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On the Molecular Mechanism of Action of Certain Substrates and Inhibitors of Ribosomal Peptidyl Transferase[1]

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Conformational analysis of a number of inhibitors and substrates of *Escherichia coli* ribosomal peptidyl transferase has indicated that they are analogs of the 3′-terminus of aminoacyl- or peptidyl-tRNA and that their primary locus of action is the acceptor site of this enzyme. The evidence available in the literature which supports the proposed scheme has been reviewed. Specific, experimentally accessible predictions of the scheme are given.

Peptidyl transferase, an integral part of the 50S bacterial and 60S mammalian ribosomal subunit (*1*, *2*), catalyses peptide-bond formation by a process involving transfer of a nascent peptide chain from peptidyl-tRNA (donor substrate) in the P-site on the ribosome to aminoacyl-tRNA (acceptor substrate) in the A-site (*3–5*, Fig. 1). Although considerable information is available on the inhibitors and substrates of bacterial peptidyl transferase shown in Fig. 2 (*6–8*), their molecular mechanism of action is unknown except in the case of puromycin (*3–5*, *9*) and closely related analogs (*11–17*).

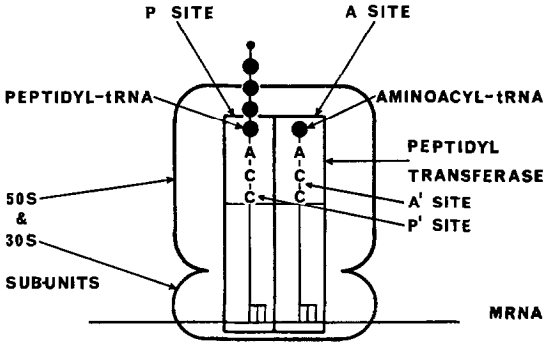
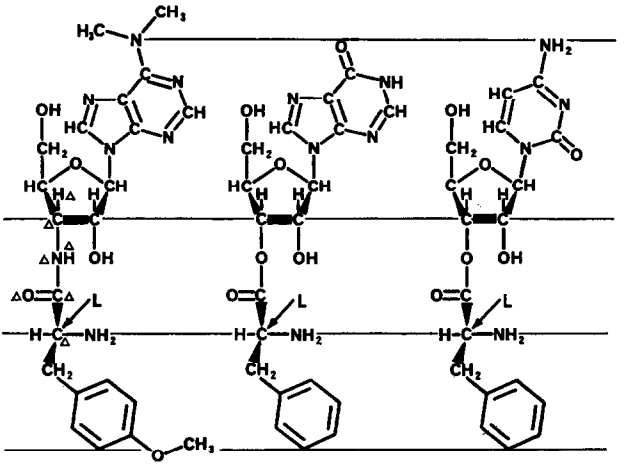


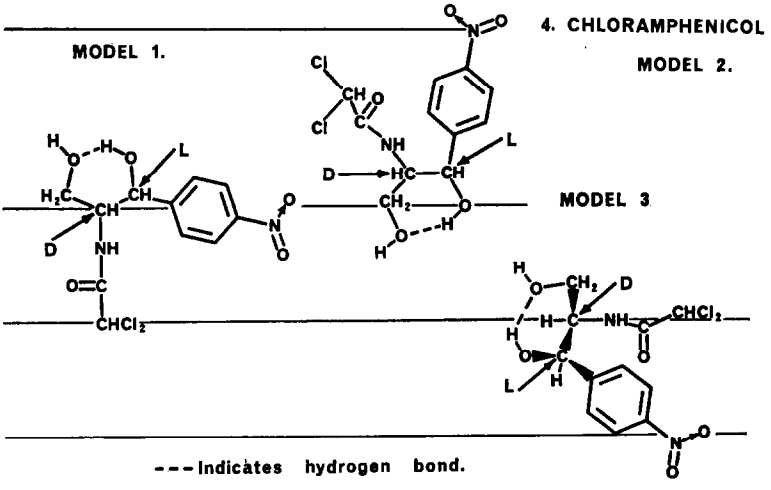
FIG. 1. Diagrammatic model of *E. coli* ribosome showing A and P sites occupied by aminoacyl- and peptidyl-tRNA and the A′ and P′ sites of peptidyl transferase occupied by the 3′-terminal portions, CpCpA-amino acid, and CpCpA-peptide, respectively.

[1] *Abbreviations:* A-amino acid, 2′(3′)-*O*-aminoacyl adenosine; A-peptide, 2′(3′)-*O*-peptidyl adenosine; A-L-phe-L-phe, 2′(3′)-*O*-(L-phenylalanyl-L-phenylalanyl) adenosine; Ac-L-phe-CH_2Cl, *N*-acetyl-L-phenylalanyl chloromethylketone; CpA-F-met, cytidylyl-(3′-5′)2′(3′)-*O*-(*N*-formyl-L-methionyl) adenosine; CpA-pro, cytidylyl-(3′-5′)-2′(3′)-*O*-L-prolyl adenosine (other N-blocked and unblocked 2′(3′)-*O*-aminoacyl oligonucleotides are similarly abbreviated); C-L-phe, G-L-phe, I-L-phe, and U-L-phe, the 2′(3′)-*O*-L-phenylalanyl derivatives of cytidine, guanosine, inosine, and uridine, respectively; TLCK, α-*N*-tosyl-L-lysyl chloromethylketone; TPCK, α-*N*-tosyl-L-phenylalanyl chloromethylketone; Z-gly-L-phe-NA, benzyloxycarbonyl-glycyl-L-phenylalanyl nitroanilide; Z-L-phe-ONP, benzyloxycarbonyl-L-phenylalanyl-*p*-nitrophenyl ester.

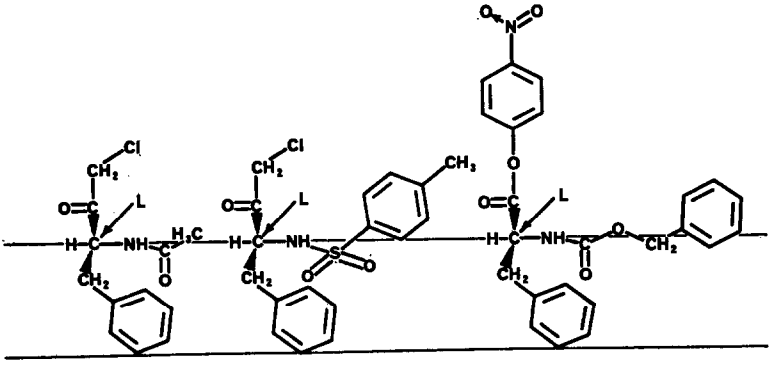
1. PUROMYCIN 2. I-L-PHE 3. C-L-PHE



△ Denotes coplanar atoms.

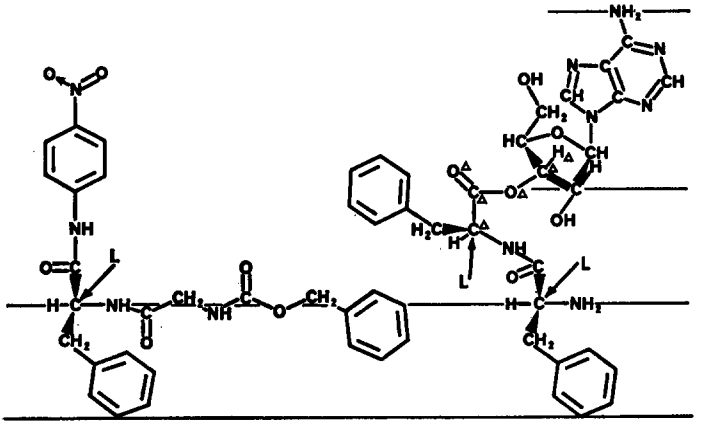


5. Ac-L-PHE-CH_2Cl 6. TPCK 7. Z-L-PHE-ONP



8. Z-GLY-L-PHE-NA

9. A-L-PHE-L-PHE

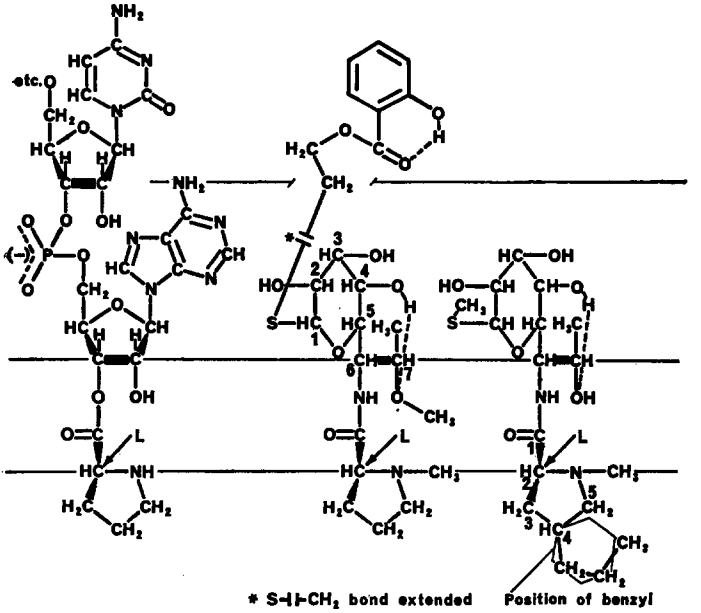


△ Denotes coplanar atoms.

CpA-L-PRO TERMINUS OF PRO-tRNA

10. CELESTICETIN

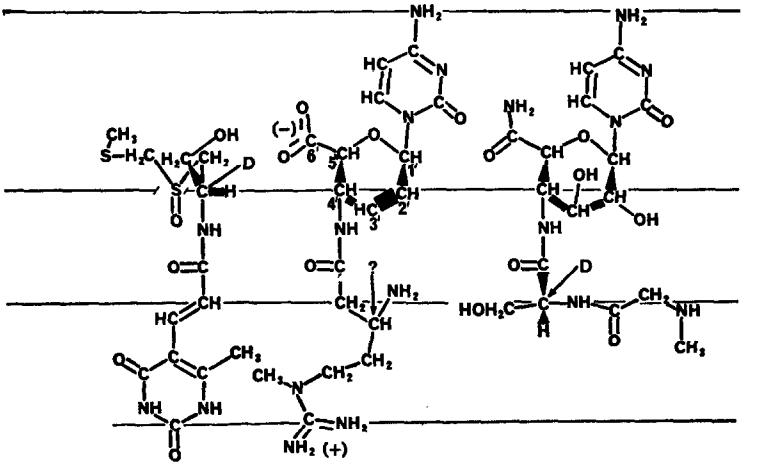
11. LINCOMYCIN



* S-H-CH_3 bond extended for clarity.

Position of benzyl group of phe-tRNA superimposed.

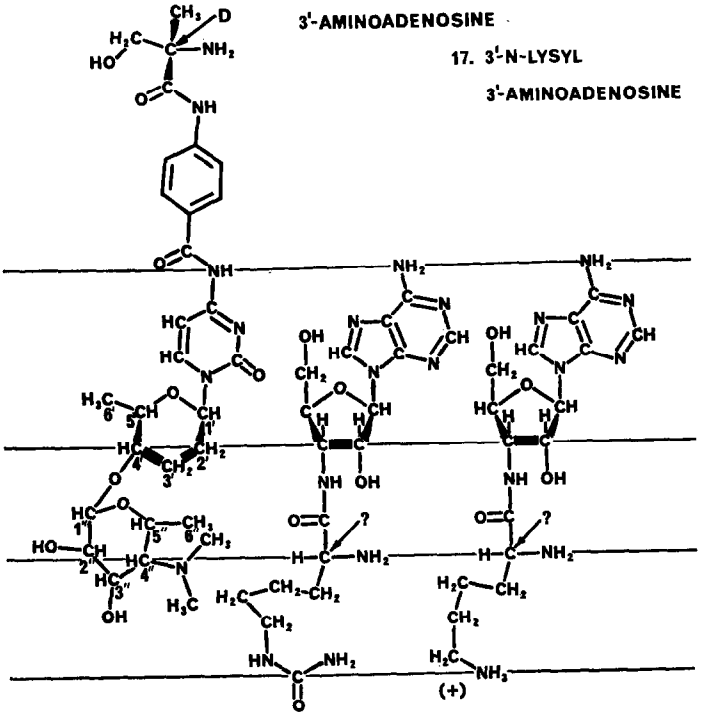
12. SPARSOMYCIN 13. BLASTICIDIN S 14. GOUGEROTIN



15. AMICETIN

16. 3'-N-HOMOCITRULLYL 3'-AMINOADENOSINE

17. 3'-N-LYSYL 3'-AMINOADENOSINE



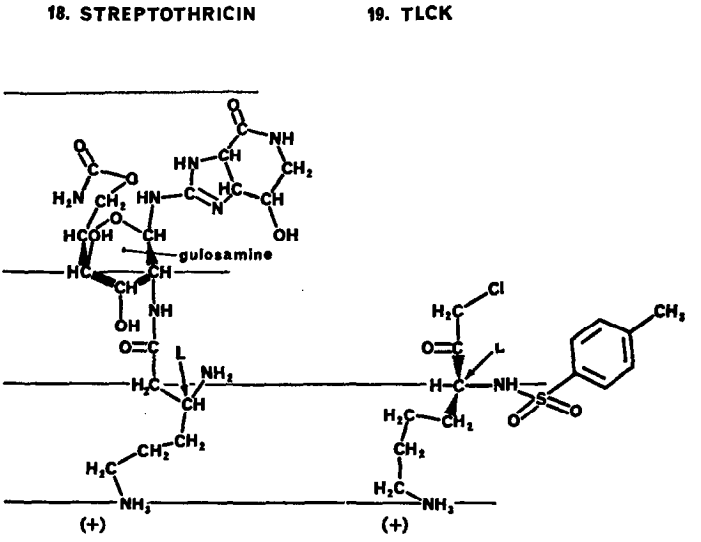


FIG. 2. Diagrammatic comparison of the structures of the various inhibitors and substrates. The comparison of structures and conformations was achieved by use of CPK and Dreiding models. The sources of the various structures are as given in the text and in addition include: amicetin, 3′-*N*-homocitrullyl 3′-aminoadenosine and 3′-*N*-lysyl 3′-aminoadenosine (*20*), blasticidin S (*30*), celesticetin (*31*), gougerotin (*32*), lincomycin (*33*), sparsomycin (*34*) and streptothricin (*35*, *36*). Conformations (where available) are as given in the text. In most cases conformations were determined by comparison with similar compounds of known conformation and by attention to the linearity of atoms involved in peptide, amide, and ester bonds (*38*, *48*, and see text).

For ease of comparison, the true positions of all atoms are not shown. The horizontal lines connecting the compounds act as a reference for comparison of structural similarities. —x indicates that x is above the plane of the paper. D, L, or ? indicates optical configuration.

In spite of the seemingly diverse structures shown in Fig. 2, it was considered possible that they were all structural analogs of some part of the 3′-terminus of aminoacyl or peptidyl-tRNA. As a result of molecular model-building studies with CPK and Dreiding models, it was found possible to arrange the conformation of these compounds (within reasonable steric limitations) such that they were stereochemically analogous to specific areas of the 3′-terminus of aminoacyl or peptidyl-tRNA. Furthermore, it is proposed that the compounds act on peptidyl transferase by binding directly to the acceptor substrate site (A′) of the enzyme (Fig. 1). Details of the proposed scheme, which are diagrammatically depicted in Fig. 2, are considered first and are followed with supporting circumstantial evidence from the literature. In addition, specific predictions which can be experimentally tested are described.

As indicated in the text, previous workers have related the structures of some of the compounds considered here to puromycin or to the 3′-terminus of aminoacyl or peptidyl-tRNA (*7*, *18–24*). The proposals were generally not given in detail and alternative hypotheses have been presented for some of the compounds (*7*, *25–29*).

RESULTS

Conformational Analysis of Inhibitors and Substrates of Peptidyl Transferase

The results of comparative model-building studies for each of the compounds depicted in Fig. 2 and CpCpA-peptide and CpCpA-amino acid are given below. The conformation of the A-amino acid section of CpCpA-amino acid was assumed to be the same as that of the crystal structure of puromycin (*37*, *109*) which was determined as the dihydrochloride.

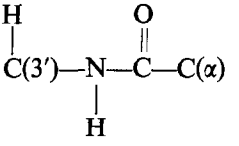
1. *Puromycin*

Puromycin has been considered as an analog of the terminal A-L-tyr of tyrosyl-tRNA (*9*) and shown to be an acceptor substrate for peptidyl transferase (*3–5*). Some of the main structural details (*37*) which were used in the present study are as follows:

(a) The nucleoside is in the anticonfiguration with $\chi = 19°$ (see Ref. *37* for definition of χ),

(b) The ribose is in the C(3′)endo-C(2′)exo configuration,

(c) The seven atoms of the arrangement



are coplanar (Fig. 2 and Ref. *38*).

2. *2′(3′)-O-L-Phenylalanyl inosine* (*I-L-phe*)

3. *2′(3′)-O-L-Phenylalanyl cytidine* (*C-L-phe*)

These compounds are analogs of the terminal A-phe of phe-tRNA and are acceptor substrates for peptidyl transferase (*14*). The low activity of these two compounds compared to that of puromycin and the inactivity of the closely related G-L-phe and U-L-phe demonstrate the importance of the purine ring and the heterocyclic 6-amino group for high activity. Presumably this reflects a higher affinity of adenine for peptidyl transferase.

4. *Chloramphenicol*

Three alternative models, discussed below, are proposed to account for the mechanism of action of chloramphenicol; model 3 is considered to be the most feasible.

(a) *Model 1* (*Fig. 2*). This model, as proposed by Coutsogeorgopoulos (*28*, *29*), considers chloramphenicol as an analog of the terminal A-amino acid of aminoacyl-tRNA in which the dichloroacetyl group in amide linkage corresponds to the aminoacyl group in ester linkage. As an extension of this model, it is possible that the propanediol chain may be an analog of the ribose moiety. However, it is not possible to assign a role for the nitrophenyl group, which is not well positioned to act as an

analog of the 3′-terminal adenine moiety of aminoacyl-tRNA. Mainly because of the latter reason, this model is considered unsatisfactory.

(b) *Model 2* (*Fig. 2*). This model is based on the previous observations of Jardetzky (*25*), which suggested that chloramphenicol was a structural analog of 5′-UMP. This interpretation has been modified and chloramphenicol is now presented as an analog of the 3′-terminal 5′-*O*-phosphoryl-adenosine of aminoacyl-tRNA (see also Ref. *27*). In this model, the dichloroacetyl group is analogous to the phosphoryl group, the *p*-nitrophenyl group (like the cytosine of C-L-phe) analogous to the adenine of A-amino acid, and the remainder of chloramphenicol analogous to the ribose of A-amino acid.

(c) *Model 3* (*Fig. 2*). Chloramphenicol is presented as an analog of the carboxyl terminus of the nascent peptide as previously proposed by Das et al. (*21*) and Hahn (*110*).

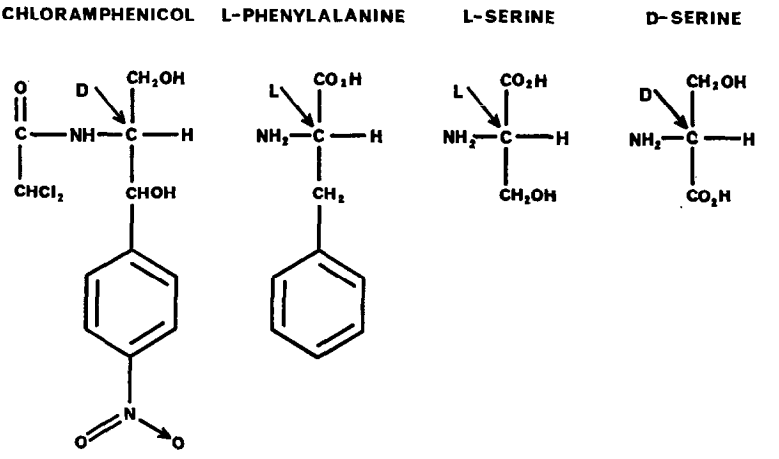


FIG. 3. Comparison of the stereochemistry (shown as Fisher projections) at C2 of chloramphenicol (*107*) and the α-carbon of L-phenylalanine, L-serine, and D-serine.

The chloramphenicol base (chloramphenicol minus dichloroacetyl group) is analogous to the carboxyl terminal amino acid of peptidyl-tRNA, and the dichloroacetyl group analogous to the second amino acid from the carboxyl terminus of the nascent peptide. In particular, the antibiotic appears to be basically a L-phenylalanine or a L-tyrosine analog as proposed by Woolley (*39*). Alternatively, it may be regarded as a L- or D-serine analog (*112*) or a combined L-phe, L-ser analog (Fig. 3). The configuration and nature of the groups at C2 of chloramphenicol are very similar to those of the groups at the C2 of the amino acids L-phe and L-ser, with the main exception being the presence of a primary hydroxyl in chloramphenicol compared to a carboxyl group in the amino acids (Fig. 3). The significance of the cyclic arrangement resulting from hydrogen bonding between the two hydroxyls of chloramphenicol is not known (*25*, *40*).

Model 3 is favoured because of the following points:

(i) The biosynthesis of chloramphenicol, like phenylalanine and tyrosine, involves the shikimic acid pathway. In addition, the chloramphenicol precursors L-*p*-aminophenylalanine and LS-threo-*p*-aminophenyl serine (*41*) are closely related structurally to L-phe and L-tyr, thus supporting the proposal that chloramphenicol is an L-phe

analog. Possibly evolution of the chloramphenicol biosynthetic pathway occurred with evolution of the peptidyl transferase active site.

(ii) Based on the observations of Calendar and Berg (*42*) that D-tyrosine is incorporated into protein at a slower rate than L-tyrosine, it appears that the acceptor substrate site of peptidyl transferase is not completely stereospecific. Consistent with this is the low but significant activity of the L-erythro isomer of chloramphenicol (inversion of optical configuration at C2; Refs. *43*, *44*).

(iii) Chloramphenicol analogs, in which the dichloroacetyl residue is replaced by various amino acids, were inhibitory on the puromycin-dependent release of polylysine from polylysyl-tRNA on *E. coli* ribosomes (*28*, *29*). According to Models 1 and 3, these analogs have increased structural similarity to the 3'-terminus of aminoacyl tRNA and of a nascent peptide, respectively. As predicted from Model 3, these analogs were not substrates for peptidyl transferase (*28*) but substrate activity may have been expected if Model 1 was correct, since the aminoacyl residue would have been in a position analogous to the *O*-methoxy-L-tyrosyl residue of puromycin (Fig. 2).

(iv) The nitrophenyl group of chloramphenicol is structurally similar to, and can be arranged in the same conformation as, the *p*-methoxyphenyl group of puromycin. The nitrophenyl group is, therefore, well suited to bind to a proposed hydrophobic site on the peptidyl transferase acceptor substrate site, which is involved in the binding of the aromatic aminoacyl R groups of phe- and tyr-tRNA and of the *O*-methyl-tyrosyl residue of puromycin during peptide bond formation (*12*, *13*, *45*).

5. N-*Acetyl*-L-*phenylalanyl-β-chloromethylketone* (*Ac*-L-*phe-*CH_2Cl)
6. N-*Tosyl*-L-*phenylalanyl-β-chloromethylketone* (*TPCK*)
7. *Benzyloxycarbonyl*-L-*phenylalanyl*-p-*nitrophenylester* (*Z*-L-*phe-ONP*)
8. *Benzyloxycarbonyl-glycyl*-L-*phenylalanyl nitroanilide* (*Z-gly*-L-*phe-NA*)

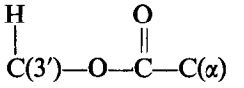
These four compounds, similarly to TLCK and chloramphenicol, are presented as analogs of the carboxyl terminus of the nascent peptide attached to tRNA as also proposed by Rychlik (*7*). The L-phe residue of these compounds is analogous to the carboxyl terminal amino acid of peptidyl-tRNA, while the tosyl group of TPCK, the acetyl group of Ac-L-phe-CH_2Cl, the glycyl residue of Z-gly-L-phe-NA, and the benzyloxycarbonyl group of Z-L-phe-ONP may be analogs of the second amino acid from the carboxyl terminus of the nascent peptide. The benzyloxycarbonyl group of Z-gly-L-phe-NA may be analogous to the third amino acid, while the nitrophenyl and nitroanilide moieties of Z-L-phe-ONP and Z-gly-L-phe-NA may be analogous to all or part of the terminal adenosine of aminoacyl-tRNA. However, studies are necessary to determine the actual contribution of each of the groups to the inhibitory activity of this series of compounds as well as TLCK.

The chloromethylketones have a similar potency as chloramphenicol (as measured by the inhibition of the puromycin-dependent release of polylysine from polylysyl-tRNA charged *E. coli* ribosomes) and are structurally similar to chloramphenicol (*7*, Fig. 2). This similarity is most striking for Ac-L-phe-CH_2Cl, especially when a comparison is made with *N*-acetyl-chloramphenicol base, an analog as potent as chloramphenicol on *E. coli in vitro* protein synthesis (*46*).

Interestingly, the chlorine atom of these inhibitors and that of clindamycin, a very potent 7(s)-chloro-derivative of 7-deoxylincomycin (*47*), can be arranged to occupy the same general region.

9. *2′(3′)-O-(L-phenylalanyl-L-phenylalanyl)-adenosine (A-L-phe-L-phe)*

This compound is presented as an analog of the terminal A-phe of phe-tRNA and has been shown to be an acceptor substrate for *E. coli* peptidyl transferase, although the product of the reaction has not been characterized (*15*). In this model, the adenine and the amino-terminal L-phe of A-L-phe-L-phe are analogous to and congruent with the adenine and L-phe (except for rotation of the α-carbonyl group) of A-L-phe. The ribose of A-L-phe-L-phe, however, is arranged in a different conformation to that of A-L-phe (Fig. 2). The six atoms of the ester grouping:



are coplanar (*38*, *48*).

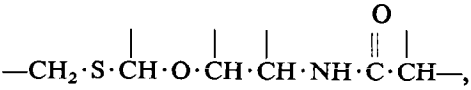
Interestingly, α-*N*-L-phenylalanyl-puromycin, a close structurally related compound, had negligible effect on the synthesis of polyphenylalanine in an *E. coli* poly (U)-primed protein-synthesizing system (*10*). This is rather surprising since models of both A-L-phe-L-phe and α-*N*-L-phenylalanyl-puromycin can be arranged in very similar conformations. The reason(s) for the difference in the activity of the two compounds is unknown.

10. *Celesticetin*

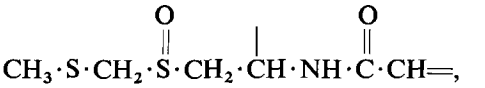
This antibiotic is presented as an analog of the CpA-L-pro terminus of prolyl-tRNA. The conformation of celesticetin is not as flexible as expected from its structure, since Slomp and Mackellar (*49*) have presented evidence for an intramolecular hydrogen bond, C4—O—H→O(CH_3)—C7, and the possibility exists for a second intramolecular hydrogen bond, as found for salicylic acid and salicylyl esters (*50*), between the O-hydroxyl and the carbonyl group of the salicylyl moiety. Within these conformational constraints, molecular models can be arranged such that the salicylyl and *N*-methyl-L prolyl residues of celesticetin occupy positions sterically analogous to those of the cytosine and L-prolyl residues, respectively, of CpA-L-pro.

As in many of the antibiotics discussed here, celesticetin contains an acylated amino group (at C6) with an adjacent hydroxyl group (at C7; the hydroxyl is methylated in this case). This arrangement appears to be stereochemically analogous to the acylated 2′, 3′-*cis*-hydroxyls of aminoacyl-tRNA, with the 7-*O*-methyl group of celesticetin being analogous to the 2′-hydroxyl and the acylated 6-amino group being equivalent to the acylated 3′-hydroxyl of aminoacyl-tRNA. (The activity of the 2′- and 3′-isomers of puromycin on *E. coli in vitro* protein synthesis indicates that the 3′-hydroxyl and not the 2′-hydroxyl of aminoacyl-tRNA is acylated during actual peptide bond formation (*10*).)

Both celesticetin and lincomycin contain a molecular grouping,



which is stereochemically related to a similar but not identical arrangement in sparsomycin,



thus suggesting a similar (in part at least) mechanism of action.

11. *Lincomycin*

An antibiotic closely related to celesticetin, lincomycin, is presented as an analog of the A-L-pro terminus of prolyl-tRNA. The main structural differences between the two antibiotics are the absence of the salicylyl group and the presence of a propyl group at 4′ of the prolyl residue. The propyl group forms a "hydrophobic tail" on the antibiotic and may bind to the hydrophobic site on the acceptor site of peptidyl transferase, which was proposed to be involved in the binding of the hydrophobic aminoacyl R groups of the various aminoacyl-tRNA molecules during peptide bond formation (*12*, *13*, *45*).

12. *Sparsomycin*

This antibiotic is presented as an analog of the terminal A-amino acid of aminoacyl-tRNA. The 6-methyluracil group shows some structural similarity to the ureido group of 3′-*N*-homocitrullyl 3′-aminoadenosine (Fig. 2). Both the 6-methyluracil and ureido groups would be uncharged at physiological pH. In addition, the structural similarity between sparsomycin, lincomycin, and celesticetin as described in 10 above, suggests a similar ribosome-binding site and mechanism of action for these three compounds.

13. *Blasticidin S*

This antibiotic is closely related to gougerotin and amicetin and is presented as an analog of the terminal A-arg of arginyl-tRNA. Similar but less detailed proposals relating the structure of blasticidin S, gougerotin, and amicetin to that of the aminoacyl-terminus of aminoacyl-tRNA have also been considered (*20*, *24*, *51*, *52*).

Although a hexose is present in blasticidin (and in gougerotin and amicetin) in contrast to the pentose of A-amino acid, the cytosine and aminoacyl moieties of blasticidin S have the same configuration of attachment (i.e., β-glycosidic and down from the plane of the hexose, respectively) and are attached at similar positions on the hexose (Cl′ and C4′) with respect to proximity to the hexose ring oxygen atom (i.e. one and two carbon atoms removed, respectively) as found for the dimethyladenine and *o*-methoxytyrosyl groups of puromycin. The hexose of blasticidin (and gougerotin and amicetin) is assumed to tend toward the more stable chair form. However, the C2′=C3′ double bond of blasticidin renders the hexose relatively inflexible as compared to a saturated hexose, and the configuration assumed is one in which the hexose ring oxygen atom is above the approximate plane of carbons 1′, 2′, 3′, and 4′. In this configuration, the C(4′)-amide linkage, C(4′)—NH—C(=O)—C(α), is congruent with the similar 3′-ester linkage of A-amino acid (or the 3′-amide linkage of puromycin), and the cytosine is coplanar with the adenine of A-amino acid (Figs. 2 and 3). The possibility of the equivalence of the cytosine and adenine heterocyclic rings considered here is supported by the observation that cytosine can substitute for the adenine of A-L-phe with retention

of substrate activity toward peptidyl transferase (see 2. above.) Possibly the C4-amino group of blasticidin (and gougerotin, amicetin, and C-L-phe) is functionally equivalent to the C6-amino group of A-amino acid or the C6-dimethylamino group of puromycin (see Fig. 4). The importance of the heterocyclic amino groups has been demonstrated by the replacement of the cytosine of blasticidin and adenine of A-L-phe by uracil with subsequent marked reduction in activity (*14*, *51*).

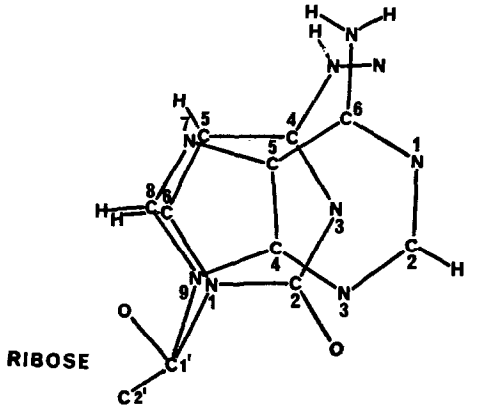


FIG. 4. The congruency of the heterocyclic rings of A-L-phe and C-L-phe. The molecular dimensions are from Voet and Rich (*54*). The cytosine of blasticidin, gougerotin, and amicetin assumes a similar arrangement to that of C-L-phe. The configuration of adenosine is assumed to be anti as found for puromycin (*37*). The anti configuration is assumed for C-L-phe since all pyrimidines so far examined exist in this form (*55*). All the heterocyclic rings are coplanar. Note that the heterocyclic amino groups are in the same general area.

Although the influence on activity of the 5′-carboxyl group has not been determined, it may be analogous to the phosphoryl residue of *p*A-amino acid of aminoacyl-tRNA, since both carry a full negative charge at physiological pH and are in the same general vicinity (Fig. 2). Perhaps the negatively charged groups are involved in a coulombic attraction with a flexible positively charged group on the ribosome (e.g., lys or arg side chain). Such an attraction could mediate the appreciable increase in activity of 2′(3′)-*O*-L-phenylalanyl-(5′-*O*-methylphosphoryl)-adenosine compared to that of A-L-phe in releasing polyphenylalanine from polyphenylalanyl-tRNA on *E. coli* ribosomes (*53*). The importance of the *β*-amino group of blasticidin for biological activity is unknown.

14. *Gougerotin*

This compound is presented as an analog of the A-ser-gly portion of gly-ser-tRNA. The conformation of the glucopyranose ring of gougerotin (and the 2′,3′,6′-trideoxyhexose of amicetin) is assumed to tend toward the more stable chair form. This does not significantly alter the arrangement of the cytosine or the 4′-aminoacyl group from that determined for blasticidin S above. The 5′-amide group is important for activity, as there is a many-fold reduction in activity on its replacement by —CH_2OH (*23*). Perhaps the amide group participates in hydrogen bonding with the guanidinium group of the

arginine side chain on the ribosome which was postulated above to be involved in coulombic attraction to the 5′-carboxyl of blasticidin S to the ribosome.

15. *Amicetin*

This antibiotic has proved difficult to classify. However, based on its structural similarity to blasticidin S and gougerotin, (amicetin contains a 4′-*O*-basically substituted 4′-hydroxyhexose with the 4′-hydroxyl in the down position and an N1-substituted cytosine in β-glycosidic linkage to C1′ of the hexose; gougerotin and blasticidin contain very similar arrangements), it is suggested that amicetin has a similar mode of action and is an analog of the A-amino acid of aminoacyl-tRNA. The basic 4″-dimethyl-amino sugar may be analogous to the R group of an arginyl or lysyl residue although this group has a low pK_a (8.05; Ref. *56*). Alternatively, this dimethylamino group may be arranged in a position similar to that of the α-amino group of puromycin (Fig. 2). The role of the α-methyl-seryl-aminobenzoyl group attached to the cytosine amino group is unknown although Levitt (*57*) has suggested that the terminal adenosine of tRNA is hydrogen bonded to another nucleoside in the tRNA molecule. The N^4-acyl group may be structurally related to this nucleoside.

16. *3′-*N*-Homocitrullyl 3′-aminoadenosine*

This compound is an analog of the terminal A-arg of arginyl-tRNA as proposed earlier by Guarino et al. (*58*), although the ureido group would be uncharged at physiological pH. The compound has been shown to be an inhibitor of *E. coli* and rat liver cell free protein synthesis (*58*).

17. *3′-*N*-Lysyl 3′-aminoadenosine*

This compound is a structural analog of the terminal A-lys of lysyl-tRNA, but it has not yet been shown to be an inhibitor of protein synthesis (*59*). More recently, Rychlik et al. (*13*) have shown that 2′(3′)-*O*-L-lysyl adenosine is an effective substrate for *E. coli* peptidyl transferase.

18. *Streptothricin*

This antibiotic is presented as an analog of the terminal A-lys of lysyl-tRNA (Fig. 2). In this model, the β-L-lysyl residue and the double heterocyclic ring of streptothricin are analogous to the L-lysyl residue and adenine of A-lys, respectively. If this model is correct, then the conformation of the 3′-terminal adenosine of aminoacyl-tRNA, once bound to peptidyl transferase, must be in the anti range (see puromycin above), since rotation of the double heterocyclic ring of streptothricin is sterically hindered.

Several antibiotics of the same general structure as streptothricin are also known. These include derivatives in which one to seven β-lysine residues (in ε-*N*-amide linkage) are attached via an amide link to the 2′-amino group of the D-gulosamine moiety (Fig. 2). These antibiotics are strong inhibitors of protein synthesis in an *E. coli* cell-free protein-synthesizing system, their activity being similar to that of chloramphenicol (*60*). The $—CH_2—O—C(=O)—NH_2$ group at C5 of the gulosamine moiety was shown to be unimportant for inhibition *in vitro* since the products of mild acid treatment of the antibiotics (which hydrolyses the above group to $—CH_2—OH$) gave a similar effect on *in vitro* protein synthesis to that of the native antibiotics (*60*).

19. *α-Tosyl-L-lysyl-β-chloromethylketone* (TLCK)

TLCK is shown as an analog of the carboxyl terminus of the nascent peptide attached to tRNA as also proposed by Rychlik (*7*). The lysyl residue is analogous to the carboxyl terminal amino acid of peptidyl-tRNA, while the tosyl group may be analogous to the second amino acid of the nascent peptide.

Supporting Evidence from the Literature

The scheme presented above is supported by, or compatible with, most of the data at present available from the literature. The data presented below are not entirely restricted to *E. coli*, although ribosomal differences between the various bacteria are likely to be small.

First, the activity of puromycin (3–5), C-L-phe (14), A-L-phe-L-phe (*15*) and 2′(3′)-*O*-L-lysyl adenosine (*13*) as acceptor substrates for peptidyl transferase indicates that these compounds are bound (transiently at least) to the A′ site of peptidyl transferase. Secondly, the inhibitory activity on the ribosomal fragment reaction (see (i)–(iii) below) of amicetin, celesticetin, chloramphenicol, gougerotin, lincomycin, and sparsomycin (*8*) indicates that the inhibitors are affecting one or more of the following processes:

(i) The binding of the donor substrate (e.g., CpApCpCpA-Ac-leu) to the P′ site.

(ii) The binding of the acceptor substrate (e.g., puromycin) to the A′ site.

(iii) The peptide bond-forming reaction.

Similarly, in a ribosomal peptide bond-forming system less resolved than the fragment reaction, the reaction of puromycin with polylysyl-tRNA bound to *E. coli* ribosomes in the presence of poly(A), the inhibition by the proteolytic inhibitors Ac-L-phe-CH_2Cl, TPCK, Z-L-phe-ONP, Z-gly-L-phe-NA and TLCK (*7*) leads to the same conclusions (i) to (iii) above, as well as the possibility of inhibition of binding of poly (A) or of some part other than the peptidyl terminus of the polylysyl-tRNA to the ribosome.

Recent data have shown that 1 m*M* chloramphenicol, celesticetin, gougerotin, amicetin, or 0.1 m*M* sparsomycin either had no effect or stimulated the binding of CpApCpCpA-Ac-leu to the P′ site (presumably) of *E. coli* ribosomes, while 1.0 m*M* lincomycin gave 64% inhibition (*61*). In addition, the binding of CpApCpCpA-phe to *E. coli* ribosomes was inhibited by chloramphenicol, celesticetin, lincomycin, sparsomycin, gougerotin, amicetin, blasticidin S, and puromycin (*62–64, 113*). The data for celesticetin (*113*) are at variance with those of Celma et al. (*64*), who found a slight stimulatory effect on CpApCpCpA-leu binding. Further evidence that chloramphenicol acts on the A′ site is the observation that the concentration at which chloramphenicol inhibited the binding of CpApCpCpA-phe was the same as that which inhibited protein synthesis *in vivo* and *in vitro* (*65*).

Further, Staehelin et al. (*66*) have shown that the ability of a series of progressively protein-deficient 50S ribosomal subparticles, α, β, and γ, to bind chloramphenicol was closely related to their ability to catalyse peptide bond formation, and that incubation with the split proteins was sufficient to restore both binding and peptidyl transferase activities to γ cores. Monro et al. (*67*) have reported similar results on the binding of chloramphenicol and lincomycin, the formation of the sparsomycin : CpApCpCpA-Ac-leu complex and peptidyl transferase activity of β, γ, and reconstituted γ cores.

Collectively, the above data suggests that the locus of action of chloramphenicol, sparsomycin, blasticidin S, gougerotin, and amicetin is the A′ site. Possibly the locus of action of lincomycin is also the A′ site while an allosteric effect is responsible for the inhibitory effect on CpApCpCpA-Ac-leu binding to the P′ site (see Ref. *64*).

Other miscellaneous evidence which supports the scheme includes the following three aspects:

(i) *Kinetic data.* Pestka (*69*) has shown that chloramphenicol and sparsomycin are competitive inhibitors of the reaction of puromycin with *N*-Ac-Phe-tRNA on *E. coli* ribosomes, while amicetin and gougerotin displayed more complex kinetics. Similarly, sparsomycin displayed competitive inhibition of the reaction of puromycin with polylysyl-tRNA bound to *E. coli* ribosomes in the presence of poly(A), while chloramphenicol and gougerotin gave more complex kinetics (*70*). However, gougerotin gave competitive inhibition of the reaction of puromycin with nascent haemoglobin peptides on rabbit reticulocyte ribosomes (*18*). Although the effects of inhibitors on the rate of protein synthesis are often difficult to interpret due to the complex nature of protein synthesis, the competitive kinetics indicate (but do not prove) that the inhibitors chloramphenicol, gougerotin, and sparsomycin bind to the same site as puromycin on peptidyl transferase.

(ii) *Inhibition of ribosomal binding of chloramphenicol and lincomycin by various antibiotics.* Amicetin, blasticidin S, celesticetin, lincomycin, and puromycin inhibited the binding of chloramphenicol to ribosomes obtained from one or more of *E. coli*, *Bacillus megaterium*, and *B. stearothermophilus* (*6*, *8*, *71–73*). Inhibition of chloramphenicol binding was not always observed, presumably because of the different affinities of the various inhibitors for ribosomes from the various sources under the conditions used. Relevant here was the inability of chloramphenicol, puromycin, and lincomycin at the concentrations tested to inhibit the binding of blasticidin S to *E. coli* ribosomes (*74*).

(iii) *Common features of action.* The many common features of the action of the inhibitors further indicate that a common ribosomal site of action is involved. Examples are as follows:

(a) The ribosomal binding of chloramphenicol (*75*), lincomycin (*B. stearothermophilus* ribosomes, Ref. *76*), CpApCpCpA-phe (*77*), and the formation of sparsomycin : CpApCpCpA-Ac-leu ribosome complex (*87*) were dependent on the presence of monovalent cations (e.g., K^+). The effect of these cations may, however, have been a general one on ribosomal conformation (*78*, and see (b) below).

(b) Removal of monovalent cations from the 50S *E. coli* ribosomal subunit led to the loss of peptidyl transferase activity, chloramphenicol and lincomycin binding, and the ability to form the sparsomycin : *N*-Ac-Leu-tRNA : 50S subunit complex. All four properties were regained by heat reactivation in the presence of monovalent cations (*79*). This effect appeared to be a local one (on peptidyl transferase?) since sedimentation and optical dispersion properties and melting profiles of the 50S particles in both states have shown no significant differences (*80*, and work of Y. Eilman quoted therein). Cerna (*81*) has recently shown that CpApCpCpA-phe did not bind to ribosomes in the inactive state while the binding of CpApCpCpA-Ac-phe was unaffected. This suggests that removal of monovalent cations specifically affects the A′ site of peptidyl transferase.

(c) The ribosomal binding of lincomycin (pH 5–9, Ref. *76*) and of chloramphenicol (pH 7–8.5, Ref. *78*) was not dependent on pH.

(d) α-Hydroxypuromycin, α-*N*-acetyl-puromycin, chloramphenicol, lincomycin, sparsomycin, gougerotin, and amicetin inhibited termination of protein synthesis, a reaction attributed to peptidyl transferase (*82–84*).

(e) Erythromycin reversed the inhibition of peptide bond formation by chloramphenicol (*85*) and by lincomycin (*86*, F. M. Chang as quoted in Ref. *6*).

(f) Sparsomycin, and to a lesser extent gougerotin, amicetin, chloramphenicol (weak effect), and celesticetin (weak effect), stimulated the binding of CpApCpCpA-Ac-leu to *E. coli* ribosomes in the presence of ethanol. The sparsomycin-induced stimulation (at 33% ethanol) was partially or completely reversed by chloramphenicol, lincomycin, gougerotin, and by amicetin (*8*, *61*, *87*). Similarly, sparsomycin and to a lesser extent chloramphenicol, gougerotin and amicetin stimulated the formation of an *N*-Ac-phe-tRNA:ribosome complex. The sparsomycin-induced complex formation was inhibited by chloramphenicol, lincomycin, gougerotin, and amicetin (*88*, *89*).

(g) Puromycin, blasticidin S, and gougerotin stimulated the T factor-dependent binding of phe-tRNA to *E. coli* ribosomes; chloramphenicol and sparsomycin, however, had no effect (*90*). Similarly, amicetin stimulated the nonenzymatic binding of aminoacyl-tRNA to the ribosome (*71*). The effect (for gougerotin, at least) appeared to be mediated by an effect on the ribosome (*90*).

(h) Stimulation of *in vitro* protein synthesis under certain conditions by puromycin (*Bacillus licheniformis* and *B. stearothermophilus* ribosomes; Ref. *91*), chloramphenicol and various analogs (*46*), and gougerotin (*92*) has been observed. These stimulatory effects are probably related to (f) and (g) above.

(i) Ribosomal mutations to resistance or sensitivity to one antibiotic lead to cross resistance or sensitivity to other antibiotics, e.g., *E. coli* mutants selected for lincomycin resistance were found to be resistant to chloramphenicol while lincomycin-sensitive mutants were sensitive to chloramphenicol (*93*). However, mutations to lincomycin sensitivity and resistance do not map together (*94*); perhaps proteins (or ribosomal RNA) other than peptidyl transferase were affected or peptidyl transferase is composed of subunits (*95*).

(j) There appears to be one 50S ribosomal site for the binding of chloramphenicol (*96–98*), lincomycin (*96*, *97*), the sparsomycin:CpApCpCpA-Ac-leu complex (*67*), and blasticidin S (*74*).

Collectively, the data indicate that the compounds of Fig. 2 are structural analogs of the 3′-terminus of aminoacyl- or peptidyl-tRNA and mediate their substrate or inhibitory activity on *E. coli* peptidyl transferase by binding directly to the A′ site of the enzyme. However, some of the compounds may also bind to the P′ site and the possibility of nondirect (allosteric) inhibition by the above compounds has not been ruled out. The direct mechanism is favoured because of the structural similarities between the compounds and the aminoacyl- and peptidyl-tRNA termini as described above.

According to the present scheme, both the stimulation of the T factor-dependent binding of phe-tRNA and the stimulation of protein synthesis by the various antibiotics referred to above are mediated by activation of ribosomes following binding of the antibiotic to the A′ site of peptidyl transferase. This leads to increased numbers of ribosomes participating in protein synthesis as has been observed in the case of gougero-

tin (*92*). However, the antibiotics exert an inhibitory effect on peptide elongation so that more smaller peptides are synthesized (*92, 99, 100*). Hence, depending on the conditions, net inhibition or stimulation can be observed.

Data not Compatible with the Proposed Scheme

The following data in the literature cannot be explained by the proposed scheme:

(a) The negligible or partial inhibition of peptide-bond formation between certain peptide donors and puromycin by chloramphenicol and lincomycin (*101–104*), e.g., 5×10^{-4} *M* chloramphenicol and 8×10^{-4} *M* lincomycin inhibited *N*-Ac-phe-puromycin formation by 0% and 9%, respectively (*101*).

(b) Lincomycin at concentrations in the range 10^{-5}–10^{-3} *M* had no significant effect on the puromycin-dependent release of peptides from *E. coli* polyribosomes (*108*).

(c) Puromycin at 10^{-2} *M* gave only 60–70% inhibition of the ribosomal binding of chloramphenicol at 10^{-6} *M* or lincomycin at 10^{-6} *M* (*97*). Similarly, high concentrations of sparsomycin (2.5×10^{-4} *M*) gave only 35% inhibition of the ribosomal binding of chloramphenicol at 1.25×10^{-6} *M*. In addition, the observed inhibition was not compatible with the dissociation constants of the antibiotics; chloramphenicol, 2.3×10^{-6} *M*; sparsomycin, $1\text{–}2 \times 10^{-7}$ *M* (*111*).

(d) Amicetin, blasticidin S, chloramphenicol, and gougerotin exhibited both competitive (at low concentrations of the antibiotics) and noncompetitive phases (at high concentrations) as inhibitors of the puromycin-dependent release of nascent peptides from *E. coli* polyribosomes (*108*).

(e) A protein-deficient 50S ribosomal subunit (β core) had partially impaired ability to bind chloramphenicol and lincomycin (63 and 31%, respectively, compared to native 50S ribosomes), while the peptidyl transferase activity was little affected (89% activity; Ref. *68*). If the A′ site was involved in binding these antibiotics, one might expect a corresponding loss of peptidyl transferase activity.

(f) Ethanol (33%) markedly stimulated the affinity of lincomycin for the ribosome (dissociation constant 20 times lower) but lowered the affinity of chloramphenicol (dissociation constant two to three times higher; Ref. *97*).

The following explanations could account for some of the above observations:

(a) The A′ site of peptidyl transferase may be affected by the type of donor substrate in the P′ site and thus inhibition of peptide bond formation in the presence of one of the antibiotics may or may not be observed (*12, 13*).

(b) Ribosomes may pass through several states during protein synthesis, e.g., initiation, elongation, termination, and various intermediate states (*104*), and the various antibiotics may specifically bind to ribosomes in one or more of these states or bind with varying affinities. Thus, for a heterogeneous ribosome population, partial inhibition of the binding of one antibiotic by another may be observed.

(c) Multiple sites for binding of some antibiotics might exist on the ribosome.

Predictions and Tests of the Proposed Scheme

The scheme outlined above leads to many specific and experimentally accessible predictions. Some of these are listed below.

1. As shown for C-L-phe and A-L-phe (*14*), compounds containing an amino group in a similar position to that of the α-amino group of puromycin may act as acceptor substrates, e.g., 3′-*N*-homocitrullyl 3′-aminoadenosine, 3′-*N*-lysyl 3′-aminoadenosine, celesticetin, and lincomycin. However, although α-*N*-dimethylpuromycin acts as an acceptor (*105*), it is possible that steric hindrance would prevent celesticetin or lincomycin being even poor acceptors. The β-amino acid-containing antibiotics, streptothricin and blasticidin S, are unlikely to be acceptors.

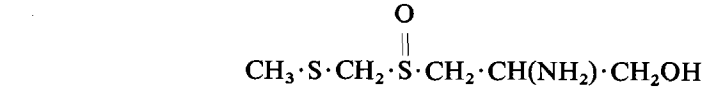
Alternatively, chemical modification so that compounds possess a free amino group in an equivalent position to that of the α-amino group of puromycin may allow acceptor activity; e.g., chloramphenicol base, if model 3 is correct; L-lysyl-chloromethylketone; L-phenylalanyl-chloromethylketone; L-phenylalanyl-*p*-nitrophenyl ester; and L-phenylalanyl-nitroanilide. In addition, chemical replacement of the 5-(*trans*-β-carboxyethylenyl)-6-methyluracil group of sparsomycin, the D-seryl-*N*-methylglycyl moiety of gougerotin or the 4,6-dideoxy-4-dimethylamino-D-glucose of amicetin with a suitable amino acid, e.g., L-phe or possibly L-lys, may confer acceptor activity on the modified antibiotics. Replacement of the β-amino acids with the corresponding α-L-amino acids in blasticidin S and streptothricin may allow acceptor activity if the native antibiotics are inactive. Certain N^4-substituted 2′(3′)-*O*-glycyl cytidine and N^6-substituted 2′(3′)-*O*-glycyl adenosine derivatives related structurally to amicetin, which contains an N^4-α-*N*-methylseryl-*p*-aminobenzoyl moiety, may also be acceptor substrates.

2. A second approach is one in which antibiotics and compounds are chemically modified to derivatives possessing inhibitory activity. For example, compounds containing an amino group in a similar position to that of the α-amino group of puromycin (see 1 above) should, on substitution with a dichloroacetyl group (or other *N*-acyl groups of active chloramphenicol derivatives–acetyl, chloroacetyl, bromoacetyl, and others; Refs. *46*, *106*), Z-glycyl or tosyl groups, be inhibitors of peptidyl transferase. Examples are: α-*N*-dichloroacetyl-puromycin, α-*N*-dichloroacetyl-α-*N*-demethylated lincomycin and α-*N*-dichloroacetyl-L-phenylalanyl chloromethylketone.

Similarly, the following combination antibiotics (i.e., antibiotics containing a part of the structure of two or more other antibiotics) may be inhibitors:

(a) Chemical substitution of 5-(*trans*-β-carboxyethylenyl)-6-methyluracil group of sparsomycin on the 3′-amino group of puromycin aminonucleoside (puromycin minus the *O*-methyl-L-tyrosine residue), on the 4′-amino group of the aminonucleonucleosides of blasticidin or gougerotin, or on the 2′(3′)-*O*-hydroxyls of cytidine, may produce active inhibitors.

(b) Similarly, chemical substitution of any of the following amino acids—*N*-methyl-L proline, *N*-methyl-4-propyl-trans-L-proline, ε-*N*-methyl-β-arginine, D-seryl-*N*-methylglycine, homocitrulline, or β-lysine—on the glucosyl amino groups of the aminonucleosides of puromycin, blasticidin, or gougerotin, on the 2′(3′)-hydroxyls of cytidine or on the amino group of the



group of sparsomycin, may produce active inhibitors.

3. All of the above compounds should compete with CpApCpCpA-phe for a single site (A′ site of peptidyl transferase) on the 50S *E. coli* ribosomal subunit.

4. Affinity-labeling derivatives of the above substrates and inhibitors should all label the same ribosomal protein(s) (peptidyl transferase and possibly neighboring ribosomal RNA and proteins).

5. It may be possible to isolate single-step ribosomal mutants which are cross resistant or cross sensitive to all or most of the above compounds.

Confirmation of any of the above suggestions would add strong support for the scheme presented. We hope that the scheme will act as a helpful guideline to the study of the mechanism of catalysis of peptide bond formation by peptidyl transferase and to the molecular mechanism of action of various antibiotics that inhibit this enzyme.

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