Preparation of 4,15-Diacetoxyscirpenol from Cultures of Fusarium sambucinum NRRL 13495†

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Filtrates of Fusarium sambucinum NRRL 13495 grown in a stagnant culture for 9 days contained up to 458 ± 60 (mean ± standard error; n = 3) µg of 4,15-diacetoxyscirpenol per liter depending on culture conditions. Extraction with ethyl acetate, chromatography on a column of silica gel, and crystallization from mixtures of ethyl acetate and hexane provided pure material in 96% yield.

Diacetoxyscirpenol (DAS) (4,15-diacetox-3-hydroxy-12,13-epoxy-trichothece-9-ene), a mycotoxin produced by several Fusarium species, was first isolated by Brian et al. (4), and its structure was determined subsequently (6, 18). Animal productivity problems thought to be related to DAS include poor growth of chickens (10), decreased egg production by hens (2), feed refusal by chickens (5), hemorrhagic bowel syndrome in swine (H. J. Kurtz, C. J. Mirocha, and R. Meade, Abstr. Proc. 4th Int. Congr. Int. Pig Vet. Soc., Abstr. No. 4, 1976), “bean hulls poisoning” in horses (20), and several poorly defined mixed toxicoses (13, 14).

Evaluation of the toxicity of DAS and its role in animal and human health has been hindered by the limited availability and the expense of the necessary amounts of pure DAS. The reported yields of DAS from several Fusarium species have varied from trace amounts to 290 µg/liter of liquid medium (Table 1). The culture conditions used for the production of DAS have varied markedly in substrate, temperature, aeration, and time of incubation (15, 18–20). Procedures for extraction and purification have been lengthy and elaborate (4, 8, 15). The present report describes a comparatively simple procedure for the consistent production of pure DAS from cultures of Fusarium sambucinum NRRL 13495. (This isolate, mislabeled as F. roseum NRRL 1181 in an earlier report [5], was identified in April 1986 as F. sambucinum by P. E. Nelson, Fusarium Research Center, Pennsylvania State University, University Park, and accessed as NRRL 13495 by the Northern Regional Research Center, Peoria, Ill.).

The fungus was maintained at 10°C as a soil culture. A portion was sprinkled on plates of potato dextrose agar (Difco Laboratories, Detroit, Mich.) that were then incubated at 25°C for 5 days to provide inocula for the experiments. Small blocks of agar (50 mm²) with adherent mycelia were used to inoculate Czapek Dox broth (Difco) supplemented with 2% Bacto-Peptone (Difco) in 2,800-ml Fernbach flasks containing 1,000 ml of medium or in 250-ml Erlenmeyer flasks containing 40 ml of medium. The flasks were stoppered with cotton. The Fernbach flasks were incubated at 29°C for 14 days, and the Erlenmeyer flasks were incubated at 29°C for 9 days, both without shaking. F. sambucinum reportedly (21) produces more DAS at 15 than at 25°C, but our use of 29°C was dictated by the availability of a 29°C rather than a 15°C incubator. In another series of experiments, the initial medium was removed aseptically after 3 and 5 days of incubation in Erlenmeyer and Fernbach flasks, respectively, and replaced with sterile Czapek Dox medium from which NaNO₃ had been omitted. The replacement cultures were incubated for an additional 6 and 9 days for Erlenmeyer and Fernbach flasks, respectively. Replacement cultures low in nitrogen reportedly have enhanced concentrations of secondary metabolites such as mycotoxins (7, 12). A total of 3 Fernbach flasks and 75 Erlenmeyer flasks were incubated statically in each experiment, and each experiment was repeated twice.

After incubation, the cultures were filtered through cheesecloth, and NaCl was added to the filtrates to provide a 5% concentration before the filtrates were extracted twice with equal volumes of ethyl acetate. The extracts from 1 liter of filtrate were combined, evaporated, redissolved in a minimal amount of warm ethyl acetate, and transferred to a small column (inside diameter, 20 mm) of silica gel (10 g of Kieselgel 60; 70/230 mesh; Merck & Co., Inc., Rahway, N.J.). The column contained the silica gel between upper and lower layers (1 cm) of sodium sulfate. Each column segment was slurry packed with chloroform. Before the extract was added, the column was rinsed with 30 ml of hexane. The less-polar fraction of the extract was eluted from the column with 100 ml of hexane-ethyl acetate (7:3) and discarded before DAS was eluted with 100 ml of hexane-ethyl acetate (4:6). The column fraction containing DAS was evaporated. The DAS in the residue was crystallized from warm ethyl acetate by using a hexane cloud point, filtered, and rinsed with hexane. After recrystallizing three times, the crystals were dried overnight at 30°C in a vacuum and weighed, and the yield was calculated.

During purification, the DAS in the fractions was monitored on thin-layer chromatograms (Silica Gel G Redi-Plate; Fisher Scientific Co., Raleigh, N.C.) that were developed in benzene-acetone (3:2 [vol/vol]). The plates were sprayed with p-anisaldehyde (17) or with 25% H₂SO₄ in methanol (1) before being heated at 110°C for 10 min. DAS was quantitated by gas chromatography with a 2-m column (Alltech Associates, Inc., Applied Science Div., State College, Pa.) of 3% OV-17 on 100/120 mesh Gas Chrom Q and a flame ionization detector. Before subsamples of the extract were injected, they were reacted with Tri Sil TBT (Pierce Chemical Co., Rockford, Ill.) for 30 min at 25°C to form the trimethylsilyl derivative. Mass spectra of the derivative were obtained by gas chromatography-mass spectrometry (HP

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TABLE 1. *Fusarium* species producing DAS

<table>
<thead>
<tr>
<th>Speciesa</th>
<th>Yield (mg/liter)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. equiseti</em></td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td><em>F. sambucinum</em></td>
<td>32</td>
<td>11</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td><em>F. graminearum</em></td>
<td>NRb</td>
<td>20</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>NR</td>
<td>9</td>
</tr>
<tr>
<td><em>F. semitectum</em></td>
<td>NR</td>
<td>15</td>
</tr>
</tbody>
</table>

*a* Species are listed as identified by Marasas et al. (13) and not as given in the original references.

*b* NR, Not reported.

5985 mass spectrometer and data system; Hewlett-Packard Co., Palo Alto, Calif.) at 70 eV. The ion detector was at 275°C, the detector was at 200°C, and the column temperature was programmed from 120 to 250°C at 10°C/min with an initial hold of 5 min and a final hold of 25 min. The carrier gas was He, and the flow rate was 1 ml/min. Samples were injected in the splitless mode on a 20-m fused silica column (SE 54; Hewlett-Packard). The mass range scanned was 40 to 600 atomic mass units. Positive chemical ionization with methane was used. Proton nuclear magnetic resonance spectra of DAS were obtained after it had been dissolved in deuteriochloroform containing tetramethyldilane as the internal reference (Aldrich Chemical Co., Inc., Milwaukee, Wis.). The nuclear magnetic resonance equipment (model QE-300; Nicolet, Fremont, Calif.) was operated at 300 MHz with a line broadening of ~0.2 Hz. Melting points were obtained with a Fisher-Johns apparatus (Fisher Scientific).

Fernbach flask cultures contained 366 ± 59 mg of DAS per liter of culture filtrate (mean ± standard error; n = 5) of which 82% was recovered as crystals (Table 2). The fractions of replacement culture from Fernbach flask cultures contained only 77 ± 8 mg of DAS per liter. This finding of less rather than more DAS in replacement cultures differs from previous work with other mycotoxins (7, 12). Replacement culture filtrates from Erlenmeyer flask, on the other hand, contained 458 ± 60 mg of DAS per liter, of which 96% was obtained as crystals. This was an improvement over filtrates from ordinary cultures in Erlenmeyer flasks, which contained 165 ± 6 mg of DAS per liter. Extracts of the replacement cultures contained fewer impurities than did extracts from ordinary cultures.

DAS recovered from the cultures had a melting point of 163.5 to 164.0°C (uncorrected) compared with 162 to 164°C (18) and 161 to 162°C (4) reported previously. The recovered DAS showed a single component on thin-layer and gas chromatography. Mass spectra of the trimethylsilyl derivative of the recovered DAS showed a molecular ion of 438, and other prominent peaks were at 439, 423, 379, 319, 289, and 229. Nuclear magnetic resonance spectra revealed resonances at 6.81 (3H, s, 14-H), 1.72 (3H, s, 16-H), 2.05 (3H, s, 15-acetyl), 2.14 (3H, s, 4-acetyl), 2.79 and 3.06 (1H each, AB, J = 4.0 Hz, 13-H), 3.68 (1H, d, J = 4.9 Hz, 2-H), 4.18 (1H, dd, J = 5.0 and 3.1 Hz, 3-H), and 5.18 (1H, d, J = 3.0, 4-H), which are in agreement with prior reports for DAS (3, 11, 98).

The present method yielded over 50% more crystalline DAS than did the method previously reported for isolation from *F. sambucinum* grown in a fermentor (18). Aside from different growth conditions, the present method also differs in details of extraction, purification, and crystallization. Steyn et al. (19) reported a yield of 604 mg/kg of corn after culturing with *F. sambucinum*, but in our experience, such cultures are more variable, and extracts of moldy grain present formidable problems during purification. We found that the present method of using liquid cultures afforded considerable savings in time, supplies, and equipment, in addition to the high yield.

**LITERATURE CITED**


