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Structural characterization of a procyanidin tetramer and pentamer from the apple by low-temperature NMR analysis

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ABSTRACT

The structures of a procyanidin tetramer and pentamer from unripe apple (*Malus pumila*) were elucidated by low-temperature NMR analysis at −34 °C. These structures were [epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (**1**)] and [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (**2**)].

Procyanidins (condensed tannins) are the polyphenol compounds in plant secondary metabolites. They have a variety of physiological functions, including antiallergic,[1,2] antiviral,[3] antiinflammatory,[4] and antioxidant[5] activities. In particular, apple procyanidins are reported to have notable functions, including antimelanogenic[6] and antitumor[7] activities, prevention of dental caries[8] and inhibition of pancreatic lipase and triglyceride absorption.[9] Procyanidins consist of a terminal unit and extension unit, which we refer to as the **A** unit and **B**–**E** units, respectively, hereafter. These units are composed of (+)-catechin or (−)-epicatechin and they are linked through a C4–C6 or C4–C8 interflavonoid bond (Fig. 1).[10,11] Procyanidins are present as a mixture of various oligomers, especially in the apple, and range from dimers to

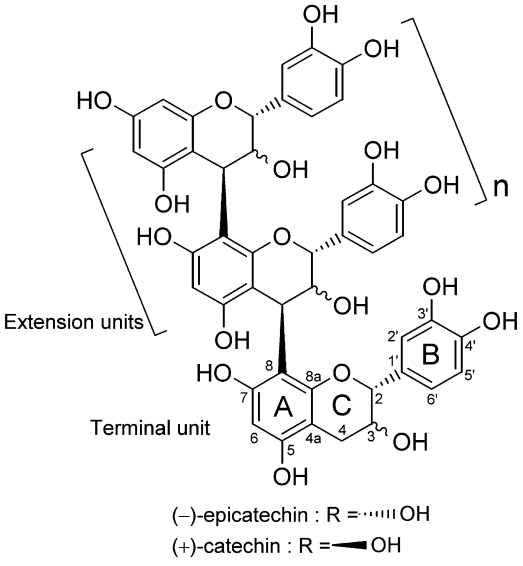

Figure 1. Structure of procyanidin.

Abbreviations: HMBC, heteronuclear multiple bond connectivity; HMQC, heteronuclear multiple quantum coherence; HPLC, high-performance liquid chromatography; MALDI-TOF/MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance; RT, retention time.

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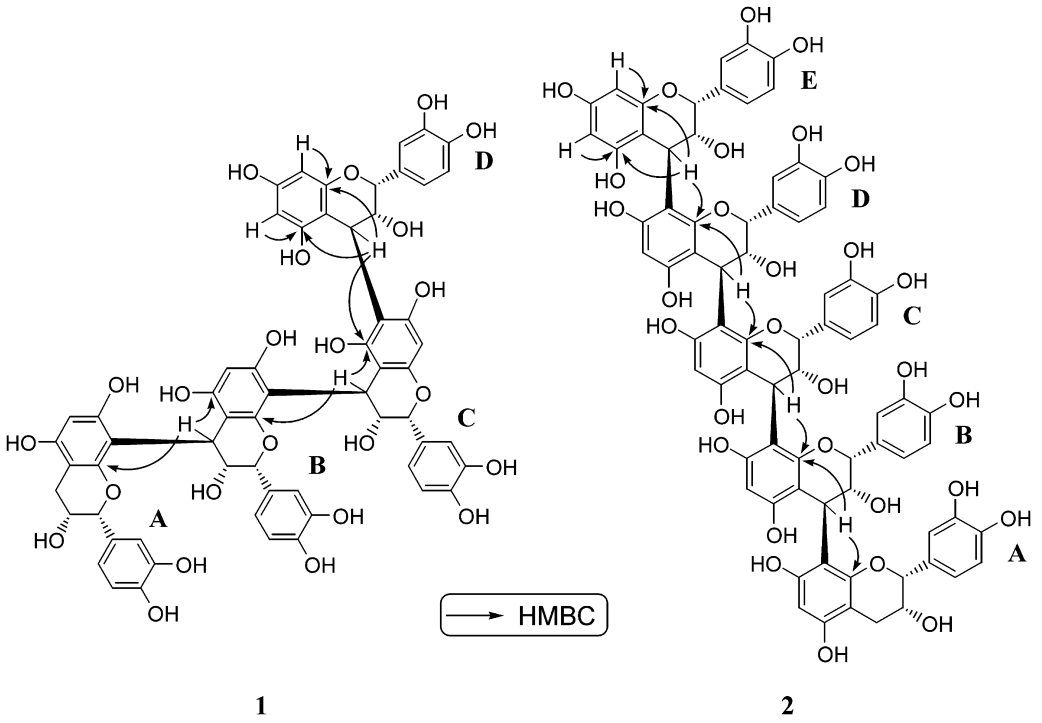

Figure 2. Structures of the procyanidins **1** and **2**. The arrows show HMBC correlations. **A**, terminal unit; **B**, **C**, **D** and **E**, extension units.

pentadecamers that can be seen by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS).[12]

Research has focused on the physiological functions related to their procyanidin structures.[13,14] Two structural factors, linkage position and degree of polymerization, might be expected to affect their functions. It is known that the dimer of epicatechin-(4β→6)-epicatechin, designated PB5, shows stronger inhibition of epidermal lipid peroxidation than epicatechin-(4β→8)-epicatechin, designated PB2. The trimer of epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin inhibited peroxidation more strongly than the dimers, PB2 and PB5.[13] Even when limited to dimers and trimers, the magnitude of physiological functions of procyanidins might differ according to their structures; that is, the position of the interflavonoid bond and their degree of polymerization. In order to elucidate the relationships between their physiological functions and the structure of procyanidin with the position of the interflavonoid bond and degree of polymerization, it is necessary to prepare purified individual molecules, followed by a determination of their structures. However, the separation and purification of the procyanidins individually had not been easy, because various polyphenols and procyanidin isomers which vary in the degree of polymerization, the constitution units, and the position of the interflavonoid bonds intricately overlapped each other on the chromatogram in the preparative reversed-phase high-performance liquid chromatography (HPLC).

Recently, effective and large-scale purification methods for procyanidins according to their degree of polymerization by normal-phase chromatography were reported,[15–18] which advanced research into the relationships between the degree of polymerization and the physiological functions of procyanidins. As such, it has become clear that physiological functions are affected by the degree of polymerization.[6,7,19–21] From each oligomer fraction of procyanidin, individual molecules with the degree of polymerization, the constitution units, and interflavonoid bonds in different positions could be purified using preparative reversed-phase HPLC.[16] Although procyanidin oligomers have been isolated and purified individually, the assignment of the position of the interflavonoid bonds for trimers remains difficult. This is because, at ambient temperature, procyanidins show broadening of ^{1}H nuclear magnetic resonance (NMR) signals due to atropisomerism, which is caused by steric interactions in the vicinity of the interflavonoid bond among the freely rotating flavonoids.[11]

To overcome this problem, Shoji et al. performed 1D NMR (^{1}H and ^{13}C) and 2D NMR (heteronuclear multiple bond connectivity (HMBC) and heteronuclear multiple quantum coherence (HMQC)) at low temperature (−20 °C) using a 600 MHz spectrometer combined with a cleavage experiment of the interflavonoid bond on the A-ring. They could determine the structures of procyanidin of

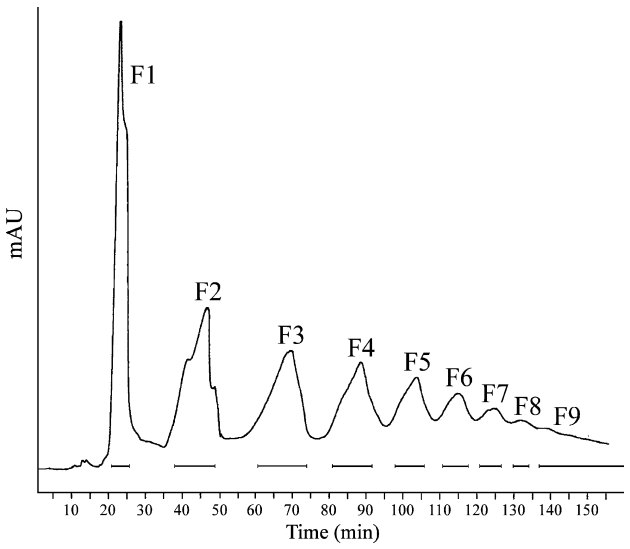

Figure 3. Elution profile of apple procyanidins separated by normal-phase chromatography detected at 280 nm.

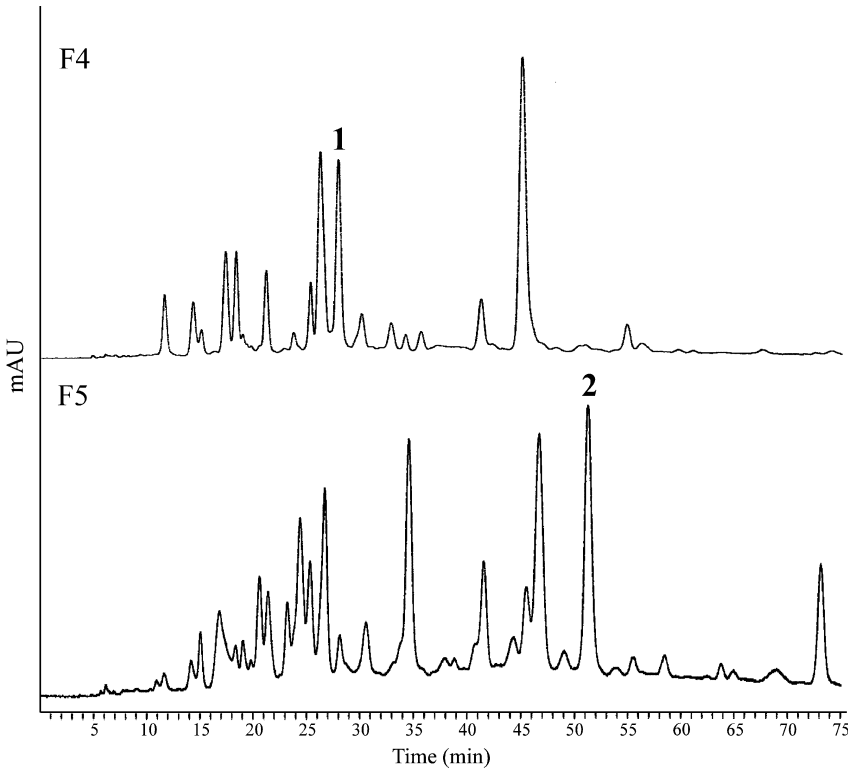

Figure 4. Elution profile of fractions F4 and F5 separated by reversed-phase HPLC. Inertsil ODS-III column (i.d. 2.1 × 250 mm; 3 μm, GL Sciences Inc.) recorded at 280 nm and 0.1% aqueous formic acid (A) and acetonitrile (B) were used as the mobile phase. The gradient program was 20% B to 30% B for 75 min at a flow rate of 0.2 mL/min.

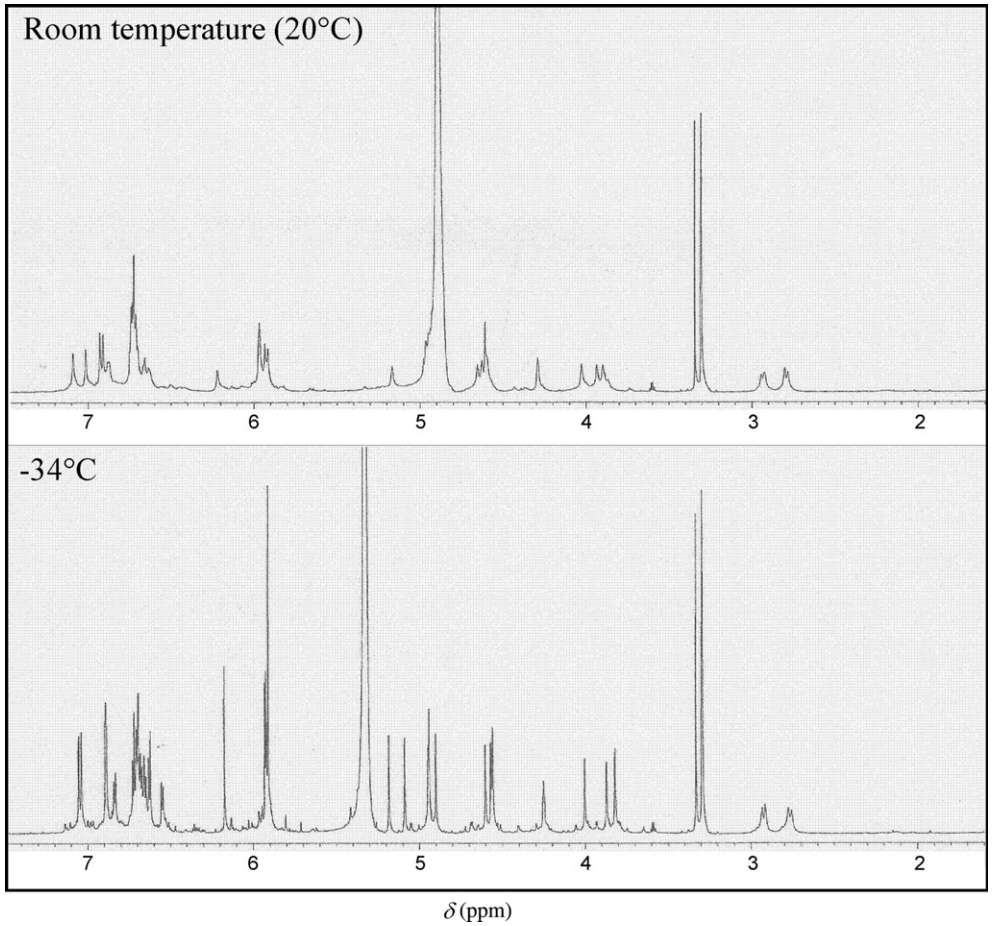

Figure 5. ^{1}H NMR spectra of procyanidin **1** at different temperature.

five trimers and one tetramer, which had not been identified previously in the apple.[22] However, more highly polymerized oligomers or complicated procyanidins could not be elucidated, because 1D NMR signals of molecules more complicated than the tetramer intricately overlapped each other using 600 MHz NMR at −20 °C. In this Letter, we performed 1D NMR (^{1}H and ^{13}C) and 2D NMR (HMBC and HMQC) similarly to the previous experiment, but using higher resolution NMR (800 MHz, ECA-800, JEOL, Tokyo, Japan) at lower temperature (−34 °C) controlled by a temperature controller (Model TC-84, FTS Systems, NY, USA) with an air supplier (AIR-JET, FTS Systems). As such, we could determine the complicated procyanidin structures of another tetramer (Fig. 2), **1** and a pentamer (Fig. 2), **2**, which were previously unidentified by NMR analysis.

Procyanidins **1** and **2** were prepared as previously reported.[16] In brief, the crude apple polyphenol fraction prepared from unripe apples (*Malus pumila* cv. Fuji) was subjected to a Diaion HP-20ss (Mitsubishi Kasei Corp., Tokyo, Japan) column. After washing with distilled water, the crude procyanidin fraction was eluted with 25% ethanol, concentrated at 45 °C, and lyophilized. The lyophilized powder was dissolved in methanol and subjected to normal-phase chromatography using an Inertsil PREP-SIL column (30 × 250 mm, GL Sciences Inc., Tokyo Japan) with hexane/methanol/ethyl acetate = 8:3:1 as solvent A and hexane/methanol/ethyl acetate = 2:3:1 as solvent B. The gradient program was as follows: 0–35 min, 0% B; 35–185 min, 0% B to 100% B at a flow rate of 24 mL/min detected at 280 nm. The peak fractions for procyanidin oligomers were collected according to the following retention time (RT): fraction F1, RT 21–26 min; fraction F2, RT 28–49 min; fraction F3, RT 61–74 min; fraction F4, RT 81–92 min; fraction F5, RT 98–106 min; fraction F6, RT 111–118 min; fraction F7, RT 121–127 min; fraction F8, RT 130–134 min, and fraction F9, RT 137–180 min (Fig. 3). Each fraction (F1–F9) was concentrated at 45 °C and lyophilized. Further purification was performed by preparative reversed-phase HPLC using an Inertsil ODS III column (25 × 250 mm, GL Sciences Inc.) with an acetonitrile/water solvent (10:90, v/v; for fraction F4) or methanol/water solvent (22:78, v/v; for fraction F5) at a flow rate of 12 mL/min. Compound **1** was isolated from tetramer fraction F4 and **2** was isolated from pentamer fraction F5 (Fig. 4).

The ^{1}H NMR spectra of procyanidin **1** at different temperatures are shown in Figure 5. At room temperature (20 °C), **1** showed the broadening of ^{1}H NMR signals, but at −34 °C ^{1}H NMR spectra obtained as sharp peaks. We therefore performed all other NMR analyses at −34 °C. To elucidate the structure of compound **1**, we first determined the 8a-position carbon signals. The 8a-position carbon signals could not be distinguished from the crowded phenolic carbon signals in the range δ_C 154–160 ppm, so we tried H–D exchange experiment using CD_3OH and CD_3OD (Fig 6). In CD_3OD, hydroxyl groups were deuterated, leading to isotopic shift of hydroxyl carbon signals on ^{13}C NMR analysis, whereas in CD_3OH, hydroxyl groups were not deuterated and showed non-isotopic

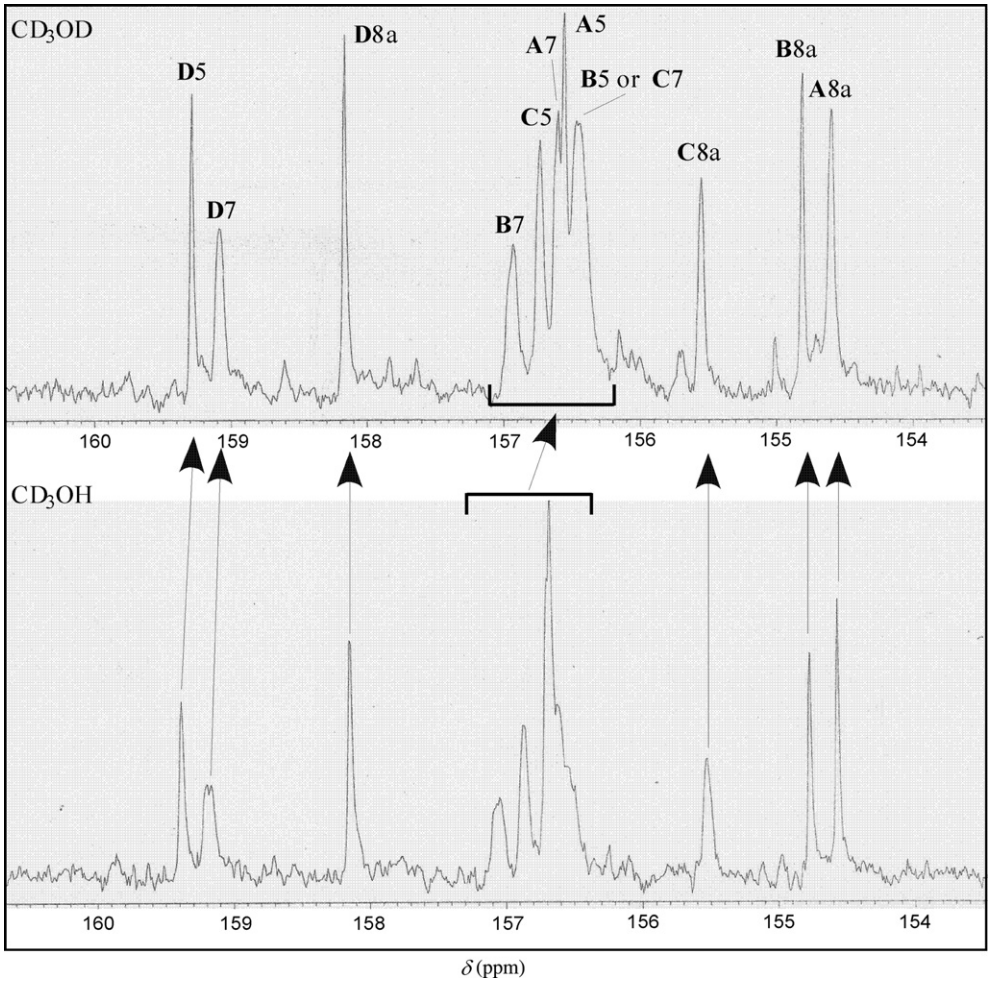

Figure 6. ^{13}C NMR spectra of procyanidin **1** in CD_3OD and CD_3OH at −34 °C. The peak number showed constitution unit (**A**–**D**) and the carbon position, that is, **A**8a; **A** unit 8a-position carbon signal.

shift of hydroxyl carbon signals on ^{13}C NMR analysis. Thus, compared with the ^{13}C NMR signals in CD_3OH and CD_3OD, the hydroxyl carbon signals showed isotopic shift. From the results previously reported, the carbon signals of procyanidin **1** in the range δ_C 154–160 ppm were predicted to be 5-, 7-, and 8a-position carbon signals.[22] In these carbon signals, the signals showing isotopic shift could be attributed to be 5- or 7-position carbon signals, and those showing non-isotopic shift could be done to be 8a-position carbon signals. As a result, four carbon signals (δ_C 154.6, 154.8, 155.6, and 158.2) showed non-isotopic shift and were selected to be the signals at the 8a-position of each unit (Fig. 6). In these signals, **D** unit 8a-position carbon signal (δ_C 158.2) was determined by the HMBC correlation from the **D** unit 8-position doublet proton signal (δ_H 5.93, J = 2). The HMBC correlation was observed between **D** unit 4-position proton signal (δ_H 4.56) and **C** unit 5-position carbon signal (δ_C 156.7), in which the carbon signal was correlated to the **C** unit 4-position proton signal (δ_H 4.57), indicating that the interflavonoid bond between the **D** and **C** units was 4β→6 (Fig. 2). Interflavonoid correlation was observed between the proton signal (δ_H 4.57) at the **C** unit 4-position and the carbon signal (δ_C 154.8) at the **B** unit 8a-position. It was also observed between the proton signal (δ_H 4.60) at the **B** unit 4-position and the carbon signal (δ_C 154.6) at the **A** unit 8a-position in the HMBC spectrum. These results suggested that the interflavonoid bonds between **C**, **B,** and **A** units were 4β→8 (Fig. 2). Mass spectrometry (MS) analysis of compound **1** in the tetramer fraction F4 (Fig. 4) showed an $[M-H]^-$ ion peak at m/z 1154 and consisted of four (−)-epicatechin units confirmed by the phloroglucinol reaction experiment.[23–25] As such, compound **1** was determined to be epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin (Fig. 2). All other ^1H and ^{13}C chemical shifts for compound **1** are shown in Table 1, and the ^{13}C NMR chart is done in Supplementary data 1.

Five possible carbon signals for the 8a-position (δ_C 154.6, 154.8, 154.9, 155.0, and 157.9) of compound **2** were selected by the H–D exchange experiment in the same way as mentioned above. In these signals, **E** unit 8a-position carbon signal (δ_C 157.9) was determined by the HMBC correlation from the **E** unit 8-position doublet proton signal (δ_H 6.00, J = 2). The HMBC correlations between 8a-position carbon signals (**D**, δ_C 154.8; **C**, δ_C 154.9; **B**, δ_C 155.0, and **A**, δ_C 154.6) and 4-position proton signals (**E**, δ_H 4.72; **D**, δ_H 4.77; **C**, δ_H 4.75, and **B**, δ_H 4.69) were observed, respectively (Fig. 2). These results suggested that all interflavonoid bonds between **E**, **D**, **C**, **B,** and **A** units were revealed to be 4β→8. B-ring proton and carbon signals from **2** (δ_H 6.91–7.12, 5H, 2′-position; δ_H 6.68–6.89, 10H, 5′- and 6′-position; δ_C 132.0–132.6, 1′-position; δ_C 114.7–115.0, 2′-position; δ_C 145.6–145.9, 3′-position; δ_C 145.1–145.3, 4′-position; δ_C 115.8–115.9, 5′-position, and δ_C 118.6–119.0, 6′-position) were not completely assignable, because several peaks intricately overlapped each other (Table 1). MS analysis of compound **2** in the pentamer fraction F5 (Fig. 4) showed an $[M-H]^-$ ion peak at m/z 1442, and the phloroglucinol reaction experiment showed that **2** consisted of five (−)-epicatechin units.[23–25] Thus, compound **2** was determined to be epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin. The ^1H and ^{13}C NMR chemical shifts for compound **2** are shown in Table 1 and the ^1H and ^{13}C NMR charts are done in Supplementary data 2. This is the first report that the structures of procyanidins **1** and **2** were completely identified by

Table 1
^1H and ^{13}C NMR spectral data for procyanidins **1** and **2** in CD_3OD at −34 °C

	Compound **1**				Compound **2**				
Position	**A** unit	**B** unit	**C** unit	**D** unit	**A** unit	**B** unit	**C** unit	**D** unit	**E** unit
^1H					*^1H*				
2	4.95	5.20	4.94	4.90	4.99	5.30[e]	5.27	5.30[e]	5.09
3	4.25	3.87	4.00	3.82	4.31	3.96	4.01	4.09	3.99
4	2.77 d (15) 2.93 d (12)	4.60	4.57	4.56	2.80 d (16) 2.95 d (16)	4.69	4.75	4.77	4.72
4a	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—
6	5.91[a]	5.91[a]	—	5.92 d (2)	5.931	5.94	5.98	5.96	5.97 d (2)
7	—	—	—	—	—	—	—	—	—
8	—	—	6.17	5.93 d (2)	—	—	—	—	6.00 d (2)
8a	—	—	—	—	—	—	—	—	—
1′	—	—	—	—	—	—	—	—	—
2′	6.88	7.04	7.05	6.89			6.91–7.12,[f] 5H		
3′	—	—	—	—	—	—	—	—	—
4′	—	—	—	—	—	—	—	—	—
5′	6.72 d (8)	6.62 d (8)	6.70 d (8)	6.695 d (8)			6.68–6.69,[g] 10H		
6′	6.83 d (8)	6.54 d (8)	6.67 d (8)	6.65 d (8)					
^{13}C					*^{13}C*				
2	79.5	76.5	76.8	77.4	79.5	76.7[h]	76.6[i]	76.6[i]	76.7[h]
3	66.9	73.1	73.3	73.2	66.7	72.8[j]	72.8[j]	73.1	73.5
4	30.0	36.9	37.4[b]	37.4[b]	30.0	37.2	37.4[k]	37.4[k]	37.0
4a	99.9	102.3	101.4	100.0	99.8	102.4	102.5	102.3	102.0
5	156.60	156.5[c]	156.7	159.3	156.6	156.2[l]	156.2[l]	156.2[l]	156.9[m]
6	96.7	97.2	108.3	96.0	97.0	97.2[n]	97.2[n]	96.8	95.9[o]
7	156.56	156.9	156.5[c]	159.1	156.7	156.8	156.9[m]	156.9[m]	158.0
8	107.5	106.0	96.2	95.7	107.6	107.3	107.4	107.0	95.9[o]
8a	154.6	154.8	155.6	158.2	154.6	155.0	154.9	154.8	157.9
1′	131.9	132.5	132.3	132.6			132.0–132.6[p]		
2′	115.0	114.7	114.9	115.3			114.7–115.0[q]		
3′	145.9	145.8	145.6[d]	145.6[d]			145.87–145.93[r]		
4′	145.6[d]	145.1	145.4	145.6[d]			145.1–145.4[s]		
5′	115.5	115.9	115.8	115.7			115.7–115.9[t]		
6′	118.8	118.2	119.0	119.5			118.6–119.0[u]		

Values in parentheses indicate coupling constants (J in Hz).
[a,e–g] Overlapped with each other.
[b–d,f–u] Assignments with the same letters are interchangeable.

using instrumental analysis with support of a phloroglucinol cleavage experiment. We will isolate and identify other procyanidin structures in fractions F4 and F5 (Fig. 4) using the same method.

Researchers have reported that physiological functions improved with an increasing degree of polymerization.[6,7,19–21] However, the relationships between physiological functions and molecular structures of compounds more complex than tetramers are unclear, because complicated procyanidin structures cannot be determined by NMR under standard conditions. In this Letter, we have succeeded in the determination of procyanidin tetramer **1** and pentamer **2** by using 800 MHz NMR at low temperature (−34 °C) combined with the phloroglucinol experiment. We believe that this analytical method might be effective in elucidating other procyanidins with more complicated structures. Furthermore, we think that individual procyanidin oligomers with an identical degree of polymerization having interflavonoid bonds in different linkage positions could be used to evaluate their physiological functions. Our results will aid in studies regarding the relationships between procyanidin oligomer structures and their physiological functions.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.08.087.

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25. One milliliter of 1% HCl–methanol was added to a mixture of the 1 mg of procyanidin and 1 mg of phloroglucinol, and the resulting mixture was stirred for 30 min at room temperature. The reaction mixture was diluted with H_2O appropriately and the resulting solution was applied to an HPLC analysis. Inertsil ODS-III column (i.d. 2.1 × 250 mm; 3 μm, GL Sciences Inc.) and 0.1% aqueous formic acid (A) and acetonitrile (B) were used as the mobile phase. The gradient program was 0% B for 5 min, a linear gradient from 0% to 13% B for 10 min and a linear gradient from 13% to 38% B for 60 min at a flow rate of 0.2 mL/min detected at 280 nm. By the results of this phloroglucinol cleavage experiments for the procyanidins **1** and **2**, the epicatechin–phloroglucinol and epicatechin were detected. These results showed that each of the procyanidins **1** and **2** was composed of only epicatechin unit.