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Design, synthesis, biophysical and primer extension studies of novel acyclic butyl nucleic acid (BuNA)†

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A novel nucleic acid analogue called acyclic (*S*)-butyl nucleic acid (BuNA) composed of an acyclic backbone containing a phosphodiester linkage and bearing natural nucleobases was synthesized. Next, (*S*)-BuNA nucleotides were incorporated in DNA strands and their effect on duplex stability and changes in structural conformation were investigated. Circular dichroism (CD), UV-melting and non-denatured gel electrophoresis (native PAGE) studies revealed that (*S*)-BuNA is capable of making duplexes with its complementary strands and integration of (*S*)-BuNA nucleotides into DNA duplex does not alter the B-type-helical structure of the duplex. Furthermore, (*S*)-BuNA oligonucleotides and (*S*)-BuNA substituted DNA strands were studied as primer extensions by DNA polymerases. This study revealed that the acyclic scaffold is tolerated by enzymes and is therefore to some extent biocompatible.

Introduction

Nucleic acid is the genetic material that stores the hereditary information passed from one generation to the next. The growing field of DNA biotechnology requires new chemically modified nucleic acid analogues that can perform advanced and superior functions. Artificial nucleotide analogues have been widely explored to modulate the properties of nucleic acids, making them promising therapeutic agents[1] and important building blocks for constructing molecular devices.[2] Researchers have investigated various artificial nucleic acid systems containing non-ribose sugars[3] as well as acyclic scaffolds. Based on the number of carbon atoms between the two phosphate groups acyclic nucleic acids can be constructed as C-2 or C-3 building blocks.[4] The straightforward chemical synthesis of acyclic phosphoramidite building blocks, facilitates the construction of fully modified artificial nucleic acids or incorporation of these nucleotides in DNA or RNA strands.[5,6] Another interesting aspect of acyclic nucleotides is the effect of different stereoisomers on the nucleic acid structure.[5i,j] It is evident from the literature that the handedness of the helical structure of acyclic nucleic acids depends upon the chirality of the nucleotide building blocks.[7] Hence, designing novel acyclic nucleotides, which are tolerated well in DNA or RNA after incorporation offers new possibilities to explore their structure and function. In 1990 Steven A. Benner and co-workers constructed the first acyclic nucleic acid analogue from glycerol derived building blocks.[4a] Extensive studies of these oligonucleotides indicated very low duplex stability (T_m = 16 °C) of alternating adenine and thymine monomers within 19 mer oligonucleotides.[4c] Similarly, 2′-deoxy-1′,2′-seco-D-ribosyl oligonucleotides were not able to form duplexes with their complementary strands.[4e] In 2005, a breakthrough was achieved by Eric Meggers and co-workers who synthesized structurally simplified glycol nucleic acid (GNA) and demonstrated its capability to form a highly stable duplex with its complementary strands.[4f] (*S*)-GNA adopts a right-handed helix and notably substituting a single thymine (*S*)-GNA nucleotide in normal DNA duplex reduces the stability of the duplex by 13 °C.[5j] (*S*)-GNA oligonucleotide does not bind with DNA but it exhibits some capability to make duplexes with complementary RNA strands.[5j] Similarly threoninol nucleic acid (*a*TNA)[4h] and serinol nucleic acid (SNA)[4i] reported as C-3 scaffold based acyclic nucleic acids, adopt A-form and B-form-like helical structures respectively. In contrast to GNA and SNA, the *a*TNA building block contains two chiral centres but surprisingly no preorganized structure was observed for the single strands. These literature findings suggest that introduction of a second chiral centre induces an unexpected structural conformation. Collectively, these reports suggest the construction of a novel acyclic oligonucleotide which is tolerated in natural nucleic acids and is capable of hybridizing with DNA/RNA. Moreover, a new structurally simplified nucleic acid may help us to understand, how self-replicating RNA could have usurped the role of genetic material over nucleic acid-like molecules.[8]

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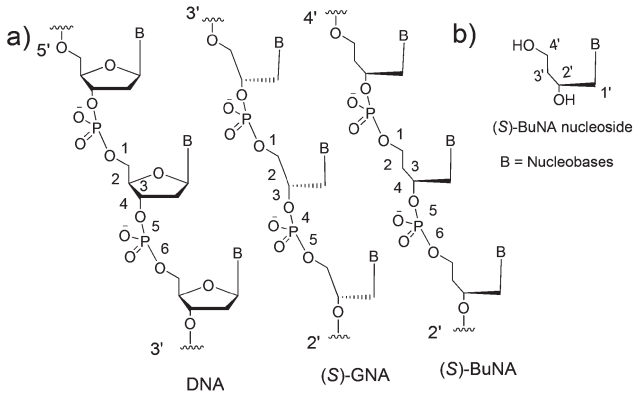
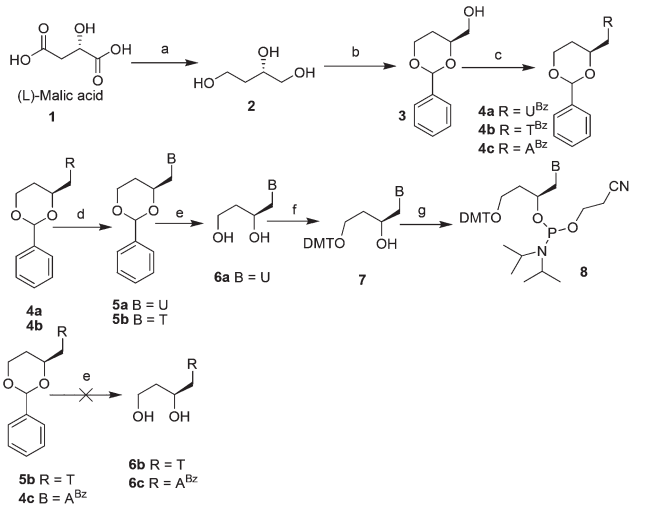


Fig. 1 (a) Chemical structures of DNA, (*S*)-GNA and (*S*)-BuNA. Numbers were assigned in order to show six-bonds-per-backbone for DNA, BuNA and five-bonds-per-backbone for (*S*)-GNA. (b) (*S*)-BuNA nucleosides.



Scheme 1 (a) Me_2S–BH_3, $B(OCH_3)_3$ dry THF, 90%. (b) Benzaldehyde dimethyl acetal, camphorsulfonic acid, dry DCM, 60%. (c) Benzoyl protected nucleobases, DEAD, PPh_3, dry THF, 40 to 60%. (d) NaOH–EtOH solution, acetic acid, 90%. (e) 10% Pd/C, H_2, MeOH, 90%. (f) DMT-Cl, dry pyridine, 80%. (g) 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, dry DCM, 70%.

When these C-2 and C-3 acyclic scaffold based nucleotides were incorporated into DNA and RNA strands, they have been found to be stable against nucleases.[4b,6] Moreover, acyclic scaffolds have been utilized to tether nucleobase surrogates (universal artificial nucleobases)[9a] or functional moieties.[9b] *a*TNA and SNA have been reported as effective C-3 scaffolds for the wedge type insertion of functional moieties working as photo switches[9c] and for tethering nucleobases.[4d,5g,h]

There are only very few acyclic scaffolds which are tolerated by enzymes in DNA strands.[10] Hence, it is important to synthesize new chiral acyclic building blocks which can be incorporated in DNA or RNA strands and can be used to construct new artificial nucleic acids. Herein, we report the synthesis of acyclic butyl nucleic acid (BuNA) with six-bonds-per-backbone of vicinal phosphodiester groups analogous to DNA and RNA (Fig. 1). Furthermore, these BuNA nucleotides were tolerated among DNA strands (primers) (Fig. 1) which was demonstrated by primer extension studies on a DNA template by Bsu and Bst DNA polymerases.

Results and discussion

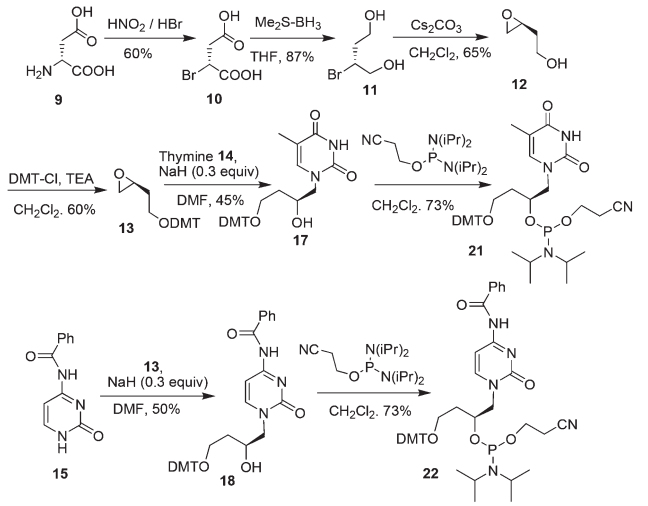
Rational design of acyclic butyl nucleic acid (BuNA)

Due to the structurally simplified structure and stable duplex formation by (*S*)-GNA, we intended to increase the back-bone length by one carbon atom, which we hoped would enable hybridization to DNA and RNA strands. The rational design of the acyclic scaffold was based on the hypothesis that keeping the number of atoms between two repeating units of monomers (Fig. 1) similar to DNA/RNA should enable the conventional Watson–Crick base-pairing to occur as in DNA/RNA. It was reported that cleavage of the C2′–C3′ bond of deoxynucleoside resulted in the (*S*)-isomer of acyclic nucleosides.[4b] In order to acquire the same configuration in BuNA as that of DNA, we synthesized the required 1,3-(*S*)-diol nucleosides[11a] from (*R*)-aspartic acid.

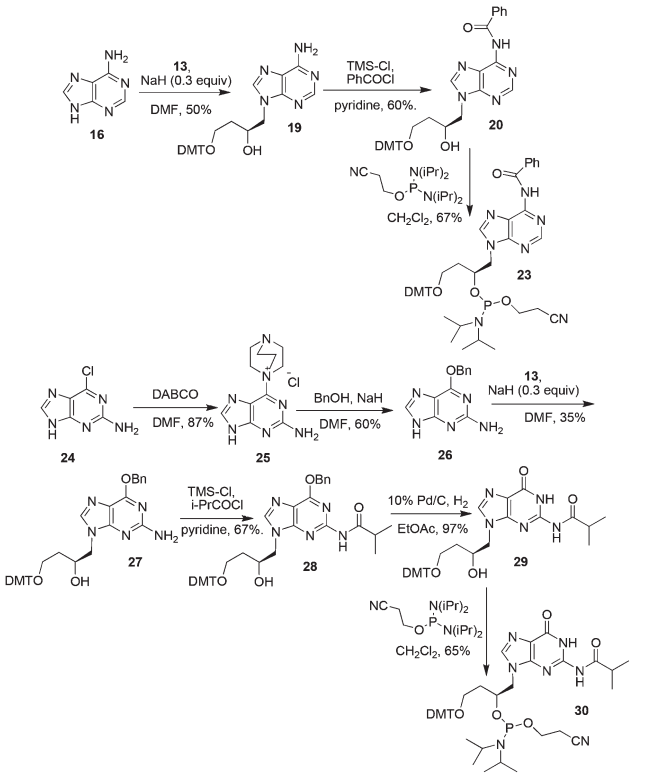
Synthesis of (*S*)-BuNA phosphoramidite building blocks

In our first attempt, (*S*)-malic acid was used as the starting material for constructing the phosphoramidite monomers as shown in Scheme 1. (*S*)-Malic acid **1** was converted to the triol by reduction with borane dimethyl sulphide complex in THF in 90% yield. Butane triol **2** was protected with benzaldehyde dimethyl acetal yielding the corresponding 1,3 protected alcohol **3** in 60% yield.[11b] The protected nucleobases and the alcohol were condensed by a Mitsunobu reaction. By this synthetic route we could condense uracil **4a**, thymine **4b** and adenine **4c** with 40–60% yield. The benzoyl group was cleaved under basic conditions to obtain **5a** and **5b** in 90% yield. Deprotection of the benzylidene protecting group was carried out by treatment with H_2 in the presence of 10% palladium/carbon. Using these conditions it was possible to deprotect only the uracil substituted compound, resulting in **6a** with 90% yield. Hydrogenation with 10% palladium/carbon of the thymine and adenine analogues to achieve nucleobase diols **6b** and **6c** was unsuccessful. Additionally, deprotection using BCl_3 led to decomposition of the substrate. The primary alcohol of the uracil nucleobase diol **6a** was converted to dimethoxy trityl chloride (DMT) alcohol **7** in 80% yield and subsequently the uracil phosphoramidite building block **8** in 70% yield. It is important to mention that we have not utilized compound **8** for oligonucleotide synthesis. In future, it might be useful for incorporation in modified siRNA.

In order to synthesis the A, T, G and C phosphoramidite building blocks, we followed an established synthetic methodology of epoxide ring opening by the nucleobase with a catalytic amount of sodium hydride in DMF as a solvent at 110 °C (Scheme 2).[4f,11d] This was achieved using (*R*)-aspartic acid **9** as the starting material. The aspartic acid was subjected to a diazotization reaction to give the corresponding bromosuccinic



Scheme 2 Synthesis of pyrimidine phosphoramidite building blocks of (*S*)-BuNA.



Scheme 3 Synthesis of purine phosphoramidite building blocks of (*S*)-BuNA.

acid **10** in 60% yield. The resulting bromosuccinic acid was converted to the bromodiol **11** by treatment with borane dimethyl sulphide complex in THF at 0 °C in 87% yield. The bromodiol was treated with cesium carbonate in dry dichloromethane and converted to epoxide **12** with 65% yield.[11c] Subsequently, this epoxide was treated with DMT chloride to afford the corresponding DMT-epoxide **13** with 60% yield. The DMT-epoxide was subjected to ring opening with nucleobase (**14**, **15** and **16**) in the presence of 0.3 equivalent of NaH in dry DMF to afford DMT-alcohols **17**, **18** (Scheme 2) and **19** (Scheme 3). Next, the exocyclic amine of compound **19** was protected by a benzoyl group to afford **20** in 60% yield. Phosphitylation of DMT-alcohols in the presence of 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite and diisopropyl ammonium tetrazolide afforded phosphoramidite building blocks **21**, **22** and **23**. In order to synthesize the guanine phosphoramidite monomer, 2-amino-6-chloropurine **24** was treated with 1,4-diazabicyclo[2.2.2]octane (DABCO) yielding the corresponding DABCO purine **25** (Scheme 3). Compound **25** was converted to O^6-benzylguanine **26** in the presence of benzyl alcohol and sodium hydride.[11e]

In order to obtain DMT-alcohol **27**, compound **26** was treated with **13** under similar conditions of epoxide ring opening. The exocyclic amine of **27** was protected by an isobutyryl group to obtain title compound **28** in 67% yield. Finally, treatment of **28** with phosphoramidite reagent and tetrazolide salt provides compound **30** in 65% yield. The regioselectivities of N-alkylation of nucleobases were confirmed by 2-D NMR (HMBC) (Fig. S4†).

Phosphoramidite monomers **21**, **22**, **23** and **30** were used to synthesize oligonucleotides (Table 1) by automated solid-phase oligonucleotide synthesis. It is noteworthy that the presence of a vicinal diol functionality in the C-2 scaffold based acyclic nucleic acid could give transesterification as a side reaction,[4b,10c] but we did not observe any side product during BuNA synthesis. Therefore, deprotection and cleavage of the

Table 1 Sequences of oligonucleotides used in this study

Entry	Oligonucleotides[a]
ON-1	4′-aaaaaaaaaaaaaaA-3′ poly(a_{14})A
ON-2	4′-ttttttttttttttT-3′ poly(t_{14})T
ON-3	4′-atatatatatatataT-3′
ON-4	4′-aaaaaaaatttttT-3′
ON-5	4′-aaaatttatattattA-3′
ON-6	4′-taataatataaatttT-3′
ON-7	4′-gtgtaataacaacaT-3′
ON-8	4′-gtgttataacaacat-2′
ON-9	4′-atgttgttattacaC-3′
ON-10	4′-atgttgttattacac-2′
ON-11	5′-AAAAAAAAAAAAAAA-3′
ON-12	5′-TTTTTTTTTTTTTTT-3′
ON-13	5′-GTGTAATAACAACAT-3
ON-14	5′-ATGTTGTTATTACAC-3′
ON-15	5′-AAAAAAAaAAAAAAA-3′
ON-16	5′-AAAaAAAAAAAaAAA-3′
ON-17	5′-TTTTTTTtTTTTTTT-3′
ON-18	5′-TTTtTTTTTTTtTTT-3′
ON-19	5′-ATGTTGTtATTACAC-3′
ON-20	5′-GTGTAATaACAACAT-3′
ON-21	5′-ATGTTGttaTTACAC-3′
ON-22	5′-GTGTAAtaaCAACAT-3′
T1	5′-CATTCACTTCCCTACGCCATGTTGTTATTACAC-3′
T2	5′-CATTCACTTCCCTACGCCAAAAAAAAAAAAAA-3′
T3	5′-CATTCACTTCCCTACGCCTTTTTTTTTTTTTT-3′

[a] Lower case letters represent (*S*)-BuNA nucleotides and upper case letters represent DNA nucleotides. 3′ notation for BuNA shows that oligonucleotides were synthesized on DNA nucleotide containing CPG solid support.

oligonucleotides from the solid support and subsequent purification can be performed by standard oligonucleotide synthesis protocols. The characterization of the resulting acyclic oligonucleotides was carried out by MALDI-TOF or LC-MS (Table S1†).

Pairing properties of (*S*)-BuNA

CD and UV-melting experiments were carried out to demonstrate the interaction between various sequences of (*S*)-BuNA with their complementary strands as well as DNA or RNA. Initially, conformational studies of poly(a_{14})A (ON-1) and poly-(t_{14})T (ON-2) were carried out individually. At 20 °C the CD spectrum of ON-1 in 10 mM phosphate buffer containing 150 mM sodium chloride at pH 7.0 revealed the preorganized structure of ON-1 with a positive cotton effect at 269 nm and a negative cotton effect at 217 nm with crossover at 247 nm (Fig. 2). Under similar experimental conditions, the CD of ON-2 was associated with the cotton effect to a much smaller extent (random coil), and showed positive peaks at 273 nm, 219 nm and negative peaks at 242 nm (Fig. 2). The conformational rigidity of ON-1 was confirmed by performing a temperature-dependent CD experiment, which showed a decrease in cotton effect at 269 nm and 217 nm (Fig. S1a†). Next, we investigated the duplex formation between ON-1 and ON-2 by mixing equimolar quantities of each strand in the same buffer. It is not unusual to obtain a different CD spectrum, when compared with their isolated components, if they are forming a duplex. Indeed, we observed similar results. Positive and negative cotton effects of single strands were transformed to give positive peaks at 273 nm, 217 nm and negative peaks at 245 nm, 203 nm (Fig. 2). This observation clearly indicates duplex formation between the two strands. Temperature-dependent CD studies demonstrated that ON-1 has a highly organized structure unlike ON-2 and therefore duplex formation resulted in loss of entropy when both single strands adopt a new conformation (Fig. S1b†). Hence,

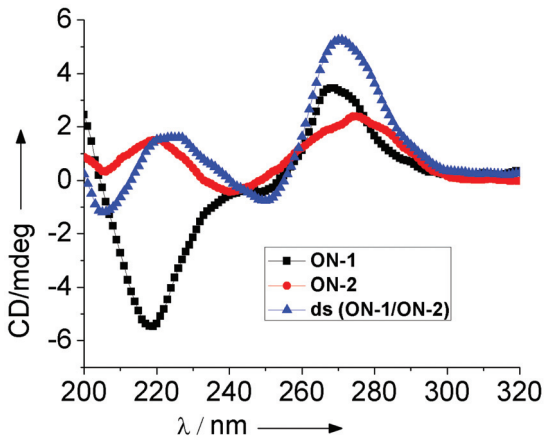


Fig. 2 CD profile of ON-1, ON-2 and their duplex. Single strand of ON-1 (20 μM) and ON-2 (20 μM) in 10 mM phosphate buffer containing 150 mM sodium chloride at pH 7. Duplex of ON-1 and ON-2 in equimolar concentration (10 μM) adopted a different conformation than single strands.

Table 2 UV-melting studies of modified (*S*)-BuNA strands and DNA

Duplex (oligo pairs)	T_m[a] (°C)	T_m[b] (°C)	T_m[c] (°C)
1 (ON-1/ON-2)	nt	22	43
2 (ON-3/ON-3)	40	nd	45
3 (ON-4/ON-4)	39	nd	43
4 (ON-5/ON-6)	37	42	40
5 (ON-8/ON-10)	21, 28	31	50

[a] UV-melting at 260 nm was carried out in 10 mM phosphate buffer containing 150 mM sodium chloride at pH 7. [b] Melting temperature in 1 M sodium chloride. [c] Melting temperature of corresponding unmodified DNA duplexes in 150 mM NaCl. nt = no melting transition was observed. nd = not determined.

no sharp melting transition was observed by UV at 260 nm with 150 mM sodium chloride. However, by increasing the salt concentration to 1 M NaCl, a sharp melting transition was observed with T_m of 22 °C (Fig. S1c†). Eschenmoser and coworkers also reported the weak pairing between poly(A_{12}) and poly(T_{12}) of α-threose nucleic acid (TNA).[12] The reported CD spectrum of a stretch of adenines of GNA was found to be completely different from BuNA.[4g] These observations clearly suggest that by introducing one additional carbon atom in the C-2 scaffold of GNA, the resulting C-3 scaffold of BuNA organizes poly adenines in a different fashion.

In order to minimize the loss of entropy in the single strands, ON-1 and ON-2, we investigated other (*S*)-BuNA oligonucleotide strands (Table 1, ON-3 to ON-10). Self-complementary (*S*)-BuNA strands ON-3 and ON-4 which contain both "a and t" acyclic nucleotides were subjected to conformational studies using CD and UV-melting under similar experimental conditions. A stable duplex formation was observed by UV-melting at 260 nm with a T_m of 40 °C and 39 °C of ON-3 and ON-4 respectively. Here we cannot rule out the formation of a hairpin-like secondary structure of self-complementary strands of BuNA similar to DNA.[13]

Next, we investigated the duplex stability of mixed sequences of "a and t" (Table 2, duplex 4) and "a, t, g and c" (Table 2, duplex 5). Duplex 4 showed a sigmoidal melting curve with T_m = 37 °C (Fig. 3a). To our surprise, introduction of "g and t" nucleotides in (*S*)-BuNA strands (duplex 5) displayed two melting transitions (Fig. 3b). In order to understand the two melting transitions, we investigated UV-melting of both single strands, which revealed the internal folding of ON-8 compared to ON-10 (Fig. S1f†). Importantly, duplex 5 displayed a single transition in the presence of 1 M NaCl. Duplex 4 showed an increase in melting temperature at 1 M sodium chloride in 10 mM phosphate buffer (Fig. 3a).

Table 2 displays the duplex stability of (*S*)-BuNA in comparison to DNA duplexes. These data reveal that BuNA duplexes show less stability compared to DNA duplexes, although they exhibit a less cooperative melting transition than DNA.

CD dependent conformational studies revealed that (*S*)-BuNA duplexes of ON-1/ON-2, self-complementary strands (ON-3 and ON-4) and mixed sequences (ON-5/ON-6 and ON-8/ON-10) were associated with A-form-like structure with positive

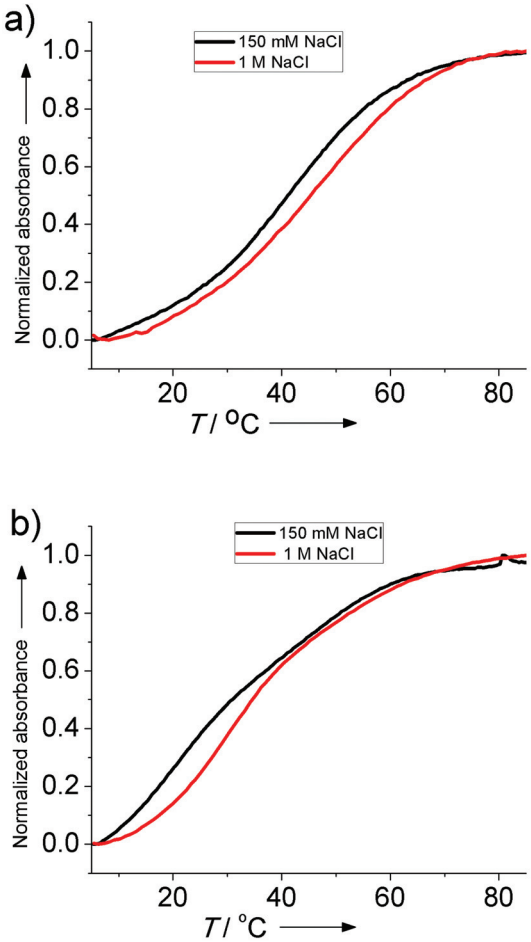


Fig. 3 UV-melting profile at 260 nm. (a) (*S*)-BuNA duplex 4 (ON-5/ON-6). (b) Duplex 5 (ON-8/ON-10). Experimental conditions: 10 mM phosphate buffer, 150 mM NaCl or 1 M NaCl, pH 7.0, 2 μM of each oligo strand.

bands at 270 nm, 223 nm and negative bands at 247 nm, 207 nm (Fig. 4). Based on previous literature reports,[7] our experimental data suggests that (*S*)-BuNA forms a left-handed helical structure.

It is evident from the literature that magnesium ions provide more stability to the folded structure of DNA and RNA duplexes therefore, (*S*)-BuNA duplexes were also investigated in the presence of magnesium chloride, which indicated that 10 mM and 100 mM magnesium ions provide more folded structures than sodium ions alone (Fig. 4). This effect of magnesium was more pronounced for duplex ON-1/ON-2 than ON-5/ON-6 (Fig. 4a and b). In the case of mixed sequences of nucleotides (a, t, g and c) ON-8/N-10 was found to be more folded than ON-5/ON-6 (containing "a and t" only). This study indicates that like natural nucleic acids BuNA also folded more effectively in the presence of divalent cations.

The preorganized structure adopted by a single strand is a very important characteristic of oligonucleotides. Therefore, we investigated temperature-dependent structural conformation studies of the (*S*)-BuNA single strand (Fig. 5a). In order to compare the helicity of the corresponding double strand (ON-8/ON-10), the CD was recorded at 10 °C for the duplex, where it showed a strong cotton effect with positive ellipticity at 275 nm, 221 nm and negative ellipticity at 243 nm, 205 nm (Fig. 5a). This observation clearly indicates that double stranded (*S*)-BuNA has a more folded and rigid structure than single strand (*S*)-BuNA. It is important to note that the CD signals disappear gradually upon increasing temperature for single and double stranded BuNA alike. These observations demonstrate that the structural conformation of mixed-sequence (a, t or a, t, g and c) of individual complimentary strands of BuNA, gain similar helical structure unlike ON-1 and ON-2 as discussed earlier (Fig. S1d and S1e†). To further demonstrate the duplex formation, the ON-8/ON-10 duplex was analysed by non-denatured polyacrylamide gel electrophoresis (PAGE) analysis. Fig. 5b clearly demonstrates the duplex formation by (*S*)-BuNA (Fig. 5, lane 4).

Next, we investigated the cross-hybridization between (*S*)-BuNA and DNA or RNA. CD studies have revealed that (*S*)-BuNA adopts a left-handed helix. Therefore, it was expected that the (*S*)-isomer of BuNA would not form a duplex with DNA or RNA. To investigate this, we carried out heteroduplex studies of BuNA with DNA and RNA by CD. The complementary parallel sequence of DNA/BuNA duplex was also studied but no hybridization was observed by these heteroduplexes (data not shown). We also investigated these heteroduplex and homoduplex pairings by non-denatured gel electrophoresis

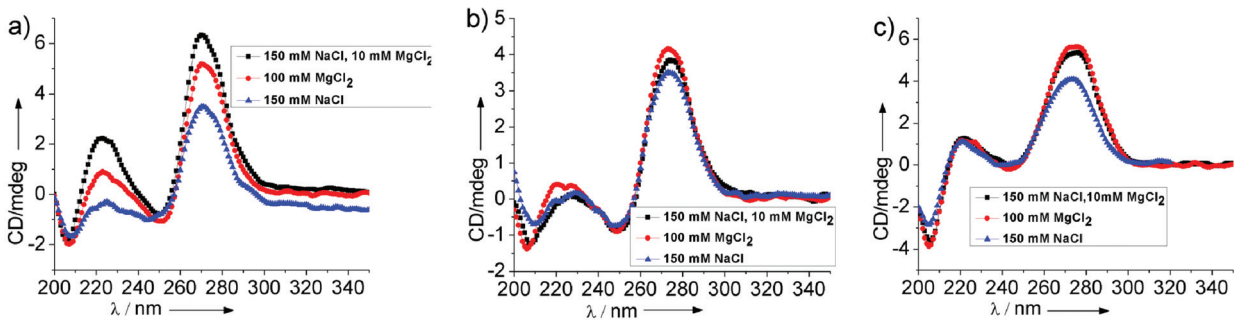


Fig. 4 Effect of magnesium ions on folding of (*S*)-BuNA duplexes. (a) ON-1/ON-2. (b) ON-5/ON-6. (c) ON-8/ON-10. Experimental conditions: [oligonucleotide] = 5 μM, 10 mM phosphate buffer containing 150 mM NaCl at pH 7.0; 10 mM tris buffer containing 100 mM $MgCl_2$ or 10 mM $MgCl_2$, 150 mM NaCl at pH 7.

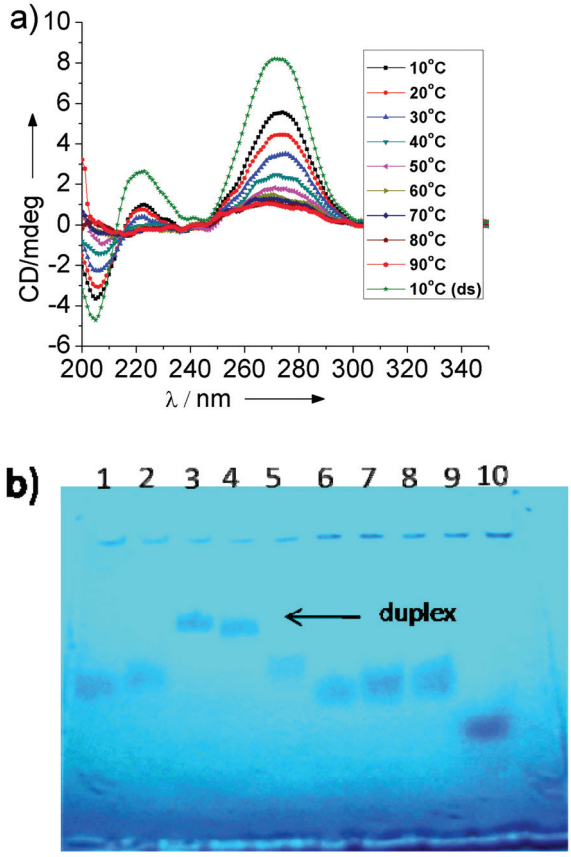


Fig. 5 Temperature-dependent CD profile and non-denatured PAGE (UV shadow) of single strands and duplexes. (a) 20 μM of ON-10 in 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7.0. Under the same experimental conditions CD was measured for the duplex (ON-8 and ON-10) containing 10 μM of each strand. (b) 20% non-denatured PAGE of single and duplex of BuNA and DNA. Lanes: 1 = ON-14, 2 = ON-10, 3 = duplex (ON-13/ON-14), 4 = duplex (ON-8/ON-10), 5 = ON-8, 6 = ON-13, hetero-duplexes 7 = (ON-10/ON-13), 8 = (ON-8/ON-14), 9 = bromophenol blue. The gel was run using 1 × 89 mM tris-borate-magnesium (TBM) buffer at 10 °C with 40 to 60 V.

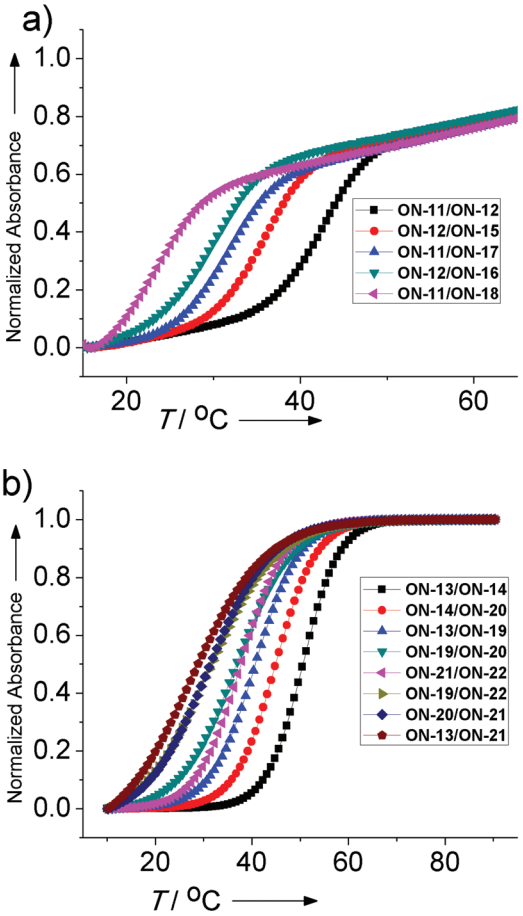


Fig. 6 UV-melting profile at 260 nm of (*S*)-BuNA modified DNA duplex. Experiment condition: 10 mM phosphate buffer, 150 mM NaCl, pH 7.0, 2 μM each oligo strand concentration. T_m values in Table 3.

(Fig. 5b) which showed the absence of oligonucleotide bands corresponding to heteroduplexes (Fig. 5b, lanes 7 and 8). Moreover, the flexible backbone of BuNA might be another reason for failing to form the heteroduplex in addition to the incompatibility of handedness. Our attempt at visualizing the gel by ethidium bromide staining did not succeed in detecting single and double stranded BuNA. Similar observations have been reported for GNA.[14] This proves the importance of the sugar moieties on the intercalation properties of DNA and RNA. These observations revealed that chirality is an important parameter in determining the handedness of the helical structure. In the literature, it has been illustrated that a peptide nucleic acid (PNA) containing all the chiral monomers can be cross-hybridized with right-handed DNA; in addition no interaction was observed with left-handed PNA.[7e]

Pairing properties of (*S*)-BuNA substituted DNA strands

In order to investigate the effect of (*S*)-BuNA nucleotide substitution in DNA strands, we measured duplex stability by UV melting at 260 nm (Fig. 6 and Table 3). Table 3 shows the effect of (*S*)-BuNA nucleotides in DNA oligomers on the melting transition. Experiments were carried out in 10 mM phosphate buffer, containing 150 mM sodium chloride at pH 7.0 with 2 μM of each oligonucleotide. Introduction of one

Table 3 UV-melting studies at 260 nm of modified DNA strands

Duplex (oligo pairs)[a]	T_m (°C)	ΔT_m[b] (°C)
1 (ON-12/ON-15)	37	−6
2 (ON-11/ON-17)	33	−9
3 (ON-13/ON-19)	41	−9
4 (ON-14/ON-20)	44	−6
5 (ON-12/ON-16)	31	−12
6 (ON-11/ON-18)	25	−18
7 (ON-13/ON-21)	28	−22
8 (ON-14/ON-22)	31	−19
9 (ON-19/ON-20)	37	−13
10 (ON-21/ON-22)	38	−12
11 (ON-20/ON-21)	31	−19
12 (ON-19/ON-22)	31	−19

[a] See Table 1 for oligonucleotide sequences. [b] Decrease in melting temperature. ΔT_m was calculated with respect to unmodified DNA duplexes T_m = 43 °C (ON-11/ON-12), T_m = 50 °C (ON-13/ON-14).

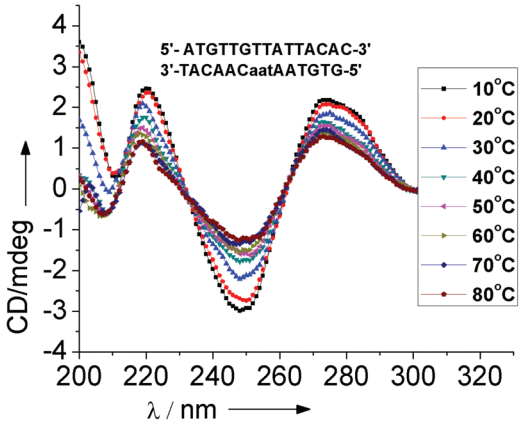


Fig. 7 Temperature-dependent CD profile of (*S*)-BuNA substituted DNA duplex (ON-13/ON-21) in phosphate buffer at pH 7 containing 150 mM NaCl.

(*S*)-BuNA thymine or one adenine nucleotide decreases the DNA duplex stability by ΔT_m/mod = −9.0 °C and ΔT_m/mod = −6.0 °C respectively (Table 3, duplex 1, 2, 3 and 4). These results suggest that (*S*)-BuNA adenine nucleotide was better tolerated than thymine nucleotide in DNA duplexes. Moreover, the destabilization effect was found to be additive for incorporation of more than one (*S*)-BuNA nucleotide (Table 3, duplex 5 and 6).

Next, we investigated the effect of three consecutive (*S*)-BuNA nucleotides in the middle of the DNA strand. This demonstrates that "tta" (ON-21) and "aat" (ON-22) incorporation destabilize the duplex by ΔT_m = −22 °C and ΔT_m = −19 °C respectively (Table 3, duplex 7 and 8).

On the other hand hybridization of complementary substituted (*S*)-BuNA nucleotides within a double stranded DNA sequence revealed a more stable duplex (T_m = 38 °C) than (*S*)-BuNA nucleotides substituted in one DNA strand (Table 3, duplex 10). To further test this hypothesis, we analysed the duplex stability of modified duplexes with one strand containing one substitution and the complementary strand containing three modifications. These duplexes were better stabilized (Table 3, duplex 11, 12) than duplex 7 and equally stabilized as duplex 8, suggesting that (*S*)-BuNA modifications were better accommodated when equal number of modifications were present in both strands and opposite to each other. These studies clearly indicate that (*S*)-BuNA nucleotides are tolerated in DNA duplexes when they are incorporated as base pairs. Furthermore, the temperature dependent CD profile showed that incorporation of three consecutive BuNA nucleotides (ON-22) resulted in a considerable decrease of the cotton effects of the DNA duplex, and still maintained a B-form helical structure (Fig. 7).

Primer extension studies of modified DNA strands

In literature acyclic universal base analogues have been incorporated in primers for PCR and sequencing.[10b] We wanted to investigate whether integrating (*S*)-BuNA nucleotides in DNA primers would disrupt their ability to participate in primer extension on a DNA template by DNA polymerases. As discussed earlier, we demonstrated that the B-form helical structure remained intact for modified DNA strands and, these results encouraged us to use these modified DNA strands as primers. For this study, we investigated nine modified primers of 15 nucleotides on 33 nucleotides of DNA templates (Fig. 8). Bst, Bsu, Taq and Therminator polymerases were employed to perform primer extension. Previously, Bst DNA polymerase has been used to demonstrate DNA polymerization on a (*S*)-GNA template,[10c] and therefore we started our studies by employing Bst as thermophilic enzyme. Moreover, structural studies of the Bst enzyme have been investigated by X-ray crystallography together with a primer/template complex.[15] Modified primer sequences containing one (*S*)-BuNA nucleotide at position 7, two nucleotides at positions 4, 12 and three consecutive nucleotides at position 7, 8, 9 were investigated. Our hypothesis was that the positions of the acyclic nucleotides exist in the DNA duplex binding region of the enzyme. When (*S*)-BuNA substituted DNA strands were employed for primer extension, it resulted in full extension of the modified primers (Fig. 8a, lanes 1, 2, 6, 7) similar to unmodified DNA primers (Fig. 8a, lanes 4, 9) by Bst polymerase. No extension was observed for fully modified primers (Fig. 8a, lanes 3, 8). It is important to note that ON-18 was found to form an unstable duplex (T_m = 25 °C) with its complementary strand, and consequently resulted in a lower yield (Fig. 8a, lane 6). Similar results were obtained when extension was carried out with Bsu polymerase (mesophilic enzyme) at 25 °C. Gel results show that primers ON-19 and ON-22 (Fig. 8b, lanes 5, 6) extended with efficiencies comparable to that of unmodified primer ON-13 (Fig. 8b, lanes 4). As in the case of Bst, fully modified BuNA primers did not result in any extension by Bsu DNA polymerase (Fig. 8b, lanes 7, 8). Even incubation at 5 °C for 12 h with Bsu polymerase did not show any extension. This study clearly suggests an incompatibility of fully modified BuNA strands with DNA polymerases. Based on the fully modified (*S*)-BuNA strands inability to engage in hybridization with normal DNA we anticipated their failure in the primer extension. Similar results were obtained for Taq and Therminator polymerase (data not shown). It is reasonable to assume that a BuNA substituted primer/template duplex is compatible with DNA polymerases for their elongation. Hence, DNA was successfully modulated by incorporation of BuNA nucleotides and still recognized by the enzymes.

Conclusions

In summary, we report the synthesis of a novel acyclic scaffold of (*S*)-butyl nucleic acid (BuNA) and demonstrate its ability to form a duplex with its complementary strands. To the best of our knowledge this is the first reported synthesis of BuNA, where monomers are conjugated through phosphodiester linkages and tethering natural nucleobases. Furthermore, we demonstrated the incorporation of (*S*)-BuNA nucleotides in DNA strands, which resulted in a destabilized duplex, known

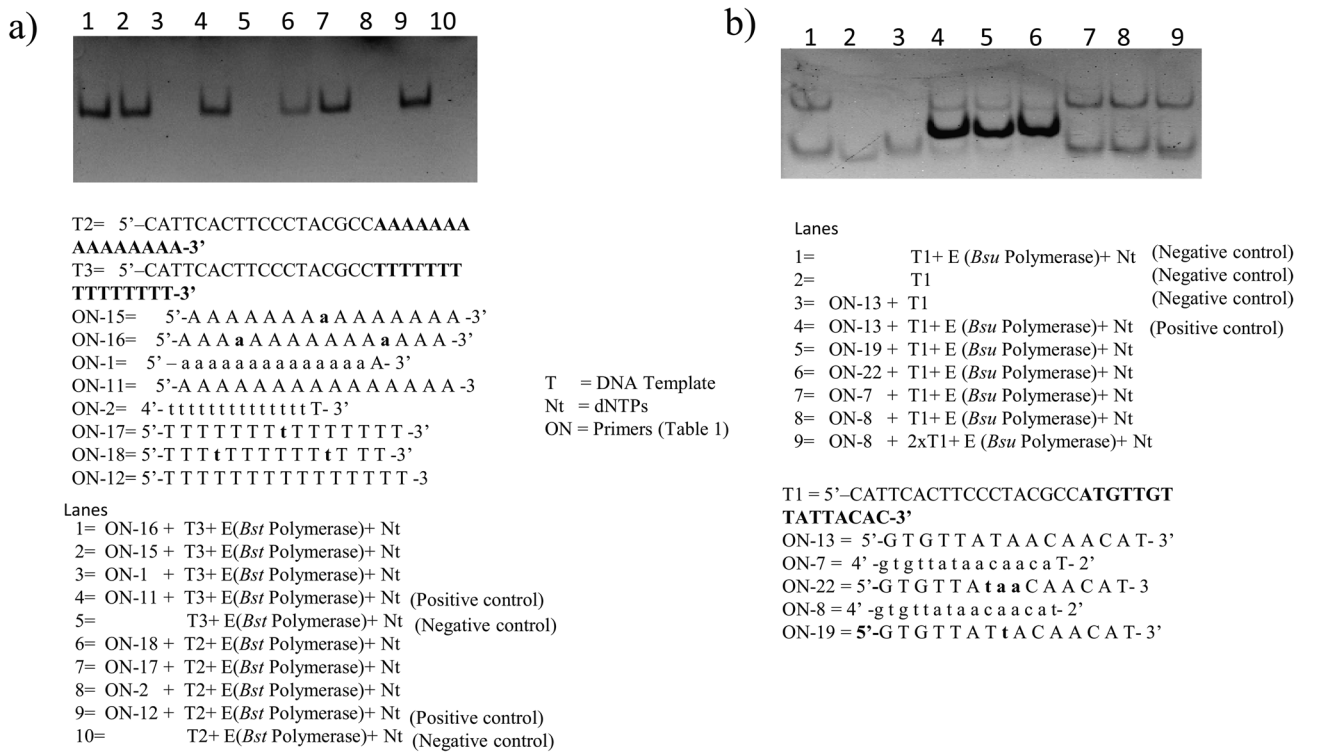


Fig. 8 Primer extension studies of (*S*)-BuNA incorporated DNA primers and fully modified BuNA primers. (a) Thermophilic enzyme (Bst polymerase) was used for primer extension studies at 55 °C. (b) Mesophilic enzyme (Bsu polymerase) was used for primer extension studies at 25 °C. Primer, enzyme, DNA template and dNTPs were incubated for 2 h at 25 °C for Bsu enzyme and at 55 °C for Bst DNA polymerase. After completion of enzymatic reaction, the mixture was loaded in non-denatured gel electrophoresis of 14% PAGE and the system was run in a cold room (10 °C) at 50 V in 1 × 89 mM tris-borate-magnesium (TBM) buffer. The gel was stained by ethidium bromide.

as an intrinsic property of acyclic scaffolds. These studies revealed that increasing the backbone length by one carbon atom of GNA resulted in a decrease in melting temperature of (*S*)-BuNA duplexes in comparison to GNA duplexes. Although (*S*)-BuNA adopts left-handed structure, it might possible that (*R*)-BuNA will adopt a right-handed structure resulting in duplex formation with natural nucleic acids. We also investigated primer extension studies of fully modified (*S*)-BuNA strands and (*S*)-BuNA substituted DNA primers by DNA polymerases. This study revealed that the (*S*)-BuNA scaffold is biocompatible with DNA polymerase and this provides a platform where other organic moieties or fluorescent functionalities can be tethered to this scaffold and can be introduced in DNA oligomers to modulate its function. In order to obtain a right handed helix of BNA the synthesis of (*R*)-BuNA is in progress.

Experimental section

General methods

CD experiments were performed in Milli-Q water using a Jasco J-815 instrument and UV experiments were carried out by a Varian Cary 300 BIO instrument. CD experiments were carried out with 1 mm path length quartz cuvettes at 20 °C, if temperature is not mentioned. CD data were collected at a scan rate 100 nm min^{-1} at 1 nm data intervals and are presented as an average of three successive scans. CD spectra were baseline subtracted and smoothed *via* a five point adjacent averaging algorithm. UV melting studies were carried out by increasing the temperature at a rate of 1 °C min^{-1}. Melting temperature (T_m) was determined by first derivatives of melting curves. Experiments were repeated three times and average values were taken for calculating reported parameters. Unmodified oligonucleotides and DNA templates were purchased from Sigma-Aldrich. DNA polymerases were purchased from New England Biolabs.

Primer extension studies

Unmodified, modified and fully modified primers (0.5 μM) and the corresponding templates (0.5 μM) were annealed in a reaction mixture of 1 × reaction buffer in 200 μl PCR tube. After brief cooling at room temperature, DNA polymerase (2 to 10 units) was added followed by the addition of dNTPs (200 μM). Reaction tubes were incubated at the required temperature for the optimal time period. Elongation of primers was analyzed by non-denatured gel electrophoresis (14% PAGE) using 1 × 89 mM tris-borate-magnesium (TBM) buffer at 10 °C with 40 to 60 V.

(*R*)-2-Bromosuccinic acid (10)

To a solution of sodium bromide (60 g, 588 mmol) in 6 N sulfuric acid (25 ml), (*R*)-aspartic acid **9** (20 g, 150 mmol) was added and cooled at 0 °C. Sodium nitrite (12.4 g, 180 mmol) was added slowly over a time period of 30 min under nitrogen pressure and temperature was maintained 0 °C. The reaction mixture was stirred for the next 3 h. Urea (4.6 g) was added and the reaction mixture stirred for 20 min. The compound was extracted with diethyl ether (3 × 100 ml). The organic layer was dried over sodium sulphate and concentrated to give a pale yellow solid in 60% yield. $[\alpha]_D^{25}$ = +5.9 (*c* = 1 in methanol). ^{1}H NMR (500 MHz, DMSO) δ 4.51 (dd, *J* = 6.3, 8.8 Hz, 1 H), 3.07 (dd, *J* = 8.5, 17.3 Hz, 1 H), 2.91–2.84 (m, 1 H); ^{13}C NMR (125 MHz, DMSO) δ 171.46, 170.59, 41.01, 40.03.

(*R*)-2-Bromobutane-1,4-diol (11)

Dry THF was added to (*R*)-2-bromosuccinic acid **10** (16.0 g, 81.6 mmol). A 2 M solution of borane dimethyl sulfide complex in THF (2.7 equiv.) was added drop by drop at 0 °C. The reaction was allowed to stir for 5 h at room temperature. After completion, the reaction was quenched with methanol drop by drop and the solvent was removed under vacuum. Three times co evaporation was carried out with methanol to give a pale yellow oil as crude product. Purification was carried out by silica gel chromatography with (5 to 10% methanol–dichloromethane) to afford a colorless oil (12.1 g, 87%) yield. $[\alpha]_D^{25}$ = +2.9 (*c* = 1 in methanol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.88 (br s, 1 H), 4.34–4.29 (m, 1 H), 3.90–3.86 (m, 1 H), 3.85–3.84 (m, 2 H), 3.82–3.77 (m, 1 H), 2.18–2.05 (m, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 67.22, 60.17, 55.03, 37.93.

(*S*)-2-(Oxiran-2-yl)ethanol (12)

Dry dichloromethane was added to (*R*)-bromodiol **11** (7.3 g, 4.43 mmol) and stirred for 5 min. 1.8 equiv. cesium carbonate (25.2 g, 77.3 mmol) was added under argon. The reaction was stirred for 72 hours at room temp. After completion, the reaction mixture was filtered and washed with dichloromethane. The filtrate was dried over magnesium sulphate and organic solvent was removed by vacuum to give a slightly pale yellow oily liquid (*S*)-epoxide (2.4 g, 60%). The next step was carried out without further purification. $[\alpha]_D^{25}$ = −2.6 (*c* = 1 in methanol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.85–3.78 (m, 2 H), 3.12–3.08 (m, 1 H), 2.81–2.80 (m, 1 H), 2.59 (d, *J* = 1.9 Hz, 1 H), 2.02–1.96 (m, 1 H), 1.77–1.70 (m, 1 H); ^{13}C NMR (125 MHz $CDCl_3$) δ 60.09, 50.64, 46.67, 34.75.

(*S*)-2-(2-(Bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)oxirane (13)

(*S*)-Epoxide **12** (3.0 g, 34 mmol) was dissolved in dry dichloromethane followed by the addition of triethylamine (8.6 g, 85 mmol) and dimethoxy trityl chloride (13.8 g, 40 mmol) at room temp under a nitrogen atmosphere. The reaction was allowed to stir overnight. After completion of the reaction, the product was filtered and the filtrate poured into a saturated aqueous sodium bicarbonate solution and extracted with dichloromethane. The organic layer was collected and washed with brine, dried over sodium sulphate and concentrated under vacuum to afford a crude yellow colored paste. The compound was purified by silica gel chromatography (2% ethyl acetate–hexane with 1.0% triethylamine) giving a pasty mass (7.5 g, 60% yield). $[\alpha]_D^{25}$ = −0.5 (*c* = 1 in dichloroethane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51–7.49 (m, 2 H), 7.40–7.37 (m, 4 H), 7.35–7.32 (m, 2 H), 7.28–7.20 (m, 1 H), 6.90–6.87 (m, 4 H), 3.82 (s, 6 H), 3.34–3.25 (m, 2 H), 3.17–3.13 (m, 1 H), 2.84–2.82 (m, 1 H), 2.56 (dd, *J* = 2.5, 5.0 Hz, 1 H), 1.89–1.85 (m, 2 H); ^{13}C NMR (125 MHz $CDCl_3$) δ 158.34, 145.05, 136.25, 129.91, 128.05, 127.69, 126.60, 113.00, 85.96, 60.36, 55.15, 55.05, 50.26, 47.13, 33.32; HRMS calcd for $C_{25}H_{26}O_4Na$ $[M + Na]^+$ 413.1729, found $[M + Na]^+$ 413.1728.

(*S*)-1-(4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-2-hydroxybutyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (17)

To a stirred solution of thymine **14** (500 mg, 3.96 mmol) in dry DMF. 0.3 equiv. NaH was added and the reaction mixture was stirred for 1 h at room temperature. DMT epoxide (1.3 g, 3.3 mmol) dissolved in dry DMF was added and the reaction mixture was stirred for 12 h at 110 °C. The solvent was removed under vacuum and ethyl acetate was added and washed with saturated bicarbonate solution. The organic phase was washed with brine and dried over sodium sulphate. The organic layer was concentrated and loaded over a silica gel column. The compound was eluted with 5 to 50% ethyl acetate–hexane with 1% triethylamine to afford a white foam product (800 mg, 45%). $[\alpha]_D^{25}$ = +22.9 (*c* = 1 in dichloroethane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33–7.30 (m, 2 H), 7.24–7.19 (m, 6 H), 7.16–7.12 (m, 1 H), 7.08 (s, 1 H), 6.78–6.74 (m, 4 H), 4.01–3.94 (m, 1 H), 3.85 (dd, *J* = 2.5, 13.9 Hz, 1 H), 3.72 (s, 6 H), 3.40 (dd, *J* = 8.2, 13.9 Hz, 1 H), 3.37–3.34 (m, 1 H), 3.25–3.19 (m, 1 H), 1.83 (s, 3 H), 1.70–1.65 (m, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.11, 158.60, 151.20, 144.40, 142.12, 135.52, 129.90, 127.99, 127.93, 126.95, 113.27, 109.75, 87.11, 70.33, 62.14, 55.22, 53.67, 33.77, 12.26; HRMS calcd for $C_{30}H_{32}N_2O_6Na$ $[M + Na]^+$ 539.2158, found $[M + Na]^+$ 539.2156.

(*S*)-*N*-(1-(4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-2-hydroxybutyl)-2-oxo-1,2-dihydropyrimidin-4-yl)benzamide (18)

N^4-Benzoyl cytosine **15** (1.0 g, 4.6 mmol) was dried under vacuum and dry DMF was added under nitrogen. 0.3 equiv. of NaH was added under nitrogen and stirred for 1 h. DMT epoxide (2.153 g, 5.5 mmol) dissolved in dry DMF was added and the reaction mixture was allowed to stir for 20 h at 110 °C. DMF was removed under vacuum and ethyl acetate was added to residue and washed with saturated sodium bicarbonate solution. The organic layer was washed with brine solution and dried over sodium sulphate. The ethyl acetate was removed by vacuum and the residue was loaded over silica gel column. The compound was eluted by (20 to 100)% ethyl acetate–hexane) with 1% triethylamine to afford a white foam solid (1.44 g, 50%). $[\alpha]_D^{25}$ = +40.6 (*c* = 1 in dichloroethane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84 (d, *J* = 7 Hz, 2 H), 7.67

(d, J = 7.5 Hz, 1 H), 7.54–7.50 (m, 1 H), 7.44–7.40 (m, 2 H), 7.32–7.29 (m, 2 H), 7.22–7.18 (m, 7 H), 7.14–7.11 (m, 1 H), 6.76–6.72 (m, 4 H), 4.15–4.07 (m, 2 H), 3.70 (s, 6 H), 3.52 (dd, J = 8.0, 13.2 Hz, 1 H), 3.35–3.31 (m, 1 H), 3.25–3.21 (m, 1 H), 1.78–1.65 (m, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 162.36, 158.59, 150.57, 144.51, 135.81, 135.67, 133.12, 129.93, 129.90, 129.02, 128.00, 127.62, 126.94, 113.29, 96.16, 87.00, 69.42, 61.90, 56.08, 55.24, 34.03; HRMS calcd for $C_{36}H_{35}N_3O_6Na$ $[M + Na]^+$ 628.2424, found $[M + Na]^+$ 628.2424.

(*S*)-1-(6-Amino-9*H*-purin-9-yl)-4-(bis(4-methoxyphenyl)-(phenyl)methoxy)butan-2-ol (19)

To a stirred suspension of adenine **16** (2.1 g, 15.5 mmol) in dry DMF, 0.3 equiv. NaH was added and reaction mixture was stirred for one hour at room temperature. DMT epoxide (6.0 g, 15.4 mmol) dissolved in dry DMF was added and the reaction mixture was stirred for 12 h at 110 °C. DMF was removed by vacuum and ethyl acetate was added, which was washed with saturated bicarbonate solution. The organic layer was washed with brine and dried over sodium sulphate. The solvent was concentrated and the compound was purified by silica gel column chromatography. The compound was eluted with 20 to 80% ethyl acetate–hexane with 1% triethylamine to afford product (4.0 g, yield 50%). $[\alpha]_D^{25}$ = +2.7 (c = 1 in dichloroethane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.32 (s, 1 H), 7.83 (s, 1 H), 7.42–7.39 (m, 2 H), 7.33–7.28 (m, 6 H), 7.25–7.21 (m, 1 H), 6.86–6.82 (m, 4 H), 5.88 (br, 2 H), 4.32 (dd, J = 2.5, 14.2 Hz, 1 H), 4.26–4.19 (m, 1 H), 4.16–4.10 (m, 1 H), 3.80 (s, 6 H), 3.44–3.40 (m, 1 H), 3.32–3.28 (m, 1 H), 1.84–1.69 (m, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.69, 155.59, 152.86, 144.70, 141.87, 135.99, 135.88, 130.04, 128.15, 127.05, 119.48, 113.37, 86.98, 69.78, 61.60, 60.53, 55.36, 50.25, 34.19; HRMS calcd for $C_{30}H_{31}N_5O_4$ $[M]^+$ 526.2454, found $[M]^+$ 526.2452.

(*S*)-*N*-(9-(4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-2-hydroxybutyl)-9*H*-purin-6-yl)benzamide (20)

Compound **19** (500 mg, 0.95 mmol) was dried well and dissolved in anhydrous pyridine under nitrogen. TMS-Cl (412.8 mg, 3.8 mmol) was added at 0 °C followed by stirring at 0 °C for 30 min and at room temperature for the next 4 h. Benzoyl chloride (200 mg, 1.42 mmol) was added drop by drop at 0 °C and the reaction was stirred for 4 h at room temperature. After completion of the reaction, water was added to stop the reaction and aqueous ammonia was added at 0 °C and stirred for 1 h. The compound was extracted with dichloromethane. The organic layer was dried over sodium sulphate and concentrated to give an yellow oily paste. The resulting oily paste was dissolved in THF and 1 M solution tetrabutylammonium fluoride in THF was added and stirred for 30 min at room temperature. Dichloromethane was added to the reaction mixture and the product was washed with water and the organic layer was washed with brine and dried over sodium sulphate. The organic layer was concentrated and loaded over a silica gel column. The compound was eluted with 20 to 90% ethyl acetate–hexane with 1% triethylamine to afford a white colored foam (400 mg, 60%). $[\alpha]_D^{25}$ = +12.2 (c = 1 in dichloroethane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.17 (br s, 1 H), 8.65 (s, 1 H), 7.99 (s, 1 H), 7.94 (d, J = 7 Hz, 2 H), 7.52–7.47 (m, 1 H), 7.44–7.38 (m, 2 H), 7.33–7.28 (m, 2 H), 7.23–7.16 (m, 6 H), 7.15–7.09 (m, 1 H), 6.76–6.70 (m, 4 H), 4.29 (dd, J = 2.5, 13.9 Hz, 1 H), 4.18–4.03 (m, 3 H), 3.69 (s, 6 H), 3.36–3.30 (m, 1 H), 3.25–3.21 (m, 1 H), 1.76–1.67 (m, 1 H), 1.66–1.56 (m, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.94, 158.79, 152.59, 152.39, 149.56, 144.72, 144.56, 135.98, 135.85, 133.96, 132.90, 130.11, 129.01, 128.18, 128.12, 127.14, 122.76, 113.47, 87.16, 69.85, 61.76, 55.43, 50.00, 34.20; HRMS calcd for $C_{37}H_{36}N_5O_6$ $[M + H]^+$ 630.2716, found $[M + H]^+$ 630.2725.

Thymine phosphoramidite (21)

Compound **17** (703 mg, 1.4 mmol) was dried under high vacuum and anhydrous dichloromethane was added followed by the addition of diisopropyl ammonium tetrazolide (120 mg, 0.5 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (633 mg, 1.5 mmol) and the reaction was allowed to stir at room temperature. After 3 h the reaction was diluted with dichloromethane and poured into saturated aqueous sodium bicarbonate. The product was extracted in dichloromethane and dried over magnesium sulphate. The product was purified by silica gel column with dichloromethane–hexanes (1 to 80%) with 1% triethylamine to afford the product (766 mg, yield 73%) as a white foam solid. ^{31}P NMR (121 MHz, $CDCl_3$) δ 149.119, 148.406; HRMS calcd for $C_{39}H_{49}N_4O_7PNa$ $[M + Na]^+$ 739.3237, found $[M + Na]^+$ 739.3234.

Cytosine phosphoramidite (22)

Compound **18** (605 mg, 1 mmol) was dried under high vacuum and anhydrous dichloromethane was added and subsequently diisopropyl ammonium tetrazolide (85 mg, 0.5 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (452 mg, 1.5 mmol) was added. The reaction was allowed to stir at room temperature. After 4 h, the reaction mixture was diluted with dichloromethane and poured in saturated aqueous sodium bicarbonate. Product was extracted in dichloromethane and dried over magnesium sulphate. The product was purified by silica gel column by dichloromethane–hexanes (10 to 60%) with 1% triethylamine to afford product as white foam (593 mg, yield 73%). ^{31}P NMR (121 MHz, $CDCl_3$) δ 149.094, 148.922; HRMS calcd for $C_{45}H_{52}N_5O_7PNa$ $[M + Na]^+$ 828.3502, found $[M + Na]^+$ 828.3505.

Adenine phosphoramidite (23)

Compound **20** (629 mg, 1 mmol) was dried under high vacuum and anhydrous dichloromethane was added followed by the addition of diisopropyl ammonium tetrazolide (85 mg, 0.5 mmol) under nitrogen. After 1 min 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (376 mg, 1.25 mmol) was added and the reaction was allowed to stir at room temperature. After 3 h the reaction mixture was diluted with dichloromethane and poured into saturated aqueous sodium bicarbonate. The product was extracted in dichloromethane and dried over magnesium sulphate. The product was purified by silica gel column using dichloromethane–hexanes (20 to

30%) with 1% triethylamine to afford the product as a white foam (560 mg, yield 67%). ^{31}P NMR (121 MHz, $CDCl_3$) δ 149.119, 148.406; HRMS calcd for $C_{46}H_{52}N_7O_6P$ $[M + H]^+$ 830.3795, found $[M + H]^+$ 830.3796.

1-(2-Amino-9*H*-purin-6-yl)-4-aza-1-azoniabicyclo[2.2.2]octyl chloride (25)

To a stirred solution of 2-amino-6-chloropurine **24** (1.0 g, 5.9 mmol) in dry DMSO, DABCO (3.64 g, 32.45 mmol) was added and the reaction mixture was stirred overnight. After completion of the reaction, the product was filtered and washed with dichloromethane. The product was dried under high vacuum to afford 87% yield as a white powder. ^{1}H NMR (500 MHz, D_2O) δ 8.25 (s, 1 H), 4.18 (t, J = 10 Hz, 6 H), 3.43 (t, J = 10 Hz, 6 H); ^{13}C NMR (125 MHz, D_2O) δ 159.08, 158.11, 151.33, 143.66, 117.03, 53.51, 44.33.

6-(Benzyloxy)-9*H*-purin-2-amine (26)

Benzyl alcohol (2.5 g, 23.1 mmol) was added to stirred solution of **25** and NaH (342 mg, 8.4 mmol) in dry DMSO. After 1 h of stirring, DABCO purine **19** (1.2 g, 4.2 mmol) was added and the reaction mixture was stirred overnight. DMSO was removed under vacuum and the compound was dissolved in ethyl acetate. The organic layer was washed with water and dried over sodium sulphate. The solvent was removed by vacuum and the solid was purified by silica gel chromatography (20 to 80% ethyl acetate–hexane) to give gave the product (600 mg, 60% yield) as a light brown powder. ^{1}H NMR (500 MHz, DMSO) δ 12.45 (br s, 1 H), 7.84 (s, 1 H), 7.51 (d, J = 7.6 Hz, 2 H), 7.40 (t, J = 7.5 Hz, 2 H), 7.34 (d, J = 7 Hz, 1 H); ^{13}C NMR (125 MHz, DMSO) δ 159.65, 155.23, 137.81, 136.77, 128.42, 128.37, 127.98, 113.51, 66.69.

(*S*)-1-(2-Amino-6-(benzyloxy)-9*H*-purin-9-yl)-4-(bis-(4-methoxyphenyl)(phenyl)methoxy)butan-2-ol (27)

To a suspension of *O*-benzyl purine **26** (2.4 g, 9.9 mmol) in dry DMF, 0.3 equiv. NaH was added and the reaction mixture was stirred for one hour at room temperature. DMT epoxide (4.2 g, 10.2 mmol) dissolved in dry DMF was added and the reaction mixture was stirred for 12 hours at 110 °C. The solvent was removed by vacuum and workup was done with ethyl acetate and saturated sodium bicarbonate solution. The organic layer was washed with brine and dried over sodium sulphate. The solvent was removed by rotavapour and loaded over a silica gel column. The compound was eluted with 20 to 80% ethyl acetate–hexane with 1% triethylamine to afford the product as a yellow foam (2.2 g, 35% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 (s, 1 H), 7.42–7.41 (m, 2 H), 7.33–7.31 (m, 2 H), 7.28–7.23 (m, 2 H), 7.23–7.18 (m, 7 H), 7.14–7.11 (m, 1 H), 6.76–6.73 (m, 4 H), 5.46 (s, 2 H), 4.75 (br s, 2 H), 4.12–4.06 (m, 2 H), 3.91–3.84 (m, 1 H), 3.70 (s, 6 H), 3.28–3.24 (m, 1 H), 3.19–3.14 (m, 1 H), 1.66–1.62 (m, 2 H); ^{13}C NMR (125 MHz, CDCl3) δ 161.17, 159.00, 158.67, 154.22, 144.85, 140.76, 136.57, 136.15, 136.05, 130.09, 128.51, 128.38, 128.19, 128.12 128.05, 126.99, 115.58, 113.34, 86.81, 69.53, 68.25, 61.27, 55.36, 50.26, 34.30; HRMS calcd for $C_{37}H_{37}N_5O_5Na$ $[M + Na]^+$ 654.2692; found $[M + Na]^+$ 654.2692.

(*S*)-*N*-(6-(Benzyloxy)-9-(4-(bis(4-methoxyphenyl)(phenyl)-methoxy)-2-hydroxybutyl)-9*H*-purin-2-yl)isobutyramide (28)

Compound **27** (740 mg, 1.2 mmol) was dried well under high vacuum and dissolved in anhydrous pyridine under nitrogen. TMS-Cl (956 mg, 8.8 mmol) was added at 0 °C and stirred at room temperature for 2 h. Isobutyryl chloride (639 mg, 5.9 mmol) was added drop by drop at 0 °C and the reaction mixture was stirred for an additional 4 h at room temperature. After completion of the reaction water was added to stop the reaction and the aqueous ammonia was added at 0 °C and stirred for 1 h. The compound was extracted with dichloromethane. The organic layer was dried over sodium sulphate and concentrated to give a yellow oily paste. The resulting oily paste was dissolved in THF and 1 M solution tetrabutylammonium fluoride in THF was added and stirred for 30 min at room temperature. The compound was washed with water in dichloromethane and further the organic layer was washed with brine and dried over sodium sulphate. The organic layer was concentrated and loaded over a silica gel column. Column chromatography was carried out with 20 to 90% ethyl acetate–hexane with 1% triethylamine to afford a white foam (842.17 mg, 67%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (br s, 1 H), 7.67 (s, 1 H), 7.44–7.41 (m, 2 H), 7.32–7.26 (m, 4 H), 7.23–7.19 (m, 8 H), 7.15–7.11 (m, 1 H), 6.76–6.73 (m, 4 H), 5.53 (s, 2 H), 4.23 (dd, J = 2.2, 14.2 Hz, 1 H), 4.13–4.07 (m, 1 H), 4.05–4.01 (m, 1 H), 3.70 (s, 6 H), 3.3–3.27 (m, 1 H), 3.21–3.17 (m, 1 H), 2.90–2.81 (m, 1 H), 1.75–1.64 (m, 2 H), 1.19 (d, J = 6.9 Hz, 6 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.74, 160.75, 158.63, 153.23, 151.62, 144.82, 142.97, 136.11, 136.02, 135.98, 130.04, 128.60, 128.32, 128.16, 128.02, 126.97, 118.08, 113.31, 86.77, 69.57, 68.88, 61.30, 55.33, 51.04, 36.08, 34.43, 19.41; HRMS calcd for $C_{41}H_{43}N_5O_6Na$ $[M + Na]^+$ 724.3111, found $[M + Na]^+$ 724.3112.

(*S*)-*N*-(9-(4-(Bis(4-methoxyphenyl)(phenyl)methoxy)-2-hydroxybutyl)-6-oxo-6,9-dihydro-1*H*-purin-2-yl)isobutyramide (29)

Compound **28** (1.7 g, 2.42 mmol) was dissolved in ethyl acetate (20 ml). 10% Pd/C (300 mg) was added under argon. The reaction was allowed to stir under hydrogen atmosphere for 5 h. The compound was filtered and washed with methanol. The organic layer was concentrated to afford the product (1.45 g, 97% yield) as a white foam solid. $[\alpha]_D^{25}$ = +5.8 (c = 1 in dichloroethane). ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.77 (br s, 1 H), 8.64 (br s, 1 H), 7.52 (s, 1 H), 7.38 (d, J = 8.20 Hz, 2 H), 7.27 (d, J = 8.20 Hz, 4 H), 7.21–7.18 (m, 2 H), 7.13–7.10 (m, 1 H), 6.76–6.73 (m, 4 H), 5.07 (br s, 1 H), 4.32–4.29 (m, 1 H), 4.09 (dd, J = 2.84, 14.19 Hz, 1 H), 3.76–3.71 (m, 1 H), 3.70 (s, 6 H), 3.34–3.30 (m, 1 H), 3.25–3.21 (m, 1 H), 2.51 (sept, J = 7 Hz, 1 H), 1.76–1.65 (m, 2 H), 1.13 (dd, J = 6.9, 18.3 Hz, 6 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.76, 158.61, 155.09, 148.33, 147.25, 145.14, 140.57, 136.34, 136.30, 130.22, 128.28, 128.01, 126.91, 120.33, 113.31, 86.68, 68.15, 60.91, 55.36, 50.67, 36.50, 34.72, 19.11, 18.88;

HRMS calcd for $C_{34}H_{37}N_5O_6Na$ $[M + Na]^+$ 634.262, found $[M + Na]^+$ 634.262.

Guanine phosphoramidite (30)

Compound **29** (617 mg, 1 mmol) was dried under high vacuum and anhydrous dichloromethane was added and subsequently diisopropyl ammonium tetrazolide (85 mg, 0.5 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (452 mg, 1.5 mmol) were added under nitrogen. The reaction was allowed to stir at room temperature. After 4 h, the reaction was diluted with dichloromethane and poured into saturated aqueous sodium bicarbonate. The product was extracted with dichloromethane and dried over magnesium sulfate. The product was purified by silica gel chromatography dichloromethane–hexanes (2 to 80%) with 1% triethylamine to afford the product as a white foam (532 mg, yield 65%). ^{31}P NMR (121 MHz, $CDCl_3$) δ 148.824, 147.865; HRMS calcd for $C_{43}H_{54}N_7O_6PNa$ $[M + Na]^+$ 834.3720, found $[M + Na]^+$ 834.3724.

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