European Journal of Medicinal Chemistry 45 (2010) 1955–1963

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Phosphaisocoumarins as a new class of potent inhibitors for pancreatic cholesterol esterase

Baojian Li, Binhua Zhou, Hailiang Lu, Lin Ma, Ai-Yun Peng*

School of Chemistry & Chemical Engineering, Sun Yat-sen University, 135 Xingangxi Lu, Guangzhou 510275, China

ARTICLE INFO

Article history:
Received 19 October 2009
Received in revised form
15 January 2010
Accepted 18 January 2010
Available online 28 January 2010

Keywords:
Cholesterol esterase
Inhibitor
Isocoumarin
Phosphaisocoumarin

ABSTRACT

Due to the importance of pancreatic cholesterol esterase (CEase) as a potential target in atherosclerosis and for the development of hypocholesterolemic agents, there are increasing interests in designing and synthesizing CEase inhibitors. In the present study, we prepared forty-five isocoumarin phosphorus analogues (i.e., phosphaisocoumarins) and investigated the inhibition of these compounds on the CEase. The results showed that some phosphaisocoumarins could act as potent inhibitors of CEase. The most potent inhibitors, compounds **9d**, **10a** and **12e** give IC_{50} values of 4.8 μM, 2.3 μM and 1.9 μM, respectively. The inhibition mechanism and kinetic characterization studies indicate that they are reversible competitive inhibitors.

1. Introduction

Since plasma cholesterol level is linked to many diseases such as coronary artery disease, cancer, obesity, and diabetes, the control of the cholesterol level has gained much attention. In the past decades, pancreatic cholesterol esterase (CEase; EC 3.1.1.13) has been studied extensively as a potential target to control the cholesterol level. It has been suggested that CEase plays important roles in the hydrolysis of dietary cholesterol esters [1–3] and the transport of free cholesterol to enterocytes [4,5], although there are some conflict reports about the roles of CEase in the absorption and transport of free cholesterol [6,7]. The gene knockout studies demonstrated that the lack of CEase reduced the intestinal absorption of dietary cholesteryl esters but had no influence on intestinal absorption of free cholesterol [2,3]. These results were also supported by the fact that some inhibitors of CEase were shown to inhibit the absorption of cholesterol esters in animal studies [8]. All the available data thus far shows that CEase has no direct role in absorption of unesterified cholesterol. It is believed that this enzyme plays an important compensatory role in providing sufficient lipolytic enzyme activities for complete triglyceride and phospholipid hydrolysis, which are required for optimal absorption of cholesterol [9,10].

In addition, CEase has been reported to be proatherogenic since it facilitated the conversion of the larger and less atherogenic lipoprotein particles to smaller and more atherogenic lipoprotein subspecies [11–13]. A recent investigation also disclosed that plaque-associated CEase had exhibited proangiogenic properties by promoting proliferation, migration, and capillary network formation by endothelial cells [14]. These findings further stimulated research interests in the inhibition of CEase as a potential drug target in atherosclerosis and for the development of hypocholesterolemic agents.

CEase and serine proteases have a similar Ser-His-Asp (Glu) catalytic triad [15,16] and they might be inhibited by the similar classes of compounds. Isocoumarins are a class of well-established mechanism-based inhibitors of a variety of serine proteases such as porcine pancreatic elastase (PPE), human leukocyte elastase (HLE), chymotrypsin, trypsin and the trypsin-like enzymes that initiate blood coagulation and the complement cascade [17–19]. Recently, Deck and coworkers [20] found that some 3-alkoxy-4-chloroisocoumarins (Fig. 1) were potent reversible inhibitors of CEase. Taking into account that phosphonic group can be regarded as bioisostere of carboxylic group and some phosphonic acid analogues of naturally occurring carboxylic acids have exhibited similar bioactivities with the carboxylic acids [21–25], we reasoned that isocoumarin phosphorus analogues (i.e., phosphaisocoumarins) (Fig. 2) [26–31] would have homologous bioactivities with isocoumarins. Furthermore, some phosphonic acid derivatives have been developed as potent irreversible inhibitors of CEase [32,33]. Herein, we decided to investigate the inhibition of CEase by phosphaisocoumarins in the present study and the results showed that some of them are potent reversible competitive inhibitors of CEase.

* Corresponding author. Tel.: +86 020 84110918; fax: +86 020 84112245.
E-mail address: cespay@mail.sysu.edu.cn (A.-Y. Peng).

0223-5234/$ – see front matter © 2010 Published by Elsevier Masson SAS.
doi:10.1016/j.ejmech.2010.01.038

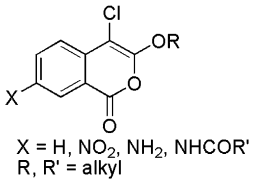


Fig. 1. Structure of 3-alkoxy-4-chloroisocoumarins.

2. Chemistry

Most of the compounds tested in this study were synthesized as previously described by our group [26–31]. The new compounds **8b**, **9b**, **10b**, **11** and **6k** were prepared by similar methods. Scheme 1 outlines the synthesis of 4-unsubstituted and 4-halophosphaisocoumarin esters. A variety of 2-(1-alkynyl)phenylphosphonic diethyl esters **4** could be easily synthesized from phenol derivatives **1** via phosphorylation, rearrangment, sulfonation, and Sonogashira coupling reactions in sequence. It was worth mentioning that aryl nonafluorobutanesulfonates (R_f = n-C_4F_9) were appropriate substrates just like aryl perfluoroalkanesulfonates (R_f = $(CF_2)_2O(CF_2)_2H$) described previously in the Sonogashira reaction [29]. 4-Unsubstituted phosphaisocoumarin esters **6** could be obtained by CuI-catalyzed cyclization of 2-(1-alkynyl)phenylphosphonic monoethyl esters **5** [26]. 4-Halophosphaisocoumarin esters **7**, **8** and **9** were prepared by halocyclization of the diethyl esters **4** with I_2 [27], $CuBr_2$ or $CuCl_2$ [30], or by halocyclization of the monoethyl esters **5** with NBS or NCS [28].

The reaction of 4-iodo-3-phenylphosphaisocoumarin ester **9g** with phenylacetylene afforded the corresponding coupling product **10a** in 83% yield [27]. While under the similar conditions, the reaction of 7-chloro-4-iodo-3-(4-ethylphenyl)phosphaisocoumarin ester **9d** and phenylacetylene gave a mixture of the coupling product **10b** in 45% yield and its acetylenic hydration derivative **11** in 26% yield (Scheme 2).

Phosphaisocoumarin acids **12a–12i** were synthesized via the reaction of phosphaisocoumarin esters with Me_3SiBr in chloroform followed by treatment with methanol, or with Me_3SiCl and NaI in acetonitrile followed by hydrolysis (Scheme 3) [31].

3. Biological results and discussion

3.1. Structure–activity correlations

The inhibition of phosphaisocoumarins on the CEase was investigated according to Hosie et al. [34] with some modifications and the results are summarized in Table 1. As shown in Table 1, the inhibitory activities were much dependent on the substituents at the positions 3, 4 and 7 of phosphaisocoumarins. Compounds with chlorine at the 7-position and *p*-nitrophenyl, *p*-methoxyphenyl or phenyl group at the 3-position (**6a**, **8b–9b**, **6c–9c**, **6d**) exhibit relatively weak inhibitory activities (IC_{50} > 25 μM). To our delight, *p*-ethylphenyl group at the 3-position and halogen group, phenylethynyl or benzoylmethyl at the 4-position could work together to improve the inhibitory effect by approximately 4–10 fold (compounds **7d–9d**, **10b**, **11** vs compounds **7c–9c**). Among them, compound **9d**, with iodine at the 4-position and a *p*-ethylphenyl group at the 3-position, displays the strongest activity (IC_{50} = 4.8 μM). However, the replacement of the aryl groups with small alkyl groups (such as cyclopropyl and *n*-butyl) at the 3-position results in inactive or poor active compounds (**6e–9e**, **6f–8f**).

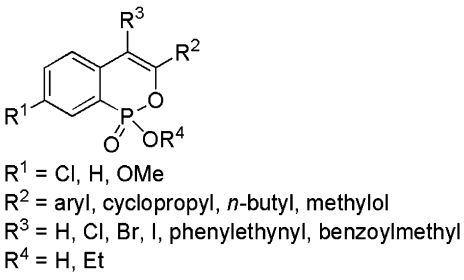


Fig. 2. Structure of phosphaisocoumarin esters and acids.

Similarly, in cases where there is no substituent at the 7-position (compounds **6g–9g**, **6h–9h**), the introduction of halogen at the 4-position can also lower the IC_{50} value. For example, the replacement of hydrogen with chlorine will improve the bioactivity by approximately 5 fold (**6g** vs **7g**). Compounds with small alkyl group at the 3-position (**6h–9h**) show no or low inhibitory activities. To our surprise, the increase in the size of the group at the 4-position going from hydrogen to phenylethynyl group is very productive by comparison of compound **10a** (IC_{50} = 2.3 μM) with compound **6g** (IC_{50} = 63.6 μM).

Compounds **6i–9i** with a methoxy group at the 7-position show weakened bioactivities compared with their counterparts with no substituent at the 7-position (**6g–9g**), indicating that the methoxy group may negatively affect the inhibitory activity. Compounds **7j**, **8j** and **6k** with a methoxy group at the 7-position and small alkyl group at the 3-position are also poor inhibitors.

Besides, we investigated the inhibition effect of phosphaisocoumarin acids (compounds **12a–12i**) on the CEase. In general, these compounds follow similar rules with the corresponding esters mentioned above. The importance of halogen at the 4-position is also observed by comparison of **12a** with **12b**, and **12d** with **12e**. It is worthwhile to point out that compound **12e** with chlorine at the 4-position gives the lowest IC_{50} value (1.9 μM) among all the compounds tested in this paper.

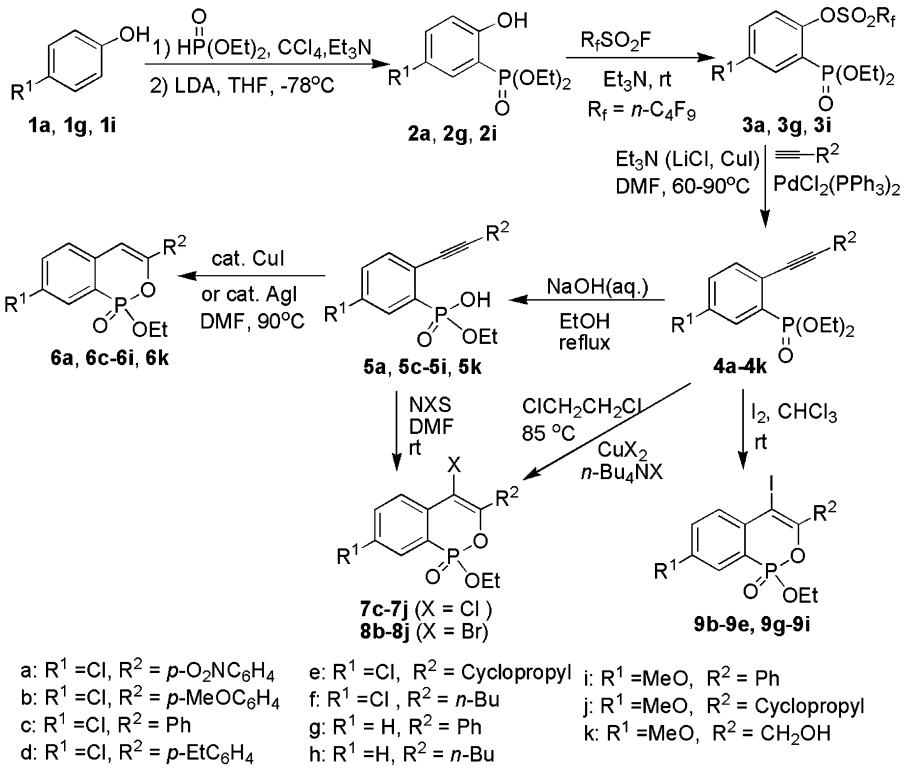
Compared with their ester counterparts, compounds **12a**, **12b**, **12e**, **12f** and **12g** show better inhibitory activities while compounds **12c**, **12d**, **12h** and **12i** are weaker inhibitors. Herein, it may indicate that the hydrophilicity is not the main factor in deciding the inhibitory effects. Additionally, the effects of different halogens are complex by comparing all the compounds with halogen at the 4-position.

Based on the above results, the following structure–activity relationships could be deduced thus far: (1) halogen or other larger (for example, phenylethynyl, benzoylmethyl) group at the 4-position, (2) an aryl group at the 3-position, and (3) no methoxy group at the 7-position are favorable for CEase inhibition. Deck et al. [20] also observed the importance of chlorine at the 4-position of isocoumarins on their inhibition of CEase, which are consistent with our results in this point.

3.2. Mechanism and kinetic characterization

The inhibition mechanism of the most potent inhibitors **9d**, **10a** and **12e** on the CEase for the hydrolysis of *p*-nitrophenylbutyrate was examined. Fig. 3 shows the relationship of enzyme activity with its concentration in the presence of different concentrations of inhibitors. The plots give a family of straight lines with different slopes, all of which pass through the origin. These results demonstrate that the inhibitory manners of compounds **9d**, **10a** and **12e** on the CEase are reversible.

To further confirm the reversible inhibitory manner of phosphaisocoumarins, compounds **9d**, **10a** and **12e** were incubated with CEase separately in the absence of *p*-nitrophenylbutyrate according to Deck et al. [35]. CEase and excess inhibitors were added to sodium

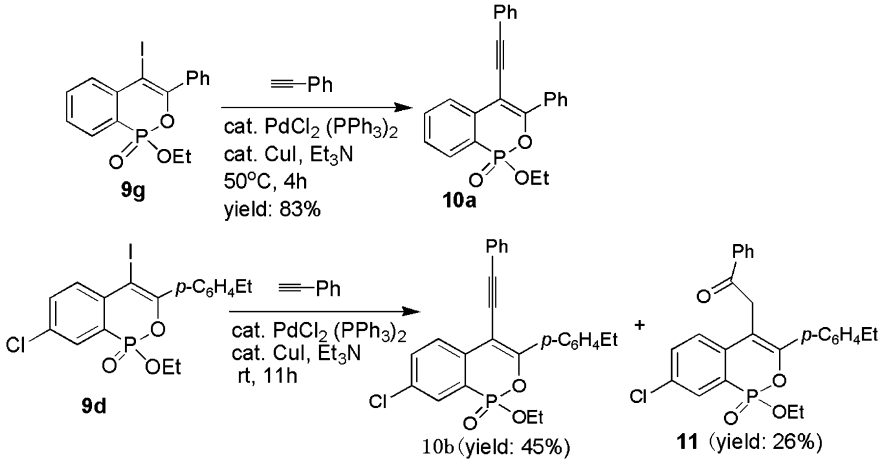


Scheme 1. Synthesis of 4-unsubstituted and 4-halophosphaisocoumarin esters **6**–**9**.

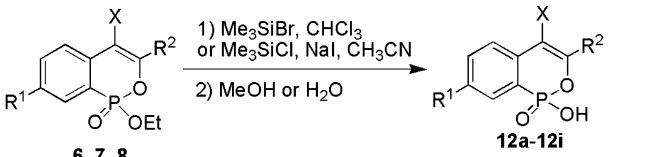
phosphate buffer (pH 7.04) containing acetonitrile (5% by volume), then they were incubated at 25 °C for up to 6 h which were detected periodically by TLC. In these processes, no new products were observed and all the added inhibitors could be finally recycled. These experimental results convinced that the phosphaisocoumarins are not irreversible but reversible inhibitors of CEase.

The kinetic behavior of phosphaisocoumarins on the CEase using *p*-nitrophenylbutyrate as substrate was then determined from Lineweaver–Burk double reciprocal plots (Fig. 4) [35]. The results indicated that they were competitive inhibitors of CEase. The dissociation constants (K_i, calculated from the plots) for **9d**, **10a** and **12e** are 4.2 μM, 1.9 μM and 1.4 μM, repectively.

Before our work, there are several similar classes of inhibitors of CEase with variable mechanistic and kinetic behaviors. For example, 3-aryl-6-chloro-2-pyrones have been developed as potential irreversible inactivators of serine proteases and CEase in early years [36]. Later, Deck et al. [35] found that 3-alkyl-6-chloro-2-pyrones were simple competitive inhibitors of CEase rather than suicide inhibitors. Thieno[2,3-*d*][1,3]oxazin-4-ones and tricyclic 1,3-oxazin-4-ones were characterized by Gütschow's group as irreversible inhibitors of CEase based on the acylation–deacylation mechanism [37,38]. Recently, Deck et al. [20] developed 3-alkoxychloroisocoumarins as potent reversible inhibitors of CEase. Hermetter and colleagues have demonstrated that some phosphonic derivatives are irreversible inhibitors of CEase [32,33]. Unlike phosphates and phosphonates are mainly known as suicide inhibitors of serine proteases and CEase, our phosphaisocoumarins in this work are reversible competitive inhibitors of CEase. This finding is interesting and may be useful for the design of new reversible phosphonates inhibitors of CEase.



Scheme 2. Synthesis of 4-(phenylethnyl)phosphaisocoumarin esters **10a**, **10b** and the derivative **11**.



6, **7**, **8**

12a-12i

12a: R^1 = Cl, R^2 = Ph, X = H
12b: R^1 = Cl, R^2 = Ph, X = Cl
12c: R^1 = Cl, R^2 = *p*-EtC_6H_4, X = H
12d: R^1 = H, R^2 = Ph, X = H
12e: R^1 = H, R^2 = Ph, X = Cl
12f: R^1 = H, R^2 = Ph, X = Br
12g: R^1 = H, R^2 = *n*-Bu, X = H
12h: R^1 = MeO, R^2 = Ph, X = H
12i: R^1 = MeO, R^2 = Ph, X = Br

Scheme 3. Synthesis of phosphaisocoumarin acids **12**.

4. Conclusions

Forty-five phosphaisocoumarins were synthesized and evaluated in vitro as inhibitors of porcine pancreatic CEase and some of them with appropriate 3-aryl and 4-halogen, 4-phenylethynyl or 4-benzoylmethyl substituents were demonstrated to be potent inhibitors of CEase. Compounds with methoxy at the 7-position or small alkyl groups (for example, cyclopropyl, *n*-butyl) at the 3-position show no or weak inhibition activity. In contrast to phosphates and phosphonates usually acting as potent irreversible inhibitors of CEase, these class of cyclic phosphonates are reversible competitive inhibitors. The present work suggested that phosphaisocoumarins might act as interesting lead compounds for developing new CEase inhibitors.

Table 1
Inhibitory effects on pancreatic Cholesterol Esterase of phosphaisocoumarin esters and acids.

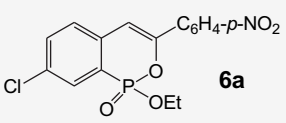
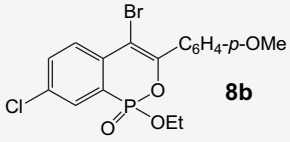
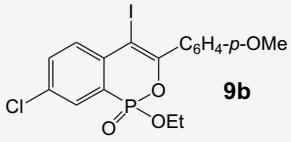
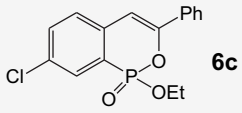
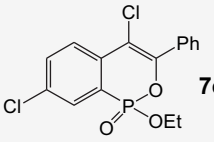
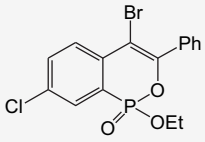
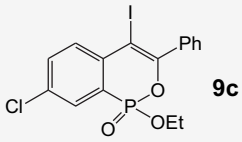
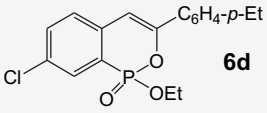
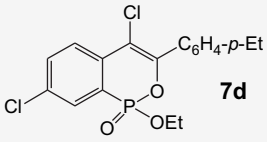
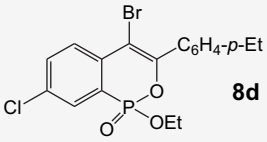
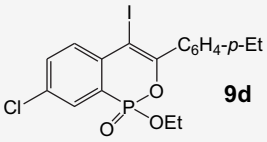
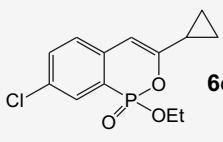
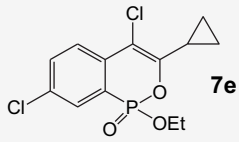
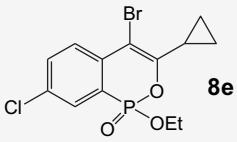
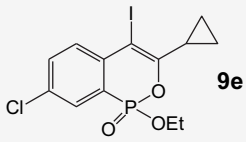
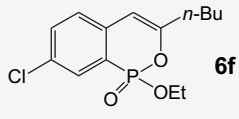
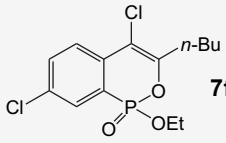
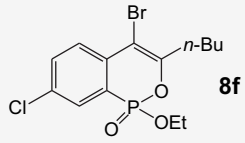
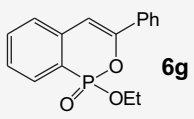
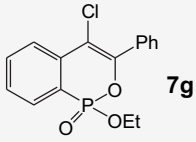
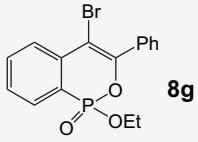
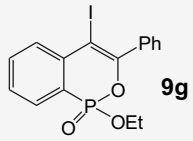
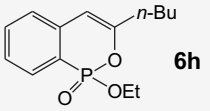
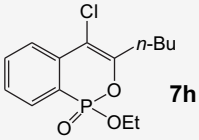
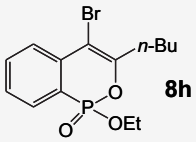
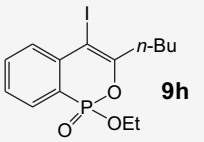
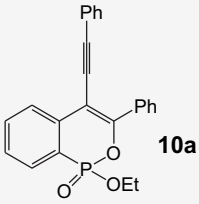
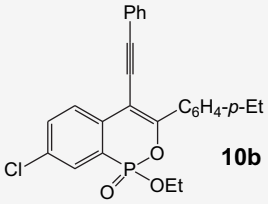
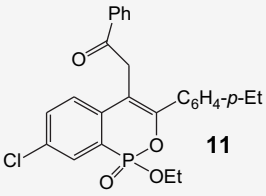
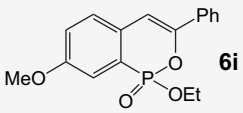
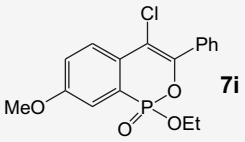
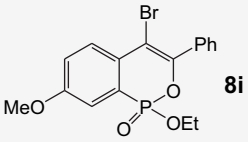
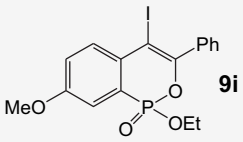
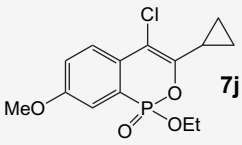
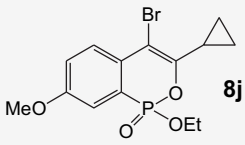
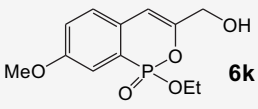
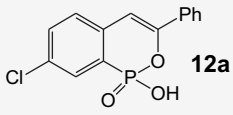
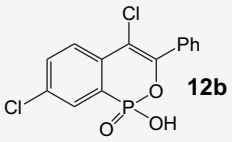
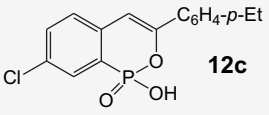
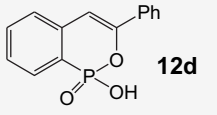
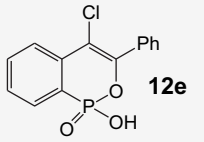
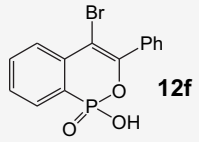
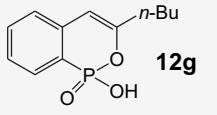
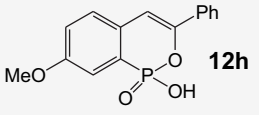
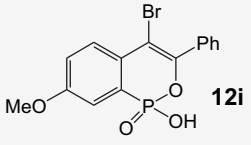
Structure	IC_{50} (μM)	Structure	IC_{50} (μM)	Structure	IC_{50} (μM)
 **6a**	>100	 **8b**	47.9	 **9b**	26.2
 **6c**	56.9	 **7c**	34.8	 **8c**	51.2
 **9c**	47.9	 **6d**	40.3	 **7d**	8.9
 **8d**	6.4	 **9d**	4.8	 **6e**	ni[a]
 **7e**	>100	 **8e**	ni	 **9e**	ni
 **6f**	43.1	 **7f**	ni	 **8f**	ni
 **6g**	63.6	 **7g**	12.7	 **8g**	18.9

Table 1 (*continued*)

Structure	IC_{50} (μM)	Structure	IC_{50} (μM)	Structure	IC_{50} (μM)
 **9g**	30.3	 **6h**	ni	 **7h**	ni
 **8h**	ni	 **9h**	ni	 **10a**	2.3
 **10b**	8.5	 **11**	5.1	 **6i**	77.4
 **7i**	89.6	 **8i**	34.4	 **9i**	54.5
 **7j**	>150	 **8j**	ni	 **6k**	79.4
 **12a**	38.7	 **12b**	14.6	 **12c**	58.4
 **12d**	96.3	 **12e**	1.9	 **12f**	14.1
 **12g**	62.6	 **12h**	102.5	 **12i**	57.4

[a] ni, no inhibition at 100 μM.

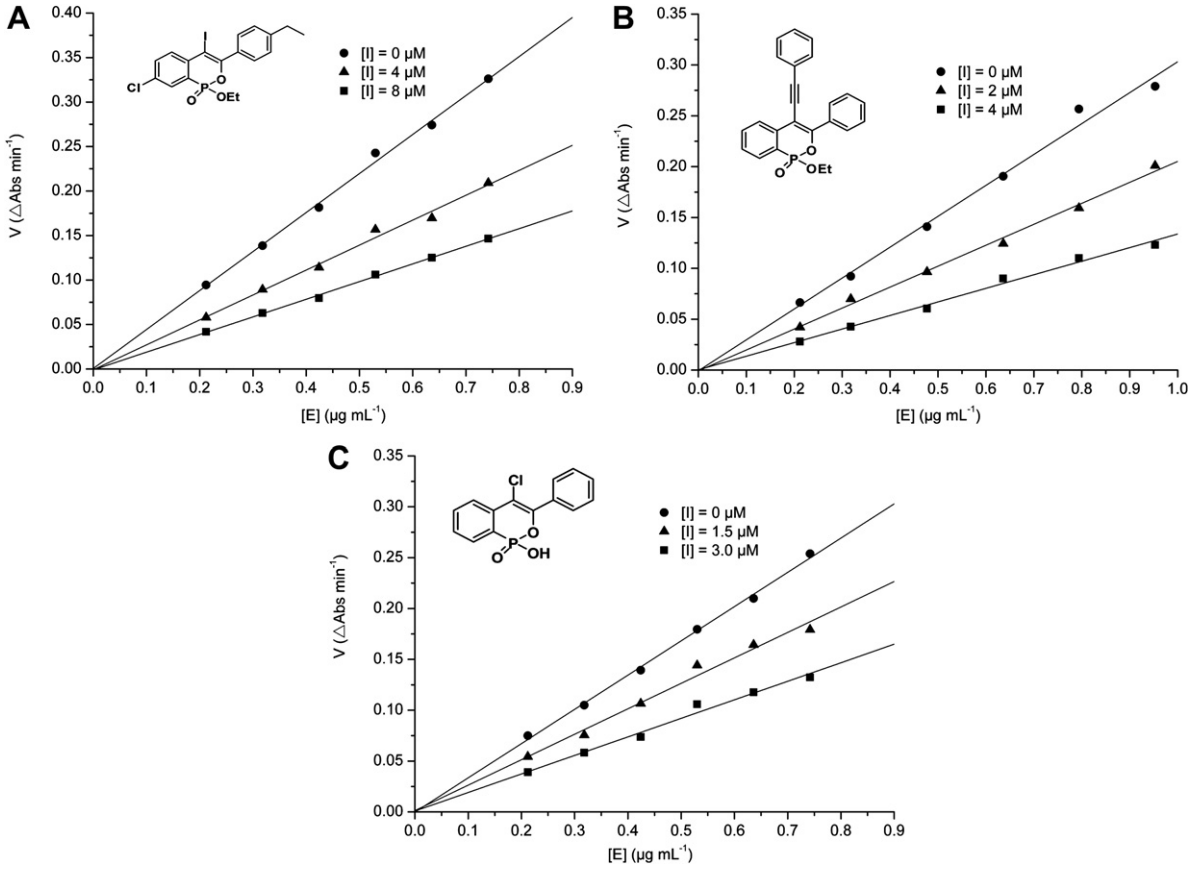


Fig. 3. Determination of the inhibition mechanism of CEase by phosphaisocoumarins with *p*-nitrophenylbutyrate as substrate. (A), (B) and (C) represent the results of compounds **9d**, **10a** and **12e**, respectively.

5. Experimental section

5.1. Chemistry

Anhydrous solvents (e.g. THF, DMF) were purified and dried according to standard procedures. Unless otherwise noted, all other reagents were obtained from commercial sources and used without further purification. NMR spectra were recorded on a Varian Mercury-Plus 300 (^{1}H 300 MHz; ^{13}C 75.4 MHz; ^{31}P 121 MHz). ESI-mass spectra were recorded on an LCMS-2010A Liquid Chromatograph mass spectrometer. HRMS were determined by a Thermo MAT95XP High Resolution mass spectrometer. IR spectra were recorded as KBr pellets on a Bruker Equinox 55 FT/IR spectrometer. Melting points were not corrected. Compounds **6a**, **6c**–**6i** [26], **7c**–**7j**, **8c**–**8j** [28,30], **9c**–**9e**, **9g**–**9i**, **10a** [27], and **12a**–**12i** [31] were prepared as described previously. Compounds **4b**, **8b**, **9b**, **6k**, **10b**, and **11** are new compounds, their synthetic procedures and spectral data are as follows.

5.1.1. 5-Chloro-2-(2-(4-methoxyphenyl)ethynyl)phenylphosphonic acid diethyl esters (*4b*)

To a mixture of **3a** (2734 mg, 5.0 mmol), $PdCl_2(PPh_3)_2$ (175 mg, 0.25 mmol), Et_3N (2.78 mL, 20.0 mmol), and DMF (15 mL) was added dropwise the *p*-methoxyphenylethyne (991 mg, 7.5 mmol) at room temperature. After stirring at 80 °C for 5 h under nitrogen, the reaction mixture was diluted with EtOAc and washed with saturated NH_4Cl until neutral and brine, dried (Na_2SO_4), and evaporated in vacuo. The residue was chromatographed on silica gel using petroleum ether/EtOAc (10:1–2:1) as eluent to give the corresponding product **4b** (1455 mg, yield: 77%) as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.99–7.94 (m, 1H), 7.41–7.53 (m, 4H), 6.86–6.89 (m, 2H), 4.07–4.28 (m, 4H), 3.82 (s, 3H), 1.33 (t, J = 7.2 Hz, 6H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 159.87, 134.07 (d, J = 10.4 Hz), 133.90 (d, J = 7.2 Hz), 133.38 (d, J = 10.5 Hz), 132.89, 131.90, 131.52 (d, J = 184.7 Hz), 124.47 (d, J = 6.5 Hz), 114.72, 113.96, 95.77, 85.71 (d, J = 8.2 Hz), 62.49 (d, J = 6.1 Hz), 55.23, 16.31 (d, J = 7.7 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ 15.69; MS (ESI): m/z (%): 373 [$(M + Na)^+$, 100]. Anal. Calcd for $C_{19}H_{20}ClO_4P$: C, 60.25; H, 5.32. Found: C, 60.30; H, 5.30. IR (KBr): 2982, 2215, 1606, 1461, 1289, 1250, 1025 cm^{-1}.

5.1.2. 4-Bromo-1-ethoxy-3-(4-methoxyphenyl)benzo[c][1,2]-oxaphosphinine 1-oxides (*8b*)

The mixture of **4b** (140 mg, 0.50 mmol) with $CuBr_2$ (447 mg, 2.0 mmol) and *n*-Bu_4NBr (17 mg, 0.05 mmol), in DCE (10.0 mL) was stirred at 85 °C for 10 h. The reaction mixture was then diluted with saturated brine and extracted with EtOAc. The combined organic phase was dried over anhydrous Na_2SO_4. After removal of the solvent in vacuo, the residue was purified by column chromatography on silica gel with petroleum ether/EtOAc (7:1–2:1) as eluent to afford **8b** (192 mg, yield: 89%) as pale yellow solid, mp: 107–109 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.63–7.94 (m, 5H), 6.95–6.98 (m, 2H), 4.25–4.35 (m, 2H), 3.89 (s, 3H), 1.39 (t, J = 6.9 Hz, 3H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 160.67, 148.36 (d, J = 12.5 Hz), 135.75 (d, J = 6.9 Hz), 134.37 (d, J = 21.6 Hz), 133.30, 131.09, 129.99 (d, J = 12.4 Hz), 128.75 (d, J = 11.1 Hz), 126.24 (d, J = 4.7 Hz), 122.48 (d, J = 178.2 Hz), 113.25 (d, J = 3.5 Hz), 102.76 (d, J = 11.7 Hz), 63.80 (d, J = 5.8 Hz), 55.40, 16.54 (d, J = 9.0 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ 8.91; MS (ESI): m/z (%): 429 [$(M + 1)^+$, 80], 431 [100]. Anal. Calcd for $C_{17}H_{15}ClBrO_4P$: C, 47.53; H, 3.52. Found: C, 47.65; H, 3.77. IR (KBr): 2958, 1592, 1469, 1269, 1240, 1021 cm^{-1}.

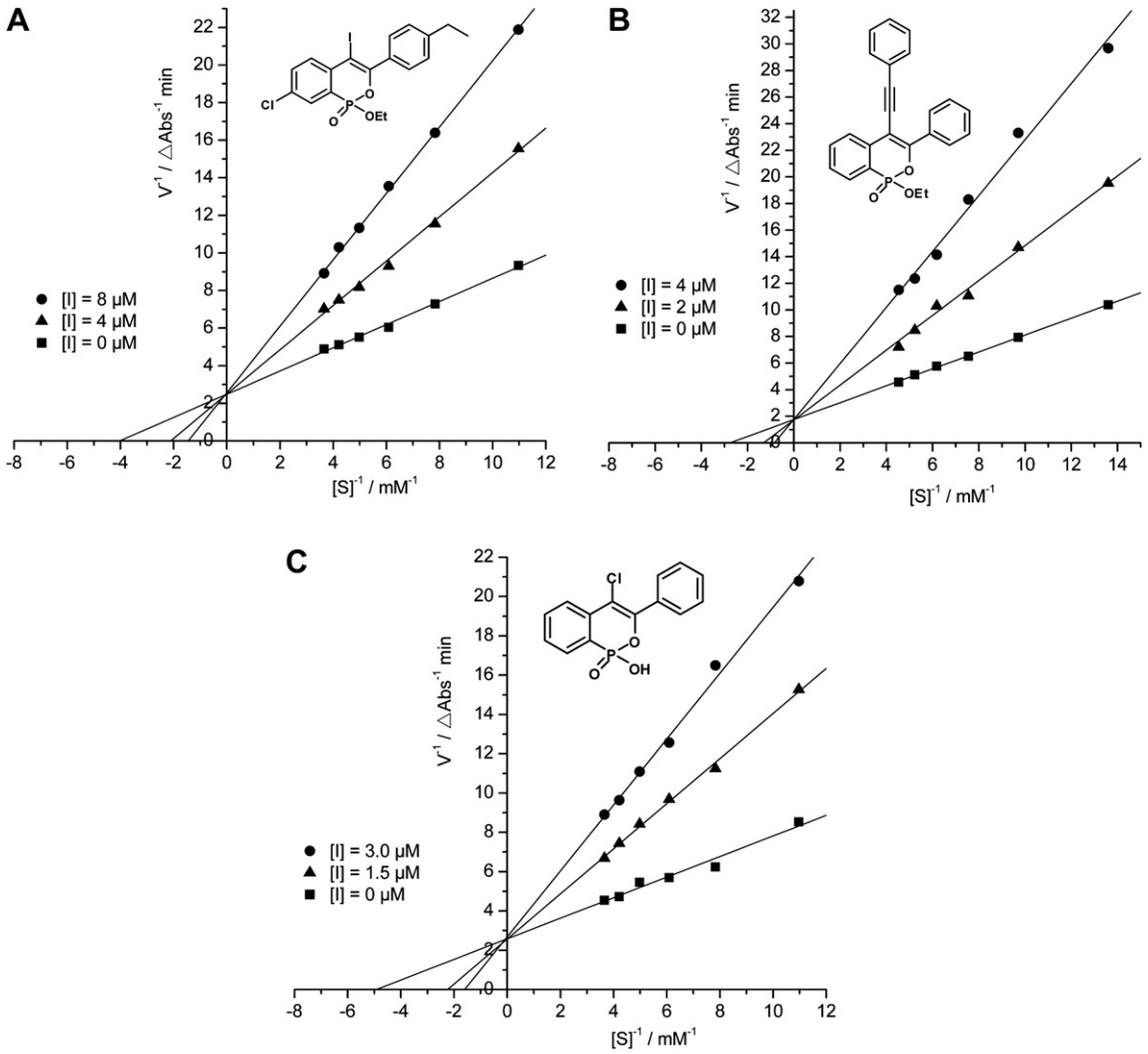


Fig. 4. Lineweaver–Burk plots for inhibition of CEase by phosphaisocoumarins with *p*-nitrophenylbutyrate as substrate. (A), (B) and (C) represent kinetics of compounds **9d**, **10a** and **12e**, respectively.

*5.1.3. 1-Ethoxy-4-iodo-3-(4-methoxyphenyl)benzo-[c][1,2]oxaphosphinine 1-oxides (**9b**)*

A mixture of **4b** (379 mg, 1.0 mmol) and I_2 (508 mg, 2.0 mmol) was dissolved in 10.0 mL $CHCl_3$. After stirring at room temperature for 18 h, the reaction mixture was then diluted with EtOAc and washed with 5% aqueous $Na_2S_2O_3$. The organic phase was washed with brine, dried (Na_2SO_4), and evaporated in vacuo. The residue was chromatographed on silica gel using petroleum ether/EtOAc (7:1–2:1) as eluent to give **9b** (308 mg, yield: 65%) as yellow solid, mp: 115–117 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.58–8.06 (m, 5H), 6.95–6.98 (m, 2H), 4.23–4.36 (m, 2H), 3.89 (s, 3H), 1.40 (t, J = 7.2 Hz, 3H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 160.66, 150.86 (d, J = 9.6 Hz), 137.40 (d, J = 3.5 Hz), 134.74 (d, J = 20.7 Hz), 133.42, 131.39, 128.83 (d, J = 7.7 Hz), 128.57 (d, J = 8.0 Hz), 126.48, 121.97 (d, J = 181.9 Hz), 113.21, 63.79 (d, J = 7.8 Hz), 55.38, 16.56 (d, J = 6.2 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ 9.27; MS (ESI): m/z (%): 477 [$(M + 1)^+$, 100]. Anal. Calcd for $C_{17}H_{15}ClIO_4P$: C, 42.84; H, 3.17. Found: C, 43.12; H, 3.41. IR (KBr): 2960, 1581, 1466, 1267, 1257, 1019 cm^{-1}.

*5.1.4. (1-Ethoxy-7-methoxy-1-oxo-1H-1λ^5-benzo[c][1,2] oxaphosphinin-3-yl)-methanol (**6k**)*

The diethyl ester **4k** [29] (500 mg, 1.68 mmol) and aq 1.0 M NaOH (10 mL, 10 mmol) were combined in EtOH (10 mL) and refluxed for 2.5 h. The mixture was evaporated in vacuo to remove the EtOH, then diluted with H_2O (20 L), cooed in an ice bath, neutralized with concd HCl, and extracted with EtOAc. The combined extracts were evaporated in vacuo to give the corresponding monoester quantitatively, which was used without further purification. To a mixture of the above monoester (130 mg, 0.48 mmol) in DMF (3 mL) was added CuI (9 mg, 0.047 mmol). After heating at 90 °C for 4 h, water was added to the mixture and the resulting aqueous mixture was extracted with EtOAc. The combined organic extract was washed with saturated NH_4Cl, brine, dried (Na_2SO_4), and evaporated in vacuo. The residue was chromatographed on silica gel using petroleum ether/EtOAc (2:1–1:1) as eluent to give **6k** (100 mg, yield: 77%) as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.01–7.26 (m, 3H), 6.03 (s, 1H), 4.20 (d, J = 3.6 Hz, 2H), 4.01–4.10 (m, 2H), 3.78 (s, 3H), 1.23 (t, J = 6.9 Hz, 3H); ^{31}P NMR (121 MHz, $CDCl_3$): δ 12.96; MS (EI): m/z: 270 (M^+, 43), 288 (93), 241 (23), 213 (58), 195 (21), 182 (44), 161 (51), 136 (23), 105 (10), 77 (25), 43 (100); Anal. Calcd for $C_{12}H_{15}O_5P$: C, 53.34; H, 5.60. Found: C, 52.98; H, 5.76; IR (film): 3381, 2928, 1605, 1495, 1242, 1033 cm^{-1}.

*5.1.5. Synthesis of **10b** and **11***

To a mixture of **9d** (210 mg, 0.44 mmol), $PdCl_2(PPh_3)_2$ (15 mg, 0.0214 mmol), CuI (15 mg, 0.079 mmol) and Et_3N (3.0 mL), was

added dropwise phenylacetylene (0.10 mol) at room temperature. After stirring at room temperature for 11 h under nitrogen, the reaction mixture was diluted with EtOAc and washed with saturated NH_4Cl until neutral and brine, dried (Na_2SO_4), and evaporated in vacuo. The residue was chromatographed on silica gel using petroleum ether/EtOAc (10:1–4:1) as eluent to give the corresponding product **10b** (89, yield: 45%) as yellow solid and **11** (5 g, yield: 26%) as yellow oil.

*5.1.5.1. 7-Chloro-1-ethoxy-3-phenyl-4-phenylethynylbenzo[c][1,2] oxaphosphinine 1-oxide (**10b**).* Pale yellow solid, mp 81–83 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.01–8.13 (m, 3H), 7.89 (dd, $J_1 = 15.3$ Hz, $J_2 = 2.1$ Hz, 1H), 7.67 (dd, $J_1 = 8.7$ Hz, $J_2 = 2.1$ Hz, 1H), 7.31–7.52 (m, 7H), 4.25–4.37 (m, 2H), 2.75 (q, $J = 7.5$ Hz, 2H), 1.38 (t, $J = 7.2$ Hz, 3H), 1.32 (t, $J = 7.5$ Hz, 3H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 154.68 (d, $J = 11.7$ Hz), 146.96, 136.18 (d, $J = 5.6$ Hz), 134.04, 133.75, 133.27, 131.13, 130.68 (d, $J = 6.7$ Hz), 128.88 (d, $J = 9.2$ Hz), 128.62, 128.60 (d, $J = 9.3$ Hz), 128.39, 127.44, 122.77, 121.68 (d, $J = 180.8$ Hz), 101.18, 96.73, 84.44, 63.62 (d, $J = 7.5$ Hz), 28.91, 16.54 (d, $J = 6.4$ Hz), 15.31; ^{31}P NMR (121 MHz, $CDCl_3$): δ 9.12; MS (ESI): m/z (%): 449 [$(M + 1)^+$, 100]. Anal. Calcd for $C_{26}H_{22}ClO_3P$: C, 69.57; H, 4.94. Found: C, 69.35; H, 4.97. IR (KBr): 2981, 2207, 1651, 1471, 1279, 1248, 1077, 1020 cm^{-1}.

*5.1.5.2. 7-Chloro-1-ethoxy-3-phenyl-4-(benzoylmethyl)benzo[c][1,2] oxaphosphinine 1-oxide (**11**).* Yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84–7.93 (m, 3H), 7.28–7.44 (m, 4H), 7.19–7.23 (m, 3H), 6.94–6.99 (m, 1H), 3.97–4.03 (m, 2H), 3.53–3.70 (m, 3H), 2.77 (q, $J = 7.8$ Hz, 2H), 1.31 (t, $J = 7.8$ Hz, 3H), 1.11 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 194.01, 151.81, 151.18 (d, $J = 11.1$ Hz), 135.22, 134.65, 134.58, 133.90 (d, $J = 20.5$ Hz), 133.21, 130.13, 129.46 (d, $J = 11.0$ Hz), 129.26, 128.57, 128.43, 127.15, 127.00 (d, $J = 5.1$ Hz), 121.95 (d, $J = 179.8$ Hz), 118.02 (d, $J = 11.2$ Hz), 62.95 (d, $J = 7.1$ Hz), 39.35 (d, $J = 4.8$ Hz), 29.15, 16.18 (d, $J = 6.7$ Hz), 15.06; ^{31}P NMR (121 MHz, $CDCl_3$): δ 9.42; MS (ESI): m/z (%): 373 [$(M + Na)^+$, 100]. Anal. Calcd for $C_{17}H_{16}ClO_4P$: C, 58.22; H, 4.60. Found: C, 58.07; H, 4.60. IR (KBr): 2966, 1667, 1603, 1474, 1288, 1264, 1027 cm^{-1}.

5.2. Inhibition of CEase

CEase (porcine) and *p*-nitrophenylbutyrate (PNPB) were from Sigma. The CEase inhibition was assayed according to Hosie et al. [34] with some modifications. CEase activity was measured by following the hydrolysis of the colorimetric substrate PNPB. According to Pietsch et al. [38], the PNPB is susceptible to nonenzymatic hydrolysis, which should be considered when the testing is more than 5 min. The testing in the present study only last for 1 min, so the effect of nonenzymatic hydrolysis was not considered here. The temperature was maintained at 25.0 ± 0.2 °C. All the compounds and PNPB were dissolved in acetonitrile. The final concentration of acetonitrile in the test solution was 2.0%. First, 0.0125 mU of CEase (0.53 μg/mL) were pre-incubated with different concentrations of inhibitor in 0.1 M sodium phosphate buffer (pH 7.04, containing 0.1 M NaCl) for 1 min. Then, the PNPB (0.1 mM) was added to the reaction mixture and the enzyme reaction was monitored for 1 min by measuring the change in absorbance at 405 nm. The measurement was performed in triplicate for each concentration and averaged before further calculation. The computer program used for the analysis of data was *Origin* 7.5.

5.3. Mechanism of CEase inhibition

The general procedure of the mechanism study is similar to the above inhibition assays. First, different concentrations of CEase (0.2–1.0 μg/mL) were pre-incubated with the inhibitor (concentration shown in Fig. 3) in sodium phosphate buffer for 1 min. Then, the PNPB (0.2 mM) was added to the reaction mixture and the enzyme reaction was monitored for 1 min by measuring the change in absorbance at 405 nm. The measurement was performed in triplicate for each concentration and averaged before further calculation.

5.4. Kinetic characterization of CEase inhibition

The general procedure of the kinetic characterization of CEase is also similar to the above inhibition assays. First, 0.0125 mU of CEase (0.53 μg/mL) were pre-incubated with the inhibitor (concentration shown in Fig. 3) in sodium phosphate buffer for 1 min. Then, different concentrations of PNPB (0.07–0.3 mM) were added to the reaction mixture and the enzyme reaction was monitored for 1 min by measuring the change in absorbance at 405 nm. Triplicate sets of data were collected for each inhibitor concentration. Kinetics of CEase inhibition was generally analyzed by Lineweaver–Burk plots [35].

Acknowledgements

This work was supported by the research grants from the National Natural Science Foundation of China (Grant No. 20602043) and Guangdong Natural Science Foundation (Grant No. 5300530).

References

[1] D.Y. Hui, Biochim. Biophys. Acta 1303 (1996) 1–14.
[2] P.N. Howles, C.P. Carter, D.Y. Hui, J. Biol. Chem. 271 (1996) 7196–7202.
[3] W. Weng, L. Li, A.M. van Bennekum, S.H. Potter, E.H. Harrison, W.S. Blaner, J.L. Breslow, E.A. Fisher, Biochemistry 38 (1999) 4143–4149.
[4] A. Lopez-Candales, M.S. Bosner, C.A. Spilburg, L.G. Lange, Biochemistry 32 (1993) 12085–12089.
[5] S.C. Myers-Payne, D.Y. Hui, H.L. Brockman, F. Schroeder, Biochemistry 34 (1995) 3942–3947.
[6] R. Shamir, W.J. Johnson, R. Zolfaghari, H.S. Lee, E.A. Fisher, Biochemistry 34 (1995) 6351–6358.
[7] R.J. Kirby, S. Zheng, P. Tso, P.N. Howles, D.Y. Hui, J. Biol. Chem. 277 (2002) 4104–4109.
[8] J.E. Heidrich, L.M. Contos, L.A. Hunsaker, L.M. Deck, D.L. Vander Jagt, BMC Pharmacol. 4 (2004) 5.
[9] K. Mackay, J.R. Starr, R.M. Lawn, J.L. Ellsworth, J. Biol. Chem. 272 (1997) 13380–13389.
[10] S.C. Young, D.Y. Hui, Biochem. J. 339 (1999) 615–620.
[11] O. Zschörnig, M. Pietsch, R. Süß, J. Schiller, M. Gütschow, J. Lipid Res. 46 (2005) 803–811.
[12] D.Y. Hui, P.N. Howles, J. Lipid Res. 43 (2002) 2017–2030.
[13] J. Brodt-Eppley, P. White, S. Jenkins, D.Y. Hui, Biochim. Biophys. Acta 1272 (1995) 69–72.
[14] O. Rebai, J. Le Petet-Thevenin, N. Bruneau, D. Lombardo, A. Verine, Arterioscl. Thromb. Vasc. Biol. 25 (2005) 359–364.
[15] W. Boland, C. Froessl, M. Lorenz, Synthesis 12 (1991) 1049–1072.
[16] A. Svendsen, in: P. Woolley, S.B. Petersen (Eds.), Lipases, Their Structure Biochemistry and Application, Cambridge University Press, Cambridge, 1994, pp. 1–21.
[17] J.C. Powers, J.L. Asgian, Ö.D. Ekici, K.E. James, Chem. Rev. 102 (2002) 4639–4750 (and references therein).
[18] F. Bihel, G. Quéléver, H. Lelouard, A. Petit, C. Alvés da Costa, O. Pourquié, F. Checler, A. Thellend, P. Pierre, J.-L. Kraus, Bioorg. Med. Chem. 11 (2003) 3141–3152.
[19] L. Pochet, R. Frédérick, B. Masereel, Curr. Pharm. Des. 10 (2004) 3781–3796.
[20] J.J. Heynekamp, L.A. Hunsaker, T.A. Vander Jagt, R.E. Royer, L.M. Deck, D.L. Vander Jagt, Bioorg. Med. Chem. 16 (2008) 5285–5294.
[21] K.B. Dillon, F. Mathey, J.F. Nixon FRS (Eds.), Phosphorus: The Carbon Copy, John Wiley & Sons, Chichester, 1998.
[22] R.G. Almquist, W.-R. Chao, C. Jennings-White, J. Med. Chem. 28 (1985) 1067–1071.
[23] R. Engel (Ed.), Handbook of Orgnophosphorus Chemistry, Marcel Dekker, New York, 1992.
[24] F.R. Hartley (Ed.), The Chemistry of Organophosphorus Compounds, Vol. 4, John Wiley & Sons, New York, 1996.
[25] L.D. Quin (Ed.), A Guide to Organophosphorus Chemistry, John Wiley & Sons, New York, 2000.
[26] A.-Y. Peng, Y.-X. Ding, J. Am. Chem. Soc. 125 (2003) 15006–15007.
[27] A.-Y. Peng, Y.-X. Ding, Org. Lett. 6 (2004) 1119–1121.
[28] A.-Y. Peng, Y.-X. Ding, Tetrahedron 61 (2005) 10303–10308.
[29] A.-Y. Peng, X.-Y. Zhang, Y.-X. Ding, Heteroat. Chem. 16 (2005) 529–534.

[30] A.-Y. Peng, F. Hao, B. Li, Z. Wang, Y. Du, J. Org. Chem. 73 (2008) 9012–9015.
[31] A.-Y. Peng, B. Li, X. Yang, J. Lin, Synthesis 15 (2008) 2412–2416.
[32] H. Schmidinger, R. Birner-Gruenberger, G. Riesenhuber, R. Saf, H. Susani-Etzerodt, A. Hermetter, ChemBioChem 6 (2005) 1776–1781.
[33] G.B. Quistad, S.N. Liang, K.J. Fisher, D.K. Nomura, J.E. Casida, Toxicol. Sci. 91 (2006) 166–172.
[34] L. Hosie, L.D. Sutton, D.M. Quinn, J. Biol. Chem. 262 (1987) 260–264.
[35] L.M. Deck, M.L. Baca, S.L. Salas, L.A. Hunsaker, D.L. Vander Jagt, J. Med. Chem. 42 (1999) 4250–4256.
[36] R.B. Westkaemper, R.H. Abeles, Biochemistry 22 (1983) 3256–3264.
[37] M. Pietsch, M. Gütschow, J. Biol. Chem. 277 (2002) 24006–24013.
[38] M. Pietsch, M. Gütschow, J. Med. Chem. 48 (2005) 8270–8288.