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Antibiotic Produced by *Fusarium equiseti* NRRL 5537

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Fusarium equiseti NRRL 5537 grown on an autoclaved white corn grit medium for 3 to 4 weeks at room temperature produced a substance in excess of 5 g/kg of substrate that inhibited some gram-positive bacteria including mycobacteria. Most *Bacillus subtilis*, *Mycobacterium phlei*, and *Staphylococcus aureus* strains were inhibited when 1 μg of the antibiotic per ml was incorporated into the culture medium. Except for *Neisseria perflava*, gram-negative bacteria, yeasts, and molds were not inhibited by 128 μg/ml. The antibiotic was recovered as a white powder, had a melting point of 65 to 66 C, and had an intraperitoneal mean lethal dose in white mice of 63 mg/kg of body weight. In thin-layer chromatographic analysis the compound appeared as a single spot in two different solvent systems. Mass spectrometry determined that the molecular weight of the antibiotic was 373 with a molecular formula of $C_{22}H_{31}NO_4$. Chemical microanalysis was in accord with the formula.

In an earlier survey, 136 strains of *Fusarium* sp. were incubated on a white corn grit medium to determine their capacity to produce T-2 toxin (3). One strain of *Fusarium equiseti* was detected that strongly inhibited gram-positive bacteria. Since *Fusarium* strains are frequently identified as the etiological organisms in mycotoxicoses, we characterized the antibiotic subtance and determined its mean lethal dose (LD_{50}) in mice.

Athough many antibiotics and mycotoxins are produced by strains of *Fusarium* species, none has the biological activity and the chemical properties of the antibiotic elaborated by *F. equiseti* NRRL 5537. The principal fusariotoxins, zearalenone (7), butenolide (11), fusariogenin (5), or the 12,13-epoxytrichothecenes (1), either lack or only exhibit weak antibacterial properties. Of the antibacterial metabolites produced by the genus *Fusarium*, the enniatins (6) are most nearly like the antibiotic of *F. equiseti* NRRL 5537 in their microorganism-inhibition spectrum. Both the enniatins and the product of NRRL 5537 inhibited *Mycobacterium phlei*, *Bacillus subtilis*, and *Staphylococcus aureus* in vitro at concentrations below 1 μg/ml. However, the enniatins and the 5537 antibiotic are grossly different in their chemical and physical properties. This report describes the production, purification, some chemical and physical properties, and also the antibacterial effects and toxicity of this antibiotic in mice.

MATERIALS AND METHODS

Microorganisms. *F. equiseti* NRRL 5537 was originally isolated from tall fescue (*Festuca arundinacea* Schreb.) and was identified by the scheme of Booth (2). All microorganisms were supplied by the ARS Culture Collection.

Production and purification. NRRL 5537 was cultured in Fernbach flasks containing 200 g of white corn grits moistened with 100 ml of water. A 50-ml amount of water was added before autoclaving, and the remaining 50 ml was added after the autoclaved grits were loosened with a stirring rod. Inoculum was prepared by incubating NRRL 5537 for 10 to 14 days at room temperature on 2 to 3 g of grits suspended in 5 ml of 1.5% agar. Copious amounts of conidia were produced on the corn grit medium. Fermentation flasks were inoculated with 1 ml of the loosened surface growth suspended in 8 ml of water. During incubation (3 to 4 weeks at room temperature) the medium was shaken daily. The fermented grits were extracted by blending the contents of each flask twice in a Waring blender jar for 1 to 2 min with 1 liter of acetone. The acetone solution was separated from the grits by filtration. Acetone from the combined extracts was removed under vacuum, and the residual watery milieu (about 500 ml/kg of substrate) containing the dissolved or suspended antibiotic was acidified to pH 2.0 with sulfuric acid. After acidification, the suspension was partitioned into hexane; six 200-ml volumes of hexane removed nearly all of the product. As the hexane evaporated at room temperature, a gel-like matrix formed; with complete evaporation, 16 to 25 g of a reddish solid product was obtained from 1 kg of white corn grits. The crude product was dissolved in 200 ml of boling hexane and filtered. Red

pigments recovered along with the antibiotic were removed from the hexane filtrate by washing (20 to 30 times) with small volumes (2 to 3 ml) of ethanol until pigment was no longer visible in the ethanol layer. The ethanol-pigment layer was removed from the hexane-antibiotic layer with a Pasteur pipette. After hexane evaporated, the primary recovery product was washed several times with 20- to 30-ml volumes of hexane.

The ethanol-pigment layers were combined, evaporated, and extracted with boiling hexane for additional recovery of product. The hexane was cleared of red pigment by washing 12 to 15 times with 2- to 3-ml portions of ethanol. The clear hexane layer was evaporated and the product was washed several times with hexane. Repeated ethanol-pigment washings were recovered and partitioned into boiling hexane until a gel no longer formed upon evaporation of the hexane. Exhaustive partitioning of the ethanol-pigment washes into hexane yielded a pink powder, the secondary recovery product.

Antibiotic assay procedure. A standard curve was prepared by diluting chromatographically pure *F. equiseti* antibiotic in acetone and adding 0.5 to 8 μg to 12.7-mm filter paper disks. The standard disks were placed on the surface of TGY agar (4), 6 ml in a standard petri dish, inoculated with 1.5×10^7 *B. subtilis* NRRL B-3284 spores and incubated overnight at 37 C. The mean inhibition zone diameter of three disks for each concentration of antibiotic was plotted semilogarithmically, and the mean response of each sample was calculated from the standard. The total amount of antibiotic produced per kilogram of grits was calculated frm the potency and the combined volume of acetone and blended grits. Antibiotic remaining in the grits was estimated from the potency and difference in volume of acetone plus grits and the volume of acetone recovered from the second extraction. Antibiotic content of other preparations obtained during purification of the product was calculated from dilutions of weighed samples and their potencies.

Physical and chemical analyses. Thin-layer chromatography was carried out on plates coated with Silica Gel G (0.5 mm) and activated at 110 C for 2 h. The developing solvent was either toluene-methanol (7:3, vol/vol) or acetonitrile-water-benzene (90:6:4, vol/vol/vol). Zones were detected by spraying developed plates with *p*-anisaldehyde (0.5 ml in 85 ml of methanol, 10 ml of glacial acetic acid, and 5 ml of concentrated sulfuric acid) and then heating at 110 C for 5 to 10 min (9). Either iodine vapor or concentrated sulfuric acid was also used to detect possible impurities.

Melting points (uncorrected) were determined with a Fisher-Johns apparatus. Infrared spectra were obtained on a Beckman model IR-8 spectrophotometer from films deposited on KRS-5 plates (Wilks, Inc.) or from solutions in a 1-mm sodium chloride cell. Mass spectra were obtained on a Nuclide high-resolution instrument (model 12—90G). Ultraviolet spectra were recorded from a Beckman DB-G with the absolute ethanol solutions of antibiotic at 25 μg/ml. Elemental analysis was carried out in a Perkin-Elmer CHN analyzer 240. Solubilities were measured by the procedure of Shriner et al. (10).

Copper salt of the antibiotic was prepared (8) and crystallized from methanol-water as clusters of fine green needles. The copper content was determined on a Perkin-Elmer 303 atomic absorption spectrophotometer.

Gas-liquid chromatography of *F. equiseti* 5537 preparations was carried out on a Bendix model 2500 chromatograph equipped with on-column flame ionization detectors. Retention times of the antibiotic and its degradation product were determined on glass columns (61 cm by 2 mm and 183 cm by 2 mm) packed with SE-30 (3%) on Supelcon AW-DMCS (Supelco, Inc.). Operating parameters were as follows: temperature, 180 or 190 C isotheramlly for 61-cm columns and programmed from 180 to 220 C at 5 C/min for 183-cm columns; carrier gas, nitrogen (40 ml/min); hydrogen (20 ml/min); air (400 ml/min); injection ports and detectors, 250 C. Samples were dissolved in absolute ethanol (10 mg/ml), and 2 μliters was used for analyses. Peak areas and retention times were obtained with a Hewlett/Packard model 3370 B integrator.

Animal toxicity. Chromatographically pure antibiotic was dissolved in warm propylene glycol and injected intraperitoneally into 25-g Swiss-Webster female mice. The LD_{50} was calculated by Weil's procedure (12) with the following notation: $K = 3$; $n = 6$; $r = 0, 1, 6, 6$; $d = 0.3010$; and $Da = 25$ mg/kg of body weight. Animals were observed for 4 weeks after treatment.

MICs for microorganisms. The *F. equiseti* antibiotic was diluted in ethanol, and penicillin G, streptomycin sulfate, and isonicotinic acid hydrazide (isoniazid) were diluted in water. Amounts up to 1 ml were added to TGY agar to provide the desired double dilution test concentrations. Minimal inhibitory concentrations (MICs) of the antimicrobial agents were determined by streaking a loopful of standardized suspensions of 24- to 48-h cultures of selected microorganisms onto the surface of 20 ml of the modified TGY agar in a standard petri dish. Microorganisms were cultured on TGY agar slants and diluted with TGY broth to yield a transmittance of about 90% at 600 nm in a Spectronic-20 spectrophotometer. All cultures grew on control plates containing 1 ml of ethanol per 100 ml of TGY agar. Bacteria were incubated at 37 C and the fungi were incubated at room temperature. Cultures were observed after 24 and 48 h, and the MIC was recorded as that concentration of antibiotic which completely inhibited growth.

RESULTS AND DISCUSSION

The trivial name equisetin is proposed for this antibiotic.

Physical and chemical properties. The primary recovery by hexane partition of the antibiotic from 16 to 25 g of crude product yielded 0.48

to 1.3 g of a white powder that inhibited the growth of *B. subtilis*, *M. phlei*, *S. aureus*, and *Neisseria perflava*. When chromatographed, the purified antibiotic was colorless and nonfluorescent; it appeared as a single spot on thin-layer chromatography plates developed with either acetonitrile-water-benzene (90:6:4, vol/vol/vol, R_f 0.53) or toluene-methanol (7:3, vol/vol, R_f 0.61). In light, equisetin was visible as a reddish spot after spraying with *p*-anisaldehyde reagent and heating to 110 C for 5 to 10 min. The antibiotic gave a positive ferric chloride test. Exposure of chromatographs to iodine vapor or spraying with concentrated sulfuric acid revealed no additional zones.

The white amorphous powder had a melting range of 65 to 66 C. It was soluble in acetone (>660 mg/ml), ethanol (>330 mg/ml), and methanol (> 220 mg/ml), and insoluble in hexane (<0.8 mg/ml) and water (<0.3 mg/ml). The ultraviolet spectrum of equisetin in ethanol is reproduced in Fig. 1. Absorption maxima occurred at 292 (ϵ = 10,760) with minor peaks of equal intensities at 250 and 235 (ϵ = 6,880).

Elemental analysis (C, 70.95; H, 8.39; N, 3.14; O, 17.52 [by difference]) and the molecular ion peak of *m/e* = 373 correspond to a formula of $C_{22}H_{31}NO_4$. The green copper salt prepared from equisetin had a melting range of 180 to 190 C. This product yielded analytical values in accord with the copper salt of a compound having the formula $C_{22}H_{31}NO_4$. [Found on material dried at 100 C in a high vacuum: C, 64.64; H, 7.15; N, 3.50; Cu, 7.85. Cu $(C_{22}H_{30}NO_4)_2$ ½ H_2O requires: C, 64.58; H, 7.17; N, 3.42; Cu, 7.77.]

The infrared spectrum of the copper salt of equisetin in chloroform (Fig. 2A) is similar to that of the copper salt of tenuazonic acid (Fig. 2B), suggesting that the antibiotic may be an *N*-methyl tetramic acid.

Gas-liquid chromatography of equisetin showed two components, the antibiotic and its degradation product. Conversion of antibiotic to a degradation product varied with temperature and residence time in the columns. Chromatographs from short columns (61 cm) at 180 C showed that the degradation peak represented only 3% of the sample (Fig. 3A). However, at 190 C, this peak increased and accounted for 6 to 8% of the sample. Chromatographs from 183-cm columns at 220 C indicated that almost half the antibiotic was converted to the degradation product (Fig. 3B).

Estimation of equisetin production and purity of recovery products. Ranges of equisetin produced by NRRL 5537 are based on seven

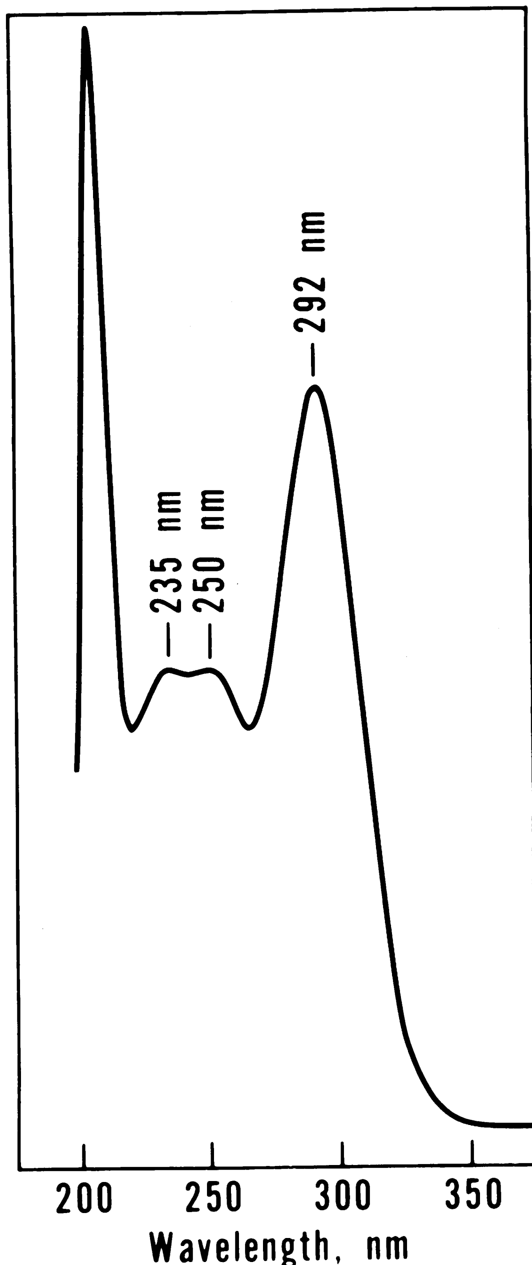

FIG. 1. *Ultraviolet spectrum of equisetin in absolute ethanol. Absorptions occur at 292, 250, and 235 nm.*

production runs. Microbial assay estimations of equisetin quantities per kilogram of fermented grits varied from a low of 4.8 to a high of 9.2 g.

Two acetone washes eluted about 90% of the equisetin from the grit medium. Six hexane extractions of the aqueous suspension remaining after evaporation of the hexane recovered

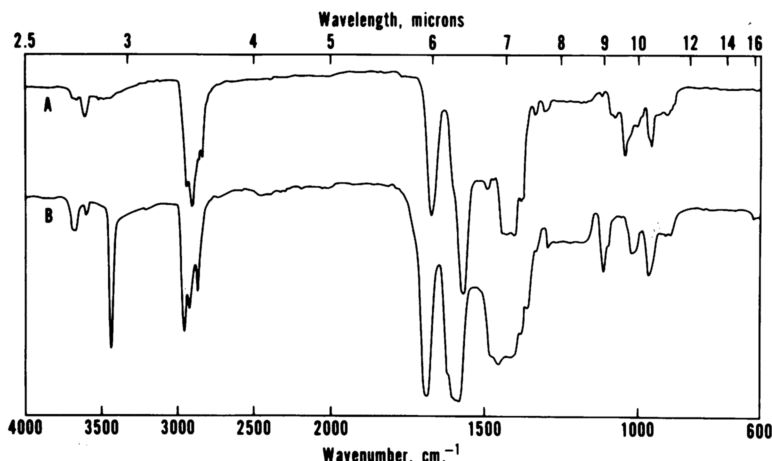

FIG. 2. *Infrared spectra of the copper salt of equisetin (A) and the copper salt of tenuazonic acid (B) in chloroform.*

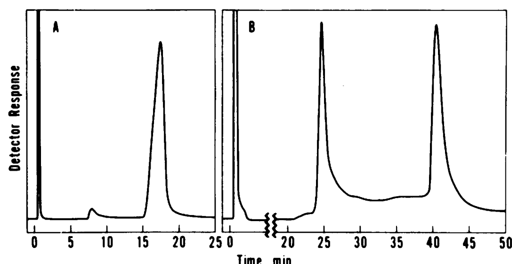

FIG. 3. *Gas chromatographs showing equisetin and its degradation product. Minor degradation (3%) occurs on a glass column (61 cm by 2 mm) packed with SE-30 and operated at 180 C (A). Almost 50% degradation occurs on a column (183 cm by 2 mm) programmed from 180 to 220 C (B).*

more than 95% of the antibiotic from the water. The 16 to 25 g of reddish solid extracted with hexane consisted of 28 to 40% equisetin, three or four red pigments, ergosterol, and other extraneous materials. In addition to the 0.48 to 1.3 g/kg of white powder that chromatographed as a single spot, amounts of a pink powder varying from 2.1 to 5.5 g/kg were recovered by exhaustive partitioning of the ethanol-pigment washes into hexane. The pink powder gave a major spot at the same R_f as equisetin and a faint red spot just above equisetin when 1 mg or more of sample was spotted. Chemical and physical properties and the antibiotic activity of the pink powder was the same as the white powder. If one ignores the slight impurity in the pink powder, recovered equisetin varied from about 2.5 to 6.2 g/kg of fermented grits.

Animal toxicity. The intraperitoneal LD_{50} dose of equisetin by Weil's procedure (12) was 63.0 mg/kg of body weight with a 95% confidence interval of 50.1 to 79.3 mg/kg. Lethal action was slow. Although mice receiving 100 or 200 mg/kg dosages were lethargic, death did not occur before the second day after treatment and deaths continued to occur during 4 days postinjection. Mice surviving 25 or 50 mg/kg doses demonstrated no visible ill effects during 4 weeks of observation. In view of the large LD_{50} dosage in mice, it is unlikely that equisetin represents a mycotoxin problem.

Microbial inhibition spectrum. Seven strains each of *B. subtilis* and *S. aureus* and two of three *M. phlei* strains were inhibited by 1 μg/ml concentrations of equisetin (Table 1). *N. perflava*, five strains, did not grow on the TGY agar containing from 1 to 4 μg of equisetin per ml. Other than *N. perflava*, none of the tested gram-negative bacteria or fungi was inhibited by equisetin in concentrations up to 128 μg/ml. Gram-negative bacteria and fungi able to grow on TGY agar containing 128 μg of equisetin per ml were *Escherichia coli* NRRL B-210, *Pseudomonas denitrificans* NRRL B-775, *Spirillum serpens* NRRL B-2052, *Vibrio tyrogenus* NRRL B-1033, *Xanthomonas campestris* NRRL B-1459, *Candida albicans* NRRL Y-477, *Rhodotorula rubra* NRRL Y-7222, *Mucor ramannianus* NRRL 1839, and *Penicillum digitatum* NRRL 1202.

A comparison of the MIC of equisetin with penicillin, streptomycin, and isoniazid for some gram-positive bacteria and *N. perflava* is given in Table 1. A lower concentration of equisetin was required for the in vitro inhibition of the tested bacteria than was required for inhibition by streptomycin or isoniazid. When compared

TABLE 1. *Minimal inhibitory concentrations of equisetin, streptomycin, penicillin, and isoniazid against susceptible bacteria*

NRRL no.	Bacterium	Antimicrobial agent MIC (μg/ml)			
		Equisetin	Streptomycin	Penicillin	Isoniazid
B-609	*Mycobacterium phlei*	1.0	2.0	16.0	4.0
B-610	*M. phlei*	1.0	2.0	16.0	4.0
B-4051	*M. phlei*	2.0	2.0	16.0	4.0
B-612	*M. smegmatis*	8.0	2.0	>128	4.0
B-2141	*M. rhodochrus*	0.5	2.0	4.0	64.0
B-543	*Bacillus subtilis*	0.5	4.0	0.008	>128[a]
B-558	*B. subtilis*	0.5	8.0	0.008	
B-644	*B. subtilis*	0.5	2.0	0.064	
B-765	*B. subtilis*	0.5	2.0	0.016	
B-972	*B. subtilis*	0.5	8.0	>128	
B-1650	*B. subtilis*	1.0	8.0	0.008	
B-3284	*B. subtilis*	1.0	4.0	0.004	
B-120	*Staphylococcus aureus*	1.0	2.0	0.032	
B-124	*S. aureus*	1.0	4.0	0.032	
B-313	*S. aureus*	0.5	2.0	0.032	
B-678	*S. aureus*	1.0	8.0	0.064	
B-1317	*S. aureus*	1.0	4.0	0.5	
B-1318	*S. aureus*	1.0	8.0	1.0	
B-2746	*S. aureus*	1.0	2.0	1.0	
B-1458	*Neisseria perflava*	2.0	2.0	0.5	
B-1788	*N. perflava*	2.0	2.0	0.25	
B-1789	*N. perflava*	4.0	2.0	2.0	
B-1790	*N. perflava*	1.0	2.0	0.25	
B-1791	*N. perflava*	4.0	4.0	0.125	

[a] *B. subtilis, S. aureus*, and *N. perflava* strains were not inhibited by 128 μg of isoniazid per ml of medium.

TABLE 2. *Tuberculostatic activity of equisetin compared with streptomycin and isoniazid*[a]

Culture	MIC (14-day readings) (μg/ml)		
	Strepto-mycin	Isoniazid	Equisetin
H37Rv (s)	1.6	0.08	25
H37Rv-SMR	>1,000	0.31	12.5
H37Rv-1NH-R	0.8	12.5	25
Erdman-TMC-107	0.8	0.16	25
PR-841	0.8	0.16	12.5
PR-1233	1.6	0.08	25
Jones, Coles	>1,000	8.0	12.5
PR-1359	12.5	16	25

[a] Tests performed by Gladys L. Hobby at the Veterans Administration Special Research Laboratory, East Orange, N. J., in a Tween-albumin liquid medium.

to penicillin, however, equisetin was far less effective against *B. subtilis, S. aureus*, or *N. perflava* strains.

In vitro MIC for eight virulent strains of *M. tuberculosis* was 12.5 to 25 μg/ml. A comparison with streptomycin sulfate and isoniazid indicated that equisetin was considerably less effective than the other two agents (Table 2) (tests made by Gladys L. Hobby at the Veterans Administration Special Research Laboratory, East Orange, N. J., in a Tween-albumin liquid medium.)

The in vitro activity of the antibiotic against gram-positive bacteria and *N. perflava*, the relative ease of production and purification, and its moderate toxicity in mice indicate that further investigations of the antimicrobial properties of this compound may be justified.

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