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Bioreductive deprotection of 4-nitrobenzyl group on thymine base in oligonucleotides for the activation of duplex formation

Hisao Saneyoshi *, Yuki Hiyoshi, Koichi Iketani, Kazuhiko Kondo, Akira Ono *

Department of Material and Life Chemistry, Faculty of Engineering, Kanagawa University, 3-27-1 Rokkakubashi, Kanagawa-ku, Yokohama 221-8686, Japan

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ABSTRACT

Oligonucleotides containing 4-*O*-(4-NO_2-benzyl)thymine residues were synthesized to assess potential prodrug-type action against hypoxic cells. These modified oligonucleotides were incapable of stable duplex formation under non-hypoxic conditions. However, following deprotection of the thymine residues under bioreductive conditions, the deprotected oligonucleotides were able to form stable duplexes with target oligonucleotides.

Various nucleobase-protecting groups have been used in the chemical synthesis of oligonucleotides.[1] In recent years, the application of protecting groups has expanded to include the control of nucleic acid functions.[2] For example, in the pioneering work by Kröck and Heckel, 2-nitrobenzyl and its congeners were installed onto thymine residues to generate light-activatable oligonucleotides.[3] Their study inspired our use of protected nucleobases to produce hypoxia-activatable oligonucleotides. Hypoxia is a characteristic property of locally advanced solid tumors resulting from the insufficient supply of oxygen from the poorly developed vasculature. Hypoxia induces increased resistance to both chemotherapy and radiation therapy.[4] Approaches to treating hypoxic tumors with drugs activated under such conditions have been reported.[5] These prodrugs are typically protected with hypoxia-labile protecting groups that mask binding sites to the target molecules. Under hypoxic conditions, these protecting groups are removed and the drugs are converted into their active forms.

In this report, we describe the synthesis of an oligonucleotide with a hypoxia-labile protecting group on one of the four nucleobases. Specifically, oligonucleotides containing 4-*O*-nitrobenzylated thymine residues as the prototype nucleobase were synthesized. The 4-NO_2-benzyl group is frequently used for hypoxia-activated prodrugs or to switch fluorescent probes on and off.[6–20] In normal cells (non-hypoxic), oligonucleotides containing 4-*O*-nitrobenzylated thymine residues (pro-oligos) do not hybridize to target RNA sequences (Fig. 1a). In hypoxic tumors, the 4-nitrobenzyl groups are deprotected via reduction of the nitro group to amino (or hydroxyamino) followed by a 1,6-elimination process to expel the active oligos (Fig. 1a and b). Thus, in the hypoxic tumor cell, a pro-oligo will be converted to an active oligonucleotide which hybridizes to the target sequence, thereby inhibiting mRNA translation.

For this study, we synthesized oligonucleotides containing 4-*O*-(4-NO_2-Bn)thymine residues, which were treated with nitroreductase for conversion into free oligonucleotides. The hybridization properties of the protected and the free oligonucleotides were investigated by thermal denaturation experiments.

Briefly, a protected thymidine **1** was reacted with triisopropylbenzenesulfonyl chloride to produce a 4-*O*-sulfonylated intermediate, which was subsequently substituted with 4-NO_2 benzyl alcohol to yield 4-benzylated derivative 2. Acetyl groups in **2** were deprotected with NH_4OH to produce **3** which was treated with 4,4′-dimethoxytrityl chloride to give **4**; then, phosphitylation of the free 3′-hydroxyl group with 2-(cyanoethoxy)-*N*,*N*-diisopropylaminochlorophosphine yielded the desired phosphoramidite derivative **5** (Scheme 1). Using the monomer unit 5, oligonucleotides

* Corresponding authors. Tel.: +81 45 481 5661; fax: +81 45 481 2781.
E-mail addresses: saneyoshih@kanagawa-u.ac.jp (H. Saneyoshi), akiraono@kanagawa-u.ac.jp (A. Ono).

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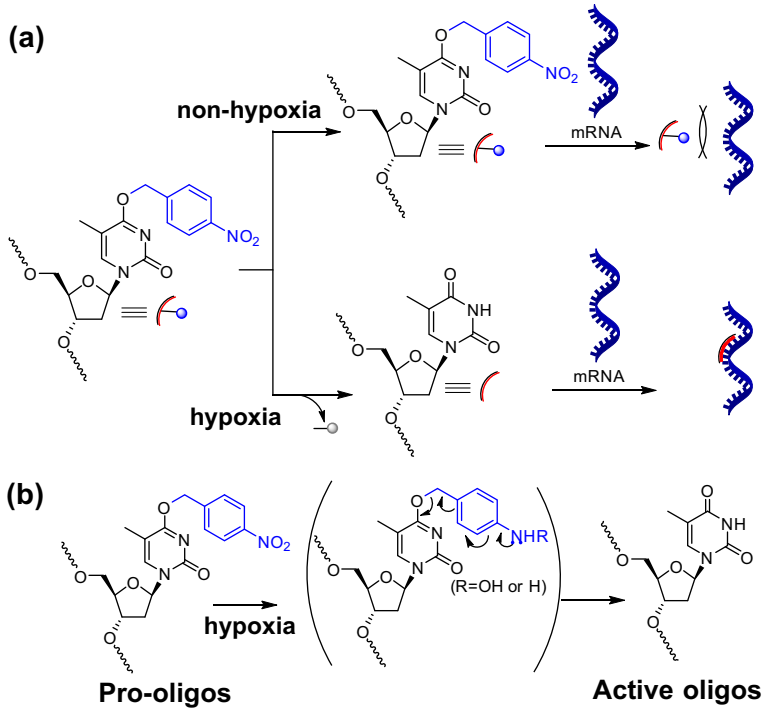
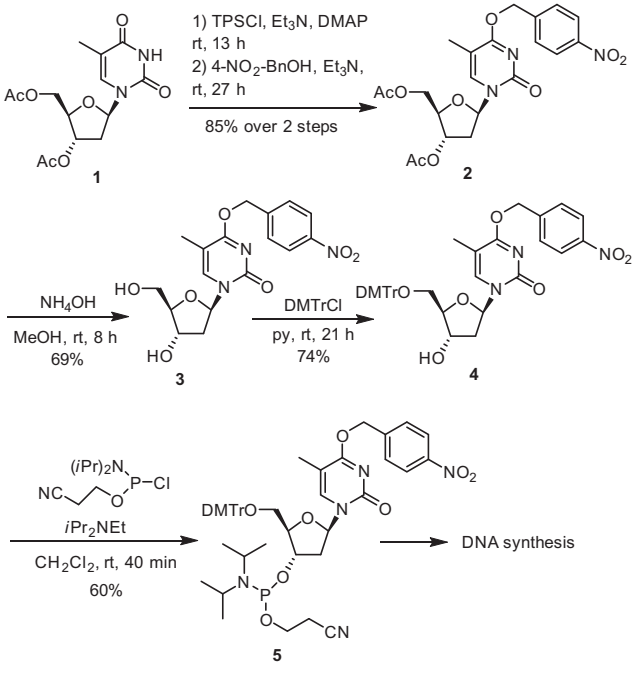


Figure 1. (a) Under non-hypoxic conditions, the pro-oligo does not hybridize to the target RNA. Under hypoxic conditions, the nitrobenzyl groups of the pro-oligo are deprotected and the free oligonucleotide forms a stable complex with the target RNA. (b) Proposed reaction process for the deprotection of 4-nitrobenzyl groups under hypoxic conditions.



Scheme 1. Synthetic route for the phosphoramidite derivative 5.

containing 4-*O*-benzylated thymine residue(s) were synthesized, deprotected, and purified using standard protocols. The purity of synthesized oligonucleotides was determined by HPLC and the structures were confirmed by MALDI-TOF mass spectroscopy (Supporting information).

As a model experiment for the deprotection of 4-NO_2-benzyl groups in oligodeoxyribonucleotides (ODNs) in hypoxic cells, ODN **1** (5′-TTXTT-3′) was treated with nitroreductase (from *Escherichia coli*) in the presence of NADH. Enzymatic reactions were monitored by HPLC. Typical HPLC profiles are shown in Figure 2. Before enzyme addition, a peak corresponding to ODN **1** was observed along with peaks corresponding to the NADH (Fig. 2b, top). After 1 min incubation with the enzyme, the peak corresponding to ODN **1** was diminished and a peak corresponding to a fully deprotected oligonucleotide (retention time 17.2 min) and contamination peak from enzyme sample (19.6 min) were observed (Fig. 2b, middle). The peak (17.2 min) was collected and the structure of the deprotected oligonucleotide was confirmed by MALDI-TOF mass spectroscopy. The other peak (19.6 min) was determined by control injection experiments described in Figure S1. ODN **1** was stable in buffer, with and without NADH or nitroreductase, for at least 1 h. Related HPLC profiles of control experiments are described in the Supplementary data (Fig. S1).

Next, a time course of the deprotection of multiple 4-nitrobenzyl groups in the mixed sequence ODN **5** (5′-CACXGCAXXGGXCAC-3′) was similarly analyzed (Fig. S2a). Before enzyme addition, a peak corresponding to ODN **5** was observed along with peak

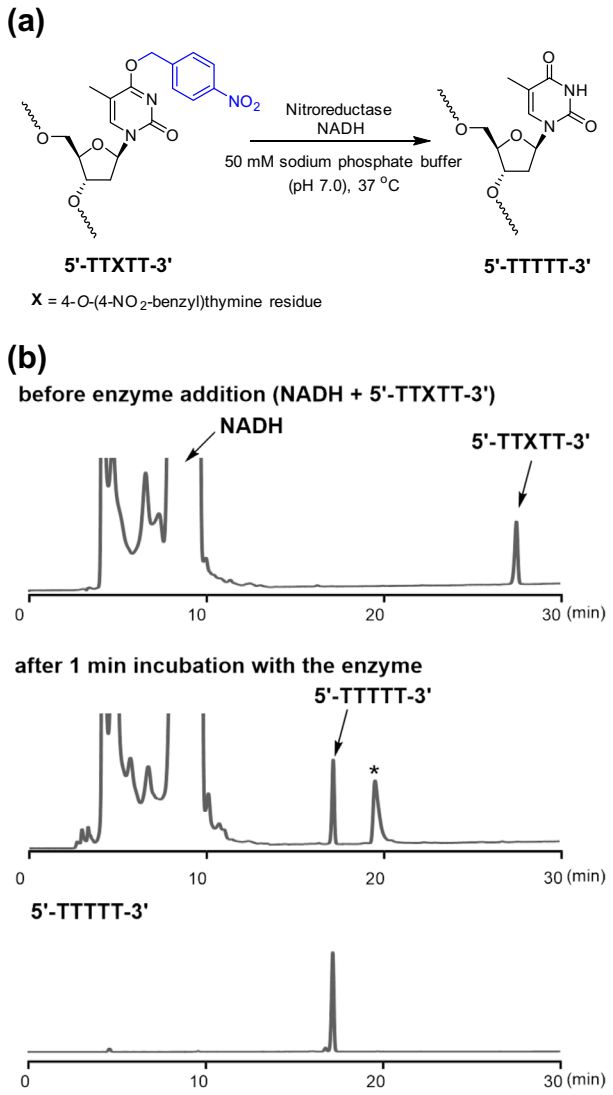


Figure 2. HPLC analysis of the bioreductive deprotection of a 4-NO_2-benzyl group on the thymine base in ODN **1**. (a) A schematic representation of enzymatic deprotection of 4-nitrobenzyl group on thymine base in ODN **1**. (b) ODN **1** (12 μM) was incubated with nitroreductase (160 μg/mL) and 10 mM NADH in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C. Top, HPLC profile of the solution before addition of the enzyme; middle, HPLC profile after 1 min incubation with the enzyme; bottom, a control oligonucleotide. *Contamination from enzyme sample.

corresponding to the NADH. After 5 min incubation with nitroreductase, the ODN **5** peak decreased and several new peaks derived from partially deprotected ODNs (retention time 26.5 min, 24.1 min, 23.4 min, 20.6 min), contamination peak from enzyme sample (19.8 min) and fully deprotected ODN (16.3 min) were observed. After 10 min, the ODN **5** peak was completely diminished and only partially deprotected ODNs and fully deprotected ODN were observed. As time proceeded, partially deprotected products were further deprotected and converted to the fully deprotected ODN (Fig. S2b). This peak was collected and the structure of the deprotected oligonucleotide was confirmed by MALDI-TOF mass spectroscopy.

Hybridization properties of the base-protected ODNs with complementary sequences were investigated by thermal denaturation experiments. Sequences of the duplexes and their melting temperatures (T_ms) are compiled in Table 1. Thermally induced denaturation profiles are shown in Figure 3. A single substitution in the central region decreased the thermal stability of duplex formation (duplex II). Three consecutive substitutions in the central region largely destabilized the thermal stability (duplex III). By distributing the protected thymine residues over the length of the sequence, the duplexes were largely destabilized, thus clear denaturation curves were not observed (duplex IV and VI).

These results demonstrate that oligonucleotides containing 4-*O*-(4-NO_2-benzyl)thymine residues have potential for use as prodrug-type oligonucleotides under bioreductive conditions. Because 4-*O*-(4-NO_2-benzyl)thymine residues are stable throughout the processes of oligonucleotide synthesis and deprotection, these residues can be incorporated into oligonucleotides for various applications. Oligonucleotide prodrugs containing 4-NO_2-benzyl groups are expected to be inactive in normal cells because the protected oligonucleotides are unable to hybridize with the target sequences. However, in hypoxic cells such as those present in locally advanced solid tumors, 4-NO_2-benzyl groups become deprotected and the resulting free oligonucleotides form stable complexes with target RNAs.

In summary, the 4-*O*-(4-NO_2-benzyl)thymines were developed as a novel bioreduction-responsive nucleobase for incorporation in oligonucleotides. This modified thymine base converted to a native thymine base under bioreductive conditions. The methods outlined in this report could be used for the development of oligonucleotide therapeutics with activity towards hypoxic cells.

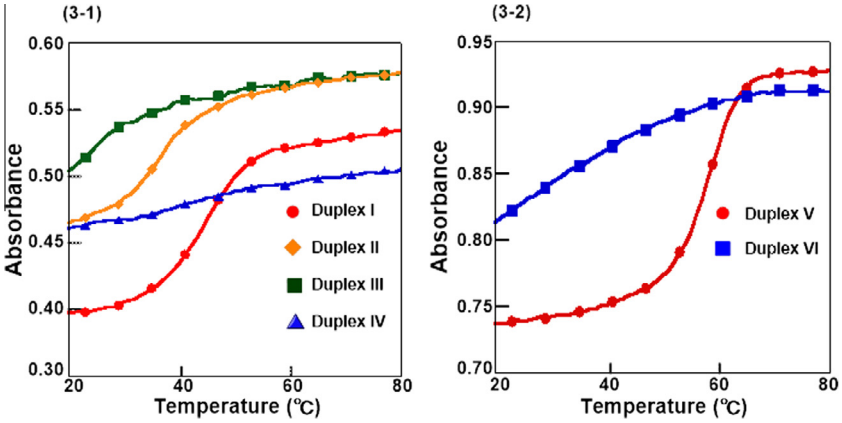


Figure 3. Thermal denaturation profiles of duplexes (I–VI). Conditions: 2 μM each of duplexes I–IV in 10 mM MOPS (pH 7.0), 1 M NaCl; *2 μM each of duplexes V and VI in 10 mM MOPS (pH 7.0), 100 mM NaCl.

Table 1
Sequences of the duplexes and their respective melting temperatures. (X = 4-*O*-(4-NO_2-benzyl)thymine)

Duplex	Sequence	T_m (°C)	ΔT_m (°C)
I	5′-TTTTTTTTTTTTT-3′ 3′-AAAAAAAAAAAAA-5′	43.6	–
II	5′-TTTTTTXTTTTTT-3′ 3′-AAAAAAAAAAAAA-5′	35.2	−8.4
III	5′-TTTTTXXXTTTTT-3′ 3′-AAAAAAAAAAAAA-5′	26.0	−17.6
IV	5′-TTTXTTXTTXTTT-3′ 3′-AAAAAAAAAAAAA-5′	n.d.	–
V*	5′-CAGTGCATTGGTCAC-3′ 3′-GTCACGTAACCAGTG-5′	57.9	–
VI*	5′-CAGXGCAXXGGXCAC-3′ 3′-GTCACGTAACCAGTG-3′	n.d.	–

* 2 μM each of duplexes V and VI in 10 mM MOPS (pH 7.0), 100 mM NaCl.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.10.025.

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