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Synthesis of bisaminoacylated pdCpAs and tandemly activated transfer RNAs

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Abstract—Described herein is the preparation of new bisacylated tRNAs and their participation in protein synthesis. It has been reported that *Thermus thermophilus* phenylalanyl-tRNA synthetase can introduce two phenylalanine moieties onto the 3′-terminal adenosine of its cognate tRNA. It is also possible to prepare bisactivated tRNAs in vitro; these participate in protein synthesis [Wang, B.; Zhou, J.; Lodder, M.; Anderson, R. D.; Hecht, S. M. *J. Biol. Chem.* **2006**, *281*, 13865]. Presently, the chemical strategy used for the synthesis of the key intermediate bisacylated pdCpAs is described. Bis-*S*-alanyl- and bis-*S*-methionyl-pdCpAs were prepared initially. Further, *S*-threonine, *S*-*allo*-threonine, *S*-homoserine, and (*S*)-(+)-2-amino-3-hydroxy-3-methylbutyric acid were coupled with the dinucleotide to define preparative methods applicable to more complex amino acids bearing additional functionality in the form of an OH group.

1. Introduction

Ribosomally mediated protein synthesis employs aminoacylated transfer RNAs for the introduction of each of the 20 proteinogenic amino acids into proteins.[1–3] There is at least one transfer RNA for each of the amino acids, as well as an activating enzyme specific for the amino acid and its cognate tRNA(s) that mediates attachment of the amino acid at the 3′-end of the tRNA in the form of an activated ester.[4] General methods for the misacylation of tRNAs,[5–7] and the site-specific incorporation of non-natural amino acids into peptides and proteins have been developed.[8–14] This method for in vitro non-natural amino acid mutagenesis is based on the expansion of the genetic code by suppressing a normal stop codon with a misacylated suppressor tRNA transcript.[15] This misacylated tRNA is obtained by the coupling of a protected 2′(3′)-*O*-aminoacylated 5′-*O*-phosphoryl-2′-(deoxy)cytidylyl(3′ → 5′) adenosine (p(d)CpA, Fig. 1A) with a tRNA transcript lacking the 3′-terminal pCpA moiety.[15] The 2′(3′)-*O*-aminoacylated pdCpA derivatives are substrates for T4 RNA ligase and can, therefore, be coupled efficiently to the truncated tRNA.[5–7,15]

Ribosomal protein synthesis is believed to involve a set of (mono)aminoacylated tRNAs that can decode individual mRNA codons during protein translation. However, Stepanov et al. have described the ability of phenylalanyl-tRNA synthetase from *Thermus thermophilus* to introduce two phenylalanine moieties onto the 3′-terminal adenosine moiety of its cognate tRNA.[16,17] Although the ability of such tandemly activated tRNAs to participate in protein synthesis was not explored, presumably due to the difficulty of obtaining sufficient material in pure form, it seemed possible that they might function in protein biosynthesis.

In fact, in a recent publication, our laboratory has described the ability of tandemly activated tRNAs to participate in protein synthesis.[18] The bisaminoacylated tRNAs employed for that study were prepared by extension of a strategy that has been used widely for the introduction of unnatural amino acids into specific, predetermined positions in proteins.[10–13]

Presently, we describe the synthesis of new bisaminoacylated pdCpA derivatives (Fig. 1B) and their use in the preparation of tandemly activated tRNAs. The general strategy for the synthesis of aminoacyl-pdCpA derivatives consists of the coupling of the N-protected amino

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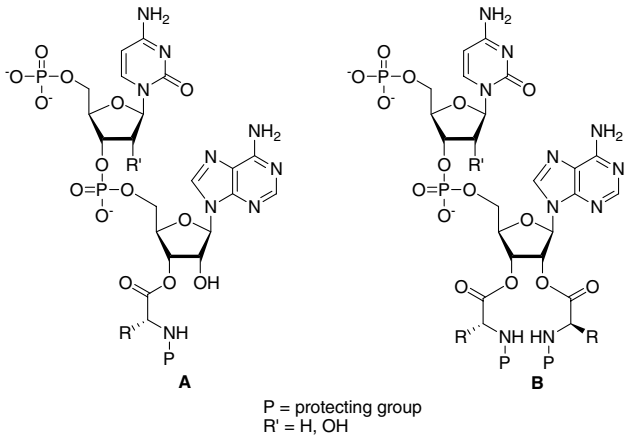


Figure 1. Structures of (A) 2′(3′)-*O*-aminoacylated dinucleotide 5′-*O*-phosphoryl-(2′-deoxy)cytidylyl(3′ → 5′)adenosine and (B) 2′,3′-bis-*O*-aminoacylated dinucleotide 5′-*O*-phosphoryl-(2′-deoxy)cytidylyl (3′ → 5′)adenosine.

acid, activated as a cyanomethyl ester, with the dinucleotide pdCpA (Schemes 1 and 2). Subsequent incubation in the presence of $tRNA_{CUA}$-C_{OH}+ T4 RNA ligase, and deblocking of the derived N-protected aminoacyl-tRNA, leads to the desired activated tRNA (Scheme 2). This method has proven successful for a wide variety of unnatural amino acids. The bisaminoacylation of tRNA has been realized in a similar manner using a bisaminoacylated dinucleotide in the ligation reaction.[18] Presently, the preparation of bisaminoacylated pdCpA derivatives bearing *S*-methionine and *S*-alanine is reported. Further, to generalize the synthetic strategy leading to bisactivated pdCpAs, more complex amino acids were studied, including *S*-threonine, *S*-*allo*-threonine, *S*-homoserine, and *S*-(+)-2-amino-3-hydroxy-3-methylbutyric acid, in which the additional OH group must be protected. Protection–deprotection strategies leading to the preparation of bisacylated pdCpAs and the final bisactivated tRNAs in good yields are described. The ability of the derived tRNAs to function as participants in protein synthesis is also illustrated.

The bisaminoacylated tRNAs described herein are important as they may represent a type of activated tRNA utilized normally in protein synthesis within certain types of organisms such as thermophilic bacteria. To the extent that this is true, the present study will enable the characterization of the way in which a newly recognized type of activated tRNA functions in protein synthesis. Additionally, bisaminoacylated tRNAs exhibit surprising stability both as chemical species and in in vitro protein biosynthesizing systems. As such, they have the wherewithal to provide significantly enhanced yields of proteins elaborated in cell free systems.

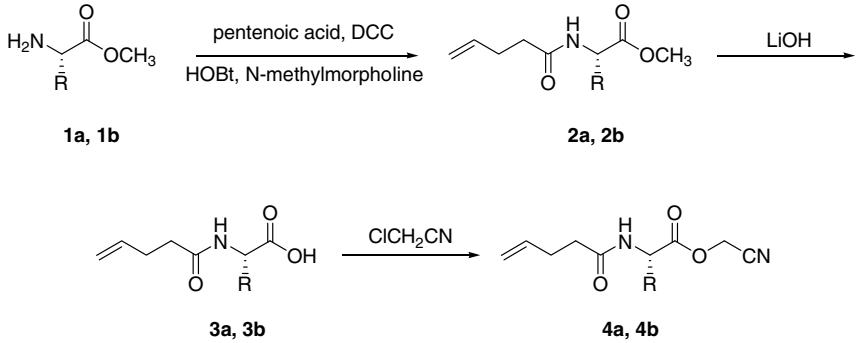
2. Results

2.1. Synthesis of alanine and methionine analogues

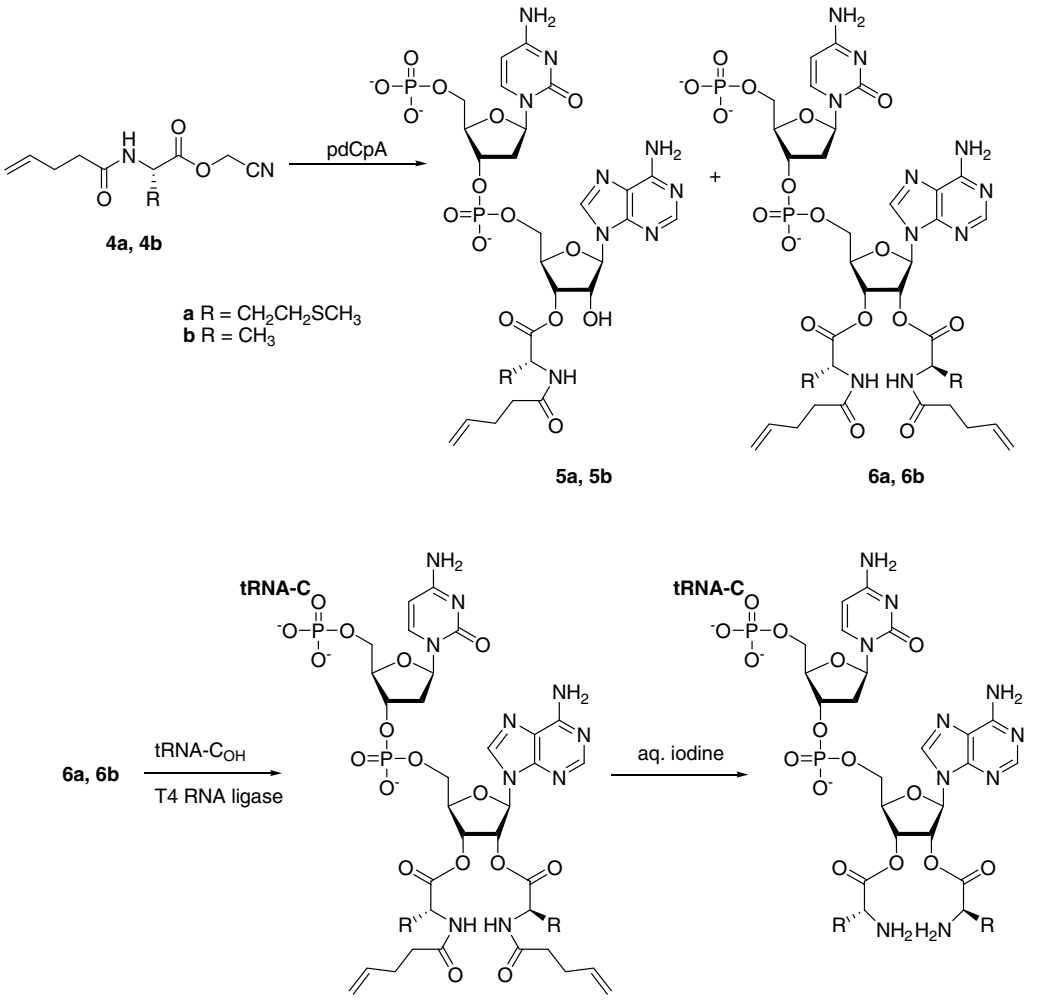
Bisacylated transfer RNAs bearing *S*-alanine and *S*-methionine were prepared as outlined in Schemes 1 and 2. *S*-methionine and *S*-alanine methyl esters (**1a** and **1b**) were protected as *N*-pentenoyl derivatives[19,20] by treatment with pentenoic acid, *N*-methylmorpholine, hydroxybenzotriazole, and *N*′,*N*′-dicyclohexylcarbodiimide. The products (**2a** and **2b**) were isolated as colorless oils in yields of 84% and 64%, respectively (Scheme 1). Following treatment with aq LiOH (25 °C, 3 h), the carboxylates (**3a** and **3b**) were treated with chloroacetonitrile and Et_3N, affording cyanomethyl esters **4a** and **4b** in yields of 58% and 12%, respectively. Each of the cyanomethyl esters was admixed with the tetra-*n*-butylammonium salt of pdCpA[15] in DMF (Scheme 2),[21] which afforded mixtures of the monoacylated pdCpA (**5a** and **5b**) and bisacylated pdCpA (**6a** and **6b**) derivatives. The yield of the latter was increased by simply extending the period of time utilized for acylation of pdCpA. The mono- and bisacylated pdCpAs were separable by C_{18} reversed phase HPLC, as illustrated in Figure 2 for the methionine derivatives. For methionine, the monoacylated derivative (**5a**) was isolated in 70% yield (based on limiting pdCpA) as a colorless solid, which was a mixture of equilibrating 2′- and 3′-*O*-aminoacyl derivatives (Fig. 2). The desired bisaminoacylated derivative (**6a**) was isolated as a colorless solid in 30% yield. The corresponding alanyl-pdCpA derivatives (**5b** and **6b**) were also obtained as colorless solids in yields of 46% and 26%, respectively.

2.2. Synthesis of threonine, *allo*-threonine, and homoserine analogues

Following the successful synthesis of bisalanyl- and bismethionyl-pdCpA derivatives the chemical prepara-



Scheme 1. Synthesis of *N*-pentenoyl-*S*-methionine cyanomethyl ester (**4a**) and *N*-pentenoyl-*S*-alanine cyanomethyl ester (**4b**).



Scheme 2. Synthesis of bismethionyl-pdCpA (**6a**) and bisalanyl-pdCpA (**6b**), and T4 RNA ligase-mediated construction of tandemly activated tRNAs.

tion of bisacylated pdCpA derivatives bearing chemically more complex amino acids was explored. The preparation of threonine and its analogues was addressed, in particular using *S*-threonine, *S*-*allo*-threonine, *S*-homoserine, and 2-amino-3-hydroxy-3-methylbutyric acid. These compounds required the development of strategies for protection and subsequent deprotection of primary, secondary, and tertiary hydroxyl groups to enable the condensation of these amino acids with pdCpA.

The same synthetic sequence was used for *S*-threonine (**7a**), *S*-*allo*-threonine (**7b**), and *S*-homoserine (**7c**) (Scheme 3); however, for *S*-(+)-2-amino-3-hydroxy-3-methylbutyric acid (**12**) a slightly modified route was used (Scheme 4).

As shown in Scheme 3, compounds **7a**–**7c** were treated with thionyl chloride and MeOH to afford the respective methyl esters (**8a**–**8c**) which were used as crude products in the next step. *N*-pentenoyl protection was carried out in a manner similar to that described for the synthesis of **2a** and **2b**, to give **9a**–**9c** in 75%, 77%, and 75% yields (over two steps), respectively. Subsequent TBS protection of the secondary (**9a** and **9b**) or primary alcohol (**9c**) was achieved using TBSCl, imidazole, and DMF to afford **10a′**–**10c′** in 91%, 92%, and 90% yields, respectively. Saponification of the methyl ester using LiOH, followed by treatment with chloroacetonitrile, triethylamine in MeCN, gave the cyanomethyl esters **11a′**–**11c′** in 85%, 87%, and 80% yields, respectively. Given that *S*-(+)-2-amino-3-hydroxy-3-methylbutyric acid (**12**, Scheme 4) contains a tertiary alcohol it seemed likely treatment with thionyl chloride in MeOH would result in dehydration. Accordingly, TMS–diazomethane was utilized to selectively methylate the carboxyl group of *N*-pentenoyl protected amino acid **13**, affording **14** in 38% yield over two steps. Attempted protection of the tertiary alcohol with TBSCl, imidazole, and DMF failed to produce the desired O-silylated product. Therefore, triethylsilyl triflate (TESOTf) was employed using 2,6-lutidine as the base to give the desired product **15** in 90% yield. The remaining steps were identical to those used for the other analogues and afforded **16** in 62% yield over two steps.

Each of the cyanomethyl esters **11a′**–**11c′** and **16** was admixed with the tetra-*n*-butylammonium salt of pdCpA[15]

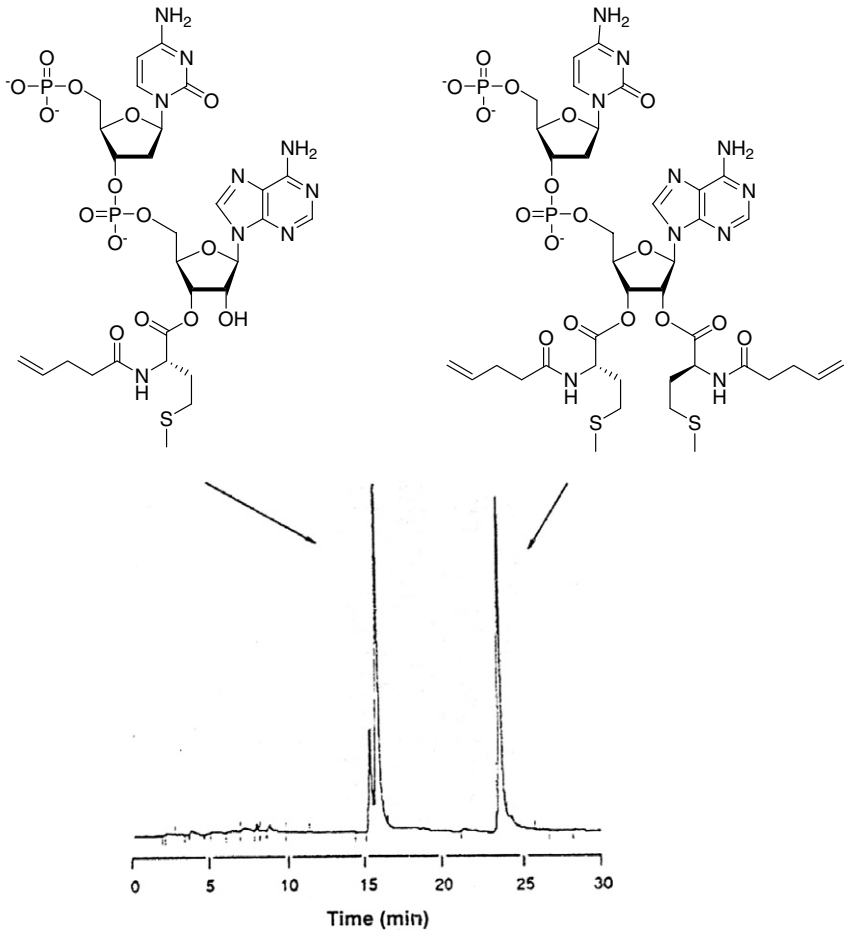
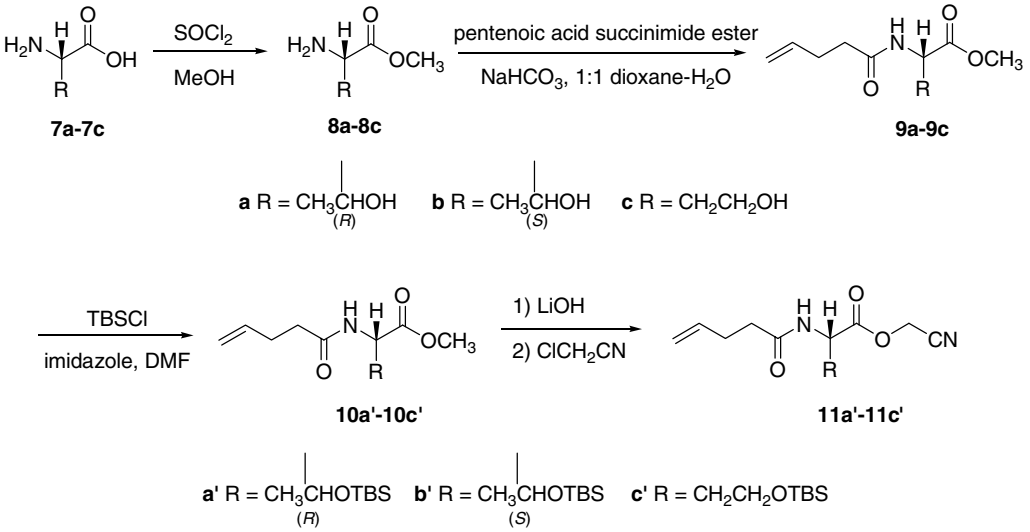
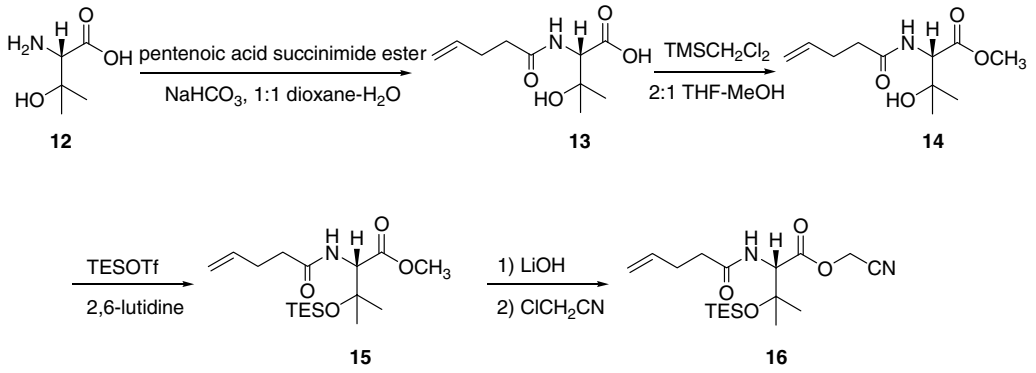


Figure 2. Separation of monomethionyl-pdCpAs from bismethionyl-pdCpA on C_{18} reversed phase HPLC. Monomethionyl-pdCpA eluted first as a mixture of (equilibrating) 2′- and 3′-*O*-aminoacyl derivatives (~16.0 and 16.5 min), followed by the bisacylated derivative (~24 min).

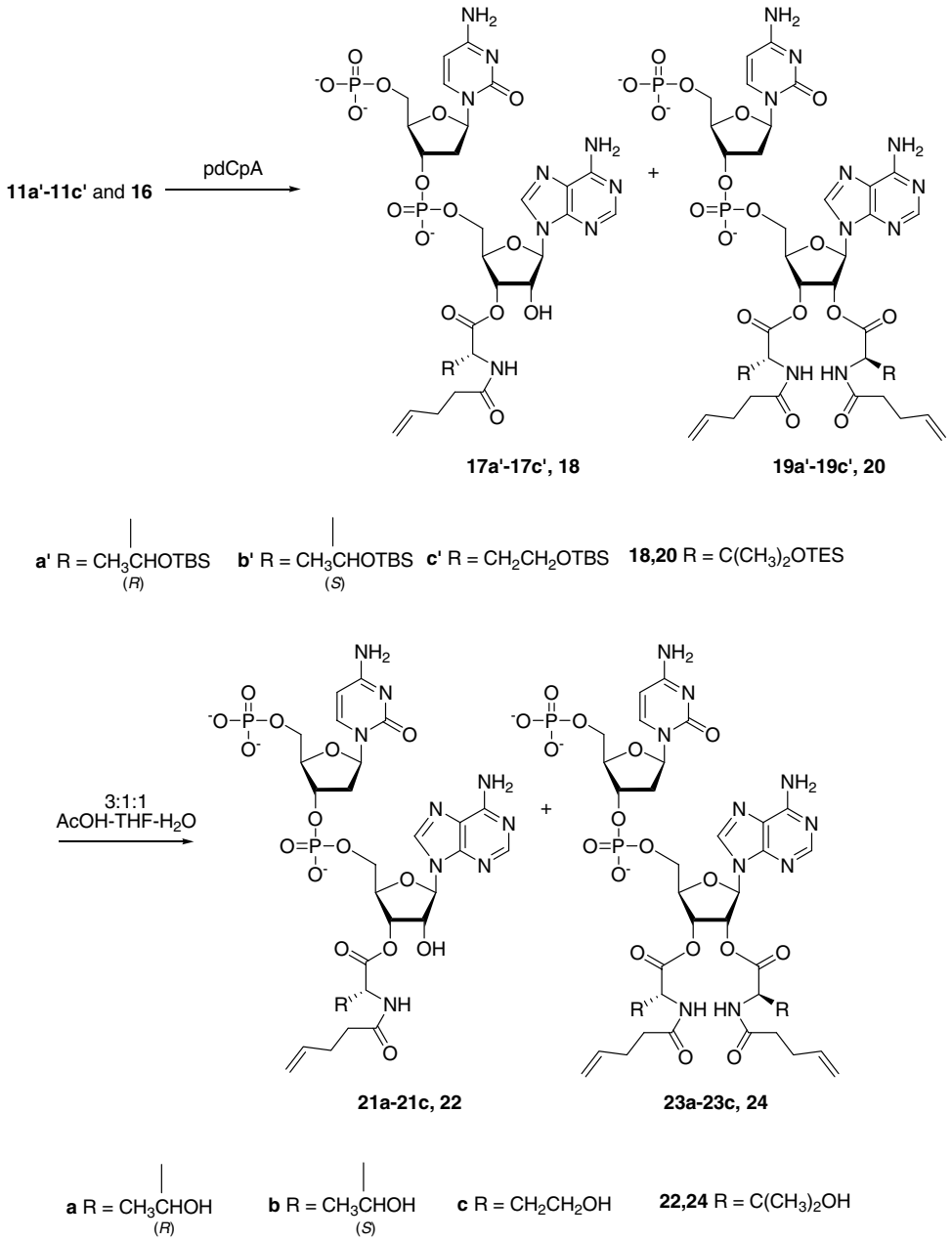


Scheme 3. Synthesis of *N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-threonine cyanomethyl ester (**11a**′), *N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-*allo*-threonine cyanomethyl ester (**11b**′) and *N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-homoserine cyanomethyl ester (**11c**′).

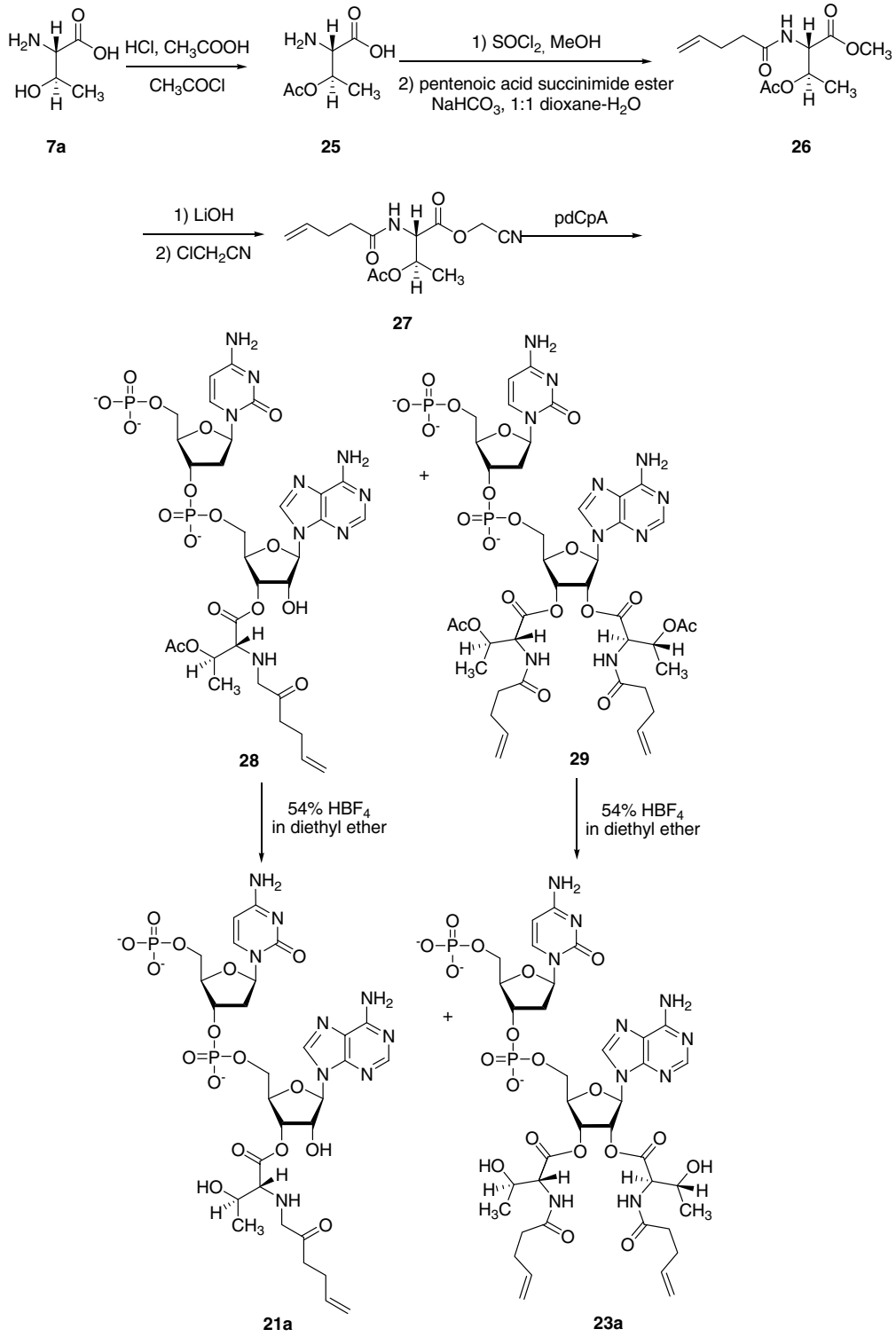
in DMF (Scheme 5), which afforded mixtures of the silyl-protected monoacylated pdCpA (**17a**′–**17c**′ and **18**) and bisacylated pdCpA derivatives (**19a**′–**19c**′ and **20**). The mono- and bisacylated pdCpA derivatives were separated by C_{18} reversed phase HPLC using a gradient of 0 → 63% acetonitrile in 50 mM NH_4OAc, pH 4.5. Monoaminoacylated compounds **17a**′–**17c**′ were obtained in yields of 65%, 55%, and 60%, respectively,



Scheme 4. *S*-*N*-((4-pentenoyl)-2-amino)-3-triethylsilyloxy-3-hydroxy-3-methylbutyric acid cyanomethyl ester (**16**).



Scheme 5. Synthesis of bis-*O*-silyl-protected threonyl-pdCpAs (**19a′**–**19c′** and **20**).



7a **25** **26** **27** **28** **29** **21a** **23a**

Scheme 6. Synthesis of bis[*O*-acetyl-*N*-(4-pentenoyl)-*S*-threonyl]-pdCpA (**29**).

while **18** was obtained in 50% yield. As expected, the bisacylated derivatives (**19a′**–**19c′** and **20**) were obtained in lower yields, about 10% in all cases. In the belief that steric hindrance due to the trialkylsilyl groups probably inhibited the formation of the bisacylated derivative, the less hindered acetyl group was tested as an alternative for the protection step. In this case, the acetyl protected *S*-threonine analogue (**25**) (Scheme 6) was prepared using hydrochloric acid, glacial acetic acid, and acetyl chloride.[22] The remaining steps, from **25** → **26** → **27** → **28** + **29**, were the same as those described above for the silyl-protected analogues. However, this route afforded monoacylated derivative **28** in 38% yield, while bisacylated **29** was obtained in 40% yield after 3 days of stirring at room temperature. The acetyl protecting group thus appears to be more suitable for the prepara-

tion of hydroxylated amino acid derivatives such as the threonine derivatives studied here.

In order to conjugate the monoacylated and bisacylated derivatives so obtained with tRNA-C_{OH} and subsequently use them in protein synthesis, the deprotection of both silyl-protected and acetyl-protected derivatives was carried out. Deprotection of TBS and TES groups was achieved by treatment of compounds **17a′**–**17c′**, **18**, **19a′**–**19c′**, and **20** (Scheme 5) with a mixture 3:1:1 AcOH/THF/H_2O, according to a reported procedure.[21] The deprotected derivatives were obtained in 50–70% yields. The acetyl groups were removed from compounds **28** and **29** using a 54% solution of HBF_4 in diethyl ether,[23] leading to deprotected derivatives **21a** and **23a** in 81% and 43% yields, respectively (Scheme 6).

2.3. Synthesis of bisactivated tRNAs

The HPLC-purified N-protected bisaminoacylated pdCpA derivatives (**6a** and **6b**) were each ligated to abbreviated suppressor $tRNA_{CUA}$ transcripts lacking the cytidine and adenosine moieties normally present at the 3′-terminus of all tRNAs.[15,24] The tRNA transcript was obtained by in vitro run-off transcription of a *Fok1*-linearized plasmid encoding the abbreviated $tRNA_{CUA}$.[25] The ligation reaction was catalyzed by T4 RNA ligase (Scheme 2).[5,7,8] The completion of the ligation reaction was verified by polyacrylamide gel electrophoresis at pH 4.5.[26] The formed *N*-pentenoyl bisacylated tRNAs were deprotected by treatment with aqueous iodine as described.[19,20]

The ability of the bisacylated tRNAs to participate in protein synthesis was investigated in a prokaryotic protein biosynthesizing system programmed with the mRNA for a dihydrofolate reductase construct in which a nonapeptide was fused at the N-terminus of wild-type DHFR. A stop codon (UAG) was introduced within the fused nonapeptide, one codon upstream from the original initiation codon (AUG) in wild-type mRNA. As shown in Figure 3, in the presence of the deprotected bismethionyl- and bisalanyl-$tRNA_{CUA}$s, full length fusion protein was obtained by suppression of the UAG codon at the position immediately upstream from the initiator AUG codon of wild-type dihydrofolate reductase. The suppression yields for bismethionyl- and bisalanyl-tRNAs were 12% and 17%, respectively. In contrast, the bisaminoacylated tRNAs containing the *N*-pentenoyl protecting groups were not able to effect suppression of the UAG codon. As is clear from Figure 3, in addition to full length protein, there was also some initiation of protein synthesis from methionine codons downstream from the UAG codon and initiation codon at position 1. While this affords bands in addition to full length DHFR fusion protein, the construct is especially valuable for judging the efficiency of the suppression reaction, since protein synthesis can proceed even in the absence of suppression.

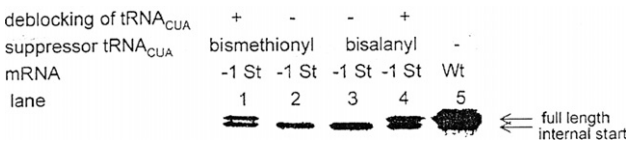


Figure 3. Introduction of alanine and methionine into *E. coli* dihydrofolate reductase by suppression of a UAG codon. The UAG codon was introduced within an N-terminal fusion peptide, one codon upstream from the initiator (AUG) codon present in wild-type mRNA.

3. Discussion

In vitro protein mutagenesis has been shown to be a powerful tool for modifying proteins in a site-specific manner, and thereby permitting the detailed study of their structure, function, and interactions. The introduction of both natural and non-natural amino acids at predetermined positions permits the analysis of these parameters at high resolution. The finding that bisaminoacylated tRNAs function well in cell free protein synthesizing systems, exhibit better stability in those systems, and can afford greater amounts of proteins under conditions limiting for the misacylated tRNA[18] has prompted us to explore methods for the efficient preparation of bisaminoacylated tRNAs. The synthesis of bisaminoacylated pdCpA derivatives, which are precursors of the tandemly activated tRNAs, was the objective of the present study.

Initially the synthesis of bismethionyl- and bisalanyl-pdCpAs (**6a** and **6b**) was investigated. These two amino acids do not contain reactive side chains with functional groups, such that the synthesis of the bisacylated analogues did not pose major obstacles, except for the relatively low yields and long reaction times. HPLC purification is also required, but pure products can be obtained readily (Schemes 1 and 2). The study of more complex amino acids such as threonine analogues was then addressed. The compounds employed (**7a**–**7c** and **12**) contain an additional side-chain hydroxyl group that can be tertiary (**12**), secondary (**7a** and **7b**) or primary (**7c**). This hydroxyl group requires differential protection, or else one may expect participation of the OH group during the pdCpA coupling reaction. The protecting group must also be removed under conditions compatible with stability of the formed bisaminoacyl-pdCpA derivatives. Schultz and co-workers reported that TBS ethers could be removed successfully from aminoacylated pdCpA derivatives using 3:1:1 AcOH/THF/H_2O at 37 °C.[27] Given this report, it seemed appropriate to use this protecting group for the O-protection of threonine and its analogues. Initial N-protection was accomplished using the pentenoyl group which had been shown previously to be removed easily using I_2 in aqueous THF.[19,20] *S*-threonine and its analogues were converted to the silylated cyanomethyl esters in 5-step sequences as shown in Scheme 3. Analogue **16**, however, was synthesized in a slightly different manner. The tertiary hydroxyl group presented two problems, namely (i) acidic treatment to form the methyl esters may result in dehydration of the tertiary alcohol and (ii) the tertiary hydroxyl group could not be successfully protected readily using standard conditions for TBS ethers given its hindered nature. Accordingly, the methyl ester was

formed using TMS–diazomethane.[28] TESOTf was then used to protect the tertiary alcohol. The coupling of the fully protected cyanomethyl esters (**11a′**–**11c′** and **16**) with pdCpA led to the formation of monoacylated derivatives (**17a′**–**17c′** and **18**, respectively) as well as the bisacylated compounds (**19a′**–**19c′** and **20**, respectively). However, the yield of the bisaminoacylated analogues was only about 10% in all cases. Since this was probably due to the presence of the sterically hindered silyl protecting groups, an *O*-acetyl group was explored as a possible alternative. Accordingly, *N*-(4-pentenoyl)-*O*-acetyl-*S*-threonine cyanomethyl ester (**27**) was obtained in 5 steps, as shown in Scheme 6, and coupled with pdCpA. In this case the yield of the desired bisaminoacylated pdCpA was much higher (40%), indicating that the *O*-acetyl protecting group is better suited to the preparation of the desired hydroxylated amino acid derivatives. While the purpose of the present study was the preparation of bisaminoacyl-tRNAs, the findings made concerning protection of threonine with an acetyl group should also find utility in the synthesis of misacylated monoaminoacylated tRNAs.

As described recently,[18] bisaminoacylated tRNAs have significant advantages for use in cell free protein synthesizing systems. These include the ability of the bisacylated tRNAs to incorporate both appended amino acids into protein in two rounds of protein synthesis, resulting in higher yields of proteins when the activated tRNAs are used at limiting concentrations. It has also been shown that bisacylated tRNAs have much greater stability in cell free protein synthesizing systems than the respective monoacylated species.[18] For example, valyl-pdCpA was 78% hydrolyzed to pdCpA + valine when maintained at pH 7.8 and 37 °C for 1 h. In contrast, 2′-*O*-alanyl-3′-*O*-valyl-pdCpA was not hydrolyzed to a detectable extent under the same conditions. An analogous finding was made for mono- and bisaminoacylated tRNAs when maintained in an in vitro protein synthesizing system for 30 min in the absence of messenger RNA.[18] This suggests that the bisaminoacylated tRNAs may find utility in continuous cell free protein synthesizing systems. In addition, the reports that at least one bisaminoacylated tRNA occurs naturally raise the possibility that such tRNAs are normal participants in protein biosynthesis in some organisms. If this proves to be correct, the present study will enable the study of the participation of bisaminoacylated tRNAs as constituents of protein synthesizing systems.

4. Experimental

4.1. Chemistry

Reagents and solvents were purchased from Aldrich Chemical Co. or Sigma Chemical Co. and used without further purification. Anhydrous grade methylene chloride, THF, DMF, and acetonitrile were purchased from VWR Scientific. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under an argon atmosphere. Flash chromatography was performed using Silicycle 40–60 mesh silica gel. Analytical TLC was performed using 0.25 mm EM silica gel 60 F_{250} plates that were visualized by irradiation (254 nm) or by staining with Hanessian's stain (cerium molybdate). ^{1}H and ^{13}C NMR spectra were obtained using a 300 MHz Varian 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; CD_3OD, δ 3.31). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, δ 77.3; DMSO-d_6, δ 39.5). 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. Phosphoroimager analysis was performed using a Molecular Dynamics 300E Phosphorimager equipped with Image Quant software. HPLC was performed using a Varian 9012 pump coupled with a Varian 2050 UV detector and an Alltech Alltima RPC_{18} column (250 × 10 mm, 5 μm).

4.1.1. *N*-4-Pentenoyl-*S*-methionyl-pdCpA (5a) and bis(*N*-4-pentenoyl-*S*-methionyl)-pdCpA (6a). To a conical vial containing 4.10 mg (15.1 μmol) of *N*-4-pentenoyl-*S*-methionine cyanomethyl ester (**4a**)[20] was added a solution of 4.10 mg (3.00 μmol) of the tris(tetrabutylammonium) salt of pdCpA[15] in 50 μL of DMF. The reaction mixture was stirred at room temperature. After 3 h the reaction mixture was diluted with 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5, to a total volume of 400 μL and purified using a semi-preparative C_{18} reversed phase column (250 × 10 mm). The column was washed with 1 → 63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). After lyophilization of the appropriate fractions both compounds were obtained as colorless solids: *N*-4-pentenoyl-*S*-methionyl-pdCpA (**5a**) (retention times 16.0 and 16.5 min, for the two positional (2′,3′) isomers): yield 1.8 mg (70%); mass spectrum (FAB), *m*/*z* 850.200 $(M+H)^+$ ($C_{29}H_{42}N_9O_{15}P_2S$ requires 850.200). Bis(*N*-4-pentenoyl-*S*-methionyl)-pdCpA (**6a**) (retention time 24 min): yield 1 mg (30%); mass spectrum (FAB), *m*/*z* 1063.280 $(M+H)^+$ ($C_{39}H_{57}N_{10}O_{17}P_2S_2$ requires *m*/*z* 1063.280).

4.1.2. *N*-4-Pentenoyl-*S*-alanyl-pdCpA (5b) and bis(*N*-4-pentenoyl-*S*-alanyl)-pdCpA (6b). To a conical vial containing 3.50 mg (16.6 μmol) of *N*-(4-pentenoyl)-*S*-alanine cyanomethyl ester (**4b**)[20] was added a solution of 4.50 mg (3.30 μmol) of the tris(tetrabutylammonium) salt of pdCpA[15] in 50 μL of DMF. The reaction mixture was stirred at room temperature. After 3 h the reaction mixture was diluted with 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5 to a total volume of 400 μL and purified using a semi-preparative C_{18} reversed phase column (250 × 10 mm). The column was washed with 1 → 63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 35 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). After lyophilization of the appropriate fractions both compounds were obtained as colorless solids: *N*-(4-pentenoyl)-*S*-alanyl-pdCpA (**5b**) (retention times 12.8 and 13.3 min, for the two positional (2′,3′) isomers) yield 1.20 mg (46%); mass spectrum (FAB), *m*/*z* 790.1932

$(M+H)^+$ ($C_{27}H_{38}N_9O_{15}P_2$ requires *m/z* 790.1962). Bis(*N*-4-pentenoyl-*S*-alanyl)-pdCpA (**6b**) (retention time 21 min) yield 0.8 mg (26%); mass spectrum (FAB), *m/z* 943.2777 $(M+H)^+$ ($C_{35}H_{49}N_{10}O_{17}P_2$ requires *m/z* 943.2752).

4.1.3. *N*-(4-Pentenoyl)-*S*-threonine methyl ester (9a). To a solution containing 500 mg (4.20 mmol) of *S*-threonine (**7a**) in 4 mL of MeOH was added 0.30 mL of thionyl chloride. The reaction mixture was heated to reflux for 1 h, then concentrated to dryness. The residue was redissolved in 4 mL of MeOH and again treated with 0.3 mL of thionyl chloride, heated to reflux for 1 h, and then concentrated. A 320-mg (1.89 mmol) portion of this crude material was dissolved in 5 mL of H_2O and 320 mg (1.89 mmol) of $NaHCO_3$ was added. To this stirred solution was added 410 mg (2.08 mmol) of 4-pentenoic acid succinimide ester[29] in 5 mL of dioxane. After stirring at room temperature overnight the reaction mixture was diluted with 10 mL of ethyl acetate and 10 mL of 1 N $NaHSO_4$, then extracted with three 20-mL portions of ethyl acetate. The combined organic extract was dried ($MgSO_4$) and concentrated under diminished pressure. The crude product was purified by flash chromatography on a silica gel column (21 × 3 cm); elution with 2:1 ethyl acetate/hexane gave **9a** as a colorless solid: yield 305 mg (75%); ^{1}H NMR ($CDCl_3$) δ 1.15 (d, 3H, J = 6.6 Hz), 2.36 (m, 4H), 3.72 (br s, 1H), 4.29 (s, 3H), 4.29 (dq, 1H, J = 6.3 and 1.8 Hz), 4.54 (dd, 1H, J = 9.0 and 1.8 Hz), 5.00 (m, 2H), 5.80 (m, 1H), and 6.60 (d, 1H, J = 9.0 Hz); ^{13}C NMR ($CDCl_3$) δ 19.88, 29.42, 35.49, 52.44, 57.21, 67.72, 115.60, 136.69, 171.52, and 173.20; mass spectrum (FAB), *m/z* 216.1235 $(M+H)^+$ ($C_{10}H_{18}NO_4$ requires *m/z* 216.1236).

4.1.4. *N*-(4-Pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-threonine methyl ester (10a′). To a solution containing 300 mg (1.40 mmol) of **9a** in 5 mL of anhydrous DMF were added 420 mg (2.79 mmol) of *O*-*tert*butyldimethylsilyl chloride (TBSCl) and 210 mg (3.08 mmol) of imidazole. The reaction mixture was stirred at room temperature under argon overnight, then diluted with 50 mL of brine and extracted with two 60-mL portions of ethyl acetate. The combined organic layer was washed with 100 mL of H_2O, dried ($MgSO_4$), and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (21 × 2 cm) using 3:1 hexanes/ethyl acetate as eluant gave **10a′** as a colorless oil: yield 420 mg (91%); ^{1}H NMR ($CDCl_3$) δ 0.02 (s, 6H), 0.83 (s, 9H), 1.12 (d, 3H, J = 6.0 Hz), 2.42 (m, 4H), 3.67 (s, 3H), 4.40 (m, 1H), 4.56 (d, 1H, J = 8.1 Hz), 5.02 (m, 2H), 5.82 (m, 1H), and 6.12 (d, 1H, J = 9.3 Hz); ^{13}C NMR ($CDCl_3$) δ −5.39, −4.48, 17.76, 20.84, 25.56, 29.39, 35.65, 52.14, 57.55, 68.75, 115.59, 136.84, 171.06, and 172.52; mass spectrum (FAB), *m/z* 330.2099 $(M+H)^+$ ($C_{16}H_{32}NO_4Si$ requires *m/z* 330.2101).

4.1.5. *N*-(4-Pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-threonine cyanomethyl ester (11a′). To a suspension containing 420 mg (1.27 mmol) of **10a′** in 5 mL of 1:1 THF/H_2O was added 160 mg (3.81 mmol) of LiOH. The reaction mixture was stirred under argon at room temperature and monitored by TLC; after 3 h the starting material had been consumed, so the reaction mixture was diluted with 60 mL of ethyl acetate and washed with 25 mL of 1 N $NaHSO_4$, dried ($MgSO_4$), and concentrated under diminished pressure. The crude residue was dissolved in anhydrous acetonitrile and 480 mg (6.35 mmol) of chloroacetonitrile was added followed by 640 mg (6.35 mmol) of triethylamine. The reaction mixture was stirred at room temperature overnight, then diluted with 50 mL of ethyl acetate and washed with 25 mL of 1 N $NaHSO_4$, dried ($MgSO_4$), and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (15 × 2 cm), elution with 2:1 hexanes/ethyl acetate, gave **11a′** as a colorless oil: yield 383 mg (85%); ^{1}H NMR ($CDCl_3$) δ 0.02 (s, 6H), 0.84 (s, 9H), 1.18 (d, 3H, J = 6.3 Hz), 2.41 (m, 4H), 4.43 (dq, 1H, J = 14.4 and 1.8 Hz), 4.60 (dd, 1H, J = 9.3 and 2.1 Hz), 4.73 (m, 2H), 5.06 (m, 2H), 5.83 (m, 1H) and 6.14 (d, 1H, J = 9.3 Hz); ^{13}C NMR ($CDCl_3$) δ −5.24, −4.28, 17.81, 20.92, 25.61, 29.31, 35.53, 48.54, 48.94, 57.56, 68.42, 115.89, 136.69, 169.45, and 172.76; mass spectrum (FAB), *m/z* 355.2052 $(M+H)^+$ ($C_{17}H_{31}N_2O_4Si$ requires *m/z* 355.2053).

4.1.6. *N*-(4-Pentenoyl)-*S*-*allo*-threonine methyl ester (9b). To a solution containing 250 mg (2.10 mmol) of *S*-*allo*-threonine (**7b**) in 2 mL of MeOH was added 0.15 mL of thionyl chloride. The reaction mixture was heated to reflux for 1 h, then concentrated to dryness. The residue was redissolved in 2 mL of MeOH and again treated with 0.15 mL of thionyl chloride, heated to reflux for 1 h, and then dried. A 350-mg (2.07 mmol) portion of this crude material was dissolved in 5 mL of H_2O and 347 mg (4.14 mmol) of $NaHCO_3$ was added. To this stirred solution was added 447 mg (2.27 mmol) of 4-pentenoic acid succinimide ester[29] in 5 mL of dioxane. After stirring at room temperature overnight the reaction mixture was diluted with 10 mL of ethyl acetate and 10 mL of 1 N $NaHSO_4$, then extracted with three 20-mL portions of ethyl acetate. The combined organic extract was dried ($MgSO_4$) and concentrated under diminished pressure. The crude product was purified by flash chromatography on a silica gel column (21 × 3 cm); elution with 2:1 ethyl acetate/hexane gave **9b** as a colorless solid: yield 342 mg (77%); ^{1}H NMR ($CDCl_3$) δ 1.14 (d, 3H, J = 6.6 Hz), 2.40 (m, 4H), 3.46 (br s, 1H), 3.70 (s, 3H), 4.13 (m, 1H), 4.66 (dd, 1H, J = 7.2 and 3.3 Hz), 5.02 (m, 2H), 5.80 (m, 1H), and 6.56 (d, 1H, J = 6.0 Hz); ^{13}C NMR ($CDCl_3$) δ 18.73, 29.35, 35.41, 52.58, 58.19, 68.93, 115.80, 136.59, 170.68, and 173.35; mass spectrum (FAB), *m/z* 216.1234 $(M+H)^+$ ($C_{10}H_{18}NO_4$ requires *m/z* 216.1236).

4.1.7. *N*-(4-Pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-*allo*-threonine methyl ester (10b′). To a solution containing 342 mg (1.60 mmol) of **9b** in 5 mL of anhydrous DMF were added 0.48 g (3.20 mmol) of TBSCl and 270 mg (4.00 mmol) of imidazole. The reaction mixture was stirred at room temperature under argon overnight, then diluted with 50 mL of brine and extracted with two 60-mL portions of ethyl acetate. The combined organic layer was washed with 100 mL of H_2O, dried ($MgSO_4$), and concentrated under diminished pressure. Purification

by flash chromatography on a silica gel column (15 × 2 cm) using 3:1 hexanes/ethyl acetate gave **10b′** as a colorless oil: yield 480 mg (92%); ^{1}H NMR ($CDCl_3$) δ 0.04 (s, 6H), 0.86 (s, 9H), 1.27 (d, 3H, J = 6.6 Hz), 2.37 (m, 4H), 3.75 (s, 3H), 4.56 (dd, 1H, J = 7.8 and 3.3 Hz), 4.58 (m, 1H), 5.10 (m, 2H), 5.86 (m, 1H), and 6.27 (d, 1H, J = 7.8 Hz); ^{13}C NMR ($CDCl_3$) δ −5.06, −4.51, 17.84, 20.70, 25.58, 29.33, 35.68, 52.05, 58.24, 70.00, 115.64, 136.83, 170.45, and 171.59; mass spectrum (FAB), m/z 330.2099 $(M+H)^+$ ($C_{16}H_{32}NO_4Si$ requires m/z 330.2101).

4.1.8. *N*-(4-Pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-*allo*-threonine cyanomethyl ester (11b′). To a suspension containing 480 mg (1.47 mmol) of **10b′** in 5 mL of 1:1 THF/H_2O was added 180 mg (4.42 mmol) of LiOH. The reaction mixture was stirred under argon at room temperature and monitored by TLC; after 3 h the starting material had been consumed, so the reaction mixture was diluted with 60 mL of ethyl acetate and washed with 25 mL of 1 N $NaHSO_4$, dried ($MgSO_4$), and concentrated under diminished pressure. The crude residue was dissolved in anhydrous acetonitrile and 550 mg (7.35 mmol) of chloroacetonitrile was added followed by 740 mg (7.35 mmol) of triethylamine. The reaction mixture was stirred at room temperature overnight, then diluted with 50 mL of ethyl acetate and washed with 25 mL of 1 N $NaHSO_4$, dried ($MgSO_4$), and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (15 × 2 cm), elution with 2:1 hexanes/ethyl acetate, gave **11b′** as a colorless oil: yield 450 mg (87%); ^{1}H NMR ($CDCl_3$) δ 0.05 (s, 6H), 0.86 (s, 9H), 1.27 (d, 3H, J = 6.6 Hz), 2.38 (m, 4H), 4.08 (m, 1H), 4.60 (dd, 1H, J = 7.8 and 3.3 Hz), 4.74 (m, 2H), 5.04 (m, 2H), 5.80 (m, 1H) and 6.20 (d, 1H, J = 7.8 Hz); ^{13}C NMR ($CDCl_3$) δ −5.10, −4.45, 17.82, 20.68, 25.55, 29.20, 35.47, 48.63, 58.09, 69.77, 113.75, 115.82, 136.60, 168.60, and 171.85; mass spectrum (FAB), m/z 355.2051 $(M+H)^+$ ($C_{17}H_{31}N_2O_4Si$ requires m/z 355.2053).

4.1.9. *N*-(4-Pentenoyl)-*S*-homoserine methyl ester (9c). To a solution containing 300 mg (2.52 mmol) of *S*-homoserine (**7c**) in 4 mL of MeOH was added 0.15 mL of thionyl chloride. The reaction mixture was heated to reflux for 1 h, then concentrated to dryness. The residue was redissolved in 4 mL of MeOH and again treated with 0.15 mL of thionyl chloride, heated to reflux for 1 h, and then concentrated under diminished pressure. The crude material obtained was then dissolved in 5 mL of water and 160 mg (1.89 mmol) of $NaHCO_3$ was added. To this stirred solution was added 200 mg of 4-pentenoic acid succinimide ester[29] in 5 mL of dioxane. After stirring at room temperature overnight the reaction mixture was diluted with 10 mL of ethyl acetate and 10 mL of 1 N $NaHSO_4$, then extracted with three 20-mL portions of ethyl acetate. The combined organic extract was dried over $MgSO_4$, and concentrated under diminished pressure. The crude product was purified by flash chromatography on a silica gel column (15 × 2 cm); elution with 9:1 methylene chloride/methanol gave **9c** as a colorless oil: yield 400 mg (75%); ^{1}H NMR ($CDCl_3$) δ 2.00–2.14 (m, 4H), 2.40–2.48 (m, 2H), 2.70 (br s, 1H), 3.54 (s, 3H), 4.00–4.06 (q, 1H, J = 9.2 and 9.0 Hz), 4.26 (t, 1H, J = 9 Hz), 4.46 (q, 1H, J = 9.2 and 9.0 Hz), 4.84–4.91 (m, 2H), 5.34–5.44 (m, 1H), and 7.09 (br s,1H); ^{13}C NMR ($CDCl_3$) δ 25.5, 49.1, 66.4, 67.1, 115.6, 115.9, 136.7, 173.3, 174.4, and 177.3; mass spectrum (CI), m/z 238.0 $(M+Na^+)$ (theoretical m/z 238.1); mass spectrum (FAB), m/z 216.1235 $(M+H)^+$ ($C_{10}H_{18}NO_4$ requires m/z 216.1236).

4.1.10. *N*-(4-Pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-homoserine methyl ester (10c′). To a solution containing 400 mg (1.86 mmol) of **9c** in 5 mL of anhydrous DMF were added 420 mg (2.79 mmol) of TBSCl and 210 mg (3.08 mmol) of imidazole. The reaction mixture was stirred at room temperature under argon overnight, then diluted with 50 mL of brine and extracted with two 60-mL portions of ethyl acetate. The combined organic phase was washed with 100 mL of water, dried over $MgSO_4$, and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (20 × 3 cm) using methylene chloride–methanol as the eluant gave **10c′** as a colorless oil: yield 560 mg (90%); ^{1}H NMR ($CDCl_3$) δ 0.09 (s, 6H), 0.99 (s, 9H), 2.25–2.40 (m, 6H), 3.69 (s, 3H), 4.18–4.26 (m, 1H), 4.42 (t, 1H, J = 9 Hz), 4.51–4.60 (m, 1H), 4.90–5.08 (m, 2H), 5.70–5.86 (m, 1H), and 7.08 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ −5.6, −4.8, 16.9, 24.8, 50.1, 65.0, 67.1, 114.6, 119.6, 135.6, 161.6, 173.0, and 174.5; mass spectrum (CI), m/z 330.0 $(M+H)^+$ (theoretical m/z 329.2); mass spectrum (FAB), m/z 330.2099 $(M+H)^+$ ($C_{16}H_{32}NO_4Si$ requires m/z 330.2101).

4.1.11. *N*-(4-Pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-homoserine cyanomethyl ester (11c′). To a suspension containing 560 mg (1.76 mmol) of **10c′** in 5 mL of 1:1 THF/water was added 222 mg (5.28 mmol) of LiOH. The reaction mixture was stirred under argon at room temperature for 3 h, then diluted with 60 mL of ethyl acetate and washed with 25 mL of 1 N $NaHSO_4$, dried over $MgSO_4$, and concentrated under diminished pressure. The crude residue was dissolved in anhydrous acetonitrile and 370 mg (8.8 mmol) of chloroacetonitrile was added, followed by 1.23 mL (8.8 mmol) of triethylamine. The reaction mixture was stirred at room temperature overnight, then diluted with 50 mL of ethyl acetate and washed with 25 mL of 1 N $NaHSO_4$, dried over $MgSO_4$, and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (15 × 2 cm), elution with 9:1 dichloromethane/methanol, gave **11c′** as a colorless oil: yield 480 mg (80%); ^{1}H NMR ($CDCl_3$) δ 0.09 (s, 6H), 0.92 (s, 9H), 2.05–2.15 (m, 2H), 2.25–2.40 (m, 6H), 2.40–2.50 (m, 2H), 3.64–3.80 (m, 1H), 4.90–5.08 (m, 2H), 5.70–5.86 (m, 1H), and 7.14 (br s); mass spectrum (FAB), m/z 355.2051 $(M+H)^+$ ($C_{17}H_{31}N_2O_4Si$ requires m/z 355.2053).

4.1.12. *N*-((4-Pentenoyl)-*S*-2-amino)-3-hydroxy-3-methylbutyric acid methyl ester (14). To a solution containing 200 mg (1.50 mmol) of *S*-(+)-2-amino-3-hydroxy-3-methylbutyric acid (**12**) in 3 mL of H_2O was added 320 mg (3.76 mmol) of $NaHCO_3$ followed by 410 mg (2.07 mmol) of 4-pentenoic acid succinimide ester in 3 mL of dioxane. The reaction mixture was allowed to

stir overnight, then acidified carefully with 1 N $NaHSO_4$ to pH 4 and extracted with two 20-mL portions of ethyl acetate. The combined organic layer was washed with 75 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. The residue (crude **13**) was dissolved in 2 mL of THF and 1 mL of MeOH, the solution was cooled to 0 °C in an ice bath, and 2.0 mL of a 2.0 M solution of TMS–diazomethane[23] was added dropwise. Upon addition the evolution of N_2 occurred immediately; after N_2 evolution ceased, the reaction was quenched by dropwise addition of AcOH. The reaction mixture was diluted with 25 mL of brine and extracted with two 25-mL portions of ethyl acetate. The combined organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (20 × 2 cm), eluting with 2:1 ethyl acetate/hexanes, gave **14** as a colorless oil: yield 130 mg (38%, 2 steps); ^{1}H NMR ($CDCl_3$) δ 1.18 (s, 3H), 1.22 (s, 3H), 2.32 (m, 4H), 3.08 (br s, 1H), 3.69 (s, 3H), 4.47 (d, 1H, J = 8.7 Hz), 4.97 (m, 2H), 5.75 (m, 1H), and 6.64 (d, 1H, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 26.62, 26.66, 29.29, 35.32, 52.06, 59.66, 71.58, 115.50, 136.65, 171.82, and 172.61; mass spectrum (FAB), m/z 230.1391 $(M+H)^+$ ($C_{11}H_{20}NO_4$ requires m/z 230.1392).

4.1.13. *N*-((4-Pentenoyl)-*S*-2-amino)-3-*O*-triethylsilyloxy-3-methylbutyric acid methyl ester (15). To a solution containing 95 mg (0.41 mmol) of **14** in 2 mL of anhydrous CH_2Cl_2 with 4 Å molecular sieves were added 220 mg (0.83 mmol) of triethylsilyl triflate (TESOTf) and 110 mg (1.03 mmol) of 2,6-lutidine. The reaction mixture was stirred at room temperature under argon for 1 h, then diluted with 50 mL of brine and extracted with two 30-mL portions of ethyl acetate. The combined organic phase was washed with 75 mL of H_2O, dried ($MgSO_4$), and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (15 × 2 cm) eluting with 2:1 hexanes/ethyl acetate gave **15** as a colorless oil: yield 130 g (90%); ^{1}H NMR ($CDCl_3$) δ 0.52 (q, 6H, J = 7.8 Hz), 0.89 (t, 9H, J = 7.8 Hz), 1.21 (s, 3H), 1.31 (s, 3H), 2.33 (m, 4H), 3.66 (s, 3H), 4.40 (d, 1H, J = 9.0 Hz), 4.98 (m, 2H), 5.78 (m, 1H), and 6.23 (d, 1H, J = 9.0 Hz); ^{13}C NMR ($CDCl_3$) δ 6.39, 6.79, 27.66, 27.86, 29.31, 35.65, 51.64, 60.89, 74.49, 115.48, 136.75, 170.63, and 171.79; mass spectrum (FAB), m/z 344.2255 $(M+H)^+$ ($C_{17}H_{34}NO_4Si$ requires m/z 344.2257).

4.1.14. *N*-((4-Pentenoyl)-*S*-2-amino))-3-triethylsilyloxy-3-methylbutyric acid cyanomethyl ester (16). To a suspension containing 130 mg (0.37 mmol) of **15** in 3 mL of 3:1:1 THF/H_2O/MeOH was added 46 mg (1.11 mmol) of LiOH. The reaction mixture was stirred under argon at room temperature and monitored by TLC; after 3 h the starting material had been consumed, so the reaction mixture was diluted with 60 mL of ethyl acetate and washed with 25 mL of 1 N $NaHSO_4$, dried ($MgSO_4$), and concentrated under diminished pressure. The crude residue was dissolved in anhydrous acetonitrile and 140 mg (1.85 mmol) of chloroacetonitrile was added, followed by 186 mg (1.85 mmol) of triethylamine. The reaction mixture was stirred at room temperature overnight, then diluted with 50 mL of ethyl acetate and washed with 25 mL of satd aq NH_4Cl, dried ($MgSO_4$), and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (15 × 2 cm), elution with 2:1 hexanes/ethyl acetate, gave **16** as a colorless oil: yield 85 mg (62%); ^{1}H NMR ($CDCl_3$) δ 0.57 (q, 6H, J = 7.8 Hz), 0.93 (t, 9H, J = 7.8 Hz), 1.28 (s, 3H), 1.36 (s, 3H), 2.35 (m, 4H), 4.42 (d, 1H, J = 8.7 Hz), 4.68 (d, 2H, J = 15.6 Hz), 4.80 (d, 1H, J = 15.6 Hz), 5.04 (m, 2H), 5.80 (m, 1H), and 6.25 (d, 1H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$) δ 6.37, 6.85, 27.60, 27.97, 29.21, 35.45, 48.42, 60.98, 74.32, 113.85, 115.74, 136.58, 168.94, and 172.19; mass spectrum (FAB), m/z 369.2209 $(M+H)^+$ ($C_{18}H_{33}N_2O_4Si$ requires m/z 369.2210).

4.1.15. *N*-4-Pentenoyl-*O*-*tert*-trialkylsilyl-*S*-aminoacyl-pdCpAs (17a′–17c′ and 18) and bis(*N*-4-pentenoyl-*O*-*tert*-trialkylsilyl-*S*-aminoacyl)-pdCpAs (19a′–19c′ and 20). To a conical vial containing 20 mg (54–56 μmol) of *N*-(4-pentenoyl)-*O*-silyl-protected-*S*-amino acid cyanomethyl esters (**11a′**–**11c′** and **16**) was added a solution of the tetrabutylammonium salt of 5.0 mg (3.64 μmol) of pdCpA[15] in 100 μL of anhydrous DMF, followed by 20 μL of triethylamine (over 4 Å molecular sieves). The reaction mixture was stirred at 25 °C and monitored by HPLC. A 5-μL aliquot of the reaction mixture was removed after 24 h and diluted with 45 μL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5. Twenty microliters of the diluted aliquot was analyzed by HPLC on a C_{18} reversed phase column (250 × 10 mm). The column was washed with 1 → 63% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 35 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The majority of the pdCpA salt had been consumed at this time so the reaction mixture was diluted to a total volume of 500 μL of 1:1 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified using the same semi-preparative C_{18} reversed phase column. After lyophilization of the appropriate fractions, *N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-threonyl-pdCpA (**17a′**) (retention time 28.4 min) was obtained as a colorless solid: yield 2.22 mg (65%); mass spectrum (MALDI-TOF), m/z 932.2766 $(M-H)^-$ ($C_{34}H_{52}N_9O_{16}P_2Si$ requires m/z 932.2777); *N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-*allo*-threonyl-pdCpA (**17b′**) (retention time 28.2 min) was obtained as a colorless solid: yield 1.88 mg (55%); mass spectrum (MALDI-TOF), m/z 933.84 (M^+) (theoretical m/z 933.28); *N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-(*S*)-homoseryl-pdCpA (**17c′**) (retention time 27.9 min) was obtained as a colorless solid: yield 2.05 mg (60%); mass spectrum (MALDI-TOF), m/z 931.2671 $(M-2H)^{2-}$ ($C_{34}H_{53}N_9O_{16}P_2Si$ requires m/z 931.2698); *N*-((4-pentenoyl)-2-amino)-3-triethylsilyloxy-3-methylbutyryl-pdCpA (**18**) (retention time 28.4 min) was obtained as a colorless solid: yield 1.74 mg (50%); mass spectrum (MALDI-TOF), m/z 947.47 (M^+) (theoretical m/z 947.30); bis-*N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-threonyl)-pdCpA (**19a′**) (retention time 33.8 min) was obtained as a colorless solid: yield 0.45 mg (10%); mass spectrum (MALDI-TOF), m/z 1253.41 $(M+Na)^+$ (theoretical m/z 1253.45), bis-*N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-*allo*-threonyl)-pdCpA (**19b′**) (retention time 34.0 min) was obtained as a colorless solid: yield 0.45 mg (10%); mass

spectrum (MALDI-TOF), *m*/*z* 1230.39 (M^+) (theoretical *m*/*z* 1230.46); bis-*N*-(4-pentenoyl)-*O*-*tert*-butyldimethylsilyl-*S*-homoseryl-pdCpA (**19c′**) (retention time 33.8 min) was obtained as a colorless solid: yield 0.45 mg (10%); mass spectrum (MALDI-TOF), *m*/*z* 1253.89 $(M+Na)^+$ (theoretical *m*/*z* 1253.45), bis-*N*-((4-pentenoyl)-2-amino)-3-triethylsilyloxy-3-methylbutyryl)-pdCpA (**20**) (retention time 33.9 min) was obtained as a colorless solid: yield 0.46 mg (10%); mass spectrum (MALDI-TOF), *m*/*z* 1257.55 $(M-H)^-$ (theoretical *m*/*z* 1257.48).

4.1.16. *N*-(4-Pentenoyl)aminoacyl-pdCpAs (21a–21c and 22) and bis-(*N*-4-pentenoyl)aminoacyl-pdCpAs (23a–23c and 24). The silyl groups were removed from compounds **17a′**–**17c′**, **18**, **19a′**–**19c′**, and **20** by treatment with 75 μL of 3:1:1 AcOH/THF/H_2O.[27] After stirring for 3 h at 25 °C the reaction mixture was diluted to a total volume of 200 μL of 1:1 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified using a semi-preparative C_{18} reversed phase column. After lyophilization of the appropriate fractions, *N*-(4-pentenoyl)-pdCpAs (**21a**–**21c** and **22**) (retention times 13.5–14.0 min) were obtained as colorless solids; bis-(*N*-(4-pentenoyl)aminoacyl)-pdCpAs (**23a**–**23c** and **24**) (retention times 15.5–18 min) were obtained as colorless solids. **21a**: yield 67%; mass spectrum (MALDI-TOF), *m*/*z* 834.04 $(M+H)^+$ (theoretical *m*/*z* 834.22); **21b**: yield 57%; mass spectrum (MALDI-TOF), *m*/*z* 834.16 $(M+H)^+$ (theoretical *m*/*z* 834.22); **21c**: yield 55%; mass spectrum (MALDI-TOF), *m*/*z* 829.50 $(M-H)^-$ (theoretical *m*/*z* 829.18); **22**: yield 60%; mass spectrum (MALDI-TOF), *m*/*z* 848.68 $(M+H)^+$ (theoretical *m*/*z* 848.24); **23a**: yield 24%; mass spectrum (MALDI-TOF), *m*/*z* 1038.69 $(M+Na)^+$ (theoretical *m*/*z* 1038.29); **23b**: yield 18%; **23c**: yield 25%; mass spectrum (MALDI-TOF), *m*/*z* 1015.76 (M^+) (theoretical *m*/*z* 1015.30); **24**: yield 20%.

4.1.17. *O*-Acetyl-*N*-(4-pentenoyl)-*S*-threonine cyanomethyl ester (27). To a solution containing 500 mg (4.20 mmol) of *S*-threonine (**7a**) which was dissolved in 1 mL of 6 N HCl, glacial acetic acid (1 mL) was added and the solution was cooled to 0 °C in an ice bath. Acetyl chloride was then added slowly. The *O*-acetoxyamino acid chloride precipitated within a few minutes; quantitative precipitation was realized by adding 2–3 vol of ether. The solid was filtered, washed with ether, and dried in vacuo. Compound **25** was obtained as a colorless oil: yield 675 mg (100%).

To 500 mg (3.10 mmol) of **25** in 5 mL of H_2O was added 521 mg (6.2 mmol) of $NaHCO_3$. To this stirred solution was added 794 mg (4.03 mmol) of 4-pentenoic acid succinimide ester[29] in 5 mL of dioxane. The reaction mixture was allowed to stir at room temperature under argon overnight, then diluted with ethyl acetate and washed with 1 N $NaHSO_4$. The organic layer was separated and the aqueous layer was back-extracted with two 25-mL portions of ethyl acetate. The combined organic phase was washed with 50 mL of brine, dried over $MgSO_4$, and concentrated under diminished pressure. The crude material was dissolved in 10 mL of acetonitrile and 1.0 mL (15.5 mmol) of chloroacetonitrile was added, followed by 2.2 mL (15.5 mmol) of triethylamine. The reaction mixture was stirred at room temperature overnight under argon. The reaction mixture was then diluted with ethyl acetate, washed with 50 mL of $NaHCO_3$, 100 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. Purification by flash chromatography on a silica gel column (20 × 3 cm) using 2:1 ethyl acetate/hexanes gave **27** as a colorless oil: yield 655 mg (75%); ^1H NMR ($CDCl_3$) δ 1.76 (d, 3H, J = 6.9 Hz), 2.25–2.35 (m, 7H), 4.22–4.25 (m, 1H), 4.77 (s, 2H), 5.02 (s, 2H), 5.65–5.75 (m, 2H), and 7.14 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.9, 25.7, 28.7, 29.6, 32.9, 35.7, 48.8, 116.5, 125.4, 136.9, 138.0,163.2, and 171.8; mass spectrum (FAB), *m*/*z* 283.1295 $(M+H)^+$ ($C_{13}H_{19}N_2O_5$ requires *m*/*z* 283.1294).

4.1.18. *O*-Acetyl-*N*-(4-pentenoyl)-*S*-threonyl-pdCpA (28) and bis-(*O*-acetyl-*N*-(4-pentenoyl)-*S*-threonyl)-pdCpA (29). To a conical vial containing 20 mg (56 μmol) of *O*-acetyl-*N*-(4-pentenoyl)-*S*-threonine cyanomethyl ester (**27**) was added a solution of the tetrabutylammonium salt of 5.0 mg (3.64 μmol) of pdCpA[15] in 100 μL of anhydrous DMF, followed by 20 μL of triethylamine (over 4 Å molecular sieves). The reaction mixture was stirred at 25 °C and monitored by HPLC. A 5-μL aliquot of the mixture was removed after 24 h and diluted with 45 μL of 1:2 CH_3CN/50 mM NH_4OAc, pH 4.5. Twenty microliters of the diluted aliquot was analyzed by HPLC on a C_{18} reversed phase column (250 × 10 mm). The column was washed with a gradient of CH_3CN (0 → 63%) in 50 mM NH_4OAc, pH 4.5, over a period of 35 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The majority of the pdCpA salt had been consumed at this point so the reaction mixture was diluted to a total volume of 500 μL of 1:1 CH_3CN/50 mM NH_4OAc, pH 4.5, and purified using the same semi-preparative C_{18} reversed phase column. After lyophilization of the appropriate fractions, 3′-*O*-acetyl-*N*-(4-pentenoyl)-*S*-threonyl-pdCpA (**28**) (retention time 15.6 min) was obtained as a colorless solid: yield 1.2 mg (38%); mass spectrum (MALDI-TOF), *m*/*z* 862.2125 $(M+H)^+$ ($C_{30}H_{42}N_9O_{17}P_2$ requires *m*/*z* 862.2114), and bis-(*O*-acetyl-*N*-(4-pentenoyl)-*S*-threonyl)-pdCpA**(29)** (retention time 20.3 min) was obtained as a colorless solid: yield 1.59 mg (40%); mass spectrum (MALDI-TOF), *m*/*z* 1088.21 $(M+2H)^{2+}$ (theoretical *m*/*z* 1088.32).

4.1.19. *N*-4-Pentenoyl-*S*-threonyl-pdCpA (21a) and bis-*N*-4-pentenoyl-*S*-threonyl-pdCpA (23a). To a conical vial containing 0.5 mg of compound **28** or compound **29** (5.73 μmol or 4.55 μmol, respectively) in 150 μL of dry methanol was added 50 μL of a 54% solution of HBF_4 in diethyl ether. The reaction mixture was stirred at room temperature for 3 h. A 10-μL aliquot of the reaction mixture was diluted with 40 μL of 1:1 acetonitrile/50 mM NH_4OAc, pH 4.5, and was analyzed by HPLC on a C_{18} reversed phase column (250 × 10 mm). The column was washed with 0% → 63% acetonitrile in 50 mM NH_4OAc, pH 4.5, over a period of 35 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The remaining reaction mixture was diluted to a total volume of 200 μL of 1:1 acetonitrile/50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed phase column.

Dinucleotide derivative **21a** (retention time ~14.0 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: yield 0.4 mg (81%); mass spectrum (MALDI-TOF) *m/z* 828.70 $(M-2H)^-$ (theoretical 828.17). Dinucleotide derivative **23a** (retention time 18.2 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: yield 0.2 mg (43%); mass spectrum (MALDI-TOF) *m/z* 1012.14 $(M-2H)^-$ (theoretical 1012.27).

4.2. Preparation of bisaminoacyl-tRNA$_{CUA}$s

Bisaminoacyl-tRNAs were prepared by a T4 RNA ligase-mediated ligation of the chemically synthesized pdCpA derivatives (**6a** and **6b**) with the abbreviated suppressor tRNA-C_{OH}.[15,24,25] Ligation reactions utilized 100 μL (total volume) of 50 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 50 μg of tRNA$_{CUA}$-C_{OH}, 1.0 A_{260} unit of *N*-pentenoyl-protected bisaminoacyl-pdCpA derivatives (5- to 10-fold molar excess), 15% DMSO, and 200 U of T4 RNA ligase. After incubation at 37 °C for 45 min, the reaction was quenched by adding 0.1 vol of cold EtOH, and the product was collected by centrifugation, washed with 70% EtOH, and dried. The tRNA was dissolved in 1 mM KOAc to a final concentration of 5 μg/μL. The ligation efficiency was estimated by gel electrophoresis at pH 4.5.[26] Deprotection of pentenoyl-containing aminoacyl-tRNAs utilized 100 μL of reaction mixtures containing 5 μg/μL of *N*-pentenoylaminoacyl-tRNA, 15% THF, and 8 mM I_2 (incubation at 25 °C for 50 min). The deprotected bisaminoacyl-tRNAs were precipitated by addition of 0.1 vol of 3 M NaOAc, pH 5.2, and then 2.5 vol of cold EtOH. The samples were collected by centrifugation. The tRNA pellets were washed with 70% EtOH, centrifuged, and dried. The deprotected bisaminoacyl-tRNAs were dissolved in 1 mM KOAc to a final concentration of 5 μg/μL and stored in aliquots at −80 °C.

4.3. Synthesis of DHFR mRNA by in vitro transcription

Plasmid pTHD(−1) was linearized with *Bam*HI and transcribed using AmpliScribe T7 transcription kit.[25] The transcribed mRNA solution was extracted successively with phenol and chloroform, then precipitated by the addition of 2.5 vol of EtOH, washed with 70% EtOH, and dried. The mRNA was dissolved in water and stored at −80 °C.

4.4. In vitro protein translation reactions

Suppression of a UAG stop codon at position −1 of DHFR was performed in an *Escherichia coli* S-30 system.[18] Translation reactions were carried out in 10–2000 μL reaction mixtures that contained the following per 100 μL: 10 μg of plasmid DNA dissolved in diethyl pyrocarbonate-treated water, 40 μL of premix [35 mM Tris–acetate (pH 7.0), 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM dithiothreitol, 11 mM magnesium acetate, 20 mM phosphoenolpyruvate, 0.8 mg/mL *E. coli* tRNA, 0.8 mM isopropyl β-D-thiogalactopyranoside, 20 mM ATP and GTP, 5 mM CTP and UTP, and 10 mM cAMP],[30] 100 μM amino acids lacking methionine, 50 μM methionine, 40 μCi of [^{35}S]-*S*-methionine, and 30 μL of S-30 extract that had been heat treated at 42 °C for 6 min.[31] Suppression reaction mixtures (100 μL) contained 25 μg of deprotected misacylated tRNA$_{CUA}$ and were incubated at 37 °C. Aliquots from in vitro translation reactions were removed for analysis by 15% SDS–PAGE.[32] Autoradiography of the gels was carried out to determine the location of ^{35}S-labeled protein. Quantification of the bands was carried out using a phosphorimager. Suppression efficiency was calculated as the percentage of the protein produced via nonsense codon suppression relative to the production of wild-type protein.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.03.088.

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