**Current Topics**

**Medicinal and Bioorganic Chemistry of Nucleosides and Nucleotides**

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**Review**

**Development of Protecting Groups for Prodrug-Type Oligonucleotide Medicines**

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In recent years, nucleic acid-based drug therapeutics have gained considerable attention for their potential in the treatment of various diseases. However, their therapeutic value is greatly hindered by the challenge of delivering them into cells. One possible strategy to improve cellular uptake is the use of “prodrug-type oligonucleotide medicine” in which negatively charged phosphodiester moieties are masked by bio-labile protecting groups. In this review, we describe our recent studies related to bio-labile protecting groups for phosphodiester moieties in the development of prodrug-type oligonucleotide medicines.

**Key words** oligonucleotide; bio-labile protecting group; drug delivery; prodrug

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1. **Introduction**

Oligonucleotide-based drug therapeutics could become effective treatment options against human diseases such as cancer and viral infections. However, the delivery of oligonucleotide drugs to their target sites of action remains a major challenge. Due to their structural aspects, oligonucleotides have poor cellular membrane permeability and are unstable against nucleases in biological fluids. To improve the cellular membrane permeability of oligonucleotide-based drugs, good drug-delivery systems (DDSs) should be developed. To date, the typical DDSs of oligonucleotide drugs are: (i) using nanoparticles as carriers, (ii) conjugating small molecules and/or polymers to improve the cellular membrane permeability of oligonucleotides; and (iii) developing prodrug-type oligonucleotide medicines. In this review, we have focused on (iii), the development of prodrug-type oligonucleotide medicines; (i) and (ii), which have been discussed in depth elsewhere, are not in the focus.

The prodrug approach to oligonucleotides is based on neutralizing the negative charges of phosphodiesters using bio-labile protecting groups for improving cellular membrane permeability. After prodrug-type oligonucleotide medicines have internalized within cells, their protecting groups are deprotected with “internal triggers” such as esterases, a reductive environment, etc. (Chart 1).

Pioneering work on prodrugs for oligonucleotides was done in the 1990s. S-Acylthioethyl groups, as esterase labile protecting groups, were originally reported as nucleotide prodrugs and later applied to the development of oligonucleotide prodrugs. The preparing of these oligonucleotides requires non canonical synthetic conditions.

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presumed that the heteroarylmethyl structure was probably unstable in DNA synthetic processes. Therefore, we investigated new protecting groups which have been found to be stable in standard DNA synthetic cycles, in order to make protected oligonucleotide preparations easier and more easily reproducible. The general design of our bio-labile protecting groups is described in Chart 2c. A typical protecting group is composed of a propyl linker connected to a benzene ring that has a heteroatom as a nucleophile. The heteroatom is protected with various trigger sensitive moieties which can respond to specific cellular triggers. The cleavage of trigger-sensitive moieties by triggers in cells gave nucleophiles such as hydroxyl, amino and mercapto groups; the intramolecular attack of these groups to the carbon near phosphotriester gave native oligonucleotides.

This design allows for a diverse set of protecting groups for phosphodiester moieties applicable to various oligonucleotide prodrugs. Moreover, substituents on the benzene ring can regulate the speed of the cyclization steps, and biocompatibility can be improved by attaching the functional molecules. Importantly, the protecting groups are highly stable in standard DNA synthetic cycles, thus the experiments can be
reproduced in many laboratories.

3. Some Examples of Bio-labile Protecting Groups

3.1. Oligonucleotide Prodrugs Bearing Photo-sensitive Protecting Groups

In order to confirm the stability of the protecting group in DNA synthetic cycles, and to detect intermediates in the deprotection pathway, we introduced a photosensitive group to the protecting group (Chart 3). Since the photoreaction was clean, we expect that the reaction pathway can be easily monitored. \(^{33}\)

A synthetic route for the protecting group and an amidite unit (a monomer unit for oligonucleotide synthesis) is shown in Chart 4. An alcohol (3) was synthesized from 2,6-dimethylphenol according to a procedure described previously, \(^{40}\) and 3 was coupled with an o-NO\(_2\) benzyl bromide to give the protecting group 4. A phosphoramidite unit (6) was synthesized using a standard synthetic procedure and incorporated into the model oligonucleotides (Chart 4).

ODN1 (Chart 4) was synthesized, deprotected, and purified using standard procedures for unmodified oligonucleotide preparation. ODN1 was obtained in good yield. By photolysis of a solution containing ODN1, the 2-nitrobenzyl group was removed to generate a phenolic intermediate which was gradually converted into the “naked” oligothymidylate. These results suggested a proposed deprotection pathway as shown in Chart 3.

3.2. Oligonucleotide Prodrugs Bearing Reduction-Activated Protecting Groups

Hypoxia in locally advanced solid tumors is a characteristic environment caused by an insufficient supply of oxygen due to poorly developed vasculature. \(^{41}\) Hence, several hypoxia-activated prodrugs using reduction-activated protecting groups have been developed and reported. \(^{42,43}\) As described above, nitrofuranyl methyl and nitrothienylmethyl groups were used for protecting phosphodiester moieties in oligothymidylates \(^{25}\) (Chart 2b). However, ODNs with mixed sequences bearing these protecting groups have not been successfully synthesized, probably because the protecting groups have inadequate stability for standard
DNA synthetic circles. Thus, we prepared a durable protecting group, the 3-(2-nitrophenyl)propyl group, as a reduction-activated protecting group for phosphodiester moieties in prodrug-type oligonucleotides35) (Chart 5).

Oligonucleotides bearing reduction-activated protecting groups were synthesized using a standard DNA synthetic protocol (Chart 6). We also synthesized an oligonucleotide containing a non-cleavable protecting group as a control (ODN 3).

Oligonucleotides bearing reduction-activated protecting groups were treated with nitroreductase (from Escherichia coli) in the presence of the reduced form of nicotinamide adenine dinucleotide (NADH). The protecting groups were deprotected to give native oligonucleotides.35) Conversely, the non-cleavable substrate (ODN 3) was completely stable under the same condition.

We evaluated the stability of oligonucleotides bearing the protecting group against exonuclease, endonuclease, and 50% human serum. Protected oligonucleotides exhibited much greater stability in 50% human serum than the full phosphodiester ODN.

Next, we evaluated the cellular uptake of the protected ODNs into HeLa cells. ODNs 6–8 were labeled with fluorescein at their 5′-ends.35) HeLa cells were treated with solutions containing the different ODNs (10 µM) for 1 h. The fixed cells were observed by confocal microscopy. The fluorescence intensity of the labeled ODNs in HeLa cells increased as the number of protecting groups increased. The maximum fluorescence intensity was observed for ODN 8. This result suggests that the oligonucleotides bearing reduction-activated protecting groups (hydrophobic groups) can be taken up into cells without the use of transfection reagents.

### 3.3. Functionalization of Prodrug-Type Oligonucleotides

It has been known that functional groups such as carbohydrates44,45) and vitamins46,47) attached to oligonucleotides induce the cellular uptake of oligonucleotides. These functional molecules, attached to the bio-labile protecting groups, can be deprotected in cells, and naked oligonucleotide drugs will then be generated (Chart 7).

We reported a method for easily preparing oligonucleotides that have functionalized bio-labile protecting groups.48) An oligonucleotide with an alkyne-linked bio-labile protecting group was synthesized, then functional groups were attached to the oligonucleotide by copper-catalyzed azide alkyne cycloaddition (CuAAC) reaction (Chart 8).

An oligonucleotide with an alkyne-linked reduction-activated protecting group is synthesized, then a coupling reaction between a functional molecule with an azide group and the alkyne group of the oligonucleotide gave a functionalized oligonucleotide.

Copper-catalyzed azide alkyne cycloaddition (CuAAC) to conjugate functional molecules and oligonucleotide on—the solid support have been reported.49–53) This strategy allowed
for the synthesis of a diverse range of oligonucleotide prodrugs from one oligonucleotide precursor: oligonucleotides with alkyne groups.

A synthetic route\(^{48}\) of an alkyne-linked reduction-activated protecting group (11) and an amidite unit (12) with a protecting group is shown in Chart 9.

A schematic representation for the synthesis of functionalized oligonucleotides is shown in Chart 10. Using amidite unit (12), an oligonucleotide with protecting groups was synthesized. Before deprotection, oligonucleotide on-support (controlled pore glass, CPG) was conjugated with various functional molecules, such as vitamins and carbohydrates. The functional-
ized oligonucleotide was treated with conc. NH₄OH for deprotection, then cleaved from the solid support (CPG).

In our experiments, the phosphoramidite unit 12 was quantitatively incorporated into oligonucleotides, and the on-support CuAAC reaction also succeeded quantitatively. Various functional molecules were conjugated in good yield in the same manner.

We investigated the deprotection reaction of functionalized reduction-activated protecting groups in oligonucleotides (Chart 11). Oligonucleotides with functional groups were treated with nitroreductase (from *E. coli*), and reactions were monitored by HPLC analysis. The functional groups were removed and naked oligonucleotides were detected.

### 4. Discussion

In the above paragraphs, protecting groups for phosphodiester groups were described. The protecting groups can be used for preparing prodrug-type DNA oligonucleotides. Also, prodrug-type oligonucleotides with 2′-O-methylribose or 2′-deoxy-2′-fluororibose can be prepared. However, prodrug-type RNA oligonucleotides cannot be prepared. This is unfortunate, since RNA type oligonucleotides, especially siRNA, have been important candidates for oligonucleotide therapeutics. For preparing prodrug-type RNA oligonucleotides, a good combination of protecting groups for the phosphodiester group and the 2′-hydroxy group should be developed (Chart 12). The protecting groups should be deprotected in the following order: first, the protecting group of the phosphodiester, then that of the 2′-hydroxy group. When the protecting group of the 2′-hydroxy group is deprotected first, the RNA strand will be degraded by reaction of the free hydroxy group to the phosphotriester group. Today, some good bio-labile protecting groups for 2′-hydroxy groups of RNA strands have been reported. However, the development of effective combinations of protecting groups has not yet been accomplished.

As previously described, as the numbers of protecting groups at the phosphodiester groups increased, cellular membrane permeability of protected oligonucleotides increased. However, the solubility of oligonucleotides decreased as the numbers of protecting groups increased. The balance between solubility and cellular membrane permeability may be important in the development of effective oligonucleotide drugs. Alternatively, by adding soluble functions to the protecting groups, the solubility of oligonucleotides could be improved (Chart 13). As described previously, functional groups such as carbohydrates and vitamins may improve the cellular membrane permeability.
uptake of oligonucleotides.

Summary

Our recent studies focused on the development of protecting groups for prodrug-type oligonucleotides. The skeleton of protecting groups is stable in standard DNA synthetic cycles, thus our experiments can be easily reproduced in any laboratories. Also, because there is some space on the benzene ring, some functional groups, such as peptides and sugars, can be connected for improving the solubility and cellular membrane permeability of these prodrug-type oligonucleotides.

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Conflict of Interest The authors declare no conflict of interest.

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