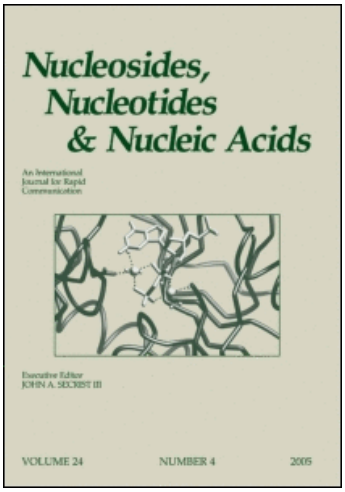

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2′-MODIFIED OLIGONUCLEOTIDES FROM METHOXYOXALAMIDO AND SUCCINIMIDO PRECURSORS: SYNTHESIS, PROPERTIES, AND APPLICATIONS

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ABSTRACT

Synthesis of 2′-modified oligonucleotides from 2′-methoxyoxalamido (MOX) and 2′-succinimido (SUC) precursors is described. Their physical and biochemical properties were assessed. Synthesized oligonucleotides were used as primers in advanced DNA sequencing protocols. An example of sequencing directly off genomic DNA template without prior cloning or PCR amplification is presented.

Chemically modified oligonucleotides, especially the ones containing 2′-modified bases, are of interest as potential diagnostic probes and therapeutics. We have developed a new synthetic strategy to access a vast number of 2′-modified oligonucleotides from the single precursor oligonucleotide. The strategy is based on the use of our proprietary methoxyoxalamido (MOX) or succinimido (SUC) precursor chemistries (1, 2). Schematically this concept is shown in Figure 1. First, the precursor oligonucleotide containing 2′-MOX/SUC substituted nucleosides is assembled. The oligonucleotide is then post-synthetically reacted with an appropriate nucleophile (primary aliphatic amine or hydroxide anion) and deprotected to

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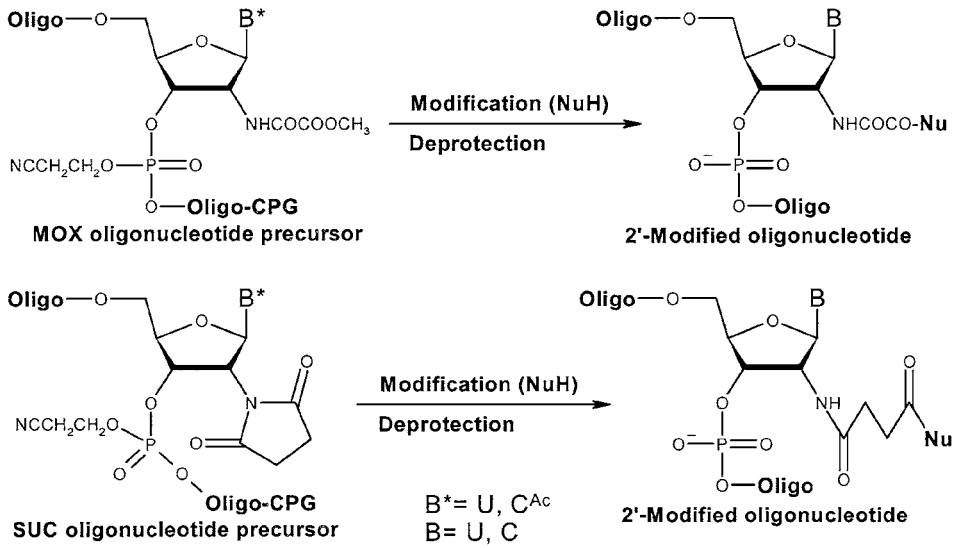

Figure 1. Schematic representation of the 2′-MOX/SUC precursor strategies.

form a modified oligonucleotide. If a mixture of different nucleophiles is applied, a library of 2′-modified oligonucleotides is produced.

OLIGONUCLEOTIDE SYNTHESIS

The monomers, 2′-MOX/SUC-2′-deoxyuridine and N^4-acetyl-2′-MOX/SUC-2′-deoxycytidine phosphoramidites, were synthesized in high yields from the corresponding 2′-amino-2′-deoxynucleosides (3). Their efficient (>97%) incorporation into an oligonucleotide during an automatic solid-phase synthesis was achieved by extending coupling time (10–15 min) and using a more powerful catalyst (5-ethylthio-1H-tetrazole, ETT). Modified oligonucleotides were synthesized on a DNA Synthesizer Expedite 8909 equipped with MOSS unit (Perceptive Biosystems) or ASM-700 (Biosset). Modifiers used in this study are listed in Table 1.

Table 1. Modifiers Used in the Study

No	Name	FW	No	Name	FW
1	Hydroxide Anion	17.01	14	N,N-Dimethyethylenediamine	88.15
4	Methylamine	31.06	22	Histamine	111.1
7	Ethanolamine	61.08	29	Spermine	202.3
9	6-Amino-1-hexanol	117.2	30	Spermidine	145.2
13	3-Dimethylaminopropylamine	102.2	35	1-(3-Aminopropyl)-imidazole	125.2

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Table 2. T_m and MS Data for Modified Oligonucleotides

Entry	Oligo	Modifier	T_m, °C	ΔT_m, °C	Width, °C	MW (calc.)	MW (MALDI)
1	T7	none	56.5	0	11.6	6454.2	6453.1
2	T71s	1	52.8	−3.7	8.2	6556.3	6555.3
3	T71s	7	53	−3.5	9	6599.4	6598.1
4	T71s	14	53.9	−2.6	8.5	6626.4	6625.3
5	T71s	29	54.2	−2.3	8.1	6740.6	6736.4
6	T71s	35	53.2	−3.3	8.4	6663.5	6662.2
7	T74s	1	40.1	−16.4	9.8	6959.5	6957.5
8	T74s	7	38.1	−18.4	9.7	7174.9	7175.2
9	T74s	14	43.1	−13.4	9.1	7310.2	7306.4
10	T74s	29	46.3	−10.2	9.8	7881.2	7886.8
11	T74s	35	39.8	−16.7	9.9	7495.3	7490.9
12	T71m	1	54.9	−1.6	8.3	6527.2	6527.8
13	T71m	14	54.9	−1.6	8.4	6597.4	6597.5
14	T71m	29	55.1	−1.4	8.4	6710.6	6711.4

EFFECT OF MODIFICATIONS ON MELTING TEMPERATURE OF OLIGONUCLEOTIDES

21-mer T71s, 5′-GTA ATA CGA CU^{s}C ACT ATA GGG-3′, containing one 2′-SUC-2′-deoxyuridine in position 11, and 21-mer T74s, 5′-GU^{s}A AU^{s}A CGA CU^{s}C ACU^s AU^{s}A GGG-3′, containing five 2′-SUC-2′-deoxyuridines in positions 5, 7, 11, 17 and 20, were synthesized. Oligonucleotide T71m, having the same sequence as T71s but 2′-MOX-2′-deoxyuridine instead of 2′-SUC-2′-deoxyuridine, was also synthesized.

After synthesis oligonucleotides were reacted with chosen modifiers, deprotected and PAGE purified. Modified oligonucleotides were then annealed with the complementary unmodified 21-mer in a buffer containing 10 mM Tris-HCl (pH8 at 25°C) and 2 mM $MgCl_2$ and the formed duplexes were melted. The data (Table 2) show that all investigated modifications decrease melting temperature. The degree of the decrease in T_m is modification dependent. The difference in T_m of oligonucleotides with modifications of a given type increased with the increase of a number of modifications (Table 2, column 5). Importantly, the width of melting transition (Table 2, column 6) was narrower in the case of all modified oligonucleotides. This implies that the modified oligonucleotides have higher annealing specificity compared to unmodified counterpart.

EFFECT OF MODIFICATIONS ON THE ENZYMATIC 5′-PHOSPHORILATION OF OLIGONUCLEOTIDES

The data on phosphorylation of the modified 16-mer $(U^{sn})_{15}T$ were U^{sn} is 2′-SUC-2′-deoxyuridine derivatized with modifier *n* (Table 1) with T_4 polynucleotide

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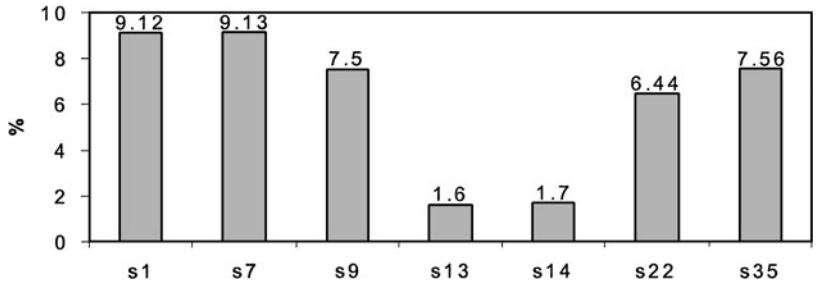

Figure 2. Phosphorylation of the modified oligonucleotides with T_4 PNK.

kinase (PNK) at 37°C for 30 min are shown in Figure 2. The degree of phosphorylation is presented as percentage in respect to phosphorylation of an unmodified oligodeoxynucleotide (100%).

The most unfavorable for the enzyme are modifications containing tertiary amino groups (modifiers **13**, **14**, Table 1). It is known that T_4 PNK acts efficiently when a natural negatively charged phosphodiester bond is at the 5′-end. Stronger inhibition of the enzyme activity by modifications s13 and s14 could be the consequence of shielding the negative charge of the phosphodiester group by the tertiary amino group which is predominantly protonated at phosphorylation condition (pH 8.0).

STABILITY OF THE MODIFIED OLIGONUCLEOTIDES IN A SERUM- AND CELL- CONTAINING MEDIUM

5′-End labeled modified 16-mers $(U^{sn})_{15}T$ were incubated in medium RPMI-1640 containing serum and serum and suspended cells at 37°C for 1-24 hours. The reaction mixtures were cleared from protein and precipitated into ethyl alcohol. The precipitates were dissolved in 8 M urea and run on 18% denatured polyacrylamide gel. Gel was autoradiographed on the X-Ray film. Bands corresponding to the intact oligonucleotide and its degraded fragments were cut out and the radioactivity of the gel slices was determined by Cherenkov's counting. 16-mer $(U^{OMe})_{15}T$, where U^{OMe} is 2′-O-methyluridine, was used as control. The data are presented in Figure 3. The stability of the modified oligonucleotides varied among different types of modifications. In general, 2′-SUC-modified oligonucleotides are more stable in a serum- and cell-containing medium compared to the 2′-O-methyl modification.

APPLICATION OF THE MODIFIED OLIGONUCLEOTIDES IN ADVANCED DNA SEQUENCING PROTOCOLS

High purity DNA samples in a substantial amount are commonly used in standard DNA sequencing protocols. To increase the outcome of sequencing reaction

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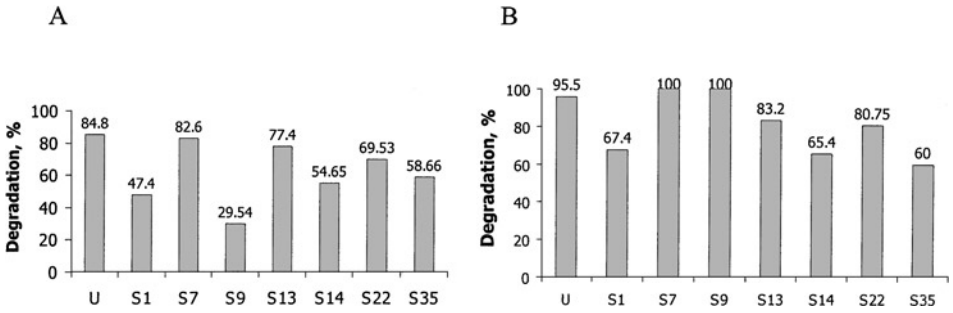

Figure 3. Degradation of the modified 16-mer $(U^{sn})_{15}T$ after 1 hr in medium RPMI-1640 containing serum (A) and serum and suspended cells (B).

we extended the number of cycles beyond 50. We used modified primers to inhibit two non-specific events that interfere with the sequencing reaction, primer-dimer extension and non-specific polymerase chain reactions (PCR).

Preliminary screening of 2′-modified T7 primers having one modification placed in positions 3–9 from the 3′-end showed that the most robust modifications are s1, s4, s7 and ml, and the most modification sensitive positions are 3 and 4. We also found that primer extension reaction is completely inhibited when DNA polymerase approaches the modified nucleoside in the template strand.

Formation the dimer from the priming oligonucleotide might be inhibited by placing at least one modified nucleoside within a potential complementary site. Placing the modification close to the 3′-end prevents non-specific primer dimer extension. Suppression of non-specific PCR by the modified primers also attributed to their improved annealing specificity. The optimal effect was found when modified nucleotide is in −5 position.

DIRECT SEQUENCING OFF MICROBIAL GENOMIC DNA TEMPLATE

Next example shown how non-specific primer dimer extension can be inhibited by the use of modified primer. Sequencing from E.coli amtB gene was primed by 26-mer 5′-GAT TTC GCG GGT GGC ACC GTG GTG CA-3′. Sequencing reaction contained 1 μg of genomic DNA, primer and Big Dye Terminator Ready Reaction Mixture (Applied Biosystems). 400 cycles of denaturation at 95°C for 5 sec, annealing at 55°C for 30 sec and extension at 60°C for 1 min were done. Samples were analyzed on ABI Prism 377 DNA Sequencer.

When non-modified primer was used, annealing of two copies of the same primer within four complementary nucleotides at the 3′-end was sufficient for extension reaction (Fig. 4, top). Expected 22 base long sequence 5′-CCA CGG TGC CAC CCG CGA AAT C-3′ was detected at the beginning of the trace. Yield of specific Sanger fragments from the template DNA was low.

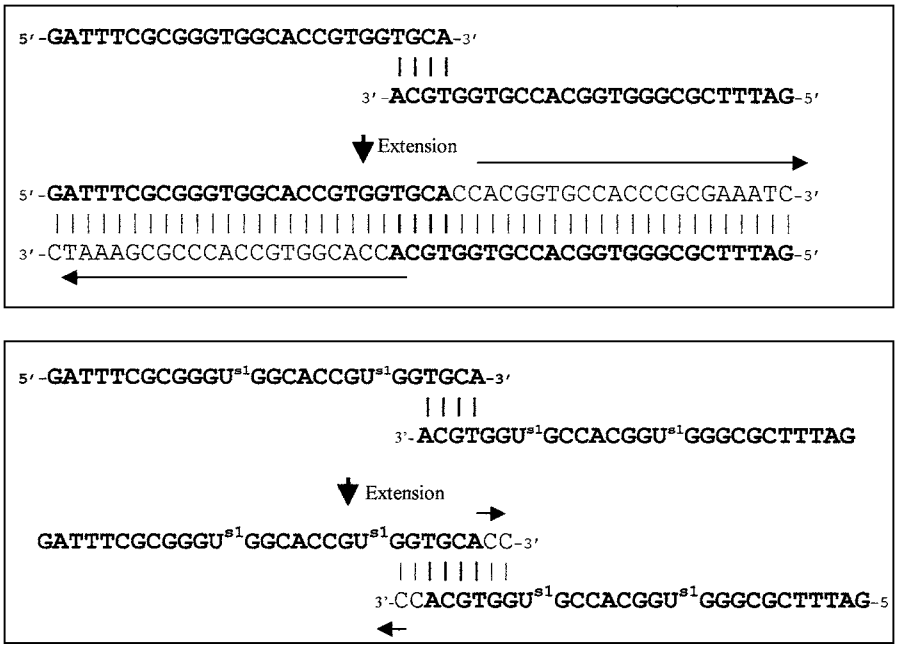

Figure 4. Inhibition of primer dimer in DNA sequencing reaction. Primer sequence is shown in bold. Horizontal arrows show extension product. U^{sl} is hydrolyzed 2′-succinimido-2′-deoxyuridine.

When modification was introduced in −15 position, shorter primer dimer extension products were observed. In this case DNA polymerase did not extend primer sequence beyond modified nucleoside (data not shown). Some improvement of the sequencing outcome was achieved.

When the second modified nucleotide was introduced in −7 position, no primer dimer extension products were detected and high quality sequence from the template DNA was obtained (Fig. 4, bottom).

SEQUENCING OFF SMALL AMOUNT OF BAC DNA TEMPLATE

Next example demonstrates inhibition of non-specific PCR amplification associated with a 400-cycle sequencing reaction. Two 35-mer oligonucleotides have been synthesized: 35T7, 5′-ACG GCC AGT GAA TTG TAA TAC GAC TCA CTA TAG GG-3′, and 35T7s7, 5′-ACG GCC AGT GAA TTG TAA TAC GAC TCA CTA U^{s7} AG GG-3′, where U^{s7} is an ethanol amine derivatized 2′-succinimido-2′-deoxyuridine. As a template for cycle sequencing reaction, we used BAC DNA containing human SEP15 gene cloned into pBeloBAC11 vector. The sequencing reaction contained 30 ng BAC DNA, 0.1 μL ThermoFidelase 2 (Fidelity Systems, Inc.), 0 or 0.1 mM 7-deaza-dGTP, 0.3 mM $MnCl_2$, 2 μL BigDye Terminator Mix and 10 pmole primer. Reaction volume was 5 μL. After initial denaturation at 95°C for 2 min, 400 cycles, each consisting of denaturation at 95°C for 5 sec and annealing and extension at 60°C for 1.5 min, were performed.

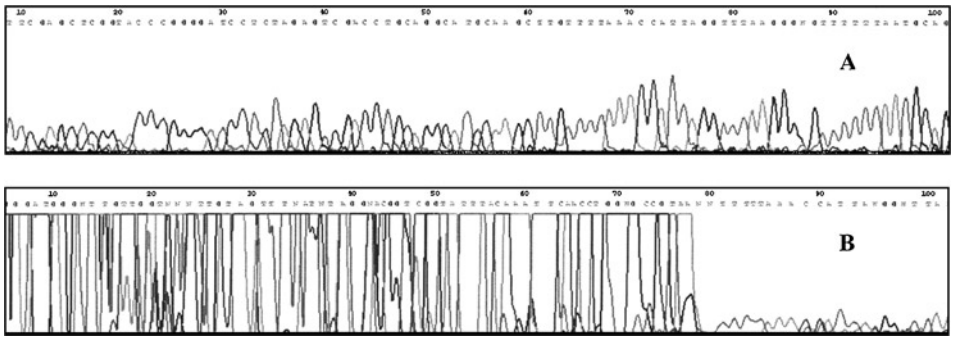

Figure 5. Sequencing traces obtained with the use of 35 base long modified (A) and non-modified (B) primer.

The reactions with modified 35T7s7 primer yielded sequence traces with 500 readable bases (Fig. 5A) while reactions with an unmodified 35T7 primer produced unidentifiable products–indication of non-specific PCR amplification (Fig. 5B).

The above examples demonstrate a number of advantages of using modified primers in cycle sequencing. First, termination of polymerase extension by the modified nucleoside placed in the fifth position of the primer prevents non-specific PCR amplification for as many as 400 thermal cycles. Second, a dramatically increased number of cycles allows to reduce consumption of the template DNA per reaction and increase sensitivity of sequencing. Thus, reliable sequencing from as low as 30 ng of BAC DNA was achieved. For comparison, in standard protocols up to 2,000 ng of BAC DNA are consumed. Third, inhibition of non-desired processes associated with cycle sequencing allows two fold increase of the primer length. The use of extended primers (30–40 base long) is critical in sequencing from long templates such as BAC and genomic DNA.

We now routinely use modified primers in our high throughput DNA sequencing efforts. Over 3,000 such primers have been synthesized and used in finishing several BAC clones from rough draft of Human Genome Project, sequencing various microbial genomic DNA templates and rapid detection of transposon insertion sites (4).

In conclusion, the described MOX/SUC precursor chemistries provide a robust way to access a vast number of 2′-modified oligonucleotides from the single precursor oligonucleotide. The properties of the modified oligonucleotides are modification dependent and, thus, can be easily controlled. Oligonucleotides with certain modifications were successfully used as primers in advanced DNA sequencing protocols.

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