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Universal hybridization using LNA (locked nucleic acid) containing a novel pyrene LNA nucleotide monomer

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A novel pyrene LNA nucleotide monomer is shown to mediate universal hybridization when incorporated into a DNA strand or a 2′-OMe-RNA/LNA chimeric strand. For the latter, high-affinity universal hybridization without compromising the base-pairing selectivity of bases neighbouring the universal pyrene LNA nucleotide monomer is documented.

Currently, there is much focus on the development of chemically modified nucleotide monomers for universal hybridization, *i.e.* so-called universal bases able to bind isoenergically with each of the natural bases.[1] The main applications of these universal bases are as primers for degenerate PCR reactions or as hybridization probes. Promising universal bases with a 2-deoxy-β-D-ribofuranosyl moiety have been reported, *e.g.* 3-nitropyrrole,[2] 5-nitroindole,[3] pyrene,[4] isocarbostyril[5] and 8-aza-7-deazaadenine[6] derivatives. While incorporation of one of these monomers into a DNA strand induces little variation in the melting temperature (T_m value) when placed opposite the four natural DNA bases (ΔT_m ~1–3 °C), decreases in the T_m value of between 4 to 10 °C per universal nucleotide monomer incorporated compared to the corresponding fully complementary reference DNA–DNA duplex are typical.[1–7] Therefore, improved binding affinity beside applicability in PCR-based applications have been considered desirable properties of an ideal universal base.[1,5]

Stimulated by the work of Kool and collaborators on hybridization using non-polar aromatic moieties as replacements of the natural bases,[4,8] we became interested in studying LNA (locked nucleic acid)[9–12] derivatives containing non-polar aromatic moieties.† The hybridization properties of LNA are characterised by very high binding affinity and strong Watson–Crick discrimination[9–12] both as fully modified LNA and as mix-meric LNA also containing, *e.g.* DNA,[9–12] RNA[13] or 2′-OMe-RNA monomers.[14] Matray and Kool showed that the pyrene nucleoside analogue **Py** (Fig. 1) when incorporated into a DNA strand paired in a universal way with the four natural bases with moderately decreased thermal affinity (−4.5 to −6.8 °C). This behaviour was explained by intercalation of the pyrene moiety within the helix.[4] As both synthetic work[15] and NMR studies[16] have indicated the importance of the nucleobases for structural organisation and thus the remarkable binding affinity of LNA, we decided to synthesise LNA containing the derivatives **Ph^L** and **Py^L** (Fig. 1, Table 1),[17] both based on the 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl moiety known to adopt a locked C3′-*endo* RNA-like furanose conformation.[9–11]

Initially, the hybridization of the oligonucleotides **ON1**–**ON6** (Table 1) towards four 9-mer DNA targets with the central base being each of four natural bases was studied by thermal denaturation experiments.‡ Compared to the DNA reference **ON1**,[10] introduction of one abasic LNA monomer **Ab^L** (**ON2**) has earlier been reported to prevent the formation of a stable duplex above 0 °C (only evaluated with adenine as the opposite base).[15] With the phenyl monomer **Ph^L** (**ON3**), thermal denaturation with T_m values in the range of 5–12 °C was observed. Thus, compared to **Ab^L**, the phenyl moiety stabilises the duplexes, but universal hybridization is not achieved—a preference for an adenine complement being observed instead. In addition, significant destabilisation compared to the **ON1**–DNA reference was observed.

The pyrene LNA nucleotide **Py^L** (**ON4**) displays more encouraging properties. Firstly, the binding affinity towards all four complements is increased compared to **ON3** (containing **Ph^L**). Secondly, universal hybridization is induced as shown by the four T_m values all being within the range 17–19 °C. With

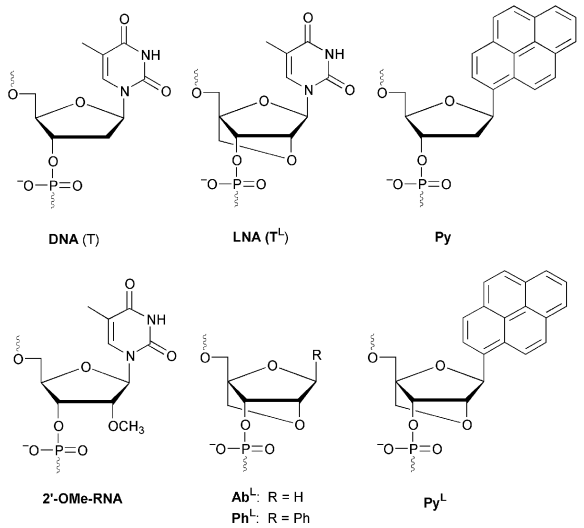

Fig. 1 Structures of selected nucleotide monomers: DNA (T), LNA (**T^L**), pyrenyl DNA (**Py**), 2′-OMe-RNA [2′-OMe(T)], abasic LNA (**Ab^L**), phenyl LNA (**Ph^L**), and pyrenyl LNA (**Py^L**). The short notations shown are used in Tables 1 and 2. For DNA, LNA and 2′-OMe-RNA, the thymine monomers are shown as examples.

Table 1 Thermal denaturation experiments (T_m/°C values shown) for **ON1**–**ON6** towards DNA complements 3′-d(CACT**Y**TACG) with each of the four natural bases in the central position[a]

		Y			
		A	C	G	T
ON1	5′-d(GTGATATGC)[10]	28	11	12	19
ON2	5′-d(GTGA**Ab^L**ATGC)[15]	< 3	nd	nd	nd
ON3	5′-d(GTGA**Ph^L**ATGC)	12	5	6	7
ON4	5′-d(GTGA**Py^L**ATGC)	18	17	18	19
ON5	2′-OMe(GTGATATGC)	35	14	19	21
ON6	2′-OMe(G**T^L**GA**Py^L**A**T^L**GC)	39	38	37	40

[a] Melting temperatures (T_m/°C values) 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^L**, **Ab^L**, **Ph^L** and **Py^L**; DNA sequences are shown as d(sequence) and 2′-OMe-RNA sequences as 2′-OMe(sequence); 'nd' denotes 'not determined'.

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respect to universal hybridization, **Py^L** thus parallels the pyrene DNA derivative **Py**,[4] but the decrease in thermal stability compared to the **ON1**–DNA reference is more pronounced for **Py^L** (~10 °C) than reported for **Py** (~5 °C in a 12-mer polypyrimidine DNA sequence[4]). It therefore appears that stacking (or intercalation) by the pyrene moiety is not favoured by the conformational restriction of the furanose ring of **Py^L**, although a comparison of the thermal stabilities of **ON2**, **ON3** and **ON4** strongly indicates interaction of the pyrene moiety within the helix.

The effect of **Py^L** could be different in an RNA-like strand, and we therefore synthesised **ON5** and **ON6**, the former being composed entirely of 2′-OMe-RNA monomers and the latter of six 2′-OMe-RNA monomers, two LNA thymine monomers **T^L**, and one central LNA pyrene monomer **Py^L**. A sequence corresponding to **ON6** but with three **T^L** monomers has earlier been shown to form a duplex with complementary DNA of very high thermal stability.[14] **ON6** is therefore suitable for evaluation of the effect of introducing high-affinity monomers around a universal base. As seen in Table 1, the 2′-OMe-RNA reference **ON5** binds to the DNA complement with slightly increased thermal stability and conserved Watson–Crick discrimination (compared to the DNA reference **ON1**). Indeed, the 2′-OMe-RNA/LNA chimera **ON6** displays universal hybridization behaviour as revealed from the four T_m values (37, 38, 39 and 40 °C). All four T_m values obtained for **ON6** are higher than the T_m values obtained for the two fully complementary reference duplexes **ON1**–DNA (T_m = 28 °C) and **ON5**–DNA (T_m = 35 °C). These data demonstrate that the pyrene LNA monomer **Py^L** displays universal hybridization behaviour both in a DNA context (**ON4**) and in an RNA-like context (**ON6**), and that the problem of decreased affinity of universal hybridization probes can be solved by the introduction of high-affinity monomers, *e.g.* 2′-OMe-RNA and/or LNA monomers.

Very recently, the effect of the universal 3-nitropyrrole DNA nucleotide on the base-pairing selectivity of neighbouring natural bases was for the first time systematically studied.[7] Reduced discriminatory ability was demonstrated in some cases suggesting caution when using this universal base in hybridization probes.[7] We therefore performed a systematic thermal denaturation study with **ON6** (Table 2). For each of the four DNA complements (monomer **Y** = A, C, G or T) used in the study shown in Table 1, **ON6**, containing a central pyrene LNA monomer **Py^L**, was hybridised with all four base combinations in the neighbouring position towards 3′-end of **ON6** (monomer **Z** = A, C, G or T, monomer **X** = T) and the same towards the 5′-end of **ON6** (monomer **X** = A, C, G or T, monomer **Z** = T). In all eight subsets of four data points, satisfactory to excellent Watson–Crick discrimination was observed between the match and the three mismatches (ΔT_m values in the range 5–25 °C).

Table 2 Thermal denaturation experiments (T_m/°C values shown) to evaluate the base-pairing selectivity of the bases neighbouring the universal pyrene LNA monomer **Py^L** in the 2′-OMe-RNA/LNA chimera **ON6**. In the target strand [3′-d(CAC**XYZ**ACG)], the central three bases **XYZ** are varied among each of the four natural bases[a]

XYZ	T_m/°C	XYZ	T_m/°C	XYZ	T_m/°C	XYZ	T_m/°C
TAA	26	TCA	22	TGA	22	TTA	29
TAC	26	TCC	29	TGC	26	TTG	31
TAG	24	TCG	24	TGG	30	TTC	32
TAT	*39*	TCT	*38*	TGT	*37*	TTT	*40*
AAT	18	ACT	27	AGT	22	ATT	28
CAT	30	CCT	31	CGT	27	CTT	35
GAT	14	GCT	28	GGT	16	GTT	27
TAT	*39*	TCT	*38*	TGT	*37*	TTT	*40*

[a] See caption below Table 1 for abbreviations and conditions used; the data for matched neighbouring bases (**X** = **Z** = T) are shown in italic.

The results reported herein have several implications for the design of probes for universal hybridization. Firstly, universal hybridization is possible with a conformationally restricted monomer as demonstrated for the pyrene LNA monomer. Secondly, universal hybridization behaviour is feasible in an RNA context. Thirdly, the binding affinity of probes for universal hybridization can be increased by the introduction of high-affinity monomers without compromising the base-pairing selectivity of bases neighbouring the universal base.§

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Notes and references

† We have defined LNA as an oligonucleotide containing one or more conformationally locked 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide monomer(s).[9]

‡ MALDI-MS ([M − H]$^-$; found/calcd.: **ON3** 2731/2733; **ON4** 2857/2857; **ON6** 3094/3093). The purity of **ON3**, **ON4** and **ON6** was verified as >80% by capillary gel electrophoretic analysis.

§ We are currently evaluating chimeric oligonucleotides containing pyrene and other known universal bases attached to various backbones (*e.g.* LNA-type monomers, ribofuranose monomers or deoxyribose monomers in 2′-OMe-RNA–LNA oligos). One purpose of these further experiments is to evaluate the possibility of obtaining similar results as for the 2′-OMe-RNA/LNA oligo **ON6** at a lower cost, *e.g.* by substituting **Py^L** with a pyrenyl-2′-OMe-ribonucleotide monomer.

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17 The synthesis of *C*-nucleosides corresponding to **Ph^L** and **Py^L**, as well as their oligomerization will be described elsewhere; B. R. Babu, A. K. Prasad and J. Wengel, manuscript in preparation.