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Incorporation of Esters into Proteins: Improved Synthesis of Hydroxyacyl tRNAs

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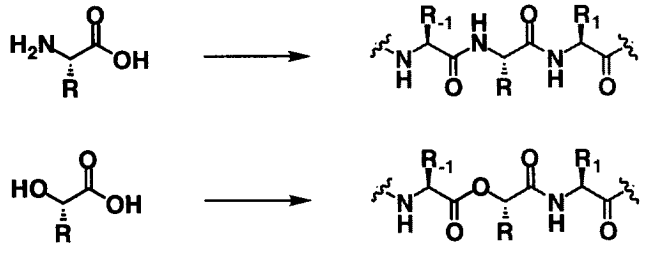
Abstract: We describe two synthetic advances in the preparation of acylated suppressor transfer RNAs which significantly enhance the ease with which α-hydroxy acids can be incorporated into protein structures using nonsense suppression methods. We demonstrate that, in most cases, protection of the α-hydroxy group in the acylation reaction is not necessary. In cases where protection of the α-hydroxy group is desired, we further demonstrate the efficacy of the TBDMS protecting group over the more standard *o*-nitrobenzyl-based protecting groups. These advances greatly simplify the protocol for incorporation of α-hydroxy acids into proteins, overcome limitations associated with photoreactive sidechains, and suggest a general approach to improved suppression yields.

The biosynthesis of proteins containing novel residues at predetermined sites using nonsense (stop codon) suppression by misacylated tRNAs is a powerful technique that can produce important insights into protein structure and function. Both *in vitro*[1] and *in vivo*[2] the method has provided valuable structure/function information on soluble and membrane bound proteins. To date, almost 100 different unnatural amino acids have been incorporated. In addition, α-hydroxy acids have been incorporated, changing the standard peptide backbone from an amide to an ester.[3,4] This provides a telling probe of hydrogen bonding and secondary structure. In addition, we have found[4] that hydroxy acid incorporation is generally very efficient - often more efficient than the analogous amino acid - and that ester incorporation is viable across a wide range of sites in proteins. We now report a substantial simplification in the synthetic protocols associated with nonsense suppression when α-hydroxy acids are used.



The site-specific incorporation of unnatural amino acids and hydroxy acids into proteins utilizes read through of a nonsense codon with an acylated suppressor tRNA. Acylated tRNAs are prepared by enzyme mediated coupling of an acylated dinucleotide (pdCpA) to tRNAs lacking the 3'terminal dinucleotide (tRNA(-CA)). A significant and time-consuming aspect of the

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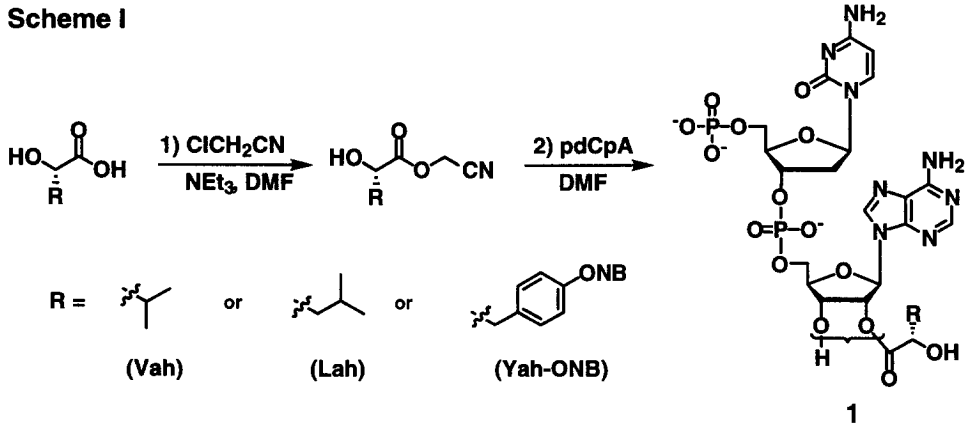
nonsense suppression methodology is the synthesis of the acylated suppressor tRNA. In the standard protocol, the α-hydroxy group (or the amine of the amino acid) is first protected as the *o*-nitrobenzyl ether (NB) or the nitroveratryloxycarbonate (NVOC), and then coupled to the dinucleotide pdCpA. This acylated dinucleotide is then ligated to the tRNA(-CA), and the protecting group (NB or NVOC) is removed photochemically immediately prior to use in a protein translation system. This protection/deprotection strategy is essential for amino acids, because the aminoacyl dinucleotide and the aminoacyl tRNA are relatively unstable due to the inductively withdrawing NH_3^+ group (Scheme I, replace OH of hydroxy acid in **1** with NH_3^+).

Scheme I



It is known that α-hydroxy analogs are more hydrolytically stable,[5] and so we reasoned that such protection may not be necessary for α-hydroxy acids. We now report that this is indeed the case. As shown in Scheme I, direct reaction of the unprotected α-hydroxy acid (the α-hydroxy analog of valine (V) is termed Vah, etc.[4]) with the dinucleotide produces the acylated dinucleotide. This product is then enzymatically ligated to the remainder of the tRNA (74 bases, made by runoff transcription), and the resulting full-length, acylated tRNA is immediately ready for protein synthesis without the need for further chemical manipulations. Noteworthy is the preparation of the tyrosine (Y) analog with the phenolic OH protected as the photocleavable *o*-nitrobenzyl group (Yah-ONB). Such "caged" sidechains represent one of the most powerful uses of the suppression methodology,[6] and the standard, photoremovable protecting groups described above for the α-amino or α-hydroxy groups are, of course, not compatible. The ability to avoid their use completely thus represents a significant advantage.[7]

In some instances, protection of the OH may still be desired. For example, with the α-hydroxy analog of alanine (Aah, lactic acid), separation by HPLC of the acylated dinucleotide from the unreacted dinucleotide proved problematic. In such instances, we find protection of the alcohol as the TBDMS ether to be superior to the NB and NVOC protecting groups (Scheme II). Following acylation of the dinucleotide, the TBDMS group can be removed with acetic acid in quantitative yield. Importantly, the crude product from the deprotection can be taken directly into the ligation step required for full-length tRNA synthesis without further purification. The byproduct of the deprotection, TBDMS alcohol, does not interfere with this enzymatic ligation reaction.

Scheme II

These findings, coupled with the increased efficiency of α-hydroxy acid incorporation and the known increase in stability of hydroxyacyl vs. aminoacyl tRNAs, suggest that certain applications of the nonsense suppression method would be better served by α-hydroxy acids rather than α-amino acids, even if backbone structure is not the major concern. Examples include studies involving biophysical probes[8], topology mapping[9], or photoreactive sidechains.[6,10] We anticipate that the findings reported here will lead to increased use of the nonsense suppression methodology.

Typical Experimental Procedures.

Cyanomethylester of Vah. To a room temperature solution of 18.4 mg (1.6 mmol) of 2-hydroxy-3-methylbutyric acid in DMF (3 mL) was add chloroacetonitrile (3 mL) followed by triethylamine (651 μL, 4.2 mmol). After stirring for ~24 hr the mixutre was poured into Et_2O and extracted one time against H_2O. The organic phase was concentrated, and remaining $ClCH_2CN$ was removed by vacuum transfer. The cyanomethylester was isolated by flash silica gel column chromatography as a colorless oil (187 mg, 76.5%). ^1H NMR (300 MHz, $CDCl_3$) δ 0.89 (d, 6.9 Hz), 1.05 (d, 6.9 Hz), 2.13 (m), 2.35 (s, broad), 4.16 (d, 3.9 Hz), 4.78 (d, 15.6 Hz), 4.87 (d, 15.6 Hz).

pdCpA coupling to Vah. To a room temperature solution of the cyanomethylester (12.3 mg, 78 μmol) in DMF (0.5 mL) was added pdCpA (31.3 mg, 26 μmol) followed by catalytic tetrabutylammonium acetate. After ~24 hr the acylated dinucleotide was separated from unreacted pdCpA by semi-preparative HPLC and lyophillized.[11] The resulting ammonium salt of the acylated dinucleotide was exchanged three times with HOAc (10 mM, 100 mL each) by repeated lypophillization to provide the free acid of the acylated dinucleotide as a white solid (3.0 mg, 16%). It is necessary to exchange the NH_4^+ counter ion for H^+ because NH_4^+ interferes with the T4 RNA ligase enzyme that is used in the next step of the protocol.

pdCpA coupling to Aah-OTBS and deprotection. To a room temperature solution of the cyanomethylester (15.3 mg, 62 μmol) was added DMF (0.5 mL) followed by pdCdA (25.3 mg, 21 μmol). After ~2 hr the acylated dinucleotide was purified by HPLC and converted to the free acid as described above (2.1 mg, 13%). The resulting TBDMS ether (447 μg) was dissolved in HOAc:THF:H_2O (3:1:1, 0.4 mL) and was placed in a 37°C incubator. After ~12 hr the mixture was lyophillized and used without further purification. Analytical HPLC was used to verify that the deprotection was complete.

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7. Protecting groups that are not removed photochemically have also been used effectively, such as Cbz, nitrophenylsulfenyl and 4-pentenoyl [Lodder, M.; Golvine, S.; Hecht, S. M. *J. Org. Chem.* **1997,** *62*, 778-779; England, P. M.; Lester, H. A.; Davidson, N.; Dougherty, D. A. *Proc. Natl. Acad. Sci. USA* **1997,** *94*, 11025 - 11030]. However, these work only with amines, not alcohols as required for α-hydroxy acids.

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11. The rate of this coupling reaction varies dramatically with the side chain of the hydroxy acid. With less bulky side chains (e.g. lactic acid) the coupling is complete in ~2 hr