# A Second Antifungal Polyacetylene Compound from Phytophthora-Infected Safflower

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ABSTRACT

Six-week-old plants of the safflower cultivar Biggs (*Carthamus tinctorius*) were wound-inoculated in the first internode with a virulent isolate of *Phytophthora drechsleri*. An aqueous solution of the methanol soluble substances, extracted from infected stems 48 hr after inoculation, was partitioned with diethyl ether. Two highly antifungal factors in the ether phase were isolated by thin-layer chromatography. One of the antifungal compounds was identified as the previously reported polyacetylene, *trans-trans*-3,11-tridecadiene-5,7,9-triyne-1,2-diol (safynol). Ultraviolet, infrared, mass, and nuclear magnetic resonance spectral data indicated that the second highly antifungal compound is *trans*-11-tridecene-3,5,7,9,-tetrayne-1,2-diol (dehydrosafynol). Adjusted for loss during chromatography, the amount of these compounds in infected stems 48 hr after inoculation was 296 μg dehydrosafynol and 3,370 μg safynol/100 g fresh-infected stems. Healthy stems contained less than 0.2 μg dehydrosafynol and 83 μg safynol/100 g fresh wt. The median effective dose ($ED_{50}$) of dehydrosafynol required to inhibit mycelial growth of *P. drechsleri* was 1.7 μg/ml. Both antifungal compounds were found in high concentration in tissues exterior to the vascular ring. Phytopathology 61:1107-1109.

*Additional key words:* antibiotic activity, disease resistance mechanisms, root and stem rot.

*Phytophthora drechsleri* Tucker incites a root and stem rot of safflower (*Carthamus tinctorius* L.). Safynol (*trans-trans*-3,11-tridecadiene-5,7,9-triyne-1,2-diol), an antifungal polyacetylene, accumulates in the hypocotyls and first internodes of safflower plants which have been wound-inoculated with *P. drechsleri* (1,2,9). The concentration of safynol increases rapidly in the first internodes of both resistant Biggs (USB) and susceptible Nebraska 10 cultivars in the first 24 hr after inoculation. In the next 72 hr, the concentration increases in the resistant cultivar and decreases in the susceptible cultivar (1).

Although safynol can account for the cessation of lesion expansion in resistant stems 96 hr after inoculation (10), it cannot account for the restriction of the lesion which occurs earlier (7, 11).

Experiments were undertaken to determine whether antifungal compounds other than safynol develop in the first internode of Biggs within 48 hr after inoculation with *P. drechsleri*. This paper describes the identity, concentration, and antibiotic activity of a new antifungal compound found in infected safflower stems.

MATERIALS AND METHODS.—Details for the growth of plants and preparation of inoculum were described previously (9). Briefly, safflower plants of the cultivar Biggs were grown in the greenhouse in steam-sterilized soil in porous 20-cm clay pots. *Phytophthora drechsleri*, isolate 201, was grown on lima bean agar in petri dishes at 27 C. Six weeks after emergence, plants were wounded with pin pricks in four vertical rows, four/row and 3 mm apart, spaced an equal distance apart around the first internode. The wounded area was covered with a strip of inoculum (lima bean agar plate cultures) held in place with a strip of aluminum foil lined with plastic film. Wounded and inoculated, and unwounded control plants were held at 30 C in a growth chamber with 1,300 ft-c (14,000 lux) of light for 16 hr/day.

Forty-eight hr after inoculation, whole cross sections (ca. 2 cm long) of infected stems, cut ca. 5 mm from the upper and lower pin pricks, and similar stem sections from noninoculated, unwounded plants were immediately extracted 4 times with methanol, 3 ml/g fresh stems, in a blender for 5 min. Tissue fragments were recovered by filtration after each extraction. The combined alcohol extracts were reduced to an alcohol-free aqueous solution by distillation in vacuo at 28 C. The aqueous solutions were extracted with peroxide-free diethyl ether. The water-saturated ether extracts were evaporated to near dryness in vacuo at 20 C, and the near-dry samples under $N_2$ gas were dissolved in 3-5 ml anhydrous ether. The ether soluble substances were applied to 250 μ silica gel layers containing 2.5% fluorescent indicator. The plates were developed with (i) benzene:ethyl acetate:formic acid, 75:24:1, v/v; (ii) chloroform:acetone:formic acid, 95:4:1, v/v; and (iii) diethyl ether:petroleum ether:formic acid, 80:19:1, v/v. The locations of compounds on plates were revealed as bands that quenched the fluorescence of the indicator under an ultraviolet lamp (254 nm).

Mycelial growth tests were used to detect antifungal activity. Silica gel from quenching as well as from non-quenching bands on thin-layer chromatography (TLC) plates was eluted with diethyl ether. Eluates were washed with water and evaporated to dryness. The resulting residues were mixed with sterile, aqueous lima bean extract at pH 5.7, and tested for inhibition of radial growth by *P. drechsleri* from 5-mm lima bean agar discs in petri dishes held for 24 hr at 25 C in the dark.

Owing to the photosensitivity of compounds isolated, all operations were performed in the dark or with so-

dium vapor photographic safety lamps at low intensities.

In some of the tests, stem sections from inoculated and from control healthy plants were frozen with liquid nitrogen, lyophilized, then extracted and chromatographed. The dry weight of each healthy sample was recorded as a percentage of the fresh weight. For each infected sample, the fresh weight before inoculation (adjusted fresh weight) was calculated: adjusted fresh weight of infected sample = (g dry weight of infected sample/g dry tissue for 100 g fresh healthy stems or stem parts) $\times 100$.

RESULTS.—*Isolation and identification of the new antifungal compound.*—Concentrated ether solutions (prepared from 50 g fresh-infected stems) were streaked across thin-layer plates in a narrow band 2 cm from the edge; then the chromatograms were developed with solvent i. Compounds eluted from a band at $R_F$ .18 to .38 completely inhibited mycelial growth. Compounds from all other bands showed little or no inhibition. Compounds obtained from $R_F$ .18 to .38 in solvent i were then chromatographed with solvent ii. Although many compounds were separated with solvent ii, only those at $R_F$ .12 to .22 completely inhibited mycelial growth. The inhibitory compounds from $R_F$ .12 to .22 on plates developed with solvent ii were separated additionally when eluted with diethyl ether and then chromatographed with solvent iii. With solvent iii, the eluates from bands at $R_F$ .50 (safynol) and $R_F$ .65 (unknown) had high antifungal activities. Compounds from all other bands lacked inhibitory activity. Chromatography of the eluate from band $R_F$ .65 (solvent iii) with solvents i, ii, or iii (and several others) indicated that the antifungal activity was due to a single compound. When safynol and the unknown compound were chromatographed alone, the $R_F$ values were, respectively, .21 and .32 for solvent i, .17 and .14 for solvent ii, and .50 and .65 for solvent iii.

The unknown inhibitory compound from plates developed with solvent iii was rechromatographed with this solvent. Eluates of the single band were evaporated to dryness, and the residue was dissolved in absolute ethanol for ultraviolet spectroscopy. Maxima were found in the ultraviolet spectrum at 375.7, 348.8, 325.2, 305.2, 288.0, 269.7, 256.4, 240.4, 231.0, 222.0, and 212.0 nm. The infrared spectrum, in $HCCl_3$, revealed the presence of hydroxyl groups (3,600 $cm^{-1}$), acetylenic linkage (2,220, 2,187, 2,150 $cm^{-1}$), and *trans*-ethylenic linkage (1,620,950 $cm^{-1}$). The nuclear magnetic resonance spectrum showed the presence of a vinyl methyl group ($\tau$ 8.14), and this was verified by double resonance. The mass spectrum showed that the compound had an empirical formula of $C_{13}H_{10}O_2$.

The ultraviolet, infrared, mass, and nuclear magnetic resonance spectral data, to be reported elsewhere in detail, indicate that the new compound is *trans*-11-tridecene-3,5,7,9,-tetrayne-1,2-diol. By analogy to the structure of safynol, *trans-trans*-3,11-tridecadiene-5,7,9-triyne-1,2-diol (2), we propose the name "dehydrosafynol" (3,4-didehydrosafynol) to simplify referral to this new compound. The concentrations of dehydrosafynol in absolute ethanol were determined by optical density at 269.7 nm ($\varepsilon = 160{,}200$).

*Loss of dehydrosafynol during chromatography.*—In three separate tests, 160, 316, and 502 µg of dehydrosafynol each were added to water. The aqueous solutions were extracted with ether and successively chromatographed with solvents i, ii, and iii with all procedures the same as those described. The amounts recovered in the three tests were 106, 195, and 321 µg, or an average of 64%.

*Concentration of dehydrosafynol and safynol in healthy, and wounded and infected, stems.*—Two samples of 30 g each of fresh unwounded and wounded and inoculated stems were lyophilized and extracted with methanol. Purification of safynol and dehydrosafynol was achieved with TLC by using solvents i, ii, and iii consecutively. The average amounts of dehydrosafynol and safynol (269 nm, $\varepsilon = 61{,}600$), adjusted for loss during isolation, were, respectively, 296 µg and 3,370 µg/100 g fresh infected stems (255 µg and 2,900 µg/100 g adjusted fresh wt), and less than 0.2 µg and 83 µg/100 g fresh healthy stems. For infected Biggs stems, the concentrations of dehydrosafynol and safynol in tissues exterior to the vascular ring were, respectively, 1.7 and 3.3 times the concentrations of these compounds in the interior (vascular ring and pith).

*Inhibition of mycelial growth of P. drechsleri with dehydrosafynol.*—Absolute ethanol containing a known amount of dehydrosafynol was added to the assay medium, and the ethanol was subsequently removed by distillation under reduced pressure. Inhibition of mycelial growth was determined by comparing the growth with that obtained in a control medium to which pure absolute ethanol had been added and subsequently removed. In three tests, the averages of the median effective doses ($ED_{50}$) of dehydrosafynol required for 50% inhibition of mycelial growth were determined as 1.7 µg/ml, 2.3 µg/ml, and 3.0 µg/ml, respectively, for measurements made at 12, 18, and 24 hr after the start of the tests. Mycelial growth was completely inhibited by dehydrosafynol at 5 µg/ml, 7.5 µg/ml, and 9 µg/ml for 12, 18, and 24 hr measurements, respectively.

DISCUSSION.—Bohlmann et al. (3) and Bohlmann & Zdero (4) described 13 naturally occurring acetylenic compounds from safflower (*C. tinctorius*), but did not report the one identified in our study. We obtained for healthy control plants an ultraviolet spectrum which was similar to the spectrum for dehydrosafynol. Further evidence to confirm the presence of dehydrosafynol in healthy plants was not obtained. However, we are sure that, if the compound is present, it occurs at a very low concentration ($< 2$ ppb). The ultraviolet spectrum of dehydrosafynol is similar to that reported by Johnson (5) for an unidentified polyacetylene from *C. tinctorius*. On ChromAR 1000 silica gel sheets (Mallinckrodt Chemical Works, Laboratory Products, New York, N.Y.) developed with $CCl_4$, Johnson's compound had $R_F$ values from .55 to .68 (5); however, we found that dehydrosafynol

did not move from the origin in this system ($R_F$ zero). We concluded that the compound reported by Johnson is not dehydrosafynol, but may be related to it.

Dehydrosafynol, $ED_{50} = 1.7$ μg/ml, is several times more fungitoxic than is safynol, $ED_{50} = 12$ μg/ml (9), and this higher toxicity may be attributed to an additional acetylenic bond in the new compound. In toxicity assays, dehydrosafynol lost its activity faster than safynol, and was presumed to be less stable than safynol.

Dehydrosafynol and safynol were found in high concentration in tissues exterior to the vascular ring. The location of these highly antifungal compounds at the portal of entry suggests that, together, they may play a role in limiting infections that occur naturally within 16-24 hr after inoculation, as reported by Klisiewicz & Johnson (7). Since infected stems often become partially dehydrated, comparisons made on a fresh weight basis could be misleading; we, therefore, reported some of our data on an adjusted fresh weight basis.

Environment markedly affects varietal resistance of safflower to *P. drechsleri* (6, 8). Studies of the accumulation of antifungal compounds in resistant and susceptible varieties as influenced by the environment or by inoculations with avirulent fungi could help further to elucidate disease resistance mechanisms in safflower.

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