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# Construction of Saccharide-modified DNAs by DNA Polymerase

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**Abstract**—Novel deoxyribonucleotide triphosphates bearing maltose or lactose groups were synthesized as substrates for DNA polymerase. The incorporation efficiencies of these modified substrates were investigated in both primer extension reactions and PCR. The stability and conformation of saccharide-modified dsDNAs were assessed by UV absorbance melting experiments and CD analysis. Enzymatic incorporation of saccharide-modified substrates can be used for the efficient production of saccharide-modified DNAs.

Glycosylated DNAs naturally occur in some phages[1] and organisms such as *Trypanosoma Brucei*.[2] The biological roles of glycosylated DNAs has been the focus of a great deal of attention because they may help protect DNA from nucleases or regulate gene expression. In addition, several groups have reported artificial DNA-carbohydrate conjugates.[3–6] Recently, DNAs with multiple saccharide residues along the helix were used to explore cooperative lectin binding[3f–3h] and to control gene expression by carbohydrate-lectin interaction.[4]

Various approaches have been used for the conjugation of saccharides with DNA, including solid-phase synthesis with glycosylated phosphoramidites[3], diazo coupling targeting the 8-position of the guanine base,[5] and chemical incorporation of saccharides at the 5'-end of oligonucleotides.[6] It can be difficult, however, to construct long, highly functionalized conjugates without structural heterogeneity using these methods. Hence, alternative methods for efficiently and site-specifically modifying DNA with saccharides are needed to expand the potential and applications of DNA-carbohydrate conjugates.

Here, we describe the synthesis of maltose- and lactose-modified dUTPs (dUTP-Mal and dUTP-Lac, respectively) and the sequence-directed incorporation of these substrates into DNA to generate DNA-carbohydrate conjugates. A DNA polymerase with the ability to incorporate modified nucleotides can be used as a convenient tool for attaching functional reporters to DNA molecules.[7] In the present study, we examined the efficiency to which our two

saccharide-modified substrates are incorporated into DNA by primer extension reactions and PCR using two DNA polymerases, KOD Dash and Taq. In addition, we explored the influence of saccharide modification on the thermal stability and conformation of the modified DNAs by UV melting and CD analysis.

The two saccharide-modified dUTPs, dUTP-Mal and dUTP-Lac, were synthesized according to the procedures shown in Scheme 1 and 2. In the first step, 6-amino-1-hexanol was converted into acrylamide derivative **2** with acryloyl chloride. Maltose octaacetate[8] and heptaacetyl-α-bromolactose [9] were separately coupled to **2** with appropriate promoters, after which the acetyl groups were deprotected. These compounds were attached at the C5 position of dUTP via a palladium-catalyzed reaction to give dUTP-Mal (**5a**) and dUTP-Lac (**5b**),[10] which were confirmed by $^{1}$H NMR and MALDI-TOF mass spectrometry.

The efficiency of enzymatic incorporation of modified nucleotides into DNA is often low for templates in which incorporation of adjacent modified nucleotides is required. The efficiency of incorporation depends on the structures and chemical properties of substituents and the polymerases used. We examined under what conditions incorporation of **5a** and **5b** at adjacent sites is possible by carrying out primer extension experiments using KOD Dash DNA polymerase or Taq DNA polymerase along with a series of template DNAs (**T1–5**; 40-mer; Table 1) with different numbers of consecutive dA nucleotides. As shown in Figure 1A, KOD Dash DNA polymerase gave full-length products for all templates (lanes **M** and **L**), suggesting that this enzyme has the high potential for incorporating up to 15 adjacent molecules of **5a** and **5b**. Compared to the corresponding unmodified products (lane **P**), the migration of the products during electrophoresis was reduced in proportion to the degree of modification. On the other hand, Taq DNA polymerase gave complete elongation products only for **T1**, which did not contain adjacent modification sites (Figure 1B). For **T2–5,** the elongations terminated mainly after incorporation of modified substrates at up to 2 adjacent sites.

We further examined the use of **5a** and **5b** in PCR. Compared to primer extension reactions with unmodified templates, PCR amplification with modified substrates requires that the polymerases have a higher fidelity and ability to incorporate nucleotides because they must be able to correctly incorporate dNTPs based on “modified” templates. Furthermore, the elongation must be completed in a relatively short time. We carried out PCR using KOD Dash DNA polymerase and a model template (**T6**; 70-mer; Table 1) that contained up to five adjacent dAs in the central region. As shown in Figure 2, **T6** was successfully amplified by PCR using **5a** or **5b** (lanes **M** and **L**). Compared to the PCR using four native dNTPs (lane **P**),

the relative efficiency of PCR using **5a** and **5b** was ~80% and ~50%, respectively. Each product amplified using the modified substrates was excised from the gels and used as a template for the next PCR with native dNTPs. The second PCRs gave 70-bp products (lanes **R1** and **R2**), indicating a reasonable conversion from modified to unmodified 70-bp fragments. These products were subjected to sequence analysis to confirm the fidelity in the two PCR. The analysis indicated that the sequences of the final PCR products were nearly identical (>99%) to the original sequence of **T6**.[11] On the other hand, Taq DNA polymerase did not give any full-length products by PCR using **T6** and **5a** or **5b** (data not shown).

Detailed studies by Sawai *et. al.*[7b,7f,7k] have shown that KOD Dash DNA polymerase accepts a broad range of thymidine analogues as substrates. Our substrates, which have bulky, nonionic saccharide residues, were also accepted by the enzyme without causing steric hindrance. Taq DNA polymerase can accept some dUTP derivatives with a primary amine or aromatic functionalities attached via an alkenyl linker,[7a,7c] but, in this study, **5a** and **5b** were not good substrates for Taq DNA polymerase due to a steric effect of the bisaccharides or structural properties of the acrylamide linker. Several groups have previously reported that other thermostable polymerases including Pwo, Pfu, and Vent DNA polymerase also have the potential for multiple incorporation of modified nucleotides.[7a,7e,7i,7j] It remains to be investigated whether **5a** and **5b** can be recognized as substrates by those enzymes.

To evaluate the influence of saccharide modification on the stability and conformation of DNA duplexes, we carried out UV absorbance melting experiments and CD analyses for unmodified and modified dsDNAs (**D1**–**4**; Table 2) containing different numbers and distributions of saccharide residues. As summarized in Table 2, only one substituent had no effect on the stability of the duplex, whereas, compared to the corresponding unmodified DNAs, the $T_m$ of modified DNAs containing more than one saccharide residue was greatly decreased, depending on the degree of modification. The detailed mechanism for the destabilization by saccharide modification is not clear, but these results suggest that it is due to steric interference of the Watson-Crick base pairings by the bulky bisaccharide residues. Our duplexes were destabilized less than the aminoglycoside-modified duplexes with methylene linkers described by Hunziker.[3a] The longer linker used in our modified nucleotides appears to have less of an effect on duplex stability.

Figure 3 shows the CD spectra of unmodified, maltose-modified, and lactose-modified duplexes for **D1**–**4**. In all samples except for **D4**, both the positive band near 280 nm and the negative band near 245 nm were reduced, depending on the degree of modification (up to

~60% and ~70% in **D3**, respectively), which might reflect strain in the base stacking. Nevertheless, these duplexes appeared to maintain a standard B-type conformation. Interestingly, CD spectra of saccharide-modified **D4**, which contain five maltose or lactose residues at every other base, include an additional negative band at 295–315 nm. Furthermore, saccharide modification shifted the maximum at 280 nm in the unmodified **D4** to a shorter wavelength by ~15 nm. This may be due to the presence of chirally oriented π-conjugation system consisting of C=C in the alkenyl linker and nucleobases in the specific sequence context of the duplex. The difference in behavior of **D4** and **D3** may be related to the alternate incorporation of five maltose or lactose residues (**D4**) causing a more moderate decrease in $T_m$ than adjacent incorporation (**D3**).

In conclusion, dUTP-Mal and dUTP-Lac can be efficiently incorporated into DNA by KOD Dash DNA polymerase, even if the template has a demanding sequence context. This method utilizing a DNA polymerase is an easy way to generate oligonucleotides containing a high density of glycosylated nucleotides. This technique should also facilitate construction of combinatorial saccharide-modified DNA libraries through PCR using randomized templates. Such a library could enable the isolation of effective glycoclusters with adequate numbers of saccharides in the correct orientation for lectin binding. We are currently assessing lectin binding for various types of saccharide-modified DNAs.

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11. The mutation rates per base in the central modified region (30 bp) were 0.31% and 0.69 % for the PCRs with **5a** and **5b**, respectively. The mutations comprised 5 base substitutions

(mainly from A/T to G/C), 3 deletions (A/T, G/C), and 1 insertion (G/C). Five of the 9 mutations were located just next to the primer regions, and only 1 deletion mutation was observed at the 5 adjacent A/T site.

**Table 1**. Sequences of templates (**T1–6**) and primers (**P1–3**) used in primer extension reactions or PCR.

| | Sequence[a,b] |
|---|---|
| **T1** | 5′-CTCT**A**TCTC**A**CTCT**A**TCTC**A**ATCGGTGCCAGTCGGATAGT-3′ |
| **T2** | 5′-TCTCTCTCTCTCTCTCTC**AA**ATCGGTGCCAGTCGGATAGT-3′ |
| **T3** | 5′-CTCTCTCTCTCTCTC**AAAAA**ATCGGTGCCAGTCGGATAGT-3′ |
| **T4** | 5′-TCTCTCTCTC**AAAAAAAAAA**ATCGGTGCCAGTCGGATAGT-3′ |
| **T5** | 5′-TCTCT**AAAAAAAAAAAAAAA**ATCGGTGCCAGTCGGATAGT-3′ |
| **T6** | 5′-CTGCTTGCTGTTCTCTTGTG**A**G**T**C**A**T**A**GC**T**AA**T**CG**AAAAA**CG**T**AA **A**TGC**T**GGAGAAGCACCGAAACAAGC-3′ |
| **P1** | 5′-ACTATCCGACTGGCACCGAT-3′ |
| **P2** | 5′-CTGCTTGCTGTTCTCTTGTG-3′ |
| **P3** | 5′-GCTTGTTTCGGTGCTTCTCC-3′ |

[a] Primer annealing sites are underlined.

[b] Letters in boldface indicate modification sites in the strand or its complementary strand.

**Table 2**. Melting Temperatures for unmodified, Mal-modified, and Lac-modified duplexes (**D1–4**).

| | Sequence | $T_m$/°C ($\Delta T_m$/modification)[a, b] X= | | |
|---|---|---|---|---|
| | | dT | dU-Mal | dU-Lac |
| **D1** | 5′-TGGCATTGAC**X**GAGAGAGAG-3′<br>3′-ACCGTAACTGACTCTCTCTC-5′ | 51 | 51 (0) | 51 (0) |
| **D2** | 5′-TGGCATTGAC**XX**AGAGAGAG-3′<br>3′-ACCGTAACTGAATCTCTCTC-5′ | 49 | 46 (-1.5) | 47 (-1) |
| **D3** | 5′-TGGCATTGAC**XXXXX**GAGAG-3′<br>3′-ACCGTAACTGAAAAACTCTC-5′ | 50 | 37 (-2.6) | 37 (-2.6) |
| **D4** | 5′-TGGCATTGAC**X**G**X**G**X**G**X**G-3′<br>3′-ACCGTAACTGACACACACAC-5′ | 56 | 49 (-1.4) | 49 (-1.4) |

[a] The measurements were performed under the following conditions: 0.8 μM duplex, 10 mM $Na_2HPO_4$/$NaH_2PO_4$, 10 mM NaCl, 1 mM EDTA, pH 7.0.

[b] The samples were prepared by polymerization reactions using KOD Dash DNA polymerase.

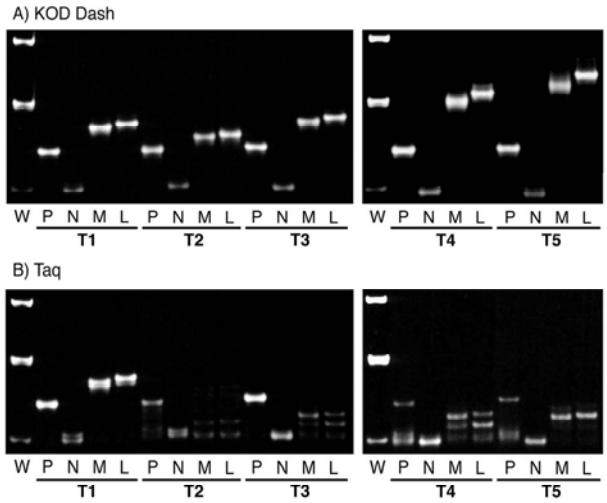



**Figure 1**. Primer extension reactions for **T1**–**5** using KOD Dash DNA polymerase (A) or Taq DNA polymerase (B). Reaction mixtures (15 μL) containing 12 pmol of template, 12 pmol of **P1**, KOD Dash or Taq buffer, 0.2 mM of each dNTP, and KOD Dash DNA polymerase (0.01 U/μL) or Taq DNA polymerase (0.025 U/μL) were incubated at 50°C for 1 h. The reaction products were analyzed by 15% polyacrylamide gel electrophoresis. The gels were stained with SYBR Gold. W, DNA marker (from top to bottom, 75 bp, 50 bp, and 40-mer + 20-mer); P, positive control (dATP+dCTP+dGTP+dTTP); N, negative control (dATP+dCTP+dGTP); M, dATP+dCTP+dGTP+dUTP-Mal; L, dATP+dCTP+dGTP+dUTP-Lac.

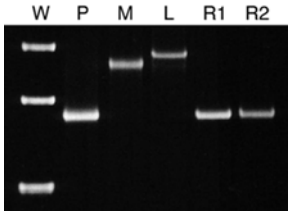



**Figure 2**. PCR for **T6** using KOD Dash DNA polymerase. The reaction mixtures (30 μL) contained 0.03 pmol of **T6**, 9 pmol of **P2** and **P3**, KOD Dash buffer, 0.2 mM of each dNTP, and KOD Dash DNA polymerase (0.025 U/μL). The thermal cycling profile included an initial denaturation step at 95°C for 5 min, followed by 15 cycles of 94ºC for 30 s, 50ºC for 30 s, and 74ºC for 3 min. The reaction products were analyzed by 12% polyacrylamide gel electrophoresis. The gel was stained with SYBR Gold. W, DNA marker (from top to bottom, 100, 75, and 50 bp); P, dATP+dCTP+dGTP+dTTP; M, dATP+dCTP+dGTP+dUTP-Mal; L, dATP+dCTP+dGTP+dUTP-Lac; R1, PCR products amplified using four native dNTPs and the amplified product (M); R2, PCR products amplified using four native dNTPs and the amplified product (L).

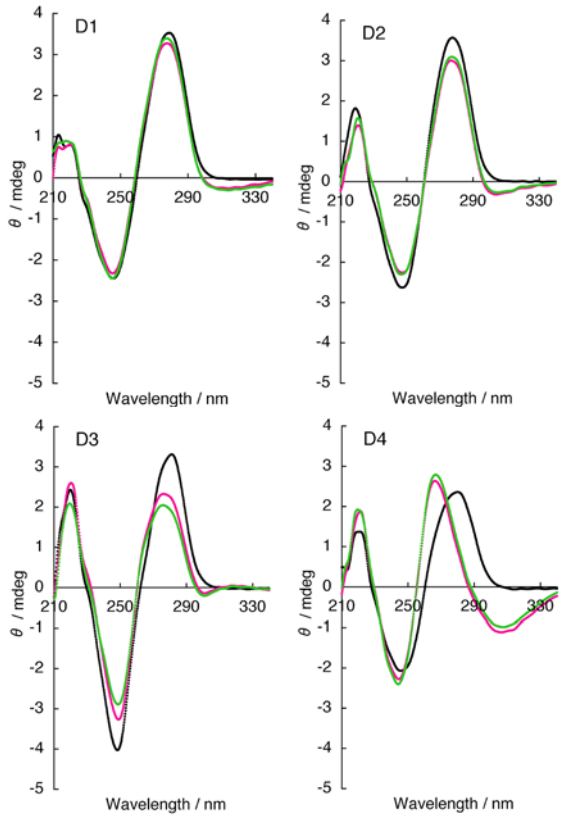
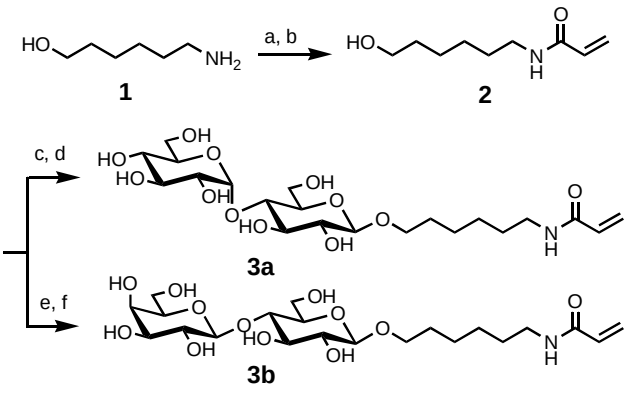
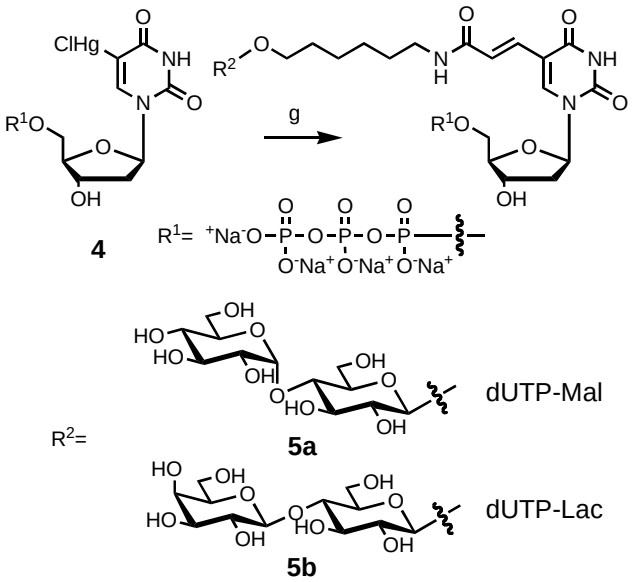



**Figure 3**. CD spectra of unmodified (black), Mal-modified (red), and Lac-modified (green) DNA (**D1**–**4**). The measurements were performed under the following conditions: 0.8 μM duplex, 10 mM $Na_2HPO_4/NaH_2PO_4$, 10 mM NaCl, 1 mM EDTA, pH 7.0, 25°C.




**Scheme 1**. Reagents and conditions: (a) acryloyl chloride, triethylamine, $CH_2Cl_2$, 45°C to rt; (b) 2 M NaOH, EtOH, rt, 69% (2 steps); (c) maltose octaacetate, $BF_3{\cdot}(C_2H_5)_2O$, dry $CH_2Cl_2$, rt; d) NaOMe, MeOH, rt; 63% (2 steps); (e) heptaacetyl-α-bromolactose, $AgClO_4$, $Ag_2CO_3$, dry $CH_2Cl_2$, 0 °C; (f) NaOMe, MeOH, rt; 32% (2 steps).




**Scheme 2**. Reagents and conditions: (g) **3a** or **3b**, $Li_2PdCl_4$, $H_2O$, rt; **5a** (trans), 44%; **5b** (trans), 26%.