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# Fluorescent 2'(3')-O-aminoacylnucleosides-acceptor substrates for ribosomal peptidyltransferase+

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ABSTRACT

2'(3')-*O*-*L*-Phenylalanylderivatives of fluorescent 1,$N^6$-ethenoadenosine and 3,$N^4$-ethenocytidine were prepared by chemical synthesis. Both compounds are good acceptor substrates in ribosomal peptidyltransferase reactions. Since these compounds cannot form Watson-Crick base pairs, the results indicate that the terminal aminoacyladenosine unit of AA-tRNA is bound to ribosomal protein on the acceptor site of peptidyltransferase and not to rRNA.

## INTRODUCTION

The highly fluorescent 1,$N^6$-ethenoadenosine and its nucleotide derivatives[3,4] have found considerable use as substrate analogues and fluorescent probes in the investigations of various biological systems[5].

As a part of our study of the involvement of the 3' terminus of tRNA in various partial reactions in protein biosynthesis, we were interested in whether the acceptor site of ribosomal peptidyltransferase would recognize the εA moiety as an analogue of the natural adenosine at the 3' terminus of AA-tRNA[6]. In such a case 2'(3')-*O*-aminoacyloligoribonucleotides containing εA at the 3' terminus should be very valuable as fluorescent probes in ribosomal systems.

The spatial outline of the base moiety of 3,$N^4$-ethenocytidine is very similar to that of adenosine; this spatial similarity allows an accommodation of the εC moiety within the binding sites of various enzymes which specifically interact with the adenine moiety[7]. Thus, the investigation of εC as another analogue for the natural adenosine terminus of AA-tRNA appeared to be an attractive proposition.

In this communication we report on the chemical synthesis of the two simplest analogues of the 3' terminus of AA-tRNA containing εA and εC, namely the 2'(3')-*O*-*L*-phenylalanylderivatives of εA and εC. We further report on the acceptor properties of these two compounds in the

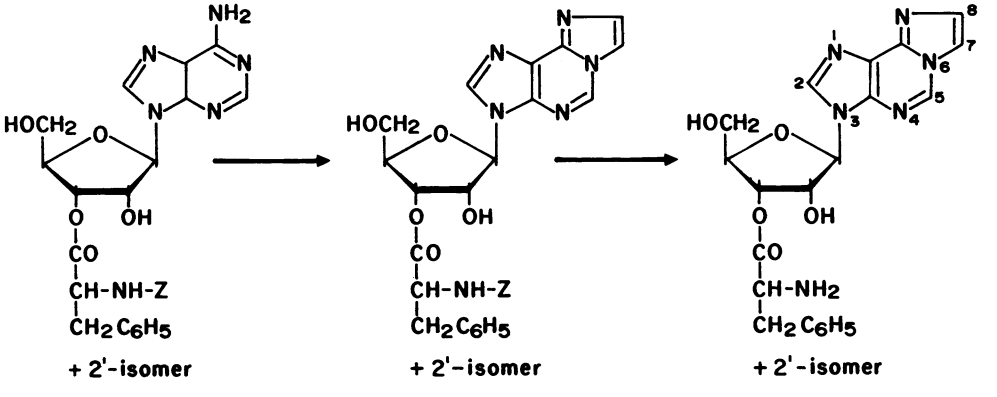
 

peptidyltransferase reaction (puromycin-like activity). We conclude that the novel ring system of εA does not severely impair the ability of εA-Phe to interact functionally with the peptidyltransferase A site in the manner of the parent A-Phe. The acceptor activity of εC-Phe is greater than that of the parent C-Phe. Since neither εA-Phe or εC-Phe are able to form classical Watson-Crick base pairs (with, for example, the appropriate bases in rRNA), the results strongly suggest that during the peptide bond formation step the terminal adenosine unit in AA-tRNA is bound to ribosomal protein and not to rRNA.

## RESULTS AND DISCUSSION

Two different methods were used for the preparation of 2'(3')-*O*-*L*-phenylalanylderivatives of εA-Phe (3) and εC (7). For the preparation of εA-Phe (3) (Scheme 1), 2'(3')-*O*-(*N*-benzyloxycarbonyl-*L*-phenylalanyl)adenosine (1) was converted to its 1,$N^6$-ethenoderivative (2) by reaction with chloroacetaldehyde in DMF-water solution[3]. Since the optimum conversion of A to εA takes place at an acidic pH at which the aminoacyl residue is stable, this reaction is not accompanied by any detectable loss of the *N*-benzyloxycarbonyl-*L*-phenylalanyl group, even at 37° for 3 days. The product 2 was isolated by preparative tlc in *ca.* 60% yield and was fully characterized. In the next step, the *N*-benzyloxycarbonyl group of 2 was removed by standard hydrogenolysis[8] on palladium catalyst in acidic medium. The final product, εA-Phe (3), was obtained in 66% yield. For reasons which are not clear at the present time, hydrogenolysis of the *N*-benzyl-

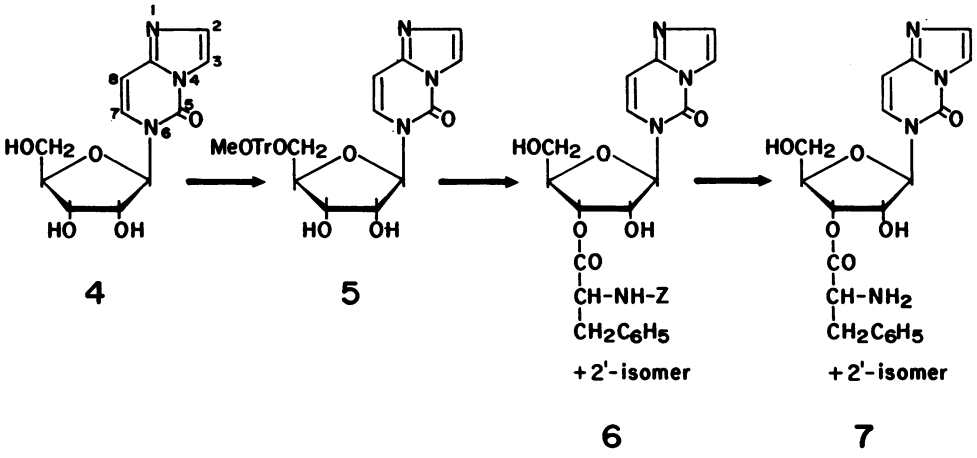
**Scheme I**



oxycarbonyl group of 2 is appreciably slower than in the comparable adenosine derivative 1; the εA moiety is left intact under the hydrogenation conditions used, as can be seen from the virtual identity of the UV spectrum of εA obtained by alkaline hydrolysis of εA-Phe (3) and that of the authentic material.

A different approach was used for the preparation of εC-Phe (7) (Scheme 2). εC (4) was first converted to its 5'-monomethoxytritylderivative (5) by the standard method in 87% yield. This compound was characterized by tlc, and by UV and NMR spectra. Derivative 5 was then aminoacylated by the general method[8], using *N*-benzyloxycarbonyl-*L*-phenylalanine and DCC in pyridine solution. After partial purification of the

**Scheme 2**




crude reaction product on preparative tlc, the 5'-*O*-monomethoxytritylgroup was removed by treatment with 80% acetic acid, and product 6 was isolated by preparative tlc in 24% yield, and was characterized by the usual methods. Only a negligible amount of the bis-aminoacyl derivative, which is usually co-synthesized with the monoaminoacylderivative[8], was detected in this experiment. Finally, routine hydrogenolysis was employed to remove the *N*-benzyloxycarbonylgroup from the phenylalanine moiety of 6 to obtain εC-Phe 7.

As was expected, both compounds εA-Phe (3) and εC-Phe (7) are unstable in alkaline medium, such as chromatographic system $S_1$, and are hydrolyzed to the parent nucleoside and phenylalanine.

The ability of εA-Phe (3) and εC-Phe (7) to function as acceptors

in the ribosomal peptidyltransferase reaction was investigated. Figure 1 shows that both compounds 3 and 7 were moderately active in the release of Ac-Phe residue from Ac-Phe-tRNA·70S ribosome·poly(U) complex. The activities of both compounds are similar, 50% release of Ac-Phe is attained at *ca.* 4 x $10^{-4}$ or 8 x $10^{-4}$ M concentration of 3 or 7, respectively. The weaker interaction of εA-Phe (3) with the peptidyltransferase A site, compared to the "natural" A-Phe, is most probably caused by the unfavorable steric influence of the imidazole ring fused at the 1,6 position of adenine. On the other hand, it is known that various substitutions of the $N^6$-aminogroup of adenine do not result in profound changes in the acceptor activity of various 2'(3')-*O*-*L*-phenylalanylpurineribosides, compared to the parent A-Phe[9]. The acceptor activity of εC-Phe (7) is much higher than that reported for C-Phe[10]. This is in agreement with the previous findings of other workers that εC is a good analogue of adenosine in various enzymatic systems, since the spatial outline of the base moiety of εC is similar to that of adenosine[7]. 1,$N^6$-Ethenoadenosine (or 3,$N^4$-ethenocytidine) cannot form Watson-Crick base pairs to uridine, since positions 1 and $N^6$ are occupied. For instance, it is known that poly(εA)

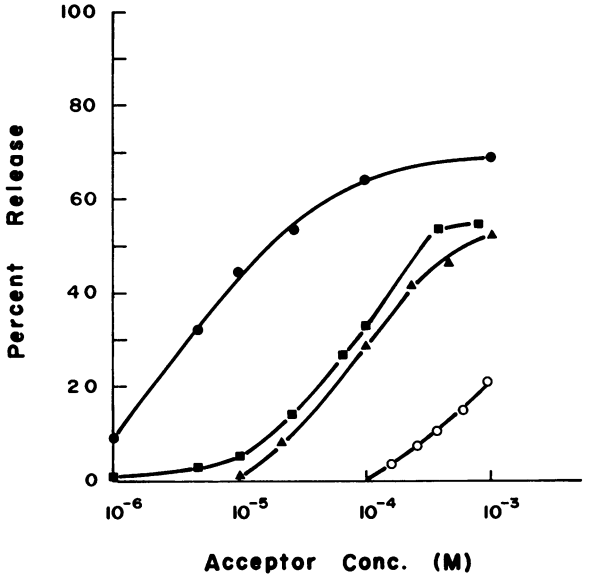



Figure 1. Acceptor activity of A-Phe (●), εA-Phe (3) (■), εC-Phe (7) (▲), and C-Phe (○) (data from ref. [9]) in the ribosomal peptidyltransferase reaction [2'(3')-*O*-*L*-phenylalanylnucleoside dependent release of the Ac[$^{14}$C]Phe residue from Ac[$^{14}$C]Phe-tRNA·70S ribosomes·poly(U) complex)].

does not form a complex with poly(U) (*cf.* [11]). Puromycin and similar models of AA-tRNA interact with acceptor site of peptidyltransferase on the 50S ribosomal subunit, the site where the aminoacyladenosine terminus of AA-tRNA also interacts[12]. Since εA-Phe and εC-Phe are able to interact with the peptidyltransferase A site, it appears that the aminoacyladenosine terminus of AA-tRNA does not use Watson-Crick pairing to 23S RNA for binding to the peptidyltransferase A site, as was recently suggested on the basis of affinity labeling experiments[14]. Thus, it is likely that the 3' terminus unit of AA-tRNA binds to the ribosomal protein(s) which is a part of the peptidyltransferase acceptor site[10]. On the other hand, our suggestion is not at variance with models giving an important structural role to 23S RNA in the peptidyltransferase A site[15].

## MATERIALS AND METHODS

### General Methods.

For general methods, see references[16,17]. All evaporations were carried out on a Buchi rotary evaporator at a temperature below 35°. Paper chromatography was performed on Whatman No. 1 paper in $S_1$ (2-propanol:conc. $NH_4OH$:water, 7:1:2) and $S_2$ (1-butanol:acetic acid:water, 5:2:3). Thin layer chromatography (tlc) was carried out as previously described in solvents $S_3$ ($CHCl_3$-MeOH, 95:5) and $S_4$ ($CHCl_3$-MeOH, 9:1). Paper electrophoresis was performed as described previously[15] on Whatman No. 1 paper in 1 M acetic acid at 30V/cm for 1.5 hr. The spots were visualized by UV light (Mineralight) and ninhydrin positive substances were detected by spraying with 0.1% ninhydrin in ethanol. Low boiling (30-60°) petroleum ether was used. Nuclear magnetic resonance (NMR) spectra were determined with a Varian A-60A spectrometer using TMS as internal standard. UV spectra were measured using a Cary 14 recording spectrophotometer. Chloroacetaldehyde was purchased from Pfaltz and Bauer, Inc. and was distilled prior to use, and $PdO/BaSO_4$ (5%) was purchased from Engelhard.

Ribosomes were prepared from late log phase *Escherichia coli* MRE 600 ($RNAse\ 1^-$) cells and were washed three times by ultracentrifugation in 0.5 M $NH_4Cl$ as described [16,17] previously. Ac[$^{14}$C]Phe-tRNA (0.4 nmol of [$^{14}$C]Phe/mg of tRNA) was prepared as described[16,17].

### 2'(3')-*O*-(*N*-Benzyloxycarbonyl-*L*-phenylalanyl)1,$N^6$,ethenoadenosine (2).

2'(3')-*O*-(*N*-Benzyloxycarbonyl-*L*-phenylalanyl)adenosine (cf.[8]) (0.54 g, 1 mM) (1) was stirred with DMF (12 ml) and 2 M aqueous chloroacetaldehyde (pH 4.7) at 37°. The pH of the reaction mixture was adjusted periodically with diluted triethylamine to pH 4.7. After 3 days, the

reaction mixture was evaporated *in vacuo* to dryness and the residue was co-evaporated with dioxane. The residue was applied to two plates (20 x 50 cm) of silica gel (loose layers) and the plates were developed by system $S_4$. Two main bands were detected, the slower fluorescent band being the major one and containing pure product 2. Both bands were eluted by system $S_4$ and eluate 1 evaporated. The faster product was rechromatographed on 3 plates (20 x 20 cm) of Stahl's silica gel and developed by system $S_4$ (3 developments) to completely separate trace impurities of starting material 1. The combined products were triturated with a mixture of chloroform-petroleum ether to obtain a chromatographically-homogeneous white solid, m.p. 185-188° (softening at 100°), yield 0.34 g (58%). UV (95% ethanol) $\lambda_{max}$ (nm) 277 (ε 5,400), 267 (ε 5,770), 261 (ε 5,400), $\lambda_{min}$ (nm) 274, 264, 252, shoulder *ca.* 295. NMR ($CD_3COCD_3$) δ, 8.88 (1, s, $H_2$), 8.25 (1, s, $H_5$), 7.85 (1, d, J = 2 Hz, $H_8$), 7.46 (1, d, J = 2 Hz, $H_7$), 7.25 (m, 10, phenyls), 6.08 (1, d, J = 7 Hz, $H_{1'}$), 5.05 (s, 2, $CH_2$ from benzyloxycarbonyl).

5'-*O*-*p*-Methoxytrityl-3,$N^4$-ethenocytidine (5).

3,$N^4$-Ethenocytidine[3] (0.91 g, 2.99 mmol) was dissolved in anhydrous pyridine (10 ml), *p*-methoxytritylchloride (0.99 g, 3.20 mmol) was added, and the reaction mixture was kept at room temperature for 24 hr, then poured onto ice and extracted with chloroform. The chloroform extract was dried over sodium sulfate and evaporated. The residue was triturated with chloroform-petroleum ether mixture to produce a pale yellow solid which was dried *in vacuo*. The yield of the chromatographically uniform product 5 was 1.41 g (87%). UV (95% ethanol) $\lambda_{max}$ (nm) 275 (ε 11,190), 235 (ε 10,390), $\lambda_{min}$ (nm) 249, shoulders (nm) 295 (ε 4,800), 281 (ε 10,390). NMR ($CD_3COCD_3$ + $D_2O$) δ 7.93 (d, 1, J = 1 Hz), 7.2 (m, 16), 6.36 (d, 1, J = 7 Hz, $H_8$), 6.16 (d, 1, J = 5 Hz, $H_{1'}$), 3.78 (s, 3, $-OCH_3$).

2'(3')-*O*-(*N*-Benzyloxycarbonyl-*L*-phenylalanyl)3,$N^4$-ethenocytidine (6).

Compound 5 (0.54 g, 1 mM) was dissolved in pyridine (3 ml), Z-Phe (0.30 g, 1 mM) was added and the solution was cooled to 0°. A cold solution of DCC (0.23 g, 1.2 mM) in pyridine (2 ml) was added and the mixture was maintained at 0° for 1 hr and then at room temperature for 20 hr. Ice was added to the purple reaction mixture, and after addition of petroleum ether, dicyclohexyl urea was filtered off and washed by pyridine. The solution was evaporated *in vacuo*, co-evaporated with toluene and the residue was extracted with petroleum ether. The residue was applied to 3 plates (20 x 50 cm) of silica gel (loose layers) and the plates were

developed by system $S_3$. Three bands were detected (in order of increasing $R_f$): starting material, monoaminoacyl and diaminoacyl derivatives (very weak band) of 5. The middle band was eluted by system $S_4$ and the eluate evaporated. Tlc in system $S_3$ indicated that the product contains a small amount of starting material. The residue was dissolved in 80% acetic acid (25 ml) and held at room temperature for 20 hr. The solution was lyophilized and the residue was co-lyophilized with dioxane. The residue was applied to two plates of loose layer silica gel (20 x 50 cm) and the plates were developed (twice) by system $S_4$. The major band (product 6) was eluted with $S_4$ and the eluate was evaporated *in vacuo*. A solid product was obtained by trituration with chloroform-petroleum ether and dried *in vacuo*. The yield of the chromatographically (tlc) uniform product was 0.14 g (24.3%). UV (95% ethanol) $\lambda_{max}$ (nm) 272 ($\varepsilon$ 11,420), $\lambda_{min}$ (nm) 234 ($\varepsilon$ 1,370), shoulders (nm) 294 ($\varepsilon$ 4,570), 282 ($\varepsilon$ 10,050). NMR ($CD_3SOCD_3$ + $D_2O$)$\delta$7.80 (m, 2), 7.35 (m, 11), 6.80 (d, 1, J = 8.5 Hz, $H_8$), 6.16 (d, 1, J = 7 Hz, $H_{1'}$).

2'(3')-*O*-*L*-Phenylalanyl-1,$N^6$-ethenoadenosine (3).

Compound 2 (24.6 mg, 0.040 mmol) was dissolved in 80% acetic acid (*ca.* 5 ml), $PdO/BaSO_4$ (110 mg) was added and the mixture was hydrogenated for 3.5 hr. at 0°, as described previously[8]. After 1.5 hr. another 210 mg of $PdO/BaSO_4$ was added. Electrophoresis (1 M acetic acid) of the reaction mixture showed complete conversion of starting material to the fast-moving product 3. The catalyst was filtered off using a Celite bed and the filtrate was freeze-dried. The residue was dissolved in 80% acetic acid (15 ml volumetric flask) and aliquots of this solution were used for determination of yield (spectrophotometrically at pH 2.0) or for electrophoretical or chromatographical analysis. Yield was 0.026 mmol (using $\varepsilon$ 274 = 10,300, cf [3]), 250/260 = 0.73, 280/260 = 1.04, 290/260 = 0.61, $\lambda_{max}$ (nm) 274, $\lambda_{min}$ (nm) 248, shoulder (nm) 267. The product 3 was uniform according to electrophoresis (1 M acetic acid; 2.3 mobility of starting material) and paper chromatography (system $S_2$) gave a positive reaction to ninhydrin spray. It is quantitatively hydrolyzed in system $S_1$ to phenylalanine and to 1,$N^6$-ethenoadenosine with a UV spectrum identical to that of an authentic sample.

Samples of 3 for biochemical investigations were prepared by freeze-drying of aliquots from stock solution. The residues were dissolved in appropriate amounts of water and the pH was carefully adjusted to *ca.* 6.6 with dilute aqueous NaOH.

2'(3')-*O*-*L*-Phenylalanyl-3,$N^4$-ethenocytidine (7).

Hydrogenation of 6 was performed as described above, starting from 33.61 mg (0.058 mmol) of 6 and 36 mg of $PdO/BaSO_4$ in 80% acetic acid (*ca.* 5 ml). Hydrogenation time was 1.5 hr. The yield of 7 was determined spectrophotometrically at pH 2.0 (using ε 288 = 12.3 at pH 2, *cf.* [3]), 0.048 mM (82%). UV (pH 2.0) $\lambda_{max}$ (nm) 288, $\lambda_{min}$ (nm) 225, shoulder (nm) 304,250. The product 7 was uniform by electrophoresis (1 M acetic acid, *ca.* 2.0 mobility of starting material) and paper chromatography (system $S_2$) positive to ninhydrin spray, and in system $S_1$ it is quantitatively hydrolyzed to εC and phenylalanine.

Assay of peptidyltransferase acceptor activity.

Peptidyltransferase activity of compounds 3 and 7 was measured essentially as previously described[16]. A typical reaction mixture contained (in 0.1 ml): 0.05 M Tris-HCl (pH 4), 0.10 M $NH_4Cl$, 0.01 M $MgCl_2$, 3.7 $A_{260}$ units of $NH_4Cl$ washed ribosomes, 10 µg of poly(U) and 0.12 $A_{260}$ units of Ac[$^{14}$C]Phe-tRNA (2000 cpm). Reactions were initiated by addition of the acceptor compounds at the concentrations indicated in Figure 1 and were allowed to incubate for 30 min at 37° before termination of the reaction. Reaction was terminated by (a) addition of cold aqueous 2.5% $CCl_3COOH$ (2 ml). The amount of unreacted Ac[$^{14}$C]Phe-tRNA was determined by measurement of $CCl_3COOH$ precipitated counts trapped by Millipore membranes as described before. The amount of Ac[$^{14}$C]Phe residue transferred from Ac[$^{14}$C]Phe-tRNA to the acceptor was determined as the difference between radioactivity retained on the filter in the absence of acceptor and that remaining on the filter in the presence of acceptor. It is expressed as a percentage of the radioactivity of Ac[$^{14}$C]Phe-tRNA added to the incubation mixture; (b) Reaction was terminated by addition of 0.1 ml of 0.1 M $Be(NO_3)_2$ and 0.3 M NaOAc (pH 5.5) saturated with $MgSO_4$. The products of the peptidyltransferase reaction were extracted with 1.5 ml of ethyl acetate as described by Monro *et al.*[18]. The radioactivity of 1.0 ml aliquots of the ethyl acetate extract was measured as described previously[19]. The amount of Ac[$^{14}$C]Phe-tRNA was determined as the difference between radioactivity extracted into ethyl acetate in the presence and absence of the acceptor. Both methods gave essentially the same results, which are summarized in Figure 1.

Analysis of peptidyltransferase reaction products.

The peptidyltransferase reaction products formed by the reaction of Ac[$^{14}$C]Phe-tRNA with the acceptor substrates were identified by paper

electrophoresis, as described previously[16] (data not shown).

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