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Oligonucleotides Bearing Cationic Groups: N^2-(3-Aminopropyl)deoxyguanosine. Synthesis, Enhanced Binding Properties and Conjugation Chemistry

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Abstract: A phosphoramidite with an aminopropyl group placed at the N^2- position of 2'-deoxyguonosine has been synthesized and incorporated into oligonucleotides. This modification shows enhanced binding properties against both DNA and RNA targets and is useful for conjugating other functionalities.

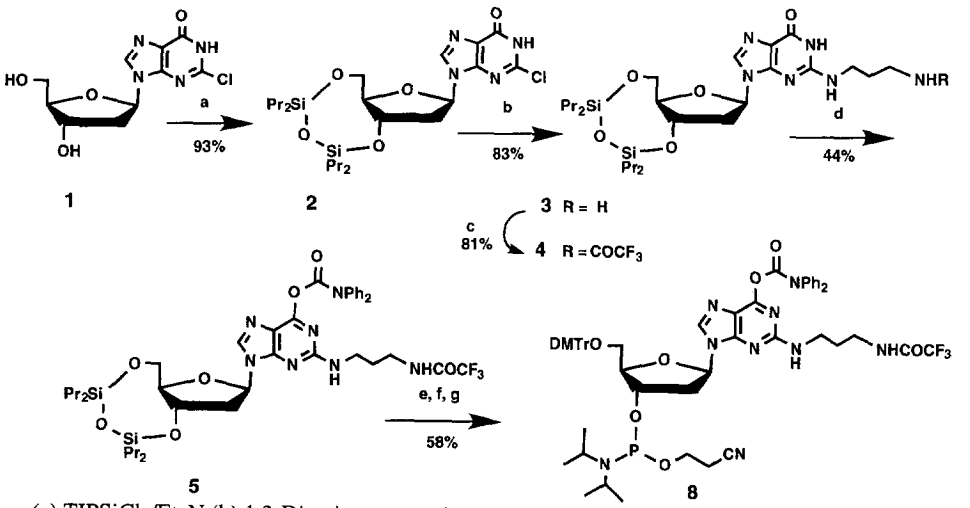
Antisense oligonucleotides should possess *in vivo* nuclease stability, adequate binding to the target mRNA, and the ability to reach appropriate target cells and tissues. In addition, they should be competent to activate RNase H to cleave the target mRNA after hybridization.[2] While several new modifications of the carbohydrate moiety and non-phosphate linkage fulfill the first three criteria, they fail to activate RNase H unless they are used in chimeric oligonucleotides containing a stretch of 2'-deoxyribo sugars with a thiophosphate backbone ("Gap Technology").[3] Base modifications, in contrast, retain RNase H activation.[4] For this reason, heterocyclic modifications are attractive as enhancers of antisense properties.

Among the various sites available within purines (N^2-, N^6- and C^8-) and pyrimidines (N^4- and C^5-), not all sites tolerate modifications. Modifications at the C^5- position of pyrimidines, which lie in the major groove, show good target binding properties[5] and maintain RNase H competency.[5] Among the minor groove nucleobase modifications, N^2- purine position can maintain RNase H activity.[6]

Recently our laboratory introduced,[6] chemistries to synthesize N^2-imidazoylpropylguanine-containing oligonucleotides and observed that, when incorporated into DNA, this oligonucleotide showed remarkable enhancement in binding to both DNA and RNA complements.[6] This enhancement may be due to hydrogen bonding interactions involving imidazole, or by a groove binding ability of the imidazole. We reasoned that replacing the imidazole with $-NH_2$, which will have a pKa of 9 or 10 and remain protonated at neutral pH, would contribute a full positive charge. A cationic group is expected to enhance binding to the target nucleic acid by electrostatic interaction with phosphate groups or the interaction with a negative potential in the helical groove. If the imidazole stabilizes the helix merely by groove-binding, however, its replacement with $-NH_2$ would reverse the observed binding enhancement. Additionally, introducing the amino group would have other chemical and pharmacokinetic advantages for antisense agents: (1) an alkyl amino group could be used for further conjugations of electrophilic functionalities at this site (active esters, aldehydes, isothiocyanates, etc.); (2) introducing a cationic group at each guanosine modification may improve the cellular uptake of oligonucleotides; (3) the presence of a protonated amino group in the minor groove may enhance the nuclease resistance for the oligonucleotides. [7]

Our rationale for introducing an aminoalkyl group was supported by the recent observation by Behr[8] that introducing two spermine residues at C^2- position of inosine provides a Tm increase of 8 to 12°/modification (3-4°/amino group) against DNA target. This indicates that a cationic group placement at N^2- position will provide strong binding properties for the oligonucleotide. Similarly, placement of a N^2-(3-imidazolepropionic acid) group enhanced RNA target binding possibly via a hydrogen bond originating from 2'-OH position.[9]

Scheme 1



(a) $TIPSiCl_2/Et_3N$ (b) 1,3-Diaminopropane/2-Methoxyethanol/100° (c) $(CF_3CO)_2O$/Pyridine (d) Ph_2NCOCl/Et_3N (e) nBu_4NF/Pyridine (f) Dimethoxytritylchloride/Pyridine (g) 2-Cyanoethyl-*N,N*-diisopropyl-chlorophosphoramidite/$(iPr)_2EtN/CH_2Cl_2$.

We chose to introduce the 3-aminopropyl group at N^2- of guanine, and used 2-chlorodeoxyguanosine (**1**) as our starting material which was prepared by the reported procedure[6]. Stirring of **1** (**Scheme 1**) with $TipSiCl_2$ in the presence of Et_3N at room temperature followed by silica gel purification provided the 3',5'-protected 2-chloronucleoside **2**. Nucleophilic displacement of the chloro group of **2** with 1,3-diaminopropane in 2-methoxyethanol readily afforded **3** as a colorless crystalline compound (mp 206-208°C). Selective protection of the primary amine functionality of **3** was achieved by reaction with trifluoroacetic anhydride at 0°C under argon atmosphere. Treatment of **4** with diphenylcarbamoyl chloride gave the fully blocked nucleoside **5**. Selective removal of the silyl protecting group of **5** with Bu_4NF provided the intermediate **6**. Dimethoxytritlyation of **6** (5' -OH) gave **7** (5' DMTrO-) which on subsequent phosphitylation afforded **8** in overall good yield. The phosphoramidite **8** was utilized on an ABI 380B DNA synthesizer as a 0.12M solution in anhydrous CH_3CN. Oligonucleotide synthesis was carried out employing the standard synthesis cycles with an extended coupling time of 10 minutes during coupling of the modified amidite. Coupling efficiency of greater than 95% was observed. The following oligonucleotides having phosphodiester inter-nucleotide linkages were synthesized and purified: Oligomer **I**: 5' GAG*CT 3'; Oligomer **II**: 5' TG*G GAG CCA TAG CGA GGC 3' Oligomer **III:** 5' TG*G GAG CCA TAG* CGA GGC 3' wherein G* represents a nucleotide functionalized with a 3-aminopropyl

group (dG_{AP}). Oligomers **II** and **III** are antisense compounds targeted against the human ICAM-1 (Intercellular Adhesion Molecule-1).

Analysis of oligonucleotide **I** by both ^{1}H and ^{31}P NMR confirmed the expected structure. The observed $J_{1'2'}$ and $J_{1'2''}$ coupling constants (7 to 9 Hz) for all anomeric protons indicate that the modified heterocycle containing sugar maintains $C_{2'}$-endo sugar puckering (DNA type). This might explain why modification at this site does not interfere with RNase H activity.

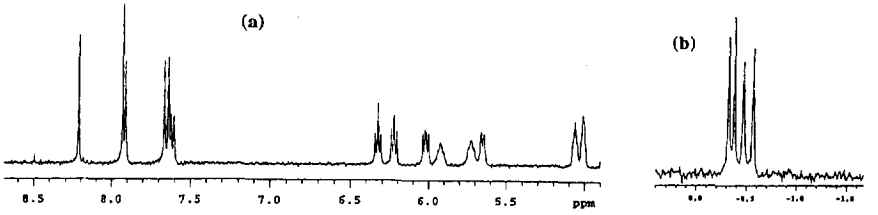
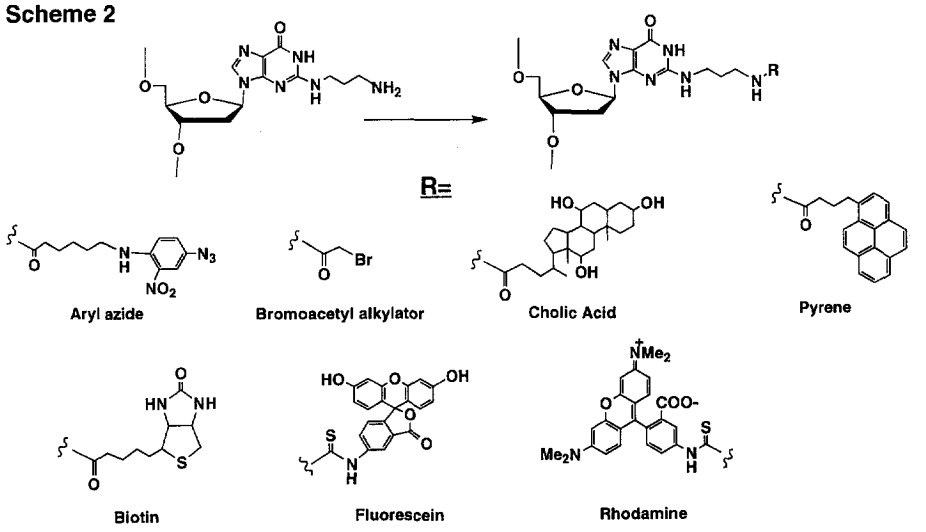
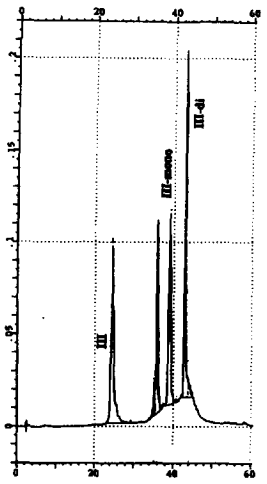


Fig. 1. a) Proton and b) Phosphorous NMR of GAG*CT at 37°C

Oligonucleotide **II** was hybridized to complementary DNA and RNA. Compared to unmodified DNA, the increase in Tm was +2.1° and +3.3° for dG_{AP} modified oligomer hybridized with DNA and RNA respectively. The enhancement in binding towards both DNA and RNA targets indicate that the origin of stability stems from the cationic nature of the functional group ($-NH-(CH_2)_3-NH_3^+$) and its proper placement rather than any change in sugar puckering or groove binding. This trend is in agreement with the observations of Schmid and Behr[8] who reported Tm changes of a C^2-spermine conjugate against DNA target. Our preliminary modeling indicates that the N^2-(3-aminopropyl) side chain ends up in the minor grooves of both RNA-DNA and DNA-DNA duplexes juxtaposed to the phosphate backbone or the base-pair regions, generating effective charge neutralization.[10]

Scheme 2





To demonstrate the reactivity and functionalization of amino group of the dG_{AP}, the oligos **II** and **III** were derivatized **(Scheme 2)** with an aryl azide (a photoactivatable crosslinking agent), bromoacetyl alkylator, cholic acid (a cellular permeability modifier), pyrene (an intercalator), and biotin, fluorescein and rhodamine (compounds used for *in vitro* and *in vivo* localization of antisense oligonucleotides) employing their active esters or isothiocyanates[11]. All of these compounds were synthesized in good to excellent yields and characterized. As shown by the HPLC analysis for the cholic acid-*N*-hydroxysuccinimide ester reaction with oligonucleotide **III**, during the reaction, two mono-conjugated products and one di-conjugated product are formed. This illustrates that multiple functionalities can be introduced into the minor groove using this chemistry.

Purine N^2- is the unique site available for minor groove modification which can offer stabilization (against both DNA and RNA targets) and still maintain RNase H activity. Furthermore, an amino tether at the guanosine moiety could be used to generate numerous chemical conjugates. It would be possible to place this heterocycle modification[12] into oligonucleotide building blocks having other favorable backbone and carbohydrate modifications to extend their therapeutic applications. We have introduced dG_{AP} into phosphorothioates and RNA mimics as potential antisense compounds and the evaluation of their pharmacokinetic and pharmacological properties is in progress.

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