Pergamon

*Tetrahedron Letters*, Vol. 38, No. 22, pp. 3875-3878, 1997
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0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)00798-3

# On the Formation of Longmers in Phosphorothioate Oligodeoxyribonucleotide Synthesis

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**Abstract**: The extent of longmer formation in phosphorothioate oligodeoxyribonucleotide synthesis through amidite chemistry on solid support depends on base composition, contact time and acidity of the promotor used for activation of the phosphoramidite. A longmer formation mechanism that involves dedimethoxytritylation of the phosphite triester intermediate is proposed.
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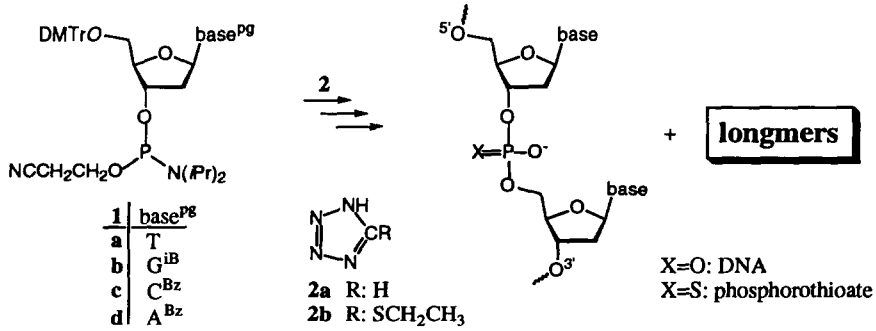
Phosphorothioate oligodeoxyribonucleosides are currently in clinical development as drugs for the treatment of diseases through an antisense mechanism of action.[1,2] Due to its high efficiency, phosphoramidite coupling[3] followed by stepwise sulfurization is the preferred method for phosphorothioate oligonucleotide solid phase synthesis.[4-6] The synthesis cycle starts with removal of the 5'-protecting group, typically 4,4'-dimethoxytrityl (DMTr), from the support bound nucleoside with an organic acid. In the subsequent coupling step, a protected phosphoramidite (**1a-d**) is activated with a weak organic acid (**2a,b**) and mixed with the solid support for a short period of time (contact time) to form a phosphite triester linkage. Sulfurization and a capping step complete the synthesis cycle. Further repetitions of this cycle yields the fully protected oligonucleotide which is cleaved from the solid support and deprotected by treatment with base, typically 30% ammonium hydroxide.




| 1 | base^Pg |
|---|---|
| a | T |
| b | G^iB |
| c | C^Bz |
| d | A^Bz |

**Scheme 1.** Oligonucleotide synthesis through phosphoramidite chemistry.

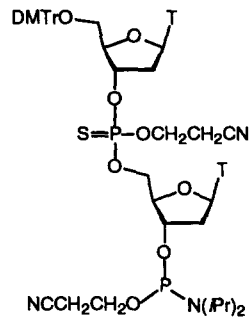
Separation-based analysis of oligonucleotide products at single base resolution is achieved by capillary gel electrophoresis (CGE). Detected process-related oligonucleotide impurities include 'shortmers' [(n-1)-, (n-2)-mers, etc.] and a small quantity of phosphodiester-containing oligonucleotides.[7] As part of our ongoing efforts to improve further the efficiency of solid phase synthesis and the quality of oligonucleotide products, we have recently been concerned with the formation of higher molecular weight products, referred to herein as 'longmers'

[(n+1)-, (n+2)-mers, etc.]. In this communication we describe for the first time a systematic investigation of the parameters which determine the extent of longmer formation and propose a mechanism for their formation.

The formation of longmers may conceivably occur in two ways, coupling of dimeric amidite synthons formed from monomers and/or by successive coupling of two monomer amidites, during a single coupling step. In both scenarios, loss of the acid labile DMTr group from the 5'-oxygen of a nucleotide is necessary. The standard reagent for activation of **1a-d** is 1*H*-tetrazole (**2a**). Among others, *S*-ethylthio tetrazole (**2b**) has been recommended, especially for RNA synthesis.[8] Both activators are organic acids of considerable acidity (**2a**: $pK_a$ 4.8; **2b**: $pK_a$ 4.28)[9], and could thus potentially cause partial detritylation of monomers or oligomers.

Activation of **1a-d** with **2a,b** is indeed accompanied by dedimethoxytritylation.[10] $^{31}P$ NMR spectra of solutions of **1a-d** and **2a,b** in $CD_3CN/CH_3CN$ were recorded over a period of 2-4 days. Resonances at 139-140 ppm in mixtures of **1** and **2**, which may be assigned to trialkyl phosphite linkages, increase in intensity over time as would be expected for dedimethoxytritylation followed by amidite coupling. At phosphoramidite concentrations ranging from 0.1-0.2 M and a 2.5-3.5 fold tetrazole excess, rates of phosphite formation were between 0.1 to 0.5 %/h. dG amidite detritylates fastest, followed by dA and dC, with T being slowest.

To assess the significance of such side reactions more directly we have conducted competition experiments between **1d** and a DMTr-T-P(*S*,*O*CE)-T phosphoramidite[7] (**3**), a model for dimer amidites formed during the activation of **1d**. We synthesized three $T_{19}$ phosphorothioate oligonucleotides through monomer coupling using the ABI standard protocol [contact time 100 s, 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (0.2 M in $CH_3CN$)], replacing **1d** (0.1 M, $CH_3CN$) in either the first coupling, coupling 9 or the last coupling with a 1:1 mixture of **1d** and **3** (0.1 M, $CH_3CN$). CGE analysis of the crude product oligonucleotides showed that the ratios of $T_{19}$ to $T_{20}$ are close to 1:1 (actual values range from 46:54 to 49:51). It appears that thymidine dimer amidites couple at rates similar to **1d**, independent of their ultimate position in the oligonucleotide. Therefore, small amounts of dimer amidites formed during the activation step could potentially contribute to (n+1)-mer content. However, considering the short mixing times of amidites and activator, on the order of seconds in an ABI 394 synthesizer, the amount of dimer amidite formed during activation appears too small to contribute substantially to the (n+1)-mer content observed.




**3**

Base-protected 3'-*O*-levulinyl- 5'-*O*-dimethoxytrityl nucleosides DMTr$O^{5'}$-dN-$O^{3'}$Lev (dN = T, $dG^{iB}$, $dC^{Bz}$, $dA^{Bz}$) (0.1 M, $CH_3CN$) treated with **2a** or **2b** undergo rapid dedimethoxytritylation as followed by HPLC (Table 1).[11] Deprotection rates are nucleobase dependent and purines are dedimethoxytritylated more rapidly than pyrimidine nucleosides. The stronger acid **2b** effects DMTr removal significantly faster than **2a**. Increasing the concentration of **2b** increases the rate of dedimethoxytritylation even further.

Both phosphoramidites and protected nucleosides are dedimethoxytritylated in the presence of **2** in a nucleobase- and activator- dependent manner. Systematic variation of these two parameters in phosphorothioate oligonucleotide synthesis on a solid support thus should translate to the resulting ratio of n-mers to longmers. We therefore synthesized a series of phosphorothioate oligonucleotides with the generic sequence $(TdNdN)_6T$ (dN = T, dG, dC, dA) on controlled-pore glass

**Table 1.** $t_{1/2}$ [h] of DMTr$O^{5'}$-dN-*O*Lev (c = 0.1 M) in presence of **2** in $CH_3CN$ at rt.

| activator, (c, [M]) | T | dN = $dG^{iB}$ | $dC^{Bz}$ | $dA^{Bz}$ |
|---|---|---|---|---|
| **2a** (0.45) | 86 | 43 | 86 | 54 |
| **2b** (0.45) | 15 | 6.5 | 33 | 15 |
| **2b** (0.90) | 5 | 2.2 | 9.2 | 4 |

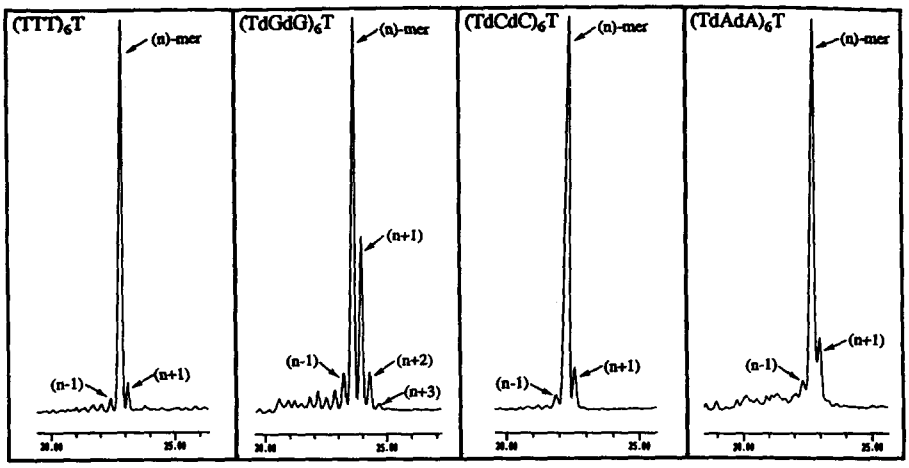



**Figure 1.** Capillary gel electropherograms of phosphorothioate oligonucleotides $(TdNdN)_6T$ synthesized on CPG with *S*-ethylthio tetrazole (0.45 M) as activator, and 3000 s contact time.

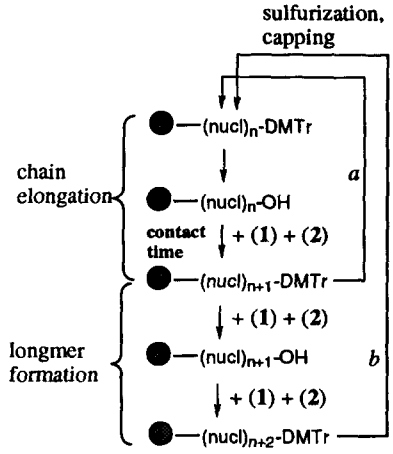
(CPG) on an ABI 394 Oligonucleotide Synthesizer. Synthesis parameters were varied over a wide range, from 100 s contact time using **2a** as activator to as long as 3000 s using **2b** as activator. To avoid purification-related changes in product composition, oligonucleotides were analyzed at the crude stage by CGE (Table 2). Authentic (n+1)-mers [$(TdNdN)_3dX(TdNdN)_3T$, dX = T or dN] coelute with the (n+1)-peak, supporting our structural assignment. With **2a** as the activator an increase in relative peak area of the (n+1)-mer from ca. 0.3-0.9 % to more than 8% is observed as the contact time was increased from 100 s to 3000 s. A very pronounced dependence on base composition is also evident. The increase of (n+1)-mer content in the order T < dC < dA < dG resembles the rate of dedimethoxytritylation: the faster the deprotection of nucleosides and nucleotides the more longmers are obtained in the oligomerization. A significant increase in the level of longmers is also observed when **2b** is used as activator instead of **2a**. (Figure 1) Under more drastic conditions, (n+2)- and (n+3)-mers begin to emerge in the electropherogram. The total quantity of (n)-mer and longmers remains more or less constant when coupling time is increased from 100 s to 3000 s, but due to longmer formation, the amount of the target (n)-mer falls. The same result is seen when comparing oligonucleotides synthesized using **2a** with those synthesized using **2b**: more longmers are formed in the synthesis using **2b** as activator, at the expense of the (n)-mer. It becomes apparent that extended coupling times and use of more acidic activators in the synthesis of standard oligo*deoxy*ribonucleosides leads to a reduction in full length material due to formation of longmers.

**Table 2.** Ratio of (n):(n+1):(n+2)-mer etc. of phosphorothioate oligodeoxyribonucleotides $(TdNdN)_6T$.[a]

| dN | activator | contact time: 100 s | 1000 s | 3000 s |
|---|---|---|---|---|
| T | **2a** | 99.7:0.3 | 99.0:1.0 | 97.4:2.6 |
| T | **2b** | 99.0:1.0 | 97.1:2.9 | 94.3:5.7 |
| dG | **2a** | 99.1:0.9 | 96.6:3.4 | 91.9:8.1 |
| dG | **2b** | 95.7:4.3 | 80.9:17.0:2.1 | 65.4:27.1:6.2:1.3 |
| dC | **2a** | 99.3:0.7 | 97.1:2.9 | 97.1:2.9 |
| dC | **2b** | 99.0:1.0 | 96.6:3.4 | 91.6:8.4 |
| dA | **2a** | [b] | [b] | 94.4:5.6 |
| dA | **2b** | [b] | [b] | 84.7:14.0:1.3 |

[a] Determined by CGE. [b] Poor resolution.

These results demonstrate that longmers [(n+1)-, (n+2)-mers, etc.] are formed during solid phase synthesis of oligodeoxyribonucleosides in a base-, activator-, and contact time-dependent manner. The longmer content increases with increasing contact time at the expense of the desired full length oligonucleotide, which is in line with our mechanistic proposal that longmers are formed by successive addition of two molecules of phosphoramidite during one coupling step (Scheme 2). The contribution of each base to the total (n+1)-mer content increases in the order T < dC < dA < dG. It may also be inferred from these results that, especially in case of sterically more demanding phosphoramidites, e.g. modified ribonucleosides, choices concerning coupling time and activator need careful consideration in order to reduce longmer formation while at the same time maintaining maximum coupling efficiency.




**Scheme 2.** Oligonucleotide synthesis on solid support (*a*) and formation of longmers through successive coupling of two phosphor amidites in one coupling step (*b*).

**Acknowledgment:** The authors like to thank Patrick Wheeler for his help (NMR).

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11. HPLC: $C_{18}$ column (Waters Nova Pak) 3.9 x 300 mm, flow rate 1.0 ml/min, $CH_3CN$ (A), $H_2O$, gradient: 0-15 min: 2 to 98% A, 15 to 25 min: 98% A, $\lambda = 254$ nm.

(*Received in USA* 27 *March* 1997; *revised* 21 *April* 1997; *accepted* 22 *April* 1997)