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Synthesis of Oligodeoxynucleotides Containing Electrophilic Groups

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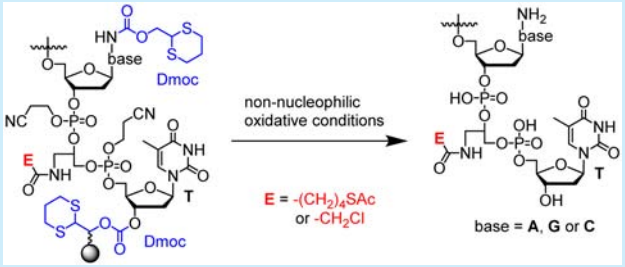
Supporting Information

ABSTRACT: By use of 1,3-dithian-2-yl-methoxycarbonyl (Dmoc) as a protecting group and linker for oligodeoxynucleotide (ODN) synthesis, deprotection and cleavage are achieved under non-nucleophilic oxidative conditions. The nucleophile-sensitive thioester and α-chloroacetyl groups are conveniently incorporated into ODN sequences. The technology could be universally useful for electrophilic ODN synthesis.



In traditional solid-phase oligodeoxynucleotide (ODN) synthesis, the amino groups on the nucleobases are protected with acyl groups that have to be removed with strongly nucleophilic reagents such as ammonium hydroxide. The widely used succinyl ester linkage is also cleaved under these conditions. For this reason, the methods are not suitable for the synthesis of ODN analogues that contain electrophilic functionalities. However, such analogues have found or are predicted to have wide applications in areas such as covalent cross-linking with messenger ribonucleic acid (mRNA) for antisense drug development,[1] analysis of nucleic acid and protein interactions by detecting cross-linked fragments using mass spectrometry,[2] and the synthesis of model compounds of sensitive nucleic acid intermediates in cells for deoxyribonucleic acid (DNA) damage and DNA methylation and demethylation studies.[3]

Current strategies for electrophilic ODN synthesis include two types. One type uses protecting groups and linkers cleavable under less basic or nearly neutral conditions. However, functions in organic chemistry suitable for the need are limited. Those used in the literature include the more base-labile phenoxyacetyl-based groups and linker,[4] the palladium-removable allyl groups,[3b,5] and the photolabile *o*-nitrobenzyl linker.[3b,6] ODN synthesis methods using these functionalities for protecting and linking still have serious drawbacks. For example, the phenoxyacetyl groups and linkers are usually cleaved with dilute K_2CO_3 in methanol or aqueous ammonium hydroxide.[4] These conditions are still strongly nucleophilic. Palladium is expensive and difficult to remove. UV irradiation can damage ODN. The second type uses traditional methods to make an ODN precursor, which is stable under nucleophilic conditions. After cleavage and deprotection, the electrophilic functionality is attached to or uncovered from the precursor.[1c−e,g−j] These methods are inconvenient, have to be developed case by case, and are not always feasible. Besides the above two strategies, enzymatic reactions have also been used to access electrophilic ODNs.[1a] Drawbacks include narrow applications and high cost. Efforts were also made to search for conditions for ODN synthesis without nucleobase protection.[7] An ideal linker cleavable under non-nucleophilic conditions remains to be developed, and the challenge of achieving complete *O*-phosphitylation over *N*-phosphitylation in the coupling step may be nontrivial.

In this paper, we report our studies on use of the 1,3-dithian-2-ylmethoxycarbonyl (Dmoc) based protecting group and linker for ODN synthesis. Previously, this and the similar 2-(phenylthio)ethyl group had been studied for peptide synthesis and thymidine protection, but they have not been tested for ODN synthesis.[8] We predicted that the Dmoc function would be stable under all ODN synthesis conditions. However, upon oxidation of the sulfides to sulfoxides or sulfones after synthesis, due to the drastically increased acidity of H-2, they could be cleaved under non-nucleophilic conditions. Using that technology, we successfully synthesized five ODNs including one containing a thioester and another containing an α-chloroacetyl function. The cleavage and deprotection were achieved in three steps under non-nucleophilic conditions. These electrophilic groups would not survive the nucleophilic conditions such as NH_4OH and K_2CO_3/MeOH, while incorporating them into ODNs is desirable due to their potential applications such as antisense drug development, sequence-specific DNA alkylation, and DNA−protein interaction studies.[1g,5a,9] We expect that the new method would be suitable for the synthesis of other electrophilic ODNs as well and, therefore, have a high impact in several research areas.

To carry out an electrophilic ODN synthesis using the Dmoc function for protecting and linking, a Dmoc linker such as that in dT-Dmoc-CPG (controlled pore glass) **1** and the phosphoramidite monomers Dmoc-dC-amidite **2**, Dmoc-dA-amidite **3**, and Dmoc-dG-amidite **4** are required (Figure 1). The corresponding dT monomer is not required because thymidine does not have an amino group, and a commercial dT amidite (**5**) can be used. The preparation of CPG **1** is shown in Scheme 1. The anion generated by treating 1,3-dithiane with *t*BuLi was

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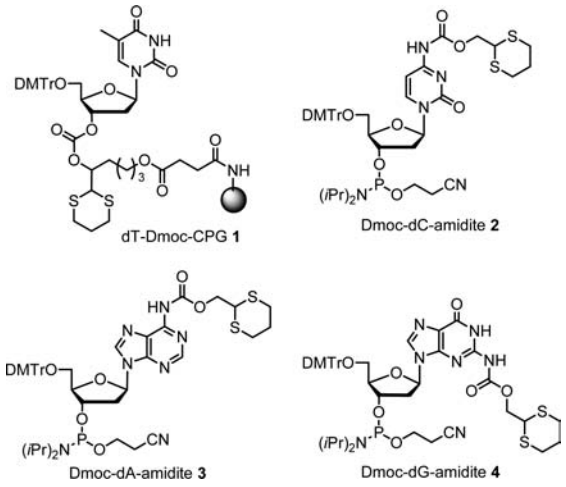
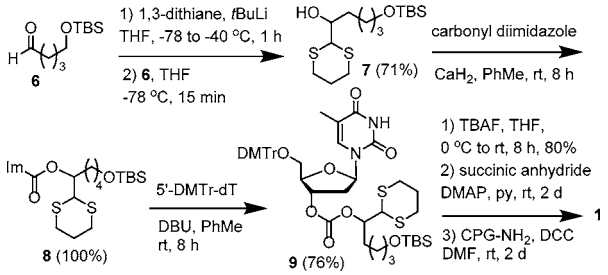


Figure 1. CPG with dT-Dmoc linker and Dmoc amidites.

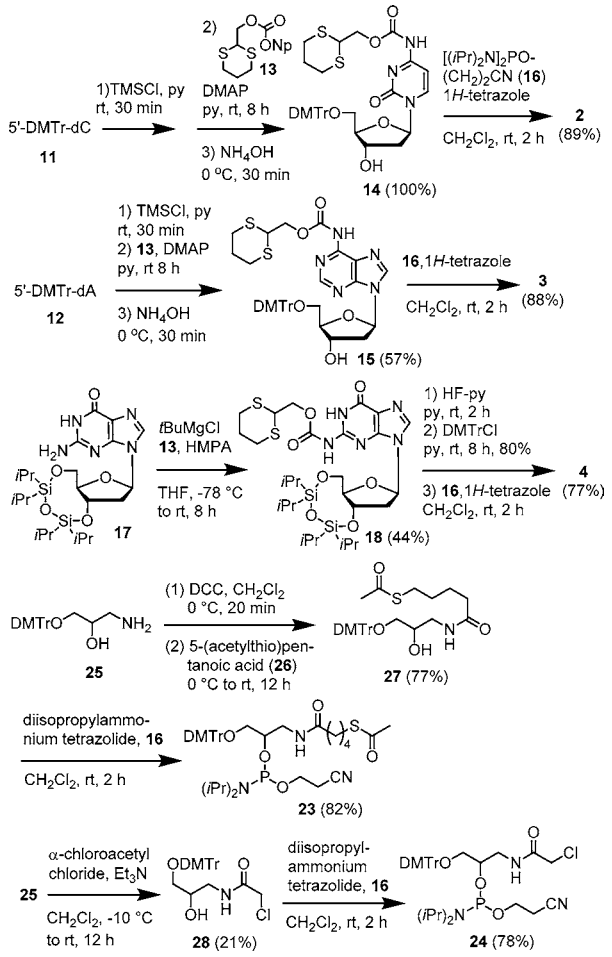
Scheme 1. Synthesis of dT-Dmoc-CPG



reacted with aldehyde **6** to give **7**. Compound **7** was converted to **8** by reacting it with 1,1′-carbonyldiimidazole in the presence of calcium hydride. Reaction of **8** with 5′-DMTr-dT using DBU as the base gave **9**. Removal of the TBS group in **9** with TBAF afforded **10** (not shown in Scheme 1). Attaching **10** to CPG to give **1** was achieved by reacting **10** with succinic anhydride followed by incubation with amino CPG in the presence of DCC. Because we installed the 1,3-dithiane moiety at the side of the linkage instead of in the linkage, the construction of **1** is quite simple.

The synthesis of Dmoc amidite **2**–**4** is shown in Scheme 2. The amino groups of 5′-DMTr-dC **11** and dA **12** were conveniently protected using (1,3-dithian-2-yl)methyl 4-nitrophenyl carbonate (**13**)[8c] by first protecting the 3′-hydroxyl group with TMSCl temporarily followed by stirring the reactants at room temperature with DMAP as the catalyst. The products **14** and **15** were obtained in 100% and 57% yields, respectively. Phosphitylation of **14** and **15** using 2-cyano-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (**16**) gave the amidite monomers Dmoc-dC-amidite **2** and Dmoc-dA-amidite **3**, respectively, in excellent yields. However, when the same acylation method was used to synthesize Dmoc-dG, no desired product could be isolated probably due to the lower basicity of the amino group of dG.[10] We tried several other conditions and finally settled with the following procedure. The *O*-protected dG **17**[11] was treated with excess *t*BuMgCl and **13** to give **18** in 44% yield. The silyl protecting group was then removed by HF–pyridine,[12] and without purification the product was reacted with DMTr-Cl to give **19** (not shown in Scheme 2) in 80% yield after flash chromatography. Compound **19** was phosphitylated using **16** to give Dmoc-dG-amidite **4** in 77% yield.

Scheme 2. Synthesis of Amidite Monomers



With dT-Dmoc-CPG **1** and amidite monomers **2**–**4** in hand, before synthesizing electrophilic ODNs, we tested the technology by synthesizing three unmodified ODNs. They were 20-mers 5′ HO-TCA TTG CTG CTT ATA CCT CT-OH 3′ (**20**), 5′ HO-TCA TTG CTG CTT AGA CCG CT-OH 3′ (**21**), and 5′ HO-TTA GTA GGA CCT ACA CCT GT-OH 3′ (**22**). The conditions were the same as those in traditional ODN synthesis using the phosphoramidite chemistry. Concentration of the amidites was 0.1 M. At the end of synthesis, the DMTr group was removed. According to the trityl assay, the coupling yields were not negatively affected by the Dmoc linker and protecting groups. For deprotection and cleavage, the 2-cyanoethyl groups were first removed by treating with DBU briefly (Scheme 3). The sulfides in Dmoc were then oxidized with $NaIO_4$ at pH 2 in 3 h.[13] The acidity of H-2 in Dmoc is now drastically increased. However, due to the acidic conditions, β-elimination did not occur at this stage as indicated by HPLC analysis of the supernatant. After removal of the supernatant, residue $NaIO_4$ was washed away with water at pH 2. Final cleavage and deprotection of ODN were then induced with an aniline solution at pH 8 (Scheme 3). The crude ODNs **20**–**22** were purified with RP HPLC. The profiles of crude and purified **20** are shown in Figure 2. Those of **21**–**22** are shown in the Supporting Information. All of the ODNs were analyzed with MALDI-TOF MS, which gave correct molecular masses (Supporting Information).

We next decided to incorporate the nucleophile-sensitive thioester and α-chloroacetyl functions into ODNs. The thioester

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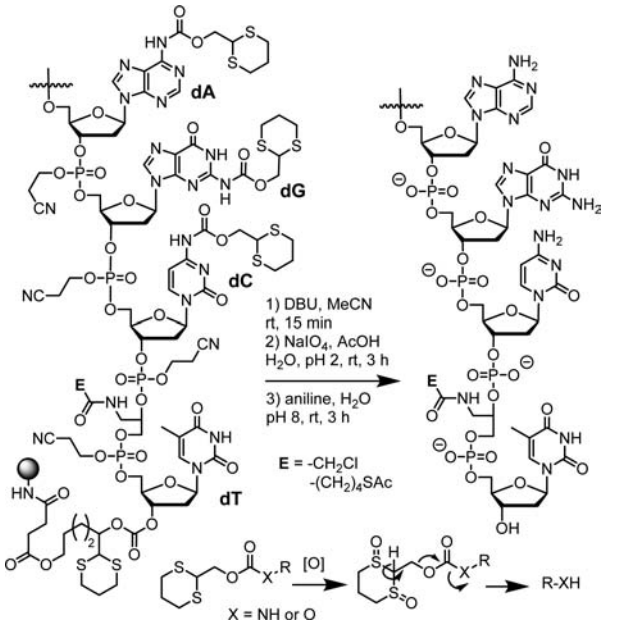
Scheme 3. ODN Deprotection and Cleavage



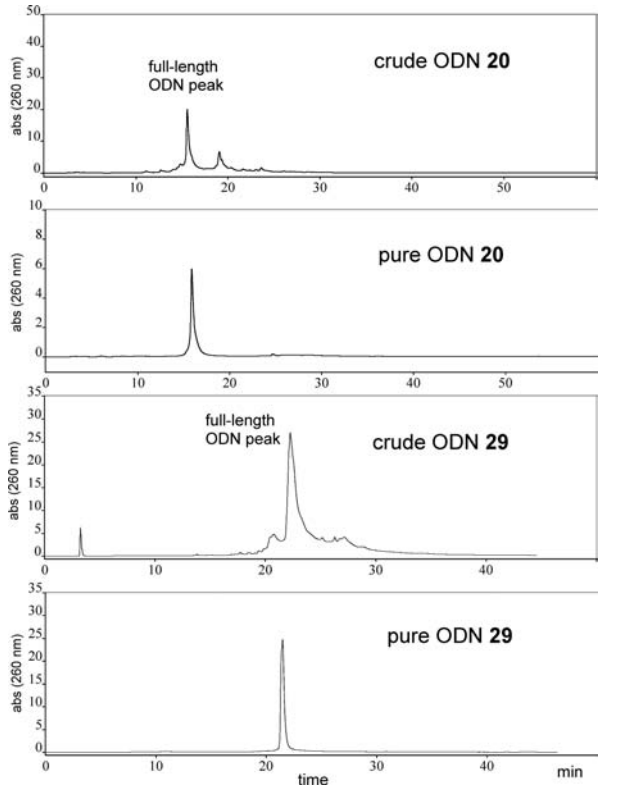


Figure 2. RP HPLC profiles of ODNs **20** and **29**. Profiles were generated by detecting absorbance at 260 nm using the linear gradient eluting system: buffer B (0−45%) in buffer A over 60 min at 0.5 mL/min flow rate. Buffer A: 0.1 M triethylammonium acetate, 5% acetonitrile. Buffer B: 90% acetonitrile. The slight discrepancy between retention times of crude and pure **29** is a result of inaccurate concentration of buffers.

function was used as a phosphate masking group in ODN prodrugs. For the application, the thioester had to be kept intact during ODN synthesis, cleavage, and deprotection.[5a,9] ODNs containing an α-chloroacetyl function could find applications in sequence-specific alkylation and cleavage of DNA and other areas.[1g−j] We chose to incorporate the electrophilic groups into the middle of the sequences because it is more challenging than attaching it to the 5′-end. The required amidites **23** and **24**, which contained the thioester and α-chloroacetyl, respectively, were prepared according to Scheme 2. Compound **25**[14] was coupled with 5-(acetylthio)pentanoic acid (**26**)[15] to give **27**, which was phosphitylated to give amidite **23**. Amidite **24** was also prepared from **25**. Acylation of **25** with α-chloroacetyl chloride gave **28**, which was phosphitylated to give **24**. Using the Dmoc-CPG **1** and amidites **2**−**4**, we successfully incorporated **23** and **24** into ODNs 5′ HO-TCA TTG CTG CTT A-**X**-A CCT CT−OH 3′ (**29**) and 5′ HO-TCA TTG CTG CTT A-**Y**-A CCT CT−OH 3′ (**30**), where **X** and **Y** are the thioester and α-chloroacetyl units introduced with **23** and **24**, respectively. The sequences were derived from **20** by replacing a T with **X** or **Y**. The conditions for ODN synthesis and cleavage and deprotection were the same as described above. No modification of the procedure was needed except that a simpler precipitation method instead of size-exclusion chromatography was used to separate ODN from small molecules after ODN cleavage and deprotection. The ODNs were purified with RP HPLC. MALDI-TOF MS analyses gave correct molecular masses (Supporting Information). The RP HPLC profiles of crude and purified **29** are in Figure 2. Those of **30** are shown in the Supporting Information.

According to trityl assays, the coupling yields using the Dmoc-amidites were excellent. To have a direct comparison of these amidites with commercial ones, we synthesized ODN **20** two times under identical conditions except that, in one time, **1**−**4** and commercial dT amidite were used and, in another, **1** and commercial dA, dC, dG and dT amidites were used (Supporting Information). In both syntheses, the CPG **1** were used and were from the same batch with identical amounts. A portion of both ODNs on the same weight of CPG were cleaved and deprotected with concentrated NH_4OH under identical conditions (the Dmoc group and linker can also be cleaved with NH_4OH). RP HPLC analysis gave similar peak areas of full-length ODN. The OD_{260} values of purified ODNs were also very close. These experiments further confirmed that the Dmoc protecting groups did not have a negative effect on ODN synthesis efficiency. Cleavage and deprotection of the ODN synthesized with Dmoc amidites were also carried out under the oxidative conditions using the same amount of CPG as the above two experiments. However, the yield of ODN obtained was lower. Both HPLC peak area and OD_{260} values were about one-fourth of those for the experiments involving NH_4OH cleavage and deprotection. The lower yield may be caused by the loss of ODN during removing aniline with Amicon centrifugal filter units.

The three-step procedure and the order of the steps for cleavage and deprotection used in new technology are important. The removal of the 2-cyanoethyl groups increases the hydrophilicity of ODNs, which is beneficial for oxidation in water in the next step. Performing oxidation under acidic conditions retains ODN on CPG, which allows easy removal of $NaIO_4$. The acidic conditions in the oxidation step did not cause any noticeable depurination because treating unmodified ODNs from the technology with concentrated NH_4OH did not give peaks of shorter ODNs in HPLC profiles. The excess aniline introduced in the last step is easy to remove due to its small size and high solubility in organic solvents. We achieved this by passing it through a size-exclusion column. We also tested a precipitation method involving adding *n*BuOH to aqueous ODN solutions.

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ODN was precipitated, and aniline remained in the supernatant, which was removed with a pipet.[16] Finally, ultrafiltration using an Amicon centrifugal filter unit also proved effective.

The Dmoc-based linker and protecting groups are well suited for electrophilic ODN synthesis. Before oxidation, H-2 in the function is not acidic in normal terms of organic chemistry (pK_a ~ 31), which enables Dmoc to be stable during ODN synthesis. After oxidation, the pK_a of H-2 is lowered to ~12. Compared to the Fmoc protecting group, in which case the pK_a of H-9 is ~22 and the group can be removed by the weak base piperidine (pK_a of conjugate acid ~11), the Dmoc linker and protecting group were predicted to be readily cleavable under nearly neutral and non-nucleophilic conditions. Our results have shown that this is indeed the case. Compared to the ODN synthesis methods that used the allyl and *o*-nitrobenzyl functionalities, the Dmoc method does not require any expensive and difficult-to-remove transition metal and DNA-damageable UV light for deprotection and cleavage. Instead, the readily available and easily removable $NaIO_4$ and aniline can accomplish the task. We have successfully shown that the technology is suitable for the synthesis of ODNs containing thioester and α-chloroacetyl amide, which we confirmed to be incompatible with the widely known mild deprotection conditions using K_2CO_3 in MeOH (Supporting Information). Besides these two electrophilic groups, other groups such as aldehydes,[13] esters, activated esters, aziridines,[1g] epoxides, alkyl halides, vinyl purines,[17] methides,[18] and maleimides could be incorporated into ODNs as well.

In conclusion, we have developed a new ODN synthesis method using the Dmoc function as the linker and protecting group. Using the method, deprotection and cleavage are carried out in three steps under non-nucleophilic conditions, and therefore, it is useful for the synthesis of electrophilic ODNs. Five sequences were successfully synthesized using the strategy. One of them contained a nucleophile-sensitive thioester, and another contained a sensitive α-chloroacetyl. The coupling yields were excellent. The products were purified with RP HPLC. MALDI-TOF MS analysis indicated that the ODNs had the correct structure. We expect that the new technology will find applications in various research fields that need electrophilic ODNs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01878.

Experimental details, ODN cleavage and deprotection protocol, NMR and MS spectra, and HPLC profiles (PDF)

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Author Contributions

†X.L., J.C., and S.S. contributed equally.

Notes

The authors declare no competing financial interest.

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