

Regular Article*Highlighted Paper selected by Editor-in-Chief***Use of ^{13}C -NMR Chemical Shifts; Application of Principal Component Analysis for Categorizing Structurally Similar Methoxyflavones and Correlation Analysis between Chemical Shifts and Cytotoxicity**Ryuichiro Suzuki,^{*a} Yoshihiro Uesawa,^b Yoshihito Okada,^b Takumi Horikawa,^b Yui Okabe,^b Masaki Aburada,^c Kunio Takahashi,^b and Kaoru Kinoshita^{*b}^aFaculty of Pharmaceutical Sciences, Josai University; 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan; ^bMeiji Pharmaceutical University; 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan; and ^cFaculty of Pharmacy, Musashino University; 1-1-20 Shin-machi, Nishitokyo, Tokyo 202-8585, Japan.

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The ^{13}C -NMR spectral data for the 15-carbon flavonoid skeleton in eleven methoxyflavones isolated from *Kaempferia parviflora* (Zingiberaceae) were processed by principal component analysis (PCA). Based on the PCA score plots, the methoxyflavones were categorized into three groups according to their structural features. The cytotoxicities of the methoxyflavones toward 3T3-L1 murine preadipocyte cells were evaluated by 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay and found to differ according to structure. The relationship between the ^{13}C -NMR chemical shifts of the methoxyflavones and their cytotoxicities was investigated using Pearson's correlation analysis. The ^{13}C -NMR signal at C-10, a quaternary carbon, was correlated with cytotoxicity. Based on these results, a structural design which lowers the ^{13}C -NMR chemical shift at C-10 would be important for the development of cytotoxic compounds. Although quantitative structure–activity and structure–property relationships are well established paradigms for predicting trends among a series of compounds, quantitative property–activity relationships have been relatively unstudied. This approach offers a new strategy for directing structure–activity relationship research.

Key words methoxyflavone; ^{13}C -NMR chemical shift; principal component analysis; cytotoxicity; Pearson's correlation analysis

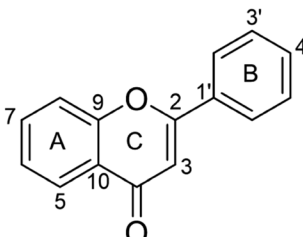
Introduction

NMR spectroscopy is an important physicochemical technique that finds wide application not only in fields such as chemistry and physics but also in medical science. In organic chemistry and natural products research, NMR is recognized as indispensable for analyzing the chemical structures of compounds, state of components, and the quality of natural medicines. ^{13}C -NMR spectroscopy enables the detection of a signal for each carbon atom in a molecule, such that the number of resonances often indicates the number of carbon atoms in that compound. Moreover, since ^{13}C -NMR chemical shifts are widely distributed across the spectrum, there is little signal overlap between carbon atoms except in cases of chemical and/or magnetic equivalence. ^{13}C -NMR signals are more easily distinguished than ^1H -NMR signals because they generally appear as singlets through the use of proton decoupling. In addition, ^{13}C -NMR chemical shifts are quite sensitive to structural features, although structurally similar compounds may have numerically similar chemical shifts. Thus, the chemical structures of unknown compounds can be determined and known compounds can be confirmed. Furthermore, the chemical shift of each carbon atom can be estimated based on the molecular structure. It is possible to estimate the structural similarity to the structure from the information of the chemical shift value obtained by measurement of ^{13}C -NMR; therefore, it is often used for structural elucidation. By comparing the ^{13}C -NMR data from numerous structurally related compounds, trends in chemical shifts induced by subtle differences in chemical structure can be detected.

Like its NMR spectrum, the biological activity of a compound also depends on its basic skeleton, functional groups, and substitution patterns. Although the overall structure of a compound affects its bioactivity, compounds with higher activity have been designed by strategically incorporating various substituents into the basic skeleton. The introduction of substituents changes not only the molecular structure but also the NMR chemical shifts for the basic skeleton. Given these facts, we sought to determine whether the NMR chemical shifts of the basic skeleton of a series of compounds would be predictive of their biological activity. If the biological effect of a compound could be related to the chemical shifts of its molecular framework, more effective compounds could be designed based on NMR data.

In this study, we used statistical methods to examine whether the ^{13}C -NMR spectral data from 11 methoxyflavones could be used to correlate their structural features and biological activities. The methoxyflavones were isolated from *Kaempferia parviflora* Wall. ex Baker (Zingiberaceae)¹ and these isolated methoxyflavones induced adipogenesis on 3T3-L1 preadipocytes by regulating transcription factors.² Several of these compounds have been reported to enhance lipolysis in mature adipocytes by activating adipose tissue triglyceride lipase and hormone-sensitive lipase, independent of peroxisome proliferator-activated receptor γ transcription.³ We considered the relationship between the ^{13}C -NMR spectral data of the methoxyflavones and their cytotoxicities toward 3T3-L1 murine preadipocyte cells. Principal component analysis (PCA) of the ^{13}C -NMR chemical shifts was conducted to categorize

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compound	C-3	C-5	C-7	C-3'	C-4'
1	OCH ₃	OH	OCH ₃		
2	OCH ₃	OCH ₃	OCH ₃		
3	OCH ₃	OCH ₃	OCH ₃		OCH ₃
4	OCH ₃	OH	OCH ₃		OCH ₃
5		OCH ₃	OCH ₃		
6		OH	OCH ₃		OCH ₃
7	OCH ₃	OH	OCH ₃	OCH ₃	OCH ₃
8	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃
9		OH	OCH ₃		
10		OCH ₃	OCH ₃		OCH ₃
11	OCH ₃	OH	OCH ₃	OH	OCH ₃

Fig. 1. Chemical Structures of Methoxyflavones Isolated from *Kaempferia parviflora*

the methoxyflavones based on their structural features. We previously reported the use of multivariate analysis, such as PCA with ¹H-NMR data in a chosen range, to analyze the cultivating places or the biological activities of various natural extracts.^{4,5} Several other studies have employed ¹³C-NMR data and multivariate analysis to distinguish the components of complex mixtures such as soy sauce.^{6,7} However, very little experimental work has combined multivariate analysis with ¹³C chemical shift data to establish predictive models. Rittner and Tasic⁸ reported α -substituent effects on the ¹³C-NMR chemical shifts in some aliphatic compounds by PCA. Verma and Hansch⁹ discussed the use of ¹³C-NMR data as a descriptor for the quantitative structure–activity/property relationship (QSAR/QSPR) paradigm in order to understand chemical–biological interactions. However, the multivariate analysis of ¹³C-NMR chemical shift data of the 15-carbon flavonoid skeleton as applied to the problem of their structure–cytotoxicity relationship has not been performed.

Results and Discussion

The eleven methoxyflavone structures and their ¹³C-NMR chemical shift values are presented in Fig. 1 and Table 1, respectively. The basic flavonoid skeleton comprises 15 carbons. Since the ¹³C-NMR chemical shift values of the framework carbons change very little among the flavonoids, the structural differences due to the substituent groups are sensitively reflected in the variation of ¹³C-NMR chemical shifts. Figure 2 shows the PCA score plots of the ¹³C-NMR spectral data for the eleven substances. PCA models are depicted as score plots and consist of two synthetic variables: principal component (PC) 1 (the greatest data variance) and PC 3 (the third greatest data variance, orthogonal to PC 2). The PCA score plot for PC 1 versus PC 3 clearly shows three independent groups (group 1: compounds 7, 8, and 11; group 2: compounds 3, 4, 6, and 10; and group 3, compounds 1, 2, 5, and 9). These groupings are related to the substituent pattern of the B ring. The compounds belonging to group 1 have methoxy or hydroxy groups at the C-3' position, whereas the compounds categorized into group 2 have only a methoxy group at C-4' in the B ring. Compounds 1, 2, 5, and 9, which belong to group 3, have no B-ring substituent.

The cell proliferation inhibitory activities of the 11 methoxyflavones on 3T3-L1 murine preadipocyte cells were examined by 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay. The results, shown in Fig. 3, indicate that the methoxyflavones differentially affect cell viability according to their structure. Overall,

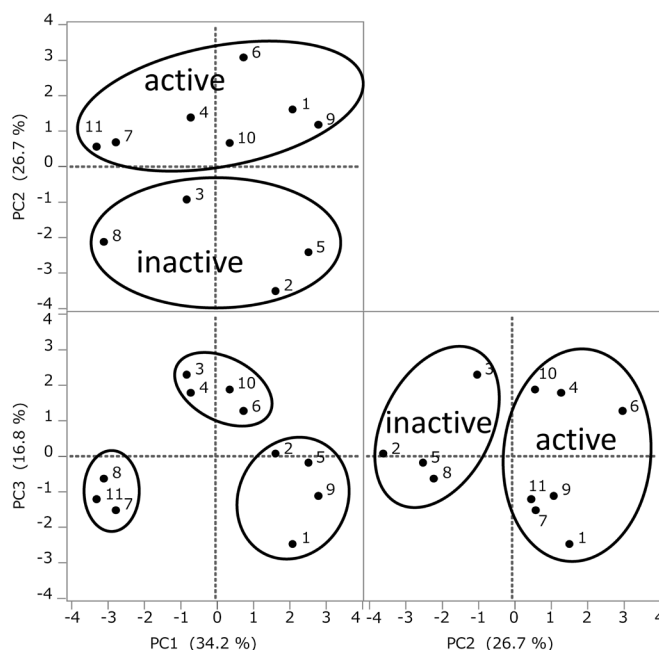


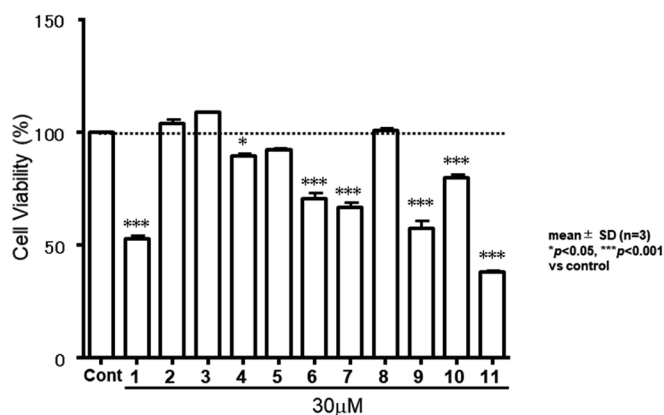
Fig. 2. Score Plot of Principal Component Analysis Derived from ¹³C-NMR Spectral Values of Methoxyflavones Isolated from *Kaempferia parviflora*

the active compounds have a hydroxyl group rather than a methoxy group at the C-5 position. Thus, compounds 2, 3, 5, and 8, which lack the C-5 hydroxy group, do not show inhibitory activity. Nevertheless, C-5-methoxylated compound 10 exerts moderate inhibitory activity on proliferation of the 3T3-L1 cells. Compound 11 is the strongest inhibitor, followed by compounds 1 and 9. The PCA score plot represented by PC 1 versus PC 2 reveals two clusters, i.e., active compounds (1, 4, 6, 7, 9, 10, and 11) and inactive compounds (2, 3, 5, and 8) (Fig. 2). Similarly, the plot of PC 2 versus PC 3 also reveals two clusters of active and inactive compounds. These score plots well describe the extent of activity: the active compounds can be divided into two groups, compounds 1, 7, 9, and 11 and compounds 4, 6, and 10, according to their activity.

The results of the bivariate relationship analysis between the chemical shifts of selected carbons in these methoxyflavones and their cell proliferation inhibitory activities using Pearson's correlation analysis are illustrated in Fig. 4. The C-10 chemical shift and cell proliferation inhibition are negatively correlated, whereas the C-4 and C-6 data are weakly

Table 1. ^{13}C -NMR Spectral Data of Methoxyflavones (1–11)

Position	1	2	3	4	5	6	7	8	9	10	11
C-2	164.1	152.8	152.5	155.8	159.8	164.2	156.5	151.9	157.9	162.0	154.9
C-3	139.5	142.0	140.9	138.8	109.0	105.8	138.7	140.7	106.0	109.1	137.5
C-4	178.7	174.3	173.9	178.6	177.5	182.6	178.5	173.4	182.6	177.6	177.4
C-5	165.4	161.2	161.0	161.9	160.8	162.8	161.7	160.4	164.1	160.8	160.3
C-6	97.8	92.7	95.6	97.8	92.7	98.3	97.7	95.3	98.4	96.0	97.2
C-7	155.7	164.2	163.7	165.3	164.0	165.6	165.2	163.4	165.8	163.9	164.5
C-8	92.1	96.1	92.3	92.1	96.1	92.9	92.0	92.1	92.9	92.8	91.7
C-9	156.7	159.1	158.6	156.6	160.5	157.9	156.5	158.2	162.3	159.8	155.6
C-10	106.1	109.8	109.4	106.5	109.2	104.8	105.8	110.4	105.9	107.6	104.6
C-1'	130.4	131.1	123.1	122.8	131.5	123.8	122.7	122.9	132.0	123.8	121.6
C-2'	128.2	128.3	129.7	130.1	125.8	128.3	110.7	110.8	126.5	127.6	114.5
C-3'	128.4	128.7	113.8	114.0	128.9	114.8	151.2	148.2	129.3	114.3	145.7
C-4'	130.7	130.5	160.8	161.6	131.1	162.4	148.6	150.4	131.5	160.6	149.7
C-5'	128.4	128.7	113.8	114.0	128.9	114.8	111.1	110.4	129.3	114.3	111.3
C-6'	128.2	128.3	129.7	130.1	125.8	128.3	122.0	121.2	126.5	127.6	119.8

Fig. 3. The Cytotoxicity of Methoxyflavones Isolated from *Kaempferia parviflora* for 3T3-L1 Cells

and positively correlated with 3T3-L1 cell proliferation. The C-10 chemical shifts range from 104 to 111 ppm, and the active compounds display lower chemical shifts. In a typical structure–activity correlation study, the hydroxyl group at C-5 would be important for predicting cell proliferation inhibitory activity. Indeed, compounds **1**, **4**, **6**, **7**, **9**, and **11**, containing a hydroxyl group at C-5, display higher inhibitory activities on cell proliferation.¹⁰ However, here we find that the presence of a hydroxyl group at C-5 is not necessary for increased activity, but rather, the introduction of a substituent that decreases the chemical shift value at C-10 is important. Compound **4**, which contains a C-5 hydroxyl group, shows moderate inhibitory activity. On the other hand, compound **10**, with a methoxy group instead of a hydroxyl group at C-5, delivers higher inhibitory activity than compound **4**. As a quaternary carbon, C-10 cannot undergo any substitution. Further, C-10 occupies the bridgehead position between the fused A- and C-rings in the flavone skeleton. It would be difficult to obtain such an insight in a typical structure–activity correlation study. Introduction of functional group to A ring of flavonoid affects chemical shift value at C-10. For example, substitution of hydroxyl group at C-5 or C-7 decrease chemical shift value at C-10. Meanwhile, chemical shift value at C-10 increase by introduction of methoxy group at C-6. Otherwise, chemical shift value at C-10 is affected by attachment of hydroxyl group at C-3.¹¹

Additional research is required to optimize this approach since the number of compounds examined in this study are not enough. We plan to consider deeply and carefully about quantitative structure–activity relationship using more test compounds including C-5 replaced other functional groups.

In general NMR spectral data are used for structure elucidation of compound. Furthermore, structurally similar compounds may have numerically similar chemical shifts. Thus, by comparing the NMR data from numerous structurally related compounds, trends in chemical shifts induced by subtle differences in chemical structure can be detected. From this viewpoint ^{13}C -NMR spectral data processed by statistical analysis were applied to structure–activity relationship research in this study. ^{13}C -NMR chemical shift values of isolated compounds were used as descriptor for quantitative structure–activity research. Using this technique, it can determine a position contributing to biological activity on carbon atom level. This method is different to general quantitative structure–activity analysis. This approach proposes a new application method of NMR spectral data.

Conclusion

The methoxyflavones isolated from *K. parviflora* were categorized according to their structural features on PCA score plots derived from their ^{13}C -NMR spectral data. Bivariate relationship analysis revealed that a decreasing chemical shift value at C-10 was related to an increase in the cytotoxicity of the methoxyflavone. As a result, multivariate analysis of ^{13}C -NMR spectral data of the common basic skeleton of the compounds and their biological activities could predict a candidate compound with potential as a therapeutic agent.

Experimental

Materials Isolation of the 11 flavonoids (Fig. 1) was performed as described previously.¹⁾

Cell Culture 3T3-L1 murine preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in a humidified atmosphere with 5% CO₂ until confluent. Two days after confluence, designated as day 0, the cells were switched to differentiation medium (DM) containing 1 μM insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone (DXM) in DMEM for another 2 d. Then, the cell culture

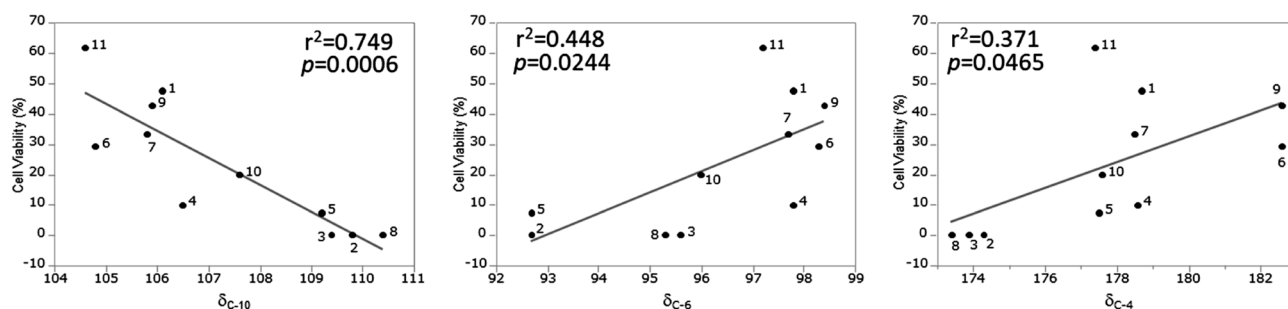


Fig. 4. Determination of Correlation Coefficient between ^{13}C -NMR Spectral Values of Methoxyflavones and Their Cytotoxicity for 3T3-L1 Cells (%)

medium was replaced with DMEM containing $1\ \mu\text{M}$ insulin, and incubation was continued for another 2 d. After day 4, the cells were maintained in DMEM with medium changes every 2 d, during which mature adipocytes containing lipid droplets formed. On day 8, when differentiation was almost complete, the cells were treated with methoxyflavones isolated from *K. parviflora*, or vehicle for up to 4 d (until day 12). All media contained 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin ($100\ \mu\text{g}/\text{mL}$).

Cell Viability Assay Cell viability was determined by MTT assay. On day 12, the confluent 3T3-L1 mature adipocytes treated with isolated methoxyflavones or vehicle in 96-well plate were switched into a $200\ \mu\text{L}$ culture medium containing 10% phosphate-buffered saline (PBS)-buffered MTT ($5\ \text{mg}/\text{mL}$) solution and incubated at $37\ ^\circ\text{C}$ for 4 h. Thereafter, the medium was removed and purple formazan crystals dissolved in dimethyl sulfoxide (DMSO) were added and absorbance was read at 595 nm using a microplate reader. Results were standardized using the vehicle group values.

^{13}C -NMR Measurements ^{13}C -NMR spectral data were recorded using a JEOL JNM-AL-400 or JEOL JNM-LA-500 spectrometer in $\text{DMSO}-d_6$ using tetramethylsilane (TMS) as an internal standard.

Statistical Analysis The ^{13}C -NMR spectral data sets obtained for the 15-carbon flavonoid skeletons were processed using JMP Pro12.2 software (SAS Institute Inc., Cary, NC, U.S.A.) for PCA and bivariate statistical analysis using Pearson's correlation analysis. The chemical shifts in the ^{13}C -NMR spectra were rounded down to one decimal place. Variables were standardized with a mean of 0 and standard deviation

of 1. All statistical analyses were performed using JMP Pro 12.2 software to identify the features contributing to group separation.

Conflict of Interest The authors declare no conflict of interest.

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