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Inhibition of Protein Synthesis by Amicetin, a Nucleoside Antibiotic*

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ABSTRACT: Amicetin is a nucleoside antibiotic in which an aminoacyl residue is attached to the amino group of cytosine, rather than to the carbohydrate moiety as is the case in the other known aminoacyl antibiotics, all of which have been shown to interfere with protein synthesis. At concentrations of amicetin which in cultures of *Escherichia coli* K12 completely prevented protein synthesis, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis continued. In a cell-free *E. coli* system, the antibiotic interfered with the polyuridylate-directed incorporation of L-phenylalanine into polyphenylalanine. Amicetin did not significantly prevent the formation of phenylalanyl-RNA, but interfered with the transfer of the soluble ribonucleic acid (s-RNA) bound amino acid to the polypeptide. These observations show that the attachment of the aminoacyl residue to the heterocyclic moiety of a nucleoside instead of to the carbohydrate portion can result in a molecule capable of interfering with protein synthesis.

A number of recently discovered antibiotics contain one or more amino acids attached to the carbohydrate moiety of a nucleoside (Fox *et al.*, 1966). The aglycone may be adenine, as in puromycin and homocitrullylaminoadenosine, or it may be cytosine, as in gougerotin and Blasticidin S. In the adenine-containing antibiotics the amino acids are attached to a 3-amino-3-deoxy-β-D-ribofuranosyl moiety (Figure 1a,b). When cytosine is the base, the amino acids are linked to a 4-amino-4-deoxyhexopyranose moiety of the D configuration (Figure 1c,d). Despite these differences, all of these antibiotics have been shown to interfere with protein synthesis (Yarmolinsky and De la Haba, 1959; Guarino *et al.*, 1963; Clark and Gunther, 1963; Nathans, 1964; Yamaguchi *et al.*, 1965).

In 1953, the antibiotic amicetin was first isolated from *Streptomyces fasciculatus* (McCormick and Hoehn, 1953) and from *Streptomyces vinaceus drappus* (DeBoer *et al.*, 1953; Caron and DeBoer, 1953). Two additional antibiotics, isolated in 1955 and named allomycin and sacromycin, were subsequently found to be identical with amicetin (Tatsuoka *et al.*, 1955; Hinuma *et al.*, 1955). The antibiotic was also found in a culture broth of *Streptomyces plicatus*, where it occurs together with two other structurally related aminoacyl nucleosides (Haskell *et al.*, 1958; Haskell, 1958). The structure of amicetin is shown in Figure 1e (Flynn *et al.*, 1953; Stevens *et al.*, 1956, 1962; Hanessian and Haskell, 1964). The α-methylseryl-*p*-aminobenzoyl residue is attached to the amino group of cytosine, rather than to the carbohydrate moiety, which consists of two hexopyranose units. Thus, amicetin differs structurally from the other known aminoacyl nucleoside antibiotics.

Early studies showed amicetin to be a strong inhibitor of mycobacteria both *in vitro* and *in vivo* (DeBoer *et al.*, 1953; Tatsuoka *et al.*, 1955). It also inhibited a number of gram-positive organisms (DeBoer *et al.*, 1953), and demonstrated intermediate activity against the KB strain of human epidermoid carcinoma cells (Smith *et al.*, 1959). Amicetin prolonged the survival time of mice with leukemia-82 (Burchenal *et al.*, 1954), but was inactive against acute leukemia in children who had previously developed resistance to methotrexate, 6-mercaptopurine, and steroids (Tan and Burchenal, 1956).

Little information concerning the mode of action of amicetin is available. Like a number of other structurally unrelated antibiotics, amicetin interfered with the resynthesis of M protein which had been removed with trypsin from a strain of *Streptococcus pyogenes* group A (Brock, 1963). Since the same concentration

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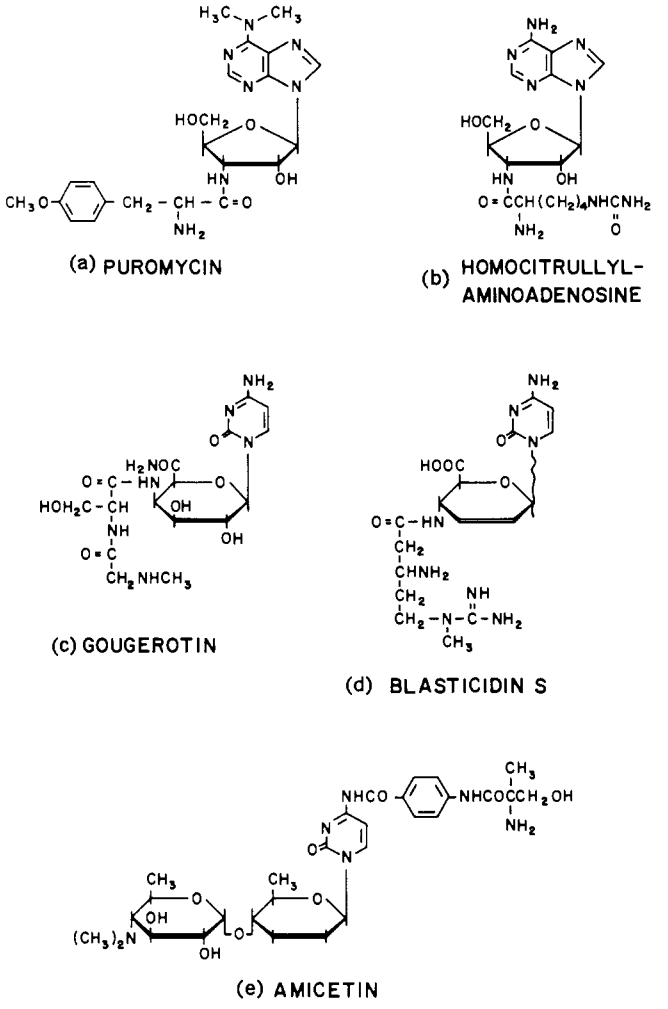

FIGURE 1: Structural representation of some aminoacyl antibiotics.

of amicetin inhibited both growth and protein synthesis, it was suggested (Brock, 1963) that the antibiotic inhibits growth because it inhibits protein synthesis.

The study reported here was carried out to determine the ability of amicetin to interfere specifically with protein synthesis both in growing cells and in a cell-free *in vitro* system of *Escherichia coli*. For comparative purposes, puromycin was examined under the same conditions.

Materials and Methods

Assay of Antimicrobial Potency and Inhibition Analysis. S. faecalis was grown in the medium of Flynn *et al.* (1951) from which uracil and the purines were omitted, and to which 1 mμg/ml of folic acid was added. *E. coli* was grown in the synthetic medium described by Gray and Tatum (1944). The assays were carried out by placing 1-ml portions of the media into 13 × 100 mm Pyrex culture tubes and adding 1 ml of water or of the solution containing the test compound. Sterilization was carried out by autoclaving for 6 min at 116–121°. Heat-labile compounds were sterilized by filtration and were then added to the autoclaved medium. The inocula were prepared from cultures of the test organisms grown in 5 ml of the basal medium for 20 hr at 37°. Following centrifugation and washing twice with isotonic saline, the cells were resuspended in enough saline to yield an optical density of 0.30 at 470 mμ as measured in a Beckman Model B spectrophotometer. A 1-ml portion of this suspension containing approximately 1.5×10^7 cells was diluted tenfold in saline, and 1 drop of this final dilution was placed in each assay tube. Incubation proceeded for 20 hr at 37°. All *E. coli* assays were carried out by shaking the cultures during incubation. The extent of growth was determined by means of a Klett-Summerson photoelectric colorimeter using a red filter (640–700 mμ).

The inhibition analyses were carried out by adding the following metabolites to the basal media containing the drug: adenine, guanine, xanthine, hypo-

xanthine, uracil, cytosine, thymine, and the corresponding ribonucleosides and deoxyribonucleosides. They were added individually or in combination at concentrations ranging from 10^{-3} to 10^{-6} M. Since *E. coli* K12 is inhibited by these concentrations of adenine or hypoxanthine, these bases were added at 10^{-7} M. DL-Alanine, L-aspartic acid, L-arginine, L-cysteine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, DL-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, DL-tyrosine, and DL-valine were added to the medium either singly or in combination at concentrations of 600 μg/ml. Glycylglycine, glycyl-DL-serine, and glycyl-DL-valine were supplied individually at 10^{-3} M concentrations. The normal vitamin complement comprising thiamine, riboflavin, pantothenic acid, nicotinic acid, *p*-aminobenzoic acid, biotin, and pyridoxine was increased 10–20-fold.

Assay of Growth, and of RNA, DNA, and Protein Synthesis. Cultures grown overnight were centrifuged, and enough cells were added to 300 ml of fresh medium to give a Klett colorimeter reading of 40. Incubation proceeded until a reading of 100 was obtained at which time the culture was divided into three 100-ml portions. Enough amicetin to give a final concentration of 10^{-3} and 10^{-4} M, respectively, was added to two flasks and the cultures were reincubated. The extent of growth was followed, and at stated intervals aliquots of 25 ml were withdrawn, chilled at once in an ice bath, and centrifuged in the cold. After washing with ice-cold saline, the pellets were frozen rapidly, and all fractions collected in this manner were assayed simultaneously for ribonucleic acid (RNA) (Brown, 1946; Volkin and Cohn, 1954), deoxyribonucleic acid (DNA) (Dische and Schwarz, 1937; Volkin and Cohn, 1954), and protein (Lowry *et al.*, 1951) content. Yeast RNA, calf thymus DNA, and bovine albumin served as standards.

Preparation of Ribosomes and Supernatant Fraction. Ribosomes, once washed, and the "supernatant" fraction 100,000*g* were prepared from frozen *E. coli* B cells, harvested in the middle of their logarithmic phase of growth, according to the procedure of Nirenberg and Matthaei (1961). Soluble ribonucleic acid (s-RNA), stripped of esterified amino acids, was obtained from *E. coli*, and this material was also used for the preparation of L-[^{14}C]phenylalanyl-RNA (von Ehrenstein and Lipmann, 1961). The composition of the complete assay is described in the footnote to Table I.

Materials. The sample of amicetin used was prepared by the Upjohn Co., batch no. 9924. Puromycin was purchased from Nutritional Biochemicals Co. Polyuridylic acid was purchased from Miles Chemical Co., and L-[^{14}C]phenylalanine (uniformly labeled) from the New England Nuclear Corp.

TABLE I: Amicetin-Dependent Inhibition of Polyuridylate-Directed Polyphenylalanine Synthesis.[a]

Conditions	Radioactivity Incorp (cpm)	% Inhibn
Complete system	4120	—
− polyuridylic acid	56	—
+ amicetin, 1 μmole/ml	1812	56.0
+ amicetin, 2 μmoles/ml	1295	68.6
+ puromycin, 1 μmole/ml	50	98.8
+ 2 × ATP	4130	—
+ 2 × ATP + amicetin, 1/μmole/ml	2095	49.2

[a] Added in the following order, the complete incubation system in a total volume of 0.25 ml contained: 25 μmoles of Tris–HCl buffer, pH 7.8, 125 μmoles of ammonium chloride adjusted to pH 7.6, 2.5 μmoles of magnesium acetate, 1.5 μmoles of 2-mercaptoethanol, 7.5 mμmoles of GTP, 0.25 μmole of ATP, 0.5 μmole of trisodium phosphoenolpyruvate, 10 μg of pyruvate kinase, 10 μg of commercial polyuridylic acid, 18 ODU at 260 mμ of s-RNA, 0.17 mg of "ribosomal" protein, 0.17 mg of "supernatant" protein, and 7.5 mμmoles of L-[^{14}C]phenylalanine (no other nonradioactive amino acids were added) with a specific activity of 10 μc/μmole. The inhibitor was added after addition of the s-RNA and before the addition of ribosomes; incubation time, 30 min at 37°. At the end of the incubation period, 0.1-ml aliquots were applied to duplicate 3MM paper disks, and the hot acid-precipitable material was assayed as described previously (Coutsogeorgopoulos and Khorana, 1964).

TABLE II: Inhibition of the Growth of *S. faecalis* and *E. coli* by Amicetin and Puromycin.

Organism	Amount (M) Needed for 50% Growth Inhibn: Amicetin	Puromycin
E. coli (K12)	3×10^{-4}	3×10^{-5}
S. faecalis (ATCC 8043)	2×10^{-5}	4×10^{-6}

Results and Discussion

Table II summarizes the extent of inhibition of the growth of *E. coli* and *S. faecalis* by amicetin and by puromycin, as determined by tube dilution assay using small inocula. *S. faecalis* is somewhat more susceptible to inhibition by the two agents than is *E. coli*, but in making such a comparison, it should be realized that the organisms are grown in two different media. However, in both test systems amicetin is an approximately tenfold weaker inhibitor of growth than is puromycin.

When added to growing cultures at concentrations

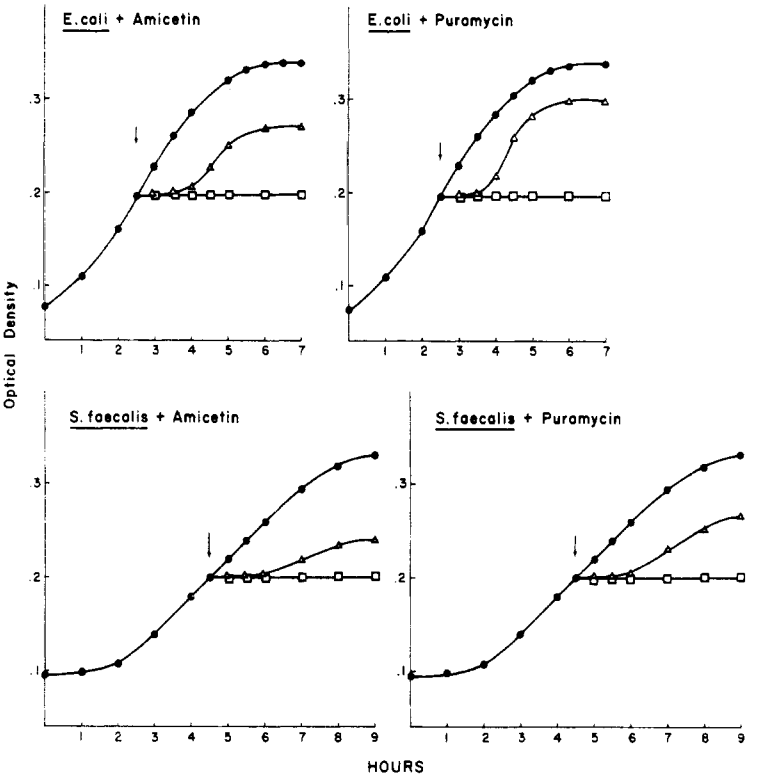

FIGURE 2: A comparison of the effect of amicetin and puromycin on the growth of *E. coli* and *S. faecalis*; concentrations: □ = 10^{-3} M, Δ = 10^{-4} M, ● = control.

of 10^{-3} and 10^{-4} M, both antibiotics immediately inhibit further growth (Figure 2). At 10^{-4} M, this stoppage is only temporary, and growth is resumed approximately 3 hr after addition of the drug. Although growth is somewhat more extensive in the presence of puromycin than it is in the presence of amicetin, maximal growth is not reached, even after prolonged incubation.

The similarity of effects of the two antibiotics on growth is paralleled by their effect on the synthesis of macromolecular components. As shown in Figure 3, addition of amicetin to logarithmically growing cultures of *E. coli* inhibits predominantly protein synthesis and affects the formation of RNA and DNA only little. While protein synthesis ceases at once, production of both nucleic acids continues, although at a slower rate. Considering the immediate cessation of growth which occurs upon addition of the drug, continuation of RNA and DNA synthesis, even at the decreased rate, clearly implicates protein synthesis as the process primarily affected by amicetin. To confirm this interference of the antibiotic with protein synthesis observed in intact cells, its effect upon polyuridylate directed polyphenylalanine synthesis in a cell-free *E. coli* system was investigated. As shown in Table I, amicetin at a 1×10^{-3} M concentration inhibited the incorporation of L-phenylalanine into polyphenylalanine by 56%, but was not as effective in preventing this inhibition as was puromycin at an equimolar concentration. Considering the total inhibition of protein synthesis in

TABLE III: Effect of Amicetin on the Formation of L-[^{14}C]Phenylalanyl-RNA.[a]

Conditions	Radioactivity Incorp (cpm)	% Inhibn
Complete system	2360	—
− s-RNA	60	—
+ amicetin, 1 μmole/ml	2200	7

[a] The complete system was the same as that described in Table I with the difference that polyuridylic acid and ribosomes were omitted; incubation time, 15 min at 37°. The samples were assayed for cold acid-precipitable material in the same manner as described for the hot acid-precipitable material (Coutsogeorgopoulos and Khorana, 1964), with the exception that the second washing with 3.5% perchloric acid was carried out at 4° for 5 min instead of 90° for 15 min. All additional perchloric acid washings were also performed at 4°.

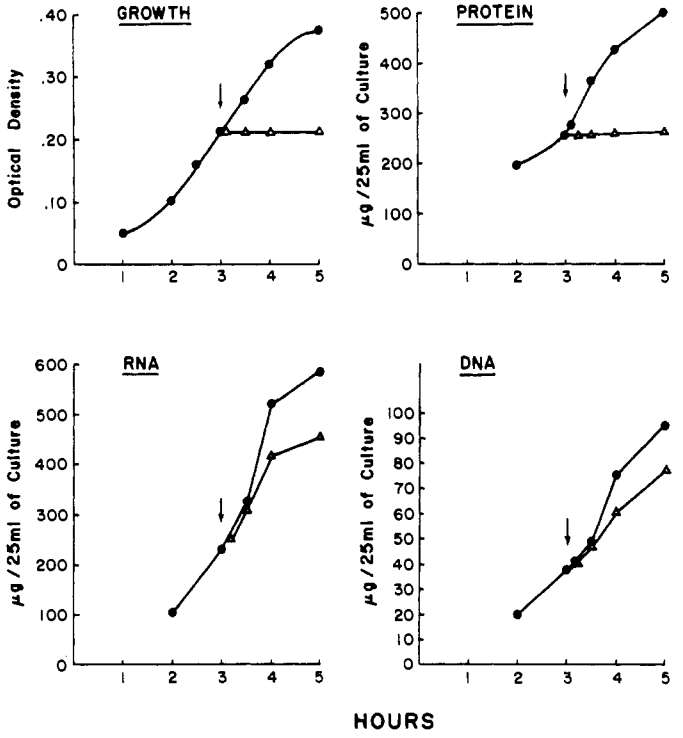

FIGURE 3: The effect of amicetin on protein, RNA, and DNA synthesis in *E. coli*. ●, control; Δ = amicetin treated culture. The arrow denotes the time at which the antibiotic was added.

intact cells by 1×10^{-3} M amicetin, it is apparent that phenylalanine incorporation does not measure the total inhibitory activity of the antibiotic. In view of the fact that the *in vitro* inhibition of polypeptide synthesis increases with increased concentrations of amicetin, it is possible that the difference between the *in vivo* and *in vitro* effectiveness of the antibiotic may result from its accumulation by the cells. To gain information concerning the possible step at which the antibiotic interferes with the *in vitro* polypeptide synthesis, its effect upon the formation of L-[^{14}C]phenylalanyl-RNA and on the transfer of the amino acid from the charged RNA to the polypeptide was examined. As shown in Table III, amicetin did not prevent significantly the formation of phenylalanyl-RNA, but as indicated in Table IV, interfered with the transfer of the s-RNA-bound amino acid to the polypeptide. Since the extent of this interference was approximately the same as when free phenylalanine was used, it appears that amicetin interferes with cell-free polypeptide formation by blocking the transfer of amino acid from aminoacyl-RNA to the ribosomal peptides. Increasing the level of ATP[1] did not affect the inhibition.

Thus, amicetin acts in a manner similar to puromycin, homocitrullylaminoadenosine, gougerotin, and Blasticidin S. None of these antibiotics affect the activation of amino acids or their transfer to s-RNA, but instead they block peptide synthesis following aminoacyl-RNA formation (Yarmolinsky and de la Haba, 1959; Guarino *et al.*, 1963; Clark and Gunther, 1963; Nathans, 1964; Yamaguchi *et al.*, 1965). This does not imply, of course, that the mechanism of action of the antibiotic is the same as that of other aminoacyl nucleosides. It remains to be determined for instance whether amicetin can function as a substitute for aminoacyl-RNA, accepting the carboxyl-activated peptide bound to the transfer ribonucleic acid (t-RNA) template, stimulating thereby the release of nascent peptide chains, much as does puromycin. Alternately, amicetin might act as do gougerotin and Blasticidin S, which have been suggested to inhibit the peptide bond formation and prevent the release of peptides from the ribosomes (Clark and Chang, 1965; Casjens and Morris, 1965; Yamaguchi *et al.*, 1965). This aspect is currently under study. Like these antibiotics, amicetin had essentially no effect on the binding of L-[^{14}C]-phenylalanine-RNA to the ribosomes in the presence polyuridylic acid as assayed by the filter membrane method of Nirenberg and Leder (1964) (Table V).

Whatever the actual mechanism, it is possible that although the aminoacyl residue is attached to the aglycone rather than to the amino sugar moiety, amicetin may nevertheless be able to function as an analog of aminoacyl-RNA. Another alternative which requires consideration is that it may act as an analog of peptidyl-RNA.

The inhibition of growth of *E. coli* or *S. faecalis* by

[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate.

TABLE IV: Inhibition of Polyphenylalanine Formation from L-[^{14}C]Phenylalanyl-RNA.[a]

Conditions	Radioactivity Incorp (cpm)	% Inhibn
Complete system	2120	—
− polyuridylic acid	184	—
+ amicetin, 1 μmole/ml	1100	48.1
+ puromycin, 1 μmole/ml	84	96.0

[a] The complete system was identical with that of Table I with the exception that ATP and s-RNA were omitted, 3.0 ODU, at 260 mμ, of L-[^{14}C]phenylalanyl-RNA were added, instead of the free radioactive amino acid. The specific activity of the L-[^{14}C]phenylalanyl-RNA used was 375 μc/μmole of charged L-[^{14}C]phenylalanine and 4000 cpm/ODU at 260 mμ of L-[^{14}C]phenylalanyl-RNA. Under our counting conditions, 1 μμmole of L-[^{14}C]phenylalanine corresponds to 500 cpm. Incubation time and assay were as described in Table III.

TABLE V: Inability of Amicetin to Affect the Binding of L-[^{14}C]Phenylalanyl-RNA to the Ribosomes of *E. Coli*.[a]

Conditions	Radioactivity Adsorbed on Millipore Filter (cpm)
Complete system	2458
− polyuridylic acid	180
+ amicetin, 1 μmole/ml	2428

[a] The complete system, in a total volume of 0.25 ml, contained 25 μmoles of Tris–HCl buffer, pH 7.2, 125 μmoles of ammonium chloride adjusted to pH 7.6, 5.0 μmoles of magnesium acetate, 20 μg of polyuridylic acid, 0.17 mg of ribosomal protein, and 3.0 ODU, at 260 mμ, of the L-[^{14}C]phenylalanyl-RNA described under Table IV. Amicetin was added before the addition of the L-[^{14}C]phenylalanyl-RNA and after the addition of the ribosomes. Incubation time was 5 min at 37°. At the end of the incubation period, the radioactivity adsorbable on Millipore filters was assayed as described by Nirenberg and Leder (1964).

amicetin or puromycin is not prevented by the supplementation of the growth media with the purines, pyrimidines, amino acids, or peptides listed above. Since many of these metabolites can readily prevent the inhibition of a variety of nucleoside analogs (Bloch and Nichol, 1964; Bloch *et al.*, 1966), their inability to prevent the inhibition of growth is an indication that the two aminoacyl nucleosides do not act as antimetabolites of either nucleosides or amino acids but function as analogs of a combined moiety.

The inhibition of protein synthesis by amicetin at the ribosomal level, and the structural comparison with compounds able to act similarly, support the previous suggestion (Fox *et al.*, 1966) that the presence of an aminoacyl group on one end of the nucleoside, and a second basic center at the other end, may be sufficient for binding of such molecules to ribosomes. Indeed, the presence of the second basic center may be required for binding, and this may well be the reason why no aminoacylamino nucleoside antibiotics containing purine or pyrimidine bases other than adenine or cytosine have been discovered as yet.

The possible metabolic role of the antibiotic has recently been discussed (Fox *et al.*, 1966), and it is of interest that the linkage between the aminoacyl residue and the base parallels the linkage found in the N^6-(aminoacyl)adenosines isolated from yeast RNA (Hall, 1964).

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