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Adaptability of nonnatural aromatic amino acids to the active center of the *E. coli* ribosomal A site

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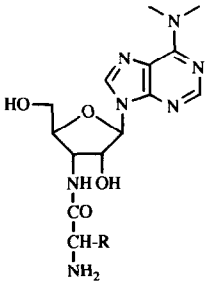
3′-*N*-Aminoacyl analogs of puromycin with nonnatural aromatic amino acids were synthesized and their inhibitory activity in *E. coli* in vitro protein synthesizing system was evaluated. The analogs with L-2-naphthylalanine, L-*p*-biphenylalanine, L-2-anthrylalanine and *trans*-L-*p*-phenylazophenylalanine were found to inhibit the protein synthesis with high efficiency. The inhibition suggests that these nonnatural amino acids are accepted by the active center of the *E. coli* ribosomal A site. A model for the adaptability of nonnatural aromatic amino acids to the active center is proposed.

Nonnatural amino acid; Puromycin; Protein biosynthesis; Ribosome; A site

1. INTRODUCTION

Until recently, the amino acid replacements in proteins are limited to 20 natural amino acids in the ordinary protein engineering. Recently, however, several groups reported techniques for the incorporation of nonnatural amino acids into proteins [1–3] by the use of chemically misacylated tRNAs [4,5]. By introducing nonnatural amino acids into biosynthetic proteins, the scope of protein engineering will be extended to include a wide variety of artificial functions. In protein biosynthesis, the selection of amino acids takes place essentially at the aminoacylation of tRNA under the control of aminoacyl tRNA synthetase. Therefore, once a tRNA is misacylated with a noncognate amino acid, the latter amino acid will be directly incorporated into proteins. This was actually shown by Noren et al. [2] and Bain et al. [3] However, they reported that the incorporation efficiency depends sharply on the type of amino acids. For instance, D-phenylalanine was exclusively rejected in their biosynthesizing system. This must be because other steps such as the peptide bond formation in ribosome, discriminate nonnatural amino acids from natural ones.

In this study, we evaluated the adaptability of nonnatural amino acids carrying a variety of aromatic groups, to the A site of *E. coli* ribosomal peptidyl transferase center. For this purpose, 3′-*N*-aminoacyl analogs of puromycin with nonnatural amino acids (Compound I) were used. Puromycin is an inhibitor of protein synthesis and known to bind to the ribosomal A site and



(I)

block the entry of aminoacyl tRNA. The amino group of bound puromycin forms a peptide bond with the carboxyl group of the peptidyl tRNA on the P site. The resulting peptide with a terminal puromycin moiety leaves the ribosome. The inhibitory activity of puromycin analogs has been examined as a measure for the adaptability of amino acids to the active center of ribosomal A site. The analogs of aromatic amino acids, L-phenylalanine and L-tyrosine were most effective [6–8], and those with *O*-benzyl-L-serine, *S*-benzyl-L-cysteine [7,8], L-homocitrulline [9] and L-lysine [10] were moderately active, whereas the analogs of other amino acids were almost inactive. The optical configuration of the amino acids was also important since the D-phenylalanyl analog was far less active than the L-isomer [6].

We examined the inhibitory activity of puromycin analogs with nonnatural amino acids carrying large aromatic groups (Fig. 1), and predicted whether or not these nonnatural amino acids can be incorporated into proteins.

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2. MATERIALS AND METHODS

Puromycin, puromycin aminonucleoside, L-1-naphthylalanine (2) and L-2-naphthylalanine (3) were purchased from Sigma. L-1-pyrenylalanine (4), L-2-pyrenylalanine (5), L-*p*-biphenylalanine (6), L-2-anthrylalanine (7), L-2-anthraquinonylalanine (8), L-9-carbazolylalanine (9), L-9-ethyl-3-carbazolylalanine (10), L-9-phenanthrylalanine (11) and L-*p*-phenylazophenylalanine (12) were synthesized in our laboratory [11]. L-$AzOC_n$ (13–16) and L-$CarC_n$ (17,18) were gifts from Professor Nishino of Kyushu Institute of Technology.

The puromycin analogs were synthesized as follows: puromycin aminonucleoside (0.01 mmol), *tert*-butyloxycarbonyl-protected amino acid (0.01 mmol) and 1-hydroxybenzotriazole hydrate (0.011 mmol) were dissolved in 0.1 ml of anhydrous dimethylformamide. To this solution, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.011 mmol) was added at 0°C and the mixture was stirred for 2 h at 0°C and 22 h at room temperature. The mixture was diluted with 2 ml of ethyl acetate and washed twice with 4% $NaHCO_3$, and twice with saturated NaCl. The organic phase was dried over $MgSO_4$ and the solvent was evaporated. The resulting solid was recrystallized from ethylacetate and *n*-hexane. For deprotection, the product was dissolved in 0.1 ml of trifluoroacetic acid at 0°C. After 30 min at 0°C, the solvent was flushed off by N_2 gas, then the deprotected product was washed with ether for several times and dried in vacuo. The purity was confirmed by reverse-phase HPLC and ^{1}H-NMR.

In the inhibition experiment, *E. coli* S-30 extract [12], mRNA encoding bacteriophage T7 gene10, and ^{14}C-labeled leucine were used. Protein synthesis was carried out at 37°C for 30 min in the presence of various concentrations of puromycin analogs. The amount of the protein synthesized was measured by filter paper disk technique [13]. The inhibitory activity of the analogs of puromycin was expressed relative to a control experiment without inhibitor.

3. RESULTS

The results of the inhibition experiments are summarized in Table I. Puromycin and L-phenylalanyl analogs showed strong inhibitory activity as reported previously [6]. Similarly, the analogs of L-2-naphthylalanine, L-*p*-biphenylalanine, L-2-anthrylalanine, and L-*p*-phenylazophenylalanine were found to inhibit with good efficiency. On the other hand, the analog of L-1-naphthylalanine showed negligible inhibitory activity, in contrast to that of L-2-naphthylalanine. The analogs of L-1-pyrenylalanine, L-2-pyrenylalanine, L-9-phenanthrylalanine, L-2-anthraquinonylalanine, L-9-carbazolylalanine and L-9-ethyl-3-carbazolylalanine were also inactive.

The inhibitory activity was markedly decreased when the *trans*-azobenzene group of the *p*-phenylazophenylalanyl analog was photoisomerized to the *cis*-form, as shown in Fig. 2. Irradiation with UV light at 350 nm resulted in conversion of about 80% of azobenzene group to the *cis*-form under photostationary state. The inhibitory activity of the puromycin analog was low under this condition. The extrapolation to 100% *cis*-form showed that the analog with *cis*-*p*-phenylazophenylalanine was virtually inactive.

The effect of spacer lengths between the C^α atom and aromatic groups was also examined. In Table IB, the results of azobenzene derivatives and carbazole deriva-

Table I

Inhibitory activity of 3′-*N*-aminoacyl analog of puromycin with a variety of aromatic natural and nonnatural amino acids in *E.coli* in vitro protein synthesizing system

	Inhibition (%)					
	Concentration (M)					
Amino acid	3×10^{-3}	1×10^{-3}	3×10^{-4}	1×10^{-4}	3×10^{-5}	1×10^{-5}
A.						
O-Methyltyrosine	–*	98.8	99.0	99.0	98.5	80.6
Phenylalanine	97.3	98.9	98.3	94.4	96.2	70.1
1-Naphthylalanine	65.6	29.2	0			
2-Naphthylalanine	99.2	90.0	92.3	93.9	74.6	28.4
1-Pyrenylalanine	–**	29.0	4.5	0		
2-Pyrenylalanine	–**	–**	0			
p-Biphenylalanine	97.8	90.4	71.6	64.5	55.4	56.3
9-Phenanthrylalanine	83.4	62.1	28.6	0		
2-Anthrylalanine	97.8	98.5	98.9	95.5	81.2	73.0
2-Anthraquinonylalanine	58.1	33.0	34.6	0		
9-Carbazolylalanine	25.5	5.7	7.1	0		
9-Ethyl-3-carbazolylalanine	0.8	11.0	4.4	0		
p-Phenylazophenylalanine	96.8	99.0	91.3	67.3	59.4	32.7
B.						
$AzOC_3$	–*	99.9	89.6	37.8	14.1	8.1
$AzOC_4$	97.8	96.5	79.7	64.5	33.8	0
$AzOC_5$	–**	15.3	8.7	0		
$AzOC_6$	–**	16.2	13.7	0		
$CarC_3$	34.6	11.5	0			
$CarC_5$	1.9	0				

*Not tested.
**Insoluble.

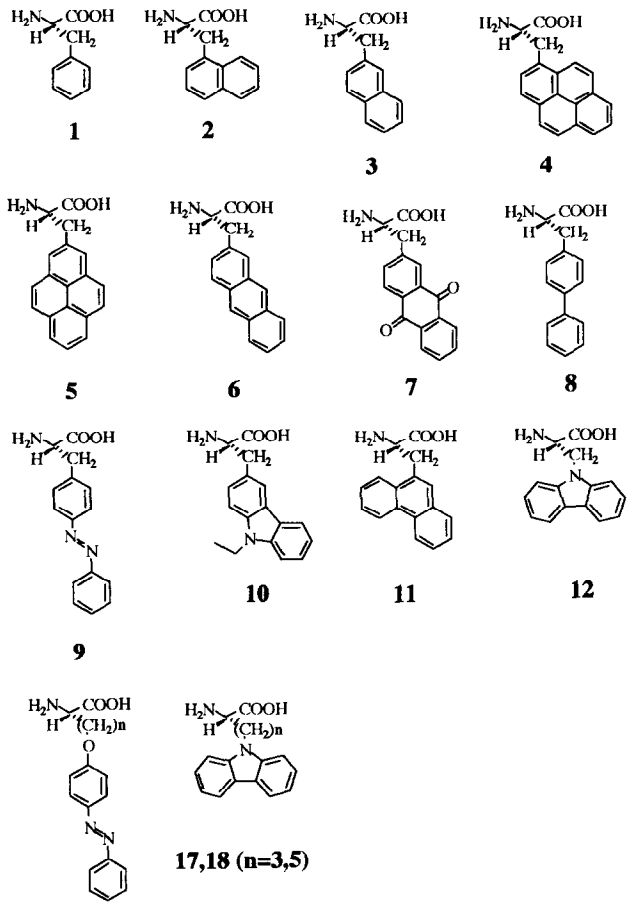

Fig. 1. Structure of aromatic natural and nonnatural amino acids used in this study.

tives are shown. The azobenzene derivatives having short spacers were active but those having long spacers were inactive. The carbazole derivatives were found to be inactive in every case.

4. DISCUSSION

The results in Table I show that the inhibitory activities of the puromycin analogs are widely different. The analogs are clearly divided into two groups depending on the inhibitory activity, those that show high activity at 10^{-5} M and those that show low activity even at 10^{-3} M.

The analogs of high inhibitory activity may bind to the active center and form peptidyl puromycin, as has been demonstrated previously for the phenylalanine analogs, tyrosine, *O*-benzylserine, and *S*-benzylcysteine. The puromycin analogs of those amino acids inhibited the protein synthesis and formed peptidyl puromycin in the ribosome [7,8]. On the other hand, those of low activity may either have low affinity for the active center, or effectively bind to the active center but do not

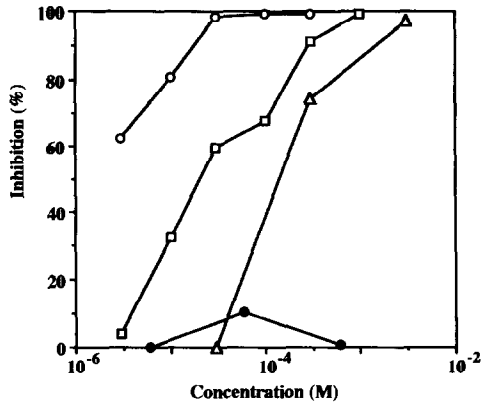

Fig. 2. Inhibitory activity of the puromycin analogs with L-*p*-phenylazophenylalanine: (□), *trans*-form (before irradiation); (△), 80% *cis*-form (after irradiation at 350 nm for 5 min); (●), 100% *cis*-form (extrapolated values); (○), puromycin as a reference.

react with peptidyl tRNA. If the latter is the case, however, a constant inhibitory efficiency that is lower than 100% will be observed after all the binding sites were occupied by the addition of an excess amount of the puromycin analog. The results in Table I exclude this possibility, because the inhibitory activity of each analog is not constant over the concentration range of 10^{-4}–10^{-3} M and is increasing with the concentration. The concentration dependence in Table I shows that the low activities are due to low affinity for the active center. Therefore, it is concluded that L-2-naphthylalanine, L-*p*-biphenylalanine, L-2-anthrylalanine, *trans*-L-*p*-phenylazophenylalanine, $AzOC_3$ and $AzOC_4$ adapt to the active center of ribosomal A site, but L-1-naphthylalanine, L-1-pyrenylalanine, L-2-pyrenylalanine, L-2-anthraquinonylalanine, L-9-carbazolylalanine, L-9-ethyl-3-carbazolylalanine, L-9-phenanthrylalanine, *cis*-L-*p*-

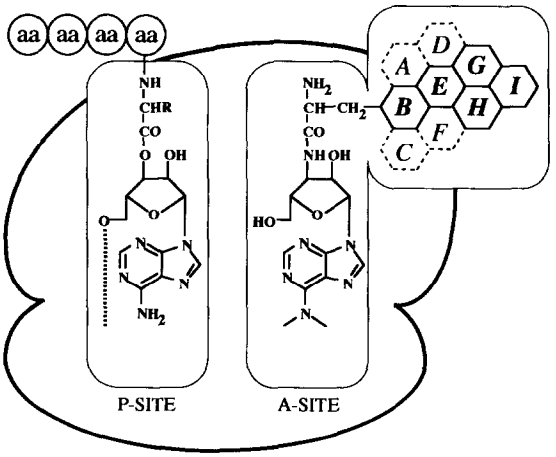

Fig. 3. A model for the adaptability of nonnatural aromatic amino acids to the active center of *E. coli* ribosomal A site. The 3′-*N*-aminoacyl analog of puromycin and peptidyl tRNA exist in ribosomal A site and P site, respectively. Nonnatural amino acids carrying benzene rings in regions B, E, G, H and I can adapt to the active center of A site, whereas those carrying benzene rings in other regions do not adapt.

phenylazophenylalanine, $AzOC_5$, $AzOC_6$, $CarC_3$ and $CarC_5$ do not adapt.

The next question was why some nonnatural amino acids adapt to the active center but others do not. In previous studies, the analogs with aromatic amino acids containing one benzene ring have been found to adapt to the active center [6–8]. However, the adaptability does not depend only on the size of aromatic group, because 2-naphthylalanine adapts but 1-naphthylalanine does not. Therefore, the active center may discriminate nonnatural amino acids not only by their size but their geometry. From a consideration of the results in Table IA, a simple model for the adaptability of aromatic nonnatural amino acids to the active center can be proposed as shown in Fig. 3. The benzene rings of arylalanine-type amino acids denoted as A, B, C, etc., can be divided into allowed regions (B, E, G, H, I) and disallowed regions (A, C, D, F). The amino acids that adapt to the active center contain benzene rings only in the allowed regions. On the other hand, those that do not adapt contain benzene rings in the disallowed regions.

Based on this model, nonnatural amino acids that adapt to the active center can be predicted. For example, L-2-phenanthrylalanine that occupies regions B, E and H may adapt, whereas L-1-phenanthrylalanine that occupies regions A, B and D will not. This model explains that the analogs with benzyl derivatives of L-serine and L-cysteine are active [7,8], because the benzene rings of these derivatives are located in region E.

The present results predict what type of nonnatural amino acids can be incorporated into proteins when they are attached to tRNA and added to the in vitro protein biosynthesizing system [1–3]. In the case of naphthylalanine, for instance, L-2-naphthylalanine may be incorporated into proteins but L-1-naphthylalanine may not. Similarly, L-2-phenanthrylalanine is a proper amino acid for the incorporation of phenanthryl group.

The results in Table IB suggest that spacer length is also an important factor in determining the adaptability. In the case of the azobenzene derivatives, spacers with $n \geq 5$ are clearly unfavorable, suggesting that azobenzene groups very remote from C^{α} atom do not adapt to the ribosomal A site. In the case of carbazole derivatives, however, no proper spacer length was found for the incorporation of 9-substituted carbazole group.

The incorporation of the photofunctional nonnatural amino acids into proteins is now in progress, and will appear in future reports.

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