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A-type proanthocyanidins from peanut skins

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Abstract

Six A-type proanthocyanidins were isolated from the water-soluble fraction of peanut skins. On the basis of spectral data, reductive cleavage with sodium cyanoborohydride, and chiral HPLC analysis, three new compounds, epicatechin-(2β→O→7, 4β → 6)-catechin, epicatechin-(2β → O → 7, 4β → 6)-*ent*-catechin and epicatechin-(2β → O → 7, 4β → 6)-*ent*-epicatechin were unambiguously identified, together with the three known compounds, proanthocyanidin A-1, proanthocyanidin A-2 and epicatechin-(2β→O→7, 4β→8)-*ent*-epicatechin. ^{13}C NMR chemical shift rules to distinguish between [2→O→7, 4→8] and [2→O →7,4→6] double-linked heptamethyl ethers of A-type proanthocyanidins are proposed. Bioassay experiments showed that these six compounds possess substantial activity against hyaluronidase.

Keywords: *Arachis hypogaea*; Leguminosae; Proanthocyanidin; Hyaluronidase; Inhibition

1. Introduction

Peanut skins are used to treat chronic hemorrhage and bronchitis in China (Jiangsu Xin Medical College, 1977) and have a high tannin content (Karchesy & Hemingway, 1986). In the course of our studies on bioactive natural compounds, the water-soluble fraction of the mature seed skins of peanut *Arachis hypogaea* L. was found to inhibit hyaluronidase. From this active fraction, six A-type proanthocyanidins were purified by monitoring its enzymatic inhibition. Among them, three were identified as new A-type proanthocyanidin dimers on the basis of: their spectral data; their conversion to monomers by reductive cleavage with sodium cynaoborohydride and from the results of chiral HPLC analyses.

2. Results and discussion

Peanut skins were extracted with boiling water with the resulting extract fractionated by adsorption on HP-20 resin eluted with water and aqueous acetone. The fraction obtained by elution with 70% aqueous acetone exhibited marked hyaluronidase inhibitory activity. This active fraction was subjected to sequential gel chromatography on Toyopearl HW-40 and Sephadex LH-20, and finally gave, following reverse phase low- and high-pressure liquid chromatographies, six compounds: compounds **1**, **2** and **4** were obtained as colorless needles, whereas compounds **3**, **5** and **6** were white amorphous powders.

All six compounds yield an orange coloration on reaction with anisaldehyde-sulfuric acid reagent (Morimoto, Nonaka, & Nishioka, 1987) which is thought to be characteristic of proanthocyanidins. The ^{1}H NMR (CD_3OD) spectra of all six compounds closely resembled each other (Table 1) and the presence of the isolated AB coupling system at δ 4.0–4.5 with $J_{3,4}$ = 3.4–3.8 Hz was ascribed as a diagnostic feature

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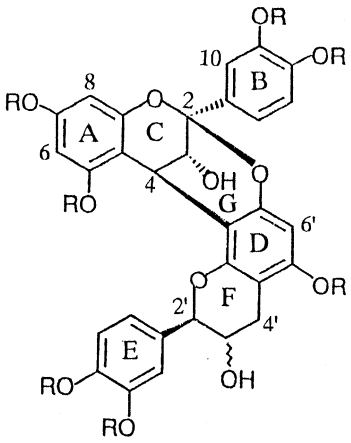

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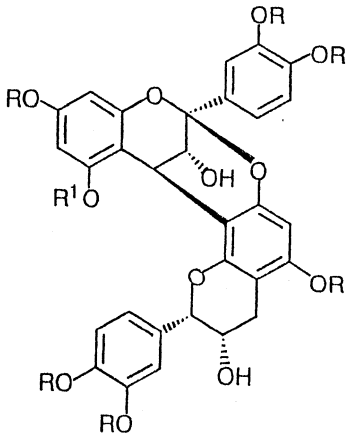
8 R = Me



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9 R = Me R^1 = H
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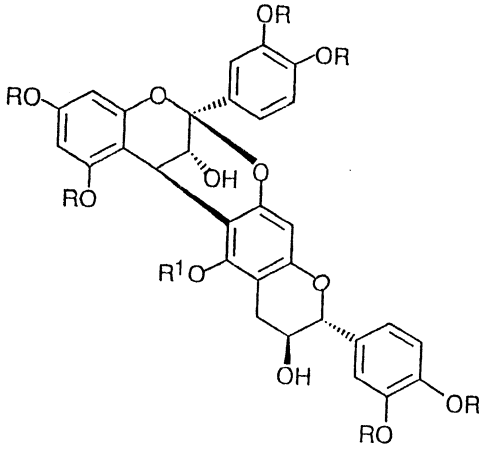

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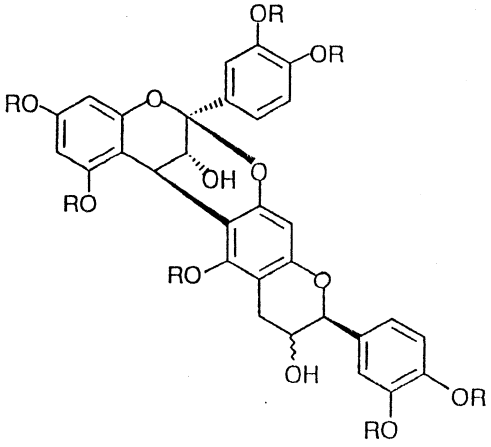
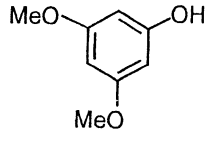


5 R = H
6 R = H
13 R = Me
14 R = Me

of the C-ring protons of A-type proanthocyanidins (Jacques, Haslam, Bedford, & Greatbanks, 1974). The *meta*-substitution pattern, as revealed by the two coupled doublets and a residual one proton singlet at around δ 6.0, as well as the two AMX coupling systems in the aromatic region (δ 6.5–7.5) were indicative of a dimeric flavanol. This class of compounds was also confirmed by the presence of 1 methylene, 13 methines and 16 quarternary carbons in the ^{13}C NMR spectrum (Table 2), and the molecular weights of 576 amu for each compound, as established by FAB–MS ($[M+1]^+$ at m/z 577).

Compound **1** was identified as proanthocyanidin A-1 and compound **2** was identified as proanthocyanidin A-2 by direct comparison of their spectral data with those of authentic samples. Data for their heptamethyl ethers also support this conclusion.

The ^{1}H NMR spectrum of **3** resembled that of **2**. The presence of the AB coupling system at δ 4.16 (1H, d, J = 3.5 Hz, H-3) and δ 4.41 (1H, d, J = 3.5 Hz,



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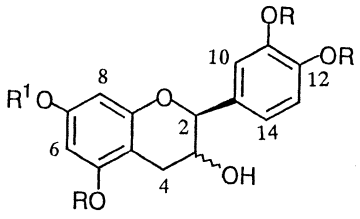

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19 R = R^1 = H
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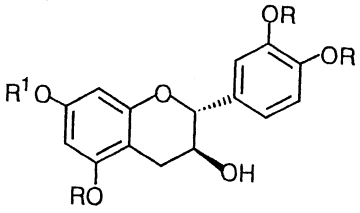
21 R = R^1 = Me



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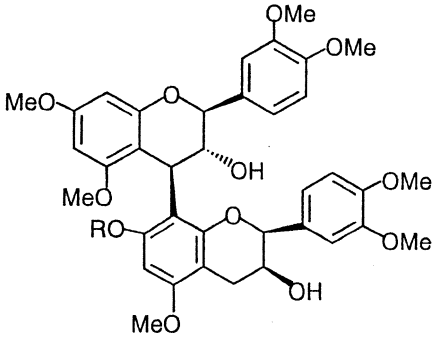

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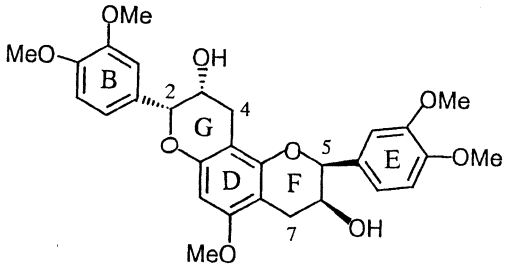

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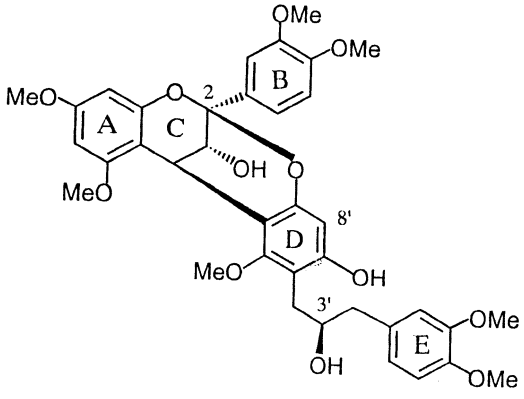

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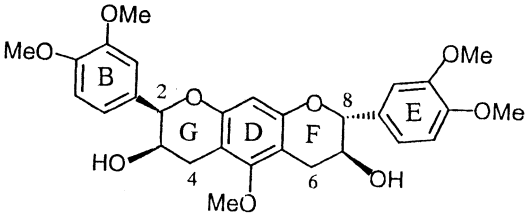

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H-4), the meta-coupled doublets at δ 5.89 and 6.07 (each 1H, d, J = 2.3 Hz, ring A), a residual one aromatic proton singlet at δ 6.08 (ring D), and two AMX systems in the aromatic region (δ 6.5–7.5) due to rings B and E confirmed the A-type proanthocyanidin dimeric structure. This doubly linked dimeric structure was also demonstrated by the one acetal carbon at δ 100.46 in its ^{13}C NMR spectrum which was assigned unequivocally by the COLOC experiment. The 2,3-*cis* relative configuration of the lower flavanol

Table 1
^{1}H NMR data (6 ppm) for compounds **1**–**6** (270 MHz; CD_3OD)

Ring	H	1	2	3	4	5	6
C	3	4.07 d (3.5)	4.05 d (3.4)	4.16 d (3.5)	4.12 d (3.5)	4. 10 d (3.5)	4.12 d (3.2)
	4	4.24 d (3.5)	4.39 d (3.4)	4.41 d (3.5)	4.29 d (3.5)	4.28 d (3.5)	4.30 d (3.2)
A	6	5. 90 d (2.4)	6. 00 d (2.3)	5.89 d (2.4)	6.03 d (2.2)	6.03 d (2.2)	6.01 d (2.3)
	8	6.05 d (2.4)	6.07 d (2.3)	6.07 d (2.4)	6.08 d (2.2)	6.09 d (2.2)	6.07 d (2.3)
B	10	7.13 d (2.4)	7.14 d (2.4)	7.15 d (2.4)	7.17 d (2.4)	7.16 d (2.4)	7.16 d (2.2)
	13	6.79 d (8.4)	6.82 d (8.2)	6.84 d (8.4)	6.82 d (8.4)	6.81 d (8.4)	6.82 d (8.1)
	14	7.01 dd (8.4, 2.4)	7.07 dd (8.2, 2.4)	7.04 dd (8.4, 2.4)	7.05 dd (8.4, 2.4)	7.06 dd (8.4, 2.4)	7.05 dd (8.1, 2.4)
F	2′	4.74 d (7.8)	4.91 br.s	5.02 br.s	4.67 d (6.5)	4.63 d (7.3)	4.80 br.s
	3′	4.16 m	4.23 m	4.23 m	4.05 m	3.99 m	4.16 m
	4′α	2.58 dd (16.5,8.1)	2.74 dd (17.3, 3.2)	2.87 m	2.81 dd (16.5, 5.4)	2.91 dd (16.7, 5.4)	2.87 m
	4′β	2.81 dd (16.5,5.4)	2.95 dd (17.3, 4.6)		2.62 dd (16.5, 8.1)	2.58 dd (16.7, 7.6)	
	6′	6.08 s	6.09 s	6.08 s			
	8′				6.09 s	6.07 s	6.09 s
D	10′	6.91 d (2.0)	7.13 d (2.2)	7.14 d (2.0)	6.80 d (2.2)	6.79 d (2.2)	6.93 d (2.2)
E	13′	6.80 d (7.8)	6.81 d (8.2)	6.81 d (8.2)	6.75 d (8.4)	6.70 d (8.4)	6.73 d (8.3)
	14′	6.80 dd (7.8, 2.0)	6.99 dd (8.2, 2.2)	6.95 dd (8.2, 2. 0)	6.68 dd (8.4, 2.2)	6.68 dd (8.4, 2.2)	6.75 dd (8.3, 2.2)

Assignments were made by combination analysis of the 1*H*–1*H* Cosy and NOE associations. *J* values (Hz) are given in parentheses.

moiety was evident from the singlet at δ 5.02 (H-2′, ring D).

Methylation of **3** with diazomethane for 24 h at room temperature yielded hexamethyl ether **9** ([M + 1]$^+$ at *m*/*z* 661) as the major product because of the hinderic effects of E ring in terminal units to HO-5 of A ring. Its methylation when continued for 4 d afforded heptamethyl ether **10**. The molecular formula

Table 2
^{13}C NMR data (6 ppm) for compounds **1**–**6** (CD_3OD, 67.5 MHz)

Carbon	1	2	3	4	5	6
2	100.34	100.21	100.46	100.57	100.61	100.57
3	67.79	68.12	67.78	68.44	68.57	67.65
4	29.23	29.31	29.27	29.74	29.72	29.79
4a	104.06	104.29	104.11	104.22	104.23	104.27
5	156.76	157.05	156.72	155.14	155.37	155.28
6	98.22	98.34	98.02	96.70	97.01	96.92
7	158.14	158.16	158.12	158.11	158.23	158.20
8	96.78	96.67	96.53	96.65	96.55	96.65
8a	154.24	154.31	154.17	154.39	154.44	154.41
9	132.30	132.50[a]	132.34[a]	132.23	132.25[a]	132.13
10	115.72	115.66	115.75	115.84	115.95	115.84
11	146.79	146.34[b]	146.23[b]	146.84	146.86[b]	145.86
12	145.65	145.69[b]	146.23[b]	146.19	146.25[b]	146.69
13	116.36	116.08	116.26	115.68	115.68	115.70
14	119.87	120.41	119.90	119.99	120.01	120.01
2′	84.49	81.81	80.87	82.51	82.83	79.96
3′	68.12	67.02	67.18	67.64	67.67	67.13
4′	28.96	29.93	29.50	27.88	28.30	29.61
4′a	103.16	102.46	101.95	103.54	103.57	102.78
5′	156.15	156.65	156.63	155.25	155.37	155.81
6′	96.58	96.53	96.60	108.78	108.76	108.85
7′	152.19	152.34	152.09	152.48	152.48	152.93
8′	106.80	107.25	106.97	96.65	96.67	96.92
8′a	151.42	152.18	151.31	151.96	152.03	152.01
9′	130.58	131.24[a]	131.44[a]	132.19	132.16[a]	132.27
10′	115.77	115.70	115.70	114.96	114.98	115.25
11′	146.79	146.80[b]	145.69[b]	146.67	145.71[b]	145.85
12′	146.34	146.03[b]	146.08[b]	146.27	146.25[b]	145.98
13′	115.72	115.97	115.25	116.18	116.17	115.93
14′	120.73	119.81	119.97	119.60	119.89	119.44

Assignments for **1**, **4** and **6** were made by HETCOR and COLOC experiments. The corresponding carbons of **2**, **3** and **5** were inferred by direct comparison. Symbol [a,b] in the same column may be interchanged.

$C_{37}H_{38}O_{12}$ was established for **10** by elemental analysis and FAB–MS ($[M+1]^+$ at m/z 675). In its ^1H NMR (Table 3) spectrum, in addition to six methoxy signals at δ 3.73–3.95, one upfield methoxyl signal at δ 3.14 was assignable to MeO-5 owing to the anisotropic shielding of E ring in the lower flavanol unit, but absence of this strongly shielded resonance in **9**. This observation not only revealed the structure of **9** as an HO-5 unchanged methyl derivative but also suggested that the two flavanol units are linked through C-4 and C-8′. This linkage was further supported by the observation of NOE associations of H-4 (δ 5.01, ring C) with the protons of the E ring in **10**. The NOE associations of H-3 (δ 4.33, ring C) with H-6′ (δ 6.20, ring D) and the protons of the B ring defined the 3,4-*trans* configuration of the C ring unambiguously (Cronje, Burger, Brandt, Kolodziej, & Ferreira, 1990) as well as helped in the assignment of the protons of B and E rings.

In addition, the two flavanol units of A-type proanthocyanidins must possess either (2α, 4α) or (2β, 4β) double interflavanyl bonds. The high-amplitude positive Cotton effect at short wavelengths ($[\theta]_{221}$ $+2.3\times10^6$) of the CD spectrum of *3* allowed assignment of the absolute configuration of C-4 as *R* (Botha, Yong, Ferreira, & Roux, 1978; Barrett et al., 1979), thus decided 2β,4β-configuration for **3**. Taking the NOE interactions into consideration, this positive Cotton effect defined epicatechin as the upper unit with 2*S*, 3*R* and 4*R* absolute configurations.

The absolute configuration of the lower terminal unit was deduced to be 2′*S* and 3′*S*, that is *ent*-epicatechin according to the nomenclature (Kolodziej et al., 1993), on the basis of the pronounced NOE effects of MeO-5 (δ 3.14)_with H-2′ (δ 4.94) of **10** (Kolodziej, Sakar, Burger, Engelshowe, & Ferreira, 1991). In contrast, in **8**, in which the lower *ent*-epicatechin (2′*S*-configuration) unit of **10** is replaced by epicatechin (2′*R*-configuration), the NOE association of MeO-5 (δ 3.48) with H-2′ (δ 5.01) was apparently absent. This absolute configuration of **3** was further confirmed by reductive cleavage with sodium cyanoborohydride in TFA (Steynberg et al., 1997) and the formation of **18** and **20** in addition to **15**, **25** and **27**.

Compound **18** was identified as the tetramethyl ether of *ent*-catechin on the basis that its ^1H NMR spectrum and retention time in HPLC on Chiralcel OD column were identical to those of authentic compound obtained by methylation of **16**. Formation of **18** was due to the upper monomeric unit of **10** because the acetal functional group was cleaved in a predominantly SN2 manner thus lead to inversion of the configuration at C-2 (Steynberg et al., 1997). Compound **20** was identified as 5, 3′, 4′-tri-*O*-methyl-*ent*-epicatechin by ^1H NMR and its molecular weight of 332 as established by mass spectra. Methylation of **20** with diazomethane generated **21**, whose structure was determined by comparison of its ^1H NMR spectrum and retention time in chiral HPLC on Chiralcel OD column with those of authentic compound; such HPLC proved to be an effective method for separating the enantiomers under the given conditions (see Section 3). The structure of the biflavanoid **25** in this experiment was expected from the reaction. Methylation of it yielded **26**. Their structures were determined by MS, CD and NMR measurements. Formation of **15** and **27** did not contribute significantly to the structure determination. Their structure determinations are not discussed in detail further, only the spectral data are listed in the experimental section. This straight forward approach lead to the unambiguous determination of **3** as epicatechin-(2β→O→7, 4β→8)-*ent*-epicatechin. This compound was previously identified in *Prunus spinosa* in the form of its heptamethyl diacetate (Antonio Gonzalez, 1994).

The presence of a doublet at δ 4.67 (d, $J=6.5$ Hz, H-2′, ring F) in the ^1H NMR spectrum of **4** suggested a 2′,3′-*trans* relative configuration of the lower flavanol moiety. In DIFNOE experiments, NOE associations similar to those of **3** were observed for **4**. However, NOE associations between H-4 and the protons of ring E were obviously lacking suggesting a [4–6] linked dimeric structure. The existence of a [2→O→7], rather than [2→O→5], ether linkage between the two flavanol units was unequivocally deduced for the NOE associations of H-8′ (δ 6.09, s, ring D) with H-2′ (δ 4.67, d, $J=6.5$ Hz, ring F) and H-10 (δ 7.17, d, $J=$ 2.4 Hz, ring B). This conclusion was also confirmed by the NOE association of the methylene signal H_2-4′ (ring F) with the methoxy function at δ 3.89 (MeO-5′, ring D) and the absence of a high field shift methoxy signal in its heptamethyl ether **12**. Methylation of **4** also yielded one hexamethyl ether **11**, its molecular weight was shown to be 660 (m/z 661, $[M+1]^+$) by FAB–MS. The absence of the NOE association of the methylene signal H_2-4′ with the methoxy function in **11** decided the structure of this hexamethylated derivative is unchanged at HO-5′.

By a combination of ^1H- and ^{13}C NMR, HETCOR and COLOC spectral analysis (Fig. 1), the signals of H and C can be definitively assigned (Tables 1–4). Careful examination of the ^{13}C NMR data of the heptamethylated ethers revealed that there are highly diagnostic difference in C-4′a, 6′ and 8′ chemical shifts that make it possible to distinguish between the [2→O→7, 4→6] and [2→O→7, 4→8] double linkage structures. In **12**, a [2→O→7, 4→6] double linked structure, the chemical shift of C-4a′, 6′ and 8′ are at ca. δ 107, 112 and 100 respectively, whereas in [2→O→7, 4→8] double linked structures the chemical shifts of these carbons are at about δ 102, 92 and 106 respectively. A methoxy carbon at 60.8 ppm, which is attributable to

Table 3
^{1}H NMR data (5 ppm) of methylated derivatives **7**–**14** ($CDCl_3$, 270 MHz)[a]

Ring	H	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
C	3	4.26 dd (3.5, 5.6)	4.27 dd (3.6, 5.9)	4.38 dd (3.6, 5.6)	4.33 dd (3.6, 5.2)	4.24 m	4.19 dd (3.5, 5.9)	4.23 dd (3.5, 5.4)	4.26 dd (3.6, 5.6)
	4	4.89 d (3.5)	4.95 d (3.6)	4.51 d (3.6)	5.01 d (3.6)	4.46 d (3.5)	4.91 d (3.5)	4.82 d (3.5)	4.81 d (3.6)
	3-OH	1.66 d (5.6)	1.74 d (5.9)	1.65 d (5.6)	1.66 d (5.2)	1.73 d (5. 1)	1.67 d (5.9)	1.72 d (5.4)	1.72 d (5.6)
A	6	6.04 d (2.0)	6.07 d (2.3)	6.11 d (2.3)	5.99 d (2.2)	6.22 d (2.2)	6.18 d (2.3)	6.17 d (2.2)	6.16 d (2.1)
	8	6.30 d (2. 0)	6.31 d (2.3)	6.30 d (2.3)	6.28 d (2.2)	6.38 d (2.2)	6.3 2 d (2.3)	6.30 d (2.2)	6.29 d (2.1)
B	10	7.22 d (2. 1)	7.31 d (1.9)	7.26 d (2.4)	7.22 d (20)	7.32 d (2.0)	7.22 d (1.9)	7.22 d (2.0)	7.21 d (1.9)
	13	6.84 d (8.4)	6.94 d (8.4)	6.99 d (8.4)	6.96 d (8.3)	6.98 d (8.2)	6.91 d (8.4)	6.94 d (8.4)	6.93 d (8.4)
	14	7.31 dd (8.4, 2.4)	7.35 dd (8.4, 1.9)	7.29 dd (8.4, 2.4)	7.26 dd (8.3, 2.3)	7.33 dd (8.2, 2.0)	7.30 dd (8.4, 1.9)	7.30 dd (8.4, 1.9)	7.30 dd (8.4, 1.9)
F	2′	5.00 d (7.4)	5.01 br.s	5.12 br.s	4.94 br.s	4.61 d (8.2)	4.60 d (8.3)	4.71 d (8.2)	5.01 br. s (8.2)
	3′	4.24 m	4.48 m	4.28 m	4.26 m	4.04 m	4.00 m	3.98 m	4.25 m
	4′α	2.72 dd (17.0, 4.8)	2.95 dd (17.1, 4.6)	3.01 dd (17.0, 1.6)	2.93 dd (17.0, 2.2)	3.12 dd (16.2, 5.4)	3.18 dd 16.0, 5.2)	3.19 dd (16.0, 5.3)	3.09 dd (16.2, 2.1)
	4′β	2.63 dd (17.0, 6.5)	2.76 dd (17.1, 2.8)	2.86 dd (17.0, 3.9)	2.82 dd (17.0, 3.2)	2.65 dd (16.2, 9.8)	2.79 dd (16.0, 9.8)	2.71 dd (16.0, 8.9)	3.04 dd (16.2, 3.3)
	3-OH	1.63 d (5.1)	1.79 d (7.6)	1.72 d (4.6)	1.85 d (5.6)	1.73 d (5.2)	1.70 d (6.2)	1.74 d (6.6)	1.67 d (5.9)
D	6′	6.21 s	6.22 s	6.26 s	6.20 s				
	8′					6.26 s	6.43 s	6.43 s	6.49 s
E	10′	6.97 d (2.4)	7.16 d (1.9)	7.07 d (2.4)	7.20 d (2.4)	7.00 d (2.0)	6.95 d (1.9)	6.89 d (1.9)	7.01 d (1.9)
	13′	6.75 d (8.2)	6.96 d (8.4)	6.92 d (8. 2)	6.93 d (8.3)	6.92 d (8.2)	6.94 d (8.4)	6.82 d (8.2)	6.89 d (8.2)
	14′	7.01 dd (8.2, 2.4)	7.34 dd (8.4, 1.9)	7.19 dd (8.2, 2.4)	7.18 dd (8.3, 2.4)	6.90 dd (8.2, 2.4)	7.01 dd (8.4, 1.9)	6.94 dd (8.2, 1.9)	6.99 dd (8.2, 1.9)
	OMe	3.94 s	3.93 s	3.94 s	3.95 s	4.04 s	3.93 s	3.91 s (×2)	3.92 s (×2)
		3.93 s	3.92 s (×2)	3.93 s	3.93 s (×3)	3.96 s	3.92 s	3.87 s (×2)	3.87s
		3.88 s	3.85	3.92 s	3.75 s	3.94 s	3.90 s (×2)	3.86s	3.88s
		3.76 s	3.76	3.91 s	3.73	3.89 s (×2)	3.89 s	3.85s	3.86s
		3.74 s	3.75	3.77 s	3.14	3.78 s	3.85 s	3.77s	3.85 s
		3.64 s	3.48	3.70 s			3.78 s		3.77 s
		3.32							

[a] Assignments were inferred on the basis of 1*H*–1*H* COSY and NOE associations in DIFNOE experiments. *J* values (Hz) are given in parentheses.

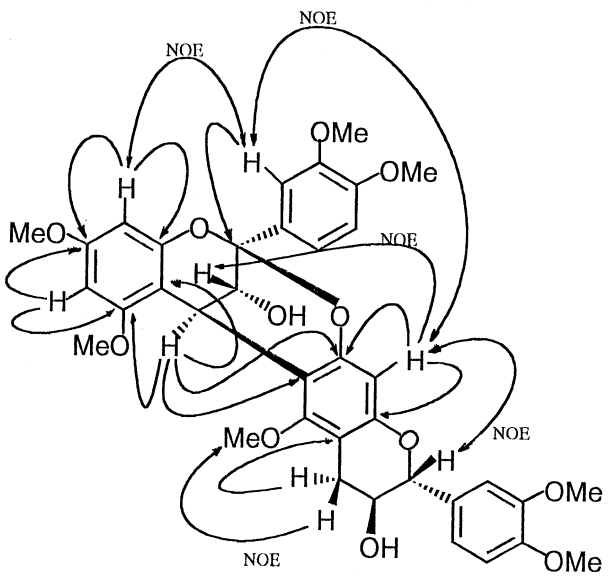

Fig. 1. NOE associations for determination of [2→7, 4→6] double linkage and COLOC experiments for determination of quaternary carbons. Arrows other than those labeled NOE correspond to COLOC correlations.

MeO-5′, is another diagnostic feature of [2→O→7, 4→6] structures. As expected from the unchanged HO-5′ in **11**, no signal at about δ 60 was apparent for this compound and the chemical shifts of C-4′a (δ 102.82), 6′ (δ 106.21), and 8′ (δ 96.42) do not differ markedly from those of **4**.

The expected NOE between H-3 and H-8′ confirmed the 3,4-*trans* relative configuration Fig. 1. The positive cotton effect at short wavelengths in the CD spectrum of **4** was consistent with the 2*S*, 3*R*, and 4*R* absolute configurations for the C ring.

With the exception of the relative chemical shift difference, the ^{1}H NMR spectrum of **5** is almost indistinguishable from that of **4**, indicating a close structural resemblance. Identical NOE observations and highly similar chemical shifts at C-4′a, 6′ and 8′ for the heptamethyl ether **13** and compound **12** suggested the [2→O→7, 4→6] double linkage structure for **5**. The positive Cotton effect at short wavelengths in the CD spectrum of **5** demonstrated 2*S*, 3*R* and 4*R* absolute configurations for the upper terminal unit. Thus **4** and **5** were shown to be stereoisomers at C2′ and C3′. The absolute configuration of the terminal units of **4** and **5** were then determined by the reductive cleavage method. From the reaction mixture of **12**, compound **18** and **23** along with **15**, **28** and **29** were obtained by PLC. Compound **18** was identified by comparison of its ^{1}H NMR spectrum and retention time in chiral HPLC with those of the authentic compound. The same approach identified **23** as 5,3′,4′-tri-*O*-methylcatechin after its conversion to **24** by methylation and subsequent chiral HPLC experiments. This straightforward method led to the unambiguous establishment of the structure of **4** as epicatechin-(2β→O→7, 4β→6)-catechin and that of **5** as epicatechin-(2β→*O*→7, 4β→6)-*ent*-catechin.

The lower terminal unit of **6** was identified as 2′, 3′-*cis*-flavan-3-ol by the presence of a singlet at δ 4.80. The same strategy as that outlined above also facilitated the determination of the interflavanol linkages and the absolute configurations for **6**. The NOEs observed with **6**, a high-amplitude positive Cotton effect at short wavelengths, and the diagnostic ^{13}C NMR spectrum of its heptamethyl ether **14** implied that **6** is also a [2→O→7, 4→6] double-linked dimer with the same configuration at upper unit as that of **5**. Direct comparison of the ^{1}H NMR spectrum of **14** with that of the heptamethyl ether of proanthocyanidin A-6 isolated from *Aesculus hippocastanum* (Nonaka, Morimoto, Kinjo, Nohara, & Nishioka, 1987) yielded a relative difference of signal pattern, thus suggested that **6** is a C2′, C3′ stereoisomer of proanthocyanidin A-6. Accordingly established the structure of **6** as epicatechin-(2β→O→7, 4β→6)-*ent*-epicatechin. This absolute structure was confirmed by the formation of **18** and **20** after reductive cleavage. Structure determinations of other product were not performed.

Hyaluronidase, as a mucosplitting enzyme, is thought to contribute to many biological functions (Chambers & Zwerfach, 1947; Cameron, Pauling, & Leibovitz, 1979; Kakegawa, Masatsumoto, & Satoh, 1992). Its inhibition by compounds **1**–**6** was assayed by the modified method of Davidson and Aronson (1969) yielding IC_{50} values of 0.35, 0.28, 0.23, 0.14, 0.22 and 0.30 mM respectively. The corresponding value for epigallocatechin gallate was 0.38 mM. No marked stereostructure-activity relationship was found in the inhibition.

These A-type proanthocyanidins from peanuts were determined to be [2→O→7, 4→8] or [2→O→7, 4→6] doubly linked dimers of flavan-3-ol. They exhibit the same absolute configurations at C2, C3 and C4. The isomerization at C2′ and C3′ of the lower terminal unit and the different interflavanol linkage points gave rise to the different compounds. In the structure determinations, DIFNOE and CD experiments proved informative with regard to configurations. Comparison of the difference in ^{13}C chemical shifts between unmethylated dimers and their heptamethylated derivatives allow the [2→O→7, 4→8] and [2→O→7, 4→6] linkages in A-type proanthocyanidins to be readily differentiated. Conversion to monomers by reductive cleavages followed by chiral HPLC analysis, also proved to be a powerful approach for stereocenters determination in the terminal units. Tannins occurred in seed skins are thought to possess fungistatic properties

Table 4
^{13}C NMR data (6 ppm) of methylated derivatives **7**–**14** ($CDCl_3$, 67.5 MHz)

Carbon	7	8	9	10	11	12	13	14
2	99.20	98.94	99.19	99.17	99.04	99.03	99.06	99.03
3	67.56	67.47	66.63	67.24	66.73	67.54	67.47	67.53
4	25.94	27.63	27.53	27.52	27.54	28.19	28.33	28.41
4a	103.73	103.73	102.62	103.89	103.50	103.45	103.57	103.43
5	158.75	159.00	155.36	158.72	156.27	156.18	156.22	156.85
6	92.81	93.22	96.80	92.68	93.26	93.11	93.27	93.27
7	160.05	159.99	160.05	159.85	160.17	160.19	160.10	160.12
8	93.20	93.47	94.47	93.13	94.69	93.40	93.35	93.35
8a	153.52	152.88	152.32	153.11	152.93	153.29	153.09	153.08
9	130.60	130.83[a]	130.36[a]	131.17	130.13[a]	129.84	130.09[a]	130.47[a]
10	110.11	110.18	110.04	110.07	110.11	110.06	109.95	109.54
11	149.75	149.98[b]	148.91[b]	149.70	149.44[b]	149.82	148.85[b]	148.85[b]
12	149.75	149.75[b]	149.57[b]	149.18	150.00[b]	149.46	149.39[b]	149.14[b]
13	111.14	110.97	111.48	111.19	111.30	111.28	111.21	110.79
14	119.55	119.37	118.45	119.47	119.65	119.56	119.53	118.58
2′	81.22	78.24	79.57	78.70	81.76	81.85	81.76	78.43
3′	67.45	65.44	66.31	66.59	68.35	68.39	68.01	66.23
4′	27.51	28.78	27.71	28.14	27.92	29.09	27.92	28.28
4′a	101.81	101.74	101.22	101.25	102.82	107.88	107.83	106.36
5′	157.46	157.66	157.94	157.73	154.26	158.43	158.68	158.70
6′	91.96	92.13	93.15	92.25	106.21	112.50	112.61	112.86
7′	151.23	151.67	151.12	151.53	151.46	151.60	151.59	151.62
8′	106.00	106.66	105.55	106.50	96.42	99.94	99.98	100.28
8′a	151.23	151.13	149.88	151.01	150.43	154.44	154.21	154.12
9′	130.60	130.33[a]	129.30[a]	130.74	130.24[a]	130.38	130.40[a]	130.51[a]
10′	109.91	109.98	109.36	109.39	110.04	110.06	109.66	109.99
11′	148.97	149.74[b]	149.57[b]	148.73	149.37[b]	148.87	149.39[b]	148.92[b]
12′	148.98	149.98[b]	149.88[b]	148.73	149.01[b]	149.61	149.79[b]	149.77[b]
13′	110.81	110.83	110.88	110.87	110.94	110.83	110.81	111.23
14′	119.17	119.20	119.47	118.38	119.89	120.12	119.78	119.55
0Me	56.05	56.06	56.10	56.13	55.57	60.86	60.84	60.89
	55.99(×2)	55.99(×3)	56.06	56.04	56.01	56.06	55.97	56.08
	55.72	55.41	55.99	55.99	56.08(×3)	55.99(×3)	55.92(×3)	55.97(×3)
	55.50	55.54	55.95	55.95	56.33	55.68	55.83	55.81
	55.42	55.29	55.65	55.61		55.45	55.51	55.43
	55.20		55.34	55.47				
				55.36				

Complete assignments for **7**, **10** and **12** were based on HETCOR and COLOC experiments. The corresponding quarternary carbons of other compounds were inferred by direct comparison. Symbol [a,b] in same column may be interchanged.

(Karchesy & Hemingway, 1986). However these polyphenols might also play an important role in preventing oxidative damage to seeds and to seed oil.

3. Experimental

Melting points were determined with a Yanaco micro-melting point apparatus and are uncorrected. Optical rotations were measured with a Jasco DIP-140 polarimeter. UV and IR spectra were determined with a Shimazu UV 240 spectrophotometer and a Jasco FT/IR-7300 spectrometer respectively. ^1H- and ^{13}C NMR spectra were recorded with a JEOL EX270 NMR spectrometer (270 MHz for ^1H NMR, 67.5 Hz for ^{13}C NMR). Chemical shifts were given in δ (ppm), based on the TMS standard. FAB–MS were performed with a JEOL JMX-HX110 mass spectrometer. CD spectra were recorded on a Jasco J-720 spectropolarimeter. Preparative HPLC was performed with a Shimazu SCL-10A system on an Intersil packed prep-ODS column (10 × 250 mm, GL Science), with a mixture of methanol and 5% THF in H_2O (20:80, v/v) as mobile phase and detection at 280 nm. A Chiralcel OD column from Diacel Chemical Co. was used for the identification of enantiomers with hexane and 2-propanol (9:1, v/v) as eluting solvents. TLC was performed on precoated aluminum sheets (Rp-18 F_{254}, 0.2 mm, Merck) with 0.1 M sodium acetate buffer (pH 4.1) and acetonitrile (7:3, v/v) with spots detected by spraying with anisaldehyde and H_2SO_4 reagents. PLC on Kieselgel 60 F_{254} (0.5 mm, Merck) was performed

for preparative purposes. Column chromatography was performed on Diaion HP-20 (Nippon Rensui), Sephadex LH-20 (Phamacia Biotech), Toyopearl HW-40 (fine grade, Tosoh), Wakogel LP60 C18 and Wakogel C-300 (Wako Chemical). Sodium cyanoborohydride was obtained from Aldrich. Hyaluronidase was obtained from bovine testes (790 units/mg solid) whereas the hyaluronic acid potassium salt came from human umbilical cord; *p*-dimethylaminobenzene-aldehyde was purchased from Sigma.

Methylation of each compound was performed with an excess of CH_2N_2 in MeOH-Et_2O for 1–4 d. After evaporation of solvent, the methylated derivatives were purified first by column chromatography on Wakogel C-300, eluted with benzene–acetone (85:15, v/v), then by crystallization from a mixture of ethyl acetate and hexane.

3.1. Extraction and isolation of phenolic compounds

Peanut skins (from 279 kg of peanuts) were extracted twice with boiling water (1000 l), each time for 2 h. The combined extracts were adsorbed on HP-20 (400 l), and components were eluted with water and 70% (v/v) aqueous acetone. The eluate with 70% acetone was concentrated to dryness under reduced pressure and the residue was then extracted with 95% (v/v) ethanol (20 l). After evaporation of the solvent under reduced pressure, the extract (190 g) was further fractionated by chromatography over Toyopearl HW 40 (bed volume, 1000 ml). Subsequent elution with mixtures of aqueous ethanol and acetone yielded 12 fractions. Fractions 8 and 10 were obtained with ethanol–acetone–water (60:15:25, v/v/v) and 50% (v/v) acetone respectively, contained mostly phenolic components as detected by TLC and showed potent hyaluronidase inhibitory activities. Fraction 8 (26 g) was subjected to further chromatography on Sephadex LH 20 (5×60 cm), with elution performed with aqueous acetone. The fractions eluted with 30% acetone contained polyphenols with flavanol moieties, as suggested by the characteristic orange colorations with anisaldehyde–H_2SO_4 reagents. These were further purified by repeated chromatography on Wakogel LP60 C18 (3.5 $\times$ 20 cm) with elution performed with 20% (v/v) methanol in water. Final purification was achieved by preparative HPLC on prep-ODS column with a mixture of MeOH–THF–water (20:5:75) as mobile phase. Compound **1** (960 mg) and **2** (16 mg) were finally crystallized as colorless needles from water and compound **3** (300 mg) was obtained as an amorphous powder. The same procedure was used to purify fraction 10 (18 g) from the Toyopearl HW 40 column to yield compound **4** (340 mg) as colorless needles and compounds **5** (53 mg), **6** (77 mg) as white powders.

3.2. Proanthocyanidin A-1 (*1*)

Colorless needles (H_2O); mp 280°C (decomp); $[\alpha]_D^{22}$ +63.87° (*c* 1.12, acetone); FAB–MS: *m/z* 577 $[M+1]^+$; Anal Calcd for $C_{30}H_{24}O_{12}\cdot 2H_2O$: C 58.82, H 4.57, Found: C 58.35, H 4.43. CD $[\theta]_{287}$ −1800, $[\theta]_{271}$ −22000, $[\theta]_{236}$ +47000sh, $[\theta]_{221}$ +73000, $[\theta]_{208}$ −11300; ^{1}HH NMR (270 MHz; CD_3OD) δ: Table 1; ^{13}C NMR (67.5 MHz; CD_3OD) δ: Table 2.

3.3. Heptamethyl ether of proanthocyanidin A-1 (*7*)

Methylation of **1** (60 mg) under the conditions described above for 3 d gave, after purification by column chromatography and crystallization from hexane–ethyl acetate (3:7, v/v), **7** (32 mg) as a crystalline powder. mp 149–150.6°C; FAB–MS: *m/z* 675 $[M+1]^+$; Anal Calcd for $C_{37}H_{38}O_{12}$: C 66.06, H 5.39; Found: C 66.17, H 5.29; ^{1}H NMR (270 MHz; $CDCl_3$) δ: Table 3; ^{13}C NMR (67.5 MHz, $CDCl_3$) δ: Table 4.

3.4. Proanthocyanidin A-2 (*2*)

Colorless needles (H_2O); mp 273°C (decomp); $[\alpha]_D$ +55.63° (*c* 1.08, acetone); FAB–MS: *m/z* 577 $[M+1]^+$; CD $[\theta]_{287}$ −2500, $[\theta]_{271}$ −17800, $[\theta]_{237}$ +46000sh, $[\theta]_{223}$ +67000, $[\theta]_{207}$ −106000; ^{1}H NMR (270 MHz, CD_3OD) δ: Table 1; ^{13}C NMR (67.5 MHz, CD_3OD) δ: Table 2.

3.5. Heptamethyl ether of proanthocyanidin A-2 (*8*)

Methylation of **2** (13 mg) as described above for 3 d, purification by column chromatography and crystallization from ethyl acetate yielded **8** (8 mg) as needles. mp 191–192.6°C; FAB–MS: *m/z* 675 $[M+1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: Table 3; ^{13}C NMR (67.5 MHz; $CDCl_3$) δ: Table 4.

3.6. Epicatechin-(2β→O→7, 4β→8)-ent-epicatechin (*3*)

White crystalline powder (H_2O); mp 260°C (decomp); FAB–MS: *m/z* 577 $[M+1]^+$; Anal Calcd for $C_{30}H_{24}O_{12}\cdot 2H_2O$: C 58.82, H 4.57; Found: C 58.73, H 4.63; CD $[\theta]_{287}$ +14200, $[\theta]_{271}$ −12500, $[\theta]_{237}$ +43000sh, $[\theta]_{221}$ +238000, $[\theta]_{207}$ −202000; ^{1}H NMR (270 MHz; CD_3OD) δ: Table 1; ^{13}C NMR (67.5 MHz; CD_3OD) δ: Table 2.

3.7. Hexamethyl ether of epicatechin-(2β→O→7, 4β→8)-ent-epicatechin (*9*)

Methylation of **3** (60 mg) as described for 24 h, purification by column chromatography, and crystallization from ethyl acetate–hexane (9:1, v/v)) yielded **9** (40

mg) as colorless needles. mp 167–168°C; FAB–MS: *m/z* 661 $[M+1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: Table 3; ^{13}C NMR (67.5 MHz; $CDCl_3$) δ: Table 4.

3.8. Heptamethyl ether of epicatechin-(2β→O→7, 4β→8)-ent-epicatechin (**10**)

Methylation of **3** (120 mg) as described above for 4 d yielded **10** (86 mg) as colorless needles after crystallization from ethyl acetate. mp 156–157°C; FAB–MS: *m/z* 675 $[M+1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: Table 3; ^{13}C NMR (67.5 MHz; $CDCl_3$) δ: Table 4.

3.9. Reductive cleavage of **10**

Sodium cyanoborohydride (40 mg) was added in portions over 30 min to a solution of **10** (46 mg) in TFA under He. After stirring for 1 h, the reaction was quenched by addition of H_2O (20 ml) and the pH was adjusted to ca. 7 with 3% (w/v) $NaHCO_3$. The mixture was then extracted three times with ethyl acetate (50, 30 and 30 ml), and the combined extract was stirred for 15 min with 3 drops of a solution of tetrabutylammonium fluoride in THF. After drying of the solution over Na_2SO_4 and evaporation of the solvent, the mixture (32 mg) was separated by PLC with benzene–acetone (8:2, v/v) to give four bands: 1 (R_f = 0.8, 2.1 mg). 2 (R_f = 0.6, 1.6 mg), 3 (R_f = 0.43, 6.2 mg), and 4 (R_f = 0.32, 3.2 mg). Band 4 was identified as the starting material **10**.

Band 1 was identified as 3,5-dimethoxyphenol (**15**) by its molecular weight of 154 (M^+) and ^{1}H NMR spectrum (270 MHz; $CDCl_3$) δ: 6.02 (2H, d, J = 8.3 Hz), 6.08 (1H, t, J = 8.3 Hz).

Band 2, which crystallized as needles from ethyl acetate, was shown to be 3′,4′,5,7-tetra-*O*-methyl-*ent*-catechin (**18**). mp 123.5–125°C; FAB–MS: *m/z* 347 $[M+1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: 7.00 (1H, dd, J = 8.2, 2.3 Hz, H-14), 6.97 (1H, d, J = 2.3 Hz, H-10), 6.90 (1H, d, J = 8.2 Hz, H-13), 6.14 (1H, d, J = 2.1 Hz, H-8), 6.11 (1H, d, J = 2.1 Hz, H-6), 4.66 (1H, d, J = 8.4 Hz, H-2), 4.07 (1H, m, H-3), 3.06 (1H, dd, J = 16.2, 5.4 Hz, H_α-4), 2.57 (1H, dd, J = 16.2, 8.9 Hz, H_β-4), 3.75 (3H, s, OMe), 3.80 (3H, s, OMe), 3.89 (6H, s, 2× OMe); R_t = 14.3 min in chiral HPLC on Chiralcel OD column (5×250 mm) with hexane-2-propanol (9:1, v/v) as mobile phase, a flow rate of 1 ml/min, and detection at 280 nm. It behaved in a manner identical to that of the authentic specimen. The R_t of its enantiomer was 32.2 min.

Further separation of band 3 on Rp-18 F_{254} TLC with methanol–THF–H_2O (7:1:3, v/v/v) yielded three bands: 3-1 (R_f = 0.52, 1.8 mg), 3-2 (R_f = 0.44, 1.1 mg) and 3-3 (R_f = 0.33, 2.1 mg). Band 3-1, a white amorphous powder, was identified as 3′,4′,5-tri-*O*-methyl-*ent*-epicatechin **20**. Its formula composition $C_{18}H_{20}O_6$ was confirmed by FAB–MS: 333 $[M+1]^+$, ^{1}H NMR (270 MHz; $CDCl_3$) δ: 7.06 (1H, d, J = 2.4 Hz, H-10), 7.05 (1H, dd, J = 8.0, 2.4 Hz, H-14), 6.92 (1H, d, J = 8.0 Hz, H-13), 6.12 (1H, d, J = 2.4 Hz, H-8), 6.07 (1H, d, J = 2.4 Hz, H-6), 4.96 (1H, s, H-2), 4.28 (1H, m, H-3), 3.91 (3H, s, OMe), 3.89 (3H, s, OMe), 3.79 (3H, s, OMe), 2.90 (2H, m, H_2-4). Methylation of **20** with CH_2N_2 in MeOH–Et_2O at −10°C for 24 h, after evaporation of the solvent, yielded **21**. FAB–MS: 347 $[M+1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: 7.07 (1H, d, J = 2.4 Hz, H-10), 7.05 (1H, dd, J = 8.0, 2.4 Hz, H-14), 6.92 (1H, d, J = 8.0 Hz, H-13), 6.20 (1H, d, J = 2.4 Hz, H-8), 6.12 (1H, d, J = 2.4 Hz, H-6), 4.97 (1H, s, H-2), 4.26 (1H, m, H-3), 3.93 (3H, s, OMe), 3.90 (3H, s, OMe), 3.80 (3H, s, OMe), 3.78 (3H, s, OMe), 2.92 (m, H_2-4). Chiral HPLC under the conditions described above for **18** differentiated **21** (R_t = 49.1 min) from its enantiomer (R_t = 58.2 min).

Band 3-3, an amorphous powder, was characterized to be 3′,4′,5,6-tetra-*O*-methyl-*ent*-catechin-[4β → 8]-3′,4′,5-tri-*O*-methyl-*ent*-epicatechin (**25**). FAB–MS: *m/z* 677 $[M+1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: 7.16 (1H, d, J = 1.8 Hz, H-10), 7.14 (1H, dd, J = 8.1, 1.8 Hz, H-14), 6.92 (1H, d, J = 8.1 Hz, H-13), 6.76 (1H, d, J = 7.8 Hz, H-13′), 6.73 (1H, d, J = 1.8 Hz, H-10′), 6.59 (1H, dd, J = 7.8, 1.8 Hz, H-14′), 6.29 (1H, s, H-6′), 5.83 (d, J = 2.6 Hz, H-8), 5.68 (d, J = 2.6 Hz, H-6), 5.13 (1H, s, H-2′), 5.12 (d, J = 4.6 Hz, H-2), 4.44 (s, H-4), 4.27 (m, H-3), 4.09 (1H, m. H-3′), 3.95 (3H, s, OMe), 3.90 (3H, s, OMe), 3.88 (3H, s, OMe), 3.86 (3H, s, OMe), 3.80(3H, s, OMe), 3.68 (3H, s, OMe), 3.49 (3H, s, OMe), 2.90 (2H, m, H_2-4′). Methylation of **25** with CH_2N_2 in MeOH–Et_2O at −10°C for 24 h yielded, after evaporation of the solvent, **26**. A white amorphous powder; FAB–MS: *m/z*: 691 $[M+1]^+$; CD $[\theta]_{286}$ +1200, $[\theta]_{273}$ −8500, $[\theta]_{213}$ +112000, $[\theta]_{200}$ −32000; ^{1}H NMR (270 MHz; $CDCl_3$) δ: 7.31 (1H, d, J = 1.8 Hz, H-10), 7.12 (1H, dd, J = 7.6, 1.8 Hz, H-14), 6.88 (1H, d, J = 7.6 Hz, H-13), 6.76 (1H, d, J = 1.8 Hz, H-10′), 6.75 (1H, d, J = 7.8 Hz, H-13′), 6.60 (1H, dd, J = 7.8, 1.8 Hz, H-14′), 6.30 (1H, s, H-6′), 5.91 (1H, d, J = 2.1 Hz, H-8), 5.68 (1H, d, J = 2.1 Hz, H-6), 5.14 (1H, d, J = 4.6 Hz, H-2), 4.93(1H, s, H-2′), 4.26 (1H, s, H-4), 4.23 (1H, m, H-3), 4.10 (1H, m. H-3′), 3.92 (3H, s, OMe), 3.90 (9H, s, OMe), 3.80(3H, s, OMe), 3.64 (3H, s, OMe), 3.52 (3H, s, OMe), 3.39 (3H, s, OMe), 2.88 (2H, m, H_2-4′).

Band 3-2 was characterized to be 9-methoxy-2,6-bis(3,4-dimethoxyphenyl)-2,3-*cis*-6,7-*cis*-3,4,7,8-tetrahydro-2H,6H-pyrano[2,3-f]chromene (**27**). A white powder; FAB–MS: *m/z* 525 $[M+1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: 6.9–7.3 (6H) for the protons of ring B and E, 6.24 (1H, s, H-8), 4.99 (2H, s, H-2 and H-6), 4.38 (1H, m, H-3), 4.35 (1H, m, H-7), 2.92–3.11 (4H, m, H_2-4 and H_2-8 at rings G and F), 3.80 (3H, s, OMe),

3.91 (3H, s, OMe), 3.92 (3H, s, OMe), 3.95 (3H, s, OMe), 3.96 (9H, s, OMe).

3.10. Epicatechin-(2β→O→7, 4β→6)-catechin (4)

Colorless needles (H_2O); mp 271–273°C (decomp); FAB–MS: *m/z* 577 $[M + 1]^+$; $[\alpha]_D$ +10.12° (*c* 1.05, acetone); Anal Calcd for $C_{30}H_{24}O_{12}\cdot H_2O$: C 60.06, H 4.27; Found: C 59.15, H 4.17; CD $[\theta]_{284}$ −3300, $[\theta]_{273}$ −11000, $[\theta]_{47}$ +48000, $[\theta]_{220}$ +29000, $[\theta]_{208}$ −150000. ^{1}H NMR (270 MHz; CD_3OD) δ: Table 1; ^{13}C NMR (67.5 MHz; CD_3OD) δ: Table 2.

3.11. Hexamethyl ether of epicatechin-(2β→O→7, 4β→6)-catechin (11)

Methylation of **4** (96 mg) as described for 12 h afforded **11** (82 mg). Colorless needles, mp 196–198°C (ethyl acetate); $C_{36}H_{36}O_{12}$; FAB–MS: *m/z* 661 $[M + 1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: Table 3; ^{13}C NMR (67.5 MHz; $CDCl_3$) δ: Table 4.

3.12. Heptamethyl ether of epicatechin-(2β→O→7, 4β→6)-catechin (12)

Continued methylation of **11** (60 mg) with CH_2N_2 in methanol for additional 48 h yielded, after purification by column chromatography, **12** (56 mg). A crystalline powder, mp 152–153°C (ethyl acetate); $C_{37}H_{38}O_{12}$; FAB–MS: *m/z* 675 $[M + 1]^+$; Anal Calcd for $C_{37}H_{38}O_{12}$: C 65.87, H 5.64; Found: C 65.72, H 5.68; ^{1}H NMR (270 MHz; $CDCl_3$) δ: Table 3; ^{13}C NMR (67.5 MHz; $CDCl_3$) δ: Table 4.

3.13. Reductive cleavage of 12

The same procedure as used for the reductive cleavage of **10** was employed for the reductive conversion of **12** (32 mg). The reaction mixture was then separated by PLC with benzene–acetone (8:2, v/v) to give 5 bands: Band 1 (R_f = 0.8, 1.8 mg), band 2 (R_f = 0.6, 1.6 mg), band 3 (R_f = 0.36, 1.4 mg), band 4 (R_f = 0.27, 3.6 mg) and band 5 (R_f = 0.15, 3.2 mg) that was identified as the starting material **12**. Band 4 was further separated on Rp-18 PLC with MeOH–H_2O (7:3, v/v) to give two bands: 4-1 (R_f = 0.56, 2.0 mg) and 4-2 (R_f = 0.47, 1.2 mg). Band 1 was characterized as **15** and band 2 as **18** by ^{1}H NMR and chiral HPLC analysis, respectively, as described above. Band 3 was identified as 3′4′5-tri-*O*-methyl-catechin (**23**). FAB–MS: *m/z* 333 $[M + 1]^+$; ^{1}H NMR (270 MHz; CD_3OD) δ: 67–7.0 (3H, protons at ring B), 6.02 (1H, d, *J* = 2.4 Hz, H-7), 5.94 (1H, d, *J* = 2.4 Hz, H-6), 4.63 (1H, d, *J* = 8.6 Hz, H-2), 4.00 (1H, m, H-3), 3.90 (3H, s, OMe), 3.87 (3H, s, OMe), 3.79 (3H, s, OMe), 2.83 (1H, dd, *J* = 17, 5.6 Hz, $H_β$-2), 2.48 (1H, dd, *J* = 17, 6.0 Hz, $H_α$-2). Its methylation gave **24**, a white powder, MS: *m/z* 347 $[M + 1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: same as **18**. R_t was 32.2 min on chiral HPLC under the same conditions as those used for separation of **18**.

Band 4-1 was identified as 3′,4′,5,7-tetra-*O*-methyl-epicatechin-[2β → 1,4β → 2]-3-*O*-methyl-4-[2-hydroxy-3-(3,4-dihydroxyphenyl)propyl]-phloroglucinol (**28**), a white powder, $C_{37}H_{40}O_{12}$. FAB–MS: *m/z* 677 $[M + 1]^+$. ^{1}H NMR (270 MHz; $CDCl_3$) δ: 7.35 (1H, dd, *J* = 8.4, 1.8 Hz, H-14), 7.26 (1H, d, *J* = 1.8 Hz, H-10), 6.94 (1H, d, *J* = 8.2 Hz, H-13), 6.74–6.83 (3H, ring E), 6.42 (1H, s, H-8′), 6.37 (1H, d, *J* = 2.0 Hz, H-8), 6.21 (1H, d, *J* = 2.0 Hz, H-6), 4.82 (1H, d, *J* = 3.5 Hz, H-4), 4.26 (1H, m, H-3), 4.18 (1H, m, H-3′), 3.03 (1H, dd, *J* = 16.5, 4.6 Hz, $H_β$-4′), 2.94 (2H, m, H_2-2′), 2.92 (1H, dd, *J* = 16.5, 7.8 Hz, $H_α$-4′), 3.78–3.93 (21H, 7× OMe). Band 4-2 was identified as 5-methoxy-2,8-bis-{3,4-dimethoxyphenyl)-2,3-*cis*-7,8-trans-3,4,6,7-tetrahydro-2H,8H-pyrano[2,3-f]-chromene (**29**), a white powder, FAB–MS: *m/z* 525 $[M + 1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: 6.65–7.05 (6H, ring B and E), 6.08 (1H, s, H-9), 4.65 (1H, s, H-2), 4.60 (1H, d, *J* = 8.4 Hz, H-8), 3.65–3.9 (15H, 5× OMe), 2.6–3.2 (4H, H_2-4 and H_2-6).

3.14. Epicatechin-(2β→O→7, 4β→6)-ent-catechin (5)

White amorphous powder (H_2O), mp 262°C (decomp). FAB–MS: *m/z* 577 $[M + 1]^+$; Anal Calcd for $C_{30}H_{24}O_{12}\cdot 2H_2O$: C 58.82, H 4.57; Found: C 58.76; H 4.70; CD $[\theta]_{283}$ +9800, $[\theta]_{273}$ −8900, $[\theta]_{247}$ +62000, $[\theta]_{220}$ +58000, $[\theta]_{208}$ +90000; ^{1}H NMR (270 MHz; CD_3OD) δ: Table 1; ^{13}C NMR (67.5 MHz; CD_3OD) δ: Table 2.

3.15. Heptamethyl ether of epicatechin-(2β→O→7, 4β→6)-ent-catechin (13)

Methylation of **5** (30 mg) as described for 3 d yielded **13** (17 mg) as an amorphous powder from ethyl acetate and hexane; mp 151–153°C. FAB–MS: *m/z* 675 $[M + 1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: Table 3; ^{13}C NMR (67.5 MHz; $CDCl_3$) δ: Table 4.

3.16. Epicatechin-(4β→6, 2β→O→7)-ent-epicatechin (6)

White amorphous powder (H_2O–MeOH), mp 280°C (decomp); FAB–MS: *m/z* 577 $[M + 1]^+$; Anal Calcd for $C_{30}H_{24}O_{12}\cdot 2H_2O$: C 58.82, H 4.57; Found: C 58.35, H 4.43; CD $[\theta]_{285}$ +8400, $[\theta]_{273}$ −12000, $[\theta]_{248}$ +68000, $[\theta]_{220}$ +66000, $[\theta]_{208}$ +224000; ^{1}H NMR (270 MHz; CD_3OD) δ: Table 1; ^{13}C NMR (67.5 MHz; CD_3OD) δ: Table 2.

3.19. Heptamethyl ether of epicatechin-(2β→O→7,4β→6)-ent-epicatechin (**14**)

Methylation of **6** (36 mg) as described for 4 d yielded **14** (21 mg) as an amorphous powder from ethyl acetate and hexane; mp 143–145°C; FAB–MS: *m/z* 675 $[M + 1]^+$; ^{1}H NMR (270 MHz; $CDCl_3$) δ: Table 3; ^{13}C NMR (67.5 MHz; $CDCl_3$) δ: Table 4.

3.18. Reductive cleavage of **14**

The same procedure as that used for the cleavage of **10** was used for the cleavage of **14** (18 mg) yielded **17** (2.1 mg) and **18** (1.2 mg). Compound **17** can be converted to **18** by further methylation. Their structures were determined by direct comparison as described above.

3.19. Inhibition of the activated hyaluronidase

The test samples (100 μl) were incubated at 37°C for 20 min with hyaluronidase (2200 units/ml) (50 μl) that had been preincubated at 37°C for 20 min in a solution of sodium acetate buffer (0.3 M, pH 4.1) (200 μl) containing 0.3 M NaCl. After addition of 100 μl of hyaluronic acid (K^+ salt, 1.8 mg/ml), the mixture was incubated for an additional 20 min at 37°C. The reaction was stopped by addition of 100 μl of 0.4 M NaOH and 100 μl of 1.5 M H_3BO_3, followed by heating the mixture at 100°C for 3 min. The absorbance value was measured at 585 nm after the addition of 3 ml of Ehrlich's reagent (0.06 M in a mixture of hydrochloric acid and acetic acid) and incubation for 20 min at 37°C. Test sample solutions were replaced by the same volume of buffer as a control. The percentage inhibition of hyaluronidase activity was calculated according to the following equation.

$$\text{Inhibition (\%)} = \frac{\text{control } A_{585} - \text{sample } A_{585}}{\text{control } A_{585}} \times 100\%$$

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