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PEPTIDYL TRANSFERASE SUBSTRATE ACTIVITY AND INHIBITION OF PROTEIN BIOSYNTHESIS BY A HYDROPHILIC-AMINOACYL ANALOGUE OF PUROMYCIN

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SUMMARY

A puromycin analogue possessing a hydrophilic amino acid, 3'-N-[S-(6-hydroxyhexyl)-L-cysteinyl]puromycin aminonucleoside, has been prepared and examined as a substrate for ribosomal peptidyl transferase. Kinetic studies indicate that this non-aromatic aminoacyl analogue is 95.6% as efficient as the parent antibiotic in the transpeptidation reaction. In addition, the analogue is an effective inhibitor of poly (U) and poly (U,C) directed protein synthesis in an Escherichia coli cell free system.

The antitumor antibiotic, puromycin, inhibits protein synthesis by substituting for the incoming coded aminoacyl-tRNA and serving as an acceptor of the nascent peptide chain of ribosome-bound peptidyl-tRNA (1-4). Since puromycin becomes linked to the released peptides by means of a peptide bond, the reaction has been used extensively in the investigation of the peptidyl transferase reaction (5). Nathans and Neidle were the first to observe that the nature of the amino acid of puromycin and its derivatives played an important role in the ability of these compounds to bind to the ribosome (6). For maximal inhibition the amino acid must be of the L-configuration and must contain an aromatic residue (6). Later reports confirmed that the substitution of the amino acid moiety of puromycin with other amino acids produced derivatives with high activity in releasing peptide from procaryotic and eucaryotic ribosomes only when the amino acid contained an aromatic ring (7-10). These studies to determine the structural requirements for puromycin activity have led to the general conclusion that there is an absolute requirement for an

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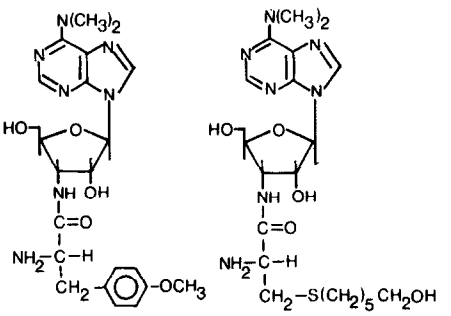

Fig. 1

aromatic amino acid in the puromycin molecule. Attempts to explain the function of the aromatic group have included a stacking of the aromatic ring with the purine ring of puromycin and the terminal adenylic acid of tRNA (12); the fortuitous binding of the aromatic ring with the binding site normally occupied by the cytosine ring of the penultimate base of tRNA (8); the optimal fit into a hydrophobic pocket of the ribosome (11); and the creation of a favorable conformation of the peptide acceptor by the aromatic amino acid at the expense of thermodynamic parameters (13). In the present communication we present the first active puromycin analogue possessing a non-aromatic, hydrophilic amino acid (Fig. 1).

MATERIALS AND METHODS

Puromycin dihydrochloride was obtained from ICN Pharmaceuticals, Inc., [^{14}C]-L-phenylalanine was obtained from New England Nuclear, Escherichia coli cell paste (B, mid log) was purchased from General Biochemicals. The polynucleotides were obtained from Miles Laboratories, and ATP, GTP, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma. Preparation of ribosomes, S-100, factors washable from ribosomes, and Ac[^{14}C]-L-Phe-tRNA were as previously described (14).

The puromycin analogue, 3'-N-[S-(6-hydroxyhexyl)-L-cysteinyl]-puromycin aminonucleoside (1) was obtained as a white solid, mp 167-168°C: spectra (infrared, ultraviolet, and proton magnetic resonsance), and elemental analysis (C,H,N) are consistent with the structure present in Fig. 1. Details of the synthetic method are being presented elsewhere (K.L.L. Fong and R. Vince, J. Med. Chem., submitted).

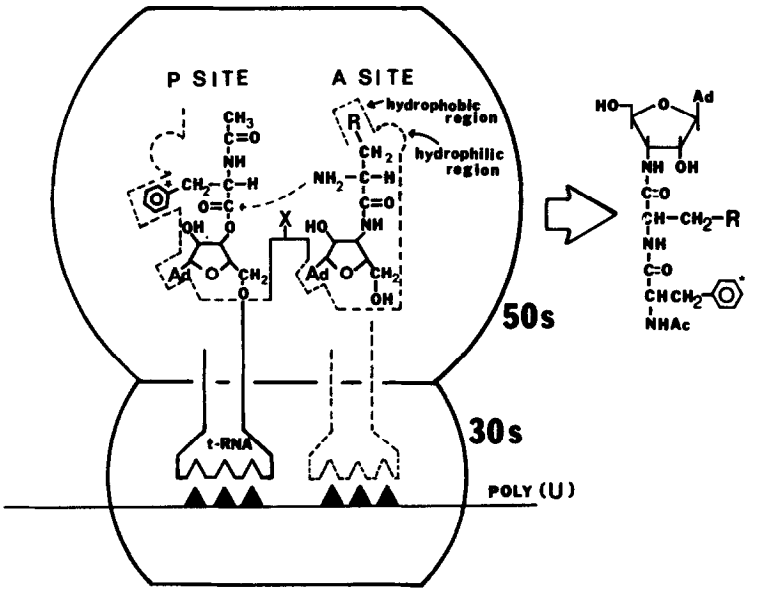

Figure 2. Schematic representation of the peptidyl transferase reaction involving transfer of N-acetyl-[^{14}C]phenylalanine from the P-site to a puromycin analogue located at the A-site. Peptide bond formation results from attack of the α-amino group of aminoacyl-tRNA (or puromycin derivatives) on the carboxyl terminal ester of peptidyl-tRNA. It is equally possible that a ribosome-peptide intermediate (via x) may first occur. The dotted lines overlapping the two subunits indicate the site normally occupied by an aminoacyl-tRNA. Ad represents an adenine or N,N-dimethyladenine in this diagram.

RESULTS AND DISCUSSION

A puromycin analogue (1) in which the p-methoxyphenylalanyl group was replaced by a S-(hydroxyhexyl)cysteinyl moiety was evaluated as a substrate for the peptidyl transferase reaction. In this assay, 1 was incubated with a preformed complex consisting of E. coli ribosomes, poly (U), and N-acetyl-[^{14}C]phenylalanyl-tRNA as illustrated in Fig. 2. Since the A-site of the ribosome must accommodate all amino acids under natural conditions, adjacent hydrophobic and hydrophilic regions are postulated in Fig. 2 to accommodate the corresponding side chains (R). Thus, the character of the R group will determine its orientation at the A-site. In the present situation the S-alkyl moiety (R) of 1 could interact with

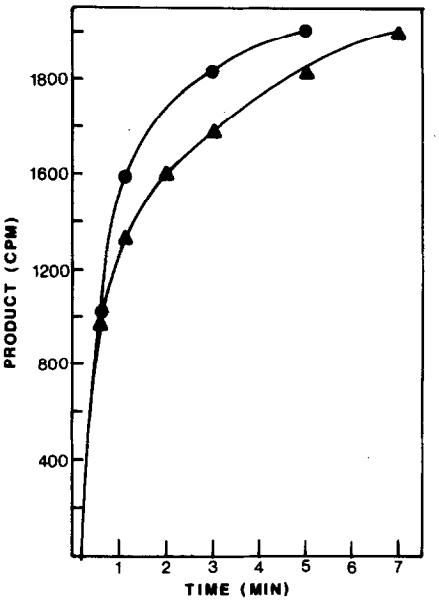

Figure 3. The course of reaction at 28° of Ac-[^{14}C]-L-Phe-tRNA as a donor substrate with puromycin (●) and 1 (▲) with E. coli ribosomes. Both compounds were tested at 2 x 10^{-4} M. The Ac-[^{14}C]Phe-tRNA was bound to the ribosomes in a reaction mixture containing 100 mM Tris-Cl (pH 7.5), 100 mM NH_4Cl (pH 7.6), 15 mM $Mg(OAc)_2$, 0.65 mM dithiothreitol, 2.8 A_{260} units of washed E. coli ribosomes, 1.2 mM GTP, 63 µg of FWR, 0.35 A_{260} units of poly (U), and 21 pmoles of Ac-[^{14}C]Phe-tRNA (464 pCi/pmole) in a total volume of 0.1 ml. The binding mixture was incubated at 28° for 8 minutes, and the peptidyl transferase reaction was initiated by the addition of 80 µl of incubation cocktail to 20 µl of puromycin or its analogue. Reactions were incubated for specified times and product formation was measured as described in reference 14.

the hydrophobic area normally occupied by the aromatic ring of puromycin. However, the six carbon chain of the S-(hydroxyhexyl)cysteinyl moiety is capable of extending beyond the reach of the natural R groups which have been used in previous studies. Evidently, a hydrophilic area is encountered beyond the hydrophobic site, and the orientation of the hydroxyl group into such a hydrophilic pocket may impart sufficient binding to enhance the formation of a complex with the ribosome.

The rate of product formation during the course of the peptidyl transferase reaction with Ac[^{14}C]-L-Phe-tRNA as a donor substrate and puromycin and 1 as acceptor substrates is illustrated in Fig. 3. The acceptor

TABLE I. Inhibition of [^{14}C]-L-Polyphenylalanine Formation

	50% inhibition (μM)	
Compound	Poly (U)	Poly (U,C)
1	269	0.59
Puromycin	178	0.50

Assay conditions are those previously described (14).

activities for the parent antibiotic and the hydrophilic aminoacyl analogue are comparable. Double reciprocal plots of initial velocities of product formation versus substrate concentrations were used to obtain the kinetic parameters, Km and Vmax. The Km and Vmax values are 0.234 mM and 3.99 pmole product/minute, respectively, for puromycin and 0.166 mM and 2.71 pmole product/minute, respectively, for 1. The ratio of Vmax/Km appears as a rate constant in the Michaelis-Menton equation at low substrate concentrations. Thus, the substrate efficiency of 1, obtained from the Vmax/Km value, is 95.6% compared with puromycin (100%).

The effect of 1 on the rate of poly (U) and poly (U,C) directed polyphenylalanine formation in an E. coli cell free system with washed ribosomes is presented in Table 1. Examination of the data reveals that 1 inhibits protein synthesis in both systems. It is noteworthy that puromycin and its analogues exhibit greater inhibitor activities in the presence of poly (U,C) than when poly (U) is used as mRNA; similar observations have been reported for puromycin and chloramphenicol (15-17). We have found that the inhibitory values obtained from poly (U,C) directed protein synthesis are consistent with polyribosome systems and reflect the effect of puromycin analogues on protein synthesis in intact cells (Vince and C.L. Ritter, unpublished).

The present study extends the exploration of the ribosome beyond the area normally occupied by the R groups of the aminoacyladenyl termini of

tRNA molecules. The discovery of the existence of hydrophilic character beyond the normal binding region, and the proper exploitation of this information has provided the first active puromycin analogue possessing a hydrophilic amino acid R-group.

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