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NOVEL FUNCTIONALIZATION OF THE SUGAR MOIETY OF NUCLEIC ACIDS FOR MULTIPLE LABELING IN THE MINOR GROOVE

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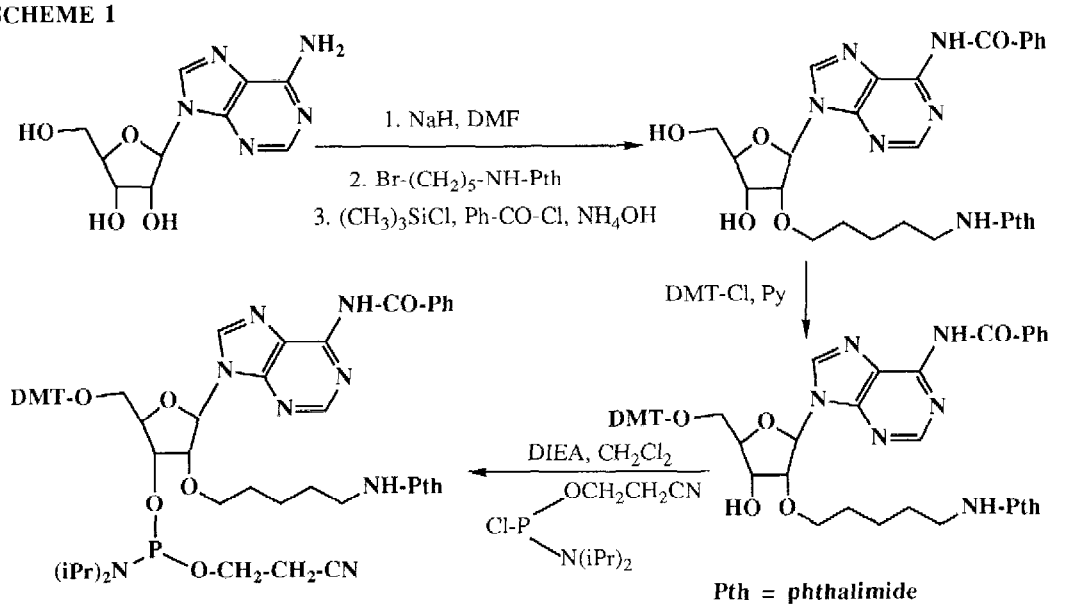
Abstract: The sugar ring of adenosine was alkylated at the 2'-O- position using N-(5-bromopentyl)phthalimide and the modified nucleoside was chemically incorporated into oligonucleotide diesters, thioates and RNA analogs. The free amino group, available after oligonucleotide deprotection, is useful for site-specific introduction of reporter groups and therapeutic agents at the 2'-position. This novel methodology permits multiple labeling of nucleic acids in the minor groove.

Antisense oligonucleotide-based therapeutics[1] and nucleic acid probe-based diagnostics[2] are two important areas of current interest in the field of nucleic acid chemistry. Both areas often require oligonucleotides carrying reactive groups such as amines either to derivatize with suitable reporter groups or to attach nucleic acid cleaving agents ("chemical nucleases"),[3] intercalators and chelating agents. The amino group with an appropriate linker has been introduced at the 5' or 3' end of the oligonucleotides,[2] at the 5-position of uracil,[4] N-4 position of cytosine,[5] N-6 position of adenosine[6] and also in the phosphodiester backbone.[7] Each of these approaches has one or more limitations. The terminal linkers place the functional groups at the ends and thus limit recognition of a given site within the double helix; the linkers attached to the bases may interfere with base pairing and/or stacking interactions;[8] the linkers attached to the backbone present chirality problems. In this communication we present a linker attached to the 2'-O-position of the sugar which is suitable for both DNA and RNA modifications and also yields itself to several conjugation reactions.

SCHEME 1



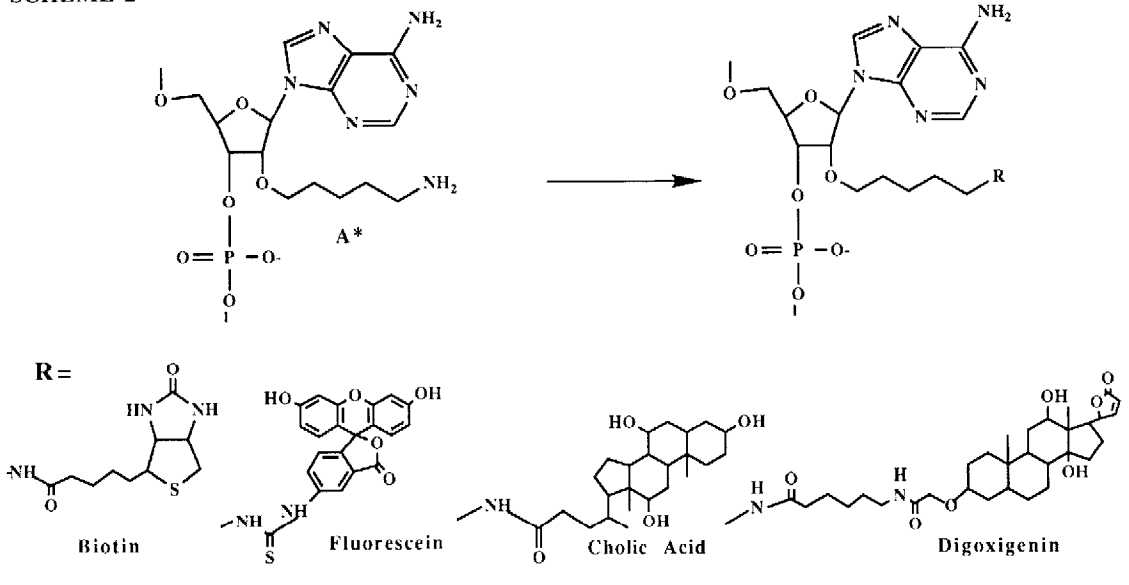
The chemistry[9] required in building up a phosphoramidite monomer which could be easily used in automated DNA synthesizers to yield the required 2'- linker is summarized in Scheme 1. Although our initial efforts are directed to the 2'-O-position of adenosine, the approach is quite general and is being extended to other nucleosides in our laboratory. Alkylation of the anion resulting from NaH/DMF treatment of adenosine at 0-5° C with N-(5-bromopentyl)phthalimide, silica column purification of the 2'-O-product (<5% 3'-O-alkylation was observed), protection of the base employing transient protection of 5' and 3' hydroxyls, tritylation and phosphitylation yielded the desired phosphoramidite with an overall yield of 21% from adenosine. It is noteworthy to point out that adenosine has been reported[10] to alkylate preferentially the 2'-O-position of the ribose ring with activated halides; the significant feature of the present alkylation is the utilization of an unactivated alkyl halide. The phosphoramidite synthesized is readily soluble in anhydrous CH_3CN and used in the synthesizer as a 0.12 M solution. Oligonucleotide syntheses were carried out in either an ABI 380B or 394 synthesizer employing the standard synthesis cycle with an extended coupling time (10 to 15 min.) during the modified phosphoramidite coupling. A coupling efficiency of >95% was observed for this step. Standard deprotection conditions (excess NH_4OH, 55° C, 24 h) yielded 2'-alkoxyamino tethered oligonucleotides which were purified by reverse phase HPLC, detritylated and further purified by size exclusion.

Oligodeoxynucleotides **I** and **II**, synthesized incorporating the 2'-O-modified adenosine (shown as **A***), are shown below. The sequence belongs to the E2 region of the bovine papilloma virus-1 (BPV-1) and is proven to have an antisense effect in gene expression.[11] The nucleoside compositions of these oligonucleotides were established by digestion with snake venom phosphodiesterase and calf-thymus alkaline phosphatase enzymes to provide their individual nucleosides which were analyzed by HPLC .

I: 5' **CTGTCTCCA*TCCTCTTCACT** 3' BPV sequence; Single Site Labeling

II: 5' **CTGTCT CCA*TCCTCTTCA*CT** 3' BPV: Double Site Labeling

SCHEME 2



Oligonucleotides **I** and **II** were reacted with a number of compounds containing functional groups possessing inherent reactivity to an amino group (Table 1). Typical examples are summarized in Scheme 2. Biotin, fluorescein and

digoxigenin are well known reporter groups used in nucleic acid probes; cholic acid confers enhanced hydrophobicity for antisense oligonucleotides for cellular uptake.[12] The conjugation reactions were carried out in an aqueous buffer at pH values between 8 and 9 under the standard conditions.[13] The reactions were followed by reverse phase HPLC and the retention times are summarized in Table 1. The conjugation yields varied between 70-90%.

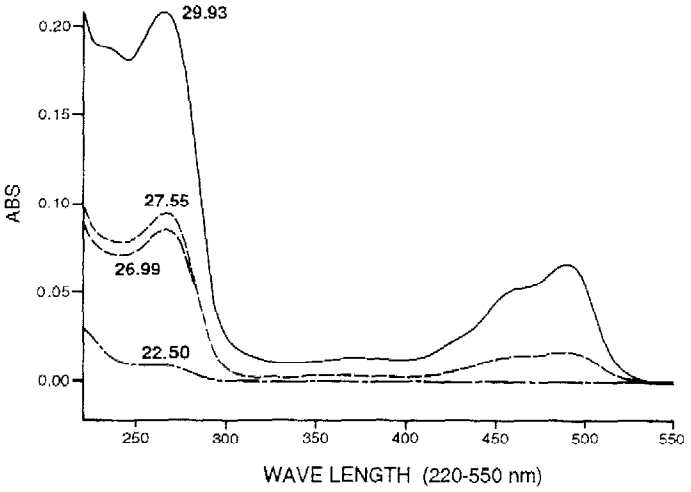
Table 1. HPLC Retention times of Oligos I and II with various Functionalities

Reagent	Retention Time (min.) I	II	
None (Parent Oligo)	21.78	22.50	
Biotin-NHS ester	23.58	24.16[a]	25.19[b]
Cholic Acid-NHS ester	30.10	30.38[a] 32.22[a]	37.00[b]
Flourescein-NCS	26.65	26.99[a] 27.55[a]	29.93[b]
Digoxigenin-NHS ester	28.06	28.14[a] 29.24[a]	33.32[b]

Conditions: Waters 600E with 991 detector; Hamilton PRP-1 column (15 x 2.5 cm); Solvent A: 50mM TEAA, pH 7.0; B: 45 mM TEAA with 80% Acetonitrile; 1.5 mL/min. flow rate; Gradient: 5% B for first 5 minutes, linear (1%) increase in B every minute afterwards. [a]Mono conjugated minor product; [b]Doubly conjugated major product.

The strength of the present methodology lies in the feasibility of multiple labeling. The multiply labeled product is conveniently synthesized and purified by HPLC. It is illustrative to look at the fluorescein reaction of the oligonucleotide II (Figure 1 shows the spectra of different HPLC fractions which are not normalized).

FIGURE 1: Spectra of Different Components of Oligonucleotide II Reaction with Fluorescein



The crude product mixture has trace amounts of **II** (22.5 min peak), two minor monolabeled components (peaks at 26.99 and 27.55), and the major product as the doubly labeled compound (29.93 min peak). As expected, *the ratio of fluorescein absorbance (at 490 nm) to oligonucleotide absorbance (at 260 nm) for the double labeled compound is twice the ratio for the single-labeled compound.* Signal amplification for diagnostic applications using multiple labeling is therefore possible; this should be a valuable method in antibody mediated biotin[14] and digoxigenin assay systems.

Finally, we have synthesized oligonucleotides containing a phosphorothioate backbone and RNA analogs carrying 2'-OMe groups, site-specifically incorporating our 2'-aminolinker. Representative examples are shown in sequences **III** and **IV**. These derivatives are already known to have either nuclease stability (thioates)[1] or enhanced hybridization properties (2'-OMe derivatives).[15] The 2'-aminolinker provides an additional handle to conjugate other functionalities such as lipophilic groups to improve membrane transport properties or nucleic acid cleaving agents.

III: 5' **TsTsGsCsTsTsCsCsA*sTsCsTsTsCsCsTsCsGsTsC** 3'

(Antisense compound directed to HPV-11 E2 region)

IV: 5' **CCCA*GGCUCAGA** 3' (HIV-1 TAR Antisense Strand with 2'-OMe groups)

In summary, we have demonstrated functionalization of the sugar ring for nucleic acid modifications and multiple labeling with reporter groups. The method shown is quite versatile as it can be extended to other nucleosides at the 2'-O-position, to DNA and RNA, and also to backbone modified oligonucleotides. Furthermore, the length of the tether could be altered in the present aminolinker by changing the alkyl chain length during the alkylation step. The 2'-aminolinker shown above sets the stage for performing minor groove modifications for biological applications . We are pursuing these studies at present.

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13. a) Biotin-NHS: 0.2 M $NaHCO_3$ buffer b) Fluo-NCS: DMF/ 1 M Na_2CO_3 -$NaHCO_3$ c) Cholic acid-NHS: 0.2 M $NaHCO_3$ buffer, 45° C d) DIG-NHS: DMF/0.1 M Borate buffer.; All reactions were left to stand overnight.
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