Synthesis and Hybridisation Properties of Phosphonamidate Ester Modified Nucleic Acid

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Received 25 January 2001

Abstract: The replacement of the two backbone oxygen atoms of the phosphodiester linkage of DNA with carbon and nitrogen in the form of a phosphonamidate ester linkage, in both possible regioisomeric forms, is described. An alkylation reaction with the 3'-*C-P-N*-5' methyl phosphonamidate regioisomer yielded the corresponding *N*-methylated analogues. For each example separation into individual phosphonamidate ester diastereoisomers was performed prior to incorporation into oligonucleotides. The effect upon duplex stability for DNA oligonucleotides containing each of these modifications with complimentary RNA is reported.

Key words: antisense, modified oligonucleotides, nucleosides, phosphorus protecting groups

The application of modified oligonucleotides for disease control, based upon the antisense concept of translational arrest, is currently an important area of medicinal chemistry research.[2] First-generation antisense oligonucleotides were based upon the phosphorothioate modification as a replacement for the enzymatically labile phosphodiester linkage of 'wild-type' DNA. Current attention is focused upon developing antisense agents with even greater in vivo stability, improved cell penetration and enhanced binding properties with complimentary RNA.[3] As part of a project to identify such novel second-generation oligonucleotide modifications, we have been interested in phosphorus containing replacements for the phosphodiester backbone linkage of 'wild-type' DNA.[4,5] In particular, the possibility of *C-P-N* replacements attracted our attention in light of the reported enhanced binding to complimentary RNA for the phosphoramidate 3'-*N-P(O$^-$)-O*-5' modification, in which nitrogen replaces the 3'-oxygen.[6] Replacement of the second oxygen atom with carbon was anticipated to be beneficial due to the increased nuclease stability associated with *C-P* backbone linkages.[4] Additionally, our own work has demonstrated enhanced hybridisation properties with the 3'-methylene phosphonate modification 3'-*C-P(O$^-$)-O*-5', in which carbon replaces the 3'-oxygen.[5] Recently, the related 3'-*N-P(Me)-O*-5' methyl phosphonamidate modification has been described, but to date no hybridisation data has been disclosed for this linkage.[7] In this letter, we present our initial results from the incorporation of the phosphonamidate ester *C-P(OR)-N* backbone modification into DNA.

Direct replacement of the phosphodiester moiety of DNA with the unsymmetrical phosphonamidate *C-P-N* linkage leads to the two regioisomeric possibilities **1** and **2**, Figure 1. Additionally, incorporation of the phosphorus moiety as the ester generates a chiral environment at phosphorus and hence diastereoisomers for each regioisomer as shown in Figure 1. The importance of phosphorus stereochemistry in determining the stability of duplexes in which such chiral modified oligonucleotides are incorporated has been described for a number of backbone modifications.[8] Therefore, a sequence was sought which would allow access to both diastereoisomers of the modifications **1** and **2**. In common with many other workers, a thymidine-thymidine dimer approach was selected for the initial evaluation requiring the preparation of phosphonamidate linked dinucleosides for incorporation into standard phosphoramidite oligonucleotide synthesis.

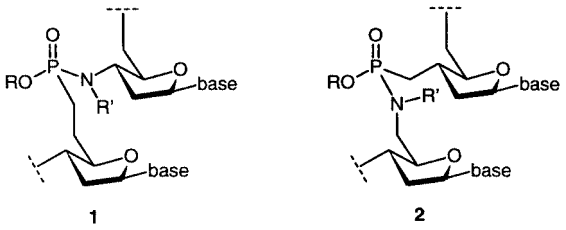

Figure 1

Our initial approach involved phosphorus to nitrogen bond construction through a Staudinger reaction with *H*-phosphinate and azido substituted nucleoside precursors.[9] A retrosynthetic analysis outlining this strategy for formation of the key phosphonamidate linkage is shown in Figure 2. This disconnection required the synthesis of the suitably protected 3'- and 5'-homologated *H*-phosphinates **3**, **4**, and **5,** the syntheses for which are described below and shown in Schemes 1 and 2. The azide coupling partners **6** and **7** were prepared from thymidine following literature procedures.[10]

Synthesis of the 5'-homologated *H*-phosphinate **3** was achieved as follows. The 5'-homologated phosphinate **8** was made available from a boron trifluoride catalysed oxetane ring opening with a phosphinate stabilised carbanion, following the procedure previously reported by this group.[4] Subsequent hydrolysis of the 3'-benzoate followed by silylation yielded **9**. Selective removal of the mixed orthoester protecting group from **9** upon treatment with trimethylsilylchloride, ethanol and chloroform at room temperature liberated the ethyl *H*-phosphinate **10**.[11] Hydrolysis with triethylamine, water and ethanol pro-

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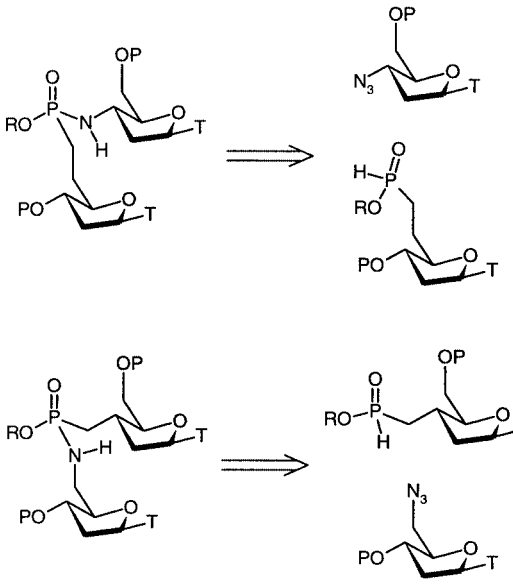
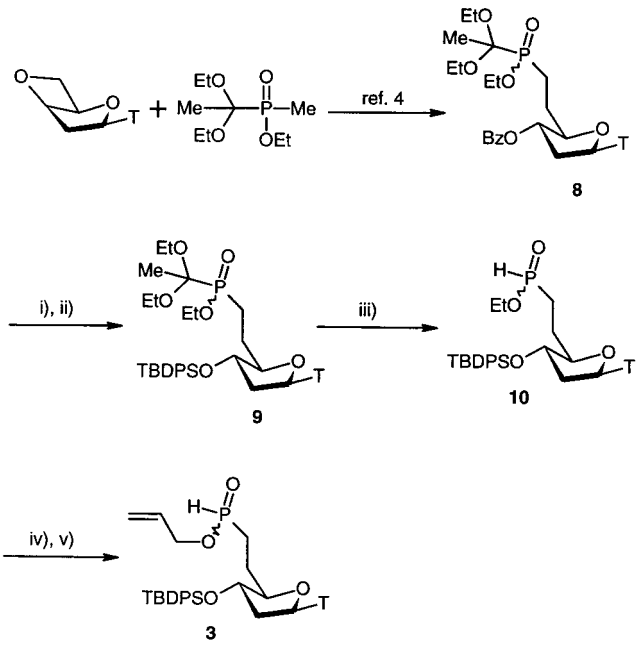


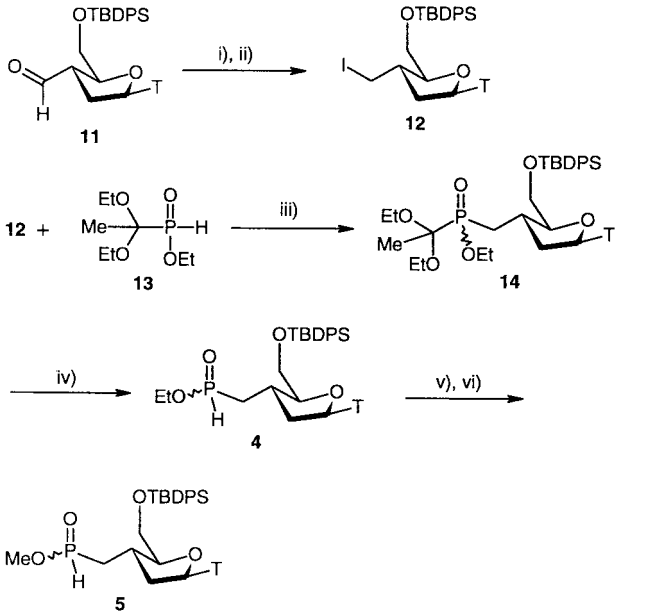
Figure 2

duced the corresponding *H*-phosphinic acid, which was re-esterified with allyl alcohol, using DCC as the condensing agent, to produce the allyl *H*-phosphinate **3**, Scheme 1.



Scheme 1 i) NaOMe, MeOH, rt (85%); ii) TBDPS-Cl, imidazole, DMF, rt (91%); iii) Me_3SiCl, EtOH, $CHCl_3$, rt (71%); iv) Et_3N, EtOH, H_2O, rt (95-98%); v) allyl alcohol, DCC, 2 mol% DMAP, THF, rt (69-73%).

The 3'-homologated *H*-phosphinates **4** and **5** were made available from the previously described 3'-formylated thymidine derivative **11**.[12] Reduction of **11** with sodium borohydride in ethanol furnished the hydroxymethyl analogue that could be readily converted to the iodide **12** upon treatment with methyltriphenoxyphosphonium iodide in DMF. The iodide **12** was then employed in an alkylation reaction with the potassium anion of the differentially protected hypophosphorous acid synthon **13** to give the ethyl phosphinate **14**.[5,11] Deprotection of **14** in an analogous manner to that described above for **10**, using in situ generated hydrogen chloride, yielded the ethyl *H*-phosphinate **4**. Subsequent hydrolysis followed by re-esterification gave the methyl *H*-phosphinates **5**, Scheme 2.



Scheme 2 i) $NaBH_4$, EtOH, rt (95-97%); ii) 1.1 equiv. $MeP^+(OPh)_3I^-$, 2 equiv. 2,6-lutidine, DMF, rt (68-76%); iii) 4 equiv. **13**, 4 equiv. KHMDS, THF, -78ºC to rt (85-94%); iv) Me_3SiCl, EtOH, $CHCl_3$, rt (70-87%); v) Et_3N, EtOH, H_2O, rt (87-92%); vi) MeOH, DCC, 2 mol% DMAP, THF, rt (75-86%).

Following the above sequences, all the mononucleoside *H*-phosphinates **3**, **4** and **5** were obtained as essentially 1:1 mixtures of phosphorus diastereoisomers as adjudged by ^{31}P NMR.

With the required intermediates in hand, dimer formation was achieved upon bis(trimethylsilyl)trifluoroacetamide catalysed reaction of the *H*-phosphinates **3**, **4** and **5** with the appropriate azidonucleoside derivative in pyridine at room temperature, as shown in Scheme 3.[9] In each case after completion of the reaction, removal of volatiles produced mixtures of the desired products and trimethylsilylated intermediates that were converted completely through to the desired products upon refluxing in a mixture of chloroform and methanol.[13] From these reactions, the three phosphonamidate dinucleosides **15**, **16** and **17** were obtained in good yields, each as 1:1 mixtures of diastereoisomers epimeric at the phosphorus centre. In the case of **15**, separation into the individual phosphorus diastereoisomers, via flash column chromatography, was readily achieved to provide the first eluting diastereoiso-

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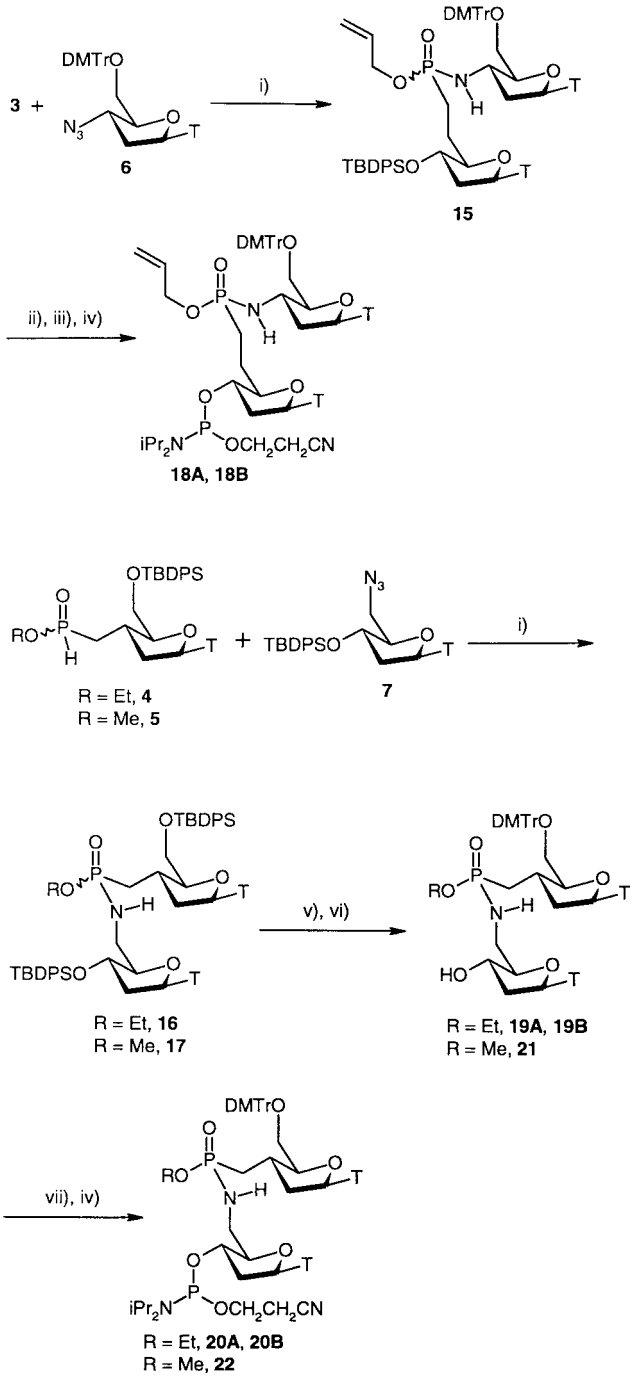
mer **15A** and second eluting isomer **15B**.[14] These individual isomers were readily desilylated with tetrabutylammonium fluoride (TBAF) in THF to yield the corresponding 3'-alcohols, which were phosphitylated to give the phosphoramidates **18A** and **18B** ready for oligonucleotide synthesis. For the 3'-*C*-*P*-*N*-5' analogues separation into the individual diastereoisomers immediately after the coupling reaction proved to be tedious. However, following desilylation of **16** with TBAF in THF and subsequent selective 5'-tritylation allowed the readily separable 3'-alcohol diastereoisomers **19A** and **19B** to be obtained.[14] Phosphitylation of these individual diastereoisomers produced the dinucleoside phosphoramidites **20A** and **20B**. In the methyl phophonamidate example **17** only minimal differences in R_f between diastereoisomers were observed throughout the analogues sequence and the mixed isomers were investigated without separation. Thus, the mixed isomer methyl phosphonamidate 5'-tritylated dinucleoside **21** was phosphitylated to give **22** as a 1:1:1:1 mixture of diastereoisomers epimeric at both the *P*(V) and *P*(III) centres.

N-Alkylation of the phosphonamidate linkage provides a site for tethering additional functionality and reducing the overall polarity, with the potential to improve cell permeability and chemical stability.[15] We selected *N*-methylation to establish the effect upon duplex stability for the 3'-*C*-*P*-*N*-5' phosphonamidate system. Utilising the reaction between *H*-phosphinate and azide to prepare the phosphonamidate linkage precluded the introduction of an *N*-alkyl substituent until after that step in the synthesis. As a consequence, competitive alkylation at the *N*-3 position of the thymine residues was anticipated to be problematic. This was overcome by selective introduction of benzyloxymethyl protecting groups at these sites in the bis-silylated intermediate **17**, upon treatment with benzyloxymethyl chloride and diazabicycloundecane (DBU) in acetonitrile at room temperature. No evidence of competitive phosphonamidate alkylation was observed during the protection of the two thymine residues. The product from this reaction could then be *N*-methylated at the desired position with an excess of methyl iodide, following deprotonation with sodium hydride. At this point in the synthesis, in contrast to the *N*-*H* precursors, separation of the phosphonamidate diastereoisomers via flash column chromatography was now readily achieved to give **23A** and **23B**.[14] Subsequent hydrogenolysis removed the benzyloxymethyl protecting groups to give the *N*-methylated analogues of dinucleoside **17**. These were then transformed into the corresponding 5'-dimethoxytrityl 3'-phosphoramidites **24A** and **24B** in an analogue manner to that described above for the *N*-*H* derivatives, as shown in Scheme 4.

Oligonucleotides were prepared incorporating each of the above seven modified dinucleosides following standard phosphoramidite coupling protocols.[16] Using this ap-



Scheme 3 i) 5 equiv. $Me_3SiNC(OSiMe_3)CF_3$, pyridine, 0 °C to rt (81-87%); ii) flash column chromatographic separation diastereoisomers, silica gel, eluent; 5% methanol in ethyl acetate; iii) 1.5 equiv. $Bu_4N^+F^-$, THF, 0 °C (83-92%); iv) 3 equiv. $(^iPr_2N)_2POCH_2CH_2CN$, 5 equiv. diisopropylammonium tetrazolide, CH_2Cl_2, rt (69-78%); v) 3 equiv. $Bu_4N^+F^-$, THF, 0 °C (96%); vi) 1.2 equiv. DMTrCl, pyidine, rt (68-88%); vii) flash column chromatography, silica gel, eluent; 10% ethanol in chloroform.

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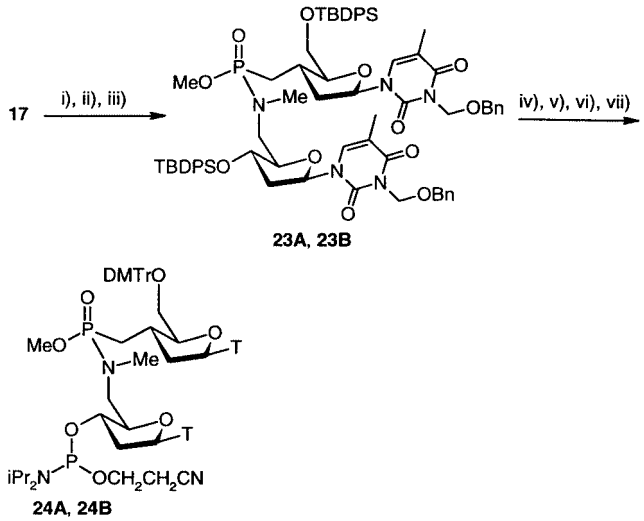

Scheme 4 i) $PhCH_2OCH_2Cl$, DBU, CH_3CN, 0 ºC (83-85%); ii) 100 equiv. MeI, NaH, toluene, 40 ºC, sealed bomb (91-95%); iii) flash column chromatographic separation diastereoisomers, silica gel, eluent; ethyl acetate; iv) 5% Pd on carbon, 1 atmosphere H_2, EtOH, rt (84-90%); v) 3 equiv. $Bu_4N^+F^-$, THF, 0 ºC (89-94%); vi) 1.2 equiv. DMTrCl, pyidine, rt (65-71%); vii) 3 equiv. $(^iPr_2N)_2POCH_2CH_2CN$, 5 equiv. diisopropylammonium tetrazolide, CH_2Cl_2, rt (63-74%).

proach 15mer and 17mer sequences, containing either one or five of the phosphonamidate modified linkages respectively, were synthesised and the melting data obtained with complimentary RNA are shown in the Table.[17]

From the melting data it is apparent that only the longer retained diastereoisomer **20B** of the 3'-*C*-*P*-*N*-5' phosphonamidate modification **2** was found to enhance duplex stability over 'wild-type' DNA. Comparison of the ΔT_m values of the methyl ester **22** with the mean value for the ethyl esters **20A** and **20B**, in the singularly modified 15mer sequence (-1.8 ºC vs. -0.23 ºC), suggests a possible beneficial effect by increasing the size of the ester residue from methyl to ethyl for the modification **2**. In contrast, modification **1**, in which carbon and nitrogen are switched, resulted in a significant reduction in hybridisation energy for both diastereoisomers. Direct comparison of the two modifications is complicated by the presence of different phosphonamidate ester residues. However, this is not anticipated to account for the majority of the difference in duplex stability between the two regioisomeric modifications. Phosphonamidate *N*-methylation with modification **2** resulted in oligonucleotides exhibiting similar affinities to their unsubstituted counterparts, but again direct comparison with individual diastereoisomers is complicated by different ester residues. However, comparing the mean ΔT_m value for *N*-methylated methyl ester diastereoisomers **24A** and **24B** with the corresponding mixed *N*-*H* diastereoisomers **22**, in the singularly modified sequence (-1.8 ºC vs. -1.8 ºC), indicates no substantial difference in affinity.

The incorporation of a less electronegative 3'-substituent in modifications **1** and **2** is anticipated to favour a 3'-*endo* sugar conformation. This *primary* structural bias has formed the basis for rationalising the origin of the enhanced hybridisation properties for a number of such backbone modifications.[18] These enhanced affinities are considered to arise from the above furanose conformational preference promoting the formation of a more stable A-type hybrid duplex. Therefore, the difference between **1** and **2** may be attributed to the facility with which the internucleotide linkage can be incorporated whilst adopting a conformation required for the formation of such an A-type duplex. Comparison of **1** with the duplex stabilising 3'-*N*-*P*-*O*-5' phosphoramidates,[6] and **2** with the duplex destabilising 3'-*C*-*P*-*C*-5' phosphinates,[4] indicates that in both instances the interchange of a 5'-heteroatom bonded to phosphorus for a carbon atom produces a marked decrease in T_m. Inclusion of such a 5'-carbon substituent results in the incorporation of two adjacent methylenes into the backbone linkage. The detrimental effect upon free energy of duplex formation arising from this structural feature most probably has key contributions due to excess conformational flexibility[19] and from perturbation of hydration due to hydrophobicity.[20]

In summary, examples of all four possible isomeric phosphonamidate ester *C*-*P*-*N* replacements for the phosphodiester linkage of DNA have been investigated. From these studies one diastereoisomer of the 3'-*C*-*P*-*N*-5' isomer was identified as enhancing the stability of duplexes with complimentary RNA relative to 'wild-type' DNA.

Table

Modified dimer incorporated	ΔT_m ºC per modification‡ **TTTttCTCTCTCTCT**	ΔT_m ºC per modification‡ **GCGttttttttTGCG**	Average ΔT_m ºC per modification
18A	-4.0	-4.4	-4.2
18B	-3.2	-3.4	-3.3
20A	-1.9	-1.5	-1.7
20B	+1.6	+0.1	+0.9
22	-1.8	not determined	-1.8
24A	-2.7	-3.2	-3.0
24B	+0.9	+0.5	+0.7

T_m for **TTTTTCTCTCTCTCT** 'wild-type' DNA with complimentary RNA 52.7 ºC.
T_m for **GCGTTTTTTTTTTTGCG** 'wild-type' DNA with complimentary RNA 50.2 ºC.
‡tt indicates modified dimer with phosphonamidate linkage.

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The introduction of an *N*-methyl residue into this modification was discovered to have no pronounced effect upon duplex stability. Further investigations with this potentially interesting phosphonamidate modification **20B**, including determination of the phosphorus stereochemistry, are reported in the following letter.[21]

Acknowledgement

We would like to acknowledge and thank Dr. U. Pieles and Dr. F. Natt for oligonucleotide synthesis as well as Dr. S. M. Freier (Isis Pharmaceuticals) for performing hybridisation experiments.

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(13) Typical procedure; Bis(trimethylsilyl)trifluoroacetamide (1.00 ml, 3.76 mmol) was added dropwise to a solution of the *H*-phosphinate **4** (430 mg, 753 μmol) and azide **7** (404 mg, 791 μmol) in anhydrous pyridine (7.5 ml) at 0 ºC. Upon completion of the addition the reaction mixture was allowed to warm room temperature and stirred for 18 h before quenching by the addition of methanol (10 ml). Removal of volatiles followed by further addition of methanol (10 ml) and evaporation yielded a crude mixture of trimethylsilylated phosphonamidate dimers. Cleavage of the trimethylsilyl residues was achieved after refluxing the crude product for 5 h in chloroform (15 ml) and methanol (5 ml). Removal of volatiles followed by flash column chromatography on silica gel, eluting with a gradient from 2% to 10% ethanol in chloroform, yielded the phosphonamidate dimer **16** as a viscous clear colourless oil (686 mg, 87%): ^{31}P NMR ($CDCl_3$, 162 MHz) δ 32.3, 31.6 ppm.
(14) Flash column chromatography was performed using Merck Silica Gel 60 (0.040-0.063mm). First eluting diastereoisomer assigned **A**, second eluting diastereoisomer assigned **B**. NMR spectra were recorded with a Bruker AC400 or Bruker DRX500 instrument. ^{31}P NMR shifts are given as ppm values relative to phosphoric acid.
15A; ^{31}P NMR ($CDCl_3$, 202 MHz) δ 35.44 ppm; ^{1}H ($CDCl_3$, 400 MHz) δ 10.16 (s, 1H), 8.96 (s, 1H), 7.65-7.58 (m, 4H), 7.52 (s, 1H), 7.44-7.18 (m, 15H), 6.94 (s, 1H), 6.81 (d, 4H, *J* = 9 Hz), 6.54 (t, 1H, *J* = 7 Hz), 6.22 (t, 1H, *J* = 7 Hz), 5.78-5.67 (m, 1H), 5.18-5.03 (m, 2H), 4.80-4.69 (m, 1H), 4.40-4.34 (m, 1H), 4.25-4.14 (m, 1H), 4.10-4.05 (m, 1H), 3.96-3.90 (m, 1H), 3.87-3.70 (m, 2H), 3.75 (s, 6H), 3.44 (dd, 1H, *J* = 2 and 11 Hz), 3.24 (dd, 1H, *J* = 2 and 11 Hz), 2.38-2.21 (m, 2H), 2.18-2.10 (m, 1H), 2.02-1.89 (m, 1H), 1.81 (s, 3H), 1.74-1.39 (m, 3H), 1.49 (s, 3H), 1.05 (s, 9H).
15B; ^{31}P NMR ($CDCl_3$, 202 MHz) δ 35.89 ppm; ^{1}H ($CDCl_3$, 400 MHz) δ 10.23 (s, 1H), 8.99 (s, 1H), 7.63-7.57 (m, 4H), 7.47 (s, 1H), 7.44-7.17 (m, 15H), 6.83 (s, 1H), 6.80 (d, 4H, *J* = 9 Hz), 6.49 (t, 1H, *J* = 7 Hz), 6.14 (t, 1H, *J* = 7 Hz), 5.92-5.80 (m, 1H), 5.31-5.14 (m, 2H), 4.80-4.71 (m, 1H), 4.44-4.34 (m, 1H), 4.31-4.22 (m, 1H), 4.07-4.00 (m, 1H), 3.91-3.82 (m, 2H), 3.76 (s, 6H), 3.74-3.66 (s, 1H), 3.38 (d, 1H, *J* = 10 Hz), 3.22 (d, 1H, *J* = 10 Hz), 2.35-2.25 (m, 1H), 2.23-2.10 (m, 2H), 1.99-1.89 (m, 1H), 1.77 (s, 3H), 1.71-1.42 (m, 3H), 1.49 (s, 3H), 1.06 (s, 9H).
19A; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 33.33 ppm; ^{1}H ($CDCl_3$, 400 MHz) δ 7.64 (s, 1H), 7.40 (d, 2H, *J* = 7 Hz), 7.32-7.24 (m, 9H), 7.23-7.16 (m, 1H), 7.09 (s, 1H), 6.84-6.77 (m, 4H), 6.04-5.97 (m, 2H), 4.44-4.37 (m, 1H), 4.10-3.72 (m, 3H), 3.76 (s, 6H), 3.52 (d, 1H, *J* = 12 Hz), 3.30-3.07 (m, 3H), 2.79-2.53 (m, 3H), 2.37-2.19 (m, 3H), 1.92-1.77 (m, 1H), 1.84 (s, 3H), 1.68-1.54 (m, 1H), 1.45 (s, 3H), 1.26-1.18 (m, 3H).
19B; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 33.26 ppm; ^{1}H ($CDCl_3$, 500 MHz) δ 7.56 (s, 1H), 7.35 (d, 2H, *J* = 7 Hz), 7.28-7.08 (m, 11H), 6.75 (dd, 4H, *J* = 1 and 7 Hz), 5.98-5.86 (m, 2H), 4.46-4.30 (m, 1H), 3.99-3.80 (m, 3H), 3.72 (s, 6H), 3.40 (d, 1H, *J* = 12 Hz), 3.27-3.09 (m, 3H), 2.89-2.80 (m, 1H), 2.77-2.66 (m, 1H), 2.61-2.54 (m, 1H), 2.36-2.15 (m, 3H), 1.87-1.75 (m, 4H), 1.63-1.51 (m, 1H), 1.42 (s, 3H), 1.16 (t, 3H, *J* = 7 Hz).
23A; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 34.18 ppm; ^{1}H ($CDCl_3$, 400 MHz) δ 7.86 (s, 1H), 7.68-7.55 (m, 8H), 7.44-7.18 (m, 23H), 6.55-6.49 (m, 1H), 6.16 (t, 1H, *J* = 7Hz), 5.51-5.42 (m, 4H), 4.71-4.64 (m, 4H), 4.07-3.91 (m, 3H), 3.83-3.78 (m, 1H), 3.75-3.70 (m, 1H), 3.39 (d, 3H, *J* = 17 Hz), 3.19-3.09 (m, 1H), 2.68-2.55 (m, 1H), 2.40-2.27 (m, 1H), 2.30 (d, 3H, *J* = 14 Hz), 2.25-2.16 (m, 1H), 2.12-2.02 (m, 1H), 1.89 (s, 3H), 1.76-1.57 (m, 4H), 1.61 (s, 3H), 1.08 (s, 9H), 1.05 (s, 9H).
23B; ^{31}P NMR ($CDCl_3$, 162 MHz) δ 35.50 ppm; ^{1}H ($CDCl_3$, 400 MHz) δ 7.68-7.56 (m, 9H), 7.46-7.17 (m, 23H), 6.52-6.45 (m, 1H), 6.04-5.99 (m, 1H), 5.50-5.39 (m, 4H), 4.72-4.65 (m, 4H), 4.07-3.92 (m, 3H), 3.74-3.63 (m, 2H), 3.40 (d, 3H, *J* = 17 Hz), 3.35-3.24 (m, 1H), 2.69-2.57 (m, 1H), 2.40-2.25 (m, 2H), 2.29 (d, 3H, *J* = 14 Hz), 2.04-1.54 (m, 4H), 1.93 (s, 3H), 1.56 (s, 3H), 1.48-1.37 (m, 1H), 1.07 (s, 9H), 1.05 (s, 9H).

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(16) Oligonucleotides were prepared using an ABI 390 DNA synthesiser following standard phosphoramidite chemistry, according to Gait, M. J. *Oligonucleotide Synthesis: A Practical Approach*, IRL Press: Oxford, 1984. For the steps involving incorporation of modified dimers, double couplings with double reaction times were employed.
(17) The thermal dematuration of DNA/RNA hybrids was performed at 260 nm using a Gifford Response II spectrophotometer (Ciba-Corning Diagnostics Corp., Oberlin, OH) absorbance vs temperature profiles were measured at 4μm of each strand in 10mM phosphate pH 7.0 (Na salts), 100mM total (Na^+) 0.1mM EDTA. T_m's were obtained from fits of absorbance vs temperature curves to a two-state model with linear slope baselines (Freier, S. M.; Albergo, T. D.; Turner, D. H. *Biopolymers* **1982**, *22*, 1107.). All values are averages of at least three experiments. The absolute error of the T_m values is ±0.5 °C.
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Article Identifier:
1437-2096,E;2001,0,04,0467,0472,ftx,en;L21700ST.pdf

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