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Oligodeoxynucleotide Probes with Multiple Labels Linked to the 4'-Position of Thymidine Monomers: Excellent Duplex Stability and Detection Sensitivity.[1]

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Abstract: A modified thymidine phosphoramidite monomer **1** has been synthesized bearing a 4'-linker arm terminating in a protected primary amine. This monomer has been incorporated once, three and four times, respectively, into short oligodeoxynucleotide probes targeting a specific sequence in the genome of *Mycobacterium tuberculosis*. These probes, following standard biotinylation, retained their ability to form hybrid duplex DNA structures and the two multiply labeled probes provided a stronger detection signal than the mono biotinylated probe.

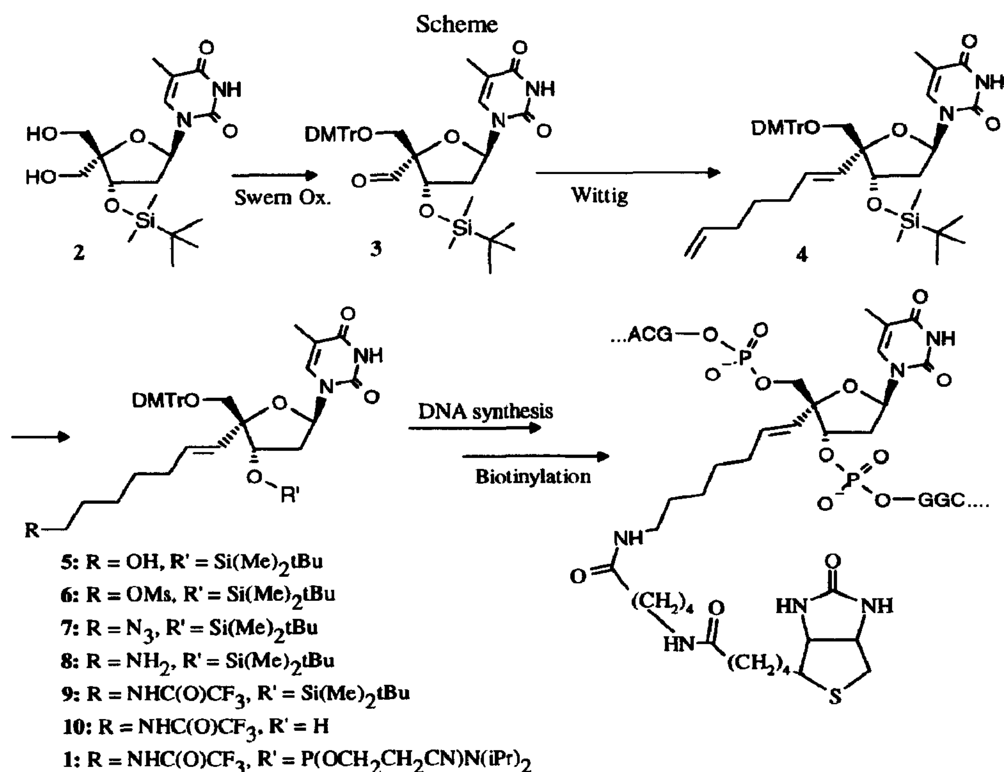
Modified oligonucleotides bearing reporter groups or stabilizing elements have become important tools for the detection of DNA sequences and for the stabilization of DNA duplexes, respectively.[3] Reporter groups such as biotin are usually attached through suitable linkers to facilitate detection and acridins, for example, are attached by similar means to achieve greater duplex stability. Typically, the linker consists of a short alkyl or heteroalkyl chain attached at either the 3'- or 5'-end of the oligonucleotide. Alternatively, the attachment point can be on the base portion such as at C-5 of a pyrimidine unit[3b] or at abasic sites within the oligonucleotide probe.[4] These methods of attachment are limited by the fact that they can only be utilized once within a short oligonucleotide sequence and/or that they have a significant negative effect on duplex formation. We describe here a novel method which circumvents these limitations and allows for the attachment of multiple reporter groups to a short oligonucleotide probe without a significant effect on duplex formation.

Inspection of a typical B-DNA model, such as the 'Dickerson dodecamer',[5] by computer modeling techniques revealed that the 4'-hydrogen of each monomeric unit points directly towards the minor groove. Attachment of an alkyl chain at these 4'-positions was possible without encountering any steric encumbrance and without the need to modify the DNA structure in any way. Additional evidence for the direct access of a 4'-substituent to the minor groove comes from the strong preference of the oxidative abstraction of the 4'-hydrogen by the DNA minor groove modifier Bleomycin.[6] The modeling studies indicated also, that attachment of an alkyl chain at the 4'-position would be preferred over attachment at the 1'-position. After completion of the work described in this report, papers reporting the attachment of reporter groups through linkers to the 1'-position of monomeric units were published.[7] Indeed, these modifications have a significant negative effect on duplex formation of the modified oligodeoxynucleotides.

In an effort to have a flexible approach to 4'-modified oligonucleotides, we planned on preparing monomeric units which could be employed in standard DNA synthesis protocols using conventional equipment and on attaching the reporter or stabilizing group after the synthesis of the oligonucleotide was complete. Thus our first target was the synthesis of the phosphoramidite **1** (Scheme), in which the 4'-linker terminates in a protected primary amine. Similar approaches have been used to attach modifiers to linkers attached at other positions in oligonucleotides, such as the 3'- and 5'-ends or at abasic units.[3] Our synthetic route starts with the known 4'-hydroxymethylthymidine derivative **2**.[8] Swern oxidation (oxalylchloride/DMSO/Et_3N) followed by treatment with 4,4'-dimethoxytritylchloride in pyridine gave the unstable 4'-formyl thymidine analog **3** (32% crude).[8] Wittig olefination with 5-hexenyltriphenylphosphonium bromide[9] (NaH, DMSO) gave diene **4** (65%). This diene was converted to the protected primary amine **9** through selective hydroboration (BH_3.Me_2S) followed by an oxidative workup with sodium perborate to provide the alcohol **5** (64%). Mesylation, displacement with sodium azide, reduction (1,3-propanedithiol) and protection of the primary amine with ethyl trifluoroacetate in the presence of triethylamine gave **9** (63% over 4 steps). Removal of the silyl protecting group (TBAF/THF, 95%) and formation of the phosphoramidite **1** (81%) under standard conditions completed the synthesis of the monomeric unit.[10]

As test cases, probes directed at a target sequence in the genome of *Mycobacterium tuberculosis*[11] were synthesized by the solid phase β-cyanoethyl *N,N*-diisopropylphosphoramidite method on an automated DNA

Scheme



synthesizer (Milligen/Biosearch 8700). At selected positions (indicated by T*), the 4'-modified thymidine phosphoramidite **7** was substituted for the unmodified thymidine reagent. Coupling efficiencies were monitored by the intensity of the trityl cation color in the trityl deprotection cycle. No difference in the coupling yield between modified or unmodified thymidine monomers was seen. The oligos were cleaved from the solid support by treatment with ammonium hydroxide, which concomitantly removed the N-trifluoroacetyl group at the end of the 4'-chain of the modified thymidines. Purification of the crude oligos was achieved by chromatography through Oligo-Pak columns (Milligen/Biosearch) followed by reverse phase HPLC. Three probes, containing one (Probe A), three (Probe B) or four (Probe C) modified thymidine units, respectively, were synthesized in addition to an unmodified probe (control) and the shared target sequence. Probes A, B and C were biotinylated with biotinyl-ε-caproic-*N*-hydroxy succinimide ester utilizing a biotin labeling kit.[12] The biotinylated probes were purified by Sephadex G-25 column chromatography followed by reverse phase HPLC. Biotinylation efficiencies were estimated spectrophotometrically as follows: Probe A: 86%; Probe B: 73%; Probe C: 38%.

The ability of the biotinylated probes to form double stranded DNA structures with the target sequence was evaluated by determining the melting temperatures (Tm) of the duplexes.[13] As shown in the table, each additional 4'-modification results in a lowering of the melting temperature by 1.2° to 1.7°. These changes are quite small and they are lower than the changes observed with other modification schemes such as the attachment of a linker to a abasic unit in the middle of a probe.[3c]

Table

Probes		Tm(°C)[13]	ΔTm/modification (°C)
Control	5'GTTCGCCTACGTGGCCTTTG3'	66.2	
A	5'GTTCGCCTACGT*GGCCTTTG3'	64.5	1.7
B	5'GTT*CGCCTACGT*GGCCTT*TG3'	62.6	1.2
C	5'GTT*CGCCT*ACGT*GGCCTT*TG3'	60.1	1.5

DNA dot blot assays were performed with the biotinylated probes A, B and C using a PCR amplified fragment from *Mycobacterium tuberculosis* (123 mer)[11] containing the target sequence as well as purified control DNA. The dot blot filters were processed according to the protocol of the BlueGene® detection system,[14] which uses a streptavidin/alkaline phosphatase conjugate for the detection of the hybridized biotinylated probes. All three probes easily detected specifically an estimated 2.5 fmole of target amplicon with no signal detected from control DNA sequences. Probes B and C gave distinctively stronger signals compared with probe A, yet the signal from probe C was approximately of the same intensity as the signal from probe B. This results shows, that multiple biotin labels per oligonucleotide probe allow for a more sensitive detection of target sequences and that this can be achieved without a loss in specificity. The result also indicates, that there are limits, probably of steric nature, to the number of labeling units which can beneficially be employed on short oligodeoxynucleotides.

The model study described in this report opens a new approach to the sequence selective, multiple modification of oligodeoxynucleotides which retain their hybridization properties. The extensive experience in these laboratories in the synthesis of 4'-modified nucleosides[15] has already paved the way for the efficient synthesis of suitable monomeric units of not just thymidine, but of 2'-deoxyadenosine, 2'-deoxycytidine and

2'-deoxyguanosine. This approach can readily be extended to the synthesis of oligodeoxynucleotides bearing stabilizing units such as acridins or to probes which carry two different elements such as a stabilizer and a label at the same time. Thus, application of this technology can be envisioned not only in the diagnostic area but also in the therapeutic field.

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