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Acylated flavonol glycosides as probing stimulants of a bean aphid, *Megoura crassicauda*, from *Vicia angustifolia*

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Abstract

A bean aphid, *Megoura crassicauda*, which feeds selectively on the plant genus *Vicia* (Fabaceae), was found to be stimulated to probe an extract solution of the host plant, narrowleaf vetch, *Vicia angustifolia* L., depositing characteristic stylet sheaths on a parafilm membrane. Two acylated flavonol glycosides were isolated as the specific probing stimulants from the extracts and characterized as quercetin 3-*O*-α-L-arabinopyranosyl-(1→6)-[2″-*O*-(*E*)-*p*-coumaroyl]-β-D-glucopyranoside and quercetin 3-*O*-α-L-arabinopyranosyl-(1→6)-[2″-*O*-(*E*)-*p*-coumaroyl]-β-D-galactopyranoside. A mixture of these compounds in the same equivalency strongly induced the probing response from *M. crassicauda*, suggesting their kairomonal roles during host recognition.

Keywords: *Vicia angustifolia*; Fabaceae; Aphid; *Megoura crassicauda*; Probing stimulants; Host selection; Acylated flavonol glycosides

1. Introduction

Megoura crassicauda Mordvilko is an oligophagous aphid which feeds selectively on *Vicia* plants such as broad bean (*Vicia faba* L.) and narrowleaf vetch (*Vicia angustifolia* L.) (Fabaceae), often causing serious damage to the plants (Moritsu, 1983). In order to understand the phytochemical basis of host selection in oligophagous aphids, we investigated the chemical factors controlling the probing behaviour of the bean aphid. Aphids in general produce proteinaceous stylet sheaths, similar to those produced in intact plant tissues through a stretched parafilm membrane, as a result of probing solutions of the host plant chemicals (Miles, 1965). The formation of stylet sheaths triggered by specific phytochemical cues in plants is suggested to be associated with the host finding behaviour of the aphids prior to ingestion of the phloem sap of specific host plants (Montllor, 1991). Here we report the isolation and structural elucidation of specific probing stimulants of *M. crassicauda* from *V. angustifolia*, one of the most widespread host plants in Japan.

2. Results and discussion

M. crassicauda actively displayed probing behaviour toward the crude aqueous extracts of a host plant, *V. angustifolia*, depositing a substantial number of thick stylet sheaths (thickness > 10 μm; length > 200 μm) on a parafilm membrane at a concentration of 1 g fresh leaf equivalent/ml (gle/ml). The aqueous extract was subjected to chromatography on an ODS column with increasing concentrations of methanol in water fractions, of which the 60% methanol eluate exhibited significant probing activity almost equivalent to that of a crude extract of *V. angustifolia*. The active eluate was further separated by a reversed phase HPLC and two major probing stimulants **1** and **2** ultimately being isolated in small quantities (yields/g leaf: **1**, 2 μg; **2**, 1.6 μg).

Both compounds **1** and **2** were suggested to be closely related flavonoid isomers with the molecular formulae of $C_{35}H_{34}O_{18}$, as deduced from the electro-spray ionization (ESI) mass spectra (positive *m*/*z* 743 $[M+H]^+$; negative *m*/*z* 741 $[M-H]^-$). Alkaline hydrolysis of compound **1** gave (*E*)-*p*-coumaric acid **3** and a quercetin diglycoside (compound **4**). Likewise, compound **2** gave (*E*)-*p*-coumaric acid **3** and a quercetin diglycoside (compound **5**). The deacylated compounds **4** and **5** were

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found as the principal flavonol diglycosides in the crude aqueous extracts of *V. angustifolia*, and were identified as quercetin 3-*O*-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside and quercetin 3-*O*-α-L-arabinopyranosyl-(1→6)-β-D-galactopyranoside, respectively, by MS and 2D NMR spectral analyses (Tables 1 and 2), acid hydrolyses, followed by D/L determination of sugars by Oshima's procedure (Oshima et al., 1982) and comparison with published data (Markham et al., 1987; Yoshimata et al., 1992; Ahn et al., 1996; Bylka and Matlawska, 1999).

The ^{1}H and ^{13}C NMR spectra of compound **1** exhibited very similar signals to those of compound **4**, with marked differences being on an acylated oxymethine proton at δ 4.93 (H-2″) in addition to signals arising from the *p*-coumaroyl moiety (Tables 1 and 2). The geometry of the *p*-coumaroyl moiety was determined to be of (*E*)-configuration from the coupling constant ($J = 15.9$ Hz) of the olefinic protons. The linkages of the two sugars were determined by the heteronuclear multiple bond correlation (HMBC) spectra, where correlations from H-1‴ (δ 3.96) of arabinose to the C-6″ (δ 67.5) of glucose, and from H-6″ (δ 3.82) of glucose to the C-1‴ (δ 103.1) of arabinose were observed. This indicated that arabinosyl moiety was linked to the C-6″ of glucose. The β-configuration of glucose and α-configuration of arabinose were evidenced by their respective J_{1-2} values for the diaxial coupling (Table 1). The anomeric signals $\delta_{C\text{-}1''}$ 98.6 and $\delta_{H\text{-}1''}$ 5.59 of the glucosyl moiety indicated the direct connection with one of the hydroxyl group of the quercetin aglycone. Comparison of the carbon shifts of the aglycone with those of published data for quercetin (Agrawal et al., 1989) revealed an upfield shift of C-3 ($\Delta\delta$ 2.8 ppm) and downfield shift of C-4 ($\Delta\delta$ 1.3 ppm), indicating glycosylation of C-3 of the aglycone. HMBC spectral analysis supported further evidence for this assignment. (*E*)-*p*-Coumaroyl ester moiety was confirmed to be attached to C-2″ of glucose by HMBC, in which a correlation was observed between the downfield-shifted methine proton H-2″ of glucose and carbonyl carbon of the coumaroyl moiety. The ^{13}C NMR spectrum of **1** also supported the C-2″ position of acylation, since both C-1″ and C-3″ signals of glucose appeared upfield by about 2.3 ppm from signals of corresponding compound **4**. Consequently, the structure of compound **1** was established as quercetin 3-*O*-α-L-arabinopyranosyl-(1→6)-[2″-*O*-(*E*)-*p*-coumaroyl]-β-D-glucopyranoside.

The ^{1}H and ^{13}C NMR signals of compound **2** were assigned by 2D NMR spectroscopic analysis as shown in Tables 1 and 2. Similar to compound **1**, carbon shifts of the aglycone and the anomeric carbon, and proton

Table 1
^{1}H NMR spectral data for compounds **1**, **2**, **4** and **5** (DMSO-d_6, 500 MHz)[a]

Position	Compound **1**	Compound **2**	Compound **4**	Compound **5**
Quercetin				
6	6.19 *d* (1.6)	6.20 *d* (1.9)	6.21 *d* (2.0)	6.23 *d* (2.0)
8	6.40 *d* (1.6)	6.41 *d* (1.9)	6.41 *d* (2.0)	6.46 *d* (2.0)
2′	7.54 *d* (2.1)	7.57 *d* (2.2)	7.58 *d* (2.0)	7.60 *d* (2.1)
5′	6.89 *d* (8.5)	6.85 *d* (8.5)	6.87 *d* (8.3)	6.86 *d* (8.5)
6′	7.57 *dd* (2.1, 8.5)	7.65 *dd* (8.5, 2.2)	7.58 *dd* (2.0, 8.3)	7.66 *dd* (8.5, 2.1)
Hexosyl	glc	gal	glc	gal
1″	5.59 *d* (8.2)	5.52 *d* (8.1)	5.37 *d* (7.6)	5.29 *d* (7.7)
2″	4.93 *dd* (9.4, 8.2)	5.22 *dd* (9.7, 8.1)	3.25 *dd* (7.6, 8.6)	3.60 *m*
3″	3.49 *m*	3.72 *dm* (3.2)	3.21 *t* (8.6)	3.41 *dd* (10.4, 4.1)
4″	3.25 *t* (10.0)	3.78 *d* (3.2)	3.10 *t* (8.6)	3.67 *d* (4.1)
5″	3.43 *dd* (10.0, 7.5)	3.70 d (3.2)	3.31 *m*	3.58 *m*
6″	3.82 *d* (10.0)	3.71 *s*	3.79 *d* (13.2)	3.69 *dd* (10.4, 5.8)
	3.50 *dd* (10.0, 7.5)		3.56 *dd* (13.2, 7.8)	3.46 *dd* (10.4, 5.8)
Arabinosyl				
1‴	3.96 *d* (7.0)	3.98 *d* (7.0)	3.96 *d* (6.8)	3.99 *d* (7.0)
2‴	3.17 *dd* (8.6, 7.0)	3.18 *dd* (8.7, 7.0)	3.15 *dd* (6.8, 9.1)	3.16 *dd* (7.0, 8.5)
3‴	2.98 *dd* (8.6, 3.5)	3.05 *dd* (7.0, 3.8)	2.94 *dd* (9.1, 4.3)	3.03 *dd* (8.5, 1.6)
4‴	3.45 *br s*	3.49 *br s*	3.41 *br s*	3.49 *br s*
5‴	3.52 *dd* (10.0, 4.0)	3.57 *dd* (12.3, 2.9)	3.49 *dd* (12.3, 4.0)	3.55 *dd* (12.3, 2.3)
	2.94 *d* (10.0)	3.05 *d* (12.3)	2.89 *d* (12.3)	3.04 *d* (12.3)
p-Coumaroyl				
2, 6	7.54 *d* (8.5)	7.53 *d* (8.5)		
3, 5	6.82 *d* (8.5)	6.79 *d* (8.5)		
7	7.59 *d* (15.9)	7.58 *d* (15.9)		
8	6.40 *d* (15.9)	6.39 *d* (15.9)		

[a] Coupling constants (J in Hz) are given in parentheses.

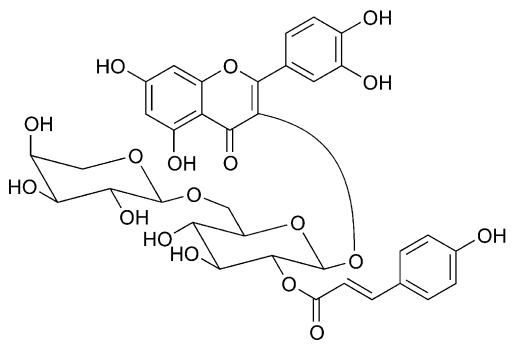
Table 2
^{13}C NMR spectral data for compounds **1**, **2**, **4** and **5** (DMSO-d_6, 125 MHz)

Position	Compound **1**	Compound **2**	Compound **4**	Compound **5**
Quercetin				
4	177.2	177.3	177.5	177.6
7	164.3	164.3	164.3	164.4
5	161.4	161.4	161.4	161.4
2	156.6	156.4	156.5	156.6
9	156.5	156.4	156.5	156.6
4′	148.8	148.7	148.7	148.7
3′	145.1	145.1	145.0	145.0
3	133.0	133.1	133.5	133.7
6′	122.0	122.4	121.8	122.2
1′	121.1	121.1	121.3	121.3
5′	116.2	116.2	116.3	116.1
2′	115.4	115.4	115.4	115.4
10	104.2	104.1	104.2	104.2
6	98.8	98.8	98.8	98.9
8	93.7	93.7	93.7	93.7
Hexosyl	glc	gal	glc	gal
1″	98.6	98.2	101.0	102.0
2″	74.0	71.1	74.1	71.2
3″	74.2	72.7	76.5	73.2
4″	70.4	68.7	70.2	68.5
5″	77.1	74.6	77.1	74.6
6″	67.5	66.6	67.5	66.7
Arabinosyl				
1‴	103.1	102.9	102.9	102.9
2‴	70.6	70.5	70.6	70.6
3‴	72.6	72.5	72.6	72.7
4‴	67.5	67.6	67.5	67.6
5‴	65.1	65.2	65.0	65.2
p-Coumaroyl				
9	165.9	165.9		
4	160.0	160.0		
7	145.1	145.1		
2, 6	130.4	130.4		
1	125.3	125.3		
3, 5	116.0	115.9		
8	114.5	114.5		

shifts of the galactosyl moiety between **2** and **5** indicated a direct connection of the galactosyl moiety at C-3 of the quercetin moiety. The signal arising from H-2″ of galactose was significantly deshielded (δ 5.22) when compared with that of corresponding signal (δ 3.60) for compound **5** as shown in Table 1, and both C-1″ and C-3″ signals of galactose appeared upfield from signals of corresponding compound **5** (Table 2), suggesting that the (*E*)-*p*-coumaroyl moiety was attached at C-2″ position of galactose. The pentose and ester linkages in **2** were confirmed by HMBC, where correlations from arabinose H-1″ (δ 3.98) to galactose C-6″ (δ 66.6); from galactose H-6″ (δ 3.71) to arabinose C-1″ (δ 102.9); and from galactose H-2″ to the coumaroyl carbonyl carbon. These observations indicated that the arabinose and the (*E*)-*p*-coumaroyl moiety were linked to the C-6″ and C-2″ position of galactose, respectively. Thus, compound **2** was determined to be quercetin 3-*O*-α-L-arabinopyranosyl-(1→6)-[2″-*O*-(*E*)-*p*-coumaroyl]-β-D-galactopyranoside.

A mixture of compounds **1** and **2** in a 1 gle/ml solution induced the strong probing activity equivalent to those of the 60% methanol eluate and crude aqueous extracts, even though each compound did not exhibit significant activity individually at 1 gle/ml concentration. The corresponding 2 gle/ml solution of each individual component elicited the significant response to the level of **1** + **2** (1 gle/ml), suggesting that the combination effect between **1** and **2** is "additive" rather than "synergistic" (Table 3).

Both **1** and **2** appear to be new compounds from plants. The α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside moiety in compound **1** has been known as vicianose and was initially characterized as a structural element of the cyanogenic glycoside vicianin contained in *V. angustifolia* seeds (Bertrand, 1906; Bertrand and Weisweiller, 1910). Although vicianoside linkages appear in various flavonoid glycosides, and quercetin vicianoside (**4**) as well as quercetin arabinosyl-(1→6)-β-galactoside (**5**) are distributed in a variety of plants (Wells and Bohm, 1988; Gil et al., 1998; Santos and Salatino, 2000; Bomfim-Patrício et al., 2001), their *p*-coumaroyl derivatives seem to provide a very unique phytochemical characteristic of the host plant. In summary, these findings add to our growing knowledge of kairomonal behaviour. Previously the effect of the chalcone glycoside, phlorizin, on the feeding behaviour of Rosaceae-feeding aphids had been reported (Klingauf,



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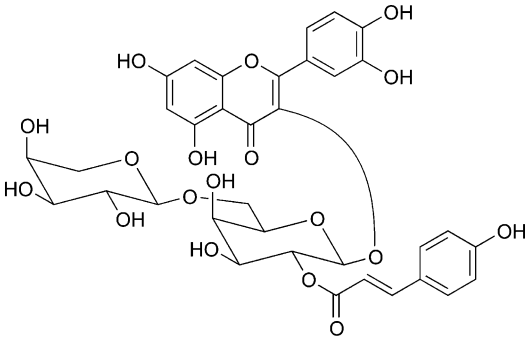

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Table 3
Probing response of *Megoura crassicauda* to a *Vicia angustifolia* extract, compounds **1** and **2**

Sample[a]	Probing activity (points) (*N*)
Control	23.8 (33) a
Crude aqueous ext.	83.8 (9) b
60% MeOH eluate	87.4 (9) b
Compound **1**	37.4 (18) a
Compound **2**	25.5 (18) a
Compounds **1** + **2**	79.8 (18) b
Compound **1** (2 gle/ml)	78.8 (9) b
Compound **2** (2 gle/ml)	66.6 (9) b

Fractions with the same letters are not significantly different at $P = 0.05$ in Kruskal–Wallis test followed by Dunn's multiple-comparison test.

[a] Dose: All fractions were examined at 1 gle/ml, except for compounds **1** and **2** (2 gle/ml).

1971; cf. Montgomery and Arn, 1974), and the quinolizidine alkaloid, sparteine, has been suggested to be s a feeding stimulant of the broom aphid, *Acyrthosiphon spartii* (Koch.), which feeds exclusively on *Cytisus scoparius* (L.) (Fabaceae) (Smith, 1966). Flavonoid glycosides have also been reported as probing stimulants of rice planthoppers (Kim et al., 1985; Adjei-Afriyie et al., 2000a,b). A systematic effort is thus needed to clarify the kairomonal roles of plant secondary metabolites together with the primary nutrients in the sequential process of feeding behaviour in these phloem-sucking insects.

3. Experimental

3.1. General

Optical rotation was measured with a Jasco DIP-370 spectropolarimeter, and UV spectra were measured with a Beckman DU-64 spectrophotometer. The MS data were recorded with a Shimadzu LCMS-2010A (probe voltage 4.5 kV, CDL temperature 250 °C) using a reversed-phase column (Cadenza CD-C18 75 mm×3.0 mm i.d.) eluted with 15–30% acetonitrile + 0.1% acetic acid in water (0.2 ml/min) with ESI-positive and ESI-negative modes. ^{1}H and ^{13}C NMR spectras were measured with a Bruker APX 500 FT-NMR spectrometer (500 MHz) and a Bruker AC 300 FT-NMR spectrometer (300 MHz) with TMS as an internal standard.

3.2. Plant material

Leaves and stems of *V. angustifolia* were collected in Sakyo-ku, Kyoto, Japan in April 2000, and identified by Dr. Reiichi Miura of Kyoto University. A voucher specimen is deposited at the Herbarium of Pesticide Research Institute, Kyoto University.

3.3. Extraction and isolation of flavonoids

Leaves and stems were extracted with 90% ethanol in water three times. The combined ethanolic solution was evaporated in vacuo, and the residue was dissolved in water and defatted five times with hexane. The crude aqueous extract (23.2 g/1000 gle) was subjected to chromatography on a reversed-phase column (100 g of Cosmosil 140C18-OPN, nacalai tesque, 210×35 mm i.d.) eluted in sequence each with an H_2O–MeOH gradient (100:0; 90:10; 80:20; 60:40; 40:60 and 0:100%, respectively). The H_2O–MeOH (40:60) was then subjected to prep. HPLC using a reversed-phase column (nacalai tesque COSMOSIL 5C18-AR 150×4.6 mm i.d.), eluted with MeOH:H_2O containing 1% acetic acid (1:1) at a flow rate of 1.0 ml/min. The active fraction eluted at the retention volume of ca. 5.5–7.5 and, this being further subjected to prep. HPLC using a reversed-phase column (YMC Pack Pro-C18, S-5 μm, 120 Å 250×6.0 mm i.d.) at 40 °C, eluted as above. Compounds **1** and **2** were isolated at $t_R = 12.8$ and 13.3 min, respectively, as a yellow solid mass. The total yield of compounds **1** and **2** from 1000 g of the leaves was ca. 2.0 and 1.6 mg, respectively. A portion of H_2O–MeOH (60:40) eluate was also subjected to prep. HPLC using a reversed-phase column (YMC Pack Pro-C18, S-5 μm, 120 Å 250×10 mm i.d.), eluted with acetonitrile:water containing 1% acetic acid (15:85) at a flow rate of 3.0 ml/min. Compounds **4** and **5** were isolated at $t_R = 23.3$ and 21.9 min, respectively, as a yellow solid mass (contents/g leaf: **4**, 12.5 μg; **5**, 33.8 μg).

3.4. Quercetin 3-O-α-L-arabinopyranosyl-(1→6)-[2″-O-(E)-p-coumaroyl]-β-D-glucopyranoside (*1*)

Yellow solid. $[\alpha]_D^{17}$ −90° (MeOH; *c* 0.73); UV λ_{max}^{MeOH} nm (log ε): 264 (4.10), 317 (4.20), 363 (3.96); LCMS (ESI-negative) *m*/*z* (rel. int.): 742 $[M]^-$ (33), 741 $[M-H]^-$ (100); LCMS (ESI-positive) *m*/*z*: 765 $[M+Na]^+$ (5), 743 $[M+H]^+$ (23), 441 [M + H−quercetin]$^+$ (100), 303 [quercetin + H]$^+$ (38). For ^{1}H and ^{13}C NMR spectral data, see Tables 1 and 2.

3.5. Quercetin 3-O-α-L-arabinopyranosyl-(1→6)-[2″-O-(E)-p-coumaroyl]-β-D-galactopyranoside (*2*)

Yellow solid. $[\alpha]_D^{17}$−46° (MeOH; *c* 0.44); UV λ_{max}^{MeOH} nm (log ε): 264 (4.04), 317 (4.12), 363 (3.92); LCMS (ESI-negative) *m*/*z* (rel. int.): 742 $[M]^-$ (33), 741$[M-H]^-$ (100); LCMS (ESI-positive) *m*/*z*: 765 $[M+Na]^+$ (5), 743 $[M+H]^+$ (20), 441 [M + H−quercetin]$^+$ (100), 303 [quercetin + H]$^+$ (12). For ^{1}H and ^{13}C NMR spectral data, see Tables 1 and 2.

3.6. Quercetin 3-O-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (4)

Yellow solid. $[\alpha]_D^{17}$ −19° (MeOH; *c* 0.13); UV λ_{max}^{MeOH} nm (log ε): 258 (4.25), 360 (4.18); LCMS (ESI-negative) *m/z*: 596 $[M]^-$ (33), 595 $[M-H]^-$ (100); LCMS (ESI-positive) *m/z*: 597 $[M+H]^+$ (71), 303 [quercetin + H]$^+$ (100). For ^{1}H and ^{13}C NMR spectral data, see Tables 1 and 2.

3.7. Quercetin 3-O-α-L-arabinopyranosyl-(1→6)-β-D-galactopyranoside (5)

Yellow solid. $[\alpha]_D^{17}$ −87° (MeOH; *c* 0.44); UV λ_{max}^{MeOH} nm (log ε): 258 (4.37), 360 (4.28); LCMS (ESI-negative) *m/z*: 596 $[M]^-$ (37), 595 $[M-H]^-$ (100); LCMS (ESI-positive) *m/z*: 619 $[M+Na]^+$ (11), 597 $[M+H]^+$ (71), 303 [quercetin + H]$^+$ (100). For ^{1}H and ^{13}C NMR spectral data, see Tables 1 and 2.

3.8. Alkaline hydrolysis of compounds 1 and 2

Compounds **1** and **2** (ca. 100 and 50 μg, respectively) were dissolved in 0.1 N KOH (100 and 50 μl, respectively) and kept for 1 h. The reaction mixtures were acidified with acetic acid, and submitted to LCMS. The deacylated compounds **1** and **2** were identified by direct comparison of LCMS data with those of compounds **4** and **5**.

Deacylated compound **1**. LCMS (ESI-negative) *m/z*: 596 $[M]^-$ (27), 595 $[M-H]^-$ (100); LCMS (ESI-positive) *m/z*: 619 $[M+Na]^+$ (100), 597 $[M+H]^+$ (77), 303 [quercetin + H]$^+$ (77).

Deacylated compound **2**. LCMS (ESI-negative) *m/z*: 596 $[M]^-$ (26), 595 $[M-H]^-$ (100); LCMS (ESI-positive) *m/z*: 635 $[M+K]^+$ (57), 619 $[M+Na]^+$ (11), 597 $[M+H]^+$ (68), 303 [quercetin + H]$^+$ (100).

(*E*)-*p*-Coumaric acid **3** liberated from both **1** and **2**. LCMS (ESI-negative) *m/z*: 163 $[M-H]^-$ (100); UV λ_{max}^{MeOH} nm: 228, 310; ^{1}H NMR spectral data (300 MHz, DMSO-d_6): δ 7.55 (1H, *d*, *J* = 15.9, H-7), 7.53 (2H, *d*, *J* = 8.6, H-2, 6), 6.89 (2H, *d*, *J* = 8.6, H-3, 5), 6.31 (1H, *d*, *J* = 15.9, H-8).

3.9. Acid hydrolysis of compounds 4 and 5

Compounds **4** and **5** (ca. 5.0 and 3.0 mg, respectively) were dissolved in 5.0 ml of 2 N HCl, heated at 90 °C for 2 h, and then partitioned between ethyl acetate and water. Aglycone quercetin was recovered from the ethyl acetate layer and identified by direct comparison with an authentic sample. ^{1}H NMR spectral data (300 MHz, DMSO-d_6): δ 7.66 (1H, *d*, *J* = 2.1, H-2′), 7.52 (1H, *dd*, *J* = 8.5, 2.2, H-6′), 6.87 (1H, *d*, *J* = 8.5, H-5′), 6.39 (1H, *d*, *J* = 2.0, H-8), 6.17 (1H, *d*, *J* = 2.0, H-6). The H_2O layers were respectively purified by HPLC (YMC-Pack Polyamine II 250×4.6 mm i.d.; 70% acetonitrile/water; 1.0 ml/min). Sugars liberated from compound **4** were identified as L-arabinose and D-glucose, from compound **5** were identified as L-arabinose and D-galactose by comparison of ^{1}H NMR spectra with those of the standard sugars and diastereoisomeric derivatization of each sugar component according to Oshima's method (Oshima et al., 1982).

3.10. Bioassay

Five apterous adult viviparae of *M. crassicauda* were starved for 2 h, and then allowed to probe a test solution (0.4 ml) of either a sample solution or distilled water (control) through a Parafilm® M (American Can Co.) membrane for 24 h under laboratory conditions (25 °C, 16 L:8 D). The stylet sheaths deposited on the parafilm membrane were observed under a microscope after being stained with a red fuchsin basic solution. The probing sheaths were classified according to the degree of branching, non-branching or branching, which were assigned coefficients of 1 and 2, respectively. The intensity of probing activity was scored as the total points.

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