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Preparation of Chemically Misacylated Semisynthetic Nonsense Suppressor tRNAs Employed in Biosynthetic Incorporation of Non-Natural Residues into Proteins

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Both run-off transcription and direct chemical synthesis were employed to prepare semisynthetic, nonhypermodified $tRNA^{Gly}$ nonsense suppressors acylated with non-natural residues. L-3-Iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA (**3**) was prepared by four separate methods, and each of the resultant suppressors was evaluated for biological activity during in vitro translation of mRNA containing a nonsense suppression site (e.g., a UAG termination codon). Direct comparison of the individual translation experiments clearly demonstrates that all four methods yield acylated tRNA products that are biologically equivalent for the their intended purpose: site-specific incorporation of non-natural residues into proteins.

Introduction

Several groups have recently embarked upon a project whose ultimate goal is to develop general techniques for the design and expression of unique proteins containing site-specific non-natural residues.[1,2] The general strategy involves engineering into a gene a termination codon (TAG, TGA, or TAA) at the desired position of incorporation, i.e., a *nonsense mutation site*. Under normal circumstances, translation of mRNA transcribed from this modified gene would result in truncated protein product, since termination codons have no corresponding tRNAs capable of participating in the ribosome-catalyzed process. However, if the translation system is provided with a semisynthetic nonsense suppressor tRNA (i.e., a tRNA whose anticodon is complementary to one of the termination codons) charged with the desired non-natural residue, incorporation of this residue at the specified site in the protein is the result.

The key to success in this project is the development of relatively straightforward procedures for the construction of such nonsense suppressor tRNAs. Based on earlier reports of "chemical misacylation" of wild-type tRNAs,[3,4] it is known that selective acylation of tRNA can be accomplished by T4 RNA ligation of a synthetic 2′(3′)-O-acylated dinucleotide (**2**) and a 3′-truncated tRNA (**1**) lacking the 3′-terminal cytidine and adenosine moieties, as depicted in Scheme I. Building on this theme, we have developed several methods for the synthesis of 2′(3′)-O-acylated nonsense suppressor tRNAs, including the tRNA portion, and have compared the biological activities of the resultant products.

Results and Discussion

A strategy to synthesize acylated nonsense suppressor tRNAs requires that three chemical components be prepared and then joined: (1) a pCpA dinucleotide,[5] (2) an appropriately protected non-natural residue, and (3) a 3′-truncated tRNA ($tRNA\text{-}C_{OH}$), which upon ligation with the acylated dinucleotide creates the intact, chemospecifically acylated tRNA. In addition, a route that yields large quantities of the final product is desirable because the acylated nonsense suppressor tRNA would act stoichiometrically in the translation of mRNAs during the in vitro protein synthesis. In this approach, illustrated retrosynthetically in Scheme II, the initial disconnection of acylated $tRNA^{Gly}_{CUA}$-dCA (**4**) leaves the 3′-truncated tRNA $tRNA^{Gly}_{CUA}\text{-}C_{OH}$ (**1**) and the acylated dinucleotide **5**, which is further simplified to an appropriately protected dinucleotide (**6**) and an N-protected non-natural residue (**7**).

Design Criteria for the Preparation of Chemically Misacylated $tRNA^{Gly}_{CUA}$-dCA. The chemical design of the

† University of California.
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(1) Bain, J. D.; Diala, E. S.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R. *J. Am. Chem. Soc.* **1989**, *111*, 8013-8014.
(2) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182-188.
(3) This simple and general strategy for contructing misacylated tRNAs was originally developed by the Hecht group: (a) Roesser, J. R.; Xu, C.; Payne, R. C.; Surratt, C. K.; Hecht, S. M. *Biochemistry* **1989**, *28*, 5185-1595. (b) Heckler, T. G.; Roesser, J. R.; Xu, C.; Chang, P.-I.; Hecht, S. M. *Biochemistry* **1988**, *27*, 7254-7262. (c) Payne, R. C.; Nichols, B. P.; Hecht, S. M. *Biochemistry* **1987**, *26*, 3197-3205. (d) Heckler, T. G.; Chang, L. H.; Zama, Y.; Naka, T.; Hecht, S. M. *Tetrahedron* **1984**, *40*, 87-94. (e) Heckler, T. G.; Zama, Y.; Naka, T.; Hecht, S. M. *J. Biol. Chem.* **1983**, *258*, 4492-4495. (f) Pezzuto, J. M.; Hecht, S. M. *J. Biol. Chem.* **1980**, *255*, 865-869. (g) Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. *J. Biol. Chem.* **1978**, *253*, 4517-4520.
(4) Heckler, T. G.; Chang, L.-H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468-1473.
(5) The following nomenclature has been employed throughout this manuscript: pCpA, 5′-*O*-phosphorylcytidylyl(3′-5′)adenosine; pdCpA (or dCA), represents the analogous deoxycytidine derivative 5′-*O*-phosphoryl-2′-deoxycytidylyl(3′-5′)adenosine; $tRNA\text{-}C_{OH}$, signifies a tRNA missing the 3′-terminal cytidine and adenosine moieties; $X\text{-}tRNA^{Y}_{Z}$, designates a tRNA normally aminoacylated with Y in vivo, containing the anticodon Z, and acylated at the 3′-terminus with the residue X (If Z is not present, the anticodon of the wild-type tRNA is employed.) $X\text{-}tRNA^{Y}_{Z}$-dCA, those tRNAs containing a deoxycytidine residue coupled to an adenosine moiety on the 3′-terminus rather than the normal all-ribose sequence.

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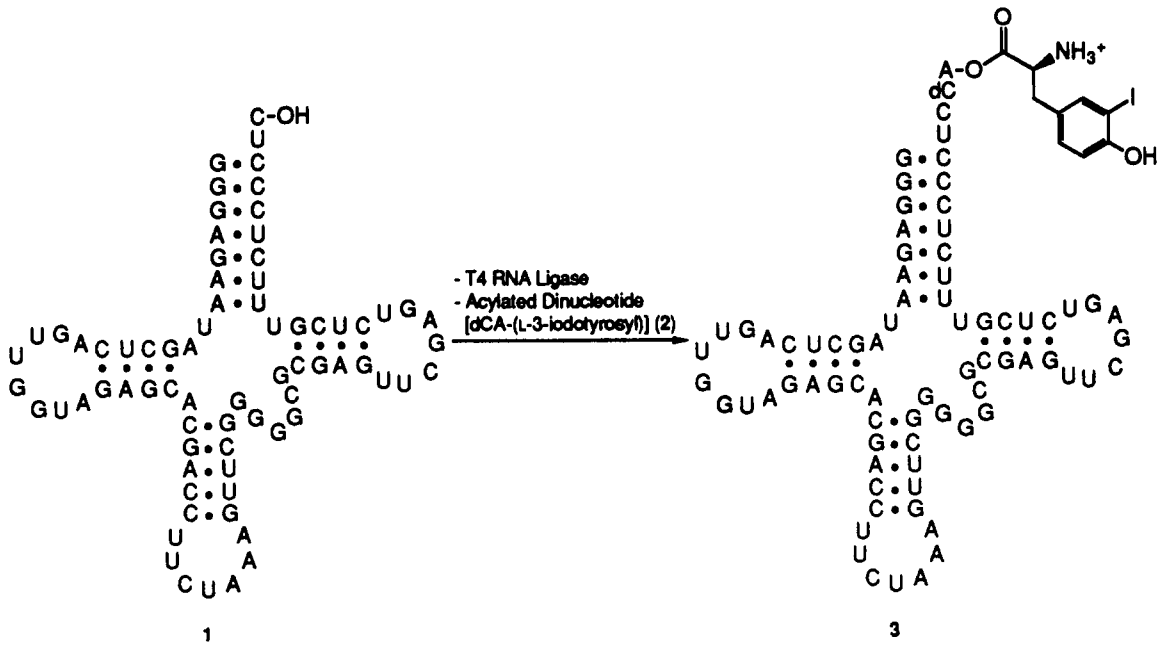
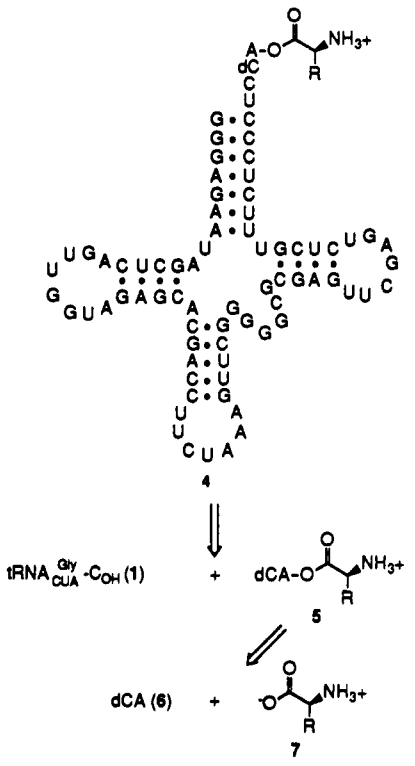
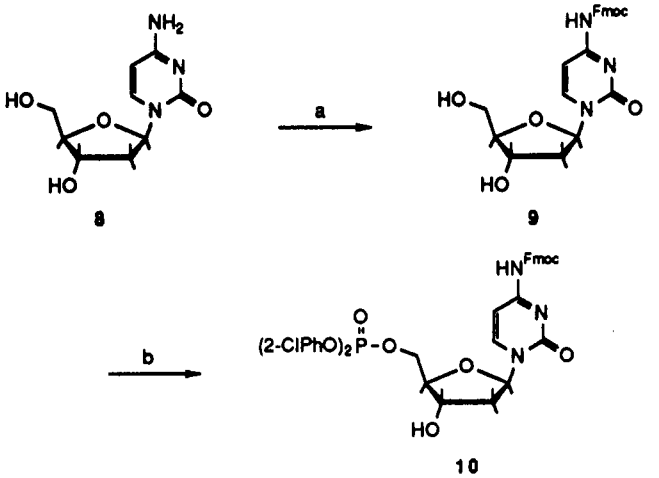
Scheme I



Scheme II



Scheme III



[a] Protection of cytidine for dinucleotide synthesis was carried out as follows: (a) i. 2′-deoxycytidine (8) monohydrochloride, TMSCl 4.1 equiv, pyridine, rt, 2 h; ii. FmocCl 1.1 equiv, rt, 3 h; (b) 9, $(2\text{-ClPhO})_2P(O)Cl$ 1.0 equiv. pyridine, 0 °C to rt, 1 h.

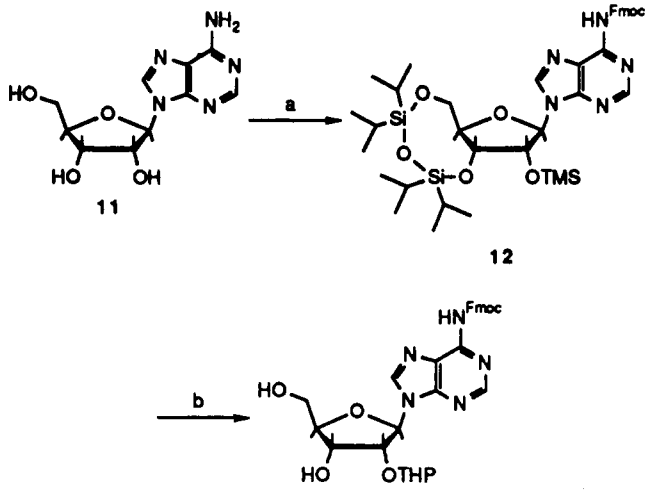
dinucleotide component was guided by several prerequisites: (1) large-scale synthesis capabilities (i.e, gram quantities of product), (2) a deprotection scheme allowing the use of Boc-, Bpoc-, Cbz-, or Fmoc-protected non-natural residues most commonly used in peptide chemistry for greater flexibility, and (3) stability of a protected dinucleotide species that could be easily stored prior to acylation with a non-natural residue. Construction of the dinucleotide component (Scheme V) required the synthesis of the key intermediates N^4-[(9-fluorenylmethyloxy)carbonyl]-5′-*O*-[bis(2-chlorophenyl)phosphoryl]-2′-deoxycytidine (**10**; Scheme III) and N^6-[(9-fluorenylmethyloxy)carbonyl]-2′-*O*-(tetrahydropyranyl)adenosine (**13a** and **13b**; Scheme IV). The use of a phosphorylated deoxycytidine derivative results in a dinucleotide RNA/DNA hybrid that has several advantages over a total RNA analogue in terms of synthetic simplicity and product stability, while maintaining compatibility with T4 RNA ligase[6] and other enzymes involved in the biosynthesis of polypeptides.[7] Specifically, several synthesis steps are eliminated since the 5′-terminus is already phosphorylated, and the relatively nonpolar product is easily purified on silica gel. The use of 2′-deoxycytidine also ensures that 2′(3′)-O-isomerization of the ribosyl–phosphodiester bond cannot occur as it does with previous methods in which a total RNA analogue has been synthesized.[2,4,8] In ad-

(6) (a) Sugino, A.; Snopek, T. J.; Cozzarelli, N. R. *J. Biol. Chem.* **1977**, *252*, 1732–1738. (b) Snopek, T. J.; Sugino, A.; Agarwal, K. L.; Cozzarelli, N. R. *Biochem. Biophys. Res. Commun.* **1976**, *68*, 417–424.

(7) (a) Khan, A. S.; Roe, B. A. *Science* **1988**, *241*, 74–79. (b) Chládek, S.; Sprinzl, M. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 371–391.

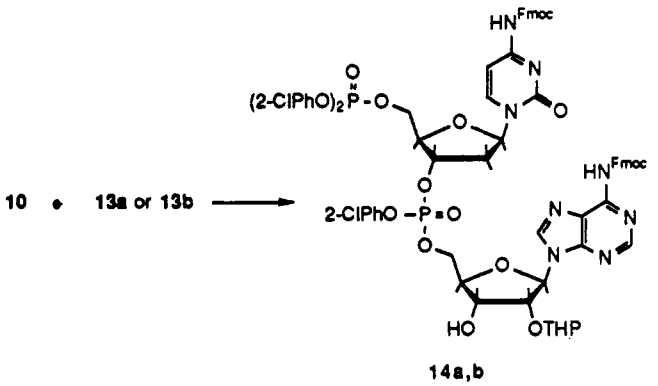
(8) Baldini, G.; Martoglio, B.; Schachenmann, A.; Zugliani, C.; Brunner, J. *Biochemistry* **1988**, *27*, 7951–7959.

Scheme IV



[a] Protection of adenosine for dinucleotide synthesis was carried out as follows: (a) i. adenosine (11), $TIPDSCl_2$ 1.1 equiv, DMF/pyridine (1:1), rt, 1 h; ii, TMSCl 2.0 equiv, rt, 1 h; iii. FmocCl 1.1 equiv, rt, 3 h; (b) i, 12, 2,3-dihydropyran, *p*-toluenesulfonic acid 0.33 equiv, 3-Å molecular sieves, dioxane, rt, 1.5 h; ii. TBAF 0.8 M, THF/pyridine/H_2O (8:1:1), rt, 1.5 h.

Scheme V

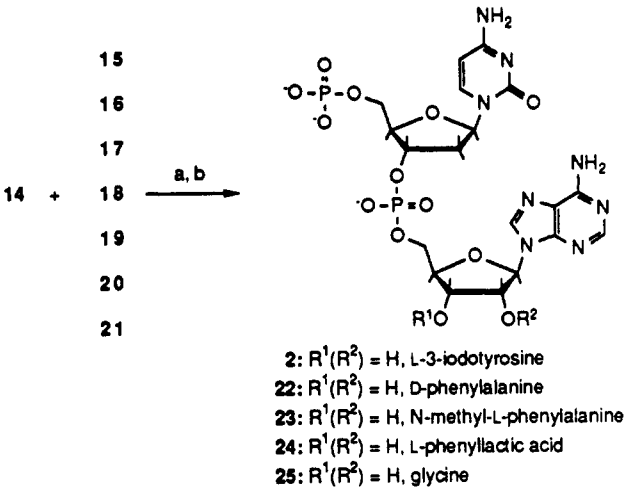


[a] Condensation of protected cytidine **10** and adenosine **13a** or **13b** to yield the dinucleotide **14a** or **14b** was carried out as follows: i. **10**, (2-ClPhO)P(O)$(OBT)_2$ 1.1 equiv, THF, rt, 30 min; ii. **13a** or **13b** 1.3 equiv, *N*-methylimidazole 0.6 equiv, rt, 30 min.

dition, stability of this phosphodiester bond ensures a longer shelf life compared to a total RNA analogue due to ubiquitous RNase activity and the extreme precautions that must be taken when working with RNA.

The other main component to be synthesized was the 3′-truncated tRNA $tRNA^{Gly}_{CUA}$-C_{OH} (1), the choice of which was determined by the desire to simplify the system as much as possible. First, it was anticipated—based upon our earlier results[1]—that a tRNA containing no hypermodified bases would be functional in an in vitro translation system. Thus, the requisite $tRNA^{Gly}_{CUA}$-C_{OH} (1) could be produced very conveniently by run-off transcription from an appropriate DNA template. Alternatively, solid-phase chemical synthesis could be employed to construct the 3′-truncated tRNA 1. Second, it was thought that minor base substitutions in the acceptor stem could be tolerated with no adverse affects. The logic behind this assumption is that the ribosome is capable of interacting with all tRNAs contained within a given translation system, and analysis of the acceptor stems of tRNAs contained within any translation system demonstrates no distinct nucleotide sequence other than the pCpCpA 3′-terminus. It stands to reason that specifc nucleotide/ribosome interactions along the acceptor stem, with the exception of the pCpCpA 3′-terminus, would be minimal due to the random nature of acceptor stem sequences within a given set of tRNAs. This assumption would allow T7 RNA polymerase run-off transcription with the optimal promoter sequence to be utilized for transcription,[9] since the last six bases of the promoter sequence are transcribed to create the 5′-terminus of the tRNA (Scheme VI). Finally, the use of a $tRNA^{Gly}$ might be expected to minimize enzymatic deacylation of the acylated $tRNA^{Gly}_{CUA}$-dCA during error correction. Mechanisms of correcting misacylated tRNAs have been explained by a "double sieve" process as sites in—or adjacent to—the synthetase receptors,[10] and thus, acylation of $tRNA^{Gly}$ with non-natural residues should yield products capable of bypassing this correction system through size exclusion.

Scheme VI



[a] Formation and deprotection of acylated dinucleotide derivatives **2** and **22–25** was carried out as follows: (a) **14a** or **14b**, N-protected non-natural residue 3.0 equiv, *N*-methylimidazole 1.5 equiv, BOP 3.0 equiv, HOBT 3.0 equiv, DMF, rt, 30 min; (b) i. 1,1,3,3-tetramethylguanidine 0.38 M, 4-nitrobenzaldoxime 0.33 M, CH_3CN, rt, 3 h; ii. 80% formic acid, 0 °C, 30 min.

Synthesis of the RNA/DNA Hybrid Dinucleotide Component. The chemical synthesis of the 2′(3′)-O-acylated tRNA **5** depicted in Scheme III is based upon a similar strategy developed by Chládek and co-workers.[11] The protected deoxycytidine derivative **10** was easily prepared by two simple transformations from readily available 2′-deoxycytidine (**8**). The initial transformation leading to the N^4-protected product N^4-[(9-fluorenylmethyloxy)carbonyl]-2′-deoxycytidine (**9**) was accomplished with minor modifications to the method of Heikkliä and Chattopadhyaya.[12] Transient protection of the 3′- and 5′-hydroxyl groups of **8** with trimethylsilyl chloride (4.1 equiv) in dry pyridine was followed by condensation with 9-fluorenylmethyl chloroformate (1.1 equiv) to afford **9** in 95% yield after workup. Conversion of **9** to the 5′-phosphoryl nucleotide analogue **10** was accomplished in modest yield (55%) by condensation of bis(2-chlorophenyl)

(9) (a) Milligan, J. F.; Groebe, D. R.; Witherell, G. W.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1987**, *15*, 8783–8798. (b) Rosa, M. D. *Cell* **1979**, *16*, 815–825.

(10) Fersht, A. R.; Dingwall, C. *Biochemistry* **1979**, *18*, 2627–2631.

(11) (a) Hagen, M. D.; Scalfi-Happ, C.; Happ, E.; Chládek, S. *J. Org. Chem.* **1988**, *53*, 5040–5045. (b) Happ, E.; Scalfi-Happ, C.; Chládek, S. *J. Org. Chem.* **1987**, *52*, 5387–5391. (c) Scalfi-Happ, C.; Happ, E.; Ghag, S.; Chládek, S. *Biochemistry* **1987**, *26*, 4682–4688.

(12) Heikkilä, J.; Chattopadhyaya, J. *Acta Chem. Scand. B* **1983**, *37*, 263–265.

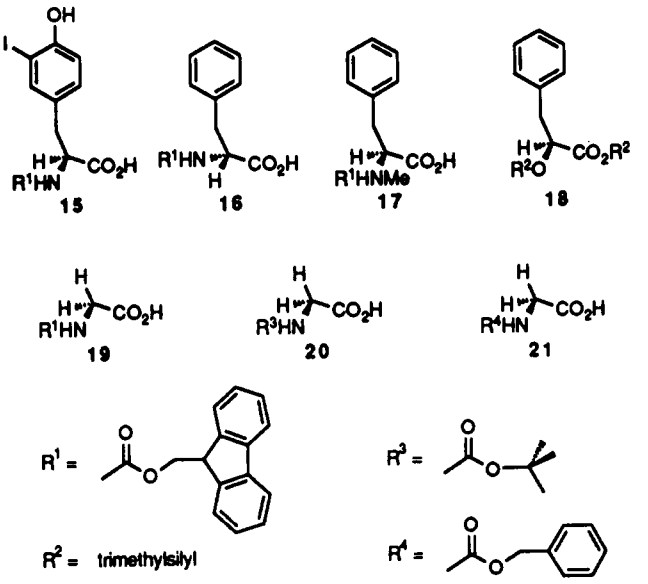
phosphorochloridate (1.0 equiv) with **9** in pyridine at 0 °C.

The other component needed for the synthesis of the dinucleotide is the appropriately protected adenosine analogue N^6-[(9-fluorenylmethyloxy)carbonyl]-2′-*O*-(tetrahydropyranyl)adenosine (**13a** and **13b**; Scheme IV). Sequential addition of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (1.1 equiv), trimethylsilyl chloride (2.0 equiv), and 9-fluorenylmethyl chloroformate (1.1 equiv) to adenosine (**11**) dissolved in a 1:1 mixture of dry pyridine and DMF gave the desired intermediate **12** in 74% yield. This addition sequence eliminates the need to isolate the intermediate *N*-[(9-fluorenylmethyloxy)carbonyl]adenosine as required by previous methods.[11] In the next step a tetrahydropyranyl group was exchanged with the trimethylsilyl group at the 2′-position by treatment with *p*-toluenesulfonic acid and dihydropyran in THF. The 3′–5′-tetraisopropyldisiloxane group was then removed from the protected adenosine intermediate with tetrabutylammonium fluoride in the presence of water and pyridine.[11b,13] This combination affects clean conversion of starting material **12** into **13** (as a mixture of diastereomers that are easily separated during purification by flash chromatography). The resultant diastereomers, which are treated separately in successive steps, show the same reactivity in both the condensation with the protected deoxycytidine analogue **10** to form the dinucleotide **14** and in the successive acylation reaction of **14** with the nonnatural residues. Thus, the use of the less expensive tetrahydropyranyl protecting group can be employed rather than the 4-methoxytetrahydropyranyl group for protection of the 2′-hydroxyl group.

The final step in the preparation of the dinucleotide was the condensation of intermediates N^4-[(9-fluorenylmethyloxy)carbonyl]-5′-*O*-[bis(2-chlorophenyl)phosphoryl]-2′-deoxycytidine (**10**) and N^6-[(9-fluorenylmethyloxy)carbonyl]-2′-*O*-(tetrahydropyranyl)adenosine (**13**) by the hydroxybenzotriazolyl phosphotriester method[11b] to give the fully protected dinucleotide **14** in good yield (~70%). Acylation with a desired non-natural residue can only occur at the 3′-hydroxyl group of **14**, thus eliminating the possibility of aminoacylation side products.[2,4,8,14]

Acylation and Deprotection of RNA/DNA Dinucleotide Hybrid. To prepare the acylated dinucleotides, we constructed a series of protected residues **15–21** that could be employed in the acylation of the dinucleotide **14**. Previously, it had been shown that the N-protecting groups (9-fluorenylmethyloxy)carbonyl (Fmoc)[1] and [2-(4,4′-biphenylyl)-2-propyloxy]carbonyl (Bpoc)[11b] were compatible with the acylation and deprotection scheme of the dinucleotide (Scheme VI). To explore the range of the N-protecting groups that could be employed for the non-natural residues, a series of N-protected, commercially available glycines **19–21** were obtained so that the common amino acid protecting groups *tert*-butyloxycarbonyl (Boc) and benzyloxycarbonyl (Cbz) could be evaluated.

Acylation of the protected dinucleotide **14** with the free acid N-protected residues **15–21**, activated just prior to use with (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate/1-hydroxybenzotriazole dissolved in a 0.2 M *N*-methylmorpholine in DMF,[15] was achieved in high yield. For the Fmoc-protected residues **15–19**, a simple two-step deprotection



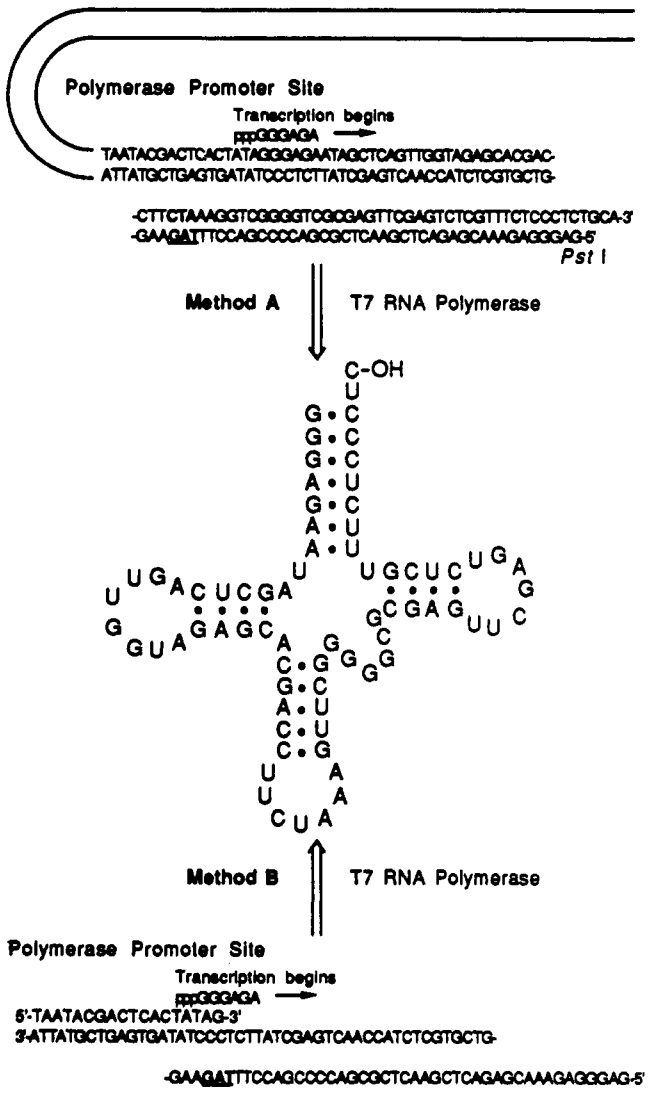
scheme was employed,[11b] followed by reverse phase HPLC using gradient elution with CH_3CN (0–40% CH_3CN in 30 min) in 5 mM ammonium formate (pH 4.5) to give the fully deprotected acylated dinucleotide **2** and **22–25** in yields ranging from 22 to 61%. This represents an improvement over previous methods, which give a diacylated product requiring extra deprotection and purification steps.[2,4,8]

Deprotection of dinucleotides acylated with Boc- or Cbz-protected residues, **20** and **21**, respectively, required a slight modification of this procedure. The first deprotection step is the same as for Fmoc-protected residues; dry oximate treatment removes both the *O*-2-chlorophenyl groups of the phosphate esters and *N*-(9-fluorenylmethyloxy)carbonyl groups from exocyclic amino groups of cytidine and adenosine.[11b] For the acylated dinucleotide dCA-(*N*-Boc)glycyl, subsequent treatment with trifluoroacetic acid[8] for 2 min at room temperature was sufficient to remove the tetrahydropyranyl and Boc groups to give **25** in 15% overall yield after purification by reverse phase HPLC. Deprotection of the acylated dinucleotide dCA-(*N*-Cbz)glycyl was accomplished by transfer hydrogenolysis[16] in a 1:1 solution of ethyl acetate/ethanol containing 10% palladium on carbon and cyclohexadiene to yield the desired deprotected dinucleotide **25** (34%). This method eliminates the often problematic reduction of the 5,6 double bond of cytidine when the Cbz group is removed with hydrogen over palladium on carbon or palladium on barium sulfate.[17]

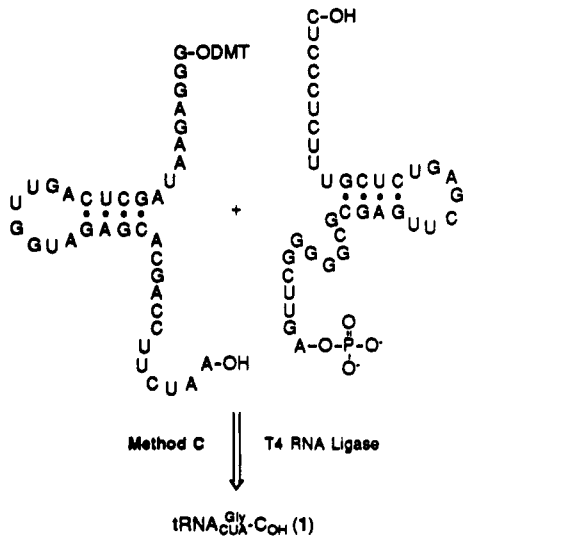
Preparation of the 3′-Truncated $tRNA^{Gly}_{CUA}$-C_{OH} (1). Four methods for the the preparation of the 3′-truncated tRNA **1** were investigated. Methods A and B (Scheme VII) employ DNA-dependent T7 RNA polymerase. The other two methods, method C (Scheme VIII) and method D, employ chemical synthesis to construct the 74-base $tRNA^{Gly}_{CUA}$-C_{OH} (**1**). For synthesis of the 3′-truncated tRNA **1** by run-off transcription, DNA templates for $tRNA^{Gly}_{CUA}$-C_{OH} (**1**) were constructed by either of two methods. The first utilizes plasmid amplification and results in an immortalized source of template, which contains both a T7 promoter site and Pst I restriction site at the 3′-end of the gene (Scheme VII, method A).

(13) Kumar, G.; Celewicz, L.; Chládek, S. *J. Org. Chem.* **1982**, *47*, 634–644.
(14) Profy, A. T.; Usher, D. A. *J. Mol. Evol.* **1984**, *20*, 147–156.
(15) Hudson, D. *J. Org. Chem.* **1988**, *53*, 617–624.
(16) Watkins, B. E.; Kiely, J. S.; Rapoport, H. *J. Am. Chem. Soc.* **1982**, *104*, 5702–5708.
(17) (a) Felix, A. M.; Heimer, E. P.; Lambros, T. J.; Tzougraki, C.; Meienhofer, J. *J. Org. Chem.* **1978**, *43*, 4194–4196. (b) Anantharamaiah, G. M.; Sivanandaiah, K. M. *J. Chem. Soc., Perkin Trans. 1* **1977**, 490–491.

Scheme VII



Scheme VIII



Overproduction of the plasmid in TB-1 cells, followed by purification and digestion by Pst I results in a linear DNA template terminating on the sense strand with a guanosine that is complementary to the 3'-terminal cytidine of tRNA$^{Gly}_{CUA}$-C$_{OH}$ (1). Run-off transcription of this Pst I-digested plasmid gives the expected 74-nucleotide product with a sequence[18] identical with that of *Escherichia coli* tRNA^{Gly} with the three modifications: (1) the transcript contains no hypermodified bases, (2) the 3'-terminal lacks the cytidine and adenosine residues, and (3) the anticodon loop contains a CUA anticodon for suppression of amber codons. Milligram quantities of this transcript are easily obtained in a single reaction and can be purified to single-nucleotide resolution on a 20% polyacrylamide preparative gel.[19] An alternative method of synthesis of the tRNA$^{Gly}_{CUA}$-C$_{OH}$ (1) utilizes a synthetic linear DNA template featuring a double-stranded promoter region and a long 5'-overhang corresponding to the transcribed sequence,[20] as seen in Scheme VII, method B. The facile production of templates is only limited by the length of time required to chemically synthesize new oligodeoxyribonucleotides. This reduces the time to generate new tRNA-C$_{OH}$s, and greatly simplifies template design (i.e., restriction sites no longer need to be designed into the structure). Milligram quantities of tRNA$^{Gly}_{CUA}$-C$_{OH}$ (1) can also be obtained with this procedure.

Complementary to these methods of run-off transcription, tRNA$^{Gly}_{CUA}$-C$_{OH}$ (1) can also be produced directly by automated synthesis techniques (methods C and D). Both tRNA$^{Gly}_{CUA}$-C$_{OH}$[21] and tDNA$^{Gly}_{CUA}$-C$_{OH}$[22] analogues have been constructed by us with standard 2-cyanoethyl phosphoramidite chemistry,[23] from commercially available (Milligen/Biosearch) oligonucleotides.

Two general strategies were employed to construct the 74-base tRNA$^{Gly}_{CUA}$-C$_{OH}$ (1). The first method (Scheme VIII, method C) requires the T4 RNA ligase-mediated condensation of 5'-half tRNA$^{Gly}_{CUA}$-A$_{OH}$ (residues 1–37) and 3'-half tRNA^{Gly}-C$_{OH}$ (residues 38–74).[24] The requisite 37-mers were synthesized with 5'-O-(4,4'-dimethoxytrityl)-2'-O-(trialkylsilyl)ribonucleoside 3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidites.[25,26] More recently, chemical synthesis (method D) of the entire tRNA-C$_{OH}$ component 1 as a single oligoribonucleotide was accomplished through the use of the more reactive 5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(trialkylsilyl)ribonucleoside 3'-*O*-(2-cyanoethyl *N,N*-di*ethyl*)phosphoramidites,[21] avoiding the enzymatic ligation step. Purification of these synthetic oligoribonucleotides was accomplished by reverse phase gradient elution with CH_3CN (10–20% CH_3CN in 40 min) in TEAA (100 mM, pH 7.0) with ultraviolet detection at 254 nm to elucidate the desired peak.

General Preparation of Chemically Misacylated tRNAs. With both the 2'(3')-O-acylated dinucleotides **2** and **22–25** and the 3'-truncated tRNA **1** in hand, construction of the intact tRNAs acylated with the desired non-natural residue could be completed. The T4 RNA ligase-mediated coupling of 20 molar equiv of 5'-*O*-phosphoryl-2'-deoxycytidylyl(3'-5')-2'(3')-*O*-(L-3-iodotyrosyl)adenosine (**2**) with tRNA$^{Gly}_{CUA}$-C$_{OH}$ (**1**) to yield L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**3**) was carried out as previously described (Scheme I).[1] The length of incubation was extended from 6 to 10 min to maximize formation of L-3-

(18) Roberts, J. W.; Carbon, J. *J. Biol. Chem.* **1975**, *250*, 5530–5541.
(19) Sampson, J. R.; Uhlenbeck, O. C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1033–1037.
(20) Francklyn, C.; Schimmel, P. R. *Nature* **1989**, *337*, 478–481.
(21) Lyttle, M. H.; Wright, P. B.; Sinha, N. D.; Bain, J. D.; Chamberlin, A. R. *J. Org. Chem.*, preceding paper in this issue.
(22) Bain, J. D.; Chamberlin, A. R. *Science*, Submitted.
(23) Tanimura, H.; Fukazawa, T.; Sekine, M.; Hata, T.; Efcavitch, J. W.; Zon, G. *Tetrahedron Lett.* **1988**, *29*, 557–578.
(24) Wittenberg, W. L.; Uhlenbeck, O. C. *Biochemistry* **1985**, *24*, 2705–2712.
(25) Sinha, N. D.; Biernat, J.; McManus, J.; Koster, H. *Nucleic Acids Res.* **1984**, *12*, 4539–4557.
(26) Wang, Y. Y.; Lyttle, M. H.; Borer, P. N. *Nucleic Acids Res.* **1990**, *18*, 3347–3352.

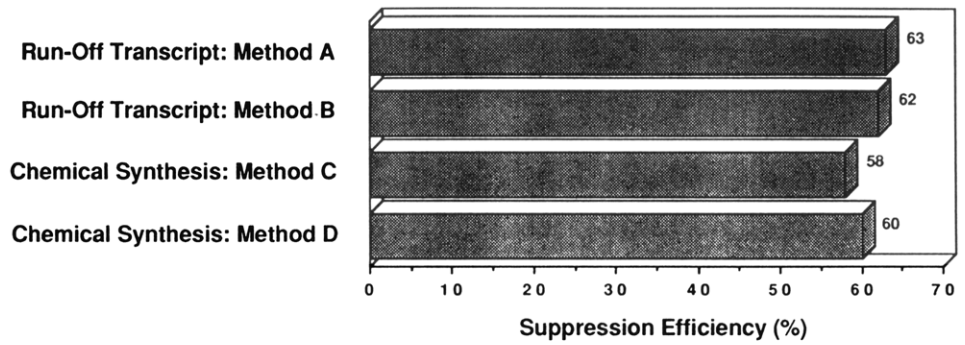


Figure 1.

iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**3**). The unreacted dinucleotide **2** was easily removed by selective precipitation of L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**3**) with 70% EtOH at −20 °C followed by chloroform/phenol extraction to remove the T4 RNA ligase. The acylated tRNA **3** was sequentially purified through Sephadex G-25 and BD-cellulose.[4] L-Phenyllactyl-tRNA$^{Gly}_{CUA}$-dCA, *N*-methyl-L-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA, D-phenylalanyl-tRNA$^{Gly}_{CUA}$-dCA, and glycyl-tRNA$^{Gly}_{CUA}$-dCA were prepared in analogous fashion.[27]

Comparison of Four Methods for the Preparation of L-3-Iodotyrosyl-tRNA$^{Gly}_{mCUA}$-dCA (3) through Evaluation in an in Vitro Translation Reaction. In order to conduct a comparison of the four methods for the preparation of acylated tRNA$^{Gly}_{CUA}$-dCA, L-3-iodotyrosyl-tRNA$^{Gly}_{CUA}$-dCA (**3**) constructed by each of these methods was evaluated in an in vitro translation reaction.[28] Within experiment error (±5%), the suppression efficiencies were indistinguishable (Figure 1). This result clearly demonstrates that all four methods yield acylated tRNA products that are biologically equivalent for the their intended purpose; site-specific incorporation of non-natural residues into proteins.

Conclusion

A general strategy has been developed for the construction of acylated tRNAs that requires the synthesis of three main chemical components: (1) the RNA/DNA hybrid dinucleotide pdCpA, (2) an N-protected residue, and (3) the 3′-truncated tRNA$^{Gly}_{CUA}$-C$_{OH}$. The use of a dinucleotide RNA/DNA hybrid offers the advantage of synthetic simplicity and product stability. In addition, judicious choice of protecting groups for the dinucleotide makes this synthetic scheme compatible with Boc-, Bpoc-, Cbz-, for Fmoc-protected non-natural residues, allowing for greater flexibility in the introduction of the non-natural residue. The final component to be synthesized with 3′-truncated tRNA. Four methods have been presented for preparation of tRNA$^{Gly}_{CUA}$-C$_{OH}$ and were shown to possess equivalent bioactivity in an in vitro translation experiment when ligated to an appropriate acylated dinucleotide. The methods described offer molecular biologists and chemists several synthetic strategies for the construction of acylated tRNAs that can be easily varied both in the sequence of the tRNA and the residue that is acylated to this component.

(27) Detailed analysis of suppression and translation efficiencies are given elsewhere (Bain, J. D.; Wacker, D. A.; Kuo, E. E.; Chamberlin, A. R. *Tetrahedron* **1991**, *47*, 2389–2400).

(28) The details of this method along with various applications have been discussed elsewhere (Bain, J. D.; Diala, E. S.; Glabe, C. G.; Wacker, D. A.; Lyttle, M. H.; Dix, T. A.; Chamberlin, A. R. *Biochemistry*. In press.).

Experimental Section

Materials. The following biological products and reagents were used: calf intestinal alkaline phosphatase and inorganic pyrophosphatase (Boehringer Mannhiem Biochemicals), RNasin (Promega Biotech), T4 polynucleotide kinase and T4 RNA ligase (New England Biolabs), T4 DNA ligase (Bethesda Research Laboratories), T7 RNA polymerase (New England Biolabs or United States Biochemicals), and restriction endonucleases *Eco*RI, *Eco*RV, *Hind*III, *Nco*I, *Pst*I, and *Xho*I (Bethesda Research Laboratories, Boehringer Mannhiem Biochemicals, or New England Biolabs). Plasmids used were either pIBI30 (International Biotechnologies Institute) or Bluescript M13$^+$ KS (Stratagene). *Escherichia coli* TB-1 cells were a generous gift from Dr. M. Cumsky (UC Irvine). Benzoylated (diethylaminoethyl)-cellulose and Poly-Prep 2-mL disposable polypropylene columns (731-1550, Bio-Rad), Sephadex G-25 Select-D columns (5301-730608/725608, 5 Prime→3 Prime, Inc), rabbit reticulocyte lysate (No. 90, Amersham), and Geneclean (BIO 101, Inc.) were purchased from the manufacturers indicated.

Solvents and reagents were dried prior to use when necessary. Procedures for acetonitrile and diethyl ether are described in the preceding paper. Chloroform was passed through a column of basic alumina (200 g/L) and then distilled from CaH_2 (5 g/L). *N*,*N*-Dimethylformamide was dried by stirring overnight at room temperature over CaH_2 (5 g/L) and distilled under diminished pressure. 1-Hydroxybenzotriazole hydrate (Aldrich) was dried before use for 72 h at 50 °C over P_2O_5 in vacuo (*WARNING*: at higher temperatures 1-hydroxybenzotriazole may explode). *N*-Methylmorpholine was dried by heating at reflux over CaH_2 (5 g/L), distilled, and then redistilled from ninhydrin (10 g/L). Piperidine was dried by heating at reflux over CaH_2 (5 g/L) for 2 h and distilled. All other solvents used were of the highest commercial grade obtainable.

Procedures for handling oligoribonucleotides are described in the preceding paper.

The following chemical reagents were purchased: (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (Advanced ChemTech), sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Calbiochem-Behring), tris(hydroxymethyl)aminomethane (Sigma), N-protected residues for peptide synthesis or N-protected non-natural residues **15**, and **19**–**21** for preparation of acylated dinucleotides **2** and **25** (Bachem, Milligen/Biosearch, or Sigma), trifluoroacetic acid (Pierce Chemical Co), nucleotides, coupling, and oxidation reagents for oligonucleotide synthesis (Milligen/Biosearch), nucleotide triphosphates (Pharmacia), *P11*-5′-(7-methyl)guanosine-*P*^{3}-5′-guanosine triphosphate, dilithium salt (Boehringer Mannhiem Biochemicals), and L-[3,4,5-^{3}H]leucine (143 Ci/mmol) and L-[^{35}S]methionine (>800 Ci/mmol) were (New England Nuclear Research Products).

The following compounds were prepared by previously reported methods: N^6-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)adenosine 3′-*O*-(2-cyanoethyl *N*,*N*-dialkylphosphoramidite), N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)cytidine 3′-*O*-(2-cyanoethyl *N*,*N*-dialkylphosphoramidite), N^2-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(triisopropylsilyl)guanosine 3′-*O*-(2-cyanoethyl *N*,*N*-dialkylphosphoramidite), and 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*O*-(2∼cyanoethyl *N*,*N*-di-

alkylphosphoramidite) were made by first synthesizing the nucleoside monomers[29] and then converting them to 2-cyanoethyl *N,N*-diisopropylphosphoramidites[26] or 2-cyanoethyl *N,N*-diethylphosphoramidites,[23] 2-chlorophenyl bis(1-hydroxybenzotriazolyl)phosphate,[30] and *N*-[(9-fluorenylmethyloxy)carbonyl]-D-phenylalanine (16).[31]

General Methods. Melting points are reported uncorrected. NMR spectra are referenced to internal tetramethylsilane for ^1H NMR, to the solvent for ^{13}C NMR, or to 3-(trimethylsilyl)propionic acid, sodium salt (DSS) when D_2O is employed as solvent. Thin-layer chromatography (TLC) was performed on 0.25-mm E. Merck precoated silica gel plates (60 F-254). Flash chromatography was performed on ICN 200–400-mesh silica gel as described by Still et al.[32] Small and medium scale purifications 920–1500 mg) were alternatively accomplished by radial chromatography using a Harrison Research Chromatotron. Radial silica gel plates of 1-, 2-, or 4-mm thickness were used consisting of Merck silica gel (60 PF_{254}) containing gypsum. High-performance liquid chromatography (HPLC) was performed on a reverse phase Vydac C-4 preparative column (5-μm packing, 10 mm i.d. × 250 mm length).

Oligodeoxynucleotides were synthesized by using 2-cyanoethyl phosphoramidite chemistry.[23] Synthesis was carried out by O. Bates and K. Burke from the protein/Nucleic Acid Analysis Lab (UC Irvine), B. Meeker from the Department of Molecular Biology (UC Irvine), or M. Lyttle (Milligen/Biosearch). Oligodeoxyribonucleotides (two to three A_{260} units of each) for gene construction were purified by electrophoresis through a 20% acrylamide/7 M urea gel, visualized by UV shadowing, and excised, and the gel was eluted twice in 10 mM Tris·HCl, pH 8.0/300 mM NaCl/1 mM EDTA/1% phenol, at 37 °C for 6 h. The eluents were combined and the DNA was EtOH-precipitated, washed with cold 70% EtOH, dried, and resuspended in distilled H_2O.

Oligoribonucleotides were synthesized (1.0-μL scale) on a Milligen/Biosearch 8700 synthesizer using 2-cyanoethyl phosphoramidite chemistry as described previously. The controlled pore glass for solid-phase-supported synthesis was the same as previously described,[27] except that the unattached ribose hydroxyl functionality was capped with an acetyl rather than a trialkylsilyl group. Synthesis of all four 37-mer oligoribonucleotides [5′-half $tRNA^{Gly}_{CUA}$-A_{OH} (residues 1–37), 5′-half $tRNA^{Gly}_{UCA}$-A_{OH} (residues 1–37), 5′-half $tRNA^{Gly}_{UUA}$-A_{OH} (residues 1–37), and 3′-half $tRNA^{Gly}$-C_{OH} (residues 38–74)] were conducted with immobilized nucleosides on 500-Å pore glass supports. The concentration of amidites was 50 mg/mL in CH_3CN and the amidite consumption was 33 mg per coupling. The cycle for RNA synthesis was similar to that used for DNA synthesis, except that the exposure time of the support to the activated RNA amidite synthons was 15 min instead of 90 s.[26] A slightly more reactive phosphoramidite variation of the compounds above was employed for synthesis of the 74-mer $tRNA^{Gly}_{CUA}$-C_{OH},[21] which allowed a 7-min coupling time. In addition, a 1000-Å pore glass support was used.

Cleavage of the oligoribonucleotides from the controlled pore glass, and deprotection of both phosphate and base moieties, was afforded by treatment with concentrated ammonium hydroxide/EtOH (75:25) for 4 h at 65 °C (2 × 1.0 mL). The supernatants were decanted, combined, concentrated by lyophilization, and resuspended in distilled H_2O (500 μL). An aliquot (200 μL) was removed and concentrated by lyophilization followed by addition of 1 M *n*-Bu_4NF in THF (300 μL). The solution was allowed to stand at room temperature overnight and then quenched by addition of an aqueous solution (500 μL) containing 50 mM NaOAc/20 mM EDTA/300 mM NaCl. The mixture was extracted once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and once with $CHCl_3$/isoamyl alcohol (24:1) and precipitated with 2.5 volumes of EtOH in −20 °C. Crude 5′-terminal DMT oligoribonucleotides were dissolved in TEAA (100 mM, pH 7.0) and injected onto a Vydac C-4 column equilibrated in TEAA (100 mM, pH 7.0) in H_2O/CH_3CN (9:1). Gradient elution with CH_3CN (10–50% CH_3CN in 50 min) in TEAA (100 mM, pH 7.0) followed by ultraviolet detection at 254 nm resolved the desired peak.

N^4-[(9-Fluorenylmethyloxy)carbonyl]-2′-deoxycytidine (9).[12] 2-Deoxycytidine monohydrochloride (8; 5.00 g, 19.0 mmol) and dry pyridine (125 mL) were added to a flame-dried round-bottom three-neck flask (250 mL) under argon fitted with a powder addition funnel containing 9-fluorenylmethyl chloroformate (5.41 g, 20.9 mmol). Trimethylsilyl chloride (8.56 g, 78.8 mmol) was added in one portion over a 5-s period and the solution stirred for 2 h at room temperature followed by addition of 9-fluorenylmethyl chloroformate over a 2-min period. The solution was stirred at room temperature for an additional 3 h and quenched with H_2O (15 mL) containing KF (100 mg). The solution was stirred at room temperature for 30 min followed by addition of EtOAc (200 mL) and 5% aqueous NH_4HCO_3 (100 mL). The layers were separated, and the organic layer was washed with H_2O (3 × 100 mL), dried over $MgSO_4$, and concentrated in vacuo to a pale yellow oil. Crystallization from CH_3CN gave 8.07 g (95%) of **9** as colorless needles: mp 158.0–160.0 °C; ^1H NMR (300 MHz, DMSO) δ 11.01 (s, 1 H), 8.31 (d, J = 7.5 Hz, 1 H), 7.83 (dd, J = 19.6, 7.4 Hz, 4 H), 7.36 (dt, J = 26.6, 7.3 Hz, 4 H), 6.99 (d, J = 7.5 Hz, 1 H), 6.14 (t, J = 6.3 Hz, 1 H), 5.28 (d, J = 4.0 Hz, 1 H), 5.07 (t, J = 4.8 Hz, 1 H), 4.37 (d, J = 7.4 Hz), 4.29–4.24 (m, 1 H), 3.88 (app d, J = 3.4 Hz, 1 H), 3.68–3.55 (m, 2 H), 2.34–2.27 (m, 1 H), 2.08–1.99 (m, 1 H); ^{13}C NMR (75 MHz, DMSO) δ 162.7, 154.2, 144.7, 143.4, 140.7, 127.8, 127.1, 125.5, 120.1, 94.2, 87.9, 86.1, 70.0, 66.9, 61.0, 46.1, 40.9; IR (KBr) 3610–2570 (br), 3287, 3069, 2951, 2916, 2874, 1744, 1650, 1560, 1497, 1450, 1404, 1328, 1279, 1208, 1100, 1078, 989, 814, 792, 760, 741 cm^{-1}; UV (9:1 CH_3CN/5 mM HCO_2NH_4, pH 4.5) λ_{max} 299, 288, 276 (sh), 265, 256 (sh), 226 (sh), 220 (sh), 208 (sh) nm; LRMS (FAB, DMSO/*p*-nitrobenzyl alcohol), *m/e* (relative intensity) 450 (MH^+, 25), 334 (47), 232 (25), 180 (19), 179 (100), 165 (24), 139 (20), 138 (54), 124 (17), 120 (30), 117 (24), 112 (19), 106 (15), 105 (15); HRMS (FAB, DMSO/*p*-nitrobenzyl alcohol) calcd for $C_{24}H_{23}N_3O_6$ (+1.0078) 450.1665, found 450.1663. Anal. Calcd for $C_{24}H_{23}N_3O_6 \cdot {}^1/_2H_2O$: C, 62.87; H, 5.27; N, 9.16. Found: C, 62.70; H, 4.99; N, 9.28.

N^4-[(9-Fluorenylmethyloxy)carbonyl]-5′-*O*-[bis(2-chlorophenyl)phosphoryl]-2′-deoxycytidine (10). N^4-[(9-Fluorenylmethyloxy)carbonyl]-2′-deoxycytidine (**9**) (4.00 g, 8.9 mmol) and dry pyridine (60 mL) were added to a flame-dried round-bottom flask (100 mL) under argon. The stirred solution was cooled to 0 °C and bis(2-chlorophenyl) phosphorochloridate (2.99 g, 8.9 mmol) was added in one portion over a 3-s period. The solution was allowed to slowly warm to room temperature, stirred for an additional 1 h, and poured into 5% aqueous NH_4HCO_3 (100 mL). Methylene chloride (100 mL) was added, the layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were combined, dired over $MgSO_4$ and concentrated in vacuo to a pale yellow solid. Flash chromatography (silica gel, step gradient: 0 to 3% MeOH in $CHCl_3$) gave 3.71 g (55%) of chromatographically pure **10** as a white solid: mp 119.5–121.5 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 8.00 (d, J = 7.5 Hz, 1 H), 7.79 (d, J = 7.5 Hz, 1 H), 7.63 (d, J = 7.4 Hz, 1 H), 7.49–7.12 (m, 16 H), 6.33 (t, J = 6.3 Hz, 1 H), 4.73–4.68 (m, 2 H), 4.53–4.41 (m, 3 H), 4.34–4.25 (m, 2 H), 2.76–2.68 (m, 1 H), 2.16 (m, 1 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.1, 146.8, 144.4, 143.9, 141.9, 131.5, 128.8, 128.6, 127.8, 127.4, 126.1, 125.7, 122.2, 122.1, 120.7, 96.0, 87.8, 85.9, 71.3, 69.5, 68.6, 47.2, 42.1; IR (KBr) 3650–2580 (br), 3408, 3074, 1749, 1656, 1561, 1499, 1479, 1400, 1212, 1058, 1035, 960, 757 cm^{-1}; UV (9:1 CH_3CN/5 mM HCO_2NH_4, pH 4.5) λ_{max} 299, 289, 277 (sh), 265, 255, 246 (sh), 226 (sh), 210 nm. Anal. Calcd for $C_{36}H_{30}Cl_2N_3O_9P$: C, 57.61; H, 4.03; N, 5.60. Found: C, 57.90; H, 4.27; N, 5.53.

N^6-[(9-Fluorenylmethyloxy)carbonyl]-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(trimethylsilyl)adenosine (12). (−)-Adenosine (11; 2.05 g, 7.67 mmol), dry DMF (25 mL), and dry pyridine (25 mL) were added to a flame-dried round-bottom flask (100 mL) under argon fitted with a powder addition funnel containing 9-fluorenylmethyl chloroformate (2.21 g, 8.53 mmol). 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (2.71 g, 8.60 mmol) was added dropwise over a 15-min period to the stirred solution. The mixture was stirred at room temperature for 1 h, followed by addition of trimethylsilyl chloride (1.64 g, 15.1 mmol)

(29) Hakimelahi, G. H.; Proba, Z. A.; Olgilvie, K. K. *Can. J. Chem.* **1982**, *60*, 1106–1113.

(30) (a) van Boom, J. H.; Wreesman, C. T. J. *Oligonucleotide Synthesis: a Practical Approach*; Gait, M. J., Ed.; IRL Press: Oxford, England, 1984; pp 153–183. (b) Wreesman, C. T. J.; Fidder, A.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1983**, *11*(23), 8389–8405.

(31) Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*(22), 3404–3409.

(32) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*(14), 2923–2925.

in one portion over a 5-s period. The mixture was stirred at room temperature for an additional 1 h, followed by addition of 9-fluorenylmethyl chloroformate over a 2-min period. The solution was stirred at room temperature for an additional 3 h and quenched with 5% aqueous NH_4HCO_3 (75 mL). Methylene chloride (75 mL) was added, the layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo to a yellow oil. Flash chromatography (silica gel, 2:1 hexanes/Et_2O) gave 4.16 g (74%) of chromatographically pure **12** as a white solid: mp 71.0–73.0 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1 H), 8.33 (s, 1 H), 7.80 (d, J = 7.4 Hz, 2 H), 7.68 (d, J = 7.3 Hz, 2 H), 7.43 (t, J = 7.3 Hz, 2 H), 7.34 (app t, J = 7.3 Hz, 3 H), 6.11 (d, J = 5.1 Hz, 1 H), 4.87 (t, J = 4.7 Hz, 1 H), 4.68–4.65 (m, 2 H), 4.45–4.43 (m, 1 H), 4.36 (t, J = 6.3 Hz, 1 H), 4.20–4.14 (m, 2 H), 3.92 (d, J = 9.0 Hz, 1 H), 1.15 (s, 28 H), 0.22 (s, 9 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.7, 151.1, 149.3, 143.5, 141.9, 141.2, 127.7, 127.1, 124.9, 119.9, 88.6, 85.7, 75.0, 71.5, 67.6, 62.4, 46.9, 18.0, 11.8, 0.24; IR (KBr) 3610–2700 (br), 3407, 2948, 2867, 1761, 1614, 1464, 1390, 1301, 1214, 1168, 1129, 1041, 1003, 944, 862, 759, 741, 697 cm^{-1}; UV (9:1 CH_3CN/5 mM HCO_2NH_4, pH 4.5) λ_{max} 296, 285, 272 (sh), 263, 255 (sh), 226 (sh), 208 nm. Anal. Calcd for $C_{37}H_{49}N_5O_7Si_2$: C, 62.92; H, 3.96; N, 1.83. Found: C, 62.97; H, 4.22; N, 1.74.

N^6-[(9-Fluorenylmethyloxy)carbonyl]-2′-*O*-(tetrahydropyranyl)adenosine (13a and 13b). *p*-Toluenesulfonic acid monohydrate (926 mg, 4.9 mmol) and dry dioxane (100 mL) were added to a flame-dried round-bottom flask (250 mL) under argon containing 3-Å molecular sieves (0.50 g) and fitted with an addition funnel containing N^6-[(9-fluorenylmethyloxy)carbonyl]-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(trimethylsilyl)adenosine (**12**; 10.7 g, 14.6 mmol), 2,3-dihydropyran (7.5 mL), and dry dioxane (50 mL). The acid solution was stirred for 2 h at room temperature followed by dropwise addition of the nucleoside solution over a 5-min period. The mixture was stirred for an additional 1.5 h at room temperature with complete conversion of the starting material as indicated by TLC (92:8 CH_2Cl_2/MeOH). The reaction mixture was neutralized by pouring into 5% aqueous NH_4HCO_3 (200 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3 × 150 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo to a white foam. The crude product was dissolved in a 0.8 M *n*-Bu_4NF solution (8:1:1 THF/pyridine/H_2O; 50 mL) and stirred for 1.5 h followed by neutralization by pouring into 5% aqueous NH_4HCO_3 (200 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3 × 150 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo to a yellow oil. Flash chromatography (silica gel, step gradient: 0 to 4% MeOH in $CHCl_3$) gave 4.25 g (51%) of a faster eluting diastereomer **13a** and 1.98 g (24%) of a slower eluting diastereomer **13b** as chromatographically pure white foams.

Faster eluting diastereomer: mp 126.5–128 °C; ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.2, 151.7, 150.1, 143.6, 142.7, 140.5, 127.7, 127.1, 125.5, 124.6, 120.1, 97.3, 86.4, 85.9, 79.2, 68.9, 66.7, 61.6, 61.1, 46.3, 45.7, 29.6, 24.8, 24.5; IR (KBr) 3680–3140 (br), 3419, 2941, 1802, 1610, 1451, 1275, 1212, 1102, 1034, 741 cm^{-1}; UV (9:1 CH_3CN/5 mM HCO_2NH_4, pH 4.5) λ_{max} 299, 288, 275 (sh), 256 (sh), 226 (sh), 208 (sh), 206 nm. Anal. Calcd for $C_{36}H_{31}N_5O_7$: C, 62.82; H, 5.45; N, 12.21. Found: C, 62.87; H, 5.62; N, 12.07.

Slower eluting diastereomer: mp 133.5–136.5 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 10.91 (s, 1 H), 8.72 (s, 1 H), 8.66 (s, 1 H), 8.30 (s, 1 H), 7.89 (d, J = 7.5 Hz, 1 H), 7.84 (app d, J = 7.2 Hz, 2 H), 7.42 (t, J = 7.2 Hz, 1 H), 7.33 (t, J = 7.2 Hz, 1 H), 6.19 (d, J = 6.4 Hz, 1 H), 5.32 (d, J = 4.9 Hz, 1 H), 5.20 (t, J = 5.3 Hz, 1 H), 4.84 (t, J = 5.1 Hz, 1 H), 4.72 (app s, 1 H), 4.48–4.28 (m, 4 H), 4.03 (app, d, J = 3.1 Hz, 1 H), 3.74–3.55 (m, 2 H), 3.14–3.01 (m, 2 H), 1.59–1.16 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.2, 151.7, 149.8, 143.6, 143.0, 140.7, 127.7, 127.1, 125.5, 123.7, 120.1, 97.0, 86.5 86.1, 77.3, 69.0, 66.8, 61.4, 60.8, 54.9, 46.3, 29.6, 24.7, 18.5; IR (KBr) 3680–3140 (br) 3385, 2941, 1755, 1615, 1466, 1306, 1212, 1100, 1034, 741 cm^{-1}. Anal. Calcd for $C_{36}H_{31}N_5O_7$: C, 62.82; H, 5.45; N, 12.21. Found: C, 62.94, H, 5.57; N, 12.39.

Representative Procedure for Synthesis of *N*-(9-Fluorenylmethyloxy)carbonyl-Protected Amino Acids. *N*-[(9-Fluorenylmethyloxy)carbonyl]-*N*-methyl-L-phenylalanine (17). A stirred solution containing *N*-methyl-L-phenylalanine (693 mg, 3.87 mmol), 10% aqueous Na_2CO_3 (10.2 mL), and dioxane (5.0 mL) in a round-bottom flask (50 mL) was cooled in an ice bath and 9-fluorenylmethyl chloroformate (1.00 g; 3.87 mmol) dissolved in dioxane (10 mL) was added in one portion over a 2-min period. The mixture was stirred in the ice bath for 1 h and then allowed to warm to room temperature. The solution was subsequently stirred for an additional 3 h, poured into H_2O (200 mL), and extracted with Et_2O (3 × 50 mL). The aqueous layer was cooled in an ice bath and the product acidified with concentrated HCl, followed by extraction with EtOAc (3 × 50 mL). The organic layers were combined, dried over $MgSO_4$, and concentrated in vacuo to a white foam. Radial chromatography (silica gel, step gradient: 0 to 1% AcOH in 1:1 hexanes/Et_2O) gave 1.41 g (91%) of **17** as a white foam: mp 55.5–57.0 °C; ^1H NMR (300 MHz, DMSO) δ 7.87 (d, J = 7.5 Hz, 2 H), 7.57–7.14 (m, 10 H), 7.04 (d, J = 6.9 Hz, 1 H), 4.78 (dd, J = 11.2, 4.7 Hz, 1 H), 4.66 (dd, J = 11.2, 4.7 Hz, 1 H), 4.31–4.12 (m, 2 H), 3.02–2.49 (m, 3 H), 2.67 (d, J = 9.8 Hz, 3 H); ^{13}C NMR (75 MHz, DMSO) δ 171.9, 155.6, 143.7, 140.7, 137.9, 128.7, 128.2, 127.6, 127.1, 126.3, 124.9, 120.1, 66.7, 60.1, 46.6, 34.2, 31.7; IR (KBr) 3700–2500 (br), 3064, 1700, 1450, 1319, 741 cm^{-1}; HRMS (CI, isobutane) calcd for $C_{25}H_{23}NO_4$ (+1.0078) 402.1705, found 402.1706. Anal. Calcd for $C_{25}H_{23}NO_4$: C, 74.80; H, 5.77; N, 3.49. Found: C, 74.80; H, 5.71; N, 3.67.

Trimethylsilyl *O*-(Trimethylsilyl)-L-phenyllactate (18). m stirred solution of phenyllactic acid (50 mg, 0.3 mmol) and hexamethyldisilazane (5 mL) was heated at reflux for 1 h in a flame-dried round-bottom flask (10 mL) with a spiral condenser under argon. The excess hexamethyldisilazane was removed in vacuo to yield **18** as a colorless oil, which was employed in the next step without further purification: ^1H NMR (300 MHz, $CDCl_3$) δ 7.29–7.39 (m, 6 H), 4.33 (dd, J = 0.03, 0.01 Hz, 1 H), 3.18 (dd, J = 0.05, 0.01 Hz, 1 H), 2.95 (dd, J = 0.05, 0.03 Hz, 1 H), 0.37 (s, 9 H), 0.01 (s, 9 H): ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.1, 138.5, 130.3, 128.8, 127.1, 74.5, 41.8, 0.3, 0.1; IR (KBr) 2956, 2899, 1740, 1253, 1182, 1133, 933, 842, 753, 698 cm^{-1}.

Representative Procedure for Dinucleotide Synthesis. 2-Chlorophenyl N^4-[(9-Fluorenylmethyloxy)carbonyl]-5′-*O*-[bis(2-chlorophenyl)phosphoryl]-2′-deoxycytidylyl(3′-5′)-[N^6-[(9-fluorenylmethyloxy)carbonyl]-2′-*O*-(tetrahydropyranyl)adenosine] (14a).[11b,30b] N^4-[(9-Fluorenylmethyloxy)carbonyl]-5′-*O*-[bis(2-chlorophenyl)phosphoryl]-2′-deoxycytidine (**10**; 500 mg, 0.7 mmol) was added to a flame-dried round-bottom flask (10 mL) under argon and coevaporated with anhydrous pyridine (3 × 2.5 mL) under the exclusion of moisture to a viscous residue followed by addition of a freshly prepared solution of 2-chlorophenyl bis(1-hydroxybenzotriazolyl) phosphate (3.8 mL of a 0.2 M solution in THF). The mixture was stirred for 30 min at room temperature and then transferred by cannula under anhydrous conditions to N^6-[(9-fluorenylmethyloxy)-carbonyl]-2′-*O*-(tetrahydropyranyl)adenosine (**13a**; 519 mg, 0.9 mmol), previously coevaporated with anhydrous pyridine (3 × 2.5 mL). Subsequent stirring for 5 min was followed by addition of *N*-methylimidazole (300 μL) and continued stirring for 30 min at room temperature. The reaction was quenched by addition of 5% aqueous NH_4HCO_3 (100 μL), diluted with CH_2Cl_2 (50 mL), and washed with 5% aqueous NH_4HCO_3 (2 × 25 mL). The mixture was then dried with $MgSO_4$ and concentrated in vacuo to a pale yellow oil, which formed a white precipitate upon dropwise addition to hexanes (50 mL). Flash chromatography (silica gel, step gradient: 0 to 5% MeOH in CH_2Cl_2) gave 718 mg (70%) of chromatographically pure **14a** as a white solid (mixture of diastereomers): ^1H NMR (500 MHz, $CDCl_3$) δ 8.76 (m, 2 H), 8.02 (m, 1 H), 7.79–7.72 (m, 3 H), 7.71–7.68 (m, 4 H), 7.59–7.57 (m, 3 H), 7.43–7.08 (m, 18 H), 7.05–6.17 (m, 2 H), 4.86–4.26 (m, 14 H), 4.25 (m, 1 H), 3.59–3.52 (m, 2 H), 3.24–3.21 (m, 2 H), 2.89–2.86 (m, 3 H), 2.66–2.64 (m, 1 H), 2.05–1.23 (m, 7 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 154.6, 152.3, 151.9, 151.0, 146.0, 145.9, 143.6, 143.5, 143.1, 142.1, 141.3, 130.8, 128.2, 128.0, 127.8, 127.2, 126.8, 126.6, 125.4, 125.2, 125.1, 124.9, 121.5 121.4 121.3, 120.1 120.0, 103.3, 100.7, 95.1, 88.4, 86.8, 83.7, 79.1, 78.8, 69.8, 69.6, 68.3, 67.9, 67.8, 63.5, 63.2, 46.9, 46.7, 36.5, 30.3, 25.3, 24.8, 19.7; UV (9:1 CH_3CN/5 mM HCO_2NH_4, pH 4.5) λ_{max} 299, 288, 276 (sh), 266, 256, 247 (sh), 227 (sh), 209 nm; LRMS (FAB, CH_2Cl_2/*p*-nitrobenzyl alcohol), *m/e* (relative intensity) 1495 (MH^+, 12), 359 (10), 358 (16), 334 (14), 261 (123), 235 (16), 192

(12), 191 (12), 180 (18), 179 (100), 178 (60), 166 (14), 165 (28), 152 (18), 124 (18), 123 (12), 122 (6), 121 (14), 120 (28), 119 (10), 115 (14), 112 (14), 106 (14), 105 (18). Anal. Calcd for $C_{72}H_{63}N_8O_{18}P_2Cl_3$: C, 57.78; H, 4.24; N, 7.49. Found: C, 57.91; H, 4.17; N, 7.43.

14b (mixture of diastereomers): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.72 (m, 2 H), 7.89–7.85 (m, 1 H), 7.78 (m, 3 H), 7.71–7.68 (m, 4 H), 7.56–7.54 (m, 3 H), 7.43–7.09 (m, 18 H), 6.26–6.24 (m, 2 H), 5.22–5.20 (m, 2 H), 4.86–4.75 (m, 3 H), 4.69–4.42 (m, 9 H), 4.25 (m, 1 H), 3.59–3.52 (m, 2 H), 3.24–3.21 (m, 2 H), 2.86–2.81 (m, 3 H), 2.76–2.68 (m, 1 H), 1.81–1.21 (m, 7 H); UV (9:1 CH_3CN/5 mM HCO_2NH_4, pH 4.5) λ_{max} 299, 288, 276 (sh), 266, 256, 247 (sh), 227 (sh), 209 nm; LRMS (FAB, CH_2Cl_2/*p*-nitrobenzyl alcohol), *m/e* (relative intensity) 1495 (MH^+, 14), 576 (10), 575 (32), 474 (100), 460 (14), 359 (18), 358 (66), 334 (16), 219 (10), 180 (12), 179 (66), 178 (52), 165 (20), 120 (16). Anal. Calcd for $C_{72}H_{63}N_8O_{18}P_2Cl_3$: C, 57.78; H, 4.24; N, 7.49. Found: C, 57.72; H, 4.19; N, 7.53.

Representative Procedure for Formation and Deprotection of Acylated Dinucleotide Derivatives. 5′-*O*-Phosphoryl-2′-deoxycytidylyl(3′-5′)-[2′(3′)-*O*-(L-3-iodotyrosyl)adenosine] (2). *N*-[(9-Fluorenylmethyloxy)carbonyl]-L-3-iodotyrosine (15; 0.005 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (0.030 mmol), 1-hydroxybenzotriazole (0.300 mmol), and dry THF (100 μL) were added to a vial (0.5 mL) under argon and allowed to stand at room temperature for 20 min. The protected dinucleotide 2-chlorophenyl N^4-[(9-fluorenylmethyloxy)carbonyl]-5′-*O*-[bis(2-chlorophenyl)phosphoryl]-2′-deoxycytidylyl(3′-5′)-[N^6-[(9-fluorenylmethyloxy)carbonyl]-2′-*O*-(tetrahydropyranyl)adenosine] (14a; 0.005 mmol) and 1-methylimidazole (0.015 mmol) dissolved in dry THF (100 μL) were added to the solution. After 2 h, the reaction was quenched by addition of saturated aqueous NaCl (300 μL) and the resultant white precipitate isolated by centrifugation. The solvent was decanted, the pellet was dissolved into CH_3CN (3 mL) and H_2O (1 mL), and the layers were separated. The H_2O layer was extracted with CH_3CN (2 × 3 mL), and the organic layers were combined and concentrated in vacuo. The protected acylated dinucleotide (0.5 mmol) was treated with a freshly prepared solution of 1,1,3,3-tetramethylguanidine (190 mg, 0.38 M) and 4-nitrobenzaldoxime (316 mg, 0.33 M) in dry CH_3CN (5.0 mL) for 3 h at room temperature. The reaction product was precipitated with Et_2O (100 mL), isolated by centrifugation, and washed with Et_2O (2 × 100 mL). The pellet was dissolved in 80% HCO_2H (12.5 mL precooled to 0 °C in ice), and after 30 min at 0 °C, Et_2O (50–75 mL) was added under stirring. The product was isolated by centrifugation and after being dissolved in 80% AcOH (1.0 mL) was reprecipitated with an excess of Et_2O (HPLC: RP C4 prep column; run 20–50% CH_3CN linear gradient over 30 min). Buffer was 5 mM ammonium formate (pH 4.5). Collected eluents were lyophilized, dissolved in 0.1 N formic acid, and lyophilized again, followed by addition of H_2O and lyophilization to yield 2.7 μmol (54%) of **2** as a white solid: ^{1}H NMR (300 MHz, D_2O) δ 8.51 (s, 1 H), 8.20 (s, 1 H), 8.07 (d, J = 7.4 Hz, 1 H), 7.61 (d, J = 2.2 Hz, 1 H), 7.10 (dd, J = 8.2, 2.3 Hz, 1 H), 6.85 (d, J = 8.1 Hz, 1 H), 6.11 (d, J = 1.7 Hz, 2 H), 6.09 (d, J = 3.7 Hz, 1 H), 5.99 (app d, J = 3.2 Hz, 1 H), 4.62 (t, J = 4.4 Hz, 1 H), 4.41–4.22 (m, 6 H), 4.07 (ddd, J = 11.0, 3.8, 2.6 Hz, 1 H), 3.98 (ddd, J = 8.0, 4.9, 3.0 Hz, 1 H), 3.91 (dd, J = 7.3, 5.4 Hz, 1 H), 3.11 (dd, J = 14.7, 5.3 Hz, 1 H), 2.99 (dd, J = 14.7, 7.5 Hz, 1 H); ^{13}C NMR (75 MHz, D_2O) δ 176.6, 168.8, 160.5, 158.2, 157.8, 155.5, 151.7, 144.5, 142.5, 142.4, 133.5, 131.1, 121.3, 118.2, 107.3, 104.2, 99.3, 91.9, 89.5, 86.8, 86.2, 77.0, 72.9, 72.3, 67.7, 65.6, 58.6, 37.6; HRMS (FAB, DMSO/*p*-nitrobenzyl alcohol) calcd for $C_{28}H_{34}N_9O_{14}P_2$ (+1.0078) 784.1856, found 784.1849. Anal. Calcd for $C_{28}H_{34}N_9O_{14}P_2\cdot H_2O$: C, 42.01; H, 4.53; N, 15.75. Found: C, 42.20; H, 4.57; N, 15.77.

5′-*O*-Phosphoryl-2′-deoxycytidylyl(3′-5′)-[2′(3′)-*O*-(D-phenylalanyl)adenosine] (22). Addition of the protected dinucleotide (**14a**; 0.005 mmol) and 1-methylimidazole (0.015 mmol) dissolved in dry THF (100 μL) to a solution containing *N*-[(9-fluorenylmethyloxy)carbonyl]-D-phenylalanine (**16**; 0.030 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (0.030 mmol), 1-hydroxybenzotriazole (0.300 mmol), and dry THF (100 μL) gave 2.3 μmol (46%) of **22** as a white solid: ^{1}H NMR (300 MHz, D_2O) δ 8.49 (s, 1 H), 8.18 (s, 1 H), 8.04 (d, J = 7.6 Hz, 1 H), 7.40–7.27 (m, 8 H), 6.11–6.08 (m, 2 H), 5.98 (app d, J = 2.6 Hz, 1 H), 4.61 (t, J = 4.3 Hz, 1 H), 4.41 (s, 1 H), 4.33–4.25 (m, 6 H), 4.09–3.97 (m, 4 H), 3.26 (dd, J = 14.4, 5.2 Hz, 1 H), 3.11 (dd, J = 15.3, 7.6 Hz, 1 H); ^{13}C NMR (75 MHz, D_2O) δ 195.9, 176.7, 168.7, 160.3, 155.4, 151.6, 144.4, 142.5, 137.7, 132.0, 131.7, 130.3, 121.2, 108.4, 99.2, 91.0, 89.5, 86.6, 86.0, 77.0, 72.9, 72.3, 67.8, 65.9, 58.7, 39.1; HRMS (FAB, DMSO/*p*-nitrobenzyl alcohol) calcd for $C_{28}H_{35}N_9O_{13}P_2$ (+1.0078) 768.1907, found 768.1914. Anal. Calcd for $C_{28}H_{35}N_9O_{13}P_2\cdot H_2O$: C, 42.81; H, 4.75; N, 16.05. Found: C, 42.66; H, 4.75; N, 16.01.

5′-*O*-Phosphoryl-2′-deoxycytidylyl(3′-5′)-[2′(3′)-*O*-(*N*-methyl-L-phenylalanyl)adenosine] (23). Addition of the protected dinucleotide (**14a**; 0.005 mmol) and 1-methylimidazole (0.015 mmol) dissolved in dry THF (100 μL) to a solution containing *N*-[(9-fluorenylmethyloxy)carbonyl]-*N*-methyl-L-phenylalanine (**17**; 0.030 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (0.030 mmol), 1-hydroxybenzotriazole (0.300 mmol), and dry THF (100 μL) gave 3.1 μmol (61%) of **23** as a white solid: ^{1}H NMR (300 MHz, D_2O) δ 8.49 (s, 1 H), 8.17 (s, 1 H), 8.02 (d, J = 7.5 Hz, 1 H), 7.39–7.25 (m, 4 H), 6.10 (t, J = 3.2 Hz, 1 H), 6.08 (s, 1 H), 5.98 (app d, J = 3.6 Hz, 1 H), 4.61 (t, J = 3.9 Hz, 1 H), 4.41 (t, J = 2.6 Hz, 1 H), 4.35–4.25 (m, 3 H), 4.12 (ddd, J = 6.6, 3.9, 2.66 Hz, 1 H), 4.03 (ddd, J = 8.1, 5.0, 3.1 Hz, 1 H), 3.85 (t, J = 6.2 Hz, 1 H), 3.20 (dd, J = 6.5, 1.8 Hz, 2 H), 2.69 (s, 3 H); ^{13}C NMR (75 MHz, D_2O) δ 175.6, 168.6, 160.2, 158.1, 155.3, 151.6, 144.4, 142.5, 137.2, 132.0, 131.7, 130.3, 99.2, 91.9, 89.5, 86.6, 85.9, 77.0, 72.9, 72.2, 67.8, 67.3, 66.0, 38.4, 34.8; HRMS (FAB, DMSO/*p*-nitrobenzyl alcohol) calcd for $C_{29}H_{37}N_9O_{13}P_2$ (+1.0078) 782.2064, found 782.2062. Anal. Calcd for $C_{29}H_{37}N_9O_{13}P_2\cdot H_2O$: C, 43.56; H, 4.92; N, 15.76. Found: C, 43.57; H, 5.13; N, 15.92.

5′-*O*-Phosphoryl-2′-deoxycytidylyl(3′-5′)-[2′(3′)-*O*-(L-phenyllactyl)adenosine] (24). Addition of the protected dinucleotide (**14a**; 0.005 mmol) and 1-methylimidazole (0.015 mmol) dissolved in dry THF (100 μL) to a solution containing *N*-[(9-fluorenylmethyloxy)carbonyl]-L-phenyllactic acid (18; 0.030 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (0.030 mmol), 1-hydroxybenzotriazole (0.300 mmol), and dry THF (100 μL) gave 1.1 μmol (22%) of **24** as a white solid: ^{1}H NMR (300 MHz, D_2O) δ 8.49 (s, 1 H), 8.18 (s, 1 H), 8.00 (d, J = 7.6 Hz, 1 H), 7.39–7.25 (m, 6 H), 6.10 (dd, J = 7.7, 7.2 Hz, 2 H), 5.97 (app d, J = 3.6 Hz, 1 H), 4.77 (app t, J = 5.4 Hz, 1 H), 4.59 (t, J = 3.7 Hz, 1 H), 4.41 (t, J = 2.9 Hz, 1 H), 4.34–4.24 (m, 4 H), 4.14 (ddd, J = 14.0, 10.2, 1.5 Hz, 1 H), 4.04 (ddd, J = 14.0, 5.1, 3.0 Hz, 1 H), 3.10 (dd, J = 14.0, 4.3 Hz, 1 H), 2.87 (dd, J = 14.0, 8.0 Hz, 1 H); ^{13}C NMR (75 MHz, D_2O) δ 183.4, 168.5, 160.0, 158.0, 155.3, 151.6, 144.3, 142.5, 140.8, 132.1, 131.2, 129.3, 99.1, 92.0, 89.5, 86.6, 85.8, 77.0, 76.1, 72.9, 72.2, 67.9, 66.2, 43.0; HRMS (FAB, DMSO/*p*-nitrobenzyl alcohol) calcd for $C_{28}H_{34}N_8O_{14}P_2$ (+1.0078) 769.1747, found 769.1753. Anal. Calcd for $C_{28}H_{34}N_8O_{14}P_2$: C, 43.76; H, 4.46; N, 14.58. Found: C, 43.58, H, 4.47; N, 14.67.

5′-*O*-Phosphoryl-2′-deoxycytidylyl(3′-5′)-[2′(3′)-*O*-(glycyl)adenosine] (25) Prepared from *N*-Fmoc-glycine (19). Addition of the protected dinucleotide (**14a**; 0.005 mmol) and 1-methylimidazole (0.015 mmol) dissolved in dry THF (100 μL) to a solution containing *N*-[(9-fluorenylmethyloxy)carbonyl]glycine (**19**; 0.030 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (0.030 mmol), 1-hydroxybenzotriazole (0.300 mmol), and dry THF (100 μL) gave 2.1 μmol (42%) of **25** as a white solid: ^{1}H NMR (300 MHz, D_2O) δ 8.50 (s, 1 H), 8.19 (s, 1 H), 8.01 (d, J = 7.5 Hz, 1 H), 6.11 (dd, J = 10.3, 5.8 Hz, 2 H), 5.98 (d, J = 3.3 Hz, 1 H), 4.60 (t, J = 4.3 Hz, 1 H), 4.41 (t, J = 2.7 Hz, 1 H), 4.33–4.23 (m, 4 H), 4.14 (ddd, J = 11.9, 3.8, 2.7 Hz, 1 H), 4.04 (ddd, J = 11.9, 4.9, 3.1 Hz, 1 H), 3.57 (s, 1 H); ^{13}C NMR (75 MHz, D_2O) δ 168.5, 160.1, 158.1, 155.3, 151.7, 144.4, 142.6, 121.2, 110.3, 107.3, 99.2, 92.0, 89.5, 86.7, 85.9, 77.0, 73.0, 72.2, 67.9, 67.8, 66.2, 66.1, 44.1; HRMS (FAB, DMSO/*p*-nitrobenzyl alcohol) calcd for $C_{21}H_{29}N_9O_{13}P_2$ (+1.0078) 678.1438, found 678.1429. Anal. Calcd for $C_{21}H_{29}N_9O_{13}P_2\cdot H_2O$: C, 36.27, H, 4.49, N, 18.13. Found: C, 36.09; H, 4.57; N, 18.23.

5′-*O*-Phosphoryl-2′-deoxycytidylyl(3′-5′)-[2′(3′)-*O*-(glycyl)adenosine] (25) Prepared from *N*-Boc-glycine (20). Addition of the protected dinucleotide (**14a**; 0.005 mmol) and 1-methylimidazole (0.015 mmol) dissolved in dry THF (100 μL) to a solution containing *N*-(*tert*-butyloxycarbonyl)glycine (**20**;

0.030 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (0.030 mmol), 1-hydroxybenzotriazole (0.300 mmol), and dry THF (100 μL) gave the protected acylated dinucleotide, which was subsequently treated with a freshly prepared solution of 1,1,3,3-tetramethylguanidine (190 mg, 0.38 M) and 4-nitrobenzaldoxime (316 mg, 0.33 M) in dry CH_3CN (5.0 mL) for 3 h at room temperature. The reaction product was precipitated with Et_2O (100 mL), isolated by centrifugation, and washed with Et_2O (2 × 100 mL). The pellet was dissolved in anhydrous TFA (0.3 mL) for 2 min at room temperature, followed by removal of the TFA by a stream of dry argon. the residue was dissolved in EtOH (2 mL) and then concentrated in vacuo at 25 °C, followed by three additional cycles of this step (HPLC: RP C4 prep column; run 20–50% CH_3CN linear gradient over 30 min). Buffer was 5 mM ammonium formate (pH 4.5). Collected eluents were lyophilized, dissolved in 0.1 N formic acid, and lyophilized again, followed by addition of H_2O and lyophilization to a white solid (0.75 μmol, 15%), which upon spectral analysis, was identical with **25** prepared from *N*-Fmoc-glycine (**19**).

5′-*O*-Phosphoryl-2′-deoxycytidylyl(3′-5′)-[2′(3′)-*O*-(glycyl)adenosine] (25) Prepared from *N*-Cbz-glycine (21). Addition of the protected dinucleotide (**14a**; 0.005 mmol) and 1-methylimidazole (0.015 mmol) dissolved in dry THF (100 μL) to a solution containing *N*-(benzyloxycarbonyl)glycine (**21**; 0.030 mmol), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (0.030 mmol), 1-hydroxybenzotriazole (0.300 mmol), and dry THF (100 μL) gave the protected acylated dinucleotide, which was subsequently treated with a freshly prepared solution of 1,1,3,3-tetramethylguanidine (190 mg, 0.38 M) and 4-nitrobenzaldoxime (316 mg, 0.33 M) in dry CH_3CN (5.0 mL) for 3 h at room temperature. The reaction product was precipitated with Et_2O (100 mL), isolated by centrifugation, and washed with Et_2O (2 × 100 mL). The pellet was dissolved in 1:1 EtOAc/EtOH (500 μL), followed by addition of 10% palladium on carbon (7 mg) and cyclohexadiene (100 μL) to the solution. The mixture was stirred for 1 h and subsequently filtered and concentrated in vacuo (HPLC: RP C4 prep column; run 20–50% CH_3CN linear gradient over 30 min). Buffer was 5 mM ammonium formate (pH 4.5). Collected eluents were lyophilized; dissolved in 0.1 N formic acid, and lyophilized again, followed by addition of H_2O and lyophilization to a white solid (1.7 μmol, 34%), which upon spectral analysis was identical with **25** prepared from *N*-Fmoc-glycine (**19**).

Preparation of $tRNA^{Gly}_{CUA}$-C_{OH} (1). Method A: Run-Off Transcription from Linearized Plasmid Template. Ten synthetic oligodeoxyribonucleotides were used to construct an insert containing both a T7 promoter site of $tRNA^{Gly}_{CUA}$-C_{OH} template. The oligodeoxyribonucleotides (200 pmol each) were combined and phosphorylated in a 25-μL reaction containing 50 mM Tris·HCl, pH 8.5/10 mM $MgCl_2$/5 mM dithiothreitol/200 μM ATP, with 20 units of T4 polynucleotide kinase. The mixture was incubated for 30 min at 37 °C, heated for 10 min at 65 °C, and allowed to cool slowly to room temperature. A vector was then prepared by digestion of plasmid pIBI30 (4 pmol) in a 10-μL reaction containing 50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/50 mM NaCl, with 10 units each of *Eco*RV and *Pst*I. The mixture was incubated for 30 min at 37 °C and the reaction terminated by heating the sample at 65 °C for 10 min. The termini of the vector were dephosphorylated by dilution of the above reaction mixture to 50 μL with 0.5 m Tris·HCl, pH 9.0/10 mM $MgCl_2$/1 mM $ZnCl_2$/10 mM spermidine, containing 10 units of calf intestinal alkaline phosphatase. Incubation for 30 min at 37 °C, followed by electrophoresis in a 1.5% agarose gel and elution using "Geneclean", yielded the purified vector. Subsequent ligation of the treated oligodeoxyribonucleotides above (1.0 pmol) and the purified vector (0.2 pmol) was carried out in a 10-μL reaction containing 50 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/20 mM dithiothreitol/50 mM NaCl/1 mM ATP, with 10 units of T4 DNA ligase at 15 °C for 12 h. The ligation mixture was used directly for transformation of *Escherichia coli* TB-1 cells. Individual colonies were used to inoculate liquid medium and grown to saturation to shake culture at 37 °C overnight. The plasmid template was purified by alkaline lysis,[33] screened for the insert using *Pst*I/*Xho*I restriction analysis and sequenced with the T7 primer using the dideoxy chain termination method of Sanger et al.[34] Template DNA was linearized by *Pst*I digestion of the plasmid (1.0 mg) in ten 50-μL reactions containing 50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/50 mM NaCl, with 100 units of *Pst*I in each reaction mixture. The mixtures were incubated for 1.5 h at 37 °C, purified by extraction once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and once with $CHCl_3$/isoamyl alcohol (24:1), and precipitated with 2.5 volumes of EtOH. RNA transcripts[19] were produced by using the linearized template (50 μg) in a 0.5-mL reaction containing 40 mM Tris·HCl, pH 8.1/20 mM $MgCl_2$/1 mM spermidine/5 mM dithiothreitol/25 μg bovine serum albumin/5 mM NTP each, with 2 units of inorganic pyrophosphatase and 12000 units of T7 RNA polymerase. The mixture was incubated for 11 h at 37 °C followed by addition of EDTA (10 μL of a 2.5 M in H_2O), extraction once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and once with $CHCl_3$/isoamyl alcohol (24:1), and precipitation with 2.5 volumes of EtOH to yield 3.5 mg of nearly pure transcript. The transcripts were purified to one-nucleotide resolution to remove minor impurities by electrophoresis through a 20% acrylamide/7 M urea gel, visualized by UV shadowing and excised, and the gel was eluted twice in 50 mM KOAc/200 mM KCl/20 mM EDTA, at 4 °C for 24 h. The eluents were combined and the RNA EtOH-precipitated (2.5 volumes), washed and resuspended in 70% EtOH, and stored in 100-μL aliquots (1 μg RNA/1 μL) at −20 °C.

Method B: Run-Off Transcription from Oligodeoxyribonucleotide Template. Two synthetic oligodeoxyribonucleotides were used to construct a DNA template containing a double-stranded promoter region and a long 5′-overhang corresponding to the transcribed sequence.[9,20] The oligodeoxyribonucleotides (0.25 μM each) were annealed and RNA transcripts were produced in a 0.5-mL reaction containing 40 mM Tris·HCl, pH 8.1/20 mM $MgCl_2$/1 mM spermidine/5 mM dithiothreitol/25 μg bovine serum albumin/5 mM NTP each, with 2 units of inorganic pyrophosphatase and 12000 units of T7 RNA polymerase. The mixture was incubated for 11 h at 37 °C followed by addition of EDTA (10 μL of a 2.5 M aqueous solution), extracted once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and once with $CHCl_3$/isoamyl alcohol (24:1), and precipitated with 2.5 volumes of EtOH to yield 2.2 mg of nearly pure transcript. The transcripts were purified to one-nucleotide resolution to remove minor impurities by electrophoresis through a 20% acrylamide/7 M urea gel, visualized by UV shadowing, and excised, and the gel was eluted twice in 50 mM KOAc/200 mM KCl/20 mM EDTA, at 4 °C for 24 h. The eluents were combined and the RNA EtOH-precipitated (2.5 volumes), washed with 70% EtOH, resuspended in 70% EtOH, and stored in 100-μL aliquots (1 μg RNA/1 μL) at −20 °C.

Method C: Representative Procedure for Construction of $tRNA^{Gly}$-C_{OH}s. Preparation of $tRNA^{Gly}_{CUA}$-C_{OH} through T4 RNA Ligase-Mediated Condensation of 5′-Half $tRNA^{Gly}_{CUA}$-A_{OH} (Residues 1–37) and 3′-Half $tRNA^{Gly}$-C_{OH} (Residues 38–74). The HPLC-purified 3′-half $tRNA^{Gly}$-C_{OH} (residues 38–74) from above was concentrated by lyophilization and dissolved in 500 μL of distilled H_2O/HOAc (1:4) to remove the 5′-DMT group. The solution was allowed to stand at room temperature for 20 min, concentrated by lyophilization, and dissolved to a final concentration of 2.3 μM in 50 mM Na^+-Hepes, pH 6.9 at 37 °C/20 mM $MgCl_2$/3.3 mM dithiothreitol/bovine serum albumin (10 μg/mL). The 5′-half $tRNA^{Gly}_{CUA}$-A_{OH} (2.3 μM final concentration) and the oligoribonucleotides were heated to 65 °C for 5 min and allowed to anneal by slow cooling to 15 °C over a 2-h period. The two half-molecules were joined as previously described.[24] T4 RNA ligase and ATP were added to a final concentration of 44 μg/mL and 10 μM, respectively. Incubation for 5 h at 15 °C was followed by precipitation with 2.5 volumes of EtOH at −20 °C. Removal of the 5′-terminal DMT groups was as described above, followed by suspension of the oligoribonucleotides in TEAA (100 mM, pH 7.0) and injection onto a Vydac C-4 column equilibrated in TEAA (100 mM, pH 7.0) in H_2O/CH_3CN (9:1). Gradient elution with CH_3CN (10–20% CH_3CN in 40 min) in TEAA (100 mM, pH 7.0) followed by ultraviolet detection at 254 nm resolved the desired

(33) Birnboim, H. C. *Methods Enzymol.* **1983**, *100*, 243–255.

(34) Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463–5467.

peak. Both $tRNA^{Gly}_{UCA}$-C_{OH} and $tRNA^{Gly}_{UUA}$-C_{OH} were produced in analogous fashion.[26]

Method D: Total Chemical Synthesis and Purification of $tRNA^{Gly}_{CUA}$-C_{OH}. The 5′-terminal DMT oligoribonucleotide was synthesized and purified as described above. The HPLC-purified oligoribonucleotide was concentrated by lyophilization and dissolved in 500 μL of distilled H_2O/HOAc (1:4) to remove the 5′-DMT group. The solution was allowed to stand at room temperature for 20 min, concentrated by lyophilization, and dissolved in TEAA (100 mM, pH 7.0) and injected onto a Vydac C-4 column equilibrated in TEAA (100 mM, pH 7.0) in H_2O/CH_3CN (9:1). Gradient elution with CH_3CN (10–20% CH_3CN in 40 min) followed by ultraviolet detection at 254 nm resolved the desired peak.

General Preparation of Chemically Misacylated tRNAs. Construction of L-3-Iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA (3). Synthesis of L-3-iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA (3) was accomplished by ligation of $tRNA^{Gly}_{CUA}$-C_{OH} (1; 20 μg) with 10 μg of 5′-*O*-phosphoryl-2′-deoxycytidylyl(3′-5′)-[2′-(3′)-*O*-(L-3-iodotyrosyl)adenosine] (2) in a 40-μL reaction containing 55 mM Na^+-Hepes, pH 7.5/15 mM $MgCl_2$/250 μM ATP/8 μg bovine serum albumin/10% DMSO, with 15 units of T4 RNA ligase. The mixture was incubated for 10 min at 37 °C and the reaction terminated by addition of 100 μL of a 250 mM NaOAc, pH 4.5/5 M NaCl/50 mM $MgCl_2$ buffer, followed by extraction once with phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and once with $CHCl_3$/isoamyl alcohol (24:1) and precipitation with 2.5 volumes of EtOH. The precipitate was dissolved in 10 mM NaOAc, pH 4.5/1 mM EDTA/100 mM NaCl (50 μL) and filtered through a Sephadex G-25 Select D column equilibrated with the same buffer. The initial isotope peak was immediately absorbed onto a BD-cellulose column (2 × 0.5 cm) that had been equilibrated at 4 °C with 50 mM NaOAc, pH 4.5/10 mM $MgCl_2$/1.0 M NaCl. Unreacted $tRNA^{Gly}_{CUA}$-C_{OH} (1) was removed by eluting the column with 10 mL of the buffer. The column was then eluted with the buffer containing 25% EtOH to effect elution of L-3-iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA (3).[4] The appropriate fractions were combined and the RNA EtOH-precipitated (2.5 volumes), washed with 70% EtOH, suspended in H_2O, lyophilized, and stored under argon at −80 °C as a fluffy white powder (7 μg). D-Phenylalanyl-$tRNA^{Gly}_{CUA}$-dCA (8 μg), *N*-methyl-L-phenylalanyl-$tRNA^{Gly}_{CUA}$-dCA (10 μg), L-phenyllactyl-$tRNA^{Gly}_{CUA}$-dCA (7 μg), and glycyl-$tRNA^{Gly}_{CUA}$-dCA (6 μg) wee produced in an analogous fashion.[25]

Rapid Screening of Unlabeled Non-Natural Residues. Translations with rabbit reticulocyte lysate were performed with a slightly modified procedure from manufacturer's instructions (Amersham). Magnesium ion and mRNA concentrations were determined as per instructions. A typical reaction (10 μL) contained lysate (9 μL), L-[^{35}S]methionine (15 μCi), L-[3,4,5-^{3}H]leucine (5 μCi), mRNA (2.0 μM), and chemically misacylated tRNA (20 μM). The mixture was incubated for 1 h at 30 °C followed by addition of synthetically prepared polypeptide standards, corresponding to the expected 8-mer and 16-mer products from the translation (10 μL of a 0.5 mM solution in 77% formic acid). The solution was immediately quenched with H_2O (1.0 mL), and the resulting precipitate was centrifuged and the solvent decanted. A cycle of resuspension in 77% formic acid (10 μL) followed by precipitation with H_2O (1.0 mL) was repeated twice. The resulting precipitate was dissolved in 77% formic acid (100 μL) followed by radioisotope detection by scintillation counting.

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Conformational Analysis of Ring A and Total Assignment of 19-Functionalized 4-En-3-one Steroids. Applicability of 2D NOE as a Crucial Technique

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The conformational analysis of ring A of 4-en-3-one steroids with three kinds of functionalizations viz. the 19-hydroxy, 19-oxo, and the 6β,19-epoxy groups, is discussed using a simple protocol—COSY 90 + NOESY + HETCOR. The 2D NOE technique is found to be of immense importance in deducing the conformational preferences of these steroids. The conformations are derived by connectivity pattern analysis and independently by torsional angle calculations employing the Karplus equation. While steroids 1 and 2 show "normal" 1α,2β half-chair conformation, steroid 3 exhibits ring A in the 1β,2α "inverted" conformation. The conformational analysis highlights aspects like interactions of the 1,3-diaxial type and the effect of tilting the 19-carbon toward ring A or ring B. A comment is made to that effect.

With the advent of techniques of modern, multiple-pulse experiments in NMR methodology, the study of NMR of steroids has seen an explosive activity.[1] The olden-days analysis of steroids structure and conformation by the resonances of the 18-H_3 and 19-H_3 groups,[2] coupled with the analysis of deshielded region of the spectrum, has now been replaced by 2D NMR methodology and computer-aided simulation.[1a,d] While the horde of pulse experiments published to date[3] reveal that NMR techniques can provide solutions with varying degrees of rigorous execution, the effort should be to extract maximum information from minimum experimentation without jeopardizing authen-

(1) (a) Schneider, H.-J.; Buccheit, U.; Becker, N.; Schmidt, G.; Siehl, U. *J. Am. Chem. Soc.* **1985**, *107*, 7027 and references cited therein. (b) Marat, K.; Templeton, J. F.; Gupta, R. K.; Shashikumar, V. P. *Magn. Reson. Chem.* **1987**, *25*, 730. (c) Wong, T. C.; Guo, W.; Bohl, M.; Hubner, M.; Luck, G.; Steiger, T.; Reck, G. *J. Chem. Soc., Perkin Trans. 2* **1988**, 765. (d) Marat, K.; Templeton, J. F.; Shashikumar, V. P. *Magn. Reson. Chem.* **1087**, *25*, 25.

(2) See, e.g.; Bhacca, N. S.; Williams, D. H. *Applications of NMR Spectroscopy in Organic Chemistry*; Holden-Day: San Francisco, London, Amsterdam, 1964.

(3) Kessler, H.; Gehrke, M.; Griesinger, C. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 490 and references cited therein.

0022-3263/91/1956-4625$02.50/0