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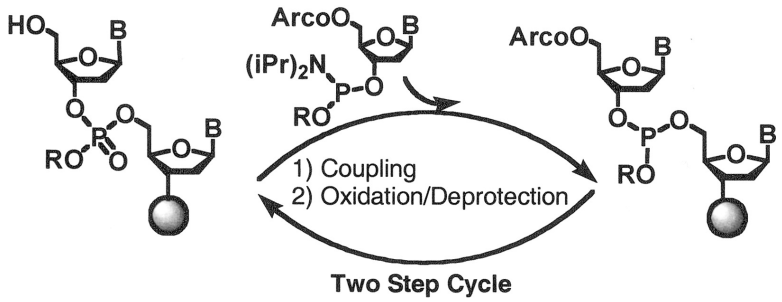

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Solid-Phase Oligodeoxynucleotide Synthesis: A Two-Step Cycle Using Peroxy Anion Deprotection

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Abstract: A novel solid-phase phosphoramidite based oligodeoxynucleotide two-step synthesis method has been developed. Keys to this method are replacement of the 5′-dimethoxytrityl blocking group with an aryloxycarbonyl and the use of *N*-dimethoxytrityl protection for the exocyclic amines of adenine and cytosine. With these modifications, coupling of each 2′-deoxynucleoside 3′-phosphoramidite to the growing oligodeoxynucleotide on the solid support can be followed by treatment with an aqueous mixture of peroxy anions buffered at pH 9.6. This reagent effectively removes the carbonate protecting group and simultaneously oxidizes the phosphite internucleotide linkage. As a consequence a new two-step synthesis cycle is possible. Oligodeoxynucleotides synthesized using this approach are identical to authentic samples when tested by a variety of analytical techniques.

Over the past 20 years, the method of choice for the chemical synthesis of oligodeoxynucleotides (ODNs[1]) has been the phosphoramidite four-step process which utilizes the reaction of deoxynucleoside phosphoramidites with a solid-phase tethered deoxynucleoside or oligodeoxynucleotide (Scheme 1).[2−4] Initially the 5′-*O*-dimethoxytrityl (DMT) group is removed from a deoxynucleoside linked to the polymer support. Step 2, elongation of a growing oligodeoxynucleotide, occurs via the initial formation of a phosphite triester internucleotide bond. This reaction product is first treated with a capping agent designed to esterify failure sequences and cleave phosphite reaction products on the heterocyclic bases. The nascent phosphite internucleotide linkage is then oxidized to the corresponding phosphotriester. In the final step of each cycle, the DMT group is removed from the growing oligodeoxynucleotide using a large excess of a weak acid, trichloroacetic acid (TCA), in an organic solvent. Further repetitions of this four-step process generate the ODN of desired length and sequence. The final product is cleaved from the solid phase and obtained free of base and the β-cyanoethyl phosphate[5,6] protecting groups by treatment of the support with concentrated ammonium hydroxide.[4] ODNs synthesized with this chemistry continue to be of satisfactory quality for most biological uses such as DNA sequencing, PCR applications, and site-specific mutagenesis.

In recent years, an impetus to develop additional strategies for the synthesis of ODNs has emerged due to several new, highly specialized uses of synthetic DNA. For example, a variety of applications in the fields of genomics and high throughput screening have fueled the demand for highly parallel, microscale synthesis of DNA and for DNA sequences attached to planar glass surfaces. An application which exemplifies this trend is DNA microarrays.[7,8] Demand has also increased for the large-scale synthesis of modified DNA useful for therapeutic applications[9−11] and for the synthesis of viruses[12] or genes.[13] Although many genes or gene fragments can be obtained by the use of various biological (cloning) or biochemical (PCR) procedures, there are increasing numbers of examples where this is difficult or impossible. These include the assembly of

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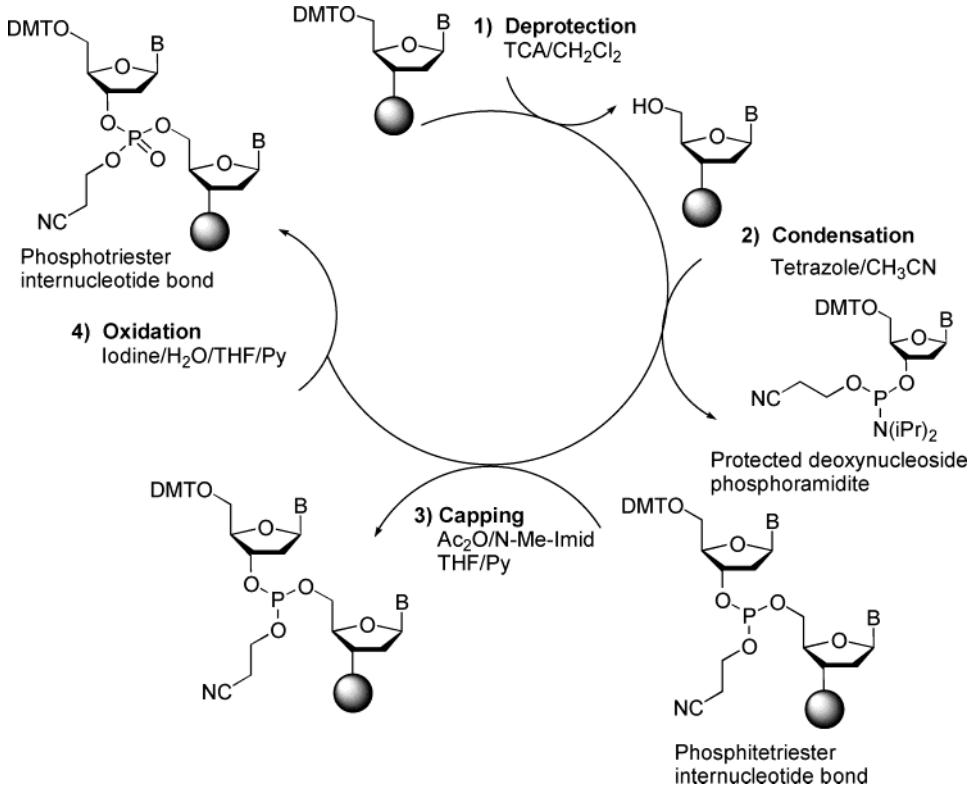
(1) Abbreviations: B, appropriately protected purine or pyrimidine base; CPG, controlled pore glass; DMT, 4,4′-dimethoxytrityl; TCA, trichloroacetic acid; ODN, oligodeoxynucleotide; ARCO, aryloxycarbonyl; MCPBA, *m*-chloroperbenzoic acid; TLC, thin-layer chromatography; TIPS, tetraisopropyldisiloxane-1,3-diyl; DCM, dichloromethane; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; DMSO, dimethyl sulfoxide; Bz, benzoyl; Fmoc, 9-fluorenylmethoxycarbonyl; ^{i}Bu, isobutyryl; DPC, *N*,*N*-diphenylcarbamoyl; *N*-Me-Imid, *N*-methylimidazole; PAP, 4-(phenylazo)phenyl; Fmol, 9-fluorenylmethyl; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

(2) Letsinger, R. L.; Lunsford, W. B. *J. Am. Chem. Soc.* **1976**, *98*, 3655−3661.
(3) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859−1862.
(4) Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* **1981**, *103*, 3186−3191.
(5) Ogilvie, K. K.; Theriault, N. Y.; Seifert, J.-M.; Pon, R. T.; Nemer, J. J. *Can. J. Chem.* **1980**, *58*, 2686−2693.
(6) Sinha, N. D.; Biernat, J.; Koster, H. *Tetrahedron Lett.* **1983**, *24*, 5843−5846.
(7) Fodor, S. A. *Science* **1997**, *277*, 393−395.
(8) Lipshutz, R. J.; Fodor, S. P. A.; Gingeras, T. R.; Lockhart, D. J. *Nature Genet. Microarray Suppl.* **1999**, *21*, 20−24.
(9) Bennett, C. F. *Biochem. Pharmacol.* **1998**, *55*, 9−19.
(10) Yuen, A.; Halsey, J.; Fisher, G.; Advani, R.; Moore, M.; Saleh, M.; Ritch, P.; Harker, G.; Ahmed, F.; Jones, C.; Polikoff, P.; Keiser, W.; Kwoh, J.; Holmlund, J.; Dorr, A.; Sikic, B. American Society of Clinical Oncology's 37th Annual Meeting, San Francisco, CA, May 12, 2001.
(11) Lacy, J.; Loomis, R.; Cheng, Y.-C. *Proc. Assoc. Cancer Res.* **2003**, *44*, Abstract 6438.
(12) Cello, J.; Paul, A. V.; Wimmer, E. *Science* **2002**, *297*, 1016−1018.
(13) Marshall, W. S.; Boymel, J. L. *Drug Discovery Today* **1998**, *3*, 34−42.

Scheme 1



nonnatural, biologically active genes[14] and genes designed for rapid, extensive modifications (cassette mutagenesis) which are useful in studying structure−function relationships.[15,16]

Each of these applications places ever more stringent requirements on this four-step chemical synthesis process. Side-reactions, which are known to occur at detectable but acceptable amounts during routine synthesis, can rise to unacceptable levels under the conditions required for these expanded applications. One example of these side reactions is repetitive exposure of the growing ODN chain to a protic acid which results in depurinated residues from deoxyadenosine and deoxyguanosine.[17−19] In addition to depurination, another related problem associated with acid detritylation is the reversible formation of dimethoxytrityl carbocation. To obtain quantitative deprotection of the DMT group, this carbocation must be flushed from the surface. Otherwise a series of failure sequences develop (retritylation of the 5′-deoxynucleoside hydroxyl due to residual carbocation) that continue to grow in number and size with each synthesis cycle. To ensure purity of synthesized oligodeoxynucleotides free of these side products, rigorous column chromatography is required to minimize mutagenesis from cloned, synthetic DNA.[15,20]

Under the standard synthesis conditions (Scheme 1), the carbocation reversibility problem is minimized by using a large excess of acid to deprotect and flush the trityl group from the solid-phase matrix. However complete removal of the dimethoxytrityl carbocation under conditions of both microscale, on planar glass surfaces, and macroscale syntheses requires even larger volume-equivalents of the protic acid solution and increased reaction times compared to those used during typical laboratory-scale synthesis on porous glass surfaces.[21−23] This excessive exposure to protic acid can cause reductions in product yield and quality primarily due to acid depurination. The presence of depurinated side products can in turn effect the performance of the ODN product in these specialized applications.[24] An alternative light-catalyzed detritylation procedure has been proposed for DNA microarray synthesis.[25,26] However with this approach, only a single exposure to acid detritylation is possible which would maximize the reversible formation of residual dimethoxytrityl containing oligodeoxynucleotides in each cycle.

As these and other applications of phosphoramidite ODN synthesis continue to evolve, further modifications of the synthesis cycle are inevitable. These may include new protecting groups, solvent changes for various steps, different surface materials, and even different synthesis cycles. The work described herein outlines the latter—an alternative synthesis approach that is potentially useful for both macroscale and microscale DNA synthesis. The key to this new approach is

(14) Caruthers, M. H. In *Protein Engineering*; Oxender, D. L., Fox, C. F., Eds.; Alan R. Liss: New York, 1987; pp 65−70.
(15) Ferretti, L.; Karnik, S. S.; Khorana, H. G.; Nassal, M.; Oprian, D. D. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 599−603.
(16) Yu, H.; Kono, M.; McKee, T. D.; Oprian, D. D. *Biochemistry* **1995**, *34*, 14963−14969.
(17) Schaller, H.; Weimann, G.; Lorch, B.; Khorana, H. G. *J. Am. Chem. Soc.* **1963**, *85*, 3821−3827.
(18) Efcavitch, J. W.; Heiner, C. *Nucleosides Nucleotides* **1985**, *4*, 267.
(19) Caruthers, M. H. *Acc. Chem. Res.* **1991**, *24*, 278−284.
(20) Fritz, H. J.; Belagaje, R.; Brown, E.; Fritz, R. H.; Jones, R.; Lees, R. G.; Khorana, H. G. *Biochemistry* **1978**, *17*, 1257−1267.
(21) Paul, C. H.; Royappa, A. T. *Nucleic Acids Res.* **1996**, *24*, 3048−3052.
(22) Septak, M. *Nucleic Acids Res.* **1996**, *24*, 3053−3058.
(23) McGall, G.; Labadie, J.; Brock, P.; Wallraff, G.; Nguyen, T.; Hinsberg, W. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13555−13560.
(24) Temsamani, J.; Kubert, M.; Agrawal, S. *Nucleic Acids Res.* **1995**, *23*, 1841−1844.
(25) Gao, X.; LeProust, E.; Zhang, H.; Srivannavit, O.; Gulari, E.; Yu, P.; Nishiguchi, C.; Xiang, Q.; Zhou, Z. *Nucleic Acids Res.* **2001**, *29*, 4744−4750.
(26) Serafinowski, P. J.; Garland, P. B. *J. Am. Chem. Soc.* **2003**, *125*, 962−965.

Table 1. 5′-Carbonate Protected 2′-Deoxythymidine Derivatives[a]

R	yield %	molecular weight	
		calculated	measured (ESI-MS)
2:4-chlorophenyl	88	396	397 (M + 1), 419 (M + Na), 431 (M + Cl)
3:9-fluorenylmethyl	90	464	465 (M + 1)
4:2-nitrophenyl	35	407	408 (M + 1)
5:4-(phenylazo)phenyl	50	466	467 (M + 1)
6:phenyl	60	362	363 (M + 1)
7:3-(trifluoromethyl)phenyl	70	431	431 (M + 1)
8:2-chloroethyl	70	348	349 (M + 1), 371 (M + Na), 383 (M + Cl)
9:1-chloroethyl	54	348	349 (M + 1)
10:4-nitrophenyl	60	407	408 (M + 1), 430 (M + Na), 442 (M + Cl)
11:4-fluorophenyl	77	380	381 (M + 1), 403 (M + Na)
12:adamantyl	42	420	421 (M + 1), 443 (M + Na)
13:2-chlorophenyl	64	396	397 (M + 1)
14:2,2,2-trichloroethyl	76	416	417 (M + 1), 439 (M + Na)
15:2-methyl-1-chloropropyl	59	376	377 (M + 1)

[a] See Scheme 2 for a definition of R.

the use of aqueous peroxy anions buffered under mildly basic conditions (pH < 10) to remove an aryloxycarbonyl (ARCO) group which is substituted for DMT in the synthesis cycle. This peroxy anion solution is both strongly nucleophilic and mildly oxidizing. The strong nucleophilicity of this reagent permits irreversible removal of the 5′-*O*-carbonate while its mild oxidative potential quantitatively oxidizes the internucleotide phosphite triester without detectable oxidation of the heterocyclic bases. This combination of desirable chemical properties into one reagent generates a new, two-step cycle which uses fewer solvents and eliminates acid-catalyzed detritylation from the synthesis procedures. As a consequence, depurination and reversibility of the detritylation step cease to exist as problems in DNA synthesis.

Results

The basis for this new, two-step approach is to use oxygen nucleophiles that have positive deviations from the Brønsted-type nucleophilicity plot. These molecules are said to exhibit an α-effect—a term describing a nucleophile alpha to an atom having a lone pair of electrons.[27,28] The most extensive class of α-effect nucleophiles are peroxy anions that include perborates, *tert*-butylperoxide (*t*-BuOOH), *m*-chloroperbenzoic acid (MCPBA), and various hydrogen peroxide salts. Our objective is to take advantage of this increased nucleophilicity to deprotect labile carbonates with hydroperoxides that have dissociation constants within the pH range 7−12.

To identify an appropriate carbonate having the desired chemical properties for DNA synthesis, a series of alkyl and aryl 5′-*O*-carbonates (**2**−**15**) of 2′-deoxythymidine were prepared from the corresponding chloroformates and 2′-deoxythymidine (Scheme 2). In all cases, the best yield for the 5′-protected 2′-deoxynucleoside (Table 1) was obtained from reactions carried out in pyridine using a slight excess of the chloroformate (1.05−1.1 equiv). Under these conditions, regioselectivity for the 5′-hydroxyl was observed with most chloroformates.[29] However, the isolated yields varied greatly and were less than for the protection of 2′-deoxynucleosides with DMT. Letsinger[30] and Tittensor[31] previously reported lower

Scheme 2

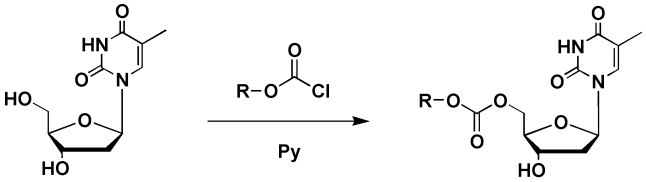


Table 2. Reaction Times for Complete Removal of Carbonate Protecting Groups with Peroxy Anion Buffers[a]

carbonate (R)	A	B	C	D	E
2:4-chlorophenyl	<1 min	<1 min	<1 min	<1 min	>12 h
3:Fmol	>60 min	−	>3 h	−	−
4:2-nitrophenyl	<1 min	<1 min	<1 min	−	−
5:PAP	<1 min	<1 min	<1 min	<1 min	>12 h
6:phenyl	<1 min	<1 min	<1 min	<2 min	>12 h

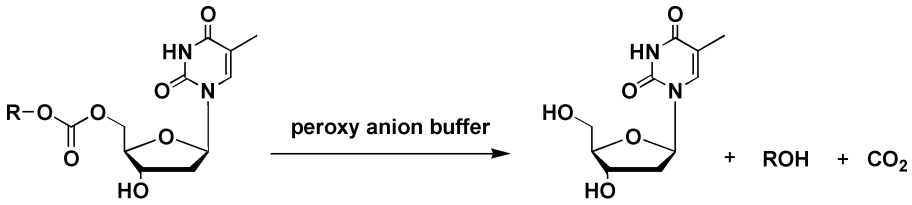
[a] Buffers: A = 3.1% LiOH/H_2O (10 mL), 1.5 M 2-amino-2-methyl-1-propanol pH 10.3 (15 mL), dioxane (50 mL), 30% H_2O_2 (12 mL), pH 12.0. B = As for A except substitute DMSO for dioxane, pH 12.0. C = As for A with MCPBA (1.78 g), pH 9.6. D = H_2O (10 mL), dioxane (50 mL), 2.5 M Tris (15 mL), 30% H_2O_2 (12 mL), MCPBA (1.78 g), pH 9.0. E = H_2O (10 mL), dioxane (50 mL), 2.5 M Tris (15 mL), *t*-BuOOH (0.1 M), pH 9.0.

yields in the synthesis of 2′-deoxynucleoside 5′-carbonates. These reduced yields were caused by the formation of 2′-deoxynucleoside alkyl or aryl carbonates followed by intramolecular elimination to form a 2′-deoxynucleoside 3′,5′-cyclic carbonate. The better leaving groups led to greater cyclic carbonate formation. Our results generally support these conclusions.

A series of initial deprotection studies (Scheme 3) were performed on selected carbonates (**2**−**6**) to assess their susceptibility to removal with peroxy anion. The protected 2′-deoxynucleosides were dissolved in various buffered peroxy anion solutions (Table 2, A−E) and cleavage rates for removal of the carbonate from 2′-deoxythymidine were monitored by TLC. As expected, the carbonate cleavage activity of these buffers was rapid only under pH conditions above the pK_a for peroxy anion formation. For example solutions at pH 9.6 (buffer C) and pH 9.0 (buffer D), where MCPBA was completely ionized, effectively cleaved four of the ARCO groups studied (**2**, **4**, **5**, **6**). Conversely *tert*-butylperoxide (buffer E) failed to remove ARCO groups under reasonably satisfactory reaction times (5−10 min). Among the carbonates that were rapidly cleaved by C and D, compound **2** was selected for further study

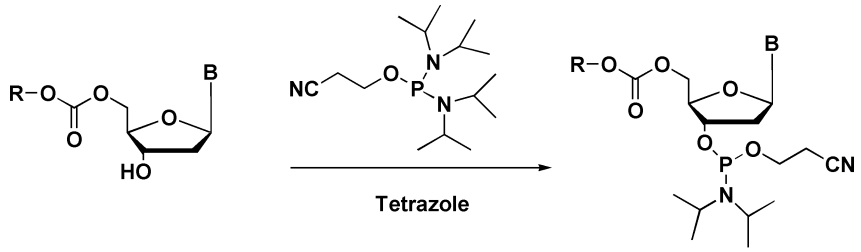
(27) McIsaac, J. E., Jr.; Subbaraman, L. R.; Subbaraman, J.; Mulhausen, H. A.; Behrman, E. J. *J. Org. Chem.* **1972**, *37*, 1037−1041.
(28) Edwards, J. O.; Pearson, R. G. *J. Am. Chem. Soc.* **1962**, *84*, 16−24.
(29) Balgobin, N.; Josephson, S.; Chattopadhyaya, J. B. *Tetrahedron Lett.* **1981**, *22*, 3667−3670.
(30) Letsinger, R. L.; Ogilvie, K. K. *J. Org. Chem.* **1967**, *32*, 296−300.
(31) Tittensor, J. R. *J. Chem. Soc. (C)* **1971**, 2656−2662.

Scheme 3



2-15 **1**

Scheme 4



2, R = 4-chlorophenyl
B = T

16, R = 4-chlorophenyl
B = T

18a-g, R = 3-(trifluoromethyl)phenyl
a, B = T
b, B = C^{Bz}
c, B = C^{Fmoc}
d, B = C^{DMT}
e, B = A^{Bz}
f, B = A^{DMT}
g, B = $G^{iBu,DPC}$

19a-g, R = 3-(trifluoromethyl)phenyl
a, B = T
b, B = C^{Bz}
c, B = C^{Fmoc}
d, B = C^{DMT}
e, B = A^{Bz}
f, B = A^{DMT}
g, B = $G^{iBu,DPC}$

as the synthesis yield was relatively high (Table 1, 88%). Furthermore, compound **5** proved unsatisfactory as a stable side product was generated under these cleavage conditions (perhaps the *N*-oxide of the carbonate).

Although extremely basic conditions (buffers A and B with ionized hydrogen peroxide, pH 12) rapidly removed carbonates, these reagents were eliminated from further consideration for several reasons. One was instability of the linkage joining oligodeoxynucleotide to controlled pore glass (CPG). When 5′-*O*-dimethoxytrityl-2′-deoxythymidine attached to CPG through the standard succinate ester[4] was treated with buffers A and B, complete cleavage of the 2′-deoxynucleoside from the support was observed in 20 min (analyzed by measuring spectrophotometrically at 498 nm the residual dimethoxytrityl carbocation released from CPG upon treatment with toluenesulfonic acid). In contrast less than 5% cleavage was found with buffers C and D after 210 min. These stability studies suggested that minimal loss of growing oligomers from the support was possible with peroxy anion reagents at pH 9−10. Another observation (TLC analysis) was that the standard amides used to protect 2′-deoxynucleoside exocyclic amines[4] were rapidly removed under the conditions of buffers A and B. This was undesirable as phosphitylation of these bases then occurs during oligodeoxynucleotide synthesis. Finally oxidation of unprotected bases and thymine was expected to be a problem at pH 12.[32,33]

The next step in assessing the utility of this chemistry was to prepare the 5′-(4-chlorophenoxycarbonyl) protected 3′-phosphoramidite of 2′-deoxythymidine (Scheme 4, **16**) and test this synthon for the preparation of oligodeoxythymidine. Initially an oligodeoxythymidylate tetramer was prepared on CPG using **16** and an automated DNA synthesizer. The standard cycle as shown in Scheme 1 was modified to use deprotection mixture D for 7 min in place of the 3% trichloroacetic acid (TCA) solution. The resulting product was compared to the same tetramer synthesized using standard 5′-DMT protected 2′-deoxythymidine-3′-phosphoramidite. These oligomers were shown to have identical yield and purity by anion-exchange HPLC and ESI-MS. Because previous research had demonstrated that MCPBA was an effective oxidant for ODN synthesis,[34] the peroxy anion deprotection solution was tested for oxidation of internucleotide phosphite triester linkages. This was accomplished by using **16**, deleting the iodine oxidation step and substituting peroxy anion solution D for TCA (Scheme 1). The results demonstrated that concomitant deprotection and oxidation produced a tetramer of identical yield and purity

(32) Subbaraman, L. R.; Subbaraman, J.; Behrman, E. J. *J. Org. Chem.* **1971**, *36*, 1256−1259.
(33) Subbaraman, L. R.; Subbaraman, J.; Behrman, E. J. *Biochemistry* **1969**, *8*, 3059−3066.
(34) Letsinger, R. L.; Groody, E. P.; Lander, N.; Tanaka, T. *Tetrahedron* **1984**, *40*, 137−143.

Scheme 5

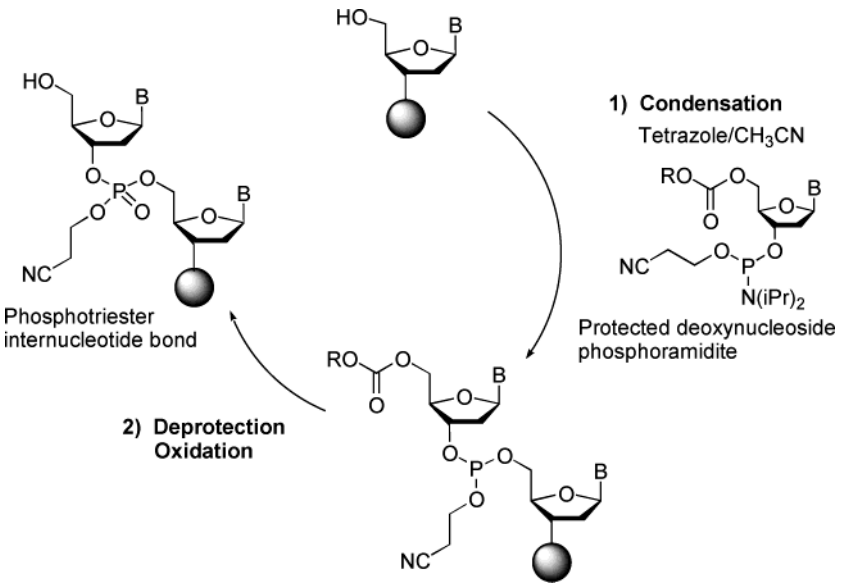


Table 3. Yield of dT_{10} as a Function of pH[a]

pH	% full length product	% stepwise yield
8.43	24.2	85.4
9.17	86.1	98.3
9.31	88.7	98.7
9.60	92.6	99.0

[a] Buffer C where the pH was adjusted with aqueous LiOH.

(anion-exchange HPLC and ESI-MS) to the same compound synthesized using DMT protected 2′-deoxythymidine-3′-phosphoramidite and the standard four-step synthesis cycle.

The decomposition of MCPBA to *m*-chlorobenzoic acid in the presence of LiOH[35] resulted in deprotection mixture D being useful for only a few hours. This not only reduced the effective peroxy anion concentration but also lowered the pH which decreased the nucleophilicity of the remaining reagent. To slow this decomposition process, it was necessary to replace Tris with 2-amino-2-methyl-1-propanol—a buffer having a pK_a near the optimum (pH 9−10) for deprotection with MCPBA. Further studies were then completed with this new reagent to maximize yield as a function of pH (aqueous lithium hydroxide was used to vary the pH).

In this experiment, 2′-deoxythymidine decanucleotides (dT_{10}) were prepared on CPG from **16** and various deprotection buffers using a two-step synthesis cycle (Scheme 5). The first step was condensation of **16** with 2′-deoxythymidine linked covalently to CPG. After appropriate washes, the peroxy anion solution as the second step in this cycle was used to remove the 5′-ARCO group and oxidize the internucleotide linkage to phosphate. The overall yield of dT_{10}, which measures the ability of each buffer at variabale pH to remove the ARCO group, was determined by collecting the products from the support under standard conditions and evaluating the product mixture by anion exchange HPLC (Table 3). For this buffer the highest stepwise yields were observed at pH 9.6. These results established buffer C (Table 2) as the preferred peroxy anion solution which was used in subsequent experiments. An increase above pH 9.6 did not lead to further improvements but actually reduced the overall yield due to cleavage of the 3′-succinyl linkage joining oligodeoxynucleotide to CPG. A final modification was to divide buffer C into a two component system (see Experimental Section). Separating lithium hydroxide from MCPBA and mixing just prior to use allowed the deprotection reagent to remain effective for at least 1 day. This two component mixture was used on an automated DNA synthesizer by either premixing the components in a single bottle or by placing these solutions in the capping port bottles where they were combined in the mixing chamber.

Following these preliminary experiments, an oligodeoxythymidine 12mer was synthesized via the approach outlined in Scheme 5. Synthesis begins with 2′-deoxythymidine linked covalently to CPG via standard procedures. The two-step cycle involved condensation of **16** with 2′-deoxythymidine in the presence of tetrazole followed by treatment for 2.5 min with the peroxy anion solution (buffer C) which oxidized the internucleotide linkage and removed the 4-chlorophenoxycarbonate protecting group. Further repetitions of this cycle followed by cleavage from the support and HPLC analysis of the total reaction mixture generated the profile shown in Figure 1, Panel A. When compared to dT_{12} prepared by the standard four-step cycle (Figure 1, Panel B), there were numerous failure sequences (oligomers less than 12 in length corresponding to 19.6% of the total UV absorbing material). To reduce the amount of these short oligomers, carbonates **7**−**15** (Table 1) were converted to their corresponding 3′-diisopropylphosphoramidites and tested for the preparation of dT_{12}. Generally these carbonates were selected because they were expected to be more labile than 4-chlorophenoxycarbonate toward α-effect nucleophiles. The resulting crude reaction mixtures were analyzed by HPLC. A comparison showed that the 3-trifluoromethylphenoxy derivative (compound **7** or **18a**) generated dT_{12} having the least amount (13.7% of the total UV absorbing material**)** of failure sequences (Figure 1, Panel C). When analyzed by MALDI-TOF spectrometry, this product was found to have the mass (Table 4) expected of d(T_{12}). There were no higher-molecular weight peaks corresponding to products having oxidized thymine. On the basis of these results, further experiments were then directed

(35) Ball, D. L.; Edwards, J. O. *J. Am. Chem. Soc.* **1956**, *78*, 1125−1129.

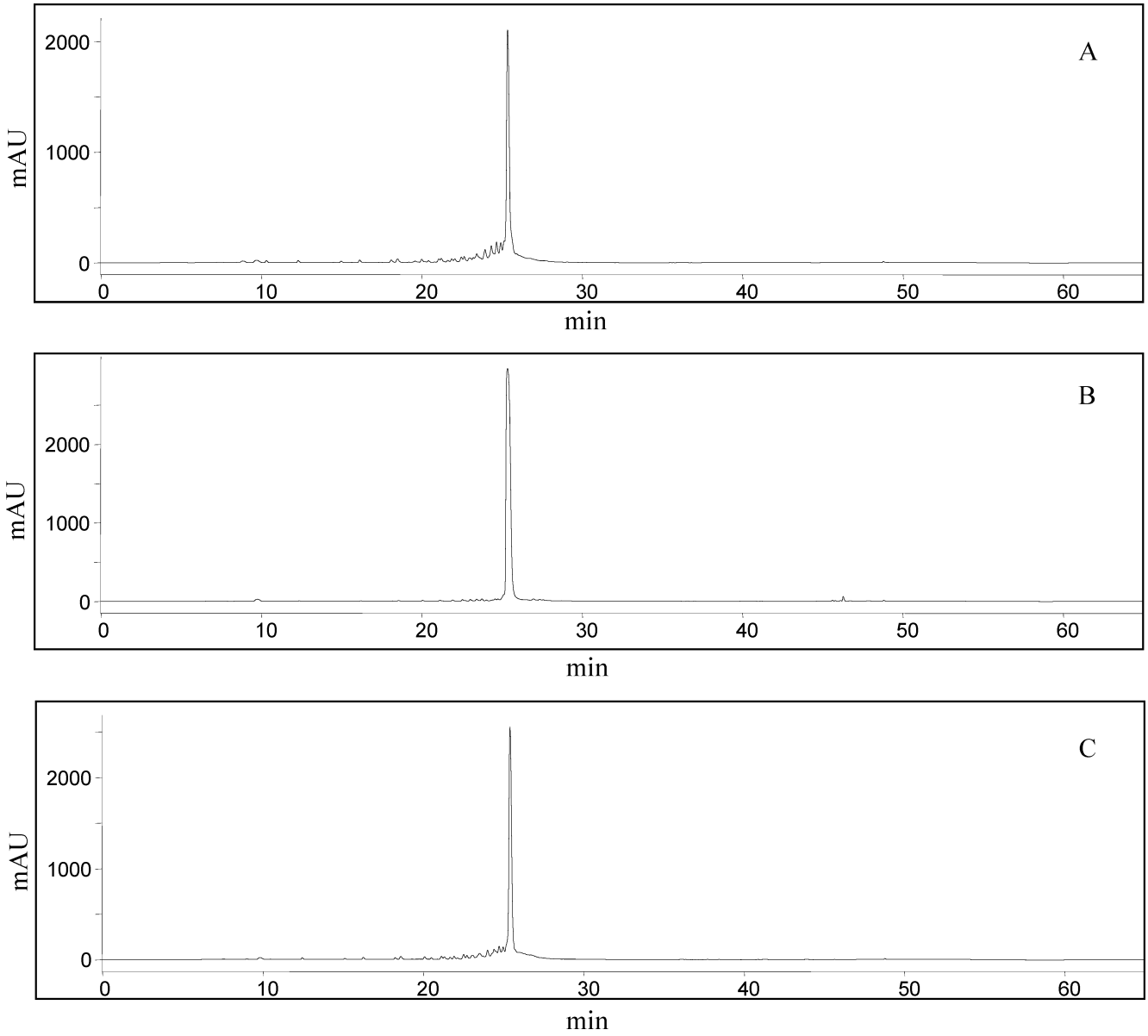


Figure 1. Reverse phase HPLC profiles from total reaction mixtures of dT_{12} prepared on CPG. (A) Two-step cycle with **16**; (B) four-step cycle with 5′-DMT-2′-deoxythymidine-3′-phosphoramidite; (C) two-step cycle with **19a**.

Table 4. MALDI-TOF Analysis of Oligodeoxynucleotides

	molecular weight	
ODN	calculated	measured (MALDI-TOF)
d(TTTTTTTTTTTT)	3059.6	3057.7 (M − 1), 3060.2 (M + 1)
d(CCCCCCCCCT)	2843.5	2842.3 (M − 1), 2844.5 (M + 1)
d(AAAAAAAAAT)	3059.6	3057.7 (M − 1), 3060.2 (M + 1)
d(GTGTGTGTGT)	3103.5	3101.9 (M − 1), 3104.3 (M + 1)
d(CTCTCTCTCT)	2903.5	2902.3 (M − 1), 2904.4 (M + 1)
d(ATATATATAT)	3023.6	3022.7 (M − 1), 3024.7 (M + 1)
d(ATGTCAACTCGTCT)	4211.7	4210.8 (M − 1), 4212.8 (M + 1)

toward the synthesis of oligodeoxynucleotides having all four standard bases.

To investigate the stability of heterocyclic base protecting groups toward the peroxy anion solution, 5′-*O*-DMT and base protected 2′-deoxynucleosides were incubated at room temperature with a large excess of buffer C and monitored by TLC to evaluate the extent of protecting group cleavage as a function of time. These stability half-lives for the 5′-*O*-DMT derivatives of N^6-benzoyl-2′-deoxyadenosine (**17a**), N^4-(9-fluorenylmethoxycarbonyl)-2′-deoxycytidine (**17b**), and N^2-isobutyryl-O^6-(*N*,*N*-diphenyl)carbamoyl-2′-deoxyguanosine (**17c**) were visually estimated to be 30 min (**17a**), 12 h (**17b**), and 24 h (**17c**). Other commonly used amide protecting groups on cytosine (acetyl and benzoyl) were even less stable. After 24 h exposure to buffer C, 5′-*O*-DMT protected 2′-deoxynucleosides were extracted from the aqueous mixture with DCM and analyzed by FAB mass spectrometry. Parent ions for the heterocyclic base protected **17b** and **17c** and corresponding heterocyclic base deprotected 5′-DMT 2′-deoxynucleosides were easily detected. For **17a,** only the heterocyclic base deprotected 5′-DMT 2′-deoxynucleoside was detected. The mass spectra did not contain unexplained parent ions such as those corresponding to oxidative side products of the heterocyclic bases. When analyzed under the same conditions, the parent ion for 5′-*O*-DMT-2′-deoxythymidine was readily observed without detectable ions corresponding to oxidized side products.

On the basis of these observations, the two-step synthesis cycle (Scheme 5) was used to prepare homopolymers of 2′-

Scheme 6

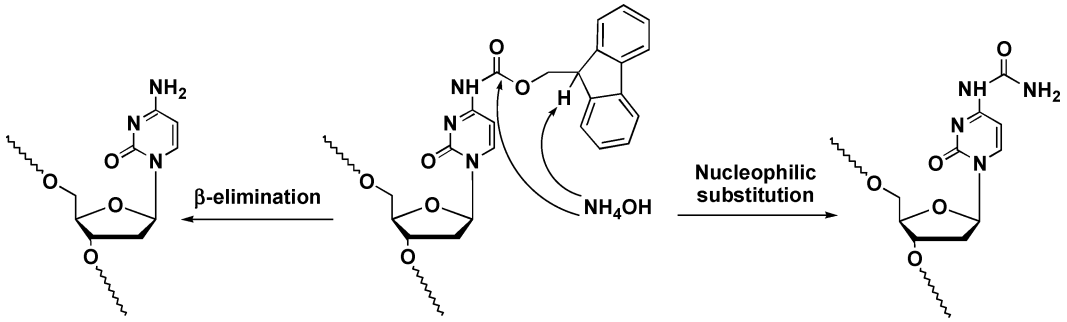


Table 5. Summary of Characterization Data for 5′-ARCO-2′-deoxynucleosides and Corresponding 3′-Phosphoramidites

			molecular weight		
compound	yield %	^{31}P NMR (ppm)	calculated	ESI-MS	HRMS (FAB)
18a	77		431.1066 (M + H)	431 (M + 1)	431.1086
18b	68		520.1332 (M + H)	520 (M + 1)	520.1315
18c	69		638.1750 (M + H)	638 (M + 1)	638.1759
18d	73		717.2298 (M +)	718 (M + 1)	717.2321
18e	69		544.1444 (M + H)	544 (M + 1)	544.1456
18f	60		740.2332 (M − H)	742 (M + 1)	740.2331
18g	80		721.2234 (M + H)	721 (M + 1)	721.2218
19a	76	149.44	631.2145 (M + H)	631 (M + 1)	631.2141
19b	45	149.73, 149.44	720.2410 (M + H)	720 (M + 1)	720.2435
19c	50	149.70, 149.40	838.2829 (M + H)	838 (M + 1)	838.2808
19d	61	149.13, 149.03	917.3377 (M +)	918 (M + 1)	917.3346
19e	65	149.19, 149.16	744.2522 (M + H)	744 (M + 1)	744.2547
19f	74	149.07, 148.98	941.3489 (M +)	942 (M + 1)	941.3510
19g	70	149.48, 149.37	921.3312 (M + H)	921 (M + 1)	921.3353

deoxycytidine (dC_{12}) and 2′-deoxyadenosine (dA_{12}) from **19b** or **19c** and **19e**, respectively (Scheme 4 and Table 5). Problems, however, were observed. Specifically, the HPLC profiles were found to be heterologous with numerous uncharacterized side products. These side products appeared to be due to removal of base protecting groups by the peroxy anion solution followed by phosphitylation of unprotected cytosine and adenine bases. This conclusion was supported by the observation that higher-quality oligomers were obtained when a pyridine hydrochloride/aniline wash (a solution known to reduce phosphitylation of unprotected bases[36]) was introduced into this cycle immediately following phosphitylation but prior to treatment with peroxy anion. Additionally when oligomers generated from **19c** were deprotected with concentrated ammonium hydroxide, a significant amount of side product with a urea modification was characterized by LC-mass spectrometry. Similar results were obtained when oligomers were synthesized by the standard four-step cycle using 5′-DMT-N^4-Fmoc-dC-3′-phosphoramidite. These results suggest that ammonia deprotection is not compatible with N^4-fluorenylmethoxycarbonyl protection of 2′-deoxycytidine. This is because ammonia attack on the protecting group leads not only to the preferred cleavage as dibenzofulvene and carbon dioxide by β-elimination but also, via nucleophilic attack on the carbonyl, elimination of 9-fluorenemethanol and generation of the stable N^4-ureido derivative (Scheme 6). This observation means that an additional non-nucleophilic base deprotection step would be required to use **19c** for oligodeoxynucleotide synthesis.

To overcome the problem of amide protecting group instability with peroxy anion (cytosine and adenine), other types of blocking groups were explored. Among those tested, the most satisfactory results were obtained by using acid labile *N*-trityl groups as these derivatives were completely stable toward peroxy anion solutions.[37] After many preliminary experiments, compounds **18d** and **18f** proved to be the most useful as the *N*-dimethoxytrityl groups could be removed in 2 h (**18d**) and 15 min (**18f**) using 3% TCA in dichloromethane (no base modification). The *N*-dimethoxytrityl 2′-deoxynucleosides were prepared from the appropriate 5′,3′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-deoxynucleosides by treatment with dimethoxytrityl chloride followed by removal of the silyl protecting group with fluoride ion. Alternatively they can be prepared by the transient, Jones silylation procedure.[40] These N-protected 2′-deoxynucleosides were converted first to the 5′-*O*-[3-(trifluoromethyl)-phenoxycarbonyl]-2′-deoxynucleosides (**18d**, **18f**) and then, using standard procedures,[41] to the 2′-deoxynucleoside-3′-phosphoramidites (**19d**, **19f**). Table 5 summarizes yields and characterization data for these synthons.

Using the two-step cycle, 2′-deoxynucleoside 3′-phosphoramidites **19d** and **19f** were tested for synthesis of oligodeoxynucleotides. In each case these synthons were joined repetitively to 2′-deoxythymidine linked to CPG through the 3′-hydroxyl to yield essentially homopolymers of 2′-deoxycytidine and 2′-deoxyadenosine. This strategy is the most rigorous test of any new synthon as problems with the chemistry will be amplified either during synthesis or deprotection. For example, when homopolymers of deoxyadenosine were prepared via the four-

(36) Gryaznov, S. M.; Letsinger, R. L. *J. Am. Chem. Soc.* **1991**, *113*, 5876−5877.

(37) Although *N*-trityl protection is common in the peptide field,[38] it has only been used sparingly in nucleic acid synthesis.[39]

(38) Gross, E.; Meinhofer, J. *The Peptides: Analysis, Synthesis, Biology*; Protection of Functional Groups in Peptide Synthesis, Vol. 3; Academic Press: New York, 1987.

(39) Schaller, H.; Khorana, H. G. *J. Am. Chem. Soc.* **1963**, *85*, 3828−3835.

(40) Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316−1319.

(41) Caruthers, M. H.; Barone, A. D.; Beaucage, S. L.; Dodds, D. R.; Fisher, E. F.; McBride, L. J.; Matteucci, M.; Stabinsky, Z.; Tang, J.-Y. *Methods Enzymol.* **1987**, *154*, 287−313.

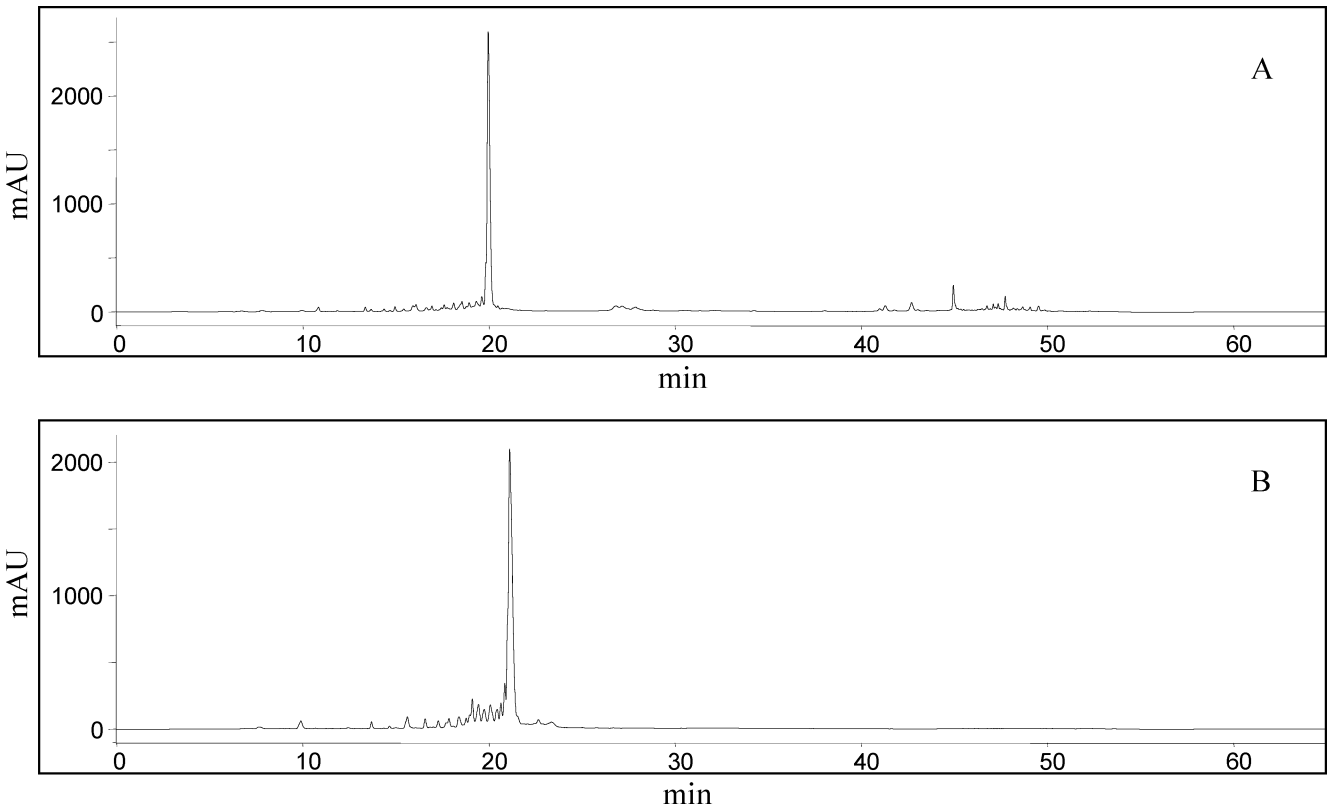


Figure 2. Reverse phase HPLC profiles from total reaction mixtures prepared on CPG. (A) synthesis of d(C_9T) via the two-step cycle using **19d**; (B) synthesis of d(A_9T) via the two-step cycle using **19f**.

step cycle, HPLC profiles clearly showed a series of peaks corresponding to depurinated oligomers.[42] Similarly when the standard thiolate deprotection step was omitted during workup of deoxythymidine homopolymers,[43] a series of *N*-methyl thymidine derivatives were easily detected via HPLC. The results presented in Figure 2 indicate that *N*-dimethoxytrityl protection on 2′-deoxycytidine and 2′-deoxyadenosine can be used in this two-step cycle to prepare homopolymers lacking unexpected side products. For example when d(C_9T) was prepared via this two-step cycle and the total reaction mixture analyzed by reverse phase HPLC (Panel A), one major product peak was observed. There were minor product peaks that eluted both before (failure sequences) and after d(C_9T). When analyzed by MALDI-TOF mass spectrometry (Table 4), the measured molecular weight of d(C_9T) corresponded to the calculated mass, and there were no peaks having increased mass which would be expected for oxidized oligodeoxynucleotides. For 2′-deoxyadenosine, the use of **19f** in the two-step cycle for synthesis of d(A_9T) also generated one major product peak (Panel B) when the total reaction mixture was analyzed by HPLC. Analysis by MALDI-TOF mass spectrometry confirmed that this oligodeoxynucleotide had the molecular weight as calculated for the expected product (Table 4). As observed with d(C_9T), there were no higher molecular weight mass peaks in this sample. These results confirmed that oxidation of the adenine base under these synthesis conditions was not a problem. There was, however, more material present in the failure sequence portion of the chromatography pattern than observed during the synthesis of d(C_9T). Recent research has demonstrated that this effect can be correlated to a slower rate of 5′-carbonate cleavage via peroxy anion due to steric hindrance associated with the *N*-dimethoxytrityl group. Additionally, trace amounts of material still retaining the dimethoxytrityl protecting group were present in both chromatography profiles as can be seen by the series of small peaks following elution of products.

Analysis of **19g** in the two-step synthesis cycle was somewhat complicated because homopolymers of 2′-deoxyguanosine tend to aggregate when analyzed by HPLC. As a consequence this synthon was tested by preparing d$(GT)_5$ where the resulting oligomer could be readily characterized by HPLC and compared with results obtained with d$(T)_{12}$, d$(AT)_5$ and d$(CT)_5$. As shown in Figure 3, Panel A, d$(GT)_5$ was the major product obtained from this synthesis (MALDI-TOF analysis, Table 4). The molecular mass was consistent with a product that had not been oxidized at any of the thymine or guanine bases. Failure sequences and trace amounts of incompletely deprotected oligomers were also present. The peak eluted late from the column was the protecting group product (*N*,*N*-diphenylcarbamate) obtained from cleavage with ammonium hydroxide. Oligomers synthesized as controls, d$(CT)_5$ and d$(AT)_5$, generated HPLC profiles (Figure 3, Panels B and C) similar to those observed for d$(C)_9$T and d$(A)_9$T (Figure 2). Characterization of d$(CT)_5$ and d$(AT)_5$ by MALDI-TOF mass spectrometry gave the expected results (Table 4) with no indication of base oxidation.

To test all four synthons simultaneously (**19a**, **19d**, **19f**, and **19g**), mixed sequence oligomers were prepared. The HPLC profile of the total reaction mixture for one example is shown in Figure 3, Panel D. Again the major peak corresponds

(42) Scaringe, S. A. Design and Development of New Protecting Groups for RNA Synthesis. Ph.D. Thesis, 1996.
(43) Urdea, M. S.; Ku, L.; Horn, T.; Gee, Y. G.; Warner, B. D. *Nucleic Acids Symp. Ser.* **1984**, *16*, 257−260.

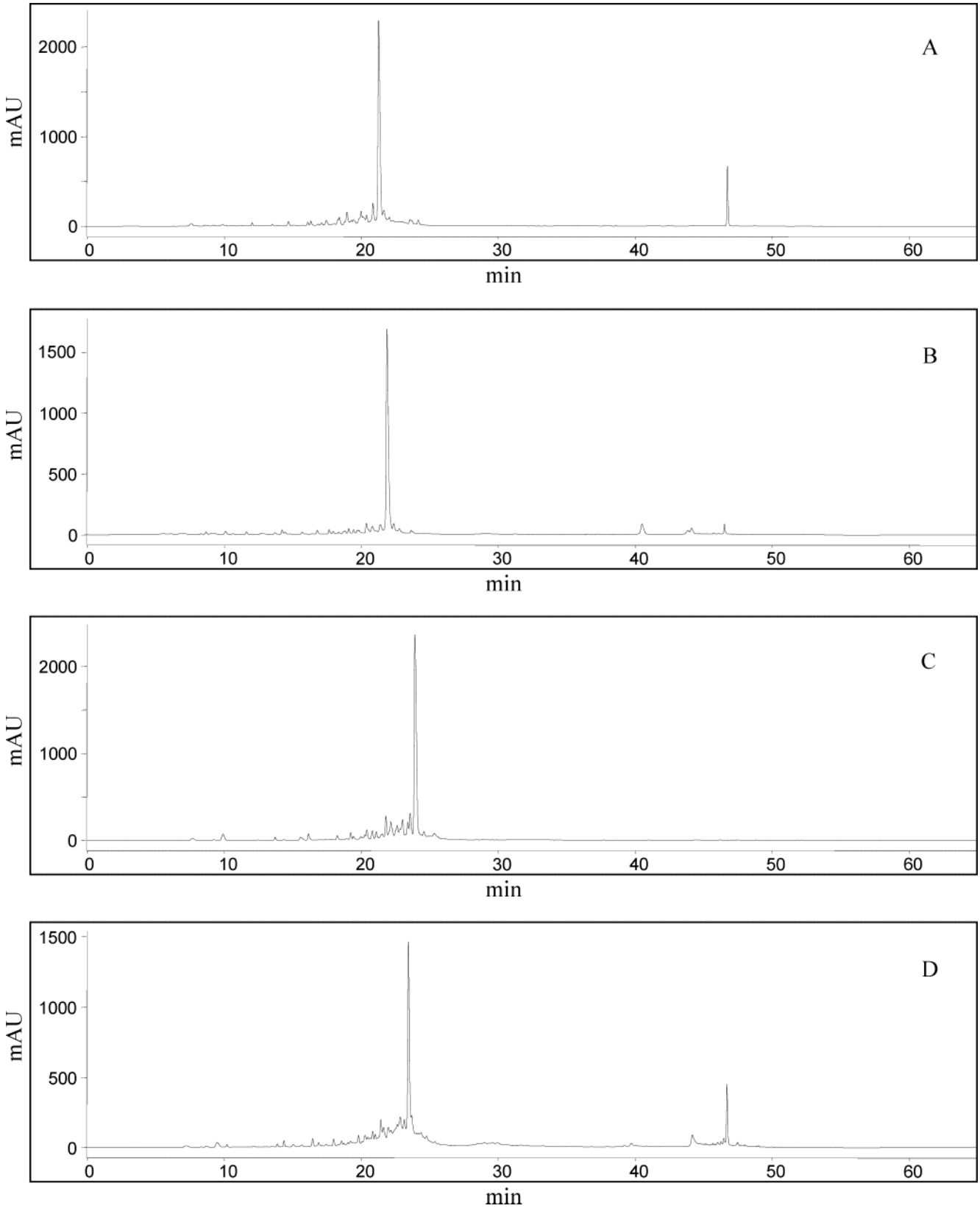


Figure 3. Reverse phase HPLC profiles from total reaction mixtures prepared on CPG. (A) synthesis of $d(GT)_5$ via the two-step cycle using **19a** and **19g**; (B) synthesis of $d(CT)_5$ via the two-step cycle using **19a** and **19d**; (C) synthesis of $d(AT)_5$ via the two-step cycle using **19a** and **19f**; (D) synthesis of d(ATGTCAACTCGTCT) via the two-step cycle using **19a**, **19d**, **19f**, and **19g**.

to product having no modified bases as characterized by MALDI-TOF (Table 4). There were also failure sequences and trace amounts of oligonucleotidic material eluting immediately after the product peak. Late in the elution profile a peak corresponding to the *N*,*N*-diphenylcarbamate was also present.

Although 2′-deoxynucleoside oxidation via peroxy anion deprotection was not detected by mass spectrometry, further experiments designed to test for oxidation were carried out by HPLC analysis of enzymatically degraded oligomers. Oligodeoxynucleotides as isolated from the major peaks shown in the HPLC profiles reproduced in Figures 2 and 3 (Panels A and D) were treated with snake venom phosphodiesterase and alkaline phosphatase followed by HPLC analysis using 2′-deoxynucleoside standards. The results as shown in Figure 4 confirm that oxidation has not occurred as only unmodified 2′-deoxynucleosides were obtained.

To further confirm the integrity of the oligodeoxynucleotides generated using this two-step cycle, additional biochemical and biophysical studies have been completed. For example when the deoxyoligonucleotide whose purification is shown in Figure 3, Panel D, d(ATGTCAACTCGTCT, forms duplexes with complementary DNA and RNA, the melting temperatures of the duplexes (45.1 and 40.1 °C, respectively) correspond to those found using a sample of the same oligomer synthesized by the four-step cycle. Similarly when the heteroduplex of this oligomer and complementary RNA is treated with RNase H1, the RNA degradation profile is identical to results obtained using DNA generated by the four-step cycle (Figure 5). These results demonstrate that ODNs synthesized using the two-step cycle have biochemical and biophysical properties analogous to results obtained using the four-step cycle.

Discussion

In conclusion we have developed an alternative strategy for the preparation of oligodeoxynucleotides. The approach utilizes an α-effect nucleophile (peroxy anion) during each synthetic cycle to simultaneously remove a 5′-carbonate and oxidize the internucleotide phosphite triester (Scheme 5). Relative to the standard approach (Scheme 1), the introduction of this reagent plus elimination of capping leads to a new two-step DNA synthesis cycle that will be useful for all major applications of synthetic DNA. There are several key features that makes this alternative cycle superior for cloning synthetic DNA, the large-scale preparation of DNA, and the synthesis of highly parallel, microscale DNA arrays. First the cyclical removal of the 5′-protecting group with peroxy anion under mildly basic conditions is essentially nonreversible and quantitative. This procedure therefore completely eliminates depurination and one series of failure sequence which are the side reactions most responsible for the high mutation frequencies in cloned, synthetic DNA and the reduced quality of ODNs synthesized in large scale or on micro-arrays. Second the two-step procedure streamlines ODN synthesis by eliminating several reagents. This should result in dramatic cost savings for the large scale synthesis of ODNs. For highly parallel, microscale DNA synthesis, this streamlined procedure allows for simpler and potentially more robust automation.

Several chemical steps leading to this new two-step cycle require further comment. One is elimination of the capping step. Removal of this procedure is possible because phosphite adducts on protected bases [*N*-dimethoxytrityl and O^6-(*N*,*N*-diphenyl)-carbamoyl] do not appear to be a problem. Presumably this is because protection of this type leads to bases that are unreactive toward phosphitylation. As a consequence, the capping step is only needed to block the further growth of failure sequences and can thus be eliminated for many applications as the repetitive coupling yields with 2′-deoxynucleoside phosphoramidite synthons is so high (>99%).[44] This is especially the case for syntheses with 2′-deoxythymidine and 2′-deoxycytidine as the repetitive yields and the quality of synthetic DNA is essentially identical to results obtained using the four-step cycle (compare Figure 1, Panel B to Figure 1, Panel C and Figure 2, Panel A). With capping in the four-step cycle (Figure 1, Panel B), the small amount of unreacted oligomer per cycle ceases to grow to longer DNA and thus the total reaction mixture appears remarkably clean. Without capping (Figure 1, Panel C and Figure 2, Panel A) in the two-step cycle, these minute amounts of unreacted oligomers continue to grow and generate visible, longer-length failure sequences. For syntheses with protected purines, a considerably larger amount of material is present in the failure sequence regions of HPLC profiles (Figure 2, Panel B and Figure 3, Panel A). Unpublished results clearly demonstrate that these lower repetitive yields are due to a slower rate of cleavage of the 5′-carbonate, a consequence of the large steric bulk of the dimethoxytrityl and *N*,*N*-diphenylcarbamoyl groups (adenine and guanine, respectively). Complete removal of carbonates must be achieved, otherwise this two-step cycle will generate a series of mutated sequences similar in composition to those from the reversibility of carbocation cleavage. Current research is focused on testing less bulky, acid labile protecting groups such as amidines.[45−47]

Several additional features of this new cycle require comment. By introducing *N*-dimethoxytrityl blocking groups on cytosine and adenine, an acid step must be used during deprotection. Although generally effective, small amounts of partially protected oligomers still remain when reaction products are analyzed by HPLC. This was especially the case with *N*-dimethoxytrityl protected cytosine (Figure 2, Panel A). Recent research has focused, with encouraging results, on designing more acid labile blocking groups for this base (*N*-trimethoxytrityl). Of particular interest as well was the observation that acid removal of dimethoxytrityl groups did not lead to depurination. Perhaps this is because the product of detritylation is 2′-deoxyadenosine which is much more stable toward acid depurination than the amide protected derivative. The latter, which is present throughout synthesis in the standard four-step cycle, must survive multiple rounds of acid exposure. Finally the use of peroxy anion does not lead to oxidized bases. This was shown by mass spectrometry analysis of synthetic ODNs and the complete degradation of these oligomers to the expected, unoxidized 2′-deoxynucleosides.

Although these results encourage us to further develop this two-step cycle, several key goals must still be attained. These include examining the mutation frequency of cloned, synthetic DNA prepared by this procedure and testing the approach in large scale oligomer synthesis. Of particular interest is recent research on glass slides where we have successfully prepared dT_{25} and shown it to be the only significant product peak when

(44) A capping step can always be added for specific applications.
(45) McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983**, *24*, 2953−2956.
(46) Froehler, B. C.; Matteucci, M. D. *Nucleic Acids Res.* **1983**, *11*, 8031−8036.
(47) McBride, L. J.; Kierzek, R.; Beaucage, S. L.; Caruthers, M. H. *J. Am. Chem. Soc.* **1986**, *108*, 2040−2048.

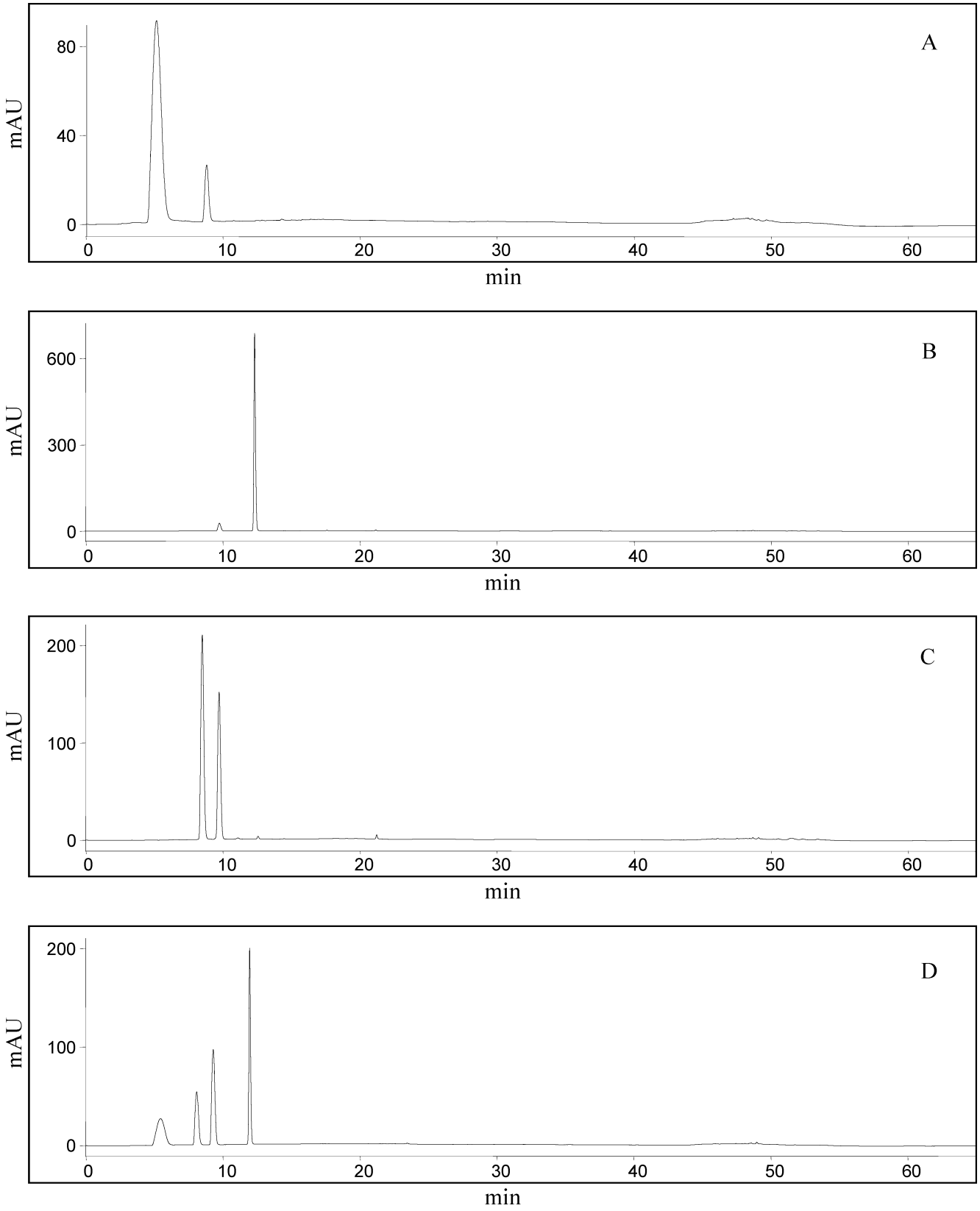


Figure 4. Reverse Phase HPLC profiles following treatment of ODNs with snake venom phosphodiesterase and alkaline phosphatase. (A) $d(C_9T)$; (B) $d(A_9T)$; (C) $d(GT)_5$; (D) d(ATGTCAACTCGTCT).

analyzed by HPLC. Current work is directed toward extending this research on glass slides to the synthesis of ODNs having all four bases.

Experimental Section

General Procedures. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purifica-

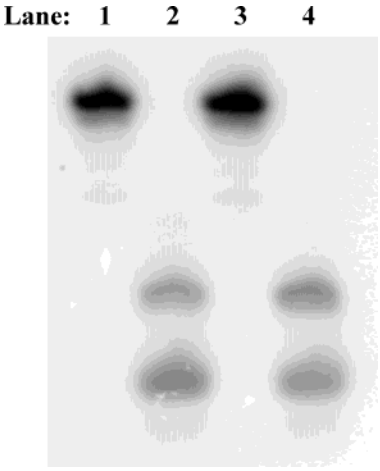


Figure 5. *E. coli* RNase H1 degradation of RNA. 5′-^{32}P-labeled-RNA was allowed to form duplexes with complementary DNAs, d(ATGTCAACTCGTCT), which were then treated with *E. coli* RNase H1 for 1 h and analyzed by PAGE. Lane 1: four-step cycle synthesized DNA, RNA; Lane 2: four-step cycle synthesized DNA, RNA, enzyme; Lane 3: two-step cycle synthesized DNA, RNA; Lane 4: two-step cycle synthesized DNA, RNA, enzyme.

tion. DCM and pyridine were distilled from calcium hydride. MCPBA was obtained 55−83% pure from Aldrich and used without further purification. Aqueous hydrogen fluoride (48%) was obtained from Mallinckrodt. 2-Amino-2-methyl-1-propanol was obtained as a 1.5 M aqueous solution (pH 10.3) from Sigma Diagnostics Inc. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (ChemGenes Corp.) was purified by distillation under high vacuum. All other DNA synthesis reagents were obtained from Glen Research Corp. Oligoribonucleotides were obtained from Dharmacon Research Inc., Louisville, CO. Medium-pressure, preparative column chromatography was performed using 230−400 mesh silica gel from EM Sciences. Thin-layer chromatography was performed on aluminum-backed silica 60 F_{254} plates from EM Sciences (Solvent A: 9/1 $CHCl_3$/methanol; Solvent B: 19/1, DCM/ethanol). Solid-phase ODN synthesis was performed on an ABI 394 automated DNA synthesizer from ABI/PE Biosystems. Reverse phase HPLC on an ODS-Hypersil column from Agilent Technologies (50 mM aqueous TEAB, pH 8.0, and acetonitrile as eluants) and ion exchange HPLC on a DNAPac column from Dionex (25 mM Tris buffer/0.05% acetonitrile, pH 8.0, and 1 M NaCl as eluants) were performed on an Agilent Technologies 1100 HPLC. NMR data were recorded on Varian Inova-400 MHz, Varian Inova-500 MHz and Bruker 400 MHz spectrometers. Tetramethylsilane was used as an internal reference for ^{1}H and ^{13}C NMR. An external capillary containing 85% H_3PO_4 was used as a reference for ^{31}P NMR. Downfield chemical shifts were recorded as positive values for ^{31}P NMR. MALDI-TOF mass data was obtained on a Voyager DE-STR instrument from Perseptive Biosystems by comparison to an internal ODN standard. ESI and FAB mass spectroscopy was performed by the University of Colorado Central Analytical Laboratories. Elemental analysis was performed by Huffman Labs, Golden, CO.

N^4-(9-Fluorenylmethoxycarbonyl)-2′-deoxycytidine (dC^{Fmoc}) was synthesized according to a published procedure.[48] 2′-Deoxyadenosine (dA), 2′-deoxycytidine (dC), 2′-deoxyguanosine (dG), N^6-benzoyl-2′-deoxyadenosine (dA^{Bz}) and N^4-acetyl-2′-deoxycytidine (dC^{Ac}), N^4-benzoyl-2′-deoxycytidine (dC^{Bz}) and N^2-isobutyryl-2′-deoxyguanosine (dG^{iBu}) were purchased from ChemGenes Corporation.

Melting Temperatures. Melting points (T_m's) were determined on a Varian Cary 1E UV−visible spectrometer. The absorbance at 260 nm was measured while the temperature of the sample was increased at a rate of 1.0 °C/min. Oligodeoxynucleotide samples prepared by the two-step cycle were separately mixed with complementary RNA and DNA in a 1 mL cuvette (1 μM duplex, 0.1 M KCl, 0.1 M KH_2PO_4, pH 7.0) and the T_m's determined as the maximum of the first derivative of the melting curve. Control experiments were performed using DNA prepared by the four-step cycle.

Peroxy Anion Buffer. The peroxy anion buffer was prepared as two components. Solution A was 3% (w/v) aqueous LiOH (10 mL), 1.5 M 2-amino-2-methyl-1-propanol in water (15 mL), and dioxane (17.5 mL). Solution B was *m*-chloroperbenzoic acid (1.78 g), aqueous 30% H_2O_2 (10 mL), and dioxane (32.5 mL). Equal volumes of solutions A and B were mixed just prior to DNA synthesis and placed on port 10 of an ABI/PE Biosystems model 394 DNA synthesizer. A fresh mixture of these two solutions was prepared daily.

Synthesis of 5′,3′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-deoxynucleosides. 2′-Deoxynucleoside (10 mmol) was coevaporated three times with pyridine. Anhydrous pyridine (35 mL) and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (3.47 g, 11 mmol) were added, and the mixture was stirred overnight at room temperature. The solution was concentrated and chromatographed on silica gel with 6% methanol in $CHCl_3$.

5′,3′-*O*-(TIPS)-2′-deoxycytidine: yield 99.9%; TLC R_f (A) 0.30; ^{1}H NMR (DMSO-d_6) δ 7.63 (dd, 1H), 7.17 (bd, 2H), 5.97 (t, 1H), 5.69 (dd, 1H), 4.48−4.44 (m, 1H), 4.04−3.91 (m, 2H), 3.73−3.70 (m, 1H), 2.36−2.18 (m, 2H), 1.07−0.90 (m, 28H); ^{13}C NMR (DMSO-d_6) δ 165.73, 154.88, 140.49, 93.84, 84.18, 83.87, 69.36, 61.37, 17.30, 17.16, 17.12, 17.08, 16.91, 16.77, 16.68, 12.79, 12.54, 12.30, 11.98; HRMS (FAB) calcd for $C_{21}H_{40}N_3O_5Si_2$. (M + H) 470.2507, found 470.2486.

5′,3′-*O*-(TIPS)-2′-deoxyadenosine: yield 92%; TLC R_f (A) 0.43; ^{1}H NMR (DMSO-d_6) δ 8.24 (s, 1H), 8.09 (s, 1H), 7.45 (bs, 2H), 6.29−6.27 (m, 1H), 5.18−5.16 (m, 1H), 3.90−3.75 (m, 3H), 2.87−2.54 (m, 2H), 1.11−0.97 (m, 28H); ^{13}C NMR (DMSO-d_6) δ 156.15, 152.31, 148.48, 139.89, 119.43, 84.34, 82.17, 71.58, 62.66, 38.98, 17.39, 17.23, 17.21, 17.17, 17.07, 16.92, 16.86, 16.81, 12.71, 12.55, 12.16, 12.02; HRMS (FAB) calcd for $C_{22}H_{40}N_5O_4Si_2$ (M + H) 494.2619, found 494.2612.

Synthesis of *N*-Dimethoxytrityl-2′-deoxynucleosides. 5′,3′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)-2′-deoxynucleoside (10 mmol) was coevaporated three times with pyridine and then dried in vacuo for 12 h. Anhydrous pyridine (30 mL) and dimethoxytrityl chloride (3.6 g, 11 mmol) were added The mixture was stirred at room temperature until TLC (Solvent A) showed complete disappearance of the 2′-deoxynucleoside substrate (16−24 h). The reaction was quenched with water/ice. Crude product was extracted with DCM, washed with a 5% aqueous solution of $NaHCO_3$, and dried with anhydrous Na_2SO_4. After filtration the organic layer was concentrated and dried in vacuo for 3 h. Hydrogen $fluoride_{aq}$ (1.4 mL, 35 mmol) was carefully added with vigorous stirring to an ice-cold solution of TEMED (7.5 mL, 50 mmol) in acetonitrile (20 mL). The TEMED-HF reagent so formed was then transferred via Teflon tubing to a flask containing crude 5′,3′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-*N*-dimethoxytrityl protected deoxynucleoside (10 mmol), and the mixture was stirred for 2.5 h. The solution was concentrated in vacuo and the residue coevaporated sequentially with pyridine, toluene, and ethanol. The crude product was purified by column chromatography using $CHCl_3$/pyridine (99.9:0.1) with a gradient of methanol (0−8%).

***N*^{4}-Dimethoxytrityl-2′-deoxycytidine:** yield 98%; TLC R_f (A) 0.30; ^{1}H NMR (DMSO-d_6) δ 8.36 (bs, 1H), 7.69 (d, 1H), 7.28−7.17 (m, 5H), 7.12 (d, 4H), 6.83 (d, 4H), 6.22 (d, 1H), 6.03 (t, 1H), 5.17 (d, 1H), 4.94 (t, 1H), 4.16−4.13 (m, 1H), 3.72 (s, 6H), 3.52−3.48 (m, 2H), 2.05−1.86 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 163.30, 157.43, 154.01, 145.13, 139.62, 137.00, 129.92, 128.51, 127.45, 126.09, 112.72, 96.34, 87.17, 84.65, 70.63, 69.31, 61.50, 54.99; HRMS (FAB) calcd for $C_{30}H_{31}N_3O_6$ (M^+) 529.2213, found 529.2200.

***N*^{6}-Dimethoxytrityl-2′-deoxyadenosine:** yield 91%; TLC R_f (A) 0.36; ^{1}H NMR (DMSO-d_6) δ 8.42 (s, 1H), 8.32 (s, 1H), 7.28−7.26

(48) Koole, L. H.; Moody, H. M.; Broeders, N. L. H. L.; Quaedflieg, P. J. L. M.; Kuijpers, W. H. A.; van Genderen, M. H. P.; Coenen, A. J. J. M.; van der Wal, S.; Buck, H. M. *J. Org. Chem.* **1989**, *54*, 1657−1664.

(m, 5H), 7.19 (d, 4H), 6.84 (d, 4H), 6.32 (t, 1H), 5.31 (d, 1H), 5.12 (t, 1H), 3.86−3.84 (m, 1H), 3.71 (s, 6H), 3.61−3.47 (m, 2H), 2.79−2.74 (m, 1H), 2.27−2.22 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 157.69, 153.68, 151.16, 148.07, 145.34, 140.37, 137.27, 129.77, 128.39, 127.71, 126.47, 121.05, 113.00, 88.04, 84.12, 70.92, 69.61, 61.83, 54.99; HRMS (FAB) calcd for $C_{31}H_{31}N_5O_5$ (M^+) 553.2325, found 553.2309.

Synthesis of *N*-Dimethoxytrityl-2′-deoxynucleosides via the Jones Procedure. 2′-Deoxynucleoside (25 mmol) was coevaporated three times with pyridine and then dried in vacuo for 12 h. Anhydrous pyridine (125 mL) and chlorotrimethylsilane (16 mL, 125 mmol) were added. After stirring this solution for 2 h, dimethoxytrityl chloride (8.59 g, 26.25 mmol) was added and the reaction mixture stirred overnight at room temperature. Water (150 mL) and concentrated ammonium hydroxide (5 mL) were added, and the reaction mixture was stirred for 30 min. The crude product was extracted with dichloromethane, and the organic phase was washed twice with a 5% aqueous solution of sodium bicarbonate and then dried with anhydrous Na_2SO_4. After filtration, the dichloromethane solution was concentrated and the product obtained by silica gel column chromatography using $CHCl_3$/pyridine (99.9:0.1) with a gradient of methanol (0−8%). Yields are 98−99%. Characterization data for the 2′-deoxyadenosine and 2′-deoxycytidine derivatives are in the previous section.

Synthesis of N^2-isobutyryl-O^6-(*N*,*N*-diphenyl)carbamoyl-2′-deoxyguanosine. N^2-isobutyryl-2′-deoxyguanosine (10 mmol) was coevaporated three times with pyridine and then dried in vacuo for 6 h. Anhydrous pyridine (330 mL), triethylamine (13 mL), and *N*,*N*-diphenylcarbamoyl chloride (2.32 g, 10 mmol) were added. The mixture was stirred overnight at room temperature. Water (6 mL) was added and the mixture evaporated under reduced pressure. The residue was coevaporated with toluene, ethanol, and DCM and then was purified by column chromatography using $CHCl_3$ with a gradient of methanol (0−5%).

Yield 90.5%; TLC R_f (A) 0.39; ^{1}H NMR (DMSO-d_6) δ 10.72 (s, 1H), 8.67 (s, 1H), 7.48−7.31 (m, 10H), 6.37 (t, 1H), 5.35 (t, 1H), 4.92 (t, 1H), 4.45 (s, 1H), 3.87 (t, 1H), 3.62−3.50 (m, 2H), 2.82−2.74 (m, 2H), 2.34−2.30 (m, 1H), 1.09 and 1.08 (2xd, 6H); ^{13}C NMR (DMSO-d_6) d 174.97, 155.06, 154.54, 152.29, 150.27, 144.04, 141.65, 129.46, 127.32, 120.54, 88.12, 83.65, 70.67, 61.61, 39.30, 34.61, 19.32, 19.30; HRMS (FAB) calcd for $C_{27}H_{29}N_6O_6$ (M + H) 533.2148, found 533.2150.

Synthesis of 5′-*O*-[3-(Trifluoromethyl)phenoxy]carbonyl-2′-deoxynucleosides. N-protected 2′-deoxynucleoside (5 mmol) was coevaporated three times with pyridine, dried in vacuo for 16 h, and dissolved in anhydrous pyridine (50 mL). The solution was cooled over a dry ice/ethanol bath and 3-(trifluoromethyl)phenyl chloroformate (1.19 g, 5.25 mmol) was added. The cooling bath was removed and the reaction mixture shaken until all the reactant was completely dissolved. After stirring overnight at room temperature, the reaction mixture was quenched with water. The product was extracted with DCM, washed with 5% aqueous solution of $NaHCO_3$, and dried with anhydrous Na_2SO_4. After concentration on a rotary evaporator, the crude product was purified by chromatography on a silica gel column using initially $CHCl_3$/benzene (9:1) followed by a gradient of methanol in $CHCl_3$ (for *N*-dimethoxytrityl analogues, 1% pyridine was added to the eluting system).

Compound 18a: yield 77.4%; TLC R_f (A) 0.42; ^{1}H NMR (DMSO-d_6) δ 11.37 (bs, 1H), 7.75−7.51 (m, 5H), 6.25−6.22 (m, 1H), 5.53 (s, 1H), 4.49−4.32 (m, 3H), 4.04−4.00 (m, 1H), 2.24−2.11 (m, 2H), 1.77 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 163.74, 152.61, 150.95, 150.51, 136.07, 131.09, 130.49, 130.23, 125.75, 123.11, 118.72, 109.87, 83.95, 83.22, 70.10, 68.55, 38.52, 12.14; HRMS (FAB) calcd for $C_{18}H_{18}N_2O_7F_3$ (M + H) 431.1066, found 431.1086.

Compound 18b: yield 68.0%; TLC R_f (A) 0.46; ^{1}H NMR (DMSO-d_6) δ 11.32 (bs, 1H), 8.19 (d, 1H), 8.01 (d, 2H), 7.78−7.51 (m, 7H), 7.39 (bs, 1H), 6.22−6.19 (m, 1H), 5.57 (s, 1H), 4.53−4.30 (m, 3H), 4.20−4.16 (m, 1H), 2.37−2.12 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 167.36, 163.20, 154.40, 152.49, 150.97, 145.12, 133.13, 132.76, 131.03, 130.47, 130.22, 128.47, 125.79, 123.09, 118.73, 96.39, 86.54, 84.01, 70.08, 68.56; HRMS (FAB) calcd for $C_{24}H_{21}N_3O_7F_3$ (M + H) 520.1332, found 520.1315.

Compound 18c: yield 69.4%; TLC R_f (A) 0.48; ^{1}H NMR (DMSO-d_6) δ 11.10 (bs, 1H), 8.10 (d, 1H), 7.93−7.33 (m, 12H), 7.06 (d, 1H), 6.18 (t, 1H), 5.55 (s, 1H), 4.49−4.13 (m, 7H), 2.36−2.00 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 162.95, 154.23, 153.18, 152.47, 150.95, 144.84, 143.46, 140.76, 131.01, 130.46, 130.20, 127.81, 127.15, 125.76, 125.59, 123.07, 120.16, 118.71, 94.59, 86.41, 83.94, 70.09, 68.57, 66.93, 46.17, 39.96; HRMS (FAB) calcd for $C_{32}H_{27}N_3O_8F_3$ (M + H) 638.1750, found 638.1759.

Compound 18d: yield 73.1%; TLC R_f (A) 0.43; ^{1}H NMR (DMSO-d_6) δ 8.43 (bs, 1H), 7.77−7.61 (m, 4H), 7.53 (d, 1H), 7.29−7.21 (m, 5H), 7.14 (d, 4H), 6.84 (d, 4H), 6.27 (d, 1H), 6.12 (t, 1H), 5.45 (d, 1H), 4.43−4.22 (m, 3H), 3.97−3.94 (m, 1H), 3.73 (s, 6H), 2.09−1.95 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 163.28, 157.45, 153.91, 152.51, 150.96, 145.09, 139.52, 136.94, 131.03, 130.46, 130.20, 129.93, 128.51, 127.44, 126.10, 125.77, 123.08, 118.75, 112.72, 96.77, 84.68, 83.06, 70.31, 69.38, 68.74, 54.97, 39.10; HRMS (FAB) calcd for $C_{38}H_{34}N_3O_8F_3$ (M^+) 717.2298, found 717.2321.

Compound 18e: yield 69.0%; TLC R_f (A) 0.43; ^{1}H NMR (DMSO-d_6) δ 11.23 (bs, 1H), 8.77 (s, 1H), 8.69 (s, 1H), 8.05 (d, 2H), 7.71−7.55 (m, 7H), 6.55 (t, 1H), 5.65 (d, 1H), 4.64−4.39 (m, 3H), 4.17 (dt, 1H), 2.99−2.94 (m, 1H), 2.49−2.43 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 165.66, 152.61, 151.96, 151.70, 150.91, 150.48, 143.28, 133.36, 132.49, 130.99, 130.44, 130.18, 128.54, 128.49, 125.75, 123.06, 122.49, 118.71, 83.90, 83.73, 70.48, 68.57, 38.42; HRMS (FAB) calcd for $C_{25}H_{21}N_5O_6F_3$ (M + H) 544.1444, found 544.1456.

Compound 18f: yield 60.6%; TLC R_f (A) 0.58; ^{1}H NMR (DMSO-d_6) δ 8.43 (s, 1H), 7.95 (s, 1H), 7.71−7.55 (m, 4H), 7.29−7.22 (m, 5H), 7.20 (d, 4H), 6.84 (d, 4H), 6.40 (t, 1H), 5.59 (d, 1H), 4.59−4.34 (m, 3H), 4.11 (dt, 1H), 3.72 (s, 6H), 2.93−2.87(m, 1H), 2.40−2.34 (m, 1H); ^{13}C NMR (DMSO-d_6) δ 157.70, 153.66, 152.59, 151.37, 150.91, 148.18, 145.35, 140.42, 137.27, 130.97, 130.44, 129.76, 128.37, 127.69, 126.46, 125.74, 123.04, 120.99, 118.70, 112.99, 83.77, 70.50, 69.59, 68.56, 54.97, 38.26; HRMS (FAB) calcd for $C_{39}H_{33}N_5O_7F_3$ (M − H) 740.2332, found 740.2331.

Compound 18g: yield 80.1%; TLC R_f (A) 0.55; ^{1}H NMR (DMSO-d_6) δ 10.75 (bs, 1H), 8.63 (s, 1H), 7.72−7.32 (m, 14H), 6.44 (t, 1H), 5.59 (d, 1H), 4.72−4.42 (m, 3H), 4.15 (dt, 1H), 2.97−2.93 (m, 1H), 2.78 (septet, 1H), 2.45−2.39 (m, 1H), 1.09, 1.08 (2xd, 6H); ^{13}C NMR (DMSO-d_6) δ 174.87, 155.13, 154.27, 152.53, 152.21, 150.93, 150.26, 144.58, 141.61, 130.95, 130.17, 129.45, 127.18, 125.73, 123.01, 120.86, 118.70, 84.25, 84.19, 70.48, 68.83, 38.26, 34.67, 19.26, 19.22; HRMS (FAB) calcd for $C_{35}H_{32}N_6O_8F_3$ (M + H) 721.2234, found 721.2218.

Synthesis of 5′-Carbonate Protected 2′-Deoxythymidines (2−15). 2′-Deoxythymidine (4.84 g, 20 mmol) was coevaporated three times with pyridine, dried in vacuo for 16 h, and then dissolved in anhydrous pyridine (200 mL). The appropriate aryl or alkyl chloroformate (22 mmol) was added and the reaction mixture was stirred overnight. The crude product was purified by silica gel column chromatography (0−10% ethanol in DCM).

Compound 2: yield 88.3%, mp 178−180 °C; TLC R_f (B) 0.22; ^{1}H NMR (DMSO-d_6) δ 7.40 (s, 1H), 7.25 (d, 2H), 7.00 (d, 2H), 6.25−6.20 (m, 1H), 4.40−3.95 (m, 4H), 2.30−2.05 (m, 2H), 1.80 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 164.4, 152.6, 150.5, 149.0, 135.5, 131.5, 129.4, 122.0, 110.9, 84.7, 83.5, 70.2, 67.5, 39.8, 12.0; Anal. Calcd for $C_{17}H_{17}ClN_2O_7$: C, 5l.5; H, 4.3; N, 7.1. Found: C, 51.3; H, 4.5; N, 7.0; ESI-MS: 397 (M + H).

Yields and molecular weight analysis (ESI-MS) are summarized in Table 1 for compounds **3**-**15**. These compounds were prepared by the procedure used to synthesize **2**.

Synthesis of 5′-*O*-[3-(trifluoromethyl)phenoxycarbonyl]-3′-*O*-[(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphino]-2′-deoxynucleoside (19a−g). The protected 2′-deoxynucleoside (3 mmol) and tetrazole (210

mg, 3 mmol) were dried separately in vacuo for 16 h. The protected 2′-deoxynucleoside was then dissolved in anhydrous DCM (30 mL), and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphane (950 mg, 3.15 mmol) was added in one portion. Tetrazole was added slowly to the reaction mixture over 1 h. The reaction mixture was then stirred for another 3 h. Triethylamine (0.5 mL) was added to neutralize the solution. The solvent was removed in vacuo, and the crude product was chromatographed with benzene containing 0.1% Et_3N followed by a gradient of ethyl acetate (0−40%) in benzene containing 0.1% Et_3N (no Et_3N for **19c**).

Compound 19a: yield 76.6%; ^{31}P NMR ($CDCl_3$) δ 149.44; ^{13}C NMR ($CDCl_3$) δ 163.90, 153.10, 153.08, 151.06, 151.03, 150.59, 150.52, 135.63, 135.54, 132.48, 132.22, 130.51, 128.52, 124.63, 123.39, 118.46, 118.43, 117.89, 111.75, 111.70, 85.15, 85.12, 83.03, 82.98, 73.29, 73.16, 72.96, 72.83, 67.95, 67.87, 58.29, 58.21, 58.14, 58.05, 43.59, 43.56, 43.49, 43.46, 39.54, 39.52, 24.81, 24.76, 24.69, 20.69, 20.64, 20.59, 12.74; HRMS (FAB) calcd for $C_{27}H_{35}N_4O_8F_3P$ (M + H) 631.2145, found 631.2141.

Compoound 19b: yield 45.7%; ^{31}P NMR ($CDCl_3$) δ 149.73, 149.44; ^{13}C NMR ($CDCl_3$) δ 166.64, 162.48, 155.05, 153.11, 151.06, 144.21, 133.41, 132.41, 132.14, 130.46, 129.23, 128.50, 127.72, 124.75, 123.37, 123.34, 118.56, 118.54, 117.86, 96.90, 87.50, 83.98, 72.54, 72.42, 67.69, 58.21, 58.07, 43.54, 43.45, 40.88, 40.85, 24.82, 24.76, 24.69, 20.60, 20.54; HRMS (FAB) calcd for $C_{33}H_{38}N_5O_8F_3P$ (M + H) 720.2410, found 720.2435.

Compound 19c: yield 50.3%; ^{31}P NMR ($CDCl_3$) δ 149.70, 149.40; ^{13}C NMR ($CDCl_3$) δ 162.55, 154.89, 153.11, 153.07, 152.39, 151.06, 151.03, 144.02, 143.37, 143.32, 141.49, 132.42, 132.16 130.46, 128.52, 128.17, 127.40, 125.13, 124.74, 124.69, 123.35, 120.34, 118.55, 118.52, 117.86, 117.81, 95.10, 95.03, 87.49, 83.92, 83.59, 72.82, 72.69, 72.59, 72.47, 68.23, 67.72, 67.65, 58.40, 58.25, 58.21, 58.05, 46.78, 43.56, 43.54, 43.46, 43.44, 40.79, 24.79, 24.74, 24.67, 20.61, 20.55; HRMS (FAB) calcd for $C_{41}H_{44}N_5O_9F_3P$ (M + H) 838.2829, found 838.2808.

Compound 19d: yield 61.2%; ^{31}P NMR ($CDCl_3$) δ 149.13, 149.03; ^{13}C NMR ($CDCl_3$) δ 165.56, 158.83, 155.28, 152.98, 152.95, 151.05, 151.01, 144.59, 140.37, 140.31, 136.36, 136.32, 132.07, 130.37, 130.00, 128.64, 128.48, 127.61, 124.68, 124.63, 123.21, 118.44, 118.41, 117.83, 113.72, 95.05, 94.98, 86.84, 86.76, 83.26, 82.97, 82.92, 73.20, 73.06, 72.75, 72.62, 70.27, 67.98, 58.37, 58.22, 58.18, 58.03, 55.38, 43.51, 43.49, 43.41, 43.39, 40.35, 24.78, 24.75, 24.72, 24.69, 20.59, 20.54, 20.49; HRMS (FAB) calcd for $C_{47}H_{51}N_5O_9F_3P$ (M^+) 917.3377, found 917.3346.

Compound 19e: yield 65.2%; ^{31}P NMR ($CDCl_3$) δ 149.19, 149.16; ^{13}C NMR ($CDCl_3$) δ 164.85, 153.08, 153.05, 152.84, 151.59, 151.04, 151.01, 149.79, 141.90, 141.85, 133.71, 132.97, 132.28, 132.01, 130.37, 129.00, 128.02, 124.75, 124.72, 123.75, 123.22, 118.52, 118.49, 117.83, 117.79, 84.93, 83.95, 83.68, 83.63, 73.66, 73.53, 73.21, 73.08, 68.01, 67.88, 58.44, 58.35, 58.28, 43.56, 43.52, 43.46, 43.42, 39.35, 39.31, 39.27, 24.83, 24.76, 24.70, 20.67, 20.62, 20.57; HRMS (FAB) calcd for $C_{34}H_{38}N_7O_7F_3P$ (M + H) 744.2522, found 744.2547.

Compound 19f: yield 74.1%; ^{31}P NMR ($CDCl_3$) δ 149.07, 148.98; ^{13}C NMR ($CDCl_3$) δ 158.45, 154.39, 153.17, 153.13, 152.57, 151.13, 151.10, 148.61, 148.57, 145.58, 138.73, 138.68, 137.61, 132.35, 132.09, 130.37, 130.27, 128.96, 128.52, 128.07, 127.00, 124.84, 124.80, 123.21, 121.70, 118.63, 118.61, 117.82, 117.78, 113.32, 84.73, 83.78, 83.48, 83.43, 73.86, 73.72, 73.34, 73.21, 70.78, 68.25, 68.15, 58.48, 58.35, 55.40, 43.57, 43.52, 43.47, 43.42, 39.03, 24.86, 24.81, 24.78, 24.76, 24.72, 24.70, 20.68, 20.62, 20.58; HRMS (FAB) calcd for $C_{48}H_{51}N_7O_8F_3P$ (M^+) 941.3489, found 941.3510.

Compound 19g: yield 70.4%; ^{31}P NMR ($CDCl_3$) δ 149.48, 149.37; ^{13}C NMR ($CDCl_3$) δ 175.20, 156.36, 156.33, 154.51, 154.46, 153.16, 153.10, 152.12, 152.10, 151.13, 151.11, 150.60, 143.12, 141.91, 132.26, 131.99, 130.36, 129.33, 128.51, 127.85, 124.85, 124.80, 123.15, 123.13, 122.06, 118.56, 118.53, 117.89, 117.86, 85.53, 85.39, 84.07, 83.90, 83.85, 74.02, 73.88, 73.57, 73.45, 68.32, 68.17, 58.43, 58.28, 43.59, 43.56, 43.49, 43.46, 38.93, 36.35, 24.83, 24.78, 24.71, 20.63, 20.60, 20.57, 20.54, 19.41, 19.38; HRMS (FAB) calcd for $C_{44}H_{49}N_8O_9F_3P$ (M + H) 921.3312, found 921.3353.

Automated Oligodeoxynucleotide Synthesis. All syntheses were performed on a 0.2 μM scale using CPG columns joined to 5′-DMT protected 2′-deoxythymidine. The DMT protecting group was removed using 3% TCA in DCM prior to automated synthesis. 5′-ARCO modified 2′-deoxynucleoside-3′-phosphoramidites (0.1 M each) in anhydrous acetonitrile were installed at ports 1−4 on the ABI/PE Biosystems model 394 DNA synthesizer. Other solutions unique to this two-step cycle were installed at ports 10 (peroxy anion buffer), 11 (DMF) and 19 (dioxane). Removal of the 5′-carbonate following each 2′-deoxynucleotide addition was completed by treatment with peroxy anion buffer (Buffer C, Table 2, unless specifically substituted in an experiment) for variable times: pyrimidines, 2.5 min; purines, 7 min). After this 5′-carbonate deprotection step, the column was washed with dioxane, DMF and acetonitrile.

Following completion of all synthesis steps (including removal of the 5′-carbonate from the final synthon added to the growing ODN), the product mixture while still joined to CPG was treated with 3% TCA in DCM at r.t. (2 h) to remove the dimethoxytrityl groups from the bases. After a wash with acetonitrile, products were removed from CPG by treatment with concentrated ammonium hydroxide for 2 h at rt. The reaction mixture free of CPG was transferred to a screw cap reactivial fitted with a Teflon−silicon seal, and the solution heated at 55 °C overnight to remove the *N*,*N*-diphenylcarbamoyl protecting group from guanine. The vial was then cooled in an ice bath, the reaction mixture transferred to an eppendorf tube, and the solution evaporated to dryness. The resulting oligomer was dissolved in water containing 5% acetonitrile and the product purified by reverse phase HPLC using an ODS−Hypersil (5 μ) column eluted at 1.5 mL/min. The eluant was 0−20% acetonitrile in 50 mM TEAB (linear gradient, 40 min). Product fractions were concentrated to dryness to remove the volatile buffer and frozen.

MALDI-TOF Analysis of Oligodeoxynucleotides. Oligodeoxynucleotides were purified by HPLC using 50 mM TEAB buffer (pH 8.0) with a 0−20% acetonitrile gradient and then dissolved in acetonitrile/ultrapure water (1:1) to give a final concentration of 0.005 A_{260} units/μL. The matrix solution was prepared by mixing 2′,4′,6′-trihydroxyacetophenone (THAP, 45 mg) and ammonium citrate (4 crystals) in acetonitrile/ultrapure water (1:1, 500 μL). The suspension was vortexed, centrifuged, and the clear matrix solution (0.3 μL) deposited on the probe. The oligodeoxynucleotide solution (0.3 μL) was then deposited on the same spot, and the plate left to dry at r.t. for 5 min. All mass spectra were acquired in both negative and positive ion modes with delayed extraction and reflector operation.

Enzymatic Hydrolysis of Oligodeoxynucleotides. In a typical experiment the reaction mixture (30 μL) containing 0.3 μg of snake venom phosphodiesterase, 4.02 μg of alkaline phosphatase, buffer (32 mM Tris-Cl, 12 mM $MgCl_2$, pH 7.5), and an oligomer (0.5 A_{260} units) was incubated at 37 °C for 4 h. Following heat denaturation of enzymes (95 °C, 3 min), the reaction mixture was diluted to 200 μL with water, centrifuged, and analyzed by reverse phase HPLC [ODS−Hypersil (5 μ) column 4.0 × 250, flow 1.5 mL/min, 0−20% acetonitrile in 50 mM TEAB (linear gradient) in 40 min].

Hydrolysis of RNA Heteroduplexes with *Escherichia coli* RNase H1. 5′-^{32}P-labeled RNA 100,000 cpm/reaction), unlabeled RNA (50 pmole) and complementary oligodeoxynucleotide (50 pmol) were added to a buffer (pH 7.8) containing 20 mM HEPES−KOH, 50 mM KCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol. The duplexes were hybridized by heating briefly to 95 °C and then incubated at 4 °C for 30 min. *E. coli* RNase H1 (Promega) was added (4 units), and the reactions were allowed to proceed for 1 h at 25 °C (28 μL total reaction volume). Aliquots of the reaction mixture (3.5 μL) were quenched with 6.5 μL of 7 M urea and 20 mM EDTA and were stored on ice until analysis by PAGE (20%, 19:1 cross-link). The developed gels were analyzed

using a Molecular Dynamics Phosphorimager (Storm 820) and ImageQuant software (version 5.1).

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