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SECALONIC ACID A, A VIVOTOXIN IN PINK ROOT-INFECTED ONION

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Key Word Index—*Pyrenochaeta terrestris*; Sphaeropsidales; *Allium cepa*; onion; pink root disease; phytotoxin; vivotoxin; ergochrome; secalonic acid A; MS-MS.

Abstract—A highly potent phytotoxic compound isolated from liquid cultures of *Pyrenochaeta terrestris* was identified as secalonic acid A. This toxic metabolite was identified as a component of infected onion roots.

INTRODUCTION

Pyrenochaeta terrestris (Hansen) Gorenz, Walker & Larson, is the etiological agent of pink root disease of onion (*Allium cepa*, Liliaceae) and other *Allium* species [1], which may also infect a range of other crop plants [2]. Infection of onion root is accompanied by reduced root growth, red pigmentation of infected areas and collapse of infected tissues [2]. Although the *in vitro* biosynthesis of several pigments by this fungus has been well studied [3, 4], the phytochemistry of toxin production has only recently received attention. For example, the pyrenocines A, B and C and the pyrenochaetic acids have been isolated and their metabolism studied [5–8]. Because of the relatively weak phytotoxicity reported for most of these compounds [5, 7] we began a systematic screening for additional phytotoxins accumulating in cultures of *P. terrestris* and/or infected onion tissue. This resulted in the isolation of a highly phytotoxic compound from the mycelium of this organism, which we identified as secalonic acid A (1) [9, 10]. Secalonic acid A as well as the isomeric secalonic acids E and G were previously known as yellow pigments from *P. terrestris* (syn. *Phoma terrestris* Hansen) [3, 9]. We have now demonstrated that compound 1 not only acts as a potent inhibitor of seedling elongation, but in addition MS-MS experiments have confirmed that secalonic acid A accumulates in *P. terrestris*-infected onion tissues.

RESULTS AND DISCUSSION

A series of solvent partitions of *P. terrestris* culture filtrate and mycelial extract showed considerable phytotoxicity in both neutral and acidic EtOAc extracts. Negligible activity was detected in the aqueous fractions. Silica gel CC of the acidic EtOAc extract yielded a yellow pigmented material which inhibited onion seedling elongation at a concentration of 2 μg/ml. Successive recrystallization yielded a purified phytotoxin with a λ_{max} at 337 nm. Ketone and hydroxyl functionality were evident in the FTIR spectrum. ^{1}H NMR (300 MHz) provided evidence for enolic hydrogen bonded protons and connectivity of aromatic and aliphatic spin systems in the molecule were clarified by double resonance experiments. Electron impact mass spectra and accurate mass measurements provided $[M]^+$ of m/z 638.1632, leading to a molecular formula of $C_{32}H_{30}O_{14}$. Due to the symmetry of the molecule (1) the ^{13}C NMR spectrum indicated only 16 carbons, ten of which appeared sp^2 hybridized. Thus, the toxin structure must be represented as one of two possible symmetrically dimeric xanthone derivatives previously isolated as secalonic acid A or its enantiomer, secalonic acid D [11, 12]. A negative optical rotation and CD spectrum demonstrated the identity of the phytotoxin with secalonic acid A (1). Subsequently the identity of secalonic acid A was confirmed by direct comparisons with authentic material. The total yield of 1 obtained from liquid shake cultures of isolate LD5 was 128.2 mg/l. of which 77.7 mg was present in the culture filtrate and the remaining 50.5 mg was associated with the mycelium (Table 1). Secalonic acid A, also known as ergochrome AA, was previously known as a metabolite of *Aspergillus ochraceus* and *Parmelia* spp. as well as *Pyrenochaeta terrestris* [13] although its phytotoxic action has not been reported heretofore.

The phytotoxicity of secalonic acid A, as determined by inhibition of onion seedling elongation, is demonstrated in Table 2. Half-maximal inhibition of seedling elongation is expressed at concentrations between 10^{-6} and 10^{-7} M. At the higher concentrations tested, most of the growth achieved during the course of bioassays occurred in the initial toxin treatment period while growth attained during the ensuing post-treatment incubation period was minimal or nonexistent. Concentrations higher than 10 μM were not assayed because of solubility constraints. Secalonic acid A is a highly potent phytotoxin, which is non-host specific in its action, as demonstrated by phytotoxicity tests against seedlings of radish (*Raphanus sativus*, Cruciferae) and pepper (*Capsicum annuum*,

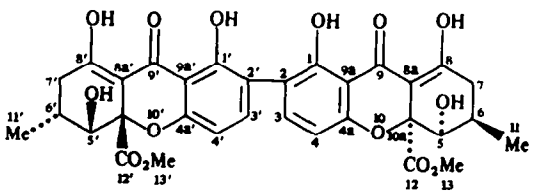

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Table 1. Growth and production of secalonic acid A (1) by virulent (LD5) and weakly virulent (11321) isolates of *P. terrestris* in liquid shake culture

	Yield (mg/l.)	
	LD5	11321
Mycelium (dry wt)	4.5×10^3	4.4×10^3
1 *ex* mycelium	50.5	8.1
1 *ex* culture filtrate	77.7	41.0
Total yields of 1	128.2	49.1

Table 2. Effect of secalonic acid A on elongation of onion seedlings

Secalonic acid A (M)	Seedling length*
10^{-5}	6
10^{-6}	32
10^{-7}	60
10^{-8}	68
10^{-9}	96
Control	100

*Determined after incubation for 96 hr and expressed as a percentage of the control value.

Solanaceae). Radish, which may act as a host of *P. terrestris* [14], shows approximately the same degree of inhibition by secalonic acid A as onion while *C. annuum*, which is also attacked by this fungus [2], appears to be slightly more sensitive to secalonic acid A than onion [unpublished results].

A weakly virulent isolate of *P. terrestris* (11321) was compared with a series of virulent isolates to determine whether secalonic acid A production by 11321 is attenuated. Isolate 11321 was found to produce far less secalonic acid A in highly aerated corn extract medium as compared with virulent isolates (e.g. LD5) (Table 1). However, this correlation was not seen from analysis of cultures of various isolates when grown at lower oxygen tension in still culture [unpublished results].

Onion seedlings infected with *P. terrestris* were examined in an attempt to determine whether secalonic acid A is produced *in vivo* during pathogenesis as well as *in vitro*. Following inoculation and incubation of onion seedlings, infected tissue was extracted and analysed by TLC and HPLC. TLC analysis on silica gel in CH_2Cl_2–MeOH–HOAc, (48 : 2 : 1) of extracts of infected roots revealed a band, not seen in chromatograms of uninfected root extracts, which co-migrated with a secalonic acid A standard to $R_f = 0.43$. During further chromatographic purification, a peak which co-chromatographed with secalonic acid A was detected during HPLC analysis of infected roots employing UV detection at 337 nm. In order to confirm the production of secalonic acid A in infected onion tissues, a partially purified fraction of *P. terrestris*-infected onion root tissue, prepared by solvent partition and CC, was subjected to analysis by mass spectrometry. Although such fractions of infected tissue, containing secalonic acid A, comprise a mixture too chemically complex for conventional analysis, the employment of a triple quadrupole mass spectrometer permitted an unambiguous demonstration that this toxin is indeed present in infected tissue (Fig. 1). The sample (I) was ionized by FAB and a 4 mu window centred at *m/z* 639 was selected in the first quadrupole. Ions of this mass were thus transmitted to the second quadrupole where they undergo collisionally activated dissociation (CAD) with N_2 at 5 mTorr. Daughter ions were separated according to mass in the third quadrupole to provide a reference spectrum for CAD of secalonic acid A. In II and III, identical *m/z* 639 mass windows were selected from ions arising from the partially purified extracts. Spectrum II shows the absence of ions resembling secalonic acid A in the extract of uninfected roots, whereas spectrum III clearly shows the presence of secalonic acid A in *P. terrestris*-infected root tissue. This result further illustrates the versatility of this type of instrument for phytochemical investigations [15–17] and at the same time represents the first reported application of the MS–MS technique to the detection and identification of trace amounts of a phytotoxin in the complex extract of an infected plant organ.

The level of secalonic acid A in extracts of *P. terrestris*-infected onion roots was estimated by integration of the peak, as monitored at 337 nm, which co-chromatographed with authentic secalonic acid A during HPLC analysis of partially purified fractions. This indicated the level of secalonic acid A in infected onion roots, including infecting fungal hyphae, to be equivalent to 0.01% of the dry wt of infected root tissue. By extrapolation, the level in fresh tissue (*ca* 15.7 μM) is at least two orders of magnitude greater than that which will inhibit seedling elongation *in vitro*, thereby indicating a possible role for secalonic acid A in pink root pathogenesis.

The potent phytotoxicity of secalonic acid A has not been previously described and its mechanism of action is unknown. Onion seedling elongation is greatly inhibited at 1 μM secalonic acid A and recovery from toxin treatment occurs slowly, if at all. However, normal development of yellow pigmentation in the hypocotyl region does not appear to be affected by toxin treatment at this level. Although the structure of secalonic acid A bears some resemblance to certain DNA interacting drugs, e.g. anthracyclines [18], DNA binding studies failed to suggest that secalonic acid A interacts significantly with DNA [DiCosmo, F., personal communication].

Biosynthetic derivatives of secalonic acid A, e.g. the enantiomer secalonic acid D [11], have been demonstrated to be toxic in mammalian systems [19] and are recognized as mycotoxins elaborated by such food spoilage fungi as *Penicillium oxalicum*, *A. ochraceus* and *A. aculeatus* [19]. The phytotoxic action of the ergochromes other than secalonic acid A has not been demonstrated. However, in view of their close structural resemblance to secalonic acid A, they would be expected to possess significant phytotoxicity. Studies on structure/activity relationships of these related compounds might be useful in obtaining some understanding of the mode of action of secalonic acid A and its related xanthone dimers [3, 12, 13] against higher plants and other organisms. The use of secalonic acid A in screening for pink root tolerant onion genotypes at the cellular level may clarify its role as a chemical determinant in pink root disease of onion.

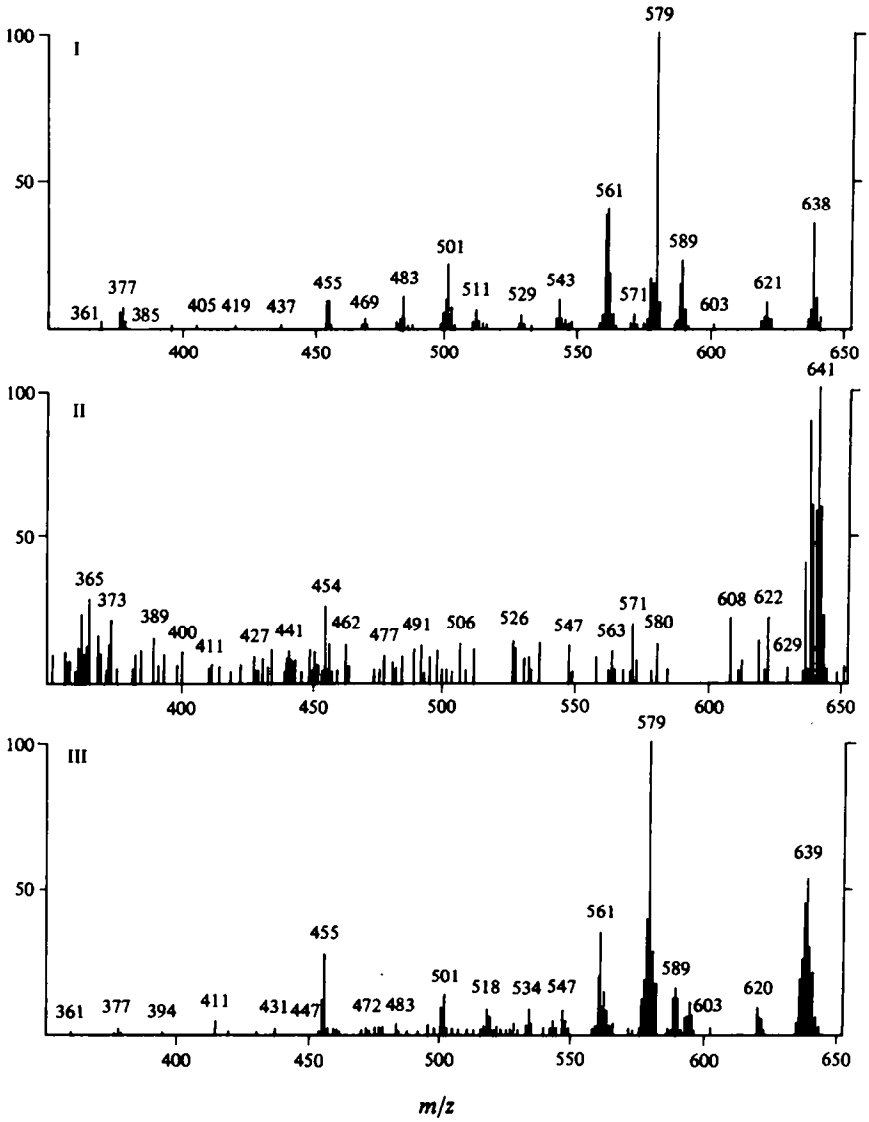

Fig. 1. Triple quadrupole mass spectrometry of pure secalonic acid A (I), extract of uninfected onion root (II) and extract from *P. terrestris*-infected onion root (III)

EXPERIMENTAL

P. terrestris isolates and culture. Isolates 11321 and LD5 were obtained from the American Type Culture Collection, Rockville, Maryland, and L. DiNitto, ARCO Seed Company, Brooks, Oregon, U.S.A., respectively. Cultures were maintained on potato dextrose agar. Liquid culture was carried out in corn extract medium composed of 50 g fr. wt of homogenized canned sweet corn and H_2O to 1 l. Batches (400 ml) in 2 l. Erlenmeyer flasks were inoculated with 10 ml of mycelial suspension and incubated at 28° for 6 days on a gyratory shaker operating at 170 rpm.

Bioassays. Onion seeds (cv. 'Early Lockyer') were surface sterilized in 15% (v/v) Clorox and germinated between two layers of moistened blotting paper at 28° for 48 hr. Seeds with radicles *ca* 2 mm long were selected and placed in 60 × 15 mm Petri dishes containing 5 ml of an appropriate concn of an aq. soln of 1. After incubation for 16 hr at 28°, seedlings were removed from the Petri dish, measured and placed between filter papers moistened with the soln of 1 at the same concn. Seedling length was then measured every 24 hr. In cases where DMSO carriers were required for solubility, its concn did not exceed 1% (v/v). 1% aq. DMSO was used as a control treatment and was not inhibitory to elongation of onion seedlings. Bioassays against radish and pepper were performed in the same manner.

Isolation of secalonic acid A. Initial isolation of the phytotoxic component was guided by onion seedling bioassays performed on each chromatographic fraction after adjustment to a concn of 25 μg/ml.

Mycelium was harvested from 400 ml of *P. terrestris* liquid culture and extracted in MeOH with stirring for 24–48 hr. The MeOH extract was reduced *in vacuo* at 30° and partitioned between EtOAc and 0.01 N HCl. The EtOAc layer showed very strong phytotoxicity against onion seedlings at 25 μg/ml, whereas the aq. layer possessed negligible activity. The dried EtOAc fraction was applied to a 2.5 × 15 cm silica gel flash CC [20] and eluted with a $CHCl_2$–HOAc (49:1) to CH_2Cl_2–MeOH–HOAc (40:10:1) gradient. Column separation was monitored by TLC (silica gel F254, 0.25 mm), CH_2Cl_2–MeOH–HOAc, (48:2:1) (R_f of 1 = 0.43). Considerable phytotoxicity was observed in a number of fractions and was conspicuous in a major yellow-

pigmented band eluting from the column at a MeOH concn of *ca* 4%. This fraction was purified by repeated crystallization from MeOH/EtOAc to yield 2 mg of 1 as bright yellow needles, mp 244–248° (decomp.). UV λ_{max}^{MeOH} nm: 247, 337; $[\alpha]_D^{20}$ −81° (*c* 0.13, $CHCl_3$); FTIR ν_{max}^{film} cm^{-1}: 3436, 1731, 1616, 1596, 1569, 1447, 1332, 1235, 1068, 1050, 1025. *cf.* lit. [9]. ^1H NMR: (300 MHz): $CDCl_3$, referenced to $CHCl_3$ = δ 7.25, as for lit. [9]. ^{13}C NMR (75 MHz, DMSO-d_6, referenced to DMSO = 39.5 ppm): δ 17.73 (C-11,11'), 29.88 (C-6,6'), 35.79 (C-7,7'), 52.82 (C-13,13'), 75.19 (C-5,5'), 85.15 (C-10a,10a'), 101.67 (C-8a,8a'), 106.28 (C-9a,9a'), 107.48 (C-4,4'), 117.27 (C-2,2'), 140.21 (C-3,3'), 158.51 (C-4a,4a'), 158.87 (C-1,1'), 170.00 (C-12,12'), 178.81 (C-8,8'), 186.54 (C-9,9'). EIMS, *m/z* (rel. int.) 638.1632 (calc. for $C_{32}H_{30}O_{14}$: 638.1635) (48), 579.1497 $[M-CO_2Me]^+$ (calc. for $C_{30}H_{27}O_{12}$: 579.1502) (100), 482 (50), 444 (89), 429 (67), *cf.* lit. [4].

Inoculation of onion seedlings with P. terrestris *and analysis of infected tissue.* Onion (cv. 'Early Lockyer') seedlings, *ca* 10 mm in length, were submerged briefly in a suspension of *P. terrestris* mycelial fragments, placed between two sheets of moistened filter paper and incubated at 28° for 8 days. Seedlings (270), showing symptoms of red pigmentation due to pink root infection, were thoroughly washed (H_2O) to remove contaminating mycelium and metabolites from the rhizoplane, extracted with MeOH and the filtrate reduced to aq. material *in vacuo* at 30°. The extract was partitioned between EtOAc and 10% HOAc to yield 10.7 mg of acidic organic extract. Crude 1 eluted from silica gel CC (1 × 12 cm), $CHCl_2$–MeOH–HOAc (47:2:1), was subjected to HPLC analysis: Waters Z Module, C_{18} reversed phase, MeCN–H_2O (41:59), 2.7 ml/min, UV detection at 337 nm. Under these conditions the major tautomeric form of secalonic acid A elutes with R_t 3.55 min. A peak with the same R_t was observed during chromatography of extracts of infected roots; 'spiking' with authentic secalonic acid A resulted in an increased integrated area of this peak.

MS-MS analysis. A similar sample of infected seedlings was extracted as above and secalonic acid A was partially purified by solvent partition and silica gel chromatography. The resulting fraction was taken up in a small quantity of DMSO for analysis on a Finnigan 4000 Q3 mass spectrometer using fast atom bombardment (FAB, 56 keV, xenon, glycerol matrix) for ionization and 5 mT N_2 as collision gas in the second quadrupole [15–17].

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