

Preparation of Translationally Competent tRNA by Direct Chemical Acylation

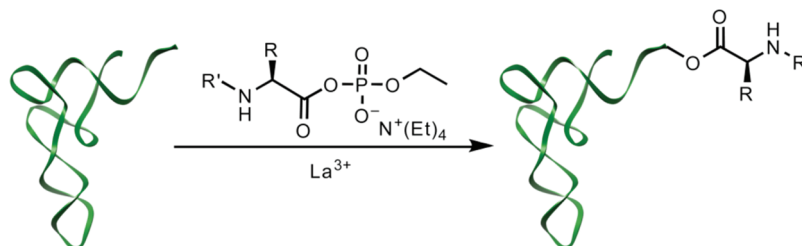
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ABSTRACT



Nonsense codon suppression for unnatural amino acid incorporation requires the preparation of a suppressor aminoacyl-tRNA. Chemical acylation strategies are general but inefficient and arduous. A recent report (*J. Am. Chem. Soc.* 2007, 129, 15848) showed acylation of RNA mediated by lanthanum(III) using amino acid phosphate esters. The successful implementation of this methodology to full-length suppressor tRNA is described, and it is shown that the derived aminoacyl-tRNA is translationally competent in *Xenopus* oocytes.

Unnatural amino acid mutagenesis provides the researcher with the ability to perform precise structure–function studies with proteins, beyond that which can be performed with the 20 natural amino acids.^{1–3} This is usually achieved through nonsense suppression, in which the site of interest is mutated to a stop codon, and an aminoacyl-tRNA bearing the appropriate anticodon delivers the unnatural amino acid during translation. The aminoacyl-tRNA is critical to this methodology, and several approaches to generating it have been developed.^{2,4,5} Nature generates aminoacyl-tRNAs via reactions of ATP-activated amino acids with the cognate tRNA, mediated by the corresponding aminoacyl-tRNA synthetase (aaRS).⁶ In some cases, the natural system can be hijacked to charge an unnatural amino acid onto a tRNA

if the difference between the unnatural and cognate amino acids is not large. Methods for generating orthogonal aaRS/tRNA pairs that accept unnatural amino acids have been shown to be a viable source of aminoacyl-tRNA. Alternatively, amino acids may be appended to tRNA using chemical synthesis. All strategies have particular advantages and disadvantages, and the chemical acylation approach is the focus of the present work.

The standard chemical aminoacylation strategy begins with chemically synthesized dinucleotide pCpA (dCA), which is then acylated with the desired amino acid.^{4,7} The derived aminoacyl-dCA is then enzymatically appended to tRNA via T4 ligase. This method provides maximal flexibility, as virtually any amino acid can be used. When combined with an appropriate expression system, the chemically synthesized aminoacyl-tRNA does not get recharged by an endogenous aaRS (an intentional aspect of the experimental design), and so the aminoacyl-tRNA is a stoichiometric reagent. Since

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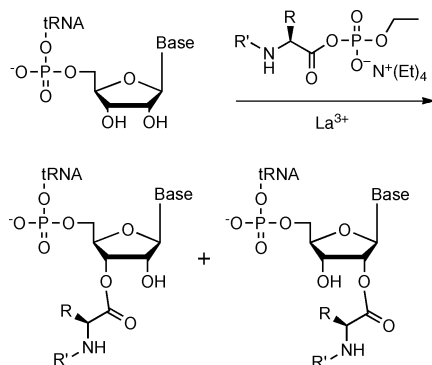
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the synthesis of the aminoacyl-tRNA is arduous, the quantity of protein that can be produced using the chemical aminoacylation strategy is limited. For the most part, the methodology has been exploited to study ion channels and neuroreceptors, where the extraordinary sensitivity of electrophysiology overcomes the quantity limitation.¹

Recently, Tzvetkova and Kluger showed selective acylation of the 3' end of RNAs through the use of La^{3+} salts and amino acids activated as ethyl phosphate esters (Scheme 1).⁸

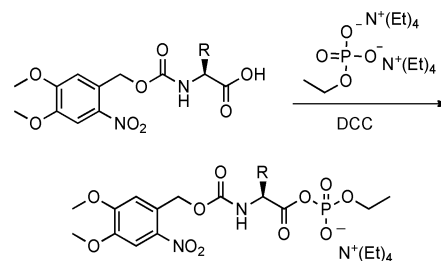
Scheme 1. La^{3+} -Mediated Acylation of the 3' End of tRNA



We will refer to this biomimetic strategy as the La^{3+} /EP approach. If direct aminoacylation of full-length tRNA can produce translationally competent tRNAs, the synthesis of dCA, one of the most laborious aspects of the chemical aminoacylation strategy, would become unnecessary. As such, we have evaluated the La^{3+} /EP strategy, focusing on two questions: (1) can we optimize the direct acylation of full-length suppressor tRNAs; and (2) are the resulting aminoacyl-tRNAs translationally competent in protein synthesis? In particular, our application of nonsense suppression to studies of neuroreceptors and ion channels requires expression in a vertebrate cell, the *Xenopus laevis* oocyte.⁴ This adds additional challenges to the methodology, compared to in vitro protein synthesis strategies.

To address these questions, we prepared a representative collection of amino acids as derivatives **1–4** (Scheme 2), in which the carboxylate is activated as an ethyl phosphate and the amino group is protected with NVOC. This protecting group has been previously established to be compatible with the *Xenopus* oocyte expression system—it is removed photochemically before use in translation.⁴ The amino acid ethyl phosphates (aaEP) **1–4** were prepared using established procedures,⁹ by coupling to bis(tetraethylammonium)ethyl phosphate using DCC (Scheme 2). As a consequence of our selected protecting groups, addition of THF as a cosolvent to CH_2Cl_2 was required to alleviate solubility issues. Protection of the phenolic OH on tyrosine was not necessary under these reaction conditions. Tetraethylammonium salts of aaEPs **1–4** were obtained by extraction into water, followed

Scheme 2. Synthesis of Amino Acid Ethyl Phosphates



R = *i*-Bu (**1**, Leu)
R = *p*-hydroxybenzyl (**2**, Tyr)

R = (**3**, Trp)

R = (**4**, F1-Trp)

by lyophilization. The resulting foamy materials were used directly without further purification.

In their report, Tzvetkova and Kluger detected acylation of RNA by careful selection of the amino acid—specifically, a fluorinated amino acid (^{19}F NMR detection) or a dansylated amino acid (fluorescence detection). Our aaEPs were not selected with these detection methods in mind, and therefore, we needed a more general method of detecting acylation. Previously, our laboratory developed a MALDI-TOF mass spectrometry method for detecting enzymatic ligation of a dCA-amino acid to a tRNA transcript lacking the 3'-terminal pCpA.¹⁰ We anticipated that the amino acids **1–4** would shift the MALDI signal sufficiently that the acylated tRNA could be detected even if conversion was not complete.

Applying the reported La^{3+} -mediated acylation conditions to uridine and cytidine with activated amino acids such as **1–4** gave 2'/3' esters in low overall conversion ($\sim 5\text{--}10\%$ as measured by LC/MS). Increasing the equivalents of the aaEPs and La^{3+} to a large excess (≤ 20 equiv) with respect to the nucleoside improved the conversion ($\sim 25\text{--}50\%$, data not shown). Using large excesses of aaEPs introduced solubility issues that could be alleviated by use of DMSO as a cosolvent. These modified conditions were then applied to a full-length tRNA (Figure 1). Very large excesses of aaEPs and La^{3+} with respect to the tRNA (>1000 equiv) were required to detect any acylation in the MALDI mass spectra. Key to the successful application of MALDI was treatment of the reaction mixture with the well-known lanthanide chelator diethylenetriamine pentaacetic acid (DTPA).¹¹ While the standard tRNA precipitation protocols removed most of

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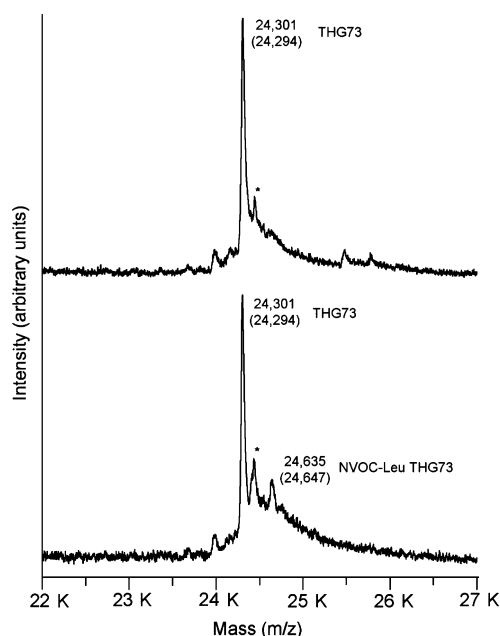


Figure 1. MALDI mass spectra of THG73 tRNA before (top) and after (below) exposure to La^{3+} -mediated acylation conditions using leucine derivative **1**. Observed masses shown, theoretical masses in parentheses. A new peak indicative of monoacylation appears, along with unacylated tRNA. Additional peak (*) at 24 438 m/z present from transcription of tRNA, which remains unaffected by the acylation conditions. Yield of acylation estimated to be 25%.

the excess La^{3+} , enough remained ($\sim 500 \mu\text{M}$),¹² presumably strongly associated with the tRNA, to disrupt the MALDI. DTPA application followed by size exclusion chromatography resulted in clean samples ($\leq 100 \text{ nM}$).^{12,13} In addition, it seems likely that injection of large amounts of La^{3+} into the *Xenopus* oocyte would be detrimental to receptor synthesis and/or function, further justifying the DTPA treatment.

Even though a large excess of aaEP was used, products arising from multiple acylations were not detected in the MALDI mass spectra; only unacylated and monoacylated tRNA were seen (Figure 1). Our previous work has shown that the aminoacyl-tRNA ionizes differently than the free tRNA, and therefore, exact quantitation of acylation via MALDI is not possible. Therefore, reference mixtures with known ratios of tRNA/aminoacyl-tRNA were prepared and evaluated by MALDI. Mass spectra of reaction products then allowed us to estimate conversion of tRNA to aminoacyl-tRNA to be 5–25%.

The monoacylation detected in the MALDI mass spectra was consistent with, but does not prove, the notion that only the 3' end of the tRNA was acylated. As further evidence, as well as to determine if these La^{3+} /EP-derived tRNAs could

be used in unnatural amino acid mutagenesis, we sought a functional test. Nonsense suppression has been extensively employed to probe the nicotinic acetylcholine receptor (nAChR) expressed in *Xenopus* oocytes, involving hundreds of experiments with scores of unnatural amino acids.^{1,14} The nAChR is a membrane-bound, ligand-gated ion channel that can be functionally interrogated by whole cell electrophysiology. Briefly, aminoacyl-tRNA and mRNA coding for the nAChR and containing a strategically positioned stop codon are microinjected into *Xenopus* oocytes. Following an incubation time of 24–48 h, expressed nAChRs are probed by application of acetylcholine (ACh) and measuring the resulting current. Channel function is typically expressed as EC_{50} , the concentration of ACh required to achieve the half-maximal response. As a negative control, injection of unacylated suppressor tRNA produces no measurable current as a result of the introduction of the stop codon for nonsense suppression.

Initial studies focused on so-called wild-type recovery experiments, in which the amino acid appended to the suppressor tRNA is the amino acid conventionally found at the site where the stop codon was inserted. This experiment should produce wild-type receptor, with behaviors indistinguishable from conventionally expressed receptor. As shown in Table 1, using three different amino acids (Leu, Tyr, and Trp), two different tRNAs (THG73¹⁵ and TQOpS'¹⁶), and two variants of the nAChR (the neuronal $(\alpha 4)_2(\beta 2)_3$ and the muscle-type $(\alpha 1)_2\beta 1\delta\gamma$), wild-type receptors were prepared. This provides strong evidence that the La^{3+} /EP methodology produces translationally competent aminoacyl-tRNAs. To confirm compatibility with unnatural amino acids, 5-fluorotryptophan (F1-Trp) was incorporated at the cation- π binding site of the muscle-type receptor (Trp149 of the α subunit).¹⁷ The 4-fold shift in EC_{50} produced in previous studies was recapitulated (Figure 2). These results clearly establish that the aminoacyl-tRNA derived from the La^{3+} /EP method produced ion channels whose functional responses match those of channels produced by the dCA method.

A notable difference between the dCA and La^{3+} /EP experiments relates to protein yield. The whole cell maximal currents measured for dCA-derived aminoacyl-tRNAs were generally higher than those from La^{3+} /EP-derived tRNAs (Table 1). We suspected that this reflects the decreased quantity of aminoacyl-tRNA being injected with the La^{3+} /EP method because of the incomplete acylation. Indeed, when we correct for the amount of acylated tRNA injected, the yields of receptor from La^{3+} /EP-derived tRNAs are comparable to dCA-derived (Table 1). It is worth reiterating that control experiments show that THG73 and TQOpS' are

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(12) As measured by IPC-MS.

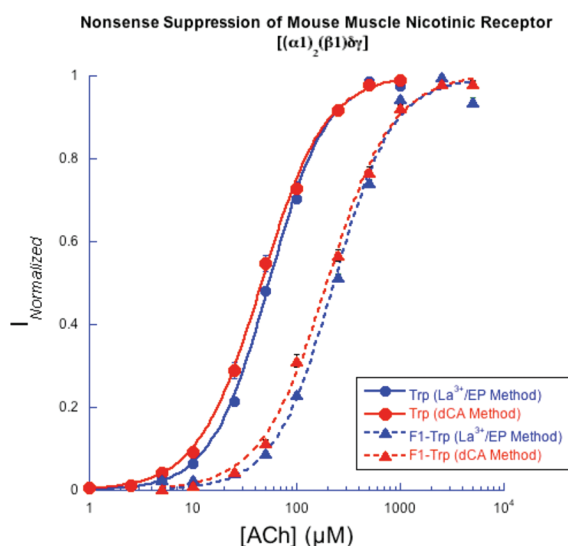
(13) Typical microinjection volume of 50 nL exposes oocytes to $\leq 5 \text{ fmol}$ of La^{3+} .

Table 1. Data for Suppression Experiments Using La³⁺/EP or dCA Methods

	Leu (1)		Tyr (2)		Trp (3)		F1-Trp (4)	
receptor	$\alpha 4_2\beta 2_3$		$\alpha 1_2\beta 1_2\delta\gamma$		$\alpha 1_2\beta 1_2\delta\gamma$		$\alpha 1_2\beta 1_2\delta\gamma$	
mutation(s)	$\alpha L9'A;\beta 119TGA$		$\alpha 1Y190TAG$		$\alpha 1W149TAG$		$\alpha 1W149TAG$	
tRNA	TQOpS'		THG73		THG73		THG73	
acylation method	La ³⁺ /EP	dCA	La ³⁺ /EP	dCA	La ³⁺ /EP	dCA	La ³⁺ /EP	dCA
oocytes (<i>N</i>)	19	18	26	16	52	15	36	27
EC ₅₀ (μM)	0.55 $\pm$ 0.02	0.47 $\pm$ 0.01	52 $\pm$ 2	44 $\pm$ 2	55 $\pm$ 2	47 $\pm$ 1	230 $\pm$ 18	200 $\pm$ 9
mean <i>I</i> _{max} (μA)	2 $\pm$ 2	4 $\pm$ 2	1 $\pm$ 1	9 $\pm$ 8	8 $\pm$ 8	15 $\pm$ 11	3 $\pm$ 3	11 $\pm$ 5
mean <i>I</i> _{max} /ng _{tRNA} ($\mu A/ng$) ^a	0.5 $\pm$ 0.3	0.8 $\pm$ 0.5	0.6 $\pm$ 0.4	1.0 $\pm$ 0.5	2 $\pm$ 2	3 $\pm$ 2	2 $\pm$ 2	0.6 $\pm$ 0.4

^a *I*_{max} values normalized to ng of acylated tRNA injected into oocytes. Acylated tRNA estimated as fraction of total tRNA injected based on percent conversion measured by MALDI-MS. The La³⁺/EP method yielded 5–25% of total tRNA acylated; the dCA method yielded 100% of total tRNA acylated.

not appreciably acylated by an endogenous aaRS, and injection of unacylated tRNA yields no meaningful signal. Injection of a mixture of acylated and unacylated tRNA from the La³⁺/EP method therefore simply produces an effective dilution of acylated tRNA.

**Figure 2.** Dose–response curves of mouse muscle nAChR, [$\alpha 1(W149TAG)$]₂ $\beta 1\delta\gamma$ when injected with suppressor tRNA. Source of tRNA indicated.

In summary, we have shown that direct acylation of tRNA is a viable alternative to the more labor-intensive dCA methodology. Although acylation yields are not high, in appropriately chosen systems, the unacylated tRNA does not interfere with ion channel characterization. The low acylation yield gives rise to a comparable reduction in protein expression, suggesting the approach will only be viable for systems that express well in the *Xenopus* oocyte or other cell of choice. Importantly, ion channels derived from aminoacyl-tRNA prepared by the La³⁺/EP method are functionally indistinguishable from channels derived from the dCA method, establishing the viability of the approach for whole-cell expression of proteins. Further work will seek to improve protein yield by improving acylation efficiency or alternatively by separation and concentration of the aminoacyl-tRNA.

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Supporting Information Available: Experimental details for tRNA acylation, oocyte protein expression, electrophysiology, and synthesis of compounds **1–4**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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