

Oligodeoxynucleotides Containing C-5 Propyne Analogs of 2'-Deoxyuridine and 2'-Deoxycytidine

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Abstract: Oligodeoxynucleotides (ODN) containing 5-(1-propynyl)-2'-deoxyuridine and 5-(1-propynyl)-2'-deoxycytidine significantly enhance double-helix formation with single strand RNA and 5-(1-propynyl)-2'-deoxyuridine significantly enhances triple-helix formation with double stranded DNA.

Oligodeoxynucleotides, as therapeutic agents, are of considerable interest in both the antigene and antisense strategies^{1,2}. Thymidine and 5-methyl-2'-deoxycytidine are known to stabilize the triple-helix and double-helix complex relative to 2'-deoxyuridine and 2'-deoxycytidine³⁻⁵. Recent measurements indicate that C-5 methylated pyrimidines show increased binding to duplex DNA as a result of entropic factors; it was postulated that this is due to displacement of highly ordered water molecules from the duplex structure.⁵ Reported herein is the use of 5-(1-propynyl)-2'-deoxyuridine (1, pdU)⁶ and 5-(1-propynyl)-2'-deoxycytidine (3, pdC) in triple-helix and double-helix formation. The propyne modification is planar with respect to the heterocycle (Figure 1) and allows for increased stacking of the bases; additionally the propyne is more hydrophobic than a methyl group to potentially allow for a further increase in the entropy of binding. The results reported below demonstrate that the triple-helix complex is stabilized by substitution with pdU and destabilized by substitution with pdC, while the double-helix is stabilized by both substitutions. pdU is more hydrophobic than thymidine (2)⁷ and, therefore, may facilitate passive diffusion of ODN's into cells, making this modification especially useful in therapeutic applications.

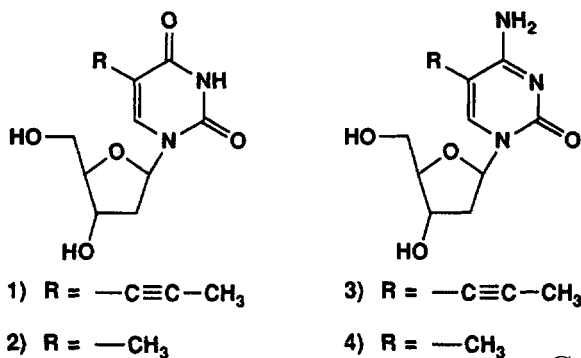


Figure 1: Structure of nucleoside analogs

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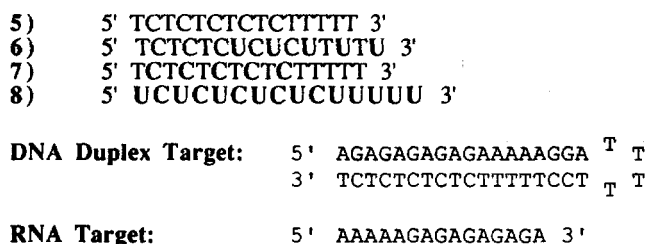


Figure 2: ODN and target sequences; U is pdU (1), C is pdC (3), T is thymidine (2), and C is 5-methyl-2'-deoxycytidine (4).

pdU and pdC were prepared from the corresponding 5-iodo-2'-deoxynucleoside and propyne with palladium catalysis by the procedure of Hobbs⁸ in 85-95 % yield. The nucleosides were protected and phosphitylated by standard procedures⁹ and incorporated into ODN's via H-phosphonate intermediates.¹⁰ Separate ODN's were prepared from pdU and pdC containing five substitutions in a 15-mer (ODN's 6 and 7, respectively, Figure 2).¹¹ The effect of 5-propyne substitutions on the stability of the triple-helix was determined by thermal denaturation (T_m) experiments with ODN's 5-7 (Figure 2) as described.¹² ODN 6, containing pdU (1), showed an increase in T_m of 2.4 °C/substitution ($\Delta T_m @ 6.6 = +12.1$ °C) relative to ODN 5 and ODN 7, containing pdC (3), showed a decrease in T_m of 3.4 °C/substitution ($\Delta T_m @ 6.6 = -17.1$ °C) relative to ODN 5 (see Table 1). The T_m of a triple-helix, in which the third strand contains 2'-deoxycytidines, is pH dependent^{12,13}, and the T_m at different pH values (from 5.8 to 7.4) was used to examine the pH dependence of the complex. With all ODN's the slope of the plot remained relatively constant (-18 to -20 °C/pH unit, Figure 3), indicating that these modifications have no significant effect on the pH dependence of triple-helix formation. The T_m of the hairpin duplex was 76.2 ± 0.4 °C and showed no pH dependence.

Table 1: Triple-helix and Double-helix T_m Utilizing C-5 Propyne Analogs

ODN	Triple-helix ^a		Double-helix (RNA) ^a	
	T_m (°C)	ΔT_m (°C/subst.)	T_m (°C)	ΔT_m (°C/subst.)
5 (control)	43.4	—	65.5	—
6 (pdU)	55.5	+2.4	74.0	+1.7
7 (pdC)	26.3	-3.4	73.0	+1.5
8 (pdU/pdC)	nd ^b	—	> 90 ^c	> 1.7

a) T_m values were assessed in 140 mM KCl/5 mM Na₂HPO₄/5 mM MgCl₂ at pH = 6.60 and the final concentration of all oligodeoxynucleotides was ~2 μ M. UV absorbance monitored at 260 nm. b) not determined. c) UV absorbance monitored at 280 nm, see reference 18.

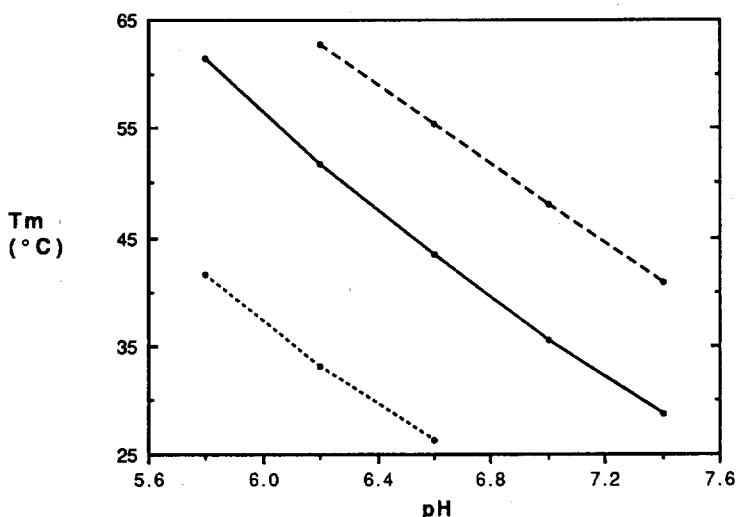


Figure 3: T_m vs pH plot for ODN's 5-7. (—), ODN 5; (----), ODN 6; (....) ODN 7.

The low T_m of ODN 7, relative to ODN's 5 and 6, can be explained in terms of basicity of the heterocycle. Titration of the hydrochloride salt of 3 and 4 showed that the pK_a of the 5-propyne analog 3 ($3.30, \pm 0.05$) is 1.05 units less than the 5-methyl derivative 4 ($4.35, \pm 0.05$).^{14, 15} The importance of protonation in triple-helix formation has been demonstrated¹⁶ and the results above indicate that a decrease in basicity of the cytosine nucleobase has a dramatic effect on the stability of the complex. Surprisingly, the large difference in pK_a 's of the cytosines (3 and 4) has no significant effect on the slope of the T_m vs pH plot (Figure 3). This is probably due to the fact that the pK_a of these heterocycles is well below the pH of the solution ($\Delta pH \sim 2-3$ units), and therefore the nucleobases are protonated to an insignificant extent prior to triple-helix formation. This is supported by data suggesting that the pK_a of cytosine nucleobases, within a triple-helix complex, is 2-3 units higher than the pK_a of the mononucleoside.¹⁷ Therefore it is likely that the pK_a of the cytosine nucleobase has no significant effect on the formation of the triple-helix complex, but has a significant effect on the stability of the complex once formed.

The stability of the DNA/RNA duplex, derived from ODN's 5-8 (see Figure 2), was also assessed by T_m analysis and the results are presented in Table 1. ODN 6, containing pdU(1), results in an increase in T_m of $1.7^\circ\text{C}/\text{substitution}$ and ODN 7, containing pdC(3), results in an increase in T_m of $1.5^\circ\text{C}/\text{substitution}$. Under these conditions ODN 8, containing complete substitution with pdU(1) and pdC(3), has a T_m greater than 90°C (approx. $1.7^\circ\text{C}/\text{substitution}$) indicating that the increase in binding affinity of these substitutions are additive.¹⁸ These results show that the double-helix complex is greatly stabilized by substitution with both pdU(1) and pdC(3) and, therefore, these analogs represent a new class of useful antisense ODN's.

One explanation for the increased stability observed with ODN's 6-8, relative to ODN 5, is increased entropy of binding. The results observed with these ODN's support the concept that the entropy of binding is further increased with the more bulky and hydrophobic 5-propyne substituent. Additionally, there may be enthalpic contributions to binding due to increased stacking interactions resulting from the propyne substituent.

We have determined that the C-5 propyne analog of 2'-deoxyuridine (1) stabilizes the triple-helix complex relative to thymidine (2) and conversely, the C-5 propyne analog of 2'-deoxycytidine (3) destabilizes the triple-helix complex, due to a decrease in pKa of the heterocycle. Both propyne analogs, pdU(1) and pdC(3), result in a sharp increase in T_m of the double-helix complex formed with RNA. The increased hydrophobicity of these propyne analogs, relative to thymidine and 5-methyl-2'-deoxycytidine, may facilitate permeation of ODN's into cells making this modification potentially valuable in therapeutic applications.

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18. 280 nm was used to monitor the T_m due to the low hypochromicity of ODN 8 at 260 nm. When the T_m experiment is repeated in the presence of 1 mM MgCl₂, with all other conditions kept constant, the T_m of the control ODN (5) is 63.0 °C and the T_m of the all propyne substituted ODN (8) is 90.0 °C; ΔT_m = 1.8 °C/substitution.

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