Phosphate analogues in the dissection of mechanism
Heidi J Korhonen¹ ̂², Louis P Conway¹ and David RW Hodgson¹

Phosphoryl group transfer is central to genetic replication, cellular signalling and many metabolic processes. Understanding the mechanisms of phosphorylation and phosphate ester and anhydride cleavage is key to efforts towards biotechnological and biomedical exploitation of phosphate-handling enzymes. Analogues of phosphate esters and anhydrides are indispensable tools, alongside protein mutagenesis and computational methods, for the dissection of phosphoryl transfer mechanisms. Hydrolysable and non-hydrolysable phosphate analogues have provided insight into the nature and sites of phosphoryl transfer processes. Kinetic isotope effects and crystallography using transition state analogues have painted more detailed pictures of transition states and how enzymes work to stabilise them.

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Introduction
The mechanisms of phosphoryl transfer between nucleophilic centres have been investigated intensely over the last half-century, with many generalisations of enzyme catalytic strategies becoming evident [¹]. Newly discovered enzymes that foster phosphoryl transfer have also regularly presented themselves, and offer fresh ground for research alongside historically challenging systems. The catalysis of phosphoryl transfer is particularly intriguing given the manifest stability of diesters, and monoester dianion systems. The delineation of the strategies employed by enzymes to provide accelerations of up to 10²¹-fold, gives enzymologists true insight into some of Nature’s most efficient catalysts [²]. Visualisation and parameterisation of the highly dynamic interactions between enzyme and substrate as they pass through to products via heavily stabilised transition states represents the long-standing challenge in this field. This opinion brings together several recent examples of phosphate ester analogues and their use in deciphering the secrets of some of Nature’s most enticingly efficient biocatalysts, in the context of ubiquitous phosphoryl transfer processes (Scheme 1).

Approaches towards understanding transfers from phosphate monoesters, diesters and phosphoanhydride systems will be included in this opinion. Both labile (reactive) and stable (inhibitory) analogues are covered, where the former usually, but not exclusively, tend to offer insight into the dynamic processes that occur during bond making and breaking between phosphorus and other nucleophilic groups. In many cases, multi-pronged strategies are adopted where parameterisations and inferences from one mechanistic tool can be supported and enhanced by others. The following three sections cover examples of phosphate monoster, diester and anhydride analogues. Initially, each section focuses on examples where the nature of the transition state and factors that stabilise it can be extracted. Thereafter, examples that offer insight into the site of bond scission though inhibition are covered. The structures of the analogues covered within each section are brought together in a table at the end of the section alongside a summary of each analogue’s application.

Monoesters
Monoesters and their analogues have been studied extensively over the last 50 or so years, however, their mechanisms of transfer, both under enzymatic catalysis and in its absence, have remained controversial [¹]. This section includes examples of kinetic studies, using heavy atom isotope effects, crystallographic studies that employ agents to mimic parts of the phosphoryl transfer process, and finally non-hydrolysable analogues that can be employed as inhibitors and active site probes for a number of purposes that will be discussed in turn.

The mechanism of protein tyrosine phosphatase 1B
A key illustration of the state of the art is the work of Brandão et al. [³], where a combination of heavy-atom isotope kinetic studies (Table 1, entry 1) complements the use of vanadate-based transition state mimicry in crystallographic studies (Table 1, entry 2) to reveal a unified view of the dynamic interactions that occur between enzyme and transferring phosphoryl group during both ‘ping’ and ‘pong’ steps of protein tyrosine phosphatase 1B. The key challenge in this area is the ability to measure and interpret the small isotope effects that arise from the use of heavy-atom systems.
A phosphorane intermediate in the active site of β-phosphoglucomutase?
A cautionary tale runs alongside crystallographic studies that suggested the unusual occurrence and apparent stability of a phosphorane during phosphate monoester transfer in the active site of β-phosphoglucomutase [4]. The β-phosphoglucomutase enzyme mediates the transfer of phosphate between hydroxyl groups within glucose, via a ping-pong mechanism. The assertion of a phosphorane intermediate, accessed through an addition-elimination mechanism sat contrary to the usual observation of more dissociative pathways. Subsequent 19-F NMR studies showed that the postulated PO₃⁻ group of the phosphorane was, in fact, a MgF₃⁻ system (Table 1, entry 3) [5*], that is difficult to distinguish from the PO₃⁻ group using X-ray diffraction alone.

Similar 19-F NMR approaches with MgF₃⁻, AlF₃ and AlF₄⁻ transition state analogue systems have been used in tandem with crystallographic and mutagenesis studies to give insight into the balance between enzyme preferences for charge balancing versus isostery in several phosphoryl transferase enzymes [6–10].

Non-hydrolysable sugar-1-phosphate analogues
Loranger et al. recently prepared l-rhamnose 1C-phosphonates (Table 1, entry 4) as potential inhibitors of bacterial nucleotidylyltransferases, which are key to the biosynthesis of viable cell walls [11]. The intention was to explore methylene (X = Y = H), monofluoromethylene (X = F, Y = H) and difluoromethylene (X = Y = F) systems as mimics of l-rhamnose 1-phosphate, however, synthetic difficulties prevented access to the monofluoro system that could potentially offer the best mimicry of the ionisation profile of the natural phosphate [12]. Thiophosphonate analogues of sugar 1-phosphates have also recently been accessed synthetically (Table 1, entry 5), where the methylene offers a non-cleavable bridge, and sulfur offers modulated metal ion-binding properties [13].
Phosphodiesters

Phosphodiester models and mimics have been used widely to understand the mechanisms of phosphodiesterases such as nucleases and ribozymes. This section discusses examples where one or more of the bridging or non-bridging oxygen atoms associated with the phosphodiester group has been exchanged for either sulfur or fluorine. The resulting analogues are often reactive, where their altered reactivity profiles are used to probe the nature of catalysis in enzyme active sites and/or binding to metal ions therein.

Phosphorothiolates in the quest for acid/base catalysis by ribozymes

Some of the most poignant recent additions to the mechanistic toolbox are the phosphorothiolates (Table 2, entry 1), where a bridging oxygen atom has been replaced by sulfur. These systems have received significant attention because synthetic advances have permitted their use in oligonucleotides [14–16]. Phosphorothiolates can also elucidate O-Mg²⁺ ion interactions through soft metal ion rescue experiments. More significantly, where a leaving group oxygen is replaced by sulfur, the enhanced leaving group properties of thiolate anions accelerate their departure, sometimes obviating the need for catalysis, and potentially making previously kinetically silent processes rate-determining. In this vein, phosphorothiolate studies have illuminated HDV [17] and VS [18] ribozyme systems alongside nucleobase substitutions to provide unequivocal evidence in support of general acid/base catalysis. Recent work in this area, primarily from the Picirilli laboratory, has been reviewed [19**,20].

More detailed pictures of RNA hydrolysis through the use of isotopes

More subtle substitution of phosphodiesters can be effected through the use of isotopomeric compounds, such as 18-O labelled species (Table 2, entry 2). Heavy atom isotope effects are challenging to determine on a practical level, however, isotopic substitutions represent the least perturbing of all possible analogues. 5'-18O and 2'-18O isotopomeric analogues of the dinucleotide 5'-UpG-3' were synthesised and the base-promoted cleavage kinetics of these phosphodiester systems were explored [21**]. Through these studies, the transition state for the 2'-O-transphosphorylation process was suggested to be late in nature, and solvent deuterium isotope effect studies suggest the prior formation of the 2'-alkoxide nucleophile rather than rate-determining general base catalysis by hydroxide ion. An extension of this, supplemented with computational studies, has been used to revisit the mechanism of ribonuclease A [22].

Fluorophosphonates: diester or monoester?

Fluorophosphonates present the possibility of concerted, diester-like transition states while offering the size and

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**Table 1**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Analogue structure</th>
<th>Acronym/name</th>
<th>Application</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="Phosphorothiolate structure" /></td>
<td>pPNPP</td>
<td>Determination of transition state structure of phosphate monoester transfer</td>
<td>[3**]</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="Vanadate structure" /></td>
<td>Vanadate</td>
<td>Transition state analogue for phosphate monoester transfer</td>
<td>[3**]</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="MgF³⁻, AlF³⁻, AlF½⁻" /></td>
<td>Phosphonate/α-halophosphonate</td>
<td>Non-scissile analogue of rhamnose-1-phosphate</td>
<td>[11]</td>
</tr>
<tr>
<td>4</td>
<td><img src="image" alt="Phosphonate/α-halophosphonate structure" /></td>
<td>Thiophosphonate</td>
<td>Non-scissile analogue of sugar-1-phosphate</td>
<td>[13]</td>
</tr>
</tbody>
</table>
hydrogen bonding characteristics of monoesters [23]. This mixed character was used to explore the promiscuous proficiencies of phosphoryl transfer by alkaline phosphatase. Comparison of aryfluorophosphonates with methyl aryl phosphate diesters (Table 2, entry 3, left) as substrates for R166A alkaline phosphatase showed the enzyme to have higher proficiency \((k_{\text{cat}}/K_M)/k_{\text{uncat}}\) towards the diester, with both systems displaying significantly reduced efficiencies compared to monoesters. This was despite the fact that the fluorophosphonates showed approximately 1000-fold higher reactivities towards hydroxide ion. This differentiation between transition states in the enzyme active site contrasts with other systems that have shown greater promiscuity [24,25], despite the deliberately small perturbation of the fluorophosphonate substrates away from native monoesters. A fluoronucleotide system (Table 2, entry 3, right) was employed alongside a number of other nucleoside-5'-phosphate analogues to demonstrate that Fhit proteins recognise and hydrolyse substrates beyond the dinucleoside triphosphates that they normally act upon [26].

**Phosphoanhydrides**

Phosphoanhydrides such as (deoxy)ribonucleoside triphosphates and sugar nucleotides are central to the actions of polymerases, kinases and glycosyl transferases *inter alia*. Phosphoanhydride analogues where bonds or atoms that are involved in enzyme-catalysed transfer have been replaced, provide substrates and inhibitors that offer mechanistic insight, however, their synthesis and isolation is particularly laborious.

Below, three specific examples of the exploitation of phosphoanhydride analogues in mechanistic studies are presented. These include non-hydrolysable systems that are key to X-ray crystallographic studies, and differentially activated/deactivated mimics for kinetic studies that can complement protein mutation studies to delineate the roles of key active site residues. Uncatalysed solution studies of substrates and analogues, in the absence of enzyme, are also essential for benchmarking catalytic acceleration factors, and to gain mechanistic insight without the complicating factors that proteins bring [27–29].

**The mechanism of RNA ligase RtcB**

RtcB is a non-canonical RNA ligase that joins a 3'-phosphate with a 5'-hydroxyl using GTP and Mn(II) ions. Desai and Raines used GTP analogues (GTPγS, GppCP, GppNHp, GTPγS and GpCP, Table 3, entry 1) to determine the site of triphosphate scission during this
### Table 3

A selection of phosphoanhydride analogues and example applications

<table>
<thead>
<tr>
<th>Entry</th>
<th>Analogue structure</th>
<th>Acronym/name</th>
<th>Application</th>
<th>Ref</th>
</tr>
</thead>
</table>
| 1     | ![GTP analogue](image) | GTPαS; X = S, Y = Z = U = O  
GpCp; Y = CH2, X = Z = U = O  
GppNHp; Z = NH, X = Y = U = O  
GTPγS; U = S, X = Y = Z = O | Studying the mechanism of RNA ligase RtcB | [30**,31] |
| 2     | ![Difluoromethylene-triphosphate](image) | α,β-Difluoromethylene-triphosphate | Evaluating the structure and mechanism of polβ | [33] |
| 3     | ![Variety of analogues](image) | X = CH₂, CHF, CHCl, CHBr, CCl₂, CBr₂, CCl₂, CFCl or CF₂ | Studying the mechanism of polβ | [34**,35,54] |
| 4     | ![N-guanosine analogues](image) | Variously:  
R₁ = Me or Bn  
R₂, R₃ = H or Me  
X = O or S  
Y₁, Y₂ = O, NH, CH₂  
N = H or 5′-guanosine  
n = 1 or 2 | Studying mRNA cap structure interactions and hydrolysis | [43] |
| 5     | ![Boranophosphate analogues](image) | boranophosphate  
2′-Ome ATP(αB); X = OH, Y = OCH₃  
3′-dATP(αB); X = H, Y = OH | Inhibitors to hepatitis C virus. | [44] |
<p>| 6     | <img src="image" alt="Boranophosphate" /> | Boranophosphate | Efficient and selective agonist to P2Y6-receptor. | [45] |
| 7     | <img src="image" alt="APCP analogues" /> | APCPP(αS) | Potential in the development of therapeutics against Alzheimer’s disease | [48] |</p>
<table>
<thead>
<tr>
<th>Entry</th>
<th>Analogue structure</th>
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<th>Application</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td><img src="image1.png" alt="Image" /></td>
<td>X = Me, or H</td>
<td>Diastereomically pure precursor for new analogues.</td>
<td>[50]</td>
</tr>
<tr>
<td>9</td>
<td><img src="image2.png" alt="Image" /></td>
<td>ApCH2OPOCH2pA</td>
<td>Inhibitor for Ap4A hydrolase</td>
<td>[51]</td>
</tr>
<tr>
<td>10</td>
<td><img src="image3.png" alt="Image" /></td>
<td>UDP-C1-phosphonate-sugar</td>
<td>Revealing UDP-apiose/UDP-xylose synthase mechanism.</td>
<td>[52]</td>
</tr>
<tr>
<td>11</td>
<td><img src="image4.png" alt="Image" /></td>
<td>α,β,γ-Bisdifluoromethylene-triphosphate</td>
<td>Nonhydrolysable nucleotide analogue</td>
<td>[53]</td>
</tr>
</tbody>
</table>
process [30**]. Their mechanism proposes the formation of a 2',3'-cyclic phosphate that is opened by nucleophilic attack of the 5'-hydroxyl of the ligating strand. The α,β-methylene analogue GpCpp proved unable to support the healing action of RtcB, suggesting that α,β-fission of GTP is critical. Further insight through crystallography using GTPoS and Mn(II) ions suggested the importance of hydrogen bonding networks and the second Mn(II) ion in orientating the triphosphate in the active site in an appropriate conformation for in-line attack by the active site His nucleophile [31]. The GMP-His intermediate is then attacked by the 3'-phosphate of the 3'-ligating strand to form a activated guanylate, and the 2'-OH then attacks to form the 2',3'-cyclic phosphate in readiness for ligation.

The mechanism and stereoselectivity of DNA polymerase β

The use of dNTP analogues in mechanistic studies was reviewed in 2010 by McKenna et al. [32], however, this team has recently augmented the dNTP analogue repertoire with a range of α,β-halomethylene-triphosphate systems (Table 3, entry 2). These systems were prepared chemoenzymatically (e.g. α,β-CF₂₃-dCTP) or using the morpholidate method (e.g. α,β-CF₂-dTTP) to study stereoelectronic effects within the triphosphate group through variation of the halo substituent and subsequent crystallographic studies in the presence of these non-hydrolysable analogues [33]. In earlier complementary works, McKenna and colleagues employed β,γ-bridge analogues that allowed perturbation of the pyrophosphate leaving group pKₐ [34**,35]. Fortunately, the β,γ-halomethylene-GTP analogues were substrates, and their kinetic activities were correlated using linear free energy relationships (LFER). Human DNA polβ incorporated β,γ-halomethylene-GTP against both cognate C and non-cognate T template residues, with the chemical step being rate-limiting in both cases. Unsurprisingly, cognate incorporation was markedly faster than non-cognate; however, individually, the two sets of kinetic data correlated under LFER analyses. Reduced activities were measured for the bulkier dihalogen substrates where the template base was also influential in the magnitude of diminished activity. The detection of even lower catalytic activity for mismpairs serves as a potential tool to explore the structural distinctions between transition states derived from cognate or non-cognate base incorporations.

The use of substituted methylene bridges, -CXY- potentially introduces an additional stereogenetic centre into β,γ-dGTP analogues (Table 3, entry 3). Crystallisation of DNA polβ in the presence of diastereomeric mixtures of each of β,γ-CHF, CMeF and CClF₃ dGTP analogues led to selective active site occupancy by the diastereomers that allowed the formation of CX-F-Arg183 hydrogen bonds [36]. Diastereomerically pure β,γ-CHF-dGTP and β,γ-CHCl-dGTP were prepared and the R and S-configurational isomers were assessed kinetically [37]. R-diastereomers proved more proficient substrates than S, with the R-β,γ-CHF-dGTP being most effective, confirming the advantageous effect of the CX-F-Arg183 interaction [38].

mRNA cap analogues

Synthetic methodologies for the preparation of mRNA cap analogues have been developed to study biotechnologically and medicinally significant cap-dependent processes (Table 3, entry 4) [39-42]. The binding and hydrolysis of 5'-cap mimics by the cap scavenger from Caenorhabditis elegans (CeDepS) were explored using a collection of methylenephosphonate, imidodiphosphate and phosphorothioate cap analogues, revealing regioselective β,γ-cleavage [43].

Other phosphoanhydride analogues

Recent examples include stereopure α-P-boranophosphate-ATPs that have shown anti-hepatitis C activity (Table 3, entry 5) [44] and selective agonism against the P2Y₆ receptor (Table 3, entry 6) [45]. Further elaboration of the boranophosphate based triphosphate to include a β,γ-dichloromethylene group provided a promising P2Y₁₁ receptor antagonist [46]. Systems combining phosphorothioate and bridging oxygen-substitutions (Table 3, entry 7) have demonstrated potential as therapeutics against Alzheimer’s disease owing to their metal ion chelation properties [47,48]. The use of sulfur-based analogues in the determination of mechanism has been reviewed recently [49]. Recent synthetic advances have also given (easier) access to: azido-phosphonate dNTPs, where bridging O-atoms have been replaced by –CHN₃ groups (Table 3, entry 8), and these analogues can be isolated as separate diastereomers [50]; and oxymethyl analogues (CH₂ insertion between O and P within anhydride linkages) for following Apₐ,Apₐ and Apₐ,N degradation and metabolism (Table 3, entry 9) [51]. Phosphonate NDP-sugar analogues, where the C1-oxygen of the glycosyl group has been replaced by methylene, have given insight into the mechanism of UDP-apiose/UDP-xylene synthase (Table 3, entry 10) [52], and bis-α,β-β,γ-CF₂-NTPs offer stericly undemanding mimics that do not hydrolyse while maintaining comparable polarity properties to their natural NTP progenitors (Table 3, entry 11) [53].

Conclusions and future

Multi-faceted approaches combining several experimental techniques and/or computational methods are currently giving some of the clearest pictures of phosphoryl transfer strategies. Most of these approaches have, in principle, been available for some time, however, experimental difficulties have precluded their exploitation. Synthesis of analogues remains a substantial obstacle, with many ‘obvious’ analogues only becoming accessible through painstaking development of challenging routes. This is particularly true of the
phosphoanhydride systems. Fortunately, several groups are working towards more convenient methodologies for the preparation of phosphoesters, anhydrides and their analogues, and details of these efforts can be accessed elsewhere [55, 56, 1], 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68*, 69]. Heavy isotope kinetic studies have proven extremely enlightening, however, the measurement of these extremely small effects (even in best case scenarios) remains the preserve of a few specialist groups. Combinations of experimental approaches with computational methods are also allowing more rigorous, quantitative assessment of observed kinetic data, where interpretations of kinetic results can often be complex. In summary, synthetic methodology, in tandem with kinetic measurements and computational dissection are providing enzymologists with an enhanced toolbox for the determination of phosphotransfer mechanisms.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


An overview of the mechanistic spectrum covered by phosphoryl transfer in solution studies and at enzyme active sites.


Contextualisation of the magnitude of catalysis required to transfer phosphoryl ester groups.


KIEs, transition state analogues and crystallography combine to give clear pictures of phosphoryl transfer to and from active site cysteine.


Kinetically and 19-F NMR studies on metal ion fluoride systems disprove the existence of a contentious phosphonate intermediate.


A phosphorothioate approach is applied alongside nucleobase substitution to give unequivocal evidence of acid/base catalysis, and the groups involved, within the HDV ribozyme.


The preparation and uses of phosphorothioate RNAs in the dissection of mechanism are reviewed.


KIE studies are used to gain the first full picture of the transphosphorylation process that occurs during hydroxide ion-catalysed RNA cleavage.


31. A range of GTP analogues was employed to demonstrate the need for α,β-cleavage of the triphosphate of GTP in the mechanism of RtcB. GTP serves as an activator for 3-phosphate RNA to form a 2',3'-cyclic phosphate terminus that is attacked by the 5'-OH of the ligating RNA strand.


36. The fidelity of the polymerase was explored as a function of pyrophosphate leaving group pK_a and linear free energy analysis showed similar values for both cognate and non-cognate base incorporations, with breaks in both relationships suggesting a change in rate-determining step for those systems with the best leaving groups.


40. Stereocap halomethylene dGTP analogues were explored with DNA polymerase β, and a unique Arg-F interaction was found. This type of interaction could allow selective targeting of cancer-related polymerases.


56. Mohamady S, Desoky A, Taylor SD: Sulfonyl imidazolium salts as reagents for the rapid and efficient synthesis of nucleoside polyphosphates and their conjugates. Org Lett 2012, 14:402-405. Sulfonyl imidazolium salts are used to activate terminal nucleoside phosphates for attack by phosphate nucleophiles to form phosphoanhydride bonds.


68. Cremosnik GS, Hofer A, Jessen HJ: Iterative synthesis of nucleoside oligophosphates with phosphoramidites. Angew Chem Int Ed Engl 2014, 53:266-269. Solvent properties are harnessed to allow the homologation of phosphates to build up polyphosphate chains using moisture tolerant phosphoramidite methods, and purification requirements are minimised or removed.