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Facile Synthesis of *N*-Acyl-aminoacyl-pCpA for Preparation of Mischarged Fully Ribo tRNA

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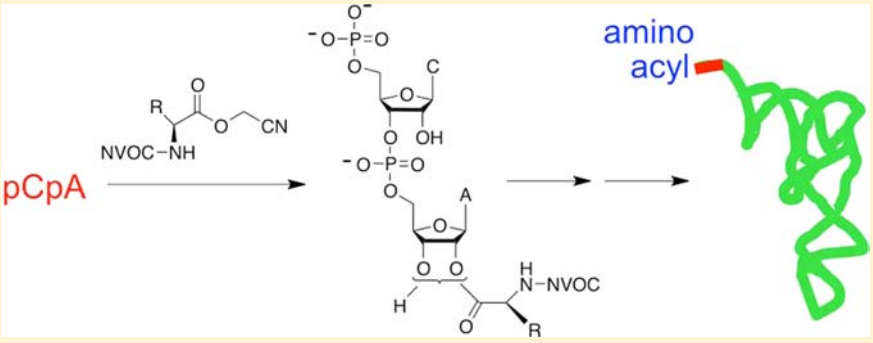
Supporting Information



ABSTRACT: Chemical synthesis of *N*-acyl-aminoacyl-pdCpA and its ligation to tRNA$^{\text{minus CA}}$ is widely used for the preparation of unnatural aminoacyl-tRNA substrates for ribosomal translation. However, the presence of the unnatural deoxyribose can decrease incorporation yield in translation and there is no straightforward method for chemical synthesis of the natural ribo version. Here, we show that pCpA is surprisingly stable to treatment with strong organic bases provided that anhydrous conditions are used. This allowed development of a facile method for chemical aminoacylation of pCpA. Preparative synthesis of pCpA was also simplified by using *t*-butyl-dithiomethyl protecting group methodology, and a more reliable pCpA postpurification treatment method was developed. Such aminoacyl-pCpA analogues ligated to tRNA$^{\text{minus CA}}$ transcripts are highly active in a purified translation system, demonstrating utility of our synthetic method.

INTRODUCTION

Unnatural amino acids charged on tRNAs have found wide application as substrates for studying the mechanism of protein synthesis,[1] investigating protein function by mutagenesis and introduction of biophysical probes,[2] and synthesizing peptidomimetics for drug discovery.[3−5] In some applications, it is advantageous to alter the 3′-terminal ribose.[6−9] However, the most flexible method in terms of the amino acid portion of unnatural aminoacyl-tRNAs and potential applications is a semisynthetic methodology based on chemical aminoacylation of dinucleotide pdCpA,[10] and its subsequent enzymatic ligation to a truncated tRNA fragment, tRNA$^{\text{minus CA}}$.[11]

The aminoacyl-pdCpA approach has been used successfully for over two decades with few improvements in the synthetic methodologies,[12] but it does have important limitations. Using a purified translation system, we found that substituting cytidine for deoxycytidine at the penultimate position in tRNA has a strong inhibitory effect when used for multiple adjacent incorporations,[13] a major problem when genetically encoding peptidomimetic synthesis for drug discovery.[14] The low yields may be related to observations that the 23S rRNA forms a Watson−Crick base pair with the first C of the tRNA CCA end in the ribosomal P site and with the second C in the A site.[15,16] One potential remedy is direct chemical acylation of tRNA,[17] but this lanthanide-catalyzed condensation was reported to occur with minimal yield. A more widely used approach is charging full-length tRNAs using activated amino acids and a "flexizyme" ribozyme,[18] but charging with some amino acids is also inefficient. This stimulated us to reinvestigate problematic syntheses of aminoacyl-pCpA that were used before introduction of the improved aminoacyl-pdCpA method.

In the original aminoacyl-pCpA method[19,20] N-protected amino acid was activated upon treatment with carbonyldiimidazole and the formed mixed anhydride was used for esterification of pCpA. This resulted in formation of bis-aminoacylated derivative in moderate yield that then had to be purified, partially deacylated, and purified again to give the desired mixture of 2′ and 3′ monoacylated products. The Schultz laboratory thus modified this Hecht laboratory method by transient protection of the exocyclic amino group on the cytosine.[21] However, this multistep method was still cumbersome and inefficient, so the Schultz laboratory moved to pdCpA—a semideoxy analogue of pCpA to simplify dinucleotide synthesis.[22] Use of pdCpA also enabled a weaker type of aminoacylation using an N-protected-amino acid

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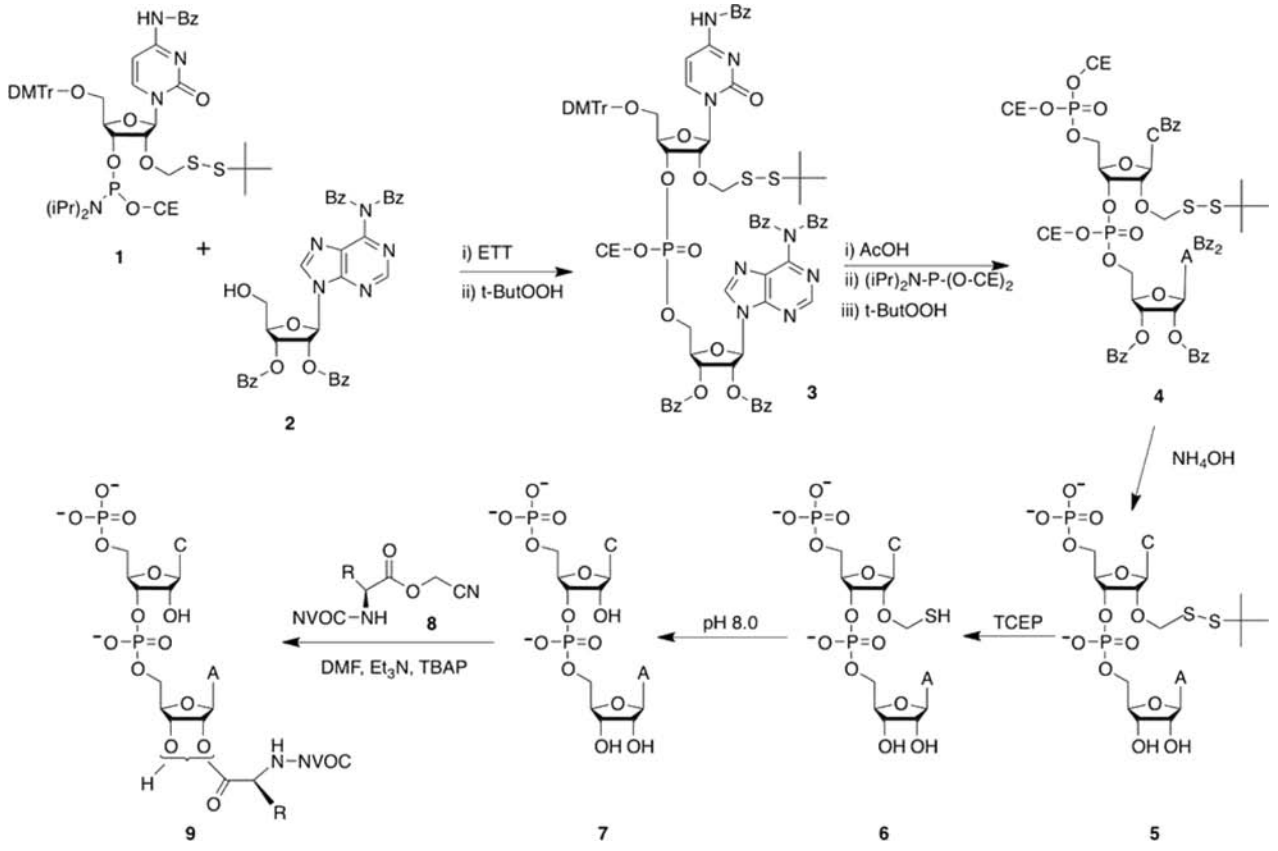
Scheme 1. Facile Synthesis of NVOC-Aminoacyl-pCpA



cyanomethyl ester under basic conditions. This weakly activated amino acid reacted with hydroxyls selectively without acylating nucleotide exocyclic amino groups, thus obviating transient protection of the cytosine.[10] We considered adapting this unprotected pdCpA aminoacylation method to unprotected pCpA, but an important concern was that the aminoacylation required basic conditions (triethylamine or tetrabutylammonium hydroxide). This risks hydrolysis of pCpA and may have disuaded researchers from ever attempting it. For example, potential degradation of pCpA during aminoacylation with amino acid cyanomethyl esters was prevented by protecting the internal 2′-OH,[23] but this method is cumbersome. However, recent work on the chemical synthesis of RNA showed that phosphodiester bond cleavage can be avoided with anhydrous conditions,[24] so we tested if the cyanomethyl ester approach might indeed be compatible with pCpA.

RESULTS AND DISCUSSION

pCpA is Stable to Base under Anhydrous Conditions. Phosphodiester linkages in RNA are notoriously susceptible to base-catalyzed hydrolysis via the adjacent 2′-OH group. So there was a concern whether or not standard base-catalyzed aminoacylation conditions used for pdCpA could be applied to the ribonucleotide dimer pCpA without protecting the cytidine 2′-hydroxyl. We tested if pCpA was stable under anhydrous conditions when dissolved in DMF:triethylamine (4:1, v/v) at 55 °C for 60 h. HPLC analysis of the reaction mixture revealed only the starting pCpA without any traces of hydrolysis products pCp, CMP, AMP, or adenosine (Supporting Information Figure S1), demonstrating surprising stability of pCpA.

Relative Nucleophilicities of the Three Hydroxyls of pCpA Obviate Internal 2′-OH Protection. Use of dinucleotide containing an extra hydroxyl group could raise a question regarding selectivity of chemical aminoacylation. This was one of the reasons why the internal 2′-OH of pCpA was protected.[23] However, misacylated side products containing 2′-O-aminoacylated cytidine were not reported in the previous attempts for pCpA acylation using carbonyldiimidazole, despite large excess of the used amino acid over dinucleotide.[19] Reactivities of different hydroxyl groups in RNAs are governed by their nucleophilicities, which are a function of their pK_a values and steric hindrance. Measurements of pK_a values in aqueous media showed that 2′,3′-terminal hydroxyls are significantly more acidic (pK_a 12.3) and thus more reactive than internal 2′-hydroxyls (pK_a 13.0−13.9).[25] It is reasonable to expect that a similar pK ratio will apply under anhydrous conditions. Increased steric hindrance at internal compared with terminal 2′-hydroxyls should also favor reaction at the terminus. We thus did not expect formation of substantial amounts of cytidine 2′-O-acylated products, at least not in quantities justifying protection of this position prior to aminoacylation, and this is indeed confirmed below. We considered this as important because acid-labile special protection of the cytidine 2′-OH group[23] limits applicability of pCpA aminoacylation to laboratories skillful in nucleic acid chemistry.

New Method for Preparative Synthesis of pCpA (7, Scheme 1). Applications of pCpA in aminoacyl-tRNA production require pCpA synthesis on the large scale. We

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therefore adapted our general DTM method, previously used for solid phase RNA syntheses[26] to solution phase synthesis. This method employs the *t*-butyl-dithiomethyl (DTM) protecting group (**1** in Scheme 1), a neutral, redox-labile, protecting group for the 2′-hydroxyl of nucleotide amidites. It has a clear advantage over all common 2′-O-silyl, fluoride-labile protecting groups because the postsynthetic treatment does not require a separate anion-exchange-based purification and desalting step.[27] Instead, a fluoride-free reaction mixture of small molecular RNA fragments can be directly and cleanly purified after the final step using a traditional silica-based reversed phase (RP) HPLC without any risk of column destruction. The phosphoramidite building block was activated by 5-ethylthio-1*H*-tetrazole (ETT), but other activators (like common tetrazole) may be used with comparable efficiency. *t*-Butyl hydroperoxide has the advantage over iodine as the oxidizing reagent because its excess can be easily evaporated, eliminating the need for an additional reducing agent that potentially interferes with dithio bonds. The following steps used published procedures for chemical phosphorylation[28] and deprotection of ribonucleotides prepared according to DTM methodology.[26]

Improved Post-Purification Treatment of pCpA for Subsequent Aminoacylation. The purified pCpA was desalted by RP HPLC, but contrary to published procedures,[28] we chose not to convert preparatively this phosphorylated dimer to the tetrabutylammonium (TBA) salt by passing it through a cation exchange column. According to the literature,[28] this extra step is necessary to guarantee proper solubility of the dinucleotide in DMF. However, we found that addition of a fixed amount of TBA phosphate to the acylation mixture (see Experimental Section) has the same effect on pCpA solubility. Additionally, we found the original procedure of cation exchange[28] prolonged preparation unduly as it demanded calculation and adjustment of the existing amount of TBA^+ ion by means of NMR. Evaporated aliquots of the dinucleotide were then dissolved in DMF and dried azeotropically by coevaporation with dry acetonitrile. We applied azeotropic drying instead of the standard drying under vacuum[28] because the drying step is often blamed for failed aminoacylations, drying under high vacuum is difficult to control and time-consuming, and azeotropic drying is known to be one of most efficient procedures for removal of small water quantities.

Efficient 2′/3′ Aminoacylation of Unprotected pCpA. Our new procedure with pCpA applied the aminoacyl cyanomethyl ester acylation methodology previously used for pdCpA, except that we were unable to obtain any acylated product without addition of triethylamine.[28] Aminoacylation of pCpA in the presence of 1% TEA, for both natural and unnatural amino acids, was selective and sufficiently efficient. (Exceeding a 3:1 ratio of activated amino acid over dinucleotide is not recommended as it increases formation of bis-acylated products.) Product purification was straightforward because the doublet peak of terminal 2′ and 3′ monoacylation products was easily separable from reaction components by HPLC. A typical example is shown in Figure 1.

Under these aminoacylation conditions, monoacylation was selective for the terminal 2′- and 3′-hydroxyl groups. Further characterization of the isolated doublet-peak product ruled out partial acylation on the internal 2′-OH or exocylic amino groups of bases as follows. Treatment of this product with nuclease P1 at pH 6 (which hydrolyzes phosphodiester linkages to give 3′-hydroxyls) converted it quantitatively to a product co-migrating with 5′-CMP reference and a new compound that appeared as a doublet with UV spectrum characteristic for NVOC-containing material (Supporting Information Figure S2). This isolated new compound, upon adjustment of pH to 9.5 (which hydrolyzes aminoacyl ester linkages, not amide linkages), decomposed completely to 5′-AMP and free N-NVOC amino acid (Supporting Information Figure S3).

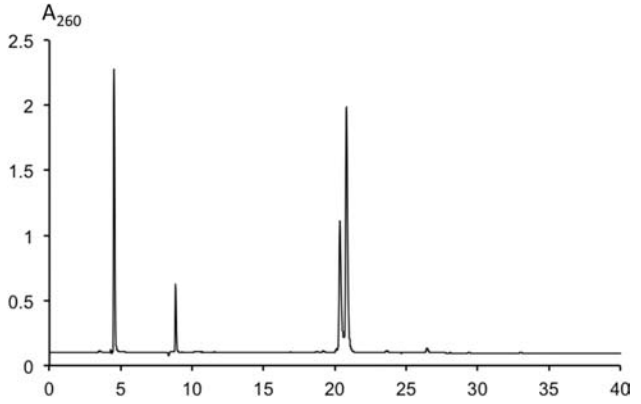

Figure 1. HPLC analysis of reaction between pCpA and N-NVOC-allylglycine cyanomethyl ester. The doublet at 20−21 min corresponds to the N-NVOC-allylglycine-pCpA monoacylation products at the terminal 2′ and 3′ positions (mass verified). The peak at 9 min corresponds to unreacted pCpA, while the peak at 4 min is the injection front. The doublet was due to interchange between vicinal 2′-O- and 3′-O-acylated isomers, as confirmed by isolating the material from each of the two peaks and finding that each fraction reruns on HPLC as a doublet peak.

Working with protected amino acids under basic conditions, especially in polar aprotic solvents like DMF, raises a question about their possible racemization. We used Marfey's method for determination of the optical purity of amino acids[29] as it is generalizable and accurate. TEA concentration, temperature, and time were optimized (1% TEA, 55 °C, 2 h) to maximize yield while minimizing the extent of alanine racemization (only 2.1% of D-Ala, Supporting Information Figure S4).

High Biochemical Activity of N-NVOC-aminoacyl-pCpA. In order to assess the biochemical compatibility of our product, N-NVOC-alanine-pCpA was ligated to unmodified transcript $tRNA^{AlaB\ minus\ CA}$ using T4 RNA ligase (ligation estimated at >90% by acid-urea polyacrylamide gel electrophoresis). The resulting conjugate was photodeprotected to give Ala-$tRNA^{AlaB}$ and then compared with native Ala-$tRNA^{Ala\_Bulk}$ (prepared by charging bulk tRNA with Ala using alanyl-tRNA synthetase) for in vitro ribosomal dipeptide formation from initiator fMet-$tRNA^{fMet}$. The dipeptide formation rates of Ala-$tRNA^{AlaB}$ ($32.3 \pm 3.7\ s^{-1}$) and native Ala-$tRNA^{Ala\_Bulk}$ ($32.6 \pm 3.9\ s^{-1}$) were indistinguishable (Figure 2), validating our facile method for *N*-acyl-aminoacyl-pCpA synthesis.

EXPERIMENTAL SECTION

Materials. *N*-Nitroveratrylooxycarbonyl (N-NVOC) protected cyanomethyl esters of AAs were prepared according to standard methodology.[28] The derivative of L-allylglycine (AllGly) was prepared as published.[30] [^{3}H]Met was from PerkinElmer. Phosphenolpyruvate (PEP), pyruvate kinase (PK), myokinase (MK), putrescine, spermidine, DTE, and nonradioactive amino acids were from Sigma-Aldrich. Other

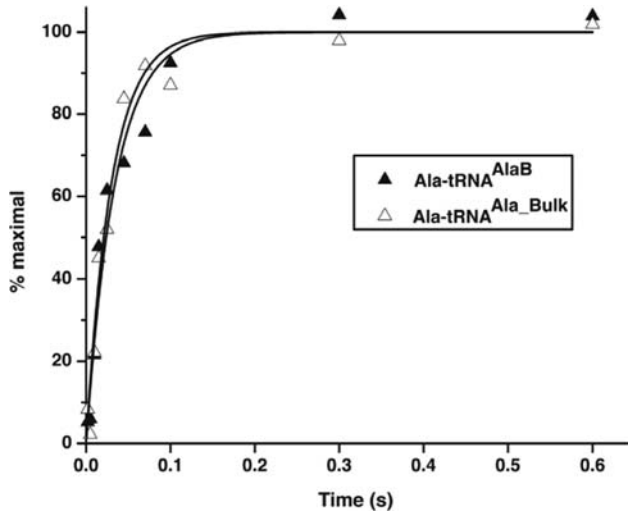

Figure 2. Kinetics of ribosomal dipeptide synthesis with Ala-tRNA^{Ala} substrates. [^{3}H]fMet-Ala dipeptide formation from [^{3}H]fMet-tRNA^{fMet} and (▲) Ala-tRNA^{AlaB} or (Δ) Ala-tRNA^{Ala_Bulk} with the GCA Ala codon in the A site. Representative plots are shown for duplicate experiments.

chemicals were analytical grade from Merck. *E. coli* MRE600 70S ribosomes, [^{3}H]fMet-tRNA^{fMet}, tRNA^{Bulk}, synthetic XR7 mRNA mMAF (codons AUG GCA UUU), and all translation factors were prepared as described.[31] Tetrabutyl ammonium phosphate (TBAP) (0.1 M) in dry DMF was prepared by titration of aqueous solution of *ortho*-phosphoric acid with tetrabutyl ammonium hydroxide to pH 8.5 and evaporation of this solution to dryness. The residue was dissolved in DMF and dried by repeated coevaporation with dry acetonitrile (5 × 50 mL). The final solution of TBAP was diluted with dry DMF to 0.1 M and stored at room temperature.

5′-Phospho-cytidyl(3′,5′)adenosine (pCpA) 7. N^6,N^6,2′-*O*,3′-*O*-Tetrabenzoyladenosine **2** (225 mg, 0.33 mmol, 1.0 equiv), prepared from adenosine,[28] was placed in a 50 mL round-bottomed flask and was dried by coevaporation with toluene (2 × 20 mL). An acetonitrile solution of amidite **1** (0.1 M, 4.0 mL, 0.4 mmol, 1.2 equiv), prepared from cytidine,[26] was added, followed by addition of an acetonitrile solution of 5-ethylthio-1*H*-tetrazole (ETT) (0.1 M, 12 mL, 3 equiv), and the mixture was stirred at RT for 15 min. A toluene solution of *t*-butyl hydroperoxide (t-ButOOH) (2 M, 5 mL) was added and the mixture was stirred for 15 min to give compound **3**. The reaction mixture was partitioned between chloroform and saturated aqueous sodium bicarbonate solution. The combined chloroform extracts were evaporated to dryness and coevaporated with toluene (2 × 50 mL). The residue was detritylated by treatment with 80% aqueous acetic acid (200 mL) for 40 min at RT and all volatile matter was evaporated. The residue was dried by coevaporation with toluene and quickly passed through a short silica-gel column using chloroform:ethanol (98:2 v/v) as eluent to obtain the TLC-pure 5′-OH dimer.

The purified dimer was evaporated, dried by coevaporation with toluene (2 × 20 mL), and subjected to reaction with di(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphoramidite (0.20 mL, 0.66 mmol) in the presence of ETT solution (0.1 M, 20 mL, 3 equiv) at RT for 15 min. The phosphitylated compound was oxidized with t-ButOOH and the reaction mixture was worked up as described above. At this stage, the TLC analysis showed that the phosphorylation reaction was practically quantitative. The final phosphorylated, fully protected product **4** was isolated after short silica-gel column chromatography using chloroform:ethanol (98:3 v/v) as eluent in the form of a white foam after evaporation of appropriate fractions. Yield: 297 mg (72%).

The benzoyl and cyanoethyl groups of compound **4** (150 mg, 0.12 mmol) were removed by treatment with excess conc. aqueous ammonia at RT overnight, and all volatile matter was evaporated. A sample of this compound was purified by HPLC and used for studies of DTM group cleavage. On the basis of these studies, the residual material was dissolved in phosphate buffer (0.4 M, pH 8.0) containing tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) (70 mg, 0.24 mmol, 2 equiv). The chromatographic analysis showed that the conversion of compound **5** to compound **6** was very fast, but the release of the remaining 2′-*O*-thiomethyl group took an additional 2 h. The final pCpA **7** was purified by preparative HPLC using LiChrosphere 100 RP 18 separation column and a gradient of acetonitrile (0−20%) in triethylammonium acetate (0.1 M, pH 7.0). The isolated material was desalted through the same preparative HPLC system using a gradient of acetonitrile (0−20%) containing 0.1% acetic acid. The amount of isolated material was measured photometrically and pCpA was divided into 5 mg portions, placed in 2 mL screw-capped Sarstedt tubes, and evaporated to dryness in SpeedVac evaporator. Colorless solid: MALDI−TOF m/z calculated from $C_{19}H_{26}N_8O_{14}P_2$ 652.10, found $(M+1)^+$ 653.16.

Acylation of pCpA with Cyanomethyl Esters of N-NVOC Protected Amino Acids 8 to Form Amino-acylated-pCpA 9. To a 2 mL screw-capped tube (Sarstedt) containing pCpA (5 mg, 7.6 μmol), anhydrous DMF (0.2 mL) and TBAP in DMF (0.1 M, 40 μL) were added and the content was stirred magnetically for 5 min. Dry acetonitrile (1 mL) was added and the mixture was dried by evaporation in a SpeedVac evaporator. The drying procedure was repeated twice and a fresh portion of dry DMF (200 μL) and dry triethylamine (2 μL) was added to the solid residue. To this solution N-NVOC-allylglycine cyanomethyl ester (9 mg, 23 μmol, 3 equiv) was added and the mixture was incubated at 55 °C for 2 h with occasional brief shaking. The tube was placed in a SpeedVac and all volatile matter was evaporated. The residue was suspended in water (0.75 mL) containing acetic acid (20 μL) and extracted by addition of ethyl acetate (3 × 0.75 mL). The agitated phases were quickly separated by centrifugation and the upper phases containing unreacted starting amino acid were removed by micropipette. The aqueous phase was diluted with water up to 2 mL and HPLC analyzed on a RP 18 column, using gradient of acetonitrile (0−80%) in ammonium acetate (0.05 M, pH 4.5) for 40 min. The monoacylated product, doublet peak appeared just before the N-NVOC amino acid single peak. The characteristic ratio of A_{265} to A_{350} for the aminoacylated pCpA (4:1) allowed for an easy discrimination between the product and N-NVOC amino acid, for which this ratio is reversed (1:3). The preparative separation was performed using the same buffer gradient but with LiChrosphere 100 RP 18 as separation column. The isolated fraction was evaporated and desalted using preparative HPLC system, and gradient of acetonitrile (0−20%) acidified with 0.1% acetic acid. HPLC-based analysis estimated 82% conversion of the starting pCpA to the **N-NVOC-AllGly-pCpA** form. UV quantitation revealed the final isolated yield of N-NVOC-AllGly-pCpA to be 81%. Light yellow solid: MALDI-TOF m/z calculated from $C_{34}H_{42}N_{10}O_{21}P_2$ 988.20, found $(M+1)^+$ 989.32,

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$(M+Na)^+$ 1011.32. Portions (0.2 μmol) of material were evaporated to dryness in separate tubes and were stored in this form at −20 °C.

N-NVOC-Phe-pCpA, yield 72% based on HPLC. Light yellow solid: MALDI-TOF *m/z* calculated from $C_{38}H_{44}N_{10}O_{21}P_2$ 1038.22, found $(M+1)^+$ 1039.23, $(M+Na)^+$ 1061.19.

N-NVOC-Ala-pCpA, yield 78% based on HPLC. Light yellow solid: MALDI-TOF *m/z* calculated from $C_{32}H_{40}N_{10}O_{21}P_2$ 962.18, found $(M+1)^+$ 963.19.

Analysis of Enantiomeric Purity of Amino Acids after Subjection to Condensation Conditions. N-NVOC-L-Ala-CM (10 mg, 27 μmol) was dissolved in a mixture containing DMF (160 μL), TBAP in DMF (0.1 M, 40 μL), and triethylamine (2 μL; 1% final). This solution was incubated at 55 °C for 2 h resembling the actual conditions used for aminoacylation of pCpA. After evaporation of volatile triethylamine, the residue was diluted with acetonitrile (20 mL) and water (80 mL) and stirred overnight at RT to hydrolyze all cyanomethyl esters. The clear solution was slightly acidified by addition of acetic acid (0.2 mL) and NVOC groups were photodeprotected by an intensive irradiation (ACE-Glass, Immersion lamp, Catalog No. 7883−14 or equivalent) of the water-cooled and stirred reaction mixture for 2 h. The deprotected mixture was concentrated and all components were partitioned between water and ethyl acetate to remove UV-absorbing products of NVOC-deprotection. HPLC analysis of the evaporated organic phase revealed the absence of unreacted N-NVOC amino acid, proving efficiency of photodeprotection. The aqueous phase was concentrated to 5 mL and a sample of 250 μL was withdrawn for amino acid analysis. It was mixed with acetonitrile (100 μL), sodium bicarbonate solution (1 M, 20 μL), and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA; Marfey's Reagent; 1% solution in acetone, 100 μL) and incubated at 40 °C for 1 h. The mixtute was neutralized by addition of hydrochloric acid (2 M, 10 μL) and analyzed on a RP 18 HPLC system using a 40 min linear gradient 50% B to 100% B, with buffer A, triethylammonium phosphate (0.05 M, pH 3), and buffer B, triethylammonium phosphate (0.05 M, pH 3)/acetonitrile (50%). The identities of all components were confirmed by separate analyses of mixtures obtained using pure optical isomeric forms of all amino acids.

Synthesis of Aminoacyl-tRNA. The 3′-CA truncated $tRNA^{Ala}$ transcript ($tRNA^{AlaB\ minus\ CA}$) was prepared as described.[32] The ligation yield of N-NVOC protected Ala-pCpA to $tRNA^{AlaB\ minus\ CA}$ with T4 RNA ligase (Thermo Scientific) was estimated by a 6.5% urea acid PAGE (pH 5.1) and the product, *N*-NVOC-Ala-$tRNA^{AlaB}$, was then purified on a Q-column as previously described.[33] The NVOC group was removed by 25 min photolysis as described in the previous paragraph except that the sample was cooled in ice water without stirring (under identical conditions, 90% of the starting material was converted to product based on HPLC[30]).

In Vitro Translation Experiments. A purified *E. coli* in vitro translation system and a quench flow apparatus (RQF-3; KinTek Corp.) were used for dipeptide formation experiments as described.[34] As 70S initiation complex was added to large molar excess over ternary complex concentration, the dipeptide formation rate was limited by ribosome concentration. Hence, the measured rate was independent of the exact tRNA concentration. The active concentration of Ala-$tRNA^{AlaB}$ and the percentage of $tRNA^{Ala}$ in bulk *E. coli* tRNA ($tRNA^{Ala\_Bulk}$) were determined by dipeptide formation experiments with other components in excess.

To set up the dipeptide formation experiment, two mixes were preincubated at 37 °C for 15 min. The ribosomal mix contained 2.4 μM MRE600 70S ribosome, 3 μM mMAF, 2 μM $[^3H]$fMet-$tRNA^{fMet}$, 3.6 μM IF1, 1.2 μM IF2, and 3.6 μM IF3 in polymix buffer supplemented with energy regeneration components. A ternary complex mix in the same buffer contained 30 μM EF-Tu, 2 μM EF-Ts, and either 0.2 μM Ala-$tRNA^{AlaB}$ or 0.2 μM $tRNA^{Ala\_Bulk}$ (total RNA $OD_{260\ nm}$ concentration was 7.48 μM), 0.4 mM L-alanine, and 0.4 μM AlaRS. Equal volumes of these two mixtures were loaded onto the quench flow apparatus for reaction followed by quick quenching with 50% formic acid (final 17%) at various time points. Sample treatment and data analysis were done as described.[34]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HPLC analyses of reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): ACF owns patent US6977150 licensed to Ra Pharmaceuticals, Inc., owns shares in the company, and is a member of its scientific advisory board.

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