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Phosphate analogues in the dissection of mechanism

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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 10^{21}-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 monester, 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.

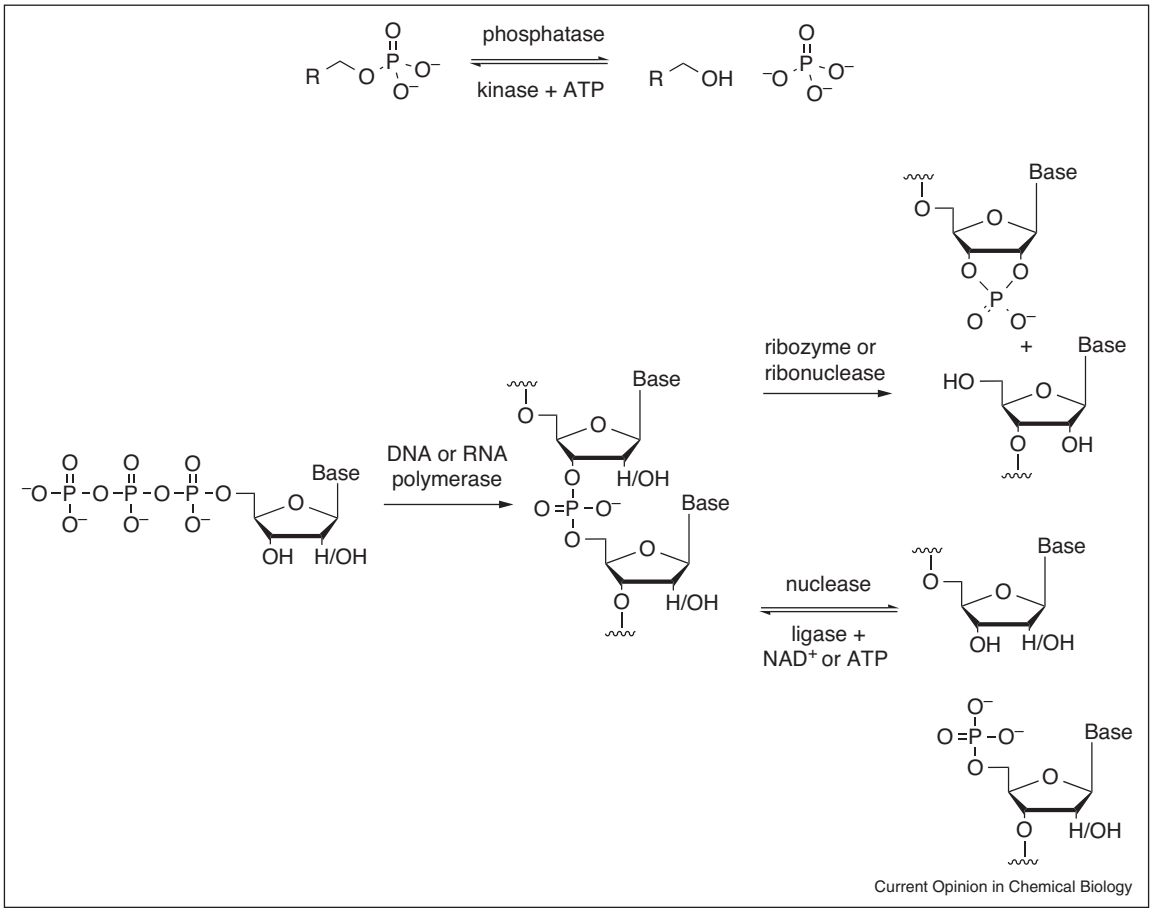
Monoesters

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.

Scheme 1



Some common phosphoryl transfer processes.

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 PO_3^- group of the phosphorane was, in fact, a MgF_3^- system (Table 1, entry 3) [5•], that is difficult to distinguish from the PO_3^- group using X-ray diffraction alone.

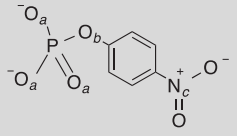
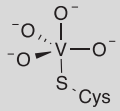
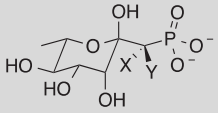
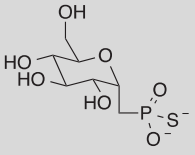
Similar 19-F NMR approaches with MgF_3^-, AlF_3 and AlF_4^- transition state analogue systems have been used in tandem with crystallographic and mutagenesis studies to give insight into the balance between enzyme preferences for charge balancing *versus* isostery in several phosphoryl transferase enzymes [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].

Table 1

A selection of phosphomonoester analogues and example applications

Entry	Analogue structure	Acronym/name	Application	Ref
1	 a,b,c=sites for isotope substitution	*p*PNPP	Determination of transition state structure of phosphate monoester transfer	[3••]
2		Vanadate	Transition state analogue for phosphate monoester transfer	[3••]
3	MgF_3^-, AlF_3, AlF_4^-	–	19-F NMR and X-ray analyses of active sites	[6–10]
4	 X=Y=H OR X=Y=F (synthesis of X=H, Y=F unsuccessful)	Phosphonate/α-halophosphonate	Non-scissile analogue of rhamnose-1-phosphate	[11]
5		Thiophosphonate	Non-scissile analogue of sugar-1-phosphate	[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-Mg^{2+} 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 Piccirilli laboratory, has been reviewed [19••,20].

More detailed pictures of RNA hydrolysis through the use of isotopes

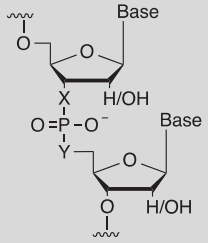
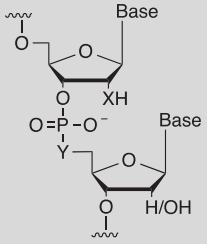
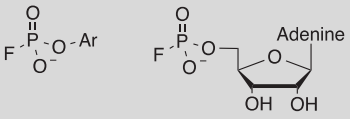
More subtle substitution of phosphodiesters can be effected through the use of isotopomeric compounds, such as 18-O labelled species (Table 2, entry 2). Heavy atom isotope effects are challenging to determine on a practical level, however, isotopic substitutions represent the least perturbing of all possible analogues. 5′-^{18}O and 2′-^{18}O 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

Table 2

A selection of phosphodiester analogues and example applications

Entry	Analogue structure	Acronym/name	Application	Ref
1	 X=S, Y=O or X=O, Y=S	Phosphorothiolate	Modulation of leaving group pK_a/metal ion interaction probe	[14–16,17•,18,19••]
2	 X=18-O, Y=16-O or X=16-O, Y=18-O	18-O isotopomers	Probing transition state structure	[21••,22]
3		Fluorophosphonate	Isosteric monoester analogue with altered perturbed TS for phosphoryl transfer	[23,26]

hydrogen bonding characteristics of monoesters [23]. This mixed character was used to explore the promiscuous proficiencies of phosphoryl transfer by alkaline phosphatase. Comparison of arylfluorophosphonates with methyl aryl phosphate diesters (Table 2, entry 3, left) as substrates for R166A alkaline phosphatase showed the enzyme to have higher proficiency ($[k_{cat}/K_M]/k_{uncat}$) towards the diester, with both systems displaying significantly reduced proficiencies 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].

Phosphoanyhydrides

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 (GTPαS, GppCP, GppNHp, GTPγS and GPcPP, Table 3, entry 1) to determine the site of triphosphate scission during this

Table 3

A selection of phosphoanhydride analogues and example applications

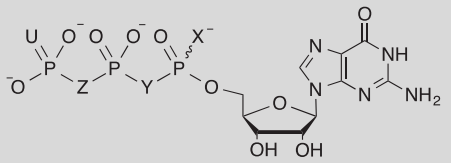
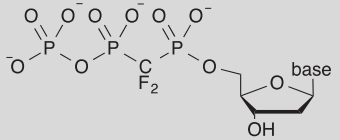
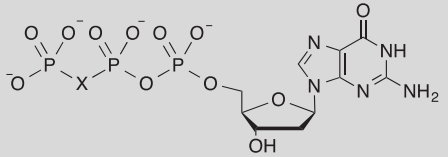
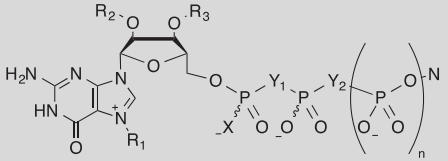
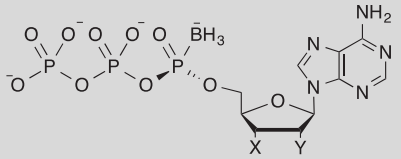
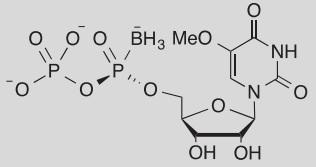
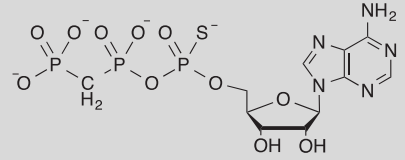
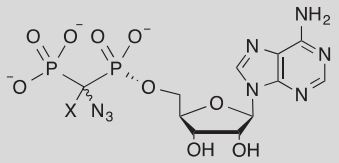
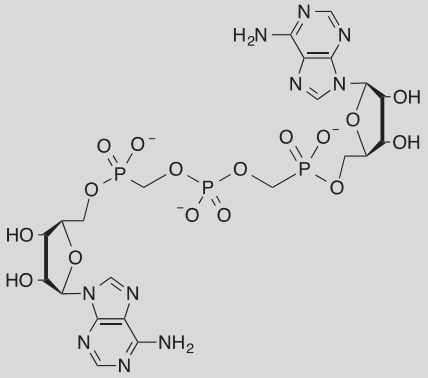
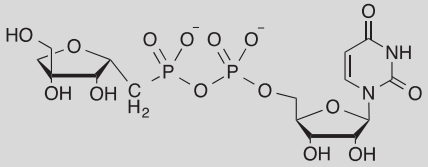
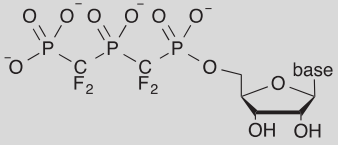
Entry	Analogue structure	Acronym/name	Application	Ref
1		GTPαS; X = S, Y = Z = U = O GpCpp; Y = CH_2, X = Z = U = O GppNHp; Z = NH, X = Y = U = O GppCp; Z = CH_2, X = Y = U = O GTPγS; U = S, X = Y = Z = O	Studying the mechanism of RNA ligase RtcB	[30••,31]
2		α,β-Difluoromethylene-triphosphate	Evaluating the structure and mechanism of polβ	[33]
3		X = CH_2, CHF, CHCl, CHBr, CCl_2, CBr_2, CCl_2, CFCl or CF_2	Studying the mechanism of polβ	[34••,35,54]
4		Variously: R_1 = Me or Bn R_2, R_3 = H or Me X = O or S Y1, Y2 = O, NH, CH_2 N = H or 5′-guanosine *n* = 1 or 2	Studying mRNA cap structure interactions and hydrolysis	[43]
5		boranophosphate 2′-OMe ATP(αB); X = OH, Y = OCH_3 3′-dATP(αB); X = H, Y = OH	Inhibitors to hepatitis C virus.	[44]
6		Boranophosphate	Efficient and selective agonist to P2Y6-receptor.	[45]
7		APCPP(αS)	Potential in the development of therapeutics against Alzheimer's disease	[48]

Table 3 (*Continued*)

Entry	Analogue structure	Acronym/name	Application	Ref
8		X = Me, or H	Diastreomerically pure precursor for new analogues.	[50]
9		$ApCH_2OpOCH_2pA$	Inhibitor for Ap4A hydrolase	[51]
10		UDP-C1-phosphonate-sugar	Revealing UDP-apiose/UDP-xylose synthase mechanism.	[52]
11		α,β-β,γ-Bisdifluoromethylene-triphosphate	Nonhydrolysable nucleotide analogue	[53]

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 GTPαS 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-CF_2-dCTP) or using the morpholidate method (e.g. α,β-CF_2–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 pK_a [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 mispairs 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 stereogenic centre into β,γ-dGTP analogues (Table 3, entry 3). Crystallisation of DNA polβ in the presence of disasterometric mixtures of each of β,γ-CHF, CMeF and CClF 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* (CeDcpS) 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 $P2Y_6$ receptor (Table 3, entry 6) [45]. Further elaboration of the boranophosphate based triphosphate to include a β,γ-dichloromethylene group provided a promising $P2Y_{11}$ 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 $–CHN_3–$ groups (Table 3, entry 8), and these analogues can be isolated as separate diastereomers [50]; and oxymethyl analogues (CH_2 insertion between O and P within anhydride linkages) for following Ap_nA and Np_nN 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-xylose synthase (Table 3,entry 10) [52], and bis-α,β-β,γ-CF_2-NTPs offer sterically 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•,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 phosphoryl transfer mechanisms.

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- • of special interest
- •• of outstanding interest

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