Synthetic Biology

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Enzymatic Synthesis of Phosphonomethyl Oligonucleotides by Therminator Polymerase**

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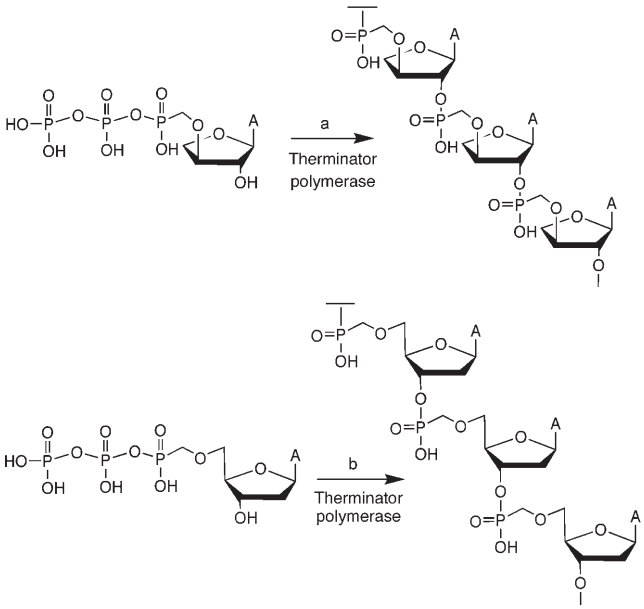
Projects in synthetic biology imply the study of the substrate specificity of natural and non-natural biopolymers with enzymatic activity and the identification of the products that are obtained. Herein we describe the enzymatic synthesis of 3′–2′ phosphonomethyl–threosyl and 5′–3′ phosphonomethyl–deoxyribosyl oligonucleotides by Therminator polymerase (Scheme 1). We demonstrate that phosphonate nucleotides can be polymerized by this enzyme with the formation of oligonucleotides with more than six internucleotide bonds.

The enzymes that catalyze the formation of the 5′–3′ phosphodiester bond in DNA and RNA are polymerases and use nucleoside triphosphates as substrates. Therminator polymerase is a mutant variant of the 9°N exo$^-$ polymerase (*Thermococcus* species 9°N-7), in which the Ala 485 residue has been replaced with a Leu residue. With this mutation in an α helix that is oriented away from the nucleotide-binding site, Therminator polymerase possesses an enhanced ability to incorporate modified substrates, such as dideoxynucleosides, ribonucleosides, and acyclic nucleosides, by using their triphosphate moiety as a substrate.[1–3]

We envisaged the synthesis of 3′–2′ phosphonomethyl–threosyl oligonucleotides to study their hybridization potential. Because of the innate stability of the phosphonate linkage, such oligonucleotides would be useful for synthetic biology.[4] As the chemical synthesis of oligonucleotides with phosphonate linkages is much more difficult than the synthesis of the parent oligomers with phosphate linkages, we viewed the application of an enzymatic route to the synthesis of these modified oligomers as an important and challenging objective to explore. The enzymatic production of phosphonomethyl–threosyl oligonucleotides with a non-natural 3′–2′ linkage has never been described.



Scheme 1. Enzymatic synthesis of phosphonomethyl oligomers by Therminator polymerase: a) phosphonomethyl–threosyladenine oligomers; b) phosphonomethyl–deoxyadenosine oligomers.

The recognition of phosphonate nucleoside diphosphates by various polymerases was tested previously when the antiviral activity of acyclic phosphonomethoxy nucleosides was investigated.[5] The isopolar and isosteric character of these compounds seemed to be of utmost importance for the recognition of the nucleoside triphosphate analogues by enzymes.[6,7] The acyclic phosphonomethoxy nucleosides, which have antiviral activity, act as chain terminators; that is, the elongation stops after the incorporation of only one phosphonate nucleoside, with the exception of the diphosphate derivative of HPMPC ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine)). Two consecutive units of the diphosphate derivative of this nucleotide analogue were incorporated into a DNA primer–template complex by HCMV (human cytomegalovirus) DNA polymerase before the elongation stopped (indirect chain termination).[8]

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α-Phosphonomethyl nucleoside diphosphates have also been studied extensively as potential substrates for polymerases. Victorova et al. showed that 2′-deoxythymidine 5′-(α-methylphosphonyl) β,γ-diphosphate was recognized by several DNA polymerases.[9] HIV reverse transcriptase was able to extend the DNA primer with eight units of the molecule. A reaction with terminal deoxynucleotidyl transferase and this nucleotide analogue resulted in the addition of one or two building blocks to the initiating primer.[10] A mixed sequence of 2′–5′-(α-methylphosphonyl)–deoxythymidine with natural deoxyribonucleosides has been synthesized in the presence of *E. coli* DNA polymerase I (Figure 1).[11]

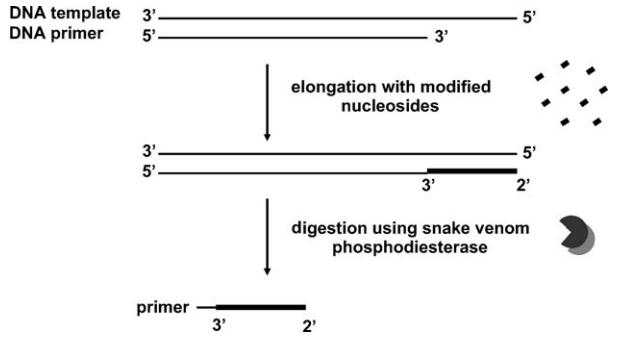

Figure 1. Elongation of the primer with phosphonate nucleosides and digestion of the elongated primer with snake-venom phosphodiesterase yields 5′-phosphorylated probes for ligase experiments.

Deoxyribo oligonucleotides with methylphosphonate linkages ($CH_3P(O)(OR)(OR')$) have been studied previously in view of potential antisense applications.[11,12] The phosphonomethyl oligomers are synthesized from the 3′ to the 5′ end by a combination of the phosphoramidite and the phosphotriester method.

5′-*O*-Phosphonomethyl ribonucleoside diphosphates have been studied by Holý et al.[13] They proved to be efficient inhibitors of *E. coli* polynucleoside phosphorylase. In a study with 5′-*O*-phosphonomethyladenine diphosphate, in which the phosphonomethyl group is in the α position, mixed di- and trinucleotides with one modified internucleotide bond could be formed by *E. coli* RNA polymerase.[14] When the phosphonomethyl group was localized in another position in the acidic part of the molecule, the analogues became very poor substrates for the DNA-dependent RNA polymerase. Cvekl et al. showed that 5′-*O*-phosphonomethyl and 3′-*O*-phosphonomethyl analogues of diribonucleotides can act as the initiating molecules in the primed abortive synthesis catalyzed by *E. coli* RNA polymerase.[15] In contrast, no elongation was observed when these dinucleotide analogues were used as the priming dimers for polymerization catalyzed by wheat-germ RNA polymerase II.[16]

As an efficient enzymatic polymerization with phosphonate nucleosides has never been described, we investigated the recognition of the diphosphate derivative of phosphonomethylthreosyladenine (PMTA) by polymerases. L-Threose nucleoside triphosphates are recognized by Vent exo⁻ DNA polymerase and HIV reverse transcriptase[17,18] and have been polymerized successfully by Therminator polymerase up to a length of more than 80 nucleotides.[19] A wide spectrum of polymerases was selected for the synthesis of these "exotic" oligonucleotides. The RNA-dependent RNA polymerase of hepatitis C virus,[20] HIV reverse transcriptase (reverse-transcriptase family), Vent exo⁻ DNA polymerase (a family B DNA polymerase), Taq DNA polymerase (a family A DNA polymerase), and terminal deoxynucleotidyl transferase (TdT, a family X DNA polymerase) could recognize the modified nucleoside. However, only Vent exo⁻ DNA polymerase could extend the primer with more than two phosphonate nucleoside diphosphates in a row; a primer + 6 product was obtained. HIV reverse transcriptase (HIV RT), the RNA-dependent RNA polymerase of hepatitis C virus, Taq polymerase, and TdT could incorporate one phosphonate nucleoside diphosphate, although weak incorporation of a second phosphonate diphosphate was visible for TdT and HIV RT (data not shown).

Remarkably, incorporation of the threosyl phosphonate nucleotides by Therminator polymerase into the DNA primer–template complex afforded a primer + 10 product (Figure 2). The elongated product was characterized by LC–ESIMS. For this purpose, the elongation reaction was

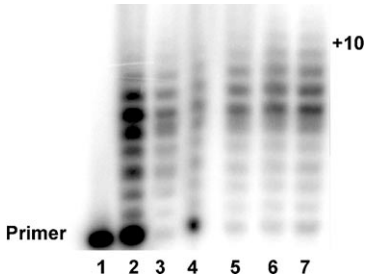

Figure 2. Incorporation of PMTApp by Therminator polymerase in P_1T_1: Lanes 2–7 correspond to reaction times before quenching of 0.5, 1, 2, 3, 4, and 24 h; lane 1 contains the primer; [P_1T_1] = 50 nM, [Therminator polymerase] = 0.4 U μL^{-1}, [PMTApp] = 100 μM.

quenched after 30 min, and the sample was analyzed. The primer + 3, primer + 4, and primer + 5 were detected (Table 1). The elongated primer was subjected to digestion with snake-venom phosphodiesterase. The primer was largely degraded, but the phosphonate oligonucleotides were found to be resistant to degradation by this 3′–5′ phosphodiesterase. Phosphonate oligonucleotides with three to five phosphonate adenine nucleosides and a deoxyguanosine monophosphate moiety at the 5′ end were detected (Figure 3).

Table 1: Calculated and measured *m/z* values for the elongated primer P_1. The phosphonate nucleosides are shown in bold.

m/z		Oligonucleotide
calculated	measured	
8736	8735	GGGTACGACTCACTATAGGGAGAGG**AAA**
9049	9048	GGGTACGACTCACTATAGGGAGAGG**AAAA**
9362	9362	GGGTACGACTCACTATAGGGAGAGG**AAAAA**

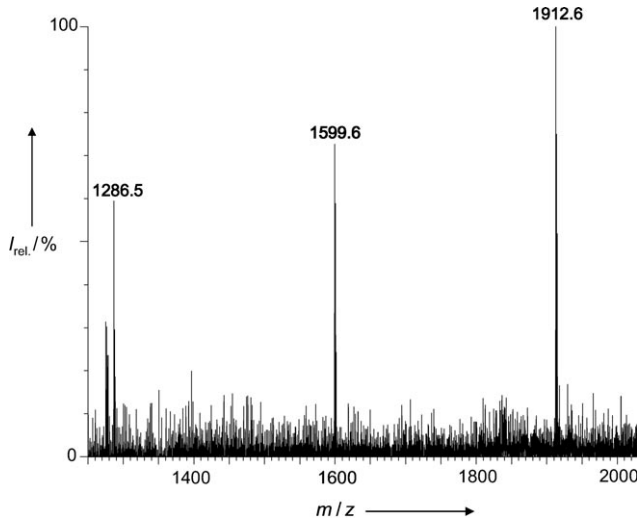

Figure 3. The deconvoluted mass spectrum after digestion of the oligonucleotides in Table 1 with snake-venom phosphodiesterase. The masses correspond to phosphonate oligomers with three to five PMTA molecules and a monophosphate guanosine moiety at the 3′ end. The *m*/*z* values for the oligomers containing four to six nucleosides are 1286.4, 1599.6, and 1912.6, respectively.

The formation of tri- to heptamers of phosphonate oligonucleotides with a deoxyguanosine monophosphate moiety at the 3′ end following degradation with snake-venom phosphodiesterase can be explained by the intrinsic endonucleolytic activity and/or residual 5′ nucleotidase activity of the enzyme (6.25% endonucleolytic activity relative to the 3′ exonucleolytic activity and/or residual 5′ nucleotidase activity of the enzyme).[21–23] This enzymatic activity degrades the DNA primer to leave a 5′-phosphorylated guanosine residue at the 3′ end of the phosphonate oligonucleotide. The exonucleolytic activity of the enzyme degrades the DNA primer and template to mononucleotides. This particular property of snake-venom phosphodiesterase could be very useful in the synthesis of modified oligonucleotides for cellular studies. Specifically the fact that a phosphorylated guanosine residue was left at the 3′ end of the phosphonate oligomer after digestion with snake-venom phosphodiesterase could provide a solution for the ligation problems often encountered with chimer construction of mixed DNA-modified nucleotide sequences. (Owing to the presence of modified nucleosides at the 5′ end, synthetic oligonucleotides are often poor substrates for kinases).

To determine whether the substrate properties of the diphosphate derivative of phosphonomethylthreosyladenine are unique for this particular sugar modification, the polymerization was carried out with the diphosphate derivative of the 5′-*O*-phosphonomethyl-2′-deoxyadenine analogue (PMdApp). Incubation with Therminator polymerase led to the formation of a product with more than 15 successive phosphonate nucleosides (see the Supporting Information). This observation is remarkable, as it shows that Therminator polymerase is not only able to synthesize oligonucleotides with five[17,18] and six internucleotide bonds, but even seven. Repetition of the experiment in the absence of PMdApp or after the substitution of the phosphonate nucleosides with pyrophosphate led to no incorporation at all and demonstrated that the observed elongation is caused by the phosphonate nucleosides (data not shown). The elongation of the primer with 5′-*O*-phosphonomethyl-2′-deoxyadenine involved the addition of more than 20 phosphonate nucleosides. As the template overhang is only 20 nucleotides long, this result demonstrates the terminal transferase activity of Therminator polymerase. The discovery that family B polymerases are able to synthesize metabolically and chemically stable phosphonate oligonucleotides with the unusual 3′–2′ linkages, as well as 5′-*O*-phosphonomethyl oligonucleotides, was also rather unexpected.

It is not clear why the elongation with the threosyl derivative stops after the incorporation of ten nucleotide analogues. One of the reasons could be that the primer elongated with phosphonate nucleosides does not hybridize well enough with the template, thus leading to a frayed duplex at the growing end of the primer–template complex. Until now, it was only possible to determine the thermal stability of phosphonate dimers because of the lack of a method for the synthesis of longer oligomers.[24]

As Therminator polymerase is able to catalyze the condensation of the diphosphate derivatives of both phosphonomethylthreosyladenine and 5′-*O*-phosphonomethyl-2′-deoxyadenine to oligomers, an alternative (to chemical synthesis) and much more straightforward method to obtain small amounts of homopolymers of these oligonucleotides has been developed. The oligonucleotides synthesized by this enzymatic approach show enhanced stability towards nucleolytic degradation: The synthetic nucleotide monomers are substrates for polymerases, but the oligomers obtained are not substrates for nucleases. An enzymatic route for the synthesis and isolation of phoshonate oligomers can be regarded as an important step towards making these non-natural polymers available for further studies.[25] The 5′-phosphorylated semisynthetic oligonucleotides obtained after phosphodiesterase degradation of the polymerization products may be used directly for incorporation in plasmids and investigation of the transliteration of this enzymatically stable potential information system.[7]

Experimental Section

Details on the experimental procedures can be found in the Supporting Information.

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