Chem. Pharm. Bull. 37(4) 1092—1094 (1989) Vol. 37, No. 4

On the Flavonoid Constituents from the Peels of *Citrus hassaku* HORT. *ex* TANAKA

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From the peels of *Citrus hassaku* HORT. *ex* TANAKA (Rutaceae), eight flavonoid aglycones were isolated and characterized as 4′,5,6,7,8-pentamethoxyflavone (I), 3′,4′,5,6,7,8-hexamethoxyflavone (II), 3,3′,4′,5,6,7,8-heptamethoxyflavone (III), 3′,4′,5,6,7-pentamethoxyflavone (IV), 4′,5,7-trimethoxyflavone (V), 3,3′,4′,5,7,8-hexamethoxyflavone (VI), 4′,5,7,8-tetramethoxyflavone (VII) and 3′,4′,5,7,8-pentamethoxyflavone (VIII), based on spectral and chemical data.

Keywords *Citrus hassaku*; Rutaceae; 4′,5,6,7,8-pentamethoxyflavone; 3′,4′,5,6,7,8-hexamethoxyflavone; 3,3′,4′,5,6,7,8-heptamethoxyflavone; 3′,4′,5,6,7-pentamethoxyflavone; 4′,5,7-trimethoxyflavone; 3,3′,4′,5,7,8-hexamethoxyflavone; 4′,5,7,8-tetramethoxyflavone; 3′,4′,5,7,8-pentamethoxyflavone

Several papers have already been published on the hypotensive effects of flavonoid glycosides from peels of citrus group fruits.[1,2] Methoxylated flavonoids also produce important physiological responses in higher animals. 4′,5, 6, 7-Tetramethoxyflavone is cytotoxic toward various strains of carcinoma cells.[3] 3′,4′,5,6,7,8-Hexamethoxyflavone and 3′,4′,5,6,7-pentamethoxyflavone decrease erythrocyte aggregation and sedimentation *in vitro* and might be useful in the dietary control of high blood viscosity syndrome.[4] In the present study, we performed exploratory analysis of flavonoid aglycones of *Citrus hassaku* HORT. *ex* TANAKA and succeeded in isolating eight new methoxylated flavonoids. In this paper, we report the separation and structural determination of these eight methoxylated flavonoids from hassaku peels.

As described in Experimental, the ether extract of fruit peels of *Citrus hassaku* HORT. *ex* TANAKA was subjected to silica-gel column chromatography, preparative thin layer chromatography (pre-TLC) and preparative high-performance liquid chromatography (pre-HPLC), and eight kinds of methoxylated flavonoids were isolated. Tables I and II list the proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectroscopic data of the flavonoids thus obtained.

Compound I, colorless needles, $C_{20}H_{20}O_7$, mp 155.5—156.5 °C. The ^{1}H-NMR spectrum showed the presence of five methoxyls and one proton singlet at δ 6.59. In the aromatic region, A_2B_2 signals (δ 7.02, d, $J=9$ Hz; 7.88, d, $J=9$ Hz) indicated substitution at the 4′-position in the B-ring. In the ^{13}C-NMR spectrum, signals of four of five methoxyl carbons were observed at low magnetic field. This suggests the presence of four methoxyls at both *ortho* positions,[5,6] and these signals were assigned to the methoxyls on the C-5, C-6, C-7 and C-8 carbons. Based on these results and values reported in the literature, compound I was identified as 4′,5,6,7,8-pentamethoxyflavone.[6]

Compound II, colorless needles, $C_{21}H_{22}O_8$, mp 137—138 °C. The ^{1}H-NMR spectrum of II showed the presence of six methoxyls and one proton singlet at δ 6.61. In the aromatic region of the spectrum, the signals of the remaining three protons appeared at δ 7.00 (1H, d, $J=8$ Hz), 7.40 (1H, d, $J=2$ Hz) and 7.57 (1H, dd, $J=2.8$ Hz) and these were assigned to the B-ring protons at 5′,2′ and 6′ positions, respectively. In the ^{13}C-NMR spectrum, the A-ring carbon signals coincided well with those of I. Compound II was, therefore, determined to be 3′,4′,5,6,7,8-hexamethoxyflavone.[6]

Compound III, pale yellow needles, $C_{22}H_{24}O_9$, mp 128.5—130 °C. The ^{1}H-NMR spectrum of III showed the presence of seven methoxyls. From the signals in the aromatic region of the spectrum, the remaining three protons of the B-ring were estimated to be at the 5′,2′ and 6′ positions. In the ^{13}C-NMR spectrum, the signals of five of seven methoxyl carbons were observed at low magnetic field. The A-ring carbon signals coincided well with those of I. Compound III was, therefore, determined to be 3,3′,4′,5,6,7,8-heptamethoxyflavone.[7]

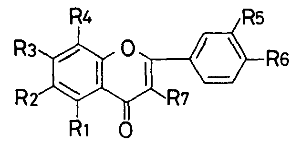
Compound IV, colorless needles, $C_{20}H_{20}O_7$, mp 179.5—180 °C. The ^{1}H-NMR spectrum showed the presence of five methoxyls and one proton singlet at δ 6.59. In the aromatic region of the spectrum, one proton signal appeared as a singlet at δ 6.80 (A-ring proton), and the B-ring proton signal pattern coincided well with that of II. In the ^{13}C-NMR spectrum, signals of two of five methoxyl carbons were observed at low magnetic field, which indicated the methoxyls to be on the C-5 and C-6 carbons. Compound IV was, therefore, determined to be 3′,4′,5,6,7-pentamethoxyflavone.[6]

Compound V, colorless needles, $C_{18}H_{16}O_5$, mp 158.5—159.5 °C. The ^{1}H-NMR spectrum showed the presence of three methoxyls and one singlet at δ 6.59. In the aromatic region of the spectrum, the signals of the remaining six protons occurred as two *meta*-coupled doublets at δ 6.38 and 6.55 (each $J=2$ Hz) for the A-ring (H-6, H-8), and two *ortho*-coupled doublets at δ 7.00 and 7.82 (each $J=9$ Hz) for the B-ring (H-3′, H-5′, H-2′, H-6′). Compound V was, therefore, determined to be 4′,5,7-trimethoxyflavone.[8]

Compound VI, pale yellow needles, $C_{21}H_{22}O_8$, mp 173—174 °C. The ^{1}H-NMR spectrum showed the presence of six methoxyls and one proton singlet at δ 6.43. The B-ring proton signal pattern coincided well with that of III. In the ^{13}C-NMR spectrum, two of six methoxyl carbons were observed at low magnetic field, and the C-6 carbon was observed at δ 92.4.[6] Based on these findings, one proton singlet at δ6.43 was assigned to H-6. The carbon signals of two methoxyls appeared at low field, and were assigned to the methoxyls on the C-3 and C-8 carbons. Compound VI was, therefore, determined to be 3,3′,4′,5,7,8-hexamethoxyflavone.[9]

Compound VII, colorless needles, $C_{19}H_{18}O_6$, mp 216—217 °C. The ^{1}H-NMR spectrum showed the presence of four methoxyls and two isolated proton singlets at δ 6.44

Table I. ^{1}H-NMR Data for the Flavonoids (δ ppm, J = Hz)



I: $R_1 = R_2 = R_3 = R_4 = R_6 = OMe$, $R_5 = R_7 = H$
II: $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = OMe$, $R_7 = H$
III: $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = OMe$
IV: $R_1 = R_2 = R_3 = R_5 = R_6 = OMe$, $R_4 = R_7 = H$
V: $R_1 = R_3 = R_6 = OMe$, $R_2 = R_4 = R_5 = R_7 = H$
VI: $R_1 = R_3 = R_4 = R_5 = R_6 = R_7 = OMe$, $R_2 = H$
VII: $R_1 = R_3 = R_4 = R_6 = OMe$, $R_2 = R_5 = R_7 = H$
VIII: $R_1 = R_3 = R_4 = R_5 = R_6 = OMe$, $R_2 = R_7 = H$

Compound	H-3	H-5	H-6	H-7	H-8	H-2′	H-3′	H-4′	H-5′	H-6′	OMe
I	6.59 s	—	—	—	—	7.88 d (J=9)	7.02 d (J=9)	—	7.02 d (J=9)	7.88 d (J=9)	3.89, 3.94×2, 4.02, 4.10
II	6.61 s	—	—	—	—	7.40 d (J=2)	—	—	7.00 d (J=8)	7.57 dd (J=2, 8)	3.96×2, 3.97, 3.98, 4.04, 4.10
III	—	—	—	—	—	7.81 d (J=2)	—	—	7.01 d (J=8)	7.84 dd (J=2, 8)	3.90, 3.95, 3.97×3, 4.00, 4.09
IV	6.59 s	—	—	—	6.80 s	7.34 d (J=2)	—	—	6.98 d (J=8)	7.52 dd (J=2, 8)	3.92, 3.95, 3.98, 4.00×2
V	6.59 s	—	6.38 d (J=2)	—	6.55 d (J=2)	7.82 d (J=9)	7.00 d (J=9)	—	7.00 d (J=9)	7.82 d (J=9)	3.89, 3.92, 3.95
VI	—	—	6.43 s	—	—	7.84 d (J=2)	—	—	7.01 d (J=8)	7.86 dd (J=2, 8)	3.90, 3.94, 3.97×2, 4.01, 4.02
VII	6.60 s	—	6.44 s	—	—	7.90 d (J=9)	7.02 d (J=9)	—	7.02 d (J=9)	7.90 d (J=9)	3.89, 3.96, 3.99, 4.01
VIII	6.60 s	—	6.43 s	—	—	7.42 d (J=2)	—	—	6.99 d (J=8)	7.58 dd (J=2, 8)	3.96×2, 3.99, 4.00, 4.03

Solvent: $CDCl_3$. s, singlet; d, doublet; dd, double doublet.

Table II. ^{13}C-NMR Data for the Flavonoids (δ ppm)

	I	II	III	IV	V	VI	VII	VIII
C-2	162.4	161.0	151.1	160.0	162.1	150.8	162.3	160.5
C-3	106.7	106.7	140.8	107.4	107.1	140.8	106.9	107.2
C-4	177.3	177.4	173.9	177.2	177.7	174.2	177.9	177.9
C-5	144.0	144.0	143.9	154.5	159.9	152.2	152.0	152.0
C-6	138.1	138.0	137.8	140.4	96.1	92.4	92.6	92.6
C-7	151.4	151.4	151.3	157.6	163.9	156.4	156.3	156.3
C-8	138.1	138.0	137.8	96.3	92.8	130.4	130.8	130.7
C-9	147.7	147.7	148.2	152.6	160.7	156.3	156.3	156.3
C-10	114.8	114.8	115.1	112.9	109.3	109.4	109.1	109.1
C-1′	123.9	124.0	123.5	124.1	123.9	123.6	123.8	124.1
C-2′	127.8	108.7	110.9	108.7	127.6	110.9	127.7	108.7
C-3′	114.4	149.3	148.8	149.3	114.4	148.7	114.5	149.3
C-4′	161.2	151.9	153.0	151.9	160.9	150.9	160.7	151.8
C-5′	114.4	111.0	111.0	111.0	114.4	111.0	114.5	111.0
C-6′	127.8	119.6	121.9	119.7	127.6	121.8	127.7	119.7
OMe	62.3	62.3	62.3	62.2	56.4	61.4	61.5	61.5
	62.0	62.0	61.9	61.6	55.7	59.9	(8)	(8)
	61.9	61.8	61.8	(5, 6)	55.5	(3, 8)	56.6	56.5
	61.7	61.7	61.7	56.3	(5, 7, 4′)	56.5	56.2	56.3
	(5, 6, 7, 8)	(5, 6, 7, 8)	59.9	56.1		56.4	55.0	56.1
	55.5	56.1	(3, 5, 6, 7, 8)	56.0		56.0	(5, 7, 4′)	56.0
	(4′)	56.0	56.0	(7, 3′, 4′)		55.9		(5, 7, 3′, 4′)
		(3′, 4′)	55.9			(5, 7, 3′, 4′)		
			(3′, 4′)					

Solvent: $CDCl_3$.

and 6.60. In the B-ring, A_2B_2 signals indicated substitution at the 4′-position. In the ^{13}C-NMR spectrum, the A-ring carbon signals coincided well with those of VI. Compound VII was, therefore, determined to be 4′,5,7,8-tetramethoxyflavone.[6]

Compound VIII, colorless needles, $C_{20}H_{20}O_7$, mp 197.5—198.5 °C. The ^{1}H-NMR spectrum showed the presence of five methoxyls and two isolated proton singlets at δ 6.43 and 6.60. The B-ring proton signals coincided well with those of IV. From the ^{13}C-NMR spectrum, the A-ring of VIII has the same structure as that of VII. Compound VIII was, therefore, determined to be 3′,4′,5,7,8-pentamethoxyflavone.[4]

As described above, eight methoxylated flavonoids were isolated from the peels of hassaku for the first time, and the structures of the compounds were identified. These methoxylated flavonoids (I, III, V—VIII) are to be tested for physiological activities.

Experimental

General Procedures All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra in KBr disk were run on a Shimadzu IR-430 spectrometer. Ultraviolet (UV) spectra were measured on a Shimadzu UV-200. Mass spectra (MS) were taken on a JEOL JMS-DX-303 mass spectrometer. NMR spectra were taken in $CDCl_3$ on a JEOL JNM-GSX-400 spectrometer, and chemical shifts are given in δ (ppm) with tetramethylsilane (TMS) as an internal standard. TLC was carried out on Silica-gel 70FM Plate-wako (Wako) with the following solvent system: benzene–acetone (3:1). Spots were detected under an FL-UV lamp (Mitamura Riken). Column chromatography was carried out on Wako-gel C-200 (Wako). For pre-TLC, Wako-gel B-O (Wako) was used, and the components were developed with benzene–acetone (1:1). For pre-HPLC, a CCPD pump, a UV-8000 detector, and an ODS-120T column (7.8 mm i.d. × 30 cm) (all from Toso) were used. Wavelength, 313 nm. Mobile phase, methanol–H_2O (75:25). Flow rate, 1.0 ml/min.

Extraction and Isolation The peels (7 kg) of commercially available *Citrus hassaku* HORT. *ex* TANAKA (produced in Misumi, Kumamoto prefecture; Cephalocitrus[2,10—12]) were sliced and extracted with ethanol under reflux. Ethanol was evaporated off under reduced pressure and the residue was partitioned between ether and H_2O. The ether layer was washed with H_2O and dried over Na_2SO_4. The residue (70 g) after the evaporation of the solvent was chromatographed on silica-gel using benzene–acetone mixture. The components (28 g) that were positive in the Mg–HCl test were fractionated by pre-TLC and pre-HPLC to give I (20 mg), II (50 mg), III (12 mg), IV (15 mg), V (7 mg), VI (8 mg), VII (9 mg) and VIII (20 mg).

4′,5,6,7,8-Pentamethoxyflavone (I): Colorless needles (MeOH), mp 155.5—156.5 °C, t_R 32.5 min, *Rf* 0.71. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1620, 1600, 1520. UV $\lambda_{\max}^{\mathrm{MeOH}}$ nm (log ε): 270 (4.10), 321 (4.25). MS *m/z* (%): 372 (M^+, 38.0), 357 ($M^+ - CH_3$, 100). Exact mass determination: 372.1231 (Calcd $C_{20}H_{20}O_7$: 372.1211)

3′,4′,5,6,7,8-Hexamethoxyflavone (II): Colorless needles (MeOH), mp 137—138 °C, t_R 24.7 min, *Rf* 0.60. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1620, 1595, 1520. UV $\lambda_{\max}^{\mathrm{MeOH}}$ nm (log ε): 246 (4.01), 268 (3.95), 330 (4.13). MS *m/z* (%): 402 (M^+, 35.5), 387 ($M^+ - CH_3$, 100). Exact mass determination: 402.1312 (Calcd $C_{21}H_{22}O_8$: 402.1315)

3,3′,4′,5,6,7,8-Heptamethoxyflavone (III): Pale yellow needles (MeOH), mp 128.5—130 °C, t_R 25.7 min, *Rf* 0.52. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1625, 1600, 1530. UV $\lambda_{\max}^{\mathrm{MeOH}}$ nm (log ε): 252 (3.96), 269sh (3.87), 342 (3.94). MS *m/z* (%): 432 (M^+, 59.0), 417 ($M^+ - CH_3$, 100). Exact mass determination: 432.1440 (Calcd $C_{22}H_{24}O_9$: 432.1420)

3′,4′,5,6,7-Pentamethoxyflavone (IV): Colorless needles (MeOH), mp 179.5—180 °C, t_R 19.6 min, *Rf* 0.54. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1625, 1595, 1510. UV $\lambda_{\max}^{\mathrm{MeOH}}$ nm (log ε): 239 (4.24), 267 (4.05), 325 (4.30). MS *m/z* (%): 372 (M^+, 34.0), 357 ($M^+ - CH_3$, 100). Exact mass determination: 372.1229 (Calcd $C_{20}H_{20}O_7$: 372.1211)

4′,5,7-Trimethoxyflavone (V): Colorless needles (MeOH), mp 158.5—159.5 °C, t_R 32.5 min, *Rf* 0.39. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1640. 1600, 1570, 1510. UV $\lambda_{\max}^{\mathrm{MeOH}}$ nm (log ε): 263 (4.25), 323 (4.30). MS *m/z* (%): 312 (M^+, 100). Exact mass determination: 312.3152 (Calcd $C_{18}H_{16}O_5$: 312.3146)

3,3′,4′,5,7,8-Hexamethoxyflavone) (VI): Pale yellow needles (MeOH), mp 173—174 °C, t_R 20.6 min, *Rf* 0.36. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1630, 1595, 1505. UV $\lambda_{\max}^{\mathrm{MeOH}}$ nm (log ε): 250 (4.15), 268 (4.13), 302sh (3.85), 350 (4.13). MS *m/z* (%): 402 (M^+, 100), 387 ($M^+ - CH_3$, 86.0). Exact mass determination: 402.1322 (Calcd $C_{21}H_{22}O_8$: 402.1315)

4′,5,7,8-Tetramethoxyflavone (VII): Colorless needles (MeOH), mp 216—217 °C, t_R 25.6 min, *Rf* 0.29. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1630, 1590, 1570, 1500. UV $\lambda_{\max}^{\mathrm{MeOH}}$ nm (log ε): 268 (4.16), 309 (4.07), 330 (4.06) MS *m/z* (%): 342 (M^+, 74.5), 327 ($M^+ - CH_3$, 100). Exact mass determination: 342.1157 (Calcd $C_{19}H_{18}O_6$: 342.1103)

3′,4′,5,7,8-Pentamethoxyflavone (VIII): Colorless needles (MeOH), mp 197.5—198.5 °C, t_R 19.6 min, *Rf* 0.26. IR $\nu_{\max}^{\mathrm{KBr}}$ cm^{-1}: 1630, 1600, 1580, 1510. UV $\lambda_{\max}^{\mathrm{MeOH}}$ nm (log ε): 243 (4.26), 270 (4.28), 337 (4.30). MS *m/z* (%): 372 (M^+, 83.0), 357 ($M^+ - CH_3$, 100). Exact mass determination: 372.1170 (Calcd $C_{20}H_{20}O_7$: 372.1211)

Acknowledgement We are grateful to Dr. S. Suzuki and Dr. K. Hisamichi of this college for MS and NMR measurements.

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