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5-Propynylamino α-deoxyuridine promotes DNA duplex stabilization of anionic and neutral but not cationic α-oligonucleotides

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This paper is dedicated to Professor Jean-Louis Imbach on the occasion of his 70th birthday.

Abstract—Incorporation of 5-propynylamino and 5-propynyl α-2′-deoxyuridine into α-oligonucleotides (α-ON) allows high-affinity targeting of complementary DNA for α-ON with anionic and neutral backbone but not for cationic α-ON, revealing clues on the role of the amino group of the propynylamino on the formation of DNA duplexes.

α-Oligonucleotides (α-ON) have a nonnatural nucleoside α-anomery and are a class of ON exhibiting unique hybridization properties. Unlike β-ON which hybridize to ss DNA or RNA with an antiparallel orientation, α-ON do it in a parallel manner without loss of affinity and specificity for their target.[1] Moreover, although the replacement of the phosphodiester backbone with any chiral phosphorus modification in β-ON induces a destabilization of their hybrids formed with nucleic acids,[2] the introduction of neutral[3] and cationic[4,5] internucleoside phosphoramidates into α-ON stabilizes them. The main dramatic stabilizing effect was observed with cationic linkages due to the electrostatic attraction between phosphates of natural targets and the cationic linkages of the modified α-ON. This strong affinity was particularly useful to employ cationic α-ON as steric blocking antisense agents.[4]

To further increase the affinity of α-ON, we considered introducing the modified nucleobase 5-propynylamino uracil. Indeed, the propynyl amino group attached to C5 of the uracil is one of the most promising modifications introduced in phosphodiester β-ON to form stable duplexes[6,7] and triplexes.[8,9] Moreover, the combination of the 5-propynylamino uracil with the positively charged 2′-aminoethoxy sugar modification[10] into the same β-ON has a synergetic effect on the hybrid stability.[11,12]

The aim of the present work was to determine the effect of 5-propynylamino α-dU **X** and 5-propynyl α-dU **Y** on the stability of duplexes formed between α-ON and a complementary parallel DNA sequence. This effect was studied with anionic phosphodiester (**I**, PO), neutral methyl phosphoramidate (**II**, PNHMe) and methoxypropyl phosphoramidate (**III**, PNHPrOMe), and with cationic dimethylaminopropyl phosphoramidate (**IV**, $PNHPrNMe_2$) α-ON (Fig. 1).

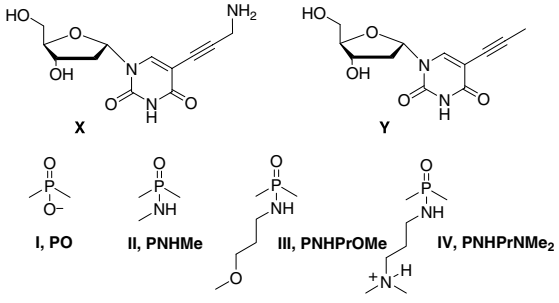

Figure 1. 5-Propynylamino α-dU **X**, 5-propynyl α-dU **Y**, and phosphodiester **I**, methyl phosphoramidate **II**; methoxypropyl phosphoramidate **III**; dimethylaminopropyl phosphoramidate **IV** internucleoside linkages.

Keywords: Oligonucleotides; α-Anomery; Phosphoramidates; Bas modification; Hybridization.
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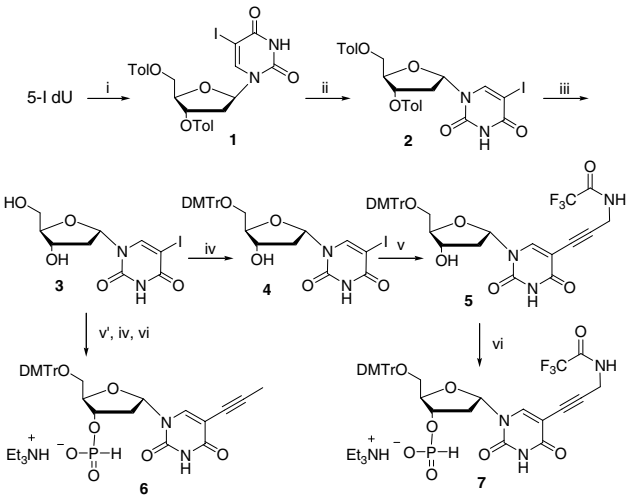

Scheme 1. Preparation of **6** and **7**. Reagents and conditions: (i) *p*-TolCl, py, rt, 85%; (ii) a—HMDS, toluene, reflux; b—TMSOTf, CH_3CN, rt, 45%; (iii) MeONa, MeOH, rt, 92%; (iv) DMTrCl, py, rt, 90%; (v) $HC{\equiv}CCH_2NHCOCF_3$, CuI, Et_3N, $Pd(PPh_3)_4$, DMF, rt, 82%; (v′) propyne, CuI, Et_3N, $Pd(PPh_3)_4$, DMF, rt, (Ref. 12); (vi) a—H_3PO_3, PivCl, py, rt; b—aq TEAB, 81%.

Synthesis of 5-propynylamino-2′-α-deoxyuridine (Scheme 1) was started with *p*-toluoylation of the hydroxyls of commercially available 5-iodo-2′-deoxyuridine yielding **1**. Epimerization of **1** into the α-anomer **2** was performed with hexamethyldisilane in toluene at reflux followed by a treatment with TMSOTf[13] yielding a ~4:1 α/β separable mixture. The α-anomer **2** was then deprotected with sodium methanolate to afford 5-iodo-2′-α-deoxyuridine **3** which was then tritylated to give **4**. The aminopropynyl moiety protected as trifluoroacetamide was next introduced into compound **5** under palladium coupling reaction.[11] Sonogashira reaction of **3** to obtain 5-propynyl α-dU **Y** was performed as previously reported.[14] Finally, 2′-deoxy-α-ribonucleoside 3′-*O*-*H*-phosphonates **6** and **7** were prepared according to published procedure.[3]

The elongation of α-ON dodecamers (Table 1) was performed on a CPG solid-support using *H*-phosphonate chemistry.[3] The stepwise coupling yield for *H*-phosphonates **6** and **7**, using pivaloyl chloride as the coupling agent, was comparable to the other protected α-nucleoside 3′-*H*-phosphonates (98%). At the end of the elongation, the oxidation of the oligonucleotide *H*-phosphonates was performed with standard iodine–pyridine–water to generate α-ON phosphodiesters, and with anhydrous CCl_4-pyridine-amine (methylamine, 3-methoxypropylamine or 3-dimethylaminopropylamine) to create phosphoramidate internucleoside linkages (PNHMe, PNHPrOMe, and $PNHPrNMe_2$, respectively) using a reported protocol.[3] After cleavage of the ON from the solid-support and their deprotection with concentrated ammonia, their purification was performed by RP-HPLC for the anionic α-PO and the neutral α-PNHMe and α-PNHPrOMe, whereas the cationic α-$PNHPrNMe_2$ was purified by cationic exchange chromatography as already reported.[4,5]

Table 1. Target and oligonucleotides synthesized

ON	Sequence 5′-3′[a]	Anomery	Internucleoside backbone[b]
Target	AAG AGG AAG AAA	β	PO
I	TTC TCC TTC TTT	α	PO
I–X1	TTC TCC **X**TC TTT	α	PO
I–X2	TTC **X**CC TTC **X**TT	α	PO
I–X4	**X**TC **X**CC **X**TC **X**TT	α	PO
I–Y1	TTC TCC **Y**TC TTT	α	PO
I–Y2	TTC **Y**CC TTC **Y**TT	α	PO
I–Y4	**Y**TC **Y**CC **Y**TC **Y**TT	α	PO
II	TTC TCC TTC TTT	α	PNHMe
II–X1	TTC TCC **X**TC TTT	α	PNHMe
II–X2	TTC **X**CC TTC **X**TT	α	PNHMe
II–X4	**X**TC **X**CC **X**TC **X**TT	α	PNHMe
III	TTC TCC TTC TTT	α	PNHPrOMe
III–X1	TTC TCC **X**TC TTT	α	PNHPrOMe
III–X2	TTC **X**CC TTC **X**TT	α	PNHPrOMe
III–X4	**X**TC **X**CC **X**TC **X**TT	α	PNHPrOMe
IV	TTC TCC TTC TTT	α	$PNHPrNMe_2$
IV–X1	TTC TCC **X**TC TTT	α	$PNHPrNMe_2$
IV–X2	TTC **X**CC TTC **X**TT	α	$PNHPrNMe_2$
IV–X2′	TTC TCC **XX**C TTT	α	$PNHPrNMe_2$
IV–X4	**X**TC **X**CC **X**TC **X**TT	α	$PNHPrNMe_2$
IV–Y1	TTC TCC **Y**TC TTT	α	$PNHPrNMe_2$
IV–Y2	TTC **Y**CC TTC **Y**TT	α	$PNHPrNMe_2$
IV–Y4	**Y**TC **Y**CC **Y**TC **Y**TT	α	$PNHPrNMe_2$

[a] Sequences of ON where **X** or **Y** are the position at which 5-propynylamino α-dU **X** or 5-propynyl α-dU **Y** were incorporated.

[b] PO, phosphodiester; PNHMe, methyl phosphoramidate; PNHPrOMe, methoxypropyl phosphoramidate; $PNHPrNMe_2$, dimethylaminopropyl phosphoramidate internucleoside linkages.

The effect of the 5-propynylamino α-dU on DNA duplex stability was evaluated by UV thermal denaturation experiments (Table 2) with a complementary β-sequence. At 0.1 M NaCl, the introduction of the 5-propynylamino uracil in anionic α-ON **I–X** induces a significant stabilization of the hybrids compared to the slight effect that 5-propynyl α-dU has (**I–Y**). The higher the number of base modifications, the larger was the stabilization (ΔT_m from 2.5 to 6.7 °C for **I–X1** to **I–X4**). However, the average stabilization per base modification slightly decreases as the number of modifications increases (ΔT_m/modif. from 2.5 to 1.7 °C for 1–4 modif.). This behavior reported for β-ON duplex containing 5-propynylamino β-dU[6,7] was explained by a destabilization resulting from the repulsion between cationic propynylamino groups. However, it is not probable here that propargylamino groups separated by two or more base pairs could interact together and the relatively low pK_a of the amine (pK_a propargylamine[6] ~8.2), which becomes harder to protonate as the number of propynylamino groups is increased, could simply explain this drop in ΔT_m/modif. The electrostatic contribution to the stabilization resulting from the protonation of the propynylamino is confirmed with melting experiments performed at 1 M NaCl. Indeed, the stabilizing effect observed at high salt concentration decreases with increasing number of propynylamino groups ($\Delta T_{m(1M\ NaCl-0.1M\ NaCl)}$ = from 14.5 °C for **I** to 10.8 °C for **I–X4**). When the NaCl concentration

Table 2. Melting temperatures (T_m values) obtained during thermal denaturation studies at different concentrations of NaCl[a]

α-ON	Backbone	0.1 M NaCl			1 M NaCl		
		T_m (°C)	ΔT_m (°C)	ΔT_m/modif.	T_m (°C)	ΔT_m (°C)	ΔT_m/modif.
I	PO	40.5	—	—	55.0	—	—
I–X1		43.0	2.5	2.5	56.5	1.5	1.5
I–X2		44.3	3.8	1.9	56.5	1.5	0.8
I–X4		47.2	6.7	1.7	58.0	3.0	0.8
I–Y1	PO	41.0	0.5	0.5	56.0	1.0	1.0
I–Y2		41.0	0.5	0.3	56.0	1.0	0.5
I–Y4		42.0	1.5	0.4	56.0	1.0	0.3
II	PNHMe	48.5	—	—	47.0	—	—
II–X1		52.5	4.0	4.0	49.5	2.5	2.5
II–X2		54.0	5.5	2.8	48.5	1.5	0.8
II–X4		56.5	8.0	2.0	49.5	2.5	0.6
III	PNHPrOMe	48.0	—	—	46.0	—	—
III–X1		51.5	3.5	3.5	47.5	1.5	1.5
III–X2		52.5	4.5	2.3	47.5	1.5	0.8
III–X4		56.0	8.0	2.0	47.0	1.0	0.3
IV	$PNHPrNMe_2$	74.5	—	—	53.5	—	—
IV–X1		71.5	−3.0	−3.0	50.0	−3.5	−3.5
IV–X2		69.5	−5.0	−2.5	49.0	−4.5	−2.3
IV–X4		nd	nd	nd	nd	nd	nd
IV–Y1	$PNHPrNMe_2$	72.0	−2.5	−2.5	53.0	−0.5	−0.5
IV–Y2		71.0	−3.5	−1.8	52.0	−1.5	−0.8
IV–Y4		69.0	−5.5	−1.4	50.5	−3.0	−0.8

[a] The melting temperatures (T_m) for dissociation of the duplexes were measured as the maximum of the first derivative of the melting curve (A_{260nm} vs temperature) recorded in 10 mM sodium cacodylate buffer, pH 7.0, using 2 μM concentrations of the two complementary strands, and a micromolar extinction coefficient of 3000 for monomer **X**. ΔT_m, change in T_m value calculated relative to the nonbase-modified ON-duplex; ΔT_m/modif., average change in T_m value per modified base.

augments, the sodium cation competes with the ammonium of the propynylamino lowering the favorable electrostatic effects resulting from the interaction with PO linkages. In comparison, as expected the stability of the duplexes formed with the propynyl α-ON **I–Y1**, **2**, **4** is as prone as the unmodified α-ON **I** to the change in the ionic strength ($\Delta T_{m(1M\ NaCl–0.1M\ NaCl)}$ ~14–15 °C).

When incorporated into neutral α-ON **II** and **III**, the 5-propynylamino α-dU **X** has a stronger effect on the stability of the duplexes ($T_m \sim 3.5$–4 °C for 1 modif.) than when integrated into anionic α-ON **I** ($T_m \sim 2.5$ °C). Like for ON **I**, introduction of more than one **X** modification has a lower but still significant impact on the T_m (ΔT_m/modif. 2 °C for 4 modif.). The stabilizing effect of the propynylamino cannot be explained here by a favorable electrostatic interaction with the neighboring phosphates of the same strand as established for natural β-ON PO.[6,7,12] Furthermore, the similar T_m observed for α-ON **II** and **III** with phosphoramidate linkages bearing short methyl or longer methoxypropyl chains suggest no steric interaction between the propynylamino group and the neutral backbones. It has been also reported for triplex formation, that the main reason for the stabilization induced by the propynylamino comes up primarily from enhanced base stacking interactions due to the amino electronegative group attached to the propyne and not from electrostatic interactions.[15] However, the lower T_m obtained at a high salt concentration (T_m 47 °C for **III–X4**) compared to those obtained at a lower salt concentration (T_m 56 °C), while the T_m of the α-ON **III** do not diverge drastically (T_m 46 and 48 °C in 1 M and 0.1 M NaCl, respectively), are clearly in favor of the behavior of protonated species. In this case, the higher the number of base modifications, the higher the sensitivity to the ionic strength ($\Delta T_{m(1M\ NaCl–0.1M\ NaCl)} = -4$ °C for **III–X1** and −9 °C for **III–X4**). A comparable behavior is observed for α-ON **II**. The stabilizing effect of the propynylamino in α-ON **II** and **III** probably results from electrostatic interactions between the amino groups and the phosphates of the complementary strand.

As regards to a possible enhanced stabilizing effect on DNA duplex hybridization, we investigated the opportunity of combining the potential stabilizing effects of cationic $PNHPrNMe_2$ linkages and the 5-propynylamino uracil base modification in an ON with improved hybridization properties. Indeed, we recently showed by molecular modeling simulations that amino groups borne by the phosphoramidate internucleoside linkages of α-ON interact efficiently with PO groups of the complementary DNA strand bridging the minor groove of duplex.[16] Considering that the 5-propynylamino is directed through the major groove[2], the two modifications are not inclined to interact together. Despite these considerations, the introduction of the base modifications into a cationic α-ON **IV** induces destabilization of the resulting hybrids (ΔT_m −3 °C for **IV–X1**). This destabilization did not depend on the position of the base modifications within the ON as two distributed

modifications in **IV**–**X2** had the same effect on T_m as two contiguous modifications in **IV**–**X2′** (not showed). It was not possible to determine the T_m value with the ON **IV**–**X4** bearing four propynylamino residues due to a low cooperativity and low hyperchromicity. It is not sure that this effect arises from the proximity of positively charged groups.

As written before, the two amines are located in different positions of the duplex. Moreover, due to the differences of pK_a between the two amino functions (pK_a *N*-dimethylamino propane[17] and propargylamine[6] ~10 and ~8.2, respectively), it is not probable that the amino of the propynylamino is protonated in α-ON **IV**–**X**. The fact that the neutral 5-propynyl induces a similar destabilization in ON **IV**–**Y** reinforces this assumption. Moreover, the neutrality of the propynylamino group in these cationic α-ON is demonstrated by the similar sensitivity of the stability to the ionic strength of all duplexes whatever the number of propynylamino or propynyl modifications ($\Delta T_{m(1M\ NaCl-0.1M\ NaCl)} \sim -21$ °C for ON **IV**–**X** and ~ -19 °C for ON **IV**–**Y**). A simple reason for the destabilization induced by the two modified bases would be that protonated and nonprotonated propynylamino behave differently. Another reason could be that the interactions between cationic phosphoramidates and phosphates of the target narrow the minor groove contracting and bending the double helix.[16] In contrast, due to enhanced base stacking, it has been reported for β-ON that the propynyl and propynylamino groups rigidify the double helix[18] and lengthen it.[19] If α-ON containing propynyl and propynylamino groups behave similarly, this rigidity may be somehow unfavorable to the flexibility required to accommodate the interactions in the duplex between cationic $PNHPrNMe_2$ linkages on one strand and PO on the other strand. However, if increased ionic strength from 0.1 M to 1 M NaCl, that reduces the interaction between the two backbones, was able to lessen the propynyl negative effect (ΔT_m −3.5 to −1.5 °C **IV**–**Y2**), this was not the case with the destabilization caused by the propynylamino which remained almost identical at the two salt concentrations. This could be explained by the stronger rigidity due to enhanced ππ stacking induced by 5-propynylamino uracil **X** compared to its propynyl analogue **Y**.

We concluded that the 5-propynylamino α-dU modification induces various effects depending on the nature of the charge owned by the backbone of α-ON. In anionic and neutral α-ON, it resulted in stabilization, whereas a destabilization was observed with cationic α-ON. We have shown the importance of the electrostatic interaction with the anionic complementary strand for α-ON neutral phosphoramidates. Possibly, the origin of the destabilization with cationic α-ON arises from a conflict in the double helix between rigidity induced by the base modification and bending due to the phosphate modification.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.11.052.

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