Biochimica et Biophysica Acta 1834 (2013) 454–463

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Review

New light on phosphate transfer from triesters ☆

Anthony J. Kirby [a], José R. Mora [b], Faruk Nome [b,*]

[a] University Chemical Laboratory, Cambridge CB2 1EW, UK
[b] INCT-Catalise, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, 88040-900, Brazil

ARTICLE INFO

Article history:
Received 31 January 2012
Received in revised form 12 April 2012
Accepted 24 April 2012
Available online 2 May 2012

Keywords:
Phosphate transfer
LFER
Hydrolysis
Density Functional Theory

ABSTRACT

The reactivity of triesters is discussed in the general context of phosphate transfer, as usually studied for the reactions of mono- and diesters. Systematic work has typically concentrated on the Linear Free Energy Relationships measuring the dependence of reactivity on the nucleophile and the leaving group, but new results indicate that it can depend equally strongly on the two non-leaving (sometimes known as spectator) groups. This conclusion is supported by first results from theoretical calculations: which also predict that a two-step mechanism can be favored over a concerted $S_N2(P)$ mechanism even for reactions involving leaving groups as good as p-nitrophenolate. This article is part of a Special Issue entitled: Chemistry and mechanism of phosphatases, diesterases and triesterases.

1. Introduction

The formation and cleavage of the P–O bond is a process of primary importance to the chemistry of life. It is catalyzed by extensive ranges of enzymes involved in metabolic pathways, in the biosynthesis and reactions of the nucleic acids, and in a host of regulation processes. Consequently the details, and especially the chemical mechanisms of these reactions, are of major practical interest. They also bear on the general problem of catalytic efficiency, since many of the reactions concerned are extraordinarily slow in the absence of enzymes: yet under physiological conditions they run at typical enzyme rates, with half-times of less than a second.

The substrates in these manifold biological processes are typically phosphate mono- or di-esters, and the group transferred PO_3^- or $ROPO_2^-$, via transition states **TS(1)** and **TS(2)**, respectively (Scheme 1).

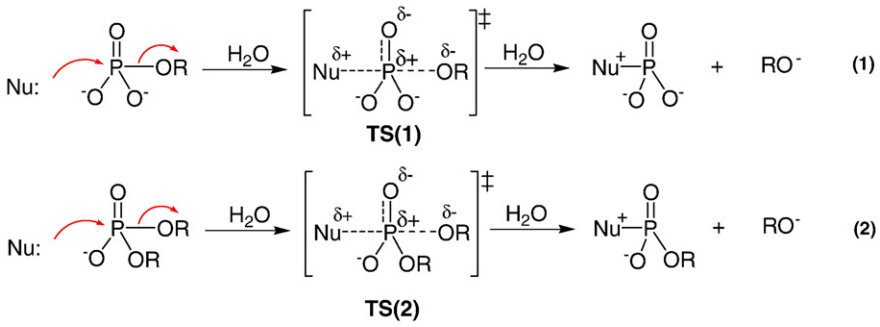
Our current understanding of the mechanisms of these processes is derived from many years of mechanistic work on simple systems: which for good practical reasons – not least the need to observe a reaction at a measurable rate – typically involved good leaving groups. The evidence is summarized and discussed in detail in excellent recent reviews, and we use their main conclusions as starting points [1–3].

Linear Free Energy Relationships (LFERs) show that reactivity in both the systems of Scheme 1 are dominated by the leaving group, as measured by the dependence on the basicity of the departing oxyanion. Thus Brønsted (leaving group) plots of log k_{obs} vs pK_{LG} for the hydrolysis of phosphate mono- and diesters and for their reactions with other nucleophiles typically show negative slopes β_{LG} in the region of unity (Table 1): consistent with charge changes on the leaving group oxygen in the transition state comparable with the full ionization of the leaving group ROH (typically a phenol). Reactivity also depends on the nucleophile, but sensitivities to pK_{nuc} (measured by LFER as β_{nuc}) are very small for the reactions of monoester dianions, consistent with bond formation to phosphorus still in its early stages: and thus a "loose" – though not completely dissociative – transition state **TS(1)**. Sensitivities (β_{nuc}) to the nucleophile are significantly greater for the reactions of diesters: these generally less reactive substrates need more substantial "leverage" from the nucleophile to set up the more advanced degree of P–O bond breaking needed to enable the departure of the leaving group: in other words, stronger bonding to the nucleophile, in a tighter transition state.

The monoanions of phosphate monoesters are a well-known special case. In principle an OH substituent should be similar in electron-withdrawing capacity to OMe: but the hydrolysis of the methyl phosphate monoanion $MeOP(OH)O_2^-$ [4] is much faster than that of dimethyl phosphate $(MeO)_2PO_2^-$; because of the mobility of the catalytically versatile proton. This is involved in the protonation of the leaving group, as indicated by the much-reduced value of $\beta_{LG} = -0.27$ at 100 °C [5]. Linear Free Energy Relationships for the hydrolysis of all the various types of phosphate ester present at significant concentrations near pH 7 are summarized in Fig. 1 in terms of observed values of β_{LG}, normalized to a common temperature of 25 °C.

* Corresponding author at: Department of Chemistry, UFSC, Florianópolis, Santa Catarina 88.040-900, Brazil. Tel.: +55 48 3721 6849, +55 48 9911 4633; fax: +55 48 3721 6850.
E-mail address: faruk.nome@ufsc.br (F. Nome).

1570-9639 © 2012 Elsevier B.V. Open access under the Elsevier OA license.
doi:10.1016/j.bbapap.2012.04.010



Scheme 1. Phosphate transfer at pH 7 from the predominant ionic forms of mono- (**1**) and diesters (**2**) to a general nucleophile Nu. The reactions involve more or less concerted $S_N2(P)$ processes (see the text).

Note that the data refer specifically to aryl phosphate esters, for which good quality data are available.

The familiar general conclusion from Fig. 1 is that diesters are the least and triesters the most reactive phosphate esters towards hydrolysis: but the behavior of monoester monoanions is special, in that the much lower slope of the line for $ArOP(OH)O_2^-$ means that it intersects with both the $(ArO)_2PO_2^-$ line at low values of pK_{LG} and the triester line at high values. So that for good leaving groups the monoester dianion is hydrolyzed faster: while extrapolation to the region of poor (alkoxide) leaving groups would indicate that for $pK_{LG} > 12.3$ the monoester monoanion will be hydrolyzed faster than the corresponding triester.

2. Reactivity of phosphate triesters

Phosphate triesters do not occur naturally in biological systems, and historically interest in their chemistry was primarily industrial, following the discovery that organophosphorus compounds like diethyl 4-nitrophenyl phosphate (marketed as ethyl paraoxon and produced as the bioactive metabolite of its P═S analogue parathion) had valuable herbicidal and insecticidal properties. Taking advantage of their anticholinesterase activities these and related compounds were developed into a range of powerful nerve gas agents. The consequent need for antidotes to organophosphorus and related poisons [7] has morphed into an important current interest in catalysts [8,9] or other means capable of destroying the huge stockpiles of these agents safely and economically [10,11].

The mid-twentieth century appearance of synthetic organophosphorus compounds in the biosphere, primarily as agricultural pesticides, appears to have set in train a natural response similar to the appearance of β-lactamases triggered by the mass use of β-lactam antibiotics. Thus, in the 1970s certain soil bacteria were found to hydrolyze a wide range of organophosphorus compounds and to contain a new class of enzyme that showed high phosphotriesterase activity [12].

The appearance of this new class of enzyme boosted interest in the reactivity of phosphate triesters, as non-natural substrates in biological systems and as a well-defined but little studied organic system. The system (**1**) selected for an early systematic investigation of "the reactivities of a wide range of nucleophiles towards the phosphorus center of a series of dialkyl substituted-phenyl phosphate esters" [13] reflects the contemporary situation nicely: the chosen compound **1** (Scheme 2) "lack the powerful anticholinesterase activity of the simple acyclic esters, but have normal chemical reactivity."

Our current understanding of the reactivity of phosphotriesters can be summarized instructively in terms of the similarities to and differences from the reactions of phosphate mono- and diesters.

2.1. The classical picture

The picture of phosphotriester reactivity that emerged from this early work revealed clear qualitative similarities to comparable reactions of carboxylic esters: though the p-nitrophenyl ester **1** (Ar = 4-nitrophenyl, half-life for spontaneous hydrolysis 2–3 years at 39 °C

Table 1
Sensitivities to nucleophile and leaving group for reactions of phosphate esters.[a]

Nucleophilic center	β_{nuc}	β_{LG}	T °C	Data sets
Monoester dianions				
N	0 ± 0.05		25–65	4
H_2O		−1.16 ± 0.07	39	3
N		−0.99 ± 0.06	39	2
Diester anions				
N	0.40 ± 0.10		39	4
H_2O		−1.16	39	1
O		−0.83 ± 0.22	25, 39	6
N		−1.09 ± 0.14	23, 39	4
Triesters				
O	0.45 ± 0.17		25, 39	11
H_2O		−0.99	39	1
O		−0.58 ± 0.18	25, 39	19

[a] Based on Tables (in Supplementary Information) of Lassila et al. (*loc. cit.*) [3].

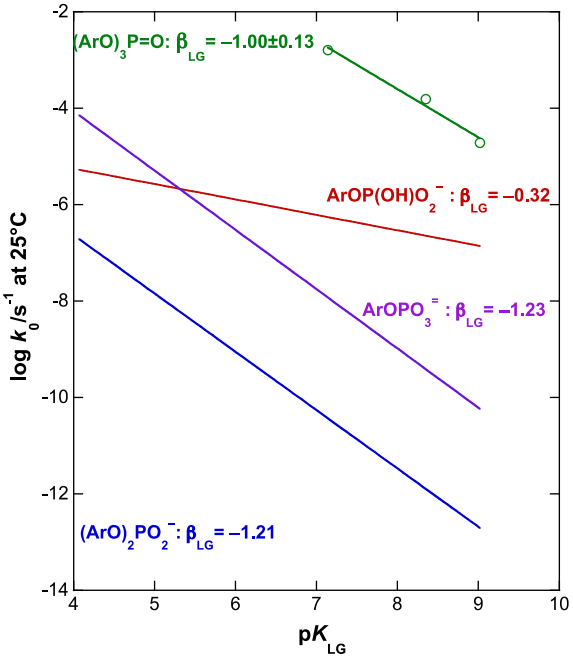
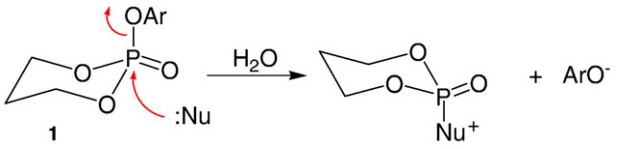


Fig. 1. LFER for the hydrolysis of the various species of phosphate ester, with β_{LG} normalized to 25 °C. The lines drawn are based on Table 1, with the cited β_{LG} corrected assuming the usual reciprocal dependence on absolute temperature. The important exception is the green line, for triesters, which shows new data points [6].



Scheme 2. The reaction of the "model" triester **1** was studied with a wide range of nucleophiles [13].

[13]) is considerably less reactive than p-nitrophenyl acetate (half-life a couple of weeks). There is a similar change in mechanism from nucleophilic (Scheme 3, Eq. 3) to general base catalysis of hydrolysis (Scheme 3, Eq. 4) as the nucleophile becomes markedly less basic than the leaving group, and α-effect nucleophiles [14] like hydroxylamine and the hydroperoxide anion show substantially enhanced reactivity: as in the case of phosphotriesters does the fluoride anion also, consistent with the formation of the stronger P – O bond compared with C – O.

Also significant was the finding that (for Reaction 3 of Scheme 3) the sensitivity to the basicity of the leaving group depends strongly on the basicity of the nucleophile (Table 2): and vice versa — the sensitivity to the basicity of the nucleophile depending similarly strongly on the leaving group, with β_{nuc} for the attack of oxyanions increasing from 0.30 to 0.48 over the small range of pK_{LG} from 4.07 to 7.14 [13].

Though this evidence for significant and variable "communication" between the nucleophile and the leaving group in the ground and transition states for Reaction 3 of Scheme 3 could also be accommodated by two-step mechanisms, the long-term consensus has clearly favored the concerted process for most reactions of triesters not involving intramolecular cyclizations. So that the observed changes are interpreted as evidence for the changing nature of the transition state: with the observed convergence of the (absolute numerical) values of β_{nuc} and pK_{LG} as consistent with a further "tightening" of the transition state for the reactions of triesters. This conclusion suggests a logical sequence: increasing tightness – i.e. stronger bonding of nucleophile and leaving group to the central phosphorus center – results from the decreasing number of negative charges – from three to two to one – that must be accommodated by **TS(1)** > **TS(2)** > **TS(3)** (Schemes 1 and 3). The greater the charge that must be stabilized the more the (effectively two) leaving groups, typically potential oxyanions, are called on to help accommodate it. Naturally poorer, less electronegative, leaving groups do this less effectively, and are a recipe for a tighter transition state: which in particularly favorable systems may exist as a pentacovalent addition (phosphorane) intermediate. Perhaps explaining, for example, the substantial amounts of retention of configuration observed in the methanolysis of the fixed conformation system **2**: which are as high as 91% with the poorest (still good!) leaving group ArO = p-$MeOC_6H_4O$ [15].

Table 2
Dependence of β_{nuc} on pK_{LG} for the reactions of nucleophiles with phosphotriester **1**.[a]

Nucleophile	pK_{nuc}	β_{LG}
Hydroxide	15.7	−0.36
$CF_3CH_2O^-$	12.4	−0.34
HOO^-	11.6	−0.35
Carbonate	10.3	−0.54
$HOPO_3^{=}$	7.2	−0.65
CH_3COO^-	4.8	−0.88
Water	−1.7	−0.99

[a] Data from Khan and Kirby, [13] for Ar = 4-nitro-, 4-acetyl-2-nitro-, 4-chloro-2-nitro- and 2,4-dinitrophenyl.

These very general conclusions are supported by a great deal of detailed experimental work, using advanced kinetic and extra-kinetic methods, including ^{18}O kinetic isotope effects on the various oxygens around phosphorus. A full presentation of the evidence and a systematic analysis of the conclusions are available in the comprehensive review of Lassila et al. [3].

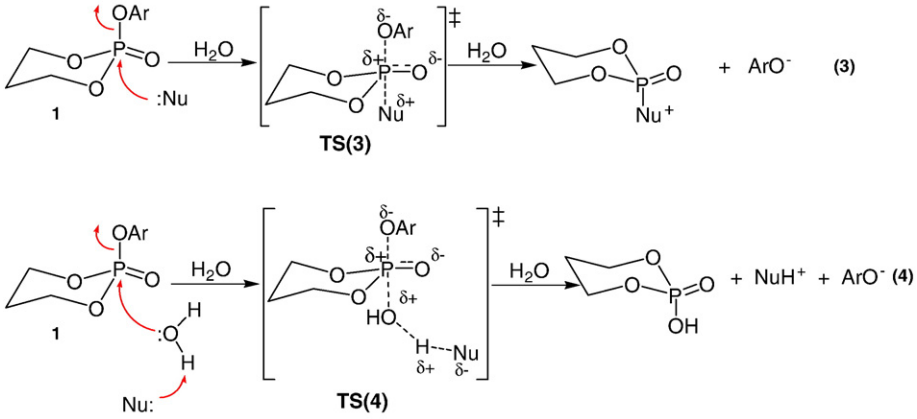
2.2. Reactivity revisited

Our discussion of triester reactivity has been based so far on comparisons with the reactions of mono- and diesters, and specifically on the effects of varying the nucleophile and the leaving group. We have seen that these effects become significantly more complex – and thus in principle more informative – for triesters: as nucleophile and leaving group are bound more tightly in the transition state (**TS(5)** in Scheme 4) they interact more strongly, as negative charge is transferred from the nucleophile, through the phosphorus center to the leaving group oxygen.

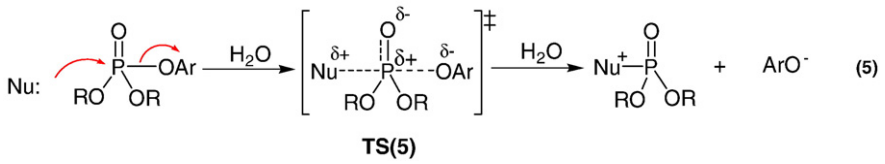
Reactivity depends on differences in free energy between ground and transition states, and in reactions of triesters the energies of these states must of course depend on all the substituents — half of which are the two non-leaving OR, groups which must occupy the equatorial positions of the trigonal bipyramidal **TS(5)**. It turns out that their effects can be very substantial. The effects of the single equatorial non-leaving group of **TS(2)** (Scheme 1) on the reactivity of diesters, by contrast, appear to be minimal [16].

This conclusion emerged very clearly from measurements of the rate of hydrolysis of the tris-2-pyridyl ester **TPP**, which is enormously faster than predicted from the LFER of Khan and Kirby (Scheme 5) [13].

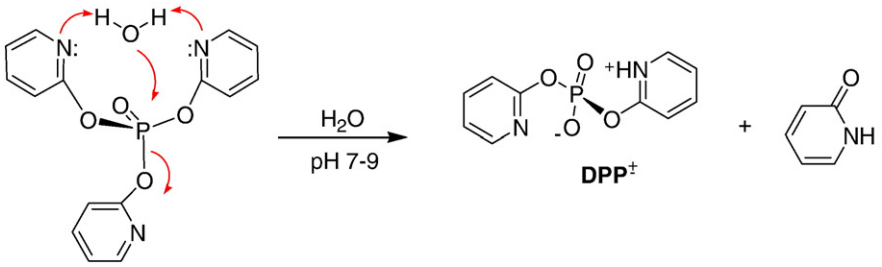
It turns out that this prediction, based on the LFER for the hydrolysis of the dialkyl aryl triester **1**, was wide of the mark. We saw in Fig. 1 that triaryl esters are hydrolyzed faster than monoester dianions, which are themselves hydrolyzed faster than diaryl esters. They are also hydrolyzed – and thus many times more reactive than – dialkyl



Scheme 3. (3) General SN(2)P reaction.(4) General basic catalysis by the nucleophile.



Scheme 4. Reactions of triesters with a general nucleophile Nu.



Scheme 5. Mechanism suggested to account for the supposed rapid hydrolysis of *TPP* [16].

aryl esters. Fig. 2 shows LFERs for the hydrolysis of a series of dialkyl aryl esters **1**, bis-trifluoroethyl aryl esters $(CF_3CH_2O)_2P(O)-OAr$ and symmetrical triaryl esters. Two conclusions stand out: reactivity increases dramatically across this series; but, nevertheless depends on the pK_a of the leaving group with the same, familiar (negative) beta leaving group (β_{LG}) of close to unity for all three series [6]. The tri-2-pyridyl ester **TPP** is hydrolyzed at the rate expected for a symmetrical triaryl ester derived from a phenol of pK_{LG} = 9.09, the nominal pK_a of 2-hydroxypyridine [6].

3. Reactivity depends significantly on the non-leaving groups

The broad spread of rates represented in Fig. 2 for any specific pK_{LG} shows clearly that increased electron-withdrawal by the two non-leaving groups increases reactivity substantially. No doubt this involves both raising the energy level of the reactant state, and also stabilizing the negative charge developing at the phosphorus center in the transition state. Thus the LFER for the hydrolysis of triaryl esters must include the effects of changing both leaving and non-leaving groups. The plot in Fig. 2 gives a reasonable straight line, suggesting that varying only the non-leaving groups should produce a new LFER: that is to say, the slope of the triaryl ester line of Fig. 2 should be equal to $\beta_{LG} + 2\beta_{NLG}$.

β_{NLG} can be obtained by measuring rates of hydrolysis for a series of compounds with the leaving group constant and the non-leaving group varied. The data available are limited, but show that the effects of the two non-leaving groups are substantial, and comparable with that of the leaving group. They also suggest strongly that both β_{NLG} and β_{LG} are greater for dialkyl aryl than for triaryl systems [6].

The interpretation of the Brønsted (non-leaving group) plot of Fig. 3 depends heavily on the data point for the hydrolysis of the bis-trifluoroethyl (the most reactive, and thus also the most accurate point of the dialkyl 4-nitrophenyl triester series). This point is a blatant outlier to plots attempting to relate the full data set to a single correlation. (As it is also in a full cognate data set of pyridyl-2-oxy

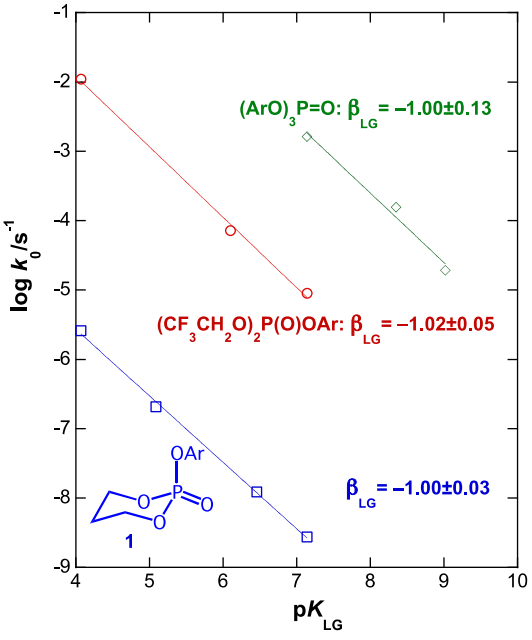


Fig. 2. LFER for the hydrolysis of three series of aryl phosphate triesters, with β_{LG} measured at (or, for series (**1**) normalized to) 25 °C.
Data from [1] (for compound **1**) and [6].

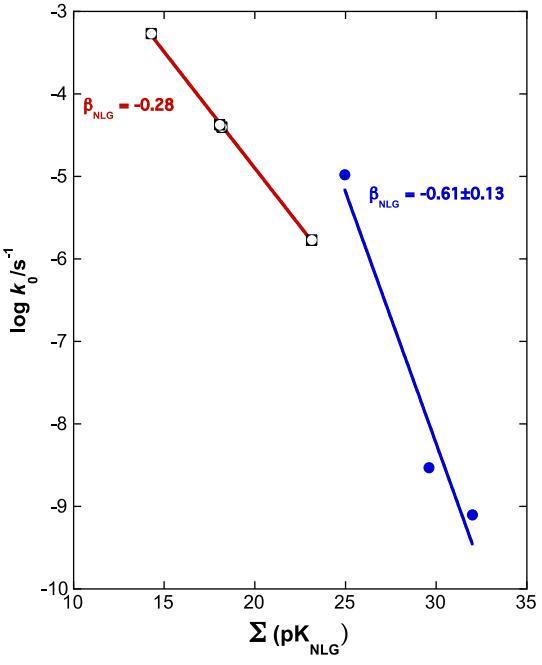
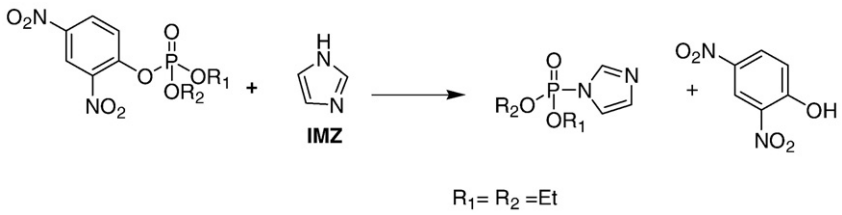


Fig. 3. Separate Brønsted (non-leaving group) plots for the hydrolysis of diaryl and ethyl aryl (open symbols), and dialkyl aryl (filled circles) 4-nitrophenyl triesters.
Data from [6].



Scheme 6. Nucleophilic catalysis by imidazole.

derivatives [6].) The clear implication of this separation of diaryl and dialkyl data sets with common aryloxy leaving groups is that reactivity depends differently, but comparably strongly, on leaving and non-leaving groups for these two types of triester. Specifically, respective values of β_{NLG} and β_{LG} of −0.61 and −0.99 are found for the spontaneous hydrolysis reactions of dialkyl aryl systems and −0.28 and −0.42 for triaryl esters [6].

Literature data for unrelated systems which support this conclusion are discussed in Section 3.3 below.

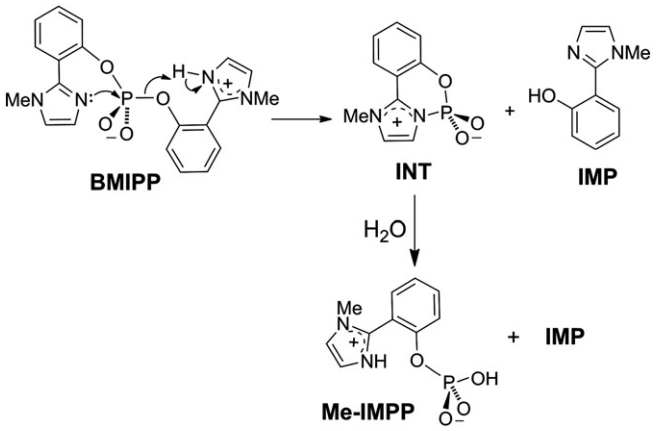
3.1. Reactions with strong nucleophiles

Brønsted (non-leaving group) plots for the reactions of hydroxide and fluoride anions with two series of triaryl esters with common leaving groups (4-nitrophenolate and 2-pyridyloxy) show markedly lower sensitivities to ΣpK_a, with observed values of β_{NLG} of the order of −0.15 [6]. For the limited data set available for these reactions there is no obvious difference in sensitivity between dialkyl and diaryl systems, as observed (Fig. 2) for spontaneous hydrolysis.

3.1.1. Reactions with imidazole

The histidine imidazole is the most versatile of the catalytic groups available to protein enzymes. At physiological pH it can act as a general acid, a general base or a nucleophile, and as a Lewis base plays important roles in the coordination, positioning and "tuning" of metal cations. The versatility of this simple functional group supports roles for histidine in the active sites of hydrolytic enzymes and in regulatory phosphorylation pathways [17].

The active site of ribonuclease A (RNAse A) depends critically on two histidine imidazoles, which work via a general acid–base pathway involving a neutral imidazole as a general base, with a second, imidazolium group acting as a general acid to assist the departure of the leaving group [17,4]. Alternatively, protonation of the phosphate diester anion offers an alternative pathway which takes advantage of the higher reactivity of phosphate triesters [4]. But imidazole also acts as a nucleophile, in the formation of the phosphohistidine intermediates which mediate the rapid and reversible phosphoryl group transfers involved in regulating many aspects of cell-life [5–9]. Despite the fact that bifunctional mimics of RNAse A provide benchmarks for the understanding of biological reactions and for the intelligent design of artificial enzymes, [18,19] models in which an imidazole group acts as a nucleophile at phosphate phosphorus are scarce, though much effort has focused on the evaluation of the reactivity of N-phosphorylimidazole [20,21] and phosphohistidines [22,23].



Scheme 7. Intramolecular catalysis in the *BMIPP*.

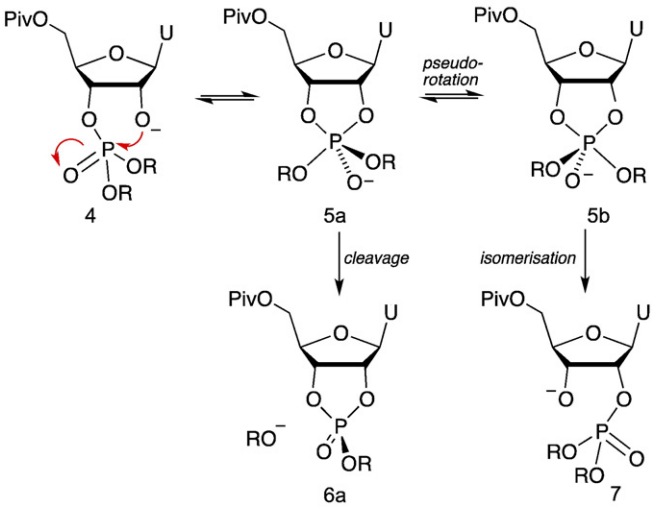
Imidazole is a very effective catalyst for the hydrolysis of phosphate triesters: the rate enhancement brought about by 1 M imidazole is 2000-fold for diethyl 2,4-dinitrophenyl phosphate (*Et_2DNPP*). Low solvent deuterium isotope effects k_{H2O}/k_{D2O} indicate that the reactions are at least primarily nucleophilic, and the entropy of activation is large and negative, also consistent with nucleophilic catalysis.

The reaction shown in Scheme 6 was followed by H NMR and ^{31}P NMR and using MS techniques. The phosphorylated intermediate was readily detected and its rate of hydrolysis to diethyl phosphate and imidazole measured as $3.33\times10^{-5}\ s^{-1}$, which corresponds to a half-life of 5.78 h at 25 °C. A series of phosphorylimidazole intermediates were characterized, some of them with half-lives, depending on the substituents, of the order of several weeks in water at 25 °C [24].

3.2. Intramolecular reactions

Of particular interest is the recently reported hydrolysis of a phosphate diester *BMIPP* bearing two imidazole groups, Scheme 7 [25,26].

This simple biomimetic model supports remarkably efficient intramolecular catalysis, and *BMIPP* is hydrolyzed up to 10^9 times faster than diphenyl phosphate. A mechanism involving general acid catalysis of nucleophilic catalysis for the hydrolysis of the reactive zwitterionic species was proposed, and the short lived cyclic intermediate *INT* was characterized using ESI-MS.



Scheme 8. Isomerization competes with cleavage in the reactions of dialkyl uridyl esters **4** (Piv is the stable pivalate protecting group) [17].

3.3. Intramolecular reactions with OH groups

Of special interest in this context are results for the series of reactions involving intramolecular cyclizations of hydroxyalkyl triester **4** (Scheme 8). These compounds undergo (reversible) base-catalyzed isomerization at pH > 3, in competition with the expected cleavage reactions, and can be understood in terms of the sequences shown in Scheme 8 [17].

The reactions involve the substrate oxyanion **4** (i.e. are primarily specific base catalyzed). The oxyanion is generated in close proximity to the triester phosphorus center, favoring the reversible addition to generate the 5-membered-ring phosphorane intermediate **5**. Given a sufficient lifetime a phosphorane anion derived from a triester can pseudorotate, with the single $P-O^-$ group always equatorial. Thus – in contrast to comparable reactions of diesters – its breakdown depends only on the leaving group capabilities of the various potential leaving groups [27]. Since these are determined by their basicities, (i) the first intermediate **5a**, with only hydroxyl and alkoxy leaving groups, shows maximum stability for the pentaoxyphosphorane anion: and (ii) its fastest reactions are the cleavage of the bonds to the 2′- and 3′-oxygens: i.e. reversion to reactant **4** and isomerization to **5b**. The slower loss of one of the exocyclic OR groups from **5a** or **5b** leads to cleavage (via the short-lived cyclic triester **6a** or its diastereoisomer **6b** (not shown)).

The isomerization of the triester **4** to its 2′-isomer **7** involves the transfer of the $(RO)_2P(O)$ group, with the non-leaving OR groups involved only indirectly in bond-making and breaking. Nevertheless their involvement is clearly substantial, as shown by the comparable values of β_{LG} and β_{isom} of -1.26 ± 0.07 and -1.10 ± 0.16, respectively [26]. In terms of β_{NLG} as defined above (plotting log k_{isom} against $\Sigma(pK_{NLG})$) this corresponds to a value of $\beta_{NLG} = -0.55 \pm 0.05$, the same, within experimental error, as that obtained (-0.61 ± 0.13, Fig. 3, above) [6] for the spontaneous hydrolysis of dialkyl aryl triesters.

4. Theoretical calculations

Theoretical calculations have been used to explore further the observed differences in reactivity between triaryl and dialkyl aryl triesters and the sensitivities to leaving and non-leaving groups [6].We focus our attention on possible transition state geometries, as well as the rate constants and thermodynamic parameters for the hydrolysis reaction. Density Functional Theory (DFT) [28,29] is used to analyze and compare the reactions of the triaryl triesters, tris-2-pyridyl phosphate (*TPP*) and dimethyl p-nitrophenyl phosphate (*DMPP*) [6,16] (Scheme 9).

Theoretical studies of the hydrolysis reactions of the triesters were performed at the B3LYP/6–31 + G(d,p) level of theory, using the GAUSSIAN 09 package implemented in Linux operating systems [28]. Transition states (*TS*) were obtained by the Quadratic Synchronous Transit (QST) protocol and their structures identified by their single imaginary frequencies. Intrinsic Reaction Coordinates (IRC) were calculated to confirm the reaction paths. Solvent effects are important and affect the results significantly. Consequently, substrate optimization was performed using both the Polarizable Continuum Model (PCM) and the SMD solvation model in all cases [30]. The reactions all proceed through characteristic van der Waals complexes ($\mathbf{R}^{\mathbf{complex}}$), involving the phosphate triester and (optimally) three water molecules, which are more stable than the separate reactants by approximately 4.2 kcal/mol.

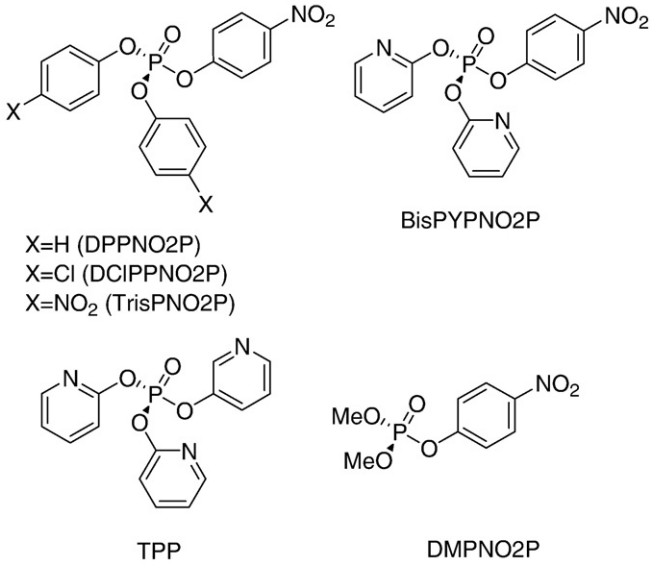
The Potential Energy Surfaces (PES) for the hydrolysis reactions of these substrates were evaluated, and stationary points characterizing van der Waals complexes, intermediates, transition states and products located, in order to obtain the activation parameters. The basic reaction involves attack of the oxygen atom of water on the central phosphorus atom of the triester and two possible mechanisms (Reactions 1 and 2 of Scheme 10) were considered. Reaction 1 is the classical $S_N2(P)$ mechanism and Reaction 2 the corresponding two step pathway, which involves a phosphorane intermediate. Table 3 compares the calculated and experimental parameters for the reaction of *DPPNO2P* with a water molecule.

There is poor agreement between the experimental and theoretical results, but the classical $S_N2(P)$ mechanism is calculated to be at least three orders of magnitude slower than the two step mechanism. Therefore, for more detailed calculations, considering specifically the effects of additional water molecules, we proceeded with the two-step mechanism 2, involving pentacoordinate phosphorane intermediates, an alternative commonly found in chemical and biological systems [31]. Mechanism 3 (which involves a six-membered cyclic transition state) shows how other water molecules could assist the hydrolysis process. Significantly, our attempted analysis of the classical $S_N2(P)$ mechanism using two water molecules resulted in the formation of the same phosphorane intermediate shown in Scheme 11.

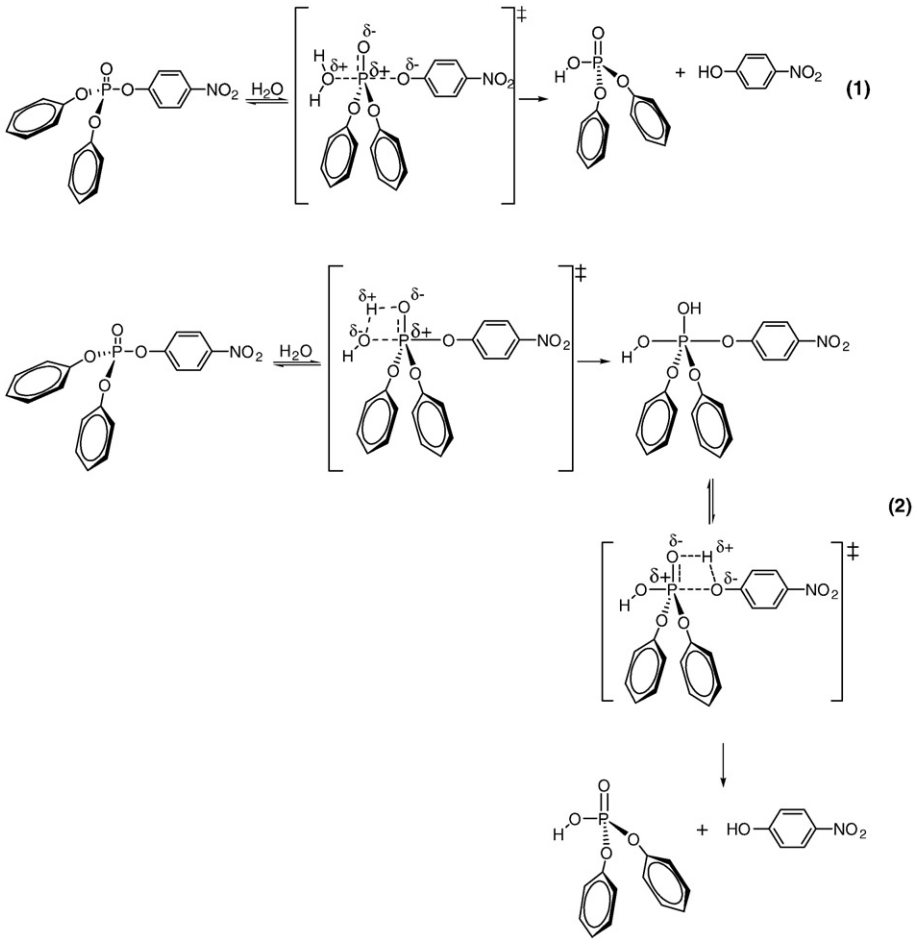
The calculations for the mechanism shown in Scheme 11 give a free energy of activation reasonably consistent with the experimental values. However, when a third water molecule is taken into account (Fig. 4), the free energy of activation to reach the transition state agrees almost perfectly with the experimental values (see Table 4). The entropy of activation would also be expected to be highly negative: as indeed it is in the only available measurements, of −35.6 e.u. for the hydrolysis of the 2,4-dinitrophenyl ester **2** [15] and −36.2 for *TPP* hydrolysis [6]. Furthermore an additional water molecule stabilizes neither the transition state nor the intermediate.

The transition states for the rate-determining step (**TS(6)**) and of the cleavage of the phosphorane intermediate (**TS(7)**) were verified by means of IRC calculations for the hydrolysis of *DPPNO2P* in the presence of three water molecules playing specific roles (Fig. 4). The IRC clearly shows a reasonably stable intermediate with well-defined participation of water molecules in the formation and cleavage of the intermediate. This result is fully consistent with proton inventory measurements and the substantial solvent deuterium isotope effect previously reported [6].

Using this transition state (**TS(6)**) as a model, theoretical calculations were performed for all the triesters shown in Scheme 8 and the results are included in Table 5. Good agreement between theoretical and experimental free energies of activation was observed for all compounds. These results show convincingly a strong dependence of



Scheme 9. Triaryl triesters compared by calculation [6].



Scheme 10. Concerted (**1**) and two-step (**2**) mechanisms for the spontaneous hydrolysis of diphenyl p-nitrophenyl phosphate.

reactivity on the non-leaving groups, as displayed in Fig. 3 for the experimental data.

As would be expected, for reactions with the same pair of non-leaving groups, the reactivity order is BisPYPNO2P > TPP, a result consistent with the difference in pK_a of the leaving group (p$K_{a\ (p\text{-nitrophenol})}$ < p$K_{a\ (pyridone)}$). Considering the common leaving group, we obtain the following order in reactivity: TrisPNO2P > BisPYPNO2P > DPPNO2P > DMPNO2P, showing that the non-leaving group plays a very significant role in the reaction, with the electron withdrawing groups accelerating the reaction by many orders of magnitude. The good agreement between the calculated and theoretical values, indicates that the "spectator" groups play important roles, increasing the electrophilicity of the phosphorus atom in the reagent and stabilizing the phosphorane intermediate.

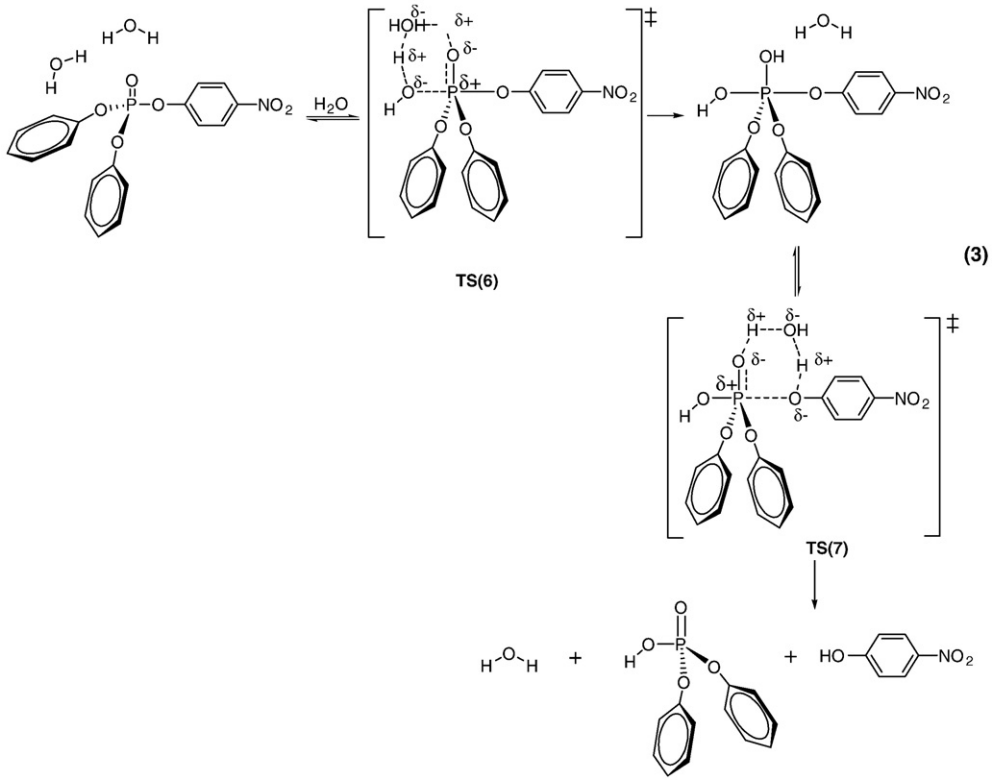
Atom numbering for the transition state (**TS(6)**) is shown in Scheme 12: the same numbering system is used for all compounds. Emphasis is given in this TS structure to the formation of $P_1 - O_2$, $H_3 - O_4$, and $H_5 - O_6$ bonds (attack of oxygen atom on the phosphorus atom) and to the rupture of $O_2 - H_3$ and $O_4 - H_5$ bonds. Table 6 summarizes structural parameters for reactant, rate-determining transition state and intermediate.

Structural analysis shows dihedral angles consistent with a trigonal bipyramidal intermediate, with the water molecules interacting through a non-planar hydrogen-bonding network. The significant decrease of the $P_1 - O_2$ distance from reactant to transition state indicates the formation of the P – O bond. The $P_1 - O_2$ distance changes from 3.806 Å to 1.778 Å for DPPNO2P, and from 5.283 Å to 1.673 Å for PYPNO2P. Although the distance between the nucleophilic water molecule and the phosphate triester differs from substrate to substrate, the $P_1 - O_2$ distance in the transition state is practically the same, at ca. 1.8 Å in each case. This result indicates that the formation of $P_1 - O_2$ bond is well advanced along the reaction coordinate in the rate-determining transition state.

The formation of the new P – O bond to the nucleophilic water is accompanied in all cases by the expected indications of the proton transfers involved in general base catalysis. A significant increase of the $O_2 - H_3$ distance, from 0.978–0.987 Å to 1.387–1.409 Å, is accompanied by a simultaneous substantial decrease in the $H_3 - O_4$ distance, from 1.823–1.869 Å to 1.091–1.097 Å, for the three triaryl phosphates (DPPNO2P, TrisPNO2P and BisPYPNO2P) with the same, p-nitrophenyl, leaving group. For the reactions of TPP and DMPNO2P the final $H_3 - O_4$ distance is similar (1.084–1.089 Å), and much shorter (3.248–3.273 Å) than in the reactant state. Evidently general base catalysis of hydrolysis in this system involves primarily just two water molecules. The $O_4 - H_5$ distance does not change significantly from reactant to transition state

Table 3
Calculated free energies of activation and rate constants the for hydrolysis of diphenyl p-nitrophenyl phosphate at 25 °C, at the B3LYP/6–31 + g(d) level of theory.

Calc. vs expt.	10^6 k (s^{-1})	$\Delta G^{\ddagger}$ (kcal/mol)
Calculated for Mechanism 1	6.25×10^{-14}	43.65
Calculated for Mechanism 2	1.22×10^{-11}	40.53
Observed experimentally	3.8	24.9



Scheme 11. Mechanism **3**. The two-step reaction (Mechanism 2 of Scheme 10) using two molecules of water.

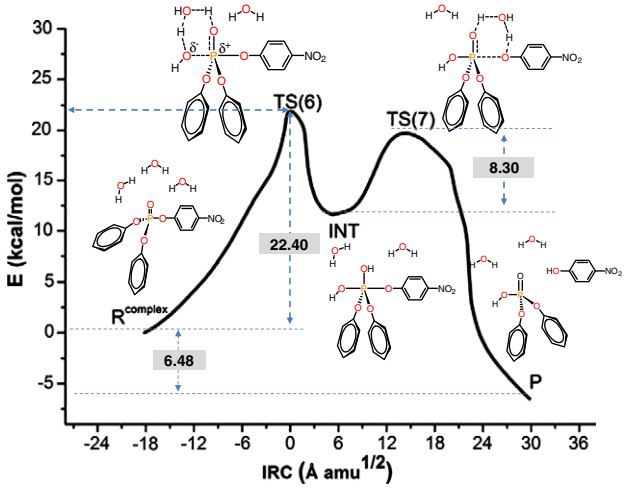


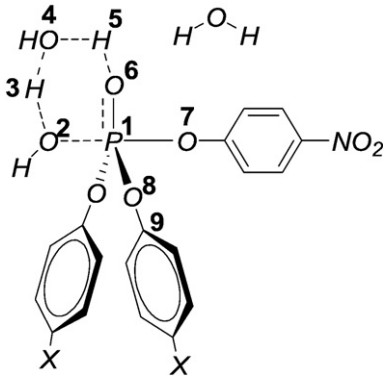
Fig. 4. Intrinsic Reaction Coordinate for the hydrolysis of diphenyl p-nitrophenyl phosphate (*DPPNO2P*), calculated for Mechanism 3 [**3** H_2O], using a step size of 0.1 bohr $amu^{1/2}$.

Table 4
Activation free energies and rate constants for hydrolysis of diphenyl p-nitrophenyl phosphate (*DPPNO2P*) with 1, 2, 3 or 4 water molecules at 25 °C at the B3LYP/6–31 + g(d) level of theory.

Calc. vs expt. [no. of water molecules]	10^6 k (s^{-1})	$\Delta G^{\ddagger}$ (kcal/mol)
Calculated for Mechanism 2 [1 H_2O]	1.22×10^{-11}	40.53
Calculated for Mechanism 3 [2 H_2O]	2.64×10^{-2}	27.79
Calculated for Mechanism 3 [3 H_2O]	2.26	25.15
Calculated for Mechanism 3 [4 H_2O]	1.46	25.42
Experimental	3.8	24.9

Table 5
Activation free energy and rate constant of hydrolysis of triaryl triesters, tris-2-pyridyl phosphate (*TPP*) and dimethyl p-nitrophenyl phosphate (*DMPNO2P*) with 3 water molecules at 25 °C at the B3LYP/6–31 + g(d) level of theory.

Compounds	Experimental	Calculated
	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol)
DPPNO2P	24.85	25.15
DClPNO2P	24.02	24.69
TrisPNO2P	21.91	22.28
BisPYPNO2P	23.36	22.23
TPP	24.46	26.14
DMPNO2P	29.59	29.97



Scheme 12. Atom numbering for the rate-determining transition state **TS(6)** for the hydrolysis of DPPNO2P. The same atom numbering system applies for all compounds listed in Table 6.

Table 6
Structural parameters of reactant (R), transition state (TS) and products (P) at B3LYP/6–31 + g(d) level of theory.

Interatomic distances (Å)							
	P_1-O_2	O_2-H_3	H_3-O_4	O_4-H_5	H_5-O_6	O_6-P_1	P_1-O_8
DPPNO2P							
R	3.806	0.985	1.869	0.982	1.855	1.491	1.595
TS	1.784	1.392	1.091	1.014	1.695	1.537	1.648
Int	1.680	2.196	0.976	1.703	1.005	1.614	1.637
DClPPNO2P							
R	5.008	0.998	1.819	0.982	1.867	1.491	1.596
TS	1.780	1.402	1.087	1.010	1.737	1.535	1.651
Int	1.673	2.812	0.973	1.673	1.008	1.611	1.641
TrisPNO2P							
R	4.825	0.987	1.822	0.981	1.881	1.488	1.598
TS	1.777	1.387	1.092	1.002	1.821	1.529	1.657
Int	1.678	1.911	0.980	3.036	0.977	1.623	1.637
BisPYPNO2P							
R	5.283	0.987	1.823	0.981	1.867	1.490	1.596
TS	1.786	1.381	1.097	1.006	1.765	1.532	1.653
Int	1.673	2.818	0.973	1.666	1.009	1.608	1.642
TPP							
R	4.055	0.978	3.248	0.973	5.352	1.489	1.602
TS	1.801	1.409	1.084	1.020	1.653	1.538	1.801
Int	1.692	1.900	0.981	3.035	0.976	1.626	1.640
DMPNO2P							
R	3.920	0.979	3.273	0.973	5.012	1.497	1.586
TS	1.799	1.400	1.089	1.027	1.616	1.545	1.631
Int	1.685	2.789	0.973	1.702	1.003	1.621	1.622

Dihedral angles (degrees) in TS					
$P_1-O_2-H_3-O_4$	$O_2-H_3-O_4-H_5$	$H_3-O_4-H_5-O_6$	$O_4-H_5-O_6-P_1$	$H_5-O_6-P_1-O_2$	$O_6-P_1-O_2-H_3$
DPPNO2P					
13.39	−1.31	−6.02	−2.64	11.95	−14.22
DClPPNO2P					
15.92	1.92	−7.11	−8.89	20.69	−22.36
TrisPNO2P					
15.16	3.13	−6.83	−9.81	21.40	−22.72
BisPYPNO2P					
11.02	4.83	−9.24	−3.41	15.65	−17.59
TPP					
12.03	2.01	−7.51	−4.55	15.21	−16.82
DMPNO2P					
14.60	−0.13	−9.00	−1.51	13.95	−16.76

and the change in H_5-O_6 distance is negligible in triaryl phosphates with the same leaving group (DPPNO2P, TrisPNO2P and BisPYPNO2P), while in TPP and DMPNO2P, this distance changes significantly. The (two) P_1-O_8 distances (bonds to the non-leaving groups) do not change significantly: the small changes observed are probably related to transition state stabilization.

5. Closing summary

Recent experimental results make clear that the reactivity of phosphate triesters depends not only on the nucleophile and leaving group capability, but also equally strongly in at least some cases on the two non-leaving (sometimes known as spectator) groups. This conclusion is confirmed by first results from the theoretical calculations: which predict that a two-step mechanism can be favored even for reactions involving leaving groups as good as p-nitrophenolate.

Acknowledgements

We are grateful to INCT-Catálise, CNPq, Capes, PRONEX and FAPESC for support of this work.

References

[1] W.W. Cleland, A.C. Hengge, Enzymatic mechanisms of phosphate and sulfate transfer, Chem. Rev. 106 (2006) 3252–3278.
[2] A.C. Hengge, Mechanistic studies on enzyme-catalyzed phosphoryl transfer, in: J.P. Richard (Ed.), Adv. Phys. Org. Chem., vol. 40, 2005, pp. 49–108.
[3] J.K. Lassila, J.G. Zalatan, D. Herschlag, Biological phosphoryl-transfer reactions: understanding mechanism and catalysis, Annu. Rev. Biochem. 80 (2011) 669–702.
[4] C.A. Bunton, D.R. Llewellyn, K.G. Oldham, C.A. Vernon, The reactions of organic phosphates. Part I. The hydrolysis of methyl dihydrogen phosphate, J. Chem. Soc. (1958) 3574–3587.
[5] A.J. Kirby, A.G. Varvoglis, The reactivity of phosphate esters. Monoester hydrolysis, J. Am. Chem. Soc. 89 (1967) 415–423.
[6] A.J. Kirby, M. Medeiros, P.S.M. Oliveira, E.S. Orth, T.A.S. Brandão, E.H. Wanderlind, A. Amer, N.H. Williams, F. Nome, Activating water: important effects of

non-leaving groups on the hydrolysis of phosphate triesters, Chem. Eur. J. 17 (2011) 14996–15004.
[7] J. Bajgar, J. Fusek, J. Kassa, K. Kuca, D. Jun, Chemical aspects of pharmacological prophylaxis against nerve agent poisoning, Curr. Med. Chem. 16 (2009) 2977–2986.
[8] R.S. Brown, A.A. Neverov, Metal-catalyzed alcoholysis reactions of carboxylate and organophosphorus esters, in: J.P. Richard (Ed.), Adv. Phys. Org. Chem, vol. 42, 2008, pp. 271–331.
[9] I.N. Ugwumba, K. Ozawa, Z.-Q. Xu, F. Ely, J.-L. Foo, A.J. Herlt, C. Coppin, S. Brown, M.C. Taylor, D.L. Ollis, L.N. Mander, G. Schenk, N.E. Dixon, G. Otting, J.G. Oakeshott, C.J. Jackson, Improving a natural enzyme activity through incorporation of unnatural amino acids, J. Am. Chem. Soc. 133 (2011) 326–333.
[10] B.M. Smith, Catalytic methods for the destruction of chemical warfare agents under ambient conditions, Chem. Soc. Rev. 37 (2008) 470–478.
[11] K. Kim, O.G. Tsay, D.A. Atwood, D.G. Churchill, Destruction and detection of chemical warfare agents, Chem. Rev. 111 (2011) 5345–5403.
[12] F.M. Raushel, H.M. Holden, Phosphotriesterase: an enzyme in search of its natural substrate, Adv. Enzymol. Relat. Areas Mol. Biol. 74 (2000) 51–93.
[13] S.A. Khan, A.J. Kirby, Reactivity of phosphate esters — multiple structure–reactivity correlations for reactions of triesters with nucleophiles, J. Chem. Soc. B (1970) 1172–1182.
[14] A.J. Kirby, D.W. Tondo, M. Medeiros, B.S. Souza, J.P. Priebe, M.F. Lima, F. Nome, Efficient intramolecular general-acid catalysis of the reactions of alpha-effect nucleophiles and ammonia oxide with a phosphate triester, J. Am. Chem. Soc. 131 (2009) 2023–2028.
[15] R. Rowell, D.G. Gorenstein, Multiple structure–reactivity correlations in the hydrolysis of epimeric 2-(aryloxy)-2-oxydioxaphosphorinanes. Stereoelectronic effects, J. Am. Chem. Soc. 103 (1981) 5894–5902.
[16] A.J. Kirby, M. Medeiros, P.S.M. Oliveira, T.A.S. Brandão, F. Nome, Activating water: efficient intramolecular general base catalysis of the hydrolysis of a phosphate triester, Chem. Eur. J. 15 (2009) 8475–8479.
[17] M. Kosonen, K. Hakala, H. Lönnberg, Hydrolysis and intramolecular transesterification of ribonucleoside 3′-phosphotriesters: the effect of alkyl groups on the general and specific acid–base-catalyzed reactions of 5′-O-pivaloyluridin-3′-yl dialkyl phosphates, J. Chem. Soc., Perkin Trans. 2 (1998) 663–670.
[18] R. Breslow, Biomimetic chemistry and artificial enzymes — catalysis by design, Acc. Chem. Res. 28 (1995) 146–153.
[19] T. Niittymaki, H. Lonnberg, Artificial ribonucleases, Org. Biomol. Chem. 4 (2006) 15–25.
[20] W.P. Jencks, M. Gilchrist, Reactions of nucleophilic reagents with phosphoramidate, J. Am. Chem. Soc. 87 (1965) 3199–3209.
[21] G.J. Lloyd, B.S. Cooperman, Nucleophilic attack by zinc (II)-pyridine-2-carbaldoxime anion on phosphorylimidazole. A model for enzymatic phosphate transfer, J. Am. Chem. Soc. 93 (1971) 4883–4889.
[22] D.E. Hultquist, R.W. Moyer, P.D. Boyer, Preparation and characterization of 1-phosphohistidine and 3-phosphohistidine, Biochemistry 5 (1966) 322–331.
[23] D.E. Hultquist, Preparation and characterization of phosphorylated derivatives of histidine, Biochim. Biophys. Acta 153 (1968) 329–340.
[24] E.S. Orth, E.H. Wanderlind, M. Medeiros, P.S.M. Oliveira, B.G. Vaz, M.N. Eberlin, A.J. Kirby, F. Nome, Phosphorylimidazole derivatives: potentially biosignaling molecules, J. Org. Chem. 76 (2011) 8003–8008.
[25] E.S. Orth, T.A.S. Brandao, H.M.S. Milagre, M.N. Eberlin, F. Nome, Intramolecular acid–base catalysis of a phosphate diester: modeling the ribonuclease mechanism, J. Am. Chem. Soc. 130 (2008) 2436–2437.
[26] E.S. Orth, T.A.S. Brandao, B.S. Souza, J.R. Pliego, B.G. Vaz, M.N. Eberlin, A.J. Kirby, F. Nome, Intramolecular catalysis of phosphodiester hydrolysis by two imidazoles, J. Am. Chem. Soc. 132 (2010) 8513–8523.
[27] R.H. Bromilow, S.A. Khan, A.J. Kirby, Intramolecular catalysis of phosphate triester hydrolysis - nucleophilic catalysis by neighboring carboxy-group of hydrolysis of diaryl 2-carboxyphenyl phosphates, J. Chem. Soc., Perkin Trans. 2 (1972) 911–918.
[28] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G.A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H.P. Hratchian, A.F. Izmaylov, J. Bloino, G. Zheng, J.L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J.A. Montgomery Jr., J.E. Peralta, F. Ogliaro, M. Bearpark, J.J. Heyd, E. Brothers, K.N. Kudin, V.N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J.C. Burant, S.S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J.M. Millam, M. Klene, J.E. Knox, J.B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Stratmann, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, R.L. Martin, K. Morokuma, V.G. Zakrzewski, G.A. Voth, P. Salvador, J.J. Dannenberg, S. Dapprich, A.D. Daniels, O. Farkas, J.B. Foresman, J.V. Ortiz, J. Cioslowski, D.J. Fox, Gaussian 09, Revision A.02, Gaussian, Inc, Wallingford, CT, 2009.
[29] A.J. Kirby, M.F. Lima, D. Silva, C.D. Roussev, F. Nome, Efficient intramolecular general acid catalysis of nucleophilic attack on a phosphodiester, J. Am. Chem. Soc. 128 (2006) 16944.
[30] a) A.V. Marenich, C.J. Cramer, D.G. Truhlar, Universal solvation model based on solute electron density and on a continuum model of the solvent defined by the bulk dielectric constant and atomic surface tensions, J. Phys. Chem. B 113 (2009) 6378–6396;
b) Junming Ho, Andreas Klamt, Michelle L. Coote, Comment on the correct use of continuum, J. Phys. Chem. A 114 (2010) 13442–13444.
[31] Z. De-Min, T. Kazunari, The hydrolysis of RNA: from theoretical calculations to the hammerhead ribozyme-mediated cleavage of RNA, Chem. Rev. 98 (1998) 991–1026.