

A New Strategy for the Synthesis of Bisaminoacylated tRNAs

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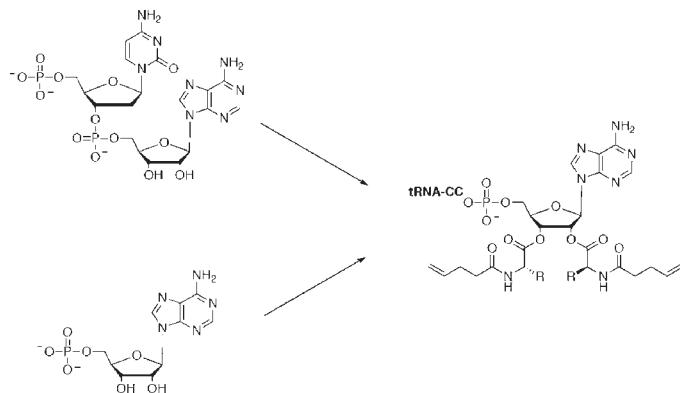
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ABSTRACT



Tandemly activated tRNAs participate effectively in protein synthesis and exhibit superior chemical and biochemical stability compared to the more commonly used singly aminoacylated tRNAs. While several bisaminoacylated tRNAs have been prepared via the T4 RNA ligase-mediated condensation of bisaminoacylated pdCpAs and abbreviated tRNA transcripts (tRNA- C_{OH}), the bisaminoacylated pdCpAs are difficult to prepare when using bulky amino acids. Described herein is a new strategy for preparing bisaminoacylated tRNAs, applicable even for bulky amino acids.

Chemically misacylated tRNAs have facilitated the introduction of non-natural amino acids into proteins at predetermined sites.^{1–6} This has enabled the detailed investigation of the biochemical and biophysical properties of numerous proteins.^{7–10} While monoaminoacylated

tRNAs are ordinarily used in protein synthesizing systems, Lavrik and co-workers¹¹ have described a phenylalanyl-tRNA synthetase from *Thermus thermophilus* that can incorporate more than one molecule of phenylalanine onto tRNA^{Ph}, producing bis- (2',3'-O-phenylalanyl)-tRNAs. Our laboratory has shown that such bisaminoacylated tRNAs can be prepared *in vitro* and employed for cell free protein synthesis; these species have the advantages of improved chemical and biochemical stabilities, as well as their ability to participate in two cycles of peptide elongation.¹² Under protein synthesizing conditions limiting

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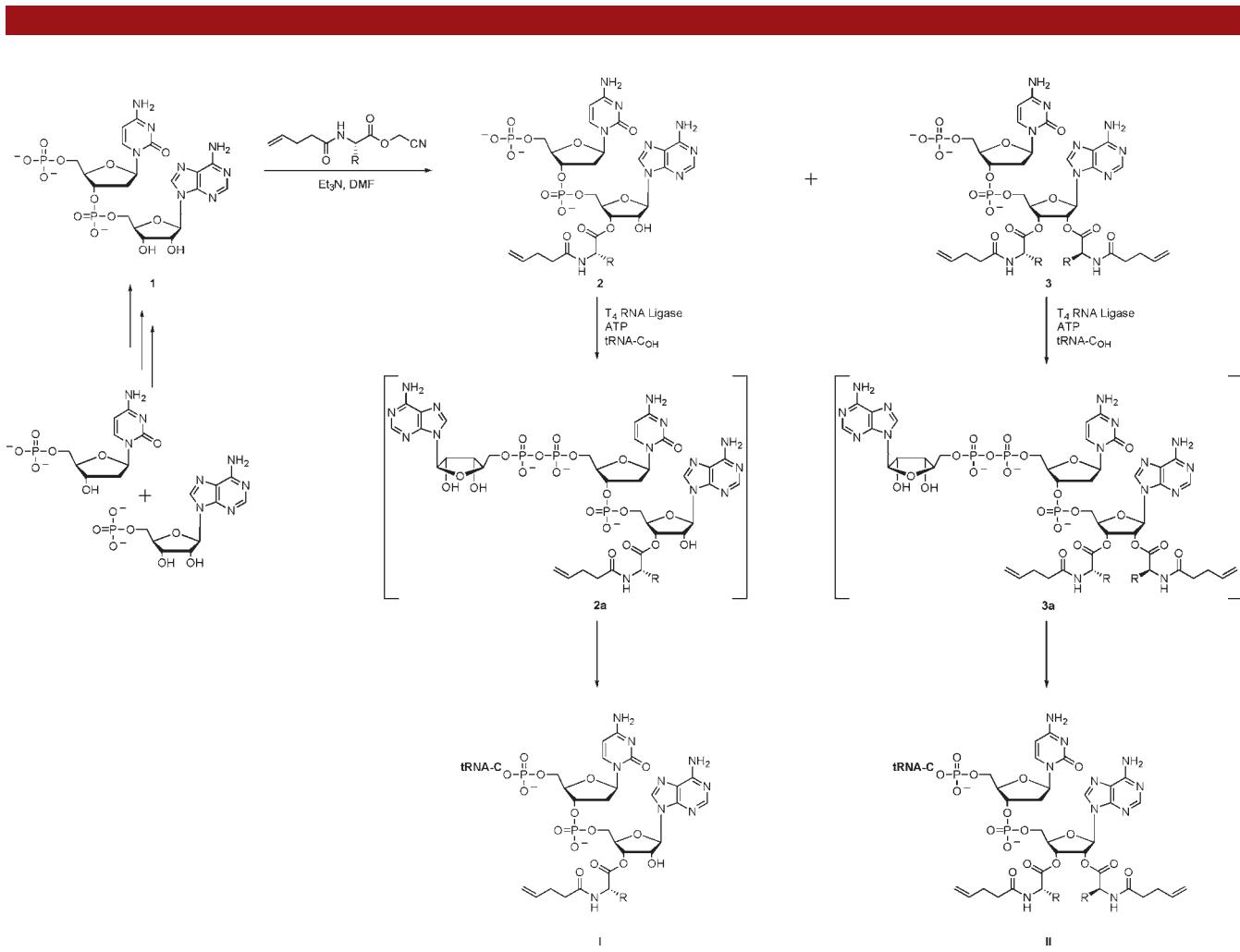


Figure 1. Preparation of mono and bisaminoacylated tRNA_{CUAs} by chemical aminoacetylation via pdCpA.

for activated tRNA, twice as much full-length protein is produced when employing bisaminoacylated tRNAs.^{12a}

The synthesis of misacylated tRNAs is outlined in Figure 1. The *N*-protected amino acid is activated as the cyanomethyl ester.¹³ The amine must be protected such that deprotection can be achieved under conditions compatible with maintenance of the labile aminoacyl moiety that results. The nitroveratryloxycarbonyl (NVOC) group can be removed by simple UV irradiation,^{13,14} while the 4-pentenoyl group can be utilized as an alternative protecting group as it is removable with aqueous iodine.¹⁵

Acylation of pdCpA (**1**) with the activated amino acid affords the monoaminoacylated pdCpA (**2**); when the activation employs a pentenoyl-protected amino acid that is not bulky, some of the bisaminoacylated pdCpA (**3**) is also obtained.¹² The acylated pdCpA intermediates **2** and **3** are subsequently used in the T4 RNA ligase-catalyzed

condensation reaction with an abbreviated suppressor tRNA (tRNA-COH) to afford the desired misacylated tRNAs (Figure 1). However, the use of bulky amino acids generally results in poor yields of **3** (Table 1). Thus, the pdCpA derivatives containing two phenylalanine or biphenylalanine moieties were accessible in modest yield, but only the monoaminoacylated pdCpAs could be obtained when dimethoxyphenylalanine or either of two regioisomers of naphthylalanine were employed. While bisaminoacylated tRNAs are potentially of great utility for the elaboration of modified proteins, the difficulty in obtaining bisaminoacylated pdCpAs (**3**) containing bulky amino acids has limited their use to date.

T4 RNA ligase is an ATP-dependent enzyme that catalyzes the formation of phosphodiester bonds from RNA donor and acceptor substrates.¹⁶ The ligation is initiated by adenylation of the 5'-phosphate moiety of the RNA donor to form a phosphoroanhydride intermediate (A5'-pp5'N...). Finally the adenylated donor is condensed with

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Table 1. Mono and Bisaminoacylation of pdCpA and AMP

	monoacyl	pdCpA	monoacyl	AMP	bisacyl
	monoacyl	bisacyl	monoacyl	monoacyl	bisacyl
	60%	30%	30%	67%	
	70%	0%	34%	54%	
	65%	0%	28%	57%	
	84%	0%	25%	67%	
	50%	40%	21%	77%	

the 3' terminal OH group of the RNA acceptor (e.g., tRNA- C_{OH}) to form a new 3'→5' phosphodiester linkage.¹⁷ Hecht and co-workers first described a “chemical aminoacylation” procedure in which a chemically synthesized aminoacylated AppA, identical with the putative intermediate in the T4 RNA ligase reaction, was utilized as a donor substrate for the enzyme, thus producing a misacylated tRNA (**5** → **5a** → **I**; Figure 2).¹⁸

It seemed possible that the bisaminoacylation of 5'-AMP with bulky amino acids might proceed more readily than of pdCpA and that the resulting bisaminoacylated AMP could still be converted to the respective AppA derivative with reasonable efficiency. As documented in Table 1, treatment of 5'-AMP with each of five bulky amino acids (*N*-pentenoyl protected derivatives, activated as the cyanomethyl esters) afforded both mono and bisaminoacylated derivatives, with the latter isolated in yields of 54–77%. All of the 5'-AMP derivatives were purified by C₁₈ reversed phase HPLC using a gradient of 0 → 65% acetonitrile in 50 mM NH₄OAc, pH 4.5. Treatment of each monoaminoacylated AMP (**5**) and bisaminoacylated AMP (**6**) with imidazolium AMP then afforded the respective aminoacylated AppA derivative in yields ranging from 81 to 94%, after purification by HPLC (Table 2). Interestingly, the coupling efficiencies did not differ dramatically for the mono and bisaminoacylated AMPs. These AppA derivatives were then ligated to tRNA-CC_{OH} via the agency of T4 RNA ligase (Figure 2), affording the

same monoaminoacylated tRNAs (**I**) and bisaminoacylated tRNAs (**II**), accessible by ligation of tRNA- C_{OH} + aminoacylated pdCpAs. The suppressor tRNA_{CUA}-CC_{OH} was obtained by *in vitro* RNA transcription from a DNA template that had been linearized with restriction enzyme *FokI*. The suppressor tRNA was structurally related to yeast tRNA^{Phe 8}.

Table 2. Mono and Bisacylated AppAs Prepared from Aminoacylated Adenosine 5'-Monophosphates

R, R'	monoaminoacylated-AppA	bisaminoacylated-AppA
	87%	90%
	94%	86%
	93%	87%
	85%	81%
	79%	81%

R = N-protected amino acid, R' = -OH or N-protected amino acid

The ability of the mono- and bisaminoacylated tRNAs prepared via aminoacylated AppA intermediates to participate in protein synthesis was investigated *in vitro* in an *E. coli* S30 coupled transcription-translation system programmed with DHFR mRNA having a UAG codon at the position corresponding to Val10. Each of the five *N*-pentenoyl protected monoaminoacylated tRNAs (**I**) and the five *N*-pentenoyl protected bisaminoacylated tRNAs (**II**) prepared via aminoacylated AppA intermediates (Table 2) was deprotected by treatment with aqueous iodine as described previously¹⁵ before introduction into the protein synthesis reaction. The synthesis of full length DHFRs in the presence of the monoaminoacylated and bisaminoacylated tRNAs is illustrated in Figures 3 and 4, respectively. Both the mono- and bisaminoacylated-tRNAs were shown to suppress the UAG codon effectively, resulting in the production of DHFR with reasonable efficiencies. Some variations were observed while employing mono- vs bisaminoacylated-tRNAs for incorporating the same amino

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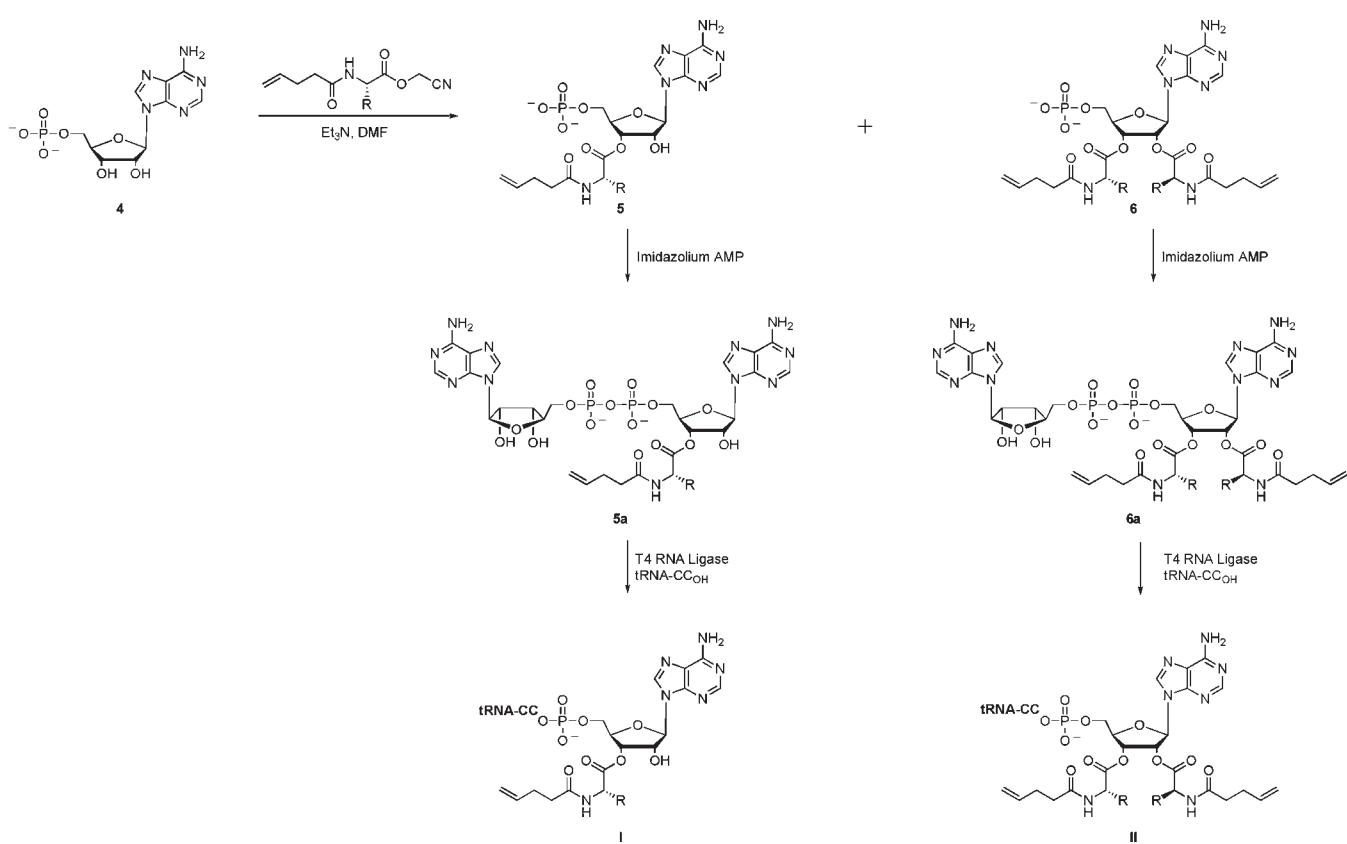


Figure 2. Preparation of mono and bisaminoacylated tRNA_{CUAs} by chemical aminoacetylation via AMP.

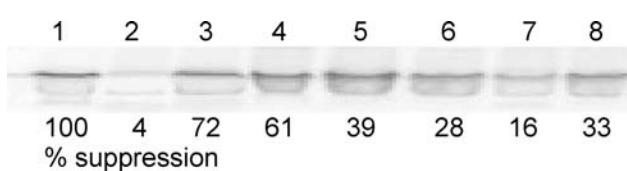


Figure 3. *In vitro* synthesis of DHFR utilizing monoacylated tRNA_{CUAs} to suppress a UAG codon at position 10 of DHFR mRNA. Lane 1, wild-type mRNA; Lane 2, no tRNA_{CUA}; lane 3, L-phenylalanyl-tRNA_{CUA} (prepared from pdCpA); lane 4, L-phenylalanyl-tRNA_{CUA}; lane 5, 3,4-dimethoxy-L-phenylalanyl-tRNA_{CUA}; lane 6, L-1-naphthylalanyl-tRNA_{CUA}; lane 7, L-2-naphthylalanyl-tRNA_{CUA}; lane 8, L-4,4'-biphenylalanyl-tRNA_{CUA}.

acid. In the case of L-1-naphthylalanine, L-2-naphthylalanine and L-biphenylalanine, the use of the bisaminoacyl-tRNAs resulted in approximately 2–3 times more DHFR than when the respective monoaminoacylated tRNAs were employed. In contrast, the yields of DHFRs were somewhat greater when monophenylalanyl-tRNA_{CUA} was employed, regardless of whether the phenylalanyl-tRNAs were prepared by the method outlined in Figure 1 or 2. This presumably reflects differences in the relative stabilities of the individual mono and bisaminoacylated tRNAs in the *in vitro* synthesizing system,¹² and plausibly also differences

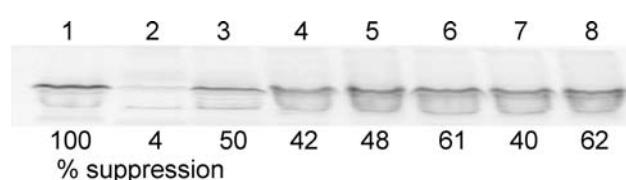


Figure 4. *In vitro* synthesis of DHFR utilizing bisaminoacylated tRNA_{CUAs} to suppress a UAG codon at position 10 of DHFR mRNA. Lane 1, wild-type mRNA; lane 2, no tRNA_{CUA}; lane 3, bis-L-phenylalanyl-tRNA_{CUA} (prepared from pdCpA); lane 4, bis-L-phenylalanyl-tRNA_{CUA}; lane 5, bis-L-3,4-dimethoxyphenylalanyl-tRNA_{CUA}; lane 6, bis-L-1-naphthylalanyl-tRNA_{CUA}; lane 7, bis-L-2-naphthylalanyl-tRNA_{CUA}; lane 8, bis-L-4,4'-biphenylalanyl-tRNA_{CUA}.

in the facility of utilization of individual bisaminoacylated tRNAs by the ribosome.

In summary, by the modification of a procedure for the misacylation of tRNAs, first described by Hecht et al.,¹⁸ it is now possible to prepare bisaminoacylated tRNAs in good yield even where bulky amino acids are involved.

Supporting Information Available. Experimental details for the preparation and characterization of all aminoacylated nucleotides. This material is available free of charge via the Internet at <http://pubs.acs.org>.