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Synthesis and biochemical properties of oligodeoxynucleotides acylated by the chemically stable 2-(trimethylsilyl)benzoyl (TMSBz) group at the 5′ or 3′ terminus

Ken Yamada, Haruhiko Taguchi, Akihiro Ohkubo, Kohji Seio, Mitsuo Sekine *

Department of Life Science, Tokyo Institute of Technology, J2-12, 4259 Nagatsuta, Midoriku, Yokohama 226-8501, Japan

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ABSTRACT

Oligodeoxynucleotides acylated with a 2-(trimethylsilyl)benzoyl (TMSBz) group at the 5′ or 3′ terminus were synthesized according to the general method used for DNA synthesis. The acylated DNA oligomers could be easily purified due to the high lipophilicity of the TMSBz group and showed enhanced hybridization ability and resistance to exonucleases.

In recent years, much attention has been paid to nucleic acid-based gene-targeting strategies.[1] A variety of chemically modified oligonucleotides were synthesized to increase the hybridization affinity for the target DNA or RNA genes.[2] Many efforts have also been paid to improve the resistance against nuclease degradation and the cell membrane permeability which have proved to be of great importance for expression of their antisense activities.[3]

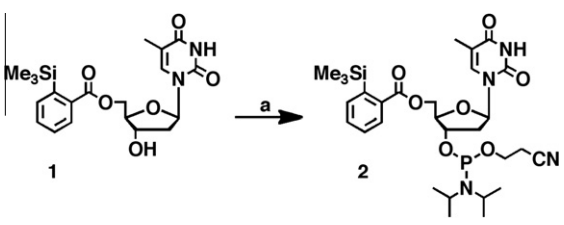
Chemical modification of oligonucleotides at their 5′ and 3′ terminus is one of the strategies widely used to improve their original properties.[4] As a new method, we focused on the 5′ or 3′ terminal modification of oligonucleotides with acyl groups that could suppress their degradation due to cellular exonucleases. However, it is necessary to use suitably designed acyl groups that tolerate basic conditions such as NH_3 aq generally used in chemical synthesis of oligonucleotides.[5]

In the previous Letter, we reported that the 2-(trimethylsilyl)benzoyl (TMSBz) group attached to the 5′, 3′, or 2′-hydroxyl group of nucleosides that was found to be extremely stable under basic conditions.[6] For example, the TMSBz group substituted at the 5′-hydroxyl group of thymidine was stable under the basic conditions of 28% ammonia at 55 °C for 24 h. These results led us to synthesize TMSBz-modified oligonucleotides using the general method where removal of the protecting groups and cleavage of oligonucleotides from CPG resins were carried out simultaneously by treatment with 28% aqueous ammonia.

In this Letter, we report a new method for the synthesis of oligonucleotides acylated by the TMSBz group and their chemical and enzymological properties.

5′-*O*-TMSBz-thymidine (**1**: ^{TMSBz}T) was prepared by Mitsunobu reaction of 2-(trimethylsilyl)benzoic acid with thymidine without protection of the 3′-hydroxyl group, as reported previously by us.[6] 5′-*O*-TMSBz-thymidine-3′-*O*-phosphoramidite unit (**2**) was synthesized in 65% yield by the reaction of compound **1** with 1.2 equiv of 2-cyanoethoxy[bis(diisopropylamino)]phosphine in CH_3CN in the presence of 0.6 equiv of diisopropylammonium 1*H*-tetrazolide at room temperature for 2 h according to the general method, as shown in Scheme 1.

To examine the stability of the TMSBz group in oligodeoxynucleotides modified with the TMSBz group, we synthesized 5′-d([^{TMSBz}T]GACTGACTGACT)-3′ (**ODN1**) using the phosphoramidite unit **2** and the other building units (where the phenoxyacetyl (Pac) group was used for A and G and the acetyl group was used for C) in an automated DNA synthesizer. The chain elongation cycle consisted of (i) detritylation, (ii) coupling, (iii) capping with phenoxyacetic anhydride (Pac_2O), and (iv) iodine oxidation.[5] After the final chain elongation, **ODN1** having the TMSBz group at the 5′-terminal site was cleaved from the solid support by treatment with aqueous ammonia at room temperature for 1 h. The protecting groups of the base and phosphate groups were simultaneously removed during this treatment. The hydrophobic property of the TMSBz group enabled us to easily purify the 5′-*O*-TMSBz-capped oligonucleotide using a Sep-Pak Plus C18 cartridge (Waters Corp.).



Scheme 1. Reagents: (a) 1*H*-tetrazole, $(i\text{-Pr})_2$NH, 2-cyanoethoxy[bis(diisopropylamino)]phosphine.

* Corresponding author. Tel.: +81 45 924 5706; fax: +81 45 924 5772.
E-mail address: msekine@bio.titech.ac.jp (M. Sekine).

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The reversed-phase HPLC profile of the **ODN1** obtained after the Sep-Pak C18 purification suggested that **ODN1** was obtained as sufficiently pure material in 42% yield so that further purification using HPLC was no longer needed, as shown in Figure 1. It should be noted that the TMSBz group remained unchanged in this successive purification procedure. **ODN1** was characterized by MALDI-TOFF mass analysis (found 4123.5, calcd 4123.8 [H+M]).

We examined if the sterically hindered TMSBz group affected the hybridization property of the original structure. As a result, it was found that the T_m value of the duplex formed between **ODN1** and its complementary oligomer was higher by 2.3 °C than that of the duplex formed between **ODN2** and its complementary strand (Table 1). This result implies that the TMSBz group at the 5′-terminus of the oligonucleotide does not disturb the formation of the duplex.

To evaluate the stabilizing effect of the TMSBz group on enzymatic digestion, the stability of the **ODN1** toward a 5′-exonuclease, bovine spleen phosphodiesterase (BSP, Sigma), was examined. The enzyme assay using BSP (0.2 U/mL) was performed in a buffer of 30 mM NaOAc at pH 6.0 at 37 °C by use of 50 μM oligonucleotide. After the enzyme was deactivated by heating 100 °C for 2 min, the solution was diluted and filtered by a 45 μm filter (Millex-HV, Millipore).[7] The mixture was analyzed by anion-exchange HPLC, as shown in Figure 2. The unmodified oligonucleotide was completely degraded in few minutes. On the other hand, the modified oligonucleotide **ODN1** remained unchanged under the same conditions even after 8 h.

To see if the chemically stable TMSBz group is also stable to esterases, the enzyme assay was carried out using pig liver esterase (PLE, Sigma). **ODN1** (11 μM) was incubated with PLE (8.9 U/mL) in a 0.2 M potassium phosphate buffer (pH 7.4 at 37 °C).[8] After the enzyme was deactivated by heating 100 °C for 2 min, the solution was diluted and filtered by a 45 μm filter (Millex-HV, Millipore). The mixture was analyzed by reversed-phase HPLC, as shown in Figure 3. It was found that the modified oligomer with the retention time of 21 min was completely resistant to PLE even after incubation for 24 h (Fig. 3). The unmodified oligomer 5′-d(TGACTGACTGACT)-3′ (**ODN2**) appeared at the retention time of 10.5 min and no new peak was observed at this area.

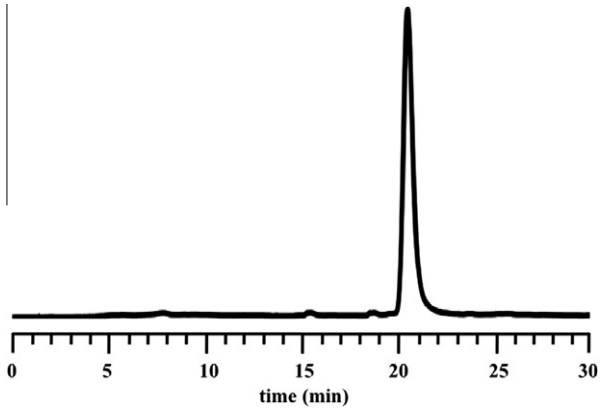


Figure 1. RP-HPLC of **ODN1** after the Sep-Pak C18 purification.

Table 1
Hybridization properties of oligonucleotides having TMSBz group at 5′-hydroxyl terminus

ODN	Sequence	T_m[a] (°C)	ΔT_m (°C)
1	5′-d([^{TMSBz}T]GACTGACTGACT)-3′	55.9	+2.3
2	5′-d(TGACTGACTGACT)-3′	53.6	none

[a] Measured in a buffer containing 10 mM sodium phosphate buffer (pH 7.0), 100 mM NaCl, 0.1 mM EDTA, and 2.0 μM duplex.

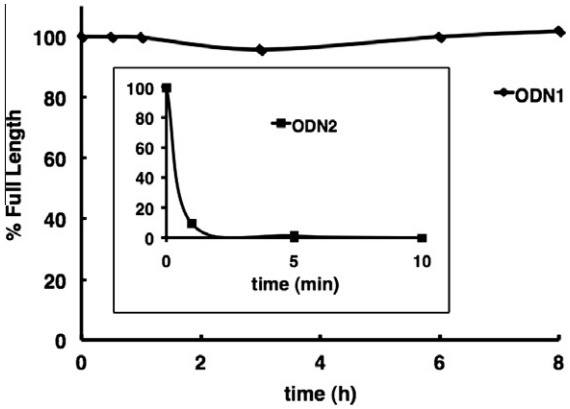


Figure 2. Time course of degradation of **ODN1** (closed diamonds) and **ODN2** with BSP (closed box).

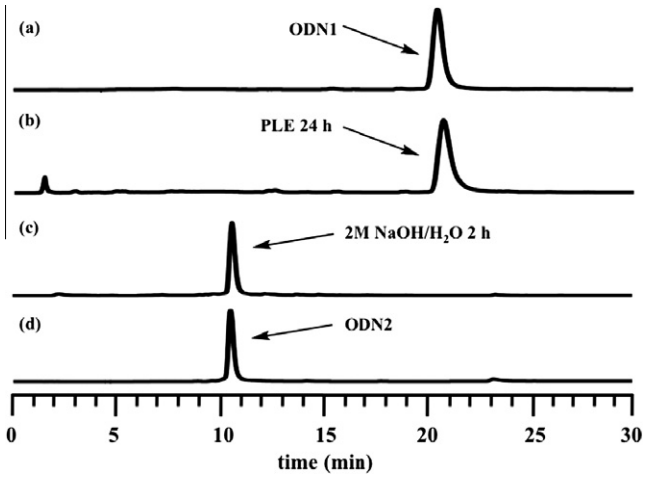
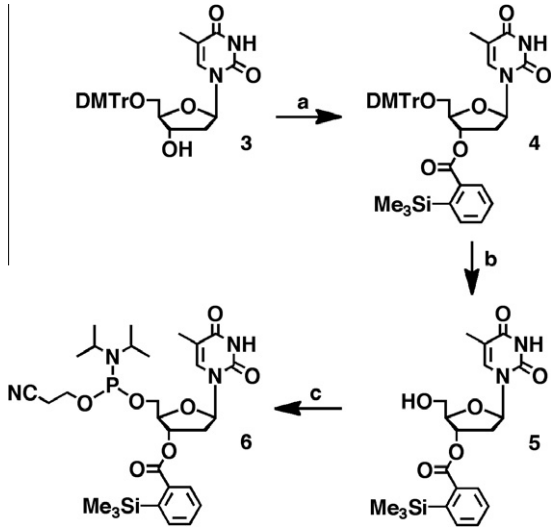


Figure 3. Reversed-phase HPLC profiles of the enzymatic reaction and alkali hydrolysis of **ODN1**: (a) **ODN1**, (b) the mixture obtained after incubation of **ODN1** with PLE in 0.2 M potassium phosphate buffer at 37 °C for 24 h, (c) the mixture obtained after treatment of **ODN1** with 2 M NaOH at room temperature for 2 h, (d) **ODN2** as the authentic sample.

Previously, we reported that the TMSBz group could be removed from compound **1** by treatment with 2 M NaOH in ethanol at room temperature for 4 h. Therefore, we tested whether the TMSBz group can be removed from **ODN1** by treatment with 2 M NaOH in aqueous solution at room temperature for 2 h. As a result, the hydrolysis of the TMSBz group was completed in 2 h and the deacylated product was observed as a single peak at 10.5 min, as shown in Figure 3.

The marked resistance of the 5′-*O*-TMSBz group against BSP led us to synthesize a 3′-*O*-TMSBz-capped oligonucleotide 5′-d(TTTTTTTTTTT[T_{TMSBz}])-3′ (**ODN3**) using 3′-*O*-DMTr-thymidine 5′-phosphoramidite unit and a 3′-*O*-TMSBz-5′-*O*-phosphoramidite unit **6** in the reverse 5′ → 3′ direction in the solid phase synthesis. The unit **6** required in the last coupling reaction was synthesized as shown in Scheme 2.

3′-*O*-[2-(Trimethylsilyl)benzoyl]thymidine (**5**), which was obtained by a two-step reaction from 5′-*O*-(4,4′-dimethoxytrityl)thymidine (**3**) according to the method previously reported,[6] was allowed to react with 2-cyanoethoxy[bis(diisopropylamino)]phosphine to give the 5′-phosphoramidite derivative **6** in 42% yield. In a manner similar to that described in the synthesis of **ODN1**, **ODN3**



Scheme 2. Reagents: (a) 2-(trimethylsilyl)benzoic acid chloride, pyridine-CH_2Cl_2 (1:1, v/v); (b) 3% TCA/CH_2Cl_2; (c) 1*H*-tetrazole, $(i\text{-Pr})_2$NH, 2-cyanoethoxy[bis(diisopropylamino)]phosphine.

was synthesized and isolated in 60% yield, as shown in Figure 4, and characterized by MALDI-TOFF mass analysis (found 4069.7; calcd: 4066.7[H+M]).

The nuclease stability of **ODN3** was evaluated by treatment with snake venom phosphodiesterase (SVP, Sigma). The enzyme assay using snake venom phosphodiesterase (5×10^{-5} U/mL) was performed in a buffer of 50 mM Tris–HCl at pH 8.5, 72 mM NaCl, and 14 mM $MgCl_2$ at 37 °C by use of 50 μM oligonucleotide.[9] After workup similar to that described in the enzyme assay using BSP, the mixture was analyzed by anion-exchange HPLC. As shown in Figure 5 the half-life $t_{1/2}$ of **ODN3** was ca. 23 min whereas the unmodified oligomer 5′-d(TTTTTTTTTTTT)-3′ (**ODN4**) degraded more rapidly with $t_{1/2}$ of 10 min. The difference in stability against SVP between **ODN3** and **ODN4** was observed, but the 3′-terminal TMSBz group was not so effective against SVP as the 5′-terminal one against BSP. In the previous studies, it was reported that when a modified base was incorporated in DNA, the 'skipping' of the modified base by snake venom phosphodiesterase was often observed.[10] On the other hand, the HPLC profile, shown in Figure 6, T_{12} appeared at 21.8 min, suggesting the predominant enzymatic cleavage of the 3′-terminal phosphodiester bond. A dimer fragment of 5′-(T[T_{TMSBz}])-3′ that should be generated by the skipping was not observed in our case (Fig. 6).

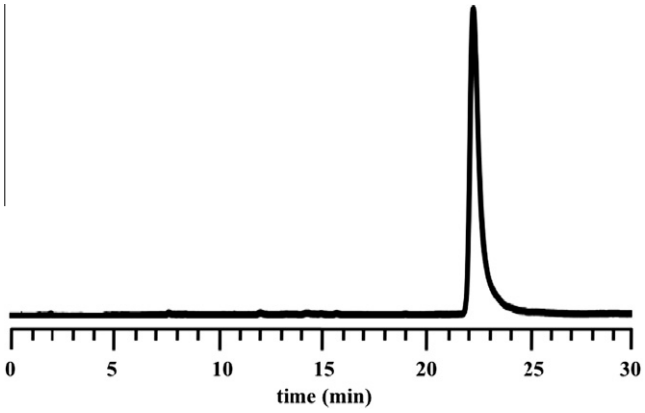


Figure 4. RP-HPLC of **ODN3** after the Sep-Pak C18 purification.

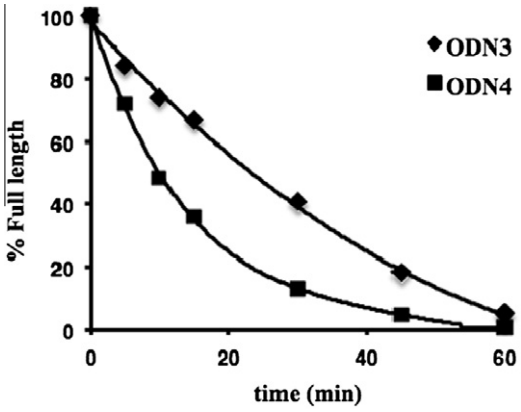


Figure 5. Time course of degradation of **ODN3** (closed diamonds) and **ODN4** with SVP (closed box).

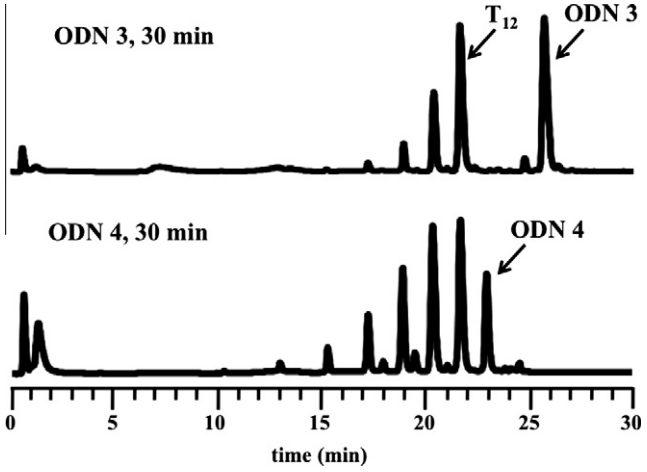


Figure 6. Anion exchange HPLC profiles of **ODN3** and **ODN4** incubated at 37 °C in the presence of snake venom phosphodiesterase (5×10^{-5} U/mL) for 30 min.

In summary, we successfully introduced the chemically stable TMSBz group at 5′- or 3′-hydroxy terminus of oligonucleotides via the general synthetic protocol using 28% aqueous ammonia. Very high lipophilicity of TMSBz group enables facile purification by Sep-Pak Plus C18 cartridge. TMSBz group attached to 5′-hydroxyl group of the oligonucleotide did not disturb the forming of DNA duplex and was found to enhance the 5′-exonuclease resistance; however, modification at 3′-hydroxyl group with TMSBz group did not show predominant increase of 3′-exonuclease resistance. Further investigation of TMSBz-modified oligonucleotides is currently in progress in our laboratory.

Acknowledgments

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