Structure of Hassmarin, a Novel Biscoumarin from a *Citrus* Plant[1)]

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The acetone extract of the root of *Citrus hassaku* Hort. *ex* TANAKA (Rutaceae) was subjected to silica gel column and preparative thin layer chromatographies to obtain a new binary coumarin (biscoumarin) named hassmarin (1). The structure, corresponding to a Diels–Alder type adduct of demethyl derivatives of phebalosin (6) and *trans*-dehydroosthol (8), was assigned on the basis of spectral analyses.

Keywords hassmarin; *Citrus*; coumarin; biscoumarin; Rutaceae

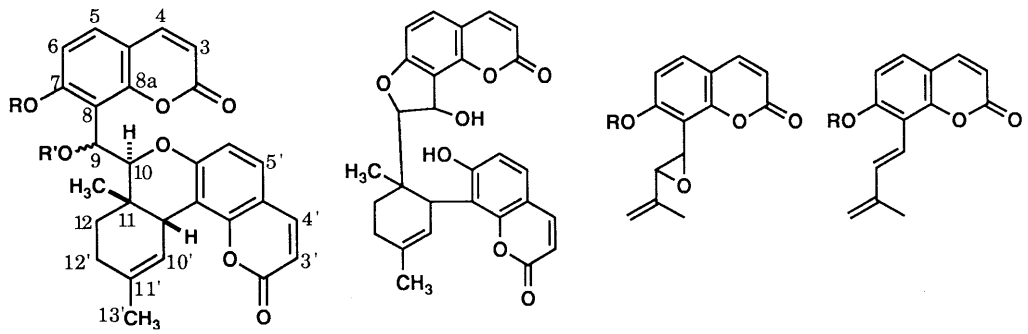
In our phytochemical studies of *Citrus* plants, many kinds of coumarins, acridone alkaloids, and flavonoids have been characterized.[2)] Further study of constituents of the root of *C. hassaku* Hort. *ex* TANAKA (Rutaceae) yielded a novel binary coumarin (biscoumarin), hassmarin, and its structure was identified as **1** by spectroscopic analyses.

Results and Discussion

The acetone extract of the root of *C. hassaku* was applied to a silica gel column, which was eluted with hexane, benzene, dichloromethane, acetone, and methanol, successively. The benzene eluate was further subjected repeatedly to preparative TLC to give hassmarin (**1**) as colorless prisms, mp 235—238 °C, $[\alpha]_D$ −3.9° ($CHCl_3$). The molecular formula $C_{28}H_{24}O_7$ was determined by high-resolution MS. The 7-oxygenated 8-substituted coumarin nuclei in the molecule were suggested by the following spectral data. a) Typical UV bands of the 7-oxygenated coumarin[3)] at λ_{max} 247, 258, and 326 nm and an IR absorption due to a lactone carbonyl group at ν_{max} 1750 cm^{-1} were observed. b) In the ^1H-NMR analysis using ^1H–^1H and ^1H–^{13}C correlation spectroscopies (COSY), two pairs of doublets due to protons of α,β-unsaturated carbonyl moieties [δ_H 6.14, 7.85 (each d, J=9.8 Hz); δ_H 6.20, 7.86 (each d, J=9.8 Hz)], and two pairs of *ortho*-located aromatic protons [δ_H 7.47 (H-5), 6.83 (each d, J=8.8 Hz, H-6); δ_H 7.38 (H-5′), 6.67 (each d, J=8.8 Hz, H-6′)] were observed. c) The ^{13}C-NMR spectrum showed two lactone carbonyl carbon signals [δ_C 161.03, 161.42]. d) In the ^1H detected multiple bond connectivity (HMBC) spectrum, ^{13}C signals having ^{13}C–^1H three σ-bonds (^3J) correlations to H-5 or H-5′ appeared at δ_C 163.54 (C-7), 152.97 (C-8a), 158.72 (C-7′), and 155.31 (C-8′a) indicating the presence of *O*-substituents at C-7 and C-7′ on the coumarin nuclei. e) Moreover, chemical shift values of C-8 (δ_C 114.31) and C-8′ (δ_C 114.79), which also showed ^{13}C–^1H three bond correlations to H-6 and H-6′, respectively, in the HMBC spectrum suggested the presence of *C*-substituents at C-8 and C-8′ on the coumarin nuclei.

Treatment of **1** with diazomethane gave the mono *O*-methyl ether (**2**) as a colorless oil. The ^1H-NMR spectrum of **2** showed a similar signal pattern to that of **1**, except for the appearance of a 3H singlet at δ_H 4.00 due to a methoxy group. In the nuclear Overhauser effect (NOE) experiment on **2**, the appearance of a 15% enhancement of the signal at δ_H 6.91 (H-6), one of the *ortho*-coupled aromatic protons, on irradiation of the methoxy signal indicated the presence of a phenolic hydroxy group at C-7 in the molecule of **1**. Treatment of **1** with acetic anhydride in pyridine afforded a diacetate (**3**) as a colorless oil, indicating the presence of an alcoholic hydroxy group in addition to a phenolic one.

The correlations of the remaining 10 carbons and the linked arrangement of the two coumarin units in the molecule were proposed in the partial structure *e* in Chart 2 based on analyses of ^1H–^1H COSY, ^1H–^{13}C COSY, and HMBC spectra of **1** as follows. a) Two 1H doublets at δ_H 6.07 and 4.32 having J=1.0 Hz were assignable to *vicinal*



1 : R=R′=H
2 : R=CH_3, R′=H
3 : R=R′=$COCH_3$

4

5 : R=H
6 : R=CH_3

7 : R=H
8 : R=CH_3

Chart 1

TABLE I. ^{1}H- and ^{13}C-NMR Data for Hassmarin (**1**) and Its Derivatives (**2** and **3**)

	1			**2**		**3**
	δ_H	δ_H[a]	δ_C	δ_H[a]	δ_C[a]	δ_H[a]
2			161.03		160.01[b]	
3	6.14 (d, 9.8)	6.16 (d, 9.5)	112.65	6.28 (d, 9.8)	113.41	6.38 (d, 9.4)
4	7.85 (d, 9.8)	7.58 (d, 9.5)	145.87	7.64 (d, 9.8)	143.75	7.66 (d, 9.4)
4a			112.75		113.76	
5	7.47 (d, 8.8)	7.31 (d, 8.4)	129.87	7.41 (d, 8.8)	126.26	7.45 (d, 8.4)
6	6.83 (d, 8.8)	6.87 (d, 8.4)	115.29	6.91 (d, 8.8)	113.78	7.07 (d, 8.4)
7			163.54		159.95[b]	
7-OR		9.03 (R=H)		4.00 (3H, s, R=Me)	56.18	2.37 (3H, s, R=Ac)
8			114.31		117.78	
8a			152.97		153.76[c]	
9	6.07 (d, 1.0)	6.06 (br s)	69.31	5.80 (d, 10.3)	67.09	6.79 (d, 3.0)
9-OR′		3.18 (d, 4.0, R′=H)		4.33 (d, 11.7, R′=H)		2.17 (3H, s, R′=Ac)
10	4.32 (d, 1.0)	4.39 (d, 2.6)	80.36	4.21 (d, 2.0)	78.87	4.48 (d, 3.0)
11			33.69		32.34	
11-Me	1.28 (3H, s)	1.25 (3H, s)	23.72	1.26 (3H, s)	22.59	1.16 (3H, s)
12	2.29 (dd, 13.7, 5.9), 1.97 (m)	2.04 (m),[c] 1.90 (m)	32.97	2.21 (m), 1.87 (m)	31.98	2.33 (m), 2.02 (m)
2′			161.42		161.38	
3′	6.20 (d, 9.8)	6.24 (d, 9.5)	113.52	6.23 (d, 9.8)	112.33	6.24 (d, 9.4)
4′	7.86 (d, 9.8)	7.61 (d, 9.5)	145.62	7.60 (d, 9.8)	144.08	7.60 (d, 9.4)
4′a			113.98		112.41	
5′	7.38 (d, 8.8)	7.20 (d, 8.4)	128.22	7.17 (d, 8.8)	128.23	7.17 (d, 8.4)
6′	6.67 (d, 8.8)	6.77 (d, 8.4)	114.56	6.70 (d, 8.8)	107.95	6.76 (d, 8.4)
7′			158.72		157.32	
8′			114.79		114.00	
8′a			155.31		153.76[c]	
9′	3.42 (br s)	3.53 (s)	43.07	3.50 (br s)	41.10	3.52 (br s)
10′	5.41 (br s)	5.39 (s)	123.83	5.41 (br s)	123.02	5.39 (br s)
11′			134.66		132.57	
11′-Me	1.68 (3H, s)	1.63 (3H, s)	23.66	1.58 (3H, s)	23.12	1.65 (3H, s)
12′	2.92 (m),[d] 2.06 (m)[d]	2.46 (m), 2.04 (m)[c]	27.44	2.34 (m), 2.01 (dd, 17.6, 5.4)	26.07	2.09 (m), 1.90 (m)

Values are δ ppm. Figures in parentheses are coupling constants (J) in Hz. Each signal corresponds to 1H, unless otherwise stated. *a*) Chemical shift values in the spectrum taken in $CDCl_3$. *b*) May be interchanged. *c*) Overlapped signal. *d*) Overlapped with HOD or the solvent signal.

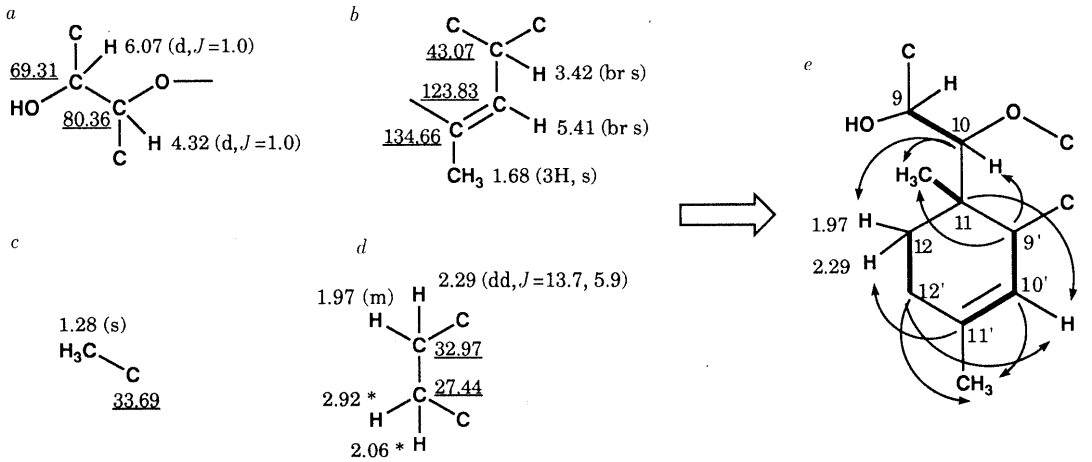


Chart 2. Partial Structures in the Hassmarin (**1**) Molecule

Chemical shift values (δ_H: normal letters; δ_C: underlined) in the ^{1}H- and ^{13}C-NMR spectra. C: sp^2 or sp^3 carbon having no H-atom. *Overlapped with HOD or the solvent signal. Arrows in formula *e*: ^{13}C–^{1}H three-bond correlations in the HMBC spectrum of **1**.

protons attached to oxygen-linked carbons. Further, the lower chemical shift value of one of the protons at δ_H 6.07 and a paramagnetic shift of this proton signals at δ_H 6.06 to 6.79 in the ^{1}H-NMR (in $CDCl_3$) spectra of **1** and **3**, respectively, suggested that this proton is attached to the hydroxylated benzylic carbon. b) The presence of broad singlets at δ_H 3.42 and 5.41 correlated with each other in the ^{1}H–^{1}H COSY and an NOE increment (5%) between the lower-field signal at δ_H 5.41 and an allyl methyl signal at δ_H 1.68 (3H, br s) suggested the presence of the moiety *b* shown in Chart 2. c) In addition to these groups, ^{1}H- and ^{13}C-NMR spectra showed the presence of a tertiary methyl group (*c*) and *vicinal* located methylenes (*d*) each bonded to sp^2 or sp^3 carbon bearing no hydrogen atom. The linked

arrangement of these moieties (*a*, *b*, *c*, and *d*) was elucidated from the results of the HMBC spectrum shown by arrows in Chart 2, indicating the presence of a monoterpene unit *e* in the molecule. Moreover, a ^3J correlation observed between the signals of the proton at δ_H 6.07 attached to the hydroxylated carbon (C-9) in *a* and an angular carbon [C-8a (δ_C 152.97)], which further correlated to H-4 (δ_H 7.85) and H-5 (δ_H 7.47) revealed the presence of the C(8)–C(9) linkage between one of the coumarin nuclei and the monoterpene unit *e*. At this stage, two plausible formulae **1** and **4** were proposed for the structure of hassmarin.

In the MS of **2**, the appearance of diagnostic fragment peaks at *m/z* 205 (100%), 281 (21%), and 282 (84%) arising from the cleavage at the benzylic position [C(9)–C(10) bond] in the molecule proved the structure of hassmarin to be represented by formula **1**. The presence of a ^3J correlation between H-9 (δ_H 5.80) attached to the hydroxylated benzylic carbon and the carbon at C-7 (δ_C 159.95) carrying the methoxy group in the HMBC spectrum of **2** also supported the above structure.

The relative stereochemistry of **1** was elucidated by NOE experiments. Irradiation of the 11-methyl signal at δ_H 1.28 gave a 4% enhancement of the signal at δ_H 3.42 (H-9′), which also showed a 3% increase on irradiation of the signal at δ_H 1.97, one of the methylene protons at C-12. Further, irradiation of the signal at δ_H 2.92, one of the methylene protons at C-12′, produced a 2% enhancement of the signal at δ_H 4.32 (H-10). Based on these results, we proposed the relative stereochemistry at C-10, C-11, and C-9′ to be as shown in formula **1**. The stereochemistry at the hydroxylated carbon (C-9) and the absolute stereochemistry remained undetermined.

Hassmarin (**1**) is considered to be formed biogenetically by a Diels–Alder type condensation between **5** and **7**, corresponding to demethyl derivatives of phebalosin (**6**)[4] and *trans*-dehydroosthol (**8**),[5] respectively, followed by cleavage of the epoxide ring by the phenolic hydroxy group of **7**. Biscoumarins having a monoterpene unit as the linkage between two coumarin nuclei are known.[6] However, hassmarin (**1**) is the first example of a biscoumarin having an ether bond as well as a monoterpene linkage.

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 acetone-d_6, 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 mass spectra were taken under electron impact (EI) conditions, unless otherwise stated, 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 $CHCl_3$ at 25 °C. Preparative TLC was done on Kieselgel 60 F_{254} (Merck).

Isolation of Hassmarin (1) from *Citrus hassaku* The acetone extract (485 g) of dried roots (3.2 kg) of *C. hassaku* Hort. *ex* TANAKA (Rutaceae) collected at Innoshima, Hiroshima, was subjected to a silica gel chromatography eluted with hexane, benzene, CH_2Cl_2, acetone and MeOH, successively. The benzene eluate was subjected repeatedly to preparative TLC with iso-Pr_2O, acetone–benzene (2 : 1), and acetone–$CHCl_3$ (1 : 9) as developing solvents to obtain **1** as colorless prisms (31.5 mg), mp 235—238 °C, $[\alpha]_D$ −3.9° ($c=0.426$, $CHCl_3$). UV λ_{max} nm: 208, 247, 258, 326. IR ν_{max} cm^{-1}: 3400 (br), 1720, 1610. ^{1}H- and ^{13}C-NMR; see Table I. MS *m/z* (%): 472 (M^+, 18), 454 (29), 310 (17), 297 (11), 287 (12), 282 (100), 279 (46), 229 (42), 213 (92), 201 (57). FAB-MS: *m/z* 473 ($M^+ + H$). HR-MS Calcd for $C_{28}H_{24}O_7$: 472.1520. Found: 472.1514. Difference NOE: irradiation of H-9, 8 and 5% enhancements of H-10 and H-12 (δ_H 2.29), respectively; irradiation of H-10, 5% enhancement of H-9; irradiation of H-12 (δ_H 1.97), 3% enhancement of H-9′; irradiation of H-12′ (δ_H 2.92), 2 and 1% enhancements of H-10 and H-9, respectively; irradiation of 11-CH_3, 4% enhancement of H-9′; irradiation of H-9′, 2% enhancement of H-10′; irradiation of H-10′, 4 and 5% enhancements of H-9′ and 11′-CH_3, respectively; irradiation of 11′-CH_3, 5% enhancement of H-10′. Significant ^3J correlations in the HMBC spectrum other than the correlations shown by arrows in Chart 2*e*: C-2–H-4; C-7–H-5; C-8–H-6; C-8a–H-4, 5, and 9; C-2′–H-4′; C-7′–H-5′; C-8′–H-6′; C-8′a–H-4′ and 5′.

***O*-Methylhassmarin (2)** An excess amount of an ether solution of diazomethane was added to a methanol solution (1 ml) of **1** (4.2 mg) and the mixture was left overnight at room temperature. The solvent was evaporated off and the residue was subjected to preparative TLC ($CHCl_3$: MeOH = 50 : 1) to obtain **2** as a colorless oil (3.9 mg). UV λ_{max} nm: 207, 220 (sh), 246, 257, 323. IR ν_{max} cm^{-1}: 3450 (br), 1725, 1600. MS *m/z* (%): 486 (M^+, 12), 282 (84), 281 (21), 267 (12), 213 (28), 205 (100), 201 (28), 199 (17), 189 (13), 187 (15), 176 (55). FAB-MS *m/z* 487 ($M^+ + H$). ^{1}H- and ^{13}C-NMR (Table I). Difference NOE ($CDCl_3$): irradiation of OCH_3 (δ_H 4.00), 15% enhancement of H-6 (δ_H 6.91). Significant ^3J (or ^2J) correlations in the HMBC spectrum ($CDCl_3$): C-7–H-9 and proton of 7-OCH_3; C-8–H-6 and H-9 (^2J); C-10–H-12 (δ_H 1.87); C-11–H_2-12 (δ_H 1.87 and 2.21, ^2J); C-12–H-13 (11-CH_3); C-7′–H-5′; C-8′–H-6′; C-9′–H-10, H-13 (11-CH_3), and H-12 (δ_H 2.21); C-10′–H-13′ (11′-CH_3); C-11′–H-12 (δ_H 2.21), H-12′ (δ_H 2.01, ^2J), and H-13′ (11′-CH_3, ^2J); C-12′–H-10′ and H-13′ (11′-CH_3); C-13′ (11′-CH_3)–H-10′.

Hassmarin Acetate (3) A mixture of **1** (5.1 mg), acetic anhydride (1.5 ml), and pyridine (1.5 ml) was stirred overnight at room temperature. MeOH (5 ml) was added to the ice-cooled and stirred solution and the mixture was evaporated to dryness. The residue was subjected to preparative TLC ($CHCl_3$: MeOH = 50 : 1) to give **3** (4.7 mg) as a colorless oil. **3**: UV λ_{max} nm: 207, 220 (sh.), 262, 286, 316. IR ν_{max} cm^{-1}: 1745, 1720, 1600. MS *m/z* (%): 556 (M^+, 10), 496 (45), 481 (13), 467 (13), 454 (11), 453 (11), 439 (10), 281 (35), 279 (100), 265 (12), 253 (11), 252 (11), 233 (11), 226 (36), 213 (76), 201 (34), 199 (21), 189 (16). ^{1}H-NMR (Table I).

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