Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.
This journal is © The Royal Society of Chemistry 2016

The Synthesis and Hydrolytic Stability of an N,S-bridging Thiophosphoramidate Analogue of Thymidylyl-3′,5′-Thymidine

-

Supplementary Information

Louis P. Conway[a], Satu Mikkola[b], AnnMarie C. O'Donoghue[a], and David R. W. Hodgson[*a]

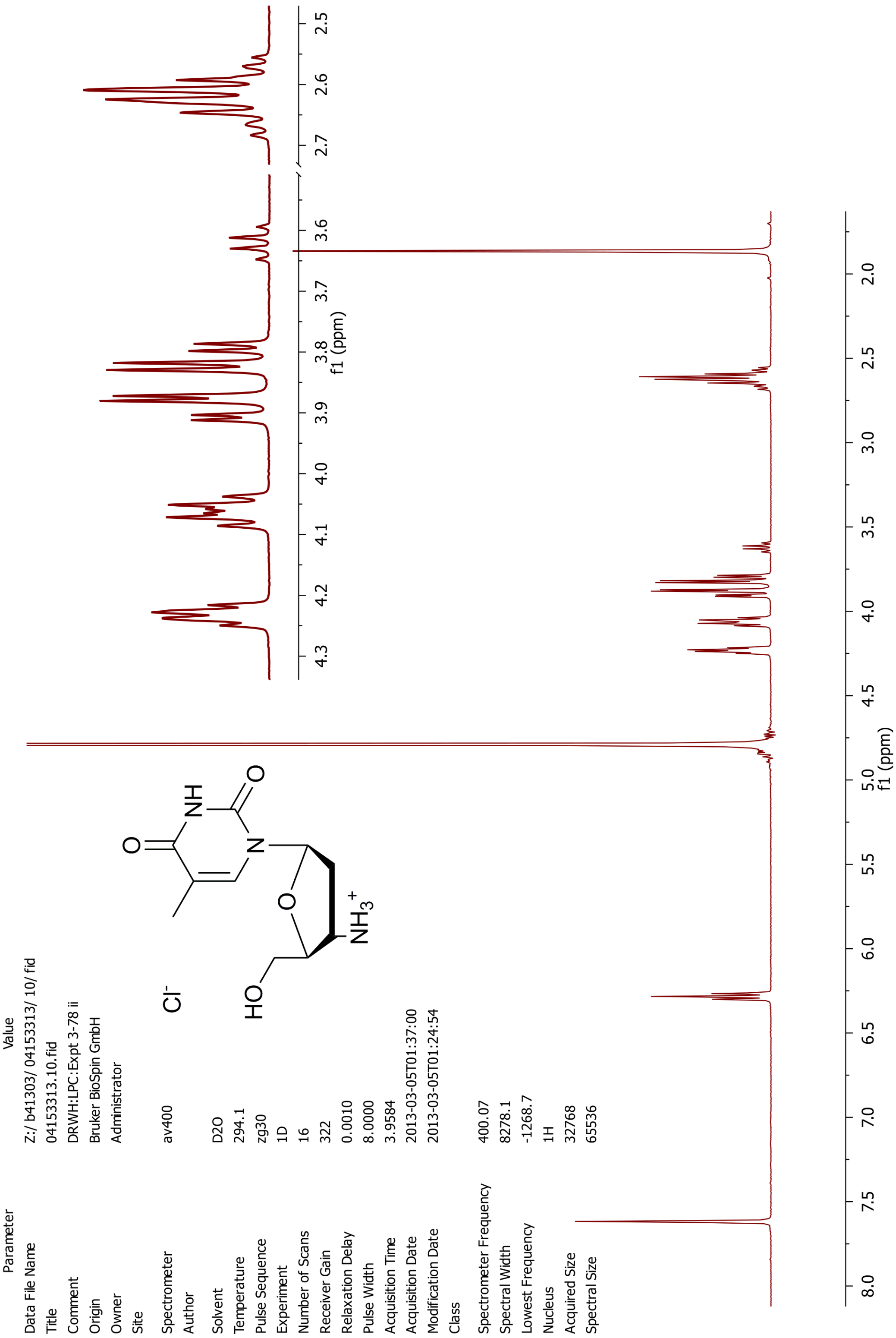
[a]Department of Chemistry, Durham University, South Road, Durham, DH1 3LE, United Kingdom
[b]Department of Chemistry, University of Turku, Vatselankatu 2, 20014 Turku, Finland
[*] *d.r.w.hodgson@durham.ac.uk*

July 5, 2016

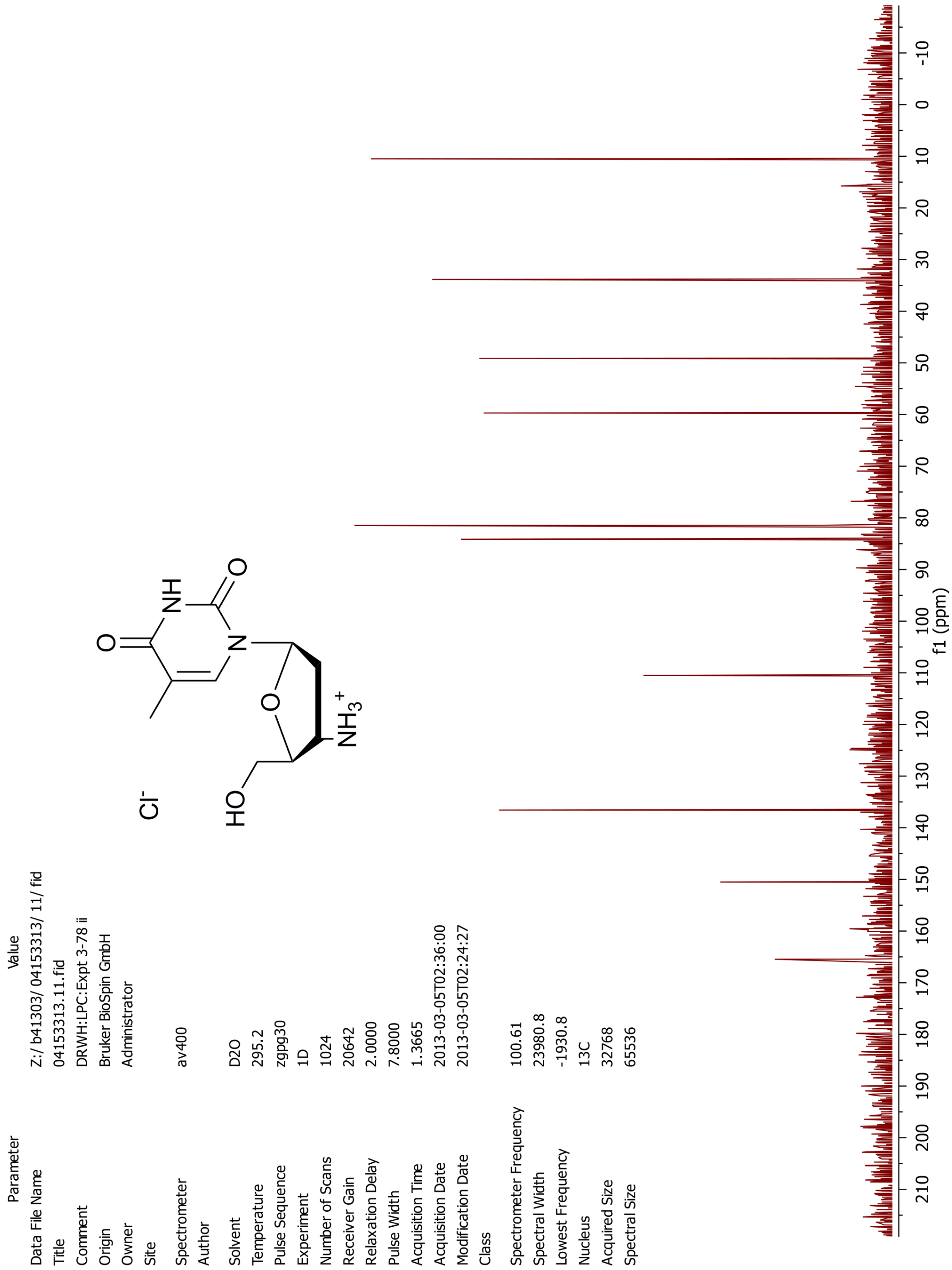
Supplementary Information

NMR Data

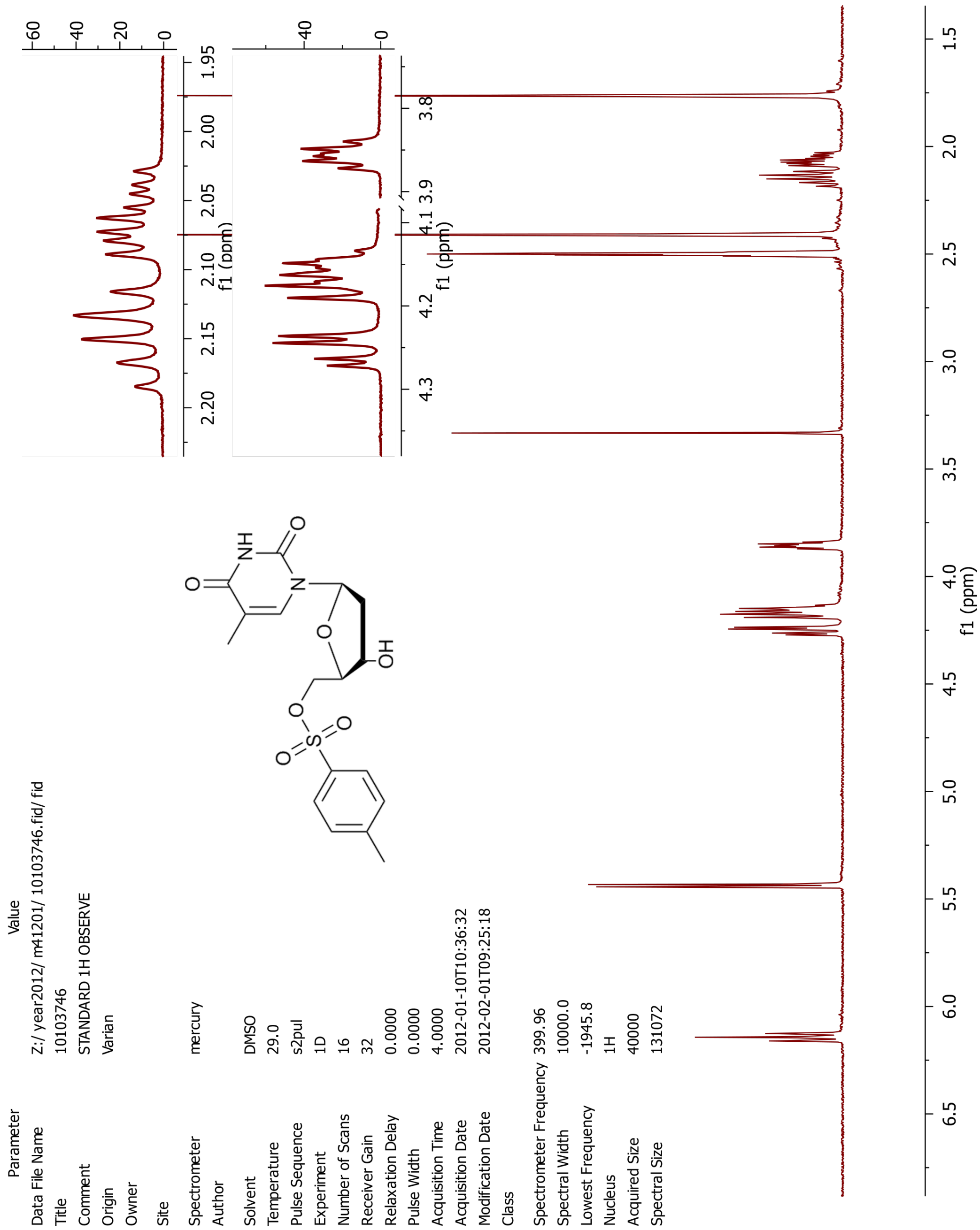
3′-Amino-3′-deoxythymidine (3), hydrochloride salt, ^{1}H NMR Spectrum



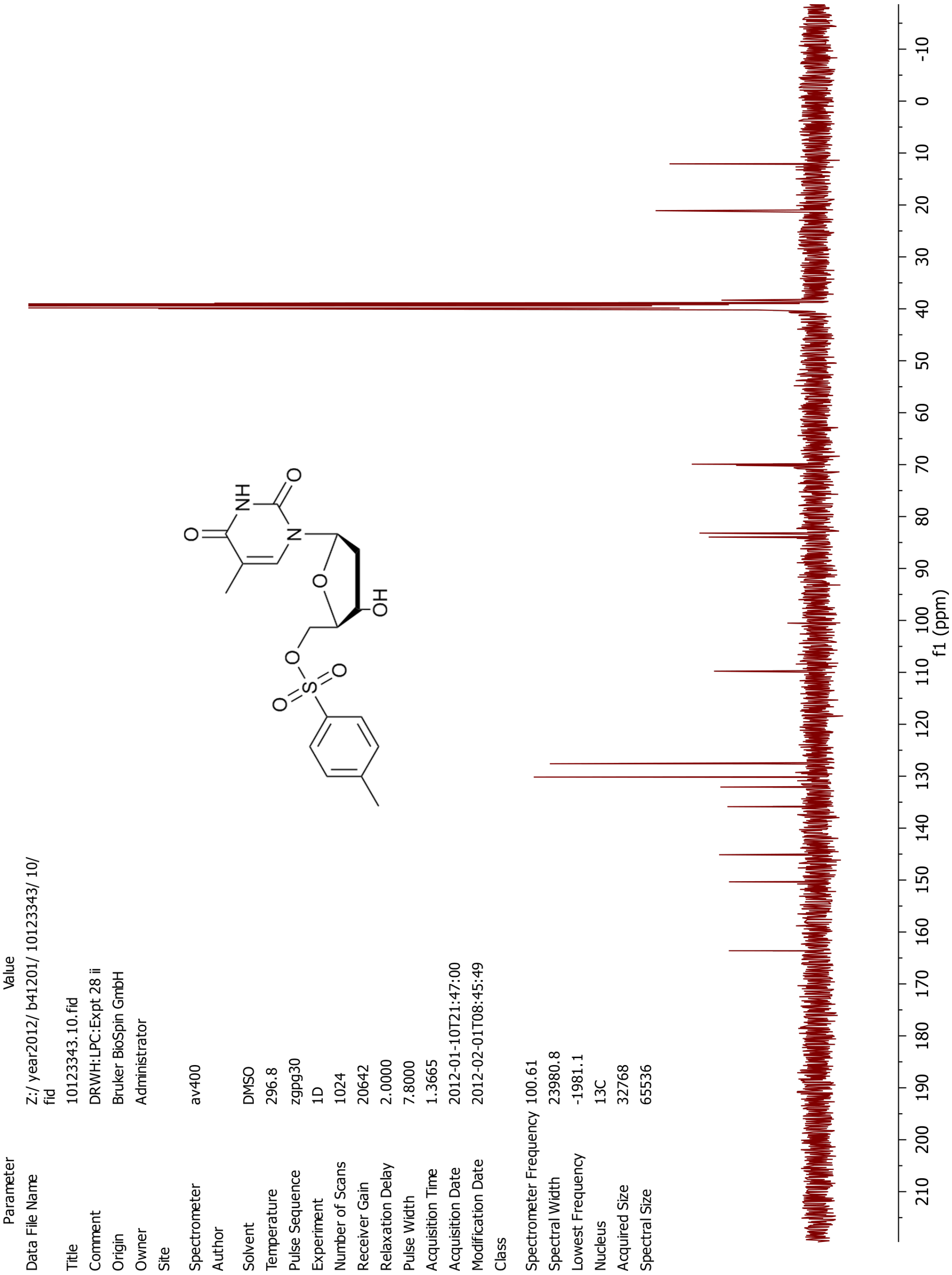
3′-Amino-3′-deoxythymidine (3), hydrochloride salt, ^{13}C NMR Spectrum



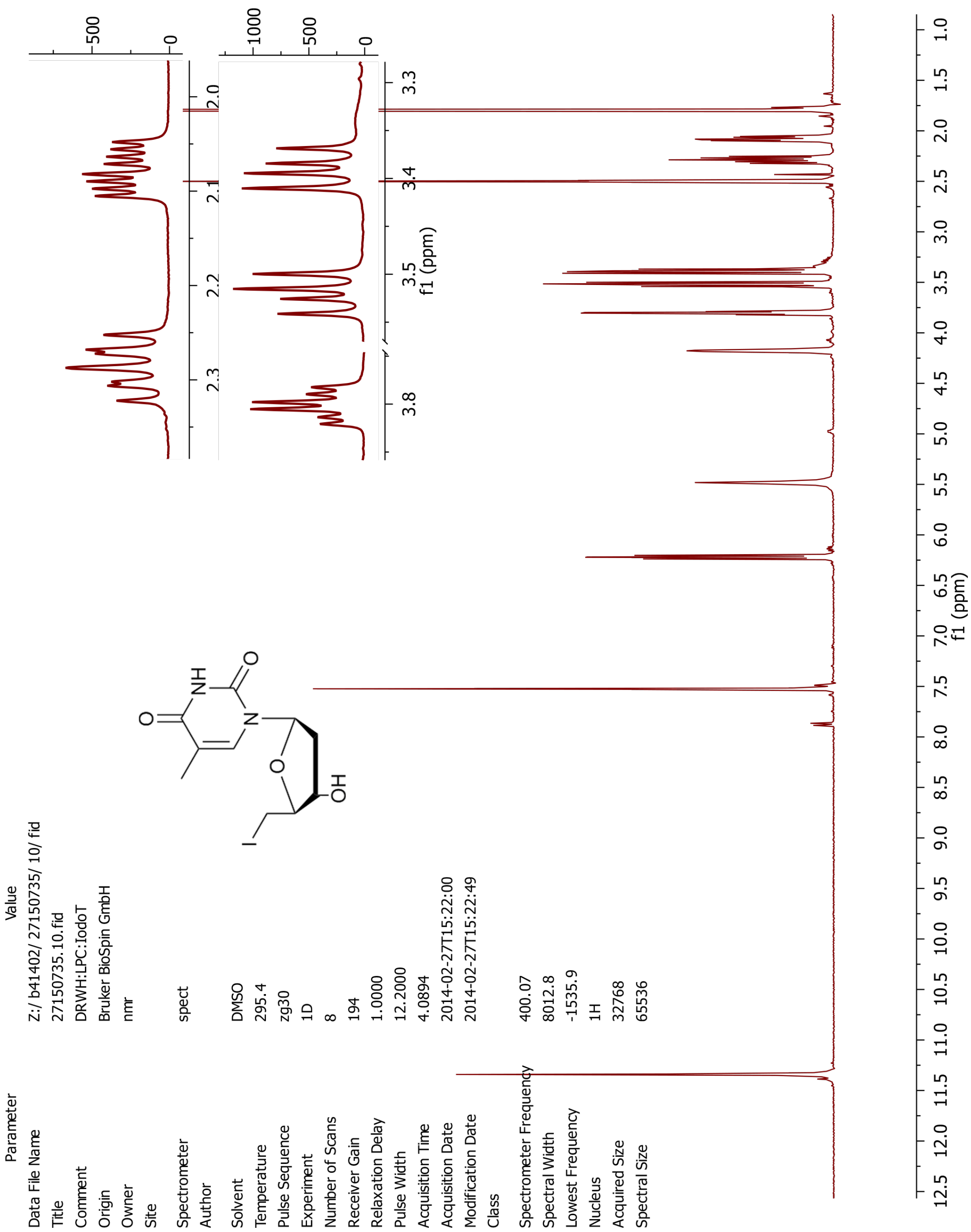
5′-Deoxy-5′-(4-toluenesulfonyl)thymidine, ^{1}H NMR Spectrum



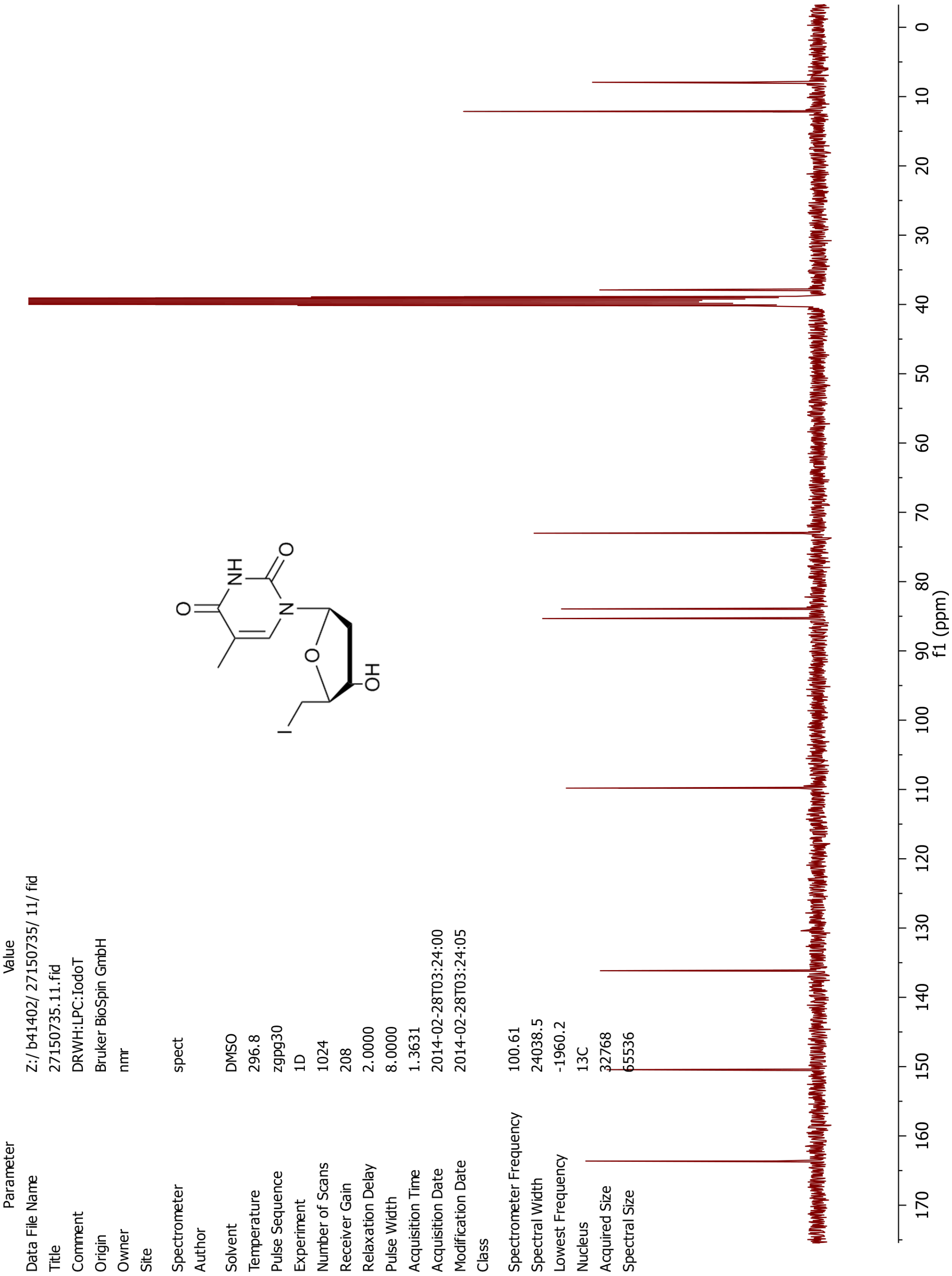
5′-Deoxy-5′-(4-toluenesulfonyl)thymidine, ^{13}C NMR Spectrum



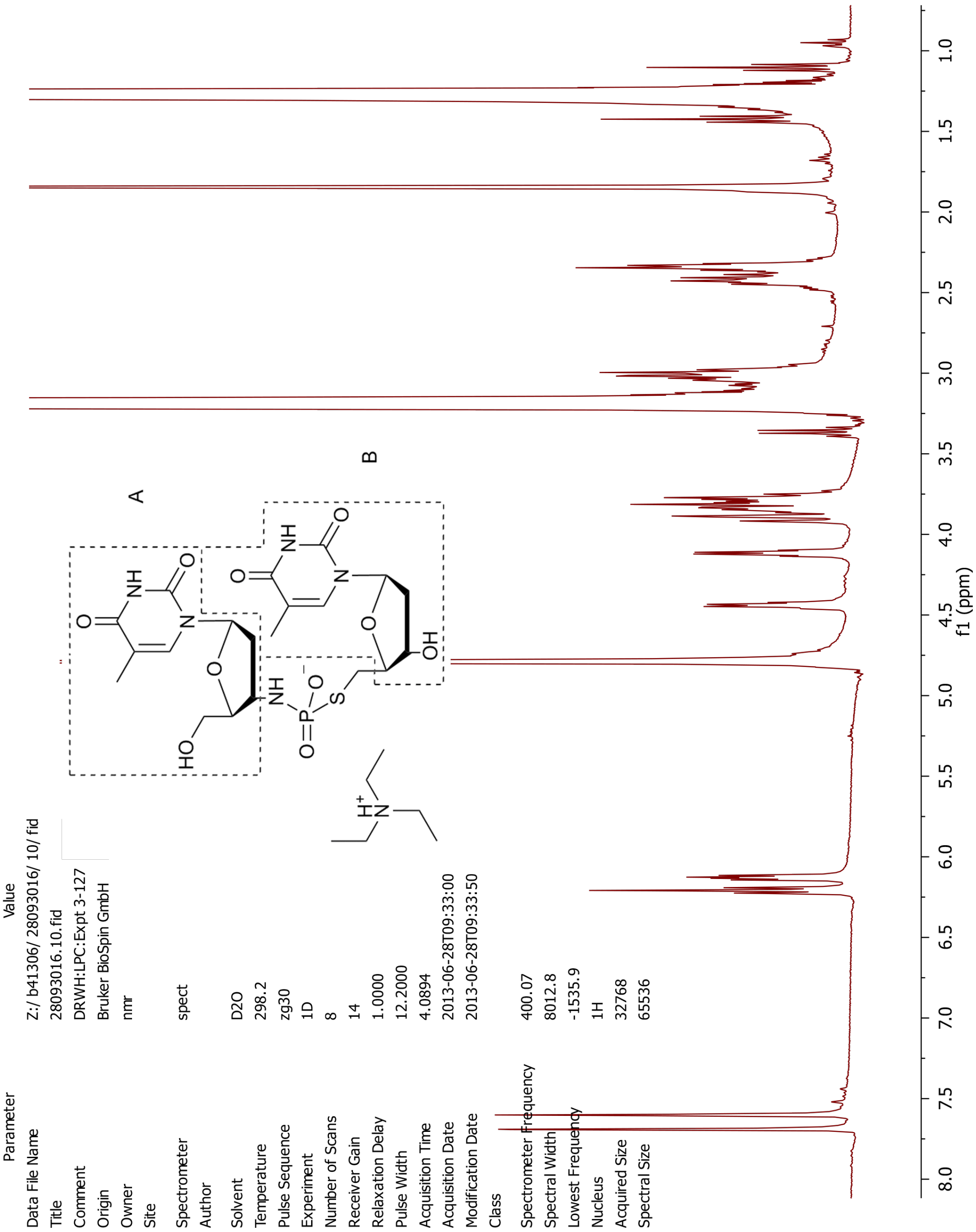
5′-Deoxy-5′-iodothymidine (5), ^{1}H NMR Spectrum



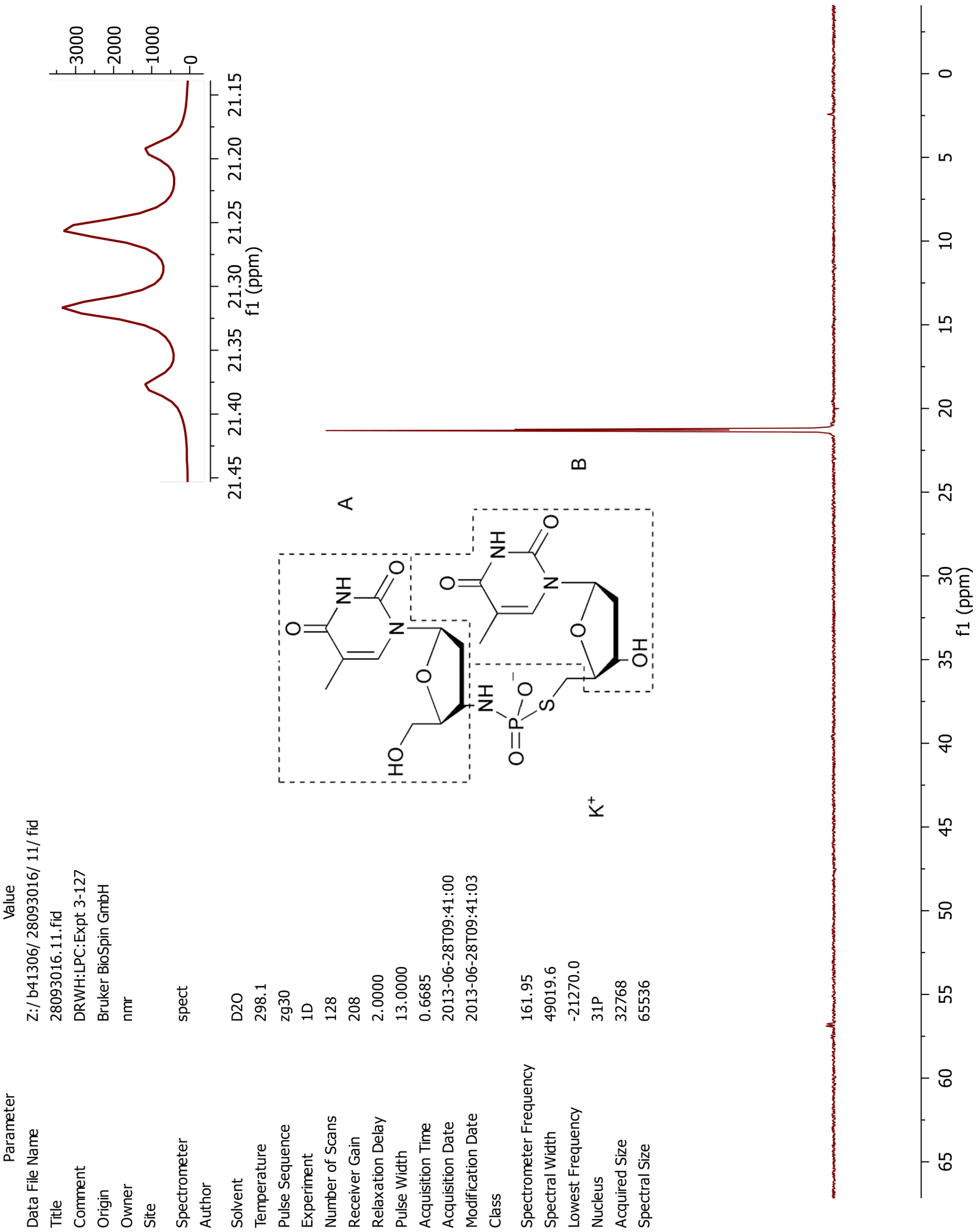
5′-Deoxy-5′-iodothymidine (5), ^{13}C NMR Spectrum



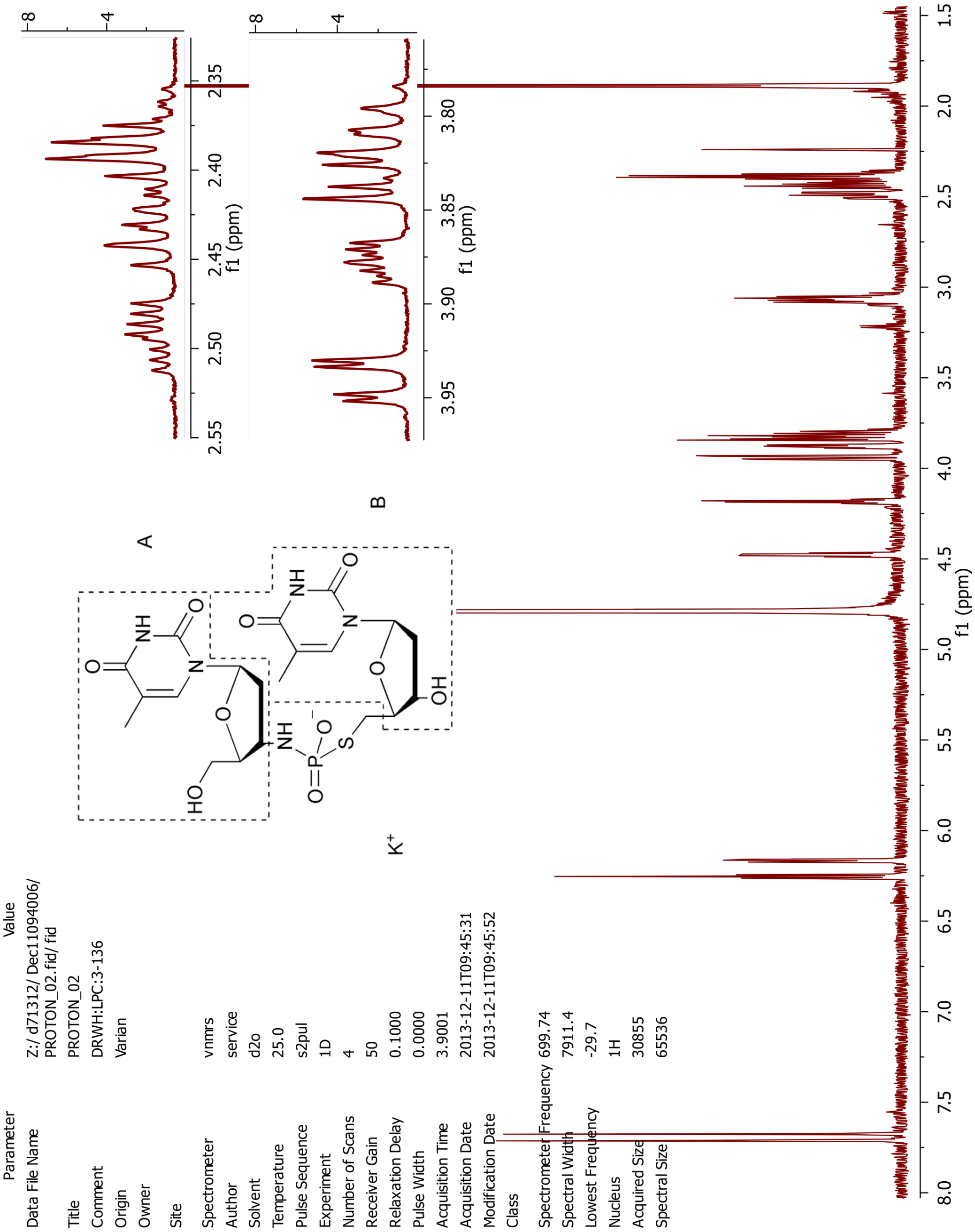
3′-Amino-3′-deoxythymidylyl-(3′→5′)-5′-deoxy-5′-thiothymidine (1), ammonium salt, ^{1}H NMR Spectrum



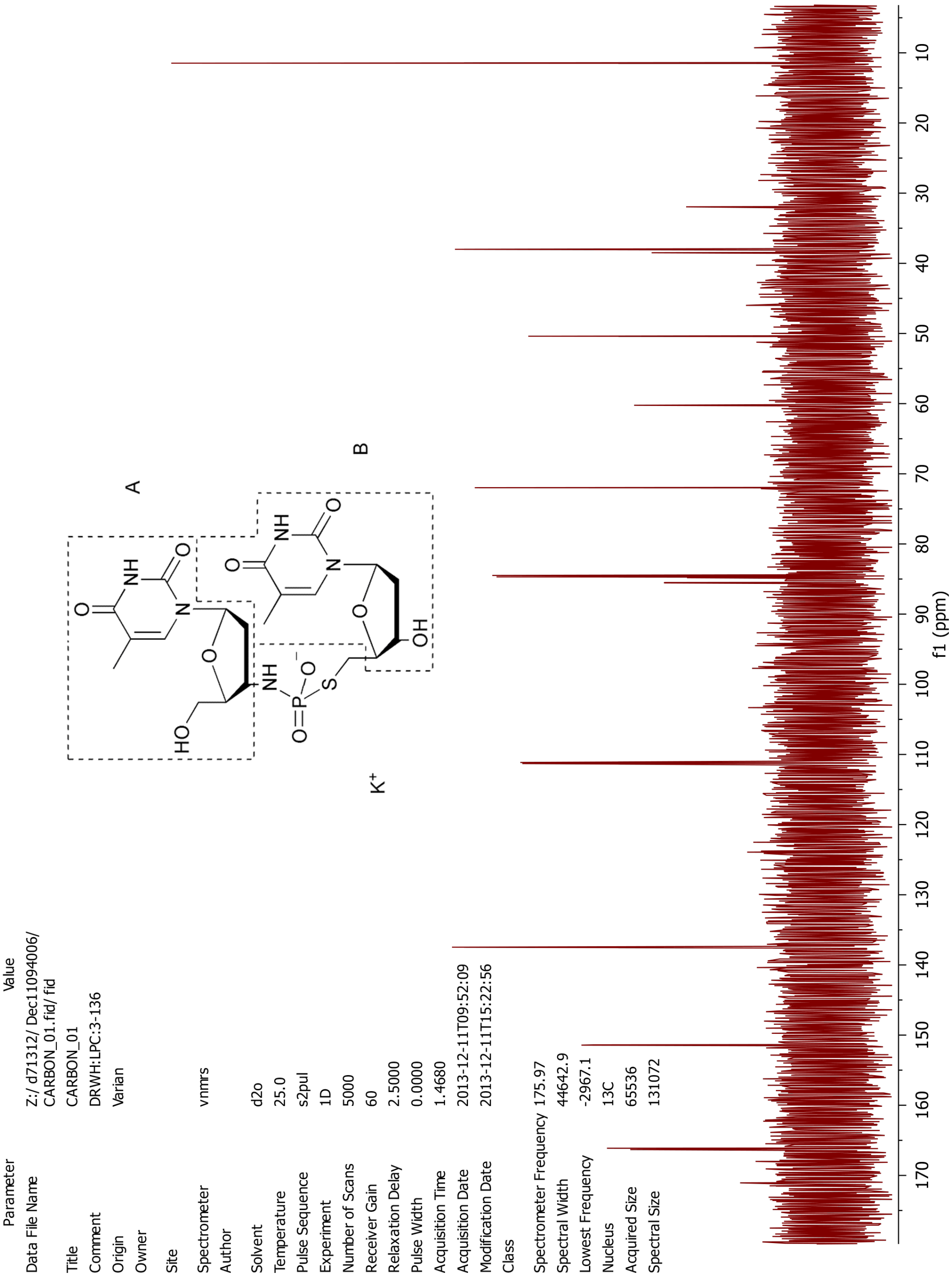
3′-Amino-3′-deoxythymidylyl-(3′→5′)-5′-deoxy-5′-thiothymidine (1), ammonium salt, ^{31}P NMR Spectrum



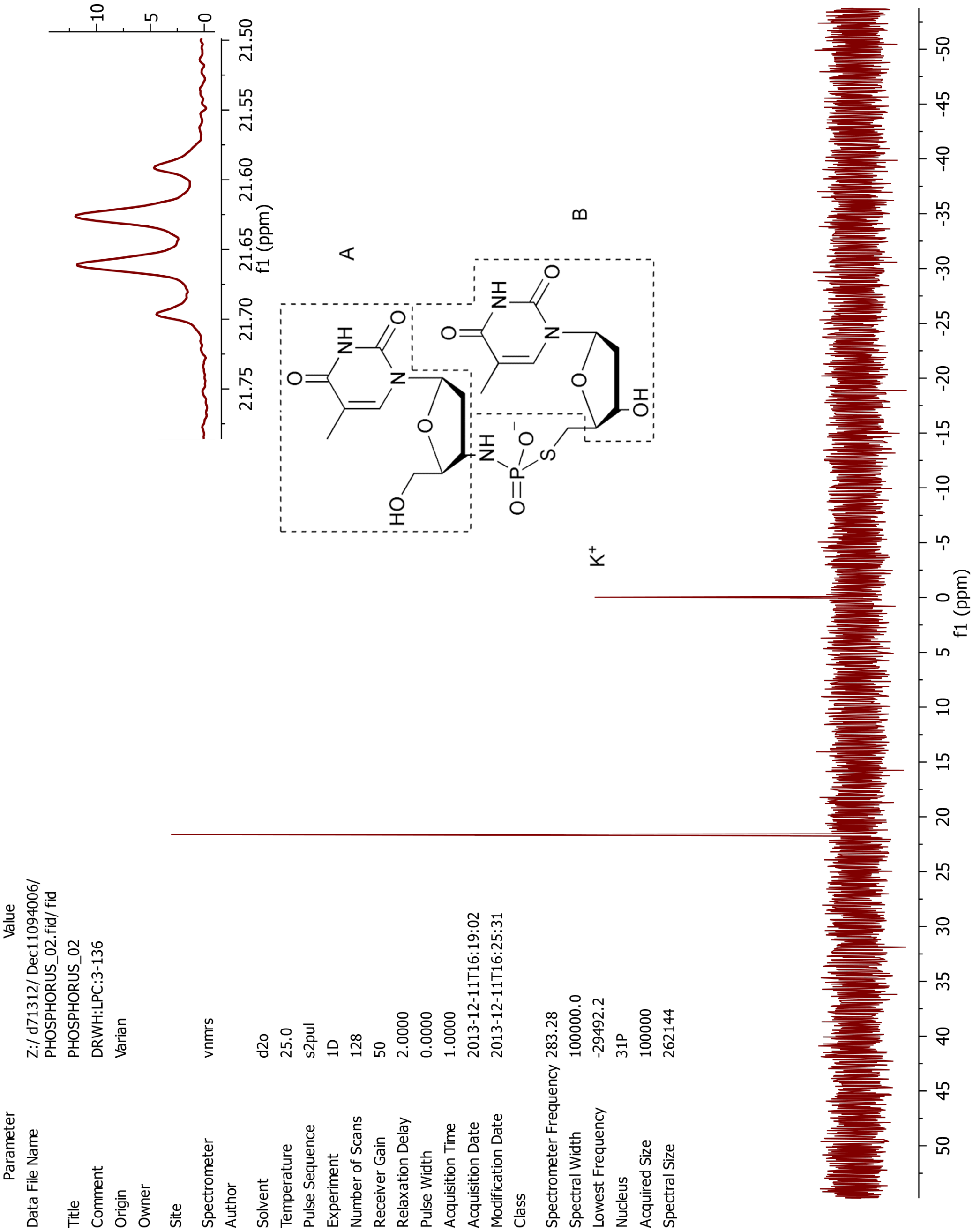
3′-Amino-3′-deoxythymidylyl-(3′→5′)-5′-deoxy-5′-thiothymidine (1), potassium salt, ^{1}H NMR Spectrum



3′-Amino-3′-deoxythymidylyl-(3′→5′)-5′-deoxy-5′-thiothymidine (1), potassium salt, ^{13}C NMR Spectrum



3′-Amino-3′-deoxythymidylyl-(3′→5′)-5′-deoxy-5′-thiothymidine (1), potassium salt, ^{31}P NMR Spectrum



Kinetic Data

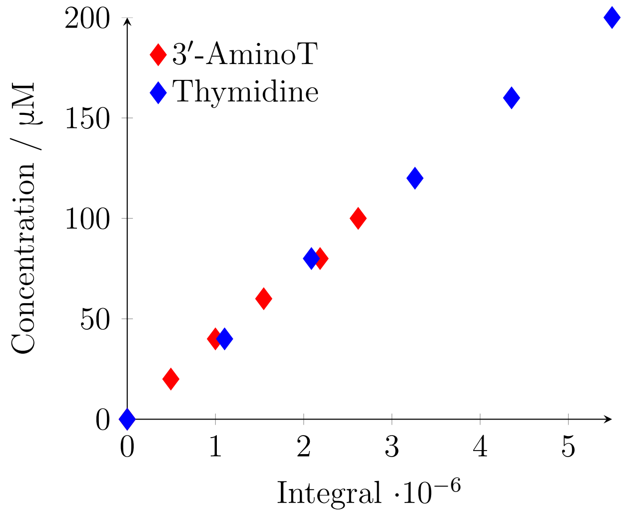
Substrate HPLC Calibration Curves

Samples of the substrate and some of the expected hydrolysis products were analysed by HPLC and the relationships between the UV/vis (254 nm) response and the concentration of the sample were investigated to ensure that they were linear. In addition, we used these data to determine the concentration of TnpsT **1**.

Concentration	**Integral** $\times 10^{-6}$	
/ μM	**3′-AminoT:**	**Thymidine:**
0	0.0000	0.0000
20	0.4945	
40	0.9994	1.1036
60	1.5470	
80	2.1858	2.0874
100	2.6177	
120		3.2613
160		4.3562
200		5.4954

Analysis

$$concentration = m \times integral$$



	3′-AminoT:	Thymidine:
Flow rate / ml min^{-1} :	0.9	0.9
m / M V^{-1} s^{-1}:	3.79 $\times 10^{-11}$	3.67 $\times 10^{-11}$
Std. error / M V^{-1} s^{-1}:	5 $\times 10^{-13}$	3 $\times 10^{-13}$
R^2:	0.9992	0.9998

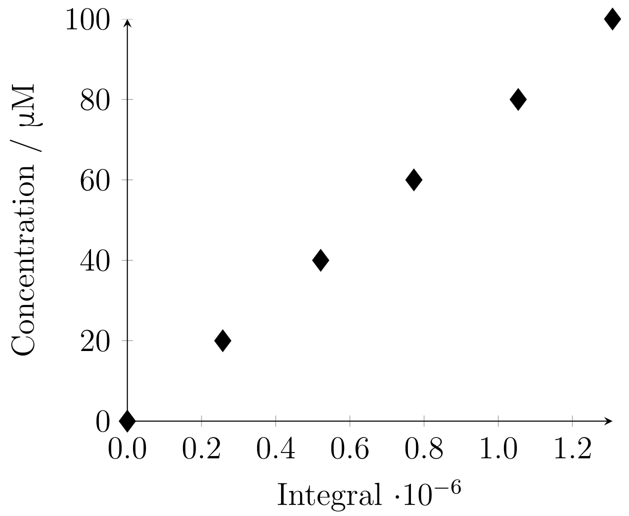
The R^2 values very close to unity indicate that the relationships can be considered to be linear over this range of concentrations.

Determination of Concentration of TnspT (1)

A 60 µg/ml solution in water of salt **1**·K^+ was prepared, and a set of serial dilutions was analysed using the conditions described above.

Concentration / µM	**Integral** $\times\ 10^{-6}$
0	0.0000
20	0.2573
40	0.5213
60	0.7726
80	1.0539
100	1.3082

$$concentration = m \times integral$$



Flow rate / ml min^{-1} :	1.0
m / M V^{-1} s^{-1}:	7.65 $\times 10^{-11}$
Std. error / M V^{-1} s^{-1}:	3 $\times 10^{-13}$
R^2:	0.99994

A linear response to serial dilution was observed, as expected. Making the assumption that $\epsilon_\lambda(TnpsT) = 2 \times \epsilon_\lambda(Thymidine)$, we determined the concentration of TnpsT **1** to be:

$$[TnpsT]_{stock} = \frac{100\,\mu M}{2} \times \frac{3.67}{7.65} = 24\,\mu M$$

HPLC Conditions and Retention Times

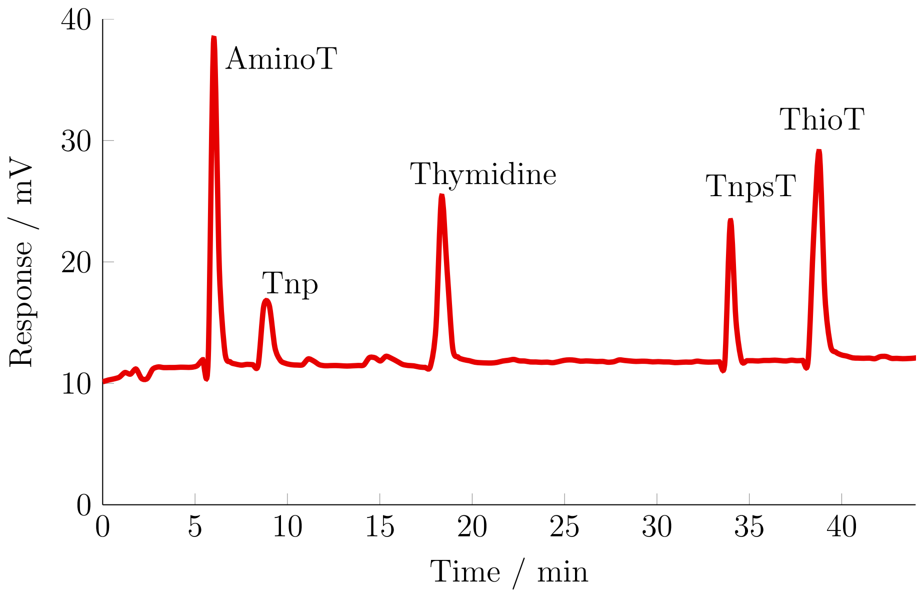
The parameters and elution method (based on the method used by Ora *et al.*)[1] recorded below were used for all the kinetic experiments analysed through HPLC. A PerkinElmer Series 200 HPLC system was used, with a Phenomenex Hyperclone 5 µ C18 column measuring 250 mm in length and 3.2 mm in diameter. Eluent A was a solution of sodium acetate (15 mM), acetic acid (45 mM), and ammonium chloride (100 mM); eluent B was acetonitrile.

Chromatograms were processed using the TotalChrom™ Chromatography Data System from PerkinElmer on Microsoft® Windows® XP.

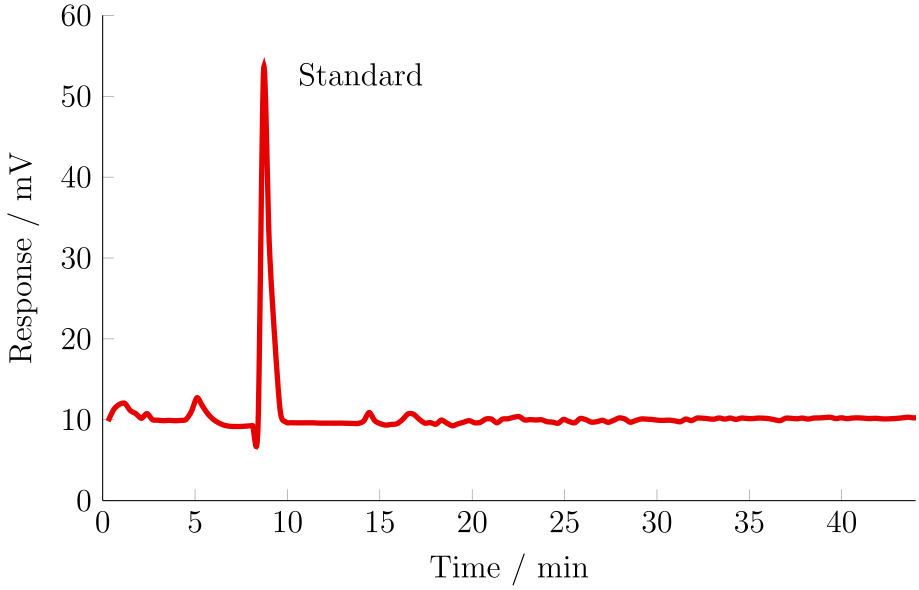
Parameter:	**Value:**	**Unit:**
Sampling Rate:	2.2727	s^{-1}
Injection Volume:	50	µl
Detector A:	254	nm
Detector B:	280	nm

Step:	**Time / min:**	**Flow / ml min^{-1}:**	**A %**	**B %**	**Curve**
0	0.5	1.00	100.0	0.0	0.0
1	10.0	1.00	100.0	0.0	0.0
2	32.0	1.00	90.0	10.0	1.0
3	12.0	1.00	90.0	10.0	0.0
4	2.0	1.00	100.0	0.0	1.0
5	3.0	1.00	100.0	0.0	0.0

Example Chromatogram of Hydrolysis Products



Chromatogram of *p*-Nitrobenzenesulphonate Standard



Compound:	**Retention Time / min:**
3′-Amino-3′-deoxythymidine	5.7
Tnp	6.6
spT	7.4
Potassium *p*-nitrobenzenesulphonate	9.8
Thymidine	18.3
TnpsT	34.5
5′-Deoxy-5′-thiothymidine	38.7

Buffer pH and Temperature

The pH values of the buffer solutions are measured at 25 °C. However, since the hydrolysis experiments were being carried out at 90 °C, the change in pH of a buffer solution between these temperatures were calculated.

The dependence of the pH of a buffer composed of a weak acid and its salt with temperature is described by the equation:[2]

$$\frac{\partial pH}{\partial T} \approx -\frac{\partial logK}{\partial T} - (2z+1)\frac{\partial log\gamma}{\partial T} \tag{1}$$

Where pH is the negative common logarithm of the activity of the hydronium ion, T is the absolute temperature, K is the acid dissociation constant of the buffer species, z describes the ionisation of the acid (see Table 1), and γ is the activity coefficient of an average univalent ion.

Table 1: The values of z for equation 1 depend on the ionisation characteristics of the buffer system[2]

Acid:	**Base:**	z
HB^{2+}	B^{+}	+1
HB^{+}	B	0
HB	B^{-}	-1
HB^{-}	B^{2-}	-2
HB^{2-}	B^{3-}	-3

The $\partial log\gamma/\partial T$ term is typically small and can be neglected, and so our equation becomes:

$$\frac{\partial pH}{\partial T} \approx -\frac{\partial logK}{\partial T} \tag{2}$$

The relationship between the acid dissociation constant and temperature is described by the van't Hoff equation:

$$\frac{\partial lnK}{\partial T} = \frac{\Delta H^{\circ}}{RT^2} \tag{3}$$

Where ΔH° is the molar enthalpy change for the dissociation of the acid and R is the gas constant.

In order to get the equation into the $\partial logK/\partial T$ form, the identity $ln(x) =$

$ln(10)log(x)$ must be substituted to give:

$$\frac{\partial logK}{\partial T} = \frac{\Delta H^\circ}{ln(10)RT^2} \tag{4}$$

Thus, if ΔH° can be found in the literature, it may be used directly. Alternatively, values for $\partial logK/\partial T$ for a number of electrolytes have been determined, in the form of $\Delta pH/^\circ C$ at 20 or 25 °C, and by substituting these into equation 5, ΔH° may be determined.

Combining equations 2 and 4, we arrive at:

$$\frac{\partial pH}{\partial T} \approx -\frac{\Delta H^\circ}{ln(10)RT^2} \tag{5}$$

Integrating this (and assuming that ΔH° is independent of temperature):

$$\int_{pH_1}^{pH_2} \partial pH \approx -\int_{T_1}^{T_2} \frac{\Delta H^\circ}{ln(10)RT^2} \partial T \tag{6}$$

$$\Delta pH \approx \frac{\Delta H^\circ}{ln(10)R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \tag{7}$$

As an example, the value of $\partial logK/\partial T$ at 25°C (298.15 K) for acetic acid is reported to be $-0.0002\ K^{-1}$. By substituting these values into equation 4, the value of $\Delta H^\circ/ln(10)R$ is found to be -18 K. The change in pH of an acetate buffer between 25 °C (298.15 K) and 90 °C (363.15 K) can then be calculated to be 0.011, indicating that acetate is relatively insensitive to temperature. Performing the same series of calculations with the $\partial logK/\partial T$ reported for the second ionisation of phosphate ($0.0024\ K^{-1}$) yields a pH change of -0.13, a more significant difference.

Equation 7 can be plotted alongside the linear extrapolation, using the autoionisation of water as an example ($\partial logK/\partial T$ of 0.033 at 25 °C), and this illustrates that while at temperatures close to 25 °C the approximation is quite good, at higher temperatures, there is a significant divergence.

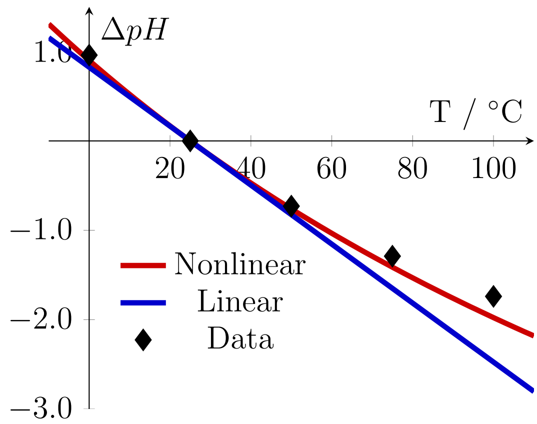


Figure 1: The linear (blue) and nonlinear (red) extrapolated relationships between temperature and ΔpH. It can be seen that there is a significant difference between the two at higher temperatures. The nonlinear equation more closely follows the measured values.

The measured values[3] do deviate from equation 7 at higher temperatures, probably due to $\Delta H°$ itself having a dependence on temperature.

At low pH values, solutions of hydrochloric acid are used, in a great enough excess over the substrate that the change in pH is negligible over the course of the reaction. Neglecting the thermal expansion of the solvent, there should be no change in pH when the solution is heated to 90 °C since the acid is effectively fully dissociated; the concentration of protons will be the same in both cases. At high pH values, when potassium hydroxide is used, the concentration of hydroxide is constant, but the concentration of protons will be dependent on the equilibrium constant for the autoionisation of water (K_W), which is temperature dependent. The values of the equilibrium constant at several temperatures can be found in the literature;[3] these can be fitted to equation 7 (see Figure 2) and the value of $\Delta H°$ can thus be determined.

The value of $\Delta H°$ is found to be -51 kJ mol^{-1}, and the change in the equilibrium constant can now be estimated for any temperature required.

The buffer pH values for the kinetic experiments were measured at 25 °C and the hydrolyses were performed at a temperature of 90 °C. The changes in pH for the various buffers used in the study, calculated as above, are tabulated

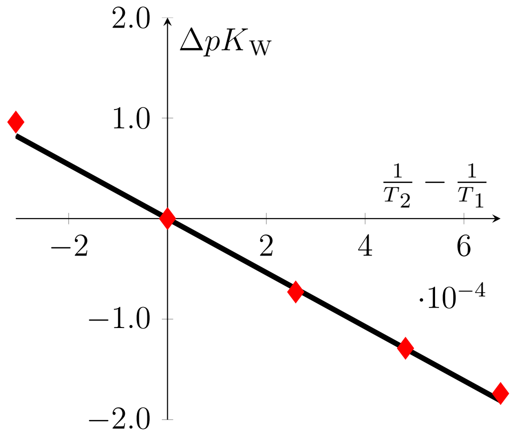


Figure 2: The literature values for the equilibrium constant for the autoionisation of water can be fitted to equation 7 and interpolated to determine the change in pK_W at any temperature in the liquid range of water at standard pressure. ΔpK_W is relative to the pK_W at T_2, 298.15 K

in Table 2.

Table 2: The change in pH from from 25 to 90 °C calculated for the various buffers used in the hydrolysis experiments

Buffer:	**∂logK/∂T**	**ΔpH**
Formate	-0.0001 (25 °C)[2]	0.01
Acetate	-0.0002 (25 °C)[2]	0.01
MES	0.011 (20 °C)[4]	-0.62
Phosphate (K_{2a})	0.0024 (25 °C)[2]	-0.13
Bicine	0.018 (20 °C)[4]	-0.93
Borate	0.0082 (25 °C)[2]	-0.44
K_W	[3]	-1.65

Sample Preparation

In this section, the procedures for the preparation of the hydrolytic experiments using TnpsT as a substrate are described. The pH of each buffer, hydrochloric acid, or potassium hydroxide solution prepared according to the following protocols was measured at 25 °C and the corresponding pH at 90 °C was calculated according to the method described in the previous section.

General Procedure for pH 3.54 - 8.67 (At 90 °C)

For the hydrolytic reactions taking place in buffered solutions, the general procedure was as follows: a solution of buffer (see below) was prepared with a concentration double that required for the experiment and an ionic strength made up to 1 M with potassium nitrate. A 1 mM solution of the standard (potassium *p*-nitrobenzenesulphonate) was also prepared and made up to IS = 1 M with potassium nitrate. In a 10 ml volumetric flask, the potassium *p*-nitrobenzenesulphonate standard solution (1 ml), the buffer solution (5 ml), and a solution of the TnpsT substrate (100 µl of a 2.4 mM solution) were combined, and the volume was made up to 10 ml with potassium nitrate solution (1 M). The solution was divided between ten ampoules, which were then sealed. The ampoules were submerged in a covered water bath, maintained at a temperature of 90.0 °C $\pm$ 0.1. Periodically, an ampoule was removed from the bath, the neck was broken, and the solution transferred to a HPLC vial for analysis.

In the case of the formate buffered solutions, 200 µl of a quench solution of dipotassium phosphate (at 10 $\times$ the concentration of the buffer solution in the ampoule) was added. For the other buffer solutions, cooling to ambient temperature was sufficient to quench the reaction.

For each buffer, this procedure was carried out using 10 mM and 100 mM concentrations of buffer. In most cases, observed rate constants were identical for 10 mM and 100 mM buffer experiments. In cases where different rate constants were observed, further experiments using 40 mM and 70 mM concentrations of buffer were performed. These results were then extrapolated to a buffer concentration of zero in order to determine the non-buffer catalysed rate constant.

Reactions at pH > 8.67 and < 3.54 (At 90 °C)

At high or low pH values, solutions of potassium hydroxide or hydrochloric acid were used to control pH. These solutions were prepared as follows: 1:1 *mol:mol* potassium *p*-nitrobenzenesulphonate / potassium chloride (1.6 mg, 5 µmol) was placed in a volumetric flask (50 ml) with a quantity of potassium nitrate sufficient to make the IS of the final solution 1 M. The required volume of 1 M hydrochloric acid or 1 M potassium hydroxide volumetric standard solution was then measured into the volumetric flask using a pipette, and water was added to make up the volume.

In the case of the potassium hydroxide solutions, 10 ml of the solution was then transferred to a 10 ml volumetric flask, and the substrate solution (100 µl, 2.4 mM) was added (for the procedure with HCl solutions, see the following section. The solution was then divided between ten ampoules which were sealed and placed in the water bath and handled from this point onwards according to the general procedure.

For each solution of this type a HCl or KOH concentration of at least 5 mM ($\geq$ 200 equivalents compared to the TnpsT substrate) was used.

Rapid Reactions (pH < 3.54 At 90 °C)

For rapid reactions (*i.e.* those carried out in HCl solutions) the short timescales involved rendered the use of a water bath and sealed ampoules impractical. Instead, an aluminium heating block was used to control temperature. One well within the block was filled with silicone oil and a thermometer was inserted, enabling the temperature of the block to be adjusted to 90.0 °C ±

0.1. Into an adjacent well was placed a small quantity of silicone oil to aid heat conduction, and a broken-necked ampoule containing hydrochloric acid solution (1 ml), the preparation of which is described in the previous section.

The solution was allowed to equilibrate for 5 min, after which time TnpsT solution (10 µl, 2.4 mM) was added by micropipette to initiate the reaction. After the required time had elapsed, quench solution (200 µl of dipotassium phosphate solution at 10 × the concentration of the HCl solution, with the exception of the 1 M HCl solution, which required 150 µl 8 M NaOH solution) was added, and the mixture was transferred to a HPLC vial. This process was repeated using a range of reaction incubation times in order to construct time profiles for kinetic analysis.

Kinetic Data for HCl Solutions

Linear regression was performed using Microsoft® Excel® 2010, on Microsoft® Windows® 7.

Time points marked with an asterisk (*) have been exluded from the analysis.

Integrals for 50 mM HCl, pH 1.32

Time / s:	Amine:	Standard:	TnpsT:	Thiol:
0		1668725	842016	0
5	222437	1736711	699473	0
10	351465	1799685	467513	68978
15	441637	1708811	320593	169838
20	454743	1717293	221233	204705
30	581509	1716614	94343	250085
60	562742	1721351	0	281536
90	579303	1718788	0	308643
180	485071	1734882	0	225346
270	270435	1780902	364761	69309

Relative Integrals for 50 mM HCl, pH 1.32

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.505	-0.684
5	0.128	0.403	-0.909
10	0.195	0.260	-1.348
15	0.258	0.188	-1.673
20	0.265	0.129	-2.049
30	0.339	0.055	-2.901
60	0.327	0.000	
90	0.337	0.000	
180	0.280	0.000	
*270	0.152	0.205	-1.586

Analysis for 50 mM HCl, pH 1.32

	Value:	Standard Error:
$\ln([\mathrm{TnpsT}]_0)$	-6.0×10^{-1}	5×10^{-2}
k_{obs} / s^{-1}	7.5×10^{-2}	3×10^{-3}
R^2	0.994	
Half-life / s	9.3	

Integrals for 5 mM HCl, pH 2.37

Time / s:	Amine:	Standard:	TnpsT:	Thiol:
0		2063195	1020409	0
30	176618	2112442	851018	0
60	271715	2120004	688949	0
90	284949	2107044	542938	0
120	376500	2120180	405840	70260
150	425299	2169817	351470	89970
180	525191	2126879	259863	79903
210	585470	2138014	215083	109019
240	540219	2125079	168982	102285
480	672987	2175757	27515	145162

Relative Integrals for 5 mM HCl, pH 2.37

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.495	-0.704
30	0.084	0.403	-0.909
60	0.128	0.325	-1.124
90	0.135	0.258	-1.356
120	0.178	0.191	-1.653
150	0.196	0.162	-1.820
180	0.247	0.122	-2.102
210	0.274	0.101	-2.297
240	0.254	0.080	-2.532
480	0.309	0.013	-4.370

Analysis for 5 mM HCl, pH 2.37

	Value:	**Standard Error:**
$\ln([\text{TnpsT}]_0)$	-7.0×10^{-1}	1×10^{-2}
k_{obs} / s^{-1}	7.68×10^{-3}	6×10^{-5}
R^2	0.9995	
Half-life / s	90.2	

Kinetic Data for pH 3.54 Formate Buffer

Integrals for 10 mM Formate Buffer

Time / s:	**Amine:**	**Standard:**	**TnpsT:**
0		1863002	1038372
600	158766	1811382	735585
1200	270427	1853054	558248
1800	352222	1847398	406960
2400	387760	1837817	334182
3000	432438	1806502	229673
3600	505999	1868529	194648
4200	502423	1884936	159793
4800	513653	1854381	114813
7200	567234	1879317	26923

Relative Integrals for 10 mM Formate Buffer

Time / s:	**Amine:**	**TnpsT:**	**ln(TnpsT):**
0		0.557	-0.585
600	0.088	0.406	-0.901
1200	0.146	0.301	-1.200
1800	0.191	0.220	-1.513
2400	0.211	0.182	-1.705
3000	0.239	0.127	-2.062
3600	0.271	0.104	-2.262
4200	0.267	0.085	-2.468
4800	0.277	0.062	-2.782
7200	0.302	0.014	-4.246

Analysis for 10 mM Formate Buffer

	Value:	**Standard Error:**
$\ln([\text{TnpsT}]_0)$	-6.4×10^{-1}	3×10^{-2}
k_{obs} / s^{-1}	4.5×10^{-4}	1×10^{-5}
R^2	0.996	
Half-life / s	1500	
Half-life / h	0.42	

Integrals for 40 mM Formate Buffer

Time / s:	Amine:	Standard:	TnpsT:
0	27454	1877886	1027287
600	283445	1894847	627732
1200	363439	1810505	317365
1800	417673	1865756	285476
2400	460377	1861908	186338
3000	444225	1761668	139665
3600	590644	1894533	80160
4200	599375	1903955	72573
4800	606714	1875177	47718
7200	622338	1871291	0

Relative Integrals for 40 mM Formate Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0	0.015	0.547	-0.603
600	0.150	0.331	-1.105
1200	0.201	0.175	-1.741
1800	0.224	0.153	-1.877
2400	0.247	0.100	-2.302
3000	0.252	0.079	-2.535
3600	0.312	0.042	-3.163
4200	0.271	0.038	-3.267
4800	0.324	0.025	-3.671
7200	0.333	0.000	

Analysis for 40 mM Formate Buffer

	Value:	Standard Error:
$\ln([\text{TnpsT}]_0)$	-7.7×10^{-1}	9×10^{-2}
k_{obs} / s^{-1}	6.2×10^{-4}	3×10^{-5}
R^2	0.984	
Half-life / s	1100	
Half-life / h	0.31	

Integrals for 70 mM Formate Buffer

Time / s:	Amine:	Standard:	TnpsT:
0	42210	1803395	948497
600	275106	1834177	524305
1200	360450	1810752	345338
1800	441468	1831797	227687
2400	478595	1828492	141365
3000	501626	1865159	96130
3600	528873	1858092	69903
4200	614538	1873930	26856
4800	621640	1878338	21844
7200	623469	1867325	0

Relative Integrals for 70 mM Formate Buffer

Time / s:	**Amine:**	**TnpsT:**	**ln(TnpsT):**
0	0.023	0.526	-0.643
600	0.150	0.286	-1.252
1200	0.199	0.191	-1.657
1800	0.241	0.124	-2.085
2400	0.262	0.077	-2.560
3000	0.269	0.052	-2.965
3600	0.285	0.038	-3.280
4200	0.328	0.014	-4.245
4800	0.331	0.012	-4.454
7200	0.334	0.000	

Analysis for 70 mM Formate Buffer

	Value:	**Standard Error:**
$\ln([\mathrm{TnpsT}]_0)$	-6.8×10^{-1}	9×10^{-2}
k_{obs} / s^{-1}	7.9×10^{-4}	3×10^{-5}
R^2	0.990	
Half-life / s	880	
Half-life / h	0.24	

Integrals for 100 mM Formate Buffer

Time / s:	Amine:	Standard:	TnpsT:
0	136088	1835852	862826
600	367776	1855558	466413
1200	408697	1820854	302063
1800	482957	1836787	165026
2400	536739	1802089	115643
3000	534078	1867048	75302
3600	592037	1880597	47813
4200	614862	1880102	0
4800	633340	1912330	0

Relative Integrals for 100 mM Formate Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0	0.074	0.470	-0.755
600	0.198	0.251	-1.381
1200	0.224	0.166	-1.796
1800	0.263	0.090	-2.410
2400	0.298	0.064	-2.746
3000	0.286	0.040	-3.211
3600	0.315	0.025	-3.672
4200	0.285	0.000	
4800	0.331	0.000	

Analysis for 100 mM Formate Buffer

	Value:	Standard Error:
$\ln([\text{TnpsT}]_0)$	-8.5×10^{-1}	5×10^{-2}
k_{obs} / s^{-1}	8.0×10^{-4}	2×10^{-5}
R^2	0.995	
Half-life / s	870	
Half-life / h	0.24	

General Catalysis Analysis for Formate Buffer

Buffer Concentration / mM:	$k_{\mathbf{obs}}$ / s^{-1}
10	4.5×10^{-4}
40	6.2×10^{-4}
70	7.9×10^{-4}
100	8.0×10^{-4}

	Value:	Standard Error:
k_0 / s^{-1}	4.4×10^{-4}	6×10^{-5}
k_{buff} / $M^{-1}s^{-1}$	4.0×10^{-6}	9×10^{-7}
R^2	0.903	
Half-life / s	1600	
Half-life / h	0.44	

Kinetic Data for pH 4.61 Acetate Buffer

Integrals for 10 mM Acetate Buffer

Time / s:	Amine:	Standard:	TnpsT:	Thiol:
0		8397616	1621528	0
3515	163529	8376815	1374020	0
7370	343582	8484302	1179757	104466
10675	422916	8513861	1016781	183900
13595	420923	8504952	833894	236256
18995	446484	6348309	740416	261372
24395	903533	8552437	186005	195374
29795	758384	8274768	471698	298874
34775	705388	8436376	391363	313518
97655	1009816	8278421	0	196920

Relative Integrals for 10 mM Acetate Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.193	-1.645
3515	0.020	0.164	-1.808
7370	0.040	0.139	-1.973
10675	0.050	0.119	-2.125
13595	0.049	0.098	-2.322
*18995	0.070	0.117	-2.149
*24395	0.106	0.022	-3.828
29795	0.092	0.057	-2.865
34775	0.084	0.046	-3.071
97655	0.122	0	

Analysis for 10 mM Acetate Buffer

	Value:	Standard Error:
$\ln([\text{TnpsT}]_0)$	-1.68	3×10^{-2}
k_{obs} / s^{-1}	4.0×10^{-5}	1×10^{-6}
R^2	0.994	
Half-life / s	17000	
Half-life / h	4.8	

Integrals for 40 mM Acetate Buffer

Time / s:	Amine:	Standard:	TnpsT:
0	154949	8501649	1438272
1800	263650	8248089	1374904
3600	368523	8673931	1273517
5460	397356	8730098	1156327
7200	383899	8551222	1049075
10800	445593	8454592	944505
16440	825242	8506709	701764
21900	929792	8445133	107234
28800	847369	8698518	371920
86400	972620	8605404	0

Relative Integrals for 40 mM Acetate Buffer

Time / s:	**Amine:**	**TnpsT:**	**ln(TnpsT):**
0	0.018	0.169	-1.777
1800	0.032	0.167	-1.792
3600	0.042	0.147	-1.919
5460	0.046	0.132	-2.022
7200	0.045	0.123	-2.098
10800	0.053	0.112	-2.192
16440	0.097	0.082	-2.495
*21900	0.110	0.013	-4.366
28800	0.097	0.043	-3.152
86400	0.113	0.000	

Analysis for 40 mM Acetate Buffer

	Value:	**Standard Error:**
ln([TnpsT]$_0$)	-1.74	2×10^{-2}
k_{obs} / s^{-1}	4.8×10^{-5}	2×10^{-6}
R^2	0.993	
Half-life / s	14000	
Half-life / h	4.0	

Integrals for 70 mM Acetate Buffer

Time / s:	Amine:	Standard:	TnpsT:
0	221836	9250739	1370111
1800	357870	8462860	1113800
3600	447075	8382452	1027973
5460	418478	8632344	885457
7200	583077	8807920	843788
10800	689137	8523983	619775
16440	776817	8229660	401528
21900	757797	8423038	293792
28800	819962	8617800	166829
86400	1025310	8384993	0

Relative Integrals for 70 mM Acetate Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0	0.024	0.148	-1.910
1800	0.042	0.132	-2.028
3600	0.053	0.123	-2.099
5460	0.048	0.103	-2.277
7200	0.066	0.096	-2.346
10800	0.081	0.073	-2.621
16440	0.094	0.049	-3.020
21900	0.090	0.035	-3.356
28800	0.095	0.019	-3.945
86400	0.122	0.000	

Analysis for 70 mM Acetate Buffer

	Value:	Standard Error:
$\ln([\text{TnpsT}]_0)$	-1.88	2×10^{-2}
k_{obs} / s^{-1}	7.0×10^{-5}	1×10^{-6}
R^2	0.997	
Half-life / s	9900	
Half-life / h	2.8	

Integrals for 100 mM Acetate Buffer

Time / s:	Amine:	Standard:	TnpsT:	Thiol:
0		8504619	1500229	0
3515	391558	8406408	890909	138014
7370	554154	8522067	667638	207539
10675	670521	8494405	525229	237612
13595	759499	8421799	399498	241875
18995	858901	8433994	275297	255533
24395	881390	7837413	203051	262795
29795	954196	8334426	0	210901
34775	963061	8485922	0	167586
97655	887185	8239523	0	959124

Relative Integrals for 100 mM Acetate Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.176	-1.735
3515	0.047	0.106	-2.245
7370	0.065	0.078	-2.547
10675	0.079	0.062	-2.783
13595	0.090	0.047	-3.048
18995	0.102	0.033	-3.422
24395	0.112	0.026	-3.653
29795	0.114	0.000	
34775	0.113	0.000	
97655	0.108	0.000	

Analysis for 100 mM Acetate Buffer

	Value:	Standard Error:
$\ln([\mathrm{TnpsT}]_0)$	-1.91	8×10^{-2}
k_{obs} / s^{-1}	7.7×10^{-5}	6×10^{-6}
R^2	0.973	
Half-life / s	9000	
Half-life / h	2.5	

General Catalysis Analysis for Acetate Buffer

Buffer Concentration / mM:	$k_{\mathbf{obs}}$ / s^{-1}
10	4.0×10^{-5}
40	4.8×10^{-5}
70	7.0×10^{-5}
100	7.7×10^{-5}

	Value:	Standard Error:
k_0 / s^{-1}	3.5×10^{-5}	4×10^{-6}
k_{buff} / $M^{-1}s^{-1}$	4.4×10^{-7}	7×10^{-8}
R^2	0.953	
Half-life / s	20000	
Half-life / h	5.5	

Kinetic Data for pH 5.68 MES Buffer

Integrals for 10 mM MES Buffer

Time / s:	Amine:	Standard:	TnpsT:
0		3052158	1699478
14400		3042009	1569030
28800	104994	3075507	1416279
43200	295546	3036299	1350318
57600	348030	3175283	1121011
100800	490947	3126050	914473
187200	707079	3086310	527249
273600	841728	3099233	309101
446400	1231040	3122664	107307
533400	1173325	3381350	69145

Relative Integrals for 10 mM MES Buffer

Time / s:	**Amine:**	**TnpsT:**	**ln(TnpsT):**
0		0.557	-0.586
14400		0.516	-0.662
28800	0.034	0.461	-0.775
43200	0.097	0.445	-0.810
57600	0.110	0.353	-1.041
100800	0.157	0.293	-1.229
187200	0.229	0.171	-1.767
273600	0.272	0.100	-2.305
446400	0.394	0.034	-3.371
533400	0.347	0.020	-3.890

Analysis for 10 mM MES Buffer

	Value:	**Standard Error:**
$\ln([\mathrm{TnpsT}]_0)$	-0.593	7×10^{-3}
k_{obs} / s^{-1}	6.21×10^{-6}	3×10^{-8}
R^2	0.9999	
Half-life / *s*	112000	
Half-life / *h*	31.0	
Half-life / *day*	1.29	

Integrals for 100 mM MES Buffer

Time / s:	Amine:	Standard:	TnpsT:
0		3015608	1706954
14400		3077453	1595214
28800		3176581	1517047
43200		3060482	1121410
57600	338848	3171542	1295226
100800	419325	3449446	1106546
187200	693082	3276010	646507
273600	857438	3036560	370006
446400	1010046	3311320	147441
533400	1186327	3179948	96592

Relative Integrals for 100 mM MES Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.566	-0.569
14400		0.518	-0.657
28800		0.478	-0.739
*43200		0.366	-1.004
57600	0.107	0.408	-0.896
100800	0.122	0.321	-1.137
187200	0.212	0.197	-1.623
273600	0.282	0.122	-2.105
446400	0.305	0.045	-3.112
533400	0.373	0.030	-3.494

Analysis for 100 mM MES Buffer

	Value:	**Standard Error:**
$\ln([\text{TnpsT}]_0)$	-5.8×10^{-1}	1×10^{-2}
k_{obs} / s^{-1}	5.56×10^{-6}	5×10^{-8}
R^2	0.9994	
Half-life / *s*	125000	
Half-life / *h*	34.7	
Half-life / *day*	1.44	

Average of 100 mM and 10 mM MES buffer k_{obs} values: 5.88×10^{-6} s^{-1}

Kinetic Data for pH 6.60 Phosphate Buffer

Integrals for 10 mM Phosphate Buffer

Time / s:	**Amine:**	**Standard:**	**TnpsT:**
0		3012915	1686985
86400		3090826	1471150
259200	342439	2968220	1021488
604800	552355	3038786	458631
777600	690532	3565864	471385
941400	770991	3243316	293507
1114200	769013	3097616	211586
1287000	811945	3071116	174762

Relative Integrals for 10 mM Phosphate Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.560	-0.580
86400		0.476	-0.742
259200	0.115	0.344	-1.067
604800	0.182	0.151	-1.891
777600	0.194	0.132	-2.023
941400	0.238	0.090	-2.402
1114200	0.248	0.068	-2.684
1287000	0.264	0.057	-2.866

Analysis for 10 mM Phosphate Buffer

	Value:	Standard Error:
$\ln([\mathrm{TnpsT}]_0)$	-6.2×10^{-1}	5×10^{-2}
k_{obs} / s^{-1}	1.83×10^{-6}	7×10^{-8}
R^2	0.9914	
Half-life / *s*	378000	
Half-life / *h*	105	
Half-life / *day*	4.37	

Integrals for 100 mM Phosphate Buffer

Time / s:	Amine:	Standard:	TnpsT:
0		3091195	1693658
86400		3136620	1440037
259200	369892	3160139	999850
604800	624089	3112808	548636
777600	688447	3026656	396416
941400	807564	3264554	337415
1114200	800326	3096372	219028
1287000	847382	3237171	161483

Relative Integrals for 100 mM Phosphate Buffer

Time / s:	**Amine:**	**TnpsT:**	**ln(TnpsT):**
0		0.548	-0.602
86400		0.459	-0.778
259200	0.117	0.316	-1.151
604800	0.200	0.176	-1.736
777600	0.227	0.131	-2.033
941400	0.247	0.103	-2.270
1114200	0.258	0.071	-2.649
1287000	0.262	0.050	-2.998

Analysis for 100 mM Phosphate Buffer

	Value:	**Standard Error:**
$\ln([\mathrm{TnpsT}]_0)$	-6.3×10^{-1}	2×10^{-2}
k_{obs} / s^{-1}	1.82×10^{-6}	3×10^{-8}
R^2	0.9983	
Half-life / s	382000	
Half-life / h	106	
Half-life / day	4.42	

Average of 100 mM and 10 mM phosphate buffer k_{obs} values: 1.83×10^{-6} s^{-1}

Kinetic Data for pH 7.39 Bicine Buffer

Integrals for 10 mM Bicine Buffer

Time / s:	Amine:	Standard:	TnpsT:
0		2148300	1307292
86400	104238	2199756	1237154
172800	162265	2172812	1131429
259200	177073	2115830	1053357
345600	265377	2147877	984923
518400	326853	2175006	847496
691200	411425	2178048	735849
864000	525665	2157935	627924
1036800	552963	2167248	540634
1209600	644530	2218031	440499

Relative Integrals for 10 mM Bicine Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.609	-0.497
86400	0.047	0.562	-0.576
172800	0.075	0.521	-0.653
259200	0.084	0.498	-0.697
345600	0.084	0.459	-0.780
518400	0.150	0.390	-0.943
691200	0.189	0.338	-1.085
864000	0.244	0.291	-1.234
1036800	0.255	0.249	-1.388
1209600	0.291	0.199	-1.616

Analysis for 10 mM Bicine Buffer

	Value:	Standard Error:
$\ln([\text{TnpsT}]_0)$	-4.8×10^{-1}	1×10^{-2}
k_{obs} / s^{-1}	9.0×10^{-7}	2×10^{-8}
R^2	0.996	
Half-life / *s*	770000	
Half-life / *h*	210	
Half-life / *day*	9.0	

Integrals for 100 mM Bicine Buffer

Time / s:	Amine:	Standard:	TnpsT:
0		2160962	1324508
86400	83216	2180873	1241694
172800	172561	2159787	1139773
259200	231624	2151164	1066353
345600	242326	2168983	989034
518400	390714	2172934	856892
691200	440459	2156941	736468
864000	560255	2153378	631954
1036800	585301	2134569	545087
1209600	651485	2208360	464536

Relative Integrals for 100 mM Bicine Buffer

Time / s:	**Amine:**	**TnpsT:**	**ln(TnpsT):**
0		0.613	-0.490
86400	0.038	0.569	-0.563
172800	0.080	0.528	-0.639
259200	0.108	0.496	-0.702
345600	0.108	0.456	-0.785
518400	0.180	0.394	-0.931
691200	0.204	0.341	-1.075
864000	0.260	0.293	-1.226
1036800	0.274	0.255	-1.365
1209600	0.295	0.210	-1.559

Analysis for 100 mM Bicine Buffer

	Value:	**Standard Error:**
$\ln([\text{TnpsT}]_0)$	-4.84×10^{-1}	7×10^{-3}
k_{obs} / s^{-1}	8.7×10^{-7}	1×10^{-8}
R^2	0.9989	
Half-life / s	800000	
Half-life / h	220	
Half-life / day	9.3	

Average of 100 mM and 10 mM bicine buffer k_{obs} values: 8.8×10^{-7} s^{-1}

Kinetic Data for pH 8.67 Borate Buffer

Integrals for 10 mM Borate Buffer

Time / s:	Amine:	Standard:	TnpsT:
0		2028390	1284514
86400	50498	1960392	1240312
172800	155490	1981424	1142461
259200	170913	1966629	1087065
345600	236837	1954921	1019262
864000	410757	2021553	707156
1382400	565758	1993645	497993
1900800	672018	1989387	338960
2419200	729964	1854456	212817
2592000	755514	1981444	202675

Relative Integrals for 10 mM Borate Buffer

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.633	-0.457
86400	0.026	0.633	-0.458
172800	0.078	0.577	-0.551
259200	0.087	0.553	-0.593
345600	0.087	0.521	-0.651
864000	0.203	0.350	-1.050
1382400	0.284	0.250	-1.387
1900800	0.338	0.170	-1.770
2419200	0.394	0.115	-2.165
2592000	0.381	0.102	-2.280

Analysis for 10 mM Borate Buffer

	Value:	Standard Error:
$\ln([\text{TnpsT}]_0)$	-4.18×10^{-1}	9×10^{-3}
k_{obs} / s^{-1}	7.16×10^{-7}	7×10^{-9}
R^2	0.9993	
Half-life / *s*	968000	
Half-life / *h*	269	
Half-life / *day*	11.2	

Integrals for 100 mM Borate Buffer

Time / s:	Amine:	Standard:	TnpsT:
0		1992483	1300266
86400	42378	1969394	1245993
172800	95656	2033986	1187240
259200	165481	2025699	1121865
345600	184571	2024400	1061050
864000	389585	2034419	768034
1382400	538873	2019683	567425
1900800	655180	2023361	409904
2419200	693175	1963368	292018
2592000	791713	1981763	271846

Relative Integrals for 100 mM Borate Buffer

Time / s:	**Amine:**	**TnpsT:**	**ln(TnpsT):**
0		0.653	-0.427
86400	0.022	0.633	-0.458
172800	0.048	0.584	-0.538
259200	0.082	0.554	-0.591
345600	0.082	0.524	-0.646
864000	0.191	0.378	-0.974
1382400	0.267	0.281	-1.270
1900800	0.324	0.203	-1.597
2419200	0.353	0.149	-1.906
2592000	0.399	0.137	-1.987

Analysis for 100 mM Borate Buffer

	Value:	**Standard Error:**
$\ln([\mathrm{TnpsT}]_0)$	-4.30×10^{-1}	6×10^{-3}
k_{obs} / s^{-1}	6.08×10^{-7}	5×10^{-9}
R^2	0.9995	
Half-life / *s*	1140000	
Half-life / *h*	317	
Half-life / *day*	13.2	

Average of 100 mM and 10 mM borate buffer k_{obs} values: 6.62×10^{-7} s^{-1}

Kinetic Data for pH KOH Solutions

Integrals for 5 mM KOH, pH 9.90

Time / s:	Amine:	Standard:	TnpsT:
0		2101459	1302862
345600	154000	2060733	1065851
691200	286624	2067771	862236
1036800	349486	2104794	688079
1382400	542729	2027073	559721
1728000	588032	2059645	461009
2073600	635070	2076066	374792
*2246400	640072	1988955	284602

Relative Integrals for 5 mM KOH, pH 9.90

Time / s:	Amine:	TnpsT:	ln(TnpsT):
0		0.620	-0.478
345600	0.075	0.517	-0.659
691200	0.139	0.417	-0.875
1036800	0.166	0.327	-1.118
1382400	0.268	0.276	-1.287
1728000	0.286	0.224	-1.497
2073600	0.306	0.181	-1.712
*2246400	0.322	0.143	-1.944

Analysis for 5 mM KOH, pH 9.90

	Value:	Standard Error:
$\ln([\mathrm{TnpsT}]_0)$	-4.7×10^{-1}	1×10^{-2}
k_{obs} / s^{-1}	5.98×10^{-7}	9×10^{-9}
R^2	0.9989	
Half-life / *s*	1160000	
Half-life / *h*	322	
Half-life / *day*	13.4	

Integrals for 50 mM KOH, pH 10.91

Time / s:	Amine:	Standard:	TnpsT:
*0	20487	2239815	1243620
86400	88042	2172057	1086538
172800	137060	2147684	1028657
259200	267019	2158397	987880
345600	272411	2144063	948256
604800	334815	2109071	854148
864000	377634	2130542	779238
1123200	439685	2079998	695064
1382400	471280	2084791	630095
2073600	565757	2025894	489438

Relative Integrals for 50 mM KOH, pH 10.91

Time / s:	**Amine:**	**TnpsT:**	**ln(TnpsT):**
* 0	0.009	0.555	-0.588
86400	0.041	0.500	-0.693
172800	0.064	0.479	-0.736
259200	0.124	0.458	-0.782
345600	0.127	0.442	-0.816
604800	0.159	0.405	-0.904
864000	0.177	0.366	-1.006
1123200	0.211	0.334	-1.096
1382400	0.226	0.302	-1.197
2073600	0.279	0.242	-1.421

Analysis for 50 mM KOH, pH 10.91

	Value:	**Standard Error:**
$\ln([\mathrm{TnpsT}]_0)$	-6.81×10^{-1}	7×10^{-3}
k_{obs} / s^{-1}	3.64×10^{-7}	7×10^{-9}
R^2	0.9976	
Half-life / *s*	1900000	
Half-life / *h*	528	
Half-life / *day*	22.0	

Integrals for 50 mM KOH, pH 10.91 Monophosphate Species

Time / s:	Tnp:	psT:	Standard:
0			2239815
86400		22633	2172057
172800			2147684
259200	39571	43340	2158397
345600	30643	34383	2144063
604800	40939	48563	2109071
864000	45701	49828	2130542
1123200	46750	60188	2079998
1382400	48601	57041	2084791
2073600	52452	59039	2025894

Relative Integrals for 50 mM KOH, pH 10.91 Monophosphate
Species

Time / s:	Tnp:	psT:
0		
86400		0.0104
172800		
259200	0.0183	0.0201
345600	0.0143	0.0160
604800	0.0194	0.0230
864000	0.0215	0.0234
1123200	0.0225	0.0289
1382400	0.0233	0.0274
2073600	0.0259	0.0291

Analysis for 50 mM KOH, pH 10.91 Monophosphate Species

A least squares fitting to the equation:

$$[Mono] = \frac{k_{\rm acc}[TnpsT]_0}{k_{\rm dec} - k_{\rm obs}} \left(e^{-k_{\rm obs}t} - e^{-k_{\rm dec}t}\right)$$

was performed, where $[Mono]$ is the concentration of the monophosphate species, and $k_{\rm acc}$ and $k_{\rm dec}$ are the rate constants for the accumulation and decomposition, respectively, of the monophosphate species. $k_{\rm obs}$ and $[TnpsT]_0$ have already been determined from the hydrolysis of TnpsT.

	Tnp:	**psT:**
$[\rm TnpsT]_0$	5.1×10^{-1}	5.1×10^{-1}
$k_{\rm obs}/s^{-1}$	3.64×10^{-7}	3.64×10^{-7}
$k_{\rm acc}/s^{-1}$	9×10^{-8}	1×10^{-7}
$k_{\rm dec}/s^{-1}$	1×10^{-6}	1×10^{-6}

Collected TnpsT Hydrolysis Results

Buffer:	**pH at 25 °C:**	**pH at 90 °C:**	**Rate / s^{-1}:**	**log(Rate):**
HCl	1.32	1.32	7.35×10^{-2}	-1.13
HCl	2.37	2.37	7.68×10^{-3}	-2.11
Formate	3.53	3.54	4.41×10^{-4}	-3.36
Acetate	4.60	4.61	3.46×10^{-5}	-4.46
MES	6.25	5.68	5.88×10^{-6}	-5.23
Phosphate	6.47	6.60	1.82×10^{-6}	-5.74
Bicine	8.32	7.39	8.81×10^{-7}	-6.05
Borate	9.11	8.67	6.62×10^{-7}	-6.18
KOH	11.55	9.90	5.98×10^{-7}	-6.22
KOH	12.56	10.91	3.64×10^{-7}	-6.44

Bibliography

[1] M. Ora, M. Murtola, S. Aho, M. Oivanen, *Org. Biomol. Chem.*, 2004, **2**, 593-600.

[2] Determination of pH: Theory and Practice, Roger Gordon Bates, Wiley, 1964

[3] A. V. Bandura, S. N. Lvov, *J. Phys. Chem. Ref. Data*, 2006, **35**, 15-30.

[4] N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, R. M. M. Singh, *Biochemistry*, 1966, **5**, 467-477.