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Isolation and Structural Elucidation of Some Procyanidins from Apple by Low-Temperature Nuclear Magnetic Resonance

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Procyanidin fractions from apple were separated according to the degree of polymerization using normal phase chromatography. Evaluation of physiological functionalities of procyanidins requires individual structural determination. However, it is difficult to elucidate the structure of procyanidins, in particular those with (+)-epicatechin (**1**) or (−)-catechin (**2**) units, and determine whether the interflavanoid bonds are 4β→8 or 4β→6 without cleavage and acetylation. Structural determination used LC-MS and low-temperature NMR. Nine procyanidins were separated by preparative HPLC consisting of three well-known procyanidins [procyanidin B1 (**3**), procyanidin B2 (**4**), and procyanidin C1 (**5**)] and six new procyanidins [epicatechin-(4β→8)-epicatechin-(4β→8)-catechin (**6**); epicatechin-(4β→6)-epicatechin-(4β→8)-catechin (**7**); epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin (**8**); epicatechin-(4β→8)-epicatechin-(4β→6)-catechin (**9**); epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin (**10**); and epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (**11**)]. Compounds **6**−**11** were detected for the first time as apple constituents.

KEYWORDS: NMR; apple; procyanidins; tetramer; trimer

INTRODUCTION

Procyanidins (condensed tannins) are a group of polymeric polyphenols widely found as secondary metabolites in plants. They provide the bitter and astringent taste (*1*) and are important for color (*2*, *3*), flavor, and longevity of beverages and foods. Procyanidins consist of (+)-catechin and (−)-epicatechin units, which are linked together through the 4β→8 or 4β→6 interflavanoid bonds (**Figure 1**). Procyanidins in plants are present in the form of oligomers and polymers, and the average degree of polymerization may vary widely.

Polyphenols in various beverages (e.g., red wine and green tea) and foods (e.g., grape (seed) and apple) have been reported to have some physiological functionalities (*4*−*7*). Procyanidins have various functionalities such as antiallergic activity (*8*−*10*), anticaries activity (*11*), anti-hypertensive activity (*12*), antioxidative activity (*6*, *13*−*15*), hair-growth promotion (*16*), and inhibitory activity against some enzymes and receptors (*17*−*19*). Therefore, much research has focused on the relationship between their degree of polymerization and structure and their physiological activity. In particular, procyanidins from apple have been reported to exhibit the above activities and also inhibition of cholera toxin (*20*) and accumulation of lipids in cells (*18*).

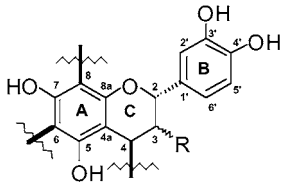

Figure 1. Structure of procyanidins.

The procyanidins in apple have been reported to include three dimers [procyanidin B1 (**3**) [epicatechin-(4β→8)-catechin], procyanidin B2 (**4**) [epicatechin-(4β→8)-epicatechin], and procyanidin B5 [epicatechin-(4β→6)-epicatechin]] and the trimer [procyanidin C1 (**5**) [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin]] (*21*−*24*). Kameyama et al. (*25*) have shown by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS) that procyanidins in apple were present as a mixture of various oligomers ranging from dimers to pentadecamers. Yanagida et al. (*26*) have reported that procyanidin fractions were prepared from apple according to the degree of polymerization by a normal phase chromatography.

Normally, characterization of procyanidins proceeds through investigations of nuclear magnetic resonance (NMR) data of their peracetate or methyl ether acetates, in conjunction with fast atom bombardment mass spectrometry (FAB-MS) and circular dichroism (CD) data. However, it is difficult to

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determine the structure of procyanidins by NMR, in particular, the position of 4β→8 or 4β→6 interflavanoid bonds, for the following reasons. At ambient temperature, procyanidins show broadening of ^{1}H NMR signals due to atropisomerism, which results from steric interactions in the vicinity of the interflavanoid bond about which the flavonoid units are free to rotate (*27*). This suggests that the T2 values of procyanidins are very small, and, therefore, it is impossible to detect cross-peaks in ^{1}H 2D NMR spectra at ambient temperature. To overcome this situation, several approaches have been adopted. Ferreira's group (*28*, *29*) synthesized phenolic permethyl ether 3-*O*-acetates, which were stable enough at high temperature to overcome the rotational barriers and yield sharp first-order spectra. The combination of the cleavage of the interflavanoid bond on the A-ring by thiolysis (*30*, *31*) or reaction with phloroglucinol (*32*−*34*) and comparison of ^{13}C NMR chemical shifts of each unit has been performed to determine the position of the interflavanoid bond. However, the procyanidins used for characterization were not intact and could not be used to study the physiological functionalities, and therefore, structure−activity relationships remained unknown.

In this investigation, we have applied ^{1}H NMR and ^{1}H 2D NMR at low temperatures (−40 and −20 °C) without acetylation and cleavage to the structural analysis of procyanidins from apple. As a result of this NMR analysis method, we have made exact distinctions of the type of units and the positions of interflavanoid bonds and obtained complete assignment of ^{1}H and ^{13}C NMR signals for five trimers and one tetramer, previously unknown in apple.

MATERIALS AND METHODS

Apparatus. ^{1}H, ^{13}C, and 2D NMR spectra were recorded with a JEOL A-600 spectrometer (JEOL, Tokyo, Japan) using 3 mm tubes with CD_3OH and CD_3OD as the solvent and tetramethylsilane (TMS) as an internal standard. ^{1}H and ^{13}C NMR were performed at 600 and 150 MHz, respectively. ^{1}H-detected heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC) spectroscopies were used to assign correlations between ^{1}H and ^{13}C signals. NMR analyses were performed at low temperatures (−40 or −20 °C) using a long-time cold dry air supplier (JEOL NM-ALAS/L unit) with a temperature controller (JEOL AVT-1A unit). Chemical shifts are given in δ values.

Liquid chromatography−mass spectrometry (LC-MS) was performed on a JEOL LC-Mate with electrospray ionization (ESI) as an ion source and operated in the positive-ion mode. The ion spray voltage was set at +50 eV. For the analysis of procyanidin fractions, high-performance liquid chromatography (HPLC) was performed by means of an HPLC system equipped with an L-6200 intelligent pump (Hitachi Ltd., Tokyo, Japan), an AS-2000 autosampler (Hitachi), and an Inertsil ODS III (GL Science Inc., Tokyo, Japan) column (i.d. 4.6 × 250 mm) at 30 °C. A mixture of 5% HCOOH and CH_3CN was used as the mobile phase, and the flow rate was 1.0 mL/min. For the first 10 min, the initial eluent used was a 5% HCOOH solution, followed by a linear gradient from 0 to 6% CH_3CN for 55 min. Subsequently, the concentration was held at 6% CH_3CN for 5 min and then returned to the initial conditions (5% HCOOH) to re-equilibrate for 10 min. Detection was performed using an L-4200 UV−vis detector (Hitachi) at 280 nm, and the injection volume was 10 μL of the procyanidin fractions (5 mg/mL).

High-resolution mass spectrometry analyses were performed on a JEOL AccuTOF JMS-T100LC spectrometer (JEOL) using a flow injection in the negative-ion mode.

Materials. (+)-Catechin (**1**) and (−)-epicatechin (**2**) were commercial samples obtained from Sigma Chemical Co., Ltd., and phloroglucinol was obtained from Wako Pure Chemical Industries Ltd. Unless otherwise stated, all other reagents and chemicals used were commercially available extrapure grade products.

Preparation of Apple Procyanidin. The crude apple procyanidin fraction was prepared from a crude apple polyphenols (CAP) fraction from unripe apples (*Malus pumila* cv. Fuji), according to the method of Yanagida et al. (*26*). Briefly, the crude procyanidin fraction was isolated from the CAP fraction using a column. Lyophilized CAP powder was dissolved in distilled water and adjusted to pH 6.5 with 5 N NaOH. The sample was applied to a Diaion HP-20ss (Mitsubishi Kasei Co., Ltd.) column, and after the column had been rinsed with distilled water, the crude procyanidin fraction was eluted with 25% ethanol. Finally, the eluate was concentrated by rotary evaporation at 45 °C and lyophilized as a crude procyanidin fraction.

Methyl Acetate Extraction of Procyanidin Oligomers. Lyophilized crude apple procyanidin powder (10 g) was suspended in methyl acetate (100 mL) and stirred for 1 h at 30 °C. The methyl acetate extract was separated and the residue re-extracted with methyl acetate three times. After the addition of distilled water, the methyl acetate fraction was concentrated by rotary evaporation at 45 °C and lyophilized.

Preparation of Procyanidin Fractions by Normal Phase Chromatography. For the preparation of procyanidin fractions according to the degree of polymerization from the methyl acetate fraction, normal phase chromatography was performed using a silica gel packed (i.d. 20 × 250 mm) column (Soken Chemical and Engineering) with hexane/acetone as the mobile phase. A sample (1 g) was applied to the silica gel column at a flow rate of 12 mL/min. For the first 30 min, the initial eluent used was hexane/acetone (35:65), followed by a linear gradient from 65 to 80% acetone for 40 min, and subsequently the concentration was held at 80% acetone for 40 min. The eluate was monitored by absorbance at 230 nm. Each procyanidin oligomer fraction (A−E) obtained was concentrated by rotary evaporation at 45 °C and lyophilized.

Purification of Procyanidins by Preparative Reversed Phase HPLC. Further purification was performed by preparative reversed phase HPLC using an Inertsil ODS III (i.d. 25 × 250 mm) column using a methanol/water solvent (15:85, v/v; for fraction B) or methanol/water solvent (20:80, v/v; for fractions C and D) at a flow rate of 12 mL/min. Two compounds were isolated from dimer fraction B, six compounds were isolated from trimer fraction C, and one compound was isolated from tetramer fraction D. The purification levels of each compound were confirmed as >95% using reversed phase HPLC.

All of the procyanidins were isolated as brownish white powders, and ^{1}H and ^{13}C NMR data at −40 or −20 °C are shown in **Tables 1**−**4**. The negative ion in the high-resolution LC-TOF mass spectra of compounds **5**−**10** gave $[M - H]^-$ and $[M - 2H + Na]^-$ at *m*/*z* 865.2 and 887.2, respectively, and the molecular formula $C_{45}H_{37}O_{18}$ was confirmed by the result of the elemental analysis. High-resolution LC-TOF (*m*/*z*): compound **5**, calcd for $C_{45}H_{37}O_{18}$ $[M - H]^-$ 865.19799; found 865.19708; compound **6**, found 865.19641; compound **7**, found 865.19560; compound **8**, found 865.19694; compound **9**, found 865.19875; compound **10**, found 865.19749. LC-TOF analysis of compound **11** gave $[M - H]^-$, $[M - 2H + Na]^-$, and $[M - 2H + K]^-$ at *m*/*z* 1153.3, 1175.3, and 1189.3, respectively, which was consistent with the molecular formula $C_{60}H_{49}O_{24}$. High-resolution LC-TOF (*m*/*z*): compound **11**, calcd for $C_{60}H_{49}O_{24}$ $[M - H]^-$ 1153.26138; found 1153.27017.

Reaction of Procyanidins with Phloroglucinol. The reaction of procyanidins with phloroglucinol was preformed according to the modified method of Foo et al. (*32*). Purified procyanidins (100 mg) and phloroglucinol (100 mg) were reacted in 1% HCl/ethanol (1 mL) for 30 min at ambient temperature. The reaction mixture was analyzed using HPLC with the equipment described above. A mixture of 5% HCOOH and CH_3CN was used as the mobile phase, and the flow rate was 1.0 mL/min. The initial eluent used was a 5% HCOOH solution, followed by a linear gradient from 0 to 25% of CH_3CN for 60 min. Detection was performed at 280 nm, and the injection volume was 10 μL of reaction mixture.

RESULTS AND DISCUSSION

In the previous study, we succeeded in the fractionation of procyanidins from apple by normal phase chromatography, and five fractions were obtained according to their degree of polymerization (**Figure 2**) (*26*). Of these fractions, two peaks in fraction A were identified as (+)-catechin (**1**) and (−)-

Table 1. ^{1}H and ^{13}C NMR Spectral Data for Procyanidin Dimers in CD_3OD at −40 °C

	^{1}H				^{13}C			
	PB1 (dimer **3**)		**PB2** (dimer **4**)		**PB1** (dimer **3**)		**PB2** (dimer **4**)	
position	A unit	B unit	A unit	B unit	A unit	B unit	A unit	B unit
2	4.96 (d, J = 5 Hz)	5.10 (br s)	4.95 (br s)	5.06 (br s)	81.4	77.0	79.9	76.9
3	4.16 (m)	3.92 (br s)	4.24 (br t)	3.79 (br s)	68.0	72.9	67.0	73.5
4	2.53 (d, J = 17 Hz) 2.58 (dd, J = 17, 4 Hz)	4.65 (br s)	2.79 (d, J = 17 Hz) 2.94 (dd, J = 17, 4 Hz)	4.61 (br s)	21.1	36.7	29.9	36.7
4a					99.9	102.1	99.6	103.9
5					155.6	157.7[a]	156.5	157.6[c]
6	5.82 (s)	5.92 (br s)	5.88 (s)	5.92 (d, J = 2 Hz)	96.5	95.4	96.8	95.6[d]
7					156.3	157.7[a]	156.6	157.8[c]
8		5.94 (br s)		5.94 (d, J = 2 Hz)	107.6	94.9	107.2	95.5[d]
8a					153.6	158.5[a]	154.5	157.7[c]
1′					132.2	132.7	132.1	132.5
2′	6.82 (br s)	6.85 (br s)	7.10 (d, J = 2 Hz)	6.83 (d, J = 2 Hz)	113.7	114.9	114.9	114.9
3′					146.0	145.4[b]	145.8	145.7
4′					145.7[b]	145.7[b]	145.5	145.4
5′	6.69 (d, J = 8 Hz)	6.68 (d, J = 8 Hz)	6.72 (d, J = 8 Hz)	6.68 (d, J = 8 Hz)	115.8	115.5	115.6	115.7
6′	6.88 (d, J = 8 Hz)	6.67 (d, J = 8 Hz)	6.85 (dd, J = 8, 2 Hz)	6.60 (dd, J = 8, 2 Hz)	119.2	119.0	118.7	119.0

[a−d] Assignments with the same letter are interchangeable.

Table 2. ^{1}H NMR Spectroscopic Data for Procyanidin Trimers in CD_3OD at −20 °C

unit	position	**PC1** (trimer **5**)	trimer **6**	trimer **7**	trimer **8**	trimer **9**	trimer **10**
A	2	4.98 (br s)	5.00 (br s)	4.78 (br s)	4.89 (br s)	4.56 (d, J = 7 Hz)	4.80 (br s)
	3	4.31 (br s)	4.18 (dd, J = 5, 11 Hz)	4.04 (dd, J = 5, 11 Hz)	4.20 (br s)	3.97 (m)	4.14 (br s)
	4	2.81 (d, J = 16 Hz) 2.94 (dd, J = 4, 16 Hz)	2.60 (dddd, J = 5, 11, 17 Hz)	2.52 (dd, J = 7, 16 Hz) 2.66 (dd, J = 4, 16 Hz)	2.76 (d, J = 17 Hz) 2.88 (dd, J = 4, 17 Hz)	2.47 (dd, J = 8, 17 Hz) 2.79 (dd, J = 4, 16 Hz)	2.74 (d, J = 16 Hz) 2.86 (dd, J = 4, 16 Hz)
	6	5.92 (s)	5.87 (s)	5.84 (s)	5.86 (s)		
	8					6.02 (s)	6.09 (s)
	2′	7.11 (d, J = 2 Hz)	6.86 (d, J = 2 Hz)	6.78 (d, J = 2 Hz)	7.08 (br s)	6.82 (d, J = 2 Hz)	6.95 (br s)
	5′	6.74 (d, J = 8 Hz)	6.71 (d, J = 8 Hz)	6.54 (d, J = 8 Hz)	6.68 (d, J = 8 Hz)	6.71 (d, J = 8 Hz)	6.74 (d, J = 8 Hz)
	6′	6.88[a]	6.88[c]	6.67 (dd, J = 2, 8 Hz)	6.75 (d, J = 2, 8 Hz)	6.74 (d, J = 8 Hz)	6.78[f] (d, J = 8 Hz)
B	2	5.23 (br s)	5.05 (br s)	5.22 (br s)	4.91 (br s)	5.01 (br s)	5.02 (br s)
	3	3.94 (br s)	4.06 (d, J = 8 Hz)	3.88 (br s)	3.84 (br s)	4.11 (br s)	4.12 (d, J = 7 Hz)
	4	4.68 (br s)	4.75 (br s)	4.46 (br s)	4.51 (br s)	4.62 (br s)	4.62 (br s)
	6	5.88 (s)	5.87 (s)			5.96 (s)	5.94 (s)
	8			6.13 (s)	6.15 (s)		
	2′	7.02 (d, J = 8 Hz)	7.04 (d, J = 2 Hz)	6.85 (br s)	6.84 (d, J = 2 Hz)	7.03 (br s)	7.03 (br s)
	5′	6.71[b]	6.68 (d, J = 8 Hz)	6.68[d]	6.67 (d, J = 8 Hz)	6.70[e]	6.72 (d, J = 8 Hz)
	6′	6.67 (d, J = 2 Hz)	6.78 (dd, J = 2, 8 Hz)	6.69[d]	6.63 (dd, J = 2, 8 Hz)	6.69[e]	6.70[f] (d, J = 8 Hz)
C	2	5.06 (br s)	5.01 (br s)	4.76 (br s)	4.86 (br s)	5.02 (br s)	5.02 (br s)
	3	3.97 (br s)	3.99 (d, J = 5 Hz)	3.87 (br s)	3.83 (br s)	3.93 (br s)	3.93 (brs)
	4	4.68 (br s)	4.67 (br s)	4.54 (br s)	4.55 (br s)	4.67 (br s)	4.67 (br s)
	6	5.97 (d, J = 2 Hz)	5.96 (d, J = 2 Hz)	6.01 (d, J = 2 Hz)	5.94 (s)	5.96 (s)	5.96 (s)
	8	6.00 (d, J = 2 Hz)	6.01 (d, J = 2 Hz)	6.05 (d, J = 2 Hz)	5.94 (s)	6.00 (s)	6.00 (s)
	2′	6.89[b] (d, J = 2 Hz)	6.89[c] (d, J = 2 Hz)	6.89 (d, J = 2 Hz)	6.87 (d, J = 2 Hz)	6.93 (br s)	6.92 (br s)
	5′	6.72[a]	6.72 (d, J = 8 Hz)	6.67[d]	6.70 (d, J = 8 Hz)	6.69[e]	6.69[f] (d, J = 8 Hz)
	6′	6.69[a]	6.70 (d, J = 2 Hz)	6.71 (d, J = 8 Hz)	6.65 (dd, J = 2, 8 Hz)	6.61 (d, J = 7 Hz)	6.64 (d, J = 8 Hz)

[a−f] Overlapped with each other.

epicatechin (**2**) by reversed phase HPLC (**Figure 3**), which are the main flavan-3-ols in apples, on the basis of direct comparison with an authentic sample and showing an $[M + H]^+$ ion peak at *m*/*z* 291 by LC-ESI/MS.

Two peaks in fraction B consisted of procyanidin dimers, showing an $[M + H]^+$ ion peak at *m*/*z* 579 by LC-ESI/MS analysis, and were purified by preparative reversed phase HPLC. Compound **3** was isolated as a brownish white powder. The ^{1}H NMR spectra of **3** at different temperatures are shown in **Figure 4**. At ambient temperature, compound **3** showed the broadening of ^{1}H NMR signals due to atropisomerism. To yield sharp first-order spectra by NMR, procyanidins have been converted into their permethyl ether 3-*O*-acetates, which are stable at high temperature (*28*, *29*), and the NMR spectra were measured at high temperature. However, ^{1}H NMR signals were obtained as sharp peaks by measurement at low temperatures (−20 or −40 °C) without the acetylation. The ^{1}H and ^{13}C NMR spectral data of **3** are shown in **Table 1**. As a result, the ^{1}H NMR spectra exhibited methylene proton signals at δ_H 2.53 and 2.58, which were assigned to the 4-position of the A unit as a (+)-catechin moiety. Next, the proton signals at the 2- and 3-positions of the A unit were assigned to δ_H 4.96 and 4.16, respectively, by ^{1}H−^{1}H shift correlation spectroscopy (COSY) from the proton signal at the 4-position of the A unit as a starting point. With the ^{13}C NMR, HMQC, and HMBC at −40 °C, the signals originating from the A unit were assigned, because long-range correlations were observed from the proton signal (δ_H 4.96) at the 2-position of the C ring to the carbon signals of the B ring and from the proton signal (δ_H 2.53 and 2.58) at the 4-position of the C ring to those of the A ring. The carbon signal at the 8a-position of the A unit was identified by the observation of an HMBC cross-peak from the proton signal (δ_H 4.96) at the 2-position of the A unit. In the B unit, the signals were assigned in the same way, after the carbon signal (δ_C 153.6) at the 8a-

Table 3. ^{13}C NMR Spectroscopic Data for Procyanidin Trimers in CD_3OD at −20 °C

	A unit						B unit						C unit					
position	**5**	**6**	**7**	**8**	**9**	**10**	**5**	**6**	**7**	**8**	**9**	**10**	**5**	**6**	**7**	**8**	**9**	**10**
2	79.4	81.5	81.9	79.2	82.5	79.5	76.6	76.7	77.4	76.9	77.0	77.0	76.7	76.6	77.0	77.3	76.7	76.8
3	66.7	68.1	68.3	67.0	68.9	67.5	72.9	72.2	72.8	73.3	71.8	71.7	73.4	73.4	72.9	73.1	73.7	73.8
4	30.0	26.3	27.3	29.8	28.6	29.6	37.1	37.0	37.2	37.1	37.8	37.0	36.9	36.9	37.5	37.3	37.7	37.0
4a	100.0	100.2	100.5	99.9	101.2	100.4	102.1	102.3	101.8	101.3	99.2	99.1	101.5	101.3	98.7	99.7	101.4	101.4
5	156.6	155.7	155.7	156.4	155.8[g]	156.4	156.4	157.1	157.1	156.7[d]	158.0	158.0	158.0	157.6	159.8	159.2	158.[h]	158.1
6	97.4	97.1	96.8	97.0	107.9	108.0	97.1	97.0	107.5	108.2	97.6	96.1	96.0[a]	95.9[b]	96.7	96.3	96.1	97.5
7	156.6	156.3	156.4	156.7[d]	155.9[g]	155.7	156.9	156.9	156.3	156.4	158.7	158.2	158.2	158.2	159.7	159.3	158.[h]	158.7
8	107.4	107.9	107.0	106.3	95.8	96.2	106.8	106.2	95.7	96.3	107.5	107.4	96.1[a]	96.2[b]	96.2	95.8	95.9	95.9
8a	154.4	153.5	153.7	154.2	154.8	155.4	154.7	154.6	155.5	155.5	154.8	154.7	157.7	158.0	157.9	158.0	157.8	158.7
1′	131.8	132.3	132.0	132.0	131.8	132.1	132.3	132.5	132.3	132.1	132.3	132.3	132.4	132.2	132.1	132.3	131.7	131.7
2′	115.0	113.9	114.0	114.8	115.0	115.1	114.9	114.8	115.0	115.0	114.9	114.9[i]	115.0	114.8	115.2	115.2	114.9	115.0[i]
3′	146.0	145.7	146.1	145.8	146.3[f]	145.9	145.9	145.8	145.7	145.9	146.0[f]	145.9[j]	145.6	146.1	145.8[c]	145.6[e]	145.9[f]	146.0[j]
4′	145.6	145.3	145.8	145.5	146.3	145.8	145.4	145.2	145.5	145.6[e]	145.6	145.5	145.4	145.6	145.8	145.6[e]	145.5	145.6
5′	115.7	115.8	115.9	115.8	115.8	115.6	115.8	115.7	115.6	115.7	115.8	115.8	115.8	115.6	115.6	115.5	115.8	115.8
6′	118.7	119.0	119.5	118.3	119.8	119.1	118.5	118.5	119.0	119.0	118.5	118.8	119.0	119.5	119.5	119.4	118.7	118.5

[a–j] Assignments with the same letter are interchangeable.

Table 4. ^1H and ^{13}C NMR Spectral Data for Procyanidin Tetramer **11** in CD_3OD at −20 °C

	^1H				^{13}C			
position	A unit	B unit	C unit	D unit	A unit	B unit	C unit	D unit
2	5.00 (br s)	5.29 (br s)	5.26 (br s)	5.09 (br s)	79.5	76.8	76.7	76.9
3	4.32 (br t)	3.97 (d, $J = 2$ Hz)	4.08 (d, $J = 2$ Hz)	3.99 (d, $J = 2$ Hz)	66.8	72.9	73.0	73.5
4	2.81 (d, $J = 17$ Hz) 2.96 (dd, $J = 4$, 17 Hz)	4.71 (br s)	4.75 (br s)	4.72 (br s)	30.0	37.3	37.4	37.1
4a					100.1	102.4	102.2	101.8
5					156.6	156.4[j]	156.4[j]	158.0
6	5.94 (s)	5.95 (s)	5.93 (s)	5.98 (d, $J = 2$ Hz)	97.2[d]	97.3[d]	97.0	96.1
7					156.7	156.9	157.0	158.2
8				6.01 (d, $J = 2$ Hz)	107.6	107.3	107.1	96.0
8a					154.6	155.0	154.8	157.9
1′					132.0	132.5	132.6	132.6
2′	7.13 (d, $J = 2$ Hz)	7.03 (d, $J = 2$ Hz)	7.09 (d, $J = 2$ Hz)	6.91[c] (d, $J = 2$ Hz)	115.1	115.0[e]	114.9	115.0[e]
3′					146.0	145.9	145.7[i]	145.7[i]
4′					145.6	145.4[h]	145.2	145.4[h]
5′	6.75[b] (d, $J = 8$ Hz)	6.69[a] (d, $J = 8$ Hz)	6.73[b] (d, $J = 8$ Hz)	6.74[b] (d, $J = 8$ Hz)	115.8	115.8[f]	116.0	115.9[f]
6′	6.91[c]	6.75[b]	6.78[b]	6.70[a]	118.9	118.7[g]	118.7[g]	119.1

[a–c] Overlapped with each other. [d–j] Assignments with same symbol are interchangeable.

position of the A unit had been established, which was assigned by HMBC correlation of the proton signal (δ_H 4.65) at the 4-position of B unit. This was the most important correlation between the proton signal (δ_H 4.96) at the 2-position of the A unit and the carbon signal (δ_C 153.6) at the 8a-position of the A unit by HMBC for the determination of the position of interflavanoid bond between A and B units. As a result, compound **3** was identified as procyanidin B1, after comparison with ^{13}C NMR data in the literature (*27*, *35*).

Compound **4** was isolated as a brownish white powder. NMR measurement in a similar way to dimer **3** provided a complete assignment of the A and B units. The ^1H and ^{13}C NMR spectral data of **4** are shown in **Table 1**. From the ^1H and ^{13}C NMR spectra, it was shown that **4** had an (−)-epicatechin unit as the A unit, because the proton signal (δ_H 2.79 and 2.94) and the carbon signal (δ_C 29.9) at the 4-position of the A unit, the carbon signal (δ_C 79.9) at the 2-position of the A unit, and the carbon signal (δ_C 156.5) at the 5-position of the A unit were assigned. It was clear that the interflavanoid bond between the A and B units was 4β→8, because of the correlation between the proton signal (δ_H 4.61) at the 4-position of the B unit and the carbon signal (δ_C 154.5) at the 8a-position of the A unit, which was assigned by correlation of the proton signal (δ_H 4.95) at the 2-position of the A unit, observed in the HMBC spectra, as seen for **3**. Therefore, compound **4** was determined to be procyanidin B2 (*36*).

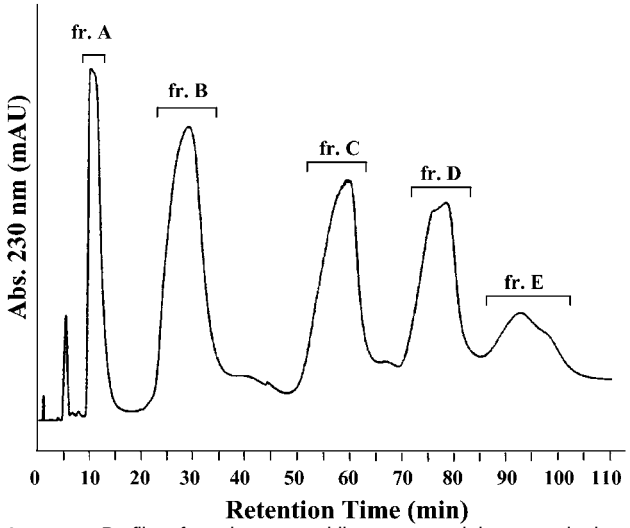

Figure 2. Profile of apple procyanidins separated by normal phase chromatography using a mixture of hexane and acetone as the mobile phase. The eluate was monitored by an absorbance at 230 nm. Fractions: A, flavan-3-ol fraction; B, procyanidin dimer fraction; C, procyanidin trimer fraction; D, procyanidin tetramer fraction; E, procyanidin pentamer fraction.

Six compounds were separated from fraction C, consisting of procyanidin trimers. The ^1H and ^{13}C NMR spectral data of

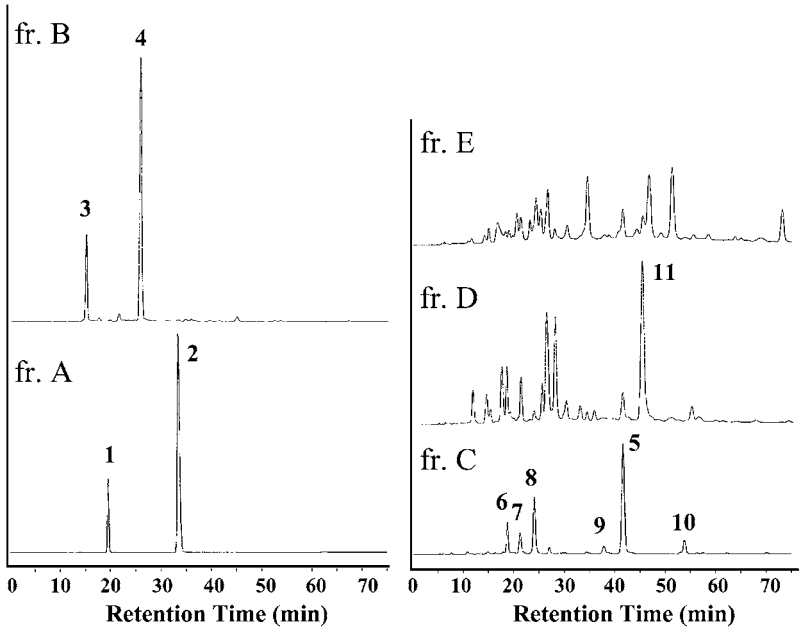

Figure 3. Reversed phase HPLC profiles of apple procyanidins fractions (A–E) separated by normal phase chromatography. Peaks: 1, (+)-catechin; 2, (–)-epicatechin; 3, procyanidin B1; 4, procyanidin B2; 5, procyanidin C1.

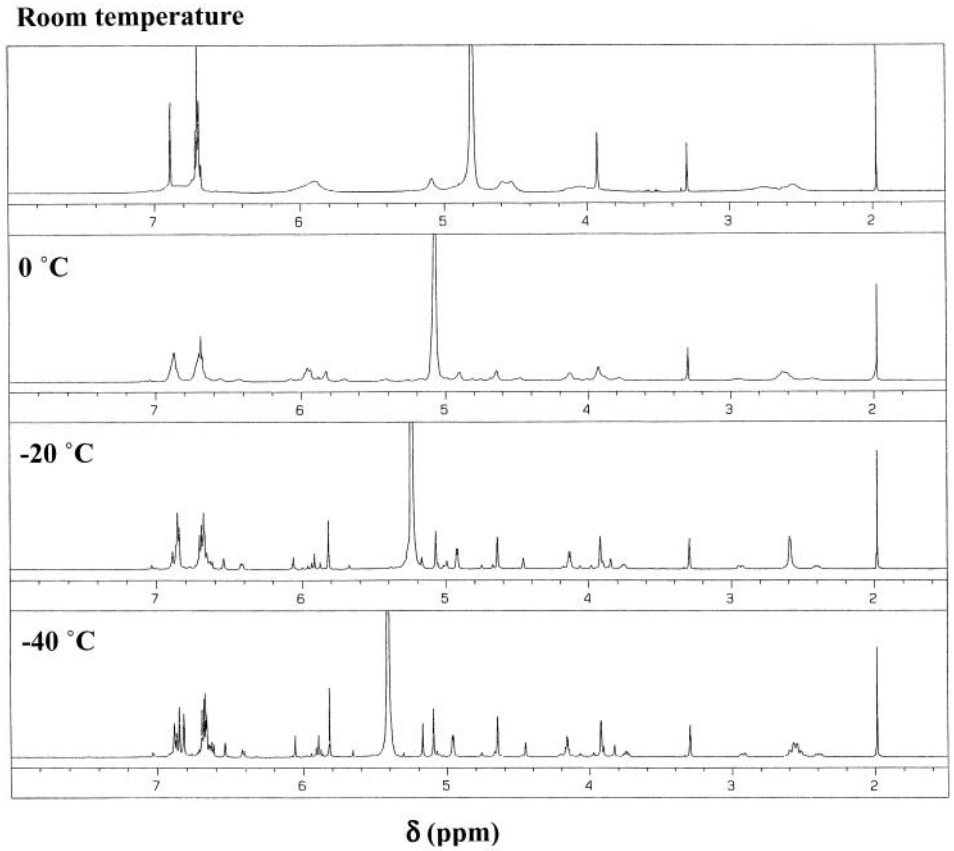

Figure 4. Effect of temperature on ^{1}H NMR spectra of procyanidin B1.

the six trimers are indicated in **Tables 2** and **3**, and the structures are shown in **Figure 5**. Compound **5**, the main compound in fraction C, was isolated using preparative HPLC and showed an $[M + H]^+$ ion peak at *m/z* 867. Following the same procedure used for the dimers, the signals were picked out as belonging to the A unit, B unit, and C unit by 1D and 2D NMR spectroscopy. Furthermore, a phloroglucinol reaction experiment was performed to confirm whether (−)-epicatechin or (+)-catechin were units contained in **5**. The result indicated that **5** was composed of three epicatechin units. All of the results showed that **5** was identical to procyanidin C1 [i.e., epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin] (*37*).

Compound **6** was isolated from fraction C using preparative HPLC and showed an $[M + H]^+$ ion peak at *m/z* 867. Compound **6** had similar NMR spectroscopic data when compared to dimer **3** for the A and partial B units and to trimer **5** for the partial B and C units. However, we could not assign the carbon signal at the 8a-position of the A unit and the position

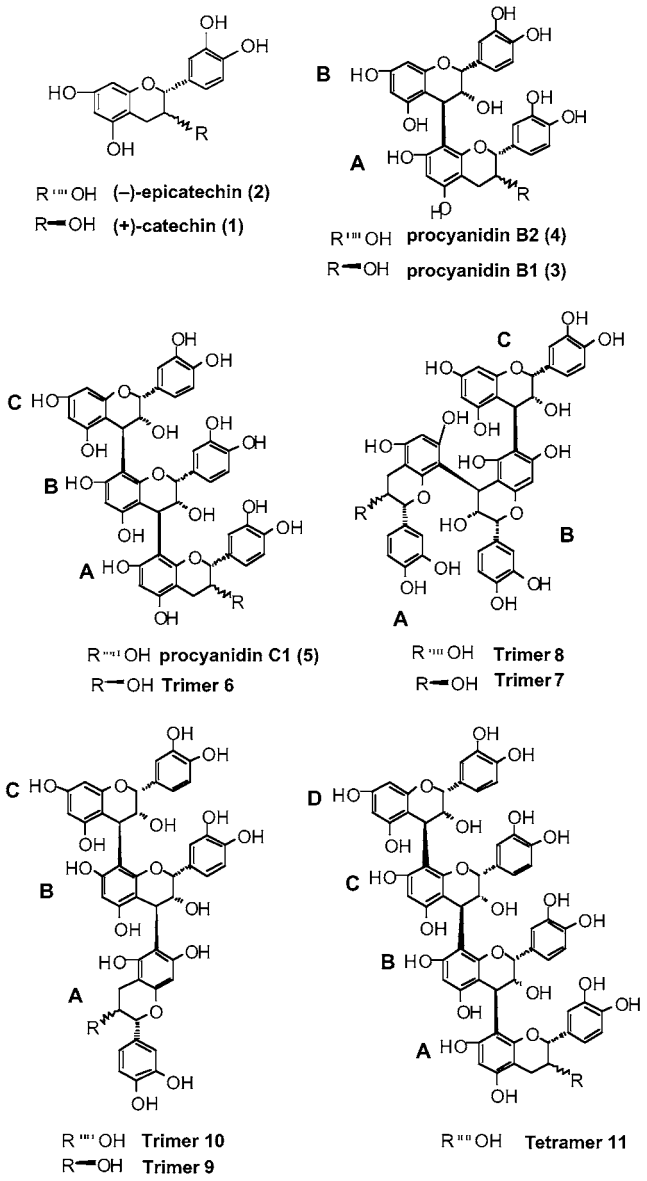

Figure 5. Structures of (+)-catechin (**1**), (–)-epicatechin (**2**), procyanidin B1 [epicatechin-(4β→8)-catechin] (**3**), procyanidin B2 [epicatechin-(4β→8)-epicatechin] (**4**), procyanidin C1 [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin] (**5**), epicatechin-(4β→8)-epicatechin-(4β→8)-catechin (**6**), epicatechin-(4β→6)-epicatechin-(4β→8)-catechin (**7**), epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin (**8**), epicatechin-(4β→8)-epicatechin-(4β→6)-catechin (**9**), epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin (**10**), and epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (**11**).

of the interflavanoid bond between the A and B units because the correlation between the proton signal (δ_H 5.00) at the 2-position of the A unit and the carbon signal at the 8a-position of the A unit was not observed in the HMBC spectrum. Therefore, we tried H−D exchange experiments in CD_3OH and CD_3OD, leading to isotopic shift of the carbon signals at the 5- and 7-positions and identifying the carbon signal at the 8a-position among the crowded phenolic carbons. As a result, three carbon signals (δ_C 153.5, 154.6, and 158.0) were selected to be the signals at the 8a-position of each unit from the nine signals observed between 155 and 159 ppm (**Figure 6**). The carbon signal (δ_C 153.5) at the 8a-position of the A unit was assigned by correlation of the proton signal (δ_H 2.60) at the 4-position of the A unit in an HMBC experiment. The proton signal at the 4-position of the B unit (δ_H 4.75) was determined

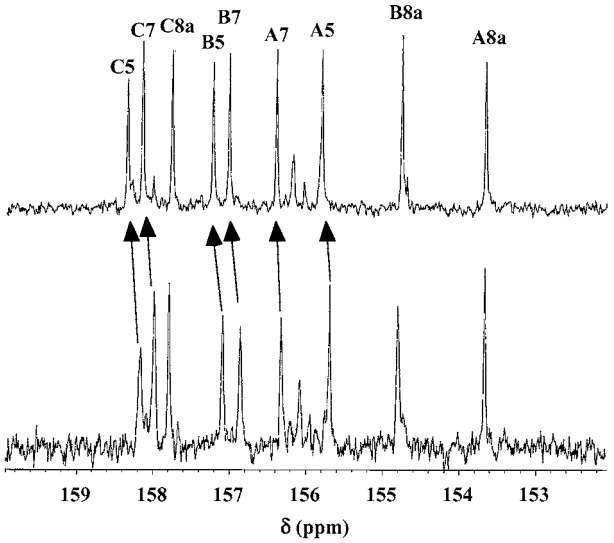

Figure 6. ^{13}C NMR spectra of procyanidin C1 in CD_3OH (A) and CD_3OD (B) at –20 °C.

by interflavanoid HMBC correlation. Assignments of the C unit signals were achieved similarly. Furthermore, the phloroglucinol reaction experiment showed that **6** consisted of two (−)-epicatechin units and one (+)-catechin unit. By comparison with the proton signals at the 4-position of the A unit (δ_H 2.81 and 2.94) of **5** to those of **3** and **4**, we assigned (+)-catechin as the A unit. As a result, **6** was determined to be epicatechin-(4β→8)-epicatechin-(4β→8)-catechin.

Compound **7** was isolated from the trimer fraction by preparative HPLC and showed an $[M + H]^+$ ion peak at *m*/*z* 867. Compound **7** consisted of two (−)-epicatechin units and one (+)-catechin unit, and the A unit was confirmed as (+)-catechin as in the trimer **6**, by observation of the proton signal at the 4-position of the A unit (δ_H 2.52 and 2.66). Three possible carbon signals for the 8a-position (δ_C 153.7, 155.5, and 157.9) were selected by the deuterium substitution method. The HMBC spectrum showed that the proton signal (δ_H 4.46) at the 4-position of the B unit was correlated to the carbon signal (δ_C 153.7) at the 8a-position of the A unit, and the proton signal (δ_H 4.76) at the 4-position of the C unit was correlated to the carbon signal (δ_C 157.1) at the 5-position of the B unit. Therefore, compound **7** was determined to be epicatechin-(4β→6)-epicatechin-(4β→8)-catechin.

Compound **8** consisted of three (−)-epicatechin units. After comparison of isotopic shifts, the correlation between the proton signal (δ_H 4.51) at the 4-position of the B unit and the carbon signal (δ_C 154.2) at the 8a-position of the A unit and the correlation between the proton signal (δ_H 4.55) at the 4-position of the C unit and the carbon signal (δ_C 156.7) at the 5-position of the B unit were observed by HMBC as with trimer **7**. Therefore, **8** was epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin.

The ^1H and ^{13}C NMR spectra of compounds **9** and **10** showed signals assigned to the 4-position of the A unit at δ_H 2.47, 2.79; δ_C 82.5; and δ_H 2.74, 2.86; and δ_C 79.5, respectively. Through the same methods described above, **9** and **10** were determined as epicatechin-(4β→8)-epicatechin-(4β→6)-catechin and epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin, respectively, due to the correlation between the proton signal at the 4-position of the B unit and the carbon signal at the 5-position of the A unit and the correlation between the proton signal at the 4-position of the C unit and the carbon signal at the 8a-position of the B unit in the HMBC spectrum. The structures of the

procyanidins can therefore be approximately estimated from a comparison of the signals at the 4-, 6-, and 8-positions.

Compound **11** was isolated from the tetramer fraction D as a brownish white powder, showing an $[M + H]^+$ ion peak at *m*/*z* 1155. The ^1H and ^{13}C NMR spectroscopic data for compound **11** are indicated in **Table 4**. The data indicate that compound **11** has an epicatechin as the A unit, because the ^1H and ^{13}C NMR spectra showed signals assigned to the 4-position of the A unit at δ_H 2.81 and 2.96 and δ_C 79.5. By the phloroglucinol reaction, compound **11** was shown to be made from four epicatechin units. After assignment of all of the signals in the A unit with 1D and 2D NMR, and H−D exchange experiments, the linkage position between the A and B units was confirmed. Repeated analysis in the same way established there were interflavanoid correlations between the proton signal (δ_H 4.71) at the 4-position of the B unit and the carbon signal (δ_C 154.6) at the 8a-position of the A unit, between the proton signal (δ_H 4.75) at the 4-position of the C unit and the carbon signal (δ_C 155.0) at the 8a-position of the B unit, and between the proton signal (δ_H 4.72) at the 4-position of the D unit and the carbon signal (δ_C 154.8) at the 8a-position of the C unit in the HMBC spectrum. As a result, compound **11** was identified as epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin.

The structures assigned for the apple procyanidins are summarized in **Figure 5**. Many authors have reported that the main procyanidins in apple are procyanidin B1 (dimer **3**), procyanidin B2 (dimer **4**), procyanidin B5 (**PB5**), procyanidin C1 (trimer **5**) and more polymerized procyanidins (*21*−*24*, *27*). In this study, we confirmed the structures of two dimers, six trimers, and one tetramer from apple. Five of the trimers (**6**−**10**) and one tetramer **11** have not previously been identified in apple.

We could not detect **PB5** in dimer fraction B. It is suggested that **PB5** probably does not exist in the varieties cultivated in Japan (e.g., cv. Fuji, Jonathan, Ohrin) or has been generated from other procyanidins by the degradation and oxidation during the making of the juice and the purification process. The reversed phase HPLC and TLC profiles of procyanidins are very complicated. The complexity is caused by the peaks of some procyanidins with the same or different degrees of polymerization having the same retention time in certain analytical conditions. In addition, **PB5** may have been misidentified in apple.

The measurement of NMR spectra at low temperature is useful to confirm the structures of procyanidins because it is not necessary to purify large amounts of the compounds to make derivatives and to perform decomposition. Cui et al. (*38*, *39*) have reported the isolation and structural elucidation of epicatechin-(4β→8)-epicatechin-(4β→6)- epicatechin and epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin by NMR analysis at ambient temperature. Because procyanidins with a (4β→6) interflavanoid bond at the terminal unit have only one conformation as proposed by Hör et al. (*37*), we believed it was possible to elucidate the structure by NMR analysis even at ambient temperature. In this study, we have succeeded in the assignment of NMR signals for the procyanidin oligomer completely and found that the kind of terminal A unit (catechin or epicatechin) and the position of interflavanoid bonds have an influence on the NMR spectra values. Therefore, it is possible to determine the structures of other procyanidins with a higher degree of polymerization. The A unit, and the linkage position between each, can be determined by comparison of NMR data observed for the trimers using only 1D NMR. That is, the proton signal at the 4-position of the (+)-catechin unit as an A unit appeared at δ_H 2.4−2.7 and δ_C 26−28 and that of the (−)-epicatechin unit appeared at δ_H 2.7−2.9 and δ_C 29−30. The carbon signals at the 2- and 5-positions of the A unit appeared at δ_C 79 and 156 for a (+)-catechin unit and at δ_C 81 and 155 for a (−)-epicatechin unit, respectively. In the case of interflavanoid bonds of procyanidin trimers, we can distinguish between 4β→8 and 4β→6 by comparison of the carbon signals at the 6-, 7-, and 8-positions of the A unit, the carbon signals at the 3-, 4a-, 7-, and 8a-positions of the B unit, and the carbon signals at the 4a-, 5-, and 7-positions of the C unit.

As procyanidins are known to possess many physiological functionalities such as antioxidative activity, much attention has been focused on the correlation between the degree of the polymerization and structure of procyanidins and their physiological activities. We believe this study will be useful in the elucidation of this relationship.

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