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## ARTICLE

# The Synthesis, Conformation and Hydrolytic Stability of an *N,S*-bridging Thiophosphoramidate Analogue of Thymidylyl-3',5'-Thymidine

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A 3'-*N,S*-bridging thiophosphoramidate analogue of thymidylyl-3',5'-thymidine was synthesised under aqueous conditions. <sup>1</sup>H NMR conformational measurements show that the 3'-*N*-substituted deoxyribose ring is biased towards the 'north', RNA-like conformation. Rate constants for hydrolysis of the analogue were measured at 90 °C in the pH range 1.3-10.9. The pH-log *k*<sub>obs</sub> profile displays a pH-independent region between approximately pH 7 and 10 (*t*<sub>1/2</sub> ~13 days). Under acidic conditions, *k*<sub>obs</sub> displays a first order dependence on [H<sub>3</sub>O<sup>+</sup>].

## A Introduction

Phosphate analogues, where oxygen atoms have been replaced by nitrogen or sulfur, are important mechanistic enzymology probes.<sup>1</sup> These heteroatom substitutions serve to alter the electrophilicity at P, change leaving group properties, and the interactions between these atoms and metal ions at the active sites of enzymes and ribozymes. Sulfur substitutions, in particular, offer the ability to study cation binding through metal ion rescue experiments, and the use of phosphorothiolates has proven the existence of general acid-base catalysed cleavage of phosphodiester bonds in ribozymes.<sup>1-5</sup> Oligonucleotides containing modified phosphate groups show increased resistance towards (ribo)nucleases and enhanced therapeutic effects.<sup>6-8</sup> Modified phosphates also offer the potential for modulating the structural properties of nucleic acid assemblies such as the i-motif.<sup>9</sup>

Substitution of the *O*-heteroatoms of phosphoryl groups has also been used to facilitate phosphorylation and nucleic acid ligation technologies. To detect single nucleotide polymorphisms and facilitate signal amplification, Kool employed the nucleophilicity of sulfur anions to allow templated ligation through *S*-alkylation.<sup>8, 10-17</sup> We have exploited the nucleophilicity of amines to facilitate the efficient aqueous *N*-phosphorylation of amines,<sup>18-20</sup> and *N*-thiophosphorylation plus *S*-alkylation of thiophosphoramidates.<sup>21-23</sup> Amine nucleophilicity has also been harnessed to promote enzyme-free templated-ligation between

3'-amino-substituted oligonucleotides and 5'-activated phosphodiester nucleosides.<sup>24, 25</sup>

We now demonstrate the application of aqueous *N*-thiophosphorylation plus *S*-alkylation towards the ligation of two nucleosides to afford thiophosphoramidate analogue **TnpsT 1** of the dinucleotide, **TpT 2** (Fig. 1). We study its conformational preference and hydrolytic stability across a broad pH range, with a view towards applying our aqueous strategies in nucleic acid ligations and bioconjugations.

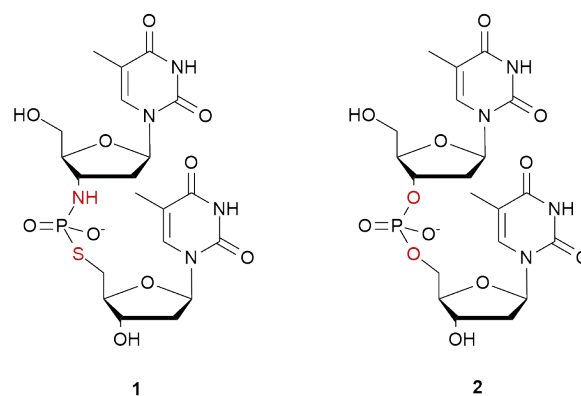


Fig. 1 3'-Amino-3'-deoxythymidylyl-(3'→5')-5'-deoxy-5'-thiothymidine (**TnpsT 1**) and its natural counterpart, thymidylyl-(3'→5')-thymidine (**TpT 2**)

## B Synthesis

3'-Amino-3'-deoxythymidyl-(3'→5')-5'-deoxy-5'-thiothymidine (**TnpsT**, **1**) was chosen as a target because starting materials for our proposed synthetic route (Scheme 1) were readily accessible. The amine, 3'-amino-3'-deoxythymidine **3** was accessed as a hydrochloride salt *via* the reduction of 3'-azido-3'-deoxythymidine (AZT). 5'-Deoxy-5'-iodothymidine **5** was prepared by 5'-tosylation of thymidine,<sup>26</sup> followed by conversion to the iodide using sodium iodide in acetone.

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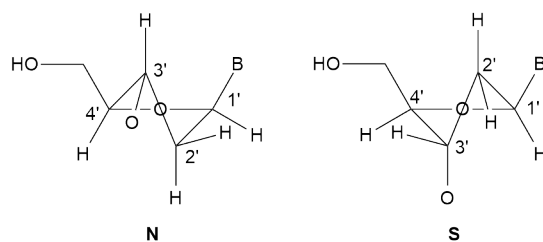
**Scheme 1** Thiophosphorylation of 3'-amino-3'-deoxythymidine **3** and subsequent reaction with 5'-deoxy-5'-iodothymidine **5**.

3'-Amino-3'-deoxythymidine **3** was *N*-thiophosphorylated using our pH-optimised protocol,<sup>22</sup> where an aqueous solution of hydrochloride salt **3.HCl** was maintained at pH 12 using KOH<sub>(aq)</sub> from an autotitrator during the addition of 1.0 equiv. of PSCl<sub>3</sub> dissolved in MeCN. This afforded crude thiophosphoramidate **4** (approximately 65% conversion by <sup>31</sup>P NMR spectroscopy).

The alkylating agent, 5'-deoxy-5'-iodothymidine **5**, showed poor solubility at neutral pH, however, deprotonation of the thymine base (p*K*<sub>a</sub> ~ 10) increased solubility markedly. Thus pH 12 was maintained throughout the addition of 5'-deoxy-5'-iodothymidine **5** to thiophosphoramidate **4**. Under these conditions, 98% of the crude thiophosphoramidate **4** was converted to **TnpsT 1** as determined by <sup>31</sup>P NMR spectroscopy. **TnpsT 1** was isolated by ion exchange chromatography in 64% yield (based on starting amine **3**) as a triethylammonium salt, which was converted to the K<sup>+</sup> salt for subsequent conformational and kinetic studies.

### C Conformational Analysis of TnpsT 1

Modified (deoxy)ribose display perturbed north/south conformational preferences (Fig. 2).<sup>27</sup> Given our long-term intention to employ our synthetic approach in nucleic acid templated ligations, that would generate ligated products containing a thiophosphoramidate motif, we sought to gauge the conformational preference of **TnpsT 1** using NMR methods.



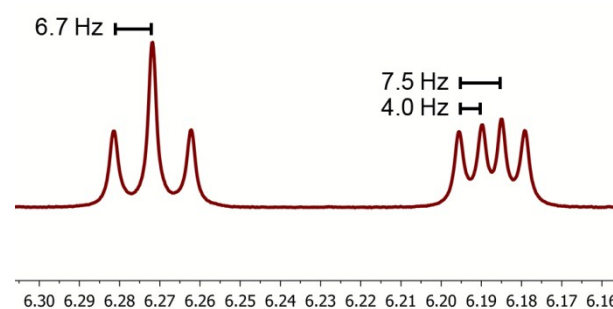
**Fig. 2** The north (N) and south (S) conformations of a deoxyribose ring.

The elucidation of solution-state ribose conformations by means of <sup>1</sup>H NMR *J*-coupling values was pioneered by Altona and Sundaralingam,<sup>28, 29</sup> and the effect on the conformation of thiophosphate-containing dinucleosides was investigated by

Beevers *et al.*<sup>30</sup> using similar methods. Rinkel and Altona found the sum of the *J*<sub>1,2'</sub> and *J*<sub>1,2''</sub> coupling constants, Σ*H1'*, to be linearly correlated with the proportion of the south conformer through Equation 1, where *P*<sub>S</sub> is the proportion of the 'south' conformer:

$$P_S = \frac{\Sigma H1' - 9.8}{5.9} \quad \text{Equation 1}$$

Many of the <sup>1</sup>H NMR signals of **TnpsT 1** are coincident, and second-order couplings complicate the extraction of *J* values of the deoxyribose protons. The signals for both C1' protons, however, are well separated, and the *J*<sub>1,2'</sub> and *J*<sub>1,2''</sub> coupling values were 7.5 and 4.0 Hz, respectively for the 3'-amino-3'-deoxythymidine (Tnp) residue of **1**. The C1'-H signal at 6.28 ppm for the 5'-deoxy-5'-thiothymidine fragment (psT) presents as an apparent triplet with a coupling constant of 6.7 Hz (Fig. 3).



**Fig. 3** The signals corresponding to the C1' protons in **TnpsT 1**. The signal at 6.27 ppm corresponds to the 5'-linked nucleoside (psT), while the signal at 6.19 ppm corresponds to the 3'-linked nucleoside (Tnp)

Application of Equation 1 yields values of 29% south for the **Tnp** ribose ring, and 61% south for the psT ribose ring. Comparison with **TpT 2** (Tp: 74.2% south; pT: 62.7% south)<sup>30</sup> indicates that the substitution of nitrogen for oxygen brings about a greater population of the 'north', C3'-endo, or 'RNA-like' conformer in the **Tnp** fragment, while the psT ribose ring retains its 'DNA-like' conformation. This is not surprising, as the conformation of the furanose ring is largely dictated by the anomeric and gauche effects, where the lower electronegativity of C3'-nitrogen compared to oxygen reduces the gauche effect of donation from the C2'-H bonding orbital into the C3'-N/O antibonding orbital and thus disfavours the south conformation. The thiophosphoramidate group is not linked directly to the ribose ring of the psT fragment and so has no apparent influence on its conformation. The **Tnp** conformational change is similar to that observed by Beevers *et al.* in the dideoxynucleoside 3'-phosphorothiolate analogue (**TspT**),<sup>30</sup> however, a more detailed analysis,<sup>31</sup> particularly in the context of extended and double-stranded nucleic acid structures will be required to confirm this result.

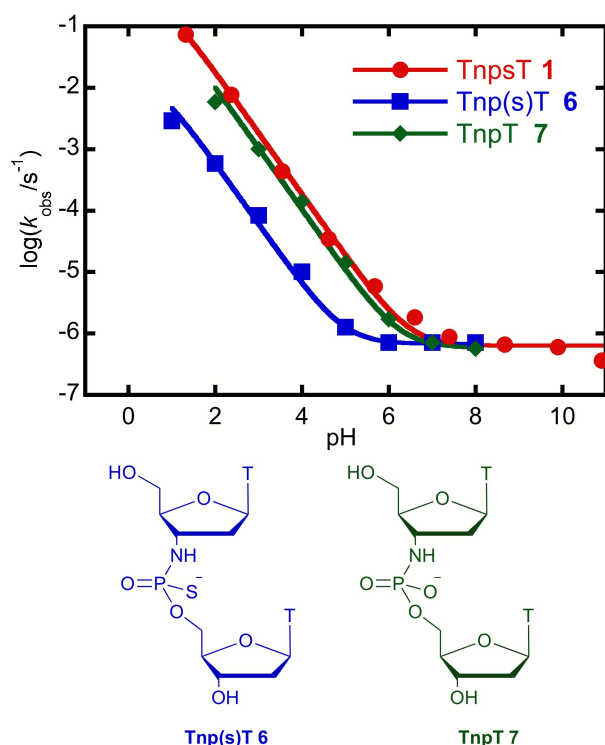
### D Kinetics and Mechanism of TnpsT Hydrolysis

#### Kinetic Experiments

Hydrolysis experiments on **TnpsT 1** were carried out at 90 °C in buffered aqueous solutions with pHs ranging from 1.32 to 10.91, at intervals of approximately one pH unit (pH values calculated at 90 °C based on values measured at 25 °C, see ESI). Aliquots of substrate **1** in each buffered solution were sealed into vials, and heated at 90 °C. Samples were removed from the heating bath or block at suitable intervals, the remaining starting material and products were resolved by HPLC, and the ratios of the integrals of the substrate and hydrolysis products relative to an internal standard were assessed.

At each pH, separate experiments were performed, using 10 and 100 mM buffer concentrations in order to check for buffer-promoted hydrolysis pathways. Acetate and formate buffered-experiments afforded rate constants that appeared to be dependent on buffer concentration. Thus, additional experiments were performed using 40 and 70 mM buffers, and  $k_{\text{obs}}$ -buffer concentration plots were extrapolated to estimate the buffer-dependent and buffer-independent rate constants (see ESI).

The log  $k_{\text{obs}}$ -pH profile for the hydrolysis of **TnpsT 1** displayed a pH-independent 'plateau' from ~pH 7 to 10 with  $t_{1/2}$  ~13 days, while at pH 1.32 the half-life was nine seconds (red trace in Fig. 4). The rapidity of reaction at lower pH values put practical limits on our ability to further explore this region. At the high pH extreme, the appearance of insoluble materials in the reaction mixture suggested etching of the glass vials, which precluded the straightforward exploration of the higher pH extreme within the format of our experimental design.



**Fig. 4** The pH- $\log(k_{\text{obs}})$  profiles for the hydrolyses at 90 °C of: **TnpsT 1**; and related systems **Tnp(s)T 6** and **TnpT 7** studied by Ora *et al.*<sup>32</sup> Kinetic data are fitted to equation 2.

$$k_{\text{obs}} = \frac{k_{\text{H}}(10^{-\text{pH}}) + k_0 K_{\text{a1}}}{10^{-\text{pH}} + K_{\text{a1}}} \quad \text{Equation 2}$$

SCHEME 2 is at the end of this document

**Scheme 2** pH-dependent speciation of **TnpsT 1**.

Data for the disappearance of **TnpsT 1** were found to fit Equation 2, where rate coefficients  $k_0$  and  $k_{\text{H}}$  represent the contributions to  $k_{\text{obs}}$  of the neutral/zwitterionic forms **1(neutral)** and monocationic form **1H<sup>+</sup>** respectively (Scheme 2). The acid dissociation constant between **1H<sup>+</sup>** and the kinetically indistinguishable neutral forms **1(neutral)** is captured in  $K_{\text{a1}}$ . A single data point at pH 10.91 suggests a potential downward trend in reactivity towards higher pHs. This may be associated with nucleobase ionisation ( $K_{\text{a2}}$ ) of **1(neutral)**, however, there are insufficient data to substantiate this hypothesis.

The reactivity on the pH plateau, with  $k_0 = 6.3 \times 10^{-7} \text{ s}^{-1}$ , is similar to the related analogues **Tnp(s)T 6** and **TnpT 7** that were studied by Ora *et al.* (blue and green traces in Fig. 4).<sup>32</sup> The lack of an observed plateau in reactivity at lower pH values limits our ability to unequivocally assign values to the interdependent variables  $k_{\text{H}}$  and  $K_{\text{a1}}$ . Based on the fitting of the available data, however, values of  $k_{\text{H}} < 0.15 \text{ M}^{-1} \text{ s}^{-1}$  and  $\text{p}K_{\text{a1}} < 1$  were estimated, which align with those observed for **TnpT 7**.

SCHEME 3 is at the end of this document

**Scheme 3** Mechanistic pathways for the hydrolysis of **TnpsT 1**.

The similarity in reactivity profiles of **TnpsT 1** and **TnpT 7** suggests that mechanisms are likely to be similar. This is borne out in product analysis studies, and illustrative examples are discussed below (Scheme 3).

At pH 7.0 and 7.7 the largest detected peak by HPLC is thymine **8**, derived from initial depyrimidinylations (route A) of either thymidine site within **TnpsT 1** and subsequent fragmentation of the resulting species, as seen by Ora *et al.* for **Tnp(s)T 6** and **TnpT 7**.<sup>32</sup> Given the remoteness of the phosphoryl-sites from the C1' sites where depyrimidinylations occur, it is unsurprising that the reactivities of **TnpsT 1**, **Tnp(s)T 6** and **TnpT 7** in the pH independent regions are similar. At pH 6, some depyrimidinylations is observed, however, P-N cleavage is now evident, with amine **3** being observed at a similar retention time to thymine **8** (routes A and B). Another product appears at a much longer retention time, with a lag in its formation. We believe this to be disulfide **11** formed from thiol **10** through oxidation, which is expected to be relatively facile at this pH, and has been reported in a similar system.<sup>33</sup> We were, however, unable to confirm this by HPLC in a MS-compatible buffer system. Thiol **10** is formed by rapid dephosphorylation of phosphorothiolate **9**, which

arises from acid promoted P-N scission. At pH 3.2, the product chromatograms are simpler, displaying only two major peaks. Amine **3** represents one of these peaks, derived from P-N scission, whereas the second peak is consistent with thiol **10**, which is formed rapidly from phosphorothiolate **9** (route B). Thiol **10** is expected to be relatively stable towards oxidation under these conditions. The overlap of the pH-log  $k_{\text{obs}}$  profiles of **TnpsT 1** and **TnpT 7** in the acidic region suggests that either the values of  $k_{\text{H}}$  and  $K_{\text{a1}}$  are identical for these species, or that ionisation and reactivity compensate each other to arrive at identical  $k_{\text{obs}}$  values.

## E Conclusions

**TnpsT 1**, which is an analogue of thymidyl-3',5'-thymidine **2**, was successfully synthesised under aqueous conditions, without protecting groups. NMR-based analyses revealed a predominantly 'north', 'RNA-like' C3'-endo conformational preference for the 3'-aza-substituted deoxyribose (Tnp) fragment of **TnpsT 1**. Hydrolytic studies on **TnpsT 1** yielded a near-identical profile to non-thio-analogue **TnpT 7**, where for pH>7, de-pyrimidinylation dominates, and P-N scission is dominant for lower pHs. The combination of simple aqueous synthesis, knowledge of conformational preference and stability of the linkage will allow us to exploit *N,S*-bridging nucleotide systems in future applications.

## F Experimental

### Synthesis

#### 3'-Amino-3'-deoxythymidine **3**, hydrochloride salt.

Adapting a literature procedure, 3'-azido-3'-deoxythymidine (1.00 g, 3.74 mmol) and triphenylphosphine (1.54 g, 5.87 mmol) were dissolved in pyridine (8 ml) and the mixture was stirred at room temperature for 1 h. Ammonia solution (30 ml, 35%) was then added, and the mixture was stirred overnight. The suspension was diluted with water (30 ml) and extracted with chloroform (3 × 30 ml) before being lyophilised. The solid residue was dissolved in ethanol (100 ml) and hydrogen chloride gas was bubbled through the solution until precipitation was observed. The precipitate was isolated by filtration, and washed with a small quantity of diethyl ether. Additional product was obtained by adding diethyl ether (500 ml) to the filtrate, collecting and washing the precipitate. The isolated solids were combined and dried under vacuum overnight yielding a total of 846 mg, 81%; mp 253-255 °C (decomp., from ethanol);  $\nu_{\text{max}}/\text{cm}^{-1}$  3392, 3032, 1694, 1644, 1470;  $\delta_{\text{H}}$  (400 MHz, (D<sub>2</sub>O) 1.86 (3H, s, C5-CH<sub>3</sub>), 2.54-2.70 (2H, m, C2'-H<sub>2</sub>), 3.81 (1H, dd, *J* 12.6, 4.6, C5'-H<sub>a</sub>), 3.89 (1H, dd, *J* 12.6, 3.4, C5'-H<sub>b</sub>), 4.06 (1H, dt, *J* 8.1, 5.5, C3'-H), 4.19-4.28 (1H, m, C4'-H), 6.28 (1H, t, *J* 6.8, C1'-H), 7.62 (1H, d, *J* 1.1, C6-H);  $\delta_{\text{C}}$  (100 MHz, (D<sub>2</sub>O) 10.5, 33.8, 49.1, 59.7, 81.5, 84.1, 110.5, 136.6, 150.5, 165.4; *m/z* 242.1142 ([M+H]<sup>+</sup>, 100%), requires 242.1141, 264.0961 ([M+Na]<sup>+</sup>, 90).

**5'-Deoxy-5'-(4-toluenesulfonyl)thymidine.** Adapting a literature procedure,<sup>26</sup> thymidine (3.92 g, 16.2 mmol) was

dissolved in pyridine (20 ml) in a round-bottomed flask, and placed in a water-ice bath. 4-Toluenesulfonyl chloride (3.83 g, 20.2 mmol), dissolved in pyridine (20 ml) was added dropwise over 10 min. Stirring was continued for a further 24 h. The solution was then poured into ice water (100 ml) and the mixture was extracted with ethyl acetate (2 × 60 ml). The organic extracts were washed with saturated sodium bicarbonate solution (40 ml), and water (40 ml) before being dried over MgSO<sub>4</sub>. The solvents were then removed under reduced pressure, and the residue was recrystallised from water to give the tosylated nucleoside, 5'-deoxy-5'-(4-toluenesulfonyl)thymidine (1.57 g, 24%).  $\delta_{\text{H}}$  (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) 1.76 (3H, s, C5-CH<sub>3</sub>) 2.02-2.09 (1H, m, C2'-H<sub>a</sub>H<sub>b</sub>) 2.11-2.19 (1H, m, C2'-H<sub>a</sub>H<sub>b</sub>) 2.41 (3H, s, CH<sub>3</sub>-Ar) 3.83-3.88 (1H, m, C3'-H), 4.12-4.20 (2H, m, C4'-H and C5'-H<sub>a</sub>H<sub>b</sub>) 4.25 (1H, dd, *J* 7.2, 3.4, C5'-H<sub>a</sub>H<sub>b</sub>) 5.44 (1H, d, *J* 4.4, OH) 6.14 (1H, app. t, *J* 7.2, C1'-H) 7.38 (1H, d, *J* 1.8, C6-H) 7.47 (2H, d, *J* 8.3, *m*-OSO<sub>2</sub>Ph) 7.79 (2H, d, *J* 8.3, *o*-OSO<sub>2</sub>Ph) 11.33 (1H, s, NH)  $\delta_{\text{C}}$  (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) 12.1, 21.1, 38.3, 69.9, 70.1, 83.1, 84.0, 109.8, 127.6, 130.2, 132.1, 135.9, 145.1, 150.3, 163.6.

#### 5'-Deoxy-5'-iodothymidine **5**.

5'-Deoxy-5'-tosylthymidine (1.57 g, 3.96 mmol) and sodium iodide (2.97 g, 19.8 mmol) were placed in a round-bottomed flask and dissolved in the minimum volume of acetone. The solution was heated at reflux for 24 h, before the solvent was removed under reduced pressure. The residue was recrystallized from water to yield the product (1.20 g, 86%).  $\delta_{\text{H}}$  (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) 1.79 (3H, s, C5-CH<sub>3</sub>) 2.07 (1H, ddd, *J* 13.5, 6.2, 3.1, C2'-H<sub>a</sub>H<sub>b</sub>) 2.29 (1H, ddd, *J* 13.5, 8.1, 6.2, C2'-H<sub>a</sub>H<sub>b</sub>) 3.39 (1H, dd, *J* 10.4, 6.3, C5'-H<sub>a</sub>H<sub>b</sub>) 3.52 (1H, dd, *J* 10.4, 6.3, C5'-H<sub>a</sub>H<sub>b</sub>) 3.80 (1H, dt, *J* 6.2, 2.8, C3'-H), 4.15-4.21 (1H, m, C4'-H), 5.49 (1H, d, *J* 4.3, OH) 6.22 (1H, dd, *J* 8.0, 6.2, C1'-H) 7.53 (1H, d, *J* 1.5, C6-H) 11.35 (1H, s, NH)

#### 3'-Amino-3'-deoxythymidyl-(3'→5')-5'-deoxy-5'-thiothymidine, triethylammonium salt **1**.Et<sub>3</sub>N<sup>+</sup>H.

First 3'-amino-3'-deoxythymidine-*N*-thiophosphoramidate **4** was prepared according to the previously reported phosphorylation procedure.<sup>22</sup> 3'-Amino-3'-deoxythymidine, hydrochloride salt **3.HCl** (139 mg, 0.500 mmol) was dissolved in water and made up to 5 ml and pH 12 with water and potassium hydroxide solution (1 M). Thiophosphoryl chloride solution (1.50 ml, 0.333 M in MeCN) was added slowly using a Hamilton<sup>®</sup> microlitre syringe, with vigorous stirring, and with the tip of the syringe below the surface of the reaction mixture. Throughout the experiment, the pH was kept constant at pH 12 using a 1.000 M solution of potassium hydroxide, added by an autotitrator equipped with a pH probe. The experiment was considered to be complete when the autotitrator needed to add negligible volumes of potassium hydroxide solution to the reaction mixture in order to maintain a constant pH. The lyophilised thiophosphoramidate was redissolved in water, and made up to 5 ml at pH 12 with water and potassium hydroxide solution (1 M). 5'-Deoxy-5'-iodothymidine **5** (352 mg, 1.00 mmol) was added to the solution with stirring, while the pH was maintained at 12 with potassium hydroxide solution (1 M) by an autotitrator. Once the 5'-deoxy-5'-iodothymidine **5** had

fully dissolved and no further addition of potassium hydroxide was required to maintain the pH, the solution was sealed to prevent losses owing to evaporation and heated to 50 °C. At intervals, aliquots were removed from the reaction vessel and analysed by  $^{31}\text{P}$  NMR spectroscopy. The *S*-alkylation process was monitored based on the disappearance of the signal for thiophosphoramidate **4** at  $\delta \sim 44$  ppm, and the appearance of a signal for **TnpsT 1** at  $\delta \sim 22$  ppm. The reaction was continued until the unalkylated thiophosphoramidate starting material had been completely consumed; the solution was then allowed to cool to room temperature. The pH of the reaction mixture was measured and found to be 10.45. The solution was lyophilised and redissolved in 1 M TEAB buffer, then purified by anion exchange chromatography with a flow rate of 5 ml/min over a DEAE-Sepharose<sup>®</sup> resin. Triethylammonium bicarbonate buffer was applied in a 0 to 0.15 M gradient over 3 h. A second chromatographic purification using the same method was required to remove all impurities to yield 3'-amino-3'-deoxythymidylyl-(3'→5')-5'-deoxy-5'-thiothymidine as its triethylammonium salt **1.Et<sub>3</sub>N<sup>+</sup>H** (212 mg, 64%).  $\delta_{\text{H}}$  (700 MHz, D<sub>2</sub>O) 1.29 (9H, t, *J* 7.3, HN<sup>+</sup>(CH<sub>2</sub>CH<sub>3</sub>)<sub>3</sub>) 1.88 (3H, d, *J* 1.2, A- or B- C5CH<sub>3</sub>) 1.89 (3H, d, *J* 1.2, A- or B- C5CH<sub>3</sub>), 2.36-2.45 (3H, m, A-C2'*H<sub>a</sub>* and B-C2'*H<sub>2</sub>*) 2.49 (1H, ddd, *J* 14.0, 8.1, 3.9, A-C2'*H<sub>b</sub>*), 3.04-3.11 (2H, m, B-C5'*H<sub>2</sub>*), 3.21 (6H, q, *J* 7.0, HN<sup>+</sup>(CH<sub>2</sub>CH<sub>3</sub>)<sub>3</sub>), 3.78-3.85 (2H, m, A-C3'*H* and A-C5'*H<sub>a</sub>*) 3.86-3.89 (1H, m, A-C5'*H<sub>b</sub>*), 4.14-4.20 (2H, m, A- and B-C4'*H*), 4.46-4.49 (1H, m, B-C3'*H*), 6.16 (1H, dd, *J* 7.4, 4.0, A-C1'*H*), 6.25 (1H, app. t, *J* 6.7, B-C1'*H*), 7.67 (1H, d, *J* 1.2, A- or B-C6*H*), 7.71 (1H, d, *J* 1.2, A- or B-C6*H*);  $\delta_{\text{C}}$  (151 MHz, D<sub>2</sub>O) 8.2 (HN<sup>+</sup>(CH<sub>2</sub>CH<sub>3</sub>)<sub>3</sub>), 11.5 (A- and B-C5CH<sub>3</sub>), 32.0, (B-C5'), 38.0 (B-C2'), 38.5 (A-C2'), 46.6 (HN<sup>+</sup>(CH<sub>2</sub>CH<sub>3</sub>)<sub>3</sub>), 58.9 (A-C3'), 60.2, (A-C5'), 72.0 (B-C3'), 84.5 (A-C1'), 84.7 (B-C1'), 84.9 (B-C4'), 85.5 (A-C4'), 111.1 (A- or B-C5), 111.3 (A- or B-C5), 137.4 (A- or B-C6), 137.5 (A- or B-C6), 151.4 (A- and B-C2), 166.1 (A- or B-C4), 166.3 (A- or B-C4);  $\delta_{\text{P}}$  (162 MHz, D<sub>2</sub>O) 21.6 (1P, app. q, *J* 9.7, HNPO<sub>2</sub>S); *m/z* 560.1207 ([M-HN<sup>+</sup>Et<sub>3</sub>]<sup>-</sup>, 100%) requires 560.1216, 279.5541 ([M-H<sub>2</sub>N<sup>+</sup>Et<sub>3</sub>]<sup>2-</sup>).

**3'-Amino-3'-deoxythymidylyl-(3'→5')- 5'-deoxy-5'-thiothymidine, potassium salt 1.K<sup>+</sup>** SP-Sepharose<sup>®</sup> resin was exchanged with potassium ions by passing a solution of potassium chloride (100 mM) over it. The triethylammonium salt of the dinucleoside (100 mg) was then dissolved in water (4 ml) and passed over the resin. The UV-active fractions were collected and lyophilised to yield the dinucleoside as its potassium salt (60 mg, 66%)  $\delta_{\text{H}}$  (700 MHz, D<sub>2</sub>O) 1.88 (3H, d, *J* 1.2, A- or B- C5CH<sub>3</sub>) 1.89 (3H, d, *J* 1.2, A- or B- C5CH<sub>3</sub>), 2.35-2.46 (3H, m, A-C2'*H<sub>a</sub>* and B-C2'*H<sub>2</sub>*) 2.49 (1H, ddd, *J* 14.0, 8.1, 4.0, A-C2'*H<sub>b</sub>*), 3.02-3.11 (2H, m, B-C5'*H<sub>2</sub>*), 3.78-3.85 (2H, m, A-C3'*H* and A-C5'*H<sub>a</sub>*) 3.88 (1H, ddd, *J* 7.3, 4.5, 2.5, A-C4'*H*), 3.94 (1H, dd, *J* 12.6, 2.5, A-C5'*H<sub>b</sub>*), 4.18 (1H, app. q, *J* 5.1, B-C4'*H*), 4.48 (1H, app. dt, *J* 6.5, 4.6, B-C3'*H*), 6.17 (1H, dd, *J* 7.5, 4.0, A-C1'*H*), 6.25 (1H, app. t, *J* 6.7, B-C1'*H*), 7.68 (1H, d, *J* 1.4, A- or B-C6*H*), 7.71 (1H, d, *J* 1.2, A- or B-C6*H*);  $\delta_{\text{C}}$  (151 MHz, D<sub>2</sub>O) 11.5, 11.5, 32.0, 38.0, 38.5, 50.4, 60.2, 72.0, 84.5, 84.7, 84.8, 84.8, 85.5, 85.6, 111.1, 111.3, 137.4,

137.5, 151.4, 151.4, 166.1, 166.3, 171.1;  $\delta_{\text{P}}$  (162 MHz, D<sub>2</sub>O) 21.6 (1P, app. q, *J* 10.0, HNPO<sub>2</sub>S); *m/z* 560.1208 ([M-K]<sup>-</sup>, 100%) requires 560.1216, 1121.2450 ([2M-2K+H]<sup>-</sup>).

**HPLC standard - potassium *p*-nitrobenzenesulfonate.** *p*-Nitrobenzenesulfonyl chloride (222 mg, 1.00 mmol) was placed in a flask with potassium hydroxide solution (2 ml, 1.000 M) and water (20 ml). The solution was heated at reflux for 3 h before being lyophilised to yield a mixture of the desired product and potassium chloride in a 1:1 molar ratio. (231 mg, 98%)  $\delta_{\text{H}}$  (400 MHz, (D<sub>2</sub>O) 8.00 (2H, d, *J* 8.9, 1H *m*- to sulfonate), 8.35 (2H, d, *J* 8.9, 1H *o*- to sulfonate);  $\delta_{\text{C}}$  (101 MHz, (D<sub>2</sub>O) 124.3, 126.9, 148.1, 149.1.

#### Hydrolysis studies

See ESI for details of the hydrolysis studies.

#### G Acknowledgements

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#### H Notes and references

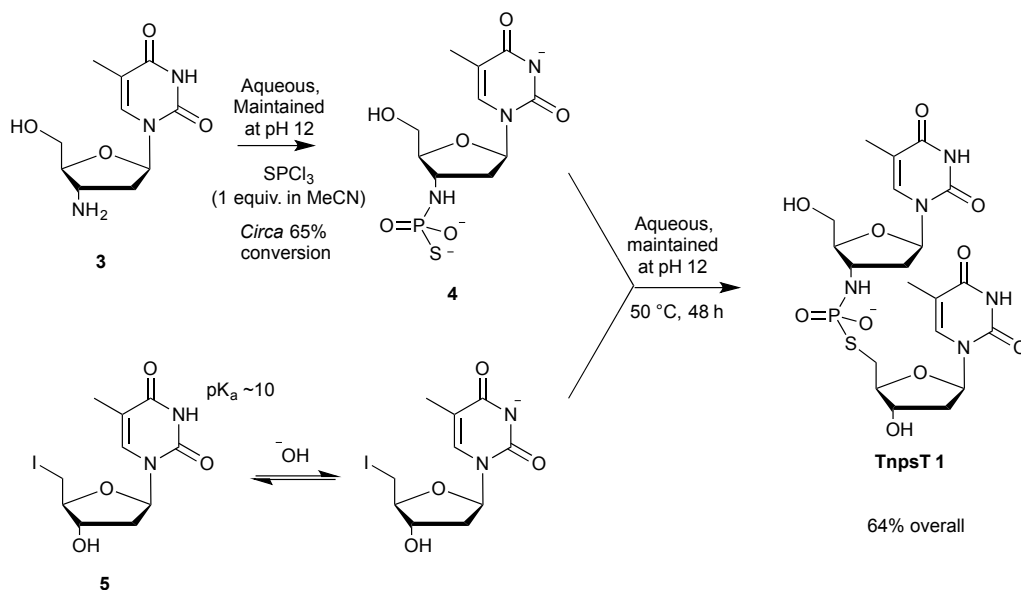
<sup>a</sup> Department of Chemistry, Durham University, South Road, Durham, DH1 3LE, UK.

<sup>b</sup> Department of Chemistry, University of Turku, Vatselankatu 2, 20014 Turku, Finland.

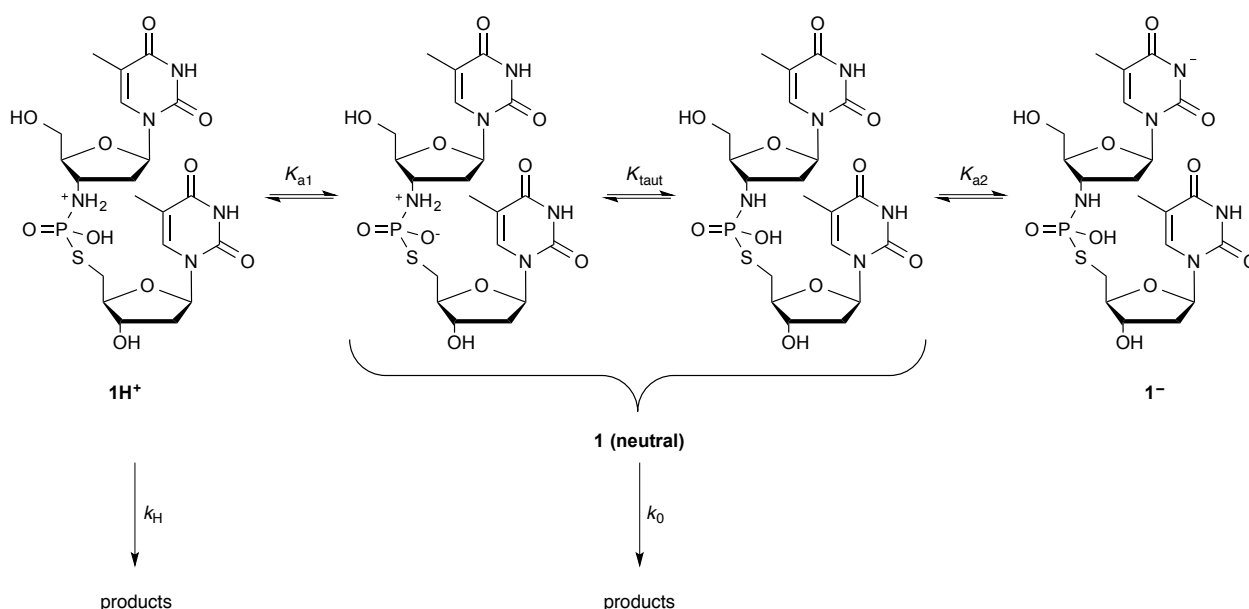
Electronic Supplementary Information (ESI) available: [<sup>1</sup>H, <sup>13</sup>C and, where applicable, <sup>31</sup>P NMR spectra of synthetic intermediates and analogue **1**, HPLC calibration data, pH-temperature corrections, further details of individual kinetics experiments, example chromatograms, and tabulated HPLC data]. See DOI: 10.1039/b000000x/

- H. J. Korhonen, L. P. Conway and D. R. W. Hodgson, *Curr. Opin. Chem. Biol.*, 2014, **21**, 63.
- N. S. Li, J. K. Frederiksen and J. A. Piccirilli, *Accounts Chem. Res.*, 2011, **44**, 1257.
- T. J. Wilson, N. S. Li, J. Lu, J. K. Frederiksen, J. A. Piccirilli and D. M. J. Lilley, *Proc. Natl Acad. Sci. USA*, 2010, **107**, 11751.
- J. E. Deweese, A. B. Burgin and N. Osheroff, *Nucleic Acids Res.*, 2008, **36**, 4883.
- K. H. Jung and A. Marx, *Cell Mol. Life Sci.*, 2005, **62**, 2080.
- R. O. Bak, A. Hendel, J. T. Clark, A. B. Kennedy, D. E. Ryan, S. Roy, I. Steinfeld, B. D. Lunstad, R. J. Kaiser, A. B. Wilkens, R. Bacchetta, A. Tsalenko, D. Dellinger, L. Bruhn and M. H. Porteus, *Hum. Gene Ther.*, 2015, **26**, A11.
- A. Hendel, R. O. Bak, J. T. Clark, A. B. Kennedy, D. E. Ryan, S. Roy, I. Steinfeld, B. D. Lunstad, R. J. Kaiser, A. B. Wilkens, R. Bacchetta, A. Tsalenko, D. Dellinger, L. Bruhn and M. H. Porteus, *Nat. Biotechnol.*, 2015, **33**, 985.
- G. Zon, *New J. Chem.*, 2010, **34**, 795.
- J. A. Brazier, J. Fisher and R. Cosstick, *Angew. Chem. Int. Edit.*, 2006, **45**, 114.
- M. K. Herrlein and R. L. Letsinger, *Nucleic Acids Res.*, 1994, **22**, 5076.
- Y. Xu and E. T. Kool, *Nucleic Acids Res.*, 1999, **27**, 875.
- S. Sando and E. T. Kool, *J. Am. Chem. Soc.*, 2002, **124**, 2096.
- H. Abe and E. T. Kool, *J. Am. Chem. Soc.*, 2004, **126**, 13980.
- S. Sando and E. T. Kool, *J. Am. Chem. Soc.*, 2002, **124**, 9686.
- S. Sando, H. Abe and E. T. Kool, *J. Am. Chem. Soc.*, 2004, **126**, 1081.
- E. M. Harcourt and E. T. Kool, *Nucleic Acids Res.*, 2012, **40**, e65.
- J. Michaelis, A. Roloff and O. Seitz, *Org. Biomol. Chem.*, 2014, **12**, 2821.

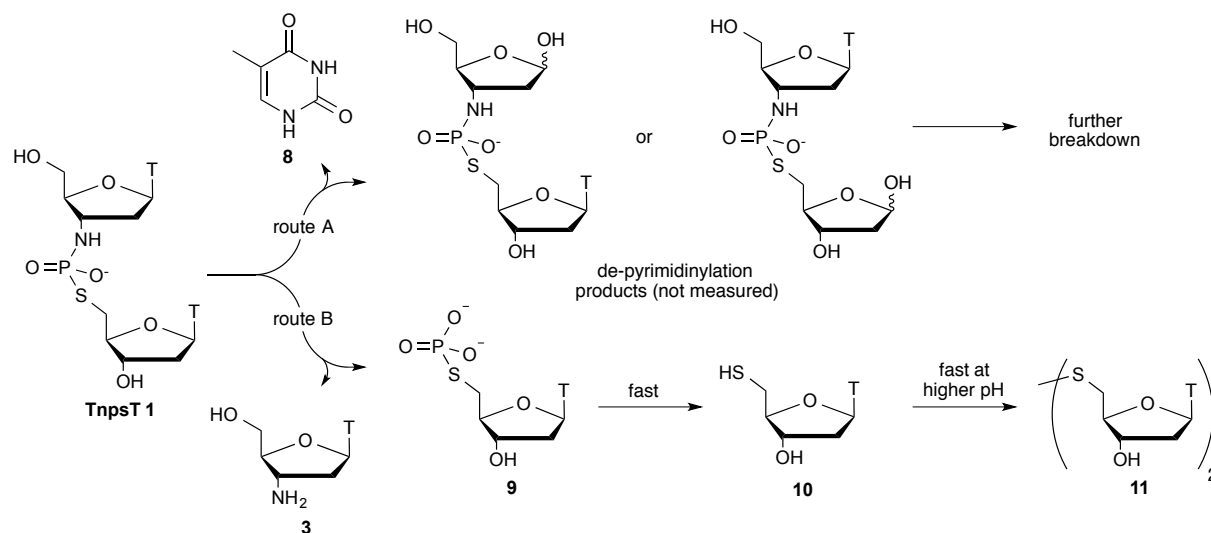
- 18 R. J. Delley, A. C. O'Donoghue and D. R. W. Hodgson, *J. Org. Chem.*, 2012, **77**, 5829.
- 19 D. Williamson and D. R. W. Hodgson, *Org. Biomol. Chem.*, 2008, **6**, 1056.
- 20 D. Williamson, M. J. Cann and D. R. W. Hodgson, *Chem. Commun.*, 2007, DOI: 10.1039/b717896d, 5096.
- 21 M. Trmcic and D. R. W. Hodgson, *Chem. Commun.*, 2011, **47**, 6156.
- 22 L. P. Conway, R. J. Delley, J. Neville, G. R. Freeman, H. J. Maple, V. Chan, A. J. Hall and D. R. W. Hodgson, *RSC Adv.*, 2014, **4**, 38663.
- 23 M. Trmcic, F. L. Chadbourne, P. M. Brear, P. W. Denny, S. L. Cobb and D. R. W. Hodgson, *Org. Biomol. Chem.*, 2013, **11**, 2660.
- 24 M. Rothlingshofer, E. Kervio, T. Lommel, U. Plutowski, A. Hochgesand and C. Richert, *Angew. Chem. Int. Edit.*, 2008, **47**, 6065.
- 25 A. Kaiser and C. Richert, *J. Org. Chem.*, 2013, **78**, 793.
- 26 D. M. Williams and V. H. Harris, *Practical Approach in Chemistry: Organophosphorus Reagents*, OUP, 1st edn., 2004.
- 27 W. Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, 1984.
- 28 C. Altona and M. Sundaralingam, *J. Am. Chem. Soc.*, 1972, **94**, 8205.
- 29 C. Altona and M. Sundaralingam, *J. Am. Chem. Soc.*, 1973, **95**, 2333.
- 30 A. P. G. Beevers, E. M. Witch, B. C. N. M. Jones, R. Cosstick, J. R. P. Arnold and J. Fisher, *Magn. Reson. Chem.*, 1999, **37**, 814.
- 31 J. Jakhilal, S. Coantic-Castex, C. Denhez, C. Petermann, A. Martinez, D. Harakat, D. Guillaume and P. Clivio, in *Chem. Commun.*, 2015, vol. 51, pp. 12381.
- 32 M. Ora, M. Murtola, S. Aho and M. Oivanen, *Org. Biomol. Chem.*, 2004, **2**, 593.
- 33 J. B. Thomson, B. K. Patel, V. Jimenez, K. Eckart and F. Eckstein, *J. Org. Chem.*, 1996, **61**, 6273.



**Scheme 1** Thiophosphorylation of 3'-amino-3'-deoxythymidine **3** and subsequent reaction with 5'-deoxy-5'-iodothymidine **5**.

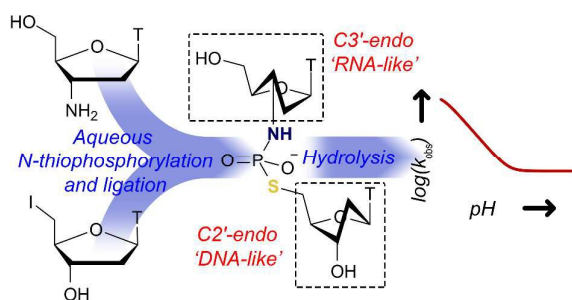


**Scheme 2** pH-dependent speciation of Tnpst **1**.



**Scheme 3** Mechanistic pathways for the hydrolysis of **TnpsT 1**.





A simple, aqueous, protecting group-free synthesis of a dinucleotide is presented, and its stability and conformation are explored.