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^{13}C-NMR SPECTROSCOPY OF BIFLAVANOIDS

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Key Word Index—^{13}C-NMR spectroscopy; naturally occurring C—C linked biflavanoids.

Abstract—The ^{13}C-NMR spectra of nine naturally occurring C—C linked biflavanoids have been assigned. The signals for the carbon atoms I-6, I-8, II-6, and II-8 appear in the region 90.0 ppm to 105.0 ppm. On the basis of the chemical shifts of these signals and their multiplicities in the off-resonance spectra, it is possible to determine the interflavonoid linkage in biflavanoids, provided that the A ring is involved. The level of oxidation of the ring C can be readily determined by a consideration of the chemical shift value of the carbonyl resonances. The position of the methoxyl substitution can also be inferred.

INTRODUCTION

Preliminary work on the ^{13}C-NMR spectral analysis of naturally occurring flavonoids [1, 2] demonstrated the suitability of this method for their structural analysis. We report in this communication on the utility of ^{13}C-NMR for the structure determination of biflavanoids*. The majority of the naturally occurring biflavanoids contain carbon–carbon linked monomers, with ring A usually being involved in the interflavonoid linkage [3, 4]. The combinations so far found in Nature are (I-6, II-6) (I-6, II-8), (I-3′, II-6), (I-8, II-8), (I-3′, II-8), and (I-3, II-8). Biflavanoids with a C—O—C linkage fall into two groups—the Hinokiflavone type (I-4′-O-II-6) and the Ochnaflavone type (I-3′-O-II-4′). Chemical degradation alone cannot differentiate whether I-6 or I-8 is involved in a C—C interflavonoid linkage. ^{1}H-NMR spectroscopy involving shifts of methoxyl signals in the spectrum of the permethyl ether, upon progressive addition of deuterobenzene [5], has been used for this purpose [6–8]. The shift of the signal occurs if one position *ortho-* to a given methoxyl group is unsubstituted. Though applied successfully in many cases, this method is restricted in its applicability. Thus in the case of hepta-*O*-methylsaharanflavone [8], one methoxyl signal does not shift at all on the addition of C_6D_6, supporting a (I-3, II-6) linked structure, in spite of the fact that a (I-3, II-8) linkage was later confirmed by synthesis. The use of the paramagnetic shift reagent Eu(fod)$_3$ helped to differentiate the signals due to H-3, H-6 and H-8 in 5,7-dimethoxyflavonoids [9] and this has been extended to the biflavanoid permethyl ethers [11, 12]. However, since both flavonoid moieties are complexed, different shifts may result from the same substituent on each nucleus. Hence a method of wider applicability is necessary for an unambiguous determination of the interflavonoid linkage in such compounds. The assignment of the signals in the ^{13}C-NMR spectra of ten oxygenated biflavanoids was achieved on the basis of off-resonance and proton coupled spectra and by analogy with published values [1] for the monomeric compounds. This method obviates the necessity of preparing the permethyl ethers which are obligatory for the ^{1}H-NMR solvent induced shift studies. As a consequence therefore, this method has potential also for the location of methoxyl substitution directly in a naturally occurring methylated biflavanoid.

RESULTS AND DISCUSSION

Linkages involving ring A only

The signals for C-6 and C-8 in the ^{13}C-NMR spectra of monomeric flavanones, flavones, and flavonols with a 5,7-dihydroxy substitution can be unambiguously differentiated by a consideration of their multiplicities in proton coupled spectra and by specific proton decoupling. For a large number of such compounds the resonances for these carbon atoms were found [1] between 90.0 ppm to 100.0 ppm. The signal for C-6 is always found to be at lower fields than C-8 in a variety of 5,7-dihydroxy compounds. This difference is small, *ca* 0.9 ppm, in the flavanones and larger, *ca* 4.8 ppm, in flavones and flavonols. In the case of permethyl-epicatechin the corresponding difference was 1.7 ppm. The signal for C-8 appeared downfield relative to that of C-6 and the assignment was confirmed by specific deuteration at C-8 [12]. On the basis of well established results [13] alkyl or aryl substitution on an aromatic nucleus should not essentially alter ($\pm$0.5 ppm) the chemical shift of the *meta*-carbon atoms. This is well exemplified by a comparison of the spectrum of pinocembrin (5,7-dihydroxyflavanone) with that of its 6-*C*-methyl and 8-*C*-methyl derivatives, as well as that of luteolin (5,7,3′,4′-tetrahydroxyflavone) and its 8-*C*-benzyl derivative. In all these compounds, the signal for the quaternary *C*-substituted carbon atom shifts by 6.0 to 9.6 ppm downfield whereas the signal for the unsubstituted carbon is not

* For nomenclature adopted, see reference [3].

Table 1. Chemical shifts (ppm, TMS = 0) of C-6 and C-8 in some 5,7-dihydroxyflavonoids [14]

Compound	δ	C-6	C-8
5,7-Dihydroxyflavanone (pinocembrin) [1]		96.1	95.1
6-*C*-Methylpinocembrin		102.1	94.7
8-*C*-Methylpinocembrin		95.7	101.9
3′,4′,5,7-Tetrahydroxyflavone (luteolin) [1]		99.2	94.2
8-*C*-Benzylluteolin		98.6	103.8
6-Hydroxyluteolin		140.4	93.6

markedly altered. Even a C-6 hydroxyl substitution, as in 6-hydroxyluteolin, only slightly alters the position of the signal for C-8 compared with that of luteolin (see Table 1).

In the spectrum of cupressuflavone (I-8, II-8 biapigenin) (see Fig. 1), there are only 13 resonances present due to the high symmetry of the molecule. The signal for I-6 and II-6 appears at 99.0 ppm whereas the signal for I-8 and II-8 shifts downfield (relative to apigenin) to 98.7 ppm, due to the substitution effect of the interflavanoid linkage. These assignments were confirmed by the proton coupled ^{13}C-NMR spectrum. Methylation of both I-7 and II-7 hydroxyl groups in cupressuflavone shifts the signal for I-6 and II-6 upfield to 95.6 ppm whereas the I-8 and II-8 signal moves downfield to 99.1 ppm. Again confirmation was achieved by taking the proton coupled spectrum in which the signal at 99.1 ppm exhibited a $^3J_{CH}$ interaction with I-6 hydrogen atom. In the hexa-*O*-methyl ether of cupressuflavone the signal for both I-8 and II-8 appears downfield relative to that of apigenin-tri-*O*-methyl ether, at 101.2 ppm whereas I-6 and II-6 are not appreciably shifted. The spectrum of agathisflavone (I-6, II-8 biapigenin) shows eight distinct resonances in the region 93.0 ppm to 104.0 ppm. The signals for the unsubstituted carbon atoms I-8 and II-6 appear at the expected values 93.7 ppm and 98.9 ppm respectively while I-6 and II-8 had their resonances at 103.6 ppm and 99.4 ppm. The downfield shifts experienced by the latter two carbon atoms of 4.7 ppm to 5.7 ppm are due to the substitution effect of the interflavonoid linkage. The other four signals between 102.8 ppm and 104.0 ppm can be assigned to the carbon atoms I-3, II-3, I-10 and II-10 respectively. There are seven signals in the region 94.0 ppm to 104.0 ppm in the spectrum of rhusflavone (naringenin I-6, II-8 apigenin). They can be assigned by analogy with agathisflavone itself and the published values for naringenin. Thus the carbon atoms II-6 and II-8 of rhusflavone and agathisflavone had almost identical chemical shift values 98.8 ppm and 99.5 ppm for the respective carbon atoms. The carbon atom I-6 resonated at 100.3 ppm. The difference in the level of oxidation of the two C rings in rhusflavone is clearly reflected in two well separated carbonyl resonances for I-4 (196.5 ppm) and II-4 (182.3 ppm) [1]. This thus

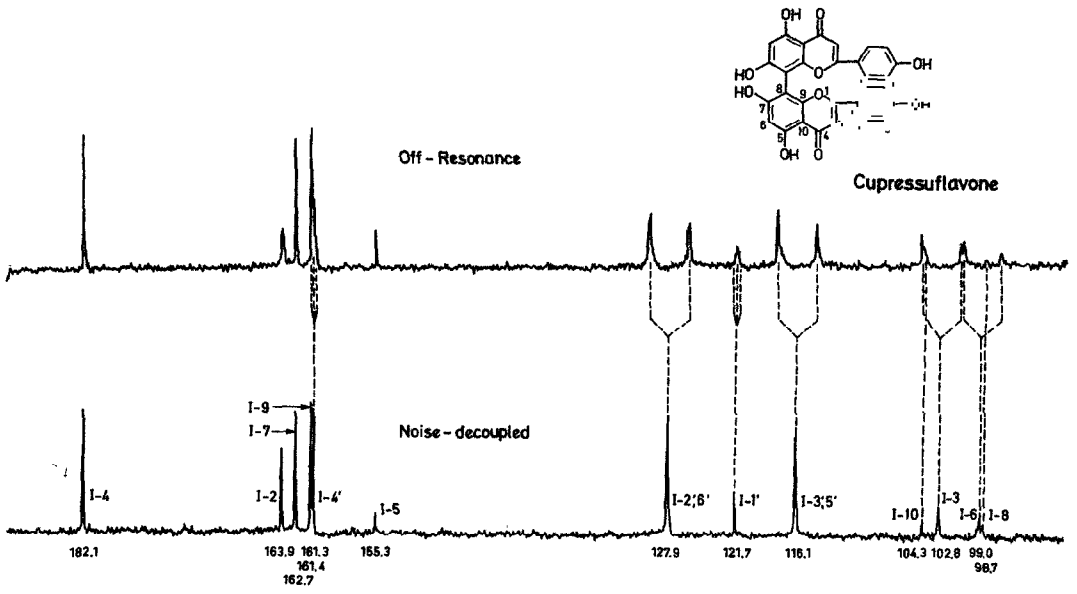

Fig. 1. ^{13}C noise decoupled and off-resonance spectra of cupressulflavone in DMSO-d_6 90°.

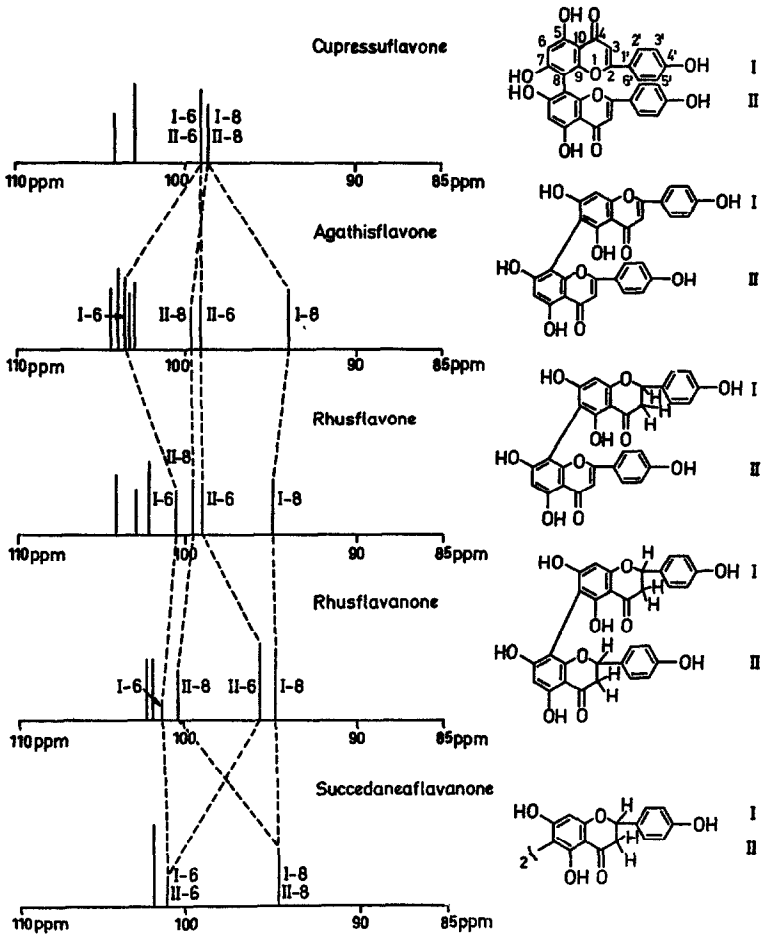

Fig. 2. Correlation of I-6, II-6, I-8 and II-8 carbon atom signals in ^{13}C-NMR spectra of A-ring linked biflavanoids.

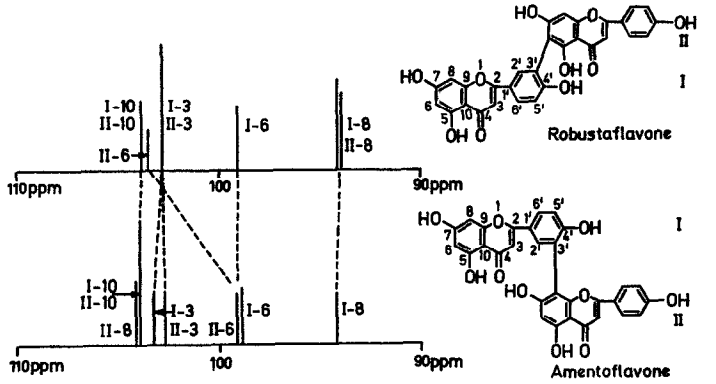

Fig. 3. Correlation of carbon atom signals in the ^{13}C-NMR spectra of amentoflavone and robustaflavone in the region 90 ppm to 110 ppm.

Table 2. ^{13}C-NMR Shifts of Biflavanoids in DMSO-d_6 (ppm, TMS = 0)

Compound	Moiety	2	3	4	5	6	7	8
Cupressuflavone	I	163.9	102.8	182.1	161.3	99.0	162.7	98.7
	II	163.9	102.8	182.1	161.3	99.0	162.7	98.7
Apigenin [1]		164.1	102.8	181.8	161.5	98.8	163.7	94.0
Genkwanin [1]		164.6	103.4	182.3	161.8	98.2	165.6	92.9
I-7,II-7-Di-*O*-methyl-cupressuflavone	I	163.9	102.7	182.2	161.2	95.6	163.2	99.1
	II	163.9	102.7	182.2	161.2	95.6	163.2	99.1
Tri-*O*-methyl-apigenin		160.4	106.8	176.0	160.0	93.3	163.7	96.2
Hexa-*O*-methyl-cupressuflavone	I	160.7	106.2	175.9	155.6	92.9	161.5	101.2
	II	160.7	106.2	175.9	155.6	92.9	161.5	101.2
Agathisflavone	I	164.1[a]	103.1[b]	182.3[e]	160.0	103.6[e]	162.9[f]	93.7
	II	163.9[a]	102.8[b]	182.1[e]	160.9	98.9	162.7[f]	99.4
Rhusflavone	I	78.7	42.2	196.5	161.8	100.3	165.2	94.8
	II	163.8	102.8	182.3	160.7	98.8	162.8	99.5
Naringenin [1]		78.4	42.0	196.2	162.9	95.9	166.7	95.0
Rhusflavanone	I	78.6[a]	42.3[b]	196.4[e]	161.8	101.2	165.2	94.6
	II	77.9[a]	42.1[b]	196.3[e]	161.8	95.7	165.2	100.3
Succedaneaflavanone	I	78.5	42.2	196.3	161.8	101.1	165.3	94.7
	II	78.5	42.2	196.3	161.9	101.1	165.3	94.7
Robustaflavone	I	164.1	102.9	181.7[a]	161.5[d]	99.0	163.7[b]	94.0[c]
	II	164.1	102.9	181.8[a]	160.1[d]	103.5	163.1[b]	93.9[c]
Amentoflavone	I	164.0[a]	103.2[b]	181.8[c]	161.6	98.9[d]	163.9[e]	94.2
	II	164.3[a]	102.8[b]	182.2[c]	160.8	99.1[d]	162.0[e]	104.1
Volkensiflavone	I	81.4	48.2	196.0	163.7	96.4	166.6	95.3
	II	163.7	102.8	181.6	160.4	98.5	162.8	100.6

Assignments bearing the same alphabetical superscript in any one spectrum may be reversed.

demonstrates the potential of ^{13}C-NMR spectroscopy when one is dealing with biflavanoids with differing oxidation levels in the C rings. The spectral region 93.0 ppm to 103.0 ppm, in the spectrum of rhusflavanone (I-6, II-8 binaringenin), comprises six resonances. The methine carbon lines for I-8 and II-6 appear as expected at 94.6 ppm and 95.7 ppm respectively. The signals at 101.2 ppm and 100.3 ppm can be assigned to I-6 and II-8 and those at 101.8 ppm and 102.2 ppm to I-10 and II-10 carbon atoms. Whereas a I-6, II-6 biapigenin is known only as a synthetic product, the corresponding I-6, II-6 binaringenin—succedaneaflavanone—is however known to occur in nature. As in the case of cupressuflavone, the spectrum of succedaneaflavanone showed only 12 signals due to the high symmetry of the molecule and the coincidence of the signals for I-5, II-5, I-9 and II-9 carbon atoms. The signal for I-6 and II-6 appears at 101.1 ppm whereas the unsubstituted I-8 and II-8 carbon atoms resonate at 94.7 ppm. The signal at 101.9 ppm for two quaternary carbon atoms can be assigned only to the I-10 and II-10 atoms in succedaneaflavanone. The signals at 101.1 ppm and 101.9 ppm were differentiated by the fact that the signal intensity of the latter was much greater than that of the former, reflecting different relaxation behaviour. The assignment of the signals for I-2, II-2, I-3, II-3, and the carbon atoms of the rings I-B and II-B in all the above compounds were made by analogy with apigenin, its methyl ethers and naringenin.

Linkages involving rings A and B

The three methine carbon atoms II-8, I-6 and I-8 of robustaflavone (I-3′, II-6 apigenin) can be easily assigned to the signals at 93.9 ppm, 99.0 ppm and 94.0 ppm respectively. The signal for II-6 carbon atom appears at 103.5 ppm. The spectral interval 116.0 ppm to 131.0 ppm consists of eight resonances of the carbon atoms of the two B rings. This is almost identical with the same spectral region of amentoflavone (I-3′, II-8 biapigenin), and the signals can be easily assigned on the basis of shifts expected for aryl substitution at I-3′ and the off-resonance spectrum. The signals for the three carbon atoms I-6, I-8, and II-6 in the spectrum of amentoflavone can also be readily identifed to be at 98.9 ppm, 94.2 ppm and 99.1 ppm respectively. The II-8 resonance appears at 104.1 ppm and is 5.6 ppm more downfield as compared with the position of the same signal in cupressuflavone.

Linkages involving I-3

The only biflavonoid included in this study with a (I-3, II-8) linkage was volkensiflavone (naringenin I-3, II-8 apigenin). The spectrum of this compound at 100° showed 22 resonances with coincidence of the signals for I-5, I-9, and II-2 (at 163.7 ppm), I-1′, I-2′, II-2′, I-6′ and II-6′ (at 128.1 ppm); II-3′ and II-5′ (at 115.9 ppm) and I-3′ and 1-5′ (at 114.6 ppm). The resonances for I-2 and I-3 appeared at 81.4 ppm and 48.2 ppm respectively and are 3.0 ppm and 6.2 ppm downfield of the corresponding ones of naringenin. These shifts are due to the β and α substituent effect of the 8-apigeninyl moiety at the carbon atom I-3. The resonances for I-6 and I-8 appear at 96.4 ppm and 95.3 ppm whereas those of II-6 and II-8 appear at 98.5 ppm and 100.6 ppm respectively. As is to be expected the two carbonyl resonances at 196.0 ppm (I-4) and 181.6 ppm (II-4) reflect the respective levels of oxida-

9	10	1′	2′	3′	4′	5′	6′	OMe
155.3	104.3	121.7	127.9	116.1	161.1	116.1	127.9	—
155.3	104.3	121.7	127.9	116.1	161.1	116.1	127.9	
157.3	103.7	121.3	128.4	116.0	161.1	116.0	128.4	—
157.7	105.0	121.6	128.8	116.3	161.8	116.3	128.8	56.0
153.9	104.3	121.0	127.9	115.9	161.2	115.9	127.9	56.5
153.9	104.3	121.0	127.9	115.9	161.2	115.9	127.9	56.5
159.3	108.5	123.2	127.6	114.4	161.9	114.4	127.6	56.1, 55.9, 55.4
159.4	108.0	122.9	126.9	114.4	161.6	114.4	127.9	56.3, 56.2, 55.4
159.4	108.0	122.9	126.9	114.4	161.6	114.4	127.9	
157.0[d]	103.8[e]	121.5[g]	128.6[h]	116.2	161.3	116.2	128.6[h]	
155.1[d]	104.0[e]	121.7[g]	128.2[h]	116.2	161.2	116.2	128.2[h]	—
162.4	101.9	129.1	128.2	115.5	157.9	115.5	128.2	
157.9	103.9	121.7	128.4	116.2	161.2	116.2	128.4	—
163.6	101.8	128.9	128.2	115.2	157.8	115.2	128.2	
162.7	101.8[d]	129.2	128.1	115.4	157.7	115.4	128.1	—
161.6	102.1[d]	129.2	127.6	115.3	157.3	115.3	128.1	—
161.9	101.9	129.3	128.3	115.4	157.8	115.4	128.3	
161.9	101.9	129.3	128.3	115.4	157.8	115.4	128.3	—
157.5	103.9	121.4[e]	127.2	120.8	159.2	116.7[f]	131.0	
156.6	103.9	121.6[e]	128.3	116.1	161.1	116.1[f]	128.3	—
157.6	104.0	121.3[f]	127.9	121.6[f]	159.6	116.4	131.6	—
154.7	104.0	120.3	128.3	116.0	161.1	116.0	128.3	—
163.7	101.7	128.1	128.1	114.6	162.0	114.6	128.1	—
157.3	103.6	121.3	128.1	115.9	161.0	115.9	128.1	—

tion of the two C rings. The presence of conformational equilibrium, due to inhibition of free rotation about the C—C bond in the interflavonoid linkage, in volkensiflavone was demonstrated by running the spectrum at 35°. Two signals of differing intensity for each of the carbon atoms I-2 (81.3 ppm and 82.4 ppm) and I-3 (48.7 ppm and 47.9 ppm) are present in the spectrum. In addition, several carbon resonances were considerably broadened.

Location of methoxyl groups

The position of a methoxyl substituent in a biflavanoid cannot be determined on the basis of the chemical shift of the methoxyl carbon as the variation is too small to be of diagnostic value. However, steric crowding as in the case of 5,6,7-tri-*O*-methyl compounds does cause a downfield shift of the methoxyl carbons by *ca* 6.0 ppm, whereas in a 5-hydroxy-6,7-dimethoxy derivative the shift is only of the order of 3.0 ppm [14]. The downfield shift of the signal for the carbon, bearing the hydroxyl group, on methylation is also variable. However, the upfield shift of the signal for the *ortho* carbon atoms is more reliable diagnostically, thus enabling an indirect determination of the site of *O*-methylation [1].

In conclusion, it is apparent that mere inspection of the chemical shift values in the spectral region 90.0 ppm to 105.0 ppm, in the ^{13}C-NMR spectrum of a biflavanoid, can give a good indication of the linkage positions if ring A is involved in a C—C linkage. This may be additionally confirmed by a normal off-resonance spectrum. In the event of any new C—C linked biflavanoid being isolated, involving the rings A in the linkage, this method will be ideal for determining the linkage positions.

EXPERIMENTAL

The ^{13}C-NMR spectra were recorded at 25.16 MHz. The deuterium signal of the DMSO-d_6 solvent was used as the lock signal. The spectral width was 5000 Hz J_{CH} couplings were usually studied at a weak hetero-decoupling level ($\Delta\nu \approx 8$ ppm and $\gamma H_2 \approx$ kHz). The quantity of biflavanoid samples used were *ca* 70–80 mg except in the case of cupressuflavone (166 mg) and its hexa-*O*-methyl ether (115 mg) where more material was available. The spectra were run between 100° to 50° in tubes of 5 mm outer diameter. The hexa-*O*-methyl ether of cupressuflavone was prepared by methylation of cupressuflavone (Me_2SO_4–K_2CO_3–Me_2CO). The other biflavanoids used in this study were isolated from the sources indicated: (±)-cupressuflavone (ex *Cupressus obtusa*), (±) I-7, II-7-di-*O*-methylcypressuflavone (ex *Araucaria rulei*), (±)-Amentoflavone (ex *Viburnum prunifolium*), (±)-agathisflavone, (−)-robustaflavone, (−)-rhusflavone, (−)-rhusflavanone, (−)-succedaneaflavanone all ex *Rhus succedanea* [15], and (∓)-volkensiflavone (ex *Garcinia multiflora*). The other flavonoids used were 6-*C*- and 8-*C*-Methylpinocembrin (ex *Melaleuca quiquenervia*) and synthesised samples of 8-*C*-benzylluteolin and 6-hydroxyluteolin.

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Note added in proof: Subsequent specific irradiation experiments and a reappraisal of the above data prompt us to reverse the C-5 and C-9 assignments in all the apigenin moieties in the biflavanoids studied. As the corresponding assignments in the apigenin derivatives studied in [1] were initially tentative we have inserted the correct values in Table 2. Since dispatch of this manuscript, our attention was drawn to the recent

publication on the ^{13}C-NMR spectra of some flavanoid derivatives (Pelter, A., Ward, R. S. and Gray, T. S. (1976) *J. Chem. Soc. Perkin I* 2475).

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