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# Allyl Group as a Protecting Group for Internucleotide Phosphate and Thiophosphate Linkages in Oligonucleotide Synthesis: Facile Oxidation and Deprotection Conditions

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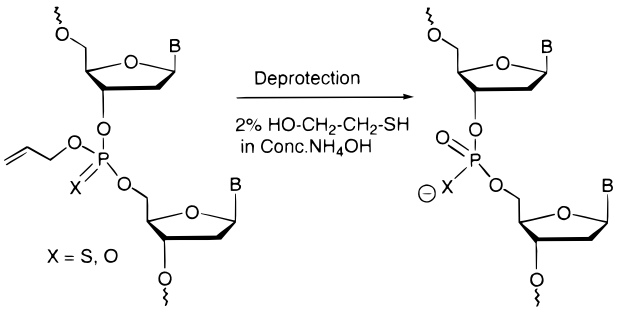
## ABSTRACT




**The allyl group, which serves as a protecting group for an internucleotide bond for both phosphates and phosphorothioates, can be easily removed by good nucleophiles under weakly basic or neutral conditions. For a practical synthesis on solid support, camphorsulfonyloxaziridine was used as the oxidizing agent for synthesizing DNA, while the Beaucage reagent was used for preparing phosphorothioate oligomers. Both types of oligonucleotides were easily deprotected by concentrated ammonium hydroxide containing 2% mercaptoethanol.**

The common procedure for the synthesis of oligonucleotides is the phosphoramidite technique[1,2] in which a nucleoside or an oligonucleotide having a free 5′-hydroxyl group is reacted with a protected $\beta$-cyanoethyl phosphoramidite monomer in the presence of a weak acid (tetrazole) to form a phosphite-linked structure. Oxidation or sulfurization of the phosphite linkage, followed by deprotection of the cyanoethyl group by base-catalyzed (concentrated ammonium hydroxide) $\beta$-elimination, ultimately yields the desired phosphodiester (P═O) or phosphorothioate (P═S) linkage.

In the course of developing chiral adjuvants for the solid support synthesis of site-specific chiral phosphorothioate oligonucleotides having high purity and a diastereomeric excess,[3] an alternative protecting group relatively stable to bases (such as DBU, amines, or fluorides) and preferably removable under neutral conditions was required. This became crucial in view of the DBU-induced rearrangement of $\beta$-cyanoethylphosphite triesters to the corresponding cyanoethyl phosphonate on a solid-phase synthesis of phosphite triesters.[4] Allyl, substituted-allyl, or *tert*-butyl groups seemed to be good candidates for our synthesis. As will be

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(1) Sinha, N. D.; Biernet, J.; Koster, H. *Tetrahedron Lett.* **1983**, *24*, 5843.
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(3) (a) Wang, J.; Just, G. *Tetrahedron Lett.* **1997**, *38*, 3797. (b) Jin, Y.; Just, G. *J. Org. Chem.* **1946**, *11*, 3647 and references cited.
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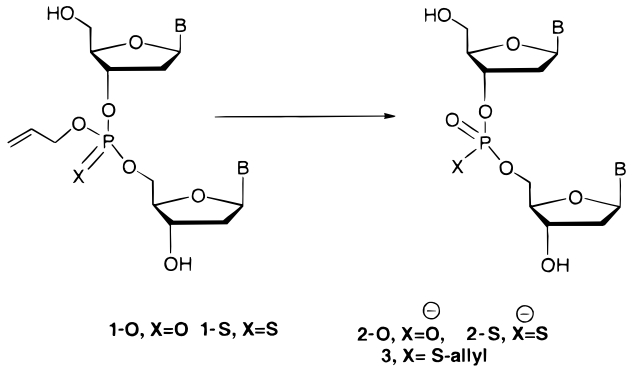
shown, the allyl group adequately fulfilled these requirements. Furthermore, allyl alcohol is much cheaper than the currently used 3-hydroxypropionitrile, the precursor of $\beta$-cyanoethyl chemistry.

Removal of the allyl group with palladium complexes has been well established due to the pioneering work of Hayakawa.[5,6] However, this method is cumbersome for a routine solid support synthesis of phosphorothioate oligonucleotides, particularly when making therapeutic compounds on a large scale. Furthermore, during deprotection, the palladium catalyst is susceptible to poisoning, especially with P═S oligonucleotides, resulting in loss of catalytic efficiency. Finally, traces of Pd(0) remain in the product after deprotection and purification. This can be potentially lethal when the product is used therapeutically. Deprotection of a T−T dimer, (3′-*O*-(allyloxycarbonyl)thymidylyl(3′-5′)thymidine), having an allyl protecting group, has also been performed using electrochemical means.[7] We reasoned that its removal by a nucleophilic displacement of an $S_N1$ or $S_N2$ type should fulfill our requirements. While our work was in progress, a paper using ammonia under rather vigorous conditions (elevated temperature) to remove the allyl group was published.[8] We report herein a simplified protocol for using allyl phosphoramidites in a routine synthesis of phosphodiester and phosphorothioate oligonucleotides.

In our initial experiments, we used both phosphoramidite and slightly modified phosphite triester[9] approaches to synthesize TT dinucleoside phosphate and phosphorothioate. It became quickly apparent that substituted allyl or *tert*-butyl groups were not suitable because of the instability of their phosphoramidites or triesters. However, the allyl phosphate (**1-O**) and the phosphorothioate (**1-S**) (Scheme 1) were easily prepared.

**Scheme 1**




We initially used concentrated ammonia or 40% aqueous dimethylamine as the deprotecting reagent. $^{31}$P NMR[10] indicated that the original two peaks (−1.22 ppm, −1.37 ppm) of allyl phosphate **1-O** became a single peak (−1.68 ppm) after treatment with ammonia for 2 h at 55 °C or with dimethylamine for ∼30 min at room temperature. In the case of the phosphorothioate **1-S**, the two peaks characteristic of the triesters at 68.49 and 68.37 ppm moved to 55.95 and 55.58 ppm. For deprotection with the same reagents, the reaction time was approximately the same as that for the phosphate **1-O**. The deprotection yields were quantitative, as indicated by $^{31}$P NMR spectra.

We next studied less basic nucleophiles such as thiophenolate and iodide. When thiophenolate[11] was used as the deprotecting reagent, the removal of the allyl group was completed at room temperature within 10 min. Deesterification of phosphate triester **1-O** could also be achieved with sodium iodide. When **1-O** was treated with an aqueous solution of sodium iodide at 55 °C for 20 h, the phosphate **2-O** and the starting material **1-O** were obtained in a ratio of 15:1. However, when phosphorothioate **1-S** was treated with a sodium iodide solution under the same reaction conditions, $^{31}$P NMR showed that the two peaks around 68 ppm were replaced by two sets of peaks around 55 and 31 ppm in a ratio of 3:1. These two sets are characteristic of the expected phosphorothioate **2-S** and of the *S*-allyl thiophosphate **3**. The identity of the latter was confirmed by reaction of **2-S** with allyl iodide. Similar results were obtained when acetone was used as a solvent.

To suppress side reactions, we used thiourea to trap the allyl iodide. With 50 equiv of thiourea present in the system, **2-O** was the only product formed in the deprotection of the phosphate. In the case of the phosphorothioate, the ratio of **2-S** to *S*-allyl thiophosphate **3** improved to 66:1.

We introduced two simple modifications of the existing oligonucleotide synthetic procedures in order to synthesize oligonucleotides using P-*O*-allyl phosphoramidites alone or in combination with $\beta$-cyanoethyl phosphoramidite precursors at selected sites. The combined use of different amidites demonstrates the simplicity and versatility of the present approach. These amidites were used in automated DNA synthesizers to synthesize oligonucleotides of the desired length and sequence. Table 1 shows selected oligonucleotides that have been synthesized.[12]

The first modification of oligonucleotide synthesis when using allyl phophoramidites relates to oxidation conditions. When an allyl protecting group is used in phosphodiester (P═O) oligonucleotide synthesis, it becomes necessary to use nonstandard oxidation conditions. Traditionally, 0.1 M iodine in water/pyridine/THF (2/20/80 v/v/v) has been used in automated DNA synthesizers.[13] Since the allyl group is reactive to iodine/water, a clean oxidation reaction does not

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(10) $^{31}$P NMR spectra were referenced externally with an 85% solution of phosphoric acid as 0 ppm.

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**Table 1.** Oligonucleotides Synthesized Using P-*O*-Allyl and *β*-Cyanoethyl Phosphoramidites

| | sequence (5′ to 3′) | backbone | mass obsd | mass calcd | HPLC $t_R$[b] |
|---|---|---|---|---|---|
| 1 | TTT TTT TTT TTT | P═S | 3765.828 | 3764.53 | 27.01 |
| 2 | TTT TTT TTT TTT | P═O | 3589.828 | 3588.11 | 19.51 |
| 3 | TGC ATC CCC CAGGCC ACC AT | P═S | 6288.688 | 6286.58 | 22.65 |
| 4 | TGC ATC CCC CAGGCC ACC AT | P═O | 5984.688 | 5980.88 | 17.98 |
| 5 | GAA CT | P═O | 742.589 | 742.60 | 19.54 |
| 6 | GAC CT | P═O | 730.588 | 730.60 | 18.73 |
| 7 | GAG CT | P═O | 750.590 | 750.60 | 17.79 |
| 8 | GAA CT | P═S | 774.549 | 774.60 | 24.65 |
| 9 | GAC CT | P═S | 762.548 | 762.60 | 24.81 |
| 10 | GAG CT | P═S | 782.550 | 782.70 | 24.47 |
| 11 | GCC CAA GCT GGC ATC CGT CA | P═O | 6060.990 | 6062.13 | 22.48 |
| 12 | GCC CAA GCT GGC ATC CGT CA[c] | P═S | 6364.170 | 6365.73 | 26.95 |
| 13 | GTA CT | P═O | 738.088 | 738.00 | 17.27 |

[a] All underlined nucleotides (N) were derived from P-*O*-allyl phosphoramidite. [b] Conditions: Waters 600E with 991 detector; Waters C4 column (3.9 × 300 mm). Solvent A: 50 mM triethylammonium acetate, pH 7.0. Solvent B: acetonitrile; 1.5 mL/min flow rate. Gradient: 5% B for first 5 min with linear increase in B to 60% during the next 55 min. [c] Antisense inhibitor of human ICAM-1(ISIS-2302).

occur. As a result, modified nonaqueous oxidation procedures have been developed, which employ *t*-BuOOH in toluene solvent.[14] However, nonaqueous *t*-BuOOH is considered to be an explosive; consequently, it is no longer commercially available.

As an alternative to *t*-BuOOH-mediated oxidation, we used the oxaziridine (1*S*)-(+)-(10-camphorsulfonyloxaziridine)[15] (Figure 1) as a source of oxygen. A 0.5 M solution of the

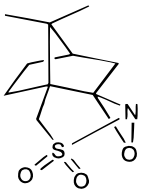
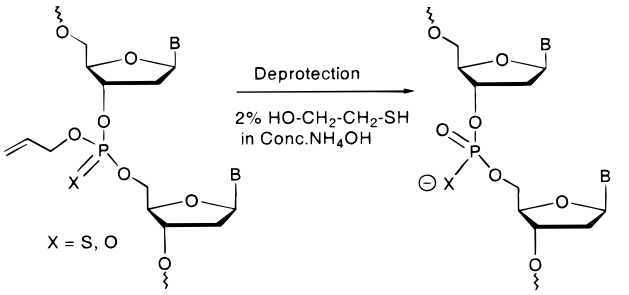


**Figure 1.** (1*S*)-(+)-(10-Camphorsulfonyloxaziridine).

oxaziridine in $CH_3CN$ gave clean oxidation without oxidizing the olefinic bond of the allyl protecting group. Phosphite to phosphate oxidation by oxaziridines, using allyl phosphoramidites, has not been reported previously.

The second synthetic modification was the deprotection conditions. For synthesizing both P═O and P═S oligomers, the allyl protecting group was cleaved in a deblocking step, using an aqueous solution of concentrated ammonium hydroxide having 2% mercaptoethanol. This cocktail deprotects the allyl group via a simple alkylation of the thiol by a simple substitution (perhaps via an $S_N2'$)[16] process (Scheme 2). While the mercaptan is presumably responsible for

**Scheme 2**




removal of the allyl group, the concentrated ammonium hydroxide deprotects the standard exocyclic amine protecting

(12) **Typical Experimental Procedure:** The allyl phosphoramidite monomers were synthesized according to reported procedures.[5] Oligonucleotides were synthesized on a Millipore Expedite Nucleic Acid Synthesis System. Coupling efficiency was found to be greater than 99% by monitoring the trityl ion released upon deblocking of 5′-OH groups. Allyl or *β*-cyanoethyl amidites were placed in dried vials and dissolved in acetonitrile to a concentration of 0.1 M. Oxidation was performed using (1*S*)-(+)-(10-camphorsulfonyl)oxaziridine (CSO, 1.0 g/8.72 mL of dry acetonitrile, 375 *μ*L) with a 3 min wait step. During phosphorothioate synthesis, the oxidation was carried out with 3*H*-1,2-benzodithiole-3-one 1,1-dioxide (225 *μ*L, Beaucage reagent, 03.4 g/200 mL of acetonitrile) with one wait step. Following the synthesis of oligonucleotides, 1 *μ*mol columns were deprotected using concentrated ammonium hydroxide (2 mL, 30%, aqueous) containing 2-mercaptoethanol (2% v/v) and heated at 55 °C for approximately 16 h. After the deprotection step, each oligonucleotide was filtered using a Gelman nylon acrodisc syringe filter (0.45 *μ*M). Each sample is then evaporated in a speed vac to remove excess concentrated ammonium hydroxide. The crude trityl-on oligomer is purified by reverse phase HPLC.

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(16) The other possibilities such as a direct $S_N2$ or $S_N1$ displacement cannot be rigorously excluded without labeling the allylic moiety. The phosphodiester would be a good leaving group for either process.

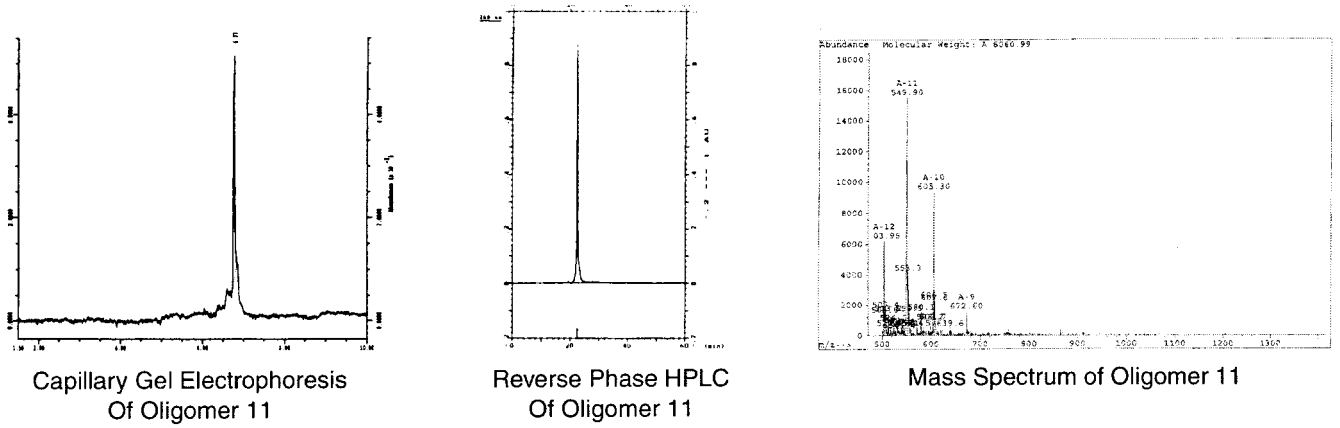


**Figure 2.** Characterization of oligonucleotide **11** (20mer, P═O) synthesized using P-*O*-allyl phosphoramidites with the modified oxidation and deprotection protocols.

groups. The presence of mercaptoethanol may quench the rearrangement of *O*-allyl to *S*-allyl observed during the TT dimer synthesis with amine-mediated deprotection.

Examination of high-resolution mass spectra, capillary gel electrophoresis, and reverse phase HPLC traces of the oligomers synthesized reveals the high purity of the compounds (Figure 2 and Table 1). The yields were comparable to those derived from $\beta$-cyanoethyl chemistry. In conclusion, we have demonstrated that the removal of the allyl protecting group in the internucleotide linkage of nucleotides can be achieved by a thiol nucleophile. The protocol reported here can be used for routine solid-phase synthesis of oligonucleotide phosphodiesters and phosphorothioates.

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