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# PRODUCTION OF THE PHYTOTOXINS RADICININ AND RADICINOL BY *ALTERNARIA CHRYSANTHEMI*

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**Key Word Index**—*Alternaria chysanthemi*; Dematiaceae; chrysanthemums; phytotoxins; radicinin; radicinol.

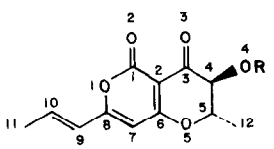
**Abstract**—A biologically active compound isolated from liquid cultures of *Alternaria chrysanthemi* in large yield has been identified as radicinin by X-ray crystallography. The structurally related compound radicinol is also identified as a metabolite of this fungus. The absolute stereochemistry of radicinin is presented.

## INTRODUCTION

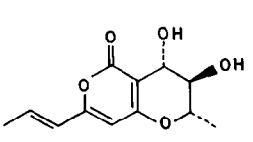
The fungus *Alternaria chrysanthemi* Simmons and Crosier (Moniliales–Dematiaceae) is the etiological agent of a leaf spot disease of shasta daisy [*Leucanthemum maximum* (Ram.) DC. (syn. *Chrysanthemum maximum* Hort.) (Compositae)] [1]. Several phytopathogenic *Alternaria* species are known to produce phytotoxic compounds, of diverse chemical structure, including a hemiquinone derivative, a penta-substituted benzene and a cyclic tetrapeptide [2]. Here we describe the insolation of two phytotoxic compounds from *A. chrysanthemi* and their identification as radicinin (**1a**) and radicinol (**2**). This is the first report of the occurrence of radicinol in the genus *Alternaria* Nees ex Fr.

## RESULTS AND DISCUSSION

Growth of the fungus for toxin production was initiated by inoculating a modified Czapek–Dox liquid medium with an agar plug from a stock culture of *A. chrysanthemi*. Routinely, culture filtrates were harvested after incubation for 25 days. Crude filtrate (pH 6.1) adjusted to pH 6.5 (1 N NaOH) caused necrosis of cuttings of Canada thistle [*Cirsium arvense* (L.) Scop. (Compositae)] within 20 hr. TLC (Si gel, $CHCl_3$–MeOH, 25 : 1) of the chloroform extract of an aliquot (10 ml) of culture filtrate revealed the presence of two short UV-quenching bands ($R_f$ 0.23 and 0.31). Both bands exhibited antifungal properties when subjected to the TLC bioassay technique of Homans and Fuchs [3]. The chloroform extract of the bulk of the culture filtrate, on standing at laboratory temperature, yielded a crystalline phytotoxic product, which was equivalent to the major TLC band ($R_f$ 0.31). The crystals were washed with cold benzene and recrystallized from hot ethanol to afford colourless needles, subsequently identified as the known fungal metabolite radicinin [4]. The yield of crystalline toxin was of the order of 0.5 g/l. Radicinin was produced by all three isolates of *A. chrysanthemi* examined.



**1a** R=H
**1b** R=*p*-Bromobenzoyl



**2**

Radicinin was described as a product of *Alternaria radicina* Meier, Drechsler and Eddy (syn. *Stemphylium radicinum* M., D. and E.), coincidentally as a yellow pigment [4] and as colourless needle-like crystals [5]. Grove [6] demonstrated the identity of stemphylone [7] (from *A. radicina*) with radicinin and elucidated its structure as **1a** on the basis of chemical and spectroscopic evidence. This structure was later verified by total synthesis of (±)-radicinin [8].

The identification of radicinin (**1a**) as a metabolite of *A. chrysanthemi* was accomplished by X-ray crystallography. The X-ray diffraction experiment, however, defined only the relative stereochemistry of the molecule, so the enantiomer shown as **1a** is arbitrary. The absolute stereochemistry of radicinin, as shown in **1a**, was established in a second X-ray crystallographic experiment, which employed its 4-*O*-*p*-bromobenzoyl ester (**1b**). The absolute stereochemistry found for **1b** and by inference for **1a** is in agreement with that previously reported [9]. In the previous study the absolute stereochemistry of radicinin was inferred from interpreting the CD spectrum of the 3,4-bis-*p*-chlorobenzoyl derivative of radicinol, in accord with the dibenzoate excition chirality rule [10]. However, the appearance of other transitions in the relevant wavelength region may render the dibenzoate rule inapplicable. In the present case, transitions due to the α-pyrone chromophore were observed, and therefore we felt that verification of the conclusion drawn from this study was required. The bond lengths for **1a** and the

two crystallographically-independent molecules of **1b** are given in Table 1.

Radicinin is reported to possess insecticidal and plant growth regulatory activity, in addition to its antifungal and phytotoxic properties [11]. The same compound is inhibitory to the growth of several Gram-positive bacteria, for example, *Staphylococcus aureus* [11] and *Clostridium* sp. [unpublished results, this laboratory]. The biological properties of radicinin are currently being investigated in more detail.

Table 1. Bond lengths and estimated standard deviations (in parentheses) for radicinin (**1a**) and the two independent molecules (A, B) of its *p*-bromobenzoyl ester (**1b**)

| Bond | Bond length, (Å) | | |
|---|---|---|---|
| | **1a** | **1b** (A) | **1b** (B) |
| C-1–C-2 | 1.438 (4) | 1.463 (12) | 1.416 (12) |
| C-1–O-1 | 1.399 (3) | 1.390 (10) | 1.402 (10) |
| C-1–O-2 | 1.196 (3) | 1.185 (10) | 1.240 (11) |
| C-2–C-3 | 1.443 (4) | 1.435 (11) | 1.465 (11) |
| C-2–C-6 | 1.379 (4) | 1.394 (13) | 1.375 (12) |
| C-3–C-4 | 1.508 (5) | 1.499 (11) | 1.519 (11) |
| C-3–O-3 | 1.218 (3) | 1.231 (10) | 1.207 (9) |
| C-4–C-5 | 1.495 (5) | 1.555 (11) | 1.463 (11) |
| C-4–O-4 | 1.408 (4) | 1.429 (8) | 1.440 (8) |
| C-5–C-12 | 1.502 (5) | 1.466 (14) | 1.544 (14) |
| C-5–O-5 | 1.453 (4) | 1.445 (10) | 1.468 (10) |
| C-6–C-7 | 1.394 (4) | 1.413 (13) | 1.396 (12) |
| C-6–O-5 | 1.345 (3) | 1.342 (11) | 1.336 (11) |
| C-7–C-8 | 1.346 (4) | 1.382 (14) | 1.300 (13) |
| C-8–C-9 | 1.438 (4) | 1.439 (13) | 1.474 (13) |
| C-8–O-1 | 1.365 (3) | 1.290 (11) | 1.421 (10) |
| C-9–C-10 | 1.312 (4) | 1.290 (13) | 1.312 (14) |
| C-10–C-11 | 1.485 (5) | 1.525 (14) | 1.479 (14) |

Pyrenphorin, a second biologically active product of *A. radicina* [12], was not detected in culture filtrates of the morphologically and pathologically distinct *A. chrysanthemi*.

In a time-course experiment, production of radicinin, radicinol, and fungal mycelium in liquid cultures of *A. chrysanthemi* were followed over a period of 31 days (Table 2). Surprisingly, radicinin production began immediately with no discernible lag phase (cf. ref. [13]), and its concentration increased rapidly to a maximum of *ca* 626 mg/l. after 20–24 days. Increase in fungal mycelium was approximately constant for the first 30 days at which time mycelial dry wt attained a value of *ca* 2.8 g/l.

After *ca* 20 days, the second antifungal constituent of *A. chrysanthemi* culture filtrates increased with the concomitant decrease in concentration of radicinin, suggesting that the two metabolites were structurally closely related, and that conversion of **1a** to **2** was readily accomplished by *A. chrysanthemi* in liquid culture. Identification of the second compound (MW 238) as radicinol (**2**) was by comparison of its spectroscopic (UV, IR, $^1$H NMR, MS) and specific rotation data with lit. values [9] and with that of authentic radicinol obtained by reduction of radicinin with sodium borohydride in methanol [9].

Radicinol has also been reported to co-occur with radicinin in the culture filtrate of one isolate (of 10) of *Cochliobolus lunatus* Nelson and Haasis, although in relatively trace amounts (2.4 mg/l.) [9]. In contrast, radicinol was produced by all three isolates of *A. chrysanthemi* examined and in concentrations of up to 413 mg/l.

Radicinol, like radicinin, exhibits phytotoxic activity, causing interveinal necrosis of cuttings of Canada thistle. Both compounds were identified (TLC, UV, MS) as constituents of necrotic lesions on chrysanthemum leaves which had been artificially infected with *A. chrysanthemi* (isolate QM 8579), suggesting a causal role for radicinin and radicinol in the foliar disease caused by *A. chrysanthemi*.

Table 2. Concentrations of radicinin and radicinol and mycelial dry wt in liquid cultures of *A. chrysanthemi*

| Age of culture (days) | Approximate concentration (mg/l.) | | Mycelial dry wt (g/l.) |
|---|---|---|---|
| | Radicinin | Radicinol | |
| 0 | < 10 | — | 0.03 |
| 1 | 15 | — | 0.12 |
| 2 | 30 | — | * |
| 3 | 49 | — | 0.28 |
| 4 | 75 | — | 0.41 |
| 5 | 127 | — | 0.52 |
| 10 | 390 | < 10 | 1.05 |
| 15 | 545 | 28 | 1.52 |
| 20 | 626 | 69 | 1.99 |
| 25 | 589 | 167 | 2.41 |
| 30 | 474 | 338 | 2.79 |
| 31 | 460 | 408 | 2.66 |

*Datum not available.
—, Not detected.

## EXPERIMENTAL

*Fungal cultures.* The three isolates of *A. chrysanthemi* (kindly provided by Professor E. G. Simmons, Dept. of Botany, University of Massachusetts) were QM 7227, QM 7228 and QM 8579. Prefix QM refers to cultures of the U.S. Army Natick Labs, Natick, Massachusetts. Isolates QM 7227 and QM 8579 are deposited with the ATCC. Cultures were maintained on 20% V-8 juice agar. Liquid shake cultures of *A. chrysanthemi* and *A. radicina* were carried out in modified Czapek–Dox medium of the following composition: 100 g sucrose, 2 g casein hydrolysate, 1.5 g $NaNO_3$, 1 g $K_2HPO_4$, 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, $H_2O$ to 1 l., pH adjusted to 6.0. 1 l. batches in 2 l. conical flasks were incubated at 24° and at 200 rpm on a gyratory shaker.

*Time-course experiment. A. chrysanthemi* (isolate QM 8579) was grown in 125 ml conical flasks containing 50 ml modified Czapek–Dox medium. A 2% (vol.) inoculum, from a 25 day liquid culture, was used for each flask and incubated as described above. At various time intervals culture filtrate was extracted with $CHCl_3$. TLC of the $CHCl_3$ phase, as already described, yielded radicinol and radicinin, concentrations of which were determined by UV absorption: log $\epsilon$, radicinin = 4.33 at 343 nm; log $\epsilon$, radicinol = 4.51 at 225 nm.

*Radicinol* (**2**). Prep. TLC (Si gel, $F_{254}$, 2.0 mm, 20 × 20 cm $Et_2O$–pentane–HOAc, 60 : 10 : 2) of mother-liquor from crystallization of **1a** (*ca* 20 mg/plate) yielded radicinol (**2**) ($R_f$ 0.21); detected as a dark quenching band under short-wavelength UV. After development **2** was eluted from the adsorbent with MeOH. Preparation of **2** usually resulted in an amorphous gum [9], but on occasion crystallized (from $CHCl_3$) as colourless needles. UV $\lambda_{max}^{EtOH}$ nm (log $\epsilon$): 225 (4.51), 262 (3.38), 271 (3.40), 319 (4.04). $[\alpha]_D^{30} = -181°$ ($CHCl_3$; *c* 6.0) (lit. [9] $[\alpha]_D = -175°$ ($CHCl_3$). MS *m/z* (rel. int.): 238 $[M]^+$ (4), 181 (69), 152 (4) 111 (33), 97 (6), 69 (74).

*p-Bromobenzoyl radicinin* (**1b**). Radicinin (250 mg) and *p*-bromobenzoyl chloride (250 mg) in 3.5 ml $C_5H_5N$ were stirred gently under $N_2$ in the dark for 2 hr. The residue after solvent evaporation was taken up in $CHCl_3$ (5 ml) and extracted × 4 with 5% aq. $NaHCO_3$. TLC of a fraction of the $CHCl_3$ phase (Si gel, MeCN–$H_2O$, 7 : 1) afforded *p*-bromobenzoyl-radicinin ($R_f$ 0.74), together with traces of unchanged radicinin ($R_f$ 0.50). *p*-Bromobenzoyl radicinin crystallized from (hot) EtOH as colourless plates; recrystallized from $Me_2CO$ or $C_6H_6$ as needles, mp 197–199°. UV $\lambda_{max}^{EtOH}$ nm (log $\epsilon$): 224 (4.24), 247 (4.41), 282 (sh) (3.53), 346 (4.26).

*Extraction of chrysanthemum leaves.* Necrotic lesions on leaves of florists' chrysanthemum (*Dendranthema morifolium* (Ramat.) Tsvel.) cv Dixie, previously inoculated with *A. chrysanthemi*, were excised and extracted with $CHCl_3$. TLC of the extract (Si gel, $CHCl_3$–MeOH, 50 : 1) gave two UV-quenching bands at the same $R_f$ values (0.10 and 0.17) as radicinol and radicinin applied as markers. UV maxima for the two bands were: (a) band at $R_f$ 0.10, as for radicinol; (b) band at $R_f$ 0.17, UV $\lambda_{max}^{EtOH}$ nm: 221, 269, 280 (sh), 343, identical with authentic radicinin obtained from fungal cultures, (cf. lit. [14] $\lambda_{max}^{EtOH}$ nm: 220.5, 270, 280, 343).

*X-ray crystallography.* Crystal structures were determined for both **1a** and **1b** using an ENRAF-NONIUS CAD-4 automatic diffractometer and the programs described by Frenz [15].

Compound **1a** is monoclinic, $a = 7.989$ (2), $b = 6.516$ (3), $c = 10.723$ (5) Å, $\beta = 91.14$ (3)°, space group $P2_1$ (No. 4), $z = 2$, molecular vol. = 279.1 Å$^3$, D (calc.) = 1.406 g/cm$^3$. Mo $K_\alpha$ radiation used ($\lambda = 0.7107$ Å). Absorption corrections not made ($\mu = 1.19$ cm$^{-1}$). 676 independent reflections for which $\theta < 25°$ and $I > 2\sigma(I)$ were used in the calculations. A trial structure was found using direct methods. The modification described by Britton and Dunitz [16, 17] was required. Refinement was made using full-matrix least squares with anisotropic thermal parameters for all the non-hydrogen atoms. Hydrogen atoms were included at their idealized positions, but were not refined, except for H (04) which was refined with an isotropic thermal parameter. Refinement converged at a conventional $R = 0.041$.

Compound **1b** is triclinic, $a = 8.052$ (5), $b = 14.614$ (11), $c = 7.937$ (6) Å, $\alpha = 91.50$ (6)°, $\beta = 105.46$ (5)°, $\gamma = 79.21$ (5)°, space group P1 (No. 1), $z = 2$, molecular vol. = 442.0 Å$^3$, D (calc.) = 1.575 g/cm$^3$. Cu $K_\alpha$ radiation used ($\lambda = 1.5418$ Å). Absorption corrections not made ($\mu = 38.0$ cm$^{-1}$, crystal size *ca* 0.2 × 0.2 × 0.2 mm). Since the absolute configuration was to be determined, the entire sphere of data was collected. 2946 reflections for which $\theta < 78°$ and $I > 3\sigma(I)$ were used in the calculations. A trial structure was found using Patterson and Fourier maps, and was refined using full-matrix least squares with anisotropic thermal parameters for the Br atoms and isotropic thermal parameters for the other non-hydrogen atoms. Hydrogen atoms were included at their idealized positions but were not refined. Refinement was carried out for both enantiomeric possibilities and convergence occurred with $R = 0.059$ for the better fit and $R = 0.065$ for the poorer fit. Also, for the better fit, Friedel pairs with large differences in *F* (calc.) agreed with the *F* (obs.) differences in *ca* 95% of the pairs examined. The correct enantiomer is as shown in structure **1b**. The two independent molecules in the unit cell were situated in a pseudo-centric arrangement but both were the same enantiomer.

An examination of the bond lengths (Table 1) shows that the bond lengths in **1a** are generally close to the average of the bond lengths for the two independent molecules of **1b**, but that the differences between the two independent molecules of **1b** are large. A half-normal probability plot [18] comparing the bond lengths between the two independent molecules suggests that the estimated standard deviations are underestimated by a factor of three by the least squares calculation. If we increase the estimated standard deviations in **1b** by a factor of three, even the worst disagreement, the bond C8-01, is less than three s.d.'s. It is common to find the least squares estimated s.d.'s to be too small, although usually not by this much. It is possible that correlation between the two pseudo-centric molecules is partly to blame, but this was not explored at the time the calculations were performed.

In both **1a** and **1b**, there are several C . . . C and C . . . O contacts between 3.2 and 3.5 Å, but none that are unusual. In particular, the hydroxyl group in radicinin (**1a**) is not involved in a hydrogen bond.

Tables of fractional coordinates, temp. factors and bond angles have been deposited with the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, U.K.

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