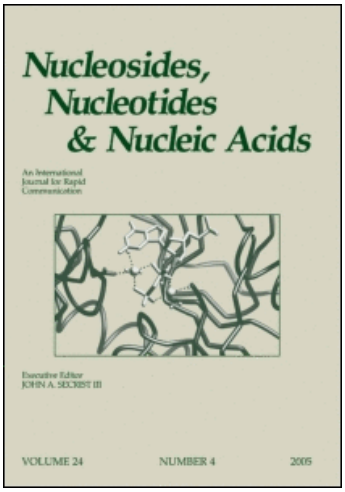

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The Fluoride Cleavable 2-(Cyanoethoxy)Methyl (CEM) Group as Reversible 3'-*O*-Terminator for DNA Sequencing-by-Synthesis—Synthesis, Incorporation, and Cleavage

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THE FLUORIDE CLEAVABLE 2-(CYANOETHOXY)METHYL (CEM) GROUP AS REVERSIBLE 3′-*O*-TERMINATOR FOR DNA SEQUENCING-BY-SYNTHESIS—SYNTHESIS, INCORPORATION, AND CLEAVAGE

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□ *A new and promising sequencing technology called sequencing-by-synthesis (SBS) enables fast determination of DNA sequences. 2′-Deoxynucleotides containing the (2-cyanoethoxy)methyl (CEM) group at the 3′-*O*-position are potential reversible terminators for the* SBS *technology. Herein we describe the synthesis, the incorporation by several polymerases, and the cleavage of this 3′-*O*-blocking group using 3′-*O*-CEM-thymidinyl-5′-*O*-triphosphate* **7** *as an example.*

Keywords DNA sequencing-by-synthesis; reversible terminator; triphosphate synthesis; 3′-O-alkylation

INTRODUCTION

Today's methods for DNA sequencing are mainly limited by the use of gel electrophoretic separation of the extension products.[1] Future demands for understanding, diagnostics, treatment, and prevention of diseases however create a need for faster and more cost effective sequencing methods. The idea of cyclic-array sequencing-by-synthesis (SBS) has the

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potential to fulfil these needs. This method is based on incorporation and immediate identification of every single nucleotide; hence, it is not based on gel electrophoresis. There are already some systems that are based on SBS-technologies such as fluorescent in situ sequencing[2] and pyrosequencing.[3] Drawbacks of these methods are that they need an amplification of the DNA to be sequenced and that they can be inaccurate concerning the length of homopolymeric sequences. A new and promising approach for the realisation of the SBS-technology without the above mentioned disadvantages is the use of 3′-*O*-modified, reversibly terminating, dye labelled nucleotides.[4,5] Here the polymerase reaction is stopped after each incorporation step and the nucleotide can be identified by the spectrum of the attached fluorescent dye. After identification the 3′-*O*-modification is cleaved and the next cycle starts. The ultimate object of this approach is the direct sequencing of single molecules of DNA arranged on an array to avoid the amplification step.

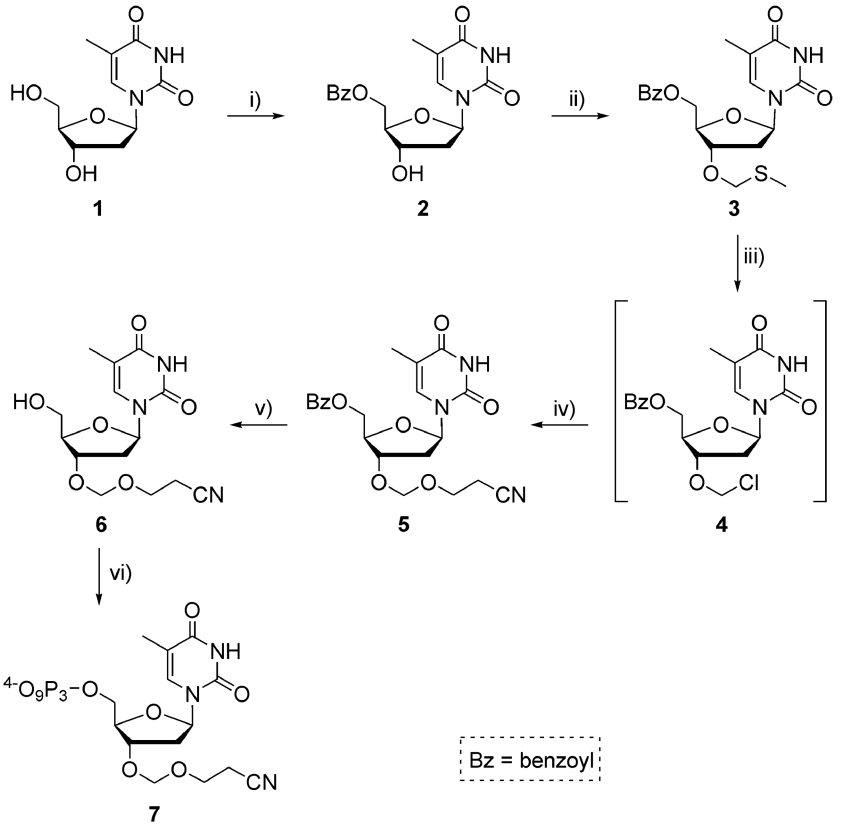
A protecting group for the 3′-*O*-position is suitable for SBS if it effectively terminates the polymerase reaction and can be removed fast and quantitative afterward. We turned our attention to a publication describing the novel (2-cyanoethoxy)methyl (CEM) 2′-*O*-protecting group for RNA synthesis.[6] They found that this group is readily removable upon treatment with tetrabutylammonium fluoride (TBAF) in dry tetrahydrofuran (THF). We asked the question if the mentioned group could be used for protection of the 3′-*O*-position in deoxynucleotides, and further, if the deprotection under such nonbiological conditions is possible without affecting the duplex structure of the DNA-template. After performing a series of experiments described herein we found the answer to be positive.

Synthesis of 3′-*O*-(2-Cyanoethoxy)methyl-thymidinyl-5′-*O*-triphosphate 7

The synthesis of the new 3′-*O*-modified nucleotide **7** is shown in Scheme 1. The formacetal moiety of the modification was introduced by using the 3′-*O*-methylthiomethyl substituted nucleoside **3**.[7] This was converted into its reactive chloro-derivative **4** which then reacted in situ with 3-hydroxypropionitrile to form the CEM group at the 3′-*O*-position of nucleoside **5**. The corresponding triphosphate **7** was synthesised according to the Ludwig-Eckstein procedure[8] and after ion exchange FPLC and RP-HPLC the pure triphosphate **7** was obtained.

Incorporation Tests

For the incorporation tests different commercially available DNA polymerases and thermostable polymerases modified by Fermentas UAB were



SCHEME 1 Synthesis of 3′-*O*-CEM-thymidinyl-5′-*O*-triphosphate **7**. i) BzCl, dry pyridine, 3.5 hours, at 0°C, 87%; ii) DMSO, AcOH, Ac_2O, 25 hours at room temperature, 81%; iii) a) Et_3N, 3 Å MS, dry DCE, 2 hours at room temperature b) SO_2Cl_2, 1 hour at 0°C; iv) 3-hydroxypropionitrile, 10 minutes at 0°C, 2 hours at room temperature, 55%; v) MeOH, 32% aq. NH_3, 5 hours at room temperature; vi) a) 1 M 2-chloro-4*H*-1,2,3-benzodioxaphosphorin-4-one in dry 1,4-dioxane, dry pyridine, dry DMF, 20 minutes at room temperature b) 0.5 M (n-$Bu_3N)_2H_2P_2O_7$, dry DMF, n-Bu_3N, 45 minutes at room temperature c) 1% I_2 in pyridine/H_2O 98:2, 30 minutes at room temperature d) 32% aq. NH_3, 15%.

used. They were incubated for 60 minutes at 37°C (buffer Tango) with 50 μM 3′-*O*-CEM-dTTP and 10 nM of the radioactively (^{33}P) labelled DNA duplex:

5′-^{33}P-TGCAGGCATGCAAGCTTGGCGTA
3′- ACGTCCGATCGTTCGAACCGCATAAAAAAAAAAAA

After 60 minutes of incubation, 100 μM dTTP were added and the reaction was allowed to proceed for additional 15 minutes at 37°C.

Figure 1 shows the results of the polymerase screening. The best results of incorporation and termination were achieved with pol6″. The

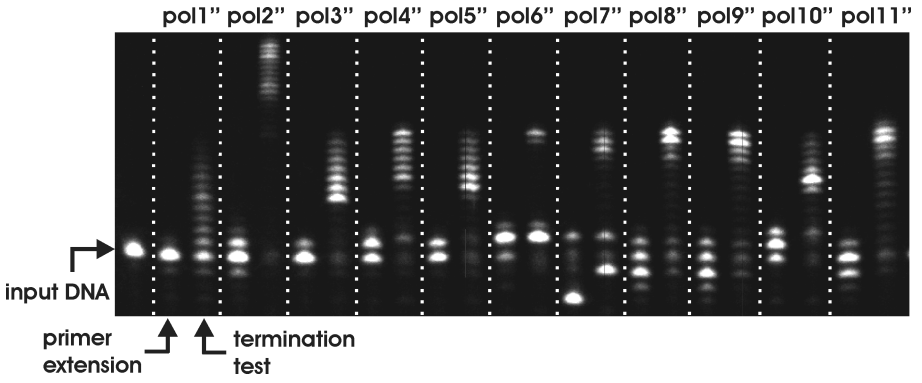


FIGURE 1 Polyacrylamid gel of the incorporation of 3′-*O*-CEM modified dTTP **7** by several polymerases, detection was carried through the radioactively (^{33}P) labelled DNA duplex.

modified nucleotide **7** is accepted very well and shows efficient terminating properties.

Cleavage Experiments

Cleavage experiments were performed using a 24-mer oligonucleotide that contains the 3′-*O*-modified thymidine at the 3′-end (5′-TGCAGGC ATGCAAGCTTGGCGTAT$_{CEM}$-3′). The 3′-*O*-modified thymidine was incorporated by the corresponding 5′-*O*-phosphoramidite made in our

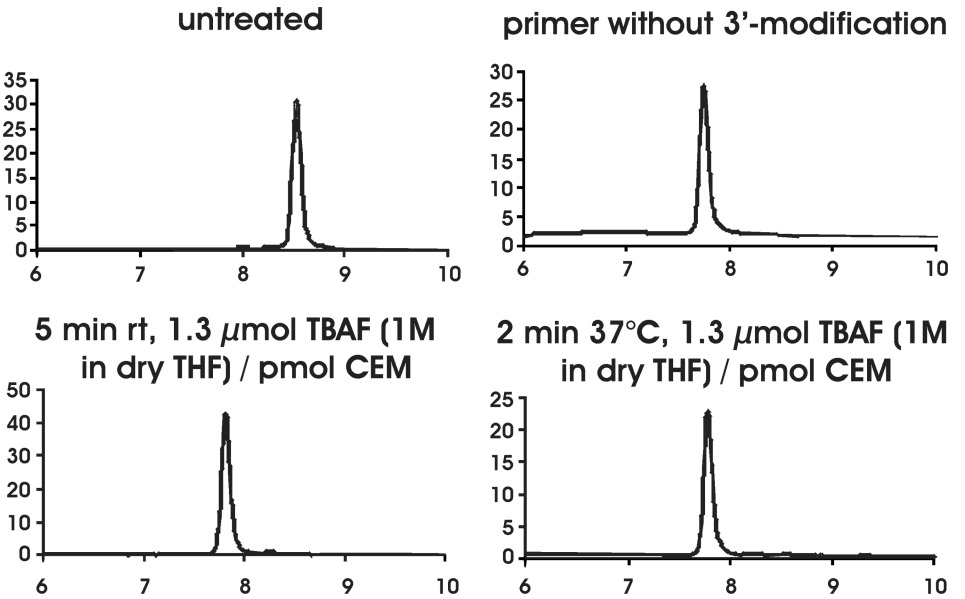


FIGURE 2 Analysis of the cleavage experiments of 3′-*O*-CEM with TBAF in dry THF by RP-HPLC (Gemini C18, Phenomenex HPLC column), buffers: A: 5% ACN in 0.1 M TEAAc, B: 80% ACN in 0.1 M TEAAc, gradient: 0–40% B in 12 minutes.

laboratory. The deprotected and HPLC purified material was treated with TBAF under the specific conditions presented for every RP-HPLC chromatogram in Figure 2. The analysis was performed on a Gemini C18, Phenomenex HPLC column using buffer A: 5% ACN in 0.1 M TEAAc and buffer B: 80% ACN in 0.1 M TEAAc, gradient: 0–40% B in 12 minutes.

The results in Figure 2 show that the cleavage proceeded very fast when treating the oligonucleotides with 1.3 μmol TBAF (1 M solution in dry THF) per pmol CEM (5 minutes at room temperature, 2 minutes at 37°C), furthermore no side product formation was observed.

Outlook

With this new 3′-*O*-CEM modified nucleotide **7** a proof of principle for the suitability of this protecting group as a reversible terminator could be achieved. A polymerase was identified that accepts the 3′-*O*-modification very well and efficient termination could be observed. The cleavage under the expected conditions also proceeded very quickly and quantitatively. At the moment the syntheses and testing of the other three modified nucleotides as well as the labelling with fluorescent dyes are in progress.

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