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Isolation and structure elucidation of tetrameric procyanidins from unripe apples (*Malus pumila* cv. Fuji) by NMR spectroscopy

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ABSTRACT

Procyanidins are plant secondary metabolites widely consumed and known to have various physiological functions, but their bioavailability and mechanism of action are still unclear especially for larger oligomers. One of the reasons is scarce information about the detailed structure of oligomeric procyanidins. As for apple, structures of procyanidin components larger than trimers are scarcely known. In this study, 11 tetrameric procyanidins including two known compounds were isolated from unripe apples (*Malus pumila* cv. Fuji) and identified by NMR spectroscopic analysis and phloroglucinol degradation. As a result, the detailed structural diversity of tetrameric procyanidins in apple was established.

1. Introduction

Polyphenols extracted from apple were found to show a wide variety of physiological functions, such as reduction of triglyceride absorption (Sugiyama et al., 2007), inhibition of melanogenesis (Shoji et al., 2005), protective effects against muscle injury (Nakazato et al., 2010), and anti-inflammatory (Yoshioka et al., 2008), anti-cancer (Miura et al., 2008), and anti-allergic activities (Akiyama et al., 2000, 2005; Tokura et al., 2005). The main components of apple polyphenols are procyanidins, which have attracted attention especially due to their positive effects on vascular health (Corder et al., 2006; García-Conesa et al., 2009). Apple procyanidins have also been confirmed to have activities on the vascular endothelium *in vitro* and *in vivo* (Caton et al., 2010; Matsui et al., 2009; Nishizuka et al., 2011). On the other hand, their mechanisms of action are still unclear. Some reports have suggested a relationship between their activity and the degree of polymerization (Caton et al., 2010; Sugiyama et al., 2007); however, the influence of differences in detailed structure has rarely been reported, especially for larger oligomers. Furthermore, investigation of the absorption of simple procyanidins has progressed along with analysis of catabolism by human gut microflora (Appeldoorn et al., 2009; Déprez et al., 2000; Stoupi et al., 2010), but there have been few studies on higher oligomeric procyanidins. This is associated with the difficulty of preparing structurally characterized procyanidins as a subject of research.

The general approach to obtain procyanidins is extraction and purification from natural resources. Natural oligomeric procyanidins are easily fractionated according to their degree of polymerization by well-established methods (Kelm et al., 2006; Shoji et al., 2003); however, identification of components is difficult owing to their complexity. Procyanidins are either oligomeric or polymeric compounds of catechin (Cat) or epicatechin (EC) through their C4 → C6 or C4 → C8 interflavonoid linkages (Fig. 1). This difference in position of the interflavonoid linkage and constitutive units gives structural diversity to the higher oligomers; thus, the number of isomers increases along with degree of polymerization (DP).

As another approach for preparation of structurally characterized procyanidins, the total synthesis (Kozikowski et al., 2003; Saito et al., 2009; Tarascou et al., 2006) or semi-synthesis (Esatbeyoglu et al., 2011) of oligomeric procyanidins can be conducted. At this time, however, the synthetic technique is limited to extension of the unit only through the C4 → C8 interflavonoid connection; the creation of various structures with C4 → C6 bonds, which exist in natural resources, remains to be achieved. Even in the case of the semi-synthetic method, precursor compounds that have C4 → C6 bonds must be prepared from natural resources.

Abbreviations: LC-ESI-TOFMS, liquid chromatography electrospray ionization time of flight mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; COSY, correlation spectroscopy; HSQC, hetero-nuclear single quantum coherence; HMBC, hetero-nuclear multiple-bond connectivity.

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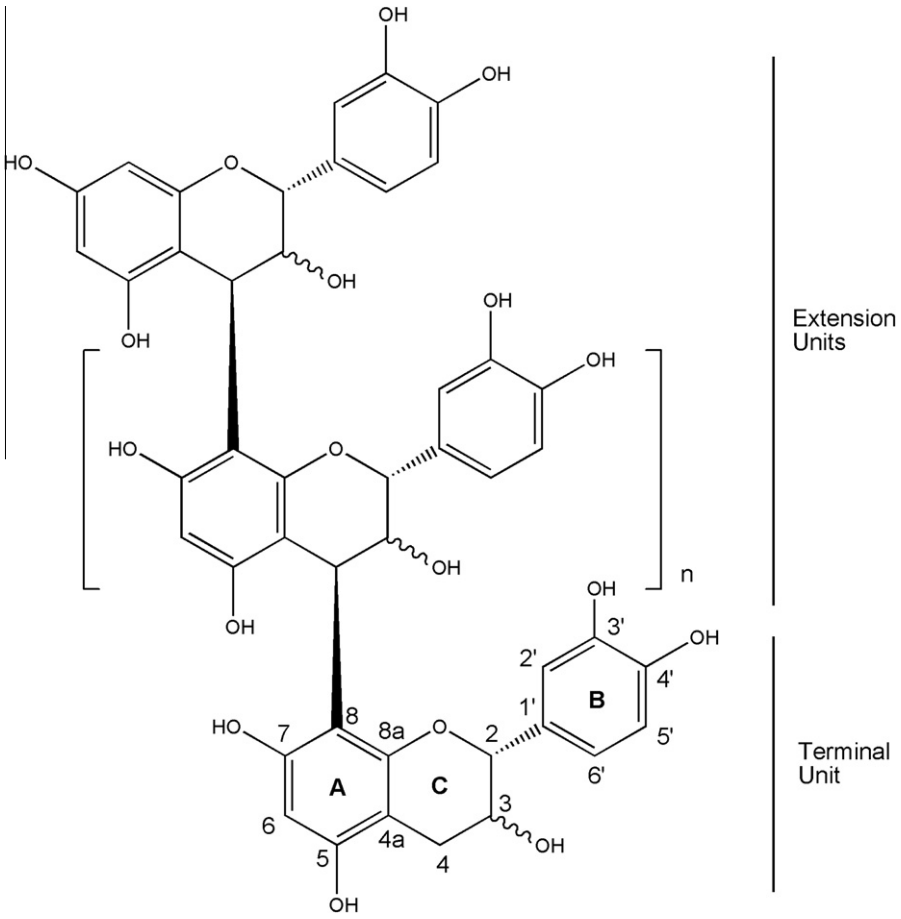

Fig. 1. General structure of procyanidins.

In consideration of this background, the investigation of the detailed structure of oligomeric procyanidins extracted from natural resources is important. In this study, 11 tetramers detected in-apple polyphenolic extract were isolated, and the structures of nine unknown compounds were determined by NMR spectroscopic and phloroglucinol degradation analyses. Combined with our previously reported studies (Abe et al., 2008; Shoji et al., 2003), this is the first study on the detailed profile of tetrameric procyanidins in apple.

2. Results and discussion

Compounds **7** and **10** were identified as EC-(4β → 6)-EC-(4β → 8)-EC-(4β → 8)-EC and EC-(4β → 8)-EC-(4β → 8)-EC-(4β → 8)-EC with reference to previous reports (Abe et al., 2008; Shoji et al., 2003), respectively. The structures of other isolated compounds were elucidated by NMR spectroscopic and phloroglucinol degradation analyses. The ^{1}H and ^{13}C chemical shifts of tetrameric procyanidins are summarized in Tables 1 and 2, and determined structures are depicted in Fig. 2.

Compound **1** was established to consist of one catechin as a terminal unit and three epicatechins as extension units by phloroglucinol degradation analysis. Structure **1** was elucidated on the basis of 1D and 2D NMR spectroscopic analysis. All NMR spectra were measured at 243 K to avoid signal broadening due to atropisomerism. First, an H-D exchange experiment was conducted with methanol-d_4 and methanol-d_3 to distinguish the C8a from C5 and C7. Among aromatic carbon signals of the A-ring in the range of δ_C of 150–160, only four resonances (δ_C 153.66, 154.71, 155.49 and 158.12 in methanol-d_4) gave no isotopic shift when measured in methanol-d_3; thus, they were determined to be C8a for each unit. Next, the A-ring and C-ring protons in each unit were assigned as follows. The H4 of the terminal unit T_1 (represented as H4-unit T_1) were only methylene protons in the procyanidins and were easily assigned according to their own coupling. The H4 of the extension units (M_1, M_2, U_1) were recognized by a HSQC correlation with C4, which showed characteristic chemical shifts in the range of δ_C of 30–40. The remaining non-aromatic protons in C-rings were assigned on the basis of COSY correlation with H4. Each A-ring proton in the δ_H range of 5.8–6.2 was linked with H4 in the same unit on the basis of the HMBC correlation to C4a. The positions of the interflavonoid bonds were investigated on the basis of HMBC correlations from protons in the A-ring or C-ring. The C8a-unit T_1 was assigned on the basis of a HMBC correlation with H2-unit T_1. The C8a-unit T_1 was also correlated with the H4-unit M_1, which indicated that unit T_1 was connected to unit M_1 through a C4 → C8 interflavonoid bond. The connection between unit M_1 and unit M_2 was determined in the same way using the correlation from both the H4-unit M_1 and the H4-unit M_2 to C8a-unit M_2. In unit M_2, both H8-unit M_2 and H4-unit M_2 showed correlations to C8a-unit M_2, which suggested that unit M_2 was connected to unit U_1 through a C4 → C6 interflavonoid bond. This connection was confirmed by correlation from both H4-unit M_2 and H4-unit U_1 to C5-unit M_2. As a result of these observations, the structure of compound **1** was elucidated as EC-(4β → 6)-EC-(4β → 8)-EC-(4β → 8)-Cat.

As for compounds **2**–**11**, only the characteristic points are explained here because the basic strategy matched that for **1**. Compound **2** consisted of one catechin as the terminal unit and three

Table 1
^{1}H NMR spectroscopic data of compounds **1**, **3**–**6**, **8**, **9**, **11** in methanol-d_4 at 243 K and **2** in methanol-d_4/deuterium oxide (25:15) at 278 K.

Unit	Position	δ (multiplicity, J)								
		1	2	3	4	5	6	8	9	11
T1	2	5.01 (d, 4.0)	3.94 (d, 9.1)	4.29	5.09	3.73 (d, 8.1)	4.94	4.99	4.59 (d, 7.3)	4.83
	3	4.20 (qbr, 4.7)	3.86 (dd, 8.8, 15.6)	3.74	4.23 (q, 4.4)	3.68 (q, 8.1)	4.34	4.24	3.99	4.15
	4	2.52 (d, 13.3)	2.15 (dd, 10.4, 15.7)	1.90	2.54 (d, 12.9)	2.28 (dd, 9.1, 17.2)	2.88 (d, 16.8)	2.83 (d, 17.3)	2.50 (dd, 8.0, 15.4)	2.75 (d, 16.1)
		2.61 (d, 12.4)	2.65 (dd, 6.0, 15.5)	2.24	2.64 (d, 13.1)	2.92 (dd, 7.1, 17.2)	2.97 (d, 13.0)	2.98 (d, 13.1)	2.78 (d, 15.8)	2.90 (d, 13.6)
	6	5.89			5.91	6.09	5.91			
	8		5.95	5.97					6.04[i]	6.11
	2′	6.82	6.91	7.00	6.87	6.55	7.16	7.10	6.83	6.97
	5′	6.69	6.87	6.76 (d, 7.7)	6.73–6.75[e]	6.65	6.73	6.72–6.74[h]	6.76	6.65–6.80[j]
	6′	6.87 (d, 7.7)	6.84[d]	6.86	6.95	6.20 (d, 8.3)	6.84	6.85 (d, 8.6)	6.72	6.79
M1	2	5.26	4.53	4.67	5.37	5.22	5.10	4.97	5.05	5.07
	3	4.04	3.92	3.86	4.14	3.80	3.81	4.03	4.13	4.14
	4	4.70	4.45	4.50	4.80	4.48	4.63	4.50	4.63	4.64
	6	5.91	6.18	6.12	5.95			5.99	6.04[i]	6.03
	8					5.52	6.18			
	2′	7.09	6.56 (d, 1.4)	6.59	7.06	6.91	6.87	7.14	7.04	7.04
	5′	6.67[a]	6.54 (d, 8.4)	6.55 (d, 8.3)	6.73–6.75[e]	6.73 (d, 8.3)	6.70	6.70	6.73	6.65–6.80[j]
	6′	6.67[a]	6.01 (d, 8.3)	6.33 (d, 8.0)	6.85	6.69	6.65 (d, 9.6)	6.56 (d, 7.7)	6.75	6.75
M2 (U2 of **8**)	2	4.94	5.32	5.45	5.29	4.98	4.99	4.88	5.24	5.25
	3	4.05	3.98	3.90	4.11	4.09	3.95	3.85	4.00	4.00
	4	4.58	4.34	4.48	4.76	4.51	4.59	4.78	4.73	4.74
	6				5.94	5.90	5.96	6.11 (d, 2.2)	5.92	5.92
	8	6.20	5.88	5.84				6.08 (d, 2.3)		
	2′	6.90[b]	7.04 (d, 1.6)	7.05	7.12	7.13	7.03	6.90	7.14	7.13
	5′	6.70	6.84d	6.74	6.73–6.75[e]	6.79 (d, 8.4)	6.67[g]	6.72–6.74[h]	6.72	6.65–6.80[j]
	6′	6.74	6.90	6.30	6.79	6.90	6.67[g]	6.52 (d, 7.7)	6.67	6.72
U1	2	4.94	4.75	4.86	5.11	5.04	5.17	5.04	5.10	5.11
	3	3.83	3.82	3.76	4.00	3.83	3.95	3.88	3.98	3.98
	4	4.57	4.31	4.36	4.74	4.55	4.69	4.68	4.72	4.72
	6	5.92[c]	6.04 (d, 2.3)	5.95	5.99	5.91[f]	5.90 (d, 2.4)	5.98	5.98 (d, 2.1)	5.99
	8	5.92[c]	6.13 (d, 2.2)	6.04	6.02	5.96[f]	5.94 (d, 2.5)	5.94 (d, 3.2)	6.01 (d, 1.9)	6.01
	2′	6.91[b]	7.10 (d, 1.6)	7.09	6.92	6.87	6.77	6.92 (d, 1.4)	6.92	6.93
	5′	6.72	6.33 (d, 8.1)	6.39 (d, 7.7)	6.73–6.75[e]	6.85 (d, 8.4)	6.69	6.72–6.74[h]	6.74	6.65–6.80[j]
	6′	6.69	6.09 (bd, 8.3)	6.15 (d, 7.7)	6.71	6.57	6.82	6.64 (d, 8.6)	6.69	6.70

[a,c–e,g–j]Overlapped with each other.
[b,f]Values with the same superscript letters are not significantly different.

epicatechins as extension units, as established by phloroglucinol degradation analysis. In the NMR spectroscopic analysis, **2** showed a more complicated spectrum than **1,** even when measured under the same conditions, which indicated that **2** existed in two conformations. This was resolved according to the method of Cui et al. (1992). By addition of deuterium oxide with three-fifths volume of methanol-d_4, the conformers of **2** converged to a single conformation, and showed a simple ^{1}H NMR spectrum at 278 K (Fig. 3). In the HMBC spectrum, the H8-unit T_1 was correlated with the C8a-unit T_1, determining that the connection between units T_1 and M_1 was C4 → C6. The connection between units M_1 and M_2 was determined to be C4 → C8 on the basis of the correlation from H4-unit M_1 and H4-unit M_2 to C8a-unit M_1. The connection between units M_2 and U_1 was determined to be C4 → C6 on the basis of the correlation from H4-unit M_2 and H4-unit U_1 to C5-unit M_2. Therefore, structure **2** was elucidated as EC-(4β → 6)-EC-(4β → 8)-EC-(4β → 6)-Cat.

Compound **3** consisted of four epicatechin units, as again established by phloroglucinol degradation analysis. It also showed a complicated ^{1}H NMR spectrum, but the signals were fairly well separated for them to be assigned. Here the assignment of the major conformer is given. The H8-unit T_1 was identified from the HMBC correlation with C4a-unit T_1, which was correlated with the H3-unit T_1. The H8-unit T_1 also correlated with C8a-unit T_1, by which the connection between units T_1 and M_1 was determined as C4 → C6. The connection between units M_1 and M_2 was determined to be C4 → C8 on the basis of the correlation from both H4-unit M_1 and H4-unit M_2 to C8a-unit M_2. The connection between units M_2 and U_1 was also determined to be C4 → C6 on the basis of the correlation from both H4-unit M_2 and H4-unit U_1 to C5-unit M_2. Therefore, structure **3** was elucidated as EC-(4β → 6)-EC-(4β → 8)-EC-(4β → 6)-EC.

Compound **4** consisted of four epicatechin units, as determined by phloroglucinol degradation analysis. The C8a-unit T_1 was as-

Table 2
^{13}C NMR spectroscopic data of compounds **1**, **3**–**6**, **8**, **9**, **11** in methanol-d_4 at 243 K and **2** in methanol-d_4/deuterium oxide (25:15) at 278 K.

Unit	posltion	δ								
		1	2	3	4	5	6	8	9	11
T1	2	81.40	82.64	79.37	81.38	83.48	79.21	79.49	82.47	79.56
	3	67.93	68.86	68.06	68.01	69.32	67.02	67.15	68.81	67.51
	4	26.05	24.31	29.45	26.00	30.62	30.26	30.04	28.42	29.68
	4[a]	99.99	102.87	101.37	99.93	101.52	99.40	101.45	101.21	100.44
	5	155.75	155.41	154.98	155.69	155.21	157.17	155.33	155.85[z]	156.37
	6	96.88	107.31	107.60	96.90	95.89	96.82	108.54	107.94[aa]	108.08
	7	156.39	154.78[e]	156.21	156.38	155.32[p]	156.89	153.89	155.85[z]	155.71
	8	107.99	95.46	95.65	108.16	108.81	107.04	107.27	97.54	96.15
	8[a]	153.66	154.55	155.09	153.06	155.92	154.61	152.58	154.86	155.50
	1′	132.18	131.33	132.66	132.33	130.93	131.96	132.07	131.98	132.30
	2′	113.68	116.30[f]	115.35	113.69	115.23	114.87	114.89	114.88	114.98[ag]
	3′	146.02	145.49	145.59[j]	146.02	145.30	145.0–146.0[u]	145.94[w]	146.16[ab]	145.2–146.1[ah]
	4′	145.66	145.92	145.53	145.5–145.7[l]	145.4–146.1[q]	145.0–146.0[u]	145.65	146.16[ab]	145.2–146.1[ah]
	5′	115.91	116.54	115.57	115.6–116.0[m]	115.35[r]	115.0–116.0[v]	115.0–116.0[x]	115.7–116.0[ac]	115.5–116.0[ai]
	6′	119.12	121.46	119.66	119.08	121.11	118.55	118.77	119.87	119.12[aj]
M1	2	76.60	77.02	77.38	76.84	76.48	76.80	77.06	77.13	77.16
	3	72.33	71.46	72.53	72.07	73.35	73.39	72.45	71.54	71.58
	4	36.78	37.81	37.85[k]	37.10	36.85	36.48	38.02	37.87	37.84
	4[a]	102.70	98.85	98.61	102.73	103.73	102.34	98.55	99.25	99.13
	5	157.16	156.64	157.40	156.98[n]	155.32[p]	155.38	157.99[y]	157.83[ad]	157.98[ak]
	6	96.63	96.83	96.26	96.90[o]	106.72	107.81	97.35	95.69	97.43
	7	156.84	156.89	157.46	156.72	154.75	155.69	158.91	158.65	158.70
	8	105.22	109.29	109.44	106.67	95.76[s]	96.37	107.82	107.94[aa]	107.89
	8[a]	154.71	155.62	156.17	154.94	155.39	155.91	154.98	154.96	154.95
	1′	132.71	131.39	131.68	132.70	130.05	132.45	131.77	131.76	131.77
	2′	114.62	115.03	114.60	114.83	115.35[r]	115.04	114.87	114.98[ae]	114.98[ag]
	3′	145.69[a]	144.52	144.88	145.78	145.4–146.1[q]	145.0–146.0[u]	146.04	145.62[af]	145.2–146.1[ah]
	4′	144.98[a]	144.49	144.96	145.19	145.4–146.1[q]	145.0–146.0[u]	145.52	145.99[af]	145.2–146.1[ah]
	5′	115.81[c]	115.60[g]	115.21	115.6–116.0[m]	115.63	115.0–116.0[v]	115.0–116.0[x]	115.7–116.0[ac]	115.5–116.0[ai]
	6′	118.19	119.90	119.74	118.64	119.39	118.55	117.99	118.62	118.58
M2	2	76.72	76.72	76.84	76.58	77.06	77.68	77.78	76.65	76.69
(U2 of **8**)	3	73.31	73.52	73.78	73.09	71.11	73.22	73.22	73.42	73.51[al]
	4	37.41	38.15	37.90[k]	37.30	37.69	38.25	37.39	37.43	37.43
	4[a]	101.09	103.89	103.57	102.12	99.49	98.33	97.88	102.03	102.03[am]
	5	156.87	155.78	156.04	156.26	157.94	158.26	159.41	156.28	156.27
	6	108.47	105.90	106.16	96.90[o]	97.09	97.02	96.78	96.85	96.83
	7	156.59	154.38	154.78	156.98[n]	158.26	158.75	160.44	156.96	156.95
	8	96.18	96.18	95.97	107.07	107.27	107.87	96.32	107.03	107.03
	8[a]	155.49	154.78[e]	155.04	154.79	154.78	155.91	158.22	154.92	154.88
	1′	132.30	132.80	133.14	132.61	131.83	132.04	131.62	132.52	132.58[an]
	2′	114.76	115.60[g]	115.68	114.70	115.88	115.09	115.08	114.80	114.81
	3′	145.0–146.0[b]	145.23[h]	145.59[j]	145.5–145.7[l]	145.4–146.1[q]	145.0–146.0[u]	146.09	145.83	145.2–146.1[ah]
	4′	145.34	145.12[i]	145.59[j]	145.06	145.4–146.1[q]	145.0–146.0[u]	145.94[w]	145.21	145.2–146.1[ah]
	5′	115.81[c]	116.35[f]	115.56	115.6–116.0[m]	115.76	115.0–116.0[v]	115.0–116.0[x]	115.7–116.0[ac]	115.5–116.0[ai]
	6′	118.95	120.16	119.99	118.50	119.76	118.83	119.24	118.24	118.31
U1	2	77.34	76.97	77.16	76.78	76.65	76.95	76.89	76.82	76.82
	3	73.20	71.80	72.74	73.53	73.76	72.41	73.89	73.51	73.51[al]
	4	37.34	37.90	37.61	36.98	36.61	35.95	37.06	36.99	36.97
	4[a]	100.35	99.00	98.55	102.00	101.96	102.45	101.70	101.99	102.03[am]
	5	159.15	159.03	159.83	157.79	157.6–157.9[t]	157.03	157.91	157.98	157.80
	6	95.65[d]	96.75	96.16	95.82	95.89	95.69	95.78	95.89	95.83
	7	158.86	158.63	159.54	157.92	157.6–157.9[t]	157.32	157.99[y]	157.83[ad]	157.98[ak]
	8	95.87[d]	96.52	95.94	95.86	95.76[s]	95.54	95.81	95.89	95.88
	8[a]	158.12	157.65	158.08	157.85	157.76	158.00	157.93	157.89	157.90
	1′	132.62	131.88	132.95	132.54	132.75	132.52	132.45	132.57	132.58[an]
	2′	115.26	116.14	115.51	114.87	114.52	114.29	114.99	114.98[ae]	114.98[ag]
	3′	145.0–146.0[b]	145.12[i]	145.59[j]	145.5–145.7[l]	145.25	145.0–146.0[u]	145.83	145.62	145.2–146.1[ah]
	4′	145.0–146.0[b]	145.23[h]	145.22	145.29	144.91	145.0–146.0[u]	145.48	145.33	145.2–146.1[ah]
	5′	115.50	115.49	115.12	115.6–116.0[m]	116.18	115.0–116.0[v]	115.0–116.0[x]	115.7–116.0[ac]	115.5–116.0[ai]
	6′	119.54	120.96	119.94	119.01	119.06	119.66	118.84	119.08	119.12[aj]

[b,c,e,g–j,l–z,aa–ad,ag–an]Overlapped with each other.
[a,d,f,k,af]Values with the same superscript letters are not significantly different.

signed on the basis of the HMBC correlation with H2-unit T_1, and the interflavonoid bonds were all determined as C4 → C8 because the C8a of units T_1, M_1 and M_2 were correlated with H4 of units M_1, M_2 and U_1 in HMBC, respectively. Thus, structure **4** was elucidated as EC-(4β → 8)-EC-(4β → 8)-EC-(4β → 8)-Cat.

Compound **5** consisted of one catechin as the terminal unit and three epicatechins as extension units, as indicated by phloroglucinol degradation analysis. In the HMBC spectrum, the H6-unit T_1 showed correlations with C5 and C7, suggesting that unit M_1 was attached to the C8-unit T_1. The connection between units M_1 and

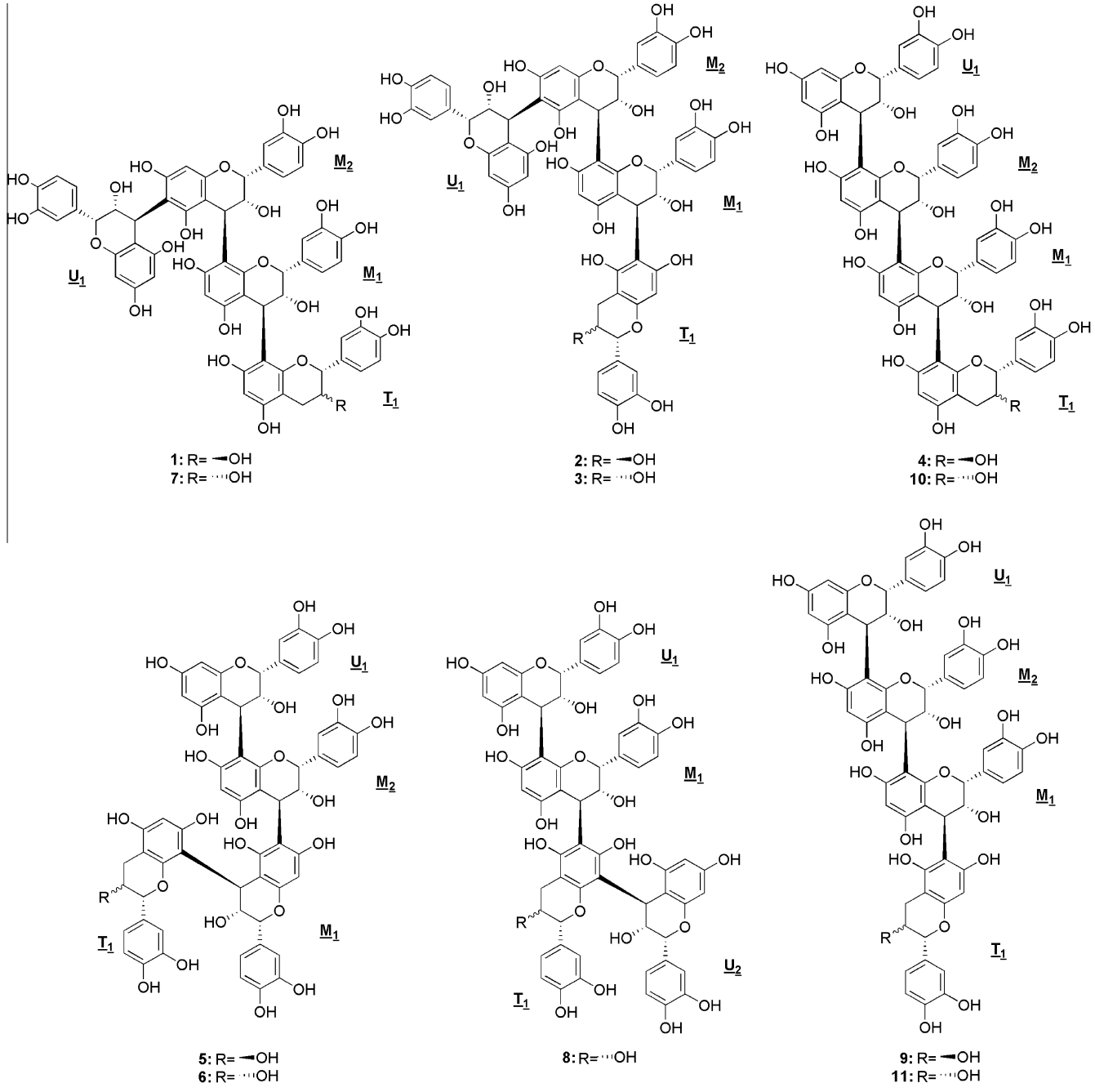

Fig. 2. Structures of isolated compounds: EC-(4β → 6)-EC-(4β → 8)-EC-(4β → 8)-Cat (**1**); EC-(4β → 6)-EC-(4β → 8)-EC-(4β → 6)-Cat (**2**); EC-(4β → 6)-EC-(4β → 8)-EC-(4β → 6)-EC (**3**); EC-(4β → 8)-EC-(4β → 8)-EC-(4β → 8)-Cat (**4**); EC-(4β → 8)-EC-(4β → 6)-EC-(4β → 8)-Cat (**5**); EC-(4β → 8)-EC-(4β → 6)-EC-(4β → 8)-EC (**6**); EC-(4β → 6)-EC-(4β → 8)-EC-(4β → 8)-EC (**7**); EC-(4β → 8)-EC-(4β → 6)-[EC-(4β → 8)]-EC (**8**); EC-(4β → 8)-EC-(4β → 8)-EC-(4β → 6)-Cat (**9**); EC-(4β → 8)-EC-(4β → 8)-EC-(4β → 8)-EC (**10**); EC-(4β → 8)-EC-(4β → 8)-EC-(4β → 6)-EC (**11**).

M_2 was determined to be C4 → C6 on the basis of the correlation between the H8-unit M_1 and the C8a-unit M_1. In the unit M_2, the C5-unit M_2 was correlated with both H4-unit M_2 and H6-unit M_2, indicating that the connection between units M_2 and U_1 was C4 → C8. Therefore, structure **5** was elucidated as EC-(4β → 8)-EC-(4β → 6)-EC-(4β → 8)-Cat.

Compound **6** consisted of four epicatechins, as established by phloroglucinol degradation analysis. Only three carbon signals showed no isotopic shift in the H-D exchange experiment; thus, overlapping of the C8a resonances seemed to occur. In the HMBC spectrum, the H6-unit T_1 showed correlations with C5 and C7, indicating that the connection between units T_1 and M_1 was C4 → C8. This was also confirmed by the correlation from H4-unit M_1 to C8a-unit T_1. The connection between units M_1 and M_2 was also determined to be C4 → C6 on the basis of the correlation from both H4-unit M_1 and H4-unit M_2 to C5-unit M_1. The connection between units M_2 and U_1 was determined to be C4 → C8 on the basis of the correlation from both H4-unit M_2 and H6-unit M_2 to C5-unit M_2. Therefore, structure **6** was elucidated as EC-(4β → 8)-EC-(4β → 6)-EC-(4β → 8)-EC.

Compound **8** consisted of four epicatechins, as demonstrated by phloroglucinol degradation analysis. Different from other tetramers, it seemed to include two ends of extension, as indicated by two sets of coupled A-ring protons (δ_H 5.94, 5.98 and δ_H 6.08, 6.11) in the ^{1}H NMR spectrum. The HMBC correlation from both H4-unit U_1 and H4-unit M_1 to C8a-unit M_1 indicated a C4 → C8

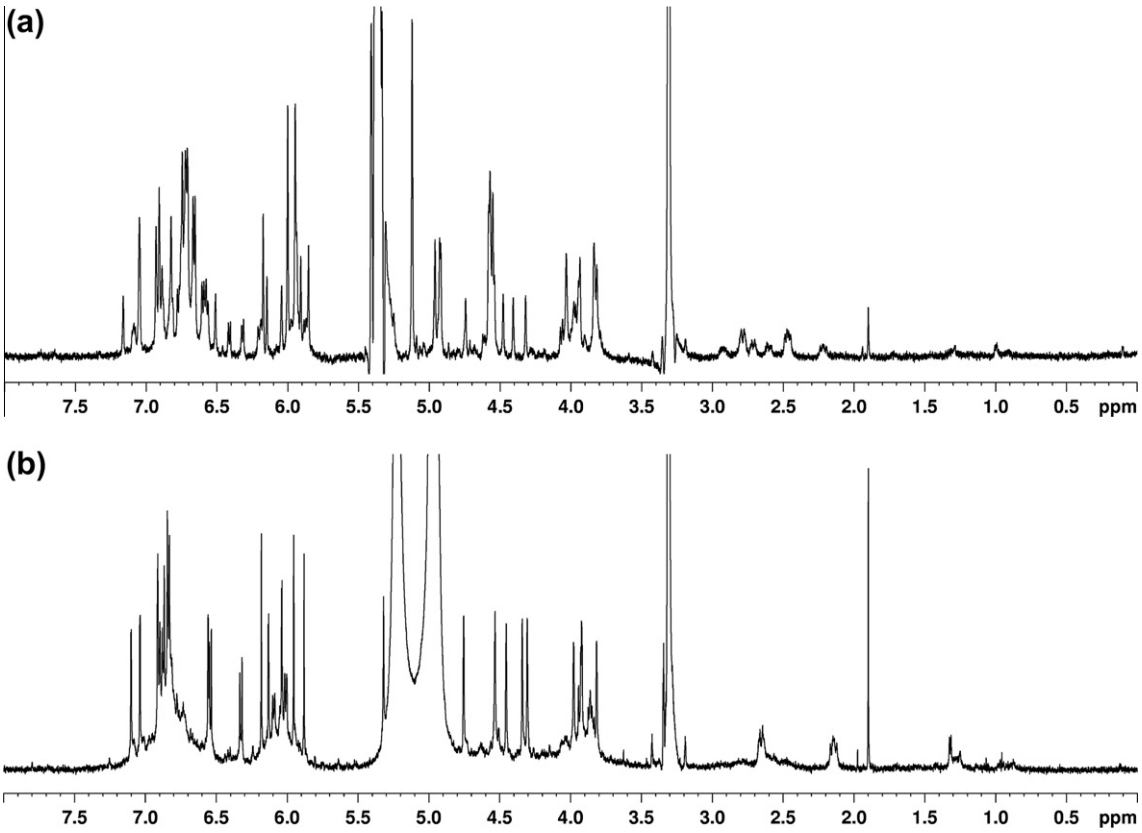

Fig. 3. Effect of solvent on the ^{1}H NMR spectrum of **2**. (a) in methanol-d_4 at 243 K, (b) in methanol-d_4/deuterium oxide (25:15) at 278 K.

connection between these units. The H4-unit M_1 showed a correlation to another three oxygen-attached aromatic carbons in the δ_C range of 153–158, which showed isotopic shifts in the H-D exchange experiment. This observation indicated that unit M_1 was never attached to C8 of the next unit. Meanwhile, the H4-unit U_2 showed an HMBC correlation to C8a-unit T_1, by which the connection between units T_1 and U_1 was determined to be C4 → C8. Moreover, both the H4-unit M_1 and the H4-unit U_2 showed a correlation to the C7-unit T_1; thus, it was confirmed that unit T_1 was attached to two extension units (units M_1–U_1 and unit U_2), and finally structure **8** was elucidated as EC-(4β → 8)-EC-(4β → 6)-[EC-(4β → 8)]-EC. Key HMBC correlations are depicted in Fig. 4.

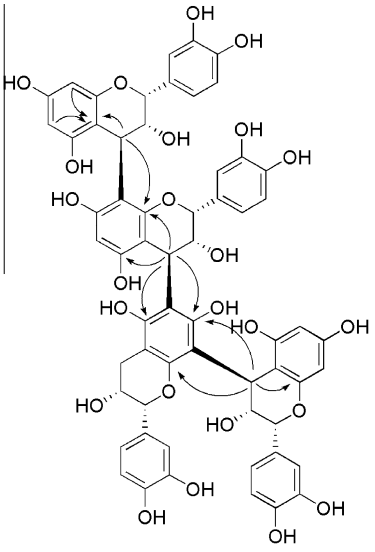

Fig. 4. Key HMBC correlations (arrow) of **8**.

Compound **9** consisted of one catechin as the terminal unit and three epicatechins as extension units, as shown by phloroglucinol degradation analysis. In the HMBC spectrum, the C8a-unit T_1 was correlated with both H2-unit T_1 and H8-unit T_1, indicating that the unit T_1 was connected to unit M_1 through a C4 → C6 interflavonoid bond. The H8-unit T_1 was severely overlapped with the H6-unit M_1 and their HMBC cross-peaks were hard to distinguish, but they were discriminable in methanol-d_3. In unit M_1, both H4-unit M_1 and H6-unit M_1 were correlated with the C5-unit M_1; thus, the connection between units M_1 and M_2 was suggested to be C4 → C8. Likewise, the connection between units M_2 and U_1 was determined to be C4 → C8 on the basis of the correlation from both H4-unit M_2 and H6-unit M_2 to C5-unit M_2. Therefore, structure **9** was elucidated as EC-(4β → 8)-EC-(4β → 8)-EC-(4β → 6)-Cat.

Compound **11** consisted of four epicatechins, as demonstrated by phloroglucinol degradation analysis. In the HMBC, the H8-unit T_1 showed a correlation with the C8a-unit T_1, indicating that the connection between units T_1 and M_1 was C4 → C6. The connection between units M_1 and M_2 was also determined to be C4 → C8 on the basis of the correlation from both H4-unit M_1 and H4-unit M_2 to C8a-unit M_1. The connection between units M_2 and U_1 was then determined to be C4 → C8 on the basis of the correlation from both H4-unit M_2 and H6-unit M_2 to C5-unit M_2. Therefore, structure **11** was elucidated as EC-(4β → 8)-EC-(4β → 8)-EC-(4β → 6)-EC.

3. Conclusion

To determine the position of interflavonoid linkages in the oligomeric procyanidins by NMR spectroscopic analysis, key observations for unambiguous elucidation are: (1) discrimination of C8a

from C5 and C7; (2) HMBC correlation from A-ring proton (H6 or H8); (3) HMBC correlation from H4 in upper and lower units. In the case of a C4 → C8 connected dimeric structure, the H4 in both upper and lower units show a HMBC correlation to C8a in the lower unit, theoretically. The clear correlations from H6 to both C5 and C7, or from both H4 and H6 to C5 in the lower unit, also indicate the connection through C8. As for the C4 → C6 connected dimer, the H4 in both upper and lower units show a HMBC correlation to C5 in the lower unit. HMBC correlation from H8 to C8a indicates the connection through C6. In the practical case of higher oligomers, signal overlapping sometimes occurs at H4, or A-ring protons and carbons. The most important thing is to pick out the evident correlation, which enables avoidance of misreading along with the theory described above.

Taken together, the detectable tetrameric procyanidins in an unripe apple extract were isolated in detail and the compounds were unambiguously identified by NMR spectroscopic and phloroglucinol degradation analyses. To our knowledge, this is the first report of the structural diversity of tetrameric procyanidins in apple. Additionally, the isolation and identification of compounds **1**, **2**, **5**, **6**, and **8** were reported for the first time. To obtain new insights on structure–activity relationship of oligomeric procyanidins, further investigations with these procyanidins are needed.

4. Experimental

4.1. General experimental procedures

^{1}H (600 MHz), ^{13}C (150 MHz), and 2D NMR spectra were measured using a Bruker AV600 equipped with a Polycold refrigerant system. LC-ESI-TOFMS analysis was conducted with an Applied Biosystems QSTAR Elite equipped with two Hitachi L-2100 pumps, a Hitachi L-2300 column oven, and a Hitachi L-2200 autosampler. Semi-preparative HPLC was conducted with a GL Science PLC761 LC system, which consists of two PU714 pumps, an UV702 UV–VIS detector, a CO705 column oven, an FC204 fraction collector, and an SC762 system controller. Optical rotations were measured in MeOH with a JASCO-DIP1000 Perkin Elmer 341 digital polarimeter. UV spectra were recorded in H_2O on a Hitachi U-2900 spectrophotometer. IR (Film) spectra were recorded on a Shimazu IRPrestige-21.

(+)-Catechin, (−)-epicatechin, and (+)-taxifolin were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Phloroglucinol was purchased from Sigma–Aldrich Co. (St. Louis, MO). All other reagents and chemicals were of special grade of Japanese Industrial Standards unless otherwise stated.

4.2. Materials

The apple procyanidin fraction was prepared from unripe apples (*Malus pumila* cv. Fuji) in the factory scale, according to the method of Shoji et al. (2003) with slight modification. Briefly, the crude apple polyphenol extract was obtained from unripe apple juice by solid phase extraction with SEPABEADS® SP-70 (Mitsubishi Chemical Corporation, Tokyo, Japan). Next, the extract was adjusted to pH 7.0 and applied to a column filled with Diaion® HP-20 (Mitsubishi Chemical Corporation, Tokyo, Japan). Adsorbed apple procyanidins were rinsed with distilled H_2O, followed by elution EtOH-H_2O (21:79, w/w). The corresponding eluate was concentrated and spray-dried to obtain the powdered apple procyanidin fraction.

4.3. Isolation

The apple procyanidin fraction (12 g) was further fractionated according to the DP by semi-preparative HPLC with a Deverosil 100diol-5 column (i.d. 20 × 250 mm, 5 μm) (Phenomenex Inc., Torrance, CA) (Kelm et al., 2006). The binary mobile phase consisted of (A) CH_3CN and (B) MeOH/H_2O (95:5, v/v). The gradient program was as follows: 0–3 min, 5% B; 3–60 min, 5–30% B; 60–63 min, 30–100% B; 63–70 min, 100% B; 70–76 min, 100–5% B; 76–86 min, 5% B. The column temperature was 35 °C and the flow rate was 12.0 mL/min. The apple procyanidin fraction was dissolved in MeOH (0.5 g/mL) and applied after filtration (0.45 μm) (GL Science, Tokyo, Japan). The eluate was monitored by absorbance at 280 nm and collected as follows: 6–10 min, as Fr.1; 15–20 min, as Fr.2; 27–32 min, as Fr.3; 37–42 min, as Fr.4; 45–50 min, as Fr.5; 52–56 min, as Fr.6; 58–62 min, as Fr.7; 63–66 min, as Fr.8; and 68–71 min, as Fr.9. The obtained amounts of nine fractions were 1.66, 1.42, 1.05, 1.02, 0.70, 0.61, 0.35, 0.25, and 1.24 g, respectively.

The Fr.4 was confirmed to include 11 tetrameric procyanidins by LC-ESI-TOFMS (Fig. 5), and an aliquot of this fraction (593 mg) was further purified to obtain isolated compounds by RP-HPLC

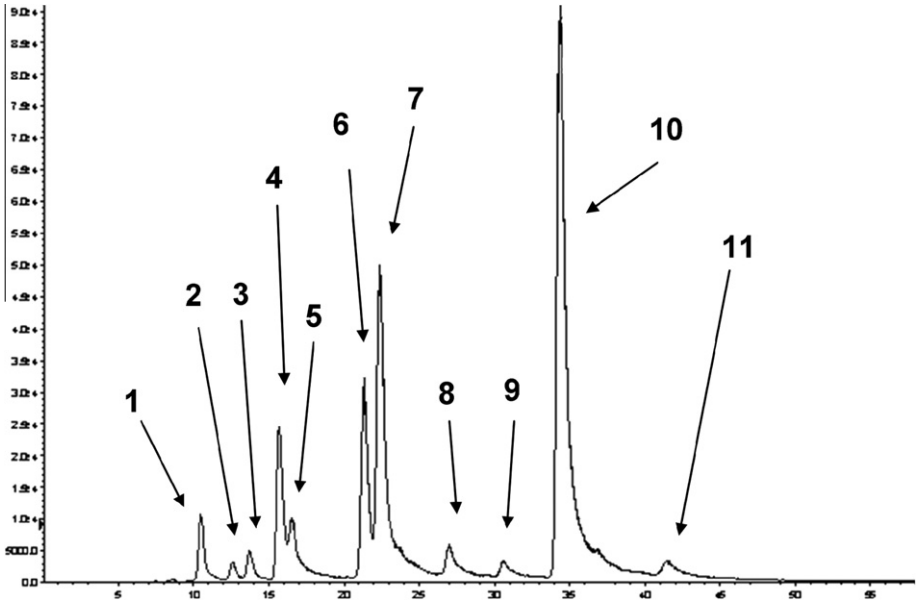

Fig. 5. Extracted ion chromatogram (m/z 1153 to 1154) of tetrameric procyanidin fraction by negative ESI-TOFMS analysis.

using Inertsil ODS-3 column (i.d. 20 × 250 mm, 4 μm) (GL Science Inc., Japan). Compounds **1**, **6**, **7**, **9**, and **10** were isolated by isocratic elution with CH_3CN-H_2O (12:88, v/v) at 12.0 mL/min and by peak collection, but others were obtained as mixed fractions. Therefore, these fractions were subjected to recycling HPLC and every remaining compound was completely isolated. The amounts of isolated compounds were described as follows: **1**, 11.4 mg; **2**, 1.2 mg; **3**, 3.1 mg; **4**, 28.9 mg; **5**, 13.6 mg; **6**, 27.4 mg; **7**, 23.9 mg; **8**, 5.7 mg; **9**, 3.7 mg; **10**, 94.8 mg; and **11**, 9.9 mg.

*4.3.1. Epicatechin-(4β → 6)-epicatechin-(4β → 8)-epicatechin-(4β → 8)-catechin (**1**)*

Pale brown amorphous solid; $[\alpha]_{24}^{D}$ = + 110 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.12) nm; IR (film) γ_{max} 3421, 1609, 1522, 1445, 1283, 1109, 1055 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2736 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

*4.3.2. Epicatechin-(4β → 6)-epicatechin-(4β → 8)-epicatechin-(4β → 6)-catechin (**2**)*

Pale brown amorphous solid; $[\alpha]_{25}^{D}$ = + 160 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.15) nm; IR (film) γ_{max} 3414, 1609, 1522, 1445, 1285, 1115, 1063 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2721 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

*4.3.3. Epicatechin-(4β → 6)-epicatechin-(4β → 8)-epicatechin-(4β → 6)-epicatechin (**3**)*

Pale brown amorphous solid; $[\alpha]_{25}^{D}$ = + 130 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.17) nm; IR (film) γ_{max} 3422, 1611, 1522, 1445, 1285, 1117, 1059 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2688 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

*4.3.4. Epicatechin-(4β → 8)-epicatechin-(4β → 8)-epicatechin-(4β → 8)-catechin (**4**)*

Pale brown amorphous solid; $[\alpha]_{25}^{D}$ = +84 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.09) nm; IR (film) γ_{max} 3377, 1609, 1522, 1447, 1285, 1107, 1061 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2751 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

*4.3.5. Epicatechin-(4β → 8)-epicatechin-(4β → 6)-epicatechin-(4β → 8)-catechin (**5**)*

Pale brown amorphous solid; $[\alpha]_{26}^{D}$ = +150 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.13) nm; IR (film) γ_{max} 3422, 1616, 1522, 1449, 1285, 1211, 1090, 1057 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2715 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

*4.3.6. Epicatechin-(4β → 8)-epicatechin-(4β → 6)-epicatechin-(4β → 8)-epicatechin (**6**)*

Pale brown amorphous solid; $[\alpha]_{26}^{D}$ = +89 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.08) nm; IR (film) γ_{max} 3422, 1609, 1522, 1447, 1285, 1109, 1057 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2705 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

*4.3.7. Epicatechin-(4β → 8)-epicatechin-(4β → 6)-[epicatechin-(4β → 8)-]epicatechin (**8**)*

Pale brown amorphous solid; $[\alpha]_{26}^{D}$ = +110 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.11) nm; IR (film) γ_{max} 3422, 1609, 1518, 1449, 1285, 1148, 1111, 1061 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2755 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

*4.3.8. Epicatechin-(4β → 8)-epicatechin-(4β → 8)-epicatechin-(4β → 6)-catechin (**9**)*

Pale brown amorphous solid; $[\alpha]_{27}^{D}$ = +130 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.13) nm; IR (film) γ_{max} 3422, 1609, 1522, 1447, 1283, 1148, 1109, 1063 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2779 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

*4.3.9. Epicatechin-(4β → 8)-epicatechin-(4β → 8)-epicatechin-(4β → 6)-epicatechin (**11**)*

Pale brown amorphous solid; $[\alpha]_{27}^{D}$ = +120 (c 0.10, MeOH); UV (water) λ_{max} (log ε) 278 (4.13) nm; IR (film) γ_{max} 3422, 1609, 1518, 1445, 1285, 1148, 1111, 1061 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESITOFMS *m/z* 1155.2677 ($[M + H]^+$, calcd for $C_{60}H_{51}O_{24}$, 1155.2692).

4.4. Phloroglucinol degradation analysis

To investigate the constitutive flavan-3-ol units of the isolated tetrameric procyanidins, acid-catalyzed degradation in the presence of phloroglucinol was conducted according to the method of Kennedy and Jones (2001). Phloroglucinol (1 g) and ascorbic acid (200 mg) were dissolved in MeOH (20 mL) containing 0.1 N HCl. Each procyanidin was reacted in this solution (5 g/L) at 45 °C for 30 min, followed by addition of a 5-fold volume of 40 mM NaOAc to stop the reaction.

Reaction products were analyzed by RP-HPLC using an Inertsil ODS-3 column (i.d. 4.6 × 250 mm, 4 μm). The binary mobile phase consisted of (A) 0.1% HCO_2H and (B) CH_3CN containing 0.1% HCO_2H. The gradient program was as follows: 0–10 min, 5% B; 10–40 min, 5–20% B; 40–40.1 min, 20–80% B; 40.1–50 min, 80% B; 50–50.1 min, 80–5% B; 50.1–65 min, 5% B. The column temperature was 35 °C and the flow rate was 1.0 mL/min. Eluate was monitored at 280 nm. Detected peaks were identified by comparing their retention times to standard compounds, such as (+)-catechin and (−)-epicatechin. Catechin-(4α → 2)-phloroglucinol was prepared from (+)-taxifolin according to the method of Kennedy and Jones (2001). Briefly, (+)-taxifolin (51.2 mg) and $NaBH_4$ (24.5 mg) were stirred in absolute EtOH (10 mL) at room temperature for 1 h. Phloroglucinol (175.5 mg) was dissolved in 0.1 N HCl (10 mL) and then combined with the reaction mixture and stirred for 30 min, followed by addition of 10 mL H_2O and extraction with EtOAc (3 × 10 mL). The extract was lyophilized to dry powder. The production of the desired compound was confirmed by LC-ESI-TOFMS analysis. The retention time of epicatechin-(4β → 2)-phloroglucinol was confirmed by phloroglucinol degradation of procyanidin B2, which had been previously isolated.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.07.011. These data include MOL files and InChiKeys of the most important compounds described in this article.

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