FERMENTATION, ISOLATION, CHARACTERIZATION AND STRUCTURE OF NITROSOFUNGIN

L. A. DOLAK, T. M. CASTLE, B. R. HANNON, A. D. ARGOUDELIS and F. REUSSER

Research Laboratories, The Upjohn Company
Kalamazoo, Michigan 49001, U.S.A.

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The new antifungal agent nitrosofungin was isolated in high yields from a mixed culture of two organisms consisting of a bacterium of the genus *Alcaligenes* (UC 9152) and *Streptomyces plicatus* UC 8272. The bacterium produces the agent, the streptomycete enhances the production by providing a precursor or an inducer. Nitrosofungin in high concentrations inhibits a broad variety of pathogenic fungi *in vitro*. The agent is relatively non-toxic in small laboratory animals and high blood levels are obtained after either oral or systemic administration. Nitrosofungin is only the second *N*-nitrosohydroxylamine isolated from microbial sources to date. It has been identified as 2-*N*-nitrosohydroxylamino-1-propanol, an acidic and highly water-soluble compound.

Our soil screening efforts resulted in the isolation of a bacterium of the genus *Alcaligenes* which produces the new antifungal agent nitrosofungin in small amounts. When the bacterium is grown in mixed culture with *Streptomyces plicatus* UC 8272 substantially higher yields of nitrosofungin are obtained. Sterile filtered fermentation broth from the streptomycete mentioned above, added to the pure bacterial culture also greatly enhances the production of nitrosofungin. Hence the streptomycete provides a precursor or an inducing substance and the bacterium produces the antifungal agent.

Results

Microorganisms

The antibiotic producing culture is a new isolate of the genus *Alcaligenes* (UC 9152) as classified by A. DIETZ and L. K. SCHADEWALD. The enhancing organism is *Streptomyces plicatus* UC 8272 as classified by A. DIETZ.

Analytical Methods

Nitrosofungin production was determined by the paper disc agar diffusion method employing *Saccharomyces pastorianus* UC 1342 as the test organism. The fermentation broth was analyzed by thin-layer chromatography on silica gel sheets (MN-polygram Sil N-HR, Brinkmann Instruments, Inc., Westbury, NY) developed with methyl ethyl ketone - acetone - water (186: 52: 20). In this system nitrosofungin had an Rf value of 0.6 and was located by bioautography against *S. pastorianus*. Thin-layer chromatography using Analtech silica gel plates with methanol gave an Rf of 0.8. Descending paper chromatography using 1-butanol - acetic acid - water (2: 1: 1) gave an Rf of 0.7 while 1-butanol - water (84: 16, with 2% piperidine added) gave an Rf of 0.2.

Fermentations

All fermentations were conducted under submerged culture conditions in 500-ml Erlenmeyer flasks containing 100 ml of culture medium. Seed cultures were prepared in a medium composed of glucose

monohydrate 25 g/liter and Pharmamedia (Traders Oil Mill Co., Fort Worth, TX) 25 g/liter. The medium was adjusted to pH 7.2 prior to sterilization. The seed flasks were inoculated with a frozen agar plug of *Alcaligenes* UC 9152 plus a similar plug of *Streptomyces plicatus* UC 8272 and incubated at 28°C for 3 days on a rotary shaker (250 rpm, 6-cm stroke). The fermentation medium contained in g/liter: glucose monohydrate 10, yeast extract (Difco) 1, Polypeptone (Becton, Dickinson and Co.) 10, globe corn starch (Corn Products, Inc.) 20. The medium was adjusted to pH 7.2 before sterilization. The flasks were inoculated with 5 ml of the mixed seed culture and incubated at 28°C on a rotary shaker. Peak antibiotic titers were usually obtained after 3~4 days. Mixed cultures produced in excess of 3.5 mg of nitrosofungin/ml whereas the bacterium alone produced only up to 0.1 mg/ml and *S. plicatus* alone produced none.

Isolation

Fermentation broth obtained as described above was filtered over a bed of diatomaceous earth. The filtrate was adjusted to pH 3 with hydrochloric acid. The antibiotic was adsorbed onto a bed of Ambersorb XE-348 (Rohm and Haas resin) and eluted with a solution of methanol - 0.1 N ammonium hydroxide (1: 1). The active eluate was adsorbed onto a bed of Dowex-1 (OH^-) and the nitrosofungin was eluted from this resin with 2 N ammonium chloride solution. The salt was removed by a cycle over a second column of XE-348 as described above. From this point on it was convenient to detect the presence of nitrosofungin in various fractions using the ultraviolet maximum at 246 nm (pH >7).

The crude nitrosofungin was loaded onto a DE52 (OH^-) column (Whatman, DEAE cellulose) and eluted from it with a water to 1 N ammonium hydroxide gradient. The fractions containing the nitrosofungin were pooled and lyophilized. The tacky solid so obtained was chromatographed over silica gel using a gradient consisting of methylene chloride - methanol (6: 1) to methanol.

Occasionally, mixed nitrosofungin salts separated as a white precipitate from the fractions containing nitrosofungin. More commonly, the appropriate fractions were pooled and concentrated on a rotary evaporator. The residue was dissolved in a minimum volume of methanol and the solution was diluted 1: 1 with 0.1 N ammonium hydroxide. The solution was warmed to 40°C and acetone was added until the cloudiness persisted. When the solution had cooled to room temperature, it was decanted from the yellow oil (if any). On cooling the decantate at 5°C overnight a white solid precipitated. This solid decomposed at 275~280°C. The elemental analysis typically showed: C 25.44, H 4.93, N 19.35, ash 22.0%. Qualitative atomic absorption showed that the major metal ions present were calcium and magnesium.

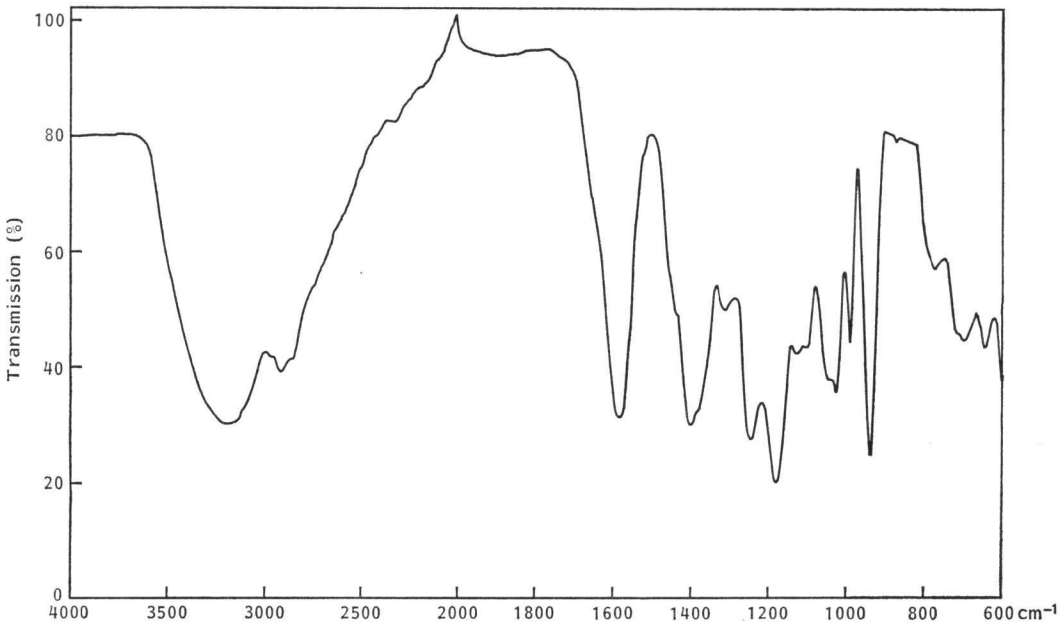
A solution of the mixed salts of nitrosofungin was percolated over a bed of Amberlite CG 120 (Ca^{++}) (Rohm and Haas). Appropriate fractions (UV analysis) were pooled and lyophilized to give calcium nitrosofungin. The calcium salt of nitrosofungin is a free-flowing, relatively non-hygroscopic, white powder. It decomposes at 275°C with no sign of liquid formation to 300°C. The elemental analysis: C 25.31, H 5.03, N 19.23. Calcd. for $C_6H_{14}N_4O_6Ca$: C 25.89, H 5.03, N 20.14.

In contrast, the sodium and ammonium salts of nitrosofungin were yellowish, hygroscopic gums.

Spectroscopic Properties of Calcium Nitrosofungin

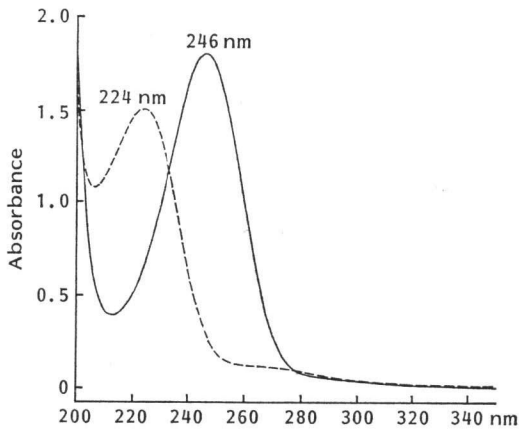
The infrared spectrum of the white powder was obtained on a KRS-5 plate (Wilks, TiBr, TiI crystal) (attenuated internal reflectance). It displayed strong absorbances, none of which was assignable to a using the FMIR technique carbonyl (Fig. 1). The ^{13}C NMR (20 MHz, DMSO-d_6) spectrum showed three lines at δ 14.95 (q), 63.13 (t) and 64.51 (d).

Fig. 1. Infrared spectrum of calcium nitrosofungin, reflectance plate method.



At pH 11 the UV spectrum showed a maximum at 246 nm (ε 17,650) and at pH 3 a maximum at 224 nm (ε 13,650) (Fig. 2). The ^{1}H NMR spectrum was taken at 30°C in DMSO-d_6 200 MHz (Fig. 3). Specific irradiation experiments showed that the methyl group at δ 1.1 was coupled to the methine proton at δ 4.18. The methine was coupled to both the methyl group and to a methylene group centered at δ 3.5. The geminal coupling constant was 11 Hz. The singlet at δ 3.5 was due to water.

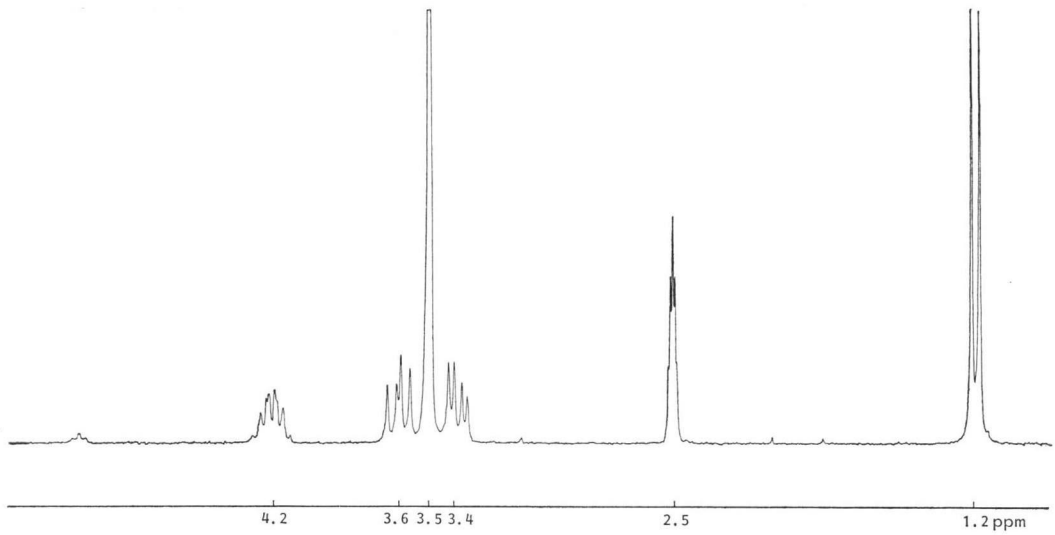
Fig. 2. UV spectrum of calcium nitrosofungin.
An aqueous solution (8.15×10^{-5} M) was adjusted to pH 11 (solid line) and to pH 2 (dashed line) with NaOH or HCl.



Mass spectroscopy with EI and FAB techniques failed to give a molecular ion using the free acid, the ammonium salt or the calcium salt of nitrosofungin. The *pKa* was 5.1 and the equivalent weight found was 142. Finally, $[\alpha]_D^{25}$ +31° (*c* 0.65, 0.01 N NaOH).

The above data show that the three carbons present in nitrosofungin are arranged methyl–methine–methylene. The UV spectrum is very similar to that of alanosine and suggests an *N*-nitrosohydroxylamine moiety. Calculations based on the elemental analyses suggested a molecular formula of $C_3H_8N_2O_3$ for the hypothetical free acid. Since the NMR spectra imply the presence of six covalently-bonded hydrogen atoms, nitrosofungin must contain two hydroxyl groups. One of them is part of the *N*-nitrosohydroxylamino moiety and the other is probably bound to the methylene group. Computer Assisted Structure Elucidation (CASE) studies[1] indicated only one probable structure

Fig. 3. 200 MHz ^1H NMR spectrum of calcium nitrosofungin at 30°C in DMSO-d_6-TMS with added D_2O.



for nitrosofungin: 2-nitrosohydroxylamino-1-propanol. This structure was proved unequivocally by reducing nitrosofungin to the known 2-amino-1-propanol (*vide infra*) and by total synthesis from 2-nitro-1-propanol[2].

The structure of nitrosofungin free acid is shown below along with that of alanosine[3]. These 3-carbon compounds are the only known microbially-produced nitrosohydroxylamines to date.

Nitrosofungin Alanosine

The Chemistry of Nitrosofungin

Nitrosofungin is unstable in acid but very stable in base. A solution of nitrosofungin in 0.1 N sodium hydroxide showed no change in UV absorbance or bioactivity after 72 hours at 60°C. On the other hand, attempts to lyophilize nitrosofungin free acid invariably led to inactivated residues.

Kinetic studies of the decomposition of nitrosofungin in acid were done by following the decrease in absorbance with time on a Hewlett-Packard HP 8450 spectrophotometer at 224 nm. The decompositions were characterized by an induction period followed by a *pseudo* first order decomposition. Acidic solutions of nitrosofungin followed Beer's Law over the range of 2.0 to 0.05 absorbance units. All the kinetic studies were done using a starting absorbance of 0.8 (3.13×10^{-5} M).

There was little difference in the reaction rate or induction periods observed when either hydrochloric or sulfuric acids were used. Both of these parameters varied with the reaction temperature, the concentration of the acid and with the age of the solutions used.

Fig. 4 shows the course of a typical decomposition run and the data are summarized in Table 1. In 0.1 N acid at 80°C the half-life is about 4 minutes in the linear region. In 1.0 N acid the rate of decomposition was reduced and the reaction stopped sooner than it did in either 0.1 or 0.01 N acid.

One of the products of the acid decomposition appears to be a dimer. A decomposition run of 2 mmol of nitrosofungin was lyophilized to give a mixture of compounds. When the mixture was subjected

Fig. 4. The decomposition of calcium nitrosofungin at 80°C in 0.1 N HCl monitored at 224 nm.

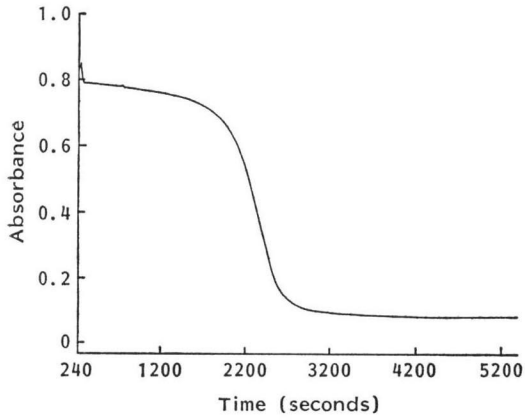


Table 1. Rates of decomposition of nitrosofungin.

T (°C)	Acid	Normality	Rate ($\times 10^5$)*
60	HCl	0.1	1.24
70	HCl	0.1	2.74
80	HCl	0.1	4.41
80	H_2SO_4	0.1	5.10
80	HCl	0.01	3.51
80	HCl	1.0	1.19

* Pseudo zero-order rate constant, mol/L-sec.

to FAB mass spectroscopy in the high resolution mode, a molecular ion was observed at *m/z* 143.0811 (theory for $C_6H_{10}N_2O_2$ is 143.0820). The decomposition products of nitrosohydroxylamines have been reported in the literature[4] and dimeric structures have been suggested.

Salts of nitrosofungin in methanol - water are inert to hydrogen at three atmospheres using either palladium or platinum catalysts. However, reduction is rapid with palladium on charcoal if the solution is acidified with hydrochloric acid. The product is 2-amino-1-propanol (identified by mass spectroscopy and by ^{13}C NMR).

Biological Properties

Nitrosofungin inhibits a wide variety of fungi when assayed in a two-fold broth dilution test (Table 2). The agent possesses a low potency as the MIC's for the organisms tested range from 0.2 to 1 mg/ml. Nitrosofungin is well tolerated in small laboratory animals. The MTD is in excess of 400 mg/kg/day in mice when administered subcutaneously for four consecutive days. The acute LD_{50} is 780 mg/kg (intraperitoneally) in mice. Following a single subcutaneous dose of 100 mg/kg in rats, a peak blood level of 216 μg/ml was attained and after a single oral dose of 100 mg/kg the peak blood level measured was 112 μg/ml.

Table 2. Minimum inhibitory concentration of nitrosofungin vs. various fungi in μg/ml.

Organism	UC	Nitrosofungin, Ca^{++}-salt (synthetic)	Nitrosofungin, fermentation prep.
Blastomyces dermatitidis	1466	400	800
Candida albicans	7163	>800	>800
Geotrichum sp.	1207	>800	>800
Microsporum canis	1395	>800	>800
Sporotrichum schenckii	1364	>800	>800
Cryptococcus neoformans	1139	200	200
Phialophora verrucosa	1807	800	400
Trichophyton interdigitale	1399	>800	>800
Trichophyton mentagrophytes	4797	>800	>800
Saccharomyces pastorianus	1342	—	1,000

The assay was performed as described by LENNETTE *et al.*[5]. Broth dilutions were made using Antibiotic Medium No. 3 (Difco). Tubes were incubated at 35°C and the end-points were read at 48 hours.

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