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Novel oligonucleotide analogues containing a morpholinoamidine unit

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ABSTRACT

Morpholinoamidines were devised as new cationic units in oligonucleotides, by combining morpholino-nucleosides (to simplify the nomenclature, we will use the term *morpholino-nucleosides* to refer to nucleoside analogues in which the ribose ring was transformed into a morpholine) with internucleoside guanidines. Here, methodology was developed to synthesize oligonucleotides containing morpholinoamidines formed by morpholino-uridine and 5′-amino-5′-deoxythymidine. Morpholinoamidine was produced by solid-phase reaction of Alloc-morpholinocarbothioamide with 5′-aminonucleoside resin and Mukaiyama's reagent activation. Two 14-*mer* oligonucleotides containing a single morpholinoamidine were synthesized and their affinity properties were investigated by forming DNA double and triple helices. Duplexes were slightly stabilized by a 3′ unit, but were less stable if internally positioned. Notably, triplexes were significantly stabilized at pH 7.0.

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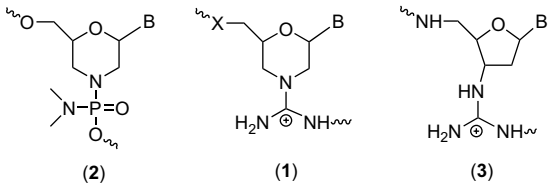
1. Introduction

As they are endowed with excellent molecular recognition and self-assembly properties, oligonucleotides have been used increasingly in pharmaceuticals, diagnostics, biotechnology and, more recently, in nanotechnology.[1] However, such a vast range of applications would be precluded if chemical synthesis had not offered the possibility of introducing modifications into oligonucleotide strands,[2] to overcome the limitations of the natural backbone, which are mainly chemical and enzymatic lability, affinity dependent on ionic media and low capacity to penetrate cells. Among many other analogues, polyarginine oligonucleotide conjugates[3] were prepared to show equal or better biophysical and pharmacodynamic properties than their anionic counterparts. Because cationic peptides are unstable in serum and are cytotoxic, other alternatives were explored to introduce cationic groups into oligonucleotides, such as tethering guanidines to phosphoramidate groups[4a] or derivatizing nucleobases[4b] and riboses.[4c] Guanidine oligonucleotides (GO) in which the internucleoside phosphate group is replaced by a guanidine are also known through the work of Bruice group.[5–8] Several types of such oligomers were synthesized[6] and showed extremely close affinity to complementary DNA and RNA sequences,[8] essentially due to favorable electrostatic interactions between guanidine and phosphate groups. GO specificity was not compromised by close affinity, and guanidinium linkages were nuclease-resistant.[7d] Although several theoretical studies suggested that GO could be useful in targeting biological sequences,[8] to date no biological assays with guanidine oligonucleotides have been reported.

We devised a novel guanidine analogue formed by morpholinoamidine units based on the beneficial effects conferred by positive charges on oligonucleotides (**1**, Fig. 1). These units resulted from the combination of two previously known backbones: nucleosides with a morpholine ring such as those forming morpholino oligonucleotides[9] (**2**) and guanidine internucleoside bonds known in guanidine oligonucleotides (**3**).

In our view, this new backbone could combine the best of both morpholino and guanidine analogues. As mentioned, guanidine groups increased oligonucleotide affinity for target sequences because of their positive charges and stability to degradation by nucleases. Morpholino oligonucleotides (MO) have promising properties as antisense agents by a non-RNase H mechanism,[9] blocking the production of miRNA[10] or altering splicing to correct



(**2**) (**1**) (**3**)

Figure 1. Oligonucleotide analogues containing morpholinoamidine (**1**), morpholino-phosphorodiamidate (**2**) and ribose guanidine units (**3**).

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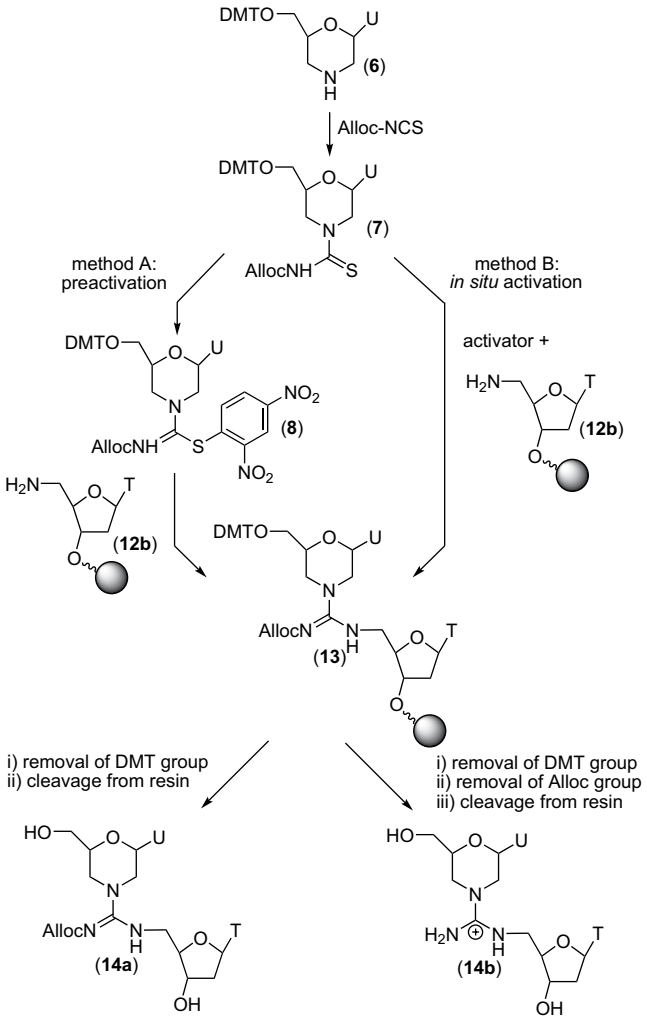
dystrophic pathologies.[11] As such, they are very useful in developmental biology.[12] Nonetheless, their main limitation is low penetration across membranes, but this could be usefully improved by coadministration[13] or by covalent conjugation with polyarginine peptides.[14] Therefore, it seems reasonable that morpholinoamidine oligonucleotides that contain guanidines immersed in backbone could also show properties of transfection into cells without being conjugated with other molecules. With regard to synthetic procedures, morpholino-nucleosides offer the advantage that they are obtained from ribonucleosides,[15] which are cheaper starting material than deoxyribonucleosides, and possess one of the amino functions needed to produce guanidine. Here, we report the preparation of oligonucleotides containing a single morpholinoamidine unit and the preliminary results on their hybridization properties to form double and triple helices.

2. Results and discussion

2.1. Oligonucleotide synthesis

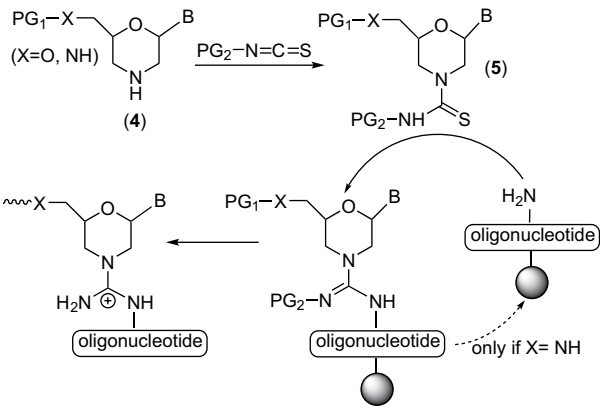
To obtain morpholinoamidine containing oligonucleotides, we devised the solid-phase methodology outlined in Scheme 1, which would permit the preparation of partially or totally modified strands. Morpholino-nucleosides (**4**) are used as nucleoside synthons, suitably functionalized to form a guanidine. Initially, we based ourselves on the solid-phase methodology for preparing GO described by Bruice,[6c–i] which employed protected thiourea derivatives (**5**) as precursors of guanidine function. Following this strategy, morpholine carbothioamides (thioureas) **5** were produced from morpholino-nucleosides by reaction with a protected isothiocyanate. At this point, thiourea protection procured activation of carbothioamide function to nucleophilic addition during guanidine formation and, when the guanidine was formed, avoided undesired reactions during oligonucleotide synthesis. Guanidine would be formed by a solid-phase reaction of a preactivated or in situ-activated morpholinocarbothioamide with an immobilized 5′-aminonucleoside. As shown in the scheme, the method could be adapted to inserting one single morpholinoamidine unit in the oligonucleotide strand by using a hydroxy morpholino-nucleoside (**4**, X=O) or adapted to building a morpholinoamidine stretch by sequential coupling of amine morpholino-nucleosides (**4** X=NH). Final deprotection and cleavage from the resin give the desired oligonucleotide.

Here, we attempted the simplest objective, which consisted of synthesizing oligonucleotides containing a single morpholinoamidine unit formed by morpholino-uridine and 5′-amino-5′-deoxythymidine. Guanidine **14b** (Scheme 2) was chosen as our first synthetic target in order to test the methodology that would permit the synthesis of more complex molecules. Two methods were studied to generate guanidine function, as outlined in Scheme 2: method A used a preactivated isothiourea intermediate (**8**), and method B activated thiourea in situ.

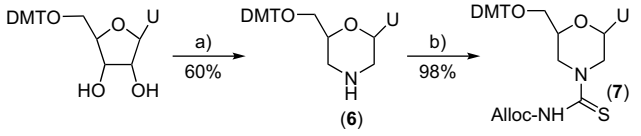


Scheme 2. Solid-phase synthesis of guanidines **14a** and **14b**.

Morpholinocarbothioamide **7** was produced as outlined in Scheme 3. First, morpholino-uridine **6** was obtained from DMT–uridine by transforming the ribose ring into a morpholine by adapting a one-step procedure described by Summerton,[15] which consisted of a first treatment of uridine with a mixture of $NaIO_4$ and NH_4HCO_3 in aqueous methanol to produce the oxidation of the 2′,3′-diol to a cyclic aminal intermediate, and in situ reduction to morpholine by addition of $NaBH_3CN$. Morpholino-uridine **6** was thus obtained after work-up and purification by column chromatography, in a moderate 60% yield from **5**. NMR spectra of morpholino-uridine **6** confirmed the correct stereochemistry of the



Scheme 1. General synthetic route to obtain oligonucleotides formed by morpholinoamidine units.



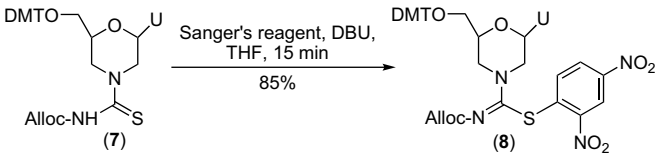
Scheme 3. Synthesis of morpholinocarbothioamide **7**. Reagents and conditions: (a) $NaIO_4$, $NaHCO_3$, MeOH, 1 h+$NaBH_3CN$, MeOH, 2 h; (b) Alloc-NCS, AcOEt, 3 h.

morpholine ring. Alloc-protected thiourea **7** was prepared by reaction of **6** with Alloc-isothiocyanate[16] in a near-quantitative yield. Our first choice was the Fmoc derivative, after the work of Bruice,[6e–i] but in preliminary essays it proved unstable and the origin of secondary products during guanidine formation. After other attempts, we finally chose the allyloxycarbonyl group (Alloc), reported elsewhere as protecting guanidines in peptides,[17] heterocycles,[18] and a dinucleotide guanidine.[6d]

At this point, we tested the solid-phase synthesis of dinucleosidic guanidine **14** (see Scheme 2). First, a solid support functionalized with the 5′-aminonucleoside (**12**) was prepared, as outlined in Scheme 4. 5′-MMT-amino-5′-deoxythymidine[19] (**9**) was incorporated on long-chain alkylamine-controlled pore glass (LCAA-CPG), following the standard methodology via monosuccinate derivative **10**, which was obtained by reaction with succinic anhydride in pyridine. Before the nucleoside was coupled, sarcosine was incorporated on solid support, in order to prevent partial cleavage of succinate linker[20] by the base employed to promote the formation of guanidine. This gave resin **11** with a functionalization of 42 μmol/g. Then, the Fmoc group was removed, and nucleoside **10** was coupled to the resin by a mixture of diisopropylcarbodiimide (DIP) and a catalytic amount of 1-hydroxybenzotriazole (HOBt) in DMF. After coupling, nucleoside functionalization was determined to be 34 μmol/g (resin **12a**).

In the current GO solid-phase synthesis, $HgCl_2$ was used to activate thiourea function to produce guanidine.[6e–i] Because of its toxicity and the production of insoluble HgS during the formation of guanidine, which should be removed by washings with ethanedithiol to prevent clogging of the solid support, we decided to explore two other methods to activate thiourea.

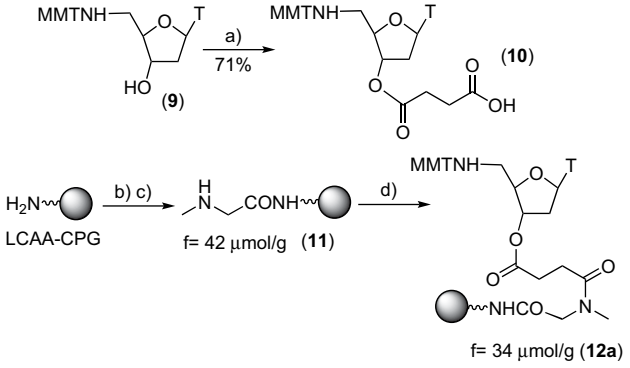
Method A (Scheme 2) was based on the use of *S*-2,4-dinitrophenylisothiourea **8** as a preactivated form of morpholinocarbothioamide **7**. Isothiourea **8** was obtained from thiourea **7** by reaction with Sanger's reagent (1-fluoro-2,4-dinitrobenzene) in the presence of DBU (Scheme 5).[21] As its stability is low, **8** was prepared just before use and purified by aqueous work-up and precipitation on ether/hexane. Essays to obtain guanidine **14** were performed at 1 μmol scale. First, free amino resin **12b** was produced by treatment with 3% TCA and subsequent neutralization with 5% triethylamine in CH_2Cl_2, after which nucleoside was coupled with equimolar amounts of nucleoside **8** and triethylamine in anhydrous THF. Coupling proved to be highly inefficient under every condition assayed, showing 10–41% conversions (data not shown). Coupling efficiency did not improve significantly by repeating the coupling steps with freshly prepared reagents, or by increasing neither coupling time and reagents concentrations from 0.01 M to 0.05 M. We reasoned that these low yields were caused by instability of intermediate **8**, so we decided to explore route B (Scheme 2), which did not require the preparation of a preactivated intermediate.



Scheme 5. Preparation of *S*-2,4-dinitrophenylisothiourea **8**.

To carry out the in situ activation of thiourea (Scheme 2), we chose 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and Mukaiyama's reagent (MR: 2-chloro-1-methylpyridinium iodide) as activators, two customary reagents in the solid-phase synthesis of guanidines.[22,23] As shown in entry 1.1 of Table 1, EDCI-mediated coupling of nucleoside was clearly inefficient (10% yield). Entries 1.2–1.6 refer to essays by using MR. A mixture of 3 equiv of nucleoside **7**, 4 equiv of triethylamine, and 3 equiv of activator resulted in a moderate 66% coupling yield after 1 h (entry 1.2). By increasing the concentrations of reagents (entry 1.3), the coupling efficiency improved up to 74%. No significant improvement was obtained by repeating coupling steps or increasing reaction time up to 12 h (entries 1.4–1.7). Product was cleaved from the resin by treatment at room temperature with concentrated ammonia for 1 h and analyzed by HPLC. Surprisingly, two products were observed in chromatograms, the main one corresponding to the desired guanidine **14a** (85%) and a minor one (15%), which was identified by MALDI-TOF spectrometry as thymidine pyridinium salt **15**. This product probably formed by S_NAr reaction of Mukaiyama's activator on 5′-amino-5′-deoxythymidine (Scheme 6), a secondary reaction which competed with the activation of thiourea. Later on, we observed that this secondary product could be nearly suppressed in the synthesis of other guanidines by reducing the molar excess of activator to 0.8 equiv and by premixing nucleoside and activator for 90 min before it was added to the resin.[24]

At this point, we assayed obtaining deprotected guanidine **14b** (Scheme 2). After the formation of guanidine **13**, the Alloc group was removed before the cleavage step with ammonia. As summarized in Table 2, different treatments previously reported to produce the Alloc group removal were assayed, by combination of the catalyst $Pd(PPh_3)_4$ with various scavenger reagents,[25–27] in order to prevent *N*-allylation, a known secondary reaction in the deprotection of Alloc-protected amines.[27] After the Alloc removal step, the crude was cleaved and analyzed by HPLC. No significant differences were observed after the different treatments, as summarized in Table 2. Apparently, each treatment produced the total and



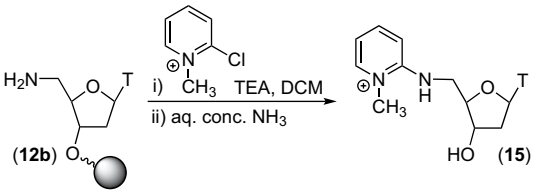
Scheme 4. Synthesis of 5′-amino-5′-deoxynucleosidyl-resin **12a**. Reagents and conditions: (a) succinic anhydride, DMAP cat., pyridine, 12 h; (b) *N*-Fmoc-sarcosine, DIP, DMF, 2 h; (c) 50% piperidine in DMF, 5 min; (d) **10**, DIP, HOBt, DMF, 2 h.

Table 1
Synthesis of guanidine **14a** via an in situ activation

Entry	Activator	Conditions[a]	No. couplings	Coupling time (h)	% Coupling[b]
1.1	EDCI	B1	1	1	10
1.2	MR	B2	1	1	66
1.3	MR	B3	1	1	74
1.4	MR	B3	2	1	68
1.5	MR	B3	3	1	71
1.6	MR	B4	1	1	76
1.7	MR	B4	1	12	73

[a] Reagents and conditions. B1: **7** (3 equiv), EDCI (6 equiv), and triethylamine (4 equiv) in CH_2Cl_2; B2: **7** (3 equiv), MR (3 equiv), and TEA (4 equiv) in CH_2Cl_2; B3: **7** (15 equiv), MR (5 equiv), and triethylamine (20 equiv) in CH_2Cl_2; B4: **7** (25 equiv), MR (25 equiv), and TEA (33 equiv) in CH_2Cl_2.

[b] Coupling yields were determined by the quantification of DMT groups on the resin and HPLC analyses of crudes after deprotection and cleavage.



Scheme 6. Secondary reaction producing pyridinium salt **15**.

clean removal of Alloc, with no trace of *N*-allylated product. Accordingly, we opted to use dimethyl malonate as scavenger in the subsequent syntheses.

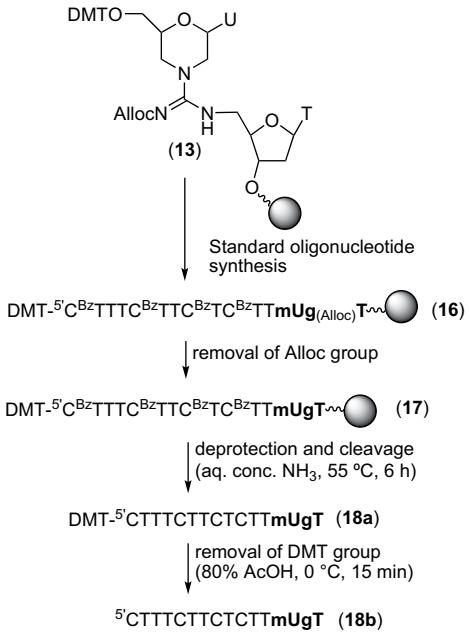
As we were able to produce a morpholinoamidine derivative by solid-phase synthesis, we decided to apply this methodology to the synthesis of oligonucleotides containing a single morpholinoamidine. In particular, we synthesized two versions of the same polypyrimidine strand, one containing a morpholinoamidine at the 3′-end (**18b**) and another modified at a central position of the strand (**23b**). Syntheses are outlined in Schemes 7 and 8, respectively.

To synthesize oligonucleotide **18b**, which contained a 3′ morpholinoamidine, we started on the guanidine resin **13** (see Scheme 7). The rest of the oligonucleotide sequence was assembled in the automatic synthesizer by using the standard phosphoramidite cycle, and keeping the 5′-DMT group to ease purification. Alloc group was first removed by a single treatment with 0.01 M $Pd(PPh_3)_4$ and 0.6 M dimethyl malonate in THF for 30 min, and oligonucleotide was deprotected and cleaved from resin by treatment with aq concd ammonia at 55 °C for 6 h. DMT-containing fractions were purified by HPLC and subsequently, the DMT group was removed by treatment with 80% AcOH. HPLC analyses (Fig. 2) showed three main bands in crude, which were isolated and characterized by MALDI-TOF spectrometry. According to MALDI-TOF analyses, the lowest t_R band corresponded to undefined truncated sequences, probably caused by inefficient capping during the synthesis; the second band was oligonucleotide **18b** (*m*/*z* 4082.7 $[M-H]^-$, calculated mass for $C_{136}H_{183}N_{35}O_{88}P_{12}$ 4085.8) and the highest t_R and main band was due to Alloc-protected oligonucleotide **18c** (*m*/*z* 4165.9 $[M-H]^-$, calculated mass for $C_{140}H_{187}N_{35}O_{90}P_{12}$ 4169.8). As the appearance of **18c** was due to incomplete removal of the guanidine-protecting group, it was deduced that a single treatment was insufficient to produce the complete removal of Alloc, most probably by either sequestration of palladium or steric effects. We observed that deprotection of guanidine improved when we increased the time of the palladium treatment from 30 min to 1 h (from 28% to 42% according to the ratio **18b**/**18c** in HPLC profiles) and carried out repetitive treatments. The best result was obtained with three treatments of 1 h (86% oligonucleotide in the crude).

Table 2
Treatments of Alloc removal to produce guanidine **14b**

Entry	Treatment	Time	Result[a]
2.1	0.01 M $Pd(PPh_3)_4$, 0.6 M dimethyl malonate in THF	30 min	Quantitative deprotection
2.2	0.01 M $Pd(PPh_3)_4$, 0.6 M trimethylsilylmorpholine, and 0.6 M trimethylsilyl acetate in THF	10 min	
2.3	0.01 M $Pd(PPh_3)_4$, 0.6 M HCO_2H, and 0.6 M $BuNH_2$ in THF	1 h	
2.4	0.01 M $Pd(PPh_3)_4$, 0.6 M $PhSiH_3$ in THF	2×10 min	
2.5	0.01 M $Pd(PPh_3)_4$, 0.6 M, $BH_3{\cdot}Me_2NH$ in THF	2×10 min	

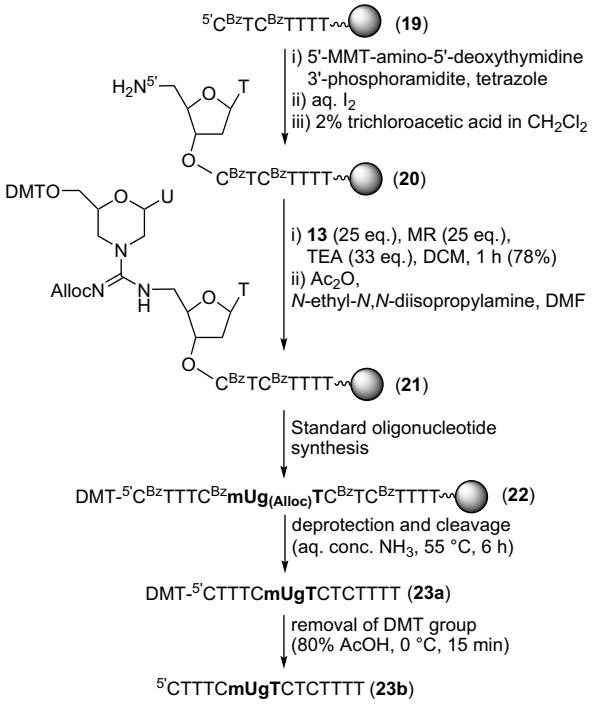
[a] The efficiency of Alloc removal was determined by HPLC analyses.



Scheme 7. Solid-phase synthesis of oligonucleotide **18b**, containing a 3′ morpholinoamidine subunit (mUg refers to the morpholino-uridine amidine unit).

These improvements meant that oligonucleotide **18b** could finally be produced at 13% yield after synthesis and purification (Fig. 2).

The synthetic route to obtain oligonucleotide **23b** with a morpholinoamidine unit in a central position of the strand is outlined in Scheme 8. First, the phosphate segment at the 3′ side of morpholinoamidine (resin **19**) was assembled by standard procedures. To obtain resin **21**, 5′-MMT-amino-5′-deoxythymidine 3′-phosphoramidite[19] was coupled, the MMT group removed and the guanidine was formed by coupling morpholinocarbothioamide **7** by activation with Mukaiyama's reagent and base (see above). DMT



Scheme 8. Solid-phase synthesis of an oligonucleotide (**23b**), containing a morpholinoamidine unit (mUg) in a central position.

determination showed that morpholinoamidine unit formed in a 78% yield. As guanidine did not form quantitatively, acetylation was performed to cap the unreacted amino function. The 5′ side sequence was then assembled by standard oligonucleotide procedures and the 5′-DMT group was kept to easy purification (resin **22**). The Alloc group was first removed by four treatments with $Pd(PPh_3)_4$ and dimethyl malonate solution (see Table 2). Finally, oligonucleotide was deprotected and cleaved of the resin by treatment with aq concd ammonia. Crude, which was more complex than in **18b** most probably because of the lower yield of guanidine formation, was purified by HPLC (Fig. 3). The DMT group was then removed by treatment with 80% AcOH and the resultant product was analyzed by HPLC to show two main bands (Fig. 3), which were collected and analyzed by MALDI-TOF spectrometry. According to mass spectra, the low t_R band corresponded to a mixture of truncated sequences; and the high t_R band, to oligonucleotide **23b** (*m*/*z* 4084.2 $[M-H]^-$). Unlike the synthesis of **18b**, no Alloc-protected oligonucleotide was detected. Oligonucleotide **23b** was obtained in 10% yield after synthesis and purification.

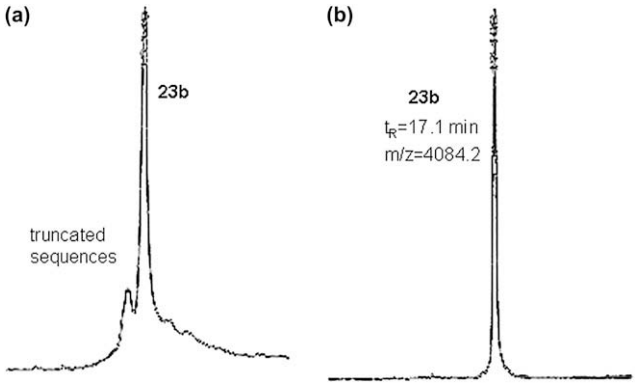
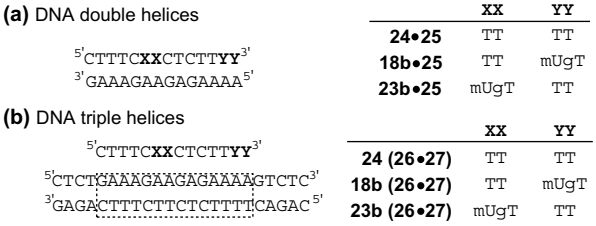


Figure 3. HPLC profiles of (a) the crude oligonucleotide **23b** after DMT removal and (b) repurified oligonucleotide **23b**. Analyses conditions: linear gradient 10–30% B in 30 min, where solvent A was 0.05 M $AcONH_4$ and solvent B was CH_3CN/water 1:1.

2.2. UV melting studies

The affinity properties of oligonucleotides **18b** and **23b** were investigated by forming the DNA double and triple helices shown in Scheme 9, and compared with the unmodified oligonucleotide **24**. For double helices, oligonucleotides **18b**, **23b**, and **24** were hybridized to polypurine complementary strand **25** at pH 7.0. Melting curves were recorded at 260 nm and T_m values were determined, to estimate thermal stability. Since magnesium competes with cationic ligands for binding to nucleic acids, the effect of its presence was also investigated, by recording curves with or without 1 mM $MgCl_2$. Results are summarized in Table 3.



	XX	YY
24•25	TT	TT
18b•25	TT	mUgT
23b•25	mUgT	TT

	XX	YY
24 (26•27)	TT	TT
18b (26•27)	TT	mUgT
23b (26•27)	mUgT	TT

Scheme 9. DNA double and triple helices studied. Segments of strands **26** and **27** targeted to form triple helix are shown in a dashed box.

According to the T_m values, modified oligonucleotides **18b** and **23b** formed duplexes of differing stability. The 3′ modified strand **18b** formed a slightly more stable duplex than unmodified **24** (ΔT_m=+1.5 °C). Therefore, a single guanidine-positive charge at the end of the strand **18b** improved the affinity to complementary strand, probably because the morpholinoamidine unity had a cohesive effect on partially hybridized edges, by favorable electrostatic interactions between the guanidinium group and phosphates. According to electrostatic effects, the T_m decreased in the presence of 1 mM $MgCl_2$ (ΔT_m=+1.0 °C) because of the sheltering of phosphates by magnesium. The increase in T_m produced by **18b** was consistent with the effect produced for an oligonucleotide containing a single UgU unit at the 3′ end of a duplex (ΔT_m=0.7–1.5 °C).[8a] On the contrary, strand **23b** with the morpholinoamidine subunit in a central position formed a less stable duplex (ΔT_m=−7.5/−8.0 °C). Here, the destabilization of duplex was most probably due to steric hindrance of morpholine-amidine, not compensated by electrostatic interactions with phosphates. In a similar context, a single UgU was reported to produce a slight increase in T_m (ΔT_m=0.3–0.6 °C).[8a] We reasoned that because of the planarity of the guanidinium group and the extended structure of the morpholine ring, the introduction of a morpholinoamidine in a central position had a disturbing effect on the regularity of duplex.

Strands **18b** and **23b** were also assayed as triple helix-forming oligonucleotides, by hybridization with 23-*mer* duplex **26**·**27**, which contains a 14-*mer* stretch targetable by the parallel motif. This type of triplex is formed by T(A.T) and C^+(G.C) triads and with a parallel orientation of one polypyrimidine strands (called the Hoogsteen or third stand) with respect to the central polypurine.[28] As third-strand cytosines need to be protonated to form C^+(G.C) triads, these triplexes are usually stable at pH <7.[29] Because of that, UV melting experiments were carried out at three different pHs. $MgCl_2$ was incorporated in buffers to procure the formation of

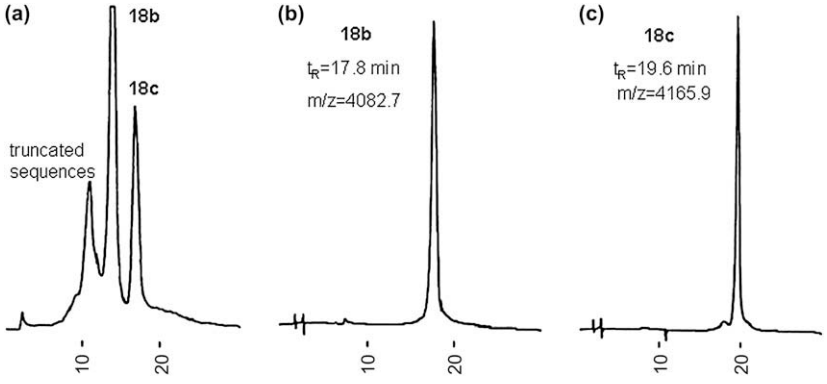


Figure 2. HPLC profiles of (a) crude oligonucleotide **18b**, (b) repurified oligonucleotide **18b** and (c) Alloc-protected oligonucleotide **18c**. Analyses conditions: linear gradient 10–30% B in 30 min, where solvent A was 0.05 M $AcONH_4$ and solvent B was CH_3CN/water 1:1.

Table 3
T_m values of duplexes

Duplex	$MgCl_2$[a] (mM)	T_m (°C)	ΔT_m[b] (°C)
24·**25**	—	42.5	—
18b·**25**	—	44.0	+1.5
23b·**25**	—	34.5	−8.0
24·**25**	1	42.5	—
18b·**25**	1	43.5	+1.0
23b·**25**	1	34.0	−7.5

[a] Experiments were performed with oligonucleotides at 1.5 μM concentration, in 40 mM NaH_2PO_4/Na_2HPO_4 and 140 mM NaCl buffer, with or without 1 mM $MgCl_2$, at pH 7.0.
[b] Differences between T_m values of modified and unmodified duplexes.

triple helices.[30] When triple helices were formed, melting curves showed two transitions. The transition at higher temperature was due to duplex **26**·**27** dissociation. Since the low temperature transition was caused by third-strand dissociation (**24**, **18b**, or **23b**), the corresponding melting temperature (T_{m1}) is a measurement of the thermal stability of triplex. Table 4 summarizes the T_{m1} values of our experiments.

As expected, the stability of the triple helix formed by unmodified strand **24** was strongly dependent on pH. Triplexes were observed at pH 6.0 and 6.5, but not at pH 7.0. Because of increased protonation of cytosine, triplex proved more stable at pH 6.0 (T_{m1}=31 °C) than at pH 6.5 (T_{m1}=27 °C). Notably, triplexes formed by the 3′ modified strand **18b** were observed at the three different pHs. At pH 7.0, the triplex was formed with a T_{m1} of 24.0 °C, whereas under the same conditions, triplex from unmodified strand **24** was not detected. Therefore, a single morpholinoamidine caused a significant increase in the stability of triplex at pH 7.0. To explain these results, we speculated on the presence of the guanidinium group decreased electrostatic repulsion between phosphate strands and compensated for the loss of hydrogen bonds and stacking interactions by the partially protonated third-strand cytosines at higher pH. This was the main reasoning to account for the extremely high stability of triplexes formed by GO.[7] The stabilizing effect was also observed at lower pHs, with ΔT_m=+5 °C over the unmodified triplex. In summary, at every pH studied, the triplex was stabilized by a single 3′ morpholinoamidine subunit in the third strand. Triplex formed by strand **23b** behaved differently from **18b**. Triplex was observed at pH 7.0, although with lower stability than for **18b** (T_{m1}=13.0 °C). Therefore, a single morpholinoamidine unit also significantly increased stability at pH 7.0 when internally positioned. Similar to the effect of **18b**, favorable electrostatic interactions between guanidine and phosphate explained the formation of triplex under these conditions. Nonetheless, the stabilization was lower than for **18b**, probably because of the steric hindrance produced by the internal morpholinoamidine. At pH 6.5, triplex increased T_{m1} up to 21.5 °C, but it was less stable than unmodified triplex (ΔT_{m1}=−5.5 °C). Notably, the triplex formed by **23b** at pH 6.0 was not only more stable than at pH 6.5, due to increased protonation of cytosines, but was a bit more stable (T_{m1}=38.0 °C) than the complex formed by **18b** (T_{m1}=36.0 °C).

Table 4
T_m values of triplexes

Triplex[a]	pH	T_{m1}[b] (°C)	ΔT_{m1}[c] (°C)
24(**26**·**27**)	7.0	nd[d]	—
	6.5	27.0	—
	6.0	31.0	—
18b(**26**·**27**)	7.0	24.0	—[e]
	6.5	32.0	+5.0
	6.0	36.5	+5.5
23b(**26**·**27**)	7.0	13.0	—[e]
	6.5	21.5	−5.5
	6.0	38.0	+7.0

[a] Experiments were performed with oligonucleotides at 1 μM concentration, in 40 mM NaH_2PO_4/Na_2HPO_4, 140 mM NaCl and 1 mM $MgCl_2$ buffer, at the pH indicated.
[b] T_{m1} referred to the triple helix dissociation.
[c] Differences between T_{m1} values of modified and unmodified triplexes at the same pH.
[d] nd: not detected.
[e] ΔT_{m1} could not be calculated because triplex **24**(**26**·**27**) was no detected at pH 7.0.

3. Conclusions

Morpholinoamidines were devised as new cationic units in oligonucleotides, by combining morpholino-nucleosides with internucleoside guanidines. Here, methodology was developed to accomplish the simplest of objectives, the synthesis of oligonucleotides containing a single morpholinoamidine unit formed by morpholino-uridine and 5′-amino-5′-deoxythymidine. Morpholinoamidine could be produced on solid-phase, by reaction of an Alloc-morpholinocarbothioamide uridine derivative with a 5′-amino-5′-deoxythymidine bound to resin, by in situ activation with Mukaiyama's reagent. When the oligonucleotide had been assembled, our procedure followed with the removal of the Alloc group from the guanidine by treatment with Pd^0, and subsequent deprotection and cleavage from the resin by aq concd ammonia. Following this methodology, two oligonucleotides containing one morpholinoamidine subunit were synthesized, the one located at the 3′ end, and the other in the middle of the strand. We investigated the affinity properties of these two oligonucleotides by forming double and triple helices with DNA complementary strands. Double helices were found to be slightly stabilized by a 3′ morpholinoamidine, but complexes were less stable when located in the middle of the sequence. Notably, triple helices were significantly stabilized at pH 7.0 by the presence of morpholinoamidine units, whereas under the same conditions unmodified strands did not produce triplex. According to our results, the stabilization effect of morpholinoamidine was probably produced by favorable electrostatic interactions between the cationic guanidinium and phosphates, whereas duplex destabilization when morpholinoamidine was internally positioned could originate in steric hindrance. In the light of these preliminary results, which revealed that a single morpholinoamidine unit can improve the affinity of oligonucleotides, we are working on optimizing and extending the synthetic methodology, to prepare further modified oligonucleotides and, gain better understanding of their properties.

4. Experimental

4.1. General

Unless otherwise indicated, all chemicals were purchased from commercial suppliers (reagent grade) and used without purification. Dry CH_3CN was obtained by distillation over CaH_2 and storage over CaH_2 lumps. CH_2Cl_2 was neutralized and dried by passing through basic Al_2O_3 and storage over CaH_2. Dry THF was obtained by distillation over sodium metal in the presence of benzophenone. DMF was bubbled with nitrogen to remove volatile contaminants and dried by storage over CaH_2. Amine-free pyridine was obtained by distillation over ninhydrin and further dried by storage over CaH_2 lumps. NMR spectra were recorded with either a Varian Unity 300 MHz or a Varian Mercury 400 MHz, respectively. UV analyses and melting curves were performed in a Jasco V-550 instrument equipped with a thermoregulated cell holder. Low resolution electrospray mass (EIMS) spectra of nucleosides were recorded in a Waters Alliance 2696 HPLC coupled to Waters Micromass ZQ MS detector. MALDI-TOF spectra of oligonucleotides were recorded in

a Perseptive Biosystems Voyager DE-RP instrument, by using 2,4,6-trihydroxyacetophenone (THAP), 2,4,6-trihydroxyacetophenone/ammonium citrate (1:1) (THAP-CA) or alpha-cyano-4-hydroxycinnamic acid (ACH) as matrixes.

4.2. Synthesis of guanidines 14a and 14b

4.2.1. Morpholino-uridine (**6**)

DMT-U (3.5 mmol) was dissolved in MeOH (100 mL), $NaIO_4$ (3.8 mmol) and NH_4HCO_3 (7.0 mmol) were added, and the mixture was left to react for 1 h. Then, the solid that formed was separated by filtration through glass wool, $NaBH_3CN$ (8.7 mmol) was added to the filtrate and left to react for additional 2 h. After that, the solution was acidified to pH 5.0 by addition of small portions of 10% citric acid, and evaporated to dryness. The residue was dissolved in AcOEt, and the corresponding organic phase was washed with water (50 mL) and brine, dried over $MgSO_4$, and the solvent was evaporated. Compound **6** was obtained as a white solid after purification by silica gel column chromatography, by elution with CH_2Cl_2/triethylamine 99:1 and increasing amounts of MeOH (up to 10%).

Yield: 60%. mp: 111–115 °C. R_f (dichloromethane/MeOH 10:1): 0.4. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.45 (1H, d, J=7.6 Hz), 7.33–7.21 (9H, m), 6.82 (4H, d, J=8.8 Hz), 5.77 (1H, d, J=7.6 Hz), 5.68 (1H, dd, J=9.7, 2.4 Hz), 3.96 (1H, m), 3.78 (6H, s), 3.24 (1H, dd, J=9.5, 4.9 Hz), 3.15 (1H, dd, J=12.1, 2.4 Hz), 3.07 (1H, dd, J=9.5, 5.1 Hz), 3.01 (1H, dd, J=12.7, 2.3 Hz), 2.58 (1H, dd, J=12.7, 10.7 Hz), 2.54 (1H, dd, J=12.1, 9.7 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 162.7, 158.5, 150.0, 144.2, 139.8, 135.7, 130.0, 128.0, 127.7, 126.3, 113.0, 102.0, 86.0, 80.0, 64.0, 55.1, 49.1, 46.4. EIMS m/z (positive mode, $[M+H]^+$) calcd for $C_{30}H_{32}N_3O_6$ 530.2, found 530.3; HRMS (positive mode, $[M+Na]^+$) calcd for $C_{30}H_{31}N_3O_6Na$ 552.2105, found 552.2098.

4.2.2. Morpholino-uridine carbothioamide (**7**)

Allyloxycarbonyl chloride (2 mmol) was dissolved in anhydrous AcOEt (5 mL) and added dropwise on a suspension of potassium thiocyanate (2.4 mmol) in anhydrous AcOEt (5 mL), and the mixture was left to react for 3 h at room temperature. After that, a solution of nucleoside **6** in anhydrous AcOEt and under argon was prepared and added dropwise on the previous mixture, and left to react for additional 2 h. After this, the solvent was removed by evaporation, the residue was redissolved in CH_2Cl_2 and the resultant organic phase was washed twice with 10% $NaHCO_3$ and brine, dried over $MgSO_4$, and evaporated to dryness. Compound **7** was obtained as a white solid, which does not need further purification according to TLC and NMR analyses.

Yield: 98%. mp: 80–82 °C. R_f (dichloromethane/MeOH/triethylamine 10:0.2:0.1): 0.3. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.42 (1H, d, J=7.6 Hz), 7.31–7.24 (9H, m), 6.82 (4H, d, J=8.8 Hz), 5.92–5.82 (2H, m), 579 (1H, d, J=7.6 Hz), 530 (1H, d, J=17 Hz), 5.22 (1H, d, J=8.8 Hz), 4.63 (1H, dd, J=12, 4.4 Hz), 4.57 (1H, dd, J=12.2, 4.8 Hz), 4.15–4.12 (1H, m), 3.77 (6H, s), 3.29–3.08 (6H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 178.4, 163.6, 160.2, 150.1, 149.9, 144.3, 139.6, 135.4, 131.2, 129.7, 128.5, 127.6, 126.2, 119.2, 113.3, 102.2, 85.7, 80.2, 67.5, 64.5, 55.2, 49.7. EIMS m/z (positive mode, $[M+H]^+$) calcd for $C_{35}H_{37}N_4O_8S$ 672.2, found 672.1. HRMS (positive mode, $[M+Na]^+$) calcd for $C_{35}H_{36}N_4O_8SNa$ 695.2146, found 695.2145.

4.2.3. 5′-MMT-amino-5′-deoxythymidin-3-yl resin (**12a**)

1 g of commercial LCAA-CPG (Sigma, 500 Å, approx. 50 μmol amino/g) was placed into a polypropylene syringe fitted with a polyethylene disc and sequentially washed with CH_2Cl_2, THF, 10% HCl, THF, DMF, 20% piperidine in DMF, 2% TCA in CH_2Cl_2, 2% *N*-ethyl-*N*,*N*-diisopropylamine in CH_2Cl_2, CH_2Cl_2, CH_3OH, and CH_3CN. *N*-Fmoc-sarcosine (Novabiochem, 50 μmol) and diisopropylcarbodiimide (50 μmol) were added to the resin, the resulting mixture was suspended in DMF and reacted for 30 min, the resin was washed with DMF and the aminoacid coupling was repeated, to render an aminoacid functionalization of 42 μmol/g. The resin was treated with a solution of Ac_2O/DMF 1:1 to cap free amino groups on the resin. Subsequently, free amine resin **11** was produced by removal of the Fmoc group with 50% piperidine in DMF and after this, 5′-MMT-amino-5′-deoxythymidine 3′-monosuccinate (**10**, 63 μmol), HOBt (63 μmol), and diisopropylcarbodiimide (336 μmol) were added to the resin and left to react for 2 h. After washings, the resin was treated with Ac_2O/DMF 1:1. The incorporation of nucleoside was determined by MMT quantification to be 34 μmol/g.

4.2.4. Guanidine **14a** (mUg(Alloc)T)

Resin **12a** (1 μmol) was placed into a cylindric polypropylene reactor normally used for the synthesis of oligonucleotides, and by the use of two syringes adapted to the reactor it was treated with 3% TCA in CH_2Cl_2 to produce the removal of MMT group, and washed with DMF and 5% triethylamine in CH_2Cl_2 to neutralize the amine. Under the best conditions (see Table 1), coupling was carried out by mixing approx. 150 μL of a solution 0.15 M of morpholinocarbothioamide 7 and 0.23 M of triethylamine and 150 μL of a solution 0.15 M of Mukaiyama's reagent, both in CH_2Cl_2 and left to react for 1 h. After this, the resin was washed with DMF and CH_2Cl_2 and the DMT group was removed with 3% trichloroacetic acid in CH_2Cl_2. In this case, the yield of guanidine formation was determined by UV quantification of DMT^+ solution (76%). The resin was treated with aq concd ammonia for 30 min to produce the cleavage. The crude was analyzed by HPLC to show guanidine **14b** (85%) and pyridium salt **15** (15%). Finally, both products were purified by HPLC and characterized by mass spectrometry.

mUg(Alloc)T (**14a**): 32% yield. Reversed-phase HPLC (Kromasil-C_{18}, linear gradient 5–100% B in 30 min, solvent A: 0.05 M $AcONH_4$, solvent B CH_3CN/water 1:1) t_R: 7.9 min. MALDI-TOF MS m/z (positive mode, ACH, $[M+H]^+$) calcd for $C_{24}H_{32}N_7O_{10}$ 577.2, found 578.0.

Pyridinium salt (**15**): 5% yield. Reversed-phase HPLC (Kromasil-C_{18}, linear gradient 5–100% B in 30 min, solvent A: 0.05 M $AcONH_4$, solvent B CH_3CN/water 1:1) t_R: 5.6 min. MALDI-TOF MS m/z (positive mode, ACH, $[M]^+$) calcd for $C_{16}H_{21}N_4O_4$ 333.2, found 333.8.

4.2.5. Guanidine **14b** (mUgT)

Guanidine was produced with the same procedure as for **14a**, with an additional deprotection step to remove the Alloc group before the cleavage step with ammonia. As a general procedure (see Table 2 for reagents), the resin contained in one synthesis reactor was vacuum dried, purged with argon, and the reactor was capped with two all plastic syringes. Then, 250 μL of a solution 1.2 M of scavenger and 250 μL of a solution 0.02 M of $Pd(PPh_3)_4$ in anhydrous THF were delivered into the reactor, and left to react for the time indicated in Table 2. After this, the resin was filtered and washed with THF, acetone, 0.1 M sodium diethyldithiocarbamate, acetone, and water to remove the traces of palladium adsorbed on the resin. Finally, the resin was treated with aq concd ammonia for 30 min to produce cleavage, and the corresponding crude was purified by HPLC.

mUgT **14b**: 29% yield. Reversed-phase HPLC (Kromasil-C_{18}, linear gradient 5–100% B in 30 min, solvent A: 0.05 M $AcONH_4$, solvent B CH_3CN/water 1:1) t_R: 5.4 min. MALDI-TOF MS m/z (positive mode, ACH, $[M+H]^+$) calcd for $C_{20}H_{28}N_7O_8$ 494.2, found 495.5.

4.3. Oligonucleotide synthesis

Unmodified oligonucleotides **24**–**27** and the phosphate fragments of modified oligonucleotides **18b** and **23b** were assembled on CPG in an automatic synthesizer (ABI 380B or Expedite) using the standard phosphite triester methodology. For the synthesis of oligonucleotide **18b**, resin **13** was first prepared as described above,

and after the guanidine formation, the phosphate stretch was assembled using the standard phosphoramidite methodology. For the synthesis of **23b**, 5′-MMT-amino-5′-deoxythymidine 3′-phosphoramidite[21] was incorporated during the oligonucleotide synthesis in the same conditions than the rest of phosphoramidites. Then, the coupling of morpholinocarbothioamide **7** was performed as described for the synthesis of guanidine **14a**. Once the guanidine was formed, the resin was returned to the synthesizer and the 5′ phosphate stretch was assembled using the standard protocol. Oligonucleotides **24**–**27** were finally deprotected and cleaved from the resin by treatment with aq concd ammonia at 60 °C (6–12 h), and purified as explained below. Oligonucleotides **18b** and **23b** were deprotected by a two-step procedure. In the first place, the oligonucleotide resin (1 μmol) was placed into a fritted syringe and was submitted under argon to three treatments with 250 μL of 1.2 M dimethyl malonate and 250 μL of 0.02 M $Pd(PPh_3)_4$ in anhydrous THF for 1 h. To produce the complete deprotection and the cleavage of the resin, the resin was placed into a vial, and treated with aq concd ammonia at 60 °C for 6 h. DMT–oligonucleotides were purified by reversed-phase HPLC (Hamilton PRP, linear gradients 10–50 or 60% B in 30 min). The collected fractions were lyophylized and treated with AcOH/H_2O 4:1 (15 min, 0 °C) to produce the removal of DMT and lyophylized. Oligonucleotides **18b** and **23b** needed to be repurified by reversed-phase HPLC (Hamilton PRP, linear gradients 10–30% B in 30 min). Oligonucleotides were quantified by UV absorption at 260 nm. ε_{260} values calculated by the nearest-neighbor method.

*Oligonucleotide **18b***: 13% yield. Reversed-phase HPLC (Kromasil-C_{18}, linear gradient 10–30% B in 30 min, solvent A: 0.05 M $AcONH_4$, solvent B CH_3CN/water 1:1) t_R: 17.8 min. MALDI-TOF MS m/z (negative mode, THAP-CA, $[M-H]^-$) calcd for $C_{136}H_{182}N_{35}O_{88}P_{12}$ 4085.8, found 4082.7.

*Oligonucleotide **23b***: 10% yield. Reversed-phase HPLC (Kromasil-C_{18}, linear gradient 10–30% B in 30 min, solvent A: 0.05 M $AcONH_4$, solvent B CH_3CN/water 1:1) t_R: 17.1 min. MALDI-TOF MS m/z (negative mode, THAP-CA, $[M-H]^-$) calcd for $C_{136}H_{182}N_{35}O_{88}P_{12}$ 4085.8, found 4084.2.

4.4. UV melting curves

Experiments were performed with oligonucleotides at 1.5 μM concentration for duplexes and 1 μM for triplexes, in 40 mM NaH_2PO_4/Na_2HPO_4 and 140 mM NaCl buffer, with or without 1 mM $MgCl_2$, at pH 7.0 for duplexes, and with 1 mM $MgCl_2$ at pH 6.0, 6.5, or 7.0 in the case of triplexes. Samples were heated at 90 °C for 5 min, allowed to cool down slowly to room temperature to induce annealing, and then kept overnight in a refrigerator (5 °C). Melting curves were recorded by heating the samples from 5 to 90 °C at a constant rate of 0.5 °C/min and monitoring the absorbance at 260 nm at a sampling rate of 6 points/min. At temperatures below 25 °C, nitrogen was flushed to prevent water condensation on cuvettes. T_m values were determined from the maxima of the first derivative of the curves, calculated with the Microcal Origin software. Experiments were repeated until coincident T_m values were obtained. The error in T_m values was estimated to be ±0.5 °C.

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Supplementary data

^1H and ^{13}C NMR spectra and mass spectra of morpholino-uridine **6** and morpholino-uridine carbothioamide **7**, and UV melting profiles of duplexes and triplexes summarized in Tables 3 and 4. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.11.069.

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