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Isolation and Identification of Flavonoids Accumulated in Proanthocyanidin-free Barley

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ABSTRACT: Flavonoids accumulated in proanthocyanidin-free near-isogenic lines iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi, developed by backcross breeding using a leading cultivar, Nishinohoshi, as a recurrent parent and a proanthocyanidin-free mutant as a nonrecurrent parent in Japan, were examined. A new flavanone, (2*RS*)-dihydrotricin 7-*O*-β-D-glucopyranoside (**1**), known flavanones (2*RS*)-dihydrotricin (**2**) and (2*RS*)-homoeriodictyol (**3**), and known flavones chrysoeriol 7-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (**4**), chrysoeriol 7-*O*-β-D-glucopyranoside (**5**), tricin (**6**), and chrysoeriol (**7**) were isolated from iso *ant* 17 of Nishinohoshi. The structures and stereochemistries of the isolated flavonoids (**1**−**7**) were elucidated on the basis of spectroscopic analyses. The concentrations of the isolated flavonoids (**1**−**7**) in iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi were similar to each other, whereas the flavonoids **1**−**5** and **7** were not detected in Nishinohoshi, an old Japanese cultivar, Amaginijo, and North American cultivar Harrington. The concentration of tricin (**6**) in Nishinohoshi was a half those in iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi. Except for iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi, the concentration of tricin (**6**) was highest in Nishinohoshi, followed by Amaginijo and Harrington. Thus, tricin (**6**), its precursor dihydrotricin (**2**), and its glucopyranoside, dihydrotricin 7-*O*-β-D-glucopyranoside (**1**), as well as chrysoeriol (**7**) and homoeriodictyol (**3**) were accumulated in iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi probably by blocking at the step of flavanone 3-hydroxylase in the procyanidin biogenetic pathway, resulting in enhancement of the alternative biogenetic pathway.

KEYWORDS: barley, *Hordeum vulgare*, proanthocyanidin-free, flavonoids

INTRODUCTION

Barley (*Hordeum vulgare*) is one of the most important cereal crops in the world. Barley grain is used principally as animal feed, malt, and food.[1] With regard to its use for food in Japan, barley grain is boiled with rice. Barely grain contains proanthocyanidins, which are responsible for beer haze in the use of malt[2] and browning in the use of rice boiled with barley.[3] To resolve these problems, proanthocyanidin-free mutants have been collected and localized to complementation groups, *Ant* genes, at the Carlsberg Laboratory in Denmark.[4] In Japan, proanthocyanidin-free cultivars and lines have been developed by backcross breeding using a leading cultivar, Nishinohoshi, as a recurrent parent and a proanthocyanidin-free mutant as a nonrecurrent parent at the National Agriculture and Food Research Organization.[5]

Proanthocyanidin-free mutants have been used for biochemical studies of flavonoid biosynthesis and for characterization of genes involved in this pathway.[4] Although *Ant* 17 and *Ant* 22 are located on different chromosomes,[6] both mutants of *ant* 17 and *ant* 22 lack catechin and proanthocyanidins but accumulate a flavanone homoeriodictyol (**3**) and a flavone chrysoeriol (**7**).[4] However, the mode of function of *Ant* 17 and *Ant* 22 is not understood. There are two possibilities. The first is that *Ant* 17 codes for one subunit of flavanone 3-hydroxylase, an assumed dimer which catalyzes the reaction from eriodictyol to dihydroquercetin, and *Ant* 22 codes for the other. The second is that mutations in *Ant* 17 and *Ant* 22 are associated with overproduction of flavonoid 3′-*O*-methyltransferase, which catalyzes the reaction from eriodictyol to homoeriodictyol (**3**).

Flavonoids have been known for their beneficial effects on health.[7] Homoeriodictyol (**3**) has been reported to be one of the bitter-masking flavanones.[8] Chrysoeriol (**7**) has been reported to show many beneficial effects on health such as antiinflammatory activity,[9] antiobesity activity,[10] antioxidant activity,[11−13] and antimutagenic activity.[14,15] However, flavonoids accumulated in the proanthocyanidin-free mutants have not yet been isolated and identified thoroughly. In this paper, we describe the isolation and structure elucidation of a new flavanone, (2*RS*)-dihydrotricin 7-*O*-β-D-glucopyranoside (**1**), known flavanones (2*RS*)-dihydrotricin (**2**) and (2*RS*)-homoeriodictyol (**3**), and known flavones chrysoeriol 7-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (**4**), chrysoeriol 7-*O*-β-D-glucopyranoside (**5**), tricin (**6**), and chrysoeriol (**7**) accumulated in iso *ant* 17 of Nishinohoshi and the determination of isolated compounds (**1**−**7**) in iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi and other cultivars.

MATERIALS AND METHODS

General Procedures. UV and CD spectra were recorded on a JASCO spectropolarimeter. IR spectra were recorded on a Perkin-Elmer Spectrum 100 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on

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Bruker Advance 800 and 500 spectrometers, respectively. HRESIMS spectra were recorded on a Bruker MicrOTOF spectrometer.

Material. Proanthocyanidin-free near-isogenic lines iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi were developed by five rounds of backcross breeding using a leading cultivar, Nishinohoshi, as a recurrent parent and proanthocyanidin-free mutants, *ant* 13.152 and *ant* 17.148 in Triumph and *ant* 22.1508 in Haruna-Nijo, respectively, as a nonrecurrent parent at the National Agricultural Research Center for Kyushu Okinawa Region. Barley grains of iso *ant* 13, iso *ant*17, and iso *ant* 22 of Nishinohoshi and a cultivar Nishinohoshi were collected at the National Agricultural Research Center for Kyushu Okinawa Region in 2008 and those of cultivars Amaginijo and Harrington in 2009. These grains were ground in a Tecator Cyclotec 1093 mill and passed through a 1 mm screen.

Comparison. Powdered grains of iso *ant* 17 of Nishinohoshi and Nishinohoshi (3 g) were extracted with MeOH (30 mL) at 25 °C for 1 day. The MeOH extracts were subjected to C_{18} HPLC [TSKgel ODS-80Ts, Tosoh Co. Ltd., 4.6 × 250 mm; eluent, $CH_3CN/MeOH/H_2O$ (15:4:31); flow rate, 0.8 mL/min; UV detection at 290 or 350 nm] to compare HPLC chromatograms of the iso *ant* 17 of Nishinohoshi and Nishinohoshi grain extracts.

Isolation. Powdered grain of iso *ant* 17 of Nishinohoshi (500 g) was extracted with MeOH (5 L) at 25 °C for 6 days. The MeOH extract (25 g) was partitioned between EtOAc and H_2O. The EtOAc layer (8 g) was separated by C_{18} HPLC [TSKgel ODS-80Ts, Tosoh Co. Ltd., 4.6 × 250 mm; eluent, $MeOH/H_2O$ (13:7); flow rate, 0.8 mL/min; UV detection at 290 and 350 nm] to afford fractions A (t_R 0.0−6.0 min), B (t_R 6.0−9.0 min), and C (t_R 9.0−12.0 min). Fraction A was further separated by C_{18} HPLC [TSKgel ODS-80Ts, Tosoh Co. Ltd., 4.6 × 250 mm; eluent, $MeCN/H_2O$ (1:3); flow rate, 0.8 mL/min; UV detection at 290 and 350 nm] to afford chrysoeriol 7-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (**4**, 4.7 mg, 0.0009% yield, t_R 7.5 min) and fraction AA (t_R 10.0−11.0 min). Fraction AA was further separated by C_{18} HPLC [TSKgel ODS-80Ts, Tosoh Co. Ltd., 4.6 × 250 mm; eluent, $MeOH/H_2O$ (11:9); flow rate, 0.8 mL/min; UV detection at 290 and 350 nm] to afford (2*RS*)-dihydrotricin 7-*O*-β-D-glucopyranoside (**1**, 3.2 mg, 0.0006% yield, t_R 6.0 min) and chrysoeriol 7-*O*-β-D-glucopyranoside (**5**, 6.6 mg, 0.0013% yield, t_R 8.0 min). Fraction B was further separated by C_{18} HPLC [SunFire, Waters Co. Ltd., 4.6 × 250 mm; eluent, $MeCN/H_2O$ (2:3); flow rate, 0.8 mL/min; UV detection at 290 nm] to afford (2*RS*)-dihydrotricin (**2**, 7.3 mg, 0.0015% yield, t_R 9.5 min) and (2*RS*)-homoeriodictyol (**3**, 2.0 mg, 0.0004% yield, t_R 10.5 min). Fraction C was further separated by C_{18} HPLC [TSKgel ODS-80Ts, Tosoh Co. Ltd., 4.6 × 250 mm; eluent, $MeCN/H_2O$ (2:3); flow rate, 0.8 mL/min; UV detection at 350 nm] to afford tricin (**6**, 5.2 mg, 0.0010% yield, t_R 11.0 min) and chrysoeriol (**7**, 3.2 mg, 0.0006% yield, t_R 12.0 min). Moreover, the HPLC epimer or enantiomer separations of **1**−**3** were confirmed by a chiral column (Chiralpak IA, Daicel Chemical Ind. Ltd., 4.6 × 250 mm).

Determination. Powdered grains of iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi, Nishinohoshi, Amaginijo, and Harrington (3 g) were extracted with MeOH (30 mL) at 25 °C for 1 day. The MeOH extracts were subjected to C_{18} HPLC [TSKgel ODS-80Ts, Tosoh Co. Ltd., 4.6 × 250 mm; eluent, $CH_3CN/MeOH/H_2O$ (1:1:3); flow rate, 0.8 mL/min; UV detection at 290 nm for **1** and 350 nm for **4** and **5**] to determine **1** (t_R 8.4 min), **4** (t_R 7.7 min), and **5** (t_R 9.6 min). The MeOH extracts were also subjected to C_{18} HPLC [TSKgel ODS-80Ts, Tosoh Co. Ltd., 4.6 × 250 mm; eluent, $CH_3CN/MeOH/H_2O$ (15:4:31); flow rate, 0.8 mL/min; UV detection at 290 nm for **2** and **3** and 350 nm for **6** and **7**] to determine **2** (t_R 18.0 min), **3** (t_R 20.5 min), **6** (t_R 21.5 min), and **7** (t_R 22.5 min). The amounts of **1**−**7** were calculated from standard curves by measurement of the areas of the peaks for them. This experiment was replicated three times.

*(2RS)-Dihydrotricin 7-O-β-D-glucopyranoside (**1**):* colorless solid; UV (MeOH) λ_{max} 278 nm (ε 51000); CD (MeOH) λ_{ext} 278 nm (Δε −1.0); ATR-FTIR ν 3902, 3750, 3260, 2920, 2626, 1698, 1618, 1523, 1462, 1431, 1352, 1285, 1244, 1173, 1141, 1036, 891, 830, 765, 730, 710, 675, 630, 567, 534, 471, 435, 410 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 517.1297 [$(M + Na)^+$; calcd for $C_{23}H_{26}O_{12}Na$, 517.1316].

Table 1. NMR Spectroscopic Data (800 MHz for ^1H NMR, 125 MHz for ^{13}C NMR, D_2O) for (2*RS*)-Dihydrotricin 7-*O*-β-D-Glucopyranoside (1)

	(2*RS*)-dihydrotricin 7-*O*-β-D-glucopyranoside (**1**)				
position	δC	mult	δH	mult (*J* in Hz)	HMBC[a]
2	81.0	CH	6.86	s	4, 9, 1′, 2′, 6′
3	44.1	CH_2	3.29	m	2, 4, 10, 1′
			2.78	m	
4	198.4	C			
5	164.7	C			
6	97.9	CH	6.19	s	4, 5, 7, 8, 10
7	166.9	C			
8	96.8	CH	6.24	s	4, 6, 7, 9, 10
9	164.4	C			
10	104.8	C			
1′	130.4	C			
2′	105.3	CH	6.86	s	2, 1′, 3′, 4′, 6′
3′	149.2	C			
4′	137.3	C			
5′	149.2	C			
6′	105.3	CH	6.86	s	2, 1′, 2′, 4′, 5′
7′	56.8	CH_3	3.87	s	2′, 3′
8′	56.8	CH_3	3.87	s	5′, 6′
1″	101.2	CH	5.05	d (7.7)	7, 2″, 3″, 5″
2″	74.6	CH	3.45	m	1″, 3″
3″	77.7	CH	3.50	m	1″, 2″, 4″
4″	71.0	CH	3.43	m	3″, 5″, 6″
5″	78.2	CH	3.55	m	1″, 4″, 6″
6″	62.4	CH_2	3.89	m	4″, 5″
			3.71	m	

[a] HMBC correlations are from proton(s) stated to the indicated carbon.

*(2RS)-Dihydrotricin (**2**):* colorless solid; UV (MeOH) λ_{max} 283 nm (ε 43000); CD (MeOH) λ_{ext} 286 nm (Δε −0.9); ^1H NMR (CD_3OD/CD_3COCD_3, 1:1) δ 2.73 (1H, dd, *J* = 3.0, 17.1 Hz, H-3a), 3.21 (1H, dd, *J* = 13.0, 17.1 Hz, H-3b), 3.87 (3H, s, H-7′), 3.87 (3H, s, H-8′), 5.40 (1H, dd, *J* = 3.0, 13.0 Hz, H-2), 5.90 (1H, d, *J* = 2.2 Hz, H-6), 5.92 (1H, d, *J* = 2.2 Hz, H-8), 6.85 (1H, s, H-2′), 6.85 (1H, s, H-6′); ^{13}C NMR (CD_3OD/CD_3COCD_3, 1:1) δ 44.1 (C-3), 56.8 (C-7′), 56.8 (C-8′), 80.8 (C-2), 96.2 (C-8), 97.0 (C-6), 103.2 (C-10), 105.2 (C-2′), 105.2 (C-6′), 130.7 (C-1′), 137.2 (C-4′), 149.1 (C-3′), 149.1 (C-5′), 164.7 (C-9), 165.3 (C-5), 168.3 (C-7), 197.6 (C-4); HRESIMS *m/z* 355.0784 [$(M + Na)^+$; calcd for $C_{17}H_{16}O_7Na$, 355.0788].

*(2RS)-Homoeriodictyol (**3**):* colorless solid; UV (MeOH) λ_{max} 279 nm (ε 47000); CD (MeOH) λ_{ext} 297 nm (Δε 3.2); ^1H NMR (CD_3OD/CD_3COCD_3, 1:1) δ 2.72 (1H, dd, *J* = 3.0, 17.1 Hz, H-3a), 3.21 (1H, dd, *J* = 13.0, 17.1 Hz, H-3b), 3.89 (3H, s, H-7′), 5.41 (1H, dd, *J* = 3.0, 13.0 Hz, H-2), 5.90 (1H, d, *J* = 2.2 Hz, H-6), 5.91 (1H, d, *J* = 2.2 Hz, H-8), 6.84 (1H, d, *J* = 8.1 Hz, H-5′), 6.97 (1H, dd, *J* = 2.1, 8.1 Hz, H-6′), 7.15 (1H, d, *J* = 2.0 Hz, H-2′); ^{13}C NMR (CD_3OD/CD_3COCD_3, 1:1) δ 44.0 (C-3), 56.5 (C-7′), 80.6 (C-2), 96.1 (C-8), 97.0 (C-6), 103.2 (C-10), 111.4 (C-2′), 116.1 (C-5′), 120.7 (C-6′), 131.5 (C-1′), 148.2 (C-4′), 148.9 (C-3′), 164.7 (C-9′), 165.3 (C-5), 168.2 (C-7), 197.6

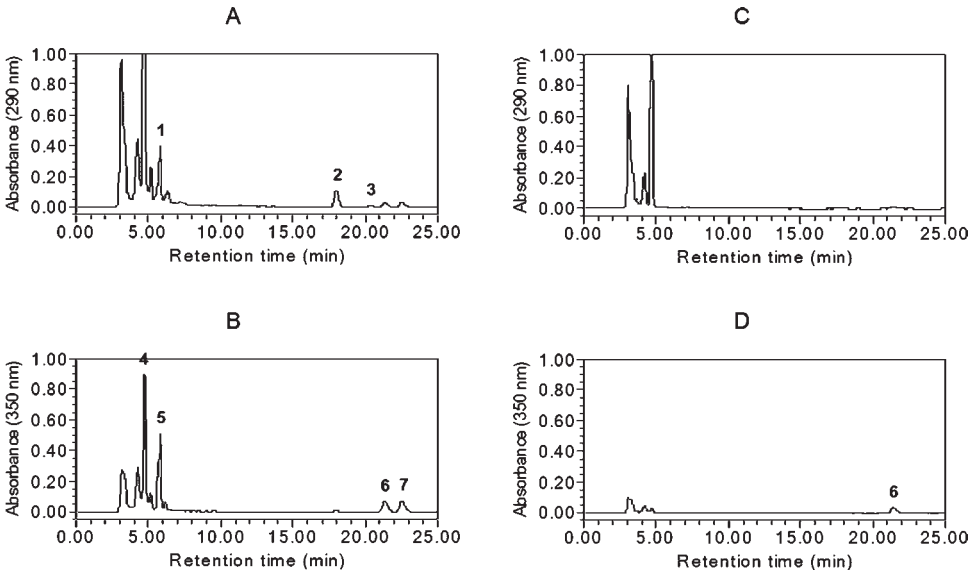
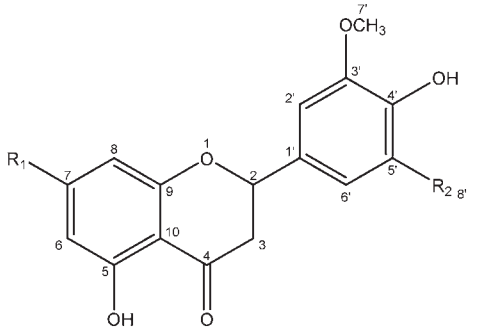
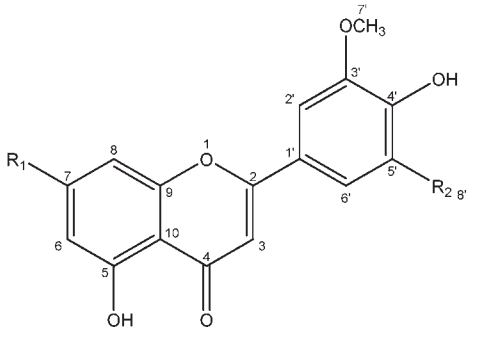


Figure 1. HPLC chromatograms of the barely line iso *ant* 17 of Nishinohoshi (A, B) and the cultivar Nishinohoshi (C, D). The MeOH extracts were subjected to C_{18} HPLC [TSKgel ODS-80Ts, Tosoh Co. Ltd., 4.6 × 250 mm; eluent, $CH_3CN/MeOH/H_2O$ (15:4:31); flow rate, 0.8 mL/min; UV detection at 290 or 350 nm] to compare HPLC chromatograms of the iso *ant* 17 of Nishinohoshi and Nishinohoshi grain extracts.



1 R_1=*O*-β-D-glucopyranoside R_2=OCH_3
2 R_1=OH R_2=OCH_3
3 R_1=OH R_2=H



4 R_1=*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] R_2=H
5 R_1=*O*-β-D-glucopyranoside R_2=H
6 R_1=OH R_2=OCH_3
7 R_1=OH R_2=H

Figure 2. Flavonoids (**1**−**7**) from the barley line iso *ant* 17 of Nishinohoshi.

(C-4); HRESIMS *m*/*z* 325.0683 [(M + Na)$^+$; calcd for $C_{16}H_{14}O_6Na$, 325.0683].

Chrysoeriol 7-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (***4***)*:* yellow solid; ^{1}H NMR (CD_3OD/CD_3COCD_3, 1:1) δ 1.18 (3H, d, *J* = 6.3 Hz, H-6‴), 3.34 (1H, m, H-4‴), 3.43 (1H, dd, 8.8, 9.7 H-4″), 3.51 (1H, m, H-2″), 3.53 (1H, m, H-3″), 3.64 (1H, dd, *J* = 6.2, 9.3 Hz, H-5‴), 3.66 (1H, dd, *J* = 6.2, 11.4 Hz, H-6a″), 3.70 (1H, dd, *J* = 3.5, 9.5 Hz, H-3‴), 3.74 (1H, ddd, *J* = 2.0, 6.3, 9.8 Hz, H-5″), 3.90 (1H, dd, *J* = 1.6, 3.5 Hz, H-2‴), 4.00 (3H, s, H-7′), 4.06 (1H, dd, *J* = 1.9, 11.3 Hz, H-6b″), 4.73 (1H, dd, *J* = 1.6 Hz, H-1‴), 5.12 (1H, d, *J* = 7.4 Hz, H-1″), 6.53 (1H, *d*, *J* = 2.2 Hz, H-6), 6.75 (1H, s, H-3), 6.81 (1H, d, *J* = 2.2 Hz, H-8), 6.99 (1H, d, *J* = 8.3 Hz, H-5′), 7.59 (1H, d, *J* = 2.1 Hz, H-2′), 7.61 (1H, dd, *J* = 2.1, 8.3 Hz, H-6′); ^{13}C NMR (CD_3OD/CD_3COCD_3, 1:1) δ 18.0 (C-6‴), 56.7 (C-7′), 67.4 (C-6″), 69.6 (C-5‴), 71.2 (C-4″), 72.0 (C-2‴), 72.4 (C-3‴), 74.0 (C-4‴), 74.7 (C-2″), 77.1 (C-5″), 77.8 (C-3″), 96.2 (C-8), 100.9 (C-6), 101.5 (C-1″), 102.0 (C-1‴), 104.6 (C-3), 107.0 (C-10), 110.8 (C-2′), 116.9 (C-5′), 121.9 (C-6′), 123.3 (C-1′), 149.4 (C-3′), 152.4 (C-4′), 158.7 (C-9), 162.9 (C-5), 164.6 (C-7), 166.4 (C-2), 183.8 (C-4); HRESIMS *m*/*z* 631.1633 [(M + Na)$^+$; calcd for $C_{28}H_{32}O_{15}Na$, 631.1633].

Chrysoeriol 7-O-β-D-glucopyranoside (***5***)*:* yellow solid; ^{1}H NMR (CD_3OD/CD_3COCD_3, 1:1) δ 3.44 (1H, dd, *J* = 8.8, 9.7 Hz, H-4″), 3.51 (1H, m, H-2″), 3.53 (1H, m, H-3″), 3.61 (1H, ddd, *J* = 2.4, 5.9, 9.7 Hz, H-5″), 3.73 (1H, dd, *J* = 5.9, 12.1 Hz, H-6a″), 3.94 (1H, dd, *J* = 2.4, 12.1 Hz, H-6b″), 3.99 (3H, s, H-7′), 5.13 (1H, d, *J* = 7.5 Hz, H-1″), 6.49 (1H, d, *J* = 2.2 Hz, H-6), 6.75 (1H, s, H-3), 6.88 (1H, d, *J* = 2.2 Hz, H-8), 6.96 (1H, d, *J* = 8.3 Hz, H-5′), 7.59 (1H, d, *J* = 2.1 Hz, H-2′), 7.61 (1H, dd, *J* = 2.2, 8.3 Hz, H-6′); ^{13}C NMR (CD_3OD/CD_3COCD_3, 1:1) δ 56.7 (C-7′), 62.5 (C-6″), 71.2 (C-4″), 74.7 (C-2″), 77.8 (C-3″), 78.4 (C-5″), 96.1 (C-8), 101.0 (C-6), 101.6 (C-1″), 104.6 (C-3), 107.0 (C-10), 110.8 (C-2′), 116.8 (C-5′), 121.9 (C-6′), 123.2 (C-1′), 149.5 (C-3′), 152.5 (C-4′), 158.8 (C-9), 162.8 (C-5), 164.7 (C-7), 166.3 (C-2), 183.8 (C-4); HRESIMS *m*/*z* 485.1045 [(M + Na)$^+$; calcd for $C_{22}H_{22}O_{11}Na$, 485.1054].

Tricin (***6***)*:* yellow solid; ^{1}H NMR (CD_3OD/CD_3COCD_3, 1:1) δ 3.97 (3H, s, H-7′), 3.97 (3H, s, H-8′), 6.21 (1H, d, *J* = 2.1 Hz, H-6), 6.51 (1H, d, *J* = 2.1 Hz, H-8), 6.72 (1H, s, H-3), 7.34 (1H, s, H-2′), 7.34 (1H, s, H-6′); ^{13}C NMR (CD_3OD/CD_3COCD_3, 1:1) δ 57.1 (C-7′), 57.1 (C-8′), 95.1 (C-8), 100.0 (C-6), 104.7 (C-3), 105.3 (C-10), 105.3 (C-2′), 105.3 (C-6′), 122.5 (C-1′), 141.2 (C-4′), 149.4 (C-3′), 149.4 (C-5′), 159.3

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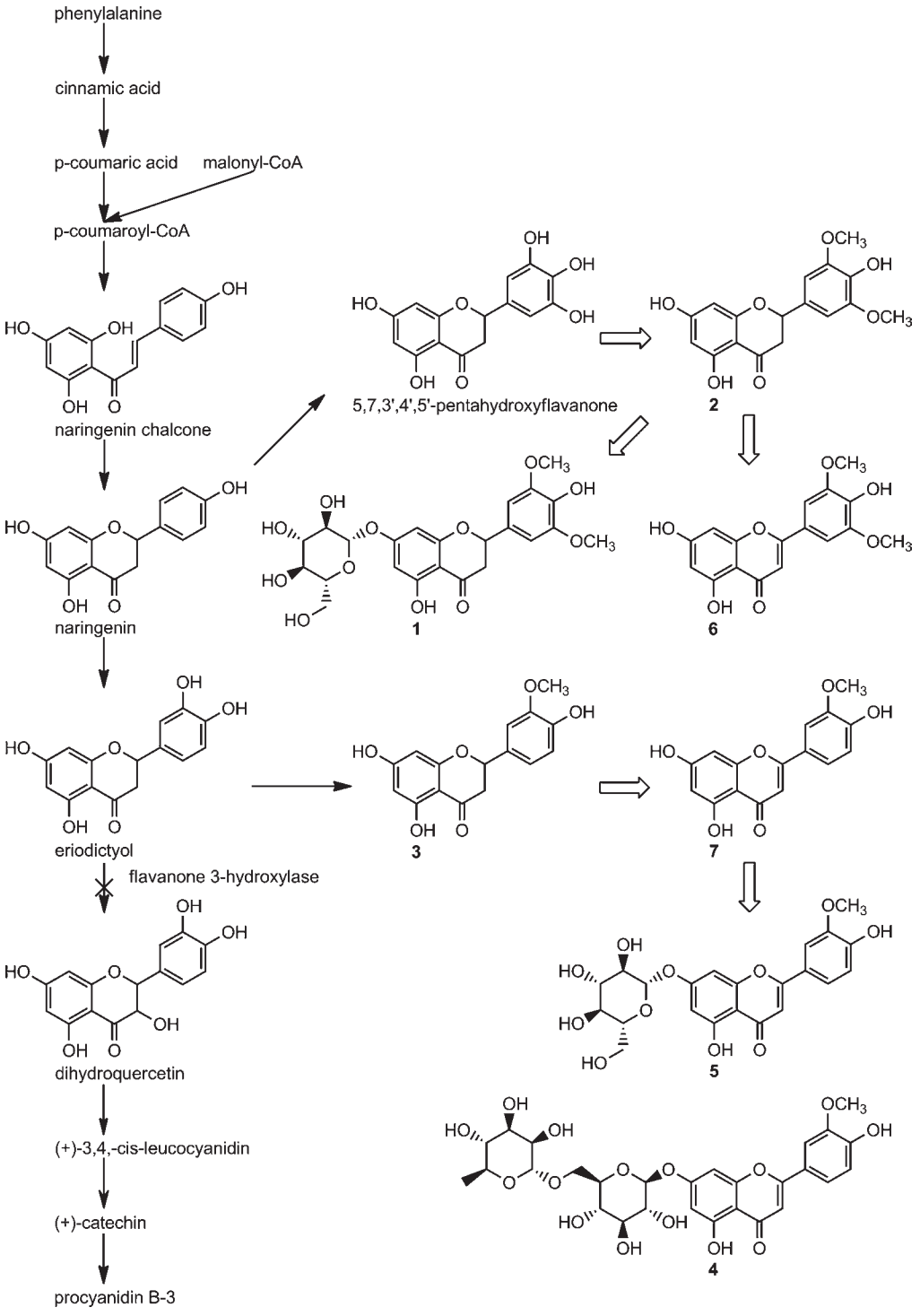

Figure 3. Plausible biogenetic pathway of flavonoids (**1**−**7**).

(C-9), 163.2 (C-5), 165.7 (C-2), 166.0 (C-7), 183.5 (C-4); HRESIMS *m*/*z* 353.0634 [(M + Na)$^+$; calcd for $C_{17}H_{14}O_7Na$, 353.0632].

Chrysoeriol (***7***): yellow solid; ^{1}H NMR (CD_3OD/CD_3COCD_3, 1:1) δ 3.99 (3H, s, H-7′), 6.21 (1H, d, *J* = 2.1 Hz, H-6), 6.49 (1H, d, *J* = 2.1 Hz, H-8), 6.68 (1H, s, H-3), 6.96 (1H, d, *J* = 8.8 Hz, H-5′), 7.57 (1H, m, H-2′) 7.58 (1H, m, H-6′); ^{13}C NMR (CD_3OD/CD_3COCD_3, 1:1) δ 56.7 (C-7′), 95.1 (C-8), 100.0 (C-6), 104.4 (C-3), 105.2 (C-10), 110.7 (C-2′), 116.7 (C-5′), 121.7 (C-6′), 123.6 (C-1′), 149.4 (C-3′), 152.0 (C-4′), 159.3 (C-9), 163.2 (C-5), 165.7 (C-2), 166.0 (C-7), 183.5 (C-4); HRESIMS *m*/*z* 323.0521 [(M + Na)$^+$; calcd for $C_{16}H_{12}O_6Na$, 323.0526].

RESULTS AND DISCUSSION

The barley line iso *ant* 17 has been developed by backcross breeding using a leading cultivar, Nishinohoshi, as a recurrent parent and a proanthocyanidin-free *ant* 17 mutant as a non-recurrent parent in Japan. The HPLC chromatograms of the iso

Table 2. Flavonoid (1−7) Concentrations (Milligrams per Kilogram, Dry Weight) in Seeds of Different Lines and Cultivars

	flavonoids						
line or cultivar	1	2	3	4	5	6	7
iso *ant* 13 of Nishinohoshi	165 ± 1[a]	62 ± 1	6 ± 0	224 ± 3	128 ± 2	42 ± 1	28 ± 1
iso *ant* 17 of Nishinohoshi	169 ± 7	53 ± 2	6 ± 0	229 ± 4	126 ± 2	40 ± 1	24 ± 1
iso *ant* 22 of Nishinohoshi	135 ± 1	38 ± 0	5 ± 0	228 ± 2	162 ± 1	39 ± 1	24 ± 1
Nishinohoshi	nd[b]	nd	nd	nd	nd	21 ± 0	nd
Amaginijo	nd	nd	nd	nd	nd	16 ± 0	nd
Harrington	nd	nd	nd	nd	nd	7 ± 0	nd

[a] Mean ± SE of results from three replicates. [b] Not detected.

ant 17 of Nishinohoshi and Nishinohoshi grain extracts were compared (Figure 1). In the extract of iso *ant* 17 of Nishinohoshi, seven major peaks (**1**−**7**) in addition to the peaks detected at retention times of 3−5 min at 290 nm were detected at 290 or 350 nm. In contrast, in the extract of Nishinohoshi, only one peak (**6**) among the seven peaks (**1**−**7**) was detected but was much smaller than that of iso *ant* 17 of Nishinohoshi. The compounds (**1**−**7**) corresponding to the seven peaks in the extract of iso *ant* 17 of Nishinohoshi were isolated.

Compound **1** had the molecular formula $C_{23}H_{26}O_{12}$ established by HRESIMS [*m*/*z* 517.1297 $(M + Na)^+$, Δ −1.9 mmu], indicating 11 degrees of unsaturation. The ^{13}C NMR and DEPT 135 spectra resolved 23 carbon signals comprising 9 carbons without a C−H bond, including 1 carbonyl, 10 methine carbons, 2 methylene carbons, and 2 methyl carbons (Table 1; Figure 2). The gross structure was elucidated by analyses of 1D and 2D NMR spectra. The ^1H and ^{13}C NMR shifts and the HMBC correlations were similar to those of dihydrotricin[16,17] except for those corresponding to a sugar moiety. The gross structure and stereochemistry of a sugar moiety were deduced from analyses of DQFCOSY and NOESY spectra and $^1H-^1H$ coupling constants. The NOESY correlations for H-1″/H-3″, H-1″/H-5″, and H-3″/H-5″ indicated that all of the H-1″, H-3″, and H-5″ were axial. The large *J*(H-1″/H-2″) value (7.7 Hz) indicated that the configuration of the anomeric center was β-form. The HMBC correlation for H-1″ to C-7 and their chemical shifts indicated the connection of C-1″ and C-7 through an oxygen atom. Moreover, the several twin signals of ^1H and ^{13}C NMR, the small Δε of the CD spectrum at 278 nm, and the separation of chiral column HPLC indicated that dihydrotricin 7-*O*-β-D-glucopyranoside (**1**) was present in a 1:1 mixture of epimers. Thus, the structure of **1** was elucidated to be (2*RS*)-dihydrotricin 7-*O*-β-D-glucopyranoside. Dihydrotricin 7-*O*-β-D-glucopyranoside (**1**) was isolated from a natural source for the first time.

The gross structures of flavanones (2*RS*)-dihydrotricin (**2**) and (2*RS*)-homoeriodictyol (**3**) were elucidated by analyses of 1D and 2D NMR spectra (Figure 2), which were in agreement with literature data.[8,16−18] The small Δε of CD spectra at 286 and 297 nm of (2*RS*)-dihydrotricin (**2**) and (2*RS*)-homoeriodictyol (**3**), respectively, and the separation of chiral column HPLC indicated that (2*RS*)-dihydrotricin (**2**) and (2*RS*)-homoeriodictyol (**3**) were present in racemic form. Although homoeriodictyol (**3**) was previously isolated from barley,[4] dihydrotricin (**2**) was isolated from barley for the first time. The gross structures of flavones chrysoeriol 7-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (**4**), chrysoeriol 7-*O*-β-D-glucopyranoside (**5**), tricin (**6**), and chrysoeriol (**7**) were elucidated by analyses of 1D and 2D NMR spectra (Figure 2), which were in agreement with literature data.[18−25] Although chrysoeriol 7-*O*-β-D-glucopyranoside (**5**), tricin (**6**), and chrysoeriol (**7**) were previously isolated from barley,[26−28] chrysoeriol 7-*O*-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside] (**4**) was isolated from barley for the first time.

Two barley lines, iso *ant* 13 and iso *ant* 22, of Nishinohoshi have been developed by backcross breeding using a leading cultivar, Nishinohoshi, as a recurrent parent and a proanthocyanidin-free *ant* 13 and *ant* 22 mutant as a nonrecurrent parent in Japan as well as a line iso *ant* 17 mutant of Nishinohoshi. In normal cultivars, which produce proanthocyanidins, procyanidin B-3 is generated from eriodictyol via dihydroquercetin, leucocyanidin, and catechin (Figure 3).[4] Flavanone 3-hydroxylase catalyzes the reaction from eriodictyol to dihydroquercetin. *Ant* 13 was reported to be a regulatory gene, coding for a trans-acting factor necessary for the transcription of at least three structural genes coding for chalcone synthase, flavanone 3-hydroxylase, and dihydroflavonol reductase.[29] *Ant* 17 may code for one subunit of flavanone 3-hydroxylase, which is believed to be a dimeric enzyme, and *Ant* 22 may code for the other.[4] The concentrations of the isolated flavonoids (**1**−**7**) in the grain extracts of iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi, Nishinohoshi, an old Japanese cultivar, Amaginijyo, and a North American cultivar, Harrington, were determined (Table 2). The concentrations of the isolated flavonoids (**1**−**7**) in iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi were similar to each other, whereas the isolated flavonoids (**1**−**5** and **7**) were not detected in Nishinohoshi, Amaginijo, and Harrington. The concentration of tricin (**6**) in Nishinohoshi was a half those in iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi. Except for iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi, the concentration of tricin (**6**) was highest in Nishinohoshi, followed by Amaginijo and Harrington. Accumulations of chrysoeriol (**7**) and its precursor homoeriodictyol (**3**) have been reported in *ant* 17 and *ant* 22 mutants,[4] whereas those of tricin (**6**), its precursor dihydrotricin (**2**), and its glucopyranoside dihydrotricin 7-*O*-β-D-glucopyranoside (**1**) have not yet been found in proanthocyanidin-free mutants. Thus, the accumulations of dihydrotricin 7-*O*-β-D-glucopyranoside (**1**), dihydrotricin (**2**), and tricin (**6**) in iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi may be caused by enhancement of the biogenetic pathway to tricin (**6**) equipped in Nishinohoshi as a result of blocking the pathway from eriodictyol to catechin via dihydroquercetin.

Homoeriodictyol (**3**) and chrysoeriol (**7**) have been reported to show many beneficial effects on health.[9−15] Dihydrotricin (**2**) has a vasodilatory effect and antioxidant activity.[17] Chrysoeriol 7-*O*-β-D-glucopyranoside (**5**) has a neuroprotective effect and antioxidant activity.[30] Tricin (**6**) has anticancer activity,[21,31,32]

antioxidant activity,[17,22] antihistaminic activity,[33] antivirus activity,[34] and neuroprotective effects.[35] Moreover, barley grain was highly evaluated as a source of dietary fiber.[36] Thus, proanthocyanidin-free barley with beneficial flavonoids as well as dietary fiber may be widely used in the food industry. The development of an effective utilization method for iso *ant* 13, iso *ant* 17, and iso *ant* 22 of Nishinohoshi other than their use in malt and rice boiled with barley is needed.

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