JOURNAL OF NATURAL PRODUCTS

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J. Nat. Prod., **1992**, 55 (4), 487-490• DOI: 10.1021/np50082a014 • Publication Date (Web): 01 July 2004

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Journal of Natural Products
Vol. 55, No. 4, pp. 487-490, April 1992

BISRADICININ: A NOVEL DIMER ELICITED IN CULTURES OF *ALTERNARIA CHRYSANTHEMI*

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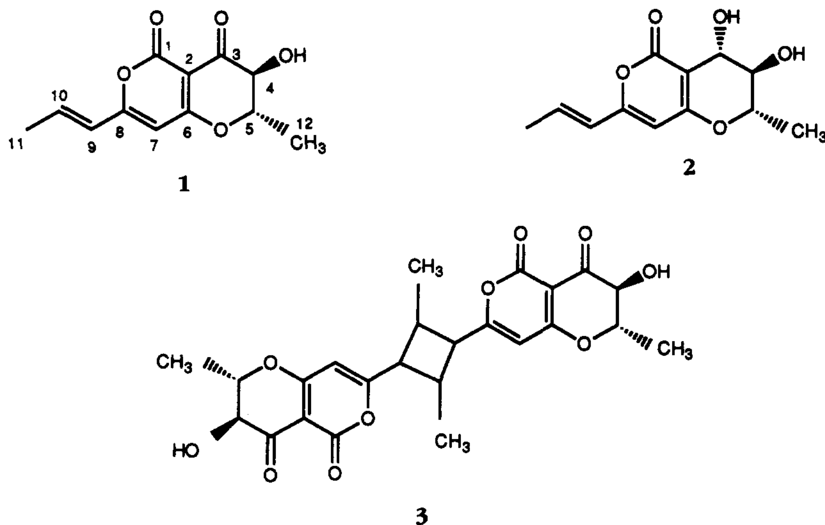
ABSTRACT.—Cultures of *Alternaria chrysanthemi* are known to produce the α-pyrones radicinin [**1**] and radicinol [**2**]. During the course of a study to monitor the ability of *A. chrysanthemi* cultures to biotransform foreign substrates it was observed that the addition of foreign substrates stimulated the production of a third α-pyrone. This compound was identified as bisradicinin [**3**], a dimer of **1**. Compound **3** was not produced in cultures of *A. chrysanthemi* grown under identical conditions without added substrate.

Alternaria chrysanthemi Simmons and Crosier (Dematiaceae) is the etiological agent of leaf spot disease of *Chrysanthemum maximum* (Ram.) DC. (Compositae) (1). Early phytochemical analyses of *A. chrysanthemi* have led to the isolation and identification of two phytotoxic compounds, radicinin [**1**] and radicinol [**2**] (2,3). To date there have been no reports on the biotransformation of foreign chemical substrates by *A. chrysanthemi*, although unidentified strains of *Alternaria* have been reported to catalyze the deacylation of phenoxymethyl penicillin (4) and the reduction of progesterone to 1-dehydroprogesterone (5). As part of an ongoing study on the ability of *A. chrysanthemi* to biotransform foreign substrates we have recently identified **3**, a novel dimer of radicinin. This compound was produced in all cultures used in biotransformation studies and was not observed in cultures grown under identical conditions but without added substrate.

Hplc analysis of EtOAc extracts of the culture medium of *A. chrysanthemi* (over a 1



to 100 day period) showed the presence of two major components **1** (Rt 3.8 min) and **2** (Rt 3.3 min). When a series of biotransformation substrates including linalool, geraniol, benzaldehyde, and acetophenone were incubated (3 mg/ml culture broth) in separate experiments with *A. chrysanthemi* cultures, hplc analysis of the EtOAc extracts of the fungal growth medium of all of the inoculated cultures showed the presence of a third compound **3** (Rt 5.28 min.). Compound **3** appeared within 3 days of the addition of foreign substrate. This compound did not occur in cultures that had no added substrate or in cultures that just contained added MeOH. Compound **3** could be isolated by cc on Si gel ($CHCl_3$/MeOH) or could be crystallized directly from an MeOH solution of the crude EtOAc extract. Compound **3** crystallized as needles (MeOH), mp 210°, and was optically active, $[\alpha]D -100°$ ($c=1$, $CHCl_3$, 25°). The molecular formula $C_{24}H_{24}O_{10}$ $[M]^+$ 473.14478, expected 473.1448, was established by mass spectral analysis.

The ^{1}H-nmr spectrum of **3** was similar to that of **1** (Table 1) with a doublet at 1.58 ppm corresponding to Me-12, a one hydrogen singlet at 5.89 ppm corresponding to H-7, a doublet of doublets centered at 3.95 ppm, and a doublet of quartets at 4.31 ppm that correspond to H-4 and H-5 respectively. The main difference between the ^{1}H-nmr spectrum of **3** and **1** appears in the chemical shift for the signals of the protons in the propenyl side chain. The propenyl methyl group resonates as a doublet of doublets upfield (δ 1.10) from the corresponding methyl group in radicinin (δ 1.96). The signal for H-9 appears as a broad doublet centered at 2.12 ppm, and the signal for H-10 appears as a doublet of triplets centered at 2.96 ppm. The shift in the frequency of the signals for H-9, H-10, and H-11 is entirely consistent with the loss of unsaturation which occurs on dimerization via the side chain. The 2D COSY spectrum of **3** clearly established the connectivity patterns between H-9, H-10, and Me-11, in addition to those between H-4, H-5, and Me-12. The ^{13}C-nmr spectrum of **3** was almost identical with that of **1** with the exception of the frequency of the signals for C-9 and C-10 (Table 1). The new signals in the spectrum of **3** at 40.27 ppm and 46.7 ppm are consistent with the expected chemical shifts (6).

In theory twelve isomers can be obtained by the dimerization of radicinin depending on whether head-to-tail or head-to-head dimerizations take place with syn or anti and with cis or trans ring junctions (7). The head-to-tail dimer will split symmetrically

TABLE 1. Chemical Shifts of Radicinin [**1**] and Bisradicinin [**3**].[a]

Position	^{1}H nmr		^{13}C nmr	
	1	**3**	**1**	**3**
1	—	—	188.1	188.6
2	—	—	97.8	97.9
3	—	—	156.0	156.8
4	4.05 (d, 12.3)	3.95 (d, 12.4)	71.8	71.9
5	4.36 (dq, 6.1, 12.2)	4.31 (dq, 5.8, 12.4)	79.4	80.2
6	—	—	174.7	175.9
7	5.86 (s)	5.89 (s)	98.2	99.3
8	—	—	162.5	169.2
9	6.05 (d, 15.6)	2.11 (bd, 5.6)	122.7	40.2
10	6.97 (dq, 6.9, 15.3)	2.96 (m)	138.3	46.7
11	1.96 (dd, 7.0, 1.4)	1.10 (d, 5.8)	18.3	18.1
12	1.65 (d, 6.2)	1.58 (d, 6.3)	17.4	17.9
OH	3.98 (s)	3.1 (bs)	—	—

[a]δ values (TMS) in $CDcl_3$.

on electron impact (Figure 1a), the asymmetrical isomer may split symmetrically and asymmetrically (Figure 1b), but the two types of splitting may not occur to the same extent (7,8). Support for the symmetrical nature of this dimer was obtained by detailed analysis of the mass spectrum. In addition to the $[M + 1]^+$ peak at *m/z* 473 and *m/z* 495 $[M + Na]^+$, the most prominent ions are at *m/z* 259 $[M + Na - 236]^+$ and at *m/z* 237 $[M - 236]^+$ corresponding to the loss of a radicinin monomer from **3**. Such fragments may be lost from either the symmetrical or the asymmetrical isomer (Figure 1). The total absence of ions at *m/z* 58, 81, 418, and 441 expected for an asymmetrical isomer favor the structure presented for **3**.

The phytoxicity of **3** was measured in a bioassay system using plant cell tissue cultures of *Nicotiana tabacum* grown on Murashige and Skoog solidified agar (9). Results were compared with those for **1** and **2** tested under the same conditions. Cultures treated with radicinin [**1**] and bisradicinin [**3**] at concentrations of 100–200 μg/ml exhibited browning and loss of viability within a ten-day period after treatment when compared to controls. Under the same conditions, radicinol [**2**] did not exhibit such severe phytotoxicity. Hplc analysis of the MeOH and aqueous solutions of **3** showed that they were stable for long periods of time (up to 30 days) suggesting that the phytotoxicity observed for **3** was not as a result of the release of monomeric **1** in the bioassay, unless such a release was catalyzed by the *Nicotiana* cells.

The occurrence of the phytotoxin **3** as an elicited or stress product, as opposed to an artifact formed by photochemical dimerization, is supported by the absence of this product from short term and long term cultures of *A. chrysanthemi* grown under identical conditions, but with no added substrate.

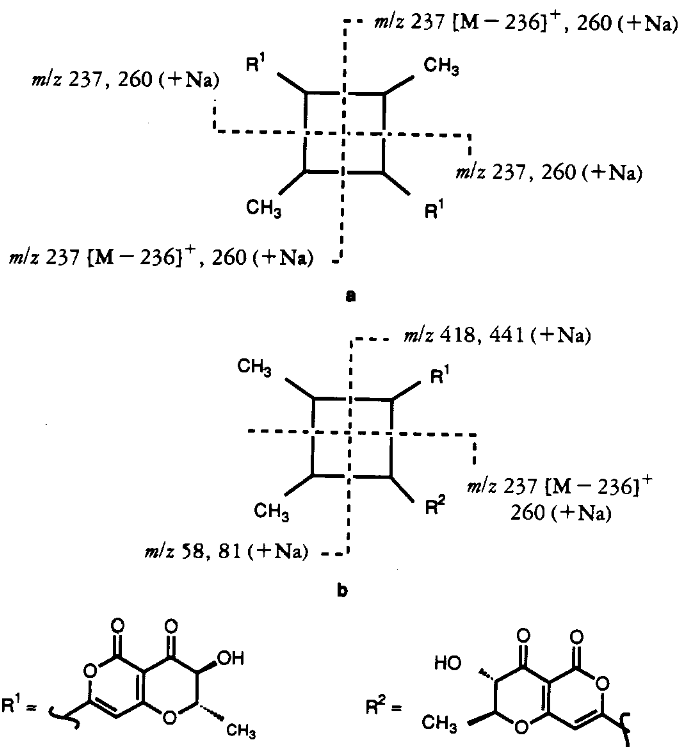


FIGURE 1. Mass spectral fragmentation expected for HT and HH dimers.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-nmr spectra were recorded at 400 MHz on a Bruker AC-400 spectrometer, using $CDCl_3$ as solvent with TMS as internal standard. Mass spectra were recorded at 70 eV. Analytical tlc was carried out on plastic sheets pre-coated (0.2 mm) with Si gel $60F_{254}$ (E. Merck, Darmstadt). Compounds were detected by visualization at 254 nm. Broth cultures of *A. chrysanthemi* (ex CBS Baarn) were prepared by inoculating 150 ml potato dextrose broth (in 250-ml conical flasks) with 4 plugs (each 4 mm in diameter) of *A. chrysanthemi* taken from the growing edge of a culture grown on half strength potato dextrose agar. Cultures were agitated on an orbital shaker at 90 rpm and grown at 25° (±2°) in a light (18 h)/dark (6 h) cycle. For biotransformation studies, foreign substrates (3 mg/ml) were added aseptically in MeOH (1 ml per 150 ml broth) to liquid cultures on day seven of the growth cycle; cultures were incubated for a further 3–20 days depending on the experiment. Cultures of *A. chrysanthemi* were also inoculated with MeOH, and these controls were examined for products by hplc. Cultures were incubated for a further 3–20 days depending on the experiment. Hplc assays were carried out on a Spherisorb 10 ODS column (250 × 4.6 mm) using a solvent system of MeCN-H_2O (3:7). The flow rate was 2 ml/min, and detection was by uv at 310 nm. Bioassays for phytotoxicity utilized plates of Murashige and Skoog agar medium supplemented with test compounds at varying concentrations (500–200 μg/ml). The plates were inoculated with cell suspension cultures of *N. tabacum* spread evenly over the surface of the medium. Plates were grown at 25° in the same light/dark cycle as the fungal cultures. Test plates were examined for phytotoxicity visually after 10 days by comparison with control plates lacking test compounds.

ISOLATION OF COMPOUNDS **1–3**.—Cultures of *A. chrysanthemi* were filtered, and fungal growth medium was extracted with EtOAc. This was dried over Na_2SO_4, filtered, and evaporated to dryness in vacuo. The resulting residue was chromatographed on a Si gel column using a $CHCl_3$/MeOH gradient as eluent. Three main fractions were thus separated to yield the following compounds, which were examined by tlc using $CHCl_3$-MeOH-H_2O (10:1:0.1).

Radicinin [**1**].—Mp 219° [lit. (3) mp 235–238°]; $[M]^+$ 236.06848 ($C_{12}H_{12}O_5$); R_f 0.32; Rt 3.8 min.

Radicinol [**2**].—Oil; $[M]^+$ 238.08413 ($C_{12}H_{14}O_5$); R_f 0.16; Rt 3.3 min.

Bisradicinin [**3**].—White needles: mp 210° (MeOH); R_f 0.10; Rt 5.28 min; ir ν max cm^{-1} (KBr) 3400, 1742, 1635, 1125; uv (MeOH) 219, 270, 340; fabms *m/z* (rel. int. %) $[M+NH_3]^+$ 495 (100), $[M]^+$ 473 (85), 259 (72), 237 (41), 219 (13).

ACKNOWLEDGMENTS

The authors thank Ms. Caroline Lowry for her assistance with fungal and plant cell culture maintenance.

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Received 23 September 1991