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BRANCHED OLIGODEOXYNUCLEOTIDES: A NEW SYNTHETIC STRATEGY AND FORMATION OF STRONG INTRA- AND INTERMOLECULAR TRIPLE HELICAL COMPLEXES

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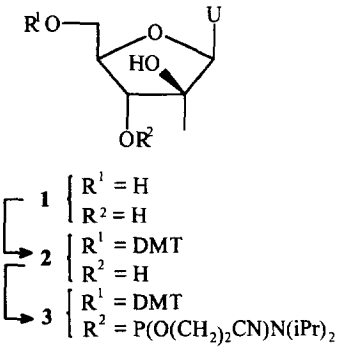
Abstract: A fully automated synthetic strategy for preparation of branched oligodeoxynucleotide (ODN) analogues with sequences of arbitrary length and base composition is described. Various novel triple helix forming branched ODNs were synthesized and their behavior during thermal denaturation at 260 nm and 284 nm studied. The intra- and intermolecular hybridization properties of the novel branched ODNs are improved compared to analogous linear ODNs.

The number of experiments involving branched RNA- or DNA-analogues has been rather limited due to synthetic limitations. However, laborious solution-phase synthesis of up to heptameric branched oligonucleotides has been accomplished[1] and few reports on solid-phase synthesis of branched oligonucleotides have been published, involving either identical sequences in two of the three branches[2] or disconnection of the solid support for deprotection before branching.[3]

For evaluation of the molecular recognition potential of branched oligodeoxynucleotides (ODNs) towards DNA and RNA, we have developed a fully automated synthetic strategy based on regioselectivity for preparation of Y-shaped ODN analogues with sequences of arbitrary length and base composition. The strategy involves solid-phase incorporation of building block **3** containing an unprotected tertiary 2'-hydroxyl group as attachment site for the third strand oriented into the major groove in DNA duplexes.

The synthesis of the phosphoramidite **3** was performed as follows (Scheme 1): Reaction of 1-(2-methyl-β-D-arabinofuranosyl)uracil **(1)**[4] with 4,4'-dimethoxytrityl chloride in dry pyridine afforded 1-(5-*O*-(4,4'-dimethoxytrityl)-2-methyl-β-D-arabinofuranosyl)uracil **(2)** in 59% yield.[5] By regioselective phosphitylation[6,7] of **2**, the nucleoside phosphoramidite **3** was obtained in 87% yield after column chromatographic purification and precipitation from petroleum ether.[8]



Reagents: See text;
DMT = 4,4'-dimethoxytrityl;
U = uracil-1-yl

Scheme 1

The fully automated strategy using phosphoramidite chemistry[9] for synthesis of the novel branched oligonucleotides **A, B, E, F, H** and **I** is outlined in Figure 1. The syntheses are controlled by use of different concentrations (and coupling times) of amidites and commences as normal for "branch A" from the solid support in the 3'- to 5'-direction until the branching amidite **3** has been incorporated.[10] To prevent unwanted branching at the unprotected tertiary 2'-hydroxyl group, the concentration of the amidites during the synthesis of "branch B" (upon detritylation of incorporated **3**) is lowered to 0.05 M. The elongation of "branch B" is terminated by an additional capping step[11] succeeding detritylation of the 5'-nucleotide. To circumvent the sterical hindrance at the unprotected 2'-hydroxyl group, the concentration of the first amidite in the synthesis of "branch C" is tripled to 0.3 M,[12] whereupon standard conditions are used to finish the synthesis of the Y-shaped ODNs.[13] During synthesis of *arabino*-oligonucleotides by application of 2'-OH unprotected *arabino*-phosphoramidite synthons, only minor amounts of chain branching was detected despite the presence of free *secondary* hydroxyl groups.[7] As phosphoramidite **3** contains an unprotected *tertiary* hydroxyl group, the applicability of **3** in the controllable regioselective branching procedure reported here is well justified. Consequently, during elongations of "branch B" the coupling yields were never larger than 100 % which would have indicated unwanted branching. In preliminary experiments however, branching was detected (>100 % coupling yields) when *normal* amidite concentrations and *longer* coupling times were used during synthesis of "branch B".

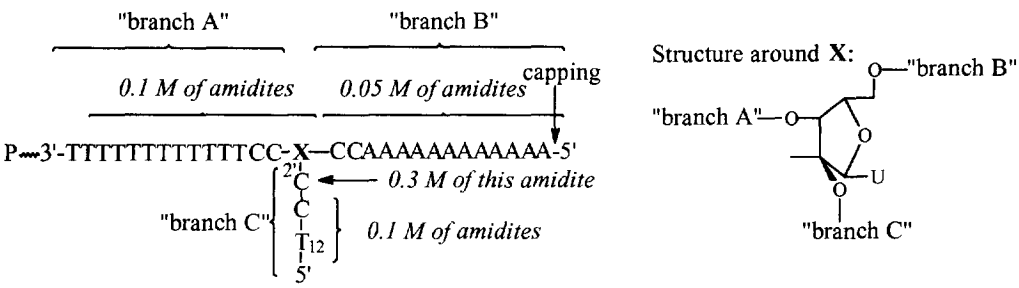


Figure 1. Strategy for synthesis of branched ODN analogue **A** using phosphoramidite **3**. The concentrations of amidites applied during automated synthesis are indicated. P = solid support; A = 2'-deoxyadenosine; C = 2'-deoxycytidine; T = thymidine; **X** = nucleotide derived from **3**

The 4,4'-dimethoxytrityl protected oligodeoxynucleotides were removed from the solid support by treatment with concentrated ammonia at 20 °C for 72 h. Subsequent purification using disposable reverse-phase cartridges[14] afforded the pure oligomers[15] (Table 1). The composition of the branched ODNs was confirmed by matrix assisted laser desorption mass spectrometry.[16] The ODNs **A** and **B** were designed for evaluation of the intramolecular hybridization properties. The sequence of ODN **A** allows formation of a 12-base triple helix[17] where one pyrimidine strand is antiparallel to the purine strand in a Watson-Crick mode and the other parallel in a Hoogsteen mode. ODN **B** consists of a 12-mer thymidine strand, a complementary 12-mer deoxyadenosine strand and a 12-mer deoxycytidine strand thus excluding the formation of an unimolecular triple helical structure.[18] The thermal denaturation profile for **A** exhibited a biphasic nature at 260 nm corresponding to dissociation first of the triplex and then the Watson-Crick duplex. The melting point at 59 °C was confirmed to be caused by triple helix dissociation by observation of a monophasic hyperchromic absorbance

change at 284 nm, known to be characteristic for dissociation of the third strand from T-A-T triplexes.[17d,17e] As expected, ODN **B** displayed a monophasic melting profile at 260 nm with a melting point at 66.0 °C and no observable transition at 284 nm. The unmodified duplex T_{12}:dA_{12} melts at 40 °C which is 26 °C lower than observed for the corresponding intramolecular duplex of analogue **A** and **B**. Apparently, the branched ODN **A** is structurally preorganized for the formation of thermodynamically favored unimolecular triple (and double) helical complexes, which may prove useful e.g. in studies on structural and conformational requirements for formation of triple helical structures.

Table 1. Sequences Synthesized and Hybridization Data

	Sequence [a]	T_m / °C [b]
A	3'-TTTTTTTTTTTTCC-X-CCAAAAAAAAAAAA-5' 5'-TTTTTTTTTTTTCC(1,2')	59 [c] ; 66 [d]
B	3'-TTTTTTTTTTTTCC-X-CCAAAAAAAAAAAA-5' 5'-CCCCCCCCCCCCCC(1,2')	66 [d]
C	3'-TTTTTTTTTTT-5'	40 [d,e]
D	3'-TTT-X-TTTTTTTT-5'	29 [d,e]
E	3'-TTT-X-TTTTTTTT-5' (2',1)CCTTTTTTT-5'	29 [d,e]
F	3'-TTT-X-TTTTTTTT-5' (2',1)CCCCTTTTTTT-5'	29 [d,e]
G	3'-TTTTTTTT-X-TTT-5'	26 [d,e]
H	3'-TTTTTTTT-X-TTT-5' 5'-TTTTTTTCC(1,2')	30 [c,e]
I	3'-TTTTTTTT-X-TTT-5' 5'-TTTTTTTCCCC(1,2')	30 [c,e]

[a] A = 2'-deoxyadenosine, C = 2'-deoxycytidine, T = thymidine, X = nucleotide derived from **3**.
[b] T_m = melting temperature measured at 260 nm. [c] Transition confirmed at 284 nm.
[d] No transition detectable at 284 nm. [e] Melting temperature of complex with dA_{12}

For evaluation of branched ODNs as a potential new class of high-affinity nucleic acid recognition probes and therapeutic agents, we synthesized ODNs **E**, **F**, **H** and **I** and recorded the melting temperatures of complexes with dA_{12} (Table 1). As references, the melting temperatures of duplexes **D**:dA_{12} and **G**:dA_{12} (29 °C and 26 °C, respectively) were used. Branched ODNs **H** and **I** were designed to induce enhanced binding affinity towards complementary dA_{12} through the formation of short bimolecular triple helical complexes. As can be seen from Table 1, regardless of the length of the deoxycytidine linker (C_2 or C_4),[19] an increase in melting temperature of 4 °C was achieved for ODNs **H** and **I** *compared to the linear reference* ***G***. This we tentatively attribute to the formation of additional Hoogsteen base pairing supported by denaturing measurements at 284 nm confirming the single monophasic transition observed. On the contrary, ODNs **E** and **F** having the two pyrimidine strands in a parallel orientation thus precluding triple helix formation, exhibit no hyperchromic absorbance change at 284 nm and no increase in melting temperature at 260 nm compared to reference **D**. The dissimilarities in melting behavior between e.g. **E** and **H** (hyperchromicities not detectable / detectable at 284 nm; unaltered / increased T_m's) strongly support the structures assigned to the branched ODNs.

In conclusion, by regioselective phosphitylation of nucleoside **2**, the phosphoramidite building block **3** was obtained and subsequently incorporated as branching point in novel branched ODNs. The synthetic strategy developed enables the synthesis of branched ODNs with arbitrary sequence length and base composition. The intra- and intermolecular hybridization properties of the novel branched ODNs are improved compared to the corresponding linear ODNs. We are currently further investigating this novel strategy for high affinity targeting of nucleic acids by branched ODNs by using other functionalized monomers as branching nucleotides.

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5. **2**: Anal. ($C_{31}H_{32}N_2O_8 \cdot 2.4H_2O$) C, H, N. ^{13}C NMR ($CDCl_3$) δ 19.7 (Me), 55.2 (OMe), 61.3 (C-5'), 73.7 (C-3'), 79.1, 80.5 (C-2', C-4'), 87.1 (C-1'), 89.6 (CAr_3), 101.7 (C-5), 113.4, 123.8, 127.0, 128.0, 128.2, 130.0, 135.3, 135.6, 136.2, 141.3, 149.5, 158.6 (Ar), 144.1 (C-6), 151.6 (C-2), 164.5 (C-4).
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7. Synthesis of *arabino*-oligonucleotides has been reported using an unprotected *arabino*-phosphoramidite. The secondary nature of this unprotected 2'-hydroxyl group induced the formation of minor amounts of 2'-*O*-amidite byproducts. Damha, M. J.; Usman, N.; Ogilvie, K. K. *Tetrahedron Lett.* **1987**, *28*, 1633.
8. **3**: ^{31}P NMR ($CDCl_3$) δ = 151.7 and 152.3 ppm. No trace of 2'-*O*-phosphitylated product was detected.
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10. The coupling yield of the modified monomer **3** was approximately 60 % (12 min coupling) compared to > 99 % for unmodified amidites (2 min couplings) as judged by the release of the dimethoxytrityl cation. The lower yield when incorporating **3** may be due to sterical hindrance from the 2'-substituents.
11. Acetic anhydride, 4-(*N,N*-dimethylamino)pyridine and 2,4,6-collidine in acetonitrile.
12. The coupling yield of this amidite (12 min coupling) was approximately 70 %.
13. The stepwise coupling yields during elongation of "branch A, B and C" were >99 %, when compared to the first monomer in the respective branch. For all unmodified monomers the standard 0.2 μmol scale synthetic cycle of the synthesizer was used.
14. Available from Cruachem Inc. The standard procedure includes detritylation.
15. The purity of the synthesized ODNs was confirmed by analytical reversed-phase HPLC.
16. Oligomer I: mass calcd. 6893.5 Da; mass found 6891.5 Da.
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18. Hybridization studies were carried out at pH 7.0 as previously reported. Prakash, G.; Kool, E. T. *J. Am. Chem. Soc.* **1992**, *114*, 3523.
19. We have obtained similar results for analogous branched ODNs containing a linker with one deoxycytidine (T_m=31 °C) and no linker (T_m=30 °C). Thus, these preliminary results do not reveal a conclusive influence from the length of the linker on the triple helix formation. Further experiments are underway to evaluate this aspect.

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