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High-Yield Solution-Phase Synthesis of Di- and Trinucleotide Blocks Assisted by Polymer-Supported Reagents

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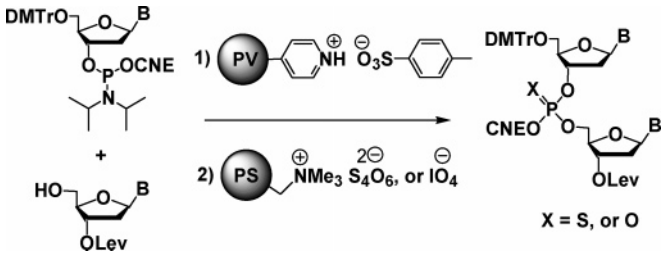
ABSTRACT



A new solution-phase phosphoramidite approach is reported for oligonucleotide synthesis employing recyclable solid-supported reagents. It uses polyvinyl pyridinium tosylate as the activator of a nucleoside-3′-*O*-phosphoramidite in the coupling step with a 5′-OH nucleoside or dinucleotide. The resulting phosphite triester was either sulfurized or oxidized using polystyrene-bound trimethylammonium tetrathionate or periodiate. This method avoids complicated purification steps, as excess reagents are easily removed by filtration.

The synthesis of large amounts of oligonucleotides and their phosphorothiate analogues is crucial for their development and their manufacture as therapeutics.[1] With the help of large-scale automatic DNA synthesizers, synthesis on solid support based on the phosphoramidite approach[2] is the standard way to produce oligonucleotides. Though very efficient, this process is not able to afford very large (multikilogram to ton) quantities in a few single batches.[3] Costly raw materials, solid supports, and technical materials partly explain this shortcoming.[4] Thus, the investigation of alternative synthetic methodologies is a matter of particular importance. A few approaches combining solid phase and solution phase have been tentatively explored.[5−7]

Scale-up of a solution-phase synthesis is more predictable and less expensive than a solid-phase synthesis but requires purification and isolation processes after each reaction step. To overcome these limitations, we have designed a new approach where polymer-bound reagents promote chemical transformations of the oligonucleotide which is in solution.[8] Indeed, solid-supported reagents reduce or even eliminate purification steps since they are simply removed from the

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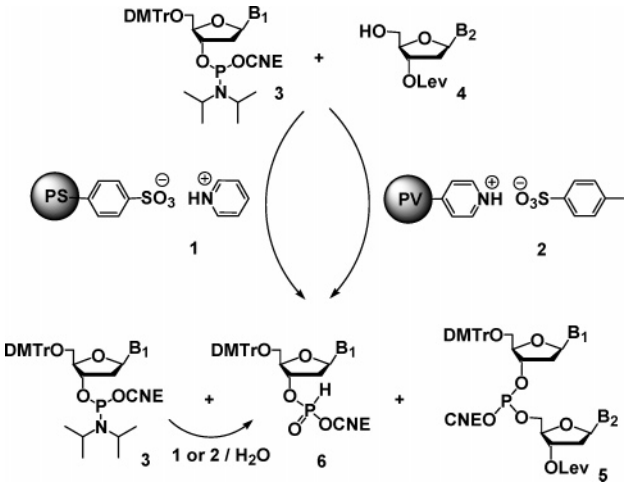
reaction mixtures by filtration.[9] This approach was applied to the synthesis of several di- and trinucleotides with oxo- and thiono-phosphotriester internucleosidic linkages.

Solid-phase synthesis using solid-phase reagents and catalysts, also called inverse solid-phase synthesis[10] or hybrid solid/solution-phase synthesis,[11] has found numerous applications in combinatorial chemistry for high-throughput screening[12−14] and in peptide chemistry.[15] In contrast, only a few examples have been reported in the oligonucleotide field. For instance, poly(3,5-diethylstyrene)-sulfonyl chloride was used as the coupling agent to create dinucleoside phosphodiester bonds according to the out-of-date phosphodiester method.[16,17] Sulfonic acid resins were proposed as alternatives of 80% acetic acid to promote detritylation of 5′-*O*-DMTr phosphodiester base-deprotected oligonucleotides.[18]

Phosphoramidite Coupling. Our examination focused on the phosphoramidite method. Tetrazole is the most extensively employed activator of the reaction between the 5′-hydroxyl function of nucleosides and nucleoside 3′-*O*-phosphoramidites leading to a phosphite triester internucleosidic linkage. Our concern is that it needs to be of high purity, involving dangerous sublimation. Because of its potential to explode,[19] tetrazole is not currently sold as a solid powder. Our inspiration came from previous works on pyridinium acidic salts that were efficiently used as safer and cheaper activators.[19,20] Polystyrene sulfonic acid resins (DOWEX 50W X8 or Amberlist 15) employed as pyridinium salt **1** and polyvinylpyridinium tosylate **2** were tested as phosphoramidite activators. One advantage of polymers **1** and **2** is that they are or could be easily prepared from commercially available resins and regenerated at the end of the reaction by simple washings. Their loading was determined by elemental analysis. The coupling reactions were followed by ^{31}P NMR and by HPLC. It can be noted that polyvinyl pyridinium hydrochloride was used for the preparation of nucleoside 3′-*O*-phosphoramidites starting from 3′-OH nucleosides and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphoramidite.[4] The low yields obtained in these reactions were explained by the low availability of the pyridinium functions on the polymer.

In our case, the coupling between phosphoramidites **3** (δ 148−150 ppm) in excess (1.5 equiv) and 5′-hydroxyl nucleosides **4** assisted by **1** or **2** (10 equiv) led to desired phosphite triesters **5** (δ 140 ppm) with concomitant disappearance of **4**, provided that the shaking of the reaction mixture was efficient (Scheme 1). However, as the reactions performed with resins **1** gave no reproducible results concerning the reaction times from one batch to another, our attention was focused on more reliable polymer **2** (1−2 h of coupling). The phosphite triesters **5** were contaminated with the excess of starting phosphoramidites **3** and with cyanoethyl *H*-phosphonate diesters **6** (δ 8 ppm) resulting from partial hydrolysis of **3** due to the water content of the resin. All our efforts to get rid of this residual water by extensive drying of the polymers (washing, coevaporation, high vacuum) were inefficient. To facilitate the purification (see below), the unreacted **3** was completely converted to **6** by addition of water without damaging **5** before the polymers were removed by filtration. The crude mixtures were directly used for the next step.

Scheme 1. Phosphoramidite Coupling Assisted by Supported Pyridinium Polymers[a]



[a] PV = polyvinyl, PS = polystyrene. B_1, B_2 = T, 2-*N*-isobutyryl G, 4-*N*-benzoyl C, 6-*N*-benzoyl A. DMTr = 4,4′-dimethoxytrityl, Lev = Levulinyl,. CNE = 2-cyanoethyl.

Oxidation and Sulfurization. Dinucleotide phosphotriesters and their thiono-phosphotriester analogues are obtained from phosphite triesters by oxidation and sulfurization, respectively. In solid-phase DNA synthesis, the oxidation could be performed in aqueous medium with conventional I_2/H_2O or in anhydrous medium with *tert*-butylhydroperoxide for example.[21] The periodate anion immobilized on an ion-exchange resin is able to oxidize triphenylphosphane in triphenylphosphane oxide.[22] In contrast to periodate salts, the efficiency of which is limited by their insolubility in nonpolar solvents, the polymer-supported periodate can be used in a diversity of solvents.[23]

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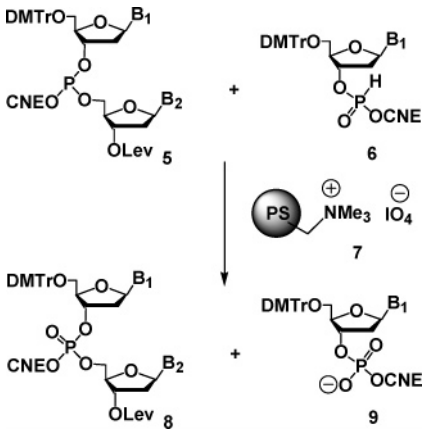
Scheme 2. Oxidation of Phosphite Triester Linkages Using a Polymer-Supported Periodate



The periodate anion was immobilized to the polymeric backbone of the anion-exchange resin Amberlyst A-26 by simple interaction with quaternary ammonium cations.[24] This reagent **7** (2 equiv) oxidizes crude mixtures obtained during the coupling reaction to provide the desired phosphotriester dinucleosides **8** (δ −2 ppm) contaminated with the 3′-phosphodiester nucleosides **9** resulting from the oxidation of the *H*-phosphonate diesters **6** (δ 0 ppm). Completion of the reaction was observed within 2 h. The resin was filtered off and the solvent evaporated.

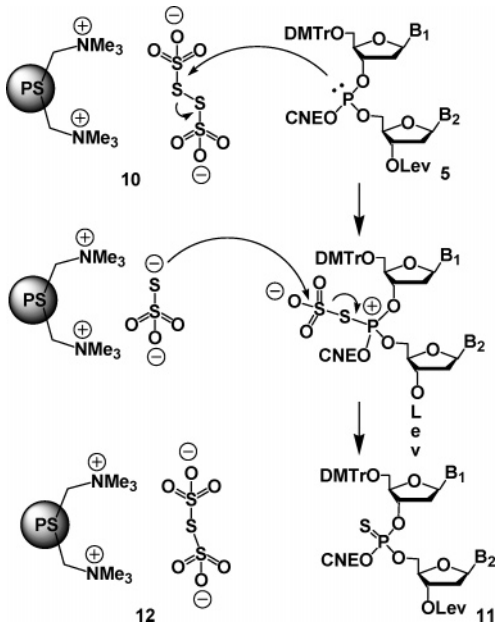
There are many sulfur-transfer reagents that have been developed in oligonucleotide chemistry; the most extensively employed is the expensive 3-*H*-1,2-benzodithiol-3-one 1,1-dioxide[25] known as Beaucage's reagent. However, during sulfurization, it produces a sulfoxide byproduct that acts as an oxidizing reagent, giving rise to the contamination of the desired thiono-phosphotriesters with unwanted phosphotriesters.

Recently, the sulfurizing agent xanthane hydride[26] was bound to a polymer, and the resulting reagent was able to sulfurize nucleoside phosphite triesters[27] but was not applied to oligonucleotides. Although efficient, one drawback of this supported reagent is the time and the cost of its elaboration. Reagents ionically attached to organic supports are cheaper than covalently linked species. Moreover, they are regenerable.

Several years ago, it was shown that inorganic tetrathionate is an effective, while sluggish, sulfurizing agent of phosphite triesters in oligonucleotide chemistry.[28] Because of its ionic nature and consequently its insolubility in nonpolar solvents, its use as a pyridinium salt was recommended in water-containing organic solutions. We took advantage of this ionic character to support the tetrathionate ion on anion exchange polymer resins. The sulfurizing reagents were easily prepared from cross-linked poly 4-vinyl pyridine or attached to the quaternary ammonium cations of the polymeric backbone of Amberlyst A-26 by simple stirring of an aqueous solution of potassium tetrathionate with the resins. The pyridinium tetrathionate was not considered further because the ionic bond between tetrathionate and pyridinum ions was not sufficiently stable. The elemental analysis of polymer-supported reagent **10** indicated a loading of 1.81 mmol of tetrathionate per gram of resin without potassium content, signifying that the dianion was bound to two quaternary ammonium cations on the resin. Generally, 5 molar equiv of **10** was able to sulfurize dinucleotide phosphite triesters **5** (δ 140 ppm) to give rise to thiono-phosphotriesters **11** (δ 68 ppm) in 2−3 h.

At the end of the sulfurization, the resin **12** was filtered off. Elemental analysis of **12** was in agreement with the conversion of 1 equiv of tetrathionate into trithionate still bound to the resin. This is in accord with the mechanism hypothesized by Efimov[28] and proposed in Scheme 3,

Scheme 3. Sulfurization of Phosphite Triester Linkages Using a Polymer-Supported Tetrathionate



involving nucleophilic attack of the phosphorus atom of the phosphite triester **5** on the disulfide bond of the tetrathionate dianion, leading to a phosphonium intermediate, and followed by nucleophilic attack by the resulting thiosulfate anion at the sulfonyl sulfur atom of the phosphonium, giving rise to the thiono-phosphotriester **11** and the supported trithionate resin **12**. It could be pointed out that the *H*-phosphonate diester side product **6** was not sulfurized. When desired (see delevunylation), the sulfurization was followed, after filtration of **12**, with the oxidation of **6** into the polar phosphodiester **9** promoted by the periodate resin **7**. The thiono-

(24) Warning: The preparation is graded as explosive by both the Method A14 of ChemG [Chemicals Act] and the German Law of Explosive Materials [2.SprengV].

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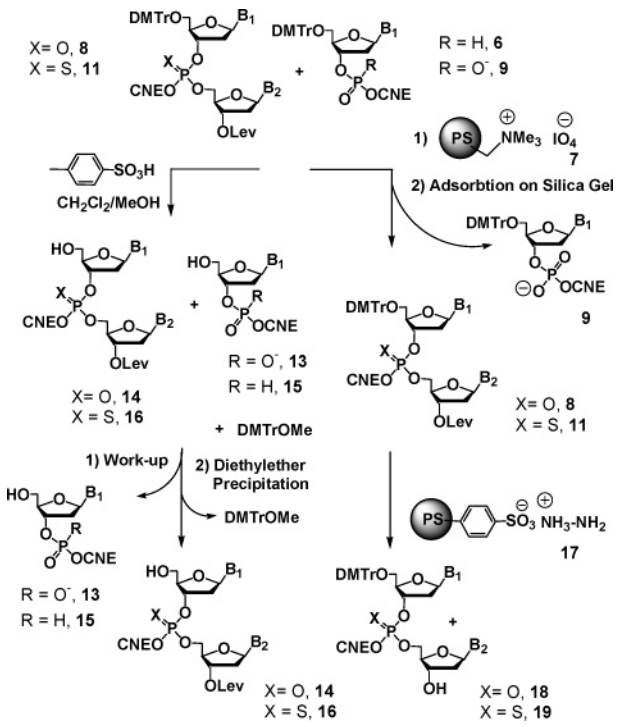
Scheme 4. Detritylation and Delevunylation of Dinucleotide Oxo and Thiono-phosphotriesters



phosphotriester **11** was not affected by this additional oxidative treatment, and **6** was removed from the reation mixture by adsorption during filtration on a small pad of silica gel.

Detritylation and Delevulinylation. As stated above, strong acid cation exchangers were proposed as acidic substitutes for the detritylation of 5′-*O*-DMTr-deprotected oligonucleotides in water.[18] One main advantage is that the trityl cation formed during the acidic treatment was trapped by the resin so that the oligonucleotide was obtained pure after filtration. The use of "in house" sulfonic acid resins in organic solvents (dichloromethane−methanol 97.5:2.5, v/v) was also described for nucleoside chemistry purposes.[29] The reactivity was significantly dependent on the nature of the polymers. It was reported that during the course of the detritylation, depurination was not observed.

All our attempts to replace usual acidic treatments for detritylation with a huge assortment of sulfonic acid resins failed. The DMTr cation was not fully trapped, but the main inconvenience was the degradation of the compounds. In our approach, the crude mixtures obtained from the oxidation or sulfurization were treated with 2% toluenesulfonic acid in CH_2Cl_2−MeOH, 7:3 v/v, at 0 °C for 0.5−1 h. Then, conventional aqueous $NaHCO_3$ workup allowed the removal of side-products 5′-OH-3′-cyanoethyl phosphodiesters **13** from desired 5′-OH phosphotriesters **14** and 5′-OH *H*-phosphonate diester **15** from 5′-OH thiono-phosphotriesters **16**. Finally, the trityl residues were withdrawn by precipitation of the crude dinucleotides in diethyl ether, and compounds **14** and **16** were obtained without chromatography.

Typical processes were run on a 1−10 mmol scale. The dinucleoside 5′-OH-3′-*O*-Lev oxo- and thiono-phosphotriester yields for the overall procedure were 81−96%, and their purity, determined by reverse-phase HPLC, was 90−96%. The identity of the compounds was confirmed by ^{31}P NMR and by MALDI-TOF MS. Several trinucleotides were obtained from 5′-OH dinucleotides **14** and **16** using exacly the same protocole: coupling, hydrolysis, filtration, oxidation or sulfurization, filtration, detritylation, aqueous workup, and precipitation.

To allow elongation from the 3′-side of building blocks, the delevulinylation reaction was performed from fully protected crudes containing oxo- or thiono-phosphotriesters **8** or **11**, respectively. Before doing it, an additional oxidation of the side products **6** into phosphodiester **9** was performed after the sulfurization step (see above). The 3′-*O*-levulinyl protecting group is usually selectively removed with a solution of hydrazine in acetic acid/pyridine.[30] We tested here the ability of a polymer-supported hydrazine **17** to remove it. This resin was prepared from the strong acid cation exchanger Amberlyst 15 by washing it with an aqueous solution of hydrazine. The deprotection of the 3′-OH function of the 5′-*O*-DMTr building blocks to afford compounds **18** and **19** was complete within 3 h.

We have developed a chemical approach to construct short oligonucleotide building blocks using polymer-supported reagents that allow us to prepare them without chromatography purification. These blocks could be either converted into phosphoramidites or *H*-phosphonates for further elongation in solution or on solid support. All the polymer-supported reagents employed in this study result from ionic interactions with the organic support. Consequently, they could be regenerated and reused, reducing their cost and their impact on the environment.

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Supporting Information Available: Selected experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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