7,8-Dihydropyrido[2,3-*d*]pyrimidin-2-one; a bicyclic cytosine analogue capable of enhanced stabilisation of DNA duplexes†

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Incorporation of a bicyclic cytosine analogue, 3-β-D-(2′-deoxyribofuranosyl)-7,8-dihydropyrido[2,3-*d*]pyrimidine, into synthetic DNA duplexes results in a greatly enhanced thermal stability (3–4 °C per modification) compared to the corresponding unmodified duplex.

Oligodeoxyribonucleotides (ODNs) containing modified bases are in widespread use as probes and primers[1] and are growing in importance for use in DNA microarray technology[2] and as therapeutic agents.[3] These applications typically rely upon the formation of nucleic acid duplexes within which the modified ODN hybridises to a defined target sequence with both high specificity and stability. In this context, the attachment of the propynyl group to the C5-position of 2′-deoxyuridine and 2′-deoxycytidine[4] (**1**; Fig. 1) increases the melting temperatures of duplexes typically between 1.5 and 2 °C per modification. For DNA–RNA hybrid duplexes in which the entire DNA strand is modified, even greater enhancement in stability per modification is observed.[5,6] Furthermore, a wide range of other modified propynyl substituents are also known to confer enhanced duplex stability.[7,8] DNA duplexes containing 7-deazapurine bases functionalised on C7 with propynyl substituents also display enhanced stabilities.[9,10] In contrast, there are very few reports describing the stability of DNA duplexes containing C5-alkenyl modified pyrimidines. In one such example concerning *E*-5-(2-bromovinyl)-2′-deoxyuridine-containing ODNs[11] no stabilisation of the DNA duplex was observed.

Despite the relative wealth of information concerning ODNs containing modified bases that lead to the duplex stabilisation mentioned above, studies of the thermodynamic driving forces for duplex stabilisation are less common. Provided the base-pairing properties of the bases are retained, the introduction of additional apolar groups is generally expected to increase stacking interactions.‡[12] Analogous to base stacking in unmodified DNA, these stacking interactions are expected to lead to an additional favourable enthalpy term for duplex formation. Indeed, enthalpy driven duplex stabilisation is found when comparing duplexes containing propynylated deoxyuridine with unsubstituted deoxyuridines.[13] Methylation of cytosines or uridines, however, leads to little change in the enthalpy of duplex formation[6,14]

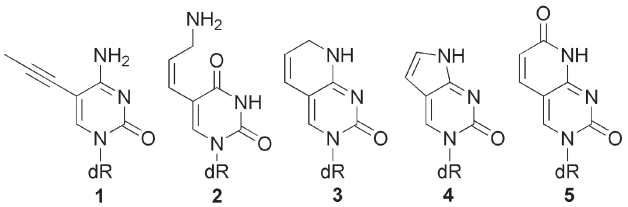

Fig. 1 C5-modified cytosine analogues **1–5**. dR = 2-deoxyribose.

During our recent studies[15,16] of the properties of DNA containing C5-amino-modified 2′-deoxyuridine analogues, we prepared the novel analogue 5-(*Z*-3-aminoprop-1-enyl)-2′-deoxyuridine (**2**) and incorporated it into synthetic ODNs.[8] However during deprotection of these ODNs using aqueous ammonia solution, we encountered the cyclisation of the nucleoside **2** to form as the major product ODNs containing the nucleoside **3**, which contains a bicyclic C5-alkenyl-modified cytosine analogue. Our initial studies of ODNs containing this bicyclic cytosine analogue revealed a remarkable enhancement of duplex stability compared to the umodified sequence when the modification was placed opposite guanine. Typically, we observed increased T_m values of up to 4 °C per modification compared to the unmodified duplex (Brazier and Williams, unpublished data). A survey of the literature revealed that the 5′-triphosphate of **3** has been described in a patent, as has the related compound in which the exocyclic alkene has been reduced.[17] However there are few experimental details and no reference to the synthesis of the corresponding phosphoramidite of **3** nor ODNs containing the modified base. Interestingly, ODN duplexes containing compound **4**, a homologue of **3**, have been described.[18] When placed opposite template guanine, compound **4** causes a slight decrease in T_m. The fluorescent 6-methyl analogue of **4** has recently also been incorporated into ODNs and oligoribonucleotides.[19] In this instance, similar T_m values were found for both modified and natural duplexes in which the analogue or cytosine was paired with guanine. The related fluorescent 2′-deoxyribonucleoside **5**[20] when placed within 10mer ODN duplexes shows a base-pairing specificity with guanine, enhanced T_ms for duplex formation, but unfortunately no thermodynamic data are reported.

The chemical synthesis of ODNs containing a single substitution of **3** can be achieved following ammonia treatment of the corresponding sequences containing analogue **2**. However, HPLC purification of such ODNs containing multiple substitutions is not feasible (Brazier and Williams, unpublished results). Consequently in order to further study the properties of ODNs

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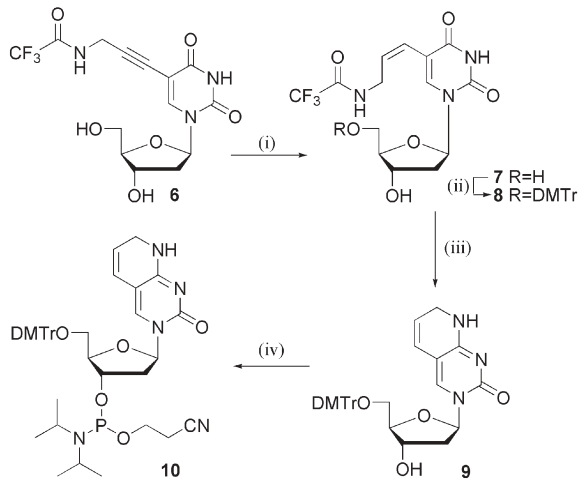

Scheme 1 Synthesis of phosphoramidite analogue. i) $NiCl_2{\cdot}6H_2O$, $NaBH_4$, MeOH, −78 °C, 30 min;[15] ii) dimethoxytrityl chloride, DMAP, pyridine, r.t.;[8] iii) NH_4OH, MeOH, r.t., 95%; iv) 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite, *N*,*N*-diisopropylethylamine, DCM, 0 °C, 2 h, 62%.

containing **3** we have now prepared its corresponding phosphoramidite and report here its synthesis, the preparation of ODNs containing **3** and the thermodynamic properties of ODN duplexes containing one or more such modified bases.

The phosphoramidite of compound **3** was prepared according to Scheme 1 (see ESI for experimental details). Thus, trifluoroacetyl protected 5-(*Z*-3-aminoprop-1-enyl)-2′-deoxyuridine **7** was obtained from 5-[3-(trifluoroacetamido)prop-1-ynyl]-2′-deoxyuridine as described.[15] The bicyclic nucleoside was then obtained following treatment of **7** with aq. ammonia.[8] However, upon treatment of **3** with dimethoxytrityl chloride, we obtained a complex mixture of products comprising several nucleosidic components as visualised by silica TLC. Consequently, compound **7** was converted into its corresponding 5′-*O*-dimethoxytrityl dervative **8**[8] which was then treated with aq. ammonia. The 5′-protected bicyclic cytosine analogue **9** was obtained in 95% yield following silica gel chromatography. Phosphitylation of **9** using 2-cyanoethyl-*N*,*N*′-diisopropyl chlorophosphoramidite furnished the phosphoramidite **10** in 62% yield.

In order to investigate the effect of the bicyclic cytosine analogue **3** on the stability of DNA duplexes, we synthesised the modified 11mer ODNs (ODN-x, where x indicates the number of modifications) containing between 1 and 4 modifications (Table 1). The complementary sequence (ODN-c) has guanine placed opposite the analogue, whilst ODN-cm possesses a mismatched adenine. In each case DNA synthesis was performed DMT-ON. ODNs were then purified by reversed phase HPLC, detritylated using 20% aqueous acetic acid, repurified by HPLC and finally dialysed. All ODNs were characterised by MALDI MS (Table 1).

DNA melting was studied by monitoring the temperature dependence of the UV absorption at 260 nm for ODN duplexes c:x0–4 and ODN mc:x0–1. In the latter case cytosine or the analogue **3** is mispaired with adenine. Normalised UV melting curves (shown in Fig. 2) and concentrations were corrected for volume expansion using Kell's density data for water,[21] pre- and post-transition baselines were fitted to the UV-absorption data and an α-plot was constructed (Fig. 1, ESI).[22] Equilibrium constants

Table 1 ODNs used in this study

		MALDI MS	
ODN[a]	Sequence	calcd	found
x0	5′-ACT CCT GCT AC-3′	3252.2	3253
x1	5′-ACT CXT GCT AC-3′	3290.2	3289
x2	5′-ACT CXT GXT AC-3′	3328.3	3328
x3	5′-AXT CXT GXT AC-3′	3366.3	3364
x4	5′-AXT XXT GXT AC-3′	3404.3	3404
c	3′-TGA GGA CGA TG-5′	3421.2	3421
mc	3′-TGA GAA CGA TG-5′	3405.2	3405

[a] ODN is labelled according to the number of modifications. c = complementary strand, mc = mismatched strand. X = modification **2**.

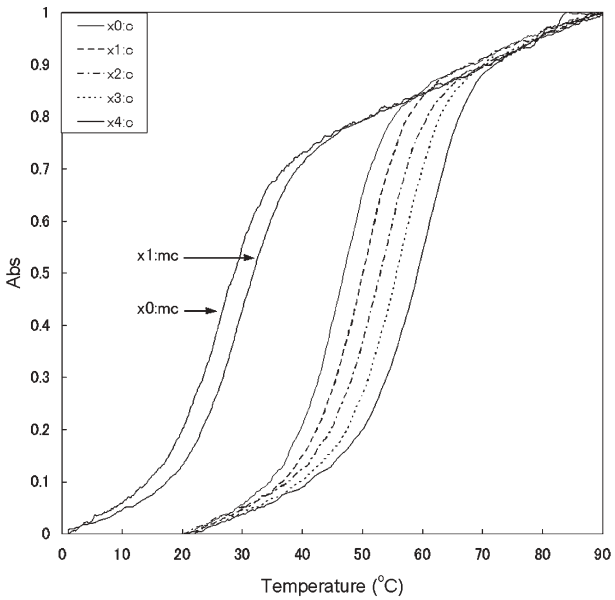

Fig. 2 Normalised thermal denaturation profiles of ODN duplexes. (See Table 1 for sequences). Conditions = 1 μM duplex concentration in 300 mM of NaCl, 10 mM of sodium cacodylate and 0.1 mM of Na_2EDTA, pH 7.0.

for duplex formation were calculated for $0.1 < \alpha < 0.9$.[22] These equilibrium constants were analysed using the Van't Hoff equation yielding enthalpy changes for duplex melting $\Delta_m H$ (Table 2).§

Table 2 shows that introducing the analogue **3** stabilises the duplex as is apparent from the increasing T_m for duplexes ODN c:x0–4. The selective recognition of template guanine by the analogue is clearly seen by the much reduced thermal stability of the duplex in which the analogue is placed opposite adenine

Table 2 Thermodynamic parameters

Duplex	T_m[a] (°C)	ΔT_m (°C)	$\Delta_m H$[b] (kJ mol^{-1})
c:x0	46.5	—	345
c:x1	49.8	3.3	354
c:x2	52.7	2.9	365
c:x3	55.8	3.1	353
c:x4	59.4	3.6	344
mc:x0	25.7	—	297
mc:x1	28.8	3.1	297

[a] T_m defined as T for which $\alpha = 0.5$ (see Fig. 1 of ESI). [b] Assuming $\Delta_m C_p = 0$ and scans at equilibrium, *i.e.* up and down scans are identical.

(*i.e.* cm:x0–1). Remarkably, Δ_mH (and therefore the entropy change for melting, Δ_mS) at the respective T_ms remains virtually constant upon introduction of the analogue for ODN c:x0–4. This behaviour is more analogous to that resulting from the introduction of methyl substituents in a DNA duplex rather than the introduction of propynes (*vide supra*).

The similarity in enthalpies and entropies of duplex melting may seem to be in contradiction with the distinctly different duplex stabilities as inferred from the increasing T_m for ODN c:x0–4. However, it should be kept in mind that Δ_mH and $T_m\Delta_mS$ relate to the T_m values for the respective oligonucleotides, whereas for a full thermodynamic analysis, enthalpy and entropy changes for different oligonucleotides should be compared at a common reference temperature, taking heat capacity changes into account.¶[23] Nevertheless, considering that for the current system, duplex stabilisation is not resulting from a more favourable enthalpy of duplex formation, enhanced stacking interactions are unlikely to be the cause of duplex stabilisation. However, classical (entropy driven) hydrophobic interactions,[24] single strand pre-organisation[5,12,25] and even duplex stabilisation by the reduction of conformational restrictions (through the availability of more hydrophobic surface available for stacking interactions) can all be reconciled with the observed thermodynamics.

The duplex stabilisation arising from the introduction of analogue **3** into ODNs is in sharp contrast to the effects of introducing analogue **4**. Presumably the geometry of the base pair formed between **4** and G is somewhat perturbed from that expected for a standard Watson–Crick base pair which in turn affects hydrogen bonding and/or base-pair stacking, thereby decreasing the T_m of the duplex.

In conclusion we have prepared and characterised ODNs containing 7,8-dihydropyrido[2,3-*d*]pyrimidin-2-one and shown that the analogue confers a greatly enhanced duplex stability. The origins of this enhanced stability, however, require further investigation.

Notes and references

‡ It should be noted that the effect of introducing substituents on the thermodynamics of duplex formation depends markedly on whether a DNA–DNA, DNA–RNA hybrid or an RNA–RNA duplex is involved (ref. 6). For example, methylating uridine in DNA–RNA hybrids leads to a less favourable enthalpy for duplex formation whereas methylating uridine in RNA–RNA duplexes leads to a more favourable enthalpy for duplex formation. Still, methylation stabilises both types of duplex. For DNA–RNA hybrids, contradictory reports on the thermodynamic reasons for enhanced duplex stability upon introduction of propyne have been published (refs. 5 and 6). In addition, different buffers appear to have an impact on the observed thermodynamics, *e.g.* refs. 10, 26.

§ Including heat capacity changes in the analysis by using the Clarke–Glew equations[27] does not significantly alter the optimised value for enthalpy changes for duplex formation and the narrow temperature ranges (individual transitions span temperature ranges of no more than 15 °C) prevent us from determining accurate values for Δ_mC_p. Heat capacity changes for duplex formation were therefore ignored in the final data analysis leading to Van't Hoff enthalpy changes for duplex melting Δ_mH given in Table 2.

¶ Order–disorder transitions, including duplex melting, are accompanied by a positive heat capacity change. See ref. 23.

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