Antibiotic Y: Biosynthesis by *Fusarium avenaceum* (Corda *ex* Fries) Sacc., Isolation, and Some Physicochemical and Biological Properties

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A compound very similar to the mycotoxin citrinin was observed on thin-layer chromatographic plates during the screening analysis of grain extracts. This compound was produced by 22 of the tested *Fusarium avenaceum* (Corda *ex* Fries) Sacc. strains isolated from wheat, triticale, barley, corn, and potatoes. A chemical test confirmed the presence of an unknown compound, which was given the preliminary name of antibiotic Y (indicating yellow fluorescence). The following properties of the new metabolite are described: spectroscopic (UV, infrared, proton nuclear magnetic resonance, fluorescence, and mass spectrometry), phytotoxic, antibiotic (inhibitory effect of bacterial growth), and toxic (toxicity to *Artemia salina*, chicken embryos, and mouse fibroblasts). Elemental analysis of the compound showed that it had the general formula C_{12}H_{16}O_{6}. In agreement with the mass spectrometric finding that the molecular ion had a molecular weight of 318. The structure of the compound is presently under study.

*Fusarium avenaceum* (Corda *ex* Fries) Sacc. has been recognized for many years as a pathogen of several plants (e.g., cereals, legumes, and potatoes), causing serious losses every year (3). However, secondary metabolites of *F. avenaceum* are not as well known as are metabolites of *F. graminearum* or *F. culmorum*. Cook et al. (6) discovered the antibiotic avenacin in *F. avenaceum* cultures.

The fungus has been recognized in some papers as a zearalenone producer (12). Recently it was found to be a producer of moniliformin (4, 11). A compound with properties similar to those of citrinin has been detected in cultures of *F. avenaceum* (5). In this paper we report the biosynthesis, isolation, purification, and some properties of this compound.

**MATERIALS AND METHODS**

Fungal strains. *F. avenaceum* (Corda *ex* Fries) Sacc. strains were isolated from wheat, triticale, barley, corn, and potatoes. Taxonomy of the strains was performed by the method of Booth (3).

Biosynthesis of antibiotic Y. *F. avenaceum* KF 58, isolated from wheat stems, was used to produce the compound by a 4-week incubation on 200-g portions of sterilized whole corn kernels in 1,000-ml Erlenmeyer flasks at 20°C and 40% water content.

(The biosynthesis, isolation, and purification of antibiotic Y are proprietary under Polish patent P251215, January 1985.)

Isolation of antibiotic Y. The compound was extracted from 1 kg of air-dried ground culture on corn kernels by using seven 1,000-ml portions of a mixture containing chloroform and 0.2 M phosphoric acid (5:1, vol/vol, pH 2). Combined chloroform extracts were evaporated to 500 ml.

Purification of antibiotic Y. (i) After isolation, antibiotic Y was extracted with seven 500-ml portions of a saturated solution of sodium bicarbonate. (ii) The combined aqueous layers were acidified with 20% HCl to pH 2 and then reextracted with four 300-ml portions of chloroform. (iii) Combined chloroform layers were evaporated to 500 ml, steps (i) and (ii) were repeated, and the final chloroform layer was dried over MgSO₄, filtered, and then evaporated to dryness. About 1 g of hardly soluble crude material, mainly consisting of antibiotic Y, was obtained. (iv) Crude antibiotic Y was recrystallized from 500 ml of boiling mixture of CH₃COOH and CH₃OH (1:3, vol/vol), and about 400 mg of antibiotic Y (melting point, 282°C) was obtained. An identical product was obtained when crude antibiotic Y was crystallized from 750 ml of boiling acetone.

Identification of antibiotic Y. Antibiotic Y was identified by thin-layer chromatography in the same way as the analysis and chemical confirmation of citrinin (7, 8). Aluminum sheets (silica gel 60, no. 5553; E. Merck AG) impregnated with 0.6 N H₂SO₄ were used; toluene-ethyl acetate-formic acid (6:3:1, vol/vol/vol) was used as a developing solvent.

Spectroscopic analysis of antibiotic Y. UV spectra were recorded on a Specord UV/VIS spectrophotometer (Carl Zeiss, Jena, German Democratic Republic). Infrared spectra were recorded on a Specord 75 IR spectrometer. Proton nuclear magnetic resonance spectra were determined on a Jeol F × 90 Q spectrometer (Jeol, Ltd., Tokyo, Japan). Mass spectra were recorded on a Jeol-JMS-D-100 spectrometer.

Fluorescence spectra were recorded on a Hitachi MPF 4 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) with a Hitachi QD15 recorder and with excitation and emission wavelengths (λₑₓ and λₑₘ) of 336 and 520 nm in emission and excitation spectra, respectively. Elemental analysis was...
performed on a Perkin Elmer 240 Elemental Analyzer (Perkin-Elmer, Ltd., Bucks., England, Switzerland).

Phytotoxicity tests. The effect of antibiotic Y on wheat and pea seedling growth was examined. Seedlings were cultivated on filter paper saturated with a 100-μg/ml solution of antibiotic Y in water. Tetraethylammonium hydroxide (25 μl) was added to 10 mg of crystalline antibiotic Y for better solubility of the compound. After 30 min, water or organic solvent was added to a final antibiotic Y concentration of 1 mg/ml.

Antibiotic properties of antibiotic Y. (i) Disk method. The effect of two levels, 3 and 30 μg, of antibiotic Y per 10-mm disk of Whatman no. 1 filter paper on bacterial growth was examined by the method of Kedzia et al. (10). Nutrient agar (1.2%; Difco Laboratories) on petri dishes was inoculated with 0.1 ml of a 17-h-old suspension containing 10^6 bacterial cells. The following strains of pathogenic bacteria isolated from plant tissues were used: Bacillus subtilis, Erwinia carotovora, and Agrobacterium tumefaciens. After 20 h of incubation at 26°C, the inhibition zones of bacterial growth were measured. Results were compared with the inhibitory effect of the same levels of streptomycin.

(ii) Serial dilution method. The MIC of antibiotic Y inhibiting the growth of Staphylococcus aureus 209P was investigated by the serial dilution method described by Kedzia et al. (10). Studies were performed at the routinely used pH 7.4 and additionally at pH 6.2, 6.4, 6.6, 6.8, 7.0, and 7.2. In all samples, solutions of antibiotic Y in phosphate buffers with the pHs mentioned above were prepared. The aim was to examine antibiotic properties at low pH values, taking into consideration the acidic character of antibiotic Y.

Toxicity toward brine shrimp (Artemia salina) larvae. The toxicity of the antibiotic Y solution was tested with A. salina eggs; the method described by Harwig and Scott (9) was used. To avoid precipitation of the compound, our own modification was introduced: instead of seawater, we used solution adjusted to pH 6.5, 7.1, and 7.4 in distilled water containing 3.0% NaCl, 0.08% KCl, and 0.6% glycine.

Chicken embryo bioassay. Evaluation of the toxicity of the antibiotic Y solution toward chicken embryos was performed by the Association of Official Analytical Chemists methods 26.073 through 26.078 (2), with fertile eggs of female White Rock and male Cornish chickens supplied by the Poultry Hatch Station, Tulce, Poland.

Toxicity toward mouse fibroblasts. Evaluation of the toxicity of antibiotic Y toward mouse fibroblasts was performed by the method described by Abbas et al. (1), with a 2-μg/ml solution of antibiotic Y in dimethyl sulfoxide and 3T3 Swiss mouse fibroblasts.

Chemicals. A 25% solution of pure tetraethylammonium pure hydroxide in methanol was supplied by Fluka AG, Buchs SG, Switzerland. Other chemicals and solvents were of analytical grade and were purchased from Państwowe Przedsiębiorstwo Obrót Chemikaliami, Gliwice, Poland.

RESULTS AND DISCUSSION

The highest yield of pure crystalline antibiotic Y (400 mg/kg of kernels) was obtained when F.avenaceumKF58 was incubated for 4 weeks on sterilized corn kernels. Both solvents used for recrystallization gave identical products; crystals of antibiotic Y with a melting point of 282°C. Thin-layer chromatography of the compound on unimpregnated plates (8) showed that it had an identical R_f value (0.41) and fluorescence to those of citrinin. It was surprising in that a Fusarium sp. could apparently produce a toxin characteristic of Penicillium and Aspergillus spp. However, when plates impregnated with 0.6 N H_2SO_4 (8) were used, different R_f values (0.38 and 0.52 for antibiotic Y and citrinin, respectively) were found, and chemical confirmatory tests (7) definitely distinguished the compounds.

Elemental analysis gave the following results: C, 56.25 and 56.23%; H, 3.18 and 3.19%; N, 0.0 and 0.0%, leading to the molecular formula C_{91} H_{115} O_{13} (calculated values are C, 56.61%; H, 3.17%), corresponding to a molecular ion of molecular weight 318 by field desorption mass spectroscopy.

The melting point (281 to 282°C) is fairly high and may indicate an acidic character due to free carboxylic group(s). The solubility of the compound decreased with every step of purification. The compound was relatively easily extracted from fungal culture with acidified chloroform, whereas pure recrystallized antibiotic Y was hardly soluble in such solvents as CHCl_3, CHCl_3/CH_3COOH, CH_3OH/CH_3COOH, acetone, dimethyl sulfoxide, ethanol, water, or saturated water solutions of Na_2CO_3 or NaHCO_3. Addition of tetraethylammonium hydroxide to crystalline antibiotic Y significantly increased the solubility. Aqueous solutions for all biological tests were prepared in this way (see phytotoxicity tests in Materials and Methods).

The following properties were observed and measured. (i) Crystals of antibiotic Y were light yellow-cream, and the solutions were yellow; they were very similar to citrinin solutions; (ii) UV characteristics (λ_max [λg]): CH_3OH: 242 nm (4.23), 265 nm (4.13), 278 nm (4.09), 347 nm (3.98), and 365 nm (4.02); (iii) fluorescence spectra in ethanol: λ_max(em) = 520 nm for λ_max of 336 nm and λ_max(em) = 277 nm for λ_max of 520 nm; (iv) infrared (KBr cm^-1): 3,400 (O-H); 3,100 to 2,700 (COO-H); 1,745 (C = O, lactone, and COOCH_3); 1,655 (COOH); and 1,610 (C = C); (v) proton nuclear magnetic resonance (dimethyl sulfoxide-tetramethylsilane): 2.47 (s, 3H), 4.01 (s, 3H), 6.25 (s, 1H), 7.25 (s, 1H), 7.45 (s, 1H), 10.49 (s, 1H).

Antibiotic Y showed no phytotoxic effect toward wheat and pea seedling growth up to a concentration of 100 μg/ml. In comparison, vomitoxin at 5 μg/ml inhibited the growth of seedlings.

Inhibitory effects of the compound on bacterial growth are presented in Tables 1 and 2 for the disk and serial dilution methods, respectively. The MIC of antibiotic Y depends very much on the pH and seems to be directly proportional to the acidic character of the compound. The inhibitory effect of the compound on bacterial growth increased more than 10-fold when the pH was lowered from 7.4 to 6.2. The MIC at pH 6.2 (0.37 μg/ml) indicates that the compound has very active antibiotic properties, even higher than those of streptomycin for S. aureus 209 P (MIC at pH 6.2, 18.8 μg/ml).

The antibiotic properties of the compound toward phytopathogenic bacteria, particularly B. subtilis and E. carotovora, were also investigated. Agrobacterium tumefaciens was found to be only slightly sensitive to

| TABLE 1. Inhibitory effect of antibiotic Y and streptomycin on bacterial growth (disk method) |
|---------------------------------|-----------------|-----------------|
| Compound                        | Conc (μg/disk)  | Bacterial growth inhibition zone (mm) for: |
|                                 |                 | B. subtilis | E. carotovora | Agrobacterium tumefaciens |
| Antibiotic Y                    | 3               | 3            | 3              | 0                        |
| Antibiotic Y                    | 30              | 4            | 4              | 1                        |
| Streptomycin                    | 3               | 10           | 6              | 0                        |
| Streptomycin                    | 30              | 12           | 8              | 2                        |
antibiotic Y. However, those species were less sensitive to antibiotic Y than to streptomycin (Table 1).

We also investigated the function of antibiotic Y in F. avenaceum cells. The high degree of antibiotic activity of the compound present in the mycelium could possibly help the fungus to survive in the presence of antagonist organisms in soil or during the invasion of plant tissues. It is possible to observe that this yellow compound is present in large amounts in some macroconidia of atypical shape (Fig. 1), and irregularly in the mycelia of strains producing high levels of antibiotic Y. This is very characteristic for these strains, and so the presence of antibiotic Y can support and confirm the taxonomy of the strain.

Investigation of the toxic effect of antibiotic Y toward A. salina gave ambiguous results. The main limitation was the solubility of the compound. When an incompletely purified extract of antibiotic Y was used, the 50% lethal dose was 40 μg/0.1 ml of larval suspension. Recrystallized antibiotic Y precipitated in combination with seawater even with the addition of tetraethylammonium hydroxide, and no toxic effect of the compound toward A. salina was observed. This difficulty was omitted by use of our own modification to the seawater preparation (see Materials and Methods). In this investigation the 50% lethal dose (expressed in micrograms per 0.1 ml of larval suspension) depended on pH and was measured at 52.0, 54.0, and 70.0 μg/0.1 ml for pH 6.5, 7.1, 7.4, respectively.

For a broad range of doses (0.5 to 200 μg of antibiotic Y per egg), the compound was found to be nontoxic in the chicken embryo bioassay. Antibiotic Y in concentrations up to 5,000 ng/ml was found to be nontoxic to 3T3 mouse cells.

Taking into consideration all the results of the biological activity of the compound, we conclude that antibiotic Y is a very interesting substance for several reasons. The very high and specific inhibitory effect on bacterial growth and the low or negligible toxicity allow the compound to be used in medical treatment. The structure of antibiotic Y and its biosynthesis pathways are currently under study.

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LITERATURE CITED