Microbial Formation of 4-Thiouracil

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A soil organism identified as *Streptomyces libani* var. *soldani* was found to produce 4-thiouracil. The product was isolated in a yield of 150 µg/ml of filtered beer and characterized by C-13 magnetic resonance and high-resolution mass spectroscopy. The product has a broad antibacterial spectrum but low specific activity.

4-Thiouracil inhibits the growth of *Lactobacillus casei* (1), causes an almost complete regression of mammary carcinoma 755 in mice (1), and is weakly active against the leukemia SR-61 system (3). Chemical synthesis of 4-thiouracil was reported in 1908 (5) and that of its nucleoside in 1958 (2). The nucleoside is present as a minor component of transfer ribonucleic acid in *Escherichia coli* (4).

We are reporting here an efficient method of microbial formation of 4-thiouracil as an alternative to the chemical synthesis.

Inoculum of *Streptomyces libani* subsp. *soldani*, NRRL 8174, was prepared by inoculating the organism into a medium containing 2.5% glucose and 2.5% Pharmamedia (Traders Oil Mill Co., Fort Worth, Tex.), adjusted to pH 7.2. The taxonomic identification of the organism was carried out by Alma Dietz (manuscript in preparation). After a 3-day incubation on a rotary shaker (250 rpm, 28°C), 5 ml of the heavily grown mycelium was inoculated into 100 ml of the fermentation medium consisting of 0.5 g of tryptone, 1 g of Brer Rabbit molasses, 1 g of glycerol, and 0.25 g of yeast extract (pH 6.4) per 500-ml Erlenmeyer flask and incubated as above.

The formation of the 4-thiouracil was determined by assaying filtered fermentation beer daily for its activity against *Bacillus subtilis*. In Table 1 we define one biounit as the amount of the antibiotic in 80 µl of filtered beer that gives a 20-mm-diameter zone of inhibition when applied on a filter paper disk (12.7 mm in diameter), placed on a layer of nutrient agar (thickness, 3 mm), inoculated with *B. subtilis* UC 564, and incubated overnight at 37°C.

A medium of the following composition was used in isolating the antibiotic: 1% glucose (technical grade), 2% dextrin, 0.1% yeast extract, and 1% tryptone adjusted to pH 7.0. One liter of this medium was dispensed in 100-ml portions in 500-ml wide-mouth Erlenmeyer

flasks, inoculated with 5% inoculum volume, and incubated on a shaker at 28°C for six days. At the end of this period, the contents of the flasks were pooled (pH 7.8) and filtered. The clear filtrate (750 ml) was adjusted to pH 7.0 with hydrochloric acid and extracted twice with equal volumes of ethyl acetate. The organic phase was washed with brine and dried with magnesium sulfate; the solvent was removed on a rotary evaporator. The residue (166.0 mg) was dissolved in 5 ml of a mixture of ethyl acetate and methanol (10:1, vol/vol), injected into a size C Merck prepacked silica gel column, and eluted isocratically with ethyl acetate at a rate of 5 ml/min, collecting 25-ml fractions.

The antibiotic-containing fractions (fractions 23-28) were pooled and concentrated to give 112.2 mg of a yellow solid for a yield of 150 µg/ml of filtered beer.

The product gave a high-resolution mass spectrum, which showed a parent ion at *m/e* 128.0055 (calculated for C₄H₄N₂OS = 128.0044). The carbon magnetic resonance spectrum (d₆-dimethyl sulfoxide, internal tetramethylsilane) was obtained on a Varian CFT-20 spectrometer.

TABLE 1. Formation of 4-thiouracil

Day	Broth pH	Biounits/ml
1	5.4	5
2	5.7	9
3	6.6	22
4	6.8	26
5	6.5	21

TABLE 2. The ¹³C chemical shifts of 4-thiouracil

Shift (ppm)	Multiplicity	Assignment
111.7	D	C-5
138.1	D	C-6
148.7	S	C-2
191.3	S	C-4

FIG. 1. Structure of 4-thiouracil.

TABLE 3. Antibacterial spectrum of 4-thiouracil

Organism	Inhibition zone ^a (diam in mm)
<i>Proteus vulgaris</i> UC 93	41
<i>Salmonella schottmuelleri</i> UC 126	41
<i>Salmonella gallinarum</i> UC 265	37
<i>Pseudomonas fluorescens</i> UC 2049	36
<i>Escherichia coli</i> UC 51	35
<i>Bacillus subtilis</i> UC 564	30
<i>Sarcina lutea</i> UC 130	26
<i>Staphylococcus aureus</i> UC 80	23
<i>Bacillus cereus</i> UC 3145	22

^a Zone of inhibition produced by the application of 100 μ g of 4-thiouracil on a paper disk (12.7 mm in diameter), which has been placed on a layer of nutrient agar (thickness, 3 mm) inoculated with the test organism.

Noise decoupled and off-resonance data are given in Table 2.

The isolated product was not separable from synthetic 4-thiouracil (Fig. 1) on Analtech silica gel plates with ethyl acetate-methanol

(10:1, vol/vol) or with pure ethyl acetate. The ultraviolet, infrared, and proton magnetic resonance spectra and elemental analysis were consistent with those expected for 4-thiouracil.

Antibiotic activity was evaluated by using agar diffusion tests and by determination of minimum inhibitory concentrations in brain heart infusion.

4-Thiouracil was found to have broad-spectrum activity in agar diffusion tests. Zones of inhibition were larger with gram-negative than with gram-positive bacteria (Table 3), but its minimum inhibitory concentrations as determined by dilution in brain heart infusion broth were 250 to 1,000 μ g/ml.

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