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Puromycin Analogs. Ribosomal Binding and Peptidyl Transferase Substrate Activity of a Carbocyclic Analog of 8-Azapuromycin†

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ABSTRACT: The synthesis of an 8-aza carbocyclic puromycin analog, 7-dimethylamino-3-{(*R*)-[(2*R*)-hydroxy-(3*R*)-(*p*-methoxyphenyl-L-alanylamino)]cyclopentyl}-*v*-triazolo[4,5-*d*]pyrimidine (**1**), is described. The previously described carbocyclic pyrimidine, 2α-acetamido-5β-(5-amino-6-chloro-4-pyrimidinylamino)cyclopentan-1α-ol (**2**), was diazotized and converted to the corresponding 8-azapurine (**4**). Treatment of **4** with aqueous dimethylamine followed by acid hydrolysis of the acetamido blocking group gave the amino nucleoside analog (**6**). Coupling of **6** with *N*-benzyloxycarbonyl-*p*-methoxyphenyl-L-alanine, followed by hydrogenolysis of the Cbz blocking group, gave a mixture of **1** and its diastereoisomer, 7-dimethylamino-3-{(*S*)-[(2*S*)-hydroxy-(3*S*)-(*p*-methoxyphenyl-L-alanylamino)]cyclopentyl}-*v*-triazolo[4,5-*d*]pyrimidine (**2**). The diastereomers were separated by chromatography. Biological testing with *Escherichia coli* ribosomes provides a comparison of the relative ribosomal binding affinities and peptidyl transferase substrate activities of puromycin and 8-aza carbocyclic puromycin. Kinetic data indicate that such compounds are capable of binding to the ribosomal acceptor site and act as peptidyl transferase substrates in a manner identical with the mechanism of action of puromycin.

A variety of analogs and isomers of the antibiotic puromycin have been prepared to define the structural requirements for protein biosynthesis inhibition. It has been well documented that puromycin binds to the ribosome and terminates protein synthesis by accepting the growing peptide

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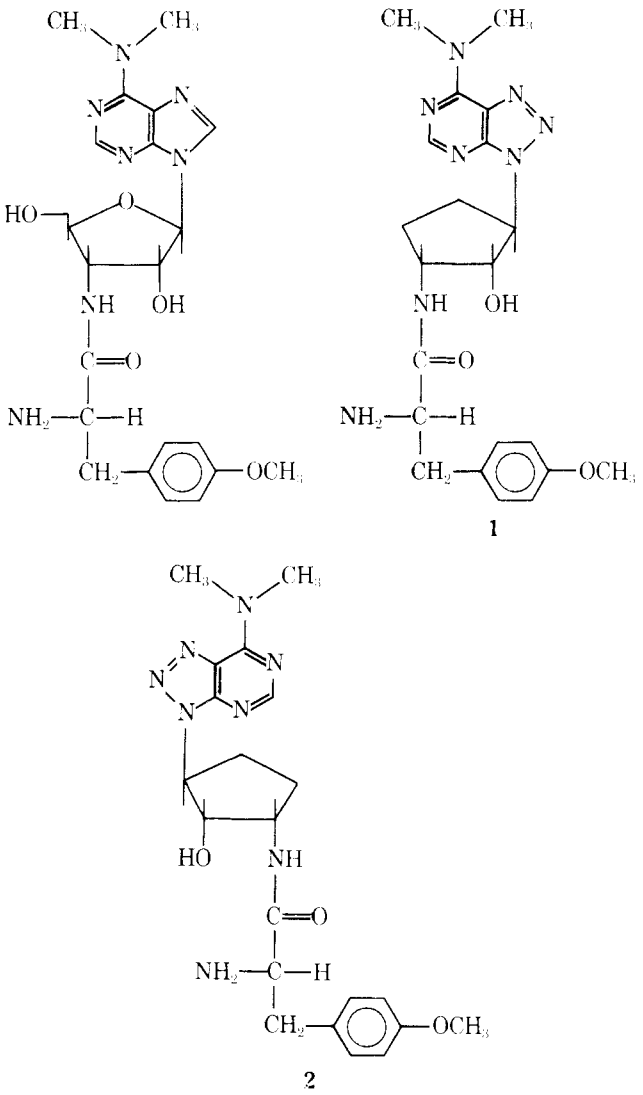


FIGURE 1: Structures of puromycin, the 8-aza carbocyclic analog, **1**, and its diastereoisomer, **2**.

chain from peptidyl-tRNA (Nathans, 1964; Traut and Monro, 1964). For this reason, puromycin has been used extensively as a tool in the investigation of the peptidyl transferase reaction. Because of its striking structural resemblance to the aminoacyladenyl terminus of aminoacyl-tRNA, all of the structural analogs have retained the classical adenosyl nucleoside structure in which an N-substituted amino sugar is attached to a purine ring through a glycosidic linkage (for example, see Nathans and Neidle, 1963; Harris *et al.*, 1971; Eckermann *et al.*, 1974). In a program involving the design of peptidyl transferase substrates and inhibitors, the difficulties encountered in preparing 3-aminoribosyl nucleosides have led to the recent development of carbocyclic puromycin analogs (Vince and Daluge, 1974; Daluge and Vince, 1972; Vince *et al.*, 1972). The facile synthesis of these analogs allows for a great degree of structural modification and also provides a series of stereoisomers which are not available from puromycin.

Since ribonucleosides are easily cleaved hydrolytically or enzymatically, many nucleosides which may be effective chemotherapeutic agents become inactive *in vivo* because of rapid cleavage of the glycosidic linkage (Roll *et al.*, 1956; Baker, 1967). Thus, the carbocyclic analogs provide a purine nucleoside moiety with a stable C–N bond due to the replacement of the furanose ring by a cyclopentane ring. Because of the nature of the carbocyclic analogs and their facile structural manipulation to obtain various active analogs, such compounds may be extremely useful in elucidating various aspects of protein synthesis. Specifically, the ability of puromycin analogs to serve as acceptors or nonsubstrate inhibitors in the transpeptidation reaction must be examined to define the characteristics of the puromycin binding site. This communication reports the preparation and biological activity of two diastereomeric nonclassical puromycin analogs (**1** and **2**) (Figure 1) in which both the purine ring and the carbohydrate moiety have been altered.

Materials and Methods

Melting points were determined on a Mel-Temp and are corrected. Optical rotations were measured at ambient temperatures with a Perkin-Elmer 141 automatic polarimeter, nuclear magnetic resonance (nmr) with a Varian A-60D spectrometer, ir with a Perkin-Elmer 237B spectrophotometer, and uv with a Beckman 25 recording spectrophotometer. Analytical results were determined by M-H-W Laboratories, Garden City, Mich. *Escherichia coli* cell paste (B, mid log) was purchased from General Biochemicals and tRNA from Calbiochem. [^{14}C]-L-Phenylalanine was obtained from New England Nuclear. The polynucleotides were purchased from Miles Laboratories and ATP, GTP, phosphoenolypruvate, and pyruvate kinase were purchased from Sigma. Puromycin dihydrochloride was obtained from Nutritional Biochemicals Co.

Preparation of Ribosomes, S-100, and Factors Washable from Ribosomes. The S-100 fraction and washed ribosomes were prepared as reported by Bodley (1969). The final ribosomal suspension was dialyzed against four changes of buffer containing 0.01 M Tris-Cl, 0.01 M $Mg(OAc)_2$, 0.05 M NH_4Cl, 0.001 M dithiothreitol (pH 7.4), and 50% gylcerol for 2 days, adjusted to 98 mg/ml, and stored at −20° for over 2 years with only slight loss in activity. The factors washable from ribosomes (FWR) were prepared as described by Fico and Coutsogeorgopolous (1972) and stored in aliquots at −70° and thawed immediately before use.

Preparation of Donor Substrate. tRNA from *E. coli* B (Calbiochem) consisting of a mixture of all tRNAs was charged with [^{14}C]phenylalanine as described by Ravel and Shorey (1971). The [^{14}C]Phe-tRNA was converted to Ac[^{14}C]-L-Phe-tRNA by the method of Haenni and Chapeville (1966). Since Ac-Phe-tRNA prepared from benzoylated DEAE-cellulose purified $tRNA^{Phe}$ gave the same results in our assays, the above preparation was used without removal of the uncharged tRNAs. The efficiency of the acetylation (100%) was checked by descending paper chromatography in butanol–acetic acid–water (78:5:17) on Whatman No. 1 or No. 4 paper. An aliquot of the aminoacyl-tRNA was hydrolyzed with 1 N NaOH for 30 min at 37°. *N*-Ac-phenylalanine and phenylalanine were used as standards. The chromatograms were cut into strips and counted in toluene with Permablend.

Inhibition of [^{14}C]-L-Polyphenylalanine Formation. Reactions were performed in a final volume of 80 μl and contained 0.1 M Tris-Cl (pH 8), 0.05 M KCl, 0.01 M β-mercaptoethanol, 0.005 M phosphoenolpyruvate, 0.001 M ATP, 0.05 mM GTP, 30 μg/ml of pyruvate kinase, 4 μl (40 μg) of S-100 fraction, 0.5 μCi/ml of [^{14}C]-L-phenylalanine (no other amino acids present), 15 mM $Mg(OAc)_2$, 1.2 mg/ml of ribosomes, and 0.5 μmP/ml of poly(U,C) (1:1). In a typ-

ical experiment a solution was made which contained all of the above components (except poly(U,C) and the desired concentration of inhibitor. The reaction was initiated by the addition of poly(U,C). Incubations were for 30 min at 37°.

Aliquots of 50 μl were removed from each tube and placed on a 2-cm square of Whatman No. 31 ET paper. The papers were collected and immersed in 400 ml of 10% trichloroacetic acid. The papers were then heated at 90° for 15 min in 5% trichloroacetic acid and then two washes each in ethanol–ether (2:1) and ether, respectively. All washes were for 5 min (except the one at 90°) with gentle stirring while the papers were suspended in a wire basket. The papers were dried and placed in counting vials containing a toluene-based liquid scintillator and counted in a Packard TriCarb Model 3375. Counting efficiency was approximately 80%. All counts were corrected by blanks in which poly(U,C) was absent. All values represent an average of triplicate determinations. The standard deviation of such replicates averaged <±6%.

Assay for Peptidyl Transferase Substrate Activity with E. coli Ribosomes. The Ac-[^{14}C]-L-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), 12 mM $Mg(OAc)_2$, 6 mM β-mercaptoethanol, 9.07 A_{260} units of ribosomes, 11.4 μg of poly(U), 1.20 mM GTP, 1.5 μl of FWR (63 μg of protein), and 0.5 A_{260} unit of Ac-[^{14}C]-L-Phe-tRNA with 2.5 pmol of [^{14}C]Phe per A_{260} unit in a total volume of 0.1 ml.

The reaction mixture was allowed to incubate for 8 min at 28° temperature and then placed in ice. Maximum binding of the tRNA was obtained during this time as indicated by adsorption of the Ac-[^{14}C]-L-Phe-tRNA–poly(U)–ribosome complex onto type HA, 0.45-μ pore size Millipore filters followed by scintillation counting. The reaction was started by the addition of 20 μl of substrate followed by incubation at 28° for a specified time. The reaction was stopped by addition of an equal volume of 1 N NaOH to each tube and the tubes were allowed to incubate at 28° for 30 min; 1 ml of ethyl acetate was added to each tube and the mixture was vortexed vigorously for 1 min. The tubes were then centrifuged at 500 rpm for 1 min and 0.5 ml of the organic layer was removed and added to 10 ml of Triton X-100–toluene (1:2) for counting.

Antimicrobial Testing. The assay procedure for the antimicrobial activities has been described previously (Daluge and Vince, 1972).

Synthesis

7-Chloro-3-[β-(2α-hydroxy-3α-acetamido)cyclopentyl]-v-triazolo[4,5-d]pyrimidine (**4**). To a solution of 2α-acetamido-5β-(5-amino-6-chloro-4-pyrimidinylamino)-cyclopentan-1α-ol (**2**) (Vince and Daluge, 1974) (800 mg, 2.78 mmol) in H_2O (35 ml) and 2 N HCl (8.5 ml) was added a solution of $NaNO_2$ (266 mg, 3.86 mmol) in H_2O (16 ml) dropwise at 0° with continuous stirring over a period of 5 min. The ice bath was removed and stirring was continued for 60 min. The solution was neutralized with solid $NaHCO_3$, saturated with NaCl, and extracted with $CHCl_3$ (4 × 100 ml). The organic extracts were combined, dried ($CaSO_4$), and evaporated to a light yellow solid. Recrystallization from absolute EtOH gave analytically pure **4**: 504 mg (61%); mp 173–175°; ir (KBr) 3400 (NH), 3275 (OH), 1650 (amide 1), 1590 and 1570 (C=C, C=N), 1550 cm^{-1} (amide 2); pmr (DMSO-d_6) δ 2.0 (s) overlapped by 1.85–2.50 (m, 7, CH_3CO and 2 CH_2), 4.18–6.50 (m, 2, H-2′, H-3′), 5.10–5.40 (bs, 1, H-1′), 7.35 (s, 1, OH), 7.83 (s, 1, NHAc), 8.25 (s, 1, purine H-2). The singlets at 7.35 and 7.83 disappeared upon addition of D_2O.

Anal. Calcd for $C_{11}H_{13}N_6O_2Cl$: C, 44.52; H, 4.42; N, 28.33. Found: C, 44.70; H, 4.52; N, 28.56.

7-Dimethylamino-3-[β-(2α-hydroxy-3α-acetamido)cyclopentyl]-v-triazolo[4,5-d]pyrimidine (**5**). A solution of **4** (1.00 g, 3.37 mmol) in 40% aqueous dimethylamine (50 ml) was allowed to react at ambient temperature for 90 min. The solution was diluted with saturated NaCl (50 ml) and extracted with EtOAc (3 × 150 ml). The combined organic layers were dried ($CaSO_4$) and evaporated *in vacuo* to a white solid. Recrystallization from EtOAc gave 726 mg (70.4%) of pure **5**: mp 159–160°; ir (KBr) 3400 (NH), 3275 (OH), 1650 (amide 1), 1620 and 1575 (C=C, C=N), 1550 cm^{-1} (amide 2); pmr (DMSO-d_6) δ 1.85 (s) overlapped by 1.85–2.35 (m, 7, CH_3CO and 2 CH_2), 3.08–4.05 (d, 6, NMe_2), 4.15–4.55 (s, 2, H-2′, H-3′), 4.85–5.28 (s, 1, H-1′), 5.30–5.45 (d, 1, OH), 7.48–7.85 (d, 1, amide NH), 8.25 (s, purine H).

Anal. Calcd for $C_{13}H_{19}N_7O_2$: C, 51.13; H, 6.27; N, 32.11. Found: C, 51.15; H, 6.36; N, 32.27.

7-Dimethylamino-3-[β-(2α-hydroxy-3α-amino)cyclopentyl]-v-triazolo[4,5-d]pyrimidine (**6**). A solution of **5** (500 mg, 1.64 mmol) in 40 ml of 2 N HCl was heated under reflux for 2 hr. The reaction mixture was evaporated *in vacuo* to a solid foam. The product was dissolved in MeOH (10 ml) and passed through a column of Amberlite IRA-400 resin (OH form) (20 ml) packed in MeOH. The basic eluate (100 ml) was evaporated to a white solid. Recrystallization from EtOH gave pure **6** as a white solid: 424 mg (95.5%); mp 129–131°; ir (KBr) 3450 (OH), 3350 and 1600 cm^{-1} (NH_2).

Anal. Calcd for $C_{11}H_{17}N_7O \cdot \frac{1}{2}H_2O$: C, 48.53; H, 6.43; N, 36.03. Found: C, 48.52; H, 6.61; N, 36.12.

7-Dimethylamino-3-{(R)-[(2R)-hydroxy-(3R)-(benzyloxycarbonyl-p-methoxyphenyl-L-alanylamino)]cyclopentyl}-v-triazolo[4,5-d]pyrimidine (**7**) *and 7-Dimethylamino-3-{(S)-[(2S)-hydroxy-(3S)-(benzyloxycarbonyl-p-methoxyphenyl-L-alanylamino)]cyclopentyl}-v-triazolo[4,5-d]pyrimidine* (**8**). To a solution of **6** (200 mg, 0.760 mmol), *N*-benzyloxycarbonyl-*p*-methoxyphenyl-L-alanine (Baker *et al.*, 1955) (262 mg, 0.797 mmol), and *N*-hydroxysuccinimide (91.7 mg, 0.797 mmol) in dry dimethylformamide (10 ml) was added dicyclohexylcarbodiimide (164 mg, 0.797 mmol). The mixture was stirred at 25° for 48 hr while protected from moisture. The dicyclohexylurea was removed by filtration and washed with EtOAc (20 ml). The filtrate was evaporated and the residue was taken up in EtOAc (100 ml), chilled, and filtered to remove remaining dicyclohexylurea. The filtrate was diluted with EtOAc (150 ml) and washed with H_2O (15 ml), half-saturated $NaHCO_3$ (15 ml), and then H_2O (2 × 30 ml). The organic layer was dried ($CaSO_4$) and evaporated *in vacuo* to a mixture of **7** and **8** as a white solid; 504 mg (98.2%). The diastereomers could not be separated by crystallization or chromatography. An analytical sample was prepared by solidification from benzene–*n*-hexane and gave a white solid: mp part at 109–110° and the remaining solid at 210–215°; ir (KBr) 3450, 3350 (OH, NH), 1775 (–NHCOO–), 1650 (amide 1), 1550 cm^{-1} (amide 2).

Anal. Calcd for $C_{29}H_{34}N_8O_5$: C, 60.61; H, 5.96; N, 19.50. Found: C, 60.47; H, 6.14; N, 19.27.

7-Dimethylamino-3-{(R)-[(2R)-hydroxy-(3R)-(p-methoxyphenyl-L-alanylamino)]cyclopentyl}-v-triazolo[4,5-

TABLE I: Inhibition of Poly(U,C) Directed [^{14}C]-L-Polyphenylalanine Formation.

Compound	% Inhibition	
	10^{-4} M	10^{-5} M
1	96.7	73.6
2	21.0	0.0
Puromycin	98.8	78.8

d]pyrimidine (**1**) *and 7-Dimethylamino-3-{(S)-[(2S)-hydroxy-(3S)-p-methoxyphenyl-L-alanylamino)]cyclopentyl}-v-triazolo[4,5-d]pyrimidine* (**2**). The diastereomeric mixture of **7** and **8** (321 mg, 0.481 mmol) was dissolved in glacial HOAc (20 ml) and shaken with 10% Pd/C under H_2 (1 atm) for 20 min. The mixture was filtered through Celite and the Celite was washed with HOAc (20 ml). Evaporation of the combined filtrates (40°, 0.25 mm) left a colorless glass. A solution of this glass in MeOH was passed slowly through a column of 20 ml of Amberlite IRA-400 resin (OH^- form) packed in MeOH. The basic eluate (100 ml) of MeOH was evaporated to a white solid mixture of **1** and **2;** yield, 173 mg (79.7%); tlc on silica gel (Eastman chromatogram sheets with fluorescent indicator) developed with 10% MeOH-$CHCl_3$ gave two spots with R_F 0.60 and 0.70. The diastereomers **1** and **2** were separated by preparative tlc (80 mg/plate) on 20 × 20 cm glass plates coated with 2 mm of silica gel F254 (E. Merck, Darmstadt) developed with 15% MeOH-$CHCl_3$. The two bands were separated and each was stirred with 20% MeOH-$CHCl_3$ for 18 hr, filtered, and evaporated giving almost quantitative recovery of the pure diastereomers as colorless glasses. The glass having R_F 0.60, assigned structure **1,** could not be crystallized and was converted to a white solid foam by evaporation from $CHCl_3$ (56°, 0.05 mm): $[\alpha]_{589}^{22}$ −90.1°, $[\alpha]_{436}^{22}$ −210° (*c* 0.223, $CHCl_3$); uv max 273 nm (ε 13,650) and 216 (sh) at pH 1; 296 nm (ε 14,864) and 217 (sh) at pH 7; 295 nm (ε 14,560) and 213 (sh) at pH 13.

Anal. Calcd for $C_{21}H_{28}N_8O_3$: C, 57.26; H, 6.41; N, 25.44. Found: C, 56.97; H, 6.39; N, 25.19.

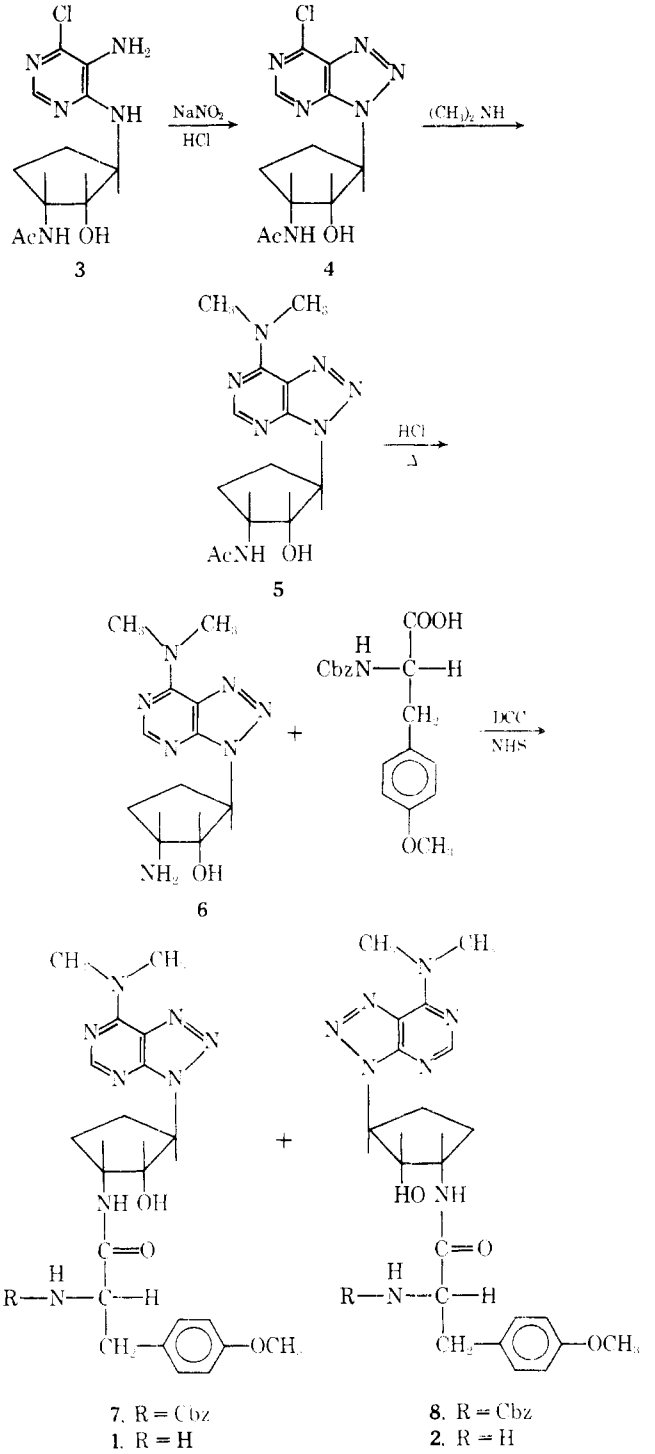
The glass having R_F 0.70, assigned structure **2,** could not be crystallized. The addition of a small amount of $CHCl_3$ followed by evaporation and drying at 56° (0.05 mm) for 24 hr gave a white solid foam: $[\alpha]_{589}^{22}$ +11.7°, $[\alpha]_{436}^{22}$ +29.4° (*c* 0.214, $CHCl_3$); uv max 273 nm (ε 13,250) and 210 (sh) at pH 1; 295 nm (ε 14,574) and 215 (sh) at pH 7; 295 nm (ε 13,867) and 215 (sh) at pH 13.

Anal. Calcd for $C_{21}H_{28}N_8O_3 \cdot H_2O$: C, 55.26; H, 6.58; N, 24.56. Found: C, 55.22; H, 6.48; N, 24.69.

Results and Discussion

Synthesis. The 8-aza carbocyclic puromycin analogs were synthesized as outlined in Scheme I. The carbocyclic pyrimidine **3** (Vince and Daluge, 1974) was diazotized with sodium nitrite and hydrochloric acid and give the corresponding 8-azapurine **4.** The 6-dimethylamino-8-azapurine derivative **5** was obtained by nucleophilic displacement of the 6-chloro group from **4** with aqueous dimethylamine. The acetamido blocking group was removed from **5** by acid hydrolysis and the amino alcohol **6** was isolated in high yield. The aminonucleoside was coupled to *N*-benzyloxycarbonyl-*p*-methoxyphenyl-L-alanine by the dicyclohexylcarbodiimide and *N*-hydroxysuccinimide method previously described (Daluge and Vince, 1972; Vince and Isakson, 1973). The resulting carbobenzoxy (Cbz) blocked diastereomers **7** and **8** could not be separated. Following hydrogenolysis of the Cbz group, separation of the diastereomers **1** and **2** by chromatography was possible. Structure **1** is assigned to the diastereomer having $[\alpha]^{22}D$ −90° and structure **2** to the diastereomer having $[\alpha]^{22}D$ +12°.

SCHEME I[a]



Biological. The effect of the 8-aza carbocyclic puromycins on the rate of poly(U,C) directed polyphenylalanine formation in an *E. coli* cell free system with washed ribosomes is presented in Table I. Examination of data reveals that carbocyclic analog **1** inhibits protein synthesis to the same degree exhibited by puromycin. However, the diaste-

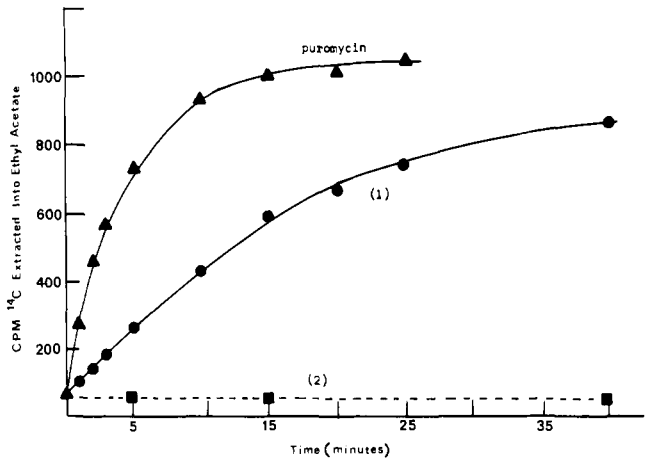


FIGURE 2: The course of reaction at 28° of Ac-[^{14}C]-L-Phe-tRNA as a donor substrate with puromycin and carbocyclic analogs with *E. coli* ribosomes. All compounds were tested at 0.35 mM as described in Materials and Methods. A control containing all of the components except substrate gave a line which coincides with the dotted line drawn for compound **2**.

reomeric analog, **2**, was relatively inactive and exhibited only 21% inhibition at the higher concentration. It is interesting to note that the carbocyclic puromycin analogs, as well as puromycin, exhibit greater inhibitory activities in the presence of poly(U,C) than when poly(U) is used as mRNA; similar observations have been reported for puromycin and chloroamphenicol (for example, see Coutsogeorgopoulos, 1966; Harris *et al.*, 1971; Almquist and Vince, 1973). We have found that the inhibitory values obtained from poly(U,C) directed protein synthesis are consistent with polyribosome systems (Pestka *et al.*, 1973) and reflect the effect of puromycin analogs on protein synthesis in intact cells (R. Vince and C. L. Ritter, unpublished).

It was anticipated that the active 8-aza carbocyclic analog **1** would act as a substrate in the peptidyl transferase reaction in a manner similar to that of puromycin. Examination of Figure 2 confirms that **1** is an acceptor for the donor acetylphenylalanyl-tRNA. However, the diastereomer **2** was not able to form the acetylphenylalanyl product. A double reciprocal plot for the transpeptidation reaction with puromycin and the active analog provided the interesting observation that both compounds exhibit the same K_m (0.22 mM) while the V_{max} for puromycin is six times greater than the V_{max} for **1**. These data indicate that the 8-aza carbocyclic analog **1** has an affinity for the ribosomal binding site equal to that of puromycin itself. A sixfold difference in rate constants for product formation is also indicated. However, since substrate activity is not a prerequisite for inhibition of the peptidyl transferase reaction, both puromycin and **1** inhibit protein synthesis to the same extent—presumably due to their equal ribosomal binding affinities.

As expected, antimicrobial testing revealed that **1** exhibited growth inhibition on the same order of magnitude as puromycin. The minimum inhibitory concentrations by a twofold serial dilution test in nutrient broth for puromycin and **1**, respectively, are (mM): *Staphylococcus aureus* (NRRL B-313), 0.128 and 0.125; *Escherichia coli* (NRRL B-210), 0.064 and 0.064.

The results represented here provide a comparison of the relative ribosomal binding affinities and peptidyl transferase substrate activities of puromycin and the nonclassical 8-aza carbocyclic puromycin. These studies clearly establish the utility of the carbocyclic compounds for exploring the ribosomal binding site. Kinetic data indicate that such compounds are capable of binding to the ribosomal acceptor site and act as peptidyl transferase substrates in a manner identical with the mechanism of action of puromycin. The Michaelis constant of **1** confirms the idea that the ribofuranosyl ring can be replaced with the more hydrolytically stable cyclopentane ring without loss of ribosomal affinity. Since there is no 5′-hydroxymethyl on the carbocyclic analog, it is evident that this moiety is not essential for binding. In addition, substitution of C-8 in the purine ring by N indicates that certain modifications of the heterocyclic moiety can be made without detriment to activity. The kinetic data also demonstrate that such modifications of the puromycin structure result in a decreased velocity of the peptidyl transferase reaction. No definite conclusions can be made at this time to explain this observation. These results, in conjunction with the phenylalanine polymerization assay, indicate that lowering of substrate activity while retaining ribosomal binding affinity does not alter the ability of puromycin-related compounds to inhibit protein biosynthesis.

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