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Process for the Capture and Reuse of the 4,4′-Dimethoxytriphenylmethyl Group during Manufacturing of Oligonucleotides

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Abstract:

In a standard amidite coupling-based automated oligonucleotide synthesis campaign, the 4,4′-dimethoxytriphenylmethyl (DMT) protecting group is discarded as waste along with large amounts of dichloromethane. Herein, we report a straightforward and simple process for complete capture and recovery of the DMT group and dichloromethane, rendering the oligonucleotide manufacturing process safer and more economical.

Introduction

The potential therapeutic use of oligonucleotides represents a new paradigm for modern drug discovery. Over the past decade, oligonucleotide-based antisense,[1] triplex,[2] ribozyme,[3] and aptamer[4] techniques have emerged as powerful tools in the discovery of more specific and effective oligonucleotide drugs. Among these technologies, the antisense approach leads the way, with a dozen or more oligonucleotides currently undergoing human clinical trials for the treatment of viral infections, cancers, and inflammatory disorders.[5] Recent advances in automated synthesis on solid support and commercialization of synthetic nucleic acid building blocks now allow the generation and screening of an unprecedented number of synthetic oligonucleotides. Manufacturing processes that allow synthesis of oligonucleotides at a reasonable cost not only are expediting the discovery of these drugs but also will enable meeting the market demand for such molecules.

It is noteworthy that a majority of oligonucleotides are made via a common procedure, popularly known as the phosphoramidite method,[6] summarized in Figure 1. Briefly, oligonucleotides are synthesized on a solid support via sequential coupling and oxidation reactions (i−v, Figure 1) in a predetermined order, all controlled by a computerized liquid delivery system. For example, synthesis can begin

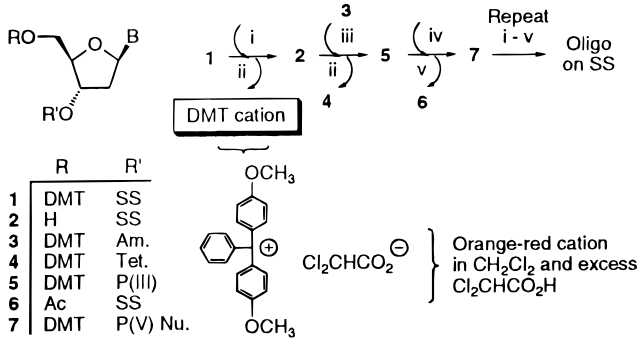

Figure 1. **Reaction, reagents, and abbreviations: i, 3% Cl_2CHCO_2H in CH_2Cl_2; ii, CH_3CN wash; iii, 0.2 M solution of 3 + 0.45 M 1*H*-tetrazole in CH_3CN; iv, oxidation step (see ref 6 for details); v, capping reagents (DMT, 4,4′-dimethoxytriphenylmethyl chloride; SS, linker solid support; Am., standard CE amidite; Tet., activated amidite; P(III), phosphite triester linkage; P(V) Nu., phosphorothioate linkage attached to a nucleoside residue via SS; B: T, C^{Bz}, or G^{iBu}).**

with a nucleoside linked to a solid support, such as **1**. Treatment with 3% dichloroacetic acid (DCA) in dichloromethane removes the 5′-*O*-(4,4′-dimethoxytriphenylmethyl) group (DMT), to provide **2** with a free 5′-OH group. In this step, the 5′-*O*-DMT group is released as a cation with a characteristic bright red-orange color. The colorful cation generated in this reaction not only allows monitoring coupling efficiency but also allows the spectrophotometric flow monitor and computer system to discontinue the flow of 3% DCA deblock solution.

Detritylation under acidic conditions is reported to be a reversible reaction.[7] Therefore, removal of all DMT cation from the solid support is crucial to the success of the deblocking step. Next, in step ii, the support is washed with dry acetonitrile (ACN) to remove traces of acid solution and any trapped DMT cation. Step iii consists of premixing amidite **3** with 1*H*-tetrazole to produce a very reactive P(III) tetrazolide intermediate[8] (**4**) that reacts almost immediately with the 5′-OH group of **2**, generating **5** with a phosphite triester internucleosidic linkage. Unreacted excess **4** is then washed from the support with dry ACN. Step iv is designed to oxidize the unstable P(III) species of **5** to a more stable P(V) internucleosidic linkage to furnish a dimer such as **7**. Capping (step v) is the final step, wherein unreacted 5′-OH groups are acylated, generating **6** to prevent further elongation of oligonucleotide attached to the support. These five

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steps are then repeated until an oligonucleotide of desired length and sequence is completed.

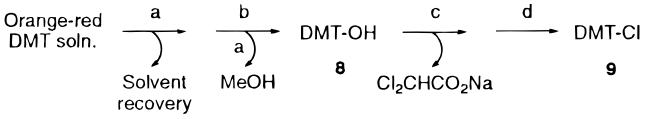
It is important to note that the amidite coupling process described creates two high-molecular-weight waste products. First, the DMT group used for 5′-OH protection amounts to approximately 35% of the weight of incoming monomeric phosphoramidite units **3**. This mass is released as the DMT cation in waste dichloromethane. Second, a 0.5 mol excess of building block **4** is also discarded in waste ACN along with 1*H*-tetrazole. Gratifyingly, methods to capture **4** for its recovery by conversion to **3** are already in place.[9] However, methods to capture the valuable DMT group and for its reconversion to useful DMT-Cl have never been published. In synthesizing a high-molecular-weight (∼6000) drug, such as an oligonucleotide, it becomes very important to maintain a high level of atom efficiency and atom economy. In this note, we describe, for the first time, a convenient process for the capture of currently wasted DMT group and its conversion to useful DMT-Cl.

Results and Discussion

There are two main reasons why the DMT group has been widely used for the protection of hydroxyl groups in carbohydrate, nucleoside, and oligonucleotide chemistry.[10] First, a triphenylmethyl group imparts crystallinity to many compounds that are low-melting solids, and second, the triphenylmethyl group is easily removed under a variety of conditions.[11] Use of the DMT group in oligonucleotide synthesis was first described by Nobel laureate Khorana[12] in 1962. After all these years, DMT is still the preferred hydroxyl protecting group for oligonucleotide synthesis, both in solid-supported[13] and in solution-phase syntheses.[14]

In a standard automated synthesis[15] of a 20-mer phosphorothioate oligonucleotide on 100 mmol scale, the 5′-*O*-DMT group is removed 19 times using a total of approximately 380 L of 3% DCA in dichloromethane. Interestingly, all of that material goes to waste and is typically incinerated at a premium price.[16] Our original manufacturing process for oligonucleotides not only generated toxic acid waste but also threw away an expensive[17] protecting group of high molecular weight. For this reason, we embarked on a project to allow (i) salvage of a protecting group which constitutes 35% of the weight of amidite **3**, (ii) neutralization of the acidic halogenated waste stream, and (iii) potential for recovery and reuse of the toxic solvent dichloromethane. The process described herein addresses all these issues and is amenable to scale-up for ton-scale manufacture scenario.

Scheme 1[a]



[a] Reagents and conditions: a, concentration under reduced pressure to an oil; b, add MeOH and 3 N NaOH solution; c, extraction of DMT-OH in organic solvent; d, a → AcCl in toluene, reflux 3 h, → a → crystallization from cyclohexane.

During a standard 60 mmol synthesis of oligonucleotide on an OligoProcess[18] reactor, one complete column volume of deblock solution was collected manually upon completion of a detritylation step (i in Figure 1). This solution is acidic (pH 0.6) and bright red-orange in color. The color is due to DMT cation present in the solution. The concentration of this cation is approximately 4.8 mmol/L, measured by its characteristic UV absorption at 504 nm. The next step in the recovery (a in Scheme 1) involves concentration of the red-orange solution under vacuum. During this step, most of the dichloromethane is removed from the solution and collected. The dichloromethane recovery is about 90% in this step, with a purity[19] of >95%. We believe that dichloromethane recovered by this process is pure enough[20] for reuse as a solvent for organic synthesis.

The residual red oil is then dissolved in a minimum volume of methanol and the solution quenched with 3 N NaOH solution (step b, Scheme 1). The rationale for use of NaOH as a quenching base is three-fold. First, it is inexpensive and environmentally safe; second, it neutralizes DCA in a very effective manner; and last, it provides hydroxide ion for generation of DMT-OH. At this point, the bright red color of the solution fades away, indicating the formation of DMT-OH, confirmed[21] by MS analysis. The pH of the solution is ∼9−10, which does not affect[22] the DMT-OH.

The basic solution is concentrated under vacuum to remove most of the methanol, providing a thick orange-yellow syrup. In the next step (c in Scheme 1), the syrup is dissolved in toluene and the solution washed with water several times. Water washing removes DCA as a soluble sodium salt, an easily disposable waste stream. The toluene solution is then concentrated to 1/10 of original volume, which helps remove trace water as an azeotrope, leaving the solution dry and ready for the chlorination step (d in Scheme 1). The literature method[23] for chlorination of DMT-OH calls for the use of neat acetyl chloride under refluxing conditions. However, we thought it was unnecessary to use such a large excess of acetyl chloride. After a few attempts, we were able to perform the chlorination with only 1/10 of

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(15) Using phosphoramidite chemistry, see ref 6 for details.
(16) The current cost of disposal by incineration of halogenated waste is $260 per 55-gallon drum.
(17) The Aldrich catalog price for DMT-Cl is $81 for 25 g.
(18) OligoProcess is the largest automated oligonucleotide synthesizer built to assemble 10−180 mmol of 20-mer oligonucleotide in less than 12 h. For more details, contact Amersham Pharmacia Biotech, Piscataway, NJ (phone 1-800-526-3593).
(19) Determined by GC.
(20) Preliminary deblocking and phosphitylation experiments with recovered dichloromethane indicated no side reactions.
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the acetyl chloride recommended in the literature report. DMT-OH solution in toluene is refluxed with acetyl chloride for 3 h, and the solution is concentrated. The residue is dissolved in cyclohexane and cooled to provide pure crystalline DMT-Cl. The ^{1}H and ^{13}C NMR, UV spectra, elemental analysis, and MS analysis of the regenerated DMT-Cl indicated that it was identical with that reported in the literature.[23] Additionally, we took this DMT-Cl and prepared 5′-*O*-DMT protected nucleosides in yield and purity similar to those obtained by the use of fresh DMT-Cl.

Conclusion

In summary, a convenient and inexpensive process for capture and regeneration of the DMT group has been developed for the first time. Overall cost, operational safety, and environmental considerations were taken into account in process development. Though this process may not be economical[24] to practice on a 100 mmol level for a 20 kg/year oligonucleotide pilot plant, it is certainly very attractive for a manufacturing plant[25] where antisense or any other oligonucleotides will be made in ton quantities per year. Furthermore, if 5′-*O*-DMT-protected nucleosides and related amidites were to be manufactured at the same site, both regenerated DMT-Cl and recaptured dichloromethane could be utilized directly on site for the synthesis of various synthons and for the preparation of deblock solution (i in Figure 1), respectively. This method of harvesting and recovery of the protecting group may also be applicable to solution-phase[26] and blockmer synthesis[27] of oligonucleotides. We believe that our method will help in reducing the ecological burden posed by the need for proper disposal of the reagent and solvent alike. Also, this is an excellent example of a multistep organic synthesis, where atom efficiency and atom economy are now maintained effectively.

Experimental Section

A complete deblocking cycle (Figure 1, step i) from a 60 mmol campaign was collected manually (12 L of 3% DCA in DCM containing triphenylmethyl cation) and concentrated under vacuum to remove most of the dichloromethane. The oily red residue was then dissolved in MeOH (1.8 L), and to this was added an aqueous NaOH solution (3 N, 1.5 L) over a period of 1 h at room temperature. The resulting solution was further stirred for 16 h at room temperature. The reaction mixture was then concentrated under vacuum to remove most of the MeOH. The remaining aqueous layer was extracted with toluene (200 mL × 3), and the organic phases were combined and dried (Na_2SO_4). A small sample (50 mL) of that toluene solution was evaporated to give 4,4′-dimethoxytriphenylmethyl alcohol[21] (DMT-OH in Scheme 1): ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.08 (s, 1H), 3.81 (s, 6H), 6.84−7.50 (m, 13H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 55.25, 86.45, 113.10, 126.70, 127.82, 128.30, 129.21, 130.15, 136.24, 144.99, 158.45; MS (FAB) *m*/*z* 343 (MNa^+). The toluene solution was then concentrated to a minimum volume (80 mL). AcCl (8.5 mL, 9.42 g, 0.12 mol) was added to the DMT-OH in toluene, and the solution was refluxed for 2 h under argon. The reaction mixture was then cooled to room temperature, and the solution was concentrated under vacuum. Cyclohexane (100 mL) was added to the residue, and the mixture was allowed to stand in a refrigerator for 16 h. The crystallized material was then collected by filtration and the product washed with cold cyclohexane (50 mL × 2) to furnish DMT-Cl (17.5 g, 89.7%): mp 122−124 °C [lit.[22] mp 119−123 °C]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.84 (s, 6H), 6.87 (d, 4H), 7.20−7.36 (m, 9H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 55.4, 82.1, 113.0, 127.8, 129.76, 131.1, 137.8, 145.8, 159.1. Anal. Calcd for $C_{21}H_{19}ClO_2$ (338.83): C, 74.44; H, 5.65. Found: C, 74.66; H, 5.82. The indentity of recaptured DMT-Cl was further confirmed by TLC cospotting with an authentic sample in three different solvents (CH_2Cl_2, R_f 0.25; CH_3CN, R_f 0.9; 30% EtOAc in hexanes, R_f 0.4). In addition, this DMT-Cl was also used for the preparation of 5′-*O*-DMT-protected nucleosides, and the products were found to be identical to the literature report.

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(24) For every kilogram of purified 20-mer antisense oligonucleotide made using an OligoProcess synthesizer, approximately 3.6 kg of DMT-Cl can be prepared. However, at this scale, the labor in recovery of DMT-Cl will be much higher than the cost of buying fresh DMT-Cl.

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