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Note

Phenolic Constituents of *Celosia cristata* L. Susceptible to Spinach Root Rot Pathogen *Aphanomyces cochlioides*

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Cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, 1), known as a host-specific attractant towards the zoospores of *Aphanomyces cochlioides*, a cause of root rot and damping-off diseases of Chenopodiaceae, was found in the Amaranthaceae plant, *Celosia cristata*, that is susceptible to the pathogen. The content of 1 in *Celosia* seedlings was quantified as 1.4 µg/g fresh weight. A new isoflavone, cristatein (5-hydroxy-6-hydroxymethyl-7,2'-dimethoxyisoflavone, 2), and five known flavonoids were also identified.

Key words: host-specific zoospore attractant; cochliophilin A; flavonoids; *Celosia cristata*; Amaranthaceae

Aphanomyces cochlioides is a soil-borne phytopathogenic Peronosporomycete which is responsible for the root rot disease of spinach (*Spinacia oleracea* L.) and damping-off disease of sugar beet (*Beta vulgaris* var. *rapa* Dum.). The presence of the potent zoospore attractant, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, 1), has been confirmed in a range of host plants in the Chenopodiaceae including sugar beet,¹ spinach² and *Chenopodium album*.³ Compound 1 is now recognized as a host-specific chemical signal^{2,4,5} for the following reasons: (A) it is distributed in limited plants compatible with the pathogen; (B) varying amounts are exuded from the host plant roots; (C) it shows highly selective activity toward the relevant pathogen; and (D) it not only attracts phytopathogenic zoospores, but also triggers zoospore encystment with subsequent cystospore germination, and causes tropism of the germinated hyphae.

We examine in the present study the zoospore attractants and related phenolics in the Amaranthaceae, *Celosia cristata*, which have been confirmed to be susceptible to *A. cochlioides*.⁶ Bioassays using intact seedlings and crude extracts from the roots and aerial parts of *C. cristata* revealed them to have clear attractant activity toward the zoospores of *A. cochlioides*. The zoospore bioassay (particle method)^{2,7}

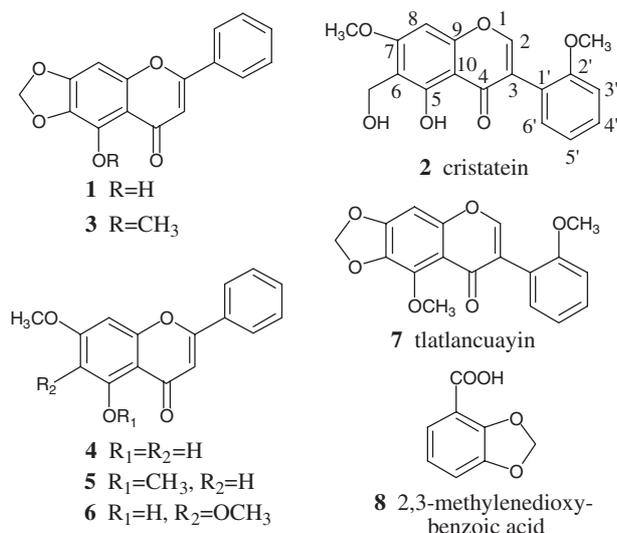
and coloring/quenching responses of phenolic compounds on F₂₅₄-type TLC plates under UV 254 and 365 nm lighting revealed the presence of cristatein (2) and tlatlancuayin (7) in the aerial parts, and cochliophilin A (1), 5-methoxy-6,7-methylenedioxyflavone (3), 5-hydroxy-7-methoxyflavone (4), 5,7-dimethoxyflavone (5), and 5-hydroxy-6,7-dimethoxyflavone (6) in the seedlings.

Compound 1, showing a characteristic methylenedioxy ¹H-NMR signal at δ 6.11, was identified by a direct comparison of its physicochemical properties with those of authentic cochliophilin A.² Compound 2 gave a molecular ion [M]⁺ at *m/z* 328.0966 by HR-EIMS, consistent with an elemental formula of C₁₈H₁₆O₆ (calcd. as 328.0947). The UV absorption maximum at 262 nm and a sharp singlet at δ_H 8.14 (isoflavone H-2) strongly suggested an isoflavone structure. The UV bathochromic shift with aluminum chloride, but no response with sodium acetate, is indicative of a free 5-OH and a blocked 7-OH.⁸ The ¹H- and ¹³C-NMR spectra showed the presence of two methoxy groups, one of which (δ_H 3.98) was NOE correlated with a singlet proton at δ_H 6.65 assignable to H-8 on the isoflavone A-ring, whilst the other (δ_H 3.80) was correlated with an *ortho*- and *meta*-coupled proton resonating at δ_H 7.09. Four aromatic protons multi-coupling at δ_H 7.00, 7.09, 7.32 and 7.39 were attributed to a 1',2'-disubstituted B-ring, based on the COSY spectrum (correlation signals between δ_H 7.00 and 7.32; δ_H 7.00 and 7.39; and δ_H 7.39 and 7.09). The remaining three protons (two methylene protons at δ_H 4.67 and one hydroxy proton at δ_H 3.49) coupled with each other with *J* = 6.2 Hz were easily assigned to a hydroxymethyl group. These substituents were unambiguously allocated by the 2D NMR technique (Table 1). The structure of new isoflavone 2 was therefore elucidated as 5-hydroxy-6-hydroxymethyl-7,2'-dimethoxyisoflavone, and was named cristatein.

Isoflavones with 2'-oxy-4'-deoxy B-rings are known as characteristic constituents of Chenopodiaceae, Amaranthaceae and Iridaceae plants. Compounds 2 and 7 are

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closely related metabolites, although they are respectively substituted with one each hydroxymethyl and 6,7-methylenedioxy groups that is rare in naturally occurring flavonoids. From a chemotaxonomical point of view, the rare 6,7-methylenedioxy-substituted flavonoids have been found in Rutaceae, Chenopodiaceae and Amaranthaceae, and sporadically in some other families (Iridaceae, Polygonaceae and Solanaceae).⁹ Although 6-methylated and 6-hydroxymethylated flavanones have been found in *Petiveria alliacea* (Phytolaccaceae),¹⁰ no correspondingly substituted isoflavone has so far been reported. The known flavonoids (**3–7**) isolated for the first time from *C. cristata* were identified by comparing their physicochemical data with those reported for **3** from *Polygonum lapathifolium*,¹¹ **4** from *Daphne oleoides*,¹² **5** from *Leptospermum scoparium*,¹³ **6** from *Polemonium viscosum*,¹⁴ and **7** from *Celosia argentea*.¹⁵ The minimum concentrations of the isolates showing zoospore-attracting activity by the particle method^{2,7} were as follows: **1**, 0.0008 ppm; **3**, 100 ppm; **4**, 0.005 ppm; **5**, 100 ppm; and **6**, 0.0005 ppm. The other *Celosia* compounds were practically inactive. The relationship between the structure and attractant activity has been reported in our earlier papers,^{14,16} and the present results are compatible with these.



The content of cochliophilin A (**1**) in *C. cristata* seedlings grown for 4 days was quantified by NMR by using the internal standard method to be at least 1.4 μg (= 4.9 × 10⁻⁹ mol)/g fresh weight. The content of **1** in spinach roots has been reported to be 5.3 μg/g fresh root, *ca.* four times higher than that in *C. cristata* roots.¹⁶ Although cochliophilin A (**1**) has previously been found only in the Chenopodiaceae,¹⁻³ the present study has now extended its range to the Amaranthaceae family. All plants in which **1** occurs are susceptible to *A. cochlioides*. Another flavone (**6**) with potent zoospore-attracting activity was also found for the first time in the host plant, *C. cristata*. Considering the isolated amounts and minimum attracting concentrations of flavones **1** and

6,¹⁴ the contribution of **6** to infection establishment between *A. cochlioides* zoospores and *C. cristata* seems to be hardly negligible.

The chromatographic and spectroscopic methods have been described in earlier papers.^{2,8} The chromatographic solvents are abbreviated as follows: C, chloroform; E, ethyl acetate; H, *n*-hexane; and M, methanol. Merck RP-18 F_{254S} and DIOL F₂₅₄ plates for HPTLC, and Sephadex LH-20 (Pharmacia), Lichroprep DIOL, and Cosmosil 75 C18-OPN were also used in the present study. The internal standard of 2,3-methylenedioxybenzoic acid (**8**) was prepared as reported.¹⁷

Zoospore bioassay. The production of zoospores and the "particle bioassay" were carried out as previously reported.^{2,7} Briefly, one particle of Chromosorb W AW (60/80 mesh) holding the test material corresponding to 4 nl of the test solution was carefully dropped into 0.4 ml of a zoospore suspension (10⁵/ml) in a plastic well (1.6 cm i.d.). The motility of the zoospores was microscopically observed, and control particles were treated with the solvent alone.

Isolation of the phenolic constituents (1–7) in young seedlings and in aerial parts of mature plants. The seeds of *Celosia cristata* L. var. *childsii* Hort. purchased from Sakata Seed Co. (Yokohama, Japan) were sown on a moistened filter paper in a Petri dish and kept in the dark for 30 h at 28 °C, and then for 70 h on a laboratory bench under ambient conditions (16–24 °C). The seedlings (361 g, fresh weight) were successively extracted with EtOAc (2 × 1000 ml) and MeOH (3 × 1000 ml). The combined extracts yielded 12 g of a residue which was further partitioned between EtOAc and water to give 2.3 g of EtOAc solubles (attractant activity at 10 ppm). These EtOAc solubles were then subjected to Cosmosil 75 C18-OPN (198 g) column chromatography. The column fraction eluted with MeOH–H₂O (9:1) yielded 120 mg of solutes (attractant activity at 1 ppm) which were rechromatographed over DIOL and eluted with H–C = 2:1 and then with H–C = 1:1. The former eluate (2.0 mg, attractant activity at 0.001 ppm) yielded **4** (*R_f* 0.53, 1.20 mg), **1** (*R_f* 0.46, 0.3 mg) and **6** (*R_f* 0.40, 0.3 mg) by preparative TLC (PTLC) on DIOL plates in H–C = 2:1, whilst the latter eluate (2.2 mg, attractant activity at 1 ppm) yielded **3** (*R_f* 0.53, 0.6 mg) and **5** (*R_f* 0.50, 1.4 mg) by PTLC in H–C = 1:1. Further related compounds were surveyed by using mature plants. The acetone extract (110 g) of the aerial parts of *C. cristata* (14 kg at the flowering stage) suspended in H₂O (600 ml) was subsequently extracted with hexane and diethyl ether. The latter fraction (27.4 g) was successively subjected to column chromatography over silica gel, Sephadex LH-20 and DIOL to isolate the two quenching substances **2** (*R_f* *ca.* 0.4) and **7** (*R_f* *ca.* 0.5) in C–E = 9:1 on silica gel plates. The earlier eluate from the DIOL column with an H–C mixture contained **7** (8.2 mg) and the later contained **2** (17.4 mg), which were respectively isolated by PTLC. Cristatein (**2**): Pale greenish solid; FD-MS *m/z* (rel. int. %): 328 [M]⁺ (100); EI-MS *m/z*

Table 1. NMR Spectroscopic Data for Cristatein (**2**) in Acetone- d_6^a

Position	δ_C	δ_H	HMBC H \rightarrow C
2	155.7	8.14 <i>s</i>	C-3, C-4, C-9, C-1'
3	122.1		
4	181.1		
5	160.3		
6	113.4		
7	164.7		
8	90.6	6.65 <i>s</i>	C-6, C-7, C-9, C-10
9	158.2		
10	106.4		
1'	120.9		
2'	158.5		
3'	111.9	7.09 br. <i>d</i> (ca. 8.3)	C-1', C-2'
4'	130.6	7.39 <i>ddd</i> (8.3, 7.4, 1.7)	C-2', C-3', C-5', C-6'
5'	120.7	7.00 <i>td</i> (7.4, 1.0)	C-1', C-3', C-4'
6'	132.3	7.32 <i>dd</i> (7.4, 1.7)	C-3, C-2', C-4', C-5'
OH-5		13.27 <i>s</i>	C-5, C-6, C-10
OCH ₃ -7	56.6	3.98 <i>s</i>	C-7
OCH ₃ -2'	56.0	3.80 <i>s</i>	C-2'
HOCH ₂ -6	52.6	4.67 <i>d</i> (6.2)	C-5, C-6, C-7
HOCH ₂ -6		3.49 <i>t</i> (6.2)	

NOESY: H-8 \leftrightarrow 7-OCH₃; H-3' \leftrightarrow 2'-OCH₃

^aTMS was used as the internal standard. Chemical shifts are shown in δ (ppm) at 270 MHz for ¹H and at 67.5 MHz for ¹³C with *J* values (Hz) in parentheses. Each proton was assigned according to the HMQC data.

(rel. int. %): 329 [M + 1]⁺ (20), 328 [M]⁺ (100), 327 [M - 1]⁺ (34), 313 (26), 312 (21), 311 (21), 299 (27), 133 (20), 131 (20). UV λ_{\max} (MeOH) nm: 262, 213; +NaOMe: 264, 209; +AlCl₃: 370, 312, 274, 212; +(AlCl₃+HCl): 366, 311, 274, 213; +NaOAc: 262. NMR data, see Table 1.

Quantification of cochliophilin A (1) in *Celosia cristata* seedlings. ¹H-NMR analyses of known amounts of **1** and internal standard **8** resulted in the following equation: $Y = 0.046X + 0.031$ (1), where *Y* is the quotient of the methylenedioxy proton signal area of **1** at δ_H 6.11 and of **8** at δ_H 5.73, and *X* is the weight of **1** (7.0–42 μ g). If a known amount of **8** is used as the internal standard and the ratio of the methylenedioxy signal intensities of **1** and **8** are determined by ¹H-NMR, the amount of **1** can be estimated by substituting the appropriate values into equation 1. This method can be used to detect several micrograms of **1** in several milligrams of a partially purified plant extract in 0.5 ml of CDCl₃ by scanning 1000–1500 times in the ¹H-NMR spectrometer.

Methanol extraction of fresh *C. cristata* seedlings (14.4 g) grown for 4 days yielded 421.8 mg of extractives which were divided into the EtOAc-soluble and EtOAc-insoluble fractions. The former (152.4 mg) was applied to a Cosmosil 75C18 OPN column (10 g), washed with 50 ml of water, and then eluted with 50 ml of MeOH to yield 21.8 mg of a fraction containing **1**. The fraction chromatographed over DIOL (4 g) yielded two eluates, the 8th (1.6 mg) and 9th (1.7 mg), each eluted with 10 ml of H-C (8:2), which were respectively subjected to a ¹H-NMR analysis in 0.5 ml of CDCl₃

containing 14 μ g of the internal standard (**8**). This gave *Y*-values of 0.40 and 0.57 for fractions 8 and 9, respectively. The absolute amounts of **1** in these two fractions were calculated to be 8.0 and 12 μ g, respectively, according to equation 1. The content of **1** in *C. cristata* seedlings was higher than 1.4 μ g/g fresh weight (= (8.0 + 12) μ g/14.4 g of seedlings), after considering the loss of **1** during partial purification.

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