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Article

Structural Elucidation of Epicatechin (4→7) 5,8,3'4'-tetrahydroxy-(2*R*,3*R*)-flavan-3-ol Isolated from the Bark of Korean Pine Tree (*Pinus densiflora*)

Yoongho Lim[1,3]*, Yeunjoong Yong[1], Sungjin Oh[2] and Hongkeun Song[2,3]

[1]*Department of Applied Biology and Chemistry, Kon-Kuk University*
[2]*Department of Forest Resource, Kon-Kuk University*
[3]*Academy of Life Science, Kon-Kuk University*

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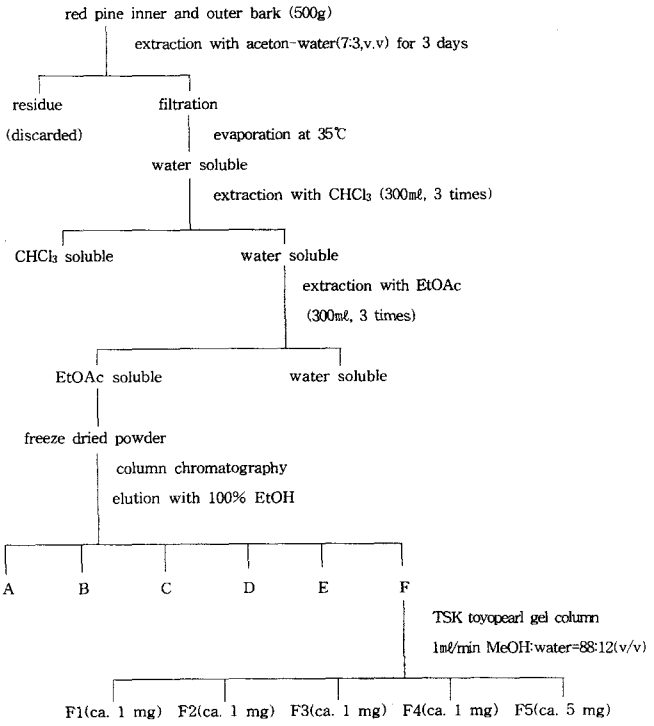
Several flavonoid dimers were isolated and elucidated from the bark of Korean pine tree (*Pinus densiflora*). One of them was postulated to be a compound, epicatechin (4→7) 5,8,3'4'-tetrahydroxy-(2*R*,3*R*)-flavan-3-ol, whose structural determination was carried out by diverse NMR techniques.

Key words : Pinus densiflora, *NMR, HMQC, HMBC, NOESY, flavanol, epicatechin.*

Flavonoids are found in most plants, and they play an important role in flowers, dyestuffs, tanning of leather, and so on.[1,2] They can be classified into 12 groups based on the oxidation level of the central pyran ring, and their structures are known to be more than 4,000.[2] However, they are still studied world-widely, because of their usefulness. The basic skeleton of flavonoid is comprised of three rings such as C6-C3-C6, which are named A, B, and C ring, respectively.[3] The compounds with a basic skeleton can be combined each other, so that dimers, trimers, and oligomers are formed. Especially, dimers are found abundantly in needle leaf trees. In this study, several dimers were isolated and elucidated from the bark of Korean pine tree (*Pinus densiflora*). Among those, one dimer was considered to be a compound, whose structural determination by NMR spectroscopy is reported here.

Materials and Methods

Isolation of flavonoid dimer. The bark of *Pinus densiflora* collected in Korea, was separated to two parts, inner and outer bark. The outer bark was ground by Willey mill and screened in 30~80 mesh. It was extracted with acetone-water (7:3, v/v) at room temperature. The acetone was removed by evaporation under the reduced pressure at 35°C and the resulting aqueous solution was partitioned with chloroform and ethyl acetate, respectively. The ethyl acetate soluble portion was dried under the reduced pressure at 35°C. The reddish colored powder obtained was loaded over a glass column packed with Sephadex LH-20 (Pharmacia Co.). This column was eluted with 100% ethanol to furnish five fractions (F1, F2, F 3, F4, and F5) by gravity. The sepatation methods were



Scheme 1. The separation method for compound F5.

*Corresponding author
Phone: 82-2-450-3760; Fax: 82-2-3436-5776
E-mail: yoongho@kkucc.konkuk.ac.kr

shown in Scheme 1. Fractions 3~5 were rechromatographed over TSK-HW40F (Toyo Pearl gel) with MeOH-H_2O (88:12, v/v) until purified compounds were obtained. Several dimers were isolated and freeze-dried for the NMR measurements. Among those, F5 was considered to be studied in detail because of its high yield.

Compound F5: mp 214°C; $[\alpha]_D^{25}$ -0.6° (c 4, MeOH); IR (KBr disk) ν_{max} 3424, 2361, 1662, 1521, 1449, 1384, 1281, 1147, 1108, 1060 cm^{-1}; FABMS *m/z* 578.1 ($C_{30}H_{26}O_{12}$)

NMR measurements. All NMR experiments were performed on a Bruker DPX 400 (9.4T). Approximately 3 mg of samples were dissolved in 500 uL of DMSO-d_6 or acetone-d_6. Chemical shifts for all spectra were referenced to TMS. All experiments were carried out in a 5 mm tube at 298 K. For the ^{1}H-NMR experiments, 16 transients were acquired with a 1 sec relaxation delay using 32 K data point, and 90° pulse was 9.7 usec, spectral width, 4200 Hz. For the ^{13}C-NMR and DEPT experiments, 10,000 transients were acquired with a 3 sec relaxation delay using 64 K data points, and 90° pulse was 9.8 usec, spectral width, 18,000 Hz. The COSY spectrum was collected with the magnitude method as described by Nagayama.[4] 256 blocks were collected with spectral width of 4,000 Hz, and 16 scans were accumulated for each block with free induction decays of 2048 data point. 16 dummy scans were used, and an acquisition time of 0.26 sec was employed. The time domain data were multiplied in the t_1 and t_2 dimension by a squared sine bell with phase shift of 0 and were zero-filled to obtain 2 K×2 K real data points. The NOESY spectrum was collected with the magnitude method as described by Turner.[5] 256 blocks were collected with spectral width of 3,500 Hz, and 16 scans were accumulated for each block with free induction decays of 2048 data point. 16 dummy scans were used, and an acquisition time of 0.29 sec was employed. The time domain data were multiplied in the t_1 and t_2 dimension by a squared sine bell with phase shift of 0 and were zero-filled to obtain 2 K×2 K real data points. The mixing time was 700 msec. The HMQC spectrum and the HMBC spectrum were collected with the methods as described by Bax[6] and Summers,[7] respectively. 256 blocks were collected with spectral width of 4,000 Hz of t_2 dimension, and that of 22,000 Hz of t_1 dimension. The number of scans for each block was 128 and data points of t_2 dimension were 4096. Dummy scans were 16, and an acquisition time, 0.5 sec. The time domain data were multiplied in the t_1 and t_2 dimension by a sine bell, and were zero-filled to obtained 2 K×2 K real data points. The delay for the long ranged coupling of HMBC was 45 msec. All 2D data were post-processed on a SGI INDY workstation with Felix program (msi).

Molecular dynamics. InsightII/Discover version 95.0 (msi) with the force field CVFF was used for the molecular dynamics calculation.

Results and Discussion

The ^{13}C-NMR spectrum of the compound indicated the existence of 30 carbons. Because a monomeric flavonoid had only 15 carbons, this compound was expected to be a dimeric flavonoid. Two peaks corresponding to big two methines with the hydroxyl group were observed in the ^{13}C-NMR spectrum so that the compound was con-

Table 1. A comparison of the ^{13}C-NMR chemical shifts of the compound F5 in acetone-d_6 and those of epicatechin (4β→8) epicatechin in acetone-d_6.

no.	δ_c of the compound F5	δ_c of epicatechin (4β→8) epicatechin[8]
1	27.2	29.1
2	36.4	36.7
3	67.4	66.2
4	72.2	72.8
5	76.5	76.5
6	81.6	79.1
7	95.2	95.9
8	95.8	96.3
9	96.5	97.3
10	100.4	100.6
11	101.9	102.2
12	107.0	107.6

Table 2. Chemical shifts of ^{1}H-NMR and ^{13}C-NMR of the compound F5 dissolved in DMSO-d_6, and their assignments.

no.	δ_c	CH_n DEPT	δ_H of directly attached protons, HMQC [*J*(Hz)]	^{13}C assignments
1	26.2	t	2.35 s	4B
2	35.3	d	4.33 d(4.9)	4T
3	66.0	d	3.85 s	3B
4	71.2	d	3.54 d(3.1)	3T
5	75.4	d	4.95 s	2T
6	80.2	d	4.72 s	2B
7	93.7	d	5.63 s	8T
8	94.5	d	5.72 s	6T
9	96.7	d	5.70 s	6B
10	98.6	s		10B
11	102.2	s		10T
12	107.2	s		7B
13	113.7	d	6.67 s	2'T
14	114.3	d	6.73 s	2'B
15	114.8	d	6.55 d(8.0)	5'B
16	114.9	d	6.45 s	5'T
17	115.2	d	6.57 d(8.0)	6'B
18	117.7	d	6.47 s	6'T
19	131.1	s		1'T
20	131.4	s		1'B
21	144.2	s		4'T
22	144.4	s		4'B
23	144.6	s		3'T
24	144.7	s		3'B
25	152.6	s		9T
26	153.7	s		5B
27	154.4	s		9B
28	155.7	s		8B
29	156.5	s		7T
30	156.6	s		5T

sidered to be a dimeric flavanol.[8] There are many kinds of dimeric flavanol compounds such as epicatechin (4→6) epicatechin, epicatechin (4→8) epicatechin, catechin (4→6) catechin, catechin (4→8) catechin, epicatechin (4→6) catechin, epicatechin (4→8) catechin, catechin (4→6) epicatechin, and catechin (4→8) epicatechin. 12 peaks between 20 ppm and 110 ppm in the ^{13}C-NMR data were compared with those of the dimeric flavanol compounds mentioned above, epicatechin (4β→8) epicatechin was the closest similar one. The ^{13}C-NMR chemical shifts of the compound and those of epicatechin (4β→8) epicatechin were listed in Table 1. Two peaks of the compound at 81.6 ppm and 95.2 ppm were different with those of epicatechin (4β→8) epicatechin at 79.1 ppm and 96.3 ppm. Therefore, experiments such as HMQC, HMBC and NOESY were carried out. The multiplicity of the carbon obtained from DEPT experiments and the chemical shifts of the correlated protons obtained from HMQC were listed in Table 2. The ^{1}H peak at 4.33 ppm was assigned to the proton of the position 4 in the top unit (4TH). The expected numbers of the peak of ^{13}C long-ranged coupled with 4TH in the HMBC spectrum were eight, which were listed in Table 3. In HMBC, however, the ^{1}H peak at 4.33 ppm was long-ranged coupled with

Table 3. The expected numbers of the peaks of ^{13}C long-ranged coupled with 4TH in the HMBC spectrum.

δc	4→6	4→7	4→8
70~80	2	2	2
95~110	2	3	2
>150	4	3	4

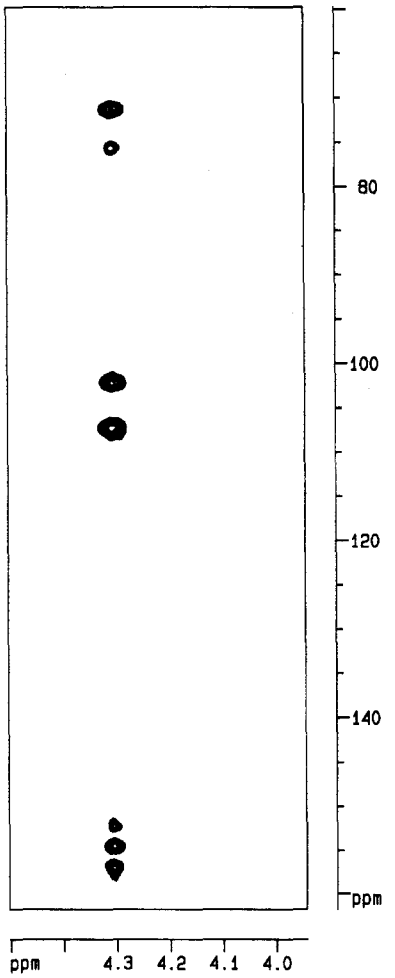


Fig. 1. The partial spectrum of HMBC in DMSO-d_6. Delay time for the long-ranged coupling = 45 msec; 4 K(t_2)×256(t_1).

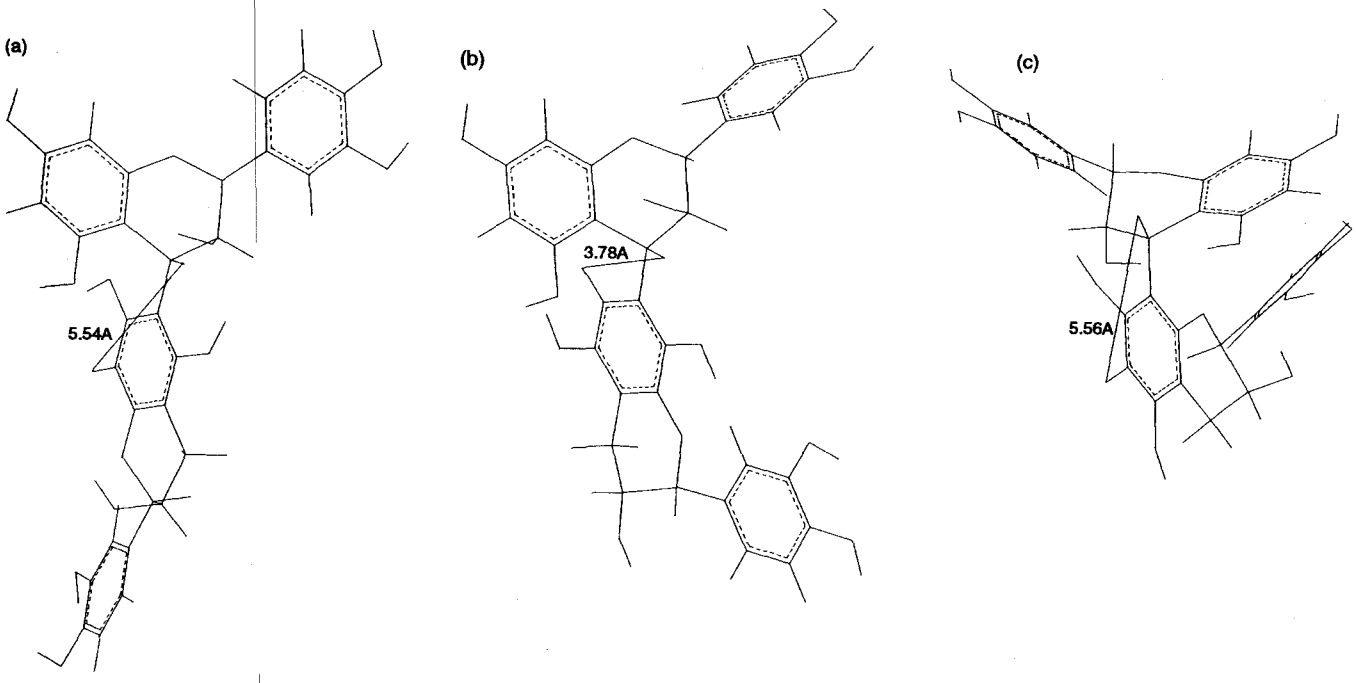


Fig. 2. The three dimensional structures of (a) epicatechin (4→6) epicatechin, (b) epicatechin (4→7) 5,8,3',4'-tetrahydroxy-(2*R*,3*R*)-flavan-3-ol, and (c) epicatechin (4→8) epicatechin, calculated by molecular dynamics.

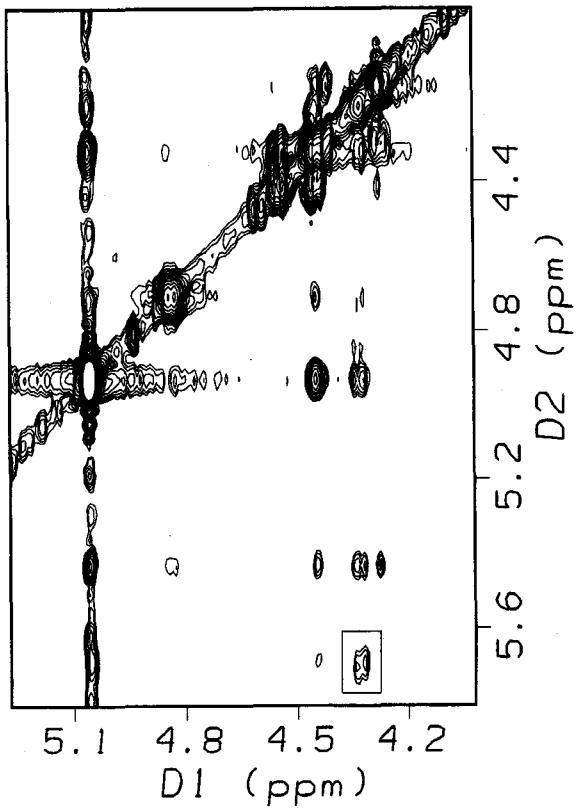


Fig. 3. The partial spectrum of NOESY in DMSO-d_6. Mixing time = 700 msec; 2 K(t_2)×256(t_1).

only seven carbon peaks at 71.2, 75.4, 102.2, 107.2, 152.6, 155.7, and 156.6 ppm as shown in Fig. 1. Several HMBC experiments were carried out with different delay times for the long-ranged coupling such as 30 ms, 45 ms, 60 ms, 70 ms, and 80 ms. But all experiments showed the same results. Therefore, the HMBC experiment was not enough to determine the connectivity between the top and the bottom units of the compound. If the protons of the hydroxyl groups were not counted, there was only one proton in the A ring of the bottom unit. The three dimensional structures of the conformers of epicatechin (4→6) epicatechin, epicatechin (4→7) 5,8,3',4'-tetrahydroxy-(2R,3R)-flavan-3-ol, epicatechin (4→8) epicatechin were obtained from the molecular dynamics calculations, whose structures were depicted in Fig. 2a, 2b, and 2c, respectively. In the case of epicatechin (4→6) epicatechin, the only one aromatic proton must be 8BH. The distance between 4TH and 8BH was calculated to be 5.54 Å. In the case of epicatechin (4→8) epicatechin, the distance between 4TH and 6BH was calculated to be 5.56 Å, whereas that of epicatechin (4→7) 5,8,3',4'-tetrahydroxy-(2R,3R)-flavan-3-ol was calculated as 3.78 Å between 4TH and 6BH. 4TH and 6BH of epicatechin (4→7) 5,8,3', 4'-tetrahydroxy-(2R,3R)-flavan-3-ol were expected to give an nOe effect. As shown in Fig. 3, the NOESY spectrum showed the cross peak of 4.33 ppm and 5.70 ppm, which could be assigned to be 4TH and 6BH, while the

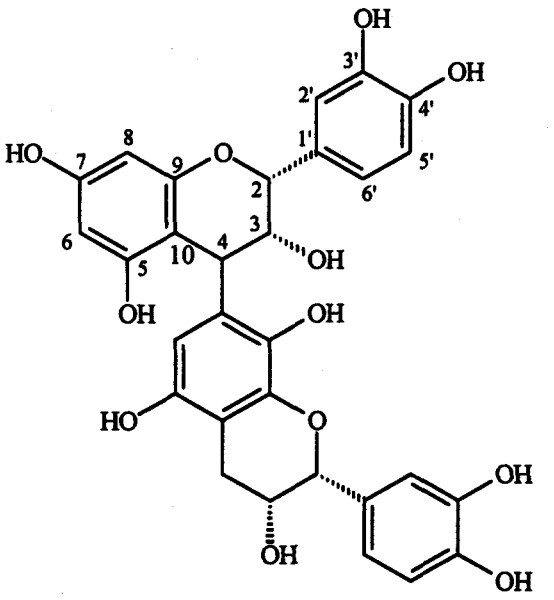


Fig. 4. The structure of the compound, (2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-4-[(2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,5,8-trihydroxy-3,4-dihydro-2H-benzo[b]oxin-7-yl]-3,4-dihydro-2H-benzo[b]oxine-3,5,7-triol.

COSY spectrum did not show the crosspeak in the same position. Therefore, it could be postulated that the compound had 4→7 conformation. Thus, it might be (2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-4-[(2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,5,8-trihydroxy-3,4-dihydro-2H-benzo[b]oxin-7-yl]-3,4-dihydro-2H-benzo[b]oxine-3,5,7-triol as shown in Fig. 4. More detailed spectral data have not yet determined. In addition, the evidence that the compound has 4→7 conformation was based only on the NOESY experiment. Therefore, unclear results should be proved by other experiments such as chemical degradation and derivatization. In this paper, however, only the possibility of the existence of the 4→7 conformer was reported.

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