EFROTOMYCIN, A NEW ANTIBIOTIC FROM *STREPTOMYCES LACTAMDURANS*

Sir:

Streptomyces lactamdurans NRRL 3802 produces a novel β-lactam antibiotic, cephamycin C. The chemical nature of cephamycin has been demonstrated to be 7-β-(D-5-amino-5-carboxyvaleramido)-3-(carbamoyloxy-methyl)-7-methoxy-3-cephem-4-carboxylic acid.[1,2]

During studies of the fermented broths of *S. lactamdurans*, an antibiotic was discovered with properties different from those described for cephamycin C. This new antibiotic was designated FR-02A, and later renamed efrotomycin.

Several characteristics of efrotomycin suggested that the agent was not a member of the cephamycin class of antibiotics. The broth from cultures of *S. lactamdurans* had a small amount of activity that could not be eliminated by treatment with a potent β-lactamase from *Enterobacter cloacae* MB2646 (HSC18410/62); cephamycin C is sensitive to hydrolysis by this enzyme.[3] Further, the enzyme-treated broth had a ratio of gram-positive to gram-negative antibacterial activity that was higher than could be expected for cephamycin C and for other members of the cephamycin family; these antibiotics have only slight activity against gram-positive organisms.[4] Paper chromatography of concentrated broth filtrate revealed two distinct, well-separated bioactive areas. When whole broth was extracted with equal volumes of water-immiscible solvents, all the efrotomycin migrated into the solvent phase whereas cephamycin C remained in the aqueous phase. The antibacterial spectrum, solvent solubility and complete indifference to the action of *E. cloacae* β-lactamase suggested that efrotomycin was not a member of the β-lactam group of antibiotics.

S. lactamdurans is maintained on agar slants of the following composition: blackstrap molasses, 1%; Dried Brewers Yeast, 1% and Difco agar, 2.5% (pH 7.0). The agar slants or lyophilized cells are used as a starter inoculum for the production of efrotomycin. To obtain optimal yields it is desirable to use two seed (inoculum) development stages. The first seed flask (250 ml Erlenmeyer) containing 40 ml of medium (1% Primary Dried Yeast, pH 7.0) is inoculated from the slant culture or directly from a lyophilized tube of *S. lactamdurans*, and allowed to incubate for 48 hours at 28°C on a rotary shaker (220 rpm, 5-cm displacement). One ml of the first seed is transferred to a second seed flask containing 10 g/liter Ardamine YEP (Yeast Products Co., Paterson, N.J., USA) at pH 7.0 and incubated for 24 hours under similar conditions. The production medium

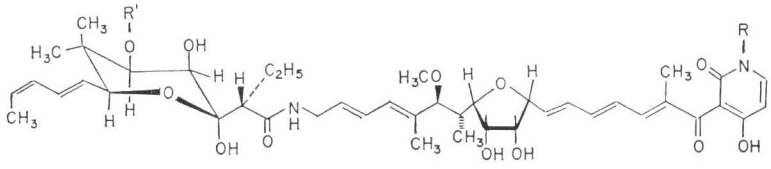
Table 1. Relative production of efrotomycin and cephamycin C by *S. lactamdurans*

Production medium	Culture age (hours)	Antibiotic production (mg/liter)	
		Cephamycin C	Efrotomycin
A	96	240	147
	120	185	147
B	96	4	77
	120	5	327

Medium A: As in text.

Medium B: Ingredients in %: cornsteep liquor, 1.8; commercial grade dextrose, 5.6; Proflo cottonseed meal, 2.8; glycerol, 1.4 v/v; dimethylformamide, 1.4; sodium thiosulfate, 0.05; and polyglycol 2000 defoamer, 0.12 v/v. Adjusted to pH 7.3 prior to sterilization. Sodium thiosulfate added aseptically from a concentrated stock solution to the sterile medium. Other conditions as in the text.

Fig. 1. Structure of antibiotics X-5108, mocimycin and efrotomycin



X-5108 (Goldinomycin) R = CH_3 ; R' = H

Mocimycin (Kirromycin) R = R' = H

Efrotomycin R = CH_3 ; R' = Disaccharide ($C_{15}H_{27}O_8$)

(medium A) contains the following ingredients in %: Primary Dried Yeast, 1.0; Distillers Solubles, 3.0; glycine, 0.05; L-phenylalanine, 0.3; cornstarch, 2.0; dimethyl formamide 1.0 v/v; Mobile par S defoamer, 0.24 v/v; and sodium thiosulfate, 0.05. The pH was adjusted to 7.0 prior to sterilization. The thiosulfate was added aseptically from a filter sterilized concentrated stock solution to the sterile production medium. Inoculum for the production phase was 2.5% of the second stage seed and was kept at this level when large volume stirred tank fermentors were used as production vessels. Incubation was for five days at 28°C.

The production of efrotomycin is followed by microbiological assay after extraction of the broth with chloroform at pH 5. A standard disc-diffusion assay is used with *Vibrio percolans* ATCC 8461 as the assay organism, and purified samples of efrotomycin serve as the reference standards. The cephamycin C content of the broth was determined with the same assay organism; in this case the aqueous phase of the extraction was used after dilution in phosphate buffer, pH 7.0. Since the assay organism is considerably more sensitive to cephamycin C than to efrotomycin, a simple dilution of the unextracted broth filtrate could also be used to measure cephamycin C without interference from efrotomycin.

In production medium A, *S. lactamdurans* NRRL 3802 produced about 150 mg/liter of efrotomycin and 250 mg/liter of cephamycin C. Production of both antibiotics reached their peaks between 96 and 120 hours.

Though both cephamycin C and efrotomycin can be produced by *S. lactamdurans*, growth media have been devised in which the production of cephamycin C can be greatly reduced and the level of efrotomycin increased significantly (Table

Table 2. Summary of physicochemical characteristics of efrotomycin

Empirical formula	$C_{59}H_{88}N_2O_{20}$
Molecular weight by mass spectral analysis	1,144
Ultraviolet absorption	
Ammonium salt in water:	λ max. 233 nm; $E^{1\%}_{1cm}=320$
	λ max. 328 nm; $E^{1\%}_{1cm}=180$
Efrotomycin in pH 7.0 phosphate buffer:	λ max. 232 nm; $E^{1\%}_{1cm}=464$
	λ max. 327 nm; $E^{1\%}_{1cm}=216$
Representative features of nuclear magnetic resonance spectrum[1]	Doublets at 1.21 (3H), 1.31 (3H), 1.74 (3H), 4.63 (1H), 5.94 (1H) and 7.32 (1H) ppm; Overlapping signals of 4 other C-methyl groups at about 0.94 ppm; Singlets at 1.65 (3H), 2.02 (3H), 3.15 (3H), 3.42 (3H), 3.45 (3H), 3.54 (3H), 3.58 (3H), and 4.87 (1H) ppm.
Characteristic of infrared absorption spectrum (frequency in cm^{-1})[2]	Broad band: 3400 Strong bands: 1640, 1460, 1380, 1080, 1020 Prominent bands: 1550, 1505, 1240, 1195, 940, 860, 720, 620
Chromatography characteristics	
Paper chromatography (isopropanol-0.1 M phosphate pH 6.0; 70: 30)	Rf=0.9
Thin-layer chromatography: Silica gel (chloroform-methanol-conc.NH_4OH; 80: 20: 1)	Rf=0.34
Sephadex LH-20: Gel filtration in methanol (as NH_4 salt)	$K_D=0.30$
Amberlite XAD-2 chromatography: 50% isopropanol-H_2O elution	D.V.[3]=3.5 to 4.0

[1] Obtained at 100 MHZ in $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard.
[2] Obtained in a Nujol mull.
[3] D.V.=Displacement volume, *i.e.*, the number of bed volumes required to elute the antibiotic.

1). Furthermore, mutants have been selected in which either the biosynthesis of efrotomycin or cephamycin C has been completely eliminated, whereas the production of the second component remains unaffected.[5] These results suggest that the biosynthetic pathways for the two antibiotics are regulated independently of each other.

Efrotomycin is found both dissolved in the culture filtrate and bound to the mycelium and undissolved nutrients in the fermented broth. For isolation of the antibiotic, 400 ml of whole broth is adjusted to pH 5 with HCl, two volumes of chloroform are added, mixed thoroughly and filtered through a Super-Cel pad. Sufficient water is added to speed the filtration. The filtrate is allowed to stand until the chloroform layer separates. The solvent layer is drawn off, washed twice with 500 ml of water, dried over anhydrous $MgSO_4$ and evaporated to dryness *in vacuo*. Petroleum ether, 500 ml, is added to the solid residue and the solid is collected by filtration, washed with 50 ml of petroleum ether and air dried to yield crude efrotomycin. The antibiotic is converted to the ammonium salt by freeze drying a solution of the crude efrotomycin in 50 ml water made basic to pH 10 with NH_4OH.

Efrotomycin may be isolated from the centrifuged or filtered cake. In this case a polar water-miscible organic solvent is preferable for the first extraction. The filter cake from 400 ml of broth is stirred in 40 ml of acetone for 30 minutes, filtered, the filter cake washed with 15-ml aliquots of acetone, and the filtrate and washings combined. The combined extract is evaporated under reduced pressure at 30°C to remove the acetone. The residue is suspended in 15 ml of water, adjusted to pH 4.0 with HCl, an equal volume of hexane is added and stirred for 10 minutes. After settling, the hexane is decanted and discarded. Two more extractions with equal volumes of hexane are made, the last being colorless. The aqueous slurry is then extracted with an equal volume of chloroform. After repeating the extraction, the chloroform fractions are combined, dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo* to dryness.

About 100 mg of the crude efrotomycin is dissolved in 0.5 ml of methanol and applied to a 75 ml bed (1.25 cm × 56 cm) of Sephadex LH-20 gel (Pharmacia Fine Chem., Inc., Piscataway, N.J.). The column is developed in methanol and the eluate is monitored with a Mecco differential refractometer. Fractions are assayed for bioactivity using disc diffusion with *V. percolans* as the indicator strain. Three mass peaks were observed in the elution profile; the third peak (K_D= 0.3) corresponded to the major share of the bioactivity. The fractions of this bioactive peak are combined and evaporated to yield about 40~50 mg of efrotomycin. This material is brought to ultimate purity by the use of Amberlite XAD-2 resin (Rohm & Haas Co., Philadelphia, Pa.). The product from the LH-20 column is dissolved in 50% isopropanol-water and acidified to pH 2 and applied to a 150 ml bed of XAD-2 (1.25 cm × 112 cm). The column is eluted with 50% isopropanol and the eluate monitored with a refractometer and for bioactivity. The antibiotic is eluted from the column at 3.5~4.0 column volumes of isopropanol. The bioactive fractions were combined and evaporated to dryness under reduced pressure. Efrotomycin obtained by this procedure is a pale yellow, noncrystalline solid of high purity and stable to normal handling.

A summary of the preliminary physicochemical characterization of pure efrotomycin is presented in Table 2. A detailed account of the structure elucidation will be presented in a separate communication.[6] Interpretation of the physical and chemical data leads to the assignment of efrotomycin as a new member of a recently described family of antibiotics (Fig. 1). In 1972, kirromycin,[7] mocimycin[8] and X-5108[9,10] were isolated from different species of *Streptomyces*. Kirromycin and mocimycin were found to have the same structure, whereas X-5108 is the N-methylated form.[11] Efrotomycin is a disaccharide derivative of X-5108 and has many physicochemical properties in common with this antibiotic. They share characteristic U. V. absorption and distinctive NMR features. In addition, the *in vitro* microbiological properties are similar.

Efrotomycin has been found to be active against a variety of microorganisms many of which are particularly important pathogens for farm animals.[12] In general, the activity of efrotomycin is more pronounced against gram-positive species than it is against gram-negative bacteria,which is also a characteristic of X-5108.[10] Efrotomycin is active *in vivo* and shows good therapeutic effectiveness in mice infected with several species of bacterial pathogens. Efficacy is obtained by oral as well as parenteral routes of administration. The antibiotic is well tolerated

in laboratory animals; LD_{50}'s of >4,000 mg/kg and >2,000 mg/kg by oral and subcutaneous routes, respectively, have been obtained.[12] Not unexpectedly, in light of its structure, efrotomycin has shown no cross resistance with commonly used antibiotics including tetracyclines, macrolides, aminoglycosides and penicillins.

In analogy to X-5108 and mocimycin,[13~15] efrotomycin has been found to have good growth enhancement activity in farm animals. This property, coupled with the substantial activity against important bacterial pathogens of animals, suggests that efrotomycin might be a useful antibiotic for veterinary medicine. Detailed reports on the microbiological and therapeutic properties of the antibiotic will be discussed in a separate report.

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