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DNA and LNA oligonucleotides containing N2′-functionalised derivatives of 2′-amino-2′-deoxyuridine

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Abstract—Synthesis of various N-acylated derivatives of 2′-amino-2′-deoxyuridine is described together with their incorporation into DNA and LNA oligonucleotides using the phosphoramidite approach on an automated DNA synthesizer. The thermal stabilities of duplexes formed by these 2′-amino-DNA-modified DNA or LNA/DNA chimeric strands and complementary DNA or RNA strands have been studied. Introduction of LNA monomers around the functionalised 2′-amino-DNA modifications results in reversal of the affinity-decreasing effect of the latter. This represents a novel general approach for design and synthesis of high-affinity functionalised oligonucleotides for biotechnological or medicinal applications.

Chemical modification of oligonucleotides (ONs) is necessary for their potential application as gene silencing agents.[1,2] The sugar moiety of the monomeric nucleotides, particularly the 2′-position, is an attractive site for modification, and conjugation of various lipophilic units to antisense ONs to optimize their pharmacokinetic properties is being intensively studied.[3] Among modified nucleotides, functionalised 2′-amino-2′-deoxynucleosides are attractive as the 2′-amino group offers a conjugation site for stable and chemoselective attachment of various entities.[4–17] Such 2′-amino-DNA nucleotides adopt a DNA-mimicking S-type furanose ring conformation but destabilise duplexes formed with DNA and RNA complements when incorporated into DNA strands.[18] A similar destabilising effect has in many cases been observed also for functionalised 2′-amino-DNA nucleotides.[4,6,10,15]

It is well established that ONs containing either LNA or 2′-amino-LNA nucleotides display high binding affinity towards complementary DNA or RNA strands,[1,19,20] and moreover that the 2′-amino functionality of 2′-amino-LNA nucleotides offers a structurally well-defined conjugation site in an LNA-type ON.[21] LNA nucleotides are known to induce N-type furanose ring conformation of neighbouring DNA monomers in LNA/DNA mixmer ONs and a concomitant B-DNA to A-DNA conformational change.[20] We have earlier demonstrated the compatibility of LNA and 2′-*N*-methyl-2′-*N*-(pyren-1-ylmethyl)-2′-amino-DNA monomers. Thus, the incorporation of LNA nucleotides apparently induced a B-DNA to A-DNA conformational change and increased binding towards an RNA target strand.[14] Encouraged by these observations, we decided to study in general the effect of LNA nucleotides on the binding affinity of DNA strands containing N2′-functionalised 2′-amino-DNA nucleotides.

Synthesis of the O5′-dimethoxytritylated 2′-amino-2′-deoxyuridine **1** was accomplished according to the published procedure.[22] Selective N-acylation of nucleoside **1** was carried out in the presence of dicyclohexyl carbodiimide (DCC) or 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) as a coupling reagent to furnish *N*-pyren-1-ylcarbonyl, *N*-(9-fluorenylmethoxycarbonyl)glycyl, *N*-adamantylacetyl, *N*-pyren-1-ylbutanoyl and *N*-palmitoyl derivatives **2a**–**2e** (Scheme 1). The 3′-hydroxy group of nucleosides **2a**–**2e** was phosphitylated using standard conditions to afford the corresponding phosphoramidite building blocks **3a**–**3e**[23] that were used for automated solid-phase synthesis of ONs. The stepwise coupling yields for amidites **3a**–**3e** using standard conditions except for extended coupling time (10-30 min, 1*H*-tetrazole or DCI as activator) were

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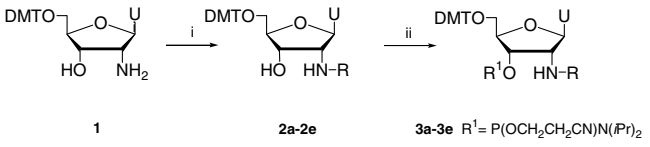

2,3	R
a	pyren-1-ylcarbonyl
b	*N*-(9-fluorenylmethoxycarbonyl)glycyl
c	adamant-1-ylacetyl
d	pyren-1-ylbutanoyl
e	palmitoyl

Scheme 1. Reagents, conditions and yields: (i) **2a**: 1-pyrenecarboxylic acid, CH_2Cl_2, DCC, rt, 12 h, 71%; **2b**: *N*-(9-fluorenylmethoxycarbonyl)glycine, CH_2Cl_2, DCC, rt, 12 h, 73%; **2c**: 1-adamantaneacetic acid, CH_2Cl_2, DCC, rt, 12h, 70%; **2d**: 1-pyrenebutyric acid, CH_2Cl_2, DCC, rt, 12 h, 70%; **2e**: palmitic acid, CH_2Cl_2, EDC, rt, 12 h, 43%; (ii) 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite, DIPEA, CH_2Cl_2, rt, 12 h (**3a**: 60%, **3b**: 58%, **3c**: 58%, **3d**: 61%, **3e**: 44%). U = uracil-1-yl; DMT = 4,4′-dimethoxytrityl.

>90% [app 99% for DNA (2 min coupling time) and LNA (6 min coupling time) amidites]. Following deprotection using standard conditions, purification (reversed-phase HPLC) and workup, the composition and purity (>80%) of the ONs were confirmed by MALDI-MS analysis and ion-exchange HPLC, respectively. The structures of the N2′-amido functionalised amino-DNA monomers studied are shown in Figure 1.

Thermal denaturation studies of 9-mer ONs **ONT**, **ONK**, **ONL**, **ONM**, **ONP**, **ONQ** and **ONS** towards complementary DNA and RNA target strands were performed in a high salt buffer (710 mM [Na^+]) and in a medium (110 mM [Na^+]) salt buffer solution in two different sequence contexts (Tables 1–3; **ONT** refers to the reference strands). As expected, the T_m values in general decreased when changing from high to medium salt

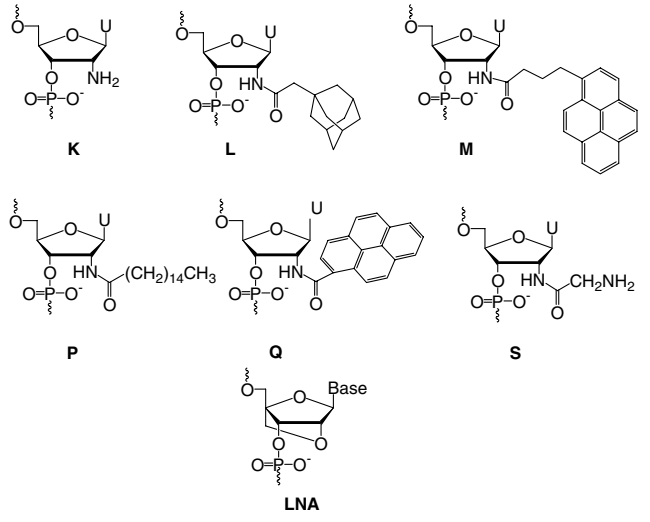

Figure 1. Structures of nucleotide monomers: 2′-amino-DNA (**K**), 2′-*N*-(adamantylacetyl)-2′-amino-DNA (**L**), 2′-*N*-(pyren-1-ylbutanoyl)-2′-amino-DNA (**M**), 2′-*N*-(palmitoyl)-2′-amino-DNA (**P**), 2′-*N*-(pyren-1-ylcarbonyl)-2′-amino-DNA (**Q**), 2′-*N*-glycyl-2′-amino-DNA (**S**) and LNA. The short notations shown are used in Tables 1–3.

Table 1. Purine-rich sequence—high salt buffer

Modification **B**:	5′-d(GTG A**B**A TGC) **ONBa**		5′-d(G**B**G A**B**A **B**GC) **ONBb**	
	DNA target	RNA target	DNA target	RNA target
T	34 (ref)	31 (ref)	34 (ref)	31 (ref)
K[14]	24 (−10)	27 (−4)	<10	<10
L	25 (−9)	<10	<10	<10
M	37 (+3)	25 (−6)	30 (−4)	<10
P	29 (−5)	22 (−9)	<10	n.d.
S	29 (−5)	21 (−10)	<10	<10

Melting temperatures [T_m values/°C (difference in T_m value relative to reference T_m value)] measured as the maximum of the first derivative of the melting curve (A_{260} vs temperature with increasing temperature [+1 °C/min]) recorded in high salt buffer (10 mM sodium phosphate, 710 mM sodium chloride and 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; G^L = guanin-9-yl LNA monomer, T^L = thymin-1-yl LNA monomer. Selected MALDI-MS *m*/*z* ([M−H]; found/calcd): **ONLa** 2930/2930; **ONMa** 3026/3028; **ONMb** 3565/3567; **ONPa** 2292/2993; **ONPb** 3471/3473; **ONSa** 2810/2811; **ONSb** 2929/2927.
n.d., not determined.

Table 2. Pyrimidine-rich sequence—high salt buffer

Modification **B**:	5′-d(GTG T**B**T **B**GC) **ONBc**		5′-d(G**T**^{L}G **T**^{L}**B**T^L **B**G^{L}C) **ONBd**	
	DNA target	RNA target	DNA target	RNA target
T	37 (ref)	36 (ref)	50 (+13)	65 (+29)
K[14]	12 (−25)	18 (−18)	43 (+6)	60 (+24)
L	<10	<10	16 (−21)	35 (−1)
M	29 (−8)	26 (−10)	36 (−1)	47 (+11)
P	<10	<10	n.d.	n.d.
Q	14 (−23)	25 (−11)	23 (−14)	39 (+3)
S	<10	<10	31 (−6)	47 (+11)

Same conditions as described in footnote of Table 1. MALDI-MS *m*/*z* ([M−H]; found/calcd): **ONLc** 3090/3089; **ONLd** 3201/3202; **ONMc** 3279/3278; **ONMd** 3391/3390; **ONPc** 3212/3214; **ONPd** 3325/3327; **ONQc** 3189/3193; **ONQd** 3304/3306; **ONSc** 2853/2851; **ONSd** 2964/2963.

buffer conditions (Table 2 vs Table 3). The incorporation of two 2′-amino-DNA monomers **K** (**ONK**) leads to significant decreases in duplex thermal stabilities when compared with the stabilities obtained with the DNA reference **ONT**.[14] Similar destabilisation in the various sequence contexts was in general found for **ONL**, **ONM**, **ONP**, **ONQ** and **ONS**, with the destabilising effect being more pronounced the more N2′-amido functionalised monomers being incorporated. **ONM** containing the 2′-*N*-pyrenylbutanoyl monomer **M** shows comparatively less destabilising effect in a B-type duplex than the remaining amino-functionalised monomers, and even a small increase when incorporated once (Table 1). Similar DNA selectivity and efficient DNA binding have been observed for other N2′-pyrene-functionalised ONs.[9,14] A similar trend was not observed for **ONQc/ONQd** containing the less flexible

Table 3. Pyrimidine-rich sequences—medium salt buffer

Modification **B**:	5′-d(GTG T**B**T **B**GC) **ONBc**		5′-d(G**T^{L}**G **T^{L}B**T^{L} **BG^{L}**C) **ONBd**	
	DNA target	RNA target	DNA target	RNA target
T	28 (ref)	28 (ref)	42 (+14)	57 (+29)
K[14]	<10	<10	31 (+3)	51 (+23)
L	<10	<10	<10	27 (−1)
M	20 (−8)	19 (−9)	28 (±0)	37 (+9)
Q	<10	18 (−10)	<10	31 (+3)
S	<10	<10	24 (−4)	40 (+12)

Melting temperatures [T_m values/°C (difference in T_m value relative to reference T_m value)] 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, 110 mM sodium chloride and 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. See caption of Table 1 for further details.

2′-*N*-pyrenylcarbonyl monomer **Q**, in analogy with data obtained for the corresponding N2′-pyrenecarbonyl 2′-amino-LNA monomer.[24]

All the modified nucleosides predominantly adopt a DNA-mimicking S-type furanose ring conformation as confirmed by coupling constant values of nucleosides **2a**–**2e** ($J_{1',2'} = 5.9$–6.3 Hz). The destabilising effect of amino-functionalised 2′-amino-DNA nucleotides has earlier been explained by steric hindrance of the bulky 2′-amido groups.[4] We expected incorporation of four RNA-mimicking LNA monomers to induce a conformational shift of the furanose rings of monomers **L**, **M**, **Q** and **S** towards N-type conformations, and therefore also an RNA-mimicking A-DNA-type conformation of **ONLd**, **ONMd**, **ONQd** and **ONSd** when participating in the duplex formation. From the thermal denaturation studies of the DNA/LNA mixmeric ONs (Tables 2 and 3) it seems that this putative conformational change from B-DNA to A-DNA relieves the steric hindrance of the 2′-amido group and leads to comparatively improved binding properties, especially towards RNA, and especially for the sterically less demanding monomers **M** and **S**. That a conformational effect is in play is supported by the reported steering effect of LNA nucleotides on neighbouring DNA nucleotides,[20] and by the effects observed earlier for N2′-pyrenylmethyl-functionalised 2′-amino-DNA/DNA/LNA mixmers.[14] These results furthermore support a recent study that concluded that 2′-amido groups structurally fit within RNA-type duplexes.[17] From the obtained results it can be concluded that the incorporation of N2′-functionalised DNA monomers into DNA/LNA mixmers constitutes a general design of easily accessible next generation ONs of potential use in biotechnology and medicine.

Monomers **Q** and **M** were recently evaluated as fluorescent probes to monitor RNA folding,[25] with the more flexible monomer **M** being the more useful construct. We have recently reported on strong hybridization-induced increases in fluorescence of N2′-pyrenylcarbonyl-functionalised 2′-amino-LNA/DNA mixmers.[24] As a continuation of this study, we have recorded fluorescence spectra of single-stranded **ONQc** and **ONQd** and of duplexes formed between these modified ONs and their complementary DNA or RNA strands (Fig. 2). The single-stranded **ONQc** or **ONQd** exhibits relatively weak fluorescence intensity only. In contrast, on hybridization with the complementary strand, the fluorescence intensity was markedly enhanced, especially in case of the LNA-modified **ONQd** hybridized with RNA. We interpret these results such that pyrene–pyrene and pyrene–nucleobase interactions within the single-stranded **ONQc** and **ONQd** significantly quench fluorescence emission, most pronounced in the case of the more flexible **ONQc**. In accordance with the results obtained for the corresponding N2′-pyrenylcarbonyl-functionalised 2′-amino-LNA oligomer,[24] it seems that the combination of the rigid amide linkage of monomer **Q** and the presence of the neighbouring conformationally locked LNA monomers induces a rigid duplex upon hybridization with the RNA complement. This leads to hybridization-induced enhanced fluorescence emission of the pyrene units explained by their fixed positioning at the brim of the minor groove thus avoiding collision-mediated quenching effects. A weak excimer band around 510 nm is observed in case of the **ONQc**-DNA or **ONQc**-RNA duplexes (Fig. 2). This can be explained by weak interaction between two pyrene fluorophores resulting from unwinding of these somewhat flexible duplexes, supported by the lack of excimer band for the more rigid duplexes containing the LNA-modified **ONQd**.

Various N2′-functionalised derivatives of 2′-amino-2′-deoxyuridine were synthesized and these building blocks incorporated into DNA strands and DNA/LNA mixmer strands. The presence of the 2′-amido-modified nucleotides within a DNA strand resulted in decreased thermal stabilities of duplexes formed with both DNA and RNA complements. The introduction of LNA monomers around these 2′-amido-modified nucleotides efficiently reversed this affinity-decreasing effect allowing efficient targeting of complementary RNA. 2′-Amino-DNA nucleosides are synthetically more easily accessible than the corresponding 2′-amino-LNA nucleosides, and the potential usefulness of 2′-amino-DNA/LNA mixmers

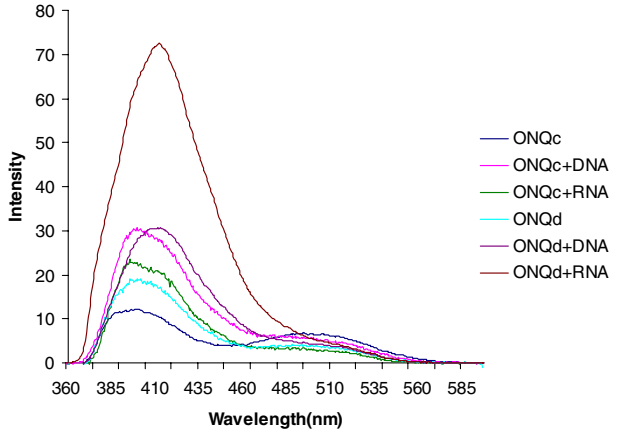

Figure 2. Steady-state fluorescence emission spectra (10 °C, high salt buffer as described in the footnote of Table 1; excitation at 340 nm).

was illustrated by the design of oligomers that display hybridization-induced increase in fluorescence. We have revealed a new and convenient approach for design and synthesis of functionalised oligonucleotides of potential use in biotechnology and medicine, and we are currently exploring this approach in our research towards optimizing RNA-targeting oligonucleotides as gene-silencing agents.

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References and notes

1. Petersen, M.; Wengel, J. *Trends Biotechnol.* **2003**, *21*, 74.
2. Kurreck, J. *Eur. J. Biochem.* **2003**, *270*, 1628.
3. Manoharan, M. *Antisense Nucleic Acid Drug Dev.* **2002**, *12*, 103.
4. Hendrix, C.; Devreese, B.; Rozenski, J.; Aerschot, A. V.; Bruyn, A. D.; Beeumen, J. V.; Herdewijn, P. *Nucleic Acids Res.* **1995**, *23*, 51.
5. Sigurdsson, S. T.; Eckstein, F. *Nucleic Acids Res.* **1996**, *24*, 3129.
6. Mitsui, T.; Nakano, H.; Yamana, K. *Tetrahedron Lett.* **2000**, *41*, 2605.
7. Beban, M.; Miller, P. S. *Bioconjugate Chem.* **2000**, *11*, 599.
8. Hwang, J.-T.; Greenberg, M. M. *J. Org. Chem.* **2001**, *66*, 363.
9. Kawai, K.; Kawabata, K.; Tojo, S.; Majima, T. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2363.
10. Krider, E. S.; Miller, J. E.; Meade, T. J. *Bioconjugate Chem.* **2002**, *13*, 155.
11. Vasil'eva, S. V.; Abramova, T. V.; Ivanova, T. M.; Shishkin, G. V.; Sil'nikov, V. N. *Russ. J. Bioorg. Chem.* **2004**, *30*, 234.
12. Bugaut, A.; Toulmé, J.-J.; Rayner, B. *Angew. Chem. Int. Ed.* **2004**, *43*, 3144.
13. Winkler, J.; Urban, E.; Losert, D.; Wacheck, V.; Pehamberger, H.; Noe, C. R. *Nucleic Acids Res.* **2004**, *32*, 710.
14. Kalra, N.; Babu, B. R.; Parmar, V. S.; Wengel, J. *Org. Biomol. Chem.* **2004**, *2*, 2885.
15. Pham, J. W.; Radhakrishnan, I.; Sontheimer, E. *Nucleic Acids Res.* **2004**, *32*, 3446.
16. Al-Rawi, S.; Ahlborn, C.; Richert, C. *Org. Lett.* **2005**, *7*, 1569.
17. Gherghe, C. M.; Krahn, J. M.; Weeks, K. M. *J. Am. Chem. Soc.* **2005**, *127*, 13622.
18. Aurup, H.; Tuschl, T.; Benseler, F.; Ludwig, J.; Eckstein, F. *Nucleic Acids Res.* **1994**, *22*, 20.
19. Singh, S. K.; Kumar, R.; Wengel, J. *J. Org. Chem.* **1998**, *63*, 10035.
20. Petersen, M.; Bondensgaard, K.; Wengel, J.; Jacobsen, J. P. *J. Am. Chem. Soc.* **2002**, *124*, 5974.
21. Sørensen, M. D.; Pedersen, M.; Wengel, J. *Chem. Commun.* **2003**, 2130.
22. McGee, D. P. C.; Vaughn-Settle, A.; Vargeese, C.; Zhai, Y. *J. Org. Chem.* **1996**, *61*, 781.
23. ^{31}P NMR data δ values (ppm, 121.5 MHz; $CDCl_3$; relative to 85% H_3PO_4 as external standard): 152.3, 151.4 (**3a**); 153.8, 151.1 (**3b**); 151.2, 150.7 (**3c**); 151.8, 150.5 (**3d**); 151.6, 150.6 (**3e**).
24. Hrdlicka, P. J.; Babu, B. R.; Sørensen, M. D.; Harrit, N.; Wengel, J. *J. Am. Chem. Soc.* **2005**, *127*, 13293.
25. Smalley, M. K.; Silverman, S. K. *Nucleic Acids Res.* **2006**, *34*, 152.