A New Diterpenoid Glucoside and Two New Diterpenoids from the Fruit of *Vitex agnus-castus*

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A new labdane-type diterpenoid glucoside and two new labdane-type diterpenoids were isolated from the fruit (chasteberry) of *Vitex agnus-castus* L. (Verbenaceae) along with 14 known compounds comprising seven labdane-type diterpenoids, one halimane-type diterpenoid, two oleanane-type triterpenoids, two ursane-type triterpenoids, one aromadendrane-type sesquiterpenoid, and one flavonoid. Their structures were characterized on the basis of spectroscopic data as well as chemical evidence. Furthermore, the antioxidative activities of the flavonoid were evaluated using five different analyses.

Key words  *Vitex agnus-castus*; Verbenaceae; chasteberry; diterpenoid; glucoside; antioxidative activity

*Vitex agnus-castus* L. (Verbenaceae) is widely distributed in Central Asia, the Mediterranean region, and Southern Europe. The fruit (chasteberry) of this plant is used as a dietary supplement for treating the hormone-imbalance syndrome in women.¹ This fruit has been reported to contain essential oils, iridoids, flavonoids, and diterpenoids.¹–⁹ Further, linoleic acid isolated from this fruit was identified as an estrogenic compound.¹⁰ In the course of our study on the constituents of Verbenaceae plants, we previously reported the isolation and structural elucidation of 14 diterpenoids, one each of norlabdane-type diterpenoid, aromadendrane-type sesquiterpenoid, and flavonoid from the fruit of *V. agnus-castus*.¹¹,¹² As part of our continuing study on the constituents of this fruit, the present paper deals with the isolation and structural elucidation of a new labdane-type diterpenoid glucoside and two new labdane-type diterpenoids along with 14 known compounds comprising seven labdane-type diterpenoids, one halimane-type diterpenoid, two oleanane-type triterpenoids, two ursane-type triterpenoids, one aromadendrane-type sesquiterpenoid, and one flavonoid.

The fruit of *V. agnus-castus* was successively percolated with hexane, acetone, and methanol (MeOH) to give hexane extract, acetone extract, and MeOH extract. The acetone extract was subjected to silica gel, Chromatorex octadecyl silica (ODS), and Sephadex LH-20 column chromatography as well as HPLC on ODS to yield 16 compounds (2–17). The successive chromatography of the MeOH extract over Diaion HP20, Sephadex LH-20, Chromatex ODS, and silica gel column yielded 1.

Compounds 4—17 were identified as (rel 5S,6R,8R,9R,10S,13S,16S)-6-acetoxy-9,13-epoxy-16-methoxy-labdan-15,16-olide (4),¹³ (rel 5R,6R,8R,9R,10S,13R,16S)-6-acetoxy-9,13-epoxy-16-methoxy-labdan-15,16-olide (5),¹³ (rel 5S,6R,8R,9R,10S,13S)-6-acetoxy-9,13-epoxy-15-methoxy-labdan-16,15-olide (6),¹³ (rel 5S,6R,8R,9R,10S,13R)-6-acetoxy-9,13-epoxy-15-methoxy-labdan-16,15-olide (7),¹³ vitexilactone (8),¹⁴ vitemagnusin C (9),¹⁵ 8-epi-sclareol (10),¹ⁱ vitexifolin D (11),¹⁴ 2,3-dihydroxy-12-ursen-28-oic acid (12),¹⁵ 2α-hydroxyursolic acid (13),¹⁵ 3-epimaslinic acid (14),¹⁷ maslinic acid (15),¹⁰ and 4α,10α-dihydroxyaromadendrane (16),¹⁵ and casticin (17),¹⁹ respectively, based on comparison of their physical and spectral data with authentic samples or those already reported (Fig. 1).

Compound 1, tentatively named vitemagnuside A, was obtained as an amorphous powder, and gave an [M+Na]+ ion peak at m/z 521 in the positive FAB-MS. The molecular formula of 1 was determined to be C_{26}H_{42}O_{9} by high-resolution (HR)-positive FAB-MS. The ¹H-NMR spectrum of 1 showed
signals due to three tertiary methyl groups (δ 1.32, 1.07, 0.95), one secondary methyl group [δ 1.11 (d, J = 6.0 Hz)], one olefinic proton [δ 7.22 (s)], and one anomeric proton [δ 4.85 (d, J = 8.0 Hz)]. The 13C-NMR spectrum of I gave 26 carbon signals including one each of carbonyl carbon (δ 174.8), oxygenated quaternary carbon (δ 76.7), oxygenated methine carbon (δ 89.1), oxygenated methylene carbon (δ 70.7), and glucopyranosyl group (δ 106.9, 75.7, 78.7, 71.7, 78.1, 62.9), and two olefinic carbons (δ 145.0, 134.8). These 1H- and 13C-NMR spectral signals were assigned with the aid of 1H-1H correlation spectroscopy (COSY), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) techniques as shown in Tables 1 and 2, and the planar structure of I, which was a labdane-type diterpenoid glucoside possessing an α-substituted butenolide ring, could be characterized as illustrated in Fig. 2. In the nuclear Overhauser and exchange spectroscopy (NOESY) spectrum of I, the key nuclear Overhauser effects (NOEs) were observed between H-3 and H2-18; H-5 and H2-18; H-8 and H2-20; and Hα-11 and H2-20 (Fig. 3). Thus, representative configurations at C-3, C-5, C-8, C-9, and C-10 were concluded to be S*, S*, R*, R*, and S*, respectively.

On acidic hydrolysis, I afforded D-glucose, which was identified by using optical rotation chiral detection in the HPLC analysis, along with several unidentified artificial aglycones. The glycosidic linkages was shown to be β by the coupling constant of the 1H-NMR spectral signal due to anomic proton. Enzymatic hydrolysis of I with β-glucosidase gave 1a, whose 1H-NMR spectrum exhibited signals due to three tertiary methyl groups (δ 1.23, 1.11, 1.03), one secondary methyl group [δ 1.13 (d, J = 6.5 Hz)], one olefinic proton [δ 7.20 (dd, J = 1.5, 1.5 Hz)], one oxygenated methylene group [δ 4.74 (d, J = 1.5 Hz)], and one oxygenated methine proton [δ 3.52 (d, J = 6.0, 10.0 Hz)]. Thus, 1a was defined to be a genuine aglycone of I. In order to determine the absolute configuration of the aglycone, the 13C-NMR spectral data of 1a were compared with those of 1a. The glycosylation shifts were observed at C-2, C-3, and C-4 of the aglycone moiety of 1 with magnitudes −1.6, +11.0, and +0.1 ppm, respectively, indicating the absolute configuration at C-3 of the aglycone to be S according to Kasai et al.20 and Seo et al.21 Consequently, the structure of I was concluded to be (3S,5S,8R,9R,10S)-3,9-dihydroxy-13(14)-labdicaen-16,15-olide 3-O-β-D-glucopyranoside.
Compound 2, tentatively named viteagnusin I, was obtained as a colorless syrup. The positive FAB-MS of 2 indicated an [M+Na]$^+$ ion peak at m/z 431; the molecular formula of 2 was concluded to be C$_{23}$H$_{36}$O$_6$ by HR-positive FAB-MS. The $^1$H-NMR spectrum of 2, which was closely analogous to that of 4, exhibited signals due to three tertiary methyl groups (δ 1.22, 0.99, 0.97), one secondary methyl group (δ 0.82 (d, J=6.5 Hz)), one acetyl group (δ 2.04), and one methoxy group (δ 3.52). The $^{13}$C-NMR spectrum of 2, which could be superimposed on that of 4, gave 23 carbon signals, including two carbonyl carbons (δ 173.6, 170.4), one acetal carbon (δ 108.0), one methoxy carbon (δ 56.4), one oxygenated methine carbon (δ 70.5), and two oxygenated quaternary carbons (δ 95.0, 86.0). These $^1$H- and $^{13}$C-NMR spectral signals were examined in detail, and the planar structure of 2, a labdane-type diterpenoid possessing one each of spiro-tetrahydrofuran ring, γ-spiro-lactone ring, methoxy group, and acetyl group, could be determined to be the same as that of 4. The relative configurations were defined on the basis of the NOESY spectrum, in which correlations were observed between H-5 and H$_3$-18; H-6 and H$_3$-18; H-8 and H$_3$-20; Ha-11 and H$_3$-20; Ha-12 and H-16; and Hb-14 and H$_3$-17 (Fig. 3). Accordingly, 2 was defined as (rel 5S,6R,8R,9S,10S,13S,16R)-6-acetoxy-9,13-epoxy-16-methoxy-labdan-15,16-olide.

Compound 3, tentatively named viteagnusin J, was obtained as a colorless syrup, and the molecular formula of 3 was concluded to be the same as that of 2 by using HR-positive FAB-MS. The $^1$H- and $^{13}$C-NMR spectra of 3 were analogous to those of 2, and the planar structure of 3 was determined to be the same as that of 2 by using 2D-NMR techniques. In the NOESY spectrum of 3, key NOEs were observed between H-5 and H$_3$-18; H-6 and H$_3$-18; H-8 and H$_3$-20; and H-16 and H$_3$-17 (Fig. 3). Furthermore, the $^1$H- and $^{13}$C-NMR spectra of 3 were different from those of 5. Therefore, 3 was defined as the 16-epimer of 5.

Although, the absolute configurations of 2 and 3 have not been confirmed, they are probably the same as that of 1 from a biogenetic point of view. However, 2—7 might be artifacts produced from aldehydes during the isolation procedures, because of co-occurrence of epimers at C-16. Since Henderson and McCrindle reported that premarrubiin (18) was converted into marrubiin (19) on distillation in vacuo, heating
for 3 h in refluxing ethanol, or dissolution in CHCl3,22) I might be also artifact.

During the course of our studies on natural antioxidants,23,24) the antioxidative properties of 17, which was isolated as a major compound in this study, was evaluated using several measurements, i.e., superoxide anion ($\mathrm{O}_2^-$) radical scavenging assay, nitrogen oxide (NO) scavenging assay, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, hydrogen peroxide ($\mathrm{H}_2\mathrm{O}_2$) scavenging assay, and metal chelating assay on ferrous ion. The scavenging activities on $\mathrm{O}_2^-$ radical, NO, DPPH radical, and $\mathrm{H}_2\mathrm{O}_2$ were ca. 1.2-fold, ca. 0.8-fold, ca. 0.4-fold, and ca. 0.1-fold of those of a standard sample, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), respectively, at a concentration of 0.50 mM and the activity of metal chelating effect was ca. 0.3-fold of that of a standard sample, ethylenediaminetetraacetic acid (EDTA) at a concentration of 0.50 mM (Table 4).

**Experimental**

All instruments and materials used were the same as cited in a previous report25) unless otherwise specified.

**Plant Material** The fruit of *Vitis aegus-castus* L. was purchased in May 2006 from Charis Seijo Co., Ltd., a commercial supplier of herbs in Tokyo, Japan and identified by one of authors (T. Nohara). A voucher specimen has been deposited at the Laboratory of Natural Products Chemistry, School of Agriculture, Tokai University.

**Extraction and Isolation** The powdered fruit of *V. aegus-castus* (1994 g) was percolated with hexane, acetone, and MeOH at room temperature, and each solvent was removed under reduced pressure to yield hexane extract (188.4 g), acetone extract (36.9 g), and MeOH extract (113.4 g), respectively. The ethyl acetate extract (188.4 g) was percolated with hexane, acetone, and MeOH at room temperature, and each solvent was removed under reduced pressure to yield ethyl acetate extract (16.8 g), acetone extract (15.4 g), and MeOH extract (10.8 g) of the MeOH extract. The MeOH extract was chromatographed over silica gel [Merck, Art. 1.09385, hexane–acetone (7:1, 5:1, 3:1, 1:1, 0:1, MeOH)] to give frs. 5.1—5.10. Fr. 5.2 (44 mg) was chromatographed over silica gel column [Merck, Art. 1.09385, hexane–acetone (20:1, 10:1, 5:1, 3:1, 1:1, 0:1)] to give frs. 3.3.1—3.3.5. Fr. 3.3.5 (6 mg) and fr. 3.3.6 (4 mg) were each subjected to HPLC using the Phenomenex Kinetex C18 (100×4.6 mm, 5 μm) column (MeOH) to give a monosaccharide fr. This fr. was analyzed by HPLC under the following conditions: column, Shodex RS-Pac DC-613, Showa Denko, Tokyo, Japan, 150 mm×4.6 mm; solvent, CH3CN–H2O (3:1); flow rate, 1.0 ml/min; column temperature, 70 °C; detector, DAD, 280 nm. After removal of the solvent under reduced pressure, the residue was extracted with MeOH, and the MeOH extract was chromatographed over silica gel [Merck Art. 1.09385, hexane–acetone (14:2:0.1, 10:2:0.1, 8:2:0.2, 7:3:0.5, 6:4:1)] to afford frs. 3.1—3.15. Fr. 3.12 (574 mg) over silica gel column [Merck, Art. 1.09385, hexane–acetone (20:1, 10:1, 5:1, 3:1, 1:1, 0:1)] to give fr. 3.3.1—3.3.5. Frs. 3.3.5.1—3.3.5.5 was each subjected to HPLC (50% MeOH, 60% MeOH, 70% MeOH, 80% MeOH, 90% MeOH, and MeOH) to yield frs. 3.1.1—3.1.4 and 1 (37 mg). Viteagnuside A (1): Amorphous powder, [ε]28 2.8 (c=0.25, MeOH). Positive FAB-MS m/z: 521 [M+Na]+. HR-FAB-MS m/z: 521.2762 (Calcd for C37H35NaO14: 521.2727). H-NMR spectral data: see Table 1. 13C-NMR spectral data: see Table 2.

**Acid Hydrolysis of 1** Compound 1 (5 mg) was heated in 2×1 HCl (1 ml) at a temperature of 95 °C for 1 h. The reaction mixture was extracted with ethyl acetate (1 ml). The aqueous layer was neutralized with Amberlite MB-3 (Organo Co., Tokyo, Japan) and then evaporated under reduced pressure to give a monosaccharide fr. This fr. was analyzed by HPLC under the following conditions: column, Shodex RS-Pac DC-613, Showa Denko, Tokyo, Japan, 150 mm×0.6 mm; solvent, CH2CN–H2O (3:1); flow rate, 1.0 ml/min; column temperature, 70 °C; detector, DAD, 280 nm; and JASCO OR-2090 plus, and JASCO CO-2060. The retention time and optical activity of the sample were identical with those of 25a (min: 7.1); optical activity: positive; of glucose. However, the ethyl acetate extract exhibited several spots by TLC, and the aglycone of 1 could not be obtained.

**Enzymatic Hydrolysis of 1** Compound 1 (10 mg) was dissolved in CH3COOH–CH3COONa buffer solution (pH 5.5, 1 ml), and β-glucosidase (from Almonds Lot. 124H40281, Sigma Chemical Co., St. Louis, U.S.A., 30 mg) was added. The mixture was left to stand at 37 °C for 14 d. After removal of the solvent under reduced pressure, the residue was extracted with MeOH, and the MeOH extract was chromatographed over silica gel [Merck Art. 1.09385, hexane–acetone (14:2:0.1, 10:2:0.1, 8:2:0.2, 7:3:0.5, 6:4:1)] to give 1a (0.2 mg) and 1b (8.5 mg). The MeOH extract was chromatographed over silica gel [Merck Art. 1.09385, hexane–acetone (7:1, 5:1, 3:1, 1:1, 0:1, MeOH)] to give frs. 5.1—5.10. Fr. 5.2 (44 mg) was chromatographed over silica gel column [Merck, Art. 1.09385, hexane–acetone (20:1, 10:1, 5:1, 3:1, 1:1, 0:1)] to give frs. 3.3.1—3.3.5. Frs. 3.3.5.1—3.3.5.5 was each subjected to HPLC using the Phenomenex Kinetex C18 (100×4.6 mm, 5 μm) column (MeOH) to give a monosaccharide fr. This fr. was analyzed by HPLC under the following conditions: column, Shodex RS-Pac DC-613, Showa Denko, Tokyo, Japan, 150 mm×4.6 mm; solvent, CH2CN–H2O (3:1); flow rate, 1.0 ml/min; column temperature, 70 °C; detector, DAD, 280 nm; and JASCO OR-2090 plus, and JASCO CO-2060. The retention time and optical activity of the sample were identical with those of 25a (min: 7.1); optical activity: positive; of glucose. However, the ethyl acetate extract exhibited several spots by TLC, and the aglycone of 1 could not be obtained.

**Assay of Scavenging Effect on O2•− Radical** The O2•− radical scavenging effect was measured by using the phenazine methosulfate (PMS) β-nicotinamide adenine dinucleotide (reduced form) (NADH) system according to previously described methods.23,24) Briefly, the reaction was started by the addition of NADH into the assay mixture containing nitroblue tetrazolium (NBT) plus test sample, then allowed to proceed for 10 min at room temperature. The absorbance of the resulting solution was measured at 570 nm. Trolox was used as a standard sample.

**Assay of Scavenging Effect on NO** The NO scavenging effect was measured by using the Griess method based on the spontaneous NO generation from sodium nitrous oxide (SNP).25,26) Briefly, the reaction was started by the addition of SNP freshly prepared into the assay mixture containing test sample, allowed for 150 min incubation at room temperature. The absorbance of the resulting solution was measured at 570 nm. Trolox was used as a standard sample.

**Assay of Scavenging Effect on DPPH Radical** The DPPH radical scavenging effect was measured based on the following method.27) Briefly, the reaction was started by the addition of DPPH into the assay mixture containing test sample, then allowed to proceed for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm. Trolox was used as a standard sample.

**Assay of Scavenging Effect on H2O2** The H2O2 scavenging effect was measured using the H2O2-dependent, horseradish peroxidase (HRP)-mediated

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**Table 4. Antioxidative Activity (%) of 17 on the Five Different Antioxidant Analyses**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Trolox</th>
<th>EDTA</th>
</tr>
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<tbody>
<tr>
<td>O2•− radical scavenging activity</td>
<td>14.53 ± 3.25</td>
<td>12.47 ± 3.80</td>
</tr>
<tr>
<td>NO scavenging activity</td>
<td>26.79 ± 2.25</td>
<td>35.40 ± 2.51</td>
</tr>
<tr>
<td>DPPH radical scavenging activity</td>
<td>36.60 ± 4.48</td>
<td>92.99 ± 0.65</td>
</tr>
<tr>
<td>H2O2 scavenging activity</td>
<td>9.88 ± 2.36</td>
<td>99.37 ± 0.17</td>
</tr>
<tr>
<td>Metal chelating activity</td>
<td>29.19 ± 4.02</td>
<td>100.1 ± 1.21</td>
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Data shown represent mean ± S.D. derived from four determinations. The final concentration of each sample tested was 0.50 mM.
ated phenol red oxidation system.26,29) Briefly, the reaction was initiated by the addition of phenol red and HRP into the assay mixture containing H$_2$O$_2$ plus test sample, allowed to proceed for 10 min at room temperature. The resulting solution, terminated with sodium hydroxide, was measured at 610 nm. Trolox was used as a standard sample.

Assay of Chelating Effect on Ferrous Metal Ions The ferrous-ion-chelating effect was measured based on the following method.30) Briefly, the reaction was started by the addition of ferrozine into the assay mixture containing test sample and ferrous chloride, then allowed to proceed for 10 min at room temperature. The absorbance of the resulting solution was measured at 562 nm. EDTA was used as a standard sample.

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References