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PREPARATION OF 2′(3′)-O-Acyl-pCpA DERIVATIVES AS SUBSTRATES FOR T4 RNA LIGASE-MEDIATED "CHEMICAL AMINOACYLATION"

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Abstract—The synthesis of 2′(3′)-O-(amino)acyl-pCpA derivatives is described and the products are characterized chemically and as substrates for T4 RNA ligase in the presence of *E. coli* $tRNA^{Phe}$–C_{OH}. The modified (N-acetylamino)acyl-tRNA's so derived are characterized by their chromatographic and biochemical properties, and by their ability to act as P-site tRNA donors in the peptidyltransferase reaction.

Although experimental verification of the adapter hypothesis[1,2] was reported more than 20 years ago,[3] and recent studies have documented in a more quantitative fashion the participation of misacylated tRNA's in protein biosynthesis,[4,5] the obvious extension of these observations to permit the elaboration of modified polypeptides via *in vitro* protein biosynthesis has not been realized. Successful implementation of this strategy, of course, would require facile access to a variety of misacylated tRNA's and a description of potential structural constraints on amino acid (analog) participation in the partial reactions of protein biosynthesis, neither of which have been available to date.

While numerous workers have described the aminoacyl-tRNA synthetase-mediated misacylation of individual tRNA's, virtually all of these studies have been concerned primarily with the process by which an aminoacyl-tRNA synthetase recognizes its cognate tRNA,[6,7] the actual mechanism of tRNA activation,[8] or the putative "proofreading" scheme ostensibly reflected in the AMP-PPi—independent deacylation of misacylated tRNA's.[9] With few exceptions,[8,10,11] the misacylated tRNA's produced in these studies contained only naturally occurring amino acids, reflecting the stringent structural requirements for amino acid activation.[8] Two reports have outlined what may represent general methods for the misacylation of tRNA's using T4 RNA ligase to mediate the addition of preaminoacylated nucleotides to tRNA's lacking one[12] or two[13] nucleotides at the 3′-terminus; the more recent of these seems especially promising.

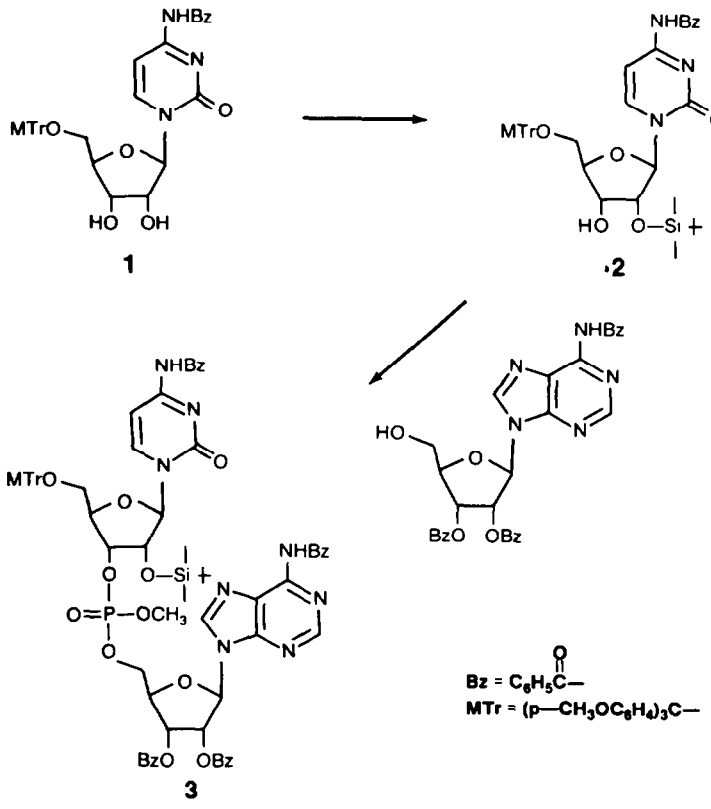
Given the broad substrate specificity of elongation factor Tu,[14,15] and the observed binding to ribosomes of tRNA species that do not participate in the peptidyltransferase reaction,[16] it seems likely that the actual process of peptide bond formation may provide the greatest constraints on the structures of modified aminoacyl-tRNA's that can be utilized to produce polypeptides *in vitro*. Many studies of the substrate specificity of peptidyltransferase have appeared, but most have employed analogs of puromycin or aminoacylated oligonucleotides and may provide limited predictive value for the behavior of the corresponding aminoacyl-tRNA's.[9] Those few reports that describe the participtation of modified aminoacyl-tRNA's in the peptidyltransferase reaction suggest that the chemical nature[17,18] and spatial orientation[9,19] of the nucleophilic group on the aminoacyl moiety of the A-site tRNA are important, as is the absolute configuration of the amino acid.[11] The availability of "chemically misacylated" tRNA's[12,13] has permitted a more precise analysis of structural requirements for participation of P-site tRNA's in the peptidyltransferase reaction,[13] and it should be possible to employ analogous techniques for dissection of events at the ribosomal A-site.

The T4 RNA ligase donor substrates utilized for the preparation of the "chemically aminoacylated" tRNA's are 2′(3′)-O-acylated derivatives of pCpA (**6**), which are obtained via chemical synthesis. Herein we describe the synthetic routes developed for their preparation, and characterization of the synthetic dinucleotides by chromatographic, chemical and enzymatic methods. Also described is the preparation of *E. coli* 3-phenylpropionyl-$tRNA^{Phe}$ by T4 RNA ligase-mediated coupling of 2′(3′)-O-(3-phenylpropionyl)-pCpA and $tRNA^{Phe}$–C_{OH}.

RESULTS AND DISCUSSION

As discussed below, the preparation of the misacylated tRNA's of interest was carried out by the condensation of $tRNA^{Phe}$–C_{OH} with chemically acylated derivatives of pCpA. To permit the utilization of a common intermediate for each of the derivatives, pCpA itself was first prepared and then acylated by the appropriate method outlined.

Synthesis of pCpA. The synthetic approach employed for the preparation of pCpA involved the coupling of N^4-benzoyl-2′-O-*t*-butyldimethylsilyl-5′-O-monomethoxytritylcytidine (**2**)[20] and N^6, $O^{2'}$, $O^{3'}$-tribenzoyladenosine with methylphosphorodichloridite.[21,22] The protected dinucleoside monophosphate (**3**) was isolated as a yellow powder in 46% yield after flash chromatography,[23] and then detritylated in the presence of several equivalents of benzenesulfonic acid. Dinucleoside phosphate **4** was isolated as a homogeneous syrup following chromatographic purification (73% yield); its UV and ^{1}H-NMR spectra were both consistent with the assigned structure. Compound **4** was then phosphorylated with phosphorous oxychloride (2:1 THF–collidine) and the crude product was treated with thiophenol (1:1 triethylamine–dioxane)[24] to effect demethylation of the phosphate ester. Silylated



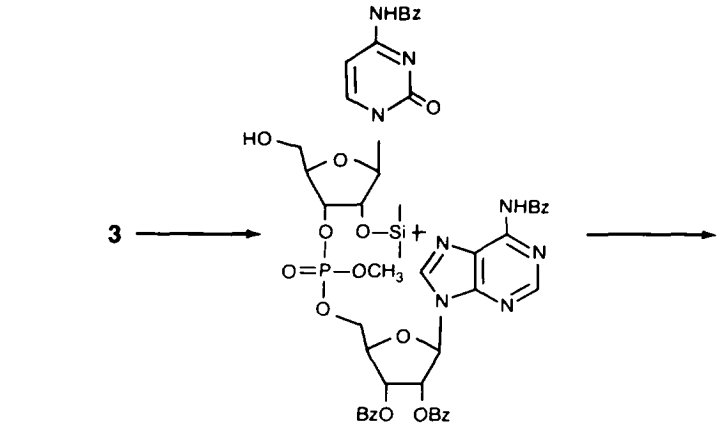
dinucleotide **5** was isolated as a white powder (overall yield 33% from **4**) following purification by preparative silica gel TLC, and was characterized by UV and ^{1}H-NMR. Desilylation was carried out via the agency of tetrabutylammonium fluoride in THF; the resulting cytidylyl(3′→5′)adenosine 5′-monophosphate (**6**; pCpA) was purified by chromatography on DEAE-cellulose. The product was isolated as a white powder in 83% yield and was characterized by UV and ^{1}H-NMR and by quantitative RNase T_2-mediated hydrolysis to a 1:1 mixture of pCp and adenosine.

2′(3′)-O-*Acyl derivatives of pCpA.* α-Aminoacyl derivatives of pCpA were prepared according to a general scheme (Method A) illustrated here for the preparation of 2′(3′)-O-(N-acetyl-L-phenylalanyl)-pCpA (**9**, R = L-$CH_3CONHCH(CH_2C_6H_5)$ CO-). Alternate procedures for the preparation of similar types of compounds have been described previously.[25,26] Treatment of dinucleotide **6** with ten equivalents each of N-carbobenzoxy-L-phenylalanine and 1, 1′-carbonyldiimidazole at 25°C for three days effected (partial) conversion to N, O-diacylated dinucleotide **7**, which was subsequently N-deacylated with mineral acid (pH 2.0, 25°, 24 h) to provide 2′(3′)-O-(N-carbobenzoxy-L-phenylalanyl)-pCpA (**8**). Purification of **8** by preparative silica gel TLC, development with 5:2:3 *n*-butanol–acetic acid–water, provided the product as a white solid. Conversion of **8** to the requisite N-acetylated aminoacyl-pCpA (**9**) was carried out by hydrogenolysis of the N-carbobenzoxy protecting group over 10% palladium-on-carbon in 0.01 N acetic acid, followed by N-acetylation with N-acetoxysuccinimide. Preparative cellulose TLC provided a pure sample of 2′(3′)-O-(N-acetyl-L-phenylalanyl)-pCpA as a white solid (70% overall yield from **7**).

The product was characterized by facile conversion to pCpA and N-acetyl-L-phenylalanine in basic solution and by quantitative ninhydrin assay of the aminoacylated dinucleotide resulting from hydrogenolysis of **8**. The position of aminoacylation was established by periodate treatment of putative 2′(3′)-O-(N-acetyl-L-phenylalanyl)-pCpA. As shown in Fig. 1 for 2′(3′)-O-(N-acetyl-D,L-β-phenylalanyl)-pCpA, when authentic pCpA was treated with periodate for 10 min, it would no longer elute from a column of aminoethylcellulose (*cf* panels A and B). Analogous treatment of the aminoacylated pCpA derivative (**9**) had no effect on its chromatographic properties on the same support (panel C), reflecting the absence of a *cis*-diol moiety.

It may be noted that the N-carbobenzoxy-L-phenylalanine derivative of pCpA (**8**) was employed as an intermediate to preclude the racemization that has been observed upon activation of N-acylated amino acids with 1, 1′-carbonyldiimidazole.[26,27] In those cases involving acylation of pCpA with racemic amino acids (e.g. N-acetyl-D, L-phenylglycine), the dinucleotide was treated directly with the N-acetylated amino acid in the presence of 1, 1′-carbonyldiimidazole.

It was found that the selective N-deacylation of diacylated dinucleotides **7** was dependent on the presence of the amino group on C^{α}. Therefore, for the preparation of 2′(3′)-O-acyl-pCpA derivatives lacking this functional group, an alternate procedure was employed. According to the alternate scheme



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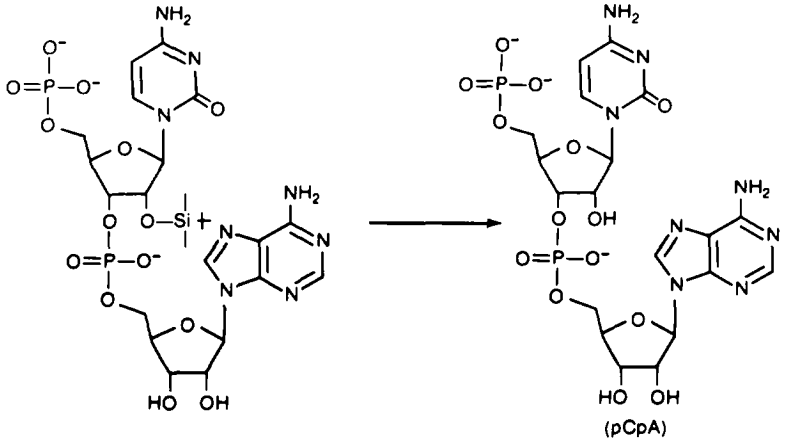

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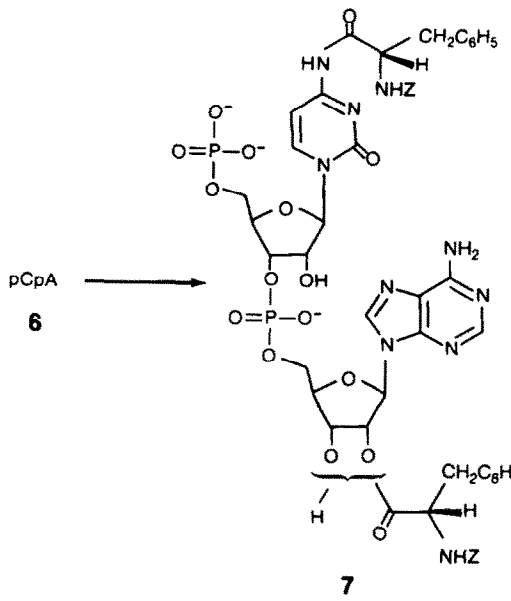
(Method B), pCpA was first converted to N^4-(N-acetyl-D, L-phenylalanyl) cytidylyl (3′→5′) adenosine 5′-phosphate (**10**) by treatment with four equivalents each of N-acetyl-D, L-phenylalanine and 1, 1′-carbonyldiimidazole for 20 hr at 25°. Dinucleotide **10** was purified by preparative cellulose TLC and characterized by UV and ^{1}H-NMR. Introduction of the desired 2′(3′)-O-acyl group was then effected by treatment of **10** with ten equivalents each of 1, 1′-carbonyldiimidazole and the requisite carboxylic acid over a period of three days in analogy with the conversion **6**→**7**. The diacylated pCpA derivatives were selectively N-deacylated in mineral acid and then purified chromatographically to provide 2′(3′)-O-acylated pCpA-derivatives as white solids (see Experimental Section for the preparation of 2′(3′)-O-(3-phenylpropionyl)-pCpA).

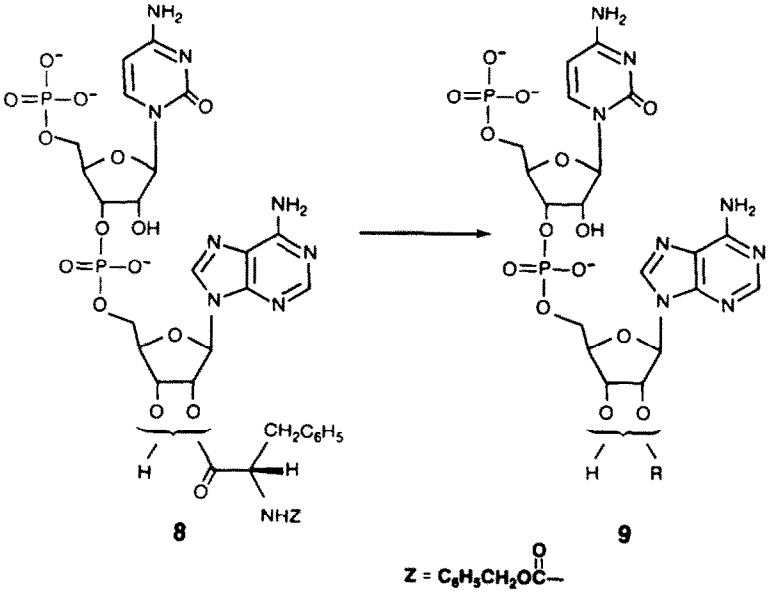
The use of the (acylated)aminoacyl moiety as a protecting group for N^4 of cytidine obviated the need for a specifically protected pCpA derivative. As shown in Table 1, the approach outlined above has been employed successfully for the synthesis of a number of 2′(3′)-O-(amino) acyl derivatives of pCpA.

"Chemical aminoacylation" of tRNA's. As illustrated in Fig. 2, admixture of 2′(3′)-O-(3-phenylpropionyl)-pCpA to an incubation mixture containing *E. coli* $tRNA^{Phe}$-C_{OH} (**12**) and T4 RNA ligase resulted in addition of the dinucleotide to the acceptor oligomer. Purification of the resulting 3-phenylpropionyl-$tRNA^{Phe}$ (**13**, R = $C_6H_5CH_2$-CH_2CO-) was carried out as shown in the Fig. by initial chromatography on DEAE-cellulose to effect separation of unreacted dinucleotide from the tRNA species (panel A), and then by rechromatography of the tRNA-containing fractions on BD-cellulose which permitted 3-phenylpropionyl-$tRNA^{Phe}$ (**13**) to be separated from unreacted $tRNA^{Phe}$-C_{OH} (**12**) (panel B).

Each of the 2′(3′)-O-acylated pCpA derivatives shown in Table 1 was employed as a substrate for T4 RNA ligase in the presence of $tRNA^{Phe}$-C_{OH} and was found to provide the respective 2′(3′)-O-acylated $tRNA^{Phe}$; the yields of isolated, purified tRNA's were 20–65% based on $tRNA^{Phe}$-C_{OH} (**12**).[13,16]

Characterization of the modified tRNA's included the demonstration that ligation of 2′(3′)-O-(N-acetyl-L-phenylalanyl)-pCpA to $tRNA^{Phe}$-C_{OH} provided a sample of N-acetylphenylalanyl-$tRNA^{Phe}$ having the same chromatographic behavior as an authentic sample, and which behaved identically to $tRNA^{Phe}$ fol-


pCpA
6
7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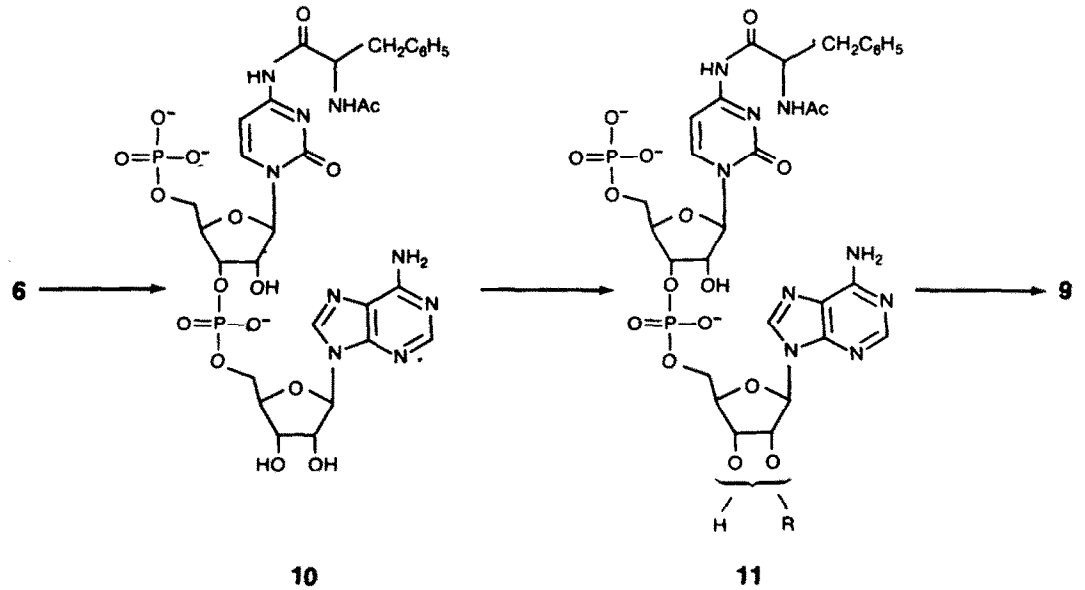
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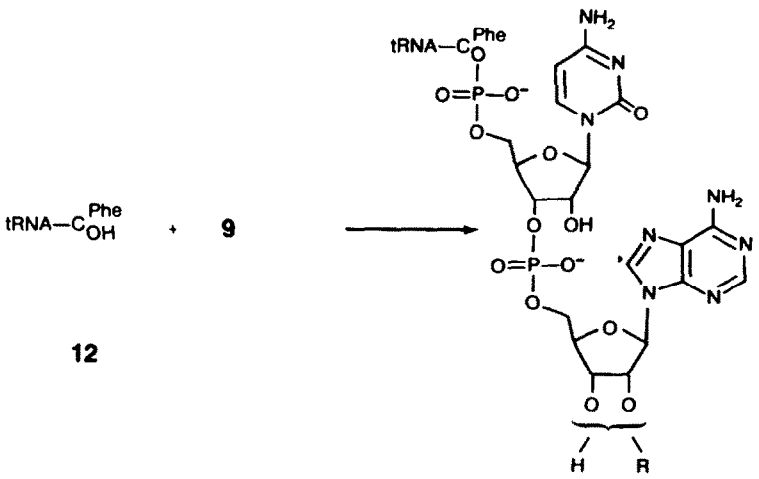

Table 1. 2′(3′)-O-Acyl Derivatives of pCpA (9)

R	Method	Rf value[a]
N-acetyl-L-phenylalanine	A	0.47
N-acetyl-D-phenylalanine	A	0.47
N-acetyl-L-tyrosine	A	0.47
N-acetyl-D-tyrosine	A	0.47
N-acetyl-D,L-phenylglycine	A	0.45
N-acetyl-D,L-β-phenylalanine	B	0.40
3-phenylpropionate	B	0.46
trans-cinnamate	B	0.44
H (6)	-	0.11

[a]Cellulose TLC, development with 5:2:3 n-butanol-acetic acid-water.

Table 2. Activation of *E. coli* $tRNA^{Phe}$'s with *E. coli* phenylalanyl-tRNA synthetase

$tRNA^{Phe}$ Substrate	Phenylalanine Acceptance (pmol/A_{260} Unit)	
	20 min	30 min
Unmodified E. coli $tRNA^{Phe}$	1110	1200
Abbreviated E. coli $tRNA^{Phe}$-C_{OH}	35	40
Alkali-treated E. coli N-acetyl-D-L-phenylalanyl-$tRNA^{Phe}$	900	940
---	0	0

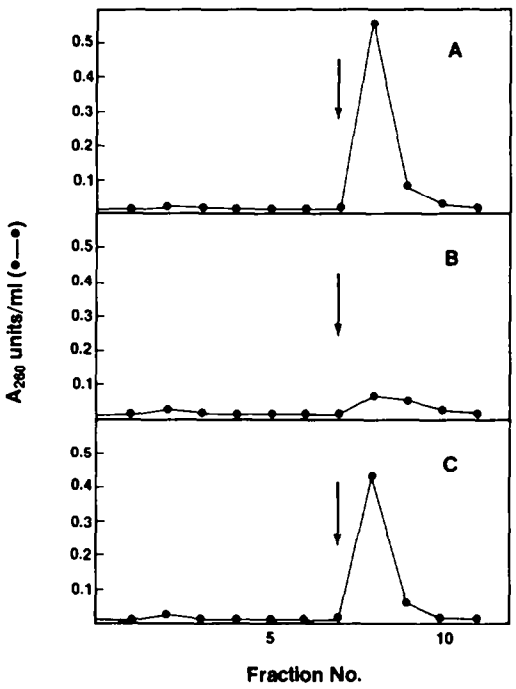

Fig. 1. Chromatography of periodate-treated pCpA derivatives on aminoethylcellulose. Samples of untreated pCpA (0.47 A_{260} unit; panel A), periodate-treated pCpA (0.47 A_{260} unit; panel B) and periodate-treated 2′(3′)-O-(N-acetyl-D, L-β-phenylalanyl)-pCpA (0.42 A_{260} unit; panel C) were applied to 1-ml columns of aminoethyl-cellulose that had been equilibrated with 50 mM NaOAc buffer, pH 6.0. Each column was washed at 25° (seven 0.7-ml fractions) with the same buffer and then with 50 mM NaOAc, pH 6.0, containing 0.25 M NaCl (four 0.7-ml fractions). Periodate oxidation was carried out at 25° in 5 mM $NaIO_4$ (35 μl) for 10 min; the reactions were quenched by addition of 20 μl of 50 mM ethylene glycol (15 min, 25°).

lowing base-catalyzed hydrolysis of the aminoacyl moiety. As expected, the alkali-treated sample of N-acetyl-L-phenylalanyl $tRNA^{Phe}$ (**13**; prepared by chemical aminoacylation) was a substrate for activation by phenylalanyl-tRNA synthetase, whereas neither the chemically aminoacylated species from which it was derived (**13**) nor $tRNA^{Phe}$–C_{OH} (**12**) could be so activated (Table 2).

Chemically aminoacylated N-acetyl-L-phenylalanyl-$tRNA^{Phe}$ was tested as a P-site tRNA in the peptidyltransferase reaction in an *E. coli* protein biosynthesizing system that employed poly (U) as a message. Upon admixture of the A-site tRNA (L-phenylalanyl-$tRNA^{Phe}$), both chemically aminoacylated and authentic N-acetyl-L-phenylalanyl-$tRNA^{Phe}$ were found to produce dipeptide to the same extent.[13] Moreover, each of the modified aminoacyl-tRNA's derived from the dinucleotides shown in Table 1 was also found to act as a donor tRNA in the peptidyl-transferase reaction, although N-acetyl-D-phenylalanyl-$tRNA^{Phe}$ and N-acetyl-D-tyrosyl-$tRNA^{Phe}$ were relatively poor donors.[13,16]

EXPERIMENTAL

Escherichia coli $tRNA^{Phe}$ (1250 pmol/A_{260} unit was converted to $tRNA^{Phe}$–C_{OH} as described.[13] T4 RNA ligase was purchased from P–L Biochemicals; one unit was defined as the amount of enzyme that catalyzed the formation of 1 nmol of phosphatase-resistant ^{32}P from 5′[^{32}P] oligo(rA)$_n$ in 30 min at 37°. *E coli* phenylalanyl-tRNA synthetase was isolated as described.[5]

N⁴-Benzoyl-2′-O-t-butyldimethylsilyl-5′-O-monomethoxy trityl-cytidine (**2**). A suspension of **1**[28] (12.2 g; 19.7 mmol), imidazole (4.0 g; 59 mmol) and t-butyldimethylsilyl chloride (3.6 g; 23.9 mmol) was stirred at room temp for 6 hr, after which the mixture was poured into ice water. The resulting syrup was dissolved in $CHCl_3$, and the $CHCl_3$ layer was washed with water, dried (Na_2SO_4) and concentrated. Purification on silica gel was effected by flash

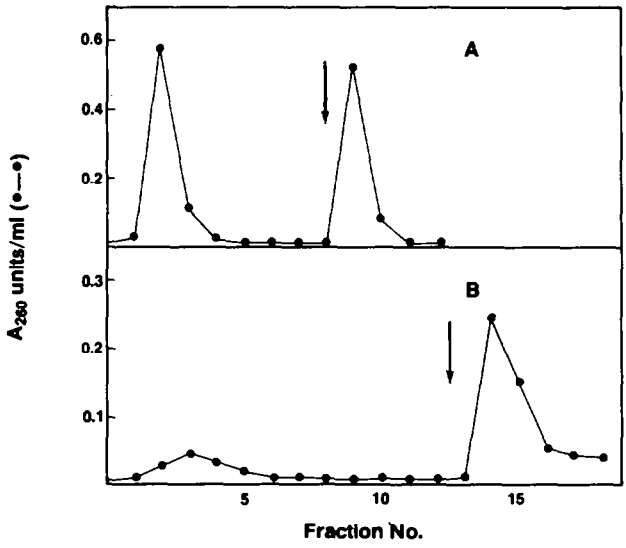

Fig. 2. Purification of 3-phenylpropionyl-$tRNA^{Phe}$. The crude reaction mixture was applied to a DEAE-cellulose column (panel A) and washed with a NaOAc buffer containing 0.25 M NaCl to effect elution of excess 2′(3′)-O-(3-phenylpropionyl)-pCpA. The tRNA species present were removed from the column by washing with the same buffer containing 1 M NaCl(↓), and were applied to a BD-cellulose column equilibrated with the same buffer (panel B). Following elution of unreacted $tRNA^{Phe}$–C_{OH}, addition of 25% ethanol to the buffer (↓) effected elution of 3-phenylpropionyl-$tRNA^{Phe}$. The details are given in the Experimental.

chromatography[23] (150 g; 1:3 n-hexane–ether), affording N^4-benzoyl-3′-O-t-butyldimethylsilyl-5′-O-monomethoxytritylcytidine (4.1 g, 28%) as well as the desired **2** (6.0 g, 42%); the latter was obtained as colorless needles from n-hexane–ether, m.p. 112–117°; λEtOH max 306 (sh), 261 and 233 nm, λ min 290, 248 and 225; *Rf* 0.48 (silica gel TLC, development with 4:1 ether–hexane); partial ^{1}H-NMR (DMSO-d_6, $(CH_3)_4Si$)δ 0.91 (s, 9), 3.71 (s, 3), 5.06 (d, 1), 5.77 (s, 1), 6.90 (d, 1) and 8.33 (d, 1).

Methyl (N^4-*benzoyl-2′-O-t-butyldimethylsilyl-5′-O-monomethoxytrityl*)*cytidylyl* (3′→5′) (N^6, $O^{2'}$,$O^{3'}$-tribenzoyl) adenosine (**3**). A soln of collidine (1.0 ml), THF (5.0 ml) and methylphosphorodichloridite[21,22] (190 μl; 2.0 mmol) was cooled to −78° and treated dropwise with a soln of cytidine derivative **2** (1.10 g; 1.5 mmol) in 7.0 ml of THF over a period of 30 min. The combined soln was stirred at −78° for 15 min, and then treated dropwise at −78° with a 7-ml THF soln containing 696 mg (1.2 mmol) of N^6, $O^{2'}$, $O^{3'}$-tribenzoyladenosine[29] over a period of 30 min. The combined soln was stirred at −78° for 15 min. and then at 25° for an additional 15 min. The mixture was then treated with I_2 (377 mg; 1.5 mmol) in 3 ml of 1:1:1 pyridine–water–THF and stirring was continued for an additional 5 min. The mixture was concentrated to dryness under diminished pressure and the residue was dissolved in $CHCl_3$. The $CHCl_3$ soln was washed with aq $NaHSO_3$ and water, then dried (Na_2SO_4) and concentrated to afford a syrup. Flash chromatography (60 g silica gel; elution with 1% MeOH in $CHCl_3$) provided dinucleoside phosphate **3** (760 mg; 46%) as a yellow powder; λ $EtOH_{max}$ 318 (sh), 261 and 230 nm, λ min 249; $R\hat{f}$ 0.58 (silica gel TLC, development with 10:9:1 EtOAc–$CHCl_3$–EtOH).

Methyl (N^4-*benzoyl-2′-O-t-butyldimethylsilyl*)*cytidylyl*-(3′→5′) (N^6,$O^{2'}$,$O^{3'}$-*tribenzoyl*)*adenosine* (**4**). Dinucleoside phosphate **3** (1.0 g; 0.7 mmol) was dissolved in a soln of 70 ml $CHCl_3$ and 30 ml EtOH containing 1.0 g (6.3 mmol) benzenesulfonic acid. The soln was maintained at 5° for 8 hr, then treated with 3 ml pyridine and concentrated to dryness. The residue was dissolved in $CHCl_3$ and the $CHCl_3$ layer was washed with water, dried (Na_2SO_4) and concentrated. The resulting syrup was purified by preparative TLC (silica gel, development with 5% MeOH in $CHCl_3$), affording detritylated dinucleoside phosphate **4** as a chromatographically homogenous syrup, yield 0.58 g (73%); $\lambda$$EtOH_{max}$ 318(sh), 261 and 230 nm, λmin 248 and 217; partial ^{1}H-NMR (DMSO-d_6, $(CH_3)_4Si$)δ 0.78 (s, 9), 5.91 (d, 1), 6.80 (d, 1), 8.42 (d, 1), 8.76 (s, 1) and 8.80 (s, 1).

2′-O-t-Butyldimethylsilylcytidylyl (3′→5′) *adenosine 5′-monophosphate* (**5**). A soln of $POCl_3$ (144 μl; 1.5 mmol) in 1.5 ml collidine was maintained at −78° and treated dropwise with **4** (336 mg; 0.3 mmol) in 3.0 ml THF over a period of 20 min. The mixture was stirred at −78° for 1 hr, and then at 0° for 30 min. The mixture was treated with 0.5 ml water, stirred at 0° for an additional 30 min and then concentrated to dryness. The residue was dissolved in $CHCl_3$, washed with water, dried ($NaSO_4$) and concentrated. The resulting syrup was treated with 0.5 ml thiophenol, 1.0 ml Et_3N and 1.0 ml dioxane and the soln was maintained at 25° for 1 hr. The mixture was concentrated to dryness and the residue was suspended in 50 ml conc NH_4OH and stirred at 25° for 20 hr. The mixture was concentrated and the residue was purified by preparative TLC (silica gel, development with 1:1:1 *i*-PrOH–NH_4OH–H_2O). Elution of material from the main band (7:3 isopropanol–H_2O) provided **5** as a white powder, yield 2200 A_{260} units (33%); λ_{max} (pH 7) 261 nm, λ_{min} 224; partial ^{1}H-NMR (D_2O)δ 0.6 (s, 9), 6.02 (m, 2), 5.80 (d, 1, J = 6 Hz), 7.8 (d, 1, J = 6 Hz), 8.17 (s, 1) and 8.4 (s, 1).

Cytidylyl (3′→5′) *adenosine 5′-monophosphate* (pCpA; **6**). A sample of **5** (3000 A_{260} units; 0.14 mmol) was dissolved in 20 ml of a THF soln containing 1 M tetrabutylammonium fluoride. The mixture was stirred at room temp for 7 hr, then diluted with 500 ml water and applied to a (30-ml; HCO_3^--form) DEAE–cellulose column. The column was washed with 500 ml water, and then with 100 ml 0.5 M NH_4^+ HCO_3^- to effect elution of the desired product. The appropriate fractions were desalted by repeated evaporation of portions of water, then dissolved in water and lyophilized to provide cytidylyl (3′→5′) adenosine 5′-phosphate as a white powder, yield 2500 A_{260} units (83%); λ max (pH 7) 261 nm, λ min 232; λ max (pH 2) 265, λ min 235; partial ^{1}H-NMR (D_2O) δ 5.72 (d, 1) 5.97 (d, 1), 6.03 (d, 1), 7.87 (d, 1), 8.17 (s, 1) and 8.40 (s, 1); Rf 0.29 (cellulose TLC, development with 55:10:35 n-PrOH–NH_4OH–H_2O).

Digestion of a sample of pCpA (**6**) with RNase T_2 (5 units/10 μl) at 37° for 24 hr converted the nucleotide quantitatively to a 1.0:1.0 mixture of pCp and adenosine.

General procedures for the synthesis of 2′(3′)-O-acyl derivatives of pCpA (**9**). The syntheses of 2′, (3′)-O-(N-acetyl-L-phenylalanyl) pCpA(**9**, R = L-$CH_3CONHCH(CH_2C_6H_5)CO$-) and 2′(3′)-O-(3-phenylpropionyl)-pCpA **9**, R = $C_6H_5CH_2CH_2CO$-) are illustrated using general methods A and B, respectively.

Method A. A soln containing 30.0 mg (0.1 mmol) N-carbobenzoxy-L-phenylalanine and 16.2 mg (0.1 mmol) 1, 1′-carbonyldiimidazole in 300 μl dry DMSO was stirred at room temp for 20 min, and then treated with 215 A_{260} units (10 μmol) of pCpA (**6**). Stirring was continued at 25° for 70 hr and the mixture was then added dropwise to 10 ml ether. The ppt was isolated by centrifugation, dissolved in 0.5 ml water, and applied to a Dowex 50W column (0.8 × 2.0 cm, H^+-form). The column was washed with water (5 ml) and the eluate was concentrated to a small volume and adjusted to pH 2.0 with 0.1 N HCl at 4°. The acidified soln was maintained at 25° for 24 hr, then neutralized with Dowex 50W (Na^+-form) and concentrated to dryness. Chromatography on a preparative TLC plate (cellulose; development with 5:2:3 n-BuOH–AcOH–water) effected purification of **8**; the major band ($R\hat{f}$ 0.53) was extracted from the plate with 0.05 N AcOH. The desired **8** was isolated as a white solid following lyophilization, yield 8.7 A_{260} units.

A soln of 4 A_{260} units of **8** in 250 μl EtOH and 250 μl 0.01 N AcOH was treated with a small amount of 10% Pd–C and the mixture was stirred at 25° under H_2 for 20 hr. The mixture was filtered and the filtrate was concentrated to dryness, after which the residue was redissolved in a soln of DMSO (200 μl) containing 0.73 mg (5 μmol) N-acetoxysuccinimide.[30,31] The mixture was stirred at 25° for 16 hr and then treated with 5 ml dry acetone. The resulting ppt was collected by centrifugation and applied to a preparative cellulose TLC plate. Development with 5:2:3 n-BuOH–AcOH–water afforded one major UV-absorbing band, the material from which was eluted from the plate with 0.01 N AcOH. Following neutralization of the AcOH soln (Dowex 50W; Na^+-form), 2′(3′)-O-(N-L-phenylalanyl)-pCpA (**9**, R = L-$CH_3CONHCH(CH_3C_6H_5)CO$-) was isolated as a white solid by lyophilization, yield 2.8 A_{260} units (70%).

Method B. A soln containing 16.6 mg (80 μmol) N-acetyl-D,L-phenylalanine and 13.0 mg (80 μmol) 1, 1′-carbonyldiimidazole in 300 μl dry DMSO was stirred at room temp for 20 min and then treated with 420 A_{260} units (20 μmol) of **6**. Stirring was continued at 25° for 20 hr and the mixture was then added dropwise to 2 ml dry acetone. The resulting ppt was isolated by centrifugation, dissolved in 0.5 ml water and applied to a preparative cellulose TLC plate. Development with 4:4:2 acetone–EtOH–water gave a chromatogram with one major UV-absorbing band; isolation of this material was effected by extraction with water (2 × 30 ml). The aqueous soln was lyophilized, affording **10**, yield 180 A_{260} units (43%); λ_{max} (pH 7) 302 and 252 nm, λ_{min} 284 and 228; A_{302}/A_{252} = 0.32; partial ^{1}H-NMR (D_2O)δ 1.86 (s, 3), 5.74 (d, 1), 6.10 (d, 1) and 7.20 (m, 5); R_f 0.52 (cellulose TLC, development with 5:2:3 n-BuOH–AcOH–water).

A soln containing 6.0 mg (40 μmol) 3-phenylpropionic acid and 6.5 mg (40 μmol) 1, 1′-carbonyldiimidazole in 150 μl dry DMSO was stirred at room temp for 20 min and then treated with 90 A_{260} units (~4 μmol) of **10**. The

mixture was stirred at 25° for 70 hr and treated with 2 ml dry acetone. The resulting ppt was isolated by centrifugation, dissolved in 0.5 ml water, and applied to a Dowex 50W column (0.5 × 0.7 cm, H^+-form). The column was washed with water and the eluate was concentrated to a small volume and acidified with 2 ml 0.01 N HCl. The soln was maintained at 25° for 24 hr and then neutralized on a Dowex 50W column (0.5 × 0.7 cm, Na^+-form). The column eluate was concentrated to a small volume and applied to a preparative cellulose TLC plate. Development with 5:2:3 n-BuOH-AcOH-water provided a chromatogram having a single major band (R_f 0.46); the material in this band was extracted with 0.01 N AcOH. Lyophilization afforded 2′(3′)-O-(3-phenylpropionyl)-pCpA (**9**, R = $C_6H_5CH_2CH_2$-CO-) as a white solid, yield 2.0 A_{260} units.

"Chemical aminoacylation" of tRNA's. The chemical aminoacylation of tRNA's is illustrated for the preparation of *E. coli* 3-phenylpropionyl-$tRNA^{Phe}$. A mixture containing 0.6 A_{260} unit (1.0 nmol) *E. coli* $tRNA^{Phe}$–C_{OH} (**12**)[13], 0.32 A_{260} unit (15 nmol) 2′(3′)-O-(3-phenylpropionyl)-pCpA (**9**, R = $C_6H_5CH_2CH_2CO$-) and 24 units T4 RNA ligase was incubated (70 μl total volume) in 55 mM Na^+-Hepes buffer, pH 7.5, containing 15 mM $MgCl_2$, 250 μM ATP, 20 μg/ml of bovine serum albumin and 10% DmSO. The incubation mixture was maintained at 4° for 16 hr and then applied to a (1.0-ml) DEAE-cellulose column that had been equilibrated at 4° in 50 mM NaOAc, pH 4.5, containing 10 mM $MgCl_2$. The column was washed at 4° with 8 ml 50 mM NaOAc, pH 4.5, containing 10 mM $MgCl_2$ and 0.25 M NaCl, and then with 4 ml of the same buffer containing 1.0 M NaCl. One-ml fractions were collected, and the fractions (9 and 10) containing the tRNA's were pooled and applied to a (1.0-ml) benzoylated diethylaminoethyl (BD)-cellulose column that had been preequilibrated at 4° with 50 mM NaOAc, pH 4.5, containing 10 mM $MgCl_2$ and 1 M NaCl. The column was washed with 12 ml of this buffer, which effected elution of unreacted $tRNA^{Phe}$–COH; 3-phenylpropionyl-$tRNA^{Phe}$ (**13**; R = $C_6H_5CH_2CH_2CO$-) was then recovered by elution with four ml of the same buffer to which 25% EtOH had been added. One-ml fractions were collected (Fig. 2) and the appropriate fractions (14 and 15) were combined, dialyzed against 5 mM NaOAc, pH 4.5, containing 1 mM $MgCl_2$ and concentrated under diminished pressure to afford 0.24 A_{260} units (40%) of 3-phenylpropionyl-$tRNA^{Phe}$.

Enzymatic aminoacylation of tRNA's. Reaction mixtures (75 μl total volume) contained 90 mM Na^+-Pipes buffer, pH 7.0, 100 mM KCl, 15 mM $MgCl_2$, 0.5 mM EDTA, 2.5 mM ATP, 10 μM [^{3}H] phenylalanine (2500 cpm/pmol) and 0.1 A_{260} unit of the tRNA of interest. The reactions were initiated by the addition of 3 μl of *E. coli* phenylalanyl-tRNA synthetase[5] and maintained at 25°; 20-μl aliquots were removed at predetermined times and quenched by addition to glass fiber disks that had been presoaked with 0.05 M cetyltrimethylammonium bromide in 1% AcOH. The dried disks were washed thoroughly with 1% acetic acid, dried and used for determination of radioactivity. The results of the experiments are shown in Table 2.

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