A General and Efficient Route for Chemical Aminoacylation of Transfer RNAs

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Abstract: A general and expedient procedure for the synthesis of aminoacyl tRNA has been developed. The preparation of aminoacyl pdCpA in one step and in high yield by reaction of the cyanomethyl active esters of N-protected α-amino acids with pdCpA is detailed. The preparation and photodeprotection of aminoacyl pdCpA derivatives containing nitroveratryl (NVOC) N-protected amino acids is also studied. The utility of the above methods for preparing aminoacyl tRNA was confirmed by enzymatically ligating NVOC-phenylalanyl pdCpA to tRNA-C_{OH} followed by photolysis to provide unprotected phenylalanyl tRNA. The phenylalanyl tRNA was shown to be competent in an in vitro protein biosynthesis system. These protocols greatly simplify the use of chemically misacylated tRNAs in the synthesis of proteins containing unnatural amino acids, as well as in studies of protein biosynthesis.

Introduction

A general biosynthetic method has recently been developed that allows unnatural amino acids with novel steric or electronic properties to be site-specifically incorporated into proteins.[1] This procedure involves substitution of the UAG codon at the site in the genetic message that corresponds to the amino acid to be altered. Because this codon is not recognized by any of the common tRNAs involved in protein synthesis it can be viewed as a "blank" in the genetic code. A suppressor tRNA is then generated (1) that recognizes and inserts the amino acid in response to the UAG codon and (2) that is not a substrate for the natural aminoacyl synthetases. Addition of suppressor tRNA that has been chemically acylated with the desired amino acid to a protein biosynthesis reaction results in specific incorporation of the amino acid into the polypeptide chain. Several mutants of *Escherichia coli* RTEM β-lactamase and T4 lysozyme containing unnatural amino acids have been generated via this approach and their catalytic properties characterized.

In order to acylate the tRNA with novel amino acids, a general method for the efficient acylation of the suppressor tRNA is required. Such a method would also prove to be an important tool for studies of the role of the 3′-acceptor stem of aminoacylated tRNAs in protein biosynthesis.[2] Although it has been reported that aminoacyl tRNA synthetases have been used to misacylate a number of tRNAs under a variety of conditions[3] we have been unsuccessful in acylating yeast $tRNA_{CUA}^{Phe}$ with a variety of phenylalanine or serine analogues using yeast phenylalanine tRNA synthetase or serine tRNA synthetase, respectively.[4] These results suggest that enzymatic misaminoacylation will not be a general approach for acylating tRNAs with unnatural amino acids. Consequently, one would like to develop a generic aminoacyl synthetase or a direct chemical method for acylating tRNAs.

Hecht and co-workers have developed one solution to this problem, which depends on the T4 RNA ligase mediated coupling of 2′(3′)-O-acylated pCpA derivatives to tRNAs missing the 3′-terminal cytidine and adenosine moieties.[5] In the initial studies, the α-amino-protecting group, which stabilizes the aminoacyl linkage,[6] was not removed from the aminoacyl tRNA. An acyl tRNA of this sort cannot function in peptide elongation until the amino acid protecting group is removed. Hecht has used these N-blocked aminoacyl tRNAs to incorporate unnatural amino acids at the amino terminus of short peptides. Brunner and co-workers subsequently demonstrated that *tert*-butyloxycarbonyl- (*t*-Boc-) protected aminoacyl pCpA can be prepared and deprotected with trifluoroacetic acid prior to ligation to give an aminoacyl tRNA with a free α-amino group that can function in peptide elongation.[7] A photoreactive analogue of Phe was substituted for Phe in trichloroacetic acid precipitable proteins synthesized in an in vitro rabbit reticulocyte system by this method. More recently, pyroglutamyl aminoacyl tRNAs have been prepared from pyroglutamyl aminoacyl dinucleotides and the α-amino group can be deprotected by pyroglutamate aminopeptidase.[8] These aminoacyl tRNAs were used as A-site acceptors in dipeptide formation. Recently, we reported that 5′-phospho-2′-deoxyribocytidylriboadenosine (pdCpA) can be acylated with nitrosulfenyl- (NPS-) or carbobenzoxy- (Cbz-) protected amino acids and can be subsequently deprotected by treatment with sodium thiosulfate or hydrogenation, respectively.[1b] The use of deoxycytidine as well as NPS and CBZ groups provides considerable simplification in the synthetic requirements for tRNA aminoacylation and a substantial improvement in yield.

Nonetheless, all of the above methods still require a number of protection and deprotection steps that result in low overall yields of aminoacyl tRNAs. Most protocols also involve coupling of the hydrolytically labile unblocked aminoacyl dinucleotide to tRNA-C_{OH}. We now report a method whereby unprotected pdCpA can be selectively aminoacylated in high yield with an N-blocked amino acid and efficiently ligated to tRNA. This N-blocked aminoacyl tRNA can then be deprotected photochemically in high yield under conditions where the unblocked aminoacyl tRNA is stable. Without further purification the aminoacylated tRNA can be directly added to the protein biosynthesis reaction.

Experimental Section

All reagents were purchased from Aldrich unless otherwise noted; the amino acids were all purchased from Sigma unless otherwise noted. Dimethylformamide and acetonitrile were dried over molecular sieves. Chloroacetonitrile was distilled prior to use. All other reagents were used without further purification unless otherwise noted.

TLC was performed on silica gel (Merck Fertigplatten, 60F-254, Article 5765) in the following solvent systems: (A) acetone/hexane (3:7, v/v); (B) acetone/hexane (1:1, v/v). All column chromatography dis-

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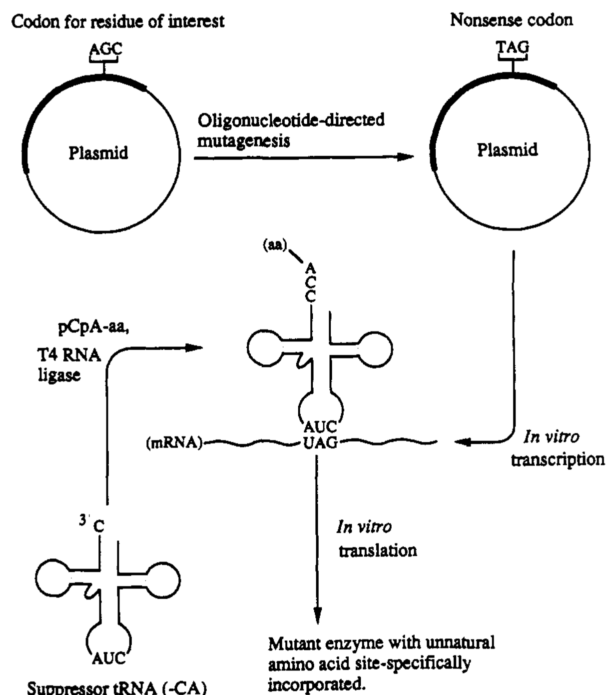

Figure 1. Strategy for biosynthetic incorporation of unnatural amino acids into proteins.

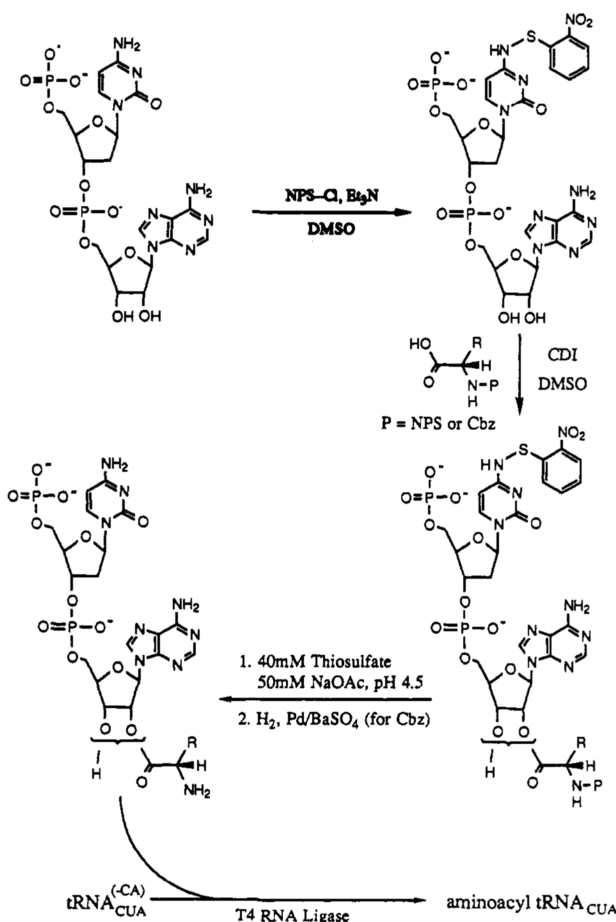

Figure 2. Strategy for the chemical aminoacylation of $tRNA_{CUA}{}^{Phe}$.

cussed was flash chromatography carried out on silica gel (Merck Kieselgel 60, Art. 9385).

Melting points (Pyrex capillary) are uncorrected. ^1H NMR spectroscopy was recorded on the UCB-400 (400 MHz), the UCB-500 (500 MHz), or the UCB-250 (250 MHz) Fourier transform NMR spectrometers at the University of California, Berkeley NMR facility. Significant ^1H NMR resonances are reported in units of ppm downfield from trimethylsilane. The internal standard for ^1H NMR spectra determined in $CDCl_3$ was Me_4Si, for those determined in DMSO, D_2O, or acetic acid, the solvent was used as the reference at δ 2.49, 4.63, and 2.05, respectively. ^{31}P NMR spectra were recorded on the UCB-400 (162 MHz) Fourier transform spectrometer; all spectra were proton decoupled and reported in units of ppm downfield from trimethyl phosphate (δ 3.086). ^{13}C NMR spectra were recorded on the UCB-500 (127 MHz) Fourier transform spectrometer; all spectra were proton decoupled and reported in units of ppm downfield from $CDCl_3$ (δ 77.0), or DMSO-d_6 (δ 39.5). IR spectra were recorded on a Mattson Polaris Fourier transform spectrophotometer using NaCl plates or KBr pellets. Fast atom bombardment mass spectra were recorded at the Unversity of California, Berkeley mass spectral laboratory on an AE1 M512 mass spectrometer, with *m*-nitrobenzyl alcohol as the matrix solvent. UV absorbance spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. High-pressure liquid chromatography (HPLC) was performed on a Waters Model 600E instrument using a Waters 490E programmable multiwave UV detector and a Waters 745 data module. Porasil columns were used for preparative (M20 10/50 ODS-3) and semipreparative (10 ODS-3) chromatography. A Rainin C18 (80-225-C15) column was used in analytical chromatography. Photodeprotections were accomplished by employing a 2000-W Hanovia mercury-xenon lamp and a Pyrex filter.

General Procedure for Formation of Cyanomethyl Esters from N-Blocked Amino Acids.[9] N-blocked (and reactive side chains protected) amino acid (3 mmol) was added to triethylamine (3 mL) and acetonitrile (3 mL) in a 25-mL round-bottom flask. The reaction mixture was cooled to 0 °C and 2 equiv of chloroacetonitrile (0.075 mL) was added. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solution was diluted with ethyl acetate (30 mL) and extracted with 0.5 N HCl (30 mL). The organic layer was then extracted with saturated aqueous sodium bicarbonate (30 mL) and saturated sodium chloride (30 mL), dried over $MgSO_4$, and concentrated under reduced pressure.

***N*-(Carbobenzyloxy)-im-benzylhistidine Cyanomethyl Ester.** The ester was recrystallized from ethyl acetate/hexane to give 1.06 g (84%) of the title compound as a white solid: mp 142–145 °C; R_f 0.47 in B; ^1H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, J = 7.5 Hz, 1 H), 7.64 (s, 1 H), 7.18–7.40 (m, 10 H), 6.92 (s, 1 H), 5.11 (s, 2 H), 5.01 (s, 2 H), 4.39 (m, 1 H), 3.31 (d, J = 11.7 Hz, 1 H), 2.86 (m, 2 H); ^{13}C NMR (127 MHz, $CDCl_3$) δ 172.02, 156.43, 137.73, 137.22, 128.61, 128.42, 127.91, 127.76, B127.65, 127.36, 117.16, 65.65, 53.94, 49.45, 49.37, 29.53; IR (film) 3205, 3034, 2946, 1754, 1713, 1499, 1434, 1268, 1166, 1058, 752, 696 cm^{-1}; mass spectrum, *m/e* 419 (MH^+). Anal. Calcd for $C_{23}H_{22}N_4O_4$: C, 66.02, H, 5.30; N, 13.39. Found: C, 66.00; H, 5.13; N, 13.25.

***N*-(Carbobenzyloxy)-*O*-benzylserine Cyanomethyl Ester.** The product was purified by silica gel chromatography, eluting with 30:70 acetone/hexane to give 0.83 g (75%) of the title compound as a clear oil: R_f 0.28 in A; ^1H NMR (500 MHz, $CDCl_3$) δ 7.12–7.32 (m, 10 H), 5.03 (s, 2 H), 4.51 (s, 2 H), 4.48 (m, 1 H) 4.35 (dd, J = 12.02, 18.19 Hz, 2 H), 3.81 (dd, J = 3.11, 6.37 Hz, 1 H), 3.60 (dd, J = 3.11, 6.39 Hz, 1 H); ^{13}C NMR (127 MHz, $CDCl_3$) δ 169.98, 155.91, 128.20, 128.16, 128.07, 128.06, 127.81, 127.70, 127.27, 126.99, 113.79, 72.84, 66.90, 64.38, 54.19; IR (film) 3427, 3032, 2945, 1727, 1520, 1454, 1210, 1069, 750, 691 cm^{-1}; mass spectrum, *m/e* 369 (MH^+). Anal. Calcd for $C_{20}H_{21}N_2O_5$: C, 65.04; H, 5.73; N, 7.59. Found: C, 64.84; H, 5.33; N, 7.21.

***N*-(Carbobenzyloxy)phenylalanine Cyanomethyl Ester.** The product was purified by silica gel chromatography, eluting with 30:70 acetone/hexane to give 0.98 g (96%) of the title compound as a clear oil: R_f 0.33 in A; ^1H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, J - 8.9 Hz, 1 H), 7.22–7.42 (m, 10 H), 5.01 (m, 4 H), 4.37 (m, 1 H), 3.09 (dd, J = 5.1, 8.7 Hz, 1 H), 2.93 (dd, J = 5.1, 8.7 Hz, 1 H); ^{13}C NMR (127 MHz, $CDCl_3$) δ 171.82, 156.47, 136.93, 136.78, 129.14, 128.35, 128.29, 127.82, 127.57, 126.64, 115.67, 65.52, 55.21, 49.49, 45.42, 36.10; IR (film) 3329, 3032, 2949, 1763, 1719, 1513, 1454, 1256, 1172, 1056, 750, 699 cm^{-1}; mass spectrum, *m/e* 339 (MH^+), 295 (base); high-resolution mass spectrum, 339.135 (MH^+).

***N,N'*-Bis(carbobenzyloxy)lysine Cyanomethyl Ester.** The product was purified by silica gel chromatography, eluting with 50:50 acetone/hexane to give 1.07 g (73%) of the title compound as a clear oil: R_f 0.61 in B; ^1H NMR (500 MHz, $CDCl_3$) δ 7.25–7.32 (m, 10 H), 5.62 (d, J = 7.69 Hz, 1 H), 5.08 (s, 2 H), 5.04 (s, 2 H), 4.70 (dd, J = 15.68, 39.93 Hz, 2 H), 4.37 (m, 1 H), 3.16 (m, 2 H), 1.84 (m, 1 H), 1.74 (m, 1 H), 1.49 (m, 2 H), 1.38 (t, J = 7.06, 2 H); ^{13}C NMR (127 MHz, $CDCl_3$) δ 171.13, 156.65, 156.01, 136.43, 135.92, 128.48, 128.43, 122.20, 128.07, 128.03, 113.92, 67.15, 66.62, 53.44, 48.83, 40.00, 29.23, 21.98; IR (film)

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3330, 2942, 1766, 1714, 1536, 1485, 1250, 1169, 1040, 752, 690 cm^{-1}; mass spectrum, m/e 454 (MH^+), 410 (base); high-resolution mass spectrum, 454.1992 (MH^+).

N-(Carbobenzyloxy)phenylalanine Phenyl Ester. N-(Carbobenzyloxy)phenylalanine (3 g, 10.1 mmol) was dissolved in ethyl acetate (30 mL) in a 100-mL round-bottom flask equipped with a magnetic stir bar. To this solution was added phenol (1 mL, 12 mmole), followed by dicyclohexylcarbodiimide (2.06 g, 10 mmol). The reaction mixture was allowed to stir at room temperature for 3 h and then was filtered. The solvent was removed under vacuum and the product recrystallized in hot ethanol + 1% acetic acid to give 3.24 g (97%) of the title compound as a white solid: R_f 0.37 in A; mp 113 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 6.98–7.38 (m, 15 H), 5.31 (d, 1 H, J = 7.9 Hz), 4.90 (m, 1 H), 3.27 (d, 2 H, J = 5.87 Hz); ^{13}C NMR (127 MHz, $CDCl_3$) δ 171.92, 156.40, 129.40, 129.37, 129.35, 128.68, 128.63, 128.47, 128.16, 128.06, 126.08, 121.15, 65.48, 64.35, 55.21, 45.43; IR (film) 1754, 1689, 1534, 1255, 1052, 756, 690 cm^{-1}; mass spectrum, m/e 376 (MH^+). Anal. Calcd for $C_{23}H_{21}NO_4$: C, 73.59; H, 5.64; N, 3.73. Found: C, 73.22; H, 5.71; N, 3.81.

N-(Carbobenzyloxy)-O-benzylserine. O-Benzylserine (0.98 g, 5 mmol) was dissolved in 2 N NaOH (5 mL) and dioxane (5 mL) in a 25-mL round-bottom flask equipped with stir bar. The solution was cooled to 0 °C and benzyloxycarbonyl chloride (1.1 equiv, 5.5 mmol, 1.16 mL) was added slowly to the reaction mixture. The reaction mixture was allowed to warm to room temperature while stirring, for 1 h. The reaction mixture was extracted four times with ether (25 mL) and excess ether was evaporated out of the aqueous phase by bubbling nitrogen through the solution. The aqueous solution was acidified to pH 1–2 by adding 4 N HCl (2.5 mL) and extracted twice with methylene chloride (30 mL). The organic phase was then washed with saturated aqueous sodium bicarbonate (30 mL) and dried over $MgSO_4$. The organics were concentrated under vacuum to give 1.44 g (87%) of the title compound: ^1H NMR (500 MHz, DMSO-d_6) δ 7.60 (d, J = 8.9 Hz, 1 H), 7.26–7.36 (m, 10 H), 5.04 (s, 2 H), 4.47 (s, 2 H), 3.4.27 (m, 1 H), 3.68 (m, 1 H); ^{13}C NMR (127 MHz, DMSO-d_6) δ 171.66, 156.07, 138.43, 137.68, 128.32, 128.18, 127.78, 126.69, 127.49, 127.44, 72.05, 69.24, 65.46, 54.19; IR (film) 3370, 3157, 3031, 2947, 1732, 1667, 1547, 1504, 1262, 1203, 1111, 1072, 753, 695 cm^{-1}; mass spectrum, m/e 330 (MH^+), 181 (base). Anal. Calcd for $C_{18}H_{19}NO_5$: C, 65.66; H, 5.82; N, 4.25. Found: C, 65.65; H, 5.74; N, 4.26.

N-NVOC-L-phenylalanine. Phenylalanine (500 mg, 3.03 mmol) and sodium carbonate (321 mg, 3.03 mmol, 1.0 equiv) were dissolved in 10 mL of H_2O. 6-Nitroveratryloxycarbonyl chloride[10] (835 mg, 3.03 mmol, 1.0 equiv) dissolved in 10 mL of dioxane was then added slowly with stirring to the aqueous solution. After being stirred at room temperature for 1 h, the reaction solution was diluted with 75 mL of CH_2Cl_2 followed by acidification with 50 mL of 1 N aqueous sodium bisulfate. The organic phase was collected and the aqueous phase was washed with CH_2Cl_2. The combined organic extracts were dried over sodium sulfate and then concentrated in vacuo to give a yellow solid. Recrystallization from ethyl acetate/hexane provided 828 mg (68%) of the titled compound as a white granular solid: mp 164–166 °C; R_f 0.45 in ethyl acetate/acetic acid 98:2, ^1H NMR (250 MHz, methanol-d_4) 7.72 (s, 1 H), 7.24 (m, 5 H), 7.07 (s, 1 H), 5.44 (d, 1 H, J = 15.5 Hz), 53.6 (d, 1 H, J = 15.5 Hz), 4.41 (dd, 1 H, J = 4.6, 9.9 Hz), 3.88 (s, 3 H), 3.86 (s, 3 H), 3.23 (dd, 1 H, J = 4.6, 14.0 Hz), 2.93 (dd, 1 H, J = 9.9, 14.0 Hz); IR (CH_2Cl_2) 3680, 3430, 2994, 1736, 1588, 1532, 1335, 1279, 1223, 1075 cm^{-1}; mass spectrum, m/e 405 (MH^+). Anal. Calcd for $C_{19}H_{20}N_2O_8$: C, 56.43; H, 4.99; N, 6.93. Found: C, 56.38; H, 4.82; N, 6.78.

N-NVOC-L-phenylalanine Cyanomethyl Ester. NVOC-L-phenylalanine (500 mg, 1.24 mmol) was dissolved in triethylamine (251 mg, 345 μL, 2.48 mmol, 2 equiv) and chloroacetonitrile (280 mg, 234 μL, 3.72 mmol, 3 equiv). The resulting solution was stirred for 24 h at room temperature, diluted with CH_2Cl_2, washed with 1 N aqueous sodium bisulfate, dried over sodium sulfate, and concentrated in vacuo to give a yellow oil. Purification by silica gel chromatography with CH_2Cl_2 followed by 10% ethyl acetate in CH_2Cl_2 as the eluent provided 520 mg (94%) of the titled compound as a white solid: mp 114–115 °C; R_f 0.29 in 50:50 ethyl acetate/hexane; ^1H NMR (250 MHz, $CDCl_3$) δ 7.69 (s, 1 H) 7.29 (m, 3 H), 7.14 (m, 2 H), 6.90 (s, 1 H), 5.45 (d, 1 H, J = 14.9 Hz), 5.28 (d, 1 H, J = 8.1 Hz), 4.80 (d, 1 H, J = 15.6 Hz), 4.73 (m, 1 H), 4.68 (d, 1 H, J = 15.6 Hz), 3.94 (s, 3 H), 3.93 (s, 3 H), 3.16 (m, 1 H); IR (CH_2Cl_2) 3430, 1771, 1735, 1588, 1532, 1335, 1279, 1223, 1167, 1075, 744, 703 cm^{-1}; mass spectrum, m/e 444 (MH), 196 (base). Anal. Calcd for $C_{21}H_{21}N_3O_8$: C, 56.91; H, 4.78; N, 9.48. Found: C, 56.83; H, 4.79; N, 9.13.

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Table I. Aminoacylation of pdCpA by Cyanomethyl Active Esters[a]

entry	active ester[b] (equiv)	reactn time, h	yield, % (HPLC)
A	**1** (5)	2.5	84
B	**1** (1.5)	10.5	74
C	**2** (5)	2.5	87
D	**3** (5)	2.5	82
E	**4** (5)	2.5	77
F	**5** (5)	2.5	68
G	**6** (5)	2.5	84

[a] All reactions were performed at 0.055 M pdCpA in anhydrous DMF at room temperature with sodium napthalenesulfonate employed as a standard. [b] **1** is N-NVOC-L-phenylalanine cyanomethyl ester, **2**, is N-NVOC-L-aspartic acid α-cyanomethyl β-6-nitroveratryl diester, **3** is N-Cbz-N-im-benzylhistidine cyanomethyl ester, **4** is N,N'-bis-Cbz-lysine cyanomethyl ester, **5** is N-Cbz-phenylalanine cyanomethyl ester, and **6** is O-benzyl-N-Cbz-serine cyanomethyl ester.

N-Boc-L-aspartic Acid α-*tert*-Butyl β-6-Nitroveratryl Diester. N-Boc-L-aspartic acid α-*tert*-butyl ester (1.0 g, 3.44 mmol) and 6-nitroveratryl alcohol (953 mg, 4.47 mmol, 1.3 equiv) were dissolved in 10 mL of freshly distilled CH_2Cl_2. Dicyclohexylcarbodiimide (781 mg, 3.78 mmol, 1.1 equiv) and (dimethylamino)pyridine (63 mg, 0.52 mmol, 0.15 equiv) were then added to the reaction solution. After the orange slurry was stirred for 2 h at room temperature, the dicyclohexyl urea was removed by filtration. The resulting orange solution was then diluted with CH_2Cl_2, washed with 1 N aqueous sodium bisulfate, dried over sodium sulfate, and concentrated in vacuo to provide a viscous yellow oil. Chromatography with 3 cm × 20 cm silica gel and with ethyl acetate/hexane/CH_2Cl_2 (30:70:1) as the eluent provided 1.19 g (71%) of the titled compound as a yellow foam: R_f 0.53 in ethyl acetate/hexane/CH_2Cl_2 (50:50:1); ^1H NMR (250 MHz, $CDCl_3$) δ 7.71 (s, 1 H), 7.00 (s, 1 H), 5.56 (d, 1 H, J = 15.0 Hz), 5.48 (d, 1 H, J = 15.0 Hz), 5.46 (m, 1 H), 3.99 (s, 3 H), 3.94 (s, 3 H), 3.01 (m, 1 H), 2.89 (dd, 1 H, J = 4.9, 16.9 Hz); IR (CH_2Cl_2) 3684, 3438, 3057, 2987, 2938, 1744, 1715, 1588, 1532, 1370, 1335, 1279, 1230, 1159, 1069 cm^{-1}; mass spectrum, m/e 485 (MH), 429, 196 (base). Anal. Calcd for $C_{22}H_{32}N_2O_{10}$: C, 54.53; H, 6.66; N, 5.78: Found: C, 54.67; H, 6.66; N, 5.81.

N-NVOC-L-aspartic Acid α-Cyanomethyl β-6-Nitroveratryl Diester. To N-Boc-L-aspartic acid α-*tert*-butyl β-6-nitroveratryl diester (1.19 g, 2.45 mmol) was added 5 mL of CH_2Cl_2, 10 mL of trifluoroacetic acid, and p-dimethoxybenzene (1.68 g, 12.2 mmol, 5 equiv). The resulting solution was stirred for 5 h at room temperature and then concentrated in vacuo to provide a yellow oil. The residual trifluoroacetic acid was removed by diluting with toluene followed by concentration in vacuo. The resulting viscous oil was dissolved in 6 mL of dioxane, and a solution of sodium bicarbonate (1.03 g, 12.3 mmol, 5 equiv) in 10 mL of H_2O was added followed by addition of a solution of NVOC-Cl (811 mg, 2.94 mmol, 1.2 equiv) in 10 mL of dioxane. The resulting orange slurry was stirred for 2 h and then diluted with CH_2Cl_2, washed with 1 N aqueous sodium bisulfate, dried over sodium sulfate, and concentrated in vacuo to give a yellow solid. Chromatography with 3 cm × 20 cm silica gel and ethyl acetate followed by 98:2 ethyl acetate/acetic acid as the eluent provided N-NVOC-L-aspartic acid β-6-nitroveratryl ester, which was converted directly to the cyanomethyl active ester (470 mg, 32% overall yield) according to the standard procedure: mp 139 °C; R_f 0.54 in ethyl acetate/CH_2Cl_2 (20:80); ^1H NMR (250 MHz, $CDCl_3$) δ 7.70 (s, 2 H), 7.03 (s, 1 H), 6.94 (s, 1 H), 5.95 (d, 1 H, J = 8.9 Hz), 5.45 (m, 4 H), 4.79 (m, 1 H), 4.76 (s, 2 H), 4.03 (s, 3 H), 3.98 (s, 3 H), 3.95 (s, 3 H), 3.94 (s, 3 H), 3.19 (dd, 1 H, J = 17.4, 4.5 Hz), 3.00 (dd, 1 H, J = 17.4, 4.4 Hz); IR (CH_2Cl_2) 3423, 3107–2840, 1771, 1735, 1581, 1525, 1469, 1335, 1279, 1223, 1195, 1180, 1068 cm^{-1}; mass spectrum, m/e 629 (M + Na) 607 (MH), 196 (base). Anal. Calcd for $C_{21}H_{21}N_3O_8$: C, 49.51; H, 4.32; N, 9.24: Found: C, 49.58; H, 3.98; N, 8.87.

General Procedure for Acylation of pdCpA (Table I). The preparation of the dinucleotide pdCpA has been previously reported.[1b] For solubility purposes the tetrabutylammonium salt of the dinucleotide pdCpA was formed by ion-exchange chromatography using Dowex 1×8-200 ion-exchange beads in the tetrabutylammonium form. (An appropriate excess of tetrabutylammonium hydroxide (~0.2 equiv) is added to an aqueous solution of the exchanged pdCpA to obtain the desired 2.2 equiv of tetrabutylammonium cation to 1 equiv pdCpA, as determined by NMR.) The product was stored as a fluffy white powder at −80 °C. Stock solutions of 0.05–0.06 M of the above compound were made in anhydrous DMF and stored at −80 °C (concentrations were determined by UV, ϵ = 23 000 cm^{-1} M^{-1}). It should be noted that stock solutions slowly decompose over time when stored at room temperature or even 0 °C.

To a flame-dried 1-dram vial, fitted with stir bar and under argon, was added exactly 50 μL of a 0.055 M stock solution of the above tetrabutylammonium salt of pdCpA in anhydrous dimethylformamide (Ald-

rich brand containing <0.005% H_2O). To the solution was added the indicated number of equivalents of the active ester, and the resulting solution was stirred at room temperature under argon atmosphere for the indicated time period. The reaction solution was then diluted with 3 mL of a 1:1 solution of 50 mM ammonium acetate, pH 4.5, and CH_3CN, followed by addition of exactly 50 μL of a sodium naphthalenesulfonate stock solution. A 10-μL aliquot of the resulting solution was then assayed by analytical HPLC [Rainin C18 (80-225-C15), 10–70% CH_3CN in 50 mM ammonium acetate, pH 4.5, over 60 min at 1 mL/min, 260 nm]. The ratio of the peak area corresponding to the aminoacyl pdCpA product relative to the peak area corresponding to the naphthalenesulfonate standard was employed to calculate the yield of the product. Cbz-protected aminoacyl pdCpA were assumed to have the same extinction coefficients as pdCpA at 260 nm (23 000 cm^{-1} M^{-1}), while yields of the NVOC-protected derivatives were corrected by assuming that the extinction coefficient at 260 nm for the NVOC-protected aminoacyl pdCpA was additive; ϵ_{260} = 23 000 cm^{-1} M^{-1} for pdCpA and ϵ_{260} = 2140 cm^{-1} M^{-1} for each 6-nitroveratryl group.

5′-Phospho-2′-deoxycytidylyl(3′–5′)-2′(3′)-*O*-[*N*-(carbobenzyloxy)-phenylalanyl]adenosine: ^{1}H NMR (400 MHz, D_2O, 2:1 mixture of monoacylated diastereomers) δ 2.09 (m, 1 H), 2.22 (m, 1 H), 2.80 (m, 1 H), 2.93 (m, 1 H), 3.89 (m, 3 H), 4.04 (m, 3 H), 4.32, 4.47 (m, 1 H), 4.55 (m, 1 H), 4.74, 4.92 (m, 1 H), 4.87 (d, H, *J* = 8.8 Hz), 5.32 (d, 1 H, *J* = 6.2 Hz), 5.68 (m, 1 H), 5.92 (d, 1 H, *J* = 8.8 Hz), 6.13 (m, 1 H), 6.88–7.42 (m, 10 H), 7.64, 7.78, 7.92 (d, H, *J* = 10.5 Hz), 8.17 (d, 1 H, *J* = 4.8 Hz), 8.40, 8.43 (s, 1 H); ^{31}P NMR (D_2O) δ 0.857 (1 P), −1.22 (1 P); mass spectrum, *m/e* 937 (MH^+).

5′-Phospho-2′-deoxycytidylyl(3′–5′)-2′(3′)-*O*-[*N*-(carbobenzyl)-*O*-benzylserinyl]adenosine: ^{1}H NMR (500 MHz, D_2O, 2:1 mixture of monoacylated diastereomers) δ 2.09 (m, 1 H), 2.22 (m, 1 H), 3.61 (m, 1 H), 3.70 (m, 1 H), 3.91 (m, 2 H), 4.03 (m, 2 H), 4.27 (m, 3 H), 4.40 (m, 1 H), 4.56 (m, 1 H), 4.87 (s, 2 H), 5.33 (m, 1 H), 5.47 (m, 1 H), 5.80 (m, 1 H), 5.92 (d, 1 H, *J* = 8.8 Hz), 5.97 (m, 2 H), 6.68–7.03 (m, 10 H), 7.70 (d, 1 H, *J* = 10.5 Hz), 7.98 (s, 1 H), 8.37 (s, 1 H); ^{31}P NMR (D_2O) δ 0.86 (1 P), −1.22 (1 P); mass spectrum, *m/e* 948 (MH^+), 970, (MNa^+).

5′-Phospho-2′-deoxycytidylyl(3′–5′)-2′(3′)-*O*-[*N*-(carbobenzyloxy)-im-benzylhistidinyl]adenosine: ^{1}H NMR (400 MHz, D_2O, 2:1 mixture of monoacylated diastereomers) δ 2.09 (m, 1 H), 2.22 (m, 1 H), 3.03 (m, 1 H), 3.20 (m, 1 H), 3.88 (m, 3 H), 4.04 (m, 3 H), 4.33, 4.37 (m, 1 H), 4.42 (m, 1 H), 5.09 (s, 2 H), 5.36, 5.42 (m, 1 H), 5.68 (d, 1 H, *J* = 8.7 Hz), 5.83 (t, 1 H, *J* = 9.0 Hz), 5.99 (m, 1 H), 6.97–7.32 (m, 10 H), 7.59 (d, H, *J* = 10.5 Hz), 7.98 (s, 1 H), 8.22 (s, 1 H), 8.32 (s, 1 H), 8.40 (s, 1 H); ^{31}P NMR (D_2O) δ 0.85 (1 P), −1.22 (1 P); mass spectrum, *m/e* 998 (MH^+).

5′-Phospho-2′-deoxycytidylyl(3′–5′)-2′(3′)-*O*-[*N*,*N*-bis(carbobenzyloxy)lysinyl]adenosine: ^{1}H NMR (400 MHz, D_2O, 2:1 mixture of monoacylated diastereomers) δ 1.35 (m, 2 H), 1.48 (m, 2 H), 1.74 (m, 1 H), 1.82 (m, 1 H), 2.09 (m, 1 H), 2.22 (m, 1 H), 3.22 (m, 2 H), 3.98 (m, 3 H), 4.27 (m, 3 H), 5.00 (s, 2 H), 5.13 (s, 2 H), 5.45, 5.58 (m, 1 H), 5.68 (m, 1 H), 6.01 (d, 1 H, *J* = 8.8 Hz), 6.11 (m, 1 H), 6.17 (m, 1 H), 7.01–7.43 (m, 10 H), 7.80 (d, H, *J* = 10.5 Hz), 8.17 (s, 1 H), 8.51 (s, 1 H); ^{31}P NMR (D_2O) δ 0.86 (1 P), −1.22 (1 P); mass spectrum, *m/e* 1033 (MH^+), 1055 (MNa^+).

5′-Phospho-2′-deoxycytidylyl(3′–5′)-2′(3′)-*O*-[*N*-(6-nitroveratryloxy)-L-phenylalanyl]adenosine: ^{1}H NMR (400 MHz, 1:1 DMSO-d_6/acetic acid-d_4, 2:1 mixture of monoacylated diastereomers, acetic acid-d_4 as reference) δ 8.84 (s, 0.66 H), 8.82 (s, 0.33 H), 8.61 (s, 0.66 H), 8.59 (s, 0.33 H), 8.24 (d, 1 H, *J* = 7.9 Hz), 7.78 (s, 1 H), 7.49 (m, 5 H), 7.18 (s, 0.66 H), 7.07 (s, 0.33 H), 6.43 (d, 0.33 H, *J* = 4.2 Hz), 6.29 (d, 1 H, *J* = 7.9 Hz), 6.27 (m, 1 H), 6.18 (d, 0.66 H, *J* = 6.4 Hz), 5.84 (t, 0.33 H, 4.7 Hz), 5.57 (m, 0.66 H), 5.53 (d, 0.66 H, *J* = 15.2 Hz), 5.51 (d, 0.66 H, *J* = 15.0 Hz), 5.47 (d, 0.33 H, *J* = 15.2 Hz), 5.40 (d, 0.33 H, *J* = 15.2 Hz), 5.06 (m, 1.66 H), 4.86 (m, 0.33 H), 4.75 (dd, 0.66 H, *J* = 5.7, 9.2 Hz), 4.66 (dd, 0.33 H, *J* = 4.4, 10.1 Hz), 4.47 (m, 0.66 H), 4.42 (m, 0.33 H), 4.36 (m, 0.66 H), 4.30 (m, 2.33 H), 3.99 (s, 1 H), 3.98 (s, 2 H), 3.96 (s, 2 H), 3.91 (s, 1 H), 3.43 (dd, 0.33 H, *J* = 3.5, 13.9 Hz), 3.37 (dd, 0.66 H, *J* = 5.6, 14.0 Hz), 3.18 (dd, 0.66 H, *J* = 9.5, 14.0 Hz), 3.06 (dd, 0.33 H, *J* = 10.4, 13.9 Hz), 2.71 (m, 1 H), 2.42 (m, 1 H); ^{31}P NMR (1:1 DMSO-d_6/acetic acid-d_4) δ 0.12 (0.33 P), 0.09 (0.66 P), −1.72 (0.33 P), −1.77 (0.66 P); mass spectrum, *m/e* 1045 (MNa^+), 1023 (MH^+).

5′-Phospho-2′-deoxycytidylyl(3′–5′)-2′(3′)-*O*-[*N*-(6-nitroveratryloxy)-D-phenylalanyl]adenosine: ^{1}H NMR (400 MHz, 4:1 acetic acid-d_4/DMSO-d_6, 2:1 mixture of monoacylated diastereomers, acetic acid-d_4 as reference) δ 7.71 (s, 1 H), 7.38–7.17 (m, 5 H), 7.07 (s, 1 H), 6.23 (m, 3 H), 5.73 (m, 0.33 H), 5.59 (m, 0.66 H), 5.44 (m, 2 H), 5.12 (t, 0.66 H, *J* = 5.3 Hz), 5.04 (m, 1 H), 4.84 (m, 0.33 H), 4.73 (dd, 0.66 H, *J* = 4.4, 9.6 Hz), 4.66 (t, 0.66 H, *J* = 7.3 Hz), 4.52 (m, 0.66 H), 4.46 (m, 1.33 H), 4.37–4.23 (m, 3 H), 3.92 (s, 3 H), 3.89 (s, 3 H), 3.44 (m, 0.66 H), 3.24 (m, 0.33 H), 3.08 (m, 1 H), 2.68 (m, 1 H), 2.35 (m, 1 H); ^{31}P NMR (4:1 acetic acid-d_4/DMSO-d_6) δ 0.79 (1 P), −1.28, −1.66 (1 P); mass spectrum, *m/e* 1045 (MNa^+), 1023 (MH^+).

5′-Phospho-2′-deoxycytidylyl(3′–5′)-2′(3′)-*O*-[*N*-(6-nitroveratryloxy)-γ-(*O*-6-nitroveratryl)-L-aspartyl]adenosine: ^{1}H NMR (400 MHz, 1:2 acetic acid-d_4/DMSO-d_6, 2:1 mixture of monoacylated diastereomers, DMSO-d_6 as reference) 8.05 (s, 0.66 H), 8.44 (s, 0.33 H), 8.17 (s, 0.66 H), 8.16 (s, 0.33 H), 8.00 (m, 1 H), 7.59 (s, 2 H), 7.08 (s, 1 H), 7.01 (s, 0.66 H), 6.98 (s, 0.33 H), 6.13 (d, 0.33 H, *J* = 4.3 Hz), 6.01 (m, 2 H), 5.92 (d, 0.66 H, *J* = 6.9 Hz), 5.60 (m, 0.33 H), 5.32 (m, 3.33 H), 5.23 (d, 0.33 H, *J* = 15.1 Hz), (d, 0.33 H, *J* = 15.1 Hz), 4.18 (m, 0.66 H), 4.76 (m, 1.66 H), 4.64 (m, 0.33 H), 4.53 (m, 0.33 H), 4.20–3.91 (m, 5 H), 3.82 (s, 2 H), 3.81 (s, 2 H), 3.80 (s, 1 H), 3.78 (s, 1 H), 3.73 (s, 3 H), 3.07 (m, 1 H), 2.96 (dd, 0.66 H, *J* = 8.2, 16.3 Hz), 2.84 (dd, 0.33 H, *J* = 8.2, 16.3 Hz), 2.44 (m, 1 H), 2.18 (m, 1 H); ^{31}P NMR (1:2 acetic acid-d_4/DMSO-d_6) δ 0.06, −1.82; mass spectrum, *m/e* 1208 (MNa^+), 1186 (MH^+), 897 (base).

Racemization Assay. No diastereomeric cross-contaminant due to racemization of the *N*-NVOC-L-phenylalanine was detected by HPLC analysis (Rainin C18 (80-225-C15) column, 0–70% CH_3CN in 50 mM ammonium acetate, pH 4.5, over 70 min, 1.0 mL/min, 260 nm) when the reaction was performed according to standard procedure. To confirm that the diastereomers did not coelute under the above HPLC conditions, a 1:1 comixture of *N*-NVOC-D-phenylalanyl pdCpA (vide infra) and *N*-NVOC-L-phenylalanyl pdCpA was analyzed by HPLC: t_r (*N*-NVOC-L-phenylalanyl pdCpA, mixture of 2′- and 3′-monoacyl derivatives) 50.46 and 51.16 min; t_r (*N*-NVOC-D-phenylalanyl pdCpA, mixture of 2′- and 3′-monoacyl derivatives) 50.46 and 51.66 min. When a 20:1 mixture of *N*-NVOC-L-phenylalanyl pdCpA and *N*-NVOC-D-phenylalanyl pdCpA was analyzed by HPLC analysis, the *N*-NVOC-D-phenylalanyl pdCpA was readily observed, thus confirming that little if any racemization had occurred in the acylation reaction.

Photodeprotection of Protected Aminoacyl pdCpAs. Photodeprotection of L-NVOC-phenylalanyl pdCpA. *N*-NVOC-L-phenylalanyl pdCpA (0.12 μmol) was dissolved in 80 μL of 1 mM potassium acetate, pH 4.5. Exactly 10 μL of the aminoacyl pdCpA solution was diluted with exactly 10 μL of a stock solution of sodium naphthalenesulfonate. The resulting solution was assayed by analytical HPLC (260 nm, 1 mL/min, 0–70% acetonitrile in 50 mM ammonium acetate, pH 4.5, over 50 min). The ratio of the *N*-NVOC-phenylalanyl pdCpA, t_r 44.43 and 45.02 min, to standard (naphthalenesulfonate), t_r 31.40 min, was 1.15:1. An aliquot (40 μL) of the remaining aminoacyl pdCpA solution was then irradiated with a 2000-W mercury–xenon lamp with a Pyrex glass filter for 7.5 min at 0 °C. The yield of the deprotected product was determined by diluting exactly 10 μL of the irradiated solution with exactly 10 μL of the above stock solution of sodium naphthalenesulfonate followed by assay by analytical HPLC (260 nm, 1 mL/min, 0–70% acetonitrile in 50 mM ammonium acetate, pH 4.5, over 70 min). The ratio of the *N*-NVOC-phenylalanyl pdCpA, t_r 30.56 min, to standard (naphthalenesulfonate), t_r 32.86 min, was 1.03:1. On the basis of the extinction coefficient at 260 nm of pdCpA (23,000 cm^{-1} M^{-1}) and of NVOC-phenylalanyl pdCpA (25 140 cm^{-1} M^{-1}) this corresponds to greater than a 95% yield of the photodeprotected product. The complete characterization of phenylalanyl pdCpA has previously been reported.[1b]

Photodeprotection of L-*N*-NVOC-Aspartyl pdCpA β-6-Nitroveratryl Ester. L-*N*-NVOC aspartyl pdCpA β-6-nitroveratryl ester (0.12 μmol) was dissolved in 2.0 mL of 1 mM potassium acetate, pH 4.5. A portion of the aminoacyl pdCpA solution, 40 μL, was diluted with 40 μL of a stock solution of sodium naphthalenesulfonate. The resulting solution was assayed by analytical HPLC (260 nm, 1 mL/min, 0–20% over 20 min followed by 20–90% acetonitrile in 50 mM ammonium acetate, pH 4.5, over 50 min). The ratio of the protected aspartyl pdCpA derivative, t_r 43.56 min, to standard (naphthalenesulfonate), t_r 31.22 min, was 1.24:1. A 40-μL sample of the aminoacyl pdCpA solution was then irradiated for 15 min at 0 °C. The yield of the deprotected product was determined by diluting the 40-μL aliquot of the irradiated solution with 40 μL of the above stock solution of sodium naphthalenesulfonate followed by assay by analytical HPLC (260 nm, 1 mL/min, 0–20% over 20 min followed by 20–90% over 50 min of acetonitrile in 50 mM ammonium acetate, pH 4.5, over 70 min). The ratio of the *N*-NVOC-phenylalanyl pdCpA, t_r 19.57 min, to standard (naphthalenesulfonate), t_r 30.93 min, was 0.96:1. Based upon the extinction coefficient at 260 nm of aspartyl pdCpA (25 000 cm^{-1} M^{-1}) and of the diprotected aspartyl pdCpA derivative (29 280 cm^{-1} M^{-1}), this corresponds to a 93% yield of photodeprotected product. Mass spectrum, *m/e* 774 (MNa^+), 752 (MH^+).

Enzymatic Ligation Reactions.[1b] Ligation reactions (80 μL) contained the following: 700 μM acyl pdCpA, tRNA $(-CA)_{CUA}$ (20 μg, which had been desalted and lyophilized after gel purification), 55 μM Hepes (Na) (pH 7.5), 250 μM ATP, 15 μM $MgCl_2$, BSA at 20 μg/μL, dimethyl

sulfoxide (DMSO) [to 10% (v/v)], and 200 units of T4 RNA ligase. The reaction mixture was incubated at 37 °C for 8 min, quenched by addition of 2.5 M sodium acetate (pH 4.5) to 10% (v/v), and then immediately extracted with phenol (equilibrated with 0.25 M sodium acetate, pH 4.5), phenol/$CHCl_3$/isoamyl alcohol (25:24:1), and $CHCl_3$/isoamyl alcohol (24:1). Acylated tRNA was then precipitated with 3 volumes of ethanol at −78 °C. The tRNA was then desalted by employing a NAP5 column (prepacked Sephadex G-25 column, Pharmacia) equilibrated with water and lyophilized. The lyophilized unpurified acyl tRNA was stored at −80 °C until immediately prior to photodeprotection.

Photodeprotection of Aminoacyl tRNA. Photodeprotection of *N*-NVOC-L-phenylalanyl tRNA was accomplished by diluting the above tRNA pellet to 1 μg/μL with 1 mM potassium acetate, pH 4.5, in a transparent microfuge tube, followed by irradiation of the solution for 7.5 min employing a 2000-W mercury–xenon lamp with a Pyrex glass filter. The solution was maintained at 0 °C during photodeprotection by placing the microfuge tube in a Pyrex vessel containing ice-cold water during the irradiation. After photodeprotection, the solution was immediately cooled to −78 °C until immediately prior to the in vitro protein biosynthesis reaction.

In Vitro Protein Synthesis. In vitro protein synthesis reactions (typically 30-μL final volume) contained, per milliter of final volume: 56.4 μmol of Tris-acetate (pH 7.4), 1.76 μL of dithiothreitol, 1.22 μmol of ATP (Na) (pH 7), 0.85 μmol each of GTP (Na), CTP (Na), and UTP (Na) (pH 7), 27 μmol of potassium phosphoenolpyruvate, (pH 7), 0.35 μmol each of the 20 amino acids; 19 μg of poly(ethylene glycol) 8000; 35 μg of folinic acid; 27 μg of pyridoxine hydrochloride, 27 μg of NADP, 27 μg of FAD, 11 μg of *p*-aminobenzoic acid, 170 μg of *E. coli* tRNA, 36 μmol of ammonium acetate, 72 μmol of potassium acetate, 9.7 μmol of calcium acetate, and 10–14 μmol of magnesium acetate (optimized for each preparation of tRNA, plasmid or S-30). Plasmid DNA (100 μg/mL) was preincubated at 37 °C for 2 min with the above components, after which suppressor tRNA suspended in 1 mM potassium acetate was added, and the resultant mixture was incubated for 1 h on a rotary shaker (200 rpm), cooled to 0 °C, and centrifuged. The supernatant was then assayed for β-lactamase activity by using the chromogenic substrate nitrocefin.[11]

Results and Discussion

Aminoacylation of pdCpA. Two obstacles must be overcome in order to efficiently aminoacylate tRNAs with unnatural amino acids: low yields associated with aminoacylation and deprotection of pdCpA (or pCpA) and the hydrolytic lability of the aminoacyl ester linkage to the tRNA. Difficulties in the selective monoacylation of pdCpA arise as a result of side reactions of the activated amino acid with cytosine and adenine, as well as diacylation of the 2′,3′-diol. [Note that it is sufficient to acylate either the 3′- or 2′-hydroxyl group of adenosine: the interconversion between the two isomers is rapid in aqueous buffer, pH 7.3, 37 °C ($t_{1/2}$ = 1–11 s^{-1}).[12]] Reaction of unprotected pdCpA has previously been attempted with N-blocked amino acids activated with carbonyldiimidazole (CDI), dicyclohexylcarbodiimide (DCC), or DCC and hydroxybenzotriazole, as well as symmetric and mixed anhydrides.[13] All of these methods resulted in complete acylation of the N^4 amino group of cytidine and partial acylation of the adenosine diol. The N^6 amino group of adenosine is less reactive and is acylated competitively with the 2′,3′-diol only when sterically hindered amino acids, such as α-methyl-D,L-phenylalanine and *N*-methyl-L-phenylalanine, are used.

Hecht and co-workers aminoacylated the unprotected dinucleotide with 10 equiv of *N*-carbobenzoxy (Cbz) amino acids in DMSO for 48 h with 10 equiv of carbonyldiimidazole as an activating agent.[5] As expected, both the N^4 of cytidine and the 2′,3′-diol of adenosine are acylated under these conditions. The molecule can be N^4-deacylated with 0.01 N HCl to afford the 2′(3′)-O-monoaminoacylated dinucleotide in 4–12% isolated yield. Hecht and co-workers subsequently acylated pCpA with pyroglutamyl-protected amino acids via the same methodology.[8] The N-protected aminoacyl dinucleotides were coupled to tRNA-C_{OH} with T4 RNA ligase and the α-amino group was deprotected enzymatically to afford the aminoacyl tRNAs in approximately 50% yield. Brunner and co-workers acylated pCpA with a *t*-Boc-protected derivative of a photoactivateable phenylalanine analogue again using carbonyldiimidazole.[7] After deprotection of the diacylated nucleotide with trifluoroacetic acid and 0.01 M HCl, a 6% yield of the monoaminoacylated dinucleotide was obtained as a mixture of 2′-5′ and 3′-5′ phosphodiester isomers. The unblocked aminoacyl dinucleotide was then efficiently ligated to tRNA at pH 7.5, 37 °C, with a 6-min coupling time.

We have recently reported methodology for selectively monoacylating pCpA and pdCpA by first blocking the exocyclic amine of cytidine with the NPS group, which can be removed under mildly reducing conditions that do not affect the labile acyl bond.[1b] The 2′,3′-diol is then acylated with Cbz or NPS N-blocked amino acids by using carbonyldiimidazole. Removal of the NPS group with sodium thiosulfate or the Cbz group by hydrogenation affords the aminoacyl dinucleotide in 35% yield. Chladek and co-workers have reported aminoacylation of 5′CpCpA in 26% overall yield by fully protecting the oligoribonucleotide prior to aminoacylation.[14] The bases were protected with the (9-fluorenylmethyloxy)carbonyl (Fmoc) group, the α-amino group of the amino acid with the 2-(4-biphenyl)isopropyloxycarbonyl group, the 2′-hydroxyls with the 4-methoxytetrahydropyranyl and the phosphate with the 2-chlorophenyl group. (Mesitylenesulfonyl)tetrazole was used to couple the α-protected amino acid in 92% yield. The acylated trinucleotide was deprotected in two steps in 28% overall yield.

All of the above protocols suffer several drawbacks that lead to low aminoacylation yields. These methods either result in diacylation of pCpA or require protection of pCpA prior to aminoacylation. The protecting groups are removed in relatively low overall yields. Moreover, most of the protocols involve coupling of free aminoacyl pCpA to tRNA-C_{OH} under conditions where the unprotected aminoacyl linkage is hydrolytically labile.[12]

Ideally one would like to develop a general method for the selective monoacylation of the 2′,3′ adenosine diol in high yield without prior protection of pdCpA. One strategy for accomplishing this transformation might be to carry out an enzymatic aminoacylation by using a nonspecific protease or lipase "in reverse" in organic solvent. Klibanov and Wong have shown that the primary hydroxyl groups on a number of sugars can be selectively acylated with various lipases when the reaction is carried out in organic solvents.[15] Trichloroethyl esters of butyrate, acetate, caprylate, and laurate as well as various 2,2,2-trichloroethyl amino acids were used as acyl donors. If the primary hydroxyl is blocked, selective monoacylation will occur at the 2′ or 3′ secondary hydroxyls of the sugar with the trichloroethyl butyrate as the acyl donor. Klibanov also showed that the protease subtilisin can selectively monoacylate A and U using trichloroethyl butyrate as the acyl donor,[16] and that the enzyme is able to acylate a hydroxyl selectively over an amine.[17]

Since the dinucleotide pdCpA has three negative charges present on the molecule, it was necessary to form the tetrabutylammonium salt (tetraethylammonium salts can also be used, but are less soluble), before the molecule was soluble at reasonable levels in organic solvents, and even then the molecule was only soluble in DMF to ~0.1 M concentration or in DMSO. We initially used Carlsburg subtilisin as the catalyst since it retains enzymatic activity in DMF and can be obtained easily in large quantities. Both the cyanomethyl and trichloroethyl esters of the amino acids were synthesized since studies by Klibanov and Wong showed that mildly activated esters were required for efficient acylation. We found that the trichloroethyl ester of phenylalanine gave virtually no product under typical reaction conditions of 0.5 M pdCpA and 0.05 M activated amino acid in dry DMF (typical reported

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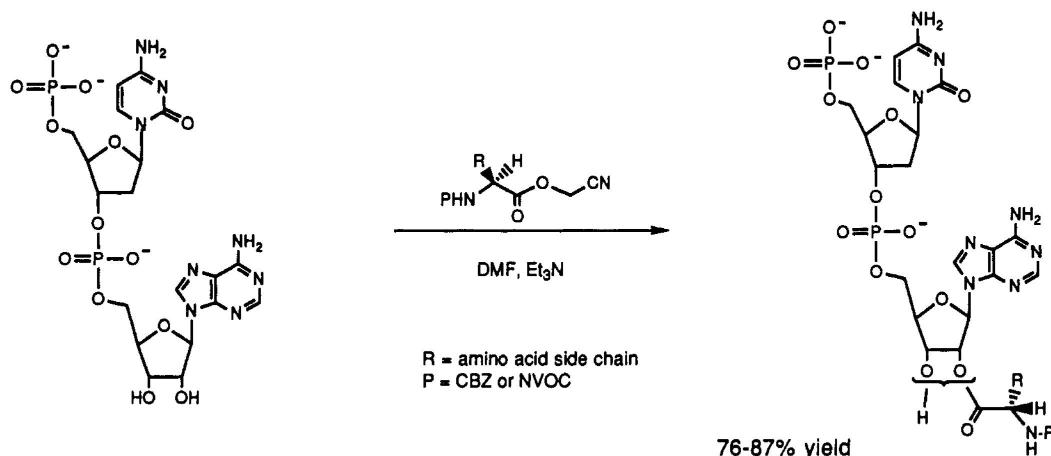

Figure 3. Acylation of pdCpA with cyanomethyl active esters.

conditions for analogous reactions were 0.2–0.5 M activated ester with 1.2–5 molar equiv excess of the acyl acceptor).[15-17] However, the cyanomethyl ester gave 60–90% conversion to monoacylated adenosine diol products as determined by HPLC analysis. No detectable amounts of diacylated or N-acylated material were observed. However, when experiments were carried out *in the absence of enzyme* the same results were obtained. Selective acylation of pdCpA therefore appears to result from selective formation or breakdown of the tetrahedral adduct formed from N and O addition to the activated ester and is not a result of enzyme specificity. The phenyl ester of Cbz-protected phenylalanine also gave reasonable yields (47%, unoptimized) of monoaminoacyl pdCpA. More strongly activated esters (*N*-hydroxysuccinimide, *p*-nitrophenyl) gave predominantly N,O-diacylated pdCpA, with no recoverable mono-O-acylated material detected.

Older stocks of cyanomethyl activated amino acids gave higher product yields than those freshly prepared. In addition, the acylation rate was dependent upon the batch of pdCpA. We therefore examined the effect of added bases, acids, and nucleophiles on the aminoacylation of pdCpA (0.01–0.05 M) by *N*-Cbz-*O*-Bn-serine (0.1–0.5 M). It was found that KCN (0.5 mM), triethylamine (0.5 mM), or 1,4-diazabicyclo[2.2.2]octane (DABCO) (0.5 mM) resulted in a modest increase in the acylation rate. Formic and acetic acid (0.1–0.5 M), $NH_4^+Cl^-$ (0.4 M), or (dimethylamino)pyridine (0.5 mM) either had no effect or decreased reaction yields compared to the uncatalyzed reaction. The most dramatic increase in the reaction rate was obtained by using stocks of pdCpA that contained greater than 2 equiv of the tetrabutylammonium counterion per pdCpA (presumably the dianion of the terminal phosphate serves as a potent base catalyst in DMF). We found that when pdCpA containing 2.2 equiv of the tetrabutylammonium counterion was employed, a convenient reaction rate was obtained at room temperature. In the case of *N*-NVOC-phenylalanine cyanomethyl ester, an 84% yield of N-protected dinucleotide was obtained as a 5:1 molar ratio of activated amino acid to dinucleotide with a 2.5-h reaction time at room temperature. No N-acylated material was detected, and appreciable amounts of O,O-diacylated material are observed only after prolonged reaction times.

Aminoacylation of pdCpA by the cyanomethyl esters of amino acids proceeds in high yield for a variety of amino acids (Table I). In the case of amino acids with reactive side-chain functional groups such as histidine, lysine, serine, and glutamic acid, protecting groups were also required for the side-chain functionality. As is the case of aspartic acid (Table I, entry C), the 6-nitroveratryl protecting groups should be generally applicable to side-chain protection. In addition, for costly unnatural amino acids, the number of equivalents of active ester can be reduced to as little as 1.2 equiv simply by extending the reaction time (Table I, entry B).

In order to determine the degree of racemization of the amino acid in the aminoacylation reaction, L-NVOC-phenylalanine cyanomethyl ester (5 equiv) was coupled to pdCpA (0.054 M) over 2.5 h at room temperature. No racemization was detected (less than 5%) by HPLC analysis of the unpurified aminoacyl pdCpA product. This result was confirmed by coinjection of the unpurified product with an authentic sample of D-NVOC-phenylalanyl pdCpA. When only 1.5 equiv of L-NVOC-phenylalanine cyanomethyl active ester was employed in the acylation reaction, 2–5% racemization was observed, probably due to the extended reaction time.

Protection of the α-Amino Group. Ideally α-amino protecting groups as well as any side-chain protecting groups on the amino acids should be efficiently removed after ligation of the aminoacyl pdCpA to tRNA-C_{OH}. Stability studies on the aminoacyl ester bond show that, under the conditions used for ligating aminoacyl pdCpA to tRNA-C_{OH} [50 mM (Na) Hepes (pH 7.5), 20 mM $MgCl_2$, 24 mM ATP, 10% (v/v) DMSO, 37 °C], aminoacyl pdCpA containing an N-blocked amino acid has a substantially longer half-life than that of the aminoacylated pdCpA containing the free amino acid ($t_{1/2}$ = 234 min for N-protected phenylalanine and 22 min for unprotected phenylalanine). Deprotection of the amino acid after ligation to the tRNA necessitates that the deprotection conditions do not modify the tRNA structure in any way that interferes with translation efficiency or synthetase recognition. For example, removal of *t*-Boc from *N*-Boc-aminoacyl pCpA led to isomerization of the phosphodiester bond[7] and, in the case of an entire tRNA, could lead to depurination.[18] Hydrogenolysis can lead to reduction of the pyrimidine bases of an intact tRNA (we have shown that hydrogenolysis over Pd/$BaSO_4$ at 1 atm reduces uridine entirely in under 2.5 h. In addition we have found it to be difficult to recover the tRNA from the Pd/$BaSO_4$ catalyst). Base-catalyzed removal is likely to lead to cleavage of the acyl linkage. The protecting group should also be applicable to a variety of function groups that might be found in the side chains of unnatural amino acids. This requirement may limit the usefulness of deprotection with proteolytic enzymes such as pyroglutamyl aminopeptidase.

Given these considerations, we turned our attention to the following protecting groups for the α-amino acid: the NPS group (removed by treatment with sodium thiosulfate), the biphenylisopropyloxycarbonyl (BPOC) group (removed by treatment with mild acid), and the nitroveratryloxy (NVOC) group (removed by photolysis). The NPS group was examined but leads to lower yields for aminoacylation of pdCpA, presumably because the α-amino group of the NPS-blocked amino acid is still somewhat nucleophilic. The BPOC group may prove to be useful, but is labile at low pH.[14,19] Because it is convenient to purify the aminoacyl dinucleotide or tRNA at a lower pH (the aminoacyl

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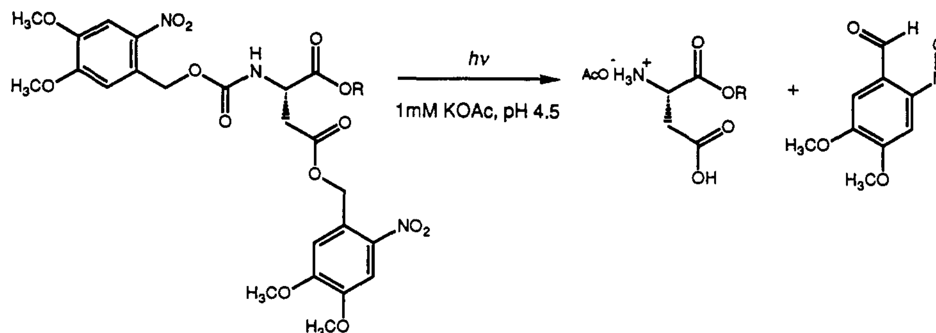

Figure 4. Photodeprotection of NVOC-protected α-amino esters.

bond is stable under these conditions) we decided to examine the NVOC protecting group. Nitroveratryloxy carbamates and esters and nitroveratryl ethers have been used to protect amino, hydroxyl, carboxyl, and phosphoryl moieties.[10,20] Deprotection can be carried out with 350-nm light, which is known not to damage RNA.[21] Because the photolysis reaction leads to nitroso and aldehyde byproducts, deprotection can be carried out under slightly acidic conditions in the case of N-blocked amines to prevent secondary reaction of the free amine with reaction photoproducts.

In order to test the applicability of this protecting group, the α-amino group of phenylalanine and both the α-amino and β-carboxyl groups of aspartic acid were protected with 6-nitroveratryl-derived protecting groups.

The α-amino group of phenylalanine was readily protected by reaction with 6-nitroveratrylcarbonyl chloride (NVOC-Cl) under standard Schotten Baumann conditions.[22] The diprotected aspartic acid derivative was prepared from *N*-Boc-aspartic acid α-*tert*-butyl ester by esterification of the β-carboxylic acid with 6-nitroveratryl alcohol [dicyclohexylcarbodiimide, (dimethylamino)pyridine][23] followed by removal of the *N*-Boc and *tert*-butyl ester protecting groups by treatment with trifluoroacetic acid and subsequent protection of the resulting amino acid with NVOC-Cl. The cyanomethyl esters of the protected amino acids were then coupled to pdCpA in high yield (Table I). The NVOC-protected aminoacyl pdCpA derivatives were of comparable stability to the Cbz-protected aminoacyl pdCpA derivatives described previously ($t_{1/2}$ = 180 min for *N*-NVOC-phenylalanyl pdCpA under ligation buffer conditions). More recently, a variety of mono- and di-NVO-protected aminoacyl pdCpA derivatives have been prepared including glutamine, leucine, homocysteic acid, glutamic acid, homocysteine, γ-fluoroglutamic acid, and α,β-diaminopropionic acid derivatives.[24]

In carrying out the photodeprotections of the aminoacyl tRNA, reaction conditions of 1 mM potassium acetate, 0 °C, pH 4.5, and aminoacyl tRNA at a concentration of 1 μg/mL were used in order to allow addition of the deprotected aminoacyl tRNA to the protein biosynthesis reaction immediately after irradiation, without any additional manipulations. These conditions had previously been determined to be optimal for addition of the aminoacyl tRNA to the protein biosynthesis reaction.[1a] In addition, the aminoacyl tRNA should be very stable to deacylation under these conditions, since a solution of phenylalanyl pdCpA in this buffer deacylated less than 3% after 2 h at room temperature.

In initially optimizing the photodeprotection reaction we employed protected aminoacyl pdCpA rather than the protected aminoacyl tRNA in order to facilitate the analysis of the reaction products. Irradiation of a 0.15 mM solution of *N*-NVOC-phenylalanyl pdCpA in 1 mM potassium acetate, pH 4.5, buffer employing a 2000-W mercury–xenon lamp (a Pyrex glass filter serves as a 300-nm cutoff filter) for 7.5 min resulted in complete deprotection in greater than 95% yield as determined by HPLC analysis with sodium naphthalenesulfonate as a standard. Irradiation of the diprotected aspartyl pdCpA derivative for 15 min under analogous conditions also resulted in complete deprotection in approximately 93% yield as determined by HPLC analysis.

The viability of the above photodeprotection conditions for the removal of the protecting groups from aminoacyl tRNA was then confirmed by ligating *N*-NVOC-phenylalanyl pdCpA to tRNA(-CA) followed by photodeprotection and addition to an in vitro protein biosynthesis system. Ligation of *N*-NVOC-phenylalanyl pdCpA to tRNA-C_{OH} was accomplished as previously reported.[1b] After irradiation of the *N*-NVOC-phenylalanyl tRNA at 1 μg/μL for 7.5 min, the deprotected aminoacyl tRNA was immediately added to an in vitro protein synthesis reaction primed with a plasmid containing a TAG mutation at the Phe66 codon of β-lactamase (pF66am).[1a] Suppression of TAG 66 with phenylalanine is a necessary condition for generating fully active β-lactamase. The suppression efficiency was analyzed by spectrophotometric determination of the rate of hydrolysis of the β-lactamase substrate, nitrocefin.[11] Suppressor tRNA prepared by ligation with phenylalanyl pdCpA afforded 7.0 ± 0.6 nitrocefin hydrolysis units (micromoles of nitrocefin hydrolyzed per minute, pH 7, 37 °C), while for suppressor tRNA aminoacylated with *N*-NVOC-phenylalanyl pdCpA followed by photodeprotection 8.4 ± 2.3 units were produced. Thus, photodeprotection can be accomplished on the full-length tRNA without diminishing the suppression efficiency.

Conclusion

A general and expedient procedure for the synthesis of aminoacyl tRNA has been developed. Aminoacyl pdCpA can be prepared in one step in 76–87% yield by reaction of the cyanomethyl active esters of N-protected α-amino acids with pdCpA. Due to the high selectivity of the cyanomethyl active ester for monoacylation of the 2′,3′-diol of the adenosine of pdCpA, protection of the base functionality of pdCpA is not required. By employing the photolabile nitroveratryl protecting groups for the protection of the α-amine and side-chain functionality of the α-amino acid, the protected aminoacyl pdCpA can be directly ligated to tRNA-C_{OH} to provide protected aminoacyl tRNA. Importantly, N protection of the α-amino group greatly enhances the stability of the labile aminoacyl bond to the ligation buffer conditions. After photochemical removal of the amino acid protecting groups, which occurs in near quantitative yield, the aminoacyl tRNA can be added directly to an in vitro protein biosynthesis reaction. This straightforward procedure for the preparation of aminoacyl tRNA should allow the rapid introduction of a variety of natural and unnatural amino acids into proteins.

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Registry No. 1, 132018-86-1; **2**, 132018-87-2; **3**, 95433-50-4; **4**, 132018-88-3; **5**, 7663-85-6; **6**, 132018-89-4; NVOC-Cl, 42855-00-5; NV-OH, 1016-58-6; pdCpA, 127067-28-1; $ClCH_2CN$, 107-14-2; Cbz-His(CH_2Ph)-OH, 21929-66-8; Cbz-Ser(CH_2Ph)-OH, 20806-43-3; Cbz-Phe-OH, 1161-13-3; Cbz-Lys(Cbz)-OH, 405-39-0; NVOC-Phe-OH, 30034-25-4; Cbz-Phe-OPh, 4865-47-8; H-Ser(CH_2Ph)-OH, 4726-96-9; H-Phe-OH, 63-91-2; BOC-Asp-OBu-*t*, 34582-32-6; BOC-Asp⇌O-NV)-OBu-*t*, 132018-90-7; BOC-Asp(O-NV)-OBu-*t*, 132018-91-8; pdCpA[3′-*O*-(Cbz-Phe)], 132018-92-9; pdCpA[2′-*O*-(Cbz-Ser-(CH_2Ph))], 132046-22-1; pdCpA[3′-*O*-(Cbz-Ser(CH_2Ph))], 132018-93-0; pdCpA[2′-*O*-(Cbz-His(CH_2Ph))], 132018-94-1; pdCpA[3′-*O*-(Cbz-His(CH_2Ph))], 132018-95-2; pdCpA[2′-*O*-(Cbz-Lys(Cbz))], 132018-96-3; pdCpA[3′-*O*-(Cbz-Lys(Cbz))], 132018-97-4; pdCpA[2′-*O*-(NVOC-Asp(O-NV))], 132018-98-5; pdCpA[3′-*O*-(NVOC-Asp(O-NV))], 132018-99-6; pdCpA[2′-*O*-(NVOC-D-Phe)], 132019-00-2; pdCpA[3′-*O*-(NVOC-D-Phe)], 132019-01-3; pdCpA[2′-*O*-(NVOC-Phe)], 132076-56-3; pdCpA[3′-*O*-(NVOC-Phe)], 132076-57-4; NVOC-D-Phe-OCH_2CN, 132019-02-4; pdCpA[2′-*O*-(H-Phe)], 132019-03-5; pdCpA[3′-*O*-(H-Phe)], 132019-04-6; pdCpA[2′-*O*-(H-asp)], 132019-05-7; pdCpA[3′-*O*-(H-Asp)], 132019-06-8.

Involvement of Side Functions in Peptide Structures: The Asx Turn. Occurrence and Conformational Aspects

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Abstract: The Asx turn is a local conformation in peptide chains that is topologically equivalent to the β turn. It is characterized by an interaction closing a 10-membered cycle and involving the C$^{\gamma}$O group of an Asn or Asp residue and the peptide NH group two residues ahead in the sequence. Its conformational features have been deduced from its statistical occurrence in crystallized proteins, and from theoretical and experimental analysis of model peptides reproducing this local conformation. Four Asx-turn types have been considered and their relative stability is discussed in relation with the sequence and the existence of longer range interactions.

In a statistical analysis of the intramolecular contacts in proteins with accurate crystal structure, Baker and Hubbard have emphasized the fact that 10% of the peptide nitrogens and 15% of the peptide carbonyls are hydrogen-bonded to side chains.[1] In return, about a quarter of the hydrogen-bonded side chains interact with peptide atoms. Furthermore, nearly half of the main-chain side-chain contacts are short- and medium-range interactions involving one of the four residues on both sides of the residue considered. These observations confirm that side chains play an important role in protein and peptide folding and could stabilize local conformations.

Among the short-range side-chain main-chain interactions, the high occurrence of Asn and Asp residues deserves to be noted, and the contact between the Asn C$^{\gamma}$O carbonyl or the Asp C$^{\gamma}O_2^-$ carboxylate and the peptide NH two residues ahead is particularly frequent (see Table 7 in ref. 1). The resulting hydrogen bond closes a 10-membered cycle topologically similar to that characterizing the well-known β turn[2] (Figure 1). Hence it has been called Asx turn (Asx standing for Asn or Asp). It was identified in the crystal structures of α-chymotrypsin, prealbumin, and carboxypeptidase A,[3,4] and also more recently in the crystal structures of some Asn-containing oligopeptides.[5-8]

Although the Asx turn is much less frequent than the β turn, it concerns ~18% of the Asn and Asp residues, and 50 Asx turns have been found in the crystal structures of 13 proteins.[1] It is often involved in β-turn- and α-helix-inducing sequences,[1,9] and possibly in posttranslational modifications such as Asn deamidation[10] and N glycosylation.[11]

No conformational analysis of the Asx turn has been reported up to now. We therefore decided to study the conformational properties of this local structure by combining a 3-fold approach: (i) statistical analysis of the Asx turns listed by Baker and Hubbard,[1] (ii) Monte Carlo analysis of Gly-Asn-Xaa-Gly (Xaa = Gly, Ala), which is the shortest sequence reproducing the Asx turn, (iii) experimental study (IR and ^{1}H NMR spectroscopies and X-ray diffraction) of model di- and tripeptides.

Due to the low precision of the data on most side chains in the crystal structures of the proteins, and the low occurrence of a given sequence in the available data set, a precise analysis of the local conformations resulting from side-chain main-chain interactions is hardly feasible with proteins. Model oligopeptides are much

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