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Isolation and characterization of wound-induced compounds from the leaves of *Citrus hassaku*

Tomonori Asai,[1] Tetsuya Matsukawa,[1,2] Atsushi Ishihara,[3] and Shin'ichiro Kajiyama[1,*]

Graduated School of Biology-Oriented Science and Technology, Kinki University, 930 Nishimitani, Kinokawa City, Wakayama 649-6493, Japan,[1] Experimental Farm, Kinki University, Yuasa, Yuasa-cho, Arida-gun, Wakayama 643-0004, Japan,[2] and Faculty of Agriculture, Tottori University, Koyama-cho, Tottori City, Tottori 680-8553, Japan[3]

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***Citrus* plants are world widely cultivated as horticultural tree crops, and nowadays their pharmacological activities have been well studied. Since research of defense responses in citrus plants have been mainly focused on the post-harvested fruits because of their commercial importance, defense mechanisms during their developmental stages have not been well understood. In the present study, two wound-induced compounds were isolated from leaves of *Citrus hassaku*, and their structures were elucidated by high-resolution electron spray ionization mass spectra (HRESIMS) and nuclear magnetic resonance (NMR) analyses. One of these compounds was identified as a known flavanone, hesperetin. The other was characterized as a novel furofuran lignan, and was named 'biscitrusnin-A'. Their antimicrobial activities were also evaluated.**

[**Key words:** *Citrus hassaku*; Mechanical wounding; Structure elucidation; Furofuran lignan; Antibacterial activity]

Citrus plants, belonging to a Rutaceae family, are widely accepted as important commercial fruit trees in the world. In recent years, some kinds of citrus fruits have been found to exhibit pharmacological activities such as hypocholesterolemic (1), hypotensive (2) and antioxidant activity (3) and received much attention as healthy food. Phenylpropanoids, flavonoids, terpenoids and alkaloids are major bioactive secondary metabolites in *Citrus* and some of them are considered to be involved in their defense responses against pathogens. For instance, polymethoxyflavones, such as nobiletin and tangeretin, have antifungal effects on *Penicillium digitatum* (4), and monoterpenes, such as α-pinene and geraniol, in *Citrus sinensis* and *Citrus paradisi* inhibit growth of *P. digitatum and Penicillium italicum* (5). The levels of defense related metabolites have been demonstrated to increase in response to environmental stresses in various plant species (6). In *Citrus*, the activities of phenylalanine ammonia lyase and chalcone synthase, which are the key enzymes of phenylpropanoid and flavonoid biosynthesis, have been reported to increase upon mechanical stress or pathogen infection (7). In addition, green leaf volatiles, which played in indirect defense mechanisms, were induced in response to stress treatments, such as wounding, and defense related phytohormones (jasmonic acid and salicylic acid) in several *Citrus* species (8).

Hassaku (*Citrus hassaku* Hort ex. Tanaka) is one of the most popular citrus species in Japan. The fruits of *C. hassaku* have been consumed not only as fresh fruit and juice but also as a source of traditional medicine (9). The secondary metabolites of *C. hassaku* have been well studied and a variety of flavonoids, limonoids and coumarins have been isolated from this plant. Coumarins including aurapten, epoxyaurapten, auraptenal, marmin were isolated from *C. hassaku* fruit (10–12), while dimeric coumarins such as nordenletin, hassamarin, neoacrimarine, claudimerine were found in its root (13–17). In spite of these increasing studies on bioactive compounds, reports of inducible compounds which react against environmental stress in *C. hassaku* are quite limited.

From this background, we focused on wound inducible secondary metabolites in *C. hassaku* leaves and showed that two metabolites were accumulated after mechanical wounding. In this paper, isolation and structural characterization of these compounds are reported.

MATERIALS AND METHODS

Plant materials Mature leaves of *C. hassaku* were collected in March 2010 at Wakayama prefecture, Japan. Leaves were cut into 5 mm square segments by surgical knife for mechanical wounding. Leaf segments were immersed in distilled water, and then, incubated for 72 h at 25°C. As a control, intact leaves were incubated under the same conditions.

Extraction, purification and isolation of wound-induced compounds Wounded leaves (183.5 g) were homogenized with Ultra-Turrax T25 (IKA, Staufen, Germany) in 10 volume of methanol. The homogenate was centrifuged at 12,000 × *g* for 15 min, and the resulting supernatant was obtained as the crude extract. The crude extract (1.10 g) was loaded onto a silica gel (Wakogel C-100, Wako, Osaka, Japan) column and eluted with a stepwise gradient of *n*-hexane/ethyl acetate (7:3 v/v), (5:5 v/v), (3:7 v/v) and (0:10 v/v). Fractions containing wound-induced compounds were subjected to preparative HPLC. A semi-preparative Inertsil ODS-3 column (6.0 mm × 250 mm, GL Sciences, Tokyo, Japan) was used and compounds were eluted with 45% methanol aqueous solution (for compound 1), and 50% methanol (for compound 2), respectively, at a flow rate of 1.8 ml/min. Finally, we obtained 5.9 mg of compound 1 and 2.1 mg of compound 2.

Spectroscopy An HPLC system consisted of a L-7200 pump and an L-4200 UV-VIS detector (Hitachi, Tokyo, Japan), which were used for detection and isolation of wound-induced compounds in *C. hassaku*. The samples were analyzed by using an Inertsil ODS-3 reverse phase column (4.6 × 150 mm, GL Sciences, Tokyo, Japan) and gradient elution with 0.1% formic acid aqueous solution and methanol at a constant flow rate of 0.8 ml/min. The linear gradient was programmed from 25% to 65%

* Corresponding author. Tel.: +81 736 77 3888; fax: +81 736 77 7011.
E-mail address: kajiyama@waka.kindai.ac.jp (S. Kajiyama).

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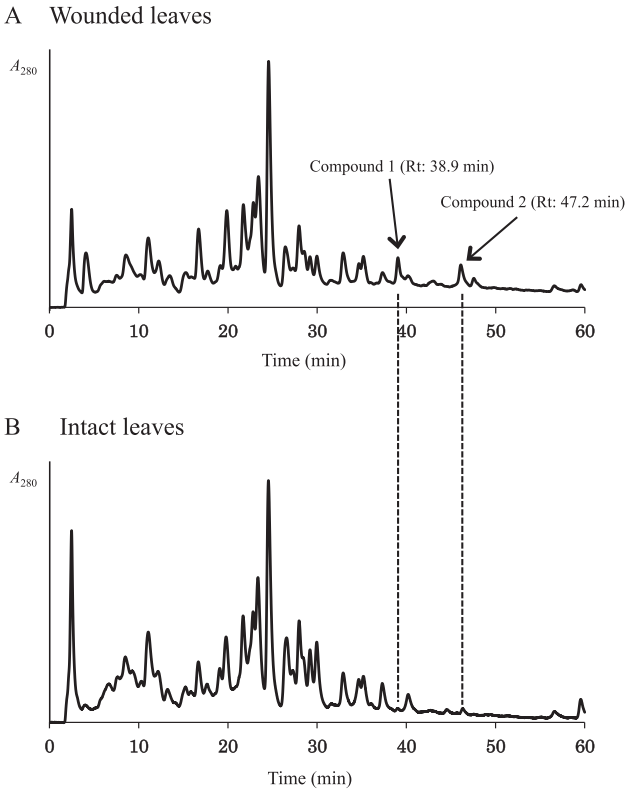

FIG. 1. HPLC chromatograms of extract from *C. hassaku* leaves; (A) wounded leaves and (B) intact leaves. Compounds were detected at 280 nm. Detected inducible peaks in wounded leaves were indicated by arrows; at 38.9 min peak (compound 1) and at 47.2 min peak (compound 2).

methanol for 60 min. The column temperature was set at 40°C, and eluted compounds were detected at 280 nm.

ESI mass spectra were recorded on a Quattro micro API mass spectrometer combined with an ACQUITY UPLC (Waters Corp., MA, USA). The samples were analyzed by using ACQUITY UPLC and gradient elution with 0.1% formic acid aqueous solution and acetonitrile at a constant flow rate of 0.2 ml/min. The linear gradient was programmed from 5% to 80% acetonitrile for 15 min, and column temperature was set at 40°C. High-resolution electron spray ionization mass spectra (HRESIMS) were recorded on Exactive spectrometer (Thermo Fisher Scientific, MA, USA).

Optical rotation was measured using P-2200 polarimeter (JASCO, Tokyo, Japan). UV spectrum was recorded on UV 2200A spectrometer (Shimadzu, Kyoto, Japan) scanning from 200 to 300 nm, and CD spectrum was measured on J820 CD spectrometer (JASCO) scanning from 210 to 350 nm. ^{1}H nuclear magnetic resonance (NMR) (600.1 MHz), ^{13}C NMR (150.9 MHz), and 2D NMR [correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC), and nuclear overhauser enhancement spectroscopy (NOESY)] spectra were obtained with a Bruker Avance II 600 MHz NMR spectrometer using a 5 mm probe, CD_3OD as solvents, and tetramethylsilane (TMS) as an internal standard.

Hesperetin (5,7,3′-trihydro-4′-methoxy flavanone, compound 1) ^{1}H-NMRδ: 2.71 (1H, dd, J = 3.0 Hz, 17.1 Hz, H-3a), 3.06 (1H, dd, J = 12.6 Hz, 17.1 Hz, H-3b), 3.86 (3H, s, H-7′), 5.32 (1H, dd, J = 3.0 Hz, 12.6 Hz, H-2), 5.88 (1H, dd, J = 1.98 Hz, H-6), 5.90 (1H, dd, J = 1.98 Hz, H-8), 6.90 (1H, d, J = 1.4 Hz, H-5′), 6.91 (1H, d, J = 1.4 Hz, H-6′), 6.93 (1H, s, H-2′). ESI-MS: m/z 301.2 $[M + H]^+$, m/z 303.2 $[M - H]^-$.

Biscitrusnin-A (4, 4′-dihydroxy-3, 3′-bis[1-(3-hydroxymethyl-1, 3-butadienyl)]-7, 9′: 7′, 9-diepoxylignan, compound 2) UV (MeOH) λ_{max} (log ε) 218 nm (3.58), 271 nm (3.38); ^{1}H-NMRδ: 3.17 (1H, m, H-8, -8′), 3.86 (1H, dd, J = 3.2 Hz, 9.1 Hz, H-9a, -9′a), 4.24 (1H, dd, J = 6.5 Hz, 9.1 Hz, H-9b, -9′b), 4.38 (2H, s, H-5, -5′), 4.72 (1H, d, J = 4.0 Hz, H-7, -7′), 5.24 (1H, s, H-4″a, -4‴a), 5.31 (1H, s, H-4″b, -4‴b), 6.78 (1H, d, J = 8.2 Hz, H-5, -5′), 6.91 (1H, d, J = 17.4 Hz, H-2″, -2‴), 6.92 (1H, d, J = 17.4 Hz, H-1″, -1‴), 7.09 (1H, dd, J = 1.9 Hz, 8.2 Hz, H-6, -6′), 7.46 (1H, d, J = 1.9 Hz, H-2, -2′). ^{13}C-NMRδ: 55.3 (C-8, -8′), 62.9 (C-5″, -5‴), 72.6 (C-9, -9′), 87.4 (C-7, -7′), 115.1 (C-4″, -4‴), 116.7 (C-5, -5′), 124.4 (C-1″, -1‴), 125.4 (C-2, -2′), 125.6 (C-3, -3′), 127.6 (C-6, -6′), 129.5 (C-2″, -2‴), 133.3 (C-1, -1′), 147.3 (C-3″, -3‴), 155.9 (C-4, -4′). ESI-MS: $[M + Na]^+$ at m/z 485.19275 calculated for $C_{28}H_{30}O_6Na$ m/z 485.19346, $[M - H]^-$ at m/z 461.19717 calculated for $C_{28}H_{29}O_6$ m/z 461.19587.

Antibacterial assay The turbidities of the culture media in 96-well microtiter plates (Sumilon, Sumitomo Bakelite Co., Tokyo, Japan) were used as a measure of microbial growth in liquid cultures in the absence or presence of various concentrations of compound 1 and compound 2. Each compound was dissolved in dimethyl sulfoxide (DMSO), and the final concentrations of DMSO in micro wells were adjusted to 1% v/v. Each of the test bacteria listed in Table 2 was pre-incubated at 30°C until growth plateaued. Then suspensions were inoculated into the NBRC 702 medium (10 g L^{-1} peptone, 2 g L^{-1} yeast extract, 1 g L^{-1} $MgSO_4 \cdot 7H_2O$, pH 7.0) containing 10, 25, 50, 100, 150, 300 μg ml^{-1} of the test compounds. At the start of culture, concentrations of the each pre-incubated culture broth were adjusted to 1% v/v. The test plates were incubated at 30°C for 36 h. After incubation, the turbidity was measured using a micro-plate reader (MPT-900Lab, Corona Electric, Ibaraki, Japan) at 610 nm wavelength. The lowest concentrations of each compound showing less than 50% growth were recorded as the minimum inhibitory concentrations (MICs).

RESULTS AND DISCUSSION

Detection of wound-induced compounds in *C. hassaku* leaves In order to detect wound-induced compounds in *C. hassaku*, crude extracts from wounded and intact leaves were subjected to HPLC analysis. Two peaks were found to be newly occurred at the retention times of 38.9 min (referred to as compound 1) and 47.2 min (referred to as compound 2) only in the

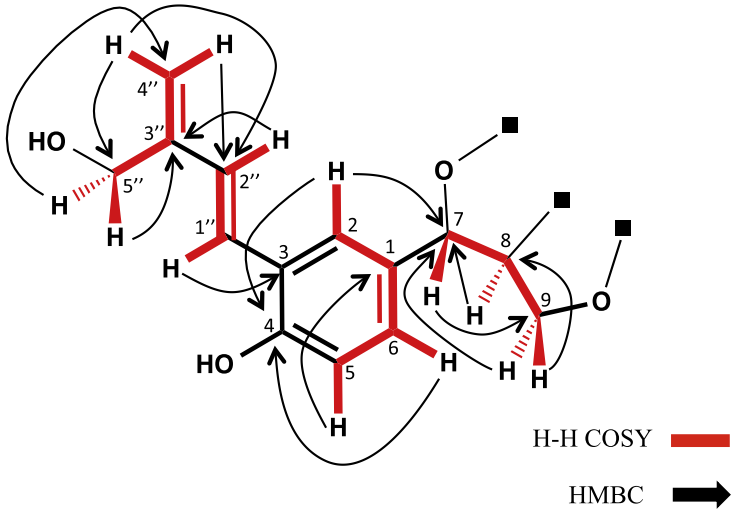

FIG. 2. ^{1}H–^{1}H and ^{1}H–^{13}C correlations for compound 2. H–H COSY coupling are indicated by red lines, and HMBC coupling are indicated by arrows (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).