LNA 5′-phosphoramidites for 5′→3′-oligonucleotide synthesis†

Andreas Stahl Madsen,*[a] T. Santhosh Kumar[a,b] and Jesper Wengel[a]

Received 29th June 2010, Accepted 10th August 2010
DOI: 10.1039/c0ob00346h

Hereby we report an efficient synthesis of LNA thymine and LNA 5-methylcytosine 5′-phosphoramidites, allowing incorporation of LNA thymine and LNA 5-methylcytosine into oligonucleotides synthesized in the 5′→3′ direction. Key steps include regioselective enzymatic benzoylation of the 5′-hydroxy group of unprotected LNA thymine, and subsequent 4,4′-dimethoxytritylation of the 3′-hydroxy group of the O5′-benzoylated LNA thymine nucleoside.

Introduction

Automated synthesis of oligonucleotides (ONs) is routinely carried out in the 3′→5′ direction using standard 3′-phosphoramidite derivatives of natural as well as modified nucleosides. Alternatively, nucleosides with 3′-*O*-dimethoxytrityl and 5′-phosphoramidite functionalities (5′-phosphoramidites) enable convenient ON synthesis proceeding in the 5′→3′ direction still using a standard ON synthesis protocol. This enables synthesis of otherwise challenging ONs, by allowing 3′-terminal incorporation of derivatives that does not offer a suitable site for attachment to a solid support, such as nucleosides without a 3′-hydroxy group (*e.g.* 2′,3′-dideoxy nucleosides), non-nucleosidic groups such as fluorophores,[1] phosphorodithioates and phosphorotrithioates,[2] thiols,[3] or chimeric syntheses where compatibility with otherwise employed chemistry dictates synthesis in the 5′→3′ direction.[4] Furthermore, in combination with standard 3′-phosphoramidites, 5′-phosphoramidites offer the opportunity to change orientation of the nucleotides during synthesis. This allows straightforward synthesis of ONs containing 3′,3′ or 5′,5′ linkages, *e.g.* for parallel hairpins[5] alternating triplex-forming ONs,[6] Hoogsteen parallel stranded DNA for targeting pyrimidine ONs,[5b,7] or multiple polarity reversals.[8]

For standard DNA monomers both 3′- and 5′-phosphoramidites are commercially available, whereas LNA (locked nucleic acid) monomers are only available as 3′-phosphoramidites (Fig. 1). LNA-modified ONs have successfully been explored for applicability within molecular biology research, diagnostics and gene silencing, generally taking advantage of the duplex-stabilizing effect of the conformationally locked LNA monomers.[9] Previously, the synthesis of the LNA thymine 5′-phosphoramidite **5** was reported,[10] but a low-yielding 4,4′-dimethoxytritylation step renders this strategy unsuitable for larger scale synthesis. We therefore wished to establish a viable synthetic route to the LNA thymine and 5-methylcytosine 5′-phosphoramidites (**5** and **9**, respectively, Scheme 1), which we hereby report with full experimental details. Furthermore, we demonstrate the efficient incorporation of 5′-phosphoramidites **5** and **9** into a short ON to give the corresponding LNA thymine and LNA 5-methylcytosine monomers ($\mathbf{T^L}$ and $\mathbf{^{Me}C^L}$, respectively Scheme 1).

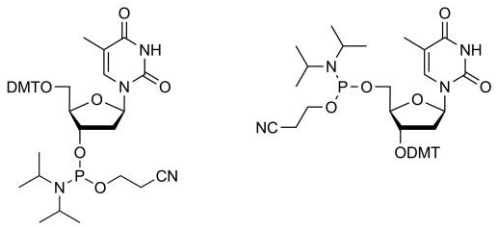


Fig. 1 Thymidine 3′-phosphoramidite building block (for standard 3′→5′ ON synthesis) and 5′-phosphoramidite building block (for reversed 5′→3′ ON synthesis), respectively.

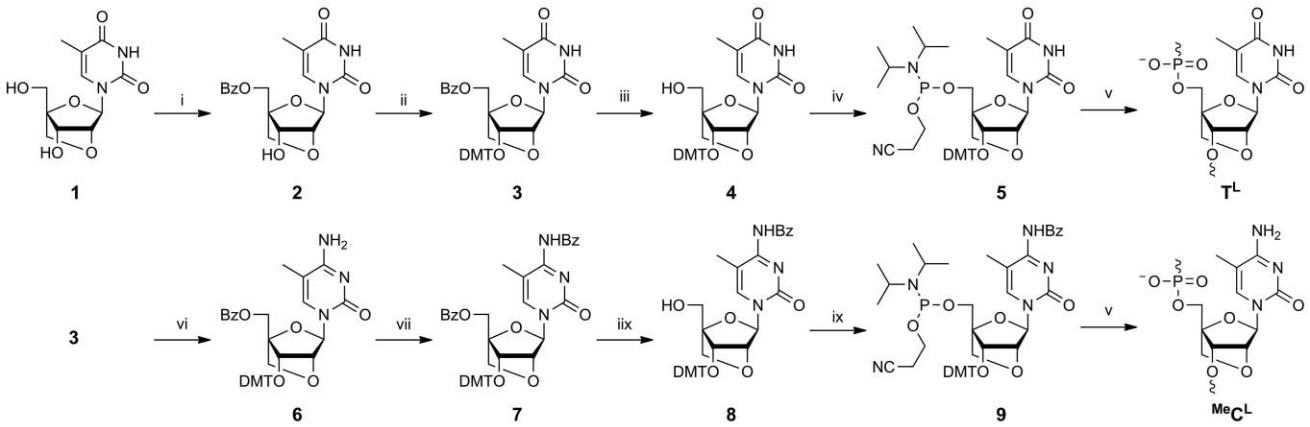
Results and discussion

We envisioned a simple synthetic strategy starting from LNA thymine diol **1**.[9a] Selective protection of the primary 5′-hydroxy group followed by O3′-dimethoxytritylation, O5′-deprotection and O5′-phosphitylation should give LNA thymine 5′-phosphoramidite **5**, whereas conversion from thymine to 5-methylcytosine and nucleobase protection at an appropriate step should give LNA 4-*N*-benzoyl-5-methylcytosine 5′-phosphoramidite **9**.

Selective protection of the primary 5′-hydroxy group over the secondary 3′-hydroxy group of nucleoside **1** has previously been accomplished by use of the bulky TBDMS group,[10] but subsequent 4,4′-dimethoxytritylation of the 3′-hydroxy group was reported to be very troublesome.[10] To circumvent this problem we decided to use the sterically less demanding benzoyl protection group. However, standard conditions (1.1 eq. benzoyl chloride in pyridine at either rt, 0 °C or −35 °C, or in CH_2Cl_2:pyridine at −70 °C) did not provide any significant selectivity (giving an approximate 3 : 2 ratio of benzoate ester **2** and the corresponding 3′-regioisomer). This rather surprising lack of selectivity is most likely a consequence of the locked 2,5-dioxabicyclo[2.2.1]heptane skeleton which forces the 3′-hydroxy group of nucleoside **1** to adopt a position which is unusually accessible, thereby leading to increased reactivity.

As a regioselective enzymatic procedure for O5′-benzoylation of 2′-deoxynucleotides has been reported,[11] we investigated the

[a]*Nucleic Acid Center, Department of Physics and Chemistry, University of Southern Denmark, (The Nucleic Acid Center is funded by the Danish National Research Foundation for studies on nucleic acid chemical biology), 5230, Odense M, Denmark. E-mail: asm@ifk.sdu.dk.; Fax: +45 6615 8780; Tel: +45 6550 2599*
[b]*Current affiliation: National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, 20892-0810, USA*
† Electronic supplementary information (ESI) available: General experimental section and copies of ^{1}H and ^{13}C NMR spectra of compounds **2–4** and **6–8** and copies of ^{31}P NMR spectra of compounds **5** and **9**. See DOI: 10.1039/c0ob00346h



Scheme 1 Synthesis of LNA 5′-phosphoramidites. Reagents and conditions: i) CH_2CHOBz, *Candida Antarctica* Lipase B (Novozyme 435®), THF, 60 °C, 72 h, 93%; ii) DMTCl, AgOTf, CH_2Cl_2:2,6-lutidine (50 : 50, v/v), rt, 20 h, 100%; iii) K_2CO_3, MeOH–CH_2Cl_2–H_2O (60 : 20 : 20, v/v), rt, 20 h, 91%; iv) $NC(CH_2)_2OP(N(i\text{-}Pr)_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, rt, 3 h, 77%; v) DNA synthesizer, vi) a) Et_3N, 1,2,4-triazole, $POCl_3$, CH_3CN, 0 °C to rt, 3 h, b) 28% aq NH_3:CH_3CN (50 : 50, v/v), rt, 2 h, 97%; vii) BzCl, pyridine, rt, 3 h, 94%; iix) 1M aq. LiOH, THF, rt, 24 h, 93%; ix) $NC(CH_2)_2OP(N(i\text{-}Pr)_2)_2$, diisopropylammonium tetrazolide, CH_2Cl_2, rt, 2 h, 81%.

potential for *Candida Antarctica* Lipase B (Novozyme 435®) to catalyze monobenzoylation of LNA nucleoside **1** using vinyl benzoate as acylating reagent. Gratifyingly, the reaction proceeded smoothly affording the desired benzoate ester **2** in 93% yield, without any detectable formation of the 3′-regioisomer. The correct connectivity for benzoate ester **2** was confirmed by NMR (including disappearance of the 5′-hydroxy signal and a downfield shift of the H5′ signal from 3.7 to 4.7 ppm). Dimethoxytritylation of the 3′-hydroxy group of alcohol **2** was unsuccessful using standard conditions (DMTCl in pyridine), however, DMTOTf[12] in dichloromethane and 2,6-lutidine afforded fully protected nucleoside **3** in quantitative yield. Furthermore, we found that synthesis and isolation of DMTOTf could be circumvented by *in situ* generation of DMTOTf from DMTCl and AgOTf in dichloromethane and 2,6-lutidine, quantitatively affording dimethoxytrityl ether **3**. Subsequent hydrolysis of the benzoate ester followed by phosphitylation afforded LNA thymidine 5′-phosphoramidite **5** in 70% yield over two steps from intermediate **3**. Conversion from thymine to 5-methylcytosine was realized by a well-established two-step methodology.[13] Thymine derivative **3** was treated with $POCl_3$, Et_3N and 1,2,4-triazole to afford the triazolothymine derivative which was converted to the corresponding 5-methylcytosine derivative **6** by treatment with aqueous ammonia in acetonitrile. Subsequent benzoylation furnished 4-*N*-benzoyl-5-methylcytosine derivative **7** in 92% yield over three chemical steps from nucleoside **3**. Selective hydrolysis of the ester affording alcohol **8**, was realized in 93% yield by treatment with aqueous LiOH in THF with no observable cleavage of the 4-*N*-benzoyl moiety. The correct constitution was confirmed by NMR, including appearance of a signal for the 5′-hydroxy group and an upfield shift of the H5′ signals (from 4.9 to 4.2 ppm). Subsequent phosphitylation afforded LNA 4-*N*-benzoyl-5-methylcytosine 5′-phosphoramidite **9** in 70% yield over five steps from intermediate **3**. The highly efficient synthetic strategy employed here, would also be expected to enable synthesis of the corresponding purine LNA phosphoramidites.

To demonstrate the applicability of 5′-phosphoramidites **5** and **9** in ON synthesis an LNA modified oligonucleotide (5′-GCA T^LAT $^{Me}C^L$AC-3′) was synthesized. To allow comparison with conventional phosphoramidites, the ON was synthesized in the 3′→5′ as well as the 5′→3′ direction using either normal 3′-phosphoramidites or 5′-phosphoramidites, respectively. For LNA 3′- and 5′-phosphoramidites, extended coupling times (15 min) resulted in stepwise coupling yields of ≥99%, as obtained for DNA 3′- and 5′-phosphoramidites (2 min coupling time). Comparable purities were obtained after purification (>90%). Composition of the product from both syntheses was verified by MALDI-MS. In agreement with the identical elution time observed for the two products by ion exchange (IE) chromatography, a mixture of the two batches eluted as a single peak, confirming that the two products are identical (Fig. 2).

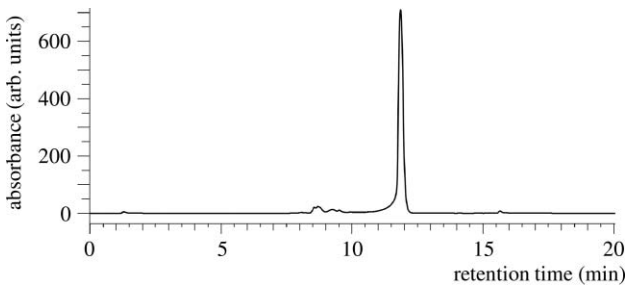


Fig. 2 IE-HPLC absorbance profile for co-injection of the 3′→5′ and 5′→3′ synthesized ON.

Experimental

(1*R*,3*R*,4*R*,7*S*)-1-Benzoyloxymethyl-7-hydroxy-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (2)

Diol **1** (1.20 g, 4.43 mmol) was dissolved in anhydrous THF (30 mL). Vinyl benzoate (3.0 mL, 21.7 mmol) and Novozyme 435® (1.20 g) were added and the mixture was stirred at 60 °C for 72 h. The reaction mixture was adsorbed directly on kieselgur and purified by silica gel column chromatography (0–6% MeOH in CH_2Cl_2) affording the desired benzoate ester **2** (1.56 g, 93%) as a white solid. R_f 0.3 (10% MeOH in CH_2Cl_2, v/v); Found: C,

56.9; H, 4.6; N, 7.3. Calcd for $C_{18}H_{18}N_2O_7 \cdot 1/4H_2O$: C, 57.1; H, 4.9; N, 7.4; δ_H (DMSO-d_6) 11.38 (br s, 1H, NH, ex), 8.00–8.04 (m, 2H, $H2_{Bz}$, $H6_{Bz}$), 7.68–7.73 (m, 1H, $H4_{Bz}$), 7.54–7.59 (m, 2H, $H3_{Bz}$, $H5_{Bz}$), 7.40 (d, J = 1.1 Hz, 1H, H6), 5.95 (br s, 1H, 3′-OH, ex), 5.48 (s, 1H, H1′), 4.76 (d, J = 12.8 Hz, 1H, $H5'_A$), 4.71 (d, J = 12.8 Hz, 1H, $H5'_B$), 4.24 (s, 1H, H2′), 4.09 (s, 1H, H3′), 4.01 (d, J = 8.0 Hz, 1H, $H5''_A$), 3.85 (d, J = 8.0 Hz, 1H, $H5''_B$), 1.60 (d, J = 1.1 Hz, 3H, 5-CH_3); δ_C (DMSO-d_6) 165.3 (COPh), 163.7 (C4), 149.9 (C2), 134.1 (C6), 133.7 ($C4_{Bz}$), 129.2 ($C2_{Bz}$, $C6_{Bz}$), 128.9 ($C3_{Bz}$, $C5_{Bz}$), 128.5 ($C1_{Bz}$), 108.6 (C5), 86.6 (C1′), 86.2 (C4′), 79.0 (C2′), 70.9 (C5″), 69.6 (C3′), 60.2 (C5′), 12.0 (5-CH_3); ESI-HRMS m/z 397.1004 ($[M + Na]^+$, $C_{18}H_{18}N_2O_7Na^+$ Calcd 397.1006).

(1*R*,3*R*,4*R*,7*S*)-1-Benzoyloxymethyl-7-(4,4′-dimethoxytrityloxy)-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (3)

Alcohol **2** (254 mg, 0.679 mmol) was co-evaporated with 1,2-dichloroethane (5 mL) and then dissolved in a mixture of anhydrous CH_2Cl_2 (4 mL) and anhydrous 2,6-lutidine (4 mL). 4,4′-Dimethoxytrityl chloride (342 mg, 1.01 mmol) and silver triflate (268 mg, 1.05 mmol (dried for 3 h at 100 °C under high vacuum)) were added at rt, resulting in a deep red solution with a white precipitate starting to form immediately. After stirring for 20 h, MeOH (0.5 mL) was added to the reaction mixture. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and then filtered through a short pad of kieselgur. The organic phase was washed successively with sat. aq $NaHCO_3$ (2 × 20 mL) and brine (20 mL), then concentrated under reduced pressure, and the residue was placed under high vacuum overnight. The resulting residue was purified by silica gel column chromatography (0–4% MeOH in CH_2Cl_2) to afford the desired dimethoxytritylated nucleoside **3** (459 mg, 100%) as a white foam. R_f 0.5 (5% MeOH in CH_2Cl_2, v/v); Found: C, 69.45; H, 5.4; N, 4.3. Calcd for $C_{39}H_{36}N_2O_9$: C, 69.2; H, 5.4; N, 4.1; δ_H (DMSO-d_6) 11.40 (s, 1H, NH, ex), 7.73–7.77 (m, 2H, $H2_{Bz}$, $H6_{Bz}$), 7.67–7.73 (m, 1H, $H4_{Bz}$), 7.47–7.53 (m, 2H, $H3_{Bz}$, $H5_{Bz}$), 7.32–7.42 (m, 2H, $H3''_{DMT}$, $H5''_{DMT}$), 7.19–7.26 (m, 7H, $H2_{DMT}$, $H6_{DMT}$, $H2'_{DMT}$, $H6'_{DMT}$, $H2''_{DMT}$, $H4''_{DMT}$, $H6''_{DMT}$), 6.92 (d, J = 1.0, 1H, H6), 6.75–6.79 (m, 2H, [$H3_{DMT}$, $H5_{DMT}$]/[$H3'_{DMT}$, $H5'_{DMT}$]), 6.71–6.75 (m, 2H, [$H3_{DMT}$, $H5_{DMT}$]/[$H3'_{DMT}$, $H5'_{DMT}$]), 5.26 (s, 1H, H1′), 4.94 (d, J = 13.0, 1H, $H5'_A$), 4.83 (d, J = 13.0, 1H, $H5'_B$), 4.37 (d, J = 8.2, 1H, $H5''_A$), 3.95 (d, J = 8.2, 1H, $H5''_A$), 3.69 (s, 3H, OCH_3), 3.66 (s, 1H, H3′), 3.62 (s, 3H, OCH_3), 2.96 (s, 1H, H2′), 1.33 (d, J = 1.0, 3H, 5-CH_3); δ_C (DMSO-d_6) 165.0 (COPh), 163.5 (C4), 158.6 ($C4_{DMT}$/$C4'_{DMT}$), 158.4 ($C4_{DMT}$/$C4'_{DMT}$), 149.4 (C2), 144.7 ($C1''_{DMT}$), 134.8 ($C1_{DMT}$/$C1'_{DMT}$), 134.6 ($C1_{DMT}$/$C1'_{DMT}$), 133.8 ($C4_{Bz}$), 132.5 (C6), 130.0 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.8 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.2 ($C3_{Bz}$, $C5_{Bz}$), 128.9 ($C1_{Bz}$), 128.8 ($C2_{Bz}$, $C6_{Bz}$), 127.8 ($C2''_{DMT}$, $C6''_{DMT}$), 127.4 ($C3''_{DMT}$, $C5''_{DMT}$), 127.0 ($C4''_{DMT}$), 113.2 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 113.1 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 108.7 (C5), 87.0 (CAr_3), 86.3 (C1′), 86.1 (C4′), 76.8 (C2′), 71.9 (C5″), 71.8 (C3′), 58.8 (C5′), 55.0 (OCH_3), 54.9 (OCH_3), 11.7 (5-CH_3); ESI-HRMS m/z 699.2321 ($[M + Na]^+$, $C_{39}H_{36}N_2O_9Na^+$ Calcd 699.2313).

(1*S*,3*R*,4*R*,7*S*)-7-(4,4′-Dimethoxytrityloxy)-1-hydroxymethyl-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (4)

Benzoate ester **3** (3.40 g, 5.02 mmol) was dissolved in a mixture of CH_2Cl_2 (20 mL), MeOH (60 mL) and H_2O (20 mL). K_2CO_3 (2.78 g, 20.1 mmol) was added, and the reaction mixture stirred at rt for 20 h whereupon additional K_2CO_3 (2.78 g, 20.1 mmol) was added. After stirring for additional 20 h, the reaction mixture was evaporated to dryness, and then partitioned between CH_2Cl_2 (200 mL) and brine (100 mL). The organic phase was evaporated to dryness and the resulting residue was purified by silica gel column chromatography (0–10% MeOH in CH_2Cl_2), affording the desired alcohol **4** (2.66 g, 91%) as a white foam. R_f 0.3 (75% EtOAc in petroleum ether (PE), v/v); Found: C, 66.1; H, 5.5; N, 4.7. Calcd for $C_{32}H_{32}N_2O_8 \cdot 1/3H_2O$: C, 66.4; H, 5.7; N, 4.8; δ_H (DMSO-d_6) 11.31 (br s, 1H, NH, ex), 7.38–7.42 (m, 2H, $H2''_{DMT}$, $H6''_{DMT}$), 7.22–7.31 (m, 4H, H6, $H3''_{DMT}$, $H4''_{DMT}$, $H5''_{DMT}$), 7.18–7.22 (m, 4H, $H2_{DMT}$, $H6_{DMT}$, $H2'_{DMT}$, $H6'_{DMT}$), 6.79–6.83 (m, 4H, $H3_{DMT}$, $H5_{DMT}$, $H3'_{DMT}$, $H5'_{DMT}$), 5.28 (t, J = 5.3, 1H, 5′-OH, ex), 5.20 (s, 1H, H1′), 4.15 (d, J = 7.9 Hz, 1H. $H5''_A$), 3.88–3.98 (m, 2H, H5′), 3.71–3.76 (m, 7H, $H5''_B$, OCH_3), 3.57 (s, 1H, H3′), 2.76 (s, 1H, H2′), 1.62 (s, 3H, 5-CH_3); δ_C (DMSO-d_6) 163.6 (C4), 158.5 ($C4_{DMT}$, $C4'_{DMT}$), 149.4 (C2), 144.9 ($C1''_{DMT}$), 135.3 ($C1_{DMT}$/$C1'_{DMT}$), 135.0 ($C1_{DMT}$/$C1'_{DMT}$), 134.0 (C6), 129.9 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.8 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 127.7 ($C3''_{DMT}$, $C5''_{DMT}$), 127.5 ($C2''_{DMT}$, $C6''_{DMT}$), 126.9 ($C4''_{DMT}$), 113.1 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 113.0 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 108.2 (C5), 88.6 (C4′), 86.8 (CAr_3), 86.1 (C1′), 76.6 (C2′), 72.0 (C5″), 71.4 (C3′), 55.9 (C5′), 55.0 (OCH_3), 12.0 (5-CH_3); ESI-HRMS m/z 595.2043 ($[M + Na]^+$, $C_{32}H_{32}N_2O_8Na^+$ Calcd 595.2051).

(1*R*,3*R*,4*R*,7*S*)-1-(2-Cyanoethoxy(diisopropylamino)-phosphinoxymethyl-7-(4,4′-dimethoxytrityloxy)-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (5)

Alcohol **4** (422 mg, 0.736 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (2 × 10 mL) and then redissolved in anhydrous 1,2-dichloroethane (50 mL), and *N*,*N*′-diisopropylammonium tetrazolide (253 mg, 1.48 mmol) and bis(*N*,*N*′-diisopropylamino)-2-cyanoethoxyphosphine (569 mg, 1.89 mmol) were added at rt. After stirring for 3 h, the reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with brine (15 mL). The aqueous phase was extracted with CH_2Cl_2 (40 mL) and the combined organic phase evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0–100% EtOAc in PE), affording the desired phosphoramidite **5** (432 mg, 77%) as a white solid. R_f 0.5 (75% EtOAc in PE, v/v); Found: C, 63.5; H, 6.4 N, 7.4. Calcd for $C_{41}H_{49}N_4O_9P$: C, 63.7; H, 6.4; N, 7.25; δ_P (DMSO-d_6) 147.9, 147.6; ESI-HRMS m/z 795.3128 ($[M + Na]^+$, $C_{41}H_{49}N_4O_9PNa^+$ Calcd 795.3129).

(1*R*,3*R*,4*R*,7*S*)-1-Benzoyloxymethyl-7-(4,4′-dimethoxytrityloxy)-3-(5-methylcytosin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (6)

Nucleoside **3** (1.70 g, 2.51 mmol) was co-evaporated with anhydrous CH_3CN (2 × 25 mL) and then redissolved in anhydrous CH_3CN (60 mL) at rt. Anhydrous Et_3N (6.0 mL, 43 mmol) and 1,2,4-triazole (2.08 g, 30.1 mmol) were added under stirring. The resulting mixture was cooled to 0 °C on an ice bath and freshly distilled $POCl_3$ (0.69 mL, 7.53 mmol) was added dropwise to give a white slurry. After 15 min the reaction mixture was allowed to reach rt. After stirring for 3 h, TLC indicated full conversion to an intermediate (R_f(**3**) = 0.6; R_f(intermediate) = 0.4, 70% EtOAc

in PE), and the reaction mixture was poured into a slurry of sat. aq $NaHCO_3$ (50 mL) and ice (25 mL) and extracted with EtOAc (3 × 25 mL). The combined organic phase was washed with brine (100 ml) and dried over Na_2SO_4. Filtration and evaporation under reduced pressure afforded this intermediate as white foam which was immediately dissolved in a mixture of 28% aq NH_3 and CH_3CN (50 : 50, 70 mL, v/v). After stirring for 2 h at rt, brine (40 mL) was added which resulted in separation into two phases. The aqueous phase was extracted with EtOAc (3 × 50 mL), the combined organic phase evaporated to dryness and the resulting residue purified by silica gel column chromatography (0–10% *i*-PrOH in CH_2Cl_2, v/v) to afford nucleoside **6** (1.65 g, 97% from **3**) as a white solid. R_f 0.3 (10% *i*-PrOH in CH_2Cl_2, v/v); Found: C, 69.6; H, 5.3; N, 6.1. Calcd for $C_{39}H_{37}N_3O_8$: C, 69.3; H, 5.5; N, 6.2; δ_H (DMSO-d_6) 7.71–7.75 (m, 2H, $H2_{Bz}$, $H6_{Bz}$), 7.68–7.71 (m, 1H, $H4_{Bz}$), 7.47–7.52 (m, 2H, $H3_{Bz}$, $H5_{Bz}$), 7.36–7.40 (m, 2H, $H2''_{DMT}$, $H6''_{DMT}$), 7.15–7.25 (m, 7H, $H2_{DMT}$, $H6_{DMT}$, $H2'_{DMT}$, $H6'_{DMT}$, $H3''_{DMT}$, $H4''_{DMT}$, $H5''_{DMT}$), 6.83 (s, 1H, H6), 6.71–6.75 (m, 2H, [$H3_{DMT}$, $H5_{DMT}$]/[$H3'_{DMT}$, $H5'_{DMT}$]), 6.68–6.71 (m, 2H, [$H3_{DMT}$, $H5_{DMT}$]/[$H3'_{DMT}$, $H5'_{DMT}$]), 5.25 (s, 1H, H1′), 4.92 (d, J = 12.9, 1H, $H5'_A$), 4.77 (d, J = 12.9, 1H, $H5'_B$), 4.34 (d, J = 8.1, 1H, $H5''_A$), 3.94 (d, J = 8.1, 1H, $H5''_B$), 3.71 (s, 3H, OCH_3), 3.63 (s, 3H, OCH_3), 3.62 (s, 1H, H3′), 3.05 (s, 1H, H2′), 1.36 (s, 3H, 5-CH_3); δ_C (DMSO-d_6) 165.3 (C4), 165.0 (COPh), 158.5 ($C4_{DMT}$/$C4'_{DMT}$), 158.4 ($C4_{DMT}$/$C4'_{DMT}$), 154.2 (C2), 144.8 ($C1''_{DMT}$), 134.8 (C6), 134.7 ($C1_{DMT}$/$C1'_{DMT}$), 134.6 ($C1_{DMT}$/$C1'_{DMT}$), 133.7 ($C4_{Bz}$), 129.9 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.8 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.2 ($C3_{Bz}$, $C5_{Bz}$), 128.9 ($C1_{Bz}$), 128.8 ($C2_{Bz}$, $C6_{Bz}$), 127.8 ($C3''_{DMT}$, $C5''_{DMT}$), 127.4 ($C2''_{DMT}$, $C6''_{DMT}$), 126.9 ($C4''_{DMT}$), 113.14 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 113.09 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 100.7 (C5), 86.9 (CAr_3), 86.8 (C1′), 85.8 (C4′), 76.8 (C2′), 71.8 (C5″), 71.5 (C3′), 58.8 (C5′), 55.0 (OCH_3), 54.9 (OCH_3), 12.8 (5-CH_3); ESI-HRMS m/z 698.2442 ([M + Na]$^+$, $C_{39}H_{37}N_3O_8Na^+$ Calcd 698.2473).

(1*R*,3*R*,4*R*,7*S*)-3-(4-*N*-Benzoyl-5-methylcytosin-1-yl)-1-benzoyloxymethyl-7-(4,4′-dimethoxytrityloxy)-2,5-dioxabicyclo[2.2.1]heptane (7)

Nucleoside **6** (3.32 g, 4.92 mmol) was co-evaporated with anhydrous pyridine (2 × 10 mL) and then redissolved in anhydrous pyridine (80 mL) at rt. Benzoyl chloride (0.90 mL, 7.75 mmol) was added and the reaction mixture stirred for 3 h and then evaporated to dryness under reduced pressure. The resulting residue was dissolved in EtOAc (150 mL) and washed successively with sat. aq $NaHCO_3$ (100 mL) and brine (100 mL). The combined organic phase was evaporated to dryness and the resulting residue purified by silica gel column chromatography (0–45% EtOAc in PE, v/v) to afford nucleoside **7** (3.66 g, 94%) as a white foam. R_f 0.6 (50% EtOAc in PE, v/v); Found: C, 70.0; H, 5.4; N, 5.1. Calcd for $C_{46}H_{41}N_3O_9 \cdot 1/2H_2O$: C, 70.0; H, 5.4; N, 5.3; δ_H (DMSO-d_6) 8.12–8.19 (m, 2H, $H2_{NBz}$, $H6_{NBz}$), 7.73–7.78 (m, 2H, $H2_{OBz}$, $H6_{OBz}$), 7.67–7.73 (m, 1H, $H4_{OBz}$), 7.58–7.63 (m, 1H, $H4_{NBz}$), 7.48–7.54 (m, 4H, $H3_{OBz}$, $H5_{OBz}$, $H3_{NBz}$, $H5_{NBz}$), 7.39–7.42 (m, 2H, $H2''_{DMT}$, $H6''_{DMT}$), 7.15–7.26 (m, 8H, H6, $H2_{DMT}$, $H6_{DMT}$, $H2'_{DMT}$, $H6'_{DMT}$, $H3''_{DMT}$, $H4''_{DMT}$, $H5''_{DMT}$), 6.75–6.79 (m, 2H, [$H3_{DMT}$, $H5_{DMT}$]/[$H3'_{DMT}$, $H5'_{DMT}$]), 6.72–6.75 (m, 2H, [$H3_{DMT}$, $H5_{DMT}$]/[$H3'_{DMT}$, $H5'_{DMT}$]), 5.35 (s, 1H, H1′), 4.98 (d, J = 13.0, 1H, $H5'_A$), 4.87 (d, J = 13.0, 1H, $H5'_B$), 4.41 (d, J = 8.2, 1H, $H5''_A$), 4.00 (d, J = 8.2, 1H, $H5''_B$), 3.68 (s, 1H, H3′), 3.67 (s, 3H, OCH_3), 3.59 (s, 3H, OCH_3), 3.05 (br s, 1H, H2′), 1.57 (s, 3H, 5-CH_3); δ_C (DMSO-d_6) 165.0 (5′-O*C*OPh), 158.6 ($C4_{DMT}$/$C4'_{DMT}$), 158.5 ($C4_{DMT}$/$C4'_{DMT}$), 144.8 ($C1''_{DMT}$), 134.7 ($C1_{DMT}$/$C1'_{DMT}$), 134.6 ($C1_{DMT}$/$C1'_{DMT}$), 133.7 ($C4_{OBz}$), 132.6 ($C4_{NBz}$), 130.0 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.8 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.2 ($C2_{NBz}$, $C6_{NBz}$, $C2_{OBz}$, $C6_{OBz}$), 128.9 ($C1_{OBz}$), 128.8 ($C3_{OBz}$, $C5_{OBz}$), 128.3 ($C3_{NBz}$, $C5_{NBz}$), 127.8 ($C3''_{DMT}$, $C5''_{DMT}$), 127.4 ($C2''_{DMT}$, $C6''_{DMT}$), 127.0 ($C4''_{DMT}$), 113.2 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 113.1 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 109.3 (C5), 87.0 (CAr_3), 86.8 (C4′), 86.4 (C1′), 76.5 (C2′), 71.9 (C5″), 71.8 (C3′), 58.9 (C5′), 54.9 (OCH_3), 54.8 (OCH_3), 12.8 (5-CH_3);[14] ESI-HRMS m/z 802.2726 ([M + Na]$^+$, $C_{46}H_{41}N_3O_9Na^+$ Calcd 802.2735).

(1*S*,3*R*,4*R*,7*S*)-3-(4-*N*-Benzoyl-5-methylcytosin-1-yl)-7-(4,4′-dimethoxytrityloxy)-1-hydroxymethyl-2,5-dioxabicyclo[2.2.1]heptane (8)

Nucleoside **7** (3.66 g, 4.69 mmol) was dissolved in THF (250 mL) at rt and aq LiOH (1.0 M, 50 mL) was added. After stirring for 40 h, the reaction mixture was partitioned between EtOAc (100 mL) and brine (100 mL) and the aqueous phase was extracted with EtOAc (2 × 100 ml). The combined organic phase was dried over $MgSO_4$ and then evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0–80% EtOAc in PE, v/v) to afford nucleoside **8** (2.96 g, 93%) as a white foam. R_f 0.4 (50% EtOAc in PE, v/v); Found: C, 68.0; H, 5.6; N, 5.9. Calcd for $C_{39}H_{37}N_3O_8 \cdot 2/3H_2O$: C, 68.1; H, 5.6; N, 6.1; δ_H (DMSO-d_6) 8.11–8.24 (m, 2H, $H2_{Bz}$, $H6_{Bz}$), 7.58–7.66 (m, 2H, $H4_{Bz}$, H6), 7.49–7.54 (m, 2H, $H3_{Bz}$, $H5_{Bz}$), 7.39–7.43 (m, 2H, $H2''_{DMT}$, $H6''_{DMT}$), 7.25–7.31 (m, 2H, $H3''_{DMT}$, $H5''_{DMT}$), 7.18–7.24 (m, 5H, $H2_{DMT}$, $H6_{DMT}$, $H2'_{DMT}$, $H6'_{DMT}$, $H4''_{DMT}$), 6.78–6.84 (m, 4H, $H3_{DMT}$, $H5_{DMT}$, $H3'_{DMT}$, $H5'_{DMT}$), 5.33 (t, J = 5.0, 1H, 5′-OH), 5.30 (s, 1H, H1′), 4.20 (d, J = 7.9, 1H, $H5''_A$), 3.93–4.03 (m, 2H, $H5'_{A+B}$), 3.80 (d, J = 7.9, 1H, $H5''_B$), 3.70 (s, 6H, OCH_3), 3.57 (s, 1H, H3′), 2.90 (s, 1H, H2′), 1.89 (s, 3H, 5-CH_3); δ_C (DMSO-d_6) 158.5 ($C4_{DMT}$, $C4'_{DMT}$), 144.9 ($C1''_{DMT}$), 135.2 ($C1_{DMT}$/$C1'_{DMT}$), 134.9 ($C1_{DMT}$/$C1'_{DMT}$), 132.5 ($C4_{Bz}$), 129.9 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.8 ([$C2_{DMT}$, $C6_{DMT}$]/[$C2'_{DMT}$, $C6'_{DMT}$]), 129.2 ($C2_{Bz}$, $C6_{Bz}$), 128.3 ($C3_{Bz}$, $C5_{Bz}$), 127.8 ($C3''_{DMT}$, $C5''_{DMT}$), 127.5 ($C2''_{DMT}$, $C6''_{DMT}$), 126.9 ($C4''_{DMT}$), 113.1 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 113.0 ([$C3_{DMT}$, $C5_{DMT}$]/[$C3'_{DMT}$, $C5'_{DMT}$]), 109.0 (C5), 89.0 (C4), 86.9 (CAr_3), 86.7 (C1′), 76.3 (C2′), 72.0 (C5″), 71.3 (C3′), 55.9 (C5′), 55.0 (OCH_3), 13.2 (5-CH_3);[14] ESI-HRMS m/z 698.2476 ([M + Na]$^+$, $C_{39}H_{37}N_3O_8PNa^+$ Calcd 698.2473).

(1*R*,3*R*,4*R*,7*S*)-3-(4-*N*-Benzoyl-5-methylcytosin-1-yl)-1-(2-cyanoethoxy(diisopropylamino)phosphinoxymethyl-7-(4,4′-dimethoxytrityloxy)-2,5-dioxabicyclo[2.2.1]heptane (9)

Nucleoside **8** (549 mg, 0.83 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (15 mL) and dissolved in anhydrous CH_2Cl_2 (50 mL) at rt. *N*,*N*′-Diisopropylammonium tetrazolide (279 mg, 1.63 mmol) and bis(*N*,*N*′-diisopropylamino)-2-cyanoethoxyphosphine (546 mg, 1.81 mmol) were added, and the reaction mixture was stirred for 3.5 h, diluted with CH_2Cl_2 (50 mL), and washed with brine (25 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 25 mL) and the combined organic phase was dried over $MgSO_4$ and then evaporated to dryness under

reduced pressure. The resulting residue was purified by silica gel column chromatography (0–40% EtOAc in PE, v/v) to afford amidite **9** (574 mg, 82%) as a white foam. R_f 0.6 (50% EtOAc in PE, v/v); Found: C, 65.6; H, 6.2; N, 7.9. Calcd for $C_{48}H_{54}N_5O_9P$: C, 65.8; H, 6.2; N, 8.0; δ_P (DMSO-d_6) 148.0, 147.6; ESI-HRMS m/z 898.3563 ($[M + Na]^+$, $C_{48}H_{54}N_5O_9PNa^+$ Calcd. 898.3551).

Oligonucleotide synthesis

ONs (5′-GCA $\mathbf{T}^{\mathbf{L}}$AT $^{\mathbf{Me}}\mathbf{C}^{\mathbf{L}}$AC-3′) were synthesized on 0.2 μmol scale using universal support polystyrene columns on an automated DNA synthesizer, following standard protocol (trichloroacetic acid in CH_2Cl_2 (3 : 97, v/v) as detritylation reagent; 0.25 M 4,5-dicyanoimidazole (DCI) in CH_3CN as activator; acetic anhydride/THF (9 : 91, v/v) and 1-methylimidazole/pyridine/THF (1 : 1 : 8, v/v) as cap solutions, and 0.02 M iodine in water/pyridine/THF (9 : 0.4 : 91, v/v) as oxidizing solution; Coupling times, DNA monomers: 2 min, LNA monomers: 15 min). After deprotection and cleavage from solid support (32% aq. NH_3, 12 h, 55 °C), purification of ONs (DMT-ON) was performed by RP-HPLC using a Waters 600 system equipped with an Xterra MS C18 (10 μm, 7.8 × 10 mm) pre-column and an Xterra MS C18 (10 μm, 7.8 × 150 mm) column using a gradient of 0–70% Buffer B in Buffer A (Buffer A: 0.05M aq. triethylammonium acetate, pH 7.4; Buffer B: 75% MeCN in buffer A (v/v)). The purified ON were detritylated (80% aq. AcOH, 20 min, rt) and precipitated (abs. EtOH, −18 °C, 12–16 h). The composition of the synthesized ONs were verified by MALDI-MS analysis, m/z ($[M+H]^+$, found, 3′→5′/found, 5′→3′/calcd): 2751/2752/2753. Purity (>90%) was verified by ion-exchange HPLC (using an a LaChrom L-7000 system equipped with a Dionex PA100 column (4 × 250 mm) using a gradient of 2–21% Buffer C and 10% Buffer D in water (Buffer C: 1.0 M aq. $NaClO_4$; Buffer D: 0.25 M aq. Tris-HCl, pH 8.0). The co-injection experiment was performed with equimolar amounts of the two batches, using the same protocol.

Conclusions

A viable and efficient synthesis of LNA thymine and LNA 4-*N*-benzoyl-5-methylcytosine 5′-phosphoramidites has been developed. Notably, the high yielding steps of regioselective O5′-benzoylation of the LNA thymine diol and subsequent quantitative 3′-*O*-dimethoxytritylation allowed synthesis of LNA thymine and LNA 4-*N*-benzoyl-5-methylcytosine 5′-phosphoramidites (**5** and **9**) in gratifying 65% (four steps) and 65% (seven steps) overall yield from LNA thymine diol **1**, respectively. ON synthesis proceeded smoothly affording the desired ON in high yield and purity.

Acknowledgements

We greatly appreciate financial support from The Danish National Research Foundation and the European Union Grant (FP-037283). We thank Mr. Brian Hermansen for assistance during synthesis, Ms. Joan Hansen for ON synthesis and workup and Ms. Tina Grubbe Hansen for ON purification.

Notes and references

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