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Synthesis and anticholinesterase activity of some new fluorogenic analogues of organophosphorus nerve agents

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Abstract

Eighteen new fluorogenic analogues of organophosphorus nerve agents were synthesised and characterised. They included analogues of tabun, sarin, cyclosarin, soman, VX, and Russian VX, with the 7-oxy-4-methylcoumarin or 7-oxy-4-(trifluoromethyl)coumarin leaving group. These analogues inhibited acetylcholinesterase (AChE) effectively *in vitro* and therefore have potential as tools for the identification of novel organophosphatases in biological systems. Analogues of VX and Russian VX with the 7-amino-4-methylcoumarin group, although poor AChE inhibitors, may have utility for screening enzyme libraries for phosphoramidases capable of cleaving P–N bonds.

Keywords: Acetylcholinesterase; Fluorogenic probes; 7-hydroxy-4-methylcoumarin; 7-hydroxy-4-(trifluoromethyl)coumarin; Organophosphorus nerve agent; Phosphotriesterase

1. Introduction

Organophosphorus (OP) compounds include pesticides and military nerve agents (NAs). The first NA, tabun, was discovered in 1936 and refinement of its structure led to the more toxic relatives, sarin, soman and cyclosarin (Scheme 1) [1]. These compounds are volatile liquids that pose mainly a vapour hazard. Two NAs with greater toxicity, VX and Russian VX, were developed during the Cold War. They are low-volatility liquids that pose mainly a skin contact hazard unless released as an aerosol.

NAs inhibit acetylcholinesterase (AChE), an enzyme which controls nerve impulse transmission, by hydrolysing acetylcholine (a neurotransmitter) to acetic acid and choline [2]. The active-site serine residue attacks the carbonyl carbon of acetylcholine to form a covalent acetyl-enzyme intermediate. The enzyme is regenerated after attack by water and release of acetic acid. NAs mimic acetylcholine and phosphylate the serine residue, losing either cyanide, fluoride or the *N*,*N*-dialkylaminoethanethiolate group. This step is fast, but regeneration of enzyme through attack by water is slow, producing an irreversibly inhibited AChE unable to hydrolyse acetylcholine.

Quantifying turnover of molecules through biological compartments such as enzymes is aided by the use of non-fluorescent (fluorogenic) substrates that produce a fluorescent motif after reaction with specific molecules [3]. Fluorogenic substrates have been developed for targeting enzymes that can hydrolyse organophosphorus compounds: coumarin phosphates[1] for protein phosphatases [4–9] and 7-(diethylphosphoryl)-6,8-difluoro-4-methylumbelliferone (DEPFMU) [10] for anti-NA enzymes (Scheme 1). Hydrolysis of DEPFMU produces 7-hydroxy-6,8-difluoro-4-methylcoumarin [10–12], a blue fluorescent dye with good photophysical properties [13] such as a high quantum yield ($\Phi = 0.89$).[2] DEPFMU has been used to target human paraoxonases that might be useful in the prophylaxis and treatment of organophosphorus insecticide and

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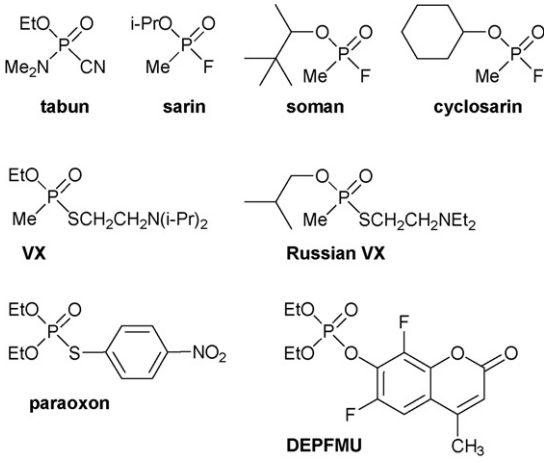
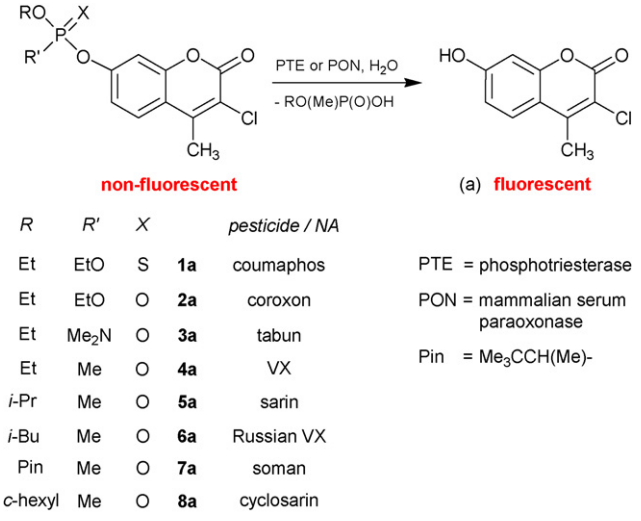
[1] Coumarin is the trivial name for 2*H*-chromen-2-one, sometimes called umbelliferone as toxic derivatives were once isolated from plants of the family Umbelliferae.

[2] Quantum yield (Φ) is the probability of luminescence occurring, expressed by the ratio of the number of photons emitted by the luminescing species to the number of photons absorbed.



Scheme 1. Structures of NAs, the pesticide paraoxon, and the fluorgenic probe 7-(diethylphosphoryl)-6,8-difluoro-4-methylumbelliferone (DEPFMU).



R	R′	X		pesticide / NA
Et	EtO	S	**1a**	coumaphos
Et	EtO	O	**2a**	coroxon
Et	Me_2N	O	**3a**	tabun
Et	Me	O	**4a**	VX
i-Pr	Me	O	**5a**	sarin
i-Bu	Me	O	**6a**	Russian VX
Pin	Me	O	**7a**	soman
c-hexyl	Me	O	**8a**	cyclosarin

PTE = phosphotriesterase
PON = mammalian serum paraoxonase
Pin = $Me_3CCH(Me)$-

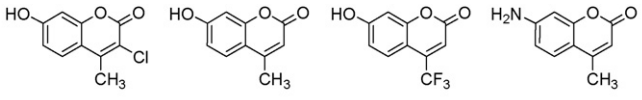
Fig. 1. Coumarin nerve agent analogues prepared previously [15].

nerve agent poisonings [14]. However, we believe it is too dissimilar in structure to the NAs to allow optimal searching for anti-NA enzymes.

To overcome these structural issues, we recently designed NA analogues based on the pesticides coumaphos **1a** and coroxon **2a** (Fig. 1) having the 3-chloro-7-oxy-4-methylcoumarin leaving group (**a**) [15]. With the exception of tabun analogue **3a** which did not inhibit AChE, analogues **4a**–**8a** were 0.7–34 times less potent inhibitors of AChE than the parent NAs and retained the same relative inhibition potency (Russian VX > cyclosarin > VX > sarin > soman). LD_{50} values for cyclosarin (0.06 mg kg^{-1}) and its analogue **8a** (0.4 mg kg^{-1}) upon subcutaneous administration to guinea pigs displayed a similar ratio [15]. The NA analogues **1a**–**8a** were suitable substrates for phosphotriesterase (PTE) from *Pseudomonas diminuta* and a mammalian serum paraoxonase (PON1), and catalytic efficiencies (k_{cat}/K_M) ranged across 10^8 M^{-1} s^{-1}. All were hydrolysed by wild-type PTE. However, wild-type PON1 hydrolysed only the non-bulky analogues efficiently [15].

In this paper, we have extended the range of fluorogenic NA analogues available for biological studies to include derivatives of 7-hydroxy-4-methylcoumarin (**b**), 7-hydroxy-4-(trifluoromethyl)coumarin (**c**) and 7-amino-4-methylcoumarin (**d**). These coumarins are commercially available and quantum yields (Φ) and wavelengths of maximum absorption (abs) and emission (em) are known (Fig. 2) [13,16].

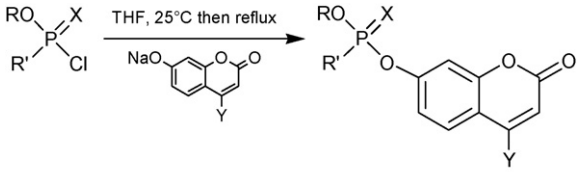
The ability of the new NA analogues to inhibit bovine erythrocyte AChE is also described. The aim was to assess which new fluorogenic analogues were suitable for biological applications involving fluorescence detection and to compare the effect on AChE inhibition in changing from a CH_3 to a CF_3 substituent in the coumarin leaving group. No studies appear to have been published on the reactivity of AChE towards organophosphorus substrates bearing fluorinated groups remote from the phosphorus centre. The main effect should be an increase in size and hydrophobicity in going from a hydrocarbon to a fluorocarbon substituent, although the possibility of the latter fluorine–hydrogen bonding with a residue close to the active site cannot be ignored. As fluorocarbon materials, with few exceptions, are man-made, there has been no evolutionary pressure for many enzymes to evolve specificity towards them, and therefore study of their interaction with enzymes should reveal information which will cast further light on the mechanism of enzyme catalysis. The study described here was partly driven by a curiosity to see how well a complex biochemical system



	(a)	(b)	(c)	(d)
Φ	0.67[a]	0.63[b]	0.34[b]	-
λ_{max} abs (nm)	350	360	385	342
λ_{max} em (nm)	460	450	501	441
pK_a	-	7.8	7.3	-
Bleaching (%) [c]	-	22	31	-

Quantum yield (a) ref. 15; (b) ref. 13; (c) Bleaching denotes the percent decrease in fluorescence intensity after 33 min of illumination in a fluorometer at the wavelength of maximum absorption (data from ref. 13)

Fig. 2. Fluorescent coumarin leaving groups.



R	R'	X		Y / isolated yield		pesticide / NA
				CH_3 **b**	CF_3 **c**	
Et	EtO	S	**1**	52%	37%	coumaphos
Et	EtO	O	**2**	40%	46%	coroxon
Et	Me_2N	O	**3**	50%	52%	tabun
Et	Me	O	**4**	21%	16%	VX
i-Pr	Me	O	**5**	27%	38%	sarin
i-Bu	Me	O	**6**	41%	29%	Russian VX
Pin	Me	O	**7**	27%	27%	soman
c-hexyl	Me	O	**8**	32%	38%	cyclosarin

Fig. 3. New fluorogenic nerve agent analogues.

(AChE) could respond to the structural change 4-CH_3 → 4-CF_3 in the coumarin leaving group, and to see whether this simple change might affect its ability to hydrolyse such compounds.

2. Results and discussion

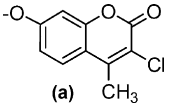
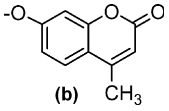
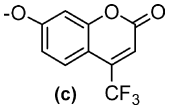
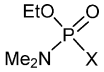
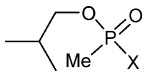
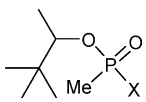
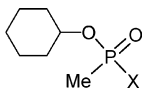
2.1. 7-Hydroxycoumarins

Analogues **1b–8b** and **1c–8c** were prepared by treating the respective phosphorus chlorides [15] with the sodium salts of the hydroxycoumarins in tetrahydrofuran (Fig. 3). Conversion usually exceeded 90% (as indicated by GC–MS analysis of crude reaction mixtures) but isolated yields were low due to decomposition during chromatography on silica gel; chromatography was necessary to obtain the products in sufficient purity (99%) for AChE inhibition studies.

First-order rate coefficients of AChE inhibition, obtained from a graph of $\log_{10}$ percentage of enzyme activity remaining 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. 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 1).

Studies by other researchers have shown that coumarin phosphates have high affinity for AChEs from various animal species [17,18], the phosphorus moiety binding to the active site and the coumarin group binding to the peripheral anionic site

Table 1
Second-order rate coefficients (k_i) for inhibition of bovine erythrocyte AChE (pH 7.4, 37 °C)

Compound	Structure	k_i (M^{-1} min^{-1})			Approximate k_i ratio		Pesticide or nerve agent	k_i (M^{-1} min^{-1}) pesticide or nerve agent[a]
		 (a)	 (b)	 (c)	**a**:**b**	**b**:**c**		
1		NA	$4.0 \pm 0.5 \times 10^3$	$1.0 \pm 0.2 \times 10^2$	–	40:1	Coumaphos	NA
2		$2.1 \pm 0.2 \times 10^4$	$2.3 \pm 0.5 \times 10^5$	$1.6 \pm 0.3 \times 10^3$	1:11	144:1	Coroxon	$2.1 \pm 0.2 \times 10^4$
3		NA	$1.2 \pm 0.2 \times 10^3$	$1.1 \pm 0.5 \times 10^3$	–	1:1	Tabun	$3.2 \pm 0.1 \times 10^5$
4		$2.2 \pm 0.2 \times 10^5$	$8.7 \pm 0.7 \times 10^4$	$13.3 \pm 0.4 \times 10^4$	3:1	1:2	VX	$6.6 \pm 2.2 \times 10^6$
5		$2.2 \pm 0.4 \times 10^4$	$2.0 \pm 0.5 \times 10^4$	$2.9 \pm 0.3 \times 10^4$	1:1	1:2	Sarin	$7.4 \pm 1.3 \times 10^5$
6		$2.1 \pm 0.5 \times 10^6$	$1.7 \pm 0.2 \times 10^6$	$6.1 \pm 0.9 \times 10^5$	1:1	3:1	Russian VX	$2.7 \pm 0.6 \times 10^7$
7		$5.7 \pm 1.2 \times 10^4$	$2.3 \pm 0.5 \times 10^5$	$4.8 \pm 0.7 \times 10^4$	1:4	5:1	Soman	$4.1 \pm 1.0 \times 10^4$
8		$1.8 \pm 0.1 \times 10^6$	$3.2 \pm 0.1 \times 10^6$	$6.3 \pm 0.7 \times 10^5$	1:2	5:1	Cyclosarin	$1.2 \pm 1.0 \times 10^7$

[a] Data taken from Ref. [15]. NA = no activity detected. X = leaving group (**a**), (**b**) or (**c**).

[18,19]. We found that potencies with respect to different substitution patterns at phosphorus followed the order expected from consideration of stereoelectronic effects of the substituents [15]. Thus, thiono analogues **1a–c** were worse inhibitors than their oxo counterparts **2a–c**, and the tabun analogues **3a–c** were worse inhibitors than the VX analogues **4a–c** (replacing Me_2N by Me rendered the phosphorus atom more electropositive and less hindered, and thus more responsive to nucleophilic attack).

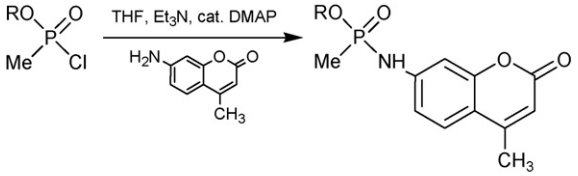
Compounds with coumarin (**a**) were equal or less potent inhibitors than those with coumarin (**b**), with the exception of VX analogue **4a** which was 2.5 times more potent than analogue **4b**. Compounds with coumarin (**b**) were also generally better inhibitors than analogues with coumarin (**c**), perhaps due a poor fit of the trifluoromethyl group in the peripheral site. Considerable controversy surrounds the issue of the size of the CF_3 group. It is clearly larger than a CH_3 group but how much larger is a matter of debate and depends to an extent on the method used to measure steric bulk. The CF_3 group is estimated from computational studies to approximate an ethyl group [20], but Taft [21], Charton [22] and Meyer [23] calculations imply that it is the size of the *s*-butyl group, *i*-butyl group, or intermediate between a methyl and ethyl group, respectively.

Whereas coumaphos **1a** and tabun analogue **3a** did not inhibit AChE, the corresponding analogues **1b** and **1c**, and **3b** and **3c**, did inhibit the enzyme. The reason for this is unclear—the leaving groups (**a–c**) might be expected to depart with equal facility due to their electronic similarity. It is possible that a chlorine atom in the 3-position of the coumarin ring exerts an unfavourable steric effect that affects its fit in the peripheral site.

The most active inhibitors were the analogues of Russian VX (**6a–c**) and cyclosarin (**8a–c**). Values of k_i measured for cyclosarin in AChEs from different animal species exceed those measured for other well-known nerve agents [24–26]. The high activity of cyclosarin and its coumarin derivatives towards bovine AChE might be due to a good fit of the cyclohexyl group into the active site [15].

2.2. 7-Aminocoumarins

Aminocoumarin analogues of VX and Russian VX, **9** and **10**, were prepared by treating the respective phosphorus chlorides [15] with 7-amino-4-methylcoumarin, triethylamine and a catalytic amount of 4-dimethylaminopyridine (DMAP) in tetrahydrofuran (Fig. 4). Yields were low due to the poor nucleophilicity of the coumarin amino group, which rendered coupling problematic, and partial decomposition of the products during chromatography on silica gel. We were unable to isolate the corresponding analogues of 7-amino-4-(trifluoromethyl)coumarin, an even less nucleophilic species.



R	*no.*	*yield*	k_i (M^{-1} min^{-1})
Et	**9**	25%	$< 5 \times 10^2$
i-Bu	**10**	29%	$8.6(\pm 3.8) \times 10^2$

Fig. 4. New fluorogenic nerve agent analogues with an aminocoumarin substituent.

Due to the poor leaving ability of the aminocoumarin group, compounds **9** and **10** were poor inhibitors of AChE, and their k_i values were much lower than those found for their hydroxycoumarin counterparts **4b** and **6b**.

2.3. Discussion

Although analogues **1b–8b** and **1c–8c** could be used to search for novel organophosphatases, their AChE k_i values did not mirror those found for the NAs (unlike the original analogues **1a–8a** whose k_i values did). Because of this, and the greater quantum yield of 7-hydroxy-3-chloro-4-methylcoumarin compared to the other coumarins (Fig. 2), analogues **1a–8a** are perhaps the most useful for applications involving detection of the fluorescent leaving group.

However, one compound in the new series – tabun analogue **3b** – has promise. Unlike **3a**, which was found to be a poor substrate for AChE (no noticeable inhibition), analogue **3b** was found to be a moderately good inhibitor. It may therefore prove useful for identifying anti-tabun enzymes. In addition, aminocoumarins **9** and **10** are potentially useful for the discovery of phosphoramidases capable of cleaving P–N bonds.

Although acute toxicity data are lacking, analogues **1b–8b** and **1c–8c** are likely to be less toxic than analogues **1a–8a** and the parent NAs. Comparative toxicity data are available only for coroxon **2a** and its analogue **2b** whose oral LD_{50}s in rats are 10 and 29 mg kg^{-1} respectively [27]. In this case, replacing coumarin (**a**) with coumarin (**b**) lowered toxicity threefold. As the dermal toxicity of coumaphos is low compared with other organophosphorus pesticides [28] and much lower than that of the NAs, compounds **1b–8b** and **1c–8c** are not expected to have high percutaneous toxicity. In terms of their ability to inhibit AChE, they were generally much worse than the parent NAs (except analogue **7b** whose k_i exceeded that of soman; refer to Table 3).

3. Conclusions

The NA analogues could be used to screen for hydrolytic enzymes in single cells and in cell arrays using electroporation [29]. Image-based methods could be used to quantify a large number of wells in a single detection procedure [30]. Large libraries of $>10^7$ mutant PTEs or PONs expressed in bacterial cells can be screened by encapsulating single cells in microdroplets in water-in-oil-in-water double emulsions and sorting the droplets [31] using fluorescence activated cell sorting (FACS).[3] Large libraries can also be screened by *in*

[3] Fluorescence activated cell sorting (FACS) is a flow cytometry application in which live fluorescent cells are excited at a specific wavelength and then sorted into physically separated sub-populations on the basis of their fluorescence emission.

Table 2
Physical and spectroscopic data for the 7-oxy-4-methylcoumaryl analogues **1b–8b** (NMR data measured in $CDCl_3$)

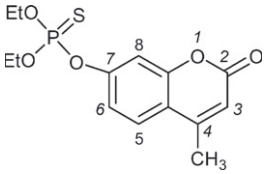
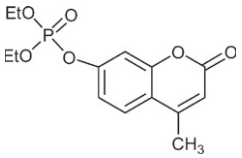
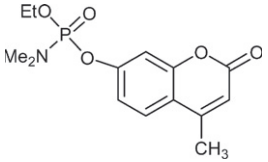
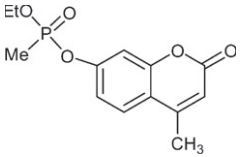
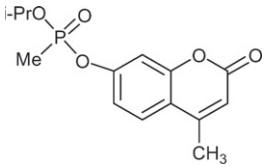
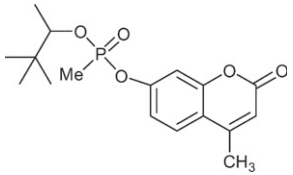
Compound	Structure	^{1}H NMR δ, J (Hz)	^{13}C NMR δ, J (Hz)	^{31}P NMR, δ
1b (mp 35–36 °C)		7.58 (1H, d, *J* 8.5, 5-H), 7.19 (1H, t, *J* 1.8, 8-H), 7.12–7.18 (1H, m, 6-H), 6.26 (1H, q, *J* 1.2, 3-H), 4.27 (4H, dq, *J* 10.1 and 7.0, 2× OCH_2), 2.43 (3H, d, *J* 1.2, 4-CH_3), 1.39 (6H, dt, *J* 7.1 and 0.8, 2× CH_3)	160.5 (s, C=O), 154.3 (s, 9-C), 153.2 (d, *J* 7.5, 7-C), 151.9 (s, 4-C), 125.5 (s, 5-C), 117.5 (d, *J* 5.2, 6-C), 117.3 (s, 10-C), 114.3 (s, 3-C), 109.7 (d, *J* 5.2, 8-C), 65.4 (d, *J* 5.8, 2× OCH_2), 18.7 (s, 4-CH_3), 15.9 (d, *J* 7.5, 2× CH_3)	+63.2
2b (liquid)		7.61 (1H, d, *J* 8.3, 5-H), 7.17–7.28 (2H, m, 6-H and 8-H), 6.25 (1H, q, *J* 1.2, 3-H), 4.35–4.20 (4H, m, 2× OCH_2), 2.45 (3H, d, *J* 1.2, 4-CH_3), 1.39 (6H, td, *J* 7.1 and 1.0, 2× CH_3)	160.2 (s, C=O), 154.1 (s, 9-C), 152.9 (d, *J* 6.3, 7-C), 151.8 (s, 4-C), 125.7 (s, 5-C), 116.9 (s, 10-C), 116.2 (d, *J* 5.2, 6-C), 113.8 (s, 3-C), 108.2 (d, *J* 5.8, 8-C), 64.8 (d, *J* 6.3, 2× OCH_2), 18.4 (s, 4-CH_3), 15.8 (d, *J* 6.9, 2× CH_3)	−6.3
3b (mp 80–81 °C)		7.57 (1H, d, *J* 8.7, 5-H), 7.27–7.20 (1H, m, 6-H), 7.18 (1H, s, 8-H), 6.23 (1H, s, 3-H), 4.24–4.13 (2H, m, OCH_2), 2.77 (6H, d, *J* 10.3, $N(CH_3)_2$), 2.43 (3H, s, 4-CH_3), 1.38 (3H, t, *J* 7.0, CH_3)	160.7 (s, C=O), 154.5 (s, 9-C), 153.9 (d, *J* 6.2, 7-C), 152.2 (s, 4-C), 125.8 (s, 5-C), 116.8 (s, 10-C), 116.7 (d, *J* 4.8, 6-C), 113.9 (s, 3-C), 108.6 (d, *J* 5.8, 7-C), 63.3 (d, *J* 5.8, OCH_2), 36.7 (d, *J* 3.8, $N(CH_3)_2$), 18.8 (s, 4-CH_3), 16.2 (d, *J* 6.7, CH_3)	+6.6
4b (mp 82–83 °C)		7.58 (1H, d, *J* 8.5, 5-H), 7.26–7.20 (1H, m, 6-H), 7.21–7.17 (1H, m, 8-H), 6.25 (1H, q, *J* 1.2, 3-H), 4.34–4.11 (2H, m, OCH_2), 2.43 (3H, d, *J* 1.2, 4-CH_3), 1.69 (3H, d, *J* 17.6, P-CH_3), 1.35 (3H, t, *J* 7.1, CH_3)	160.4 (s, C=O), 154.4 (s, 9-C), 153.0 (d, *J* 8.1, 7-C), 151.8 (s, 4-C), 125.8 (s, 5-C), 117.0 (s, 10-C), 116.9 (d, *J* 4.6, 6-C), 114.0 (s, 3-C), 108.9 (d, *J* 5.2, 8-C), 62.8 (d, *J* 6.9, OCH_2), 18.6 (s, 4-CH_3), 16.3 (d, *J* 6.3, CH_3), 11.5 (s, *J* 145.4, P-CH_3)	+28.7
5b (mp 43–44 °C)		7.58 (1H, d, *J* 8.6, 5-H), 7.24 (1H, ddd, *J* 8.6, 2.4 and 1.2, 6-H), 7.20 (1H, dd, *J* 2.4 and 1.3, 8-H), 6.25 (1H, q, *J* 1.3, 3-H), 4.93–4.76 (1H, m, OCH), 2.43 (3H, d, *J* 1.3, 4-CH_3), 1.67 (3H, d, *J* 17.6, P-CH_3), 1.38 (3H, d, *J* 6.2, CH_3), 1.31 (3H, d, *J* 6.2, CH_3)	160.6 (s, C=O), 154.5 (s, 9-C), 153.2 (d, *J* 8.1, 7-C), 152.0 (s, 4-C), 125.9 (s, 5-C), 117.1 (d, *J* 4.6, 6-C), 117.1 (s, 10-C), 114.1 (s, 3-C), 109.1 (d, *J* 4.6, 8-C), 72.1 (d, *J* 6.9, CH), 24.1 (d, *J* 4.6, CH_3), 23.9 (d, *J* 4.6, CH_3), 18.8 (s, 4-CH_3), 12.3 (d, *J* 145.4, P-CH_3)	+27.6
6b (liquid)		7.52–7.60 (2H, m, 2× 5-H), 7.15–7.31 (4H, m, 2× [6-H and 8-H]), 6.25 (2H, br. s, 2× 3-H), 4.49–4.28 (2H, m, 2× OCH), 2.43 (6H, s, 2× 4-CH_3), 1.68 (3H, d, *J* 17.6, P-CH_3), 1.67 (3H, d, *J* 17.6, P-CH_3), 1.35 (3H, d, *J* 6.3, CH_3), 1.19 (3H, d, *J* 6.5, CH_3), 0.94 (9H, s, $C(CH_3)_3$), 0.90 (9H, s, $C(CH_3)_3$)	160.5 (s, 2× C=O), 154.4 (s, 2× 9-C), 153.2 (d, *J* 8.1, 7-C), 153.2 (d, *J* 7.5, 7-C), 151.9 (s, 2× 4-C), 125.7 (s, 2× 5-C), 117.2 (d, *J* 4.0, 6-C), 117.0 (s, 2× 10-C), 116.9 (d, *J* 5.2, 6-C), 114.1 (s, 3-C), 114.0 (s, 3-C), 109.1 (d, *J* 5.2, 8-C), 109.0 (d, *J* 5.2, 8-C), 83.0 (d, *J* 7.5, OCH), 82.6 (d, *J* 7.5, OCH), 35.0 (d, *J* 6.9, 2× *C*$(CH_3)_3$), 25.6 (s, C(*C*$H_3)_3$), 25.5 (s, C(*C*$H_3)_3$), 18.7 (s, 2× 4-CH_3), 17.0 (s, 2× CH_3), 12.7 (d, *J* 146.0, P-CH_3), 11.8 (s, d, *J* 147.1, P-CH_3)	28.3/27.4[a]

Table 2 (*Continued*)

Compound	Structure	^{1}H NMR δ, J (Hz)	^{13}C NMR δ, J (Hz)	^{31}P NMR, δ
7b (liquid)		7.58 (1H, d, *J* 8.7, 5-H), 7.27–7.21 (1H, m, 6-H), 7.18 (1H, dd, *J* 2.2 and 1.2, 8-H), 6.25 (1H, q, *J* 1.2, 3-H), 3.97 (1H, ddd, *J* 9.7, 7.3, 6.7, OCH_AH_B), 3.85 (1H, dt, *J* 9.7, 6.7, OCH_AH_B), 2.43 (3H, d, *J* 1.2, 4-CH_3), 2.05–1.87 (1H, m, CH), 1.70 (3H, d, *J* 17.6, P-CH_3), 0.95 (6H, d, *J* 6.7, 2× CH_3)	160.5 (s, C=O), 154.4 (s, 9-C), 153.1 (d, *J* 8.1, 7-C), 151.9 (s, 4-C), 125.8 (s, 5-C), 117.0 (s, 10-C), 116.9 (d, *J* 4.0, 6-C), 114.1 (s, 3-C), 108.9 (d, *J* 5.2, 8-C), 72.6 (d, *J* 6.9, OCH_2), 29.1 (d, *J* 6.3, CH), 18.7 (s, 4-CH_3), 18.6 (d, *J* 1.2, 2× CH_3), 11.3 (d, *J* 145.4, P-CH_3)	+28.7
8b (mp 83–84 °C)		7.57 (1H, d, *J* 8.7, 5-H), 7.26–7.22 (1H, m, 6-H), 7.19 (1H, dd, *J* 2.3 and 1.3, 8-H), 6.25 (1H, q, *J* 1.3, 3-H), 4.64–4.49 (1H, m, c.hexyl OCH), 2.43 (3H, d, *J* 1.3, 4-CH_3), 1.94–2.01 (1H, m, c.hexyl), 1.91–1.84 (1H, m, c.hexyl), 1.80–1.67 (2H, m, c.hexyl) 1.68 (3H, d, *J* 17.6, P-CH_3), 1.62–1.44 (3H, m, c.hexyl), 1.40–1.17 (3H, m, c.hexyl)	160.5 (s, C=O), 154.4 (s, 9-C), 153.1 (d, *J* 8.2, 7-C), 151.9 (s, 4-C), 125.7 (s, 5-C), 117.0 (d, *J* 4.3, 6-C), 116.9 (s, 10-C), 114.0 (s, 3-C), 109.0 (d, *J* 4.8, 8-C), 76.8 (d, *J* 7.2, c.hexyl OCH), 33.7 (d, *J* 4.3, c.hexyl), 33.5 (d, *J* 3.8, c.hexyl), 25.0 (s, c.hexyl), 23.5 (s, c.hexyl), 18.7 (s, 4-CH_3), 12.4 (d, *J* 145.4, P-CH_3)	+27.6

[a] Diastereoisomeric pair. Pin = pinacolyl group, –CH(Me)CMe_3.

vitro compartmentalization (IVC) [32] where single genes are transcribed and translated *in vitro* in aqueous microdroplets in double emulsions, which are sorted using FACS to select genes encoding enzymes with improved catalytic activity [33]. The NA analogues may also be applicable to high-throughput screening of novel biological hydrolytic enzymes or catalysts using conventional techniques or microfluidic devices [34].

4. Experimental

4.1. Materials and analytical equipment

7-Hydroxy-4-methylcoumarin was obtained from Lancaster Synthesis (Morecambe, UK), 7-hydroxy-4-(trifluoromethyl)-coumarin from Apollo Scientific (Stockport, UK) and 7-amino-4-methylcoumarin and other reagents from Sigma–Aldrich (Gillingham, UK). Triethylamine was distilled from CaH_2 and stored over CaH_2. Anhydrous solvents were used: tetrahydrofuran (THF) was distilled from sodium/benzophenone. Organophosphorus precursors were prepared as described previously [15]. Thin layer chromatography was performed on MK6F silica gel 60 Å plates (Whatman, USA) 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 121.5 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 141.3 ppm) for ^{31}P spectra.

Safety warning. The compounds described have significant anticholinesterase activity and must be handled with care in an efficient fume cupboard. They must not be allowed to come into contact with skin. Rubber gloves must be worn and glassware decontaminated in aqueous bleach.

4.2. LC–MS and LC–MS^n experiments

4.2.1. Material

Ammonium formate was purchased from Fluka (Gillingham, UK) and HPLC grade methanol from Aldrich (Gillingham, UK). Water from a Milli-Q system (Millipore, UK) was used for dilutions and the mobile phase.

4.2.2. LC conditions

A Thermo Finnigan Surveyor LC system was fitted with a C_{18} column (150 mm × 2 mm i.d., 5 μm, 100 Å) from Columbus (Phenomenex, Macclesfield, UK). A linear gradient elution was performed at a flow rate of 0.2 ml min^{-1}, with a mobile phase of 20 mM ammonium formate in water (solvent A) and 20 mM ammonium formate in methanol (solvent B). The gradient was 5% B (0–5 min), 5–90% B (5–10 min), held at 90% B (10–15 min), returned to the initial composition 5% B

Table 3
Physical and spectroscopic data for the 7-oxy-4-(trifluoromethyl)coumarin analogues **1c**–**8c** (NMR data measured in $CDCl_3$)

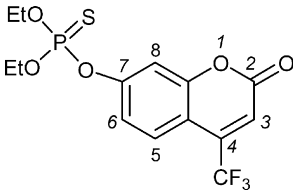
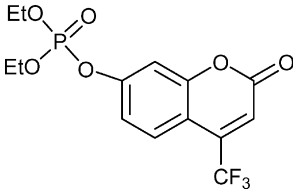
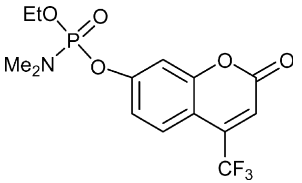
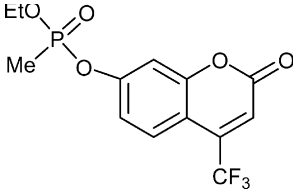
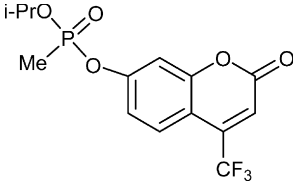
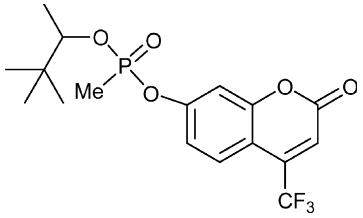
Compound	Structure[a]	^{1}H NMR δ, J (Hz)	^{13}C NMR δ, J (Hz)	^{31}P NMR δ
1c (mp 57–58 °C)		7.69–7.77 (1H, m, 5-H), 7.28 (1H, dd, J 2.3 and 1.6, 8-H), 7.22 (1H, ddd, J 8.9, 2.3 and 1.3, 6-H), 6.76 (1H, s, 3-H), 4.28 (4H, dq, J 10.1 and 7.1, 2× OCH_2), 1.40 (6H, td, J 7.1 and 1.0, 2× CH_3)	158.5 (s, C═O), 155.1 (s, 9-C), 154.1 (d, J 6.9, 7-C), 141.1 (q, J 33.1, 4-C), 126.4 (s, 5-C), 121.4 (q, J 275.8, CF_3), 118.4 (d, J 5.2, 6-C), 114.9 (q, J 5.8, 3-C), 110.7 (s, 10-C), 110.1 (d, J 5.2, 8-C), 65.6 (d, J 5.8, 2× OCH_2), 15.9 (d, J 7.5, 2× CH_3)	+62.8
2c (liquid)		7.68–7.76 (1H, m, 5-H), 7.33–7.23 (2H, m, 6-H and 8-H), 6.76 (1H, s, 3-H), 4.35–4.19 (4H, m, 2× OCH_2) and 1.40 (6H, td, J 7.1 and 1.1, 2× CH_3)	158.5 (s, C═O), 154.2 (d, J 5.2, 7-C), 141.1 (q, J 33.0, 4-C), 126.7 (s, 5-C), 121.4 (q, J 275.7, CF_3), 117.4 (d, J 4.6, 6-C), 114.9 (q, J 4.6, 3-C), 109.1 (d, J 4.0, 8-C), 65.2 (d, J 5.2, 2× OCH_2), 16.1 (d, J 5.8, 2× CH_3)	−6.4
3c (mp 37–38 °C)		7.70 (1H, dd, J 8.7 and 1.7, 5-H), 7.24–7.31 (2H, m, 6-H and 8-H), 6.74 (1H, s, 3-H), 4.25–4.13 (2H, m, OCH_2), 2.78 (6H, d, J 10.4, $N(CH_3)_2$), 1.39 (3H, td, J 7.1 and 0.9, CH_3)	158.8 (s, C═O), 155.5 (s, 9-C), 155.0 (d, J 6.7, 7-C), 141.3 (q, J 33.1, 4-C), 126.6 (s, 5-C), 121.5 (q, J 275.9, CF_3), 117.6 (d, J 5.3, 6-C), 114.6 (q, J 5.4, 3-C), 110.2 (s, 10-C), 109.2 (d, J 5.3, 8-C), 63.5 (d, J 5.8, OCH_2), 36.7 (d, J 3.8, $N(CH_3)_2$), 16.2 (d, J 6.7, CH_3)	+6.6
4c (mp 68–69 °C)		7.71 (1H, dd, J 8.7 and 1.8, 5-H), 7.34–7.30 (1H, m, 8-H), 7.25–3.32 (1H, m, 6-H), 6.75 (1H, s, 3-H), 4.37–4.10 (2H, m, OCH_2), 1.71 (3H, d, J 17.7, P-CH_3), 1.36 (3H, t, J 7.1, CH_3)	158.5 (s, C═O), 155.4 (s, 9-C), 154.1 (d, J 8.1, 7-C), 141.1 (q, J 33.0, 4-C), 126.7 (s, 5-C), 117.9 (d, J 4.6, 6-C), 121.4 (q, J 275.8, CF_3), 114.8 (q, J 5.8, 3-C), 110.5 (s, 10-C), 109.5 (d, J 5.2, 8-C), 63.0 (d, J 6.3, OCH_2), 16.3 (d, J 6.3, CH_3), 11.7 (d, J 144.8, P-CH_3)	+29.0
5c (liquid)		7.70 (1H, dd, J 8.6 and 1.7, 5-H), 7.33–7.28 (1H, m, 8-H), 7.24–7.33 (1H, m, 6-H), 6.74 (1H, s, 3-H), 4.74–4.92 (1H, m, OCH), 1.68 (3H, d, J 17.7, P-CH_3), 1.38 (3H, d, J 6.2, CH_3), 1.31 (3H, d, J 6.2, CH_3)	158.7 (s, C═O), 155.5 (s, 9-C), 154.3 (d, J 7.5, 7-C), 141.3 (q, J 33.5, 4-C), 126.8 (s, 5-C), 121.5 (q, J 276, CF_3), 118.0 (d, J 4.6, 6-C), 114.9 (q, J 5.8, 3-C), 110.5 (s, 10-C), 109.7 (d, J 4.6, 8-C), 72.4 (d, J 6.3, OCH), 24.1 (d, J 5.2, CH_3), 24.0 (d, J 4.0, CH_3), 12.5 (d, J 146.0, P-CH_3)	+27.9
6c (liquid)		7.66–7.75 (2H, m, 2× 5-H), 7.34–7.31 (2H, m, 2× 8-H), 7.35–7.27 (2H, m, 2× 6-H), 6.75 (2H, s, 2× 3-H), 2× 4.49–4.32 (2H, m, 2× OCH), 1.70 (3H, d, J 17.6, P-CH_3), 1.69 (3H, d, J 17.6, P-CH_3), 1.35 (3H, d, J 6.3, CH_3), 1.21 (3H, d, J 6.5, CH_3), 0.95 (9H, s, $C(CH_3)_3$), 0.90 (9H, s, $C(CH_3)_3$)	158.6 (s, 2× C═O), 155.4 (s, 2× 9-C), 154.5 (d, J 7.5, 7-C), 154.3 (d, J 8.1, 7-C), 141.2 (q, J 33.3, 2× 4-C), 126.6 (s, 2× 5-C), 118.0 (d, J 4.6, 6-C), 117.9 (d, J 4.6, 6-C), 121.4 (q, J 275.4, 2× CF_3), 114.8 (q, J 5.5, 3-C), 114.7 (q, J 5.5, 3-C), 110.4 (s, 10-C), 110.3 (s, 10-C), 109.7 (d, J 4.6, 8-C), 109.5 (d, J 5.2, 8-C), 83.3 (d, J 7.5, OCH), 82.8 (d, J 8.1, OCH), 35.0 (s, $C(CH_3)_3$), 34.9 (s, $C(CH_3)_3$), 25.5 (s, 2× $C(CH_3)_3$), 17.0 (s, 2× CH_3), 12.8 (d, J 146.0, P-CH_3), 11.9 (d, J 147.1, P-CH_3)	+28.5 and 28.4[b]

Table 3 (*Continued*)

Compound	Structure[a]	^{1}H NMR δ, *J* (Hz)	^{13}C NMR δ, *J* (Hz)	^{31}P NMR δ
7c (mp 51–52 °C)		7.71 (dd, *J* 9.0 and 1.3, 5-H), 7.35–7.23 (2H, m, 6-H and 8-H), 6.75 (1H, s, 3-H), 3.97 (1H, ddd, *J* 9.8, 7.4 and 6.6, OCH_ACH_B), 3.84 (1H, dt, *J* 9.8, 6.6, OCH_ACH_B), 1.87–2.06 (1H, m, CH), 1.72 (3H, d, *J* 17.7, P-CH_3), 0.95 (6H, d, *J* 6.7, CH_3)	158.5 (s, C=O), 155.4 (s, 9-C), 154.2 (d, *J* 7.5, 7-C), 141.1 (q, *J* 32.9, 4-C), 126.7 (s, 5-C), 121.4 (q, *J* 275.2, CF_3), 117.9 (d, *J* 4.6, 6-C), 114.8 (q, *J* 5.6, 3-C), 110.5 (s 10-C), 109.5 (d, *J* 5.2, 8-C), 72.8 (d, *J* 6.9, OCH_2), 29.1 (d, *J* 6.3, CH), 18.6 (s, 2× CH_3), 11.5 (d, *J* 146.0, P-CH_3)	+29.0
8c (mp 53–54 °C)		7.71 (1H, dd, *J* 8.6 and 1.7, 5-H), 7.33–7.29 (1H, m, 8-H), 7.31–7.25 (1H, m, 6-H), 6.75 (1H, s, 3-H), 4.65–4.49 (1H, m, c.hexyl OCH), 2.05–1.83 (2H, m, c.hexyl), 1.70 (3H, d, *J* 17.7, CH_3), 1.82–1.62 (2H, m, c.hexyl), 1.62–1.44 (3H, m, c.hexyl), 1.43–1.17 (3H, m, c.hexyl)	158.6 (s, C=O), 155.4 (s, 9-C), 154.2 (d, *J* 8.1, 7-C), 141.1 (q, *J* 33.1, 4-C), 126.6 (s, 5-C), 121.4 (q, *J* 275.2, CF_3), 117.9 (d, *J* 4.6, 6-C), 114.9 (q, *J* 5.8, 3-C), 110.4 (s, 10-C), 109.5 (d, *J* 4.6, 8-C), 77.2 (d masked, c.hexyl OCH), 33.7 (d, *J* 4.0, c.hexyl), 33.6 (d, *J* 3.5, c.hexyl), 24.9 (s, c.hexyl), 23.5 (2C, s, c.hexyl), 12.5 (d, *J* 146.0, P-CH_3)	+27.9

[a] The CF_3 group appeared as a singlet with a chemical shift of −64.7 ppm in the ^{19}F NMR spectra.
[b] Diastereoisomeric pair.

(15–20 min) and then equilibrated at 5% B (20–25 min). Samples were dissolved in methanol and injected (10 μl) at a concentration of 10 μg ml^{-1}.

4.2.3. MS conditions

MS analysis was performed using a Finnigan Deca XP+ ion trap MS system fitted with an atmospheric pressure ionization (API) interface, operated in positive APCI mode. The discharge current was set at 2 μA, capillary temperature at 150 °C and vaporizer at 400 °C. The sheath gas flow was maintained at 60 (arbitrary units) and the auxiliary gas at 20 (arbitrary units). Mass spectra were collected over a scan range of *m/z* 50–500 with source CID set between 15 and 20 V. MS^2 product ion spectra of protonated molecular ions (±2 *m/z*) were obtained with the ion trap collision energy set between 25 and 35%, activation *Q* at 0.25, and the activation time at 60 ms.

4.3. Measurement of AChE k_i values

4.3.1. Materials

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). Deionised water was used to make the solutions and for washing the reaction vessel, electrode, stirrer and delivery tubes.

4.3.2. 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.

4.3.3. Method

AChE was dissolved in 0.1 M NaCl-containing pH 7.4 phosphate buffer (5×10^{-3} M) to a specific activity of 5 μM units ml^{-1}. The solution was stored in the refrigerator when not in use. AChI solutions (100 and 10 mM) and standard NaOH were made up 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 ml NaCl solution (0.1 M) and 0.5 ml AChE solution (5 μM units ml^{-1}). AChI (0.10 ml 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

Table 4
LC–MS data for $R^1R^2P(X)O$-coumarins **1b–8b** and **1c–8c** and $R^1R^2P(X)NH$-coumarins **9** and **10**, including *m/z* ratios with relative abundance in brackets

Compound	R^1	R^2	X	M_W	$[MH]^+$	$[M + NH_4]^+$	$[MH - R]^+$	$[MH - O_2R^1R^2PX]^+$
1b[a] (R_T 19.44 ± 0.05)	EtO	EtO	S	328	329 (100), 330 (16), 331 (6), 332 (1)	346 (9), 347 (1)	301 (MS^2 only)	–
2b (R_T 17.55 ± 0.05)	EtO	EtO	O	312	313 (100), 314 (15)	330 (19), 331 (2)	285 (MS^2 only)	177 (2)
3b (R_T 17.48 ± 0.05)	EtO	Me_2N	O	311	312 (100), 313 (15)	329 (23), 330 (3)	284 (MS^2 only)	177 (1)
4b (R_T 16.62 ± 0.05)	EtO	Me	O	282	283 (100), 284 (14)	300 (41), 301 (6)	255 (MS^2 only)	177 (6), 178 (1)
5b (R_T 17.29 ± 0.05)	*i*-PrO	Me	O	296	297 (100), 298 (15)	314 (37), 315 (5)	255 (3)	177 (7), 178 (1)
6b (R_T 19.02 ± 0.05)	PinO	Me	O	338	339 (100), 340 (18)	356 (42), 357 (6)	255 (17), 256 (2)	177 (11), 178 (1)
7b (R_T 18.04 ± 0.05)	*i*-BuO	Me	O	310	311 (100), 312 (14)	328 (27), 329 (4)	255 (1)	177 (3), 178 (1)
8b (R_T 18.77 ± 0.05)	CyO	Me	O	336	337 (100), 338 (17)	354 (32), 355 (5)	255 (8), 256 (1)	177 (8), 178 (1)
1c[a] (R_T 21.32 ± 0.05)	EtO	EtO	S	382	383 (65), 384 (9), 385 (4), 386 (1)	400 (100), 401 (13)	355 (MS^2 only)	–
2c (R_T 18.83 ± 0.05)	EtO	EtO	O	366	367 (57), 368 (8)	384 (100), 385 (14)	339 (MS^2 only)	–
3c (R_T 18.88 ± 0.05)	EtO	Me_2N	O	365	366 (74), 367 (10)	383 (100), 384 (13)	338 (MS^2 only)	–
4c (R_T 18.12 ± 0.05)	EtO	Me	O	336	337 (32), 338 (9)	354 (100), 355 (12)	309 (MS^2 only)	–
5c (R_T 18.69 ± 0.05)	*i*-PrO	Me	O	350	351 (100), 352 (14)	368 (61), 369 (8)	309 (13), 310 (2)	–
6c (R_T 20.34 ± 0.05)	PinO	Me	O	392	393 (11), 394 (2)	410 (100), 411 (14)	309 (14), 310 (2)	–
7c (R_T 19.34 ± 0.05)	*i*-BuO	Me	O	364	365 (44), 366 (6)	382 (100), 383 (12)	309 (2)	–
8c (R_T 20.11 ± 0.05)	CyO	Me	O	390	391 (39), 392 (7)	408 (100), 409 (14)	309 (5), 310 (1)	–
9 (R_T 16.20 ± 0.05)	EtO	Me	O	281	282 (100), 283 (14)	299 (72), 300 (8)	254 (MS^2 only)	176 (15), 177 (2)
10 (R_T 17.65 ± 0.05)	*i*-BuO	Me	O	309	310 (100), 311 (17)	327 (64), 328 (8)	254 (MS^2 only)	176 (24), 177 (3)

R_T = retention time in min; Pin = pinacolyl; ^{12}C and ^{13}C isotopes of major *m/z* are given; relative abundances <1 not included; fragments only observed in MS^2 are marked (MS^2 only); – denotes no fragment detected; [a]Denotes sulfur-containing compound (^{32}S and ^{34}S isotopes given).

1 min, after which the appropriate phosphorus compound in solution (0.01–0.1 ml)[4] was added. Four different inhibitor concentrations were investigated.

4.4. Synthesis of analogues (*1b–8b*) and (*1c–8c*)

A solution of the appropriate 7-hydroxycoumarin (10 mmol) in THF (30 ml) was added via cannula to a stirred suspension of sodium hydride (0.48 g, 20 mmol) in THF (20 ml). 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 (10 mmol) in THF (5 ml) was added dropwise via cannula to the 7-hydroxycoumarin sodium salt suspension stirred at 0–5 °C. After 12 h, chloroform (55 ml) and water (55 ml) were added. The mixture was stirred and the two phases separated. The organic layer was washed with aqueous K_2CO_3 solution (55 ml, 0.15 mol dm^{-3}), separated and dried ($MgSO_4$). The drying agent was filtered off and the filtrate concentrated. Column chromatography on silica gel, eluting with 1:1 hexane–ethyl acetate (7:3 hexane–ethyl acetate in the case of **7c**), gave the fluorogenic analogues in 98% purity as colourless liquids or white solids. Products were characterised by multinuclear NMR spectroscopy (Tables 2 and 3) and by LC–MS^n experiments (Table 4).

[4] The compounds investigated are solids that are not very soluble in propan-2-ol, the solvent usually used in this procedure. Approximately 50% of the final solution volume of acetone was used to dissolve the material, which was then made up using either ethanol or propan-2-ol.

4.5. O-Ethyl N-(4-methyl-2-oxo-2H-chromen-7-yl)methylphosphonamidate (*9*)

A solution of 7-amino-4-methylcoumarin (0.5 g, 2.85 mmol) and triethylamine (0.4 ml, 2.86 mmol) in THF (50 ml) was added dropwise to a stirred solution of ethyl methylphosphonochloridate (0.41 g, 2.86 mmol) in THF (10 ml). A catalytic amount of DMAP (50 mg) was added and the mixture was heated under reflux for 7 h under argon. The mixture was allowed to cool to room temperature. It was concentrated, dissolved in CH_2Cl_2 (100 ml) and washed with aqueous $NaHCO_3$ (20 ml, 10%, w/v). The organic layer was separated and dried ($MgSO_4$). Filtration of the drying agent and evaporation of solvent from the filtrate gave the crude product. Chromatography on silica gel, eluting with 98:2 then 95:5 CH_2Cl_2–isopropanol, gave the title compound as a glassy syrup (0.2 g, 25%). δ_H (300 MHz) 7.48 (1H, d, $^4J_{HH}$ 8.5, 8-H), 6.98–6.92 (3H, m, NH, 5-H and 6-H), 6.15 (1H, q, $^4J_{HH}$ 1.2, 3-H), 4.21 (1H, ddq, $^3J_{PH}$ 10.1, $^2J_{HH}$ 7.7 and $^3J_{HH}$ 7.2, CH_AH_B), 4.01 (1H, ddq, $^3J_{PH}$ 10.1, $^2J_{HH}$ 7.7 and $^3J_{HH}$ 7.2, CH_AH_B), 2.40 (3H, d, $^4J_{HH}$ 1.2, 4-CH_3), 1.70 (3H, d, $^2J_{HP}$ 16.9, P-CH_3), 1.36 (3H, t, $^3J_{HH}$ 7.2, CH_3). δ_C (75 MHz) 161.2 (C=O), 155.1 (9-C), 152.4 (4-C), 144.3 (6-C), 125.9 (8-C), 114.0 (d, $^2J_{PC}$ 6.9, 7-C), 113.9 (10-C), 112.2 (3-C), 103.8 (d, $^4J_{PC}$ 5.2, 5-C), 60.6 (d, $^2J_{PC}$ 6.9, OCH_2), 18.6 (4-CH_3), 16.1 (d, $^3J_{PC}$ 6.9, CH_3), 12.5 (d, $^1J_{PC}$ 133.2, P-CH_3). δ_P 28.2. CI-MS *m/z* (rel. abundance) = 282 (M + 1, 60%).

4.6. O-Isobutyl N-(4-methyl-2-oxo-2H-chromen-7-yl) methylphosphonamidate (*10*)

A solution of 7-amino-4-methylcoumarin (0.43 g, 2.45 mmol) and triethylamine (0.38 ml, 2.76 mmol) in THF (60 ml) was added dropwise to a stirred solution of isobutyl

methylphosphonochloridate (0.47 g, 2.76 mmol) in THF (10 ml). A catalytic amount of DMAP (30 mg) was added and the mixture was heated under reflux for 9 h under argon. The mixture was allowed to cool to room temperature. It was diluted with water, extracted with CH_2Cl_2 (100 ml) and the organic extracts were combined and dried ($MgSO_4$). Filtration and concentration of the filtrate gave crude product (85% pure by ^{31}P NMR and GC–MS). Chromatography on silica gel, eluting with ethyl acetate, gave the title compound as a glassy syrup (0.22 g, 29%). δ_H (500 MHz) 8.09 (1H, d, $^2J_{PH}$ 5.7, NH), 7.48 (1H, d, $^4J_{HP}$ 8.4, 8-H), 7.03–7.00 (2H, m, 5-H and 6-H), 6.14 (1H, q, $^4J_{HH}$ 1.2, 3-H), 3.93 (1H, dt, $^3J_{HP}$ 9.7 and $^3J_{HH}$ 6.7, CH_AH_B), 3.65 (1H, dt, $^3J_{PH}$ 9.7 and $^3J_{HH}$ 6.7, CH_AH_B), 2.40 (3H, d, $^4J_{HH}$ 1.2, 4-CH_3), 1.98 (1H, m, $CHMe_2$), 1.73 (3H, d, $^2J_{HP}$ 16.8, P-CH_3), 0.96 (3H, d, $^3J_{HH}$ 6.7, CH_3), 0.95 (3H, d, $^3J_{HH}$ 6.7, CH_3). δ_C (125 MHz) 161.2 (C=O), 154.9 (9-C), 152.5 (4-C), 144.7 (6-C), 125.6 (8-C), 114.0 (d, $^2J_{PC}$ 7.7, 7-C), 113.6 (10-C), 111.9 (3-C), 103.6 (d, $^4J_{PC}$ 4.8, 5-C), 70.2 (d, $^2J_{PC}$ 6.7, CH_2), 28.8 (d, $^3J_{PC}$ 6.7, $CHMe_2$), 18.7 (2× CH_3), 18.5 (4-CH_3), 12.2 (d, $^1J_{PC}$ 132.9, P-CH_3). δ_P 28.9. CI-MS *m/z* (rel. abundance) = 310 ($M + 1$, 52%).

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