Oligodeoxynucleotides incorporating structurally simple 5-alkynyl-2′-deoxyuridines fluorometrically respond to hybridization†

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Selected 5-ethynyl derivatives of 2′-deoxyuridine are shown to fluorometrically respond to hybridization and selectively base-pair to adenine whilst maintaining duplex stability.

With advances in genome sequencing, there is an increasing need for probes and other molecular tools for clincal diagnostics, environmental and agricultural monitoring, and research use. Of the various techniques used for detection of hybridization events, fluorescence-based methods are popular because of their sensitivity, rapidity and possibility of automation.

Over the last several years, there has been much research into fluorescent derivatives of nucleosides for use as hybridization probes. Nucleoside derivatives that remain capable of recognition of a complementary base have been termed "base discriminating fluorescent" nucleosides,[1] or fluorescent DNA base replacements.[2] Within the various and diverse structures of fluorescent nucleosides possessing a modified nucleobase, two general subclasses of fluorophores can be differentiated based on the nature of the chromophore. One manifestation is achieved by appending a relatively large fluorophore to the nucleobase[3] while the other approach relies on transforming the base-pairing moiety itself into a fluorophore.[4] Potential advantages of the latter approach are that the chromophore is minimized and one can also take advantage of the predictable change in environment of the nucleobase between the single-stranded state and duplex states.

Recently, there has also been some interest in minimized chromophores based on derivatization of uracil, such as the compact 5-biaryl derivatives, used for the detection of abasic sites.[5] It has long been recognized that the bicyclic uracil derivative furanouracil, that is prepared from 5-alkynyluracil, is fluorescent, Fig. 1.[6] However, upon formation of the fused ring, its hydrogen bonding properties change such that it is no longer complementary to adenine. Only quite recently has it been clearly described that structurally simple 5-phenylethynyl-derivatives of uracil are intrinsically fluorescent and that a large extrinsic chromophore is not a structural requirement.[7,8] Although structurally modest 5-alkynyluracil derivatives (*e.g.* alkyl, phenyl) have been extensively studied in nucleic acid analogs, their fluorescence properties and use as fluorescent labels has been largely overlooked. Herein, we report that simple, compact acyclic precursors to furanouracil

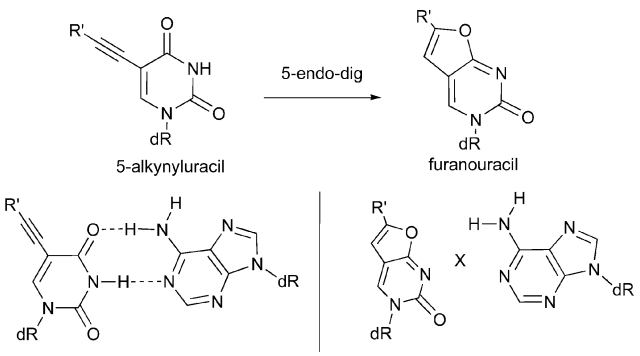
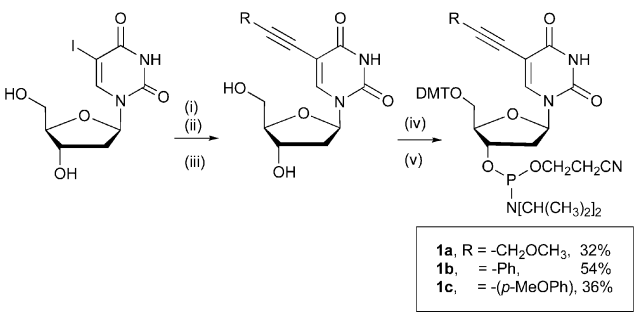


Fig. 1 Structure of 5-alkynyluridine and its proposed base-pairing (left) compared to the cyclic derivative furanouridine. dR = 2′-deoxyribose.

are fluorescent, that the fluorescence is responsive to the state of hybridization and the modified bases maintain the fidelity of base-pairing, Fig. 1.

Previously, we have reported that N1-unsubstituted and N1-alkyl derivatives of 5-alkynyluracils are potential fluorophores for use in the oligonucleotide analog peptide nucleic acid (PNA).[8] Since the chemistry and use of oligodeoxynucleotides are much better developed and more accessible to the research community, as compared to PNA chemistry, we pursued the study of the same modifications in DNA. These fluorophores are readily prepared according to Scheme 1.



Scheme 1 Synthesis of the (2′-deoxy-β-D-*ribo*-pentofuranosyl)-5-alkynyluridine derivatives suitable for oligonucleotide synthesis, *via* the bis-(acetylated) nucleoside. (i) Ac_2O, py, 24 h. (ii) RC≡CH, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, rt, 24 h. (iii) py–EtOH (1 : 5, v/v), 1 M NaOH, 4 °C. (iv) DMT-Cl, py, 5 h. (v) Cl(i-Pr_2N)$POCH_2CH_2CN$, CH_2Cl_2, NEt_3. Overall chemical yield from the common intermediate 5-iodo-5′,3′-*O*-bis(acetyl)-2′-dexoyuridine, is shown.

Firstly, the commercially available 5-iodo-2′-deoxyuridine was acetylated and then employed as a substrate in the Sonogashira coupling with a small selection of terminal alkynes.[9] Subsequent

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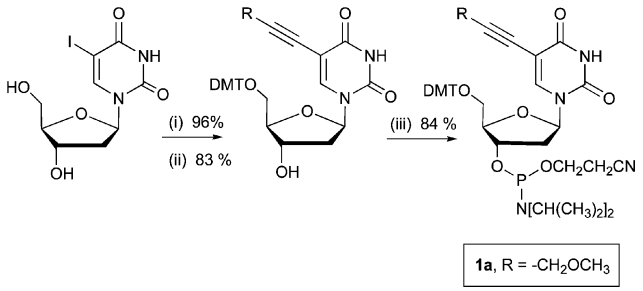
saponification gave the nucleoside derivative, which, after standard transformations, yielded the phosphoramidite reagent.

Although these conditions worked well, the coupling with methyl propargyl ether gave a product with better aqueous solubility than the corresponding 5-phenylethynyl nucleosides and this resulted in losses during the aqueous workup. To circumvent this difficulty, dimethoxytritylation of 2′-deoxy-5-iodouridine was done first, followed by cross-coupling as previously reported, Scheme 2.[10] This method gave a better yield of **1a**, was one step shorter and was suitable for the other alkynes used, leading to improved overall yields as compared to the approach in Scheme 1.§



Scheme 2 Synthesis of the (2′-deoxy-β-D-*ribo*-pentofuranosyl)-5-alkynyluridine derivatives suitable for oligonucleotide synthesis *via* the 5′-dimethoxytrityl nucleoside (i) DMT-Cl, py, 5 h. (ii) $CH_3OCH_2C{\equiv}CH$, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, rt, 24 h. (iii) $Cl(i\text{-}Pr_2N)POCH_2CH_2CN$, CH_2Cl_2, NEt_3.

The three modified nucleosides were incorporated into oligodeoxynucleotides, using standard conditions with the exception of extended coupling time (3 min), Table 1. Each modification is tolerated, having a negligible effect on the overall duplex stability while maintaining excellent mismatch discrimination—properties that are in accord with literature precedent.[11]

The base-modified nucleosides were also placed at either the 3′- or 5′-terminus and showed no deleterious effect on duplex stability, Table 2. The thermal denaturation temperature (*T*m) data support the conclusion that the expected hydrogen bonding is in operation and that the 5-substitution does not substantially affect the tautomeric form of the uracil.

Table 1 Thermal denaturation data for DNA sequences[a]

		*T*m/°C[a]			
		Target strand (3′ → 5′)			
	Central modification	GCG-TTA-**X**-ATT-GCG			
	DNA sequence (5′ → 3′)	X = A	X = G	X = C	X = T
		6	**7**	**8**	**9**
2	CGC-AAT-**T**-TAA-CGC	51.5	42.5	39.5	40.5
3	CGC-AAT-^{MME}**U**-TAA-CGC	52.0	42.0	40.0	41.5
2	CGC-AAT-^{Ph}**U**-TAA-CGC	51.5	40.0	40.5	39.0
5	CGC-AAT-^{MeOPh}**U**-TAA-CGC	49.5	39.5	39.5	38.5

In bold: modified nucleobase and complement.[a] Ionic conditions: 100 mM NaCl, 10 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.0. ^{MME}U = (2′-deoxy-β-D-*ribo*-pentofuranosyl)-5-methoxymethylethynyluridine. ^{Ph}U = (2′-deoxy-β-D-*ribo*-pentofuranosyl)-5-phenylethynyluridine. $^{MeOPh}U$ = 2′-deoxy-β-D-*ribo*-pentofuranosyl)-5-(*para*-methoxy)phenylethynyluridine.

Table 2 Thermal denaturation data for DNA sequences[a]

	5′ Terminus modification	Target strand (3′ → 5′)
	(5′ → 3′)	**A**GG-TCG-CGT-TG
10	**T**CC-AGC-GCA-AC	53.0
11	^{MME}**U**CC-AGC-GCA-AC	52.5
12	^{Ph}**U**CC-AGC-GCA-AC	53.5
13	^{MeOPh}**U**CC-AGC-GCA-AC	53.0
		Target strand (3′ → 5′)
	3′ Terminus modification	CGG-ATT-GAA-GGC-CTC-TAC-**A**
14	GCC-TAA-CTT-CCG-GAG-ATG-**T**	63.0
15	GCC-TAA-CTT-CCG-GAG-ATG-^{MME}**U**	63.0
16	GCC-TAA-CTT-CCG-GAG-ATG-^{Ph}**U**	62.0
17	GCC-TAA-CTT-CCG-GAG-ATG-^{MeOPh}**U**	63.0

In bold: modified nucleobase and complement.[a] See Table 1, footnote.

Table 3 Spectral data for modified 2′-deoxyuridines

	Modification	ε^a (260 nm)	λ/nm[b] Excitation	λ/nm Emission
1a	^{MME}U	4100	350	450
1b	^{Ph}U	14075	320	400
1c	^{MeOPh}U	19450	330	450

[a] Extinction coefficients were determined for the nucleoside by Beer's plot. [b] Excitation and emission data are reported for single-stranded oligomers **3**, **4** and **5**, representing the modifications **1a**, **1b**, and **1c**, respectively.

The fluorescence properties of the oligomers were subsequently examined according to the parameters shown in Table 3, under identical conditions as the thermal denaturation studies (concentration and ionic strength) to maintain the same *T*m value as observed in the thermal denaturation experiments.

From our earlier synthetic studies on cytosine nucleobase derivatives,[12] we expected the 5-methoxymethylethynyl-derivatized uracil (**1a**) to demonstrate relatively poor fluorescence emission in comparison to the phenylethynyl cogeners (**1b**,**c**). Although this was found, oligomer **3** also demonstrated an excellent ability to fluorometrically report hydridization. As illustrated in Fig. 2, at 2 μM strand concentration, the single-stranded species (grey line) has little fluorescence, whereas in the presence of complementary DNA (**6**), a six-fold increase in fluorescence intensity is observed.

We reasoned that we could improve on the weak fluorescence, yet maintain a compact fluorophore by employing an unsaturated substituent on the alkyne. Thus, compound **1b** was prepared and incorporated into an oligomer (**4**). Indeed, compared to **3**, the emission is more intense, yet a loss in the fluorescent-based discrimination between the single-stranded **4** (grey line) and its matched complex (**4** + **6**, black line) was observed (2-fold change), Fig. 3.

To maintain the compactness of the overall chromophore, we were inspired to investigate the *p*-methoxyphenylethynyl substituent (**1c**). This substituent proved to impart the combined

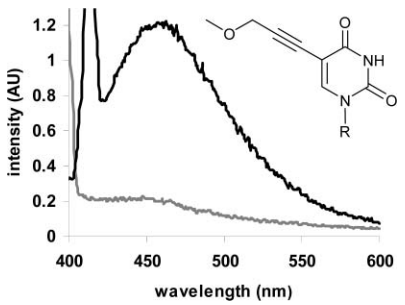


Fig. 2 Steady state fluorescence response of a 5-methoxymethylethynyluracil-containing oligonucleotide (**3**) in the presence of complementary oligonucleotide **6** (black) as compared to single-strand (grey). Oligonucleotides at 2.0 μM in 100 mM NaCl, 10 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.0, 22 °C.

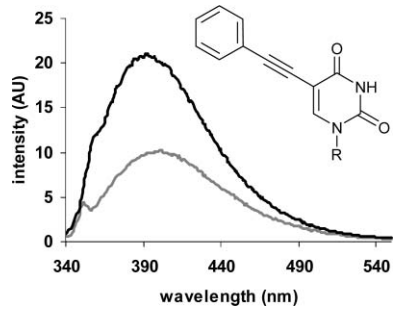


Fig. 3 Steady state fluorescence response of a 5-phenylethynyluracil-containing oligonucleotide (**4**) in the presence of complementary oligonucleotide **6** (black) as compared to single-strand (grey). Oligonucleotides at 2.0 μM in 100 mM NaCl, 10 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.0, 22 °C.

desirable properties of relatively high emission and a large change in fluoresence upon hybridization. In this instance, a six-fold increase in fluorescence intensity was observed when oligomer **5** bound complementary DNA (**6**), Fig. 4.

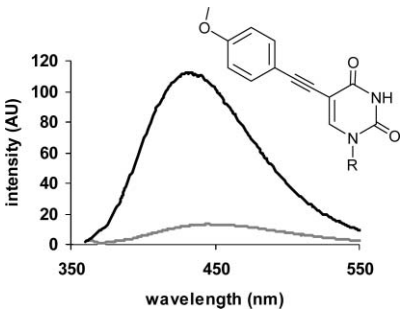


Fig. 4 Steady state fluorescence response of a 5-(*p*-methoxyphenylethynyl)uracil-containing oligonucleotide (**5**) in the presence of complementary oligonucleotides **6** (black) as compared to single-strand (grey). Oligonucleotides at 2.0 μM in 100 mM NaCl, 10 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.0, 22 °C.

The fluorescence reporting ability of these nucleobases was examined when placed at either 5′- or 3′-terminal positions (oligomers **10–17**). Generally, the fluorescence signal of the terminally modified oligonucleotides was unresponsive to state of hybridization. Taken altogether, the fluorescence response is consistent with the idea that the modified base experiences less quenching in the rigid, hydrophobic base stack environment compared to the unstructured and solvent exposed single-strands or terminal positions that are susceptible to end-fraying.

The fluorescence response of the centrally modified oligomers to mismatches is interesting.† Even though the experimentally determined thermal denaturation data (Table 1) and calculated *T*m data[13] indicate roughly equal thermodynamic stability of the mismatched hybrid duplexes, irrespective of the nature of the mismatch, the fluorescence data infers different environments for the chromophore. For each mismatched duplex, when the 5-modified nucleobase is across from guanine (*e.g.* duplexes **3** + **7**, **4** + **7**, **5** + **7**), the emission spectrum is more similar to the single-strand spectrum than the duplex, that is, it suffers from an almost equivalent amount of quenching. Whereas, for the oligomers bearing the phenylethynyl-modified bases (**4**, **7**), a mismatch to thymine produces less quenching. Finally, a mismatch to cytosine produces spectra that are more similar to the fully matched duplex than any other mismatch or the single-strand. These results suggest that the chromophore is sensitive to its local environment and may be useful for examining changes in structure that are more subtle than complete denaturation/renaturation. Currently, we present a phenomenological description of the fluorescence response; further studies are needed to characterize the photophysical basis of these observations.

In conclusion, we show that structurally simple and compact 5-alkynyl-derivatized 2′-deoxyuridines are fluorescent reporters of hybridization events when placed at internal positions in oligodeoxynucleotides. In addition, the chromophores are responsive to their local structure/environment and therefore have potential use in the examination of nucleic acid conformation or nucleic acid–ligand interactions.

Notes and references

§ Sonogashira cross-coupling was also successful using the unprotected nucleoside, as reported in ref. 6.

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