A9145, A NEW ADENINE-CONTAINING ANTIFUNGAL ANTIBIOTIC

I. DISCOVERY AND ISOLATION

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A9145 is a new water-soluble antifungal antibiotic produced by a strain of *Streptomyces griseolus* (NRRL 3739). This basic antibiotic has an apparent molecular weight of about 510, contains adenine and a possible sugar moiety, and forms crystalline organic and inorganic salts. A9145 is active against *Candida* species, *Saccharomyces pastorianus*, plant disease fungi, and *Trypanosoma* species. It has an LD_{50} (subcutaneous) of 185 mg/kg in mice.

In the search for new antibiotics a new strain of *Streptomyces griseolus* (NRRL 3739) was found to produce an unusual antifungal activity. This organism was isolated from a soil sample collected in the Ivory Coast region of Africa. In preliminary screening the fermentation broth inhibited the growth of *Candida tropicalis* and *Saccharomyces pastorianus*, but was inactive against *Neurospora crassa*, *Fusarium moniliforme*, and *Trichophyton mentagrophytes*.

The fermentation, isolation, physical-chemical properties, and biological properties of this antifungal antibiotic, designated A9145, are presented in this paper.

Fermentation

Stock cultures of *Streptomyces griseolus* (WAKSMAN and HENRICI) (NRRL 3739) were maintained as lyophilized pellets and then transferred for growth on BENNETT's modified agar medium[1] for 5~7 days at 30°C.

Vegetative inoculum was grown in a medium of the following composition: glucose, 15 g; soybean grits, 15 g; corn steep solids, 5 g; $CaCO_3$. 2 g; NaCl, 5 g; tap water, 1,000 ml. Inoculated medium, 100 ml/500 ml Erlenmeyer flask, was incubated 48 hours at 30°C on a reciprocal shaker at 108 two-inch strokes per minute. The resulting culture was used to provide a 5 % inoculum for the fermentation flasks. The fermentation medium was composed of glucose, 10 g; edible molasses, 20 g; peptone (Difco), 5 g; $CaCO_3$, 2 g; tap water, 1,000 ml. Incubation was carried out on either a reciprocal shaker operating at 108 two-inch strokes per minute or a rotary shaker which described a circle two-inches in diameter at a speed of 250 rpm for 72 hours at 30°C. Additional fermentation studies were presented by BOECK *et al.*[2]

Saccharomyces pastorianus (ATCC 2366) was used as the assay organism in a turbidimetric assay.[2]

Isolation Procedure

After the addition of filter aid (Hyflo Super-Cel), the fermentation broth was filtered, and the activity in the filtrate was adsorbed onto a cation-exchange resin column (IRC-50, hydrogen cycle).

The column was washed with water, and the activity was eluted with 0.05 N H_2SO_4. This active eluate was applied directly to a carbon column, and A9145 was subsequently eluten with a 30% aqueous acetone solution. The active fractions were combined and applied to a Dowex 50×4 (ammonium cycle) column, and the activity was eluted with 0.15 N NH_4OH. The eluate was concentrated to a low volume, two volumes of methanol were added, and this mixture was added to 20 volumes of acetone to precipitate A9145. After filtering and drying, the crude preparation was dissolved in a small volume of water, and the solution applied to a cellulose column packed in *n*-butanol-acetic acid-water (5 : 2 : 3). The column was developed with this solution; the active fractions were concentrated to a low volume and A9145 was precipitated by addition of methanol and acetone as outlined above to yield a white powder.

Chromatography

The R_f values of A9145 on paper chromatograms were 0.47 using a methanol-0.1 NH_4Cl (3 : 1) system and 0.19 using a butanol-saturated water system. The R_f value on cellulose thin-layer chromatography was 0.33 using a butanol-acetic acid-water (5 : 2 : 3) system. *Saccharomyces pastorianus* was used as the indicator organism. Ultraviolet light and ninhydrin spray were also used to detect the antibiotic.

Preparation of Crystalline Salts

The hydrochloride and sulfate salts of A9145 were prepared by dissolving the antibiotic in methanol-water (2 : 1), adding the appropriate dilute acid to about pH 4.0 and precipitating with excess acetone. The salts were crystallized from water-ethanol. A9145 hydrochloride (mp 195~197°C); A9145 sulfate (mp 220~222°C).

Organic salts were prepared by adding a saturated aqueous solution of organic acid to an aqueous solution of A9145. The insoluble salt which formed was filtered and recrystallized from hot water: A9145 *p*-hydroxyazobenzene sulfonate (mp 220~222°C); A9145 picrate (mp 160~162°C).

Acetylation

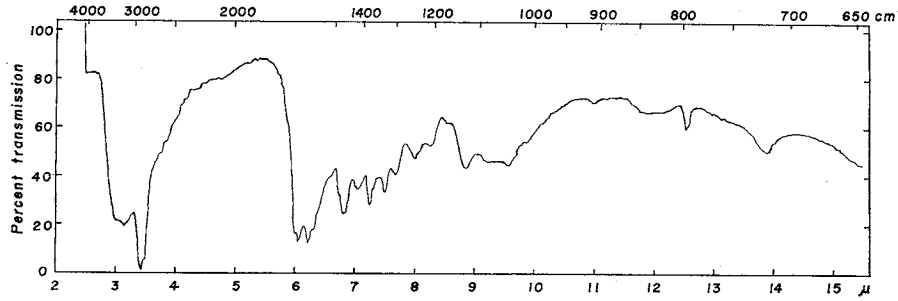
A9145 reacted with acetic anhydride to form two different products depending on the conditions. Acetic anhydride-methanol yielded a product containing three acetyl groups, while acetic anhydride-pyridine yielded a product with six acetyl groups. The acetylated derivatives were not biologically active.

Characterization of A9145

A9145 is a weakly basic compound exhibiting pK_a's of 2.9, 3.9, 8.9, and 10.2 (66% DMF) and has an apparent molecular weight of about 510 as calculated from the titration data; $[\alpha]_D^{25}$ −2.61° (*c* 5, H_2O). The antibiotic is soluble in water, very slightly soluble in methanol and ethanol, and insoluble in organic solvents. A9145 is stable at room temperature in aqueous solution from pH 1 to 11.

The compound exhibited ultraviolet absorption maxima at 206 nm ($E_{1\,cm}^{1\%}$ 520) and 258 nm ($E_{1\,cm}^{1\%}$ 325) in neutral solutions; single maximum at 256 nm ($E_{1\,cm}^{1\%}$ 325) in basic solutions. The infrared spectrum run in Nujol mull is shown in Fig. 1 giving peaks at the following frequencies:

Fig. 1. Infrared spectrum of A9145 in nujol mull



3.0, 3.15, 5.85, 6.00, 6.02, 6.07, 6.22, 6.30, 6.74, 6.83, 7.07, 7.26, 7.32, 7.52, 7.69, 8.01, 8.27, 8.88, 9.20, 9.57, 11.0, 11.8, and 12.56 μ.

Elemental analysis gave: C, 46.47; H, 6.8; N, 22.28, O, 26.22 %.

Acid hydrolysis (6 N HCl, 100°C, 24 hours) of A9145 yielded adenine hydrochloride and glycine. The glycine was attributed to degradation of adenine. The NMR spectrum showed peaks which were assigned to sugar hydroxyl protons and adenine nucleus protons. Structure studies will be reported later.

Biological Properties

A9145 was inhibitory to *Ceratostomella ulmi*, *Helminthosporium sativum*, and *Penicillium expansum* at a concentration of 100 μg/ml in an agar dilution assay, In a paper disc plate assay, 100 μg/ml of A9145 produced a 35 mm zone and a 25 mm zone of inhibition against *Saccharomyces pastorianus* and *Candida tropicalis*, respectively. The antibiotic was quite active both *in vitro* and *in vivo* against *Candida albicans*; these experiments are discussed in detail by GORDEE *et al.*[3]

A9145 was quite active against foliar plant diseases when applied to the plant as a spray at concentrations of 50~400 ppm. These diseases and causative organisms were: powdery mildew (*Erysiphe polygoni*); bean rust (*Uromyces phaseoli* var. *typico*); anthracnose (*Colletotrichum lagenarium*), crown gall (*Argobacterium tumefaciens*); and bacterial blight (*Xanthomonas phaseoli* var. *sojensis*).

The antibiotic was effective against infections of *Trypanosoma rhodesiense*, *Trypanosoma gambiense*, and *Trypanosoma congolense* in mice at 10 mg/kg injected subcutaneously. The acute toxicity (LD_{50}) by subcutaneous injection in mice was 185 mg/kg.

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