Nucleic Acid Containing 3′-*C*-*P*-*N*-5′ Ethyl Phosphonamidate Ester and 2′-Methoxy Modifications in Combination; Synthesis and Hybridisation Properties

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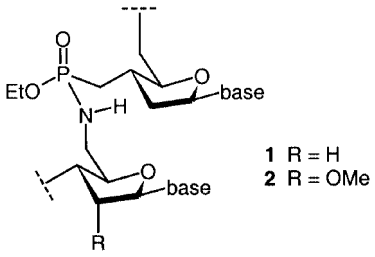
Abstract: The preparation of thymidine-thymidine and thymidine-5-methylcytidine dinucleosides containing a 3′-*C*-*P*-*N*-5′ ethyl phosphonamidate ester linkage, with defined phosphorus stereochemistry, in combination with a 2′-methoxy substituent in the lower sugar residue, is described. Incorporation of these dinucleosides into DNA oligonucleotides and the effect upon duplex stability with complimentary RNA is reported.

Key words: antisense, modified oligonucleotides, nucleosides, phosphorus, stereoselective

Significant effort continues to be expended on the identification of modified oligonucleotides for antisense applications.[2] The primary focus being upon: enhancing nuclease resistance, increasing the affinity for complementary RNA and improving cellular uptake by reducing the overall charge. Changes to all of the nucleic acid structural elements, base, sugar and phosphate linkage, have been investigated individually, and in a smaller number of instances, in combination.[3] Through the appropriate amalgamation of stabilising modifications, oligonucleotides have been produced with impressive affinities for their RNA targets. Such modifications are particularly effective for the flanking sequences of gapmer oligonucleotides, where they complement a central unmodified, or phosphorothioate, RNase H supporting region.[4] In these applications the flanking modifications are primarily responsible for the overall stability and affinity of the antisense oligonucleotide. Previously we have described DNA incorporating thymidine-thymidine (T-T) dimers modified with the neutral 3′-*C*-*P*-*N*-5′ (R_p)-ethyl phosphonamidate ester linkage **1**, which stabilise hybrid duplexes to the extent of ΔT_m +0.9 °C per modification.[5,6] The enhanced hybridisation properties are rationalised as arising from a 3′-endo conformational preference in the upper sugar of the modified dinucleoside promoting the formation of a more stable A-type hybrid duplex. To enhance the affinity of oligonucleotide sequences containing dinucleosides with this linkage further, the incorporation of a complementary 3′-endo conformational bias in the lower sugar residue would be anticipated to reinforce the preference for an A-type duplex. As a way to achieve this the incorporation of a 2′-electronegative substituent has been well described as an effective route to the desired ribose conformation.[7] In particular, 2′-methoxy substituents have been demonstrated to be well suited to this role, both individually,[8] and as rationalised above, in combination with both a 4′-*C*-aminomethyl sugar modification and a carboxamide backbone linkage.[9] In this letter we present our findings for combination of the 3′-*C*-*P*-*N*-5′ ethyl phosphonamidate ester linkage and 2′-methoxy modifications, and extend the scope of investigation beyond linking T-T residues (Figure).



Figure

To evaluate the outcome of combining the two modifications fully our initial approach was to prepare both diastereoisomeric 3′-*C*-*P*-*N*-5′ ethyl phosphonamidate ester T-T dinucleosides substituted with a 2′-methoxy moiety in the lower sugar ring **2**. A sequence involving a Staudinger reaction, between nucleosides bearing *H*-phosphinate and azide functionality, in the key dinucleoside forming step was selected, and is shown in Scheme 1. To initiate the sequence, the previously described 3′-homologated *H*-phosphinate **3** was employed as a 1:1 mixture of diastereo-isomers epimeric at phosphorus.[5] The remaining coupling partner 5′-azido-5′-deoxy-2′-methoxythymidine **4** was prepared from 2′-methoxythymidine following reported procedures.[5,10] Thus, activation of the *H*-phosphinate **3** with bis(trimethylsilyl)trifluoroacetamide (BSTFA) facilitated reaction with azide **4** to produce the dinucleoside diastereoisomers **5** and **6** in good yield, after removal of trimethylsilyl residues. At this stage separation via flash column chromatography allowed the first eluting (S_p)-diastereoisomer **5** and second eluting (R_p)-diastereoisomer **6** to be isolated, vide infra.[11] These individual isomers were separately desilylated upon treatment with tetrabutylammonium fluoride in THF to give the corresponding diols. Subsequent selective 5′-

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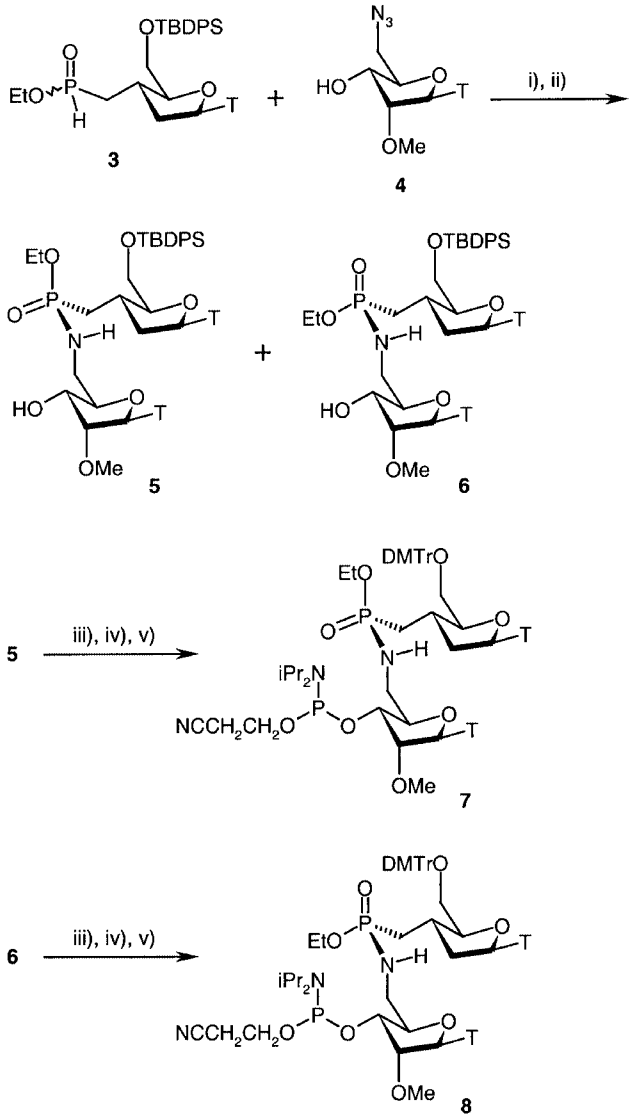
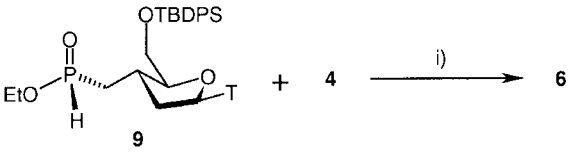
tritylation followed by phosphitylation of the 3′-alcohol under standard conditions, yielded the phosphoramidites **7** and **8** ready for oligonucleotide synthesis.



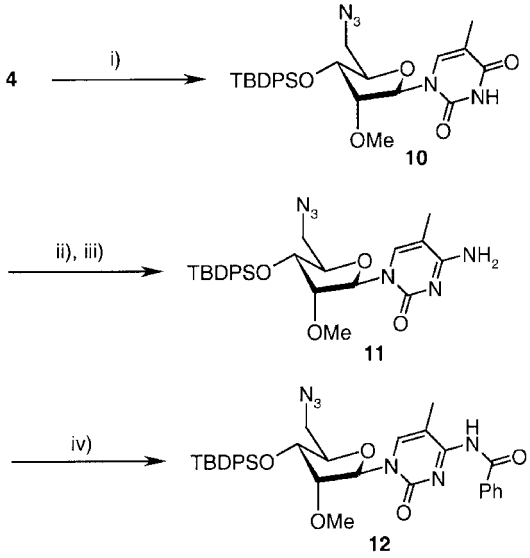
Scheme 1 i) 6 equiv $Me_3SiNC(OSiMe_3)CF_3$, pyridine, 0 ºC to r.t., then $CHCl_3$, CH_3OH, 4 h, reflux (77%); ii) flash column chromatographic separation of diastereoisomers, silica gel, eluant; 10% ethanol in chloroform; iii) 1.3 equiv $Bu_4N^+F^-$, THF, 0 ºC (67–80%); iv) 1.4 equiv DMTr-Cl, pyridine, r.t. (62–87%); v) 3 equiv (*i*-$Pr_2N)_2POCH_2CH_2CN$, 5 equiv diisopropylammonium tetrazolide, CH_2Cl_2, r.t. (60–70%).

Assignment of the phosphorus stereochemistry in the separated dimers **5** and **6**, was made following coupling of **9**, the (S_P)-*H*-phosphinate diastereoisomer of **3**, with the azide **4**, as shown in Scheme 2. Reaction under the BSTFA promoted conditions employed for the preparation of **5** and **6** have previously been shown to proceed with retention of phosphorus configuration.[6] Hence, the product derived from **9** is the (R_P)-phosphonamidate, which could be retrospectively correlated with the second eluting isomer **6**, obtained after separation in the sequence shown above in Scheme 1.



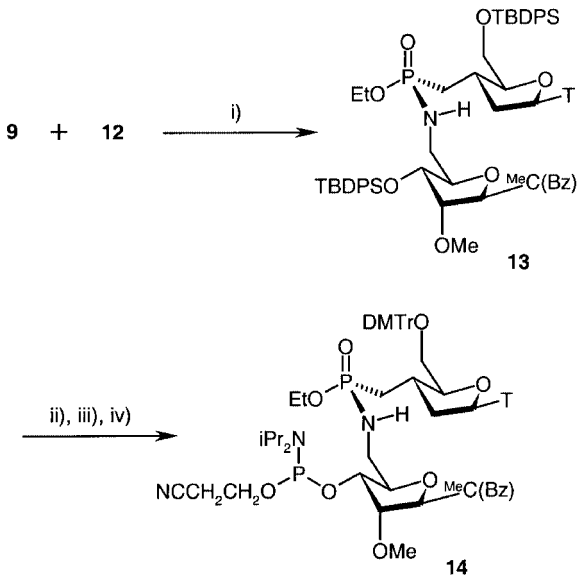
Scheme 2 i) 6 equiv $Me_3SiNC(OSiMe_3)CF_3$, pyridine, 0 ºC to r.t., then $CHCl_3$, CH_3OH, 4 h, reflux (73%).

To explore the potential utility of the 3′-*C*-*P*-*N*-5′ (R_P)-ethyl phosphonamidate ester modification further for antisense applications, a study into the stabilising effect upon sequences in which it is located beyond the linking of adjacent thymidines is required. One well studied oligonucleotide sequence incorporates seven consecutively linked dinucleosides, including modifications between thymidine and cytidine residues.[3a] To investigate this possibility a 5′-azido-5′-deoxy-2′-methoxy-5-methylcytidine derivative was prepared from the thymidine derived azide **4**, as shown in Scheme 3. Silylation of **4** yielded the 3′-protected alcohol **10**, in a suitable form for subsequent manipulation of the 4-keto group of the pyrimidine moiety. This was achieved utilising a standard sequence, of activation as the 4-(1,2,4-triazoyl) intermediate followed by displacement with ammonia to produce the 5-methyl cytidine derivative **11**.[12] Protection of **11** with a slight excess of benzoyl chloride delivered **12**, the required precursor for the Staudinger coupling reaction.



Scheme 3 i) 1.1 equiv TBDPS-Cl, imidazole, DMF, r.t. (>95%); ii) 10 equiv 1,2,4-triazole, 5 equiv $POCl_3$, pyridine, 50 °C (>95%); iii) 0.88 equiv NH_3, 1,4-dioxan, r.t. (>95%); iv) 1.2 equiv PhCOCl, 3 equiv Et_3N, Et_2O, r.t. (92%).

Azide **12** underwent a BSTFA mediated coupling with the (S_P)-*H*-phosphinate **9** to yield the anticipated dinucleoside **13** with >95% retention of stereochemical integrity at the phosphorus centre, as shown in Scheme 4.[13] Repeating the same sequence of alcohol group manipulation, as employed in the preparation of **7** and **8**, involving desilyla-



Scheme 4 i) 5 equiv $Me_3SiNC(OSiMe_3)CF_3$, pyridine, 0 ºC to r.t., then $CHCl_3$, CH_3OH, 4 h, reflux (89%); ii) 3.0 equiv $Bu_4N^+F^-$, THF, 0 ºC (74%); iii) 1.3 equiv DMTr-Cl, pyridine, r.t. (87%); iv) 3 equiv $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, 5 equiv diisopropylammonium tetrazolide, CH_2Cl_2, r.t. (77%).

tion, selective 5′-tritylation followed by 3′-phosphitylation, gave the thymidine-5-methylcytidine (T-^{Me}C) phosphoramidite **14** ready for oligonucleotide synthesis.

Oligonucleotide sequences were prepared incorporating the modified dinucleosides **7**, **8** and **14** utilising standard phosphoramidite coupling protocols.[14] Following this strategy a 17mer and a 15mer sequence, containing five and one or seven modified linkages respectively, were synthesised and the melting data with complimentary RNA determined.[15] The results are shown in the Table and compared with data for the singularly modified 3′-*C*-*P*-*N*-5′ ethyl phosphonamidate ester linkage **1**.[5]

Comparison of the melting data indicates that incorporation of the 2′-methoxy-substituent into the lower sugar residue to give **2** produced the anticipated increase in hybrid duplex stability for both diastereoisomeric forms of the phosphonamidate linkage, when compared with **1**. The S_P increasing by 2.6 °C per modification and the R_P increasing by 1.4 °C per modification, in comparative T-T containing sequences. This increase for the (R_P)-diastereoisomer being similar to the incorporation of a single 2′-methoxy-modification into the above 15mer DNA sequence, of ΔT_m +1.2 °C.[16] The reason for the relatively large increase in ΔT_m for the (S_P)-ethyl phosphonamidate linkage is unclear. However, the anticipated preference for the (R_P)-diastereoisomer to provide the most stable modification is conserved, and is rationalised as arising from a more favourable orientation of the ethyl residue away from the backbone axis in the hybrid duplex.[17]

Incorporation of seven T-T and T-^{Me}C dimers to give the 15mer sequence with alternating (R_P)-phosphonamidate/phosphodiester linkages produced a ΔT_m value of +3.1 °C per modification. Correcting this value for the established stabilising effect for the introduction of a 5-methyl residue into cytidine, of +0.5 °C per modification,[18] normalises to a slightly greater ΔT_m value when compared to that observed with the exclusively T-T containing sequences, of +2.7 °C per modification. Hence, extending the scope of the (R_P)-phosphonamidate linkage to T-^{Me}C containing sequences resulted in no loss of stabilisation, and suggests that the modification may be suitable for general application. Additionally, the presence of consecutively modified (R_P)-phosphonamidate dinucleosides produced the sequence with the highest ΔT_m value. Indicating no loss of the desired stabilising properties upon incorporation into a highly modified oligonucleotide, again suggesting the possibility for application across a broad range of sequences.

In summary, T-T and T-^{Me}C dinucleosides combining both diastereoisomeric forms of the ethyl phosphonamidate linkage and a 2′-methoxy substituent in the lower sugar residue have been synthesised and incorporated into DNA oligonucleotides. Hybridisation data with complementary RNA has validated the proposed combination of these structural elements to produce a highly stabilising modification, and in the case of the (R_P)-diastereoisomer, displaying many attributes required for antisense applications.

Table Melting data for DNA oligonucleotides containing modifications **1** and **2** with complementary RNA

Modified dimer incorporated	ΔT_m (ºC) per modification[a] **TTTttCTCTCTCTCT**[c]	ΔT_m (ºC) per modification[a] **GCGtttttttttTGCG**[d]	ΔT_m (ºC) per modification[a] **tttttctctctctT**	Average ΔT_m (ºC) per modification[b]
(S_P)-diastereoisomer **1**	–1.9	–1.5	–	–1.7
(R_P)-diastereoisomer **1**	+1.6	+0.1	–	+0.9
(S_P)-diastereoisomer **2**	+0.8	+0.9	–	+0.9
(R_P)-diastereoisomer **2**	+2.5	+2.0	+3.1	+2.3

[a] Lower case indicates modified dimer with phosphonamidate linkage.
[b] Average for sequences containing exclusively T-T modified dimers.
[c] T_m for **TTTTTCTCTCTCTCT** 'wild-type' DNA with complementary RNA 52.7 °C.
[d] T_m for **GCGTTTTTTTTTTTGCG** 'wild-type' DNA with complementary RNA 50.2 °C.

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(11) Reaction conditions were as described in ref.[5] Flash column chromatography was performed using Merck Silica Gel 60 (0.040–0.063 mm). NMR spectra were recorded with a Brucker AC400 instrument. Key distinguishing ^{1}H resonances for each diastereoisomer are assigned. ^{31}P NMR shifts are given as ppm values relative to phosphoric acid. Mass spectroscopy was carried out using a Fisons Instruments VG Platform II spectrometer. A reaction carried out on a 3.11 mmol scale gave;
5: White amorphous foam; 1.07 g; ^{31}P NMR ($CDCl_3$, 162 MHz): δ = 32.52 ppm; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 9.67 (br s, 1 H), 9.52 (s, br, 1 H), 7.69–7.61 (m, 4 H), 7.45–7.34 (m, 7 H), 7.13 (s, 1 H), 6.10–6.02 (m, 1 H, H1′ upper sugar), 5.50 (d, *J* = 2 Hz, 1 H, H1′ lower sugar), 4.26–3.64 (m, 8 H), 3.50 (s, 3 H), 3.36–3.17 (m, 3 H), 2.74–2.61 (m, 1 H), 2.51–2.42 (m, 1 H), 2.32–2.20 (m, 1 H), 2.04–1.82 (m, 2 H), 1.84 (s, 3 H), 1.74–1.59 (m, 1 H), 1.58 (s, 3 H), 1.27 (t, *J* = 7 Hz, 3 H), 1.04 (s, 9 H). MS (ES+): *m/z* (%) = 840(27) [M + H], 862(100) [M + Na].
6: White amorphous foam; 0.94 g; ^{31}P NMR ($CDCl_3$, 162 MHz): δ = 32.81 ppm; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 9.38 (br s, 1 H), 9.17 (s, br, 1 H), 7.70–7.62 (m, 4 H), 7.44–7.34 (m, 7 H), 7.13 (s, 1 H), 6.14–6.06 (m, 1 H, H1′ upper sugar), 5.55 (d, *J* = 2 Hz, 1 H, H1′ lower sugar), 4.24–3.64 (m, 8 H), 3.51 (s, 3 H), 3.50–3.18 (m, 3 H), 2.80–2.68 (m, 1 H), 2.50–2.39 (m, 1 H), 2.34–2.23 (m, 1 H), 2.01–1.84 (m, 1 H), 1.84 (s, 3 H), 1.77–1.58 (m, 2 H), 1.61 (s, 3 H), 1.20–1.10 (m, 3 H), 1.04 (s, 9 H). MS (ES+): *m/z* (%) = 840(8) [M + H], 862(100) [M + Na].

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(13) Reaction conditions were as described in ref.[5] A reaction carried out on a 1.46 mmol scale gave;
13: White amorphous foam; 1.54 g; ^{31}P NMR ($CDCl_3$, 162 MHz): δ = 32.70 ppm; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.62 (s, 1 H), 8.15 (d, 2 H, *J* = 7 Hz), 7.58–7.44 (m, 8 H), 7.35–7.31 (m, 1 H), 7.30–7.17 (m, 16 H), 7.08 (s, 1 H), 5.96–5.90 (m, 1 H, H1′ upper sugar), 5.42 (d, 1 H, *J* = 2 Hz, H1′ lower sugar), 3.97–3.89 (m, 1 H), 3.86–3.77 (m, 2 H), 3.69–3.53 (m, 4 H), 3.10 (s, 3 H), 2.98–2.81 (m, 2 H), 2.76–2.63 (m, 1 H), 2.59–2.48 (m, 1 H), 2.29–2.20 (m, 1 H), 2.03–1.94 (m, 1 H), 1.91 (s, 3 H), 1.76–1.60 (m, 1 H), 1.44 (s, 3 H), 1.38–1.14 (m, 1 H), 1.07–0.99 (m, 3 H), 0.94 (s, 9 H), 0.91 (s, 9 H). Minor (S_P)-diastereoisomer; ^{31}P NMR ($CDCl_3$, 162 MHz): δ = 31.71 ppm; ^{1}H NMR ($CDCl_3$, 400 MHz): key distinguishing resonances δ = 5.90–5.83 (m, 1 H, H1′ upper sugar), 5.29 (d, 1 H, *J* = 2 Hz, H1′ lower sugar).

(14) (a) 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**. (b) For the steps involving incorporation of modified dimers, double couplings with double reaction times were employed.

(15) (a) The thermal denaturation 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 4 μm of each strand in 10 mM phosphate pH 7.0 (Na salts), 100 mM total (Na^+) 0.1 mM 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. (b) 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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