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LNA guanine and 2,6-diaminopurine. Synthesis, characterization and hybridization properties of LNA 2,6-diaminopurine containing oligonucleotides

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Abstract—LNA guanine and 2,6-diaminopurine (D) phosphoramidites have been synthesized as building blocks for antisense oligonucleotides (ON). The effects of incorporating LNA D into ON were investigated. As expected, LNA D containing ON showed increased affinity towards complementary DNA (ΔT_m +1.6 to +3.0 °C) and RNA (ΔT_m +2.6 to +4.6 °C) ON. To evaluate if LNA D containing ON have an enhanced mismatch sensitivity compared to their complementary LNA A containing ON thermal denaturation experiments towards singly mismatched DNA and RNA ON were undertaken. Replacing one LNA A residue with LNA D, in fully LNA modified ON, resulted in higher mismatch sensitivity towards DNA ON ($\Delta\Delta T_m$ −4 to >−17 °C). The same trend was observed towards singly mismatched RNA ON ($\Delta\Delta T_m$ D–a = −8.7 °C and D–g = −4.5 °C) however, the effect was less clearcut and LNA A showed a better mismatch sensitivity than LNA D towards cytosine (ΔT_m +5.5 °C).

1. Introduction

The promising aspects of using analogues of natural nucleic acids in antisense oligonucleotides (ON) has stimulated much interest in this field during recent years. Locked nucleic acid (LNA)[‡] was introduced in 1998[1–3] as a novel class of conformationally restricted ON analogues. It is well established that LNA is the single nucleic acid modification that contributes the highest increase in affinity (T_m) ever obtained in Watson–Crick hydrogen bonding.[4] The 2′,4′-linked bicyclic structure provide high affinity and high specificity to both fully and partially LNA modified ON. LNA monomers can be mixed with DNA and RNA monomers and in principle all other known nucleic acid analogues based on the phosphoramidite oligomerization approach, for example, phosphorothioates and 2′-*O*-alkyl modified RNA.[5] These unique properties positions LNA as a versatile probe in nucleic acid chemistry.

In order to design and synthesize LNA therapeutics it is essential to have efficient and robust syntheses of the necessary LNA phosphoramidites. The LNA thymine, 5-methylcytosine and adenine monomers are now routinely synthesized on a 100+g scale, however the essential LNA guanine monomer has until recently caused problems due to several troublesome synthetic steps. In particular the Vorbrüggen coupling[6,7] of the guanine nucleobase (or derivatives thereof) to the carbohydrate moiety has been problematic, inevitably leading to mixtures of the N7 and N9 regioisomers.[8] Many different strategies have been devised to enhance the selectivity in favour of the N9 isomer.[9–12] We found a method employing 2-amino-6-chloropurine in the Vorbrüggen coupling particularly appealing for several reasons.[9,13–15] Besides potentially being able to furnish a regiospecific coupling a number of other advantages could be envisaged: (1) guanine and guanine nucleosides are very polar compounds, complicating synthetic work

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‡ LNA is defined as an ON containing one or more 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide monomers (LNA monomers).[1]

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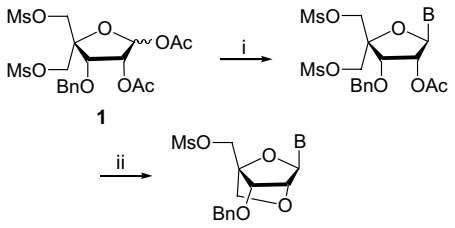
and the use of the less polar 2-amino-6-chloropurine was likely to be an advantage; (2) 2-amino-6-chloropurine is cheap and commercially available in large quantities; (3) 2-amino-6-chloropurine can be transformed into a number of different nucleobase analogues such as 6-arylpurines,[16] 2-aminopurine,[17,18] 6-thiopurine[19,20] and 2,6-diaminopurine[21,22] essentially making it a convergent synthesis of a whole range of LNA purine monomers. The potential of 6-substituted purines as nucleoside drugs[23] and the ability to modulate the properties of ON by introducing modified nucleobases[24] are areas of considerable interest and has recently also turned our attention towards modified nucleobases in a LNA context.

Here we present the first regio- and stereospecific synthesis of the LNA G phosphoramidite employing 2-amino-6-chloropurine, unambiguously showing that the N9 isomer is the only product of the Vorbrüggen coupling. In addition, the synthesis of the LNA 2,6-diaminopurine (LNA D) phosphoramidite and ON containing this novel LNA monomer has been accomplished. To evaluate LNA D containing ON potential as probes for antisense ON, mismatch discrimination experiments of LNA D containing ON towards singly mismatched DNA and RNA sequences have been performed, and compared to LNA A containing ON. Moreover, the hybridization properties of LNA D containing ON, towards complementary RNA and DNA sequences, have been studied in an effort to gain a better understanding of what effects govern 2,6-diaminopurines ability to stabilize certain duplexes.

2. Results and discussion

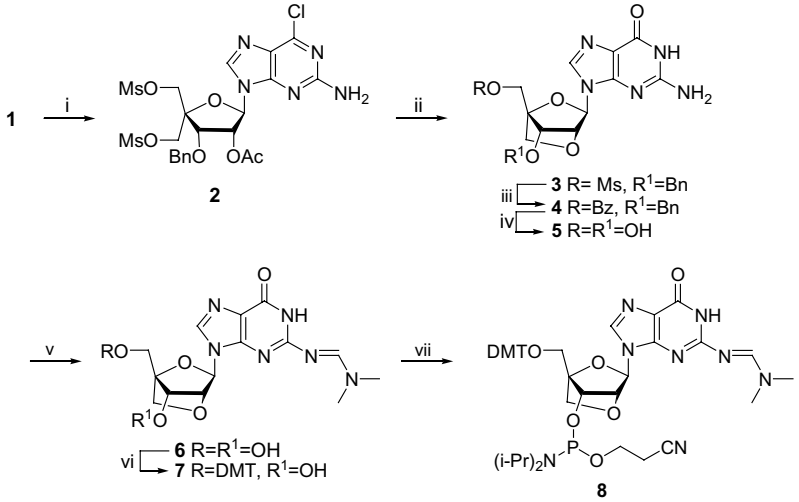
The convergent synthesis of LNA monomers relies on the coupling of different nucleobases to the same coupling sugar **1** (Scheme 1) followed by ring closure to give the bicyclic nucleosides.[8] The coupling of **1** with 2-*N*-isobutyrylguanine has been reported previously.[8] Although conditions favouring the formation of the LNA guanine N9 isomer are employed, significant amounts (approximately 10–15%) of the N7 isomer were always detected in our hands and the N9 and N7 isomers were very difficult to separate. Moreover, 2-*N*-isobutyrylguanine is tedious to synthesize and purify.[12]



Scheme 1. (i) Vorbrüggen coupling; (ii) ring closing to form the bicyclic LNA skeleton. B = nucleobase or nucleobase analogue.

We decided to use 2-amino-6-chloropurine in the Vorbrüggen coupling with **1** to see if we could improve on the selectivity (Scheme 2). A detailed analysis of the obtained nucleoside **2** by HPLC–MS, ^{1}H and ^{13}C NMR (vide infra) revealed the exclusive formation of one isomer. The formation of the β-anomer was expected due to the C2 acetate group in the coupling sugar (**1**), allowing only attack from the β-face. X-ray analysis confirmed that the desired N9 regioisomer was the exclusive product (Fig. 1). As previously demonstrated by other researchers[9] the regiospecific formation of the N9 isomer can be achieved when 2-amino-6-chloropurine is coupled to a carbohydrate moiety under thermodynamic conditions (e.g., TMS-triflate, refluxing dichloroethane).[11]

Nucleoside **2** is a versatile compound allowing the synthesis of a wide range of LNA nucleobase analogues. Either the ring closure to form the bicyclic LNA skeleton with concomitant formation of the guanine nucleo-



Scheme 2. Reagents and conditions: (i) 2-Amino-6-chloropurine, *N*,*O*-bis(trimethylsilyl)acetamide, TMS-triflate, 1,2-dichloroethane; (ii) $HOCH_2CH_2CN$, NaH, THF; (iii) NaOBz, DMSO, 100 °C; (iv) (a) MsOH, CH_2Cl_2, (b) Amberlyst A-26 (OH^- (aq), EtOH); (v) $(CH_3O)_2CHN(CH_3)_2$, (vi) DMT-Cl, pyridine (vii) $NC(CH_2)_2OP(N(^iPr)_2)_2$, 4,5-dicyanoimidazole, MeCN, CH_2Cl_2. DMT = 4,4′-dimethoxytrityl.

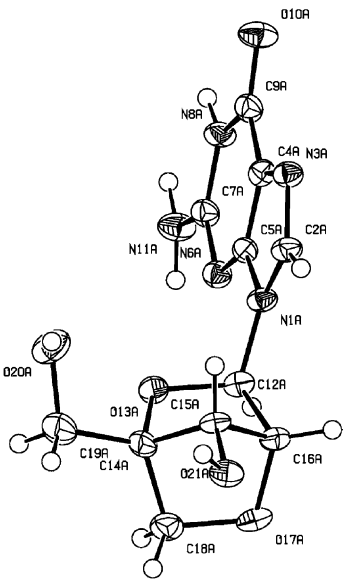


Figure 1. X-ray structure of nucleoside **5**.

base can be performed (Scheme 2) or alternatively exclusive formation of the bicyclic structure without affecting the nucleobase can be carried out allowing for later modification of the purine to a wide range of nucleobase analogues, for example, 2,6-diaminopurine (Scheme 4).

It has been reported that 2-amino-6-chloropurines upon treatment with aqueous NaOH undergo substitution of the chlorine atom to give guanine.[25,26] These conditions are very similar to those employed for the ring closure of the LNA bicyclic skeleton (aq LiOH or NaOH in THF).[8] As expected, upon treatment with aq NaOH compound **2** rapidly formed the bicylic system. However, the substitution reaction on the purine was very slow even under forcing conditions and was difficult to drive to completion. To increase the reaction rate we clearly needed a better nucleophile. 3-Hydroxypropionitrile is a useful nucleophile for this particular reaction, giving the desired guanine nucleobase after β-elimination of acrylonitrile.[27,28] In most cases triethylamine or DBU is used to promote the formation of the alkoxide in situ. We decided to use slightly different conditions from the previously published procedures[27,28] pre-forming 3 equiv of the alkoxide of 3-hydroxypropionitrile by treatment with NaH, followed by the addition of nucleoside **2**. These conditions gave a very rapid ring closure, usually complete in less than 15 min, followed by a somewhat slower conversion of 2-amino-6-chloropurine to guanine yielding nucleoside **3** in >80% yield after 4 h. Substitution of the mesylate on C5′ of nucleoside **3** with sodium benzoate produced nucleoside **4** that was debenzylated with $Pd(OH)_2$–C and ammonium formate, conditions that also cleaved the 5′-benzoyl group producing the fully deprotected LNA guanine **5**. Unfortunately, the reaction was sluggish taking 2–3 days and up to 0.5 equiv Pd-catalyst to go to completion. This prompted us to investigate other conditions for the deprotection of the 3′-benzyl group. Using methanesulfonic acid in dichloromethane[29] resulted in a very efficient removal of the benzyl group. However, the published work-up procedure with triethylamine resulted in inseparable mixture of nucleoside and triethylammonium salts. The use of a basic anion exchange resin (Amberlyst A-26) as a scavenger to remove the acid solved this problem, but the basic conditions resulted in the premature hydrolysis of the 5′-benzoyl group, giving nucleoside **5**. Due to the very polar nature of nucleoside **5** it precipitated under the conditions employed and required large volumes of solvent to wash it off the resin. This problem was however solved by washing the resin with ethanol prior to use. Now the 5′-benzoyl nucleoside was effectively removed from resin devoid of all methanesulfonic acid. The EtOH solution containing the benzoylated nucleoside was then treated with aqueous NaOH resulting in rapid hydrolysis of the 5′-benzoyl group producing nucleoside **5**. The structure of the fully deprotected nucleoside **5** was confirmed by X-ray crystallographic analysis (Fig. 1).

We have previously experienced problems in the synthesis of ON where the guanine residues have been protected with the commonly employed isobutyryl protection group, requiring longer deprotection times, and as a result being incompatible with the synthesis of ON containing base sensitive groups. Therefore, we decided to introduce the dimethylformamidine protection group, which is cleaved very rapidly using standard ON deprotection protocols. Hence, the 2-amino group on the crude nucleoside **5** was protected as the dimethylformamidine giving nucleoside **6**, followed by protection on the 5′-hydroxy group with 4,4′-dimethoxytrityl chloride producing nucleoside **7** in 84% yield from **4**. Phosphitylation of the 3′-hydroxy group gave the phosphoramidite **8** in 88% yield ready for the automated incorporation into ON.

2.1. NMR analysis of the N7 and N9 regioisomers

In order to have an easy method for determining the regiochemistry of guanine nucleosides in the future we did a comparative NMR study on the 2-*N*-isobutyryl protected nucleosides **9** and **10** (Fig. 2). The route based on the Vorbrüggen coupling of 2-*N*-isobutyrylguanine, as previously published by Koshkin et al.[8] was used to obtain a mixture of **9** (N9 isomer) and **10** (N7 isomer) in

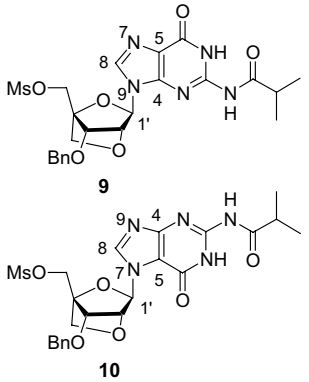


Figure 2. The N9 isomer (**9**) and the N7 isomer (**10**).

Table 1. The most diagnostic NMR signals for compounds **9** and **10**

	9 (N9 isomer)	**10** (N7 isomer)
H8	7.74	8.12
H1′	5.83	6.24
C2, C4 and C6	147.3, 147.9 and 155.2	148.4, 153.2 and 158.0
C5	121.5	110.8

^{1}H NMR run at 400 MHz and ^{13}C NMR run at 100 MHz in $CDCl_3$. All values are in ppm.

a 9:1 ratio. A pure sample of the N9 isomer **3** (Scheme 2) was prepared as described above and then acylated with isobutyryl anhydride to give **9** (Fig. 2). Comparison of the NMR spectra of the mixture with the pure sample confirmed that the major product in the regioselective coupling of 2-*N*-isobutyrylguanine was the N9 isomer (**9**).

The most diagnostic NMR signals for the two regioisomers **9** and **10** are shown in Table 1. Comparison of **9** and **10** by ^{1}H NMR spectrometry showed a significant upfield shift for H1′ and H8 in nucleoside **9** (Table 1). However, using the H1′ proton for identifying regioisomers is unreliable because the chemical shift is highly dependent on the carbohydrate moiety, making the diagnostic value of the signals limited, unless the carbohydrate moiety is unchanged.

Brand et al.[20] synthesized a wide range of alkylated purines and studied the shift of the H8 proton between the two regioisomers. They found in full agreement with the observations of Kjellberg and Johansson[30] that the H8 signal of the N9 regioisomers is shifted upfield relative to the H8 signal of the N7 regioisomers. The same is observed for **9** and **10** with a difference of approximately 0.4 ppm. However, the chemical shift values again cannot be used as a general method for assigning the regioisomers, because they are highly dependent on the alkyl group/carbohydrate moiety attached to the purine.

^{13}C NMR proved a more useful tool for assigning the two regioisomers. According to Chenon et al.[31] and Bailey and Harnden[32] the chemical shift difference between the C4 and C5 atoms is very pronounced for N7 and N9 regioisomers. They reported that shifting the substituent from N9 to N7 resulted in a significant downfield shift of the C4 resonance that approximately equalled the upfield shift of the C5 resonance. In the case of regioisomers **9** and **10** an upfield shift of approximately 10 ppm for C5 in nucleoside **9** compared to **10** was observed. Therefore, we anticipated a downfield shift of the same magnitude for C4 when going from nucleoside **9** to **10**. In our case it is difficult to distinguish between C2, C4 and C6 without using advanced NMR techniques (e.g., INADEQUATE). However, upon examination it is seen that two of the signals are largely unaffected and the remaining signal is observed shifting downfield approximately 10 ppm (approximately 147–158 ppm, Table 1). On the basis of the data in the literature[31,32] it is reasonable to assume that C4 corresponds to either the signal at 147.3 or 147.9 ppm in the N9 isomer and the signal at 158.0 ppm in the N7 isomer (Table 1).

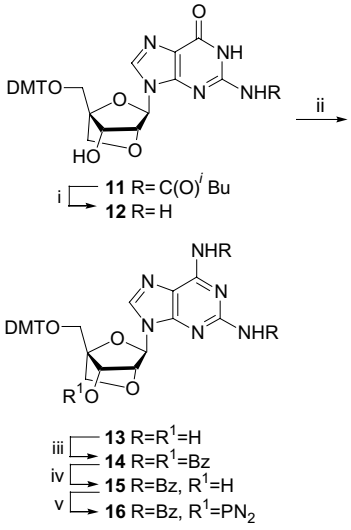
Using this simple correlation between the chemical shifts as a guideline ^{13}C NMR spectrometry proves to be a simple and fast method for determining which regioisomer is obtained, and in our experience the trend is general for a wide variety of guanine nucleosides.

2.2. Synthesis of LNA 2,6-diaminopurine

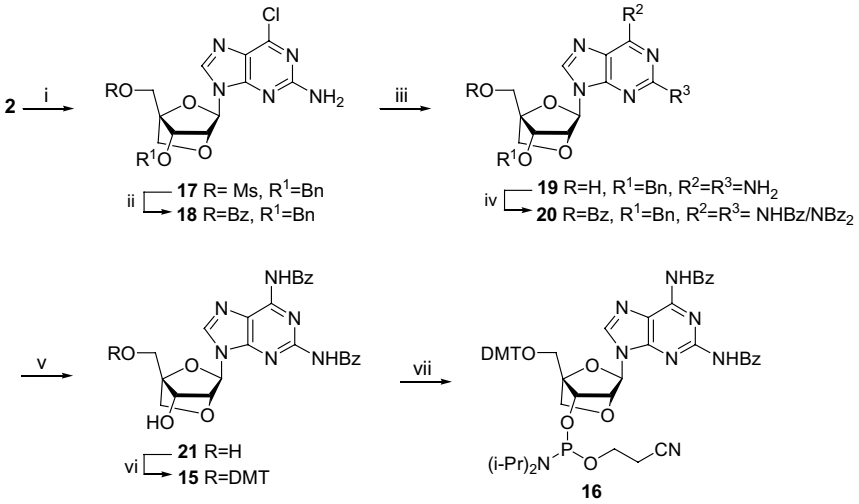
2,6-Diaminopurine nucleosides can be synthesized from guanine nucleosides according to the procedure by Gryaznov and Schultz.[33] As anticipated this procedure worked equally well for the conversion of LNA G to LNA D (Scheme 3). Initially, this gave us the desired LNA D phosphoramidite **16** for our studies. However, because the starting material (LNA G) is the product of a long linear reaction sequence, adding further steps in a linear fashion to obtain LNA D was undesirable.

During our work on the synthesis of the LNA G nucleoside (vide supra) we discovered that treatment of nucleoside **2** (Scheme 4) with aqueous NaOH resulted in a fast ring closing reaction to give the bicyclic nucleoside **17**, leaving the 2-amino-6-chloropurine largely unaffected.

This in turn became a useful way of synthesizing the LNA D nucleoside. Treatment of nucleoside **2** with aq LiOH in THF produced nucleoside **17** in 90% yield. Displacement of the mesylate on C5′ of **17** with sodium benzoate in DMF was as expected a fast reaction giving nucleoside **18** in a 91% yield. Treatment of **18** with a saturated solution of ammonia in MeOH in a sealed glass tube at 90 °C and 90 psi resulted in the formation



Scheme 3. Reagents and conditions: (i) Satd methanolic ammonia; (ii) (a) $(TFA)_2O$, pyridine, 0 °C, (b) NH_3 (55% from **11**); (iii) BzCl, pyridine; (iv) 2 M aq NaOH, THF, MeOH, H_2O (6:4:1, v/v/v) (81% from **13**); (v) $NC(CH_2)_2OP(N(^iPr)_2)_2$, 4,5-dicyanoimidazole, MeCN, DMF (95%). DMT = 4,4′-dimethoxytrityl, PN_2 = 2-cyanoethoxy(diisopropylamino)phosphinyl.



Scheme 4. Reagents and conditions: (i) LiOH (aq), THF; (ii) BzONa, DMF; (iii) satd methanolic ammonia (80 °C/60 psi); (iv) BzCl, pyridine; (v) (a) MsOH, CH_2Cl_2, (b) Amberlyst A-26 (OH^-, aq); (vi) DMT-Cl, pyridine (vii) $NC(CH_2)_2OP(N(^iPr)_2)_2$, 4,5-dicyanoimidazole, MeCN, DMF. DMT = 4,4′-dimethoxytrityl.

of the 2,6-diaminopurine nucleoside **19** together with the undesired 6-*O*-methyl nucleoside by substitution with methoxide. To favour the formation of nucleoside **19** and suppress the formation of the 6-*O*-methyl substituted nucleoside we investigated the reaction at a range of temperatures and pressures. Lowering the reaction temperature to 60 °C and the pressure to 36 psi resulted in the exclusive formation of the desired nucleoside **19** but the reaction was too slow (30% conversion after 12 h) to be of practical use. Eventually, we found that a temperature of 80 °C and a pressure of 60 psi for 4 h resulted in the formation of nucleoside **19** in an acceptable yield with a minimum of the 6-*O*-methyl nucleoside and some unreacted starting material **18**. Longer reaction times did not increase the conversion of starting material **18** to diaminopurine **19**, but resulted in degradation of the product. Due to the very polar nature of **19** it was used without further purification in the following step where it was treated with benzoyl chloride in pyridine to give a mixture the tetra- and penta-benzoylated nucleosides **20** in a 1:1 ratio (65% yield from **18**). In order to confirm the identity of the material a sample of the product was treated with diluted ammonia in methanol for 5 min resulting in the removal of one/two benzoyl group(s). The tribenzoylated product was identified as the desired nucleoside **20** (Scheme 4, $R^2 = R^3 = NHBz$) by NMR spectrometry. Because we expected the imide benzoyl groups to be hydrolyzed during the work-up after the debenzylation step (vide infra) it was deemed unnecessary to remove them at this point and the mixture **20** was used without further purification.

During the work-up of the product from the debenzylation of the LNA G nucleoside (vide supra) we discovered that it was necessary to wash the Amberlyst resin (employed as an acid scavenger) to avoid hydrolysis of the 5′-benzoyl group. We hoped we could use this to our advantage in the synthesis of nucleoside **21**. Debenzylation of nucleoside **20** with methanesulfonic acid, as described above, gave nucleoside **21**. Treating crude **21** with untreated Amberlyst A-26 resin hydrolyzed both the 5′-benzoyl group and the imide benzoyl groups, as expected, without affecting the two amides on the purine. Crude **21** was then protected with 4,4′-dimethoxytrityl chloride to give nucleoside **15** in 74% yield from **20**. In the final step the 3′-hydroxy group was phosphitylated to produce the phosphoramidite **16** in a 95% yield ready for the automated incorporation into ON.

Benzoyl protection groups on the nucleobase were employed, to make the synthesis of the monomers easier,[§] even though it has been reported that they can be difficult to remove from the synthesized ON.[34] Slower deprotection for the LNA D containing ON was indeed observed, but complete deprotection was always achieved within 18 h at 65 °C.

2.3. Oligonucleotides containing LNA D

It has been established that D–T basepairs compared to A–T basepairs stabilize some duplexes and destabilize others.[35] In general, the D–T basepair contributes a larger increase in duplex stability when incorporated into RNA duplexes than observed for the complementary DNA duplexes. It has been suggested that the lower increase in stability in DNA duplexes is due to the 2-amino groups effect on structural features such as hydration of the minor groove, helical conformations

[§] Attempts at preparing the dimethylformamidine and dibutylformamidine protected 2,6-diaminopurine phosphoramidites were unsuccessful, due to the loss of the formamidine protection groups during the course of reactions and purifications. We were not able to obtain any pure formamidine protected diaminopurine nucleoside at any time, due to the loss of the protection groups during chromatography, and we were unable to crystallize any of the nucleosides.

and base stacking.[36,37] As expected, this effect is clearly manifested when 2,6-diaminopurine monomers are incorporated into ON that promote formation of A-type duplexes, such as, for example, phosphoramidates,[38] hexitol nucleic acids (HNA)[37] and threofuranosyl(3′→2′)oligonucleotides (TNA),[39] showing a higher affinity towards RNA complements than towards DNA complements. The observed effect is attributed to the fact that the carbohydrates are in the north conformation thus producing A-type duplexes, which may be essential for formation of the third hydrogen bonding interaction in the D–T base pair, as well as producing a more favourable helical conformation, and thus improved base stacking and hydration of the duplex.

It is well known that LNA nucleosides adopt an extreme north conformation,[40] and LNA would therefore be the perfect nucleoside analogue to test this hypothesis. We decided to compare the duplex stability of ON containing LNA D towards complementary DNA and RNA sequences to see if we would obtain higher thermal stabilities towards RNA sequences compared to DNA sequences (Table 2).

The results in Table 2 clearly show a significant increase in duplex stability when substituting adenine with diaminopurine, as expressed by higher T_m values, both towards DNA and RNA complements. In perfect agreement with the literature (vide supra) all duplexes showed the largest increases in thermal stability towards RNA complements. However, to be able to conclusively attribute this to an effect arising solely from the carbohydrates extreme north conformation (resulting in an A-type duplex) comparison of iso-sequential ON containing a nucleoside analogue that adopts an extreme south conformation (resulting in a B-type duplex) is necessary. To this end we are currently synthesizing the α-L-LNA 2,6-diaminopurine monomer that Wengel and co-workers have shown adopt an extreme south type confirmation.[41] A much lower increase in duplex stability is expected when this monomer is incorporated, and this would demonstrate the close relationship between sugar conformation and the ability of the 2,6-diaminopurine nucleobase to hybridize satisfactorily to complementary DNA and RNA strands, giving us a better understanding of what governs the ability of 2,6-diaminopurine to stabilize helices.

Table 2. Duplex melting temperatures (T_m). D containing ON versus complementary DNA and RNA sequences

	Sequence	$T_m/\Delta T_m$ (°C)	
		5′-d(act ata cg)-3′	5′-r(act ata cg)-3′
1	5′-cgt ata gt-3′	23.0	20.8
2	5′-cgt dta gt-3′	26.8/3.8	26.0/5.2
3	5′-cgt dtd gt-3′	31.3/4.1	33.9/6.6
4	5′-cgt AtA gt-3′	42.2	45.4
5	5′-cgt DtA gt-3′	43.8/1.6	48.0/2.6
6	5′-cgt DtD gt-3′	48.2/3.0	54.5/4.5
7	5′-CGT ATA GT-3′	58.8	66.8
8	5′-CGT DTA GT-3′	61.0/2.2	71.4/4.6
9	5′-CGT DTD GT-3′	63.4/2.3	75.6/4.4

Lower case bases are DNA or RNA; a = adenine, c = cytosine, t = thymine, g = guanine, d = 2,6-diaminopurine. Upper case bases are LNA: A = adenine, C = 5-methyl cytosine, T = thymine, G = guanine, D = 2,6-diaminopurine. ON were synthesized by phosphoramidite chemistry. Buffer for melting temperature (T_m) determination: 100 mM phosphate buffer, 1 M NaCl, 4 μM for each ON strand, melting starting at 5 °C, under argon flow. ΔT_m is the increase per a substituted for d or A substituted for D.

Table 3. Duplex melting temperatures (T_m)

	Sequence	5′-d(act aXa cg)-3′			
		X = t	X = a	X = c	X = g
1	5′-cgt ata gt-3′	23.0	<10	<10	9.8
2	5′-cgt dta gt-3′	26.8	5.6	5.9	6.1
3	5′-cgt AtA gt-3′	42.2	9.0	17.0	18.0
4	5′-cgt DtA gt-3′	43.8	<10	22.0	7.0
5	5′-CGT ATA GT-3′	58.8	27.0	27.3	33.7
6	5′-CGT DTA GT-3′	61.0	<10	<10	31.4

See Table 2 legend for details.
A and D containing ON versus mismatched DNA sequences.

Table 4. Duplex melting temperatures (T_m)

	Sequence	5′-r(act aXa cg)-3′			
		X = t	X = a	X = c	X = g
1	5′-cgt ata gt-3′	20.8	<10	<10	<10
2	5′-cgt dta gt-3′	26.0	<10	<10	<10
3	5′-cgt AtA gt-3′	45.4	17.0	24.0	25.8
4	5′-cgt DtA gt-3′	48.0	27.0	28.0	25.5
5	5′-CGT ATA GT-3′	66.8	44.7	46.7	48.9
6	5′-CGT DTA GT-3′	71.4	40.6	56.8	49.0

See Table 2 legend for details.
A and D containing ON versus mismatched RNA sequences.

One of the properties of the 2,6-diaminopurine nucleobase, compared to adenine, is a better mismatch discrimination,[35] which in particular has been applied successfully in technologies working by the principle of non-self complementarity.[42] To investigate if this principle could be applied using LNA D, thermal denaturation experiments against all three singly mismatch DNA sequences (Table 3) and singly mismatched RNA sequences (Table 4) were undertaken.

The T_m determinations of DNA sequences (1 and 2, Table 3) and DNA sequences containing LNA monomers (3 and 4, Table 3) show no detectable effect upon replacement of adenine for 2,6-diaminopurine.[¶] However, for the fully LNA modified ON 5 and 6 a large effect is seen, showing a much greater mismatch sensitivity towards all singly mismatched sequences for ON 6, with ΔT_m (ON 5 vs 6) −4 to >−17 °C.

The T_m determinations of DNA sequences (1 and 2, Table 4) show no detectable effect upon replacement of

[¶] The low T_m values for sequences 1 and 2 (X = t, Tables 3 and 4) makes it impossible to detect any difference between the mismatched sequences (X = a, c and g, Tables 3 and 4). Making the DNA sequences longer resulted in a very high T_m of the iso-sequential LNA ON making it impossible to determine them accurately. Hence, it was necessary to make short sequences, to be able to determine T_m values for the fully LNA modified ON.

adenine for 2,6-diaminopurine, because of the low T_m of the mismatched sequences.[¶] ON 3 and 4 also do not show any improved discrimination upon introduction of D.

However, in the case of the fully LNA modified ON (5 and 6, Table 4) an improved discrimination is observed for X = a ($\Delta\Delta T_m$ = −8.7 °C) and X = g ($\Delta\Delta T_m$ = −4.5 °C). Somewhat unexpected, in the case of X = c, ON 5 has a better mismatch sensitivity than ON 6 ($\Delta\Delta T_m$ = +5.5 °C), showing that the result of introducing D in place of A does not necessarily mean an improved mismatch discrimination. The overall effects on the specificity of exchanging adenine for diaminopurine is, at least not in this RNA model, pronounced.

In general the substitution of LNA A with LNA D results in enhanced affinity towards DNA and more pronounced towards RNA. The apparent higher specificity of LNA D towards mismatches in DNA could make the monomer a suitable tool in DNA targeting.

3. Conclusion

An efficient synthesis of the LNA G phosphoramidite has been developed taking advantage of the regiospecific Vorbrüggen coupling of 2-amino-6-chloropurine to the carbohydrate moiety, eliminating the issue of traces of the N7 regioisomer in the final product. The guanine nucleobase was protected with the dimethylformamidine protection group allowing for the use of fast ON deprotection protocols when this is required. Moreover, the NMR spectra of both the N7 and N9 regioisomers after the Vorbrüggen coupling (**9** and **10**) have been studied in detail, providing a simple tool for the assignments LNA guanine nucleosides by NMR spectrometry.

The 2-amino-6-chloropurine nucleoside (**2**) obtained in the Vorbrüggen coupling proved to be a versatile synthon for the preparation of a wide range of LNA analogues with modified nucleobases, as exemplified by the synthesis of the LNA 2,6-diaminopurine (D) phosphoramidite.

In full agreement with the literature, the extreme north conformation of the LNA residues resulted in a higher increase in thermal stability towards complementary RNA strands rather than towards complementary DNA strands. This effect is probably due to a more favourable helical conformation, and thus improved base stacking, hydration of the duplex and base-pairing between D and T.

The mismatch sensitivity of LNA D compared to LNA A containing ON's has been evaluated towards DNA and RNA. Towards RNA no clear difference was seen between LNA A and LNA D. Towards DNA the fully modified LNA ON showed enhanced mismatch discrimination towards singly mismatched DNA when LNA A was replaced with LNA D.

4. Experimental

For reactions conducted under anhydrous conditions glassware was dried overnight in an oven at 150 °C and was allowed to cool in a dessicator over anhydrous KOH. Anhydrous reactions were carried out under an atmosphere of argon using anhydrous solvents. Solvents were HPLC grade, of which DMF, pyridine, acetonitrile and dichloromethane were dried over molecular sieves (4 Å from Grace Davison) and THF was freshly distilled from Na·benzophenone to a water content below 20 ppm. TLC was run on Merck silica 60 F_{254} aluminium sheets. Dry column vacuum chromatography (DCVC) was performed according to the published procedure.[43]

Synthesis of nucleoside **13** was carried out in a heavy-wall borosilicate pressure glass tube from Ace glass fitted with a #15 Ace thred PTFE plug and a pressure gauge. ^1H and ^{13}C NMR spectra were recorded at, respectively, 400 and 100 MHz with solvents as internal standard (δ_H: $CDCl_3$ 7.26 ppm, DMSO-d_6 2.50 ppm; δ_C: $CDCl_3$ 77.0 ppm, DMSO-d_6 39.4 ppm. D_2O with 1% 1,4-dioxane as internal standard δ_H: 3.75 ppm; δ_C: 67.2). ^{31}P NMR was run at 121 MHz with 85% H_3PO_4 as external standard. J values are given in Hz. Assignments of NMR spectra are based on 2D spectra and follow the standard carbohydrate/nucleoside nomenclature (the carbon atom of the 4′-*C*-substituent is numbered C1″) even though the systematic compound names of the bicyclic nucleoside derivatives are given according to the von Baeyer nomenclature. Crude compounds were used without further purification if they were ⩾95% pure by TLC and HPLC–MS. HPLC was performed with an Agilent 1100 series HPLC on a Xterra column from Waters (MS C18, 3.5 μm, 2.1 × 150 mm), with the following gradient 0–0.5 min, 50% A in B; 0.5–3.0 min, 50–100% A in B; 3.0–7.0 min, 100% B, 7.0–8.0 min, 100–50% A in B, 8.0–12.0 min, 50% B (solvent A: 0.1% concd NH_4OH in H_2O. Solvent B: 20% A in acetonitrile). Elemental analyzes were obtained from the University of Copenhagen, Microanalytical Department. Crystal X-ray crystallographic data of nucleoside **5** was obtained from University of Copenhagen, Centre for Crystallographic Studies.[‖]

4.1. 9-(2-*O*-Acetyl-3-*O*-benzyl-5-*O*-methanesulfonyl-4-*C*-methanesulfonyloxymethyl-β-D-*erythro*-furanosyl)-2-amino-6-chloropurine (2)

1,2-Di-*O*-acetyl-5-*O*-methanesulfonyl-4-*C*-methanesulfonyloxymethy-3-*O*-benzyl-D-*erythro*-pentofuranose (**1**) (19.0 g, 37.2 mmol) was dissolved in 1,2-dichloroethane (250 mL) and 2-amino-6-chloropurine (6.9 g,

[‖] Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no CCDC 216314. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

40.9 mmol) was added followed by *N,O*-bis(trimethylsilyl)acetamide (18.2 mL, 74.4 mmol). The reaction mixture was refluxed until it became a clear solution (1 h) and cooled to rt. Trimethylsilyl triflate (13.5 mL, 74.4 mmol) was added over 15 min and the reaction mixture was refluxed for 3 h. The reaction mixture was allowed to cool to rt and was poured into satd aq $NaHCO_3$ (500 mL). $CHCl_3$ (300 mL) was added and the mixture was stirred for 30 min and transferred to a separatory funnel. The phases were separated and the aq phase was extracted with $CHCl_3$ (3×250 mL). The combined organic phases were washed with satd aq $NaHCO_3$/brine (1:1, 500 mL), dried (Na_2SO_4), filtered and evaporated in vacuo to give a red foam. The product was purified by DCVC (i.d. 10 cm; 100 mL fractions; 50–100% EtOAc in *n*-heptane (v/v) 10% increments; 1–10% MeOH in EtOAc (v/v) 1% increments). Fractions containing nucleoside **2** were pooled and evaporated in vacuo to give a white foam (20.7 g, 90%). R_f = 0.75 (5% MeOH in EtOAc, v/v). ESI-MS *m/z* found 620.1 ($[MH]^+$, calcd 620.1). ^{1}H NMR ($CDCl_3$): δ 7.76 (s, 1H, H8), 7.40–7.32 (m, 5H, Ar–H), 6.00 (d, 1H, *J* 3.3, H1′), 5.84 (dd, 1H, *J* 5.9 and 3.3, H2′), 5.44 (br s, 2H, NH_2), 5.19 (d, 1H, *J* 6.0, H3′), 4.78 (d, 1H, *J* 10.4, CH_2), 4.68–4.61 (m, 3H, H2′, CH_2), 4.41–4.38 (m, 2H, CH_2), 3.03 (s, 3H, Ms), 3.00 (s, 3H, Ms), 2.11 (s, 3H, Ac). ^{13}C NMR ($CDCl_3$): δ 169.6 ($CH_3C(O)$), 159.1 (C2), 152.4, 152.1 (C4, C6), 141.5 (C8), 136.5, 128.6, 128.5, 128.3 (Ph), 125.9 (C5), 88.3 (C1′), 84.2 (C4′), 78.0 (C3′), 74.6 (PhCH_2), 74.0 (C2′), 67.5, 67.2 (C5′ and C1″), 37.8, 37.5 (2×Ms), 20.6 (CH_3C(O)). Elemental analysis: calcd for $C_{22}H_{26}ClN_5O_{10}S_2$: C, 42.6; H, 4.2; N, 11.3. Found: C, 42.8; H, 4.2; N, 10.9.

4.2. (1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-1-methanesulfonyloxymethyl-3-(guanin-9-yl)-2,5-dioxabicyclo[2.2.1]heptane (3)

3-Hydroxypropionitrile (3.65 mL, 53.5 mmol) was dissolved in THF (75 mL) and cooled to 0 °C. Sodiumhydride (60% in mineral oil, 2.61 g, 65.3 mmol) was added in portions and the temperature was allowed to reach rt. The mixture was stirred for 30 min at rt and cooled to 0 °C. Compound **2** (7.37 g, 11.9 mmol) was dissolved in THF (75 mL) and added dropwise over 20 min and the temperature was allowed to reach rt. After 4 h the reaction was quenched by addition of HCl (1 M, aq)/brine (1:9, 200 mL) and the mixture was transferred to a separatory funnel. The phases were separated and the aqueous phase was extracted with EtOAc (3×100 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated in vacuo to give a yellow oil. The product was purified by DCVC (i.d. 6 cm; 100 mL fractions; 0–20% MeOH in EtOAc (v/v) 1% increments). Fractions containing nucleoside **3** were pooled and evaporated in vacuo to give an off-white solid (4.74 g, 86%). R_f = 0.31 (10% MeOH in EtOAc v/v). ESI-MS *m/z* found 464.1 ($[MH]^+$, calcd 464.1). ^{1}H NMR (DMSO-d_6): δ 10.75 (br s, 1H, NH), 7.78 (s, 1H, H8), 7.36–7.26 (m, 5H, Ar–H), 6.64 (br s, 2H, NH_2), 5.80 (s, 1H, H1′), 4.78 (d, 1H, *J* 12.1, H5′), 4.75 (s, 1H, H2′ or H3′), 4.68 (s, 2H, PhCH_2), 4.59 (d, 1H, *J* 11.9, H5′), 4.41 (s, 1H, H2′ or H3′), 4.03 (d, 1H, *J* 8.1, H1″), 3.89 (d, 1H, *J* 8.1, H1″), 3.24 (s, 3H, Ms). ^{13}C NMR (DMSO-d_6): δ 156.7 (C6), 154.0 (C2), 150.5 (C4), 137.6 (Ph), 134.1 (C8), 128.4, 127.8, 127.7 (Ph), 116.7 (C5), 85.0 (C4′), 84.5 (C1′), 77.7, 77.0 (C2′ and C3′), 71.7, 71.4 (PhCH_2 and C1″), 66.1 (C5′), 36.9 (Ms). Elemental analysis: calcd for $C_{19}H_{21}N_5O_7S \cdot 0.25H_2O$: C, 48.8; H, 4.6; N, 15.0. Found: C, 48.6; H, 4.4; N, 14.7.

4.3. (1*R*,3*R*,4*R*,7*S*)-3-(Guanin-9-yl)-7-benzyloxy-1-benzoyloxymethyl-2,5-dioxabicyclo-[2.2.1]heptane (4)

Compound **3** (14.92 g, 32.2 mmol) was dissolved in DMSO (400 mL) and NaOBz (14.02 g, 96.6 mmol) was added. The reaction mixture was heated to 100 °C with stirring. After 3.5 h the reaction was allowed to cool to rt and EtOAc (400 mL) and H_2O/satd aq $NaHCO_3$ (3:1, 500 mL) was added. The solution was transferred to a separatory funnel and the phases were separated. The aq phase was extracted with dichloromethane (3×300 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated in vacuo to give a brown oil. The product was diluted with EtOH (96%, 300 mL) and boiled for 10 min with decolorizing charcoal. The slurry was filtered through a plug of Celite and hot water (500 mL) was added to the solution until it became turbid. The mixture was allowed to cool to rt and was then cooled to 5 °C. The product **4** was filtered off, washed with cold water and ether to give a tan solid (14.97 g, 95%). R_f = 0.49 (10% MeOH in EtOAc v/v). ESI-MS *m/z* found 490.2 ($[MH]^+$, calcd 490.2). ^{1}H NMR (DMSO-d_6): δ 10.66 (br s, 1H, NH), 7.93 (d, 2H, *J* 7.1, Bz), 7.70 (s, 1H, H8), 7.67 (d, 1H, *J* 7.5, Bz), 7.53 (d, 2H, *J* 7.8, Bz), 7.35–7.24 (m, 5H, Ar–H), 6.57 (br s, 2H, NH_2), 5.82 (s, 1H, H1′), 4.80–4.65 (m, 5H, H2′ or H3′, H5′ and PhCH_2), 4.50 (s, 1H, H2′ or H3′), 4.11 (d, 1H, *J* 8.1, H1″), 3.97 (d, 1H, *J* 8.1, H1″). ^{13}C NMR (DMSO-d_6): δ 165.3 (Ph*C*(O)), 156.7 (C6), 153.9 (C2), 150.4 (C4), 137.7 (Ph), 133.9 (Bz), 133.7 (C8), 129.3, 129.1, 128.9, 128.3, 127.7, 127.6 (Ar), 116.7 (C5), 85.0, 84.9 (C1′ and C4′), 77.6, 77.0 (C2′ and C3′), 71.9, 71.3 (PhCH_2 and C1″), 60.5 (C5′). Elemental analysis: calcd for $C_{25}H_{23}N_5O_6 \cdot 0.5H_2O$: C, 60.2; H, 4.8; N, 14.0. Found: C, 60.0; H, 4.7; N, 14.2.

4.4. (1*S*,3*R*,4*R*,7*S*)-3-(Guanin-9-yl)-7-hydroxy-1-hydroxymethyl-2,5-dioxabicyclo-[2.2.1]heptane (5)

Compound **4** (489 mg, 1.0 mmol) was suspended in dichloromethane (4 mL) and cooled 0 °C with stirring. Methanesulfonic acid (1.30 mL, 20 mmol) was added to give a clear solution. After 30 min the reaction mixture was allowed to reach rt. After stirring for an additional 3.5 h the reaction was diluted with dichloromethane (4 mL) and cooled to 0 °C. Amberlyst A-26 resin (20 mL) pre-washed with NaOH (1 N (aq), 3×20 mL) and EtOH (30 mL) was suspended in EtOH (30 mL), cooled to 0 °C and the reaction mixture was added slowly. After stirring for 30 min the resin was removed by filtration and washed with warm EtOH (4×20 mL). Fractions

containing the product were combined and the pH was adjusted to 11 with NaOH (1 N, aq). After 10 min the solution was neutralized with HCl (1 N, aq). The EtOH was removed in vacuo and the remaining aqueous solution was lyophilized to give nucleoside **5**. The crude product was purified by HPLC (Xterra column from Waters (PrepRP$_{18}$, 5 μm, 7.8×50 mm), with the following gradient 0–6.0 min, 0–2% B in A; 6.0–8.0 min, 2–4% B in A; 8.0–10.0 min, 4–100% B in A (Solvent A: 0.1 M NH_4OAc in H_2O, pH = 8. Solvent B: acetonitrile) and recrystallized from water to give **5** as white needles (145 mg, 49%). R_f = 0.04 (10% MeOH in EtOAc v/v). ESI-MS m/z found 296.3 ([MH]$^+$, calcd 296.1). ^{1}H NMR (D_2O): δ 7.86 (s, 1H, H8), 5.85 (s, 1H, H1′), 4.60 (s, 1H, H2′ or H3′), 4.42 (s, 1H, H2′ or H3′), 4.03 (d, 1H, J 8.6, H1″), 3.97 (d, 1H, J 8.5, H1″), 3.96 (s, 2H, H5′). ^{13}C NMR (D_2O): δ 160.8 (C6), 153.7 (C2), 150.6 (C4), 136.1 (C8), 114.5 (C5), 88.3 (C4′), 84.9 (C1′), 79.4 (C2′), 71.4 (C3′), 70.1 (C1″), 56.7 (C5′). Elemental analysis: calcd for $C_{11}H_{13}N_5O_5 \cdot H_2O$: C, 42.2; H, 4.8; N, 22.4. Found: C, 41.9; H, 4.8; N, 22.2.

4.5. (1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-(2-*N*-(dimethylamino)methylidene)-guanin-9-yl)-2,5-dioxabicyclo[2.2.1]heptane (7)

Compound **4** (2.23 g, 4.56 mmol) was suspended in dichloromethane (18 mL) and cooled to 0 °C with stirring. Methanesulfonic acid (5.9 mL, 91.2 mmol) was added to give a clear solution. After 30 min the reaction mixture was allowed to reach rt and the reaction was stirred for an additional 3.5 h. The reaction mixture was diluted with dichloromethane (18 mL) and cooled to 0 °C. Amberlyst A-26 resin (100 mL) pre-washed with NaOH (1 N (aq), 3×100 mL) and EtOH (150 mL) was suspended in EtOH (100 mL), cooled to 0 °C and the reaction mixture was added slowly. After stirring for 30 min the resin was removed by filtration and washed with warm EtOH (4×50 mL). Fractions containing the product were combined and the pH was adjusted to 11 with NaOH (1 N, aq). After stirring for 10 min the solution was neutralized with HCl (1 N, aq). The EtOH was removed in vacuo and the remaining aqueous solution was lyophilized to give compound **5**. The crude product was suspended in DMF (30 mL) and *N*,*N*-dimethylformamide dimethyl acetal (1.23 mL, 9.1 mmol) was added. The reaction was heated to 55 °C for 30 min and the DMF was removed in vacuo. The resulting slurry was co-evaporated from pyridine (2×25 mL) and suspended in pyridine (50 mL). 4,4′-Dimethoxytrityl chloride (2.32 g, 6.84 mmol) was added and the reaction mixture was stirred for 3 h at rt. Most of the pyridine was removed in vacuo and the residue was dissolved in EtOAc (200 mL) and washed with half satd aq $NaHCO_3$ (2×100 mL) and brine (100 mL). The combined aqueous phases were extracted with EtOAc (2×100 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated in vacuo to give a brown foam. The product was purified by DCVC (i.d. 4 cm; 100 mL fractions; 2×50% dichloromethane in *n*-heptane (v/v); 0–10% MeOH in dichloromethane (v/v) 1% increments). Fractions containing nucleoside **7** were pooled and evaporated in vacuo to give a white foam (2.48 g, 84%). R_f = 0.43 (1%, Et_3N and 10% MeOH in dichloromethane, v/v/v). ESI-MS m/z found 653.3 ([MH]$^+$, calcd 653.3). ^{1}H NMR (DMSO-d_6): δ 11.36 (s, 1H, NH), 8.57 (s, 1H, N=CH), 7.89 (s, 1H, H8), 7.43–7.39 (m, 2H, Ar–H), 7.34–7.22 (m, 7H, Ar–H), 6.92–6.87 (m, 4H, Ar–H), 5.90 (s, 1H, H1′), 5.73 (d, 1H, J 4.7, H3′), 4.47 (s, 1H, OH), 4.40 (d, 1H, J 4.6, H2′), 4.35 (d, 1H, J 7.1, H5′), 4.31 (d, 1H, J 7.1, H5′), 3.74 (s, 6H, OCH_3), 3.51 (d, 1H, J 10.8, H1″), 3.32 (d, 1H, J 10.8, H1″), 3.14 (s, 3H, NCH_3), 3.03 (s, 3H, NCH_3). ^{13}C NMR (DMSO-d_6): δ 158.2 (Ar), 158.2, 157.6, 157.4 (C2, C6 and N=*C*H), 149.0 (C4), 144.8 (Ar), 135.5 (C8), 135.3, 129.8, 129.7, 127.9, 127.7, 126.8 (Ar), 119.9 (C5), 113.3 (Ar), 86.7, (C4′), 85.5 (C1′), 85.1 (*C*Ph$_3$), 79.3 (C3′), 71.7 (C5′), 70.7 (C2′), 59.9 (C1″), 55.1, 55.0 (2×OCH_3), 40.7, 34.7 ($N(CH_3)_2$). Elemental analysis: calcd for $C_{35}H_{36}N_6O_7$: C, 64.4; H, 5.6; N, 12.9. Found: C, 64.2; H, 5.5; N, 13.0.

4.6. (1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-(2-cyanoethoxy(diisopropylamino)phosphinoxy)-3-[2-*N*-((*N*′,*N*′-dimethylamino)methylidene)-guanin-9-yl]-2,5-dioxabicyclo[2.2.1]heptane (8)

Compound **7** (3.20 g, 4.90 mmol) was dissolved in dichloromethane–DMF (2:1, 70 mL) and 4,5-dicyanoimidazole in acetonitrile (1.0 M, 3.43 mL, 3.43 mmol) was added. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (1.72 mL, 5.39 mmol) was added dropwise to the reaction mixture. After 4 h the reaction was diluted with dichloromethane (70 mL) and transferred to a separatory funnel and extracted with satd aq $NaHCO_3$ (2×50 mL) and brine (50 mL). The combined aqueous phases were extracted with dichloromethane (100 mL). The combined organic phases were dried (Na_2SO_4), filtered and concentrated in vacuo to give a yellow foam. Purification by DCVC (i.d. 4 cm; 100 mL fractions; the column was pretreated with 1% Et_3N in *n*-heptane (v/v); 80–100% EtOAc in *n* -heptane (v/v) 5% increments; 1–10% MeOH in EtOAc (v/v) 1% increments) gave nucleoside **8** (3.68 g, 88%) as a white solid. R_f = 0.37 (1%, Et_3N and 10% MeOH in CH_2Cl_2, v/v/v). ESI-MS m/z found 853.2 ([MH]$^+$, calcd 853.4). ^{31}P NMR ($CDCl_3$) δ 149.7, 147.6.

4.7. (1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-1-methanesulfonyloxymethyl-3-(2-*N*-isobutyrylguanin-9-yl)-2,5-dioxabicyclo[2.2.1]heptane (9)

^{1}H NMR ($CDCl_3$): δ 12.13 (s, 1H, NH), 9.62 (s, 1H, NH), 7.74 (s, 1H, H8), 8.12 (s, 1H, H8), 7.35–7.24 (m, 5H), 5.83 (s, 1H, H1′), 4.71–4.45 (m, 6H, H2′, H3′, H5′, PhCH_2–), 4.12 (d, 1H, J 7.9, H1″), 3.93 (m, 1H, H1″), 3.06, (s, 3H, Ms), 2.77 (heptet, 1H, J 6.9, $(CH_3)_2CH$–), 1.27, 1.25 (s, 6H, $(CH_3)_2$CH–). ^{13}C NMR ($CDCl_3$): δ 179.4 (NHC=O), 155.2 (C6), 147.9, 147.3 (C2, C4), 136.2 (C8), 128.5, 128.2, 127.9 (Ph), 121.5 (C5), 86.9 (C4′), 85.4 (C1′), 77.6 (Ph*C*H$_2$), 72.53, 72.45 (C2′, C3′), 72.1 (C1″), 64.6 (C5′), 37.7 (Ms), 36.2 ($(CH_3)_2CH$–), 19.0, 18.9 ($(CH_3)_2$CH–).

4.8. (1*R*,3*R*,4*R*,7*S*)-7-Benzyloxy-1-methanesulfonyloxymethyl-3-(2-*N*-isobutyrylguanin-7-yl)-2,5-dioxabicyclo[2.2.1]heptane (10)

^{1}H NMR ($CDCl_3$): δ 12.47 (s, 1H, NH), 10.80 (s, 1H, NH), 7.35–7.20 (m, 5H), 6.24 (s, 1H, H1′), 4.84–4.48 (m, 6H, H2′, H3′, H5′, PhCH_2–), 4.16 (d, 1H, *J* 8.0, H1″), 3.97 (d, 1H, *J* 8.1, H1″), 3.13 (s, 3H, Ms), 2.94 (heptet, 1H, *J* 6.9, $(CH_3)_2CH$–), 1.31–1.24 (m, 6H, $(CH_3)_2$CH–). ^{13}C NMR ($CDCl_3$): δ 180.6 (NHC=O), 158.0 (C4), 153.2 (C6), 148.4 (C2), 140.5 (C8), 128.8, 128.5, 128.1 (Ph), 110.8 (C5), 88.7 (C4′), 86.1 (C1′), 77.8 (PhCH$_2$), 72.5, 72.22, 72.25 (C1″, C2′, C3′), 64.8 (C5′), 37.9 (Ms), 36.2 ($(CH_3)_2$CH–), 19.4, 19.3 ($(CH_3)_2$CH–).

4.9. (1*R*,3*R*,4*R*,7*S*)-3-(2-Amino-6-chloropurine)-7-benzyloxy-1-methanesulfonyloxymethyl-2,5-dioxabicyclo[2.2.1]heptane (17)

To a solution of compound **2** (10.1 g, 16.3 mmol) in THF (160 mL) aq LiOH (1.0 M, 82 mL, 82 mmol) was added. The reaction was stirred at ambient temperature for 45 min and glacial acetic acid (5 mL, 87 mmol) was added. The reaction mixture was transferred to a separatory funnel with brine (200 mL) and extracted with EtOAc (200 + 2×100 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated in vacuo to give a yellow liquid. The product was dissolved in dichloromethane and toluene was added. The solution was evaporated to dryness in vacuo to give a yellow foam that was purified by DCVC (i.d. 10 cm; 100 mL fractions; 50–100% EtOAc in *n*-heptane (v/v) 10% increments; 1–8% MeOH in EtOAc (v/v) 1% increments). Fractions containing nucleoside **17** were combined and evaporated in vacuo to give a white foam (7.03 g, 90%). R_f = 0.41 (100% EtOAc). ESI-MS *m*/*z* found 482.0 ([MH]$^+$, calcd 482.1). ^{1}H NMR ($CDCl_3$): δ 7.83 (s, 1H, H8), 7.33–7.26 (m, 5H, Ar–H), 5.91 (s, 1H, H1′), 5.28 (br s, 2H, NH_2), 4.73 (s, 1H), 4.68–4.59 (m, 4H), 4.31 (s, 1H), 4.18 (d, 1H, *J* 7.9), 4.00 (d, 1H, *J* 8.1) (3×CH_2, H2′ and H3′), 3.10 (s, 3H, Ms). ^{13}C NMR ($CDCl_3$): δ 159.0, 152.3, 151.6, 139.2 (C2, C4, C6 and C8), 136.5, 128.6, 128.4, 127.9 (Ar), 125.5 (C5), 88.6, 85.4, 76.9, 72.6, 72.2, 64.0, 53.8 (3×CH_2, C1′, C2′, C3′ and C4′), 37.8 (Ms). Elemental analysis: calcd for $C_{19}H_{20}ClN_5O_6S \cdot 0.5$EtOAc: C, 48.0; H, 4.6; N, 13.3. Found: C, 48.4; H, 4.5; N, 13.3.

4.10. (1*R*,3*R*,4*R*,7*S*)-3-(2-Amino-6-chloropurine-9-yl)-7-benzyloxy-1-benzoyloxymethyl-2,5-dioxabicyclo-[2.2.1]heptane (18)

Compound **17** (6.9 g, 14.3 mmol) was dissolved in DMF (300 mL) and NaOBz (4.13 g, 28.6 mmol) was added. The reaction mixture was heated to 100 °C with stirring. After 4 h the warm reaction mixture was poured into water (250 mL) and allowed to cool. The solution was transferred to a separatory funnel with brine (200 mL) and extracted with dichloromethane (200 + 2×100 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated in vacuo to give a yellow gum. The product was purified by DCVC (i.d. 10 cm; 100 mL fractions; 0–100% EtOAc in *n*-heptane (v/v) 10% increments; 0.5–3% MeOH in EtOAc (v/v) 0.5% increments). Fractions containing nucleoside **18** were pooled and evaporated in vacuo to give a white foam (6.63 g, 91%). R_f = 0.53 (20% *n*-heptane in EtOAc v/v). ESI-MS *m*/*z* found 508.0 ([MH]$^+$, calcd 508.1). ^{1}H NMR ($CDCl_3$): δ 7.56 (br d, 2H, *J* 7.1, Bz), 7.78 (s, 1H, H8), 7.60 (br t, 1H, *J* 7.4, Bz), 7.46 (br t, 2H, *J* 7.7, Bz), 7.30–7.24 (m, 5H, Ph), 5.93 (s, 1H, H1′), 5.25 (br s, 2H, NH_2), 4.80 (d, 1H, *J* 12.8, CH_2), 4.77 (s, 1H, H2′ or H3′), 4.67 (d, 1H, *J* 11.9, CH_2), 4.65 (d, 1H, *J* 12.6, CH_2), 4.56 (d, 1H, *J* 11.9, CH_2), 4.27 (d, 1H, *J* 7.9, CH_2), 4.25 (s, 1H, H2′ or H3′), 4.08 (d, 1H, *J* 8.1, CH_2). ^{13}C NMR ($CDCl_3$): δ 166.0 (PhC(O)), 159.2, 152.4, 151.8 (C2, C4 and C6), 139.1 (C8), 136.7, 133.7, 129.7, 129.3, 128.8, 128.7, 128.4, 127.9 (Ar), 125.8 (C5), 86.7 (C1′), 86.0 (C4′), 77.5, 77.4 (C2′ and C3′), 72.8, 72.6, 59.8 (3×CH_2). Elemental analysis: calcd for $C_{25}H_{22}ClN_5O_5$: C, 59.1; H, 4.4; N, 13.8. Found: C, 58.8; H, 4.3; N, 13.5.

4.11. (1*R*,3*R*,4*R*,7*S*)-3-(2,6-Tribenzoyl-2,6-diaminopurine-9-yl)-7-benzyloxy-1-benzoyloxymethyl-2,5-dioxabicyclo-[2.2.1]heptane and (1*R*,3*R*,4*R*,7*S*)-3-(2,6-tetrabenzoyl-2,6-diaminopurine-9-yl)-7-benzyloxy-1-benzoyloxymethyl-2,5-dioxabicyclo-[2.2.1]heptane (20)

Compound **18** (1.0 g, 1.97 mmol) was dissolved in MeOH saturated with ammonia (50 mL) and transferred to a heavy-wall borosilicate pressure glass tube fitted with a threaded PTFE plug and a pressure gauge. The reaction was heated to 80 °C (60 psi) for 4 h. The reaction mixture was cooled to 0 °C before opening the pressure tube. The reaction mixture was transferred to a round bottom flask and the solvent removed in vacuo to give a brown oil. The crude product was evaporated from pyridine (2×25 mL) and dissolved in pyridine (50 mL). Benzoyl chloride (1.37 mL, 11.8 mmol) was added and the reaction stirred at rt for 16 h. Most of the solvent was removed in vacuo, the residue dissolved in EtOAc (100 mL) and transferred to a separatory funnel. The organic phase was extracted with half satd aq $NaHCO_3$ (2×100 mL) and brine (100 mL). The combined aqueous phases were extracted with EtOAc (2×100 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated in vacuo to give a brown oil. The product was purified by DCVC (i.d. 4 cm; 100 mL fractions; 25–75% EtOAc in *n*-heptane (v/v) 5% increments). Fractions containing nucleosides **20** were pooled and evaporated in vacuo to give a pink foam (1.1 g, 65% 1:1 mixture of the tetra- and penta benzoylated nucleosides).

4.12. (1*R*,3*R*,4*R*,7*S*)-3-(2,6-Tribenzoyl-2,6-diaminopurine-9-yl)-7-benzyloxy-1-benzoyloxymethyl-2,5-dioxabicyclo-[2.2.1]heptane

R_f = 0.57 (30% *n*-heptane in EtOAc, v/v). HPLC: T_R = 6.49 min. ESI-MS *m*/*z* found 801.2 ([MH]$^+$, calcd 801.3).

4.13. (1*R*,3*R*,4*R*,7*S*)-3-(2,6-Tetrabenzoyl-2,6-diaminopurine-9-yl)-7-benzyloxy-1-benzoyloxymethyl-2,5-dioxabicyclo-[2.2.1]heptane

R_f = 0.67 (30% *n*-heptane in EtOAc, v/v). HPLC: T_R = 7.37 min. ESI-MS *m/z* found 905.1 ($[MH]^+$, calcd 905.3).

4.14. (1*R*,3*R*,4*R*,7*S*)-3-(2,6-*N*,*N*′-Dibenzoyl-2,6-diaminopurine-9-yl)-7-benzyloxy-1-benzoyloxymethyl-2,5-dioxabicyclo-[2.2.1]heptane

The mixture **20** was treated with diluted ammonia in MeOH for 5 min producing the tribenzoylated nucleoside. R_f = 0.37 (30% *n*-heptane in EtOAc, v/v). HPLC: T_R = 5.60 min. ESI-MS *m/z* found 697.2 ($[MH]^+$, calcd 697.2). ^{1}H NMR (DMSO-d_6): δ 11.22 (s, 1H, NH), 11.07 (s, 1H, NH), 8.40 (s, 1H, H8), 8.07 (d, 2H, *J* 7.3, Bz), 8.0 (d, 2H, *J* 7.5, Bz), 7.94 (d, 2H, *J* 7.1, Bz), 7.68–7.47 (m, 9H, Bz), 7.31–7.22 (m, 5H, Ar–H), 6.14 (s, 1H, H1′), 5.07 (s, 1H), 4.88 (d, 1H) (H2′, H3′), 4.80 (s, 2H, PhCH_2), 4.74 (d, 2H, *J* 4.6, H5′), 4.18 (d, 1H, *J* 8.2, H1″), 4.05 (d, 1H, *J* 8.1, H1″). ^{13}C NMR (DMSO-d_6): δ 165.6, 165.3 (NHC=O), 152.4, 152.2, 150.9 (C2, C4 and C6), 142.0, 141.9 (Ph), 137.8 (C8), 134.2, 133.6, 132.5, 132.0, 129.3, 128.8, 128.5, 128.2, 128.0, 127.5 (Ar), 123.5 (C5), 85.9, 85.2 (C1′, C4′), 78.2, 77.1 (C2′, C3′), 72.0, 71.3 (C1″, Ph*C*H_2), 60.7 (C5′).

4.15. (1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-(2,6-*N*,*N*′-dibenzoylaminopurin-9-yl)-2,5-dioxabicyclo[2.2.1]heptane (15)

Compound **20** (650 mg, 0.76 mmol) was dissolved in dichloromethane (3 mL) and cooled to 0 °C. Methanesulfonic acid (1.00 mL, 20 mmol) was added and after 1 h the temperature was allowed to reach rt. After an additional 3 h the reaction was diluted with dichloromethane (4 mL) and cooled to 0 °C. Amberlyst A-26 resin (40 mL) washed with NaOH (1 N (aq), 3×20 mL) was suspended in EtOH (30 mL), cooled to 0 °C and the reaction mixture was added slowly. After stirring for 10 min the resin was filtered off and washed with EtOH (3×25 mL). The solution was neutralized with HCl (1.0 N, aq) and concentrated in vacuo. The residue was lyophilized to give crude **21**. The product was evaporated from pyridine (2×25 mL) and dissolved in pyridine (35 mL). 4,4′-Dimethoxytrityl chloride (515 mg, 1.52 mmol) was added and the reaction mixture stirred overnight. Most of the pyridine was removed in vacuo and the residue dissolved in EtOAc (50 mL) and washed with half satd aq $NaHCO_3$ (2×50 mL) and brine (50 mL). The combined aqueous phases were extracted with EtOAc (2×50 mL). The combined organic phases were dried (Na_2SO_4), filtered and evaporated in vacuo to give a brown foam. The product was purified by DCVC (i.d. 2 cm; 50 mL fractions; 75–100% EtOAc in *n*-heptane (v/v) 5% increments; 1–10% MeOH in EtOAc (v/v) 1% increments). Fractions containing nucleoside **15** were pooled and evaporated in vacuo to give a white foam (452 mg, 74%). R_f = 0.18 (EtOAc). ESI-MS *m/z* found 805.2 ($[MH]^+$, calcd 805.3). ^{1}H NMR (DMSO-d_6): δ 11.23 (s, 1H, NH), 11.03 (s, 1H, NH), 8.40 (s, 1H, H8), 8.07 (d, 2H, *J* 7.3, Bz), 7.99 (d, 2H, *J* 7.3, Bz), 7.68–7.50 (m, 6H, Bz), 7.44–7.40 (m, 2H, Ar–H), 7.35–7.21 (m, 7H, Ar–H), 6.92–6.88 (m, 4H, Ar–H), 6.08 (s, 1H, H1′), 5.75 (d, 1H, *J* 4.6, H3′), 4.65 (s, 1H, OH), 4.42 (d, 1H, *J* 4.7, H2′), 4.02 (d, 1H, *J* 7.9, H5′), 3.97 (d, 1H, *J* 7.9, H5′), 3.73 (s, 6H, OCH_3), 3.59 (d, 1H, *J* 11.9, H1″), 3.31 (d, 1H, *J* 11.0, H1″). ^{13}C NMR (DMSO-d_6): δ 165.5, 165.4 (C=O), 158.0 (Ar), 152.3, 152.1, 150.7 (C2, C4 and C6), 144.6 (Ar), 140.9 (C8), 135.4, 135.1, 134.1, 133.1, 129.6, 129.5, 128.3, 128.0, 127.8, 127.5, 126.6 (Ar), 123.3 (C5), 113.1 (Ar), 87.0 (C4′), 85.4, 85.2 (C1′ and O*C*Ph_3), 78.9 (C3′), 71.7 (C5′), 70.8 (C2′), 59.9 (C1″), 54.9 (OCH_3). Elemental analysis: calcd for $C_{46}H_{40}N_6O_8 \cdot H_2O$: C, 67.1; H, 5.1; N, 10.2. Found: C, 66.9; H, 4.9; N, 10.1.

4.16. (1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-(2-cyanoethoxy(diisopropylamino)phosphinoxy)-3-[2,6-*N*,*N*′-dibenzoylaminopurin-9-yl]-2,5-dioxabicyclo-[2.2.1]heptane (16)

Compound **15** (490 mg, 0.60 mmol) was dissolved in DMF (10 mL) and 4,5-dicyanoimidazole in acetonitrile (1.0 M, 0.42 mL, 0.42 mmol) was added with stirring. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (0.21 mL, 0.64 mmol) was added dropwise to the reaction mixture. After 3 h the reaction was diluted with EtOAc (50 mL) and transferred to a separatory funnel and extracted with half satd aq $NaHCO_3$ (2×50 mL) and brine (50 mL). The combined aq phases were extracted with EtOAc (50 mL). The organic phases were pooled and dried (Na_2SO_4). After filtration the organic phase was concentrated in vacuo to give a yellow foam. Purification by DCVC (i.d. 2 cm; 20 mL fractions; the column was pretreated with 1% Et_3N in *n*-heptane (v/v); 50–100% EtOAc in *n*-heptane (v/v) 5% increments) gave nucleoside **16** (570 mg, 95%) as a white solid. R_f = 0.45 (EtOAc). ESI-MS *m/z* found 1005.3 ($[MH]^+$, calcd 1005.4). ^{31}P NMR ($CDCl_3$). δ 150.4, 149.8.

4.17. Oligonucleotide synthesis

ON were synthesized using phosphoramidite chemistry on an Expedite 8900/MOSS synthesizer (Multiple Oligonucleotide Synthesis System) on a 1 μmol scale. The β-D-LNA monomers were obtained from Proligo (Boulder, CO). After completed ON synthesis (DMT–ON), the ON were cleaved from the solid support using aqueous ammonia for 1–2 h at rt, and further deprotected for 4 h at 65 °C. ON containing 2,6-diaminopurine required deprotection for 18 h at 65 °C. The crude ON were purified by reverse phase HPLC. After removal of the DMT-group, the ON were characterized by RP-HPLC and the structure was further confirmed by ESI-MS.

4.18. UV melting analysis

Equimolar solutions of ON and their complementary DNA or RNA (final concentration, 4 μM) were mixed and dissolved in 100 mM phosphate buffer, 1 M NaCl, pH = 7, heated to 95 °C for 2 min and then cooled down to rt. Meltings were recorded on a Lambda 20 Perkin–Elmer UV–vis spectrometer attached to a PTP6 Peltier Temperature Programmer in 1 cm path-length quartz cells at 260 nm using a heating rate of 1 °C/min, a response of 0.2 s and a slit of 2 nm with a starting temperature of 5 °C. T_m values were obtained from the maxima of the first derivatives of the melting curves.

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