A RESTING CELL SYSTEM FOR EFROTOMYCIN BIOSYNTHESIS

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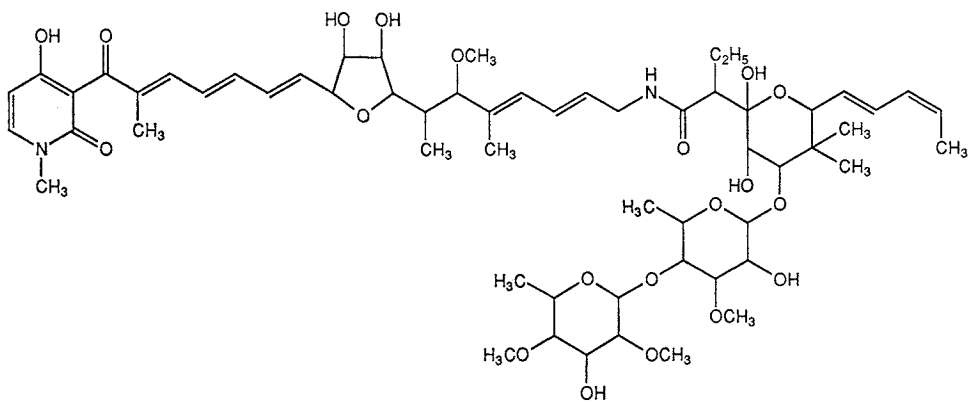
Efrotomycin, a modified polyketide antibiotic with utility as a growth permittant in the animal industry, is produced by *Nocardia lactamdurans*. A resting cell system has been developed to facilitate biosynthetic studies. Washed cells harvested from oil-based medium at 64 hours and resuspended in buffer produce up to 700 mg/ml efrotomycin in 60 hours when supplemented with a carbon source, optimally glucose. No evidence of carbon or nitrogen repression was observed. Productivity declines progressively with cell age but becomes less dependent on ongoing protein synthesis. The protein synthesis dependent and independent systems were used to study carbon utilization, incorporation of labeled precursors and to examine inhibitors of efrotomycin biosynthesis. A system derived from an efrotomycin non-producer was used to examine the glycosylation of the aglycone, aurodox, to efrotomycin through a monosaccharide form, 6'-deoxyallosyl aurodox.

Efrotomycin is an antibiotic of the elfamycin family with utility as a growth permittant in swine and chickens[1]. It is the disaccharide of aurodox or X-5108, as it was first called[2], and exhibits the pattern of labeling by acetate, propionate, butyrate and methyl groups of methionine established for aurodox[3]. The structure of the disaccharide moiety has recently been established as 6-deoxy-4-*O*-(6-deoxy-2,4-di-*O*-methyl-α-L-mannopyranosyl)-3-*O*-methyl-β-D-allopyranose attached *via* an α linkage from the allose C_1 to the hexahydropyran substructure of aurodox (Fig. 1)[4]. The three *O*-methyl groups on the sugar residues are derived from the methyl group of methionine and the rest of the carbon probably arises from glucose units.

The origin of the pyridone ring and of the peptide bond are unknown. No intermediates have been isolated and the sequence of macrolide condensations, glycosylations, and methylation is unknown. To facilitate biosynthetic studies a resting cell system has been developed. It has provided a simple

Fig. 1. The structure of efrotomycin.



means of examining the incorporation of isotopically labeled precursors, the effect of compounds on biosynthesis and the conversion of aurodox to efrotomycin.

A preliminary account of this work has been reported elsewhere[5].

Materials and Methods

Cultures

Nocardia lactamdurans, MA5887, was maintained on slants or as frozen vegetative mycelia in NYG seed medium containing in g/liter; nutrient broth 4, yeast extract 2, and glucose 10. After a 20-hour seed stage in NYG medium at 26°C, cells were grown in YEME containing in g/liter; yeast extract 10, malt extract 10, and glucose 10, or a soy bean oil-based production medium for 64 hours at 26°C or longer as noted. The cells were harvested by centrifugation at 3,000 rpm for 15 minutes, washed twice with distilled water, and resuspended at approximately 30 mg dry weight/ml in 0.1 M Tris-HCl, pH 8.0. This suspension, at 2 times final concentration, can be stored for up to 24 hours at 4°C without loss of synthetic capacity.

Resting Cell Incubation

Incubations were performed in volumes ranging from 0.5 ml (16×125 mm tubes) to 2.5 ml (25×180 mm tubes) or up to 40 ml in a 250-ml Erlenmeyer flask at 26°C at 220 rpm in a gyratory shaker with the addition of a carbon source, 1.2% glucose, as noted, and with 0.1 μg/ml vitamin B_{12}. There is no increase in dry cell weight for cells harvested at age 40~120 hours during the resting cell incubation period.

Inhibition of protein synthesis by chloramphenicol was assessed by the rate of incorporation of [^{35}S]methionine (New England Nuclear) at a concentration of 1 μg/ml and a specific activity of 124 μg/μmol. After incubation at 30°C, with shaking, for various lengths of time, 0.1 ml of cells was placed on Whatman 3 mm filter paper. The filters were washed successively in cold 10% TCA, hot 10% TCA, twice in cold 3% perchloric acid, twice in ethanol, dried, and counted in a scintillation counter.

Glutamine synthetase was assayed as described for *Streptomyces cattleya*[6] and lipase by radioactive tripalmitate hydrolysis[7].

Analytical Procedures

Efrotomycin was assayed by a colorimetric procedure using $AlCl_3$[8]. The aglycone, aurodox, and its monosaccharide react equally in the $AlCl_3$ color reaction. The three forms can be separated and assayed by HPLC using a Hamilton PRP-1 column, and isocratic elution with 0.03 M ammonium phosphate pH 7.0 and acetonitrile (67 : 33) at ambient temperature and a flow rate of 1.2 ml/minute. The relative retention times for the monosaccharide, aurodox and the disaccharide, efrotomycin are 0.43, 0.57 and 1.0. The forms may also be separated by TLC on silica plates containing F_{254} indicator developed in chloroform - methanol (4 : 1). The Rf values of monosaccharide, aurodox and efrotomycin are 0.49, 0.62 and 0.69, respectively. Duplicate determinations on a single sample agree within 3%. All the determinations cited are the results of analyzing duplicate samples and agreed within 5%.

A non-producing mutant (np15) was obtained following UV treatment at 1% survival of fragmented cells. The suspension for mutagenesis was prepared by gentle removal with glass beads of aerial mycelium grown for 7 days at 28°C on confluently plated 1/3 ELG agar containing in g/liter; yeast extract 0.33, NZ-Amine Type E (Sheffield Chemical) 0.66, meat extract (Difco) 0.33, glycerol 10, sucrose 20, $CaCl_2 \cdot 2H_2O$ 1.47, $MgCl_2 \cdot 6H_2O$ 2.03, agar 20. Survivors were plated on 1/3 ELG agar and then patched onto isolated wells of the same medium composition. A 7-mm plug of each was removed at 7 days for bioassay with the indicator organism *Bacillus cereus* (Difco). Zones of inhibition of 21±1 mm are seen with MA5887. np15 was selected as it did not produce any inhibitory component. It does not synthesize any efrotomycin-like products when assayed by either HPLC or TLC subsequent to growth in liquid medium.

Aurodox was obtained from Dr. PHILLIP MILLER, then at Hoffmann-LaRoche, Inc., Nutley,

NJ 07110, U.S.A. Thiolactomycin was obtained from Chugai Pharmaceutical Co., Ltd., Takada, Toshima-ku, Tokyo 171, Japan.

Results

Derivation of Resting Cell System

Early studies on washed cells derived from medium YEME gave rise to a system producing 50 μg/ml efrotomycin after 48 hours incubation. This could be increased to 200 μg/ml by the addition of an exogenous carbon supply, optimally 1.2% glucose, but was not further increased by any additional supplementation of amino acids. A more productive system was derived from cells harvested from a soy bean oil-based complex production medium. Productivity, stimulation by exogenous carbon source and dependence upon continuing protein synthesis vary significantly with cell age. The kinetics of efrotomycin synthesis (over 64 hours incubation period), are shown in Fig. 2, without added carbon source and with glucose or soy bean oil. The only other stimulatory additive is vitamin B_{12}. At 0.1 μg/ml, it stimulates glucose-supplemented production by 15~20% (Fig. 2), and non or oil supplemented production by 5~10%.

The ability of other carbon sources to support production is shown in Table 1, for incubation carried out for 64 hours. Dextrin and all the disaccharides tested are inactive, and no sugar is more active than glucose. In contrast to cells grown in YEME medium, cells pregrown in the oil-based complex medium are capable of using oil as a carbon source, although with a lag relative to glucose (Fig. 2). The ability to use oil depends upon the induction of lipase during the growth phase prior to harvesting of the cells. If soy bean oil is replaced in the growth medium by glycerol, lipase is not induced and oil cannot be

Fig. 2. Production of efrotomycin (μg/ml) by oil-derived cells obtained after 64 hours of growth harvested at 64 hours.

● No added carbon source, ▲ soy bean oil 40 mg/ml, □ glucose 12 mg/ml, ■ glucose 12 mg/ml+ vitamin B_{12} 0.1 μg/ml.

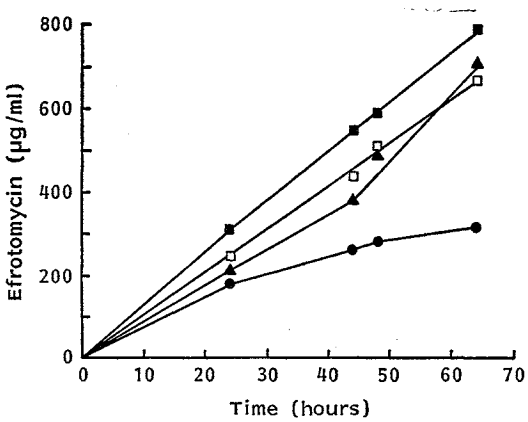


Table 1. The effect of various carbon sources on efrotomycin synthesis by MA5887.

	Addition (%)	Production of efrotomycin at 64 hours % relative to glucose[a]
No addition		46
Glucose	1.2	100
Mannose	1.2	72
Mannitol	1	89
Sorbose	1	50
Sorbitol	1	74
Galactose	1	85
α-Methyl glycoside	1	47
Fructose	1.2	76
Arabinose	1.2	83
Ribose	1.2	83
Melibiose	1	77
Lactose	1	48
Sucrose	1	47
Maltose	1	25
Dextrin	1	50
Glycerol	2	72
Oil	2	106

[a] Glucose supplemented productivity was 660 μg/ml. The results are an average of two experiments.

Each was added at its optimal level to cells harvested at 64 hours and incubated for 64 hours after carbon source addition.

Table 2. Production of aurodox, monosaccharide (MS) and efrotomycin by resting cells.

Age of cells (hours)	Initial glucose (%)	Productivity (μg/ml)				Stimulation by glucose
		Efrotomycin	MS	Aurodox	Total	
29	—	26	12	64	102	
	1.2	34	31	35	100	1.0
40	—	68	14	28	110	
	1.2	97	39	20	156	1.5
48	—	126	9	7	142	
	1.2	148	105	—	253	1.8
64	—	125	6	6	137	
	1.2	230	30	—	260	1.9
96	—	171	15	10	196	
	1.2	200	35	—	235	1.2
120	—	173	17	—	190	
	1.2	175	33	—	208	1.1

Cells were harvested at various ages and incubated with and without glucose for 16 hours under resting cell conditions.

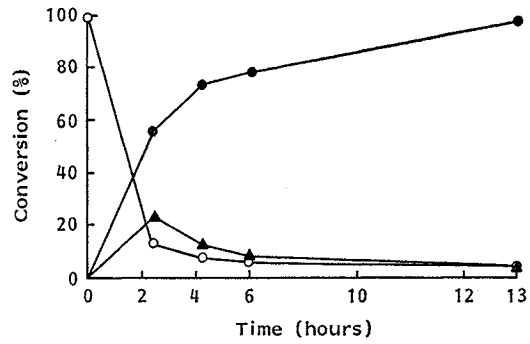
utilized by washed cells.

Glycosylation Reactions

Total productivity and the degree of glycosylation found upon analysing efrotomycin-related products vary with harvest age (Table 2). Cells harvested at 40 hours and younger show little glucose stimulation and a relatively high proportion of the underglycosylated forms, aurodox and its monosaccharide. The monosaccharide appears in HPLC analysis of incubations as an early eluting peak with an retention time (R_T) of 3.2 minutes. It was identified (B. ARISON and J. SMITH; personal communication) by NMR and mass spectrophotometric analysis as 6′-deoxyallosyl aurodox, a monosaccharide lacking the 3′-methyl group present in efrotomycin. Very little aurodox is seen in cells harvested at 48 hours or older even in the absence of glucose, but appreciable amounts of the monosaccharide are seen longer than 16 hours of resting cell incubation at all harvest ages. With longer resting cell incubation periods, for cells harvested at 64 hours or later, essentially all the product is fully glycosylated.

Fig. 3. Conversion of aurodox (○), to 6′-deoxyallosyl aurodox (▲) and efrotomycin (●) in resting cells of np15.

Each form is expressed as a percentage of the total HPLC-analyzed material.



A resting cell system derived from a non-producing mutant of MA5887, np15, converts aurodox to efrotomycin through an intermediate form identical with that found in resting cell incubations as described above. The kinetics of this conversion is shown in Fig. 3 for 72 hours resting cells prepared from np15. Cells harvested at 96 or 120 hours show similar but faster conversion. 6′-Deoxyallosyl aurodox added to this non-producing mutant cells under resting conditions is fully converted to efrotomycin, presumptive evidence that this is an intermediate.

Dependence of Resting Cell System on Protein Synthesis

Chloramphenicol (50 μg/ml) inhibits the incorporation of [^{35}S]methionine into protein by 98%

Table 3. Effect of chloramphenicol on resting cell synthesis of efrotomycin as a function of the age of cells employed.

Age of cells (hours)	Inhibition by chloramphenicol (%)
29	85
40	72
48	65
64	47
96	35
120	21

Production is the sum of efrotomycin and underglycosylated forms after 16 hours resting cell incubation, without exogenous carbon source. Chloramphenicol, when used, is at 50 μg/ml.

Table 4. IC_{50} for various inhibitors of efrotomycin synthesis.

	IC_{50} (mM)
Methylation inhibitors	
Methionine	3.0
Ethionine	0.05
Sinefungin	0.15
Dihydroxypyridine	0.36
Fatty acid synthesis inhibitors	
Cerulenin	0.14
Thiolactomycin	None
Membrane perturbants	
Phenethyl alcohol	0.2%
Toluene	0.3%
SDS	0.2%
Lecithin	0.2%
Ethanol, methanol	3.0%
Miscellaneous	
Phosphate	2.0
Arsenate	0.15
Arsenite	0.1
Co^{2+}	0.025
Cd^{2+}	0.1
Zn^{2+}	0.18
EDTA	0.4
O-Phenanthroline	0.5

Cells were harvested at 96 hours, and incubations performed for 16 hours in the absence of exogenous carbon source, and presence of 50 μg/ml chloramphenicol.

in *N. lactamdurans* within 2 minutes of addition, and is more than 95% inhibitory for the subsequent 18 hours. After 24 hours about 20~40% recovery of [^{35}S]methionine incorporation is seen, possibly due to inactivation of chloramphenicol to a subinhibitory level since full inhibition can be maintained by a second addition of 25 μg/ml chloramphenicol at 18 hours. The uninhibited protein synthetic rate varied little over the age range shown in Table 3, diminishing only slightly with 120 hours cells compared with younger cells. Table 3 shows the effect of inhibiting protein synthesis on efrotomycin productivity in resting cells without exogenous carbon source. The effect in the presence of glucose or oil was similar. Thus at all ages maximal synthesis of efrotomycin is dependent upon ongoing protein synthesis. However, in cells harvested at 64 hours or later, this dependence is reduced to less than 50%. Such cells, treated with chloramphenicol, are suited to a study of inhibitors or activators of efrotomycin synthesis which are relatively independent of effects on protein synthesis.

As shown above, there is no evidence for glucose inhibition of efrotomycin synthesis under resting cell conditions. Similarly, no evidence was found for nitrogen repression. NH_4Cl up to 50 mM is completely without effect under these conditions, although amounts as low as 5 mM NH_4Cl cause rapid, reversible and nearly complete inactivation of glutamine synthetase, demonstrating its ready access to the cell. Phosphate is inhibitory with an IC_{50} of 2 mM (Table 4). Thus of these common repressors of secondary metabolism only phosphate has a clear inhibitory effect in the relative absence of protein synthesis.

Stimulation and Inhibition of Efrotomycin Synthesis

Resting cells harvested at 96 hours, treated with 50 μg/ml chloramphenicol and incubated with no exogenous carbon source were used to search for effectors of efrotomycin synthesis. No amino acid, either singly or in combinations stimulates production beyond that shown by glucose or oil

addition. Among vitamins, cofactors and trace elements examined, only vitamin B_{12} at 0.1 μg/ml is stimulatory, increasing efrotomycin synthesis by 15~20% over the whole time course (Fig. 2). Vitamin B_{12} is now routinely added to resting cell incubation. The addition to the resting cell incubation of trace elements used in the growth media (Fe^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Mo^{2+}, Cu^{2+} and BO_4^{2-}) have no effect. Failure to add Fe^{2+} at 50 mg/liter to the pregrowth media results in 5-fold lower efrotomycin productivity even though growth is barely affected.

The IC_{50} for a variety of inhibitors are listed in Table 4. Oxidative phosphorylation inhibitors, arsenite and arsenate were inhibitory. Cobalt, cadmium and zinc are the only inhibitory metals found. The metal chelators EDTA and *o*-phenanthroline cause lysis of the cells at only slightly higher levels than their IC_{50} (about 2-fold), suggesting that the inhibitory effects seen are due to non-specific membrane disruption. All membrane perturbants tested are inhibitory at fairly low concentrations.

The fatty acid synthesis inhibitor, cerulenin, inhibits efrotomycin synthesis with an IC_{50} of 0.14 mM (30 μg/ml), a sensitivity similar to that reported for the synthesis of several macrolides by ŌMURA[9]. Degradation of cerulenin to subinhibitory levels occurs under resting cell conditions as noted in some other systems. Thiolactomycin is reported[10] to be a selective inhibitor of type II fatty acid synthetases, the monomeric acyl carrier protein dependent synthase present in many bacteria. Type I synthases, the multimeric form found in mammals and yeast, are unaffected. Thiolactomycin has no effect on efrotomycin synthesis in resting cells. It is apparently taken up by the cell, since growth of *N. lactamdurans* is inhibited by 5 μg/ml.

The inhibition of efrotomycin synthesis by thymine and thymidine (Table 5) appears to be a specific effect on the efrotomycin pathway by thymine itself or its degradation products. Uridine reverses thymidine inhibition by preventing thymidine degradation to thymine. Thymine inhibition can be about 50% reversed by β-alanine, a degradation product of uracil, an effect which appears related to the flow of substrates to early efrotomycin biosynthetic steps and not to CoA synthesis because CoA synthesis may be fully inhibited under resting cell conditions by 40 μg/ml sodium salicylate, a known inhibitor of pantoate synthesis[11], without any effect on efrotomycin synthesis.

Efrotomycin contains 9 methyl groups derived from methionine (Fig. 1)[12], four *O*-linked, four *C*-linked and one *N*-linked groups. Several methylation inhibitors affect overall synthesis as shown in Table 4. Ethionine is profoundly inhibitory, without causing the synthesis of any new UV absorbing, efrotomycin related species; sinefungin leads to the accumulation of reduced amounts of a demethyl product, 20-*O*-demethyl-efrotomycin (A. KEMPF and K. WILSON; personal communication).

In *Streptomyces avermitilis*, methylation inhibition is less profound and leads to the accumulation of a demethyl, or ethyl (when ethionine is used) analogues[13].

Table 5. Effect of purine and pyrimidine bases and nucleosides on efrotomycin synthesis in resting cells.

Nucleosides and bases	IC_{50} (mM)
Cytidine	None
Uridine	None
Thymidine[a]	0.4
Adenosine	0.5
Guanosine	None
Cytosine	None
Uracil	None
Thymine[b]	0.16
Adenine	0.7

[a] Reversed by 1 mM uridine or cytidine.
[b] Partially reversed by 2.5 mM β-alanine.
Conditions are as in Table 4.

Isotopic Labeling

An example of the utility of the resting cell system is shown in Table 6. Propionate was

used as precursor, in the most productive system, to prepare highly labeled efrotomycin for metabolic studies in animals. Lines 1~4 represent preliminary trials in single flasks to illustrate the reproducibility of the system. Line 5 represents the final large scale conditions to produce efrotomycin labeled to a specific activity approaching that of the precursor. Previous attempts to label in complex media showed much greater isotopic dilution. Label distribution had been confirmed prior to the final preparation by [^{13}C]propionate incorporation at a single site (C-7), as was demonstrated for aurodox[3] (H. E. MERTEL *et al.*; personal communication).

Table 6. Extent of [^{14}C]propionate incorporation into efrotomycin by resting cells.

[1-^{14}C]Propionate, added		Isolated efrotomycin (μg/mmol)	RMSA[a]
μCi/ml	Specific activity (μg/mmol)		
0.5	10	0.06	0.006
200.0	14.5	3.7	0.26
500.0	14.5	6.4	0.44
1,000.0	14.5	8.8	0.61
1,000.0	58.0	49.0	0.84

[a] RMSA: Relative molar specific activity, efrotomycin/propionate.

Cells were harvested at 64 hours, and supplemented with 1.2% glucose. Incubation was for 72 hours except for the first line (16 hours). This experiment was performed before the routine addition of vitamin B_{12}.

Discussion

The resting cell system described here is highly productive, reaching 750 μg/ml of efrotomycin in 65 hours when supplemented with glucose and vitamin B_{12}. Our results indicate that efrotomycin synthesis, unlike a number of other secondary metabolites, is insensitive to glucose inhibition. Production of efrotomycin in complex or defined media, however, is repressed by glucose (unpublished results), suggesting that the glucose-sensitive step(s) lie outside the direct biosynthetic pathway to efrotomycin. Soy bean oil under conditions appropriate for lipase induction in the growth period is as effective a carbon source as glucose, after an initial lag period.

A system relatively independent of protein systhesis may be derived by the addition of chloramphenicol to cells harvested at 96~120 hours without severe loss of biosynthetic capability of the cells to produce efrotomycin. Using such a system a variety of agents were screened for their effects on antibiotic synthesis. No amino acid has any significant effect on synthesis, apparently none being direct precursors of any portion of efrotomycin. Of the vitamins, only B_{12} has any effect, stimulating synthesis by about 15%. Several metals and all membrane perturbants tested are inhibitors at low concentrations. Thymine or its breakdown products are inhibitors in a manner partially reversed by β-alanine, but unrelated to pantothenate biosynthesis. Cerulenin, as expected from its ability to inhibit polyketide biosynthesis, inhibits efrotomycin biosynthesis but thiolactomycin, an inhibitor of acyl carrier protein dependent fatty acid synthesis, is without activity, supporting the view that polyketide condensation is catalysed by a macrolide synthetase differing mechanistically from fatty acid synthetase. Several methylation inhibitors depress overall efrotomycin synthesis severely, rather than causing the accumulation of demethyl products, suggesting that early mandatory steps involving methylation may exist in the pathway.

The resting cell as described also has utility of studying terminal steps in efrotomycin synthesis, as exemplified here by glycosylation. Aurodox fed to a non-producing mutant is converted first to a monosaccharide and then to the disaccharide efrotomycin. Finally, the resting cell system is well suited for the preparation of highly radioactive labeled efrotomycin in a simple system with minimal precursor dilution.

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