2) and 20*S*,22*R*, 23*S*,24*R*-16β,23;22,25-diepoxy-cycloartane-3β,23,24-triol 3-*O*-(6-*O*-*trans*-isoferuloyl-β-D-glucopyranosyl)-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-xylopyranoside (3), were isolated from a commercial Cimicifuga Rhizome. Their structures were determined by two dimensional (2D) NMR spectroscopic analysis and chemical evidence. These compounds suppressed the proliferation of lymphocytes in mouse allogeneic mixed lymphocyte reaction.

Key words Cimicifuga Rhizome; cycloartane glycoside; immunosuppressive activity; *Cimicifuga* sp.; Ranunculaceae

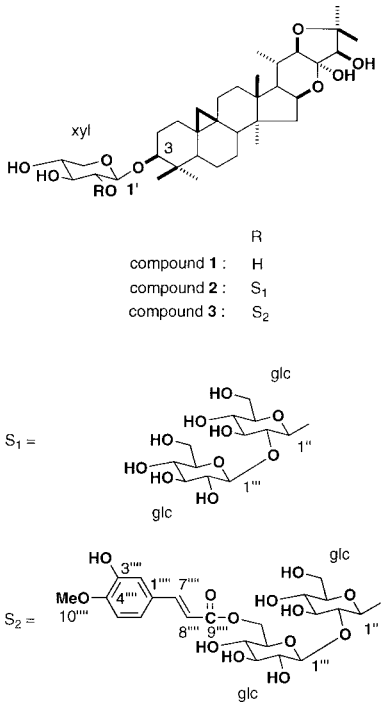
Cimicifuga Rhizome, originated from a rhizome of the genus *Cimicifuga* plants, have been used as anti-inflammatory, analgesic and antipyretic remedies in Chinese traditional medicine. *Cimicifuga* species have been extensively investigated and many cycloartane glycosides have been isolated.[1)] During our investigation on the chemical constituents in Ranunculaceous plants, we have now isolated two new cycloartane triglycosides, compounds **2** and **3**, as well as the known compound **1**, from a commercial Cimicifuga Rhizome. We report the structural elucidation of the cycloartane glycosides and immunosuppressive activity in mouse allogeneic mixed lymphocyte reaction.[2)]

Results and Discussion

The methanolic extract of Cimicifuga Rhizome was partitioned between chloroform-soluble, water-soluble and insoluble portions. The insoluble portion was separated by MCI gel CHP20P, Sephadex-LH20 and silica gel column chromatographies and finally HPLC to give two new cycloartane triglycosides (**2** and **3**), together with the known compound (**1**).

Compound (**1**) was obtained as a white powder, $[\alpha]_D$ −2.4° (MeOH). In the negative-ion FAB-MS of **1**, a quasi-molecular ion peak was observed at *m/z* 619 $[M-H]^-$, while its positive-ion FAB-MS showed a quasi-molecular ion peak at *m/z* 643 $[M+Na]^+$. The ^{1}H-NMR spectra displayed one cyclopropane methylene at δ 0.21 (d, J=4.3 Hz) and 0.48 (d, J=4.3 Hz), six quaternary methyls at δ 0.86, 1.05, 1.21, 1.34, 1.68 and 1.77, a secondary methyl at δ 1.23 (J=6.1 Hz), an anomeric proton at δ 4.87 (d, J=7.3 Hz). The above ^{1}H-NMR data of **1** was similar to those of cycloartane glycoside from *Cimicifuga acerina* and *C. simplex.* The compound was identical with cimiaceroside B by comparing its physical and spectra data with literature values.[4)]

Compound (**2**) was obtained as a white powder, $[\alpha]_D$ −8.5° (MeOH). In the positive-ion FAB-MS of **2**, a quasi-molecular ion peak was observed at *m/z* 967 $[M+Na]^+$. The ^{1}H-NMR spectra displayed one cyclopropane methylene at δ 0.20 (d, J=3.7 Hz) and 0.48 (d, J=3.7 Hz), six quaternary methyls at δ 0.85, 1.11, 1.20, 1.26, 1.70 and 1.76, a secondary methyl at δ 1.22 (J=6.7 Hz), three anomeric protons at δ 4.89 (d, J=7.3 Hz), 5.38 (d, J=7.3 Hz) and 5.42 (d, J=7.9 Hz). The above ^{1}H-NMR data of **1** was similar to those of compound **1**. In the ^{1}H- and ^{13}C-NMR spectra of **2**, the signals due to the aglycone moiety were in good agreement with those of **1**, although the signals due to the sugar moiety were not identical. On acid hydrolysis, **2** afforded D-glucose and D-xylose whose structures were confirmed by ^{1}H-NMR data [xylose H-1′—6′: δ 4.89 (d, J=7.3 Hz), 4.13 (dd, J=7.3, 9.2 Hz), 4.42 (dd, J=9.2, 9.2 Hz), 4.11 (overlapped), 3.74 (dd, J=10.3, 11.0 Hz), 4.33 (dd, J=4.9, 11.0 Hz); glucose H-1″—6″: δ 5.42 (d, J=7.9 Hz), 4.14 (dd, J=7.9, 9.2 Hz), 4.30 (dd, J=9.2, 9.2 Hz), 4.22 (dd, J=9.2, 9.2 Hz), 3.86 (m), 4.38 (dd, J=4.7, 11.5 Hz), 4.46 (br d, J=10.4 Hz); glucose H-1‴—6‴: δ 5.38 (d, J=7.3 Hz), 4.10 (dd, J=7.3, 9.2 Hz), 4.18 (dd, J=9.2, 9.2 Hz), 4.18 (dd, J=9.2, 9.2 Hz), 3.96 (m), 4.32 (dd, J=4.8, 11.6 Hz), 4.56 (br d, J=11.6 Hz)] and specific rotations using chiral detec-



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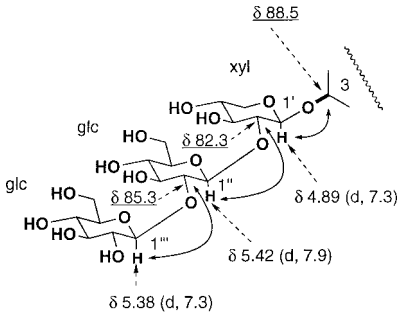

Fig. 1. ^{1}H–^{13}C Long-Range Correlation of the Saccharide Moieties of **2**

J values (Hz) in the ^{1}H-NMR spectrum are given in parentheses. Underlined values indicate ^{13}C-NMR chemical shifts.

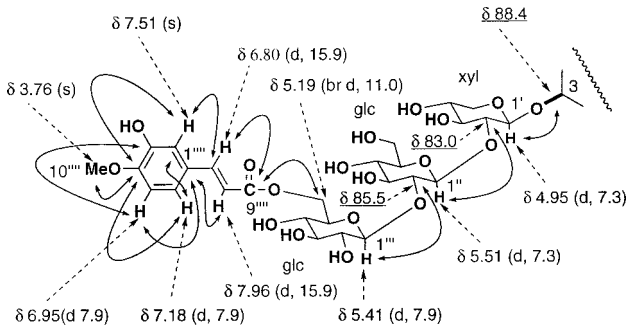

Fig. 2. ^{1}H–^{13}C Long-Range Correlation of the Saccharide Moieties of **3**

J values (Hz) in the ^{1}H-NMR spectrum are given in parentheses. Underlined values indicate ^{13}C-NMR chemical shifts.

tion in HPLC analysis.[5] In the meantime, the negative FAB-MS of **2** gave a $[M-H]^-$ ion peak at *m/z* 943 along with fragment peaks at *m/z* 781 [*m/z* 943−162 (glucose unit)]$^-$, 619 [*m/z* 781−162 (glucose unit)]$^-$ and 487 [*m/z* 619−132 (xylose unit)]$^-$. These evidence suggested that its sugar moiety was composed of a glucosyl–glucosyl–xylosyl unit. The NMR data could be assigned with the aid of ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), total correlation spectroscopy (TOCSY) and heteronuclear multiple bond correlation spectroscopy (HMBC) experiments. In the HMBC experiment, the anomeric proton signals at δ 4.89 (H-1′), 5.42 (H-1″) and 5.38 (H-1‴) showed long-range correlations with the carbon signals at δ 88.5 (C-3), 82.3 (C-2′) and 85.3 (C-2″), respectively (Fig. 1). From the above evidence, the structure of **2** was concluded to be 20*S*,22*R*,23*S*,24*R*-16β,23;22,25-diepoxy-cycloartane-3β,23,24-triol 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-xylopyranoside.

Compound (**3**) was obtained as a white powder, $[\alpha]_D$ −35.4° (MeOH). In the positive-ion FAB-MS of **3**, a quasi-molecular ion peak was observed at *m/z* 1143 $[M+Na]^+$. On acid hydrolysis, **3** afforded D-glucose and D-xylose together with several unidentified artificial sapogenols.[5] The NMR spectra of **3** were essentially analogous with those of **2** and suggestive of a cycloartane glycoside of the same type. In the meantime, the negative FAB-MS of **3** gave a peak at *m/z* 1119 due to $[M-H]^-$, which was higher by 176 mass units than that of **2**. Furthermore, the ^{1}H-NMR [δ 3.76 (3H, s), 6.80 (1H, d, *J*=15.9 Hz), 6.95 (1H, d, *J*=7.9 Hz), 7.18 (1H, d, *J*=7.9 Hz), 7.51 (1H, s), 7.96 (1H, d, *J*=15.9 Hz)], ^{13}C-NMR [δ 55.8 (CH_3), 112.1 (CH), 115.8 (CH), 116.5 (CH), 121.4 (CH), 128.6 (C), 145.6 (CH), 148.5 (C), 150.9 (C), 167.6 (C)] and HMBC spectra (Fig. 2) indicated the presence of one additional mole of a *trans*-isoferuloyl group in **3**. Besides, the negative FAB-MS of **2** gave fragment peaks at *m/z* 943 [*m/z* 1119−176 (isoferuloyl unit)]$^-$, *m/z* 781 [*m/z* 943−162 (glucose unit)]$^-$, 619 [*m/z* 781−162 (glucose unit)]$^-$ and 487 [*m/z* 619−132 (xylose unit)]$^-$. These evidences suggested the composition of an isoferuloyl–glucosyl–glucosyl–xylosyl unit. In the ^{1}H-NMR spectra of **3**, the signal due to the terminal glucose H-6‴ was shifted downfield by 0.60 and 0.63, as compared with those of **2** to appear at δ 4.32 (dd, *J*=4.8, 11.6 Hz) and 4.56 (br d, *J*=11.6 Hz). The above data clearly accounted for linkage of the *trans*-isoferuloyl moiety to the terminal glucose C-6‴. The NMR data of **3** could be assigned with the aid of ^{1}H–^{1}H COSY, HMQC, TOCSY and HMBC techniques. In the HMBC experiment, the anomeric proton signals at δ 4.95 (H-1′), 5.51 (H-1″), 5.41 (H-1‴) and 5.19 (H-6‴) showed long-range correlations with the carbon signals at δ 88.4 (C-3), 83.0 (C-2′), 85.5 (C-2″) and 167.6 (C-9‴′), respectively (Fig. 2). Thus, the structure of **3** was elucidated.

Table 1. ^{13}C-NMR Data for **2** and **3** (Pyridine-d_5)

	2	**3**		**2**	**3**
C-1	32.0	32.1	C-1′	104.8	105.1
2	29.8	30.0	2′	82.3	83.0
3	88.5	88.4	3′	76.6	77.7
4	41.3	41.3	4′	70.1	71.7
5	47.4	47.4	5′	66.0	66.5
6	21.0	21.1	C-1″	103.2	103.3
7	26.4	26.4	2″	85.3	85.5
8	47.5	47.7	3″	77.8	77.4
9	19.7	19.7	4″	71.5	71.1
10	26.6	26.6	5″	77.8	77.8
11	26.3	26.2	6″	62.7	62.9
12	33.4	33.5	C-1‴	106.4	106.3
13	45.2	45.3	2‴	76.3	76.1
14	46.8	46.8	3‴	77.8	77.9
15	43.3	43.4	4‴	71.1	70.4
16	72.3	72.4	5‴	79.2	76.4
17	52.3	52.4	6‴	62.5	64.4
18	20.6	20.7	C-1⁗		128.6
19	30.2	30.4	2⁗		115.8
20	34.7	34.8	3⁗		148.5
21	17.5	17.5	4⁗		150.9
22	86.9	86.9	5⁗		112.1
23	106.0	106.0	6⁗		121.4
24	83.3	83.3	7⁗		145.6
25	83.6	83.6	8⁗		116.5
26	24.8	24.8	9⁗		167.6
27	27.7	27.8	10⁗		55.8
28	19.7	19.7			
29	25.7	25.7			
30	15.4	15.4			

As previously reported , the presence of a large number of cycloartane glycosides, possessing a monosaccharide unit, was a common feature of the genus *Cimicifuga* plants.[1] We now have described for the first time a cycloartane glycoside possessing a trisaccharide unit. Furthermore, compounds **1**—**3** were evaluated for their immunosuppressive activity in mouse allogeneic mixed lymphocyte reaction.[2] These compounds suppressed the proliferation of lymphocytes and the 50% inhibitory concentrations (IC_{50}) were calculated from

the dose-dependent curve. Compounds **1**, **2** and **3** showed potent immunosuppressive activity in mouse allogeneic mixed lymphocyte reaction (IC_{50} 1.03×10^{-4} M, 5.56×10^{-5} M and 9.96×10^{-5} M, respectively). Immunosuppressive activity of **1**—**3** was much of the same value, independent of the sugar moiety.

Experimental

Optical rotations were taken with a JASCO DIP-1000 automatic digital polarimeter. The NMR spectra were measured with a JEOL alpha 500 NMR spectrometer and chemical shifts are given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. The FAB-MS was recorded with a JEOL HX-110 spectrometer. HPLC was carried out using a TSK gel-120A (7.8 mm i.d.×30 cm) column with a Tosoh CCPM pump, Tosoh RI-8010 detector and JASCO OR-2090 detector. Absorbance spectra were recorded on a Bio-Rad Microplate Reader Model 550. TLC was performed on pre-coated Kieselgel 60 F_{254} (Merck), and detection was achieved by spraying with 10% H_2SO_4 followed by heating. Column chromatography was carried out on Kieselgel (230—400 mesh, Merck), Sephadex-LH20 (Pharmacia Find Chem. Co. Ltd.) and MCI gel CHP20P (Mitsubishi Chemical Ind.).

Plant Material Cimicifuga Rhizome was purchased from Uchida Wakanyaku Co., Ltd. This dried rhizome originated from Heilungkiang Province in China.

Extraction and Isolation Cimicifuga Rhizome (20 kg) was extracted with MeOH at room temperature for six months. The MeOH extract (2 kg) was partitioned between chloroform-soluble (1046 g), water-soluble (941 g) and insoluble (12 g) portions. The insoluble portion was subjected to MCI gel CHP20P column chromatography with MeOH–H_2O (50→60→70→80→90%) to afford ten fractions (fr. 1—fr. 10). Fraction 4 (334 mg) was further separated by Sephadex-LH20 column chromatography with MeOH to afford two fractions (fr. 11—fr. 12). Fraction 11 was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5), followed by HPLC with MeOH–H_2O (3 : 1), to furnish compound **2** (10 mg). Fraction 5 (305 mg) was further separated by Sephadex-LH20 column chromatography with MeOH to afford two fractions (fr. 13—fr. 14). Fraction 14 was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (9 : 1 : 0.1), followed by HPLC with MeOH–H_2O (7 : 3), to furnish compound **3** (6 mg). Fraction 9 (961 mg) was further separated by silica gel column chromatography with $CHCl_3$–MeOH–H_2O (9 : 1 : 0.1) to afford three fractions (fr. 15—fr. 17). Fraction 16 was subjected to HPLC with MeOH–H_2O (4 : 1), to furnish compound **1** (24 mg).

Compound **2**: A white powder, $[\alpha]_D^{25}$ −8.5° (c=0.5, MeOH). Pos. FAB-MS (m/z): 967 $[M+Na]^+$. Neg. FAB-MS (m/z): 943 $[M-H]^-$, 781 $[M-H-hexose]^-$, 619 $[M-H-hexose-hexose]^-$, 487 $[M-H-hexose-hexose-pentose]^-$. ^{1}H-NMR (pyridine-$d_5$): δ: 0.20, 0.48 (each 1H, d, J=3.7 Hz, H_2-19), 0.85 (3H, s, H_3-28), 1.11 (3H, s, H_3-30), 1.20 (3H, s, H_3-18), 1.22 (3H, d, J=6.7 Hz, H_3-21), 1.26 (3H, s, H_3-29), 1.70 (3H, s, H_3-27), 1.76 (3H, s, H_3-26), 2.26 (1H, m, H-20), 3.38 (1H, dd, J=4.3, 11.6 Hz, H-3), 3.89 (1H, d, J=11.0 Hz, H-22), 4.17 (1H, s, H-24), 4.96 (1H, q, J=7.9 Hz, H-16); xyl-1′ to xyl-5′, 4.89 (1H, d, J=7.3 Hz), 4.13 (1H, dd, J=7.3, 9.2 Hz), 4.42 (1H, dd, J=9.2, 9.2 Hz), 4.11 (1H, overlapped), 3.74 (1H, dd, J=10.3, 11.0 Hz), 4.33 (1H, dd, J=4.9, 11.0 Hz); glc-1″ to glc-6″, 5.42 (1H, d, J=7.9 Hz), 4.14 (1H, dd, J=7.9, 9.2 Hz), 4.30 (1H, dd, J=9.2, 9.2 Hz), 4.22 (1H, dd, J=9.2, 9.2 Hz), 3.86 (1H, m), 4.38 (1H, dd, J=4.7, 11.5 Hz), 4.46 (1H, br d, J=10.4 Hz); glc-1‴ to glc-6‴, 5.38 (1H, d, J=7.3 Hz), 4.10 (1H, dd, J=7.3, 9.2 Hz), 4.18 (1H, dd, J=9.2, 9.2 Hz), 4.18 (1H, dd, J=9.2, 9.2 Hz), 3.96 (1H, m), 4.32 (1H, dd, J=4.8, 11.6 Hz), 4.56 (1H, br d, J=11.6 Hz). ^{13}C-NMR (pyridine-d_5): Table 1.

Compound **3**: A white powder, $[\alpha]_D^{25}$ −35.4° (c=0.3, MeOH). Pos. FAB-MS (m/z): 1143 $[M+Na]^+$. Neg. FAB-MS (m/z): 1119 $[M-H]^-$, 943 $[M-H-isoferuloyl\ unit]^-$, 781 $[M-H-isoferuloyl\ unit-hexose]^-$, 619 $[M-H-isoferuloyl\ unit-hexose-hexose]^-$, 487 $[M-H-isoferuloyl\ unit-hexose-hexose-pentose]^-$. ^{1}H-NMR (pyridine-$d_5$): δ: 0.18, 0.47 (each 1H, d, J=3.7 Hz, H_2-19), 0.85 (3H, s, H_3-28), 1.15 (3H, s, H_3-30), 1.21 (3H, d, J=6.7 Hz, H_3-21), 1.23 (3H, s, H_3-18), 1.31 (3H, s, H_3-29), 1.68 (3H, s, H_3-27), 1.77 (3H, s, H_3-26), 2.26 (1H, m, H-20), 3.39 (1H, dd, J=4.3, 11.6 Hz, H-3), 3.90 (1H, d, J=10.4 Hz, H-22), 4.18 (1H, s, H-24), 4.97 (1H, q, J=7.8 Hz, H-16), 3.76 (3H, s, isoferuloyl H-10‴), 6.80 (1H, d, J=15.9 Hz, isoferuloyl H-8‴), 6.95 (1H, d, J=7.9 Hz, isoferuloyl H-5‴), 7.18 (1H, d, J=7.9 Hz, isoferuloyl H-6‴), 7.51 (1H, s, isoferuloyl H-2‴), 7.96 (1H, d, J=15.9 Hz, isoferuloyl H-7‴); xyl-1′ to xyl-5′, 4.95 (1H, d, J=7.3 Hz), 4.10 (1H, dd, J=7.3, 9.2 Hz), 4.38 (1H, dd, J=9.2, 9.2 Hz), 4.12 (1H, overlapped), 3.79 (1H, dd, J=10.3, 11.0 Hz), 4.31 (1H, dd, J=4.8, 11.0 Hz); glc-1″ to glc-6″, 5.51 (1H, d, J=7.3 Hz), 4.18 (1H, dd, J=7.3, 9.2 Hz), 4.32 (1H, dd, J=9.2, 9.2 Hz), 4.21 (1H, dd, J=9.2, 9.2 Hz), 3.88 (1H, m), 4.38 (1H, dd, J=4.8, 11.5 Hz), 4.48 (1H, br d, J=10.3 Hz); glc-1‴ to glc-6‴, 5.41 (1H, d, J=7.9 Hz), 4.17 (1H, dd, J=7.9, 9.2 Hz), 4.24 (1H, dd, J=9.2, 9.2 Hz), 4.12 (1H, dd, J=9.2, 9.2 Hz), 3.96 (1H, overlapped), 4.92 (1H, dd, J=4.8, 11.6 Hz), 5.19 (1H, br d, J=11.0 Hz). ^{13}C-NMR (pyridine-d_5): Table 1.

Sugar Analysis A solution each of compound (**2** or **3**) (1 mg) in 2 N HCl–dioxane (1 : 1, 2 ml) was heated at 100 °C for 1 h. The reaction mixture was diluted with H_2O and evaporated to remove dioxane. The solution was neutralized with Amberlite MB-3 and passed through a SEP-PAK C_{18} cartridge to give a sugar fraction. The sugar fraction was concentrated to dryness *in vacuo* to give a residue, which was dissolved in CH_3CN–H_2O (3 : 1, 250 μl). The sugar fraction was analyzed by HPLC under the following conditions: column, Shodex RS-Pac DC-613 (6.0 mm i.d.×150 mm, Showa-Denko, Tokyo, Japan); solvent, CH_3CN–H_2O (3 : 1); flow rate, 1.0 ml/min; column temperature, 70 °C; detection, refractive index (RI) and optical rotation (OR). t_R (min) of sugars were as follow. **2**: D-xylose 5.5 (+), D-glucose 7.4 (+). **3**: D-xylose 5.5 (+), D-glucose 7.4 (+). [reference: D-xylose 5.5 (positive optical rotation: +), D-glucose 7.4 (positive optical rotation: +)].

Biological Assays Animals: Male BALB/cAnNCrj and C57BL/6NCrj mice were purchased from Japan Charles River Laboratories, Kanagawa, Japan. All animals were used at 5 weeks of age.

Cell Culture Medium: RPMI-1640 (Life Technologies, Inc., Rockville, MD, U.S.A.) was supplemented with 2 mM L-glutamine, penicillin at 100 U/ml, streptomycin at 100 μg/ml, 25 nM HEPES and $NaHCO_3$ at 2 mg/ml. Fetal bovine serum (Life Technologies, Inc., Rockville, MD, U.S.A.) was heat-inactivated at 56 °C for 30 min and added to the medium as indicated.

Mouse Allogeneic Mixed Lymphocyte Reaction: Mouse allogeneic mixed lymphocyte reaction was carried out by culturing BALB/c mouse spleen cells (4×10^5 cells, responder) and an equal number of C57BL/6 mouse spleen cells treated with mitomycin C at 40 μg/ml for 30 min at 37 °C (stimulator) in 200 μl of RPMI-1640 medium containing 5×10^{-5} M 2-mercaptoethanol, 10% fetal bovine serum and a variable amount of test substance. The cells were placed in a 96-well flat-bottomed microtest plate (No. 3072 Falcon, Becton Dickinson, Lincoln Park, NJ, U.S.A.) and cultured for 4 d at 37 °C in an atmosphere of 5% CO_2. After 4 d, 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8)[3] solution was added to each well and cultured for 1 d at 37 °C in an atmosphere of 5% CO_2. The absorbance at a wavelength of 450 nm was measured with a microplate reader. The results were expressed as IC_{50} values. WST-8 assay was performed using a cell counting kit-8 (Dojindo Laboratories).

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