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Constituents of a Fern, *Davallia mariesii* MOORE. II.[1)] Identification and ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectra of Procyanidin B-5, Epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin, and Epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin

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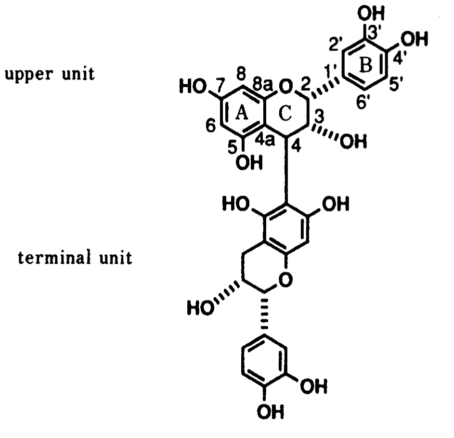
Procyanidin B-5 (1), epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin (2), and epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin (3), which showed an inhibitory effect toward protein kinase C, were isolated from the rhizomes of *Davallia mariesii* MOORE. Detailed analyses of their ^{1}H- and ^{13}C-nuclear magnetic resonance spectra were carried out by the use of two-dimensional nuclear magnetic resonance techniques.

Keywords *Davallia mariesii*; Davalliaceae; condensed tannin; procyanidin B-5; epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin; epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin; ^{1}H-NMR; ^{13}C-NMR; 2D NMR; protein kinase C inhibitor

In previous papers,[1,2)] we reported the isolation and structure elucidation of davallialactone, eriodictyol-7-*O*-β-D-glucuronide, davalliosides A and B, and (−)-epicatechin-5-*O*-β-D-glucopyranoside from the rhizomes of a fern, *Davallia mariesii* MOORE (Davalliaceae), which is used in Korea as a folk medicine "Sin Seong Cho"[3)] and in China as a traditional medicine "Gu Sui Bu".[4)] In a continued study, we have isolated procyanidin B-5 (**1**),[5)] epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin (**2**),[6)] and epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin (**3**),[7)] which showed an inhibitory activity toward protein kinase C. This paper describes the identification and proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) signal assignments of these



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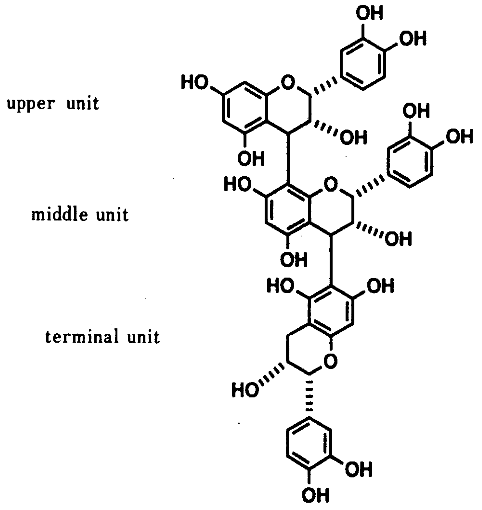

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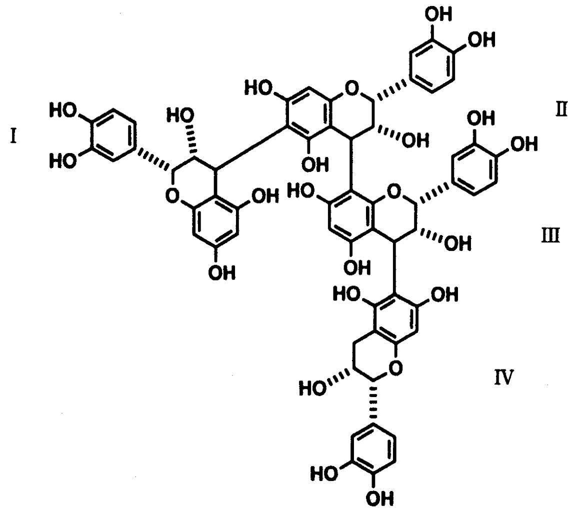

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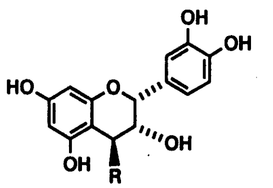

4 : R=H
5 : R=$SCH_2C_6H_5$

Chart 1

procyanidins.

The ethyl acetate-soluble fraction of the aqueous acetone extract of dried rhizomes of *D. mariesii* was subjected to repeated column chromatography over Sephadex LH-20, giving compounds **1**, **2**, and **3** along with caffeic acid, 4-*O*-*β*-D-glucopyranosylcaffeic acid, 4-*O*-*β*-D-glucopyranosyl-*p*-coumaric acid, davallialactone, and the 7-*O*-*β*-D-glucuronide of (±)-eriodictyol.[1]

Compound **1**, amorphous powder, $[\alpha]_D^{24}$ +94.7° (acetone), and compound **2**, amorphous powder, $[\alpha]_D^{32}$ +104.5° (MeOH), had the molecular formulae $C_{30}H_{26}O_{12}$ and $C_{45}H_{38}O_{18}$, respectively. They showed ultraviolet (UV) absorptions at around 220 and 280 nm, and gave a dark-blue color with ferric chloride reagent and an orange-red color with anisaldehyde-sulfuric acid reagent, which are typical of proanthocyanidins.[6] The ^{1}H- and ^{13}C-NMR spectra suggested that both **1** and **2** may be epicatechin derivatives (Table I), and eventually they were identified as procyanidin B-5 (**1**)[5] and epicatechin-(4*β*→8)-epicatechin-(4*β*→6)-epicatechin (**2**),[6] respectively, by direct comparison with authentic samples.

Compound **3** was obtained as a slightly brown-colored amorphous solid and showed $[\alpha]_D^{22}$ +168.5° (MeOH). The positive ion fast atom bombardment mass spectrum (FAB-MS) showed the quasi-molecular ion peak at *m/z* 1155 $[M+H]^+$ and the combustion data agreed with the formula $C_{60}H_{50}O_{24} \cdot 2H_2O$. It showed UV and infrared (IR) absorptions similar to those of **1** and **2**, suggesting that **3** is also a condensed tannin. Acid-catalyzed degradation of **3** with toluene-α-thiol[5,8] afforded (−)-epicatechin (**4**) and (−)-4*β*-benzylthioepicatechin (**5**)[8] in a molar ratio of 1 : 3, suggesting that **3** is a (−)-epicatechin tetramer. Finally, **3**

Table I. 400 MHz ^{1}H and 100 MHz ^{13}C NMR Data for **1**, **2**, and **4** in Methanol-d_4 (Coupling Constants in Parenthesis)

Position[a]	**1**				**2**[b]		**4**			
	δ_H	δ_C	^{1}H L. r. coupled ($^3J_{CH}$	$^2J_{CH}$)[c]	δ_H	δ_C	δ_H	δ_C	^{1}H L. r. coupled ($^3J_{CH}$	$^2J_{CH}$)[c]
2(u)	4.91 br s	78.0 d			5.07 br s	77.7 d				
3(u)	4.02 br s	73.4 d		4(u)	4.02 br s	74.3 d				
4(u)	4.56 br d (1.2)	38.4 d			4.73 br s	38.0 d				
4a(u)	—	101.4 s			—					
5(u)	—	158.7 s	4(u)	6(u)	—					
6(u)	6.06 d (2.4)	96.9 d		8(u)	6.08 br s	97.0 d				
7(u)	—	160.3 s		6(u), 8(u)	—					
8(u)	5.99 d (2.4)	97.5 d		6(u)	5.97 br s	98.4 d				
8a(u)	—	160.2[d] s	4(u)	8(u)	—					
1′(u)	—	133.1 s	5′(u)	2(u), 2′(u)	—					
2′(u)	6.89 d (2.0)	116.0 d	2(u), 6′(u)		6.97[e] br d (1.5)	116.0 d				
3′(u)	—	146.7 s	5′(u)	2′(u)	—					
4′(u)	—	146.4 s	2′(u), 6′(u)	5′(u)	—					
5′(u)	6.73 d (8.2)	116.7 d			6.76 d (8.0)	116.8 d				
6′(u)	6.68 dd (8.2, 1.5)	120.0 d	2(u), 2′(u)		6.72 dd (8.0, 1.5)	119.9 d				
2(m)					5.10 br	77.9 d				
3(m)					4.16 br	72.6 d				
4(m)					4.69 br s	38.5 d				
6(m)					6.03[f] br s					
2′(m)					7.07 br s	115.9 d				
5′(m)					6.77 d (8.0)	116.7 d				
6′(m)					6.78 br d (8.0)	119.6 d				
2(t)	4.81 br s	80.5 d			4.81 br s	80.3 d	4.81 br s	80.6 d	4	
3(t)	4.15 br s	68.2 d			4.19 br s	68.2 d	4.18 ddd (4.6, 3.0, 1.5)	68.3 d		4
4(t)	2.68 br d (16.5) 2.89 dd (16.5, 4.5)	30.4 t			2.76 br d (16.0) 2.83 br d (16.0)	30.3 t	2.73 dd (16.8, 3.0) 2.85 dd (16.8, 4.6)	30.0 t		
4a(t)	—	101.4 s	3(t), 8(t)	4(t)	—		—	100.9 s	3, 6, 8	4
5(t)	—	157.0[f] s	4(u)		—		—	158.7 s	4	6
6(t)	—	108.9 s		4(u)	—		5.93 d (2.4)	97.2 d	8	
7(t)	—	156.6[f] s	4(u)		—		—	158.4 s		6, 8
8(t)	6.07 s	97.4 d			6.07[f] br s		5.91 d (2.4)	96.7 d	6	
8a(t)	—	156.2[d] s	4(t)		—		—	158.1[g] s	4	8
1′(t)	—	133.1 s	3(t), 5′(t)	2(t), 2′(t)	—		—	133.0 s	5′	2
2′(t)	6.97[e] d (1.8)	116.1 d	2(t), 6′(t)		6.99[e] d (2.0)	116.0 d	6.97[e] d (1.8)	116.1 d	6′	2
3′(t)	—	146.7 s	5′(t)	2′(t)	—		—	146.7 s	5′	
4′(t)	—	146.5 s	2′(t), 6′(t)	5′(t)	—		—	146.5 s	2′, 6′	
5′(t)	6.76 d (8.2)	116.7 d			6.77 d (8.0)	116.7 d	6.75 d (8.2)	116.7 d		
6′(t)	6.80[e] dd (8.2, 1.8)	120.2 d	2(t), 2′(t)		6.81[e] dd (8.0, 2.0)	120.2 d	6.79[e] dd (8.2, 1.8)	120.2 d	2, 2′	

δ Values in ppm and coupling constants in hertz. *a*) (u), (m), and (t) indicate the upper, middle, and terminal epicatechin unit, respectively. *b*) Signals due to the methines and methylene of the terminal unit, except for C_8 methine, were assigned unambiguously based on the results of ^{1}H–^{1}H and ^{1}H–^{13}C COSY spectra. The other ^{1}H- and ^{13}C-signals were divided into two groups by means of ^{1}H–^{1}H and ^{1}H–^{13}C COSY, but the allocation to the upper and middle units was based on the comparison with the NMR data of **1** and **4**. *c*) $^2J_{CH}$ and $^3J_{CH}$ indicate the protons coupled with the carbon through two and three bonds, respectively, which were detected in the long-range ^{1}H–^{13}C COSY or HMBC spectra. *d*) Assignments are based on the LSPD experiments under irradiation at H-2 in each epicatechin unit. *e*) Long-range coupling was observed with H-2 of the corresponding epicatechin unit in the ^{1}H–^{1}H COSY. *f*) Assignments may be interchanged in each column. *g*) Assignment is based on the results of the LSPD and the long-range H–C J-resolved spectrum under irradiation at H-2 ($^3J_{CH}$ = 1 Hz).

was identified as epicatechin-$(4\beta\rightarrow6)$-epicatechin-$(4\beta\rightarrow8)$-epicatechin-$(4\beta\rightarrow6)$-epicatechin[7] by direct comparison with an authentic sample.

Compounds **1**, **2**, and **3** inhibited protein kinase C with IC_{50} values of 14.2, 4.6, and 1.5 μM, respectively.[9] Although microbial metabolites such as staurosporine, UCN-01, and polymixin B and antipsychotic drugs such as trifluoperazine and chlorpromazine have been reported as protein kinase C inhibitors,[10] our results provide the first example of condensed tannins as inhibitors of this enzyme.

Though **1** and **2** are well known and their ^{1}H- and ^{13}C-NMR data have already been reported,[6,11] previous studies were limited to analyses of the methine and methylene signals in the C-rings of epicatechin units. Compound **3** has recently been isolated from *Davallia divaricata* Blume,[7] but no NMR study on this compound has been done yet. Thus, we carried out detailed analyses of the ^{1}H- and ^{13}C-NMR spectra of these compounds by means of two-dimensional (2D) NMR techniques.

Analyses of the ^{1}H- and ^{13}C-NMR spectra of **1** with the aid of ^{1}H–^{1}H and ^{1}H–^{13}C shift correlation spectroscopy (COSY) led to unambiguous assignments of all ^{1}H- and ^{13}C-signals of methylene and methine groups except those due to the 6- and 8-methine groups in the upper unit. As can be seen in Fig. 1, the ^{1}H-signals due to 2-H, 3-H, and 4-H(H_2) of the C-rings and 2′-H, 5′-H, and 6′-H of the B-rings are evidently correlated with each other and the combination of rings B and C in each epicatechin unit is revealed by the appearance of significant long-range ^{1}H–^{1}H correlations between 2(t)-H (δ 4.81, C-ring) and 2′(t)- and 6′(t)-H (δ 6.97 and 6.80, respectively; B-ring) in the terminal unit. The ^{13}C-signals due to protonated carbons were assigned readily by means of ^{1}H–^{13}C COSY (Table I).

As to quaternary carbon signals, those of C-1′, C-3′, and C-4′ of the B-rings were readily assigned by the use of long-range ^{1}H–^{13}C COSY ($^{1r}J_{CH}=8$ Hz), as shown in Fig. 2. However, carbons of the A-rings in **1** failed to give significant correlation peaks in the spectrum, probably because the carbon signals were broadened by restricted rotation about the interflavonoid linkage [C-4(u)—C-6(t)].[5,12] Only three quaternary carbons appeared as sharp singlets at δ 101.4 [$2\times$C, C-4a(u) and C-4a(t)] and at δ 156.2 [C-8a(t)], both of which showed long-range correlations with 4(t)-H_2 (δ 2.68 and 2.89). Of these, the former could be assigned to C-4a(u) and C-4a(t) based on the chemical shift value.[11] On the other hand, the latter signal was allotted to C-8a(t) by application of the long-range selective proton decoupling (LSPD) method under irradiation at H-2 (δ 4.81) in the terminal epicatechin unit. Similarly, the LSPD experiment under selective irradiation of 2(u)-H (δ 4.91) enabled us to assign the ^{13}C-signal at δ 160.2 to C-8a in the upper epicatechin unit (Table I).

Then, we measured the ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) spectrum[13,14] of **1** in order to assign the remaining quaternary carbon signals in the δ 156—161 ppm region. As shown in Fig. 3, C-8a(u) (δ 160.2) shows long-range correlation with the *meta*-coupled benzene proton at δ 5.99 (d, $J=2.4$ Hz), indicating that this proton is H-8(u) and its counterpart at δ 6.06 (*d*, $J=2.4$ Hz), is H-6(u). Thus, the carbons at δ 158.7 and δ 160.3 which show long-range correlations with 4(u)- and 6(u)-H and

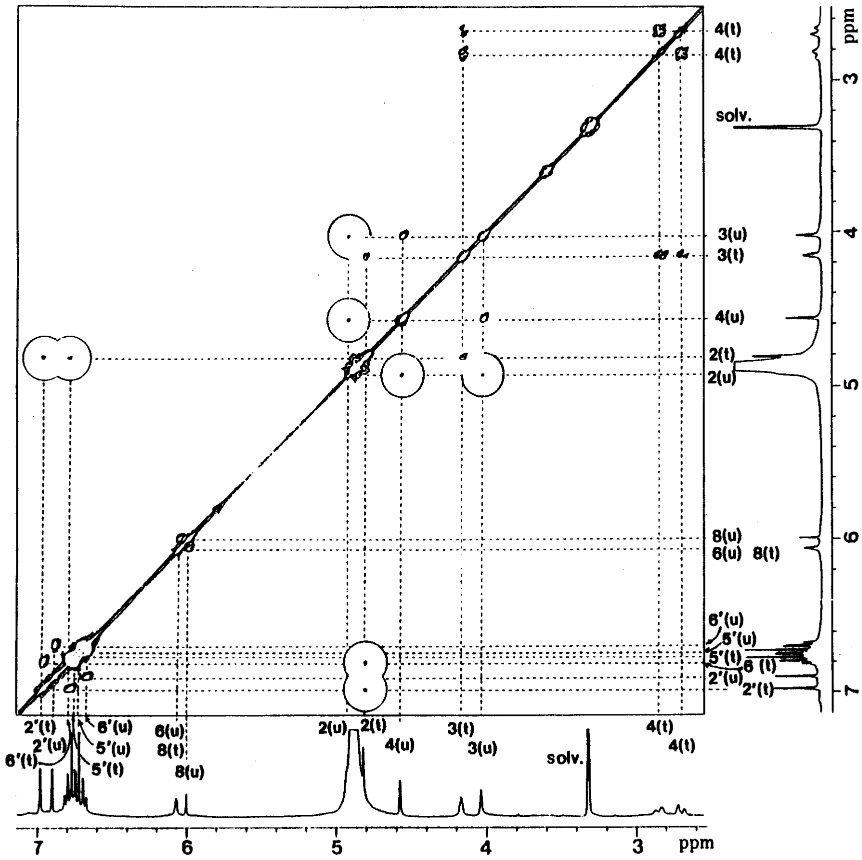

Fig. 1. ^{1}H–^{1}H COSY Spectrum of **1** in Methanol-d_4 (Sample, 48 mg; 2 h Run)

Open circles indicate significant but weak peaks at this threshold level.

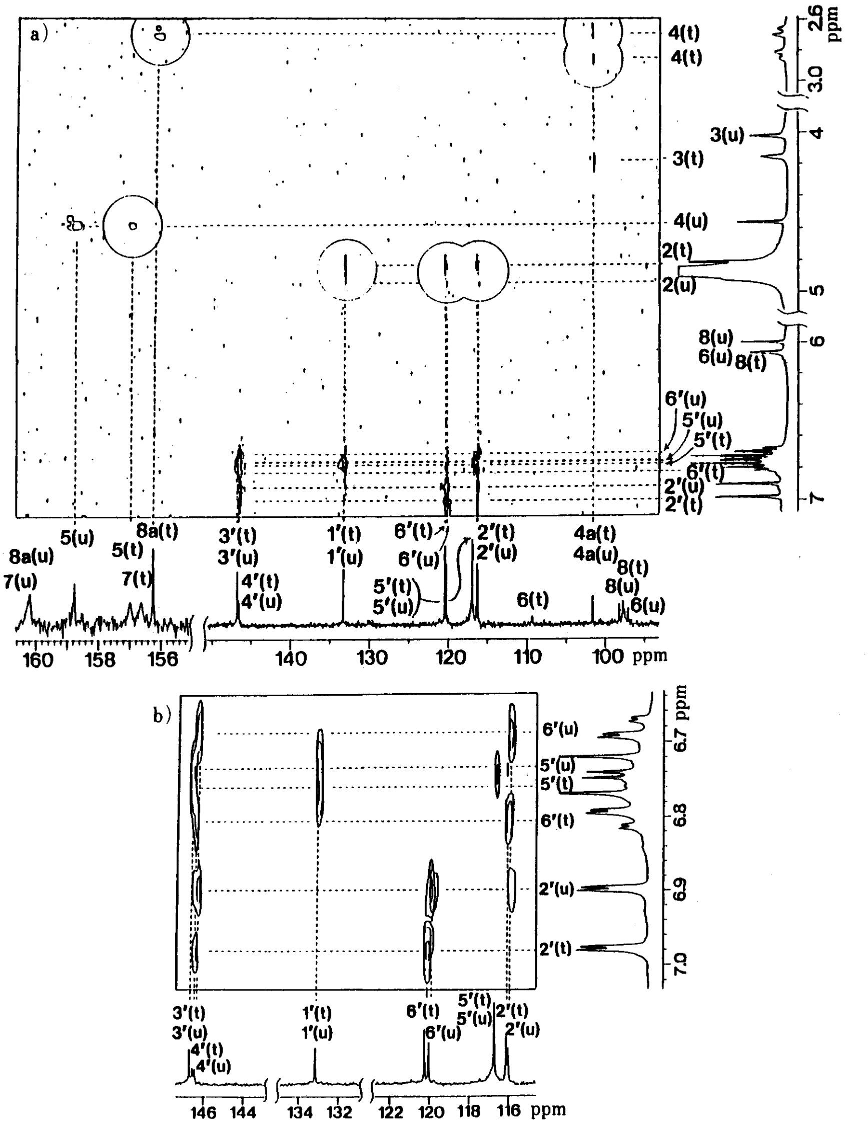

Fig. 2. Long-Range ^{1}H–^{13}C COSY Spectrum of **1** in Methanol-d_4 (Sample, 48 mg; ${}^{1r}J_{CH}=8$ Hz; 74 h Run)
a) Aromatic carbon region. b) Ring B region.

with 8(u)- and 6(u)-H, respectively, are assigned to C-5(u) and C-7(u), respectively. The other two carbons at δ 156.6 (br) and δ 157.0 (br), which are correlated only with 4(u)-H, are assigned to C-7(t) and C-5(t), respectively, by analogy with those of epicatechin (**4**) (Table I).

On the other hand, the remaining quaternary carbon at δ 108.9 [br, C-6(t)] showed a correlation peak with 4(u)-H (Table I). Although this carbon failed to give a correlation peak with 8(t)-H, it is assigned to C-6 in the terminal unit, which is the carbon connecting with the upper unit.

The ^{1}H-NMR spectrum of epicatechin-(4β→8)-epicatechin-(4β→6)-epicatechin (**2**) showed broadening of several signals due to restricted rotation about the interflavonoid linkages. Nevertheless, it could be analyzed with the aid of ^{1}H–^{1}H COSY, by which all the protons of the B/C rings in each unit were discriminated. For instance, a four-spin system of ring C and a three-spin system of ring B in the terminal unit exhibited distinct correlation peaks with each other, and the relation between these two spin systems was disclosed by the significant long-range correlation peak observed between 2-H (ring C) and 2'-H (ring B) (see Table I). However, it was not possible to determine which of the other two sets of B/C ring protons belongs to the upper

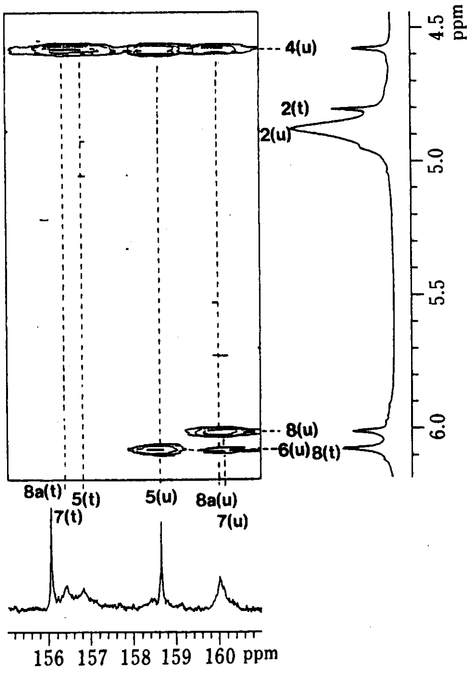

Fig. 3. Enlarged HMBC Spectrum of Low-Field Region of **1** in Methanol-d_4 (Sample, 73 mg; ${}^{1r}J_{CH}=6$ Hz; 40 h Run)

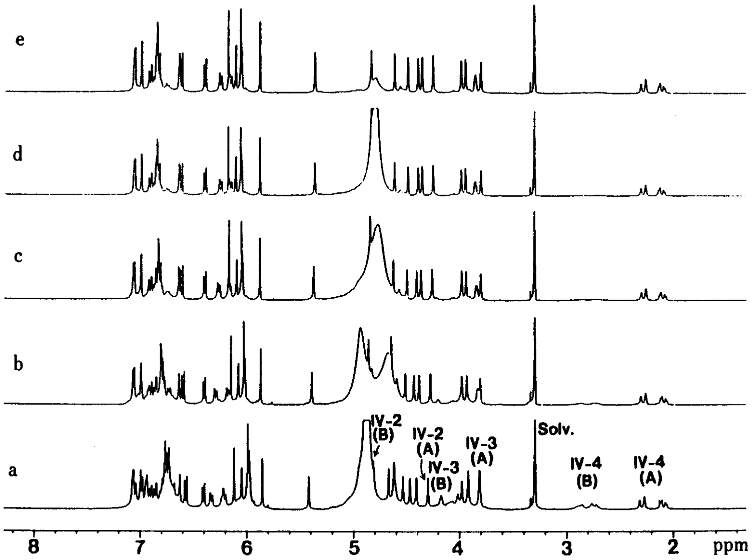

Fig. 4. ^{1}H-NMR Spectra of 3

a: Spectrum in CD_3OD. b, c, and d: Spectra in CD_3OD–D_2O (25:5), (25:10), and (25:15), respectively. e: Spectrum in CD_3OD–D_2O (25:15) under irradiation of the H_2O signal at δ4.97.

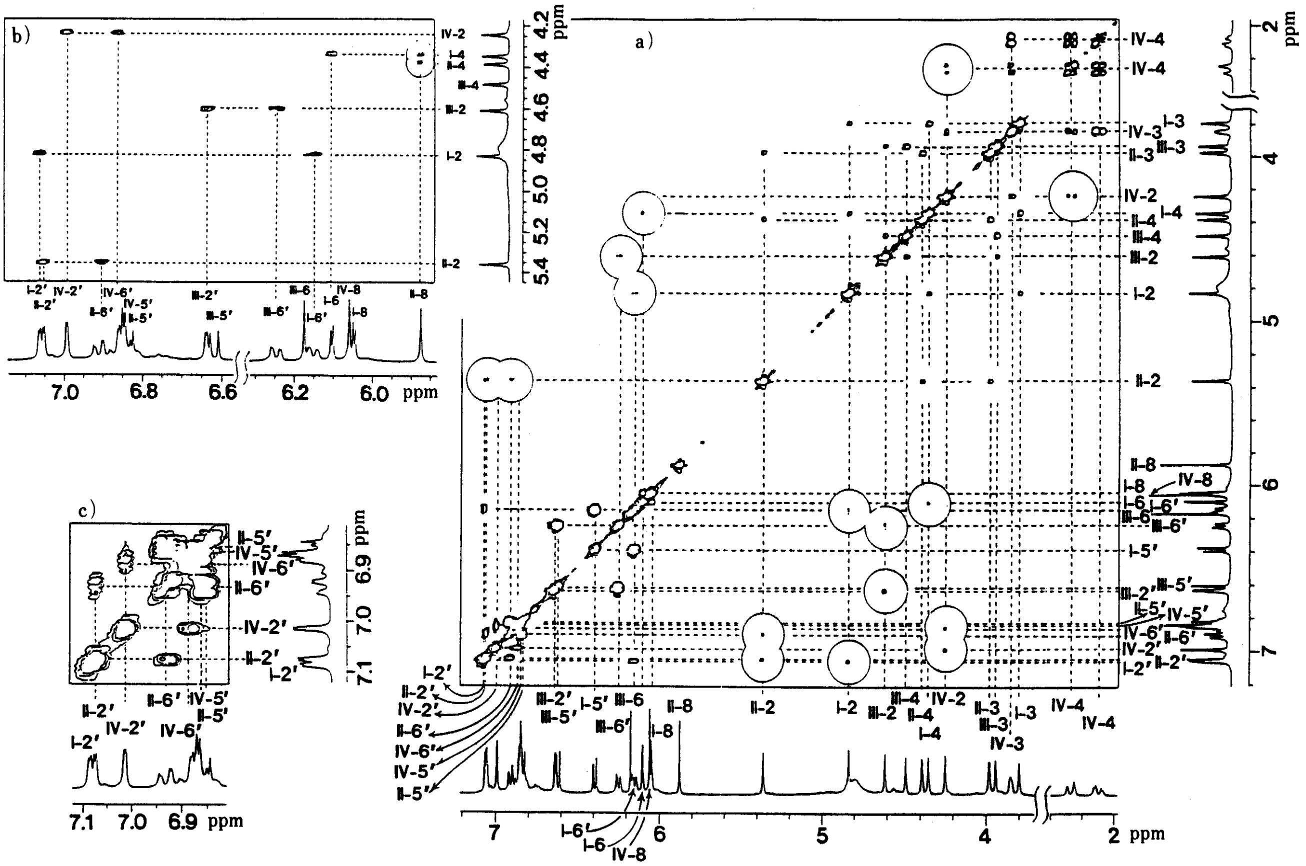

Fig. 5. ^{1}H–^{1}H COSY Spectrum of 3 in CD_3OD–D_2O (25:15) (Sample, 33 mg; 2 h Run)

a) Whole region. b) Cross peaks between ring C and rings A/B protons. c) Cross peaks of ring B protons.

and which to the middle unit in 2, and thus signal assignments of the protons in the upper and middle units were based on a comparison with the ^{1}H-NMR data of 1 and 4 (Table I). Assignments of the A-ring protons in 2 were also based on a comparison with those of 1 and 4.

On the other hand, carbon signals of methine and methylene groups in 2, except for methine carbons of the A-rings,[15] were assigned by correlation with the relevant proton(s) with the aid of ^{1}H–^{13}C COSY (Table I). However, signal assignments of the quaternary carbons were unsuccessful, because the signals were broadened by the rotational isomerism.[5,12]

The ^{1}H- and ^{13}C-NMR spectra of 3 were also complicated due to the rotational isomerism.[7] As can be seen in Fig. 4, the ^{1}H-NMR spectrum in methanol-d_4 reveals two sets of AB-type signals due to methylene protons (4-H_2 of unit IV)

TABLE II. 400 MHz ^{1}H- and 100 MHz ^{13}C-NMR Data for **3**

Units	No.	In CD_3OD–D_2O (25 : 15)				In $CD_3OD^{a)}$			
		δ_H (J in Hz)	δ_C	^{1}H L. r. coupled ($^3J_{CH}$ $^2J_{CH}$)$^{b)}$		Major conformer A		Minor conformer B	
						δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
I	2	4.83 br s	77.78 d			4.88 br s			
	3	3.79 br s	73.2 d			3.82 br s			
	4	4.34 br s	38.3 d			4.41 br s			
	4a	—	100.0 s	I3, I6, I8	I4	—			
	5	—	158.4 s	I4	I6	—			
	6	6.10 d (2.4)	97.3 d	I8		6.04 d (2.3)		6.12	
	7	—	159.5 s		I6, I8	—			
	8	6.04 d (2.4)	97.7 d	I6		5.97 d (2.3)		5.99 d (2.3)	
	8a	—	159.7$^{c)}$ s	I4	I8	—			
	1′	—	133.3 s	I5′	I2	—			
	2′	7.06$^{d)}$ d (1.5)	116.5 d	I2, I6′		7.06$^{d)}$ d (1.5)			
	3′	—	145.9 s	I5′	I2′	—			
	4′	—	145.8 s	I2′, I6′	I5′	—			
	5′	6.39 d (8.2)	116.3 d		I6′	6.40 d (8.2)			
	6′	6.14$^{d)}$ dd (8.2, 1.5)	121.0 d	I2, I2′		6.21 dd (8.2, 1.5)			
II	2	5.36 br s	77.5 d			5.41 br s			
	3	3.98 br s	74.2 d			3.98 br s			
	4	4.38 br s	38.7 d			4.47 br s			
	4a	—	104.9 s	II3, II8	II4	—			
	5	—	156.5 s	II4, I4		—			
	6	—	106.9 s	II8, I3	I4	—			
	7	—	155.1 s	I4	II8	—			
	8	5.87 s	97.2 d			5.85 s			
	8a	—	155.7 s	II4	II8	—			
	1′	—	133.6 s	II5′	II2	—			
	2′	7.05$^{d)}$ d (1.8)	116.6 d	II2, II6′		7.05$^{d)}$ d (1.8)			
	3′	—	145.9 s	II5′	II2′	—			
	4′	—	145.98 s	II2′, II6′	II5′	—			
	5′	6.82 d (8.3)	117.1 d			6.75 d (8.2)			
	6′	6.90$^{d)}$ dd (8.3, 1.8)	121.0 d	II2, II2′		6.90 dd (8.2, 1.8)			
III	2	4.61 br s	77.84 d			4.67 br s			
	3	3.93 d (1.8)	72.5 d			3.92 br s			
	4	4.48 br s	38.6 d			4.54 br s			
	4a	—	100.0 s	III3, III6	III4	—			
	5	—	157.4 s	III4	III6	—			
	6	6.17 s	97.8 d			6.12 s			
	7	—	157.6 s	II4	III6	—			
	8	—	110.2 s	III6, II3	II4	—			
	8a	—	156.7 s	III4, II4		—			
	1′	—	132.3 s	III5′	III2	—			
	2′	6.63$^{d)}$ d (1.8)	116.0 d	III2, III6′		6.63$^{d)}$ d (1.2)			
	3′	—	145.44 s	III5′	III2′	—			
	4′	—	145.41 s	III2′, III6′	III5′	—			
	5′	6.61 d (8.3)	116.6 d			6.57 d (8.2)			
	6′	6.24$^{d)}$ dd (8.3, 1.8)	121.0 d	III2, III2′		6.34$^{d)}$ dd (8.2, 1.2)			
IV	2	4.24 br s	80.1 d			4.30 br s	80.3 d	4.81 br s	80.5 d
	3	3.84 br s	68.3 d			3.82 br s	68.8 d	4.18 br	68.3 d
	4	2.09 dd (16.9, 4.6) 2.26 br d (16.9)	29.9 t			2.10 dd (16.5, 4) 2.28 br d (16.5)	30.2 t	2.74 br d (16.5) 2.88 br d (16.5)	30.4 t
	4a	—	102.6 s	IV3, IV8	IV4	—		—	
	5	—	156.5 s	IV4, III4		—		—	
	6	—	108.4 s	IV8, III3	III4	—		—	
	7	—	155.3 s	III4	IV8	—		—	
	8	6.05 s	96.9 d			5.99 s			
	8a	—	155.6 s	IV4	IV8	—		—	
	1′	—	133.1 s	IV5′	IV2	—		—	
	2′	6.99$^{d)}$ d (1.5)	116.3 d	IV2, IV6′		7.00$^{d)}$ d (1.5)		6.98$^{d)}$ d (1.5)	
	3′	—	146.04 s	IV5′	IV2′	—		—	
	4′	—	146.04 s	IV2′, IV6′	IV5′	—		—	
	5′	6.83 d (8.2)	117.1 d			6.76 d (8)		6.76 d (8)	
	6′	6.86$^{d)}$ dd (8.2, 1.5)	120.8 d	IV2, IV2′		6.86$^{d)}$ dd (8, 1.5)		6.81$^{d)}$ dd (8, 1.5)	

a) Assignments are based on the result of ^{1}H–^{1}H COSY and on the comparison with the data in CD_3OD–D_2O (25 : 15). *b*) $^2J_{CH}$ and $^3J_{CH}$ indicate the protons coupled with the carbon through two and three bonds, respectively, which were observed in the HMBC spectrum. *c*) Assignment is based on the long-range C–H J-resolved spectrum obtained under irradiation at 2-H in the unit I. *d*) Long-range ^{1}H–^{1}H coupling was observed with 2-H of each unit in the ^{1}H–^{1}H COSY.

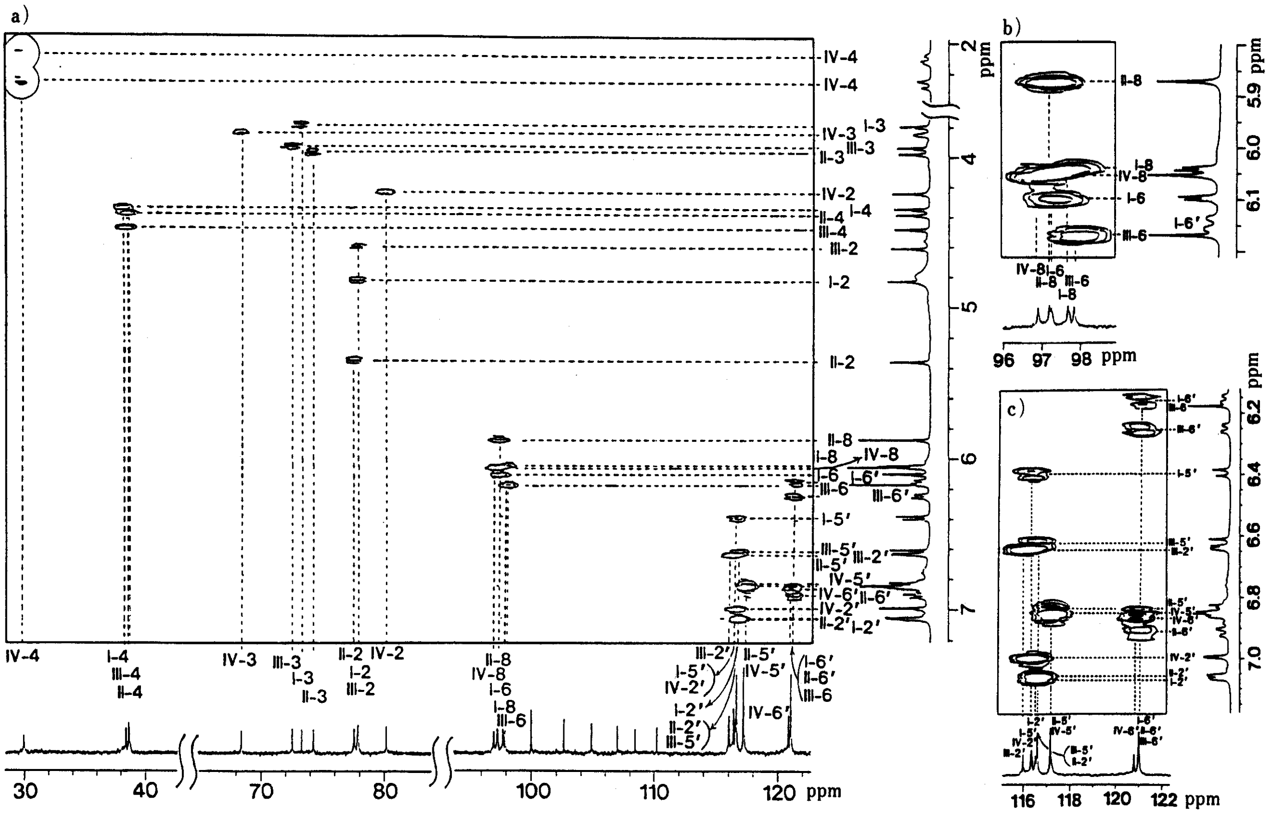

Fig. 6. HMQC Spectrum of 3 in $CD_3OD–D_2O$ (25 : 15) (Sample, 33 mg; $^1J_{CH}=135$ Hz; 15 h Run)

a) Whole region. b) Cross peaks of ring A methine groups. c) Cross peaks of ring B methine groups.

centered at δ 2.81 and 2.20 in an approximate ratio of 5 : 6.[16) In parallel, the ^{13}C-NMR spectrum also shows double lines for several carbon signals.[16) This problem was solved by addition of deuterium oxide (D_2O), causing a gradual increase of the amount of the major conformer (A). Finally, in a mixture of methanol-d_4–D_2O (25 : 15), the spectra showed simple patterns, which may be ascribed to a single conformer (A) (see Fig. 4).

The ^{1}H–^{1}H COSY (Fig. 5) shows correlation peaks due to four sets of 1,3,4-trisubstituted benzene protons (ring B) and four sets of ring C protons. The combination of rings B and C in each epicatechin unit can readily be determined by detection of ^{1}H–^{1}H long-range couplings between 2-H and 2′- and 6′-H (Table II). Thus, the proton signals of the lower terminal epicatechin unit (unit IV), for instance, were readily assigned by tracing the ^{1}H–^{1}H correlation peaks starting from the characteristic AB-type signals due to 4-H_2 (δ 2.09 and 2.26). Further, the proton at δ 4.34 (4-H in unit I) shows a correlation peak, possibly due to the long-range coupling through five bonds,[17) with the proton at δ 6.10 (d, $J=2.4$ Hz, 6-H in unit I), which is *meta*-coupled with the proton at δ 6.04 (d, $J=2.4$ Hz). This suggests that the proton at δ 4.34 is 4-H of the upper terminal epicatechin unit (unit I). Thus, all ^{1}H-signals of the upper terminal epicatechin unit were discriminated unequivocally. Also, in the ^{1}H–^{1}H COSY spectrum (Fig. 5b), the proton at δ 4.38 (4-H in unit II) shows a weak but significant cross peak due to long-range coupling[17) with the proton at δ 5.87 (8-H in unit II), which, in turn, is correlated with 4-H of unit I. This suggests that these two protons are 4-H (δ 4.38) and 8-H (δ 5.87) of the second upper terminal epicatechin unit (unit II), and thus all ^{1}H-signals of the second upper terminal epicatechin unit were assigned reasonably (Table II). The remaining ^{1}H-signals were properly ascribed to the protons of the third epicatechin unit (unit III), but assignments of 6-H of unit III and also of 8-H of unit IV were uncertain at this stage.

The ^{13}C-signals due to protonated carbons in 3 were correlated with the corresponding proton(s) by the ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) method[14,18) (Fig. 6), and the assignments were established unequivocally.

Next, we measured the HMBC spectrum of 3 in order to analyze the signals due to quaternary carbons. As can be seen in Fig. 7, the carbon signal at δ 100.0 (2 × C) shows long-range correlations with 3-H, 4-H, 6-H, and 8-H of unit I and also with 3-H and 4-H of unit III and the singlet benzene proton at δ 6.17, while the carbon at δ 102.6 shows correlation peaks with 3-H and 4-H_2 of unit IV and with the benzene proton at δ 6.05. Thus, the former carbon signal should be assigned to C-4a's of units I and III, and the latter signal is ascribed to C-4a of unit IV. Further, the protons at δ 6.17 and at δ 6.05 are ascribed to 6-H of unit III and 8-H of unit IV, respectively. Likewise, the carbon at δ 104.9 was assigned to C-4a of unit II. On the other hand, the quaternary carbons at δ 106.9, 108.4, and 110.2 show long-range correlations with 8(II)-H, 3(I)-H, and 4(I)-H, with 8(IV)-H, 3(III)-H, and 4(III)-H, and with 6(III)-H, 3(II)-H, and 4(II)-H, respectively. This allows us to assign these carbons to C-6's of units II and IV and C-8 of unit III, respectively. Similarly, the quaternary carbon signals at δ 132.3 [C-1′(III)], 133.1 [C-1′(IV)], 133.3 [C-1′(I)], and 133.6 [C-1′(II)] could be assigned based on long-range correlations with the 2-H and 5′-H of the

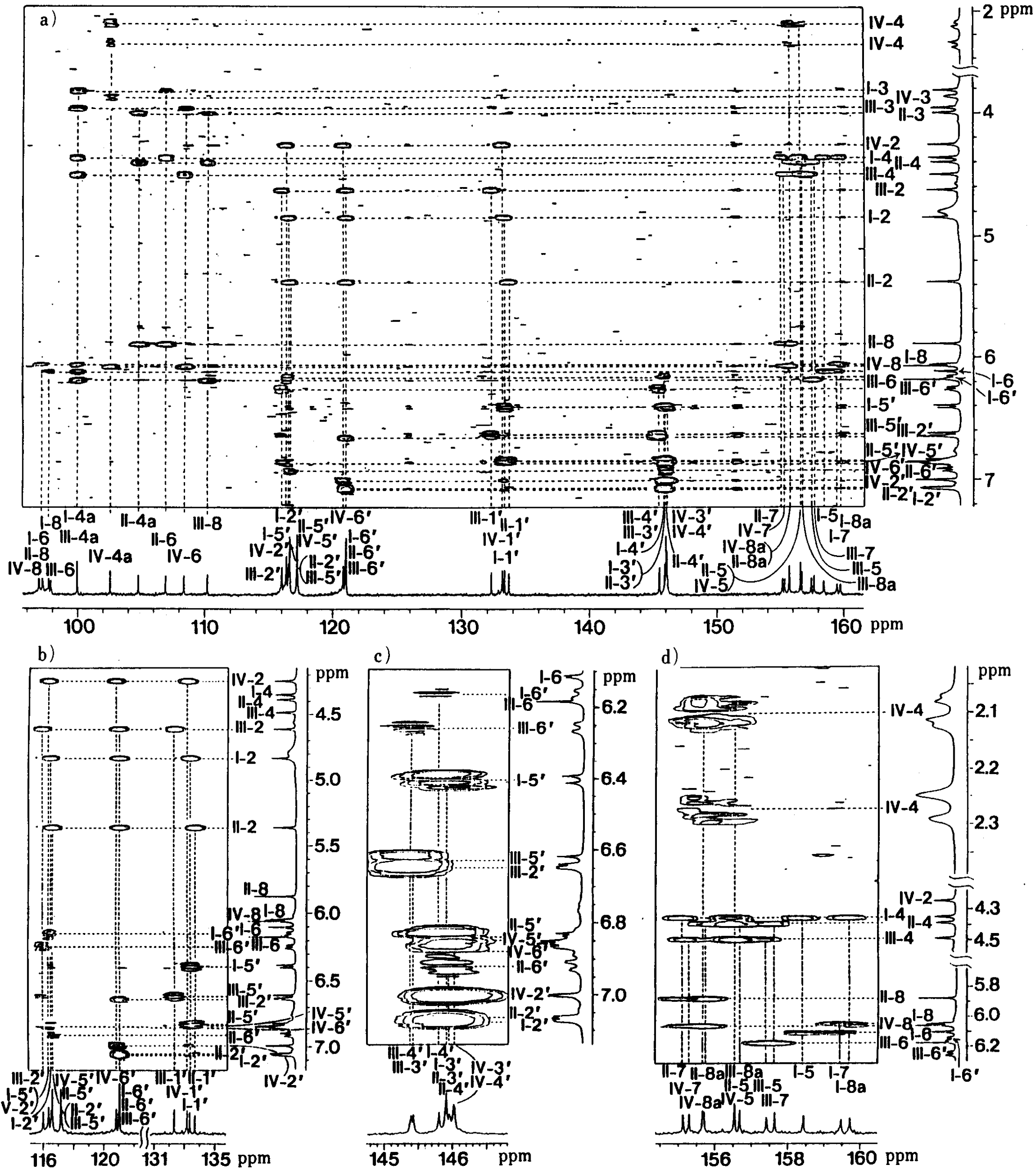

Fig. 7. HMBC Spectrum of 3 in CD_3OD–D_2O (25 : 15) (Sample, 55 mg; ${}^{lr}J_{CH}=6$ Hz; 60 h Run)

a) Whole region. b) δ_C 115—135 ppm region. c) δ_C 144.8—146.9 ppm region. c) δ_C 154.5—160.5 ppm region.

corresponding units.

The ^{13}C-signals due to oxygenated quaternary carbons in 3 appeared in the δ 145—146 ppm region and in the δ 155—160 ppm region. Signals of the former group (δ 145—146 ppm) were ascribed to C-3′ and C-4′ carbons of the B-rings by careful analyses of long-range ^{1}H–^{13}C correlation peaks, as can be seen in Fig. 7. Signals of the latter group (δ 155—160 ppm) were also examined in the same way and assigned to C-5, C-7, and C-8a carbons of the A-rings, except for C-5 and C-8a carbons of the upper terminal unit (unit I), both of which showed long-range correlations with 4(I)-H (δ 4.34).

At this stage, we measured the long-range C–H J-resolved 2D NMR (LRCJR)[19] spectrum under selective irradiation at 2(I)-H (δ 4.83), where the carbon at δ 159.7 was split into a doublet ($J=2$ Hz). Therefore, this signal was unambiguously assigned to C-8a (I), and the remaining one (δ 158.4) to C-5 (I). Furthermore, these carbons, C-8a (I) and C-5 (I), showed long-range correlations with the protons at δ 6.04 [d, $J=2.4$ Hz, 8(I)-H] and at δ 6.10 [d, $J=2.4$ Hz, 6(I)-H], respectively, in the HMBC spectrum (Fig. 7). This allows us to assign these proton signals unequivocally, and further leads to the exact assignments of the C-6 and C-8 methine carbons (δ 97.3 and 97.7, respectively) of the upper terminal unit.

It should be noted that the C-2 and C-3 methine carbons of the lower terminal units (4β-6 linked form) in **1**, **2**, and **3** resonate at around δ 80 and δ 68, respectively, the chemical shift values being identical with those of epicatechin (**4**),[20] while those of other units resonate in the δ 77—78 ppm and δ 73—74 ppm regions, respectively (Tables I and II). Similarly, the 2-H and 3-H signals of the lower terminal units in dimeric and trimeric procyanidins (**1** and **2**) have the same chemical shift values (δ 4.81 and δ 4.15—4.19) as those of epicatechin (**4**),[21] while those of other units have slightly larger values (Table I). However, in the case of the tetramer (**3**), chemical shifts of ring-C protons of the lower terminal unit (uint IV) depend on the solvent employed. In methanol-d_4 solution, there are two conformers (see Fig. 4), which probably arise from restricted rotation about the central interflavonoid linkage.[5b)] It is of interest that the conformer A is preferred in polar solvents and shows the signals due to ring-C protons at a relatively higher region, whereas the conformer B is preferred in less polar solvents and shows the corresponding signals at the same region as epicatechin (**4**).

Experimental

Optical rotations were measured on a JASCO DIP-140 digital polarimeter, and UV spectra with a Shimadzu 202 UV spectrophotometer in MeOH solutions. IR spectra were taken with a JASCO IR-2 spectrometer in KBr discs. Positive and negative ion FAB-MS's were obtained with a JEOL D-300, JMS-DX300, or JMS-SX102 mass spectrometer using a direct inlet system. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX400 spectrometer with tetramethylsilane as an internal standard and chemical shifts are recorded in δ values. Methanol-d_4 was used as the solvent, because signal overlapping was less than in acetone-d_6. Multiplicities of ^{13}C-NMR signals were determined by means of the distortionless enhancement by polarization transfer (DEPT) method and are indicated as s (singlet), d (doublet), and t (triplet). 2D NMR spectra were measured by the use of JEOL standard pulse sequences (^{1}H–^{1}H COSY: VCOSYN; ^{1}H–^{13}C COSY: VBDCHSHF, $^1J_{CH}=140$ Hz; long-range ^{1}H–^{13}C COSY: VCHSHF, $^1J_{CH}=140$ Hz, $^{lr}J_{CH}=8$ Hz; HMQC: VHMQCDBBH, $^1J_{CH}=135$ Hz; HMBC: VHMBC, $^1J_{CH}=135$ Hz, $^{lr}J_{CH}=6$ Hz) and collected data were treated by using JEOL standard software. Column chromatography was done with Sephadex LH-20 (Pharmacia). Thin-layer chromatography (TLC) and preparative TLC were carried out on precoated Merck Kieselgel 60 F_{254} plates with EtOAc–EtOH–H_2O (12:2:1 or 10:2:1) or MeOH–$CHCl_3$ (8:2) as developing solvents, and spots were detected under UV light, or by using $FeCl_3$ and anisaldehyde-H_2SO_4 reagents.

Isolation of Procyanidins As described in a previous paper,[1)] fraction DA-3 (EtOAc-soluble fraction from the aqueous acetone extract, 20 g) was chromatographed on a Sephadex LH-20 column with EtOH and EtOH–MeOH (1:1) to give fifteen fractions. Among these, fraction 7 (EtOH eluate, 109 mg) gave procyanidin B-5 (**1**) as a slightly brown-colored amorphous powder, $[\alpha]_D^{24}$ +94.7° ($c=1.7$, acetone). UV λ_{max} nm (log ε): 218 (4.45), 281 (3.86). IR ν_{max} cm^{-1}: 3350 (OH), 1605, 1515, 1450 (aromatic ring). Negative ion FAB-MS (matrix, glycerol) m/z: 577 $[M-H]^-$. ^{1}H- and ^{13}C-NMR: Table I.

Fraction 9 (EtOH eluate, 273 mg) furnished epicatechin-(4$\beta\rightarrow$8)-epicatechin-(4$\beta\rightarrow$6)-epicatechin (**2**) as a slightly brown-colored amorphous powder, $[\alpha]_D^{32}$ +104.5° ($c=1.0$, MeOH). UV λ_{max} nm (log ε): 221 (4.46), 281 (4.10). IR ν_{max} cm^{-1}: 3350 (OH), 1600, 1520, 1440 (aromatic ring). Positive ion FAB-MS (matrix, *m*-nitrobenzyl alcohol) m/z: 867 $[M+H]^+$. Negative ion FAB-MS (*m*-nitrobenzyl alcohol) m/z: 865 $[M-H]^-$. *Anal.* Calcd for $C_{45}H_{38}O_{18}\cdot 3H_2O$: C, 58.70; H, 4.82. Found: C, 58.91; H, 4.82. ^{1}H- and ^{13}C-NMR: Table I.

Fraction 14 [EtOH–MeOH (1:1) eluate, 1.3 g] was chromatographed again on a Sephadex LH-20 column (bed, 3.5 × 64 cm) using methanol–H_2O with increasing amounts of methanol (10:90→70:30). A fraction eluted with methanol–H_2O (60:40) gave epicatechin-(4$\beta\rightarrow$6)-epicatechin-(4$\beta\rightarrow$8)-epicatechin-(4$\beta\rightarrow$6)-epicatechin (**3**) (365 mg) as a slightly brown-colored amorphous solid, $[\alpha]_D^{22}$ +168.5° ($c=0.6$, MeOH). UV λ_{max} nm (log ε): 222 (4.82), 281 (4.15). IR ν_{max} cm^{-1}: 3450—3300 (br, OH), 1600, 1500, 1435 (aromatic ring), 1260—1020. Positive ion FAB-MS [matrix, glycerol + thioglycerol (1:1)] m/z: 1155 $[M+H]^+$. *Anal.* Calcd for $C_{60}H_{50}O_{24}\cdot 2H_2O$: C, 60.81; H, 4.47. Found: C, 60.78; H, 4.51. ^{1}H- and ^{13}C-NMR: Table II.

Complete Thiolytic Degradation of 3 A mixture of **3** (58 mg), toluene-α-thiol (1.5 ml), and acetic acid (2 ml) in EtOH (10 ml) was refluxed for 24 h with stirring. The reaction mixture was concentrated under reduced pressure and the residue was chromatographed over Sephadex LH-20 (bed, 1.5 × 3 cm). After removal of toluene-α-thiol with CH_2Cl_2, elution with MeOH gave a mixture of products. This was subjected to preparative TLC with $CHCl_3$–MeOH (8:2) and separated into two fractions. The less polar fraction afforded (−)-epicatechin (**4**) (9.4 mg) as an amorphous solid, $[\alpha]_D^{26}$ −43.5° ($c=0.9$, MeOH). MS m/z (%): 290 (M^+, 83), 151 (50), 139 (100), 123 (36), 91 (25). ^{1}H- and ^{13}C-NMR: Table I. The more polar fraction gave (−)-4β-benzylthioepicatechin (**5**) (42 mg) as an amorphous powder, $[\alpha]_D^{25}$ −28.2° ($c=1.0$, acetone). Positive ion FAB-MS (matrix, glycerol) m/z: 413 $[M+H]^+$. ^{1}H-NMR (methanol-d_6) δ: 3.85 (1H, dd, $J=2.3$, 1.1 Hz, 3-H), 3.95 (2H, s, $-SC\underline{H}_2-$), 4.05 (1H, d, $J=2.3$ Hz, 4-H), 5.23 (1H, br s, 2-H), 5.90 (1H, d, $J=2.1$ Hz, 8-H), 5.96 (1H, d, $J=2.1$ Hz, 6-H), 6.75 (1H, d, $J=8.2$ Hz, 5′-H), 6.67 (1H, dd, $J=8.2$, 1.8 Hz, 6′-H), 6.93 (1H, d, $J=1.8$ Hz, 2′-H), 7.21 (1H, tt, $J=7.0$, 2.0 Hz, 4″-H), 7.29 (2H, t, $J=7.0$ Hz, 3″,5″-H), 7.41 (2H, dd, $J=7.0$, 2.0 Hz, 2″,6″-H). ^{13}C-NMR (methanol-d_6) δ: 38.7 (t, $-S\underline{C}H_2-$), 44.7 (d, C-4), 72.4 (d, C-3), 76.3 (d, C-2), 96.5 (d, C-8), 97.6 (d, C-6), 100.9 (s, C-4a), 116.0 (d, C-2′), 116.7 (d, C-5′), 120.0 (d, C-6′), 128.7 (d, C-4″), 130.3 (2C, d, C-2″, 6″), 130.8 (2C, d, C-3″,5″), 132.8 (s, C-1′), 141.2 (s, C-1″), 146.5 (s, C-4′), 146.7 (s, C-3′), 158.0 (s, C-8a), 159.6 (s, C-5), 159.8 (s, C-7). These ^{1}H- and ^{13}C-signals were assigned by the use of ^{1}H–^{1}H and ^{1}H–^{13}C COSY and the HMBC spectrum.

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References and Notes

1) Part 1: C.-B. Cui, Y. Tezuka, T. Kikuchi, H. Nakano, T. Tamaoki, and J.-H. Park, *Chem. Pharm. Bull.*, **32**, 3218 (1990).
2) C.-B. Cui, Y. Tezuka, T. Kikuchi, H. Nakano, and J.-H. Park, *Chem. Pharm. Bull.*, **38**, 2620 (1990).
3) J.-H. Park, *Korean. J. Pharmacogn.*, **18**, 191 (1987).
4) Chiang Su New Medical College, "Dictionary of Chinese Crude Drugs," Vol. II, Shanghai Scientific Technologic Publisher, Shanghai, 1978, p. 1658.
5) *a*) R. S. Thompson, D. Jacques, E. Haslam, and R. J. N. Tanner, *J. Chem. Soc., Perkin Trans. 1*, **1972**, 1387; *b*) A. C. Fletcher, L. J. Porter, E. Haslam, and R. K. Gupta, *ibid.*, **1977**, 1628.
6) E. Ezaki-Furuichi, G. Nonaka, I. Nishioka, and K. Hayashi, *Agric. Biol. Chem.*, **50**, 2061 (1986).
7) T.-H. Hwang, Y. Kashiwada, G. Nonaka, and I. Nishioka, *Phytochemistry*, **29**, 279 (1990).
8) F. Hsu, G. Nonaka, and I. Nishioka, *Chem. Pharm. Bull.*, **33**, 3293 (1985).
9) It should be noted that **1**, **2**, and **3** showed an inhibitory action toward protein kinase C, but had no effect on protein kinase A at a concentration of 40 μg/ml.
10) T. Tamaoki, H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita, *Biochem. Biophys. Res. Commun.*, **135**. 397 (1986); I. Takahashi, E. Kobayashi, K. Asano, M. Yoshida, and H. Nakano, *J. Antibiotics*, **40**, 1782 (1987); G. J. Mazzei, N. Katoh, and J. F. Kuo, *Biochem. Biophys. Res. Commun.*, **109**, 1129 (1982); R. C. Schatzman, B. C. Wise, and J. F. Kuo, *ibid.*, **98**, 669 (1981); T. Mori, Y. Takai, R. Minakuchi, B. Yu, and Y. Nishizuka, *J. Biol. Chem.*, **255**, 8378 (1980).
11) G. Nonaka, O. Kawahara, and I. Nishioka, *Chem. Pharm. Bull.*, **30**, 4277 (1982).
12) K. Weinges, H.-D. Marx, and K. Goritz, *Chem. Ber.*, **103**, 2336 (1970); I. C. du Preez, A. C. Rowan, D. G. Roux, and J. Feeney, *Chem. Commun.*, **1971**, 315.
13) A. Bax and S. Subramanian, *J. Magn. Reson.*, **67**, 565 (1986).
14) M. F. Summers, L. G. Marzilli, and A. Bax, *J. Am. Chem. Soc.*, **108**, 4285 (1986).
15) Methine carbons of A-rings in **2** appeared as broad signals in the δ 96.5—98.5 ppm region and failed to give significant correlation peaks in the ^{1}H–^{13}C COSY spectrum. Attempts at selective ^{1}H-decoupling experiments were also unsuccessful.
16) It is noteworthy that there are also two conformers in acetone-d_6 solution, where the ratio of conformer A to B is about 1:2. However, addition of D_2O increased the relative amount of conformer A. In acetone-d_6–D_2O (6:3) solution the ratio of A and B becomes equal and finally in acetone-d_6–D_2O (6:16) solution conformer B practically disappears.
17) E. Pretsch, T. Clerc, J. Seibl, and W. Simon, "Tabellen zur Struktur-

aufklarung Organischer Verbindungen mit Spektroskopischen Methoden," Springer-Verlag, Berlin, 1981, p. H120, H205-210, H245.

18) A. Bax and M. F. Summers, *J. Am. Chem. Soc.*, **108**, 2093 (1986).

19) H. Seto, K. Furihata, N. Otake, Y. Itoh, S. Takahashi, T. Haneishi, and M. Ohuchi, *Tetrahedron Lett.*, **25**, 337 (1984).

20) We carried out a reexamination of the ^{1}H- and ^{13}C-NMR spectra of epicatechin by means of 2D NMR methods, and found that previous assignments of C-3′ and C-4′ signals in dimethyl sulfoxide-d_6 solution and those of C-5 and C-7 signals in methanol-d_4 solution should be revised. See P. K. Agrawal, M. C. Bansal, L. J. Porter, and L. Y. Foo, "Carbon-13 NMR of Flavonoids," ed. by P. K. Agrawal, Elsevier Science Publishers B. V., Amsterdam, 1989, p. 446; C. Cui, Y. Chen, S. Yao, G. Cu, and Y. Xian, *Zhong Cao Yao*, **18**, 297 (1987).

21) It was reported that 2-H and 3-H of the lower terminal unit linked at the 6-position resonate at the same region as those of epicatechin. See reference 6.