THE MYXOPYRONINS, NEW INHIBITORS OF BACTERIAL RNA SYNTHESIS FROM *MYXOCOCCUS FULVUS* (MYXOBACTERALES)†,*

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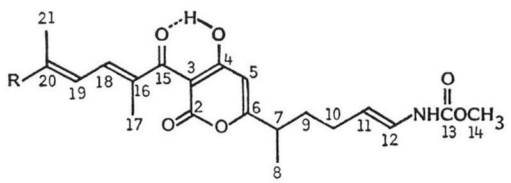
From the culture supernatant of the myxobacterium, *Myxococcus fulvus* strain Mx f50, an antibiotic activity was isolated which blocked growth of many Gram-positive and several Gram-negative bacteria, but not of yeasts and fungi. The activity consisted of two closely related compounds, myxopyronins A and B. The myxopyronins appear to be new antibiotics, and seem to specifically inhibit bacterial RNA polymerase.

In this article, we present data on a new antibacterial compound, myxopyronin (Fig. 1)[1], which was discovered in a screening program for antibiotics from myxobacteria. After myxothiazol[2], myxovirescin[3], myxovalargin[4], and myxalamid[5], this is now the fifth new antibiotic which we could isolate from these unusual and little studied organisms. In addition, we found two antibiotics, pyrrolnitrin[6] and althiomycin[7], that were already known from other bacteria. We describe here the fermentation and some physico-chemical and biological properties of the antibiotic, while the elucidation of its chemical structure is published elsewhere[1].

Fig. 1. The chemical structures of myxopyronins A and B[1].



A: R=*n*-C_8H_7
B: R=*n*-C_4H_9

Production of the Antibiotic

Myxococcus fulvus strain Mx f50 (=*M. fulvus* HR4) was isolated in 1977 from rabbit dung collected near Calpe, Alicante, Spain. It is deposited at the German Collection of Microorganisms in Göttingen (DSM 2549).

The bacterium was cultivated in the following medium: 0.6% peptone from casein, tryptically digested (Merck, Darmstadt; or Serva, Heidelberg); 0.05% yeast extract (Difco); 0.2% $MgSO_4 \cdot 7H_2O$; 0.04% $CaCl_2 \cdot 2H_2O$; pH adjusted to 7.2 before autoclaving. In this medium, the organism grew as a homogeneous cell suspension.

Batch cultures of 200 ml in 1-liter Erlenmeyer flasks were incubated on a rotary shaker at 160 rpm at 30°C. Fermentations were performed in 70-liter or 300-liter bioreactors (Giovanola Frères, Manthey, Switzerland) agitated by a circulating pump stirrer system. Antifoam (Silikon Merck) was added in a concentration of 0.02%. The fermentor cultures were started from shake cultures (7 to 10%,

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v/v; late log phase). The temperature was kept at 30°C, while the pH was not regulated.

The pO_2 was adjusted to 95 to 100% saturation at the beginning of the fermentation. This was achieved with a stirring rate of about 400 per minute, and an aeration rate of 0.1 v/v/minute. In the course of the fermentation, the pO_2 decreased and after 30 to 35 hours reached about 40 to 50% saturation. After this time, the pO_2 began to rise slowly. In case that the pO_2 fell too fast, or reached a value under 30%, the stirrer rate was slightly raised. The fermentation was stopped after about 40 hours by separating the cells from the supernatant by centrifugation. By this time, the wet weight of the cell mass was 4.5~5.6 g/liter, and the pH about 8. The antibiotic was exclusively in the supernatant.

The antibiotic concentration in the culture broth went strictly parallel to the optical density (OD, measured at 623 nm) and to the cell mass. After the stationary phase was reached, no further production took place. The antibiotic content of the broth was then 5 to 10 mg/liter. Higher yields were obtained in shake flasks (up to 26 mg/liter), but, for unknown reasons, these values could not be achieved in the bioreactor. In the beginning of our studies, antibiotic production was even lower and, in addition, further degenerated in the course of a few transfers. This was due to a heterogeneity in the strain, and by cloning we obtained from a large number of low- and non-producers some colonies with the production capacity mentioned above. The selected clones remained stable later on.

Isolation of the Antibiotic

The antibiotic was extracted from 600 liters of culture broth with ethyl acetate (1/10 volume) using a three step counter current extraction plant (Westphalia, Oelpe, FRG). The ethyl acetate was removed under reduced pressure at 40°C. The residue was dissolved in 500 ml of methanol - water (95: 5), and the solution extracted twice with 200 ml of heptane to remove contaminating material. The methanol phase was then evaporated to dryness, the residue (8.7 g) dissolved in 100 ml of methanol, and chromatographed on a Sephadex LH-20 column (6×80 cm) with methanol as the mobile phase. The biological activity of the fractions was determined by the agar diffusion assay using paper discs and *Staphylococcus aureus* as indicator organism. The residue of the combined active fractions (4.25 g) was dissolved in 20 ml of methanol and purified by reversed phase HPLC, with methanol - water - acetic acid (80: 20: 4) as the eluant (200 ml/minute), using a Waters Prep LC 500 chromatograph equipped with two RP-8 cartridges. Detection was *via* the refractive index. The main peak (retention time, 6.0 minutes) which contained the antibiotic activity was collected and neutralized with saturated aqueous sodium bicarbonate. From this solution, the antibiotic was extracted with methylene chloride. After evaporation of the solvent, the remaining antibiotic substance (2.9 g) was 95% pure. It consisted of two components, myxopyronins A and B, which were separated and further purified by preparative HPLC with methanol - water - acetic acid (70: 30: 4) as the eluant (6.4 ml/minute), using a Waters Pump M6000A, Rheodyne Valve 7125, and a LiChrosorb RP-18 (10 μm) column (16 mm×25 cm, Knauer). Detection was *via* UV absorption at 280 nm. The retention time of myxopyronin A was 11.0 minutes, and that of myxopyronin B 15.0 minutes. The quantitative relation between myxopyronins A and B varied from experiment to experiment; usually, myxopyronin A was the dominating compound (about 90% of the total).

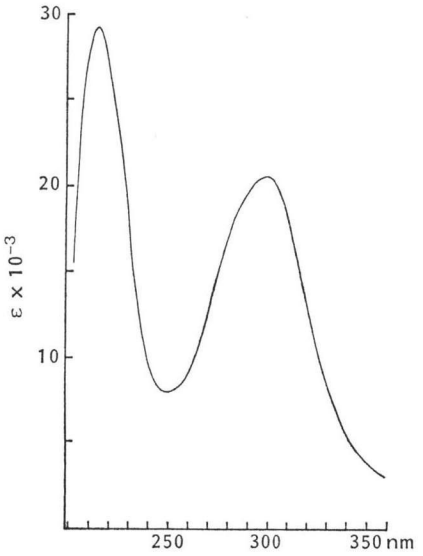
Physico-chemical Properties

The UV spectra (Fig. 2) were recorded with a Zeiss DMR 21 spectrophotometer in methanol Uvasol (Merck) as the solvent. They were identical for myxopyronins A and B. The IR spectra (Fig. 3) were measured in chloroform solution with a Perkin-Elmer 297 IR spectrophotometer. They, too, were identical for myxopyronins A and B. The molecular formulas were determined by high resolution mass

spectrometry using the peak-matching method with a resolution of $M/\Delta M = 15{,}000$ (10% valley) and a Kratos MS 902 S mass spectrometer. Myxopyronin A had the molecular formula $C_{23}H_{31}NO_6$ (found 417.2151, calcd 417.2152), and myxopyronin B $C_{24}H_{33}NO_6$ (found 431.2306, calcd 431.2308).

Thin-layer chromatograms on aluminium sheets precoated with Silica gel 60 F_{254} (Merck) showed the following Rf values: with dichloromethane - methanol (95: 5) as the solvent, Rf 0.8; with toluene - acetone (80: 20), Rf 0.4; with dichloromethane - 2-propanol (98: 2), Rf 0.4; and with hexane - acetone - acetic acid (80: 20: 4), Rf 0.4. Detection was by spraying with glacial acetic acid containing 1% anisaldehyde and 2% sulfuric acid which gave blue to violet spots after heating at 110°C. The result of the structure elucidation[1] is shown in Fig. 1.

Fig. 2. Electronic absorption spectrum of myxopyronin in methanol.



Biological Properties

While the antibiotic was active against most Gram-positive bacteria tested, only few Gram-negatives and no yeasts or molds were sensitive (Table 1). *M. fulvus* Mx f50 was inhibited by its own antibiotic at an MIC of 25 ~ 50 μg/ml (myxopyronin A). In general, myxopyronin B was more active than myxopyronin A.

Myxopyronin A+B had no acute toxicity for mouse up to a concentration of 100 mg/kg (s.c.). Neither was the antibiotic active in elimination studies in mouse with infections of *Escherichia coli* (applied dose 100 mg/kg s.c.), *Staphylococcus aureus* (30 mg/kg s.c.), or *Streptococcus pyogenes* (30 mg/kg s.c.).

To get an idea of the mode of action, protein, RNA and DNA synthesis were studied in *S. aureus* in presence and absence of the antibiotic. In

Fig. 3. The infrared spectra of myxopyronins A and B in chloroform.

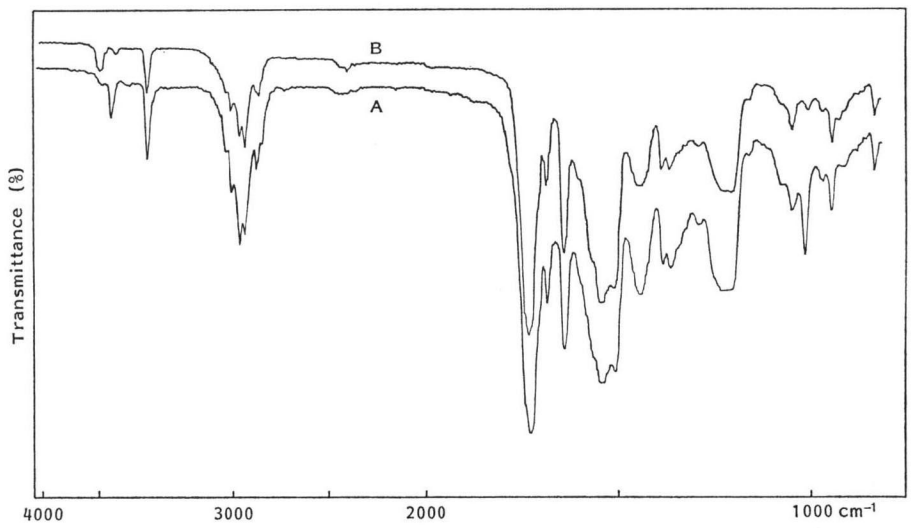


Table 1. The antibiotic spectrum of myxopyronin.

	Test organism	Diameter of the inhibition zone[1] (mm)				MIC[2]	
		Concentration of myxopyronin A[3]				Myxopyronin A (μg/ml)	Myxopyronin B (μg/ml)
		1	2	5	10		
Gram-positive bacteria	*Mycobacterium phlei*	0	0	0	0		
	Corynebacterium mediolanum	0	9	13	17		
	Arthrobacter simplex	0	9	10	12		
	Bacillus megaterium	0	10	13	17	6	0.8
	B. subtilis	0	0	8	9	50	12.5
	Brevibacterium ammoniagenes	0	0	8	10		
	Staphylococcus aureus	11	16	19	22	1.0	0.3
	Micrococcus luteus	0	0	8	10	12	3.1
Gram-negative bacteria	*Acinetobacter calcoaceticus*	20	24	28	30	0.8	
	Agrobacterium tumefaciens	10	16	20	24	0.8	1.3
	Escherichia coli	0	0	0	0	>100	
	Serratia marcescens	0	0	0	0		
	Proteus mirabilis					>100	
	Salmonella typhimurium	0	0	0	0	>100	
	Pseudomonas fluorescens	0	0	0	0		
	P. aeruginosa					>100	
	Myxococcus fulvus Mx f50	0	0	7	9	50	25
Yeasts and mold	*Saccharomyces cerevisiae*					>100	
	Candida albicans	0	0	0	0		
	Schizosaccharomyces pombe	0	0	0	0		
	Mucor hiemalis	0	0	0	0		

[1] The agar diffusion test using paper discs of 6 mm diameter was performed as described before[4]. The initial cell density of the test organisms was 10^5/ml, with *M. fulvus* it was 10^7/ml.

[2] The serial dilution assay was performed as described before[4].

[3] μg/disc.

these experiments pure component A was used. *S. aureus* was grown in shake culture at 37°C in the following medium: 0.3% peptone from casein; 0.2% $MgSO_4 \cdot 7H_2O$; 0.05% $CaCl_2 \cdot 2H_2O$; and 0.05% yeast extract. As soon as an OD of 0.4 (623 nm, 1 cm) was reached, the culture was diluted with fresh medium to give an OD of 0.1, and was then divided into a series of parallel cultures. After an adaptation period of 10 minutes, the radioactive precursors were added (either [*U*-^{14}C]leucine, or [2-^{14}C]uracil, or [*U*-^{14}C]thymidine, at 0.1 μCi/ml each; all radiochemicals were purchased from Amersham-International, Braunschweig). The cultures were incubated for 6 minutes, then either the antibiotic (3 μg/ml, dissolved in methanol) or a corresponding amount of methanol were added, and the first sample (0.5 ml) was taken. Additional samples followed in 5 minutes, later in 10 minutes intervals. Each sample was pipetted into 2 ml of cold 3% perchloric acid (PCA), the precipitate was collected on a Whatman GF/B glass fiber filter and washed with 4×5 ml of PCA and 2×4 ml of 95% ethanol. After drying of the filters, the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

As shown in Fig. 4, DNA synthesis was not affected by the antibiotic. However, incorporation of leucine (Fig. 5) and particularly of uracil (Fig. 6) became strongly reduced a few minutes after addition of myxopyronin. At the end of 40 minutes, the uracil incorporated in presence of the antibiotic was only about 10% that of the control without the antibiotic. The inhibition of leucine incorporation was

Fig. 4. Effect of myxopyronin A on DNA synthesis in *Staphylococcus aureus* measured as incorporation of [^{14}C]thymidine into TCA insoluble material.

At the time indicated by the arrow the antibiotic (3 μg/ml) was added to the experimental culture (open circles). Closed circles: control culture without the antibiotic.

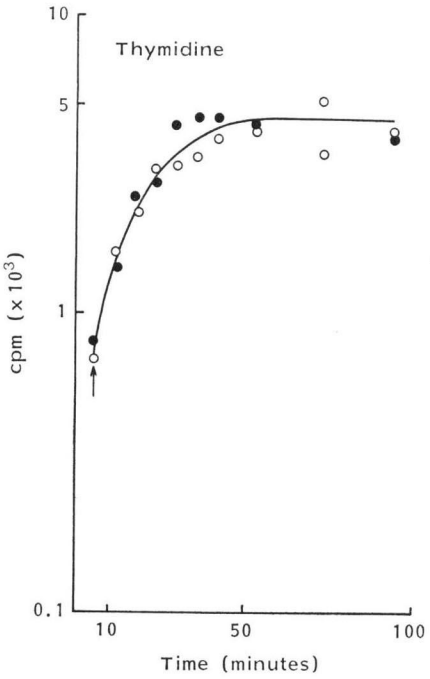
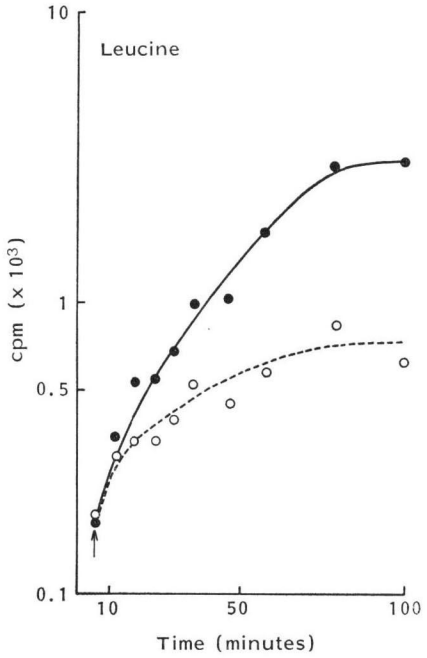


Fig. 5. Effect of myxopyronin A (3 μg/ml) on protein synthesis in *Staphylococcus aureus* measured as incorporation of [^{14}C]leucine into TCA insoluble material.

Symbols as in Fig. 4.



clearly delayed with respect to uracil incorporation (Fig. 7). Thus, the results of the incorporation experiments suggested that RNA synthesis was the primary site of action of myxopyronin.

Consequently, the effect of myxopyronin A on DNA-dependent RNA polymerase (E. C. 2.7.7.6) from *E. coli* (Boehringer, Mannheim) was studied *in vitro* using the method of BURGESS[8] with the following modifications: The concentration of the nucleotide triphosphates was 0.55 mM each, [^{14}C]UTP was added at 0.675 μCi/ml, calf thymus DNA (Boehringer, Mannheim) at a concentration of 130 μg/ml, the enzyme activity was one unit per assay, the total volume of the assay 200 μl. The reaction mixture minus the nucleotide triphosphates was incubated at room temperature for 5 minutes. Then the reaction was started by adding the nucleotide triphosphates. After 15 minutes at 37°C, the reaction was stopped by pouring the mixture onto 2 ml of cold 5% trichloroacetic acid (TCA) containing 0.01 M $Na_4P_2O_4$. The precipitate was transferred to a membrane filter (Sartorius, Göttingen, 0.45 μm pore size) and washed with 3×5 ml of cold 5% TCA solution. After drying, the radioactivity of the filters was determined in a liquid scintillation spectrometer. Fig. 8 shows the inhibition of the enzyme activity by increasing amounts of the antibiotic. The dose required to inhibit the enzyme activity by 50% was 0.6~0.8 μg of myxopyronin A per unit of enzyme (3~4 μg/ml).

To test whether myxopyronin acts specifically on prokaryotic RNA polymerase, an *in vitro* assay was performed with wheat germ RNA polymerase II (Miles Laboratories, Elkhart, Indiana) according to the instructions by Miles except that 0.54 μCi [^{14}C]UTP/ml was used. The reaction mixture of 240 μl

Fig. 6. Effect of myxopyronin A (3 μg/ml) on RNA synthesis in *Staphylococcus aureus* measured as incorporation of [^{14}C]uracil into TCA insoluble material.

Symbols as in Fig. 4.

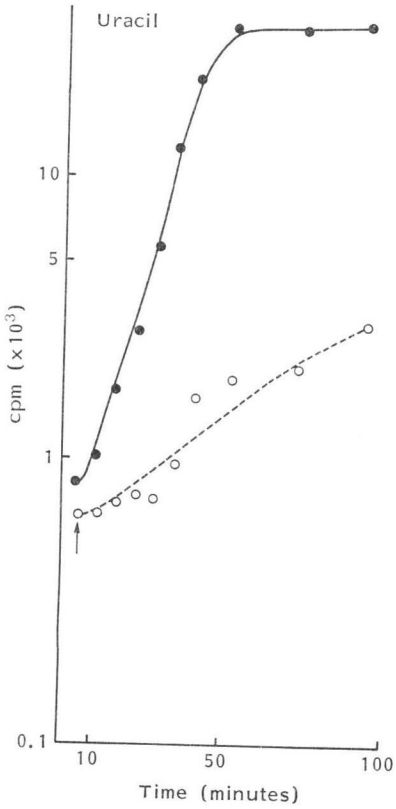


Fig. 7. The kinetics of inhibition of RNA (triangles) and protein (circles) synthesis in *Staphylococcus aureus* by myxopyronin A (3 μg/ml).

The curves give the incorporation of precursor by the inhibited culture in percent of the incorporation by the control without the antibiotic.

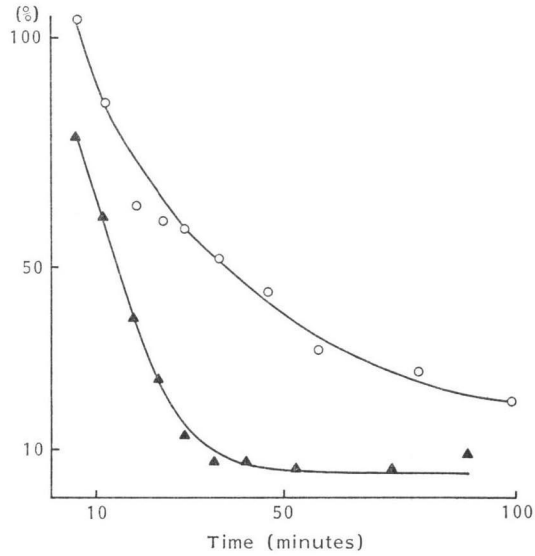


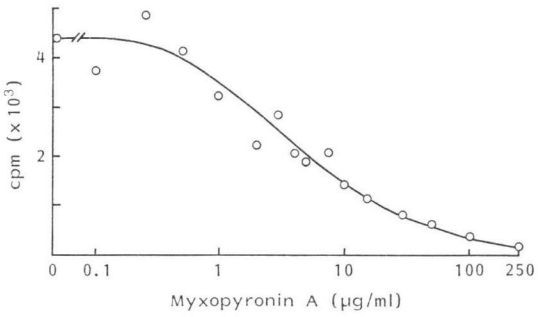
Table 2. The activity of wheat germ RNA-polymerase II in presence of myxopyronin A or α-amanitin*.

Myxopyronin A (μg/ml)	α-Amanitin (μg/ml)	Activity (%)
0		100
12.4		122
24.8		115
207		97
	1	0

* For experimental details, see text.

Fig. 8. The dose-response curve of DNA-dependent RNA polymerase from *Escherichia coli* with myxopyronin A.

The incorporation of [^{14}C]uracil into TCA insoluble material was taken as a measure for enzyme activity.



contained 1 unit of enzyme, the incubation time was 15 minutes, the temperature 25°C. From Table 2 it can be seen that this eukaryotic RNA polymerase was not effected by up to 200 μg of myxopyronin A per ml. As a control, the enzyme was incubated with α-amanitin[9] (Boehringer, Mannheim): 1 μg/ml (about 1 μM) were sufficient to inhibit the enzyme completely.

Discussion

Judging from the physico-chemical data and the recently elucidated chemical structure[1], myxopyro-

nin appears to be a new antibiotic. It is produced by *M. fulvus* strain Mx f50 as two homologues, myxopyronins A and B, the latter containing an additional methyl group (Fig. 1). Myxopyronin seems to be relatively rare, for so far we have not found another strain synthesizing this compound.

The inhibition spectrum of myxopyronin is rather narrow (Table 1). Gram-positive bacteria were usually inhibited; there were, however, remarkable differences in sensitivity even within one genus, as shown by *Bacillus megaterium* and *B. subtilis*. Most Gram-negative bacteria did not respond, still a few of them turned out to be very sensitive to myxopyronin. Yeasts and molds were totally resistant. In general, component B was more active than component A.

The inhibition spectrum becomes understandable, if one looks into the mechanism of action of the new antibiotic. Incorporation studies with labeled precursors suggested that myxopyronin inhibits RNA synthesis and, as a result, with a certain delay also protein synthesis. This hypothesis was corroborated by studies on isolated DNA-dependent RNA polymerase from *E. coli* which was strongly inhibited by myxopyronin. It should be mentioned, however, that neither with whole cells (*S. aureus*) nor with isolated RNA polymerase myxopyronin would block RNA synthesis completely: Even at high concentrations of the antibiotic (250 μg/ml), there remained a certain, if low residual activity.

While the MIC for *E. coli* was above 100 μg/ml, *E. coli* polymerase was strongly inhibited *in vitro* already at 1~10 μg/ml (Table 1 and Fig. 8). This discrepancy can be explained best by a restricted transport of the antibiotic into the cell, rather than by an inactivation of the antibiotic by the bacterium. This explanation is suggested by the fundamental difference in sensitivity between Gram-positive and Gram-negative bacteria, and by the superior activity of myxopyronin B, which chemically is slightly modified only.

The effect of myxopyronin seems to be restricted to prokaryotic RNA polymerases, for wheat germ RNA polymerase II did not respond to the antibiotic at all, up to a concentration of 200 μg/ml (Table 2). This may also indicate that the polymerase itself is the target, and not some other, more basic reaction. It remains to be seen, however, whether the other eukaryotic RNA polymerases are also resistant to myxopyronin. It is not yet known which step of RNA synthesis myxopyronin interferes with[10]. In any case, it is now obvious that the antibiotics produced by myxobacteria not only have completely different chemical structures, but also differ in their modes of action, inhibiting the electron transport in the respiratory chain[5,11], bacterial cell wall synthesis[3], and bacterial protein synthesis[4].

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