Chemical synthesis of biologically active oligoribonucleotides using β-cyanoethyl protected ribonucleoside phosphoramidites

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ABSTRACT

The preparation of fully protected diisopropylamino-β-cyanoethyl ribonucleoside phosphoramidites with regioisomeric purity > 99.95% is described. It is demonstrated that the combination of standard DNA protecting groups, 5′-*O*-DMT, *N*-Bz (Ade and Cyt), *N*-*i*Bu (Gua), β-cyanoethyl for phosphate, in conjunction with TBDMS for 2′-hydroxyl protection, constitutes a reliable method for the preparation of fully active RNA. Average stepwise coupling yields in excess of 99% were achieved with these synthons on standard DNA synthesizers. Two steps completely deprotect the oligoribonucleotide and workup is reduced to a fifteen minute procedure. Further, it is shown that the deprotected oligoribonucleotides are free from 5′-2′ linkages. This methodology was applied to the chemical synthesis of a 24-mer microhelix, a 35-mer minihelix and two halves of a catalytic 'Hammerhead Ribozyme'. These oligoribonucleotides were directly compared in two distinct biochemical assays with enzymatically (T7 RNA polymerase) prepared oligoribonucleotides and shown to possess equal or better activity.

INTRODUCTION

The importance of RNA in biological systems has long been recognized and thus the roles of tRNA, mRNA and rRNA in cellular processes are under constant study. Recent work (1,2) has illustrated the potential of certain RNA sequences to act as catalysts in splicing and ligation reactions. Structurally, RNA has been shown to assume a variety of interesting and biologically important conformations (3). Research in all of these areas has demonstrated the need for a practical means to synthesize sequence-specific RNA oligonucleotides.

There are presently two approaches to the synthesis of RNA oligoribonucleotides: primer-template directed synthesis utilizing phage T7 RNA polymerase (4) and chemical synthesis using protected ribonucleoside phosphoramidites (5–7), analogous to deoxyoligonucleotide synthesis. The T7 system has proved useful as a simple means of producing specific RNA sequences, however, it is highly length and sequence dependent. Oligoribonuclotides shorter than 12 bases give poor yields and are difficult to purify to homogeneity. Sequences which do not begin with a guanosine at the 5′-end have proven exceedingly difficult to make. Further, conditions often have to be customized for each particular sequence to optimize yields. It has also been observed that the T7 polymerase undergoes abortive initiations and may add an undesired base, thus complicating purification (4). Lastly, T7 polymerase may only incorporate four separate bases into an oligoribonucleotide, *e.g.* it is not possible to incorporate an additional nonstandard nucleoside base, such as inosine or 2-aminopurine (8), or a deoxyribonucleoside, at a specific position if the oligoribonucleotide contains all four standard bases.

An alternative to the T7 method is the chemical synthesis of RNA, which has, to a certain extent, paralleled the methodology of DNA synthesis. However, the need to protect the additional 2′-hydroxyl group in RNA has hindered the development of a practical method to synthesize RNA. A variety of protecting group strategies have been developed and studied (9–13), illustrating several difficulties in choosing a protection scheme. First, there is the potential of forming undesired 5′-2′ internucleotidic bonds, usually resulting from the incorporation of isomerically impure ribonucleoside phosphoramidites. Second, the 2′-protecting group must be stable through all stages of oligoribonucleotide synthesis, and conditions for the deprotection of the oligoribonucleotides should not cause nucleoside base modification, migration of the phosphate linkage, nor degradation. Lastly, the fully deprotected oligoribonucleotide must be fully functional in biochemical assays.

The problems in RNA chemical synthesis have been largely overcome by the use of alkylsilyl protecting groups for the 2′-hydroxyl (5,6). The use of the TBDMS group in conjunction with the phosphoramidite method has led to the synthesis of molecules up to 77 nucleotides in length which have modest biological activity (6). However, no chemical synthesis has provided fully functional RNA to date. As a result, the T7 system has proven to be the more reliable and practical method for synthesizing oligoribonucleotides.

We demonstrate here that the protection scheme using

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conventional deoxyribonucleoside phosphoramidite protecting groups: DMT for the 5′-hydroxyl, Bz for adenosine and cytidine and *i*Bu for guanosine exocyclic amino groups, and β-cyanoethyl for phosphate protection, in conjunction with *t*-butyldimethylsilyl for 2′-hydroxyl protection is an effective method for the synthesis of RNA. The synthesis and deprotection methodologies have been developed and refined such that the RNA produced, when tested using a combination of assays, is free of base modification, 5′-2′ linkages, and unremoved protecting groups. Most importantly, the RNA is completely functional in two independent biochemical systems and is recognized as well as or better than the same sequences generated by the T7 RNA polymerase system. The chemical system described here meets all of the above mentioned criteria without suffering from the length and sequence limitations of the T7 RNA polymerase system.

RESULTS AND DISCUSSION

Synthesis of Protected Ribonucleoside Phosphoramidites

A principal consideration in the chemical synthesis of the ribonucleoside phosphoramidites is contamination of the desired 2′-*O*-silyl-3′-*O*-phosphoramidite, **2a-d**, with undesired 3′-*O*-silyl-2′-*O*-phosphoramidite, **4a-d**. Syntheses performed with **2a-d** contaminated with **4a-d** yield oligoribonucleotides containing 5′-2′ linkages. Thus, the purity of ribonucleoside phosphoramidite is crucial to successful syntheses.

There are several stages during the synthesis of **2a-d** where care must be exercised to ensure that 2′-*O*-phosphoramidite is not present in the final product. During the silylation reaction, the 2′- and 3′-hydroxyl groups are not markedly distinguishable, thus a mixture of 2′-*O*-silyl, **1a-d**, and 3′-*O*-silyl regioisomers, **3a-d**, are generated (14). These isomers can be clearly resolved by TLC, and purified by silica gel chromatography. By TLC analysis as little as 0.06% of the wrong isomers, **3a-d**, in the presence of the correct isomer, **1a-d** can be detected. This level of detection was ascertained by performing a serial dilution of **3a-d** and mixing with **1a-d**. Purified compounds **1a-d** were obtained from Chemgenes Inc. with an isomeric homogeneity of > 99.94% as determined by TLC.

Synthesis of the phosphoramidites **2a-d** by phosphitylation of the silyl isomer was accomplished using (*N,N*-diisopropylamino)(cyanoethyl) phosphonamidic chloride as the phosphitylating agent (Figure 1). This reaction is usually performed in THF with DMAP as a catalyst and DIEA as the base scavenger (5). However, it has been shown that the silyl group can migrate under basic conditions and in some protic solvents (15). Thus, we examined the extent of isomerization under the phosphitylation conditions as described below.

Pure 5′-*O*-DMT-2′-*O*-silyl isomers **1a-d** were subjected to a number of reaction conditions, in the absence of the phosphitylating reagent, and analyzed by TLC. In THF alone isomerization could not be detected even after 24 hr. However, detectable isomerization, *i.e.* > 0.1%, was found within minutes for all other combinations of DMAP and DIEA. Thus, these phosphitylation conditions are basic enough to cause isomerization of the 2′-*O*-silyl isomer before it is completely phosphitylated. It was therefore necessary to either phosphitylate by an alternate method or find a base and catalyst which would not isomerize the silyl group. The use of bis(*N,N*-diisopropylamino)(2-cyanoethoxy)phosphine as the phosphitylating reagent was considered but has been shown to cause considerable isomerization (16), possibly *via* the strong base diisopropylamine which is generated during the reaction. A number of alternate bases were tested to determine if they cause isomerization. The bases DABCO, DBU, purine, and diisopropylamine all caused isomerization within minutes under the conditions of the assay. The bases pyridine, 2,6-lutidine and 2,4,6-collidine gave no detectable isomerization, even after 24 hr. However, if a base is insufficiently basic, the phosphoramidite will decompose during the reaction. This was observed in reactions containing pyridine or 2,6-lutidine as the base. We finally determined that 2,4,6-collidine as a base and *N*-methylimidazole as a catalyst do not isomerize **1a-d** and gave excellent yields, with no noticeable increase in degradation compared to the standard DIEA and DMAP system.

Having determined that our starting materials **1a-d** were > 99.94% pure, we proceeded to ascertain the level of isomeric impurity in the final phosphoramidite product. Thus, we synthesized and purified each 3′-*O*-silyl-2′-*O*-phosphoramidite, **4a-d**, by the same procedure as for the 3′-*O*-phosphoramidites **2a-d** (Figure 1). The introduction of a chiral phosphorus center produced two phosphoramidite diastereomers. Thus a 2′/3′ mixture of phosphoramidites contained four diastereomers. Separation of 2′/3′ isomer mixtures for each nucleoside by TLC was largely unsuccessful. Only in the case of the cytidine could the 2′-*O*-phosphoramidite **4b** diastereomers be marginally resolved from the desired 3′-*O*-phosphoramidite diastereomers **2b**. However, the presence of even 5% of **4b** relative to **2b** could not be accurately detected. The adenosine, guanosine and uridine 3′-*O*-phosphoramidtes **2a,c,d** could not be resolved from their corresponding 2′-*O*-ribonucleoside phosphoramidites **4a,c,d** (data not shown). Further, silica gel chromatography using a wide variety of conditions could not separate 3′-*O*-phosphoramidites free of contaminating 2′-*O*-ribonucleoside phosphoramidites.

Although the phosphoramidite isomers were inseparable by TLC analysis, the members of each pair of 2′- and 3′-*O*-phosphoramidite diastereomers could be observed independantly by ^{31}P NMR analysis as shown in Table 1 by the Δδ values. To determine the sensitivity of ^{31}P NMR, pure adenosine 3′-*O*-phosphoramidite samples **2a** were contaminated with 10%, 1%, 0.5%, 0.1%, 0.05%, and 0.01% of the adenosine 2′-*O*-phosphoramidite **4a** and analyzed. Below 1% impurity, the instrumental integration could not accurately quantitate the amount of contamination. However, the 2′-*O*-phosphoramidite diastereomer peaks could still be detected down to the 0.05% level. The 0.01% contaminated sample showed no detectable 2′-*O*-phosphoramidite. This type of analysis was performed for the remaining three phosphoramidites **2b-d** and gave similar results. Thus, reaction of the 2′-*O*-silyl nucleosides, **1a-d**, under the specified phosphitylation conditions produced phosphoramidites, **2a-d**, which were > 99.95% isomerically pure.

Finally, we have fully characterized the protected ribonucleoside phosphoramidites **2a-d** by ^{1}H NMR. Table 2 contains the chemical shift information for both diastereomers and Table 3 contains the relevant coupling constants.

Solid Phase Synthesis of Oligoribonucleotides

To test our methodology with biochemical assays we prepared the sequences **S**, **R**, μ-Helix (μH), and m-Helix (**mH**), shown in Table 4. These sequences were chosen because they have been previously prepared by the T7 transcription method and have well

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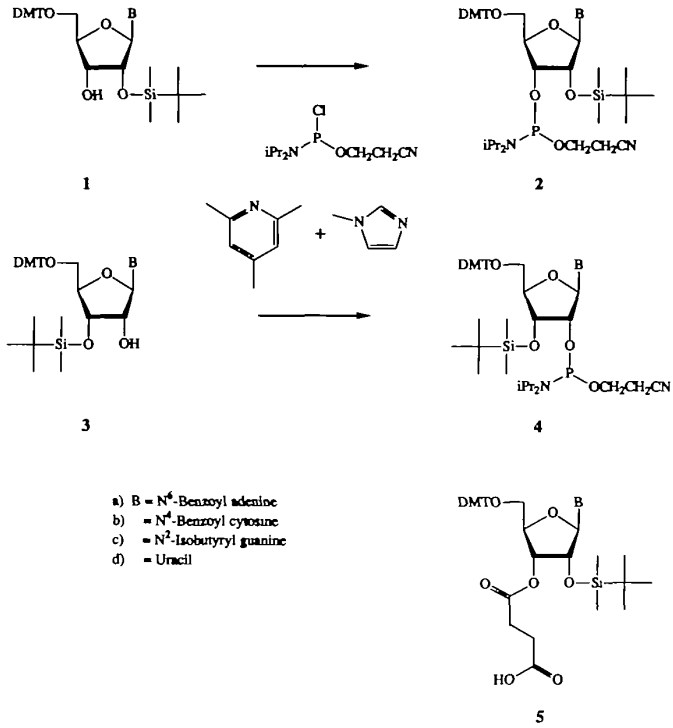

Figure 1: Synthetic scheme.

Table 1 ^{31}P Chemical Shifts and Difference Shifts (ppm)

	2'Si, 3'P Down Field	2'Si, 3'P Up Field			3'Si, 2'P Down Field	3'Si, 2'P Up Field		$\Delta\delta_1$	$\Delta\delta_2$
2 a	151.926	149.913		4 a	151.347	151.010		-0.579	+1.097
2 b	150.925	150.166		4 b	152.143	148.744		+1.218	-1.422
2 c	151.742	149.721		4 c	151.368	150.552		-0.374	+0.831
2 d	150.743	150.326		4 d	151.010	150.204		+0.267	-0.122

$\Delta\delta_1$ & $\Delta\delta_2$ are the differences between the downfield and upfield shifts of the two regioisomers, respectively.

characterized biochemical activities (*vide infra*). They are also of sufficient length to demonstrate the efficacy of the procedure as it relates to coupling efficiency. All solid phase syntheses were performed on commercially available DNA Synthesizers (see experimental). Syntheses were conducted on a 0.5 μmol scale using derivatized 1000Å CPG (average loading = 10−15 μmol g^{-1}) solid supports. These supports were prepared by the coupling of compounds **5a-d** to LCAA-CPG. A thirty-fold excess of ribonucleoside phosphoramidite and a 400 fold excess of tetrazole were used. Other reagents and details are described in the experimental section.

Deprotection, Purification, and Characterization of Chemically Synthesized Oligoribonucleotides

Deprotection

There are several variables which must be considered during the deprotection of chemically synthesized oligoribonucleotides. It is necessary that the protecting groups be completely removed and that no nucleoside base modification or phosphodiester linkage isomerization occur. Using the ensemble of protecting groups described above, only two steps were required to completely deprotect the oligoribonucleotide. First, ammonia treatment of the support bound oligoribonucleotide removes the *N*-acyl protecting groups, eliminates the β-cyanoethyl phosphate protecting group, and cleaves the RNA from the CPG support. Second, TBAF removes the TBDMS groups.

The standard conditions for the first deprotection step have been a 3:1 mixture of NH_4OH and ethanol for 16 hr at 55 °C (5). Ethanol was included to increase the solubility of the silyl protected oligoribonucleotide. It was subsequently found that the addition of ethanol strongly suppressed hydrolysis of the silyl protecting group (17). In the absence of ethanol, NH_4OH removed a significant proportion of silyl groups which led to cleavage of the neighboring phosphodiester linkage. It has recently been reported that the use of methanolic ammonia at room temperature also avoids the loss of silyl groups during the ammonolysis step (18).

These observations led us to extend the observed ameliorative effect of alcoholic NH_3. For ammonia deprotection at higher temperatures we prepared ethanolic ammonia, which is milder and less volatile than methanolic ammonia. To test the ability of ethanolic ammonia to completely remove the *N*-acyl protecting

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Table 2 ^{1}H Chemical Shifts (ppm)

	H8 or H6	H2 or H5	$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5'}$	$H_{5''}$	O-CH_3	P-O-CH_2-	-CH_2-CN	tBu-Si	Me-Si	Me-Si
2 a	8.70 s	8.23 s	6.08 d	5.09 dd	4.43 m	4.37 m	3.62 m	3.33 dd	3.76 s	3.92 m	2.64 t	0.74 s	-0.04 s	-0.22 s
	8.68 s	8.20 s	6.03 d	5.06 dd	4.41 m	4.35 m	3.58 m	3.28 dd	3.76 s	3.86 m	2.63 t	0.74 s	-0.07 s	-0.24 s
2 b	8.49 bs	4.35 bs	5.98 d	4.41 bs	4.32 m*	4.28 m*	3.65 m	3.63 m	3.81 s	3.86 m	2.58 t	0.90 s	0.23 s	0.14 s
	7.85 bs	4.34 bs	5.86 d	4.40 bs	4.30 m*	4.26 m*	3.53 dd	3.48 dd	3.80 s	~3.6 m	2.38 t	0.89 s	0.22 s	0.13 s
2 c	7.87 s	-	5.89 d	4.87 dd	4.30 d	4.33 bs	3.51 dd	3.12 dd	3.76 s	4.00 m	2.83 t	1.14 s	0.005 s	-0.09 s
	7.78 s	-	5.65 d	5.24 dd	4.25 d	4.17 bs	3.48 dd	2.96 dd	3.74 s	3.97 m	2.77 t	0.77 s	-0.22 s	-0.24 s
2 d	7.98 d	5.29 d	5.96 d	4.42 dd	4.32 bs*	4.29 bs*	~3.6 m*	~3.5 m*	3.779 s	3.92 m	2.63 t	0.89 s	0.12 s	0.10 s
	7.90 d	5.25 d	5.88 d	4.33 dd	4.30 bs*	4.20 bs*	~3.6 m*	~3.5 m*	3.785 s	3.90 m	2.62 t	0.87 s	0.11 s	0.09 s

* = not resolved, bs = broad singlet, s = singlet, d = doublet, dd = doublet of doublets, m = multiplet, - = N.A.

Table 3 Coupling Constants (Hz)

	$J_{1'-2'}$	$J_{2'-3'}$	$J_{3'-4'}$	$J_{4'-5'}$	$J_{4'-5''}$	$J_{5'-5''}$	J_{H-H} CH_2-CH_2-CN	J_{H5-H6}
2 a	6.4	6.0	NR	~5	~3	~7	5.1	-
2 b	1.2	3.2	NR	~2	~2	7.7	6.1	~3
2 c	8.0	4.6	~0	~1	2.5	11	6.5	-
2 d	5.3	4.5	NR	NR	NR	NR	4.3	7.7

NR = not resolved, - = N.A.

Table 4

Name	Sequence 5'-3'	Length
S	GCG CCG AAA CAC CGU GUC UCG AGC	24
R	GGC UCG ACU GAU GAG GCG C	18
μ-Helix	GGG GCU AUA GCU CUA GCU CCA CCA	24
m-Helix	GGG GCU AAG CGG UUC GAU CCC GCU UAG CUC CAC CA	35
1.	TTTTTrATTTT (2% 5'-2')	10
2.	TTTTTrATTTT	10
3.	TTTTTrGTTTT	10

groups, we subjected N^2-Bz guanosine to the reagent for 12 hr at 55 °C. Under these conditions guanosine is completely deprotected. Removal of the Bz protecting group from guanosine has a half life more than twice that for the removal of *i*Bu from guanosine (19). In our system the removal of the latter is the rate limiting deprotection step in the ammonia deprotection.

To assay the extent of silyl goup loss during treatment with ethanolic ammonia, we synthesized the dimers rU(2'-Si)pdT and rU(2'-Si)pdC, which were then cleaved from the support at room temperature in ethanolic ammonia and purified by TLC followed by HPLC (see experimental). Equal aliquots were treated with NH_4OH, 3:1 NH_4OH:ethanol, and ethanolic ammonia at 55 °C for 16 hr. Following removal of the ammonia solutions *in vacuo* the reaction products were separated by reverse-phase HPLC. In the case of rU(2'-Si)dC the retention time (t_R) of the protected dimer was 30.5 min. NH_4OH alone resulted in considerable loss of the silyl group producing rUdC (t_R= 27.2 min) coupled with cleavage of the dimer to yield dC (t_R=25.7 min). The 3:1 mixture showed much less silyl protecting group loss, in agreement with a previous study (17). In the case of ethanolic ammonia no detectable silyl group loss could be detected by HPLC. This result was further confirmed by the appearance of the crude sequences by gel analysis (*vide infra*). As a result of these observations all oligoribonucleotides were deprotected with ethanolic ammonia at 55 °C for 16 hr.

The second step in the deprotection the oligoribonucleotides is removal of the TBDMS groups from the 2'-hydroxyls with a 1.0 M solution of TBAF in THF. In the study by Stawinski *et al.* (17) it was shown that removal of the silyl group from dimer models was not accompanied by phosphate migration nor phosphate cleavage and that the silyl groups were quantitatively removed in ~6 hours. The authors did note, however, that longer deprotection times would be beneficial for longer RNA sequences to ensure complete deprotection. Therefore, we treated our oligoribonucleotides with 50 equivalents of TBAF per TBDMS group for 24 hr. Using base composition analysis, presented below, we determined that the silyl groups were completely removed without modification of the RNA. Although deprotection of the silyl groups is an efficient reaction, it is complicated by the difficulty of removing the excess TBAF. This has classically been accomplished using a time consuming Sephadex desalting column. However, we have found that the excess TBAF may be efficiently removed through the use of an anion exchange cartridge and organic salt buffers, thus reducing the desalting step to a simple 15 min procedure (see experimental).

Purification & Characterization

The oligoribonucleotide products were purified to complete homogeneity using polyacrylamide gel electrophoresis (PAGE). In all cases the desired full-length product was the major species.

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Following elution from the gel, the product was desalted on a reverse-phase C18 cartridge and lyophilyzed. A small aliquot was then analyzed by PAGE to verify purity. Figure 2a shows a UV shadowing photograph of crude minihelix (**mH**) and microhelix (μH). Figure 2b shows an autoradiogram of purified 5′-end labeled **mH** and μH. The profile of the crude sequences, comprised of only a single major band, is indicative of the efficacy of the synthesis and deprotection methods. The overall yield of purified RNA from μmol of nucleoside (attached to CPG) to the purified final product was ~20−30%.

To determine that the nucleoside bases had been fully deprotected and that no base modifications occurred, we employed a 2D TLC base composition assay as previously described (20). Complete digestion of the oligoribonucleotides by ribonuclease T_2 followed by labeling with γ-^{32}P ATP, nuclease P_1 digestion, and 2D TLC analysis on cellulose plates showed only the four expected 5′-nucleotides.

To determine the level, if any, of 5′-2′ internucleotide bond formation an additional enzymatic assay was used. We synthesized three DNA/RNA hybrid decamers TTTTTrNTTTT containing at the rN position: 1) adenosine in which 2% of the incorrect isomer **4a** was added to the coupling reaction to simulate the effect of having the wrong isomer incorporated, 2) adenosine, and 3) guanosine, (Table 4, Sequences **1−3** respectively). 5′-End labeling and digestion of these hybrid oligonucleotides by a 5′-3′ linkage specific RNA endonuclease should result in the formation of only a single labeled product of six nucleotides in length and the concommitant disappearence of the starting material. Digestion of these three hybrids with ribonuclease T_2 completely cleaved the parent 10-mers containing adenosine and guanosine as shown in Figure 2c, lanes 4 and 6. In the case of the 2% contaminated oligonucleotide the impurity may be clearly discerned by the presence of uncleaved decamer (Figure 2c, lane 2). The latter result indicates that the 5′-2′ linkages are not cut by our enzyme preparation and that the level of detection is much greater than 2%. DNA/RNA hybrids containing cytidine and uridine nucleotides are not efficiently cut by either ribonuclease T_2 or pancreatic ribonuclease (data not shown). Thus this assay could not be used for hybrids containing cytidine or uridine. Nevertheless the results obtained with the three purine ribonucleotide containing hybrids demonstrate that the conditions for the phosphitylation of the ribonucleosides, and synthesis and deprotection of oligoribonucleotides did not lead to the formation of 5′-2′ linkages.

Having ascertained the chemical purity of the oligoribonucleotides, the biological activity of the chemically synthesized oligoribonucleotides was compared to that of the same sequences prepared by the T7 system in two independant biochemical systems. The first, which involves aminoacylation of small RNA molecules which are fragments of *E. coli* alanine tRNA (21), illustrates the fidelity of a protein-RNA interaction. The second, which is a catalytic hammerhead RNA cleavage system, illustrates the fidelity of an RNA-RNA interaction.

Aminoacylation of an RNA microhelix

The extent of aminoacylation of a microhelix^{Ala} (μH in Table 4) synthesized chemically was compared to that of the same molecule prepared using T7 RNA polymerase. In these reactions, the RNAs were present at a final concentration of 1 A_{260} ml^{-1} and alanine synthetase was added at greater than stoichiometric concentrations (4.7 μM). The maximum extent of aminoacylation, shown in Figure 3a, was reached in < 5 minutes, allowing the

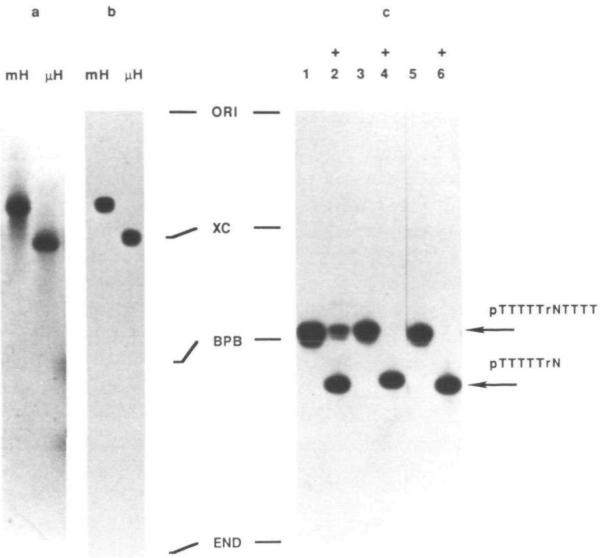

Figure 2: 20% polyacrylamide/7 M urea gels of: **a)** UV shadowing photograph of crude **mH** (35-mer) and μH (24-mer). **b)** Autoradiogram of ^{32}P 5′-end labeled and purified **mH** (35-mer) and μH (24-mer). **c)** Autoradiogram of ^{32}P 5′-end labeled DNA/RNA hybrid sequences **1−3**, in the absence, −, or presence, +, of ribonuclease T_2. Lanes: 1 & 2) 2% impurity adenosine hybrid **1**, 3 & 4) adenosine hybrid **2**, 5 & 6) guanosine hybrid **3**. The positions of the origin (ORI), xylene cyanol FF dye (XC), bromophenol blue dye (BP) and the end (END) are indicated.

total concentration of alanine specific substrate molecules prepared by either method to be determined. The plateau values were 3633 pmoles alanine A_{260}^{-1} for a typical chemically synthesized substrate and 1770 pmoles alanine A_{260}^{-1} for the transcribed species. The value for the chemical substrate compared favorably to the value of 3132 pmoles A_{260}^{-1} obtained for the enzymatically prepared substrate described previously (21).

The second-order rate constants (k_{cat}/K_m) allow the catalytic efficiency of the chemically and enzymatically prepared materials to be compared (see Figure 3b). Initial rates of aminoacylation of both of these substrates with alanine were obtained over a 50-fold range of concentrations from 1 μM to 50 μM of RNA. In these experiments, the concentration of alanine synthetase was 4 nM, which is a catalytic concentration with respect to the RNA concentration. From the slopes of these graphs, values of 1.1×10^4 M^{-1}sec^{-1} and 6.6×10^3 M^{-1} sec^{-1}, were obtained for the chemical and enzymatically synthesized materials, respectively. These values are in good agreement with the value of 7.7×10^3 M^{-1} sec^{-1} reported previously (21).

Hammerhead Ribozyme

In a catalytic system developed by Uhlenbeck (22), a 24-mer substrate molecule, **S** (Table 4), anneals to a 19-mer ribozyme molecule, **R** (Table 4) which upon addition of Mg^{2+}, cleaves the 24-mer substrate specifically at cytidine residue 18. In that study the oligoribonucleotides were prepared using the T7 polymerase method. We therefore tested our chemically synthesized **S** and **R** molecules in a similar manner.

The chemically synthesized **S** and **R** molecules were 5′-end labeled and the full length products isolated by PAGE. The reaction should convert **S** (the 24-mer substrate) to an end-labeled

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18-mer **P** (product) and an unlabeled 6-mer. The experiments were performed with an **S/R** ratio = 1. The results are shown in Figure 4. In lanes 6,7,9, and 10 respectively, 20 mM Mg^{2+} was included to initiate the reaction, resulting in almost complete cleavage of the substrate. In addition a time course reaction (not shown) indicated the $t_{1/2}$ of the reaction was 2.9 ± 0.2 min, which compares favourably to the cited value of 3.0 min (22). To test whether there were any intrinsically inactive substrate molecules an incubation with a 5-fold excess of **R** to **S** was also performed for 6 hr at 37 °C. Overexposure of the resulting autoradiogram showed no detectable **S** molecules (data not shown). In the original description of this system it was noted that approximately 15% of the **S** molecules synthesized by T7 polymerase were uncleavable due to either chemical modification of the DNA template and/or misincorporation by the T7 polymerase (22). Our results indicate that the chemically synthesized system yields more active substrate molecules than the T7 system, allowing for a more detailed study of small ribozyme systems.

Conclusions

Although some of the protecting groups used in this work have been previously described for RNA synthesis, no complete method for chemically synthesizing sequence specific oligoribonucleotides has been generally accepted as reliable and efficient. Skepticism with chemical methods has focused on the potential presence of 5′-2′ linkages, nucleoside base modification, incomplete deprotection, and strand cleavage. Further, the biological activity of the chemically synthesized oligoribonucleotides was never well established and characterized. As a result, the T7 RNA polymerase system quickly took hold as the preferred method for making oligoribonucleotides, despite the RNA length and sequence limitations.

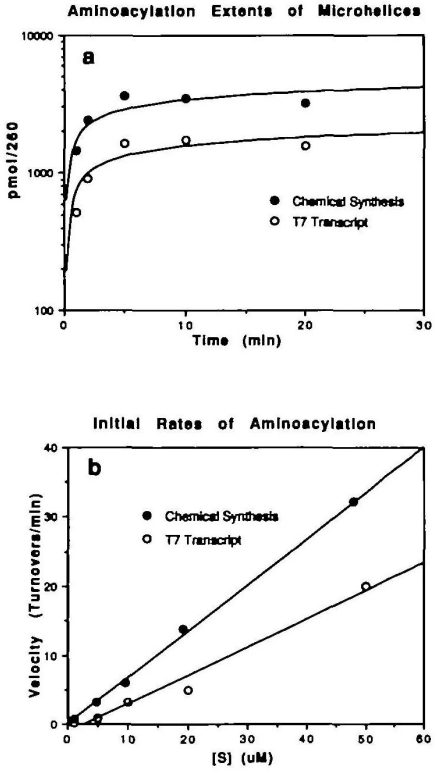

Figure 3: a) Extent of aminoacylation of 24-mer microhelices. b) Concentration dependent initial rates of aminoacylation of 24-mer microhelices.

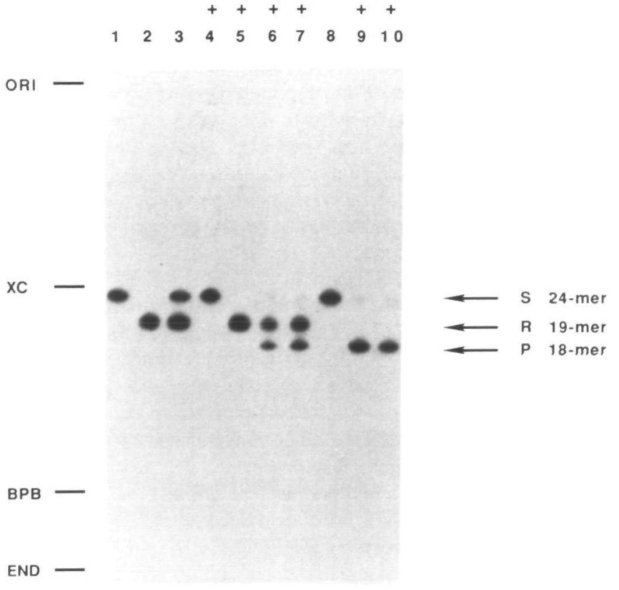

Figure 4: Autoradiogram of 20% polyacrylamide/7 M urea gel of ^{32}P 5′-end labeled **S** and **R** molecules. All reactions were at 37 °C unless noted. Incubation buffer: 50 mM Tris pH 7.5, 0.5 mM EDTA (+ 20 mM Mg^{2+} where indicated). A + indicates the addition of Mg^{2+}. Lanes: 1) **S**, 2) **R**, 3) **S** + **R**, 4) **S** + Mg^{2+}, 5) **R** + Mg^{2+}, 6) **S** + **R** + Mg^{2+}, 7) **S** + **R** + Mg^{2+} (55 °C), 8) **S** + **R**(cold), 9) **S** + **R**(cold) + Mg^{2+}, 10) **S** + **R**(cold) + Mg^{2+} (55 °C). The positions of the origin (ORI), xylene cyanol FF dye (XC), bromophenol blue dye (BP) and the end (END) are indicated.

In this paper we have shown that the ribonucleoside phosphoramidites **2a-d** are > 99.95% isomerically pure. We have further demonstrated that the protecting group strategy used led to the successful preparation, deprotection, and isolation of RNA. The product RNA contained only correct 5′-3′ linkages and was free from detectable nucleoside base modifications. Comparison of chemically synthesized oligoribonucleotides with the same sequences prepared by the well accepted T7 system in two distinct biological assays demonstrated that, biologically, the chemical method produces oligoribonucleotides of equal or better quality, without many of the disadvantages of the T7 system.

EXPERIMENTAL

General Materials and Methods

THF used for the preparation of the nucleoside phosphoramidite reagents was continuously refluxed from Na/benzophenone and distilled prior to use. DMF was distilled from CaH_2 under reduced pressure and stored over 4 Å activated molecular sieves. Pyridine (Baker low-water HPLC) was further dried by shaking over KOH until the slurry was snowy white, then distilled. The distillate was then refluxed and distilled from phthallic anhydride. 2,4,6-Collidine (Aldrich) was dried over 4 Å activated molecular sieves. Chromatographic solvents were Baker HPLC grade. All other chemicals were reagent grade or better.

All reactions for the preparation of the nucleoside phosphoramidite reagents were carried out in septum fitted, oven

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dried, and argon purged round bottom flasks. All liquid reagents and solutions were transferred or added *via* syringe. (*N,N*-diisopropylamino)(cyanoethyl) phosphonamidic chloride was generously donated by Pharmacia and ChemGenes.

Ethanolic ammonia was prepared by bubbling NH_3 gas through absolute ethanol at 0°C. The solution was stored at −20°C and replenished after every fifth use (*N.B.* care must be exercised to exclude moisture). 1M TBAF/THF was obtained from Aldrich and was used for all deprotections. TEAB solution was prepared by bubbling CO_2 through mixture of TEA and water until a single homogeneous phase was formed and the pH reached 7.2 for the 0.1 M and 8.2 for the 2M solutions and then sterile filtered. All H_2O used in the deprotections was sterilized by autoclaving at 120°C for 2 hr. All glass and plasticware were autoclaved. 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 15% or 20% acrylamide/7M urea and were run at 300−1500 V using TBE buffer. 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 detect the labeled RNA.

Enzymes were obtained from Boehringer Mannheim, New England Biolabs, Sigma, and Pharmacia.

Ultraviolet (UV) spectra and colorimetric trityl determinations were obtained on a Hewlett Packard 8451A spectrophotometer.

NMR Analysis

^1H and ^{31}P nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-300 spectrometer equipped with a Varian 5 mm broad band probe. ^1H NMR spectra were referenced to the internal $CHCl_3$ signal in the samples, $\delta=7.24$ ppm. ^{31}P NMR chemical shifts quoted are downfield from 85% H_3PO_4 (external). $CDCl_3$, obtained from Cambridge Isotopes, was used as lock. Conditions for the determination of isomeric impurities: a) 1 ml of 130 mM ribonucleoside phosphoramidite, which corresponds to ~125 mg in 1 ml $CDCl_3$, b) 2048 transients (~30 min) were collected for each sample, c) spectral window from 145−155 ppm, and d) for the low percentage determinations, a series of dilutions beginning with a 5 mg portion of **4a-d** dissolved in 50 μl $CDCl_3$ was prepared and the appropriate amount added to the 1 ml sample.

TLC Analysis

The best results were obtained for thin-layer chromatographic data (R_f values) by loading 0.5−2.0 mg of compound and running the solvent front for 10 cm on pre-run Merck Kieselgel 60 F_{254} analytical glass backed sheets. Note that the silyl isomers isomerize in methanol but isomerization is not a factor with TLC solvent systems using methanol. Silyl isomers **1 a,b,d** were developed in Solvent A. The guanosine isomer **1c** was best resolved in Solvent B.

The ribonucleoside phosphoramidites tend to degrade with extended exposure to silica gel in the absence of base. Triethylamine in the solvent system minimizes this problem but reduces resolution. We have therefore included solvent systems which give the best resolution of silyl starting material, product diastereomers and degradation products for monitoring the progress of the phosphitylation reaction. We have also included TEA solvent systems which resolve the phosphoramidites from degradation products for analysis of the phosphoramidtes after purification.

The dimers rU(2′-Si)pdT and rU(2′-Si)pdC were cleaved from the support at room temperature in ethanolic ammonia and purified on 20 cm long cellulose glass-backed sheets in solvent **J**. The desired product was scraped off and eluted with water and further purified by HPLC (next section).

	Higher Resolution Solvent Systems				TEA Solvent Systems		
Base	Solvent	R_f	R_f	SM R_f	Solvent	R_f	R_f
2a	C	0.83	0.71	0.57	F	0.62	0.62
2b	D	0.71	0.55	0.65	F	0.73	0.59
4b	D	0.51	0.43	0.14	–	–	–
2c	E	0.61	0.61	0.42	G	0.72	0.72
2d	A	0.61	0.61	0.78*	H	0.42	0.42

SM = starting material **1a-d**, R_f = for phosphoramidite diastereomers, * 3′-*O*-acetyl-2′-*O*-silyl (see next section)

Solvent Systems:				
	A	50:50:1	/	$CHCl_3$:Et_2O:MeOH
	B	55:20:25	/	Hex:CH_2Cl_2:Acetone
	C	80:20	/	CH_2Cl_2:Et_2O
	D	35:65	/	Hex:EtOAc
	E	40:60	/	Hex:EtOAc
	F	50:40:10	/	Hex:EtOAc:TEA
	G	30:60:10	/	Hex:Acetone:TEA
	H	40:50:10	/	Hex:EtOAc:TEA
	I	95:5	/	CH_2Cl_2:MeOH
	J	70:30	/	Ethanol:1M NH_4OAc pH 7.0

HPLC Analysis

Analytical HPLC was performed on a Rainin Microsorb C8 column (4.6 mm×25 cm) using a binary buffer system consisting of Buffer A (100mM TEAA pH 7.0) and Buffer B (acetonitrile). A gradient of 100% A (10 min), followed by 0−50% B over 35 min at a flow rate of 1 ml min^{-1} was used to elute the dimers and monomer products.

Synthesis of 5′-*O*-(Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl) ribonucleoside 3′-*N,N*-Diisopropyl(cyanoethyl) phosphoramidites (2a-d). Dry *N*-Acyl-5′-*O*-DMT-2′-*O*-silyl-ribonucleoside **1a-d** (10 mmol, 1 eq) was dissolved in 30 ml dry THF. 2,4,6-Collidine (75 mmol, 7.5 eq) was added followed by *N*-methylimidazole (5 mmol, 0.5 eq). (*N,N*-diisopropyl-amino)(cyanoethyl) phosphonamidic chloride (25 mmol, 2.5 eq) was then added dropwise over 5 min at room temperature. The reaction was complete after one hour as determined by TLC. Uridine starting material could not be separated from the phosphoramidite product by TLC. To verify that reaction went to completion, after workup, a small aliquot was treated with acetic anhydride in pyridine for 15 min. The 3′-*O*-acetyl-2′-*O*-silyl compound was resolved from the phosphoramidite by TLC (see TLC section above). The reaction was diluted with 100 ml ethyl acetate, washed first with 150 ml 5% sodium bicarbonate followed by saturated sodium chloride. The aqueous washes were back extracted with 50 ml EtOAc and the combined organic phases were dried over Na_2SO_4. The solvent was removed *in vacuo* yielding a viscous oil. Coevaporation (× 5) with 50 ml toluene afforded the crude phosphoramidite as an offwhite foam or oil. The ribonucleoside phosphoramidites were further purified by silica gel chromatography yielding a white foam in 75−85% yields. The NMR characteristics of **2a-d** are listed in Tables 1−3.

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Synthesis of 5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl) ribonucleoside 3′-*O*-succinates (5a-d). Pure 5′-*O*-DMT-2′-*O*-silyl-ribonucleoside (2.5 mmol) was dissolved in 15 ml pyridine. Triethylamine (15 mmol, 6 eq) and DMAP (0.5 mmol, 0.2 eq) were added followed by succinic anhydride (7.5 mmol, 3 eq). The reaction was complete after 48 hr of shaking at room temperature. The pyridine was removed *in vacuo* and the residue was then coevaporated with toluene (3×50 ml). The residue was resuspended in methylene chloride, washed with 5% bicarbonate and dried over Na_2SO_4. The presence of ~5% 2′-O-succinate was detected, probably due to isomerization of the silyl starting material by DMAP. The crude succinates were purified by silica gel chromatography. Yields = 65−75%. R_f values in solvent system I: **5a** = 0.62, **5b** = 0.62, **5c** = 0.47, **5d** = 0.55.

Derivatization of Controlled Pore Glass Supports

1000 Å LCAA-CPG (CPG Inc., NJ) was derivatized with compounds **5a-d** following a published protocol (23). Final nucleoside loadings of 10−15 μmol g^{-1} were obtained.

Automated Synthesis of Oligoribonucleotides

All syntheses were conducted on either a Gene Assembler Plus (Pharmacia), or a Cyclone (Milligen/Biosearch) synthesizer using standard protocols with an extended 12 min coupling step. A 30 fold excess (150 μl of 0.1 M = 15 mg, ~15 μmol) of **2a-d** and a 400 fold excess of tetrazole (400 μl of 0.5 M = 200 μmol) relative to CPG-bound 5′-hydroxyl was used in each coupling cycle. Synthesis scale was 0.5 μmol. Average coupling yields on the GA Plus, monitored by an online colorimeter, were ~99.0% and on the Cyclone 97−98%, determined by colorimetric quantitation of the trityl fractions. Reaction columns for 0.5 μmol syntheses were Milligen/Biosearch 1.0 μmol columns. Oligonucleotide synthesis reagents: 1) for GA plus: Detritylation solution was 2% TCA in ethylene dichloride; capping was performed with 20% *N*-Methyl imidazole in THF and 10% acetic anhydride/10% 2,6-lutidine in THF; oxidation solution was 0.02 M I_2, 1% lutidine, 10% water in THF. Baker Bio-Analyzed grade acetonitrile was further dried over activated 4 Å molecular sieves. Tetrazole solution (0.5 M in acetonitrile) was obtained from Applied Biosystems. 2) for Cyclone: All standard DNA synthesis ancillary reagents were used.

Deprotection of Oligoribonucleotides

The CPG-bound oligoribonucleotide was transfered from the synthesis column to a 4 ml glass screw top vial. 1 ml of ethanolic ammonia was added and heated at 55 °C for 16 hr. After cooling to −20 °C, the ethanolic ammonia was removed from the CPG beads and the CPG was washed with 0.5 ml of 50:50/ethanol:water which was then added to the ethanolic ammonia. The combined supernatants containing the oligoribonucleotide were dried to a white powder. To remove the silyl protecting groups, the ammonia-deprotected oligoribonucleotide was resuspended in 50 μl of 50:50/ethanol:water and 600 μl of 1M TBAF/THF and left at room temperature for 12−24 hr. The solution was then added directly to 10 ml of 0.1M TEAB and loaded onto a Qiagen 500 anion exchange cartridge (Qiagen Inc., Studio City, CA) prewashed with 10 ml of 0.1 M TEAB. After washing the cartridge with 5 ml 0.1M TEAB, the RNA was eluted with 7 ml of 2M TEAB and dried down to a white powder.

Gel Purification of Fully Deprotected Oligoribonucleotides

The oligomers were first checked by analytical PAGE (0.75 mm×20 cm×45 cm). 1 ODU of oligoribonucleotide in 5 μl H_2O was added to 5 μl of deionized formamide and the total 10 μl solution was loaded into a 1 cm wide lane. Following electrophoresis the gels were photographed by placing the gel over a fluorescent TLC plate and illuminating the gel with a UV lamp. The desired sequence was established according to electrophoretic mobility and purified by preparative electrophoresis using 1.5 mm thick gels with a single 12 cm wide lane. After electrophoresis the desired band was excised, crushed, placed into a sterile 5 ml test tube, and covered with 50 mM NH_4OAc pH 7.0. The tube was covered and kept at 37 °C O/N. The supernatant was then removed and the gel pieces washed with an additional 1 ml of the extraction buffer. The combined washings were filtered through a 0.45 micron filter and loaded onto a 1 gram size Sep-Pak C18 cartridge (Waters-Millipore) prewashed with 5 ml each of acetonitrile, 50% acetonitrile/0.1M TEAB and 0.1M TEAB. After washing the cartridge with 5 ml of 0.1 M TEAB, the RNA was eluted in 5 ml 35:35:30 acetonitrile/methanol/water and dried down to a white powder.

Ribonuclease T_2 Digestion of DNA/RNA Hybrids

The hybrid decamers **1−3** were deprotected as described above. 100 pmol each of the crude sequences were 5′-end labeled with γ-^{32}P ATP (24) and purified by 20% PAGE. Each reaction contained 2 pmol of decamer, in 2μl H_2O, 8 μl NH_4OAc pH 4.6, and 2 μl of 0.05 U μl^{-1} ribonuclease T_2, + reactions, or 2 μl NH_4OAc pH 4.6, - reactions. The reactions were incubated for 1.5 hr. @ 37 °C and quenched with 10 μl formamide. The reactions were then directly loaded onto a 20% PAGE and analyzed by autoradiography.

Aminoacylation Assays

Microhelix RNA was synthesized with T7 RNA polymerase and a synthetic DNA template using a double stranded promoter region and a long 5′-overhang corresponding to the transcribed sequence, as described (4). T7 RNA polymerase was purified according to Grodberg and Dunn (25). Transcription reactions were incubated for four hr at 37 °C in a buffer containing 40 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol, 1 mM spermidine, 50 mg ml^{-1} bovine serum albumin, 80 mg ml^{-1} polyethylene glycol (relative molecular mass [M_r] = 8000), 20 mM $MgCl_2$, 4 mM of each nucleotide, and 2.5 U ml^{-1} of T7 RNA polymerase. The DNA concentration in each strand was 0.25 mM. The reactions were terminated by phenol/chloroform extraction, precipitated with ethanol, and then fractionated on denaturing 20% polyacrylamide gels. The RNA samples were isolated from the gel by diffusion, precipitated, dried, and then dissolved in TE buffer.

The aminoacylation assays were carried out in a buffer containing 50 mM HEPES (pH 7.5), 100 mg ml^{-1} bovine serum albumin, 20 mM potassium chloride 10 mM $MgCl_2$, 20 mM β-mercaptoethanol, 4 mM ATP, 20 mM alanine, 2.4 mM [2,3-^{3}H] alanine, and varying concentrations of RNA substrates. The reactions were initiated by adding purified alanine synthetase, and then terminated by spotting aliquots of the reaction on Whatman 3MM filter pads that had been been presoaked in 5% trichloroacetic acid. After three washes in 5% trichloroacetic acid and one in 95% ethanol, the pads were dried and the bound radioactivity was quantitated by scintillation counting.

Hammerhead Ribozyme Assays

100 pmol of both **S** and **R** were 5′-phosphorylated using polynucleotide kinase and γ-^{32}P ATP (24). The 5′-end labeled RNA was then purified on a 20% PAGE and the desired band

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excised, eluted, and desalted as described above. The samples were divided into ~2 pmol aliquots for each reaction. The ratio of **S/R** = 1 was used for all experiments. The control buffer consisted of 50 mM Tris pH 7.5, 0.5 mM EDTA and the cleavage buffer contained, in addition, 20 mM Mg^{2+}. The samples, in 10 μl buffer, were incubated for 90 min at 37 °C and quenched by the addition of 10 μl formamide. In the case of the time course experiment, seven samples were used at 0, 1, 2, 4, 8, 16, and 32 min. The reactions were analyzed on a 20% PAGE by autoradiography.

Abbreviations

Ade = adenine, Bz = benzoyl, CPG = controlled pore glass, Cyt = cytosine, dC = deoxycytidine, DABCO = 1,4-diazabicyclo[2.2.2]octane, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DIEA = diisopropylethylamine, DMAP = dimethylaminopyridine, DMT = dimethoxytrityl, DNA = deoxyribonucleic acid, Gua = guanine, *i*Bu = *i*-butyryl, LCAA-CPG = long chain alkylamine-CPG, NH_4OH = 30% aqueous ammonium hydroxide, PAGE = polyacrylamide gel electrophoresis, rN = ribonucleotide, RNA = ribonucleic acid, Si = silyl = *t*-butyldimethylsilyl, T = thymidine, TBAF = tetra-*n*-butylammonium fluoride, TBDMS = *t*-butyldimethylsilyl, TCA = trichloroacetic acid, TEA = triethylamine, TEAA = triethylammonium acetate, TEAB = triethylammonium bicarbonate, THF = tetrahydrofuran, Ura = uracil.

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