

THE MECHANISM OF PRUMYCIN ACTION

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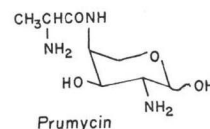
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Examination of the effects of prumycin on sensitive *Botrytis cinerea* and *Sarcina lutea* showed that inhibition of protein synthesis in both organisms and reduction in ribonucleic acid synthesis in *Botrytis cinerea* are among the mechanisms of action. The inhibition of RNA biosynthesis in *Botrytis cinerea* is probably a secondary effect resulting from the termination of protein synthesis rather than a direct inhibition. In a cell-free protein-synthesizing system obtained from crushed *Botrytis cinerea* cells, incorporation of L-phenylalanine-¹⁴C into polyphenylalanine (with polyuridylic acid as synthetic messenger) was inhibited by prumycin. Ribosome binding studies indicated that prumycin did not effect its inhibition at the ribosomal stage of protein synthesis.

Prumycin,^{1,2)} a new antifungal antibiotic with the structure established as 4-N-[D-alanyl]-2, 4-diamino-2, 4-dideoxy-L-arabinose,^{3,4)} inhibits the growth of fungi such as *Botrytis fabae*, *Botrytis cinerea*, *Sclerotinia cinerea* and *Sclerotinia sclerotiorum*, and some bacteria such as *Sarcina lutea* and *Vibrio comma*.³⁾

We have studied the effects of prumycin on the biosynthesis of protein, DNA, RNA and cell walls in *Botrytis cinerea* and *Sarcina lutea* and wish to report our observations.



Materials and Methods

Cultures. *Botrytis cinerea* KF 184 was obtained from Kitasato Institute in Tokyo, Japan. The culture was maintained on potato-dextrose agar described below. *Sarcina lutea* ATCC #9341 was purchased from the American Type Culture Collection and maintained on Difco nutrient agar (weekly transfer).

Labelled Chemicals. D-Glucosamine-1-¹⁴C HCl (4.8 mCi/mM), uridine-2-¹⁴C (52.7 mCi/mM), thymidine-2-¹⁴C (56 mCi/mM), and L-leucine-1-¹⁴C (44.8 mCi/mM) were purchased from the New England Nuclear Corporation (Boston, Massachusetts). L-Phenylalanine-¹⁴C (U) (5.3 mCi/mM) was purchased from Amersham/Searle (Arlington Heights, Illinois).

Biological Chemicals. Adenosine 5'-triphosphate, guanosine 5'-triphosphate, phospho (enol) pyruvate, ribonucleic acid (from baker's yeast), and pyruvate kinase were purchased from Sigma Chemical Co. (St. Louis, Missouri). Potassium uridylylate was purchased from Schwarz/Mann (Orangeburg, New York).

Growth Media. For *Botrytis cinerea* potato-dextrose broth, prepared by boiling 1 kg of chopped potatoes in 5 liters of water for 30 minutes, straining the mash through gauze and autoclaving after the addition of 2 % dextrose, and HAYDUCK's chemically defined media (for incorporation experiments) were used. For *Sarcina lutea*, nutrient broth and a media containing 1.1 % MgSO₄, 7 % H₂O, 0.1 % NaCl, 0.1 % (NH₄)₂HPO₄, 0.05 % KH₂PO₄ and 1.0 % glucose were employed.

Growth of *Botrytis cinerea*. The organisms were grown on a New Brunswick Scientific Shaker (model G76-speed setting #5) at 27°C for 72 hours in 50-ml flasks containing 20 ml of

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potato-dextrose broth. The cells were harvested by centrifugation and inoculated into potato-dextrose broth (20 ml of broth in 50-ml flasks) containing various concentrations of prumycin. A quantitative estimate of growth was determined by collecting aliquots of the growing culture on previously tared filter paper discs.

Sarcina lutea. The cells were grown on the shaker described previously at 30°C for 24 hours in 50-ml flasks containing 20 ml of Difco nutrient broth. The cells were harvested by centrifugation and suspended in Difco nutrient broth (20 ml) containing various concentrations of prumycin. Growth of the organisms was observed as a change in optical density at 700 nm on a Bausch and Lomb spectrophotometer (Spectronic 20).

Incorporation of Thymidine-2-¹⁴C and Uridine-2-¹⁴C into Nucleic Acid.

Botrytis cinerea. Cells grown for 72 hours in 100 ml of potato-dextrose broth (250 ml flask) at 27°C were collected by centrifugation and washed with sterile distilled water and sterile HAYDUCK's solution. The cells were resuspended in 20 ml of sterile HAYDUCK's solution in 50-ml Erlenmeyer flasks at a concentration of 2 mg dry mycelium/ml. After 2 hours of incubation on a shaker at 27°C, labelled uridine or thymidine was added to give a final nucleotide concentration of 0.1 μ mole/ml (2 μ Ci). The effect of prumycin on nucleotide incorporation was measured by adding 0.1 or 5.0 μ g/ml of antibiotic at the same time the labelled nucleotide was added. Control flasks receiving no antibiotic were included.

The flasks were shaken for 4 hours at 27°C and samples assayed by a modified SCHMIDT-THANNHAUSER-SCHNEIDER technique^{1,5,6)} at various times as follows: each sample (20 ml) was centrifuged and the cells washed once with distilled water (10 ml). The cells were suspended in 10 ml of distilled water and heated in a boiling water bath for 5 minutes, then centrifuged. Cold 10% w/v trichloroacetic acid (TCA) 2.5 ml was added, the cells shaken at 0°C for 30 minutes and centrifuged. The sediment was washed once with 1 ml of 10% TCA, then twice with 5 ml of 95% ethanol. The mycelial mass was then suspended in 3 ml of 5% TCA and heated at 90°C for 15 minutes with frequent stirring. The insoluble protein residue was centrifuged off, leaving the nucleic acid dissolved in the TCA supernatant. For labelled thymidine studies, the DNA content of the TCA was determined by the diphenylamine,⁷⁾ assay using calf thymus DNA as the standard, while for labelled uridine assays, the RNA was determined by the orcinol procedure⁷⁾ using yeast RNA as the standard.

Radioactivity was measured by adding 0.5 ml portions of the TCA solution containing the labelled nucleic acid to 15 ml of BRAY's solution⁸⁾ and counting the solution in a Packard Tri-Carb Liquid Scintillation Spectrometer (model 574).

Sarcina lutea. Cells grown for 24 hours in 100 ml of Difco nutrient broth (250-ml flask) on a shaker at 30°C as previously described were harvested by centrifugation, washed with the synthetic media (10 ml), and resuspended in that media (20 ml) at a cell concentration of about 1 mg/ml. The cells were incubated on a shaker at 30°C for 2 hours before the addition of nucleotides and prumycin (6.0 and 20 μ g/ml). Incubation was then continued on a shaker bath at 30°C for 2 hours and samples assayed as described for *Botrytis cinerea* nucleotide incorporation.

Incorporation of D-Glucosamine-1-¹⁴C into Cell Wall. Reaction conditions for label incorporation into cell wall were the same as described for nucleotide incorporation with both organisms. In place of labelled nucleotide, D-glucosamine-1-¹⁴C was added to a concentration of 0.1 μ mole/ml (2 μ Ci total) for *Botrytis cinerea*, and 0.2 μ mole/ml (8 μ Ci total) for *Sarcina lutea*. The cell wall fraction of each sample was separated by the PARK-HANCOCK method⁹⁾ and the radioactivity determined by adding weighed aliquots of cell wall material obtained, to BRAY's solution and measuring the radioactivity in a scintillation spectrometer.

Incorporation of L-Leucine-1-¹⁴C into Protein Fractions. The assay procedure was identical to that described for nucleotide incorporation except that each flask contained 0.1 μ mole/ml (2 μ Ci total) of L-leucine-1-¹⁴C. The protein insoluble residue remaining after hot 5% TCA

treatment was dissolved in 1 ml of 1 N NaOH at 90°C for 30 minutes. Protein concentration was determined by the biuret method⁷⁾ using bovine serum albumin as the standard, and radioactivity determined in BRAY'S solution by a scintillation spectrometer.

Effect of Prumycin on the Cell-Free Incorporation of Polyuridylic Acid Directed Poly-Phenylalanine Synthesis. *Botrytis cinerea* cells were grown for 48 hours on a shaker bath at 27°C in 100 ml of potato-dextrose broth in a 250-ml Erlenmeyer flask. The cells were harvested by centrifugation and washed with a buffer containing tris (hydroxymethyl) amino-methane-acetate (pH 7.6); 0.06 M; magnesium acetate, 0.005 M; potassium chloride, 0.01 M; and 2-mercaptoethanol, 0.06 M, (hereby designated as tris-buffer). The cells were centrifuged and the mycelial mat was transferred to a pre-cooled mortar at 0°C and covered with twice its weight of acid washed, chloride-free, glass beads, 0.2-mm diameter (Schwarz Bio Research) at the same temperature. The cells were ground for 2~3 minutes, 4 ml of the tris-buffer added, and the extract centrifuged in a Sorvall RC2-B centrifuge at 15,000×g for 30 minutes. The supernatant was dialyzed against 200 volumes of the tris-buffer for 18 hours, and used as the protein synthesizing enzyme and ribosome source.

To assay for prumycin binding to *Botrytis cinerea* ribosomes, the ribosomes were separated from the dialyzed extract by centrifugation at 105,000×g for 90 minutes on a Beckman L2-658 ultracentrifuge with a 50 Ti rotor. The ribosome pellet was washed with tris-buffer, recentrifuged at 105,000×g for 90 minutes, and resuspended in 1 ml of tris-buffer. A portion of the ribosome preparation (0.2 ml) was added to 6.8 ml of tris-buffer containing 100 µg/ml of prumycin. The solution was centrifuged at 105,000×g for 90 minutes and the ribosomes redissolved in 0.1 ml of tris-buffer. A similar treatment was carried out on 0.2 ml of ribosome preparation with no antibiotic added.

Both ribosome preparations were assayed for their protein-synthesizing capability by adding 0.05 ml of ribosome solution (15 µg) to a cell-free synthesizing system containing 0.15 ml (60 µg) of the ribosome-free enzyme solution obtained after the first ultracentrifugation step.

To determine the ability of the various extracts to incorporate ¹⁴C-phenylalanine (U) into protein, a system containing the following components in a total volume of 0.5 ml was used: tris-acetate (pH 7.6), 30 µmoles; magnesium acetate, 2.5 µmoles; potassium chloride, 5 µmoles; 2-mercaptoethanol, 3 µmoles; ATP, 2 µmoles; GTP, 0.5 µmoles; phospho (enol) pyruvate, 5 µmoles, phospho (enol) pyruvate kinase, 25 µg; transfer RNA, 250 µg; polyuridylic acid, 100 µg; ¹⁴C-phenylalanine (U), 10 µmoles (0.5 µCi); enzyme extract (containing ribosomes), 100 µg.

Incorporation was allowed to proceed at 30°C for 30 minutes, the tubes diluted to 1 ml with distilled water, and the protein precipitated by the addition of 1 ml of 10% TCA. The tubes were heated in a boiling water bath for 15 minutes, cooled, and the precipitate collected on 25 mm diameter Millipore membrane-filters with a pore size of 45 microns. The membrane filters were washed three times with 10 ml of 5% TCA, dried for 15 minutes at 100°C, and added to 15 ml of BRAY'S solution. The solution was counted for radioactivity using the Packard Scintillation Spectrometer.

The effects of prumycin on cell-free synthesis was determined by adding various concentrations of the antibiotic to the incubation mixture before the incubation was begun.

Results

Botrytis cinerea

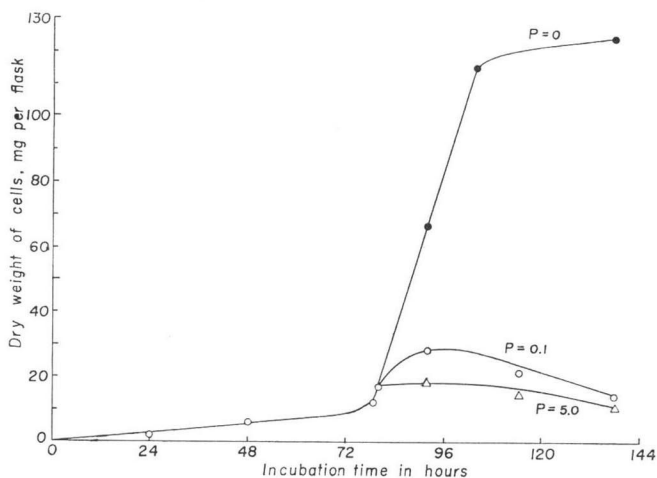
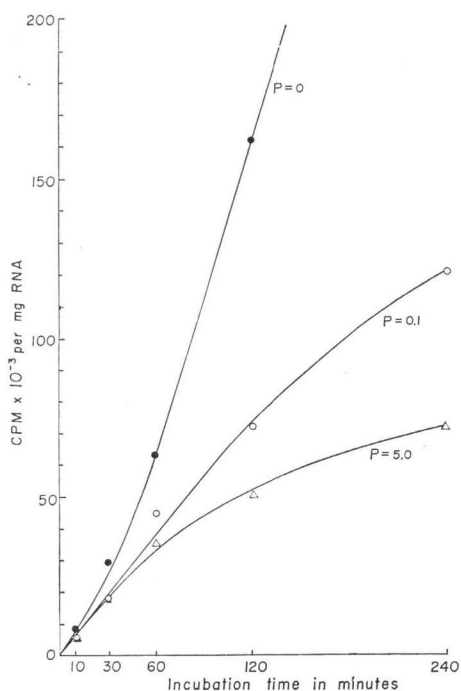
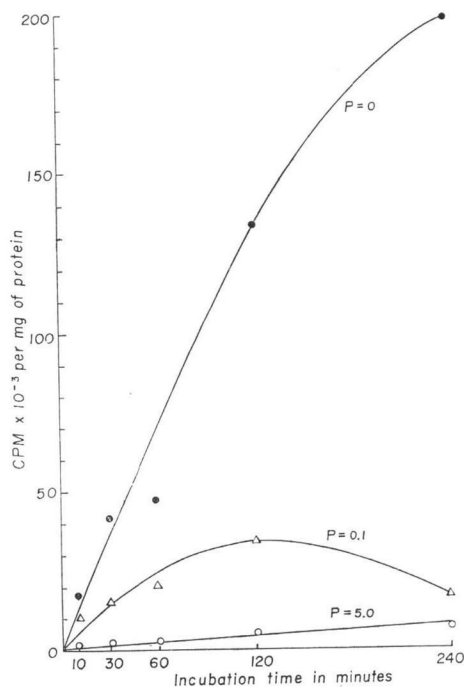
Growth:

The effect of prumycin on the growth of *Botrytis cinerea* is shown in Fig. 1. When the antibiotic was added to the culture in early log phase, immediate inhibition was observed at a concentration of 5.0 µg/ml. At a concentration of 0.1 µg/ml, growth continued for about 1 generation (2~3 hours) before the cells were completely inhibited.

Effect of Prumycin on Macromolecular Synthesis:

There was no significant effect by prumycin on the incorporation of ^{14}C -thymidine into DNA or ^{14}C -glucosamine into cell wall material at a prumycin concentration of $0.1\ \mu\text{g}/\text{ml}$. At an antibiotic concentration of $5.0\ \mu\text{g}/\text{ml}$, DNA synthesis was inhibited by 10% of control and glucosamine incorporation by 20%.

The effect of prumycin on RNA biosynthesis can be seen in Fig. 2. A decrease in incorporation of ^{14}C -uridine into RNA is observed with prumycin concentrations of 0.1 and $5.0\ \mu\text{g}/\text{ml}$

Fig. 1. Effect of prumycin on the growth of *Botrytis cinerea*Fig. 2. Effect of prumycin on ^{14}C -uridine incorporation into *B. cinerea* RNAFig. 3. Effect of prumycin on ^{14}C -leucine incorporation into *B. cinerea* protein

compared to cells grown in the absence of the antibiotic. The reduction in labelled RNA is gradual however, and does not seem to represent a direct inhibition of RNA biosynthesis.

The effect of prumycin on ^{14}C -L-leucine incorporation into protein can be seen in Fig. 3. At both antibiotic concentrations, a marked inhibition of protein synthesis occurs relative to that of cells grown in the absence of prumycin. At a concentration of $5.0\ \mu\text{g}/\text{ml}$ (minimum inhibitory concentration is $0.1\sim 0.3\ \mu\text{g}/\text{ml}$ in broth) a 90% inhibition occurs within 10 minutes of antibiotic addition. Inhibition is somewhat less at a concentration of $0.1\ \mu\text{g}/\text{ml}$, being 60% at 10 minutes and about 90% after 4 hours.

Sarcina lutea

Growth:

The effect of prumycin on the growth of *Sarcina lutea* can be seen in Fig. 4. Bacterial growth continued for about two generations at $6\ \mu\text{g}/\text{ml}$, and for one generation at $20\ \mu\text{g}/\text{ml}$ prumycin (the minimum inhibitory concentration is $5\ \mu\text{g}/\text{ml}$ in broth).

Effect of Prumycin on Macromolecular Synthesis:

There was no significant effect on the incorporation of ^{14}C -thymidine into DNA, ^{14}C -uridine into RNA or ^{14}C -glucosamine into cell wall material at prumycin concentrations of 6.0 and $20\ \mu\text{g}/\text{ml}$.

The effect of prumycin on ^{14}C -L-leucine incorporation into protein can be seen in Fig. 5. At a prumycin concentration of $20\ \mu\text{g}/\text{ml}$, about a 50% decrease in protein biosynthesis compared to that of cells grown in the absence of prumycin is observed.

Effect of Prumycin on the Cell-free Synthesis of Poly-phenylalanine:

Fig. 4. Effect of prumycin on the growth of *Sarcina lutea*

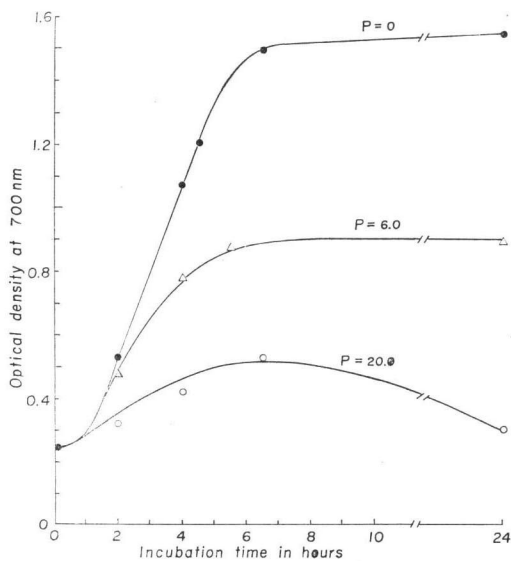


Fig. 5. Effect of prumycin on ^{14}C -leucine incorporation into *S. lutea* protein

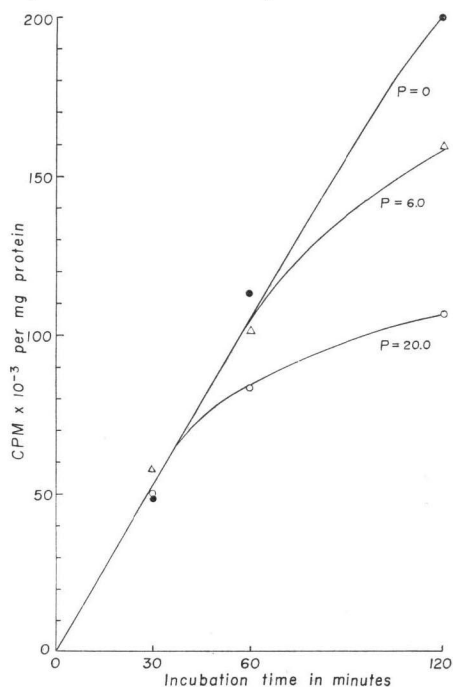


Table 1. Prumycin inhibition of cell-free protein synthesis from ground cells of *B. cinerea*

Sample treatment	Counts/min. of poly- ¹⁴ C phenylalanine
Complete system	3,520
Complete plus prumycin 5 μ g/ml	3,275
Complete plus prumycin 25 μ g/ml	1,952
Complete plus prumycin 50 μ g/ml	1,572
Complete plus chloramphenicol 50 μ g/ml	724
minus poly-uridylic acid	733

Table 2. The effect of prumycin on *B. cinerea* ribosomes

Sample treatment	Counts/min. of poly- ¹⁴ C phenylalanine	
	1	2
Complete plus normal ribosomes	2,169	3,524
Complete plus ribosomes exposed to 100 μ g/ml prumycin	2,507	3,044
Minus ATP generating system normal ribosomes	531	1,556
Ribosomes - no enzyme	858	—
Enzyme - no ribosomes	970	—

The effect of prumycin on cell-free protein synthesis is summarized in Table 1. There is a dose-response relationship between poly-uridylic acid directed synthesis and the amount of prumycin added. At a prumycin concentration of 50 μ g/ml, an inhibition of nearly 70 % is observed. As a comparison, chloramphenicol at the same concentration inhibits the system by 100 % of control.

The effect of prumycin on *Botrytis cinerea* ribosomes is summarized in Table 2. Ribosomes exposed to 100 μ g/ml of prumycin were as competent in carrying out protein synthesis as the ribosomes not so exposed.

Discussion and Summary

Prumycin is an antifungal antibiotic effective at very low concentrations against species of *Botrytis*, *Sclerotinia*, and *Sarcina lutea*. In determining the mechanism of action of prumycin, the classical techniques of measuring macromolecular metabolism — DNA, RNA protein, and cell wall biosynthesis — have been employed utilizing labelled precursors. Similar experiments have been done in the past with many antibiotics including chloramphenicol,^{10,11)} tetracycline,¹⁰⁾ streptomycin,¹²⁾ puromycin,¹³⁾ griseofulvin,^{14,15)} and cerulenin¹⁶⁾ to yield information concerning the mechanism of action of these antibiotics. The inhibition of cell-free protein synthesis utilizing poly-uridylic acid as a synthetic messenger for labelled phenylalanine has also been used in the past for proof of an antibiotic's effectiveness in inhibiting protein synthesis.^{17,18,19)}

Prumycin's effect on *Sarcina lutea* seems also to be a result of protein synthesis inhibition. Neither DNA, RNA, or cell wall biosynthesis is inhibited by the antibiotic. Protein synthesis is inhibited in whole cell experiments to the same level that growth of the organism is inhibited, indicating that the inhibition of *Sarcina lutea* is due to prumycin interference with protein synthesis.

The inhibition of cell-free protein synthesis by prumycin is further proof of its inhibitory action on *Botrytis cinerea* protein synthesis. In an effort to further narrow prumycin's site of action, the ribosome binding experiments were performed. That the ribosomes were unaffected by exposure to high concentrations of prumycin indicates that the antibiotic does not inhibit at the ribosome level under these conditions. Tentative experiments on amino acid activation and activated amino-acid-transfer to RNA have suggested that prumycin is effective in inhibiting one or both of these reactions. Further experimentation must be carried out, however, before a definitive statement can be made concerning ribosome binding or pre-ribosomal antibiotic inhibition.

The data clearly demonstrates that prumycin inhibits protein synthesis in *Botrytis cinerea*. A rapid and complete inhibition of protein synthesis in whole cells is observed with a prumycin concentration of 5.0 μ g/ml. At 0.1 μ g/ml, protein synthesis continues slowly for a short time

before complete inhibition. The results with both antibiotic concentrations on protein synthesis parallel exactly the antibiotics effect on cell growth seen in Fig. 1.

As stated, neither DNA nor cell wall biosynthesis in *Botrytis cinerea* are significantly affected by prumycin. However, RNA synthesis is inhibited. The inhibition is not as rapid nor as complete as that observed with protein synthesis, and is most likely the result of RNA sensitivity to other biochemical events, *i.e.*, protein synthesis.

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