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Remarkable Enhancement of Binding Affinity of Heterocycle-Modified DNA to DNA and RNA. Synthesis, Characterization and Biophysical Evaluation of N^2-Imidazolylpropylguanine and N^2-Imidazolylpropyl-2-aminoadenine Modified Oligonucleotides.

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Abstract: Oligonucleotides containing novel N^2-Imidazolylpropylguanine and N^2-Imidazolylpropyl-2-aminoadenine moieties were synthesized and studied for their hybridization and biophysical properties. Interestingly, these heterocyle modified oligonucleotides showed a remarkable enhancement of heteroduplex binding affinity when hybridized to complementary DNA.

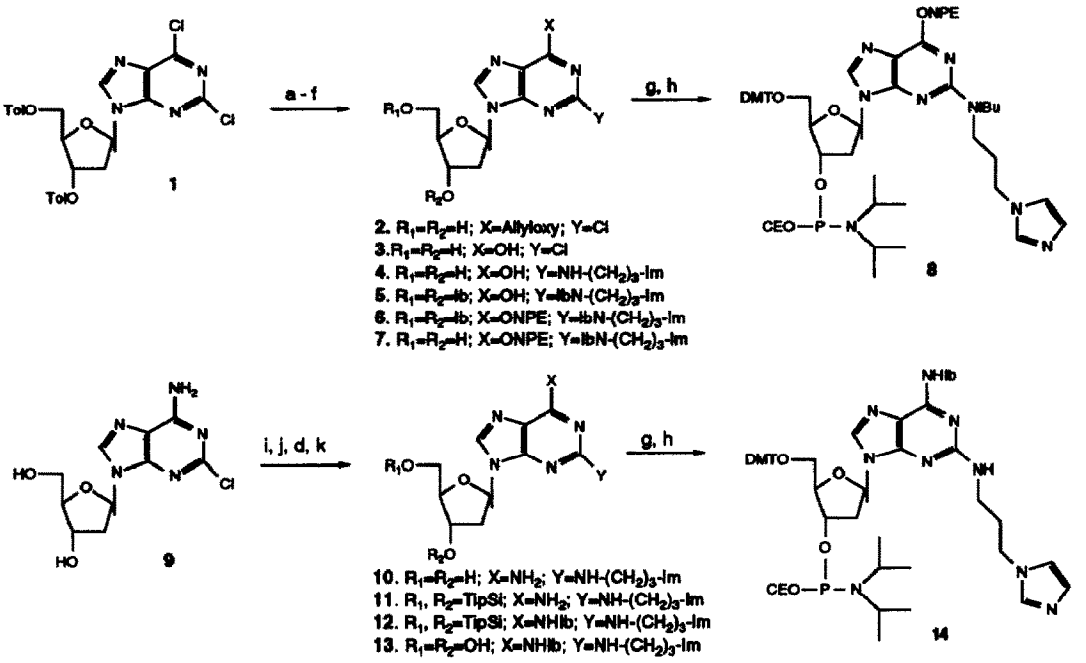
Modified oligonucleotides are of interest as antisense therapeutic agents.[1] Although this relatively new drug discovery concept is presently thought to be of great therapeutic potential, a number of problems have yet to be solved.[1] For example, the precise event(s) that terminates or in some way interferes with an essential RNA function after sequence-specific binding of an oligonucleotide to a target RNA is unclear. Antisense effects are thought to result as a consequence of simply binding of the oligonucleotide to targeted RNA or binding and subsequent cleavage of targeted RNA by endogenous RNase H.[1,2] Both mechanisms require nuclease resistant-oligonucelotides which effectively bind to target RNA. Unfortunately, first generation modifications such as phosphorothioates, methylphosphonates, and phosphoramidates, although providing nuclease resistance, compromised heteroduplex binding affinity.[1] The RNase H mode of action requires that the heteroduplex formed between the RNA target and the DNA be bound and cleaved by the enzyme. However, all reported modifications of the sugar-phosphate backbone, with the exception of phosphorothioates and phosphorodithioates, obliterate the RNase H terminating event.[1,3]

We are pursuing several approaches to develop antisense oligonucleotides with precise terminating events. One is based on the concept that antisense oligonucleotides containing heterocycle-modifications, rather than sugar-phosphate modifications, can be resistant to nucleolytic degradation, yet on hybridization to target RNA provides a heteroduplex that supports RNase H-mediated cleavage.[7] Another approach is directed to the development of sequence-specific chemical cleavers of RNA. This concept requires the attachment of pendent groups with acid/base properties to oligonucleotides; when hybridized to RNA, the pendent groups of the oligonucleotides would be accessible, via the minor groove, to the 2'-hydroxyl and phosphodiester linkages of the targeted RNA.

Obviously, pendent groups designed to support either enzymatic or chemical cleavage of RNA must be compatible with the requisite hybridization step. We have focused on pendent groups on the N^2-position of guanine and 2-aminoadenine as these groups should protrude into the minor groove of a DNA-RNA

heteroduplex and were not expected to adversely affect binding affinities. Thus, our initial research in this area has been the examination of bulk tolerance of a N^2-[3-(1H-imidazol-1-yl)propyl] moiety as determined by hybridization studies. This moiety serves as a prototype substituent with general acid/base properties capable of extending into the minor groove. We describe the attachment of the 3-(1H-imidazol-1-yl)propyl moiety to the 2-amino group of deoxyguanosine and 2-aminodeoxyadenosine, and, subsequent incorporation of these novel nucleosides into oligonucleotides for hybridization studies and biophysical characterization.

Scheme I[a]



[a](a) NaH/Allyl alcohol. (b) Pd/C/H_2/EtOH. (c) 1-(3-Aminopropyl)imidazole/2-Methoxyethanol/90°C. (d) IbCl/TEA/Py. (e) 2-(p-nitrophenyl)ethanol/Ph_3P/DEAD/Dioxane. (f) NH_4OH/CH_3OH. (g) DMTCl/TEA/Py. (h) 2-Cyanoethyl N,N-diisopropyl-chlorophosphoramidite/ N,N-diisopropylethylamine/ CH_2Cl_2. (i) 1-(3-Aminopropyl)imidazole / 2-Methoxyethanol/125°C. (j) TipSiCl/TEA/PY. (k) Bu_4NF/THF.

Our synthetic route to N^2-substituted purine phosphoramidites (**8**) and (**14**) is outlined in **Scheme I**. 2-Chloro-9-(2-deoxy-ß-D-*erythro*-pentofuranosyl)inosine (**3**), a versatile, key intermediate, was obtained by heating 2,6-dichloro-9-(2-deoxy-b-D-*erythro*-pentofuranosyl)purine (**1**)[8] with NaH in allyl alcohol, followed by hydrogenation of the intermediate **2** with Pd/C at atmospheric pressure.[9] Displacement of the 2-chloro atom of **3** with 1-(3-aminopropyl)imidazole gave **4**. The isobutyryl derivative of **4** (**5**) was subjected to the Mitsunobu reaction condition[10] in the presence of 2-(*p*-nitrophenyl)ethanol to provide fully protected nucleoside **6**. Selective removal of the 2'- and 3'-isobutyryl groups in **6**, followed by dimethoxytritylation[11] and phosphitylation[12] afforded the deoxyguanosine amidite synthon **8** in 73% yield. 2-Chloro-deoxyadenosine (**9**)[13] was treated with 1-(3-aminopropyl)imidazole to provide N^2-substituted 2-aminodeoxyadenosine (**10**) which was protected by sequential treatment with TipSiCl[14] and IbCl to afford **12**.

Removal of the TipSi protecting group of **12** with Bu_4NF[15] and subsequent dimethoxytritylation and phosphitylation provided the deoxyadenosine amidite synthon **14**.

Incorporation of amidites **8** and **14** into oligonucleotide sequences was accomplished via automated DNA synthesis protocols.[16] Enzymatic degradation and subsequent HPLC analysis of the modified oligonucleotides indicated the expected ratios of the nucleoside components.[17] A 21-mer oligonucleotide [5'-d(GCCGAGGTCCATGTCGTACGC)] was modified with one, three or seven N^2-[3-(1H-imidazol-1-yl)propyl)dGs or with one or three N^2-[3-(1H-imidazol-1-yl)propyl]-2-NH_2 dAs and hybridized to complementary DNA or RNA.[18] Compared to unmodified DNA, the average ΔTm/mod was +2.0° and +0.3 for dG modified oligonucleotides hybridized with DNA and RNA, respectively; the average ΔTm/mod was +2.7° and +0.6 for dA modified oligonucleotides hybridized with DNA and RNA, respectively. The average enhancement of binding affinity of several different N^2-imidazolylpropyl dG and N^2-imidazolylpropyl-2-amino-dA modified oligonucleotides hybridized to DNA is 2.7°/mod (3 different sequences, 16 incorporations) and 2.5°/mod (3 different sequences, 12 incorporations), respectively. The relative specificity of hybridization of dG or N^2-imidazolylpropyl dG to cytidine versus A, G and U(T) mismatches on an RNA or DNA complement shows that N^2-modified dG is more specific to its complement cytidine than the corresponding unmodified dG; N^2-imidazolylpropyl-2-NH_2 dA is as specific as dA against DNA or RNA. Incorporation of three N^2-imidazolylpropyl dGs or 2-NH_2-dAs at the n-1, n-2 and n-3 positions at the 3' end of a 15-mer provided an increase in stability ($T_{1/2}$ = 9 and 16h, respectively) to nucleolytic degradation in fetal calf serum compared to the unmodified oligomer ($T_{1/2}$ = 1h).[19] The capped N^2-modified dG sequence was stabilized by 2.8 °C/mod against DNA and 0.9 °C/mod against RNA and the 2-NH_2-dA sequence was stabilized by 2.6°C/mod against DNA and 1.5 °C/mod against RNA.

Molecular modeling simulations of oligomers containing the N^2-imidazolylpropyl functionality suggest that the imidazole binds in the minor groove proximate to the phosphate backbone, stabilizing the DNA-DNA duplex[20]. In the case of a DNA-RNA duplex, the imidazolylpropyl group does not bind specifically since the minor groove is broad and the phosphates are turned away. However, other hydrogen bond donors and acceptors are accessible in the minor groove, which may account for the increased stabilization of the modified DNA-RNA duplex.

A 21-mer having imidazolylpropyl modified dG in 7 positions (1, 4, 6, 7, 13, 16 and 20) and another 21-mer having imidazolylpropyl modified 2-NH_2-dA in 5 positions (1, 5, 8, 11, and 18) support HeLa cell extract RNase H dependent cleavage.[21] Furthermore, a 17-mer phosphorothioate containing a modified dG at the primary cleavage site of HeLa cell extract RNase H did not prevent cleavage by the enzyme.[22] These data suggest that heteroduplexes formed between N^2-imidazolylpropyl dG or 2-NH_2-dA modified oligonucleotides and RNA are recognized by RNase H.

We conclude that the N^2 position of dG and 2-NH_2-dA is a novel site to attach potential RNA cleaving moieties as well as other moieties that may enhance pharmacokinetic properties of antisense oligonucleotides without affecting RNase H degradation of target RNA. In addition, a remarkable enhancement of heteroduplex binding affinity is observed when hybridizing N^2-imidazolylpropyl dG and N^2-imidazolylpropyl-2-NH_2 dA modified oligonucleotides to complementary DNA.

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