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Branched Oligonucleotides Containing Bicyclic Nucleotides as Branching Points and DNA or LNA as Triplex Forming Branch

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Abstract—Various Y-shaped branched oligonucleotides containing a 2′-*O*,3′-*C*-ethylene linked or 2′-*O*,4′-*C*-methylene linked bicyclic nucleotide as branching point were synthesized on an automated DNA synthesizer. Thermal denaturation experiments at 260 and 284 nm showed increased thermal stabilities of complexes formed between these Y-shaped oligonucleotides and complementary DNA compared with those formed with the corresponding linear reference. The most significant effect was observed when LNA (locked nucleic acid) monomers were used in the triplex forming branch.

Recognition of single-stranded DNA and/or RNA using branched (Y-shaped) oligonucleotides (ONs) has been studied by several groups.[1] In our search for Y-shaped branched ON that could serve as a probe for high-affinity targeting of complementary ONs we have earlier used derivatives **V** (Fig. 1) as branching monomers in the model system depicted in Figure 2.[1c–e] The rationale behind this model system is that hybridization takes place through Watson–Crick base-pairing between the target sequence and the Y-shaped ON, more precisely the part composed of the linear sequence involving the 5′- and 3′-oxygen atoms of the branching monomers. The remaining part of the Y-shaped ON ('the branch') participates in triple helix formation via Hoogsteen base-pairing in the major groove. The arabinofuranosyl branching monomers **V**, however, proved rather inefficient in forming such bimolecular complexes with complementary ONs.[1c–e] As this, at least in part, can be explained by a detrimental effect ($\Delta T_m = -10\,^{\circ}C$) on the thermal stability of duplexes caused by the incorporation of one arabinofuranosyl monomer in a linear ON, and an unfavorable geometry for the attachment of a third ON strand at the 2′-OH position, we decided to evaluate the branching monomers **X** and **Y** (Fig. 1). As these contain functionalized bicyclic furanose moieties structurally based on bicyclic monomers which earlier have been shown to hybridize satisfactorily (2′,3′-BcNA)[2] or excellently (LNA; locked nucleic acid; Figure 1)[3] with complementary ONs we were hoping to improve the binding affinity of the branched ONs. The additional functional groups of monomers **X** and **Y** (a *C*-hydroxymethyl branch at the 2′-*O*,3′-*C*-ethylene linker for **X** and a 3′-*C*-(2″-hydroxyethyl) branch for **Y**) are, according to results from modelling, X-ray studies[4] and NMR studies[5] of duplexes containing structurally closely related monomers, oriented towards the major groove of a B-type duplex. The synthesized Y-shaped ONs consist of a 12-mer polypyrimidine sequence (duplex forming segment) and a third strand ('the branch') of varying length and composition (Table 1). The results disclosed in this report show **X** and **Y** to be interesting branching monomers and LNA to bind very efficiently in the triplex mode.

Synthesis of the phosphoramidite building block **5** for automated synthesis of the branched ONs containing monomer **X** was achieved as depicted in Scheme 1. Protection of the primary hydroxy group ('3″-OH group') of nucleoside **1**[2b] was performed by the formation of its levulinate **2** in 81% yield by reaction with levulinic anhydride[6] in the presence of 4-(*N*,*N*-dimethylamino)pyridine in anhydrous pyridine. Debenzylation of nucleoside **2** afforded the diol **3** in 94% yield and subsequent selective protection of the primary 5′-hydroxy function gave the nucleoside **4** in 90% yield using 4,4′-dimethoxytrityl chloride (DMTCl) in anhydrous pyridine. The phosphoramidite **5**, ready for automated ON synthesis, was obtained in 50% yield from **4** by phosphitylation of the 3′-hydroxy group with 2-cyanoethyl *N*,*N*-diisopropylaminophosphoramidochloridite

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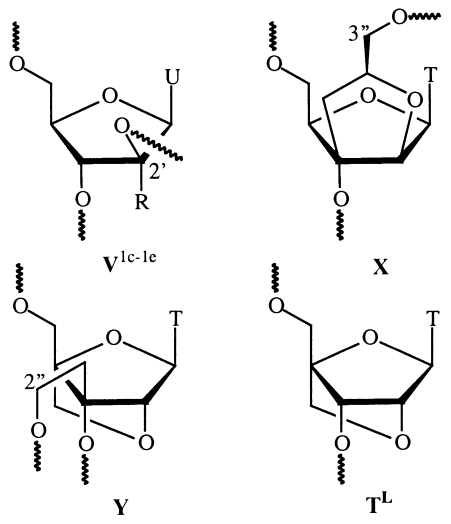
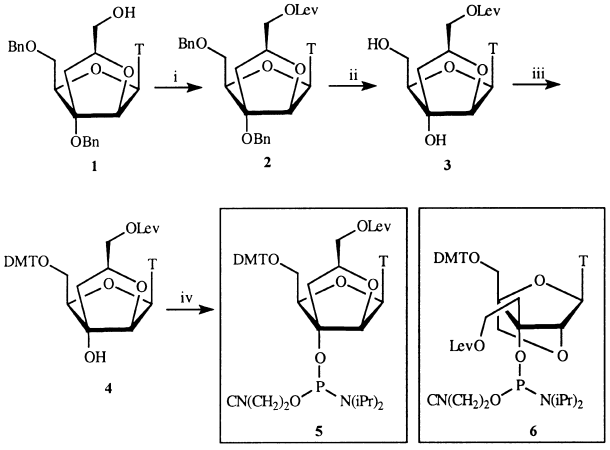


Figure 1. Structure of branching nucleotides **V**,[1c–e] **X** and **Y**, and structure of an LNA thymine monomer ($\mathbf{T^L}$). U = uracil-1-yl. T = thymin-1-yl. R = H or CH_3.

in the presence of *N*,*N*-diisopropylethylamine in anhydrous dichloromethane followed by column chromatography.[7] Using similar strategies for levulinoylation of the 2″-hydroxy group and phosphitylation of the 3′-hydroxy group, the 3′-*C*-levulinoyloxyethyl branched LNA phosphoramidite derivative **6** was synthesized.[7,8]



Scheme 1. (i) Levulinic anhydride, DMAP, pyridine; (ii) H_2, $Pd(OH)_2$/C, EtOH; (iii) DMTCl, pyridine; (iv) 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, *N*,*N*-diisopropylethylethylamine, CH_2Cl_2. DMT = 4,4′-dimethoxytrityl. Lev = levulinoyl. T = thymin-1-yl.

The ONs **A**–**H** (Table 1) were synthesized using standard phosphoramidite chemistry[9] with cycles including detritylation, coupling, capping and oxidation. The syntheses were initiated by constructing the linear strand using standard amidite concentration (0.033 M in anhydrous acetonitrile) and coupling time (1 min). However, when the amidite **5** was incorporated, a 0.042 M solution and a coupling time of 30 min were applied. For incorporation of amidite **6**, a 0.050 M solution and a coupling time of 2×60 min were applied (coupling, washing, coupling with a fresh solution of amidite, capping, oxidation and detritylation). The oxidation was performed with *tert*-butyl hydroperoxide for the amidites **5** and **6** to prevent strand cleavage,[10] and with iodine for the other nucleotides. A separate end-capping cycle (consisting of detritylation and acetylation) was used to block further elongation at the 5′-end, thus preparing for branching. The support was subsequently treated with a 0.5 M solution of hydrazine hydrate in a pyridine:acetic acid:water buffer (4:3:0.35, v:v:v) for 5 min to remove the levulinoyl protecting group. The first nucleotide in the branch was subsequently attached at the 3″-hydroxy group (in the case of branching nucleotide **X**) or at the 2″-hydroxy group (in the case of branching nucletide **Y**) by 5 min couplings at standard amidite concentration whereupon the remaining part of the branch was synthesized using standard conditions. The 5′-*O*-DMT group of the latest incorporated nucleotide was removed as the last step on the synthesizer for ONs **A**–**F** whereas the 5′-*O*-DMT group was kept on for ONs **G** and **H**. The coupling yields determined spectrophotometrically at 498 nm (quantification of the amount of DMT cation released during each detritylation step) were >99% for commercial amidites, ~95–98% for the modified amidite **5**, and ~76% for amidite **6**. The ONs were cleaved from the solid support and deprotected by treatment with 32% aqueous ammonia for 16 h at room temperature. The crude ONs were precipitated from ethanol to give pure ONs **A**–**F** (Table 1)[11] or reversed phase purified (COP columns, Cruachem; procedure includes detritylation) to give pure ONs **G** and **H**.[11] The composition of the ONs was confirmed by matrix-assisted laser desorption mass spectrometry.[12]

The hybridization properties of ONs **A**–**H** towards single stranded DNA (A_{12}) were evaluated (Table 1). The melting temperatures (T_m values) of the non-branched duplexes **A**:A_{12} (31.5 °C) and **G**:A_{12} (31.0 °C) were used as references. The branched ONs **B**, **C**, **E**, **F** and **H** were designed to bind complementary ONs through the formation of bimolecular complexes as shown in Figure 2. In all cases, only one melting transition (hyperchromic shift) was detected and the formation of a structure involving a triple helical segment was confirmed if a melting transition was detected not only at 260 nm but also at 284 nm.[13]

The ONs **B**, **C** and **H** have the potential of forming eight Hoogsteen basepairs in addition to the Watson–Crick duplex. In analogy with our earlier results, and the results of Kool et al.[14] on circular DNA, it was expected that only one melting transition, i.e., cooperativity between the two binding modes, would be observed leading to stabilization. Compared to the reference duplexes **A**:A_{12} and **G**:A_{12}, the Y-shaped ONs **B**, **C** and **H** showed slightly improved binding affinities towards A_{12} as indicated by the T_m values increased by +1, +1.5 and +3.0 °C, respectively. The involvement of a triple helix in the complexes formed with ONs **B**, **C** and **H**, but *not* with the references **A** and **G**, was confirmed by transitions observed at 284 nm.[13] It appears from the T_m results that the presence of a C_2-linker has no significant influence on the thermal stability (**B**:A_{12} versus **C**:A_{12}). The geometry for formation of the bimolecular complexes is apparently slightly more favourable with the branching point **Y** than with **X**, but for synthetic reasons the latter was evaluated further in this preliminary study.

The ON **D** containing a $T_2GT_3GT_2$-5′ branch with two mismatched 2′-deoxyguanosine monomers was not expected to be able to participate in triplex formation. Accordingly, no sign of a dissociation at 284 nm could be observed. The decreased T_m values of the complex **D**:A_{12} (−3.5 °C) compared with the reference **A**:A_{12} strongly indicate that the attachment of a (binding or non-binding) branch at the 3″-OH position has a destabilizing effect on the duplex which further substantiates the participation of the branches in complex formation for ONs **B**, **C**, **E**, **F** and **H**, and also the stabilization resulting thereof.

Despite the fact that the branch for ONs **E** and **F** has been shortened to five nucleotides, both appear from hybridization studies at 284 nm to be involved in complex formation during binding towards A_{12}. The T_m value obtained with five unmodified thymidines in the branch (**E**:A_{12}; $T_m = 30.0$ °C) was as expected lower than for ONs **B** and **C**, each containing eight thymidines in the branch, but higher than for ON **D** with a non-binding branch. The ON **F**, containing three thymidines and two LNA thymine monomers ($\mathbf{T^L}$) in the branch display a dramatically increased T_m value against A_{12} (39.0 °C), and triple helix involvement was confirmed at 284 nm. This result emphasizes, and expands, the unique potential of LNA to recognize complementary nucleic acids. We chose not to evaluate the corresponding ON containing five LNA thymine monomers in the branch because of the possibility of formation of alternative binding complexes, e.g., a duplex involving the $\mathbf{T^L}_5$ branch. LNA monomers are known to exist in a conformationally locked 3′-*endo* (N-type) furanose conformation,[4,15] and the strong triplex binding with LNA is in line with earlier reports on increasing stability of triple helices with increasing N-type conformation of the participating nucleotide monomers.[16]

Table 1. ONs synthesized and thermal denaturation studies towards A_{12}[a]

	Sequence[b]	T_m/°C[c]
A	3′-TTTTTTTT-$_3$**X**$_5$-TTT-5′	31.5[e]
B	3′-TTTTTTTT-$_3$**X**$_5$-TTT-5′ 5′-TTTTTTTT-$_{3''}$	32.5[d]
C	3′-TTTTTTTT-$_3$**X**$_5$-TTT-5′ 5′-TTTTTTTTCC-$_{3''}$	33.0[d]
D	3′-TTTTTTTT-$_3$**X**$_5$-TTT-5′ 5′-TTGTTTGTT-$_{3''}$	28.0[e]
E	3′-TTTTTTTT-$_3$**X**$_5$-TTT-5′ 5′-TTTTT-$_{3''}$	30.0[d]
F	3′-TTTTTTTT-$_3$**X**$_5$-TTT-5′ 5′-TT^{L}TT^{L}T-$_{3''}$	39.0[d]
G	3′-TTTTTTTT-$_3$**Y**$_5$-TTT-5′	31.0[e]
H	3′-TTTTTTTT-$_3$**Y**$_5$-TTT-5′ 5′-TTTTTTTT-$_{2''}$	34.0[d]

[a]Recorded in 10 mM $MgCl_2$, 100 mM NaCl, 10 mM Tris HCl, pH 7.0.
[b]A = 2′-deoxyadenosine monomer; C = 2′-deoxycytidine monomer; G = 2′-deoxyguanosine monomer; T = thymidine monomer; $\mathbf{T^L}$ = LNA thymine monomer; **X** = modified bicyclic nucleotide derived from amidite **5**; **Y** = modified bicyclic nucleotide derived from amidite **6**.
[c]Melting points were determined as the maximum of the first derivative of the absorption versus temperature curve at 260 nm. Observed hyperchromicity ratios: **A**: 1.27; **B**: 1.28; **C**: 1.29; **D**; 1.23; **E**: 1.27; **F**: 1.31; **G**: 1.27; **H**: 1.22.
[d]Transition confirmed at 284 nm. Observed hyperchromicity ratios: **B**: 1.07; **C**: 1.08; **E**: 1.04; **F**: 1.08; **H**: 1.04.
[e]No transition detectable at 284 nm.

In summary, the two functionalized bicyclic furanose phosphoramidite building blocks **5** and **6** were incorporated as branching points in Y-shaped ONs. The thermal stabilities of complexes with the branched ONs were increased compared to those with the linear references, and triple helix involvement was confirmed. These results underline the potential of using the branched constructs for efficient nucleic acid recognition. Interesting results from the present study include that even a five nucleotide branch is sufficient for stabilization via triple helix formation, and that LNA is able to bind very efficiently in the triplex mode.

Acknowledgements

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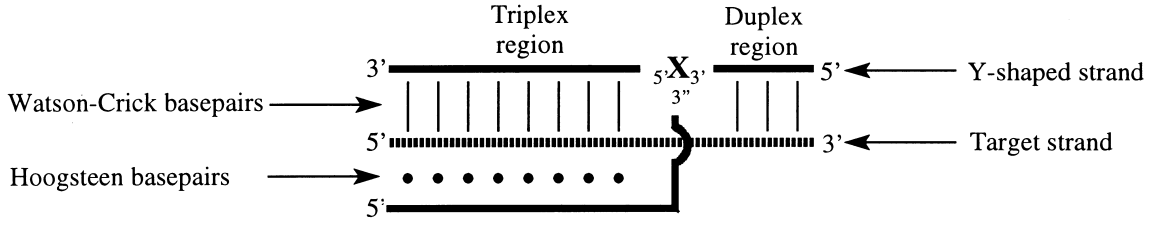

Figure 2. Model system used. Schematic representation of the structure of the complexes formed between branched ONs and complementary ONs. The branching monomer can be **X** as shown or $\mathbf{V}^{1c-e}$ or **Y**.

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