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The biosynthetic origin of the pyridone ring of efrotomycin

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SUMMARY

Nocardia *lactamdurans* has been shown to catabolyse uracil via the reductive pathway. The end product of this pathway, β-alanine, is incorporated into the pyridone ring of efrotomycin. Support for this proposal includes: (1) reversal of thymine inhibition of efrotomycin biosynthesis by dihydrouracil and *N*-carbamoyl-β-aline, two intermediates of the catabolic pathway; (2) incorporation of [5,6-^{3}H]-uracil into efrotomycin with a relative molar specific activity of approximately 0.5, close to the theoretical maximum; and (3) ^{13}C coupling at C_4 and C_5 of efrotomycin after feeding resting cells with [4,5-^{13}C]-uracil. Our results do not rule out the possibility of an alternative source of β-alanine or the coexistence of uracil catabolism via oxidative reactions.

INTRODUCTION

Efrotomycin (Fig. 1) [18] is a member of the elfamycin class of antibiotics. Representatives of this class of compounds share a common mode of action, namely inhibition of protein synthesis due to interaction with elongation factor Tu (reviewed in [13]). Glycosylation at C_{31} distinguishes efrotomycin from earlier members of this group, aurodox [1], heneicomycin [19] and kirrothricin [14]. In common with these antibiotics, efrotomycin contains a chain-terminating 4-hydroxy pyridone moiety.

The biosynthesis of aurodox was studied in detail by using both ^{14}C [8] and ^{13}C-labelled substrates [9]. The polyketide nature of the antibiotic was clearly demonstrated by using ^{13}C-labelled substrates; 13 molecules of acetate, 1 butyrate and 1 propionate were incorporated into the molecule. In the case of efrotomycin the labelling pattern is similar. In addition, the peptide nitrogen has been shown to originate from glycine [11]. In none of these studies was the origin of the pyridone ring established.

The first improved *Nocardia lactamdurans* mutant isolated in a traditional strain improvement program was retrospectively shown to be resistant to 5-fluorouracil (FU) and to accumulate uracil (≥ 5 mM) during growth

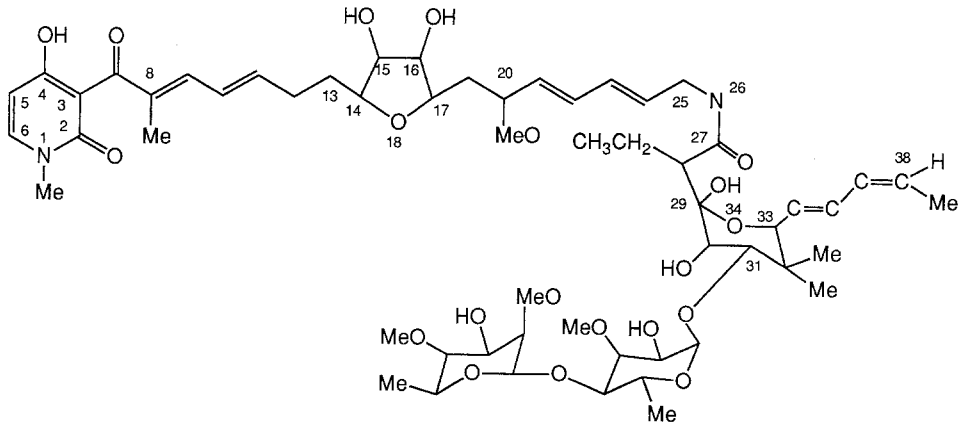

Fig. 1. Chemical structure of efrotomycin. The numbering system is taken from Dewey et al. [4].

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in a chemically defined medium. During the later stages of the fermentation, after growth had ceased, uracil was gradually depleted to undetectable levels. This phenotype was subsequently reproduced in a series of spontaneous FU-resistant mutants. All mutants exhibited improved productivity. A rough correlation was found between the peak uracil concentration and efrotomycin titer [6]. Resistance and uracil overproduction could be attributed, at least in part, to derepression of the pyrimidine biosynthetic pathway [10]. In addition, fermentations which fail to yield optimal concentrations of efrotomycin are often found to have high concentrations of uracil in the medium.

The data presented below demonstrate the importance of uracil in efrotomycin biosynthesis, and strongly suggest that β-alanine derived from the reductive catabolism of uracil is incorporated into the pyridone moiety of efrotomycin.

MATERIALS AND METHODS

Bacteria and culture conditions

N. lactamdurans MA6384 was isolated after several rounds of nitrosoguanidine mutagenesis of *N. lactamdurans* MA2908. It differs from the original soil isolate by virtue of resistance to FU. MA6462 was derived from MA6384 by selection for resistance to 5-fluorodeoxyuridine (FUdR) by virtue of the loss of thymidine kinase (Tdk) [3].

Cells were grown routinely in a defined medium consisting of (per liter): NH_4Cl, 2 g; K_2HPO_4, 1.3 g; NaCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; aspartic acid, 0.5 g; monosodium glutamate, 8.25 g; sodium citrate, 0.5 g; inositol, 0.2 g; $CaCO_3$, 25 mg; $FeSO_4 \cdot 7H_2O$, 25 mg; $MnSO_4 \cdot 5H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; and $CuSO_4 \cdot 5H_2O$, 2 mg. The pre-sterile pH was adjusted to 7.0 with HCl. Glucose and glycerol were added after sterilization to final concentrations of 2% (w/v) each. The incubation temperature was 30 °C. Cultures were started from frozen (−70 °C) vegetative mycelia, representing mid-late log phase cells, prepared in defined medium. After 24 h, a 1/20 dilution was made into fresh medium and incubation was continued for the indicated period of time.

Efrotomycin biosynthesis

All efrotomycin biosynthetic studies were performed with washed cell suspensions [12]. After 72 h of growth, the cells were washed and resuspended in 1/2 culture volume of water. The reaction was begun by adding the aqueous cell suspension to an equal volume of 0.1 M Tris · HCl (pH 8) containing 0.1 μg/ml vitamin B_{12}. Other additions were as described in the text.

HPLC techniques

Efrotomycin was isolated by isocratic HPLC in a solvent system consisting of 60% of phosphate buffer (2.72 g/l KH_2PO_4 and 3.66 g/l Na_2HPO_4) and 40% acetonitrile. The column temperature was maintained at 35 °C.

^{14}C-Labelled material was isolated at a flow rate of 1.5 ml/min from a 150 × 4.1 mm PRP-1 column (Hamilton Co.). Material eluting between 5.3 and 6.3 min was collected and checked for purity after concentration. ^{13}C-Labelled efrotomycin was obtained from a preparative (250 × 21.5 mm) PLRP-S column at a flow rate of 5 ml/min. Material eluting between 48.5 and 57.7 min was collected and verified to be efrotomycin.

Uracil catabolism by cell free extracts

Cells were harvested after 72 h of growth, washed with 10 volumes of buffer consisting of: 50 mM Tris, 2 mM dithiothreitol, 10 mM $MgCl_2$ and 10% (w/v) glycerol (pH 7.5). Washed cells were resuspended in 1 volume of the same buffer supplemented with 1 mM phenylmethyl sulfonyl fluoride and 2 μg/ml leupeptin. Spheroplasts were prepared by the addition of lysozyme to a final concentration of 2.5 mg/ml and incubated for 75 min at room temperature. At this time, octyl-β-D-glucoside was added to 4 mg/ml and incubation was continued for an additional 10 min. During the incubation period the cells were gently mixed with a magnetic stirrer. After cooling for 60 min in an ice bath, the spheroplasts were lysed by sonication. The 100000 × g supernatant was used as a source of crude enzyme.

The reaction was initiated by adding 10 μl of crude enzyme to 40 μl of a solution containing: 150 nmol glucose 6-phosphate, 15 nmol NADPH, 0.3 unit glucose 6-phosphate dehydrogenase, 1 μC [2-^{14}C]-uracil (specific act. = 54.5 μCi/μmol). The reaction was terminated at the indicated time by immersion for 2 min in a boiling water bath. The products of the reaction were separated by TLC [15]. Quantitation of the chromatogram was performed with a Bioscan, $^{14}CO_2$ release was estimated by the difference between the initial and final dpm in the reaction mixture.

Isotopes

Radioactive uracil, [5,6-^{3}H]-uracil (45.7 Ci/mmol) and [2-^{14}C]-uracil (54.4 mCi/mmol) was obtained from New England Nuclear. [4,5-^{13}C]-uracil (99% pure) was purchased from Cambridge Isotope Laboratory.

RESULTS

The effect of thymine and thymidine on efrotomycin biosynthesis

A simple and reproducible method of inhibiting the efrotomycin fermentation in synthetic medium is by the addition of 1 mM thymine or thymidine. The inhibition is accompanied by the accumulation of uracil. This is probably due to an apparent inability of the cells to metabolize uracil in the later stages of the fermentation rather than a defect in uracil biosynthesis (data not shown). The inhibition of efrotomycin biosynthesis by thymine can be duplicated with resting cells (Table 1).

The data are expressed as the concentration of compounds needed to inhibit efrotomycin biosynthesis by 50%. The IC_{50} is approx. 0.2 mM in thymine for all strains tested. Thymine and thymidine (TdR) are equally potent inhibitors of efrotomycin biosynthesis by resting cells. The fact that MA6462, a Tdk-negative strain, shows the same sensitivity as its parent (MA6384) argues that phosphorylation to the nucleotide level is not required for inhibition. Although deoxyadenosine increases the incorporation of thymine into DNA [3], it does not potentiate the inhibitory effect of thymine. Among thymidine analogs which were tested and found to be inactive were thymine arabinoside and thymine riboside, neither of which is considered to be a substrate for the thymidine phosphorylase and thus not likely to yield free thymine. Taken together, the data imply that thymine is the actual inhibitor.

TABLE 1

The effect of thymine and thymidine on efrotomycin biosynthesis by resting cells of *N. lactamdurans*

Strain	Phenotype[b]	IC_{50} (mM)[a]		
		T[c]	T + AdR	TdR
MA2908	wt	0.17	0.18	nd[d]
MA6384	FU^R	0.15	0.16	0.19
MA6462	Tdk^-	0.16	0.17	0.23

[a] Concentration needed to inhibit efrotomycin production by 50%.

[b] wt, wild type; FU^R, fluorouracil-resistant; Tdk^-, thymidine kinase-negative.

[c] Abbreviations: T, thymine; TdR, thymidine; AdR, deoxyadenosine.

[d] Not determined.

Thymine inhibition of uracil catabolism by resting cells

The simplest explanation for the above observations is that the inhibition of uracil catabolism by thymine interferes with efrotomycin biosynthesis. The inhibition of uracil catabolism by thymine has been noted previously [17]. The data in Fig. 2 were obtained from resting cells prepared after 72 h of growth in defined medium. The ordinate represents total radioactivity remaining in 1 ml of whole broth after exposure to [2-^{14}C]-uracil. In the absence of added thymine the loss of radioactivity was rapid,

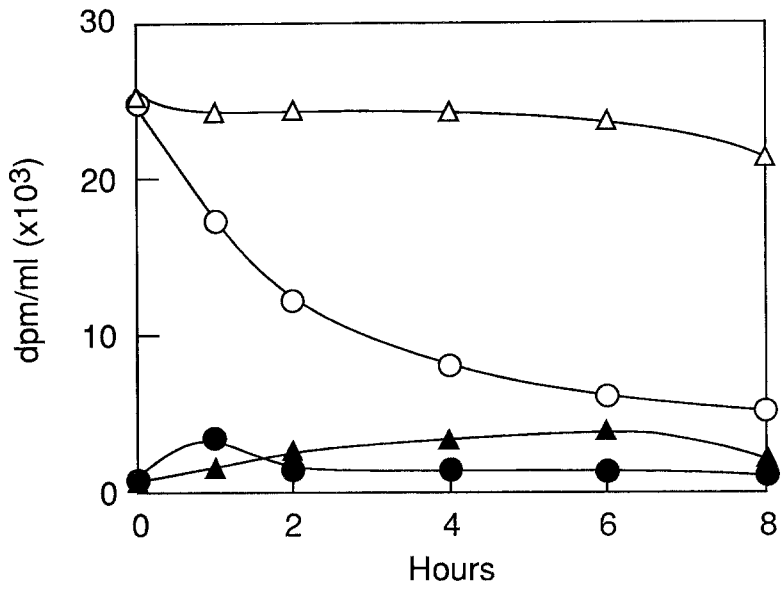

Fig. 2. Inhibition of uracil catabolism by thymine. [2-^{14}C]-uracil 0.1 mM (specific activity = 0.1 μCi/μmol) was added to a resting cell suspension and the recovery of radioactivity from whole broth (open symbols) was monitored. Cell-associated radioactivity (closed symbols) was determined by filtration. Circles, no additions; triangles, addition of 1 mM thymine.

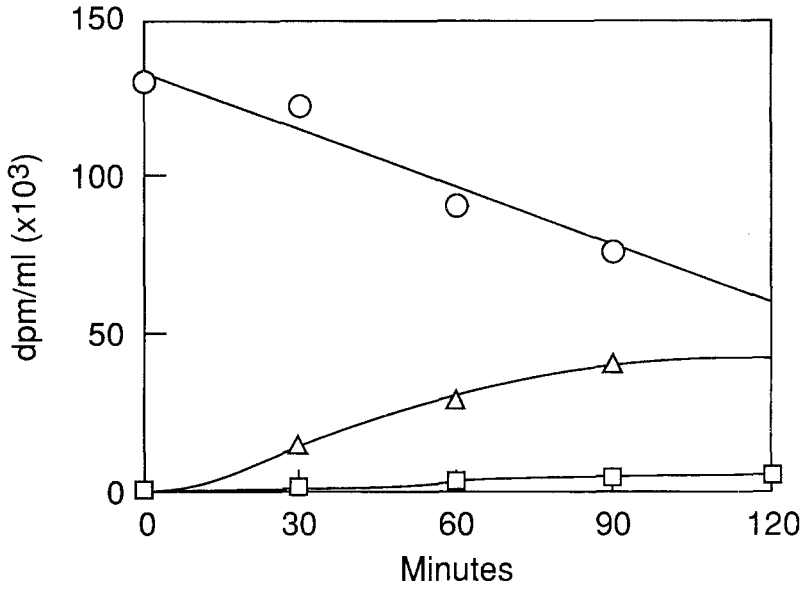

Fig. 3. Catabolism of [2-^{14}C]-uracil by cell free extracts of *N. lactamdurans*. The reaction mixed contained 100 μg of protein and 180 nmol of [2-^{14}C]-uracil (sp. act = 55 μCi/μmol) in a total volume of 50 μl. The reaction was terminated by heating for 2 min in a boiling water bath, centrifuged and 2.5 μl analysed by TLC as described in MATERIALS AND METHODS. Circle, uracil; square, dihydrouracil; and triangle, *N*-carbamoyl-β-alaline.

about 70% of the input radioactivity was lost, as $^{14}CO_2$, in the first 4 h. Of the remaining radioactivity, approx. 20% is retained by 0.2-μm glass fiber filters, and represented about 6% of the initial radioactivity.

The addition of 1 mM thymine had a profound effect on uracil catabolism. Loss of radioactivity was virtually eliminated by thymine addition. The amount of radioactivity retained on filters peaked at about 15% at 6 h. The increased level of cellular incorporation may be accounted for by the higher concentration of radioactivity which persists throughout the incubation period.

Uracil catabolism by cell free extracts

Uracil catabolism can be either oxidative or reductive in nature (reviewed in [16]). The reductive pathway with β-alanine as one of the end products [2] was thought to be the most likely catabolic route (see Fig. 4). In order to demonstrate the presence of this pathway in *N. lactamdurans*, a crude enzyme preparation (100000 × *g* supernatant) was allowed to react with [2-^{14}C]-uracil in the presence of an NADPH generating system and the products of the reaction separated by the TLC system of Traut and Loechel [15]. Due to the loss of the C_2 of uracil as CO_2, β-alanine can not be detected in this assay. Figure 3 summarizes the results of a typical experiment. Uracil was catabolysed with a $t_{1/2}$ of about 75 min and two intermediates of the reductive pathway, dihydro-uracil

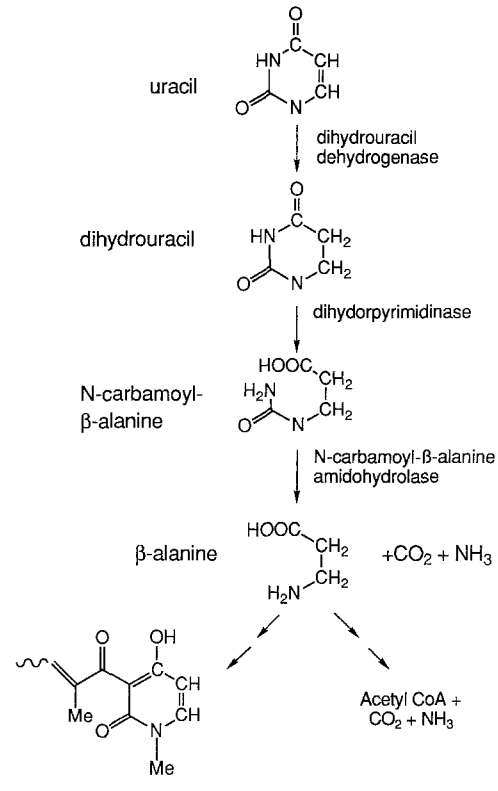

Fig. 4. Postulated pathway for the incorporation of uracil into efrotomycin.

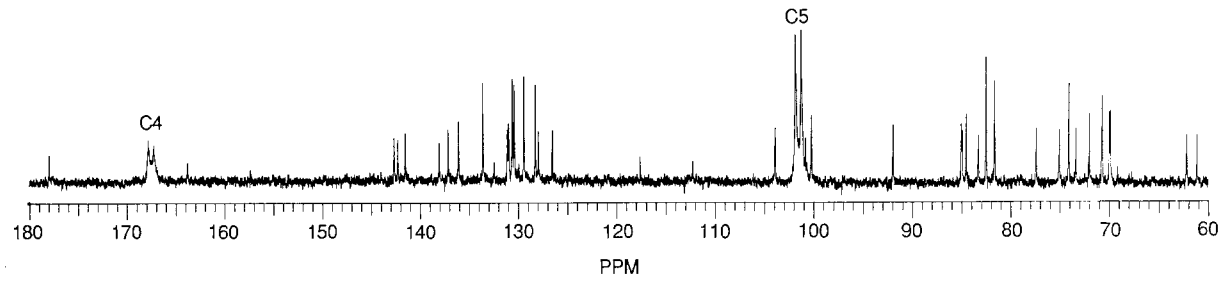

Fig. 5. ^{13}C-NMR spectrum of efrotomycin labelled with [4,5-^{13}C]-uracil.

(DHU) and *N*-carbamoyl-β-alanine (NCβA) were demonstrated. Independent experiments demonstrated the release of $^{14}CO_2$ was established by trapping with alkali (data not shown).

Having established the existence of the reductive pathway, the hypothesis outlined in Fig. 4 was formulated. It was proposed that β-alanine derived from the catabolism of uracil is incorporated into the nitrogen and carbons 4, 5 and 6 of the pyridone ring. Tests of this proposal are presented below.

Reversal of thymine inhibition by catabolites of uracil

The addition of 1 mM thymine or thymidine to washed cell suspensions inhibited efrotomycin biosynthesis by about 90% (Table 2). If inhibition is due to interference with uracil catabolism, it is expected that one or more of the intermediates of the reductive pathway should circumvent inhibition. At a concentration of 1 mM both DHU and NCβA overcame the adverse effects of thymine and thymidine; partial reversal was seen at 0.1 mM.

The addition of β-alanine (0.1 mM) resulted in only slight reversal of thymine inhibition. The interpretation of this observation is complicated by the fact that β-alanine itself is an inhibitor at the higher concentration. Dihydrothymine (DHT), the product of thymine reduction, had no effect on efrotomycin biosynthesis and did not prevent the inhibition caused by thymine or thymidine.

The incorporation of [5,6-^{3}]-uracil into efrotomycin

Washed cell suspensions incubated in the presence of [5,6-^{3}H]-uracil were extracted with ethyl acetate, an aliquot was removed to estimate the efficiency of incorporation and the remainder analyzed by HPLC. Center cuts of the efrotomycin peak were collected and the specific activity determined. Concentration was estimated by using A_{323nm} = 373 and radioactivity was determined in a liquid scintillation counter equipped with automatic

TABLE 2

Reversal of thymine and thymidine inhibition of efrotomycin biosynthesis by metabolites of uracil. Studies with washed cell suspensions of *N. lactomdurans* MA6462

Additions	mM	Percentage of control[a]		
		None	Inhibitors (1 mM)	
			Thymidine	Thymine
None		100.0	8.2	7.3
DHU[b]	1.0	92.7	99.0	108.7
	0.1	104.2	43.4	38.1
NCβA	1.0	111.6	112.3	101.8
	0.1	112.1	71.9	77.2
β-Alanine	1.0	63.7	22.1	23.3
	0.1	105.2	17.3	16.0
DHT	1.0	91.2	9.7	9.7
	0.1	117.5	7.2	11.3

[a] The control reaction produced 60 μg/ml efrotomycin after 21 h at 30 °C.

[b] Abbreviations: DHU, dihydrouracil; NCβA, *N*-carbamoyl-β-alanine; DHT, dihydrothymine.

TABLE 3

The incorporation of [5,6-^{3}H]-uracil into efrotomycin

Hours	Efrotomycin (μg/ml[a])	dpm/ml ($\times 10^{-5}$)[b]	Percentage incorporated	RMSA[c]
1.5	7	0.13	1.8	0.22
3.0	16	0.69	9.3	0.46
4.5	28	0.98	13.1	0.49
6.0	39	1.02	13.6	0.40
22.0	136	1.26	16.7	0.13

[a] Efrotomycin titer determined by HPLC.

[b] Total ethyl acetate extractable radioactivity.

[c] RMSA, molar specific activity of isolated efrotomycin/molar specific activity of uracil. Determined by using the center cut of the efrotomycin peak obtained by HPLC and an input specific activity determined to be 5.46×10^6 dpm/μmol.

quench correction. The results are summarized in Table 3. The specific activity of efrotomycin ($M_r = 1145$) was compared to that of the added uracil. Between about 3 and 6 h the molar specific activity of efrotomycin ($M_r = 1145$) relative to uracil approached 0.5.

Incorporation of [4,5-^{13}C]-uracil into efrotomycin

In order to confirm that incorporation of tritium was occurring in the pyridone ring and was not due to isotopic scrambling, efrotomycin was labelled with ^{13}C by incubation of a cell suspension with [4,5-^{13}C]-uracil. Since extensive catabolism was predicted labelled uracil was added incrementally at 0, 7 and 12 h. A total of 4.2 mg was added to 90 ml of cell suspension (3 × 30 ml in 250-ml flasks) and incubation continued for a total of 23 h. Efrotomycin (10.9 mg) was isolated by HPLC as described above.

The spectrum is reproduced in Fig. 5. Two relatively intense doublets, at 167.5 ppm and 101.5 ppm, representing C_4 and C_5 respectively, are noted. The degree of enrichment is estimated to be 10–15-fold. The coupling constant of 57 Hz is identical to that seen for these two carbon atoms when doubly labelled acetate is used to label efrotomycin (J. Nielsen and B. Arison, unpublished). The coupling indicates that carbons 4 and 5 of uracil are incorporated as a unit into the pyridone moiety of efrotomycin. These carbon atoms are expected to remain together during the catabolism of uracil to β-alanine.

DISCUSSION

While thymine was known for some time to be a potent inhibitor of the efrotomycin fermentation (Kaplan and Nielsen, unpublished), the significance of the finding remained obscure until the observation was made that it was also a potent inhibitor of uracil catabolism. Given the additional fact that the addition of thymine to fermentations in chemically defined media resulted in the accumulation of uracil (Darland and Greene, unpublished), it seemed possible that the two effects were related. The resting cell system [12] was ideally suited for testing this notion.

Thymine and thymidine are effective inhibitors of efrotomycin biosynthesis by resting cells with an IC_{50} of approx. 0.2 mM. The non-metabolizable analogs of TdR, thymine riboside and arabinoside, have no inhibitory effect. The susceptibility of the Tdk$^-$ mutant (MA6462) to TdR is identical to its parent (MA6384) arguing that the conversion to the nucleotide level is not necessary. The effectiveness of thymine is not improved by the inclusion of AdR even though this procedure is an effective method of stimulating the incorporation of thymine into DNA [3] presumably by increasing the availability of deoxyribose-1-phosphate [7]. This suggests that the conversion of thymine to thymidine is not necessary. Thus, it appears that thymine is the actual inhibitor. The possibility that a catabolite of thymine is responsible for the inhibition can not be ruled out, but is felt to be unlikely since dihydrothymine has no effect on efrotomycin biosynthesis.

The existence of the reductive pathway of uracil catabolism [2] has been established by demonstrating the production of two intermediates of this pathway, dihydrouracil and *N*-carbamyl-β-alanine. The production of β-alanine is implied by virtue of the rapid loss of radioactivity, as $^{14}CO_2$, from [2-^{14}C]-uracil. The inhibition of efrotomycin biosynthesis by thymine is overcome by either DHU or NCβA. This is consistent with the hypothesis that uracil catabolites are utilized in the biosynthesis of efrotomycin.

Contrary to predictions made by the model (Fig. 4), β-alanine was relatively ineffective in reversing the inhibitory effect of thymine. The ineffectiveness is probably due to the inhibitory effect of β-alanine itself at high concentrations. No rigorously tested model exists to explain the inhibition, it has been shown that the decarboxylation of β-alanine by whole cells and cell free extracts is quite rapid (Darland and Rosenbach, unpublished). The isolation of mutants defective in this reaction could provide insight into the mechanism of inhibition.

The incorporation [5,6-^{3}H]-uracil into efrotomycin by resting cells was performed in the absence of an external carbon source in order to minimize isotope dilution due to uracil synthesis. Under these conditions incorporation of tritium was rapid and efficient. Between 3 and 6 h the relative molar specific activity approached 0.5. The stereochemistry of uracil catabolism has been studied in detail by Gani and Young [5]. They established that the hydrogens on carbons 5 and 6 of uracil are retained, giving rise to carbons 2 and 3 of β-alanine. Loss of two of the four hydrogens from these carbons is expected to occur upon formation of the pyridone ring. A RMSA of tritium incorporation of 0.5 is consistent with either random loss of hydrogens or selective loss from one or the other of the carbon atoms.

Exposure of resting cells to [4,5-^{13}C]-uracil conclusively demonstrated the incorporation of C_4 and C_5 of uracil into the pyridone ring of efrotomycin. The 10–15-fold enrichment at both positions and the coupling constant of 57 Hz proves that the C–C bond was not broken during incorporation. The most likely route is via β-alanine. The inability to obtain either [2,3-^{13}C]- or [3-^{13}C, ^{15}N]-β-alanine precluded a direct test of the hypothesis. The efficiency of [1-^{14}C]-β-alanine incorporation into efrotomycin was low due to extensive decarboxylation and the inhibitory effect of β-alanine incorporation discussed above. The isolation of a β-alanine activating enzyme

from *N. lactamdurans* [11] provides independent support for the model.

In conclusion, the above data offer a ready explanation for the linkage uracil metabolism and efrotomycin biosynthesis referred to in earlier work [6]. It now appears likely that efrotomycin biosynthesis by the original soil isolate may have been limited by a relative scarcity of uracil. The isolation of fluorouracil-resistant mutants and the overproduction of uracil overcame this rate-limiting step.

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