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New cholesterol esterase inhibitors based on rhodanine and thiazolidinedione scaffolds

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ABSTRACT

We present a new class of inhibitors of pancreatic cholesterol esterase (CEase) based on 'priviledged' 5-benzylidenerhodanine and 5-benzylidene-2,4-thiazolidinedione structural scaffolds. The lead structures (5-benzylidenerhodanine **4a** and 5-benzylidene-2,4-thiazolidinedione **4b**) were identified in an in-house screening and these inhibited CEase with some selectivity over another serine hydrolase, acetylcholinesterase (AChE) (**4a**, CEase IC_{50} = 1.76 μM vs AChE IC_{50} = 5.14 μM and **4b**, CEase IC_{50} = 5.89 μM vs AChE IC_{50} >100 μM). A small library of analogs (**5a**–**10a**) containing a core amino acid in place of the glycerol group of the lead structures, was prepared to explore other potential binding interaction with CEase. These analogs inhibited CEase with IC_{50} values ranging from 1.44 to 85 μM, with the majority exhibiting some selectivity for CEase versus AChE. The most potent compound of the library (**10a**) had 17-fold selectivity over AChE. We also report molecular docking (with CEase) and detailed kinetic analysis on the amino acid analogs to further understand the associated structure–activity relationships.

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1. Introduction

Pancreatic cholesterol esterase (CEase, EC 3.1.1.13) is a member of the α/β hydrolase family of proteins that catalyzes the hydrolysis of cholesterol esters into free cholesterol in the lumen of the small intestine.[1,2] It is also thought to play a role in the transport of cholesterol from micelles to the enterocyte,[1] although there are conflicting reports on this.[3] Given the combined roles of CEase in the absorption and transport of cholesterol, its inhibition is of much interest as a target for developing treatments of hypercholesterolemia and associated diseases such as coronary heart disease.[4] Despite this need, existing inhibitors of CEase are somewhat limited to standard classes of mechanism-based inhibitors[5] such as boronic acids, aryl haloketones, aryl phosphates and various heterocycles.[6–15] These inhibitors target the enzyme's catalytic triad (Ser-His-Asp)[16] which is common to other members of the α/β hydrolase family of proteins.

This family of proteins share a common α/β hydrolase fold that consists of a mostly parallel 5 to 14-stranded β sheet surrounded by several α helices,[16] and a high degree of overall structural homology. For example, the reported structures of carboxyesterases (EC 3.1.1.1), acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8)[17–20] reveal a high degree of similarity in the N-terminal domain, especially around the active-site serine.[21] It is interesting to note that an X-ray structure of pancreatic CEase has been solved at 1.6 Å by molecular replacement based on a truncated model of *Torpedo californica* acetylcholinesterase.[16] This similarity of structure is consistent with reports that inhibitors of CEase also inhibit AChE,[22] with some inhibitors of AChE approved as therapeutics. For example, rivastigmine (Exelon), donepezil (Aricept) and galanthamine (Reminyl) are currently used for the treatment of Alzheimer's disease.[23,24] Selectivity of inhibition therefore presents a challenge and an important consideration, if one is to develop pharmaceuticals based on the inhibition of CEase and/or AChE. Despite this need, there are few studies[6,7] that address this issue within the α/β hydrolase family, particularly with regards to CEase. There is a need for new classes of inhibitors of CEase that are amenable to the preparation of a range of derivatives that will provide an opportunity to explore selectivity of inhibition.

In this paper we present a new class of CEase inhibitors based on 'privileged'[25,26] 5-benzylidenerhodanine and 5-benzylidene-2,4-thiazolidinedione structural scaffolds (see Fig. 1) that exhibit some selectivity for CEase over AChE. The scaffolds were chosen as a starting point since compounds containing them are known to be pharmacologically active;[27] for example as inhibitors of protein tyrosine phosphatases.[28] Certain representatives are of

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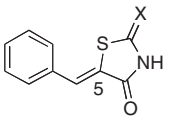


Figure 1. 5-Benzylidenerhodanine (X = S) and 5-benzylidene-2,4-thiazolidinedione (X = O) core structures.

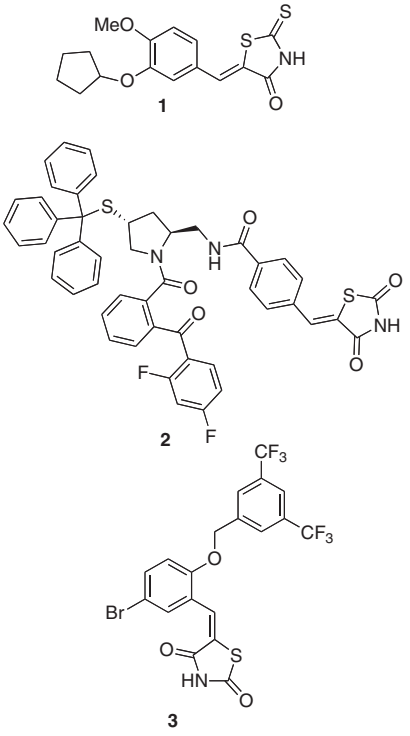


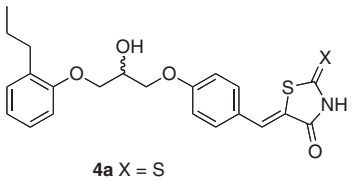
Figure 2. Inhibitors of PDE4[29] (**1**), phospholipase A_2 (**2**)[30,31] and protein tyrosine phosphatases (**3**)[32] bearing the rhodanine and 2,4-thiazolidinedione scaffolds.

particular significance to our study as inhibitors of several esterases and lipases, including PDE4[29] (**1**), phospholipase A_2 (**2**)[30,31] and protein tyrosine phosphatases (**3**)[32] (Fig. 2).

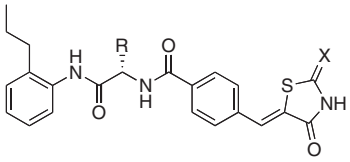
2. Results and discussion

2.1. Inhibitor design

Figure 3 shows the compounds that were prepared in the study. The 5-benzylidenerhodanine (**4a**) and the corresponding 5-benzylidene-2,4-thiazolidinedione (**4b**) were identified as our lead structures based on an in-house initial screening of a small library of available rhodanines and thiazolidines against CEase and AChE. The rhodanine **4a** was shown to inhibit CEase with some selectivity over AChE (CEase IC_{50} = 1.76 μM vs AChE IC_{50} = 5.14 μM, see Table 1). The oxo-analogue **4b**, which has been reported to have antibacterial activity via an unknown mechanism,[33] had slightly reduced potency against CEase, but significantly improved selectivity over AChE (CEase IC_{50} = 5.89 μM vs AChE IC_{50} >100 μM, see Table 1). Given these preliminary results we proposed a small library of analogs of **4a** and **4b**, where the glycerol group is replaced with an amino acid core, in order to introduce structural diversity and potential sites for interaction with CEase (see structures **5**–**10** in Fig. 3). The 5-benzylidene and 2-propylphenyl groups of **4a**/**4b** were retained in all structures to allow direct comparison across the series. We chose to incorporate mainly hydrophobic amino



4a X = S
4b X = O



	X	R
5a	S	CH_2Ph (Phe)
5b	O	CH_2Ph (Phe)
6a	S	Me (Ala)
6b	O	Me (Ala)
7a	S	CH_2OH (Ser)
7b	O	CH_2OH (Ser)
8a	S	$CH_2OC(Me)_3$ (Ser*O*t*Bu*)
8b	O	$CH_2OC(Me)_3$ (Ser*O*t*Bu*)
9a	S	CH_2Ph-4-OH (Tyr)
10a	S	CH_2OCH_2Ph (SerOBn)

Figure 3. 5-Benzylidenerhodanines and 5-benzylidene-2,4-thiazolidine-diones prepared.

acids since the catalytic triad of CEase is known to be flanked by hydrophobic domains, see later for a discussion.[34] Specifically, Phe (**5a** and **5b**), Ala (**6a** and **6b**), Ser*O*t*Bu* (**8a** and **8b**) and SerOBn (**10a**) were introduced into the base structures. More polar residues, Ser and Tyr, were also investigated (**7a**, **7b** and **9a**) to further explore possible hydrogen bonding interactions. Derivatives **5**–**10** have the added advantage of ease of synthesis compared to **4a** and **4b**.

2.2. Chemistry

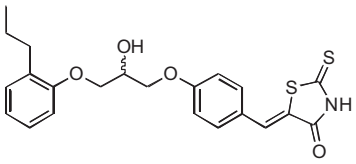
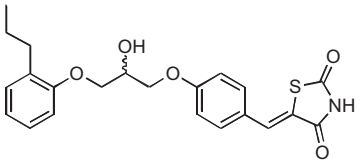
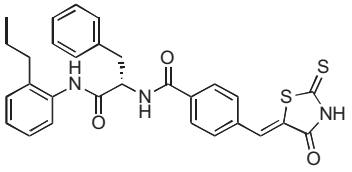
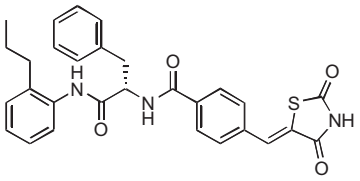
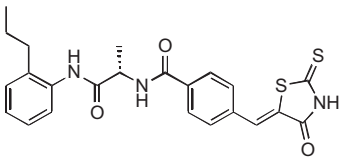
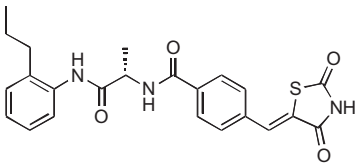
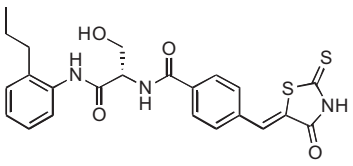
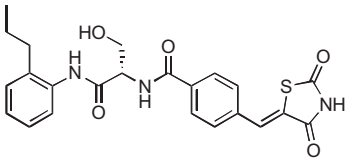
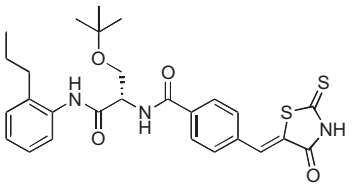
The 5-benzylidene-2,4-thiazolidinedione (**4b**) was prepared by Knoevenagel condensation of the aldehyde **11**[33] with 2,4-thiazolidinedione in ethanol, containing piperidine, as shown in Scheme 1.[35] The novel 5-benzylidenerhodanine (**4a**) was prepared from rhodanine under the same conditions as shown in Scheme 1. ^{1}H NMR analysis of both derivatives revealed a single olefinic resonance, consistent with the formation of a single isomer, which was assigned to have the thermodynamically more stable *Z* configuration.[36,37]

The amino acid-based examples **5**–**10** were prepared as outlined in Schemes 2 and 3. The amines **14a**–**e** (prepared as shown in Scheme 2) were separately coupled with **15a** or **15b**,[30] in the presence of *O*-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxybenzotriazole (HOBT) and diisopropylethylamine (DIPEA) to give **5**, **6** and **8**–**10** (Scheme 3). The *tert*-butyl ether protecting groups of **8a** and **8b** were then removed on treatment with 3 M HCl in dioxane to give the serine derivatives **7a** and **7b**.

2.3. Enzyme inhibition and structure–activity relationships

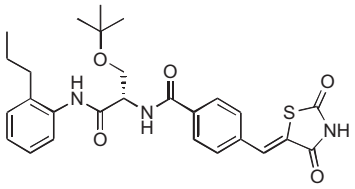
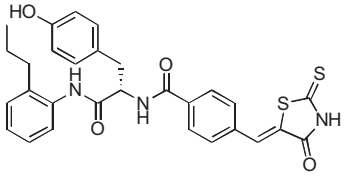
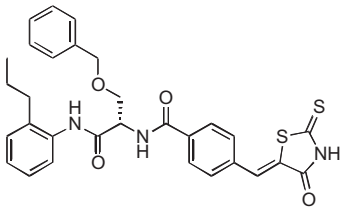
Derivatives **4**–**10** were assayed against both porcine pancreatic CEase and *Electrophorus electricus* AChE with the results given in Table 1. As discussed earlier, compound **4a** inhibited CEase with an IC_{50} of 1.76 μM, while compound **4b** was slightly less potent but more selective over AChE. All the amino acid-based examples

Table 1
Inhibitory activity against CEase and AChE

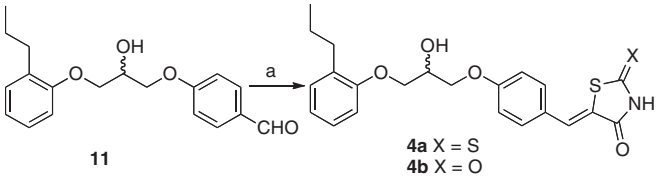
	Compound	CEase		AChE[a]
		IC_{50}[a] (μM)	$v_{[I]\to\infty}$(%)[b]	IC_{50} (μM) [a]
4a		1.76 ± 0.17	18%	5.14 ± 0.72[c]
4b		5.89 ± 0.42	17%	>100
5a		40		43
5b		40		14
6a		50		>100
6b		54		>100
7a		31		>100
7b		5.70 ± 0.43	12%	>100
8a		6.07 ± 0.43		21.6 ± 0.6[c]

(*continued on next page*)

Table 1 (*continued*)

Compound		CEase		AChE[a]
		IC_{50}[a] (μM)	$v_{[I]\to\infty}$(%)[b]	IC_{50} (μM) [a]
8b		85		66
9a		14.5 ± 1.3		57
10a		1.44 ± 0.57	25%	25

[a] Limits without standard error were determined from duplicate experiments at a single inhibitor concentration. Values without standard error were from quadruplicate experiments at a single inhibitor concentration. Values with standard error were from duplicate experiments with five different inhibitor concentrations.
[b] Residual activity at infinite inhibitor concentration.
[c] A three-parameter model was used for non-linear regression.



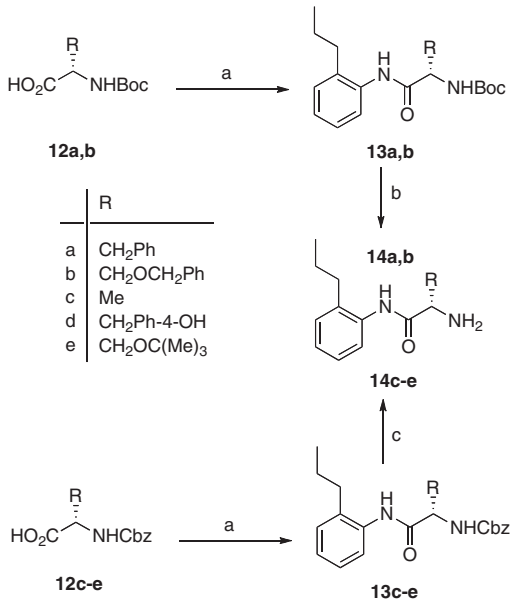
Scheme 1. a) piperidine, rhodanine or 2,4-thiazolidinedione, EtOH.

were active against CEase, with compounds **7b**, **8a** and **10a** being the most potent. Some limited structure-activity relationships can be drawn. A large hydrophobic amino acid side chain appears to be well tolerated in **8a**, **9a**, **10a**. Among the more hydrophilic serine-based compounds, the thiazolidinedione **7b** shows enhanced potency compared to the rhodanine analogue **7a**. The activity of **7a** (but not of **7b**) was significantly improved on protecting the hydroxyl group as the hydrophobic *tert*-butyl ether (**8a**). It is noteworthy that we identified inhibitors (**4b**, **7b**, **10a**) that show selectivity for CEase over AChE (greater than 15-fold). However, these derivatives are significantly less potent than the known phosphorylated flavones[15], chloropyrones[10] and chloroisocoumarins[9] inhibitors.

Compounds **15–16** (Fig. 4) were also assayed against both CEase and AChE, with all being inactive (IC_{50} values greater than 100 μM for both enzymes). As such, the benzylidenerhodanine and benzylidenethiazolidinedione groups alone are not sufficient for activity and a structural extension is required to inhibit these enzymes.

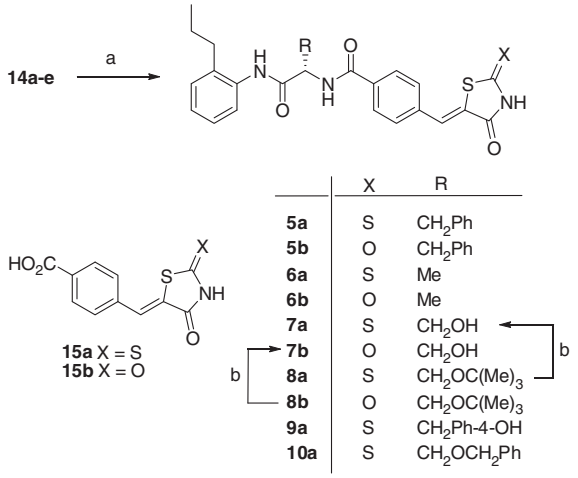
2.4. Docking studies

The inhibitors **4–10** were docked with bovine CEase (PDB entry: 1AQL[38]) and AChE from *Mus musculus* (PDB entry: 2JEY[39]) in order to gain some insight into the inhibitory results shown in Table 1. These X-ray crystallography structures (1AQL and 2JEY) were

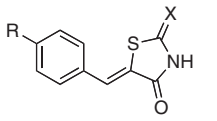


Scheme 2. (a) EDCI, HOBT, DIPEA, 2-propylaniline, DMF; (b) 10% TFA, DCM; (c) 5% Pd/C, cyclohexene, ethyl acetate.

chosen since a structure of porcine CEase and a high-resolution structure of *E. electricus* AChE (used in the assays) were not available. The two pdb files required minimal editing before application of AutoDock[40,41] and importantly, both contain ligands, taurocholate in the case of CEase and an active site bound oxime in the case of AChE. An alignment of the two structures using PyMol[42] revealed a strong similarity (see Fig. 5) and a calculated RMSD of 2.12 Å. This result is consistent with the observation that some inhibitors of CEase also inhibit AChE.[22]



Scheme 3. (a) **15a** or **15b**, HATU, HOBT, DIPEA, DMF; (b) 10% TFA, DCM.



15a R = CO_2H, X = S
15b R = CO_2H, X = O
16a R = H, X = S
16b R = H, X = O

Figure 4. Benzylidenerhodanine and benzylidenethiazolidinedione analogs lacking the amino acid or glycerol functionality.

Docking studies were then carried out using AutoDock4 by performing 25 docking runs on each inhibitor using the Lamarckian Genetic Algorithm[40] and a maximum of 500,000 energy evaluations. Meaningful results were obtained for CEase, with predicted binding energies ranging from −10.2 to −7.0 kcal/mol. However, despite the similarity of structure, AutoDock4 was unable to place any of the inhibitors within the active site of AChE. Thus the following discussion on docking is limited to CEase, with some comparative analysis.

Based on the docking results, two groups of inhibitors of CEase were apparent. Group 1 (**4a**, **4b**, **6a**, **6b**, **7a** and **7b**) interact similarly with CEase, with the thiazolidinedione and rhodanine moieties binding in the vicinity of residues Ala113, Ala117, Leu124 and Tyr125 (see Fig. 6). This commonality of binding is reflected by the observation that both classes similarly inhibit CEase, see Table 1. The 2-propylphenyl moieties of these inhibitors all bind to a hydrophobic pocket defined by Ala108, Leu110, Leu272, Leu285 and Phe324 (see box in Fig. 6, residues not shown), which is distant from the catalytic triad. A similar pocket, found in the structure of human CEase, is known to bind the hydrophobic fatty acid portion of the native cholesterol ester substrates.[43]

Group 2 inhibitors (**5a**, **5b**, **8a**, **8b**, **9a** and **10a**) all contain a relatively large hydrophobic amino acid side chain (R in Fig. 3). For **5a** (R = CH_2Ph) and **10a** (R = CH_2OCH_2Ph), these groups occupy a hydrophobic pocket defined by Leu282, Leu392 and Trp227, see Figure 7. The 2-propylphenyl groups of each compound reside just outside this pocket. Compounds **5b** and **9a** (not shown) bind to CEase in a similar fashion. The docking of compound **8a** and **8b** to CEase proved interesting, with its O*t*Bu moiety orientated towards Ala436 and the 2-propylphenyl functionality rotated 180° away from the hydrophobic pocket (Fig. 8).

The group 2 compounds are by and large more potent toward AChE than are the group 1 compounds, see Table 1. This finding might be helpful for further investigations directed towards selectivity issues of esterase inhibitors.

2.5. Kinetic analysis of CEase inhibition

The kinetics of this new class of CEase inhibitors were studied in detail using compound **4a.** Interestingly, this compound did not completely inhibit CEase at high concentrations as plots of the rate of the enzymatic reaction versus the inhibitor concentration, [I], did not become asymptotic to the *x*-axis (see Fig. 9). Therefore, a residual activity at infinite concentration of **4a** ($v_{[I]\to\infty}$ = 18%) had to be considered. Such a behavior was also observed for benzylidenethiazolidinediones **4b** and **7b** as well as benzylidenerhodanine **10a** which showed values $v_{[I]\to\infty}$ of 12–25%. Occurrences of such residual activities at an infinite inhibitor concentration has recently been described for the inhibition of CEase[44] and related α/β hydrolases, such as AChE[7] and butyrylcholinesterase.[45] To characterize the inhibition of CEase by **4a**, a kinetic model that represents the general modifier mechanism[46] was considered (Scheme 4). In this model the substrate (S) and inhibitor (I) bind to the enzyme (E) at different sites. The dissociation constant of EI is K_i, and the dissociation constant of the ternary complex, ESI, to form ES and I is αK_i. Product formation from ES is governed by the catalytic constant k_P. The complex ESI is still functional with a decreased rate of product formation governed by βk_P. If

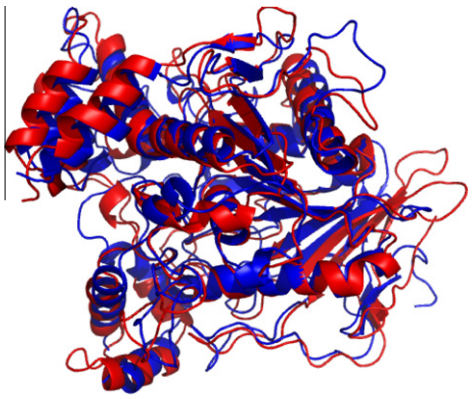


Figure 5. Overlay of bovine CEase (PDB entry: 1AQL, red) and *Mus musculus* AChE (PDB entry: 2JEY, blue).

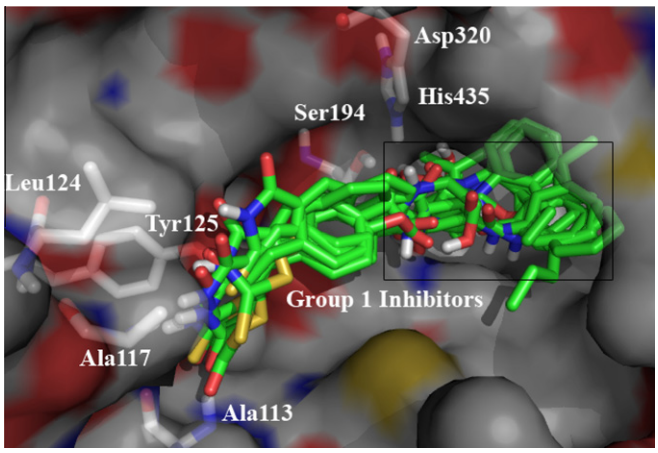


Figure 6. Docking of group 1 inhibitors (green) with CEase (PDB: 1AQL). The gray (hydrophobic), blue (basic) and red (acidic) surface represents the active site of the enzyme. The catalytic triad is shown (Ser194, Asp320 and His435). The rectangular box highlights the hydrophobic pocket.

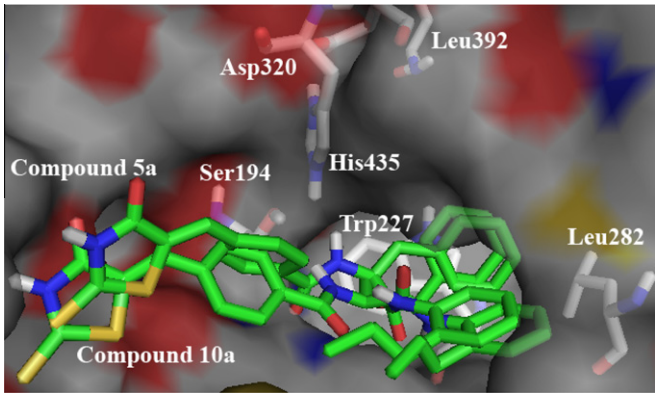


Figure 7. Docking of **5a** and **10a** within the active site of CEase. The catalytic triad is shown. Trp227, Leu282, Leu392 define a key hydrophobic pocket.

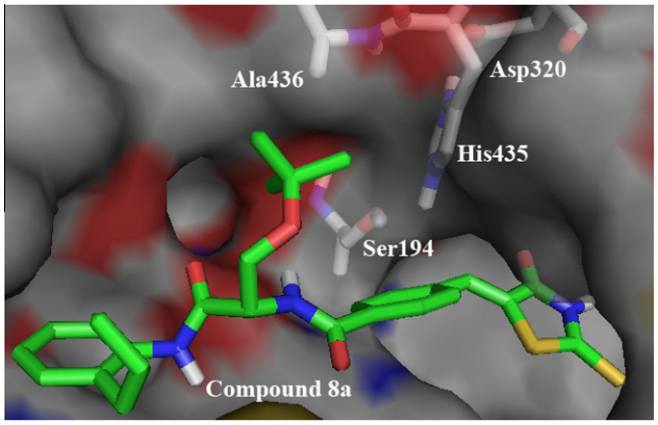


Figure 8. Docking of **8a** within the active site of CEase, with Ala436 highlighted. The catalytic triad is shown.

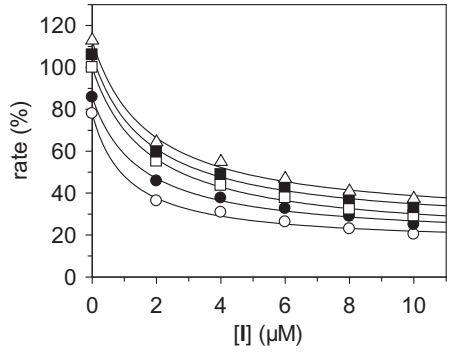
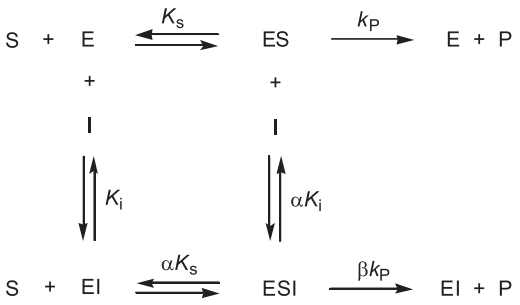


Figure 9. Inhibition of CEase by compound **4a**. The data are mean values of quadruplicate experiments with substrate concentrations of [S] = 300 μM (open triangles), 250 μM (closed squares), 200 μM (open squares), 150 μM (closed circles), and 100 μM (open circles). At a substrate concentration of 200 μM, values IC_{50} = 1.76 ± 0.17 μM and $v_{[I]\to\infty}$ = 17.8 ± 1.9% were obtained by nonlinear regression according to Eq. 5.

$0 < \beta < 1$, the type of inhibition is referred to as 'hyperbolic mixed-type inhibition'.

In order to obtain the kinetic parameters K_i, α, and β, CEase inhibition by compound **4a** was analyzed using the specific velocity plot according to Eq. 1[47] where v_0 and v are the rates in the absence and presence of inhibitor and $\sigma = [S]/K_m$. Values v_0/v were plotted versus $\sigma/[1+\sigma]$, and the intercepts on the ordinate axis for



Scheme 4. Kinetic model of the general modifier mechanism.

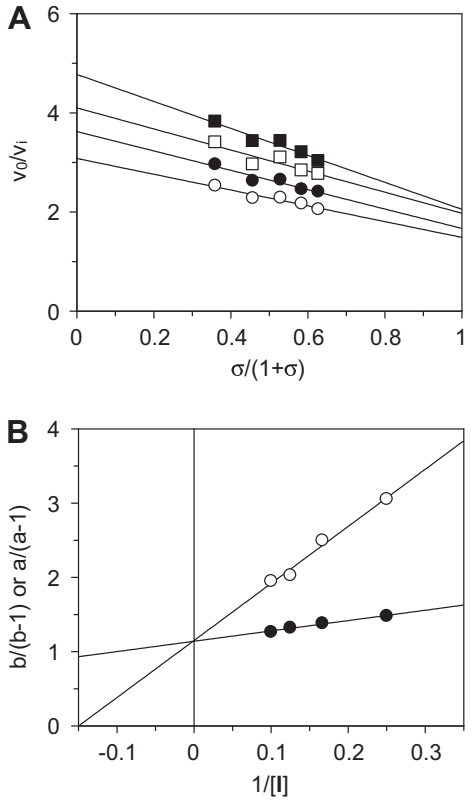


Figure 10. (A) Specific velocity plot for the inhibition of CEase by compound **4a**. Rates measured at [I] = 4 μM (open circles), 6 μM (closed circles), 8 μM (open squares), and 10 μM (closed squares) were used. The values $\sigma/[1+\sigma]$, where $\sigma = [S]/K_m$, were calculated using the separately determined K_m value of 178 μM and [S] = 100, 150, 200, 250, and 300 μM. Linear regression according to Eq. 1 gave values a and b. (B) Replots $a/(a-1)$ versus 1/[I] (closed circles) and $b/(b-1)$ versus 1/[I] (open circles) for the inhibition of CEase by **4a**. Linear regression according to Eq. 2 gave a slope $\alpha K_i/(\alpha-\beta)$ = 1.39 μM and an intercept $\alpha/(\alpha-\beta)$ = 1.14; linear regression according to Eq. 3 gave a slope $\alpha K_i/(1-\beta)$ = 7.68 μM and an intercept $1/(1-\beta)$ = 1.15. Values β = 0.13 (using the intercept of Eq. 3) and α = 5.5 (quotient of αK_i and K_i) were calculated. A value K_i = 1.22 μM resulted from the quotient of slope and intercept of the plot $a/(a-1)$ versus 1/[I].

$\sigma/[1+\sigma] = 0$, as well as $\sigma/[1+\sigma] = 1$, that is, values a and b, were used in replots according to Eqs. 2 and 3.

$$\frac{v_0}{v_i} = \frac{\left(\frac{1}{\alpha K_i} - \frac{1}{K_i}\right)[I]}{1 + \frac{\beta[I]}{\alpha K_i}} \times \frac{\sigma}{1+\sigma} + \frac{1 + \frac{[I]}{K_i}}{1 + \frac{\beta[I]}{\alpha K_i}} \quad (1)$$

$$\frac{a}{a-1} = \frac{\alpha K_i}{\alpha - \beta} \times \frac{1}{[I]} + \frac{\alpha}{\alpha - \beta} \quad (2)$$

$$\frac{b}{b-1} = \frac{\alpha K_i}{1 - \beta} \times \frac{1}{[I]} + \frac{\alpha}{1 - \beta} \quad (3)$$

From these replots, the values K_i and αK_i were obtained. The parameter α was then calculated from K_i and αK_i whereas β was obtained from the intercept of Eq. 3.

Analysis of CEase inhibition by **4a** on the basis of Eqs. 1–3 revealed hyperbolic mixed-type inhibition with a pronounced competitive component (α >1). This is shown by the common intersection point of the lines in the specific velocity plot at $\sigma/[1+\sigma] > 1$ (Fig. 10A) and the intercepts of the replots being greater than one (Fig. 10B).[47] On the basis of these replots, the parameters K_i = 1.22 μM, α = 5.5 and β = 0.13 were determined. The value α indicates a 5- to 6-fold higher affinity of **4a** to free CEase than to the substrate-bound enzyme. However, the parameter β represents a relative efficiency of ESI conversion to product (at saturating [I]),[48] which was found to be 13% compared to that of ES. This value is in agreement with the value $v_{[I]\to\infty}$ = 18% calculated under the assumption of an infinite inhibitor concentration. While the molecular docking results (Fig. 6) suggest that **4a** might interact with the active site in the EI complex, the arrangement of the corresponding ESI complex might be different to allow for a residual catalytic activity.

The observed residual activity of CEase in the presence of high concentrations of **4a** (Fig. 9) might be explained with the so-called 'side-door', a secondary pore adjacent to the active site. This side door in CEase and related carboxylesterases as found by crystallographic studies potentially acts as an alternative opening allowing for the traffic of substrates and products.[49–53]

3. Conclusion

CEase plays an important role in the absorption and transport of cholesterol and is as such an interesting target for developing treatments for diseases such as hypercholesterolemia and coronary heart disease. The design of potent inhibitors of CEase, which show selectivity against other members of the α/β hydrolase family, is a significant challenge. The present study contributes one possible solution to this problem. The 'priviliged' rhodanine and thiazolidinedione scaffolds were chosen, as substances bearing them are known to inhibit structurally homologous proteins such as PDE4 and protein tyrosine phosphatases. A series of inhibitors with a central glycerol or amino acid unit and an additional 2-propylphenyl moiety was investigated and possible binding interactions with CEase were explored using molecular docking and kinetic studies. Most of the compounds displayed some selectivity for CEase over the structurally homologous AChE. The optimum compound (**10a**) had an IC_{50} value of 1.44 μM against CEase, with 17-fold selectivity for CEase versus AChE. The paper presents a new class of CEase inhibitors, with significant scope for further optimization of potency and selectivity against other members of the α/β hydrolase family.

4. Experimental

4.1. General chemistry

All reagents and solvents were from standard commercial sources and of reagent grade. Reactions were monitored by ascending TLC using precoated plates (silica gel 60 F_{254}, 250 μm, Merck, Darmstadt, Germany), spots were visualized with ultraviolet light at 254 nm and sulfuric acid–vanillin spray. Column chromatography was performed with silica gel (40–63 μm, 60 Å, Davisil, Grace, Germany). Melting points were recorded uncorrected on a Reichert Thermovar Kofler microscope. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 (300 MHz) or a Varian Inova 600 MHz. Chemical shifts are given in ppm (δ) relative to the residue signals, which in the case of DMSO-d_6 were 2.50 ppm for ^1H and 39.55 ppm for ^{13}C and $CDCl_3$ were 7.26 ppm for ^1H and 77.23 ppm for ^{13}C. Structural assignment was confirmed with COSY, ROESY, HMQC and HMBC. High-resolution mass spectra (HRMS) were recorded on a Thermo Fisher Scientific LTQ orbitrap FT MS equipment (Δ <2 ppm). Analytical reversed-phased HPLC measurements were performed on a HP Series 1100 with a Phenomenex Gemini C18 5 μM (250 × 4.60 mm), tested samples were dissolved in dimethylformamide and eluted at a flow rate of 1 mL/min with a linear gradient of 40–90% CH_3CN in 0.01% TFA/H_2O over 15 min, followed by 90% CH_3CN in 0.01% TFA/H_2O over 10 min. Purity of tested samples was > 95% or as reported. Compounds **4b**,[33] **11**,[33] **16a**[54] and **16b**[55] were synthesized according to reported procedures.

4.2. General procedures

4.2.1. General procedure A (13a–e)

The *N*-protected amino acid **12a-e** (1 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.2 mmol), HOBt (1.2 mmol), DIPEA (4.75 mmol) and 2-propylaniline (1.2 mmol) were suspended in anhydrous DMF (5 mL). The reaction mixture was stirred at rt overnight under nitrogen. 1 M HCl (50 mL) was added and the mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic phases were washed with 1 M HCl (50 mL), brine (50 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography on silica gel to yield **13a-e**.

4.2.2. General procedure B (14a,b)

The *N*-Boc-amino acid **13a,b** (0.3 mmol) was suspended in 10% trifluoroacetic acid in anhydrous dichloromethane (5 mL). After stirring overnight the reaction mixture was concentrated in vacuo. The residue was diluted with methanol (5 mL). Water was added and the precipitate was collected by filtration and dried in vacuo to yield **14a** and **14b**.

4.2.3. General procedure C (14c–e)

The *N*-Cbz-amino acid **13c–e** (0.4 mmol) and cyclohexene (2.0 mmol) in ethyl acetate (2 mL) was stirred at rt under nitrogen. 10% Pd/C (0.16 mmol) was added and the reaction was refluxed for 2 h. After cooling to rt the mixture was filtered and washed with ethyl acetate. The filtrate was dried over anhydrous Na_2SO_4 and concentrated in vacuo to yield the desired amine to yield **14c**–**e**.

4.2.4. General procedure D (5a, 5b, 6a, 6b, 8a, 8b, 9a, and 10a)

The carboxylic acids **15a**,[31] or **15b**[31] (0.22 mmol), HATU (0.24 mmol), HOBT (0.24 mmol), DIPEA (0.95 mmol) and amino acid anilide **14a–e** (0.20 mmol) were suspended in anhydrous DMF (2 mL). The reaction mixture was stirred at rt overnight under nitrogen. 1 M HCl (10 mL) was added and the mixture was extracted with ethyl acetate (3 × 25 mL). The combined organic phases were washed with 1 M HCl (50 mL), brine (50 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography on silica gel to yield **5a**, **5b**, **6a**, **6b**, **8a**, **8b**, **9a**, and **10a**.

4.3. Spectral data

4.3.1. (*Z*)-5-[[4-[(2*R*,*S*)-2-Hydroxy-3-(2-propylphenoxy) propoxy]phenyl]methylene]-2-thioxo-thiazolidin-4-one (4a)

To a solution of rhodanine (31 mg, 0.23 mmol) in anhydrous ethanol (10 mL), aldehyde **11** (73 mg, 0.23 mmol) and piperidine (4.6 μL, 0.046 mmol) were added and refluxed under nitrogen for 8 hrs. The reaction was cooled to rt, diluted and precipitated with glacial acetic acid (3 mL). The mixture was filtered and washed with cold water (2 × 15 mL) and ethanol (2 × 15 mL). The

precipitate was dissolved in toluene and concentrated in vacuo to give **4a** Yield: 50 mg, 50%. mp: 161–163 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.92 (br s, 1H) 7.57–7.54 (m, 3H), 7.17–7.09 (m, 4H), 7.00 (d, J = 8.4 Hz, 1H), 6.89 (t, J = 7.2 Hz, 1H), 5.43 (br d, J = 4.8 Hz, 1H), 4.25–4.02 (m, 5H), 2.54–2.49 (m, 6H, signal overlap with DMSO), 1.56 (tq, J = 7.5, 7.2 Hz, 2H), 0.86 (t, J = 7.5, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 196.2, 170.9, 156.2, 133.6, 131.1, 130.3, 129.6, 127.0, 125.8, 123.3, 121.4, 115.5, 112.5, 72.3, 69.7, 68.8, 31.4, 23.6, 14.9. HRMS calcd for $C_{22}H_{22}NO_4S_2$ m/z [M-H]$^-$: 428.0990; found: 428.0984. HPLC t_R = 16.94 min (96.94%).

4.3.2. *N*-[(1*S*)-1-Benzyl-2-oxo-2-(2-propylanilino)ethyl]-4-[(*Z*)-(4-oxo-2-thioxo-thiazolidin-5-ylidene)methyl]benzamide (5a)

Reaction of **15a** (58 mg) with **14a** (73 mg) according to general method D gave **5a** (60 mg, 57%). mp: 208–211 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.09 (s, 1H), 8.85 (d, J = 8.4 Hz, 1H), 7.92 (d, J = 8.4 Hz, 2H), 7.59 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 7.2 Hz, 2H) 7.31–7.35 (m, 1H), 7.28–7.31 (m, 3H), 4.90–4.94 (m, 1H), 3.69 (br s, 1H), 2.89 (dd, J = 4.8 Hz, 13.8 Hz, 1H), 2.81–2.86 (m, 1H), 2.13–2.22 (m, 2H), 1.14 (tq, J = 7.2 Hz, 7.8 Hz, 2H), 0.51 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 198.6, 170.1, 165.6, 163.2, 137.9, 137.0, 136.3, 135.2, 133.5, 129.2 (×2), 128.9, 128.3, 127.9, 126.1, 125.7, 125.6, 125.5, 125.3, 55.2, 36.7, 32.6, 22.6, 13.6. HRMS calcd for $C_{29}H_{28}N_3O_3S_2$ m/z [M+H]$^+$ 530.1569; found 530.1567. HPLC t_R = 16.11 min (97.40%).

4.3.3. *N*-[(1*S*)-1-Benzyl-2-oxo-2-(2-propylanilino)ethyl]-4-[(*Z*)-(2,4-dioxothiazolidin-5-ylidene)methyl]benzamide (5b)

Reaction of **15b** (54 mg) with **14a** (73 mg) according to general method D gave **5b** (71 mg, 69%). mp: 175–178 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.52 (br s, 1H), 8.84 (d, J = 7.8 Hz, 1H), 7.95 (d, J = 8.4 Hz, 2H), 7.69 (s, 1H), 7.65 (d, J = 8.4 Hz, 2H), 7. 42 (d, J = 7.8 Hz, 2H), 7.28–7.34 (m, 2H), 7.11–7.21 (m, 3H), 6.53 (br s, 1H), 4.92–4.96 (m, 1H), 3.23 (dd, J = 4.8 Hz, 13.8 Hz, 1H), 3.10–3.26 (m, 1H), 2.46–2.55 (m, 2H), 1.47 (tq, J = 7.2 Hz, 7.8 Hz, 2H), 0.84 (t, J = 7.2 Hz). ^{13}C NMR (150 MHz, DMSO-d_6): δ 171.4, 170.5, 169.8, 165.8, 138.2, 136.7, 136.6, 135.4, 134.3, 129.5 (×2), 129.3, 128.2, 126.4, 126.1, 125.7, 49.8, 36.1, 32.9, 23.0, 13.9. HRMS calcd for $C_{29}H_{28}N_3O_4S$ m/z [M+H]$^+$ 514.1795; found 514.1801. HPLC t_R = 14.85 min (98.16%).

4.3.4. *N*-[(1*S*)-1-Methyl-2-oxo-2-(2-propylanilino)ethyl]-4-[(*Z*)-(4-oxo-2-thioxo-thiazolidin-5-ylidene)methyl]benzamide (6a)

Reaction of **13c** (134 mg) according to general method C gave **14c** (56 mg, 69%). mp: 124–125 °C. ^{1}H (300 MHz, CD_3OD): δ 9.86 (br s, 1H), 8.09 (d, J = 7.8 Hz, 1H), 7.02–7.23 (m, 3H), 3.63 (q, J = 7.2 Hz, 1H), 2.58 (t, J = 7.8 Hz, 2H), 1.63 (qt, J = 7.8 Hz, 7.2 Hz, 2H), 1.42 (d, J = 7.2 Hz, 3H) 0.98 (t, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD): δ 173.5, 135.3, 132.0, 129.5, 126.6, 124.2, 121.4, 51.3, 33.5, 22.8, 21.5, 13.9.

Reaction of **15a** (58 mg) with **14c** (41 mg) according to general method D gave **6a** (54 mg, 60%). mp: 144–146 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.32 (s, 1H), 8.73 (d, J = 6.6 Hz, 1H), 8.01 (d, J = 8.4 Hz, 2H), 7.60 (d, J = 8.4 Hz, 2H), 7.37 (d, J = 7.8 Hz, 1H), 7.31 (s, 1H), 7.09–7.19 (m, 3H), 6.51 (s, 1H), 4.64–4.69 (m, 1H), 2.49–2.55 (m, 2H), 1.43–1.51 (m, 2H), 1.46 (d, J = 7.2 Hz, 3H), 0.83 (t, J = 7.8 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 171.4, 165.7, 137.5, 136.4, 135.5, 133.5, 129.4, 129.3, 128.2, 126.0, 125.8, 125.4, 124.1, 49.7, 32.9, 22.9, 18.3, 13.8. HRMS calcd for $C_{23}H_{24}N_3O_3S_2$ m/z [M+H]$^+$ 454.1254; found 454.1258. HPLC t_R = 13.14 min (97.66%).

4.3.5. 4-[(*Z*)-(2,4-Dioxothiazolidin-5-ylidene)methyl]-*N*-[(1*S*)-1-methyl-2-oxo-2-(2-propylanilino)ethyl]benzamide (6b)

Reaction of **15b** (54 mg) with **14c** (41 mg) according to general method D gave **6b** (65 mg, 74%). mp: 168–170 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.32 (s, 1H), 8.74 (d, J = 6.6 Hz, 1H), 8.02 (d, J = 8.4 Hz, 2H), 7.66 (d, J = 8.4 Hz, 2H), 7.64 (s, 1H), 7.37 (d, J = 7.2 Hz, 1H), 7.19 (d, J = 7.2 Hz, 1H), 7.09–7.17 (m, 2H), 6.51 (br s, 1H), 4.65–4.70 (m, 1H), 2.46–2.55 (m, 2H), 1.42–1.50 (m, 5H), 0.83 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 171.4, 170.4, 168.0, 165.7, 136.9, 136.4, 135.5, 134.0, 129.4, 129.2, 128.2, 127.0, 126.0, 125.8, 125.4, 49.6, 32.9, 22.9, 17.5, 13.8. HRMS calcd for $C_{23}H_{24}N_3O_4S$ m/z [M+H]$^+$ 438.1482; found 438.1489. HPLC t_R = 11.02 min (98.43%).

4.3.6. *N*-[(1*S*)-1-(Hydroxymethyl)-2-oxo-2-(2-propylanilino)ethyl]-4-[(*Z*)-(4-oxo-2-thioxo-thiazolidin-5-ylidene)methyl]benzamide (7a)

Compound **8a** (47 mg, 0.1 mmol) was suspended in 10% trifluoroacetic acid in dichloromethane (5 mL) at rt. After stirring overnight the reaction mixture was concentrated in vacuo and dissolved in methanol (5 mL). Water (50 mL) was added and the precipitate was filtered and air dried to give a off-white powder (15 mg, 37%). mp: 165–168 °C. ^{1}H NMR (600 MHz, DMSO-d_6): 13.9 (s, 1H), 9.45 (s, 1H), 8.79 (d, J = 7.8 Hz, 1H), 8.03 (d, J = 7.8 Hz, 2H), 7.66 (d, J = 7.8 Hz, 2H), 7.51 (s, 1H), 7.36 (d, J = 7.8 Hz, 1H), 7.21 (d, J = 7.2 Hz, 1H), 7.11–7.20 (m, 2H), 4.76–4.80 (m, 1H), 3.71–3.77 (m, 2H), 2.55–2.58 (m, 2H), 1.44 (qt, J = 7.2 Hz, 6.6 Hz, 2H), 1.19 (s, 9H), 0.78 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 168.6, 165.9, 138.1, 136.6, 136.5, 135.3, 134.3, 129.8, 129.4, 128.7, 128.4, 128.2, 127.5, 127.4, 127.3, 126.0, 125.9, 125.6, 72.2, 69.4, 32.8, 22.8, 13.8. HRMS calcd for $C_{23}H_{24}N_3O_4S_2$ m/z [M+H]$^+$ 470.1203; found 470.1269. HPLC t_R = 11.03 min (95.30%).

4.3.7. 4-[(*Z*)-(2,4-Dioxothiazolidin-5-ylidene)methyl]-*N*-[(1*S*)-1-(hydroxymethyl)-2-oxo-2-(2-propylanilino)ethyl]benzamide (7b)

Compound **8b** (45 mg, 0.1 mmol) was suspended in 10% trifluoroacetic acid in dichloromethane (5 mL) at rt. After stirring overnight the reaction mixture was concentrated in vacuo and dissolved in methanol (5 mL). Water (50 mL) was added and the precipitate was filtered and air dried to give a white powder (20 mg, 50% yield). mp: 116 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 8.54 (s, 1H), 7.94 (d, J = 8.2 Hz, 2H), 7.83 (d, J = 8.2 Hz, 1H), 7.78 (s, 1H), 7.55 (d, J = 7.8 Hz, 2H), 7.47 (d, J = 6.0 Hz, 1H), 7.24–7.08 (m, 3H), 4.87–4.75 (m, 1H), 4.09 (dd, J = 9.0, 1.8 Hz, 1H), 3.60 (t, J = 9.0 Hz, 1H), 2.60 (t, J = 7.2 Hz, 2H), 1.69–1.59 (m, 2H), 0.99–0.93 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 169.0, 166.6, 166.4, 166.3, 136.1, 135.0, 134.6, 133.8, 132.4, 130.2, 129.6, 128.1, 127.9, 126.6, 125.6, 124.7, 123.7, 75.3, 61.4, 33.2, 22.9, 13.9. HRMS calcd for $C_{23}H_{24}N_3O_5S$ m/z [M+H]$^+$ 454.1437; found 454.1452. HPLC t_R = 14.52 min (95.00%).

4.3.8. *N*-[(1*S*)-1-(*tert*-Butoxymethyl)-2-oxo-2-(2-propylanilino)ethyl]-4-[(*Z*)-(4-oxo-2-thioxo-thiazolidin-5-ylidene)methyl]benzamide (8a)

Reaction of **15a** (58 mg) with **14e** (55 mg) according to general method D gave **8a** (92 mg, 88%). mp: 156–160 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.16 (s, 1H), 8.31 (d, J = 7.8 Hz, 1H), 8.02 (d, J = 8.4 Hz, 2H), 7.59 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.4 Hz, 1H), 7.51 (s, 1H), 7.10–7.20 (m, 3H), 4.85 (dd, J = 5.7 Hz, 13.4 Hz, 1H), 3.76–3.86 (m, 2H), 2.55–2.60 (m, 2H) 1.50–1.63 (m, 2H), 1.24 (s, 9H), 0.9 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 196.7, 172.6, 167.5, 164.6, 135.3, 134.2, 134.2, 133.8, 132.9, 129.7, 128.3, 127.9, 126.8, 126.1, 124.5, 123.9, 123.6, 71.9, 60.2, 53.2, 31.5, 25.9, 21.4, 12.4. HRMS calcd for $C_{27}H_{32}N_3O_4S_2$ m/z [M+H]$^+$ 526.1829; found 526.1840. HPLC t_R = 16.41 min (97.22%).

4.3.9. *N*-[(1*S*)-1-(*tert*-Butoxymethyl)-2-oxo-2-(2-propylanilino) ethyl]-4-[(*Z*)-(2,4-dioxothiazolidin-5-ylidene) methyl]benzamide (8b)

Reaction of **15b** (54 mg) with **14e** (55 mg) according to general method D gave **8b** (87 mg, 85%). mp: 141–143 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 12.59 (br, 1H), 9.40 (s, 1H), 8.57 (d, J = 7.8 Hz, 1H), 8.01 (d, J = 7.8 Hz, 2H), 7.23 (s, 1H), 7.68 (d, J = 7.8 Hz, 2H), 7.35 (d, J = 7.8 Hz, 1H), 7.20 (d, J = 7.2 Hz, 1H), 7.16 (t, J = 7.2 Hz, 1H), 7.11 (dd, J = 7.2 Hz, 7.8 Hz, 1H), 4.75–4.78 (m, 1H), 3.69–3.76 (m, 2H), 2.52–2.55 (m, 2H), 1.49 (tq, J = 7.2 Hz, 6.6 Hz, 2H), 1.15 (s, 9H), 0.84 (t, J = 6.6 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 169.1, 169.0, 165.7, 136.5, 136.4, 135.5, 134.5, 129.5, 129.3, 128.7, 128.3, 125.9, 125.8, 125.6, 73.0, 61.6, 54.8, 31.3, 27.3, 22.7, 13.9. HRMS calcd for $C_{27}H_{32}N_3O_5S$ m/z $[M+H]^+$ 510.2057; found 510.2068.

4.3.10. *N*-[(1*S*)-1-[(4-Hydroxyphenyl)methyl]-2-oxo-2-(2-propylanilino)ethyl]-4-[(*Z*)-(4-oxo-2-thioxo-thiazolidin-5-ylidene)methyl]benzamide (9a)

Reaction of **15a** (58 mg) with **14d** (60 mg) according to general method D gave **9a** (34 mg, 31%). mp: 262–265 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.90 (s, 1H), 9.57 (s, 1H), 8.96 (d, J = 7.8 Hz, 1H), 8.21 (d, J = 8.4 Hz, 2H), 7.98 (d, J = 8.4 Hz, 2H), 7.79 (d, J = 8.4 Hz, 2H), 7.68 (m, 3H), 7.31 (d, J = 7.8 Hz, 1H), 7.09–7.25 (m, 3H), 6.66 (d, J = 9.0 Hz, 1H), 4.95–4.99 (m, 1H), 3.25–3.31 (m, 1H, overlap with H_2O), 3.15–3.20 (m, 1H), 2.51–2.56 (m, 2H), 1.44–1.52 (m, 2H), 0.85 (t, J = 7.8 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 195.6, 170.3, 165.7, 163.9, 149.1, 137.9, 136.7, 135.4, 135.1, 130.6, 130.5, 130.3, 130.2, 129.9, 129.4, 128.7, 127.3, 126.0, 125.7, 121.5, 55.4, 36.3, 32.9, 22.9, 13.9. HRMS calcd for $C_{29}H_{28}N_3O_4S_2$ m/z $[M+H]^+$ 546.1516; found 546.1524. HPLC t_R = 14.77 min (95.38%).

4.3.11. *N*-[(1*S*)-1-(Benzyloxymethyl)-2-oxo-2-(2-propylanilino)ethyl]-4-[(*Z*)-(4-oxo-2-thioxo-thiazolidin-5-ylidene)methyl]benzamide (10a)

Reaction of **15a** (58 mg) with **14b** (85 mg) according to general method D gave **10a** (95 mg, 85%). mp 112–115 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.48 (s, 1H), 8.79 (d, J = 7.2 Hz, 1H), 8.03 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 8.4, 2H), 7.48 (s, 1H), 7.27–7.36 (m, 5H), 7.10–7.19 (m, 3H), 6.51 (br, 1H), 4.97 (dd, J = 7.2 Hz, 13.2 Hz, 1H), 4.57–4.61 (m,2H), 3.84–3.90 (m, 2H), 2.45–254 (m, 2H), 1.41–1.46 (m, 2H), 0.78 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 196.0, 168.7, 165.9, 138.1, 136.7, 136.5, 135.4, 134.2, 129.8 (×2), 129.4, 129.3, 128.7, 128.5, 128.3, 128.2, 127.5, 127.4, 126.8, 126.0, 125.8, 125.6, 72.2, 55.8, 42.1, 32.8, 22.8, 13.9. HRMS calcd for $C_{30}H_{30}N_3O_4S_2$ m/z $[M+H]^+$ 560.1672; found 560.1680. HPLC: 16.63 min (94.22%).

4.3.12. *tert*-Butyl *N*-[(1*S*)-1-benzyl-2-oxo-2-(2-propylanilino) ethyl]carbamate (13a)

Reaction of **12a** (264 mg) with 2-propylaniline (160 mg) according to general method A gave **13a** (243 mg 59%). mp: 75–78 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.82 (d, J = 8.4 Hz, 1H), 7.65 (s, 1H), 7.24–7.33 (m, 5H), 7.19 (dd, J = 7.2, 7.8 Hz, 1H), 7.12 (d, J = 7.2 Hz), 7.08 (t, J = 7.2 Hz, 1H), 5.08 (br, 1H), 4.47–4.49 (m, 1H), 3.19 (d, J = 7.2 Hz, 2H), 2.27–2.38 (m, 2H), 1.43–1.50 (m, 12H) 0.89 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 169.5, 155.6, 136.6, 134.7, 133.1, 129.5, 129.3, 128.8, 127.1, 126.7, 125.2, 122.9, 80.7, 56.7, 38.0, 33.0, 28.3, 22.9, 13.9. HRMS calcd for $C_{23}H_{31}N_2O_3$ m/z $[M+H]^+$ 383.2330; found 383.2329.

4.3.13. Benzyl *N*-[(1*S*)-1-methyl-2-oxo-2-(2-propylanilino) ethyl]carbamate (13c)

Reaction of **12c** (222 mg) with 2-propylaniline (160 mg) according to general method A gave **13c** (282 mg, 83%). mp: 128–131 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.95 (br s, 1H), 7.84 (d, J = 7.8 Hz, 1H), 7.30–7.36 (m, 5H), 7.20 (dd, J = 7.2, 7.8 Hz, 1H), 7.16 (d, J = 7.2 Hz, 1H), 7.10 (dd, J = 7.2, 7.2 Hz, 1H), 5.28 (br s, 1H), 5.17 (d, J = 12.6 Hz, 1H), 5.15 (d, J = 12.6 Hz, 1H), 4.36–4.45 (m, 1H), 2.49–2.52 (m, 2H), 1.53–1.63 (m, 2H), 1.49 (d, J = 7.2 Hz, 3H), 0.95 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.3, 156.4, 135.8, 134.8, 133.4, 129.7, 128.6, 128.4, 128.1, 126.7, 125.3, 123.2, 67.4, 51.2, 33.4, 23.1, 17.7, 13.9; HRMS calcd for $C_{20}H_{25}N_2O_3$ m/z $[M+H]^+$ 341.1864; found 341.1860.

4.3.14. Benzyl *N*-[(1S)-1-[(4-hydroxyphenyl)methyl]-2-oxo-2-(2-propylanilino)ethyl]carbamate (13d)

Reaction of **12d** (314 mg) with 2-propyl-aniline (160 mg) according to general method A gave **13d** (198 mg, 46%). mp: 125–129 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.28 (s, 1H), 7.92 (d, J = 8.1 Hz, 1H), 7.07–7.37 (m, 8H), 5.51 (br s, 1H), 4.64 (d, J = 11.7 Hz, 1H), 4.57 (d, J = 11.7 Hz, 1H), 4.39–4.50 (m, 1H), 4.04 (dd, J = 3.6 Hz, 9.3 Hz, 1H), 3.69 (dd, J = 6.6 Hz, 9.3 Hz, 1H), 2.45 (t, J = 7.5 Hz, 2H), 1.40–1.51 (m, 2H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.2, 155.3, 136.8, 134.6, 132.6, 129.3, 128.2, 127.7, 127.6, 126.4, 124.7, 122.5, 80.2, 73.3, 69.4, 54.1, 32.9, 27.9, 22.5, 13.5. HRMS calcd for $C_{26}H_{29}N_2O_4$ m/z $[M+H]^+$ 433.2126; found 433.2122.

4.3.15. Benzyl *N*-[(1*S*)-1-(*tert*-butoxymethyl)-2-oxo-2-(2-propylanilino)ethyl]carbamate (13e)

Reaction of **12e** (294 mg) with 2-propylaniline (160 mg) according to general method A gave **13e** (280 mg, 68%). mp: 114–116 °C. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.29 (br s, 1H), 7.83 (d, J = 8.1 Hz, 1H), 7.37–7.32 (m, 5H), 7.21 (t, J = 7.2 Hz, 1H), 7.12 (d, J = 7.2 Hz, 1H), 7.11 (t, J = 7.2 Hz, 1H), 5.85 (br s, 1H), 5.19 (s, 2H), 4.50–4.32 (m, 1H), 4.04–3.87 (m, 1H), 3.56–3.50 (m, 1H), 2.55 (t, J = 7.8 Hz, 2H), 1.55–1.63 (m, 2H), 1.23 (br s, 9H) 0.96 (t, J = 7.8 Hz, 3H). ^{13}C NMR (600 MHz, $CDCl_3$): δ 168.7, 156.2, 156.2, 134.8, 133.5, 129.5, 128.6, 128.3, 128.2, 126.7, 125.3, 123.4, 67.2, 61.8, 55.5, 33.3, 27.5, 22.9, 13.9. HRMS calcd for $C_{24}H_{33}N_2O_4$ m/z $[M+H]^+$ 413.2441; found 413.2435.

4.3.16. (2*S*)-2-Amino-3-phenyl-*N*-(2-propylphenyl) propanamide trifluoroacetate salt (14a)

Reaction of **13a** (114 mg) according to general method B gave **14a** (67 mg, 57%). mp: 172–176 °C. ^{1}H NMR (600 MHz, CD_3OD): δ 7.16–7.40 (m, 9H), 4.41 (t, J = 7.8, 1H), 3.34 (dd, J = 14.4 Hz, 7.2 Hz, 1H) 3.21 (dd, J = 13.9 Hz, 7.2 Hz, 1H), 2.37–2.47 (m, 2H), 1.52 (qt, J = 7.8 Hz, 7.2 Hz, 2H), 0.90 (t, J = 7.8 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ 169.4, 139.1, 136.1, 135.7, 131.2, 131.1, 130.7, 129.5, 128.6, 127.9, 127.8, 56.5, 39.3, 34.4, 24.7, 14.7. HRMS calcd for $C_{18}H_{23}N_2O$ m/z $[M+H]^+$ 283.1803; found 283.1810.

4.3.17. (2*S*)-2-Amino-3-benzyloxy-*N*-(2-propylphenyl) propanamide trifluoroacetate salt (14b)

Reaction of **12b** (295 mg) with 2-propylaniline (160 mg) according to general method A gave **13b** (308 mg, 81%). mp: 94–99 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.28 (s, 1H), 7.92 (d, J = 8.1 Hz, 1H), 7.07–7.37 (m, 8H), 5.51 (br s, 1H), 4.64 (d, J = 11.7 Hz, 1H), 4.57 (d, J = 11.7 Hz, 1H), 4.39–4.50 (m, 1H), 4.04 (dd, J = 3.6 Hz, 9.3 Hz, 1H), 3.69 (dd, J = 6.6 Hz, 9.3 Hz), 2.45 (t, J = 7.5 Hz, 2H), 1.40–1.51 (m, 11H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.2, 155.3, 136.8, 134.6, 132.6, 129.3, 128.2, 127.7, 127.6, 126.4, 124.7, 122.5, 80.2, 73.3, 69.4, 32.9, 27.9, 22.5, 13.5.

Reaction of **13b** (119 mg) according to general method B then gave **14b** (126 mg, 99%). ^{1}H NMR (600 MHz, CD_3OD): δ 7.20–7.41 (m, 9H), 4.65–4.69 (m,, 2H), 4.37 (dd, J = 4.2 Hz, 5.4 Hz, 1H), 3.99 (dd, J = 4.2 Hz, 10.2 Hz, 1H), 3.96 (dd, J = 5.4 Hz, 10. 2 Hz, 1H), 2.55 (dt, J = 2.4 Hz, 7.8 Hz, 2H), 1.55 (tq, J = 7.2 Hz, 7.8 Hz, 2H)

0.88 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ 167.2, 139.0, 138.5, 135.4, 131.5, 131.0, 129.6, 129.2, 128.2, 127.7, 127.6, 75.2, 69.8, 55.3, 34.9, 24.9, 14.8. HRMS calcd for $C_{19}H_{25}N_2O_2$ m/z $[M+H]^+$ 313.1910; found 313.1916.

4.3.18. (2*S*)-2-Amino-3-(4-hydroxyphenyl)-*N*-(2-propylphenyl) propanamide (14d)

Reaction of **13d** (172 mg) according to general method C gave **14d** (83 mg, 70%). mp: 120–122 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.50 (d, J = 7.2 Hz, 1H), 7.07–7.19 (m, 5H), 6.71–6.75 (m, 2H), 3.69 (dd, J = 7.2 Hz, 6.3 Hz, 1H), 3.03 (dd, J = 6.3 Hz, 13.5 Hz, 1H), 2.84 (dd, J = 7.2 Hz, 13.5 Hz, 1H), 2.42 (t, J = 7.8 Hz, 2H), 1.49 (qt, J = 7.8 Hz, 7.5 Hz, 2H), 0.89 (t, J = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, CD_3OD): δ 175.9, 157.5, 137.2, 136.2, 131.5, 130.7, 129.4, 127.4, 127.0, 125.9, 116.4, 58.2, 41.5, 34.3, 24.3, 14.3; HRMS calcd for $C_{18}H_{23}N_2O_2$ m/z $[M+H]^+$ 299.1754; found 299.1754.

4.3.19. (2*R*)-2-Amino-3-*tert*-butoxy-*N*-(2-propylphenyl) propanamide (14e)

Reaction of **13e** (163 mg) according to general method C gave **14e** (95 mg, 86%). ^{1}H NMR (600 MHz, CD_3OD): δ 9.68 (s, 1H), 8.08 (dd, J = 7.8 Hz, 1H), 7.21 (dd, J = 7.2, 6.6 Hz, 1H), 7.16 (d, J = 7.8 Hz), 7.05 (dd, J = 7.2, 6.6 Hz, 1H), 3.74–3.59 (m, 3H), 2.62 (t, J = 7.8 Hz, 2H), 1.88 (br s, 2H), 1.75–1.57 (m, 2H), 1.20 (br s, 9H) 1.04 (t, J = 7.2 Hz, 3H). ^{13}C NMR (150 MHz, CD_3OD): δ 170.2, 134.4, 131.1, 128.5, 125.7, 123.3, 120.6, 72.4, 62.6, 55.1, 32.7, 26.5, 21.9, 13.0. HRMS calcd for $C_{16}H_{27}N_2O_2$ m/z $[M+H]^+$ 279.2065 found 279.2067.

4.4. Biological assays

4.4.1. General methods and material

Enzymatic assays were done on a Varian Cary 50 Bio UV/VIS spectrometer and a Varian Cary 100 Bio UV/VIS spectrometer, both with a cell holder equipped with a constant temperature water bath. Cholesterol esterase (CEase) from porcine pancreas, acetylthiocholine iodide (ATCh), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium taurocholate (TC), and *p*-nitrophenyl butyrate (pNPB) were obtained from Sigma (Steinheim, Germany). Acetylcholinesterase (AChE) from *E. electricus*, anhydrous DMSO, and acetonitrile (HPLC grade) were purchased from Fluka (Deisenhofen, Germany).

4.4.2. CEase inhibition assay

Porcine cholesterol esterase inhibition was assayed spectrophotometrically at 405 nm at 25 °C as previously described.[7] Assay buffer was 100 mM sodium phosphate, 100 mM NaCl, pH 7.0. A stock solution of CEase (122 μg/mL) was prepared in 100 mM sodium phosphate buffer, pH 7.0 and kept at 0 °C. A 1:122 dilution was done with the same buffer immediately before starting the measurement. TC (12 mM) was dissolved in assay buffer and kept at 25 °C. A stock solution of pNPB (20 mM) was prepared in acetonitrile. The final concentration of acetonitrile was 3%, of DMSO 3%, of the substrate pNPB 200 μM, and of TC 6 mM, which corresponds to the duodenal bile salt concentration.[56] Assays were performed with a final concentration of 10 ng/mL of CEase which corresponded to an initial rate of 5.0 μM/min. Into a cuvette containing 430 μL assay buffer, 500 μL of the TC solution, 10 or 20 μL acetonitrile, 30 or 20 μL DMSO, 10 μL of the pNPB solution, and 10 μL of a inhibitor solution in either actonitrile or DMSO were added and thoroughly mixed. After incubation for 5 min at 25 °C, the reaction was initiated by adding 10 μL of the enzyme solution (1 μg/mL) and followed over 6 min. IC_{50} values were calculated from the linear steady-state turnover of the substrate.

Compounds **5a**, **5b**, **6b** and **8b** were analyzed in quadruplicate experiments, **6a** and **7a** in sixtuplicate experiments, and compounds **15a**–**16b** in duplicate experiments, each at a single inhibitor concentration (50 μM). IC_{50} values were calculated using Eq. 4, where v_0 and v are the rates in the absence and the presence of inhibitor.

$$IC_{50} = \frac{[I]}{\left(\frac{v_0}{v} - 1\right)} \tag{4}$$

For inhibitors **4a**, **4b**, **7b**, **8a**, **9a** and **10a** five different concentrations were used in duplicate experiments at a single concentration of pNPB (200 μM). IC_{50} values for **4a**, **4b**, **7b** and **10a**, resulted from plots of rates versus [I] and non-linear regression according to Eq. 5, where $v_{[I]\to\infty}$ is the residual activity at infinite concentration of the inhibitor. The IC_{50} value calculated by Eq. 5 corresponds to the concentration of the inhibitor which reduces the rate of the enzyme-catalyzed reaction to a velocity $(v_0 - v_{[I]\to\infty})/2 + v_{[I]\to\infty}$, *i.e.* the velocity half between v_0 and $v_{[I]\to\infty}$. Data $v_{[I]\to\infty}$ are given as relative velocities with respect to v_0 at a substrate concentration of 200 μM.

$$v = \frac{(v_0 - v_{[I]\to\infty})}{\left(1 + \frac{[I]}{IC_{50}}\right)} + v_{[I]\to\infty} \tag{5}$$

IC_{50} values for **8a** and **9a** resulted from plots of rates versus [I] and non-linear regression according to Eq. (6).

$$v = \frac{V_0}{\left(1 + \frac{[I]}{IC_{50}}\right)} \tag{6}$$

Substrate dependency of CEase inhibition by **4a** was analyzed in quadruplicate experiments at five different inhibitor concentrations (2–10 μM) with five different substrate concentrations (100–300 μM). The rate of the uninhibited reaction in the presence of a substrate concentration of 200 μM was set 100% in these experiments.

4.4.3. AChE inhibition assay

Inhibition of acetylcholinesterase from *E. electricus* was assayed spectrophotometrically at 412 nm at 25 °C as previously described.[7] Assay buffer was 100 mM sodium phosphate, 100 mM NaCl, pH 7.3. The enzyme stock solution (~100 U/mL) in assay buffer was kept at 0 °C. Appropriate dilutions were prepared immediately before starting the measurement. ATCh (10 mM) and DTNB (7 mM) were dissolved in assay buffer and kept at 0 °C. The final concentration of acetonitrile was 6%, of ATCh 500 μM, and of DTNB 350 μM. Assays were performed with a final concentration of ~30 mU/mL of AChE which corresponded to an initial rate of ~0.8 μM/min. Into a cuvette containing 830 μL assay buffer, 50 μl of the DTNB solution, 55 μL acetonitrile, 5 μL solution of the test compound, and 10 μL of an enzyme solution (~3 U/mL) were added and thoroughly mixed. After incubation for 15 min at 25 °C, the reaction was initiated by adding 50 μL of the ATCh solution. IC_{50} values were calculated from the linear steady-state turnover of the substrate.

Compounds **5a**, **8b**, **9a**, and **10a** were analyzed in quadruplicate experiments, compound **5b** in a sixtuplicate experiment, and compounds **4b**, **6a**, **6b**, **7a**, **7b** and **15a**–**16b** in duplicate experiments, each at a single inhibitor concentration (5–25 μM) and IC_{50} values were calculated with Eq. (4). Inhibitors **4a** and **8a** were analyzed in duplicate experiments at five different concentrations using a three-parameter Eq. 7.

$$v = \frac{v_0}{\left(1 + \frac{[I]}{IC_{50}}\right)^X} \tag{7}$$

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