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Solid-Phase Synthesis, Hybridizing Ability, Uptake, and Nuclease Resistant Profiles of Position-Selective Cationic and Hydrophobic Phosphotriester Oligonucleotides

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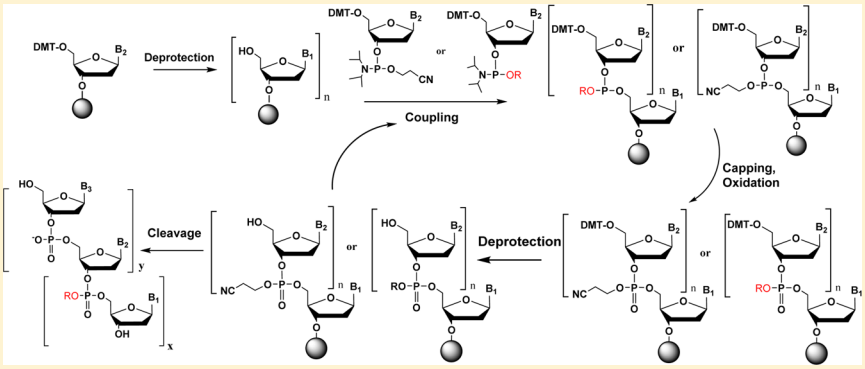
Supporting Information



ABSTRACT: Analogues of oligonucleotides and mononucleotides with hydrophobic and/or cationic phophotriester functionalities often generate an improvement in target affinity and cellular uptake. Here we report the synthesis of phosphotriester oligodeoxyribonucleotides (ODNs) that are stable to the conditions used for their preparation. The method has been demonstrated by introducing phosphoramidite synthons where *N*-benzyloxycarbonyl (Z) protected amino alcohols replace the cyanoethyl group. After synthesis these ODNs were found to be stable to the condition required to remove base labile protecting groups and the ODNs from the solid support. Moreover the use of 1-(4,4-dimethyl-2, 6-dioxocyclohex-1-ylidene) ethyl (Dde) in place of Z protection on the amino alcohol has allowed us to introduce cationic aminoethyl phosphotriester modifications into ODNs. Melting temperatures of duplexes containing cationic or hydrophobic Z modified ODNs indicate that the backbone-phosphotriester modifications minimally affect duplex stability. Nuclease stability assays demonstrate that these phosphotriesters are resistant toward 5′- and 3′-exonucleases. Fluorescently labeled 23-mer ODNs modified with four cationic or hydrophobic Z phosphotriester linkages show efficient cellular uptake during passive transfection in HeLa and Jurkat cells.

INTRODUCTION

Chemically modified oligodeoxynucleotides[1−5] (ODNs) are used for applications in diagnostics,[6] biomedical research, therapeutics,[7] forensics,[8] and material sciences.[9−11] For therapeutic applications, the modifications are introduced to confer properties such as enhanced nuclease stability,[12] improved pharmacokinetics, stronger and more selective binding to complementary DNA/RNA,[13] and improved cellular uptake.[14] To date a large number of ODNs have been tested for biological activity with encouraging results that have stimulated much activity in this area. A particularly challenging problem that still remains is to identify successful and versatile methodologies for the synthesis of modified ODNs in order to increase cellular uptake, possibly to direct potential therapeutic ODNs to specific cell types, and also to sites within cells where these ODNs are biologically active.

One analog that has appeared attractive for applications as a therapeutic ODN is the phosphotriester derivative of DNA. This is because ODNs having phosphotriester linkages would be less negatively charged than natural DNA, hydrophobic, potentially increase hybridization with complementary sequences, and provide a nucleotide nonspecific site within DNA for the introduction of useful functionality (cell receptor agonists, oligosaccharides, cationic amino acids, trafficking of an ODN to a biologically active site, and others).[15−18,19,20] The synthesis of phosphate triester modified DNA has been explored for many years dating from 1971.[18] In addition to simple triesters such as methyl and ethyl, others have been investigated such as l,l-dimethyl-2,2,2-trichloroethyl,[21] isopropyl,[22] neopentyl,[23] *n*-butyl,[24] 2,2,2-trifluoroethyl,[25] phenyl,[26] and dodecyl.[27] To date the most successful synthesis strategies have involved preparing 2′-deoxynucleoside 3′-phosphoramidites where the phosphotriester functionality is contained within these synthons. However, such a strategy is difficult to implement as many conventional solid-phase synthesis parameters must be altered. These include introducing new nucleobase and phosphorus protecting groups,[21a,25,26,28] changing the standard

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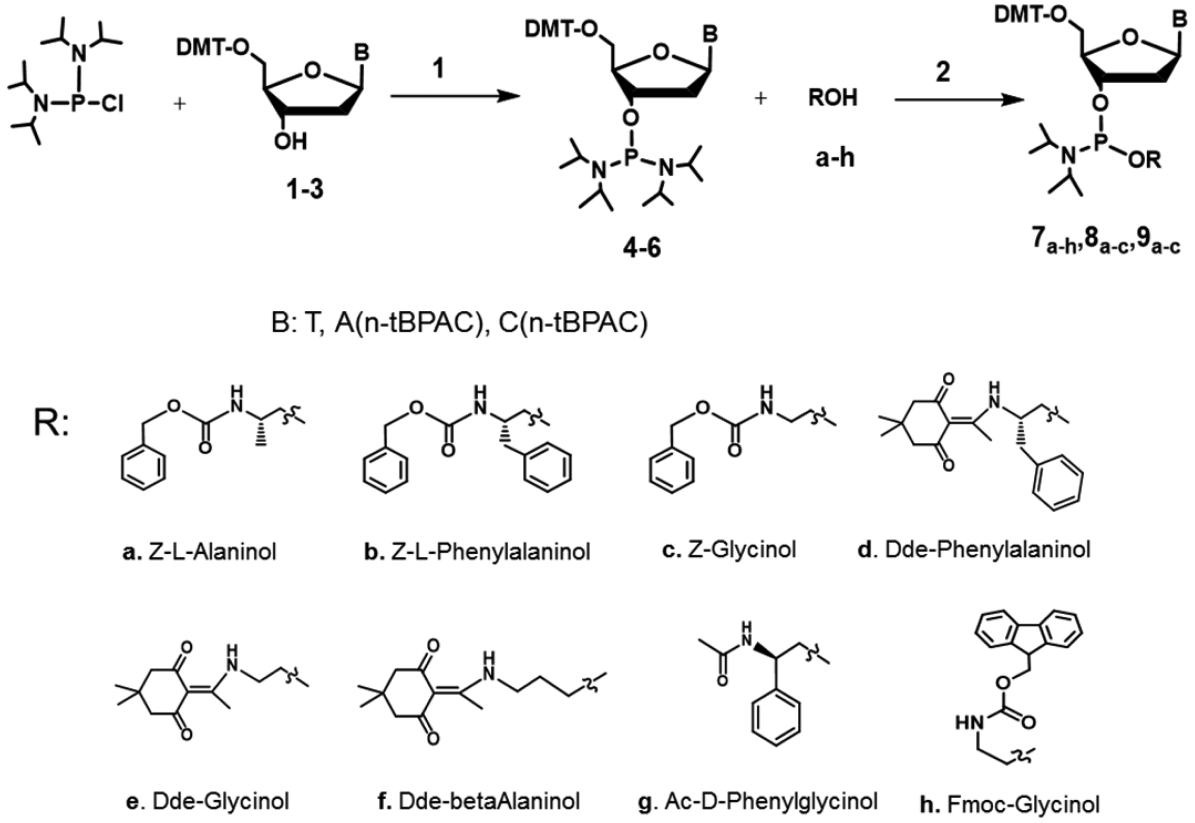
Scheme 1. Synthesis of Appropriately Protected 2′-Deoxynucleoside 3′-Phosphoramidites[a]



[a](1) Dichloromethane + 3 equiv diisopropylethylamine. (2) N-protected amino alcohol (R) + 1 equiv 5-ethylthio-1H-tetrazole. B: Thymine (**1**, **4**, 7_{a-h}), *N*-*tert*-butylphenoxyacetyladenine (**2**, **5**, 8_{a-c}), *N*-*tert*-butylphenoxyacetylcytosine (**3**, **6**, 9_{a-c}). R: (a) Z-L-Alaninol; (b) Z-L-Phenyalaninol; (c) Z-Glycinol, where Z is benzyloxycarbonyl; R: (d) Dde-L-Phenylalaninol; (e) Dde-Glycinol; (f) Dde-*β*-alaninol, where Dde is 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl, R: (g) *N*-Acetylphenyglycinol; R: (h) *N*-Fmoc-Glycinol, where Fmoc is *N*-Fluorenylmethyloxycabonyl.

succinyl linker of the solid support,[26] and, in some cases, altering the coupling and oxidation synthesis steps.[26] For most successful strategies reported to date, except for a few cases,[20,27] only small ODNs containing 10 nucleotides or less and having no more than three sites modified have been reported.

Recently an approach has been proposed that appears to have solved many of the challenges associated with the use of phosphotriesters as possible therapeutic ODNs. This method is based upon earlier work developed by Imbach and his collaborators where they first proposed the use of *S*-acyl-2-thioethyl (SATE) phosphotriesters as monophosphate nucleotide prodrug inhibitors of HIV and Hepatitis B viruses.[29] Imbach's group later introduced this same strategy for synthesizing SATE phosphotriester modified ODNs, but in order to synthesize mixed ODNs they changed the linker of the solid support and the protecting groups on the nucleobases.[30] The research of Meade et al. extends this work to mixed sequence ODNs containing a large number of SATE-type phosphotriesters. These ODNs are not only taken up by cells at 25−100 nM concentration but are converted to biologically active phosphodiester ODNs via cytoplasmic thioesterases.[20] These observations demonstrate for the first time the ability to synthesize and self-deliver ODN phosphotriester conjugates that are intracellularly converted into charged phosphodiester siRNAs that induce biological activity *in vivo*.

In this contribution we describe the synthesis of ODNs carrying amino alkyl phosphotriesters. These ODNs are prepared using standard phosphoramidite chemistry with ultra-mild nucleobase protecting groups. Of particular interest were observations that these ODNs were remarkably stable to the standard solid-phase and ammonia deprotection conditions and form duplexes with complementary, unmodified DNA that are either stabilizing or minimally destabilizing. These phosphotriesters were not degraded by exonucleases and undergo cellular uptake in the absence of lipid transfecting agents.

RESULT AND DISCUSSION

Preparation of N-Protected Amino Alkyl 2′-Deoxynucleoside 3′-Phosphoramidites. In order to introduce the N-protected amino alkyl phosphotriester linkage at any selected site within an ODN, N-protected amino alkyl 3′-phosphoramidite synthons of 2′-deoxyribonucleosides (**7**−**9**, Scheme 1) were prepared from commercially available N-protected amino acid alcohols (a−h, Scheme 1). Synthesis begins by phosphitylation of the 5′-*O*-dimethoxytrityl-2′-deoxyribonucleosides (compounds **1**−**3**) with bis(*N*,*N*-diisopropylamino) chlorophosphine in dichloromethane containing 3 equiv of diisopropylethylamine in order to generate the bis-(diisopropylamino)-3′-phosphorodiamidites (compounds **4**−**6**). After column chromatography, **4**−**6** were allowed to react with 5-ethylthio-1H-tetrazole and the appropriate N-protected amino alcohols to yield 5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside-3′-*O*-(N-protected aminoalkyl)phosphoramidites (Scheme 1, Synthons 7_{a-h}, 8_{a-c}, and 9_{a-c}). These synthons were obtained in high purity after column chromatography with

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Table 1. 2′-Deoxyoligothymidine Phosphotriester Modified ODNs (Z Protected Amino Alcohol Triester Linkages)[a]

Entry	ODN sequence	Obs mass (calc mass)	Yield (%)
ODN1	TT$_a$TTTTTTT	[M-2H]$^{2-}$ 1584.2 (1584.5)	99
ODN2	T$_a$T$_a$T$_a$TTTTTTT	[M-2H]$^{2-}$ 1775.3 (1775.6)	99
ODN3	TT$_b$TTTTTTT	[M-2H]$^{2-}$ 1622.2 (1622.5)	99
ODN4	T$_b$T$_b$T$_b$TTTTTTT	[M-2H]2^{-} 1889.9 (1889.6)	95
ODN5	TT$_c$TTTTTTT	[M-2H]$^{2-}$ 1577.2 (1577.5)	99
ODN6	TT$_c$TT$_c$TT$_c$TT$_c$TT	[M-2H]$^{2-}$ 1842.8 (1843.1)	97
ODN7	TT$_b$TTT$_a$TTTTT	[M-2H]2- 1717.8 (1718.1)	97

[a]Subscripts between nucleotides refer to the type of triester linkage at that site (a, Z-L-Alaninol, b, Z-L-Phenyalaninol, c, Z-Glycinol, see Scheme 1). The remaining internucleotide linkages without subscripts are phosphate diesters.

Table 2. Mixed Sequence 2′-Deoxyoligonucleotide Phosphotriester Modified ODNs (Z Protected Amino Alcohol Triester Linkages)[a]

Entry	ODN sequence	Obs mass (calc mass)	Yield (%)
ODN8	AT$_b$CAGCT$_b$GTT	[M-2H]$^{2-}$ 1794.3 (1794.6)	94
ODN9	AT$_c$TAGAT$_c$GTT	[M-2H]$^{2-}$ 1704.8 (1704.7)	97
ODN10	AT$_c$T$_b$AGAT$_c$GT$_b$T	[M-2H]$^{2-}$ 1967.4 (1971.7)	95
ODN11	T$_a$GTAAACCATGATGTGCTGCT$_a$A	[M-4H]$^{4-}$ 1781.8 (1781.9)	90
ODN12	T$_a$GTAAACCAT$_a$GAT$_a$GTGCTGCT$_a$A	[M-4H]$^{4-}$ 1877.6 (1877.4)	86
ODN13	T$_a$GT$_a$AAACCAT$_a$GAT$_a$GTGCT$_a$GCT$_a$A	[M-4H]$^{4-}$ 1973.1 (1973.0)	84
ODN14	T$_a$GTAAAC$_a$CATGA$_a$TGTGCTGCT$_a$A	[M-4H]$^{4-}$ 1877.5 (1877.4)	80
ODN15	T$_a$GTA$_a$AAC$_a$CATGA$_a$TGTGC$_a$TGCT$_a$A	[M-4H]$^{4-}$ 1973.1 (1973.0)	76
ODN16	T$_b$GTAAACCATGATGTGCTGCT$_b$A	[M-4H]$^{4-}$ 1819.8 (1820.5)	75
ODN17	T$_b$GTAAACCAT$_b$GAT$_b$GTGCTGCT$_b$A	[M-4H]$^{4-}$ 1953.6 (1953.4)	75
ODN18	T$_b$GT$_b$AAACCAT$_b$GAT$_b$GTGCT$_b$GCT$_b$A	[M-4H]$^{4-}$ 2087.2 (2087.0)	65
ODN19	T$_b$GTAAAC$_b$CATGA$_b$TGTGCTGCT$_b$A	[M-4H]$^{4-}$ 1953.6 (1953.4)	65
ODN20	T$_b$GTA$_b$AAC$_b$CATGA$_b$TGTGC$_b$TGCT$_b$A	[M-4H]$^{4-}$ 2087.4 (2087.0)	60
ODN21	T$_c$GTAAACCATGATGTGCTGCT$_c$A	[M-4H]$^{4-}$ 1774.8 (1774.9)	90
ODN22	T$_c$GTAAACCAT$_c$GAT$_c$GTGCTGCT$_c$A	[M-4H]$^{4-}$ 1863.5 (1863.4)	87
ODN23	T$_c$GT$_c$AAACCAT$_c$GAT$_c$GTGCT$_c$GCT$_c$A	[M-4H]$^{4-}$ 1952.1 (1951.9)	85

[a]Subscripts between nucleotides refer to the type of triester linkage at that site (a, Z-L-Alaninol, b, Z-L-Phenyalaninol, c, Z-Glycinol, see Scheme 1). The remaining internucleotide linkages without subscripts are phosphate diesters.

average yields ranging from 60−80%. Characterization of the products by NMR and mass spectral analyses (see Experimental Section) confirmed the assigned structures.

Solid-Phase Synthesis. Modified ODNs (1.0 μmol each) having phosphotriester internucleotide linkages were synthesized on solid supports using N-protected (aminoalkyl)-

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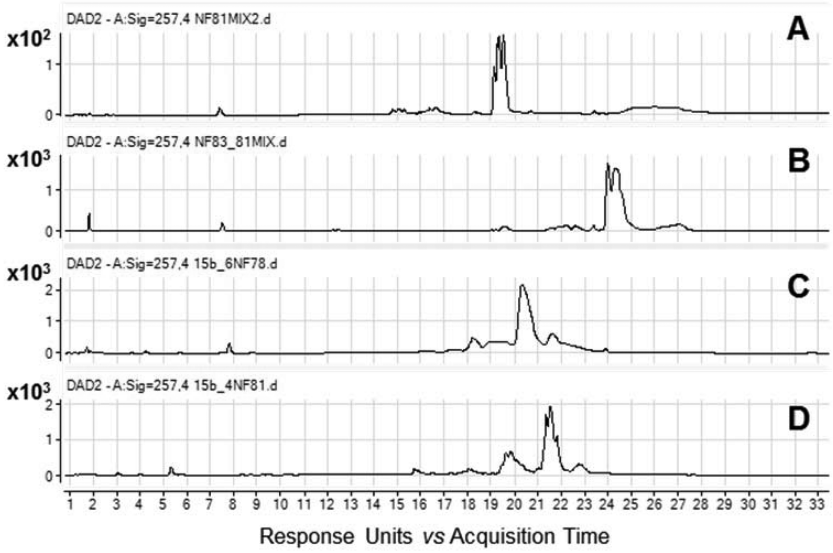
Table 3. Mixed Sequence 2′-Deoxyoligonucleotide Phosphotriester Modified ODNs (Amino Alcohol Triester Linkages)[a]

Entry	ODN sequence	Obs mass (calc mass)	Yield (%)
ODN24	T_d,GTAAACCATGATGTGCTGCT_d,A	$[M-4H]^{4-}$ 1752.7 (1752.9)	90
ODN25	T_d,GTAAACCAT_d,GAT_d,GTGCTGCT_d,A	$[M-4H]^{4-}$ 1819.5 (1819.4)	88
ODN26	T_d,GT_d,AAACCAT_d,GAT_d,GTGCT_d,GCT_d,A	$[M-4H]^{4-}$ 1886.1 (1885.9)	85
ODN27	T_e,GTAAACCATGATGTGCTGCT_e,A	$[M-4H]^{4-}$ 1707.7 (1707.8)	87
ODN28	T_e,GTAAACCAT_e,GAT_e,GTGCTGCT_e,A	$[M-4H]^{4-}$ 1729.0 (1729.4)	85
ODN29	T_e,GT_e,AAACCAT_e,GAT_e,GTGCT_e,GCT_e,A	$[M-4H]^{4-}$ 1750.5 (1750.9)	80
ODN30	T_f,GTAAACCATGATGTGCTGCT_f,A	$[M-4H]^{4-}$ 1714.7 (1714.8)	89
ODN31	T_f,GTAAACCAT_f,GAT_f,GTGCTGCT_f,A	$[M-4H]^{4-}$ 1743.5 (1743.4)	85
ODN32	T_f,GT_f,AAACCAT_f,GAT_f,GTGCT_f,GCT_f,A	$[M-4H]^{4-}$ 1771.97 (1771.94)	80

[a]Subscripts between nucleotides refer to the type of triester linkage at that site d′,e′, and f′ are, respectively d, e, and f in Scheme 1 without the Dde protecting group (d′, L-Phenylalaninol, e′, Glycinol, f′, β-alaninol). The remaining internucleotide linkages without subscripts are phosphate diesters.



	Sequence	Length	Number of modifications
A	AT$_b$CAGCT$_b$GTT (ODN8)	10	2
B	AT$_c$T$_b$AGAT$_c$GT$_b$T (ODN10)	10	4
C	T$_a$GT$_a$AAACCAT$_a$GAT$_a$GTGCT$_a$GCT$_a$A (ODN13)	22	6
D	T$_b$GTAAACCAT$_b$GAT$_b$GTGCTGCT$_b$A (ODN17)	22	4

Figure 1. Comparison of ODN8, ODN10, ODN13, and ODN17 LC profiles (LC spectra using a C18 column). Subscripts between nucleotides refer to the type of triester linkage (a, Z-L-Alaninol, b, Z-L-Phenyalaninol, c, Z-Glycinol, see Scheme 1) at that site.

diisopropylaminophosphoramidites of 5′-*O*-dimethoxytrityl-2′-deoxythymidine **7**$_{a-h}$, *N*-*tert*-butylphenoxyacetyl-2′-deoxyadenosine **8**$_{a-c}$, *N*-*tert*-butylphenoxyacetyl-2′-deoxycytidine **9**$_{a-c}$, and the 3′-*O*-(2-cyanoethyl)diisopropylaminophosphoramidites of 5′-*O*-dimethoxytrityl-2′-deoxythymidine, *N*-phenoxyacetyl-2′-deoxyadenosine, *N*-acetyl-2′-deoxycytidine, and *N*-isopropyl-phenoxyacetyl-2′-deoxyguanosine. These synthons were introduced using the standard DNA solid-phase synthesis cycle with 5-ethylthio-1H-tetrazole (0.25 M in anhydrous acetonitrile) as activator and a reaction time of 25 s. Post-synthesis, ODNs were cleaved from the solid support by treatment with 28−30% ammonia for 2 h at room temperature. The cleavage mixture was discarded, and the solid-support dried *in vacuo* using a SpeedVac. The ODN products adsorbed on the support were dissolved in water and analyzed by Accurate-Mass Q-TOF LC/MS. Sequences, mass, and yields (refers to the purity of the

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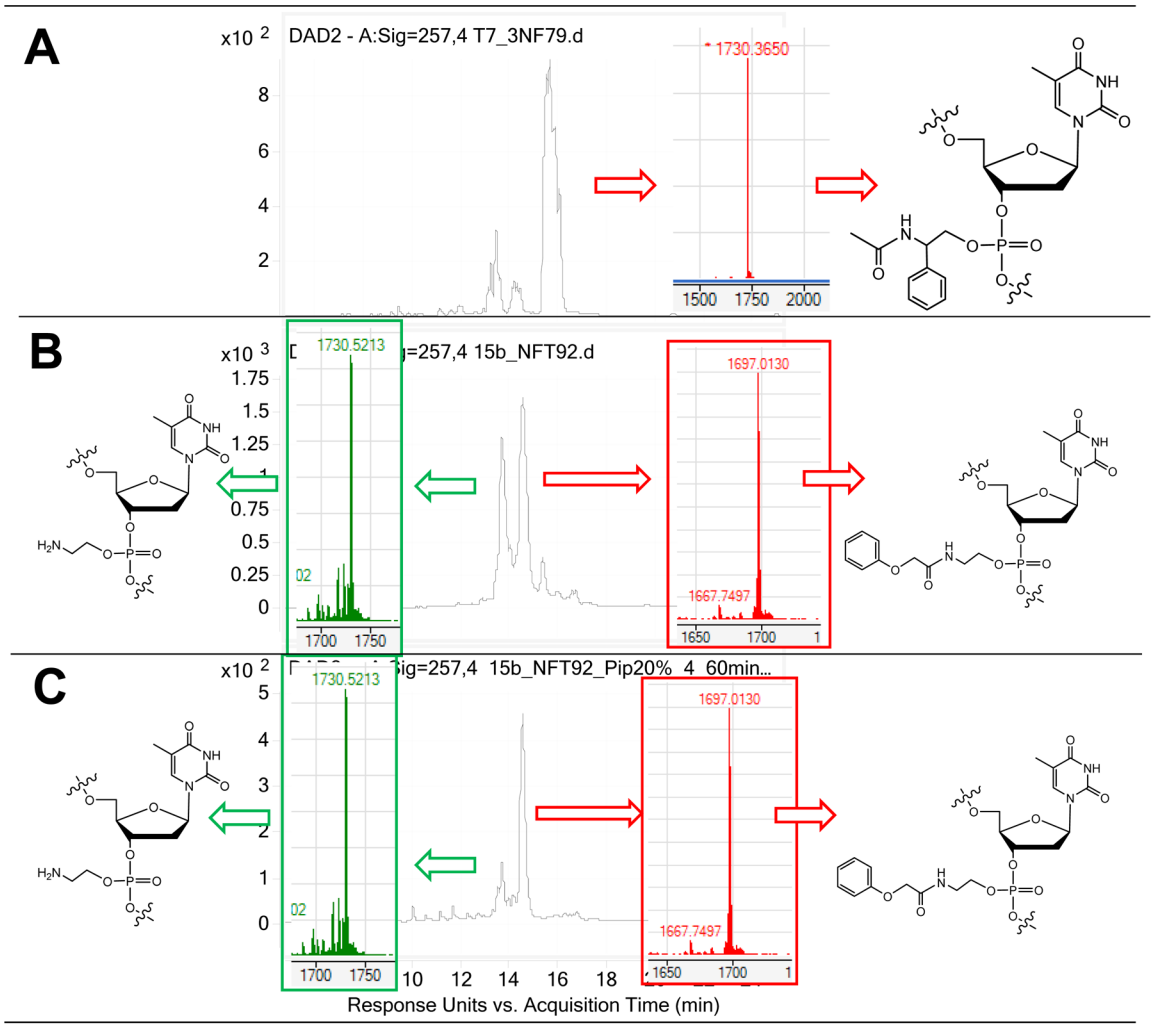

	sequence
A	$\mathbf{T_gT_gT_g}$TTTTTT
B	TG$\mathbf{T_h}$AAACCATGATGTGCTGCTA
C	TG$\mathbf{T_h}$AAACCATGATGTGCTGCTA

Figure 2. LC spectra (C18 column) of (A) d(T_{10} with 3 Ac-D-phenylglycinol phosphotriester linkages cleaved with ammonia for 2 h, (B) 22mer with 1 Fmoc-glycinol after a 10 min piperidine treatment before 2 h ammonia cleavage, and (C) 22mer with 1 Fmoc-glycinol after a 60 min piperidine treatment before 2 h ammonia cleavage.

crude product from LC-MS spectra evaluation, see Supporting Information (SI)) for ODNs are presented in Tables 1, 2, and 3.

In order to verify the compatibility of these synthons with the phosphoramidite synthesis method, including in particular ammonia cleavage, a series of 2′-deoxyoligothymidine ODNs having the Z-N-substituted aminoethyl phosphotriester modifications (ODNs 1−7) were synthesized. LC-MS analyses showed that the resulting phosphate triesters were stable to ammonia treatment for 2 h at room temperature (see SI).

As a next step, mixed base sequence ODNs having Z-amino alcohols (Scheme 1, R: a−c) and the standard base protecting groups (*N*-benzoyl adenine, *N*-benzoyl cytosine, and *N*-isobutyroyl guanosine) were prepared. However, ammonia treatment at 55 °C for 16 h., which is required to remove the protecting groups from dA, dC, and dG, led to hydrolysis of the Z-amino alcohol phosphotriester linkages. When 2′-deoxynucleoside 3′-(2-cyanoethyl)phosphoramidites which have the ultra-mild phenoxyacetyl (Pac) protecting group for dA, 4-isopropylphenoxyacetyl (*i*Pr-Pac) for dG, and acetyl (Ac) for dC were used in conjunction with the Z-amino alcohol diisopropylaminophosphoramidites having *tert*-butylphenoxyacetyl (*t*BPAC) protection on dA and dC (Scheme 1, $\mathbf{8}_{a-c}$, $\mathbf{9}_{a-c}$), cleavage from the support and base deprotection in ammonia at room temperature could be carried out in 2 h. Using this approach, ODNs 8−23 (Table 2) having various amino alcohols were synthesized and tested for stability toward ammonia.

As shown in the HPLC profiles presented in Figure 1, the ODNs were the major peaks and are broad due to the presence of multiple chiral phosphorus centers. Based on mass spectra analysis, impurities from hydrolysis at phosphorus of the Z-

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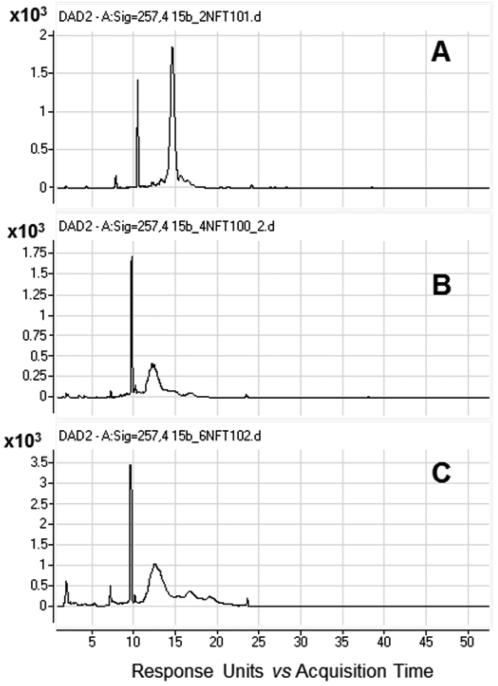
amino alcohols were also observed. As expected, these hydrolysis side products increased as more phosphotriester linkages were introduced. Additionally Z-amino alcohols having aromatic side chains (ODNs 16−20) generated more hydrolysis side products than the alkyl Z-amino alcohols. However, even when side products were detected, the overall yields of product ODNs were never lower than 60%, and all ODNs could be isolated free of impurities.

These encouraging results led us to develop synthesis strategies that would lead to ODNs free of protecting groups on the amino alcohols. This type of triester analog would be less electronegative and retain a more hydrophobic character than unmodified DNA. Perhaps as well, these ODNs might have a different and more favorable intracellular profile than analogs that have previously been studied. Initially a $(dT)_{10}$ having *N*-acetylphenylglycinol phosphotriester linkages was prepared and tested for removal of the acetyl groups using a 2 h treatment with ammonia. However, these conditions were insufficient, and the recovered ODN retained the acetyl protecting group (Figure 2A). A similar result was observed with Fmoc protection. When a mixed sequence ODN having Fmoc protected aminoethyl phosphotriester linkages was first treated with 20% piperidine in DMF and then with ammonia for 2 h, the phenoxyacetyl group on adenine migrated to the amino alcohol. After 10 min with piperidine, 40% of the amino alcohol triesters had been converted to the Pac derivative and after 1 h-90% (Figure 2B,C). Substitution of 4-*tert*-butyl phenoxy acetyl or benzoyl for adenine nucleobase protection was also unsatisfactory. Migration of the protecting group to the amino alcohol was observed with the 4-*tert*-butyl phenoxy acetyl, and the benzoyl group was not completely removed with ammonia after 2 h.

However, protection of the amino alcohol with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl (Dde) proved to be satisfactory. This group was removed under normal cleavage conditions (2 h in ammonia at room temperature) without migration of the adenine nucleobase phenoxyacetyl protecting group. With these Dde protected amino alcohol synthons, mixed base ODN 22mers having from 2 to 6 phenyalaninol, *β*-alaninol, and glycinol phosphotriester linkages (ODNs 24−32, Table 3) were prepared.

The LC-MS analyses of total reaction mixtures from the synthesis of three ODNs having free amino alcohol phosphotriester modifications are shown in Figure 3. Of interest were the observations that retention times did not change due to an increasing number of modifications and the product peaks were broader when compared to the Z-amino alcohol analogs.

Thermal Denaturation Studies with Amino Alcohol Phosphotriester ODNs. In order to measure the effect of various amino alcohols on the stability of DNA duplexes, ODNs 22 nucleotides in length with the same sequence and different amino alcohols were synthesized. Each ODN carried two, four, or six amino alcohol triester linkages (Z-glycinol, Z-alaninol, Z-phenylalaninol, glycinol, phenylalaninol, and *β*-alaninol) where these modifications were always located at the same relative positions. Each ODN was mixed with the complementary DNA sequence in a 1:1 ratio under low salt conditions (10 mM sodium phosphate, 10 mM NaCl, pH 7.3) at an overall duplex concentration of 1 μM. The samples were heat denatured at 90 °C for 5 min, cooled to 15 °C at a rate of 1 °C/min, and maintained at 15 °C for 5 min. Melting was performed by heating the duplexes from 15 to 90 °C at 1 °C/min with the absorbance (260 nm) being recorded at 1 min intervals. Melting temperatures (T_m) were determined at the maximum of first derivative plots. When the T_ms were measured from 90 to 15 °C, the same T_ms were observed. A summary of these duplex stabilities is shown in Table 4.



Sequence

A	$\mathbf{T_e}$,GTAAACCATGATGTGCTGC$\mathbf{T_e}$,A (ODN27)
B	$\mathbf{T_d}$,GTAAACCA$\mathbf{T_d}$,GA$\mathbf{T_d}$,GTGCTGC$\mathbf{T_d}$,A (ODN25)
C	$\mathbf{T_f}$G$\mathbf{T_f}$AAACCA$\mathbf{T_f}$GA$\mathbf{T_f}$GTGC$\mathbf{T_f}$GC$\mathbf{T_f}$A (ODN32)

Figure 3. LC spectra (using a C18 column) of crude 22mers having (A) two amino ethyl phosphotriester linkages, (B) four 2-benzylalaninol phosphotriester linkages, and (C) six amino propyl phosphotriester linkages. These LC profiles were obtained after removal of the Dde protecting group. The sharp peak at 10 min is the side product formed during removal of Dde protecting group and does not contain 2′-deoxyoligonucleotide.

For most ODNs, duplex destabilization is about 0.7−0.9 °C/modification with two Z-amino alcohol phosphotriester linkages (ODNs 11, 16, 21) and increases to more than 1 °C/modification with four and six of these linkages (ODNs 12, 13, 17, 18, 22, 23). With amino alcohol phosphotriesters having a free amino group (ODNs 24−32), the loss in stability is generally much less, and in two ODNs (ODN24, phenylalaninol with 2 modifications and ODN31, *β*-alaninol with four modifications), either there was no loss or a small increase in stability. The minimum loss in stability with the amino alcohol phosphotriesters is very encouraging as all contain multiple P-chiral linkages and therefore a large number of diastereomers. With most analogs that have been studied (triesters, amidates, thioates, and others) these types of linkages generally lead to a reduction in duplex stability. Perhaps the amino alcohols, which would be positively charged at neutral pH, form zwitterions with adjacent internucleotide phosphates, and these internal salt bridges lead to stabilization of the duplex.

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Table 4. Mixed Sequence ODN Melting Temperatures[a]

	duplex	modifications	T_m	ΔT_m
ODN11	11/x	2	54.2	−1.4
ODN12	12/x	4	51.5	−4.1
ODN13	13/x	6	49.5	−6.1
ODN16	16/x	2	54.5	−1.1
ODN17	17/x	4	50.4	−5.2
ODN18	18/x	6	48.8	−6.8
ODN21	21/x	2	53.8	−1.8
ODN22	22/x	4	51.5	−4.1
ODN23	23/x	6	48.9	−6.7
ODN24	24/x	2	55.6	0
ODN25	25/x	4	54.1	−1.5
ODN26	26/x	6	53.3	−2.3
ODN27	27/x	2	55.2	−0.4
ODN28	28/x	4	52.2	−3.4
ODN29	29/x	6	51.2	−4.4
ODN30	30/x	2	54.2	−1.4
ODN31	31/x	4	56.2	0.6
ODN32	32/x	6	53.3	−2.3
–	x′/x	0	55.6	0

[a]The sequences for ODNs 11−33 are defined in Tables 2 and 3. x = 5′-TAG CAG CAC ATC ATG GTT TAC A-3′; x′ = 5′-TGT AAA CCA TGA TGT GCT GCT A-3′. All T_ms represent an average of at least three experiments; and ΔT_m is the difference in T_m when compared to the T_m of the unmodified duplex.

Nuclease Resistance. 2′-Deoxyoligothymidine ODNs, having one phosphotriester linkage (Z-alaninol, phenylalaninol), at the final 5′ or 3′ internucleotide linkage, were synthesized and tested for stability in the presence of calf-spleen phosphodiesterase (CSPDE) and snake venom phosphodiesterase (SVPDE).

In presence of CSPDE, the unmodified polythymidylate 10mer was partially degraded after 15 min and completely converted to mononucleotides after 45 min (Figure 4A). In contrast, ODNs carrying a hydrophobic (Figure 4B) or a cationic (Figure 4C) phosphotriester linkage at the 5′-internucleotide linkage were not degraded even after 24 h. Similarly with SVPDE and under conditions where an unmodified 2′-deoxythymidine 10mer was degraded in 2 min (Figure 5A), ODNs carrying Z-alaninol or phenylalaninol phosphotriester linkages at the 3′ terminal internucleotide linkage were degraded only after 1 h (Figure 5B and 5C).

Flow Cytometry. Preliminary studies on the cell uptake of ODNs 33−36 were carried out on HeLa and Jurkat cells, and the results quantified by fluorescence-assisted cell sorting (FACS). Exponentially growing HeLa cells maintained as subconfluent monolayers in Dulbecco's modified eagle medium (DMEM) were seeded on 12-well culture plates 24 h before transfection. These cells and a fresh medium premixed with the ODNs at various concentrations (0.5, 1.0, and 3.0 μM) were incubated for 16 h. The cells were then rinsed three times with D-PBS (Dulbecco's phosphate buffered saline), trypsinized, and resuspended in D-PBS. Jurkat cells, maintained in Roswell Park Memorial Institute Medium (RPMI) were seeded in 12-well plates and incubated for 24 h. For transfection of Jurkat cells, ODNs at various concentrations (0.5, 1.0, and 3.0 μM) were added to each well, and the cells were incubated for 16 h. Cells were then pelleted and resuspended two times with D-PBS. Fluorescence intensity was analyzed by comparison to the autofluorescence of cells incubated with medium during the 16 h transfection. For these studies (Table 5), 23mer ODNs having four phosphotriester modified linkages (17% modified) were used (Z-glycinol, Z-alaninol, phenylalaninol, and β-alaninol).

As shown in Table 5, these ODNs were taken up by adherent (HeLa) and suspension (Jurkat) cells without lipids. Transfection was also concentration dependent. In particular ODNs with Z-protected amino alcohol triester linkages (ODN33, Z-Alaninol; ODN34, Z-glycinol) were taken up in HeLa cells (>70% for all the concentration tested except for ODN33 at 0.5 μM). For Jurkat cells the uptake for ODN33 and ODN34 at 3.0 μM was respectively 85% and 66%. Certain ODNs having amino alcohol triesters such as the phenylalaninol oligomer (ODN35) demonstrated a good uptake (46% at 0.5 μM and 94% at 3.0 μM) in HeLa cells. However, ODN36 with β-alaninol triester linkages, was taken up only 19% by HeLa cells at 0.5μM and 67% at 3.0 μM. Interestingly with Jurkat cells, ODN36 had a higher percentage uptake (59% at 0.5 μM and

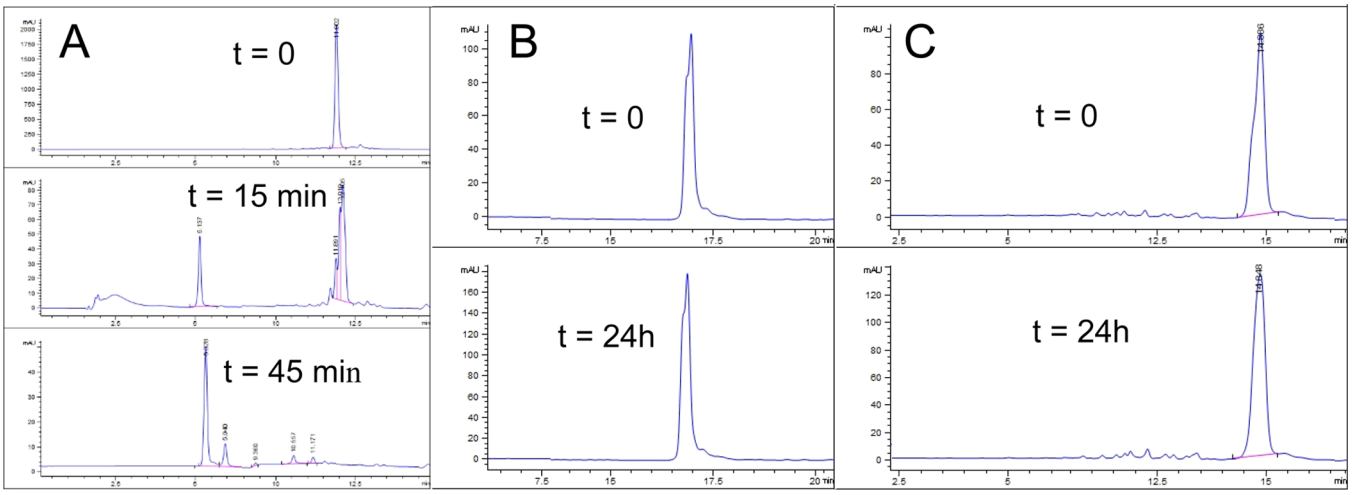

Figure 4. Time-dependent HPLC elution profiles (using a C18 column) of ODNs treated with CSPDE. (A) dT_{10}, (B) d(T_aTTTTTTTTT), and (C) d($T_{d'}$TTTTTTTTT). Subscripts refer to the type of triester linkage (a, Z-L-Alaninol; d′ refers to d in Scheme 1 without the Dde protecting group).

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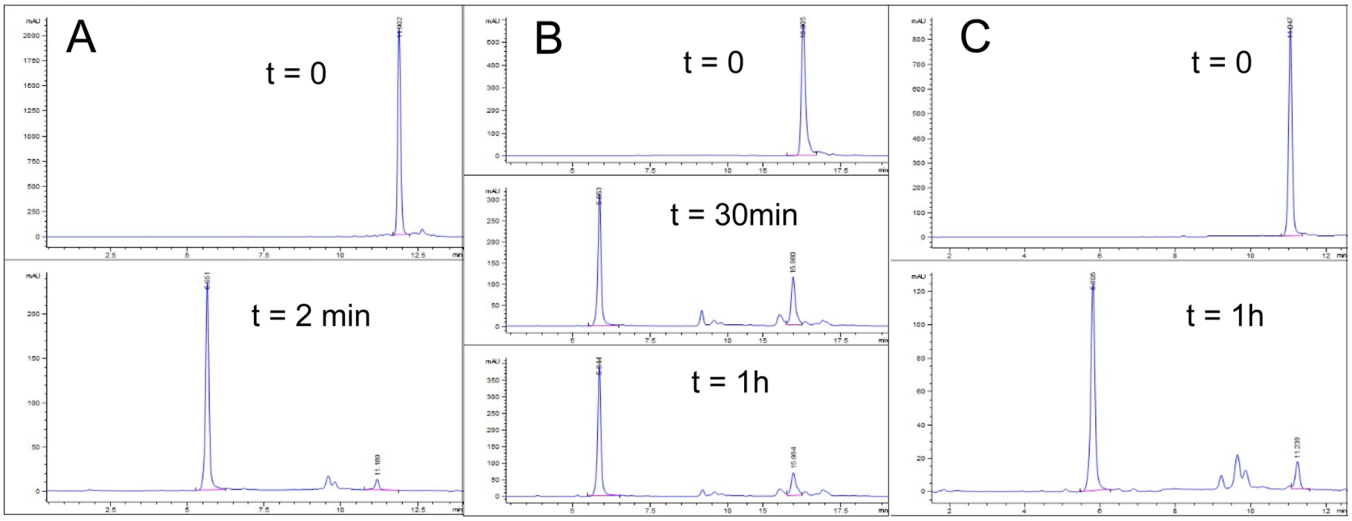

Figure 5. Time-dependent HPLC elution profiles (using a C18 column) of ODNs treated with SVPDE. (A) dT_{10}. (B) d(TTTTTTTTT$_a$T), and (C) d(TTTTTTTTT$_{d'}$T). Subscripts refer to the type of triester linkage (a, Z-L-Alaninol; d′ refers to d in Scheme 1 without the Dde protecting group).

Table 5. FACS Analysis of Mixed Sequence Phosphotriester Modified ODNs[a]

	sequence					
ODN33	5′-F-TGT$_a$AAACCAT$_a$GAT$_a$GTGCTGCT$_a$AT-3′					
ODN34	5′-F-TGT$_c$AAACCAT$_c$GAT$_c$GTGCTGCT$_c$AT-3′					
ODN35	5′-F-TGT$_{d'}$AAACCAT$_{d'}$GAT$_{d'}$GTGCTGCT$_{d'}$AT-3′					
ODN36	5′-F-TGT$_{f'}$AAACCAT$_{f'}$GAT$_{f'}$GTGCTGCT$_{f'}$AT-3′					
	HeLa uptake (%)			Jurkat uptake (%)		
	0.5 μM	1.0 μM	3.0 μM	0.5 μM	1.0 μM	3.0 μM
ODN33	44.55 ± 4.82	78.68 ± 1.60	88.19 ± 0.55	53.29 ± 1.76	64.25 ± 2.96	85.26 ± 2.52
ODN34	70.58 ± 5.23	85.17 ± 1.62	92.59 ± 2.12	26.05 ± 0.07	58.32 ± 1.20	66.15 ± 0.96
ODN35	46.00 ± 1.17	85.69 ± 2.81	94.57 ± 2.45	30.70 ± 2.15	55.84 ± 1.63	69.56 ± 2.53
ODN36	19.32 ± 2.57	24.35 ± 4.74	66.96 ± 1.68	59.25 ± 1.03	71.95 ± 0.14	86.58 ± 3.84

[a]Results are expressed as the percentage of HeLa and Jurkat cells that are transfected. Experiments were carried out in triplicate. F represents a fluorescein tag on the 5′ position. Subscripts between nucleotides refer to the type of triester linkage (see Scheme 1) at that site. d′and f′ are respectively d and f in Scheme 1 without the Dde protecting group d′, L-Phenylalaninol, f′, β-alaninol. The remaining internucleotide linkages without subscripts are phosphate diesters.

86% at 3.0 μM) than the more hydrophobic ODN35. Unmodified DNA failed to transfect these cells.

CONCLUSIONS

This manuscript outlines a versatile and general method for synthesizing both hydrophobic and cationic phosphotriester modified ODNs. The approach begins with 2′-deoxynucleoside 3′-phosphoramidite synthons where an N-protected amino alcohol replaces the standard β-cyanoethyl group. These synthons are compatible with the standard 2′-deoxyoligonucleotide solid-phase phosphoramidite synthesis methodology where ultra-mild nucleobase protecting groups are used. Cationic internucleotide phosphotriester modified ODNs were successfully synthesized by using the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl (Dde) to protect the amino function of the amino alcohols. Following synthesis the Dde group was selectively removed under the conditions used to deprotect the nucleotide bases and to cleave the ODN from the support. Thermal denaturation studies of ODNs having up to six phosphotriester internucleotide modifications demonstrated that the binding affinity of amino alcohol phosphotriester linkages do not seriously affect the binding of these modified 2′-deoxyoligonucleotides to complementary DNA. Additionally phosphotriester modified ODNs show uptake in HeLa and Jurkat cells in the absence of lipid transfection reagents and are stable to 5′ and 3′ exonucleases when compared to the corresponding unmodified DNAs. These preliminary results prompt us to continue investigations of Z-protected and various aminoethyl phosphotriester ODNs for biological studies, including applications in the diagnostic and therapeutic areas.

EXPERIMENTAL SECTION

General. Commercially available DNA synthesis reagents as well as Ultramild Phosphoramidites were purchased from a chemical supplier. 5′-*O*-Dimethoxytrityl-2′-deoxyribonucleosides were purchased from a chemical supplier (compounds 1−3 Scheme 1). All Z, Dde, Ac, and Fmoc-amino alcohols as defined in Scheme 1 (compounds a−h) were purchased from a chemical supplier and used to prepare the corresponding 2′-deoxynucleoside 3′-phosphoramidites ($\mathbf{7}_{a-h}$, $\mathbf{8}_{a-d}$ $\mathbf{9}_{a-c}$). Medium-pressure, preparative column chromatography was performed using 60 Å standard grade silica gel.

Chemical shifts are given in ppm with positive shifts downfield. ^{1}H and ^{13}C chemical shifts were referenced relative to the signal from residual protons of a lock solvent (^{1}H: 5.32 ppm for CD_2Cl_2; ^{13}C: 54.00 ppm for CD_2Cl_2). ^{31}P Chemical shifts are referenced to 0.0 ppm in the ^{1}H NMR spectrum according to the standard IUPAC method.

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General Procedure for Synthesis of 4−6. The general procedure for the synthesis of 5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside 3′-*O*-aminoalkylphosphoramidites (**4−6**, Scheme 1) is as follows: In a flame-dried 250 mL Schlenk flask equipped with a magnetic stir bar and septum, bis(*N*,*N*-diisopropylamino) chlorophosphine (0.019 mol, 5.20 g) was dissolved under argon in anhydrous dichloromethane (50 mL). To this solution, *N*,*N*-diisopropylethylamine (0.057 mol, 3.0 equiv) and the appropriately base-protected 5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside (1−3) (1.0 equiv) were added. The reaction mixture was stirred under argon at room temperature. The ^{31}P NMR spectrum after 30 min indicated complete conversion to product. The solvent was removed *in vacuo* and compounds **4**,[31] **5**, and **6** were isolated by chromatography using a solvent containing 2% triethylamine and a gradient of ethyl acetate:hexane (1:1 to 9:1).

5′-*O*-Dimethoxytrityl-N^6-*tert*-butylphenoxyacetyl-2′-deoxyriboadenosine 3′-*O*-*N*,*N*,*N*′,*N*′-*tetrakis*(1-methylethyl)-phosphorodiamidite (5). Yield: 95% (18.02 g); ^{31}P NMR ($CDCl_3$): δ 116.11 (s); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.43 (1H, bs), 8.76 (1H, s), 8.21 (1H, s), 7.43−7.18 (16H, m), 7.02 (2H, d), 6.80 (4H, m), 6.53 (1H, m), 4.85 (2H, s), 4.64 (1H, m), 4.43 (1H, m), 3.79 (6H, s), 3.61−3.47 (4H, m), 3.44−3.41 (1H, m), 3.37−3.34 (1H, m), 2.89−2.82 (1H, m), 2.77−272 (1H, m), 1.33 (9H, s), 1.21−1.14 (24H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.7, 158.5, 154.8, 152.4, 151.5, 148.2, 145.2, 144.5, 141.8, 135.7, 135.7, 130.0, 128.1, 127.8, 126.8, 126.6, 114.5, 113.1, 86.7, 86.5, 85.3, 74.3, 74.1, 68.3, 64.1, 55.2, 44.8, 44.7, 44.6, 40.0, 34.2, 31.5, 24.56, 24.49, 24.48, 24.41, 24.11, 24.05, 23.99, 20.6. HRMS (ESI/Q-TOF) *m*/*z*: [M + H]+ Calcd for $C_{55}H_{72}N_7O_7P$ 974.5304; Found 974.5304.

5′-*O*-Dimethoxytrityl-N^4-*tert*-butylphenoxyacetyl-2′-deoxyribocytidine 3′-*O*- *N*,*N*,*N*′,*N*′-*tetrakis*(1-methylethyl)-phosphorodiamidite (6). Yield: 90% (16.65 g); ^{31}P NMR ($CDCl_3$): δ 116.69 (s); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.17 (1H, bs), 8.28 (1H, d), 7.43−7.24 (16H, m), 7.13 (1H, d), 6.91−6.83 (6H, m), 6.30−6.27 (1H, m), 4.60 (2H, s), 4.54−4.48 (1H, m), 4.37−4.34 (1H, m), 3.81 (6H, s), 3.60−3.42 (6H, m), 2.31−2.24 (1H, m), 1.31 (9H, s), 1.19−1.09 (15H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.3, 161.1, 158.6, 158.6, 155.1, 154.4, 145.4, 145.1, 144.2, 135.5, 135.2, 130.19, 130.06, 128.2, 127.95, 127.05, 126.7, 114.1, 113.24, 113.22, 95.7, 88.0, 86.90, 86.75, 86.70, 73.21, 73.02, 67.4, 63.2, 55.2, 44.91, 44.78, 44.46, 44.34, 41.54, 41.49, 34.2, 31.4, 24.69, 24.6, 24.44, 24.35, 24.11, 24.05, 23.97, 23.92. HRMS (ESI/Q-TOF) *m*/*z*: [M + H]+ Calcd for $C_{54}H_{72}N_5O_8P$ 950.5192; Found 950.5194.

General Procedure for Synthesis of 7−9. 5′-*O*-Dimethoxytrityl-2′-deoxyribonucleoside 3′-*O*-phosphorodiamidite (**4−6**) (2.58 mmol, 2 g) and the corresponding alcohol (a−h) (1.0 equiv) were placed in a 100 mL round-bottom flask, anhydrous dichloromethane (30 mL) was added, and the reaction mixture stirred under argon at room temperature. 1H-Ethylthiotetrazole (0.25 M) dissolved in anhydrous acetonitrile (1.0 equiv) was added dropwise to the mixture via a syringe over a period of 15 min, and the mixture was allowed to stir under nitrogen at room temperature until TLC analysis (ethyl acetate:hexane:triethylamine 6:4:0.2) showed complete conversion of the starting material to a product having a higher mobility. The solvent was removed *in vacuo*, and the product was isolated by chromatography using a solvent containing 2% triethylamine and a gradient of ethyl acetate:hexane (1:1 to 9:1).

5′-*O*-Dimethoxytrityl-2′-deoxyribothymidine 3′-*O*-*N*-carboxybenzyl-(1-methyl)aminoethylphosphoramidite (7a). Yield: 70% (1.59 g); ^{31}P NMR ($CDCl_3$): δ 148.44, 148.09 (d); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (1H, bs), 7.64 (1H, d), 7.42−7.24 (14H, m), 6.84 (4H, m), 6.41 (1H, m), 5.08 (2H, d), 4.64 (1H, m), 4.15 (1H, m), 3.81 (6H, s), 3.60−3.43 (6H, m), 3.31(1H, m), 2.57−2.45 (1H, m), 2.31 (1H, m), 1.46 (3H, s), 1.30−1.04 (15H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 163.5, 158.7, 155.7, 150.1, 144.2, 136.59, 135.68, 135.24, 130.2, 130.1, 128.47, 128.21, 128.03, 127.2, 113.2, 111.2, 86.9, 85.62, 85.52, 84.79, 73.94, 73.76, 73.00, 72.82, 67.0, 66.87, 66.53, 63.4, 62.9, 55.2, 47.4, 43.24, 43.1, 40.15, 40.11, 24.5, 17.8, 11.7. HRMS (ESI/Q-TOF) *m*/*z*: [M + H]+ Calcd for $C_{48}H_{59}N_4O_{10}P$ 883.4042; Found 883.4063.

5′-*O*-Dimethoxytrityl-2′-deoxyribothymidine 3′-*O*-*N*-carboxybenzyl-(1-benzyl)aminoethylphosphoramidite (7b). Yield: 65% (1.61 g); ^{31}P NMR ($CDCl_3$): δ 148.71, 148.11 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (1H, bs), 7.65 (1H, d), 7.42−7.20 (19H, m), 6.83 (4H, m), 6.42 (1H, m), 5.09 (2H, m), 4.67 (1H, m), 4.16 (1H, m), 3.80 (6H, s), 3.64−3.43 (6H, m), 3.33(1H, m), 2.95−2.76 (2H, m), 2.54−2.46 (1H, m), 2.36−229 (1H, m), 1.43 (3H, s), 1.23−1.06 (12H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 163.5, 158.7, 155.7, 150.1, 144.2, 137.7, 136.5, 135.7, 135.2, 130.1, 130.1, 129.43, 129.40, 128.61, 128.47, 128.20, 128.16, 127.99, 127.16, 126.58, 126.48, 113.3, 111.2, 86.9, 85.5, 84.7, 73.9, 66.6, 62.8, 55.2, 52.8, 44.5, 43.2, 40.2, 37.7, 24.7, 24.46, 23.66, 22.62, 11.7. HRMS (ESI/Q-TOF) *m*/*z*: [M + H]+ Calcd for $C_{54}H_{63}N_4O_{10}P$ 959.4355; Found 959.4376.

5′-*O*-Dimethoxytrityl-2′-deoxyribothymidine 3′-*O*-*N*-carboxybenzyl-aminoethylphosphoramidite (7c). Yield: 80% (1.79 g); ^{31}P NMR ($CDCl_3$): δ 148.45, 147.61 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (1H, bs), 7.64 (1H, d), 7.42−7.24 (14H, m), 6.84 (4H, m), 6.42 (1H, m), 5.09 (2H, d), 4.64 (1H, m), 4.17 (1H, m), 3.80 (6H, s), 3.61−3.49 (6H, m), 3.32(2H, m), 2.56−2.46 (1H, m), 2.35−2.25 (1H, m), 1.43 (3H, s), 1.33−1.06 (12H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 163.5, 158.7, 156.4, 150.1, 144.2, 136.5, 135.3, 130.1, 128.47, 128.18, 128.08, 127.98, 113.2, 111.1, 86.9, 85.7,, 84.8, 74.1, 73.90, 73.30, 66.7, 63.4, 62.64, 62.48, 55.3, 44.5, 43.19, 42.96, 40.1, 24.6, 23.7, 22.6, 11.7. HRMS (ESI/Q-TOF) *m*/*z*: [M + H]+ Calcd for $C_{47}H_{57}N_4O_{10}P$ 869.3885; Found 869.3885.

5′-*O*-Dimethoxytrityl-2′-deoxyribothymidine 3′-*O*-*N*-Dde-(1-benzyl)aminoethylphosphoramidite (7d). Yield: 65% (1.66 g); ^{31}P NMR ($CDCl_3$): δ 148.58, 148.08 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.32 (1H, bs), 7.64 (1H, d), 7.43−7.10 (15H, m), 6.83 (4H, m), 6.42 (1H, m), 4.76 (0.5H, m), 4.66 (0.5H, m), 4.14 (1H, m), 3.81 (6H, s), 3.74−3.47 (6H, m), 3.34(1H, m), 3.09−2.72 (2H, m), 2.55−2.27 (9H, m) 1.43 (3H, s), 1.18−0.59 (18H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 198.8, 196.6, 172.9, 158.7, 150.1, 144.3, 136.6, 135.7, 135.35, 135.28, 130.1, 129.2, 128.7, 128.2, 127.98, 127.15, 127.04, 113.2, 111.1, 107.7, 86.9, 85.7, 84.8, 73.6, 64.6, 63.4, 60.4, 56.0, 55.2, 53.5, 52.2, 43.1, 40.1, 38.6, 30.0, 28.3, 24.50, 17.7, 14.2, 11.7. HRMS (ESI/Q-TOF) *m*/*z*: [M + H]+ Calcd for $C_{56}H_{69}N_4O_{10}P$ 989.4825; Found 989.4830.

5′-*O*-Dimethoxytrityl-2′-deoxyribothymidine 3′-*O*-*N*-Dde-aminoethylphosphoramidite (7e). Yield: 75% (1.81 g); ^{31}P NMR ($CDCl_3$): δ 148.43, 148.10 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.44 (1H, bs), 7.65 (1H, d), 7.43−7.23 (9H, m), 6.83 (4H, m), 6.44 (1H, m), 4.70 (1H, m), 4.15 (1H, m), 3.80 (6H, s), 3.73−3.43 (8H, m), 2.61, 2.53 (4H, 2s), 2.53−2.47 (1H, m), 2.39−2.35 (1H, m), 2.32 (3H, m), 1.43 (3H, s), 1.31−1.00 (18H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ198.9, 196.7, 173.5, 158.7, 150.2, 144.3, 135.76, 135.70, 135.42, 135.27, 130.1, 128.2, 127.96, 127.14, 113.2, 111.11, 86.9, 85.7, 85.4, 84.7, 63.2, 61.2, 60.4, 55.2, 53.6, 52.2, 44.2, 43.3, 40.1, 30.0, 28.2, 24.5, 22.9, 21.1, 18.0, 14.2, 11.7, 11.7. HRMS (ESI/Q-TOF) *m*/*z*: [M + H]+ Calcd for $C_{49}H_{63}N_4O_{10}P$ 899.4355; Found 899.4366.

5′-*O*-Dimethoxytrityl-2′-deoxyribothymidine 3′-*O*-*N*-Dde-aminopropylphosphoramidite (7f). Yield: 75% (1.84 g); ^{31}P NMR ($CDCl_3$): δ 147.53, 147.30 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.25 (1H, bs), 7.65 (1H, d), 7.43−7.31 (9H, m), 6.86 (4H, m), 6.43 (1H, m), 4.66 (1H, m), 4.17 (1H, m), 3.81 (6H, s), 3.73−3.43 (8H, m), 2.58, 2.52 (4H, 2s), 2.48−2.28 (5H, m), 2.01−1.94 (1H, m), 1.83−1.81 (1H, m), 1.43 (3H, s), 1.32−1.04 (18H, m). δ ^{13}C NMR (101 MHz, $CDCl_3$): δ 173.7, 173.6, 163.5, 158.7, 150.1, 144.2, 135.7, 135.4, 135.2, 130.15, 130.09, 128.2, 128.1, 128.0, 127.2, 113.3, 111.2, 111.2, 107.9, 86.2, 85.77, 85.56, 84.84, 73.9, 73.7, 73.3, 73.2, 63.4, 63.2, 60.2, 60.17, 60.07, 60.0, 53.4, 43.0, 43.0, 40.2, 40.1, 30.7, 30.1, 28.3, 24.5, 24.5, 24.4, 23.7, 22.66, 22.62, 17.9, 17.8, 11.7. HRMS (ESI/Q-TOF) *m*/*z*: [M + H]+ Calcd for $C_{50}H_{65}N_4O_{10}P$ 913.4512; Found 913.4517.

5′-*O*-Dimethoxytrityl-2′-deoxyribothymidine 3′-*O*-*N*-acetyl-(1-phenyl)aminoethylphosphoramidite (7g.). Yield: 80% (1.83 g); ^{31}P NMR ($CDCl_3$): δ 148.42, 147.69 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.82 (1H, bs), 7.63 (1H, d), 7.42−7.24 (14H, m), 6.85 (4H, m), 6.41−6.36 (1H, m), 5.23−5.12 (1H, m), 4.62−4.54 (1H, m),

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4.16 (1H, m), 3.81 (6H, s), 3.64−3.20 (6H, m), 2.52−2.40 (1H, m), 2.32−2.14 (1H, m), 2.06 (3H, s), 1.43 (3H, s), 1.23−1.06 (12H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.4, 163.7, 158.8, 150.3, 144.2, 139.3, 135.6, 135.4, 135.32, 135.27, 130.12, 128.4, 128.1, 128.0, 127.4, 127.2, 127.0, 113.3, 111.1, 86.9, 85.6, 84.78, 84.39, 73.2, 66.0, 65.82, 63.4, 63.0, 60.4, 55.3, 53.7, 53.5, 45.2, 43.15, 43.10, 43.02, 40.0, 24.66, 24.59, 24.56, 24.49, 24.42, 24.35, 23.4, 23.26, 23.00, 22.97, 22.93, 21.1, 14.2, 11.8, 11.78. HRMS (ESI/Q-TOF) *m/z*: [M + H]+ Calcd for $C_{47}H_{57}N_4O_9P$ 853.3937; Found 853.3959.

5′-*O*-Dimethoxytrityl-2′-deoxyribothymidine 3′-*O*-*N*-(fluorenylmethyloxycarbonyl)aminoethylphosphoramidite (7h). Yield: 70% (1.80 g); ^{31}P NMR ($CDCl_3$): δ 148.53, 147.98 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.47 (1H, bs), 7.78−7.23 (18H, m), 6.84 (4H, m), 6.45 (1H, m), 4.66 (1H, m), 4.39 (2H, d), 4.16 (3H, m), 3.80 (6H, s), 3.60−3.19 (8H, m), 2.62−2.58 (1H, m), 2.32 (1H, m), 1.43 (3H, s), 1.23−1.16 (12H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 163.6, 158.7, 156.3, 150.2, 144.25, 144.21, 143.96, 143.90, 141.3, 135.62, 135.28, 130.2, 130.1, 128.19, 128.15, 127.98, 127.67, 127.65, 127.17, 127.01, 125.1, 125.0, 112.0, 113.2, 111.28, 111.18, 87.0, 85.7, 85.5, 84.8, 74.1, 74.0, 73.3, 73.1, 66.75, 66.64, 63.5, 62.98, 62.61, 62.45, 60.4, 55.2, 47.2, 43.2, 43.10, 42.98, 42.06, 40.1, 24.66, 24.58, 24.50, 14.2, 11.7. HRMS (ESI/Q-TOF) *m/z*: [M + H]+ Calcd for $C_{54}H_{61}N_4O_{10}P$ 957.4203; Found 957.4202.

5′-*O*-Dimethoxytrityl-N^6-*tert*-butylphenoxyacetyl-2′-deoxyriboadenosine 3′-*O*-*N*-carboxybenzyl-(1-methyl)-aminoethylphosphoramidite(8a). Yield: 80% (2.27grams); ^{31}P NMR ($CDCl_3$): δ 148.64, 148.48 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.42 (1H, bs), 8.73 (1H, s), 8.18(1H, s), 7.42−7.18 (16H, m), 7.01 (2H, d), 6.80 (4H, m), 6.44 (1H, m), 5.07 (2H, s), 4.85 (2H, s), 4.75 (1H, m), 4.33 (1H, m), 3.80 (6H, s), 3.62−3.54 (5H, m) 3.44−3.40 (1H, m), 3.36−3.31 (1H, m), 2.73−2.66 (1H, m), 2.64−258 (1H, m), 1.33 (9H, s), 1.32−1.13 (15H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.7, 158.5, 155.7, 154.8, 152.4, 151.5, 148.2, 145.3, 144.5, 142.0, 136.5, 135.7, 130.0, 128.5, 128.43, 128.1, 127.8, 126.8, 126.64, 123.2, 114.5, 113.2, 86.5, 86.0, 84.9, 74.0, 73.5, 72.9, 72.76, 68.3, 66.5, 63.5, 63.2, 60.4, 55.2, 47.5, 44.5, 43.2, 43.1, 39.4, 34.2, 31.5, 24.6, 24.5, 23.7, 22.6, 17.9, 14.2. HRMS (ESI/Q-TOF) *m/z*: [M + H]+ Calcd for $C_{60}H_{72}N_7O_{10}P$ 1082.5156; Found 1082.5170.

5′-*O*-Dimethoxytrityl-N^6-*tert*-butylphenoxyacetyl-2′-deoxyriboadenosine 3′-*O*-*N*-carboxybenzyl-(1-benzyl)-aminoethylphosphoramidite (8b). Yield: 75% (2.24 g); 31P NMR ($CDCl_3$): δ 148.48, 148.23 (d). ^{1}H NMR (400 MHz, CDCl3): δ 9.42 (1H, bs), 8.72 (1H, s), 8.17 (1H, s), 7.43−7.21 (21H, m), 7.02 (2H, d), 6.78 (4H, m), 6.50−6.47 (0.5H, m), 6.42−6.38 (0.5H, m), 5.08 (2H, s), 4.85 (2H, s), 4.79−4.77 (1H, m), 4.33 (1H, m), 3.79 (6H, s), 3.66−3.54 (4H, m) 3.47−3.32 (2H, m), 2.97−2.82 (3H, m), 2.70−2.59 (1H, m), 1.33 (9H, s), 1.22−1.13 (12H, m). ^{13}C NMR (101 MHz, CDCl3): δ 166.7, 158.5, 155.7, 154.8, 152.4, 152.4, 151.5, 148.2, 145.3, 144.5, 142.08, 141.96, 137.6, 136.5, 135.6, 130.0, 129.5, 129.4, 128.52, 128.49,, 128.11, 128.08, 127.8, 126.9, 126.64, 126.49, 123.2, 114.5, 113.1, 86.5, 86.0, 85.0, 74.1, 68.3, 66.6, 63.5, 55.2, 53.0, 43.30, 43.18, 39.4, 37.6, 34.2, 31.5, 24.62, 24.59, 24.54. HRMS (ESI/Q-TOF) *m/z*: [M + H]+ Calcd for $C_{66}H_{76}N_7O_{10}P$ 1158.5470; Found 1158.5471.

5′-*O*-Dimethoxytrityl-N^6-*tert*-butylphenoxyacetyl-2′-deoxyriboadenosine 3′-*O*-*N*-carboxybenzylaminoethylphosphoramidite (8c). Yield: 80% (1.75 g); ^{31}P NMR ($CDCl_3$): δ 148.12, 148.01 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.42 (1H, bs), 8.74 (1H, s), 8.18(1H, s), 7.41−7.18 (16H, m), 7.01 (2H, d), 6.79 (4H, m), 6.47 (1H, m), 5.09 (2H, s), 4.84 (2H, s) 4.75 (1H, m), 4.35 (1H, m), 3.78 (6H, s), 3.62−3.53 (4H, m), 3.43, 3.34 (4H, 2m), 2.73,2.64 (2H, 2m), 1.33 (9H, s), 1.32−1.13 (12H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.7, 158.5, 154.8, 152.4, 151.4, 148.2, 145.3, 144.5, 141.9, 136.5, 135.6, 130.0, 128.5, 128.1, 127.8, 126.89, 126.64, 114.5, 113.1, 86.47, 86.10, 85.95, 84.9, 73.2, 68.3, 66.7, 63.5, 63.2, 62.8, 62.6, 55.2, 44.5, 43.19, 43.06, 34.2, 31.5, 24.64, 24.57, 23.4, 23.7, 22.7, 22.6. HRMS (ESI/Q-TOF) *m/z*: [M + H]+ Calcd for $C_{59}H_{70}N_7O_{10}P$ 1068.5000; Found 1068.4900.

5′-*O*-Dimethoxytrityl-N^4-*tert*-butylphenoxyacetyl-2′-deoxyribocytidine 3′-*O*-*N*-carboxybenzyl-(1-methyl)-aminoethylphosphoramidite (9a). Yield: 65% (2.32 g); ^{31}P NMR ($CDCl_3$): δ 149.53, 148.73 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.42 (1H, bs), 8.31 (1H, d), 7.42−7.18 (16H, m), 7.14 (1H, d) 6.92−6.84 (6H, m), 6.31−6.23 (1H, m), 5.10 (2H, m), 4.66−4.58 (1H, m), 4.59 (2H, s), 4.23 (1H, m), 3.82 (6H, s), 3.61−3.36 (5H, m), 2.85−2.81 (1H, m), 2.74−2.69 (1H, m), 2.32−2.24 (1H, m), 1.31 (9H, s), 1.28−1.06 (15H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.2, 161.0, 158.7, 155.7, 155.1, 154.3, 145.5, 145.1, 145.0, 144.1, 135.3, 135.1, 135.1, 130.21, 130.14, 128.48, 128.45, 128.18, 128.00, 127.1, 126.7, 114.1, 113.3, 95.9, 87.3, 87.09, 86.94, 85.82, 85.76, 72.5, 72.3, 70.8, 707, 67.3, 66.5, 62.4, 61.7, 55.2, 47.4, 43.2, 43.2, 43.1, 41.4, 41.3, 34.2, 31.4, 24.7, 24.6, 24.5, 23.69, 23.66, 22.6, 22.6, 21.1, 17.8. HRMS (ESI/Q-TOF) *m/z*: [M + H]+ Calcd for $C_{59}H_{72}N_5O_{11}P$ 1058.5044; Found 1058.5046.

5′-*O*-Dimethoxytrityl-N^4-*tert*-butylphenoxyacetyl-2′-deoxyribocytidine 3′-*O*-*N*-carboxybenzyl-(1-benzyl)-aminoethylphosphoramidite (9b). Yield: 60% (1.50 g); ^{31}P NMR ($CDCl_3$): δ 149.62, 148.70 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.02 (1H, bs), 8.32 (1H, d), 7.43−7.30 (21H, m), 7.14 (1H, d) 6.92−6.83 (6H, m), 6.32−6.29 (1H, m), 5.09 (2H, m), 4.69−4.63 (1H, m), 4.59 (2H, s), 4.24 (1H, m), 3.81 (6H, s), 3.66−3.51 (4H, m) 3.47−3.32 (2H, m), 2.91−2.70 (3H, m), 2.34−2.27 (1H, m), 1.33 (9H, s), 1.29−1.13 (12H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.2, 161.1, 158.7, 155.8, 155.6, 155.0, 154.4, 145.4, 145.0, 144.1, 137.8, 137.6, 136.62, 136.49, 135.3, 135.2, 135.1, 135.1, 130.2, 130.1, 130.1, 129.4, 129.4, 128.5, 128.5, 128.4, 128.2, 128.1, 128.0, 127.1, 127.1, 126.7, 126.5, 126.5, 114.1, 113.3, 95.9, 87.3, 87.0, 85.8, 85.5, 72.5, 72.3, 70.6, 67.4, 66.5, 64.1, 63.9, 63.6, 63.4, 62.40, 61.6, 55.2, 52.9, 43.3, 43.2, 41.4, 40.9, 37.7, 37.5, 34.2, 31.5, 24.8, 24.60, 24.48. HRMS (ESI/Q-TOF) *m/z*: [M + H]+ Calcd for $C_{65}H_{76}N_5O_{11}P$ 1134.5358; Found 1134.5354.

5′-*O*-Dimethoxytrityl-N^4-*tert*-butylphenoxyacetyl-2′-deoxyribocytidine 3′-*O*-*N*-carboxybenzylaminoethylphosphoramidite (9c). Yield: 65% (1.49 g); ^{31}P NMR ($CDCl_3$): δ 148.96, 147.91 (d). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.06 (1H, bs), 8.27 (1H, d), 7.42−7.27 (16H, m), 7.14 (1H, d) 6.92−6.83 (6H, m), 6.33−6.24 (1H, m), 5.08 (2H, m), 4.67−4.63 (1H, m), 4.59 (2H, s), 4.22 (1H, m), 3.82 (6H, s), 3.61−3.50 (4H, m), 3.43, 3.34 (4H, 2m), 2.85, 2.74 (1H, 2m), 2.32, 2.23 (1H, 2m), 1.33 (9H, s), 1.32−1.13 (12H, m). ^{13}C NMR (101 MHz, $CDCl_3$): δ 161.0, 158.7, 155.1, 154.3, 145.4, 145.09, 144.98, 144.1, 135.3, 135.1, 130.19, 130.1, 128.5, 128.44, 128.17, 128.00, 127.1, 126.7, 114.1, 113.3, 95.9, 87.3, 87.1, 86.9, 86.9, 85.9, 72.7, 72.6, 67.4, 66.6, 62.6, 61.8, 55.2, 44.5, 43.2, 43.1, 43.0, 42.2, 42.1, 41.3, 34.2, 31.4, 24.7, 24.6, 24.59, 24.57, 24.52, 24.45, 23.69, 23.67, 22.65, 22.63. HRMS (ESI/Q-TOF) *m/z*: [M + H]+ Calcd for $C_{58}H_{70}N_5O_{11}P$ 1044.4888. Found 1044.4886.

Solid-Phase Synthesis. Solid-phase synthesis was carried out using an ABI automated DNA synthesizer. The synthetic protocol was based on the conventional phosphoramidite method (Scheme S1). The 5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside 3′-*O*-aminoalkyl-phosphoramidites ($\mathbf{7}_{a-h}$, $\mathbf{8}_{a-c}$, $\mathbf{9}_{a-c}$) were used for coupling at positions within the growing ODN where phosphotriester modifications were to be incorporated. To generate 2′-deoxyribonucleoside internucleotide phosphate linkages, commercially available 5′-*O*-dimethoxytrityl-2′-deoxynucleoside 3′-*O*-β-cyanoethylphosphoramidites of N4-acetyl-2′-deoxycytidine, N6-phenoxyacetyl-2′-deoxyadenosine, N2−4-isopropyl-phenoxyacetyl-2′-deoxyguanosine, and 2′-deoxythymidine were purchased from a chemical supplier and used without further purification. All appropriately protected 2′-deoxyribonucleoside 3′-*O*-β-cyanoethyl-phosphoramidites were dissolved in anhydrous acetonitrile at a concentration of 100 mM and placed on the appropriate ports of the synthesizer. Prior to synthesis, the 5′-*O*-dimethoxytrityl group on the support bound 2′-deoxyribonucleoside was removed with 3% dichloroacetic acid in dichloromethane. The coupling wait time was 25 s for the 5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside 3′-*O*-amino-alkylphosphinoamidites ($\mathbf{7}_{a-h}$, $\mathbf{8}_{a-c}$, $\mathbf{9}_{a-c}$). All commercially available 2′-deoxyribonucleoside phosphoramidites were used according to the manufacturer's recommendations. The activator was 5-ethylthio-1H-tetrazole (0.25 M in anhydrous acetonitrile). After each condensation step, the support was washed with acetonitrile for 40 s, and Unicap Phosphoramidite was used according to the manufacturer's recom-

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mendations for capping failure sequences. The standard oxidation reagent (0.02 M iodine in THF/water/pyridine) was used for oxidation. Post-synthesis the CPG covalently linked to the ODN was removed from the column and placed in a 1.5 mL screw cap, conical glass reaction vial. Cleavage of the ODN from the support with aqueous ammonia (28−30%) was allowed to proceed for 2 h at room temperature. The cleavage mixture was discarded, and the solid-support evaporated to dryness in a SpeedVac. The ODN product adsorbed on the support was dissolved in 5 mL water, analyzed by LC-MS, and purified by RP-HPLC.

Melting Temperature Conditions. UV−vis measurements were performed on a UV−vis spectrophotometer equipped with a thermoelectrically controlled multicell holder. Samples of DNA and RNA were prepared in a buffer containing 10 mM NaCl and 10 mM sodium phosphate (pH 7.2). The solutions were heated to 90 °C for 5 min followed by cooling to 15 °C at a rate of 1 °C min-1, equilibrated for 5 min, and then heated to 90 °C at the same rate. The absorbance at 260 nm was recorded throughout at intervals of 0.5 °C. The derivative of the heating curve was calculated, and the temperature corresponding to the maximum of the derivative curve was determined to be the melting temperature.

Nuclease Stability Experiments. Snake venom phosphodiesterase I (SVPDE) and calf spleen phosphodiesterase II (CSPDE) were purchased from a chemical supplier. For SVPDE hydrolysis experiments, 1× of Tris-EDTA buffer (pH 8.0), 10 μL of 0.1 M $MgCl_2 \cdot H_2O$, ODNs (65 μM), and 0.015 U of SVPDE (2 mg) were mixed, the total volume adjusted to 100 μL, and the mixture was incubated at 37 °C; 20 μL aliquots of the reaction mixture were removed at the indicated time points, quenched by the addition of 1.0 M EDTA, and stored in dry ice until analyzed by analytical RP-HPLC. For the CSPDE assay, 5 μL of 5 M ammonium acetate buffer (pH 6.8), 2.5 mM EDTA (38 μL), ODNs (55 μM), and 0.03 U of CSPDE were mixed, the volume adjusted to 100 μL, and the mixture was incubated at 37 °C. Twenty microliter aliquots of the reaction mixtures were removed at the indicated time points, quenched by the addition of 2.0 M urea, and stored in dry ice until analyzed by analytical RP-HPLC.

Cellular Uptake Studies. Preparation of Cells. HeLa cells and Jurkat cells were obtained from a chemical supplier and serially maintained at monolayer cultures in a humidified atmosphere of 5% carbon dioxide at 37 °C in Dulbecco's modified eagle's medium (HeLa) and Roswell Park Memorial Institute Medium (Jurkat), containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL). These cultures were adjusted to the appropriate density, seeded onto 12-well culture plates, and incubated for 24 h prior to ODN transfection. HeLa and Jurkat cells were used at passages 8−10 and 7−9, respectively.

Transfection Experiments. For transfection experiments, 8×10^4 HeLa cells/well (12-well plates) were incubated in DMEM containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) for 24 h. The concentration of 5′-fluorescein-labeled ODN dissolved in HyPure Molecular Biology grade water was measured by UV spectroscopy. The media was removed, and the cells were transfected with the appropriate ODN in DMEM to give the required final concentration. The cells were then incubated at 37 °C for 16 h. After incubation, the media was removed from the wells, and cells were washed three times with 0.5 mL D-PBS. Cells were then treated for 3 min at 37 °C with a pre-warmed solution of trypsin-EDTA (1×) until all cells became round and detached from the plates. The cells from each plate were then placed in 1 mL D-PBS and pelleted by centrifugation at 1000 rpm for 5 min. The pellets were washed and resuspended in D-PBS and kept at 0 °C in the dark until analyzed by flow cytometry. Jurkat cells (2×10^5 cells) were grown in suspension in 12-well plates and incubated in Roswell Park Memorial Institute Medium (RPMI) containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) for 24 h. The cells were transfected with the ODNs and incubated at 37 °C for 16 h. After incubation, the cells were pelleted by centrifugation at 1000 rpm for 5 min, resuspended in D-PBS, and washed twice with D-PBS. The final pellet was resuspended in 100 μL of D-PBS and kept at 0 °C in the dark until used for flow cytometric analysis.

Flow Cytometry. Flow cytometric data on at least 10,000 cells per sample were acquired on a Moflow flow-cytometer equipped with a single 488 nm argon laser, 530/40 nm emission filter. Raw flow cytometry data was manipulated and visualized using Summit 4.3 software. Fluorescence intensity of the 5′-fluorescein tag was analyzed for cells presenting higher fluorescence than the background. The background was defined as the auto fluorescence of cell.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b01512.

NMR spectra of the phosphoramidites, LC-MS spectra of ODNs synthesized, melting temperature curves (PDF)

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Notes

The authors declare no competing financial interest.

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