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Selective Phosphate Protection: A Novel Synthesis of Double-Labeled Oligonucleotides

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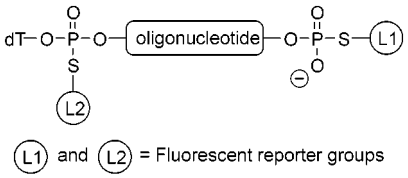
ABSTRACT



A novel, selective labeling of oligonucleotides with two different reporter groups is described. The oligonucleotide is synthesized using a stable 2-(4-methoxybenzamido)ethyl protection for a selected internucleosidic thiophosphate (PS) and a labile 2-(*N*-isopropyl-4-methoxybenzamido)-ethyl for the 3′-terminal PS and internucleosidic phosphates. The latter group and the base protection are removed, and the 3′-terminal PS is labeled. The former protection is then cleaved by a prolonged ammonolysis, and the second reporter is introduced at the internucleosidic PS.

Recently, oligonucleotides selectively labeled with two different reporter groups or other modifiers have attained widespread interest. While one of the modifications is a conjugated peptide or other pendant of biological importance, the other often is a reporter group for nonradioactive detection.[1] Alternatively, an oligonucleotide precursor can be labeled with two different reporter groups to allow a two-color detection of a product, the double-labeled oligonucleotide. If the reporter groups are appropriate donors and acceptors, the labeled oligonucleotides display hybridization-dependent fluorescence.[2] These molecular beacons are widely used for real-time monitoring of hybridization in in vitro[3] and in vivo[4] test systems.

The synthesis of double-labeled oligonucleotides is most conveniently performed with the aid of dye-labeled solid supports and phosphoramidites.[5,6] Alternatively, oligonucleotides that bear an amino and an activated thiol group at opposite ends are synthesized using a variety of modified phosphoramidites[7,8] and solid supports,[9,10] and the chemoselective labeling is performed postsynthetically.[11,12]

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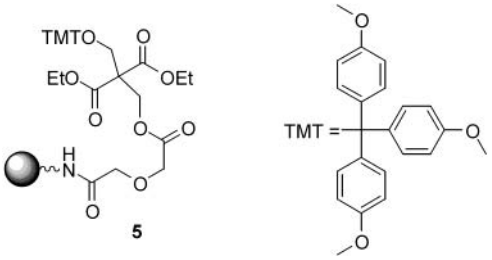
The primary disadvantage of these methods is that reporter groups commercially available as phosphoramidites, solid supports, or chemoselective reagents are restricted to a limited number of well-established dyes, whereas a variety of thiol reactive fluorescent alkylating reagents is available on the market. Their use in the labeling of internucleosidic and 3′-terminal thiophosphate (PS) groups in oligonucleotides is well documented.[12,13] However, because these functions possess similar reactivity, the selective introduction of two different reporter groups on oligonucleotides using a conventional approach is precluded.

In this communication, we report a novel method for the preparation of oligonucleotides with two different thiophilic reporter groups placed at the 3′-terminal and internucleosidic PS moieties. To perform selective labeling, a recently developed strategy allowing chemoselective deprotection of internucleosidic phosphate groups was used.[14] An oligonucleotide **1** bearing a 3′-terminal PS group and an internucleosidic PS residue with a stable protecting group PG1 was synthesized and then reacted selectively at the 3′-terminus with the first reporter group L1 to give **2** (Scheme 1). The protecting group PG1 was subsequently removed with concentrated aqueous ammonium hydroxide. The oligonucleotide **3** thus obtained was labeled with the second reporter group L2 at the internucleosidic PS group to give the oligonucleotide **4** labeled with two different reporter groups.



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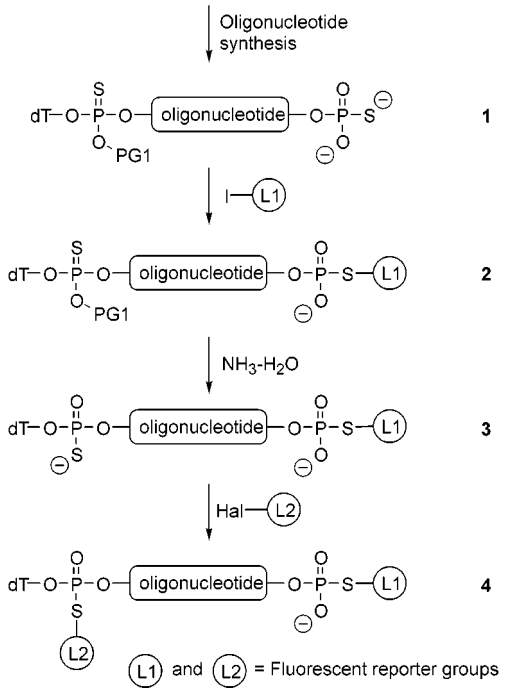
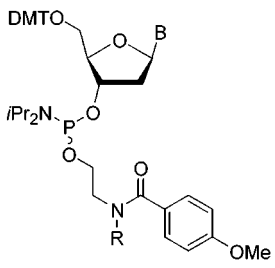
The efficient synthesis of the modified oligonucleotide **1** was achieved using an altered version of the standard phosphoramidite method. The oligonucleotide chain assembly was carried out on a solid support **5**, thus permitting the release of the 3′-terminal PS group under mild, nonorthogonal conditions.[15]

The preparation of the partially protected oligonucleotide **1** required the use of two phosphate protecting groups that could be removed in dramatically different rates, thus possibly permitting chemoselective deprotection.

Scheme 1. Preparation of Double-Labeled Oligonucleotides



Recently, we reported a novel protecting strategy for internucleosidic phosphate (PO) and PS moieties.[14,16] Of the various groups investigated, the 2-(*N*-isopropyl-4-methoxybenzamido)ethyl group (PG) was used in the present work as a labile protecting group. In our studies, we observed that oligonucleotides synthesized using the phosphoramidite building blocks **6**−**9** were already largely deprotected in the course of oligonucleotide synthesis. The remaining protecting groups were removed by brief treatment with pyridine/MeOH or aqueous ammonium hydroxide.[14] Consequently, a modified synthetic protocol was necessary to maintain the high coupling efficiency of **6**−**9**.[14,17]



6-10

	B	R
6	Thy	*i*Pr
7	^{bz}Ade	*i*Pr
8	^{bz}Cyt	*i*Pr
9	^{ibu}Gua	*i*Pr
10	Thy	H

To introduce a PS moiety protected with a stable 2-(4-methoxybenzamido)ethyl group (PG1), the phosphoramidite **10** was used. In contrast to PG, the removal of PG1 required prolonged heating with concentrated aqueous ammonium hydroxide.

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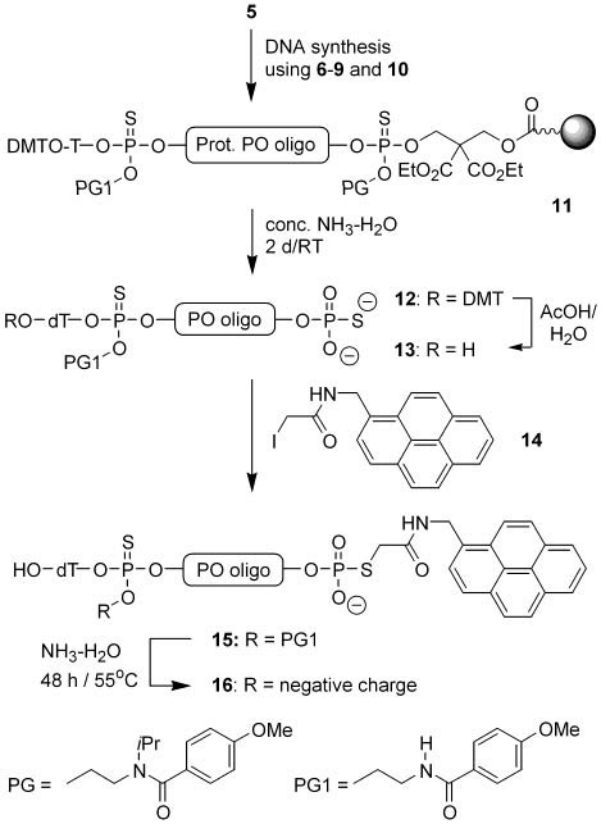
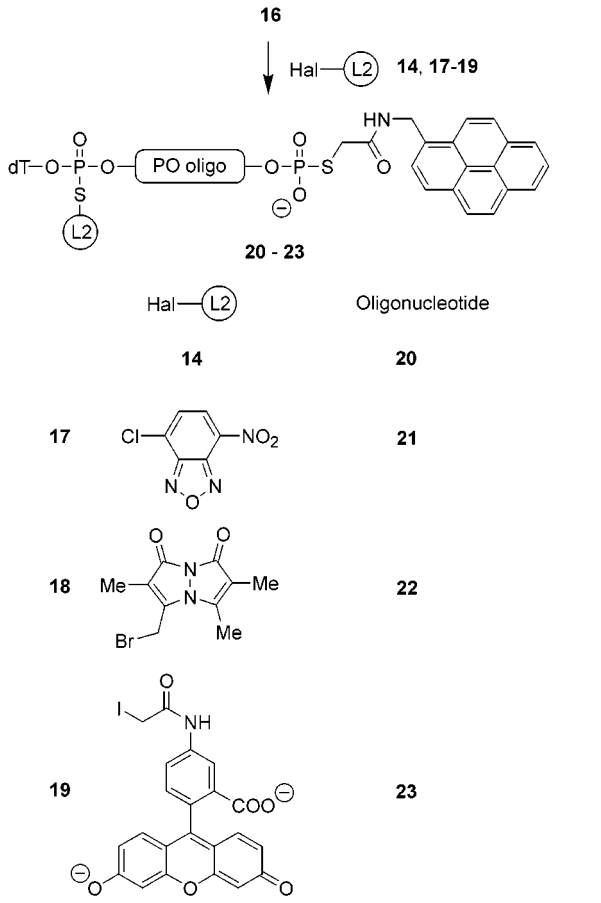
With these considerations in mind, we first synthesized the modified oligonucleotide **11** (Scheme 2). To form a 3′-terminal PS linkage to the solid support, the building block **6** was coupled to **5**, and the phosphite triester formed was sulfurized with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide.[18] The chain assembly was then carried out using a modified elongation cycle in which the standard detritylation subroutine was followed by washing with a solution of neutralizer (0.1 M DMAP and 0.1 M 1*H*-tetrazole in MeCN) as previously described.[17] For the coupling step, compounds **6**−**9** (0.2 M in MeCN, 6 min) were used, and the following oxidation was carried out with *t*-BuOOH (10% in MeCN, 10 min). The final coupling with **10** (0.1 M in MeCN) was followed by sulfurization to give the solid-support-bound **11**, which was treated with concentrated aqueous ammonium hydroxide (36 h/rt). The product **12** contained a single internucleosidic PS moiety protected with PG1 while the internucleosidic PO groups, 3′-terminal PS group, and nucleic bases were deprotected. As evidenced by HPLC, the loss of PG1 resulting from this treatment did not exceed 10%. The completely deprotected oligonucleotide, formed as an impurity, was readily removed by HPLC purification on a reverse-phase column. The purified **12** was detritylated with 10% aqueous AcOH, desalted to furnish **13** as a triethylammonium salt, and characterized by HPLC-ESMS.

Scheme 2. Introduction of the 3′-Terminal Reporter Group[a]



[a] PO oligonucleotide: 5′-GCATC$_5$AG$_2$C$_2$AC$_2$AT-3′

Scheme 3. Introduction of the Internucleosidic Reporter Group[a]



[a] PO oligonucleotide: 5′-GCATC$_5$AG$_2$C$_2$AC$_2$AT-3′.

To incorporate the 3′-terminal pyrene reporter group, **13** was alkylated with iodoacetamide **14** (Scheme 2). Under optimized conditions, the labeling was carried out using 0.125 mM **13**, 6 mM **14**, and 50 mM ethyldiisopropylammonium acetate (pH 7.0) in 75% aqueous DMSO for 4 h at 37 °C. The product, **15**, was isolated by reverse-phase HPLC in 70% yield and characterized by ESMS.

To introduce the second reporter group at the internucleosidic PS moiety, the 2-(4-methoxy benzamido)ethyl protecting group PG1 in **15** was first removed with concentrated aqueous ammonium hydroxide (55 °C/48 h) to give **16** in quantitative yield. In agreement with previously reported observations,[19] no loss of the 3′-terminal reporter group resulting from this treatment was observed by HPLC analysis of the deprotection mixture.

The second labeling reaction at the internucleosidic PS group of **16** was then carried out, as depicted in Scheme 3.

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The oligonucleotide **16** was treated with a variety of thiophilic alkylating agents, **14** and **17**−**19**. In the case of the poorly soluble **14**, the conditions described above for the preparation of **15** were used. For **17**−**19**, the alkylation was performed as recommended in the literature.[12] After isolation by reverse-phase HPLC, the double-labeled oligonucleotide conjugates **20**−**23** were obtained in yields of 70−90%, which are typical for the alkylation reaction. The homogeneity of **20**−**23** was verified by analytical HPLC, and their structures were confirmed by ESMS. In addition, the UV−vis spectrum of **23** demonstrated the absorbance characteristic for both the pyrene (315, 329, and 346 nm) and fluorescein (462 and 492 nm) chromophores.

A rapid, versatile attachment of various reporter groups to oligonucleotides and screening of the conjugates is of crucial importance for the development of novel applications. The data presented here suggest that the preparation of a variety of oligonucleotide conjugates is dramatically facilitated by the selective protection of internucleosidic PO and PS groups. The utility of the proposed methodology was demonstrated using a combination of the labile 2-(*N*-isopropyl-4-methoxybenzamido)ethyl group PG and the stable 2-(4-methoxybenzamido)ethyl group PG1. Taking advantage of chemoselective deprotection, we synthesized an oligonucleotide bearing a single protected PS linkage and converted it to oligonucleotide conjugates labeled with two different reporter groups. While one of the labels is placed at the 3′-terminal PS residue, positioning of the second reporter group may vary depending on the requirements of a particular application.

Supporting Information Available: Experimental details and ESMS data for compounds **12**, **13**, **15**, **16**, and **20**−**23**, HPLC profiles for **12** and **20**−**23**, and UV−vis spectra for **16**, **20**, and **23**. This material is available free of charge via the Internet at http://pubs.acs.org.

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