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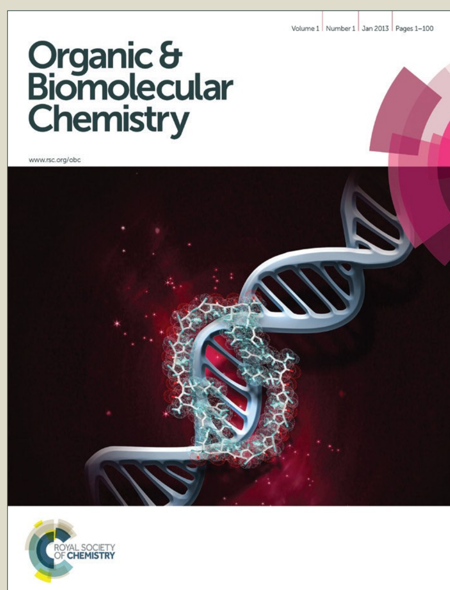

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Tetrahydrofuranyl and Tetrahydropyranyl Protection of Amino Acid Side-Chains Enables Synthesis of a Hydroxamate-Containing Aminoacylated tRNA†

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†Electronic supplementary information (ESI) available: compound characterization data are presented.

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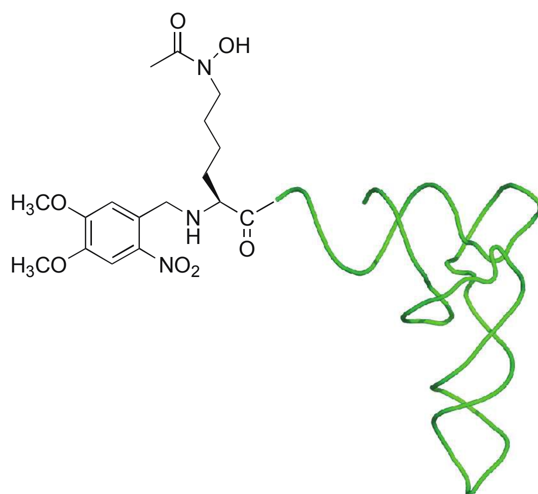

An *O*-protection strategy using tetrahydrofuranyl or tetrahydropyranyl has enabled the addition of a hydroxamate-containing unnatural amino acid to a suppressor tRNA, allowing for subsequent site-specific incorporation of the amino acid into the transcription factor, TFIIIA.

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Abstract

The ability to specifically engineer metal binding sites into target proteins has far-reaching consequences ranging from the development of new biocatalysts and imaging reagents to the production of proteins with increased stability. We report the efficient tRNA-mediated incorporation of the hydroxamate containing amino acid, N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine, into a transcription factor (TFIIIA). Because this amino acid is compact, hydrophilic, and uncharged at physiological pH, it should have little or no effect on protein folding or solubility. The N^{ε}-hydroxy group of the hydroxamate is refractory to photodeprotection and required the identification of reagents for *O*-protection that are compatible with the synthesis of acylated tRNA. Tetrahydrofuranyl and tetrahydropyranyl *O*-protecting groups can be removed using mild acid conditions and allowed for an orthogonal protection strategy in which deprotection of the amino acid side chain precedes ligation of an acylated dinucleotide to a truncated suppressor tRNA. These protecting groups will provide a valuable alternative for *O*-protection, especially in cases where photodeprotection cannot be used.

Introduction

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Strategies for the preparation of tRNA acylated with unnatural amino acids have expanded the repertoire of chemical moieties that can be precisely placed within the structure of a protein.[1] A description of the most commonly used *in vitro* procedure to prepare tRNA aminoacylated with an unnatural amino acid is presented in Scheme 1A.[2-4] The activated cyanomethyl ester of an N^{α}-protected amino acid is reacted with the hybrid dinucleotide, pdCpA, which is then joined enzymatically to a truncated tRNA lacking the two terminal 3' nucleotides. The advent of these techniques, however, has necessitated the development of new protecting groups that can be removed from the amino acid under conditions that will not induce hydrolysis of the labile aminoacyl linkage nor harm the nucleic acid. Unfortunately, the number of reagents that meet these requirements is limited, due mostly to complications that arise during chemical deprotection. Protection of nucleophilic side chains is required during the aminoacylation reaction to prevent formation of side products.[5] Nucleophilic groups on the side-chain of the amino acid can also compete effectively with the 2' and 3' hydroxyl groups of the adenosine nucleotide for reaction at the α-carbonyl carbon of the cyanomethyl ester.[5] To avoid significant deacylation of the tRNA, side chain protecting groups must be removed using mild conditions. For convenience, deprotection of both the N^{α} and the amino acid side chains is generally done simultaneously by utilizing protecting groups with related scissile susceptibility. For example, photolytic side chain protecting groups include the NVOC (6-nitroveratryloxycarbonyl) group for amines, 6-nitroveratryl ethers for hydroxyls and 6-nitroveratryl esters for side chain acids.[5, 6] This deprotection problem for side chains has also been circumvented using protecting groups that can be removed enzymatically (pyroglutamyl group) or chemically (4-penteneoyl group).[7, 8]

We report here the successful use of two new groups for *O*-protection of a novel hydroxamate containing amino acid that can be used to site-specifically place a metal binding moiety into proteins. Both tetrahydrofuranyl and tetrahydropyranyl groups were used to protect the N^{ε}Dhydroxyl of the hydroxamate, N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine **1** (Scheme 1B). Both protecting groups can be efficiently removed from the aminoacylated amino acid in weakly acidic solution. The resulting aminoacyled tRNA exhibited good biosynthetic activity in an *in*

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vitro transcription/translation reaction, suppressing an amber stop codon in the coding sequence of transcription factor IIIA (TFIIIA) with 40% efficiency. The tetrahydrofuranyl and tetrahydropyranyl groups should find general utility for the protection of a variety of nucleophilic moieties in the side-chains of both native and unnatural amino acids. Since these groups are removed chemically, they provide a valuable orthogonal alternative in those cases, *i.e.,* light sensitive amino acids, when photodeprotection is not possible.

The hydroxamate containing amino acid described here is smaller and more hydrophilic than other metal-chelating unnatural amino acids that have been reported.[9-12] Thus, we expect that incorporation of N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine should have fewer adverse effects on the proper folding of proteins either *in vitro* or *in vivo.* Moreover, there should be fewer steric restrictions on the placement of this amino acid within a given protein, due to its compact size.

Results and discussion

Aminoacyl bond formation is a critical step in the pathway for the preparation of tRNA charged with an unnatural amino acid (Scheme 1). The cyanomethyl ester of an amino acid reacts quite specifically with the either the 2' or 3'-hydroxyl position on the hybrid dinucleotide, 5'-phospho-2'-deoxycytidylyl(3',5')adenosine (pdCpA), requiring no protection of the N4 position of the cytidine.[4, 5, 13] However, a nucleophilic functionality such as a hydroxyl, carboxyl, amide, or sulfhydryl group in the side-chain of the amino acid will readily react with the active ester, thereby preventing any significant acylation of the dinucleotide. Although there are several protecting groups that may be used to mask such functionalities, the subsequent removal of these groups from the amino acid, when present on the charged tRNA, poses special problems. The aminoacyl bond is especially susceptible to hydrolysis even at neutral pH. Protecting groups such as carbobenzoxy (Cbz), nitrosulfenyl (NPS), and biphenylisopropyloxycarbonyl (Bpoc) are removed under conditions that leave the aminoacyl linkage intact; however, yields of deprotected acylated tRNA can be low and side-reactions with the RNA are possible in some cases.[14, 15] NVOC derivatives, which are removed by irradiation,

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have therefore been the most widely used protecting groups for N^{α} and other nucleophilic positions in activated amino acids.

The side-chain hydroxamate group in the amino acid N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine prevents aminoacylation of suppressor tRNA by the method outlined in Scheme 1. We have tested various protecting groups for this position in order to identify those that are compatible with the reaction of activated amino acid with pdCpA and subsequent deprotection of aminoacylated tRNA. Since the N^{α} position is normally protected with the photoactivatable NVOC group, we tested the use of a second nitroveratryl group to protect the side-chain hydroxyl with the expectation that both protecting groups could be removed simultaneously. The reaction of N^{α}-(6-nitroveratryl)-N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine with 6-nitroveratryl-*O*-tosyl alcohol proceeded poorly with appreciable hydrolysis of the NVOC group from the N^{α} position. Consequently, a synthetic scheme was devised in which protection of the side-chain hydroxyl precedes protection of the N^{α} position (Scheme 2). Suitably protected hydroxamate precursors were prepared either through the formation of a nitrone or through epoxidation of a Schiff base according to published syntheses.[16] Following removal of the *O*-acyl group by amidolysis, the hydroxamate **2** was reacted with 6-nitroveratryl-*O*-tosyl alcohol. The Boc and *t*-butyl protecting groups were removed by treatment with trifluroacetic acid and the resulting *O*-protected hydroxamate **3** purified by reverse phase HPLC.

Photolytic removal of the *O*-nitroveratryl protecting group was studied using **3**. The rate of photolysis was measured using analytical reverse phase HPLC to quantitate the amount of **3** remaining after irradiation. The conditions for photodeprotection were identical to those used to remove NVOC from the N^{α} position. After 30 min of irradiation, more than 80% of the protected hydroxamate remained; moreover reverse phase TLC and FAB mass spectrometry experiments showed that the major deprotection product was N^{ε}-acetyl-L-lysine and not the hydroxamate. Thus, photolysis unexpectedly resulted in the cleavage of the N–O, rather than the C–O, bond. A subsequent series of experiments established that N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine **1** remains unaltered during a 30 min irradiation, indicating that the generation of N^{ε}-acetyl-L-

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lysine does not proceed through a mechanism involving the deprotected hydroxamate as an intermediate (L. J. Lambert, unpublished results). This suggested that it could still be possible to use NVOC group for protection of the N^{α} position but that a more suitable strategy was required to protect the hydroxamate side chain.

The ether linkage of **3** was replaced with a carbamate-like linkage **4** (Scheme 3) in an effort to separate the N^{ε} oxygen of the hydroxamate **1** from the reacting benzylic position of the nitroveratryl protecting group.[17-20] However, reaction of the tetrabutylammonium salt of pdCpA with 5.0 equivalents of **4** led to the formation of several products upon analysis by HPLC. Preliminary characterization of some of these products indicated that they included the cyanomethyl ester of the hydroxamate missing the N^{ε} NVOC group and dinucleotide nonspecifically acylated with the NVOC group at both N and O positions. Formation of the latter products is analogous to the *N*- and *O*- acylation of dinucleotide by the *N*-hydroxysuccinimidyl active ester of amino acids reported by Robertson *et al.* and is consistent with the notion that the hydroxmate active ester is a better leaving group than the cyanomethylester.[5]

We tested protection of **1** with a silyl protecting group, *tert*-butyldimethylsilyl (TBDMS), with the expectation that increased steric bulk would reduce reactivity at the N^{ε} oxygen (Scheme 3). Similar protection of aryl hydroxyls has been reported previously.[21] Aminoacylation reactions with the hydroxamate protected with TBDMS, **6**, yielded a mixture of *N* and *O* silylated dinucleotide, a complication similar to that encountered with the NVOC-protected amino acid **5**.

The requirement for a more robust linkage that could be efficiently removed after aminoacylation under mild conditions led us to tetrahydrofuranyl (THF) and tetrahydropyranyl (THP) protecting groups.[22, 23] The full synthesis is described. The cyanomethyl ester of the unprotected hydroxamate **5** was prepared in two steps after NVOC protection of the amine (Scheme 4). The reaction of the NVOC protected N^{α}-(6-nitroveratryloxy)-N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine cyanomethyl ester, **5**, with dihydropyran yielded the *O*-protected product, **7a**,

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in 85% yield after purification by chromatography on silica gel. A similar acetal exchange reaction with 2-tetrahydrofuranyl diphenylacetate was used to prepare the *O*-tetrahydrofuranyl protected hydroxamate **7b**. This latter compound was used to identify conditions for deprotection of the hydroxamate without hydrolysis of the aminoacyl bond. TLC resolved **7a/7b** from the ferric chloride-positive free hydroxamate **5**; whereas, both deacylation and deprotection could be monitored simultaneously by reverse phase HPLC. Table 1 summarizes the conditions tested for removal of the THP group.

Dilute trifluoroacetic acid, used to cleave the Bpoc protecting group from the N^{α} position of aminoacylated pdCpA[24], did not remove the THP group and prolonged incubation led to significant deacylation. However, incubation in a 3:1:1 acetic acid:water:DMF mixture proved quite effective for the removal of both THF and THP protecting groups with half-lives of 1.8 and 2.9 h respectively. Thus, the aminoacylation reaction could be followed by incubation in weak acid to deprotect only the hydroxamate side chain. Cyanomethyl ester mediated aminoacylation of the pdCpA by the THF protected hydroxamate **7b** was followed by the addition of mild acid until the side chain was deprotected, with the 2'3'-monoacylated product **9** subsequently purified by HPLC (Figure 1). This aminoacylated hybrid dinucleotide **9** was conjugated to truncated tRNA and, just before use, subjected to photodeprotection.

The biological activity of the charged suppressor tRNA was tested using an *in vitro* protein synthesis extract (*E. coli* S30) programed with a plasmid encoding the transcription factor TFIIIA (Figure 2). The coding sequence of the open reading frame contains an amber stop codon (TAG) at amino acid position 199 that normally encodes an arginine residue. Full-length TFIIIA is 344 amino acids.[25] The protein products of the translation reaction were analyzed by western blot using an antibody against TFIIIA. The reaction containing suppressor tRNA acylated with N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine without photodeprotection produced no full-length protein (*lane* 4); whereas, deprotection of N^{α} led to a suppression efficiency of 40% as measured by densitometry (*lane* 5). This robust activity demonstrates that deprotection of the amino acid in mild acid has no adverse effects on the biological activity of the aminoacylated dinucleotide

Metals play a variety of roles in the structure and function of proteins.[26] The widespread occurrence of assorted zinc-binding motifs is just one example of the means by which metals enable and stabilize the folding and higher order structure of proteins.[27] Redox active metals such as iron and copper mediate diverse activities ranging from electron and oxygen transport to ribonucleotide reduction and deamination.[28-31] Similarly, magnesium participates directly in the chemistry of several enzyme-catalyzed reactions, including the polynucleotide polymerases and nucleases.[32] It is well appreciated that the ability to engineer metal binding sites into proteins holds the potential to expand their function, creating, for example, novel imaging reagents, spectroscopic probes, and catalysts.[33-35]

The specific positioning of metals in native proteins is accomplished by either of two means: through the precise configuration of amino acid side chains that form a coordination sphere or through the utilization of a prosthetic group. *De novo* design of new metal binding sites into proteins using either of these strategies has inherent difficulties that have been discussed at length. [33-36] The first instance requires that the three-dimensional arrangement of amino acids used to create the binding site does not perturb the native structure of the protein. The second instance faces the same challenge, if a binding site for the prosthetic group is desired. Alternatively, direct linkage of a prosthetic group to an amino acid side chain is restricted to compatible sites on the surface of the protein. Given these appreciable technical challenges, there has been increasing effort to use unnatural amino acids to create novel metalloproteins either by solid-state synthesis of a polypeptide or by the genetically based strategy employed here. There is a small number of cases where a metal-binding amino acid has been incorporated site-specifically using the latter method.[10-12] In these cases, the chelating moieties have been generally large and often hydrophobic, meaning there is the potential to either disrupt or, at least, perturb proper folding of the protein. Additionally, the hydrophobic nature of these moieties can potentially lead to protein aggregation at high concentrations as well as diminish the solubility of the acylated tRNA used to incorporate the amino acid during ribosome-based synthesis. The

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hydroxamate-containing amino acid described here obviates these complications and, thus, represents an important addition to the family of metal-binding unnatural amino acids.

Conclusions

Naturally occurring hydroxamates are compact ligands that possess high single-ligand affinity for several metal cations, including Fe(III), Zn(II), and Cu(II).[37] Their chemical properties are especially well suited for protein engineering since they are polar, yet uncharged at physiological pH, suggesting that they will have little tendency to perturb native protein structure through either hydrophobic or electrostatic interactions. We have demonstrated that hydroxamates can be protected with groups (tetrahydrofuranyl and tetrahydropyranyl) whose efficient removal is compatible with the mild deprotection conditions imposed by the acylated dinucleotide. This opens the way for site-specific incorporation of this family of chelators into proteins of interest.

Experimental section

General procedures and methods

All organic reactions were conducted under nitrogen using baked glassware. All chemicals were purchased from Aldrich Chemicals or Sigma Chemicals unless otherwise noted. All solvents were distilled before use and reaction solvents underwent further distillation over CaH_2 unless otherwise noted. Routine molecular biology and organic techniques followed established protocols.[38, 39] Analytical thin-layer chromatography was performed on 60 F_{254} (Whatman) plates; reverse phase thin layer silica gel chromatography was performed using C-18 F_{254} (Whatman) plates. Flash chromatography utilized 200-400 mesh silica gel (Acros). ^{1}H and ^{13}C NMR spectra were measured on a General Electric GN-300 spectrophotometer using

tetramethylsilane as a reference unless otherwise noted. High pressure liquid chromatography was performed using a Waters Model 510 pump equipped with a 486 Tunable absorbance detector using columns as described in the methods section. Mass Spectroscopy was performed by the Notre Dame Mass Spectroscopy facility. Absorbance measurements were made using a Beckman DU30 spectrophotometer. Densitometric analysis was performed using a Molecular Dynamics Computing Densitometer Model 300 B. All restriction enzymes were obtained from either Promega or New England Biolabs. Other enzymes came from the indicated sources. ESI contains data on compound characterization using NMR, MS, IR, and HPLC.

6-Nitroveratryl-*O*-tosyl-alcohol. 6-Nitroveratryl alcohol (0.60 g, 2.8 mmol) was dissolved in 3 mL of CH_2Cl_2 that had been distilled over CaH_2 under nitrogen. The stirring reaction was supplied with *p*-toluenesulfonic acid anhydride (1.06 g, 3.2 mmol) and the reaction cooled on ice. Heat evolved upon addition of 270 μL of dry pyridine and the yellow solution was stirred under nitrogen for 1 h. The reaction mixture was concentrated by rotary evaporator, dissolved in ethyl acetate and filtered to remove *p*-toluenesulfonic acid. The filtrate was concentrated and subjected to chromatography (D = 2.0 cm, 10 g silica gel, 1:1 v/v ethyl acetate: hexanes, 8 mL fractions). The pure fractions were combined and concentrated to yield a light-yellow precipitate (0.89 g, 2.4 mmol, 85%). TLC R_f 0.40 (1:1 EtOAc: hexanes) ^{1}H NMR ($CDCl_3$) δ 2.46 (s, 3H), 3.95 (s, 3H), 3.99 (s, 3H), 5.48 (s, 2H), 7.15 (s, 1H), 7.36 (s, 1H), 7.39 (s, 1H), 7.70 (s, 1H) 7.84 (s, 1H) 7.87 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.7, 56.4, 56.6, 68.6, 108.0, 109.8, 125.6, 128.0, 130.1; HRMS (FAB) Calcd for $C_{16}H_{17}N_1O_7S_1$ (MH^+) 367.0726, Found: 367.0714; IR (thin film) 2948, 2360, 1715, 1582, 1520, 1456, 1328, 1278, 1228, 1070 cm^{-1}.

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N^{ε}-Acetyl-N^{ε}-hydroxy-*O*-(6-nitroveratryl ether)-L-lysine (3). N^{α}-Boc-N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine-*tert*-butyl ester **(2)** (0.46 g, 1.3 mmol) was dissolved in 3 mL of dry CH_3CN and Cs_2CO_3 (0.59 g, 1.81 mmol) was added as a pulverized powder. To the stirring suspension was added 6-nitroveratryl-*O*-tosyl alcohol (0.67 g, 1.82 mmol) and the reaction stirred at room temperature under nitrogen for 1 h in the dark. The ferric chloride test of the major product was negative indicating masking of the hydroxamate. The reaction was diluted in 20 mL of CH_2Cl_2 and extracted with an equal volume of 0.2 M citric acid. The sample was dried over Na_2SO_4, filtered and purified using silica gel chromatography (D = 2 cm, 15 g silica gel, 100 mL 10% step gradient, 20-80% v/v EtOAc: hexanes, 8 mL fractions). Peak fractions were pooled and concentrated to a yellow solid (0.55 g, 0.96 mmol, 74%) and used without further purification. TLC R_f = 0.40 (6:4 v/v hexanes: EtOAc). ^{1}H NMR ($CDCl_3$) δ 1.23 (m, 2H), 1.39 (s, 9H), 1.40 (S, 9H), 1.50-1.75 (m, 6H), 2.28 (s, 3H), 3.83 (t, 2H), 3.97 (s, 3H), 4.06 (s, 3H), 4.33 (m, 1H), 5.05 (d, 1H), 5.22 (s, 2H), 7.10 (s, 1H) 7.72 (s, 1H). The *O*- protected hydroxamate (0.55 g, 0.96 mmol) was dissolved in neat trifluoroacetic acid (4 mL) added in 0.5 mL portions over 5 min. The reaction proceeded for 0.5 h. Analytical HPLC showed product formation eluting at 12.9 min (Waters Nova Pac ® 3.9 X 1500 mm, 0.8 mL/min 10-99% over 30 min, CH_3CN in 50 mM NaOAc, pH 4.5). The reaction was concentrated with use of the rotary evaporator to a dark brown oil. This impure material was subjected to silica gel chromatography (D = 2 cm, 5 g silica gel). The oil was loaded on the column, washed with CH_2Cl_2 to elute residual organics and the product eluted from the column with 20% v/v methanol in CH_2Cl_2. Polar impurities remained. Purification to homogeneity was done using preparative HPLC; the product eluted at 15.3 min. (Waters Prep LC Module, Prep Nova Pac ® HR C18 6 µm 60 Å, size, 5 mL/min 10-99% over 30 min, CH_3CN in 10 mM AcOH: water; 0.1 g of impure sample per injection). Concentration of three 8 mL fractions yielded the pure *O*-protected amino acid **(3)** (0.30 g, 0.75 mmol, 78%) ^{1}H NMR (D_2O) δ 1.44 (m, 2H), 1.67 (m, 2H), 1.88 (m, 2H), 2.09 (s, 3H), 3.72 (t, 2H, J = 3 Hz), 3.96 (s, 3H), 4.00 (s, 3H), 5.27 (s, 2H), 7.21 (s, 1H), 7.80 (s, 1H; ^{13}C NMR (D_2O) 20.3, 22.6, 28.5, 31.0, 45.6, 55.6, 57.2, 57.4, 109.8, 114.8, 140.6, 141.9, 175.6; HRMS (FAB) Calcd for

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$C_{17}H_{25}N_2O_8$ (MH^+) 385.1611, Found: 385.1592; IR (thin film) 3439, 1623, 1525, 1457, 1410, 1278, 1064 cm^{-1}.

***N*$^{\alpha}$-NVOC-*N*$^{\varepsilon}$-Acetyl-*N*$^{\varepsilon}$-hydroxy-*O*-(6-nitroveratryl ether)-L-lysine-cyanomethyl ester (5).** Crude *N*$^{\varepsilon}$-acetyl-*N*$^{\varepsilon}$-hydroxy-*O*-(6-nitroveratryl ether)-L-lysine (**4**) (0.58 g) was dissolved in 3 mL of 50:50 w/w phenol:$CHCl_3$ in a round bottom flask. The flask was immersed in a water bath at room temperature. To this was added 6-nitroveratryloxycarbonyl chloride (0.62g, 2.2 mmol) and triethylamine (0.45 g, 0.62 mL, 4.5 mmol). The exothermic reaction was stirred at room temperature for 1 h under nitrogen. Monitoring of the reaction by TLC showed consumption of the ninhydrin positive starting material to produce the *N*-protected product. The reaction was supplemented with 20 mL of CH_2Cl_2 and extracted with an equal volume of 0.2 M citric acid. The organic layer was dried over Na_2SO_4, filtered and subjected to silica gel chromatography (D = 2.0 cm, 15 g silica gel, eluted with CH_2Cl_2) to yield 0.34 g of light yellow solids TLC R_f = 0.1 (9:1 v/v EtOAc: hexanes).

A portion of these solids (0.19 g, 0.28 mmol) was dissolved in 1.0 mL of CH_3CN in a round bottom flask. Distilled triethylamine (86 mg, 0.11 mL, 0.84 mmol) and chloroacetonitrile (63 mg, 0.84 mmol) were added to the reaction. The reaction was stirred at room temperature under nitrogen in the dark for 3 h. Addition of more triethylamine (57 mg, 0.07 mL, 0.55 mmol) and chloroacetonitrile (63 mg, 0.84 mmol) at 3 h gave complete conversion of the starting material as determined by TLC. The solvent was removed by rotary evaporation and the material subjected to silica gel chromatography (D = 2 cm, 10 g silica gel, 90:10 v/v EtOAc: hexanes) to yield 0.151 g of yellow oil. Addition of toluene (approximately 1 mL) to form an azeotrope with which to remove residual solvents gave 0.124 g (**5**) (0.182 mmol, 65%) of a course yellow solid. TLC R_f = 0.4 (9:1 v/v EtOAc: hexanes); ^{1}H NMR ($CDCl_3$) δ 1.3-1.9 (m, 6H), 2.16 (s, 3H), 3.6-3.7 (m, 2H), 3.95 (s, 3H), 3.97 (s, 3H), 4.01 (s, 3H), 4.02 (s, 3H), 4.38 (m, 1H), 4.77 (dd, 2H, J1 = 21 Hz, J2 = 25 Hz), 5.23 (d, 2H, J = 3.3 Hz), 5.51 (dd, 2H, J1 = 15 Hz, J2 = 37 Hz), 5.67 (d 1H, J = 1.5 Hz), 7.04 (s, 1H), 7.71 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.2, 22.7, 27.0, 31.4, 49.7, 54.3, 57.2, 57.3, 64.8, 75.5, 108.8, 108.9, 110.5, 111.6, 114.6, 125.9, 128.9,

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129.0, 129.7, 140.8, 148.8, 149.4, 154.4, 156.3, 171.7; FABMS 678 $(M + H)^+$. HRMS (FAB) Calcd for $C_{29}H_{35}N_5O_{14}$ (MH^+) 678.2259, Found: 678.2261; IR (thin film) 3358, 3294, 1759, 1720, 1654, 1581, 1522, 1277, 1857 cm^{-1}.

N^{α}-NVOC-N^{ε}-Acetyl-N^{ε}-hydroxy-L-lysine. N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine **(1)** (0.453 g, 2.2 mmol) was dissolved in 10 mL of aqueous 0.4 M Na_2CO_3. 6-Nitroveratryl carbonyl chloride (0.75 g, 2.7 mmol) was dissolved in 10 mL of 1,4 dioxane and added to the stirring mixture drop wise. Reaction was continued at room temperature for 30 min. The reaction was diluted in 30 mL of 0.1 M Na_2CO_3 and extracted twice with 50 mL of EtOAc. The aqueous phase was acidified with concentrated HCl to pH 2.0 and extracted twice with 30 mL of ethyl acetate. The organic layer was dried over Na_2SO_4, filtered and concentrated to 0.37 g of the N-protected hydroxamate as a yellow foam (0.83 mmol, 37%). Purification for analysis of was done on 10 mg portions using preparative HPLC. The product eluted at 16 min. (Waters Prep LC Module, Prep Nova Pac ® HR C18 6 µm 60 Å, size, 5 mL/min, 20-99% CH_3CN in 10 mM AcOH: water over 30 min). After collection of product fractions, the product was lyophilized to yellow powder. R_f = 0.3 (Reverse Phase TLC 1:1 v/v, 50 mM NaOAc, pH 4.5: CH_3CN, UV and ferric chloride positive) ^1H NMR (D_2O) δ 1.38 (m, 2H), 1.7-1.9 (m, 4H), 2.11 (s, 3H), 3.61 (t, 3.61), 3.94 (s, 3H), 3.98 (s, 3H), 5.43 (dd, 2H, J1 = 30 Hz, J2 = 10 Hz), 7.14 (s, 1H), 7.79 (s, 1H); ^{13}C NMR (CD_3OD) 56.8, 108.3, 154.3, 155.5, 159.9, 160.6, 162.2, 175.1, 180.3 FABMS (M + H) 444. HRMS (FAB) Calcd for $C_{18}H_{25}N_3O_{10}$ (MH^+) 444.1618, Found: 444.1613; IR (thin film) 3371, 2943, 1714, 1582, 1520, 1278, 1070 cm^{-1}.

N^{α}-NVOC-N^{ε}-Acetyl-N^{ε}-hydroxy-L-lysine-cyanomethyl ester (5). N^{α}-NVOC-N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine (155 mg, 0.35 mmol) was dissolved in 1 mL of distilled DMF. Chloroacetonitrile (131 mg, 109 µL, 1.7 mmol) and triethylamine (88 mg, 120 µL, 0.87 mmol) were added. The reaction was stirred at room temperature for 6 h under nitrogen. Development of the ferric chloride positive product was closely monitored by TLC. The sample was diluted in 10 mL of EtOAc and extracted with an equal volume of 0.2 M citric acid. Drying over Na_2SO_4 was followed by filtration and concentration via rotary evaporator to give an oil. The sample

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was subjected to silica gel chromatography (D =2 cm, 30 g, 50-70% v/v acetone: hexanes as 10% in 100 mL increments). The ferric chloride positive product fractions (8 mL per fraction) were pooled and concentrated to 111 mg (0.23 mmol, 65%) of a yellow oil **(5)**. R_f = 0.3 (1: 1 v/v acetone: hexanes, UV/ ferric chloride positive, starting material Rf = 0.1). ^{1}H NMR δ 1.3 - 2.0 (m, 10 H), 2.1 (s, 3H), 3.5- 3.7 (m 2H), 3.96 (s, 3H), 4.01 (s, 3H), 4.46 (s, 1H), 4.80 (dd, 2H, J1 = 26 Hz, J2 = 12 Hz), 5.51 (dd, 2H, J1 = 19 Hz, J2 = 12 Hz), 7.00 (s, 1H), 7.71 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 18.6, 20.5, 21.6, 22.3, 26.92, 31.5, 49.1, 56.4, 64.3, 108.1, 110.0, 113.9, 127.4, 139.8, 148.3, 153.7, 156.1, 164.4, 171.1, 172.8 FABMS 483 (M + H); HRMS (FAB) Calcd for $C_{20}H_{26}N_4O_{10}$ (MH^+) 483.1727, Found: 483.1714; IR (thin film) 3311, 2940, 1761, 1724, 1619, 1582, 1522, 1278, 1221, 1071 cm^{-1}.

***N*$^{\alpha}$-NVOC-*N*$^{\varepsilon}$-Acetyl-*N*$^{\varepsilon}$-hydroxy-*O*-(NVOC)-L-lysine cyanomethyl ester (4).** *N*-6-acetyl-*N*-6-hydroxy-L-lysine **1** (0.25g, 1.2 mmol) was dissolved in 2 mL of 1:1 (w/w) phenol: $CHCl_3$. 6-Nitroveratryl carbonyl chloride (NVOC-Cl) (1.0 g, 3 mmol) was added slowly to the stirring reaction. The reaction vessel was immersed in a water jacket kept at room temperature and triethylamine (0.32 g, 0.41 mL, 3.0 mmol) was immediately added. Reaction for 1 h showed complete protection of the hydroxamate as determined by a negative ferric chloride test on TLC. The sample was diluted in $CHCl_3$ (less than 2 mL) and applied to silica gel (D = 2 cm, 15 g silica gel) packed in CH_2Cl_2 and the column washed until phenol had been completely eluted. The sample was eluted from the column with methanol and concentrated by rotary evaporation to a crude oil. The sample was dissolved in 20 mL of EtOAc and extracted with an equal volume of 0.2 M citric acid. The organic layer was dried over Na_2SO_4, filtered and concentrated to an impure yellow oil of the doubly protected NVOC compound *N*$^{\alpha}$-NVOC-*N*$^{\varepsilon}$-acetyl-*N*$^{\varepsilon}$-hydroxy-*O*-(NVOC)-L-lysine (0.11 g). R_f = 0.5 (1: 1 v/v acetone: hexanes) ^{1}H NMR ($CDCl_3$) δ 4.40 (m, 1H), 4.05 (s, 3H), 4.00 (s, 6H), 3.95 (s, 3H).

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The N^{α}-NVOC-N^{ε}-acetyl-N^{ε}-hydroxy-*O*-(NVOC)-L-lysine (0.11 g) was dissolved in 1.0 mL of CH_3CN. To this was added chloroacetonitrile (311 mg, 260 μL, 4.1 mmol) and triethylamine (124 mg, 160 μL, 1.23 mmol) and the reaction stirred in darkness under a nitrogen atmosphere for 4 h. The sample was diluted with 15 mL of CH_2Cl_2 and extracted against an equal volume of 0.2 M citric acid. The sample was dried over Na_2SO_4, filtered and concentrated by rotary evaporation. The product **4** was applied to a silica gel column (D = 2 cm, 10 g silica gel, 1: 1 v/v acetone: hexanes) and concentrated to 45 mg of material. This material was further purified by chromatography on a silica gel column (D = 1 cm, 5 g slica gel, 70% v/v ethyl acetate in hexanes). The product was suspended in dry DMF to make a 42 mM stock solution **4**. ^{1}H NMR ($CDCl_3$) δ 4.6 (dd, 2H), 4.4 (m, 1H), 4.04 (s, 6H), 3.94 (s, 3H), 3.90 (s, 3H) HRMS (FAB) Calcd for $C_{30}H_{36}N_5O_{16}$ (MH^+) 722.2157, Found 722.2157.

N^{α}-NVOC-N^{ε}-Acetyl-N^{ε}-hydroxy-*O*-(*tert*-butyl dimethylsilyl ethoxy ether)-L-lysine-cyanomethyl ester (6). N^{α}-NVOC-N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine cyanomethyl ester (**5**) (18 mg, 0.037 mmol) was dissolved in 0.40 mL of distilled DMF. Imidazole (56 mg, 0.08 mmol) and *tert*-butyldimethylsilyl chloride (88 mg, 0.06 mmol) were added and the reaction stirred at room temperature for 20 min. The reaction was diluted with 5 mL of EtOAc and extracted with two equal portions of 0.1 M $NaHCO_3$. The sample was dried over Na_2SO_4, filtered, concentrated to a clear oil and purified by silica gel chromatography (D = 1 cm, 7 g silica gel, 1:1 acetone: hexanes with 1 % triethylamine). Concentration to white solids yielded 15 mg (0.025 mmol, 68%) of (**6**). R_f = 0.8 (1: 1 v/v acetone: hexanes, UV/ weakly ferric chloride positive); ^{1}H NMR δ 0.12 (s, 6H), 0.97 (s, 9H), 1.1-2.0 (m, 10H), 2.10 (s, 3H), 3.55 (m, 1H), 4.8 (m, 1H), 3.96 (s, 3H), 4.03 (s, 3H), 4.37 (m, 1H), 4.77 (dd, 2H, J1 = 27 Hz, J2 = 16 Hz), 5.54 (dd 2H, J1= 50 Hz, J2 =

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21 Hz), 5.8 (m, 1H), 7.06 (s, 1H), 7.73 (s, 1H); ^{13}C NMR ($CDCl_3$) -4.0, -3.8, 18.5, 21.9, 22.5, 26.33, 26.37, 49.6, 54.4, 57.1, 57.3, 64.7, 79.1, 108.8, 110.3, 114.6, 128.8, 140.1, 148.7, 154.4, 156.4, 171.8; FABMS 597 (M + H); HRMS (FAB) Calcd for $C_{28}H_{43}N_4O_{10}Si_1$ (MH^+) 597.2592, Found: 597.2604; IR (thin film) 3986, 2933, 1761, 1523, 1279, 836 cm^{-1}.

N^{α}-NVOC-N^{ε}-Acetyl-N^{ε}-hydroxy-*O*-(tetrahydropyranyl ether)-L-lysine cyanomethyl ester (7a). N^{α}-NVOC-N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine cyanomethyl ester (**5**) (0.1 g , 0.21 mmol) was dissolved in 3.0 mL of distilled chloroform. The stirring mixture was placed in an ice bath and 1.0 mL 1,1 dihydropyran and 50 mg of p-toluenesulfonic acid (0.25 mmol) was added. The mixture evolved heat and became a dark brown color. The reaction was diluted in 20 mL of EtOAc and extracted with equal volumes of 0.1 M $NaHCO_3$ and water. The organics were dried over Na_2SO_4, filtered and concentrated by rotary evaporator to a brown oil. The sample was dissolved in 1 mL of chloroform and subjected to silica gel chromatography (D = 2.5 cm, 30 g silica gel, 1:1 acetone: hexanes, 1% triethylamine). The peak fractions (8 mL per fraction) were pooled and concentrated to 0.045 g (0.08 mmol, 38% yield) of diastereomers **7a** as a light yellow glaze. R_f = 0.64 (1: 1 v/v acetone: hexanes, UV/ ferric chloride positive upon heating); ^1H NMR δ 1.4-1.8 (m, 12H), 2.14 (s, 3H), 3.6 (m, 1H), 3.7 (m, 1H), 3.96 (s, 3H), 4.03 (s, 3H), 4.38 (m, 1H), 4.88 (dd, 2H, J1 = 26 Hz, J2 = 16 Hz), 4.91 (m, 1H), 5.64 (m, 2H), 5.80 (m, 1H), 7.06 (s, 1H), 7.72 (s, 1H); ^{13}C NMR ($CDCl_3$) 10.2, 20.5, 21.6, 21.7, 22.7, 25.5, 26.7, 29.8, 31.1, 31.3, 47.4, 49.6, 54.5, 57.1, 57.2, 64.4, 64.6, 105.1, 105.4, 108.8, 110.4, 114.6, 128.8, 140.2, 148.7, 154.5, 156.4, 171.9; FABMS 567 (M + H); HRMS (FAB) Calcd for $C_{25}H_{35}N_4O_{11}$ 567.2302 (MH^+), Found: 567.2265; IR (thin film) 3286, 2945, 1759, 1726, 1654, 1633, 1522, 1277, 1070 cm^{-1}.

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***N*$^{\alpha}$-NVOC-*N*$^{\varepsilon}$-Acetyl-*N*$^{\varepsilon}$-hydroxy-*O*-(tetrahydrofuranyl ether)-L-lysine cyanomethyl ester (7b)**. The cyanomethyl ester (**5**) (111 mg, 0.23 mmol) was dissolved in dry dimethylformamide (0.5 mL). To this was added 2-tetrahydrofuranyl diphenylacetate (0.32 g, 1.1 mmol) and catalytic *p*-toluenesulfonic acid (22 mg, 0.11 mmol).[40-42] The reaction was stirred under nitrogen for 30 min. Thin layer chromatography showed approximately 50% conversion to the THF protected hydroxamate **(7b)**. More 2-tetrahydrofuranyl diphenylacetate (0.32 g, 1.1 mmol) was added and the reaction was allowed to continue until observed to be complete by TLC. The reaction was diluted with 20 mL of EtOAc and extracted three times with 0.1 M $NaHCO_3$ to remove residual diphenylacetic acid. The organic layer was dried over Na_2SO_4, filtered and concentrated by rotary evaporation to an oil. The sample was dissolved in chloroform and subjected to silica gel chromatography (D = 2 cm, 20 g, 1:1 v/v acetone: hexanes, 1% triethylamine). Concentration yielded 87 mg (0.16 mmol, 69%) of diastereomeric yellow solids **(7b)**. $R_f = 0.3$ (1:1:1 v/v/v acetone: ethyl acetate: hexanes, UV/ became ferric chloride positive on heating, starting material (**5**). $R_f = 0.1$); ^{1}H NMR 1.2-2.05 (m, 12H), 2.10 (s, 3H), 3.5-3.9 (m, 3H), 3.95 (s, 3H), 4.02 (s, 3H), 4.35 (m, 1H), 4.762 (dd, 2H, J1 = 26 Hz, J2 = 16 Hz), 5.40 (s, 1H), 5.55 (dd, 2H, J1 = 45 Hz, J2 = 15 Hz), 5.8 (m, 1H) 7.05 (s, 1H), 7.71 (s, 1H); ^{13}C NMR ($CDCl_3$) 21.5, 22.6, 24.2, 26.5, 31.1, 31.7, 49.6, 54.4, 57.1, 57.3, 64.7, 69.0, 69.1, 108.8, 109.4, 110.4, 114.6, 128.8, 140.1, 148.7, 154.4, 156.4, 171.8; FABMS 553 (M + H) 483 major fragment; HRMS (FAB) Calcd for $C_{24}H_{32}N_4O_{11}$ 553.2146 (MH^+), Found: 553.2141; IR (thin film) 3305, 2948, 1760, 1726, 1643, 1582, 1521, 1277, 1071 cm^{-1}.

***N*$^{\alpha}$-NVOC-*N*$^{\varepsilon}$-Acetyl-*N*$^{\varepsilon}$-hydroxy-*O-(*tetrahydropyranyl ether)-L-lysine-pdCpA (8a).** A stock solution (0.1 M) of cyanomethyl ester **7a** was made in dry DMF. The cyanomethyl ester (50 µL, 2.8 mg, 5 µmol) was combined in an Eppendorf tube with 50 µL of a DMF stock

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solution (1.7 mg, 55 mM) of the tetrabutylammonium salt of pdCpA.[5] The aminoacylation at 37°C was monitored by analytical HPLC (C18 Waters Nova-Pac® 3.9 X 1500 mm using a linear gradient of 1-25% CH_3CN over 30 min in 50 mM NaOAc pH 4.5 at 0.8 mL/min monitored at 260 nm). The 2' and 3' *O*-acyl products eluted at 25 min. For the purpose of characterization, the aminoacyl product **8a** was purified in a single step of semi-preparative HPLC. The coupling reaction was diluted with an equal volume of 50 mM NaOAc, pH 4.5 and was isolated by semi-preparative reverse phase HPLC (Waters Prep LC Module, Prep Nova Pac® HR C18 6 μm 60 Å. The purification method employed a gradient of 1-50% CH_3CN in 20 mM acetic acid, pH 4.5 at 5 mL/min for 60 min with monitoring at 260 nm). The product eluted at 17 min. The peak fractions (8 mL) were pooled and frozen in plastic test tubes before being lyophilized overnight in darkness to yield a yellow powder **8a**. Yield as determined by OD_{260} ($\varepsilon = 25000\ cm^{-1}M^{-1}$) was 85% (2.7 mg). Mass spectrum (MALDI) m/z 1145, $C_{42}H_{55}N_{11}O_{23}P_2$ expected mass 1145, (M + Na) 1168, 1061 major fragment.

***N^α*-NVOC-*N^ε*-Acetyl-*N^ε*-hydroxy-*O-(*tetrahydrofuranyl ether)-L-lysine-pdCpA (8b).** A stock solution (0.1 M) of cyanomethyl ester **7b** was made in dry DMF. 50 μL (2.8 mg, 5 μmol) was combined in an Eppendorf tube with 50 μL of a DMF stock solution (1.7 mg, 55 mM) of the tetrabutylammonium salt of pdCpA.[5] The aminoacylation at 37°C was monitored by analytical HPLC as described for compound **8a.** The 2' and 3' *O*-acyl products **8b** eluted at 25 min. The aminoacyl product **8b** was purified using methods identical to those described for compound. Yield as determined by OD_{260} ($\varepsilon = 25000\ cm^{-1}M^{-1}$) was 45% (1.4 mg). Mass spectrum (MALDI) m/z 1104 fragment with tetrahydrofuranyl protection removed, $C_{41}H_{55}$-$N_{11}O_{23}P_2$ 1131.

***N^α*-NVOC-*N^ε*-acetyl-*N^ε*-hydroxy-L-lysine-pdCpA (9).** The unpurified aminoacylation coupling reactions 100 μL with tetrahydrofuranyl or tetrahydropyranyl protection (**8a** or **8b**) were dissolved in 300 μL of glacial acetic acid and 100 μL of water. Reaction at 37°C was monitored by analytical HPLC in a manner identical to that for the

synthesis of compound **8a/8b.** The deprotected 2' and 3' *O*-acyl isomers eluted at 21 min. Hydrolysis of the tetrahydrofuranyl and tetrahydropyranyl forms was observed to be quantitative with a $t_{1/2}$ of 1.8 h and 2.9 h, respectively. Preparative HPLC using the methods used to purify compound **8a/8b** were used to acquire the deprotected form **9** which eluted at 15 min. Mass spectrum (MALDI) m/z 1067, expected for $C_{37}H_{49}N_{11}O_{22}P_2$ 1061.

***In vitro* synthesis of tRNA Phe (CUA)-CA and enzymatic ligation**

Plasmid pYΦ2 (gift from Dr. P.G. Schultz, Scripps Institute, CA) was used to prepare suppressor tRNA by runoff transcription.[4] Ligation of the aminoacylated dinucleotide was performed as follows. tRNA (7.0 µg, 0.3 nmol) was suspended in 10 mM Tris-HCl pH 8.0 (17 µL) and was combined with RNA ligase buffer (final concentration of 50 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM ATP), 3.5 µL DMSO, 3.1 mM aminoacylated dinuclotide **9**, and 220 U T4 RNA ligase (New England Biolabs) in a final volume of 35 µl. The mixture was incubated at 37 °C for 20 min. The ligation reaction was stopped by the addition of 6.1 µL of 2.5 M NaOAc pH 4.5 followed by extraction with phenol (pre-equilibrated with NaOAc, pH 4.5) and then chloroform:isoamyl alcohol (24:1). The RNA was precipitated with ethanol and the pellet washed with 70% ethanol; the sample was stored at -80 °C until needed.

Photodeprotection of tRNA (CUA) aminoacylated with NVOC-protected amino acids

Photoprotection was carried out using a 400 W Hg (Xe) lamp (Oriel) with a 6 cm focal length Pyrex lens. Aminoacylated tRNA was resuspended in 1.0 mM KOAc pH 4.5 in a microcentrifuge tube, placed in ice water, and irradiated with the focused beam for 5 min. Quantitative removal of NVOC protecting groups was observed under these conditions. After

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photodeprotection the charged tRNA was supplied immediately to an *in vitro* protein synthesis reaction.

In vitro protein synthesis using S30 extract

The S30 extract was prepared from *E. coli* strain D10.[43] The plasmids used for this experiment encode full TFIIIA (pV-1) or encode a nonsense mutant with a TAG stop codon at amino acid position 199 (pV-199). Transcription/translation extract was mixed with 1.0 μg of plasmid DNA and 10 μg of photodeprotected aminoacylated tRNA and incubated at 37°C on a rotating wheel for 1.5 h. The protein synthesis reaction was stopped by the addition of 45 μL of acetone. After 15 min on ice, the precipitated protein products were pelleted by centrifugation. The pellet was solubilized in SDS loading dye and loaded on a 10% SDS-PAGE polyacrylamide gel and resolved by electrophoresis. The gel was blotted to a nitrocellulose membrane overnight and analyzed by western blotting using an antibody against TFIIIA.

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Table 1 Optimization for hydroxamate deprotection with preservation of aminoacyl linkage

Acid Conditions	Time	Temp °C	Deprotection (TLC)	Deacylation (HPLC)
1:1 CH_3CN:H_2O:TFA pH 2.7	24 h	23	−	−
88% Formic Acid	45 min	23	+	−
100% Acetic Acid	24 hr	37	−	+
88% Acetic Acid	24 hr	37	−	+
3:1:1 Acetic Acid: H_2O: DMF	7 hr	37	+	+

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Scheme 1. A) The general strategy for *in vitro* suppression of an amber TAG stop codon using suppressor tRNA chemically aminoacylated with an unnatural amino acid.[5] The enzymatic ligation of aminoacylated hybrid dinucleotide pdCpA to tRNA(-CA) is used to construct the full length tRNA. After deprotection, this charged tRNA is used in a transcription/translation reaction programmed with a plasmid encoding the protein of interest. The protein coding sequence contains the stop codon at the desired site of substitution with an unnatural amino acid. B) The hydroxamate chelate N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine **1**.

Scheme 2. Preparation of (6-nitroveratryl ether) protected hydroxamate.

Scheme 3. NVOC and Silyl protection of hydroxamate.

Scheme 4. Tetrahydrofuranyl and Tetrahydropyranyl protection of side chain hydroxamates.

Figure 1. Acid deprotection of aminoacylated pdCpA. Dinucleotide aminoacylated with THF protected hydroxamate **8b** was incubated in 3:1:1 acetic acid:water:DMF (v/v/v) at 37 °C. The progress of the deprotection reaction was monitored as a function of time by analyzing aliquots by HPLC (Waters Nova-Pac 3.9 × 1500 mm developed with a linear gradient from 1-99% CH_3CN over 30 minutes in 50 mM NaOAc, pH 4.5, at 0.8 mL/min monitored at 260 nm). The protected aminoacylated dinucleotide **8b** elutes as a doublet at 24 - 25 min, trace amounts of pdCpA at 10.9 min, and deprotected product **9** at 21 – 22 min. (A) t = 0; (B) t = 3 h; (C) t = 5 h. Asterisks indicate an injection artifact.

Figure 2. Site-specific incorporation of N^{ε}-acetyl-N^{ε}-hydroxy-L-lysine into transcription factor IIIA. Synthesis of TFIIIA in *E. coli* transcription/translation (S30) extract was monitored by western blot. Lanes: 1, native TFIIIA: 2, complete transcription/translation mixture without plasmid encoding TFIIIA; 3, reaction programmed with plasmid containing a stop codon at position 199 without suppressor tRNA; 4, reaction programmed with plasmid and suppressor tRNA acylated with hydroxamate **1** that was not photodeprotected; 5, reaction programmed with plasmid and photodeprotected suppressor tRNA acylated with hydroxamate **1.** The positions of truncated (Δ) and full-length TFIIIA are indicated. An *asterisk* indicates a 30 kDa fragment that arises from the full-length protein by proteolytic cleavage.[44]

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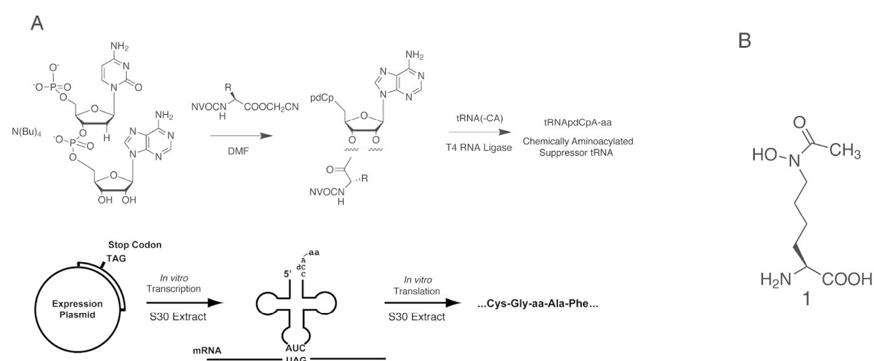

Scheme 1. A) The general strategy for in vitro suppression of an amber TAG stop codon using suppressor tRNA chemically aminoacylated with an unnatural amino acid.5 The enzymatic ligation of aminoacylated hybrid dinucleotide pdCpA to tRNA(-CA) is used to construct the full length tRNA. After deprotection, this charged tRNA is used in a transcription/translation reaction programmed with a plasmid encoding the protein of interest. The protein coding sequence contains the stop codon at the desired site of substitution with an unnatural amino acid. B) The hydroxamate chelate Nε-acetyl-Nε-hydroxy-L-lysine 1.
279x215mm (300 x 300 DPI)

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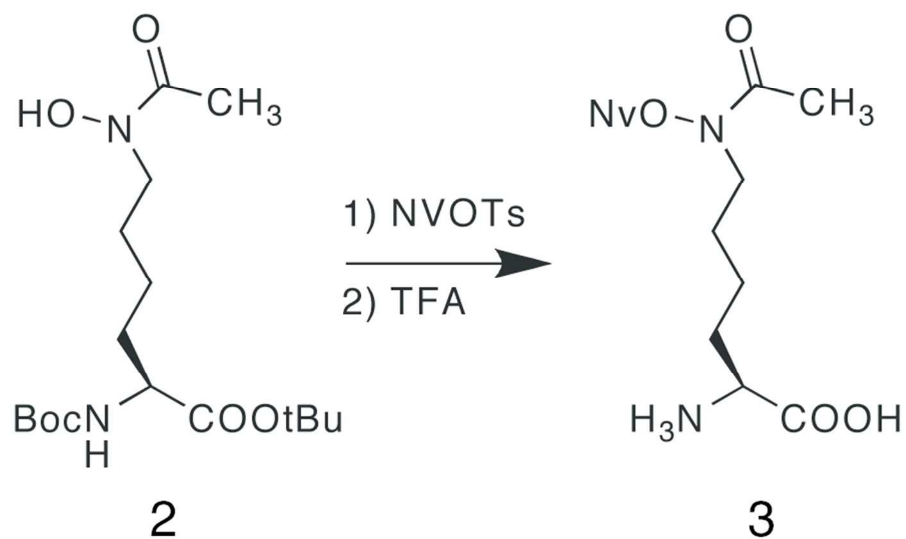

Scheme 2. Preparation of (6-nitroveratryl ether) protected hydroxamate.
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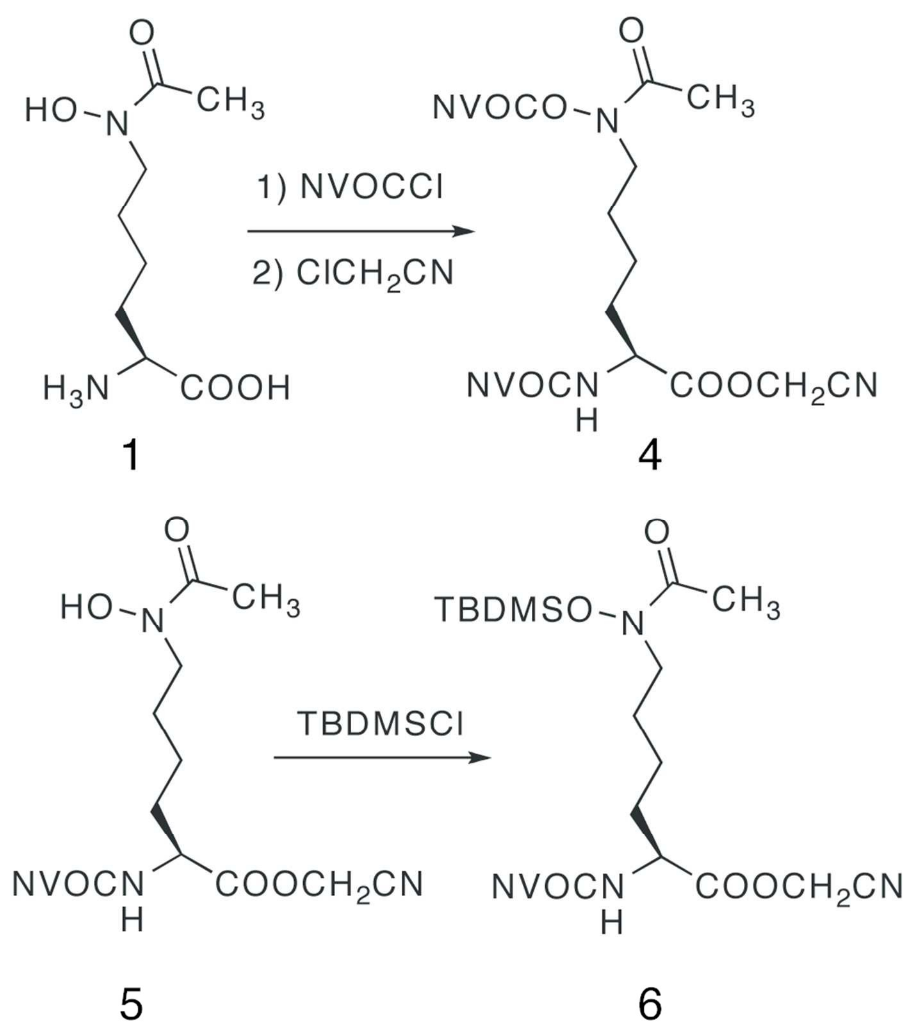

Scheme 3. NVOC and Silyl protection of hydroxamate.
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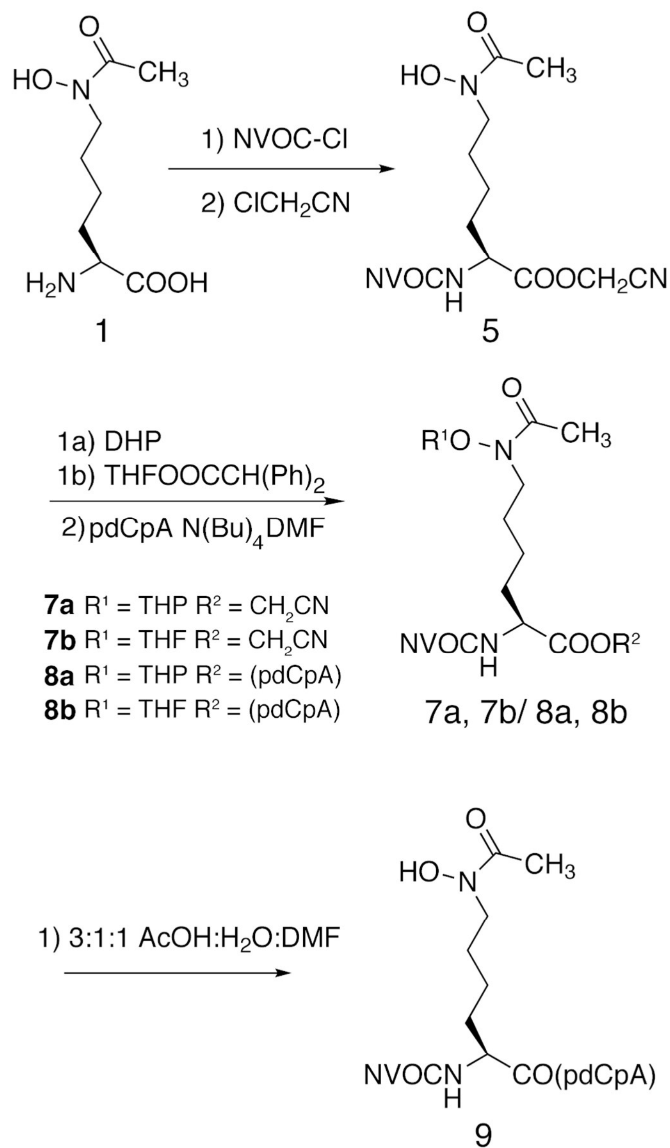

Scheme 4. Tetrahydrofuranyl and Tetrahydropyranyl protection of side chain hydroxamates.
93x152mm (300 x 300 DPI)

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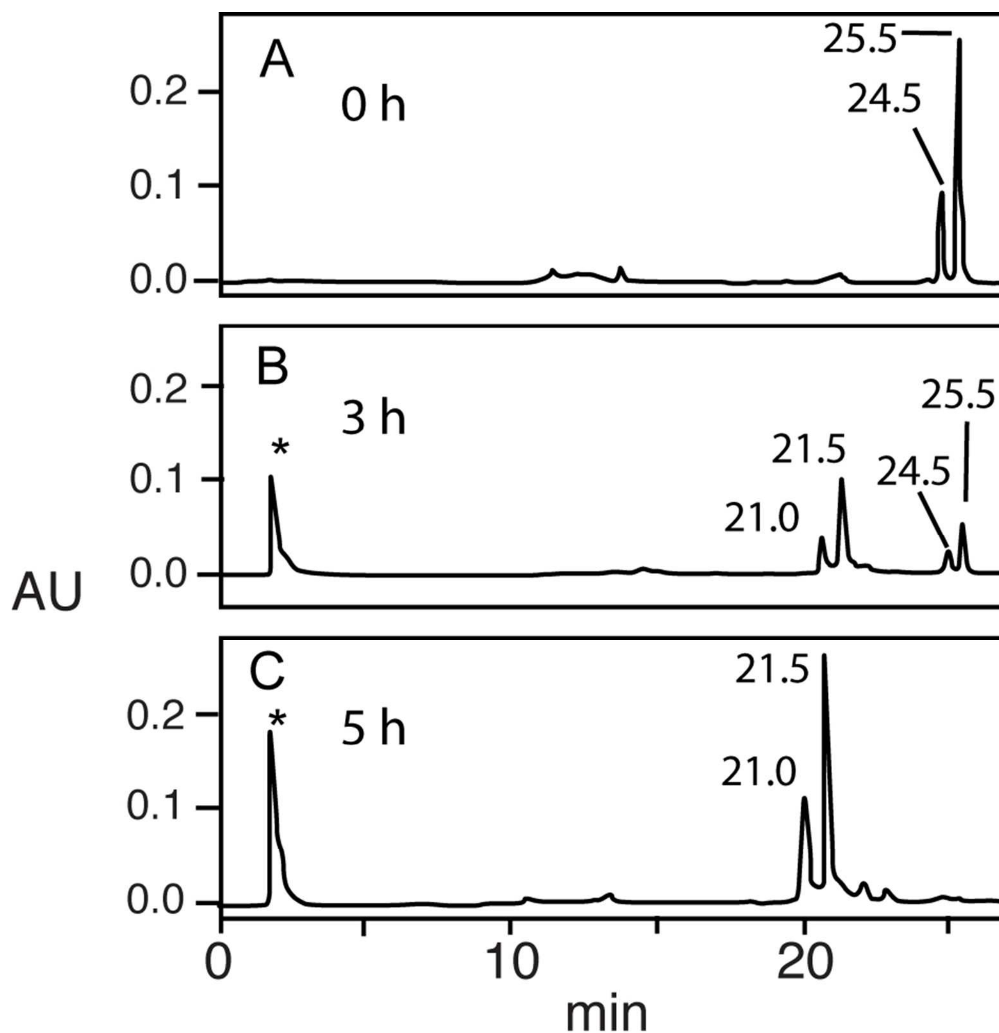

Figure 1. Acid deprotection of aminoacylated pdCpA. Dinucleotide aminoacylated with THF protected hydroxamate 8b was incubated in 3:1:1 acetic acid:water:DMF (v/v/v) at 37 °C. The progress of the deprotection reaction was monitored as a function of time by analyzing aliquots by HPLC (Waters Nova-Pac 3.9 × 1500 mm developed with a linear gradient from 1-99% CH_3CN over 30 minutes in 50 mM NaOAc, pH 4.5, at 0.8 mL/min monitored at 260 nm). The protected aminoacylated dinucleotide 8b elutes as a doublet at 24 - 25 min, trace amounts of pdCpA at 10.9 min, and deprotected product 9 at 21 – 22 min. (A) t = 0; (B) t = 3 h; (C) t = 5 h. Asterisks indicate an injection artifact.
80x83mm (300 x 300 DPI)

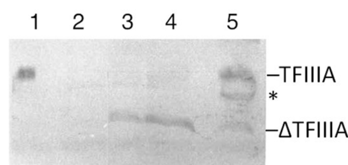

Figure 2. Site-specific incorporation of Nε-acetyl-Nε-hydroxy-L-lysine into transcription factor IIIA. Synthesis of TFIIIA in E. coli transcription/translation (S30) extract was monitored by western blot. Lanes: 1, native TFIIIA: 2, complete transcription/translation mixture without plasmid encoding TFIIIA; 3, reaction programmed with plasmid containing a stop codon at position 199 without suppressor tRNA; 4, reaction programmed with plasmid and suppressor tRNA acylated with hydroxamate 1 that was not photodeprotected; 5, reaction programmed with plasmid and photodeprotected suppressor tRNA acylated with hydroxamate 1. The positions of truncated (Δ) and full-length TFIIIA are indicated. An asterisk indicates a 30 kDa fragment that arises from the full-length protein by proteolytic cleavage.44
121x91mm (300 x 300 DPI)

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