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Versatile Strategy for Oligonucleotide Derivatization. Introduction of Lanthanide(III) Chelates to Oligonucleotides

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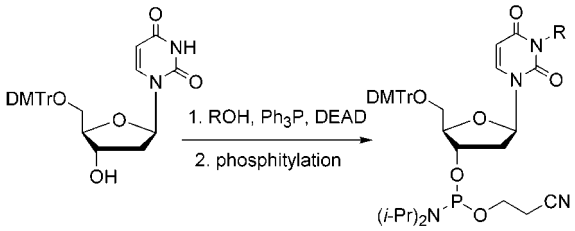
ABSTRACT



R = a linker containg a protected functional or conjugate group

Novel nucleosidic phosphoramidite blocks were synthesized by a Mitsunobu reaction between 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)uridine and a primary alcohol containing a conjugate group in its structure (a protected functional group, an organic dye, or a precursor of a lanthanide(III) chelate) followed by phosphitylation. They were used in machine-assisted DNA synthesis in the standard manner. A slighty modified deprotection procedure was used for the preparation of oligonucleotide conjugates tethered to lanthanide(III) chelates. For the latter application one non-nucleosidic block was also synthesized.

Synthetic oligonucleotides tethered to various ligands have been used as research tools in molecular biology.[1] They have been applied to genetic analysis and used to elucidate the mechanism of gene function. Oligonucleotides carrying reporter groups have had widespread use for automated DNA sequencing, hybridization affinity chromatography, and fluorescence microscopy. Oligonucleotide−biotin conjugates are widely used as hybridization probes.

The fluorescent label monomers for solid phase oligonucleotide chemistry are most commonly organic dyes, and several of those blocks are even commercially available. However, such labels and labeled biomolecules suffer from many drawbacks such as Raman scattering, low water solubility, and concentration quenching. Thus multilabeling of oligonucleotides with organic fluorophores may not enhance the detection sensitivity to the degree needed in several applications. For these type of applications lanthanide(III) chelates are labels of choice because they do not suffer from these phenomena.[2]

Multilabeling of oligonucleotides can be performed by three alternative methods:[3] (i) by coupling several base- or carbohydrate-tethered nucleosidic building blocks to the growing oligonucleotide chain, (ii) by functionalization of the internucleosidic phosphodiester linkages, or (iii) by using several multifunctional non-nucleosidic building blocks during the oligonucleotide chain assembly. All of these methods have their own drawbacks. Since the double helix formation of DNA is based on hydrogen bonding between the complementary base residues, tethers attached to the base moieties often weaken these interactions. This problem is easily

(1) For reviews, see: Iyer, R. P.; Roland, A.; Zhou, W.; Ghosh, K. *Curr. Opin. Mol. Ther*. **1999**, *1*, 344. Uhlman, E.; Peyman, A. *Chem. Rev*. **1990**, *90*, 543. English, U.; Gauss, D. H. *Angew. Chem., Int. Ed. Engl*. **1991**, *30*, 613.

(2) Hemmilä, I.; Dakubu, S.; Mukkala, V.-M.; Siitari, H.; Lövgren, T. *Anal. Biochem*. **1984**, *137*, 335.

(3) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 6123. Wojczewski, C.; Stolze, K.; Engels, J. W. *Synlett* **1999**, 1667.

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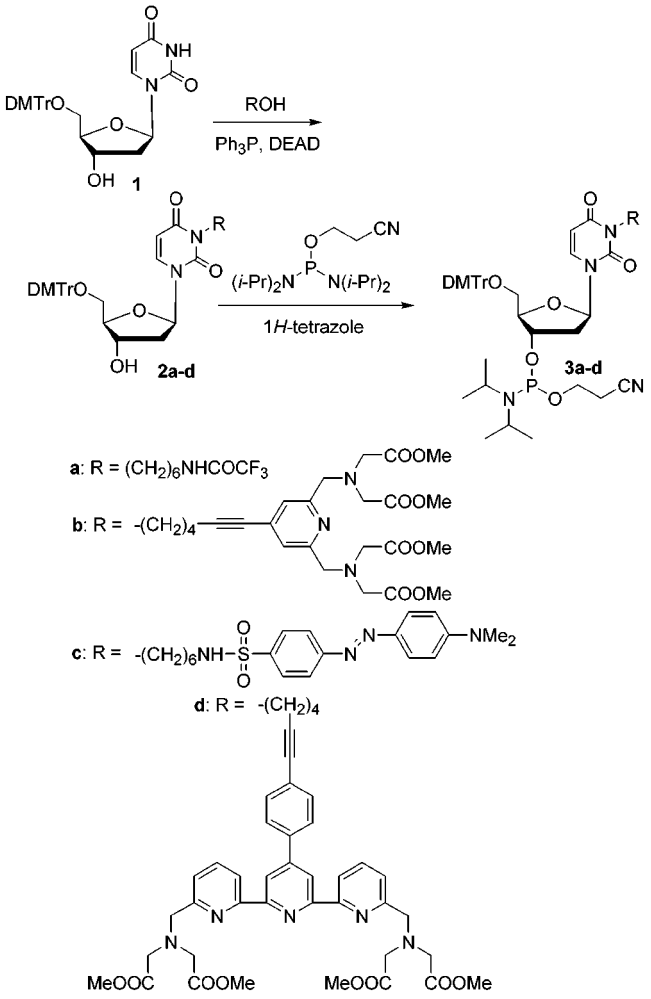
overcome by using the tethered nucleosides at the 3′- or 5′-terminus of the coding sequence or by using labels linked to C5 of pyrimidine residues. Introduction of tethers to the phosphate backbone often gives rise to new chiral centers and makes the purification of these analogues difficult. Introduction of the tether arm to the carbohydrate moiety, in turn, often decreases the coupling efficiency of the phosphoramidite. Although design of non-nucleosidic blocks may look attractive on paper, very often their syntheses suffer from complexity, low coupling yields, and problems associated with the storage and handling of the phosphoramidites. For several applications design of base-tethered nucleosidic building blocks is often the method of choice.

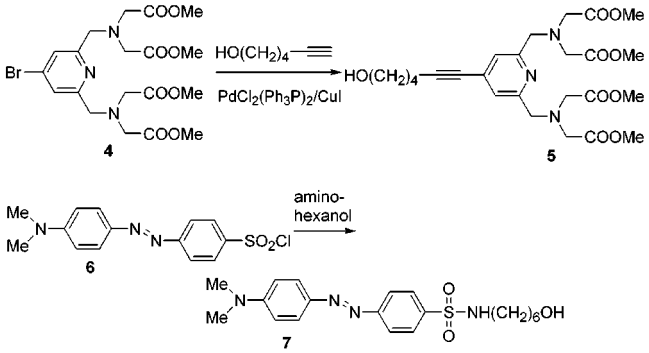
The present synthetic strategy for the preparation of nucleosidic phosphoramidite building blocks is shown in Scheme 1. Accordingly, reaction of 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)uridine (**1**) with a primary alcohol under Mitsunobu conditions[4] gives the *N*3-derivatives, **2**, phosphitylation of which results in the desired phosphoramidites, **3**. The key steps are discussed below in detail.

Scheme 1. Present Strategy for the Preparation of Nucleosidic Phosphoramidites



Synthesis of the Alcohols. The ligand, **5**, was synthesized by palladium-catalyzed coupling of 4-bromo-2,6-bis[*N*,*N*-bis(methoxycarbonylmethyl)aminomethyl]pyridine (**4**)[5] to 5-hexynol (Scheme 2). The dabsyl derivative, **7**, was obtained by allowing dabcyl chloride (**6**) to react with an excess of 6-aminohexanol in dichloromethane.

Scheme 2



Synthesis of the terpyridine structure, **15**, is outlined in Scheme 3. In general, the synthesis was analogous to that published for the corresponding 4-amino derivative.[6] Accordingly, 4-bromobenzaldehyde was condensed with 2-acetylpyridine by a Claisen−Schmidt reaction to give the (*E*)-prop-2-enone, **8**. Reaction of this with *N*-[2-(pyrid-2′-yl)-2-oxoethyl]pyridinium iodide, **9**, and ammonium acetate in methanol yielded the terpyridine derivative, **10**, in high yield. The terminal pyridine moieties were then oxidized with 3-chloroperbenzoic acid to *N*,*N*″-dioxides to give rise to **11**. The modified Reissert−Henze reaction yielded the 6,6′-dicarbonitrile **12**, which was reduced with borane to the corresponding bis(aminomethyl) compound, **13**. It was carboxymethylated to the tetraester, **14**, with methyl bromoacetate in the presence of diisopropylethylamine and potassium iodide as a catalyst. Finally, reaction of the bromide **14** with 5-hexynol in the presence of Pd(II) and Cu(I) gave the ligand **15**.

Synthesis of the Phosphoramidites and Solid Supports. To demonstrate the versatility of the present method for nucleoside derivatization **1** was allowed to react with N^6-trifluoroacetamidohexanol as well as the alcohols **5**, **7**, and **15** in the presence of triphenyl phosphine and DEAD. The nucleoside derivatives **2a**−**d** were obtained in high yield. Their structures were confirmed on NMR analyses,

(4) Mitsunobu, O. *Synthesis* **1981**, 1.

(5) This compound was synthesized as described for the corresponding *tert*-butyl ester: Takalo, H.; Mukkala, V.-M.; Mikola, H.; Liitti, P.; Hemmilä, I. *Bioconjugate Chem.* **1994**, *5*, 278.

(6) Mukkala, V.-M.; Helenius, M.; Hemmilä, I.; Kankare, J.; Takalo, H. *Helv. Chim. Acta* **1993**, *76*, 1361.

(7) Mitsunobu reaction between 3′,5′-*O*-protected dU and 5-trifluoroacetamidopentan-1-ol has been reported; see: Brossette, T.; Le Faou, A.; Vaillex, A.; Criminon, C.; Grassi, J.; Mioskowski, C.; Lebeau, L. *J. Org. Chem.* **1999**, *64*, 5083.

(8) Dahlén, P.; Liukkonen, L.; Kwiatkowski, M.; Hurskainen, P.; Iitiä, A.; Siitari, H.; Ylikoski, J.; Mukkala, V.-M.; Lövgren, T. *Bioconjugate Chem.* **1994**, *5*, 268.

(9) Kwiatkowski, M.; Samiotaki, M.; Lamminmäki, U.; Mukkala, V.-M.; Landegren, U. *Nucleic Acids Res.* **1994**, *22*, 2604.

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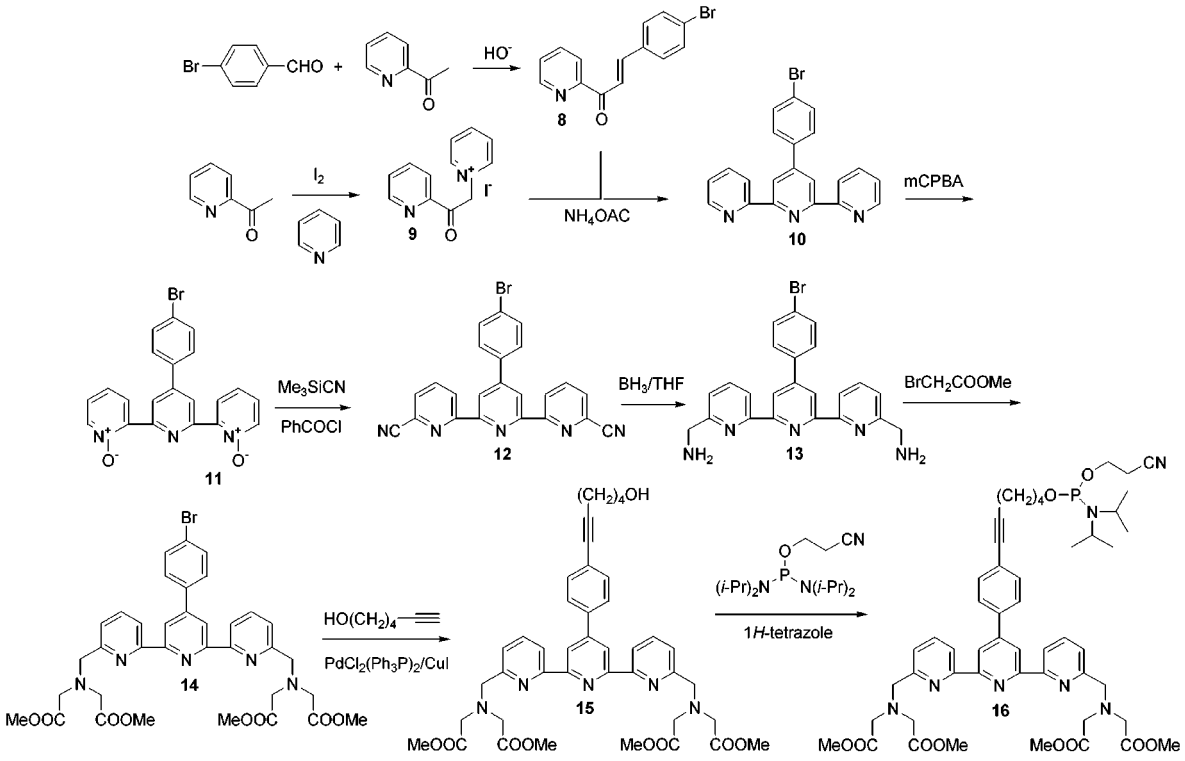
Scheme 3



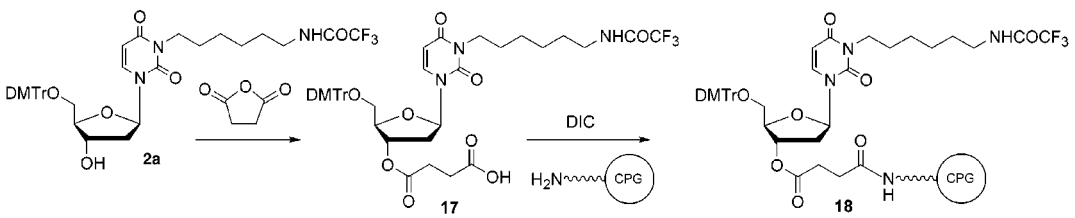
and the site of alkylation was in all cases undoubtedly *N*3 (see Supporting Information). It is worth noting that under the reaction conditions employed 3′-*O*-protection of the nucleoside is not required.[7] Finally, treatment of **2a**−**d** with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite in the presence of 1*H*-tetrazole yielded the corresponding phosphoramidites, **3a**−**d**. Purification was performed on silica gel or by precipitation from cold (−70 °C) hexanes.

To attach the labels to the 3′-terminus of oligonucleotides, synthesis of the corresponding modified solid support is needed. As an illustrative example **2a** was allowed to react with succinic anhydride, giving the corresponding succinate, **17**, which was immobilized on a long chain alkylamine CPG polymer support with *N*,*N*′-diisopropylcarbodiimide (Scheme 4).

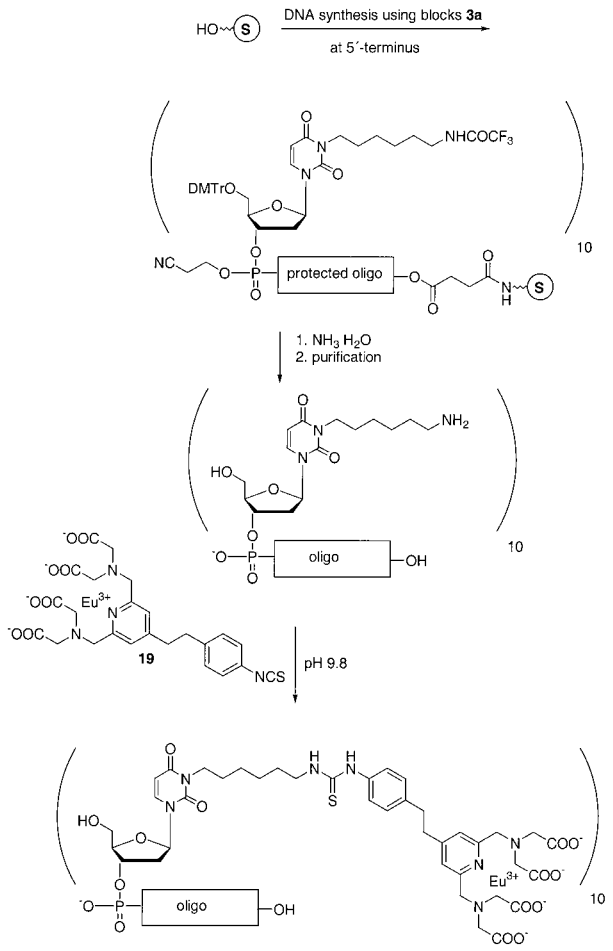
Synthesis of the Oligonucleotides. To demonstrate the applicability of the building blocks **3a**−**d** for oligonucleotide synthesis, several model sequences were synthesized using standard machine-assisted phosphoramidite chemistry. These blocks were coupled to the 5′-terminus of the oligomers. No differences in coupling efficiency between **3a**−**d** and commercial nucleoside monomers were detected. The oligonucleotide conjugates synthesized in the aid of blocks **3a** and **3c** were deprotected by conventional ammonialysis. An oligonucleotide bearing 10 primary amino groups was further labeled with a nonluminescent europium(III) chelate, **19**,[5] in solution[8] according to Scheme 5.

Direct introduction of lanthanide(III) chelates to oligonucleotides required a modified deprotection strategy[9] as described in Scheme 6. Accordingly, after completion of the oligonucleotide synthesis the fully protected oligomer was initially treated with sodium hydroxide (0.1 M; 4 h at room temperature) to ensure total hydrolysis of the ester protecting groups followed by ammonialysis to complete the base deprotection. Treatment of the deblocked oligomer with europium(III) citrate converted the oligonucleotide conjugate to the corresponding europium(III) chelate.

Scheme 4. Synthesis of the Solid Support with the Aid of **2a**



Scheme 5. Introduction of Europium(III) Chelates to Oligonucletides in the Aid of Block **3a**



For several applications, introduction of only a single label molecule to the 5′-terminus the oligonucleotide structure is needed. For these applications the ligand structure can be simplified by omitting the nucleobase, resulting in non-nucleosidic phosphoramidite building blocks such as **16**. This block was successfully coupled to the 5′-terminus of a 45-mer oligonucleotide. Deprotection strategy was similar to that for nucleosidic lanthanide blocks **3b** and **3d**. The oligonucleotides labeled with lanthanide chelates were used in DELFIA[10]-based DNA hybridization assays as well as LANCE fluorescence quenching assays (Helicase, RNase, Molecular Beacons). These results will be published elsewhere.

The current method for oligonucleotide derivatization has the following advantages: (i) Because the coupling reaction between the nucleoside and the tether molecule is performed under mild reaction conditions (at ambient temperature in dry THF) a wide range of tethers and label molecules can be introduced. The only requirement is that the tether molecule has a primary hydroxyl group in its structure and other functional groups are protected. (ii) The nucleosidic blocks are solid materials, which makes their storage and handling convenient (by contrast, the non-nucleosidic block **16** is an oil). (iii) The blocks can be incorporated into the oligonucleotide structure in high efficiency using standard protocols of machine-assisted DNA chemistry. In is worth noting that since the labels attached to the N3 positon of uracil residues naturally weaken hydrogen bonds of in the dublex, these labels should be used only up- or downstream of the coding sequence.

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Supporting Information Available: Experimental procedures and characterization data for the compounds prepared. This material is available free of charge via the Internet at http://pubs.acs.org.

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Scheme 6. Introduction of Lanthanide(III) Chelates to Oligonucleotides in the Aid of Block **3b**