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3′-Immobilized Probes with 2′-Caps: Synthesis of Oligonucleotides with 2′-*N*-Methyl-2′-(anthraquinone carboxamido)uridine Residues

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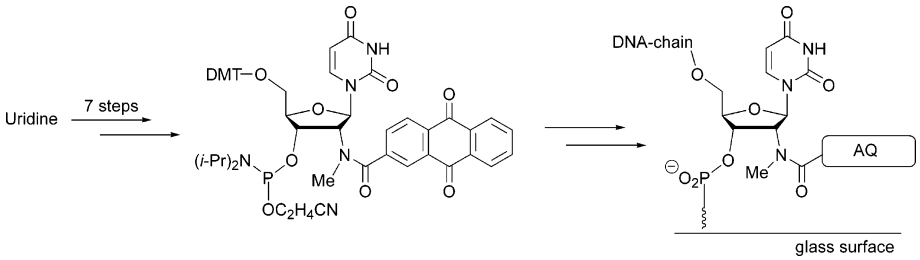
ABSTRACT



A synthesis for oligodeoxynucleotides with a 3′-terminal 2′-*N*-methyl-2′-acylamido-2′-deoxyuridine residue was developed. Unlike their unmethylated counterparts, these oligodeoxynucleotides can be stably immobilized on aldehyde-displaying glass surfaces to provide DNA microarrays. An anthraquinone carboxamido group as a 2′-substituent doubled the capture efficiency of an immobilized tetradecamer.

Double helices between complementary DNA strands[1] are exquisitely predictable structures that follow base pairing rules and play pivotal roles in practical applications.[2,3] The extended, linear structure of duplexes, which differs markedly from the globular structure of most soluble proteins, causes different levels of base pairing fidelity in different regions of the duplex, however. While mismatches in the interior of duplexes, where neighboring base pairs provide tight constraints, significantly lower duplex stability, mismatches at the termini, where few neighbors are affected and fraying occurs frequently, have minimal effects. The smallest effect of mismatches on duplex stability is found for the terminal base pair, particularly when one of the nucleobases is adenine or thymine/uracil. Here, mismatches can cause decreases in free energy of duplex formation of less than 1 kcal/mol.[4]

Nature, in certain instances, has chosen to accept the promiscuous binding at the termini of duplexes, e.g., by developing a degenerate code that compensates for mismatches at the termini of codon:anticodon duplexes.[5] Biomedical applications call for high fidelity, however, to avoid false positives. Lengthening the probes to make the terminal base pairs less significant is not promising, as the penalty for single mismatches will drop, the likelihood of partial complementarity with other targets will increase, and the kinetics of duplex formation will be slowed. Instead, one might chemically modify the probes to increase pairing fidelity.

One known type of modification that increases base pairing fidelity employs short stretches of minor groove binders covalently linked to PCR primers.[6] These do not significantly affect the termini, however. To achieve better discrimination at the termini, nonnucleosidic appendages that bridge the terminus and create a chemical environment resembling the interior of duplexes could be employed. Examples of such bridging appendages or "molecular caps" that bind to

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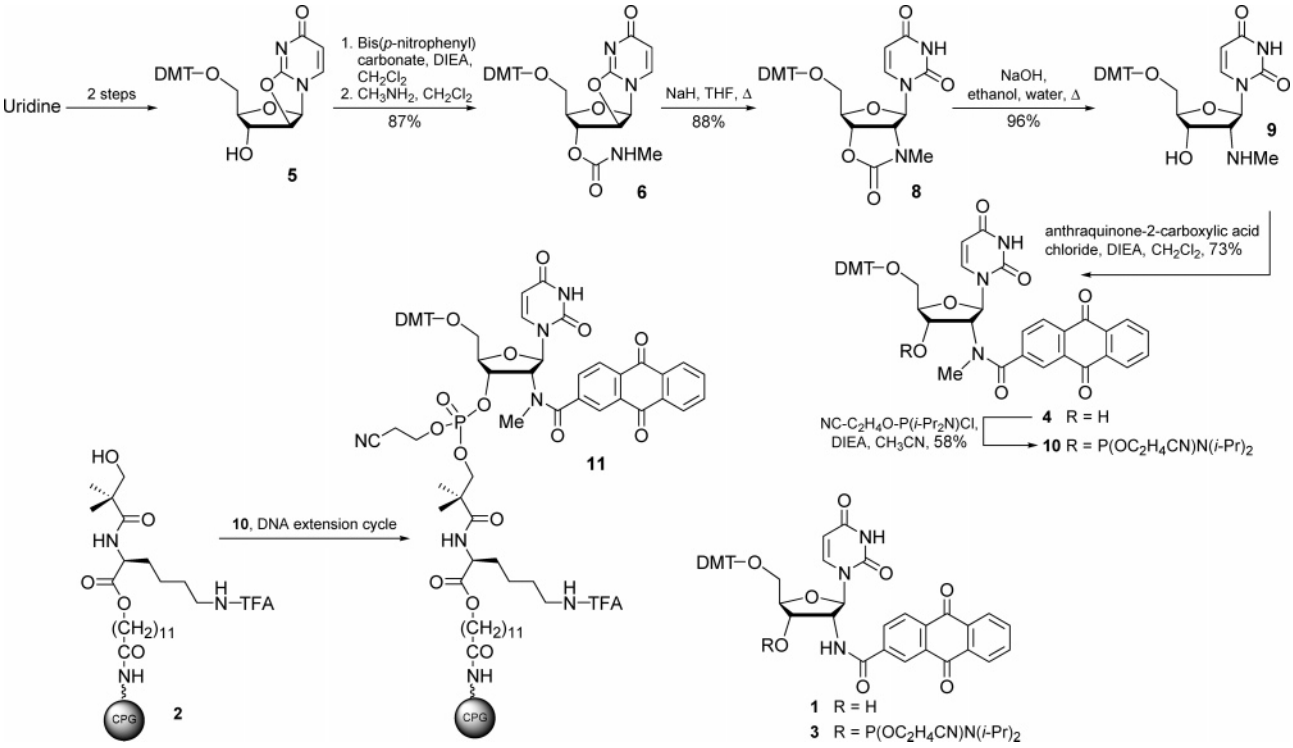
Scheme 1



duplexes with correct canonical base pairs exist.[4,7,8] Besides increasing base pairing fidelity, the molecular caps allow a tuning of duplex stability.

Probes on DNA microarrays[9] are not commonly employed with caps, however. This is unfortunate, as the massive multiplexing of DNA microarray experiments, where up to 2.5×10^5 hybridization equilibria compete with each other, calls for exquisite selectivity in duplex formation. Recent studies have led to caps that increase capture efficiency for hybridization probes 3′-immobilized on glass surfaces.[7c,10] The caps tested to date were all attached to the 5′-position of the probes. For the 3′-termini, similar caps have been lacking, as these require nucleosides providing both a link to the surface and a molecular cap. The example of RNA shows how much a simple 2′-substituent in oligonucleotides can complicate oligomer syntheses.

Here we present a synthesis of a deoxyuridine derivative that functions as a cap-bearing 3′-terminal residue of oligonucleotide probes on DNA microarrays. This residue carries both a cap at its 2′-position and a phosphoramidite group reactive toward a linker for immobilization on derivatized glass surfaces at its 3′-position. The cap employed is an anthraquinone. Anthraquinones have recently been shown to be excellent caps for termini when attached as 2′-acylamido groups of 3′-terminal deoxyuridine residues.[11] They give melting point increases of up to 14 °C per residue and increase base pairing fidelity at the terminus. Other anthraquinone derivatives covalently linked to oligonucleotides are being explored for their charge transport capabilities.[12] Different modes of attaching anthraquinone residues to DNA[13] or PNA[14] exist. Unconjugated anthraquinones can act as photonucleases,[15] and certain anthraquinone derivatives bind G quadruplex DNA,[16] making them a particularly versatile class of nucleic acid ligands.

Our attempts to generate 2′-capped hybridization probes for immobilization on microarrays started with the synthesis of **1** (Scheme 1). This nucleoside features the anthraquinone carboxamido cap and a free 3′-hydroxyl group for phosphi-

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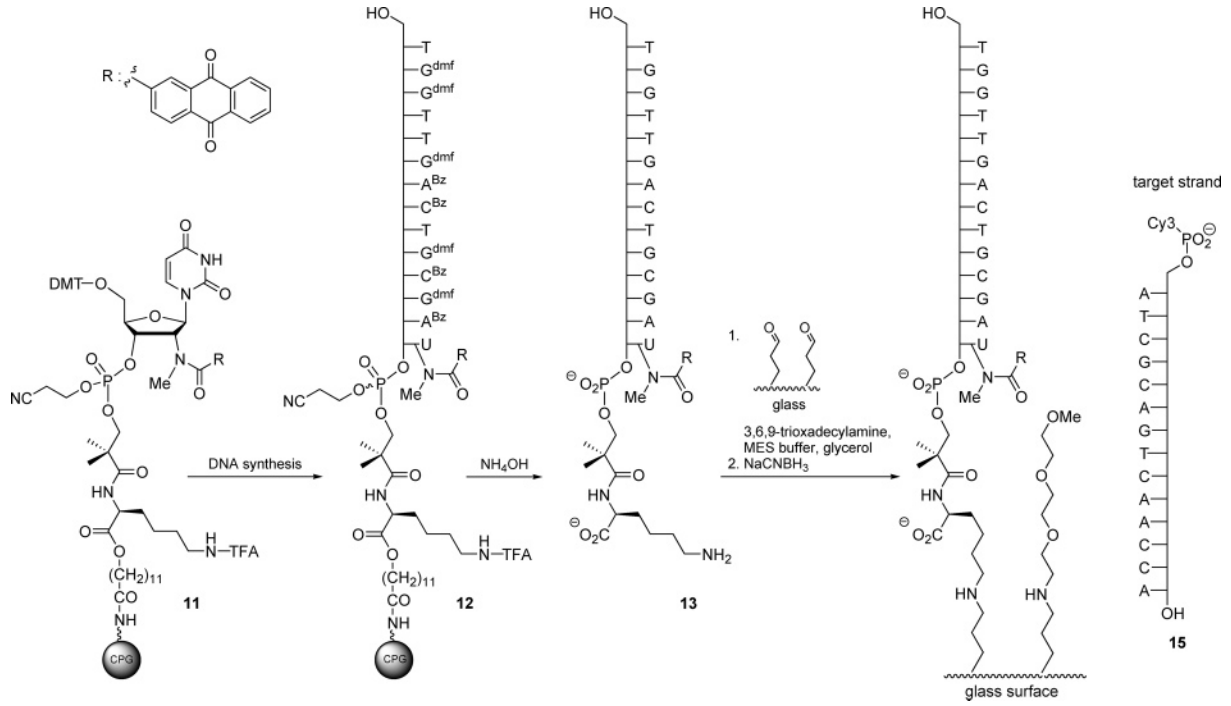
Scheme 2



tylation and subsequent coupling to linker-bearing support **2**.[10] All attempts to convert **1** to phosphoramidite **3** in high yield and to generate 3′-capped oligonucleotides for covalent immobilization failed. Probably the amido functionality at the 2′-position, particularly in the presence of base, was reactive toward the neighboring 3′-phosphoramidite or phosphotriester. An attack of the oxygen of the 2′-amido group on the phosphorus can occur via a six-membered ring, and an attack of the (deprotonated) amido nitrogen can occur via a five-membered ring. Other base-induced nucleophilic attacks of amido groups on 3′-esters of nucleosides are known.[17]

To eliminate the high nucleophilicity of the amido groups in the presence of bases, we decided to prepare 2′-*N*-methyl derivative **4** (Scheme 1). The synthesis of **4** started with the conversion of uridine to 5′-DMT-protected anhydro derivate **5** (Scheme 1) in 51% yield, following literature protocols.[18] Two routes were considered for introducing the methylamino functionality at the 2′-position. One would involve opening of **5** with an azide,[19] reduction to a 2′-amine, and *N*-methylation, possibly under Eschweiler−Clarke conditions, as described for an aminodeoxyadenosine derivative.[20] The other involves an intramolecular attack of the nitrogen of a 3′-attached urethane moiety. The latter route has been employed for an *N*-ethyl nucleoside, formed by reacting the 3′-hydroxyl group with alkyl isocyanates.[21] For our substrate, we wished to avoid both methylation on the nucleoside level and the use of highly toxic methyl isocyanate. Our first attempt to prepare **6** therefore involved *N*-methyl carbamic acid NHS ester (**7**) as reagent, but no conversion of **5** was observed under several conditions. When the more reactive bis(*p*-nitrophenyl) carbonate[22] or its monochloro derivate[23] were used, a carbonate was successfully generated. The in situ reaction with methylamine gave **6** in 87% overall yield. To induce isomerization via intramolecular ring opening, carbamate **6** was treated with NaH, which led to the formation of oxazolidinone **8** in 88% isolated yield. Compound **8** was hydrolyzed to *N*-methylated amine **9** using conditions similar to those employed for other nucleosides.[24]

Several methods were tested for coupling of anthraquinone carboxylic acid to the secondary amine of **9**. Activation of

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the acid to an acid chloride with oxalyldichloride and addition to the aminonucleoside gave higher yields than preactivation with "uronium salt" agents such as HBTU.[25] With the former method, amide **4** was obtained in 73% yield. Despite the steric bulk of the 2′-substituent, **4** could be converted to phosphoramidite **10** using 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in 58% yield after chromatography.

With key building block **10** in hand, 3′-capped oligonucleotides were prepared using standard phosphoramidite chemistry. A tetradecamer sequence containing all four nucleotides that has been well studied in the context of microarrays[7c,10] was prepared starting from lysine-bearing, controlled pore glass **11**[26] (Scheme 2). One-step HPLC purification of **13** gave a sample suitable for immobilization on aldehyde-bearing glass slides via reductive amination.[10] Unmodified control strand 5′-TGGTTGACTGCGAT-DP-Lys-3′ (**14**), where DP denotes the dimethylhydroxypropionic acid linker,[26] was spotted alongside **13**, and the remaining free surface sites were reacted with a triethyleneglycol amine, following a protocol reported earlier.[7c] Incubation with target strand 5′-Cy3-ATCGCAGTCAACCA-3′ (**15**), washing with buffer solutions (1 × SSC/0.2% SDS and 1 × SSC), and scanning with a microarray reader gave fluorescence images such as the one shown in Figure 1. It can be discerned that the cap increases the affinity for the target strand, as evidenced by the high fluorescence signal from the spots displaying 3′-capped **14**. This effect was observed reproducibly and over a range of hybridization temperatures (Table SI, Supporting Information).

Both modified probe **13** and control strand **14** feature the same 3′-substituent for immobilization. The amino group for immobilization is 15 atoms away from the position at which the two oligonucleotides differ in structure. Further, modified probe **13** is slightly more sterically hindered. The increase in fluorescence signal was observed in four independent experiments, making it unlikely that local changes in surface coating are responsible for the effect observed. Taken together, this leads us to believe that **13** does indeed capture its target strand more efficiently than **14**.

An independent hybridization experiment, performed on-chip under the same experimental conditions, employed 3′-labeled target strand 5′-ATCGCAGTCAACCA-Cy3-3′ (**16**). This strand was prepared using 5′-phosphoramidites so that the fluorophore could be incorporated in the last step of the solid-phase synthesis. When a slide displaying **13** and **14** on its surface was incubated with **16** at 70 °C, followed by washing, and scanning, a fluorescence image was obtained that closely resembles the one shown in Figure 1 (see Figure S7, Supporting Information). Again, **13** captured the target significantly more strongly, while unmodified **14** gave only 41% as much signal. The slightly greater capture efficiency in this case might be due to the absence of low-level fluorescence quenching that could occur when Cy3 label and anthraquinone are at the same terminus of the duplex.

These results show that an oligonucleotide probe enforced with 3′-terminal anthraquinone carboxamido cap can be successfully prepared and employed in microarray-based hybridization experiments. Since the anthraquinone cap gives the strongest melting point increase among all caps tested in our work, with a ΔT_m of up to 14 °C per residue,[11] it is not surprising that the increase in signal for spots featuring **13** over those with unmodified control (**14**) is so substantial for a tetradecamer. It was previously shown that the 2′-anthraquinone carboxamido cap does not require blunt ends to exert its duplex-stabilizing effect;[4] therefore, capturing longer target strands with overhangs at either terminus can be expected to occur with equally high efficiency. The 2′-appended cap may now be combined with 5′-caps[7] and possibly other fidelity- and affinity-enhancing[27] modifications. Thus, some of the weaknesses of DNA microarrays such as the low target affinity of A/T-rich sequences, low base pairing fidelity at the termini, lability toward exonuclease attack, and difficult-to-obtain electric signals for binding may be alleviated. The synthetic route described here is short, as it avoids protecting group changes, and may provide a blueprint for the synthesis of other 2′-modified oligonucleotides to be employed in functional nucleic acid constructs immobilized via a 3′-phosphodiester.

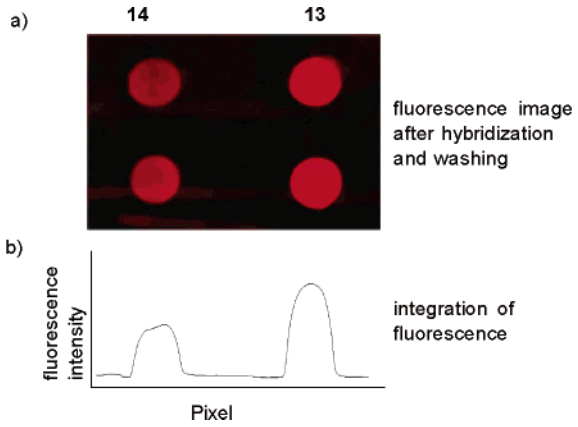

Figure 1. Fluorescence of spots of a DNA microarray after incubation at 70 °C with fluorescent complementary strand Cy3-ATCGCAGTCAACCA (**15**) and washing (a) Fluorescence image, with spots featuring control strand **14** (left) and 3′-capped strand **13** (right). Spotting was in duplicate to demonstrate reproducibility. (b) Integration of fluorescence over the area vertically above.

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Supporting Information Available: Experimental procedures, Table SI, and NMR or MALDI spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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