mmHg); ^{1}H NMR ($CDCl_3$) δ −0.05 (s, 9), 0.74 (s, 9), 0.74–2.53 (br m, 8), 4.12 (br s, 1); ^{13}C NMR ($CDCl_3$) δ −0.8 (CH_3Si), 21.8, 25.2, 32.7, 32.8, 35.3, 40.8 ($SiCHCH_2CH_2CHCH_2$), 27.5 ($(CH_3)_3C$), 68.8 (CHO-H).

***r*-5-*tert*-Butyl-*c*-2-(trimethylsilyl)cyclohex-*t*-yl Trifluoroacetate (6-O(CO)CF$_3$).** The ester was synthesized by the same method as that used for the trifluoroacetate **3** from the corresponding alcohol (**6**-OH, 0.228 g, 1 mmol). An elimination reaction to form 4-*tert*-butylcyclohexene occurred quickly, but the ^{1}H NMR spectrum prior to decomposition revealed a broad signal at δ 5.36 indicative of the trifluoroacetate.

***r*-5-*tert*-Butyl-*c*-2-(trimethylsilyl)cyclohex-*t*-yl 3,5-Dinitrobenzoate (6-ODNB).** 3,5-Dinitrobenzoyl chloride (0.282 g, 1.2 mmol) was dissolved in 5 mL of pyridine, and the solution was cooled with an ice bath. A solution of the alcohol **6**-OH (0.228 g, 1.0 mmol) in 1 mL of pyridine was added dropwise with stirring. The mixture was then stirred for 4 h at 0 °C. Water (5 mL) was added and the mixture was extracted with diethyl ether (4 × 15 mL). The combined organics were dried over $MgSO_4$ and filtered. Evaporation of the solvent gave a solid that was recrystallized from absolute ethanol: 0.22 g, 60%; mp 96–98 °C (dec); ^{1}H NMR ($CDCl_3$) δ 0.2 (s, 9), 1.0 (s, 9), 0.7–2.0 (m, 7), 5.65 (m, 1), 9.1–9.3 (m, 3, arom); IR (KBr) 1713 cm^{-1} (C=O). A sample was recrystallized from hexane for elemental analysis. Anal. Calcd for $C_{20}H_{30}N_2O_6Si$: C, 56.87; H, 7.11; N, 6.64; Si, 6.65. Found: C, 56.65; H, 7.08; N, 6.46; Si, 6.22.

Solvents. Ethanol was distilled from magnesium ethoxide as described by Lund and Bjerrum.[27] Commercial 2,2,2-trifluoroethanol was distilled from aqueous K_2CO_3, dried with P_2O_5, and fractionally distilled through a 10-in. vacuum-jacketed column packed with glass helices, bp 73.5–74.5 °C. Ethanol mixtures are quoted in volume percentage and trifluoroethanol mixtures in weight percentage.

Kinetic Methods. Rates in aqueous solvents were determined conductometrically with an Industrial Instruments Model RC 16B2 conductivity bridge. The conductivity cell (Industrial Instruments) had black Pt electrodes, cell constant 0.42 cm^{-1}, and a volume of approximately 35 mL. In a typical experiment, enough substrate to make a solution approximately 10^{-3} M was added to the cell, which contained almost 20 mL of solvent. The cell was then stoppered and equilibrated in a constant-temperature bath (Haake Model NB22) for at least 5 min with stirring. Solvolyses were followed by taking at least 10 readings approximately equally spaced in conductance over 1-3 half-lives. The raw conductance data were then fitted to a first-order rate equation by means of a least-squares computer program. The precision of the fit to first-order kinetics was satisfactory over up to 3 half-lives in the aqueous ethanol and aqueous trifluoroethanol solvents.

Product Studies. A 0.2–0.5 M solution of the substrate in the desired solvent (1 mL) was prepared in a Pyrex tube (0.8 mm in diameter). The tubes were sealed and placed in a constant-temperature bath at 90 °C. The dinitrobenzoates were mostly insoluble at room temperature but totally soluble at 90 °C. After 5 days, the tubes were cooled in an ice bath and opened. A filtration was then performed to remove any solid product formed. The mixtures were then analyzed with GC–MS: 20-m column with 3% Carbowax on Chromosorb W, FID detector, gas flow 10 mL/min. Under these conditions, all the components were well separated and the product alkene and alcohol had differences in retention times as much as 6 min. Cyclohexanol is characterized by peaks at *m/e* 100, 82, 67, and 57, cyclohexene by peaks at *m/e* 82, 67, and 54, and 4-*tert*-butylcyclohexene by peaks at *m/e* 138, 82, 67, and 57.

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Automated Chemical Synthesis of Long Oligoribonucleotides Using 2′-O-Silylated Ribonucleoside 3′-*O*-Phosphoramidites on a Controlled-Pore Glass Support: Synthesis of a 43-Nucleotide Sequence Similar to the 3′-Half Molecule of an *Escherichia coli* Formylmethionine tRNA[1]

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Abstract: The synthesis of silyl-protected ribonucleoside 3′-*O*-phosphoramidites and the preparation of the controlled-pore glass supports needed for the solid-phase chemical synthesis of oligoribonucleotides are described. These reagents were evaluated in the synthesis of a series of oligomers consisting of the pentadecameric homopolymers of adenosine, cytidine, guanosine, uridine, and various sequences of mixed-base composition. Different reactivities were observed for the morpholino- and (diisopropylamino)phosphoramidites under actual synthesis conditions, and these were attributed to the relative ease of their activation by tetrazole as determined by ^{31}P NMR. A comparison of the methyl- and cyanoethyl-protected phosphoramidites indicated that, although the cyanoethyl group was easier to remove, there was no significant difference between the two in terms of the quality of the final sequence. The purity of the 3′-phosphoramidites is easily determined by NMR. A full protocol for the deprotection, handling, and purification of synthetic oligoribonucleotides is also described. The results of these studies were applied to the successful chemical synthesis of the 43-mer 5′-CAU AAC CCG AAG AUC GUC GGU UCA AAU CCG GCC CCC GCA ACC A-3′, corresponding to the 3′-terminus of a formylmethionine (fMet) tRNA of *Escherichia coli*, in which the modified bases have been substituted by their unmodified parent nucleosides. The 43-mer was fully characterized by polyacrylamide gel electrophoresis, enzymatic RNA sequencing, HPLC analysis of an enzymatic digest, and terminal nucleotide analysis, both 5′ and 3′, using enzymatic digestion followed by two-dimensional chromatography. The effectiveness of alkylsilyl ethers for the protection of the 2′-hydroxyl of the ribose ring, when used in conjunction with the phosphoramidite method for the formation of the phosphotriester linkages in RNA synthesis, is clearly established.

Intense interest in the development of facile, rapid, and high-yield RNA synthesis strategies has been fueled by its unique and sometimes unexpected biological activities, i.e., self-splicing RNA[2] antisense RNA in the translational control of the expression of genes,[3] the formation of lariats during the splicing of eukaryotic mRNA,[4] recombinant RNA technology,[5] and involvement of a

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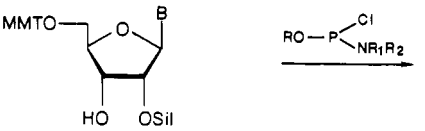
chloroplast glutamate tRNA in chlorophyll biosynthesis,[6] in addition to the classical roles of messenger RNA (mRNA) and transfer RNA (tRNA) in transcription and translation of the genetic code.

A major problem in the chemical synthesis of oligoribonuclides is the presence of the 2′-hydroxyl group in the ribose ring, which requires selective protection and may sterically hinder the 3′-position during the formation of the internucleotide linkage. The selection of a suitable protecting group for the 2′-OH is a nontrivial matter, as it must be stable under the conditions of coupling, deprotection of the 5′-protecting group (to allow for chain extension), oxidation in the case of phosphite triester chemistries, capping following the coupling, deprotection of the exocyclic amino-protecting groups, deprotection of the phosphate-protecting groups, and in the case of polymer-supported syntheses, cleavage from the solid support. Finally, it must be removed under very mild conditions so as to prevent the attack of the released 2′-hydroxy function on the adjacent phosphodiester linkage. For these reasons, there has been a lag in the development of strategies for the synthesis of RNA compared with those already well established in the synthesis of DNA.

The use of the *tert*-butyldimethylsilyl (TBDMS) or triisopropylsilyl (TIPS) ether as a 2′-hydroxyl protecting group[7] has greatly facilitated the synthesis of oligoribonucleotides. Both solution-[8] and solid-phase strategies,[9] when used in conjunction with either the chlorophosphite[7a,8] or phosphoramidite[10] coupling procedures, are effective for the synthesis of short fragments. The TBDMS and TIPS ethers are sufficiently stable to both acidic and basic conditions yet are easily removed with tetrabutylammonium fluoride in THF.

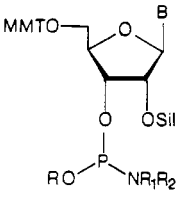
Other workers have employed acetyl 2′-OH protecting groups such as the tetrahydropyranyl (THP),[11] 4-methoxytetrahydropyranyl (MTHP),[12] and 3-methoxy-1,5-dicarbomethoxypentanyl (MDMP)[13] with some success. All of these groups are, however, acid sensitive, which renders them incompatible with acid-labile 5′-protecting groups such as substituted trityl ethers.[14] [monomethoxytrityl (MMT) or dimethoxytrityl (DMT)]. The trityl groups have become standards in oligonucleotide synthesis because of the absorption in the visible spectrum of the released trityl cations under acidic conditions, which allows for the monitoring of coupling efficiencies during the synthesis.[14] Reese and Skone,[15] as well as Gait et al.[16] recently demonstrated the incompatibility of the MTHP group with either 9-phenylxanthen-9-yl-(pixyl or Px) or DMT-protected ribonucleosides due to the undesired removal of the MTHP moiety, and subsequent migration or cleavage of the phosphotriester linkages, during the acidic removal of the 5′-protecting group. Other workers have investigated the use of substituted aryl ethers such as *o*-nitrobenzyl[17] and *p*-methoxybenzyl[18] as 2′-OH protecting groups with limited success due to difficulties encountered in the removal of the protecting group at the termination of the synthesis. The use of 2′-*O*-THP-protected ribonucleosides for the synthesis of several ribooctamers on a solid support has been recently demonstrated by Caruthers.[19]

Scheme I. Synthesis of Ribonucleoside Phosphoramidites



1a: B = N^6-benzoyladenine; Sil = TBDMS*
b: B = N^4-benzoylcytosine; Sil = TBDMS
c: B = N^2-benzoylguanine; Sil = TIPS**
d: B = uracil; Sil = TBDMS

*tert-butyldimethylsilyl, **triisopropylsilyl



2a-d: R_1R_2 =  R = CH_3; B = uracil; Sil = TBDMS

3a-d: $R_1 = R_2$ = *i*-Pr; R = CH_3; B = uracil; Sil = TBDMS
e: R = CH_2CH_2CN; B = uracil; Sil = TBDMS

We have introduced ribonucleoside 5′-*O*-MMT-2′-*O*-TBDMS-3′-*O*-phosphoramidites (Scheme I) for the synthesis of short- to medium-length ribonucleotides.[10] In this paper, we describe the full details of the synthesis of long oligoribonucleotides, the preparation of the nucleoside phosphoramidites, and in the case of guanosine, the preparation of the silylated precursor. We show the 3′-phosphoramidites are obtained pure from the 2′-silylated ribonucleosides, thus demonstrating that silyl groups do not migrate under the phosphorylation conditions. The 3′-phosphoramidites are readily distinguished from the 2′-phosphoramidites by NMR. We have determined the optimal conditions (type of phosphoramidite and synthesis cycle) required to achieve high coupling yields. We have also compared the use of cyanoethyl[20a-c] and methyl[20d,e] phosphate protecting groups in terms of the overall integrity of the final nucleotide sequence. Conditions under which the protected oligomer can be deprotected and purified without chemical or enzymatic degradation of the final product have been determined. The results of this study have been used successfully to synthesize a 43-mer similar in sequence to the 3′-half of a formylmethionine (fMet)tRNA molecule from *Escherichia coli*.

Results and Discussion

Synthesis of Protected Ribonucleoside Phosphoramidites. For the preparation of the deoxynucleoside phosphoramidites a large number of alkylamines have been used to constitute the phos-

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Table I. NMR Characteristics of Ribonucleoside Phosphoramidites

	^{1}H NMR chemical shifts, δ									^{31}P NMR
compd	$H_{1'}$ ($J_{1'-2'}$)	$H_{2'}$ (m)	$H_{3'}$ (m)	$POCH_3$ (J_{P-H})	OCH_3 MMT (s)	H_6 or H_8	*t*-Bu (s)	MeSi (s)	SiMe (s)	(s), δ
2a	6.06 (d, 5.8)	5.07	4.48	3.46 (d, 12.3)	3.77	8.70 (s)	0.77	−0.04	−0.20	146.26
	6.04 (d, 5.8)	5.00	4.34	3.28 (d, 12.3)		8.67 (s)	0.75	−0.05	−0.23	145.92
2b	5.80 (s, 0)	4.32	4.33	3.36 (d, 12.7)	3.81	8.55 (d)	0.90	0.25	0.13	147.43
	5.76 (s, 0)			3.23 (d, 12.7)		8.50 (d)	0.90	0.24	0.13	143.77
2c[b]	5.76 (d, 6.1)	5.28	4.44	3.44 (d, 12.9)	3.61	7.72 (s)	(0.85–0.95)			147.99
	5.68 (d, 6.1)	5.24	4.39	3.06 (d, 12.9)		7.76 (s)				146.75
2d	5.87 (d, 2.9)	4.40	4.28	3.67 (d, 12.3)	3.78	8.05 (d)	0.88	0.13	0.13	146.84
	5.82 (d, 2.9)			3.26 (d, 12.3)			0.88	0.10	0.10	144.05
3a	6.14 (d, 4.0)	5.04	4.02	3.41 (d, 13.1)	3.76	8.69 (s)	0.75	−0.02	−0.24	151.30
	6.00 (d, 4.0)	4.98		3.20 (d, 13.1)		8.65 (s)	0.74	−0.04	−0.20	149.48
3b	5.89 (d, 2.0)	4.22	4.25	3.31 (d, 13.3)	3.81	8.55 (d)	0.91	0.25	0.15	149.97
	5.86 (d, 2.0)			3.23 (d, 13.3)		8.45 (d)	0.89	0.24	0.13	148.70
3c[b]	5.79 (d, 7.0)	5.27	4.40	3.38 (d, 13.4)	3.61	7.78 (s)	(0.84–0.92)			153.71
	5.61 (d, 7.0)	5.22	4.34	2.91 (d, 13.4)	3.60					148.96
3d	5.91 (d, 3.0)	4.35	4.22	3.39 (d, 13.2)	3.78	7.94 (d)	0.89	0.13	0.13	150.34
	5.78 (d, 3.0)			3.24 (d, 13.2)		8.03 (d)	0.87	0.13	0.10	150.29
3e[c]	5.96 (d, 5.1)	4.35	4.29	3.53 (m, NR)[d]	3.778	7.91 (d)	0.88	0.11	0.11	150.50
	5.87 (d, 3.4)				3.782	7.98 (d)	0.87	0.09	0.09	150.07

[a] *J* values are quoted in hertz. ^{1}H NMR spectra are referenced to internal $CHCl_3$ (δ 7.24 downfield from TMS). ^{31}P NMR spectra are referenced to external H_3PO_4. All samples were dissolved in $CDCl_3$. [b] Contains triisopropylsilyl protection and the range of the 21-proton multiplet is given. [c] Contains cyanoethyl phosphate protection, the δ given is that of the $POCH_2$ protons. For CH_2CN δ 2.74 (t, J_{H-H} = 6.0). [d] NR = not resolved.

Table II. Physical, Chromatographic, and UV Properties of Ribonucleoside Phosphoramidites

compd	isolated yield, %	chromatogr solvent[a]	TLC solvent[a]	R_f	mp, °C	UV (max; min),[b] nm
2a	75	40/58/2	40/58/2, 3×	0.47	89–91	278; 254
2b	77	40/58/2	50/43/7	0.62	105–107	304, 288; 261, 254
2c	65	40/58/2	40/58/2, 3×	0.49	106–110	288; 262
2d	85	50/44/6	50/43/7	0.50	92	263; 246
3a	96	40/58/2	A	0.64, 0.54	78–80	279; 255
3b	90	40/58/2	A	0.50	78–80	289; 246
3c	83	55/35/10	A	0.50, 0.42	92–94	285; 272
3d	99	50/46/4	A	0.64	69–71	262; 247
3e[c]	98		A	0.46	76–79	259; 245

[a] Ratio of CH_2Cl_2/hexanes/NEt_3; solvent A, $Et_2O/CHCl_3$ (1/1). [b] UV spectra were recorded in 95% EtOH. [c] The cyanoethyl-protected derivative **3e** was not purified by chromatography.

phorus–nitrogen bond.[21-24] The two most widely used are the morpholino and the diisopropylamino groups. The former was found to be exceptionally stable, while the latter appears to give higher stepwise coupling yields.[21] We first compared these two amidites by using methyl phosphate protection.

The preparation of the protected ribonucleosides **1a,b,d** has been previously described.[25] In the case of **1c**, N^2-benzoyl-5′-*O*-(monomethoxytrityl)guanosine[25] (1 equiv) was treated with triisopropylsilyl chloride[7c] (2 equiv) and imidazole (2.7 equiv) in anhydrous DMF overnight. Following workup, the resulting yellow gum was dissolved in a minimum of CH_2Cl_2 and the solution added dropwise to a 78/22 mixture of ether/hexanes. The desired 2′-O-silylated compound **1c** selectively precipitated out in 62% overall yield, thereby obviating the need for chromatographic separation of the silylated isomers. The isomeric purity of the 2′-*O*-TIPS-G derivative **1c** was confirmed by ^{1}H and ^{29}Si NMR spectroscopy.[26] This methodology of selective precipitation works only for compound **1c**; attempts to apply it to TBDMS-silylated guanosine were not successful.

The general procedure for the preparation of the protected 2′-O-silylated nucleoside 3′-*O*-phosphoramidites is shown in Scheme I. In the case of the ribonucleoside methyl-*N*-morpholinophosphoramidites **2a–d**, a modification of the procedure developed by Dörper and Winnacker[23] for deoxyribonucleosides was used. The protected ribonucleosides **1a–d** were dissolved in dry distilled $CHCl_3$ to which was added diisopropylethylamine followed by the phosphitylating reagent morpholinomethylphosphonoamidic chloride[23] at room temperature. The reactions were complete after 2 h. In the case of the preparation of the ribonucleoside *N,N*-diisopropylmethylphosphoramidites **3a–d**, this procedure was unsatifactory. Therefore an alternate method was developed that involved the dropwise addition of a THF solution of the protected ribonucleoside (**1a–d**) to a stirred THF solution of diisopropylethylamine, (*N,N*-diisopropylamino)methylphosphonamidic chloride[22] [or (*N,N*-diisopropylamino)(cyanoethyl)phosphonamidic chloride[32a] in the case of **3e** vide infra], and a catalytic amount of DMAP at room temperature. As in the former case, the reactions required 2–3 h to go to completion. In both preparations, the workup was a simple extraction between ethyl acetate and saturated brine. The crude products were purified by silica gel chromatography using CH_2Cl_2/hexane/NEt_3 mixtures as eluent. The spectroscopic and physical properties of **2a–d** and **3a–d** are shown in Tables I and II, respectively. The

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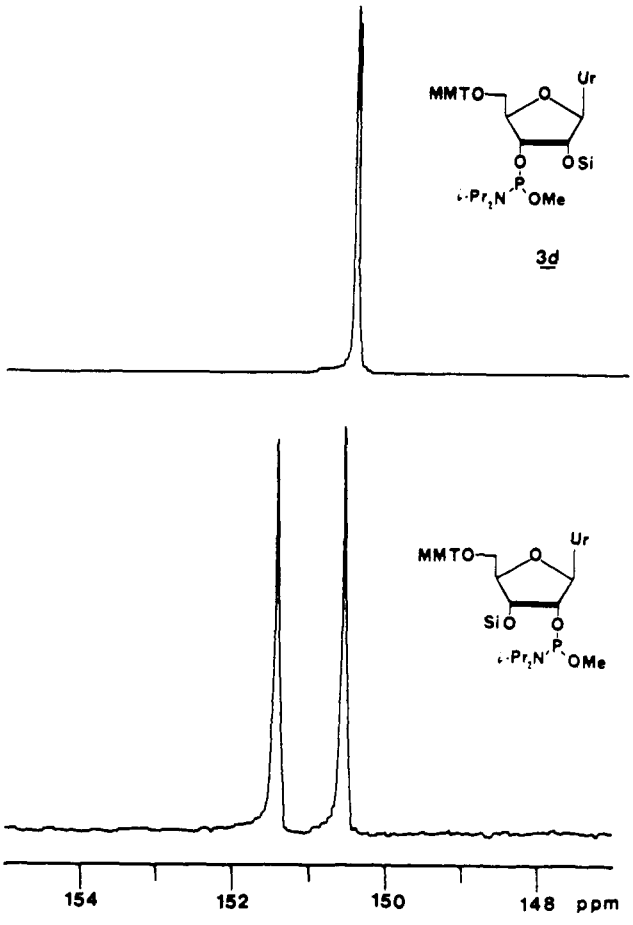
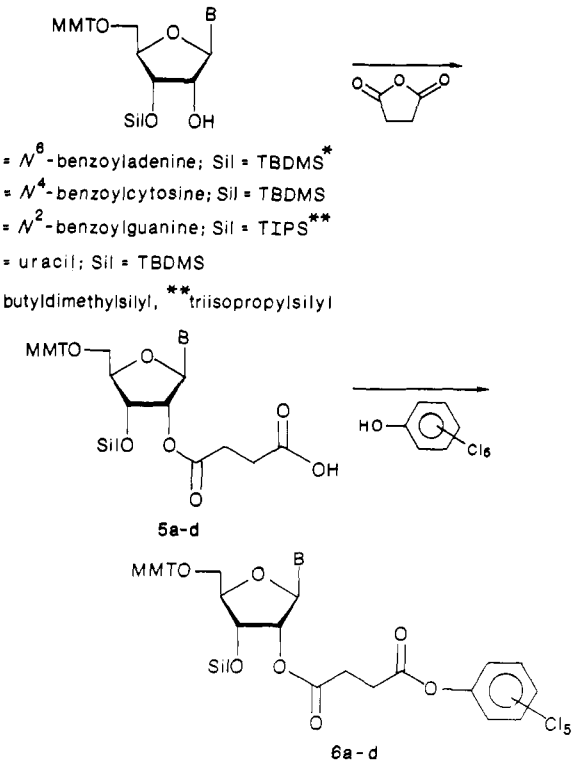


Figure 1. ^{31}P NMR of uridine 2′- and 3′-phosphoramidites. The 3′-phosphoramidite (**3d**) is after one passage through a silica gel column; the 2′-phosphoramidite is the crude reaction product before chromatography.

NMR data clearly show that there is, as expected, only one pair of diastereomeric signals in both the phosphorus and proton spectra for all of the ribonucleoside phosphoramidites, thereby (see below) establishing the isomeric purity of these compounds. In the case of the purine ribonucleoside phosphoramidites **3a** and **3c**, the individual diasteromers could be separated during chromatography, with the faster moving products having the downfield ^{31}P NMR signal. The separation of these diastereomers was, however, not necessary since treatment with tetrazole results in complete epimerization at the phosphorus center.[27]

Some authors[19,28] have implied that the silyl groups described here may not be stable to the conditions of phosphorylation. Reese,[28] Pfleiderer,[29] and ourselves[30] noted that the alkylsilyl groups do migrate in protic solvents such as methanol and in aqueous solutions such as pyridine/water. Obviously, no one would choose these solvents, since either alcohol or water would minimize the possibility of the desired phosphorylation of nucleosides. On the other hand we have previously shown, as has the Pfleiderer group, both using HPLC analysis, that the silyl groups are stable in dry solvents[30] including nonprotic bases such as anhydrous pyridine for at least 44 h.[29] Reese reported[28] that a 2′-silylated adenosine underwent isomerization in dry pyridine at 36 °C with a half-life of 19 h, conditions that hardly reflect against the use of the silyl group in nucleotide synthesis. In addition, we have clearly[8,9] demonstrated the use of 2′-silylated ribonucleosides for the synthesis of ribonucleotides that contain only 3′-5′ linkages. Similar proof has been demonstrated independently by Pfleiderer[29] and more recently by Garegg.[31] We include in this paper (Table I) the ^{1}H and ^{31}P NMR chemical shifts for the 3′-phosphoramidites **3**. The chemical shifts of the anomeric protons provide a clean diagnostic proof of isomer purity, since they are different than those of the 2′-phosphoramidites (within the limits of NMR detection, generally accepted as being to ~1%). The ^{31}P chemical shifts are also clearly distinguishable. We show in Figure 1 the ^{31}P NMR of the products obtained in the separate preparations of the isomeric uridine phosphoramidites. It can clearly be seen that each sample is isomerically pure, thus demonstrating unequivocally that the silyl groups are stable to the phosphorylation conditions. The results are similar for all the phosphoramidites described here. A full NMR (^{29}Si, ^{31}P, ^{13}C, and ^{1}H) analysis of silylated ribonucleosides, their phosphoramidites, and ribonucleotides prepared from them will be described elsewhere. As a final proof, the nucleotides prepared from compounds **3** have been completely characterized as described below.

Scheme II. Preparation of Ribonucleoside (Pentachlorophenyl)succinyl Esters



We have also evaluated the cyanoethyl protecting group as an alternative to the more commonly used methyl group. There have been a number of reports[32,33] suggesting that the use of cyano-protected deoxyribonucleoside phosphoramidites gave rise to equivalent or increased coupling efficiencies and better product yields upon deprotection, relative to the methyl protection, in the synthesis of deoxynucleotides. To evaluate this possibility in oligoribonucleotides synthesis, we prepared the cyanoethyl-protected phosphoramidite **3e** in a manner analogous to that of the methyl-protected (diisopropylamino)phosphoramidites, as is shown in Scheme I. The spectroscopic and physical properties of **3e** are shown in Tables I and II. All of the ribonucleoside phosphoramidite compounds are stable, like their deoxy counterparts, and may be stored for over 1 year if kept at <0 °C.

Preparation of Controlled Pore Glass Solid Supports. In order to attach the terminal-protected ribonucleoside to commercially available long-chain alkylamine CPG,[34] it was necessary to prepare the active 3′-*O*-TBDMS- (or TIPS-) ribonucleoside 2′-*O*-(pentachlorophenyl)succinates **6a–d** shown in Scheme II. The method used was a modification of a procedure originally developed by Itakura et al.[35] for thymidine derivatization on polyacrylmorpholide resin. The 3′-silylated nucleosides **4a–d** were used, since they are produced during the silylation of the ribonucleosides and are not

(33) Nielson, J.; Marugg, J. E.; Taagaard, M.; van Boom, J. H.; Dahl, O. *Recl: J. R. Neth. Chem. Soc.* **1986**, *105*, 33–34.
(34) Gough, G. R.; Brunden, M. J.; Gilham, P. T. *Tetrahedron Lett.* **1981**, *22*, 4177–4180.
(35) Miyoshi, K.; Miyake, T.; Hozumi, T.; Itakura, K. *Nucleic Acids Res.* **1980**, *8*, 5473–5489.

Table III. Automated Synthesis Cycles

step	reagent[a]	(step) time, min cycle A		cycle B		cycle C	
1	5% trichloroacetic acid/dichloroethane	(1)	3.50	(1)	3.50	(1)	2.00
2	acetonitrile	(2)	0.75	(2)	0.75	(2)	0.67
3	0.15 M nucleoside phosphoramidite + 0.5 M tetrazole/acetonitrile (2/1)	(3)	0.25	(3)	0.25	(3)	0.25
4	recycle	(4)	14.75	(4)	14.75	(4)	14.75
5	0.1 M I_2 THF/pyridine/H_2O (7/2/1)	(5)	0.50	(6)	1.50	(6)	1.50
6	0.25 M Ac_2O/DMAP/collidine/THF	(6)	1.50	(5)	0.50	(5)	0.50
		(2)	1.25	(2)	1.25	(2)	0.67
						(6)	0.50
						(2)	0.50
total cycle time			22.50		22.50		21.45

[a] flow rate 5 mL min^{-1}.

needed for the synthesis of RNA containing normal 3′–5′ phosphodiester linkages. Compounds **4a–d** were treated with succinic anhydride and a catalytic amount of DMAP in pyridine for 3 days. TLC analysis indicated that the reaction was complete. Following workup, the crude ribonucleoside monosuccinates **5a–d** were treated with pentachlorophenol and dicyclohexylcarbodiimide in DMF at room temperature for 3 days. The crude products **6a–d** were precipitated from CH_2Cl_2 in hexane in almost quantitative yields and were used without further purification.

The derivatization of the CPG supports followed a slight modification of a procedure previously described for the preparation of deoxynucleoside-functionalized CPG.[36] Commercially available LCAA-CPG (Pierce Chemical Co., 100 μmol g^{-1} of amino groups) was used in all cases. Support (1 g) was treated with 3 equiv of **6a–d** in anhydrous pyridine to which was added 0.2 mL of triethylamine. The slurry was shaken for 4 days, filtered, and washed successively with pyridine, CH_2Cl_2, and Et_2O. The supports were dried overnight, and the loadings of bound ribonucleoside were determined by detritylation of 5-mg portions of the support with 5% trichloroacetic acid (TCA)/dichloroethane (DCE) by use of an extinction coefficient of 56 μmol^{-1} cm^{-1} mL. The loadings achieved were between 18 and 20 μmol g^{-1}. These supports were used for the syntheses of the pentadecamer and shorter sequences. The CPG support, derivatized with adenosine (25 μmol g^{-1}), used for the synthesis of the 43-mer was prepared by the same procedure except that the CPG was subjected to an acidic pretreatment using 5% TCA in DCE for 2 h.

Solid-Phase Synthesis of Oligoribonucleotides. All of our solid-phase syntheses were carried out using an in-house built, computer-controlled, automated synthesizer[10] (full details appear in the Experimental Section) and were monitored by the spectrophotometric quantitation of released monomethoxytrityl cation at 478 nm. The final trityl group was removed on the polymer in all cases. Tetrazole, which is commercially available, easily sublimed, dried, and gives rapid and essentially quantitative activation of the appropriate phosphoramidite, was the activator employed in all of the syntheses.

Our first syntheses were carried out to evaluate the relative effectiveness of the two different types of phosphoramidite synthesized above, the morpholino and the diisopropylamino derivatives, and the effect of amidite concentration on coupling yield. For these syntheses the coupling cycle used is shown in Table III and is referred to as cycle A (the general solid-phase procedure is illustrated in Scheme III). Three octamers of uridylic acid were synthesized under the following conditions: 0.05 M **3d**, sequence 1; 0.1 M **3d**, sequence 2; 0.1 M **2d**, sequence 3; all in the presence of 0.17 M tetrazole as activator. The average and overall coupling yields are summarized in Table IV, and they indicated that the most effective procedure employed the (diisopropylamino)phosphoramidites at a concentration of 0.1 M.

The difference noted in the coupling yields of the nucleoside phosphoramidites **2d** and **3d** appears to be related to the ease of

Scheme III. Solid-Phase Synthesis

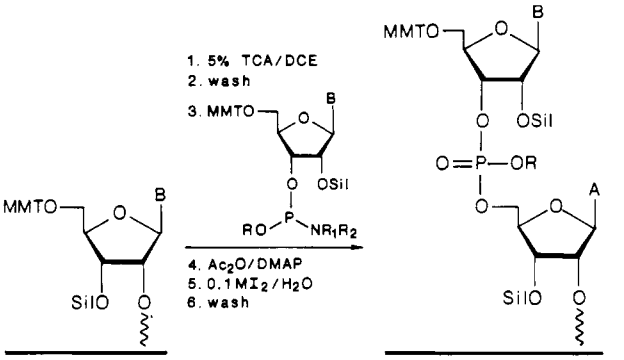


Table IV. Coupling Yields[a]

sequence no.	sequence (5′–3′)	cycle	av yield, %
1[b]	UUU UUU UU	A	92
2	UUU UUU UU	A	95
3[c]	UUU UUU UU	A	90
4	UUU UUU UUU UUU UUU	A	98
5[d]	UUU UUU UUU UUU UUU	A	96
6	CCC CCC CCC CCC CCC	A	98
7	AAA AAA AAA AAA AAA	A	96
8	CGG CCC CCG AAC CCA	A	98
9	GGG GGG GGG GGG GGG	B	97
10	CAU AAC CCG AAG AUC GUC GGU UCA AAU CCG GCC CCC GCA ACC A	C	98

[a] Nucleoside phosphoramidites **3a–d**; concentrations were 0.1 M unless otherwise noted. [b] 0.05 M **3d**. [c] 0.1 M **2d** (morpholidite). [d] 0.1 M **3e** (cyanoethyl phosphate protection).

activation of the two species with tetrazole. This was investigated by ^{31}P NMR. In both cases 1 equiv of the ribonucleoside phosphoramidite **2d** or **3d** was treated with 3 equiv of tetrazole, and the formation of the intermediate tetrazolide was monitored at δ 126. In the case of the diisopropylamino compound **3d**, the activation was complete in <1 min. In contrast, the formation of the tetrazolide was not detectable with the morpholidite derivative **2d** even after 30 min. A number of other activators were investigated by ^{31}P NMR, including *p*-nitrophenyltetrazole, dichloroimidazole, hydroxybenzotriazole, and 3-nitrotriazole, but none of these gave better results than those obtained with tetrazole. Our observations for the morpholidite derivative are in agreement with those of Froehler and Matteucci[37] who were unable to detect any activated species using a thymidine morpholidite. These results indicate that the use of the (diisopropylamino)phosphoramidites **3a–d** is preferred for oligoribonucleotide synthesis.

In order to fully assess the efficacy of the procedure, we undertook the synthesis of a series of pentadecamers consisting of

(36) Gait, M. J., Ed. *Oligonucleotide Synthesis—A Practical Approach*; IRL: Oxford, 1984; p 4749.

(37) Froehler, B. C.; Matteucci, M. D. *Tetrahedron Lett.* **1983**, *24*, 3171–3174.

Table V. Deprotection and Purification Protocol

step	procedure	reagents and conditions
1	removal of $POCH_3$	thiophenol/NEt_3/dioxane (1/2/2), 1 h
2	cleavage from CPG and debenzoylation	15 M NH_4OH/EtOH (3/1), 55 °C, 16 h
3	desilylation	1 M TBAF/THF, 4 h
4	desalting	Sephadex G-25F size-exclusion column
5	purification	polyacrylamide gel electrophoresis
6	desalting	

the four homopolymers of adenylic, cytidylic, guanylic, and uridylic acid and a pentadecamer of mixed base composition corresponding to the 3′-end of the fMet tRNA molecule, 5′-CGG CCC CCG CAC CCA-3′ (sequences 7, 6, 9, 4, and 8, respectively, in Table IV). Compounds **3a–d** were used, and the coupling results and cycle used are shown in Table IV. In all but the homoguanylic acid sequence, the coupling cycle A (Table III) was used. In a study of reactions that may occur at the O^6-position of guanosine during oligonucleotide synthesis,[38] it was found that cycle A was not satisfactory for the synthesis of sequences containing a large number of guanosine residues. In these cases it was necessary to reverse the order of oxidation and capping to give cycle B, which is shown in Table III. In all cases, the average coupling yields were excellent, ranging from 96 to 98%.

The cyanoethyl-protected amidite **3e** was used in the synthesis of a second pentadecamer of uridylic acid (sequence 5) using cycle A. The average coupling yield from the cyanoethyl amidite **3e** was slightly lower, 96%, when compared with the 98% yield obtained with the methyl amidite **3d**. As will be discussed below, the yield following deprotection was slightly higher in the case of the cyanoethyl-protected oligomer. Although there was not a significant difference between the two quantitatively, the cyanoethyl derivative was easier to deprotect, since the removal of the cyanoethyl group could be accomplished at the same time as the debenzoylation–polymer cleavage step using NH_4OH/EtOH (3/1) at 55 °C. A thiophenoxide treatment was necessary for removal of the methyl phosphate protecting group. It appears that either protecting group is suitable for oligoribonucleotide synthesis.

Having established the necessary criteria for the efficient synthesis of oligoribonucleotides, we synthesized the 43-mer, sequence 10, similar to the 3′-end of the fMet tRNA with the phosphoramidite reagents **3a–d** and coupling cycle C shown in Table III. In this coupling cycle, there are two capping steps, one to dry the column prior to detritylation and coupling and another for the decomposition of the guanine O^6-adducts[38] and capping of any unreacted 5′-hydroxyls. The average coupling yield was 97.9% to give an overall yield of 40.6% (Table IV). These coupling yields are equivalent to those currently obtained in deoxynucleotide synthesis.

Deprotection, Isolation, and Characterization of Chemically Synthesized Oligoribonucleotides. The deprotection of the protected oligoribonucleotide must avoid chain cleavage and/or isomerization of the phosphodiester linkages. Another aspect of the deprotection of synthetic RNA, which is often overlooked, is the necessity for the sterile equipment and reagents. The ubiquitous nature of RNase requires that caution be exercised when working with RNA, a fact not often considered or reported in the chemical literature. A protocol for the preparation of reagents and equipment needed in the deprotection of synthetic RNA appears in the Experimental Section.

All of the sequences described above were treated in a similar manner with the exception of sequence 5, which contained cyanoethyl phosphate protection and therefore did not require the thiophenoxide step. The protocol is summarized in Table V. The first step involved the removal of the methyl phosphate protecting groups from the phosphotriesters using thiophenoxide ion (thiophenol/NEt_3/dioxane (1/2/2), 1 h)[20d] to generate the diester linkages. The CPG supports were washed with 95% EtOH and then treated with a solution of NH_4OH/EtOH (3/1) at 55 °C for 16 h to affect the concommitant cleavage of the sequence from the support and removal of the exocyclic amino benzoyl protecting groups, leaving only the 2′-*O*-silyl ethers intact. The silyl groups were removed by treating the residue produced in the previous step with 1 M TBAF in THF (35–40 equiv/silyl group) for 3–4 h. The reaction was then quenched with an equal volume of 0.05 M NH_4OAc (sterile), and the total volume was applied directly to a Sephadex G-25F size-exclusion column packed and run with 0.05 M NH_4OAc for desalting and removal of other organic impurities. The column was monitored by a UV detector (254 nm), and the desired fully deprotected oligomers eluted with the void volume. The overall yields ranged from 20 to 40%. Comparison of the overall yields showed syntheses using cyanoethyl phosphate protection to be 5% higher than those employing methyl protection. The resulting solutions of crude oligomers were evaporated to dryness and reconstituted as 1 ODU 5 μL^{-1} solutions.

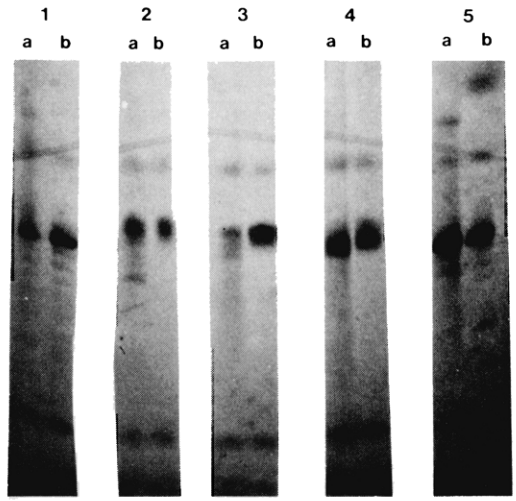


Figure 2. 20% polyacrylamide/7 M urea gel electrophoresis of fully deprotected oligoribonucleotides. Lanes: 1, AAA AAA AAA AAA AAA; 2, CCC CCC CCC CCC CCC; 3, UUU UUU UUU UUU UUU (methyl phosphate protection); 4, UUU UUU UUU UUU UUU (cyanoethyl phosphate protection); 5, CGG CCC CCG AAC CCA. (a) Crude, 1 ODU; (b) pure, 0.5 ODU.

Each oligomer was first checked by analaytical (0.75 mm thick) polyacrylamide gel electrophoresis (PAGE) of 1 ODU samples of the crude product. The products on the sizing gels were visualized by UV shadowing of the gel over a fluorescent TLC plate. Figure 2 shows the 20% acryamide gels of the pentadecamer sequences 7, 6, 4, 5, and 8, lanes 1–5, respectively, (a) crude and (b) after purification. These sequences were purified by preparative gel electrophoresis, 1.5-mm gel thickness, in which the desired band was sliced out and extracted with either NH_4OAc buffer or H_2O alone. Following extraction, the supernatant was desalted on a Sephadex G25F column as for desilylation (vide supra). The results for sequences 1, 2, 3, and 9 were similar (not shown). It is clear from the gel results that the crude sequences were already quite pure, indicating the effectiveness of our methodology.

The 43-mer, sequence 10, was deprotected in the same manner, but following the desilylation step the oligomer was 5′-labeled using [γ ^{32}P]ATP and polynucleotide kinase[39] and applied to a 12% PAGE. The resulting autoradiogram is shown in Figure 3. The position of the xylene cyanol and bromophenol blue dye markers are indicated. Based on these markers, the radioactive oligomer migrates as a 43-mer. The lack of other bands testifies to the integrity of the synthetic oligomer.

In order to confirm the veracity of the purified sample, i.e., to ensure that no base modification or phosphodiester linkage isomerization had occurred, a number of other analytical methods

(38) (a) Pon, R. T.; Usman, N.; Damha, M. J.; Ogilvie, K. K. *Nucleic Acids Res.* **1986**, *14*, 6453–6470. (b) Pon, R. T.; Damha, M. J.; Ogilvie, K. K. *Ibid.* **1985**, *13*, 6447–6465.

(39) Donis-Keller, H.; Maxam, A. M.; Gilbert, W. *Nucleic Acids Res.* **1977**, *4*, 2527–2535.

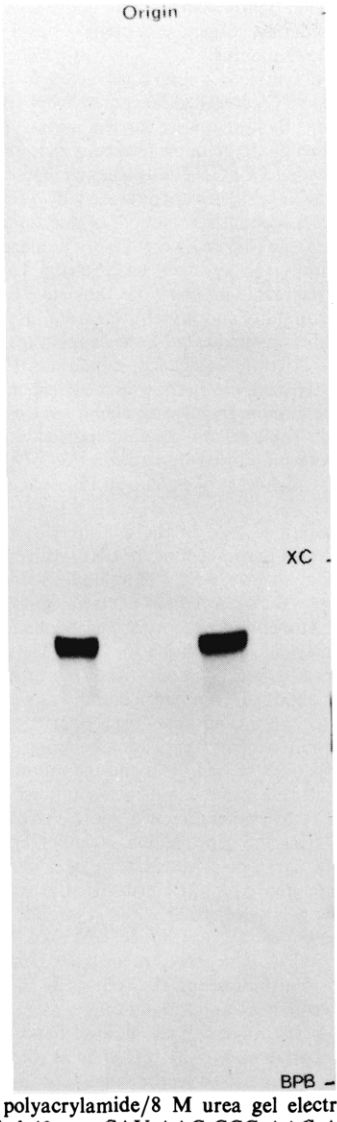


Figure 3. 12% polyacrylamide/8 M urea gel electrophoresis of the [γ^{32}P]ATP-labeled 43-mer CAU AAC CCG AAG AUC GUC GGU UCA AAU CCG GCC CCC GCA ACC A. Duplicate samples.

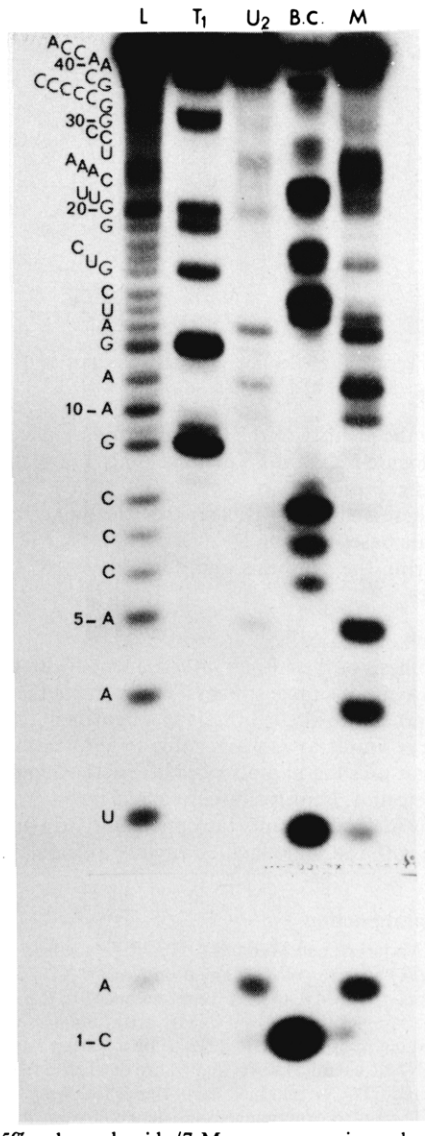


Figure 4. 15% polyacrylamide/7 M urea sequencing gel of 5′-[γ^{32}P]-ATP-labeled sequence 10. Enzymes are discussed in the text, L = ladder.

were employed. These included reversed-phase HPLC analysis of the enzymatic digests of the oligomers,[40] enzymatic sequencing,[41] and terminal nucleotide analyses,[42,43] which are described in the following paragraphs.

The purified pentadecameric homopolymers of A, C, G, and U were treated with spleen phosphodiesterase, under conditions that do not lead to degradation of 2′–5′-linked nucleotides, to generate a mixture of nucleotides (Np) and a nucleoside (N), which was directly injected onto an ODS-2 analytical column run with a 1% NH_4OAc (pH 5.9) buffer. Integration of the peaks corresponding to the two products Np and N gave the expected ratio of 14:1. In the case of the mixed sequences 8 and 10, 0.2 OD unit of the purified oligomers was subjected to enzymatic degradation by snake venom phosphodiesterase (SVPD)/alkaline phosphatase (Alk. P.) to give a mixture of the four ribonucleosides, which was analyzed by HPLC on a Whatman Partisil 5-μ C-8 column to give the correct ratio of A/C/G/U, 3:1:1 :0 for sequence 8 and 2:2.8:1.3:1 for sequence 10. In the degradations that used SVPD/Alk. P. it was noted that if the enzymatic digestion was allowed to proceed for more than 30 min a fifth peak would begin to appear in the HPLC analysis and would grow with time. This was found to be the result of the conversion of adenosine to inosine by adenosine deaminase, which is a contaminant in SVPD/Alk. P. preparations. The identity of the inosine peak was confirmed by comparison with a known standard and added further evidence that the sequences were indeed unmodified.

A more rigorous analysis of the 43-mer was carried out to ensure that the sequence was correct. The 5′-labeled oligomer was purified by preparative gel electrophoresis, and following desalting, an enzymatic sequencing procedure was applied.[41] The nucleases T_1, U_2, Phy M, and BC specific for G, A, A + U, and U + C, respectively, were used, and the products of the partial digests were run on a 15% acrylamide/7 M urea sequencing gel. The autoradiogram of the gel is shown in Figure 4 and substantiates the expected sequence. In addition, a sample of the 43-mer labeled at the 3′-terminus with [^{32}P]pCp and RNA ligase[41] was subjected to a similar enzymatic degradation and PAGE. These results (not shown) confirm the sequence as determined with the 5′-labeled sample.

A final analysis to unequivocally prove the identity of the terminal nucleotides was performed, since often the RNA termini are of utmost of importance for biochemical activity such as ligation to other pieces of RNA or aminoacylation. The purified oligomer was labeled at either the 5′- or 3′-end, and the molecule was then completely degraded enzymatically.[42] The resulting digest was then analyzed by two-dimensional cellulose TLC to

(40) (a) McLaughlin, L. W.; Cramer, F.; Sprinzl, M. *Anal. Biochem.* **1981**, *112*, 60. (b) Pon, R. T. Doctoral Thesis, McGill University, 1984.
(41) Nicoghosian, K.; Gu, X. R.; Cedergren, R. J. *FEBS Lett.* **1985**, *193*, 255–260.
(42) Peattie, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 1760–1764.
(43) (a) Silberklang, M.; Gillum, A. M.; RajBhabdary, U. L. *Meth. Enzymol.* **1979** *59*, 58–109. (b) Sprinzl, M.; Gruter, F.; Gauss, D. H. *Nucleic Acids Res.* **1979**, *6*, r1.

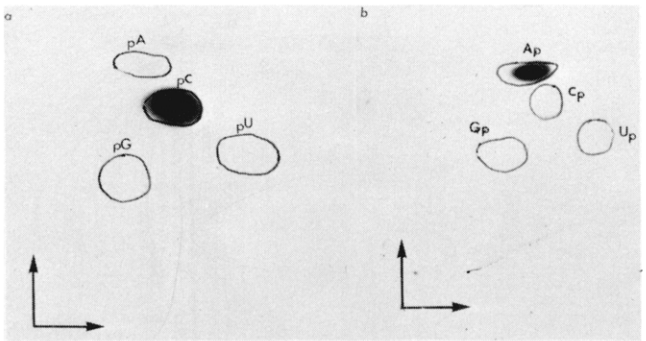


Figure 5. Terminal nucleotide analysis of sequence 10. (a) [γ^{32}P]-ATP-labeled 5′-end; (b) [^{32}P]pCp-labeled 3′-end.

determine the identity of the labeled terminal nucleotide.[43] The results in Figure 5 show the 5′-terminal to be C and the 3′-terminus to be A as expected.

From these analyses it is clear that the ingegrity of both the heterocyclic bases and the 5′–3′-phosphodiester linkages is preserved during the synthesis and deprotection of the oligoribonucleotides.

Conclusions

The synthesis of the 43-mer, in conjunction with the protecting group and synthesis cycle studies, demonstrates the effectiveness of our approach to the synthesis of oligoribonucleotides. The procedure is simple to use, amenable to automation in any synthesizer that uses the phosphoramidite methodology, and yields, upon deprotection, isomerically correct and intact products in good yield. This method presents the opportunity to synthesize RNA molecules with the same facility already available for DNA sequences.

Experimental Section

General Materials and Methods. ^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectra were obtained on Varian XL-200 and XL-300 spectrometers. ^{1}H NMR spectra were referenced to the internal $CHCl_3$ signal in the samples (δ 7.24). ^{31}P NMR activation and coupling studies were carried out in 5-mm NMR tubes using a Varian 5-mm broad-band probe. ^{31}P NMR chemical shifts quoted are downfield from 85% H_3PO_4 (external), and CD_3CN, obtained from Merck Isotopes, was used as lock reference. The actual experiments were done in freshly distilled CH_3CN using oven-dried glassware under argon. Ultraviolet (UV) spectra were recorded on a Carey 17 or Hewlett Packard 8451A spectrophotometer. Melting points (mp) were determined on a Fisher-Johns melting point apparatus and are reported uncorrected. Thin-layer chromatographic data (R_f values) were obtained with Merck Kieselgel 60 F 254 analytical sheets developed with chloroform/ether (1/1, solvent A). Preparative chromatography was performed on E. Merck Kieselgel 60 (230–400 mesh) using 20 g silica g^{-1} crude compound, and the solvents are shown in Table II.

HPLC grade acetonitrile was first dried over activated (~600 °C) 4-Å molecular sieves and then continuously refluxed over CaH_2. THF for the capping solution and dichloroethane for the detritylation solution were reagnet grade and were dried over activated 4-Å molecular sieves prior to use. THF used for the preparation of the nucleoside phosphoramidite reagents was continuously refluxed from Na/benzophenone and distilled piror to use. $CHCl_3$ was distilled from P_2O_5 and stored over activated 4-Å molecular sieves. Collidine and DMF were distilled from CaH_2. DMAP was recrystallized from THF and dried in vacuo over P_2O_5 prior to use. Tetrazole was obtained from the Aldrich Chemical Co. and was stored in vacuo over P_2O_5 or sublimined prior to the synthesis. Chromatographic solvents were fractionally distilled prior to use with the exception of NEt_3. All other chemicals were reagent, or better, grade and were used as is.

All reactions for the preparation of the nucleoside phosphoramidite reagents were carried out in septum-fitted, oven-dried, and argon-purged Hypovials (Pierce). All liquid reagents and solutions were transferred or added via syringe. (*N*,*N*-diisopropylamino)(cyanoethyl)phosphonamidic chloride was generously donated by American BioNuclear (Emeryville, CA). Bottles for the delivery of freshly distilled CH_3CN and capping solution were oven dried prior to use.

The capping solution was prepared just prior to the start of a synthesis by mixing equal volumes of 0.5 M Ac_2O/THF and 0.5 M DMAP/collidine/THF stock solutions. These stock solutions were prepared in advance and stored in tightly sealed bottles under argon for no more than 1 month. The oxidation reagent was prepared as a stock solution and could be stored for 3 months.

The automated synthesizer was constructed in our laboratory and consisted of an IBM PC controlled reagent delivery system and a Waters M-45 HPLC pump. Syntheses were carried out in 2.4-mm i.d. × 50-mm length stainless steel guard columns packed with approximately 100 mg of nucleoside-derivatized CPG. The synthesizer control program and the computer–machine interface were provided by Bio Logicals. In the automated syntheses acetonitrile solutions of nucleoside phosphoramidite (0.15 M) and tetrazole (0.5 M) were freshly prepared and delivered in disposable polypropylene syringes (Aldrich). The 2/1, nucleoside phosphoramidite/tetrazole mixing ratio thus gave final concentrations of 0.1 M phosphoramidite and 0.17 M tetrazole. Prior to the start of a synthesis the column was treated with the capping reagent under manual control for 3 min. A 1-mL recycle loop containing 100 μmol of amidite and 170 μmol of tetrazol was used, which corresponds to a 20–30-fold excess of activated amidite to polymer-bound nucleoside. The synthesis cycles are shown in Table III and the coupling yields appear in Table IV. The syntheses were monitored by quantitation of the released MMT cation at 476 nm, which was collected on a Pharmacia Frac 100 fraction collector.

All aqueous solutions used in the deprotections were sterilized by treatment with diethyl pyrocarbonate[44] (DEP, Aldrich) as a 1% solution followed by autoclaving at 120 °C. The solutions were preserved by the addition of 0.001% NaN_3 to prevent bacterial growth. All glass- and plasticware were autoclaved except for the Sephadex columns, which were treated with a 1% DEP solution for 2 h. Glassware, e.g., pipettes, was silanized prior to sterilization with Sigmacote (Sigma). 1 M TBAF/THF was obtained from Aldrich and was used for all deprotections. Sephadex G-25F was obtained from Pharmacia Canada and was also sterilized by autoclaving. Sephadex columns, 37 × 0.9 cm, were packed with sterile 0.05 M NH_4OAc and monitored with a Pharmacia UV-1 UV monitor at 254 nm. Aqueous solutions of the deprotected oligomers were dried by evaporation in a Speed-Vac concentrator (Savant Instruments). During the deprotection, strictly sterile conditions were used, including the use of disposable latex gloves in addition to the precautions mentioned above for the sterilization of reagents, etc.

Electrophoretic gels were either 20% acrylamide/7 M urea (for sequences of <25 bases) or 16% acrylamide/8 M urea (for the 43-mer) and were run at 400–500 V. The gels were visualized and photographed by UV shadowing over a fluorescent TLC plate. In the case of the radioactively labeled sequences autoradiography was used to visualize the results. For the preparative gels the desired band was sliced out and extracted by incubation in 0.05 M NH_4OAc at 37 °C overnight. The supernatants were then desalted on the same Sephadex G-25 columns as used for the TBAF desaltings. The resulting solutions were lyophilized 3 × from sterile H_2O. Fully deprotected oligomers were stored ~−20 °C.

Enzymes were obtained from Boehringer Mannheim and Pharmacia. HPLC analyses were carried out on a SpectraPhysics SP8000 HPLC equipped with a 254-nm UV detector. HPLC columns, 4.6 mm ID × 250 mm length, were either Whatman ODS-2 10 μ [mobile phase, 1% NH_4OAc (pH 5.9) flow rate 2 mL min^{-1}] or Whatman Partisil 5 C-8 5 μ [mobile phase, 0.05 M KH_2PO_4, 2 mM PIC-A (Waters-Millipore) (pH 4.9)/methanol (93/7), flow rate 1 mL min^{-1}] and were obtained from Chromatographic Specialties. Spectroscopic and physical properties, chroamtographic solvent composition, and isolated yields of the ribonucleoside phosphoramidites **2a-d** and **3a–e** appear in Tables I and II.

Synthesis of N^2-Benzoyl-5′-*O*-(monomethoxytrityl)-2′-*O*-(triisopropylsilyl)guanosine (1c). N^2-Benzoyl-5′-*O*-monomethoxytrityl)-guanosine[25] (6.04 g, 9.16 mmol, 1 equiv was dissolved in 25 mL of anhydrous DMF under an Ar atmosphere to which were added imidazole (1.71 g, 25.18 mmol, 2.7 equiv) and triisopropylsilyl chloride (3.58 g, 18.6 mmol, 2 equiv) with stirring. The reaction mixture was stirred overnight, quenched with 5% $NaHCO_3$, and concentrated in vacuo. The resulting residue was coevaporated with toluene, dissolved in CH_2Cl_2, and extracted with H_2O. Following a second coevaporation with toluene the residue was dissolved in a minimum amount of CH_2Cl_2 and the solution added dropwise to 1800 mL of 78/22 Et_2O/hexanes with stirring. After 15 min, isomerically pure **1c** precipitated from the solution and was filtered off and dried, yield = 4.11 g (62%). The supernatant contained mostly the 3′-*O*-TIPS isomer and some remaining **1c**, which could be recovered by column chromatography using an isocratic elution with 90/5/5 $CHCl_3/CH_2Cl_2/NEt_3$:[26] mp 125–127 °C; UV, λ_{max} 295, 265 nm; ^{1}H NMR ($CDCl_3$) δ 7.71 (s, 1, H_8), 5.74 (d, J = 7.7 Hz, 1, $H_{1'}$), 5.36 (m, 1 $H_{2'}$), 4.32 (m, 1, $H_{3'}$), 3.63 (s, 3, OCH_3), 0.95–0.89 (m, 21,

(44) Ehrenberg, L.; Fedorcsak, F. *Prog. Nucleic Acid Res. Mol. Biol.* **1976**, *16*, 189–262.

$Si[CH(CH_3)_2]_3$); ^{29}Si NMR ($CDCl_3$) δ 18.3 [downfield from TMS, the 3′-isomer (δ 17.6), obtained in 25% yield, had a chemical shift δ 0.7 upfield from the 2′-isomer, and the two could be clearly differentiated[26]]; R_f (solvent A) 0.60.

Synthesis of Protected Ribonucleoside *N*-Morpholinophosphoramidites (2a–d). 5′-*O*-(Monomethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*N*-Morpholinomethylphosphoramidite (2d). To an Ar-purged, sealed 10-mL Hypovial containing 1.26 g (2 mmol, 1 equiv) of protected ribonucleoside **1d** was added 6 mL of dry, distilled $CHCl_3$ followed by 1.4 mL (1.04 g, 8 mmol, 4 equiv) of diisopropylamine. To this solution was added, dropwise with stirring, 0.3 mL (0.55 g, 3.0 mmol, 1.5 equiv) of morpholinomethylphosphonamidic chloride over 90 s. The reaction was allowed to stir at room temperature for 2 h and was then diluted with 70 mL of EtOAc. The solution was washed 5x with saturated brine and the organic phase dried over Na_2SO_4. Removal of the solvent in vacuo afforded the crude product as a foam, which was then chromatographed on a silica gel column with the appropriate solvent (Table II). The purified compound was obtained as a white foam.

N^6-Benzoyl-5′-*O*-(monomethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)adenosine 3′-*N*-Morpholinomethylphosphoramidite (2a). As for the preparation of **2d**; 1.52 g (2 mmol, 1 equiv) of **1a** was dissolved in 6 mL of $CHCl_3$, to which was added 1.4 mL (1.04 g, 8 mmol, 4 equiv) of diisopropylethylamine; 0.35 mL (0.64 g, 3.5 mmol, 1.75 equiv) of the phosphitylating reagent was added dropwise. after 2 h the reaction was worked up and purified as for **2d**.

N^4-Benzoyl-5′-*O*-monomethoxytrityl-2′-*O*-(*tert*-butyldimethylsilyl)-cytidine 3′-*N*-morpholinomethylphosphoramidite (2b): as for **2d**; 1.46 g (2 mmol, 1 equiv) of **1b**, 6 mL of $CHCl_3$, 1.4 mL (1.04 g, 8 mmol, 4 equiv) of dissopropylamine, 0.35 mL (0.64 g, 3.5 mmol, 1.75 equiv) of the phosphitylating reagent. After 2 h the reaction was worked up and purified as for **2d**.

N^2-Benzoyl-5′-*O*-(monomethoxytrityl)-2′-*O*-(triisopropylsilyl)-guanosine 3′-*N*-morpholinomethylphosphoramidite (2c): as per **2d**; 0.84 g (1 mmol, lequiv) of **1c**, 2.5 mL of $CHCl_3$, 0.7 mL (0.5 g, 4 mmol, 4 equiv) of diisopropylethylamine, 0.2 mL (0.36 g, 2 mmol, 2 equiv) of the phosphitylating reagent. The reaction was diluted with 35 mL of EtOAc, worked up and purified as for **2d**.

Synthesis of Ribonucleoside (*N*,*N*-Diisopropylamino)phosphoramidites (3a–d). N^6-Benzoyl-5′-*O*-(monomethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)adenosine 3′-*N*,*N*-Diisopropylmethylphosphoramidite (3a). To a stirred THF (20 mL) solution of 6.13 mL (35 mmol, 4 equiv) diisopropylethylamine, 2.19 mL (11.38 mmol, 1.3 equiv) (*N*,*N*-diisopropylamino)methylphosphonamidic chloride, and a catalytic amount (215 mg, 1.75 mmol, 0.2 equiv) of DMAP was added, dropwise, a solution of the protected ribonucleoside **1a** (6.63 g, 8.75 mmol, 1 equiv in 20 mL of THF) at room temperature with stirring. After being stirred for 3 h, the reaction mixture was worked up by diluting with 250 mL of EtOAc and washing the organic phase 5x with saturated brine. The organic phase was dried over Na_2SO_4 and the solvent removed in vacuo. Following chromatography, the pooled fractions were coevaporated first with 95% EtOH and then with Et_2O, providing the product as a white foam.

N^4-Benzoyl-5′-*O*-(monomethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)cytidine 3′-*N*,*N*-Diisopropylmethylphosphoramidite (3b). As for the synthesis of **3a**; to a stirred THF (25 mL) solution of 7.0 mL (40 mmol, 4 equiv) of diisopropylethylamine, 2.51 mL (13.0 mmol, 1.3 equiv of (*N*,*N*-diisopropylamino)methylphosphonamidic chloride, and a catalytic amount (244 mg, 2.0 mmol, 0.2 equiv) of DMAP was added, dropwise, a solution of the protected ribonucleoside **1b** (7.33 g, 10.0 mmol, 1 equiv in 20 mL of THF) at room temperature with stirring. After being stirred for 3 h, the reaction mixture was worked up and purified as for **3a**.

N^2-Benzoyl-5′-*O*-(monomethoxytrityl)-2′-*O*-(triisopropylsilyl)-guanosine 3′-*N*,*N*-Diisopropylmethylphosphoramidite (3c). As for the synthesis of **3a**; to a stirred THF (10 mL) solution of 3.21 mL (18.4 mmol, 6 equiv) of diisopropylethylamine and 1.42 mL (7.35 mmol, 2.4 equiv) of (*N*,*N*-diisopropylamino)methylphosphonamidic chloride was added, dropwise, a solution of the protected ribonucleoside **1c** (2.50 g, 3.06 mmol, 1 equiv in 20 mL of THF at room temperature with stirring. No DMAP was used in this case. After being stirred overnight, the reaction mixture was diluted with 100 mL of EtOAc and worked up and purified as for **3a**.

5′-*O*-(Monomethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*N*,*N*-Diisopropylmethylphosphoramidite (3d). As for the synthesis of **3a**; to a stirred THF (15 mL) solution of 6.6 mL (37.7 mmol, 4 equiv) of diisopropylethylamine, 2.36 mL (12.25 mmol, 1.3 equiv) of (*N*,*N*-diisopropylamino)methylphosphonamidic chloride, and a catalytic amount (233 mg, 1.88 mmol, 0.2 equiv) of DMAP was added, dropwise, a solution of the protected ribonucleoside **1d** (5.94 g, 9.42 mmol, 1 equiv in 20 mL of THF) at room temperature with stirring. After being stirred for 3 h, the reaction mixture was worked up and purified as for **3a**.

5′-*O*-(Monomethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*N*,*N*-Diisopropyl(cyanoethyl)phosphoramidite (3e). As for the synthesis of **3a**; to a stirred THF (7 mL) solution of 1.4 mL (8 mmol, 4 equiv) of diisopropylethylamine, 0.55 mL (0.615 g 2.6 mmol, 1.3 equiv) of (*N*,*N*-diisopropylamino)(cyanoethyl)phosphonamidic chloride, and a catalytic amount (49 mg, 0.4 mmol, 0.2 equiv) of DMAP was added, dropwise, a solution of the protected ribonucleoside **1d** (1.26 g, 2 mmol, 1 equiv) in 7 mL of THF) at room temperature with stirring. After being stirred for 3 h, the reaction mixture was worked up as for **3a**. Following workup, the crude **3e** was redissolved in Et_2O and the solution passed through a fine sintered glass funnel. Removal of the Et_2O in vacuo yielded **3e** as a white foam, which was pure as determined by 200-MHz ^1H NMR, therefore the product was not further purified.

Synthesis of 5′-*O*-(Monomethoxytrityl)-3′-*O*-silylated 2′-*O*-Succinate Ribonucleosides (5a–d). N^6-Benzoyl-5′-*O*-(monomethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)adenosine 2′-*O*-Succinate (5a). **4a** 3.03 g, 4 mmol, 1 equiv), 1.20 g (12 mmol, 3 equiv) of succinic anhydride, and 244 mg (2 mmol, 0.5 equiv) of DMAP were dissolved in 20 mL of anhydrous pyridine in an argon-purged, oven-dried 50-mL flask. The reaction was allowed to stir for 2 days at ambient temperature. TLC analysis (solvent A) showed the complete disappearance of the starting material and the formation of a single, more polar product. The pyridine was removed in vacuo, and the residue was then coevaporated with toluene (3 × 50 mL). The dark brown residue was taken up in 100 mL of CH_2Cl_2 and washed with saturated brine. The organic phase was dried over Na_2SO_4, and following removal of the solvent in vacuo, the crude product was obtained as a light brown foam in quantitative yield. **5a**: ^1H NMR ($CDCl_3$) δ 6.26 (d, J = 4.0 Hz, 1, $H_{1'}$), 3.75 (s, 3, OCH_3), 2.53–2.59 (m, 4, CH_2CH_2), 0.85 (s, 9, Si-*t*-Bu), 0.02 (s, 3, $SiCH_3$), −0.02 (s, 3, $SiCH_3$); R_f (solvent A) 0.18.

N^4-Benzoyl-5′-*O*-(monomethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)cytidine 2′-*O*-Succinate (5b). As for **5a**; 2.93 g (4 mmol, 1 equiv) of **4b**, 1.20 g (12 mmol, 3 equiv) of succinic anhydride, and 244 mg (2 mmol, 0.5 equiv) of DMAP were dissolved in 20 mL of anhydrous pyridine in an argon-purged, oven-dried 50-mL flask. The reaction was allowed to stir for 3 days at ambient temperature. The same workup was employed as in the case of **5a**, and the crude product was obtained in quantitative yield as a foam. **5b**: ^1H NMR ($CDCl_3$) δ 5.91 (d, J = 1.0 Hz, 1, $H_{1'}$), 3.80 (s, 3, OCH_3), 2.57–2.72 (m, 4, CH_2CH_2), 0.86 (s, 9, Si-*t*-Bu), 0.18 (s, 3, $SiCH_3$), 0.05 (s, $SiCH_3$); R_f (solvent A) 0.19.

N^2-Benzoyl-5′-*O*-(monomethoxytrityl)-3′-*O*-(triisopropylsilyl)-guanosine 2′-*O*-Succinate (5c). As for **5a**; 3.26 g (4 mmol, 1 equiv) of **4c**, 1.20 g (12 mmol, 3 equiv) of succinic anhydride, and 244 mg (2 mmol, 0.5 equiv) of DMAP were dissolved in 20 mL of anhydrous pyridine in an argon-purged, oven-dried 50-mL flask. The reaction was allowed to stir for 3 days at ambient temperature. The same workup was employed as in the case of **5a**, and the crude product was obtained in quantitative yield as a foam. **5c**: ^1H NMR ($CDCl_3$) δ 5.95 (d, J = 4.0 Hz, 1, $H_{1'}$), 3.64 (s, 3, OCH_3), 2.58–2.67 (m, 4, CH_2CH_2), 0.92–0.94 (m, 21, $Si(i\text{-}Pr)_3$); R_f (solvent A) 0.12.

5′-*O*-(Monomethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)uridine 2′-*O*-Succinate (5d). As for **5a**; 2.52 g (4 mmol, 1 equiv) of **4d**, 1.20 g (12 mmol, 3 equiv) of succinic anhydride, and 244 mg (2 mmol, 0.5 equiv) of DMAP were dissolved in 20 mL of anhydrous pyridine in an argon-purged, oven-dried 50-mL flask. The reaction was allowed to stir for 3 days at ambient temperature. The same workup was employed as in the case of **5a**, and the crude product was obtained in quantitative yield as a foam. **5d**: ^1H NMR ($CDCl_3$) δ 5.91 (d, J = 3.0 Hz, 1, $H_{1'}$), 3.78 (s, 3, OCH_3), 2.63–2.65 (m, 4, CH_2CH_2), 0.84 (s, 9, Si-*t*-Bu), 0.07 (s, 3, $SiCH_3$), 0.06 (s, 3, $SiCH_3$); R_f (solvent A) 0.18.

Synthesis of 5′-*O*-(Monomethoxytrityl)-3′-*O*-silylated 2′-*O*-(pentachlorophenyl)succinyl Ribonucleosides (6a–d). N^6-Benzoyl-5′-*O*-(monomethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-*O*-[(pentachlorophenyl)succinyl]adenosine (6a). **5a** (3.43 g, 4 mmol, 1 equiv), 1.6 g (6 mmol, 1.5 equiv) of pentachlorophenol, and 122 mg (1 mmol, 0.25 equiv) of DMAP were placed in a dry, argon-purged 100-mL flask. To this was added 1.65 g (8 mmol, 2 equiv) of DCC followed by 30 mL of dry DMF. The reaction mixture was stirred for 3 days at room temperature. TLC analysis (solvent A) showed the complete disappearance of **5a** and the formation of a nonpolar product. The reaction was quenched by the addition of 95% EtOH, and the solvents were removed in vacuo. The resulting dark brown residue was coevaporated with toluene (3 × 50 mL) and was then dissolved in 160 mL of CH_2Cl_2 and washed with water. The CH_2Cl_2 layer was dried over Na_2SO_4 and the solvent removed in vacuo. The residue was taken up in 30 mL of CH_2Cl_2 and filtered through a fine sintered glass funnel to remove the DCU byproduct. The solution was concentrated and the filtration repeated. The residue was dissolved in 8 mL of CH_2Cl_2 and precipitated in 200 mL of hexanes × 2 to yield 2.88 g (65%) of **6a** as a light brown powder. **6a**: ^1H NMR ($CDCl_3$) δ 6.23 (d, J = 4.0 Hz, 1, $H_{1'}$), 3.76 (s, 3, OCH_3), 2.58–2.63 (m,

4, CH_2CH_2), 0.83 (s, 9, Si-*t*-Bu), 0.03 (s, 3, $SiCH_3$), −0.04 (s, 3, $SiCH_3$); R_f (solvent A) 0.51.

N^4-Benzoyl-5′-*O*-(monomethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-*O*-[(pentachlorophenyl)succinyl]cytidine (6b): As for **6a**; 3.34 g (4 mmol, 1 equiv) of **5b**, 1.6 g (6 mmol, 1.5 equiv) of pentachlorophenol, and 122 mg (1 mmol, 0.25 equiv) of DMAP to which was added 1.65 g (8 mmol, 2 equiv of DCC followed by 30 mL of dry DMF. The reaction mixture was stirred for 3 days at room temperature. The reaction was worked up as for **6a**. In this case the precipitation was carried out as follows. The residue was dissolved in 6 mL of CH_2Cl_2 and precipitated in 400 mL of hexanes only once to yield 3.0 g (69.2%) of **6b** as a light brown powder. **6b**: ^{1}H NMR ($CDCl_3$) δ 5.98 (d, J = 1.0 Hz, 1, $H_{1'}$), 3.80 (s, 3, OCH_3), 2.65–2.92 (m, 4, CH_2CH_2), 0.86 (s, 9, Si-*t*-Bu), 0.18 (s, 3, $SiCH_3$), 0.05 (s, $SiCH_3$); R_f (solvent A) 0.62.

N^2-Benzoyl-5′-*O*-(monomethoxytrityl)-3′-*O*-(triisopropylsilyl)-2′-*O*-[(pentachlorophenyl)succinyl]guanosine (6c): As for **6a**; 3.21 g (3.5 mmol, 1 equiv) of 1.4 g (5.25 mmol, 1.5 equiv) of pentachlorophenol, and 106 mg (0.875 mmol, 0.25 equiv) of DMAP to which was added 1.44 g (7 mmol, 2 equiv) of DCC followed by 26 mL of dry DMF. The reaction mixture was stirred for 3 days at room temperature and worked up as for **6a**. In this case the precipitation was carried out as follows. The residue was dissolved in 6 mL of CH_2Cl_2 and precipitated in 400 mL of hexanes only once to yield 3.6 g (88.2%) of **6c** as a light brown powder. **6c**: ^{1}H NMR ($CDCl_3$) δ 5.98 (d, J = 4.0 Hz, 1, $H_{1'}$), 3.65 (s, 3, OCH_3), 2.51–2.72 (m, 4, CH_2CH_2), 0.80–0.96 (m, 21, Si(*i*-Pr)$_3$); R_f (solvent A) 0.41.

5′-*O*-(Monomethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-2′-*O*-[(pentachlorophenyl)succinyl]uridine (6d): As for **6a**; 2.93 g (4 mmol, 1 equiv) of **5d**, 1.6 g (6 mmol, 1.5 equiv) of pentachlorophenol, and 122 mg (1 mmol, 0.25 equiv) of DMAP to which was added 1.65 g (8 mmol, 2 equiv) of DCC followed by 30 mL of dry DMF. The reaction mixture was stirred for 3 days at room temperature. The reaction was worked up as for **6a**. In this case the precipitation was carried out as follows. The residue was dissolved in 6 mL of CH_2Cl_2 and precipitated in 400 mL of hexanes only once to yield 2.45 g (62.4%) of **6d** as a light brown powder. **6d**: ^{1}H NMR ($CDCl_3$) δ 5.95 (d, J = 4.0 Hz, 1, $H_{1'}$), 3.78 (s, 3, OCH_3), 2.57-2.75 (m, 4, CH_2CH_2), 0.84 (s, 9, Si-*t*-Bu), 0.07 (s, 3, $SiCH_3$), 0.06 (s, 3, $SiCH_3$); R_f (solvent A) 0.70.

Derivatization of Controlled-Pore Glass Supports. A mixture of 0.75 mmol of the appropriate protected (pentachlorophenyl)succinyl nucleoside derivative **6a–d**, 2.5 g of long-chain alkylamine CPG, 8 mL of anhydrous pyridine, and 0.2 mL of triethylamine was shaken in a dry, argon-purged 50-mL flask for 4 days. The slurry was then filtered through a fine sintered glass funnel and successively washed with pyridine (100 mL), $CHCl_3$ (100 mL), and Et_2O (100 mL). The derivatized supports were then dried overnight in a methanol drying pistol. The loading of each support was determined by detritylation of 5-mg samples with 3% TCA/DCE solution. The loadings were as follows: adenosine 18.4 μmol g^{-1}, cytidine 17.2 μmol g^{-1}, guanosine 20.1 μmol g^{-1}, uridine 19.2 μmol g^{-1}.

Deprotection of Oligoribonucleotides. The general procedure for the deprotection of chemically synthesized oligomers is discussed above (see Table V). The deprotection of the 43-mer is described as an example.

Deprotection of *E. coli* fMet tRNA Half Molecule (Sequence 10). CPG (51 mg) bearing the protected sequence was placed in a sterile 5-mL polypropylene test tube to which was added 0.5 mL of thiophenol/triethylamine/dioxane (1/2/2). The slurry was allowed to stand for 1 h, after which the excess reagent was removed with a sterile glass pipette. The support was washed with 95% EtOH by adding the EtOH, agitating the slurry, centrifuging the test tube, and then withdrawing the washings with a sterile pipette. The washing procedure was repeated five times. In the same test tube 3 mL of concentrated NH_4OH/95% EtOH (3/1) was added to the support and the tube sealed with a septum (the septum was taped to the tube with commonly available electrical tape). The tube was placed in a thermostated oil bath at 55 °C for 16 h to effect the concomitant removal of the sequence from the support and the removal of the exocyclic amino benzoyl protecting groups. After the tube was cooled in an EtOH/dry ice bath, the washings were removed with a sterile silanized pipette and placed in a sterile 15-mL test tube. The support was further washed with 3/1 NH_4OH/95% EtOH (4 × 2 mL). The combined washings were frozen in an EtOH/dry ice bath and evaporated in a Speed-Vac evaporator overnight. The resulting residue was treated with 0.6 mL of 1 M TBAF/THF for 4 h. The reaction was quenched with an equal volume of sterile 0.05 M NH_4OAc and applied directly to a Sephadex G-25F column. The compound was eluted with 0.05 M NH_4OAc as described in the general materials and methods section. Quantitation of the major fraction showed the presence of 160 ODU of crude product. The NH_4OAc solutions were evaporated, and the fully deprotected oligomer was transferred to a 1.5-mL Eppendorf tube and reconstituted as a 1 ODU 5 μL^{-1} solution.

Characterization and Purification of Fully Deprotected Ribonucleotides. The oligomers were first checked by analytical PAGE (0.75 mm × 20 cm × 20 cm). To 1 ODU in 5 μL of H_2O was added 5 μL of loading buffer containing 2% xylene cyanol (XC) and 2% bromophenol blue (BPB) in deionized formamide, and the total 10-μL solution was loaded into a single lane (10 lanes/gel). Following electrophoresis, the gels were wrapped in commercially available plastic wrap and photographed by placing the gel over a fluorescent TLC plate and illuminating the gel with a UV lamp. Once the presence of the desired sequence was established according to electrophoretic mobility, the sequence was purified by preparative electrophoresis using 1.5-mm thick gels and a single 12-cm-wide lane. After electrophoresis the desired band was excised, cut into small pieces, placed into a sterile 5-mL test tube, and covered with 0.05 M NH_4OAc. The tube was covered and kept at 37 °C overnight. The supernatant was then removed, and the gel pieces were washed with an additional 1 mL of the extraction buffer. The combined washings were directly applied to a Sephadex G25-F column for desalting to give 10 ODU of pure 43-mer.

Enzyme Degradation of HPLC Analysis of the Enzymatic Digest. The purified 43-mer (0.4 ODU) was treated with 10 μL of SVPD/10 μL of alkaline phosphatase for 30 min in 65 μL of buffer solution (50 mM TRIS-HCl, 10 mM $MgCl_2$, pH 8) in a 1.5-mL Eppendorf tube. The reaction was centrifuged and 5–10-μL aliquots were injected directly onto a C-8 column. The ribonucleoside ratio was determined by dividing the area of each peak by the appropriate extinction coefficient.[40b] The conversion of adenosine to inosine was confirmed by comparison with a standard and by incubating a mixture of the four ribonucleosides in the SVPD/Alkaline phosphatase system. In the case of the homopolymer sequences, 0.2 ODU of the purified oligomer was treated with 10 μL of spleen phosphodiesterase in 55 μL of buffer solution (0.5 M NH_4OAc, pH 6.5) for 30 min. The digest was centrifuged and injected onto an ODS-2 column. The ratio of NP to N was obtained by dividing the area of each peak by the appropriate extinction coefficient.[40b]

Terminal Nucleoside Analysis. 3′-end labeling of the oligoribonucleotides was carried out by the method described by Peattie[42] using [^{32}P]pCp and RNA ligase. 5′-labeling was accomplished by the method of Donis-Keller et al.[39] with [γ^{32}P]ATP and polynucleotide kinase. The 3′-labeled oligomer was treated with a mixture of T_1, T_2, and pancreatic ribonucleases; the resulting solution was subjected to two-dimensional cellulose TLC analysis using, in the first dimension, 5/3 (v/v) of isobutyric acid/0.05 M ammonium hydroxide and in the second dimension, 70/15/15 (v/v/v) isopropanol/concentrated HCl/H_2O.[43] The identity of the labeled 3′-terminal nucleotide was determined by comparison with authentic 3′-mononucleotides. The 5′-labeled sample was digested with nuclease P_1, which generates 5′-mononucleotides, and analysis was performed by TLC using the same solvent system as above. Identification of the 5′-terminal nucleotide was accomplished by comparison with authentic 5′-mononucleotide samples.

RNA Sequence Analysis. The rapid gel-sequencing method originally described by Donis-Keller et al.[39] was used as modified in our laboratory.[41] The 5′- or 3′-labeled oligomer was treated with either T_1 (G specific), U_2 (A specific), Phy M (U and A specific), or *Bacillus cereus* (C and U specific) ribonucleases under partial hydrolysis conditions, and the products were analyzed on 15% PAGE. Autoradiography allowed the detection of the different fragments.

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