Effects of Hydroxy and Methoxy Substituents on NMR Data in Flavonols

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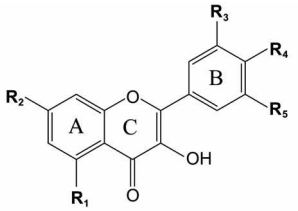
Kaempferide, kaempferol, and galangin belong to flavonol, one of flavonoid classes. Even they have the same moiety as flavonol, their binding affinities for a hexahistidine-tagged C-terminal nucleotide-binding domain are different with each other.[1] The dissociation constants of flavonol, kaempferide, kaempferol, and galangin are 10.1, 4.5, 6.7, and 5.3 mM, respectively. Galangin contains two more hydroxyl groups than flavonol, and kaempferol has three more hydroxyl groups. Kaempferide differs with kaempferol in only one substituent: 4'-hydroxyl group of kaempferol is switched to 4'-methoxyl group in kaempferide. Likewise, galangin and kaempferide show different enzymatic kinetic parameters. V_{max}/K_m, for cytochrome P450 isomers CYP2C9 and CYP1A1 which take an important role in oxidative metabolism of galangin and kaempferide. The values of V_{max}/K_m of galangin for CYP2C9 and CYP1A1 were 59.0 and 10.2 μL/min/mg, respectively, and those of kaempferide were 5.1 and 1.9, respectively.[2] While kaempferide has 4'-methoxyl group, galangin does not have it. As a result, it can be considered that the substitution of hydroxyl or/and methoxyl groups on flavonols results in significant changes of the biological activities. Flavonol derivatives are being found still from natural sources. One of the best methods to identify them is NMR spectroscopy.[3,4] Since the substitution of hydroxyl or/and methoxyl groups causes the chemical shift changes in the ^{1}H and ^{13}C NMR spectra, the elucidation of the effects of substituents on the chemical shifts in flavonol derivatives may help us predict the structures of the unknown compounds based on the simple one dimensional NMR experiments.

In order to elucidate the substituent effects of chemical shifts on flavonol derivatives, the NMR experiments of flavonol (**1**) and nine flavonol derivatives (**2-10**) were carried out in this study. Of them, the ^{13}C NMR data of three derivatives were already published.[5,6,7] Because only the partial NMR data of others have been known, however, the complete assignments of their ^{1}H and ^{13}C NMR data are reported here. One of the ^{13}C NMR data published previously showed the incorrect ^{13}C NMR data, thus their corrected values were determined in this study.

The structures and nomenclatures of flavonol derivatives **1-10** are shown in Figure 1. As mentioned above, the NMR data of three derivatives **2**, **8**, and **9** have been previously reported. We found that the NMR data of derivative **8** were partially incorrect. The ^{13}C chemical shifts of the derivatives **8**, 5,7,4'-trihydroxy-3'-methoxyflavonol, were reported by Kumari *et al.*[6] Whereas C-3' and C-4' of **8** were assigned to the signals at 149.4 and 147.9 ppm in Kumari's data, respectively, we found them to be at 147.4 and 148.9 ppm, respectively. That is, the order of the ^{13}C chemical shifts of C-3' and C-4' was opposite in the Kumari's data. In order to decide whether our assignments are correct or not, the interpretation of the NMR data was carried further out. The ^{1}H peak at 3.84 ppm suggested the presence of 3'-methoxy proton. In the HMBC spectrum, 3'-methoxy proton was long-range coupled to the ^{13}C peak at 147.4 ppm. Therefore, 147.4 ppm should be assigned to C-3'. The ^{13}C chemical shifts of C-4' can be known through the HMBC spectrum of H-2' and H-6'. The H-2' and H-6' signals showed a long-range coupling with two ^{13}C peaks at 146.7 and 148.9 ppm. Those peaks should be assigned to C-2 and/or C-4', respectively. Because 3-hydroxy proton peak at 9.41 ppm was long-range coupled to the ^{13}C peak at 146.7 ppm in



Derivative	nomenclature	R1	R2	R3	R4	R5
1	flavonol	H	H	H	H	H
2	7-hydroxy-3',4'-dimethoxyflavonol	H	OH	OMe	OMe	H
3	7-hydroxy-3',4',5'-trimethoxyflavonol	H	OH	OMe	OMe	OMe
4	7,4'-dihydroxy-3'-methoxyflavonol (geraldol)	H	OH	OMe	OH	H
5	5,7-dihydroxy-4'-methoxy-flavonol (kaempferide)	OH	OH	H	OMe	H
6	5,7-dihydroxy-3',4',5'-trimethoxyflavonol	OH	OH	OMe	OMe	OMe
7	5,7,3'-trihydroxy-4'-methoxyflavonol (tamarixetin)	OH	OH	OH	OMe	H
8	5,7,4'-trihydroxy-3'-methoxy-flavonol (isorhamnetin)	OH	OH	OMe	OH	H
9	5,3',4'-trihydroxy-7-methoxy-flavonol (rhamnetin)	OH	OMe	OH	OH	H
10	5,7,4'-trihydroxy-3',5'-dimethoxyflavonol	OH	OH	OMe	OH	OMe

Figure 1. Structures and nomenclatures of ten flavonol derivatives.

HMBC. however. it was assigned to C-2 and the ^{13}C peak at 148.9 ppm should be assigned to C-4'.

Since the complete ^{1}H and ^{13}C chemical shifts of the remaining six derivatives (**3-7**, **10**) were not reported yet, we carried out their assignments completely. Derivative **3** is 7-hydroxy-3',4',5'-trimethoxyflavonol. Fifteen peaks were observed in the ^{13}C NMR spectrum. The DEPT experiments of derivative **3** gave nine singlets, four doublets and two quartets. The most downfield shifted peak was 176.3 ppm which was assigned ketone group (C-4). In the HMBC spectrum. C-4 showed a long-ranged coupling with the ^{1}H peak at 7.95 ppm, so it could be assigned to H-5. In the COSY spectrum. because the cross-peak between 7.95 and 6.93 ppm was observed. the ^{1}H peak at 6.93 ppm should be H-6. H-6 shows two coupling constants of 2.1 and 8.6 Hz. Since H-5 shows the coupling constant of 8.6 Hz. the ^{1}H peak with the *meta*-coupling constant of 2.1 Hz should be H-8 which was found at 7.01 ppm. In HMBC, the H-5 signal showed a long-ranged coupling with C-4 and two ^{13}C peaks at 156.4. and 162.5 ppm. Those peaks should be assigned to C-7 and/or C-9. respectively. However. H-6 was long-range coupled to the ^{13}C peak at 162.5 ppm, so that it was C-7 and the ^{13}C peak at 156.4 ppm should be C-9. The ^{13}C peak at 114.1 ppm showed a long-ranged coupling with two proton signals, H-6 and H-8. so this carbon peak should be assigned to C-10. The A-ring was completely determined. The B-ring signals were easily assigned by consideration of symmetry of the C-1' ~ C-4' axis. Since the ^{1}H peak at 7.50 ppm showed double intensities. it could be assigned to H-2'/H-6'. The ^{1}H peak at 3.86 ppm showed also double intensities (6H), it could be assigned to 3'-methoxy/5'-methoxy proton. Those carbon peaks corresponding to H-2'/H-6' and 3'-methoxy/5'-methoxy proton were confirmed based on the HMQC spectrum. H-2'/H-6' showed a long-ranged coupling with two ^{13}C peaks at 138.9 and 143.9 ppm in the HMBC spectrum. Those peaks should be assigned to C-2 and/or C-4', respectively. However, methoxy proton at 3.75 ppm was long-range coupled to the ^{13}C peak at 138.9 ppm, so that it was C-4' and the ^{13}C peak at 143.9 ppm should be C-2. Since the exchangeable proton at 9.31 ppm showed a long-ranged coupling with C-2, C-4, and another singlet carbon at 138.1 ppm, this singlet carbon and exchangeable proton could be assigned to C-3 and 3-OH, respectively. The remaining singlet carbon at 126.7 ppm was assigned to C-1'. The complete assignments of the ^{1}H and ^{13}C chemical shifts of 7-hydroxy-3',4'.5'-trimethoxyflavonol (**3**) are listed in Tables 1 and 2, respectively. In the same manner, the NMR data of the remained five hydroxymethoxyflavonol derivatives. **4-7**. **10**, were assigned and their ^{1}H and ^{13}C NMR data are listed in Tables 1 and 2, respectively.

We investigated the substitution effect of the hydroxyl and methoxyl groups on the ^{1}H and ^{13}C NMR chemical shift changes of flavonol derivatives based on the elucidation of Tables 1 and 2. Generally, the substitution of hydroxyl and methoxyl groups affects the *ortho*- and *para*-position of flavonol to move the upfield.[8] Especially, the substitution

Table 1. The ^{1}H chemical shifts of ten flavonol derivatives **1-10**

	δ of ^{1}H (J, Hz)									
position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
5	8.10 (dd, 1.5, 8.0)	7.93 (d, 8.7)	7.95 (d, 8.6)	7.93 (d, 8.8)	–	–	–	–	–	–
6	7.42 (m)	6.92 (dd, 2.1, 8.7)	6.93 (dd, 2.1, 8.6)	6.91 (dd, 2.2, 8.8)	6.19 (d, 2.0)	6.21 (d, 2.0)	6.19 (d, 2.0)	6.19 (d, 2.1)	6.34 (d, 2.2)	6.20 (d, 2.0)
7	7.76 (ddd, 1.5, 7.2, 8.5)	–	–	–	—	–	–	–	–	–
8	7.71 (d, 8.5)	6.98 (d, 2.1)	7.01 (d, 2.1)	6.97 (d, 2.2)	6.45 (d, 2.0)	6.52 (d, 2.0)	6.42 (d,2.0)	6.47 (d, 2.1)	6.69 (d, 2.2)	6.52 (d, 2.0)
2'	8.20 (dd, 1.4, 7.2)	7.77 (d, 1.9)	7.50 (s)	7.77 (d, 2.1)	8.12 (d, 9.0)	7.47 (s)	7.67 (m)	7.75 (d, 2.1)	7.72 (d, 2.2)	7.50 (s)
3'	7.54 (m)	–	–	–	7.09 (d, 9.0)	–	–	–	–	–
4'	7.42 (dd, 7.2, 7.2)	–	–	–	–	–	–	–	–	–
5'	7.54 (m)	7.12 (d, 8.5)	–	6.94 (d, 8.5)	8.12 (d, 9.0)	–	7.65 (m)	6.94 (d, 8.5)	6.89 (d, 8.5)	–
6'	8.20 (dd, 1.4, 7.2)	7.80 (dd, 1.9, 8.5)	7.50 (s)	7.70 (dd, 2.1, 8.5)	7.09 (d, 9.0)	7.47 (s)	7.08 (d, 8.4)	7.68 (dd, 2.1,8.5)	7.57 (dd, 2.2, 8.5)	7.50 (s)
3-OH	9.60 (s)	9.15 (s)	9.31 (s)	9.66 (s)	9.47 (s)	9.64 (s)	9.42 (s)	9.41 (s)	9.47 (s)	9.46 (s)
5-OH	–	–	–	–	12.43 (s)	12.34 (s)	12.44 (s)	12.45 (s)	12.48 (s)	12.43 (s)
7-OH	–	10.72 (s)	10.69 (s)	10.74 (s)	10.83 (s)	10.89 (s)	10.78 (s)	10.83 (s)	–	10.77 (s)
3'-OH	–	–	–	–	–	–	9.30 (s)	–	9.30 (s)	–
4'-OH	–	–	–	9.08 (s)	–	–	–	9.77 (s)	9.66 (s)	9.14 (s)
7-OMe	–	–	–	–	–	–	–	–	3.86 (s)	–
3'-OMe	–	3.83 (s)	3.86 (s)	3.83 (s)	–	3.84 (s)	–	3.84 (s)	–	3.84 (s)
4'-OMe	–	3.84 (s)	3.75 (s)	–	3.83 (s)	3.74 (s)	3.84 (s)	–	–	–
5'-OMe	–	–	3.86 (s)	–	–	3.84 (s)	–	–	–	3.84 (s)

Table 2. The ^{13}C chemical shifts of ten flavonol derivatives **1-10**

	δ of ^{13}C									
position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
2	145.1	144.4	143.9	144.9	146.3	145.7	146.1	146.7	147.4	146.5
3	139.1	137.6	138.1	137.3	136.1	136.9	136.0	135.9	136.1	136.0
4	173.0	172.0	172.1	172.0	176.1	176.2	175.8	175.9	176.0	175.9
5	124.8	126.4	126.4	126.4	160.8	160.8	160.6	160.7	160.4	160.7
6	124.5	114.7	114.8	114.7	98.3	98.4	98.1	98.3	97.5	98.2
7	133.6	162.3	162.5	162.3	164.1	164.2	163.8	164.0	165.0	164.0
8	118.3	102.0	102.2	102.1	93.6	93.9	93.3	93.7	92.0	93.7
9	154.5	156.3	156.4	156.3	156.3	156.3	156.1	156.2	156.1	156.2
10	121.3	114.2	114.1	114.2	103.7	103.2	102.9	103.1	104.1	103.0
1'	131.3	123.9	126.7	122.6	123.3	126.3	123.3	122.1	121.9	120.8
2'	127.6	110.9	105.4	111.7	129.4	105.6	114.5	111.8	115.3	105.9
3'	128.5	148.4	152.7	147.4	114.1	152.8	146.2	147.4	145.1	147.8
4'	129.8	150.0	138.9	148.4	160.6	139.3	149.2	148.9	147.9	138.2
5'	128.5	111.5	152.7	115.5	114.1	152.8	111.7	115.6	115.6	147.8
6'	127.6	121.1	105.4	121.4	129.4	105.6	119.6	121.8	120.1	105.9
7-OMe	–	–	–	–	–	–	–	–	56.1	–
3-OMe	–	55.6	56.1	55.6	–	56.2	–	55.8	–	55.2
4-OMe	–	55.6	60.2	–	55.4	60.3	55.5	–	–	–
5-OMe	–	–	56.1	–	–	56.2	–	–	–	55.2

effect of hydroxyl and methoxyl groups on the ^{13}C NMR spectra can be assessed by comparing the ^{13}C chemical shifts of two isomeric flavonols, isorhamnetin (**8**) and tamarixetin (**7**), because the only one difference of these compounds is the substituted position of hydroxyl and methoxyl groups at C-3' and C-4'. The substitution effect of hydroxyl and methoxyl groups obviously shows that the *ortho* effect of the methoxyl substituent on the ^{13}C chemical shifts is more upfield of 3.3 ± 0.6 ppm than that of the hydroxyl substituent. However, the *para* effect of the hydroxyl substituent on the ^{13}C chemical shifts appears more upfield of 1.7 ± 0.5 ppm than that of the methoxyl substituent. Another evidence of the *para* substitution effect of hydroxyl and methoxyl groups on the ^{13}C NMR spectra shows the comparison of the chemical shifts of C-1' of derivatives, **3**, **6**, and **10**. The chemical shifts of B-ring are usually not affected with C-ring. Therefore, the substitution group of C-4' can only affect the chemical shifts of C-1' of the above derivatives. The table 2 shows that the *para* effect of the hydroxyl substituent on the ^{13}C chemical shifts of the derivative **10** appears more upfield of 5.7 ± 0.2 ppm than that of the methoxyl substituent of derivatives **3** and **6**. The derivatives **2** and **9** show the same result as the *para* substitution effect. The substituents of C-3' do not affect the chemical shifts of C-1' due to the *meta* position. As a result, the hydroxyl group of C-4' of the derivative **9** causes the chemical shift of C-1' to move more upfield of 2 ppm than the methoxyl group of that of the derivative **2**. The results obtained in this study provide a useful tool for structural analysis of various flavonol derivatives. In conclusion, the substitution effect of the hydroxyl and methoxyl groups on the ^{1}H and ^{13}C NMR data can be useful for identifying novel derivatives isolated from natural sources.

Experimental Section

Flavonol (**1**) and nine flavonol derivatives, 7-hydroxy-3',4'-dimethoxyflavonol (**2**), 7-hydroxy-3',4',5'-trimethoxyflavonol (**3**), 7,4'-dihydroxy-3'-methoxyflavonol (geraldol) (**4**), 5,7-dihydroxy-4'-methoxyflavonol (kaempferide) (**5**), 5,7-dihydroxy-3',4',5'-trimethoxyflavonol (**6**), 5,7,3'-trihydroxy-4'-methoxyflavonol (tamarixetin) (**7**), 5,7,4'-trihydroxy-3'-methoxyflavonol (isorhamnetin) (**8**), 5,3',4'-trihydroxy-7-methoxyflavonol (rhamnetin) (**9**), and 5,7,4'-trihydroxy-3',5'-dimethoxyflavonol (**10**), were purchased from INDOFINE chemical company, Inc. (Hillsborough, NJ). Their structures and nomenclatures are shown in Figure 1. Since they were supplied from the company at the purity of 98%, the chemicals were used for the experiments without further purification.

All NMR measurements were performed using Bruker Avance 400 spectrometer (9.4 T, Karlsruhe, Germany). The concentrations of the NMR samples were about 50 mM in DMSO-d_6. The ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC, and HMBC experiments were carried out at 298 K. The number scans and data points for the ^{1}H NMR were 16 and 32 K, respectively. Its 90° pulse was 10.2 μsec with a spectral width of 12 ppm. The ^{13}C NMR and DEPT spectra were obtained with a spectral width of 210 ppm using 64 K data points. Their 90° pulses were 10.3 sec. All two-dimensional spectra were acquired with 2.048 data points for t_2 and 256 for t_1 increments using magnitude mode. The long-ranged coupling time for HMBC was 70 msec. All NMR data were processed using NMRPIPE and analyzed with Sparky.[9]

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