A cyclic dinucleotide with a four-carbon 5′-*C*-to-5′-*C* connection; synthesis by RCM, NMR-examination and incorporation into secondary nucleic acid structures

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A 5′-*C*-allylthymidine derivative was prepared from thymidine by the application of a stereoselective allylation procedure and its 5′(*S*)-configuration was confirmed. From this nucleoside derivative, appropriately protected building blocks were prepared and coupled using standard phosphoramidite chemistry to afford a dinucleotide with two 5′-*C*-allylgroups. This molecule was used as a substrate for a ring-closing metathesis (RCM) reaction and after deprotection, a 1 : 1 mixture of *E*- and *Z*-isomers of a cyclic dinucleotide with an unsaturated 5′-*C*-to-5′-*C* connection was obtained. Alternatively, a hydrogenation of the double bond and deprotection afforded a saturated cyclic dinucleotide. An advanced NMR-examination confirmed the constitution of this molecule and indicated a restriction in its overall conformational freedom. After variation of the protecting group strategy, a phosphoramidite building block of the saturated cyclic dinucleotide with the 5′-*O*-position protected as a pixyl ether and the phosphate protected as a methyl phosphotriester was obtained. This building block was used in the preparation of two 14-mer oligonucleotides with a central artificial bend due to the cyclic dinucleotide moiety. These were found to destabilise duplexes, slightly destabilise bulged duplexes but, to some extent, stabilise a three-way junction in high Mg^{2+}-concentrations.

Introduction

The construction of chemically modified nucleic acid fragments with altered conformational behaviour is a powerful tool in chemical biology and in the development of nucleic acid based therapeutics and diagnostics.[1,2] For instance, oligonucleotides containing nucleoside monomers with restricted carbohydrate moieties can form duplexes with significantly increased stability[2–4] and are currently under intensive investigation as antisense therapeutics.[5,6] On the other hand, conformationally restricted models of other secondary structural elements[7] have gained much less attention,[8,9] though a few restricted dinucleotides have been synthesised and investigated,[10–15] *e.g.* as a model of the anticodon loop in a bacterial tRNA.[12]

Recently, we have applied the ring-closing metathesis (RCM) methodology[16–18] using the ruthenium-based precatalysts developed by Grubbs and co-workers,[19]§ in the construction of conformationally restricted dinucleotide[20–27] and trinucleotide structures.[22] We formulated a general strategy (see Fig. 1) by which terminal double bonds are incorporated by various methods and on various positions in dinucleotides that are subsequently used as substrates for RCM-reactions. These are envisioned to mimic, modulate and even stabilise other secondary nucleic acid structures than duplexes like bulges or three-way junctions (Fig. 1).

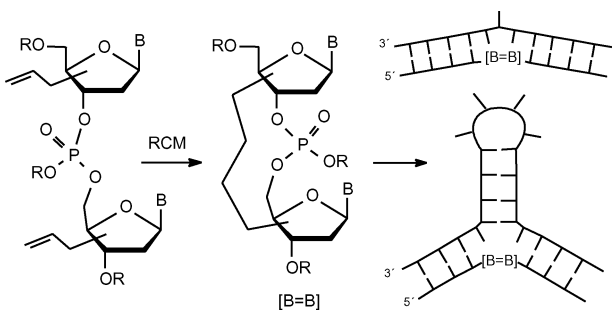

Fig. 1 General RCM-based strategy towards cyclic dinucleotides and secondary nucleic acid structures like bulges and three-way junctions. B = a nucleobase, R = varying protecting groups.

A number of cyclic dinucleotides have been obtained by this general strategy.[20–29] As the first example, four stereoisomeric dinucleotides with unsaturated 7-membered rings in the internucleoside linkage were obtained from RCM-reactions on substrates with an allyl phosphotriester linkage and an adjacent 5′-*C*-vinyl group.[20,21] Also cyclic dinucleotides in which the ring is based on the combination of an allyl phosphotriester and a 5-allyluracil

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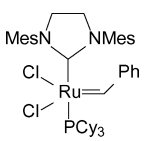
§ RCM and metathesis reactions in general have been recently reviewed.[16–18] Grubbs' 2nd generation catalyst (Mes = 2,4,6-trimethylphenyl, Cy = cyclohexyl):[19]



moiety have been efficiently obtained giving 14-membered ring systems.[22,23,25] A trinucleotide with two allyl phosphotriester linkages has been used as an efficient RCM-substrate and a cyclic trinucleotide with a 13-membered ring was obtained albeit as a mixture of 8 stereoisomers.[22] Recently, a series of 2′-*C* to phosphate connections revealing cyclic dinucleotides with 7–10-membered rings have been obtained in medium to high yields.[27] In the mentioned examples, phosphotriester internucleoside linkages were obtained revealing two problems; (1) the chirality of the phosphorus leads to stereoisomeric products and (2) the allylic phosphotriester moiety demonstrates a high basic lability.[23] The latter problem, however, has been significantly reduced by saturation of the allylic moiety using a tandem RCM-hydrogenation protocol[30] in which Grubbs' catalyst is active in both reactions.[23,26] Hence, one example has been more comprehensively studied, that is a cyclic dinucleotide with a 2′-*C*-$(CH_2)_4$O–P connection.[26] The major R_P isomer was found to be chemically stable and incorporated at a central position in two 14-mer oligonucleotide sequences. A large destabilisation of duplex structures was, expectedly, observed, but a stabilisation of a three way-junction with a complementary RNA-hairpin was also revealed.[26]

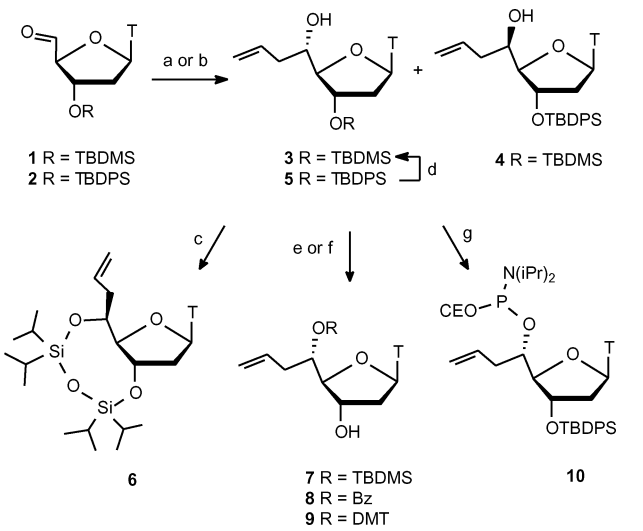
In order to obtain more chemically stable and convenient molecules, cyclic dinucleotides with an intact, charged and achiral phosphodiester internucleoside linkage have been approached. Two adjacent 5-allyluracil moieties have been combined in a cyclic dinucleotide with a very large 20-membered ring, however in this case in a relatively low yield.[25] In a more promising approach, we investigated dinucleotide substrates on which terminal double bonds are placed on the 4′-*C* or 5′-*C* positions. In the preliminary study,[24] a 5′-*C*-allyl group was found to be significantly more reactive than an 4′-*C*-vinyl group, and a fully protected cyclic dinucleotide with an unsaturated four-carbon 5′-*C*-to-5′-*C* connection was obtained. In the present paper, the development of an appropriate protecting strategy, and the successful preparation and NMR-analysis of a saturated cyclic dinucleotide and its incorporation into oligonucleotides is presented.

Results and discussion

Chemical synthesis

Construction of 5′-*C*-allylthymidine building blocks. The introduction of a 5′-*C*-allyl group via the 3′-silylated thymidine aldehyde derivatives **1** and **2** (Scheme 1) has been demonstrated before in the literature. Thus, a Grignard reaction with **1** using allylMgCl and CuCN has afforded both 5′-epimers **3** and **4** as a 1 : 1 mixture in 63% yield.[31,32] On the other hand, a stereoselective procedure starting with **2** applying allyltrimethylsilane and BF_3:OEt_2 afforded exclusively the 5′(*S*)-configurated 5′-*O*-TMS-protected analogue of **5** in an impressive 95% yield after silylation.[33] We decided to repeat and evolve these procedures and to confirm the configuration of the products.

Firstly, we found that the oxidation step for obtaining **1** or **2** was most reliably and efficiently performed by the use of Dess–Martin periodinane[34,35] in both cases compared to alternative IBX,[36] Swern or Moffatt oxidations.[37,38] Subsequently, we attempted to improve the Grignard reaction by the use of allylMgBr and $CeCl_3$[39] but we obtained a similar 1 : 1 epimeric mixture of **3** and **4** in only 45% yield. On the other hand, the stereoselective method using



Scheme 1 *Reagents and conditions*: a, allylMgBr, $CeCl_3$, THF, 47%; b, allyltrimethylsilane, BF_3:OEt_2, CH_2Cl_2, 73% **5**; c, (i) TBAF, THF, (ii) $TIPDSCl_2$, Pyr, 52%; d, (i) TBAF, THF, (ii) TBDMSCl, imidazole, DMF, 61% **3** and 2% **7**; e, (i) BzCl, Pyr, 71%, (ii) TBAF, THF, 63%; f, (i) DMT-triflate, 2,6-lutidine, CH_2Cl_2, (ii) TBAF, THF, 58%; g, $NC(CH_2)_2OP(N(iPr)_2)_2$, 4,5-dicyanoimidazole, CH_3CN, 80%; T = thymin-1-yl, TBDMS = *tert*-butyldimethylsilyl, TBDPS = *tert*-butyldiphenylsilyl, TIPDS = tetraisopropyldisilyl, DMT = 4,4′-dimethoxytrityl, CE = 2-cyanoethyl.

allyltrimethylsilane[33] was repeated to give only a single isomer **5** in 71% overall yield. We also attempted this reaction with the TBDMS-protected derivate **1** but in this case, a 9 : 1 epimeric mixture of **3** and **4** was obtained in 78% yield.

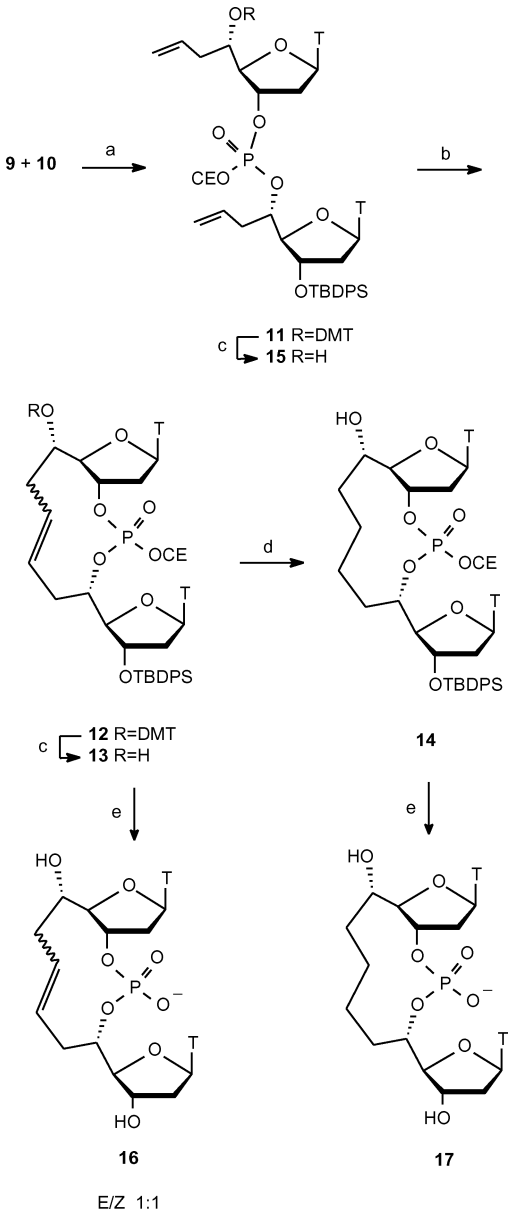
In the original paper,[33] **5** was reported to be the 5′(*S*)-isomer. This was determined by the conversion of **5** to another product which has been alternatively obtained from a different precursor determined by X-ray crystallography.[33] In order to confirm this important determination in a simple way, we converted the 9 : 1 mixture of **3** and **4** to the conformationally restricted 3′,5′-disiloxane protected derivative **6** which was subsequently examined by NMR spectroscopy in an NOE-difference experiment. Large mutual contacts between H-5′ and H-4′ as well as the lack of contacts between H-5′ and H-3′/H-6 confirmed the 5′(*S*)-configuration of **3**. This way of determining 5′-configuration has been used before with other 5′-alkylated thymidine derivatives where both isomers have been examined.[21,31,38,40] Wang and Middleton determined the configurations of **3** and **4** by the conversion to the two 3′,5′-*O*-di(*t*-butyl)silyl derivatives and subsequent NOE-difference spectroscopy.[32] However, direct comparison of our NMR-data of **3** and **4** with the reported data[32] was not reliable due to intense spectral overlap. Finally, the 5′(*S*)-configuration of **5** was confirmed, indirectly, by converting **5** to **3**. Hence, a desilylation and a subsequent treatment with TBDMSCl and imidazole gave the isomer **3** as the only major product in 61% yield. This also proved an extraordinary difference in reactivity between the two secondary 3′- and 5′-alcohols as the 5′-silylated isomer **7** was obtained as the minor product in only 2% yield.

In the further preparation of dinucleotides, we applied the 5′(*S*)-configurated compound **5** and considered different protecting group strategies. In our preliminary study, we prepared the

benzoyl ester for the 5′-position and obtained **8** after desilylation (Scheme 1).[24] However, the benzoyl group is not orthogonal to the cyanoethyl group that is planned as a protecting group for the phosphodiester moiety. We decided, therefore, to investigate the DMT-group, which might be envisioned as a permanent protecting group useful in the eventual incorporation of the dinucleotide into oligonucleotide sequences. Tritylation of the secondary 5′-OH group of **5** was hereafter accomplished. However, the use of DMT-chloride and pyridine as a standard condition turned out to be insufficient, and the DMT-protection was performed by the use of DMT-triflate prepared according to a procedure by Leumann and co-workers.[41] Subsequent desilylation gave the 3′-*O*-deprotected derivative **9** in a reasonable overall yield. In order to prepare a dinucleotide by standard phosphoramidite chemistry, 5′-phosphoramidite derivative **10** was prepared from **5** in a good yield.

Construction of cyclic dinucleotides. A dinucleotide **11** was obtained by the coupling of the alcohol **9** with the phosphoramidite **10** using standard coupling conditions, *i.e.* 1*H*-tetrazole as the activator (Scheme 2). Oxidation of the intermediate phosphite to a phosphotriester was performed by *tert*-butyl hydrogenperoxide instead of the standard iodine solution in order to avoid reactions with the double bonds. This afforded the dinucleotide **11** in a 3 : 1 mixture of phosphorous epimers. This surprising ratio might be rationalised from the fact that **10** is a sterically hindered phosphoramidite of a secondary alcohol. The dinucleotide **11** with two terminal double bonds was hereafter examined as a substrate for RCM reactions. The DMT-group was found to be completely stable when using the standard metathesis conditions, *i.e.* 5–10 mol% of Grubbs' 2nd generation catalyst§ in refluxing dichloromethane. Only when heating the reaction mixture in other solvents and/or under microwave conditions, was some detritylation observed. This result is in contrary to former results in our group, where RCM reactions with DMT-protected substrates have been impossible due to detritylation and subsequent quenching of the catalyst.[22] Hence, the cyclic dinucleotide **12** was obtained as a mixture of four stereoisomers (ratio: ~8 : 6 : 4 : 1) in a high yield after a successful ring-closing reaction.

In order to obtain a saturated cyclic dinucleotide, a tandem RCM-hydrogenation procedure[30] was attempted. We have recently obtained very successful results with other dinucleotide substrates by the application of Grubbs' 2nd generation catalyst§ as a combined metathesis–hydrogenation catalyst, where the latter hydrogenation was carried out under a 1000 psi pressure.[23,25] However, when this was attempted with the present dinucleotide substrate **11**, only the detritylated, ring-closed but still unsaturated product **13** was obtained. Standard hydrogenation of the cyclic dinucleotide **12** was unsuccessful after attempting Pd/C or $Pd(OH)_2/C$ at atmospheric or high pressure, thus, detritylation was the dominating result. However, performing the detritylation after the RCM reaction under standard acidic conditions to give **13** and, subsequently, performing a standard hydrogenation under a high pressure afforded the dinucleotide **14** in a reasonable yield. No starting material was left as indicated by MALDI-MS. An attempt of performing both the RCM and the hydrogenation after detritylation was also attempted. Hence, the deprotected dinucleotide **15** was obtained after a standard acidic detritylation of **11** and used as a substrate for RCM. However, this was less efficient, and a mixture of ring-closed products and unidentified side-products was obtained.

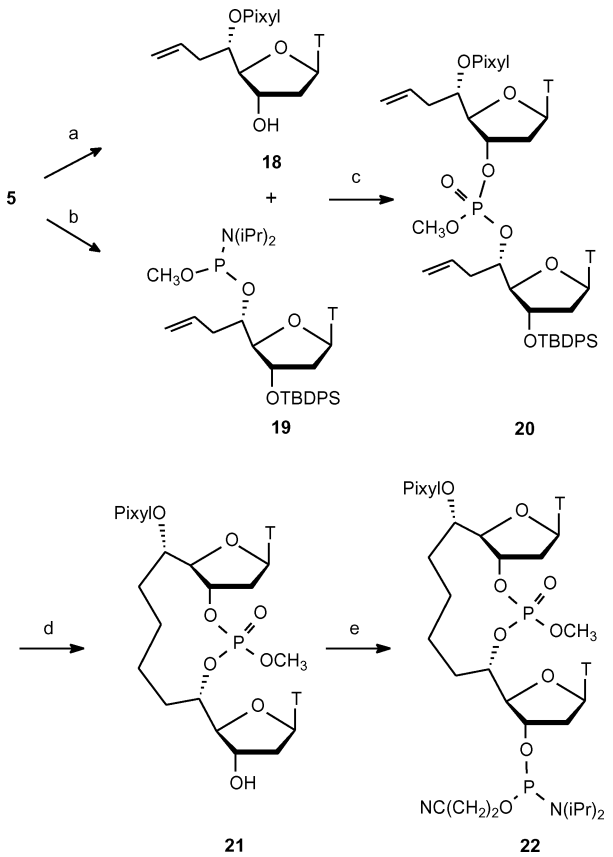


Scheme 2 *Reagents and conditions*: a, (i) 1*H*-tetrazole, CH_3CN, (ii) *t*-BuOOH, toluene, 63%; b, Grubbs' 2nd gen. catalyst§, CH_2Cl_2, 74%; c, CF_3COOH, Et_3SiH, CH_2Cl_2, 86% **13**, 80% **15**; d, H_2 (1000 psi), $Pd(OH)_2/C$, CH_3OH, 63%; e, (i) TBAF, THF, (ii) 32% aq. NH_3.

Finally, a complete deprotection of **13**, and **14**, to give the cyclic dinucleotide compounds **16** and **17**, respectively, was performed by desilylation followed by treatment with saturated ammonia and HPLC-purification. **16** was isolated as a 1 : 1 mixture of *E*/*Z* isomers as judged from ^{31}P NMR. The equimolar ratio of *E*/*Z* isomers in **16** compared with the ~8 : 6 : 4 : 1 ratio of isomers in **12** reflects that whereas the major phosphorus epimer of **11** affords a 6 : 8 mixture of *E* : *Z* products in the RCM-reaction, the other epimer affords the opposite 4 : 1 mixture of *E* : *Z* products. The saturated cyclic dinucleotide **17** was obtained as a single isomer and subsequently examined by NMR spectroscopy and dynamic simulations (see below).

Preparing the cyclic dinucleotide for incorporation into oligonucleotides. A clear experience from the RCM-hydrogenation protocol is that the DMT-group cannot be used as a permanent protecting group. Furthermore, attempts to reprotect the saturated cyclic dinucleotide **14** failed even using the more active DMT-triflate[41] as a tritylation agent. An alternative to the DMT group is the pixyl group, which can be used in standard automated oligonucleotide synthesis. Pixylation is usually more efficient than DMT-protection.[40] However, preliminary attempts to convert **12** into a 3′-*O*-phosphoramidite building block demonstrated other difficulties in removing the TBDPS-group without affecting the cyanoethyl-group. Therefore, a complete revision of the protecting group strategy was obviously necessary in order to obtain an efficient incorporation of **17** into oligonucleotides. Methyl phosphotriesters have been used before for phosphate protection in oligonucleotide synthesis being much more stable than the corresponding cyanoethyl protected phosphotriesters,[42] though they demand a different deprotection procedure.[42,43]

Two new building blocks for constructing the dinucleotide were prepared from the key intermediate **5** (Scheme 3). Pixylation[40] was indeed remarkably more efficient than DMT-protection affording **18** in quantitative yield after desilylation. A methyl protected phosphoramidite **19** was also efficiently obtained. The coupling of **18** and **19** afforded the dinucleotide **20** in a good yield. This dinucleotide was a good substrate for the RCM-reaction. However, this proved most efficient in 1,2-dichloroethane as the solvent and a partial depixylation was observed under the reaction conditions. Thus, both pixylated and depixylated fractions were collected in a combined 69% yield. These fractions were recombined for the following hydrogenation, which proceeded very efficiently using Adams' catalyst under pressure leading to a saturated but, this time, completely depixylated intermediate. Therefore, a repixylation of the reaction mixture was introduced. This was again very efficient and, hence, the pixylation has been efficient where a DMT-protection has failed. After a straightforward desilylation using TBAF and acetic acid showing no problems with either the pixyl or the methyl phosphotriesters, the appropriately protected cyclic dinucleotide **21** was obtained in 27% yield over the four steps from **20**. The use of Et_3N–3HF or TBAF without acid both failed to give a pure removal of the TBDPS-group. Phosphitylation of **21** gave the phosphoramidite **22** as the building block for introducing the cyclic dinucleotide moiety into oligonucleotide sequences.



Scheme 3 *Reagents and conditions*: a, (i) pixyl-Cl, pyridine, (ii) TBAF, THF, 99% (2 steps); b, $CH_3OP(Cl)N(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2, 75%; c, (i) 1*H*-tetrazole, CH_3CN, (ii) *t*-BuOOH, toluene, 75%; d, (i) Grubbs' 2nd gen. catalyst§ $(CH_2Cl)_2$, (ii) H_2 (1000 psi), PtO_2, CH_3OH, (iii) pixyl-Cl, 2,6-lutidine, CH_2Cl_2, (iv) TBAF, AcOH, THF, 27% (4 steps); e, $EtN(iPr)_2$, $NC(CH_2)_2OP(Cl)N(iPr)_2$, CH_2Cl_2, 37%. Pixyl = 9-phenylxanthen-9-yl.

NMR analysis and modelling

In order to further investigate the structural and dynamic properties of the cyclic dinucleotide **17**, a 1000 ps molecular dynamics simulation was performed. During the simulation, time averaged coupling constant and distance restraints were employed. The restraints were obtained from 1D ^{1}H NMR and NOESY spectra. Due to severe overlap of especially the linker protons, no restraints were used in this part of the molecule. In order to analyze and compare the results, a similar simulation was performed for the analogous unmodified dinucleotide.

The molecular dynamics simulations showed that compared to the unmodified dinucleotide, the cyclic dinucleotide is considerably more restricted in its motions. With respect to whether the dinucleotide is more prone to adopt a bent configuration, however, the simulations yielded inconclusive results. A clear result from the simulation is, that the "upper" O–C5′–C4′–C3′ torsional angle of **17** remains in a *trans* conformation throughout the simulation, whereas the corresponding angle of the unmodified dinucleotide remains in the *gauche*+ conformation that is also usually found in duplexes. The very large $^3J_{H4'H5'}$ coupling constant of 10.1 Hz observed for **17** confirms the preferred *trans* conformation. The simulation of **17** as well as the observed $^3J_{H1'H2'}$ constants indicated for both nucleoside parts in the dinucleotide the same preference for *S*-type conformations as normally seen for 2′-deoxynucleosides. Fig. 2 shows an energy minimized snapshot taken during the simulation of the cyclic dinucleotide.

Synthesis and evaluation of oligonucleotides

Two 14-mer oligonucleotide sequences each with the cyclic dinucleotide **22** incorporated once in the middle were constructed. These contain either solely standard 2′-deoxynucleotides (**24**) or a mixture of standard 2′-deoxynucleotides and LNA-monomers (**26**), *i.e.* a DNA and an LNA sequence, respectively (Table 1). Thus, LNA is defined as an oligonucleotide sequence containing one or several of the LNA-monomers, which are nucleosides with a bicyclic carbohydrate moiety, locked in the *N*-type conformation.[6,44] Hereby, the LNA-monomers secure an overall A-type or A-type-like duplex conformation as well as a

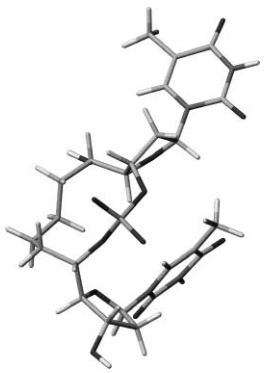


Fig. 2 An energy minimized snapshot of the dinucleotide **17** taken during the simulation.

Table 1 Prepared oligodeoxynucleotide sequences and their MS-data

	ODN sequences[a]	MW (found/calc.)[b]
23	5′-GCTCACTTCTCCCA	—
24	5′-GCTCAC[T=T]CTCCCA	4186.8/4188.5 $[MH]^+$
25	5′-G**C**^{L}T**C**^{L}A**C**^{L}TT**C**^{L}T**C**^{L}**C**^{L}A	—
26	5′-G**C**^{L}T**C**^{L}A**C**^{L}[T=T]**C**^{L}T**C**^{L}**C**^{L}A	4438.9/4440.6 $[MH]^+$

[a] [T=T] refers to the incorporation of **22**. **C**^{L} refers to the LNA 5-methylcytidine monomer. [b] HiResESI-MS positive mode.

significantly increased duplex stability.[6,44] Unmodified DNA and LNA sequences, **23** and **25**, used for comparison, are the same as those used in our former studies.[26,45]

The incorporation of **22** into oligonucleotides was performed by automated solid-phase synthesis using a standard protocol including a prolonged coupling time for **22** of 20 minutes, a subsequent double capping procedure and a slightly prolonged coupling time for the proceeding standard phosphoramidite. A coupling yield of >95% was detected for **22**. The pixyl group was removed by a standard acidic treatment after the coupling of **22**. In the deprotection protocol following the completed synthesis, a treatment with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate[43] was introduced in order to remove the methyl protecting group of the phosphate.[42,43] After completed deprotection, the constitution and purity of **24** and **26** were confirmed by MALDI-MS (see Table 1) and ion-exchange HPLC.

The hybridisation properties of the oligonucleotides with complementary DNA and RNA sequences were evaluated by thermal stability experiments (Table 2 and 3). First, the duplexes formed between **23–26** and their fully matching DNA and RNA compliments were studied (Table 2). As expected, large drops in duplex stability were observed. Comparing **23** and **24**, the cyclic dinucleotide induced a drop in T_m of 16 °C in a DNA–DNA duplex, and, comparing **25** and **26**, 20 °C in an LNA-modified duplex. The latter must be A-type like and is, as expected, significantly more stable due to the LNA-monomers.[6,44] Also the DNA–RNA duplex was significantly destabilised by the cyclic dinucleotide with a drop in T_m of 14 °C. These results are comparable to the results obtained with our firstly studied cyclic dinucleotide (with a 2′-*C*-$(CH_2)_4$O–P connection)[26] and demonstrate the expected distortion of a standard nucleic acid duplex structure by the introduction of a bending cyclic dinucleotide moiety.

Next, the hybridisation properties of oligonucleotides **23–26** with bulged DNA and RNA complements were studied (Table 2, see also Fig. 1). Thus, 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 still significant drop in stability of 5.2 to 11.4 °C when comparing the modified sequences, **24** and **26**, with their unmodified counterparts, **23** and **25**. These results are again comparable to the results obtained in our former study,[26] however, the destabilisation is slightly more pronounced with the present cyclic structure. Nevertheless, the observation that the introduction of a bulge in a duplex reduces the stability with 8–12 °C whereas the same bulge in distorted duplexes containing **17** reduces the stability by only 0.5–3 °C, is still valid.

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
23	54.4	60.4	43.1	49.8
24	38.1 (−16.3)	46.5 (−13.9)	36.6 (−6.5)	44.6 (−5.2)
25	79.7	>90	69.4	84.9
26	58.6 (−20.1)	80.5 (<−9.5)	58.0 (−11.4)	77.4 (−7.5)

	Complements with an intrastrand stem-loop[d,e]					
	DNA			RNA		
	0 mM	5 mM	10 mM	0 mM	5 mM	10 mM
23	26.5	30.2	31.5	38.4	42.7	44.0
24	25.7 (−0.8)	30.0 (−0.2)	32.0 (+0.5)	38.2 (−0.2)	42.6 (−0.1)	44.5 (+0.5)
25	54.5	57.1	58.8	74.1	78.0	>80
26	51.9 (−2.6)	55.6 (−1.5)	57.5 (−1.3)	73.5 (−0.6)	77.4 (−0.6)	>80

[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 $MgCl_2$.

Table 3 Hybridisation data for the oligonucleotides **23** and **24** with bulged intrastrand stem-loop DNA-complements[a,b]

	Intrastrand stem-loop with 1 × C bulge from 5′-end.[c]			Intrastrand stem-loop with 1 × C bulge from 3′-end.[d]			Intrastrand stem-loop with 2 × C bulge from 5′-end.[e]			Intrastrand stem-loop with 2 × C bulge from 3′-end.[f]		
	0 mM	5 mM	10 mM	0 mM	5 mM	10 mM	0 mM	5 mM	10 mM	0 mM	5 mM	10 mM
23	17.0	20.7	25.0	26.0	30.3	32.1	n.d.[g]	—[h]	—[h]	26.3	31.2	33.1
24	n.d.[g]	n.d.[g]	n.d.[g]	23.9 (−2.1)	27.6 (−2.6)	29.4 (−2.7)	n.d.[g]	—[h]	—[h]	22.9 (−3.4)	27.3 (−3.9)	28.7 (−4.4)

[a] See Table 2, note *a*. [b] Increasing concentration of Mg^{2+} by addition of $MgCl_2$. [c] 3′-CGAGTGA-**CGCGTTTTCGCG**-C-AGAGGGT. [d] 3′-CGAGTGA-C-**CGCGTTTTCGCG**-AGAGGGT. [e] 3′-CGAGTGA-**CGCGTTTTCGCG**-CC-AGAGGGT. [f] 3′-CGAGTGA-CC-**CGCGTTTTCGCG**-AGAGGGT, bold sequences are stem-loops. [g] n.d. = no well-defined transition. [h] — = not determined.

The stability of a three-way junction (TWJ) was also studied. This is composed of a standard stable stem-loop sequence with two single stranded regions being complementary to the oligonucleotides **23–26** with the cyclic dinucleotide in the branching point (see Fig. 1). First, we studied a TWJ in which no bulges are included, *i.e.* all nucleobases are intended to participate in Watson–Crick base-pairing or, alternatively, to leave two unpaired nucleobases on opposite positions. This TWJ was in general not very stable with a T_m of 26.5 °C in a complete DNA-context and 38.4 °C as 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 by 3–5 °C, and a smaller further stabilisation with a 10 mM Mg^{2+} concentration of approx. 2 °C was observed. The TWJ was in general found to be unaffected or slightly destabilised by the cyclic dinucleotide concerning the overall thermal stability. Thus, small drops in T_m of 0.6–2.6 °C were seen when comparing **25** with **26**, *i.e.* an LNA-sequence context. On the other hand, even smaller differences were observed when comparing **23** and **24** tending to a small increase in stability of 0.5 °C with **24** when increasing the Mg^{2+} concentration. For the unmodified sequences, some variations in the T_m's compared to our former measurements of the same TWJ[26] were seen. This is due to relatively broad transitions reflecting a not very well-defined structure of the TWJ.

In order to study a more stable or at least more well-defined structure, we incorporated an additional bulge into the TWJ. Hence, one or two additional cytosines were inserted into the target DNA-sequence on either side of the stem-loop (see Table 3). Thus, the optimal constitution of natural TWJ's seems to be involving two costacking bases in a bulge at one of the three strands.[46,47] In our first investigation, only DNA-compliments were prepared. As evident from the T_m's, the unmodified TWJ's with one or two cytosines in a bulge from the 3′-end of the target stem-loop display the same stability as the first TWJ ($T_m \approx$ 26 °C). Indeed, more sharp transitions and thereby more well-defined structures were observed. The cyclic dinucleotide, on the other hand, destabilises this in both cases with the drop in T_m of 2.1–4.4 °C (comparing **23** and **24**) increasing with the Mg^{2+}-concentration. The TWJ's with the cytosine(s) in a bulge from the 5′-end of the target stem-loop are significantly less stable, and with the cyclic dinucleotide incorporated, no well-defined transitions could be observed. Due to the general destabilisation induced by the cyclic dinucleotide, no further studies with RNA-compliments nor with the LNA-sequences **25** and **26** were undertaken.

The present results cannot, unfortunately, demonstrate any significant positive influence by the introduction of the cyclic moiety of **17** into secondary nucleic acid structures. However, the tolerance of the cyclic structure within one of the TWJ's studied ($\Delta T_m = +0.5$ °C, Table 2) indicates that a TWJ being more significantly stabilised by **17** might be found. A reason for the present results could be that the right sequence context has not been found. Thus, no intriguing difference was observed with the bulged TWJ's (Table 3) either. Also the sequence constitution can be discussed. Only seven base pairs were used in each duplex flanking the TWJ. A more well-defined structure might have been obtained by longer flanking duplex sequences. On the other hand, this was attempted by using the inherent stabilisation obtained by using an LNA-sequence. The relative differences between modified and unmodified TWJ's are undoubtedly similar despite the higher level of thermal stability.

It is well known that TWJ's are very flexible and inhomogen in their structure.[46] Therefore, the idea of inducing some conformational restriction by a cyclic dinucleotide and thereby a better defined structure was intriguing. Nevertheless, the applied conformational restriction is not very strong. The 11-membered ring introduced here is large and relatively flexible. Nevertheless, the philosophy was to modulate/decrease the conformational freedom to some extent leaving an oligonucleotide preferring a bent to a linear organisation. Leaving the double bond unsaturated as in **16** could be a preferable alternative concerning the degree of conformational restriction. On the other hand, two isomers would have to be handled which seems to be a difficult task. A simple alteration of **17** could be to invert one or both of the 5′-configurations. If the 5′(*R*)-configuration (as in compound **4**) has been used, completely different results might have been obtained. The O–C5′–C4′–C3′ torsional angle is strongly dependent on the 5′-configuration and, as confirmed by the NMR-examination, more distorted compared to a standard B-type duplex with a 5′(*S*)-configuration than with a 5′(*R*)-configuration. There is no doubt from the results of incorporating **17** into standard duplexes (Table 2) that the duplexes are indeed significantly distorted. Another important aspect of incorporating the cyclic dinucleotide structure **17** into nucleic acid structures is its hydrophobic nature. Thus, the butylene chain might exclude structural water and thereby actually oppose any gain of conformational restriction. Furthermore, the cyclic structure might force phosphates into proximity giving a high charge density. This might explain why the positive influence on stability of adding Mg^{2+} ions is relatively higher for the modified compared to the unmodified TWJ's.

Two other modifications have been introduced in the same sequence context; (1) another cyclic dinucleotide (with the 2′-*C*-$(CH_2)_4$O-P connection in a 9-membered ring)[26] and (2) a nucleoside with an additional base (2′-deoxy-2′-*C*-(2-(thymin-1-yl)ethyl)uridine)[45] incorporated at the same position as **17** (*via* **22**) in sequence **24** and **26**. The former demonstrated results which are more or less comparable to the data presented herein for **17**. The destabilisation of duplexes and bulged duplexes was slightly more pronounced in the case of **17**, and the stabilisation of the TWJ with no C-bulges that was actually seen in our former study[26] was less pronounced with **17**. In the other approach, in which the stabilisation of TWJ's was attempted by an additional nucleobase instead of a large ring and with sequences thereby being one nucleoside shorter in the branching point, more promising results have been obtained.[45] Here, small stabilisations of the TWJ's were in general seen in the DNA–DNA and DNA–RNA contexts compared to either two thymidines (as in **23**) or one in the branching point.[45] Furthermore, much more relative stabilisation of the TWJ in an LNA–DNA context was gained by the addition of Mg^{2+} indicating a more dynamic structural context. Thus, the introduction of an additional binding potential in the oligonucleotide (an additional nucleobase[45] or an intercalating moiety[48]) seems to be a more powerful approach for targeting a stem-loop and to stabilise a TWJ than the introduction of a large cyclic moiety bending the oligonucleotide. The only way of improving the latter approach seems to be a target oriented design of the bend needed for the specific TWJ.

Conclusion

In summary, our RCM-based strategy for introducing large internucleotide connections in dinucleotides has proved efficient in the construction of a cyclic dinucleotide with a 5′-*C*-$(CH_2)_4$-5′-*C* connection forming an 11-membered ring. Thus both the deprotected cyclic dinucleotide **17** and its corresponding phosphoramidite building block **22** were isolated in reasonable overall yields. In the right sequence context, oligonucleotides containing **17** might stabilise secondary nucleic acid structures. In general, the present RCM-based strategy can with the appropriate effort in modelling and design be a general tool towards the development of oligonucleotide structures that are preorganised for recognising RNA secondary structures. New nucleic acid based molecular structures based on this concept can reveal a new impact for modulating RNA function for future therapeutic uses and nanobiotechnology.

Experimental

All reagents were used as supplied. When necessary, all 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 obtained on a Varian Gemini 2000 spectrometer. ^{1}H NMR spectra were recorded at 300 MHz, ^{13}C NMR spectra were recorded at 75,5 MHz and ^{31}P NMR spectra were recorded at 121.5 MHz. Values for δ are in ppm relative to tetramethylsilane as an internal standard or 85% H_3PO_4 as an external standard. Assignments of NMR-signals when given are based on 2D spectra and follow standard carbohydrate and nucleoside style; *i.e.* the carbon atom next to a nucleobase is assigned C-1′. However, compound names for bicyclic compounds are given according to the von Baeyer nomenclature. Fast-atom bombardment mass spectra (FAB-MS) were recorded in positive ion mode on a Kratos MS50TC spectrometer and MALDI mass spectra as well as accurate mass determinations were performed on an Ionspec Ultima Fourier Transform mass spectrometer. Microanalyses were performed at The Microanalytical Laboratory, Department of Chemistry, University of Copenhagen.

Preparation of 5′(*S*)-*C*-allyl-3′-*O*-(*tert*-butyldimethylsilyl)thymidine (3) and 5′(*R*)-*C*-allyl-3′-*O*-(*tert*-butyldimethylsilyl)thymidine (4)

The aldehyde **1** (466 mg, 1.31 mmol) was coevaporated with anhydrous CH_2Cl_2 and dissolved in the same solvent (10 cm^3). The solution was stirred at 0 °C. Allyltrimethylsilane (1.05 cm^3, 6.58 mmol) and BF_3:OEt_2 (0.820 cm^3, 6.67 mmol) were added, whereby the reaction mixture went from weak yellow to orange-red. The reaction mixture was stirred for 1 h at 0 °C and then diluted with CH_2Cl_2 (60 cm^3). The solution was washed with a saturated aqueous solution of $NaHCO_3$ (2 × 25 cm^3) and brine (25 cm^3). The organic phase was dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using EtOAc and petroleum ether (1 : 1 v/v) as the eluent to give the product (407 mg, 78%) as a white foam and a 9 : 1 mixture of the 5′-epimers **3** and **4**, respectively; R_f 0.46 (EtOAc and petroleum ether, 3 : 1 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) (major isomer **3**) 8.73 (1H, br s, NH), 7.52 (1H, d, *J* 1.0 Hz, H-6), 6.18 (1H, t, *J* 6.8 Hz, H-1′), 5.84 (1H, m, H-7′), 5.23–5.15 (2H, m, H-8′), 4.50 (1H, m, H-3′), 3.82 (1H, m, H-5′), 3.76 (1H, m, H-4′), 2.43–2.28 (3H, m, H-2′, H-6′), 2.17 (1H, ddd, *J* 13.3, 6.2, 3.4 Hz, H-2′), 1.92 (3H, d, *J* 1.0 Hz, CH_3), 0.90 (9H, s, $C(CH_3)_3$), 0.08 (6H, s, $Si(CH_3)_2$). δ_C (75 MHz; $CDCl_3$; Me_4Si) (major isomer **3**) 163.7 (C-4), 150.3 (C-2), 137.2 (C-7′), 134.1 (C-6), 118.8 (C-8′), 110.9 (C-5), 88.8, 86.8 (C-1′, C-4′), 73.0, 70.1 (C-3′, C-5′), 40.3, 39.0 (C-6′, C-2′), 25.7 ($C(CH_3)_3$), 17.9 ($C(CH_3)_3$), 12.6 (CH_3), −4.6, −4.8 ($Si(CH_3)_2$); MS(FAB) *m*/*z* 397 ($M + H^+$).

Preparation of 5′(*S*)-*C*-allyl-3′-*O*-(*tert*-butyldiphenylsilyl)thymidine (5)

The aldehyde **2** (6.25 g, 13.1 mmol) was dissolved in anhydrous CH_2Cl_2 (150 cm^3) and stirred at 0 °C. Allyltrimethylsilane (10.4 cm^3, 65.4 mmol) and BF_3:OEt_2 (8.3 cm^3, 65.4 mmol) were added, whereby the reaction mixture went from weak yellow to orange-red. The reaction mixture was stirred for 5 h at 0 °C and then quenched by the dropwise addition of a saturated aqueous solution of $NaHCO_3$ (60 cm^3). The layers were separated and the organic phase was dried ($MgSO_4$). The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography using EtOAc and petroleum ether (1 : 2 v/v) as the eluent to give the product (5.00 g, 73%) as a white foam; R_f 0.55 (EtOAc and petroleum ether, 3 : 1 v/v); mp 83–84 °C. δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.75 (1H, br s, NH), 7.69–7.36 (11H, m, H-6, Ph), 6.30 (1H, dd, *J* 6.1, 8.1 Hz, H-1′), 5.63 (1H, m, H-7′), 5.11–5.02 (2H, m, H-8′), 4.48 (1H, m, H-3′), 3.83 (1H, br s, H-4′), 3.11 (1H, m, H-5′), 2.30–2.11 (4H, m, H-2′, H-6′), 1.86 (3H, d, *J* 1.1 Hz, CH_3), 1.09 (9H, s, $C(CH_3)_3$). δ_C (75 MHz; $CDCl_3$; Me_4Si)

163.7 (C-4), 150.3 (C-2), 137.1 (C-7′), 135.8, 135.7 (Ph), 134.1 (C-6), 133.4, 133.1, 130.1, 130.0, 127.9 (Ph), 118.5 (C-8′), 110.8 (C-5), 88.9 (C-4′), 86.8 (C-1′), 74.6 (C-3′), 70.0 (C-5′), 39.9, 38.9 (C-6′, C-2′), 26.9 (C(CH_3)$_3$), 19.0 (C(CH_3)$_3$), 12.5 (CH_3). MS(MALDI) m/z 543 (M + Na^+), HRMS 543.2288 (calcd for $C_{29}H_{36}N_2O_5SiNa$ 543.2286).

Preparation of 5′(*S*)-*C*-allyl-3′,5′-*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxandiyl)thymidine (6)

Compounds **3** and **4** (9 : 1 mixture) (66.0 mg; 0.167 mmol) were dissolved in anhydrous THF (3.0 cm^3) and a 1 M solution of TBAF in THF (0.360 cm^3) was added. The mixture was stirred for 2.5 h at room temperature and then concentrated under reduced pressure. The residue was dissolved in EtOAc (20 cm^3) and washed with a saturated aqueous solution of $NaHCO_3$ (2 × 10 cm^3) and brine (10 cm^3). The combined aqueous phases were extracted with more EtOAc (3 × 10 cm^3). The combined organic phases were dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was coevaporated with anhydrous pyridine and redissolved in the same solvent (1.0 cm^3). The solution was stirred at 0 °C and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.111 cm^3, 0.347 mmol) was added. The solution was stirred at room temperature for 18 h and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (30 cm^3) and washed with water (15 cm^3), a saturated aqueous solution of $NaHCO_3$ (2 × 15 cm^3) and brine (15 cm^3). The organic phase was dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using MeOH and CH_2Cl_2 (1 : 50 v/v) as the eluent to give the product (91 mg, ~52% calculated from an estimated content of hydrolysed disiloxane reagent) as an oil; R_f 0.56 (MeOH and CH_2Cl_2, 1:9 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.91 (1H, br s, NH), 7.49 (1H, d, J 1.1 Hz, H-6), 6.06 (1H, dd, J 7.4, 1.6 Hz, H-1′), 5.84 (1H, m, H-7′), 5.23–5.04 (2H, m, H-8′), 4.42 (1H, m, H-3′), 4.04 (1H, m, H-5′), 3.67 (1H, dd, J 8.4, 2.8 Hz, H-4′), 2.39–2.62 (3H, m, H-2′, H-6′), 2.21 (1H, ddd, J 13.3, 7.4, 1.6 Hz, H-2′), 1.92 (3H, d, J 1.1 Hz, CH_3), 1.17–0.90 (28H, m, SiCH, CH(CH_3)$_2$). δ_C (75 MHz; $CDCl_3$; Me_4Si) 163.9 (C-4), 150.1 (C-2), 135.2 (C-7′), 133.7 (C-6), 118.1 (C-8′), 110.3 (C-5), 85.1, 83.6 (C-1′, C-4′), 68.4, 67.3 (C-3′, C-5′), 39.8, 38.1 (C-6′, C-2′), 17.6, 17.5, 17.3, 17.2, 17.1, 17.0, 16.9, 16.9 (CH(CH_3)$_2$), 13.5, 13.1, 12.8, 12.6, 12.5 (CH(CH_3)$_2$, CH_3); MS(FAB) m/z 525 (M + H^+).

Preparation of 5′(*S*)-*C*-allyl-3′-*O*-(*tert*-butyldimethylsilyl)thymidine (3) and 5′(*S*)-*C*-allyl-5′-*O*-(*tert*-butyldimethylsilyl)thymidine (7)

Compound **5** (402 mg, 0.77 mmol) was dissolved in anhydrous THF (12 cm^3) and added a 1 M solution of TBAF in THF (1.25 cm^3). The mixture was stirred for 80 min at room temperature and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography using MeOH and CH_2Cl_2 (1 : 20 v/v) as eluent to give 5′(*S*)-*C*-allylthymidine (172 mg, 92%) as a white solid; R_f 0.64 (MeOH and CH_2Cl_2, 1 : 9 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.56 (1H, br s, H-6), 6.24 (1H, dd, J 7.3, 6.5 Hz, H-1′), 5.85 (1H, m, H-7′), 5.23–5.16 (2H, m, H-8′), 4.58 (1H, m, H-3′), 3.91 (1H, dd, J 3.0, 2.3 Hz, H-4′), 3.85 (1H, m, H-5′), 2.44–2.34 (3H, m, H-2′, H-6′), 2.29 (1H, ddd, J 13.7, 6.5, 3.4 Hz, H-2′), 1.92 (3H, s, CH_3); MS(MALDI) m/z 305 (M + Na^+). This diol (202 mg, 0.715 mmol) was dissolved in anhydrous DMF (5 cm^3), imidazole (227 mg, 3.33 mmol) and TBDMSCl (344 mg, 2.28 mmol) were added. The mixture was stirred at room temperature for 4 h, then EtOAc (20 cm^3) was added and washed with brine (2 × 20 cm^3). The organic phase was dried (Na_2SO_4) and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using MeOH and CH_2Cl_2 (1 : 50 v/v) as the eluent to give the products as white solids. **7** (4.9 mg, 1.7%); R_f 0.82 (MeOH and CH_2Cl_2, 1 : 14 v/v); δ_H (300 MHz; DMSO-*d*6; Me_4Si) 11.34 (1H, br s, NH), 7.57 (1H, br s, H-6), 6.18 (1H, m, H-1′), 5.81 (1H, m, H-7′), 5.25 (1H, m, 3′-OH), 5.16–5.05 (2H, m, H-8′), 4.18 (1H, m, H-3′), 3.89 (1H, m, H-5′), 3.78 (1H, m, H-4′), 2.50 (1H, m, H-6′), 2.40 (1H, m, H-6′), 2.25 (1H, m, H-2′), 2.05 (1H, m, H-2′), 1.77 (3H, d, J 1.1 Hz, CH_3), 0.87 (9H, m, C(CH_3)$_3$), 0.12 (3H, s, SiCH_3), 0.09 (3H, s, SiCH_3). **3** (188 mg, 66%); R_f 0.55 (MeOH and CH_2Cl_2, 1 : 14 v/v); δ_H (300 MHz; DMSO-*d*6; Me_4Si) 11.30 (1H, br s, NH), 7.87 (1H, s, H-6), 6.17 (1H, dd, J 8.0, 6.5 Hz, H-1′), 5.84 (1H, m, H-7′), 5.21 (1H, d, J 5.2 Hz, 5′-OH), 5.11–5.05 (2H, m, H-8′), 4.44 (1H, m, H-3′), 3.69 (1H, br s, H-4′), 3.65 (1H, m, H-5′), 2.29–2.19 (2H, m, H-6′), 2.15 (1H, m, H-2′), 2.05 (1H, ddd, J 13.7, 6.5, 2.9 Hz, H-2′), 1.77 (3H, s, CH_3), 0.87 (9H, m, C(CH_3)$_3$), 0.08 (6H, s, SiCH_3); δ_C (75 MHz; $CDCl_3$; Me_4Si) 163.6 (C-4), 150.3 (C-2), 137.2 (C-7′), 134.1 (C-6), 118.8 (C-8′), 110.9 (C-5), 88.9, 86.9 (C-1′, C-4′), 73.0, 70.1 (C-3′, C-5′), 40.3, 39.1 (C-6′, C-2′), 25.7 (C(CH_3)$_3$), 18.0 (C(CH_3)$_3$), 12.6 (CH_3), −4.6, −4.8 (Si(CH_3)$_2$); MS(MALDI) m/z 419 (M + Na^+).

Preparation of 5′(*S*)-*C*-allyl-5′-*O*-benzoylthymidine (8)

Compound **5** (200 mg, 0.384 mmol) was dissolved in anhydrous pyridine (4 cm^3) and added benzoylchloride (0.09 cm^3, 0.77 mmol). The reaction mixture was stirred at room temperature for 8 h and then quenched by the addition of water (0.5 cm^3). The mixture was diluted with EtOAc (20 cm^3) and washed with a saturated aqueous solution of $NaHCO_3$ (2 × 10 cm^3) and water (10 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by column chromatography using MeOH and CH_2Cl_2 (1 : 19 v/v) as the eluent to give 5′(*S*)-*C*-allyl-5′-*O*-benzoyl-3′-*O*-(*tert*-butyldiphenylsilyl)thymidine (175 mg, 71%) as a white solid; found C 69.30, H 6.41, N 4.57, $C_{36}H_{40}N_2O_6Si$ requires C 69.20, H 6.45, N 4.48; mp 75–76 °C; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.09 (9H, s, C(CH_3)$_3$), 1.73 (1H, m, H-2′), 1.84 (3H, d, J 1.1, CH_3), 2.43–2.21 (3H, m, H-6′, H-2′), 4.07 (1H, m, H-4′), 4.33 (1H, m, H-3′), 4.79 (1H, m, H-5′), 5.09–5.00 (2H, m, H-8′), 5.68 (1H, m, H-7′), 5.98 (1H, dd, J 5.6, 8.4, H-1′), 7.34–7.48 (9H, m, H-6, Ph), 7.69–7.53 (5H, m, Ph), 7.90–7.85 (2H, m, Ph), 8.83 (1H, br s, NH); δ_C (75 MHz; $CDCl_3$; Me_4Si) 12.4 (CH_3), 19.0 (C(CH_3)$_3$), 26.9 (C(CH$_3$)$_3$), 35.8 (C-6′), 40.7 (C-2′), 72.6 (C-5′), 73.8 (C-3′), 84.7 (C-1′), 87.2 (C-4′), 111.1 (C-5), 118.8 (C-8′), 127.9, 128.0, 128.5, 129.3, 130.1 (Ph), 132.4, 132.8, 133.3 (Ph, C-7′), 135.0, 135.0, 135.6, 135.7 (C-6, Ph), 150.2 (C-4), 163.7 (C-2), 165.6 (PhCO); MS(MALDI) m/z 647 (M + Na^+), HRMS 647.2559 (calcd for $C_{36}H_{40}N_2O_6SiNa$ 647.2548). This compound (295 mg, 0.460 mmol) was dissolved in anhydrous THF (15 cm^3) and TBAF (1.0 M in THF, 0.46 cm^3) was added. The reaction mixture was stirred at room temperature for 45 min and then a suspension of DOWEX in pyridine, water and MeOH (3 : 1 : 1 v/v/v, 10 cm^3) was added. The mixture was stirred

for 8 h and then filtered. The filter was rinsed with pyridine, water and MeOH (3 : 1 : 1 v/v/v, 2 cm^3), and the combined filtrates were concentrated under reduced pressure. The residue was purified by column chromatography using EtOAc and petroleum ether (1 : 1 v/v) as the eluent to give the product (117 mg, 63%) as a white solid; found C 62.19, H 5.81, N 7.32, $C_{20}H_{22}N_2O_6$ requires C 62.17, H 5.74, N 7.25; mp 167–168 °C; δ_h (300 MHz; DMSO-*d*6; Me_4Si) 1.75 (3H, s, CH_3), 2.21–2.04 (2H, m, H-2′), 2.65–2.46 (2H, m, H-6′), 3.95 (1H, m, H-4′), 4.30 (1H, m, H-3′), 5.03–5.19 (2H, m, H-8′), 5.35 (1H, m, H-5′), 5.47 (1H, d, *J* 4.7, OH), 5.83 (1H, m, H-7′), 6.20 (1H, t, *J* 6.7, H-1′), 7.43 (1H, s, H-6), 7.60–7.50 (2H, m, Ph), 7.69 (1H, m, Ph), 7.92–8.06 (2H, m, Ph), 11.34 (1H, br s, NH); δ_c (75 MHz; DMSO-*d*6; Me_4Si) 12.1 (CH_3), 35.5 (C-6′), 39.3 (C-2′), 70.4 (C-3′), 72.8 (C-5′), 83.7 (C-1′), 86.3 (C-4′), 109.7 (C-5), 118.3 (C-8′), 128.8, 129.1, 129.6 (Ph), 133.5, 133.5 (C-7′, Ph), 135.4 (C-6), 150.3 (C-4), 163.6 (C-2), 165.4 (Ph*C*O); MS(MALDI) *m*/*z* 409 ($M + Na^+$).

Preparation of 5′(*S*)-*C*-allyl-5′-*O*-(4,4′-dimethoxytrityl)thymidine (9)

Compound **5** (293 mg, 0.562 mmol) was dissolved in a mixture of anhydrous 2,6-lutidine and anhydrous CH_2Cl_2 (1 : 1 v/v, 4 cm^3). DMT-triflate (532 mg, 1.18 mmol) was added and the reaction mixture was stirred at room temperature. Additional amounts of DMT-triflate were added after 18 h and 24 h (174 mg, 0.39 mmol and 261 mg, 0.58 mmol, respectively). After 48 h the solution was washed with a saturated aqueous solution of $NaHCO_3$ (23 cm^3). The aqueous layer was extracted with CH_2Cl_2 (15 cm^3), the combined organic extracts were dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was coevaporated with toluene and redissolved in anhydrous THF (22.5 cm^3) and TBAF (1.0 M in THF, 1 cm^3) was added. The reaction mixture was stirred at room temperature for 3 h. An additional amount of TBAF (1.0 M in THF, 1 cm^3) was added and the reaction mixture was stirred for another 2 h. Dowex 50 W × 2 in pyridine, MeOH and water (3 : 1 : 1 v/v, 8 cm^3) was added and the solution was stirred at room temperature overnight. The resin was filtered off and the filtercake was washed with pyridine, MeOH and water (3 : 1 : 1 v/v, 8 cm^3). The filtrate was diluted with CH_2Cl_2 (15 cm^3). The combined organic phases were washed with water (7.5 cm^3), dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using EtOAc and petroleum ether (3 : 4 v/v) and then MeOH and CH_2Cl_2 (1 : 49 v/v) as eluents to give the product (190 mg, 58%) as a beige foam; R_f 0.30 (MeOH and CH_2Cl_2, 1 : 19 v/v); mp 118–120 °C. δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.79 (1H, br s, NH), 7.86 (1H, d, *J* 1.1 Hz, H-6), 7.46–7.24 (9H, m, Ph), 6.85–6.81 (4H, m, Ph), 6.32 (1H, t, *J* 7.2 Hz, H-1′), 5.41 (1H, m, H-7′), 4.97–4.86 (2H, m, H-8′), 4.38 (1H, m, H-3′), 3.82 (1H, m, H-4′), 3.79 (6H, s, OCH_3), 3.41 (1H, m, H-5′), 2.45–2.19 (4H, m, H-2′, H-6′), 2.01 (1H, d, *J* 4.2 Hz, OH), 1.83 (3H, s, CH_3). δ_C (75 MHz; $CDCl_3$; Me_4Si) 163.7 (C-4), 158.7 (Ph), 150.2 (C-2), 145.8, 136.4, 136.1, 135.6, 133.9, 130.3, 130.2, 128.1, 127.8, 127.1 (C-6, C-7′, Ph), 117.8 (C-8′), 113.1 (Ph), 111.2 (C-5′), 87.3 ($C(Ar)_3$), 86.7 (C-4′), 84.1 (C-1′), 73.6 (C-5′), 71.6 (C-3′), 55.2 (OCH_3), 41.0 (C-2′), 36.3 (C-6′), 12.4 (CH_3). MS(MALDI) *m*/*z* 607 ($M + Na^+$), HRMS 607.2425 (calcd for $C_{34}H_{36}N_2O_7Na$ 607.2415).

Preparation of 3′-*O*-(*tert*-butyldiphenylsilyl)-5′-(cyanoethoxy(diisopropylamino)phosphinyl)-5′(*S*)-*C*-allylthymidine (10)

Compound **5** (1.95 g, 3.74 mmol) was coevaporated with anhydrous CH_3CN (3 × 40 cm^3) and redissolved in anhydrous CH_2Cl_2 (50 cm^3). To the mixture was added a 1.0 M solution of 4,5-dicyanoimidazole in CH_3CN (2.5 cm^3, 2.5 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphordiamidite (1.3 cm^3, 3.97 mmol). The reaction mixture was stirred at room temperature for 4 h. An additional amount of 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphordiamidite (0.5 cm^3, 1.52 mmol) was added and the reaction mixture was stirred for another 18 h. The solution was diluted with CH_2Cl_2 (200 cm^3), washed with a saturated aqueous solution of $NaHCO_3$ (2 × 50 cm^3) and brine (50 cm^3). The combined organic phases were dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using EtOAc and petroleum ether (1 : 1 v/v) as eluent to give the product (2.2 g, 80%) as a white foam; R_f 0.59 (EtOAc and petroleum ether, 3 : 1 v/v). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.57 (m, NH), 7.69–7.56 (m, H-6′, Ph), 7.49–7.35 (m, Ph), 6.53–6.48 (m, H-1′), 5.63–5.51 (m, H-7′), 5.10–5.03 (m, H-8′), 4.46 (d, *J* 5.1 Hz, H-3′), 4.36 (d, *J* 5.1 Hz, H-3′), 4.01–3.97 (m, H-4′), 3.80–3.58 (m, CH_2OP), 3.53–3.23 (m, H-5′, $HC(CH_3)_2$), 2.58–2.52 (m, CH_2CN), 2.39–2.24 (m, H-2′, H-6′), 2.13–2.08 (m, H-6′), 1.98–1.82 (m, H-2′), 1.88 (s, CH_3), 1.87 (s, CH_3), 1.14–0.90 (m, $C(CH_3)_3$, $HC(CH_3)_3$). δ_p ($CDCl_3$) 151.14, 151.05. MS(MALDI) *m*/*z* 743 ($M + Na^+$), HRMS 743.3334 (calcd for $C_{38}H_{53}N_4O_6PSiNa$ 743.3364).

Preparation of [5′-*O*-(5′(*S*)-*C*-allyl-3′-*O*-(*tert*-butyldiphenylsilyl)thymidinyl]-[3′-*O*-(5′(*S*)-*C*-allyl-5′-*O*-(4,4′-dimethoxytrityl)thymidinyl] cyanoethylphosphate (11)

The alcohol **9** (548 mg, 0.937 mmol) and the phosphoramidite **10** (1.288 g, 1.787 mmol) were mixed, coevaporated with a mixture of anhydrous CH_3CN and anhydrous CH_2Cl_2 (2 : 1 v/v, 3 × 30 cm^3) and redissolved in the same mixture (45 cm^3). The solution was added a 0.45 M solution of 1*H*-tetrazole in CH_3CN (9.0 cm^3, 4.05 mmol) and stirred at room temperature for 3 h. The reaction mixture was cooled to 0 °C, added a 3.0 M solution of *t*-BuOOH in toluene (1.5 cm^3, 3.0 mmol) and stirred at room temperature for 12 h. The solution was diluted with CH_2Cl_2 (100 cm^3), washed with a saturated solution of $NaHCO_3$ (2 × 50 cm^3) and brine (50 cm^3). The combined organic phases were dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using MeOH and CH_2Cl_2 (1 : 99–1 : 14 v/v) to give the product (716 mg, 63%) as a white foam; R_f 0.27 (MeOH and CH_2Cl_2, 1 : 14 v/v); found C 64.38, H 6.08, N 5.62, $C_{66}H_{74}O_{14}N_5PSi\cdot H_2O$ requires C 64.00, H 6.18, N 5.65. δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.05 (br s, NH), 8.86 (br s, NH), 8.72 (br s, NH), 7.91 (s, H-6), 7.80 (s, H-6), 7.65–7.61 (m, Ph), 7.49–7.21 (m, H-6, Ph), 6.86–6.80 (m, Ph), 6.52 (dd, *J* 5.0, 9.0 Hz, H-1′), 6.47 (dd, *J* 5.1, 9.0 Hz, H-1′), 6.32 (dd, *J* 5.2, 9.0 Hz, H-1′), 6.20 (t, *J* 7.2 Hz, H-1′), 5.58–5.19 (m, H-7′), 5.11–4.82 (m, H-8′), 4.79–4.74 (m, T1-H-3′), 4.32–4.28 (m, T2–H-3′), 4.05–3.73 (m, H-4′, T2–H-5′, OCH_2, OCH_3), 3.33–3.29 (m, T1-H-5′), 2.42–1.98 (m, H-2′, H-6′, CH_2CN), 1.89–1.76 (m, H-2′, CH_3), 1.14–1.00

(m, $C(CH_3)_3$). δ_p ($CDCl_3$) −1.43, −1.71 (1 : 3). MS(MALDI) *m/z* 1242 ($M + Na^+$), HRMS 1242.4612 (calcd for $C_{66}H_{74}N_5O_{14}PSiNa$ 1242.4631).

Preparation of (*E*,*Z*)-(3*R*/*S*)-(1*S*,5*S*,10*S*,11*R*,13*R*)-3-(2-cyanoethoxy)-10-(4,4′-dimethoxytrityl)oxy-3-oxo-5-(3(*S*)-(*tert*-butyldiphenylsilyl)oxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)-13-(thymin-1-yl)-3-phospha-2,4,12-trioxabicyclo[9.3.0]tetradec-7-ene (12)

Compound **11** (338 mg, 0.277 mmol) was coevaporated with anhydrous CH_2Cl_2 (3 × 25 cm^3) and redissolved in the same solvent (15 cm^3). To the solution was added a solution of Grubbs' 2nd generation catalyst§ (16 mg, 0.019 mmol) in CH_2Cl_2 (5 cm^3) and the reaction mixture was stirred at 40 °C for 4 h. Then the temperature was lowered to 30 °C and the reaction mixture was stirred for 16 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography using MeOH and CH_2Cl_2 (1 : 49–1 : 9 v/v) to give the product (246 mg, 74%) as a white foam; R_f 0.41 (MeOH and CH_2Cl_2, 1 : 14 v/v). δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.57–8.28 (m, NH), 7.68–7.65 (m, Ph), 7.54–7.08 (m, H-6, Ph), 6.87–6.72 (m, H-1′, Ph), 6.60–6.48 (m, H-1′), 6.02–5.95 (m, H-1′), 5.78–5.71 (m, $CH_2CH{=}CHCH_2$), 5.30–5.04 (m, $CH_2CH{=}CHCH_2$), 4.93–3.61 (m, H-3′, H-4′, H-5′, CH_2OP, CH_3O), 2.64–1.49 (m, H-2′, CH_3, CH_2CN, $CH_2CH{=}CHCH_2$), 1.09–1.08 (m, $C(CH_3)_3$). δ_p ($CDCl_3$) 0.37, −1.03, −6.51, −8.06 (4 : 1 : 8 : 6). MS(MALDI) *m/z* 912 ($M + Na^+$ − DMT), HRMS 912.3003 (calcd for $C_{43}H_{52}N_5O_{12}PSiNa$ 912.3012).

Preparation of (*E*,*Z*)-(3*R*/*S*)-(1*S*,5*S*,10*S*,11*R*,13*R*)-3-(2-cyanoethoxy)-10-hydroxy-3-oxo-5-(3(*S*)-(*tert*-butyldiphenylsilyl)oxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)-13-(thymin-1-yl)-3-phospha-2,4,12-trioxabicyclo[9.3.0]tetradec-7-ene (13)

Compound **12** (196 mg, 0.164 mmol) was dissolved in CH_2Cl_2 (10 cm^3). To the solution was added Et_3SiH (60 μL, 0.364 mmol) and a 1% solution of TFA in CH_2Cl_2 (1.0 cm^3, 0.129 mmol), whereby the reaction mixture went from weak yellow to orange-red. The reaction mixture was stirred at room temperature for 45 min. The solution was diluted with CH_2Cl_2 (100 cm^3) and washed with a saturated solution of $NaHCO_3$ (2 × 40 cm^3). The organic phase was dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using MeOH and CH_2Cl_2 (1 : 19 v/v) to give the product (126 mg, 86%) as a white foam; R_f 0.34, 0.39 (MeOH and CH_2Cl_2, 1 : 19 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.94–8.78 (m, NH), 8.63 (m, NH), 7.71–7.64 (m, Ph), 7.50–7.43 (m, Ph), 7.38–6.97 (m, H-6, Ph), 6.58–6.41 (m, H-1′), 6.11–5.89 (m, H-1′), 5.68–5.60 (m, $CH_2CH{=}CHCH_2$), 5.44–5.26 (m, $CH_2CH{=}CHCH_2$), 5.09–4.30 (m, H-3′, H-4′, H-5′), 4.16–3.87 (m, H-4′, H-5′, CH_2OP), 3.78–3.57 (m, T1–H-5′), 3.07 (br s, OH), 2.88 (br s, OH), 2.81–2.26 (m, H-2′, CH_2CN, $CH_2CH{=}CHCH_2$), 2.20–1.78 (m, H-2′, CH_3, $CH_2CH{=}CHCH_2$), 1.10–1.07 (m, $C(CH_3)_3$). δ_p ($CDCl_3$) 0.44, −0.76, −6.00, −7.46 (4 : 1 : 8 : 6). MS(MALDI) *m/z* 912 ($M + Na^+$), HRMS 912.2998 (calcd for $C_{43}H_{52}N_5O_{12}PSiNa$ 912.3012).

Preparation of (3*R*/*S*)-(1*S*,5*S*,10*S*,11*R*,13*R*)-3-(2-cyanoethoxy)-10-hydroxy-3-oxo-5-(3(*S*)-(*tert*-butyldiphenylsilyl)oxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)-13-(thymin-1-yl)-3-phospha-2,4,12-trioxabicyclo[9.3.0]tetradecane (14)

Compound **13** (119 mg, 0.134 mmol) was dissolved in MeOH (10 cm^3) and $Pd(OH)_2/C$ (23.2 mg, 0.033 mmol) in MeOH (3 cm^3) was added. The reaction mixture was bubbled with H_2 for 5 min. and another 2 cm^3 of MeOH was added. The reaction mixture was placed in an autoclave at 1000 psi H_2 at 55 °C for 16 h. The mixture was filtered through celite and after washing the celite with MeOH (50 cm^3), the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using MeOH and CH_2Cl_2 (1 : 14 v/v) to give the product (75 mg, 63%) as a white foam; R_f 0.37, 0.41 (MeOH and CH_2Cl_2, 1 : 9 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.37 (br s, NH), 9.32 (br s, NH), 9.14 (br s, NH), 8.98 (br s, NH), 7.69–7.63 (m, Ph), 7.50–7.40 (m, H-6, Ph), 7.22 (br s, H-6), 7.07 (br s, H-6), 6.49 (dd, *J* 5.4, 8.5 Hz, H-1′), 6.36 (t, *J* 6.9 Hz, H-1′), 6.09–6.02 (m, H-1′), 5.03 (T1-H-3′), 4.76 (T1-H-3′), 4.47 (T2-H-3′), 4.28–3.68 (m, T2–H-3′, H-4′, H-5′, CH_2O), 2.93 (br s, OH), 2.72–2.47 (m, H-2′, CH_2OP), 2.35–2.28 (m, H-2′), 1.96–0.99 (m, H-2′, CH_3, $(CH_2)_4$, $C(CH_3)_3$). δ_p ($CDCl_3$) −0.23, −4.79 (1 : 2.4). MS(MALDI) *m/z* 914 ($M + Na^+$), HRMS 914.3130 (calcd for $C_{43}H_{54}N_5O_{12}PSiNa$ 914.3168).

Preparation of [5′-*O*-(5′(*S*)-*C*-allyl-3′-*O*-(*tert*-butyldiphenylsilyl)thymidinyl]-[3′-*O*-(5′(*S*)–*C*-allyl)thymidinyl] cyanoethylphosphate (15)

Compound **11** (48 mg, 0.039 mmol) was dissolved in CH_2Cl_2 (4 cm^3). To the solution was added Et_3SiH (13 μL, 0.079 mmol) and a 1% solution of TFA in CH_2Cl_2 (0.23 cm^3, 0.030 mmol), whereby the reaction mixture went from weak yellow to orange-red. The reaction mixture was stirred at room temperature for 20 min. The solution was diluted with CH_2Cl_2 (30 cm^3) and washed with a saturated solution of $NaHCO_3$ (2 × 10 cm^3). The organic phase was dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using MeOH and CH_2Cl_2 (1 : 33 v/v) to give the product (29 mg, 80%) as a white foam; R_f 0.44 (MeOH and CH_2Cl_2, 1 : 9 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.00 (br s, NH), 8.90 (br s, NH), 8.83 (br s, NH), 8.78 (br s, NH), 7.67–7.62 (m, Ph), 7.54–7.39 (m, H-6, Ph), 6.55–6.50 (m, H-1′), 6.19–6.13 (m, H-1′), 5.82–5.51 (m, H-7′), 5.27–4.96 (m, T1-H-3′, H-8′), 4.35–4.27 (m, T2–H-3′), 4.20–3.93 (m, H-4′, T2–H-5′, CH_2OP), 3.84–3.73 (m, T1–H-5′), 2.76–2.53 (m, H-2′, H-6′, CH_2CN), 2.41–2.14 (m, H-2′, H-6′), 1.91–1.81 (m, H-2′, CH_3), 1.10–1.07 (m, $C(CH_3)_3$). δ_p ($CDCl_3$) −1.42. MS(MALDI) *m/z* 940 ($M + Na^+$), HRMS 940.3369 (calcd for $C_{45}H_{56}N_5O_{12}PSiNa$ 940.3325).

Preparation of (*E*,*Z*)-(1*S*,5*S*,10*S*,11*R*,13*R*)-3,10-dihydroxy-5-(3(*S*)-hydroxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)-3-oxo-13-(thymin-1-yl)-3-phospha-2,4,12-trioxabicyclo[9.3.0]tetradec-7-ene (16)

Compound **13** (11.8 mg, 0.013 mmol) was dissolved in anhydrous THF (1 cm^3) and TBAF (1.0 M in THF, 0.030 cm^3) was added to the solution. After stirring for 1 h, the solvent was removed under reduced pressure. The residue was redissolved in 32% NH_3(aq) and the solution was stirred at 55 °C for 20 h. The solvent was removed

under reduced pressure, and the residue was subjected to HPLC-purification (C_{18} column, buffer A (CH_3CN and 0.1 M NH_4HCO_3, 1 : 19 v/v) and buffer B (CH_3CN and 0.1 M NH_4HCO_3, 3 : 1 v/v)); δ_H (300 MHz; D_2O; Me_4Si) 7.95 (s, H-6), 7.85 (s, H-6), 7.58 (br s, H-6), 6.44 (m, H-1′), 6.30 (m, H-1′), 5.73–5.69 (m, $CH_2CH{=}CHCH_2$), 5.13–4.52 (m, H-3′, H-4′, H-5′), 4.12–3.52 (m, H-3′, H-4′, H-5′), 2.64–2.23 (m, H-2′, $CH_2CH{=}CHCH_2$), 1.97–1.92 (m, CH_3). δ_p (D_2O) −0.39, −1.23 (1 : 1).

Preparation of (1*S*,5*S*,10*S*,11*R*,13*R*)-3,10-dihydroxy-5-(3(*S*)-hydroxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)-3-oxo-13-(thymin-1-yl)-3-phospha-2,4,12-trioxabicyclo[9.3.0]tetradecane (17)

Compound **14** (25.4 mg, 0.029 mmol) was dissolved in anhydrous THF (3 cm^3) and TBAF (1.0 M in THF, 0.030 cm^3) was added to the solution. After stirring for 45 min the solvent was removed under reduced pressure. The residue was redissolved in 32% NH_3(aq) and the solution was stirred at 55 °C for two days. The solvent was removed under reduced pressure, and the residue was subjected to HPLC-purification (C_{18} column, buffer A (CH_3CN and 0.1 M NH_4HCO_3, 1 : 19 v/v) and buffer B (CH_3CN and 0.1 M NH_4HCO_3, 3 : 1 v/v)); δ_H (500 MHz; D_2O; Me_4Si) 7.81 (s, H-6), 7.52 (s, H-6), 6.36 (t, *J* 6.5 Hz, H-1′), 6.26 (dd, *J* 7.5, 6.7 Hz, H-1′), 4.76 (1H, m, T1-H-3′), 4.67 (1H, m, T2-H-3′), 4.43 (1H, m, T2-H-5′), 4.04 (1H, dd, 10.1, 3.4 Hz, T1-H-4′), 3.99 (1H, m, T2-H-4′), 3.82 (1H, m, T1–H-5′), 2.56–2.32 (4H, m, T1-H-2′, T2-H-2′), 2.09–1.93 (2H, m, $(CH_2)_4$), 1.96 (3H, s, CH_3), 1.91 (3H, s, CH_3), 1.87–1.62 (4H, m, $(CH_2)_4$), 1.46–1.38 (2H, m, $(CH_2)_4$). δ_p (D_2O) 0.12.

Preparation of 5′(*S*)-*C*-allyl-5′-*O*-pixylthymidine (18)

Compound **5** (2.20 g, 4.23 mmol) was coevaporated with anhydrous pyridine (3 × 15 cm^3) and redissolved in anhydrous pyridine (20 cm^3). 9-Chloro-9-phenyl-9*H*-xanthen (pixyl chloride) was added, and the reaction mixture was stirred for 12 h. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (50 cm^3) and washed with a saturated aqueous solution of $NaHCO_3$ (2 × 20 cm^3). The aqueous phase was extracted with CH_2Cl_2 (3 × 20 cm^3) and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was coevaporated with toluene and redissolved in anhydrous THF (20 cm^3) and TBAF (1.0 M in THF, 7.8 cm^3) was added. The reaction mixture was stirred for 12 h at room temperature. The reaction mixture was partitioned between water (100 cm^3) and EtOAc (100 cm^3). The separated aqueous phase was extracted with EtOAc (2 × 25 cm^3). The combined organic phases were washed with brine (50 cm^3), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using 0.1% Et_3N in CH_3OH and CH_2Cl_2 (1 : 99 v/v) to give the product as a white foam (2.20 g, 99%); R_f 0.30 (MeOH and CH_2Cl_2, 1 : 19 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 7.65 (1H, d, *J* 1.2 Hz, H-6), 7.48–7.20 (9H, m, Ph), 7.09–6.99 (4H, m, Ph), 6.16 (1H, t, *J* 6.3 Hz, H-1′), 5.47–5.33 (m, 1H, H-7′), 4.85 (1H, dd, *J* 9.9, 1.5 Hz, H-8′), 4.71 (1H, dd, *J* 17.1, 1.5 Hz, H-8′), 3.64–3.55 (2H, m, H-4′, H-3′), 3.40–3.25 (1H, m, H-5′), 2.22–1.93 (6H, m, H-2′, H-6′, CH_3). δ_C (75 MHz; $CDCl_3$; Me_4Si) 164.1 (C-4), 152.2, 151.9, 150.5 (C-2, arom), 147.0, 135.8, 134.0, 131.9, 130.9, 130.1, 130.0, 128.1, 127.9, 127.8, 127.4, 123.9, 123.6, 123.1 (C-6, C-7′, arom), 118.0, 116.9, 116.7, 110.9 (C-8′, arom, C-5), 86.5, 84.3, 77.4, 72.6, 71.0 (C-1′, C-3′, C-4′, C-5′, $C(Ar)_3$), 40.6 (C-2′), 36.8 (C-6′), 12.9 (CH_3). MS(MALDI) *m/z* 561 ($M + Na^+$).

Preparation of 5′(*S*)-*C*-allyl-3′-*O*-(*tert*-butyldiphenylsilyl)-5′-(methoxy(diisopropylamino)phosphinyl)thymidine (19)

To a solution of **5** (2.75 g, 5.29 mmol) in anhydrous CH_2Cl_2 (20 cm^3) at 0 °C was added diisopropylethylamine (1.81 cm^3, 10.6 mmol) and $(i\text{-}Pr)_2NP(Cl)OCH_3$ (1.53 cm^3, 7.93 mmol). The reaction mixture was stirred at room temperature for 3 h and quenched with methanol (5 cm^3). The reaction mixture was diluted with EtOAc (100 cm^3), washed with a saturated aqueous solution of $NaHCO_3$ (3 × 30 cm^3) and brine (3 × 30 cm^3), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using 0.1% Et_3N in EtOAc and petroleum ether (1 : 3 v/v) to give the product as a white foam (2.70 g, 75%) R_f 0.30 (EtOAc and petroleum ether, 3 : 1 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 8.80–8.76 (1H, br s, NH), 7.75–7.59 (5H, m, H-6, Ph), 7.46–7.32 (6H, m, Ph), 6.60–6.45 (1H, m, H-1′), 5.65–5.45 (1H, m, H-7′), 5.10–4.95 (2H, m, H-8′), 4.58 (1/2H, d, *J* 5.1 Hz, H-3′), 4.50 (1/2H, d, *J* 5.1 Hz, H-3′), 3.99–3.94 (1H, m, H-4′), 3.48–3.20 (3H, m), 3.25 (3H, d, *J* 13.5 Hz, OCH_3), 3.07 (3H, d, *J* 13.5 Hz, OCH_3), 2.40–2.20 (3H, m), 2.00–1.80 (4H, m), 1.14–0.90 (21H, m). δ_P ($CDCl_3$) 152.10, 150.44. MS(MALDI) *m/z* 704 ($M + Na^+$), HRMS 704.3228 (calcd for $C_{36}H_{52}N_3O_6PSiNa$ 704.3255).

Preparation of [5′-*O*-(5′(*S*)-*C*-allyl-3′-*O*-(*tert*-butyldiphenylsilyl)thymidinyl]-[3′-*O*-(5′(*S*)-*C*-allyl-5′-*O*-pixylthymidinyl] methylphosphate (20)

To a solution of **18** (1.00 g, 1.90 mmol) and **19** (1.30 g, 1.90 mmol) in CH_3CN and CH_2Cl_2 (2 : 1 v/v, 50 cm^3) was added 1*H*-tetrazole (0.45 M in CH_3CN, 4.65 cm^3, 2.09 mmol) and the reaction mixture was stirred for 12 h at room temperature. The reaction mixture was cooled to 0 °C and *tert*-butylhydroperoxide (5.5 M in decane, 0.52 cm^3, 2.85 mmol) was added. The reaction mixture was stirred at room temperature for 3 h. CH_3OH (10 cm^3) was added and the solution was diluted with CH_2Cl_2 (50 cm^3). The reaction mixture was washed with a saturated aqueous solution of $NaHCO_3$ (3 × 25 cm^3) and brine (25 cm^3), dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by silica gel column chromatography using 0.1% Et_3N in EtOAc and petroleum ether (3 : 1 v/v) to give the product as a white foam (1.61 g, 75%): R_f 0.15 (EtOAc and petroleum ether, 3 : 1 v/v); δ_H (300 MHz; $CDCl_3$; Me_4Si) 9.40–9.10 (m, NH), 7.80–6.90 (m, H-6, Ph), 6.56–6.49 (m, H-1′), 6.19–6.09 (m, H-1′), 6.02–5.97 (m, H-1′), 5.53–5.26 (m, H-7′), 5.15–4.62 (m, T1-H-3′, H-8′), 4.37–4.32 (m, T2-H-3′), 3.98–3.26 (m, H-4′, H-5′, OCH_2), 3.44 (d, $J = 11.4$ Hz, OCH_3), 3.43 (d, $J = 11.4$ Hz, OCH_3), 2.40–1.65 (m, H-2′, H-6′, CH_3), 1.10–1.06 (m, $C(CH_3)_3$). δ_P ($CDCl_3$) −0.01, −0.88 (1 : 1.8). MS(MALDI) *m/z* 1157 ($M + Na^+$), HRMS 1157.4119 (calcd for $C_{62}H_{67}N_4O_{13}PSiNa$ 1157.4104).

Preparation of (3*R*/*S*)-(1*S*,5*S*,10*S*,11*R*,13*R*)-3-methoxy-10-pixyloxy-3-oxo-5-(3(*S*)-(*tert*-butyldiphenylsilyl)oxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)-13-(thymin-1-yl)-3-phospha-2,4,12-trioxabicyclo[9.3.0]tetradecane (21)

To a solution compound **20** (1.16 g, 1.02 mmol) in anhydrous 1,2-dichloroethane was added Grubbs' 2nd generation catalyst (60.8 mg, 0.072 mmol) and the reaction mixture was stirred at 60 °C for 88 h. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography using a gradient of EtOAc (0–100%) in 0.1% Et_3N in petroleum ether to give the two ring-closed intermediates (308 mg, 27%, R_f 0.15 (EtOAc); δ_P ($CDCl_3$) 2.10, 0.64, −4.95, −6.30 (5 : 1.3 : 1 : 4.2); MS(MALDI) *m*/*z* 1129 (M + Na^+), HRMS 1129.3815 (calcd for $C_{60}H_{63}N_4O_{13}PSiNa$ 1129.3791) and (353 mg, 42%, R_f 0.05 (EtOAc); MS(MALDI) *m*/*z* 873 (M + Na^+), HRMS 873.2893 (calcd for $C_{41}H_{51}N_4O_{12}PSiNa$ 873.2903)) as well as unreacted starting material (110 mg, 10%, one isomer only). The two ring-closed intermediates were combined in a solution in anhydrous methanol (1 cm^3) and added Adams' catalyst (PtO_2) (60 mg, 0.26 mmol). The solution was degassed several times with argon and placed under a hydrogen atmosphere. The reaction mixture was stirred for 12 h at room temperature and filtered through celite. The solvent was removed under reduced pressure to give a crude saturated and depixylated intermediate (621 mg, MS(MALDI) *m*/*z* 875 (M + Na^+), HRMS 875.3077 (calcd for $C_{41}H_{53}N_4O_{12}PSiNa$ 875.3059)). To a solution of this intermediate (100 mg, 0.111 mmol) in anhydrous CH_2Cl_2 was added 2,6-lutidine (68 μL, 0.59 mmol) and pixyl chloride (51.5 mg, 0.18 mmol). The reaction mixture was stirred at room temperature for 2 h, and the solvent was removed under reduced pressure. The residue was coevaporated with toluene to give the crude pixylated intermediate (150 mg). To a stirred solution of this intermediate (100 mg, 0.09 mmol) in THF at 0 °C was added a mixture of glacial acetic acid (10.3 μL, 0.18 mmol) and TBAF (1.0 M in THF, 271 μL). The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography using 0.1% Et_3N in MeOH and CH_2Cl_2 (1 : 24 v/v) to give the product as a white foam (25 mg, 27% from **20**): R_f 0.33 (MeOH and CH_2Cl_2, 1 : 9 v/v); δ_P ($CDCl_3$) 1.35, −3.63 (3 : 1); MS(MALDI) *m*/*z* 893 (M + Na^+), HRMS 893.2704 (calcd for $C_{44}H_{47}N_4O_{13}PNa$ 893.2769).

Preparation of (3*R*/*S*)-(1*S*,5*S*,10*S*,11*R*,13*R*)-3-methoxy-10-pixyloxy-3-oxo-5-(3(*S*)-(2-cyanoethoxy)(diisopropylamino)phosphin)oxy-5(*R*)-(thymin-1-yl)tetrahydrofuran-2(*R*)-yl)-13-(thymin-1-yl)-3-phospha-2,4,12-trioxabicyclo[9.3.0]tetradecane (22)

To a stirred solution of compound **21** (80 mg, 0.092 mmol) in anhydrous CH_2Cl_2 were added diisopropylethylamine (23.6 μL, 0.138 mmol) and i-$Pr_2NP(Cl)OCH_2CH_2CN$ (24.6 μL, 0.11 mmol) at 0 °C. The mixture was stirred at room temperature for 6 h and then partitioned between CH_2Cl_2 (2 × 10 cm^3) and a saturated aqueous solution of $NaHCO_3$ (10 cm^3). The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using EtOAc and petroleum ether (1 : 2 v/v) and then EtOAc and MeOH (99 : 1 v/v) to give the product as a white foam (36 mg, 37%): R_f 0.40 (MeOH and CH_2Cl_2, 1 : 9 v/v); δ_P ($CDCl_3$) 150.40, 150.04, 150.00, 1.20, 0.99, −4.08.

NMR spectroscopy of 17

1D 500 MHz ^1H NMR spectra were recorded at 10, 23 and 25 °C with 20032 points and a spectral width of 5000 Hz. A phase sensitive NOESY spectrum with a mixing time of 700 ms was recorded at 23 °C with 2 K points in the t_2-dimension using spectral widths of 5000 Hz in both dimensions and 48 transients for each of the 600 t_1-experiments. Phase sensitive DQF-COSY spectra were recorded at 23 and 25 °C with 4 K points sampled in F2, 5000 Hz spectral widths in both dimensions and 512 t_1-experiments, each recorded with 48 transients. All spectra were processed using NMRpipe.[49] Cross peak intensities were measured using the program Sparky.[50]

Restrained molecular dynamics simulations on 17

MD calculations were performed using the AMBER 7.0 program package[51] on an SGI/O2 workstation. Distance restraints were derived from the NOESY spectra using the ISPA approach. Atomic charges around the modified phosphorus were calculated using the restrained electrostatic potential (RESP) procedure.[52] The unmodified dinucleotide and the cyclic dinucleotide **17** were following a short energy minimisation subjected to 1000 ps molecular dynamics simulation at 300 K. In case of the cyclic dinucleotide, the calculations were performed both with and without time averaged NMR restraints.

Preparation of oligodeoxynuclotides

Oligonucleotide synthesis was carried out by using an Expedite™ 8909 nucleic acid synthesis system from PerSeptive Biosystems Inc. following the phosphoramidite approach. Synthesis of oligonucleotides **24** and **26** 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 phosphoramidite **22**. The synthesis followed the regular protocol employing standard CPG supports. However, for the LNA monomer, a prolonged coupling time of 4 min was used, and for **22**, a manual coupling in 20 min followed by 2 × 45 s capping was used. Coupling yields for **22** were >95%. The 5′-*O*-DMT-ON oligonucleotides were treated with a 1 M solution of disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate[43] in DMF for 30 min, rinsed with ethanol and then removed from the solid support by treatment with concentrated aqueous ammonia at room temperature for 72 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 × 150 mm column; buffer A: 0.5 M triethylammonium acetate, pH 7.4; buffer B: CH_3CN and water (3 : 1 v/v); 0–2 min 100% A, 2–40 min from 100% A to 70% B, 30% A, 40–50 min from 70% B, 30% A to 100% B. All fractions containing 5′-*O*-DMT-protected oligonucleotide (retention time 25–30 minutes) were collected, concentrated and diluted with water (1 cm^3). 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 96% ethanol, dried and dissolved in water. MALDI MS *m/z* (M + H) found/calcd: **24** 4186.8/4188.5; **26** 4438.9/4440.6.

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 **17** 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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