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PHYTOTOXINS FROM *ALTERNARIA ALTERNATA*, A PATHOGEN OF SPOTTED KNAPWEED[1]

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ABSTRACT.—The fungus *Alternaria alternata* was found to be a host-selective pathogen of spotted knapweed, *Centaurea maculosa*, a major weed pest in rangelands of the northwestern United States and southwestern Canada. Examination of the extracts of fungal cultures revealed three classes of phytotoxins—diketopiperazines, tetramic acids, and perylenequinones. The isolation, identification, and phototoxic activity of the tetramic acid and perylenequinones are described; two of the perylenequinones, alterlosins I and II, are new compounds. Members of the three classes were compared individually and in combination with respect to phytotoxicity and host specificity.

Spotted knapweed (*Centaurea maculosa*) is the number one weed problem in western Montana and poses a significant threat throughout the northwestern United States and southwestern Canada. Since its introduction into the United States in the early 1900s, this hardy member of the Compositae has invaded over two million acres of prime rangeland in Montana with an estimated 70% decline in forage production. Similar losses have occurred in British Columbia, Idaho, and Washington (1). Current attempts at control of this highly competitive weed involve the application of Tordon 22K (picloram), which is detrimental to both crop and ornamental plants (2). Biological control is an attractive alternative for solving this problem.

Fungi have long been recognized as instigators of plant disease, sometimes with catastrophic results, as witnessed by the Southern corn blight epidemic of 1970 (3). We are interested in exploiting this natural propensity for disease induction as a potential source of host-specific toxins in the fight against economically undesirable plants. Until now, plant pathogens capable of elaborating phytotoxins with any degree of specificity at the species or cultivar level were known only from crop plants. Crop plants represent a genetically homogeneous base, susceptible to the spread of a particular pathogen, while weeds represent a heterogeneous genetic base that may resist the spread of most pathogens.

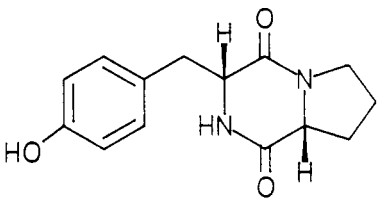
We recently disclosed the isolation, identification, and synthesis of maculosin [**1**], the first host-specific phytotoxin for a weed pest (4) from extracts of the fungus *Alternaria alternata* Lam. This diketopiperazine was accompanied by several other phytotoxic fractions in the fungal extracts. Herein we report the isolation, structure elucidation, and biological testing of a series of perylenequinones, including the two new phytotoxins alterlosins I and II, and a tetramic acid from the organic soluble extracts of *A. alternata*.

RESULTS AND DISCUSSION

In the course of isolating maculosin [**1**] from the EtOAc-soluble extracts of the fungal culture (4), we consistently encountered an additional phytotoxic fraction which contained no diketopiperazines. Further gel permeation chromatography of this mate-

[1]Contribution #J2196 from the Montana Agricultural Experiment Station.

rial provided tenuazonic acid [**2**], a broad spectrum cytotoxin known from a number of *Alternaria* cultures (5).



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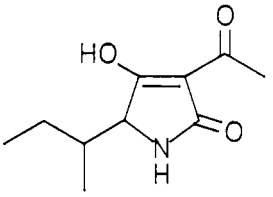

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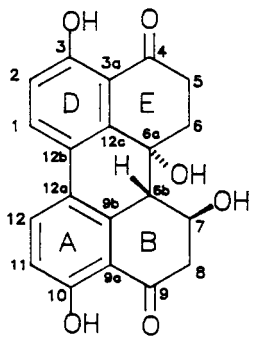
The CH_2Cl_2-soluble extracts also exhibited more phytotoxicity than could be accommodated by the moderately active diketopiperazine fractions contained therein. Assays of fractions obtained by size exclusion chromatography indicated activity in a fraction containing a mixture of aromatic compounds. Separation of these closely related compounds proved to be challenging, but resolution of the four components of the mixture was achieved by centrifugal countercurrent chromatography. Subsequent analysis of spectral data led to identification of these compounds as perylenequinones **3–6**.

Compound **3** was found to have a molecular formula of $C_{20}H_{16}O_6$ by mass spectral analysis; an ir absorption near 1645 cm^{-1} indicated a conjugated, possibly hydrogen-bonded carbonyl. These data, together with the ^1H-nmr spectrum and decoupling information, corresponded to altertoxin I, reported by Stack *et al.* (6) as a mutagen (Ames test) from *A. alternata*. Okuno *et al.* (7) also found this compound in cultures of *A. alternata* and reported it to be phytotoxic to lettuce.



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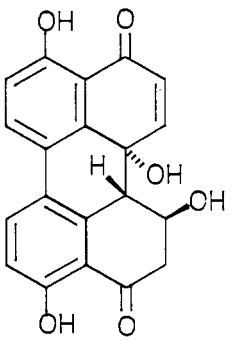

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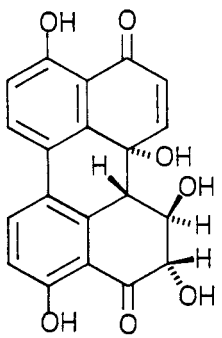

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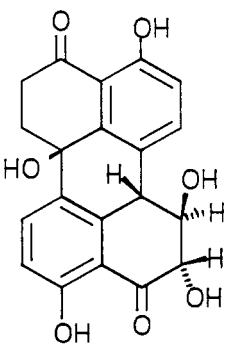

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Perylenequinone **4** was assigned a molecular formula of $C_{20}H_{14}O_6$ on the basis of ms. The ^{1}H-nmr spectral differences from **3** suggested a double bond in the "E" ring. This compound was identical to alteichin, reported by Robeson *et al.* (8) from *Alternaria eichorniae*. Okuno *et al.* (7) also found this compound in their study of *A. alternata*.

Alterlosin I [**5**] had a mol wt of *m/z* 366.0752, which required a molecular formula of $C_{20}H_{14}O_7$. Inspection of the ^{1}H-nmr data (Table 1) indicated a conjugated olefin and a 1,2 diol as part of a system of three contiguous methine carbons. Coupling constants associated with these methines indicated that the three protons were mutually *trans* with typical axial-axial interactions ($J \sim 10$ Hz). Difference nOe experiments also supported these assignments, as irradiation of H-6b induced no enhancement in H-7. Compound **5** had not been reported in the literature, and it is the first member of this series with a *trans* 1,2 diol.

TABLE 1. ^{1}H-nmr Data for Alterlosins I and II.[a]

Alterlosin I [5]		Alterlosin II [6]	
Proton[b]	δ[c]	Proton[b]	δ[c]
H-1, H-12	7.85 (d, 8.4)	$H\text{-}1_{ax}$	2.46 (dt, 4, 13.9)
	7.92 (d, 8.4)	$H\text{-}1_{eq}$	3.10 (m)
H-2, H-11	7.07 (d, 8.4)	$H\text{-}2_{ax}$	3.10 (m)
	7.12 (d, 8.4)	$H\text{-}2_{eq}$	2.67 (dt, 15.4, 4)
H-5	6.39 (d, 10.1)	H-5	7.11 (d, 8.4)
H-6	7.84 (d 10.1)	H-6, H-12	7.82 (d, 8.4)
H-6b	3.37 (d, 10.1)		7.84 (d, 8.4)
H-7	4.48 (dd, 10.8, 10.1)	H-6b	3.18 (dd, 9.1, 1.2)
H-8	4.36 (d, 10.8)	H-7	4.40 (dd, 9.1, 8.7)
		H-8	4.43 (d, 8.7)
		H-11	7.09 (dd, 8.4, 1.2)

[a]Recorded in $CDCl_3$ at 250 MHz.
[b]Numbering scheme as delineated by Stack *et al.* (6).
[c]Chemical shift (multiplicity, coupling constants in Hz).

Alterlosin II [**6**] represented a distinct departure from the system type established by the other members of this series. While mass spectral measurements delineated the molecular formula $C_{20}H_{16}H_7$, several factors indicated that the structure was not the dihydro analogue of **5**. Although two contiguous methylene carbons were present rather than the olefinic system in **5** and the *trans* 1,2-diol system was also present, the associated chemical shifts and coupling constants were different enough to suggest some alternation in conformation and/or anisotropy effects (Table 1).

The uv absorption for alterlosin II appeared at 348 nm, rather than in the typical range of 358–380 nm (7). This suggested a disruption of the biphenyl chromophore; $NaBH_4$ reduction of **6** gave a product with a λ max 268 nm ($\epsilon = 7850$). In our hands, phenol plus $NaBH_4$ gave a λ max 270 nm; in contrast, Stack *et al.* (6) reported that $NaBH_4$ reduction of the biphenyl chromophore gave a product with λ max at 229 and 214 nm. These data suggested that the biphenyl system had been replaced by a diagonal array of the aromatic rings, as had been observed previously by Stack *et al.* (6) and Arnone *et al.* (9).

Difference nOe experiments supported the proposed structure and were pivotal in assigning some resonances and the relative stereochemistry. Irradiation of H-6b did not enhance $H\text{-}1_{ax}$; while the lack of an nOe response generally bears little significance, this result does tend to corroborate the diagonal array of aromatic rings, because the biphenyl alternative would be expected to show an effect similar to that observed in **3**

(20%). Irradiation of the OH proton (on C-12b) at δ 3.76 induced a 7% enhancement of H-6b and a 6% enhancement of one of the resonances at δ 3.10, indicating that all three were axial and allowing us to unravel assignments for the isolated four spin system at C-1 and C-2 as shown in Table 1.

All four of the perylenequinones were tested against knapweed to ascertain their relative phytotoxic behavior. Alterlosins I [**5**] and II [**6**] induced necrotic lesions on knapweed at test concentrations of 10^{-4} M, with **6** inducing larger necrotic lesions compared to the small flecks induced by **5**. Compounds **3** and **4** were not phytotoxic to knapweed at any test concentration.

During the course of our investigation we had isolated and identified four compounds from three distinct chemical classes with toxicity to knapweed: the two novel perylenequinones **5** and **6**, tenuazonic acid [**2**], and maculosin [**1**]. We were interested in determining the relative phytotoxicities of these four metabolites, comparing both activity levels and host specificity to knapweed. The coexistence of the three classes of phytotoxins within the fungal extract also raised some interesting questions concerning

TABLE 2. Determination of Host Specificity of *A. alternata* Phytotoxins.[a]

Test plant	Maculosin [1]			Tenuazonic Acid [2]			Alterlosin II [6]	
	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}
Dicots								
Centaurea maculosa (spotted knapweed)	+++	++	+	+++	++	+	++	+
Lactuca sativa (lettuce)	−	−	−	+++	++	+	+	−
Citrus limon (lemon)	−	−	−	++	+	+	−	−
Lycopersicon esculentum (tomato)	−	−	−	+	+	−	−	−
Malus sylvestris (apple)	−	−	−	+	+	−	−	−
Helianthus annuus (sunflower)	−	−	−	+	+	−	−	−
Cucumis sativus (cucumber)	−	−	−	++	+	−	[c]	[c]
Euphorbia esula (leafy spurge)	−	−	−	++	+	−	−	−
Bidens pilosa (marigold)	−	−	−	−	−	−	[c]	[c]
Taraxacum officinale (dandelion)	−	−	−	++	+	+	−	−
Artemisia tridentata (sagebrush)	−	−	−	+	−	−	−	−
Cirsium arvense (Canadian thistle)	−	−	−	+	−	−	−	−
Euphorbia splendens (roundleaf spurge)	−	−	−	+	+	−	−	−
Monocots								
Sorghum halapense (Johnson grass)	−	−	−	++	+	+	+	−
Poa annua (bluegrass)	−	−	−	++	+	+	[c]	[c]
Avena sativa (park oat)	−	−	−	++	++	+	[c]	[c]
Agropyrens repens (quack grass)	−	−	−	++	+	+	[c]	[c]
Digitaria ischaemum (crabgrass)	−	−	−	+	+	−	−	−
Zea mays (corn)	−	−	−	++	++	+	[c]	[c]

[a]+++, weeping necrotic lesion >4 mm from inoculation site; ++, necrotic lesion 2–4 mm; +, necrotic lesion 0.5–2 mm; −, no lesion present or fleck <0.5 mm.

[b]Concentrations in molarity (M).

[c]Not tested.

the possibility of a synergistic relationship among the various toxins which we attempted to explore.

The three most active metabolites were tested to establish the magnitude of their phytotoxicity to knapweed as well as their relative toxicity to a variety of monocots and dicots (Table 2). Maculosin and tenuazonic acid were clearly the most active of the phytotoxins, with maculosin displaying the most host specificity. Alterlosin II displayed reasonable phytotoxicity and, though not as specific as maculosin, was host selective.

We then exlored the possibility of synergistic phytotoxicity by comparing the effects of the two most active phytotoxins, **1** and **2**, which also showed the most disparate host-specificity. The analysis involved leaf assays with three different weed plants—leafy spurge, knapweed, and Johnson grass—all three of which were susceptible to tenuazonic acid. The plants were all retested with each toxin at 10^{-3}, 10^{-4}, and 10^{-5} M. Tenuazonic acid and maculosin were then combined to total concentrations of 10^{-4} M and 10^{-5} M (each 0.5×10^{-4} and 0.5×10^{-5} M) and applied to the test plants. It is interesting that the effective toxicity to knapweed was doubled when tenuazonic acid and maculosin were applied to the test plants with the combined molarities indicated, while that to Johnson grass and leafy spurge was halved (Table 3).

Plant pathogenic organisms often express several phytotoxins, and each may play a different role in host-pathogen interactions (10). It is reasonable to suspect that each toxin may target a particular host mechanism and that the elaboration of more than one phytotoxin by a fungus may facilitate a multiphase attack on the host. Our studies suggest that two toxins may actually enhance one another's deleterious effects on a given plant host, increasing the pathogenicity of the fungus to a target plant.

TABLE 3. Study of Synergy in the Phytotoxicity of Maculosin [**1**] and Tenuazonic Acid [**2**].[a]

Test plant	Compound[b]							
	1			**2**			Mixture	
	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	10^{-4}	10^{-5}
Knapweed[c]	+++	++	+	+++	++	+	+++	++
Leafy spurge[d]	++	+	+	−	−	−	+	−
Johnson grass[e]	++	++	+	−	−	−	+	+

[a]+++, weeping necrotic lesion >4 mm from inoculation site; ++, necrotic lesion 2–4 mm; +, necrotic lesion 0.5–2 mm; −, no lesion present or fleck <0.5 mm.

[b]Concentration in mole/liter (M).

[c]*Centaurea maculosa*.

[d]*Euphorbia esula*.

[e]*Sorghum halapense*.

The majority of studies on phytotoxins have been conducted on the effects of toxins on hybrid crop plants characterized by extreme genetic similarity, which renders them susceptible to the onslaught to pathogenic fungi. Weeds are a genetically diverse lot and may actually vary from acre to acre in a well-established biome. History has not recorded the devastating loss of any major weed due to a microbial pathogen. Throughout this study we attempted to use knapweed test plants grown from seeds from a variety of geographic locations, and some variation was seen in phytotoxicity results depending on the knapweed source. Knapweed seeds were collected from plants found near Missoula, Bozeman, and Butte, Montana. Plants grown from these various seeds exhibited some response variability to toxin challenges. In all cases, plants grown from seeds collected near the initial collection site were the most sensitive to the phytotoxins. The results reported in this study represent an average of all of the plants tested.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on a Bruker WM250 spectrometer, using $CDCl_3$ as solvent and internal standard. Mass spectra were obtained with VG Instruments MM16F and 7070 EHF spectrometers. The detection, collection, culture, and extraction of *A. alternata* have been described (4). The nicked leaf assay for phytotoxicity has also been delineated elsewhere (11).

ISOLATION OF TENUAZONIC ACID [**2**].—Size exclusion chromatography of the EtOAc-soluble extracts of *A. alternata* on Bio-Beads S-X4 with hexane-CH_2Cl_2-EtOAc (4:3:1) gave twelve fractions. Fraction 5 yielded the host-specific phytotoxin maculosin [**1**] (4). Fraction 7, also phytotoxic, was permeated through Sephadex LH-20 with CH_2Cl_2-MeOtBu-iPrOH (1:1:1); the second fraction was identified as tenuazonic acid [**2**] (150 mg from 5 liters culture), by comparison of uv, ir, and nmr data with literature values (5).

ISOLATION OF THE PERYLENEQUINONES **3–6**.—The CH_2Cl_2-soluble extracts of *A. alternata* were chromatographed on Sephadex LH-20 with CH_2Cl_2-MeOH (1:1); fraction 3 was phytotoxic. This material was permeated through Sephadex LH-20 again, this time with MeOH-MeCN (1:1). Fraction 2 was phytotoxic but was still a mixture that resisted further separation by gel permeation chromatography. However, centrifugal countercurrent chromatography provided baseline resolution of four perylenequinones, **3–6**, using the upper phase of a $CHCl_3$-MeOH-H_2O (25:34:20) system as the mobile phase. Compounds **3** and **4** were identified by comparison of their spectral data with previously reported data (6–8).

ALTERLOSIN I [**5**].—Compound **5**: 2.9 mg from 5 liters culture; mp 191–193°; $[\alpha]_D$ +122° (c = 0.21, MeOH); ir ν max ($CHCl_3$) 3400 (broad), 1646, 1600, 1486, 1457, 1369, 1336, 1231, 1170, 1062, 1018, 951 cm^{-1}; uv λ max (MeOH) 256 nm (ϵ = 31,500), 285 (15,700), 366 (5100); ms *m/z* $[M]^+$ ($C_{20}H_{14}O_7$) 366.0752, $[M-H_2O]^+$ 348, $[M-2H_2O]^+$ 330, 314.

ALTERLOSIN II [**6**].—Compound **6**: 1.8 mg from 5 liters culture; mp 185–187°; $[\alpha]_D$ +131° (c = 0.20, MeOH); ir ν max (neat) 3400 (broad), 1632, 1600, 1473 cm^{-1}; uv λ max (MeOH) 255 nm (ϵ = 30,400), 348 (5000); ms *m/z* $[M]^+$ ($C_{20}H_{16}O_7$) 368.0669, $[M-2H_2O]^+$ 352, $[M-3H_2O]^+$ 334, $[M-4H_2O]^+$ 316 (100%).

ACKNOWLEDGMENTS

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