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A versatile reagent for the synthesis of 5′-phosphorylated, 5′-thiophosphorylated or 5′-phosphoramidate-conjugated oligonucleotides

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This work is dedicated to Professor Jean-Louis Imbach for his 70th birthday

Abstract—We report the synthesis of a new phosphorylating reagent that is easily accessible and allows not only the chemical synthesis of 5′-phosphorylated and 5′-thiophosphorylated oligonucleotides but also the 5′-conjugation through a phosphoramidate linkage. 5′-Amino-linker and 5′-alkyne oligonucleotides were obtained and the latter was conjugated by a 1,3-dipolar cycloaddition (click chemistry) with a galactosylated azide derivative to afford 5′-galactosyl oligonucleotide with high efficiency.

1. Introduction

Several applications of oligonucleotides in molecular biology require the presence of 5′-phosphate group or conjugation of ligands. A number of reagents are commercially available such as **2**,[1] **3**[2] or reported in the literature such as **4**,[3,4] **5**[5] and **6**[6] for the preparation of 5′-phosphate or 5′-thiophosphate oligonucleotides. More recently, a new reagent **7**[7] was reported for the conjugation of 5′-phosphoramidate to oligonucleotides or 5′-phosphorylation but not for the preparation of 5′-thiophosphorylated oligonucleotides (Fig. 1). All of these reagents **2**–**7** are useful for oligonucleotide modifications but none of them could give a simultaneous access to 5′-phosphorylation, 5′-thiophosphorylation and 5′-phosphoramidates conjugation. It is worth mentioning that reagent **6** could be used for 5′-phosphoramidate conjugation although Lartia and Asseline[6] have not reported that application. However, its introduction would require the use of *H*-phosphonate chemistry with specific ancillary reagents and solvents, which is less convenient than the standard phosphoramidite chemistry.

We report here a convenient approach for the 5′-terminal phosphorylation, thiophosphorylation and conjugation of oligonucleotides through a phosphoramidate linkage. The 5′-modification was introduced by means of a novel and simple reagent, namely 2-cyanoethyl *tert*-butyl *N*,*N*-diisopropylphosphoramidite **1**. It has been rapidly and easily prepared in a single step from commercially available 2-cyanoethyl tetraisopropylphosphorodiamidite[8] and *tert*-butanol in dichloromethane in the presence of diisopropylammonium tetrazolide as activating agent (Scheme 1).

After its incorporation at the 5′-end of an oligonucleotide using phosphoramidite chemistry, the resulting phosphite triester was not oxidized but directly treated with dichloroacetic acid (DCA) to afford a *H*-phosphonate diester linkage through an Arbuzov-like mechanism.[9,10] This *H*-phosphonate diester linkage could be

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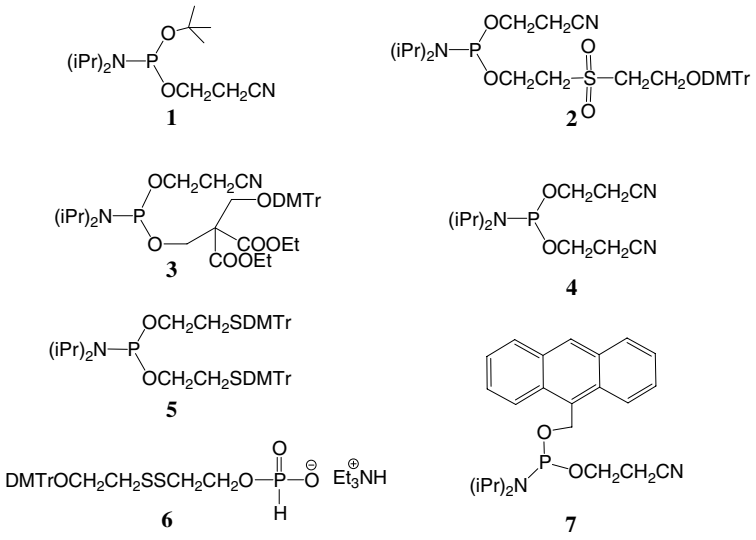
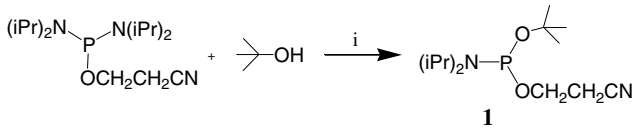


Figure 1. Novel phosphoramidite reagent **1** and previously reported reagents **2**–**7** for the 5′-phosphorylation of oligonucleotides.

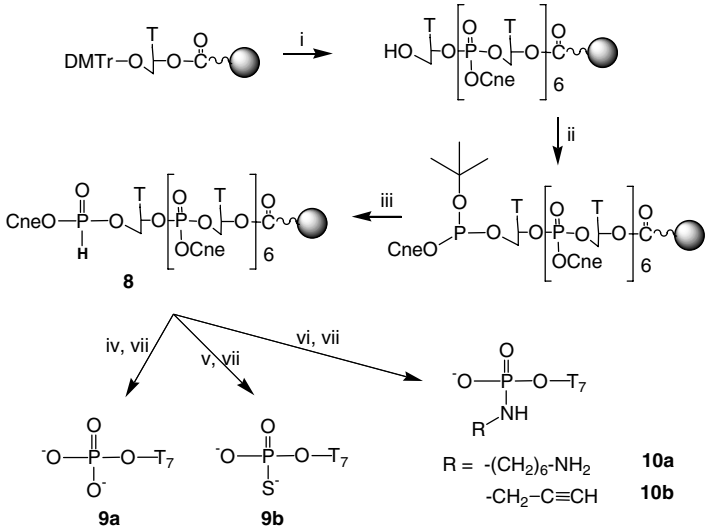


Scheme 1. Reagents: (i) CH_2Cl_2, diisopropylammonium tetrazolide.

further oxidized to phosphoro-, thiophosphoro- or phosphoramidate-diesters[11] according to standard procedures.

Hepta-thymidines were synthesized on a commercial CPG solid support loaded with an initial thymidine according to the phosphoramidite chemistry using benzylthiotetrazole (BMT) as coupling activator. At the end of the elongation sequence, reagent **1** (0.15 M in acetonitrile) was coupled for 60 sec and a subsequent treatment with DCA (3% in CH_2Cl_2) was used as a standard detritylation step. The resulting supported 5′-cyanoethyl-*H*-phosphonate diester T_7 **8** was obtained after washing with acetonitrile and drying with an argon flush (Scheme 2).

5′-Monophosphate T_7 **9a** was obtained by treatment of the supported oligonucleotide **8** with a commercial oxidizing solution (0.1 M I_2 in THF/pyridine/water) for 15 min, washing with acetonitrile and treatment with concentrated ammonia for 2 h (Scheme 2). 5′-Monophosphorothioate T_7 **9b** was obtained by treatment of **8** with a 5% solution of elemental sulfur in CS_2/pyridine/triethylamine[12] for 15 min, washing with CS_2/pyridine then acetonitrile, and treatment with concentrated ammonia for 2 h (Scheme 2). After evaporation, the crude products were analyzed by HPLC C_{18} reverse phase and characterized by MALDI-TOF mass spectrometry (Fig. 2).[13]



Scheme 2. Reagents and conditions: (i) automated synthesis of the T_7 oligonucleotide; (ii) **1** (0.15 M) + BMT (0.3 M) in CH_3CN, 60 s; (iii) DCA (3% in CH_2Cl_2), 120 s then CH_3CN wash; (iv) I_2 (0.1 M), THF/pyridine/water (90:5:5, v/v/v), 15 min; (v) S_8 (5%), CS_2/pyridine/Et_3N (95:95:10, v/v/v), 15 min; (vi) CCl_4, 1,6-diaminohexane or propargylamine, BSA, pyridine, 2 h; (vii) concd ammonia, 2 h. Grey ball: LCAA-CPG.

Amidative oxidation[14] was performed by CCl_4 in the presence of either 1,6-diaminohexane or propargylamine (Scheme 2). The diamine yielded a 5′-amino linker T_7 that could further react with activated derivatives (e.g., FITC or Fluorescein-NHS[15]) while propargylamine afforded a 5′-alkyne T_7, which was further conjugated with a galactosyl azide derivative through 1,3-dipolar cycloaddition.[16] Amidative oxidations were initially

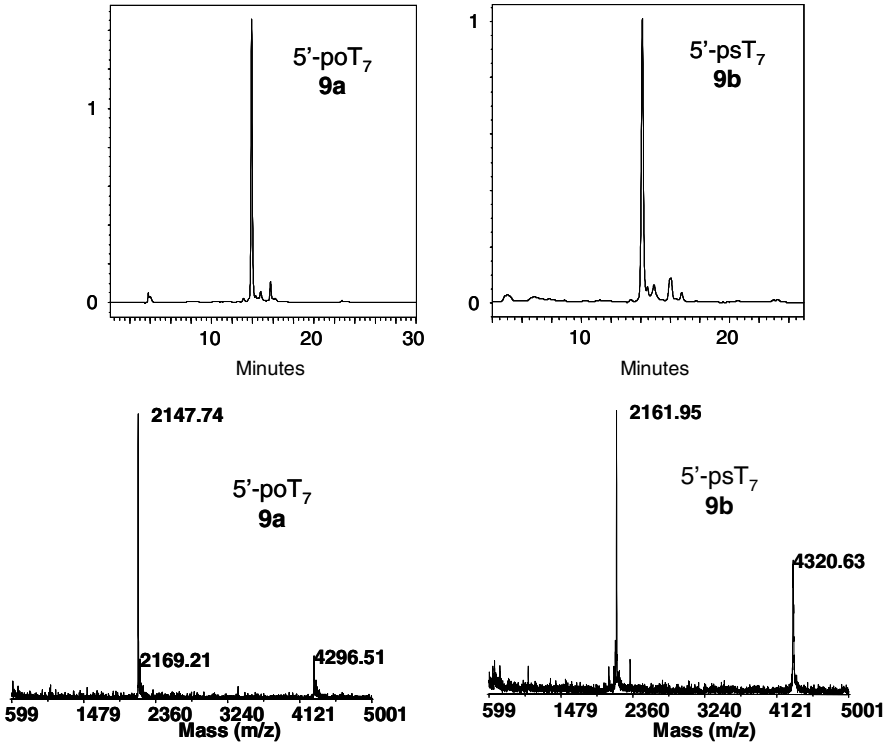


Figure 2. HPLC and MALDI-TOF profiles of the crude reaction mixtures obtained for **9a** (left) and **9b** (right).

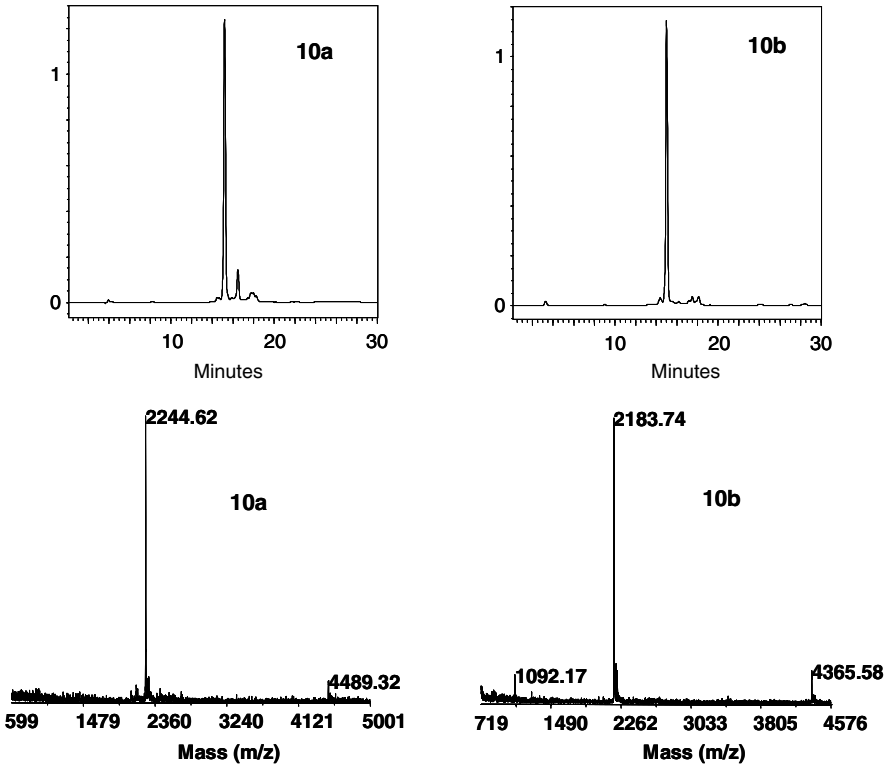


Figure 3. HPLC and MALDI-TOF profiles of the crude reaction mixtures obtained for **10a** (left) and **10b** (right).

carried out in CCl_4/pyridine (1:1, v/v) with 3000 M equiv of amine at room temperature. After deprotection, HPLC analyses showed the formation of the desired 5′-phosphoramidate T_7 but contaminated with ~10% of 5′-*H*-phosphonate monoester T_7 due to a partial removal of the cyanoethyl protecting group from the *H*-phosphonate moiety. It is known that the resulting *H*-phosphonate monoester cannot be further oxidized due to the difficulty to generate the reactive trivalent monoester species.[17] To avoid the formation of this impurity, the amidative oxidation was then performed in the presence of an additional 500 M equiv of *N*,*O*-bis(trimethylsilyl)acetamide (BSA) for the conversion of the *H*-phosphonate monoester traces into the corresponding silylated *H*-phosphonate diester, which could then be oxidized.[18]

HPLC profile of the crude reaction mixtures (Fig. 3) showed a major peak ($t_R = 15.0$ min) of the expected phosphoramidate **10a** and an additional peak at higher retention time ($t_R = 16.5$ min, 4.5%) corresponding to the dimeric T_7 due to the reaction of the free amine of **10a** with another chain of supported oligonucleotide **8**. However, dimeric T_7 impurity is easy to purify and cannot further react with activated derivatives (e.g., FITC or NHS molecules). The phosphoramidate conjugate **10a** was characterized by MALDI-TOF mass spectrometry (Fig. 3).[13]

Amidative oxidation of **8** carried out in the presence of propargylamine with BSA and subsequent deprotection afforded **10b**[13] in high yield and high purity (Fig. 3). The resulting 5′-alkyne T_7 **10b** was then conjugated, in solution, with tetraacetyl-β-D-galactosyl azide derivative **11**[19,20] by a Cu(I)-catalyzed 1,3-dipolar cycloaddition under microwave activation (Scheme 3).[20]

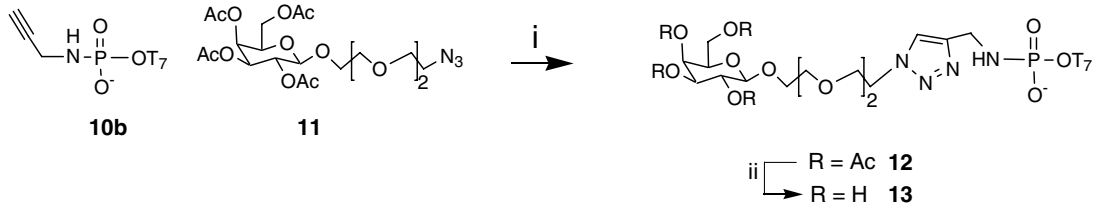
HPLC analysis of the crude reaction mixture showed a complete conversion of the alkyne into the crude triazole conjugate **12**[13] (Fig. 4), which was purified by preparative HPLC. Acetyl protecting groups were then removed affording the pure carbohydrate-triazole-oligonucleotide conjugate **13**, which was characterized by MALDI-TOF mass spectrometry.[13]

In conclusion, the synthesis of 5′-phosphorylated and 5′-thiophosphorylated oligonucleotides and also 5′-conjugated oligonucleotides through a phosphoramidate linkage was performed with high efficiency thanks to the use of phosphoramidite reagent **1**. The main advantage of this reagent is that we only used ancillary reagents of standard phosphoramidite chemistry to form the intermediate *H*-phosphonate diester linkage, which can then be oxidized not only into phosphoro-, thiophosphoro- or phosphoramidate-diesters but also into selenophosphoro-,[21,22] and boranophosphoro-diesters.[23]

2. Experimental

2.1. Synthesis of 2-cyanoethyl *tert*-butyl *N*,*N*-diisopropylphosphoramidite 1

To a solution of 2-cyanoethyl tetraisopropylphosphorodiamidite (1.143 mL, 3.6 mmol) and diisopropylammonium tetrazolide (0.315 g, 1.84 mmol) in anhydrous



Scheme 3. Reagents and conditions: (i) $CuSO_4$, sodium ascorbate, H_2O, MeOH, microwaves (100 W), 30 min, 60 °C; (ii) concd NH_4OH, rt, 2 h.

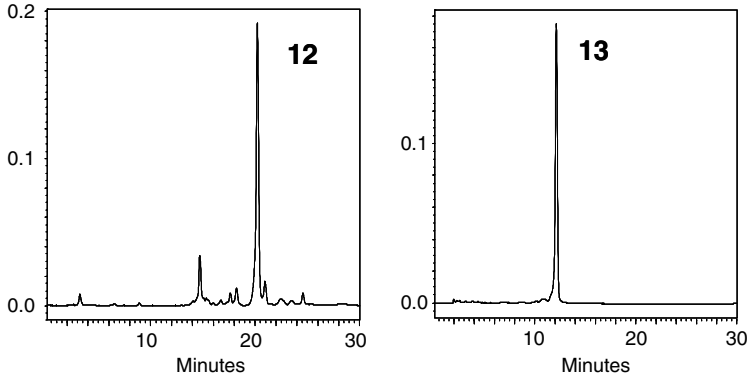


Figure 4. HPLC profiles of the crude carbohydrate-triazole-oligonucleotide conjugate **12** (left) and the pure deprotected carbohydrate-triazole-oligonucleotide conjugate **13** (right).

dichloromethane (15 mL) was added anhydrous *tert*-butanol (0.671 g, 2.2 mmol). The resulting mixture was stirred overnight at room temperature, diluted with ethyl acetate (50 mL) and washed with brine (2×100 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated to dryness. The residue was purified by flash column chromatography (silica gel; gradient 0–20% dichloromethane in cyclohexane with 5% Et_3N) to afford the resulting phosphoramidite **1** (0.642 mg, 65% yield) as a colourless oil. TLC (cyclohexane/CH_2Cl_2/Et_3N; 5:4:1; v/v/v) R_f: 0.70; ^{31}P NMR (200 MHz, CD_3CN): 139.2 ppm; ^1H NMR (200 MHz, CD_3CN): δ ppm: 1.17–1.18 and 1.20–1.23 (2d, 12H, $2CH(CH_3)_2$), 1.36 (s, 9H, $C(CH_3)_3$), 2.61–2.67 (tr, 2H, CH_2CN), 3.47–3.78 (m, 4H, $2CH(CH_3)_2$, OCH_2).

2.2. General procedure for amidative oxidation

Amine (3000 M equiv) and BSA (500 M equiv) in CCl_4/pyridine (900 μL, 1:1, v/v) were applied to a column containing the solid-supported oligonucleotide **8** for 2 h at room temperature with frequent shaking and finally washed with pyridine and then CH_2Cl_2.

2.3. Procedure for 1,3-dipolar cycloaddition

A solution of **10b** (0.16 μmol), $CuSO_4$ (0.4 M equiv) and sodium ascorbate (2.0 M equiv) in water (115 μL), and **11** (0.8 μmol in 100 μL of MeOH) were placed in a sealed tube and introduced into a microwave synthesizer (Initiator™ from Biotage) set at 60 °C, 100 W for 30 min.

Acknowledgement

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