3-METHYLPSEUDOURIDINE AS A FERMENTATION PRODUCT

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3-Methylpseudouridine (β isomer) has been identified in fermentation broths of *Nocardia lactamdurans*. It accumulates at quite high levels following the accumulation of extracellular uracil in strains exhibiting increased levels of *de novo* pyrimidine biosynthetic enzymes. It is labeled by exogenous uracil, and appears to result from an irreversible modification of one of the components of the elevated pyrimidine pool. Its methyl group is labeled efficiently by [*methyl*-^{14}C]methionine.

Nocardia lactamdurans produces efrotomycin[1], which is a modified polyketide of the kirromycin class of antibiotics[2]. The first improved efrotomycin producer in the genealogy derived from the original soil isolate excretes uracil under some conditions and is resistant to the pyrimidine analog 5-fluorouracil. The cause for this pyrimidine overproduction phenotype was demonstrated to be an approximately 10-fold elevation in the enzyme levels of the *de novo* pyrimidine synthetic pathway[3,4]. The pathway in the original soil isolate, MA 2908, is not regulated by pyrimidine feedback control[4]. Thus when the six enzymes are elevated coordinately, as they are in improved efrotomycin strains and in a set of spontaneous fluorouracil-resistant mutants, UMP synthesis is increased and pyrimidine excretion, in the form of uracil, takes place. We have noted the appearance of a new compound in the extracellular medium as the uracil level reaches its maximum value. This new compound, accumulating as uracil disappears, has been identified by NMR, MS and UV spectroscopy as 3-methylpseudouridine (3-MΨU) reported prior to now only as a chemically synthesized methylation product of pseudouridine[5]. 1-MΨU has been identified as a fermentation product of *Streptomyces platensis* var. *clarensis*[6].

Experimental

The strains of *N. lactamdurans* used are MA 2908, the original soil isolate for efrotomycin, derivatives of MA 2908 spontaneously resistant to 500 μg/ml 5-fluorouracil (FUR 1-44, 1-22, 1-24)[3] and MA 4820, the first improved strain in the efrotomycin genealogy, which is also 5-fluorouracil resistant. Cultures were grown in a defined medium containing in g/liter; glucose 20, glycerol 20, monosodium glutamate 8.25, NH_4Cl 2.0, aspartic acid 0.5, NaCl 0.5, K_2HPO_4 1.33, $MgSO_4 \cdot 7H_2O$ 0.5, gelatin 3, inositol 0.2, $CaCO_3$ 0.025, $FeSO_4 \cdot 7H_2O$ 0.025, $ZnSO_4 \cdot 7H_2O$ 0.010, $CuSO_4 \cdot 5H_2O$ 0.002, $MnSO_4 \cdot H_2O$ 0.005, pH 7.0.

Uracil and the new metabolite were separated and visualized by TLC on silica plates (EM Reagents) in $CHCl_3$ - MeOH, 4 : 1 (uracil Rf 0.54; the unknown Rf 0.43). They were quantitated using a reflectance scanning spectrophotometer (Shimadzu) with uracil and uridine standards. The molar extinction coefficient of uridine (1×10^4 at pH 7) was used for 3-MΨU after its identity was known. It was isolated by preparative TLC using several extractions with methanol to elute the UV-absorbing material from the silica. Final purification was performed by HPLC on a semipreparative PRP-1 column (Hamilton 7.0 mm i.d. $\times$ 30 cm), isocratically in 40 mM ammonium acetate pH 5.0, 1% methanol at room temperature and a flow rate of 2 ml/minute. The retention time was 30 minutes. UV

spectra were taken with a Beckman DU7 spectrophotometer. Pseudouridine (α and β forms) were obtained from Sigma.

[2-^{14}C]Uracil (57.4 mCi/mmol) was obtained from New England Nuclear, and L-[*methyl*-^{14}C]-methionine (56.7 mCi/mmol) from Amersham. *In vivo* incorporation experiments were performed with cells harvested at 60 hours, washed twice with distilled water and resuspended in fresh synthetic medium at the same cell density (18~20 mg dry weight/ml). After isotope addition the culture (0.5 ml) was incubated at 30°C capped 16×125 mm tubes with shaking at 220 rpm for the specified period. 3-MΨU was isolated by HPLC. Efrotomycin was isolated also by HPLC on a PRP-1 column using isocratic elution with 0.03 M ammonium phosphate pH 7.0 and acetonitrile (67 : 33) and a flow rate of 1.2 ml/minute[7]. Peaks were collected and counted after the addition of 10 ml Scintiverse fluor/0.5 ml eluant.

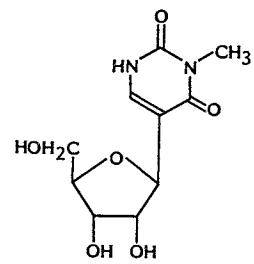
Results and Discussion

A new metabolite accumulates in the medium during the time course of uracil excretion by strains of *N. lactamdurans* expressing elevated levels of pyrimidine biosynthetic enzymes. It was isolated as described above and identified as 3-*N*-MΨU by combined NMR and mass spectrophotometric analyses.

The mass spectral observation that the molecular ion, at 258, was 14 mass units greater than pseudouridine led to the proposal of an *N*-methyl analog. A search of our substructural file for spectra containing *N*-methyl groups flanked by two carbonyls or by a carbonyl and a carbon-carbon double bond located a dozen examples with a combined chemical shift range between δ 3.2 and 3.8. The observed chemical shift of δ 3.30 (Table 1) is consistent with an *N*-methyl in either environment, but favors the 3- rather than the 1-methyl isomer based on the chemical shift differences between the 6-H and the *N*-methyl peaks in the new compound and 1-MΨU. These differences are not large but assume greater significance when contrasted against the close correspondence of 1′-H in all three compounds and 6-H in the new compound and β-pseudouridine. The β configuration was indicated by the virtual superposition of all the sugar proton signals with those in 5β-D-ribofuranosyluracil. All chemical shifts including that for the base proton agree to within +0.015 ppm.

Nuclear Overhauser effect suppressed (NOESY) maps on the 3-MΨU isolate obtained with mixing times of 0.6, 1.2 and 1.8 seconds showed cross peaks and hence proximity between the following protons: Base proton with 1-H, 2-H and 3-H (weak); 1-H with 2-H, 3-H (weak) and 4-H; 2-H with 3-H; 4-H with 3-H and both 5′-Hs.

The UV spectrum of the new nucleoside, shown in Fig. 1, clearly establishes it as different from 1-MΨU which has a 1,5 disubstituted (thymidine-like) chromophoric system, absorbing maximally at 270 nm at pH 7 and at pH 1 and



3-MΨU

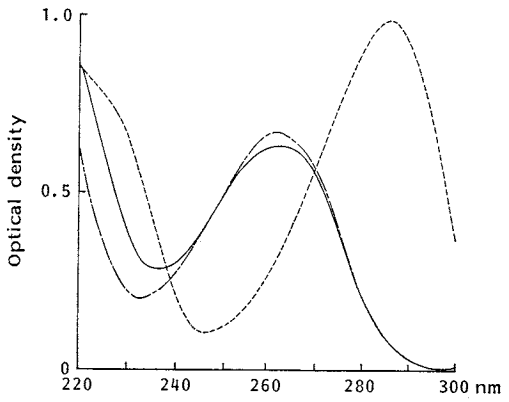
Table 1. NMR shifts of pseudouridine, 1-MΨU and new nucleoside (D_2O)[a].

	6-H	1′-H	2′-H	3′-H	4′-H	5′-H	5′-H	NCH_3
β-Pseudouridine	7.68	4.69	4.30	4.16	4.03	3.85	3.74	—
Unknown	7.67	4.72	4.29	4.15	4.03	3.86	3.75	3.30
1-MΨU[6]	7.81	4.71	|——3.95~4.4——|			|——3.85——|		3.42

[a] Chemical shifts are in ppm relative to the internal deuterated TSP (sodium trimethylsilylpropionate-2,2,3,3-d_4).

at 267 nm at pH 10. The new compound has maxima at 263 nm at pH 7 and pH 1, but exhibits a large shift at pH 10 to a maximum of 285 nm. Such a shift is characteristic of 3 substitutions[8]. The maximum absorption of 3-methyluracil shifts from 259 nm at pH 7 to 282 nm at pH 10. Thus, we conclude, from the spectral data combined with NMR and MS evidence, that the new nucleoside is β-3(*N*)-MΨU.

Fig. 1. UV spectrum of 3-MΨU at pH 7 (——), pH 2 (—·—), pH 12 (------).



The time course of appearance of 3-MΨU in strain MA 4820 grown in synthetic medium is shown in Fig. 2A. It is first detectable in the medium soon after the onset of uracil excretion and continues at a nearly linear rate even after extracellular uracil levels decline. The decline is the result of uptake and reutilization *via* breakdown to β-alanine (unpublished observations). That the reductive catabolic pathway[9] is active in *N. lactamdurans* was shown by the isolation of $^{14}CO_2$ from [2-^{14}C]uracil in cell-free extracts of 72 hours cultures, results not shown[10]. Thus while uracil appears to play the role of reversible nitrogen excretory product in these non-pyrimidine repressible bacteria, 3-MΨU accumulates irreversibly as long as there is residual extracellular uracil. In FUR 1-44 with less highly expressed *de novo* pyrimidine synthetic enzymes[4], uracil excretion is less and is reversed sooner in the fermentation cycle (Fig. 2B). 3-MΨU accumulation ceases after the exhaustion of extracellular uracil, but its level remains constant thereafter. MA 2908, with basal expression of pyrimidine synthetic enzymes[4], can be made to synthesize 3-MΨU upon the addition of uridine: 0.6, 1.2 and 2.4 mg/ml uridine added at inoculation prompted the synthesis of 3-MΨU, reaching plateau values of 25, 90 and 112 μg/ml, respectively. Uridine addition after growth was over gave very similar results. Uridine was used because it is more soluble than uracil. In *N. lactamdurans* exogenous uridine is rapidly converted to uracil and excreted until being taken up and catabolized as shown in Fig. 2.

Fig. 2. The time course of uracil and 3-MΨU excretion by *Nocardia lactamdurans* MA 4820 (A) and by strain FUR 1-44[3] (B).

Each was grown in synthetic medium. ○ Uracil, ● 3-MΨU.

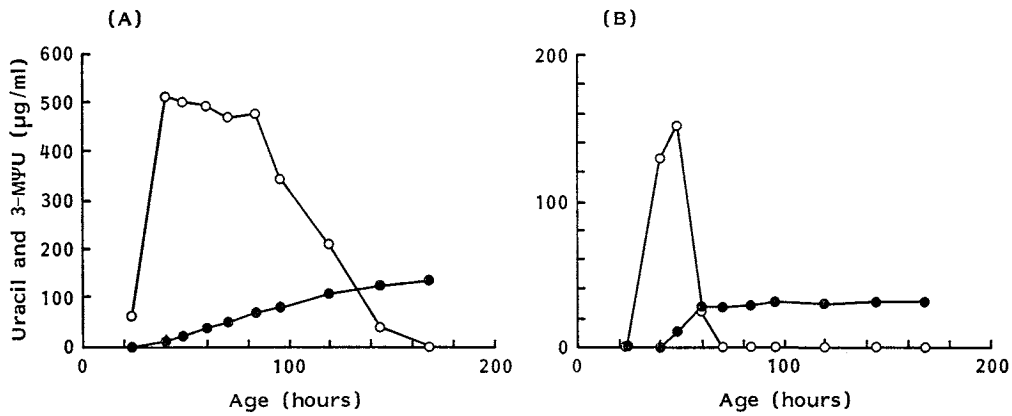


Table 2. Labeling of 3-MΨU by [2-^{14}C]uracil and [*methyl*-^{14}C]methionine.

Precursor	Specific activity (mCi/mmol)	Labeling time (hours)	3-MΨU[a]		
			dpm	μg	Specific activity (mCi/mmol)
[2-^{14}C]Uracil	57.4	3	4,740	1.1	0.54
	57.4	6	10,840	2.4	0.56
			Specific activity (mCi/mmol)		
			Uracil	3-MΨU	Efrotomycin
[*Methyl*-^{14}C]methionine[b]	11.8	4	<0.001	8.9	59
[*Methyl*-^{14}C]methionine+4×serine[c]	11.8	4	<0.001	8.0	71

[a] In 0.5 ml incubation. 1 μCi of [2-^{14}C]uracil was added.
[b] 1.2 μCi [*methyl*-^{14}C]methionine was added in each 0.5 ml incubation.
[c] On a molar basis.

3-MΨU can be labeled by exogenous uracil. [2-^{14}C]Uracil added to washed cells of MA 4820 showed linear incorporation of ^{14}C into 3-MΨU (Table 2). Since degradation of uracil proceeds *via* reductive decarboxylation with the loss of C-2 as CO_2, this incorporation cannot be the result of degradation and resynthesis through the *de novo* pathway, but must be the result of labeling of the internal uracil/uridine/UMP pool, with subsequent modification of one of these components to form the ribosylated, methylated product. [*Methyl*-^{14}C]methionine added to washed cells labeled 3-MΨU and efrotomycin to the degree expected from their relative content of methyl groups (Table 2). Efrotomycin contains 9 methionine-derived methyl groups, 6 in aurodox[11] and 3 on the disaccharide moiety[12]. The relative molar specific activity of efrotomycin was about 9 times that of 3-MΨU made in the same incubation. Incorporation into neither efrotomycin nor 3-MΨU was diluted by a 4-fold excess of serine which is an excellent precursor of the C-1-tetrahydrofolate pool (the methyl donor in thymine). This evidence suggest that the source of the methyl group in 3-MΨU is the same as that for the multiple residues in efrotomycin and is the *S*-adenosylmethionine pool.

Attempts to establish a cell-free system for 3-MΨU synthesis have failed so far. Sonicates of 60 hours washed cells, with *S*-adenosylmethionine and either ribose-1-phosphate or phosphoribosyl pyrophosphate as methyl and ribosyl donors respectively have given no incorporation of labeled uracil or uridine into 3-MΨU, despite the addition of a variety of protease inhibitors, glycerol and other protective agents. Further attempts will be made to establish the identity of the substrate for the possibly concerted ribosylation and methylation leading to a novel nucleoside as fermentation product.

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