Rational Design of α-Keto Triglyceride Analogues as Inhibitors for *Staphylococcus hyicus* Lipase[†,‡]

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ABSTRACT: We have synthesized a series of α-keto triglyceride analogues as inhibitors for the lipase from *Staphylococcus hyicus* (SHL). Hydrolysis at positions 1 and 2 was prevented by replacement of the ester bonds by nonhydrolyzable ether, carbamoyl, or amide bonds, and an α-keto fatty acid was introduced at position 3. Such compounds act as competitive inhibitors of SHL. Inhibition must be caused by the presence of the α-keto functions, since the compounds containing an ester or a hydroxyl group in position 3 did not inhibit the enzyme. We propose that our inhibitors react with the active site Ser of the lipase, hereby mimicking the tetrahedral intermediate occurring in substrate hydrolysis. We conclude that the localization of the α-keto triglycerides is very important for inhibition because only those compounds which are insoluble in water are lipase inhibitors. In addition, other specific protein−inhibitor interactions, probably with the carbonyl oxygen at position 1 and/or 2, improve inhibitor binding. This makes the compounds with amide or carbamoyl groups at positions 1 and 2 better inhibitors than their ether counterparts. The inhibitory power could be improved further by replacing the oxygen at position 3 by an amido group. The resulting inhibitor 1,2-diethylcarbamoyl-3-amido-α-ketododecanoylglycerol has a K_i* value of 0.008 mol %, indicating that it binds approximately 125-fold tighter than the substrate. These results illustrate that effective lipase inhibitors can be designed by combining an α-keto group with good micellar solubility and optimal protein−inhibitor interactions.

Lipases (glycerol ester hydrolases, EC 3.1.1.3) are active at the lipid−water interface where they degrade water-insoluble triglycerides. Not only triglycerides but also numerous synthetic lipids and phospholipids are hydrolyzed by lipases, often with stereo, positional, and/or chain length selectivity (*1*, and references therein). Moreover, lipases are very stable in organic solvents, and under these conditions, they can catalyze (trans)esterification reactions (*2*, *3*). These properties make lipases suitable biocatalysts for the application in organic synthesis and as detergent additives, and at present, these enzymes are produced on a bulk scale for industrial purposes. The challenge for industry is the optimization and the de novo design of lipase substrate specificities, for catalyzing specific reactions. To achieve this, one requires a better understanding of how these enzymes function and, moreover, how they interact with substrate molecules at the molecular level.

The determination of the first X-ray structure of a lipase from human pancreas has substantially improved our understanding of lipases (*4*). This structure confirmed the presence of a "classical" Ser-Asp-His catalytic triad, comparable to the active site of proteases, but in the lipase, the active site was blocked by surface loops, thereby making it inaccessible. The subsequently published structure of this lipase bound to a substrate micelle revealed that this interaction results in the structural rearrangement of these loops, now making the active site accessible to substrate molecules (*5*). Comparable conformational changes were also observed for many other lipases, either complexed with active site-directed inhibitors (*6*, *7*), bound to micelles (*8*), or crystallized under specific conditions (*9*, *10*). As a result, the molecular basis for the opening of the active site is thoroughly understood today, but it is the next step in the catalytic event that determines the selectivity of the enzyme. In this step, the substrate molecule specifically interacts with the protein, resulting in the optimal orientation for chemical hydrolysis by the catalytic residues. These highly specific enzyme−substrate interactions are only poorly understood because the current understanding is based on X-ray structures of lipases inhibited by small monoalkyl organophosphonates. These structures revealed the presence of a so-called oxyanion hole, where during catalysis the negatively charged transition state intermediate is stabilized by specific hydrogen bonds with the protein. The alkyl moiety of the inhibitor appeared to be bound in either a hydrophobic binding pocket inside the protein or a groove on the surface of the protein (*11*−*13*). These observations provided the basis for the first models of lipase−triglyceride interaction and stereoselectivity, but for a full understanding of all protein−substrate interactions, the inhibitor ideally should have a structure that is as close

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as possible to the natural substrate. In this respect, the recent series of syntheses of phosphonate derivatives which resemble real triglycerides was a major step forward in the design of lipase inhibitors (*14*, *15*). These compounds are potent, covalent inhibitors of lipases, and inhibition occurs via a nucleophilic substitution reaction of the active site Ser with the phosphonate ester. This reaction can easily be monitored spectrophotometrically, since *p*-nitrophenol (PNP) is the leaving group. Drawbacks of these inhibitors are (1) the fact that the phosphorus atom is the second chiral center in these compounds, which complicates both kinetic and structural analyses, (2) possible steric hindrance by the bulky PNP group which may influence the mode of interaction between the enzyme and the inhibitor, and (3) the occurrence of an irreversible side reaction which may result in the formation of a negatively charged monoester and the loss of the diglyceride moiety in the complex (*16*−*18*).

We here report the synthesis of a novel class of triglyceride analogues, which are competitive rather than covalent inhibitors. The synthesis of these inhibitors is based on the observation that α-keto carboxylic acid analogues of peptides are good inhibitors for proteases (*19*, *20*). Moreover, it has recently been reported that a tricarbonyl derivative of arachidonic acid is a good inhibitor for intracellular phospholipase A_2 (PLA_2)[1] (*21*). Since we intended to mimick the natural substrate as close as possible, we introduced an α-keto fatty acid into diglyceride analogues. The inhibitory power of the resulting α-keto triglycerides with the lipase from *Staphylococcus hyicus* (SHL) was assessed, and the mechanism of inhibition was studied. Modifications which are introduced to improve the inhibitory power of these compounds highlight the importance of solubility and chemical structure in the rational design of lipase inhibitors.

MATERIALS AND METHODS

Materials. Triton X-100 (TX100) was obtained from Serva, and *p*-nitrophenyl butyrate (PNPB) was from Sigma. 1-Bromobutane, *n*-butyl chloride, sodium hydride, and sodium borohydride were purchased from Acros Chimica. *rac*-Diaminopropionic acid was purchased from Fluka. α-Ketobutyric acid and ethyl isocyanate were obtained from Aldrich. Benzyl bromide, thionyl chloride, and triethylamine were obtained from Merck. Trityl chloride was from Janssen Chimica. All other chemicals used were of analytical grade. Recombinant SHL was purified to homogeneity after overexpression in *Escherichia coli* as described previously (*22*). Lipase concentrations were determined spectrophotometrically at 280 nm using an absorption coefficient of 14.5.

General Methods. Thin-layer chromatography (TLC) was carried out on silica gel plates from Merck (60F254). Melting points (mp) were determined on a Leitz melting point microscope and are uncorrected. ^{1}H NMR measurements were carried out on a 360 MHz Bruker machine. Mass data were obtained on a JMS SX 102/102 FAB spectrometer.

Synthesis of Inhibitors. The synthesis of the inhibitors is described as Supporting Information which is available via the Internet.

Lipase Inhibiton Assays. The enzymatic activity of SHL was determined spectrophotometrically with as a substrate mixed micelles of PNPB (1 mM) and TX100 (100 mM) in a buffer composed of 50 mM Hepes (pH 7), 10 mM $CaCl_2$, and 50 mM NaCl. The inhibitor was added from a concentrated stock solution in acetonitrile (10 mM) at a maximal concentration of 0.5 mM. The percentage of acetonitrile present in the assay mixture never exceeded 5%. The enzymatic activity of SHL was not changed in the presence of such low amounts of acetonitrile. The total reaction volume was 1 mL, and the reaction temperature was 21 °C. The reaction was started by the addition of enzyme. Activities were calculated from the increase in absorbance at 400 nm using a molar extinction coefficient of 2480 M^{-1} cm^{-1} for *p*-nitrophenol, which is the experimentally determined value under the assay conditions that were used.

Determination of Inhibitor Solubility in the Absence and Presence of Detergent. Stock solutions (50 mM) of inhibitor were prepared in acetonitrile. A small aliqout (50 μL) of this stock solution was added to 1 mL of a buffer containing 50 mM Hepes (pH 7), 10 mM $CaCl_2$, and 50 mM NaCl, either in the absence or in the presence of 100 mM TX100. The mixtures were vigorously stirred for 30 min, and thereafter centrifuged at 10000*g* for 10 min to remove any insoluble inhibitor. Subsequently, the amount of inhibitor present in the supernatant was determined using hydroxylamine and ferric perchlorate reagents as described by Hall and Shaefer (*23*). Absorbances were measured at 510 nm. Calibration curves for each inhibitor were lineair over a concentration range from 0 to 8 mM.

RESULTS

The design of a first-generation of α-keto inhibitors was based on the structure of tributyrin, which is a good substrate for SHL (*22*). The ideal triglyceride inhibitor contains only one α-keto group, whereas at the other two positions, normal ester bonds are maintained. Such a combination of substrate and inhibitor properties in the same molecule may, however, complicate kinetic analyses. Moreover, the presence of hydrolyzable bonds is a serious problem in structural studies whose aim is to elucidate interactions between the inhibitor and the enzyme. Therefore, we synthesized diglyceride analogues containing nonhydrolyzable ether (**1**), carbamoyl (**2**), or amide (**3**) bonds at positions 1 and 2. Different nonhydrolyzable bonds were used, since in the case of PLA_2 inhibitors it has been reported that the chemical structure of these bonds is important for the potency of the inhibitors (*24*). Short acyl chains were retained in each case. Subsequently, these diglycerides were esterified at position 3 with either butyric acid or α-keto butyric acid. These compounds were synthesized as racemic mixtures, since SHL exhibits a low stereopreference (*25*). The chemical structures are given in Figure 1.

In an initial experiment, SHL was incubated in the presence of an excess of either the ester (**1b**) or the α-keto derivative (**1c**) of compound **1**. TLC analysis showed that the ester was completely hydrolyzed after overnight incubation, yielding the parent diglyceride and butyric acid. In contrast, the α-keto compound was resistant to enzymatic hydrolysis (not shown).

Not only tributyrin but also *p*-nitrophenol esters of fatty acids are rapidly degraded by SHL. With the latter substrates,

[1] Abbreviations: SHL, *Staphylococcus hyicus* lipase; PNP, *p*-nitrophenol; PLA_2, phospholipase A_2; TX100, Triton X-100; PNPB, *p*-nitrophenyl butyrate; TLC, thin-layer chromatography; THF, tetrahydrofuran; DMAP, (dimethylamino)pyridine; DCC, *N*,*N*′-dicyclohexylcarbodiimide.

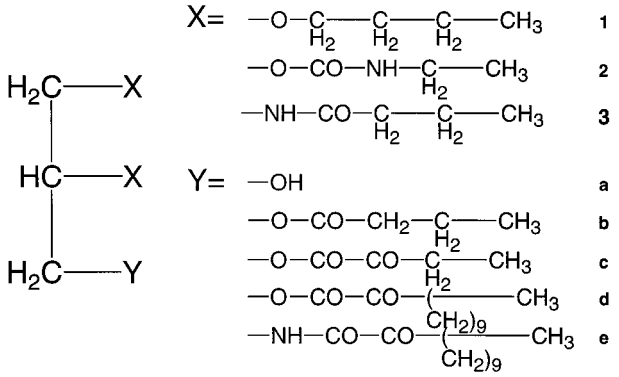

FIGURE 1: Chemical structures of the α-keto triglyceride analogues used in this study.

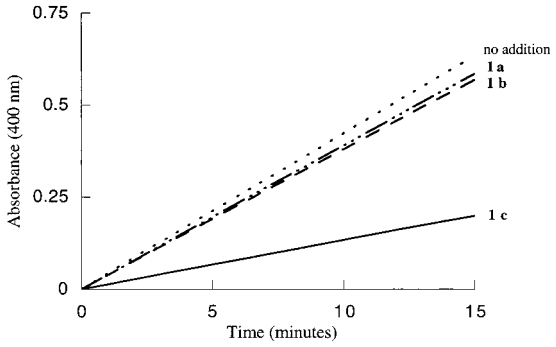

FIGURE 2: Reaction progress curves of substrate hydrolysis by SHL in the presence and absence of inhibitor. The increase in absorbance at 400 nm due to PNPB hydrolysis was assayed in the absence of inhibitor (···), in the presence of inhibitor **1c** (—), and in the presence of control compound **1a** (—···—) or **1b** (- - -). The substrate (1 mM) was incorporated into TX100 micelles (100 mM), and 0.25 mM inhibitor or control compound was included. The reaction buffer contained 50 mM Hepes (pH 7), 10 mM $CaCl_2$, and 50 mM NaCl, and the experiment was carried out at 21 °C. The reaction was started by the addition of 900 ng of SHL.

the activity can easily be followed by the increase in absorbance at 400 nm due to the release of the *p*-nitrophenol anion. In Figure 2, a typical reaction progress curve for the hydrolysis of *p*-nitrophenol butyrate (PNPB) incorporated in TX100 micelles is given. The increase in absorbance is linear for at least 15 min, and from this curve, an activity of 57 units/mg was calculated. In the presence of 0.25 mM **1c**, the slope of the reaction curve decreased 4-fold, resulting in an activity of only 14 units/mg. The compound containing a normal ester bond at position 3 (**1b**) and the parent diglyceride containing a hydroxyl group (**1a**) did not significantly inhibit SHL activity (Figure 2). The small decrease in activity in the presence of the latter compounds probably results from a dilution of the substrate in the interface. The inhibition of enzymatic activity by compound **1c** is a general property because the same extent of inhibition was found with tributyrin as with the substrate in a pH-stat assay (data not shown). These results show that triglyceride analogues containing an α-keto ester inhibit the activity of SHL. Moreover, it shows that this inhibition is specifically associated with the presence of the α-keto group.

The mode of inhibition was further investigated by the determination of the degree of inhibition at different inhibitor concentrations. In these experiments, the concentration of TX100 was fixed at 100 mM for two reasons. First, since

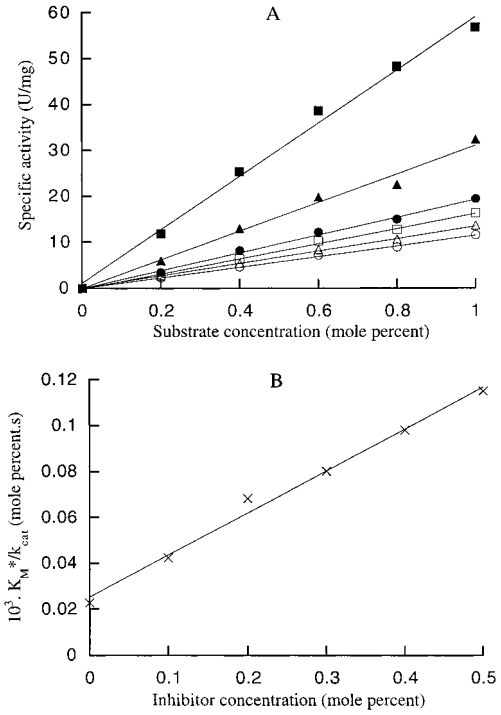

FIGURE 3: (A) Concentration-dependent inhibition of SHL by inhibitor **1c**. Activities were determined at PNPB concentrations varied between 0 and 1 mol % with a fixed TX100 concentration of 100 mM. The inhibitor concentrations were 0 (■), 0.1 (▲), 0.2 (●), 0.3 (□), 0.4 (△), and 0.5 mol % (○). Reactions were performed in a buffer composed of 50 mM Hepes (pH 7), 10 mM $CaCl_2$, and 50 mM NaCl at a temperature of 21 °C. Reactions were started by the addition of SHL. Each point represents the average of duplicate measurements, and the variation was ≤10%. (B) Replot of the reciprocal of the specificity constants calculated from the curves depicted in panel A. Further details of the procedure are described in the text.

the activity of lipases is very sensitive to the physical state of the substrate, it is important that the properties of the substrate interface are not changed upon addition of inhibitor. In our experiments, the concentration of substrate together with inhibitor never exceeded 1.5 mM, which comprises only 1.5 mol % of the total interface. At this low interfacial concentration, the physical properties of the micelle are mainly determined by the TX100 molecules. Hence, any decrease in activity in the presence of inhibitor can be ascribed to a direct effect of the inhibitor on the enzyme and not to changes in the quality of the interface. Second, we have shown before that all the lipase is present at the interface at a concentration of 100 mM TX100 (*22*), and hence, the inhibition involves only micelle-bound lipase. This considerably simplifies the kinetic analysis of our data, since the preceding step of adsorption of the enzyme to the micelle plays no role.

In an initial experiment, the activity of SHL was determined at PNPB concentrations varied between 0 and 1 mol %. Subsequently, this experiment was repeated in the presence of five different inhibitor concentrations, ranging from 0.1 to 0.5 mol %. From the resulting curves, which are shown in Figure 3A, it is evident that the inhibition of SHL depends on the concentration of the inhibitor. From each curve, it is possible to calculate the specificity constant for substrate hydrolysis (k_{cat}/K_M), both in the absence and in the presence of different inhibitor concentrations. In the

general case of competitive inhibition, one can write eq 1:

$$\left(\frac{k_{cat}}{K_M}\right)_{obs} = \frac{k_{cat}/K_M}{1 + \frac{[I]}{K_i}} \quad (1)$$

where $(k_{cat}/K_M)_{obs}$ is the observed specificity constant for substrate hydrolysis in the presence of inhibitor and k_{cat}/K_M is the specificity constant in the absence of inhibitor, both with the dimension M^{-1} s^{-1}. [I] is the concentration of inhibitor, and K_i is the dissociation constant for the enzyme−inhibitor complex, both expressed as moles per liter. For the inhibition of lipases in the presence of an interface, it is important to realize that the competition between substrate and inhibitor occurs in the micellar interface, which is a two-dimensional diffusion process. Concentrations should therefore not be expressed three-dimensionally, but as the mole fraction of substrate or inhibitor present in the interface. In addition, the enzyme−substrate and enzyme−inhibitor complexes are present at the interface, and the interfacial K_M and K_i are therefore indicated with an asterisk. Incorporation of the notations and rewriting of eq 1 give eq 2:

$$\left(\frac{K_M^*}{k_{cat}}\right)_{obs} = \frac{K_M^*}{k_{cat}} + \frac{K_M^*/k_{cat}}{K_i}[I] \quad (2)$$

From this equation, it follows that a graph of $(K_M^*/k_{cat})_{obs}$ versus [I] should result in a straight line with K_M^*/k_{cat} as a *Y*-axis intersection and $(K_M^*/k_{cat})/K_i^*$ as a slope. Plotting the values obtained from Figure 3A in this way yields a straight line with an *R* value of 0.996 (Figure 3B). These results strongly indicate that inhibition indeed occurs via a mechanism of competitive inhibition. From the slope of the line, a K_i^* value of 0.14 mol % was calculated. This means that the presence of one inhibitor molecule per seven substrate molecules gives rise to about 50% inhibition.

Interestingly, when we used compounds **2c** and **3c** which contain the α-keto ester at position 3, but a carbamate or amide, respectively, at positions 1 and 2, no inhibition was observed. To exclude the possibility that the rates for the association of the carbamate and amide compounds with SHL are slow compared to that of the ether inhibitor, we preincubated SHL with either compound **2c** or compound **3c** for 1 h prior to activity assays. Also, using these modified conditions, no significant inhibition of lipase was observed.

Another explanation for the absence of inhibition by compounds **2c** and **3c** could be associated with the localization of the enzyme and the inhibitor. In this respect, it is important to realize that under our assay conditions all lipase is present at the interface (see above). To investigate the localization of the inhibitors, we determined the solubilities of compounds **1c**−**3c**, both in the absence and in the presence of TX100 micelles. In the absence of micelles, 2.5 μmol of the carbamate (**2c**) and amide (**3c**) compounds were added to 1 mL of buffer. It appeared that both compounds nearly completely dissolve in buffer to concentrations of 2.4 and 2.3 mM, respectively (Table 1). In contrast, the ether compound (**1c**) only dissolves to a concentration of 1.2 mM. In the presence of micelles, however, not only compounds **2c** and **3c** but also compound **1c** completely dissolves to a concentration of 2.5 ± 0.15 mM. Because we did not add larger amounts of these compounds, the actual solubility limit might be higher. On the basis of these data, it is very likely that **1c** (partially) incorporates into the micelles, whereas **2c** and **3c** are mainly dissolved in the water phase, explaining the lack of inhibition by these latter compounds.

Table 1: Solubilities and Inhibitor Dissociation Constants of α-Keto Triglyceride Analogues

compd	chemical bonds at positions 1 and 2	α-keto acid position 3	solubility (mM)	K_i^* (mol %)[a]
1c	ether	butyric	1.2	0.14
2c	carbamoyl	butyric	2.4	*b*
3c	amide	butyric	2.3	*b*
1d	ether	dodecanoic	0.4	0.16
2d	carbamoyl	dodecanoic	0.3	0.05
3d	amide	dodecanoic	0.6	0.05
2e[c]	carbamoyl	dodecanoic	0.4	0.008

[a] K_i^* values were determined as described in the text using as a substrate mixed micelles of PNPB and TX100. The accuracy for the given values is 10%. [b] No significant inhibition of SHL by these compounds was observed. [c] Note that the oxygen at position 3 is changed into an amido group (Figure 1).

To verify this hypothesis, we increased the acyl chain length at position 3 by introducing α-keto dodecanoic acid instead of α-keto butyric acid. This was done for the ether and the carbamate as well as for the amide compound, resulting in compounds **1d**−**3d** (see Figure 1). This additional hydrophobicity should lower the solubility in water of the three inhibitors and should target these molecules to the micelle. That this is indeed the case is confirmed by the data in Table 1, which show that all three long-chain compounds have only a low solubility in buffer. They completely dissolve in the presence of TX100 (data not shown). These compounds are therefore predominantly present in the micelle and available for the lipase. The K_i^* values of these long-chain inhibitors were determined as described for compound **1c** and are summarized in Table 1. In contrast to the short-chain compounds, all three long-chain compounds inhibit SHL, irrespective of the nature of the chemical bond at positions 1 and 2. No inhibition was observed with the compounds containing a normal ester, or with the parent diglycerides (not shown).

These results show that only those triglyceride analogues which incorporate into the micelle are SHL inhibitors. Interestingly, there is a variation in the inhibitory power which cannot be explained by a difference in solubility. The carbamate and amide inhibitors, which like the substrate contain a carbonyl group at positions 1 and 2, are 3-fold better inhibitors than the ether compound. This observation shows that interactions with the carbonyl at position 1 and/or 2 may contribute to the binding of these inhibitors. On the basis of this observation, we wondered whether the inhibitory properties of the α-keto inhibitors could further be improved. Therefore, the oxygen at position 3 of inhibitor **2d** was replaced by an amido group, resulting in compound **2e** (see Figure 1). This compound turned out to be the best inhibitor that we have synthesized so far. Using the method described above, a K_i^* value of 0.008 mol % was determined, which means that one molecule of this inhibitor present per 125 substrate molecules causes 50% inhibition.

DISCUSSION

Although α-keto inhibitors have been described for proteases (*19*, *20*), little is known about their mechanism of

action. For activated fluoromethyl ketones, however, the mechanism of inhibition is well-established. It has been demonstrated that fluoromethyl ketone substrate analogues of esterases (*26*−*28*), proteases (*27*, *29*), and recently also cytosolic PLA_2 (*30*) are potent inhibitors of these enzymes. Inhibition is generally ascribed to the presence of the electron-withdrawing fluorine atoms, resulting in an activated ketone which is highly susceptible to nucleophilic attack by the enzyme. This mechanism of inhibition was subsequently confirmed by structural studies. The X-ray structures of elastase and chymotrypsin inhibited by trifluoromethyl ketones revealed the presence of a covalent bond between the active site Ser and the inhibitor (*31*, *32*). NMR studies strongly suggest that these activated ketones are bound as the anion of the hemiketal, thus mimicking the transition state of substrate hydrolysis (*33*, *34*).

On the basis of several lines of evidence, we propose that our triglyceride analogues inhibit SHL in a similar way, where the α-keto oxygen fulfills the role of the electron-withdrawing group. First, SHL, which is a member of the lipase family, belongs to the same superfamily of serine hydrolases which are all inhibited by fluoromethyl ketones. Second, our α-keto triglycerides are competitive inhibitors, just as are the fluoromethyl ketones. This means that both classes of inhibitors compete with the substrate for the active site, which suggests that they interact with the enzyme like real substrates do. Third, we unambiguously showed that the absence of the activating α-keto oxygen, like in the normal ester or in the diglyceride, results in a loss of inhibition of SHL.

An important difference in inhibition kinetics between our inhibitors and fluoromethyl ketones was also observed. Inhibition of serine hydrolases by fluoromethyl ketones is characterized by biphasic reaction progress curves, which are indicative of slow-binding inhibition (*35*, *36*). The slow development of inhibition has been ascribed to the occurrence of relatively slow conformational changes in the enzyme−inhibitor complex, and/or reorientations of the inhibitor in the complex, preceding the formation of a tightly inhibited complex (*29*, *37*). However, crystallographic studies of proteases inhibited by slow-binding trifluoromethyl ketones did not provide any evidence for major conformational changes as a result of inhibitor binding (*31*, *32*). Alternatively, slow-binding inhibition has been associated with a low effective inhibitor concentration. Since these inhibitors bind in the nanomolar range, only small amounts of inhibitor are added in inhibition assays. Moreover, it is important to realize that activated ketones are highly susceptible to hydration by water. For fluoromethyl ketones, it was shown that these compounds are essentially completely hydrated in aqueous solution, whereas the nonhydrated form is the inhibitory species (*29*). For these reasons, the effective inhibitor concentration during inhibition assays may become very low, and diffusion-limited association between enzyme and inhibitor could account for the slow development of inhibition (*37*, *38*).

In contrast to this complex kinetic behavior of fluoromethyl ketones, reaction curves in the presence of our inhibitors are linear over time. It is not unlikely that the occurrence of instantaneous inhibition is related to the fact that our inhibitors are present in micelles. This micellar solubilization may increase the effective inhibitor concentration in two ways. First, by incorporation into the micelles, the inhibitor is targetted to the micelle-bound lipase, and thus, the concentration of the available inhibitor is increased. Second, as a result of the hydrophobic environment inside the micelle, the level of hydration might be lower. Finally, it should be noted that our inhibitors bind in the micromolar range, and therefore are added at higher concentrations compared to the nanomolar range of fluoromethyl ketones.

The results of this study allow us to define the structural properties which should be combined to obtain good lipase inhibitors. First, one of the hydrolyzable ester bonds should be changed into an inhibitory group, which in our inhibitors is realized by the presence of an α-keto function. In contrast to the previously described phosphonate inhibitors (*14*, *15*), this is a very subtle modification which does not involve the introduction of an extra chiral center, or of any bulky group. Second, the inhibitor should dissolve into the micellar phase. This is exemplified by the observation that highly water-soluble α-keto triglycerides fail to inhibit SHL. By simply increasing the hydrophobicity, and hereby targetting these compounds to the micelle, we obtained good inhibitors. It should be noted that the increase in chain length in position 3 does not drastically increase the affinity of SHL for the inhibitor, since the short- and long-chain ether compounds are equally good inhibitors. Third, specific protein−inhibitor interactions other than with the α-keto function also play an important role. This conclusion is based on the observation that the long-chain triglycerides, although all predominantly present in the micelle, differ in their inhibitory powers. Interestingly, the carbamate and amide inhibitors are about 3-fold more potent than the inhibitor containing ether bonds. This observation suggests that the presence of the carbonyl oxygens is important for inhibition, probably via the existence of specific interactions with the enzyme. In this respect, it is relevant to note that Stadler and co-workers showed that the nature of the chemical bond at position 2 of substrate molecules is very important for stereoselective catalysis by lipases (*39*). The results of these authors and our results suggest that similar interactions exist in our inhibitors just like in real substrates, which further supports the notion that α-keto triglycerides are good substrate mimicks for lipases.

In inhibitor **2e**, all the properties described above are combined. Moreover, an additional change of the oxygen at position 3 into an amide group made it the best inhibitor for SHL. The increased extent of inhibition as a result of this latter modification could result either from the formation of an additional protein−inhibitor interaction that increases the extent of inhibitor binding or from a changed electrostatic effect on the α-keto function that improved the reactivity of this group. In summary, the stepwise modification of α-keto triglyceride analogues resulted in competitive inhibitors which bind in the low micromolar range. These inhibitors are highly similar to real substrates, from both a structural and a kinetic point of view. They may therefore be useful in cocrystallization experiments with any lipase, in increasing our knowledge of enzyme−substrate interactions at the molecular level. Our next aim is to further improve these inhibitors by "fine-tuning" the length of the acyl chains in each position for an optimal fit into the enzyme's binding pocket.

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SUPPORTING INFORMATION AVAILABLE

Description of the synthesis of the inhibitors used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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