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Conformationally controlled high-affinity targeting of RNA or DNA by novel 2′-amino-DNA/LNA mixmers and pyrenyl-functionalized 2′-amino-DNA

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9-Mer DNA sequences containing 2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA monomers display significantly increased affinity towards DNA complements whereas the corresponding 2′-amino-DNA monomer has a detrimental effect on duplex stability. These effects are efficiently reversed by incorporation of four LNA nucleotides inducing a B-DNA to A-DNA conformational change.

High-affinity targeting of RNA can be achieved using chemically modified oligonucleotides (ONs) that structurally mimic RNA with furanose rings restricted or locked in *N*-type conformations.[1,2] The development of ON analogues capable of high-affinity targeting of DNA has proven significantly more difficult, but continued efforts in this direction are needed to improve the fidelity of DNA diagnostics and to enable *in vivo* targeting of DNA. 2′-Amino-DNA nucleotides are known to adopt an *S*-type furanose ring conformation and therefore to conformationally mimic DNA nucleotides, but their incorporation into DNA sequences destabilizes duplexes formed with DNA or RNA complements.[3] However, the ease of synthesis of 2′-amino-2′-deoxyuridine derivative **1**,[4] the improved DNA-binding properties resulting after incorporation of a 2′-amino-2′-deoxy-2′-*N*-(pyren-1-ylpropanoyl)uridine monomer into a DNA sequence,[5] the likely protonation of the N2′ atom at neutral pH, and the opportunity of mixing 2′-amino-DNA and LNA monomers, prompted us to study the effect of the 2′-amino-2′-deoxyuridine monomer **X** and the novel 2′-amino-2′-deoxy-2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)uridine monomer **Z** in DNA and LNA sequence contexts (Fig. 1). As LNA monomers are known to induce *N*-type furanose ring conformations of neighboring DNA monomers in LNA/DNA mixmers and a B-DNA to A-DNA conformational change,[6] we were aiming at studying the effect of monomers **X** and **Z** in sequences adopting a B-DNA conformation (Table 1, **ON3**, **ON5** and **ON8**–**ON11**) or an A-DNA conformation (Table 1, **ON4** and **ON6**).

The novel phosphoramidite building block **6** (Scheme 1), suitable for incorporation of monomer **Z** into ONs, was synthesized starting from the known uridine derivative **1**.[4] Reductive alkylation of **1** by reaction with 1-pyrenecarboxaldehyde and sodium cyanoborohydride furnished 2′-*N*-pyren-1-ylmethyl derivative **2** that was detritylated using 2% $Cl_2CHCOOH$ in CH_2Cl_2 in the presence of triethylsilane to afford nucleoside **3**. To avoid the need of additional N2′-protection during ON synthesis, the tertiary amine derivative **4** was obtained by N2′-methylation using Eschweiler–Clarke conditions (formaldehyde in formic acid). The best overall yield was obtained by performing the reductive alkylation on the O5′-dimethoxytritylated nucleoside **1** and the N2′-methylation under acidic conditions, with the latter step preceded by a controlled detritylation step. The 5′-*O*-dimethoxytrityl group was reintroduced to give nucleoside **5**‡ followed by standard O3′-phosphitylation to afford the desired phosphoramidite **6**§ that was used for automated solid phase synthesis of **ON5**, **ON6**, **ON10** and **ON11**. Incorporation of the monomer **X** during the synthesis of **ON3**, **ON4**, **ON8** and **ON9** was accomplished using a known 2′-*N*-trifluoroacetyl phosphoramidite derivative of 2′-amino-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)uridine.[7] The stepwise coupling yield for all phosphoramidite derivatives was >99% using 1*H*-tetrazole as catalyst and coupling times of 2 min (DNA phosphoramidites), 6 min (LNA phosphoramidites[8]) and 10 min (**6** and the phosphoramidite[7] for incorporation of the monomer **X**). Following standard deprotection, purification and work-up, the composition of the synthesized ONs was confirmed by MALDI-MS analysis and the purity by capillary gel electrophoresis (or HPLC).

The hybridization properties of the synthesized 9-mer ONs were evaluated by measuring thermal denaturation temperatures (T_m values) against complementary DNA and RNA at medium

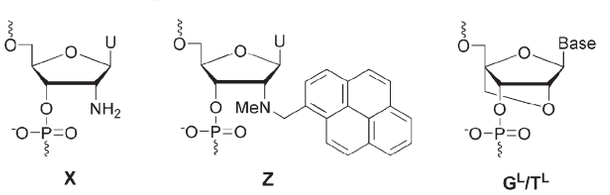
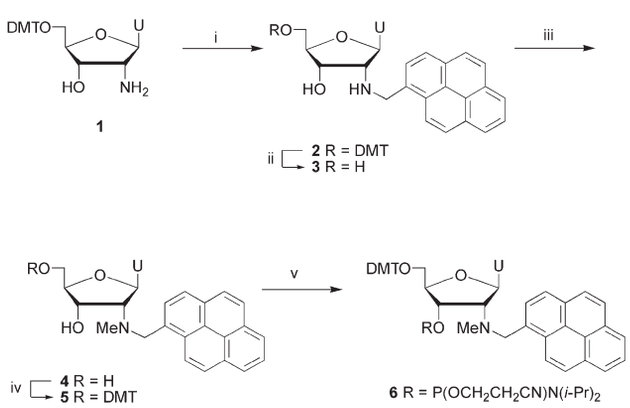


Fig. 1 Structures of nucleotide monomers: 2′-amino-DNA (**X**), 2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA (**Z**), G-LNA (**G**$^{\mathbf{L}}$, Base = guanin-9-yl), T-LNA (**T**$^{\mathbf{L}}$, Base = thymin-1-yl); U = uracil-1-yl; the short notations shown are used in Table 1.



Scheme 1 *Reagents and conditions*: i) 1-pyrenecarboxaldehyde, MeOH, $NaCNBH_3$, 0 °C then rt, 16 h, 70%; ii) 2% $Cl_2CHCOOH$ in CH_2Cl_2, Et_3SiH, rt, 6 h, 68%; iii) CH_2O, HCOOH, 80 °C, 1.5 h, 53%; iv) DMTCl, DMAP, pyridine, rt, 12 h, 65%; v) 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, DIPEA, CH_2Cl_2, rt, 12 h, 43%. U = uracil-1-yl; DMT = 4,4′-dimethoxytrityl.

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Table 1 Thermal denaturation experiments (T_m values shown) for **ON1**–**ON11** towards DNA and RNA complements[a]

		110 mM [Na+]			710 mM [Na+]	
		DNA	RNA	MM-DNA[b]	DNA	RNA
ON1	5′-GTGTTTTGC	28	29		37	36
ON2	5′-G^{L}GT^{L}TT^{L}TG^{L}C	42	57		50	65
ON3	5′-GTGT**X**T**X**GC	<10	<10		12	18
ON4	5′-G^{L}GT^{L}**X**T^{L}**X**G^{L}C	31	51		43	60
ON5	5′-GTGT**Z**T**Z**GC	34	27	26/34/30	40	31
ON6	5′-G^{L}GT^{L}**Z**T^{L}**Z**G^{L}C	25	39		32	44
ON7	5′-GTGATATGC	27	25	11/19/12[8]	34	31
ON8	5′-GTGA**X**ATGC	19	20	<10/<10/<10	24	27
ON9	5′-G**X**GA**X**A**X**GC	<10	<10		<10	<10
ON10	5′-GTGA**Z**ATGC	42	29	22/38/31	47	33
ON11	5′-G**Z**GA**Z**A**Z**GC	51	28	32/48/41	56	29

[a] Melting temperatures (T_m values/°C) 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) or in high salt buffer (10 mM sodium phosphate, 700 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 1.0 μM concentrations of the two complementary strands assuming identical extinction coefficients for modified and unmodified nucleotides, and using a micromolar extinction coefficient of 22.4 for a pyrene unit; A = adenin-9-yl DNA monomer, C = cytosin-1-yl DNA monomer, G = guanin-9-yl DNA monomer, T = thymin-1-yl DNA monomer; see Fig. 1 for structures of **T^{L}**, **G^{L}**, **X** and **Z** monomers. [b] In the column 'MM-DNA' are listed T_m values recorded for mis-matched DNA target strands containing a single mis-matched nucleotide; the values are listed in the following order (indicating the mis-matched nucleotide in the central position of the target DNA): C/G/T; MALDI-MS *m/z* ([M−H]; found/calc.): **ON3**, 2735/2737; **ON4**, 2846/2849; **ON5**, 3196/3194; **ON6**, 3306/3306; **ON8**, 2753/2754; **ON9**, 2758/2756; **ON10**, 2982/2982; **ON11**, 3438/3441.

(110 mM [Na+]) or high (710 mM [Na+]) salt conditions in two different 9-mer sequence contexts (Table 1). **ON1** and **ON7** are DNA reference strands and **ON2** is an LNA (DNA/LNA mixmer) reference strand. As expected, a general increase in T_m value was observed by changing from medium to high salt conditions, and all changes in T_m values discussed below therefore refer to medium salt conditions unless otherwise stated. Introduction of four LNA nucleotides induces a typical increase in thermal stability (**ON2** relative to **ON1**), especially pronounced towards complementary RNA (+14 °C towards DNA and +28 °C towards RNA). As expected,[3] introduction of two 2′-amino-DNA monomers **X** (**ON3**) induces a strong decrease in duplex thermal stability relative to DNA reference **ON1**, and only at high salt conditions were melting transitions above 10 °C detected. Remarkably, by exchanging four DNA monomers with LNA monomers around the two 2′-amino-DNA monomers **X** (**ON4**), the affinity-decreasing effect of the latter is counter-balanced, and thermal stabilities approaching those of the LNA reference **ON2**, especially towards the RNA complement, are achieved. An important implication of these results is that mixmers of LNA, 2′-amino-DNA and DNA monomers are promising antisense molecules. These novel molecular constructs enable innovative medicinal chemistry focused on variations in overall molecular charge, lipophilicity and conjugation chemistry towards further improving the antisense potency of LNA.[9,10]

Strikingly different results were obtained for the novel 2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA monomer **Z**. In a DNA sequence context (**ON5**), favorable binding to the DNA complement is evident, both relative to the DNA binding of the DNA reference **ON1**, and relative to the RNA binding of **ON5**. This binding preference is reversed upon exchange of four DNA monomers with LNA monomers around the two **Z** monomers (**ON6**), and the affinity-decreasing effect of −9 °C towards the DNA complement resulting from the introduction of four LNA monomers is unprecedented pointing to a remarkable conformational influence on hybridization.

The predominance of the *S*-type furanose ring conformation of 2′-amino-DNA monomers[3] was confirmed herein for nucleosides **1** ($J_{1',2'}$ = 7.1 Hz) and **5** ($J_{1',2'}$ = 6.5 Hz). It can therefore be anticipated that the furanose rings of monomers **X** and **Z** in the DNA contexts (**ON3**, **ON8**, **ON9**, and **ON5**, **ON10**, **ON11**, respectively) likewise adopt *S*-type conformations. The detrimental effect of the 2′-amino-DNA monomers on the thermal stability of the duplexes has earlier been explained by steric hindrance imposed by the presence of the 2′-amino substituent when attached to a nucleotide adopting an *S*-type furanose ring conformation.[3] The strong DNA binding obtained for **ON5** can be explained by stabilizing interactions of the pyrenyl moieties of the **Z** monomers with the duplex (groove interaction or intercalation) corroborated by similar effects obtained for ONs containing a 2′-*O*-(pyren-1-ylmethyl)-RNA monomer,[11] a 1-*O*-(pyren-1-ylmethyl)glycerol monomer,[12,13] or a pyren-1-ylcarbonyl-functionalized 4′-*C*-piperazinomethyl-DNA monomer.[14] This stabilizing interaction is most pronounced when the duplex adopts a B-type conformation, *i.e.* when both strands are DNA (or DNA-mimicking) sequences. Incorporation of the four RNA-mimicking LNA monomers (**ON4** and **ON6**) is expected to induce a conformational shift of the furanose rings of monomers **X** and **Z** towards *N*-type conformations and therefore also an RNA-mimicking A-DNA conformation of **ON4** and **ON6** when participating in duplex formation. Apparently, this conformational change from B- to A-DNA relieves the steric hindrance of the 2′-amino group and induces the very satisfactory binding properties of the LNA/DNA/2′-amino-DNA mixmer **ON4**, especially towards RNA. However, for **ON6** containing two 2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA monomers **Z** the change from B- to A-DNA duplex conformation (**ON5** to **ON6**), impeding the stabilizing interaction between the pyrene units and the duplex, leads to decreased affinity towards DNA.

The efficient DNA targeting by the 2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA/DNA mixmer **ON5** prompted us to synthesize **ON8**–**ON11** (Table 1). Thermal denaturation experiments furnished results pointing in similar directions as those obtained for **ON3** and **ON5**. Thus, incorporation of one or three monomers **X** induced strong destabilization whereas one or three monomers **Z** enforced significant stabilization of duplexes with complementary DNA. Relative to the corresponding DNA reference **ON7**, remarkable increases in T_m values per **Z** monomer were obtained (+15 and +8 °C). The unusual DNA-targeting ability of 2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA/DNA mixmers is underlined by the fact that the thermal stability of the duplex **ON11**:DNA is higher than that of the corresponding duplex formed between an LNA/DNA mixmer (three LNA monomers) and DNA (T_m = 44 °C).[8]

For practical uses the base-pairing selectivity is another very important aspect, and we therefore determined the thermal denaturation temperatures of duplexes formed between

ON5, **ON10** and **ON11** and mis-matched DNA complements in a comparison with DNA reference **ON7** and the 2′-amino-DNA/DNA mixmer **ON8**. Watson–Crick base-pairing selectivity was observed for **ON7**[8] and **ON8**, but not for **ON5**, as for the latter similar T_m values were obtained against A (matched) and G nucleotides in the central position of the complement and only relatively weakly decreased T_m values against C and T nucleotides). It is, however, encouraging that the base-pairing selectivity for **ON10** and **ON11** containing one and three **Z** monomer(s), respectively, follows the Watson–Crick pattern with the least discrimination towards the mis-matched complement with a G nucleotide in the central position (corresponding to a wobble base pair), a similar trend to that seen for the DNA reference **ON7**.

The generality of the pronounced affinity-enhancing effect of monomer **Z** needs to be further evaluated, but the preliminary data presented herein point to a stronger effect in a purine-rich sequence context (**ON10** and **ON11**) than in a pyrimidine-rich one (**ON5**). The differences in base-pairing selectivity suggest the possibility of different modes of interaction of the pyrene moieties, and we therefore measured steady-state fluorescence emission spectra. Despite similar absorbances at the excitation wavelength (340 nm), a pronounced fluorescence quenching is observed (Fig. 2) for **ON5** but not for **ON10** or **ON11** (both for the single-stranded state and for duplexes with DNA). One possible explanation for these striking differences is that intercalation of the pyrene units leads to strong fluorescence quenching in the case of **ON5**, whereas the pyrene moieties are engaged in groove interactions in the case of **ON10** and **ON11**.

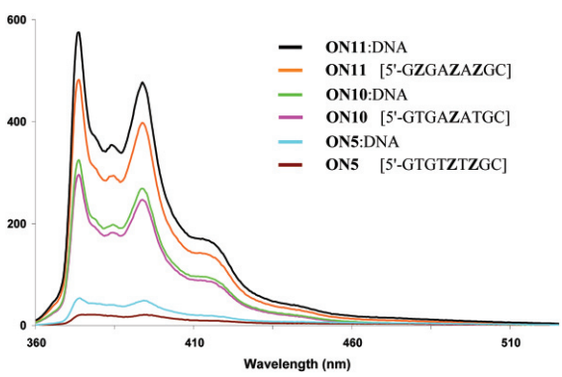

Fig. 2 Steady-state fluorescence emission spectra (19 °C; medium salt buffer as described in the footnote to Table 1; excitation at 340 nm).

In summary, high-affinity nucleic acid targeting under conformational control has been demonstrated for 2′-amino-DNA/LNA mixmers in A-type duplexes (RNA targeting) and for 2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA/DNA mixmers in B-type duplexes (DNA targeting). These results enable optimization of RNA targeting *in vivo* by novel antisense molecules composed of 2′-amino-DNA/LNA mixmers, and stimulate further research towards studying and exploring 2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA/DNA mixmers for efficient DNA targeting *in vitro* and *in vivo*.

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Notes and references

‡ 2′-Amino-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)uridine (**5**). δ_C ($CDCl_3$) 163.2, 158.8, 158.8, 150.4, 149.9, 144.3, 140.6, 136.1, 135.3, 135.1, 131.4, 131.3, 130.8, 130.5, 130.3, 130.2, 129.9, 128.5, 128.3, 128.1, 127.7, 127.4, 127.3, 126.2, 125.5, 125.2, 124.8, 124.6, 123.8, 123.1, 113.4, 103.1, 87.4, 85.1, 84.8, 70.7, 70.5, 63.7, 58.9, 55.4, 40.1. MALDI-HRMS *m/z* 796.2974 ($[M + Na]^+$, $C_{48}H_{43}N_3O_7Na^+$ calc. 796.2993).

§ 2′-Amino-3′-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)uridine (**6**). δ_P ($CDCl_3$) 151.7, 150.4. MALDI-HRMS *m/z* 996.4102 ($[M + Na]^+$, $C_{57}H_{60}N_5O_8PNa^+$ calc. 996.4072).

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