2′-*O*-Aminopropyl Ribonucleotides: A Zwitterionic Modification That Enhances the Exonuclease Resistance and Biological Activity of Antisense Oligonucleotides†

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Oligonucleotides containing 2′-*O*-aminopropyl-substituted RNA have been synthesized. The 2′-*O*-(aminopropyl)adenosine (APA), 2′-*O*-(aminopropyl)cytidine (APC), 2′-*O*-(aminopropyl)-guanosine (APG), and 2′-*O*-(aminopropyl)uridine (APU) have been prepared in high yield from the ribonucleoside, protected, and incorporated into an oligonucleotide using conventional phosphoramidite chemistry. Molecular dynamics studies of a dinucleotide in water demonstrates that a short alkylamine located off the 2′-oxygen of ribonucleotides alters the sugar pucker of the nucleoside but does not form a tight ion pair with the proximate phosphate. A 5-mer with the sequence ACT*U*C has been characterized using NMR. As predicted from the modeling results, the sugar pucker of the APU moiety is shifted toward a C3′-endo geometry. In addition, the primary amine rotates freely and is not bound electrostatically to any phosphate group, as evidenced by the different sign of the NOE between sugar proton resonances and the signals from the propylamine chain. Incorporation of aminopropyl nucleoside residues into point-substituted and fully modified oligomers does not decrease the affinity for complementary RNA compared to 2′-*O*-alkyl substituents of the same length. However, two APU residues placed at the 3′-terminus of an oligomer gives a 100-fold increase in resistance to exonuclease degradation, which is greater than observed for phosphorothioate oligomers. These structural and biophysical characteristics make the 2′-*O*-aminopropyl group a leading choice for incorporation into antisense therapeutics. A 20-mer phosphorothioate oligonucleotide capped with two phosphodiester aminopropyl nucleotides targeted against C-*raf* mRNA has been transfected into cells via electroporation. This oligonucleotide has 5−10-fold greater activity than the control phosphorothioate for reducing the abundance of C-*raf* mRNA and protein.

Introduction

Antisense therapy has stimulated interest in preparation of modified DNA and RNA oligonucleotides which address the limitations of the unmodified phosphodiester backbone of naturally occurring nucleic acids. *In vivo*, oligonucleotides are degraded by nucleases, have low cellular permeation, and reduce affinity of short (∼20-mer) strands for their RNA target.[1−3] Modification of the phosphodiester backbone via replacement of equatorial oxygen(s) with sulfur generates the phosphorothioate and phosphorodithioate oligonucleotides, with a dramatic enhancement in nuclease resistance. More extensive atomic replacements yield the MMI, PNA, guanidyl, and amide backbones.[4−7] Oligonucleotides containing these modifications have reduced negative charge wherever the achiral phosphodiester is replaced by a neutral or positively charged linkage. Such backbone incorporations favorably enhance the properties of the resulting molecules, producing higher affinity for complementary DNA or RNA, greater specificity of base pairing, and reduced enzymatic degradation compared to phosphodiester or phosphorothioate oligodeoxynucleotides. However, recognition of duplexes with complementary RNA by enzymes such as RNAse H is lost.[8−10]

The total net charge on a nucleic acid *in vivo* also can be reduced by incorporating *C*5-(aminohexyl)pyrimidines to form zwitterionic molecules.[11,12] Placement of alkylamines in the major groove does not alter the stability of the DNA duplex formed with a complementary strand. Any potential stabilization of the duplex through reduction of unfavorable electrostatic interactions between strands apparently is offset by new unfavorable steric or entropic interactions involving the long alkyl side chains off the nucleobases. No data on stability or uptake have been reported.

Chemistry

We have investigated an alternative form of zwitterionic RNA where a cationic group is introduced into the minor groove of the duplex by derivitization of the ribose sugar at the C2′-position. The synthesis of precursors for incorporation of alkylamine-substituted nucleotides using conventional phosphoramidite chemistry is complicated by the need to protect the amine with a functional group which is stable to the conditions of oligomerization and can be removed quantitatively. We have explored incorporation of 2′-aminopropyl and 2′-aminopentyl units via phthalimide protection as well as the more labile trifluoroacetyl-protected deri

† Abbreviations: ES, electrospray; AP, aminopropyl; PAGE, polyacrylamide gel electrophoresis; DMT, dimethoxytrityl; CE, capillary electrophoresis; MMI, methylenemethylimino; PNA, peptide nucleic acid; SVPD, snake venom phosphodiesterase.

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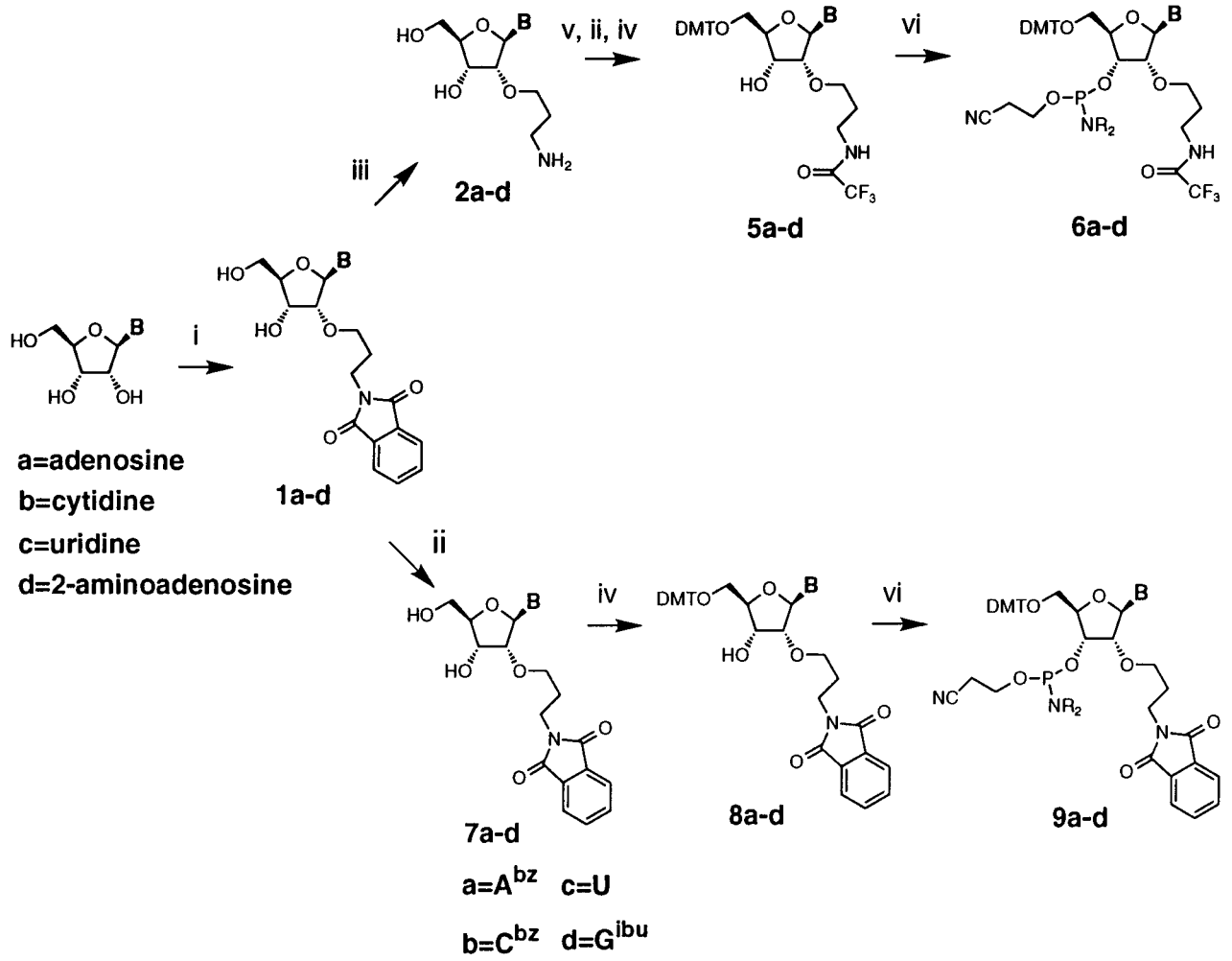
Scheme 1. Route for Synthesis of Protected 2′-*O*-Aminopropyl Nucleoside Phosphoramidites[a]



[a] (i) NaH/R-Br/DMF/5 °C/16 h; (ii) Bz-Cl/pyridine/20 °C/8 h; (iii) EtOH/H_2NNH_2/16 h; (iv) DMT-Cl/pyridine/20 °C/16 h; (v) CF_3CO_2Et/MeOH/TEA/20 °C/16 h; (vi) $C_9H_{18}N_3OP/CH_2Cl_2$/20 °C/16 h.

vatives of the amine. As shown in Scheme 1, the synthetic route starts by taking advantage of the acidity ($pK_a \sim 12$) of the 2′-hydroxy proton of the purine nucleosides. The reaction of adenosine and 2-aminoadenosine with *N*-(3-bromopropyl)phthalimide results in the isolation of the 2′-substituted nucleosides. The 2′,3′-stannyl complex of uridine provides entry to the 2′-*O*-alkylated product.[13] In the case of cytidine, a mixture of the 2′/3′-isomers was obtained and the pure 2′-product was not isolated until the DMT stage. Conversion of the alkylated 2-aminoadenosine into the 2′-*O*-(aminopropyl)guanosine is accomplished using adenosine deaminase.[14] The trifluoroacetyl-protected nucleosides are generated from the 2′-*O*-phthalimido nucleosides. Hydrazine readily cleaves the phthaloyl group, and the resulting primary amine is acylated directly with trifluoroethyl acetate. Tetrabutylammonium fluoride has been employed in the transient protection of the heterocycle instead of ammonium hydroxide to assure the integrity of the trifluoroacetyl group.[15] Conventional benzoyl and isobutyryl groups were used to protect the amino functionality of the nucleosides, which were subsequently converted to the 5′-*O*-DMT derivatives and finally the bis(*N*,*N*-diisopropyl)phosphoramidites.

The properties of 2′-*O*-aminopropyl (AP) oligonucleotides have been analyzed using molecular dynamics, NMR, hybridization with RNA complement, enzymatic digestion, and incorporation into cells. We demonstrate that the AP nucleosides can be incorporated into oligonucleotides in high yield via the phthalimide-protected phosphoramidites. Molecular dynamics analyses of the hydration and electrostatic interaction of the amine with a phosphate linkage have been performed. NMR studies of a pentanucleotide containing a single AP uridine suggest that the amino group rotates freely relative to the phosphodiester backbone and is not bound via inner-sphere coordination. Hybridization studies for oligomers substituted with aminopropyl and pentylamino residues as point substitutions and in fully modified sequences show that the alkylamine side chains reduce the T_m of a duplex with RNA complement by an amount comparable to an alkyl chain of the same length. However, incorporation of two AP units at the 3′-terminus of an oligomer dramatically enhances the exonuclease stability of the strand compared to phosphodiester or phosphorothioate-substituted oligonucleotides. A 2′-*O*-aminopropyl-modified oligonucleotide has been targeted against C-*raf* mRNA in cells using electroporation. Compared to the control phosphorothioate, this compound demonstrates enhanced potency for reducing mRNA levels and protein levels in A549 cells. Hence, aminopropyl oligonucleotides will be useful in the design of novel antisense therapeutics with favorable properties *in vivo*.

Table 1. Sequence and Structure of Oligonucleotides[a]

oligomer	sequence
A	ACT*U*C
B	*A*CCGAGGAT*C*AT*C*TCGT*A*CGC
C	GG*A*CCGG*AA*GGT*A*CGAG
D	CGACTATGCAA*A*A*A*C
E	CGACGATGCAAGTA*C*
F	CTCGTACCATTCCGGTCC
G	GAGCTCCCAGGC
H	*GAGCUCCCAGG*C
I	*CGACUAUGCAAGUA*C
J	*GGACCGGAAGGUACGA*G
K	*UUCUC*GCCCGCTCC*UCCUCC*C (full P=S)
L	*UUCUC*GCCCGCTCC*UCCUCC*C (P=S gap)
M	*UUUUU*TTTTTTTTT*UUUUU*T[b]
N	UUUUUUUUUUUU*UUUU*
O	TTTTTTTTTTTTTT*UUUU*T
P	TCCCGCCTGT*GACAUGCAUU*
Q	TCCCGCCTGTGACATGCATT

[a] Italics indicate a 2′-*O*-aminopropyl-substituted nucleotide in the sequence. For **H**, italics indicate 2′-*O*-methyl nucleotide substitutions. Underlined residues contain internucleotide phosphorothioate linkages. [b] Italics indicate a 2′-*O*-pentylamino-substituted nucleotide in the sequence.

Results

Synthesis of 2′-*O*-Aminopropyl Oligonucleotides. Two protection strategies have been evaluated for incorporation of aminopropyl nucleotides into the corresponding amidites, as shown in Scheme 1. Initial attempts were performed using the trifluoroacetamide of the amine. Utilization of this group has been described previously by Switzer et al.[11] The synthetic route begins with 2′-*O*-alkylation of adenosine, 2-aminoadenosine, and cytidine using the 3-*N*-phthalimidopropyl halide to yield compounds **2a**−**d**. The phthalimido moiety is removed with hydrazine, and the primary amine is quantitatively converted to the trifluoroacetamide. Alkylation of uridine proceeds through the 2′,3′-stannyl oxide. The 2′-*O*-(3-*N*-phthalimidopropyl)guanosine is generated by enzymatic conversion of the 2-aminoadenosine derivative using adenosine deaminase. Subsequent protection of the heterocycles with benzoyl or isobutyryl groups followed by addition of a dimethoxytrityl group at O5′ gives compounds **5a**−**d**. Conversion to the phosphoramidite using the bis(*N*,*N*-diisopropylamino)phosphine provided phosphoramidites **6a**−**d**. Incorporation of the amidites into oligonucleotides listed in Table 1 proceeded smoothly.

In our hands, the trifluoroacetyl group was removed very quickly upon treatment with ammonia during oligonucleotide deprotection. ES-MS analysis of the oligonucleotide products demonstrated the presence of multiple side products. As shown in Figure 1 for **M**, a series of adducted ions corresponding to *n**53.1 Da (*n* = 1−4) could be detected at higher *m*/*z* for each charge state of the oligonucleotide. The 53.1 Da increase in molecular mass corresponds to addition of C_3H_3N, readily rationalized by Michael addition of the primary amine to acrylonitrile generated during deprotection of the cyanoethyl-protected phosphate triesters. Prolonged deprotection (24 h) with ammonia at 55 °C did not reduce the quantity of the adduct or change the observed ion intensities and values of *m*/*z*.

Observation of the acrylonitrile adducts in five oligomers prepared using the trifluoroacetyl protecting group led us to evaluate a different deprotection strategy for the aminopropyl functionality. The phthalimide-protected phosphoramidites **9a**−**d** could be incorporated into oligomers with >98.5% stepwise coupling efficiency using two sequential coupling steps. Removal of the phthalimide group from the product oligonucleotide via ammonium hydroxide treatment was ineffective, leaving partially ring-opened phthalamide functionality bound to the amine group. A second deprotection strategy was developed, using an initial ammonium hydroxide treatment followed by reaction with 10% aqueous methylamine.[16] In the initial treatment, the cyanoethyl groups could be removed from the phosphate to prevent alkylation of the nascent primary alkylamine with acrylonitrile, and the benzoyl groups could be removed rapidly from the exocyclic amine of C nucleotides to preclude substitution of the exocyclic amine of C with methylamine.[17] Further reaction of the oligomer with 10% aqueous methylamine cleanly deprotects the isobutyryl groups from guanosine and the phthalimide groups from the alkylamine side chains. PAGE and ES-MS analyses showed that the aminopropyl and aminopentyl groups could be incorporated cleanly into oligomers under these conditions.

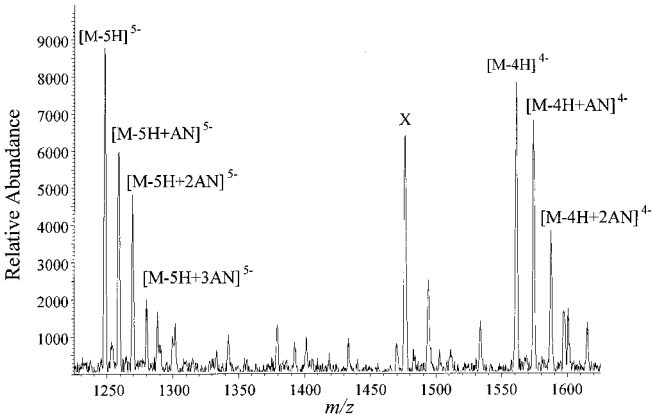


Figure 1. ES-MS spectrum for propylamine oligonucleotide **M** prepared using TFA protection of the primary amine. The $[M - 5H]^{5-}$ and $[M - 4H]^{4-}$ charge states are shown between *m*/*z* 1200 and 1650. The ions originating from acrylonitrile adducts (+53 Da) are indicated as +AN. Molecules with 1−4 acrylonitrile adducts can be observed in the mass spectrum for both charge states. No acrylonitrile adducts were observed for oligonucleotides prepared using the phthaloyl protection/deprotection strategy. The samples were desalted via precipitation from 10 M ammonium acetate prior to analysis.

Molecular Dynamics. The 2′-*O*-aminopropyl group has the potential to interact with any of the electronegative groups on surrounding nucleotides. We have investigated the probability for such interactions using a *U*T dinucleotide model system that could be modeled in the presence of explicit solvent molecules. The simulation was performed in a 20.0 Å^3 box of pre-equilibrated water molecules. Data from the first 20 ps of the calculation have been attributed to the equilibration process and were not included in the final analysis. The system remains energetically stable after the initial equilibration phase for the next 150 ps. The computed structural properties (radial distribution functions; data not shown) of the solvent model used in the present study are in close agreement with literature results employing different water models.[18] Over the time course of the simulation, the distance from the terminal nitrogen to the equatorial oxygens of the phosphate varied randomly between 3.2 and 6.5 Å. No binding of the amino group was detected to neighboring base carbonyl or sugar O4′ atoms.

Table 2. Proton NMR Chemical Shifts, Coupling Constants, and Pseudorotation Parameters for **A**

	chemical shift (ppm)				coupling constant (Hz)					
residue	H8/H6/H5	H1′	H2′, H2″	H3′	^3J(H1H2)	^3J(H2H3)	^3J(H3H4)	P_N	P_S	%N
A	8.01	6.24	2.64, 2.57	4.60	8.0, 6.0	5.6	2.4	15	168	15.0
C	7.62	6.06	2.37, 2.09	4.69	7.9, 6.1	5.6	nd[a]			
	5.70									
T	7.43	6.03	2.33, 2.14	4.71	8.0, 6.0	5.6	nd			
	1.67									
U	7.69	5.82	4.04	4.57	5.2	4.8	≤4.4	1.5	170.5	40.0
	5.74									
C	7.72	6.11	2.26, 2.14	4.38	6.8, 6.8	4.8	2.6	4.0	164.5	14.0
	5.91									
propylamine	3.62									
	1.80									
	2.98									

[a] Could not be determined accurately due to spectral overlap.

The pseudorotation of the 2′-*O*-aminopropylU sugar was calculated from the internal dihedrals as a function of time. The psuedorotation lies predominately in the C3′-endo form but does transit to a C2′-endo pucker. This interconversion is independent of the motion of the propylamine side chain. The upper sugar tends toward a C4′-exo conformation, although this may be an artifact of having no constraints on the 5′-end of the dinucleotide.

NMR Spectroscopy. The results obtained from molecular modeling have been confirmed using NMR in a larger model, 5-mer oligonucleotide **A** containing an AP uridine. A pK_a value of 9.4 has been determined for the propylamine group in the oligomer from observation of the proton chemical shifts of the propyl protons as the pH was titrated between 5.0 and 11.0. The ^{31}P NMR chemical shifts for the phosphates were invariant as a function of pH between 5.0 and 9.0.

The coupling constants for the sugar protons have been measured directly from a 1D spectrum or a TOCSY spectrum obtained with 0.5 Hz resolution in the acquisition dimension and are presented in Table 2. The pseudorotation parameters for the AP uridine have been determined from the values of the H1′−H2′, H2′−H3′, and H3′−H4′ scalar coupling constants. These values have been used to generate a fit to a two-state model for psuedorotation using a version of PSEUDOROT modified for 2′-*O*-alkyl substituents.[19] <Sen>The AP uridine exists as 40% C3′-endo conformer (P = 1.5°) and 60% C2′-endo (P = 170.5°). Variations of ±0.4 Hz in the value of the H3′H4′ coupling constant used in the calculation of the pseudorotation parameters shifted P_N by ±17° and P_S by ±5° but did not alter the %N conformation for the propylamine sugar. The 3′-flanking DNA residue adopted a C2′-endo conformation (%N = 14.0) with P_N = −4.0° and P_S = 164.5°. Predominantly C2′-endo conformations are observed for the remaining DNA nucleotides.

A NOESY spectrum was acquired with a 500 ms mixing time at 20 and 37 °C. At 20 °C, interproton NOE cross-peaks from the H5 and H6 base protons of C and U have the same sign as the diagonal, and few NOE cross-peaks are observed from sugar protons, while the cross-peaks among the protons of the propyl arm are inverted relative to the diagonal. A ROESY spectrum obtained with a 100 ms mixing time generates off-diagonal peaks which are inverted relative to the diagonal. Assuming that $\omega^*\tau(c) = 1.0$ and given the proton resonance frequency of 400 MHz, the effective correlation time $\tau(c)$ for the sugar protons can be calculated to be ~8.0×10^{-10} s at this temperature. In NOESY spectra obtained at 37 °C, the signs of the cross-peaks between the H5,H6 base protons and the sugar protons all invert relative to the diagonal.

Table 3. Hybridization Data for Oligonucleotides with AP Point Substitutions against RNA Complement

	sequence						
substitution	**B**	**C**	**D**	**E**	**F**	**G**	**H**
DNA, P=S	−0.38	nd	−0.52	−0.80	−0.65	−0.73	nd
2′-*O*-methyl[a]	−0.01	+0.96	+0.91	+0.31	+0.03	+0.67	+2.13
2′-*O*-propyl[a]	−0.36	+0.24	+0.84	−1.02	−1.14	−0.10	+2.00
2′-*O*-propylamine	−0.40	+0.27	+1.09	−1.08	−1.78	+1.13	+1.88

[a] Values are listed as ΔT_m values calculated versus the unmodified DNA control. Data taken from ref 10; nd = not determined.

Table 4. Hybridization Data for Fully Substituted Oligonucleotides against RNA Complement

	sequence	
substitution	**I**	**J**
DNA, P=S	−0.52	nd
2′−*O*−methyl[a]	+1.29	+1.49
2−*O*−propyl	+0.99	+1.10
2−*O*−propylamine	+0.43	+0.39

[a] Values are listed as ΔT_m values calculated versus the unmodified DNA P=O control; nd = not determined.

Hybridization Studies. Hybridization studies have been conducted for both point substitutions in sequences with a DNA background and fully modified sequences against RNA complement. Oligomer sequences are listed in Table 1, while ΔT_m values are listed in Table 3. As a point modification in six deoxynucleotide sequences, AP A is stabilizing compared to P=S DNA in four cases and destabilizing in two cases. In sequence **C** where AP A residues are adjacent, the modification provides some stabilization. Duplex stabilization by the AP A relative to dA and 2′-*O*-alkyl A is observed in sequences **D** and **G**, where the AP A is flanked on the 3′-side by a purine. As point substitutions in a 2′-*O*-methyl background (sequence **H**), the AP A modification is stabilizing compared to DNA but slightly destabilizing compared to the 2′-*O*-methyl and 2′-*O*-propyl modifications. When incorporated into fully modified sequences **I** and **J**, the AP nucleotides are stabilizing compared to DNA by +0.4 °C but less stabilizing than the 2′-*O*-methyl and -propyl derivatives, listed in Table 4. The effect of incorporating 2′-substituents in the wings of phosphorothioate and phosphodiester chimeric oligonucleotides has been investigated. As shown in Table 5, the aminopropyl group does not destabilize the fully

Table 5. Hybridization Data for Chimeric Oligonucleotides Containing Modified Wings against RNA Complement

substitution	sequence K	sequence L
2′-*O*-methyl[a]	+0.95	nd
2-*O*-propyl	−0.93	+0.00
2-*O*-propylamine	+0.01	−0.46

[a] Values are listed as ΔT_m values calculated versus the unmodified DNA P=O control; nd = not determined.

Table 6. Rates of Degradation of Oligonucleotide **O** by SVPD

modification	sequence	$t_{1/2}$ SVPD (N → *N* − 1; min)
P=O	$T_{14}U_4T$	4 ± 1
P=S	$T_{14}U_4T$	360
2′-AP P=O	$T_{17}UT$	20
AP P=O	$T_{16}U_2T$	600
AP P=O	$T_{15}U_3T$	1440 ± 60
AP P=O	$T_{14}U_4T$	>1440
Pr P=O	$T_{14}U_4T$	7
1 AP, 3 Pr	$T_{14}U_4T$	≤15
2 AP, 2 Pr	$T_{14}U_4T$	800
3 AP, 1 Pr	$T_{14}U_4T$	1200

P=S sequence **K**, in contrast to the oligomer containing 2′-*O*-propyl nucleotides. For comparison, the 2′-*O*-methyl nucleotides provide some stabilization in sequence **K**. However, some destabilization from the AP nucleotides is observed in oligomer **L** with phosphodiester wings relative to the propyl derivative. Finally, the salt dependence of the T_m value has been measured for sequence **M** containing five 2′-*O*-pentylamino residues. As observed by Hashimoto et al., incorporation of pendant amino groups reduces the slope of $\Delta T_m/\Delta[Na^+]$ by 2.5%/amino substitution compared to a control deoxynucleotide sequence.[12]

Nuclease Digestion. The 3′-exonuclease resistance of oligomers containing the propylamine modification has been determined using snake venom phosphodiesterase (SVPD).[20] All oligomers were 5′-end-labeled with [^{32}P]ATP and T4 polynucleotide kinase. Studies were performed with sequence **O**, $dT_{(14+N)}\boldsymbol{U}_{(4-N)}dT$, where $N = 0-4$ and ***U*** represents 2′-*O*-(aminopropyl)-uridine. In all cases, a sequential degradation from the 3′-terminus was observed. Under the conditions used in the assay, the $t_{1/2}$ for a dT_{19} phosphodiester was ∼4 min, while the $t_{1/2}$ value for a dT_{19} phosphorothioate was ∼360 min. The $t_{1/2}$ values are presented in Table 6. In the molecule with a single AP substitution, the 3′-terminal dT residue is cleaved with a $t_{1/2}$ of 20 min. A gap is observed in the gel at the position where the zwitterionic AP U residue is lost, as a result of the increase in the charge/mass ratio. Surprisingly, incorporation of two sequential AP U nucleotides increases the $t_{1/2}$ for removal of the 3′-dT from 20 to 600 min. Further incorporation of sequential AP U residues generates more exonuclease resistance with $t_{1/2}$ values of ≤1440 and >1440 min for three and four incorporations, respectively. A gel showing the course of degradation of the oligomers containing 1−4 AP U incorporations is presented in Figure 2.

The origins of this increase in nuclease resistance was explored in a second series of experiments. No change in the rate of degradation of the 3′-dT was observed in molecule **O** between pH 7.0 and 9.5. The possibility that a conformational change or steric effect from incorporating 2′-substituted RNA into the oligomer instead of DNA was ruled out by incorporating varying numbers of AP U residues in a 2′-propyluridine background in oligomer **N**. As listed in Table 6, the $t_{1/2}$ values were similar to those observed for the oligomers with dT substitutions. The possibility that the on-rate for SVPD to the 3′-terminus of the oligomer with multiple AP U incorporations was altered was explored in a series of oligomers where the location of the AP U "block" was moved internally. Normal kinetics were observed in all cases for digestion of 3′-dT residues up to the first dT adjacent to a AP-containing nucleotide. A kinetic analysis of the competition for degradation between 3′-(*p*-nitrophenyl)-phosphoryl dT demonstrated that oligomer **O** is not a better competitive inhibitor of enzyme activity than a comparable deoxyphosphodiester oligomer (data not shown).

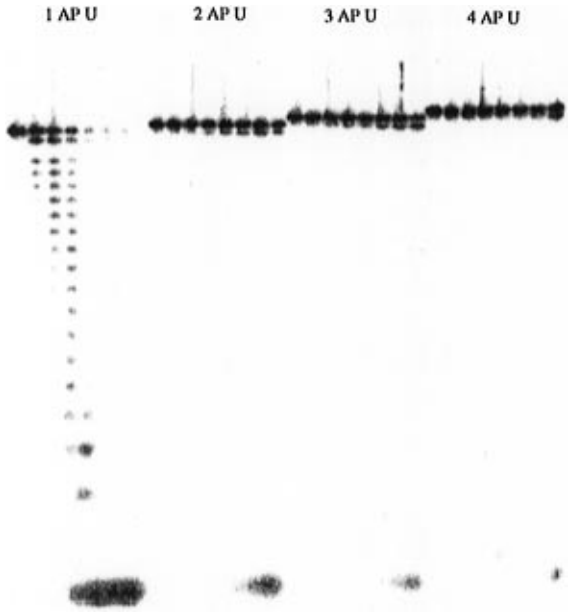


Figure 2. Scan of a PAGE gel showing time course of SVPD-catalyzed degradation of oligonucleotide **O** with 1−4 2′-*O*-(aminopropyl)uridine residues. The samples in each lane were obtained at 0, 0.5, 1, 2, 4, 8, and 24 h time points.

Inhibition of C-*raf* mRNA and Protein Expression. A 20-mer phosphorothioate oligonucleotide (**Q**) previously demonstrated to reduce the levels of C-*raf* mRNA and protein was prepared (**P**) with nine AP nucleotides at the 3′-terminus.[27] Attempts to transfect **P** into A549 cells with varying concentrations of cationic lipids proved unsuccessful. However, both **P** and **Q** could be transfected efficiently using electroporation methods.[28] As shown in Figure 3, a 250 nM concentration of **P** lowered the level of C-*raf* mRNA by 50% 24 h following administration, while a 10-fold higher concentration of **Q** was required to effect the same reduction. The reduction in levels of C-*raf* protein was more pronounced, where a ∼150 nM concentration of **P** produced a 50% reduction in protein at 48 h, compared to >300 μM **Q**.

Discussion

The design of antisense oligonucleotides is complicated by the need to improve properties such as resistance to nuclease degradation while maintaining cellular activity. Generation of chimeric oligomers which mix different chemistries has proven to be an effective approach.[21] This strategy keeps the favorable properties of a negatively charged deoxynucleotide "gap" which

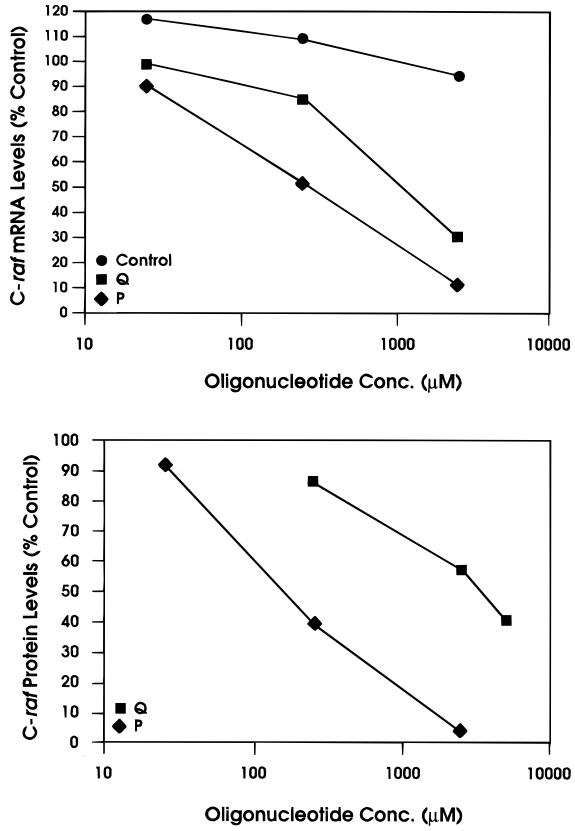


Figure 3. Inhibition of C-*raf* kinase mRNA and protein expression in A549 cells. Upper: quantitation of C-*raf* mRNA levels following electroporation with increasing concentrations of 20-mer PS oligonucleotides **P** (◆; 3′-AP_2), **Q** (■; no AP), and a scrambled control (●) phosphorothioate. Total RNA was prepared 24 h after treatment and analyzed for C-*raf* and G3PDH mRNA levels by Northern blot analysis. Lower: quantitation of C-*raf* protein levels in A549 cells treated with varying concentrations of **P** (◆) and **Q** (■). Cells were treated with ODN, protein extracts were prepared 48 h later, and C-*raf* protein levels were determined as described in the Experimental Section.

can provide a binding domain for enzymes such as RNAse H, while the surrounding "wings" can have reduced net charge or other characteristics which provide improved uptake and pharmacokinetics, alter affinity for specific proteins, or increase nuclease resistance.[22] In the wings, positioning of positively charged groups in the minor groove rather than the major groove decreases their proximity to the density of negative charge of the phosphate backbone and is straightforward synthetically. A short 2−3-carbon alkyl chain will have less entropy and less disruption of hydration of a duplex and should have superior properties compared to a longer alkyl linker. The distance from the amino group to hydrogen-bonding/electrostatic functionality of bases on the other strand is longer with a short alkyl chain, which should minimize nonspecific steric interactions. Hence, a short linker chain positioned in the minor groove might be expected to overcome the problems associated with an amine situated in a major groove location off C5 of pyrimidine nucleotides.

Synthesis. A convenient route to the preparation of oligonucleotides containing 2′-*O*-alkylamino moieties via phthalimido protection and differential deprotection has been developed. Amidites of all four bases can be prepared using trifluoroacetyl or phthaloyl protecting groups.[23] In our hands, however, the TFA amide proved to be incompatible with the conditions of DNA synthesis. Early deprotection of the primary amine allows Michael addition to acrylonitrile generated during removal of the cyanoethyl protecting groups from the phosphates. A differential deprotection strategy can be employed when the amine is incorporated as an alkylphthalimido moiety. The cyanoethyl and benzoyl protecting groups are removed rapidly during a preliminary treatment with ammonia. Subsequent treatment with methylamine rapidly removes the phthalimido protecting groups and the isobutyryl protecting groups on the exocyclic amine of **G**.[16]

Molecular Modeling and NMR. In the MD study, both the propylamine and the phosphate backbone are coordinated to waters of hydration. The protonated propylamine is not attracted toward the phosphate by electrostatic forces during the 150 ps of the simulation and does not form an inner-sphere coordination complex. The lack of a strong electrostatic interaction of the propylamine with anions is confirmed following addition of sodium chloride to the simulation. The position of the chloride anion equilibrates without strong binding to the propylamine. These results suggest that the propylamine side chain is of a correct length to minimize the formation of tight ion pairs and hydrogen-bonding interactions with neighboring functionality in the oligomer.

The MD study also shows that the AP sugar adopts a conventional northern conformation centered around 0°. During the course of the simulation, one movement toward an O4′-endo conformation was observed, but the conformation rapidly fell back to the more stable C3′-endo sugar pucker. This sugar conformation minimizes potential steric interactions between the propylamine side chain and the H5′,H5″ sugar protons on the 3′-nucleotide.

The solution dynamics of the propylamine side chain have been confirmed in ^{1}H NMR studies, which show a greater mobility for the AP protons compared to the protons of the sugar−phosphate backbone. The comparison of cross-peak intensities from the ROESY and NOESY spectra for a pentanucleotide containing a single AP substitution suggests that the propylamine group has a 2−4-fold shorter correlation time than the oligonucleotide backbone.[24] This short correlation time is consistent with uncorrelated motion between the phosphate and the AP chain and confirms the modeling results on the longer NMR time scale. In addition, the ^{31}P NMR chemical shift is invariant over a pH range where the amino group titrates, suggesting that the amine does not bind via inner-sphere coordination to the flanking phosphate. Formation of a tight ion pair between the amine and a desolvated phosphate would be undesirable, since hydration of the phosphates may be critical for solubility.

The sugar pucker for the AP unit is altered from conventional RNA as evidenced from the large H1′−H2′ coupling constant compared to RNA or 2′-*O*-Me derivatives.[9] The coupling constants can be fit to a two-state exchange model between C3′-endo and C2′-endo sugar puckers, with a 40% proportion of a C2′-endo conformation. This conformation can be accommodated in the single-stranded oligomer, but the C2′-endo sugar pucker for an AP nucleotide would be difficult to fit into an 'A'-form duplex with RNA complement, where the alkyl

chain would have bad steric contacts with the proximate 3′-residue. The destabilization of the C3′-endo conformation by the side chain, combined with the incompatibility of a C2′-endo conformation in a duplex, may account in part for the reduced T_m of the AP-containing oligomers. No NOE is detected from the side chain to protons of flanking sugars, showing that the AP does not bind to O4 of the 3′-sugar ring. In a duplex with RNA, the AP side chain will traverse a broad region in the vicinity of the flanking phosphate. The pH determination for the AP side chains shows that the amine moiety will stay protonated in a zwitterionic form under physiological conditions.

Hybridization. Substitution of AP nucleotides into DNA oligomers produces stabilization of a duplex with complementary RNA roughly equivalent to alkyl chains of the same length. Duplexes with uniformly AP-modified sequences are less stable than 2′-*O*-methyl or 2′-*O*-propyl sequences but still superior to DNA sequences. In a 2′-*O*-methyl sequence, substitution of AP residues generates no destabilization. In a winged phosphorothioate oligonucleotide, the AP nucleotides stabilize superior to a 2′-*O*-propyl wing but less than a 2′-*O*-methyl wing. In an oligomer with phosphodiester wings, substitution with propylamine residues is destabilizing relative to the other sugar alkyls. Incorporation of five pentylamine residues reduces the salt dependence of the T_m similar to C5-substituted pyrimidines with amino groups, suggesting that binding of cations and anions to the strands of the propylamine-substituted strand:RNA duplex during the dissociation process generates an unfavorable entropic effect. Substitution of aminopropyl residues into the sequence provides improved affinity compared to phosphorothioate oligonucleotides.

Nuclease Resistance. Capping of a phosphodiester oligonucleotide with two propylamine units provides nearly complete resistance to 3′-exonuclease degradation. These capped oligomers are ~1000-fold more resistant than a phosphodiester and have greater resistance than a P=S linkage. This resistance to exonuclease degradation is independent of pH between pH 5 and 9. The increase in resistance is not just a steric effect, since a propyl or longer pentyl side chain confers only a mild increase in resistance.[10] The sugar conformation does not play a role, since incorporation of propyl-substituted residues does not produce a dramatic increase in nuclease resistance. The net charge of the oligo is not critical, since the digestion is rapid up to the first residue 3′ from the AP nucleotide. These data suggest that location of a positive charge in the general proximity of the phosphate is sufficient to inhibit enzymatic activity, presumably through introduction of an unfavorable electrostatic interaction with a hydrated metal ion or positively charged amino acid side chain which catalyzes the cleavage process. While SVPD can cleave through one AP residue, a two-AP nucleotide cap provides an oligomer with nearly complete 3′-exonuclease resistance.

Inhibition of C-*raf* mRNA and Protein. An antisense oligomer (**P**) targeted against human C-*raf* kinase containing nine 2′-*O*-aminopropyl modifications has been found to be at least 10-fold more potent in reducing C-*raf* mRNA and protein expression as compared with a phosphorothioate ODN of the same sequence when transfected into cultured cells by electroporation. These results are both sequence-specific and target-specific, consistent with an antisense mechanism of action. Oligonucleotide **P** contained nine phosphodiester linkages, and these results demonstrate that the greatly enhanced nuclease stability observed for AP oligonucleotides in cell-free systems using SVPD translates into enhanced stability and antisense activity in cells. Thus, antisense oligonucleotides containing 2′-*O*-AP modifications are attractive alternatives for the design of effective, nuclease-stable antisense molecules with reduced phosphorothioate content. Since the human C-*raf* antisense sequence employed in this study has previously been shown to be a potent antitumor agent in animals when synthesized as a phosphorothioate ODN, future studies examining the antitumor properties of AP analogs of this and additional sequences are highly warranted.

Conclusions

The 2′-*O*-propylamine ribonucleotides are incorporated readily into antisense oligonucleotides using conventional phosphoramidite monomers and a phthalimido protecting group for the amine. A differential deprotection strategy with an initial ammonia treatment followed by reaction with methylamine cleanly and rapidly removes all of the protecting groups. The propylamine side chain is mobile and does not form a tight ion pair with the flanking 3′-phosphate in molecular dynamics simulations or an NMR analysis of chain motion. Oligonucleotides incorporating propylamine groups have reduced net charge but maintain favorable solubility properties.

No dramatic increase in affinity of oligomers containing propylamine sugars for RNA complement is observed compared to simple alkyl side chains. This result suggests that no cratic release of cations from the phosphate backbone is produced, which would provide stabilization. The shift in the sugar pucker of 2′-substituted nucleotides toward C3′-endo geometries may limit the location of the propylamine nucleotides to the wings of gapmers to retain RNAse H activity.

The enhanced exonuclease resistance of propylamine-containing oligomers may be related to an electrostatic repulsion between the side chain and functional groups at the catalytic site of the enzyme, which must dehydrate the phosphate and bring in a positively charged catalyst such as a hydrated metal ion or charged amino acid side chain prior to cleavage. The inability of SVPD to cleave the propylamine-capped phosphodiester is not just a result of steric hindrance or an unfavorable nonspecific electrostatic interaction.

The potency of PS oligonucleotides is enhanced through incorporation of two phosphodiester 2′-AP nucleotides at the 3′-terminus. These capped oligonucleotides reduced levels of mRNA in cells more efficiently and dramatically reduced protein expression relative to the PS ODN of the same sequence. The increase in nuclease resistance without the penalty in affinity for RNA complement induced by phosphorothioates, and demonstration of improved cellular potency, makes oligomers containing phosphodiester-linked 2′-*O*-aminopropyl nucleotides leading candidates as antisense therapeutic agents *in vivo*.

Experimental Section

General Methods. Bulk chemicals were purchased from Sigma or Aldrich and were of the highest available purity. Ultraviolet−visible spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer with a Peltier temperature controller accessory. Melting points (uncorrected) were determined in an open glass capillary on a Thomas-Hoover apparatus. Kieselgel 60 F254 plates from E. Merck were used for TLC, with detection by UV light or H_2SO_4/MeOH and heat. Flash chromatography was performed using silica gel (40 μm; J. T. Baker). Elemental analyses were performed by Quantitative Technologies (Bound Brook, NJ). ^{1}H NMR spectra were recorded on a Gemini 200 (200 MHz) spectrometer (Varian Associates, Palo Alto, CA) using tetramethylsilane as an internal standard. ^{31}P and ^{19}F NMR studies utilized phosphoric acid and trifluoroacetic acid as internal references, respectively.

2′-*O*-(Propylphthalimido)adenosine (1a). Sodium hydride (60%) (4.5 g, 112 mmol) was added to a solution of adenosine (20.0 g, 75 mmol) in dry dimethylformamide (550 mL) at 5 °C under N_2 atmosphere. After 1 h, *N*-(3-bromopropyl)phthalimide (23.6 g, 86 mmol) was added and the temperature raised to 30 °C and held for 16 h. After cooling, ice was added and the reaction mixture concentrated to a gum. The gum was partitioned between water and ethyl acetate and extracted thoroughly (4 × 300 mL). The organic extracts were dried and concentrated *in vacuo*, and the resulting gum was subjected to chromatography on silica gel (95/5 CH_2CL_2/MeOH) to afford a white solid. Recrystallization from MeOH afforded 5.7 g of the 2′-isomer (17%): mp 123−124 °C; ^{1}H NMR (DMSO) δ 1.70 (m, 2H, CH_2), 3.4−3.7 (m, 6H, C5′CH_2, OCH_2, PhthCH_2), 3.95 (q, 1H, C4′H), 4.30 (q, 1H, C3′H), 4.46 (t, 1H, C2′H), 5.15 (d, 1H, C3′OH), 5.41 (t, 1H, C5′OH), 5.95 (d, 1H, C1′H), 7.35 (s, 2H, NH_2), 7.8 (brs, 4H, Ar), 8.08 (s, 1H, C2H), 8.37 (s, 1H, C8H). Anal. ($C_{21}H_{22}N_6O_6$) C,H,N.

2′-*O*-(Aminopropyl)adenosine (2a). A solution of **1a** (8.8 g, 19 mmol), 95% ethanol (400 mL), and hydrazine (10 mL, 32 mmol) was stirred for 16 h at room temperature, whereupon the reaction mixture was filtered and the filtrate concentrated *in vacuo*. Water (150 mL) was added, and the pH was adjusted to 5.0 with HOAc. The aqueous layer was extracted with ethyl acetate (2 × 30 mL) and concentrated *in vacuo* to afford 7.1 g of the product as the HOAc salt (95%): ^{1}H NMR (DMSO) δ 1.70 (m, 2H, CH_2), 2.76 (m, 2H, CH_2NH_2), 3.55−3.67 (m, 4H, C5′-CH_2, OCH_2), 4.0 (q, 1H, C4′H), 4.30 (q, 1H, C3′H), 4.47 (t, 1H, C2′H), 6.0 (d, 1H, C1′H), 7.39 (s, 2H, NH_2), 8.16 (s, 1H, C2H), 8.41 (s, 1H, C8H). Anal. ($C_{13}H_{20}N_6O_4$·HOAc) C,H,N.

2′-*O*-[[*N*-(Trifluoroacetyl)amino]propyl]adenosine (3a). A solution of **2a** (6.0 g, 16 mmol) in methanol (50 mL) and triethylamine (15 mL, 107 mmol) was treated with ethyl trifluoroacetate (18 mL, 151 mmol). After 16 h, the reaction mixture was concentrated *in vacuo* and the resultant gum subjected to chromatography on silica gel (9/1 EtOAc/MeOH) to afford 3.3 g of the product (54%): mp 200−201 °C; ^{1}H NMR (DMSO) δ 1.7 (m, 2H, CH_2), 3.20 (m, 2H, CH_2NHCO), 3.37−3.67 (m, 4H, C5′CH_2, OCH_2), 4.00 (q, 1H, C4′H), 4.35 (q, 1H, C3′H), 4.49 (t, 1H, C2′H), 5.26 (d, 1H, C3′OH), 5.43 (t, 1H, C5′OH), 6.02 (d, 1H, C1′H), 7.38 (s, 2H, NH_2), 8.16 (s, 1H, C2H), 8.40 (s, 1H, C8H), 9.34 (s, 1H, NHCO); ^{19}F NMR (DMSO) δ −75.48. Anal. ($C_{15}H_{19}F_3N_6O_5$) C,H,N.

6-*N*,*N*-Dibenzoyl-2′-*O*-[[*N*-(trifluoroacetyl)amino]propyl]adenosine (4a). Compound **3a** (12.6 g, 30 mmol) was converted to **4a** using a modification of the procedure of Ti et al.[15] Tetrabutylammonium fluoride was utilized instead of ammonium hydroxide in the workup. Concentration of the reaction mixture followed by chromatography on silica gel (1/1 EtOAc/MeOH) afforded 6.8 g of the product as a foam (36%): ^{1}H NMR (DMSO) δ 1.72 (m, 2H, CH_2), 3.20 (m, 2H, CH_2-NHCO), 3.45−3.68 (m, 4H, C5′CH_2, OCH_2), 4.03 (q, 1H, C4′H), 4.37 (q, 1H, C3′H), 4.53 (t, 1H, C2′H), 5.18 (t, 1H, C5′OH), 5.34 (d, 1H, C3′OH), 6.17 (d, 1H, C1′H), 7.45−7.83 (m, 10H, Ar), 8.73 (s, 1H, C2H), 8.90 (s, 1H, C8H), 9.37 (s, 1H, NHCO); ^{19}F NMR (DMSO) δ −75.48. Anal. ($C_{28}H_{27}F_3N_6O_7$) C, H, N.

5′-*O*-(Dimethoxytrityl)-6-*N*,*N*-dibenzoyl-2′-*O*-[[*N*-(trifluoroacetyl)amino]propyl]adenosine (5a). 4,4′-Dimethoxytrityl chloride (95%) (3.6 g, 10 mmol) was added to a solution of **4a** (3.4 g, 5.4 mmol) in dry pyridine (100 mL) at room temperature and stirred for 16 h. The solution was concentrated *in vacuo* and purified by chromatography on silica gel (99/1 EtOAc/TEA) to afford 3.8 g of the product as a foam (75%): ^{1}H NMR (DMSO) δ 1.74 (m, 2H, CH_2), 3.23 (m, 2H, CH_2NHCO), 3.60−3.73 (m, 10H, $ArOCH_3$, C5′CH_2, OCH_2), 4.09 (m, 1H, C4′H), 4.51 (m, 1H, C3′H) 4.66 (m, 1H, C2′H), 5.34 (d, 1H, C3′OH), 6.19 (d, 1H, C1′H), 6.80−7.78 (m, 23H, ArH), 8.64 (s, 1H, C2H), 8.78 (s, 1H, C8H), 9.35 (s, 1H, NHCO); ^{19}F NMR (DMSO) δ −75.48. Anal. ($C_{50}H_{45}F_3N_6O_9$) C, H, N.

5′-*O*-(Dimethoxytrityl)-6-*N*,*N*-dibenzoyl-2′-*O*-[[*N*-(trifluoroacetyl)amino]propyl]adenosine-3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (6a). A solution of **5a** (6.5 g, 7 mmol) in dry methylene chloride was treated with bis(*N*,*N*-diisopropylamino)cyanoethyl phosphite (1.1 equiv) and (*N*,*N*-diisopropylamino)tetrazolide (catalytic amount) at room temperature for 16 h. The reaction was concentrated *in vacuo* and subjected to chromatography on silica gel (6/4 EtOAc/hexane, 1% TEA) to afford 6.7 g of the product as a foam (78%): ^{1}H NMR ($CDCl_3$) 1.0−1.31 (m, 12H, CH_3), 1.85 (m, 2H, CH_2), 2.36 (t, 2H, CH_2NH), 2.61 (t, 2H, CH_2CH), 3.21−3.91 (m, 14H, $AROCH_3$, C_4, H, C_5, CH_2, CHN, OCH_2, OCH_2), 4.38 (m, 1H, C_3, H), 4.62 (m, 1H, C_2, H), 6.15 (d, 1H, C_1, H), 6.77−7.82 (m, 23H, ARH) 8.28 (S, 1H, C_2H), 8.57 (S, 1H, C_8H); ^{31}P NMR (CD_3CN) δ 150.87; ($CDCl_3$) δ 150.55, 150.83; ^{19}F NMR (CD_3CN) δ −77.1; ($CDCl_3$) δ −81.6. Anal. ($C_{59}H_{62}F_3N_8O_{10}P$) C,H,N.

6-*N*-Benzoyl-2′-*O*-(propylphthalimido)adenosine (7a). Compound **1a** (3.0 g, 6.6 mmol) was converted to **7a** using benzoyl chloride via the transient protection procedure described above. After workup, concentration of the reaction *in vacuo* followed by chromatography on silica gel (9/1 EtOAc/MeOH) afforded 2.74 g of the product as a foam (74%): ^{1}H NMR (DMSO) δ 1.81 (m, 2H, CH_2), 3.45−3.7 (m, 6H, C5′, OCH_2, PhthCH_2), 3.93 (m, 1H, C4′H), 4.3 (m, 1H, C3′H), 4.5 (q, 1H, C2′H), 5.12 (t, 1H, C5′OH), 5.20 (d, 1H, C3′OH), 6.1 (d, 1H, C1′H), 7.5−8.06 (m, 9H, Ar), 8.68 (s, 1H, C2H), 8.71 (s, 1H, C8H), 11.2 (s, 1H, NH). Anal. ($C_{28}H_{26}N_6O$) C,H,N.

5′-*O*-(Dimethoxytrityl)-6-*N*-benzoyl-2′-(propylphthalimido)adenosine (8a). Compound **8a** was prepared from **7** (2.7 g, 5.0 mmol) following the procedure described for compound **5a**. Chromatography on silica gel (99/1 EtOAc/TEA) afforded 3.1 g of the product (75%): ^{1}H NMR (DMSO) δ 1.87 (m, 2H, CH_2), 3.19−3.70 (m, 6H, C5′CH_2, OCH_2, PhthCH_2), 4.07 (m, 1H, C4′H), 4.43 (m, 1H, C3′H), 4.63 (m, 1H, C2′H), 5.25 (d, 1H, C3′OH), 6.13 (d, 1H, C1′H), 6.8−8.08 (m, 22H, Ar), 8.60 (s, 1H, C2H), 8.61 (s, 1H, C8H), 11.1 (s, 1H, NH). Anal. ($C_{49}H_{44}N_6O_9$) C,H,N.

***N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(propylphthalimido)adenosine-3′-*O*-(2-cyanoethyl *N*,*N*′-diisopropylphosphoramidite) (9a).** Compound **9a** was prepared from **8a** (2.7 g, 3.2 mmol) according to the procedure for compound **6a**. Chromatography on silica gel (1/1 EtOAc/hexane, 0.5% TEA) afforded 2.7 g of the product (80%): ^{1}H NMR ($CDCl_3$) δ 1.0−1.34 (m, 12H, CH_3), 1.85 (m, 2H, CH_2), 2.63 (t, 2H, CH_2CN), 3.24−3.89 (m, 17H, $AROCH_3$, C_4, H, C_5, CH_2, OCH_2, OCH_2, CHN, CH_2NPHTH), 4.35 (m, 1H, C_3, H), 4.60 (m, 1H, C_2, H), 6.1 (d, 1H, C_1, H), 6.72−7.91 (m, 22H, ARH), 8.21 (S, 1H, C_2H), 8.59 (S, 1H, C_8, H); ^{31}P NMR (CD_3-CN) δ 150.88, 151.22. Anal. ($C_{58}H_{61}N_8O_{10}P$) C,H,N.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-O-(propylphthalimido)-uridine-3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (9b). The 2′-(propylphthalimido)uridine nucleoside was prepared as described for **7a**, except that the alkylation proceeded through the stannyl derivative. The nucleoside was protected as the 5′-DMT derivative via reaction with DMT-Cl in pyridine and purified by chromatography on silica gel. This material was converted to the phosphoramidite using bis(*N*,*N*-diisopropyamino)cyanoethyl phosphite.[15] Following aqueous extraction with bicarbonate, a solution of **9b** was triturated into petroleum ether. The resulting precipitate was collected and evaporated twice from acetonitrile: ^{1}H NMR (CD_3CN) δ 0.99−1.21 (m, 12H, CH_3), 1.82 (m, 2H, CH_2), 2.51 (t, 2H, CH_2-NH), 2.64 (t, 2H, CH_2CN), 3.2−4.19 (m, 14H, OCH_2, C_5, CH_2, CHN, $AROCH_3$, C_4, H, C_3, H), 4.35−4.61 (m, 1H, C_2, H), 5.23

(d, 1H, CH═), 5.82 (d, 1H, C_1, H), 6.82−7.52 (m, 14H, ARH), 7.61 (brs, 1H, NHCO), 7.75 (d, 1H, CH═), 8.85 (brs, 1H, ArCH); ^{31}P NMR (CD_3CN) δ 154.87, 155.66.

***N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-(3-*N*-phthalimidopropyl)cytidine-3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) (9c).** The 2′-(3-*N*-phthalimidopropyl)-cytidine nucleoside was prepared following the protocol of Guinosso.[15,23] The nucleoside was protected as the 5′-DMT derivative via reaction with DMT-Cl in pyridine and purified by chromatography on silica gel. This material was converted to the *N*-benzoyl derivative and the phosphoramidite using bis-(*N,N*-diisopropylamino)cyanoethyl phosphite. Following aqueous extraction with bicarbonate, a solution of **9c** was triturated into petroleum ether. The resulting precipitate was collected and evaporated twice from acetonitrile: ^{1}H NMR ($CDCl_3$) δ 1.11−1.32 (m, 12H, CH_3), 1.99 (m, 2H, CH_2), 2.76 (t, 2H, CH_2-CN), 3.42−4.21 (m, 16H, OCH_2, OCH_2, CHN, C_5, CH_2, CH_2-NPHTH, $AROCH_3$, C_4, H, C_3, H), 4.52 (m, 1H, C_2, H), 6.01 (S, 1H, C_1, H), 6.90 (d, 1H, CH═), 7.0−7.92 (m, 19H, ARH, ArNH), 8.63 (d, 1H, CH═); ^{31}P ($CDCI_3$) δ 150.35.

***N*-Isobutyryl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(3-*N*-phthalimidopropyl)guanosine-3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) (9d).** The 2′-(3-*N*-phthalimidopropyl)guanosine nucleoside was prepared following the protocol of Guinosso.[15,23] The nucleoside was protected as the 5′-DMT derivative via reaction with DMT-Cl in pyridine, converted to the *N*-isobutyryl derivative, and purified by chromatography on silica gel. This material was converted to the phosphoramidite using bis(*N,N*-diisopropylamino)cyanoethyl phosphite. Following aqueous extraction with bicarbonate, a solution of **9d** was triturated into petroleum ether. The resulting precipitate was collected and evaporated twice from acetonitrile: ^{1}H NMR ($CDCl_3$) δ 0.90−1.3 (m, 18H, CH_3), 1.98 (m, 2H, CH_2), 2.16 (m, 1H, CHCO), 2.65 (t, 2H, CH_2CN), 3.29−3.97 (m, 16H, OCH_2, OCH_2, CH_2NPHTH, CHN, $C_{51}CH_2$, $AROCH_3$), 4.31−4.54 (m, 3H, C_2, H, C_3, H, C_4, H), 5.90 (d, 1H, C_1, H), 6.79−7.5 (m, 17H, ARH), 7.85 (S, 1H, C_8H); ^{31}P NMR ($CDCI_3$) δ 150.496, 150.885.

DNA Synthesis. DNA phosphoramidites were purchased from Millipore (Boston, MA). Oligonucleotides were synthesized on controlled pore glass solid support using conventional phosphite triester chemistry with an Applied Biosystems (Foster City, CA) 380B synthesizer. Phosphorothioate backbones were prepared using Beaucage reagent during the oxidation step.[25] Cleavage from support and deprotection were accomplished by treatment with concentrated ammonium hydroxide for 15 h at 55 °C unless otherwise stated. The purity of oligonucleotides was monitored using capillary electrophoresis (model 270A; ABI, Foster City, CA) or gel chromatography and confirmed using electrospray mass spectrometry.

Hybridization Stability. Absorbance versus temperature curves of duplexes were measured at a 4 μM strand concentration in a 10 mM phosphate buffer at pH 7.4 containing 100 mM Na^+ and 0.1 mM EDTA. The T_m was determined from fits of data to a two-state model with linear sloping baselines.[10] The salt dependence of the T_m for duplexes were determined using Na^+ concentrations ranging from 0.005 to 0.6 M.

ES-MS. All experiments were performed in negative ionization mode using a 5998 quadrupole MS instrument with a 59987A electrospray unit (Hewlett-Packard, Palo Alto, CA). Samples were desalted via ethanol precipitation from 10 M ammonium acetate at −20 °C.[26] Typically, a 0.1 A_{260} unit sample of oligo was dissolved in 100 μL of a solution containing 1/1 2-propanol/water. The sample was then infused at a rate of 7.0 μL/min. Spectra were obtained over 5 min and signal-averaged without filtering. At least three ions with differing values of m/z were identified, and the HP software deconvolution algorithm was employed to calculate an average molecular mass for the molecule.

Molecular Dynamics. The following protocol has been used in the present molecular dynamics study. A dinucleotide was located in a cubical box of length 20.0 Å. Solvating this solute with pre-equilibrated water molecules and removing bad contacts to obtain a density of approximately 1 g/cm^3 left a total of 232 waters in the central box. A salt concentration of approximately 0.2 M was mimicked by the addition of one Na^+ ion and one Cl^- ion by randomly displacing two water molecules. An all-atom CFF91 force field was employed in our calculations (Biosym Technologies, San Diego, CA). A three-point flexible water model was used, in which each atom possessed a partial point charge (O, −0.82 eu; H, +0.41 eu). Electrostatic interactions were computed via Coulomb's law, using a dielectric constant of 1.0. The cutoff used for the intermolecular interactions was 9.0 Å. Periodic boundary conditions were applied in all directions. The simulation was carried out with a time step size of 1 fs, for a total of 150 ps at constant volume and constant temperature. The simulation was initiated by 1000 steps of steepest descent minimization. Initial velocities were assigned from a Maxwell−Boltzmann distribution at 300 K.

Nuclear Magnetic Resonance. All two-dimensional NMR experiments were performed using a 400 MHz Unity (Varian Associates, Palo Alto, CA) spectrometer. ^{1}H NMR spectra were referenced relative to internal TMS, while ^{31}P NMR spectra were acquired at 161.4 MHz using H_3PO_4 as an external standard. Two-dimensional NOESY, ROESY, and TOCSY spectra were acquired using conventional pulse sequences.

Nuclease Digestion. Oligonucleotide derivatives of sequence **O** were purified by polyacrylamide gel electrophoresis followed by desalting with Poly-pak cartridges (Glen Research, Sterling, VA). Oligomers were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Stability assays were performed using 5×10^{-2} or 5×10^{-3} U/mL SVPD (USB, Cleveland, OH) and 1 μM solutions of oligonucleotide at 37 °C in a buffer of 50 mM Tris-HCl, pH 8.5, with 72 mM sodium chloride and 14 mM magnesium chloride. The enzyme was active for >24 h under these conditions. Aliquots of the reaction mixture were removed at the indicated times, quenched by addition to an equal volume of 80% formamide gel loading buffer, and heated for 2 min at 95 °C. Quantitation was performed by reading intensities from a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The listed values of $t_{1/2}$ were determined for loss of the 3′-terminal nucleotide.

Oligonucleotide Treatment of Cells. The human bladder carcinoma cell line T24 (American Type Tissue Collection, Bethesda) grown in T175 flasks to 75% confluency were trypsinized and resuspended in complete tissue culture media (McCoys 5A media supplemented with 10% fetal bovine serum) at a density of 1×10^7 cells/mL. A total of 3.6×10^6 cells were employed for each treatment by combining 360 μL of cell suspension with oligonucleotide at the indicated concentrations to reach a final volume of 400 μL. Cells were then transferred to an electroporation cuvette and electroporated using an Electrocell Manipulator 600 instrument (Biotechnologies and Experimental Research Inc.) employing 350 V, 100 μF, at 13 Ω. Electroporated cells were then transferred to conical tubes containing 5 mL of complete media, mixed by inversion, and plated onto 10 cm culture dishes. Analysis of mRNA levels by Northern blot was initiated 20 h following electroporation, while analysis of protein levels by Western blot was initiated 48 h following electroporation.

Northern Blot and Western Blot Analyses. For determination of mRNA levels by Northern blot, total RNA was prepared from cells by the guanidinium isothiocyanate procedure.[27] Total RNA was isolated by centrifugation from cell lysates. RNA was quantitated and normalized to levels of glycerol-3-phosphate dehydrogenase mRNA using a Molecular Dynamics Phosphoimager as described previously.[27]

For determination of protein levels by Western blot, cellular extracts were prepared using 250 μL of RIPA extraction buffer/10 cm dish. A 25 μg lot of protein was then separated by electrophoresis on a 10% SDS−polyacrylamide minigel (Bio-Rad). Once transferred, membranes were treated for 2 h with a monoclonal antibody that specifically recognizes C-*raf* kinase protein (Transduction Laboratories) at a dilution of 1:1000 followed by incubation with 5 μCi of ^{125}I-labeled goat anti-mouse antibody (ICN Radiochemicals) for 1 h. Labeled proteins were visualized and quantitated by PhosphorImage analysis.

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