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Synthesis of pdCpAs and transfer RNAs activated with derivatives of aspartic acid and cysteine

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ABSTRACT

Described herein is the preparation of new aminoacylated derivatives of the dinucleotide pdCpA, and of transfer RNAs. The focus of the present work is the synthesis of amino acid analogs related to aspartic acid and cysteine species that have important functional roles in many proteins. The activated aminoacyl-tRNAs prepared can be utilized for the elaboration of proteins containing modified aspartic acid and cysteine derivatives at predetermined sites. Of particular interest is definition of functional group protection strategies that can be used for the preparation of the aminoacylated pdCpAs and tRNAs.

1. Introduction

The introduction of unnatural amino acids into proteins mediated by misacylated suppressor transfer RNAs (tRNAs) has been developed into a powerful technique for studying protein structure, function, dynamics, and intermolecular interactions.[1–4] This procedure involves the use of site-directed mutagenesis to replace the codon for the amino acid of interest with a nonsense codon such as TAG,[1] TAA[5] or TGA,[6] or else by some other unique codon.[7,8] A suppressor tRNA containing the cognate anticodon is chemically misacylated with the desired unnatural amino acid,[1,9–11] and then is added to an in vitro transcription/translation system containing the modified plasmid (Scheme 1). The general strategy of T4 RNA ligase-mediated ligation of a synthetic aminoacyl-pdCpA[12] with an abbreviated tRNA lacking the 3′-terminal cytidine and adenosine moieties[13] has proven to be successful for a wide variety of unnatural amino acids, permitting their incorporation into proteins.[14–19] Recently, modification of the 23S rRNA of the *Escherichia coli* ribosome has also permitted D-amino acids to be incorporated into proteins.[20,21]

Among the twenty proteinogenic amino acids, aspartic acid and cysteine are especially interesting, since they function in the active sites of many enzymes.[22–26] For the interactions between enzymes and their substrates, aspartic acid can bind to substrate functional group to orient them for catalysis,[22] or provide the means to support acid–base function for the requisite transformations.[24–26] Analogously, the active site sulfhydryl group of cysteine participates as a nucleophile, notably in the cysteine proteases.[27–29] To facilitate the study of proteins containing functionally important aspartic acid and cysteine moieties, we have prepared a series of aminoacyl derivatives of pdCpA and $tRNA_{CUA}$ containing aspartic acid and cysteine derivatives. Particular attention has been focused on oxidized cysteine and homocysteine derivatives that may be regarded as S analogs of aspartic (and glutamic) acid. Also critical for the present study was definition of those side chain functionalities requiring chemical protection during the several chemical and biochemical transformations leading to the misacylated tRNAs, and the development of suitable strategies for each of these analogs.

2. Results

2.1. Synthesis of tRNAs activated with aspartic acid and cysteine analogs

Activated tRNAs bearing aspartic acid analogs were prepared as outlined in Scheme 2. L-Aspartic acid γ-methyl ester (**1a**) and L-asparagine (**1b**) were treated with 6-nitroveratryl chloroformate (NVOC-Cl) in 1:1 dioxane/H_2O in the presence of two equivalents of $NaHCO_3$ to afford the respective *N*-6-nitroveratryl derivatives. These *N*-6-nitroveratryl amino acid derivatives were then treated with chloroacetonitrile and triethylamine in acetonitrile to obtain the desired cyanomethyl esters (**2a** and **2b**, respectively). The products (**2a** and **2b**) were isolated as yellow oils in yields of 53% and 25%, respectively. Each of the cyanomethyl esters was combined with the tetra-*n*-butylammonium salt of pdCpA[12] in DMF. The crude product mixture was fractionated by C_{18} reversed phase

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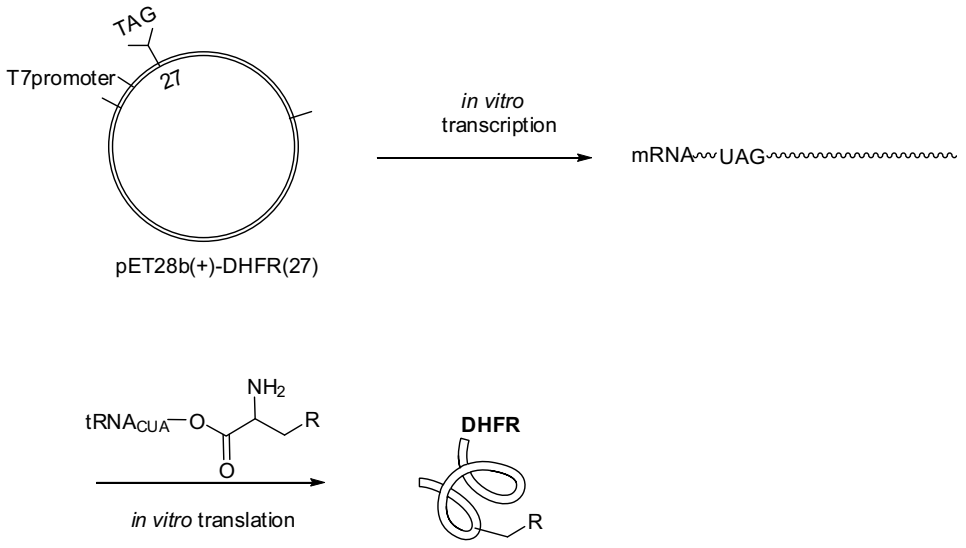
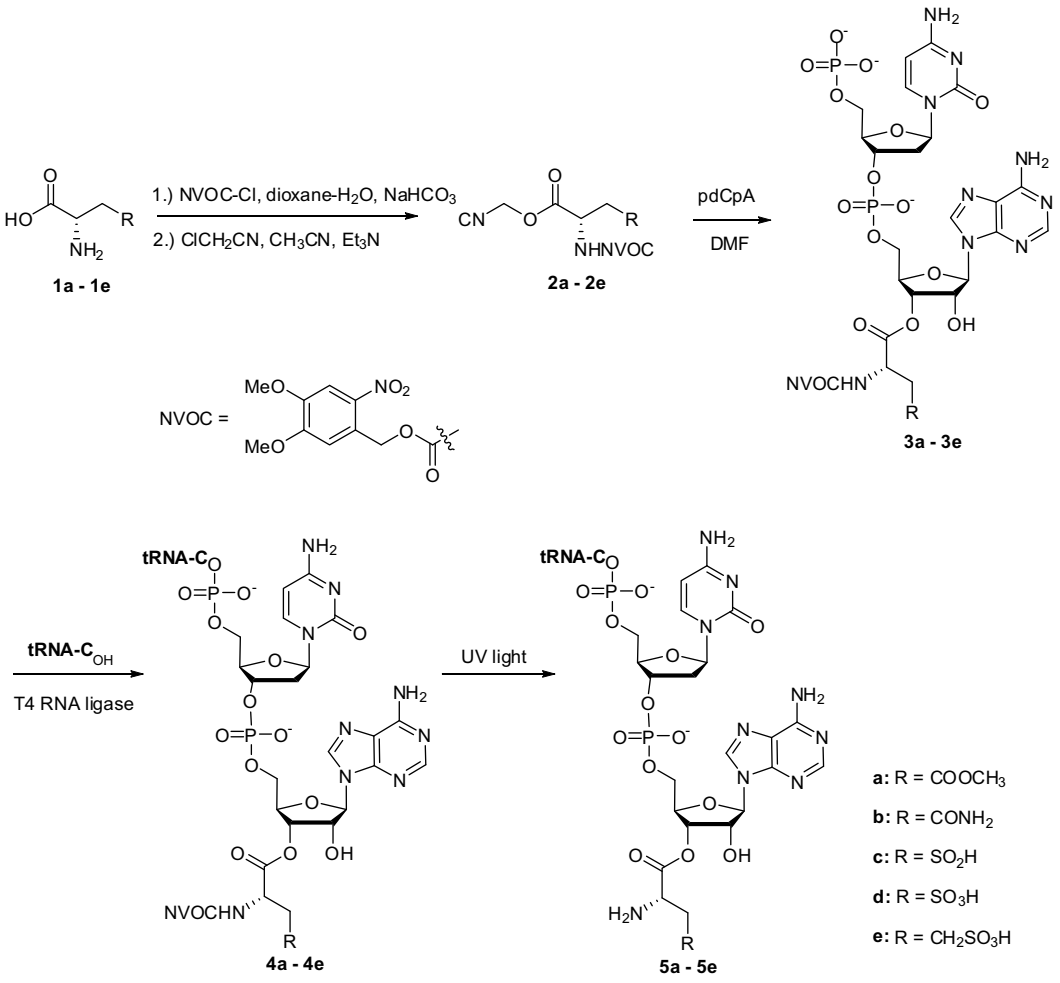


Scheme 1. Strategy employed for effecting the site-specific incorporation of unnatural amino acids into proteins. The elaboration of DHFRs altered at position 27 is used as an example.



Scheme 2. Synthesis of tRNAs activated with analogs of aspartic acid.

HPLC to obtain monoacylated pdCpAs (**3a** and **3b**) in yields of 36% and 23%, respectively. The N-protected monoaminoacylated pdCpA derivatives (**3a** and **3b**) were then ligated to abbreviated suppressor tRNA$_{CUA}$-C$_{OH}$ transcripts (i.e., lacking the cytidine and adenosine moieties at the 3′-terminus) to obtain N-protected aminoacylated tRNA$_{CUA}$s (**4a** and **4b**) (Scheme 2). The ligation reac-

tion was catalyzed by T4 RNA ligase; the efficiency of ligation was estimated by gel electrophoresis at pH 5.2[30] (Fig. 1). More than 95% of the suppressor tRNA-C_{OH} transcript[13] underwent ligation in each case. Each of the N-protected monoaminoacylated tRNAs (**4a** and **4b**) was irradiated with high intensity UV light for several minutes, affording activated tRNAs bearing L-aspartic acid-γ-methyl ester (**5a**) and L-asparagine (**5b**), respectively. Thus the γ-methyl ester and amide derivatives of aspartic acid were accessible without employing any additional protection of the side chain functional groups.

For L-cysteinesulfinic acid (**1c**), L-cysteic acid (**1d**), and L-homocysteic acid (**1e**), each of which contains a strongly acidic group in the side chain, it seemed possible that their intrinsic reactivity might permit the preparation of the aminoacyl-pdCpA derivatives without the need to employ side chain protection, especially if the transformations were performed under somewhat more basic conditions. Accordingly, treatment with 6-nitroveratryl chloroformate (NVOC-Cl) in 1:1 dioxane/H_2O was carried out in the presence of 4 equivalents of $NaHCO_3$; the crude products were treated with chloroacetonitrile and triethylamine in acetonitrile to obtain the desired cyanomethyl esters (**2c**, **2d**, and **2e**) in yields of 33%, 31%, and 59%, respectively. The cyanomethyl esters were treated with the tri-*n*-tetrabutylammonium salt of pdCpA[12] in dry DMF in the presence of triethylamine. Following purification by C_{18} reversed phase HPLC, monoacylated pdCpAs **3c**, **3d**, and **3e** were obtained in yields of 14%, 27%, and 13%, respectively.

As for the aspartyl-pdCpAs, T4 RNA ligase-mediated condensation of aminoacylated pdCpA derivatives **3c**–**3e** with $tRNA_{CUA}$-C_{OH} afforded N-protected aminoacyl-$tRNA_{CUA}$s **4c**, **4d**, and **4e** in yields of 93%, 97%, and 96%, respectively. Brief (5-min) irradiation with a 500 W mercury–xenon lamp at 0 °C then effected removal of the NVOC protecting group, affording fully deprotected cysteinesulfinyl-$tRNA_{CUA}$ (**5c**), cysteinesulfonyl-$tRNA_{CUA}$ (**5d**), and homocysteinesulfonyl-$tRNA_{CUA}$ (**5e**), respectively. As anticipated, protection of the amino acid side chain functional groups proved to be unnecessary.

2.2. Synthesis of tRNAs activated with homocysteine

In comparison with tRNAs **5a**–**5e**, it seemed clear that the preparation of homocysteinyl-$tRNA_{CUA}$ (**11**) would require protection of the side chain SH group. As shown in Scheme 3, L-homocysteine (**6**) was treated with butyl 1-thiobutane-1-sulfinate in ethanol,[31] forming the S-*n*-butyl disulfide derivative. This was treated with 6-nitroveratryl chloroformate (NVOC-Cl) in 1:1 dioxane/H_2O in the presence of two equivalents of $NaHCO_3$, affording the *N*-6-nitroveratryl derivative **7** in 33% yield. Compound **7** was treated with chloroacetonitrile and triethylamine in acetonitrile to obtain cyanomethyl ester **8** in 69% yield. The cyanomethyl ester was treated with the tri-*n*-tetrabutylammonium salt of pdCpA[12] in dry DMF. Following purification by C_{18} reversed phase HPLC, the acylated pdCpA **9** was obtained in 28% yield. The aminoacylated pdCpA derivative **9** was ligated to the abbreviated $tRNA_{CUA}$-C_{OH} transcript[13] via the agency T4 RNA ligase to afford N,S-diprotected homocysteinyl-tRNA **10**. The efficiency of ligation was about 95%. The S-protecting group, *n*-butanethiol, was removed by treatment with 100 mM dithiothreitol (DTT); the NVOC group was then removed using high intensity UV light, affording the activated $tRNA_{CUA}$ bearing homocysteine (**11**).

2.3. Participation of the activated tRNAs in protein synthesis

The ability of the activated tRNAs to participate in protein synthesis was investigated initially in a cell-free protein biosynthesizing system. A plasmid encoding *E. coli* dihydrofolate reductase (DHFR), [32] which catalyzes the reduction of 7,8-dihydrofolic acid (DHF) to 5,6,7,8-tetrahydrofolic acid (THF) was used for this experiment.[33] Since aspartic acid 27 is an essential active site residue in DHFR, a stop codon (TAG) was introduced into the plasmid at position 27 of the DHFR gene.[32] Each of the misacylated tRNAs prepared here was found to be incorporated into position 27 of DHFR (Table 1). This is illustrated in Figure 2 for the tRNA activated with cysteic acid (**5d**). As shown in the figure, in the presence of cysteinesulfonyl-$tRNA_{CUA}$, a full length DHFR containing this modified amino acid was obtained in 45% yield (lane 3) relative to the amount of wild-type DHFR obtained using the unmodified DHFR mRNA (lane 1). In the absence of tRNA **5d**, minimal non-specific readthrough of the nonsense code was obtained (lane 2).

3. Discussion

In previous publications, we have described methods for preparing pdCpA derivatives and tRNAs activated with amino acids bearing functionalized side chains. These have included analogs of lysine[34] and arginine,[35] hydroxylated amino acids[36] and glycosylated amino acids.[19] Most of the amino acid analogs have required side chain protection to permit their introduction into pdCpA derivatives and tRNAs. For a limited number of aspartic acid derivatives studied previously, it was found that the presence of side chain protection could sometimes facilitate the incorporation of the modified amino acid into protein from the aminoacylated suppressor tRNA,[23,37] and even produce a caged protein useful for studying protein function.[23]

In the present study, we investigated methods for the activation of pdCpA and $tRNA_{CUA}$ with two aspartic acid derivatives, aspartic acid γ-methyl ester (**1a**) and asparagine (**1b**), bearing side chain functionality which seemed unlikely to require additional protection. Also studied was homocysteine (**6**) whose side chain SH group seemed likely to require protection, and three amino acids (cysteinesulfinic acid (**1c**), cysteic acid (**1d**), and homocysteic acid (**1e**)) whose requirement for side chain protection seemed less certain. The syntheses of activated pdCpAs and tRNAs bearing cysteinesulfinic acid, cysteic acid and homocysteic acid were investigated as outlined in Scheme 2. These three amino acids contain a strongly acidic functionality (–SO_3H or –SO_2H) in their side chains. The conjugate bases are rather weakly reactive, such that the initial transformations (to afford **2c**–**2e**) were simply carried out with an excess of $NaHCO_3$. However, the presence of the –SO_3H or –SO_2H groups did increase the polarity of the compounds and made the products difficult to purify by silica gel column chro-

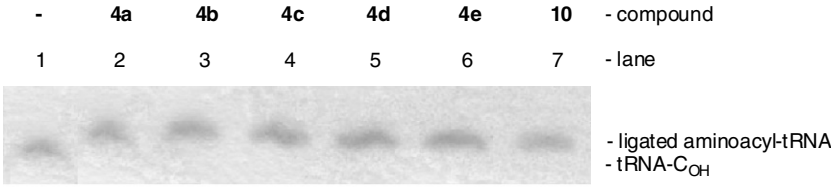
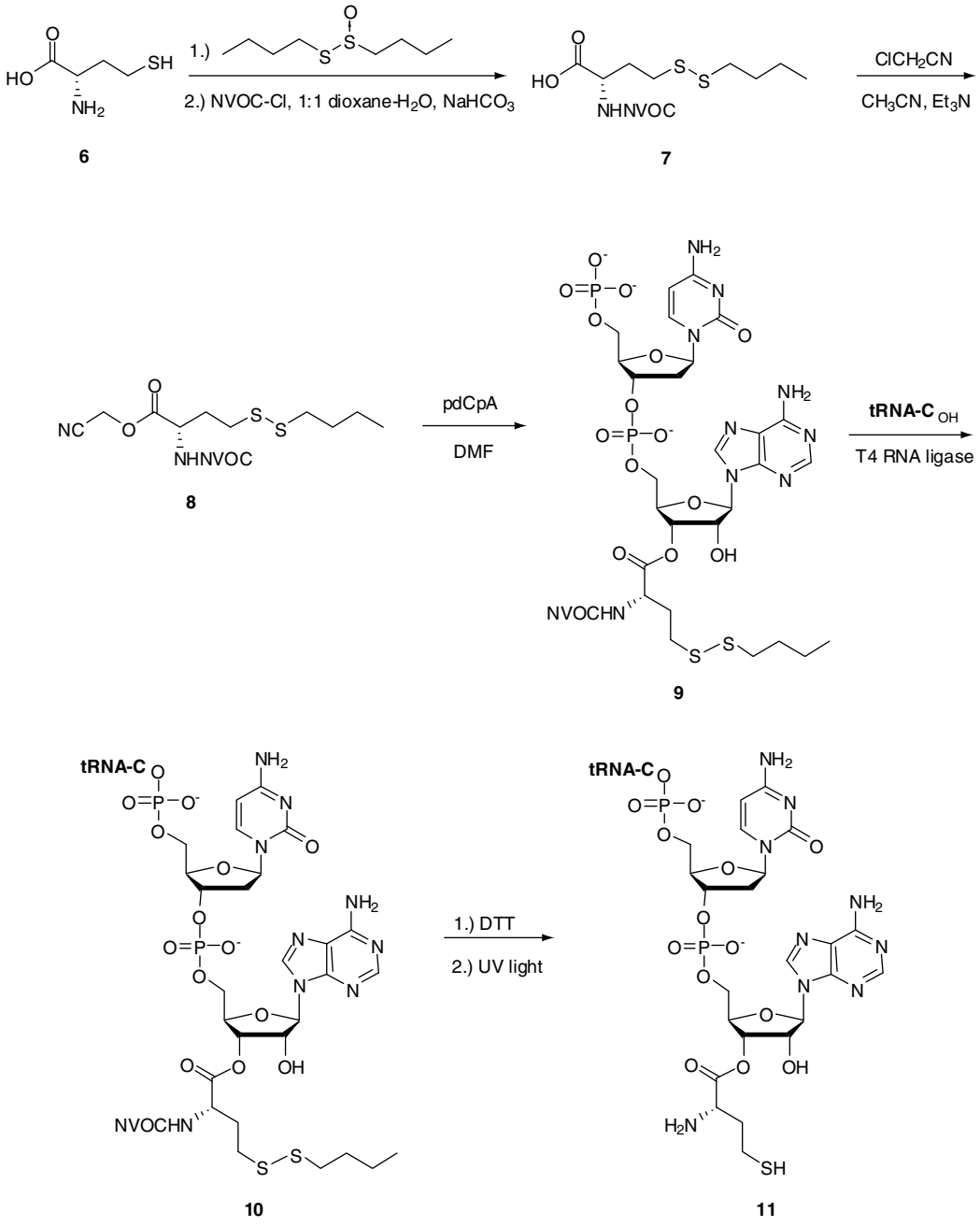


Figure 1. Acidic polyacrylamide gel (8%) illustrating the ligation of analogs of aspartyl-pdCpA and cysteinyl-pdCpA with $tRNA_{CUA}$-C_{OH}.



Scheme 3. Synthesis of $tRNA_{CUA}$ activated with homocysteine.

Table 1
Suppression efficiencies for DHFR synthesis at position 27 with six aminoacyl-$tRNA_{CUA}$s

Amino acid	Suppression efficiency (%)
No aminoacyl-tRNA	3
Aspartic acid methyl ester (**1a**)	35
Asparagine (**1b**)	13
Cysteinsulfinic acid (**1c**)	26
Cysteic acid (**1d**)	24
Homocysteic acid (**1e**)	15
Homocysteine (**6**)	22

matography. Thus, they were not purified after introduction of the NVOC protecting group. After the carboxyl groups were protected as cyanomethyl esters, the products could be purified by silica

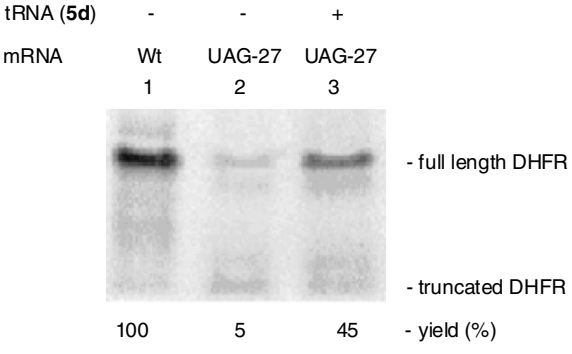


Figure 2. SDS–polyacrylamide gel (10%) illustrating the incorporation of cysteic acid into DHFR at position 27 by suppression of a UAG codon.

gel column in spite of the polar nature of the products. When treated with the tri-*n*-tetrabutylammonium salt of pdCpA[12] in dry DMF in the presence of triethylamine, NVOC-cysteinsulfinyl-pdCpA (**3c**), NVOC-cysteinesulfonyl-pdCpA (**3d**) and NVOC-homocysteinesulfonyl-pdCpA (**3e**) were obtained, albeit in low yields. These N-protected pdCpA derivatives were ligated to $tRNA_{CUA}$-C_{OH} using T4 RNA ligase to afford **4c**–**4e**. Deprotection of the NVOC groups by photolysis with a mercury–xenon lamp afforded smooth conversion to the deprotected aminoacylated $tRNA_{CUA}$s (**5c**–**5e**).

The synthesis of homocysteinyl-pdCpA and $tRNA_{CUA}$ was accomplished following protection of the SH group as the *n*-butyldisulfide by treatment with butyl 1-thiobutane-1-sulfinate.[31] Following synthesis of the fully protected homocysteinyl-tRNA, deprotection was achieved by successive treatments with dithiothreitol to reduce the disulfide moiety to an SH group, and photolysis to remove the NVOC group. The protocol used for the dithiothreitol treatment of the misacylated tRNA was optimized using cyanomethyl ester **8** as a model compound. The amino acid product resulting from cleavage of the disulfide bond in **8** was isolated and its composition was verified by mass spectrometry.

All of the aminoacyl-$tRNA_{CUA}$s prepared in this study were found to suppress a UAG codon at position 27 of DHFR mRNA with reasonable efficiencies. This is illustrated in Figure 2 for cysteinylsulfonyl-tRNA (**5d**) which produced DHFR containing cysteic acid at position 27 with an efficiency ~45% relative to the cell-free synthesis of wild-type DHFR from the unmodified mRNA. Thus, for tRNAs **5a**–**5e**, the presence of relatively polar side chain residues did not preclude reasonably efficient elaboration of the proteins modified at position 27. This result is more favorable than those noted previously for the incorporation of aspartic acid analogs into the same position of DHFR in earlier protein synthesis experiments.[32] It may be noted that oxidized cysteine analogs are of importance both at the amino acid[38,39] and protein levels,[40,41] so the ability to protect and activate such species is of potential importance.

4. Experimental

4.1. Synthesis of aminoacylated pdCpAs and tRNAs

Reagents and solvents for chemical synthesis were purchased from Aldrich Chemical Co. or Sigma Chemical Co. and used without further purification. Butyl 1-thiobutane-1-sulfinate was synthesized as previously reported.[31] All reactions involving air- or moisture-sensitive reagents or intermediates were performed under Ar. Flash chromatography was performed using Silicycle silica gel (40–60 mesh). Analytical TLC was performed using EM silica gel 60 F_{254} plates (0.25 mm), and was visualized by UV irradiation (254 nm). ^{1}H and ^{13}C NMR spectra were obtained using a 300 MHz Varian NMR instrument. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^{1}H resonance of the solvent ($CDCl_3$, δ 7.26; D_2O, δ 4.79). ^{13}C NMR spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, δ 77.3). Splitting patterns are designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were obtained at the Michigan State University—NIH Mass Spectrometry Facility. HPLC was carried out using a Varian 9012 pump, a Varian 2050 UV detector and an Alltech Alltima reversed phase C_{18} column (250 × 10 mm, 5 μm). High intensity UV light deprotection was performed using a 500 W Oriel mercury–xenon lamp.

T4 RNA ligase was purchased from New England Biolabs. [^{35}S]Methionine (1000 Ci/mmol, 10 μCi/μL) was purchased from Amersham Corporation. Protease inhibitor (complete, EDTA-free) was obtained from Boehringer Mannheim Corp. Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics.

4.1.1. *N*-(6-Nitroveratryloxycarbonyl)-γ-*O*-methyl-L-aspartic acid cyanomethyl ester (2a)

To a solution of 220 mg (1.1 mmol) of L-aspartic acid γ-methyl ester (**1a**)[42] and 185 mg (2.2 mmol) of $NaHCO_3$ in 2 mL of 1:1 H_2O/dioxane was added 330 mg (1.2 mmol) of 6-nitroveratryl chloroformate. The reaction mixture was stirred at room temperature for 1 h, then acidified with 1 N $NaHSO_4$ and extracted with three 10-mL portions of ethyl acetate. The organic phase was dried ($MgSO_4$), filtered, and concentrated. The crude product was dissolved in 5 mL of acetonitrile, then treated with 0.65 mL (4.7 mmol) of Et_3N and 0.6 mL (9.7 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 12 h under Ar. The reaction mixture was concentrated under diminished pressure, diluted with 10 mL of water and extracted with three 10-mL portions of ethyl acetate. The organic phase was dried ($MgSO_4$), filtered, and concentrated. The crude product was purified on a silica gel column (30 × 4 cm); elution with 1:1:0.1 ethyl acetate/hexanes/methanol gave **2a** as a yellow oil: yield 250 mg (53%); ^{1}H NMR ($CDCl_3$) δ 2.90–2.94 (m, 1H), 3.03–3.07 (m, 1H), 3.76 (s, 3H), 3.95 (s, 3H), 4.01 (s, 3H), 4.75 (t, 1H, J = 3.9 Hz), 4.80 (s, 2H), 5.45–5.64 (m, 2H), 5.92 (d, 1H, J = 8.7 Hz), 7.01 (s, 1H), and 7.72 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 49.65, 50.41, 52.63, 56.60, 56.77, 64.41, 76.89, 77.32, 77.74, 108.32, 109.95, 169.71, 169.77, 170.56, 170.66, 171.32, and 171.59; mass spectrum (FAB), *m/z* 426.1151 $(M+H)^+$ ($C_{17}H_{20}N_3O_{10}$ requires *m/z* 426.1149).

4.1.2. *N*-(6-Nitroveratryloxycarbonyl)-γ-*O*-methylaspartyl-pdCpA (3a)

To a solution of 3.0 mg (2.2 μmol) of pdCpA[12] in 150 μL of DMF was added 15 mg (35 μmol) of aspartic acid derivative **2a**. The reaction mixture was stirred at room temperature under Ar. A 10-μL aliquot of the reaction mixture was removed after 3 h, diluted with 100 μL of 1:2 CH_3CN/50 mmol NH_4OAc, pH 4.5, and analyzed by HPLC on a C_{18} reversed phase column. The column was washed with 1 → 63% CH_3CN/50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min. After an additional 12 h, the reaction mixture was diluted with 1.5 mL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified by HPLC on a C_{18} reversed phase column using the same conditions described above. Lyophilization afforded **3a** (t_R = 26.8 min) as a colorless oil: yield 0.8 mg (36%); mass spectrum (FAB), *m/z* 1003.1880 $(M-H)^-$ ($C_{34}H_{41}N_{10}O_{22}P_2$ requires *m/z* 1003.1872).

4.1.3. *N*-(6-Nitroveratryloxycarbonyl)-γ-*O*-methylaspartyl-tRNA (4a)

To a solution of 100 μL (total volume) of 100 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 2.0 A_{260} units of *N*-NVOC-γ-*O*-methylaspartyl-pdCpA (**3a**), 15% DMSO, and 200 U of T4 RNA ligase was added 100 μg of suppressor $tRNA_{CUA}$-C_{OH}.[13] After incubation at 37 °C for 30 min, the reaction mixture was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of EtOH. The reaction mixture was incubated at −20 °C for 30 min, centrifuged at 10,000*g* at 4 °C for 30 min, and then the supernatant was carefully decanted. The pellet was washed with 50 μL of 70% EtOH to obtain *N*-NVOC-γ-*O*-methylaspartyl-tRNA (**4a**). The efficiency of ligation (96%) was determined following 8% denaturing polyacrylamine gel electrophoresis at pH 5.2 for 4 h at 110 V.[30]

The transcription of suppressor $tRNA_{CUA}$-C_{OH} was carried out using an Ampliscribe T7 transcription kit in a buffered reaction mixture (200 μL) containing 7.5 mM each of ATP, CTP, GTP, and UTP, 10 mM dithiothreitol, 30 μg of *Fok 1*-linearized DNA template

and 20 μL of T7 RNA polymerase. The transcription reaction was run at 37 °C for 6 h. The incubation mixture was then treated with 10 μL of RNase-free DNase I and incubated at 37 °C for 15 min. The reaction mixture was treated with 20 μL of 3 M NaOAc, followed by 600 μL of EtOH. Following incubation at −20 °C for 30 min, the reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was then carefully decanted. The elaborated $tRNA_{CUA}$-C_{OH} transcript was dissolved in 200 μL of 0.1 M NaOAc, pH 5.2, and loaded onto a 400 μL DEAE-Sepharose CL-6B column. The column was washed with 400 μL of 0.1 M NaOAc, pH 5.2, and eluted successively with 400-μL portions of 0.1 M NaOAc, pH 5.2, containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and then 0.9 M NaCl. Each of the 400 μL eluates was treated with 900 μL of 2-propanol and incubated at 4 °C for 30 min. The reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was then carefully decanted. The pellets were washed with 100 μL of 70% ethanol, redissolved in 20 μL of RNase-free water, and analyzed by 8% denaturing polyacrylamine gel electrophoresis (100 V, 2 h). The fractions (0.5–0.8 M NaCl) tRNA-C_{OH} containing were pooled and stored at −80 °C.

4.1.4. γ-*O*-Methylaspartyl-$tRNA_{CUA}$ (5a)

A solution containing 100 μg of **4a** in 30 μL of water was irradiated with a 500 W mercury–xenon lamp at 0 °C for 5 min. Then 3 μL of 3 M NaOAc was added and the tRNA was precipitated by the addition of 100 μL of EtOH. The reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was carefully decanted. The pellet was washed with 20 μL of 70% ethanol and then dissolved in 30 μL of water to obtain γ-*O*-methylaspartyl-tRNA (**5a**).

4.1.5. *N*-(6-Nitroveratryloxycarbonyl)-L-asparagine cyanomethyl ester (2b)

To a solution of 100 mg (0.76 mmol) of L-asparagine (**1b**) and 135 mg (1.6 mmol) of $NaHCO_3$ in 20 mL of 1:1 H_2O/dioxane was added 220 mg (0.8 mmol) of 6-nitroveratryl chloroformate. The reaction mixture was stirred at room temperature for 1 h and then acidified with 1 N $NaHSO_4$. The resulting solid was filtered and dried. The crude product was dissolved in 20 mL of acetonitrile, then treated with 0.65 mL (4.7 mmol) of Et_3N and 0.6 mL (9.7 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 12 h under Ar. The reaction mixture was concentrated under diminished pressure, diluted with 10 mL of water, and extracted with three 10-mL portions of ethyl acetate. The combined organic phase was dried ($MgSO_4$), filtered, and concentrated. The crude product was purified on a silica gel column (30 × 4 cm); elution with 1:1:0.1 ethyl acetate/hexanes/methanol gave **2b** as a yellow oil: yield 78 mg (25%); ^{1}H NMR ($CDCl_3$) δ 2.84–2.92 (m, 1H), 3.13–3.23 (m, 1H), 3.91 (s, 3H), 3.97 (s, 3H), 4.40 (s, 2H), 4.47–4.54 (m, 1H), 5.40 (s, 2H), 5.46 (s, 2H), 6.27 (d, 1H, *J* = 7.2 Hz), 6.94 (s, 1H), and 7.62 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 26.18, 35.55, 50.51, 56.63, 56.82, 64.76, 108.38, 110.59, 113.70, 127.19, 139.77, 148.52, 153.95, 155.92, 172.80, and 174.45; mass spectrum (FAB), *m/z* 411.1149 $(M+H)^+$ ($C_{16}H_{19}N_4O_9$ requires *m/z* 411.1152).

4.1.6. *N*-(6-Nitroveratryloxycarbonyl)-asparaginyl-pdCpA (3b)

To a solution of 3.0 mg (2.2 μmol) of pdCpA[12] in 150 μL of DMF was added 15 mg (36 μmol) of asparagine derivative **2b**. The reaction mixture was stirred at room temperature under Ar for 3 h. A 10-μL aliquot of the reaction mixture was diluted with 100 μL of 1:2 CH_3CN/50 mmol NH_4OAc, pH 4.5, and the aliquot was analyzed by HPLC on a C_{18} reversed phase column. The column was washed with 1 → 63% CH_3CN/50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min. After an additional 12 h, the reaction mixture was diluted with 1.5 mL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified by HPLC on a C_{18} reversed phase column using the same conditions described above. Lyophilization afforded **3b** (t_R = 25.6 min) as a colorless oil: yield 0.5 mg (23%); mass spectrum (FAB), *m/z* 988.1878 $(M-H)^-$ ($C_{33}H_{40}N_{11}O_{21}P_2$ requires *m/z* 988.1875).

4.1.7. *N*-(6-Nitroveratryloxycarbonyl)-asparaginyl-tRNA (4b)

To a solution of 100 μL (total volume) of 100 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 2.0 A_{260} units of *N*-NVOC-asparaginyl-pdCpA (**3b**), 15% DMSO, and 200 U of T4 RNA ligase was added 100 μg of suppressor $tRNA_{CUA}$-C_{OH}.[13] After incubation at 37 °C for 30 min, the reaction mixture was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of EtOH. The reaction mixture was incubated at −20 °C for 30 min, centrifuged at 10,000*g* at 4 °C for 30 min, and then the supernatant was carefully decanted. The pellet was washed with 50 μL of 70% EtOH to obtain *N*-NVOC-asparaginyl-tRNA (**4b**). The ligation efficiency was 95%.

4.1.8. Asparaginyl-$tRNA_{CUA}$ (5b)

A solution containing 100 μg of **4b** in 30 μL of water was irradiated with a 500 W mercury–xenon lamp at 0 °C for 5 min. Then 3 μL of 3 M NaOAc was added and the tRNA was precipitated by the addition of 100 μL of EtOH. The reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was carefully decanted. The pellet was washed with 20 μL of 70% EtOH and then dissolved in 30 μL of water to obtain asparaginyl-$tRNA_{CUA}$ (**5b**).

4.1.9. *N*-(6-Nitroveratryloxycarbonyl)-cysteinesulfinic acid cyanomethyl ester (2c)

To a solution of 55 mg (0.32 mmol) of L-cysteinesulfinic acid monohydrate (**1c**) and 100 mg (1.2 mmol) of $NaHCO_3$ in 10 mL of 1:1 H_2O/dioxane was added 90 mg (0.33 mmol) of 6-nitroveratryl chloroformate. The reaction mixture was stirred at room temperature for 1 h, then concentrated a small volume and washed with three 10-mL portions of ethyl acetate. The aqueous phase was lyophilized to obtain a yellow solid. The crude product was dissolved in 5 mL of acetonitrile, then treated with 1 mL (7.2 mmol) of Et_3N and 0.7 mL (11 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 16 h under Ar. The reaction mixture was concentrated under diminished pressure, diluted with 5 mL of water, and extracted with three 10-mL portions of ethyl acetate. The combined organic phase was dried ($MgSO_4$), filtered, and concentrated. The crude product was purified on a silica gel column (30 × 4 cm); elution with 1:1:0.2 ethyl acetate/hexanes/methanol gave **2c** as a yellow oil: yield 45 mg (33%); ^{1}H NMR (D_2O) δ 3.43–3.48 (m, 2H), 3.63–3.70 (m, 1H), 3.78 (s, 3H), 3.85 (s, 3H), 4.98 (s, 2H), 5.15–5.25 (m, 2H), 6.82 (s, 1H), and 7.43 (s, 1H); ^{13}C NMR (D_2O) δ 50.24, 50.70, 50.97, 56.10, 56.43, 64.13, 107.86, 109.07, 115.28, 127.96, 138.38, 147.16, 153.42, 156.79, and 170.40; mass spectrum (FAB), *m/z* 430.0559 $(M-H)^-$ ($C_{15}H_{16}N_3O_{10}S$ requires *m/z* 430.0556).

4.1.10. *N*-(6-Nitroveratryloxycarbonyl)-cysteinesulfinyl-pdCpA (3c)

To a solution of 3.0 mg (2.2 μmol) of pdCpA[12] in 150 μL of DMF and 6 μL of triethylamine was added 15 mg (35 μmol) of cysteinesulfinic acid derivative **2c**. The reaction mixture was stirred at room temperature under Ar for 8 h. A 10-μL aliquot of the reaction mixture was diluted with 100 μL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and analyzed by HPLC on a C_{18} reversed phase column. The column was washed with 1 → 63% CH_3CN/50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min. After an additional 40 h, the reaction mixture was diluted with 1.5 mL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified by HPLC on a C_{18} re-

versed phase column using the same conditions described above. Lyophilization afforded **3c** (t_R = 22.8 min) as a colorless oil: yield 0.3 mg (14%); mass spectrum (FAB), *m/z* 1009.1441 $(M-H)^-$ ($C_{32}H_{39}N_{10}O_{22}P_2S$ requires *m/z* 1009.1436).

4.1.11. *N*-(6-Nitroveratryloxycarbonyl)-cysteinesulfinyl-tRNA$_{CUA}$ (4c)

To a solution of 100 μL (total volume) of 100 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 2.0 A_{260} units of *N*-NVOC-cysteinesulfinyl-pdCpA, 15% DMSO, and 200 U of T4 RNA ligase was added 100 μg of suppressor tRNA$_{CUA}$-C$_{OH}$.[13] After incubation at 37 °C for 30 min, the reaction mixture was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of EtOH. The reaction mixture was incubated at −20 °C for 30 min, centrifuged at 10,000*g* at 4 °C for 30 min, and then the supernatant was carefully decanted. The pellet was washed with 50 μL of 70% EtOH to obtain *N*-NVOC-cysteinsulfinyl-tRNA$_{CUA}$ (**4c**). The efficiency of ligation was 93%.

4.1.12. Cysteinesulfinyl-tRNA$_{CUA}$ (5c)

A solution containing 100 μg of **4c** in 30 μL of water was irradiated with a 500 W mercury–xenon lamp at 0 °C for 5 min. Then 3 μL of 3 M NaOAc was added and the tRNA was precipitated by the addition of 100 μL of EtOH. The reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was carefully decanted. The pellet was washed with 20 μL of 70% EtOH and then dissolved in 30 μL of water to obtain cysteinesulfinyl-tRNA$_{CUA}$ (**5c**).

4.1.13. *N*-(6-Nitroveratryloxycarbonyl)-cysteic acid cyanomethyl ester (2d)

To a solution of 100 mg (0.59 mmol) of L-cysteic acid (**1d**) and 200 mg (2.4 mmol) of $NaHCO_3$ in 5 mL of 1:1 H_2O/dioxane was added 170 mg (0.62 mmol) of 6-nitroveratryl chloroformate. The reaction mixture was stirred at room temperature for 1 h, then concentrated to a small volume and washed with three 10-mL portions of ethyl acetate. The aqueous phase was lyophilized to give a yellow solid. The crude product was dissolved in 5 mL of acetonitrile, then treated with 0.65 mL (4.7 mmol) of Et_3N and 0.6 mL (9.7 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 16 h under Ar. The reaction mixture was concentrated under diminished pressure, diluted with 5 mL of water and extracted with three 10-mL portions of ethyl acetate. The organic phase was dried ($MgSO_4$), filtered, and concentrated. The crude product was purified on a silica gel column (30 × 4 cm); elution with 1:1:0.2 ethyl acetate/hexanes/methanol gave **2d** as a yellow oil: yield 81 mg (31%); ^{1}H NMR (D_2O) δ 3.44–3.48 (m, 2H), 3.62–3.65 (m, 1H), 3.74 (s, 3H), 3.81 (s, 3H), 4.98 (s, 2H), 5.06–5.12 (m, 2H), 6.74 (d, 1H, *J* = 6.9 Hz), and 7.34 (d, 1H, *J* = 7.8 Hz); ^{13}C NMR (D_2O) δ 50.27, 50.98, 56.05, 56.42, 57.61, 64.05, 107.67, 108.82, 115.34, 128.03, 138.17, 147.06, 153.39, 156.69, and 170.42; mass spectrum (FAB), *m/z* 446.0510 $(M-H)^-$ ($C_{15}H_{16}N_3O_{11}S$ requires *m/z* 446.0506).

4.1.14. *N*-(6-nitroveratryloxycarbonyl)-cysteinesulfonyl-pdCpA (3d)

To a solution of 3.0 mg (2.2 μM) of pdCpA[12] in 150 μL DMF and 6 μL of triethylamine was added 15 mg (34 μmol) of cysteic acid derivative **2d**. The reaction mixture was stirred at room temperature under Ar for 8 h. A 10-μL aliquot of the reaction mixture was diluted with 100 μL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and the aliquot was analyzed by HPLC on a C_{18} reversed phase column. The column was washed with 1 → 63% CH_3CN/50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min. After an additional 40 h, the reaction mixture was diluted with 1.5 mL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified by HPLC on a C_{18} reversed phase column using the same conditions described above. Lyophilization afforded **3d** (t_R = 23.2 min) as a colorless oil: yield 0.6 mg (27%); mass spectrum (FAB), *m/z* 1025.1391 $(M-H)^-$ ($C_{32}H_{39}N_{10}O_{23}P_2S$ requires *m/z* 1025.1385).

4.1.15. *N*-(6-Nitroveratryloxycarbonyl)-cysteinesulfonyl-tRNA$_{CUA}$ (4d)

To a solution of 100 μL (total volume) of 100 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 2.0 A_{260} units of *N*-NVOC-cysteinesulfonyl-pdCpA, 15% DMSO, and 200 U of T4 RNA ligase was added 100 μg of suppressor tRNA$_{CUA}$-C$_{OH}$.[13] After incubation at 37 °C for 30 min, the reaction mixture was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of EtOH. The reaction mixture was incubated at −20 °C for 30 min, centrifuged at 10,000*g* at 4 °C for 30 min and then the supernatant was carefully decanted. The pellet was washed with 50 μL of 70% EtOH to obtain *N*-NVOC-cysteinesulfonyl-tRNA$_{CUA}$ (**4d**). The ligation efficiency was 97%.

4.1.16. Cysteinesulfonyl-tRNA (5d)

A solution containing 100 μg of 4**d** in 30 μL of water was irradiated with a 500 W mercury–xenon lamp at 0 °C for 5 min. Then 3 μL of 3 M NaOAc was added and the tRNA was precipitated by the addition of 100 μL of EtOH. The reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was carefully decanted. The pellet was washed with 20 μL of 70% EtOH and then dissolved in 30 μL of water to obtain cysteinesulfonyl-tRNA$_{CUA}$ (**5d**).

4.1.17. *N*-(6-Nitroveratryloxycarbonyl)-homocysteic acid cyanomethyl ester (2e)

To a solution of 100 mg (0.55 mmol) of L-homocysteic acid (**1e**) and 200 mg (2.4 mmol) of $NaHCO_3$ in 10 mL of 1:1 H_2O/dioxane was added 160 mg (0.58 mmol) of 6-nitroveratryl chloroformate. The reaction mixture was stirred at room temperature for 1 h, then concentrated to a small volume and washed with three 10-mL portions of ethyl acetate. The aqueous phase was lyophilized to give a yellow solid. The crude product was dissolved in 5 mL of acetonitrile, then treated with 1 mL (7.2 mmol) of Et_3N and 0.7 mL (11 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 16 h under Ar. The reaction mixture was concentrated under diminished pressure, diluted with 5 mL of water, and extracted with three 10-mL portions of ethyl acetate. The combined organic phase was dried ($MgSO_4$), filtered, and concentrated. The crude product was purified on a silica gel column (30 × 4 cm); elution with 1:1:0.2 ethyl acetate/hexanes/methanol gave **2e** as a yellow oil: yield 150 mg (59%); ^{1}H NMR (D_2O) δ 2.06–2.22 (m, 2H), 3.02 (t, 2H, *J* = 7.8 Hz), 3.69 (s, 3H), 3.77 (s, 3H), 4.46–4.51 (m, 1H), 4.90–5.06 (m, 4H), 6.68 (s, 1H), and 7.24 (s, 1H); ^{13}C NMR (D_2O) δ 26.19, 47.36, 49.16, 50.14, 52.85, 56.03, 56.39, 107.53, 108.66, 115.55, 127.85, 138.06, 147.03, 153.34, 156.96, and 171.62; mass spectrum (FAB), *m/z* 460.0667 $(M-H)^-$ ($C_{16}H_{18}N_3O_{11}S$ requires *m/z* 460.0662).

4.1.18. *N*-(6-Nitroveratryloxycarbonyl)-homocysteinesulfonyl-pdCpA (3e)

To a solution of 3.0 mg (2.2 μmol) of pdCpA[12] in 150 μL of DMF and 6 μL of triethylamine was added 15 mg (33 μmol) of homocysteine derivative **2e**. The reaction mixture was stirred at room temperature under Ar for 8 h. A 10-μL aliquot of the reaction mixture was diluted with 100 μL of 1:2 CH_3CN/50 mmol NH_4OAc, pH 4.5, and analyzed by HPLC on a C_{18} reversed phase column. The column was washed with 1 → 63% CH_3CN/50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min. After an additional 40 h, the reaction mixture was diluted with 1.5 mL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified by HPLC on a C_{18} reversed

phase column using the same conditions described above. Lyophilization afforded **3e** (t_R = 23.8 min) as a colorless oil: yield 0.3 mg (13%); mass spectrum (FAB), *m/z* 1039.1550 $(M-H)^-$ ($C_{33}H_{41}N_{10}O_{23}P_2S$ requires *m/z* 1039.1542).

4.1.19. *N*-(6-Nitroveratryloxycarbonyl)-homocysteinesulfonyl-tRNA$_{CUA}$ (4e)

To a solution of 100 μL (total volume) of 100 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 2.0 A_{260} units of *N*-NVOC-homocysteinesulfonyl-pdCpA, 15% DMSO, and 200 U of T4 RNA ligase was added 100 μg of suppressor tRNA$_{CUA}$-C$_{OH}$.[13] After incubation at 37 °C for 30 min, the reaction mixture was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of EtOH. The reaction mixture was incubated at −20 °C for 30 min, centrifuged at 10,000*g* at 4 °C for 30 min and then the supernatant was carefully decanted. The pellet was washed with 50 μL of 70% EtOH to obtain *N*-NVOC-homocysteinesulfonyl-tRNA$_{CUA}$ (**4e**). The ligation efficiency was 96%.

4.1.20. Homocysteinesulfonyl-tRNA$_{CUA}$ (5e)

A solution containing 100 μg of **4e** in 30 μL of water was irradiated with a 500 W mercury–xenon lamp at 0 °C for 5 min. Then 3 μL of 3 M NaOAc was added and the tRNA was precipitated by the addition of 100 μL of EtOH. The reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was carefully decanted. The pellet was washed with 20 μL of 70% EtOH and then dissolved in 30 μL of water to obtain homocysteinesulfonyl-tRNA$_{CUA}$ (**5e**).

4.1.21. *N*-(6-Nitroveratryloxycarbonyl)-homocysteinyl *n*-butyl disulfide (7)

To a suspension of 460 mg (3.4 mmol) of homocysteine (**6**) in 100 mL of anhydrous EtOH was added 1.2 g (6.2 mmol) of freshly prepared butyl 1-thiobutane-1-sulfinate[31] in 40 mL of chloroform. The reaction mixture was stirred at 40 °C overnight, and then 100 mL of water was added. The solution was concentrated to 20 mL under diminished pressure and was washed with ethyl ether. The aqueous layer was maintained at 4 °C overnight. The precipitated white solid was collected and dried in vacuo. The crude product was dissolved in 30 mL of 1:1 H_2O/dioxane and was treated with 500 mg (6.0 mmol) of $NaHCO_3$ and 750 mg (2.7 mmol) of 6-nitroveratryl chloroformate. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was adjusted to pH < 3 with 1 N HCl and extracted with three 30-mL portions of ethyl acetate. The combined organic phase was dried ($MgSO_4$), filtered, and concentrated. The crude product was applied to a silica gel column (30 × 4 cm); elution with 1:1:0.1 ethyl acetate/hexanes/methanol gave **7** as a yellow oil: yield 520 mg (33%); ^{1}H NMR ($CDCl_3$) δ 0.93 (t, 3H, *J* = 7.5 Hz), 1.37–1.45 (m, 4H), 1.70–1.74 (m, 2H), 3.09–3.22 (m, 4H), 3.92 (s, 3H), 3.95 (s, 3H), 4.33–4.36 (m, 1H), 5.46 (s, 2H), 7.03 (s, 1H), 7.64 (s, 1H), 8.02 (s, 1H), and 9.95 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.88, 21.17, 22.02, 25.61, 33.14, 37.08, 55.87, 56.56, 56.82, 64.12, 108.18, 109.98, 128.39, 139.52, 148.13, 156.32, 163.50, and 177.21; mass spectrum (FAB), *m/z* 461.1055 $(M-H)^-$ ($C_{18}H_{25}N_2O_8S_2$ requires *m/z* 461.1052).

4.1.22. *N*-(6-Nitroveratryloxycarbonyl)-homocysteinyl *n*-butyl disulfide cyanomethyl ester (8)

To a solution of 100 mg (0.22 mmol) of *N*-(6-nitroveratryloxycarbonyl)-homocysteinyl *n*-butyl disulfide (**7**) in 5 mL of acetonitrile was added 1.0 mL (7.2 mmol) of Et_3N and 0.70 mL (11.3 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 16 h under Ar. The reaction mixture was concentrated under diminished pressure, diluted with 5 mL of water and extracted with three 10-mL portions of ethyl acetate. The organic phase was dried ($MgSO_4$), filtered, and concentrated under diminished pressure. The crude product was applied to a silica gel column (30 × 4 cm); elution with 1:1 ethyl acetate/hexanes gave (**8**) as a yellow oil: yield 75 mg (69%); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, *J* = 6.9 Hz), 1.32–1.40 (m, 2H), 1.56–1.63 (m, 2H), 2.06–2.13 (m, 1H), 2.30–2.36 (m, 1H), 2.63–2.72 (m, 4H), 3.91 (s, 3H), 3.96 (s, 3H), 4.51–4.58 (m, 1H), 4.77–4.79 (m, 2H), 5.39–5.54 (m, 2H), 5.77 (br, 1H), 6.96 (s, 1H), and 7.64 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 13.90, 21.80, 31.28, 31.41, 34.28, 38.73, 49.51, 52.92, 56.61, 56.76, 64.33, 108.33, 110.13, 114.23, 127.87, 139.75, 148.34, 153.89, 155.81, and 171.07; mass spectrum (FAB), *m/z* 500.1165 $(M-H)^-$ ($C_{20}H_{26}N_3O_8S_2$ requires *m/z* 500.1161).

4.1.23. *N*-(6-Nitroveratryloxycarbonyl)-homocysteinyl-pdCpA *n*-butyl disulfide (9)

To a solution of 3.0 mg (2.2 μmol) of pdCpA[12] in 150 μL of DMF was added 15 mg (30 μmol) of homocysteine derivative **8**. The solution was stirred at room temperature under Ar for 8 h. A 10-μL aliquot of the reaction mixture was diluted with 100 μL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and analyzed by HPLC on a C_{18} reversed phase column. The column was washed with 1 → 63% CH_3CN/50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min. After 12 h, the reaction mixture was diluted with 1.5 mL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified by HPLC on a C_{18} reversed phase column using the same conditions described above. Lyophilization afforded **9** (t_R = 23.0 min) as a colorless oil: yield 0.7 mg (28%); mass spectrum (FAB), *m/z* 1079.2052 $(M-H)^-$ ($C_{37}H_{49}N_{10}O_{20}P_2S_2$ requires *m/z* 1079.2041).

4.1.24. Mono-*N*-(6-nitroveratryloxycarbonyl)-homocysteinyl-tRNA$_{CUA}$ *n*-butyl disulfide (10)

To a solution of 100 μL (total volume) of 100 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 2.0 A_{260} units of NVOC-homocysteinyl-pdCpA *n*-butyl disulfide (**9**), 15% DMSO, and 200 U of T4 RNA ligase was added 100 μg of suppressor tRNA$_{CUA}$-C$_{OH}$.[13] After incubation at 37 °C for 30 min, the reaction mixture was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of EtOH. The reaction mixture was maintained at −20 °C for 30 min, centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was carefully decanted. The pellet was washed with 50 μL of 70% EtOH to obtain NVOC-homocysteinyl-tRNA *n*-butyl disulfide (**10**). The efficiency of ligation was 95%.

4.1.25. Homocysteinyl-tRNA$_{CUA}$ (11)

A solution containing 100 μg of **10** in 100 μL of water was treated with 10 μL of 1 M DTT and then incubated at 4 °C for 12 h. Then 11 μL of 3 M NaOAc was added. The tRNA was precipitated by the addition of 400 μL of EtOH. The reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was carefully decanted. The pellet was dissolved in 30 μL of water and irradiated with a 500 W mercury–xenon lamp at 0 °C for 5 min. Then 3 μL of 3 M NaOAc was added and the tRNA was precipitated by the addition of 100 μL of EtOH. The reaction mixture was centrifuged at 10,000*g* at 4 °C for 30 min and the supernatant was carefully decanted. The pellet was washed with 20 μL of 70% EtOH and then dissolved in 30 μL of water to obtain homocysteinyl-tRNA$_{CUA}$ (**11**).

4.2. In vitro translation of modified DHFRs

The DHFR plasmid containing a TAG codon at position 27 was utilized in an in vitro protein synthesis system[43] as exemplified below for cysteinyl-tRNA$_{CUA}$ (**5d**). The reaction mixture (200 μL total volume) containing 20 μg of plasmid DNA, 80 μL of premix (35 mM Tris–acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 11 mM magnesium acetate, 20 mM phospho-(enol)pyruvate, 0.8 mg/mL of *E. coli* tRNA,

0.8 mM isopropyl β-D-thiogalactopyranoside, 20 mM ATP and GTP, 5 mM CTP and UTP, and 4 mM cAMP),[44] 100 μM of each of the 20 amino acids, 30 μCi of [^{35}S]methionine, 10 μg/μL of rifampicin, 80 μg of cysteinesulfonyl-tRNA$_{CUA}$ (**5d**), and 60 μL of bacterial S-30 extract from *E. coli* strain BL21(DE3) was incubated at 37 °C for 45 min. As a positive control, wild-type DHFR was expressed using the same system; as a negative control, in vitro translation of the modified DHFR was also carried out in the absence of any misacylated tRNA$_{CUA}$. Aliquots were taken for electrophoretic analysis using 10% SDS–PAGE. Autoradiography of the gel was carried out to determine the location of the ^{35}S-labeled protein; quantification of the bands was carried out using a phosphorimager.

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