Cucurbitane Glucosides from *Momordica charantia* Leaves as Oviposition Deterrents to the Leafminer, *Liriomyza trifolii*

Daniel Bisrat Mekuria*, Takehiro Kashiwagi, Shin-ichi Tebayashi, and Chul-Sa Kim

Department of Bioresources Science, Faculty of Agriculture, Kochi University, B200 Monobe, Nankoku 783-8502, Japan. Fax: +81-88-864-5186. E-mail: daniel_bisrat98@yahoo.com

* Author for correspondence and reprint requests

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The American serpentine leaf mining fly, *Liriomyza trifolii*, whose larva feeds on more than 120 plant species is well characterized by its high degree of polyphagy. Observations on the oviposition behavior by *L. trifolii* demonstrated that among cucurbitaceous plants, *Momordica charantia* is rarely attacked by *L. trifolii.* The methanol extract of *M. charantia* leaves strongly deterred the females from ovipositing on kidney bean leaves treated at a concentration of 1 g leaf equivalent extract/ml. Analysis of the polar fraction of the methanol extract of *M. charantia* leaves resulted in the isolation of a novel cucurbitane glucoside, 7-*O*-β-D-glucopyranosyl-3,23-dihydroxycucurbita-5,24-dien-19-al, named momordicine IV, along with another known compound, momordicine II. Momordicine II and IV deterred oviposition by *L. trifolii* significantly when bioassays were carried out on kidney bean leaves treated at 75.6 and 20.3 μg/cm^2 leaf surface, respectively. There was no synergistic effect on oviposition deterrent when the two compounds were combined in their natural abundance.

Key words: Liriomyza trifolii, *Momordica charantia*, Oviposition Deterrent, Momordicine IV

Introduction

Host selection by insects consists of a series of five steps: host-habitat finding, host finding, host recognition, host acceptance, and host suitability (Kogan, 1994). These steps lead to acceptance or rejection of a resource (Strom *et al.*, 1999). Host plant resistance is one method under examination to reduce the impact of pests on agriculture. *Liriomyza trifolii* (Burgess) is a major leafminer pest on a wide variety of vegetables, floricultures, and ornamental plants. This insect feeds on over 120 plant species (Minkenberg and van Lenteren, 1986). The females possess ovipositors, which are specially adapted to puncture the plants, and they use them to pierce the leaf surface, making holes through which exuding sap can be obtained (Murphy and Lasalle, 1999). Oviposition by *L. trifolii* occurs in the leaf mesophyll tissue. The larvae, hatching from the eggs, feed in the leaf mesophyll tissue and form a serpentine mine. Their mining activity can reduce significantly the photosynthetic capacity of the plant (Dogimont *et al.*, 1999). It has been reported that the pest status of *L. trifolii* has been strengthened by resistance to a wide spectrum of insecticides (Leibee, 1981). Thus, researchers have been seeking alternative means of pest control. Among cucurbitaceous plants, *M. charantia* is rarely attacked by phytophagous insects (Yasui *et al.*, 1998; Abe and Matsuda, 2000).

In a previous paper (Mekuria *et al.*, 2005), we reported the isolation and characterization of two oviposition deterrents from the less polar fraction of *M. charantia* leaf extract, namely momordicine I and 7,23-dihydroxy-3-*O*-malonylcucurbita-5,24-dien-19-al. As part of our ongoing study on oviposition deterrents from *M. charantia*, we have reinvestigated the methanol extract of *M. charantia* leaves. Working on the polar fraction of the methanol extract of *M. charantia* leaves led to the isolation of momordicine II and a newly identified compound, momordicine IV, as oviposition deterrents against females of *L. trifolii.*

Materials and Methods

Instruments

^{1}H and ^{13}C NMR data were recorded on a JEOL JNM-L400 Spectrometer in pyridine-d_5 with TMS as an internal standard. LC-MS spectra were measured on a Shimadzu LC-MS 2010 Liquid Chromatography Mass Spectrometer using an electrospray ionization (ESI) mode. HPLC was carried out with a Hitachi L-6200 Intelligent Pump equipped with a Hitachi L-4000 UV Detector (HPLC) and a Hitachi D-2500 Chromato Integra-

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tor. A Wakogel C-300 type of silica gel (Kyoto, Japan) was used for column chromatography.

Plant material

Seeds of *M. charantia* (var. Satsuma futonaga reishi) were purchased from Maekawa Shubyou Co. Ltd., Kochi, Japan. The seeds were initially sown in 8-cm-diameter pots (2 seeds per pot) with holes in the bottom, with soil consisting of vermiculite. Plants were grown in the glasshouse for seven weeks until four to six true leaves were fully expanded. *M. charantia* seedlings were then transplanted and grown in the open field at the Faculty of Agriculture, Kochi University. All plants were grown in the glasshouse or in the field without any application of pesticides. Fresh leaves were collected for extraction from matured *M. charantia* (fruiting-stage already passed) after three months and a half of transplanting to open field.

Insect and rearing methods

Liriomyza trifolii insects were initially provided by Kochi Prefecture Agricultural Research Center. Laboratory colonies of *L. trifolii* were reared successively on 10- to 14-day-old kidney bean seedlings, which were changed twice a week. They were maintained at (27 ± 2) °C, a relative humidity of 60–70% with a 16 h:8 h (L:D) photoperiod. In all experiments five *L. trifolii* females, 24-h-old after emerging, were collected randomly from the rearing unit, in which several hundred individuals had been reared, and were used for bioassay.

Bioassay on kidney bean leaves treated with crude extract and its constituents by the leaf dipping method

All test sample solutions were prepared at 1 g of fresh leaf equivalent/ml methanol. Kidney bean leaves were soaked in test sample solution for 30 s and then the solvent was removed quickly by air drying. A filter paper was set in a petri dish and moistened with distilled water to maintain humidity. Control leaves were treated in the same manner with only methanol. Treated and control leaves were then placed individually in petri dishes exposing their upper surface. Five adult female flies, at least 24-h-old, were added into the 50-ml screw vial (28 mm Ø) and placed on treated and control leaves by turning the screw vial tube upside down allowing them to oviposit for 24 h. All assays were conducted in a controlled room at 27 °C, with a relative humidity of 60–70% and a 16 h:8 h (L:D) illumination. Each test was replicated six times; data shown are mean values. The extent of oviposition deterrent activity was evaluated by counting the number of leaf puncture scars left by adult females of *L. trifolii.*

Extraction and isolation

Fresh leaves (2.3 kg) of *M. charantia* were cut into pieces and extracted with methanol (3 × 15 l) at room temperature for 3 d under darkness. The combined extracts were filtered and then the solvent was removed under reduced pressure (82 g; 3.6% on extraction). The residue was re-dissolved in water (1.5 l) and successively partitioned with hexane (2 × 1.5 l; 3.2 g), diethyl ether (2 × 1.5 l; 5.7 g) and water-saturated butanol (2 × 1.5 l; 39.9 g), respectively. Each fraction was dried under reduced pressure and then submitted for bioassay at the concentration of 1 g of leaf equivalent/ml. Three fractions were collected when the butanol fraction (20 g) was chromatographed over a silica gel column, eluting in sequence with an increasing concentration of methanol in ethyl acetate to obtain an ethyl acetate (fraction 1; 2 g), a 50% methanol in ethyl acetate (fraction 2; 8 g), and a methanol (fraction 3; 9.5 g) fraction. Further separation of the active 50% MeOH fraction (4 g) by HPLC led to the isolation of compounds **1** (1.6 g) and **2** (460 mg). HPLC was performed on a Cosmosil-5C_{18}-AR-II column (Nacalai Tesque, Inc., Kyoto, Japan; 250 mm × 10 mm i.d.) at 1.5 ml/min flow rate, using an isocratic solvent system of 80% methanol in water over 30 min. Compound **1** and **2** were detected at 210 nm (UV), and eluted at Rt = 22.4 min and 29.1 min, respectively.

*Momordicine II (**1**)*

$[\alpha]_D^{22}$ + 46.7° (*c* 0.3, MeOH). – Positive-ion ESI-MS: *m/z* = 657 $[M + Na]^+$, 673 $[M + K]^+$. – Negative-ion ESI-MS: *m/z* = 633 $[M - H]^-$. – ^{1}H NMR (pyridine-d_5): δ = 0.86, 0.88, 1.16, 1.47 (each 3H, *s*), 1.19 (3H, *d*, *J* = 6.6 Hz, H_3-21), 1.69, 1.75 (each 3H, *s*, H_3-26 and H_3-27), 1.18, 1.53, 1.57, 1.58, 1.93, 1.94, 1.96, 2.71 (each 1H), 1.35, 1.55, 1.93 (each 2H), 2.06 (1H, *m*, H-20), 2.36 (1H, *s*, H-8), 2.71 (1H, *m*, H-10), 3.80 (1H, *brs*, H-3), 3.88 (1H, *brs*, Glc-5), 4.01 (1H, *brt*, Glc-2), 4.21 (1H, *d*, Glc-3), 4.22 (1H, *d*, Glc-4), 4.33 (1H, *d*, *J* = 4.6 Hz, H-7), 4.34 (1H, *dd*, *J* = 9.1, 5.2 Hz, Glc-6_a), 4.46 (1H, *dd*, *J* = 9.1, 1.9 Hz, Glc-6_b), 4.94 (1H, *d*, *J* = 7.8 Hz,

H-23), 4.94 (1H, *d*, *J* = 7.8 Hz, Glc-1), 5.61 (1H, *d*, *J* = 7.8 Hz, H-24), 6.27 (1H, *d*, *J* = 4.4 Hz, H-6), 10.73, (1H, *s*, H-19). – ^{13}C NMR (pyridine-d_5): see Table I.

Momordicine IV (7-O-β-D-glucopyranosyl-3,23-dihydroxycucurbita-5,24-dien-19-al, ***2****)*

$[\alpha]_D^{22}$ + 90.0° (*c* 0.1, MeOH). – Positive-ion ESI-MS: *m/z* = 657 $[M + Na]^+$, 673 $[M + K]^+$. – Negative-ion ESI-MS: *m/z* = 633 $[M - H]^-$. – ^{1}H NMR (pyridine-d_5): δ = 0.75, 0.89, 1.12, 1.43 (each 3H, *s*), 1.10 (3H, *d*, *J* = 6.6 Hz, H_3-21), 1.68, 1.70 (each 3H, *s*, H_3-26 and H_3-27), 1.16, 1.50, 1.53, 1.55, 1.90, 1.94, 1.96, 2.65 (each 1H), 1.35, 1.55, 1.93 (each 2H), 2.05 (1H, *m*, H-20), 2.53 (1H, *s*, H-8), 2.69 (1H, *m*, H-10), 3.81 (1H, *brs*, H-3), 3.88 (1H, *brs*, Glc-5), 4.02 (1H, *brt*, Glc-2), 4.27 (1H, *d*, Glc-3), 4.22 (1H, *d*, Glc-4), 4.41 (1H, *dd*, *J* = 12.4, 5.6 Hz, Glc-6_a), 4.61 (1H, *dd*, *J* = 12.4, 1.2 Hz, Glc-6_b), 4.62 (1H, *d*, *J* = 4.1 Hz, H-7), 4.78 (1H, *dt*, $J_1 = J_2$ = 8.1 Hz, J_3 = 1.9 Hz, H-23), 4.95 (1H, *d*, *J* = 8.0 Hz, Glc-1), 5.60 (1H, *d*, *J* = 8.4 Hz, H-24), 6.16 (1H, *d*, *J* = 4.1 Hz, H-6), 10.56 (1H, *s*, H-19, aldehyde). – ^{13}C NMR (pyridine-d_5): see Table I.

Enzymatic hydrolysis of compound ***2***

A solution of compound **2** (20 mg) and β-glucosidase (4 mg; Oriental Yeast Co., Ltd., Tokyo, Japan) in 3 ml of water (1.5 ml) was kept stirring for 12 h at 34–37 °C. The reaction mixture was concentrated to dryness, and chromatographed on an octadecylsilyl-silica gel (ODS) open column eluting with water (fraction 1) and methanol (fraction 2) to afford a product identical to **3** [^{1}H, ^{13}C NMR and by comparing its Rt (HPLC) and R_f values (TLC) with the standard sample].

Compound ***3***

^{1}H NMR (pyridine-d_5): δ = 0.86, 0.95, 1.18, 1.50 (each 3H, *s*), 1.15 (3H, *d*, *J* = 6.4 Hz, H_3-21), 1.68, 1.70 (each 3H, *s*, H_3-26 and H_3-27), 1.16, 1.53, 1.57, 1.58, 1.93, 1.94, 1.96, 2.70 (each 1H), 1.35, 1.55, 1.93 (each 2H), 2.09 (1H, *m*, H-20), 2.38 (1H, *s*, H-8), 2.70 (1H, *m*, H-10), 3.82 (1H, *brs*, H-3), 4.30 (1H, *d*, *J* = 4.6 Hz, H-7), 4.74 (*dt*, $J_1 = J_2$ = 8.4 Hz, J_3 = 2.4 Hz, H-23), 5.52 (1H, *d*, *J* = 8.4, H-24), 6.20 (1H, *d*, *J* = 4.6 Hz, H-6), 10.66, (1H, *s*, H-19). – ^{13}C NMR (pyridine-d_5): see Table I.

Results and Discussion

Effect of kidney bean leaves treated with crude extract and its constituents on adult L. trifolii oviposition

In a previous study, we established that *L. trifolii* females deterred from ovipositing on *M. charantia* leaves (Mekuria *et al.*, 2005). As shown in Fig. 1, we found that kidney bean leaves treated with the methanol extract of *M. charantia* leaves significantly reduced the number of leaf punctures [methanol extract: (2.57 ± 0.27) marks/cm^2; control: (57.52 ± 3.83) marks/cm^2, mean ± S.E.]. This result showed the existence of a methanol-soluble oviposition deterrent in the leaves of *M. charantia.* During the bioassay, the female flies landed briefly on kidney bean leaves treated with *M. charantia* leaf extract. Upon landing, females walked over the treated leaf, and attempted to insert their ovipositors into the treated leaf. Once the ovipositors were inserted into the treated leaf, females remained agitated and occasionally retracted their ovipositor, and attempted to do somewhere else. However, once the ovipositor inserted into an untreated kidney bean leaf, the females stayed motionless, and swelling of the basis of the ovipositor could be seen. When the methanol extract of *M. charantia* was partitioned into hexane, diethyl ether, butanol and water fractions, the butanol fraction [(2.88 ± 0.51) marks/cm^2] decreased the number of oviposition marks, in a similar manner

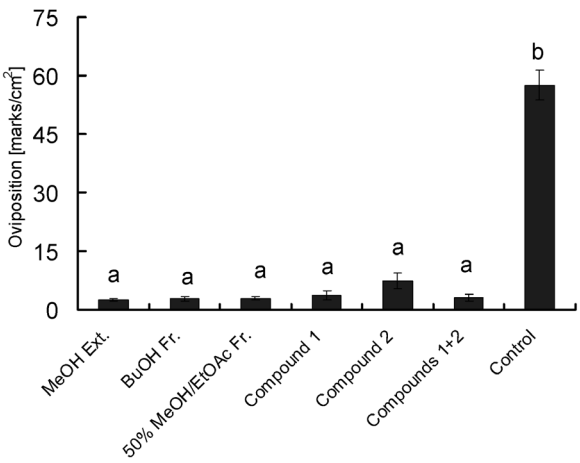
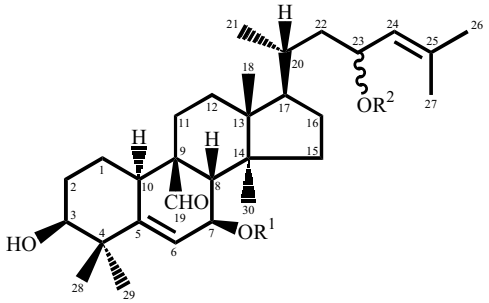


Fig. 1. Oviposition responses of various test solutions of bitter gourd leaf extract to adult females of *L. trifolii*. Values with different letters are significantly different (Tukey-Kramer multiple range test, $P < 0.05$).

to the initial methanol extract. However, the other fractions failed to deter oviposition at 1 g fresh leaf equivalent/ml [hexane fraction, (40.67 ± 1.62) marks/cm^2; diethyl ether fraction, (52.04 ± 2.33) marks/cm^2; water fraction, (55.90 ± 2.73) marks/cm^2; mean ± S.E.]. These results indicated that *M. charantia* leaves contained polar oviposition deterrents, which were predominately extractable with butanol during liquid-liquid partition. Fractionation of the butanol extract on silica gel and reassaying of the fractions indicated that active component(s) were present in the 50% methanol in ethyl acetate fraction [(2.91 ± 0.49) marks/cm^2, mean ± S.E.]. The other two fractions, ethyl acetate and methanol fractions produced no significant decline in oviposition [ethyl acetate fraction, (49.18 ± 3.06) marks/cm^2; methanol fraction, (53.72 ± 3.48) marks/cm^2]. Continuing working on the polar fraction (50% ethyl acetate/methanol fraction) by HPLC resulted in the isolation of **1** and **2** along with the previously reported compounds momordicine I and 7,23-dihydroxy-3-*O*-malonylcucurbita-5,24-dien-19-al (Mekuria *et al.*, 2005). *L. trifolii* was deterred from ovipositing on kidney bean leaves treated with compounds **1** (75.6 μg/cm^2) and **2** (20.3 μg/cm^2) at gle/ml [**1**: (3.65 ± 1.07) marks/cm^2; **2**: (7.32 ± 1.96) marks/cm^2]. There was no synergistic effect observed on oviposition deterrence when **1** and **2** were combined [(3.03 ± 0.89) marks/cm^2]. This suggests that the dose responses of each compound at the concentration 1 g leaf equivalent extract/ml may be close to the saturation point (constant) response, fitting to the baseline or maximum response. In this study, the contents of **1** and **2** in the fresh leave of *M. charantia* were 2.7 mg/g (79.8 μg/cm^2; by calculation) and 0.8 mg/g (22.4 μg/cm^2; by calculation), respectively. This result demonstrated that cucurbitane glucosides in the leaves of *M. charantia* deterred the female from ovipositing on it.

Structure determination

Compound **1** is obtained as an amorphous solid, and is present at about 0.27% in fresh weight of leaves of *M. charantia.* This compound gave a positive reaction in the Liebermann-Burchard test. Pseudomolecular ions of **1** were observed at *m/z* 657 $[M + Na]^+$ and 673 $[M + K]^+$ in the positive-ion ESI-MS spectrum. Compound **1** also gave a negative pseudomolecular ion at *m/z* 633 $[M - H]^-$, indicating the molecular formula $C_{36}H_{58}O_9$, which



1: R^1 = H; R^2 = Glc (Momordicine II)
2: R^1 = Glc; R^2 = H (Momordicine IV)
3: R^1 = H; R^2 = H (Momordicine I)

Fig. 2. Structures of compounds **1**, **2** and **3**.

was in good agreement with ^{1}H and ^{13}C NMR data. Based on the above spectroscopic evidence, the structure of **1** was concluded as momordicine II (23-*O*-*ß*-D-glucopyranosyl-3,7-dihydroxycucurbita-5,24-dien-19-al, Fig. 2). Carbon and proton assignments of **1** were in good agreement with those signals reported for the same compound in the literature (Yasui *et al.*, 1998; Abe and Matsuda, 2000).

Compound **2** gave pseudomolecular ions at *m/z* 657 $[M + Na]^+$ and 673 $[M + K]^+$ in the positive-ion ESI-MS spectrum, corresponding to the molecular formula $C_{36}H_{58}O_9$, which was in good agreement with ^{1}H and ^{13}C NMR data. The ^{13}C NMR and DEPT (Table I) spectra of **2** showed signals for 36 different carbon atoms, assigning to seven methyl groups, eight methylene groups, fourteen methines including two olefinic carbon atoms, six quaternary carbon atoms and an aldehyde carbonyl carbon atom. The pseudomolecular ions of **2** in the positive-ion ESI-MS were identical with those of **1**, in which both **1** and **2** appeared 162 mass units higher than **3**. Compound **2** showed a pattern of signals in the ^{1}H and ^{13}C NMR spectra similar to that of compound **3**, except the presence of additional signals due to a glucose moiety. The presence of an *O*-glucose unit in **2** was confirmed by conversion of **2** to **3** by enzymatic hydrolysis using *ß*-glucosidase.

The placement of the glucose moiety with the hydroxyl group at C-7 in **2** was suggested by its ^{13}C NMR spectrum, in which a glycosidation shift of + 5.87 ppm relative to that of the same in **3** was observed (65.47 ppm in **3** to 71.34 ppm in **2**). Confirmation of the position of the attached of

Table I. ^{13}C NMR spectral data of compounds **1**–**3** in C_5D_5N.

C	**1**	**2**	**3**
1	21.74	21.35	21.27
2	29.88	29.26	29.40
3	75.61	75.39	75.43
4	41.76	41.72	41.59
5	145.69	147.23	145.38
6	124.27	121.95	123.63
7	65.70	71.34	65.47
8	50.57	44.30	49.12
9	50.64	51.08	50.99
10	36.83	36.14	36.35
11	22.68	22.08	22.20
12	29.60	28.89	29.02
13	45.89	45.62	45.21
14	48.26	47.99	48.13
15	34.92	34.23	34.36
16	27.81	27.43	27.58
17	51.24	50.72	50.66
18	14.93	14.40	14.47
19	207.50	206.92	207.49
20	32.66	32.37	32.38
21	19.41	18.65	18.58
22	43.75	44.89	44.89
23	75.29	64.93	64.69
24	129.11	131.53	131.56
25	132.23	130.49	130.52
26	18.25	17.64	17.72
27	26.21	25.73	25.74
28	25.82	25.25	25.29
29	27.31	26.76	26.81
30	18.21	17.57	17.60
7-Glc-1	–	101.35	–
7-Glc-2	–	74.74	–
7-Glc-3	–	78.57	–
7-Glc-4	–	71.54	–
7-Glc-5	–	78.46	–
7-Glc-6	–	62.69	–
23-Glc-1	104.16	–	–
23-Glc-2	75.66	–	–
23-Glc-3	78.90	–	–
23-Glc-4	71.81	–	–
23-Glc-5	78.25	–	–
23-Glc-6	62.96	–	–

Assignments are in ppm.

the *O*-glucose unit was obtained from the ^1H detected heteronuclear multiple bond connectivity (HMBC) spectrum in which ^3J correlations were observed between the proton at C-7 (H-7, δ 4.62) and the anomeric carbon signal at δ 101.35, as well as the anomeric proton at δ 4.95 and the carbon signal at C-7 (δ 71.34). Other important ^3J correlations from the HMBC spectrum can be summarized as follows: (i) Correlation between the proton at C-3 (H-3, δ 3.81) and a quaternary carbon atom (C-5, δ 147.23); (ii) cross peak between the oxymethine proton at C-7 (H-7, δ 4.62) and a quaternary carbon atom (C-5, δ 147.23); (iii) correlation between the oxymethine proton at C-23 (H-23, δ 4.78) and a quaternary carbon atom (C-25, δ 130.49). The configuration of the glucosidic linkage at C-7 was determined as β-form based on the coupling constant of the anomeric proton (δ 4.95, d, J = 8.0). Therefore, the structure of this new triterpene glucoside **2** is deduced to be 7-*O*-β-D-glucopyranosyl-3,23-dihydroxycucurbita-5,24-dien-19-al, for which the trivial name momordicine IV is proposed (Fig. 2).

It was previously suggested that polarity, such as glucopyranose moiety might have been the factors contributing to the antifeedant activity of *M. charantia* (Yasui *et al.*, 1998). In this study the authors have noticed that the triterpenoid glucosides **1** and **2** showed stronger oviposition deterrency than the corresponding aglycone, momordicine I (**3**). The reason for this difference in activity might be also due to difference in their polarity, and the glucopyranose moiety might be one of the important factors contributing to stronger activity. *L. trifolii* is clearly causing serious damage to a wide range of crops. Also a wide range of insecticides is used to manage this insect but insecticide-resistance populations are fast developing and adding a new problem for farmers. In a previous study, we established that the leaves of *M. charantia* are rejected by the ovipositing females. Thus, the compounds described in this and previous studies may serve as a guide for the design of safe, environmentally compatible behavioral control methods for *L. trifolii*, which will decrease our reliance on use of insecticides.

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