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Formation of 4,4′-dimethoxytrityl-*C*-phosphonate oligonucleotides

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Abstract—Incomplete sulfurization during solid-phase synthesis of phosphorothioate oligonucleotides using phosphoramidite chemistry was identified as the cause of formation of two new classes of process-related oligonucleotide impurities containing a DMTr-*C*-phosphonate (DMTr = 4,4′-dimethoxytrityl) moiety. Phosphite triester intermediates that failed to oxidize (sulfurize) to the corresponding phosphorothioate triester react during the subsequent acid-induced (dichloroacetic acid) detritylation with the DMTr cation or its equivalent in an Arbuzov-type reaction. This leads to formation of DMTr-*C*-phosphonate mono- and diesters resulting in oligonucleotides modified with a DMTr-*C*-phosphonate moiety located internally or at the 5′terminal hydroxy group. DMTr-*C*-phosphonate derivatives are not detected when optimized sulfurization conditions are employed.

Oligonucleotides find widespread application in diagnostics, molecular biology, and as therapeutic agents. Recently, DNA and RNA analogs have emerged as potential drugs for treatment of diseases through antisense mechanisms of action.[1–4] The most advanced drug candidates are phosphorothioate oligonucleotides (PS-oligonucleotides) in which one of the non-bridging oxygens of the phosphate diester (PO) internucleotide linkage is replaced by a sulfur. Automated synthesis of PS-oligonucleotides is performed on a solid support (derivatized CPG, crosslinked polystyrene), using commercially available phosphoramidites as the starting materials.[5] The typical synthesis cycle (Scheme 1) consists of four chemical reactions that are separated by rinsing steps designed to remove excess reagents. The four reactions are:

- Acid-induced removal of the 4,4′-dimethoxytrityl (DMTr) protecting group to liberate the 5′-hydroxyl group of the support-bound oligonucleotide (reaction *a*).
- Extension of the oligonucleotide chain by coupling of a protected nucleoside phosphoramidite in the presence of a weak acid (reaction *b*).
- Incorporation of a sulfur atom by oxidative sulfurization of the trialkyl phosphite triester intermediate ($P^{(III)}$ species) to form a phosphorothioate triester ($P^{(V)}$ species) (reaction *c*).
- Capping of unreacted 5′-hydroxyl groups to prevent elongation of failure sequences (reaction *d*).

Repetition of this synthesis cycle allows for assembly of PS-oligonucleotides on scales ranging from a few nanomoles to several hundreds of millimoles. Cleavage and separation of the oligonucleotide from the solid-support matrix, deprotection steps, purification, and isolation of the final product complete the synthesis (reactions *e*). This synthesis cycle was used to prepare the target PS-oligodeoxyribonucleotide **1**, the nucleotide sequence of which is shown here: PS-d(GCCCAAGCTGGCATCCGTCA).[6] Despite recent advances in preparative oligonucleotide chemistry, the development of improved methods for the synthesis of therapeutic grade oligonucleotides remains an area of intense research.[7–12] Various reagents have been proposed for the conversion of oligonucleotide phosphite triester species to the corresponding phosphorothioate triesters.[13–29] Recently, we demonstrated that phenylacetyl disulfide (PADS) is a more efficient and economical reagent than 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent).[29] Solvent composition, concentration of the reagent, molar excess, and contact time are crucial parameters to

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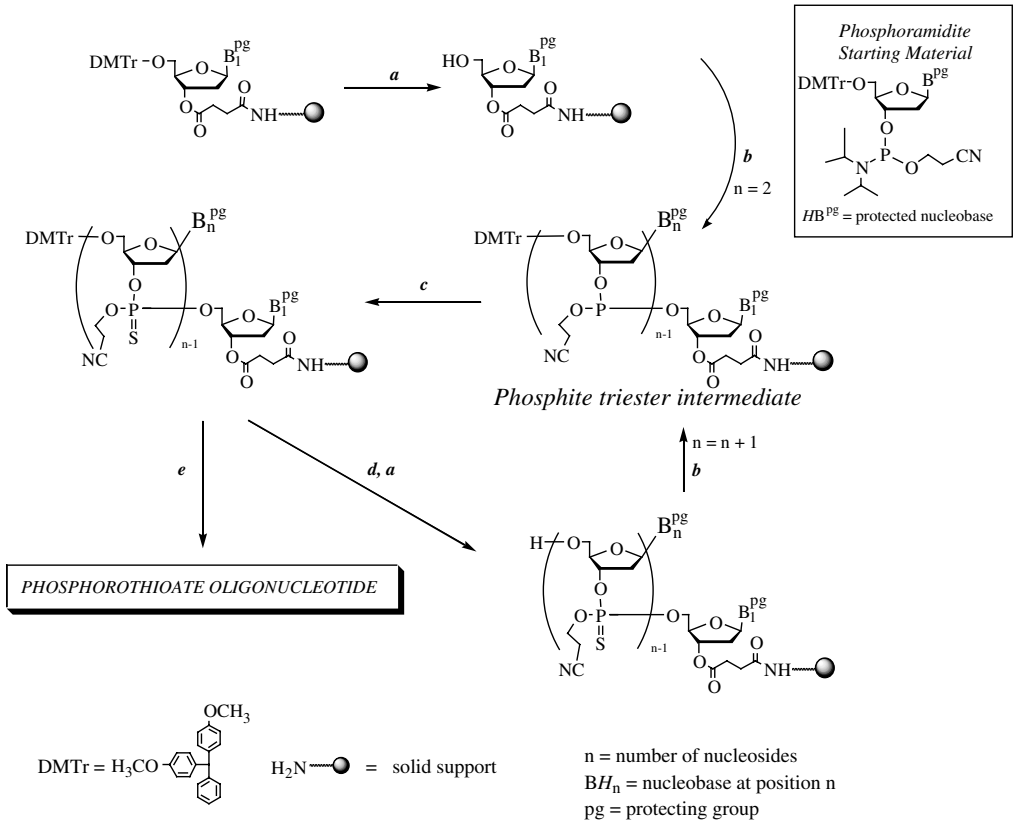


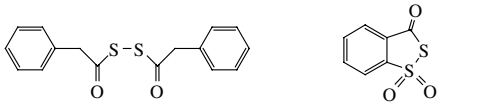
a, Deprotection; *b*, Coupling; *c*, Sulfurization; *d*, Capping; *e*, Cleavage, deprotection, purification, isolation

Scheme 1.

achieve high-efficiency sulfurization. During optimization of reaction parameters of the sulfurization step we noticed that less than optimal reaction conditions led to additional oligonucleotide products. In this report, we describe the structure elucidation of these compounds and suggest a potential mechanism of formation.

One of the crucial steps in PS-oligonucleotide synthesis is the incorporation of the sulfur atom in the internucleotide linkage by oxidative sulfurization of the $P^{(III)}$ trialkyl phosphite triester intermediate to form a $P^{(V)}$ trialkyl phosphorothioate triester linkage (Scheme 1, reaction *c*). PADS has been used as a sulfurization agent for the synthesis of phosphorothioate oligonucleotides.[27] We found that this reagent works most efficiently in a solvent system of acetonitrile/3-picoline.[29] Reagent concentration, number of molar equivalents, and sulfurization contact times were optimized for solid-phase synthesis. On large scale commercial synthesis, PADS has proven to be superior in terms of efficiency, consistency of performance, commercial availability, and cost when compared to the previously employed 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) (Scheme 2).[17]

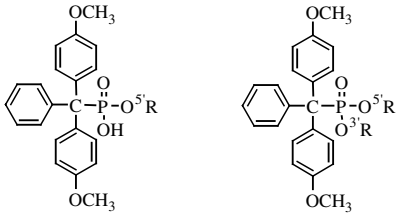
The excellent sulfurizing properties of PADS (0.2 M in 3-picoline/acetonitrile 1:1, v/v, 6.5 equiv based on synthesis scale) are reflected in the high phosphorothioate diester-to-phosphate diester ratio and the good overall



Phenylacetyl disulfide (PADS) Beaucage Reagent

Scheme 2.

oligonucleotide yields obtained. In an effort to optimize economics and lab productivity (throughput), we reduced the molar equivalents and contact time during the sulfur-transfer step. Under suboptimal sulfurization conditions (less than 1.7 equiv) we observed formation of two new classes of process-related oligonucleotide products **2** and **3** eluting by RP-HPLC after the main 'DMTr-off' peak **1** (**2** and **3** are referred to in the following as 'late-eluters'). A detailed description of the analytical data leading to the proposed structures shown in Scheme 3 follows. Knowledge of the chemical structure of actual or potential process-related impurities, as well as a better understanding of their origins and mechanisms of formation is crucial to the design and execution of both lab scale and commercial scale synthesis. It leads to improvements in oligonucleotide quality and yield, facilitates process optimization and troubleshooting and forms a solid foundation of a science-based assessment of a synthetic process.



DMTr-*C*-phosphonate monoester **2**

DMTr-*C*-phosphonate diester **3**

Scheme 3.

Oligonucleotide **1** elutes on RP-HPLC as a single broad peak at a retention time (t_R) of 11.8 min. When suboptimal sulfurization conditions were used, additional, partially resolved peaks (late-eluters **2** and **3**) appeared in the chromatogram of the purified and detritylated product[11,12] at longer retention times (t_R 14.3–16.5 min). The photodiode array detector showed an absorbance maximum near 260 nm for all peaks, typical for oligonucleotides. Due to the number of partially resolved peaks we concluded that several species were present and that the late-eluting compounds might consist of a family of oligonucleotide derivatives with a common covalent modification. For comparison of relative HPLC retention times, we co-injected oligonucleotide **5′-*O*-DMTr-1**, which partially overlapped (t_R 16.0–17.0 min) the late-eluting peaks, suggesting that thelate-eluting compounds possibly possessed a large hydrophobic group (Fig. 1). Additional treatment of the original sample with acetic acid confirmed that DMTr removal from the 5′ terminus was complete and excluded acid-labile 5′-*O*-DMTr-modified oligonucleotides as structural candidates for the late-eluters. Preparative RP-HPLC yielded a sample of the late-eluters that was used for further structure elucidation.

Capillary gel electrophoresis (CGE) separates oligonucleotides based on their mass-to-charge ratio. CGE analysis of the late-eluting peaks showed the presence of multiple peaks indicating the presence of several oligonucleotides of different lengths (data not shown). All components migrated faster than **1**, suggesting that the late-eluters were a mixture of shorter (less than 20 nucleotides) oligonucleotides. ^{1}H NMR spectroscopy of the isolated mixture (in D_2O) showed additional broad resonances at 3.8 ppm (OCH_3) and between 6.7 and 7.15 ppm (aromatic protons). These resonances are not seen in **1** and are reminiscent of a 4,4′-dimethoxytrityl group typically used for 5′-protection of the nucleoside phosphoramidite. From the integration values it was estimated that one DMTr group per oligonucleotide was present. ^{31}P NMR spectroscopy has been shown to be very useful for phosphorus functional group analysis of oligonucleotide derivatives.[30,31] The proton decoupled ^{31}P NMR spectrum (in D_2O) of the late-eluters showed multiple resonances at 56 ppm (91.6%), typical of phosphorothioate diester linkages. In addition, resonances were observed at 24 ppm (6.1%) characteristic of alkyl phosphonate monoesters. The presence of small amounts of alkyl phosphonate diester (1.0%) and phosphate diester (0.6%) at 29–30 and 0 ppm, respectively, was also evident. The ratio of phosphorothioate linkages to the newly observed alkyl phosphonate monoester groups was 15:1, corresponding to, on average, a 16-mer phosphorothioate oligonucleotide with a single phosphonate moiety attached. HPLC retention time, CGE analysis, and ^{1}H and ^{31}P NMR spectroscopy, coupled with the presence of an 'acid-stable' DMTr group, suggested that the late-eluters consisted of a family of oligonucleotides possessing a 4,4′-dimethoxytrityl phosphonate [DMTr-P(O)O] modification.[31,32] The chemical structures of the proposed DMTr-*C*-phosphonate monoesters **2** and DMTr-*C*-phosphonate diesters **3** are shown in Scheme 3.

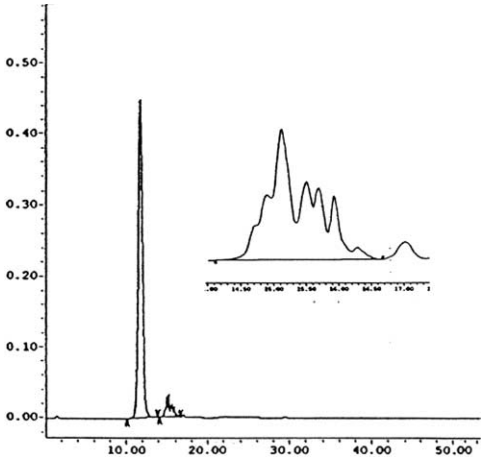


Figure 1. Reversed-phase HPLC trace of **1** (t_R 11.8 min) and late-eluters **2** and **3** (insert, t_R 14.3–16.5 min). **2** and **3** consist of multiple overlapping peaks. For comparison, **5′-*O*-DMTr-1** elutes at t_R 16.0–17.0 min (data not shown).

Figure 2 shows the LC/MS data (*m/z* range 1000–1800) obtained for the late-eluters. The *m/z* values were deconvoluted to give masses 6085 (**2c**), 5780 (**2d**), 5474 (**2e**), 5144 (**2f**), 4166 (**2i**), and 3845 (**2k**), respectively (Table 1). The observed mass difference between the two highest masses, **2c** and **2d** is 305 amu and corresponds to a dC phosphorothioate nucleotide [PS-dC, (305 calcd)]. Additionally, the mass differences found between the highest mass and successive lower masses correspond to a PS-d(CC) dimer, [611, (610 calcd)], PS-d(CCA) trimer [941, (940 calcd)], PS-d(CCAAGC) hexamer [1919, (1920 calcd)], and PS-d(CCAAGCT) heptamer [2240, (2240 calcd)], respectively. In addition, ions corresponding to DMTr-*C*-phosphonate diesters **3** were identified, albeit at lower abundances. Masses 5990 (**3c**), 5684 (**3d**), 5375 (**3e**), 5048 (**3f**), 4720 (**3g**), and 4373 (**3h**) were assigned to the corresponding deletion sequences containing an internal DMTr-*C*-phosphonate diester modification. These sequences are part of a heptamer located at the 5′-side of oligonucleotide **1** (PS-d(GC***CCAAGCT***GGCATCCGTCA). It is notable that **3a** was not found in this particular sample despite the fact that it is the most abundant phosphonate diester species in the crude material. This observation is most likely a result of the RP-HPLC purification step, which is capable of separating components with zero, one and two DMTr (e.g., **3a** species) groups from each other.

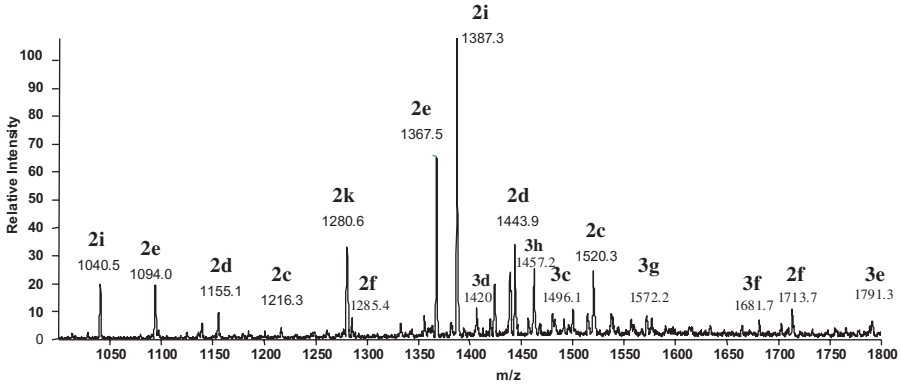


Figure 2. Electrospray ionization mass spectrum of late-eluters **2** and **3**. Shown are −5, −4 and −3 charge states.

Table 1. Comparison of masses found in the late-eluters **2** and **3** and deletion sequences of **1**

Compound, mass	**1** and its 5′-deletion sequences, mass (calcd)	Δ*m*
2a, (not found)	GCCCAAGCTGGCATCCGTCA, 6368	
3a, (not found)[a]		271
2b, (not found)	CCCAAGCTGGCATCCGTCA, 6023	
3b, (not found)		
2c, 6085	CCAAGCTGGCATCCGTCA, 5718	367
3c, 5990		272
2d, 5780	CAAGCTGGCATCCGTCA, 5412	368
3d, 5684		272
2e, 5474	AAGCTGGCATCCGTCA, 5107	367
3e, 5375		268
2f, 5144	AGCTGGCATCCGTCA, 4778	366
3f, 5048		270
2g, (not found)	GCTGGCATCCGTCA, 4449	
3g, 4720		271
2h, (not found)	CTGGCATCCGTCA, 4103	
3h, 4373		270
2i, 4166	TGGCATCCGTCA, 3798	367
2k, 3845	GGCATCCGTCA, 3478	367

[a] **5′-*O*-DMTr-3a** (6941 amu) is the most abundant DMTr-*C*-phosphonate diester **3** in crude product.

To aid structural determination, the late-eluters were converted to the corresponding family of phosphate diester oligonucleotides using an iodine/*N*-methyl imidazole procedure.[33] We confirmed the number of PS linkages in individual components of **2** by calculating the mass difference between the component of **2** and its corresponding phosphate diester form **PO-2** that was obtained following replacement of sulfur by oxygen (Table 2). The assignment of corresponding sequences of **2** and **PO-2** was made based on the analogous mass differences in **PO-2** components. The mass differences found between the highest mass and successive lower masses of **PO-2** corresponded to the PO-dC monomer [289, (289, calcd)]. PO-d(CC) dimer, [578, (578 calcd)], PS-d(CCA) trimer [891, (892 calcd)], PS-d(CCAAGC) hexamer [1823, (1824 calcd)], and PS-d(CCAAGCT) heptamer [2127, (2128 calcd)], respectively. Table 1 shows that **2** and **3** comprise a series of 5′-truncated oligonucleotides of **1** with a common mass difference of 367 ± 1 amu (for DMTr-*C*-phosphonate monoester derivatives **2**) and 271 ± 2 amu (for DMTr-*C*-phosphonate diester derivatives **3**) between the experimentally found masses and the assigned deletion sequences. In summary, the data presented above suggests that late-eluters **2** and **3** are families of 5′-deletion sequences of **1** [(*n*-2, 3, 4, 5, 8, 9)-mers] containing a 5′-terminal DMTr-*C*-phosphonate monoester or an internally located DMTr-*C*-phosphonate diester group, respectively.

To provide further support for the above structural assignments, **PO-2** was enzymatically digested to a mixture of nucleosides using snake venom phosphodiesterase and alkaline phosphatase. HPLC analysis of the digest showed, in addition to the four natural nucleosides [dC (5.7 min), dG (11.9 min), T (12.6 min), dA (14.3 min), 91.1 area %], peaks at significantly longer retention times (25.3–26.7 min, 8.9 area % combined) (Fig. 3). LC/MS analysis of these latter peaks revealed masses (charge state −1) of 592.2 (**4a**), 607.2 (**4b**), 616.2 (**4c**), and 632.2 (**4d**), respectively. The mass difference of 366 amu (dC+366, T+366, dA+366, dG+366) between the four natural nucleosides and the modified nucleosides supported our earlier notion of a DMTr-P(O)O modification [$C_{21}H_{19}O_4P$, 366.4 (calcd)].

Table 2. Determination of oligonucleotide length and number of internucleotide linkages of **2** through exchange of sulfur for oxygen

Mass of **2**	Mass of **PO-2**	Δm (**2**−**PO-2**)	Number of S exchanged	Oligonucleotide length
6085 (**2c**)	5811	274	17	18
5780 (**2d**)	5522	258	16	17
5474 (**2e**)	5233	241	15	16
5144 (**2f**)	4920	224	14	15
4166 (**2i**)	3988	178	11	12
3845 (**2k**)	3684	161	10	11

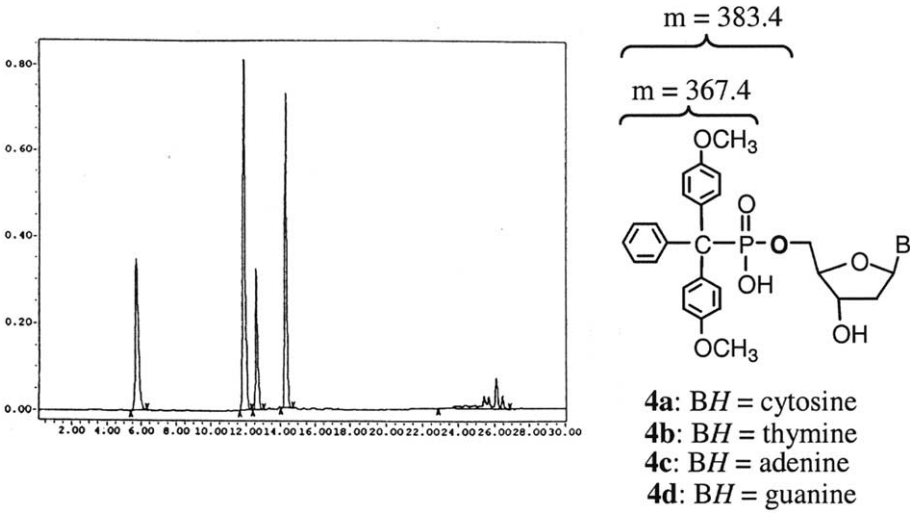


Figure 3. (Left) Reversed-phase HPLC trace of the enzyme digest of the phosphate diester forms (**PO-2**, **PO-3**) of late-eluters **2** and **3**. In addition to unmodified nucleosides [dC (5.7 min), dG (11.9 min), T (12.6 min), dA (14.3 min), 91.1 area %], we also observe peaks **4a**–**d** at significantly longer retention times (25.3–26.7 min, 8.9 area % combined) with masses corresponding to dN+366 amu. (Right) Structural proposal for **4a**–**d**.

MS/MS analysis of the nucleoside derivatives **4a**–**d** gave, in each case, predominant fragments corresponding to the heterocyclic base and to an ion with $m/z = 383$ [DMTr-P(O)(OH)O, $C_{21}H_{20}O_5P$, 383.4 (calcd)]) indicating that the DMTr phosphonate monoester is attached to the sugar and not to the nucleobase.

Collision-induced dissociation (CID) of oligonucleotides is a powerful technique for sequencing oligonucleotides and was employed in the present context to determine the position of DMTr-*C*-phosphonate monoester group attachment of a selected species (**2c**) of the late-eluters.[34] The MS/MS experiment was performed by isolating the $[M-4H]^{4-}$ charge state at $m/z = 1520$ of **2c**, and comparing the fragmentation to the corresponding fragmentation pattern of the $[M-4H]^{4-}$ charge state of **1-d(GC)** at $m/z = 1428$. The resulting spectra are presented in Figure 4. Fragmentation from the 3′-end is identical for both ions. Both the w7 (charge state −2 at $m/z = 1123$) and the w3 (charge state −1, at $m/z = 971.3$) fragments are readily identified in both spectra, thus excluding positioning of the DMTr-*C*-phosphonate modification at the 3′-terminus of **2c**. Fragmentation from the 5′-terminus leads to the a5-b fragment [charge state −1, at $m/z = 1731$ for **2c** and 1365 for **1-d(GC)**]. Similarly, the a4-b fragment [charge state −1, at $m/z = 1402$ for **2c**, 1036 for **1-d(GC)**] and the a3-b fragment [charge state −1 at $m/z = 1073$ for **2c** and 707 for **1-d(GC)**] are detected. The mass difference of 366 amu in corresponding fragment ions of the a-series clearly demonstrates that the phosphonate modification is located toward the 5′-side of **2c**. The presence of four modified nucleosides in the enzyme digest, the fact that the modification is located at the 2-deoxyribose sugar and MS/MS data locating the modification at the 5′-side of the oligonucleotides clearly points to the 5′-terminal hydroxy group as the site of DMTr-*C*-phosphonate monoester attachment.

Based on the data described above we identified the structures of **2** as 5′-deletion sequences of **1** modified at the *5′-terminal hydroxy group* with a 4,4′-dimethoxytrityl-*C*-phosphonate monoester group. Similar chromatographic behavior combined with mass spectral data support the proposed 4,4′-dimethoxytrityl-*C*-phosphonate diester structure of **3**.

We propose that late-eluters **2** and **3** arise according to the mechanism shown in Scheme 4. Under suboptimal sulfurization conditions a fraction of the trialkyl phosphite triesters **5** formed in the preceding coupling reaction fail to sulfurize. These phosphites can react during the subsequent detritylation step with the DMTr cation or its equivalent[31,35] to give phosphonium ion **6** that can then undergo several fates.[36] Elimination of the cyanoethyl group or reaction with traces of water or the anion of dichloroacetic acid and cleavage of the bond marked **a** leads to DMTr-*C*-phosphonate **7**. Compound **7** is presumably extended in subsequent synthesis cycles to give late-eluters **3**. Reaction of **6** and scission of the bond marked **b** leads to component **8**, wherein the

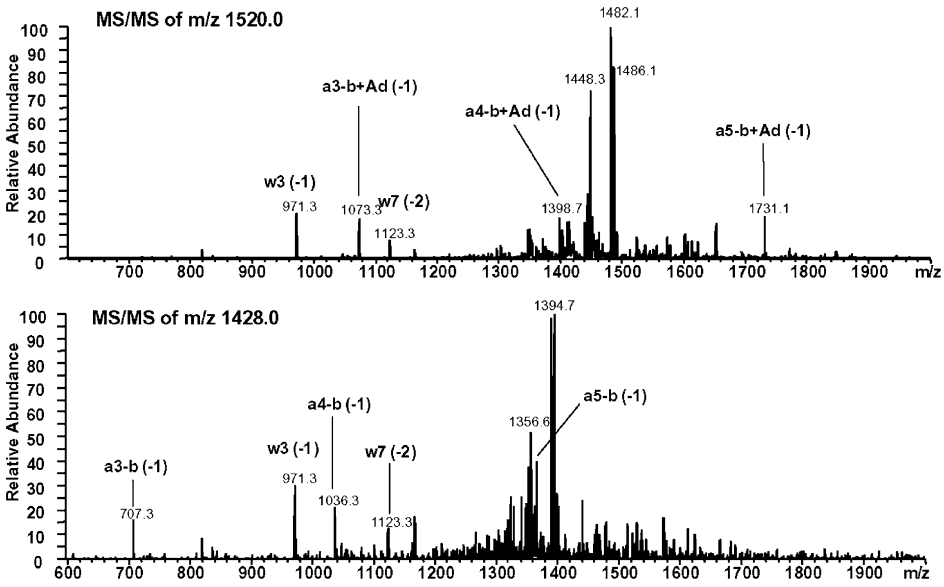
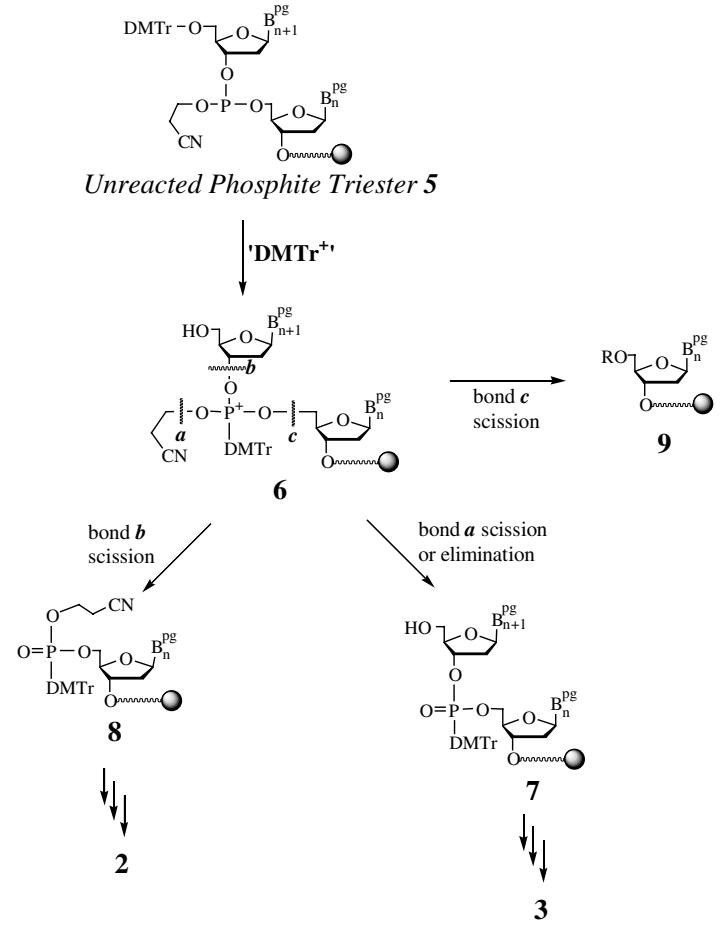


Figure 4. Collision-induced dissociation mass spectrum of **1-d(GC)** at $m/z = 1428$ (lower) and **2c** (upper) establish that the DMTr-*C*-phosphonate modification is located at the 5′-terminus.



Scheme 4.

DMTr-*C*-phosphonate diester moiety is located at the 5′-hydroxy group. This effectively caps the molecule and prevents any further coupling. The cyanoethyl group of **8** is removed during ammonolysis at the end

of synthesis to give late-eluters **2**. Note that the third option open to **6**, that is, cleavage of the bond marked **c**, results in loss of the phosphonate grouping. It should be noted that the reactions as described in Scheme 4 predict that when oligonucleotides are synthesized in a 'DMTr-On mode,' formation of the specific late-eluter **2b**, which lacks the 5′-terminal residue of the parent oligonucleotide, is not possible. The results obtained above were consistent with this prediction.

Suboptimal sulfurization conditions during solid-phase synthesis of phosphorothioate oligonucleotides lead to formation of process-related impurities **2** and **3** containing 'acid-stable' DMTr groups. A combination of traditional and state-of-the art analytical methods led to the conclusion that oligonucleotides **2** consist of a group of deletion sequences of **1** that terminate at the 5′-terminus in a DMTr-*C*-phosphonate monoester group. Oligonucleotides **3** containing an internal DMTr-*C*-phosphonate diester moiety were also identified. Although the work described above deals exclusively with the synthesis of phosphorothioate oligonucleotides, the conclusions reached would be expected to apply in a broader sense to any oligonucleotide synthesis in which a stepwise oxidation step is followed by an acid-catalyzed DMTr removal.

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