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Isolation and Structure of Whiskey Polyphenols Produced by Oxidation of Oak Wood Ellagitannins

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Three new phenolic compounds named whiskey tannins A and B and carboxyl ellagic acid were isolated from commercial Japanese whiskey, along with gallic acid, ellagic acid, brevifolin carboxylic acid, three galloyl glucoses, a galloyl ester of phenolic glucoside, 2,3-(*S*)-hexahydroxydiphenoylglucose, and castacrenin B. Whiskey tannins A and B were oxidation products of a major oak wood ellagitannin, castalagin, in which the pyrogallol ring at the glucose C-1 position of castalagin was oxidized to a cyclopentenone moiety. These tannins originated from ellagitannins contained in the oak wood used for barrel production; however, the original oak wood ellagitannins were not detected in the whiskey. To examine whether the whiskey tannins were produced during the charring process of barrel production, pyrolysis products of castalagin were investigated. Dehydrocastalagin and a new phenolcarboxylic acid trislactone having an isocoumarin structure were isolated, along with castacrenin F and ellagic acid. However, whiskey tannins were not detected in the products.

KEYWORDS: Ellagitannin; castalagin; oak; oxidation; whiskey

INTRODUCTION

In whiskey production, distilled spirits are aged for several years in oak barrels, and the constituents of the wood dissolve into the spirit to determine its color, flavor, and taste. Although oak woods contains C-glycosidic ellagitannins, such as castalagin, vescalagin, grandinin, and roburins A−E (*1*), the polyphenols in whiskey are different from the original oak wood tannins. First, tannins decompose during the toasting or charring process in barrel production, in which the inside of the barrel is scorched with fire (*2*). In addition, during the aging process, oxygen molecules penetrate into the spirits through the barrel wood and oxidize the solutes. Therefore, the polyphenols in whiskey are a mixture of products generated through a complex chemical process. To clarify the structures of polyphenols in commercial whiskey, the present study first compared the polyphenol concentration of some commercial bottled Japanese whiskey, and then polyphenols were isolated from the whiskey with the highest polyphenol content. In addition, pyrolysis of castalagin, the major C-glycosidic ellagitannin of oak wood, was examined as a mimic of decomposition during the charring process in barrel production and the products were characterized.

MATERIALS AND METHODS

Materials. Castalagin was isolated as a white microcrystalline powder from Japanese chestnut heart wood (*Castanea crenata*, Fagaceae) (*3*). Commercial pure malt whiskey was supplied through the courtesy of Suntory Limited, Osaka, Japan.

General Procedures. Ultraviolet (UV) spectra were obtained with a JASCO V-560 UV−vis spectrophotometer (JASCO Co., Tokyo, Japan), and optical rotations were measured with a JASCO DIP-370 digital polarimeter. ^{1}H, ^{13}C NMR, ^{1}H−^{1}H correlation spectroscopy (COSY), ^{1}H NMR nuclear Overhauser and exchange spectroscopy (NOESY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond connectivity (HMBC) spectra were recorded in acetone-d_6, MeOH-d_4, DMSO, and a mixture of acetone-d_6/D_2O (0.8 mL/2 drops) at 27 °C with a Varian Unity Plus 500 spectrometer (Varian, Inc., Palo Alto, CA) operating at 500 MHz for ^{1}H and at 125 MHz for ^{13}C. Coupling constants are expressed in hertz, and chemical shifts are given on a δ (parts per million) scale. HMQC (J_{CH} = 140 Hz), HMBC ($^{n}J_{CH}$ optimized for 8 Hz), and NOESY (mixing time = 0.50 s) experiments were performed using standard Varian pulse sequences. Mass spectra were recorded on a JEOL JMS-700N spectrometer (JEOL Ltd., Tokyo, Japan) or an Voyager-DE PRO (Applied Biosystems, Foster City, CA); glycerol or *m*-nitrobenzyl alcohol was used as the matrix for fast atom bombardment mass spectroscopy (FAB-MS) measurements, and 2,5-dihydroxybenzoic acid

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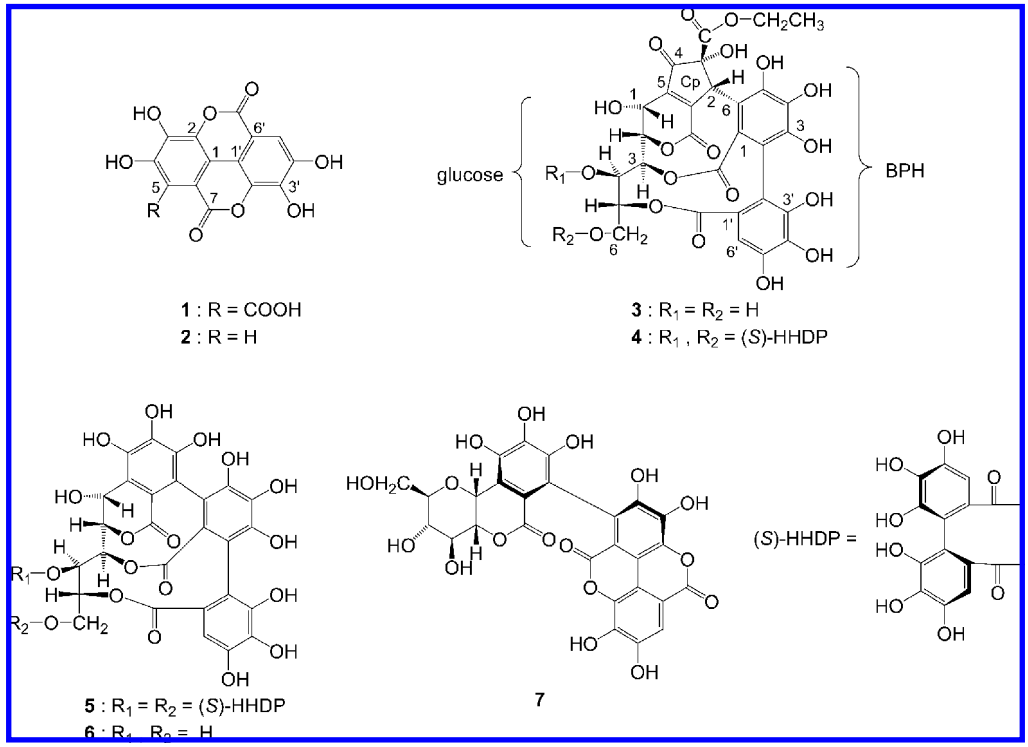

Figure 1. Structures of compounds **1**−**7**.

was used as the matrix for matrix-assisted laser desorption ionization MS (MALDI-TOF-MS).

Column chromatography was performed using a Diaion HP20SS, MCI gel CHP20P (75−150 μm) (Mitsubishi Chemical Co., Tokyo, Japan), Sephadex LH-20 (25−100 μm) (GE Healthcare Bio-Sciences, Uppsala, Sweden), TSK gel Toyopearl HW-40F (TOSOH Co. Tokyo, Japan), Funacel microcrystalline cellulose (Funakoshi, Co., Tokyo, Japan), and Chromatorex ODS (Fuji Silysia Chemical Ltd., Kasugai, Japan). Thin-layer chromatography (TLC) was performed on 0.2 mm precoated Kiesel gel 60 F_{254} plates (Merck Ltd. Japan, Tokyo, Japan) with toluene/ethyl formate/formic acid (1:7:1, v/v) or chloroform/methanol/water (14:6:1, v/v). TLC was also performed on a 0.1 mm precoated Cellulose F plate (Merck) with 2% acetic acid. Spots were detected by UV illumination and by spraying with 2% ethanolic $FeCl_3$ or 10% sulfuric acid reagent, followed by heating. Analytical HPLC was performed on a 250 × 4.6 mm i.d. Cosmosil $5C_{18}$-AR II column (Nacalai Tesque, Inc. Kyoto, Japan) with gradient elution of 4−30% (39 min) and 30−75% (15 min) of CH_3CN in 50 mM H_3PO_4 (flow rate, 0.8 mL/min; detection, JASCO photodiode array detector MD-910).

Measurement of Total Polyphenol. Total polyphenol concentrations of four commercial whiskeys were compared by the Folin−Ciocalteu method (*4*). The whiskeys were diluted 5, 10, 25, and 50 times, and each sample solution (0.5 mL) was mixed with water (1.5 mL) and phenol reagent (0.5 mL) (Kanto Chemical Co., Tokyo, Japan) and kept at room temperature for 3 min. To the mixture was added 10% Na_2CO_3 (0.5 mL), and after 1 h, absorption at 700 nm was measured. A calibration curve was made using recrystallized gallic acid as standard.

Fractionation of Whiskey. Commercial whiskey (12 L), a pure malt whiskey produced by Suntory Limited, Japan, was directly applied to a Diaion HP20SS column (40 cm × 5 cm i.d.). Most of the phenolic compounds passed through the column, and nonpolar compounds adsorbed on the gel were eluted with MeOH to give a solid (3.1 g). The first fraction that contained phenolic compounds was concentrated by a rotary evaporator to give a brown syrup (33.6 g), which was successively partitioned with Et_2O and EtOAc to give an Et_2O fraction (1.35 g), an EtOAc fraction (2.7 g), and a water-soluble fraction (26.0 g). The Et_2O fraction gave precipitates when dissolved in MeOH, which was collected by filtration (47.3 mg) and identified as ellagic acid (**2**) (**Figure 1**) by co-HPLC and comparison of UV absorption (*5*). The EtOAc-soluble fraction (2.7 g) was suspended in 50% MeOH, and insoluble precipitates of **2** (156 mg) were collected by filtration; the filtrate was applied to an MCI gel CHP-20P column (35 cm × 3.5 cm i.d., 0−100% MeOH) and a Chromatorex ODS column (20 cm × 1.5 cm i.d., 0−40% MeOH) to give lyoniresinol (*6*) (42 mg). The water-soluble fraction was subjected to MCI gel CHP20P column chromatography (25 cm × 3.5 cm i.d.) with H_2O, which contained increasing proportions of MeOH (10% stepwise elution from 0 to 100%, each 200 mL), to give nine fractions. Fraction 1, mainly composed of sugars, was further fractionated by Sephadex LH-20 column chromatography (20 cm × 3 cm i.d.) with H_2O/MeOH (0−40%, 10% stepwise, and then 40−100%, 20% stepwise, each 200 mL) to give fraction 1-1 (sugars and related compounds), gallic acid (53 mg), and fraction 1-2 (851 mg). Fraction 1-2 was applied to a Chromatorex ODS column (25 cm × 3 cm i.d.) with 0−40% MeOH (5% stepwise elution, each 200 mL) to give 6-*O*-galloyl glucose (*7*) (122 mg) and a mixture of 3-*O*-galloyl glucose and 2,3-(*S*)-hexahydroxydiphenoyl (HHDP)-D-glucose, which was purified by chromatography on a Toyopearl HW40F column (17 cm × 1 cm i.d.) with 0−5% MeOH (5% stepwise elution, each 100 mL) to give 3-*O*-galloyl glucose (*8*) (33 mg) and 2,3-(*S*)-HHDP-D-glucose (*9*) (6 mg). Fraction 3 (663 mg) was separated by Sephadex LH-20 chromatography (20 cm × 2.5 cm i.d.) with 0−100% MeOH (0−40%, 5% stepwise, and then 40−100%, 10% stepwise each 100 mL) to give 2,3-di-*O*-galloyl glucose (*10*) (7.6 mg) and a fraction containing whiskey tannin A (**3**), which was finally purified by Chromatorex ODS chromatography (18 cm × 2 cm i.d.) with 0−30% MeOH (5% stepwise, each 100 mL) to give **3** (21.8 mg). Fraction 4 was successively subjected to Sephadex LH-20 (0−100% MeOH), Avicel cellulose (2% AcOH), and MCI gel CHP-20P (0−20% MeOH) column chromatography to give brevifolin carboxylic acid (5.0 mg) (*11*). Fraction 5 (961 mg) was separated by Sephadex LH-20 (22 cm × 2.5 cm i.d., with 0−100% MeOH), Avicel cellulose (12 cm × 1.5 cm i.d., 2% AcOH), MCI gel CHP-20P (15 cm × 1.0 cm i.d., 0−100% MeOH), and Toyopearl HW40F (25 cm × 3.0 cm i.d., 0−100% MeOH) chromatography to give brevifolin carboxylic acid (1.6 mg), 3-methoxy-4-hydroxyphenol 1-*O*-(6′-*O*-galloyl)-β-D-glucopyranoside (*12*) (4.0 mg), carboxyl ellagic acid (**1**) (5.9 mg), whiskey tannin B (**4**) (7.1 mg), and castacrenin B (**7**) (*3*) (4.8 mg). Fraction 8 was separated by Sephadex LH-20 column chromatography (23 cm × 3.0 cm i.d., 0−100% MeOH)

Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR Data for **3** and **4**[a]

		3		4	
	position	^{1}H	^{13}C	^{1}H	^{13}C
glucose	1	5.35 (dd, 3.0, 6.0)	63.1	5.45 (dd, 3.2, 6.4)	62.8
	2	5.22 (br d, 6.0)	77.0	4.99 (d, 6.4)	76.5
	3	5.85 (br d, 7.1)	69.4	5.97 (d, 7.8)	66.4
	4	4.29 (dd, 7.1, 9.2)	68.4	5.42 (t, 7.8)	69.6
	5	4.95 (ddd, 3.2, 4.6, 9.2)	75.1	5.45 (ddd, 2.5, 4.1, 7.8)	70.6
	6	3.89 (dd, 4.6, 12.4)	62.2	4.92 (dd, 4.1, 12.6)	64.8
		3.80 (dd, 3.2, 12.4)		3.97 (dd, 2.5, 12.6)	
Cp	1		154.3		154.3
	2	5.34 (d, 3.0)	44.5	5.39 (d, 3.2)	44.6
	3		83.7		82.8
	4		200.2		200.0
	5		142.6		142.7
	6		163.1		162.4
	7		170.6		170.7
BPH	1, 1′		125.6, 125.7		124.9−126.3
	2, 2′		113.6, 114.7		113.7, 114.3
	3, 3′		144.5, 144.3[b]		144.4−145.8
	4, 4′		135.3, 136.6		135.1, 137.1
	5, 5′		145.8, 145.5[b]		144.4−145.8
	6		110.7		110.4
	6′	6.70 (s)	108.2	6.70 (s)	108.2
	7		167.2		166.2
	7′		168.6		167.5
HHDP	1, 1′				115.1, 115.9
	2, 2′				124.9−126.3
	3, 3′				144.4−145.8
	4, 4′				136.1, 136.8
	5, 5′				144.4−145.8
	6			6.83 (s)	108.3
	6′			6.63 (s)	107.6
	7				167.6
	7′				168.7
$-OCH_2$		4.18 (dq, 7.1, 14.2)	63.0	4.22 (dq, 7.1, 10.8)	63.0
		4.17 (dq, 7.1, 14.2)		4.17 (dq, 7.1, 10.8)	
$-CH_3$		1.18 (3H, t, 7.1)	14.1	1.20 (3H, t, 7.1)	14.2

[a] Measured in acetone-d_6 + D_2O, δ in ppm, J in hertz. [b] Assignments may be interchanged within the same column.

to give ellagic acid (889 mg). Known compounds were identified by spectroscopic comparison with authentic samples or data described in the literature.

*Carboxyl ellagic acid (**1**):* yellow powder, FAB-MS *m/z* 347 $[M + H]^+$, 369 $[M + Na]^+$, 385 $[M + K]^+$; high-resolution (HR)-FAB-MS 347.0030 $[M + H]^+$, ($C_{15}H_7O_{10}$ requires 347.0038); UV λ_{max} nm (log ϵ) 368 (4.06), 254 (4.69); ^{1}H NMR (DMSO-d_6) δ 7.47 (1H, s, H-5); ^{13}C NMR (DMSO-d_6) δ 166.8 (C-8), 159.0 (C-7′), 157.7 (C-7), 148.3 (C-4, 4′), 145.2 (C-3), 139.5 (C-3′), 136.5 (C-2′), 136.1 (C-2), 121.2 (C-5), 112.12 (C-6), 112.07 (C-6′), 110.4 (C-1), 110.4 (C-5′), 107.9 (C-1′).

*Whiskey tannin A (**3**):* yellow amorphous powder; $[\alpha]_D$ −77.8 (*c* 0.1, MeOH); IR ν_{max} cm^{-1} 3457, 1746, 1614; UV λ_{max}(MeOH) nm (log ε) 221 (4.55); FAB-MS *m/z* 677 $[M + H]^+$, 677 $[M + Na]^+$; HR-FAB-MS *m/z* 677.0997 $[M + H]^+$ (calcd for $C_{29}H_{25}O_{19}$ 677.0990); CD (*c* 4.4 × 10^{-5} M) $\Delta\varepsilon$ (nm) −4.2 (273), −0.8 (255), −17.1 (234); ^{1}H and ^{13}C NMR data, see **Table 1**.

*Whiskey tannin B (**4**):* yellow amorphous powder; $[\alpha]_D$ −74.9 (*c* 0.1, MeOH); IR ν_{max} cm^{-1} 3417, 1731, 1614, 1517; UV λ_{max}(MeOH) nm (log ε) 218 (4.80); FAB-MS *m/z* 1001 $[M + Na]^+$; HR-FAB-MS *m/z* 1001.0869 $[M + Na]^+$ (calcd for $C_{43}H_{30}O_{27}Na$ 1001.0872); CD (*c* 3.5 × 10^{-5} M) $\Delta\varepsilon$ (nm) −15.0 (265), 10.2 (238); ^{1}H and ^{13}C NMR data, see **Table 1**.

Pyrolysis of Castalagin. Castalagin (**5**) (1.0 g) was first dissolved in a small amount of MeOH and then dried under reduced pressure. Dried solid was heated in a microwave oven at 190 °C for 70 min. The resulting mixture was separated by MCI gel CHP-20P (23 cm × 2.5 cm i.d., 0−100% MeOH, 10% stepwise elution) and Sephadex LH-20 (20 cm × 1.5 cm i.d., 20−100% MeOH) column chromatography to yield dehydrocastalagin (*13*) (153 mg), **10** (20 mg), ellagic acid (158 mg), and castacrenin F (*14*) (4 mg), along with recovery of **5** (280 mg). The structure of dehydrocastalagin was determined on the basis of NMR experiments including HSQC and HMBC spectra and finally identified by comparison of the NMR data with those described in the literature. Castacrenin F was identified by comparison of the NMR data and HPLC with those of authentic samples.

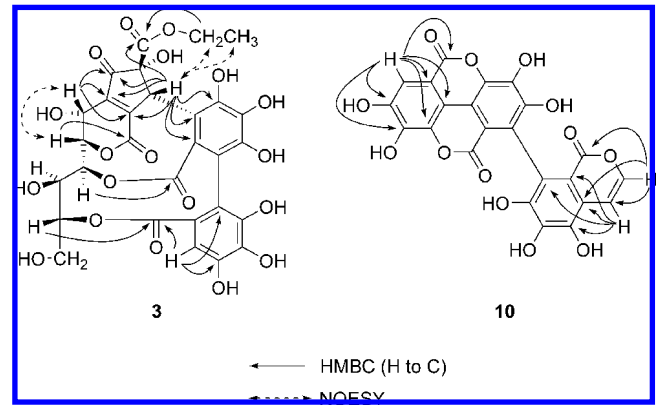

Figure 2. Selected HMBC and NOESY correlations for **3** and HMBC correlations for **10**.

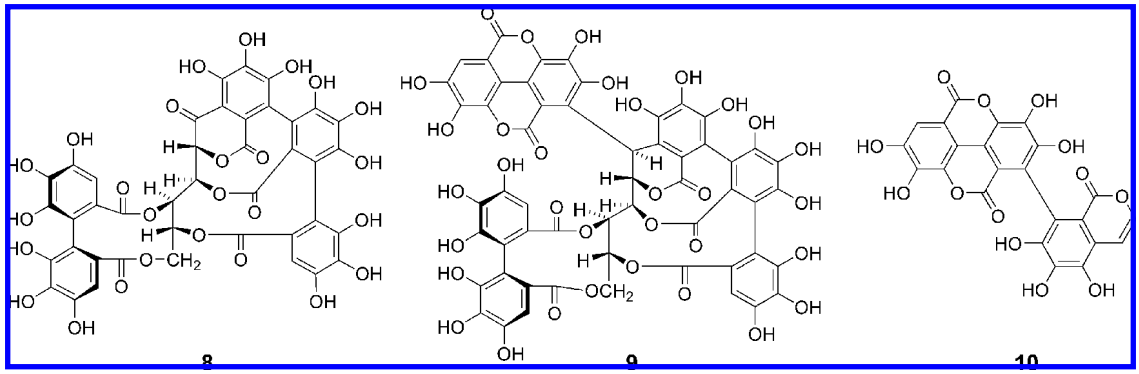

Figure 3. Structures of pyrolysis products of **5**.

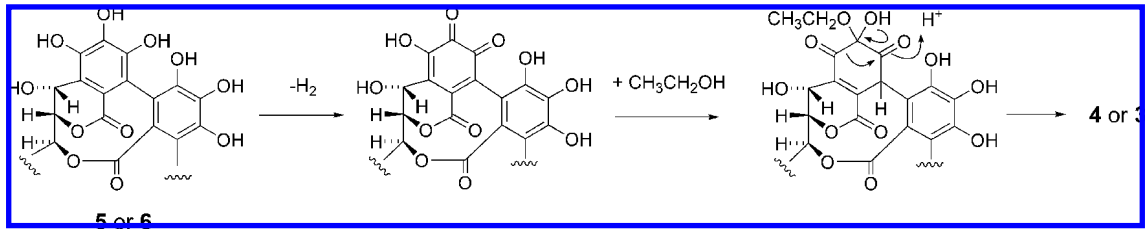

Figure 4. Possible production mechanism for **3** and **4**.

*Compound **10**:* yellow amorphous powder, $[\alpha]_D$ −2.6 (*c* 0.05, MeOH); MALDI-TOF-MS *m/z* 495 $[M + H]^+$, 517 $[M + Na]^+$; IR ν_{max} cm^{-1} 3389, 1724, 1605; UV λ_{max} (MeOH) nm (log ε) 370 (4.02), 251 (4.69); ^1H NMR (DMSO-d_6) δ 7.57 (1H, s, H-5′), 7.20 (1H, d, J = 5.6 Hz, H-3″), 6.87 (1H, d, J = 5.6 Hz, H-4″); ^{13}C NMR (DMSO-d_6) δ 161.2 (C-1″), 160.1 (C-7′), 158.5 (C-7), 148.5 (C-4′), 146.8, 144.7, 140.0, 139.0, 136.9 (C-2, C-3, C-4, C-6″, C-7″), 142.5 (C-3″), 139.7 (C-3′), 139.4 (C-5″), 137.4 (C-2′), 124.2 (C-5), 121.9 (C-4a″), 117.4 (C-8″), 113.9 (C-6), 113.7 (C-6′), 113.0 (C-8a″), 111.2 (C-5′), 109.4 (C-1′), 109.0 (C-1), 102.3 (C-4″). Anal. Calcd for $C_{23}H_{10}O_{13}\cdot{}^9/_4H_2O$: C, 51.65%; H, 2.73%. Found: C, 51.95%; H, 3.12%.

RESULTS AND DISCUSSION

The total polyphenol content of four commercial Japanese whiskeys was compared by the Folin−Ciocalteu method (*4*). The highest value of 417 ± 44.1 mg/L was observed in a vatted whiskey that contained a malt whiskey aged in charred new oak barrels. A representative Japanese single-malt whiskey also contained a high concentration of polyphenols, 236.4 ± 1.2 mg/L. Concentrations of the remaining two blended whiskeys were relatively low (82.2 ± 17.7 and 78.7 ± 16.8 mg/L). The whiskey that showed the highest polyphenol concentration was selected for further chemical study on whiskey polyphenols. The whiskey was directly passed through a Diaion HP20SS column to remove nonpolar constituents, and then the fractions that contained polyphenols were successively partitioned with Et_2O and EtOAc. Most of the polyphenols remained in the aqueous layer. The Et_2O and EtOAc layer contained ellagic acid, which was the major polyphenol of the whiskey. Lyoniresinol (*6*), a lignan composed of two sinapylalchohol units, was isolated from the EtOAc layer. Chromatographic separation of the aqueous layer yielded gallic acid, ellagic acid (**2**) (*5*), 6-*O*-galloyl glucose (*7*), 3-*O*-galloyl glucose (*8*), 2,3-di-*O*-galloyl glucose (*10*), 2,3-(*S*)-HHDP-D-glucose (*9*), brevifolin carboxylic acid (*11*), 3-methoxy-4-hydroxyphenol 1-*O*-(6′-*O*-galloyl)-β-D-glucopyranoside (*12*), and castacrenin B (*3*), together with three new compounds named carboxyl ellagic acid (**1**) and whiskey tannins A (**2**) and B (**3**). The known compounds were identified by comparison of their NMR spectroscopic data with those from authentic samples or with those described in the literature. From another Japanese single-malt whiskey (total polyphenol = 236.4 ± 1.2 mg/L), 3-*O*- and 6-*O*-galloyl glucoses were also isolated by similar chromatographic separation (data not shown) together with ellagic acid. Isolation of these simple galloyl glucoses was somewhat surprising because the occurrence of these compounds in oak wood has not been reported so far. Brevifolin carboxylic acid is an oxidation product of the HHDP group of ellagitannins (*11*). 3-Methoxy-4-hydroxyphenol 1-*O*-(6′-*O*-galloyl)-β-D-glucopyranoside is a phenolic glycoside originally isolated from acorns of *Quercus mongolica* and the bark of *Quercus acutissima* (*12*). Castacrenin B (**7**) is a dehydrative degradation product of castalagin (**5**), a major oak wood ellagitannin, and was first isolated from heart wood of the Japanese chestnut tree (*3*). Castalagin and related ellagitannins that were reported to be present in the original oak wood (*1*) were not detected in any of the four whiskey samples examined in this study.

Carboxyl ellagic acid (**1**) was obtained as a yellow powder, which was hardly soluble in water and MeOH. The UV absorption at 254 and 368 nm was similar to that of ellagic acid (**2**). FAB-MS showed $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$ at *m/z* 347, 369, and 385, respectively, which indicated that the molecular weight was 346. The molecular weight was 44 mass units larger than that of **2**, and the molecular formula, $C_{15}H_6O_{10}$, was confirmed by HR-FAB-MS. The ^1H NMR spectrum resembled that of **2** and showed an aromatic proton singlet at δ 7.47 (H-5′). The ^{13}C NMR spectrum was also related to that of **2**. However, in contrast to the symmetrical structure of **2**, the spectrum of **1** showed signals attributable to six oxygenated aromatic carbons [δ 148.3 (2C, C-4, 4′), 145.2 (C-3′), 139.6 (C-3), 136.5 (C-2), 136.1 (C-2′)], six nonoxygenated aromatic carbons [δ 121.2 (C-5′), 112.1 (C-6′), 112.0 (C-6), 110.4 (2C, C-5, 1′), 107.9 (C-1)], and three carboxyl carbons [δ157.7 (C-7), 159.0 (C-7′), 166.8 (C-8)]. An unusual upfield shift of two of the three carboxyl carbon signals indicated that these were ascribable to $\alpha,\beta,\gamma,\delta$-unsaturated δ-lactone, similar to those of **2**. When the molecular weight was taken into account, the remaining carboxyl carbon signal (δ 166.8) was assignable to the α,β-unsaturated carboxyl group. The location of the carboxyl group was concluded to be at C-5 of **2**, on the basis of the results of th HMBC experiment: the H-5′ signal was correlated with C-1′, C-2′, C-3′, C-4′, and C-6′, and the

remaining aromatic and carboxyl carbons did not correlate with any proton signals. Thus, the structure of **1** was deduced to be 5-carboxyl ellagic acid.

Whiskey tannin A (**3**) was obtained as a yellow amorphous powder and gave a blue coloration with the $FeCl_3$ reagent. The molecular formula $C_{29}H_{24}O_{19}$ was determined by HR-FAB-MS. The ^{1}H NMR spectrum exhibited an aromatic proton singlet signal (δ 6.70, H-6′) and aliphatic proton signals attributable to six methine, two methylene, and one methyl group (**Table 1**). The presence of an ethoxyl group was indicated by the appearance of mutually coupled methyl and methylene proton signals. The ^{1}H−^{1}H COSY correlations of the remaining one methylene and five methine protons revealed the presence of a polyalchohol unit, which was closely related to the open-chain glucose unit of castalin (**6**) (*3, 15*). As well as their chemical shifts, comparison of the coupling constants of **3** (**Table 1**) with those of **6** ($J_{1,2}$ = 5 Hz, $J_{2,3}$ = 1 Hz, $J_{3,4}$ = 7 Hz, $J_{4,5}$ = 7 Hz, $J_{5,6a}$ = 3 Hz, $J_{5,6b}$ = 6 Hz) strongly suggested that the polyalcohol moiety was an open-chain glucose (*3*). In addition, on the basis of the ^{1}H NMR chemical shifts and HMBC correlations (**Figure 2**), it was apparent that hydroxyl groups at C-2, C-3, and C-5 of the glucose were acylated. However, in contrast to the three pyrogallol rings of **6**, signals arising from only two pyrogallol moieties were observed in the spectrum of **3**. Instead, **3** showed carbon signals due to a carbonyl [δ 200.2 (Cp-4)], a carboxyl [δ 170.6 (Cp-7)], and two olefinic [δ 142.6 (Cp-5) and 154.3 (Cp-1)] and two aliphatic carbons [δ 44.5 (Cp-2) and 83.7 (Cp-3)]. In the HSQC spectrum, the methine carbon resonating at δ 44.5 (Cp-2) showed a ^1J correlation peak with an aliphatic proton singlet at δ 5.34 (Hc_p-2). This methine proton signal showed ^2J and ^3J HMBC correlation peaks with Cp-1, Cp-4, Cp-5, and aromatic carbon signals of a pyrogallol ring (**Figure 2**). Furthermore, the Hc_p-2 and methylene protons of the aforementioned ethoxyl group were correlated to an ester carboxyl carbon (δ 170.6, Cp-7). Although the oxygen-bearing quaternary carbon (δ 83.7, Cp-3) did not show any HMBC correlation peaks, these spectroscopic observations indicated that the ethoxyl group was connected to the Cp-3 carbon through the Cp-7 ester carboxyl carbon. In addition, the HMBC correlations of the Cp-1−Cp-5 (**Figure 2**) indicated the presence of a cyclopentenone ring structure. The connection of the Cp-5 to the glucose H-1 was apparent from the HMBC correlations of the glucose H-1 with Cp-1, Cp-5, and Cp-4. A homoallyl long-range coupling (J = 3.0 Hz) between glucose H-1 and Cp-2 methine proton was also observed. Chemical shift of the remaining ester carbonyl carbon signal at δ 163.1 (Cp-6) indicated that this carbonyl was conjugated with a double bond, and this ester carbon was shown to form a δ-lactone ring with the glucose C-2 hydroxyl group, by observation of the HMBC correlation between this ester carbon and glucose H-2. Moreover, glucose H-3 and H-5 also showed correlation peaks with ester carbonyl signals at δ 167.2 and 168.6, respectively, and the latter signal was correlated with the aromatic singlet signal at δ 6.70. On the basis sof these results, the planar structure was constructed as shown in **Figure 1**, and this was supported by comparison of the spectroscopic data with those of rhoipteleanin H (*16*).

As for stereostructure, the ^{1}H−^{1}H coupling constant between glucose H-1 and H-2 (J = 6.0 Hz) of **3** indicated that the configuration of glucose C-1 of this compound was the same as that of **6** (J = 5 Hz) and different from that of vescalin (J < 1 Hz), the C-1 epimer of **6**. This implied that the glucose C-3 carbon of **3** was oriented to the α-side of the δ-lactone ring formed between C-2 and Cp-6 (**Figure 2**). As shown by the HMBC correlation illustrated in **Figure 2**, the glucose C-3 hydroxyl group formed another lactone ring with the carboxyl group attached to the hexahydroxybiphenyl moiety (BPH C-7). This implied that the glucose C-3 and the biphenyl moiety were placed on the same side of the molecule. Assuming that **3** was derived from **6** and, therefore, that the glucose was the D-form, on the basis of the similarity of the planar structure of **3** and **6**, the configuration at Cp-2 methine carbon was deduced to be *R* as shown in **Figure 2**. The configuration of the quaternary carbon Cp-3 was determined to be *R* because the Cp-2 methine proton showed NOESY correlations with the ethoxyl protons (**Figure 2**). On the basis of these observations, the structure of whiskey tannin A was concluded to be represented by the formula **3**. Atropisomerism of the BPH group could not be determined clearly in this study. The CD spectrum showed a large negative Cotton effect at 234 nm and a small positive Cotton effect at 255 nm, which suggested that the biphenyl bond was *R*-configuration (*17*). However, this result was inconsistent with the configurations reported for **5** and **6** (*18*). The CD curve of **3** was different from those of typical ellagitannins and was apparently affected by the coexisting unsaturated carbonyl of the cyclopentenone ring.

Whiskey tannin B (**4**) was isolated as a tan amorphous powder and exhibited an $[M + Na]^+$ peak at *m/z* 1001 in FAB-MS, which was 302 mass units larger than that of **3**. The difference coincided with the molecular mass of **2**. The ^{1}H and ^{13}C NMR spectra were closely related to those of **3** and showed signals due to an open-chain glucose, an ethoxyl group, and a cyclopentene ring moiety. The HMBC correlations of the signals supported the presence of the same partial structures in **3** and **4**. In addition, the appearance of two aromatic singlet signals (δ 6.63, 6.83) in the ^{1}H NMR spectrum and signals arising from two pyrogallol and two carboxyl carbons in the ^{13}C NMR spectrum indicated the presence of an additional HHDP group in **4**. This was consistent with the above-mentioned FAB-MS results. Compared to those of **3**, large low-field shifts of glucose H-4 (δ 5.42) and H-6 (δ 3.97, 4.92) were observed, and these protons were correlated with the carboxyl group (δ 167.6, 168.7) of the HHDP moiety in the HMBC spectrum. This observation showed that the HHDP group was attached to H-4 and H-6 of the open-chain glucose. In contrast to that of **3**, the CD spectrum of **4** exhibited large positive and negative Cotton effects at 238 and 265 nm, respectively, and this strongly suggested that the HHDP group had an *S*-biphenyl bond (*17*). Thus, the structure of whiskey tannin B was concluded to be represented by the formula **4**.

A production mechanism of whiskey tannins A and B was proposed as illustrated in **Figure 4**. The pyrogallol ring attached to the glucose C-1 position was oxidized, and subsequent benzylic acid-type rearrangement after addition of ethanol formed the ethoxycarbonyl cyclopentenone moiety. To examine whether this oxidation occurred during the charring process of barrel making, pyrolysis products of **5** (**Figure 3**) at 190 °C were investigated. The major products were identified to be ellagic acid (**2**) and dehydrocastalagin (**8**) (*13*), which was accompanied by castacrenin F (**9**) (*14*) and a new product **10**. Production of **9** suggested electrophilic substitution of the C-1 of C-glycosidin ellagitannins to the HHDP group or ellagic acid.

The ^{1}H NMR spectrum of **10** showed an aromatic singlet at δ 7.57 (H-5′) and two mutually coupled (J = 5.6 Hz) olefinic proton signals at δ 6.87 and 7.20. The chemical shift of the aromatic singlet was similar to those of **1** and **2**, and the HMBC correlations of this signal strongly suggested the presence of an ellagic acid moiety in **10** (**Figure 2**). This was supported by

the appearance of UV absorption at 370 and 251 nm and by comparison of the ^{13}C NMR signals with those of **1**. The ^{13}C NMR spectrum showed three carboxyl carbon signals at δ 158.5, 160.1, and 161.2, two of which were attributable to the δ-lactone of ellagic acid moiety (δ 158.5, C-7; 160.1, C-7′). The remaining one (δ 161.2, C-1″) showed HMBC correlations with one of the olefinic protons at δ 6.87 (H-3″). In addition, HMBC correlations of the two olefinic protons shown in **Figure 2** revealed a trihydroxyisocoumarin unit. Because this compound was the degradation product of **5**, these spectroscopic data allowed us to construct the structure of **10**, as shown in **Figure 3**. The $[M + H]^+$ peak at *m/z* 495 in MALDI-TOF-MS supported the structure. The two olefinic methine carbons of the isocoumarin unit originated from the glucose C-1 and C-2. In molecules **8**−**10**, the pyrogallol ring attached to the glucose C-1 was not oxidized. No products having structures related to **3** and **4** were detected among the reaction products. Thus, the results suggested that **3** and **4** in the commercial whiskey were produced by oxidation of **5** and **6** during the aging process in barrels. Related oxidation of the pyrogallol ring of **5** in model aqueous ethanol solutions was demonstrated (*19*), and the spectroscopic data of one of the products was closely related to **4**. The oxidation product of the in vitro model experiment may be identical to whiskey tannin B (**4**).

In conclusion, two new ellagitannins named whiskey tannins A (**3**) and B (**4**) were isolated from Japanese whiskey, which were produced by oxidation of oak wood ellagitannins during the aging process in barrels. Another product, carboxyl ellagic acid (**1**), was also presumed to be an oxidation product of ellagitannin, although the origin of the carboxyl group was not clear. Probably, it came from the ellagic acid moiety attached to the glucose C-1 of C-glycosidic ellagitannins, such as castacrenin F (**9**), or from triphenyl compounds, such as compound **10**. Brevifolin carboxylic acid was also isolated, and this compound is known to be an oxidation product of HHDP ester groups (*11*). In addition, it was very interesting that simple galloyl glucoses, which have not been reported in oak wood, were found in two commercial Japanese whiskeys. In this study, we were conscious of the presence of significant amounts of an uncharacterized phenolic substance in the whiskey, which was detected as a broad hump on the baseline upon HPLC analysis and a broad spot on cellulose TLC. Condensation of ellagitannins with coexisting compounds possibly occurs (*20*). Further chemical study on the substance is now in progress.

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