Isolation and Characterization of Stemphylin, a Chromone Glucoside from Stemphylium botryosum1

Received for publication July 12, 1974 and in revised form December 12, 1974

ISAAC BARASH2, ARTHUR L. KARR, JR.3, AND GARY A. STROBEL4
Department of Plant Pathology, Montana State University, Bozeman, Montana

ABSTRACT
A phytotoxic compound was isolated from a liquid culture medium of Stemphylium botryosum, a pathogen of lettuce. The toxin is an amorphous yellow solid with absorbance maxima at 218, 268, and 427.5 nm and exhibits a bathochromic shift in alkaline pH. It has a molecular weight of 370 and an empirical formula of C19H22O9. Glucose and aromatic pigments are detected after acid hydrolysis. Based on its spectral and chemical properties, the proposed structure of the toxin is 3-hydroxy-2,2-dimethyl-5-a-d-glucopyranoside-2,3-dihydrochromone, and it has been given the trivial name stemphylin. A linear relationship exists between lesion area and amount of toxin applied to a young lettuce leaf. The relationship between toxin production and the development of disease symptoms is discussed.

Leaf spot of lettuce caused by Stemphylium botryosum Wallr. is of major economic importance in many parts of the world (6). The pathogen is extremely destructive to lettuce and tomato fields in Israel (9), and this has prompted breeding programs for developing resistant varieties of both crops at the Volcani Institute. The usefulness of phytotoxins associated with disease symptoms in screening programs for resistance as well as for basic understanding of host parasite interactions has been recently reviewed by Strobel (11). In a preliminary communication (2) we have reported the isolation of three phytotoxins from defined liquid cultures of S. botryosum by means of ion exchange chromatography. The present work describes the purification procedure, characterization, and biological activity of the anionic toxin.

MATERIALS AND METHODS
Culturing. A culture of Stemphylium botryosum pathogenic on lettuce was obtained from the Volcani Institute of Agricul-

1 This work was supported in part by National Science Foundation Grant GB 43192 and United States Department of Agriculture Cooperative State Research Service Grant C145-2575, and is paper No. 538 of the Montana Agricultural Experiment Station.
2 On sabbatical leave from the Department of Botany, Tel-Aviv University, Israel.
3 Present address: Department of Plant Pathology, University of Missouri, Columbia, Mo. 65201.
4 Public Health Service Research Career Development Awardee 1K4-GM42, 475-05, from the National Institute of General Medical Sciences.
of medium were removed from the mycelial mats by straining through Whatman No. 1 filter paper. The filtrate was concentrated to 50 ml by flash evaporation at 45 C. Acetone (150 ml) at —20 C was added, and the precipitate—removed by centrifugation at 20,000 g for 20 min at 4 C—was discarded. The acetone was removed from the supernatant liquid by flash evaporation, and the volume was reduced to 5 ml. This solution was extracted three times with 10-ml aliquots of water-saturated 1-butanol. The water phase was discarded, and the butanol phases were combined and taken to dryness by flash evaporation at 50 C. The residue of the butanol phase was stored under vacuum over P2O5. The dried material was dissolved in 5 ml of water, was adjusted to pH 7 with 1 N NaOH, and passed through a column of Dowex 50W H+ form (1 x 9 cm). The effluent containing the toxin was adjusted to pH 7 and concentrated to 1 ml. The toxin solution was applied to a Bio-Gel P-2 column (2.5 x 98 cm) and was eluted with distilled H2O. Peaks of the various pigments were detected by measuring the absorbance at 450 nm. The region to toxin activity was located by using the leaf bioassay method. The amount of toxin was determined according to a standard curve after a characteristic toxin spectrum in the UV region had been established. The toxin solution was pooled, concentrated to 5 ml, and applied to Dowex 1 x 8 formate form (1 x 5 cm). The column was washed with 100 ml of distilled H2O. Toxin elution was performed by gradient increase in formic acid concentration from 0 to 2 M, each reservoir containing 250 ml. The formic acid solution was evaporated under reduced pressure and the tubes were stored under vacuum over P2O5. The residue was taken up in 1 ml of H2O and the toxin concentration was determined as described above. The toxin preparation from this latter procedure was taken as the purified anionic toxin used in this study.

**Analytical Methods.** Ultraviolet absorption spectra of the toxin preparations were taken in a Beckman Model 25 K recording spectrophotometer. Infrared spectra were obtained on a Beckman Microspec Spectrophotometer using a micropellet of KBr. Formation of KBr pellet was achieved by grinding a portion of the toxin with the KBr and then drying under vacuum over P2O5. Nuclear magnetic resonance spectra were obtained in a Varian Model T-60 spectrometer. The toxin was dissolved in dimethyl-d8 sulfoxide (99.5% deuterium). A Varian Model CH-5 was used to obtain the mass spectrum. Chemical elemental analyses and vapor pressure osmometry were carried out by Galbraith Laboratories, Knoxville, Tenn.

**Chemicals.** All the chemicals or solvents used were either analytical reagent grade or spectral grade. Acetone and 1-butanol were distilled before use and water was distilled and deionized. p-Glucose-U-15C was purchased from New England Nuclear. Dimethyl-d8 sulfoxide was obtained from Bio-Rad.

**RESULTS**

**Isolation of Toxin.** Bio-Gel and ion-exchange chromatography were employed in the purification of *S. botryosum* toxin. Four pigments were separated by Bio-Gel P-2 column chromatography (Fig. 1). The first three pigments appeared as brown bands in the column, whereas the pigment coincidental with toxic activity was a yellow band. The eluted fractions were taken to dryness under reduced pressure and the residue was redissolved in 0.2 ml of H2O before being tested for biological activity. The amount of the recovered toxin from 1 liter of culture medium was 765 μg as determined by the standard curve. The position of the toxin in the elution profile resulted from interactions with the Bio-Gel and was not representative of the actual mol wt.

The toxin was applied to Dowex 1 x 8 anion exchange resin (formate form). Elution of the toxin from the column was achieved by a gradient increase in formic acid concentration (Fig. 2). Fractions eluted between 0.15 to 0.45 M formic acid contained the toxin. Approximately 40% of the toxin obtained from the Bio-Gel column was recovered after the ion exchange step.

**Criteria for Purity.** The purified toxin (about 50 μg) was subjected to paper chromatography in solvents A through C and to TLC in solvents B, and D through H. The papers were sprayed with reagents I, IV, and VII through IX, and TLC plates with reagents I through VIII. The toxin preparation migrated as a single colored component in all of the solvent systems (Table I). The compound retained its yellow color after the spray treatments, with the exception of reagents IV and X.

![Fig. 1. Fractionation of the toxin preparation by Bio-Gel P-2 chromatography. Fractions of 7 ml were collected after the void volume. Absorbance at 450 nm (○); toxin (●).](image)

![Fig. 2. Chromatography of the toxin mixture on Dowex 1 x 8 column (Fig. 1). Fractions of 4.2 ml were collected. Toxin (○); formic acid (●).](image)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Paper</th>
<th>TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.43</td>
<td>0.59</td>
</tr>
<tr>
<td>C</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>0.41</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>0.95</td>
</tr>
<tr>
<td>G</td>
<td></td>
<td>0.62</td>
</tr>
<tr>
<td>H</td>
<td></td>
<td>0.18</td>
</tr>
</tbody>
</table>

*Table I. Thin Layer and Paper Chromatography of Stemphylin*
in which it reacted to yield a brown spot. In all systems no other compound appeared.

Quantitative chromatography on paper (solvents A through C) and TLC (solvents B, and D through F) indicated that the biological activity coincided with the recovered yellow compound. The absorbance ratio $A_{280}/A_{380}$ of the toxin remained constant during the quantitative paper and TLC.

**Chemical Properties.** The purified toxin is highly soluble in methanol and dimethyl-$d_6$ sulfoxide and partially soluble in water, acetone, ethanol, and ether. It is a yellow solid with a decomposition point of 188 C. Elemental analysis showed the compound to be composed of C, H, and O, with no detectable N. The absence of unsubstituted carboxyl or phenolic functional groups were inferred from the lack of reaction of the toxin with aniline-glucose and ferric chloride spray reagents (14), respectively.

The toxin was subjected to hydrolysis in a sealed tube under N$_2$ in trifluoracetic acid (1) for 2 hr at 121 C. The hydrolyzate contained a mixture of two yellow and two brown compounds which were separated by paper chromatography (ethylacetate-pyridine-H$_2$O: 8:2:1, v/v). The hydrolyzate also contained a noncolored compound which gave a positive reaction with silver nitrate reagent (12). The compound was identified as glucose by paper co-chromatography with labeled glucose and by gas-liquid partition chromatography of the trimethylsilyl derivative. The conditions of acid hydrolysis did not result in complete removal of glucose from the toxin. The results from acid hydrolysis suggest that the compound is a glucoside.

Approximately 10 $\mu$g of the toxin were dissolved in 0.1 ml of 0.5 m tris-HCl buffer, pH 7.1. To the yellow solution we added 20 $\mu$g of NaBH$_4$ and the solution immediately turned colorless, suggesting that reduction of a chromophoric group had occurred. Further, we added 10 $\mu$g of sodium bismuthate to the solution, and within 20 sec the solution again became yellow, indicating that oxidation of the chromophoric group had taken place.

**Spectral Properties.** The toxin exhibits UV absorption maxima at 218 nm and 268 nm (Fig. 3) in addition to a weak absorption maximum at 427.5 nm. A bathochromic shift was observed at a basic pH. These results indicate an aromatic structure for the aglycone portion of the glucoside. A linear relation was established between toxin concentrations and absorbance both at 268 nm and 218 nm (Fig. 4). The $A_{380}/A_{268}$ ratio of the pure toxin was 2.405 and could be used as a criterion of purity. The molar extinction coefficients for a 1-cm light path were: $A_{218} = 19,200$ and $A_{268} = 7,330$.

The IR spectrum of the toxin is shown in Figure 5. The strong absorption at 2.8 $\mu$m and broad peak at 9.4 $\mu$m are due to stretch vibrations of hydroxy groups in the sugar moiety of the toxin. The peak at 6.15 $\mu$m is typical of a conjugated carbonyl functional group (7). The aromatic nature of the aglycone is further confirmed by absorption bands in the longer wavelengths (10–14 $\mu$m).

The mol wt of the compound was estimated by vapor pressure osmometry to be 350 $\pm$ 20. This estimation was helpful in deciding which peaks in the mass spectrum were related to the toxin. A series of temperature-dependent peaks appeared around 370 mass units and below, with several peaks at much higher mol wt appearing only at higher probe temperatures. On this basis, the peak at m/e 370 was assigned at the mol ion (Fig. 6). A peak at 369 was attributed to the loss of 1 hydrogen atom. The peak at 355 could result from the loss of CH$_3$ from the parent compound. The intense peak at 342 can be accounted for by a loss of CO from the parent molecule. The base peak at 207 most likely represents the aglycone fragment carrying with it the glycosidic oxygen. Assuming a mol wt of 370 and the elemental analysis data below, the empirical formula of the compound is

$$C_{12}H_{15}O_3$$

Calculated: C 55.1 H 5.9 O 39.0

Found: C 52.4 H 5.0 O 40.3

Because of its high solubility in dimethylysulfoxide the NMR

---

5 Abbreviation: NMR: nuclear magnetic resonance.
spectrum of the toxin (Fig. 7) and all other reference compounds were obtained in the deuterated form of this solvent. At the outset, interpretation of the NMR spectra was complicated by: (a) difficulty in completely removing H2O from the sample, (b) instability of the toxin in dimethyl sulfoxide and, (c) the slow exchange of a portion of the aromatic protons. Chemical shifts were calculated by assuming that the chemical shift of the methyl protons of dimethyl sulfoxide was 2.62 ppm relative to trimethylsilane. Integration of peaks was carried out by assuming that the peak at 4.02 ppm was due to 6 non-exchangeable protons on the sugar ring. This assignment was supported in part by the chemical shift of the protons on the glucose portion of salicin occurring at 3.5 ppm. The singlet at 1.39 ppm was due to 6 equivalent protons on saturated carbons and was assigned to 2 symmetric methyl functional groups in the aglycone portion of the molecule. The peak at 4.45 ppm integrating for 1 proton had the identical chemical shift for the anomeric proton of methyl α-D-glucopyranoside. Further, it had a coupling constant of two cycle which is also in agreement with an α-linkage assignment according to Van der Veen (13). The three protons of the broad peaks centered around 7 ppm were due to an unsaturated region of the molecule. Presumably these protons are the aromatic protons of the toxin since the aromatic protons of salicin as well as p-coumaric acid also have chemical shifts in this portion of the spectrum. The broad peak at 5.2 ppm has the correct chemical shift of a proton adjacent to O2 functionalities. However, since it integrates for more than 1 proton, the peak may represent a breakdown product of the toxin or a toxin dimethylsulfoxide complex. The peak centering at 4.8 ppm is assigned to DHO.

Based on the available evidence the proposed structure of the toxin is 3-hydroxy-2,2-dimethyl-5-α-D-glucopyranoside-2,3-dihydrochormone, as shown in Figure 8, and the compound has been given the trivial name stemphylin.

**Biological Activity Studies.** The application of the pure toxin on lettuce leaves was followed by the development of a limited sunken brown lesion within 14 hr. The symptoms caused by the toxin were identical to those produced by the fungus (Fig. 9). A linear correlation was detected between toxin concentration and lesion area in the concentration ranges of 0.5 to 2.5 μg (Fig. 10). Visible symptoms could be observed at toxin concentrations as low as 6 × 10⁻⁶ mol.

Host range studies were performed by inoculation of leaves with 10 μg of pure toxin. In addition to lettuce (*Lactuca sativa* L.), other plants such as *Vicia sativa* L., *Medicago sativa* L., *Nicotiana tabacum* L., *Lycopersicon esculentum* Mill., and *Triticum vulgare* Vill. were also affected by the toxin. Brown necrotic spots developed on all these plants during 24 hr. However, the tomato leaves appeared to be highly resistant to the toxin and only traces of a detrimental effect were detected.

**DISCUSSION**

The structure proposed for the toxin of *S. botryosum* discussed in this report is in agreement with the best available
group. The fact that two methyl groups are present and attached to the nonaromatic portion of the aglycone moiety is witnessed in the NMR spectrum (Fig. 7). The methyl protons of two methyl groups bonded to a nonaromatic nonprotonated carbon commonly resonate as one peak near 1–2 ppm (3). That a carbonyl functional group is present in the molecule is witnessed by the parent peak –28, which is common in the mass spectrum for compounds having this functionality (4). Further, a strong carbonyl absorption band at 6.15 μm is characteristic for the chromone carbonyl absorption (7). It is likely that if the aglycone were a coumarin or quinone derivative, this band would appear at lower or higher wavelengths, respectively (7).

An OH group is assigned to carbon 3. NMR data are supportive of a proton with the expected chemical shift being on this position. Because of its proximity to the carbonyl group, the OH would tend to lend some acidity to the molecule accounting for its ability to adhere to an anion exchange resin and for the parent peak –1 in the mass spectrum. Further, this group could tend to tautomerize with the C-4 carbonyl making the molecule behave somewhat like a quinone. This would effectively lend coloration to the compound and explain why it becomes colorless upon reduction, possesses a pronounced bathochromic shift in the UV common for hydroxylated chromones (10) and is easily reoxidized back to a colored compound.

The assignment of the glucoside linkage to position 5 of the chromone nucleus is a matter of convention since the few known chromone derivatives are commonly oxygenated at position 5 and occasionally at both positions 5 and 7 (8). Substitution at position 8 would invariably result in an increase in the absorption maximum (8). Substitutions at position 6 are uncommon.

From our previous (2) and present reports it appears that S. botryosum produces several phytotoxic compounds capable of producing symptoms normally associated with the lettuce leaf spot disease. Stemphylin, the subject of this report, is present in the anionic fraction and forms brown necrotic zonate spots (Fig. 9) as opposed to the scorching and subsequent death of the entire leaf caused by the isolated neutral toxin (2). Both symptoms were described under natural conditions of infection by S. botryosum (6). Anionic and neutral fractions obtained by the procedure outline in this report from stemphylin-infected lettuce leaves produced identical symptoms with those of the stemphylin and the neutral toxin, respectively (Barash and Strobel, unpublished results). The identity of the toxins in the infected tissue remain to be examined before their role in symptom development can be established.

Acknowledgments—We wish to thank Mr. Ken Lipkowitz and Dr. Arnold Craig of Montana State University for their help in gathering mass spectral data and in reviewing the manuscript. The help of Dr. Lou Friedelman of the Brookhaven National Laboratory is also gratefully appreciated.

LITERATURE CITED


