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Oligodeoxynucleotides containing amide-linked LNA-type dinucleotides: synthesis and high-affinity nucleic acid hybridization

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Received (in Cambridge, UK) 6th December 2001, Accepted 29th January 2002
First published as an Advance Article on the web 12th February 2002

Synthesis of three different amide-linked LNA-type dinucleotides and their incorporation into 9-mer and 17-mer oligodeoxynucleotides is described; compared to the reference DNA–RNA duplex, incorporation of one of the three dimers (5′-DNA*LNA dimer) induced significantly increased duplex thermostabilities.

In the chemical search for ideal antisense molecules, the exchange of the phosphordiester moiety by various amide-linked non-phosphorus internucleoside linkages has been studied.[1–7] Whereas amide linkages confer stability towards nucleolytic degradation, only a few have shown increased thermal stability of duplexes formed with RNA target strands.[1,2,6,7] Unfortunately, synthesis of the necessary building blocks for most of the more promising of the known amide-linked dinucleotides is rather troublesome requiring, *e.g.*, stereoselective introduction of C3′-alkyl substituents and/or C2′-substituents.[2,6,7] Among the more easily accessible amide-linked dinucleotides are several containing an additional atom in the internucleoside linkage compared to the natural C3′-O-P(=O)(OH)-O-CH_2-C4′ four-atom linkage,[3,4] *e.g.*, the T*T dimer[3] shown in Fig. 1 with an C3′-O-CH_2-CH_2-NH-C(=O)-C4′ linkage. This linkage was conveniently introduced by condensing two easily available thymidine building blocks, *i.e.* the deacetylated derivative of **1** and nucleoside **3** (Fig. 2).[3] The T*T dimer introduced once or twice into a 17-mer oligodeoxynucleotide sequence induced a small decrease in the T_m value compared to the corresponding unmodified DNA–DNA duplex (see Table 1, **ON16** and **ON17**).[3]

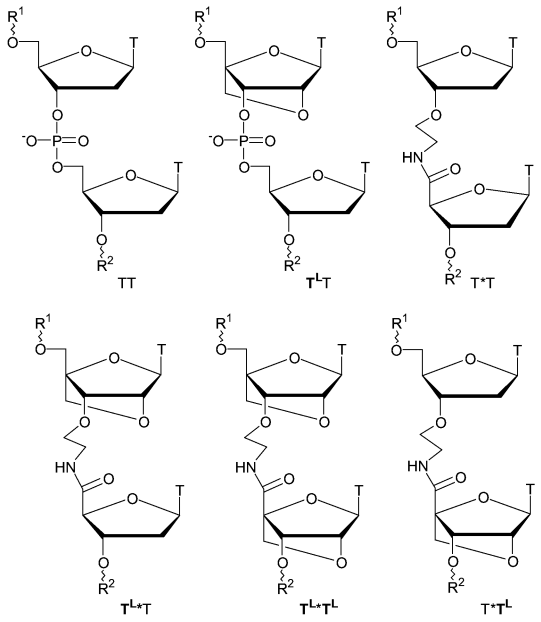

Fig. 1 Structures of a DNA*DNA phosphordiester dimer (TT), an LNA*DNA phosphordiester dimer (**T**$^{\mathbf{L}}$T), a DNA*DNA amide-linked dimer (T*T), an LNA*DNA amide-linked dimer (**T**$^{\mathbf{L}}$*T), an LNA*LNA amide-linked dimer (**T**$^{\mathbf{L}}$*T$^{\mathbf{L}}$) and a DNA*LNA amide-linked dimer (T*T$^{\mathbf{L}}$). T = thymin-1-yl.

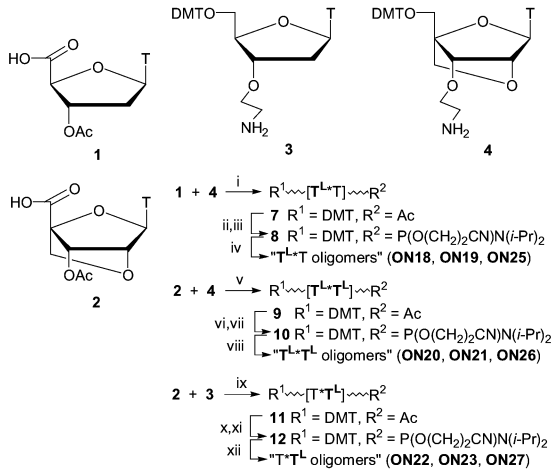

Fig. 2 *Reagents and conditions*: i), v), ix) DPPA, Et_3N, DMF [i) 64%], [v) 60%], [ix) 46%]; ii), vi), x) sat. methanolic ammonia [ii) 81%], [vi) 74%], [x) 75%]; iii), vii), xi) $ClP(O(CH_2)_2CN)N(i\text{-}Pr)_2$, DIPEA, DCM [iii) 34%], [viii) 31%], [xii) 50%]; iv), viii), xii) DNA synthesizer.

Table 1 Thermal denaturation experiments towards complementary single-stranded DNA and complementary single-stranded RNA[a]

Sequence (**ON**)[b]	T_m/°C DNA	T_m/°C RNA
5′-d(CACCAACTTCTTCCACA) (**13**)	57.5	57.5
5′-d(–C**T**$^{\mathbf{L}}$TCTTC–)[b] (**14**)	58.0 (+0.5)	61.0 (+3.5)
5′-d(–C**T**$^{\mathbf{L}}$TC**T**$^{\mathbf{L}}$TC–)[b] (**15**)	60.0 (+1.3)	65.0 (+3.8)
5′-d(–C[T*T]CTTC–)[b] (**16**)	[c](−2.5)	n.d.
5′-d(–C[T*T]C[T*T]C–)[b] (**17**)	[c](−1.5)	n.d.
5′-d(–C[**T**$^{\mathbf{L}}$*T]CTTC–)[b] (**18**)	49.5 (−8.0)	53.5 (−4.0)
5′-d(–C[**T**$^{\mathbf{L}}$*T]C[**T**$^{\mathbf{L}}$*T]C–)[b] (**19**)	43.0 (−7.3)	50.5 (−3.5)
5′-d(–C[**T**$^{\mathbf{L}}$*T$^{\mathbf{L}}$]CTTC–)[b] (**20**)	51.5 (−6.0)	57.5 (±0)
5′-d(–C[**T**$^{\mathbf{L}}$*T$^{\mathbf{L}}$]C[**T**$^{\mathbf{L}}$*T$^{\mathbf{L}}$]C–) (**21**)	47.5 (−5.0)	58.0 (+0.3)
5′-d(–C[T*T$^{\mathbf{L}}$]CTTC–)[b] (**22**)	59.0 (+1.5)	61.0 (+3.5)
5′-d(–C[T*T$^{\mathbf{L}}$]C[T*T$^{\mathbf{L}}$]C–)[b] (**23**)	61.0 (+1.8)	64.5 (+3.5)
5′-d(GTGTTTTGC) (**24**)	31.0	30.5
5′-d(GTG[**T**$^{\mathbf{L}}$*T]TTGC) (**25**)	18.0 (−13.0)	23.0 (−7.5)
5′-d(GTG[**T**$^{\mathbf{L}}$*T$^{\mathbf{L}}$]TTGC) (**26**)	17.5 (−13.5)	31.0 (+0.5)
5′-d(GTG[T*T$^{\mathbf{L}}$]TTGC) (**27**)	35.0 (+4.0)	39.5 (+9.0)

[a] Melting temperatures [T_m values (ΔT_m values per modification)] measured as the maximum of the first derivative of the melting curve (A_{260} *vs.* temperature) recorded in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 1.5 μM concentrations of the two strands; A = adenine monomer, C = cytosine monomer, G = guanine monomer, T = thymine monomer; See Fig. 1 for structures of **T**$^{\mathbf{L}}$, T*T, **T**$^{\mathbf{L}}$*T, **T**$^{\mathbf{L}}$*T$^{\mathbf{L}}$ and T*T$^{\mathbf{L}}$; 'n.d.' denotes 'not determined'. [b] For the sequences **ON14**–**ON23**, only the central fragment is shown but their full base sequence is identical to the 17-mer unmodified sequence **ON13**. [c] ΔT_m values taken from ref. 3. The absolute T_m values of **ON16** and **ON17**[3] are not directly comparable to those recorded herein because of the use of slightly different buffer conditions.

DOI: 10.1039/b111000d

Stimulated by the unprecedented binding affinity of LNA (locked nucleic acids),‡ [8–11] we decided to evaluate the effect of introducing locked furanose conformations in oligonucleotides containing non-phosphorus linkages. Due to its relatively straightforward synthesis we chose to study the T*T system (Fig. 1) as the first amide-linked LNA-type oligonucleotide, more precisely the three dimers $\mathbf{T^L}$*T, $\mathbf{T^L}$*$\mathbf{T^L}$ and T*$\mathbf{T^L}$ (Fig. 1). To synthesize the three amide-linked phosphoramidite derivatives **8**, **10** and **12**, the four monomeric building blocks **1**,§ **2**,§ **3**[3] and **4**§ were prepared and condensed two by two under mild neutral conditions using diphenyl phosphorazidate (DPPA) as condensing agent following procedures described previously[3] furnishing the three amide-linked dinucleosides **7**, **9** and **11**. Subsequently, deacetylation and phosphitylation afforded the dimeric phosphoramidite building blocks **8**, **10** and **12**¶ suitable for incorporation of dimers $\mathbf{T^L}$*T, $\mathbf{T^L}$*$\mathbf{T^L}$ and T*$\mathbf{T^L}$, respectively, into oligonucleotides (Fig. 2).

Oligonucleotides **ON13**-**ON15** and **ON18**-**ON27** (Table 1) were synthesized in 0.2 μmol scale on an automated DNA synthesizer using the phosphoramidite approach.[12] Standard procedures were used but with modifications as described previously[13] when coupling the LNA-T amidite[9] [leading to incorporation of '$\mathbf{T^L}$' (**ON14** and **ON15**)], and amidites **8**, **10** and **12**. The coupling yields for all types of amidites were ≥98% with coupling times of 2 min (deoxynucleotide amidites), 10 min (LNA-T amidite and amidites **8** and **12**), and 20 min (amidite **10**). Cleavage from the solid support and removal of the protection groups was performed using concentrated ammonia (55 °C, 12 h) to give **ON13**–**ON15** and **ON18**–**ON27** after precipitation from ethanol. Satisfactory purities (>80%) were verified by capillary gel electrophoresis and the compositions by MALDI-MS analysis.‖

The hybridization properties towards complementary single-stranded DNA and RNA strands were evaluated by thermal denaturation studies (Table 1). Relative to the reference DNA–DNA and DNA–RNA duplexes, the data shown in Table 1 for **ON13**–**ON17** reveal a limited decrease in T_m value resulting from incorporation of the DNA*DNA dimer (not evaluated towards RNA), and increased T_m values towards DNA or RNA resulting from incorporation of the LNA-T monomer $\mathbf{T^L}$. The data obtained for **ON18**–**ON23** demonstrate strikingly different effects of the three amide-linked dimers $\mathbf{T^L}$*T (**ON18** and **ON19**), $\mathbf{T^L}$*$\mathbf{T^L}$ (**ON20** and **ON21**) and T*$\mathbf{T^L}$ (**ON22** and **ON23**). Thus, with an LNA-type monomer at the 5'-end of the dimer ($\mathbf{T^L}$*T), substantially decreased binding affinity towards both DNA and RNA was obtained. The dimer with two LNA-type monomers ($\mathbf{T^L}$*$\mathbf{T^L}$) likewise induced a significant decrease in T_m value towards DNA but had no effect when hybridized towards RNA. Finally, a moderate affinity increase towards DNA, and a significant affinity increase towards RNA (ΔT_m/mod = +3.5 °C), resulted from incorporation once or twice of the dimer with one LNA-type monomer positioned at the 3′-end of the dimer (T*$\mathbf{T^L}$). The results obtained for the 9-mer sequence (**ON24**–**ON27**) follow the same trends, but due to the reduced sequence length the effects per modification were more pronounced (*e.g.*, ΔT_m/mod = +4.0 °C and + 9.0 °C for T*$\mathbf{T^L}$). For all oligomers containing one of the three LNA-type amide-linked dimers, a weak ($\mathbf{T^L}$*T and T*$\mathbf{T^L}$) or moderate ($\mathbf{T^L}$*$\mathbf{T^L}$) RNA-selectivity was observed.

An important result from the present work is that it is possible to convert a destabilizing non-natural amide internucleoside linkage into a strongly stabilizing one by exchanging a DNA monomer with an LNA-type monomer [with a locked C3′-*endo* type furanose conformation] in the 3′-end of the dimer. It is furthermore noteworthy that this stabilization is accomplished despite the fact that the linker used herein contains an additional atom compared to the natural phosphordiester linker, and that incorporation of the LNA-type monomer in the 5′-end of the dimer is strongly destabilizing. Previous studies on the effect on introducing 2′-substituents (2′-fluoro and 2′-OMe substituents; and thus conformational restriction) in the 5′-end positioned monomer and/or the 3′-end positioned monomer of optimized amide-linked dinucleosides likewise showed that the positive effect of introducing 2'-substituents is by far larger at the 3′-end of the dimer than at the 5′-end.[6,7] However, introduction of these 2′-substituted analogues at the 5′-end of the dimer was reported to be neutral or even slightly stabilizing.[6,7] The highly variable effects observed for the three different dimers ($\mathbf{T^L}$*T, $\mathbf{T^L}$*$\mathbf{T^L}$ and T*$\mathbf{T^L}$) support our previous conclusion that conformational tuning of neighboring unmodified deoxynucleotide monomers by the presence of one (or several) LNA monomers in an oligomer is much more pronounced in the 3′-direction than in the 5′-direction.[14] Thus, no favorable conformational tuning in the 5′-direction is apparently possible for the LNA*DNA ($\mathbf{T^L}$*T) dimer or the LNA*LNA ($\mathbf{T^L}$*$\mathbf{T^L}$) dimer. We are continuing our studies on the hybridization properties and structural characteristics of oligomers containing the three LNA-type amide-linked dimers introduced herein.

We thank The Danish Research Agency and The Danish National Research Foundation for financial support, Ms Britta M. Dahl for oligonucleotide synthesis, and Dr Michael Meldgaard, Exiqon A/S, for MALDI-MS analysis.

Notes and references

† A research center funded by The Danish National Research Foundation for studies on nucleic acid chemical biology.

‡ We have defined LNA as an oligonucleotide containing one or more conformationally locked 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide monomer(s) ('LNA monomer(s)').

§ Synthesis of compounds **1**, **2** and **4** will be reported elsewhere.

¶ ^{31}P NMR data: δ (CH_3CN) 150.4, 148.8 (amidite **8**); δ ($CHCl_3$) 149.7, 149.4 (amidite **10**); δ ($CHCl_3$) 149.5, 149.2 (amidite **12**).

‖ MALDI-MS ([M − H]$^-$ measured/[M − H]$^-$ calculated): 5038/5038 (**ON18**), 5043/5043 (**ON19**), 5066/5066 (**ON20**), 5098/5099 (**ON21**), 5038/5042 (**ON23**), 2739/2740 (**ON25**), 2766/2768 (**ON26**), 2737/2740 (**ON27**).

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