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Stabilisation of a nucleic acid three-way junction by an oligonucleotide containing a single 2′-*C* to 3′-*O*-phosphate butylene linkage prepared by a tandem RCM-hydrogenation method†

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A cyclic dinucleotide with a butylene linker between the upper 2′-*C* position and the 3′-*O*-phosphate linkage was synthesised from simple nucleoside building blocks *via* a tandem ring-closing metathesis and hydrogenation procedure. The major of two phosphorus epimers was incorporated into an oligodeoxynucleotide, as well as into an LNA–DNA mixmer oligonucleotide. These were evaluated as parts in three different secondary structures, a duplex, a bulged duplex and a three-way junction, with both DNA and RNA complements. In the DNA : RNA hybrid molecule, the oligodeoxynucleotide containing this single 2′-*C* to 3′-*O*-phosphate butylene linkage was found to stabilise a three-way junction.

Introduction

The design of synthetic nucleic acid fragments mimicking secondary structures of DNA and RNA has been mostly concentrated on the stabilisation of A- and B-type duplexes by *e.g.* conformationally restricted nucleoside building blocks.[1–3] However, cyclic dinucleotides mimicking other secondary structures have also been explored. As prime examples, Sekine and co-workers have made cyclic dinucleotides with connections between the 2′-*O* position and the pyrimidine 5-*C* position of the two adjacent nucleosides and used these in oligonucleotides as artificial models of bend motifs *e.g.* U-turns in RNA.[4,5] For similar purposes, the same group also introduced a series of 5′-mononucleotides and corresponding dinucleotides with two or three carbon linkages between the pyrimidine 5-*C* position and the phosphate moiety.[6–8] Furthermore, nucleic acid fragments with intra- or interstrand linkages obtained by disulfide bridges have been explored.[9–12] Also, direct alkyl cross-links between the *N*-2 positions of adjacent guanines have been prepared, forming cyclic dinucleotide moieties within oligonucleotide sequences.[13]

We have recently formulated a general strategy for the synthesis of conformationally restricted nucleic acid fragments based on a ring-closing metathesis (RCM) methodology.§[14–16] Thus, a series of dinucleotides with terminal double bonds incorporated in various positions has been prepared and, especially, Grubbs 2nd generation catalyst§[17,18] has subsequently been applied in the preparation of a selection of cyclic dinucleotide structures.[19–24] A seven-membered ring connecting the internucleotide phosphate linkage and the adjacent 5′-*C*-position was our first example of a dinucleotide analogue obtained by this strategy.[19,20] A corresponding six-membered ring was later obtained using a different strategy by Le Clézio *et al.*,[25] who also very recently introduced other constrained dinucleotides with six-membered rings involving the phosphates.[26] With our strategy, large ring connections have been made in dinucleotides between the internucleotide phosphate linkage and 5-*C* of an adjacent pyrimidine nucleobase,[21,22,24] between two adjacent pyrimidines,[24] between 4′-*C* and 5′-*C* positions[23] and, finally, between the two phosphates in a trinucleotide.[21] A large ring connecting the nucleobase and the phosphate with a four carbon linkage in an allylic phosphortriester moiety was found to be very unstable towards basic conditions.[22] However, this was solved by the preparation of a saturated linker by employing the tandem RCM–hydrogenation protocol[22] first introduced by Grubbs and co-workers.[27] The synthesis of phosphorus containing heterocycles *via* RCM has been recently reviewed.[28]

Herein, we describe the synthesis of a new type of cyclic dinucleotide and its incorporation into oligodeoxynucleotide sequences. The synthesis was based on the tandem RCM–hydrogenation protocol with a dinucleotide substrate containing an allyl phosphortriester linkage and an easily available 2′-*C*-allyl-2′-deoxyuridine building block. The cyclic moiety was expected to introduce a bend motif in the oligonucleotides and, subsequently, to distort the recognition of complementary DNA and RNA in standard duplex formation. On the other hand, a potential stabilisation of other secondary structures like bulges and three-way junctions was envisioned and studied.

Results and discussion

Chemical synthesis

The dinucleotide was constructed on the basis of known methodology for preparing 2′-allyl-2′-deoxynucleosides.[29,30] Hence, the nucleoside **1**[29] was protected as a 5′-silyl ether **2** in a high yield (Scheme 1). The 5′-*O*-phosphoramidite **4** was easily prepared from 3′-*O*-*tert*-butyldimethylsilylthymidine **3** by an allyl phosphordiamidite reagent and 1*H*-tetrazole as the activator. As an alternative activator, dicyanoimidazole[31] afforded the same yield. The nucleoside building blocks **2** and **4** were coupled by treatment with 1*H*-tetrazole followed by oxidation to give, after purification, the diallylic dinucleotide **5** in a high yield as a mixture of two phosphorus epimers in an approximately 2 : 1 ratio. This epimeric mixture was subjected to a standard RCM protocol using Grubbs 2nd generation catalyst§[17,18] to give the two epimers **6R** and **6S** in 40 and 20% isolated yields, respectively. The formation of cyclic structures was confirmed by NMR and MS, demonstrating the loss of terminal double bonds and the loss of the mass of ethylene. The designation of

† Electronic supplementary information (ESI) available: additional experimental data. See http://www.rsc.org/suppdata/ob/b5/b502720a/

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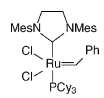
§ RCM and metathesis reactions in general have been recently reviewed.[14–16] Grubbs 2nd generation catalyst (Mes = 2,4,6-trimethylphenyl, Cy = cyclohexyl):[17,18]



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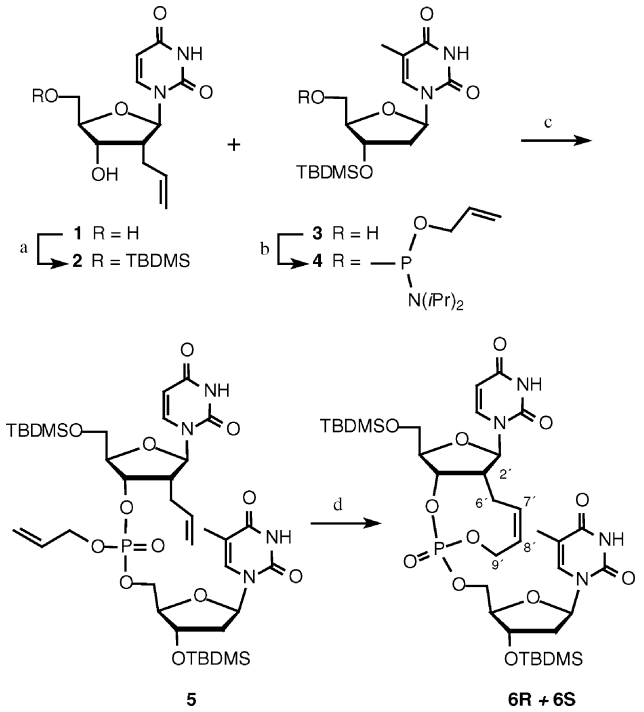
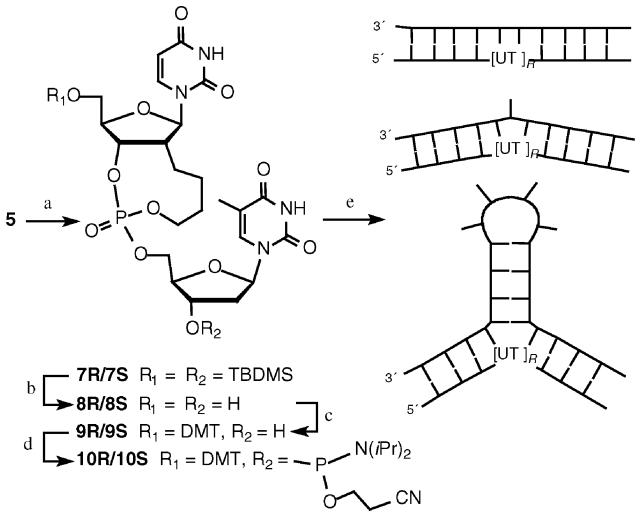


Scheme 1 *Reagents and conditions*: a) TBDMSCl, $AgNO_3$, pyridine, 85%; b) $CH_2{=}CHCH_2OP(N(iPr)_2)_2$, 1*H*-tetrazole, CH_3CN, 66%; c) i) 1*H*-tetrazole, CH_3CN, ii) *t*BuOOH, toluene, CH_3CN, 85%; d) Grubbs 2nd gen. catalyst,§ CH_2Cl_2, 40% **6R**, 20% **6S**, 16% mixture. TBDMS = *t*-butyldimethylsilyl.

phosphorus configuration to the two epimers was performed by more advanced NMR spectroscopy as described in detail below.

The cyclic structure of **6** is based on an allylic phosphortriester functionality. A similar entity in another cyclic dinucleotide was found to be unstable towards weak bases such as ammonia and pyridine.[22] Therefore, the formation of the saturated and predictably much more stable[22] analogues of **6** was strongly preferred. Hence, the application of the tandem RCM–hydrogenation protocol[22,24,27] with **5** as the substrate afforded the saturated cyclic dinucleotides **7R** and **7S** in 48% and 17% isolated yields, respectively, after a chromatographic separation (Scheme 2). As the elucidation of the exact configuration of



Scheme 2 *Reagents and conditions*: a) Grubbs 2nd gen. catalyst,§ CH_2Cl_2, *then* 1000 psi H_2, 48% **7R**, 17% **7S**; b) 90% TFA (aq.), 100% (**8R**), 85% (**8S**); c) DMTCl, 2,6-lutidine, DMSO, 64% (**9R**), 53% (**9S**); d) $NC(CH_2)_2OP(N(iPr)_2)_2$, 4,5-dicyanoimidazole, CH_3CN, 81% (**10R**), 57% (**10S**); e) incorporation into a duplex, a bulged duplex and a three-way junction, respectively (see Table 2). $[UT]_R$ reflects the incorporation of **10R** (see Table 1 and 2). TBDMS = *t*-butyldimethylsilyl, DMT = 4,4′-dimethoxytrityl.

the phosphorus epimers turned out to be impossible and as approximately the same ratio of epimers was obtained from the RCM reaction as well as from the RCM-hydrogenation tandem protocol, the major epimer of **7** was depicted to be of R_P-configuration and the minor of S_P-configuration, as elucidated carefully for the respective epimers of **6** (see below).

Both **7R** and **7S** were deprotected by an acidic treatment to give **8R** and **8S** in high yields. The major product **8R** was tested for its reactivity towards a treatment with 32% aq. ammonia. MALDI-MS and NMR showed no formation of an ammonia adduct after 24 h at room temperature. This result encouraged us to proceed towards incorporation of the two epimeric cyclic and uncharged dinucleotides into oligonucleotide sequences. Hence, **8R** and **8S** were protected as their 5′-*O*-dimethoxytrityl (DMT) ethers, **9R** and **9S**, by the application of DMT-Cl and an unusual solvent mixture, DMSO and 2,6-lutidine. This was applied after the failure of the more standard combination of DMT-Cl and $AgNO_3$ in pyridine. Using the cyanoethyl phosphordiamidite reagent, the phosphoramidites **10R** and **10S** were synthesised in good yields as equimolar epimeric mixtures as judged from NMR.

NMR study of the epimers of 6 and 8

Due to a significant overlap of ^{1}H NMR signals, especially in the saturated 2′-*C* to phosphate linkage, all attempts of determining the exact phosphorus configuration of the two epimers of **8** (or **7**) were unsuccessful. However, the determination was successfully performed on the unsaturated and protected epimers of **6**.

All protons in the two phosphorus epimers of compound **6** were assigned with combined use of DQF-COSY and NOESY spectra. The cross peaks in the 700 ms NOESY spectra have negative signs relative to the diagonal peaks, indicating rapid rotational motion of the molecules. In the following, all atom labelling follows the numbering shown in Scheme 1. At a first glance the NMR spectra of the two compounds were quite similar, with the majority of spins differing less than 0.1 ppm in the two compounds. The largest change in chemical shift was seen for H-3′ of the uridine, which is shifted 0.36 ppm downfield in the isomer assigned (*vide infra*) to be **6R** relative to **6S**. Additional effects were observed for the H-9′a and H-9′b protons that differ by 0.13 and 0.18 ppm, respectively, in the two epimers. A number of coupling constants were extracted from high quality 1D spectra. The two protons on the double bonded carbons, H-7′ and H-8′, display a coupling constant of 10.5 or 10.8 Hz consistent with the double bond being in the *cis* configuration.

The 700 ms NOESY experiments revealed a number of important structural clues regarding the configuration of the phosphorus in the two dinucleotides. For **6R**, cross peaks between T-CH_3 and H-9′a and H-9′b were seen, the cross peaks between H-8′ and H-9′a/b have the same intensity and no cross peak can be observed between H-6′ and H-9′ protons. In **6S**, no cross peaks are observed between T and any signal of the upper nucleoside. The H-8′-H-9′a cross peak is significantly more intense than the H-8′-H-9′b cross peak and a strong cross peak is found between H-6′b and H-9′b. All the observed NOESY cross peaks were integrated and converted into distances by utilizing the isolated spin pair approximation (ISPA). In total 14 and 19 noncovalently fixed distances were obtained for **6R** and **6S**, respectively, in addition to four structurally relevant coupling constants. After several runs of structure calculation and careful inspection of the resulting structures and the NOESY spectra, a further eight repulsive restraints were added for both epimers. For each of the compounds two starting structures were employed, differing only in the chirality of the phosphorus atom. A total of 20 separate structure calculations were performed with each starting structure and the final result was compared with respect to force field energies, energy penalties due to violation of the imposed restraints and the violation of restraints. Compound **6R** was found to be consistent with the structure having R_P

configuration around phosphorus as evaluated by an average forcefield energy 10% lower for the R_P configuration, fewer violations and a much lower energy penalty from constraints. Similarly, **6S** is characterized by having a force field energy approximately 10% lower when an S_P starting structure is used relative to an R_P starting structure and fewer violations were observed. By inspection of the calculated lowest energy structures of the two epimers, some of the differences in the chemical shifts can now be rationalized. The R_P configuration in **6R** brings the phosphate oxygen into close proximity of the H-3′ proton and can explain the downfield shift of 0.36 ppm relative to **6S**. In **6S** the *O*-5′ of the 5′-neighboring thymidine was seen to be quite close to H-9′b, which is consistent with the downfield shift of 0.18 ppm seen in this compound.

No immediate information about the conformational behaviour of **8R** and **8S** is given from the modelling on **6R** and **6S**. This is a natural consequence of the introduction of a saturated linker and hereby more conformational freedom in **8R** and **8S**. Nevertheless, and despite the overlap of signals in the ^{1}H NMR spectra of **8R** and **8S**, some observations concerning the conformational behaviour of the molecules are worth mentioning. The same difference in chemical shift for the uridine H-3′ proton observed for **6R** relative to **6S** is found between **8R** and **8S**. Thus, a similar difference in conformation between the two epimers as the one found between **6R** and **6S** is indicated. Very small $J_{H3'H4'}$ coupling constants (<1 Hz) were observed for the uridine nucleoside in both epimers **8R** and **8S** (as well as **6R** and **6S**), indicating a strong preference for an *S*-type sugar pucker. This was expected due to the 2′-*C*-alkyl group and the cyclic moiety. Finally, in NOESY spectra obtained for **8R** and **8S** in DMSO-*d*6, positive signs were observed for most signals of the uridine nucleoside compared to negative signs for the signals of the thymidine nucleoside. This indicates a slower dynamic and, despite the long and saturated linker, a much stronger conformational stiffness for the uridine part compared to the less restricted thymidine moiety.

Preparation and evaluation of oligonucleotides

The two amidites **10R** and **10S** were incorporated into four different oligonucleotides by standard automated solid phase DNA-synthesis using 10 min coupling time for **10R** and **10S** followed by prolonged capping time. For both, the coupling efficiencies were in the range of 86–98%. The oligonucleotides prepared for this study are shown in Table 1. Thus, the two central thymidines of the standard sequence **11** were replaced by the cyclic dinucleotide **8R** in **12** and **8S** in **15**. Also an LNA–DNA mixmer sequence **13** was studied, securing an overall preference for A-type duplex formation for this sequence¶[33,34] Replacing the central TT in this sequence with either **8R** or **8S** gave **14** and **16**, respectively. The constitution of the oligonucleotides was verified by MALDI-MS (Table 1) in addition to clean HPLC-profiles showing **11–14** as pure compounds. On the other hand, MALDI for the two oligonucleotides with the S_P-isomer, **15** and **16**, demonstrated an ammonia adduct as the primary product as evidenced by an increase in mass of 17 Daltons. Hence, the two epimers demonstrate a significantly different stability towards ammonia and **15** and **16** were excluded from further studies. Therefore, only the influence of the R_P-isomer (the major isomer) of **8** on secondary nucleic acid structures was examined.

¶ LNA (*locked nucleic acid*) has been introduced as oligonucleotides containing the LNA monomers with a bicyclic carbohydrate moiety locked in an *N*-type conformation[2,32,33] and with the ability of tuning the overall duplex conformation towards the A-type.[33,34] LNA sequences recognise complementary DNA and RNA with significantly increased affinity compared to unmodified oligonucleotides.[32,33] LNA monomers:

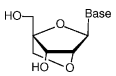


Table 1 Prepared oligodeoxynucleotide sequences and their MS-data

	ODN sequences[a]	MW (found/calcd)[b]
11	5′-GCTCACTTCTCCCA	4135[c]/4136 $[MH]^+$
12	5′-GCTCAC$[UT]_R$CTCCCA	4175[c]/4176 $[MH]^+$
13	5′-GC^LTC^LAC^LTTC^LTC^LCC^LA	4387[c]/4388 $[MH]^+$
14	5′-GC^LTC^LA$C^L$$[UT]_R$$C^LTC^LCC^L$A	4423[c]/4428 $[MH]^+$
15	5′-GCTCAC$[UT]_S$CTCCCA	4192[d]/4193 $[MNH_4]^+$
16	5′-GC^LTC^LA$C^L$$[UT]_S$$C^LTC^LCC^L$A	4444[d]/4445 $[MNH_4]^+$

[a] $[UT]_{R/S}$ refers to incorporation of **10R** or **10S**. C^L refers to the LNA 5-methylcytidine monomer. [b] MALDI-MS positive mode. [c] Only one major peak. [d] Also minor signals from the expected $[MH]^+$.

Hybridisation properties of oligonucleotides **11–14** with complementary DNA and RNA sequences were evaluated by thermal stability examinations (Table 2). First, the standard duplexes formed between **11–14** and their fully matched complements were examined and large drops in duplex stability were observed. Comparing **11** and **12**, the cyclic dinucleotide induced a drop in T_m of 15 °C in a DNA : DNA duplex compared to a standard sequence. The same was evident in an LNA-modified (and therefore A-type)[33,34] duplex, which was significantly more stable due to the LNA-monomers.¶[2,32,33] Also a DNA : RNA duplex was strongly destabilised, with a drop in T_m of 12.3 °C. These results demonstrate the expected distortion of standard nucleic acid duplexes by the cyclic dinucleotide structure.

For further elucidation of the secondary structure induced by the cyclic dinucleotide **8R**, we investigated the hybridisation properties of oligonucleotides **11–14** with bulged DNA and RNA complements (see also Scheme 2). An additional guanosine residue was incorporated in between the two adenosines opposite the cyclic dinucleotide residue. The subsequent T_m measurements revealed a smaller but significant drop in stability of 4.9 to 6.6 °C when comparing the modified sequences, **12** and **14**, with their unmodified counter parts, **11** and **13**. In other words, the introduction of a bulge in a duplex drops the stability by 8–12 °C, whereas in the distorted duplexes containing **8R** the difference is only 0.5–3.3 °C.

Finally, we examined the stability of a three-way junction (TWJ) composed of a standard stable stem-loop sequence with two single stranded regions being complementary to the oligonucleotides **11–14** with the cyclic dinucleotide in the branching point (see Scheme 2). The TWJ was in general not very stable with a T_m of 26.4 °C in a complete DNA-context and 36.8 °C with a DNA : RNA hybrid, though significantly more stable with six LNA-monomers (Table 2). A 5 mM concentration of Mg^{2+} was in general found to increase the thermal stability with 6–8 °C. A smaller further stabilisation with a 10 mM Mg^{2+} concentration of 2–3 °C was observed. When comparing the data of **11** with **12**, the TWJ was found to be stabilised with the cyclic dinucleotide, especially with the stem-loop sequence being RNA with an increase in T_m of 2.2 °C rising to 2.7 °C with the addition of Mg^{2+}. In the LNA-containing sequences, the TWJ is slightly destabilised with a drop in T_m of only 0.3–0.5 °C, although this is amplified by the increase of Mg^{2+} concentration in the context of a DNA-complement.

The structural basis for the observed stabilisation of a TWJ by the artificial bend motif is not immediately deducible from the hybridisation data. The Mg^{2+} mediated stabilisation of the TWJ is more or less uniform and no indications of a different Mg^{2+} induced conformational shift with the modified sequences are indicated. On the other hand, the present results demonstrate the potential for mimicking and stabilising secondary structures like TWJs by the introduction of simple artificial distortions like cyclic dinucleotides. The bend motif induced in an oligonucleotide by **8R** might not be optimal for any of the secondary structures investigated in this study as, after all, only a slight stabilisation of the TWJ was seen. On the other hand, a significant structural stabilisation might be found in

Table 2 Hybridisation data for the prepared oligonucleotides with DNA/RNA-complements[a]

	Fully matched complements[b]		Complements with G-bulges[c]	
	DNA	RNA	DNA	RNA
11	54.1	60.2	42.1	49.7
12	39.1 (−15.0)	47.9 (−12.3)	36.1 (−6.0)	44.8 (−4.9)
13	76.3	>90	67.5	82.6
14	61.7 (−14.6)	79.3 (<−10.7)	61.2 (−6.3)	76.0 (−6.6)

	Complements with an intrastrand stem-loop[d,e]					
	DNA			RNA		
	0 mM	5 mM	10 mM	0 mM	5 mM	10 mM
11	26.4	32.8	35.2	36.8	44.6	46.5
12	27.1 (0.7)	33.4 (0.6)	36.8 (1.6)	39.0 (2.2)	47.2 (2.6)	49.2 (2.7)
13	52.2	59.2	62.5	71.3	79.3	80.8
14	51.9 (−0.3)	58.2 (−1.0)	60.0 (−2.5)	70.8 (−0.5)	78.8 (−0.5)	80.7 (−0.l)

[a] Melting temperatures (T_m values/°C) obtained from the maxima of the first derivatives of the melting curves (A_{260} *vs.* temperature) recorded in a medium salt buffer (Na_2HPO_4 (15 mM), NaCl (100 mM), EDTA (0.1 mM), pH 7.0) using 1.0 μM concentrations of each strand. All T_m values are given as averages of double determinations. ΔT_m values are given in brackets. [b] DNA 3′-CGAGTGAAGAGGGT, RNA 3′-CGAGUGAAGAGGGU. [c] DNA 3′-CGAGTGA**G**AGAGGGT, RNA 3′-CGAGUGA**G**AGAGGGU. [d] DNA 3′-CGAGTGA**CGCGTTTTCGCG**AGAGGGT, RNA 3′-CGAGUGA**CGCGUUUUCGCG**AGAGGGU, bold sequences are stem-loops. [e] Increasing concentration of Mg^{2+} by addition of $MgCl_2$.

a more optimised combination of **8R** and a secondary nucleic acid structure. Powerful stabilisation of secondary structures, comparable to the stabilisation of duplexes obtained by LNA and others,[1–3] might also be obtained by other dinucleotide structures prepared by our RCM-based strategy. Thus, other examples are in progress. For example, cyclic dinucleotides connecting both furanose moieties directly thereby introducing a stronger bend motif and, presumably, a stronger effect on the overall conformational restriction of the secondary structure, compared to the restriction given by **8R**. Therefore, the appropriate molecular modelling combined with a selection of cyclic structures can reveal a general tool for the design of conformationally restricted and stable nucleic acid fragments. This can play a strong future role in the design of nucleic acid based architectures for therapeutics and nanoscale engineering.

Conclusions

In conclusion, we have synthesised a cyclic dinucleotide **8R** by a convenient method based on RCM and incorporated this into oligonucleotides. Hence, an example of an artificial bend nucleic acid motif has been introduced and found to significantly distort nucleic acid duplexes but slightly stabilise a three-way junction. This confirms the value of the general RCM-based strategy for mimicking and stabilisation of nucleic acid secondary structures.

Experimental

All commercial reagents were used as supplied. When necessary, reactions were performed under an atmosphere of nitrogen. Column chromatography was carried out on glass columns using silica gel 60 (0.040–0.063 mm). NMR spectra were recorded on a Varian Gemini 2000 spectrometer or a Varian UNITY 500 spectrometer. ^{1}H NMR spectra were recorded at 300 or 500 MHz, ^{13}C NMR spectra were recorded at 62.5 MHz and ^{31}P NMR spectra were recorded at 121.5 MHz. The values for δ are in ppm relative to tetramethylsilane as internal standard or 85% H_3PO_4 as external standard. HRMALDI and ESI mass spectra were recorded on an Ionspec Ultima Fourier Transform mass spectrometer with a DHB-matrix. Assignments of NMR spectra are based on ^{1}H, ^{1}H-COSY, ^{1}H, ^{13}C-COSY and/or DEPT spectra and follow standard carbohydrate and nucleoside style; *i.e.* the carbon atom next to a nucleobase is assigned C-1′. Carbons in the linker next to C-2′ are assigned C-6′ to C-9′, as depicted in Scheme 1. However, compound names for bicyclic compounds are given according to the von Baeyer nomenclature.

Preparation of 2′-*C*-allyl-5′-*O*-*tert*-butyldimethylsilyl-2′-deoxyuridine (2)

2′-*C*-Allyl-2′-deoxyuridine **1**[29] (0.510 g, 1.90 mmol) was co-evaporated twice with anhydrous pyridine (20 cm^3) and then redissolved in anhydrous pyridine (30 cm^3). TBDMSCl (0.340 g, 2.26 mmol) and $AgNO_3$ (0.390 g, 2.30 mmol) were added and the reaction mixture was stirred for 25 h at room temperature. The reaction was quenched by the addition of methanol (3 cm^3) and concentrated under a reduced pressure. The residue was redissolved in dichloromethane (50 cm^3), filtered through celite and the filtrate was washed with water (50 cm^3) and dried (Na_2SO_4). The solvent was removed by destillation under a reduced pressure and the residue was purified by dry column chromatography (0–6% methanol–EtOAc) to give the product as a white powder (0.617 g, 85%); R_f 0.60 (10% methanol–EtOAc); δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.11 (6H, s, $SiCH_3$), 0.92 (9H, s, $C(CH_3)_3$), 2.20–2.30 (2H, m, 2′-CH_2, H-2′), 2.47 (1H, m, 2′-CH_2), 3.79 (1H, dd, *J* 2.0, 11.4 Hz, H-5′a), 3.89 (1H, dd, *J* 2.4, 11.4 Hz, H-5′b), 4.11 (1H, m, H-4′), 4.33 (1H, d, *J* 4.4 Hz, H-3′), 5.05 (1H, dd, *J* 1.5, 10.1 Hz, C*H*$_2$=CH), 5.13 (1H, dd, *J* 1.5, 17.1 Hz, C*H*$_2$=CH), 5.71 (1H, d, *J* 8.1 Hz, H-5), 5.77 (1H, m, CH_2=C*H*), 6.17 (1H, d, *J* 8.4 Hz, H-1′), 7.88 (1H, d, *J* 8.1 Hz, H-6), 9.20 (1H, br s, NH); δ_C (75 MHz; $CDCl_3$; Me_4Si) −5.49, −5.39 ($SiCH_3$), 18.42 (*C*$(CH_3)_3$), 25.98 (C(*C*$H_3)_3$), 28.44 (C-2′), 49.75 (2′-CH_2), 64.28 (C-5′), 73.90 (C-3′), 87.15 (C-4′), 88.20 (C-1′), 102.93 (C-5), 117.08 (*C*H_2=CH), 135.30 (CH_2=*C*H), 140.51 (C-6), 150.86 (C-2), 163.38 (C-4); HiRes MALDI FT-MS *m*/*z* (M + Na) found/calc. 405.1818/405.1816.

Preparation of 3′-*O*-*tert*-butyldimethylsilylthymidin-5′-yl-*O*-allyl-*N*,*N*′-diisopropylaminophosphoramidite (4)

3′-*O*-(*tert*-Butyldimethylsilyl)thymidin **3** (0.795 g, 2.23 mmol) was dissolved in CH_3CN (4 cm^3). Allyloxybis(diisopropylamino)phosphine (0.97 g, 3.35 mmol) was added. A 0.45 M solution of 1*H*-tetrazole in CH_3CN (7.5 cm^3, 3.4 mmol) was added over a period of 5 min. The reaction mixture was stirred for 1 h, filtrated and diluted with dichloromethane (40 cm^3). The solution was washed with a saturated aq. solution of $NaHCO_3$ (20 cm^3) and brine (4 cm^3), dried (Na_2SO_4) and concentrated

under a reduced pressure. The residue was purified by dry column chromatography (0–100% EtOAc–0.5% Et_3N–petrol ether) to give the product as an oily mixture of two phosphorus epimers (0.800 g, 66%); R_f 0.60 (75% EtOAc–petrol ether); δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.08 (6H, m, $SiCH_3$), 0.89 (9H, br s, $C(CH_3)_3$), 1.17–1.22 (12H, m, $CH(CH_3)_2$), 1.91 (3/2H, d, *J* 1.1 Hz, CH_3), 1.93 (3/2H, d, *J* 1.1 Hz, CH_3), 1.99–2.14 (1H, m, H-2′a), 2.20–2.29 (1H, m, H-2′b), 3.55–3.69 (2H, m, $CH(CH_3)_2$), 3.75–3.93 (2H, m, H-5′), 4.00–4.05 (1H, m, H-3′), 4.11–4.22 (2H, m, $POCH_2$), 4.42–4.47 (1H, m, H-4′), 5.12–5.32 (2H, m, CH_2=CH), 5.87–5.97 (1H, m, CH_2=CH), 6.31–6.37 (1H, m, H-1′), 7.58 ($\frac{1}{2}$H, 7.58, d, *J* 1.1 Hz, H-6), 7.75 ($\frac{1}{2}$H, d, *J* 1.1 Hz, H-6), 8.10 (1H, br s, NH); δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) 148.92, 149.06. ESI MS *m/z* (M_2 + Na) 1109.

Preparation of allyl (2′-*C*-allyl-5′-*O*-*tert*-butyldimethylsilyl-2′-deoxyuridin-3-yl) (3′-*O*-*tert*-butyldimethylsilyl thymidin-5′-yl) phosphate (5)

Compound **2** (0.610 g, 1.59 mmol) and compound **4** (1.12 g, 2.07 mmol) were mixed and coevaporated with anhydrous CH_3CN (20 cm^3). The mixture was redissolved in anhydrous CH_3CN (24 cm^3) and a 0.45 M solution of 1*H*-tetrazole in CH_3CN (4.3 cm^3, 1.9 mmol) was added over a period of 10 min. The reaction mixture was stirred for 2.5 h generating the intermediate phosphite (R_f 0.60 (75% EtOAc–petrol ether)). Then, a 3 M solution of *t*-BuOOH in toluene (2.6 cm^3, 7.8 mmol) was added and the reaction mixture was stirred for 40 min. The reaction was quenched by the addition of methanol (3 cm^3). The mixture was concentrated under a reduced pressure and the residue was purified by dry column chromatography (0–100% EtOAc–Petrol ether) to give the product as a mixture of two phosphorus epimers as a white foam (1.14 g, 85%); R_f 0.44 (75% EtOAc–Petrol ether); δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.10–0.12 (12H, m, CH_3), 0.89–0.91 (18H, m, $C(CH_3)_3$), 1.93–1.95 (3H, m, T-CH_3), 2.09–2.50 (5H, m, T-H-2′, U-H-2′, 2′-CH_2), 3.83–3.90 (2H, m, U-H-5′), 4.00–4.05 (1H, m, T-H-4′), 4.18–4.31 (2H, m, T-H-5′), 4.33–4.45 (2H, U-H-4′, T-H-3′), 4.55–4.64 (2H, m, $POCH_2$), 4.88–4.95 (1H, m, U-H-3′), 4.98–5.13 (2H, m, 2′-CH_2CH=CH_2), 5.28–5.43 (2H, m, $POCH_2CH$=CH_2), 5.62–5.78 (2H, m, 2′-CH_2CH=CH_2, U-H-5), 5.87–6.01 (1H, m, $POCH_2CH$=CH_2), 6.13–6.17 (1H, m, U-H-1′), 6.24–6.30 (1H, m, T-H-1′), 7.33–7.40 (1H, m, T-H-6), 7.79–7.84 (1H, m, U-H-6), 9.12–9.37 (2H, m, 2 × NH); δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) −0.22, −0.09; HiRes MALDI FT-MS *m/z* (M + Na) found/calc. 863.3372/863.3460.

Preparation 3*R*- and 3*S*-(1*S*,9*R*,10*R*,12*R*)-3-(3(*S*)-(*tert*-butyldimethylsilyl)oxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)methoxy)-3-oxo-12-(*tert*-butyldimethylsilyl)oxymethyl-10-(thymin-1-yl)-2,4,11-trioxa-3-phosphabicyclo[7.3.0]dodec-6-ene (6R and 6S)

Compound **5** (0.052 g, 0.062 mmol) was dissolved in dichloromethane (6 cm^3). Grubbs 2nd gen. catalyst§ (2.6 mg, 3 μmol) was added and the reaction mixture was refluxed for 24 h. An additional amount of Grubbs 2nd gen. catalyst (2.0 mg, 2 μmol) was added and the reaction mixture was refluxed for another 24 h. The mixture was concentrated under a reduced pressure and the residue was purified by column chromatography (66–100% EtOAc–petrol ether followed by 5% methanol–EtOAc) to give the two phosphorus epimers **6R** and **6S** as isolated products, as well as a **6R/S** mixture (0.008 g, 16%, approx. 7 : 3 ratio):

6R (0.020 g, 40%). R_f 0.56 (10% methanol–EtOAc); δ_H (500 MHz; $CDCl_3$; Me_4Si) 0.11–0.12 (12H, m, $SiCH_3$), 0.90–0.91 (18H, m, $C(CH_3)_3$), 1.95 (3H, s, CH_3), 2.08 (1H, m, H-6′a), 2.16 (1H, dt, *J* 6.7, 12.0 Hz, T-H-2′a), 2.31 (1H, ddd, *J* 3.5, 6.7, 12.0 Hz, T-H-2′b), 2.41 (1H, m, U-H-2′), 2.88 (1H, q, *J* 12.3 Hz, U-H-6′b), 3.87 (2H, m, U-H-5′), 4.05 (1H, m, T-H-4′), 4.22–4.31 (3H, T-H-5′, U-H-4′), 4.39 (1H, ddd, *J* 6.0, 12.0, 16.2 Hz, H-9′a), 4.47 (1H, dt, *J* 3.5, 6.7 Hz, T-H-3′), 4.83 (1H, dt, *J* 8.8, 12.0 Hz, H-9′b), 4.89 (1H, dd, *J* 4.9, 9.4 Hz, U-H-3′), 5.75 (1H, d, *J* 8.3 Hz, U-H-5), 5.78 (1H, ddd, *J* 6.0, 10.8, 12.3 Hz, H-7′), 5.91 (1H, ddd, *J* 6.0, 8.8, 10.8 Hz, H-8′), 6.21 (1H, d, *J* 9.0 Hz, U-H-1′), 6.28 (1H, t, *J* 6.7 Hz, T-H-1′), 7.36 (1H, s, T-H-6), 7.87 (1H, d, *J* 8.3 Hz, U-H-6), 9.07 (1H, s, NH), 9.37 (1H, s, NH). δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) −0.26. HiRes MALDI FT–MS *m/z* (M + Na) found/calc. 835.3126/ 835.3141.

6S (0.010 g, 20%). R_f 0.40 (10% methanol–EtOAc); δ_H (500 MHz; $CDCl_3$; Me_4Si) 0.11–0.12 (12H, m, $SiCH_3$), 0.90 (18H, s, $C(CH_3)_3$), 1.96 (3H, s, CH_3), 2.06 (1H, m, H-6′a), 2.11 (1H, m, T-H-2′a), 2.30 (1H, ddd, *J* 3.5, 6.7, 12.0 Hz, T-H-2′b), 2.30 (1H, m, U-H-2′), 2.86 (1H, q, *J* 12.3 Hz, H-6′b), 3.71 (1H, dd, *J* 2.0, 12.0 Hz, U-H-5′a), 3.81 (1H, dd, *J* 2.0, 12.0 Hz, U-H-5′b), 4.01 (1H, dt, *J* 3.5, 7.0 Hz, T-H-4′), 4.21 (1H, m, T-H-5′a), 4.24 (1H, m, U-H-4′), 4.26, (1H, m, H-9′a), 4.29 (1H, m, T-H-3′), 4.32 (1H, m, T-H-5′b), 4.57 (1H, dd, *J* 4.4, 10.0 Hz, U-H-3′), 5.01 (1H, dt, *J* 10.5, 11.0 Hz, H-9′b), 5.72 (1H, d, *J* 8.2 Hz, U-H-5), 5.86 (1H, ddd, *J* 6.1, 10.5, 12.3 Hz, H-7′), 5.96 (1H, dt, *J* 6.5, 10.5 Hz, H-8′), 6.19 (1H, d, *J* 9.7 Hz, U-H-1′), 6.23 (1H, t, *J* 6.7 Hz, T-H-1′), 7.25 (1H, s, T-H-6), 7.78 (1H, d, *J* 8.0 Hz, U-H-6), 8.66 (1H, s, NH), 8.73 (1H, s, NH). δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) −0.52. HiRes MALDI FT-MS *m/z* (M + Na) found/calc. 835.3105/835.3141.

Preparation of 3*R*- and 3*S*-(1*S*,9*R*,10*R*,12*R*)-3-(3(*S*)-(*tert*-butyldimethylsilyl)oxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)methoxy)-3-oxo-12-(*tert*-butyldimethylsilyl)oxymethyl-10-(thymin-1-yl)-2,4,11-trioxa-3-phosphabicyclo[7.3.0]dodecane (7R and 7S)

Compound **5** (0.199 g, 0.236 mmol) was dissolved in dichloromethane (23 cm^3). Grubbs 2nd gen. catalyst§ (10 mg, 12 μmol) was added and the reaction mixture was refluxed for 48 h. The reaction mixture was transferred to a Parr-bomb and subjected to 1000 psi H_2 at 50 °C for 12 h. The mixture was concentrated under a reduced pressure and the residue was purified by column chromatography (60–100% EtOAc–petrol ether followed by 2–5% methanol–EtOAc) to give the two phosphorus epimers **7R** and **7S** as isolated products:

7R (0.092 g, 48%). R_f 0.60 (10% methanol–EtOAc); δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.10–0.14 (12H, m, $SiCH_3$), 0.90–0.93 (18H, m, $C(CH_3)_3$), 1.60–1.75 (2H, m, H-8′), 1.80–1.95 (4H, m, H-6′, H-7′) 1.95 (3H, s, CH_3), 2.15 (1H, m, T-H-2′a), 2.25–2.35 (2H, m, U-H-2′, T-H-2′b), 3.89 (2H, br s, U-H-5′), 4.03 (1H, m, T-H-4′), 4.18–4.32 (4H, T-H-5′, H-9′a, U-H-4′), 4.38–4.51 (2H, m, T-H-3′, H-9′b), 5.21 (1H, m, U-H-3′), 5.73 (1H, d, *J* 8.1 Hz, H-5), 6.07 (1H, d, *J* 8.3 Hz, U-H-1′), 6.31 (1H, t, *J* 6.6 Hz, T-H-1′), 7.41 (1H, s, T-H-6), 7.84 (1H, d, *J* 8.1 Hz, U-H-6), 9.06 (1H, s, NH), 9.31 (1H, s, NH). δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) 0.44. HiRes MALDI FT-MS *m/z* (M + Na) found/calc. 837.3323/837.3298.

7S (0.032 g, 17%). R_f 0.47 (10% methanol–EtOAc); δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.10–0.12 (12H, m, $SiCH_3$), 0.90–0.92 (18H, m, $C(CH_3)_3$), 1.55–1.90 (6H, m, H-6′, H-7′, H-8′), 1.96 (3H, s, CH_3), 2.00–2.35 (3H, m, T-H-2′, U-H-2′), 3.73–3.87 (2H, m, U-H-5′), 4.04 (1H, m, T-H-4′), 4.17–4.33 (5H, T-H-5′, U-H-4′, T-H-3′, H-9′a), 4.51 (1H, m, H-9′b), 4.83 (1H, dd, *J* 4.8, 9.6 Hz, U-H-3′), 5.71 (1H, dd, *J* 1.8, 8.1 Hz, H-5), 6.04 (1H, d, *J* 9.1 Hz, U-H-1′), 6.25 (1H, m, T-H-1′), 7.28 (1H, d, *J* 1.0 Hz, T-H-6), 7.78 (1H, d, *J* 8.1 Hz, U-H-6), 8.48 (1H, s, NH), 8.51 (1H, s, NH). δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) −1.62. HiRes MALDI FT-MS *m/z* (M + Na) found/calc. 837.3262/837.3298.

Preparation of (1*S*,3*R*,9*R*,10*R*,12*R*)-3-(3(*S*)-hydroxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)methoxy)-3-oxo-12-hydroxymethyl-10-(thymin-1-yl)-2,4,11-trioxa-3-phosphabicyclo[7.3.0]dodecan (8R)

Compound **7R** (0.088 g, 0.108 mmol) was dissolved in a 90% aq. solution of TFA (2 cm^3). The mixture was stirred for 90 min,

an additional amount of 90% TFA solution added (1 cm^3) and stirred for another 200 min. The reaction mixture was concentrated under a reduced pressure and the residue was coevaporated with absolute ethanol and purified by column chromatography (10–30% ethanol–EtOAc) to give the product as a white powder (0.064 g, 100%). R_f 0.20 (20% ethanol–EtOAc); δ_H (300 MHz; DMSO-*d*6; Me_4Si) 1.45–1.85 (6H, m, H-6′, H-7′, H-8′), 1.80 (3H, s, CH_3), 2.08–2.16 (2H, m, T-H-2′), 2.36 (1H, m, U-H-2′), 3.56–3.63 (2H, m, U-H-5′), 3.93 (1H, m, T-H-4′), 4.09–4.28 (6H, m, U-H-4′, T-H-5′, H-9′, T-H-3′), 5.15 (1H, dd, *J* 5.2 Hz, *J* 9.5 Hz, U-H-3′), 5.32 (1H, t, *J* 5.1 Hz, 5′-OH), 5.47 (1H, d, *J* 4.3 Hz, 3′-OH), 5.71 (1H, dd, *J* 2.2 Hz, *J* 8.1 Hz, U-H-5), 5.82 (1H, d, *J* 9.4 Hz, U-H-1′), 6.20 (1H, t, *J* 6.8 Hz, T-H-1′), 7.49 (1H, d, *J* 1.1 Hz, T-H-5), 7.86 (1H, d, *J* 8.1 Hz, U-H-6), 11.31 (1H, s, T-NH), 11.38 (1H, d, *J* 2.2 Hz, U-NH); δ_P (121.5 MHz; DMSO-*d*6; H_3PO_4) 0.80; HiRes MALDI FT-MS *m*/*z* (M + Na) found/calc. 609.1556/ 609.1568.

Preparation of (1*S*,3*S*,9*R*,10*R*,12*R*)-3-(3(*S*)-hydroxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)methoxy)-3-oxo-12-hydroxymethyl-10-(thymin-1-yl)-2,4,11-trioxa-3-phosphabicyclo[7.3.0]dodecan (8S)

Compound **7S** (0.220 g, 0.271 mmol) was dissolved in a 90% aq. solution of TFA (10 cm^3) and the mixture was stirred for 3 h at room temperature and at 60 °C for 1 h, an additional amount of 90% TFA solution added (2 cm^3) and stirred for another 4 h. The reaction mixture was concentrated under a reduced pressure and the residue was coevaporated with absolute ethanol and purified by column chromatography (0–20% ethanol–EtOAc) to give the product as a white powder (0.135 g, 85%). R_f 0.23 (20% ethanol–EtOAc); δ_H (300 MHz; DMSO-*d*6; Me_4Si) 1.40–1.88 (6H, m, H-6′, H-7′, H-8′), 1.80 (3H, s, CH_3) 2.08–2.16 (2H, m, T-H-2′), 2.38 (1H, m, U-H-2′), 3.44–3.62 (2H, m, U-H-5′), 3.94 (1H, m, T-H-4′), 4.01–4.42 (6H, m, T-H-5′, U-H-4′, T-H-3′, H-9′), 4.88 (1H, m, U-H-3′), 5.28 (1H, t, *J* 5.0 Hz, 5′-OH), 5.43 (1H, d, *J* 4.0 Hz, 3′-OH), 5.70 (1H, d, *J* 8.3, U-H-5), 5.82 (1H, d, *J* 9.7 Hz, U-1′), 6.21 (1H, m, T-H-1′), 7.50 (1H, s, T-H-5), 7.83 (1H, d, *J* 8.3 Hz, U-H-6), 11.33 (1H, s, NH), 11.38 (1H, s, NH); δ_P (121.5 MHz; DMSO-*d*6; H_3PO_4) −1.44; HiRes MALDI FT-MS *m*/*z* (M + Na) found/calc. 609.1564/609.1568.

Preparation of (1*S*,3*R*,9*R*,10*R*,12*R*)-3-(3(*S*)-hydroxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)methoxy)-3-oxo-12-(4,4′-dimethoxytrityl)oxymethyl-10-(thymin-1-yl)-2,4,11-trioxa-3-phosphabicyclo[7.3.0]dodecan (9R)

Compound **8R** (0.080 g, 0.136 mmol) was coevaporated three times with anhydrous pyridine. 4,4′-Dimethoxytritylchloride (DMT-Cl) (0.052 g, 0.153 mmol), DMSO (0.6 cm^3) and 2,6-lutidine (0.7 cm^3) were added and the reaction mixture was stirred at room temperature for 46 h. An additional amount of DMT-Cl (0.007 g, 0.02 mmol) was added and the reaction mixture was stirred for another 2 h. The reaction was quenched by the addition of water (10 cm^3) and the mixture was extracted with dichloromethane (3 × 15 cm^3). The combined organic phases were washed with a saturated aq. solution of $NaHCO_3$ (30 cm^3) and brine (30 cm^3), dried (Na_2SO_4) and concentrated under a reduced pressure. The residue was purified by dry column chromatography (0–8% methanol–1% pyridine–EtOAc) to give the product as a white foam (78 mg, 64%). R_f 0.43 (20% ethanol–EtOAc); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.45–2.00 (6H, m, H-6′, H-7′, H-8′), 1.90 (3H, s, CH_3), 2.16 (1H, m, T-H-2′a), 2.36–2.40 (2H, m, T-H-2′b, U-H-2′), 3.43–3.56 (2H, m, U-H-5′), 3.78 (6H, s, OCH_3), 4.09 (1H, m, T-H-4′), 4.20–4.35 (4H, m, T-H-5′, U-H-4′, H-9′a), 4.41 (1H, m, H-9′b), 4.51 (1H, m, T-H-3′), 5.30 (1H, m, U-H-3′), 5.39 (1H, d, *J* 8.3 Hz, U-H-5), 6.03 (1H, d, *J* 9.3 Hz, U-H-1′), 6.32 (1H, t, *J* 6.6 Hz, T-H-1′), 6.81–6.86 (4H, m, Ph), 7.18–7.39 (10 H, m, T-H-6, Ph), 7.53 (1H, d, *J* 8.3 Hz, U-H-6), 9.71 (1H, s, T-NH), 9.92 (1H, s, U-NH); δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) 0.52; HiRes ESI FT-MS *m*/*z* (M + Li) found/calc. 895.3126/895.3137.

Preparation of (1*S*,3*S*,9*R*,10*R*,12*R*)-3-(3(*S*)-hydroxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)methoxy)-3-oxo-12-(4,4′-dimethoxytrityl)oxymethyl-10-(thymin-1-yl)-2,4,11-trioxa-3-phosphabicyclo[7.3.0]dodecan (9S)

The same procedure as in the preparation of **9R** was used with **8S** (0.125 g, 0.213 mmol) to give the product as a white foam (100 mg, 53%). R_f 0.40 (20% ethanol–EtOAc); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.45–2.00 (6H, m, H-6′, H-7′, H-8′), 1.85 (3H, s, CH_3), 2.10–2.20 (1H, m, T-H-2′a), 2.30–2.45 (2H, m, T-H-2′b, U-H-2′), 3.43–3.50 (2H, m, U-H-5′), 3.79 (6H, s, OCH_3), 3.90–3.96 (2H, m, T-H-5′), 4.10–4.40 (4H, m, T-H-4′, U-H-4′, T-H-3′, H-9′a), 4.53 (1H, m, H-9′b), 4.89 (1H, m, U-H-3′), 5.47 (1H, d, *J* 8.3 Hz, U-H-5), 5.99 (1H, d, *J* 8.9 Hz, U-H-1′), 6.07 (1H, t, *J* 6.6 Hz, T-H-1′), 6.82–6.87 (4H, m, Ph), 7.19–7.35 (10H, m, T-H-6, Ph), 7.59 (1H, d, *J* 8.3 Hz, U-H-6), 9.33 (1H, s, T-NH), 9.40 (1H, s, U-NH); δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) −1.24; HiRes ESI FT-MS *m*/*z* (M + Li) found/calc. 895.3167/895.3137.

Preparation of the 3′-*O*-phosphoramidite of 9R (10R)

Compound **9R** (0.076 g, 0.086 mmol) was coevaporated with anhydrous pyridine and dissolved in dichloromethane (1 cm^3). A 1 M solution of 4,5-dicyanoimidazole in CH_3CN (0.1 cm^3) and 2-cyanoethyl-*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (0.035 g, 0.116 mmol) were added and the reaction mixture was stirred for 30 min. The mixture was diluted with dichloromethane (10 cm^3) and washed with a saturated aq. solution of $NaHCO_3$ and brine (10 cm^3), dried (Na_2SO_4) and concentrated under a reduced pressure. The residue was purified by dry column chromatography (50–100% EtOAc–1% pyridine–petrol ether) to give the product as an off-white foam and a mixture of two epimers (75 mg, 81%); R_f 0.55 (10% methanol–1% pyridine–EtOAc); δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) 0.37, 0.42, 149.95, 150.40; HiRes ESI FT-MS *m*/*z* (M + Li) found/calc. 1095.4247/1095.4216.

Preparation of the 3′-*O*-phosphoramidite of 9S (10S)

The same procedure as in the preparation of **10R** was used with **9S** (0.072 g, 0.081 mmol) to give the product as a white foam (50 mg, 57%); R_f 0.53 (20% ethanol–EtOAc); δ_P (121.5 MHz; $CDCl_3$; H_3PO_4) −1.53, −1.47, 150.23, 150.29.

NMR analysis of compounds 6R and 6S. All NMR experiments were performed on a Varian UNITY 500 spectrometer at 25 °C in $CDCl_3$. 1D ^1H NMR spectra of **6R** and **6S** were recorded with 22 208 points and a spectral width of 5500 Hz. Phase sensitive NOESY spectra with a mixing time of 700 ms were recorded for **6R** and **6S** with 2 K points in the t_2-dimension using spectral widths of 5500 Hz in both dimensions and 16 transients for each of the 680 t_1-experiments. Phase sensitive DQF-COSY spectra were recorded with 4 K points sampled in F2, 5500 Hz spectral widths in both dimensions and 640 t_1-experiments, each recorded with 16 transients. All spectra were processed using NMRpipe.[35] Cross peak intensities were measured using the program SPARKY.[36]

Restrained molecular dynamics simulations. All MD calculations were performed using the AMBER 6.0 program package[37] on an SGI/O2 workstation. Due to the lack of suitable angular parameters in the AMBER 6.0 forcefield to describe the modifications at the phosphorus atom, a series of *ab initio* calculations were performed using the Gaussian 98 program[38] and the 3–21G and 6–31G(d,p) basis sets. These *ab initio* energy optimised structures were used to measure ideal angles to be included in the force field calculations. Atomic charges around the modified phosphorus were calculated using the restrained electrostatic potential (RESP) procedure.[39]

Distance restraints were derived from the NOESY spectra using the ISPA approach. After carefully evaluating the preliminary energy minimized structures, additional restraints were later included in form of broad repulsive restraints. All distance restraints were incorporated into the refinement as distance bounds with bounds of the calculated ISPA value ±0.5 Å. Two torsional restraints were included to ensure *cis* configuration of the double bond. In addition, six chirality restraints at the chiral atoms in the two deoxyribose sugar rings were included in each calculation.

A simulated annealing protocol was utilized to obtain average structures of the four compounds. For each compound, two different starting structures, differing only in the configuration at the phosphorus atom, were used. Each structure was then initially energy minimized before being subjected to 80 ps of molecular dynamics in time-steps of 1 fs : 4 ps at 800 K, followed by cooling to 200 K over 76 ps. Finally, a restrained energy minimization was performed for each structure. For all distance restraints the force constant used was $K = 20$ kcal mol^{-1} Å^2 and for coupling constants the force constant was 10 kcal mol^{-1} Hz^2. A distance dependent dielectric constant, $\varepsilon = 4r$, was used and the non-bonded cut-off was 20 Å.

Preparation of oligodeoxynuclotides

Oligonucleotide synthesis was carried out by using an Expedite™ 8900 Nucleic acid synthesis system from PerSeptive Biosystems Inc. following the phosphoramidite approach. Synthesis of oligonucleotides **11–16** was performed on a 0.2 µmol scale by using 2-cyanoethyl phosphoramidites of standard 2′-deoxynucleosides as well as the LNA–^{me}C monomer in combination with the modified phosphoramidites **10R** and **10S**. The synthesis followed the regular protocol emploing standard CPG supports. However, for the LNA monomer a prolonged coupling time of 4 min was used and for **10R** and **10S** a manual coupling in 10 min, followed by 2 × 45 s capping. Coupling yields for **10R** and **10S** were in the range of 86–98%. The 5′-*O*-DMT–ON oligonucleotides were removed from the solid support by treatment with concentrated aq. ammonia at room temperature for 48 h, which also removed the protecting groups. Purification using reversed-phase HPLC was performed on a Waters Prep LC 4000 system using a X_{terra} prep MS C_{18}; 10 µm; 7.8 × 300 mm column; A buffer: 3800 mL 0.1 M NH_4HCO_3 and 200 mL CH_3CN; B buffer: 750 mL CH_3CN and 250 mL NH_4HCO_3; 0–4 min. 100% A, 4–15 min. linear gradient 0–100% B, 15–20 min. 100% B, 20–22 min. 0–100% A, 22–25 min. 100% A. All fractions containing 5′-*O*-DMT protected oligonucleotide (retention time 14–17 min) were collected, concentrated and diluted with water (1 mL). The oligonucleotides were precipitated by treatment with 100 µL 80% CH_3COOH for 30 min, followed by the addition of 100 µL UHQ water, 50 µL 3 M CH_3COONa (aq.) and 600 µL 99.9% ethanol. The mixture was left at −18 °C for 1 h followed by centrifugation for 20 min at 4 °C. The supernatant was removed and the oligonucleotide was washed with cold 99.9% ethanol, dried and dissolved in water. MALDI MS *m/z* (M + H) found/calcd: **11** 4135/4136; **12** 4175/4176; **13** 4387/4338; **14** 4423/4428. For **15–16**, for which **10S** was employed, ring-opened ammonia-adducts were found. MALDI MS *m/z* (M + NH_4) found/calcd: **15** 4192/4193; **16** 4444/4445.

Melting experiments

UV melting experiments were carried out on a Perkin-Elmer Lambda 35 UV/VIS spectrometer with a PTP-6 Peltier system and data were processed with Templab version 2.00 and UV WINLAB version 2.85.04. Samples were dissolved in a medium salt buffer containing Na_2HPO_4 (15 mM), NaCl (100 mM) and EDTA (0.1 mM), pH 7.0 with 1 µM concentrations of the two complementary sequences. The extinction coefficients were calculated assuming the extinction coefficients for the dinucleotide **8R** to equal two thymidines. The increase in absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 10 to 90 °C at a rate of 1.0 °C min^{-1}. The melting temperature was determined as the local maximum of the first derivatives of the absorbance *versus* temperature curve. All melting curves were found to be reversible. For melting experiments with Mg^{2+} concentrations of 5 or 10 mM, $MgCl_2$ (1.0 µL and 2.0 µL, respectively, of a 5.0 M solution) was added to the samples.

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