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On the stability of furanopyrimidin-2-one bases in oligonucleotides†

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The fluoresceinated furanopyrimidin-2-one nucleobase 4 incorporated into an oligonucleotide undergoes unexpectedly facile hydrolytic ring-opening in aqueous buffer at slightly elevated temperatures.

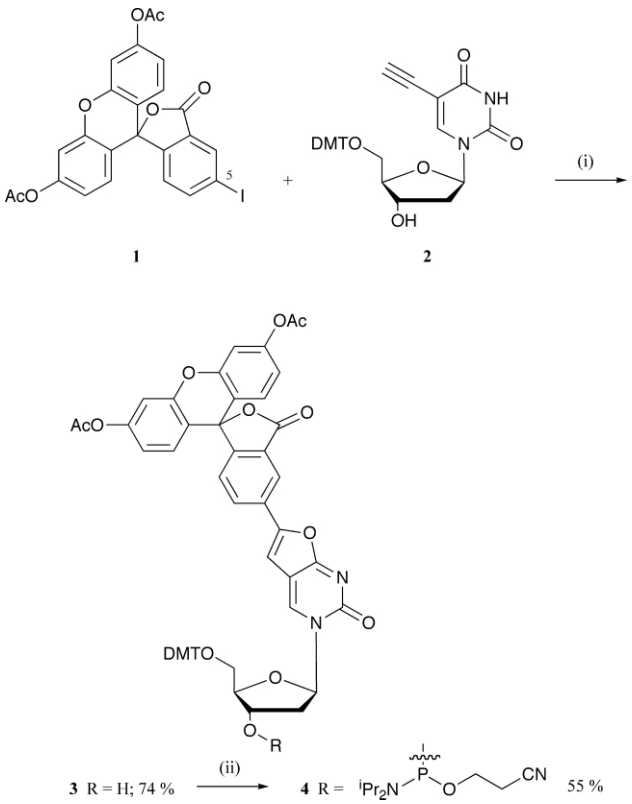
2′-Deoxy-5-alkynyluracil bases **A** are useful analogs of uracil and thymidine which are conveniently prepared *via* Sonogashira reactions[1] of 5-iodouracil.[2] Their alkyne substituents tend to be directed away from the helical structure of double strand (ds)-DNA hence decreasing the likelihood that they will perturb its structure and function.[3] However, 5-alkynyluracil bases tend to cyclize onto the alkyne substituent giving the corresponding furanopyrimidin-2-ones **B**.[4] These derivatives are also interesting, particularly for their applications in medicinal chemistry.[5–7] The most recent phase of research in this area was initiated by Yu *et al.* who incorporated furanopyrimidin-2-ones into oligonucleotides and studied the stabilities of modified ds-DNA produced in this way *via* thermal denaturation experiments.[8] Reported here is a potential side reaction that can compromise data obtained from that kind of approach.

Our interest in this area began when attempting to couple 5-iodofluorescein **1**[9] with the 5-alkynyluracil derivative **2**.[10] The non-cyclized Sonogashira products were formed when the reaction was performed at near ambient temperature for relatively short times (cat. $Pd(PPh_3)_4$, CuI, NEt_3, THF, 40 °C, 0.5 h; 83%; not shown),[11] but longer reaction times at higher temperatures favoured formation of the cyclic product **3** (Scheme 1).

The next step was to investigate the properties of the fluoresceinated furanopyrimidin-2-one in DNA. Consequently, compound **3** was converted to the corresponding phosphoramidite **4** *via* standard methods, then incorporated into the oligonucleotide **5** *via* solid phase syntheses.[12]

In their work on oligonucleotides modified with a furanopyrimidin-2-one base, Yu *et al.* reported thermal denaturation data that implied this type of analog anneals with highest affinity to a G complement, *i.e.* the base behaves as a C analog. However, the data in Fig. 1 indicate the ds-DNA sequence **6** dissociates at 11 °C less than **7**; this melting temperature is comparable to the T:G mismatched system **8**. Conversely, when the modified base was set opposite an A residue in the duplex **9** then the melting temperature was within 0.8 °C of the T:A matched situation **10** and 11.8 °C greater than the A:A mismatched one in **11**. These results strongly imply the modified base in oligonucleotide **5** resembled a T residue more than a C.

A standard procedure for measuring thermal denaturation was used to obtain the data shown in Fig. 1.[10] This involved first heating the sample in phosphate buffer (pH 7.2) to 90 °C for 5 min, then

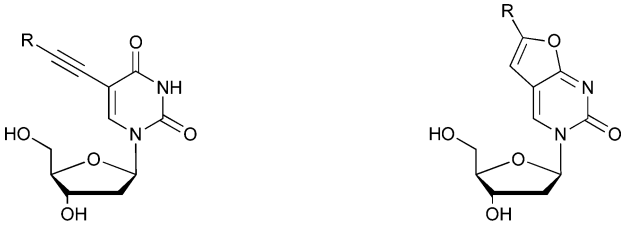


Scheme 1 Preparation of **3** and **4**, *Reagents and conditions*: (i) cat. $Pd(PPh_3)_4$, CuI, NEt_3, THF, 55 °C, 12 h; (ii) $ClP(N^iPr_2)(OCH_2CH_2CN)$, Et N^iPr_2, CH_2Cl_2, 25 °C, 4 h.

5'-TCA ACG ZAG CTG A-3'
5

5'-TCA ACG ZAG CTG A-3' **3'-AGT TGC GTC GAC T-5'** **6** T_m = 46.0 °C	**5'-TCA ACG ZAG CTG A-3'** **3'-AGT TGC ATC GAC T-5'** **9** T_m = 50.3 °C
5'-TCA ACG CAG CTG A-3' **3'-AGT TGC GTC GAC T-5'** **7** T_m = 57.0 °C	**5'-TCA ACG TAG CTG A-3'** **3'-AGT TGC ATC GAC T-5'** **10** T_m = 51.1 °C
5'-TCA ACG TAG CTG A-3' **3'-AGT TGC GTC GAC T-5'** **8** T_m = 45.1 °C	**5'-TCA ACG AAG CTG A-3'** **3'-AGT TGC ATC GAC T-5'** **11** T_m = 38.5 °C

Fig. 1 Sequence of **5** where ***Z*** is the position at which **4** was incorporated, the ds-DNA oligomers that were made from it, and their melting temperatures.



† Electronic supplementary information (ESI) available: experimental procedures for the preparation of the new compounds, protocols for the thermal denaturation and enzyme digestion experiments. See http://www.rsc.org/suppdata/cc/b4/b402559h/

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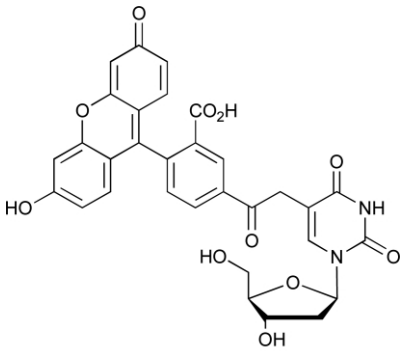
cooling it rapidly to 0 °C; it was then heated from 0 °C to 90 °C in small temperature increments over 6 h while the absorbance was recorded. To test if this protocol caused a chemical change in **5**, it was repeated on this oligonucleotide alone. The molecular ion detected before this treatment was 4296 Da (MALDI), and the calculated mass was 4297 Da, these readings are within experimental error. However, after the thermal denaturation cycle the observed mass was 4315 Da, *representing an increase of approximately 18 units*.

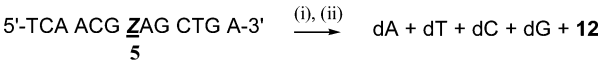
A literature search revealed that a nucleoside containing a furanopyrimidin-2-one base had been treated with 2 M NaOH and was found to open at the furan ring.[13] When the fluorescein-labelled furanopyrimidin-2-one **3** was subjected to the same conditions (2 M NaOH, THF, 25 °C, 6 h), it was found to ring-open in a similar way giving the ketone **12**.



12

Finally, in a key experiment, the ss-oligonucleotide that had been subjected to the thermal denaturation sequence described above, was then digested with a combination of enzymes to cleave all the phosphodiester bonds. HPLC analyses of the digest indicated it contained dA, dT, dC, dG, and one extra peak. Co-injection of the digest with standard samples indicated this extra peak did not correspond to the furanopyrimidin-2-one (*i.e.* compound **3** without DMT protection), but it did co-elute with **12** (Scheme 2).

It is clear that experiments with modified nucleosides can be complicated by the formation of furanopyrimidin-2-ones and their hydrolytic ring-opening reactions. Curiously, there are many



Scheme 2 Thermal denaturation and enzymatic degradation of **5**. *Reagents and conditions*: (i) 90 °C, phosphate buffer pH 7.2, 5 min then 0 °C to 90 °C over 6 h; (ii) phosphodiesterase 1, alkaline phosphatase, and nuclease, $MgCl_2$, Tris HCl pH 7.5, 37 °C, 24 h.

syntheses of 2′-deoxy-5-alkynyluracil derivatives in the literature where this cyclization has not been observed. However, work by Yu *et al.*[8] and others[14] indicate that the ferrocene-substituted compounds **B**, R = Fc, seem to undergo this cyclization readily. Conversely, that same ferrocenyl derivative seems to be stable to thermal denaturation conditions (though Yu *et al.* did note that some unidentified materials were observed on enzyme digestion of their modified oligonucleotides) while the fluorescein-substituted compounds featured here appear to be more vulnerable to this ring-opening process. We conclude that the substituent R may have a relatively strong influence on the ease with which structures **A** convert to the cyclized forms **B**, and on subsequent ring-opening reactions. Overall, these studies indicate that thermal denaturation data of modified oligonucleotides containing furanopyrimidin-2-ones should be interpreted with care, and medicinal chemists working with these types of nucleosides as pharmaceutical leads might consider hydrolytic ring-opening of the nucleobase as a possible metabolic pathway *in vivo*.

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