Analogues with Fluorescent Leaving Groups for Screening and Selection of Enzymes That Efficiently Hydrolyze Organophosphorus Nerve Agents

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Enzymes that efficiently hydrolyze highly toxic organophosphorus nerve agents could potentially be used as medical countermeasures. As sufficiently active enzymes are currently unknown, we synthesized twelve fluorogenic analogues of organophosphorus nerve agents with the 3-chloro-7-oxy-4-methylcoumarin leaving group as probes for high-throughput enzyme screening. This set included analogues of the pesticides paraoxon, parathion, and dimefox, and the nerve agents DFP, tabun, sarin, cyclosarin, soman, VX, and Russian-VX. Data from inhibition of acetylcholinesterase, in vivo toxicity tests of a representative analogue (cyclosarin), and kinetic studies with phosphotriesterase (PTE) from *Pseudomonas diminuta*, and a mammalian serum paraoxonase (PON1), confirmed that the analogues mimic the parent nerve agents effectively. They are suitable tools for high-throughput screens for the directed evolution of efficient nerve agent organophosphatases.

Introduction

Organophosphorus (OP) compounds are used as pesticides and chemical warfare (CW) agents.[1] The discovery of the pesticide dimefox occurred around the time of the development of the first nerve agent (NA) tabun, which was weaponised but not used during the Second World War (Figure 1). Diisopropyl fluorophosphate (DFP) was investigated as a potential war gas in the same period,[2] while the development of the more potent NAs sarin, soman, and cyclosarin followed later.[3] By the late-1950s, more toxic NAs with a different structure were synthesized, including VX and its isomer Russian-VX.[4] These and other related NAs pose a threat to military personnel and civilian populations.

OP compounds can inhibit acetylcholinesterase (AChE),[5] an enzyme that controls nerve impulse transmission by hydrolyzing acetylcholine to acetic acid and choline. Hydrolysis of acetylcholine by AChE involves an active-site serine residue initiating a nucleophilic attack on the carbonyl carbon of acetylcholine to form a covalent acetyl-enzyme intermediate, concurrent with the release of free choline from the active site. The free enzyme is regenerated in a second step via a hydrolytic attack by water and the release of acetate. The NAs mimic the natural substrate of AChE, they phosphorylate the active site serine residue while losing either a cyanide, fluoride, or *N*,*N*-dialkylaminoethanethiolate group. This first step is fast, but the regeneration of the free enzyme through the nucleophilic attack by water is extremely slow, creating a phosphorylated AChE unable to hydrolyze acetylcholine.[6] Inhibitors having a secondary ester group, once bound to AChE, are prone to 'aging', a term describing cleavage of the PO−C bond with loss of a carbenium ion, sometimes within minutes (half-life < 2 min in the case of soman).[2,3] The negative charge on the bound inhibitor renders the phosphorus atom resistant to attack by nucleophiles such as oximes, thus preventing reactivation of AChE.

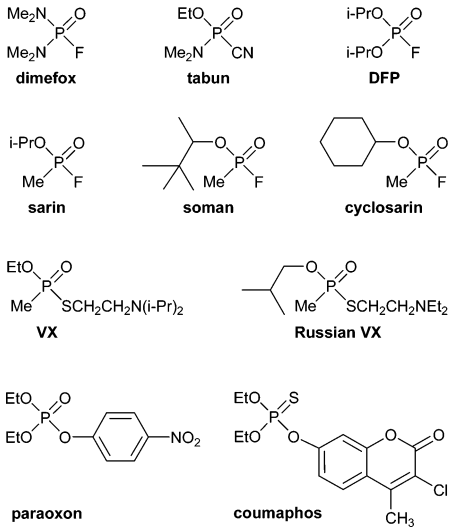

Figure 1. Structures of the main military nerve gases, their prototypes dimefox and diisopropyl fluorophosphate (DFP), and the pesticides paraoxon and coumaphos.

A number of enzymes have been identified that can catalyze the hydrolysis of OP compounds, including CW agents. Two of the best characterized are the phosphotriesterase (PTE) from *Pseudomonas diminuta*[7] and PON1, a member of the serum paraoxonase family.[8]

With its best substrate, the pesticide paraoxon, the turnover rate (k_{cat}) of PTE is high (>2280 s^{-1}) and its catalytic efficiency (k_{cat}/K_M) of 6.2×10^7 M^{-1} s^{-1} is close to the limit set by the diffusion-controlled encounter of the enzyme and the substrate.[1,9] PTE can also catalyze the hydrolysis of the NAs sarin and soman, and VX-type NAs. However, the catalytic efficiency of PTE toward NAs is 10^3 to 10^5-fold lower than for paraoxon (Table 1). If it was possible to engineer a variant PTE which could efficiently hydrolyze NAs, it would have several potential

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Table 1. Catalytic Efficiencies of Human BuChE(G117H), PON1(Q191), and PTE[a]

	k_{cat}/K_M (M^{-1} s^{-1})		
	BuChE[b] (G117H)	PON1 (Q191)	PTE
paraoxon	1.8×10^2	1.1×10^4	6.2×10^7
sarin	0.6	1.5×10^4	8.0×10^4
soman	ND	4.7×10^4	9.6×10^3
VX	2.2	ND[c]	6.9×10^2

[a] Data from refs 1, 46, 55−57. [b] The 'catalytic efficiencies' of BuChE are k_{cat}/K_d values. [c] ND = not determined.

applications: in decontamination of areas exposed to CW agents,[10] destruction of NA stockpiles,[11] "active" fabrics and filter elements for personal protection,[12,13] and sensors for CW agents.[14]

It could also be attractive to use enzymes which hydrolyze NAs for medical treatment of individuals exposed, or at risk of exposure, to CW agents. For such purposes, however, it is desirable to use a human enzyme, such as PON1, to prevent an adverse immune response to the enzyme. PON1 catalyzes the hydrolysis of sarin over 10^4 times more efficiently than the engineered human butyrylcholinesterase currently advocated as a prophylactic anti-nerve agent scavenger[15,16] (Table 1).

Rational modifications of the substrate-binding cavity of PTE have proved that it is possible to manipulate PTE substrate specificity and a variant PTE with a small (33%) increase in rate of VX hydrolysis compared to wild-type PTE has been reported.[17] In another site-directed mutagenesis study, variant PTEs were isolated with 11-fold and 16-fold increases in k_{cat} for NPPMP (an analogue of sarin) and demeton-S (an analogue of VX), respectively.[18] However, the catalytic efficiency of the best of these PTE variants toward CW agents is still orders of magnitude lower than with paraoxon as a substrate. The hydrolysis of chiral OPs by PTE is stereoselective, which is significant, as the major CW agents (including sarin, soman, and VX) are racemic mixtures with substantial differences in toxicity of the individual enantiomers. Wild-type PTE hydrolyses analogues of the more toxic S_p-enantiomer of sarin 10-fold more slowly than the R_p-enantiomer. A variant PTE with reversed stereoselectivity on phosphotriester substrates was also found to have reversed stereospecificity for chiral sarin analogues, hydrolyzing the S_p-enantiomer 10 times more efficiently than the R_p-enantiomer.[19,20] However, this enzyme showed no significant improvement in the overall rate of hydrolysis of the racemate.

To generate enzymes that efficiently catalyze the hydrolysis of NAs, it is best to move beyond simple site-directed mutagenesis to the selection of large repertoires of mutated genes: a PTE variant with an ~1000-fold increase in activity (k_{cat}/K_M) toward the most toxic stereoisomer (S_pS_c) of a chromogenic analogue of soman has been isolated from a library of 400 mutated PTE genes.[21] Using in vitro compartmentalization (IVC) of a library of $\sim 10^7$ genes in aqueous microdroplets in water-in-oil emulsions, it was possible to select a PTE variant (PTE-h5) with an even faster turnover rate for paraoxon than wild-type PTE.[22]

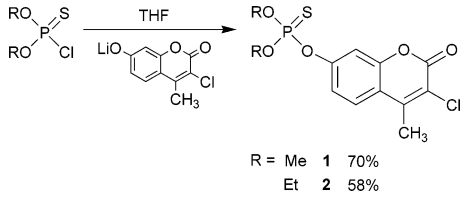
The screening of large gene libraries is facilitated by a simple chromogenic or fluorogenic enzyme assay.[23] Chromogenic analogues of sarin and soman used to study and screen PTE variants replace the leaving group with a *p*-nitrophenol moiety.[20] Fluorogenic substrates, on the other hand, are much more sensitive. The fluorogenic paraoxon analogue 7-*O*-(diethylphosphoryl)-3-cyanocoumarin (DEPCyC) has proven very effective for screening *E. coli* colonies containing PON variants with increased paraoxon hydrolyzing activity that are expressed efficiently.[24,25] Fluorogenic NA analogues also open up the possibility of using fluorescence-activated cell sorting (FACS) machines to sort rapidly (at up to 10^5 s^{-1}) very large repertoires of genes or bacteria compartmentalized in double emulsions.[26−28]

In this study, to enable the screening of large repertoires of mutant PTEs and PONs, we describe the synthesis of a novel series of analogues of nerve agents, related in structure to the pesticide coumaphos,[29] in which the leaving groups have been replaced with the fluorescent 3-chloro-7-oxy-4-methylcoumarin moiety.[30−32] The departure of the coumarin group allows real-time measurement of fluorescence and acquisition of kinetic data. The ability of these derivatives to inhibit bovine erythrocyte AChE and the LD_{50} of a representative derivative in an in vivo toxicity assay were determined. The compounds were used for hydrolysis studies with wild-type PTE, the evolved PTE-h5 (I106T/F132L),[22] a mammalian high-yield soluble PON1, G3C9[25] (referred to here as PON1-wt), and two evolved variants of the latter, 2.4PC (L69V/S193P/V346A) and 3.2PC (L69V, S138L, S193P, N287D).[33] The results of this study comprise the first step toward a systematic high-throughput screen for PTE and PON variants capable of efficiently hydrolyzing NAs.

Results

Synthesis. Analogues **1** and **2** (coumaphos)[29,34] were prepared by treating the appropriate dialkyl chlorothiophosphate with the lithium salt of 3-chloro-7-hydroxy-4-methylcoumarin in tetrahydrofuran (Scheme 1).

Scheme 1

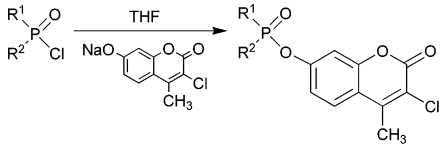


The ease of synthesis of the corresponding oxo analogues **3** and **4** and derivatives **5** to **12** depended on the availability of chloridates **a**−**j**. Chloridates **f**−**h** were made by chlorinating the respective dialkyl methylphosphonates with oxalyl chloride or phosgene; diisobutyl methylphosphonate was prepared by treating methylphosphonic dichloride $MeP(O)Cl_2$ with two molar equivalents of sodium isobutoxide.[35] Pinacolyl and cyclohexyl phosphonochloridates **i** and **j** were prepared by the action of methylphosphonic dichloride on a molar equivalent of the respective alcohols in ether. Coupling of the chloridates was achieved using the sodium salt of the hydroxycoumarin (Scheme 2). The compounds were isolated in >99% purity by column chromatography as low-melting white solids. They were characterized by multinuclear NMR spectroscopy and by LC-MS and $LC\text{-}MS^n$ experiments (Supporting Information).[36]

Second-Order Rate Coefficients of Inhibition of AChE. First-order rate coefficients of inhibition, obtained from a graph of log percentage of AChE remaining activity versus time (data not shown), were plotted against inhibitor concentration. Second-order rate coefficients of inhibition (k_i) were then calculated from the gradient of the best straight line produced. The error values associated with the k_i values were calculated from the deviations from the best straight line in the graph of first-order rate coefficient of inhibition versus inhibitor concentration (Table 2).

Compounds **1** and **2** (coumaphos) did not inhibit AChE. They contain the P═S bond and are less reactive than substrates that contain the P═O bond; the phosphorus atom of the former is

Scheme 2

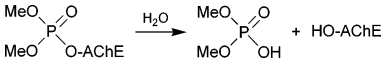


	R^1	R^2	number	yield
a	MeO	MeO	**3**	62%
b	EtO	EtO	**4**	65%
c	i-PrO	i-PrO	**5**	66%
d	Me_2N	Me_2N	**6**	52%
e [a]	EtO	Me_2N	**7**	80%
f	EtO	Me	**8**	69%
g	i-PrO	Me	**9**	62%
h	i-BuO	Me	**10**	57%
i	PinO	Me	**11**	56%
j	c-hexyl	Me	**12**	70%

[a] Tabun (P−CN) was used instead of the chloridate (P−Cl).

less positively charged than that of the latter as S is less electronegative than O, which reduces vulnerability to nucleophilic attack. In mammals[37] and bacteria,[38] coumaphos is oxidized[39] to the oxo analogue, an inhibitor of AChE responsible for coumaphos toxicity. Dimethyl phosphate analogue **3** failed to irreversibly inhibit AChE. A reduction in its inhibition rate with time was observed and complete inhibition was never achieved, consistent with data for other dimethyl phosphate inhibitors where spontaneous reactivation of inhibited AChE by water occurs[40] (Scheme 3). It was not possible to use higher concentrations of inhibitor due to its limited aqueous solubility.

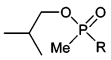
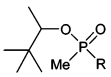
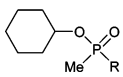
Scheme 3



With longer or branched alkoxy groups on phosphorus, complete inhibition of AChE is usually observed; this was seen with diethyl analogue **4** (coroxon) as noted earlier[41] and with diisopropyl analogue **5**. Analogue **4** was approximately 10 times more potent an inhibitor than analogue **5**. AChE inhibited by these two compounds contains a more electron-rich and hindered phosphorus atom than AChE inhibited by the dimethyl analogue and is much less susceptible to reactivation.

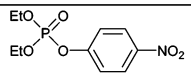
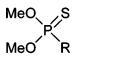
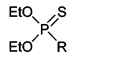
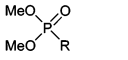
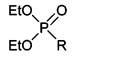
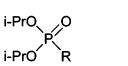
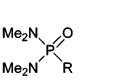
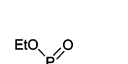
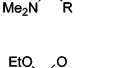
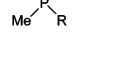
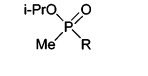
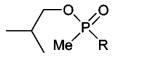
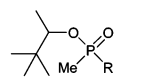
Replacement of isopropoxy groups with dimethylamino groups gave dimefox analogue **6**, which did not inhibit AChE. Dimethylamino substituents deactivate phosphorus compounds to nucleophilic attack because of steric hindrance and strong electron donation, which renders the phosphorus atom inaccessible and negatively charged. Even tabun analogue **7**, containing only one dimethylamino group, was insufficiently reactive to inhibit AChE.

Table 2. Rate Coefficients for Inhibition of Bovine Erythrocyte AChE (pH 7.4, 37 °C)[a]

Compound	Structure	k_i (M^{-1} min^{-1})	Pesticide / nerve agent	k_i (M^{-1} min^{-1})	Ratio of k_i nerve agent / k_i
1		*0*	methyl-parathion	ND	-
2		*0*	parathion	ND	-
3		*0*	methyl-paraoxon	ND	-
4		$2.1(\pm0.2) \times 10^4$	paraoxon	ND	-
5		$2.3(\pm0.5) \times 10^3$	DFP	$6.8(\pm1.3) \times 10^3$	3
6		*0*	dimefox	< 18	-
7		*0*	tabun	$3.2(\pm0.1) \times 10^5$	-
8		$2.2(\pm 0.2) \times 10^5$	VX	$6.6(\pm2.2) \times 10^6$	30
9		$2.2(\pm 0.4) \times 10^4$	sarin	$7.4(\pm1.3) \times 10^5$	34
10		$2.1(\pm 0.5) \times 10^6$	Russian-VX	$2.7(\pm0.6) \times 10^7$	13
11		$5.7(\pm 1.2) \times 10^4$	soman	$4.1(\pm1.0) \times 10^4$	0.7
12		$1.8(\pm 0.1) \times 10^6$	cyclosarin	$1.2(\pm 0.2) \times 10^7$	7

[a] *0* = no activity detected. ND = not determined. R = 3-chloro-7-oxy-4-methylcoumaryl (refer to Scheme 1).

Table 3. Efficiency of Hydrolysis of Substrates by the Phosphotriesterases (k_{cat}/K_M in M^{-1} s^{-1})[a]

Compound	Analogue of	Structure	PTE-wt	PTE-h5	PON1-wt	2.4PC	3.2PC
Paraoxon	-		7.6(±0.6) × 10^7	1.8(±0.1) × 10^7	5.9(±0.29) × 10^3	1.6 (±0.2) × 10^4	3.9(±0.1) × 10^4
1	methyl-parathion		6.5(±0.5) × 10^5	6.4(±0.4) × 10^3	*0*	*0*	*0*
2	parathion		1.1(±0.1) × 10^7	2.4(±0.6) × 10^5	*0*	*0*	*0*
3	methyl-paraoxon		3.6(±0.3) × 10^5	3.2(±0.2) × 10^2	4.9(±0.3) × 10^2	7.8(±0.4) × 10^2	1.8(±0.1) × 10^3
4	paraoxon		2.7(±0.3) × 10^6	3.0(±0.2) × 10^5	5.3(±0.2) × 10^2	1.8(±0.1) × 10^3	6.2(±0.1) × 10^4
5	DFP		2.2(±0.2) × 10^5	6.8(±0.4) × 10^4	*0*	1.3(±0.1) × 10^2	2.1(±0.1) × 10^1
6	dimefox		0.094 (±0.012)	*0*	*0*	*0*	*0*
7	tabun		1.2(±0.1) × 10^3	16(±2)	4.28(±0.1)	21(±0.2)	53(±0.5)
8	VX		3.5(±0.1) × 10^5	7.8(±0.3) × 10^3	6.9(±0.1) × 10^4	3.7(±0.1) × 10^3	3.6(±0.1) × 10^4
9	sarin		2.6(±0.1) × 10^5	9.6(±1.6) × 10^3	7.7(±0.2) × 10^3	1.7(±0.1) × 10^3	5.6(±0.1) × 10^3
10	Russian-VX		3.4(±0.2) × 10^5	1.5(±0.1) × 10^4	5.6(±0.1) × 10^4	2.2(±0.1) × 10^3	3.5(±0.1) × 10^4
11	soman		7.8(±0.5) × 10^3	1.6(±0.1) × 10^2	63(±0.3)	53(±0.1)	16(±0.5)
12	cyclosarin		5.7(±0.1) × 10^5	2.0(±0.1) × 10^4	9.0(±0.1) × 10^2	1.8(±0.1) × 10^2	4.5(±0.1) × 10^2

[a] *0* = No activity detected. R = 3-chloro-7-oxy-4-methylcoumaryl (refer to Scheme 1).

Incorporation of a *P*-methyl bond in place of a *P*-alkoxy or -dialkylamino group, as expected, produced strong inhibitors. Phosphonate **8** was approximately 10 times better an inhibitor than corresponding phosphate **4** (same difference between **9** and **5**). Ethyl analogues were approximately 10 times better inhibitors than isopropyl counterparts (compare **4** and **5**, then **8** and **9**). A 10-fold and 95-fold increase in inhibitory power over ethyl analogue **8** and isopropyl analogue **9** was observed for isobutyl compound **10**, where the isopropyl group was separated from phosphorus by a methylene bridge. Branching at the α-carbon, as in soman analogue **11**, resulted in reduction in inhibitory potency, yet cyclohexyl analogue **12** ranked alongside the isobutyl analogue as one of the most potent inhibitors tested. The high activity of the cyclosarin derivative might be due to a better fit of the cyclohexyl group into the AChE binding pocket.

In Vivo Toxicity of Cyclosarin Analogue 12. Limited information is available on the in vivo toxicity of coumaphos-related compounds. LD_{50} values have been reported for oral (56 mg kg^{-1} for coumaphos and 10−12 mg kg^{-1} for coroxon)[42,43] and dermal application (860 mg kg^{-1} for coumaphos) to rats.[44] To access the in vivo toxicity of the fluorogenic analogues, the LD_{50} of cyclohexyl analogue **12**, one of the most potent AChE inhibitors, was measured and found to be 0.4 mg kg^{-1} by subcutaneous administration to the guinea pig (the LD_{50} of cyclosarin under the same test protocol was 0.06 mg kg^{-1}). Analogue **12** is therefore approximately seven times less acutely toxic than cyclosarin in this animal model.

Kinetic Data for Reactions with Organophosphatases. The NA analogues, like most of the NAs (except sarin), have poor aqueous solubility. However, 1% v/v dimethylformamide (DMF) in the reaction buffer solubilized enough substrate to allow accurate measurements (up to ∼10 μM of coumaphos). To assess the effect of such concentration of solvent on the performance of the enzymes, their kinetic parameters with paraoxon as substrate were determined in the presence and absence of DMF (Supporting Information). It was found that 1% v/v DMF in the reaction mixture tended to increase the K_M slightly, but not significantly.

The specificity constants (k_{cat}/K_M) of the organophosphatases versus racemic mixtures of analogues **1**−**12** were determined by measuring the initial rate of increase in fluorescence (ex. 350 nm, em. 460 nm) at different substrate concentrations. All

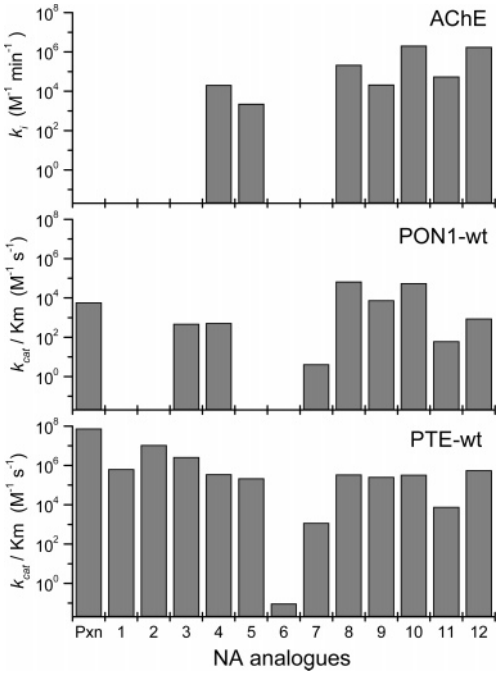


Figure 2. Activity profiles of AChE, PON1-wt, and PTE-wt toward the NA analogues (Pxn = paraoxon).

the compounds were hydrolyzed by PTE-wt, and this enzyme was the most efficient hydrolase for all the compounds tested (Table 3).

Unlike AChE, both PTE-wt and PTE-h5 were able to hydrolyze thiono analogues **1** and **2** (coumaphos) with the latter being the best substrate for PTE out of the series synthesized in this study. The values obtained for analogue **2** were essentially the same as those obtained with commercial coumaphos ($k_{cat}/K_M = 9.4 \pm 0.37 \times 10^6$ M^{-1} s^{-1} with PTE-wt). In general, PTE usually hydrolyses P═O compounds better than P═S compounds.[45] However, PTE-wt hydrolyzed analogue **2** four times more efficiently than analogue **4** (coroxon).

The diisopropyl phosphate analogue **5** was not hydrolyzed as efficiently as coroxon. Compounds with a dimethylamino substituent were hydrolyzed poorly: dimefox analogue **6** and tabun analogue **7** were much less reactive than the other analogues tested.

The replacement of a *P*-alkoxy group with a *P*-methyl group gave substrates with lower reactivity toward the PTEs, the reverse of the situation found with AChE (e.g. diethyl phosphate analogue **4** was hydrolyzed approximately 8 times faster than ethyl methylphosphono analogue **8** by PTE-wt). Efficiencies of hydrolysis for VX analogue **8**, sarin analogue **9**, Russian-VX analogue **10**, and cyclosarin analogue **12** were comparable (Figure 2). In contrast, the PTEs were much less efficient at hydrolyzing soman analogue **11**, presumably because the binding pocket cannot easily accommodate the bulky pinacolyl side-chain.

Compared with PTE-wt, the variant PTE-h5, which was evolved to hydrolyze a paraoxon analogue, with a bulky caged-biotin substituted for one of the ethyl groups,[22] showed diminished activity toward all the NA analogues.

Regarding the PONs, the catalytic efficiencies with analogues **3** and **4** (coroxon) were consistent with mutants 2.4PC and 3.2PC being evolved to hydrolyze more efficiently a very similar substrate, 7-*O*-(diethylphosphoryl)-3-cyanocoumarin (DEPCyC),[33] which is identical to analogue **4** except for a different coumarin leaving group: 3.2PC had a 117-fold higher k_{cat}/K_M (6.2×10^4 M^{-1} s^{-1}) than the PON1-wt with analogue **4** as substrate. No activity was detected with analogues **1** and **2** (coumaphos), highlighting that the PONs can discriminate between oxono and thiono pairs.

Like the PTEs, the PONs hydrolyze compounds bearing dimethylamino groups (**6** and **7**) very inefficiently and clearly hydrolyze analogue **4** more efficiently than diisopropyl phosphate analogue **5**, for which there was no detectable hydrolysis by PON1-wt, though some hydrolysis was detected with the evolved 2.4PC and 3.2PC variants. Substrates bearing *P*-methyl groups were well accepted as PON substrates. In fact, the ethyl methylphosphono analogue **8** was hydrolyzed around 130 times more efficiently than diethyl phosphate analogue **4** by PON1-wt.

Overall, bulky side chains hinder PON activity. With Russian-VX analogue **10**, which bears the isobutyl group, the activity is comparable to VX analogue **8**. However, the activity drops 100-fold for cyclosarin analogue **12** with the bulky cyclohexyl group, and 1000-fold for soman analogue **11** with the pinacolyl group.

Discussion

We describe the synthesis of 12 fluorogenic nerve agent (NA) analogues with the 3-chloro-7-oxy-4-methylcoumarin leaving group. Included in this series are analogues of pesticides (paraoxon, parathion, and dimefox) and nerve agents (DFP, tabun, sarin, cyclosarin, soman, VX, and Russian-VX).

AChE inhibition assays show an overall congruency between the functionality of each NA and its analogue (Table 2). The analogues are between 0.7 and 34-times less potent inhibitors than the corresponding NAs, retaining the same relative inhibition potency order (Russian VX > cyclosarin > VX > sarin > soman > DFP). The exception is tabun analogue **7**, in which the lack of detectable inhibition reflects the great leaving facility of the cyanide group of tabun. The in vitro AChE inhibition data is supported by the similar LD_{50} values obtained with cyclosarin (0.06 mg kg^{-1}) and the cyclosarin analogue **12** (0.4 mg kg^{-1}) upon their subcutaneous administration to guinea pigs.

The fluorogenic analogues proved to be suitable substrates for both types of organophosphatases used, bacterial phosphotriesterases (PTEs) and mammalian serum paraoxonases (PONs). Their catalytic efficiencies (k_{cat}/K_M) ranged across 10^8 M^{-1} s^{-1}, illustrating the functional and structural diversity of the NA analogues and the promiscuity of the enzymes for such compounds. All the analogues were hydrolyzed by PTE-wt: parathion analogue **2** (coumaphos) was the most reactive and the dimethylamino bearing analogues of dimefox and tabun were the least reactive. Compared with PTE-wt, the variant PTE-h5, which was evolved to hydrolyze a paraoxon biotin analogue,[22] showed an overall diminished activity toward the NA analogues. PON1-wt[25] hydrolyzed nonbulky analogues efficiently and clearly resolved the more toxic oxo phosphonates from the thio analogues. The evolved PON1 variants, 2.4PC and 3.2PC,[33] also evolved to hydrolyze a (different) paraoxon analogue, had enhanced activity toward paraoxon analogue **4** and DFP analogue **5** compared to PON1-wt.

The AChE in vitro inhibition and in vivo toxicity data, strongly suggest that the fluorogenic NA analogues mimic the NAs quite well. Hence, they have great potential as probes in high throughput screening: the sensitivity of fluorogenic assays is very high and the NA analogues have low volatility compared to the NAs (data not shown), allowing their safe manipulation using standard laboratory precautions.

In this study, all the compounds were assayed as racemic mixtures. However, some enantiomers are known to be more

toxic than others (e.g. sarin, the S_p-enantiomer of sarin is substantially more toxic than the R_p enantiomer[19]). Hence, we are currently working on the preparation of the pure enantiomers.

If it were possible, for example, to engineer a PTE variant that hydrolyzed a NA as efficiently as it hydrolyses paraoxon, it is estimated that just 2.5 kg of enzyme, immobilized within a polyurethane foam matrix, may be sufficient to degrade 30000 tons of nerve agent in one year.[11] Similarly, with a PON variant which hydrolyzed sarin as efficiently as PTE hydrolyses paraoxon, it is estimated that only ~25 μg of such variant might be required to detoxify ~1 LD_{50} sarin in a 70 kg man in 3.9 min.[46]

We believe that in order to create PTEs or PONs that are highly efficient catalysts for CW agent hydrolysis, it is necessary to screen large repertoires of mutants in a process of directed evolution. One of the most important potential uses of the fluorogenic NA analogues presented here is as probes for screening mutant organophosphatase libraries for variants with improved NA hydrolyzing activity.

The NA analogues can be used to screen libraries of PTEs or PONs in several ways. If the enzymes are overexpressed in bacteria they can be used to screen libraries of up to ~10^7 bacterial colonies on plates.[24,25] Larger libraries of >10^7 mutants expressed in bacterial cells can be screened by encapsulating the single cells in aqueous microdroplets in water-in-oil-in-water double emulsions and sorting the droplets using a fluorescence activated cell sorter (FACS).[19,26] Large libraries can also be screened by in vitro compartmentalization (IVC)[47] where single genes are transcribed and translated in vitro in aqueous microdroplets in double emulsions, which are sorted using a FACS to select genes encoding enzymes with improved catalytic activity.[28] It is important to note that although the fluorogenic analogues are useful for optimizing enzymes, they are surrogates and can only mimic the nerve agents. However, this does not invalidate our approach.

Conclusions

A series of fluorogenic nerve-agents analogues have been synthesized that appear to be good mimics of the corresponding nerve agents. The catalytic efficiency of the organophosphatases PTE and PON1 toward the analogues was determined using the fluorescent leaving group signal. The synthesis and characterization of these fluorogenic nerve agent analogues comprises the first step toward the selection of PTE and PON1 variants capable of efficiently hydrolyzing nerve agents.

Experimental Section

General Synthetic Procedures. Material. 3-Chloro-7-hydroxy-4-methylcoumarin was obtained from Lancaster Synthesis Ltd (Morecambe, UK). Other reagents were obtained from Aldrich (Gillingham, UK) and used as received. Triethylamine was distilled from CaH_2 and stored over CaH_2. Tabun of >98% purity was prepared at Dstl Porton Down. Anhydrous solvents were used: tetrahydrofuran (THF) was distilled from sodium/benzophenone.

Apparatus. Thin-layer chromatography was performed on MK6F silica gel 60 Å plates (Whatman) with detection by UV light (λ = 254 nm). Sorbsil C30 40/60 silica was used for column chromatography. Melting points were determined on an Electrothermal apparatus and are uncorrected. NMR spectra were obtained on a JEOL Lambda 500 instrument (operating at 500 MHz for ^{1}H, 125 MHz for ^{13}C, and 202 MHz for ^{31}P spectra) or a JEOL Lambda 300 instrument (operating at 300 MHz for ^{1}H, 75 MHz for ^{13}C, and 122 MHz for ^{31}P spectra) as solutions in $CDCl_3$, with internal reference $SiMe_4$ for ^{1}H and ^{13}C, and external $(MeO)_3P$ (δ 140 ppm) for ^{31}P spectra.

Safety Warning. Phosgene is toxic and must be used in a fume cupboard. Tabun is a potent anti-cholinesterase and must be handled by a trained worker under the terms of the Chemical Weapons Convention.[48] Analogues **1**−**12** are also anticholinesterases and must be handled with care; rubber gloves must be worn and glassware decontaminated in aqueous bleach.

Synthesis of P═S analogues (1 and 2). *n*-Butyllithium (8.75 cm^3, 14 mmol of a 1.6 M solution in hexanes) was added dropwise by syringe to a stirred solution of 3-chloro-7-hydroxy-4-methylcoumarin (2.95 g, 14 mmol) in THF (25 cm^3). After addition, the solution was stirred for 1 h. A solution of the appropriate dialkyl chlorothiophosphate (14 mmol) in THF (25 cm^3) was added dropwise. After addition, the mixture was left at room temperature for 12 h. The solvent was removed to yield a liquid residue, which was dissolved in chloroform (50 cm^3) and washed with aqueous K_2CO_3 (100 cm^3, 5% w/v). The aqueous layer instantly colored yellow. The chloroform layer was separated and dried ($MgSO_4$). The drying agent was filtered off, and the filtrate was concentrated to give a yellow liquid. Column chromatography on silica gel eluting with 49:1 chloroform−methanol gave, after trituration with ether and drying, the title compounds as pale yellow solids.

Ethyl Methylphosphonochloridate (f). A solution of oxalyl chloride (14 g, 0.11 mol) in chloroform (20 cm^3) was added dropwise to a stirred solution of diethyl methylphosphonate (17 g, 0.11 mmol) in chloroform (40 cm^3) at room temperature. Effervescence was observed shortly after addition. When addition was complete, the mixture was stirred for another 20 h. Analysis of an aliquot by GC-MS indicated complete conversion to the chloridate. The solvent was removed to give a liquid. Fractionation under reduced pressure gave the title compound as a colorless liquid (12.5 g, 80%). Bp 62−64 °C/8 mmHg. Lit.[49] bp 40−41 °C/1 mmHg. δ_P 40.8.

Isopropyl Methylphosphonochloridate (g). Phosgene was bubbled slowly through diisopropyl methylphosphonate (10 g, 56 mmol) for 2 h. A stream of argon was passed into the liquid product to drive out residual hydrogen chloride. Fractionation under reduced pressure gave the title compound as a colorless liquid (6.6 g, 76%). Bp 46 °C/3 mmHg. Lit.[49] bp 47 °C/3 mmHg. δ_P 39.2.

Isobutyl Methylphosphonochloridate (h). Phosgene was bubbled through diisobutyl methylphosphonate[35] (4.7 g, 22.6 mmol) for 2 h. The reaction mixture was degassed with argon for 30 min before being placed under vacuum. The product was isolated in quantitative yield and in high purity and no further purification was required. δ_P 31.1.

Pinacolyl Methylphosphonochloridate (i). A mixture of pinacolyl alcohol (15.7 g, 153.6 mmol) and triethylamine (15.5 g, 153.6 mmol) in ether (100 cm^3) was added dropwise to a stirred solution of methylphosphonic dichloride (20.4 g, 153.5 mmol) in ether (300 cm^3) under argon at 0−5 °C. A catalytic amount of 4-(dimethylamino)pyridine (50 mg) was added. After addition, the solution was allowed to warm to room temperature and was left for 12 h. The precipitate of triethylamine hydrochloride was filtered off, and the filtrate was concentrated to yield a liquid. Distillation under reduced pressure gave the title compound as a colorless liquid (21.5 g, 71% yield). Bp 57 °C/0.4 mmHg. δ_P 40.2 and 39.6.

Cyclohexyl Methylphosphonochloridate (j). Prepared using the method used for pinacolyl methylphosphonochloridate but starting from cyclohexanol. Yield 63%. Bp 69 °C/0.1 mmHg. δ_H 4.77−4.6 (1H, m, OCH), 2.07−1.95 (2H, m, β-CH_2), 1.97 (3H, d, *J* 17, P−CH_3), 1.84−1.69 (2H, m, β-CH_2), 1.68−1.18 (6H, m, CH_2). δ_C 77.9 (d, *J* 8, OCH), 33.4 (d, *J* 4, β-CH_2), 32.9 (d, *J* 5, β-CH_2), 24.8 (s, δ-CH_2), 23.4 (s, γ-CH_2), 20.6 (d, *J* 130, P−CH_3). δ_P 39.3.

Synthesis of P═O Analogues (3-12). A solution of 3-chloro-7-hydroxy-4-methylcoumarin (10 mmol) in THF (30 cm^3) was added dropwise to a stirred suspension of sodium hydride (0.24 g, 10 mmol) in THF (20 cm^3). After addition, the mixture was heated under reflux for 30 min and then allowed to cool to room temperature. A solution of the appropriate phosphorus chloride **a**−**d** and **f**−**j** or tabun **e** (10 mmol) in THF (5 cm^3) was added dropwise with stirring over 10 min to the sodium hydride suspension at 0−5 °C. After addition, the mixture was left at room temperature for

12 h. The solvent was removed to yield a liquid residue, which was dissolved in chloroform (50 cm^3) and washed with aqueous K_2CO_3 (100 cm^3, 5% w/v). The aqueous layer instantly colored yellow. The chloroform layer was separated and dried ($MgSO_4$). The drying agent was filtered off, and the filtrate was concentrated to give a yellow liquid. Column chromatography on silica gel gave, after trituration with ether and drying, the title compounds as white solids. Eluting solvents and NMR data appear in Table 4.

Inhibition of AChE. Material. Bovine erythrocyte AChE (EC 3.1.1.7) and acetylcholine iodide (AChI) were obtained from Sigma Chemicals (Dorset, UK) and standard solutions of NaOH from BDH Laboratory Supplies (Leicester, UK). Deionized water was used to prepare the solutions and for washing the reaction vessel, electrode, stirrer, and delivery tubes.

Apparatus. Inhibition experiments were performed at 37 °C and pH 7.4 using a Metrohm automatic titration apparatus to pH-stat the reaction. The apparatus comprised a 713 pH Meter, a 614 Impulsomat, and two 765 Dosimats (one containing 10 mM NaOH solution to maintain the pH at 7.4, the other containing 10 mM AChI solution to maintain substrate concentration). The reaction was monitored using a Kipp and Zonen y−t pen recorder. A Haake DC10 was used to control the water bath temperature and circulate water (at 37 °C) to the jacket surrounding the reaction vessel.

Method. AChE was dissolved in 100 mM NaCl containing pH 7.4 phosphate buffer (5×10^{-3} M) to a specific activity of 5 μM units cm^{-3}. The solution was stored in the refrigerator when not in use. AChI solutions (100 mM and 10 mM) and standard NaOH were prepared and used throughout the week; they were refrigerated when not in use. Experiments were performed at 37 °C in water with an ionic strength of 100 mM in NaCl. The reaction vessel initially contained 5 cm^3 NaCl solution (0.1 M) and 0.5 cm^3 AChE solution (5 μM units cm^{-3}). AChI (0.10 cm^3 of 10^{-1} M) was then added, giving a total AChI concentration of 1.8×10^{-3} M. The pH-stat was set to maintain the reaction at pH 7.4 by adding NaOH solution (0.01 M). The rate of addition was monitored on a y−t pen recorder. AChI solution (10^{-2} M) was added at the same rate to maintain the substrate concentration. This process was allowed to run for approximately 1 min, after which the appropriate organophosphorus compound, in solution (0.01−0.1 cm^3),[50] was added. Four different inhibitor concentrations were investigated.

LD_{50} Determination. Male albino Dunkin Hartley guinea pigs (Harlan, UK) weighing 200−300 g were used. The animals were group housed and had access to Harlan Teklad diet and water ad libitum prior to experimentation. Cyclosarin analogue **12** was dissolved in DMSO (5 mg cm^{-3}) on the day of administration. Subsequent dilutions were made in DMSO, and the compound was administered subcutaneously to the scruff in a dose volume of 500 μL kg^{-1}. Animals were housed in small groups following dosing and observed for up to 8 h before return to the Experimental Animal House overnight. Signs of poisoning were consistent with cholinesterase inhibition. There was a delay of 1−2 h before any signs of poisoning were observed at doses close to the 24 h LD_{50} which was determined as 0.45 mg kg^{-1} (95% confidence limits 0.32−0.58 mg kg^{-1}) by the Dixon staircase method.[51] All experiments were performed in compliance with the Animals (Scientific Procedures) Act 1986 and approved by the Home Office UK.

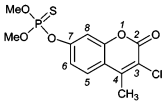
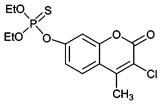
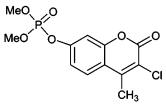
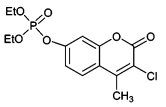
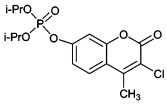
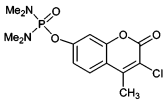
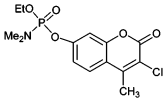
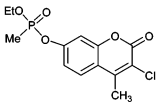
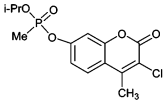
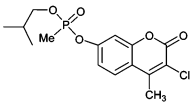
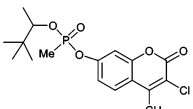
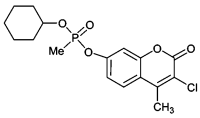
General Procedures on Protein Biochemistry. Material. Paraoxon, coumaphos, and protamine sulfate were purchased from Sigma-Aldrich (Dorset, UK). Dimethylformamide (DMF), calcium chloride ($CaCl_2$), zinc chloride ($ZnCl_2$), and sodium sulfate were from BDH Laboratory Supplies (Leicester, UK). Ampicilin sodium salt, isopropyl β-D-thiogalactoside (IPTG), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic sodium salt (HEPES sodium salt) and dithiothreitol (DTT) were from Melford Laboratories (Chelsworth, UK). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and sodium chloride (NaCl) were from Fisher Scientific (Loughborough, UK). Centriprep concentrators were from Millipore (Bedford, MA). Precast 10% PAGE gels were from Invitrogen (Paisley, UK).

Substrate Quantification. Paraoxon stocks were prepared in 100% DMF and their concentration checked by absorbance at 274 nm ($\epsilon_{274} = 8.9 \times 10^3$ M^{-1} cm^{-1}). For quantification of the synthesized analogues, their absorbance at 315 nm in 100% DMF was compared to a standard curve obtained using commercial coumaphos ($\epsilon_{315} = 8.9 \times 10^3$ M^{-1} cm^{-1}).

Protein Production and Purification. Flasks containing one liter of 2xTY, 100 μg cm^{-3} ampicillin, were inoculated with a saturated culture of *E. coli* C41[52] containing pIVEX-*opd* and pIVEX-opd-*h5* plasmids[22] and incubated at 37 °C and 250 rpm, until $OD_{600\ nm} \sim 0.7$. The cultures were then induced with 1 mM IPTG and supplemented with 1 mM $ZnCl_2$, the temperature was lowered to 18 °C, and the cultures were incubated for a further 40 h. Cell pellets were resuspended in 50 mM HEPES pH 8.5 and lysed by sonication, and the soluble fraction was recovered. PTE and PTE-h5 were purified as described elsewhere[53] with the following modifications: after protamine and ammonium sulfate fractioning, a Superdex-75 column (Pharmacia-Pfizer) equilibrated with 50 mM HEPES pH 8.5, 50 mM NaCl, and 5 mM DTT was used in the gel filtration step. Fractions were pooled based on absorbance at 280 nm and enzymatic activity (hydrolysis of 0.25 mM paraoxon, see later). Subsequently, using a Vision WorkStation (Applied Biosystems), the pooled fractions were loaded into a tandem of POROS 20HQ and 20PI Perfusion Chromatography columns, equilibrated previously with 50 mM HEPES pH 8.5 and 5 mM DTT and eluted with a NaCl gradient 0−400 mM in 50 column volumes. The elution buffer was changed to 50 mM HEPES pH 8.5, 50 mM NaCl, using Centricon bench centrifuge filters with a 10 kDa cutoff. PTE and PTE-h5 were quantified by absorbance at 280 nm ($\epsilon_{280} = 34.4 \times 10^3$ M^{-1} cm^{-1}), while a purity above 95% was ensured by PAGE (not shown). Aliquots were dispensed, fast frozen in liquid nitrogen, and stored at −80 °C. PONs cloned in a pET32-trx (Novagen) system were expressed in *E. coli* Origami B DE3 (Novagen).[54] Cell pellets were harvested and resuspended in 50 mM Tris pH 8.0, 1 mM $CaCl_2$, 50 mM NaCl, 0.1 mM DTT, and 1 μM Pepstatin A (PL Buffer). Cells were lysed by sonication and the recovered supernatant incubated with 0.1% (v/v) Tergitol for 2.5 h. The solutions were further fractioned with 55% (*w*/*v*) ammonium sulfate, and the pellets were resuspended in ice-cold PL Buffer with 0.1% (v/v) Tergitol and dialyzed consecutively against PL Buffer and 50 mM Tris pH 8.0, 1 mM $CaCl_2$, 50 mM NaCl, and 0.1% (v/v) Tergitol (PA Buffer) at 4 °C. The solutions were incubated with Ni-NTA resin (2 cm^3, Qiagen) in 300 mM NaCl, 20 mM imidazole, and PA Buffer at 4 °C for 16 h. The resin was further washed with 300 mM NaCl, 35 mM imidazole, and PA Buffer and eluted with 150 mM imidazole and PA Buffer. The eluted fractions were dialyzed against PA Buffer, supplemented with 0.02% sodium azide, and stored at 4 °C. Purity above 90% was ensured by PAGE (not shown) and quantified using a bicinchoninic acid (BCA) colorimetric assay (Pierce) using BSA as a standard (not shown).

Steady-State Measurements. The hydrolysis of paraoxon was followed by the change in absorbance at 405 nm using a SpectraMAX 190 Microplate reader (Molecular Devices) at 25 °C. Initial velocities (v) were measured at different paraoxon concentrations $[S]_0$ (ranging from 2 nM to 5 mM) with a fixed concentration of enzyme $[E]_0$ ([PTE] = 0.75 nM; [PTE-h5] = 0.37 nM; [PON1-wt], [2.4PC] and [3.2PC] = 25 nM). The kinetic parameters k_{cat} and K_M were obtained by fitting the initial rate (v) at each paraoxon concentration $[S]_0$ to the Michaelis−Menten equation ($v = [E]_o[S]_o k_{cat}/[S]_o + K_M$) using the Levenberg−Marquardt algorithm as implemented in KaleidaGraph (Synergy). The hydrolysis of the NA analogues was followed by the change in fluorescence at 460 nm (ex. = 350 nm, cutoff = 455 nm) using a SpectraMAX GeminiXS Microplate reader (Molecular Devices) at 25 °C. Initial velocities were measured at five different substrate concentrations (0.5, 1, 1.5, 2, 2.5 μM) with amounts of enzyme varied to give a reliable signal (PTE-wt and PTE-h5 between 0.37 and 1.5 μM; PON1-wt, 2.4PC and 3.2PC between 75 and 375 μM). As $[S]_o \ll K_M$, the k_{cat}/K_M values were obtained by plotting substrate concentration versus initial rates, $v \approx [E]_o[S]_o(k_{cat}/K_M)$. 50mM HEPES pH 8.0 was used as base buffer and supplemented with 1 mM $CaCl_2$ whenever PON1-wt, 2.4PC, or 3.2PC were used. Neither $ZnCl_2$

Table 4. Physical and Spectroscopic Data for Compounds **1**−**12** (NMR data measured in $CDCl_3$)

Compound	Structure	^{1}H NMR δ, J/Hz, TMS	^{13}C NMR δ, J/Hz, $CDCl_3$	^{31}P NMR δ, J/Hz, $P(OMe)_3$
1 [a] mp 107-108°C (lit.[29] mp 108°C)		7.62 (1H, dd, *J* 9.2 and 0.8, 5-H), 7.23-7.16 (2H, m, 6-H and 8-H), 3.89 (6H, d, *J* 13.9, 2 × OCH_3), 2.59 (3H, s, 4-CH_3)	156.6 (s, C=O), 152.9 (d, *J* 7.5, 7-C), 152.1 (s, 9-C), 147.2 (s, 4-C), 125.9 (s, 5-C), 120.3 (s, C-Cl), 118.0 (d, *J* 5.2, 6-C), 117.2 (s, 10-C), 109.6 (d, *J* 5.2, 8-C), 55.4 (d, *J* 5.8, 2 × OCH_3), 16.2 (s, 4-CH_3)	+66.7
2 [b] mp 92-93°C (lit.[41] mp 93°C)		7.62 (1H, d, *J* 8.3, 5-H), 7.25-7.16 (2H, m, 6-H and 8-H), 4.27 (4H, dq, *J* 10.0 and 7.1, 2 × OCH_2), 2.59 (3H, s, 4-CH_3), 1.39 (6H, td, *J* 7.1 and 1.0, 2 × CH_3)	156.9 (s, C=O), 153.2 (d, *J* 6.9, 7-C), 152.1 (s, 9-C), 147.4 (s, 4-C), 126.0 (s, 5-C), 120.3 (s, C-Cl), 118.3 (d, *J* 5.2, 6-C), 117.1 (s, 10-C), 109.7 (d, *J* 5.2, 8-C), 65.6 (d, *J* 5.8, 2 × OCH_2), 16.4 (s, 4-CH_3), 16.0 (d, *J* 7.5, 2 × CH_3)	+63.1
3 [c] mp 122-123°C		7.62 (1H, d, *J* 8.8, 5-H), 7.27 (1H, ddd, *J* 8.8, 2.4 and 0.9, 6-H), 7.23 (1H, dd, *J* 2.4 and 1.0, 8-H), 3.91 (6H, *J* 11.3, 2 × OCH_3), 2.59 (3H, s, 4-CH_3)	156.7 (s, C=O), 152.8 (d, *J* 6.2, 7-C), 152.2 (s, 9-C), 147.2 (s, 4-C), 126.2 (s, 5-C), 120.3 (s, C-Cl), 117.0 (s, 10-C), 116.9 (d, *J* 4.8, 6-C), 108.6 (d, *J* 5.3, 8-C), 55.2 (d, *J* 6.2, 2 × OCH_3), 16.3 (s, 4-CH_3)	−4.0
4 [a] mp 68-69°C (lit.[41] mp 71-72°C)		7.58 (1H, d, *J* 8.7, 5-H), 7.22 (1H, ddd, *J* 8.8, 2.4 and 1.0, 6-H), 7.18 (1H, dd, *J* 2.4 and 1.0, 8-H), 4.26-4.17 (4H, m, 2 × OCH_2), 2.54 (3H, s, 4-CH_3), 1.34 (6H, td, *J* 7.1 and 1.0, 2 × CH_3)	156.8 (s, C=O), 153.1 (d, *J* 6.7, 7-C), 152.2 (s, 9-C), 147.4 (s, 4-C), 126.3 (s, 5-C), 120.2 (s, C-Cl), 117.2 (d, *J* 5.3, 6-C), 116.9 (s, 10-C), 108.6 (d, *J* 5.8, 8-C), 65.2 (d, *J* 6.2, 2 × OCH_2), 16.4 (s, 4-CH_3), 16.2 (d, *J* 6.7, 2 × CH_3)	−6.3
5 [d] mp 65-66°C		7.61 (1H, d, *J* 8.7, 5-H), 7.30-7.27 (1H, m, 6-H), 7.26-7.23 (1H, m, 8-H), 4.88-4.71 (2H, m, 2 × OCH), 2.59 (3H, s, 4-CH_3), 1.39 (6H, d, *J* 6.2, 2 × CH_3), 1.35 (6H, *J* 6.2, 2 × CH_3)	156.8 (s, C=O), 153.4 (d, *J* 6.3, 7-C), 152.2 (s, 9-C), 147.3 (s, 4-C), 126.0 (s, 5-C), 120.0 (s, C-Cl), 117.2 (d, *J* 5.8, 6-C), 116.6 (s, 10-C), 108.5 (d, *J* 5.8, 8-C), 74.2 (d, *J* 5.8, 2 × OCH), 23.6 (d, *J* 5.2, 2 × CH_3), 23.5 (d, *J* 4.6, 2 × CH_3), 16.2 (s, 4-CH_3)	−8.0
6 [d] mp 103-104°C		7.59 (1H, d, *J* 8.8, 5-H), 7.30-7.24 (1H, m, 6-H), 7.19-7.15 (1H, m, 8-H), 2.75 (12H, d, *J* 10.2, 2 × $N(CH_3)_2$), 2.58 (3H, s, 4-CH_3)	156.9 (s, C=O), 154.1 (d, *J* 6.3, 7-C), 152.3 (s, 9-C), 147.4 (s, 4-C), 125.9 (s, 5-C), 119.6 (s, C-Cl), 117.4 (d, *J* 4.6, 6-C), 116.1 (s, 10-C), 108.4 (d, *J* 5.8, 8-C), 36.6 (d, *J* 4.0, 2 × $N(CH_3)_2$), 16.2 (s, 4-CH_3)	+17.1
7 [d] mp 68-69°C		7.60 (1H, d, *J* 8.8, 5-H), 7.27 (1H, ddd, *J* 8.8, 2.3 and 1.0, 6-H), 7.21 (1H, dd, *J* 2.3 and 1.0, 8-H), 4.27-4.08 (2H, m, OCH_2), 2.77 (6H, d, *J* 10.3, $N(CH_3)_2$), 2.58 (3H, s, 4-CH_3), 1.38 (3H, td, *J* 7.1 and 1.0, CH_3)	156.8 (s, C=O), 153.7 (d, *J* 6.3, 7-C), 152.2 (s, 9-C), 147.4 (s, 4-C), 126.0 (s, 5-C), 119.8 (s, C-Cl), 117.2 (d, *J* 5.2, 6-C), 116.4 (s, 10-C), 108.4 (d, *J* 5.8, 8-C), 63.2 (d, *J* 5.8, OCH_2), 36.6 (d, *J* 3.5, $N(CH_3)_2$), 16.2 (s, 4-CH_3), 16.1 (d, *J* 6.9, CH_3)	+6.6
8 [a] mp 82°C		7.61 (1H, d, *J* 8.8, 5-H), 7.29 (1H, ddd, *J* 8.8, 2.3 and 1.1, 6-H), 7.22 (1H, dd, *J* 2.2 and 1.3, 8-H), 4.26 (1H, ddq, *J* 10.2, 8.8 and 7.1, OCH_AH_B), 4.18 (1H, ddq, *J* 10.2, 8.0 and 7.1, OCH_AH_B), 2.58 (3H, s, 4-CH_3), 1.69 (3H, d, *J* 17.8, P-CH_3), 1.35 (3H, t, *J* 7.1, CH_3)	156.8 (s, C=O), 152.1 (d, *J* 7.0, 7-C), 152.2 (s, 9-C), 147.3 (s, 4-C), 126.2 (s, 5-C), 120.2 (s, C-Cl), 117.7 (d, *J* 3.8, 6-C), 116.9 (s, 10-C), 109.0 (d, *J* 5.3, 8-C), 62.9 (d, *J* 6.2, OCH_2), 16.5 (s, 4-CH_3), 16.3 (d, *J* 8.6, CH_3), 11.7 (d, *J* 145.4, P-CH_3)	+28.9
9 [a] mp 83-84°C		7.61 (1H, d, *J* 8.8, 5-H), 7.29 (1H, ddd, *J* 8.8, 2.4 and 1.2, 6-H), 7.23 (1H, dd, *J* 2.4 and 1.3, 8-H), 4.84 (1H, dseptet, *J* 8.0 and 6.2, OCH), 2.59 (3H, s, 4-CH_3), 1.68 (3H, d, *J* 17.7, P-CH_3), 1.38 (3H, t, *J* 6.2, CH_3), 1.30 (3H, d, *J* 6.2, CH_3)	156.7 (s, C=O), 153.1 (d, *J* 7.5, 7-C), 152.3 (s, 9-C), 147.2 (s, 4-C), 126.0 (s, 5-C), 120.0 (s, C-Cl), 117.7 (d, *J* 4.0, 6-C), 116.7 (s, 10-C), 109.0 (d, *J* 5.2, 8-C), 72.1 (d, *J* 6.3, OCH), 24.0 (d, *J* 4.6, CH_3), 23.8 (d, *J* 4.6, CH_3), 16.2 (s, 4-CH_3), 12.3 (d, *J* 145.4, P-CH_3)	+27.8
10 [a] mp 62°C		7.59 (1H, d, *J* 8.8, 5-H), 7.26 (1H, ddd, *J* 8.8, 2.3 and 1.0, 6-H), 7.19 (1H, dd, *J* 2.4 and 1.3, 8-H), 3.94 (1H, ddd, *J* 9.7, 7.3, and 6.7, OCH_AH_B), 3.82 (1H, dt, *J* 9.7 and 6.5, OCH_AH_B), 2.55 (3H, s, 4-CH_3), 1.98-1.89 (1H, m, CH), 1.68 (3H, d, *J* 17.6, P-CH_3), 0.92 (6H, d, *J* 6.8, 2 × CH_3)	156.7 (s, C=O), 153.0 (d, *J* 8.2, 7-C), 152.2 (s, 9-C), 147.3 (s, 4-C), 126.1 (s, 5-C), 120.1 (s, C-Cl), 117.6 (d, *J* 3.4, 6-C), 116.8 (s, 10-C), 108.9 (d, *J* 4.3, 8-C), 72.7 (d, *J* 6.7, OCH_2), 29.1 (s, CH), 18.6 (s, 2 × CH_3), 16.3 (s, 4-CH_3), 11.4 (d, *J* 145.4, P-CH_3)	+28.8
11 [d] mp 97-98°C		7.61 (1H, d, *J* 8.8, 5-H), 7.60 (1H, d, *J* 8.8, 5-H), 7.36-7.27 (2H, m, 2 × 6-H), 7.26-7.20 (2H, m, 2 × 8-H), 4.48-4.31 (2H, m, 2 × OCH), 2.59 (3H, s, 4-CH_3), 2.58 (3H, s, 4-CH_3), 1.69 (3H, d, *J* 17.6, P-CH_3), 1.68 (3H, d, *J* 17.6, P-CH_3), 1.35 (3H, d, *J* 6.3, $CHCH_3$), 1.18 (3H, d, *J* 6.3, $CHCH_3$), 0.94 (9H, s, $C(CH_3)_3$), 0.89 (9H, s, $C(CH_3)_3$)	156.9 (s, 2 × C=O), 153.4 (d, *J* 7.5, 7-C), 153.2 (d, *J* 7.5, 7-C), 152.3 (s, 2 × 9-C), 147.4 (s, 2 × 4-C), 126.1 (s, 2 × 5-C), 120.1 (s, C-Cl), 120.0 (s, C-Cl), 117.9 (d, *J* 4.0, 6-C), 117.7 (d, *J* 4.6, 6-C), 116.8 (s, 10-C), 116.7 (s, 10-C), 109.2 (d, *J* 5.2, 8-C), 109.0 (d, *J* 5.2, 8-C), 83.2 (d, *J* 7.5, OCH), 82.8 (d, *J* 7.5, OCH), 35.1 (s, $C(CH_3)_3$), 35.0 (s, $C(CH_3)_3$), 25.6 (s, 2 × $C(CH_3)_3$), 17.1 (s, 2 × $CHCH_3$), 16.4 (s, 4-CH_3), 12.9 (d, *J* 146.0, P-CH_3), 11.9 (d, *J* 147.1, P-CH_3)	28.5 and 27.6 [e]
12 [d] mp 112-113°C		7.60 (1H, d, *J* 8.8, 5-H), 7.31-7.27 (1H, m, 6-H), 7.23-7.22 (1H, m, 8-H), 4.61-4.49 (1H, m, c.hexyl OCH), 2.58 (3H, s, 4-CH_3), 2.03-1.93 (1H, m, c.hexyl), 1.90-1.82 (1H, m, c.hexyl), 1.81-1.71 (2H, m, c.hexyl), 1.68 (3H, *J* 17.6, P-CH_3), 1.61-1.43 (3H, m, c.hexyl), 1.41-1.16 (3H, m, c.hexyl)	156.8 (s, C=O), 153.0 (d, *J* 8.2, 7-C), 152.2 (s, 9-C), 147.3 (s, 4-C), 126.1 (s, 5-C), 120.0 (s, C-Cl), 117.7 (d, *J* 4.3, 6-C), 116.7 (s, 10-C), 109.0 (d, *J* 5.3, 8-C), 77.0 (d, (masked), c.hexyl OCH), 33.7 (d, *J* 3.8, c.hexyl), 33.6 (d, *J* 3.4, c.hexyl), 25.0 (s, c.hexyl), 23.6 (s, c.hexyl), 16.3 (s, 4-CH_3), 12.4 (d, *J* 145.4, P-CH_3)	+27.8

[a] Chromatography eluent: 49:1 chloroform−methanol. [b] Recrystallized from hexane. [c] Chromatography eluent: 24:1 chloroform−methanol, then chloroform. [d] Chromatography eluent: chloroform. [e] Diastereomeric pair. Pin = pinacolyl group, $CH(Me)CMe_3$.

nor $KHCO_3$ (PTE cofactors) were added when PTE-wt and PTE-h5 were assayed because no enhancement in activity was observed in titration trials (data not shown). Whenever mentioned, DMF was present at a final concentration of 1% v/v. The hydrolysis rates of the fluorogenic NA analogues in the absence of enzyme varied according to their structure, but even for the least stable substrate, was still $\sim 10^{10}$ lower than the rate catalyzed by PTE (Supporting Information).

The emission and excitation spectra were measured in 50 mM HEPES at pH 8.0. The emission spectrum was obtained with an excitation at 350 nm. Conversely, the excitation spectrum was obtained by reading fluorescence emitted at 460 nm. Under these conditions, the maximal excitation and emission wavelength were 375 and 465 nm, respectively. The quantum yield (Φ) of the fluorogenic leaving group, 3-chloro-7-hydroxy-4-methylcoumarin, was obtained by the comparative method of Williams et al.,[58] taking as standard 7-diethylamino-4-methylcoumarin (Sigma Chemicals Ltd, Dorset, UK) for which the quantum yield is known ($\Phi = 0.73$).[59] The Φ value for 7-hydroxy-3-chloro-4-methylcoumarin was 0.67.

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Supporting Information Available: Additional synthetic and NMR data for organophosphorus intermediates; LC-MS data for the fluorogenic analogues and their hydrolysis rates in the absence of enzyme. This material is available free of charge via the Internet at http://pubs.acs.org.

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