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Original article

Synthesis and biological evaluation of phosphorylated flavonoids as potent and selective inhibitors of cholesterol esterase

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ABSTRACT

A series of phosphorylated flavonoids were synthesized and investigated in vitro as inhibitors of pancreatic cholesterol esterase (CEase) and acetylcholinesterase (AChE). The results showed that most of the synthesized compounds exhibited nanomolar potency against CEase, much better than the parent flavonoids. Furthermore, these phosphorylated flavonoids demonstrated good to high selectivity for CEase over AChE, which only showed micromolar potency inhibition of AChE. The most selective and potent inhibitor of CEase (**3e**) had IC_{50} value of 0.72 nM and 11800-fold selectivity for CEase over AChE. The structure–activity relationships revealed that the free hydroxyl group at position 5 and phosphate group at position 7 of the phosphorylated flavonoids are favorable to the inhibition of CEase. The inhibition mechanism and kinetic characterization studies indicated that they are irreversible competitive inhibitors of CEase.

1. Introduction

Pancreatic cholesterol esterase (CEase; EC 3.1.1.13) is an important serine hydrolase that plays significant roles in the absorption of dietary cholesterol. Inhibition of CEase has attracted much attention in the last decades as a potential approach to treat hypocholesterolemia and atherosclerosis by limiting the bioavailability of dietary cholesterol [1]. Several classes of potent CEase inhibitors have been developed [2], including 6-chloro-2-pyrones [3], thieno [1,3]-oxazin-4-ones [1,4], carbamates [5], aryl phosphates and phosphonates [6], chloroisocoumarins [7], phosphaisocoumarins [8], and thiazolidinediones [9] (Fig. 1). However, most of these inhibitors are not highly selective and they could also inhibit other serine hydrolases, such as acetylcholinesterase (AChE; EC 3.1.1.7), butyrylcholinesterase (BChE, EC 3.1.1.8), *Pseudomonas* species lipase (PSL, EC 3.1.1.3), chymotrpsin (CT, EC 3.4.21.1) and trypsin (EC 3.4.21.4) [1,3,5d,5f,5h]. One main reason for the poor selectivity is that all serine hydrolases as well as serine proteases share the similar catalytic triad of Ser-His-Asp (Glu) and mechanism of acylation–deacylation [10]. Therefore, these enzymes usually can be inhibited by the same class of compounds and it is challenging to improve the selectivity when developing inhibitors of them.

Nevertheless, some selective inhibitors of certain serine hydrolase have been designed and developed. For example, acetylcholinesterase (AChE) is one of the most studied serine hydrolases as a viable target to treat Alzheimer's disease (AD) and many high selective inhibitors of AChE with nanomolar to picomolar potency have been identified, three of which have been used clinically to treat AD [11]. In contrast, only a few selective inhibitors of CEase were reported. In 1999, Deck's group [3a] found that 6-chloro-3-(1-ethyl-2-cyclohexyl)-2-pyranone could effectively inhibit CEase with a *Ki* value of 25 nM and its selectivity for CEase over chymotrypsin was greater than 1000-fold. Recently, Gütschow and Pietsch developed two CEase inhibitors with micromolar potency and medium selectivity for CEase over AChE; one is a 1,3-oxazin-4-one [1], and the other is a thiazolidinedione [9]. Apparently, it is still desirable to develop novel inhibitors of CEase with high potency and high selectivity.

Flavonoids, including flavones, flavonols, isoflavones and flavanones, are a large class of polyphenolic compounds widely distributed in herbs and foods of plant origin, and they exhibit diversified biological activities, such as antioxidant, antiviral, anticancer and enzyme inhibition [12]. Recently, we prepared five fully phosphorylated flavones and found that they displayed excellent

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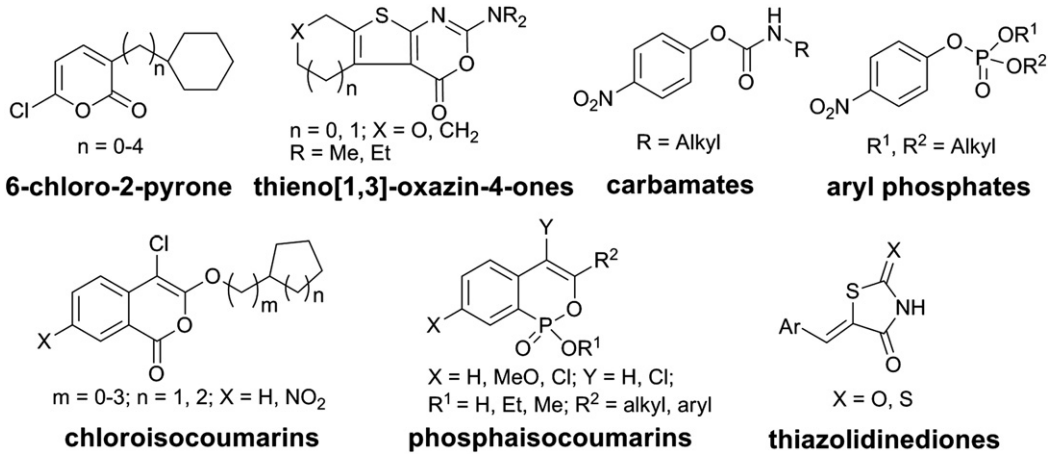


Fig. 1. Structures of some reported inhibitors of CEase.

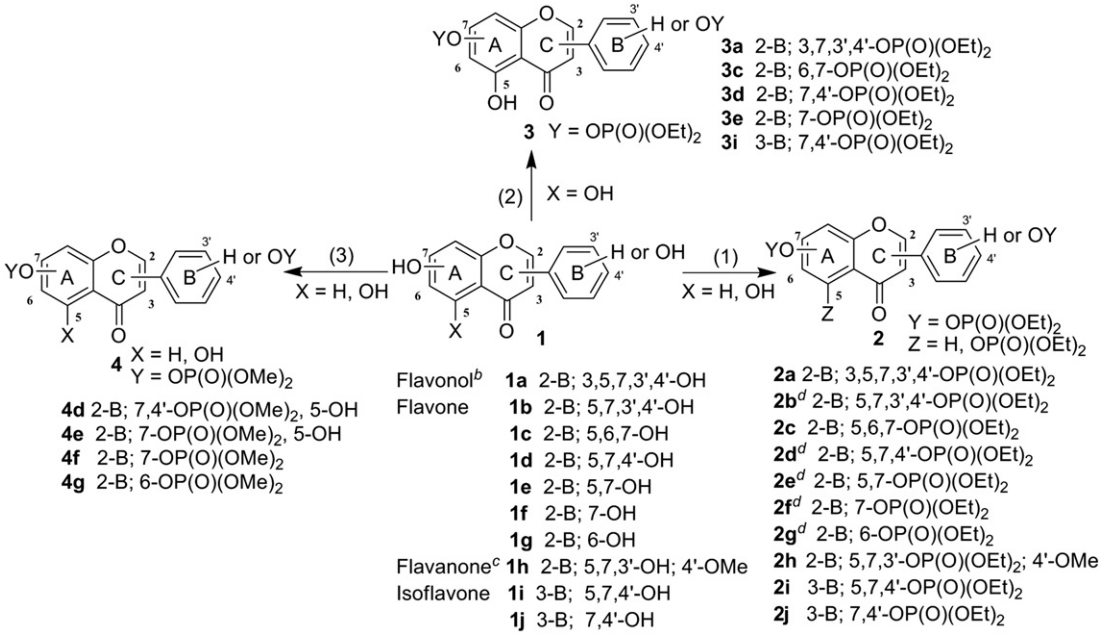
CEase inhibitory activities with IC_{50} values in the nanomolar range [13]. This work indicated that we may develop inhibitors of CEase with better activities by investigating more kinds of phosphorylated flavonoids. Furthermore, aryl phosphates, especially those with good leaving groups, such as diethyl 4-nitrophenyl phosphate (paraoxon), are well-known irreversible inhibitors of many serine hydrolases and proteases and widely used as insecticides [6a,14]. Taking into account that many phosphate insecticides are toxic and the neurotoxicity of them are mainly associated with their AChE inhibition [14], we think it necessary to examine whether these flavonoid phosphate derivatives can selectively inhibit CEase over AChE so as to preliminarily know about their application prospect.

In this paper, we presented a series of fully and partially phosphorylated flavonoids as inhibitors of CEase with low nanomolar potency and high selectivity for porcine CEase over AChE from Electrophorus electricus. The highest selectivity reached to over 10000-fold. Besides, we examined the inhibition mechanism and kinetics, the results indicated that they are irreversible competitive inhibitors of CEase.

2. Results and discussion

2.1. Chemistry

The synthesis of the phosphorylated flavonoids **2**–**4** was outlined in Scheme 1. According to our previous procedure [13], the fully phosphorylated flavonoids **2a**–**2j** could be obtained by the treatment of **1a**–**1j** with $ClP(O)(OEt)_2$, Et_3N and 4-dimethylamiopryidine (DMAP) in THF at room temperature for 24 h. To accelerate the reaction process, we carried out these reactions under reflux conditions, and found that all the reactions completed within 3 h (condition (1), Scheme 1). During these



[a] Reagents and conditions: (1) DMAP, Et_3N, $ClP(O)(OEt)_2$, reflux, 3 h; (2) $HP(O)(OEt)_2$, Et_3N, CCl_4, room temperature, 24 h. (3) $HP(O)(OMe)_2$, Et_3N, CCl_4, room temperature, 24 h. [b] 2-B means the B ring is linked to position 2, and 3-B means the B ring is linked to position 3. [c] For flavanones **1h** and **2h**, the bond between position 2 and 3 is single bond instead of double bond. [d] These compounds have been reported in Ref. 13.

Scheme 1. General synthetic procedure for the preparation of compounds **2**, **3** and **4**.[a].

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courses, we noticed that for those parent flavonoids with a 5-hydroxy group, the fully phosphorylated products **2** were produced at first, but they would gradually dephosphorylate the 5-phosphate to give the corresponding compounds **3**. Compounds **3** became the main products when the reaction time was prolonged to over 4 h in refluxing THF or when they were purified through column chromatography using regular silica gel. This result can be explained by the formation of the hydrogen bond between the 5-hydroxy group and the nearby carbonyl group at position 4, which makes compounds **3** more stable [15]. To get compounds **2** with high purity and avoid the undesired dephosphorylation reaction, it is important to control the reaction time within 3 h under reflux conditions and purify them through column chromatography on deactivated silica gel [16]. Under these control conditions, the transformation of compounds **2** into compounds **3** was negligible, and compounds **2** could be obtained in pure and good yields (see Experimental section). For example, the isolated yield of **2c** was 73% when using deactivated silica gel, while only a mixture of **2c** and **3c** were obtained when using the regular silica gel.

For the preparation of the phosphorylated flavonoids **3** with a 5-hydroxy group, the above procedure is not convenient and effective. Compounds **3** could be synthesized readily by the reaction of compounds **1** and $HP(O)(OEt)_2$ using the classic Atherton–Todd reaction [17] (condition (2), Scheme 1). Under these conditions, the hydroxy group at position 5 is stable enough to resist the phosphorylation reaction.

Several methyl phosphorylated compounds **4** were prepared under condition (3) (Scheme 1). As $ClP(O)(OMe)_2$ is not easily commercially available, we chose $HP(O)(OMe)_2$ as the phosphorylation reagent. In this case, no matter using DMAP as catalyst or increasing the equivalent of $HP(O)(OMe)_2$, the 5-OH could not be phosphorylated. Besides, when the starting material having more than three hydroxyl groups, the reaction did not lead to the desired product but to an unknown compound with high polarity ($R_f = 0$, CH_2Cl_2:MeOH = 10:1) according to TLC. In the end, four methyl phosphorylated compounds **4** were synthesized under typical Atherton–Todd reaction conditions.

2.2. Biology

2.2.1. Structure–activity relationship (SAR) studies

The inhibition of CEase by compounds **1**–**4** was determined spectrophotometrically by the Hosie assay [18] with some modification as described before [6a,13]. The inhibition of AChE was assayed according to the Ellman et al. method [19] and other literatures [6a,20]. The IC_{50} values of all compounds are summarized in Table 1. Based on the data shown in Table 1, the preliminary structure–activity relationships could be summarized as follows.

(1) Phosphorylation of the phenolic groups of flavonoids significantly improved their inhibitory potency against CEase. The parent flavonoids **1** showed weak or no activities on CEase. However, all tested derivatives (**2**–**4**) except **4g** exhibited good to excellent inhibitory activities against CEase, and seven of them had IC50 values below 5.0 nM. For example, **3d** showed an IC_{50} value of 4.18 nM toward CEase that was 7223-fold better than that of the parent compound **1d** ($IC_{50} = 35.7$ μM). Among all compounds examined, **3e** was the best CEase inhibitor ($IC_{50} = 0.72$ nM). The above results may be attributed to the facts that these derivatives have better lipophilicity than the parent compounds [1,21] and the tetrahedral phosphates can mimic the transition-states of the hydrolysis of cholesterol esters [22].

(2) Comparing inhibition of CEase and AChE, all phosphate derivatives examined only showed micromolar inhibition of AChE and most of them demonstrated good to high selectivity for CEase over AChE. Compounds **2e**, **3e**, **2f** and **3i** were 1157-, 11800-, 873-, and 1735-fold more efficient for inhibition of CEase than that of AChE (see Table 1). The possible reasons for the superior inhibition of CEase are as follows. On one hand, the catalytic triad of AChE is located at the bottom of a deep and narrow cavity [23], while CEase has a larger active site binding pocket than AChE [24]. The phosphorylated flavonoids are relatively bulky that makes them easier to enter the larger active site pocket of CEase. On the other hand, these phosphate derivatives are not good electrophiles with good leaving groups, and the nucleophilicity of AChE is less than that of CEase according to Lin and coworkers' research [5d].

(3) Ethyl phosphorylated flavonoids were more active than the methyl derivatives in inhibiting CEase. For example, compounds **2f**, **3d** and **3e** have IC_{50} values of 2.44 nM, 3.18 nM and 0.72 nM against CEase, being 283-, 65-, and 236-fold more potent than the methyl counterparts **4f**, **4d** and **4e.** This is probably due to the fact that the ethyl phosphates have better lipophilicity than the methyl ones.

(4) Phosphorylated isoflavones inhibited CEase more effectively than the phosphorylated flavones. For example, the only difference between **2d** and **2i**, **3d** and **3i** is the B benzene ring at position 2 or 3. The isoflavone derivatives **3i** and **2i** were 2- to 6-fold more active than the flavone derivatives **3d** and **2d** (**3i** vs **3d**, **2i** vs **2d**).

(5) The position of the phosphate group has much effect on the inhibitory activities and the presence of the 7-phosphate and 5-OH is beneficial to the inhibition of CEase. From Table 1, we can see that all potent inhibitors of CEase ($IC_{50} < 5.0$ nM) have a 7-phosphate group, indicating that the introduction of a phosphate group at position 7 is important to the high inhibition of CEase. Besides, compounds with a 5-OH were

Table 1
Inhibition of CEase and AChE by flavonoids and the phosphorylated derivatives.

Compound	IC_{50} (nM)		Selectivity for CEase over AChE (fold)
	CEase	AChE	
1a	4720 ± 110	58200 ± 500	12.3
2a	40.4 ± 2.2	3380 ± 600	83.7
3a	36.7 ± 1.9	Ni[a]	–
1b	28100[b]	Ni	–
2b	3.89[b]	170 ± 40	43.7
1c	12300 ± 800	62100 ± 1000	5.05
2c	1340 ± 150	Ni	–
3c	430 ± 50	Ni	–
1d	35700[b]	Ni	–
2d	26.1[b]	280 ± 60	10.7
3d	4.18 ± 0.15	970 ± 20	232
4d	270 ± 60	6600 ± 450	24.4
1e	Ni[b]	Ni[b]	Ni
2e	3.76[b]	4350 ± 120	1160
3e	0.72 ± 0.06	8520 ± 1010	11800
4e	170 ± 30	6730 ± 240	39.6
1f	Ni[b]	Ni	
2f	2.44[b]	2130 ± 670	873
4f	690 ± 120	4190 ± 240	6.07
1g	Ni[b]	Ni	–
2g	390[b]	12600 ± 2900	32.3
4g	Ni	Ni	–
1h	Ni	Ni	–
2h	20.6 ± 0.4	2940 ± 290	143
1i	20900 ± 1600	Ni	–
2i	4.62 ± 0.56	1260 ± 20	273
3i	1.85 ± 0.01	3210 ± 190	1735
1j	Ni	Ni	–
2j	25.7 ± 1.4	7650 ± 240	298

[a] Ni, no inhibition at 100 μM.
[b] Data taken from Ref. [13].

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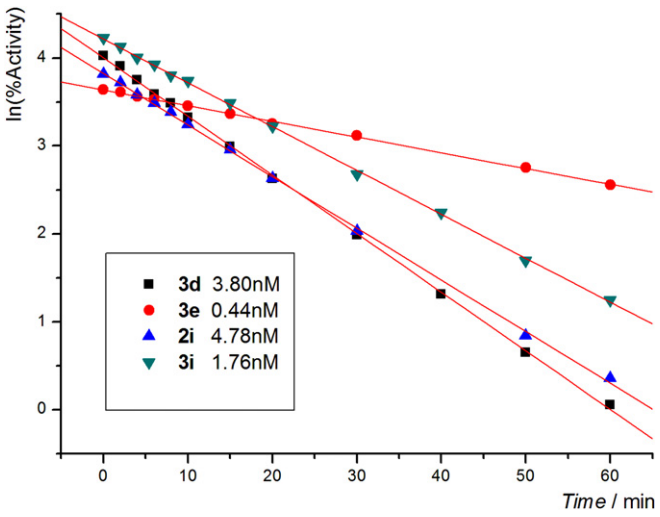

Fig. 2. Time-dependent inhibition of CEase by compounds **3d**, **3e**, **2i** and **3i**. CEase was preincubated with each inhibitor. At various incubation time intervals, the substrate (3.0 mg/mL, 5 μL) was added to the incubation mixture, and the remain CEase activity was immediately assayed. In the absence of inhibitor, no deactivation of enzyme was observed during the time scale shown in the plot.

generally about 2- to 6-fold more active than their counterparts with a 5-phosphate (**2c** vs **3c**, **2d** vs **3d**, **2e** vs **3e**, **2i** vs **3i**). On the contrary, the presence of a phosphate group at position 6 or 3 has little or adverse effect on the inhibition of CEase (**2c** vs **2e**, **2a** vs **2b**, **3c** vs **3e**).

2.2.2. *Mechanism and kinetic characterization*

To determine whether phosphorylated flavonoids could produce irreversible inhibition of CEase or not, four most potent inhibitors were preincubated with the enzyme and the residual activities of CEase were measured according to the literature protocol [25]. As indicated in Fig. 2, **3d**, **3e**, **2i** and **3i** (all at nanomolar concentration) inactivated CEase progressively; the enzyme activity decreased as the preincubation time increased. The four compounds were all characterized as active-site irreversible inhibitors of CEase since they were time-dependent and followed the first-order kinetics over the observed time period, which met some criteria proposed by Abeles and Maycock [26]. In addition, no enzyme activity was recovered after dialysis of the assay solution, which further proved that they are irreversible inhibitors.

The kinetic behavior of compounds **3d** and **3e** on the CEase (Fig. 3) were then determined from Lineweaver–Burk double reciprocal plots [25]. The results demonstrated that on a short time scale, they were competitive inhibitors of CEase. The Michaelis–Menten constant K_m value was calculated to be 130 ± 10 μM from the intercept of the *X*-axis when [I = 0] of the plots in Fig. 3, which is in agreement with the literature results [5b,5c]. The inhibition constant K_i values (calculated from the plots) for the inhibition of CEase by compounds **3d** and **3e** were 3.81 ± 0.33 nM and 0.40 ± 0.03 nM, respectively. These kinetic results are not surprising. Tsou and Tian [27] have systematically investigated the kinetics of irreversible inhibition of enzymes. They found that competitive, noncompetitive, and uncompetitive inhibitions could also be applied to irreversible inhibition and diisopropyl fluorophosphate was as an irreversible competitive inhibitor of α-chymotrypsin.

Carbamates [5d], aryl phosphates and phosphonates [14c,22] are well known irreversible inhibitors of many serine hydrolases and proteases, their inhibition usually includes reversible binding with the enzyme and irreversible carbamylation or phosphorylation of the active site. Similarly, the inhibition of CEase by the phosphorylated flavonoids might involve reversible binding the inhibitors with the enzyme followed by phosphorylation of the active-site serine to give phosphorylated enzyme irreversibly (Fig. 4).

3. Conclusions

In conclusion, a series of phosphorylated flavonoids were synthesized and identified as potent and selective inhibitors of CEase. Most of the synthesized compounds showed nanomolar inhibitory potency toward CEase and only micromolar inhibition of AChE, indicating good to high selectivity between the two enzymes. The inhibition mechanism and kinetic characterization studies suggested that they inactivate CEase in an irreversible competitive manner.

Among all compounds examined, **3e** was the best inhibitor of CEase with IC_{50} as low as 0.72 nM. The structure–activity relationships indicated that the 7-phosphate and 5-OH of the phosphorylated flavonoids are favorable to the inhibition of CEase. Moreover, ethyl phosphorylated flavonoids were more active than the methyl ones, and the phosphorylated isoflavones exhibited better inhibition activity on CEase.

The present work suggested that the phosphorylated flavonoids with excellent inhibitory activity and good selectivity for CEase

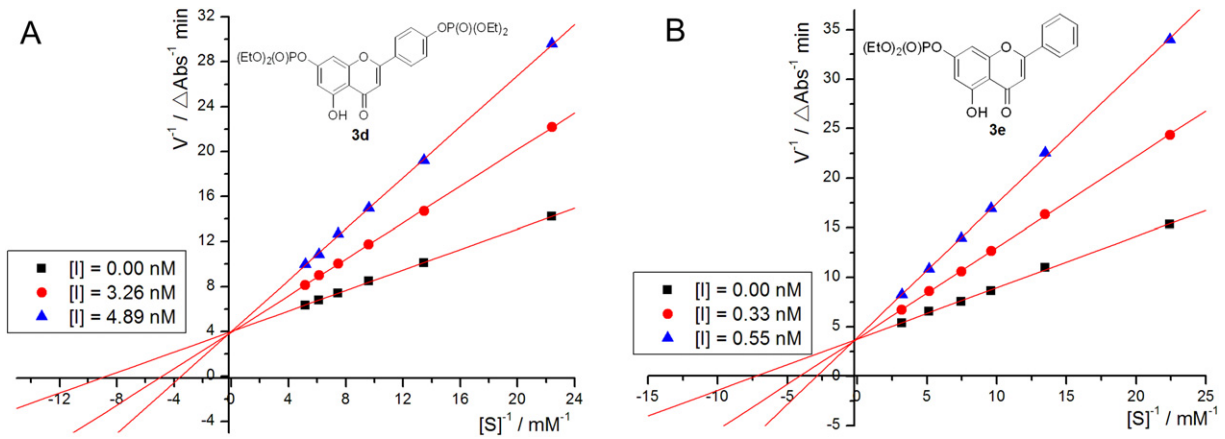

Fig. 3. Lineweaver–Burk plots for inhibition of CEase by **3d** and **3e** with *p*-nitrophenylbutyrate (*p*NPB) as the substrate. (A) and (B) represent kinetics of compounds **3d** and **3e**, respectively. $K_i = [I]/[K_m\,(app)/K_m - 1]$. K_m (app) determined with inhibitor and substrate and K_m with substrate alone.

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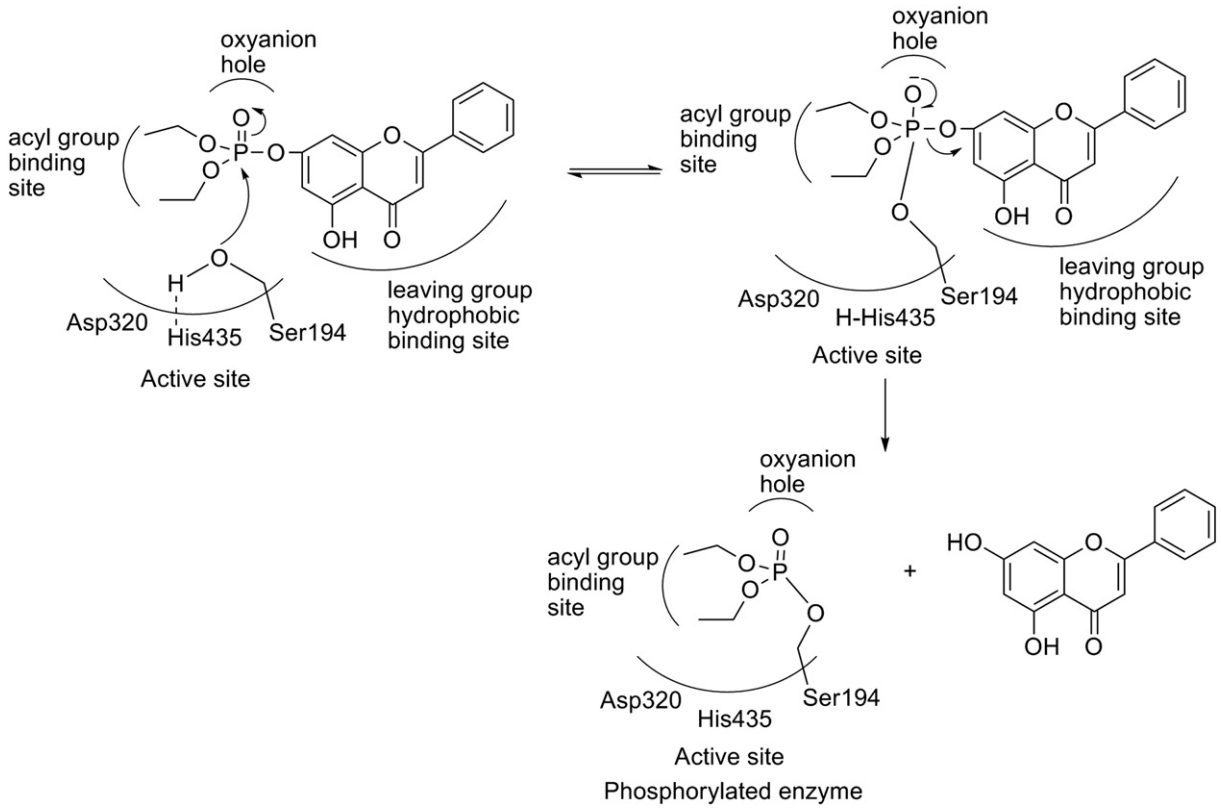

Fig. 4. Proposed mechanism of inhibition of CEase by compound **3e**.

over AChE (such as **3e**) might act as lead compounds for developing new, efficient and selective CEase inhibitors.

4. Experimental section

THF was dried according to standard procedures. Unless otherwise noted, all other reagents were obtained from commercial sources and used without further purification. NMR spectra was recorded on a Varian Mercury-Plus 300 (^{1}H 300 MHz; ^{13}C 75.4 MHz; ^{31}P 121 MHz). ESI-mass spectra was recorded on an LCMS-2010A Liquid Chromatograph mass spectrometer. IR spectra was recorded as KBr pellets on a Bruker Equinox 55FT/IR spectrometer. HRMS was determined by a Thermo MAT95XP High Resolution mass spectrometer. Melting point was measured on a SGWX-4 Micro Melting Point Apparatus and not corrected. Thin layer chromatography was conducted on Kieselgel 60 F254. The column chromatography was performed on deactivated 200–300 mesh silica gel, which was pretreated with a 0.025 M solution of KH_2PO_4 in 1:l H_2O/MeOH, filtered and oven-dried at 120 °C for 12 h [16]. Elemental analysis was determined at Vario EL Elemental Analyzer, and the results were within 0.4% of the theoretical values. The compounds without analytical purity were confirmed >95% pure via HPLC methods that was performed on Shimadzu LC-10AT. The synthesis and CEase inhibition of compounds **2b**, **2d**–**2g** had been reported previously by our group [13], and the characterization data were in agreement with the published data. Compounds **2a** and **2j** was reported in a patent by one of our authors [28] and compounds **2c**, **2h**, **2i**, **3a**, **3c**, **3d**, **3i**, **4d**–**4g** are new compounds; their synthetic procedures and spectral data are as follows. CEase (porcine) was from Worthington. AChE (Electrophorus electricus) and pNPB were from Sigma. S-acetylthiocholine iodine (ATCh) was from Alfa. Enzymatic assays were done on a Shimadzu UV-2450 scanning spectrophotometer. Origin 7.5 was used to analyze the enzyme assay data.

4.1. Chemistry

4.1.1. General procedure for the synthesis of compound *2*

Compound **1** (1.0 mmol) was added to a solution of 20 mL THF, DMAP (3.0 mmol per OH group) and Et_3N (3.0 mmol per OH group), and the mixture was stirred until dissolved. A solution of $ClP(O)(OEt)_2$ (4.0 mmol per OH group) with 5 mL THF was then added dropwise with vigorous stirring in an ice-water bath over 30 min. After stirring at 70 °C for 3 h under nitrogen, the reaction mixture was concentrated, diluted with EtOAc (3 × 30 mL), washed with 0.5 M HCl (3 × 15 mL), 5% (w/v) NaOH (3 × 15 mL) and brine, and dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by column chromatography on deactivated silica gel with petroleum ether/EtOAc (1:1–1:4), CH_2Cl_2/MeOH (100:1–60:1) as eluant to give the corresponding product **2**.

*4.1.1.1. 2-[3,4-Bis[(diethoxyphosphinyl)oxy]phenyl]-4-oxo-4H-1-benzopyran-3,5,7-triyl phosphoric acid hexaethyl ester (**2a**).* Yellow oil. Yield: 56%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (s, 1H), 7.69 (d, J = 9.5 Hz, 1H), 7.50 (d, J = 8.7 Hz, 1H), 7.20 (d, J = 10.9 Hz, 2H), 4.36–4.02 (m, 20H), 1.25 (dt, J = 39.2, 7.1 Hz, 30H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.8, 156.6, 153.8 (d, J = 5.4 Hz), 152.0 (d, J = 6.2 Hz), 150.2 (d, J = 6.0 Hz), 143.6 (t, J = 5.7 Hz), 141.0 (t, J = 7.1 Hz), 134.4 (d, J = 7.1 Hz), 126.3, 126.2, 122.0, 120.9, 113.2 (d, J = 6.1 Hz), 109.6, 105.2, 65.4–64.4 (m), 16.0 (d, J = 6.4 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ −4.02, −5.31, −5.80, −6.37, −6.77; MS (ESI): m/z: 1005 $(M + Na)^+$, 983 $(M + H)^+$. Anal. Calcd for $C_{35}H_{55}O_{22}P_5$: C, 42.78; H, 5.64. Found C, 42.50; H, 5.54; IR (film, cm^{-1}): 2986, 2924, 1661, 1478, 1386, 1282, 1160, 1029.

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*4.1.1.2. 4-Oxo-2-phenyl-4H-1-benzopyran-5,6,7-triyl phosphoric acid hexaethyl ester (**2c**).* Yellow oil. Yield: 73%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89–7.82 (m, 2H), 7.69 (s, 1H), 7.55–7.48 (m, 3H), 6.70 (s, 1H), 4.48–4.29 (m, 12H), 1.41 (t, J = 7.0 Hz, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.5, 161.6, 153.1, 146.7, 141.2, 131.9, 131.3, 130.4, 128.6, 125.7, 114.8, 107.9 (d, J = 9.1 Hz), 106.0, 65.7–64.3 (m), 15.9 (d, J = 4.5 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ −4.77 (d, J = 3.6 Hz), −5.81 (d, J = 3.6 Hz), −6.59; MS (ESI): m/z: 701 $(M + Na)^+$, 679 $(M + H)^+$. HRMS m/z calcd for $C_{27}H_{37}O_{14}P_3$ (M^+), 678.1391; found, 678.1391. IR (film, cm^{-1}): 2986, 2926, 0.1654, 1452, 1358, 1287, 1107, 1027.

*4.1.1.3. 2-[3-(Diethoxyphosphinyl)oxy]phenyl]-4-oxo-4H-1-benzopyran-5,7-diyl phosphoric acid tetraethyl ester (**2h**).* Yellow oil. Yield: 80%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (d, J = 1.9 Hz, 1H), 7.17 (d, J = 9.0 Hz, 1H), 6.95 (d, J = 8.5 Hz, 1H), 6.90 (d, J = 1.0 Hz, 1H), 6.77 (d, J = 2.3 Hz, 1H), 5.36 (dd, J = 13.2, 2.8 Hz, 1H), 4.40–4.14 (m, 12H), 3.87 (s, 3H), 3.02 (dd, J = 16.5, 13.2 Hz, 1H), 2.77 (dd, J = 16.5, 2.9 Hz, 1H), 1.42–1.32 (m, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 188.1, 163.2, 155.5 (d, J = 6.1 Hz), 151.2 (d, J = 6.0 Hz), 150.9 (d, J = 4.5 Hz), 139.8 (d, J = 6.8 Hz), 130.4, 123.4, 119.4, 112.6, 110.6 (d, J = 6.5 Hz), 106.77, 105.7 (d, J = 16.9 Hz), 78.7 (d, J = 7.2 Hz), 64.8 (dd, J = 33.5, 6.1 Hz), 56.0, 45.3, 16.5–15.8 (m); ^{31}P NMR (121 MHz, $CDCl_3$): δ −5.00, −6.38, −6.58; MS (ESI): m/z: 733 $(M + Na)^+$, 749 $(M + K)^+$, 711 $(M + H)^+$. Anal. Calcd for $C_{28}H_{41}O_{15}P_3$: C, 47.33; H, 5.82. Found C, 47.12; H, 5.67; IR (film, cm^{-1}): 2986, 2924, 1696, 1612, 1517, 1434, 1380, 1276, 1148, 1027.

*4.1.1.4. 3-[4-(Diethoxyphosphinyl)oxy]phenyl]-4-oxo-4H-1-benzopyran-5,7-diyl phosphoric acid tetraethyl ester (**2i**).* Yellow oil. Yield: 73%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.83 (s, 1H), 7.47 (d, J = 8.4 Hz, 2H), 7.27 (d, J = 1.3 Hz, 3H), 7.24 (d, J = 1.0 Hz, 1H), 4.41–4.19 (m, 12H), 1.43–1.34 (m, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.2, 157.5, 153.1 (d, J = 5.8 Hz), 151.1, 150.4–150.1 (m), 150.0, 129.9, 127.6, 125.0, 119.3, 113.9 (d, J = 6.2 Hz), 109.5, 105.1, 65.1–63.9 (m), 15.7; ^{31}P NMR (121 MHz, $CDCl_3$): δ −5.26, −6.26, −6.45; MS (ESI): m/z: 701 $(M + Na)^+$, 717 $(M + K)^+$, 679 $(M + H)^+$. HRMS m/z calcd for $C_{27}H_{37}O_{14}P_3$ (M^+), 678.1391; found, 678.1391. IR (film, cm^{-1}): 2986, 2923, 1654, 1433, 1371. 1282, 1170, 1029.

*4.1.1.5. 3-[4-(Diethoxyphosphinyl)oxy]phenyl]-4-oxo-4H-1-benzopyran-7-yl phosphoric acid diethyl ester (**2j**).* Yellow oil. Yield: 92%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.28 (d, J = 8.7 Hz, 1H), 7.98 (s, 1H), 7.54 (d, J = 8.3 Hz, 2H), 7.41 (dd, J = 2.1, 1.0 Hz, 1H), 7.32–7.26 (m, 3H), 4.34–4.18 (m, 8H), 1.39 (dd, J = 7.1, 4.8, 1.1 Hz, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.1, 156.6, 154.4, 154.3, 152.9 (d, J = 5.6 Hz), 150.5 (d, J = 6.8 Hz), 130.1, 128.1, 124.4, 121.3, 119.9, 118.0, 108.4, 64.8 (dd, J = 33.7, 6.0 Hz), 16.5–15.9 (m); ^{31}P NMR (121 MHz, $CDCl_3$): δ −5.28, −5.89; MS (ESI): m/z: 549 $(M + Na)^+$, 565 $(M + K)^+$, 527 $(M + H)^+$. HRMS m/z calcd for $C_{23}H_{28}O_{10}P_2$ (M^+), 526.1152; found, 526.1154. IR (film, cm^{-1}): 2986, 2922, 1650, 1441, 1368, 1278, 1175, 1030.

*4.1.2. General procedure for the synthesis of compound **3***

Compound **1** (1.0 mmol) was added to a solution of 20 mL THF and 5 mL Et_3N (35.6 mmol), and the mixture was stirred until dissolved. A solution of $HP(O)(OEt)_2$ (1.1 equiv. per OH; the numbers of OH were calculated one less than that of compound **1**) and 5 mL CCl_4 (35.6 mmol) was added dropwise with vigorous stirring in an ice-water bath over 30 min. The reaction proceeded for 24 h at room temperature. The resulting salt of triethylamine was filtered. The filtrate was evaporated *in vacuo*, and 10 mL of water was added. The solution was extracted with EtOAc (3 × 30 mL), washed with 1 M HCl (3 × 15 mL), saturated $NaHCO_3$ solution (3 × 15 mL) and brine, and dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by column chromatography on deactivated silica gel with petroleum ether/EtOAc (1:1–1:4), CH_2Cl_2/MeOH (100:1–60:1) as eluant to give the corresponding product **3**.

*4.1.2.1. 2-[3,4-Bis[(diethoxyphosphinyl)oxy]phenyl]-5-hydroxy-4-oxo-4H-1-benzopyran-3,7-diyl phosphoric acid tetraethyl ester (**3a**).* Yellow oil. Yield: 40%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.22 (s, 1H), 7.95 (d, J = 1.0 Hz, 1H), 7.79 (d, J = 8.7 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 6.93–6.90 (m, 1H), 6.68 (d, J = 2.1 Hz, 1H), 4.36–4.10 (m, 16H), 1.64–1.13 (m, 24H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.2, 161.6, 156.0 (d, J = 5.8 Hz), 155.6, 154.6 (d, J = 5.7 Hz), 143.9, 141.2 (t, J = 6.7 Hz), 132.4 (d, J = 7.2 Hz), 126.5, 126.2, 122.2, 121.1, 108.0, 103.5, 98.8, 65.0, 16.0; ^{31}P NMR (121 MHz, $CDCl_3$): δ −3.92, −5.27, −5.79, −6.27; MS (ESI): m/z: 845 $(M - H)^-$. Anal. Calcd for $C_{31}H_{46}O_{19}P_4$: C, 43.98; H, 5.48. Found C, 43.90; H, 5.71; IR (film, cm^{-1}): 3077, 2986, 2913, 1655, 1509, 1446, 1346, 1279, 1158, 1030.

*4.1.2.2. 5-Hydroxy-4-oxo-2-phenyl-4H-1-benzopyran-5,7-diyl phosphoric acid tetraethyl ester (**3c**).* Yellow solid. Yield: 75%. M.p.: 119–120 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 13.03 (s, 1H) 7.86 (dd, J = 7.5, 2.1 Hz, 2H), 7.69 (s, 1H), 7.557.48 (m, 3H), 6.70 (s, 1H), 4.47–4.28 (m, 8H), 1.41 (t, J = 7.0 Hz, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 182.6, 164.8, 152.9, 152.6, 148.4, 132.4, 130.6, 129.0, 126.3, 125.7 (d, J = 8.6 Hz), 108.3–108.1 (m), 105.4 (d, J = 11.3 Hz), 98.6 (d, J = 5.3 Hz), 65.1 (dd, J = 35.3, 6.4 Hz), 16.8–15.8 (m); ^{31}P NMR (121 MHz, $CDCl_3$): δ −4.28, −6.33; MS (ESI): m/z: 541 $(M - H)^-$. Anal. Calcd for $C_{23}H_{28}O_{11}P_2$: C, 50.93; H, 5.20. Found C, 51.22; H, 4.92; IR (KBr, cm^{-1}): 3081, 2989, 1620, 1454, 1360, 1290, 1168, 1053.

*4.1.2.3. 2-[4-(Diethoxyphosphinyl)oxy]phenyl]-5-hydroxy-4-oxo-4H-1-benzopyran-7-yl phosphoric acid diethyl ester (**3d**).* Yellow oil. Yield: 57%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.72 (s, 1H), 7.88 (d, J = 8.7 Hz, 2H), 7.38 (d, J = 8.3 Hz, 2H), 6.99 (d, J = 1.3 Hz, 1H), 6.67 (s, 1H), 6.64 (d, J = 1.8 Hz, 1H), 4.38–4.16 (m, 8H), 1.48–1.31 (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 182.33, 163.5, 161.9, 156.7, 155.8 (d, J = 5.7 Hz), 153.5 (d, J = 6.2 Hz), 128.0, 127.3, 120.6, 108.1, 105.7 (d, J = 11.9 Hz), 103.8 (d, J = 10.6 Hz), 98.9 (d, J = 10.2 Hz), 65.0 (dd, J = 11.1, 6.0 Hz), 16.9–15.7 (m); ^{31}P NMR (121 MHz, $CDCl_3$): δ −5.65, −6.15; MS (ESI): m/z: 541 $(M - H)^-$. HRMS m/z calcd for $C_{23}H_{28}O_{11}P_2$ (M^+), 542.1101; found, 542.1100. IR (film, cm^{-1}): 3078, 2986, 1655, 1496, 1343, 1281, 1156, 1030.

*4.1.2.4. 5-Hydroxy-4-oxo-2-phenyl-4H-1-benzopyran-7-yl phosphoric acid diethyl ester (**3e**).* Pale yellow solid. Yield: 66%. M.p.: 84–85 °C (lit [29]., 87–89 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.74 (s, 1H), 7.89 (dd, J = 7.7, 1.6 Hz, 2H), 7.55 (s, 2H), 7.53 (s, 1H), 7.00 (s, 1H), 6.72 (s, 1H), 6.66 (s, 1H), 4.34–4.22 (m, 4H), 1.41 (t, J = 7.1 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 182.5, 164.5, 162.0, 156.9, 155.9, 132.0, 130.9, 129.1, 126.3, 108.3, 106.0, 103.8 (d, J = 5.3 Hz), 99.0 (d, J = 4.3 Hz), 65.1 (d, J = 5.8 Hz), 16.2 (d, J = 6.8 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ −6.16; MS (ESI): m/z: 389 $(M - H)^-$. IR (KBr, cm^{-1}): 3081, 2984, 1623, 1495, 1346, 1285, 1150, 1027.

*4.1.2.5. 3-[4-(Diethoxyphosphinyl)oxy]phenyl]-5-hydroxy-4-oxo-4H-1-benzopyran-7-yl phosphoric acid diethyl ester (**3i**).* Yellow oil. Yield: 37%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.75 (s, 1H), 7.95 (s, 1H), 7.51 (d, J = 8.5 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 6.90 (d, J = 1.4 Hz, 1H), 6.69 (d, J = 2.1 Hz, 1H), 4.33–4.20 (m, 8H), 1.45–1.35 (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 180.5, 162.3, 156.8, 155.7 (d, J = 5.9 Hz), 153.6, 150.7 (d, J = 6.6 Hz), 130.0, 126.8, 123.2, 120.0, 108.6, 103.6, 98.7, 64.8 (dd, J = 29.9, 5.4 Hz), 16.1; ^{31}P NMR (121 MHz, $CDCl_3$): δ −5.32, −6.19; MS (ESI): m/z: 565 $(M + Na)^+$, 581 $(M + K)^+$, 543 $(M + H)^+$. HRMS m/z calcd for $C_{23}H_{28}O_{11}P_2$ (M^+), 542.1101; found, 542.1102. IR (film, cm^{-1}): 3073, 2986, 2918, 1652, 1507, 1443, 1366, 1247, 1170, 1033.

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*4.1.3. General procedure for the synthesis of compound **4***

Compound **1** (1.0 mmol) was added to a solution of 20 mL THF and 5 mL Et_3N (35.6 mmol), and the mixture was stirred until dissolved. A solution of $HP(O)(OMe)_2$ (1.1 equiv. per OH; the numbers of OH were calculated one less than that of compound **1**) and 5 mL CCl_4 (35.6 mmol) was added dropwise with vigorous stirring in an ice-water bath. The reaction proceeded for 24 h at room temperature. The resulting salt of triethylamine was filtered. The filtrate was evaporated *in vacuo*, and 10 mL of water was added. The solution was extracted with EtOAc (3 × 30 mL), washed with 1 M HCl (3 × 15 mL) until neutral, saturated $NaHCO_3$ solution (3 × 15 mL) and brine, and then dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified by column chromatography on deactivated silica gel CH_2Cl_2/MeOH (60:1–30:1) with as eluant to give the corresponding product **4**.

*4.1.3.1. Dimethyl 5-hydroxy-4-oxo-2-phenyl-4H-chromen- 4′, 7-yl phosphate (**4d**).* Pale yellow solid. Yield: 53%. M.p.: 117–119 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.68 (s, 1H), 7.86 (d, J = 8.7 Hz, 2H), 7.36 (d, J = 8.5 Hz, 2H), 6.94 (dd, J = 2.1, 0.8 Hz, 1H), 6.64 (s, 1H), 6.62 (d, J = 2.1 Hz, 1H), 3.89 (dd, J = 11.4, 2.0 Hz, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 182.3, 163.4, 162.0, 156.7, 155.6 (d, J = 6.0 Hz), 153.4 (d, J = 6.2 Hz), 128.1, 127.5, 120.5 (d, J = 4.4 Hz), 108.2, 105.8 (d, J = 3.7 Hz), 103.7, 98.9, 55.2 (t, J = 6.6 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ −3.48, −3.95; MS (ESI): m/z: 485 $(M - H)^-$. Anal. Calcd for $C_{19}H_{20}O_{11}P_2$: C, 46.93; H, 4.15. Found C, 47.06; H, 4.37; IR (KBr, cm^{-1}): 3080, 2958, 1658, 1499, 1346, 1289, 1159, 1058.

*4.1.3.2. 5-Hydroxy-4-oxo-2-phenyl-4H-1-benzopyran-7-yl phosphoric acid dimethyl ester (**4e**).* Pale yellow solid. Yield: 58%. M.p.: 112–113 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.76 (s, 1H), 7.90 (dd, J = 7.9, 1.7 Hz, 2H), 7.60–7.50 (m, 3H), 7.00 (dd, J = 2.2, 0.9 Hz, 1H), 6.74 (s, 1H), 6.66 (dd, J = 2.2, 0.7 Hz, 1H), 3.93 (d, J = 11.4 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 182.5, 164.5, 162.1, 156.9, 155.6 (d, J = 5.8 Hz), 132.1, 130.7, 129.0, 126.3, 108.3, 106.0, 103.7, 98.9, 55.2 (d, J = 6.1 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ −3.89; MS (ESI): m/z: 361 $(M - H)^-$. Anal. Calcd for $C_{17}H_{15}O_7P$: C, 56.36; H, 4.17. Found C, 56.08; H, 4.12; IR (KBr, cm^{-1}): 3077, 2962, 1621, 1493, 1345, 1293, 1144, 1026.

*4.1.3.3. 4-Oxo-2-phenyl-4H-1-benzopyran-7-yl phosphoric acid dimethyl ester (**4f**).* Pale yellow solid. Yield: 48%. M.p.: 100–101 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.20 (d, J = 8.5 Hz, 1H), 7.88 (s, 2H), 7.51 (s, 5H), 6.79 (s, 1H), 3.93 (d, J = 11.4 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.2, 163.4, 156.7, 154.2 (d, J = 6.4 Hz), 131.5, 131.2, 128.9, 127.5, 126.1, 121.0, 117.7 (d, J = 5.1 Hz), 108.8 (d, J = 4.0 Hz), 107.4, 55.2 (d, J = 6.1 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ −3.67; MS (ESI): m/z: 347 $(M + 1)^+$, 369 $(M + Na)^+$, 385 $(M + K)^+$. Anal. Calcd for $C_{17}H_{15}O_6P$: C, 58.97; H, 4.37. Found C, 58.77; H, 4.66; IR (KBr, cm^{-1}): 2959, 1644, 1446, 1371, 1286, 1154, 1048.

*4.1.3.4. 4-Oxo-2-phenyl-4H-1-benzopyran-6-yl phosphoric acid dimethyl ester (**4g**).* Pale yellow solid. Yield: 54%. M.p.: 85–87 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.95 (s, 1H), 7.92–7.85 (m, 2H), 7.64–7.55 (m, 2H), 7.54–7.47 (m, 3H), 6.79 (s, 1H), 3.90 (d, J = 11.3 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.3, 163.4, 153.0, 147.4 (d, J = 6.8 Hz), 131.6, 131.3, 128.9, 126.3 (d, J = 4.0 Hz), 126.1, 124.7, 119.8, 115.6 (d, J = 4.9 Hz), 106.9, 55.1 (d, J = 6.1 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ −3.00; MS (ESI): m/z: 347 $(M + 1)^+$, 369 $(M + Na)^+$, 385 $(M + K)^+$. Anal. Calcd for $C_{17}H_{15}O_6P$: C, 58.97; H, 4.37. Found C, 59.04; H, 4.61; IR (KBr, cm^{-1}): 2923, 1634, 1467, 1361, 1270, 1178, 1034.

4.2. Biology

4.2.1. CEase inhibition assay

CEase inhibition was assayed spectrophotometrically according to Hoise et al. [18] with some modification as previously described [6a,13]. CEase activity was measured by following the hydrolysis of the colorimetric substrate pNPB. Assay buffer was 0.1 M sodium phosphate buffer (pH 7.04, containing 0.1 M NaCl). The temperature was maintained at 25.0 ± 0.2 °C. The inhibitors and the substrate were dissolved in acetonitrile. The final concentration of acetonitrile was 1.5%. The inhibitors were pre-incubated with CEase (3.25 U/mL, 5 μL) for 15 min. The substrate (3.0 mg/mL, 5 μL) was then added to initiate the enzyme reaction (1.0 mL in a final total volume), which was monitored immediately for 1 min by measuring the absorbance at 405 nm. Each inhibitor was assayed with six different concentrations around the IC_{50} values that were roughly estimated in the first round of experiments. The measurement was performed in triplicate for each concentration and averaged before further calculation.

4.2.2. AChE inhibition assay

AChE inhibition was assayed spectrophotometrically at 37.0 ± 0.2 °C according to the method of Ellman et al. [19] and other literature [6a,20]. AChE activity was measured by following the hydrolysis of the colorimetric substrate ATCh. Assay buffer was 0.1 M sodium phosphate buffer (pH 7.04, containing 0.1 M NaCl). The temperature was maintained at 37.0 ± 0.2 °C. The inhibitors and the substrate were dissolved in acetonitrile. The final concentration of acetonitrile was 1.5%. The inhibitors were pre-incubated with AChE (1.0 U/mL, 10 μL) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (3.0 mg/mL, 50 μL) as chromogenic agent for 15 min. ATCh (3.0 mg/mL, 10 μL) was then added to initiate the enzyme reaction (1.0 mL in a final total volume), which was monitored immediately for 1 min by measuring the absorbance at 412 nm. Each inhibitor was assayed with six different concentrations around the IC_{50} values that were roughly estimated in the first round of experiments. The measurement was performed in triplicate for each concentration and averaged before further calculation.

4.2.3. Mechanism of CEase inhibition

Four most potent inhibitors (**3d**, **3e**, **2i** or **3i**) were chosen to examine their inhibition mechanisms of CEase. CEase (3.25 U/mL, 5 μL) was preincubated with each inhibitor at a certain concentration near the IC_{50} value at 25.0 ± 0.2 °C. At various incubation time intervals (0–60 min), the substrate (3.0 mg/mL, 5 μL) was added to the above mixture (1.0 mL in a final total volume), and the remain CEase activity was immediately monitored for 1 min by measuring the absorbance at 405 nm. The measurement was performed in triplicate for each time interval and averaged before further calculation.

4.2.4. Kinetic characterization of CEase inhibition

Compounds **3d** and **3e** were chosen to determine the kinetic characterization of CEase according to the literature method [25]. CEase activity was measured by varying the concentration of the substrate. CEase (3.25 U/mL, 5 μL) was preincubated with each inhibitor at two known concentrations or with no inhibitor at 25.0 ± 0.2 °C for 15 min. The enzyme reaction was initiated by the addition of the substrate (1.0 mL in a final total volume), and the enzyme activity was monitored for 1 min by measuring the absorbance at 405 nm. Triplicate sets of data were collected for each inhibitor concentration. The inhibition type, K_m (without inhibitor) and K_i (with inhibitor) were determined by Lineweaver–Burk double reciprocal plots.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.025.

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