Structural Elucidation of Citrumarins: Four Novel Binary Coumarins Isolated from *Citrus* Plants

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Four binary coumarins, namely citrumarin-A (1), -B (2), -C (3), and -D (4), were isolated from root of *Citrus hassaku* and/or Swingle Citrumelo, and their structures were elucidated by spectroscopic methods.

Keywords citrumarin; coumarin; *Citrus*; bicoumarin; binary; Rutaceae

Many kinds of coumarins and acridone alkaloids have been isolated from *Citrus* plants.[1-3] In our continuing studies on constituents of *Citrus* plants,[4,5] we isolated four novel binary coumarins, namely citrumarin-A (**1**), -B (**2**), -C (**3**), and -D (**4**). We report here the isolation and structural elucidation of these novel bicoumarins from the root of Swingle Citrumelo[6] (*Poncirus trifoliata* × *Citrus paradisi*) or *Citrus hassaku* hort. ex TANAKA collected at Innoshima, Hiroshima.

Structures of Citrumarin-A (1) and -B (2) Citrumarin-A (**1**) was isolated from the roots of original seedlings of Swingle Citrumelo[6] as an amorphous powder, $[\alpha]_D$ ±0° (chloroform). The ultraviolet (UV) and infrared (IR) absorptions at λ_{max} 212, 256, 266, 284 and 326 nm, and at ν_{max} 1720, 1715, 1620, and 1600 cm^{-1}, respectively, suggested the presence of a 7-oxygenated coumarin nucleus[1] in the molecule. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, the appearance of two pairs of AB-type signals at δ_H 6.28 and 7.65 (each doublet, J=9.4 Hz) due to an α- and β-protons on α,β-unsaturated carbonyl moiety, respectively, and at δ_H 6.89 and 7.35 (each doublet, J=8.7 Hz) assignable to *ortho* located aromatic protons, and a methoxy group signal at δ_H 3.97 (3H, singlet), coupled with the observation of a 16% nuclear Overhauser enhancement (NOE) of the proton at δ_H 6.89 (H-6) on irradiation of the methoxy proton at δ_H 3.97, suggested the presence of a 7-methoxy-8-substituted coumarin skeleton in the molecule. The appearance of other AB-type signals at δ_H 6.11 and 8.16 (each doublet, J=9.4 Hz) having a typically deshielded β-proton[1] (δ_H 8.16) of an α,β-unsaturated carbonyl moiety suggested the presence of another coumarin nucleus having an *O*-substituent at C(5).[1] The high-resolution mass spectrum (HR-MS) showed the molecular formula $C_{34}H_{34}O_7$, which was considered to give rise to two characteristic ions from the two halves of the molecule at *m/z* 312 and 242, corresponding to the formulae

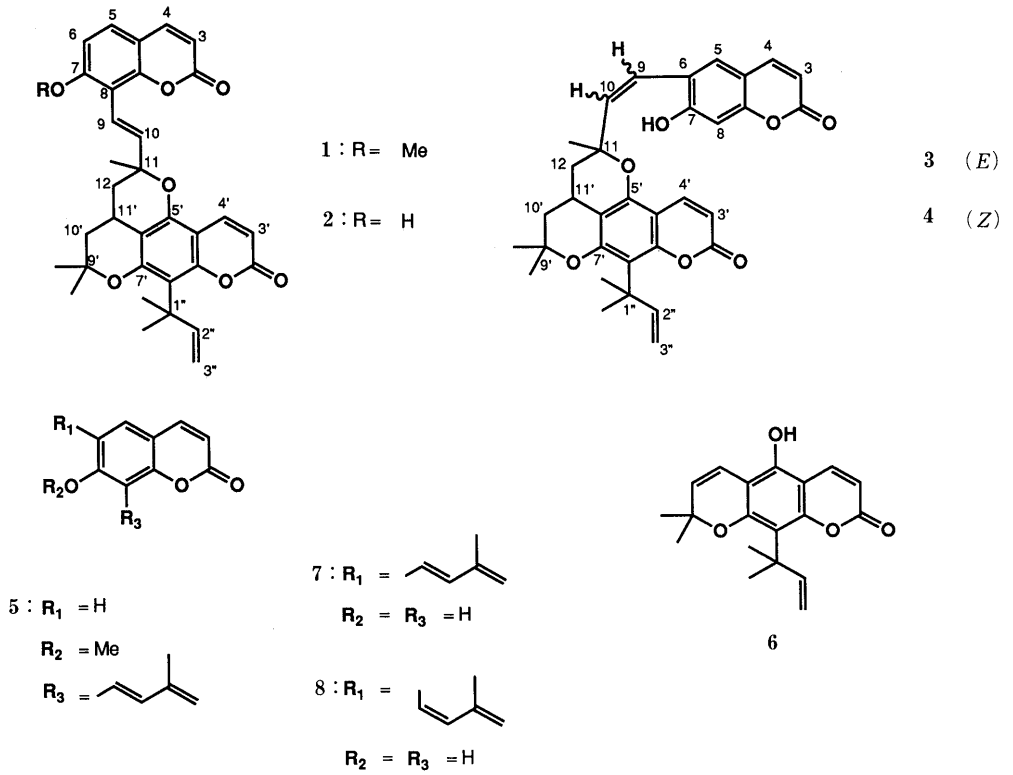

Chart 1

TABLE I. ^{1}H-NMR Data for Citrumarin-A (**1**), -B (**2**), -C (**3**), and -D (**4**)

H-No.	Citrumarin-A (**1**)	Citrumarin-B (**2**)	Citrumarin-C (**3**)	Citrumarin-D (**4**)
H-3	6.28 (d, 9.4)	6.25 (d, 9.4)	6.26 (d, 9.4)	6.16 (d, 9.4)
H-4	7.65 (d, 9.4)	7.64 (d, 9.4)	7.66 (d, 9.4)	7.06 (d, 9.4)
H-5	7.35 (d, 8.7)	7.25 (d, 8.7)	7.51 (s)	7.14 (s)
H-6	6.89 (d, 8.7)	6.95 (d, 8.7)		
7-OCH_3	3.97 (3H, s)			
H-8			6.94 (s)	6.92 (s)
H-9	7.12 (d, 16.5)	7.10 (d, 16.5)	7.00 (d, 16.1)	6.47 (d, 12.8)
H-10	7.05 (d, 16.5)	7.00 (d, 16.5)	6.46 (d, 16.1)	6.02 (d, 12.8)
11-CH_3	1.67 (3H, s)	1.66 (3H, s)	1.66 (3H, s)	1.50 (3H, s)
H-12[a]	1.4 (overlapped), 1.91 (dd, 13.4, 5.0)	1.4 (overlapped), 1.90 (dd, 13.4, 5.0)	1.4 (overlapped), 1.91 (dd, 13.1, 4.7)	1.35 (overlapped), 1.85 (dd, 13.1, 4.7)
H-3′	6.11 (d, 9.4)	6.11 (d, 9.8)	6.10 (d, 9.4)	5.72 (d, 9.4)
H-4′	8.16 (d, 9.4)	8.19 (d, 9.8)	8.14 (d, 9.4)	7.34 (d, 9.4)
9′-CH_3	1.41 (3H, s), 1.47 (3H, s)	1.41 (3H, s), 1.46 (3H, s)	1.41 (3H, s), 1.47 (3H, s)	1.35 (3H, s), 1.44 (3H, s)
H-10′[a]	1.6 (overlapped), 2.15 (dd, 13.4, 4.4)	1.6 (overlapped), 2.16 (dd, 13.4, 4.4)	1.6 (overlapped), 2.09 (dd, 13.1, 4.7)	1.7 (overlapped), 2.01 (dd, 13.1, 4.7)
H-11′	3.01 (m)	3.01 (m)	3.00 (m)	2.89 (m)
1″-CH_3	1.62 (3H, s), 1.63 (3H, s)	1.62 (6H, s)	1.60 (3H, s), 1.63 (3H, s)	1.57 (3H, s), 1.60 (3H, s)
H-2″	6.28 (dd, 17.5, 10.4)	6.25 (dd, 17.5, 10.4)	6.27 (dd, 17.2, 10.4)	6.21 (dd, 17.5, 10.4)
H-3″	4.84 (d, 10.4), 4.91 (d, 17.5)	4.82 (d, 10.4), 4.89 (d, 17.5)	4.83 (d, 10.4), 4.90 (d, 17.2)	4.79 (d, 10.4), 4.85 (d, 17.5)

Spectra were measured at 270 MHz in $CDCl_3$. All signals correspond to 1H, unless otherwise stated. Values in δ (ppm). Multiplicities are indicated by the usual symbols. The coupling constants (J values) in parentheses are in Hz. *a*) Assignment of H-12 and H-10′ may be interchanged.

$C_{19}H_{20}O_4$ and $C_{15}H_{14}O_3$, respectively, again indicating a binary coumarin structure for citrumarin-A.

Furthermore, the ^{1}H-NMR spectrum showed signals due to a 1,1-dimethylallyl moiety [δ_H 1.62 (3H, s), 1.63 (3H, s), 4.84 (1H, d, J=10.4 Hz), 4.91 (1H, d, J=17.5 Hz), and 6.28 (1H, dd, J=17.5, 10.4 Hz)], three quaternary methyls attached to oxygenated carbons [δ_H 1.41, 1.47, 1.67 (each 3H, s)], and *trans* olefinic protons [δ_H 7.12, 7.05 (each 1H, doublet, J=16.5 Hz)]. The analysis of the H–H correlation spectroscopy (COSY) spectrum and coupling constants of the remaining signals at δ_H 3.01 (1H, m), 2.15 (1H, dd, J=13.4, 4.4 Hz), 1.91 (1H, dd, J=13.4, 5.1 Hz) and overlapped signals at δ_H 1.6 and 1.4, indicated the presence of the partial structure [–C–CH_2–CH–CH_2–C–].

On the other hand, citrumarin-B (**2**) was isolated as colorless prisms, mp 243—245 °C, $[\alpha]_D$ +2.9° (chloroform), from the root of *C. hassaku*. The ^{1}H-NMR spectrum of citrumarin-B (**2**) showed an analogous signal pattern to that of citrumarin-A (**1**) (Table I), except for the lack of a methoxy signal in the spectrum of **1**. Treatment of citrumarin-B (**2**) with methyl iodide in acetone in the presence of potassium carbonate gave a mono-*O*-methyl ether, which was found to be identical with citrumarin-A (**1**).

Further information on the structure were obtained from analyses of ^{1}H- and ^{13}C-NMR spectra in dimethyl sulfoxide (DMSO-d_6) using the H–H and H–C COSY and ^{1}H detected heteronuclear multiple bond connectivity (HMBC) spectra of citrumarin-B (**2**). Assignments of carbons and protons (Experimental) were made on the basis of the H–C COSY and HMBC results shown by arrows in Fig. 1. The location of the 1,1-dimethylallyl moiety at C(8′) in the lower coumarin unit was shown by the presence of the cross peak between C(8′) at δ_C 112.08 and 1″-methyl protons at δ_H 1.59 in the three-bond H–C connectivities in the HMBC spectrum. The *trans* olefinic group at C(8) was determined from the three-bond correlations of C(7)–H(9), C(8a)–H(9),

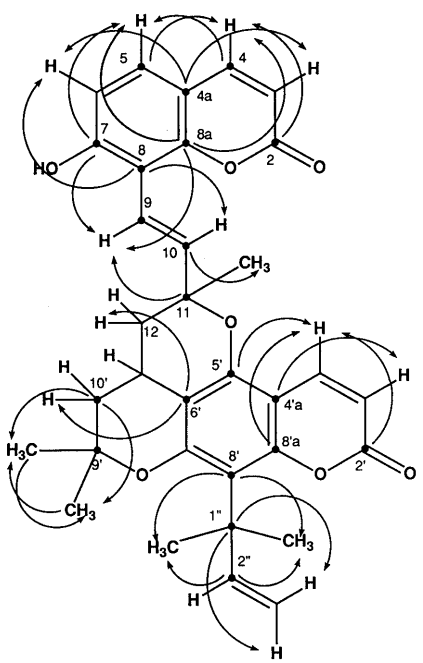

Fig. 1. C–H Long-Range Correlations in the HMBC Spectrum of Citrumarin-B (**2**)

and C(8)–H(10). The structure of the dimethyldihydropyran ring and the linking of the two coumarin units were confirmed by cross peaks of C(6′)–H(10′) and H(12), C(11)–H(9), C(10′)–geminal methyl protons (9′-CH_3), and C(10)–the lower field 11-methyl protons at δ_H 1.57 in HMBC spectrum. These structural features were supported by the observation of characteristic fragment ions at m/z 312 ($C_{19}H_{20}O_4$) and 228 ($C_{14}H_{12}O_3$) accompanied with an ion corresponding to the loss of a methyl radical [m/z 213 (base peak)], which were rationalized to occur by retro-Diels–Alder type cleavage of a dihydrobenzopyran ring in the molecule.

From the aforementioned results together with other correlations in the HMBC spectrum shown in Fig. 1, the structures of citrumarin-A and -B were concluded to be **1** and **2**, respectively, except for the stereochemistry. Citrumarin-A (**1**) is considered biogenetically to correspond to the dimer of *trans*-dehydroosthol (**5**)[7] and nordentatin (**6**).[8]

Structures of Citrumarin-C (3) and -D (4) Citrumarin-C (**3**) and -D (**4**) were isolated as colorless oils having $[\alpha]_D$ +7.8° (chloroform) and −15.4° (chloroform), respectively, from the root of *C. hassaku*, and were both found to have the same molecular formula, $C_{33}H_{32}O_7$, as that of citrumarin-B (**2**), by HR-MS analyses. The UV and IR spectra (Experimental) of citrumarin-C (**3**) and -D (**4**) showed typical absorptions of 7-oxygenated coumarin analogues.[1] In the electron-impact mass spectrum (EI-MS), both compounds showed a closely similar fragment pattern (Experimental) to that of **2**, including characteristic fragment ions at *m/z* 228 ($C_{14}H_{12}O_3$) and 312 ($C_{19}H_{20}O_4$), accompanied with strong ions at *m/z* 213 and 297 corresponding to the loss of a methyl radical, respectively, suggesting these citrumarins to be structural isomers of each other.

In the ^{1}H-NMR spectra of citrumarin-C (**3**) and -D (**4**) (Table I), proton signals due to a 1,1-dimethylallyl group, α- and β-protons of two α,β-unsaturated carbonyl systems as two pairs of AB-type doublets (J=9.4 Hz), three methyls attached to oxygenated carbons, and five protons in a five-spin system as in **2** were observed.

The remaining proton signals in the ^{1}H-NMR spectra were two one-proton singlets at δ_H 7.51 and 6.94 in citrumarin-C (**3**) and at δ_H 7.14 and 6.92 in citrumarin-D (**4**) due to *para* located aromatic protons, instead of *ortho* AB-type doublets (J=8.7 Hz) in the spectrum of **2**. Other AB-type doublets at δ_H 7.00 and 6.46 (J=16.1 Hz), and δ_H 6.47 and 6.02 (J=12.8 Hz) in the spectra of **3** and **4**, respectively, were assignable to *trans*- and *cis*-oriented olefinic protons, respectively. Based on these spectral data, the structures of citrumarin-C and -D should be as depicted by formulae **3** and **4**, respectively, corresponding to the dimer of nordentatin (**6**) and des-*O*-methylcitrubunin (**7**)[9] and its *cis* analogue (**8**), respectively. The stereochemistries of **3** and **4** remain to be determined.

Experimental

Melting points were measured on a micromelting point hot-stage apparatus (Yanagimoto). ^{1}H- and ^{13}C-NMR spectra were recorded on GX-270 (JEOL) and GX-400 (JEOL) spectrometers, respectively, in $CDCl_3$, unless otherwise stated. Chemical shifts are shown in δ values (ppm) with tetramethylsilane (TMS) as an internal reference. HMBC spectra were measured at J=8 Hz on the GX-400. All MS were measured under EI conditions, using an M-80 (Hitachi) or a JMS-HX-110 (JEOL) spectrometer having a direct inlet system. UV spectra were recorded on a UVIDEC-610C double-beam spectrophotometer (JASCO) in methanol, IR spectra on an IR-810 (JASCO) in $CHCl_3$, and optical rotations on a DIP-181 (JASCO) in $CHCl_3$. The preparative TLC was done on Kieselgel 60 F_{254} (Merck).

Isolation of Citrumarin-A (1) The dried root (250 g) of original seedlings of Swingle Citrumelo[6] [*Poncirus trifoliata* (L.) RAF. × *Citrus paradisi* MACF.] (Rutaceae) collected at Taiwan and grown in the orchard of Okitsu Branch, Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Shimizu, Shizuoka, was extracted with acetone at room temperature. The acetone extract was subjected to silica gel column chromatography with appropriate mixtures of hexane, benzene, and acetone, successively. The benzene–acetone (10:1) fraction was further subjected to preparative TLC using appropriate mixtures of hexane, benzene, isopropyl ether, and acetone as developing solvents to give citrumarin-A (**1**) (2.0 mg) along with other components.[10]

Isolation of Citrumarin-B (2), -C (3), and -D (4) An acetone extract (485 g) of dried root (3.2 kg) of *Citrus hassaku* hort. ex TANAKA (Rutaceae) collected in Innoshima, Hiroshima,[4] was chromatographed over silica gel with appropriate mixtures of hexane, benzene, dichloromethane, acetone, and methanol, successively. The dichloromethane and dichloromethane–acetone fractions were subjected to preparative TLC using acetone–benzene (2:8) and/or ethyl acetate–benzene (1:1 or 3:7) as developing solvents to obtain citrumarin-B (**2**) (56.4 mg), -C (**3**) (18 mg), and -D (**4**) (7.4 mg), respectively.

***O*-Methylation of Citrumarin-B (2)** An acetone solution (5 ml) of **2** (2.5 mg) was treated with added methyl iodide (0.1 ml) in the presence of anhydrous K_2CO_3 (100 mg), and the mixture was refluxed for 2.5 h. Then, the solvent was evaporated and the residue was subjected to preparative TLC (benzene:ethyl acetate=4:1) to give the mono-*O*-methyl ether (2.5 mg), which was found to be identical with **1** by comparisons of IR and ^{1}H-NMR spectra.

Citrumarin-A (1) Colorless amorphous powder. $[\alpha]_D$ ±0° (c=0.0925, $CHCl_3$). UV λ_{max} nm: 212, 256, 266, 284, 326. IR ν_{max} cm^{-1}: 1720, 1715, 1620, 1600. HR-MS Calcd for $C_{34}H_{34}O_7$: 554.2302. Found: 554.2355. Calcd for $C_{19}H_{20}O_4$: 312.1359. Found: 312.1352. Calcd for $C_{15}H_{14}O_3$: 242.0943. Found: 242.0963. EI-MS *m/z* (%): 554 (M^+, 22), 351 (10), 313 (13), 312 (27), 297 (36), 243 (25), 242 (100), 241 (12), 227 (27), 211 (30), 189 (12).

Citrumarin-B (2) Colorless prisms. mp 243—245 °C from acetone. $[\alpha]_D$ +2.9° (c=0.084, $CHCl_3$). UV λ_{max} nm: 212, 257, 266, 286, 325. IR ν_{max} cm^{-1}: 3300, (br), 1720, 1715, 1620, 1600. HR-MS Calcd for $C_{33}H_{32}O_7$: 540.2145. Found: 540.2102; Calcd for $C_{19}H_{20}O_4$: 312.1359. Found: 312.1331; Calcd for $C_{14}H_{12}O_3$: 228.0786. Found: 228.0826. EI-MS *m/z* (%): 540 (M^+, 20), 351 (18), 312 (33), 297 (80), 269 (11), 241 (15), 228 (12), 213 (100), 185 (10). ^{1}H-NMR (DMSO-d_6) δ_H: 8.09 (1H, d, J=9.8 Hz, H-4′), 7.95 (1H, d, J=9.3 Hz, H-4), 7.45 (1H, d, J=8.8 Hz, H-5), 7.08 (2H, s, H-9,10), 6.93 (1H, d, J=8.8 Hz, H-6), 6.25 (1H, d, J=9.3 Hz, H-3), 6.22 (1H, dd, J=17.6, 10.3 Hz, H-2″), 6.10 (1H, d, J=9.8 Hz, H-3′), 5.41 (1H, br s), 4.85 (1H, d, J=17.6 Hz, H-3″), 4.79 (1H, d, J=10.3 Hz, H-3″), 3.03 (1H, m, H-11′), 2.23 (1H, br d, J=9.3 Hz, H-10′ or 12), 2.01 (1H, br d, J=8.3 Hz, H-12 or 10′), 1.59 (6H, s, 1″-CH_3), 1.57 (3H, s, 9′-CH_3), 1.48 (1H, overlapped, H-10′ or 12), 1.42 (3H, s, 9′-CH_3), 1.39 (1H, overlapped, H-12 or 10′), 1.39 (3H, s, 11-CH_3). ^{13}C-NMR (DMSO-d_6) δ_C: 160.05 (C-2), 111.07 (C-3), 144.97 (C-4), 111.32 (C-4a), 128.00 (C-5), 112.81 (C-6), 159.30 (C-7), 110.08 (C-8), 152.80 (C-8a), 115.93 (C-9), 138.97 (C-10), 78.75 (C-11), 29.01 (11-CH_3), 37.31 (C-12 or 10′), 160.05 (C-2′), 108.93 (C-3′), 139.10 (C-4′), 101.78 (C-4′a), 148.00 (C-5′), 104.01 (C-6′), 155.58 (C-7′), 112.08 (C-8′), 152.74 (C-8′a), 77.21 (C-9′), 37.55 (C-10′ or 12), 22.08 (C-11′), 40.33 (C-1″), 150.09 (C-2″), 107.36 (C-3″), 29.70 (9′-CH_3), 25.72 (9′-CH_3), 29.56 (1″-CH_3), 24.84 (1″-CH_3).

Citrumarin-C (3) Colorless oil. $[\alpha]_D$ +7.8° (c=0.0705, $CHCl_3$). UV λ_{max} nm: 210, 259, 336. IR ν_{max} cm^{-1}: 3400 (br), 1720, 1715, 1620, 1600. HR-MS Calcd for $C_{33}H_{32}O_7$: 540.2146. Found: 540.2151; Calcd for $C_{19}H_{20}O_4$: 312.1359. Found: 312.1337; Calcd for $C_{14}H_{12}O_3$: 228.0786. Found: 228.0787. EI-MS *m/z* (%): 540 (M^+, 8), 351 (12), 312 (35), 297 (100), 269 (11), 241 (17), 228 (24), 213 (100), 185 (21).

Citrumarin-D (4) Colorless oil. $[\alpha]_D$ −15.4° (c=0.1445, $CHCl_3$). UV λ_{max} nm: 210, 258, 340. IR ν_{max} cm^{-1}: 3400 (br), 1720, 1715, 1620, 1600. HR-MS Calcd for $C_{33}H_{32}O_7$: 540.2146. Found: 540.2185; Calcd for $C_{19}H_{20}O_4$: 312.1359. Found: 312.1328; Calcd for $C_{14}H_{12}O_3$: 228.0786. Found: 228.0836. EI-MS *m/z* (%): 540 (M^+, 15), 351 (11), 312 (27), 297 (50), 229 (12), 213 (100), 185 (15).

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