Cimicifuga racemosa NUTT., commonly going by the name of black cohosh, is a herb indigenous to North America and Europe, and its rhizomes have long been used for the treatment of a variety of ailments such as diarrhea, sore throat, and rheumatism by Native Americans.¹ Now, black cohosh has become a well-known alternative herbal medicine with health benefits in treating painful menstrual periods and menopausal disorders not only in the United States but also in European countries.² Black cohosh has been revealed to contain triterpene monoglycosides with the cycloartane skeleton,³ isoflavones,⁴ alkaloids,⁵ and phenylpropanoids,⁶ among which the cycloartane glycosides are its main secondary metabolites and are considered to partially contribute to the pharmacological effects of this herbal medicine.⁷ The present investigation is part of a series of studies on the chemical constituents of the herbal medicines.⁸ As a result, a total of twelve cycloartane glycosides (1—12), including four new compounds (4—6, 12), were isolated from the MeOH extract of C. racemosa. This paper deals with the structural assignments of the new glycosides on the basis of two-dimensional (2D) NMR data, and chemical methods. The cytotoxic activities of the isolated compounds against human oral squamous cell carcinoma (HSC-2) cells and normal human gingival fibroblasts (HGF) are also reported.

**Results and Discussion**

The rhizomes of C. racemosa (dry weight of 5.2 kg) was extracted with hot MeOH. The MeOH extract was subjected to column chromatography over porous-polymer polystyrene resin (Diaion HP-20), silica gel, and octadecylsilanized (ODS) silica gel, as well as preparative HPLC, to give compounds 1—12. Compounds 1—3 and 7—11 were identified as cimigenol 3-O-α-L-arabinopyranoside (1),⁹ 25-O-methoxy CIMigenol 3-O-α-L-arabinopyranoside (2),¹⁰ 12β-hydroxy CIMigenol 3-O-α-L-arabinopyranoside (3),¹¹ 27-deoxyactein (7),¹² actein (8),¹³ cimiracemoside F (9),¹⁴ cimiracemoside G (10),¹⁴ and cimiracemoside H (11),¹⁴ respectively.

Compound 4 was isolated as an amorphous solid. Its molecular formula was derived as C₃⁷H₅₈O₁₁ by data from the positive-ion FAB-MS, which showed an [M+Na]⁺ ion at m/z 701. The ¹³C-NMR spectrum, with a total of 37 carbon signals, and the results of elemental analysis were consistent with the deduced formula. The IR spectrum showed an ab-
The 1H-NMR spectrum of 4 showed signals for a cyclopropyl methylene group at \( \delta 0.62 \) and 0.38 (each 1H, d, \( J=4.1 \) Hz), six tertiary methyl groups at \( \delta 1.71, 1.69, 1.42, 1.27, 1.21, \) and 1.00 (each 3H, s), a secondary methyl group at \( \delta 1.39 \) (3H, d, \( J=5.8 \) Hz), and an anomeric proton at \( \delta 4.79 \) (1H, d, \( J=6.9 \) Hz). These 1H-NMR data and 13C-NMR spectral features of 4 were quite similar to those of 3. In addition, the presence of an acetyl group in 4 was shown by the IR (1734 cm\(^{-1}\)) and 13C-NMR spectra. Alkaline hydrolysis of 4 with Na\(_2\)CO\(_3\) solution yielded 3, indicating that 4 was a monoacetate of 3. When the 13C-NMR spectrum of 4 was compared with that of 3, the signal due to C-25 was shifted to a lower field by 12.1 ppm, whereas the signals assignable to C-24, C-26, and C-27 occurred, respectively, at higher fields by 3.3, 2.4, and 3.7 ppm. Thus, the acetyl moiety was revealed to be linked to the aglycon C-25 hydroxyl group, and the structure of 4 was formulated as 25-O-acetyl-12β-hydroxycimigenol 3-O-\( \alpha-L\)-arabinopyranoside.

Compound 5 was shown to have the molecular formula \( C_{35}H_{56}O_{11} \) on the basis of the positive-ion FAB-MS (\( m/z \) 675 [M+Na]+), 13C-NMR spectrum, and elemental analysis. The 1H-NMR spectrum of 5 contained signals for a cyclopropyl methylene group at \( \delta 0.65 \) and 0.34 (each 1H, d, \( J=4.1 \) Hz), six tertiary methyl groups at \( \delta 1.52, 1.49\times2, 1.28, 1.23, \) and 1.00 (each 3H, s), and an anomeric proton at \( \delta 6.78 \) (1H, d, \( J=7.0 \) Hz). Enzymatic hydrolysis of 5 with naringinase furnished the genuine aglycon (\( C_{29}H_{48}O_{7} \)) and \( L\)-arabinose. Comparison of the 1H- and 13C-NMR spectra of 5a with those of 12β-hydroxycimigenol\(^{11}\) showed their considerable structural similarity. However, the molecular formula of 5a was higher by one oxygen atom than that of 12β-hydroxycimigenol, and complete acetylation of 5a gave the corresponding pentaacetate (5b). This indicated the presence of one more hydroxyl group in addition to the C-3β, C-12β, C-15β, and C-25 hydroxyl groups. When the 1H-NMR spectrum of 5a was compared with that of 12β-hydroxy-
and comparison with that of revealed to be included in the structure of signals at of This chemical evidence allowed the structural determination of the C-20 hydroxyl group was evident. The C-20α configuration was ascertained by nuclear Overhauser effect (NOE) correlations from H-20 (δ 2.18) to Me-18 (δ 1.49) and H-22β (δ 2.41) in the phase-sensitive NOE correlation spectroscopy (NOESY) spectrum. In the 1H-detected heteronuclear multiple-quantum coherence (HMQC) spectrum of 5, a long-range correlation was observed from the anomeric proton of the α-L-arabinopyranosyl group at δ 4.78 to the aglycon C-3 carbon at δ 88.4. Accordingly, the structure of 5a, a new triterpene sapogenin, was shown to be (20R,23R,24S)-16α,23:24di-epoxy-9,19-cyclostan-3β,12β,15β,21,25-pentol (12b,21-dihydroxycimigenol), and consequently, the structure of 5 was established as 12b,21-dihydroxycimigenol 3-O-α-L-arabinopyranoside.

Compound 6 was analyzed for C_{37}H_{58}O_{11} by combined positive-ion FAB-MS (m/z 685 [M+Na]+), 13C-NMR spectrum with distortionless enhancement by polarization transfer (DEPT) data, and elemental analysis. The 1H-NMR spectrum of 6 showed a pair of cyclopropene methylene proton signals at δ 0.58 and 0.32 (each 1H, d, J = 4.0 Hz), six tertiary methyl proton signals at δ 1.40, 1.37, 1.30, 1.25, 1.21, and 1.05 (each 3H, s), a secondary methyl proton signal at δ 1.26 (3H, d, J = 6.6 Hz). In addition, a secondary hydroxyl group, a carbonyl group, an acetylxylo group, and an epoxy ring, as well as an α-L-arabinopyranosyl moiety, were revealed to be included in the structure of 6 by analysis of its 1H- and 13C-NMR spectra. Thus, 6 was presumed to be an α-L-arabinopyranoside of a shengmanol derivative. Chemical conversion of 6 to the corresponding cimigenol saponin was carried out according to the method reported by Kusano et al. After deacetylation of 6 with Na₂CO₃ solution, the hydrolysate was treated with 2.5% AcOH at 95°C to furnish 1. This chemical evidence allowed the structural determination of 6 as 23-O-acetylshengmanol 3-O-α-L-arabinopyranoside.

Compound 12 was deduced as C_{37}H_{58}O_{11} from its FAB-MS, 13C-NMR spectral, and elemental analysis data. The 1H-NMR spectrum showed signals characteristic of the 9,19-cycloartenyl derivative. Analysis of the 13C-NMR spectrum of 12 and comparison with that of 11 implied that the aglycon of 12 was identical to that of 11, but differed from 11 in terms of the monosaccharide constituent. Instead of the signals for a xylose moiety, five signals assignable to an α-L-arabinopyranosyl residue were observed at δ 107.3 (CH), 72.9 (CH), 74.6 (CH), 69.5 (CH), and 66.7 (CH₃). Hydrolysis of 12 with naringinase gave L-arabinose and an aglycon (12a), which was identical to the product obtained by enzymatic hydrolysis of 11. The glycosidic linkage of the arabinosyl group to C-3 of the aglycon was ascertained by an HMBC correlation from the H-1 aglycone proton at δ 4.77 (d, J = 7.0 Hz) to the C-3 aglycon carbon at δ 88.1. Thus, the structure of 12 was shown to be (22R,23R,24R)-12β-acyloxy-16β,23:22,25-diepoxy-23,24-dihydroxy-9,19-cyclostan-3-β-yl α-L-arabinopyranoside.

The isolated compounds were evaluated for their cytotoxic activities against HSC-2 cells and HGF. The results are shown in Table 3. It is suggested that slight differences in the

<table>
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<th>Compounds</th>
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<td>HSC-2</td>
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aglycon structures exerted some effects on the cytotoxic activities. Although it did not exhibit any apparent cytotoxicity against HSC-2 cells even at the sample concentration of 400 μM, the 25-O-methyl derivative (2) of 1 dose-dependently reduced the viable cell number and its IC50 value was calculated to be 30 μM. The C-27 hydroxy derivative (8) of 7, which is the main secondary metabolite of C. racemosa, was more cytotoxic than 7. In the cinamycorogenin derivatives (9–12), the cytotoxicity of the 7(8)-dehydro saponins (9, 10) was more potent than that of the corresponding saturated saponins (11, 12). It is notable that 10 showed about 15-fold higher cytotoxic activity against HSC-2 tumor cells than against normal HGF.

### Experimental

NMR spectra were recorded on a Bruker DRX-500 (500 MHz for 1H-NMR, 125 MHz for 13C-NMR) spectrometer using standard Bruker pulse programs. Assignment of the 1H and 13C nuclear magnetic resonance (NMR) spectra was achieved through 1D- and 2D-correlation experiments (COSY, NOESY, and HMBC) on a Varian Unity INOVA spectrometer. Mass spectra were determined using a JEOL JMS-HX110A or a JEOL-700SE spectrometer. The specific optical rotation was determined using a Polarimeter- polarimeter model P-1020 (JASCO, Tokyo, Japan) and a P-2000S (JASCO, Tokyo, Japan). Infrared spectra were recorded on a JASCO FT/IR-4100 spectrometer using pressed KBr pellets. Ultrasound-assisted extraction (UAE) was carried out with an ultrasonic processor (Model VCX750, Qsonica, Stamford, CT, USA).

### Plant Material

The plant material was provided by Tohoku Pharmaceutical Co., Ltd., Chiba, Japan. A small amount of the sample is preserved in our laboratory (00-09-01).

### Extraction and Isolation

The plant material (dry weight, 5.2 kg) was extracted with hot MeOH (21 l) for 3 h twice. The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (445 g) was passed through a Diaion HP-20 column, successively eluting with 30%, 50%, 70%, and 100% MeOH, to afford 7.2 g of crude material. Column chromatography of the 50% MeOH eluate portion (14 g) on silica gel and elution with stepwise gradient of CHCl3–MeOH (9 : 1 to 4 : 1) gave 1 (0.6 mg). The 100% MeOH eluate fraction was purified by silica gel column chromatography eluting with CHCl3–MeOH (30 : 1; 10 : 1; 5 : 1) and ODS silica gel with MeOH–H2O (8 : 5) to give 8 (0.8 mg). The 20% MeOH eluate fraction was analyzed by HPLC under the following conditions: column, Capcell Pak NH2 UG80 (4.6 mm i.d. × 250 mm, ODS, 5 μm); solvent, MeCN–H2O (17 : 3); flow rate, 1.0 ml/min; detection, refractive index (RI) and optical rotation (OR). Identification of l-arabinose was carried out by comparison of its retention time and OR with those of an authentic sample.

### Complete Acetylation of 5a

Compound 5a (3.3 mg) was acetylated with a mixture of Ac2O (1.0 ml) and pyridine (1.0 ml) in the presence of 4-(dimethylaminomethyl)pyridine (1.0 mg) as a base catalyst, and the crude acetate was chromatographed on silica gel eluting with hexane–Me2CO (4 : 1) to afford the corresponding pentaacetate (5b) (1.6 mg).

### Enzymatic Hydrolysis of 12a

Compound 12a (3.9 mg) was dissolved in MeOH (0.2 ml) and added to 1% Na2CO3 solution (1.0 ml) and was stirred at room temperature for 12 h. The reaction mixture was neutralized by addition of 5% AcOH (0.6 ml) and extracted with EtOAc (3.0 ml/3). After removal of EtOAc, the residue was dissolved in a mixture of 1,4-dioxane (0.6 ml) and 5% AcOH (0.6 ml), and was heated at 95 °C for 2 h under an Ar atmosphere. The reaction mixture was concentrated and subjected to column chromatography on silica gel eluting with CHCl3–MeOH (9 : 1) to give 1 (0.6 mg).

### Transformation of 6 into 1

Compound 6 (1.5 mg) was dissolved in MeOH (0.2 ml) and added to 1% Na2CO3 solution (1.0 ml), which was stirred at room temperature for 12 h. The reaction mixture was neutralized by addition of 5% AcOH (0.6 ml) and extracted with EtOAc (3.0 ml/3). After removal of EtOAc, the residue was dissolved in a mixture of 1,4-dioxane (0.6 ml) and 5% AcOH (0.6 ml), and was heated at 95 °C for 2 h under an Ar atmosphere. The reaction mixture was concentrated and subjected to column chromatography on silica gel eluting with CHCl3–MeOH (9 : 1) to give 1 (0.6 mg) by passing it through an Amberlite IR-120B (Organo, Tokyo, Japan) and Toyopak IC-SP M cartridge (Tosoh) eluting with 20% MeOH.

### Enzymatic Hydrolysis of 12

Compound 12 (7.6 mg) was subjected to enzymatic hydrolysis using naringinase as described for 5 to give an aglycon (12a) (4.3 mg) and a sugar fraction. HPLC analysis of the sugar fraction indicated the presence of l-arabinose (1.0 mg). The 20% MeOH eluate fraction was analyzed by HPLC under the following conditions: column, Capcell Pak NH2 UG80 (4.6 mm i.d. × 250 mm, 5 μm); solvent, MeCN–H2O (17 : 3); flow rate, 1.0 ml/min; detection, refractive index (RI) and optical rotation (OR). Identification of l-arabinose was carried out by comparison of its retention time and OR with those of an authentic sample.

### Enzymatic Hydrolysis of 12a

Compound 12a (1.0 mg) was subjected to enzymatic hydrolysis using naringinase as described for 5 to give an aglycon (12a) (4.3 mg) and a sugar fraction. HPLC analysis of the sugar fraction indicated the presence of l-arabinose (1.0 mg). The 20% MeOH eluate fraction was analyzed by HPLC under the following conditions: column, Capcell Pak NH2 UG80 (4.6 mm i.d. × 250 mm, 5 μm); solvent, MeCN–H2O (17 : 3); flow rate, 1.0 ml/min; detection, refractive index (RI) and optical rotation (OR). Identification of l-arabinose was carried out by comparison of its retention time and OR with those of an authentic sample.

### Enzymatic Hydrolysis of 10

Compound 10 (2.0 mg) was subjected to enzymatic hydrolysis using naringinase as described for 5 to give an aglycon (10a) (0.8 mg) and a sugar fraction. HPLC analysis of the sugar fraction indicated the presence of l-arabinose (0.3 mg). The 20% MeOH eluate fraction was analyzed by HPLC under the following conditions: column, Capcell Pak NH2 UG80 (4.6 mm i.d. × 250 mm, 5 μm); solvent, MeCN–H2O (17 : 3); flow rate, 1.0 ml/min; detection, refractive index (RI) and optical rotation (OR). Identification of l-arabinose was carried out by comparison of its retention time and OR with those of an authentic sample.
flask. The explants were incubated in α-MEM supplemented with 30% FBS and antibiotics. When outgrowth of the cells was observed, the medium was replaced twice until the cells reached confluence. The cells were detached from the monolayer by trypsinization and recultured in 100 cm² tissue culture flasks until confluent monolayers were again obtained. Cells between the fifth and seventh passages were used.

Assay for Cytotoxic Activity Cells were trypsinized and inoculated at $6 \times 10^3$ per each 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson, San Jose, CA, U.S.A.), and incubated for 24 h. After washing once with PBS, they were treated for 24 h without or with test compounds. They were washed once with PBS and incubated for 4 h with 0.2 mg/ml MTT in DMEM supplemented with 10% FBS. After the medium was removed, the cells were lysed with 0.1 ml dimethyl sulfoxide (DMSO), and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using Labsystems Multiskan® (Biochromatic, Helsinki, Finland) connected to a Star/DOT Matrix printer JL-10. The IC₅₀ value, the concentration that reduces the viable cell number by 50%, was determined from the dose–response curve.

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References