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Studies on the Constituents of Useful Plants. V.[1] Multisubstituted Flavones in the Fruit Peel of *Citrus reticulata* and Their Examination by Gas-Liquid Chromatography[2]

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Thirteen kinds of flavones, including two new flavones, were isolated from the fruit peel of *Citrus reticulata* Blanco (Rutaceae) on the basis of thorough examination of the components in the chloroform extract of the peel. The structures of these flavones were confirmed by analysis of the spectral data and by structural conversion or total synthesis. β-Sitosterol, limonin, hesperidin, ferulic acid, and 5,5′-oxydimethylene-bis(2-furaldehyde) were also isolated as components other than flavones. The multisubstituted flavones obtained were examined by gas-liquid chromatography, and may provide a good marker for chemotaxonomical studies of *Citrus* species.

Keywords——*Citrus reticulata* Blanco; Rutaceae; 5-hydroxy-7,8,4′-trimethoxyflavone; 4′-hydroxy-5,6,7,8-tetramethoxyflavone; 5,5′-oxydimethylene-bis(2-furaldehyde); ^{13}C-NMR; GLC of polyoxygenated flavones

Studies on the constituents of *Citrus* plants can be divided roughly into two groups; one consists of so-called chemotaxonomical studies,[4] directed to the examination of flavanone glycosides[5] and essential oils[6] as markers. The other group consists of chemical studies on multisubstituted flavones characteristic of *Citrus* species.[7] In the present series of studies, we have carried out exhaustive examinations of multisubstituted flavones in the peel of mature fruit of *Citrus reticulata* Blanco (Rutaceae)[8] and have utilized gas–liquid chromatography (GLC) as a new approach to chemotaxonomical studies of the *Citrus* species with these flavones as a marker.

Structural Determination

The chloroform extract of the fruit peel of *Citrus reticulata* was repeatedly subjected to silica gel column chromatography, and 13 kinds of flavones (**1a**—**4f**, excluding **3c**) were isolated (Chart 1). The structures of the flavones other than **2c** and **4f** were estimated from the spectral data, and confirmed by direct comparison with authentic specimens (**2a**, **3a**,[9] **4c**,[10] and **4d**[8a])

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3) Location: *Mitahora-higashi 5-6-1, Gifu 502, Japan.*
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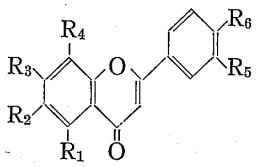

1a : $R_1=R_3=R_6=OMe$, $R_2=R_4=R_5=H$
2a : $R_1=R_3=R_4=R_5=R_6=OMe$, $R_2=H$
2b : $R_1=R_3=R_4=R_6=OMe$, $R_2=R_5=H$
2c : $R_1=OH$, $R_3=R_4=R_6=OMe$, $R_2=R_5=H$
2d : $R_1=R_6=OH$, $R_3=R_4=OMe$, $R_2=R_5=H$
3a : $R_1=R_2=R_3=R_5=R_6=OMe$, $R_4=H$
3b : $R_1=OH$, $R_2=R_3=R_5=R_6=OMe$, $R_4=H$
3c*: $R_1=R_2=R_3=R_6=OMe$, $R_4=R_5=H$
4a : $R_1=R_2=R_3=R_4=R_5=R_6=OMe$
4b : $R_1=OH$, $R_2=R_3=R_4=R_5=R_6=OMe$
4c : $R_1=R_3=R_6=OH$, $R_2=R_4=R_5=OMe$
4d : $R_1=R_2=R_3=R_4=R_6=OMe$, $R_5=H$
4e : $R_1=OH$, $R_2=R_3=R_4=R_6=OMe$, $R_5=H$
4f : $R_6=OH$, $R_1=R_2=R_3=R_4=OMe$, $R_5=H$
4g*: $R_1=R_6=OH$, $R_2=R_3=R_4=OMe$, $R_5=H$

* These were prepared for identification.

Chart 1

and by structural conversion, such as methylation (apigenin→**1a**, **2d**→**2b**, 5,3′-dihydroxy-6,7,-4′-trimethoxyflavone[9]→**3b**, 4′-hydroxy-5,6,7,8,3′-pentamethoxyflavone[10]→**4a**) or by partial demethylation (4′-hydroxy-5,7,8-trimethoxyflavone→**2d**, **4a**→**4b**, **4d**→**4e**). **2c** and **4f** are flavones isolated for the first time from a natural source, and their structures were confirmed by total synthesis.

2c was obtained as yellow needles, mp 220—221°, and the presence of a hydroxyl at the 5-position was suggested by the bathochromic shift of 70 nm in its ultraviolet (UV) spectrum on the addition of aluminum chloride, and by its stability in hydrochloric acid. The proton magnetic resonance (PMR) spectrum of **2c** showed signals due to three methoxyls, and a signal at 6.36 ppm due to 3-H and one at 6.51 ppm due to 6-H or 8-H, and a characteristic A_2B_2 signal due to 4′-substitution in the B ring. Thus, **2c** was presumed to be 5-hydroxy-7,8,4′-trimethoxyflavone or 5-hydroxy-6,7,4′-trimethoxyflavone.[11] Complete methylation of **2c** gave **2b**, which was different from **3c**, so that **2c** was presumed to be 5-hydroxy-7,8,4′-trimethoxyflavone, and this structure was confirmed by its total synthesis by the following route.

Condensation of 2-hydroxy-3,4,6-trimethoxyacetophenone and *p*-benzyloxybenzoic acid gave an ester (**5**), mp 124—127°, and Baker-Venkataraman rearrangement of this compound afforded a *β*-diketone (**6**), mp 104—108°. Dehydrative cyclization of **6** in the presence of sulfuric acid resulted in debenzylation[12] with cyclization, and 4′-hydroxy-5,7,8-trimethoxyflavone (**7**), mp 258—260°, was obtained. A part of **7** was partially demethylated to afford 5,4′-dihydroxy-7,8-dimethoxyflavone (**2d**) and the remainder was completely methylated to give 5,7,8,-4′-tetramethoxyflavone (**2b**). Partial demethylation of **2b** gave 5-hydroxy-7,8,4′-trimethoxyflavone which was identical with **2c**.

4f was obtained as colorless needles, mp 196—198°, and formed a monoacetate, mp 154°, on acetylation. The PMR spectrum of **4f** revealed the presence of four methoxyls (3.85, 3.90, 3.95, and 4.04 ppm), and A_2B_2 signals indicating substitution at the 4′-position in the B ring. These results suggest a flavone oxygenated in the 5,6,7,8, and 4′ positions, like tangeritin (**4d**). The hydroxyl group was probably located at the 4′-position, judging from the bathochromic shift (56 nm) in the presence of sodium methoxide. This was confirmed by the ^{13}C-nuclear magnetic resonance (NMR) spectrum.

In the ^{13}C-NMR spectra of flavones, the signals of methoxyl groups in adjacent positions show a downfield shift (+6 ppm) compared with isolated methoxyl groups.[13] In the ^{13}C-NMR spectrum of **4d**,[14] the signals at 62.0 and 61.8 ppm can be assigned to methoxyls at the 5-, 6-,

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Table I. ^{13}C-NMR Chemical Shifts of **4d**, **4e**, and **4f**

	2	3	4	5	6	7	8	9	10
4d	162.3	106.4	175.8	143.8	138.0	151.1	138.0	147.7	115.0
4e	163.6	106.0	182.1	144.9	135.8	152.1	132.5	148.1	114.6
4f	161.4	106.0	167.3	144.1	138.4	151.4	138.4	148.1	116.7

	1′	2′, 6′	3′, 5′	4′	OCH_3
4d	123.6	128.0	115.0	160.7	62.0 61.8 55.8
4e	122.7	128.0	114.6	162.4	61.1 60.2 55.3
4f	122.0	128.5	114.9	161.4	62.4

Chemical shifts are expressed in ppm from TMS. Solvent: Me_2SO.

7-, and 8-positions and that at 55.8 ppm would be the signal of the methoxyl carbon at the 4′-position. The same considerations apply to **4e**. In the case of **4f**, the signal at higher field disappeared and a signal appeared at 62.4 ppm. These results indicate that the four methoxyls in **4f** are located in adjacent positions, *i. e.*, at the 5-, 6-, 7-, and 8-positions, and that the hydroxyl is at the 4′-position. Further, since methylation of **4f** gave tangeritin (**4d**) and partial demethylation gave xanthomicrol[15] (**4g**), **4f** was considered to be 4′-hydroxy-5,6,7,8-tetramethoxyflavone,[16] this was confirmed by synthesis through the following route.

2-Hydroxy-3,4,5,6-tetramethoxyacetophenone, obtained by alkaline decomposition of nobiletin (**4a**), was condensed with *p*-benzyloxybenzaldehyde in the presece of alkali to give 4-benzyloxy-2′-hydroxy-3′,4′,5′,6′-tetramethoxychalcone (**8**), mp 87—89°. **8** was boiled with 85% phosphoric acid to give 4′-benzyloxy-5,6,7,8-tetramethoxyflavanone (**9**), mp 115—116°, then DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) oxidation[17] gave 4′-benzyloxy-5,6,7,8-tetramethoxyflavone (**10**), mp 136—139°, and finally debenzylation of **10** provided 4′-hydroxy-5,6,7,8-tetramethoxyflavone, mp 197—199°, which was identical with **4f**.

The flavones isolated from the fruit peel of *C. reticulata* in the present series of studies can be divided into four groups according to the mode of substitution in the A ring; 5,7-dioxygenated type (group 1), 5,7,8-trioxygenated type (group 2), 5,6,7-trioxygenated type (group 3), and 5,6,7,8-tetraoxygenated type (group 4). Substitution in the B ring is also systematic, being at the 4′- or 3′- and 4′-positions. Consequently, many flavones with various combinations of substitutions in the A and B rings may occur. It is possible that the fruit peel of *C. reticulata* contains minor flavones with such combinations of substitutions, although they were not detected in the present series of experiments.

In addition to these flavones, hesperidin, β-sitosterol, limonin, ferulic acid, and 5,5′-oxydimethylene-bis(2-furaldehyde)[18] were isolated from the fruit peel of *C. reticulata* and identified by comparison with authentic specimens.

Gas-Liquid Chromatography of Multisubstituted Flavons

There have been few reports on the GLC of flavones,[19] and none on multisubstituted flavones, as far as we are aware. Consequently, GLC of the isolated multioxygenated flavones of known structure was examined.

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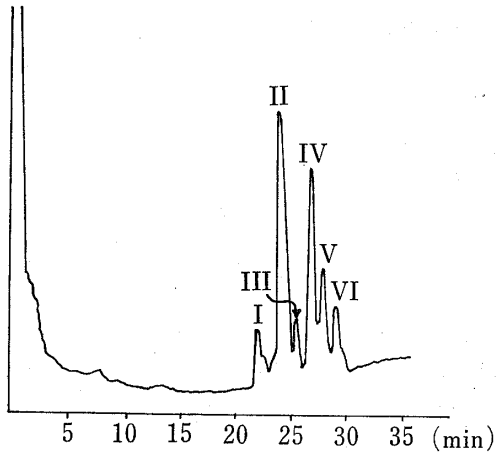

Fig. 1. Gas Chromatogram of Multioxygenated Flavones

Stationary phase: 1% OV-17,
Column: 3 mm×2 m,
Temp.: 180—320°,
Carrier gas flow: H_2 50 ml/min,
Detector: FID.
I, **2c**. II, **1a**, **3c**, **4d**, **4e**. III, **2b**. IV, **3b**, **4b**. V, **3a**, **4a**. VI, **2a**.

The GLC pattern of **1a** to **4g** showed 6 peaks (I to VI) which were not separated completely. The correlation between the flavone structure and the relative retention time (t_R) may be summarized as follows. (a) No flavones having a hydroxyl at a position other than the 5-position were detected (**2d**, **4c**, **4f**, **4g**). (b) Retention times of flavones having one methoxyl in the ring B were shorter than those of flavones with two methoxyls (**2c**, **4e**, *etc. vs.* **3b**, **4a**, **4b**, *etc.*). (c) Introduction of a methoxyl at the 6-position resulted in shorter retention times (**2b** *vs.* **4d**, **2a** *vs.* **4a**). (d) The retention time did not change with the introduction of a methoxyl into the 8-position (**3c**→**4d**, **3a**→**4a**). (e) When the mode of substitution in the ring B was the same, the retention times of 5,6,7-trisubstituted flavones were shorter than those of 5,7,8-trisubstituted flavones (**3c**→**2b**, **3a**→**2a**)

These results suggest that GLC would be useful as a tool for the structure elucidation of flavones and for chemotaxonomical studies using flavone derivatives of *Citrus* species as a marker, in that the presence or absence and contents of various flavones can be determined by GLC. This is now being examined, in combination with the use of high-speed liquid chromatography.

Experimental

Melting points are all uncorrected. UV spectra were determined with a Hitachi 323 spectrophotometer, PMR with a Hitachi R-20B, using trimethylsilane as an internal standard, at 60 Hz, with chemical shifts in δ values (ppm), ^{13}C-NMR with a JEOL FX-60FT spectrometer operating at 25.15 MHz, spectral width 4000 Hz, 4096 data points, and mass spectra with a JEOL JMS-D300. The gas–liquid chromatograph used was a JEOL JGC-20KF machine. TLC was carried out with kieselgel G (Merck), developed with benzene–Me_2CO (3: 1) and colored with 10% H_2SO_4. Wakogel C-200 (Wako Pure Chemicals, Ltd., Tokyo) was used for column chromatography.

Extraction and Isolation of Fruit Peel Components of *Citrus reticulata*——Dried peel (1.2 kg) of fruit of *C. reticulata* was extracted with 3 liters of $CHCl_3$ for 40 hr on a water bath. The extract was concentrated under reduced pressure and extracted with hexane to remove essential oils and liposoluble pigments. The aqueous layer was further extracted with BuOH and the BuOH extract was concentrated under reduced pressure. The concentrated extract was subjected to silica gel chromatography, and the column was developed successively with 5: 1; 3: 1, and 1: 1 mixtures of benzene and Me_2CO.

General Procedure for Methylation——A mixture of flavone and 1.5 volumes of MeI (the volume was changed in accordance with the number of hydroxyl groups) in Me_2CO was boiled in the presence of K_2CO_3 on a water bath for 5 hr. The filtered reaction mixture was concentrated and the residue was recrystallized from MeOH.

General Procedure for Partial Demethylation——A mixture of 1 mol of flavone and 1.1 mol of anhyd. $AlCl_3$ dissolved in dry nitrobenzene was allowed to stand at room temperature overnight. The reaction mixture was poured into a mixture (1: 1) of ice water and conc. HCl, and nitrobenzene was removed by steam distillation. The precipitate was collected and recrystallized from AcOEt-hexane.

2-(4′-Benzyloxybenzoyloxy)-3,4,6-trimethoxyacetophenone (**5**)——A mixture of 450 mg (2 mmol) of 2-hydroxy-3,4,6-trimethoxyacetophenone, 450 mg (2 mmol) of *p*-benzyloxybenzoic acid, and 1.5 ml of $(CF_3CO)_2O$ in 20 ml of benzene was stirred at room temperature for 4 hr. **5** was obtained as colorless prisms (from benzene), mp 124—127°. PMR ($CDCl_3$) δ: 2.45 (3H, s, $COCH_3$), 3.73, 3.84, 3.90 (9H, each s, 3×OCH_3), 5.13 (2H, s, $OCH_2C_6H_5$), 6.43 (1H, s, H-5), 7.02 (2H, d, J=8.8 Hz, H-3′,5′), 7.40 (5H, s, benzyl protons), 8.11 (2H, d, J=8.8 Hz, H-2′,6′).

2-Hydroxy-4′-benzyloxy-3,4,6-trimethoxydibenzoylmethane (**6**)——A mixture of 800 mg (1.8 mmol) of **5** and 1.0 g of powdered KOH in 20 ml of pyridine was stirred at 110° for 20 min, cooled, and acidified with conc. HCl. This acid solution was extracted with 50 ml of AcOEt, then the extract was washed with

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water, dried over Na_2SO_4, and concentrated under reduced pressure, **6** was obtained as yellow needles (from MeOH), mp 104—108°.

4′-Hydroxy-5,7,8-trimethoxyflavone (7)——A solution of 720 mg (1.65 mmol) of **6** dissolved in 30 ml of AcOH was warmed to 110°, then 30 ml of conc. H_2SO_4–AcOH mixture (1 : 10) was added, and the whole was stirred for 7 min. After cooling, ice water was added to this mixture, causing crude crystals to precipitate out. A small amount of contaminating 4′-benzyloxy-5,7,8-trimethoxyflavone was separated by column chromatography, and the product was debenzylated by the conventional method of hydrogenation using a Pd-C catalyst. **7** was obtained as colorless needles (from MeOH), mp 258—260°.

5-Hydroxy-7,8,4′-trimethoxyflavone——Partial demethylation of **7** by the general procedure gave 5,4′-dihydroxy-7,8-dimethoxyflavone, and methylation of this compound gave 5-hydroxy-7,8,4′-trimethoxyflavone as pale yellow needles (from AcOEt), mp 223—224°. UV λ_{max}^{MeOH} nm: 275, 350. This compound was identical with **2c**.

5-Hydroxy-7,8,4′-trimethoxyflavone (**2c**)——This flavone from the natural source formed yellow needles (from AcOEt), mp 220—221°. PMR ($CDCl_3$) δ: 3.82 (3H, s, OCH_3), 3.89 (6H, s, $2\times OCH_3$), 6.36 (1H, s, H-3), 6.51 (1H, s, H-6), 6.96 (2H, d, J=9.3 Hz, H-3′, 5′), 7.85 (2H, d, J=9.3 Hz, H-2′,6′). UV λ_{max}^{MeOH} nm: 276, 301, 350 (sh); $\lambda_{max}^{+AlCl_3}$ 276, 316, 350, 420 (sh); $\lambda_{max}^{+AlCl_3+HCl}$ 276, 318, 360, 420.

A mixture of 100 mg of **2c**, 2 ml of Ac_2O, and 100 mg of anhyd. AcONa was refluxed for 1 hr, then the cooled mixture was poured into ice-water. The crude crystals were collected by suctional filtration and recrystallized from MeOH to give the monoacetate, mp 167—170°. PMR ($CDCl_3$) δ: 2.41 (3H, s, $COCH_3$), 3.88, 3.96, 3.99 (9H, each s, $3\times OCH_3$), 6.52, 6.65 (2H, each s, H-3, 6), 7.02 (2H, d, J=9.2 Hz, H-3′, 5′), 7.86 (2H, d, J=9.2 Hz, H-2′, 6′).

Methylation of **2c** by the general procedure gave 5,7,8,4′-tetramethoxyflavone (**2b**), mp 207—210° (MeOH). PMR ($CDCl_3$) δ: 3.85, 3.91, 3.99 (12H, each s, $4\times OCH_3$), 6.40 (1H, s, H-3), 6.53 (1H, s, H-6), 6.98 (2H, d, J=8.9 Hz, H-3′, 5′), 7.86 (2H, d, J=8.9 Hz, H-2′, 6′).

4′-Hydroxy-5,6,7,8-tetramethoxyflavone (**4f**)——This flavone from the natural source formed colorless needles (from benzene), mp 196—198°. PMR ($CDCl_3$) δ: 3.85, 3.90, 3.95, 4.04 (12H, each s, $4\times OCH_3$), 6.55 (1H, s, H-3), 7.00 (2H, d, J=9.0 Hz, H-3′, 5′), 7.70 (2H, d, J=9.0 Hz, H-2′, 6′). UV λ_{max}^{MeOH} nm: 272, 328; $\lambda_{max}^{+AlCl_3}$ 272, 328; λ_{max}^{+MeONa} 261 (sh), 384.

Acetylation of **4f** by the usual procedure gave the monoacetate as colorless needles, mp 154°. PMR ($CDCl_3$) δ: 2.32 (3H, s, $COCH_3$), 3.94 (6H, s, $2\times OCH_3$), 3.99 (3H, s, OCH_3), 4.08 (3H, s, OCH_3), 6.63 (1H, s, H-3), 7.30 (2H, d, J=8.9 Hz, H-3′, 5′), 8.00 (2H, d, J=8.9 Hz, H-2′, 6′).

Partial demethylation of 200 mg of **4f** gave xanthomicrol as yellow needles, mp 227—229° (reported[14] mp 227—230°). PMR (DMSO-d_6) δ: 3.84, 3.92, 4.02 (9H, each s, $3\times OCH_3$), 6.87 (1H, s, H-3), 6.92 (2H, d, J=8.9 Hz, H-3′, 5′), 7.90 (2H, d, J=8.9 Hz, H-2′, 6′).

Methylation of 100 mg of **4f** gave tangeritin, mp 156—157°.

4-Benzyloxy-2′-hydroxy-3′,4′,5′,6′-tetramethoxychalcone (**8**)——A solution of 0.6 g (2.3 mmol) of 2-hydroxy-3,4,5,6-tetramethoxyacetophenone and 0.5 g (2.3 mmol) of *p*-benzyloxybenzaldehyde dissolved in 50 ml of 80% EtOH containing 8.0 g of KOH was stirred at room temperature overnight. The mixture was acidified to 20% HCl and extracted with AcOEt. The extract was dried over Na_2SO_4, concentrated under reduced pressure, and the residue was recrystallized from MeOH to 1.0 g of **8** as reddish-orange needles, mp 87—89°.

Table II. Physical Properties and Mass Spectra of Flavones in *C. reticulata*

No.	mp (°C)	*Rf*	t_R (min)	MS (*m/e*)
1a	156—158	0.17	24.8	312 (M^+) (100%), 295, 283
2a	204	0.10	29.4	372 (M^+), 357 (100%), 342
2b	211—212	0.11	26.0	342 (M^+), 327 (100%), 313
2c	220—221	0.85	22.4	328 (M^+), 313 (100%), 285
2d	292—294	0.03	—	314 (M^+) (100%), 298, 166
3a	176—177	0.43	28.4	372 (M^+), 357 (100%), 341
3b	190—191	0.68	27.2	358 (M^+) (100%), 343, 181
3c	158	0.51	24.8	342 (M^+), 327 (100%), 311
4a	138	0.51	28.4	402 (M^+), 387 (100%), 371
4b	143—145	0.78	27.2	388 (M^+), 375 (100%), 358
4c	210—212	0.24	—	360 (M^+), 345 (100%), 330
4d	153—154	0.62	24.8	372 (M^+), 357 (100%), 343
4e	176—178	0.84	24.8	358 (M^+), 343 (100%), 211
4f	196—198	0.29	—	358 (M^+), 343 (100%), 329
4g	227—229	0.51	—	344 (M^+), 329 (100%), 314

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4′-Benzyloxy-5,6,7,8-tetramethoxyflavone (9)——A solution of 1.0 g (2.2 mmol) of **8** dissolved in 100 ml of EtOH containing 10 g of 85% H_3PO_4 was boiled for 50 hr, concentrated under reduced pressure, and extracted with H_2O and AcOEt. The AcOEt extract was purified by column chromatography and 450 mg of **9** was obtained as colorless needles (from benzene), mp 115—116°. *Anal.* Calcd for $C_{26}H_{26}O_7$: C, 69.32; H, 5.82. Found: C, 69.32; H, 5.78. From the column chromatographic separation, 460 mg of starting **8** was recovered.

4′-Benzyloxy-5,6,7,8-tetramethoxyflavone (10)——A solution of 450 mg (1 mmol) of **9** and 450 mg (2 mmol) of DDQ dissolved in 20 ml of dry dioxane was boiled for 10 hr. The reduced hydroquinone was removed by filtration, and the residue was recrystallized from MeOH to give **10** as pale yellow needles, mp 136—139°. Yield, 350 mg. PMR ($CDCl_3$) δ: 3.90, 3.95, 3.97, 4.05 (12H, each s, $4\times OCH_3$), 5.02 (2H, s, $OCH_2C_6H_5$), 6.65 (1H, s, H-3), 7.02 (2H, d, $J=8.9$ Hz, H-3′, 5′), 7.35 (5H, br. s, benzyl protons), 7.80 (2H, d, $J=8.9$ Hz, H-2′, 6′). *Anal.* Calcd for $C_{26}H_{24}O_7$: C, 69.64; H, 5.39. Found: C, 69.68; H, 5.37.

4′-Hydroxy-5,6,7,8-tetramethoxyflavone——A suspension of 200 mg (0.4 mmol) of **10** and 350 mg of Pd-C in 100 ml of AcOEt was subjected to catalytic hydrogenation and 4′-hydroxy-5,6,7,8-tetramethoxyflavone was obtained as colorless needles (from benzene), mp 197—199°. Yield, 120 mg. *Anal.* Calcd for $C_{19}H_{18}O_7$: C, 63.69; H, 5.06. Found: C, 63.55; H, 5.06. UV λ_{max}^{MeOH} nm: 271, 327. This product was identical with **4f** obtained from the natural source.

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