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Synthesis of Oligonucleotides with a 2′-Cap at the 3′-Terminus via Reversed Phosphoramidites

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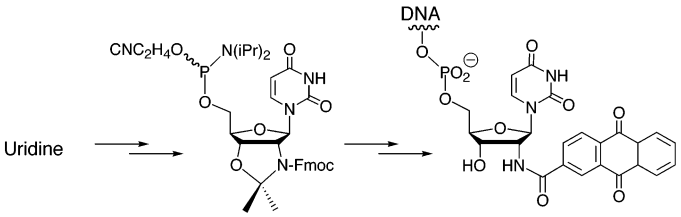
ABSTRACT



A method is presented for the synthesis of single compounds or small combinatorial libraries of oligonucleotides with 2′-acylamido-2′-deoxyuridine residues at the 3′-terminus. Selection experiments identified the residue of anthraquinone-2-carboxylic acid as a "molecular cap" that increases the UV melting point of the duplex (5′-ACGCGU-3′)$_2$ by up to 28 °C compared to the unmodified control duplex.

Oligonucleotides with modifications at the termini play important roles in molecular biology, biotechnology, and the emerging field of molecular medicine. Among the most common non-nucleic acid moieties appended to termini are chromophores such as the "big dyes" used to label strands produced in the chain terminator method of DNA sequencing,[1] cyanine dyes for labeling strands employed in DNA chip experiments,[2] and the fluorophore/quencher combinations employed in molecular beacons.[3] Further, linkers for the immobilization of oligonucleotides on flat surfaces are central to the preparation of oligonucleotide microarrays.[4] Non-nucleic acid moieties appended to the termini can also be used to increase the affinity for target strands[5] or modulate the biological fate of oligonucleotides.[6] Even mRNAs have been terminally modified to allow conjugation to proteins.[7]

Recently, it was demonstrated that carboxylic acid residues directly appended to an oligonucleotide with a terminal aminodeoxynucleotide can have substantial effects on the stability of DNA duplexes.[8] Work in these laboratories has focused on 5′-modified oligonucleotides, but oligonucleotides with a 3′-terminal 2′-deoxy-2′-nalidixoylamidouridine residue (**1NA**, Figure 1) have also been described.[9] The duplex (5′-ACGCGU−NA-2′)$_2$, where NA is a nalidixic acid residue, has a UV melting point (T_m) up to 22 °C higher than that of unmodified control duplexes (ACGCGU)$_2$ and (ACGCGT)$_2$. This melting point increase is lower than that of the best

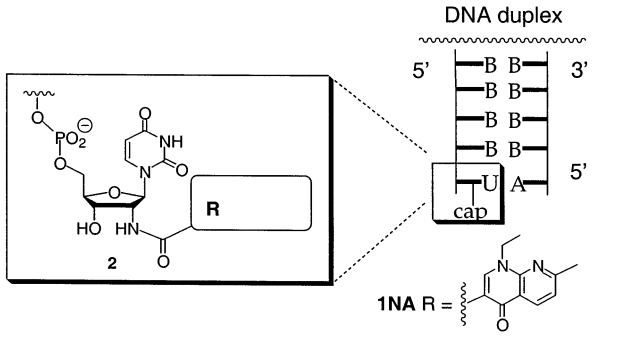


Figure 1.

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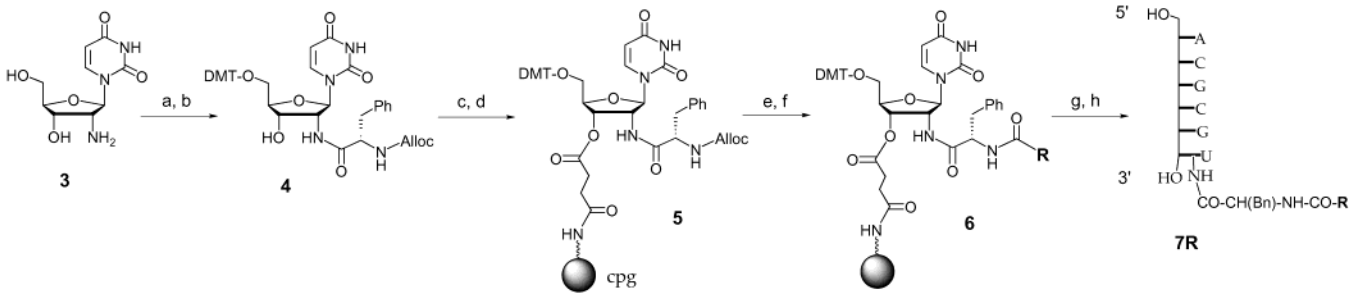
Scheme 1[a]



[a] Reaction conditions: (a) Alloc-Phe-OH, HBTU, DIEA, DMF; (b) DMT-Cl, DMAP, TEA, pyridine; (c) succinic anhydride, DMAP, pyridine; (d) LCAA-cpg, HBTU, HOBT, DIEA, DMF; (e) $Pd(PPh_3)_4$, $[Et_2NH_2]^+HCO_3^-$, PPh_3, CH_2Cl_2; (f) R-CO_2H, HBTU, HOBT, DIEA, DMF; (g) DNA synthesis via 3′-phosphoramidite protocol; (h) NH_4OH.

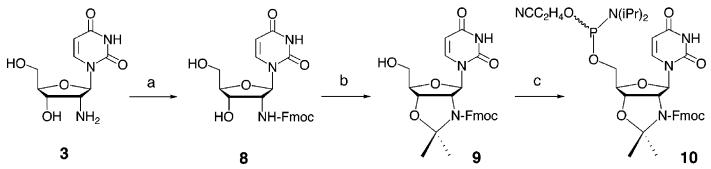
5′-cap identified to date, where a ΔT_m of up to 27.8 °C has been realized.[10] Therefore, it was decided to search for 3′-terminal uridine residues of general structure **2** (Figure 1), where the residue R provides greater duplex stabilization than the residue of nalidixic acid. This search was to involve combinatorial synthesis and spectrometrically monitored selection assays (SMOSE).[21] Here we report on a new route to oligonucleotides with 2′-acylamido-2′-deoxyuridine residues at their 3′-termini that allows for combinatorial syntheses and on the identification of a residue with a greater duplex-stabilizing effect than nalidixic acid.

2′-Acylamidonucleotides have previously been incorporated in oligoribonucleotides and oligodeoxyribonucleotides.[9−14] Two routes to small combinatorial libraries of oligonucleotides with 3′-terminal residues of general structure **2** were tested (Schemes 1−3). Both start from 2′-amino-2′-deoxyuridine (**3**).[15−17] The first route (Scheme 1) produced oligonucleotides where a phenylalanine residue links the acid "cap" to the aminouridine and employed conventional 3′-phosphoramidites for the unmodified portion of the DNA. The phenylalanine residue was inserted to avoid O-to-N acyl migration of the succinyl linker to the solid support. Such a migration could occur via a five-membered intermediate similar to that of ribonuclease-catalyzed hydrolysis of RNA strands[18] during the coupling of activated carboxylic acids. The route, involving protected nucleoside **4**, amino-acylated solid support **5**, and support-bound coupling product **6**, produced modified strands of general structure **7R**, where **R** were the residues of nalidixic acid (NA) and anthraquinone-2-carboxylic acid (AQ) (Figure 1 and Scheme 3). Attempts to prepare small combinatorial libraries via mixed couplings[21] by this route were thwarted by massive side reactions discovered in the MALDI-TOF mass spectra of crudes. These were presumably due to the low reactivity of the amino groups located close to the surface of the solid support and side reactions during DNA synthesis following the installation of the acid residues.

Therefore, an alternative route employing "reversed phosphoramidites", i.e., 5′-phosphoramidites,[19] was established. This synthesis involves chain assembly from the 5′- to the 3′-terminus of the DNA strand (Schemes 2 and 3). It places the amino group of the 2′-amino-2′-deoxyuridine residue at the distal end of the DNA chain attached to the solid support, ensuring high reactivity in solid-phase-based couplings to the amine and avoids side reactions with phosphoramidites during chain extension. The route required the synthesis of 5′-phosphoramidite with 2′,3′-protection of 2′-amino-2′-deoxyuridine (**3**). Several protection schemes were tested, including one where the 5′-hydroxyl was first protected with a dimethoxytrityl group, followed by installation of Alloc groups on the 3′-hydroxyl and 2′-amino groups. This route proved to be low-yielding because dimethoxytritylation of **3** was not sufficiently selective and resulted in concomitant protection of the 2′-amino group. Even the silylation of the 5′-position of **3** with a TBDPS group as a first step was unsatisfactory due to a lack of regio- and chemoselectivity. Best results were obtained with the route shown in Scheme 2. It involved 2′-protection of **3** with an Fmoc-group to give **8**, and *N*,*O*-acetal formation to give **9**. Phosphitylation of this double-protected nucleoside gave **10** in 54% overall yield from **3**.

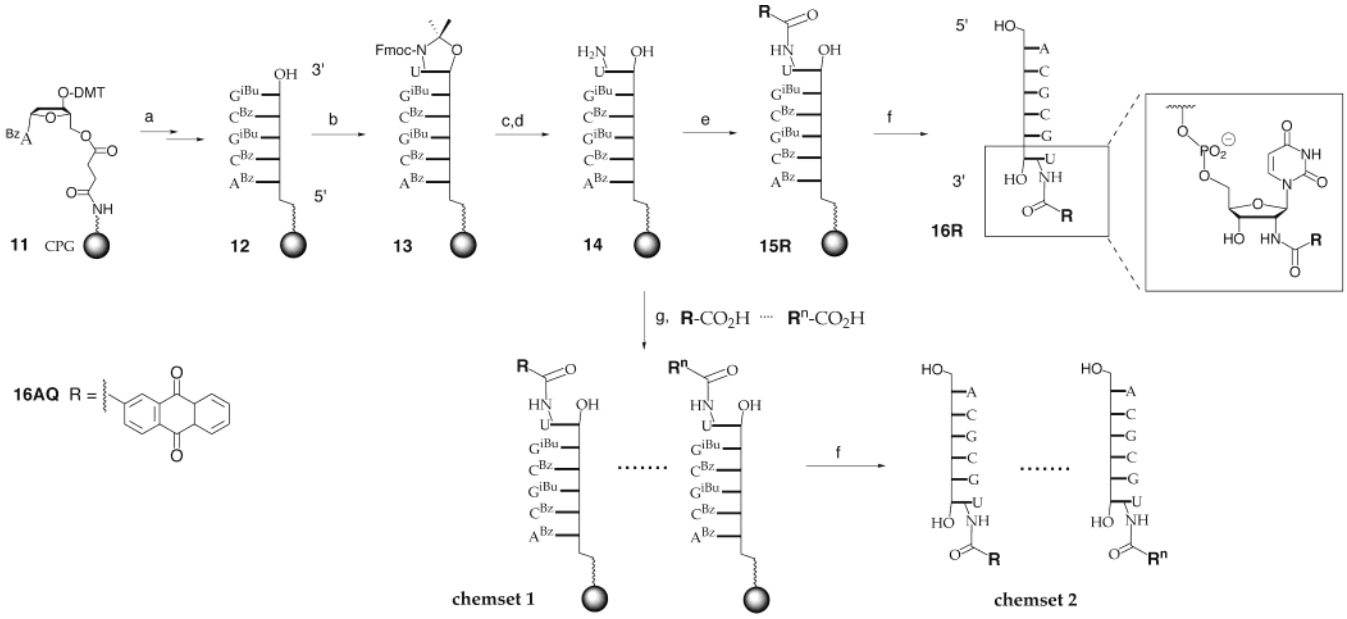
Starting from commercial support **11** and using 5′-phosphoramidites, chain assembly via **12** allowed incorporation of **10** in oligonucleotides in high yield, producing **13**, the isopropylidene group of which was made acid labile by removal of the Fmoc group with piperidine (Scheme 3).

Scheme 2[a]



[a] Reaction conditions: (a) Fmoc-OSu, DMF, 74%; (b) 2,2-dimethoxypropane, CSA, acetone, 96%; (c) NCC_2H_4O-P(N*i*Pr_2)$_2$, DIPAT, CH_3CN, 76%.

Scheme 3[a]



[a] Reaction conditions: (a) DNA synthesis with reverse phosphoramidites; (b) coupling cycle with **10**; (c) piperidine, DMF; (d) AcOH, H_2O; (e) R−CO_2H, HBTU, HOBT, DIEA, DMF; (f) NH_4OH; (g) carboxylic acid mixture, HBTU, HOBT, DIEA, DMF.

Treatment with 20% aqueous acetic acid for 15 min gave amino alcohol **14** without residual 2′/3′-protected species, as detected in MALDI-TOF mass spectra of deprotected crudes. Coupling of activated carboxylic acids to **14** proceeded in high yield, generating compounds of general structure **15R**, whose deprotected crudes again showed no uncoupled DNA hexamers. This encouraged us to perform combinatorial couplings with small reactivity-adjusted mixtures of carboxylic acids.[20] The mixtures employed are shown in Figure S9 (Supporting Information). When **14** was allowed to react with these mixtures of activated carboxylic acids, seven libraries of general structure **chemset 1** were formed, deprotection of which with NH_4OH gave **chemsets 2**. The MALDI-TOF mass spectra of the crude libraries are shown in Figures S1−7 (Supporting Information).

The crude oligonucleotide libraries of general structure **chemset 2** were then employed in nuclease survival selection experiments.[21] These subject small chemical libraries to the

Table 1. UV Melting Points and Hyperchromicities of DNA Duplexes

buffer	duplex[a]	T_m (°C)[b]	ΔT_m (°C)[c]	hyperchromicity (%)[b]	ref
150 mM NH_4OAc	(ACGCGT)$_2$ (**17**)$_2$	33.6 ± 1.0		9.0 ± 0.3	9
	(ACGCGU−NA)$_2$ (**16NA**)$_2$	48.0 ± 0.9	+14.4	16.5 ± 0.4	9
	(ACGCGU−Phe−NA)$_2$ (**7NA**)$_2$	48.1 ± 0.8	+14.5	18.3 ± 1.0	this work
	(ACGCGU−Phe−AQ)$_2$ (**7AQ**)$_2$	50.9 ± 1.0	+17.3	11.5 ± 0.6	this work
	(ACGCGU−AQ)$_2$ (**16AQ**)$_2$	53.6 ± 0.5	+20.0	13.5 ± 0.9	this work
1000 mM NH_4OAc	(ACGCGT)$_2$ (**17**)$_2$	33.9 ± 0.7		8.5 ± 0.2	9
	(ACGCGU)$_2$ (**10**)$_2$	34.1 ± 0.8	+0.2	9.0 ± 0.2	9
	(ACGCGU−NA)$_2$ (**16NA**)$_2$	50.5 ± 2.0	+16.6	13.7 ± 1.9	9
	(ACGCGU−Phe−NA)$_2$ (**7NA**)$_2$	49.6 ± 0.2	+15.7	16.5 ± 0.7	this work
	(ACGCGU−Phe−AQ)$_2$ (**7AQ**)$_2$	52.0 ± 0.3	+18.1	9.5 ± 0.9	this work
	(ACGCGU−AQ)$_2$ (**16AQ**)$_2$	56.2 ± 2.7	+22.3	19.1 ± 1.4	this work
10 mM phosphate buffer	(ACGCGT)$_2$ (**17**)$_2$	20.6[d]		6.3[d]	9
	(ACGCGU−NA)$_2$ (**16NA**)$_2$	35.2[d]	+14.6	16.6[d]	9
	(ACGCGU−AQ)$_2$ (**16AQ**)$_2$	48.0 ± 0.4	+27.4	9.4 ± 0.4	this work
150 mM NaCl, 10 mM phosphate buffer	(ACGCGT)$_2$ (**17**)$_2$	31.7 ± 0.7		7.9 ± 2.0	9
	(ACGCGU−NA)$_2$ (**16NA**)$_2$	52.6 ± 1.3	+20.9	17.5 ± 1.7	9
	(ACGCGU−AQ)$_2$ (**16AQ**)$_2$	56.3 ± 0.2	+24.6	8.6 ± 1.3	this work
1 M NaCl, 10 mM phosphate buffer	(ACGCGT)$_2$ ((**17**)$_2$	31.1 ± 0.3		6.6 ± 1.4	9
	(ACGCGU−NA)$_2$ (**16NA**)$_2$	53.1 ± 0.3	+22.0	16.7 ± 0.7	9
	(ACGCGU−AQ)$_2$ (**16AQ**)$_2$	59.2 ± 0.3	+28.1	9.8 ± 0.2	this work

[a] Sequences are given from the 5′- to the 3′-terminus. [b] Average of four melting points ± SD at a 3.5 ± 0.6 μM strand concentration. [c] Melting point difference to control strand. [d] Average of two melting curves.

attack of single-strand-specific nucleases. In the present case, calf spleen phosphodiesterase (E.C. 3.1.16.1) was employed, which digests single-stranded oligonucleotides from the 5′-terminus, i.e., the terminus where the strands are unmodified. Modified strands with 2′-appendages strongly stabilizing their duplexes could be expected to be in the single-stranded state to the smallest extent, thus resisting nuclease attack the longest. To specifically select for strands with "caps" better than the known nalidixic acid residue, 1 equiv of compound **16NA** was added to every library. The MALDI-TOF mass spectra of mixtures undergoing nuclease selection were screened for peaks of full length oligonucleotides surviving longer than **16NA**. While the libraries produced with mixtures 1−6 did not yield compounds exceeding **16NA** in lifetime, the selection of the library prepared with mixture 7 did show that **16AQ** was a "lone survivor" (Figure S8, Supporting Information).

Accordingly, **16AQ** was synthesized individually, HPLC purified, and subjected to UV melting experiments under various buffer conditions (Table 1). Compared to control duplex $(ACGCGT)_2$ $(\mathbf{17})_2$, that of lead compound $(ACGCGU{-}NA)_2$ $(\mathbf{16NA})_2$, and those of the two phenylalanine-linked hybrids $(ACGCGU{-}Phe{-}NA)_2$ $(\mathbf{7NA})_2$ and $(ACGCGU{-}Phe{-}AQ)_2$ $(\mathbf{7AQ})_2$, the duplex of **16AQ** melted at higher temperatures under all conditions tested. The melting point increase (ΔT_m) was greatest in phosphate buffered saline solution, reaching +28.1 or +14 °C per modification at 1 M NaCl and 10 mM phosphate buffer. This is the highest melting point increase observed to date for an acylamido/deoxy-modified DNA hexamer, slightly above the 27.8 °C realized for a 5′-appended quinolone, which reached this value at 10 mM salt, i.e., conditions less favorable for hybridizations on DNA chips.[22,23]

It is interesting to note that the "winner cap" identified in this exploratory combinatorial study has been tested as a 5′-cap in our earlier work[21b] and did not come up as a strongly stabilizing residue. Nor did cholic acid, the residue of which is known to tightly bind to terminal base pairs when appended to the 5′-terminus as an acylamido substituent,[8b] produce a hit, suggesting that the duplex-stabilizing effect of the anthraquinone carboxylic acid residue is not an unspecific effect due to its hydrophobicity. Modeling and force field minimizations performed with an anthracene carboxylic acid moiety suggest that a residue of this shape can stack on the terminal base pair formed by the aminodeoxyuridine without disruption of base pairing.[24]

In conclusion, the results presented here show that oligonucleotides with 2′-acylamido-2′-deoxyuridine residues at the 3′-terminus can be prepared via a short and rugged route allowing for the generation of small combinatorial libraries. Anthraquinone carboxylic acid as the building block for the acyl portion of a modified hexamer leads to significantly enhanced duplex stability.

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Supporting Information Available: Experimental procedures and MALDI-TOF mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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