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STRUCTURAL ELUCIDATION OF POLYMETHOXYFLAVONES FROM SHIFT REAGENT PROTON NMR MEASUREMENTS*

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Key Word Index—Polymethoxyflavones; ^{1}H NMR; shift reagents; Pr(fod)$_3$; structural elucidation.

Abstract—The quantitative shift reagent behavior of polymethoxylated flavones in the presence of Pr(fod)$_3$ shows that for structural elucidation of these molecules the degree of substitution in the neighborhood of the carbonyl group can be determined from the number of signals that are strongly shifted and broadened. The induced shifts of the remaining signals are of complementary help and even the resonances of individual methoxyl groups can be ascribed.

INTRODUCTION

In an earlier work [1], we described the quantitative behavior of all possible monomethoxyflavones and that of 3,5-dimethoxyflavone in the presence of Pr(fod)$_3$. This shift reagent turned out to be a convenient one for the study of these compounds, since good association occurs and the upfield induced shifts of aromatic protons rarely require one to offset the spectrometer. The experimental data allow one to estimate the lanthanide atom position during pseudocontact interaction with the flavone carbonyl group. It was found from these model compounds that four groups of flavones have to be considered for the elucidation of substitution patterns, in contrast to other workers who deduced [2–5] general rules using flavones always having a 5-OMe.

In order to test the validity of our predictions, we now describe a shift reagent study of a representative sample of polymethoxyflavones derived from natural products in which we have also been able to ascribe the individual methoxyl resonances of the molecules studied.

RESULTS AND DISCUSSION

The chemical shifts of the individual aromatic protons and those of the methoxyl groups attached to the flavones are summarized in Table 1. The values in the 6–8 ppm region correspond to the protons on sp^2 carbons, while those around 4 ppm are due to the methoxyl groups. The assignment of H-3 and of the aromatic protons could be done directly from the spectra by considering the multiplicity of the signals, the magnitude of their coupling constants [6], the effect of methoxy groups at the various positions of the flavone skeleton and taking into account the spectra of all possible monomethoxyflavones. These assignments were also confirmed after the shift reagent experiments. The substitution patterns of the flavones studied, include representatives of most of the commonly occurring natural products [7–9] and in general the chemical shifts are in agreement with predictions considering the effects of introducing methoxyl groups, although it is worth mentioning that in zapotin (5,6,2′,6′-tetramethoxyflavone) the signal of H-3 is shifted to higher fields, mainly due to a through space effect of the methoxyl groups at the 2′ and 6′ positions.

The assignment of the individual methoxyl resonances could be achieved in some cases from the data of monomethoxyflavones and in others after performing the shift reagent experiments. Since the methoxyl signal in 3-methoxyflavone appears at 3.85 ppm while that of 7-methoxyflavone appears at 3.93 ppm, in the spectrum of 3,7,3′,4′-tetramethoxyflavone the signal at 3.83 is ascribed to the substituent at C-3 and that at 3.90 to the 7-OMe. The remaining 6H singlet is ascribed to the 3′ and 4′ methoxyls. The shift reagent behavior confirms these assignments. Furthermore, all compounds having a substitution pattern at the B ring with methoxyl groups at 3′ and 4′ showed a single resonance peak in the 3.98–3.95 ppm region for both groups. In 5,6,2′,6′-tetramethoxyflavone the B ring substituents appear at 3.80 ppm. The A ring substituents were ascribed from the shift reagent treatment, which shows that the 5-OMe is found at 4.00 ppm and the 6-OMe at 3.93 ppm.

Three of the studied compounds have methoxyl groups in the 5 and 7 positions and different degrees of substitution at the B ring. The 5-methoxyl signal appears always in the 3.95–3.97 ppm region while the 7-OMe is found between 3.90 and 3.92 ppm in agreement both with the shift reagent behavior and the chemical shift of the monomethoxyflavones, in which the 5-OMe is at 4.00 ppm and the 7-OMe at 3.93 ppm.

A group of five substances possess substituents at 3, 5 and 7 with further methoxyls at the B ring. Since the substituent signals of 3,5-dimethoxyflavone, which are included in Table 1 have been ascribed unambiguously from isotope labeling experiments [1] and the spectrum

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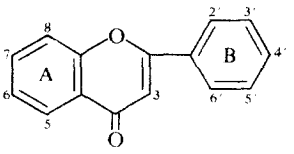
Table 1. Proton chemical shifts of flavones (in ppm from internal TMS)



Compound	Position									
	3	5	6	7	8	2′	3′	4′	5′	6′
Flavone*	6.82	8.23	7.40	7.68	7.55	7.91	7.50	7.54	7.50	7.91
3-OMe*	3.85	8.13	7.39	7.48	7.20	7.95	7.39	7.42	7.39	7.95
3,7,3′,4′-$(OMe)_4$	3.83	8.13	6.98	3.90	6.93	7.72	3.98	3.98	6.99	7.73
5-OMe*	6.77	4.00	6.83	7.62	7.13	7.90	7.60	7.64	7.60	7.90
5,6,2′,6′-$(OMe)_4$	6.27	4.00	3.93	7.23	7.23	3.80	6.62	7.38	6.62	3.80
5,7-$(OMe)_2$	6.65	3.96	6.37	3.92	6.56	7.87	7.47	7.52	7.47	7.87
5,7,4′-$(OMe)_3$	6.58	3.95	6.35	3.90	6.57	7.82	7.00	3.87	7.00	7.82
5,7,3′,4′-$(OMe)_4$	6.57	3.97	6.34	3.92	6.54	7.31	3.95	3.95	6.94	7.48
3,5-$(OMe)_2$	3.92	4.02	6.77	7.55	7.07	8.08	7.48	7.53	7.48	8.08
3,5,7-$(OMe)_3$	3.92	3.97	6.36	3.92	6.53	8.11	7.47	7.52	7.47	8.11
3,5,7,4′-$(OMe)_4$	3.88	3.95	6.33	3.88	6.48	8.08	7.02	3.88	7.02	8.08
3,5,7,2′,4′-$(OMe)_5$	3.80	3.95	6.32	3.87	6.42	3.83	6.58	3.87	6.60	7.35
3,5,7,3′,4′-$(OMe)_5$	3.92	3.98	6.32	3.92	6.48	7.67	3.98	3.98	6.93	7.68
3,5,7,3′,4′,5′-$(OMe)_6$	3.90	3.95	6.32	3.91	6.48	7.35	3.95	3.95	3.95	7.35
3,5,6,7,4′-$(OMe)_5$	3.92	4.03	3.93	3.98	6.75	8.07	7.02	3.92	7.02	8.07
3,5,6,7,3′,4′-$(OMe)_6$	3.87	4.02	3.92	3.97	6.77	7.65	3.97	3.97	6.98	7.67

* From ref. [1].

of 5,7-dimethoxyflavone has been discussed already, the signals due to the methoxyls at C-3, C-5 and C-7 were assigned as shown in Table 1. The B ring methoxyls of this group of flavones were ascribed from symmetry considerations in the case of 3′,4′,5′-substitution, from the previously discussed cases for 4′ and for 3′,4′-substitutions and from the shift reagent treatment for the 2′,4′-substitution, since the 2′-OMe is shifted more than the 4′-OMe in the corresponding monomethoxyflavones.

Finally, there are two flavones having methoxyl groups at 3, 5, 6 and 7, with further groups in the B ring. The 3-OMe group is ascribed in the 3.90 ppm region and the signal near 4.02 ppm is due to the 5-OMe, in agreement with previous values. The substituents at 6 and 7 could be ascribed after the shift reagent experiments. It is interesting to note that on comparing the methoxyl data of Table 1 with all monomethoxyflavones [1] even in the case of the highly substituted A ring compounds there is good chemical shift agreement. Furthermore, in all cases in which the assignments of methoxyl signals were done from the shift reagent measurements, very good agreement between predicted and experimental signals was always found. These assignments were done by extrapolating the induced chemical shifts to zero shift reagent concentration on plots (Fig. 1) relating the induced shifts with the shift reagent to substrate ratio. To our knowledge this appears to be the first systematic assignment of methoxyl signals in substituted flavones.

In order to gain satisfactory data that account for the quantitative behavior of the polymethoxylated flavones in the presence of the $Pr(fod)_3$ shift reagent, the experimental chemical shifts obtained after each of five successive additions of 0.01 mmol of the shift reagent to solutions containing 0.1 mmol of the corresponding flavone in deuterochloroformic solutions, were plotted as shown in Fig 1. A careful analysis of the plots shows that a great similarity in the behavior of the various protons of the molecules is obtained on going from a given molecule to others, thus allowing the assignment of each signal, or alternatively the deduction of a given substitution pattern.

The chemical shifts of the individual protons and of the methoxyl signals after each shift reagent addition, were treated in a least-square adjustment and the results are summarized in Table 2. They correspond therefore to a 1:1 molar ratio of shift reagent to substrate. Their regression coefficients were in general higher than 0.993 although for three lines only 0.985 was obtained. This shows that there is a linear behavior in the shift reagent concentration range studied and that satisfactory correlations were obtained. For an easier evaluation of the values included in Table 2, those corresponding to methoxyl signals are underlined.

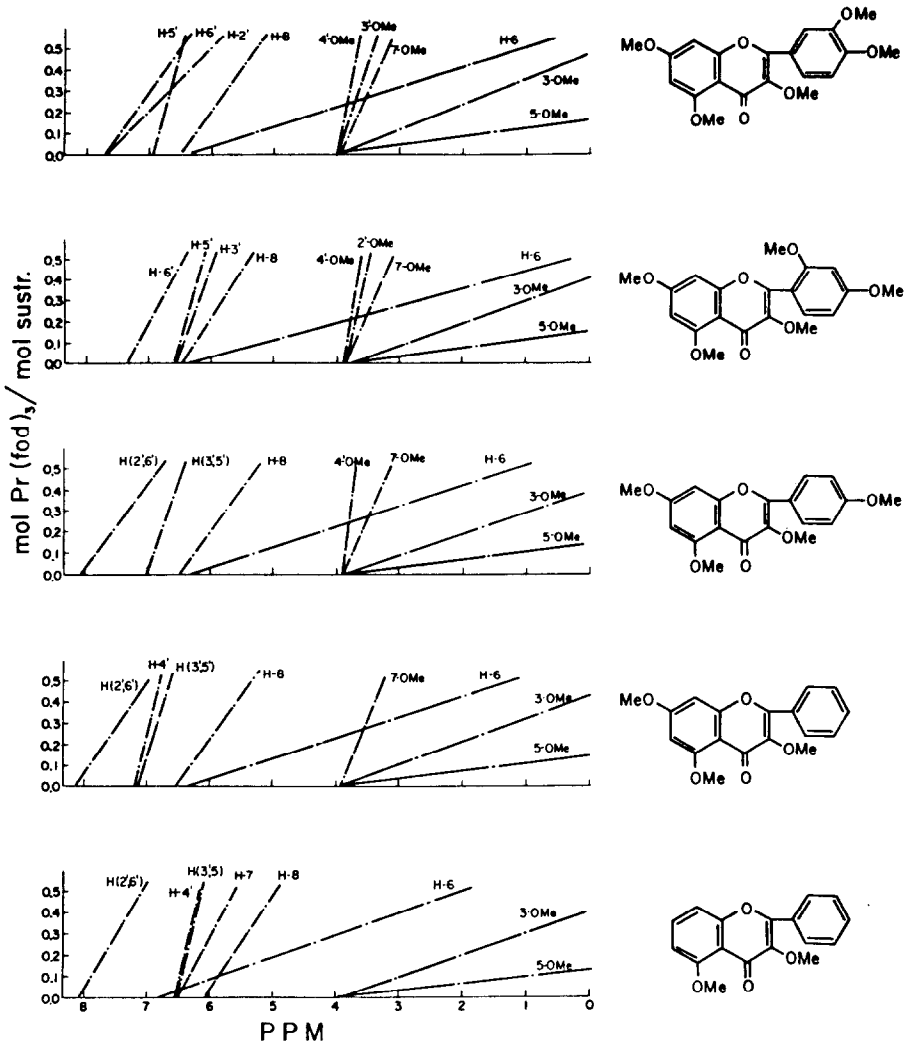


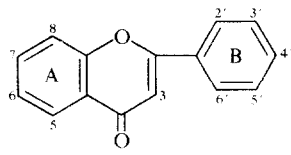
Fig. 1. The behavior of some polymethoxyflavones in the presence of $Pr(fod)_3$.

By comparing the lanthanide-induced shifts of 3-methoxyflavone and of 3,7,3′,4′-tetramethoxyflavone with those of flavone, it becomes evident that the introduction of a methoxyl group at C-3 changes the shifting order. Thus, while in the parent molecule the A ring protons are more shifted than those of the B ring, this situation reverses in flavones with a 3-OMe and is particularly distinctive for the 2′ and 6′ positions. This is due to the fact that in 3-methoxyflavones the lanthanide atom interaction occurs simultaneously at the carbonyl and the methoxyl oxygens in a bidentate type association, which is further substantiated since the 3-OMe signal broadens in such a way that its height corresponds approximately to 40% of that of other methoxyl groups.

When the effect of the shift reagent is evaluated for flavones that possess a methoxyl group at C-5 and are unsubstituted at the 3-position, the 5-OMe signal is shifted and broadened much more than the methoxyl group in 3-substituted flavones. The magnitude of the shift is indicated in Table 1, while the broadening effect of the shift reagent is so severe that the height of the signal is only about 10% of that of the other methoxyl groups of the same substrate. The bidentate type interaction for 5-MeO flavones has a six-center geometry, while that of 3-MeO flavones corresponds to only a five-center geometry and therfore more severe broadening and bigger induced shifts are obtained for 5-methoxy than for 3-methoxy substituents. Furthermore, while in the parent molecule H-3 is shifted further than H-8, which in turn is shifted further than H-6, in 5-MeO flavones the situation is completely different, since now H-6 is shifted more than H-3, which in turn is shifted more than H-8. This is also in agreement with the bidentate type complexation. The B ring protons in 5-methoxyflavones show only small shifts which are of similar magnitude to those of the parent molecule, the methoxyl groups at the 3′ (or 5′) and 4′ positions are moderately shifted, while those groups at the 2′ and 6′ positions are not shifted.

In those flavones that possess methoxyl groups at both the 3 and the 5 positions, the shift reagent treatment causes severe broadening of both of these substituents, the 5-OMe group being much more shifted and broadened than the 3-OMe, while the signals due to other parts of the molecules behave as in the case of 5-methoxyflavones that

Table 2. Induced chemical shifts for a 1:1 molar ratio of flavone $Pr(fod)_3$ (in ppm)



Compound	Position									
	3	5	6	7	8	2'	3'	4'	5'	6'
Flavone*	15.3	16.8	3.2	2.9	4.2	2.1	0.4	0.4	0.4	2.1
3-Me*	21.5	11.2	2.1	1.7	2.6	7.5	2.7	2.5	2.7	7.5
3,7,3',4'-$(OMe)_4$	18.6	9.4	1.8	2.9	2.8	7.1	2.3	1.1	2.9	5.5
5-OMe*	6.7	23.0	9.5	3.0	2.0	1.3	0.6	0.6	0.6	1.3
5,6,2',6'-$(OMe)_4$	4.8	25.0	5.8	3.5	2.3	0.1	0.4	0.3	0.4	0.1
5,7-$(OMe)_2$	6.9	26.8	10.8	1.7	2.4	1.4	0.6	0.5	0.6	1.4
5,7,4'-$(OMe)_3$	6.0	25.7	10.5	1.7	2.4	1.2	0.5	1.9	0.5	1.2
5,7,3',4'-$(OMe)_4$	5.7	25.7	10.4	1.7	2.3	1.2	0.6	0.3	0.5	1.1
3,5-$(OMe)_2$	9.8	31.6	9.5	1.8	2.2	2.0	0.7	0.7	0.7	2.0
3,5,7-$(OMe)_3$	9.3	28.2	10.4	1.4	2.4	2.2	1.0	0.8	1.0	2.2
3,5,7,4'-$(OMe)_4$	9.6	27.0	10.6	1.4	2.4	2.5	1.1	0.4	1.1	2.5
3,5,7,2',4'-$(OMe)_5$	9.1	26.5	9.8	1.4	2.1	0.7	1.0	0.5	0.9	1.8
3,5,7,3',4',-$(OMe)_5$	8.7	27.3	10.7	1.5	2.4	2.9	1.1	0.7	0.9	2.4
3,5,7,3',4',5'-$(OMe)_6$	7.5	27.0	10.0	1.4	2.1	2.0	0.8	0.6	0.8	2.0
3,5,6,7,4'-$(OMe)_5$	7.1	†	6.1	0.4	1.9	2.0	0.7	0.4	0.7	2.0
3,5,6,7,3',4'-$(OMe)_6$	7.5	†	7.3	1.0	2.2	2.0	0.7	0.4	0.8	1.9

* From ref. 1.

† The signal broadened so severely that this induced shift cannot be determined. Values underlined correspond to OMe groups.

are unsubstituted at C-3. This is also in agreement with the preferred geometry of complexation. The 3-OMe signal broadens as in the case of flavones having a proton at C-5 and a 3-MeO group, while the signal due to the 5-OMe group can hardly be seen over the baseline of the spectrum after the addition of only 0.1 mol of shift reagent per mol of substrate. Therefore, in those molecules having methoxyl groups at 3 and 5 simultaneously, only small amounts of shift reagent should be added.

This situation is particularly true for compounds with methoxyl groups at the 3, 5 and 6 positions in which addition of 0.1 mol $Pr(fod)_3$/mol substrate completely precludes the observation of the 5-methoxyl group. This fifth group of flavones has to be studied at much lower shift reagent to substrate ratios in order to determine the presence of all methoxyl groups. The lower trace of Fig. 2 corresponds to the 60 MHz proton NMR spectrum of 20 mg ($\sim$0.05 mmol) of 3,5,6,7,3',4'-hexamethoxyflavone in 0.3 ml $CDCl_3$. Addition of only 1 mg (0.001 mmol) of $Pr(fod)_3$ changed the spectrum to that shown in the central trace, in which the 5-methoxyl group appears as a very broad signal that can hardly be seen under the other methoxyl signals and where both the 3 and 6 methoxyls are also broad. The addition of a second mg of shift reagent (total 0.04 mol $Pr(fod)_3$/mol flavone) provided the upper trace of Fig. 2. After a further addition of shift reagent, the signal of the 5-OMe could not be detected and therefore the induced shifts of that group, in flavones with substitution at 3, 5 and 6 are not given in Table 2. When the spectra of 3,5,6,7,4'-pentamethoxyflavone and of 3,5,6,7,3',4'-hexamethoxyflavone are measured at higher concentrations of shift reagent, the broad signal due to the 3 and 6 methoxyls is resolved into two broad peaks. We tentatively assign the one most shifted to the 3-OMe group since the behavior of other signals in the group of 3,5,6 substituted molecules resembles more those having methoxyls at both 3 and 5, than other cases. Interestingly the broadening of the 6-methoxyl signal occurs only when two additional groups are present at 3 and 5, since in the case of 5,6,2',6'-tetramethoxyflavone, although the shift of the 6-MeO is large, the signal is not broadened by the shift reagent.

In conclusion it is possible to determine the structure of flavones from proton NMR measurements in the presence of shift reagents. The substitution in the neighborhood of the carbonyl group is deduced from the number of signals that broaden and its quantitative induced shifts, thus leading to five groups of flavones: (a) with protons at 3

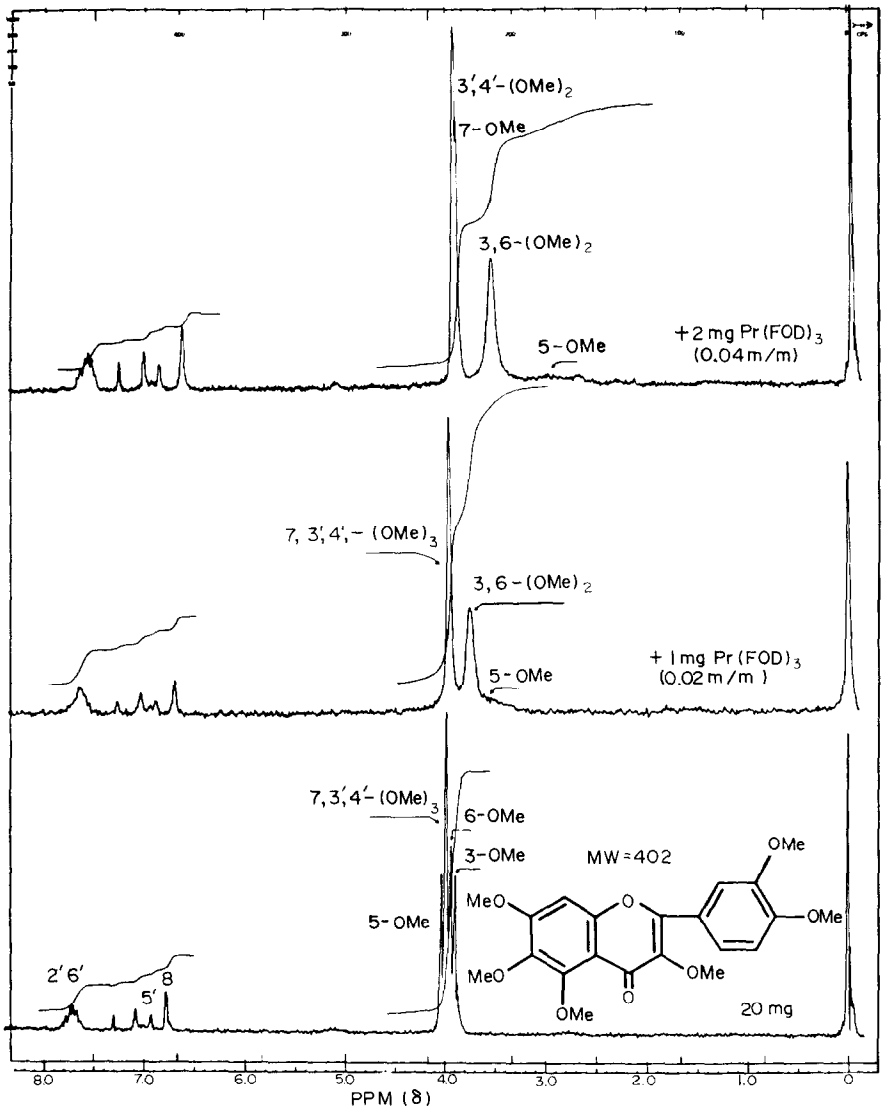


Fig. 2. ^{1}H NMR spectra of 3,5,6,7,3',4'-hexamethoxyflavone.

and 5; (b) proton at 5 and methoxyl at 3; (c) proton at 3 and methoxyl at 5; (d) methoxyls at 3 and 5; and (e) methoxyls at 3, 5 and 6. The remaining substitution pattern is deduced from the multiplicity of aromatic protons and the quantitative induced shifts of all signals. The distinction between free and methylated hydroxyl groups can be achieved by methylation using mixtures of dimethylsulphate with its hexadeuteroanalog, as has already be done in the case of 3,5-dimethoxyflavone [1] and even the position of sugar residues could be deduced by methylation with partially labeled methyl iodide reagents (CH_2DI and CHD_2I) which are commercially available. Complementary information could in some cases also been obtained using europium perchlorate for hydroxyflavones [10].

EXPERIMENTAL

General. The ^{1}H NMR spectra were obtained on Varian Associates A-60 and XL-100A-FT-16K spectrometers in $CDCl_3$ and tetramethylsilane as internal standard. The least-square adjustments were done on a Hewlett–Packard 9100A desk computer.

Polymethoxylated flavones. The completely methylated flavones were obtained by Me_2SO_4/K_2CO_3 treatment of hydroxylated molecules, followed by column chromatography purification using EtOAc washed alcoa F-20 alumina. This procedure was used for fisetin (3,7,3',4'-tetrahydroxyflavone), chrysin (5,7-dihydroxyflavone), acacetin (5,7-dihydroxy-4'-methoxyflavone), 5,7-dihydroxy-3',4'-dimethoxyflavone, galangin (3,5,7-trihydroxyflavone), kaemferol or robigenin (3,5,7,4'-tetrahydroxyflavone), morin (3,5,7,2',4'-pentahydroxyflavone), quercetin (3,5,7,3',4'-pentahydroxyflavone), myricetin (3,5,7,3',4',5'-hexahydroxyflavone) which were commercially available and for eupaletin [11] (3,5,4'-trihydroxy-6,7-dimethoxyflavone) and eupatoletin [11] (3,5,3',4'-tetrahydroxy-6,7-dimethoxyflavone) which were isolated from *Eupatorium lingustrinum*. Zapotin [12] (5,6,2',6'-tetramethoxyflavone) was isolated from *Casimiroa edulis*. No impurities were detected in the NMR spectra of the polymethoxyflavones.

Shift reagent study. Solutions containing 0.1 mmol of the individual flavone in 0.3 ml of $CDCl_3$ were prepared. After recording the corresponding spectra, 0.01 mmol of $Pr(fod)_3$ were added, the spectra recorded again and the procedure repeated until five shift reagent additions were completed. In the case of the 3,5,6 substituted molecules, only 0.05 mmol of flavone with individual additions of 0.002 mmol of shift reagent were also used. The chemical shifts of each individual signal in each spectrum were plotted as shown in Fig. 1. For the pulse-FT spectra 1 mg of flavone was used and additions of ~0.1 mg shift reagent were done.

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