Pergamon

Tetrahedron Letters 42 (2001) 8943–8946

TETRAHEDRON LETTERS

Linker phosphoramidite reagents for oligonucleotide synthesis on underivatized solid-phase supports

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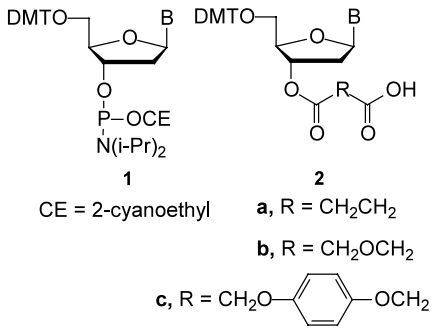
Received 2 October 2001; revised 23 October 2001; accepted 24 October 2001

Abstract—Linker phosphoramidite reagents containing a protected nucleoside with a cleavable 3′-ester linkage to either succinic acid, diglycolic acid, or hydroquinone-*O*,*O*′-diacetic acid (*Q-Linker*) allow the 3′-terminal nucleoside of an oligonucleotide sequence to be attached to underivatized 'Universal' amino or hydroxyl supports. After solid-phase oligonucleotide synthesis, treatment with NH_4OH cleaves the 3′-ester link to the support and produces oligonucleotides with only 3′-OH ends. No changes to existing phosphoramidite synthesis cycles, cleavage, or deprotection methods are required. © 2001 Published by Elsevier Science Ltd.

1. Introduction

High-throughput DNA synthesizers using multiple columns or 96-well plates allow large numbers of oligonucleotides to be rapidly synthesized.[1–3] However, sorting supports pre-derivatized with the first nucleoside into the desired columns or wells is tedious and susceptible to error. 'Universal' supports based on special cyclic[4–10] or acyclic[11,12] diol structures which allow the first base to be added using conventional phosphoramidite reagents **1** have been developed to eliminate this task. However, this approach introduces a terminal 3′-phosphate linkage which is unsatisfactory for many oligonucleotide applications. Removal of the terminal phosphate group requires post-synthesis modifications to the cleavage and deprotection steps which can increase processing time and reduce yield. The dephosphorylation step is also not quantitative and a mixture of 3′-phosphate and 3′-OH products may result.

Synthesis of the above 'Universal' supports also requires a multi-step synthesis, often of significant complexity, to prepare the diol linker and attach it to the support. Therefore, these materials are also much more expensive than supports prederivatized with nucleosides **2a**–**c**.[13–17]



1 CE = 2-cyanoethyl

2

a, R = CH_2CH_2

b, R = CH_2OCH_2

c, R = CH_2O–C$_6$H$_4$–OCH_2

We believe the simplest possible 'Universal' supports are the same underivatized amino or hydroxyl functionalized supports which are the starting materials for conventional prederivatized supports. These materials are inexpensive, widely available, and can be used directly in automated synthesis as long as a rapid, on-line method is available for adding the first nucleoside residue.

However, a cleavable linkage to the surface of the support is required for eventual product release. As mentioned above, phosphate linkages are not ideal because of the problems involved in dephosphorylation. Instead, ester linkages, such as those in prederivatized supports with dicarboxylic acid linker arms like succinic,[13,14] diglycolic[15] or hydroquinone-*O*,*O*′-diacetic (*Q-Linker*)[16,17] acid are much better.

Originally we developed a strategy which rapidly coupled nucleoside-3′-*O*-carboxylates **2a**–**c** to underivatized supports using *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 4-

Keywords: solid-phase synthesis; oligonucleotides; linker arms; phosphoramidite reagents; *Q-Linker*; universal support.

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0040-4039/01/$ - see front matter © 2001 Published by Elsevier Science Ltd.
PII: S0040-4039(01)01996-7

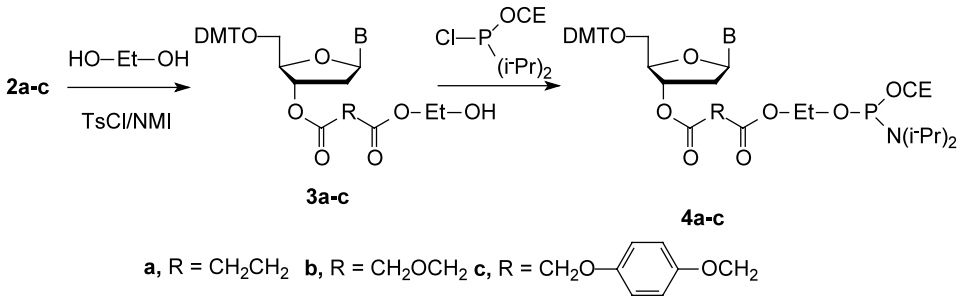
aminopyridine (DMAP) as part of the automated synthesis.[18] This method was part of our work to develop reusable supports for large scale oligonucleotide synthesis.[17] Although, the HBTU/DMAP coupling was fast and efficient, the method is better suited for large-scale synthesis rather than small-scale high-throughput oligonucleotide synthesis. This is because a second coupling reagent and cycle is required in addition to the phosphoramidites. Additionally, automated synthesizers which can accommodate five extra reagents (four nucleosides and the HBTU/DMAP solution) are not commonly available for small-scale oligonucleotide synthesis.

In this communication, we report on new linker phosphoramidite reagents **4a–c** which eliminate the HBTU coupling reagent while still retaining a cleavable 3′-ester linkage to the nucleoside. These new reagents allow rapid, automated coupling using conventional phosphoramidite coupling cycles. They can easily be used on most DNA synthesizers with no changes to the synthesizer's operation. The linker phosphoramidites readily couple to either underivatized amino or hydroxyl supports, which serve as 'Universal' supports. Since conventional phosphoramidites **1** are no longer used to attach the first nucleoside, no unwanted 3′-phosphorylated products are produced and all of the post-synthesis difficulties related to 3′-dephosphorylation are eliminated.

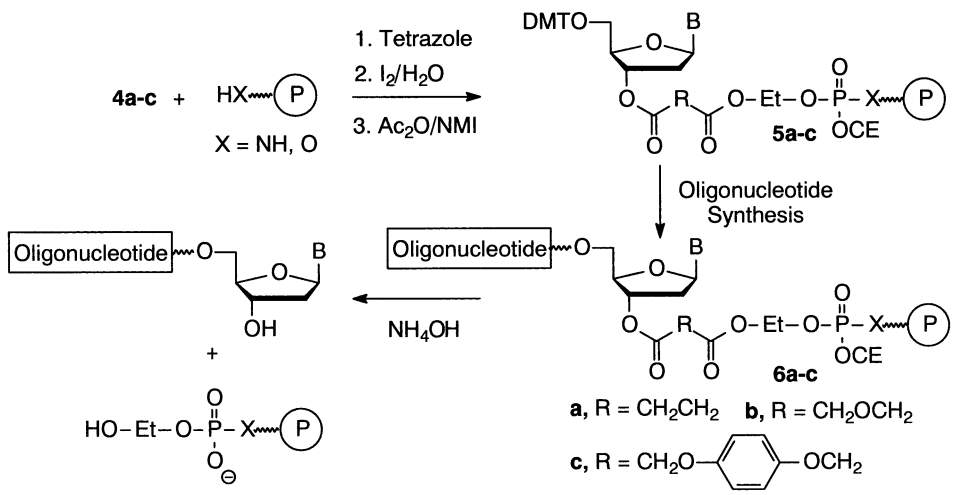
2. Results and discussion

Synthesis of the linker phosphoramidites **4a–c** is simple and straightforward (Scheme 1). Succinic anhydride, diglycolic anhydride, or hydroquinone-*O*,*O*′-diacetic acid (*Q-Linker*) is attached to the 3′-position of a protected nucleoside using existing methods. The terminal carboxyl group of **2a–c** is then converted into an hydroxyl function by coupling an intermediate spacer to the end of the linker arm to yield **3a–c**. In our initial studies, ethylene glycol was used as the spacer between the carboxylic acid linker and the phosphoramidite group because of its simplicity and low cost. However, other longer diols can be used to increase the length of the linkage to the solid-phase support. Finally, nucleoside **3a–c** is converted into phosphoramidite derivative **4a–c** using a phosphitylation reagent such as 2-cyanoethyl-*N*,*N*′-diisopropylamino-phosphorochloridite or bis-(diisopropylamino)-2-cyanoethylphosphine.[19–22]

The linker phosphoramidite reagents **4a–c** are activated by tetrazole and react with solid-phase supports in the same manner as conventional phosphoramidites **1**.[23] After oxidation with I_2 and H_2O, either phosphoramidate **5** (X = NH) or phosphate linkages **5** (X = O) to the support are formed (Scheme 2). Both of these linkages are quite stable. For example, permanent phosphate triester and diester linkages to various solid-phase surfaces are commonly used for permanently immobilized



Scheme 1.



Scheme 2.

oligonucleotide probes.[24] Phosphoramidate linkages are less common and less stable (especially to acid), but their properties have been well characterized.

In previous work by Gryaznov and Letsinger,[25] diester phosphoramidate linkages to LCAA-CPG were used in the synthesis of 3′-terminally phosphorylated oligonucleotides by taking advantage of the difference in stability towards acidic hydrolysis of diester phosphoramidate and monoester phosphoramidate linkages. The diester phosphoramidate linkage, which was similar to **5**, was stable to the acid conditions used for detritylation, but upon conversion to a monoester phosphoramidate (by removal of the cyanoethyl protecting group), the linkage became susceptible to slow (4 h) cleavage by aqueous acetic acid. Oligonucleotides with internucleotide phosphoramidate linkages have also been widely prepared by solution-phase[26] and solid-phase synthesis[27–29] and again, the diester phosphoramidate linkages are sufficiently stable during synthesis. Therefore, the stability of the diester phosphoramidate linkage formed by coupling our new reagents directly to commonly used LCAA-CPG was not expected to be problematic.

The nucleoside loading obtained on long chain alkylamine controlled pore glass (LCAA-CPG) supports containing ~100 μmol/g of amino groups (the same as used for conventional derivatization) varied between 32–45 μmol/g when reagents **4a** and **4b** were used at 0.1–0.15 M concentrations. A lower nucleoside loading (17–21 μmol/g) was obtained with **4c**. Coupling to an hydroxyl derivatized support, glycerol-CPG, containing an initial loading of 90 μmol/g produced higher nucleoside levels. 0.1 M solutions of **4a** and **4b** produced 54–58 μmol/g, while **4c** gave a loading of 26 μmol/g. Further characterization of how different surface properties, such as the initial surface loading and pore size affect the nucleoside loading are in progress. However, small scale oligonucleotide synthesis is typically performed with loadings of 20–40 μmol/g, and this level of derivatization is easily obtainable.

After the coupling and oxidation steps, unreacted amino sites on the support were acetylated (capped) with acetic anhydride and *N*-methylimidazole to prevent further coupling to the surface. The efficiency of this step was noted by the absence of any excess dimethoxytrityl cation (i.e. trityl yields of >100%) from the subsequent coupling and detritylation cycle.[13] Automated oligonucleotide synthesis was performed without any modifications.

The product was cleaved from the support using NH_4OH, which hydrolyzed the 3′-ester linkage within **6a–c**. The time required for cleavage depended upon the dicarboxylic acid used in the linker phosphoramidite. Recovery of ~95% of the product was obtained by treating supports **6a–c** with NH_4OH for 60, 40, and 2 min, respectively.

A 20-mer sequence, dACCTTATGTATCATACACAT, was prepared on conventionally derivatized succinyl LCAA-CPG and with reagents **4a** and **4c**. The amount of crude product released from each synthesis was calculated as the number of A_{260} units per μmol of starting nucleoside. The control with the prederivatized succinyl support yielded 112 A_{260} units/μmol, while the syntheses from **4a** and **4c** produced 135 and 137 A_{260} units/μmol, respectively. Thus, there was no decrease in the amount of product which would indicate premature cleavage of the phosphoramidate linkages by the detritylation conditions (5% dichloroacetic acid in 1,2-dichlorethane) used.

Oligonucleotide 17-mers (dGTAAAACGACGGCCAGT) produced using **4a–c** on both LCAA-CPG and glycerol-CPG were characterized by comparison to the same sequence made on conventional prederivatized LCAA-CPG. Capillary gel electrophoresis (CGE) analysis of the crude products showed virtually identical results. In addition, a 17-mer bearing a terminal 3′-phosphate residue was intentionally made using a 3′-phosphate CPG. Co-injection of the products from **4a–c** with the above showed that no 3′-terminally phosphorylated impurities were detectable in the products from the linker phosphoramidites. MALDI-TOF mass spectrometry also clearly confirmed the complete removal of all protecting groups and linkers since the m/z observed for **4a** (5228.2), **4b** (5228.2), and **4c** (5225.7) were all well within the expected accuracy (± 0.1%) for the calculated $[M+H]^+$ value of 5227.7.

Modifying the phosphoramidite reagent used to add the first base is a novel approach to eliminating prederivatized solid-phase supports and has several advantages. First, only inexpensive underivatized supports are required instead of the more expensive diol derivatized universal supports. Secondly, the 3′-ester linkage is hydrolyzed to yield a 3′-OH end without unwanted 3′-phosphates using the same conditions as prederivatized supports with similar dicarboxylic acid linkers. Unlike some other universal supports, the method does not require deprotection with additional reagents, treatment time, or elevated temperatures. Product yield is also not reduced. When reagent **4c** containing the *Q-Linker* is employed particularly fast cleavage (2 min) is also possible. Finally, the linker phosphoramidite reagents have very similar properties (i.e. reactivity, stability, etc.) to other phosphoramidite reagents and are therefore completely compatible with all existing automated DNA synthesizers.

These properties make the linker phosphoramidites very well suited for use in high-throughput DNA synthesizers, which may need to produce several hundred to several thousand oligonucleotides per day. In such circumstances, any method which can save time or reduce costs is valuable. Additionally, we are also developing a tandem synthesis method for the solid-phase synthesis of multiple oligonucleotides in a single run.[30] Use of linker phosphoramidite reagents in this approach will be reported separately.

3. Experimental

3.1. Synthesis of a nucleoside with an ethylene glycol spacer (3a)

5′-Dimethoxytritylthymidine-3′-*O*-succinyl hemiester **2a** (2 mmol) was dissolved in anhydrous acetonitrile (30 ml) and pyridine (16 mmol). *p*-Toluenesulfonyl chloride (3.9 mmol, 0.74 g) and *N*-methylimidazole (7.2 mmol, 0.6 ml) were added. The solution was stirred at room temperature (10 min) and then ethylene glycol (200 mmol, 11 ml) was slowly added dropwise via syringe. The reaction was stirred 30 min after completion of the addition. Then the solvent was evaporated off, the residue was redissolved in chloroform, washed with saturated aqueous sodium bicarbonate (1×) and then water (2×). The crude material was purified by silica gel column chromatography using a 0–2% methanol in chloroform. Yield of **3a**, 76%.

3.2. Synthesis of nucleoside linker phosphoramidites 4a

A solution of **3a**, B = T (1.34 mmol) and diisopropylethylamine (5.2 mmol) in chloroform (8 ml) was stirred at room temperature in a septum sealed vessel. 2-Cyanoethyl-*N*,*N*′-diisopropylchlorophosphoramidite (1.75 mmol) was added and the reaction was stirred (1 h). The solution was diluted with chloroform (50 ml) and washed with aqueous sodium chloride (4×) and water (1×). The solution was then concentrated and purified by silica gel chromatography using first dichloromethane/hexane/triethylamine 42:53:5 to 40:50:10 and then 5% triethylamine/chloroform. Yield of **3a**, 89%. ^{31}P NMR ($CDCl_3$): δ 150.754, δ 150.269.

Acknowledgements

We thank Maria Loskot, Wade Stout, Fady Girgis and Marko Markicevic for technical assistance and Isis Pharmaceuticals for financial assistance.

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