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Studies toward the site specific incorporation of sugars into proteins: Synthesis of glycosylated aminoacyl–tRNAs

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Abstract

A series of glycosylated serine derivatives was synthesized from peracetylated sugars and Fmoc-protected serine; these were chemically esterified with the tris-(tetrabutylammonium) salt of pdCpA. The fully protected and deprotected glycosylated aminoacyl pdCpAs were ligated enzymatically to an abbreviated tRNA (tRNA–C_{OH}) to provide the title compounds that are key intermediates in the elaboration of glycoproteins using readthrough of a nonsense codon.

Keywords: Glycoprotein; Monosaccharide; S-serine; Site-specific glycosylation; Transfer RNA

1. Introduction

The majority of cell-surface and secreted proteins are glycosylated with carbohydrates covalently linked through either a nitrogen atom (supplied by the amino acid asparagine) or an oxygen atom (supplied by serine or threonine).[1] The carbohydrate moiety of a glycoprotein is believed to participate directly in recognition events, but may also influence the properties of the protein.[2–6] These include effects on the catalytic activities,[7] protection from proteolytic degradation[8] and alteration of the peptide backbone conformation and folding.[9]

Investigation of the structure and function of individual glycoproteins has been complicated by the fact that naturally occurring glycoproteins exist in a number of glycoforms that possess the same peptide backbone but differ in both the nature and the sites of glycosylation. Only in a few studies have the isolation of pure glycoforms been successful, and then only by extensive chromatographic separations.[10] In order to study the biological processes mediated by the carbohydrate constituents of glycoproteins, single glycoforms which have the same mono or oligosaccharide attached to a specific protein glycosylation site need to be available.

Recent advances in carbohydrate chemistry and peptide solid-phase synthesis have made possible the synthesis of glycopeptides bearing reasonably complex glycans.[11,12] However, the assembly of glycopeptides larger than 60 amino acids is still challenging. The most frequently adopted strategies for protein glycosylation take advantage of the inherent reactivity of lysine or cysteine side chain.[13] However, these methods are not residue spe-

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cific and result in multiple sites of glycosylation.

In an attempt to increase the selectivity of protein glycosylation, various novel approaches have been reported in recent years. For example, using a combined site-directed mutagenesis and chemical modification strategy, Jones and co-workers prepared a small library of protected and deprotected mono- and diglycosylated proteins.[14] The resulting linkages differed structurally from the natural ones and thus might have limited applications in structure–function studies. Wong and co-workers reported the synthesis of a single unnatural glycoform of RNAse B using a series of protease and glycotransferase-catalyzed reactions.[15] In a similar manner, endoglycosidase-catalyzed transglycosylations have been applied to the synthesis of a Mn_6-$GlcNAc_2$ glycoprotein.[16] However, these elegant enzymatic methods still require the presence of a N-glycan linkage from the outset as a recognition site for the enzymes involved, and they afford no control over the site of glycosylation. Another approach for the synthesis of modified O-linked glycoproteins using chemoselective ligation has been reported by Bertozzi and co-workers.[17] The same group recently described the total synthesis of the antimicrobial O-linked glycoprotein diptericin by native chemical ligation, based on the condensation of two glycopeptide fragments prepared by solid-phase synthesis.[18] Very recently, Tolbert and Wong have used an intein-mediated ligation for the incorporation of a single carbohydrate into the C-terminus of a protein.[19]

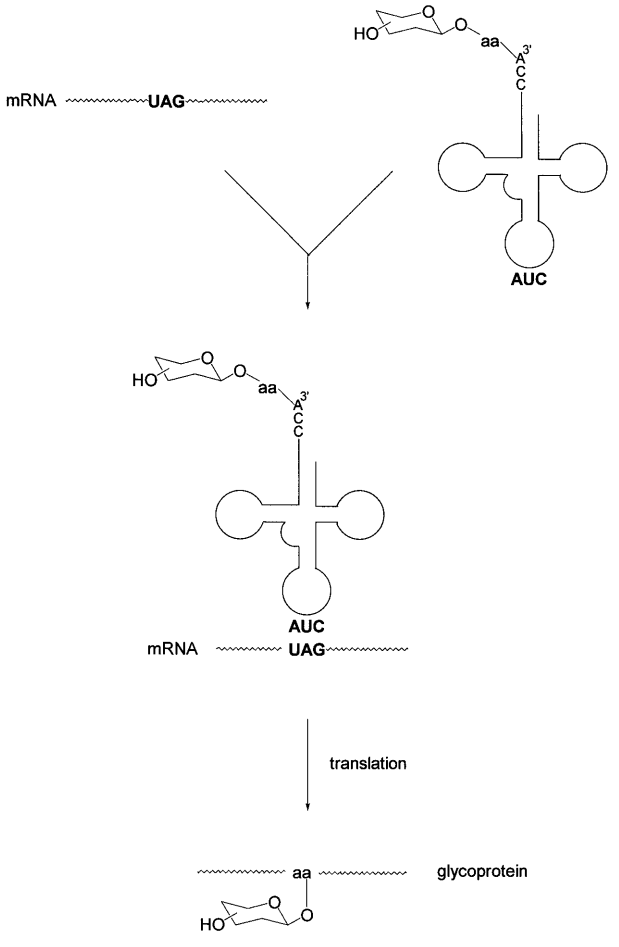


Fig. 1. Preparation of glycoproteins by in vitro suppression of a nonsense codon with a glycosylated aminoacyl–$tRNA_{CUA}$.

The biosynthesis of proteins containing unnatural amino acids at predetermined sites via nonsense (stop codon) suppression by misacylated tRNAs is a powerful technique that can produce important insights into protein structure and function.[20] The extension of this technique to glycosylated amino acids would afford the attractive possibility of producing proteins glycosylated at predetermined sites (Fig. 1). Although glycosylation of proteins is a post-translational process in nature, some recent experiments have shown that it is possible to obtain glycoproteins with monosacharides attached at specific sites using this method.[21] From a synthetic point of view, in addition to the problems concerning the stability of the tRNA molecule and the protecting groups that are used, the sugar moiety presents its own technical problems. Especially in the case of O-linked sugars, the acid and base lability of the glycosidic bond complicates the choice of the protecting groups for the hydroxyl groups. In this paper, we report a general strategy for the synthesis of O-glycosylated aminoacyl–pdCpAs and their ligation to truncated tRNAs (Fig. 2). The glycosylated aminoacyl–tRNAs so obtained can be used in an in vitro translation system to produce the desired glycoproteins.

2. Results and discussion

Synthesis of glycosylated serine pdCpA esters.—The key intermediate in the prepara-

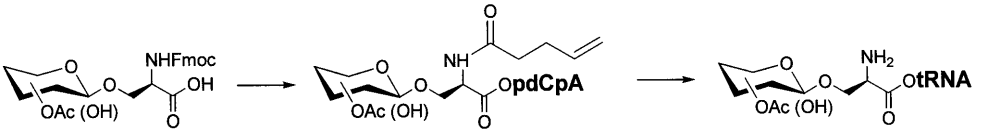


Fig. 2. Synthesis of glycosylated seryl–pdCpAs and ligation to tRNA.

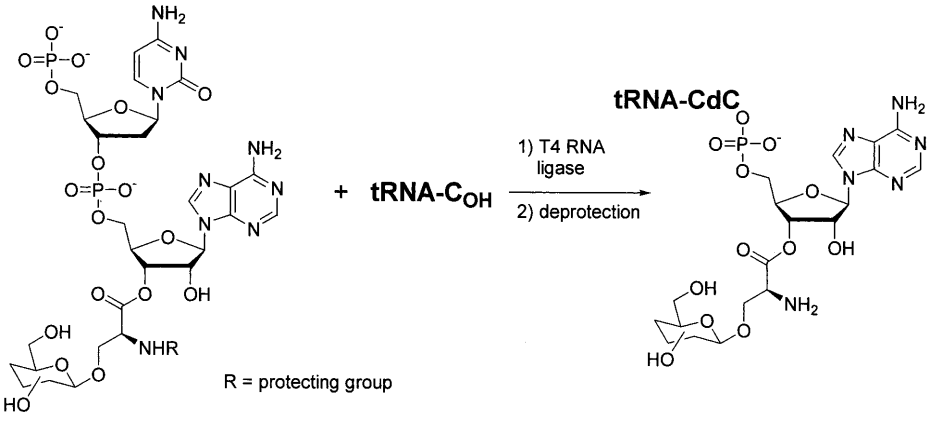


Fig. 3. Preparation of misacylated tRNAs by chemical aminoacylation. The glycosylated aminoacyl moiety is shown arbitrarily attached to the 3′-OH group of ribose.

tion of glycosylated aminoacyl–tRNAs is the glycosylated aminoacyl-pdCpA, which is employed in the enzymatic ligation with an abbreviated tRNA transcript (Fig. 3).

Glycosylated serine derivatives **2a–d** were prepared as shown in Scheme 1 using a known procedure.[22] Fmoc-*S*-serine was coupled to peracetylated β-D-glucose, β-D-galactose and α-D-mannose (**1a–c**) using boron trifluoride diethyl etherate as the promoter in acetonitrile. Glycosides **2a–c** were obtained in low to moderate yields (30–57%); the formation of 1,2-trans isomers undoubtedly resulted from neighboring-group participation of the C-2 acetyl donor functionality. This transformation may well proceed via a glycosyl ester formed by reaction between the carboxyl group of the amino acid and the glycosyl donor. In the presence of excess Lewis acid, the glycosyl ester would rearrange to the desired *O*-glycoside.[22]

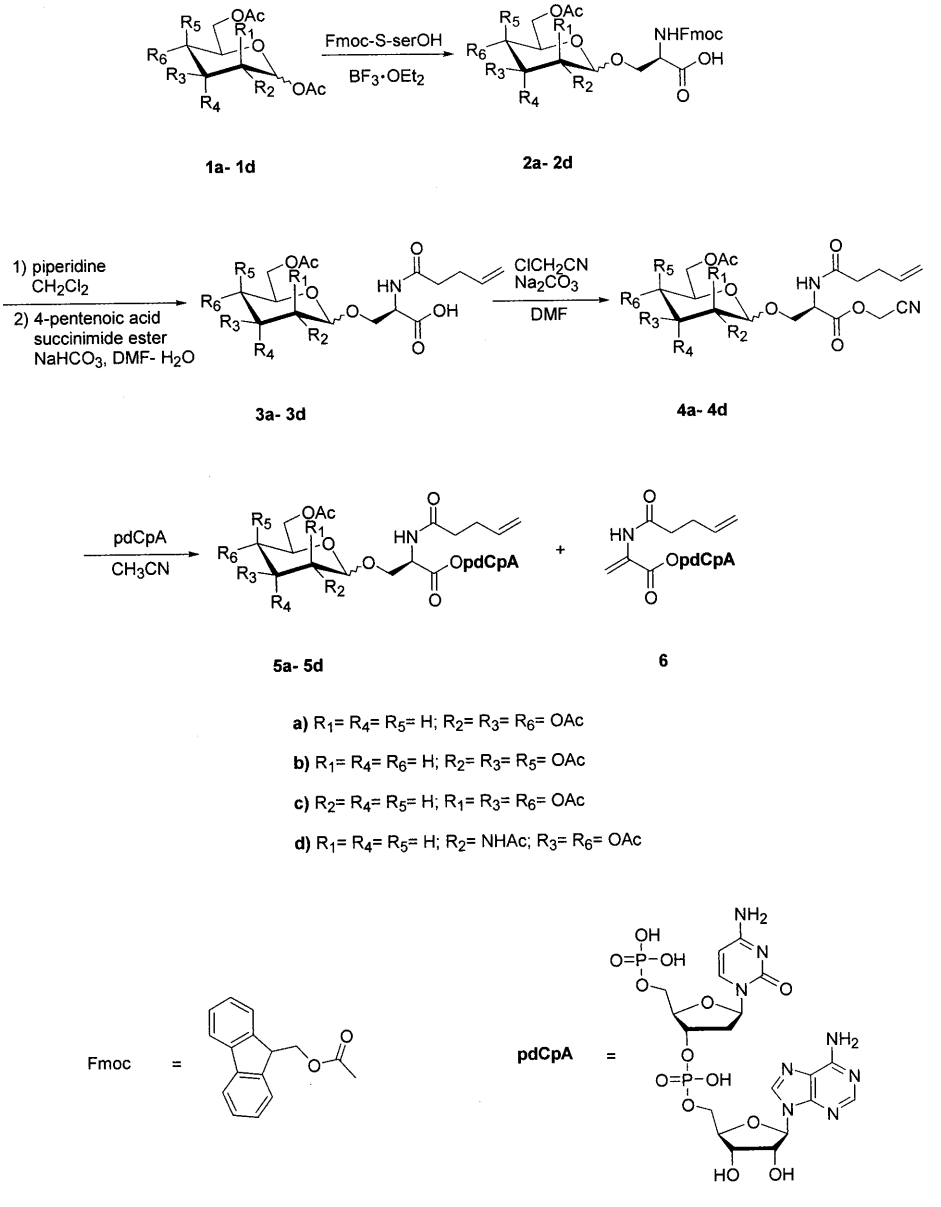
In the next step, treatment of glycosylated Fmoc serine derivatives **2a–c** with piperidine in dichloromethane resulted in the selective deprotection of the amine to give the free amino acid. The amino group was reprotected with the 4-pentenoyl group using 4-pentenoic acid succinimide ester as the acylating agent in the presence of sodium bicarbonate in aqueous *N*,*N*-dimethylformamide. The 4-pentenoyl group[23] was chosen because its removal after glycosylaminoacyl–pdCpA ligation to tRNA–C_{OH} could be effected under conditions compatible with the integrity of the activated tRNA molecule.[24] Compounds **3a–c** were obtained in 63, 82 and 50% yields, respectively. We also investigated the possibility of using the *N*-(4-pentenoyl)-*S*-serine cyanomethyl ester as glycosyl acceptor,[25] thereby shortening the synthetic route to **3a–c**. Unfortunately, all attempts to couple this compound with different glycosyl donors in the presence of different promoters failed. This may have been due to the low reactivity of amide N-protected serine as a consequence of a hydrogen bonding interaction between the hydroxyl group and the amide hydrogen.[26]

For the synthesis of 2-acetamido-2-deoxy-β-D-glucose serine derivative **2d**, a modified route was used.[27] The oxazoline derived from 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose (**1d**) was prepared in situ using an excess of boron trifluoride diethyl etherate, and then treated with the Fmoc–*S*-SerOH to give compound **2d** in 61% yield. Removal of the Fmoc protecting group with

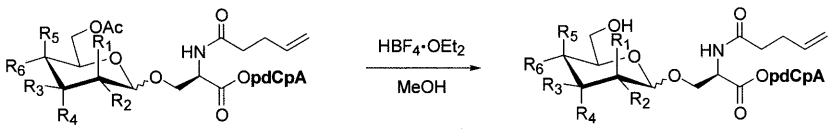
piperidine, and subsequent protection of the liberated amine with the 4-pentenoyl group gave compound **3d** in 63% yield.

For the activation of the free acids of **3** as cyanomethyl esters, compound **3b** was used as a model and was treated with chloroacetonitrile in acetonitrile in the presence of triethylamine.[28] The cyanomethyl ester **4b** was obtained in only 18% yield in an impure form. Using *N*,*N*-dimethylformamide as solvent and sodium carbonate as the base, the reaction proceeded smoothly and the activated ester **4b** was obtained in 64% yield. In a similar manner, acids **3a**, **3c** and **3d** treated with chloroacetonitrile under the same conditions gave **4a**, **4c** and **4d** in 66, 67 and 53% yields, respectively.

The next step was the O-aminoacylation of pdCpA. This was carried out using the method reported by Schultz and co-workers.[28] Treatment of galactose serine derivative **4b** with the tris-(tetrabutylammonium) salt of pdCpA in dry *N*,*N*-dimethylformamide gave compound **6**, resulting from the loss of the sugar moiety via β-elimination, as the major product. The desired galactosyl aminoacyl pdCpA (**5b**) was also obtained, albeit only in low yield. The reaction proceeded very slowly and



Scheme 1.



5a- 5d

a) R_1= R_4= R_5= H; R_2= R_3= R_6= OAc

b) R_1= R_4= R_6= H; R_2= R_3= R_5= OAc

c) R_2= R_4= R_5= H; R_1= R_3= R_6= OAc

d) R_1= R_4= R_5= H; R_2= NHAc; R_3= R_6= OAc

7a- 7d

a) R_1= R_4= R_5= H; R_2= R_3= R_6= OH

b) R_1= R_4= R_6= H; R_2= R_3= R_5= OH

c) R_2= R_4= R_5= H; R_1= R_3= R_6= OH

d) R_1= R_4= R_5= H; R_2= NHAc; R_3= R_6= OH

Scheme 2.

the competition between aminoacylation and elimination apparently favored the latter. This was probably due to the slightly basic character of *N*,*N*-dimethylformamide. Using the same conditions for the activated esters, **4a**, **4c** and **4d** gave virtually identical results. To aminoacylate pdCpA efficiently with glycosylated amino acids, we substituted acetonitrile for *N*,*N*-dimethylformamide. Interestingly, the reaction between the activated esters and the tris-(tetrabutylammonium) salt of pdCpA generally proceeded more rapidly than in *N*,*N*-dimethylformamide and reached completion within 90 min, except in the case of ester **4a** for which the reaction required 24 h. Glycosylated aminoacyl–pdCpA derivatives **5a**–**d** were obtained in 14, 23, 25 and 55% yields, respectively, and were purified by HPLC. In each case, the elimination product **6** was also isolated in 20–26% yields.

The next goal was deprotection of the carbohydrate moiety without cleavage of the glycosidic bond (β-elimination) or hydrolysis of the aminoacyl bond. This proved to be a challenging problem since almost all methods used for the removal of acetate groups require basic conditions. However, a selective enzymatic hydrolysis of the carbohydrate acetates using lipase WG has been reported and applied successfully to different glycosylated amino acid conjugates.[29] The mildness of the conditions used (pH 7.0, 37 °C) prompted us to apply this method to the glycosylated aminoacyl–pdCpAs. Thus, a mixture of pdCpA derivative **5b** and lipase WG in a phosphate buffer, pH 7.0, were incubated at 37 °C. After 30 min a new product was detected by HPLC and isolated. It was tentatively identified as resulting from the loss of only one acetyl group [(FAB), *m*/*z* 1093 $(M+H)^+$, 1115 $(M+Na)^+$]. However, longer reaction times (ca. 1 h) led to the hydrolysis of the aminoacyl bond, and pdCpA was detected by HPLC as the major product, along with other minor peaks probably corresponding to the glycosylated aminoacyl–pdCpA lacking two, three or four acetates.

As a consequence of this discouraging result, we turned our attention to the acid-catalyzed deacetylation. Tetrafluoroboric acid in methanol was reported to effect the chemoselective removal of *O*-acetyl groups in the presence of *O*-benzoyl groups and was reported to be a superior catalyst to HCl, which has been the standard reagent for this type of transformation.[30] To test this reaction for deprotection of compounds **5**, pdCpA derivative **5b** in methanol was treated with a solution of 54% tetrafluoroboric acid in diethyl ether. After 3 h at room temperature, a major product was detected by HPLC (t_R 12–13 min) along with some minor byproducts at t_R 13.5 and 15 min. This major product was isolated and identified as *N*-(4-pentenoyl)-3-*O*-(β-D-galactopyranosyl)-*S*-seryl–pdCpA (**7b**) [(FAB) *m*/*z* 968 $(M+H)^+$, 990 $(M+Na)^+$] (Scheme 2).

In the same manner, compounds **5a**, **5c** and **5d** were successfully deacetylated to give **7a**, **7c** and **7d**, respectively, all of which have been characterized by LRMS and HRMS. During the acid-catalyzed deprotection, some pdCpA was formed, but no products resulting from hydrolysis of the phosphate backbone or cleavage of the glycosidic bond were detected.

Ligation to tRNA and N-deprotection.—The effective formation of aminoacylated suppressor tRNA from the T7 transcript of suppressor tRNA–C_{OH} and pdCpA-coupled unnatural amino acids is a key step in the elaboration of proteins having unnatural amino acids. We analyzed all glycosylated seryl-pdCpA derivatives for their ability to form aminoacyl–tRNA derivatives. As noted by RajBhandary and co-workers,[31] gel analysis of aminoacyl–tRNAs at pH 5.0 differentiates the aminoacylated and unacylated forms of tRNA and thereby readily gives qualitative information about the stability of these aminoacyl esters. Fig. 4 shows that compounds **5a**–**d** and **7a**–**d** all efficiently formed aminoacyl esters with tRNA and that the aminoacylated forms of tRNA differentiate well in this assay from abbreviated tRNA (tRNA–C_{OH}) and full length (non-aminoacylated) tRNA. Not unexpectedly, protected (acetylated) monosaccharides demonstrated larger mobility shifts in this assay compared to their deacetylated counterparts and the elimination product. The latter can be distinguished readily from the glycosylated aminoacyl–tRNAs, as shown in Fig. 5.

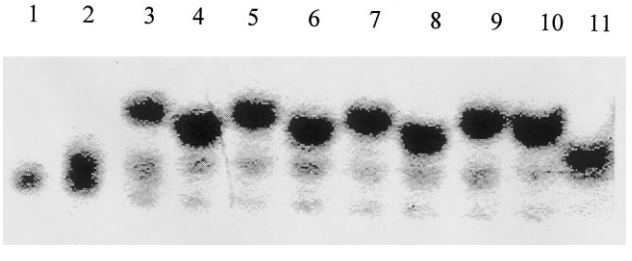


Fig. 4. Analysis of *N*-4-pentenoyl protected aminoacylated RNA^{Phe} on a denaturing acid gel. Lane 1, abbreviated $tRNA^{Phe}$–C_{OH}; lane 2, mixture of full length and abbreviated $tRNA^{Phe}$–C_{OH}. O-Acetylated sugars. Lane 3, glucose; lane 5, mannose; lane 7, galactose; lane 9, *N*-acetylglucosamine. O-Deacetylated sugars. Lane 4, glucose; lane 6, mannose; lane 8, galactose; lane 10, *N*-acetylglucosamine; lane 11, elimination product.

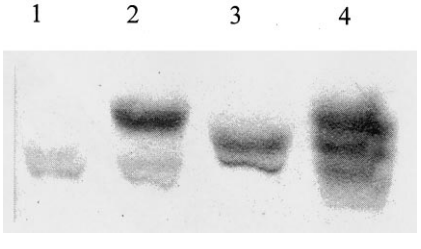


Fig. 5. Difference in mobility shift between $tRNA^{Phe}$, glycosylated aminoacyl–tRNA and dehydroalanyl–tRNA on a denaturing acid gel. Lane 1, mixture of full length and abbreviated $tRNA^{Phe}$s; lane 2, mannosylserine; lane 3, elimination product; lane 4, mixture of mannosylserine and elimination product.

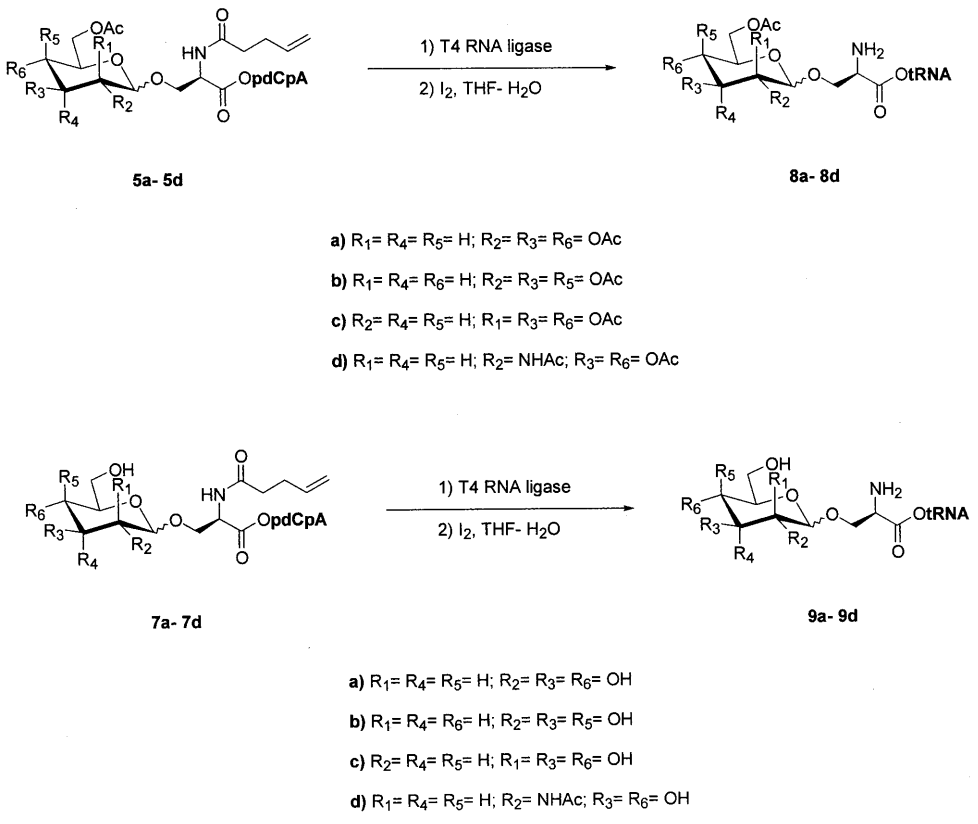
Finally, the removal of the 4-pentenoyl protecting group from the amine, using iodine in aqueous THF, gave free aminoacylated tRNAs **8a**–**d** and **9a**–**d** (Scheme 3). These are much less stable than their N-protected counterparts, but still can be analyzed on acid gels (Fig. 6).

Labeling of misacylated suppressor tRNAs by dansylhydrazine.—To verify the presence of the sugar moiety in the misacylated tRNAs, mannosylseryl–tRNA and glucosylseryl–tRNA were analyzed according to the procedure of Eckhardt et al. with minor modifications.[32] Glycosylated tRNAs were oxidized with periodate to generate aldehydic functions that were condensed with dansylhydrazine. Dansyl fluorophore emission was specific for those samples containing oxidized sugar residues. As shown in Fig. 7, only mannosylseryl–tRNA and glucosylseryl–tRNA showed clear dansyl fluorescence, whereas a valyl–tRNA subjected to the same treatment, showed no dansyl fluorescence. If the periodate oxidation step was omitted (e.g., for mannosylseryl–tRNA), dansyl fluorescence was also not observed. These results indicated that the carbohydrate moiety actually is covalently attached to the tRNA.

The incorporation of the glycosylated serine derivatives into proteins is currently under investigation and will be reported elsewhere.

3. Experimental

Materials and methods.—^1H and ^{13}C NMR spectra were recorded on a General Electric QE-300 spectrometer. Moisture-sensitive reactions were conducted under Ar in oven-dried glassware. All chemical reagents were purchased from Aldrich Chemical Co. or Sigma Chemical Co. and used without further purification. Acetylated monosaccharides and wheat germ lipase were obtained from Sigma Chemical Co. Fmoc amino acids were purchased from Novabiochem. Acetonitrile and



Scheme 3.

CH_2Cl_2 were distilled from CaH_2; *N*,*N*-dimethylformamide (DMF) was distilled from CaH_2 under diminished pressure. Triethylamine was distilled from P_2O_5. Analytical thin-layer chromatography was performed on Silica Gel 60 F_{254} (E. Merck) plates and visualized using iodine or sulfuric acid. Flash chromatography was performed using 230–400 mesh silica gel. Elemental analyses were carried out by Atlantic Microlab Inc., Norcross, GA. High-resolution mass spectra were recorded at the Nebraska Center for Mass Spectrometry.

T4 RNA ligase (1 unit is defined as the amount of enzyme required to convert 1 nmol of 5′-phosphoryl termini in 5′-[^{32}P]rA_{20} to phosphatase-resistant form in 30 min at 37 °C at a 5′-termini concentration of 10 mM), endonuclease *Fok*I (1 unit is defined as the amount of enzyme required to completely digest 1 μg of λ DNA at 37 °C in 50 μL of assay buffer) were obtained from New England Biolabs. Kits for plasmid isolation were purchased from either Qiagen or PGC Scientific. AmpliScribe transcription kits and T7 RNA polymerase were purchased from Epicentre Technologies (Madison, WI); [^{35}S] methionine (1000 Ci/mmol, 10 mCi/mL) was from Amersham Corporation. Tris-acrylamide, bis-acrylamide, urea, ammonium persulfate, TEMED, EDTA, and Sephacryl S-200 were purchased from Sigma Chemical Co. as were periodic acid and sodium metabisulfite. Plasmid pYR8 coding for sequence of yeast suppressor $tRNA^{Phe}$ was constructed by Dr S. Mamaev.[33] Dansylhydrazine (5-dimethylaminonaphthal-

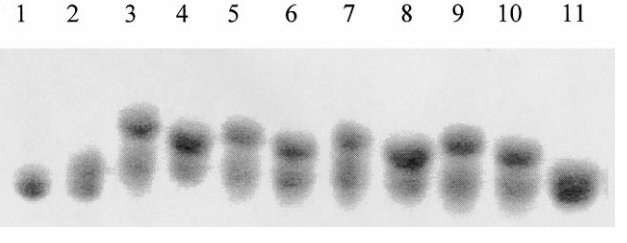


Fig. 6. Analysis of *N*-deprotected aminoacyl–$tRNA^{Phe}$s on a denaturing acid gel. Lane 1, abbreviated $tRNA^{Phe}$–C_{OH}; lane 2, mixture of full length and abbreviated $tRNA^{Phe}$. O-Acetylated sugars. Lane 3, glucose; lane 5, mannose; lane 7, galactose; lane 9, *N*-acetylglucosamine. O-Deacetylated sugars. Lane 4, glucose; lane 6, mannose; lane 8, galactose; lane 10, *N*-acetylglucosamine; lane 11, elimination product.

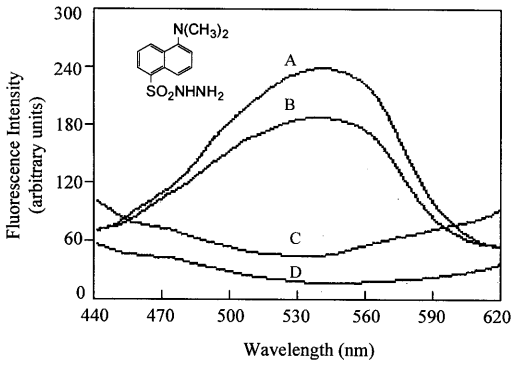


Fig. 7. Fluorescence spectra of misacylated tRNAs labelled with dansylhydrazine. (A) mannosylseryl–tRNA; (B) glucosylseryl–tRNA; (C) valyl–tRNA; (D) mannosylseryl–$tRNA_{CUA}$, no periodate treatment.

ene-1-sulfonylhydrazine) was obtained from Molecular Probes, Inc.

N - *(9 - Fluorenylmethoxycarbonyl) - 3 -* O-*(2,3,4,6-tetra-*O-*acetyl-β-*D-*glucopyranosyl)-*S-*serine* (**2a**).—To a solution of 509 mg (1.3 mmol) of β-D-glucose pentaacetate (**1a**) and 515 mg (1.56 mmol) of *N*-Fmoc-*S*-serine in 15 mL of dry CH_3CN was added 0.49 mL (0.55 g, 3.9 mmol) of $BF_3{\cdot}OEt_2$. The reaction was stirred at 25 °C under an Ar atmosphere for 24 h. The reaction mixture was diluted with 30 mL of CH_2Cl_2 and washed successively with 50 mL of 0.5 N aq $NaHSO_4$ and 30 mL of H_2O. The organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm); elution with a gradient of MeOH in CH_2Cl_2 (2–10%) afforded glycoside **2a** as a white foam (257 mg, 30%); $[\alpha]_D^{23}$ +17.5° (*c* 0.85, $CHCl_3$); lit. $[\alpha]_D$ +21° (*c* 0.91, $CHCl_3$);[22] R_f 0.36 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (500 MHz, acetone-d_6) δ 1.94, 1.96, 1.99, 2.01 (4 s, 12 H), 3.96 (m, 2 H), 4.11 (dd, 1 H, *J* 12.5 Hz), 4.82 (d, 1 H, *J* 7.5 Hz), 4.89 (dd, 1 H, *J* 9 and 8.5 Hz), 5.03 (t, 1 H, *J* 9.7 Hz), 5.27 (t, 1 H, *J* 9.5 Hz), 6.42 (d, 1 H, *J* 8.1 Hz).

N - *(9 - Fluorenylmethoxycarbonyl) - 3 -* O-*(2,3,4,6-tetra-*O-*acetyl-β-*D-*galactopyranosyl)-*S-*serine* (**2b**).—To a solution of 1.8 g (4.61 mmol) of β-D-galactose pentaacetate (**1b**) and 1.89 g (5.53 mmol) of *N*-Fmoc-*S*-serine in 50 mL of dry CH_3CN was added 1.75 mL (1.96 g, 13.83 mmol) of $BF_3{\cdot}OEt_2$. The reaction mixture was stirred at 25 °C under an Ar atmosphere for 2.5 h. The reaction mixture was diluted with 75 mL of CH_2Cl_2 and washed successively with 110 mL of 0.5 N aq $NaHSO_4$ and 60 mL of H_2O. The organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (30 × 4 cm); elution with a gradient of MeOH in CH_2Cl_2 (2–10%) afforded glycoside **2b** as a white foam (1.75 g, 57%); $[\alpha]_D^{24}$ +0.3° (*c* 0.92, $CHCl_3$); lit. $[\alpha]_D$ +0.8° (*c* 0.6, $CHCl_3$);[22] R_f 0.36 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (300 MHz, acetone-d_6): δ 1.92, 1.97, 2.11 (3 s, 12 H), 3.96 (dd, 1 H, *J* 4 and 10.5 Hz), 4.19–4.35 (complex, 3 H), 4.42 (m, 2 H), 4.47 (m, 1 H), 4.74 (d, 1 H, *J* 6.1 Hz), 5.12 (m, 2 H), 5.39 (d, 1 H, *J* 1.9 Hz), 6.39 (1 H, *J* 8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.03, 47.57, 56.56, 61.48, 67.43, 69.32, 71.16, 102.10, 120.51, 125.58, 127.63, 128.27, 141.74, 144.16, 144.34, 170.52, 170.73, 171.00.

N-*(9-Fluorenylmethoxycarbonyl)-3-*O-*(2,3,4,6-tetra-*O-*acetyl-α-*D-*mannopyranosyl)-*S-*serine* (**2c**).—To a solution of 0.97 g (2.5 mmol) of α-D-mannose pentaacetate (**1c**) and 0.98 g (3 mmol) of *N*-Fmoc-*S*-serine in 30 mL of dry CH_3CN was added 0.95 mL (1.06 g, 7.5 mmol) of $BF_3{\cdot}OEt_2$. The reaction mixture was stirred at 25 °C under an Ar atmosphere for 15 h. The reaction mixture was diluted with 60 mL of CH_2Cl_2 and washed successively with 50 mL of 0.5 N aq $NaHSO_4$ and 30 mL of H_2O. The organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (25 × 4 cm); elution with a gradient of MeOH in CH_2Cl_2 (2–10%) afforded glycoside **2c** as a white foam (0.94 g, 57%); $[\alpha]_D^{23}$ +49° (*c* 0.8, $CHCl_3$); R_f 0.36 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (500 MHz, acetone-d_6): δ 1.93, 2.00, 2.01, 2.10 (4 s, 12 H), 4.05 (m, 1 H), 4.12–4.27 (complex, 6 H), 4.34 (m, 1 H), 4.41 (dd, 1 H, *J* 7.5 and 10.5 Hz), 4.54 (m, 1 H), 4.93 (bs, 1 H), 5.22–5.27 (complex, 2 H), 5.30 (dd, 1 H, *J* 3.5, 10.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.33, 21.16, 21.02, 47.56, 62.78, 69.38, 69.81, 76.92, 98.54, 120.46, 125.64, 127.59, 128.21, 141.73, 170.26, 170.73, 171.25; LRMS (FAB), *m/z* 658 $(M+H)^+$, 680 $(M+Na)^+$; HRMS (FAB), *m/z* 680.1932 $(M+H)^+$ ($C_{32}H_{35}NO_{14}Na$ requires 680.1955).

N-*(9-Fluorenylmethoxycarbonyl)-3-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)*-S-*serine* (**2d**).—To a solution of 480 mg (1.23 mmol) of β-D-glucosamine pentaacetate (**1d**) and 4 Å molecular sieves in 8 mL of dry CH_2Cl_2 was added 0.48 mL (0.54 g, 3.81 mmol) of $BF_3{\cdot}OEt_2$. The reaction mixture was stirred at 25 °C under an Ar atmosphere for 20 h. Triethylamine (160 μL) was added at 0 °C, and stirring was continued for 30 min. A solution of 416 mg (2.54 mmol) of *N*-Fmoc-*S*-serine in 3 mL of a mixture of 1:2 CH_3CN–CH_2Cl_2 was added, and the reaction mixture was stirred at rt under Ar for 48 h. The reaction mixture was quenched by the addition of 1 mL of Et_3N, diluted with 15 mL of CH_2Cl_2 and filtered through Celite. The filtrate was evaporated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm); elution with a gradient of MeOH in CH_2Cl_2 (2–15%) afforded glycoside **2d** as a white foam (0.49 g, 61%); $[\alpha]_D^{23}$ −5.5° (*c* 0.9, $CHCl_3$); lit. $[\alpha]_D$ −7.9°;[22] lit. $[\alpha]_D$ +27.8°;[27] silica gel TLC R_f 0.27 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.67, 1.85, 1.91, 1.95 (4s, 12 H), 3.59–3.75 (m, 5 H), 4.10–4.25 (m, 6 H), 4.65 (d, 1 H, *J* 8.4 Hz), 4.76 (t, 1 H, *J* 9.6 Hz), 5.02 (t, 1 H, *J* 9.6 Hz).

N-*(4-Pentenoyl)-3-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)*-S-*serine* (**3a**).—*N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine (**2a**) (172 mg, 0.26 mmol) in 4 mL of CH_2Cl_2 was treated with 1 mL of piperidine (0.86 g, 10.1 mmol) at 25 °C for 35 min. The reaction mixture was diluted with 20 mL of toluene, and the solvents were concentrated under diminished pressure (bath temperature < 30 °C). The residue was dissolved in 2 mL of DMF, and 61.9 mg (0.314 mmol) of 4-pentenoic acid succinimide ester was added followed by a solution of 26.3 mg (0.314 mmol) of $NaHCO_3$ in 1 mL of H_2O. The reaction mixture was stirred vigorously at 25 °C for 16 h. The reaction mixture was poured onto 3 g of ice, acidified with 3 mL of 1 N aq $NaHSO_4$ and extracted with three 5-mL portions of CH_2Cl_2. The combined organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm); elution with a gradient of MeOH in CH_2Cl_2 (10–30%) afforded *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine (**3a**) as a white foam (72 mg, 63%); $[\alpha]_D^{25}$ +18.8° (*c* 0.82, $CHCl_3$); R_f 0.24 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.06, 2.09, 2.12, 2.15 (4 s, 12 H), 2.38 (m, 2 H), 2.48 (m, 2 H), 3.76 (m, 1 H), 3.95 (dd, 1 H, *J* 3.5 and 10.5 Hz), 4.21 (dd, 1 H, *J* 4.5 and 12.5 Hz), 4.26 (dd, 1 H, *J* 3.2 and 10.5 Hz), 4.34 (dd, 1 H, *J* 1.5 and 12.5 Hz), 4.58 (d, 1 H, *J* 7.5 Hz), 4.83 (m, 1 H), 4.99 (dd, 1 H, *J* 7.5 and 9.5 Hz), 5.05 (dd, 1 H, *J* 1.5 and 11.5 Hz), 5.12 (m, 1 H), 5.28 (t, 1 H, *J* 9.5 Hz), 5.77 (m, 1 H), 7.01 (d, 1 H, *J* 7.5 Hz); LRMS (FAB), *m/z* 518 $(M+H)^+$, 540 $(M+Na)^+$; HRMS (FAB), *m/z* 540.1669 $(M+Na)^+$ ($C_{22}H_{31}NO_{13}Na$ requires 540.1693).

N-*(4-Pentenoyl)-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)*-S-*serine* (**3b**).—*N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-*S*-serine (**2b**) (410 mg, 0.61 mmol) in 5.5 mL of CH_2Cl_2 was treated with 1.3 mL of piperidine (1.1 g, 13.1 mmol) at 25 °C for 30 min. The reaction mixture was diluted with 30 mL of toluene, and the solvents were evaporated under diminished pressure (bath temperature < 30 °C). The residue was dissolved in 6.8 mL of DMF, and 184 mg (0.91 mmol) of 4-pentenoic acid succinimide ester was added, followed by a solution of 79 mg (0.91 mmol) of $NaHCO_3$ in 2.7 mL of H_2O. The reaction mixture was stirred vigorously at 25 °C for 16 h. The reaction mixture was poured onto 10 g of ice, acidified with 10 mL of 1 N aq $NaHSO_4$ and extracted with three 15-mL portions of CH_2Cl_2. The combined organic phase was dried ($MgSO_4$), filtered and evaporated under diminished pressure. The residue was purified by chromatography on a silica gel column (25 × 3 cm); elution with a gradient of MeOH in CH_2Cl_2 (10–40%) afforded *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-*S*-serine (**3b**) as a white foam (252 mg, 82%); $[\alpha]_D^{23}$ +0.33° (*c* 1, $CHCl_3$); R_f 0.24 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.03, 2.10, 2.12, 2.19 (4 s, 12 H), 2.39 (m, 2 H), 2.47 (m, 2 H), 3.95 (m, 2 H), 4.11 (dd, 1 H, *J* 6.5 and 11.5 Hz), 4.25 (m,

2 H), 4.53 (d, 1 H, *J* 7.5 Hz), 4.82 (m, 1 H), 5.04–5.16 (m, 3 H), 5.43 (ψd, *J* 2 Hz), 5.78 (m, 1 H), 6.98 (d, 1 H, *J* 8 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.03, 21.07, 21.16, 21.29, 29.77, 35.85, 54.69, 61.45, 67.50, 69.36, 71.18, 101.77, 116.15, 137.38, 170.47, 170.61, 171.04; HRMS (FAB), *m*/*z* 540.1685 $(M + Na)^+$ ($C_{22}H_{31}NO_{13}Na$ requires 540.1693).

N-*(4-Pentenoyl)-3-*O*-*(2,3,4,6-tetra*-O*-acetyl-α-*D*-mannopyranosyl)*-S-*serine* (**3c**).—*N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-*S*-serine (**2c**) (375 mg, 0.57 mmol) in 5 mL of CH_2Cl_2 was treated with 1.25 mL of piperidine (1.07 g, 12.6 mmol) at 25 °C for 30 min. The reaction mixture was diluted with 20 mL of toluene, and the solvent was evaporated under diminished pressure (bath temperature < 30 °C). The residue was dissolved in 7 mL of DMF and 169 mg (0.85 mmol) of 4-pentenoic acid succinimide ester was added, followed by a solution of 74 mg (0.85 mmol) of $NaHCO_3$ in 2.5 mL of H_2O. The reaction mixture was stirred vigorously at rt for 16 h. The mixture was poured onto 10 g of ice, acidified with 10 mL of 1 N aq $NaHSO_4$ and extracted with three 15-mL portions of CH_2Cl_2. The combined organic phase was dried ($MgSO_4$), filtered and evaporated under diminished pressure. The residue was purified by chromatography on a silica gel column (25 × 3 cm); elution with a gradient of MeOH in CH_2Cl_2 (10–40%) afforded *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-*S*-serine (**3c**) as a colorless oil (148 mg, 50%); $[\alpha]_D$ +2.7° (*c* 1, $CHCl_3$); R_f 0.24 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.00, 2.03, 2.09, 2.13 (4 s, 12 H), 2.93 (m, 4 H), 3.90 (m, 1 H), 3.99 (d, 1 H, *J* 2.7 Hz), 4.12 (dd, *J* 2.1 and 12.5 Hz), 4.22 (dd, 1 H, *J* 5.5 and 12.2 Hz), 4.81 (bs, 1 H), 4.88 (m, 1 H), 5.01–5.29 (complex, 4 H), 5.75 (m, 1 H), 7.28 (d, 1 H, *J* 7.7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.71, 29.31, 35.27, 62.20, 65.91, 68.72, 69.30, 97.97, 115.59, 136.94, 169.67; LRMS (CI) *m*/*z* 518 $(M + H)^+$, 540 $(M + Na)^+$; Anal. Calcd for $C_{22}H_{31}NO_{13}\cdot H_2O$: C, 49.34; H, 6.16. Found: C, 49.33; H, 5.70.

N-*(4-Pentenoyl)-3-*O*-*(2-acetamido-2-deoxy-3,4,6-tri*-O*-acetyl-β-*D*-glucopyranosyl)*-S-*serine* (**3d**).—*N*-(9-Fluorenylmethoxycarbonyl)-3-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine (**2d**) (130 mg, 0.19 mmol) in 1.6 mL of CH_2Cl_2 was treated with 0.4 mL of piperidine (0.34 g, 3.99 mmol) at 25 °C for 35 min. The reaction mixture was diluted with 15 mL of toluene, and the solvent was evaporated under diminished pressure (bath temperature < 30 °C). The residue was dissolved in 2.5 mL of DMF, and 59 mg (0.29 mmol) of 4-pentenoic acid succinimide ester was added, followed by a solution of 25 mg (0.29 mmol) of $NaHCO_3$ in 1 mL of H_2O. The reaction mixture was stirred vigorously at rt for 18 h. The reaction mixture was poured onto 2 g of ice, acidified with 2 mL of 1 N aq $NaHSO_4$ and extracted with three 10-mL portions of CH_2Cl_2. The combined organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 3 cm); elution with a gradient of MeOH in CH_2Cl_2 (10–50%) afforded *N*-(4-pentenoyl)-3-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine (**3d**) as a white foam (65 mg, 63%); $[\alpha]_D^{23}$ −3.9° (*c* 1, $CHCl_3$); R_f 0.12 (4:1 CH_2Cl_2–MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.06, 2.10, 2.11, 2.16 (4 s, 12 H), 2.38 (m, 4 H), 3.74 (m, 1 H), 3.95 (dd, 1 H, *J* 3.7 and 10.2 Hz), 4.08 (dd, 1 H, *J* 8.5 and 10.5 Hz), 4.23 (m, 2 H), 4.30 (dd, 1 H, *J* 2 and 12.5 Hz), 4.57 (d, 1 H, *J* 8.0 Hz), 4.82 (t, 1 H, *J* 3.5 Hz), 5.03–5.22 (m, 3 H), 5.75 (m, 1 H), 6.58 (d, 1 H, *J* 9.5 Hz), 7.23 (d, 1 H, *J* 7.4 Hz); LRMS (CI), *m*/*z* 517 $(M + H)^+$; HRMS (FAB), *m*/*z* 539.1837 $(M + Na)^+$ ($C_{22}H_{32}N_2O_{12}Na$ requires 539.1853).

N-*(4-Pentenoyl)-3-*O*-*(2,3,4,6-tetra*-O*-acetyl-β-*D*-glucopyranosyl)*-S-*serine cyanomethyl ester* (**4a**).—To a solution containing 145 mg (0.28 mmol) of *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine (**3a**) and 118 mg (1.12 mmol) of anhyd Na_2CO_3 in 3 mL of freshly distilled DMF was added 142 μL (169 μg, 2.4 mmol) of chloroacetonitrile, and the reaction mixture was stirred at 25 °C under an Ar atmosphere for 18 h. The reaction mixture was poured onto 5 g of ice, acidified with 5 mL of 1 N aq $NaHSO_4$ and extracted with three, 10-mL portions of CH_2Cl_2. The combined organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was

purified by chromatography on a silica gel column (15 × 3 cm); elution with a gradient of EtOAc in hexane (50–90%) afforded *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine cyanomethyl ester (**4a**) as a colorless oil (103 mg, 66%); $[\alpha]_D^{23}$ +7.5° (*c* 1.1, $CHCl_3$); (R_f 0.39 7:3 EtOAc–hexane); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.99, 2.01, 2.05, 2.08 (4 s, 12 H), 2.38 (m, 4 H), 3.70 (m, 1 H), 3.88 (dd, 1 H, *J* 3.46 and 10.4 Hz), 4.13–4.29 (m, 3 H), 4.52 (d, 1 H, *J* 8.1 Hz), 4.79–4.88 (m, 3 H), 4.92 (dd, 1 H, *J* 7.9 and 9.4 Hz), 5.01–5.11 (m, 3 H), 5.18 (t, 1 H, *J* 9.4 Hz), 5.82 (m, 1 H), 6.34 (d, 1 H, *J* 7.7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.20, 20.29, 20.34, 20.38, 28.85, 34.88, 49.02, 51.86, 61.24, 67.66, 67.96, 70.67, 71.70, 71.97, 99.99, 113.51, 115.48, 136.22, 168.13, 169.02, 169.76, 170.23, 171.96; LRMS (EI) *m*/*z* 556 (M^+); Anal. Calcd for $C_{24}H_{32}N_2O_{13}$: C, 51.80; H, 5.80. Found: C, 52.05; H, 5.91.

N-*(4-Pentenoyl)-3-*O*-*(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)*-S-*serine cyanomethyl ester* (**4b**).—To a solution containing 0.7 g (1.35 mmol) of *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-*S*-serine (**3b**) and 0.57 g (5.4 mmol) of anhyd Na_2CO_3 in 14 mL of freshly distilled DMF was added 0.68 mL (0.8 g, 10.8 mmol) of chloroacetonitrile, and the reaction mixture was stirred at 25 °C under an Ar atmosphere for 16 h. The reaction mixture was poured onto 10 g of ice, acidified with 10 mL of 1 N aq $NaHSO_4$ and extracted with three 20-mL portions of CH_2Cl_2. The combined organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm); elution with a gradient of EtOAc in hexane (50–70%) afforded *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-*S*-serine cyanomethyl ester (**4b**) as a colorless oil (0.48 g, 64%); $[\alpha]_D^{23}$ +6.3° (*c* 1.2, $CHCl_3$); R_f 0.39 (7:3 EtOAc–hexane); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.97, 2.04, 2.06, 2.14 (4 s, 12 H), 2.36 (m, 4 H), 3.89 (m, 2 H), 4.11 (m, 2 H), 4.21 (dd, 1 H, *J* 3.5 and 10.0 Hz), 4.47 (d, 1 H, *J* 7.7 Hz), 4.80 (m, 3 H), 4.96–5.14 (complex, 3 H), 5.36 (d, 1 H, *J* 2.7 Hz), 5.82 (m, 1 H), 6.34 (d, 1 H, *J* 7.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.96, 21.04, 21.10, 21.25, 29.69, 35.85, 49.80, 52.76, 61.71, 67.36, 68.53, 69.12, 71.04, 71.62, 101.35, 116.31, 137.09, 169.01, 170.0, 170.42, 170.57, 170.81, 172.75; LRMS (CI) *m*/*z* 556 (M^+); Anal. Calcd for $C_{24}H_{32}N_2O_{13}\cdot 0.5\ H_2O$: C, 50.97; H, 5.88. Found: C, 51.00; H, 5.82.

N-*(4-Pentenoyl)-3-*O*-*(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)*-S-*serine cyanomethyl ester* (**4c**).—To a solution containing 87 mg (0.17 mmol) of *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-*S*-serine (**3c**) and 72 mg (0.67 mmol) of anhyd Na_2CO_3 in 2 mL of freshly distilled DMF was added 172 μL (205 μg, 1.34 mmol) of chloroacetonitrile, and the reaction mixture was stirred at 25 °C under an Ar atmosphere for 18 h. The reaction mixture was poured onto 5 g of ice, acidified with 5 mL of 1 N aq $NaHSO_4$ and extracted with three 5-mL portions of CH_2Cl_2. The organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (25 × 1 cm); elution with a gradient of EtOAc in hexane (50–100%) afforded *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-*S*-serine cyanomethyl ester (**4c**) as a colorless oil (63 mg, 67%); $[\alpha]_D^{23}$ +51° (*c* 1.2, $CHCl_3$); R_f 0.41 (7:3 EtOAc–hexane); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.95, 2.01, 2.06, 2.10 (4 s, 12 H), 2.38 (m, 4 H), 3.91 (m, 2 H), 3.99 (dd, 1 H, *J* 3.5 and 10.8 Hz), 4.76 (d, 1 H, *J* 1.2 Hz), 4.82 (s, 2 H), 4.92 (m, 1 H), 5.02–5.21 (complex, 5 H), 5.81 (m, 1 H), 6.62 (d, 1 H, *J* 7.7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.12, 21.15, 21.19, 21.26, 29.71, 35.69, 50.00, 52.79, 62.82, 66.51, 69.08, 69.32, 69.67, 69.75, 98.84, 114.28, 116.37, 137.22, 169.12, 170.15, 170.39, 170.45, 171.09, 172.84; LRMS (CI) *m*/*z* 557 $(M+H)^+$; Anal. Calcd for $C_{24}H_{32}N_2O_{13}\cdot 0.5\ H_2O$: C, 50.97; H, 5.88. Found: C, 51.16; H, 5.75.

N-*(4-Pentenoyl)-3-*O*-*(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)*-S-*serine cyanomethyl ester* (**4d**).—To a solution containing 50 mg (0.096 mmol) of *N*-(4-pentenoyl)-3-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine (**3d**) and 41 mg (0.38 mmol) of anhyd Na_2CO_3 in 1 mL of freshly distilled DMF was added 50 μL (59 μg, 0.78 mmol) of chloroacetonitrile, and

the reaction mixture was stirred at 25 °C under an Ar atmosphere for 20 h. The reaction mixture was poured onto 2.5 g of ice, acidified with 2.5 mL of 1 N aq $NaHSO_4$ and extracted with three 5-mL portions of CH_2Cl_2. The combined organic phase was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10×3 cm); elution with a gradient of MeOH in EtOAc (0–12%) afforded *N*-(4-pentenoyl)-3-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine cyanomethyl ester (**4d**) as a colorless gum (28 mg, 53%); $[\alpha]_D^{23}$ −5.5° (*c* 0.8, $CHCl_3$); R_f 0.32 (EtOAc); ^1H NMR (300 MHz, $CDCl_3$): δ 1.97, 2.01, 2.02, 2.08 (4 s, 12 H), 2.40 (m, 4 H), 3.60 (m, 1 H), 3.70 (m, 1 H), 3.85 (dd, 1 H, *J* 3.1, 11.2 Hz), 4.12 (dd, 1 H, *J* 2.5 and 12.5 Hz), 4.25 (m, 2 H), 4.77 (d, 2 H, *J* 13.5 Hz), 4.83 (m, 1 H), 4.93 (d, 1 H, *J* 8.0 Hz), 5.04 (m, 2 H), 5.27 (q, 1 H, *J* 9.2 and 10.4 Hz), 5.83 (m, 1 H), 6.04 (d, 1 H, *J* 7.7 Hz), 6.92 (d, 1 H, *J* 8.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.25, 20.30, 20.41, 23.16, 28.94, 29.33, 34.80, 48.84, 51.95, 55.03, 61.51, 68.00, 68.18, 71.23, 71.83, 100.08, 113.69, 115.28, 136.44, 168.44, 169.05, 170.38, 171.27, 172.42, HRMS (FAB), *m*/*z* 556.2129 ($C_{24}H_{34}N_3O_{12}$ requires 556.2142).

N-*(4-Pentenoyl)*-*3*-O-*(2,3,4,6-tetra*-O-*acetyl-β*-D-*glucopyranosyl)*-S-*serine pdCpA ester* (**5a**).—To a conical vial containing 10.5 mg (18.7 μmol) of *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine cyanomethyl ester (**4a**) was added a solution of 5.1 mg (3.75 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 60 μL of freshly distilled CH_3CN. The reaction mixture was stirred at rt for 24 h. A 2-μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN–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 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). The remaining reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **5a** (t_R 21–22 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: (0.6 mg, 14%); LRMS (FAB), *m*/*z* 1136 $(M+H)^+$, 1158 $(M+Na)^+$; HRMS (FAB), *m*/*z* 1136.2874 $(M+H)^+$, ($C_{41}H_{56}N_9O_{25}P_2$ requires 1136.2862). Another product which had t_R = 14–15 min was also isolated and identified as the deglycosylated derivative **6** (0.8 mg, 27%); LRMS (FAB), *m*/*z* 788 $(M+H)^+$, 810 $(M+Na)^+$; HRMS (FAB), *m*/*z* 788.1834 $(M+H)^+$, ($C_{27}H_{36}N_9O_{15}P_2$ requires 788.1806).

N-*(4-Pentenoyl)*-*3*-O-*(2,3,4,6-tetra*-O-*acetyl-β*-D-*galactopyranosyl)*-S-*serine pdCpA ester* (**5b**).—To a conical vial containing 12.5 mg (22.4 μmol) of *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-*S*-serine cyanomethyl ester (**4b**) was added a solution of 5.2 mg (3.8 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 60 μL of freshly distilled CH_3CN. The reaction mixture was stirred at rt for 1.5 h. A 2-μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN–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 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). The remaining reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **5b** (t_R 21–22 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: (1 mg, 23%); LRMS (FAB), *m*/*z* 1136 $(M+H)^+$, 1158 $(M+Na)^+$; HRMS (FAB), *m*/*z* 1136.2871 $(M+H)^+$ ($C_{41}H_{56}N_9O_{25}P_2$ requires 1136.2862). The elimination product **6** (t_R 14–15 min) was also isolated (0.6 mg, 20%).

N-*(4-Pentenoyl)*-*3*-O-*(2,3,4,6-tetra*-O-*acetyl-α*-D-*mannopyranosyl)*-S-*serine pdCpA ester* (**5c**).—To a conical vial containing 12.5 mg (22.4 μmol) of *N*-(4-pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-*S*-serine cyanomethyl ester (**4c**) was added a solution of 5.2 mg (3.8 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 60 μL of freshly distilled CH_3CN. The reaction mixture was stirred at rt for 1.5 h. A 2-μL aliquot of the reaction mixture was diluted with 58 μL of

1:1 CH_3CN–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 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). The remaining reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **5c** (t_R 21–22 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: (1.1 mg, 25%); LRMS (FAB), *m/z* 1136 $(M + H)^+$, 1158 $(M + Na)^+$; HRMS (FAB), *m/z* 1136.2862 $(M + H)^+$ ($C_{41}H_{56}N_9O_{25}P_2$ requires 1136.2862). The elimination product **6** (t_R 14–15 min) was also isolated (0.8 mg, 26%).

N - *(4 - Pentenoyl) - 3* - O - *(2 - acetamido - 2-deoxy-3,4,6-tri*-O-*acetyl-β*-D-*glucopyranosyl)*-S-*serine pdCpA ester* (**5d**).—To a conical vial containing 10.6 mg (19.1 μmol) of *N*-(4-pentenoyl)-3-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine cyanomethyl ester (**4d**) was added a solution of 5.2 mg (3.8 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 70 μL of freshly distilled CH_3CN. The reaction mixture was stirred at rt for 2 h. A 2-μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN–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 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). The remaining reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **5d** (t_R 17–18 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: (2.4 mg, 55%); LRMS (FAB), *m/z* 1135 $(M + H)^+$, 1157 $(M + Na)^+$; HRMS (FAB), *m/z* 1135.3001 $(M + H)^+$ ($C_{41}H_{57}N_{10}O_{24}P_2$ requires 1135.3020). The elimination product **6** (t_R 14–15 min) was also isolated (0.8 mg, 26%).

N-*(4-Pentenoyl)-3*-O-*(β*-D-*glucopyranosyl)*-S-*serine pdCpA ester* (**7a**).—To a conical vial containing 0.4 mg (0.35 μmol) of *N*-(4-pentenoyl) - 3 - *O* - (2,3,4,6 - tetra - *O* - acetyl - β - D-glucopyranosyl)-*S*-serine pdCpA ester (**5a**) in 160 μL of dry MeOH was added 40 μL of a 54% solution of HBF_4 in diethyl ether. The reaction mixture was stirred at rt for 3 h. A 10-μL aliquot of the reaction mixture was diluted with 40 μL of 1:1 CH_3CN–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 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). The remaining reaction mixture was diluted to a total volume of 300 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **7a** (t_R 12–13 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: (0.2 mg, 58%); LRMS (FAB), *m/z* 968 $(M + H)^+$, 990 $(M + Na)^+$; HRMS (FAB), *m/z* 968.2473 $(M + H)^+$, ($C_{33}H_{48}N_9O_{21}P_2$ requires 968.2440).

N - *(4 - Pentenoyl) - 3* - O - *(β* - D - *galactopyranosyl)*-S-*serine pdCpA ester* (**7b**).—To a conical vial containing 0.8 mg (0.70 μmol) of *N*-(4-pentenoyl) - 3 - *O* - (2,3,4,6 - tetra - *O* - acetyl - β - D-galactopyranosyl)-*S*-serine pdCpA ester (**5b**) in 320 μL of dry MeOH was added 80 μL of a 54% solution of HBF_4 in diethyl ether. The reaction mixture was stirred at rt for 3 h. A 10-μL aliquot of the reaction mixture was diluted with 40 μL of 1:1 CH_3CN–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 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). The remaining reaction mixture was diluted to a total volume of 300 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **7b** (t_R 12–13 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: (0.3 mg, 44%); LRMS (FAB), *m/z* 968 $(M + H)^+$, 990 $(M + Na)^+$; HRMS (FAB), *m/z* 968.2452 $(M + H)^+$, ($C_{33}H_{48}N_9O_{21}P_2$ requires 968.2440).

N-*(4-Pentenoyl)-3*-O-*(α*-D-*mannopyranosyl)*-S-*serine pdCpA ester* (**7c**).—To a conical vial containing 1.7 mg (1.5 μmol) of *N*-(4-

pentenoyl)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-*S*-serine pdCpA ester (**5c**) in 360 μL of dry MeOH was added 90 μL of a 54% solution of HBF_4 in diethyl ether. The reaction mixture was stirred at rt for 2 h 45 min. A 10-μL aliquot of the reaction mixture was diluted with 40 μL of 1:1 CH_3CN–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 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). The remaining reaction mixture was diluted to a total volume of 300 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **7c** (t_R 12–13 min) was recovered from the appropriate fractions by lyophilization as a colorless solid (0.9 mg, 62%) LRMS (FAB), m/z 968 $(M+H)^+$, 990 $(M+Na)^+$; HRMS (FAB), m/z 968.2437 $(M+H)^+$, ($C_{33}H_{48}N_9O_{21}P_2$ requires 968.2440).

N-*(4-Pentenoyl)-3-*O*-*(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)*-S-*serine pdCpA ester* (**7d**).—To a conical vial containing 1.8 mg (1.5 μmol) of *N*-(4-pentenoyl)-3-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-*S*-serine pdCpA ester (**5d**) in 360 μL of dry MeOH was added 90 μL of a 54% solution of HBF_4 in diethyl ether. The reaction mixture was stirred at rt for 3 h. A 10-μL aliquot of the reaction mixture was diluted with 40 μL of 1:1 CH_3CN–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 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). The remaining reaction mixture was diluted to a total volume of 300 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. Dinucleotide derivative **7d** (t_R 13–14 min) was recovered from the appropriate fractions by lyophilization as a colorless solid: (0.7 mg, 43%); LRMS (FAB), m/z 1009 $(M+H)^+$, 1031 $(M+Na)^+$; HRMS (FAB), m/z 1009.2738 $(M+H)^+$, ($C_{35}H_{51}N_{10}O_{21}P_2$ requires 1009.2710).

In vitro transcription of 5′-monophosphorylated abbreviated suppressor yeast $tRNA^{Phe}_{CUA}$.—Synthesis of an abbreviated suppressor $tRNA^{Phe}_{CUA}$ ($-C_{OH}$) programmed by *Fok*I restriction fragment from plasmid pYRNA8 [33] was carried out using Ampliscribe T7 RNA polymerase transcription kit with additional 20 mM GMP and 5% formamide at 42 °C for 12 h. A tRNA preparation was phenol–$CHCl_3$ treated, purified on Sephacryl S-200 column in buffer containing 0.1 M NaCl, 1 mM EDTA and 10 mM Tris–HCl, pH 8.0, then precipitated with 3 vols of cold EtOH in the presence 0.3 M sodium acetate, pH 5.3, washed with 70% ethanol, dried, and finally dissolved in water and maintained at − 20 °C.

Chemical misacylation of suppressor $tRNA–C_{OH}$.—Chemical misacylation reactions were carried out in 50 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 100 μg suppressor $tRNA–C_{OH}$, 1.0 A_{260} unit protected aminoacyl–pdCpA (5–10-fold molar excess), 20–25% dimethyl sulfoxide and 200 units of T4 RNA ligase (100 μL total incubation volume). After incubation at 37 °C for 60 min, the reaction was quenched by the addition of 0.1 vol of 3 M sodium acetate, pH 5.0, and the tRNA was precipitated with 4 vols of EtOH. The efficiency of ligation was estimated by gel electrophoresis in 8% PAGE–7 M urea–0.1 M NaOAc, pH 5.0.[31] Pentenoyl-protected aminoacyl–tRNAs were deprotected at rt for 20 min at a tRNA concentration of 1 μg/μL in 5–10 mM I_2 (from a stock solution containing 100 mM I_2 dissolved in 1:1 THF–H_2O). Following deblocking, the solution was centrifuged, and the cleared supernatant was adjusted to 0.3 M AcONa, pH 5.0, and treated with 4 vols of EtOH to precipitate the acylated tRNA and remove trace amounts of I_2. After washing the tRNA pellet with 70% EtOH, the deprotected aminoacyl–tRNA was dried under diminished pressure and dissolved in 1 mM KOAc, pH 5.0, for in vitro suppression experiments or in formamide, 50 mM NaOAc, pH 5.0, containing 0.025% xylene cyanole for electrophoretic analysis.

Electrophoretic analysis.—The extent of aminoacylation of tRNA was estimated from an 8% PAGE–7 M urea gel in 0.1 M NaOAc

buffer, pH 5.0.[31] In this assay, the mobility of tRNA was roughly the same as that of xylene cyanole. For detection RNA was stained with ethidium bromide at a concentration of 0.2 μg/mL, or with 0.25% methylene blue in 7% AcOH, and destained in water.

Labeling of misacylated suppressor tRNAs by dansylhydrazine.—Each of the synthesized misacylated suppressor $tRNA_{CUA}$s (0.5 nmol) was incubated for 2 h at 25 °C in 100 μL of 0.5% (w/v) periodic acid. To this reaction mixture was added 170 μL of 0.5% sodium metabisulfite in 5% aq AcOH. After 1 h incubation at 25 °C, the reaction mixture was quenched by the addition of 30 μL of 3 M AcONa, pH 5.0. The tRNA was precipitated with 2.5 vols of EtOH, collected by centrifugation, washed with 70% EtOH and dried. The tRNA was then incubated with 100 μL of fresh dansylhydrazine solution. The latter was prepared by dissolving 10 mg of dansylhydrazine in 3.3 mL of absolute EtOH followed by dilution to 10 mL with 0.1 M AcONa, pH 5.0. After incubation for 2 h at 25 °C, excess dansylhydrazine was removed by EtOH precipitation of the tRNA. The tRNA was then purified and concentrated to 4 μM in 1 mM potassium acetate by using a microconcentrator (Microcon YM-10, Millipore Corporation). Emission spectra at 534 nm (Fig. 7) were measured using a Hitachi F2000 fluorescence spectrophotometer (excitation at 336 nm) in a total assay volume of 100 μL containing 0.4 nmol of the aminoacylated tRNA.

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