



A role of uroleuconaphins, polyketide red pigments in aphid, as a chemopreventor in the host defense system against infection with entomopathogenic fungi

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Abstract

Four red polyketide pigments, uroleuconaphins A₁ (**1**) and B₁ (**2**) and their glucosides **3** and **4**, were isolated from the red goldenrod aphid *Uroleucon nigrotuberculatum*. Although these red pigments exist only as glucosides **3** and **4** in the intact insect body, **3** and **4** convert instantly to aglycones **1** and **2** at death. Pigments **1** and **2** inhibited the growth of *Lecanicillium* sp. (Ascomycota: Cordycipitaceae) and **1**, **2**, and **3** were active against *Conidiobolus obscurus* (Entomophthoromycota; Entomophthorales); these fungal species are pathogenic. We therefore regard aphid pigments **1–4** as chemopreventive agents that aid in the resistance of infection by entomopathogenic fungi at the level of the individual aphid and/or at the species level.

Introduction

Aphids have novel polyketide pigments [1–9], such as furanaphin [1, 2] uroleuconaphins [3–6], and viridaphin A₁ glucoside [7, 8], which affect body color and predator–prey interactions [10]. Furthermore, since aphid pigments possess interesting biological activities such as cytotoxicity [1, 3, 6, 7] and antibacterial activities [7], we hypothesized that aphid pigments may protect the aphids from pathogenic species such as viruses, bacteria, and fungi [7]. As part of our continuing efforts toward testing this hypothesis and elucidating the biological functions of aphid pigments, we

focused on two entomopathogenic fungi: *Lecanicillium* sp. (Ascomycota: Cordycipitaceae) and *Conidiobolus obscurus* (Entomophthoromycota: Entomophthorales). Although *C. obscurus* is a known pathogen of several aphids [11], no field observations of the infection of the red goldenrod aphid *Uroleucon nigrotuberculatum*, which has red-pigment uroleuconaphins [3], with this fungus have been reported. In contrast, infection of this aphid with *Lecanicillium* sp. is frequently observed; sometimes the fungus causes outbreaks of epizootic disease in populations of this aphid. We therefore evaluated the growth-inhibition activity of the red pigments of this aphid against both fungal species.

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Results and discussion

Red pigments **1** and **2** (Fig. 1a, c) were obtained from an ether extract of live *U. nigrotuberculatum* (Fig. 1d), as reported previously [3]. Surprisingly, we were unable to isolate **1** and **2** from an ethanol extract of the live aphids; instead, we obtained very polar red pigments whose structures were determined to be the β -D-glucosides of **1** and **2** (compounds **3** and **4**, respectively; Fig. 1b, c). We established the structures of **3** and **4** as follows. The aphids (24.8 g) were crushed with a pestle and washed repeatedly with ethanol. The combined ethanol extract was concentrated and subjected to silica gel column

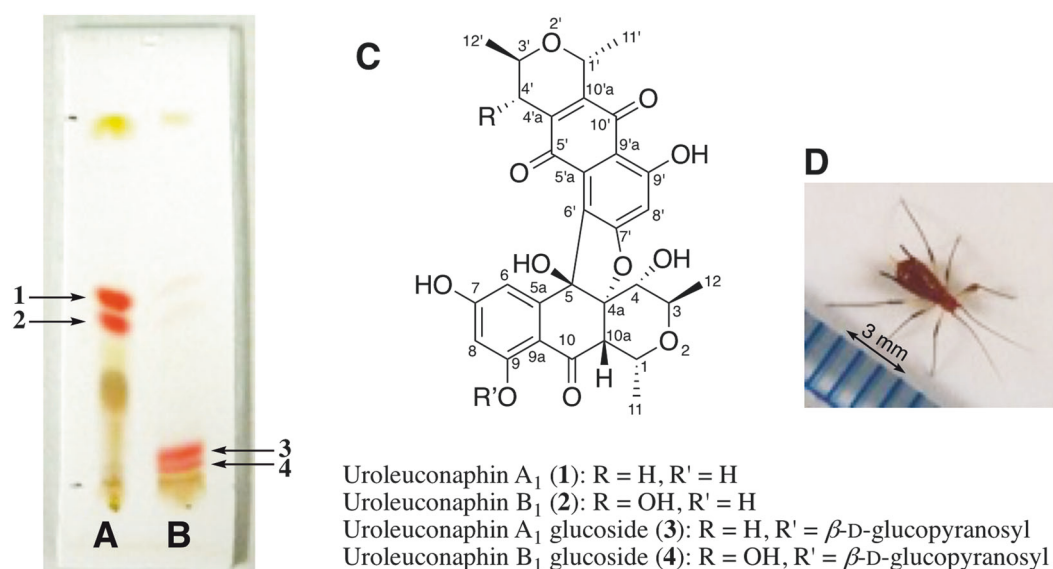


Fig. 1 **a** TLC (CHCl₃:methanol = 10:1) of ether extract of the aphid. **b** TLC (CHCl₃:methanol = 10:1) of ethanol extract of the aphid. **c** The structures of uroleuconaphins A₁ (**1**) and B₁ (**2**) and their β-D-glucosides **3** and **4**. **(d)** *Uroleucon nigrotuberculatum*

chromatography (CHCl₃: ethanol = 20: 1, 10: 1, 6: 1) and high performance liquid chromatography to yield molecules **3** (88 mg) and **4** (13 mg) as red solids. Since the density of the aphid's body is ~1.06 g/mL (Experimental Section), the molar concentrations of **3** and **4** in the aphid body were approximately 5.2 and 0.8 mM, respectively.

The molecular formula of **3** was established as C₃₆H₃₈O₁₆ by FABHRMS (*m/z* 749.2058, calculated for C₃₆H₃₈O₁₆Na). The IR spectrum of **3** indicated the presence of hydroxy groups (3396 cm⁻¹, br) and a conjugated carbonyl moiety (1668 cm⁻¹). The ¹H and ¹³C NMR data in MeOH-*d*₄ are shown in Table 1. The presence of a β-glucopyranosyl linkage was suggested by the ¹H NMR (methanol-*d*₄) signals at δ 4.76 (*J* = 7.6 Hz); by the ¹³C NMR (methanol-*d*₄) signals at δ 62.5, 71.2, 74.8, 77.2, 78.6, and 104.5. The HMBC correlation between the anomeric proton (δ H 4.76) of the glucose and C-9 (δ C 160.6) of the aglycone moiety revealed the position of the sugar linkage (Fig. 2). Furthermore, detailed NMR studies (¹H NMR, ¹³C NMR, HMBC, HMQC) of **3** comparing with that of **1** [3] suggested that **3** was the monoglucoside of **1**. This conclusion was confirmed by hydrolysis of the glycosyl linkages of **3**, which was carried out under acidic conditions (0.5 M H₂SO₄ in dioxane-H₂O) to yield D-glucose and under enzymatic conditions (naringinase) [12] to yield aglycone **1**.

The molecular formula of **4** was established as C₃₆H₃₈O₁₇ by FABHRMS (*m/z* 765.2007, calculated for C₃₆H₃₈O₁₇Na). The structure of **4** was determined by detailed experiments in the same as **3** (Table 1 and Fig. 2).

Compounds **3** and **4** could not be obtained from the ethanol extracts of aphids that died due to infection with

Lecanicillium sp. or that starved to death; only aglycones **1** and **2** were obtained. These findings suggest that 1) red pigments exist as glucosides **3** and **4** in the live aphid; 2) the aphid must contain a glucosidase that can quickly hydrolyze **3** and **4** to yield aglycones **1** and **2**; 3) the ethanol used for pigment extraction must deactivate the glucosidase instantly to prevent the hydrolysis of glucosides **3** and **4** to aglycones **1** and **2**; 4) the ether used for pigment extraction must be unable to deactivate the glucosidase to yield **1** and **2**; and 5) although the glucosidase is inactive when the aphid is alive, it must become active instantly to produce aglycones **1** and **2** when death begins. Actually, we could confirm the generation of aglycone **1** using TLC, when a separately prepared pure sample of glucoside **3** was mixed with a residue of crushed aphid's body, which was obtained after pigment extraction with ether (Fig. 3). Furthermore, the existence of some kind of glucosidase was suggested by earlier studies of pigments in the aphid *Eriosoma lanigerum* [13]. With these observations and conjectures in mind, we evaluated the growth inhibition activities of compounds **1–4** against the insect pathogens *Lecanicillium* sp. and *C. obscurus*, in order to elucidate the biological functions of these aphid pigments.

The activities of each pigment were evaluated by measuring the inhibition of growth of fungal colonies on medium mixed with each pigment. Although glucoside **3** caused concentration-dependent growth inhibition of *C. obscurus* (Fig. 4a), glucoside **4** did not (Fig. 4b). As the molar concentration of **3** in the aphid body was quite high (5.2 mM as mentioned above), the aphid may be resistant to *C. obscurus* due to the presence of **3**. Thus, aphid pigment **3** may be a chemopreventive agent that aids in the resistance

Table 1 ^{13}C NMR (150 MHz) and ^1H NMR (600 MHz) data for compounds **3** and **4** in methanol- d_4 .

Position	3		4	
	δ_{C} , mult.	δ_{H} (J in Hz)	δ_{C} , mult.	δ_{H} (J in Hz)
1	69.9, CH	4.53, qd (6.9, 5.5)	69.9, CH	4.51, qd (6.9, 5.4)
3	65.9, CH	3.93, qd (5.9, 9.9)	66.0, CH	3.95, qd (6.0, 9.9)
4	73.5, CH	4.01, d (9.9)	73.5, CH	4.03, d (9.9)
4a	95.2, C		95.2, C	
5	80.7, C		80.8, C	
5a	128.6 or 150.1, C		129.5 or 150.0, C	
6	109.3, CH	6.96, d (2.3)	109.2, CH	6.97, d (2.3)
7	165.6, C		165.6, C	
8	107.0, CH	6.76, d (2.3)	107.0, CH	6.75, d (2.3)
9	160.6, C		160.4, C	
9a	113.4, C		113.5, C	
10	193.9, C		193.9, C	
10a	51.3, CH	3.49, d (5.5)	51.5, CH	3.48, d (5.4)
11	16.6, CH_3	1.70, d (6.9)	16.7, CH_3	1.72, d (6.9)
12	19.1, CH_3	1.22, d (5.9)	19.1, CH_3	1.23, d (6.0)
1'	68.4, CH	4.96, qd, (6.9, 1.3)	67.2, CH	4.80–4.90, (overlapped with H_2O)
3'	64.0, CH	4.08–4.12, m	70.7, CH	4.01, m
4'	31.1, CH_2	2.89, dd (19.1, 3.3) 2.23, dd (19.1 10.4)	66.4, CH	4.48, br s (6.0)
4'a	143.6, C		143.0, C	
5'	190.1, C		190.9, C	
5'a	128.6 or 150.1, C		129.5 or 150.0, C	
6'	133.8, C		133.6, C	
7'	167.6 or 167.9, C		167.5 or 168.1, C	
8'	105.1, CH	6.53, s	105.1, CH	6.55, br s
9'	167.6 or 167.9, C		167.5 or 168.1, C	
9'a	110.6, C		111.0, C	
10'	187.6, C		187.6, C	
10'a	149.0, C		150.7, C	
11'	20.0, CH_3	1.57, d (6.9)	19.6, CH_3	1.60, d (6.7)
12'	21.7, CH_3	1.35, d (6.2)	18.1, CH_3	1.33, d (6.3)
1''	104.5, CH	4.76, d (7.6)	104.3, CH	4.76, d (7.6)
2''	74.8, CH	3.51, dd (9.2, 7.6)	74.7, CH	3.51, dd (9.2, 7.6)
3''	77.2, CH	3.45, dd (9.2, 8.5)	77.2, CH	3.45, dd (9.2, 8.5)
4''	71.2, CH	3.37–3.42, (overlapped)	71.2, CH	3.37–3.42, (overlapped)
5''	78.6, CH	3.37–3.42, (overlapped)	78.6, CH	3.37–3.42, (overlapped)
6''	62.5, CH_2	3.89, dd (11.9, 1.9) 3.70, dd (11.9, 5.2)	62.5, CH_2	3.89, dd (11.9, 1.7) 3.70, dd (11.9, 5.2)

of infection at the level of the individual aphid. Moreover, aglycones **1** and **2** also inhibited fungal growth in a concentration-dependent fashion (Fig. 4c, d) and the activities of them were more potent than that of **3**. Although, the

biological functions of these aglycones for the living aphid are unclear, they may function as a secondary system for resistance against the fungi. Further experiments are necessary.

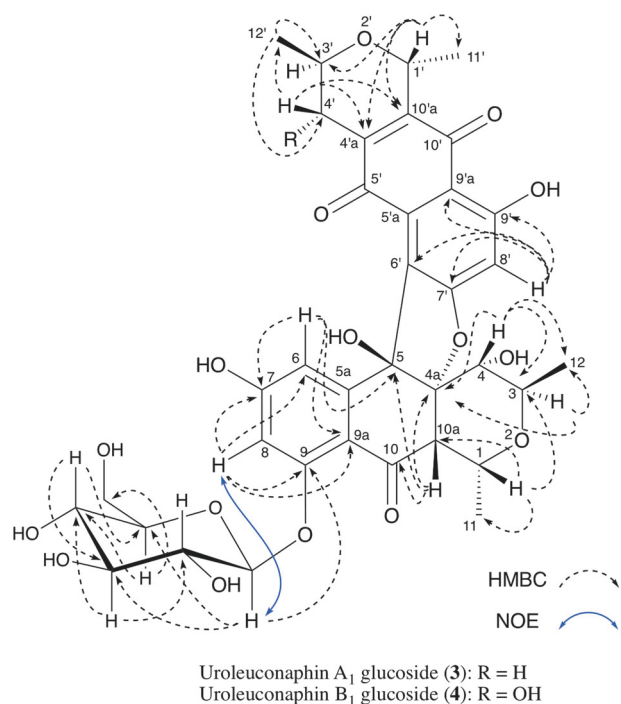


Fig. 2 Selective HMBC and NOE correlations of glucosides

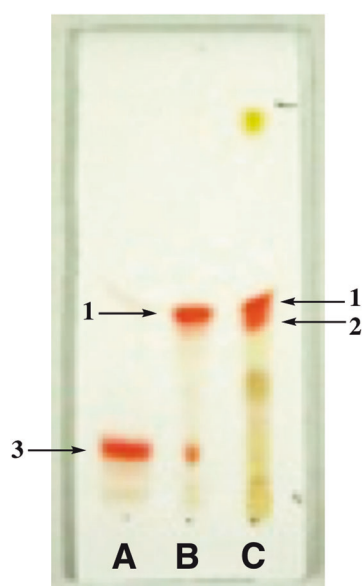


Fig. 3 TLC using CHCl₃:methanol (10:1) as solvent. **a** Glucoside **3**. **b** Ether extract of a mixture of **3** and the aphid residue, which was prepared as follows. A few red aphids were crushed in ether, and the ether fraction was removed. Insoluble material in the ether (aphid residue) was washed with ether until the red color (compound **1** and **2**) disappeared. A separately prepared, pure sample of glucoside **3** was added to the aphid residue in ether and mixed vigorously. TLC of this solution indicated that nearly all glucoside **3** was converted to aglycone **1**. **c** Ether extract of the live aphid

On the other hand, although glucosides **3** and **4** were not active against *Lecanicillium* sp. (Fig. 5a, b), aglycones **1**

and **2** inhibited growth at concentrations above 0.06 mM (Fig. 5c, d). These results indicate that this aphid is susceptible to infection by *Lecanicillium* sp. due to the inactivity of **3** and **4** against this fungus. However, when death by infection begins, aglycones **1** and **2** are produced and inhibit the growth of this ascomycete. Hence, we hypothesize that death of the individual aphid retards ascomycete growth on the aphid cadaver, lowering the rate of transmission to other individual aphids in the same colony and transmission to other aphid colonies. Although future investigation of the role of aglycones **1** and **2** in reducing horizontal transmission are necessary, we predict that the colony (at the species level) may be protected to some extent from infection with *Lecanicillium* sp. based on self-sacrifice of individual aphid.

Thus, aphid pigments **1–4** may be chemopreventive agents that aid in the resistance against infection by some entomopathogenic fungi. Hence, the aphid polyketide pigments assume the host defense system of individual level and/or species level of the aphid to increase the aphid's inclusive fitness.

Several recent studies have shown that bacterial endosymbionts protect host aphids from insect pathogens [14–16]. However, the mechanism of this protection remains unclear. Here, we found that aphid pigments do not only affect body color, but also preserve the aphid from infection by insect pathogens. Recently, we reported that a colorless aphid, *Cryptomyzus* sp., harbors unpigmented polyketides with cytotoxic activity [17]. We hypothesize that a large majority of aphids have polyketides (pigments or unpigmented molecules) that may function as chemopreventive agents. And we have another question. How did aphids acquire the enzymes required for polyketide synthesis (polyketide synthetase) through evolution? Did bacterial endosymbionts affect this evolutionary process? Further investigations are needed.

Experimental section

General experimental procedures

UV spectra were measured on a JASCO V-650 spectrophotometer. Optical rotations were measured on JASCO P-1030 polarimeters. IR spectra were measured on a JASCO FT/IR-410 spectrophotometer. ¹H NMR spectra were acquired with Varian Unity-600 (600 MHz) and Varian Unity-500 (500 MHz) spectrophotometers with tetramethylsilane as the internal standard in methanol-*d*₄. ¹³C NMR spectra were measured on Varian Unity-600 (150 MHz) and Varian Unity-500 (125 MHz) spectrophotometers; chemical shifts were referenced to the residual solvent signal (methanol-*d*₄: δ_C 49.0 ppm). Signal

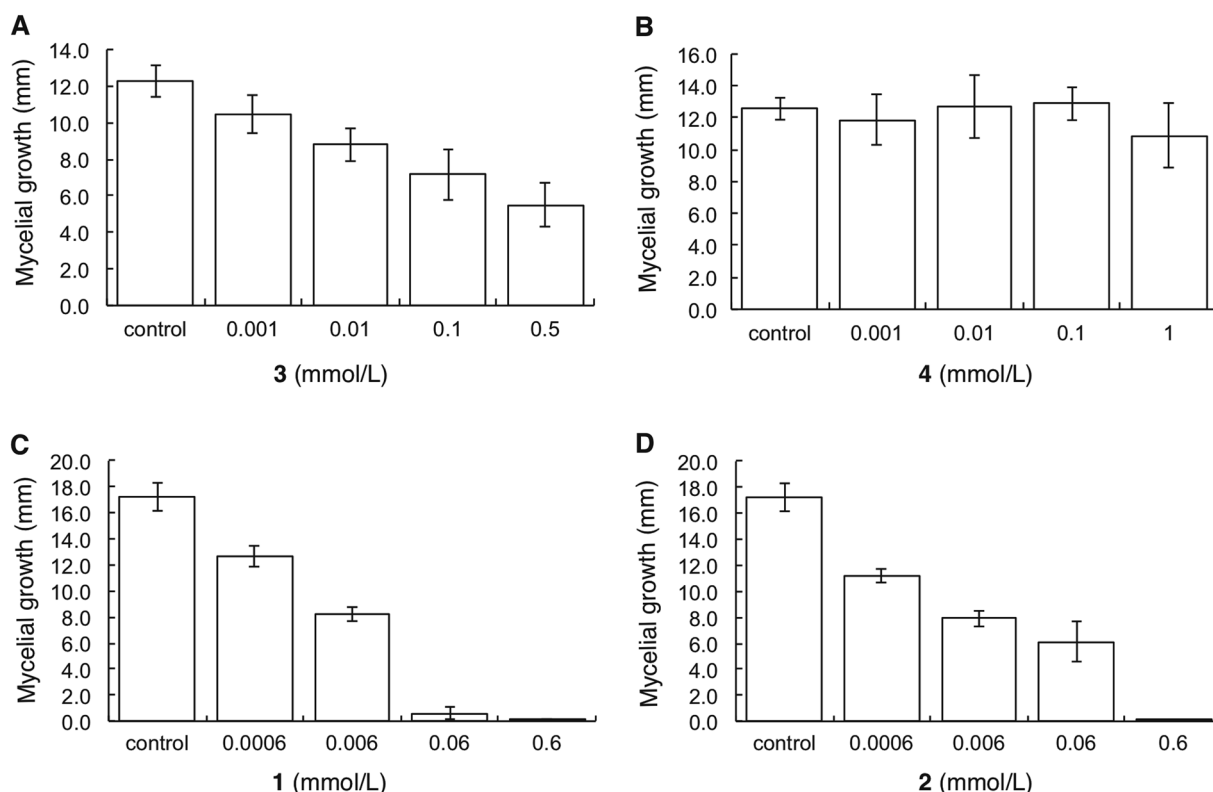


Fig. 4 Growth inhibition of pigments against *C. obscurus*. **a** Effect of glucoside **3**. **b** Effect of glucoside **4**. **c** Effect of aglycone **1**. **d** Effect of aglycone **2**. Each value is the mean and standard deviation ($n = 5$)

multiplicities were established via distortionless enhancement by polarization transfer. Mass spectra, including high-resolution mass spectra, were acquired with a JEOL JMS-700 spectrophotometer. The TLC analysis was performed with Merck pre-coated silica gel plates (60 F). Column chromatography was conducted with silica gel 60 N (Kanto Chemical Co. Inc, 63–210 μm). Preparative HPLC was carried out on a JASCO 880-PU pump unit equipped with an 880-UV detector (λ 254 nm) on a normal-phase column (Cosmosil 5SL-II, 28 \times 250 mm); the column was eluted with CHCl_3 :methanol (7:1) at a flow rate of 10.0 mL/min.

Reagents

Naringinase (from *Aspergillus niger*) was purchased from Sigma-Aldrich Inc. Amberlite IRA96SB was purchased from Organo Co. Ltd. Ethanol, dimethyl sulfoxide (DMSO), and D-(+)-glucose were purchased from Wako Pure Chemical Industries, Ltd. Methanol and dichloromethane were purchased from Kanto Co. Ltd. Acetic anhydride, pyridine, *n*-butanol, ether, chloroform (for HPLC), methanol (for HPLC), toluene, ethyl acetate, dioxane, 0.5 M H_2SO_4 aq., 2 M HCl aq., Na_2SO_4 , and phthalate pH standard solution (pH 4.01) were purchased from Nacalai Tesque Inc. Pyridine was used after distillation from CaH_2 . Agar was purchased

from Kyokuto Pharmaceutical Industrial Co. Ltd. BactoTM Peptone was produced by Difco and purchased from Wako Pure Chemical Industries, Ltd.

Material. Insect

Red goldenrod aphids (*U. nigrotuberculatum*) were collected as they fed on the Canadian goldenrod (*S. altissima*) in Tokushima Prefecture, Japan, in May 2010. A voucher specimen for this aphid was prepared by Sigeru Takahashi and deposited in the Faculty of Agriculture of Utsunomiya University under code no. 10005.

Material. Entomopathogenic fungi

C. obscurus was isolated from a diseased aphid (species unknown) on *Sonchus oleraceus* at Tsukuba, Ibaraki, Japan on May 28, 2010, using Sabouraud's dextrose agar supplemented with 1% yeast extract and subcultured on Nemoto's medium (200 mL Sabouraud's dextrose agar with one egg yolk). *Lecanicillium* sp. was isolated from a diseased *U. nigrotuberculatum* on *S. altissima* at Tsukuba, Ibaraki, Japan on June 24, 1996, using Sabouraud's dextrose agar supplemented with 1% yeast extract and maintained in distilled water at 5 $^\circ\text{C}$.

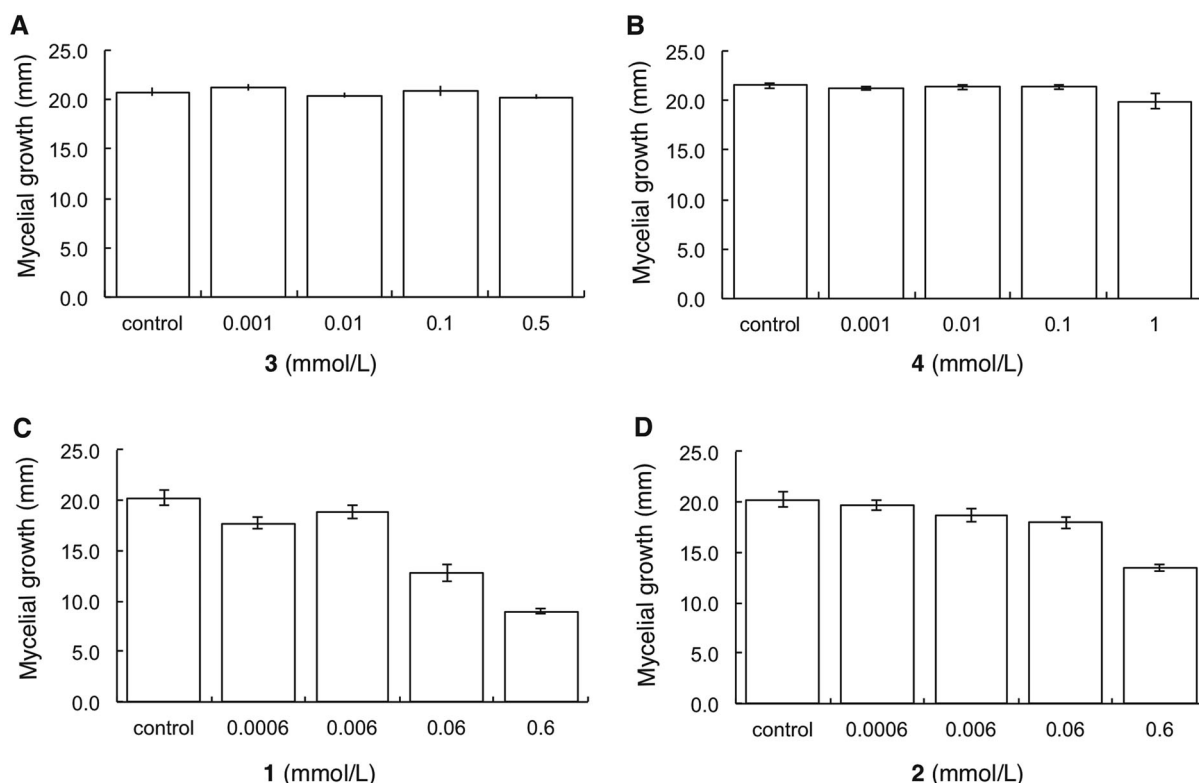


Fig. 5 Growth inhibition of pigments against *Lecanicillium* sp. **a** Effect of glucoside **3**. **b** Effect of glucoside **4**. **c** Effect of aglycone **1**. **d** Effect of aglycone **2**. Each value is the mean and standard deviation ($n = 5$)

Extraction, isolation and spectroscopic analyses of compounds **3** and **4**

U. nigrotuberculatum was removed from *S. altissima* with a soft paintbrush and collected in a plastic Erlenmeyer flask equipped with a plastic funnel. The aphids (24.8 g) were crushed with a pestle and washed repeatedly with ethanol. The ethanol supernatant was separated by decanting. The residue was washed with several aliquots of fresh ethanol. The combined ethanol solutions were evaporated under reduced pressure to yield crude extracts (3.0 g), which were subjected to silica gel column chromatography using CHCl_3 :ethanol (20:1, 10:1, 6:1) and repeated HPLC using CHCl_3 :methanol (7:1) to yield red pigments **3** (88 mg) and **4** (13 mg). Aphids (0.435 g) were immersed in 6.0 mL of ethanol. Since the total volume of the mixture was 6.41 mL, aphid density is calculated to be ~ 1.06 g/mL.

Uroleuconaphin A₁ glucoside (3): red amorphous powder; $[\alpha]_D^{21} + 770$ (c 0.017, methanol); UV (methanol) λ_{\max} ($\log \epsilon$) 273 (4.27), 494 (3.64) nm; IR (neat) ν_{\max} 3396 (-OH), 1668, 1635, 1612, 1443, 1409, 1282 cm^{-1} ; NMR data were provided in Table 1; fast atom bombardment mass spectrometry (FAB-MS) m/z 749 $[\text{M} + \text{Na}]^+$; FAB-HRMS m/z 749.2062 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{36}\text{H}_{38}\text{O}_{16}\text{Na}$, 749.2058).

Uroleuconaphin B₁ glucoside (4): red amorphous powder; $[\alpha]_D^{19} + 995$ (c 0.094, methanol); UV (methanol) λ_{\max} ($\log \epsilon$) 274 (4.27), 492 (3.62) nm; IR (neat) ν_{\max} 3364 (-OH), 1666, 1608, 1514, 1445, 1410, 1384 cm^{-1} ; NMR data were provided in Table 1; FAB-MS m/z 765 $[\text{M} + \text{Na}]^+$; FAB-HRMS m/z 765.2001 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{36}\text{H}_{38}\text{O}_{17}\text{Na}$, 765.2007).

Determination of the sugar structure of molecule **3**

A solution of **3** (9.2 mg) in 0.5 M H_2SO_4 aq. (1 mL) and dioxane (1 mL) was heated at 100 °C for 1.5 h. After cooling, the reaction mixture was neutralized with Amberlite IRA96SB. The resin was filtered off and washed with a small amount of water. *n*-Butanol (1 mL) was added to the filtrate and the mixture was evaporated. The residue was dissolved in H_2O (3 mL) and the mixture was washed with ethyl acetate and separated. A small amount of *n*-butanol was added to the aqueous layer and the resulting solution was evaporated *in vacuo* into syrup. A pyridine (400 μL) solution of the syrup was treated with 100 μL (large excess) acetic anhydride at ambient temperature for 24 h. After the addition of 2 M HCl aq. (2 mL), the mixture was extracted with CH_2Cl_2 (three extractions with 3 mL) and the combined organic layers were dried over Na_2SO_4 . After

evaporation, the residue was purified via silica gel column chromatography (2 g, *n*-hexane:ethyl acetate = 3:1) to yield 1.4 mg of acetate as a colorless powder. This acetate was identified as D-glucose pentaacetate by comparison with a standard sample ($^1\text{H NMR}$ and $[\alpha]_{\text{D}}^{20}$ value $[\alpha]_{\text{D}}^{20} + 35.7$ (*c* 0.15, CHCl_3)) and a pentaacetate of standard L-glucose ($[\alpha]_{\text{D}}^{22} - 43.8$ (*c* 2.1, CHCl_3)).

Determination of the sugar structure of molecule 4

The sugar structure of molecule 4 was determined via the method described for molecule 3. Molecule 4 (5.4 mg) yielded 0.6 mg of D-glucose pentaacetate as a colorless powder ($[\alpha]_{\text{D}}^{20} + 26.8$ (*c* 0.17, CHCl_3)) and was compared with a pentaacetate of standard L-glucose ($[\alpha]_{\text{D}}^{22} - 43.8$ (*c* 2.1, CHCl_3)).

Hydrolysis of glucosides 3 and 4 using naringinase

Toluene (10 mL) and naringinase (200 mg) were added to a mixture of 3 (6.4 mg) and phthalate buffer (pH 4.01, 15 mL); the resulting mixture was heated at 37 °C for 2 h. After cooling, the organic layer was separated. The aqueous layer was extracted with toluene (two extractions with 2 mL). The combined organic layers were washed with water (3 mL) and evaporated in vacuo to yield aglycone 1 quantitatively as a red solid. Aglycone 2 was obtained from 4 via the same procedure, with 84% yield. The structures of these compounds were established by comparison with samples that had been isolated previously from the ether extract of aphids.

Inhibition of the growth of entomopathogenic fungi

The activities of each pigment were evaluated by measuring the inhibition of growth of fungal colonies on medium mixed with each pigment. Pigments were dissolved in a very small volume of DMSO, diluted with distilled water, and sterilized by filtration through a 0.2- μm membrane filter, Sabouraud's dextrose agar (10 g Bacto Peptone, 40 g dextrose, 15 g agar in 1000 mL distilled water) was autoclaved for 15 min and cooled to 50 °C. The pigment solution was mixed in the medium before the agar solidified to generate agar with the indicated final concentrations, poured into a 35 × 10 mm culture dish, and solidified. Sabouraud's dextrose agar without pigments was used as a control.

Each fungal species was shaker-cultured at 25 °C for 5 days to obtain a mycelial suspension. Using a 2-mm platinum loop, the mycelial suspension was inoculated onto the center of a plate of agar containing pigment. Six replicates were performed for each concentration of each pigment. After inoculation, the dishes were sealed with

Parafilm and incubated at 25 °C in total darkness for 10 days.

Two perpendicular diameters of the colony were measured with a caliper. The difference between the diameter of the colony without inoculum and the diameter of the colony with inoculum was determined.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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