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A Introduction

Phosphate analogues, where oxygen atoms have been replaced by nitrogen or sulfur, are important mechanistic enzymology probes. 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. Oligonucleotides containing modified phosphate groups show increased resistance towards (ribo)nucleases and enhanced therapeutic effects. Modified phosphates also offer the potential for modulating the structural properties of nucleic acid assemblies such as the i-motif.

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. We have exploited the nucleophilicity of amines to facilitate the efficient aqueous N-phosphorylation of amines, and N-thiophosphorylation plus S-alkylation of thiophosphoramidates. Amine nucleophilicity has also been harnessed to promote enzyme-free template-ligation between 3′-amino-substituted oligonucleotides and 5′-activated phosphodiester nucleosides.

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.

B Synthesis

Fig. 1 3′-Amino-3′-deoxythymidylyl-(3′→5′)-5′-deoxy-5′-thiothymidine (TnpsT 1) and its natural counterpart, thymidylyl-(3′→5′)-thymidine (TpT 2)
3’-Amino-3’-deoxythymidyl-1(3’→5’)-5’-deoxy-5’-thiothyridine (TnpS, 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’-iodothyridine 5 was prepared by 5’-tosylation of thymidine,\(^{26}\) followed by conversion to the iodide using sodium iodide in acetone.

**Scheme 1** Thio phosphorylation of 3’-amino-3’-deoxythymidine 3 and subsequent reaction with 5’-deoxy-5’-iodothyridine 5.

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)\(^{20}\) 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 thio phosphoryramidate 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 Beever et al. in the dideoxynucleoside 3’-phosphorothiolate analogue (TspT),\(^{20}\) however, a more detailed analysis,\(^{31}\) particularly in the context of extended and double-stranded nucleic acid structures will be required to confirm this result.

**D Kinetics and Mechanism of TnpS Hydrolysis**

**Kinetic Experiments**

\[ P_S = \frac{\Sigma H' - 9.8}{5.9} \] (Equation 1)
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 remaining starting material and products were resolved by HPLC, and the ratios of the integrals of the substrate and products relative to an internal standard were calculated at 90 °C based on values measured at 25 °C, see ESI. 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 kobs-buffer concentration plots were extrapolated to estimate the buffer-dependent and buffer-independent rate constants (see ESI).

The log kobs-pH profile for the hydrolysis of TnpsT 1 displayed a pH-independent ‘plateau’ from ~pH 7 to 10 with t1/2 ~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.

At pH 7.0 and 7.7, the largest detected peak by HPLC is thymine 8, derived from initial depyrimidinylitation (route A) of either thymidine site within TnpsT 1 and subsequent fragmentation of the resulting species, as seen by Ora et al. for Tnps(s)T 6 and TnpsT 7 that were studied by Ora et al. (blue and green traces in Fig. 4).22 The lack of an observed plateau in reactivity at lower pH values limits our ability to unequivocally assign values to the interdependent variables kH and Kd. Based on the fitting of the available data, however, values of kH < 0.15 M⁻¹s⁻¹ and pKd < 1 were estimated, which align with those observed for TnpsT 7.

The similarity in reactivity profiles of TnpsT 1 and TnpsT 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 depyrimidinylitation (route A) of either thymidine site within TnpsT 1 and subsequent fragmentation of the resulting species, as seen by Ora et al. for Tnps(s)T 6 and TnpsT 7.22 Given the remoteness of the phosphoryl-sites from the C1′ sites where depyrimidinylitations occur, it is unsurprising that the reactivities of TnpsT 1, Tnps(s)T 6 and TnpsT 7 in the pH independent regions are similar. At pH 6, some depyrimidinylitation 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.23 We were, however, unable to confirm this by HPLC in a MS-compatible buffer system. Thiol 10 is formed by rapid dephosphorylation of phosphothiolate 9, which

**Fig. 4** The pH-log(kobs) profiles for the hydrolyses at 90 °C of: TnpsT 1, and related systems Tnps(s)T 6 and TnpsT 7 studied by Ora et al. Kinetic data are fitted to equation 2.

**Equation 2**

\[
k_{\text{obs}} = \frac{k_H(10^{-\text{pH}})+k_Dk_{\text{A}}} {10^{-\text{pH}}+k_{\text{A}}}
\]

Data for the disappearance of TnpsT 1 were found to fit Equation 2, where rate coefficients kH and kD represent the contributions to kobs of the neutral/zwitterionic forms (neutral) and monocationic form (1H⁺) respectively (Scheme 2). The acid dissociation constant between 1H⁺ and the kinetically indistinguishable neutral forms (neutral) is captured in Kd. 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 (Kd) of (neutral), however, there are insufficient data to substantiate this hypothesis.

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

The reactivity on the pH plateau, with kH = 6.3 × 10⁻⁷ s⁻¹, is similar to the related analogues Tnps(s)T 6 and TnpsT 7 that were studied by Ora et al. (blue and green traces in Fig. 4).22 The lack of an observed plateau in reactivity at lower pH values limits our ability to unequivocally assign values to the interdependent variables kH and Kd. Based on the fitting of the available data, however, values of kH < 0.15 M⁻¹s⁻¹ and pKd < 1 were estimated, which align with those observed for TnpsT 7.

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

The similarity in reactivity profiles of TnpsT 1 and TnpsT 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).
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 $k_{obs}$ profiles of TnpsT 1 and Tnp 7 in the acidic region suggests that either the values of $k_a$ and $k_o$ are identical for these species, or that ionisation and reactivity compensate each other to arrive at identical $k_{obs}$ 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 Tnp 7, where for pH=7, de-pyrimidination 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'-deoxynucleoside 3, hydrochloride salt.

Adapting a literature procedure, 3'-azido-3'-deoxynucleoside (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 x 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); $\nu_{max}$ cm$^{-1}$ 3392, 3032, 1694, 1644, 1470; $\delta$ (400 MHz, D$_2$O) 1.86 (3H, s, C5-CH$_3$), 2.54-2.70 (2H, m, C2'-H$_2$), 3.81 (1H, dd, J 12.6, 4.6, C5'-H$_3$), 3.89 (1H, dd, J 12.6, 3.4, C5'-H$_3$), 4.06 (1H, dt, J 8.1, 5.5, C3'-H$_3$), 4.19-4.28 (1H, m, C4'-H$_3$), 6.28 (1H, t, J 6.8, C1'-H$_3$), 7.62 (1H, dd, J 1.1, C6-H$_3$); $\delta$ (100 MHz, D$_2$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)$^+$, 100%), requires 242.1141, 264.0961 [(M+Na)$^+$, 90].

5'-Deoxy-5'-[4-toluenesulfonyl]thymidine. Adapting a literature procedure, 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 x 60 ml). The organic extracts were washed with saturated sodium bicarbonate solution (40 ml), and water (40 ml) before being dried over MgSO$_4$. 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%). $\delta$ (400 MHz, (CD$_3$)$_2$SO) 1.76 (3H, s, C5-CH$_3$) 2.02-2.09 (1H, m, C2'-H$_3$H$_3$) 2.11-2.19 (1H, m, C2'-H$_3$H$_3$) 2.41 (3H, s, CH$_3$Ar) 3.83-3.88 (1H, m, C3'-H$_3$), 4.12-4.20 (2H, m, C4'-H and C5'-H$_3$H$_3$) 4.25 (1H, dd, J 7.2, 3.4, C5'-H$_3$H$_3$) 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$_2$Ph) 7.79 (2H, d, J 8.3, o-OSO$_2$Ph) 11.33 (1H, s, NH) $\delta$ (400 MHz, (CD$_3$)$_2$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%). $\delta$ (400 MHz, (CD$_3$)$_2$SO) 1.79 (3H, s, C5-CH$_3$) 2.07 (1H, dd, J 13.5, 6.2, 3.1, C2'-H$_3$H$_3$) 2.29 (1H, dd, J 13.5, 8.1, 6.2, C2'-H$_3$H$_3$) 3.39 (1H, dd, J 10.4, 6.3, C5'-H$_3$H$_3$) 3.52 (1H, dd, J 10.4, 6.3, C5'-H$_3$H$_3$) 3.80 (1H, dt, J 6.2, 2.8, C3'-H$_3$) 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'-deoxynucleoside 3, hydrochloride salt 1. Et$_3$N$^+$H

First 3'-amino-3'-deoxynucleoside-5'-thiophosphoramidate 4 was prepared according to the previously reported phosphorylation procedure. 3'-Amino-3'-deoxynucleoside, hydrochloride salt 3.HCl (1.38 mg, 0.500 mmol) was dissolved in water and made up to 5 ml at pH 12 with potassium hydroxide solution (1 M). Thiophosphoryl chloride solution (1.50 ml, 0.333 M in MeCN) was added slowly using a Hamilton® 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 1.00 M solution of potassium hydroxide, added by an autotrigger equipped with a pH probe. The experiment was considered to be complete when the autotrigger needed to add negligible volumes of potassium hydroxide solution to the reaction mixture in order to maintain a constant pH. The lyophilised phosphoramidate was redisissolved 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 autotrigger. 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 $^3$P NMR spectroscopy. The S-alkylation process was monitored based on the disappearance of the signal for thioephosphoramidate 4 at $\delta$ $\approx$ 44 ppm, and the appearance of a signal for TnpST 1 at $\delta$ $\approx$ 22 ppm. The reaction was continued until the unalkylated thioephosphoramidate 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® resin. Triethylammonium bicarbonate buffer was applied to 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'-$\rightarrow$5')-5'-deoxy-5'-thiophymidine as its triethylenammonium salt 1. Et$_3$N$^+$H (212 mg, 64%). $\delta$ (700 MHz, D$_2$O) 1.29 (9H, t, J 7.3, $\text{HN}$(CH$_2$CH$_3$)$_3$) 1.88 (3H, d, J 1.2, A-or B- C$^5$H$_3$) 1.89 (3H, d, J 1.2, A-or B-C$^5$H$_3$), $\delta_c$ (151 MHz, D$_2$O) 8.2 (HN$^+$2CH$_2$CH$_3$)), 11.5 (A-and B-C$^5$H$_3$), 32.0, (B-C$_3$'), 38.0 (B-C$_3'$), 46.6 (HN$^+$2CH$_2$CH$_3$)), 58.9 (A-C$'3$), 60.2, (A-C$'5$), 72.0 (B-C$'3$), 84.5 (A-C$'1$), 84.7 (B-C$'1$), 84.9 (B-C$'4$), 85.5 (A-C$'4$), 111.1 (A-or B-C$'5$), 111.3 (A-or B-C$'5$), 137.4 (A-or B-C$'6$), 137.5 (A-or B-C$'6$), 151.4 (A-and B-C$'2$), 166.1 (A-or B-C$'4$), 166.3 (A-or B-C$'4$), 171.1; $\delta_p$ (162 MHz, D$_2$O) 21.6 (1P, app. q, J 10.0, HNPO$_2$S); m/z 560.1208 ([M-H$^+$Na$^+$]$,^{100\%}$) requires 560.1216, 1121.2450 ([2M-2K$^+$H$^-$]).

**HPLC standard - potassium p-nitrobenzensulfonate, 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%) $\delta_p$ (400 MHz, D$_2$O) 8.00 (2H, d, J 8.9, 1H m- to sulfonate), 8.35 (2H, d, J 8.9, 1H o- to sulfonate); $\delta_c$ (101 MHz, D$_2$O) 124.3, 126.9, 148.1, 149.1.

**Hydrolysis studies**

See ESI for details of the hydrolysis studies.

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**H Notes and references**

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Electronic Supplementary Information (ESI) available: [H $^3$C and where applicable, $^3$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/


**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 TnpsT1.
A simple, aqueous, protecting group-free synthesis of a dinucleotide is presented, and its stability and conformation are explored.