Insect Antifeedant Flavonoids from *Gnaphalium affine* D. Don

Masanori Morimoto,* Sumiko Kumeda, and Koichiro Komai

Department of Agricultural Chemistry, Kinki University, Nakamachi, Nara 3327-204, Japan

The antifeedant flavonoids, 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone (**1**), 5-hydroxy-3,6,7,8-tetramethoxyflavone (**2**), 5,6-dihydroxy-3,7-dimethoxyflavone (**3**), and 4,4′,6′-trihydroxy-2′-methoxychalcone (**4**), have been isolated from cudweed *Gnaphalium affine* D. Don (Compositae). Four natural flavonoids showed insect antifeedant activity against the common cutworm (*Spodoptera litura* F.). These flavonoids were detected in small amounts in the plant by HPLC analysis, but these natural compounds had strong antifeedant activity against the common cutworm. On the other hand, **4** was detected in a large amount in the plant, but this compound had only a slight activity. Therefore, these natural compounds were regarded as one of the plant's defensive systems against phytophagous insects along with the woolly plant surface. As for the structure−activity relationship, it is an advantage for antifeedant activity to have no oxy-substituents on the B-ring of the flavonoid but have an ether linkage such as a pyran in the chemical structure.

Keywords: *Gnaphalium affine; 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone; 4,4′,6′-trihydroxy-2′-methoxychalcone; antifeedant; Spodoptera litura*

INTRODUCTION

A part of the screening of natural products from wild plants in Japan is the potential application for the control of phytophagous pests. Additionally, there is the chance of finding compounds for the development of agrochemicals. As a result of a screening test, we recognized that the hexane and ether extracts of *Gnaphalium affine* have potential antifeedant activity against a polyphagous insect, the common cutworm, *Spodoptera litura*. This plant species is used as a medicinal plant in the Asian region, for astringent and vulnerary purposes. As a plant physiological feature, this plant and related species belong to the winter annual weeds in Japan and have many short hairs on the plant surface. Recently, some constituents in this plant have been investigated as inhibitory agents of bovine LAR and platelet aggregation (Tachibana et al., 1995). One of the constituents of *G. affine*, 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone (**1**), showed anticancer activity (Kingston et al., 1979). There were reports of the isolation of the polymethylated flavonoids from the plant, including *Gnaphalium* spp. (Torrenegra et al., 1980; Guerreiro et al., 1982). Antifeedants occurring in the Compositae, sesquiterpenes in *Senecio* spp., polyacetylenes in *Rudbeckia* spp., and chromenes in *Encelia* spp. have been previously reported (González-Coloma et al., 1995; Guillet et al., 1997; Isman and Proksch, 1985). We have isolated four antifeedants from *G. affine* and have described their related compounds for the investigation of insect antifeedant activity.

MATERIALS AND METHODS

General. The ^{1}H and ^{13}C NMR spectra were measured using a JEOL 270EX (270 MHz) spectrometer. Mass spectra were taken at 70 eV (probe) using a Shimadzu GCMS 9100-MK. FT-IR were recorded using a Shimadzu FT-IR 8020D. The compounds were separated by column chromatography on silica gel, Fuji silysia chemicals BW-127ZH and BW-300 (Fuji Silysia Chemical, Ltd., Japan), and gel filtration, Sephadex HL-20 (Amersham Pharmacia Biotech Inc., Sweden). TLC silica gel plates with a fluorescent indicator (Merck Silica Gel 60 F_{254}, 0.25 mm thick) were used. HPLC for the quantitative analysis of the flavonoids was carried out using a Shimadzu LC-9A system. The column used was an Inertsil ODS, 4.6 mm i.d. × 250 mm (GL Science Inc., Japan).

Insect Bioassays. Common cutworms (*S. litura*) were reared on an artificial diet (Insecta LF, Nihon Nosan Kogyo Co., Japan) in a controlled room environment at 26.5 °C and 60% humidity.

Choice Leaf-Disk Bioassay. The experimental setting was based on that described by Escoubas et al. (Escoubas et al., 1993). Leaf-disks, 2 cm diameter, were prepared with a cork borer from fresh sweet potato (*Ipomoea batatas*) leaves that had been cultivated in the Kinki University farm without agrochemicals.

Two disks were treated with a specific amount of plant extracts or test compounds in an acetone solution, and two other disks with acetone only were used as the control. The four disks were set in alternating positions in the same Petri dish. After complete removal of the solvent, 15 larvae (third instar) were released into the dish. The dishes were then kept in an insect rearing room at 26.5 °C in the dark for 2−5 h. Partially consumed leaf-disks were taped onto photocopier paper for monotone data conversion. The monotone data were photocopied, determined to contain no errors, and then converted to digital data files using a digital scanner. Digital data analyses were performed on a Macintosh computer using the public-domain NIH Image program (developed by the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov, or on a floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI). For each experiment, the data file of an intact disk was measured and compared to that of the treated disk. For evaluation of the antifeedant activity of the extracts and test compounds, we adopted the antifeedant index (AFI): AFI = % of treated disks consumed/(% of treated disks consumed + % of control disks consumed) × 100. The control disks were set at an AFI value of 50. The data were evaluated by probit analysis. A straight line was fitted to the points obtained by bioassay, and the ED_{50}

* Author to whom correspondence should be addressed.

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was calculated as the dose corresponding to midpoint between complete inhibition and no effect by the computer program.

TLC Bioassay. The TLC plates were cut into 4 × 10 cm pieces. The samples containing carrier solvent were applied to the plate as a band, and the plate was developed in the appropriate solvent system for good separation. Distilled water was added to the artificial diet to make a paste, which was then applied to the surface of the developed TLC plate. This TLC plate was then placed in a plastic cup with 50 larvae (fourth instar) for 1 d at 26.5 °C. The uneaten area was compared with the *Rf* values of the compounds detected on the reference TLC plate developed under the same conditions. Moreover, residual silica gel was found on the plate after the TLC plate assay was removed and extracted with ethyl acetate. The extract of residual silica gel after the TLC plate assay was developed in the same solvent system, detected with UV light (254 nm) and 50% sulfuric acid heated to 150 °C to confirm the compounds with antifeedant properties contained in the silica gel residue (Escoubas et al., 1992).

Extraction and Isolation. Plant material of cudweed (*G. affine*) was collected at the flowering stage from March to July in Nara and Kyoto Prefecture, Japan. The fresh whole body of the cudweed (3.7 kg) was extracted with hexane. The concentrated extract had any paraffins removed with methanol. The yellow oil (11.9 g) was separated by silica gel (Fuji Silysia Chemical, BW-127ZH) column chromatography with 5:1 hexane/ethyl acetate. The fraction including the antifeedants was rechromatographed on the silica gel (Fuji Silysia Chemical, BW-300) with the same solvent system. The final purification of the antifeedants was performed by gel filtration chromatography (Pharmacia, Shepadex LH-20) with methanol. Three antifeedant flavonoids, 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone (**1**), 5-hydroxy-3,6,7,8-tetramethoxyflavone (**2**), and 5,6-dihydroxy-3,7-dimethoxyflavone (**3**) were isolated from this extract (Imre et al., 1984; Bohlmann et al., 1979, 1980). After extraction with hexane, the plant residue was extracted with ether and treated in the same manner. Thus we separated out a constituent, 4,4′,6′-trihydroxy-2′-methoxychalcone (**4**) (Bohlmann et al., 1979). For evaluation of the structure−activity relationship, one of the methylated flavonoids, tangeretin (**5**), was isolated from the hexane extract of *Citrus auranetium*. The peel of this citrus contains the known 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone (**1**) (Sarin et al., 1960). However, we did not detect this compound in the hexane extract of *C. auranetium*. These natural compounds were characterized by spectral analyses.

5-Hydroxy-3,6,7,8,4′-pentamethoxyflavone (1): yellow powder (hexane); mp 122−123 °C; UV (MeOH) λ_{max} 275, 325 nm; ^{1}H NMR ($CDCl_3$) δ 12.20 (1H, s, Ar−O*H*), 8.09 (2H, d, J = 9.0 Hz, Ar−*H*), 6.97 (2H, d, J = 9.0, Ar−*H*), 4.03 (3H, s, OCH_3), 3.88 (6H, s, OCH_3 × 2), 3.83 (3H, s, OCH_3), 3.80 (3H, s, OCH_3); EIMS *m*/*z* (relative intensity) 388 (M^+, 66.6%), 373 (M−CH_3, 100%).

5-Hydroxy-3,6,7,8-tetramethoxyflavone (2): yellow powder (hexane); mp 98−100 °C; UV (MeOH) λ_{max} 278, 352 nm; ^{1}H NMR ($CDCl_3$) δ 12.62 (1H, s, Ar−O*H*), 8.13 (3H, m, Ar−*H*), 7.54 (2H, m, Ar−*H*), 4.10 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.97 (3H, s, OCH_3); EIMS *m*/*z* (relative intensity) 358 (M^+, 66.9%), 343 (M−CH_3, 100%).

5,6-Dihydroxy-3,7-dimethoxyflavone (3): yellow crystals (hexane); mp 178 °C; UV (MeOH) λ_{max} 274, 346 nm; ^{1}H NMR ($CDCl_3$) δ 12.37 (1H, s, Ar−O*H*), 8.11 (3H, m, Ar−*H*), 7.54 (2H, m, Ar−*H*), 6.44 (1H, s, Ar−*H*), 6.34 (1H, s, Ar−O*H*), 4.00 (3H, s, OCH_3), 3.88 (3H, s, OCH_3); EIMS *m*/*z* (relative intensity) 314 (M^+, 66.9%), 299 (M−CH_3, 100%).

4,4′,6′-Trihydroxy-2′-methoxychalcone (4): yellow powder ($CHCl_3$); mp 248 °C; UV (MeOH) λ_{max} 245, 366 nm; ^{1}H NMR ($CDCl_3$) δ 13.93 (1H, s, Ar−O*H*), 10.30 (2H, s, Ar−O*H* × 2), 7.91 (1H, d, J = 13.0 Hz, CH═C(β)*H*), 7.91 (1H, d, J = 13.0 Hz, CH═C(α)*H*), 7.58 (2H, d, J = 8.0 Hz, Ar−*H*), 6.85 (2H, d, J = 8.0 Ar−*H*), 6.00 (1H, s, Ar−*H*), 5.91 (1H, s, Ar−*H*), 3.87 (3H, s, OCH_3); EIMS *m*/*z* (relative intensity) 286 (M^+, 82.1%), 271 (M−CH_3, 41.5%), 167 (100%).

Tangeretin (5): white needles (hexane); mp 152 °C; ^{1}H NMR($CDCl_3$) δ 7.96 (2H, d, J = 9.0 Hz, Ar−*H*), 7.10 (2H, d, J = 9.0 Hz, Ar−*H*), 6.69 (1H, s, *H* −3), 4.18 (3H, s, −OCH_3), 4.10 (3H, s, −OCH_3), 4.06 (3H, s, −OCH_3), 4.03 (6H, s, −OCH_3 × 2), 3.97 (3H, s, −OCH_3); EIMS *m*/*z* (relative intensity) 372 (M^+, 31.6%), 357 (M−CH_3, 100%).

Quantitative Analysis of Flavonoids in the Plants. For measurement of the amount in the plants, the flavonoids were analyzed by HPLC. The analysis was carried out using a Shimadzu LC-9A system. The column used was an Inertsil ODS,. 4.6 mm i.d. × 250 mm (GL Sciences Inc., Japan). One of the flavonoids, quercetin, was used as the internal standard for the quantitative analysis by HPLC. We investigated methylated flavonoids for six extracts from *Gnaphalium* spp. in Japan at 285 nm by a UV detector.

SYNTHESIS OF DERIVATIVES

Selective Demethylation of Methyl Ether Flavones. A solution of a methylated flavone in acetonitrile was mixed with a solution of anhydrous aluminum bromide in acetonitrile under reflux conditions in a nitrogen atmosphere. Water was added to the mixture 10 min later. The mixture was then extracted with ethyl acetate and dried over anhydrous sodium sulfate. The solvent was removed by a rotary evaporator under reduced pressure. The resulting demethyl derivative was separated (Horie et al., 1993, 1995).

Flavonoid Methyl Ethers. The appropriate molarity of methyliodide corresponds to the number of phenols in the chemical structure of the parent compound used for the reactions with acetone containing potassium carbonate. The methylation was performed through the night at room temperature, and the liquid turned brown with formation of iodine. After completion of the reaction, water was added, and the solution was extracted with ethyl acetate and dried over anhydrous sodium sulfate. The solvent was removed by a rotary evaporator under reduced pressure and purified in an ordinary manner. These derivatives were characterized by spectral analyses.

5-Demethyltangeretin (6): white powder (hexane); mp 175 °C; ^{1}H NMR ($CDCl_3$) δ 12.57 (1H, s, Ar−O*H*), 7.90 (2H, d, J = 9.0 Hz, Ar−*H*), 7.04 (2H, d, J = 9.0 Hz, Ar−*H*), 6.60 (1H, s, Ar−*H*), 4.11 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 3.90 (3H, s, OCH_3); EIMS *m*/*z* (relative intensity) 358 (M^+, 56.8%), 343 (M−CH_3, 100%).

2′,4′-Dihydroxy-4,6′-dimethoxychalcone (7): pale orange needles ($CHCl_3$); mp 194−195 °C; UV (MeOH) λ_{max} 208, 366 nm; ^{1}H NMR (hexane) δ 14.43 (1H, s, Ar−O*H*), 7.79 (2H, s, −C*H*═C*H*−), 7.55 (2H, d, J = 8.0 Hz, Ar−*H*), 6.92 (2H, d, J = 8.0 Hz, Ar−*H*), 6.10 (1H, d, J = 2.5 Hz, Ar−*H*), 5.96 (1H, d, J = 2.5 Hz, Ar−*H*), 5.23 (1H, s, Ar−O*H*), 3.91 (3H, s, OCH_3), 3.82 (3H, s, OCH_3); EIMS *m*/*z* (relative intensity) 300 (M^+, 100%), 285 (M−CH_3, 15.6%).

2′-Hydroxy-4,4′,6′-trimethoxychalcone (8): yellow needles (hexane); mp 113 °C; UV (MeOH) λ_{max} 208, 362 nm; ^{1}H NMR ($CDCl_3$) δ 13.93 (1H, s, Ar−O*H*), 7.79 (2H, s, −C*H*═C*H*−), 7.55 (2H, d, J = 8.0 Hz, Ar−*H*), 6.92 (2H, d, J = 8.0 Hz, Ar−*H*), 6.10 (1H, d, J = 2.5 Hz, Ar−*H*), 5.96 (1H, d, J = 2.5 Hz, Ar−*H*), 3.91 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.82 (3H, s, OCH_3); EIMS *m*/*z* (relative intensity) 314 (M^+, 100%), 299 (M−CH_3, 12.2%).

4,2′,4′,6′-Tetramethoxychalcone (9): yellow needles (hexane); mp 119−121 °C; UV (MeOH) λ_{max} 245, 366 nm; ^{1}H NMR ($CDCl_3$) δ 7.46 (2H, d, J = 8.0 Hz, Ar−*H*), 7.32 (1H, d, J = 13.0 Hz, CH═C(α)*H*), 6.86 (1H, d, J = 13.0 Hz, CH═C(β)*H*), 6.78 (2H, d, J = 8.0 Hz, Ar−*H*), 6.14 (2H, s, Ar−*H*), 3.85 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.75 (6H, s, OCH_3 × 2); EIMS *m*/*z* (relative intensity) 328 (M^+, 66.9%), 313 (M−CH_3, 100%).

RESULTS AND DISCUSSION

Insect Antifeedant Activities of Natural Flavonoids and Their Derivatives. During the choice leaf-disk bioassay for evaluation of insect antifeedants, we initially recognized the insect antifeedant activities of the hexane and ether extracts from *G. affine*. Active

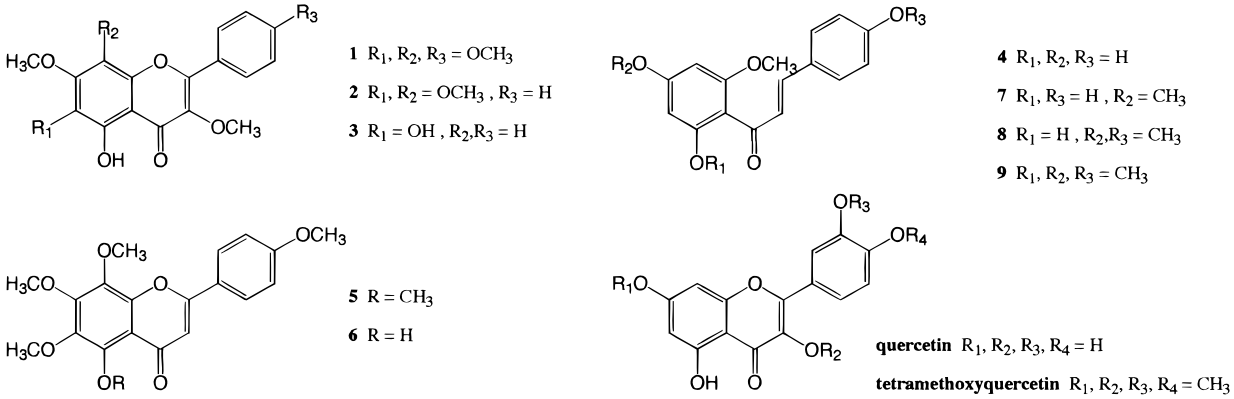

Figure 1. Structures of test flavonoids.

compounds were present in the TLC bioassay of these extracts; these natural compounds have been isolated by column chromatography and other procedures. Based on the spectral analyses, the antifeedants have the features of polymethylated flavonoids. We have evaluated the antifeedant activity of methylated flavonoids by methylation. Therefore, we evaluated not only these natural flavonoids from *G. affine*, but also the authentic flavonoids, tangeretin, quercetin, and rotenone. The flavonol quercetin is well-known to be widespread in plants and acts as a plant defense system toward some insect species. However, this compound was recognized to have no effect on the common cutworm in our bioassay. Quercetin was then altered to the methylated derivatives because the antifeedants in *G. affine* are polymethylated flavonoids. However, the methylated quercetin did not increase the inhibition of the common cutworm to feeding. This fact suggested that not all of the methylated flavonoids have insect antifeedant activity. There are significant relationships between insect antifeedant activity and the chemical structures of the flavonoids. The 5-hydroxy-3,6,7,8-tetramethoxyflavone (**2**) and 5,6-dihydroxy-3,7-dimethoxyflavone (**3**) were shown to have the strongest insect antifeedant activities. On the other hand, 5-hydroxy-3,6,7,8,4′-pentamethoxyflavone (**1**) was found to have less activity than the previous two flavonoids. These flavonoids were polymethylated, excluding the phenol by hydrogen bonding with the carbonyl group at the 5-position of the flavones; **2** and **3**, especially, have only a hydrogen substituent on the B-ring (Figure 1). Based on the bioassay evaluation, introduction of a methyl ether on the B-ring of the flavonoid decreases the insect antifeedant activity. This hypothesis is supported by the comparison of the test compounds, the methylated quercetin and methylated flavonoids in *G. affine*, which have a number of diverse substituents on the B-ring (Table 1).

With respect to the antifeedant activities of the chalcones, all the methylated derivatives have less activity than the parent compound. The natural compound **4** had the strongest antifeedant activity of these chalcones (Table 2). It was important for feeding inhibition of the common cutworm that the correct number of methyl ethers be introduced on the aromatic moiety.

In comparison, the chalcone **4** had a weaker activity than the three flavonoids in *G. affine*, but the amount of chalcone was larger than these flavonoids in the plant. This fact suggested that both acted as one defensive system for the plant against phytophagous insects.

Table 1. Antifeedant Activity of the Flavonoids from *G. affine* and Authentic Flavonoids against *S. litura* Larvae in a Choice Leaf-Disk Bioassay

	antifeedant activity	
test flavonoid	ED_{50} (mol/cm^2)	pED_{50} (mol/cm^2)
5-hydroxy-3,6,7,8,4′-pentamethoxyflavone (**1**)	1.1×10^{-7}	6.96
5-hydroxy-3,6,7,8-tetramethoxyflavone (**2**)	2.0×10^{-8}	7.70
5,6-dihydroxy-3,7-dimethoxyflavone (**3**)	2.5×10^{-8}	7.60
tangeretin (**5**)	2.8×10^{-7}	6.55
5-demethyltangeretin (**6**)	1.8×10^{-7}	6.74
rotenone	1.5×10^{-7}	6.82
quercetin	inactive	
tetramethoxyquercetin	inactive	

Table 2. Antifeedant Activity of the Chalcone from *G. affine* and Its Derivatives against *S. litura* Larvae in a Choice Leaf-Disk Bioassay

	antifeedant activity	
test chalcone	ED_{50} (mol/cm^2)	pED_{50} (mol/cm^2)
4,4′,6′-trihydroxy-2′-methoxychalcone (**4**)	3.8×10^{-7}	6.42
2′,4′-dihydroxy-4,6′-dimethoxychalcone (**7**)	2.1×10^{-5}	4.68
2′-trihydroxy-4,4′,6′-trimethoxychalcone (**8**)	9.0×10^{-6}	5.05
4,2′,4′,6′-tetramethoxychalcone (**9**)	5.2×10^{-7}	6.28

A comparison of flavones and chalcones for insect antifeedant activity showed the importance of the chemical structure. The important structural feature was the ether linkage system consisting of the pyran ring in the chemical structure. This fact was supported by the difference in antifeedant activity for the two categories of flavonoids (Tables 1 and 2). Some of antifeedants occurring in the other plant species also have ether linkages in their chemical structures. For instance, the furan moiety is contained in the azadirachtins and clerodan diterpenes, and the pyran moiety is contained in the precocens and rotenoids (Klocke et al., 1989; Kato et al., 1972; Bowers et al., 1976). The isoflavones also have strong antifeedant activity against some phytophagous insect species, and rotenoids are included in this category (Lane et al., 1985). Harborne discussed the flavonoid insect relationships in his book (Harborne and Grayer, 1993). These relationships include both allomones and kairomones.

Quantitative Analysis of the Flavonoids in Plants. It was shown by HPLC that these flavonoids (**1**−**3**) as antifeedants were present at levels of 4.5×10^{-4}%, 8.7×10^{-4}%, and 1.8×10^{-3}% on a fresh weight basis, respectively. Although we have analyzed other *Gnaphalium* spp. in Japan, i.e., *G. japonicum* Thumb., *G. pensylvanicum* Willd., G. *spicatum* Lam., *G. pur-*

pureum L., and *G. calviceps* Ferm., these flavonoids were not detected in these plant species by the HPLC analysis.

In summary, three polymethylated flavonoids and one chalcone, which were isolated from *G. affine*, were shown to be antifeedants. Two flavonoids (**2**, **3**) showed the highest antifeedant activity, while **1** and the chalcone showed less feeding inhibition. A comparison of their chemical structures showed that the introduction of a methyl ether excluding the B-ring in the flavonoid structure increased the antifeedant activity. However, the introduction of a methyl ether at the 3- and 5-position on the flavonoid was not relative to the rate of antifeedant activity. Moreover, it was advantageous for the effect of insect feeding inhibition that an agent had an ether moiety linkage in the chemical structure.

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