Control over the Localization of Positive Charge in DNA: The Effect on Duplex DNA and RNA Stability

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The development of antisense therapies to inhibit the expression of specific genes requires the generation of oligonucleotides (ODNs) that are physiologically stable, nontoxic, and able to penetrate into cells, while maintaining stringent base-pairing fidelity for unique DNA sequences.[1] Modifications to the bases, deoxyribose ring, and phosphate backbone have been generated to address some of these issues.[2] Among the potential structural changes, alterations of the phosphate backbone have received the most attention and have had the greatest impact on antisense technology. In fact, backbones made up of phosphorothioates have been tested in vivo as antisense reagents because they are DNase resistant and the resulting RNA−DNA complex is a substrate for RNase H.[3] However, phosphorothioate-based ODNs are complex diastereomeric mixtures, cause toxicity by poorly understood mechanisms, and most importantly, base-pair with reduced stability.[4]

Oligomers with 5-(3-aminopropyn-1-yl)-2′-deoxyuridine substitutions (Figure 1, **X**) were synthesized[5] in order to enhance the physiological stability of the oligomer, enhance duplex formation, and maintain Watson−Crick recognition. The amino group, which is ionized at physiological pH,[6] is capable of forming a salt bridge with the nonbridging major groove oxygen on the phosphate of the 5′-nucleotide (Figure 2a).[7] Such a salt bridge structure should significantly increase the stability of the duplex by lowering the electrostatic repulsion between the anionic strands.

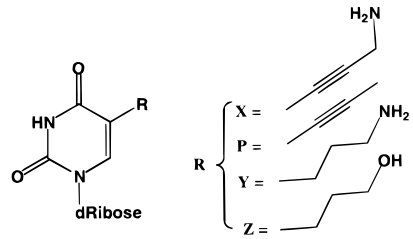

Figure 1. Structures of base modifications.

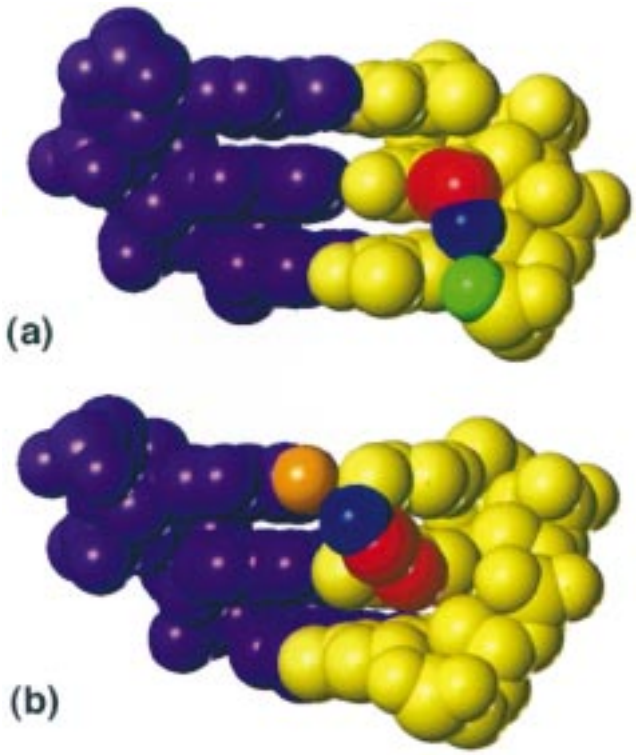

Figure 2. Minimized structures of ODN with **X** (6) and **Y** (14b) side chain modifications (see Figure 1 for structures) in a *d*(purine)$_3$-*d*(pyrimidine)$_3$ sequence: purple, purine strand (5′−3′ top to bottom); yellow, pyrimidine strand (3′−5′ top to bottom); red, alkyl or alkyne side chain; blue, terminal amino group; green (Figure 2a), nonbridging oxygen of the 5′-phosphate; orange (Figure 2b), O^6-position on G in purine strand.

In addition, the 3-aminopropyn-1-yl side chain has been proposed to stabilize DNA due to enhanced base stacking and hydrophobic interactions as has been observed with neutral 5-propyne-substituted pyrimidines.[8]

UV melting studies on oligomers with one or more **X** substitutions confirm that there is a very significant increase in T_M (Table 1). At 100 mM NaCl, a single substitution (ODN-2) results in a 4.5 °C increase, whereas an oligomer with four **X** residues (ODN-3) shows an *11.9 °C increase* (Table 1*)*. This increase exceeds that observed with the neutral propyne side chain[8] (Figure 1, **P**) by 1 °C per residue in the same sequence (ODN-4 and -5). The introduction of flexible 3-aminopropyl side chains, **Y** (Figure 1), (ODN-6) decreases the T_M at 100 mM NaCl by 2.4 °C, and the presence of neutral 3-hydroxypropyl appendages, **Z**, (ODN-7) results in a duplex structure that is 7 °C less stable (Table 1). These data with the flexible **Y** and **Z** (Figure 1) side chains are similar to those reported for 5-butane-substituted

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(5) 5-(3-Aminopropyn-1-yl)-2′-deoxyuridine was prepared by coupling 5-iodo-2′-deoxyuridine with 1-aminoprop-2-yne (protected as the *N*-phthalimide) in the presence of [(PPh)$_3$]$_4$Pd(0) (Gibson, K. J.; Benkovic, S. J. *Nucleic Acids Res.* **1987**, *15*, 6455−6467. Alternative methods have also been reported (Cruickshank, K. A.; Stockwell, D. L. *Tetrahedron Lett.* **1998**, *29*, 5221−5224 and Haralambis, J.; Chai, M.; Tregear, G. W. *Nucleic Acids Res.* **1987**, *15*, 4857−4876). The deoxynucleoside is converted into the 5′-*O*-dimethoxytrityl-3′-*O*-phosphoramidite by standard procedures (Beaucage, S. L. *Methods in Molecular Biology, Protocols for oligonucleotides and analogues. Synthesis and properties*; Agrawal, S., Ed.; Humana: Totowa, NJ, 1993; Vol. 20, pp 33−61). The structure was confirmed by ^{1}H NMR and mass spectrometry. All phosphoramidites were incorporated into oligomers using standard chemistry on an ABI synthesizer, purified by reverse phase HPLC, and shown to be homogeneous by polyacrylamide gel electrophoresis. ODNs with 3-aminopropyn-1-yl, 3-aminopropyl and 3-hydroxypropyl side chains were characterized by sequential hydrolysis and HPLC analysis.
(6) The pK_a of 1-aminoprop-2-yne is 8.15 (Perrin, D. D. *Dissociation constants of organic bases in aqueous solution*. Butterworths: London, 1965).
(7) The duplex was constructed in Sybyl (Tripos Inc, St. Louis, MO) using Arnott coordinates (Arnott, S.; Hukins, D. W. L. *Biochem. Biophys. Res. Commun*. **1972**, *47*, 1504−1509). Because of the rigid linkage, the potential interactions of the amino group can be determined by rotation around the C2−C3 bond of the 3-aminopropyn-1-yl side chain. The minimum distance between the amine nitrogen and the nonbridging phosphate oxygen on the 5′-nucleotide is 2.71 Å (Figure 2), which compares to a minimum of >3.5 Å for the immediate 5′-oxygen.
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Table 1. Thermal Stability of ODNs

ODN	duplex[b]	T_M (°C) at [NaCl] (mM)[a] 50	100	200	500
1	5′-TGTATAGGGAGAGAAAG-3′ 3′-TCCCTCTCTTTC-5′	40.7	44.0	50.0	54.3
2	5′-TGTATAGGGAGAGAAAG-3′ 3′-TCCCTC**X**CXTTC-5′	44.5	48.5	52.5	56.0
3	5′-TGTATAGGGAGAGAAAG-3′ 3′-TCCC**X**C**X**C**X**T**X**C-5′	52.5	55.9	57.5	60.3
4	5′-TGTATAGGGAGAGAAAG-3′ 3′-TCCCTC**P**CTTTC-5′	n.d.[c]	47.2	n.d.	n.d.
5	5′-TGTATAGGGAGAGAAAG-3′ 3′-TCCC**P**C**P**C**P**T**P**C-5′	n.d.	52.1	n.d.	n.d.
6	5′-TGTATAGGGAGAGAAAG-3′ 3′-TCCC**Y**C**Y**C**Y**T**Y**C-5′	39.0	41.6	44.2	46.2
7	5′-TGTATAGGGAGAGAAAG-3′ 3′-TCCC**Z**C**Z**C**Z**T**Z**C-5′	34.7	37.0	40.1	45.3
8	5′-TGTATAGGGAGTGAAAG-3′ 3′-TCCCTC̄**X**CTTTC-5′	31.8	n.d.	n.d.	n.d.
9	5′-TGTATAGGGAGGGAAAG-3′ 3′-TCCCTC̄**X**CTTTC-5′	34.8	n.d.	n.d.	n.d.
10	5′-TGTATAGGGAGCGAAAG-3′ 3′-TCCCTC̄**X**CTTTC-5′	30.9	n.d.	n.d.	n.d.
11	5′-AGCGG*AAAA*GCACC-3′ 3′-TCGCCTTTTCGTCC-5′	n.d.	58.8	n.d.	n.d.
12	5′-AGCGG*AAAA*GCACC-3′ 3′-TCGCC**XXXX**CGTCC-5′	n.d.	69.0	n.d.	n.d.

[a] Temperature ramped from 15 to 80 °C at a rate of 1°/min and UV-monitored at 260 and 280 nm. Thermal T_M's were calculated using the first derivative method. Conditions: 2.5 μM duplex, 10 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, and NaCl as indicated.[b] See Figure 1 for structures of **X**, **Y**, and **Z**. [c] Not determined.

nucleotides and indicate that any stabilizing electrostatic effect of the tethered cationic amine in **Y** is countered by the destabilizing steric effect of the aliphatic side chain.[9] In previous studies, 5-(*ω*-*N*-aminoalkyl)carbamoyl-2′-deoxyuridine substitutions have caused position-dependent increases in T_M's, although the increase is ≤1 °C per modification at 100 mM salt.[10] Clearly the location of the cationic charge determines the magnitude of the stabilization. The 4 °C increase per **X** residue compares to ∼3° increase in T_M when an oligomer with a neutral R_p-methylphosphonate residue is paired with its complementary natural oligodeoxynucleotide.[11] The S_p-isomer binds inefficiently to its complement.[12]

If the stabilization of duplex DNA by **X** substitution(s) has an electrostatic component, then the T_M's of the oligomers should not be as sensitive as unmodified ODNs to ionic strength. The relationship between salt concentration vs T_M for the different ODNs is shown in Table 1. As anticipated, the ΔT_M over the range of NaCl concentration is smaller for ODN-3 (7.8 °C) vs unmodified duplex ODN-1 (13.6 °C). Previous work showed that an ODN fully substituted with **Y** side chains is completely insensitive to salt concentration.[9a]

To confirm that the fidelity of base pairing was not compromised by the **X** side chain, the effect on duplex stability of the three potential mismatches opposite the modified residue was measured. The studies (Table 1) show that the T_M drops by 10−14 °C upon the introduction of a mismatch opposite **X**, which is in the range of that observed for mismatches involving thymine residues.[13]

We have previously used the DNA methylation pattern induced by methanediazonium ion (generated from *N*-methyl-*N*-nitrosourea) to probe the location of cationic side chains.[14] Using this approach, which is based on the repulsion of the positively charged methylating agent by the tethered ammonium ion, it was demonstrated that flexible **Y** substitutions regioselectively protect DNA from methylation toward the 3′-direction, a result that is consistent with molecular modeling studies (Figure 2b).[14b] A similar methylation analysis was done with the **X**-substituted ODNs. No significant quantitative or qualitative effect on DNA methylation at the N7-G position was observed with ODN-2; however, all Gs in ODN-3 with four **X** side chains are methylated less efficiently than those in unmodified ODN-1 or ODN-2, which has a single modified side chain (data not shown). The general decrease in methylation is similar to that which is observed when the salt concentration is increased[15] and is probably due to a reduction in the electrostatic attraction between the anionic ODNs and the positively charged methanediazonium ion. Therefore, the methylation protection data is consistent with the locations of the rigid **X** (Figure 2a) and flexible **Y** (Figure 2b) side chains.

To demonstrate the potential of the **X**-modified nucleotides to stabilize RNA−DNA complexes, a chimeric 14mer was synthesized, and the stability of duplexes with natural DNA (ODN-11) and DNA containing four of the **X** substitutions opposite the RNA bases (ODN-12) was measured. The T_M data show that the **X** modification increases the melting of ODN-12 by 10.2 °C in comparison to unmodified ODN-11(Table 1).

In conclusion, we have demonstrated that the introduction of rigid 3-aminopropyn-1-yl side chains at the 5-position of deoxyuridine results in ODNs with a marked increase in duplex DNA and RNA−DNA stability but with no decrease in base-pairing fidelity. These modified oligomers should be considered as potential antisense molecules and useful reagents to study the effect of charge neutralization on DNA structure.[16] The possibility that ODNs with **X** side chains will be less susceptible to nuclease degradation and more efficient at penetrating into cells as a result of the internal neutralization of backbone charge via salt bridging is under investigation.

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Supporting Information Available: (A) ^{1}H NMR spectra (300 MHz) of **X**-phosphoramidite in $CDCl_3$; (B) base composition analysis of ODN-2 and -3 from acid hydrolysis on reverse phase HPLC; (C) CD spectra of ODN-1, -2, -3, -6, -7; (D) T_M melting curve data for ODNs in Table 1; (E) denaturing polyacrylamide sequencing gel of ODN-1, -2, -3 and -6 reaction with *N*-methyl-*N*-nitrosourea, and the densitometry analysis (36 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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