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### An Efficient Oxidizing Reagent for the Synthesis of Mixed Backbone Oligonucleotides via the H-Phosphonate Approach

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Abstract—The mixture of carbon tetrachloride, *N*-methyl morpholine (NMM), pyridine and water in acetonitrile has been exploited for the oxidation of dinucleoside H-phosphonate diesters to the corresponding phosphates. The system is found to be inert to the phosphoramidate (P-N) and the phosphorothioate (P-S) linkages and has successfully been applied to the solid phase synthesis of mixed-backbone oligonucleotides (MBOs).

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### Introduction

The synthesis of oligonucleotides by the phosphoramidite<sup>1</sup> and the H-phosphonate<sup>2</sup> methodologies has acclerated the development of oligonucleotides as potential therapeutics and diagnostic agents and for the appplications in the field of molecular biology. A significant potential for success to control the natural cellular functions, is the use of the synthetic oligonucleotides with modified backbones that are complementary to the gene or mRNA responsible for those functions. Some of the important examples of the phosphate modifications being investigated are the phoshorothioates, phosphoramidates and phosphotiesters. These modifications in the oligonucleotide can lead to enhanced cell membrane permeability, increased resistance to exo and/or endonuclease activity and increased stability of oligonucleotide/targeted sequence duplex. The nucleoside H-phosphonates provide a convenient route to these types of modifications with the advantage that a common intermediate would be required to obtain any of the various modifications.

The synthetic cycle for the H-phosphonate chemistry is relatively simple as compared to the phosphoramidite approach since the oxidation step is carried out at the end of the synthesis, before the final detritylation. The efficiency of the oxidation is therefore a highly critical issue. The oxidation procedure with the 0.1 M  $I_2$  in Py/  $H_2O/THF$  (5/5/90) provides inconsistent oxidation of the internucleotide H-phosphonate linkages.<sup>3</sup> Results have been improved by using a two step oxidation, beginning with iodine solution containing a weak base like N-methyl imidazole followed by a solution containing a strong base like triethyl amine.<sup>3</sup> Additionally, iodine solution containing water looses the ability to oxidize support bound oligo H-phosphonate after a couple of days. Hence mixing the fresh iodine solution prior to oxidation is advised.<sup>3</sup> An alternative method for oxidation is a solution of 2% iodine in Py/H<sub>2</sub>O (98:2,v/v).<sup>4</sup> However this oxidation strategy by aqueous iodine in pyridine is problematic because the aqueous condition brings about partial hydrolysis of the internucleotidic H-phosphonate linkage.<sup>3</sup> de Vroom et al.<sup>5</sup> has developed an alternative method of oxidation using tert-butyl hydroperoxide (TBHP) in the presence of N,O-bis(trimethylsilyl) acetamide (BSA). In this case BSA, not only activates the nucleoside H-phosphonate but also acts as a scavenger of all traces of water in the oxidation reaction. However the excess use of BSA. converts TBHP to the less reactive trimethylsilyl tertbutyl hydroperoxide, and consequently causes retarding of the oxidation reaction.

As the oligonucleotide-based applications continue to evolve, synthetic methodologies need to be refined to meet the needs. The use of phosphorothioate oligodeoxynucleotides (PS-oligos), has resulted in the first antisense drug Fomivirsen<sup>6</sup> (vitravene). However, these PS-oligos are not optimal antisense molecules and their

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drawbacks have been revealed in the development process of the drugs.<sup>7</sup> Therefore at the current stage of technology, the mixed backbone oligonucleotides (MBOs) are considered as the best choice as the second generation antisense compounds.<sup>8</sup>

As part of our interest in the field of oligonucleotide synthesis<sup>9</sup> we have optimized  $CC1_4/H_2O/NMM/Py/CH_3CN^{10}$  oxidizing mixture to the H-phosphonate chemistry and in turn to the synthesis of MBOs containing the phosphorothioate and phosphoramidate linkages. The system is efficient and fast in converting nucleoside H-phosphonate diesters to the corresponding

phosphates both in the solution phase and the solid phase synthesis of oligonucleotides.

### **Results and Discussion**

In order to explore the role of CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/ CH<sub>3</sub>CN as an oxidizing reagent in the H-phosphonate chemistry, preliminary experiments were carried out in the solution phase. The unoxidized dithymidyl H-phosphonate diester 1 (0.01 mmol) ( $\delta$  = 12.1 ppm) was subjected to oxidation with CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/1.0/ 1.0/6.0/1.0, v/v) (Scheme 1). The addition of CCl<sub>4</sub>/H<sub>2</sub>O/



Figure 1. <sup>31</sup>P NMR spectra of the dithymidyl phosphate, 2 synthesized by using: (a) CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/1.0/1.0/6.0/1.0, v/v), (b) with 0.035 M I<sub>2</sub> in Py/THF/H<sub>2</sub>O (1/300/33, v/v), (c) 0.2 M iodine in Py/H<sub>2</sub>O (98:2, v/v).

NMM/Py/CH<sub>3</sub>CN to the unoxidized dithymidyl H-phosphonate diester resulted in almost immediate (<5 min) and quantitative conversion to dithymidyl phosphate 2 ( $\delta$ =2.9 ppm), with no detectable side products as judged by <sup>31</sup>P NMR spectroscopy (Fig. 1(a)). A similar experiment was performed with 0.035 M I<sub>2</sub> in Py/THF/H<sub>2</sub>O (1/300/33, v/v). The oxidation reaction in this case did not lead to complete formation of 2. Two products were formed, one of them was the phosphate

(3.0 ppm), and the other a side product ( $\delta = -9.8$  ppm) (Fig. 1(b)).

However, when 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2,v/v) was added, the oxidation was immediate (<5 min) and quantitative, with no detectable side products (Fig. 1C). These results indicate that the same combination of 0.035 M I<sub>2</sub> in Py/THF/H<sub>2</sub>O (1/300/33, v/v) which is conventionally used in the phosphoramidite approach,



Figure 2. RP-HPLC ( $^{31}P$  NMR is shown in the inset) of d(AA), 3 synthesized by: (a)  $I_2$  in  $Py//H_2O$  method (positive control), (b) no oxidation method (negative control), (c)  $CCl_4/H_2O/NMM/Py/CH_3CN$  method.

cannot be used as an oxidant in the H-phosphonate chemistry which demands 0.2 M iodine in  $Py/H_2O$  (98:2, v/v). In contrast, the CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/ 1.0/1.0/6.0/1.0, v/v) mixture can be used as a versatile oxidizing reagent for the solution phase oxidation of nucleoside phosphite triester to the corresponding phosphotriester via the phosphoramidite approach<sup>10</sup> as well as for the conversion of the nucleoside H-phosphonate diesters to corresponding phosphodiesters via the H-phosphonate approach.

In order to check the suitability of the developed  $CCl_4/H_2O/NMM/Py/CH_3CN$  mixture on solid phase, the dimer d(AA) (3) was synthesized by coupling of  $dA^{bz}$  H-phosphonate with  $dA^{bz}$  lcaa CPG 500 Å using the

standard protocols for the H-phosphonate chemistry. The oxidation was effected by the CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/ CH<sub>3</sub>CN (2.5/0.2/1.0/6.0/9.3, v/v) system for 45 min.<sup>11</sup> In order to check the oxidizing efficiency of the system, control experiments were performed. One control experiment (positive control) involved the oxidation of the unoxidized d(A<sub>pH</sub>A) dimer supported on lcaa CPG-500 Å with the conventionally used 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) for 30 min. In the second control experiment (negative control), the d(Ap<sub>H</sub>A) dimer supported on lcaa CPG-500 Å was not oxidized at all. After performing the standard deprotection under basic conditions, the crude deprotection mixtures of the three experiments were analyzed by RP-HPLC and <sup>31</sup>P NMR spectroscopy and were compared with each other. The



Figure 3. RP-HPLC profile (ESI-MS shown in the inset) of d(GTTAAGACTTTTAC), 4 synthesized by CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN method. ESI-MS of 4 *MW*<sub>found</sub>: 4234.8 *MW*<sub>calcd</sub>: 4237.



Figure 4. Solution phase stability of d(TpsT), 5 in: (a) CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN system and (b) I<sub>2</sub> in Py/H<sub>2</sub>O system.



 $\label{eq:Figure 5. Solution phase stability of $d(T_{pN}T)$ 6, in: (a) CCl_4/H_2O/NMM/Py/CH_3CN system and (b) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (b) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (c) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (c) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (c) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (c) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (c) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (c) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (c) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 6, in: (c) CCl_4/H_2O/NMM/Py/CH_3CN system and (c) I_2 in Py/H_2O system. Solution phase stability of $d(T_{pN}T)$ 7, in Py/H_2O system and $d(T_{pN}T$ 

RP-HPLC analysis of the 3 synthesized by  $I_2$  in Py/H<sub>2</sub>O showed the oxidized dimer 3, as the major peak at retention time 7.76 min, its <sup>31</sup>P NMR showed signal at  $\delta = 0.93$  ppm arising due to the phosphodiester linkages (Fig. 2A). The negative control showed two peaks one at retention time 5.95 min (adenosine) and the other at retention time 6.48 min arising due to the formation of H-phosphonate, with <3% formation of 3 (retention time 7.79 min), while its <sup>31</sup>P NMR showed two signals at  $\delta = 6.9$  and 8.3 ppm (Fig. 2B). The RP-HPLC analysis of the crude deprotection mixture by  $CCl_4/H_2O/$ NMM/Py/CH<sub>3</sub>CN method, showed the major peak at retention time 7.76 min (expected product 3), with no evidence for the formation of the H-phosphonate arising due to the cleavage of the unreacted H-phosphonate diester linkages under basic conditions used to release the oligonucleotide from the solid support (Fig. 2(c)). Also, <sup>31</sup>P NMR of its crude deprotection mixture indicated only one signal at  $\delta = 0.9$  ppm (shown in the inset of Fig. 2C). These experiments clearly indicate that the conversion of oligonucleotide H-phosphonate diesters to the corresponding phosphates results in quantitative conversion in case of the optimized CCl<sub>4</sub>/H<sub>2</sub>O/NMM/  $Py/CH_3CN$  system. In case of  $I_2$  in  $Py/H_2O$  system ca. 2% formation of H-phosphonate (retention time 6.4 min) was detected. It has been reported that the oxidation of H-phosphonate with the  $I_2$  in Py/H<sub>2</sub>O system is slow and it is preferable to do it in an automated mode using a three reagent combination in a DNA synthesizer.12

The oxidizing system has successfully been applied to the synthesis of 14 mer oligo d(GTTAAGACTTTTAC) (4). The RP-HPLC profile of the crude deprotection mixture of **4** is shown in Figure 3. All the fractions were characterized simultaneously by ESI-MS analysis (ESI-MS of the major fraction, **4** is shown in the inset of Fig. 3).

In order to assess the chemical stability of the  $CCl_4/H_2O/NMM/Py/CH_3CN$  system towards the phosphorothioate

linkages in the backbone, the dimer d(TpsT) (5) (1.0  $A_{260}$  units) ( $\delta = 57.0$  and 57.4 ppm) was stirred for 16 h with  $CCl_4/H_2O/NMM/Py/CH_3CN$  (2.5/1.0/1.0/6.0/1.0, v/v) mixture at ambient temperature. A similar experiment was carried out under identical scale, conditions and protocols using 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v). In order to judge the potential occurrence of desulfurization by these oxidizing reagents, the <sup>31</sup>P NMR analysis of the reaction mixtures were carried out. The <sup>31</sup>P NMR of the crude reaction mixture using  $CCl_4/H_2O/$ NMM/Py/CH<sub>3</sub>CN reagent (Fig. 4(a)) indicated ca. 6% desulfurization  $\delta = 0.9$  ppm). However, the crude reaction mixture using  $I_2$  in Py/H<sub>2</sub>O (98:2, v/v) method indicated complete desufurization of 5, to give d(TpoT) as the only detectable product ( $\delta = 0.96$  ppm) (Fig. 4(b)) as judged by the <sup>31</sup>P NMR analysis. The desulfurization process by  $I_2$  in Py/H<sub>2</sub>O has been reported previously in literature.13

Similarly in order to check the compatibility of the CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/C<sub>3</sub>CN system towards the phosphoramidate linkages in the backbone, the dimer  $d(T_{pN}T)(6)$  (1.0 A<sub>260</sub> units) = 12.08 and 12.00 ppm) was stirred individually for 16 h with the CCl<sub>4</sub>/H<sub>2</sub>O/NMM/ Py/CH<sub>3</sub>CN (2.5/1.0/1.0/6.0/1.0, v/v) and 0.2 M I<sub>2</sub> in  $Py/H_2O$ ) (98:2, v/v) oxidizing reagents, as described previously. The <sup>31</sup>P NMR analysis of the crude reaction mixtures were then carried out. The dimer 6 stirred with the CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN did not show any cleavage of the phosphoramidate linkages as judged by the phosphorus NMR (Fig. 5(a)). However, the phosphoramidate linkages were completely cleaved by  $I_2$  in  $Py/H_2O$  reagent to give the phosphodiester signal at  $\delta = 0.97$  ppm which was the only detectable product (Fig. 5(b)). These solution phase analysis clearly indicate that the CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN oxidation method is far superior than the conventionally used  $I_2$ in  $Py/H_2O$  in retaining the phosphorothioate and the phosphoramidate linkages in the oligonucleotide backbone.



 $\label{eq:Figure 6. RP-HPLC profile of d(TpoTpsT), \ensuremath{\textit{7}}\xspace{1.5}\xspace{$ 



 $\label{eq:Figure 7. RP-HPLC profile of d(TpoT_{PN}T), \mbox{7 synthesized by: (a) } CCl_4/H_2O/NMM/Py/CH_3CN \mbox{ method and (b) } I_2 \mbox{ in } Py//H_2O \mbox{ method.}$ 





Scheme 1. Solution phase oxidation of dithymidyl H-phosphonate diester 1 with CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN system.

Encouraged by these positive results on the dimers 5 and 6 we decided to investigate the use of the  $CCl_4/$ H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN method to the synthesis of trimers d(TpoTpsT) (7) and d(Tpo<sub>PN</sub>T) (8) [where PN corresponds to P-NH-(CH<sub>2</sub>)<sub>3</sub>N(CH<sub>3</sub>)<sub>2</sub> linkages] on solid phase using the standard protocols for the H-phosphonate chemistry (Scheme 2). After the standard deprotection step, the crude deprotection mixtures were analysed by RP-HPLC. The RP-HPLC of 7 synthesized by CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/0.2/1.0/6.0/9.3, v/v) method showed ca. 2% desulfurization (retention time 9.11 min), while the expected product 7 was obtained as the major peak at retention time 9.8 and 10.35 min (two peaks are due to the diasteriomers resulting from the chiral phosphorus centre) (Fig. 6(a)). The RP-HPLC of 7 synthesised by  $0.2 \text{ M I}_2$  in Py/H<sub>2</sub>O (98:2, v/v) method however resulted in nearly complete loss of sulfur to give the desulfurized trimer, d(TpoTpoT) as the major peak at retention time 9.15 min. The expected product 7 obtained was ca. 4% (Fig. 6(b)).

Similarly the trimer **8** was synthesized by using CCl<sub>4</sub>/ $H_2O/NMM/Py/CH_3CN$  (2.5/0.2/1.0/6.0/9.3, v/v) and 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) method on solid phase. The RP-HPLC analysis of the crude deprotection mixtures were then carried out. The CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN method, although was stable towards the phosphoramidate linkages in the solution phase, in the solid phase cleaved ca. 2% of the phosphoramidate linkages (retention time 9.0 min), while **8** was obtained as major peak (retention time 10.2 and 10.4 min) as the mixture of diasteriomers (Fig. 7(a)). As one could expect from the solution phase experiments, **8** surprisingly did not undergo extensive loss of the phosphoramidate linkage on solid phase by I<sub>2</sub> in Py/H<sub>2</sub>O method.





The trimer 8 sufferd ca. 7% loss of the phosphoramidate linkages, while the major fraction was eluted at retention time 10.2 and 10.4 min (Fig. 7(b)).

Presently, a number of 2'-modifications that offer the dual advantage of high nuclease resistance and enhanced binding affinity have been reported.14 The 2'-O-(2-methoxyethyl) (2'-O-MOE),<sup>15</sup> with the phosphodiester (PO) backbone provides sufficient nuclease resistance, at approximately the same level as the 2'-O-deoxyphosphorothioate modification. A typical approach entails the use of the 2'-O-alkylated phosphodiester to replace part of the PS linkers in the antisense compounds, leading to a MBO with reduced phosphorothioate content. The preliminary experiments have shown that these MBOs exhibit more acceptable safety profiles than those of the PS oligos.<sup>16</sup> As a consequence of which, the antisense oligos containing PS and PO linkages in the backbone are presently undergoing human clinical trials.<sup>17</sup> Taking into account the superior attributes of the developed CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/ CH<sub>3</sub>CN (2.5/0.2/1.0/6.0/9.3, v/v) oxidation method and the importance of the MBOs containing both phosphorothioate and phosphodiester linkages, we have extended our developed methodology to the synthesis of d(TpoTpsTpsTpsTpsTpsTpsTpsT) (9). The expected product was obtained at retention time 31.0 min (Fig. 8), which was simultanesouly characterized by ESI-MS (shown in the inset of Fig. 8). The LC-MS profile of 9 indicated that the overall desulfurization was less than 4% (desulfurized 8 mer oligo retention time 30.35 min, containing 2 phosphodiester and 5 phosporothioate linkages in the backbone).

#### Conclusion

In the present study we have developed  $CCl_4/H_2O/NMM/Py/CH_3CN$  mixture as a mild and efficient oxidizing reagent, that can quantitatively convert nucleoside H-phosphonate diesters to the corresponding phosphates both in the solution phase as well as in the solid phase synthesis of oligonucleotides. The data presented suggests that this oxidation method can serve as an advantageous replacement for the conventionally used aqueous iodine in pyridine oxidation method, particularly in the synthesis of Mixed Backbone Oligonucleotides.

#### Experimental

### Materials

All the nucleoside H-phosphonates, Icaa CPG-500 Å were purchased from Glen Res. (Virginia). Acetonitrile (HPLC grade, Spectrochem India Ltd.) was distilled and stored over activated 3 Å molecular sieves (Aldrich Chemical Co.). Pyridine (E. Merck, India) was dried by refulxing over KOH and was stored over activated 3 Å molecular sieves. Pivaloyl chloride (AR grade, Spectrochem India Ltd.) was used as received. The solid phase oligonucleotide syntheses were performed manually in a 2.5 mL Hamilton gas tight syringe with a glass wool plug at the inlet.<sup>18</sup>

#### Methods

<sup>31</sup>P NMR analysis: the <sup>31</sup>P NMR was performed on a Bruker AMX 500 instrument. The experiments were carried out in an NMR sample tube (5 mm×180 mm) at 25 °C with proton decoupling at 202 Mz for <sup>31</sup>P NMR using 85% H<sub>3</sub>PO<sub>4</sub> as external reference. In each measurement, data accumulation was carried out 64 times with a pulse width of 10  $\mu$ s, an acquisition time of 0.196 s, and a pulse delay of 3.0 s. The spectrum was acquired with 16384 data points for the spectral width of 41666 Hz.

RP-HPLC techniques: the RP-HPLC analysis of oligonucleotides 3 along with the positive and negative control experiments were performed on a Licrosphere RP-18 (e) 5  $\mu$ m, (125×4 mm) column using a Merck Hitachi LC pump L-7100 which was connected to a diode array detector (model L-7455). The system used was A: 0.1 M TEAA, pH = 7.0, B: 30%  $CH_3CN$  in 0.1 M TEAA (pH = 7.0) using a gradient A-B in 20 min, flow rate: 1.5 mL min<sup>-1</sup>. RP-HPLC analysis of oligonucleotides 7 and 8 were performed on a HP spherisorb ODS-2, 5  $\mu$ m, (125×4 mm) column using a Varian Prostar LC pump to which was attached a Dynamax absobance detector (model UV-D11). The solvent system and the conditions were same as described above. The RP-HPLC analysis of the oligonucleotides 4 and 9 were performed on a Waters YMC-Pack<sup>TM</sup> ODS-AQ<sup>TM</sup> S-5 column (2.0×250 mm) using a Waters HPLC system (600 E system controller, 996 Photodiode Array Detector). All these RP-HPLC fractions were simultaneously characterized by electrospray ionization mass spectrometry (ESI-MS) using a Hewlett-Packard 59987A electrospray quadrupole mass analyzer using negative polarity.

### Solution phase synthesis of dithymidyl H-phosphonate diester (1)

To a well stirred solution of dT-H-phosphonate TEA salt (0.1 mmol) and 3'-O-acetyl thymidine (0.11 mmol) dissolved in 1.0 mL of anhydrous pyridine, was added pivaloyl chloride (0.1 mmol). The reaction mixture was stirred at ambient temperature for 15 min. The reaction mixture was monitored by TLC (ethyl acetate/pet ether = 8:2). After completion of the reaction, the reaction mixture was concentrated and was subjected to column chromatography to get the product 1 (isolated yield 85%).

<sup>31</sup>P NMR (CD<sub>3</sub>CN) 1,  $\delta = 12.1$  ppm.

## Solution phase synthesis of dithymidyl phosphonate (2) using CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN oxidation method

The dithymidyl H-phosphonate diester 1 (10 mg, ca. 0.01 mmol)  $\delta = 12.1$  ppm) was dissolved in CD<sub>3</sub>CN (200 µL) and was transferred into a NMR sample tube. The 2.0 mL of CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/1.0/1.0/ 6.0/1.0, v/v) mixture was added at ambient temperature to the above solution. The reaction was monitored by <sup>31</sup>P NMR. After 5 min, quantitative formation of 2 ( $\delta = 2.9$  ppm) was observed as the only detectable product.

## Solution phase synthesis of 2 using 0.035 M I<sub>2</sub> in Py/THF/H<sub>2</sub>O (1/300/33, v/v) oxidation method

The solution of 1 (10 mg, ca. 0.01mmol) in CD<sub>3</sub>CN (200  $\mu$ L) was transferred into a NMR sample tube. To this solution was added 2.0 mL of 0.035 M I<sub>2</sub> in Py/THF/ H<sub>2</sub>O (1/300/33, v/v) at ambient temperature, the progress of the reaction was monitored by <sup>31</sup>P NMR spectroscopy. After 5 min formation of 2 ( $\delta$ =3.0 ppm) along with the formation of another product at  $\delta$ =-9.8 ppm was detected.

### Solution phase synthesis of 2 using $0.2 \text{ M I}_2$ in Py/H<sub>2</sub>O (98:2, v/v) oxidation method

As previously described, to the solution of 1 (10 mg, ca. 0.01 mmol) dissolved in CD<sub>3</sub>CN (200  $\mu$ L) was added 2.0 mL of 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v). After 5 min quantitative formation of 2 ( $\delta$ =2.5 ppm) was observed as the only detectable product.

### Synthesis of oligodeoxynucletides (ODNs)

### Solid phase synthesis of d(AA) (3) using CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN oxidation method (protocol 1).

The DMT-A<sup>bz</sup> loaded 1caa CPG 500 Å (22 mg, 1  $\mu$ mole, loading 44.3  $\mu$ mol/g) was poured into a well dried 2.5 mL Hamilton gas tight syringe with a glass wool

plug at the inlet. The support was initially washed with CH<sub>3</sub>CN ( $3 \times 0.5$  mL). The DMT protecting group was removed by washing with dichloroacetic acid (DCA)/  $CH_2Cl_2$  (3/100, v/v, 1.0 mL). Additional washes with  $DCA/CH_2Cl_2$  (8×0.5 mL) were performed and all the orange effluents were collected for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency. The support was washed successively with Py/ CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN (4×0.5 mL). The dA<sup>bz</sup>-H-phosphonate solution (10 mg, ca. 0.012 mmol) in dry CH<sub>3</sub>CN (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 µL, ca. 0.075 mmole) was drawn into the syringe and was agitated for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry CH<sub>3</sub>CN  $(3 \times 0.5 \text{ mL})$ . The internucleoside H-phosphonate linkages were then oxidized with 2.0 mL of  $CCl_4/H_2O/$ NMM/Py/CH<sub>3</sub>CN (2.5/0.2/1.0/6.0/9.3, v/v) for 45 min. Washings were performed with  $Py/CH_3CN$  (1/4, v/v,  $3 \times 1.5$  mL) and dry CH<sub>3</sub>CN ( $3 \times 1.5$  mL). The terminal DMT protecting group was removed by washing with dichloroacetic acid (DCA)/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with  $DCA/CH_2Cl_2$  (8×0.5 mL) followed by subsequent washings with Py/CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN (4×0.5 mL). The support bound oligonucleotide was then completely deprotected using concentrated NH<sub>4</sub>OH, 55 °C, 16 h.

RP-HPLC of 3 (retention time 7.76 min).

<sup>31</sup>P NMR (D<sub>2</sub>O) **3**,  $\delta = 0.9$  ppm.

The negative control experiment was performed exactly identical to protocol 1. The only difference was that the oxidation step was omitted. The support bound oligonucleotide was then completely deprotected using concentrated  $NH_4OH$ , 55 °C, 16 h.

**RP-HPLC** of **3** (retention time 7.79 min), adenosine (retention time 5.95 min), dA-H-phosphonate (retention time 6.4 min).

<sup>31</sup>P NMR (D<sub>2</sub>O) dA-H-phosphonate,  $\delta = 6.9$  and 8.3 ppm.

## Solid phase synthesis of 3 using 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) oxidation method (positive control)

The dimer was synthesized by coupling  $dA^{bz}$ -H-phosphonate with the support bound  $dA^{bz}$  loaded 1caa CPG 500 Å as described in protocol 1. At the end of the synthesis, before the final detritylation, the internucleoside H-phosphonate linkages were oxidized with 2.0 mL of 0.2 M solution in Py/H<sub>2</sub>O (98:2, v/v) for 30 min. The support was then washed with CH<sub>3</sub>CN/Py (4/1, v/v,  $6 \times 1.5$  mL) and with CH<sub>3</sub>CN ( $3 \times 1.5$  mL). After the removal of the DMT group, the support bound oligonucleotide was then completely deprotected using concentrated NH<sub>4</sub>OH, 55 °C, 16 h.

**RP-HPLC** of **3** (retention time 7.76 min), dA-H phosphonate (retention time 6.4 min).

<sup>31</sup>P NMR (D<sub>2</sub>O) **3**,  $\delta = 0.9$  ppm.

## Solid phase synthesis of d(GTTAAGACTTTTAC) (4) using $CCl_4/H_2O/NMM/Py/CH_3CN$ oxidation method

The DMT-C<sup>bz</sup> loaded 1caa CPG 500 Å (21 mg, 1 umole, loading 46.9 (umol/g) was poured into a well dried 2.5 mL Hamilton gas tight syringe with a glass wool plug at the inlet. The support was initially washed with CH<sub>3</sub>CN ( $3 \times 0.5$  mL). The DMT protecting group was removed by washing with dichloroacetic acid (DCA)/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL). Additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> ( $8 \times 0.5$  mL) were performed and all the orange effluents were collected for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency. The support was then washed with Py/CH<sub>3</sub>CN  $(1/4, v/v, 1 \times 0.5 \text{ mL})$  and dry CH<sub>3</sub>CN (4×0.5 mL). The dA<sup>bz</sup>-H-phosphonate solution (10 mg, ca. 0.012 mmol) in dry CH<sub>3</sub>CN (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 L, ca. 0.075 mmole) was drawn into the syringe and was agitated for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry CH<sub>3</sub>CN ( $3 \times 0.5$  mL). The terminal DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> ( $8 \times 0.5$  mL) followed by subsequent washings with Py/CH<sub>3</sub>CN (1/4, v/v,  $1 \times 0.5$  mL) and dry CH<sub>3</sub>CN ( $4 \times 0.5$  mL). This cycle was repeated by coupling of the appropriate nucleosidic unit, until the desired length of the oligonucleotide was achieved. The oxidation step was then carried out with 2.0 mL of CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/0.2/1.0/6.0/9.3, v/v) for 45 min at the end of the synthesis and before final detritylation. After the final detritylation and washings steps, the oligonucleotide was completely deprotected using concentrated NH<sub>4</sub>OH, 55 °C, 24 h.

RP-HPLC of 4 (retention time 27.8 min).

ESI-MS of 4, *M*<sub>wfound</sub>: 4234.8 *M*<sub>w calcd</sub>: 4237.

## Solution phase stability of d(TpsT) (5) in $CCl_4/H_2O/NMM/Py/CH_3CN$ oxidizing system

The DMT-T loaded 1caa CPG 500 Å (20 mg, 1 µmol, loading 48.3  $\mu$  mol/g) was poured into a well dried 2.5 mL Hamilton gas tight syringe with a glass wool plug at the inlet. The support was initially washed with CH<sub>3</sub>CN  $(3 \times 0.5 \text{ mL})$ . The DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL). Additional washes with  $DCA/CH_2Cl_2$  (8×0.5 mL) were performed and all the orange effluents were collected for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency. The support was washed successively with Py/CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry  $CH_3CN$  (4×0.5 mL). The dT-H-phosphonate solution (10 mg, ca. 0.015 mmol) in dry  $CH_3CN$  (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 µL, ca. 0.075 mmol) was drawn into the syringe and was agitated for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry CH<sub>3</sub>CN ( $3 \times 0.5$  mL). The internucleotidic H-phosphonate linkages were sulfurized by using 2.0 mL mixture of 10% S<sub>8</sub> in CS<sub>2</sub>/Py/TEA (35/35/1) for

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3 h. Washings were performed with Py/CH<sub>3</sub>CN (1/4, v/ v, 6×1.5 mL) and dry CH<sub>3</sub>CN (3×1.5 mL). The terminal DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> (8×0.5 mL) followed by subsequent washings with Py/CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN (4×0.5 mL). After performing the deprotection with concentrated NH<sub>4</sub>OH, 5°C, 16 h, the oligo was purified by RP-HPLC. The purified oligo 5 (1.0 A<sub>260</sub> units) was then stirred in a Wheaton glass vial for 16 h at ambient temperature with 1.0 mL of CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/1.0/1.0/ 6.0/1.0, v/v) mixture. The reaction mixture was lipholyzed and was then analyzed by <sup>31</sup>P NMR spectroscopy.

<sup>31</sup>P NMR (D<sub>2</sub>O) **5**,  $\delta = 57.4$  and 57.0 ppm; desulfurized dimer d(TpoT)  $\delta = 0.9$  ppm.

### Solution phase stability of 5 in 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) oxidizing system

As described above the purified oligo 5 (1.0  $A_{260}$  units) was stirred in a Wheaton glass vial for 16 h at ambient temperature with 1.0 mL of 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) mixture. After lipholyzation the <sup>31</sup>P NMR analysis was performed.

 $^{31}$ P NMR (D<sub>2</sub>O) desulfurized dimer d(TpoT)=0.9 ppm

## Solution phase stability of $d(T_{PN}T)$ (6) in CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN oxidizing system

The DMT-T loaded lcaa CPG 500 Å (20 mg, 1 µmol, loading 48.3 (µmol/g) was poured into a well dried 2.5 mL Hamilton gas tight syringe with a glass wool plug at the inlet. The support was initially washed with CH<sub>3</sub>CN  $(3 \times 0.5 \text{ mL})$ . The DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL). Additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> ( $8 \times 0.5$  mL) were performed and all the orange effluents were collected for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency. The support was washed successively with Py/CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN ( $4 \times 0.5$  mL). The dT-H-phosphonate solution (10 mg, ca. 0.015 mmol) in dry  $CH_3CN$  (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 µL, ca. 0.075 mmol) was drawn into the syringe and was agitated for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry CH<sub>3</sub>CN ( $3 \times 0.5$  mL). The dithymidyl H-phosphonate diester linkages were converted to the phosphoramidate linkages by using 2.0 mL mixture of 15% N,N-dimethyl-1,3- propane diamine in CCl<sub>4</sub> for 1 h. Washings were performed with Py/CH<sub>3</sub>CN (1/4, v/v,  $3 \times 1.5$  mL) and dry CH<sub>3</sub>CN ( $3 \times 1.5$  mL). The terminal DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> ( $8 \times 0.5$  mL) followed by washings with  $Py/CH_3CN$  (1/4, v/v, 1×0.5 mL) and dry  $CH_3CN$  (4×0.5 mL). After performing the deprotection with concentrated NH<sub>4</sub>OH, 55 °C, 16 h, the oligo was

purified by RP-HPLC. The purified oligo 5 (1.0  $A_{260}$  units) was then stirred in a Wheaton glass vial for 16 h, at ambient temperature with 1.0 mL of CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/1.0/1.0/6.0/1.0, v/v) mixture. The <sup>31</sup>P NMR analysis was carried out after lipholization of the reaction mixture.

<sup>31</sup>P NMR (D<sub>2</sub>O) **6**,  $\delta = 12.08$  ppm and 12.02 ppm.

### Solution phase stability of 6 in 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) oxidizing system

As described above, the purified oligo **6** (1.0  $A_{260}$  units) was stirred with 1.0 mL of 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) mixture in a Wheaton glass vial for 16 h at ambient temperature. The lipholyzed reaction mixture was subjected to <sup>31</sup>P NMR analysis.

<sup>31</sup>P NMR (D<sub>2</sub>O) desulfurized dimer d(TpoT)  $\delta = 0.97$  ppm.

### Solid phase synthesis of d(TpoTpsT) (7) using CCl<sub>4</sub>/ H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN oxidation method

The DMT-T loaded lcaa CPG 500 Å (20 mg, 1 µmole, loading 48.3 (µmol/g) was poured into a well dried 2.5 mL Hamilton gas tight syringe with a glass wool plug at the inlet. The support was initially washed with CH<sub>3</sub>CN  $(3 \times 0.5 \text{ mL})$ . The DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL). Additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> (8×0.5 mL) were performed and all the orange effluents were collected for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency. The support was washed successively with  $Py/CH_3CN$  (1/4, v/v, 1×0.5 mL) and dry  $CH_3CN$  (4×0.5 mL). The dT-H-phosphonate solution (10 mg, ca. 0.015 mmol) in dry CH<sub>3</sub>CN (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8  $\mu$ L, ca. 0.075 mmol) was drawn into the syringe and was agitated for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry  $CH_3CN$  (3×0.5 mL). The internucleotidic H-phosphonate linkages were sulfurized by using 2.0 mL mixture of 10% S<sub>8</sub> in CS2/Py/TEA (35/35/1) for 3 h. Washings were performed with Py/CH<sub>3</sub>CN (1/4, v/v, 6×1.5 mL) and dry  $CH_3CN$  (3×1.5 mL). The terminal DMT protecting group was removed by washing with DCA/ CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with  $DCA/CH_2Cl_2$  (8×0.5 mL), followed by subsequent washings with Py/CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN (4×0.5 mL). The next coupling step was then effected by drawing the dT-H-phosphonate solution (10 mg, ca. 0.015 mmol) in dry CH<sub>3</sub>CN (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 µL, ca. 0.075 mmol) into the syringe and agitating the mixture for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry CH<sub>3</sub>CN ( $3 \times 0.5$  mL). The internucleoside H-phosphonate linkages were then oxidized with 2.0 mL of  $CCl_4/H_2O/NMM/Py/CH_3CN$  (2.5/0.2/ 1.0/6.0/9.3, v/v) for 45 min. Washings were performed with  $Py/CH_3CN$  (1/4, v/v, 3×1.5 mL) and dry  $CH_3CN$  (3×1.5 mL). The terminal DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> (8×0.5 mL) followed by subsequent washings with Py/CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN (4×0.5 mL). The support bound oligonucleotide was then completely deprotected using concentrated NH<sub>4</sub>OH, 55 °C, 16 h.

**RP-HPLC** of 7 (retention time 9.8 and 10.3 min); desulfurized trimer d(TpoTpoT) retention time 9.1 min.

## Solid phase synthesis of 7 using 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) oxidation method

The synthesis of 7 was carried out identically as for the synthesis described above by the  $CCl_4/H_2O/NMM/Py/CH_3CN$  oxidation method. The only difference was that the oxidation reaction was carried out at the end of the synthesis, before final detritylation by 2.0 mL of 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) method for 30 min instead of  $CCl_4/H_2O/NMM/Py/CH_3CN$  method. The support was then washed with  $CH_3CN/Py$  (4/1, v/v, 6×1.5 mL) and with  $CH_3CN$  (3×1.5 mL). After the removal of the DMT group, the support bound oligonucleotide was then completely deprotected using concentrated NH<sub>4</sub>OH, 55 °C, 16 h.

**RP-HPLC** of 7 (retention time 9.9 and 10.3 min), desulfurized trimer d(TpoTpoT) (retention time 9.1 min).

# Solid phase synthesis of d(TpoTPNT) (8) using CCl<sub>4</sub>/ $H_2O/NMM/Py/CH_3CN$ oxidation method

The DMT-T loaded lcaa CPG 500 Å (20 mg, 1 µmol, loading 48.3 µmol/g) was poured into a well dried 2.5 mL Hamilton gas tight syringe with a glass wool plug at the inlet. The support was initially washed with CH<sub>3</sub>CN  $(3 \times 0.5 \text{ mL})$ . The DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL). Additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> ( $8 \times 0.5$  mL) were performed and all the orange effluents were collected for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency. The support was washed successively with Py/CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry  $CH_3CN$  (4×0.5 mL). The dT-H-phosphonate solution (10 mg, ca. 0.015 mmol) in dry  $CH_3CN$  (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 µL, ca. 0.075 mmol) was drawn into the syringe and was agitated for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry  $CH_3CN$  (3×0.5 mL). The dithymidyl H-phosphonate diester linkages were converted to the phosphoramidate linkages by using 2.0 mL mixture of 15% N,N-dimethyl-1,3-propane diamine in  $CCl_4$  for 1 h. Washings were performed with Py/CH<sub>3</sub>CN (1/4, v/v,  $3 \times 1.5$  mL) and dry CH<sub>3</sub>CN ( $3 \times 1.5$  mL). The terminal DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with DCA/  $CH_2Cl_2$  (8×0.5 mL) followed by subsequent washings with  $Py/CH_3CN$  (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN  $(4 \times 0.5 \text{ mL})$ . The next coupling step was then effected by drawing the dT-H-phosphonate solution (10 mg, ca. 0.015 mmol) in dry CH<sub>3</sub>CN (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 µL, ca. 0.075 mmol) into the syringe and agitating the mixture for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry CH<sub>3</sub>CN ( $3 \times 0.5$ mL). The internucleoside H-phosphonate linkages were then oxidized with 2.0 mL of CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/ CH<sub>3</sub>CN (2.5/0.2/1.0/6.0/9.3, v/v) for 45 min. Washings were performed with Py/CH<sub>3</sub>CN (1/4, v/v, 3×1.5 mL) and dry CH<sub>3</sub>CN (3×1.5 mL). The terminal DMT protecting group was removed by washing with DCA/ CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with  $DCA/CH_2Cl_2$  (8×0.5 mL) followed by subsequent washings with Py/CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN (4×0.5 mL). The support bound oligonucleotide was then completely deprotected using concentrated NH<sub>4</sub>OH, 55 °C, 16 h.

**RP-HPLC** of **8** (retention time 10.2 and 10.4 min); desulfurized trimer d(TpoTpoT) (retention time 9.08 min).

## Solid phase synthesis of 8 using 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) oxidation method

The synthesis of **8** was carried out identically as for the synthesis described above by the CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN oxidation method. The only difference was that the oxidation reaction was carried out at the end of the synthesis, before final detritylation by 2.0 mL of 0.2 M I<sub>2</sub> in Py/H<sub>2</sub>O (98:2, v/v) method for 30 min instead of CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN method. The support was then washed with CH<sub>3</sub>CN/Py (4/1, v/v, 6×1.5 mL) and with CH<sub>3</sub>CN (3×1.5 mL). After the removal of the DMT group, the support bound oligonucleotide was then completely deprotected using concentrated NH<sub>4</sub>OH, 55 °C, 16 h.

**RP-HPLC** of **8** (retention time 10.2 and 10.4 min), desulfurized trimer d(TpoTpoT) (retention time 9.04 min).

## Synthesis of d(TpoTpsTpsTpsTpsTpsTpsTpsT) (9) using CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN oxidation method

The DMT-T loaded 1caa CPG 500 Å (20 mg, 1 µmol, loading 48.3  $\mu$ mol/g) was poured into a well dried 2.5 mL Hamilton gas tight syringe with a glass wool plug at the inlet. The support was initially washed with CH<sub>3</sub>CN  $(3 \times 0.5 \text{ mL})$ . The DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL). Additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub> ( $8 \times 0.5$  mL) were performed and all the orange effluents were collected for subsequent spectroscopy (448 nm) and calculation of the coupling efficiency. The support was washed successively with Py/CH<sub>3</sub>CN (1/4, v/v,  $1 \times 0.5$  mL) and dry CH<sub>3</sub>CN ( $4 \times 0.5$  mL). The dT-H-phosphonate solution (10 mg, ca. 0.015 mmol) in dry  $CH_3CN$  (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 µL, ca. 0.075 mmol) was drawn into the syringe and was agitated for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry CH<sub>3</sub>CN ( $3 \times 0.5$  mL). This cycle was repeated

until the oligonucleotide,  $d(T_{PH}T_{PH}T_{PH}T_{PH}T_{PH}T_{PH}T)$ (where PH corresponds to the H-phosphonate diester linkages in the backbone) was achieved. The internucleotidic H-phosphonate linkages were sulfurized by using 2.0 mL mixture of 10% S8 in CS<sub>2</sub>/PyTEA (35/35/ 1) for 3 h. Washings were performed with Py/CH<sub>3</sub>CN  $(1/4, v/v, 6 \times 1.5 \text{ mL})$  and dry CH<sub>3</sub>CN  $(3 \times 1.5 \text{ mL})$ . The terminal DMT protecting group was removed by washing with dichloroacetic acid (DCA)/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, 1.0 mL) and additional washes with DCA/CH<sub>2</sub>Cl<sub>2</sub>  $(8 \times 0.5 \text{ mL})$  followed by subsequent washings with Py/ CH<sub>3</sub>CN (1/4, v/v, 1×0.5 mL) and dry CH<sub>3</sub>CN (4×0.5 mL). The last dT nucleosidic unit was added by drawing the dT-H-phosphonate solution (10 mg, ca. 0.015 mmol) in dry CH<sub>3</sub>CN (0.25 mL) and dry pyridine (0.25 mL) containing pivaloyl chloride (8 µL, ca. 0.075 mmol) into the syringe and agitating the mixture for 2 min. The coupling agents were ejected from the syringe, and the support was washed with dry CH<sub>3</sub>CN ( $3 \times 0.5$  mL). The internucleoside H-phosphonate linkage was then oxidized with 2.0 mL of CCl<sub>4</sub>/H<sub>2</sub>O/NMM/Py/CH<sub>3</sub>CN (2.5/0.2/1.0/6.0/9.3, v/v) for 45 min. Washings were performed with  $Py/CH_3CN$  (1/4, v/v, 3×1.5 mL) and dry  $CH_3CN$  (3×1.5 mL). The terminal DMT protecting group was removed by washing with DCA/CH<sub>2</sub>Cl<sub>2</sub> (3/100, v/v, v)1.0 mL) and additional washes with DCA/CH<sub>2</sub>C<sub>12</sub> ( $8 \times 0.5$ mL) followed by subsequent washings with Py/CH<sub>3</sub>CN (1/ 4, v/v,  $1 \times 0.5$  mL) and dry CH<sub>3</sub>CN ( $4 \times 0.5$  mL). The support bound oligonucleotide was then completely deprotected using concentrated NH<sub>4</sub>OH, 55 °C, 16 h.

RP-HPLC of 9 (retention time 31.0 min).

ESI-MS spectrum of 9, Mw<sub>calc</sub>: 2459.95 Mw<sub>found</sub>: 2466.

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#### **References and Notes**

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