ANTIBIOTICS PRODUCED BY *STREPTOMYCES FICELLUS* I. FICELLOMYCIN

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Ficellomycin is a new basic antibiotic produced by *Streptomyces ficellus*. Ficellomycin, $C_{13}H_{24}N_6O_3$, inhibits the growth of gram-positive bacteria *in vitro* and is effective in the treatment of experimental *Staphylococcus aureus* infections in mice.

Cultures of *Streptomyces ficellus** grown in a complex medium were found to contain several antibacterial agents which were characterized by their tlc behavior and their antibacterial spectrum. One of these antibiotics, which exhibits high activity against *Staphylococcus aureus*, was isolated and designated ficellomycin (U-47, 929). The present communication deals with the production, isolation and chemical characterization of ficellomycin. Work related to the co-produced antibiotics will be reported in subsequent communications.

Experimental

Assay and Testing Procedures

Antibiotic production and purification was measured by a microbiological disc-plate assay procedure[1] with *Staphylococcus aureus* or *Penicillium oxalicum* as the assay organisms.

Thin-Layer Chromatographic Procedures

Thin-layer chromatograms were run on silica gel G using 95% ethanol - water (75: 25, v/v) as the solvent system. The antibiotics present in the fermentation broth or in preparations obtained during purification were detected by bioautography on *S. aureus* or *P. oxalicum*-seeded agar trays.

Spectroscopic Methods

Proton magnetic resonance spectra were recorded on a Varian XL-100-15 spectrometer operating at 100 MHz. Solutions (*ca.* 0.4 ml, *ca.* 0.25 M) of the compounds in D_2O were used.

Carbon magnetic resonance spectra were recorded on a Varian XL-100-15 spectrometer operating in the FOURIER transform (FT) mode. Pmr and cmr chemical shifts are reported as ppm relative to tetramethylsilane.

Complete high resolution mass spectra (HR-MS) were obtained on the CEC-21-110B spectrometer using a photographic plate as detector.

The field desorption mass spectrum was obtained on a Varian-MAT CH-5-DF spectrometer.

Fermentation Procedures

Shake Flask Fermentations: Seed cultures of *S. ficellus* were prepared in a medium consisting of glucose monohydrate, 25 g/liter and Pharmamedia, 25 g/liter (Trader's Oil Mill Co., Fort Worth, Texas, U.S.A.). The cultures were incubated at 28°C for 72 hours on a rotary shaker. Fermentation medium consisting of glucose monohydrate, 15 g/liter; starch, 40 g/liter; Blackstrap molasses, 20 g/liter (Knappen Molasses Company, Chicago, Illinois, U. S. A.); Pharmamedia, 25 g/liter; and calcium

* Taxonomic studies were done by Miss ALMA DIETZ of The Upjohn Company. The organism is designated *Streptomyces ficellus* UC®5438.

carbonate, 8 g/liter was adjusted to pH 7.2 and inoculated at a rate of 5% (v/v) with the 72-hour seed culture. Fermentations were incubated at 28°C on a rotary shaker and analyzed for antibiotic production by paper and tlc chromatography and bioactivity determination. Peak titers were obtained after 72~96 hours of incubation.

Tank Fermentations: *S. ficellus* was used to inoculate a series of 500-ml Erlenmeyer flasks, each containing 100 ml of preseed medium consisting of glucose monohydrate, 25 g/liter and Pharmamedia, 25 g/liter. The flasks were incubated at 28°C for 72 hours on a rotary shaker. Three flasks (300 ml) of the preseed culture were then used to inoculate a tank containing 250 liters of seed medium consisting of glucose monohydrate, 10 g/liter; cornsteep liquor, 10 g/liter (CPC International, Englewood Cliffs, New Jersey, U. S. A.); Pharmamedia, 2 g/liter and Wilson's liquid peptone No. 159, 10 g/liter (Wilson Protein Technology, Division of Wilson Pharm. and Chem. Co., Calumet City, Illinois, U. S. A.); Pre-sterilization pH 7.2. The seed tank was agitated at an impeller speed of 400 rpm. Sterile air was supplied at the rate of 10 liter/minute and the tank was kept at 28°C for 48 hours. This seed tank was used to inoculate a tank containing 5,000 liters of the medium consisting of glucose monohydrate, 15 g/liter; Blackstrap molasses, 20 g/liter; starch, 40 g/liter; Pharmamedia, 25 g/liter; calcium carbonate, 8 g/liter and UCON antifoam, 1 liter (Union Carbide, Chemical Division, 1042 West 7 Mile Road, Detroit, Michigan); pre-sterilization pH 7.2. The fermentor was agitated at an impeller speed of 240 rpm and sterile air was supplied to the fermentor at the rate of 80 liters/minute. The fermentor was maintained at 28°C and the culture broth was harvested after 72 hours.

Isolation of Ficellomycin

Florisil Chromatography: The whole broth (*ca.* 5,000 liters) was filtered with the aid of diatomaceous earth. The filter cake was discarded. Part of the clear filtrate (2,000 liters) was passed at a rate of 5 liters/minute over a florisil column prepared from 80 kg of acid washed florisil*. The spent broth was discarded. The column was washed with 150 liters of water at a rate of 5 liters/minute. The aqueous wash was also discarded. The column was then eluted with 900 liters of 50% aqueous acetone. Fractions containing ficellomycin were concentrated to an aqueous solution which was freeze-dried to give 599 g of crude ficellomycin which was purified as described below.

Amberlite XAD-4 Chromatography: Crude ficellomycin, 944 g, obtained as described above, was dissolved in 95 liters of 10% aqueous sodium chloride solution. The solution was adjusted to pH 10 and passed over a column (23-cm internal diameter) containing 27 liters of Amberlite XAD-4 (Rohm and Haas Co., Philadelphia, Pa., U. S. A.) at a flow rate of 100 ml/minute. The spent was found bioinactive and was discarded. The column was washed with 72 liters of water. This fraction did not contain ficellomycin and was also discarded. The column was then eluted with a mixture of methanol-water (20: 80 v/v). Seventeen 4-liter fractions were collected and analyzed by tlc and bioassay. Fractions containing ficellomycin were combined and the solution was concentrated to dryness to give 346 g of a purified preparation of ficellomycin. Pure antibiotic was obtained by the chromatography described below.

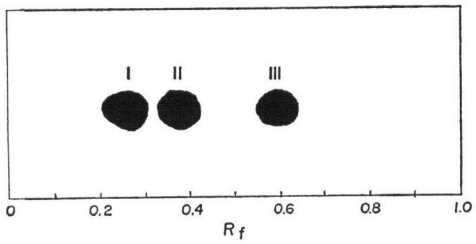
Dowex-1 (OH^-) Chromatography: The column was prepared from 300 ml of Dowex-1 (X-4) in the hydroxide form. Twenty g of ficellomycin preparation, obtained as described above, was dissolved in 200 ml of water (pH found, 10.4). This solution was passed through the column at a flow rate of 5 ml/minute. The column was eluted with water. Fractions (20-ml each) were tested for bioactivity against *S. aureus* and analyzed by tlc. Ficellomycin was located by spraying the tlc plates with ninhydrin in addition to bioautography on *S. aureus*-seeded agar trays. Fractions 26~50 were combined and freeze-dried to give 8.3 g of pure ficellomycin the properties of which are described below.

Discussion and Results

Cultures of *Streptomyces ficellus* contain several compounds exhibiting antibacterial activity. Of these, the components designated I, II and III (Fig. 1) are the main antibacterial components.

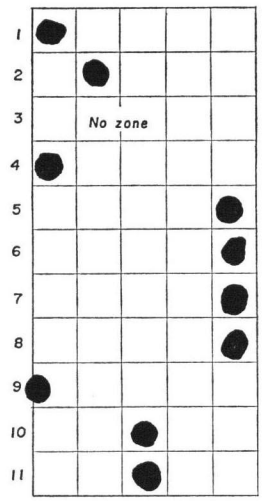
* Acid washed florisil is prepared by stirring commercial florisil with 3 N aqueous sulfuric acid. The mixture is allowed to stand at room temperature for 20 hours, then is washed with deionized water until pH is *ca.* 5.

Fig. 1. Thin-layer chromatography* of culture filtrates of *Streptomyces ficellus*



* Silica gel G; 95% Ethanol - water (75: 25). Antibiotics I, II, III were detected by bioautography on *S. aureus* (I) or *S. lutea* (II) or *E. coli* (III)-seeded agar.

Fig. 2. Paper-chromatographic mobility of ficellomycin.



Solvent systems: 1) 1-butanol - water (84: 16); 2) 1-butanol - water (84: 16) and 0.25% *p*-toluene - sulfonic acid; 3) 1-butanol - acetic acid - water (2: 1: 1); 4) 2% piperidine (v/v) in 1-butanol - water (84: 16); 5) 1-butanol - water (4: 96); 6) 1-butanol - water (4: 96)+0.25% *p*-toluene - sulfonic acid; 7) 0.5 M phosphate buffer pH 7.0; 8) 0.075 M NH_4OH saturated with methyl isobutyl ketone, lower phase; 9) benzene - methanol - water (1: 1: 2); 10) 1-butanol - water (84: 16) and 2% *p*-toluene sulfonic acid; 11) methanol - 15% aqueous sodium chloride (4: 1).

The paper used is impregnated with 0.1 M Na_2SO_4.

Ficellomycin (component I) is characterized by high *in vitro* activity against *S. aureus* and *P. oxalicum* and lack of activity against gram-negative organisms. Component II is highly active against *Sarcina lutea*, slightly active against *S. aureus* and inactive against *P. oxalicum*. Component III is active against gram-positive and gram-negative organisms (*S. aureus, E. coli*) and inactive against *P. oxalicum*. Therefore, the production and isolation of the three antibiotics was followed by testing against assay organisms sensitive to the antibiotic understudy. Specifically, production of ficellomycin was followed by assaying against *S. aureus* and *P. oxalicum* and by paper or thin-layer chromatography. The paper chromatographic pattern of ficellomycin in several solvent systems is presented in Fig. 2.

Ficellomycin was isolated by a sequence of chromatographic procedures. Florisil chromatography separated ficellomycin from component III. Amberlite XAD-4 chromatography yielded ficellomycin preparations free of component II. Pure ficellomycin was obtained as an amorphous colorless material, $[\alpha]_D^{25}+39°$ (*c* 1, water) by chromatography over Dowex-1 (X-4) in the hydroxide form. The antibiotic is soluble in water and lower alcohols. It is insoluble in ketones, halogenated or saturated hydrocarbon solvents and ethyl acetate or other ester type solvents.

Ficellomycin forms a penta-trimethylsilyl-derivative, $C_{13}H_{19}N_6O_3 \cdot 5Si(CH_3)_3$; molecular weight, calcd. 672.3886; found (HRMS), 672.3891. The molecular formula of ficellomycin, therefore, is $C_{13}H_{24}N_6O_3$, mol. weight 312. The field desorption mass spectrum showed the molecular ion at *m/e* 312 in agreement with the data obtained by high resolution mass spectroscopy of the trimethylsilyl-derivative of ficellomycin. The bar graph of the low resolution mass spectrum of the TMS-derivative of ficellomycin is presented in Fig. 3.

The ir spectrum (Fig. 4) shows absorptions at 3180~3370 and at 1645, 1510 cm^{-1} assigned to –OH, –NH– and amide functions. The UV spectrum of ficellomycin showed end absorption only. The

pmr spectrum (Fig. 5) showed no absorptions below δ 5.0 indicating the absence of olefinic or aromatic hydrogens in the molecule. Absorptions centered at δ 1.0 (d, 6H) are assigned to the methyl protons of an isopropyl group. Spin decoupling studies established the presence of fragment A (Fig. 6) in ficellomycin. The α-CH- appears as a doublet at δ 3.3 (1H) while the β-CH- is part of a

Fig. 3. Mass spectrum of the TMS-derivative of ficellomycin

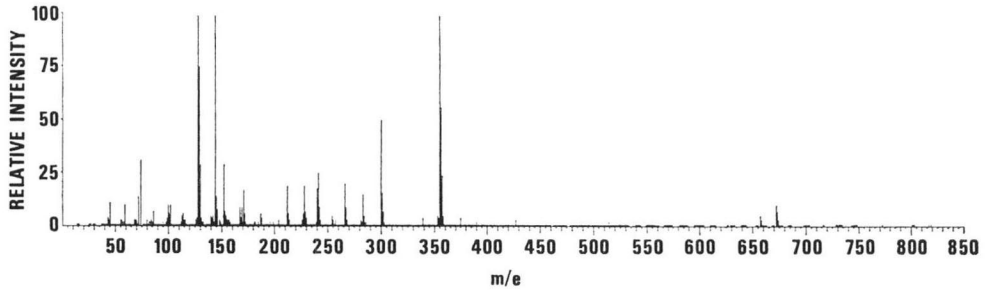


Fig. 4. Infrared spectrum of ficellomycin (in Nujol mull)

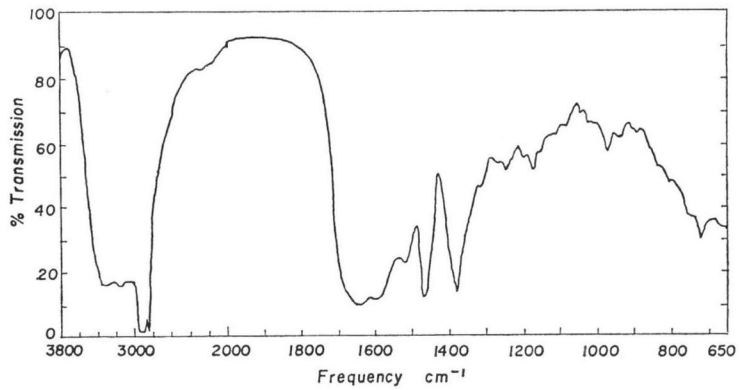


Fig. 5. Proton magnetic resonance spectrum of ficellomycin

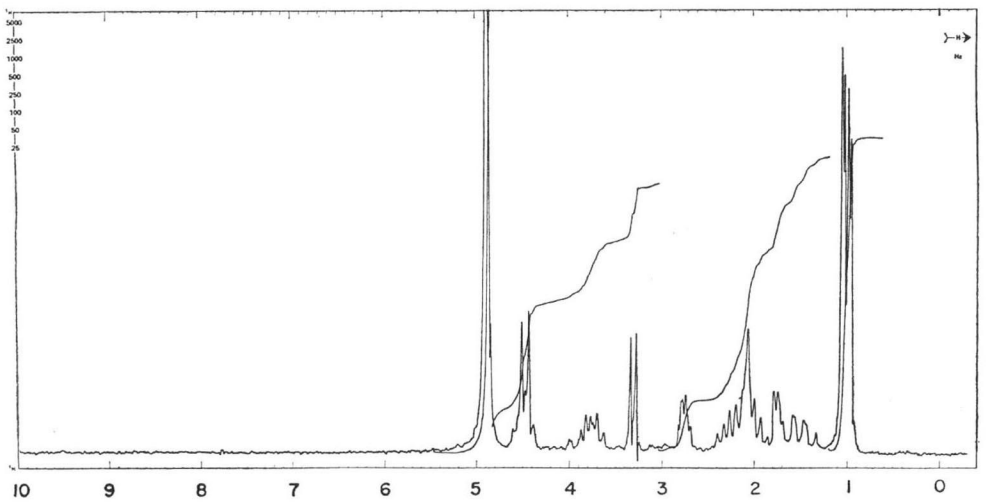
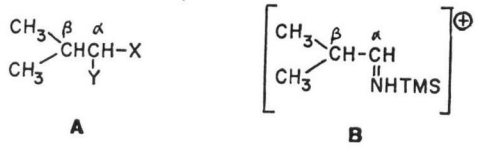


Fig. 6.



A B

complex absorption centered at δ 2.15. The chemical shift of the α-methine proton combined with cmr data (doublet at δ 61.39 and singlet at δ 176.51 or 176.91, Table 1) suggested that X and Y in A (Fig. 6) are –C=O and –NH– or NH_2, NH–
respectively, thereby indicating the presence of valine in ficellomycin.

This conclusion is in agreement with high resolution mass spectral data. The presence of an ion at *m/e* 144 in the mass spectrum of ficellomycin·5TMS is assigned to fragment B (Fig. 6) (Calcd. for $C_7H_{18}NSi$, 144.1208; found, 144.1209). This indicates that valine is linked to the remaining part of ficellomycin by a peptide linkage; the amino group of valine is free in agreement with the positive response of the antibiotic to ninhydrin. Furthermore the presence of valine in ficellomycin is supported by the hydrolytic studies discussed below. The pmr specturm also shows absorption at δ, 1.4 to 2.4 (*ca*, 5H); δ 2.75 (d, d of d, 1H)*; δ 3.80 (d, d of d, 1H)* and δ 4.5 (d and m, 2H)*.

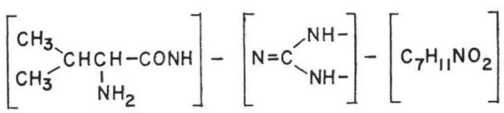
Table 1. Carbon-13 magnetic resonance spectrum[1] of ficellomycin.

Chemical shift, δ[2]	Multiplicity[3]	Assignment
18.04	q	CH_3–(valine)
19.67	q	CH_3–(valine)
22.34	t	$-CH_2-$
27.98	t	$-CH_2-$
32.85	d	>CH–(valine)
39.67	d	>CH–
52.62	d	>CH–NH–
57.43	d	>CH–NH–
61.39	d	>CH–NH–(valine)
63.27	d	>CH–
157.51	s	$-N\diagdown\atop-N\diagup$>C=NH
176.51	s	$-C\diagdown^{O}_{NH-}$ (valine)
176.91	s	$-C\diagdown^{O}_{NH-}$

1 Spectrum obtained in D_2O.
2 In ppm relative to TMS. Dioxane was used as an internal standard (δ, 67.4).
3 Multiplicity in off-resonance proton-decoupled spectrum: q=quartet; t=triplet; d=doublet; s=singlet.

Absorptions observed in the cmr spectrum of ficellomycin are tabulated in Table 1. Five of the 13 carbons have been assigned to the valine fragment. A singlet at δ 157.51 has been tentatively assigned to a guanidino-type carbon[3]. The presence of a guanidino group in ficellomycin is also indicated by the positive SAKAGUCHI test and the strongly basic character of the antibiotic and its behavior in acid or base hydrolysis. Ficellomycin treated with 6 N aqueous hydrochloric acid (reflux, 24 hours) yielded valine and other unidentified degradation product (s), without evolution of either carbon dioxide or ammonia. On the other hand, treatment of ficellomycin with saturated aqueous barium hydroxide solution (reflux, 24 hours) gave, in addition to valine, one mole of carbon dioxide (isolated as barium carbonate) and ammonia (isolated as ammonium chloride) per mole of the antibiotic. Valine, isolated by both acid or base hydrolysis was found identical (pmr, $[\alpha]_D$, ir) to authentic L-valine. On the basis of the data discussed the following partial structure can be written for ficellomycin.

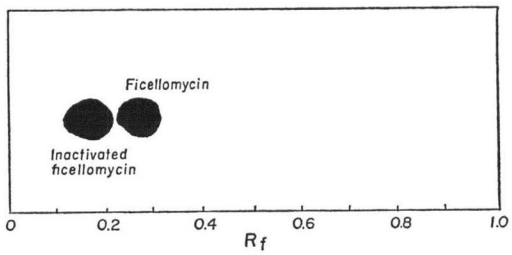
Ficellomycin is stable in aqueous solutions at pH 8~10. At lower pH the antibiotic is slowly transformed to a bioinactive material which has been designated "inactivated ficellomycin". This transformation can be followed



* d,d of d=doublet of doublet of doublets: d=doublet; m=multiplet.

by tlc (Fig. 7). The inactivated antibiotic shows mass spectroscopic behavior identical to that of ficellomycin. This indicates that "inactivated ficellomycin" and ficellomycin have identical molecular formulas and therefore the transformation of ficellomycin to "inactivated ficellomycin" involves intramolecular changes rather than fragmentation of the ficellomycin molecule. The pmr and cmr spectra of "inactivated ficellomycin" differ substantially from those of ficellomycin. These spectra will be discussed in a later communication on the structure of ficellomycin.

Fig. 7. Thin-layer-chromatographic* separation of ficellomycin and "inactivated ficellomycin"



* Silica gel G; 95% Ethanol - water (75: 25). Antibiotics were detected by a ninhydrin reagent.

Biological Properties of Ficellomycin

Ficellomycin shows *in vitro* activity against *S. aureus* including strains resistant to penicillin, streptomycin, neomycin, macrolides and lincosaminide-antibiotics. The antibiotic was effective in the treatment of experimental *S. aureus* infections in mice (subcutaneously, CD_{50} *ca.* 7.6 mg/kg). Ficellomycin was also active in treatment of infections caused by *S. aureus* strains resistant to either streptomycin or erythromycin (mice, subcutaneously, CD_{50} of 10 and 11 mg/kg, respectively).

Ficellomycin was inactive against a variety of fungi (except *P. oxalicum*) and viruses *in vitro*. The acute LD_{50} values for ficellomycin in mice were *ca.* 800 mg/kg by both intraperitoneal or intravenous administration.

Acknowledgement

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