Isolation of Some Glycosides as Aroma Precursors in Young Leaves of Japanese Pepper (*Xanthoxylum piperitum* DC.)

Lihua Jiang,† Hiroshi Kojima,‡ Kumi Yamada,† Akio Kobayashi,† and Kikue Kubota*,†

Laboratory of Food Chemistry, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan, and Food R & D Center, Japan Tobacco Inc., 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227, Japan

To clarify the formation mechanism for the major alcoholic aroma compounds in young leaves of Japanese pepper, the glycosides were isolated as aroma precursors. The presence of glycosides of the main alcoholic aroma constituents was indirectly determined by enzymatic hydrolysis and trifluoroacetylation (TFA) of the glycoside-containing fraction. After Amberlite XAD-2 column chromatography, ODS flash chromatography, and high-performance liquid chromatography (HPLC), two new compounds, namely, (3*S*,6*S*)-*cis*-linalool-3,7-oxide *β*-D-glucopyranoside and 2-methylpropanyl 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside, were isolated. In addition, (3*S*,6*R*)-*cis*-linalool-3,6-oxide *β*-D-glucopyranoside, which absolute configuration was the first determined, and six known glycosides, citronellyl *β*-D-glucopyranoside, linalyl 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside, (*Z*)-3-hexenyl *β*-D-glucopyranoside, benzyl 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside, dendranthemoside A, and 3,6-dihydroxy-5,6-dihydro-*β*-ionol 9-*β*-D-glucopyranoside, were isolated. All of these glycosides were isolated for the first time from the leaves of Japanese pepper. Their structures were established on the basis of spectral data and chemical evidence. The ratios of stereoisomers of the aglycon moieties of citronellyl *β*-D-glucopyranoside and linalyl 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside were investigated by a chiral GC analysis and compared with those of free citronellol and linalool in the aroma concentrate.

Keywords: *Japanese pepper; Xanthoxylum piperitum DC.; glycoside; aroma precursor; (3S,6S)-cis-linalool-3,7-oxide β-D-glucopyranoside; 2-methylpropanyl 6-O-β-D-apiofuranosyl-β-D-glucopyranoside; (3S,6R)-cis-linalool-3,6-oxide β-D-glucopyranoside*

INTRODUCTION

Japanese pepper (*Xanthoxylum piperitum* DC.) is commonly used in Japanese dishes as a spice that imparts a characteristic aroma and pungent taste. The aroma composition of the leaves and fruits has been reported by several authors (*1*−*4*). It has become apparent that the oxygenated monoterpenes, citronellal and citronellol, are the major contributors to the characteristic aroma. In contrast, young leaves, which have a fresher and less pungent flavor than the fruit, are often utilized as a seasonal herb after being slapped by hand or crushed by a mortar. In our previous study, the main aroma compounds such as citronellal, citronellol, (*Z*)-3-hexenol, and linalool, were dramatically increased by slapping and crushing the young leaves, and these alcoholic compounds were presumed to be liberated by the action of endogenous enzymes (*5*).

Many investigations have reported that glycosidic precursors of the alcoholic aroma compounds were present in many plant materials and that these aroma compounds are released by hydrolysis of the glycosides and play an important role in the flavor of these plants (*6*). Kojima et al. (*4*) have also reported that the alcoholic aroma compounds from young leaves of Japanese pepper, citronellol, (*Z*)-3-hexenol, and benzyl alcohol were liberated from the crude glycoside-containing fraction by enzymatic hydrolysis. We measured the activity of *β*-D-glucosidase in acetone powder prepared from fresh leaves in our previous report (*5*). Analysis of all these results indicated that the major alcoholic aroma compounds produced in young leaves of Japanese pepper were mainly liberated by enzymatic hydrolysis of the corresponding glycosides. However, there has not yet been a report relating to the isolation and structural elucidation of these glycosides.

In this present study, our aim was to provide unequivocal evidence to clarify the aroma formation mechanism in the young leaves of Japanese pepper. Some glycosides of the main aroma compounds were isolated (shown in Figure 1), after confirming their presence by an enzymatic hydrolysis of the crude glycoside fraction and by analyzing their trifluoroacetyl (TFA) derivatives. In addition, the ratio of the stereoisomers of their aglycons was compared with that of the free aroma compounds.

MATERIALS AND METHODS

Materials. Fresh young leaves of Japanese pepper obtained in Shizuoka and Gifu prefectures were preserved at −28 °C after being freeze-dried for 24 h.

Chemicals. The trifluoroacetylating reagent [*N*-methyl bis-(trifluoroacetamide), MBTFA] and phenyl *β*-D-glucopyranoside were purchased from Tokyo Kasei Industries Co. (Tokyo, Japan). (±)-Citronellol was purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan), and (*RS*)-linalool (>98%) was obtained from Kanto Chemical Ltd. (Tokyo, Japan). Four

* To whom correspondence should be addressed (telephone +81-3-5978-5758; fax +81-3-5978-5759; e-mail kubota@cc.ocha.ac.jp).

† Ochanomizu University.

‡ Japan Tobacco Inc.

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isomers of linalool oxide, *trans*-linalool-3,6-oxide, *cis*-linalool-3,6-oxide, *trans*-linalool-3,7-oxide, and *cis*-linalool-3,7-oxide, were provided by Hasegawa Perfume Ltd. (Tokyo, Japan). Polyclar AT, which was used to remove the polyphenols, was purchased from Gokyo Industries Ltd. (Osaka, Japan).

Extraction of the Glycoside Fraction. This fraction was extracted according to Kojima et al. (*4*). Briefly, freeze-dried leaves from a fresh sample were immersed overnight in methanol and then extracted with an 80% methanol aqueous solution to deposit the protein. After the methanol had been evaporated under reduced pressure, the residual aqueous solution was washed with pentane/ether (2:3) to deposit the pigment. The aqueous phase was treated with Polyclar AT, and the solution was concentrated and submitted to Amberlite XAD-2 adsorption column chromatography. After the free sugars, amino acids and volatile compounds had been removed with water and then with pentane/ether, the glycoside fraction was successively eluted with ethyl acetate and methanol, and the two fractions were each concentrated and dried. The fraction prepared for prior to analysis as the TFA derivative was eluted directly with methanol without using ethyl acetate.

Analysis of the Volatile Compounds Released from the Glycoside Fraction. *Preparation of the Crude Enzyme System*. An acetone powder of the crude enzyme system was prepared according to the method described in our previous report (*5*).

Hydrolysis of the Glycoside Fraction. The combined fraction of the ethyl acetate and methanol eluate was hydrolyzed with acetone powder. The glycoside fraction (0.15 g) and 1.5 g of acetone powder were incubated at 37 °C in 40 mL of a sodium citrate buffer (50 mM at pH 5.0) while shaking in a water bath for 24 h. The liberated volatile compounds was extracted by ethyl ether and analyzed by GC and GC-MS. Two solutions without the substrate or enzyme were used as blanks.

GC and GC-MS Analyses. The GC analysis was conducted with a Hewlett-Packard 5890 series II gas chromatograph coupled with a flame ionization detector (FID), in which a DB-WAX capillary column (J&W, 60 m × 0.25 mm i.d.) was connected to the GC instrument. The analytical conditions were as follows: helium carrier gas flow rate, 1.0 mL/min; split ratio, 30:1; injector temperature, 200 °C; column temperature, held at 60 °C for 4 min and then raised to 220 °C at a rate of 2 °C/min; and detector temperature, 220 °C. The GC-MS analysis was conducted with the Hewlett-Packard 5890 series II gas chromatograph coupled to an HP 5972 mass spectrometer. The conditions used for the GC part of the analysis were the same as those described above.

Trifluoroacetylation of the Extracted Crude Glycosides. The method used for trifluoroacetylation was that reported by Wang et al. (*7*). Briefly, the crude glycosides (10 mg), including phenyl β-D-glucopyranoside which had been added as an internal standard to the fresh young leaves before their extraction, were treated with 20 μL of anhydrous pyridine and 25 mg of MBTFA under nitrogen and then heated at 60 °C for 50 min. The GC-MS analysis was similar to that just described, except for using a DB-5 column (J&W, 60 m × 0.25 mm i.d.) instead of the DB-WAX column. The analytical conditions were as follows: column temperature, held at 130 °C for 2 min and then raised to 280 °C at a rate of 2 °C/min; injector temperature, 280 °C; detector temperature, 300 °C; helium carrier gas flow rate, 1.0 mL/min; and split ratio, 15:1.

Isolation of the Glycosides. The crude glycoside fraction (1 g) eluted by ethyl acetate was fractionated by ODS flash chromatography (ODS-SS-1020T, 200 mm × 30 mm i.d.; Senshu Scientific Co., Tokyo, Japan) with stepwise elution of 5, 20, 30, 40, 50, and 100% MeOH/H_2O (1 L each). After the presence of glycosides had been checked by TLC analysis, each fraction was concentrated and then subjected to HPLC on an ODS column (Pegasil ODS, 250 mm × 20 mm i.d.; Senshu Scientific Co., Tokyo, Japan). The flow rate for gradient elution of CH_3CN/H_2O was set at 7 mL/min, and UV detection was at 210 nm.

Spectral Analyses. HRFAB-MS spectral data were measured with glycerol as the matrix by a JEOL MStation JMS-700 instrument. ^1H and ^{13}C NMR spectra were obtained with JEOL JNM-GX 270 and 400 spectrometers. 2D ^1H-^1H double quantum filtered-direct chemical shift correlation spectroscopy (DQF-COSY), inverse detected ^1H-^{13}C heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) data for compounds **2**, **6**, and **7** were measured with a Bruker AVANCE 500 spectrometer and with a Bruker DRX400 instrument for **8** and **9**. Methanol-d_4 with 0.05% tetramethylsilane (TMS, 99.8%, Isotec) or D_2O (99.8%, Merck) with 3-(trimethylsilyl)propionic acid sodium salt (TSP, >99%, Acros) were used as the solvent and internal standard, respectively.

Compound **1**. HRFAB-MS: 317.1980 $[M - H]^-$ ($C_{16}H_{29}O_6$, requires 317.1964); ^1H NMR (270 MHz, CD_3OD/TMS, ppm, *J* in Hz) δ 0.90 (3H, d, $J = 6.6$, H-10), 1.15−1.20 (1H, m, H-4a), 1.23−1.32 (2H, m, H-4b and 2a), 1.40−1.67 (2H, m, H-2b and 3), 1.60 (3H, s, H-8), 1.67 (3H, s, H-9), 2.01 (2H, m, H-5), 3.15 (1H, dd, $J = 7.6, 8.9$, H-2′), 3.24−3.34 (3H, overlapped with solvent, H-3′, 4′ and 5′), 3.54−3.60 (1H, m, H-1a), 3.66 (1H, dd, $J = 5.8, 11.7$, H-6′a), 3.86 (1H, dd, $J = 1.9, 11.5$, H-6′b), 3.91−3.95 (1H, m, H-1b), 4.24 (1H, d, $J = 7.6$, H-1′), 5.10 (1H, t, H-6).

Compound **2**. HRFAB-MS: 447.2220 $[M - H]^-$ ($C_{21}H_{35}O_{10}$, requires 447.2230); $[\alpha]_{589}^{27}$ −51.2° (MeOH, *c* 0.26); ^1H NMR (270 MHz, D_2O/TSP, ppm, *J* in Hz) δ 1.39 (3H, s, H-10), 1.62 (3H, s, H-8), 1.69 (3H, s, H-9), 1.57−1.74 (2H, m, H-4), 2.00 (2H, m, H-5), 3.23 (1H, t, H-2′), 3.36 (1H, t, H-4′), 3.43 (1H, t, H-3′), 3.49−3.55 (1H, m, H-5′), 3.64−3.70 (1H, overlapped with H-5″, H-6′a), 3.67 (2H, s, H-5″), 3.89 (1H, d, $J = 9.9$, H-4″a), 3.98 (1H, d, $J = 3.0$, H-2″), 4.00 (1H, overlapped with H-4″a and 2″, H-6′b), 4.06 (1H, d, $J = 10.2$, H-4″b), 4.53 (1H, d, $J = 7.9$, H-1′), 5.09 (1H, d, $J = 3.3$, H-1″), 5.20 (2H, m, H-6), 5.29 (H, d, $J = 16.8$, H-1a), 5.30 (H, d, $J = 11.5$, H-1b), 5.88 (2H, dd, $J = 10.6, 18.1$, H-2).

Compound **3**. HRFAB-MS: 333.1950 $[M + H]^+$ ($C_{16}H_{29}O_7$, requires 333.1913); $[\alpha]_{589}^{28}$−30.2° (MeOH, *c* 0.58); ^1H NMR (270 MHz, CD_3OD/TMS, ppm, *J* in Hz) δ 1.23 (3H, s, H-9), 1.26 (3H, s, H-8), 1.30 (3H, s, H-10), 1.68−1.92 (4H, m, H-4 and 5), 3.14 (1H, dd, $J = 7.7, 9.0$, H-2′), 3.25−3.37 (3H, overlapped with solvent, H-3′, 4′ and 5′), 3.63 (1H, dd, $J = 5.3, 11.9$, H-6′a), 3.82 (1H, dd, $J = 2.1, 11.7$, H-6′b), 4.08 (1H, t, H-6), 4.56 (1H, d, $J = 7.9$, H-1′), 4.98 (1H, dd, $J = 1.7, 10.7$, H-1a), 5.22 (1H, dd, $J = 1.7, 17.5$, H-1b), 6.00 (1H, dd, $J = 10.6, 17.5$, H-2).

Compound **4**. HRFAB-MS: 331.1793 $[M - H]^-$ ($C_{16}H_{27}O_7$, requires 331.1757); $[\alpha]_{589}^{26}$−4.1° (MeOH, *c* 0.41); ^1H NMR (270 MHz, CD_3OD/TMS, ppm, *J* in Hz) δ 1.11 (3H, s, H-10), 1.20 (3H, s, H-9), 1.36 (3H, s, H-8), 1.54−1.62 (1H, m, H-4a), 1.73−1.87(1H, m, H-5a), 1.96−2.03 (1H, m, H-5b), 2.13−2.19 (1H, m, H-4b), 3.13 (1H, t, H-2′), 3.27−3.34 (3H, overlapped with solvent, H-3′, 4′ and 5′), 3.41 (1H, dd, $J = 11.2, 4.3$, H-6), 3.66 (1H, d, $J = 12.2$, H-6′a), 3.85 (1H, d, $J = 12.2$, H-6′b), 4.32 (1H, d, $J = 7.6$, H-1′), 5.00 (2H, dd, $J = 11.2, 17.1$, H-1), 5.96 (1H, dd, $J = 11.2, 18.1$, H-2).

Compound **5**. HRFAB-MS: 261.1313 $[M - H]^-$ ($C_{12}H_{21}O_6$, requires 261.1339); $[\alpha]_{589}^{26}$ −40.6° (MeOH, *c* 0.19); ^1H NMR (270 MHz, CD_3OD/TMS, ppm, *J* in Hz) δ 0.90 (3H, t, H-6), 2.07 (2H, quin, H-5), 2.38 (2H, quar, H-2), 3.16 (1H, dd, $J = 7.9, 8.9$, H-2′), 3.25−3.34 (3H, overlapped with solvent, H-3′, 4′ and 5′), 3.53 (1H, m, H-1a), 3.65 (1H, m, H-6′a), 3.85 (2H, m, H-1b and 6′a), 4.26 (1H, d, $J = 7.6$, H-1′), 5.33−5.47 (2H, m, H-3 and 4).

Compound **6**. HRFAB-MS: 401.1409 $[M - H]^-$ ($C_{18}H_{25}O_{10}$, requires 401.1448); $[\alpha]_{589}^{28}$ −80.0° (MeOH, *c* 0.60); ^1H NMR (400 MHz, D_2O/TSP, ppm, *J* in Hz) δ 3.31 (1H, t, $J = 7.9, 9.1$, H-2′), 3.42 (1H, t, $J = 7.3, 8.9$, H-4′), 3.46 (1H, t, $J = 8.9, 7.0$, H-3′), 3.56 (1H, m, H-5′), 3.68 (1H, s, H-5″), 3.74 (1H, dd, $J = 6.1, 11.9$, H-6′a), 3.91 (1H, d, $J = 10.4$, H-4″a), 4.03 (1H, d, $J = 3.0$, H-2″), 4.05 (1H, overlapped with H-4″b and 2″, H-6′b), 4.07 (1H, d, $J = 10.0$, H-4″b), 4.53 (1H, d, $J = 7.9$, H-1″), 4.74−4.78 (1H, overlapped with solvent, H-7b), 4.79 (1H, d, $J = 11.6$, H-7a), 5.13 (1H, d, $J = 3.3$, H-1″), 7.41−7.49 (5H, m, from C_6H_5).

Compound **7**. HRFAB-MS: 367.1606 $[M - H]^-$ ($C_{15}H_{27}O_{10}$, requires 367.1604); $[\alpha]_{589}^{26}$ −114.7° (MeOH, *c* 0.17); ^1H NMR

(270 MHz, D_2O/TSP, ppm, J in Hz) δ 0.90 (3H, d, J = 2.3, H-4), 0.93 (3H, d, J = 2.3, H-3), 1.91 (1H, m, H-2), 3.28 (1H, t, H-2′), 3.40 (1H, t, H-4′), 3.44−3.48 (1H, m, H-1a), 3.50 (1H, t, H-3′), 3.57 (1H, m, H-5′), 3.68 (1H, s, H-5″), 3.65−3.71 (1H, m, H-1b), 3.73 (1H, dd, J = 6.3, 11.5, H-6′a), 3.90 (1H, d, J = 10.2, H-4″a), 4.00 (1H, d, J = 3.3, H-2″), 4.03 (1H, overlapped with H-4″b and 2″, H-6′b), 4.07 (1H, d, J = 10.2, H-4″b), 4.45 (1H, d, J = 7.9, H-1′), 5.13 (1H, d, J = 3.3, H-1″).

Compound **8**. HRFAB-MS: 389.2184 $[M - H]^-$ ($C_{19}H_{33}O_8$, requires 389.2175); $[\alpha]_{589}^{27}$ −39.4° (MeOH, *c* 0.72); ^{1}H NMR (400 MHz, CD_3OD/TMS, ppm, J in Hz) δ 0.84 (3H, d, J = 6.8, H-13), 0.87 (3H, s, H-12), 0.97 (3H, s, H-11), 1.25 (3H, d, J = 6.4, H-10), 1.50 (1H, m, H-4a), 1.56 (1H, m, H-2a), 1.67 (1H, m, J = 12.0, H-2b) 1.81 (1H, m, H-4b), 1.95 (1H, m, H-5), 3.13 (1H, dd, J = 7.8, 9.0, H-2′), 3.26−3.35 (3H, overlapped with solvent, H-3′, 4′ and 5′), 3.66 (1H, m, H-6′a), 3.86 (1H, m, H-6′b), 3.95 (1H, m, H-3), 4.30 (1H, m, H-9), 4.35 (1H, d, J = 7.8, H-1′), 5.55 (1H, d, J = 15.7, H-7), 5.73 (1H, dd, J = 6.0, 15.7, H-8).

Compound **9**. HRFAB-MS: 389.2135 $[M - H]^-$ ($C_{19}H_{33}O_8$, requires 389.2095); $[\alpha]_{589}^{27}$ −52.0° (MeOH, *c* 0.33); ^{1}H NMR (400 MHz, CD_3OD/TMS, ppm, J in Hz) δ 0.88 (3H, d, J = 6.8, H-13), 0.87 (3H, s, H-12), 0.95 (3H, s, H-11), 1.29 (3H, d, J = 6.6, H-10), 1.40 (1H, m, H-2a), 1.41 (1H, m, H-4a), 1.66 (1H, m, J = 12.0, H-2b) 1.70 (1H, m, H-4b), 1.97 (1H, m, H-5), 3.14−3.20 (1H, dd, J = 7.8, 9.0, H-5′), 3.16−3.22 (1H, m, H-2′), 3.25−3.35 (3H, overlapped with solvent, H-3′ and 4′), 3.64 (1H, dd, J = 6.0, 11.9, H-6′a), 3.80 (1H, m, H-3), 3.86 (1H, dd, J = 2.2, 11.9, H-6′b), 4.35 (1H, d, J = 7.8, H-1′), 4.50 (1H, m, H-9), 5.56 (1H, dd, J = 6.0, 15.7, H-8), 5.67 (1H, d, J = 15.7, H-7).

Chiral GC Analysis. Isolated glycosides **1**, **2**, **3**, and **4** were hydrolyzed by Rohapect D5L in the same manner as that described previously. The liberated aglycon moieties were analyzed by GC as already described. An SP α-DEX 120 column (30 m × 0.25 mm i.d.) for **1** and a CP-cyclodextrin-β-2, 3, 6-M-19 column (50 m × 0.25 mm i.d.) for **2**, **3,** and **4** were used in place of the DB-WAX column. The oven temperature was held isothermally at 95 °C for **1**, **2,** and **3** and at 115 °C for **4**. The other analytical conditions were as follows: helium carrier gas flow rate, 1.0 mL/min; split ratio, 30:1; injector temperature, 200 °C; and detector temperature, 220 °C.

RESULTS AND DISCUSSION

Presence of the Glycosides of the Main Aroma Compounds. It has been demonstrated in our previous work that the oxygenated monoterpenes, citronellal, citronellol, and linalool, and C_6 compounds, (*Z*)-3-hexenol, (*Z*)-3-hexenal, and (*E*)-2-hexenal, mainly contributed to the flavor characteristics of young leaves of Japanese pepper. Furthermore, it has been proposed that the formation of the major alcoholic aroma compounds was primarily by enzymatic hydrolysis of the corresponding glycosides (*5*). Although Kojima et al. have already described that alcoholic aroma compounds such as (*Z*)-3-hexenol, citronellol, and benzyl alcohol can be liberated from the glycoside-containing fraction by endogenous enzymatic action (*4*), we identified additional alcoholic aroma compounds in our previous study (*5*). Consequently, we again investigated the constituents from the hydrolysis of glycosides with acetone powder prepared from fresh leaves. The results are summarized in Table 1. It is apparent that (*Z*)-3-hexenol, citronellol, linalool, benzyl alcohol, *cis*-linalool-3,6-oxide, *cis*-linalool-3,7-oxide, and 2-methylpropanol were released by this action. These results suggested the possibility that more alcoholic aroma compounds existed as glycosides in the young leaves of Japanese pepper.

In addition, trifluoroacetylation (TFA) of the extracted glycoside was conducted as a more direct method for the identification of glycosides. The results of the GC-MS analysis are summarized in Figure 2 and Table 2. The amount of each glycoside in Table 2 was calculated by comparing the peak areas to that of the internal standard. Comparing the mass spectra and the retention indices of the TFA derivatives of the extracted glycosides with the data of the authentic samples in the literatures reported by Wang et al. (*7*) and Voirin et al. (*8*), seven glycosides were each identified (Table 2). Moreover, the amount of glycosides implied that the alcoholic aroma formed under mechanical stimulus was closely correlated with the quantities of the corresponding glycosides, especially for the formation of (*Z*)-3-hexenol in the slapped leaves (*5*).

Table 1. Major Volatile Compounds Liberated from the Glycosides of Young Leaves of Japanese Pepper by Acetone Powder

compound	KI[a]	peak area[b]
2-methylpropanol	1104	21
1-penten-3-ol	1204	5
(*Z*)-2-penten-3-ol	1327	21
(*Z*)-3-hexenol	1411	56
cis-linalool-3,6-oxide	1494	133
linalool	1583	2
cis-linalool-3,7-oxide	1784	96
citronellol	1789	14
methyl salicylate	1805	14
exo-2-hydroxycineole	1888	91
benzyl alcohol	1896	218
2-phenylethanol	1928	7

[a] Kovats index on DB-Wax. [b] Yields of volatile compounds are presented by comparing their peak areas with that of the internal standard, ethyl decanoate, which is assigned the numerical value of 100.

Isolation and Structural Elucidation of the Glycosides. The ethyl acetate fraction was used to isolate the glycosides, because this fraction included more aroma compounds released by the hydrolytic action than the methanol fraction (data not shown). Repeated ODS flash chromatography and HPLC enabled nine glycosides to be isolated. Compounds **1**, **2**, and **3** were isolated from the 50%, 40%, and 30% MeOH/H_2O fractions, while compounds **4**−**9** were obtained from the 20% MeOH fraction. Their structures were elucidated by NMR, mass spectroscopic, and chemical evidence.

The sugar moieties of compounds **1**−**7** were divided in two groups, because they each exhibited similar NMR spectra. One group consisted of compounds **1**, **3**, **4,** and **5** and the other of compounds **2**, **6,** and **7**.

The ^{13}C NMR and HRFAB-MS spectra indicated that the sugar moiety of compounds **1**, **3**, **4**, and **5** was a hexose unit. In the ^{1}H NMR spectrum, the signals of H-2′-5′ at δ 3.15−3.37 and an anomeric proton with β-configuration (δ 4.24−4.56, d, J = 7.6 or 7.9, H-1′) were observed. These data show that the sugar moiety was β-D-glucopyranose, this structure also being ascertained by the ^{13}C NMR spectral data except for a small difference in shift of the anomeric carbon (Table 3).

The aglycon moiety of **1** was assumed to be a monoterpenoid consisting of 10 signals, from the ^{13}C NMR spectrum and supported by HRFAB-MS data. In the ^{1}H NMR spectrum, one doublet and two singlets of methyl groups (H-10, 8, and 9) were observed, the latter two indicating that they were adjacent to a double bond. A broad triplet at δ 5.10 (H-6) also corresponded to the presence of a double bond. These spectral data suggested the aglycon of **1** to be citronellol. A comparison with the reported data (*9*−*11*) enabled **1** to be identified as citronellyl 6-*O*-β-D-glucopyranoside.

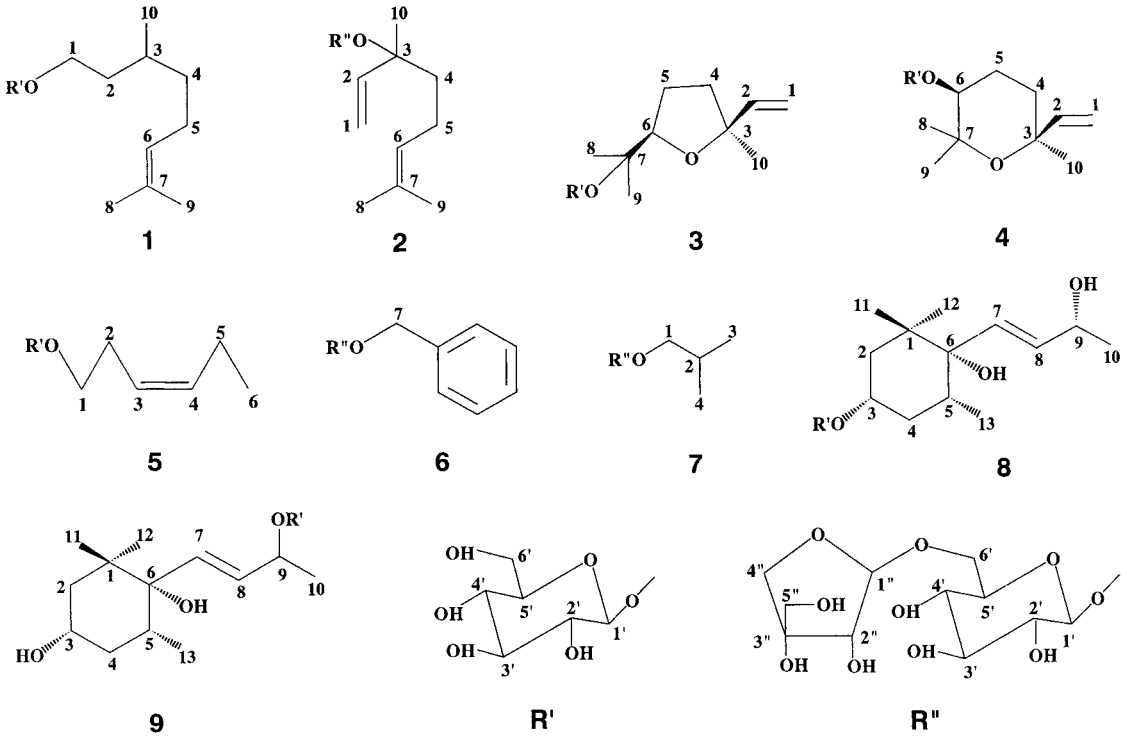

Figure 1. Structures of the glycosides isolated from the young leaves of Japanese pepper.

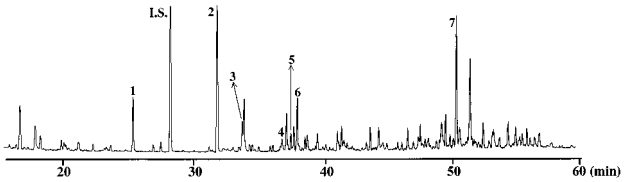

Figure 2. Gas chromatogram of TFA derivatives of the glycosides extracted from the fresh leaves of Japanese pepper. 1−7 represent TFA derivatives of the *β*-D-glucopyranosides of (*Z*)-3-hexenol, benzyl alcohol, (3*S*,6*R*)-*cis*-linalool-3,6-oxide, (3*S*,6*R*)-*trans*-linalool-3,7-oxide, (3*S*,6*S*)-*cis*-linalool-3,7-oxide, and citronellol and of the 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside of benzyl alcohol, respectively.

Table 2. Identification and Contents of Glycosides Extracted by Using TFA Derivatives from Young Leaves of Japanese Pepper

peak no.	compound[a]	RRT[b]	ref	content (mg/100 g of fresh leaves)[d]
1	(*Z*)-3-hexenyl glucoside	0.897	[c]	1.9
2	benzyl glucoside	1.127	[c]	7.3
3	(3*S*,6*R*)-*cis*-linalool-3,6-oxide glucoside	1.195	[c]	1.2
4	(3*S*,6*R*)-*trans*-linalool-3,7-oxide glucoside	1.292	[c]	0.8
5	(3*S*,6*S*)-*cis*-linalool-3,7-oxide glucoside	1.321	[c]	1.1
6	citronellyl glucoside	1.339	[e]	2.7
7	benzyl apiosyl glucoside	1.784	[f]	7.1

[a] Glucoside: *β*-D-glucopyranoside; apiosyl glucoside: 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside. [b] RRT, relative retention time to that of phenyl *β*-D-glucoside in a DB-5 column. [c] Data are cited from ref *7*. [d] Contents were calculated by comparing the peak areas of the TFA derivatives with that of the internal standard, phenyl *β*−D-glucopyranoside, which was added at 16.9 mg/100 g of fresh leaves. [e] Data are cited from our laboratory records. [f] Data are cited from ref *8*.

The aglycon moiety liberated from the hydrolysis of **1** with glycosidase was submitted to chiral GC to elucidate the absolute configuration at the C-3 position. The result is shown in Figure 3. It is known that the elution order proceeds from (*R*)- to (*S*)-citronellol (*12*). The ratio of (*R*)- and (*S*)-citronellol (77:23) was relatively similar to that of free citronellol (74:26) extracted directly with ethyl ether from the fresh young leaves, by a comparison with authentic racemic citronellol. This indicates that the free citronellol liberated under mechanical stimulus was closely correlated with compound **1**.

The aglycon moiety of **3** was also presumed to be a monoterpenoid from the molecular formula of $C_{16}H_{28}O_7$. In the ^{1}H NMR spectrum, a cyclic structure was found from the appearance of a multiplet signal of two methylene groups at *δ* 1.68−1.92 (H-4 and 5). A double doublet proton signal (*δ* 6.00, H-2) and two AB-type proton signals (*δ* 4.98, H-1a and *δ* 5.22, H-1b) indicated the existence of a terminal vinyl group. With the three methyl group signals (*δ* 1.26, 1.23, and 1.30, H-8, 9, and 10), the aglycon moiety of **3** was assumed to be linalool-3,6-oxide. According to the reported elution order (*13*), the chiral GC analysis of the aglycon of **3** from enzymatic hydrolysis confirmed that the aglycon moiety was (3*S*,6*R*)-*cis*-linalool-3,6-oxide (Figure 4). Therefore, compound **3** was characterized as (3*S*,6*R*)-*cis*-linalool-3,6-oxide *β*-D-glucopyranoside. The isolation of the *β*-glucoside of *cis*-linalool-3,6-oxide from wine have been reported (*14*), but its absolute configuration of aglycon at C-3 and 6 position has not been assigned. This study is the first determined its absolute structure.

The aglycon structure of **4** was identified in a manner similar to that of **3**. The molecular formula was found to be $C_{16}H_{28}O_7$. Based on the ^{1}H and ^{13}C NMR spectral data and on the optical GC analysis as for **3**, the aglycon moiety of **4** was identified as (3*S*,6*S*)-*cis*-linalool-3,7-oxide (Figure 4). Thus, **4** was elucidated as (3*S*,6*S*)-*cis*-linalool-3,7-oxide *β*-D-glucopyranoside, this being a new natural plant compound.

The ^{13}C NMR and HRFAB-MS spectral data showed that the aglycon moiety of **5** was a C_6 alcohol. The

Table 3. ^{13}C NMR Data for the Glycosides Isolated from Young Leaves of Japanese Pepper

	1	2	3	4	5	6	7	8	9
	CD_3OD/TMS 270 MHz	D_2O/TSP 400 MHz	CD_3OD/TMS 270 MHz	CD_3OD/TMS 270 MHz	CD_3OD/TMS 270 MHz	D_2O/TSP 400 MHz	D_2O/TSP 270 MHz	CD_3OD/TMS 400 MHz	CD_3OD/TMS 400 MHz
C-1	69.1	119.1	111.8	111.4	70.4		80.0	40.4	40.4
2	37.8	144.4	145.2	147.3	28.8	139.4	30.6	42.5	45.8
3	30.6	84.5	84.4	74.8	125.7	131.5	21.2	75.6	67.5
4	38.5	43.0	38.7	33.7	134.3	131.4	21.2	38.1	39.9
5	26.5	25.1	28.1	25.8	21.6	131.3		35.5	35.6
6	125.8	127.3	85.2	85.7	14.7			78.3	78.2
7	131.8	136.3	80.1	77.1		74.5		133.7	138.8
8	17.8	19.7	23.4	30.1				135.5	132.6
9	25.9	27.6	22.7	22.0				69.2	75.2
10	20.0	24.6	25.9	32.3				24.2	22.5
11								25.1	25.2
12								25.8	25.8
13								16.5	16.9
C-1′	104.3	100.1	98.3	106.2	104.2	104.1	105.2	102.6	100.5
2′	75.1	76.0	75.3	75.3	75.1	75.9	75.8	75.1	75.0
3′	78.1	78.7	78.0	78.1	78.1	78.6	78.5	78.0	78.3
4′	71.6	72.7	71.6	71.6	71.6	72.5	72.5	71.7	71.7
5′	77.9	77.2	77.6	77.7	77.9	77.6	77.4	77.8	78.1
6′	62.8	70.7	62.8	62.8	62.7	70.4	70.5	62.8	62.9
C-1″		111.9				111.8	111.6		
2″		79.6				79.5	79.3		
3″		82.2				82.2	82.1		
4″		76.4				76.5	76.3		
5″		66.5				66.4	66.3		

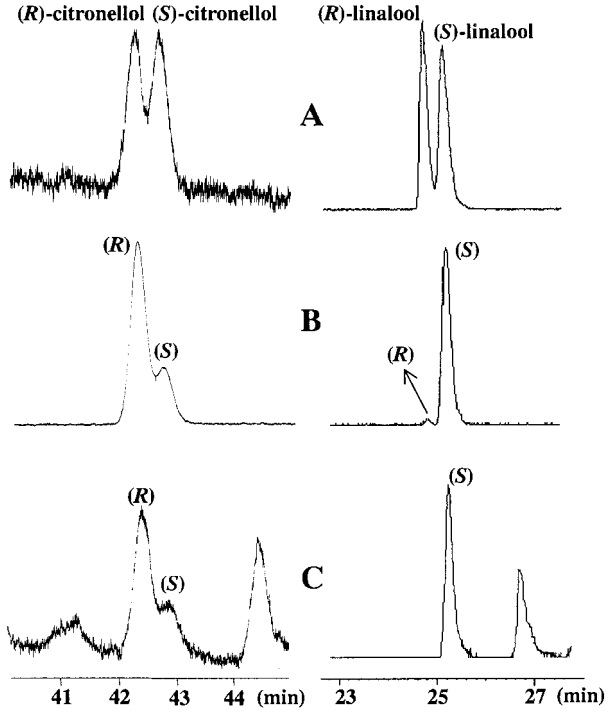

Figure 3. Comparative gas chromatograms of the aglycons of compounds **1** (left side) and **2** (right side) and their corresponding free aroma compounds in the chiral column: (A) authentic racemic samples; (B) aglycon moiety; and (C) free aroma compounds extracted from the young leaves by ethyl ether.

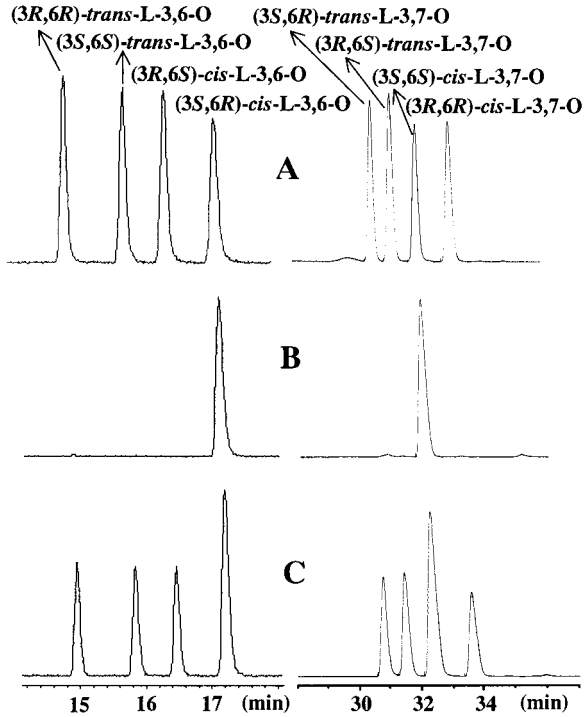

Figure 4. Chiral gas chromatograms of the aglycon fractions of compound **3** (left side) and **4** (right side): (A) authentic racemic samples; (B) aglycon moiety; and (C) mixture of A and B. L-3,6-O: linalool-3,6-oxide; L-3,7-O: linalool-3,7-oxide.

analysis of the ^{1}H and ^{13}CNMR spectral data and a comparison with reference data (*15*, *16*) identified compound **5** as (*Z*)-3-hexenyl *β*-D-glucopyranoside. This result strongly suggests that (*Z*)-3-hexenol formed in slapped leaves was liberated from the corresponding glucoside by enzymatic action as proposed in our previous study (*5*).

The sugar moiety of the group of compounds **2**, **6**, and **7** was found to consist of a hexose and a pentose from the 11 carbon signals containing two anomeric carbons in the ^{13}C NMR spectra. On the basis of the ^{1}H and ^{13}C NMR spectra, and DQF-COSY and HMQC experiments, the sugar moiety was confirmed to consist of a *β*-D-apiofuranose and a *β*-D-glucopyranose. Additionally, a cross-peak between H-1″ and H-6′ detected in the HMBC experiment enabled the sugar moiety of this group to be established as 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranose.

The aglycon moiety of **2** had a terminal vinyl group (H-1a, 1b, and 2), a double bond which was adjacent to two methyl groups (H-8 and 9), and a multiplet proton (H-6) in the ^{1}H and ^{13}C NMR spectra. Additionally, the

identification of a methyl group adjacent to a quaternary carbon (H-10) and two methylene groups (H-4 and 5) indicated that the aglycon of **2** was linalool. Moreover, a cross-peak between H-3 and H-1′ detected in the HMBC spectrum concluded **2** as linalyl 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside, a structure that corresponded well with the published data (*17*, *18*).

The optical isomer of linalool liberated from **2** was analyzed as **1**. The ratio of (*R*)- and (*S*)-linalool was 2:98, very similar to that of free linalool of only (*S*)-form (Figure 3). The flavor note of (*S*)-linalool, with a more citruslike aroma, was different from that of (*R*)-linalool with a woody lavender note (*19*). This result suggested that the citruslike and floral flavor produced from the young leaves in our previous report (*5*) were closely associated with (*S*)-linalool released from **2**.

The aglycon moiety of **6** was identified as benzyl alcohol from the prominent benzyl group and a methylene group in the ^{1}H and ^{13}C NMR spectra. The DQC-COSY, HMQC, and HMBC experiments enabled **6** to be established as benzyl 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside. This compound was also confirmed by comparing with the reference data (*20*, *21*).

The ^{1}H and ^{13}C NMR spectra of the aglycon moiety of **7** was simple. Four signals from two methyl groups at δ 21.2 (H-3 and 4), one methine group at δ 30.6 (H-2), and one anomeric carbon at δ 80.0 (H-1) were observed, apart from the 11 signals of the sugar moiety in the ^{13}C NMR spectrum. DQF-COSY, HMQC, and HMBC experiments identified **7** as 2-methylpropanyl 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside, this compound also being a new glycoside from a plant source.

Finally, the structures of compounds **8** and **9** were elucidated. The molecular formula of **8** was found to be $C_{19}H_{34}O_8$ from the HRFAB-MS data. Among 19 carbon signals observed in the ^{13}C NMR spectrum, six were attributed to *β*-D-glucopyranose as the sugar moiety. The ^{1}H and ^{13}C NMR spectral data indicated the remaining structure to have a cyclic configuration, four methyl groups, one double bond, and two hydroxyl groups. In addition, after analyzing the results from DQF-COSY, HMQC, HMBC, and NOESY (nuclear Overhauser enhancement) experiments, **8** was confirmed to be dendranthemoside A, a previously known compound isolated from *Dendranthema shiwogiku* and *Alangium premnifolium* (*22*, *23*).

Compound **9** was determined to have the molecular formula $C_{19}H_{34}O_8$ by HRFAB-MS, this being the same as **8**. The sugar moiety was confirmed to be *β*-D-glucopyranose from the ^{1}H and ^{13}C NMR spectral data and tested by HPLC in comparison with authentic sugars after the enzymatic hydrolysis (data not shown). The aglycon moiety was indicated to be a positional isomer of that of **8** by the ^{1}H and ^{13}C NMR spectra and DQF-COSY, HMQC, HMBC, and NOESY experiments. The glucosylation-induced downfield shift was observed at the C-9 carbon atom, this being similar to that of alangionoside A (*23*). However, different chemical shifts at C-7, 8, 9, 10, and 1′ were observed. Since the absolute structure at C-7 and 8 was indicated to be of trans configuration by the coupling constant (J = 15.8 Hz), the chiral center at C-9 is considered to be responsible for the differences. The absolute structure at C-9 of compound **9** is not clear, although that of alangionoside A is known to have an *R* configuration. Similar data to those for **9** have been reported by Ramadan et al. (*24*).

This isolation of glycosides as aroma precursors is the first instance for young leaves of Japanese pepper. These results suggest that the nonvolatile precursors of the main alcoholic aroma, citronellol, linalool, and (*Z*)-3-hexenol, responsible for the citruslike, floral, and fresh grassy flavor, exist in combination with at least two different sugar moieties, i.e., *β*-D-glucopyranose and 6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranose, in young leaves of Japanese pepper. Furthermore, these glycosides can be hydrolyzed by the action of endogenous enzymes that seem to be activated by external stimuli like mechanical pressure. The role of the glycosides of C_{13}-norisoprenoids, considered to be carotenoid biodegradation products, in flavor formation has been discussed in detail in a model degradation study (*25*), although their real role in plants still needs to be clarified. As described in Introduction section, many glycosides with the alcohol aglycon in the diverse plants have been isolated, and it had been also confirmed that their alcohol aglycons can be released by endogenous glycosidases. Moreover, based on the determination of the activity of *β*-D-glucosidase in our previous study (*5*) and the results of the isolation of glycosides and the enzymatic hydrolysis in the present study, we conclude that complex and diverse glycosides exist and play an important role in the aroma formation in young leaves of Japanese pepper.

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