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PAPER

A new synthesis of fully phosphorylated flavones as potent pancreatic cholesterol esterase inhibitors†

Guoping Peng,[a] Yidan Du,[b] Yingling Wei,[b] Jingming Tang,[b] Ai-Yun Peng*[b] and Liqun Rao*[a]

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Five flavones possessing one to four phenolic groups were fully phosphorylated efficiently and the obtained compounds showed excellent pancreatic cholesterol esterase (CEase) inhibitory activities with IC_{50} in the nanomolar range, which were much more potent than their parent compounds. The inhibition mechanism and kinetic characterization studies indicate that they are irreversible competitive inhibitors.

Introduction

Pancreatic cholesterol esterase (CEase; EC 3.1.1.13) is a nonspecific α/β hydrolase that can catalyze the hydrolysis of cholesterol esters, triacylglycerides, phospholipids and lipid-soluble vitamin esters prior to their absorption. Since CEase plays important roles in the dietary cholesterol absorption, inhibition of CEase has aroused much interest as a new potential approach to treat hypercholesterolemia.[1,2]

Many efforts have been made in the development of potent inhibitors of CEase. Several classes of CEase inhibitors have been reported, including substituted 6-chloro-2-pyrones,[3,4] 1,3-oxazin-4-ones,[5,6] carbamates,[7–9] phosphonates,[10,11] high molecular weight sulfated polysaccharides[12] and 3-alkoxychloroisocoumarins.[13] In our previous work, we have shown that some phosphaisocoumarins are reversible competitive inhibitors of CEase.[14] However, most of the above inhibitors (Fig. 1) are not potent and safe enough for practical use. Therefore, it is still desirable to develop novel CEase inhibitors with more potent activities and lower side effects.

In the present study we designed and synthesized a series of phosphorylated flavones as potential inhibitors of CEase on the basis of the following considerations. First, there exists some similarity in the skeleton structure between cholesterol and flavones (Fig. 2), and so phosphorylated flavones may act as alternate substrate-based inhibitors of CEase. Second, the target phosphates have tetrahedral structures that may mimic the transition states of the hydrolysis of cholesterol esters and thereby inhibit the activity of CEase. Third, phosphorylated flavones may act as prodrugs to inhibit CEase. Flavones are a class of polyphenolic compounds that are widely distributed in plant foods and many studies have shown that flavones have the potential to become drugs especially in cancer and cardiovascular disease prevention and therapy.[15–17] However, most of the flavones show poor solubility not only in water but also in lipids, which reduces their absorption in *vivo* and limits their further practical applications. Phosphates are often used as prodrugs to improve the bioavailabilities of the parent agents and several phosphorylated flavones have been reported as anticancer agents,[18] but the phosphorylated flavones containing

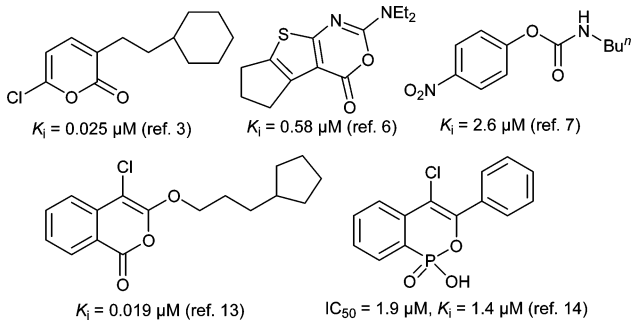


Fig. 1 Structures and inhibitory activities of some potent CEase inhibitors.

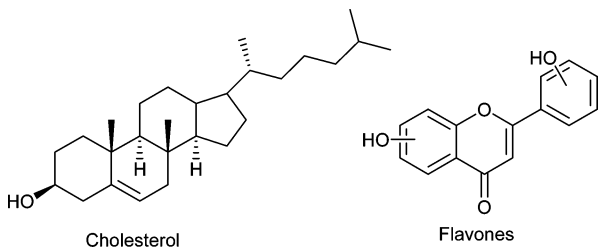


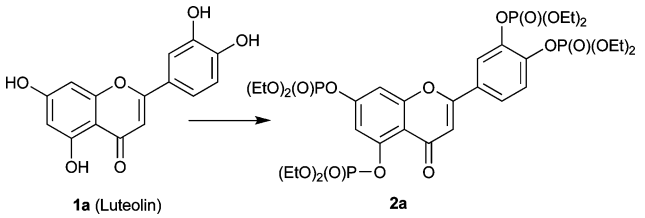
Fig. 2 Structures of cholesterol and flavones.

[a]*College of Bioscience and Biotechnology, Hunan Agricultural University, Furong District, Changsha, 410428, China. E-mail: raoliqun@163.com; Fax: 86 0731 84612870; Tel: 86 0731 84673601*

[b]*School of Chemistry & Chemical Engineering, Sun Yat-sen University, 135 Xingang.xi Lu, Guangzhou, 510275, China. E-mail: cespay@mail.sysu.edu.cn; Fax: 86 20 84112245; Tel: 86 020 84110918*

† Electronic supplementary information (ESI) available: NMR spectra for **2a–2e**; procedure for enzyme assays and graphs for determination of inhibitors IC_{50} values. See DOI: 10.1039/c0ob00640h

Table 1 Optimization of reaction conditions toward fully phosphorylated flavones[a]



Entry	Catalyst (equiv.)	Solvent	Phosphorylated reagent (equiv.)	Isolated yield (%)
1	—	CCl_4	$HP(O)(OEt)_2$ (12)	\[b]
2	—	CCl_4 + DMF	$HP(O)(OEt)_2$ (20)	\[b]
3	—	CCl_4 + THF	$HP(O)(OEt)_2$ (20)	\[b]
4	—	THF	$ClP(O)(OEt)_2$ (12)	\[b]
5	DMAP (8)	THF	$ClP(O)(OEt)_2$ (12)	**2a** (91)

[a] All reactions were carried out at room temperature for 24 h with the addition of 8 equiv. of Et_3N. [b] The reaction led to a complicated mixture.

more than two phenolic groups and their inhibitory activities on CEase have not been reported so far.

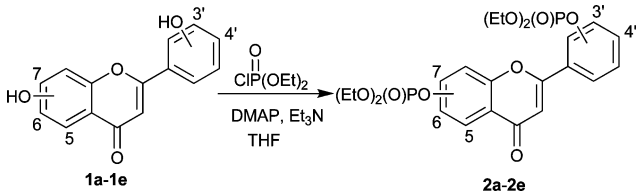
Results and discussion

Chemistry

We initiated this study with the phosphorylation of luteolin (**1a**) possessing four phenolic groups and the results are summarized in Table 1. The Atherton–Todd reaction has proved to be a useful method to phosphorylate flavonoids with one or two phenolic groups.[19,20] However, under typical Atherton–Todd reaction conditions, the reaction of **1a** with $HP(O)(OEt)_2$ and Et_3N in CCl_4 only led to a complicated mixture (entry 1). Taking into account that this result is probably caused by the incomplete phosphorylation and the weak solubility of luteolin in CCl_4, we tried to optimize the reaction by increasing the amount of $HP(O)(OEt)_2$ and adding a polar solvent (*e.g.* DMF), but all attempts lead to unfavorable results (entries 2, 3). Since it has been reported that DMAP (4-dimethylaminopyridine) can catalyze the full sulfation of flavones, then we turned our attention to the use of DMAP as a catalyst. Gratifyingly, we finally found that when **1a** was treated with 3.0 equiv of $ClP(O)(OEt)_2$, 2.0 equiv of Et_3N, 2.0 equiv of DMAP per hydroxy in THF, the starting material **1a** was consumed completely and a single tetraphosphorylated product **2a** was isolated in 91% yield.

With these optimized conditions in hand, the reactions of five flavones **1a–1e** with excess $ClP(O)(OEt)_2$, Et_3N and DMAP were carried out in THF at room temperature and the results are summarized in Table 2. This reaction is very efficient and all flavones tested could be converted to the desired products **2a–2e** in good to excellent yields. It is also worthy to note that the purification is very simple and convenient since only a single most non-polar fully phosphorylated flavone was formed in the end for each reaction. Among these products, compounds **2a**, **2b** and **2e** are new compounds; compounds **2c** and **2d** have been prepared using the Atherton–Todd reaction by Zhao's group.[19,20] However, no yield was given for compound **2c** in the literature,[19] although **2d** was obtained in 92% yield.[20a]

Table 2 DMAP-catalyzed synthesis of fully phosphorylated flavones[a]

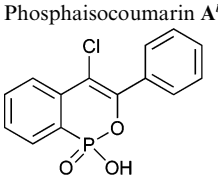


Entry	Substrate	Product	Isolated yield (%)
1	**1a** (5,7,3′,4′-OH)	**2a** (5,7,3′,4′-$OP(O)(OEt)_2$)	91
2	**1b** (7,3′,4′-OH)	**2b** (7,3′,4′-$OP(O)(OEt)_2$)	83
3	**1c** (5,7-OH)	**2c** (5,7-$OP(O)(OEt)_2$)	86
4	**1d** (7-OH)	**2d** (7-$OP(O)(OEt)_2$)	80
5	**1e** (6-OH)	**2e** (6-$OP(O)(OEt)_2$)	74

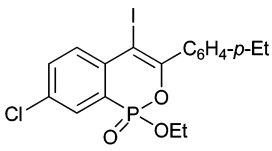
[a] All reactions were carried out at 2.5 mmol scale in THF (30 mL) with DMAP (2 equiv. per OH), Et_3N (2 equiv. per OH), $ClP(O)(OEt)_2$ (3 equiv. per OH) at room temperature for 24 h.

Table 3 Inhibitory effects on CEase of flavones **1a–1e** and **2a–2e**

Compound	IC_{50}/nM	Compound	IC_{50}/nM
2a	3.89	**1a**	28 100
2b	26.1	**1b**	35 700
2c	3.76	**1c**	ni[a]
2d	2.44	**1d**	ni
2e	390	**1e**	ni
Phosphaisocoumarin **A**[b]	1900	Phosphaisocoumarin **B**[b]	4800



Phosphaisocoumarin **A**



Phosphaisocoumarin **B**

[a] ni, no inhibition at 100 μM. [b] In line with data in ref. 14.

Biology

Structure–activity correlations. The inhibitory activity of **2a–2e** and **1a–1e** against CEase was investigated according to Hosie *et al.*[21] with some modifications and the results are summarized in Table 3. Compared with phosphaisocoumarins **A**, **B** (Table 3) and other known CEase inhibitors (Fig. 1), phosphorylated flavones showed excellent inhibitory activity toward CEase. According to the data in Table 3, the following structure–activity relationships could be deduced.

(1) Phosphorylation of flavones can significantly enhance the inhibition of CEase. The parent flavones (**1a–1e**) showed weak (IC_{50} > 25 μM) or no inhibition activities against CEase, while their phosphorylated derivatives (**2a–2e**) displayed excellent CEase inhibitory activities with IC_{50} in the nanomolar range. For example, tetra-phosphorylated luteolin **2a** has an IC_{50} value of 3.89 nM, and was 7224-fold more potent than luteolin **1a** (IC_{50} = 28.1 μM).

(2) The position of the phosphate group has a large effect on the inhibitory activity. For example, compound **2d** with a phosphoryl group at positon 7 (IC_{50} = 2.44 nM) was nearly 160-fold more active than **2e** with a phosphoryl group at positon 6 (IC_{50} = 390 nM). All results showed that introduction of a phosphoryl group at positon

7 is a very important beneficial factor. In addition, phosphoryl groups at position 4′ and/or 3′ are unnecessary or even adverse since both **2a** and **2c** showed nearly the same activity and **2b** exhibited less activity.

Mechanism and kinetic characterization. To determine whether compounds **2** are irreversible or reversible inhibitors of CEase, we measured residual activity of CEase after incubation with the potent inhibitors **2a** and **2d** according to the literature protocol.[22] The results showed that **2a** and **2d** (both at nanomolar concentrations) inhibit CEase in a progressive manner; the enzyme activity decreases as the incubation time increases (Fig. 3). Both compounds were characterized as irreversible inhibitors of CEase since they meet some of the criteria proposed by Abeles and Maycock.[23] For example, the inhibition was time-dependent and followed the first-order kinetics over the observed time period (Fig. 3).

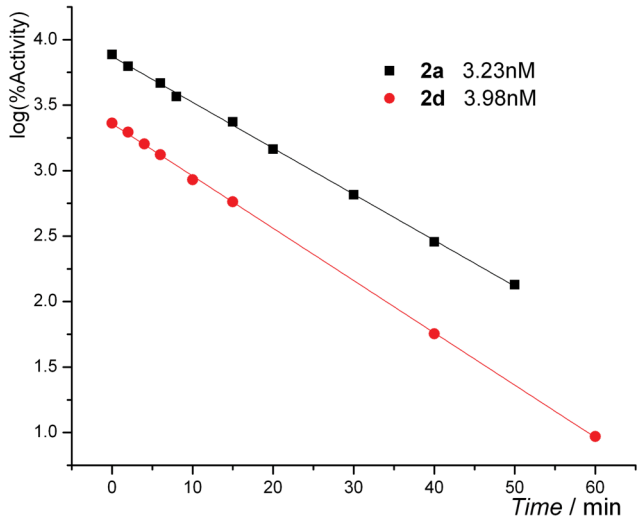


Fig. 3 Time-dependent inhibition of CEase by compounds **2a** and **2d**.

The kinetic behavior of compounds **2a** and **2d** on the CEase using *p*-nitrophenylbutyrate as the substrate was then determined from Lineweaver–Burk double reciprocal plots (Fig. 4).[4] The results indicated that on a short time scale they were competitive inhibitors of CEase, suggesting that these inhibitors and the substrate might competitively bind with the same site of the free enzyme. The K_i and K_m values for **2a** (calculated from the plots) are 3.73 nM and 0.11 mM, and the K_i and K_m values for **2d** are 1.17 nM and 0.19 mM. It is apparent that the K_m value is significantly greater than the K_i value for both compounds, indicating that the affinity between the enzyme and the inhibitor is far higher than the affinity between the enzyme and the substrate. These results may explain why phosphorylated flavones **2** show such good inhibitory activities against CEase.

Conclusion

In conclusion, the present investigation not only developed a new effective and convenient way to synthesize fully phosphorylated flavones, but also provided a new class of potent irreversible competitive CEase inhibitors. The results showed that all the synthesized compounds had much more potent inhibitory activities than the parent flavones. Compounds **2a**, **2c** and **2d** were found to be the most potent inhibitors with IC_{50} value of 3.89 nM, 3.76 nM and 2.44 nM, respectively, indicating that introduction of a phosphoryl group at position 7 of flavone was beneficial to the inhibition of CEase. This work suggested that phosphorylated flavones might act as interesting lead compounds for developing new effective and safe CEase inhibitors which may have good prospects for practical application in the treatment of hypercholesterolemias.

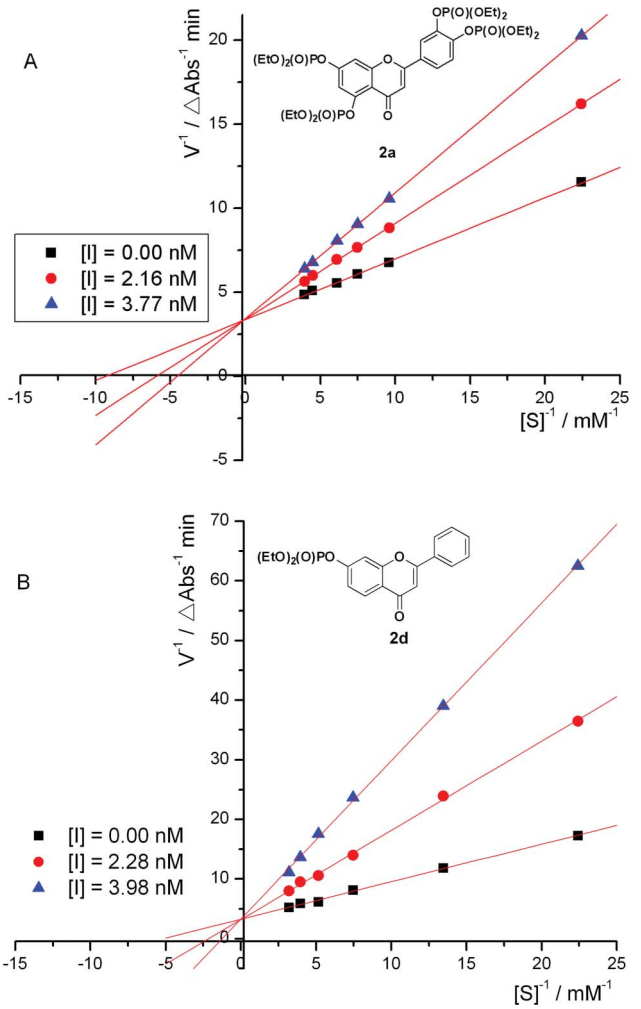


Fig. 4 Lineweaver–Burk plots for inhibition of CEase by **2a** and **2d** with *p*-nitrophenylbutyrate (*p*NPB) as the substrate. (A) and (B) represent kinetics of compounds **2a** and **2d**, respectively. $K_i = [I]/[K_m(app)/K_m - 1]$. K_m(app) determined with inhibitor and *p*NPB and K_m with *p*NPB alone.

Experimental

General

Anhydrous 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 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 a LCMS-2010A Liquid Chromatograph mass spectrometer. IR spectra were recorded as KBr pellets on a Bruker Equinox 55

FT/IR spectrometer. Melting points were not corrected. Column chromatography was performed on 200–300 mesh silica gel. Thin-layer chromatography was conducted on Kieselgel 60 F254.

General procedure for the synthesis of 2a–2e. To a stirring solution of **1** (2.5 mmol), DMAP (2.0 mmol per –OH group), Et_3N (2.0 mmol per –OH group) in anhydrous THF (30.0 mL), a solution of $ClP(O)(OEt)_2$ (DEPC, 30 mmol) in anhydrous THF (20 mL) was added dropwise in an ice-water bath over 30 min. After stirring at room temperature for 24 h under nitrogen, the reaction mixture was diluted with EtOAc and washed with 0.5 M HCl, 5% (*w*/*v*) K_2CO_3, brine and water, and then 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 (4 : 1–2 : 1) as eluent to give the corresponding product **2**.

Diethyl 4-oxo-2-phenyl-4*H*-chromen-3′,4′,5,7-yl phosphate (2a). Oil. Yield: 91%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85 (s, 1H), 7.53–7.65 (m, 2H), 7.33 (s, 1H), 7.24 (s, 1H), 6.59 (s, 1H), 4.26–4.40 (m, 16H), 1.34–1.40 (m, 24H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 175.7, 159.9, 157.8, 153.8 (d, J = 3.4 Hz), 150.3 (d, J = 6.6 Hz), 144.2, 141.8, 128.2, 123.4, 121.7, 119.4, 113.7 (d, J = 5.5 Hz), 110.2 (d, J = 6.3 Hz), 109.0, 105.7 (d, J = 5.4 Hz), 65.3, 65.2, 65.1, 65.0, 16.1 (m, 4C); ^{31}P NMR(121 MHz, $CDCl_3$) δ: –6.65, –6.47, –5.88, –5.43. MS: *m*/*z* 853(M^+, 100); Anal. Calcd. for $C_{31}H_{46}O_{18}P_4$: C, 44.83; H, 5.58. Found C, 44.93; H, 5.74; IR (film, cm^{-1}): 2919, 1654, 1419, 1330, 1273, 1105, 1029.

Hexaethyl tri-phosphoric ester of apigenin (2b). Oil. Yield: 83%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.75–7.79 (m, 2H), 7.15–7.30 (m, 4H), 6.54 (s, 1H), 4.12–4.36 (m, 12H), 1.20–1.35 (m, 18H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 175.7, 160.7, 157.7, 153.6 (d, J = 5.1 Hz), 153.1 (d, J = 6.2 Hz), 150.1 (d, J = 8.7 Hz), 127.6, 127.3, 120.3, 113.6 (d, J = 6.3 Hz), 109.9, 108.3, 105.5, 65.1, 65.0, 64.8, 64.7, 16.1 (m, 4C); MS (ESI): *m*/*z* (%): 678 [$(M+1)^+$, 100]. Anal. Calcd for $C_{27}H_{37}O_{14}P_3$: C, 47.80; H, 5.50. Found C, 47.54; H, 5.70. IR (film, cm^{-1}): 2936, 1656, 1479, 1383, 1286, 1030, 988.

Tetraethyl bis-phosphoric ester of chrysin (2c). A pale yellow solid. Yield: 86%; Mp: 79–81 °C (lit.[19] 83–84 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77–7.86 (m, 2H), 7.41–7.53 (m, 3H), 7.31–7.40 (m, 1H), 7.17–7.28 (m, 1H), 6.64 (s, 1H), 4.24–4.40 (m, 8H), 1.31–1.40 (m, 12H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 175.9, 161.7, 157.8, 153.7 (d, J = 8.4 Hz), 150.2 (d, J = 7.1 Hz), 131.5, 130.7, 128.8, 125.9, 113.7 (d, J = 8.6 Hz), 109.9, 108.6, 105.6 (d, J = 3.7 Hz), 65.2, 65.1, 16.1, 16.0; ^{31}P NMR (121 MHz, $CDCl_3$): δ –6.28, –6.52. MS (ESI): *m*/*z* (%): 526 [M^+, 100]. IR (film, cm^{-1}): 2986, 1651, 1438, 1380, 1278, 1156, 1031, 979.

Diethyl flavone-7-yl phosphate (2d). A pale yellow solid. Yield: 80%; Mp: 61–63 °C (lit.[20] 60–61 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.20 (d, J = 8.7 Hz, 1H), 7.91–7.94 (m, 2H), 7.53–7.57 (m, 4H), 7.25–7.29 (m, 1H), 6.84 (s, 1H), 4.24–4.34 (m, 4H), 1.41 (dt, J_1 = 7.2 Hz, J_1 = 1.5 Hz, 6H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 177.3, 163.5, 156.8, 154.6 (d, J = 6.9 Hz), 131.6, 131.4, 128.9, 127.4, 126.1, 121.0, 117.8 (d, J = 7.5 Hz), 108.9 (d, J = 4.3 Hz), 107.5, 65.1 (d, J = 7.6 Hz), 16.1 (d, J = 7.5 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ –5.87. MS (ESI): *m*/*z* (%): 374 [M^+, 100]. IR (film, cm^{-1}): 2985, 1642, 1444, 1374, 1280, 1158, 1040, 976.

Diethyl flavone-6-yl phosphate (2e). A pale yellow solid. Yield: 74%. Mp: 67–68 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.88–7.97 (m, 3H), 7.49–7.65 (m, 5H), 6.81 (s, 1H), 4.21–4.31 (m, 4H), 1.39 (t, J = 7.2 Hz, 6H); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 177.4, 163.4, 153.0, 147.7 (d, J = 5.1 Hz), 131.6, 131.4, 128.9 (d, J = 3.0 Hz), 126.3, 126.1, 124.7, 119.6, 115.7 (d, J = 3.3 Hz), 106.9, 64.9 (d, J = 6.3 Hz), 16.2 (d, J = 6.0 Hz); ^{31}P NMR (121 MHz, $CDCl_3$): δ –5.24; MS (ESI): *m*/*z* (%): 375 [$(M+1)^+$, 100]. Anal. Calcd for $C_{19}H_{19}O_6P$: C, 60.96; H, 5.12. Found C, 60.96; H, 5.40. IR (KBr, cm^{-1}): 2988, 1643, 1498, 1393, 1261, 1235, 1181, 1026.

Assay procedure and determination of inhibitor IC_{50}

CEase (porcine) was from Worthington, *p*-nitrophenyl butyrate (*p*NPB) was from Sigma. The CEase inhibition was assayed according to Hosie *et al.*[21] with some modifications. CEase activity was measured by following the hydrolysis of the colorimetric substrate *p*NPB. The temperature was maintained at 25.0 ± 0.2 °C. All compounds and *p*NPB were dissolved in acetonitrile. The final concentration of acetonitrile in the test solution was 1.5%. First, 3.25 U of CEase (2.35 μg mL^{-1}) were preincubated with different concentrations of inhibitor in 0.1 M sodium phosphate buffer (pH 7.04, containing 0.1 M NaCl) for 15 min. Then, the *p*NPB (0.07 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.

Mechanism of CEase inhibition

2a and **2d** were assayed in 0.1 M sodium phosphate buffer (pH 7.04, containing 0.1 M NaCl) using *p*-nitrophenyl butyrate (*p*NPB) as the substrate to determine whether they are reversible or irreversible inhibitors. The temperature was maintained by a temperature controllable water bath kept at 25 ± 0.2 °C. The inhibitors and the enzyme were preincubated in the absence of substrate for different time periods. Then, the *p*NPB (0.10 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 time interval and averaged before further calculation.

Kinetic characterization of CEase inhibition

The general procedure of the kinetic characterization of CEase is also similar to the above inhibition assays. First, 3.25 U of CEase (2.35 μg mL^{-1}) were preincubated with the inhibitor in sodium phosphate buffer for 15 min. Then, different concentrations of *p*NPB 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.[4]

Acknowledgements

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