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4,4′-Dimethoxytrityl group derived from secondary alcohols: Are they removed slowly under acidic conditions?

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Abstract—Removal of 4,4′-dimethoxytrityl (DMT) groups from primary and secondary hydroxyl functionality was investigated. It was observed that deblocking of DMT group from secondary hydroxyl group of molecules attached to solid support under acidic conditions occurred relatively slowly compared to primary hydroxyl group. Marginal difference in rate of detritylation was observed between DMT group attached to 5′-hydroxyl group of deoxyribonucleoside and 2′-*O*-methoxyethylribonucleoside when attached to one kind of support. Removal of DMT from nucleoside attached to OligoPrep solid support was found to be slow.

1. Introduction

The use of antisense oligonucleotides as therapeutic agents represents a new paradigm in drug discovery and development.[1–4]Antisense drugs, which are short DNA oligonucleotide strands, are designed to inhibit the production of disease-causing proteins and can be used to treat a wide range of infections, inflammatory, and cardiovascular diseases, as well as cancer. A number of pharmaceutical companies, large and small, are actively engaged in the discovery and development of antisense drugs.[5]

Automated synthesis of phosphorothioate oligonucleotides is performed on a solid support using commercially available DNA/RNA synthesizer and phosphoramidite coupling chemistry.[6–8] The typical synthesis cycle consists of four chemical reactions that are separated by a rinsing step to remove excess reagents:

(a) removal of acid-labile 5′-*O*-DMT protecting group from support-bound nucleoside/nucleotide;
(b) coupling of a cyanoethyl-protected phosphoramidite monomer in the presence of an acidic activator;
(c) oxidative sulfurization of resulting phosphite triester to form phosphorothioate triester;
(d) capping of unreacted 5′-hydroxyl groups.

Repetition of synthesis cycle allows for assembly of phosphorothioate oligonucleotides on scales ranging from micromoles to nearly a mole within hours. Cleavage from support, deprotection, purification, and isolation afford the desired oligonucleotide drug as an amorphous hygroscopic solid.

Despite recent advances in oligonucleotide chemistry, the development of improved synthetic methods for synthesis of therapeutic grade oligonucleotides remains an area of intense research. Recently, we have reported several advances in synthesis of oligonucleotides aimed at improving the yield as well as quality, and many of these advances have already been incorporated into the routine manufacture of oligonucleotides here at Isis Pharmaceuticals.[9–25] Still, there is room to reduce the cost of drugs if these drugs were to compete against low-cost small molecule drugs for some large market such as treatment of diabetics.

Recently, we have been working on reducing the volumes of solvents and reagents used during oligonucleotide synthesis such as detritylation solution without compromising the quality (viz., increasing the $(n-1)$-mer level caused by inefficient detritylation). Another area of intense research is the design of new polystyrene-based solid supports[26,27] aimed at lowering the cost and improving the quality of oligonucleotides. Currently, Amersham-Pharmacia Biotech's polystyrene PS200 bead is the only support routinely used for efficient synthesis of phosphorothioate oligonucleotides,[28] even though other supports such as controlled-pore glass

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(CPG), OligoPrep, and Primer HL30 are commercially available. These supports differ in physical properties and perform differently. One such example is the detritylation step. It is well known that during detritylation, the haloacetic acid binds strongly to DNA as well as to solid support.[29,30] This binding depends on the nature of solid support. To understand the behavior of these supports toward detritylation as well as to study the difference between DMT groups attached to hydroxyl groups under different environments, a project was initiated in our laboratories. We investigated various DMT-removal scenarios by varying the nature of monomer attached to the support (deoxy, 2′-*O*-methoxyethylribo, and a non-nucleoside), type of support (PS200, OligoPrep, HL30, and Nittomar 90), and reactivity (primary and secondary). Results are summarized below.

2. Results and discussion

Amersham-Pharmacia Biotech's (APB) polystyrene PS200 (**2**, **6**, and **10**), dextran-coated HL30 supports (**1**, **5**, **9**, and **11**), Merck's OligoPrep (**3**, **7**), and Nittomar 90, a new polystyrene support (**4**, **8**), were used for comparison. Three kinds of hydroxyl groups (one primary and two secondary) protected with DMT groups were studied. In total, 11 different supports were evaluated for detritylation. These supports are shown in Figure 1.

Recently, we reported the use of a non-nucleoside based universal linker molecule (UnyLinker) for efficient synthesis of oligonucleotides as a means of alleviating the need to make and load various nucleoside succinates to solid supports.[31] During evaluation of this chemistry, we noticed unacceptable levels of $(n-1)$-mer formation and reasoned that removal of DMT from the secondary hydroxyl group could be incomplete. Extended detritylation, followed by oligonucleotide synthesis, afforded good quality of phosphorothioate oligonucleotide.[32] Hence, supports (**10**, **11**) containing this UnyLinker molecule were also included in the current detritylation studies.

2.1. Detritylation condition

Historically, removal of DMT group was performed using a solution of 3% trichloroacetic acid in dichloromethane. Later, the condition was changed to 3% dichloroacetic acid in dichloromethane. For manufacture of antisense drugs, a large volume of dichloromethane is needed and this solvent is not environmentally friendly. Recently, we reported[17,19,20] that halogenated solvent could be replaced with inexpensive and industrially acceptable solvent such as toluene and still not compromise on quality. Later, we further improved the synthesis by replacing the solution of 3% dichloroacetic acid with 10% dichloroacetic acid in toluene. Currently, this condition is routinely used for manufacture of our antisense drugs.[10]

Detritylation was performed on an Akta 100 DNA/RNA synthesizer using a fine line 35 glass column. Since loadings of monomers were different and all experiments were performed at 500-μmol scale, bed heights varied according to loading. However, back-pressure was not observed during the detritylation studies.[33] The detritylation of various supports was plotted for comparison, as shown in Figure 2. Some interesting observations could

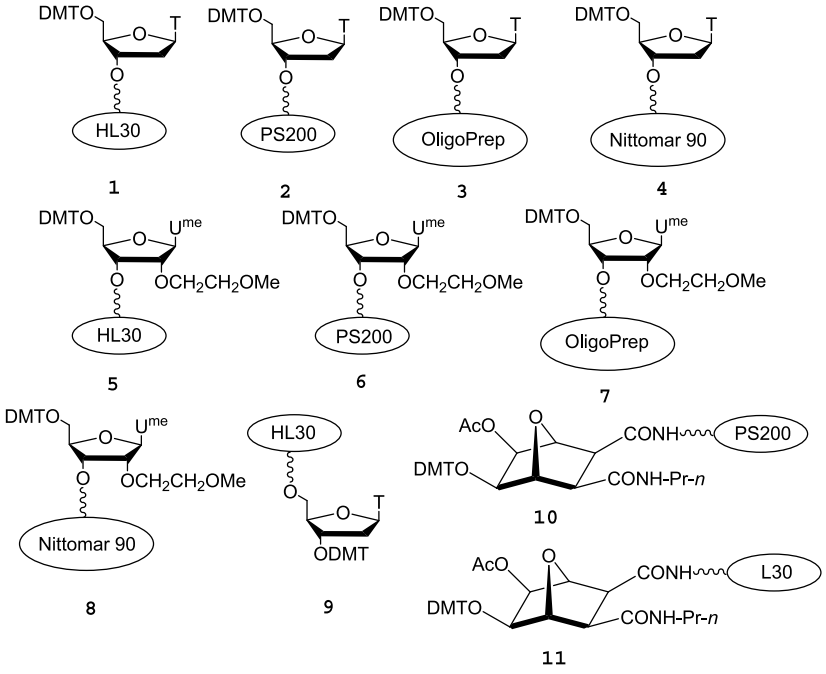

Figure 1. Various solid supports evaluated for detritylation. 5′-DMT thymidine loaded onto HL30 (**1**), polystyrene PS200 (**2**), OligoPrep (**3**), polystyrene Nittomar 90 (**4**), 5′-DMT-2′-*O*-methoxyethyl-5-methyluridine loaded onto HL30 (**5**), polystyrene PS200 (**6**), OligoPrep (**7**), polystyrene Nittomar 90 (**8**), 3′-DMT thymidine loaded onto HL30 (**9**), and UnyLinker molecule loaded onto polystyrene PS200 (**10**) and HL30 (**11**) supports.

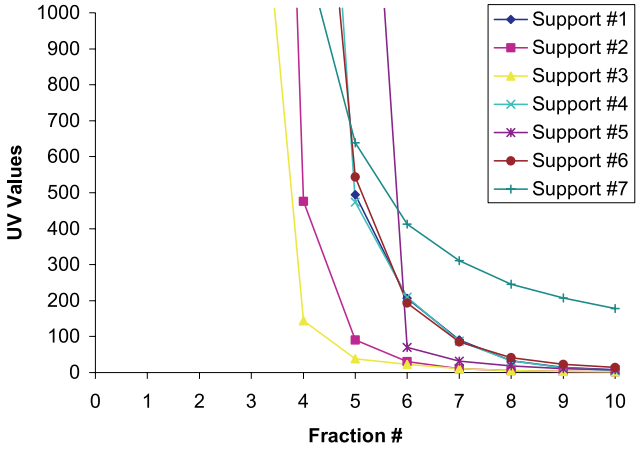

Figure 2. Chart showing the removal of DMT group from nucleosides attached to various supports: #1, HL30 T support; #2, PS200 T support; #3, OligoPrep T support; #4, Nittomar 90 T support; #5, HL30 MOE meU support; #6, PS200 MOE meU support; #7, OligoPrep MOE meU support; #8, Nittomar 90 MOE meU support; #9, 3′-DMT T on HL30 support; #10, UnyLinker on PS200 support; and #11, UnyLinker on HL30 support.

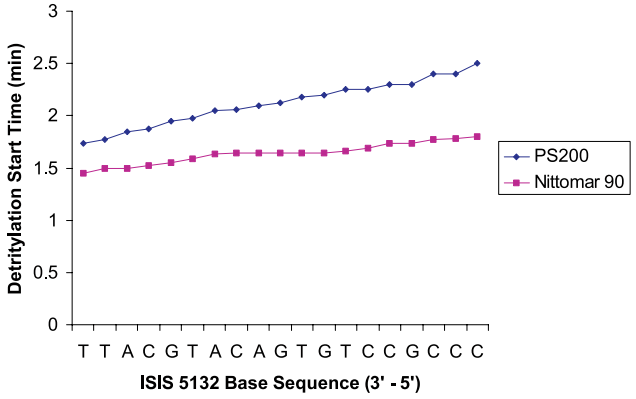

Figure 3. Detritylation start time versus nucleotide position in oligonucleotide.

be made. Removal of DMT group from 2′-*O*-methylethyl-5-methyluridine is slower compared to thymidine when attached to swellable supports like PS200 or OligoPrep but not in rigid supports like HL30. Removal of DMT group from 2′-*O*-methoxyethyl-5-methyluridine attached to OligoPrep was so slow that the reaction was not even complete at the end of fraction collection. Thus, solid supports appear to play a major role during the detritylation step.

To magnify this difference, two supports were chosen and compared by synthesizing a 20-mer phosphorothioate oligonucleotide. ISIS 5132 [5′-TCC-CGC-CTG-TGA-CAT-GCA-TT] was chosen as an example. Synthesis was performed on APB Akta 10 DNA/RNA synthesizer at 150-μmol scale synthesis using β-cyanoethyl phosphoramidite synthon (1.75 equivalents, 0.2 M in CH_3CN). 1*H*-Tetrazole (0.45 M in CH_3CN) was used as activator and phenylacetyl disulfide (PADS) (0.2 M in 3-picoline–CH_3CN 1:1, v/v) as sulfur transfer reagent.[16,23] Capping reagents were made to the recommended Pharmacia recipe: Cap A, *N*-methylimidazole–CH_3CN (1:4 v/v); Cap B, acetic anhydride–pyridine–CH_3CN (2:3:5, v/v/v). Pharmacia PS200 Primer support and Nittomar 90 solid support were used for comparison. Amidite and tetrazole solutions were prepared using anhydrous CH_3CN (water content ca. 10 ppm) and were dried further by addition of activated 4 Å molecular sieves (~50 g/L).

During our experimentation, we noticed that more than one column volume of deblock solution must be passed through the synthesis column before the orange color of DMT cation is first observed in the eluent. The first fractions of deblock solution that pass through the synthesis column are largely depleted of acid (due to binding to DNA and support) and contain a significant amount of DMT groups.[34] Occurrence of orange color (DMT cation) is directly associated with the elution of dichloroacetic acid. A plot of time when dimethoxytrityl absorption is first detected in UV absorbance monitor versus base position shows a steady increase in detritylation start time with increasing number of nucleotides bound to support (Fig. 3). From the difference in delay between first and 19th detritylation (the 20th nucleotide was synthesized as DMT-on), it may be estimated that on average ca. 2 mmol of acid per mmol of nucleotide are absorbed. Thus, from data presented in Figure 3, it appears that detritylation is faster in case of Nittomar 90 as compared to PS200 solid support.

Table 1. Removal of 5′-*O*-DMT group from nucleosides attached to various supports

Fraction #	Support #1	Support #2	Support #3	Support #4	Support #5	Support #6	Support #7	Support #8	Support #9	Support #10	Support #11
1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	4361.2	2892.7	0.0	0.0	5981.8	2581.5	0.0	0.0	259.1	0.0
3	7915.5	4771.3	2933.1	6767.3	9426.2	1705.0	1516.4	4759.8	5901.2	1473.8	6530.2
4	2170.6	476.2	1184.2	981.9	1660.6	143.9	1040.7	4563.3	2643.3	4519.0	2091.7
5	494.0	90.4	638.8	214.1	161.1	37.8	986.0	365.5	473.6	2222.1	544.2
6	205.8	30.9	412.9	55.6	34.8	22.8	813.5	64.5	209.2	69.8	193.5
7	90.5	10.9	311.2	19.0	13.4	10.4	664.1	20.5	87.2	31.3	84.7
8	33.2	5.2	245.8	8.0	5.4	5.3	541.2	7.9	33.0	18.1	41.6
9	14.6	2.8	207.8	4.2	2.5	2.9	427.9	3.9	13.5	10.7	22.7
10	7.5	2.3	177.6	2.7	1.8	1.9	331.2	2.4	5.6	7.9	14.5

#1, HL30 T support; #2, PS200 T support; #3, OligoPrep T support; #4, Nittomar 90 T support; #5, HL30 MOE meU support; #6, PS200 MOE meU support; #7, OligoPrep MOE meU support; #8, Nittomar 90 MOE meU support; #9, 3′-DMT T on HL30 support; #10, UnyLinker on PS200 support; #11, UnyLinker on HL30 support. Numbers in the table represent absorbance units at 412 nm (A_{412}).

3. Experimental

An Amersham-Pharmacia Biotech Akta 100 DNA/RNA synthesizer was used for all experiments. Ten percent of dichloroacetic acid in toluene (flow rate = 9 ml/min) was used for deblocking of DMT groups. To eliminate differences in loading and also to have consistency in amounts of DMT groups removed, all experiments were carried out at 500-μmol scale. Initially, the support was washed with acetonitrile (100 ml) and then deblock solution was passed through. Ten milliliter fractions were collected and DMT absorbance was measured by UV at 412 nm. Appropriate dilutions were made to obtain the readings within limits. Table 1 shows the UV absorbance readings of DMT group from various supports used for detritylation.

In summary, we have demonstrated that removal of DMT group is directly related to the nature of solid support used. DMT groups attached to secondary hydroxyl groups detritylate slower than primary hydroxyl groups. The polymeric properties of solid support should be kept in mind while designing a cycle for optimal synthesis to obtain best quality and yield.

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