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Quinone methide based organophosphate hydrolases inhibitors: *Trans* proximity labelers versus *Cis* labeling activity-based probes

Artem Dubovetskyi[1], Kesava Phaneendra Cherukuri[1], Yacov Ashani[1], Anna Meshcheriakova[1], Eitan Reuveny[1], Gili Ben-Nissan[1], Michal Sharon[1], Laura Fumagalli[2] and Dan S. Tawfik[1,*]

[1] Department of Biomolecular Sciences, The Weizmann Institute of Science, Rehovot 76 100, Israel; [2]Dipartimento di Scienze Farmaceutiche, Università degli Studi di Milano, via Mangiagalli 25, I-20133, Milano, Italy.

* Corresponding author; E-mail: dan.tawfik@weizmann.ac.il

Quinone methide (QM) chemistry is widely applied including in enzyme inhibitors. Typically, enzyme-mediated bond-breaking releases a phenol product that rearranges into an electrophilic QM that in turn covalently modifies protein side-chains. However, the factors that govern the reactivity of QM-based inhibitors, and their mode of inhibition, have not been systematically explored. Foremost, enzyme inactivation may occur in *cis*, whereby one of the QM molecules inactivates the very same enzyme molecule that released it, or by *trans*, if the released QMs diffuse away and inactivate other enzyme molecules. We examined QM-based inhibitors for enzymes exhibiting phosphoester hydrolase activity. We tested different phenolic substituents and benzylic leaving groups, thereby modulating the rates of enzymatic hydrolysis, phenolate-to-QM rearrangement, and the electrophilicity of the resulting QM. By developing assays that distinguish between *cis* versus *trans* inhibition, we have identified certain combinations of leaving groups and phenyl substituents that lead to inhibition in the *cis* mode, while other combinations led to *trans* inhibition. Our results suggest that *cis* acting QM-based substrates may be used as activity-based probes for identifying various phospho- and phosphono-ester hydrolases, and potentially other hydrolases.

Inhibitors, and mechanism-based inhibitors in particular, are a key element in the enzymology toolbox, with broader uses spanning from cell biology to medicinal chemistry. In this context, quinone methide-based inhibitors were described for many classes of enzymes, foremost for hydrolytic enzymes[1-7]. Enzyme-mediated breakdown, typically hydrolysis, of an inert substrate leads to the release of a phenol product that rearranges into an electrophilic QM that then modifies nucleophilic side-chains within the enzyme's active-site (**Figure 1**).

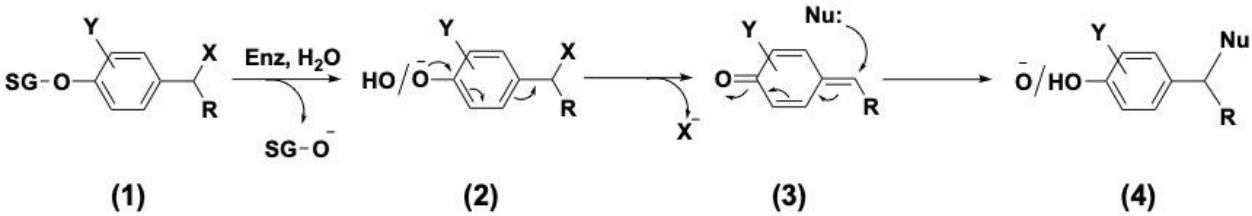

Figure 1. A general scheme of enzyme mediated quinone methide (QM) release and inhibition. Enzymatic cleavage of the substrate group (SG; *1*) releases a phenolate leaving group (or phenol, depending on the product's pK_a and whether the enzyme protonates the leaving group; (*2*). This phenol/ate product subsequently rearranges into a QM (*3*) that reacts with nucleophiles to form a covalent adduct (4). Occurrence of the last step with a side-chain within the enzyme's active-site leads to enzyme labeling and inactivation.

QM-based inhibitors could, in principle, act as activity-based probes[1],[2],[8-10], namely, as mechanism-based inhibitors that enable selective labeling, isolation and identification of enzymes[3], [11-13]. Single turnover suicide inhibitors are ideal activity-based probes, but these are available for relatively few classes of enzymes (*e.g.* activated phosphonates for serine hydrolases[14],[15]). In other cases, inhibition may be based on release of an electrophile that reacts with a nucleophilic side-chain in the enzyme's active site (e.g. Refs[11],[12]). However, in oppose to single turnover suicide inhibitors, inhibition by QM-based inhibitors is statistical. The occasional turnover may result in the QM being formed and covalently modified within the very same active site in which it was generated (*cis* labeling). However, most turnovers result in the dissociation of the phenol/ate product or of its rearranged QM product. Once released from the active-site, the QM can be quenched by water molecules and other

nucleophiles (*e.g.* by the buffer), but it may also react with other enzyme molecules present in the same solution (*trans* labeling; **Figure 2**).

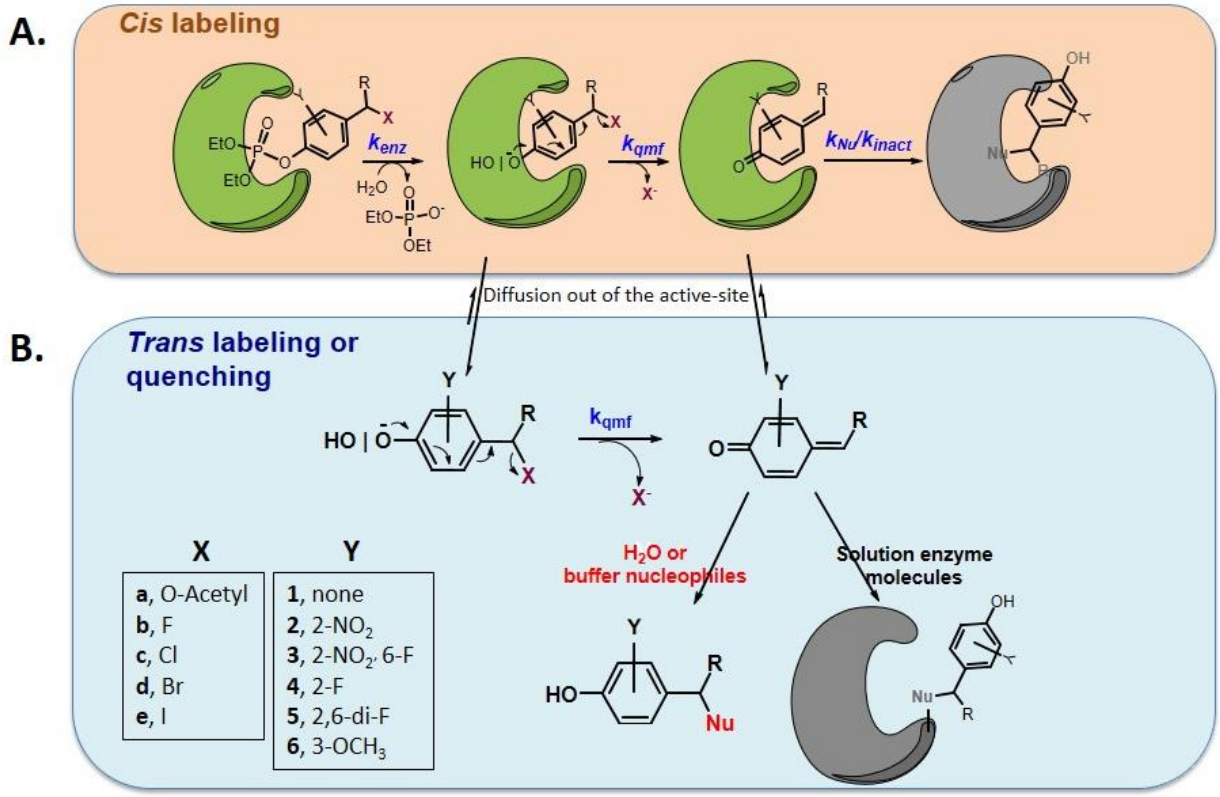

Figure 2. *Cis* and *trans* modes of inhibition by QM-releasing diethyl phosphoesters (DEP-QMs). A. In the *cis* mode, the QM is formed and is covalently trapped within the very same active site in which it was generated (ochre zone). **B**. Alternatively, the phenol/ate, or its QM product, may diffuse out of the active site in which they were generated and react with other enzyme molecules (causing inhibition, or merely modifying their surfaces) or be quenched by buffer and/or water molecules (blue zone). **Inset**: A series of leaving groups (X) and ring substituents (Y) were applied as to modulate the rate of enzymatic cleavage of the DEP-QM substrate (k_{enz})**,** the rate QM formation (k_{qmf}), and the rate of alkylation of the released QM (k_{Nu}) which in turn leads to enzyme inhibition (k_{inact}). The benzylic side-chain, R, was either H (series **1**, **2** and **6**) or methyl group (series **3**, **4** and **5**) but this modification had little effect on the efficacy of inhibition (**Figure S2**).

Inhibition by *cis* (**Figure 2A**) is crucial for the application of QMs as activity-based probes. Conversely, *trans* inhibition may be of useful in proximity labeling, *i.e.*, for labeling interacting proteins with a tractable tag[16-19]. However, although multiple QM-based enzyme inhibitors have been described, the vast majority were tested with purified enzyme samples, and typically in buffers that are not efficient nucleophiles[2],[4-7],[20-24]. Thus, their mode of inhibition remains unknown, as are the parameters that govern it. Aiming to develop activity-based probes for phospho- and phosphono-ester hydrolases (broadly addressed as organophosphates hydrolases, OPHs), we tested a QM-based inhibitor design that has also been commonly applied with hydrolases[2],[4],[6],[25],[26] (with a fluoro-benzyl leaving group (X=F; R=(C=O)NH); *4-(2-(butylamino)-1-fluoro-2-oxoethyl) phenyl diethyl phosphate*; **Figure S1**). We observed inhibition in buffers with low nucleophilicity, at high inhibitor concentrations, as originally reported[23]. However, inhibition was completely abolished when buffers that can act as nucleophiles were used (**Figure S1**) suggesting that inhibition occurred via the *trans* mode (**Figure 2B**).

Can effective *cis* inhibition be achieved with QM-based inhibitors? What are the factors that govern the efficiency and the mode of inhibition? To address these questions, we have tested a series of phosphor- and phosphono-ester substrates that release phenolate products that can rearrange to give the respective QM. These substrates contained different substituents and leaving groups (**Figure 2B**). In doing so, we modulated the rate of enzymatic hydrolysis (k_{enz} which relates to k_{cat}/K_M of the DEP-QM substrate and its applied concentration), the rate of phenol/ate-to-QM rearrangement (k_{qmf}), and the rate of the enzyme's inactivation due to its modification by the QM (k_{inact}). We have also developed assays that rigorously distinguish between *cis* versus *trans* inhibition. Although the rates of these individual steps cannot be changed in isolation, namely without affecting the other steps, the wide range of modifications, and testing these modifications with multiple enzymes, allowed to derive mechanistic conclusions. We thus show that the phenol/ate release rate, the rate of QM formation, and the QM's electrophilicity could be modulated to

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a degree that neither of these individual steps limits the rate of enzyme inactivation. We further show that while some substrates exhibited *trans* labeling, specific combinations of electron-withdrawing ring substituents and good benzylic leaving groups provided relatively efficient inhibition in the *cis* mode. We thus demonstrate selective labelling of various OPHs in the *cis* mode, and also facile detection of labelled enzymes with a commercially-available antibody.

RESULTS AND DISCUSSION

The enzymes tested. We first tested three different enzymes that exhibit phosphotriesterase, or OPH (organophosphate hydrolase) activity: rePON1, *Bd*-PTE and *Sb*-PTE. To ensure the generality of our analysis, the chosen enzymes differ by orders of magnitude in their catalytic efficiency for paraoxon (diethyl, p-nitrophenyl phosphate) – a benchmark for OPH activity, and also in how sensitive their rate is to changes in the phenolate leaving group's pK_a (paraoxon's leaving group ≈ 7.14; Ref.[27]).

The first enzyme, RePON1-G3C9 (hereafter rePON1), is a recombinant variant of human serum paraoxonase PON1[28]. PON1 is a calcium dependent lactonase with moderately efficient promiscuous OPH activity. The k_{cat}/K_M value of rePON1's hydrolysis of paraoxon $1.2x10^4$ $s^{-1}M^{-1}$ (k_{cat} = 1.1 s^{-1})[28]; other phosphotriesters and phosphonodiesters can be hydrolyzed, but PON1's k_{cat} decreases with the increase of the phenolic leaving group's pK_a ($\beta_{LG} \approx -1.6$)[29].

The second enzyme, *Bd*-PTE, is a bi-metallo phosphotriesterase isolated from *Brevundimonas diminuta* (now reclassified as *Sphingopyxis wildii*[30]. *Bd*-PTE evolved in soil bacteria toward hydrolysis of organophosphates such as paraoxon, and accordingly, its activity with paraoxon is nearly 3-orders-of-magintidue higher than rePON1's: $k_{cat} = 1.17x10^3$ s^{-1}; $k_{cat}/K_M = 1.95x10^7$ $s^{-1}M^{-1}$ (Ref.[31]). *Bd*-PTE's k_{cat} is highly sensitive to the product's leaving group pK_a ($\beta_{LG} \approx -2.2$)[32].). Here, we used mutant version of *Bd*-PTE, dubbed dPTE2, a variant with essentially the same catalytic properties as wild-type *Bd*-PTE yet with improved stability[31] (addressed for simplicity as *Bd*-PTE hereafter).

The third enzyme is a manganese dependent phosphotriesterase from *Sphingobium* sp. TCM1 (*Sb*-PTE)[33],[34]. Its activity with paraoxon is higher than rePON1's but not nearly as high as *Bd*-PTE's (k_{cat} = 28 s^{-1}; $k_{cat}/K_M = 7.7x10^4$ $s^{-1}M^{-1}$ (Ref.[35])). This enzyme has, however, a unique ability to hydrolyze substrates with poor leaving group with essentially the same rate as activated ones[34].

Design, synthesis and stability of phosphoester QM-releasing substrates. The QM-releasing substrates were designed to have the same diethylphosphoryl moiety (DEP) yet differ in the phenolic group substituent (**Y**) and in the benzylic leaving group (**X**; **Figure 2B**). The benzylic leaving groups primarily modulate the rate of formation of the QM via 1,6-rearrangement of the enzyme released phenol/ate product (k_{qmf}, **Figure 1, 2**). In principle, the rate becomes faster as the benzylic leaving group's pK_a decreases (considering the conjugate acid – acetate would be the slowest and iodide the fastest). The phenolic ring substituents may, however, exert multiple effects. Firstly, electron-withdrawing substituents at the ortho positions (**Y** = **2**-**5**; **Figure 2B**) are expected to increase the rate of enzymatic hydrolysis ($k_{enz,}$ **Figure 2**) and thus accelerate the rate of release of the phenol/ate product. As mentioned above, both PON1 and *Bd*-PTE are highly sensitive to the pK_a of the phenolic leaving group, while *Sb*-PTE is not expected to show such dependency. Secondly, the phenolic substituents also affect the rate of the subsequent step, QM formation (k_{qmf}). Electron-withdrawing groups at ortho position (**Y** = **2**-**5**) may reduce k_{qmf} (Ref.[20]), while electron-donating (e.g. **Y** = **6**) should increase it[36],[37]. Thirdly, electron-withdrawing substituents, and especially *o*-nitro, are expected to boost the QM's electrophilicity and thereby increase the rate of enzyme inactivation (k_{inact}). The variability of modifications examined here therefore allowed us to systematically modulate the three steps leading to enzyme inactivation and examine which modifications yield the highest efficacy of inhibition, and inhibition in the *cis* mode. Note that the effects of the QM's leaving group (**X**) and of the ring substituents (**Y**) on the three steps leading to enzyme inactivation may be complementary but also contradictory – *e.g*. *o*-nitro may accelerate the first (k_{enz}) and last step (k_{inact}) but inhibit the second one (k_{qmf}).

The DEP-QM substrates were synthesized as described in the **METHODS**. The ones with more activated leaving groups (**X** = **Br, I**) showed limited stability in DMSO solutions. Decomposition occurred not by cleavage of the P-O ester bond but possibly due to a nucleophilic attack of DMSO on the benzylic carbon[20],[38], as substrates with electron-withdrawing phenyl ring substituents were generally more stable (series **3**, **4** and **5**; that also turned out to be the most efficient inhibitors). Acetonitrile was therefore used for stock

solutions, affording storage stability at -20 °C over months. Further, when diluted from acetonitrile stock solutions into assay buffers, the DEP-QM substrates, including those with an activated bromide leaving group, remained largely intact (≥90%) for the assay duration (≤ 2 h; **Table S1**). Solubility also varied and the maximal concentration achieved in the enzyme inhibition reactions across the entire set of DEP-QMs was 1.5 mM while using 10% DMSO as cosolvent.

Inhibition efficacy. We first tested inhibition under a fixed enzyme concentration and varying DEP-QM inhibitors concentrations. We applied the lowest possible enzyme concentration given that the inhibition reaction mix had to be further diluted for assaying the enzyme's residual activity using a chromogenic substrate. We observed a wide range of inhibition efficacies from no inhibition at the highest inhibitor concentration that could be applied, to complete inactivation at the μM range. Since the three enzymes had to be applied at different concentrations in accordance with their OPH activity, and given the DEP-QM's inhibition mode (elaborated below), the inhibition efficacy was compared as a function of the substrate-to-enzyme concentration ratio ($[S]_0/[E]_0$, or the partition ratio[39],[40] (**Figure 3**). The log($I_{PR}50$), the logarithm of the substrate-to-enzyme ratio that gave rise to 50% enzyme inhibition was used to compare the various DEP-QM's and enzymes (**Table S2**).

The factors determining inhibition efficacy. As expected, the inhibition efficacy of DEP-QM substrates was highly dependent on both the benzylic leaving group (**X**) and the phenolic ring substituents (**Y**). The rate of QM formation (k_{qmf}) turned out to be the most dominant factor, as indicated by consistently higher inhibition efficacy (lower log($I_{PR}50$) values) with increased reactivity of the benzylic leaving group, going from acetate to iodide (**Figure 3**, **Table S2**). However, high rate of QM formation is necessary but not sufficient for effective inhibition. Introducing electron-withdrawing phenolic ring substituents significantly increased inhibition efficacy – in *Bd*-PTE, for example, the unsubstituted substrates (series **1**) gave no detectable inhibition at the maximal substrate/enzyme ratio that could be applied, whereas inhibition was observed with all 2-nitro substrates (series **2**). Electron-withdrawing ring

substituents increase the QM's electrophilicity, and hence increase the likelihood that it will inactivate the enzyme. However, electron-withdrawing phenolic ring substituents also affect the rate of enzyme hydrolysis (k_{enz}). Thus, the unsubstituted substrates might not show inhibition because they are not hydrolyzed at the tested enzyme concentration.

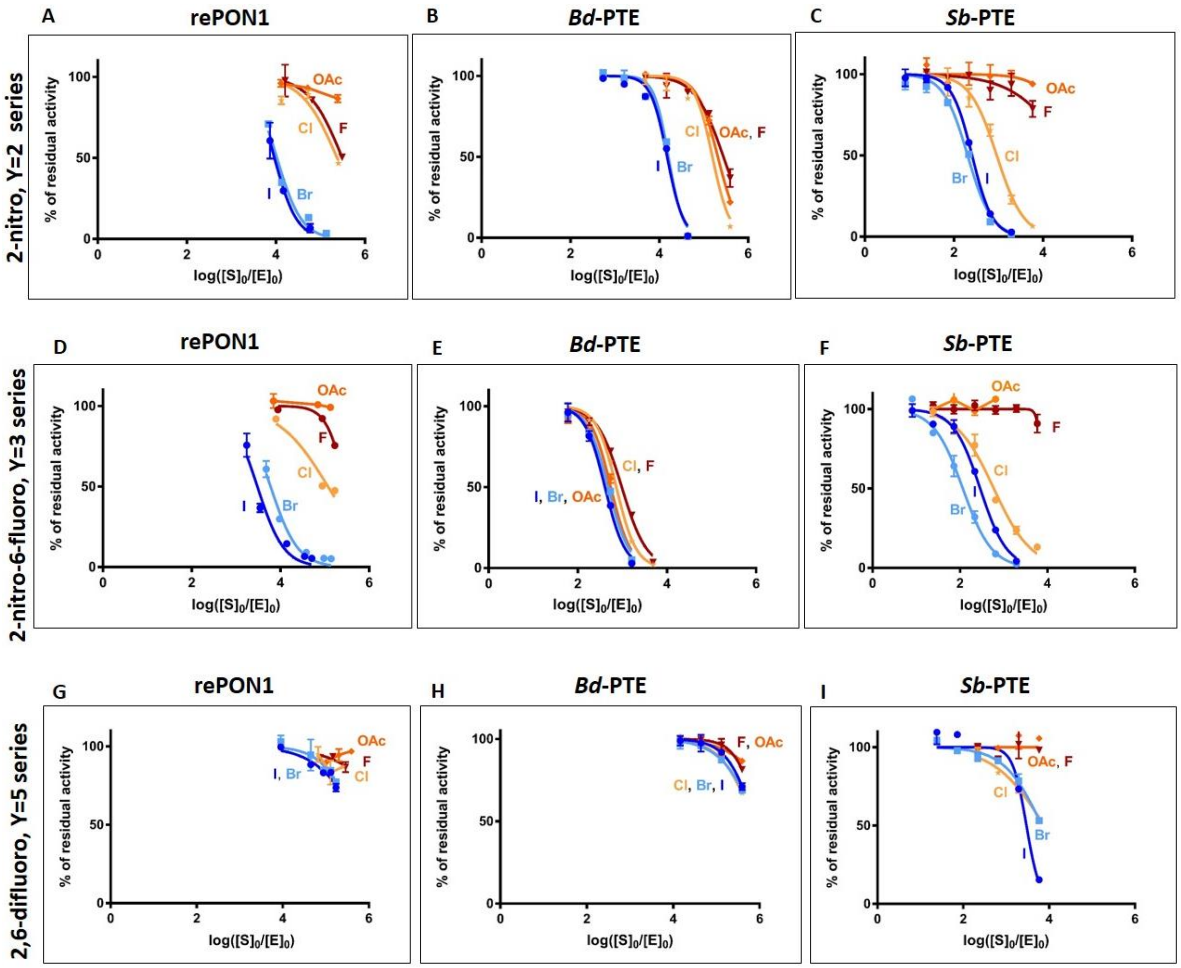

Figure 3. Inhibition by **DEP-QM substrates.** Assays were performed at a fixed enzyme concentration and varying substrate concentrations, and the enzyme's residual activities were plotted as the logarithm of the substrate-to-enzyme concentration ratio ($[S]_0/[E]_0$). The columns correspond to the three tested enzymes, and the rows display substrates with different phenyl ring substituents (**Y**), while the various benzylic leaving groups (**X**) are displayed in each plot. Enzyme concentrations in the inhibition reactions were: 3.8 nM for *Bd*-PTE, 12-24 nM for

rePON1, and 225 nM for *Sb*-PTE; the reaction medium contained exogenous nucleophiles (Tris buffer *plus* a ≥1000-fold excess of a competing protein). The residual activity was tested with paraoxon and compared to the untreated enzyme. Every point reflects 3 repeats, and the standard deviation is shown as error bar.

In order to examine whether the tested DEP-QMs are utilized as substrates, we have determined their enzymatic hydrolysis rates. As expected, DEP-QMs with electron-withdrawing phenolic ring substituents were rapidly hydrolyzed, and conversely, the enzyme could be fully inhibited by the time the substrate has been consumed. For example, the kinetic parameters of *Bd*-PTE towards the series **2** substrates (**Y** = **2-NO$_2$**; **Table S3**) indicate that with the applied enzyme (3.8 nM) and substrate concentrations (≤ 1.5 mM), these substrates are largely consumed during 30 min incubation (the calculation is provided as Footnote *c* of **Table S3;** the QM leaving group, **X**, has no significant effect on the enzymatic rates). However, for the slower *Sb*-PTE, it appears that only ~20% of the 1.5 mM substrate was consumed under the assay conditions despite the relatively high enzyme concentration applied (225 nM; **Table S3**). Nevertheless, this level of hydrolysis is sufficient because complete inhibition of *Sb*-PTE was observed with all series **2** substrates. For other substrates that do not yield a chromogenic phenolic leaving group, we have determined the fraction of hydrolyzed substrate by HPLC. As expected by the lower phenolate pK_a (7.30)[27], the 2,6-difluoro DEP-QM substrates were fully consumed by 3.8 nM *Bd*-PTE in 30 min, whereas only ~20% of the 2-fluoro substituted **4a** (phenolate pK_a = 8.81; Ref.[27]) were consumed (**Table S4**). The unsubstituted series were the slowest substrates, and the assays indicate that given the applied enzyme concentrations and incubation times < 1% of the series **1** substrates are consumed by *Bd*-PTE and *Sb*-PTE (**Table S4, Table S5**) or by rePON1 (**Figure S3**). Thus, lack of inhibition by these substrates may also derive from very slow product formation. In this respect, the partial inhibition of rePON1 by **1d** and **1e** is hard to explain – although the log(I_{PR}50) values indicate a high substrate-to-enzyme ratio (log(I_{PR}50) > 4, **Table S2**), given that the substrate remained largely intact, inhibition actually occurred within a few

turnovers. One possible explanation is that hydrolysis of **1d** and **1e**, the rearrangement to the QM, and the nucleophilic attack by an active-site residue, occur in rePON1 in a concerted manner.

Overall, it appears that electron-withdrawing ortho phenolic ring substituents are critical for efficient inhibition, because they promote substrate hydrolysis (higher k_{enz}) and also induce higher QM electrophilicity (higher k_{inact}). Enhanced electrophilicity seems primarily due to a resonative effect. This is apparent by comparison of the 2-nitro and 2,6-difluor series (**2** and **5**, respectively) that have roughly the same electron-withdrawing impact as indicated by their nearly identical phenolate pK_a values (pKa 2,6-difluorophenol[27] is 7.30 and of 2-nitrophenol[41], 7.23). Nonetheless, the 2-nitro DEP-QMs exhibited ~50-fold higher inhibition efficacy with all three enzymes (Δlog(I_{PR}50) = 1.7 comparing **2d** to **5d**; **Table S2**). Finally, electron-withdrawing substituents may slow down QM formation due to the stabilization of the phenolate form (reactions were performed at pH 8.0, and the phenolate pK_a values for series **2**, **3** and **5** are ≤7.3). However, with no exception, substrates with electron-withdrawing phenolic substituents were more effective inhibitors compared to the corresponding unsubstituted substrates. Further, the **3** series with the lowest phenolate pK_a (~4.9 as predicted by ChemDraw) are better inhibitors than the **2** series (phenolate pK_a = 7.23; Ref.[41]). It seems therefore that the slowing down of QM formation due to electron-withdrawing ring substituents is relatively minor, and can also be readily compensated by activated leaving groups (**X** = **Br**, **I**). Further, substitution with the meta-methoxy group (series **6**) is known to dramatically accelerate the rate of QM formation[36],[37] , yet these DEP-QMs exhibited poor inhibition at low enzyme concentration (**Figure 4B**). Poor inhibition is due to the meta-methoxy phenol group having a high pK_a and hence yielding a poor substrate (slow k_{enz}), but also due to their less electrophilic QM's (lower k_{inact} or k_{Nu}).

In summary, using a combination of activated leaving group, typically, **X** = **Br** or **I**, and resonative electron-withdrawing phenolic ring substituents (**Y** = **2-NO_2** or combined **2-F** and

6-NO_2 substituents) enabled inhibition all three enzymes with substrate-to-enzyme molar ratios as low as 120 ($log(I_{PR}50) \geq 2.07$; **Figure 3**, **Table S2**).

***Cis* versus *trans* inhibition.** That effective inhibition correlates with high rate of QM formation suggests that the QMs are reacting with the very same active site in which they are generated – *i.e.*, *cis* mode of inhibition. If so, the presence of competing nucleophiles in the reaction buffer, be they proteins or small molecules, should not hinder enzyme inactivation. We thus compared inhibition in a nucleophile-containing buffer *plus* competing protein at >1000 molar excess to the tested enzyme (Tris-BSA, or Tris-lysozyme buffers) to inhibition in a buffer that is less nucleophilic and with no competing protein (HEPES). Indeed, the inhibition by substrates with electron-withdrawing ring substituents (**Y** = **2-NO_2,** combined **2-F** and **6-NO_2** or **2-F** and **6F**) was both effective and resistant to the effect of exogenous nucleophiles (**Figure 3**, **Figure S4**). In contrast, substrates lacking such substituents (Y= m-OCH_3, or no substituent) were not only sensitive to exogenous nucleophiles (**Figure S1**, **Figure S5**), but also tended to be poor inhibitors at low enzyme concentrations (**Figure 4B**).

To further establish the mode of inhibition, we compared the substrate titrations presented above to a reverse titration, *i.e.*, varying the enzyme's concentration under a fixed substrate concentration. A *cis* mode of inhibition means a partition between inhibition (the phenol/ate product rearranges into the QM and reacts with an active-site side-chain) and the release of the phenol/ate product to give a free enzyme that in turn performs another catalytic turnover. The fraction of inhibited enzyme is therefore determined by the number of turnovers per active-site, and not by the inhibitor concentration per se[39],[42],[43]. Accordingly, the titration curves looked essentially identical when the substrate or the enzyme were titrated (**Figure 4A**). In contrast, for DEP-QMs with the m-methoxy phenolic substituent (**Y** = **6**), titration of the enzyme (*Bd*-PTE) under a fixed substrate concentration gave a different pattern whereby inhibition increased with increasing enzyme concentration (**Figure 4B**). In

accordance with exogenous nucleophiles leading to loss of inhibition (**Figure S5)**, these DEP-QMs likely act in the *trans* mode.

Finally, the ratio of rate constants of product release and inhibition, k_{enz}/k_{inact}, should correspond to the ratio of concentrations of the released product and the inhibited enzyme. As shown in **Figure S6**, the apparent first-order rate constant for enzyme inactivation measured with **2d** was k_{inact} =0.024±0.005 s^{-1}. The apparent first-order rate of product release was found to be k_{enz} = 77.4±12.6 s^{-1} (determined with the acetate analogue (**2a**) that barely induces enzyme inactivation). Taking into account that **2a** is 2.4 times better substrate for *Bd*-PTE than **2d** (**Table S3**), the k_{enz}/k_{inact} ratio would be ~1,500 – in the same range as ratio of product to inhibited enzyme (~3700) and also in agreement with the $[S]_0/[E]_0$ ratio for **2d** ($10^{4.2}$ ≈ 15,800; **Table S2**; $[S]_0/[E]_0$ should be higher than $[P]/[E]_I$ due to incomplete substrate consumption by the inactivated enzyme).

Overall, the three tests described above show that the combinations of activated benzylic leaving groups (**X** = **Br**, **I**) and electron-withdrawing phenolic substituents (**Y** = 2-**NO_2**, or **2-F**, **2-NO_2**) allows effective inhibition towards the *cis* mode. However, other DEP-QMs (Y = m-OCH_3, or no substituent), including those with leaving groups such as **X** = fluoride that have been routinely used[23],[25],[26], inhibit via the *trans* mode. That said, while the results shown so far indicate that *cis* inhibition occurs, even with these substrates (e.g. **2d**, **X** = **Br**; **Y** = 2-**NO_2**), the vast majority of enzymatic turnovers result in dissociation of the product (be it the phenolate, or its QM product). The released QMs, or those formed in solution, could be labelling other proteins in *trans*. As shown later, such *trans* labeling occurs with **2d**, yet with much lower efficiency compared to cis labelling (**Figure 6**).

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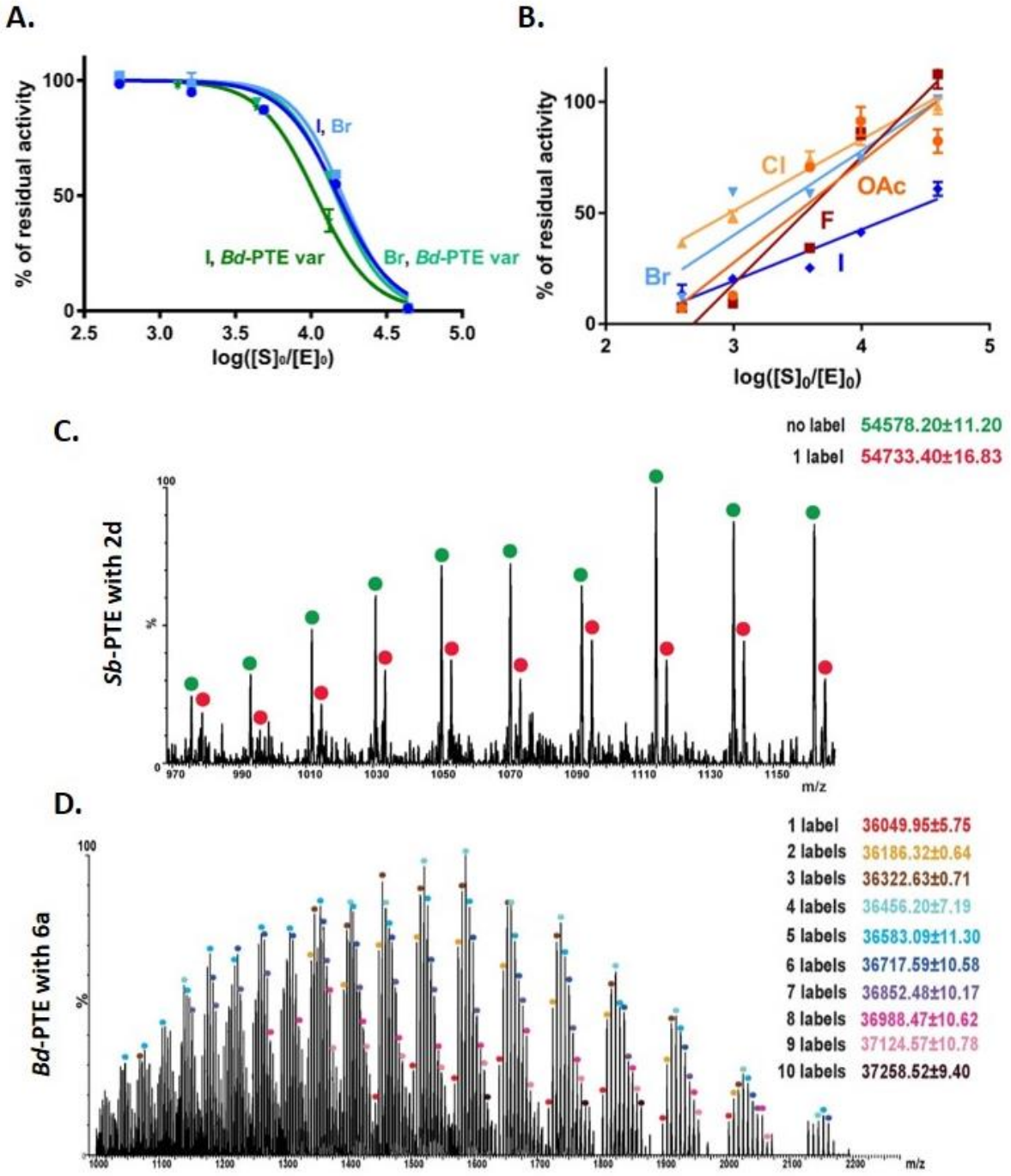

Figure 4. Validating the *cis* and *trans* modes of inhibition.

A. Varying of substrate concentration under a fixed enzyme concentration (blue lines; *Bd*-PTE, 3.8 nM; DEP-QM's **2d** and **2e**, as in **Figure 3**) gives essentially the same dependency as the reverse titration, *i.e.*, different enzyme concentrations at a fixed substrate concentration (green lines). Curve fits are as in **Figure 3**. The reverse

titration of *Bd*-PTE was done in Tris-BSA buffer at *Bd*-PTE's concentration of 3.8 nM up to 127 nM, and 0.167 mM of substrates **2d** or **2e**.

B. The enzyme *Bd*-PTE (38 nM – 3.8 µM) was incubated in HEPES for 30 min with DEP-QM substrates (Y=**6** series; X=**a**, **b**, **c**, **d**, **e**) at 1.5 mM final concentration (or with 10% DMSO as a control). The reaction mixtures were subsequently diluted to 3.8 nM Bd-PTE final concentration in HEPES and the enzyme's residual activity was determined with 1mM paraoxon (described in **SUPPORTING METHODS**). The residual activity represents the mean of three parallel measurements and the standard deviations are shown as error bars. As can be seen, inhibition decreases at higher enzyme/substrate ratio (the opposite trend is observed with *cis* inhibition, as shown in **A** and in **Figure 3**).

C. Mass spectrometry data of *Sb*-PTE treated with **2d**. Green circles correspond to non-labeled *Sb*-PTE, and red circles to the labeled enzyme. The mass difference between labeled and non-labeled (155 Da±17 Da) corresponds to the expected mass of the QM adduct (153.04 Da). The enzyme (11.5 µM) was incubated with **2d** (1.5 mM) in Tris buffer, and was inhibited to 10% residual activity. The untreated *Sb*-PTE shows the only mass spectra of non-labeled enzyme. Note that while >90% inhibition was observed, the labeled peak is smaller than the unlabeled one. This is likely due to the enzyme preparation containing an inactive enzyme (due to loss metal or misfolded states).

D. *Bd*-PTE treated with **6a** (a variant of *PTE* was used; see methods). The red, yellow, brown, etc. circles, correspond to the enzyme labeled by a different number of QM adducts. The mass difference between labeled species (136 Da ±11.3 Da) corresponds to the expected QM adduct (138.07 Da). Enzyme concentration was 7.2 µM and the concentration of **6a** was 1.5 mM. The reaction was conducted in HEPES buffer and the enzyme was inhibited to 54 % residual activity. We sought to also compare *Bd*-PTE with **2d**; however, due to its high hydrolysis rates *Bd*-PTE is applied in nM concentrations that are insufficient for MS analysis

Mass spectrometry indicating covalent inhibition. To confirm the covalent labeling of the enzymes, and thereby the feasibility of these QM precursors as activity-based probes or proximity labelling, mass spectrometry measurements were conducted using *Sb*-PTE as the model enzyme and **2d** as a characteristic *cis* inhibitor. Following incubation with **2d** that led to 90% inhibition, the enzyme was HPLC purified and analyzed by mass spectrometry. The mass difference between the untreated and **2d**-treated enzyme corresponded to a single modification by the released QM (**Figure 4C**). This finding supports the *cis* mode of inhibition, which is anticipated to yield a single modification, and also indicates that the QM modification is stable enough to serve as an activity-based probe. In contrast, when the same procedure was applied with a DEP-QM substrate that acts in *trans* (**6a**, with *Bd*-PTE; **Figure 4B, Figure S5**), up to 10 modifications per protein were observed (**Figure 4D**).

QM-based inhibitors of phosphonate monoester hydrolases (PMHs). In principle, the *cis*-inhibiting groups identified above should be applicable to other hydrolases. To examine their generality, we have generated a substrate analogous to **2d** for enzymes that hydrolyze phosphomonoesters (and typically also phosphodiesters)[44]. This substrate showed low stability and could not be purified to homogeneity (see **SUPPORTING METHODS**). Nonetheless, even when applied as crude, it was found to be a highly effective inhibitor with two different PMHs (**Figure 5**).

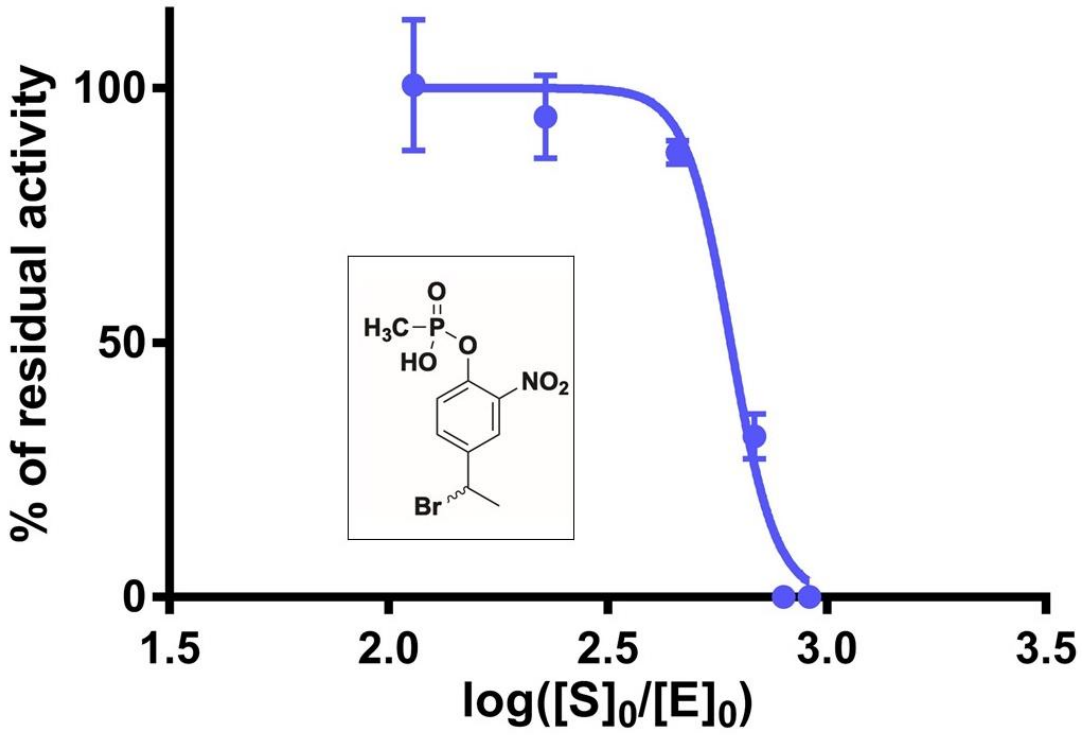

Figure 5. **Inhibition of phosphonate monoester hydrolase/phosphodiesterae from *Rhizobium leguminosarum* (*Rl*-PMH).** The phosphonate monoester QM substrate (*inset*) was added as a crude mix (X = Br/OH at ~0.8/1 ratio; at 1 mM up to 8 mM total concentration) was incubated with *Rl*-PMH (8.77 µM). The inhibition partition ratio was found to be: log(I_{PR}50) is 2.78. The reaction was conducted in Tris 25 mM pH 8.0, NaCl 25 mM solution for 30 min at the room temperature. The residual activity was tested with bis-(4-nitrophenyl) phosphate and compared to untreated *Rl*-PMH. Every point reflects two repeats, and the standard deviation is shown as error bars.

Antibody-mediated identification of phosphotriesterase *cis* labeling and background *trans* labeling. To further confirm the *cis* labelling of *Bd*-PTE, and also assess the level of *trans* labelling that may occur in parallel to *cis* labeling, we performed Western blot analysis of the reaction mixtures. Fortuitously, the best labeling QMs attach an o-nitrophenyl moiety to the labelled enzymes (**Figure 2**), and we found that this moiety could be readily and selectively identified with commercially available anti-3-nitrotyrosine monoclonal antibodies. We could thus detect labeling by DEP-QM substrate **2d** by Western blots (**Figure 6).** *Bd*-PTE showed intense labeling, thus confirming **2d**'s labeling efficiency and stability. Further, the specificity

of the labeling could be demonstrated by including a competitor protein, lysozyme, at 3,500 molar excess compared to *Bd*-PTE. As shown above, *Bd*-PTE inhibition by **2d** is not interrupted by a competitor protein (**Figure S4**; **Table S2**). Accordingly, the labeling of *Bd*-PTE as detected by Western blot was not significantly affected by addition of lysozyme. Further, lysozyme was labeled to roughly the same degree as *Bd*-PTE, suggesting that under these reaction conditions, cis labeling with **2d** is ~1,000-fold more efficient than the background, *trans* labeling. Finally, the Western blot experiment also shows that no labeling is seen when **2d** is incubated with lysozyme without *Bd*-PTE, indicating that this DEP-QM substrate does not modify proteins in a non-selective manner.

The ability to detect labeling by **2d** with a commercially available antibody (and in principle by any other o-nitro QM substrate) further enhances the generality of these QM probes (while the synthesis of the DEP-QM substrates described here is relatively simple, derivatives with a biotin or Click handle is considerably more complicated to make).

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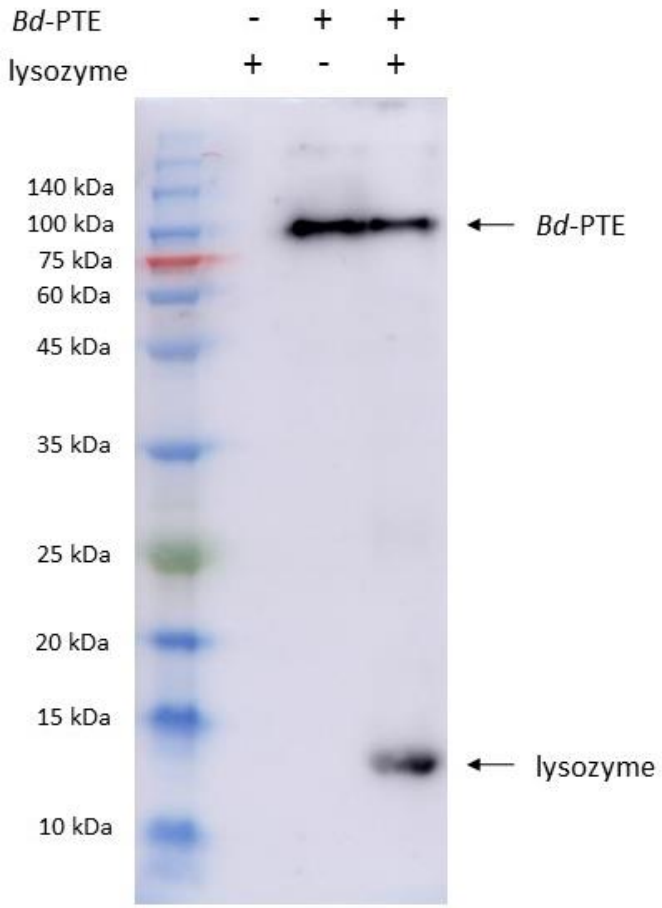

Figure 6. Western blot analysis of QM-modified *Bd*-PTE. DEP-QM **2d** was incubated with or without 20 nM *Bd*-PTE in Tris buffer, and with or without lysozyme (70 µM). The reaction mixtures were dialyzed against urea to remove noncovalently attached products and were subsequently run on SDS-PAGE. The gel was blotted using a commercially available anti-nitrotyrosine antibody. The MBP fused *Bd*-PTE (78 kDa) shows clear labeling by the 2-nitrophenyl group. Some cross-labeling of lysozyme (14.5 kDa) can be seen in the sample containing *Bd*-PTE, yet no labeling is seen when **2d** is incubated with the lysozyme without *Bd*-PTE.

CONCLUDING REMARKS

Covalent, irreversible mechanism-based enzyme inhibition can occur via different modes. Inhibitors that act within a single turnover are known, but many generally applicable inhibitors are statistical: substrate conversion releases a highly reactive species, a radical[45], or an electrophile[11],[12], which can react with protein side-chains thereby resulting in covalent labeling and enzyme inactivation. Diffusion of the reactive species out of the active site may lead to labeling in *trans*. Activity-based probes, however, should act via *cis* inhibition, because diffusion and *trans* modification would result in non-specific labeling. We show that the reactivity of QM-releasing substrates can be tuned to obtain higher inhibition efficacy and labeling by the *cis* mode. The generality of the derived conclusions with respect to optimal QM-precursor design was validated with three enzymes that share the same enzymatic activity (phosphortriesterase) yet widely differ in mechanism and level of activity (**Figure 3**), as well as with two different phosphonate monoester hydrolases (**Figure 5**).

We found that the benzylic leaving group (**X**) allows to modulate the rate of QM formation (k_{qmf}) and highly activated leaving groups, such as **Br** or **I**, are critical for efficient inhibition (**O-Acetyl** < **F** < **Cl** < **Br** < **I**; **Figure 3**) and for ensuring a *cis* mode of inhibition. However, at some point k_{qmf} ceases to be the rate-limiting step as suggested by the observation that for most DEP-QM's, inhibition efficacy with **X** = **Br** and **I** was the same (**Figure 3**, **Table S2**). The QM's electrophilicity (k_{inact} or k_{Nu}) is another critical factor that could be optimized by introducing electron-withdrawing ortho phenolic substituents (**Y**: **unsubstituted** ≈ **2-F** < **2,6-F,F** << **2-NO$_2$** < **2-F, 6-NO$_2$**). The phenyl substituents also affect the enzymatic rate, *i.e.*, the rate of phenol/ate product formation (k_{enz}, which is a function of k_{cat} and K_M). Indeed, in the absence of electron-withdrawing groups, substrate conversion barely occurs. However, comparison of substrates that are largely consumed within the applied assay time and enzyme concentration (Y = **2**-**5**) indicates that QM electrophilicity is the key factor. We further observed that high QM electrophilicity is promoted primarily via a resonative effect (the **2-NO$_2$** substrates (series **2**) is far more potent compared to the **2-F,6-F** substrate (series **5**) despite nearly identical phenol pK_a values). Tradeoffs between QM formation rate and QM electrophilicity may occur (phenolic electron-withdrawing groups that improve the

latter may reduce the former). However, it appears that the undesirable side-effect of reducing the rate of QM formation is insignificant compared to the gain in QM electrophilicity.

We further show that efficient enzyme inhibition largely corresponds with the *cis* mode of inhibition. Inhibition in *cis* was demonstrated via kinetics in multiple ways: failure of exogenous nucleophiles, including competing proteins, to abolish inhibition (**Figure S4A**, **B**); kinetic partition between product release and inactivation (**Figures 4A**, **S6**); and the stoichiometry of labeling. Indeed, **2d**, that was found to act in *cis* in the kinetic assays also showed a single QM modification per enzyme molecule by mass spectrometry (**Figure 4C**). That multiple labeling, and background *trans* labeling, were not observed (**Figure 4C**, **Figure 6**, respectively) suggests that once diffused out of the active-site, the QMs generated by **2d** are rapidly quenched by the solvent thus minimizing *trans* inhibition of other enzyme molecules. In contrast, **6a**, a DEP-QM substrate that exhibited kinetics that are compatible with a *trans* mode of labeling, showed multiple labeling (**Figure 4D**) as observed with other QM-based inhibitors[23],[25],[26]. In fact, *trans* labelling by **6a** occurs with relatively high efficiency – roughly 1 out 50 product molecules yield a covalent adduct. This system, namely *Bd*-PTE plus DEP-QM **6a**, might therefore be useful for proximity labeling[16],[17],[18],[19] (or for labeling of all proteins present in a given compartment[46]). However, in the crowded cellular environment, the feasibility of proximity labeling depends on how selective labelling is with respect to proteins in the vicinity of the labelling enzyme – a factor that was not examined in the experiments described here.

Finally, we have also demonstrated the ability to selectively detect enzyme labeling using an anti-nitrotyrosine antibody. The latter opens the road for future applications as activity-based probes, namely for detection and identification of enzymes that are of utility in the detoxification and degradation of organophosphates (pesticides, nerve agents, flame retardants)[47], and of other types of hydrolases.

METHODS

Synthesis of DEP-QM substrates. The DEP-QM substrates were synthesized following published procedures[5],[21] with some modifications. Foremost, 4'-hydroxy benzaldehyde/acetophenone derivatives served as the starting material instead of the previously reported 4-hydroxy benzyl alcohol derivatives (Ref.[48]). Specific derivatization of the latter at the phenolic hydroxyl to obtain the DEP-4-benzyl alcohol derivatives has proven challenging. Thus, commercially available, or synthesized 4'-hydroxy aldehyde/keto derivatives were used as starting materials. These were readily phosphorylated, reduced to the DEP-4-benzyl alcohol derivatives, and then modified to obtain the different leaving groups (**X**=**OH to X**= **a**-**e**).

	a (iii)	b (iv)	c (v)	d (vi)	e (vii)
X	OAc	F	Cl	Br	I

	Z	Z'	Y	R
1	H	H	H	H
2	NO_2	H	H	H
3	F	NO_2	H	CH_3
4	F	H	H	CH_3
5	F	F	H	CH_3
6	H	H	OCH_3	H

i) DABCO, dry THF, rt, 3h ii) $NaBH_4$, MeOH:$CHCl_3$ (1:1), -78°C, 1h iii) Ac_2O, DMAP, DCM, rt, 30min iv) DAST, DCM, 0°C, 30min v) PPh_3, NCS, DCM, 0°C-rt, 1h vi) PPh_3, NBS, DCM, 0°C-rt, 1h vii) KI, P_2O_5, ACN, rt, 2h (or) PPh_3, NIS, DCM, 0°C-rt, 1h.

Scheme 1. The synthesis of the DEP-QM substrates.

Detailed synthetic procedures and characterization are provided in **Supporting Information**. Briefly, phosphorylation of the respective 4'-hydroxy-keto/aldehyde derivatives was achieved using diethyl phosphoryl chloride and DABCO as a base (Scheme 1, step *i*). Subsequent reduction of the 4'-DEPketo/aldehyde moiety with $NaBH_4$ yielded the DEP-4-benzyl alcohol derivatives (Scheme 1, step *ii*). These DEP-4-benzyl alcohol derivatives were then converted to 4-benzyl O-acetyl derivatives (X=a) using acetic anhydride and 4-(dimethylamino) pyridine (DMAP) (Scheme 1, step *iii*), to 4-benzyl fluoro derivatives (X=b) using diethylaminosulfurtrifluoride (DAST) (Scheme 1, step *iv*), to 4-benzylchloro (X=c) and 4-benzyl bromo (X=d) derivatives by using N(C/B)S & PPh_3 (Scheme 1, step *v, vi* respectively), and to 4-benzyl Iodide derivatives by using either NIS & PPh3 or KI & P_2O_5 (Scheme 1, step *vii*). All compounds were purified using silica gel column chromatography with EtOAc,

Hexane gradients. NMR (^{1}H, ^{13}C, ^{31}P, ^{19}F) and mass spectrometry (ESI-MS) were used to characterize and determine the purity of all the synthesized DEP-QM substrates.

For all other Methods, including the production of enzymes, assay substrates and buffers, measuring inhibition efficacy and kinetics, mass spectrometry, and Western Blotting, see Supporting Information.

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Keywords: enzymes; inhibitors; irreversible inhibitor; phosphotriesterase; phosphonate monoester hydrolase

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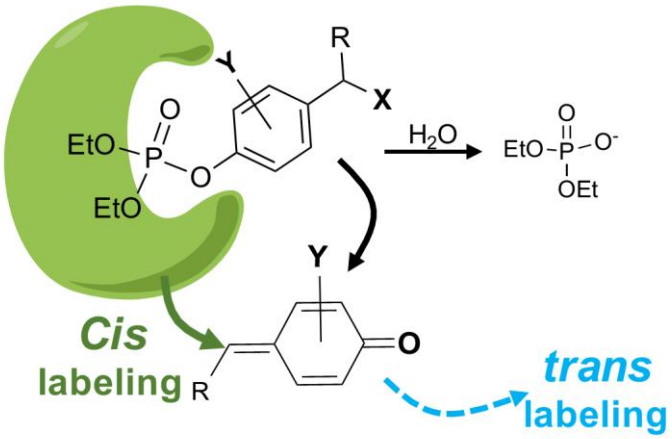

Phosphoester substrates that release electrophilic quinone-methides were applied as activity-based probes. Their reactivity, and mode of labeling, were systematically investigated with the aim of achieving cis labeling.