Alternate Substrate Inhibition of Cholesterol Esterase by Thieno[2,3-d][1,3]oxazin-4-ones

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In a kinetic study, the interaction of bovine pancreatic cholesterol esterase (CEase) with fused 1,3-oxazin-4-ones and 1,3-thiazin-4-ones was investigated, and the compounds were characterized as alternate substrate inhibitors. Inhibition assays were performed in the presence of sodium taurocholate with p-nitrophenyl butyrate as chromogenic substrate. Strong active site-directed inhibition was detected for 2-diethylaminothieno[2,3-d][1,3]oxazin-4-ones with a cycloaliphatic chain at positions 5,6. The most potent inhibitors, compounds 3 and 4, exhibited $K_I$ values of 0.58 and 1.86 $\mu$M, respectively. An exchange of the ring oxygen by sulfur and the removal of the cycloaliphatic moiety as well as the replacement of the thiophene ring by benzene led to a loss of inhibitory potency. CEase has the capability to catalyze the hydrolysis of representatives of fused 1,3-oxazin-4-ones as well as the highly stable 1,3-thiazin-4-ones by using an acylation-deacylation mechanism. Hydrolyses were performed in the presence of a high enzyme concentration, and products were identified spectrophotometrically and by means of high performance liquid chromatography. The kinetic parameters $V_{\text{max}}$ and $V_{\text{max}}/K_m$ for the CEase-catalyzed turnover were determined. The compounds whose enzyme-catalyzed hydrolysis followed first-order kinetics ($K_m > 25 \mu$M) failed to inhibit CEase. On the other hand, inhibitors 3 (initial concentration of 25 $\mu$M) and 4 (20 $\mu$M) were hydrolyzed by CEase under steady-state conditions in the first phase of the reaction. Rate-limiting deacylation was demonstrated in nucleophilic competition experiments with ethanol as acyl acceptor wherein the conversion of compound 3 was accelerated up to an ethanol concentration of 1.5 M. The characterization of these compounds (i.e. 3 and 4) as alternate substrate inhibitors is not only based on the verification of the CEase-catalyzed hydrolysis. It also rests upon the concurrence of $K_I$ values obtained in the inhibition assay compared with separately determined $K_m$ values of their enzyme-catalyzed consumption, as could be predicted from the kinetic model used in this study.

Bile salt-dependent lipase, also referred as cholesterol esterase (CEase)1 (Sterol esterase, EC 3.1.1.13), is found in the pancreatic secretion of a wide range of species as well as in lactation of mammals. Pancreatic CEase once secreted into the duodenum and activated by primary bile salts catalyzes the hydrolysis of a broad spectrum of substrates including cholesterol esters, triacylglycerides, phospholipids, and esters of lipid-soluble vitamins (1, 2). Most probably, the role of CEase extends beyond that of simply hydrolyzing dietary lipids. Circulating CEase may function as a cholesterol transfer protein (3) and may have a deleterious effect in atherosclerosis processes, because it has been reported to convert the larger and less atherogenic low density lipoprotein to the smaller and more atherogenic low density lipoprotein subspecies (4). However, the role of plasmatic CEase in atherogenesis and the relationship of the enzyme to various pathological conditions are not clearly established so far (2). CEase belongs to the $\alpha/\beta$-hydrolase fold family of proteins whose members, mostly serine esterases, share secondary and tertiary structural features (5, 6). Ester hydrolysis is catalyzed by the operation of a catalytic triad (Ser-194, His-435, and Asp-320 in the case of CEase numbered for the rat enzyme). This triad is stereochemically convergent with catalytic triads of serine proteases, and like serine proteases, serine esterases of the $\alpha/\beta$-hydrolase fold family use an acylation-deacylation mechanism. Both the acylation and deacylation stages transit tetrahedral intermediates that are stabilized by a tripartite oxanion hole (5–7).

In recent years, much attention has focused on the inhibition of CEase as a potential target particularly for the development of hypocholesterolemic agents. Sharing a common mechanism for substrate hydrolysis, CEase and serine proteases might be expected to be inhibited by the same classes of mechanism-based inhibitors that indeed have been demonstrated for boronic and borinic acids, aryl haloketones, aryl phosphates and phosphonates, and carbamates (8). Aryl and cholesteryl carbamates comprise the most studied class of CEase inhibitors (8–11). A detailed characterization of transient inhibition by aryl carbamates was reported by Feaster et al. (9). Inhibition occurs because of rapid carbamylation of the active site serine followed by slow decarbamylation. Biphasic time courses reflect the progressive loss of enzyme activity in a nonlinear phase and a subsequent steady-state phase of the reaction. From their kinetic mechanism, such inhibitors are best described as pseudo-substrate inhibitors or as alternate substrate inhibitors (8, 12).

The incorporation of a scissile CO–O or CO–N bond into a ring system has frequently been used in the design of mechanism-based inhibitors of serine proteases, e.g. of leukocyte elastase (13–18). This concept has found less attention for the development of CEase inhibitors. However, 6-chloro-2-pyrones, representatives of a known class of mechanism-based inhibitors of serine proteases, have recently been described as potent CEase inhibitors (19). Although having the potential to act as alternate substrates or suicide inhibitors, competitive inhibition of CEase was postulated.

In the kinetic study presented in this paper, fused 1,3-oxa-
zin-4-ones and 1,3-thiazin-4-ones were investigated as inhibitors of CEase. The acylation-deacylation mechanism by which 3,1-benzoazoxin-4-ones and analogous thieno[1,3]oxacin-4-ones interact with various serine proteases is well documented (18, 20–24). Their potency to inhibit serine proteases derives from the ratio of the acylation and deacylation rates. So far, these alternate substrate inhibitors of serine proteases have not been investigated as inhibitors of CEase. The aim of this study was (i) characterization of fused 1,3-oxazin-4-ones and 1,3-thiazin-4-ones as inhibitors of CEase, (ii) investigation of a CEase-catalyzed turnover of these heterocyclic compounds, and (iii) examination to what extent inhibitory potency can be concluded from the kinetic parameters of the enzyme-catalyzed conversion.

**EXPERIMENTAL PROCEDURES**

**Materials and Instruments**—CEase from bovine pancreas (41 units/mg), sodium taurocholate (TC), and p-nitrophenyl butyrate (pNPB) were obtained from Sigma (Steinheim, Germany). Compounds 1–6 and 8 were prepared as described elsewhere (22, 23, 25). The synthesis and structure elucidation of compound 7 was done using general methods and instruments as reported previously (22). Spectrophotometric assays were done on a Perkin-Elmer Lambda 16 UV-visible spectrophotometer with a cell holder equipped with a constant temperature water bath. Incubation experiments were performed using an Eppendorf thermomixer comfort. Analytical HPLC was performed on a Bischoff chromatograph 2200 with an UV detector Lambda 1000. A 5-μm Phenomenex Jupiter 250 × 4.6-mm column was used at a flow rate of 0.5 ml/min.

**Kinetic Parameters of the Non-enzymatic Hydrolysis**—Alkaline hydrolysis of the compounds was followed spectrophotometrically at a fixed wavelength (compound 1 = 348 nm; compounds 2–4 = 353 nm; and compounds 5 and 6 = 385 nm) by monitoring the disappearance of the compounds at 25 °C in 50 mM CAPS, pH 9.5, for compound 1 and pH 11.25 for all other compounds, respectively. Stock solutions of the compounds were prepared in Me2SO. The final inhibitor concentration was 1.8 μM/min, and the final Me2SO concentration was 5%. The reactions were monitored for at least two half-lives. For compounds 5 and 6 (5 μM each), a final Me2SO concentration of 15% was used, and reactions were followed for 50 h. Curves were analyzed as first-order reactions.

Inhibition of CEase by Compounds 1–6—A series of fused 1,3-oxazin-4-ones and analogous 1,3-thiazin-4-ones was evaluated as inhibitors of bovine pancreatic cholesterol esterase. Detailed investigations were performed with 2-diethylamino derivatives 1–6 whose structures are shown in Table I. The reactions were followed over the period of 1–6 min after initiation by the addition of the enzyme. Progress curves were characterized by a linear steady-state turnover of the substrate, and values of a linear regression were fitted to an equation of competitive inhibition to obtain K₈ values (Table I).

The enzatic conversion of the compounds was followed spectrophotometrically at 25 °C. Into a cuvette containing assay buffer, 500 μl of TC solution, 50 μl of acetonitrile, and 10 μl of an inhibitor solution were added, thoroughly mixed, and incubated for 2 min at 25 °C. The reaction was initiated by adding a volume (9–12 μl) of an enzyme solution (122 μg/ml). This volume was adjusted by the determination of a 1.6250 dilution that converts pNPB (200 μM) with a rate of 1.8 μM/min. The entire volume was 1 ml containing the following concentrations: 6 mM TC, CEase (adjusted activity), 6% acetonitrile, and 10–25 μM inhibitor. The reactions were analyzed by monitoring UV-visible spectra in fixed time intervals or by following the time course at a fixed wavelength. For the latter experiments, the absorption maxima of the compounds were used for the molar extinction coefficients. The final wavelength was determined separately in triplicate experiments. Control experiments to prove the stability of the compounds were performed by adding 100 mM sodium phosphate buffer, pH 7.0, instead of the enzyme solution.

**RESULTS**

**Inhibition of CEase by Compounds 1–6**—The Hanes-Woolf plot [S]/v versus substrate concentration is shown in Fig. 2. The relevant relationship is given in Equation 1

\[
[S]/v = \left[1 + (|I|K_{I})\right]/[S]/V_{max} = \left[1 + (|I|K_{I})/K_{m}V_{max}\right]
\]

where [S], V_{max}, and K_{m} are the concentration and the Michaelis-Menten constants of pNPB, respectively, and v is the relative steady-state velocity (v = v/v₀ × 100%). I is the concentration of inhibitor 3, and K_{I3} and K_{I2} are inhibition constants to estimate the type of inhibition. Linear regression gave values for slopes and vertical intercepts that were replotted against [I] to calculate K_{m} and K_{I3}, respectively. A formally competitive inhibition could be deduced, and values of K_{I3} = 650 μM were obtained. The experiment resulted from the inhibition assay for compound 3 (K_{I} = 0.58 μM) (Table I).

**Product Identification of the CEase-catalyzed Turnover of Compounds 1–6**—The possibility that fused 1,3-oxazinones and 1,3-thiazinones are substrates of CEase was checked for compounds 1–6. The solutions of each compound were incubated with CEase at 25 °C in the presence of TC. The enzyme...
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Enzyme inhibition was assayed in the presence of the chromogenic substrate pNPB (200 μM). Progress curves were monitored at 405 nm and analyzed by linear regression. \( K_i \) values were determined using the equation \( v_\text{i} = \frac{v_\text{vi}}{[I]/K_i} + 1 \), where \( v_\text{vi} \) and \( v_\text{i} \) are the steady-state velocities and the velocity in the absence of the inhibitor, [I] is the inhibitor concentration, and \( K_i \) is the apparent inhibition constant. \( K_i \) values were calculated from equation \( K_i = K_i^0([S]/K_m^0) + 1 \), where \([S]\) is the concentration of the chromogenic substrate pNPB. \( K_m^0 \) for pNPB was separately determined. CEase-catalyzed conversion of heterocyclic compounds 1, 2, 3, 5 (each 25 μM), 4 (20 μM), and 6 (10 μM) was monitored in the absence of pNPB at the absorption maxima indicated. The conversions of 1, 2, and 5 were analyzed as first-order reactions. Initial velocities were calculated using equation \( v_\text{vi} = k[I] \), where \( v_\text{vi} \) is the initial velocity of the transformation, \( k \) is the first-order rate constant, and \([I]_0 \) is the initial concentration of the heterocyclic compound. Progress curves of the conversion of 3 and 4 were analyzed as described in the text. Alkaline hydrolysis was followed spectrophotometrically at a fixed wavelength and analyzed as first-order reaction. NI, no inhibition, which refers to a rate of ≥94% of the reaction in the absence of the inhibitor; ND, not determined.

### Table I
Inhibition constants and kinetic parameters of the CEase-catalyzed turnover and alkaline hydrolysis of fused 1,3-oxazin-4-ones and 1,3-thiazin-4-ones

| Compound | \( K_i \) (µM) | \( \lambda_{\text{max}} \) (nm) | \( v_\text{max}^{1/4} \) (µM min\(^{-1}\)) | \( v_\text{max}^{1/4}K_m^{1/4} \) (min\(^{-1}\)) | log \( k_{\text{cat}} \) |
|----------|----------------|-----------------|----------------|--------------------------|------|
| 1        | 53\(^a\)       | 352             | >13\(^b\)      | 0.51                     | 0.98 |
| 2        | NI\(^f\)       | 331             | >2.1\(^b\)     | 0.085                    | -0.91\(^e\) |
| 3        | 0.58 ± 0.05\(^d\) | 349             | 0.093          | 0.17                     | -1.03\(^c\) |
| 4        | 1.86 ± 0.05\(^e\) | 346             | 0.015          | 0.0092                   | -1.49\(^f\) |
| 5        | NI\(^f\)       | 371             | >0.023\(^b\)   | 0.0010                   | -2.00 |
| 6        | NI\(^f\)       | 393             | ND\(^e\)       | ND\(^e\)                 | -2.55 |

\(^a\) The average of duplicate experiments determined at a single inhibitor concentration of 25 μM.  
\(^b\) The limits were defined according to \( v_\text{max}^{1/4} > v_\text{vi} \).  
\(^c\) The data from Ref. 23.  
\(^d\) The average of duplicate experiments determined at five different inhibitor concentrations (2–10 μM).  
\(^e\) The average of duplicate experiments determined at six different inhibitor concentrations (2.5–25 μM).  
\(^f\) The average of duplicate experiments determined at a single inhibitor concentration of 10 μM.  
\(^e\) 96% of the substrate was detected after incubation for 24 h as determined by HPLC.

Concentration was ~60-fold higher compared with the inhibition experiments in the presence of pNPB. Reactions were monitored spectrophotometrically or by means of HPLC. Compound 3 indeed underwent an enzymatic turnover to form the corresponding thiophenecarboxylic acid 7 (see structure in Fig. 3). This could be concluded, because the final UV spectra obtained for the reaction with CEase (Fig. 3A) were identical with that of the reference compound 7 synthesized. Accordingly, the final spectra of the CEase-catalyzed conversion of compound 4 (data not shown) revealed the thiophenecarboxylic acid 8 (see structure in Fig. 3) as the product. The formation of compound 8 as the only product of the CEase-catalyzed transformation of compound 4 was additionally demonstrated by HPLC analysis using the synthesized thiophenecarboxylic acid 8 as reference. Enzyme-catalyzed hydrolysis of compounds 1 and 2 (Fig. 4A) and 4 was also examined spectrophotometrically. To a minor extent, compound 6 was converted by CEase. This reaction was monitored by HPLC. The acylation-deacylation mechanism as concluded from these results is shown exemplary for compounds 3 and 4 in Fig. 3.

**Kinetic Parameters of the CEase-catalyzed Turnover of Compounds 1–6** —The enzymatic transformation of the compounds was investigated with an equal CEase activity adjusted toward pNPB prior to all experiments. Kinetic parameters are given in Table I. The first-order rate constants obtained for compounds 1, 2, and 5 refer to \( V_\text{max}^{1/4}K_m^{1/4} \) (27). \( V_\text{max}^{1/4} \) and \( K_m^{1/4} \) are the Michaelis-Menten parameters for the heterocyclic compounds as substrates of CEase. Michaelis-Menten constants could be ascertained being \( K_m^{1/4} > 25 \) μM for 1, 2, and 5. The kinetic analysis for the CEase-catalyzed hydrolysis of compound 2 is illustrated in Fig. 4. The determination of kinetic parameters of the enzymatic hydrolysis of compound 3 to form 7 is depicted in Fig. 5. The reaction approximated a zero-order kinetics until the conver-
The aim of the current study was the kinetic characterization of the interaction of pancreatic cholesterol esterase with fused 1,3-oxazin-4-ones and 1,3-thiazin-4-ones. Based on preliminary studies (data not shown), we intended to focus on 2-diethylamino derivatives. Benzoxazine 1 is a poor inhibitor of CEase with a $K_i$ value of 53 μM (Table I). The replacement of the unchanged compound still was detected. Nucleophilic Competition—The effect of ethanol on the CEase-catalyzed transformation of compounds 2 and 3 was investigated by monitoring the reactions at the absorption maxima of both compounds. The conversion of compound 2 (Fig. 6A) was significantly inhibited by ethanol. Transformations were analyzed as first-order reactions. Initial velocities were plotted as relative values against ethanol concentration (Fig. 6B) to allow for the determination of an apparent inhibition constant of ethanol ($K_i$ = 530 μM) by non-linear regression using an equation of competitive inhibition. A different feature was found in the case of compound 3. Transformations followed zero-order kinetics (Fig. 7A), and an increase of maximum velocities up to an ethanol concentration of 1.5 M was observed. A plot of relative maximum velocities versus ethanol concentration is shown in Fig. 7B. Thus, an inhibitory effect of ethanol as it could be concluded from the above mentioned experiment is more than compensated. This result indicated a nucleophilic activation by ethanol to affect deacylation as the rate-determining step that deacylation are shown. Reference substances 7 and 8 were prepared by alkaline hydrolysis of compounds 3 and 4, respectively.
ment of the benzene unit by a (substituted) thiophene ring (compounds 2–4) resulted in a strong enzyme inhibition by thieno[1,3]oxazinones with cycloaliphatic moieties (compounds 3 and 4). An exchange of the ring oxygen by sulfur (compound 3 versus 6) led to a loss of inhibition.

Active site-directed inhibition as shown for compound 3 (Fig. 2) is a prerequisite for alternate substrate inhibition. Clear evidence for this type of inhibition was provided by the findings that the inhibitors themselves undergo an enzymatic hydrolysis as shown exemplary in Fig. 3. A similar mode of interaction of fused 1,3-oxazinones and 1,3-thiazinones has been reported for the inhibition of several serine proteases including chymotrypsin, leukocyte elastase, and chymase (18, 20–24). Typically, a time-dependent inhibition was observed, and the rates of acylation and deacylation, respectively, were available by using slow binding kinetics (28).

In this inhibition study, CEase-catalyzed pNPB hydrolysis gave straight lines of the reaction progress, indicating that a steady-state was already reached at the beginning of the measurements. A relief from steady-state attributed to enzyme-catalyzed turnover of the inhibitor did not occur within 6 min. The kinetic model of the inhibition assay is part ([Nu] = 0) of

FIG. 4. Kinetics of the CEase-catalyzed hydrolysis of compound 2. Reaction was performed in 100 mM sodium phosphate, 100 mM NaCl, pH 7.0, with 6 mM TC and 6% acetonitrile. Initial concentration of compound 2 was 25 μM. A, depletion of compound 2 is illustrated by monitoring UV-visible spectra in 4-min intervals. The arrow indicates the initial spectrum. B, hydrolysis was followed at 331 nm and analyzed as first-order reaction. A first-order rate constant of 0.065 ± 0.00002 min⁻¹ that corresponds to $V_{\text{max}}/K_{\text{m}}$ was obtained by nonlinear regression. Control reaction in the absence of CEase is shown to demonstrate the stability of compound 2.

FIG. 5. Kinetics of the CEase-catalyzed hydrolysis of compound 3. Reaction was performed in 100 mM sodium phosphate, 100 mM NaCl, pH 7.0 with 6 mM TC and 6% acetonitrile. Initial concentration of compound 3 was 25 μM. A, depletion of compound 3 is illustrated by monitoring UV-visible spectra at 20-min intervals. The arrow indicates the initial spectrum. B, hydrolysis of compound 3 to form 7 was followed at 349 nm. Control reaction in the absence of CEase is shown to demonstrate the stability of compound 3. C, plot $(\ln[I_0] - \ln[I])/t$ versus $(\ln[I_0] - \ln[I])/t$ according to an integrated form of the Michaelis-Menten equation, $(\ln[I_0] - \ln[I])/t = V_{\text{max}}/K_{\text{m}} - [K_{\text{m}}/\ln[I_0] - \ln[I]]/t$, where $[I_0]$ and [I] are the initial concentration of compound 3 and the concentration at the time t. The values for 160–320 min were obtained from the data shown in B. Linear regression gave a negative slope of 0.56 ± 0.004 μM that corresponds to $K_{\text{m}}$ and an intercept of $V_{\text{max}} = 0.093 ± 0.00004$ μM min⁻¹.
concentration. The control for [EtOH] reactions in the absence of CEase were performed for each ethanol concentration (0.25–2 M). Progress curves were fitted as first-order reactions, and initial velocities, \( v_i \), were calculated from equation \( v_i = k_0 \left[ I_0 \right] \). Control reactions in the absence of CEase were performed for each ethanol concentration. The control for [EtOH] = 1.5 M is shown, \( B \), the values for relative initial velocities obtained from fits to the data shown in \( A \) (initial velocity in the absence of ethanol = 100% activity) were plotted against the concentrations of ethanol. Non-linear regression according to an equation of competitive inhibition gave an apparent inhibition constant of \( K_i' = 0.53 \pm 0.09 \mu M \).

Fig. 8. Inhibitor constants, \( K_i \), are governed by Equation 2.

\[
K_i = (k_{-1}h_1j_1 + k_{-1}h_2j_1 + k_{-1}h_3j_1 + k_{-1}h_4j_1) / [E_0] \]  
(Eq. 2)

The rates of the enzyme-catalyzed inhibitor consumption, \( v_i \), were determined in the absence of both pNPB ([S] = 0) and ethanol ([Nu] = 0). The kinetic model is part of Fig. 8, and \( V_{\text{max}}^{-1} \) and \( V_{\text{max}}^{-1}/K_m^{-1} \) are related by Equations 3 and 4.

\[
V_{\text{max}}^{-1} = k_{-1}h_2j_1[E_0](k_1j_1 + k_{-1}h_2j_1 + k_{-1}h_3j_1) \\ V_{\text{max}}^{-1}/K_m^{-1} = k_{-1}h_2j_1[E_0](k_{-1}h_2j_1 + k_{-1}h_3j_1 + k_1j_1) 
\]  
(Eq. 3)

On the basis of these expressions, the kinetic results of 2-diethylamino-substituted compounds will be discussed. Benzoxazinone 1 is a comparable good substrate for CEase, whereas the replacement of the benzene unit by thiophene leads to a 6-fold decrease in \( V_{\text{max}}^{-1}/K_m^{-1} \) (compound 1 versus 2, Table I). The reaction of both compounds with CEase followed first-order kinetics, and thus neither EI nor E-I did accumulate within the time course studied. The influence of the benzene-thiophene replacement on the affinity of the compound toward the active site is assumed to be rather small. Different rates simply reflect a reduced chemical reactivity of the thiophene-derived compound 2. An enhanced electron density at the thiophene C-atoms results in a decreased carbonyl activity in the case of compound 2 (22, 23). The second-order rate constant \( k_{\text{OH}} \) of the alkaline hydrolysis can be used to estimate intrinsic reactivity toward nucleophiles and is two orders of magnitude lower for compound 2 compared with 1.

The replacement of the ring oxygen of compound 2 by sulfur results in a further enhanced chemical stability and decreased carbonyl reactivity in the case of compound 5, which shows a 10-fold lower \( k_{\text{OH}} \) value (compound 2 versus 5, Table I). This effect is attributed to a stronger resonance stabilization for the thiolactone-containing heterocycles as discussed elsewhere for...
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3,1-benzoazin-4-ones and analogous 3,1-benzothiazin-4-ones (21). However, it was demonstrated in this study that CEase does catalyze the hydrolysis of the highly stabilized thieno[1,3]thiazinone 5. The $V_{\text{max}}/K_{\text{m}}$ for this reaction is strongly decreased compared with the hydrolysis of compound 2. As in the cases of compounds 1 and 2, the reaction followed first-order kinetics over the entire measuring time, and intermediate enzyme-inhibitor complexes did not accumulate. In accordance with these results, compound 1 showed only weak inhibition, and compounds 2 and 5 did not inhibit the CEase-catalyzed hydrolysis of the chromogenic substrate pNPB (Table I).

An enlargement of the structure 2 by a cycloaliphatic moiety (compounds 3 and 4) has a strong impact on the kinetic parameters of the CEase-catalyzed conversion. Specific interactions with the enzyme account for these effects, because the $k_{\text{OH}}^-$ values are similar. The reactions of 3 and 4 are characterized by remarkably decreased $V_{\text{max}}^{-1}$ values in comparison with the enzymatic hydrolysis of compound 2 (Table I). These findings are interpreted in terms of a decelerated hydrolytic deacylation that probably leads to an accumulation of E-I as the enzyme species responsible for enzyme inhibition by compounds 3 and 4 in the presence of pNPB. Additional support for this assumption is provided by a comparison of the kinetic data of compounds 3 and 6. The thiazinone 6 does not inhibit CEase in the presence of pNPB and undergoes a very slow enzyme-catalyzed conversion. Thus, the differences are supposed to result from a slow formation of E-I in the case of the thiazinone 6 according to its low $k_{\text{OH}}^-$. For the structurally analogous but more reactive oxazinone 3, an accelerated formation of E-I is assumed, whereas a strong impact on the affinity to form EI is not expected to come from the O–S exchange.

As the structures 2–4 become more space-filling, deacylation rates decrease in this order. The conclusion can be rationalized as a result of pronounced shielding from an attack of external water on the acyl enzyme.

The above results indicated hydrolytic deacylation of E-I as the rate-limiting step in the CEase-catalyzed turnover of the potent inhibitors 3 and 4. We tested this conclusion by monitoring the reaction of 3 in the presence of an additional nucleophile by providing a parallel pathway $k_{\text{a}}[\text{Nu}]$ of deacylation. The reactions were performed with different ethanol concentrations, [Nu], in the absence of pNPB ([S] = 0), and the kinetic model is part of Fig. 8. The conversions were analyzed as zero-order reactions to obtain $V_{\text{max}}^{-1}$ values (Fig. 7A). If deacylation is slow ($k_{\text{a}} = k_{\text{Nu}}$) or fast ($k_{\text{a}} = k_{\text{a}}^1$), a linear independence of $V_{\text{max}}^{-1}$ from [Nu] can be predicted. This was observed for ethanol concentrations up to 1.5 M (Fig. 7B). Thus, it could be shown that ethanol as an additional acyl acceptor increases the rate of deacylation. At high concentrations of ethanol, deacylation might no longer be rate-determining. Moreover, it has to be taken into account that ethanol acts as an inhibitor of CEase, contrary affecting the nucleophilic activation.

Increasing ethanol concentrations reduce the rates of the CEase-catalyzed hydrolysis of compound 2 (Fig. 6). Because the reactions followed first-order kinetics, intermediates did not accumulate, and both $k_{\text{a}}$ and $k_{\text{a}}^{-1}$ are much lower than $k_{\text{a}}$ and $k_{\text{a}}^{-1}$ [Nu]. Under such conditions, the first-order rate constants ($V_{\text{max}}/K_{\text{m}}$) are invariant with respect to [Nu]. Therefore, the inhibitory effect of ethanol on the activity of bovine pancreatic CEase shown in Fig. 6 is independent from the nucleophilic activation.

Our results are in agreement with previous reports on the effects of nucleophiles on the CEase activity toward common non-cyclized substrates. Lombardo and Gyl (29) have studied the influence of nucleophiles on the activity of human pancreatic CEase. Strong inhibition of the hydrolysis rates of methyl butyrate and triacetin by n-butanol was attributed to rate-limiting acylation, whereas the data for 4-nitrophenyl acetate and n-propylthiol acetate conversion were interpreted in terms of a nucleophile competition. Both acylation and deacylation step were partly rate-determining for the latter substrates. A nucleophile binding site was postulated to be evident when 4-nitrophenyl acetate was the substrate. Stout et al. (30) have investigated the kinetics of the pNPB conversion catalyzed by porcine and bovine pancreatic CEase. It was shown by nucleophilic trapping for pNPB as for p-nitrophenyl esters with various acyl chains (31) that hydrolysis is rate-limited by deacylation. An addition of alcohols led to similar increases of $V_{\text{max}}$ and $K_{\text{m}}$; however, at higher concentrations of ethanol (2 M) and n-butanol (0.2 M), respectively, $K_{\text{m}}$ increased more rapidly than did $V_{\text{max}}$ and competitive inhibition was assumed (30).

From the kinetic feature discussed above, compound 2 was shown to be a suitable substrate to determine an inhibitory effect of nucleophiles on the activity of CEase. Compound 2 is highly stable, and its depletion can easily be followed spectrophotometrically. This led us to the first determination of an apparent inhibition constant of $K_{\text{a}}^1 = 530 \mu M$ for inhibition of CEase by ethanol (Fig. 6B).

A final consideration is addressed to possible predictions of the inhibitory potency of the compounds by using the kinetic parameters (Table I) of their enzymatic and non-enzymatic hydrolysis. The values for $k_{\text{OH}}^{-1}$ can be used to judge stability toward non-specific nucleophiles but do not reflect inhibitory potential. Expectedly, the $V_{\text{max}}/K_{\text{m}}$ values as estimation for enzyme specificity do not correlate with the $K_{\text{m}}$ values obtained. However, for $K_{\text{a}}^{-1}$, the form is Equation 5 obtained by a combination of Equations 3 and 4.

$$K_{\text{a}}^{-1} = (k_{\text{a}}^{-1} + k_{\text{a}}^{-1} + k_{\text{a}}^{-1})$$

(Eq. 5)

This equation is just the expression derived for $K_{\text{a}}$ as determined in the inhibition assays (Table I) (see Equation 2). Our data are in good agreement with this prediction ($K_{\text{a}} = K_{\text{a}}^{-1}$), thus confirming the kinetic model of alternate substrate inhibition: the $K_{\text{m}}$ values of 0.56 and 1.65 $\mu M$, respectively, were determined for the CEase-catalyzed hydrolysis of compounds 3 and 4, respectively; the $K_{\text{m}}$ values as obtained in the presence of pNPB are 0.58 and 1.86 $\mu M$, respectively. Moreover, the failure to inhibit CEase (i.e., compounds 1, 2, and 5) is consistent with $K_{\text{m}}$ values of $>25 \mu M$. Therefore, it can be concluded that if the alternate substrate mechanism is ensured, a single turnover
experiment in the absence of a chromogenic substrate is sufficient to determine $K_v$. We are currently investigating this methodology for alternate substrate inhibitors of other serine esterases.

Our experimental data are in agreement with the classification of alternate substrate inhibitors as stable analogs that have the potential for conversion to products during a normal course of catalysis with a rate of one or more steps that has become extremely slow (12). The separately determined kinetic parameters for the consumption of the alternate substrate inhibitor are operative in the inhibition assay system but where consumption might practically not occur. For example, inhibitor 3 was assayed at concentrations 2–10 μM over 6 min, and for this period of time a product formation of less (because of substrate competition) than 9 nM could be estimated by considering $V_{max}^1$ and the enzyme concentrations used.

In summary, we have analyzed alternate substrate inhibitors of CEase based on derivations of a kinetic system including a one-substrate, two-products, two-step irreversible reaction for substrate hydrolysis and a one-substrate, one-product, one-step irreversible reaction for the hydrolysis of the alternate substrate inhibitors. Strong inhibition was achieved by introducing a cycloaliphatic moiety into the thieno[2,3-$d$][1,3]oxazin-4-one skeleton, which results in a remarkable decrease of the deacylation rate. These derivatives (i.e. compounds 3 and 4) show high chemical stability and act as true alternate substrate inhibitors of pancreatic cholesterol esterase.

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