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Design of an effective mechanism-based inactivator for a zinc protease

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ABSTRACT **(*R*)-2-Benzyl-5-cyano-4-oxopentanoic acid (compound 4) was studied as a mechanism-based inactivator (suicide substrate) for the zinc protease carboxypeptidase A (CPA; peptidyl-L-amino-acid hydrolase, EC 3.4.17.1). This compound was designed rationally based on the knowledge of the active site topology and the reported stereospecific proton exchange on ketonic substrate analogue (*R*)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid [Sugimoto, T. & Kaiser, E. T. (1978) *J. Am. Chem. Soc.* 100, 7750–7751] by CPA. It is suggested that enzymic deprotonation on the C-5 methylene moiety may result in the transient formation of a ketenimine as the key intermediate that partitions between turnover and enzyme inactivation. The enzyme inactivation exhibited pseudo-first-order kinetics, was irreversible, and could be fully prevented in the presence of the reversible inhibitor benzylsuccinate. The inactivation rate constant, k_{inact}, was evaluated to be 0.083 ± 0.003 min^{-1} and k_{cat} was measured at 1.78 ± 0.06 min^{-1}. In turn, a partition ratio of 28 ± 3 was calculated. The reversible inhibitor constant (K_i) was measured at 1.8 ± 0.5 μM, indicative of a high affinity for compound 4 shown by CPA; however, K_m for the turnover process was determined at 4.93 ± 0.43 mM. Kinetic analysis and labeling by the radioactive form of the inactivator suggested that the stoichiometry for protein modification by compound 4 approaches a 1:1 ratio.**

Zinc proteases constitute a large and growing subfamily of these metalloenzymes. Traditionally, carboxypeptidase A (CPA; peptidyl-L-amino-acid hydrolase, EC 3.4.17.1) has served as the prototypic zinc protease, as it has been the subject of many studies (1–5). While the mechanistic details of this enzyme are still subject to investigation, much is known about the active site and the role of zinc in catalysis that allows for a rational design of a mechanism-based inactivator for this enzyme.

In earlier investigations on the catalytic role of Glu-270 in CPA, it was shown that the enzyme carried out a proton abstraction from the pro-*R* position α to the carbonyl group in compound **1** (6, 7). Subsequent studies showed that this enzymic enol formation was accompanied by α,β-elimination of the good leaving groups in compounds **2** (8) and **3** (9). Products of the elimination reactions did not trap an active site residue in a Michael fashion, as it was originally intended. As a result compounds **2** and **3** and their elimination products served as reversible competitive inhibitors for CPA.

We wish to report here our studies of compound **4** as the first mechanism-based inactivator for CPA. This molecule was prepared based on the expectation that it would undergo an enzyme-mediated isomerization to an α-ketoketenimine species, which would trap an active site nucleophile of CPA.

MATERIALS AND METHODS

Infrared spectra were obtained on a Perkin–Elmer 1420 spectrometer or a Mattson Cygnus 25 FT-IR instrument. Kinetic measurements were carried out on a Perkin–Elmer Lambda-5 spectrometer. ^{1}H NMR spectra were recorded on a Nicolet 360 MHz instrument using tetramethylsilane as the internal standard. Mass spectra were obtained on a VG 7250 double-focusing mass spectrometer. Scintillation counting was carried out on a Beckman LS3801 instrument. (*R,S*)-Benzylsuccinic acid and CPA were purchased from Sigma. α-Chymotrypsin was a Boehringer Mannheim product. All other chemicals were purchased from Aldrich. Enzyme activity was monitored according to the method of Suh and Kaiser using the chromogenic ester substrate *O*-(*trans*-*p*-chlorocinnamoyl)-L-3-phenyllactate (10). A radioactive form of compound **4** was prepared, with ^{14}C incorporated at the nitrile moiety by allowing (*R*)-benzylsuccinic anhydride to react with the lithium anion of *t*-butyldimethylsilyl-[^{14}C]cyanoacetate, followed by acid workup (11). The desired compound **4** was purified from its regioisomer (*R*)-3-benzyl-5-[^{14}C]cyano-4-oxopentanoic acid on a semipreparative C_8 column. The identity of radiolabeled compound **4** was verified by its comparison to authentic material synthesized by an independent route, as detailed below. The specific activity was 50 dpm/nmol. Inactivation experiments with radioactive compound **4** and the workup were performed according to the methodology published previously (12).

(*R,S*)-Dimethyl Benzylsuccinate (Compound 7). Acetyl chloride (4.5 ml, 64 mmol) was added to 100 ml of anhydrous methanol over 5 min at 0°C under a nitrogen atmosphere. The solution was stirred for 15 min and then was warmed to room temperature. (*R,S*)-Benzylsuccinic acid (compound **6**; 6 g, 28.8 mmol) was added, and the solution was refluxed for 3 hr. Subsequently, the solution was concentrated *in vacuo* to dryness. The residual oil was taken up in ethyl acetate (200 ml), washed with saturated $NaHCO_3$ and brine, and then dried over anhydrous $MgSO_4$. The solution was evaporated to dryness to afford the product as an oil (6.5 g, 96%). ^{1}H NMR (C^2HCl_3) δ 7.31–7.14 (m, 5H), 3.67 (s, 3H), 3.64 (s, 3H), 3.15–3.02 (m, 2H), 2.80–2.64 (m, 2H), 2.41 (dd, J = 4.9, 16.8 Hz, 1H); IR (neat) 3029, 2953, 1731 cm^{-1}; CI MS m/z 237 (100%, M + H).

Methyl (*R*)-2-Benzyl-3-carbomethoxypropionic acid (Compound 8). A solution of α-chymotrypsin (EC 3.4.21.1, 800 mg) in 60 ml of water was allowed to react with compound **7** (6.55 g, 29 mmol), which was suspended in 100 ml of water at 23°C. A constant pH of 7.2 was maintained by titrating the reaction mixture with 0.1 M NaOH using a pH-Stat. After 18 hr, 140 ml of the NaOH solution was consumed, indicating 50% hydrolysis of the racemic substrate. The solution was washed with ethyl ether and the aqueous phase was acidified to pH 2.0 with 1 M HCl. The resultant solution was concentrated *in*

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Abbreviations: CPA, carboxypeptidase A; THF, tetrahydrofuran.

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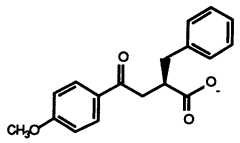
[‖]Deceased July 18, 1988.



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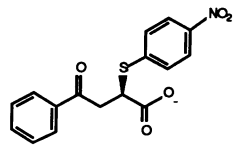

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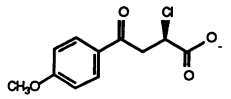

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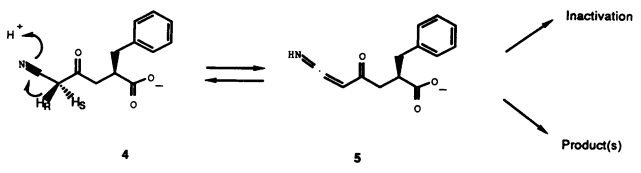
vacuo to dryness. The residue was sonicated in the presence of ethyl ether, and subsequently the solution was filtered, washed with brine, and dried over anhydrous $MgSO_4$. Evaporation of the organic solution to dryness provided the optically active product as an oil (3.05 g, 98%). ^1H NMR (C^2HCl_3) δ 7.33–7.05 (m, 5H), 3.65 (s, 3H), 3.16 (m, 2H), 2.78 (m, 1H), 2.66 (dd, J = 16.9 and 8.6 Hz, 1H), 2.41 (dd, J = 16.9, 4.7 Hz, 1H); IR (neat) 3500–3300 (br), 1738, 1713 cm^{-1}; CI MS m/z 223 (100%, M + H).

Methyl (*R*)-3-benzyl-4-hydroxybutanoate (Compound 9). A solution of BH_3 in tetrahydrofuran (THF) (10 ml of a 1.0 M solution, 10 mmol) was added dropwise to a stirred solution of compound **8** (1.70 g, 7.6 mmol) in 10 ml of THF at 0°C under a nitrogen atmosphere. The solution was stirred for 15 min at 0°C, and then the flask was placed in the freezer for 16 hr. The reaction was quenched at 0°C with methanol (5 ml), stirred for 30 min at 0°C, and then stirred for an additional 30 min at room temperature. More methanol (10 ml) was added and the solvent was removed *in vacuo* (three times). The residue was dissolved in ethyl ether (100 ml), washed with saturated $NaHCO_3$ and brine and was dried over $MgSO_4$. The solvent was evaporated *in vacuo* to afford the product as an oil (1.36 g, 86%). ^1H NMR (C^2HCl_3) δ 7.32–7.16 (m, 5H), 3.64 (s, 3H), 3.63–3.49 (m, 2H), 2.72 (dd, J = 13.6, 6.6 Hz, 1H), 2.59 (dd, J = 13.5, 6.7 Hz, 1H), 2.46–2.33 (m, 3H); IR (thin film) 3453 (br), 3026, 2950, 1732 cm^{-1}; CI MS m/z 209 (100%, M + H).

(*R*)-4-Benzyl-2-(*R,S*)-cyanomethyl-2-(*R,S*)-hydroxytetrahydrofuran (Compound 10). A solution of acetonitrile (0.64 ml, 12.3 mmol) in 10 ml of dry THF was added dropwise to a solution of *n*-butyllithium (5.4 ml of a 2.5 M solution in hexane, 13.5 mmol) in 20 ml of dry THF at −78°C under an atmosphere of nitrogen. A white precipitate formed after addition of the solution; the mixture was stirred for 1 hr at −78°C. A solution of compound **9** (0.64 g, 3.1 mmol) in 10 ml of THF was added dropwise, and the reaction mixture was stirred for 1 hr at −78°C, followed by an additional hour at 0°C. Subsequently, the reaction was quenched with 5% HCl (7 ml) and the solution was diluted with ethyl ether (50 ml).



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Scheme I

The organic phase was separated, and the aqueous layer was extracted with a 20-ml portion of ethyl ether. The combined organic extracts were washed with brine and then dried over $MgSO_4$. The solvent was evaporated and the residue was flash-chromatographed with 1% *i*-propanol/30% ethyl acetate in hexane as eluent, to give 645 mg of compound **10** as an oil (97%). The product was a 7:3 mixture of two isomers as determined by NMR. ^1H NMR (C^2HCl_3) δ 7.32–7.14 (m, 5H), 4.14 (m, 0.7H), 4.03 (m, 0.3H), 3.80 (m, 0.3H), 3.68 (m, 0.7H), 2.93–2.69 (m, 5H), 2.24 (m, 1H), 1.93 (m, 0.3H), 1.74 (m, 0.7H); IR (thin film) 3418 (br), 2930, 2258 cm^{-1}; CI MS m/z 218 (60%, M + H).

(*R*)-2-Benzyl-5-cyano-4-oxopentanoic acid (Compound 4). A solution of compound **10** (26 mg, 0.12 mmol) in 1.2 ml of acetic acid was added very slowly to a solution of 1.0 ml of Jones reagent (0.4 ml of $H_2Cr_2O_7$/0.2 ml of H_2SO_4/0.4 ml of H_2O in 1.6 ml of acetic acid). After addition was completed, the solution was stirred for 1 hr. The reaction mixture was diluted with 10 ml of water and the aqueous solution was extracted with 2 ml of ethyl ether (three times). The combined organic layer was washed with brine and dried over $MgSO_4$. The solvent was evaporated, the residue was redissolved in toluene (5 ml), and the solution was concentrated *in vacuo*. Flash chromatography of the residue with 0.5% acetic acid/35% ethyl acetate in hexane as eluent afforded 14 mg of compound **4** as an oil (50%). The compound was subsequently purified further by HPLC; t_R = 15.9 min (Vydac; 10-μm ODS, 1 × 25 cm, 5–95% linear acetonitrile gradient in 0.1% aqueous trifluoroacetic acid over 30 min, 3 ml/min, 254 nm); ^1H NMR (C^2HCl_3) δ 7.34–7.16 (m, 5H), 3.37 (br s, 2H), 3.32–3.17 (m, 2H), 2.83–2.76 (m, 2H), 2.54–2.48 (dd, J = 4.4, 17.4 Hz, 1H); IR (thin film) 3200 (br), 2916, 2264, 1731, 1713 cm^{-1}; CI MS m/z 232.082 (20%, M + H) (expected 232.089).

Measurements of the Initial Rates of Inactivation. A series of inactivation experiments were carried out with inactivator concentrations within the range of 1.0 to 10.0 mM. Typically, a solution of the inactivator in *p*-dioxane was added to an enzyme solution in 50 mM Mops/0.5 M NaCl, pH 7.0, to afford a final concentration of 2.0 μM enzyme in 10% *p*-dioxane for any given inactivator concentration. The solutions were incubated at room temperature. At 2.5-min time intervals 15-μl samples of the inactivation mixture were removed and diluted into 135 μl of 10 mM Mops/0.5 M NaCl, pH 7.0. A 100-μl/portion of the resultant solution was added to 900 μl of assay mixture and the decrease in the absorbance at 320 nm was monitored for the first 30 sec of the reaction immediately.

To demonstrate that the inactivation was active site directed, a competition experiment with benzylsuccinate, a CPA inhibitor (13), was carried out. A 100-μl solution of 0.1 μM (*R,S*)-benzylsuccinate, 1.9 μM enzyme, and 5 mM compound **4** in 50 mM Mops/0.5 M NaCl, pH 7.0, supplemented with 10% *p*-dioxane was stirred gently at room temperature. Samples of the inactivation mixture (15 μl) were removed and diluted 1:10 with the same buffer at various time intervals. A 100-μl portion of the enzyme dilution was added to a 900-μl assay mixture and the activity was monitored at 320 nm.

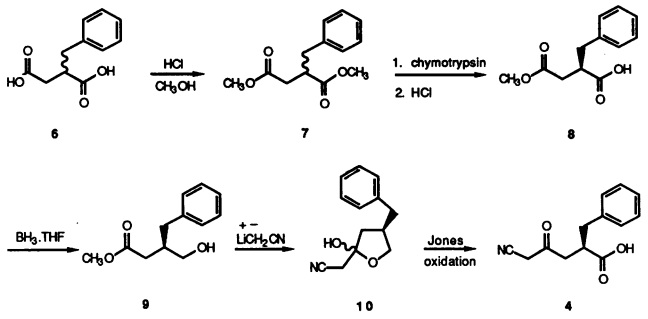
Determination of the Reversible Inhibitor Constant for Compound 4. Reversible inhibition of the hydrolytic activity of CPA by compound **4** was studied according to the method of Dixon (14). Two concentrations of the substrate ester (see above), 500 and 100 μM, were used to ascertain the mode of inhibition as well. A series of assay mixtures containing both the substrate and various concentrations of the inactivator (10–50 μM) were prepared in 50 mM Mops/0.5 M NaCl, pH 7.0, supplemented with 5% *p*-dioxane. A sample of the enzyme was added to afford a final enzyme concentration of

200 nM in a total vol of 500 μl. The enzymic activity was measured immediately.

Measurement of the Partition Ratio. A series of solutions (200 μl each), containing 2.0 μM enzyme and various molar equivalents of compound **4**, were prepared to give $[I]_o/[E]_o$ ratios of 1.0 to ≈2600 in 50 mM Mops/0.5 M NaCl, pH 7.0, supplemented with 5% *p*-dioxane and were stirred gently at 4°C for 16–17 hr. Subsequently, 15-μl samples of each solution were diluted into 135 μl of the same buffer. A 100-μl portion of each dilution was added to 900 μl of the assay mixture and the activity was measured immediately. Control experiments were carried out in the same manner in the absence of the inactivator.

RESULTS

Synthesis of Compound 4. The synthesis of compound **4** is shown in Scheme **II**, with (*R,S*)-benzylsuccinic acid (compound **6**) used as starting material. Dimethyl ester of benzylsuccinic acid (compound **7**) was prepared in 96% yield by refluxing compound **6** in methanolic HCl. Subsequently, chymotrypsin was used to selectively hydrolyze the methyl ester proximal to the benzylic group to give the monoester compound **8** (15), with the desirable *R* stereochemistry at the methine carbon in nearly quantitative yield. Reduction of the carboxylic acid moiety of compound **8** with BH_3·THF afforded compound **9** in 86% yield. Reaction of the lithium salt of acetonitrile with the methyl ester of compound **9** gave a ketone, which readily cyclized to give the hemiketal compound **10** in 97% yield, as a 7:3 mixture of diasterimers. Finally, transformation of compound **10** to **4** was effected by Jones oxidation under acidic conditions.



Scheme II

Kinetic Analysis. Eq. **1** depicts a minimal kinetic scheme for the processing of compound **4** by CPA. Compound **4** is represented by I, and EI is the Michaelis complex, which rearranges to the electrophilic species EI* (i.e., **5**). As stated above, EI* may turn over to product(s)†† (P) or may lead to enzyme inactivation (E_{inact}). We have assumed that product(s) P may be reversible inhibitors of the enzyme in our analyses.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EI^* \begin{matrix} \overset{k_3}{\nearrow} E_{inact} \\ \underset{k_4}{\searrow} E + P \end{matrix} \overset{K_P}{\rightleftarrows} EP \qquad [1]$$

The rate expressions for the processes outlined in Eq. **1** are given below:

$$-\frac{d[I]}{dt} = \frac{k_{cat}([E]_o - [E_{inact}])[I]}{K_m(1 + [P]/K_P) + [I]} \qquad [2]$$

$$\frac{d[E_{inact}]}{dt} = \frac{k_{inact}([E]_o - [E_{inact}])[I]}{K_m(1 + [P]/K_P) + [I]} \qquad [3]$$

where, $K_m = K_a/(1 + K_b)$, $k_{inact} = k_3/(1 + K_b)$, $k_{cat} = k_4/(1 + K_b)$, $K_a = (k_{-1}k_{-2} + k_{-1}k_3 + k_{-1}k_4 + k_2k_3 + k_2k_4)/k_1k_2$, and $K_b = (k_{-2} + k_3 + k_4)/k_2$.

While the kinetic scheme of Eq. **1** is much too complicated to allow for a meaningful analysis of each kinetic parameter, we will attempt to gain insight into a number of these parameters in the following sections.

Initial Rates of CPA Inactivation by Compound 4. At the outset of the enzymatic processing of compound **4**, one may assume that $[E_{inact}]_o$ and [P] are essentially equal to zero and that $[I] = [I]_o$. These assumptions simplify Eq. **3** to give Eq. **4**.

$$v_o = \frac{k_{inact}[E]_o[I]_o}{K_m + [I]_o} \qquad [4]$$

$$\frac{v_o}{[E]_o} = \frac{k_{inact}[I]_o}{K_m + [I]_o} = k_{obs} \qquad [5]$$

The ratio of $v_o/[E]_o$ equals the observed first-order inactivation rate constant of the enzyme (Eq. **5**). A double-reciprocal plot of k_{obs} vs. $[I]_o$ provides a linear presentation, allowing for the evaluation of k_{inact} and K_m.

A series of experiments was carried out at different inactivator concentrations (1–10 mM), during which the enzyme concentration (20 μM) was kept constant. Enzyme inactivation obeyed saturation kinetics within this range of inactivator concentrations. A double-reciprocal plot of the inactivation rate, for the first 20% loss of activity, as a function of inactivator concentrations was linear (Fig. 1). The kinetic parameters k_{inact} and K_m were evaluated from Fig. 1 to be 0.083 ± 0.003 min^{-1} and 4.93 ± 0.43 mM, respectively. When the inactivation was attempted in the presence of (*R,S*)-benzylsuccinate, a potent competitive inhibitor of carboxypeptidase (13), a complete protection of enzyme from inactivation was observed.

The rate of enzyme inactivation (k_{obs}) is directly proportional to the concentration of the inactivator. This is expressed by Eq. **6**, where *n* is the order of inhibitor in its reaction with the enzyme and *k'* is a constant. The order of the inactivation reaction (*n*) can be determined for the inactivator from a plot of log k_{obs} vs. log [I] (16).

$$k_{obs} = k'[I]_o^n \qquad [6]$$

A slope of 0.8 (Fig. 2), strongly suggests that CPA interacts with a single molecule of compound **4** in the course of inactivation. Furthermore, we carried out an inactivation experiment with a radioactive form of compound **4**, where

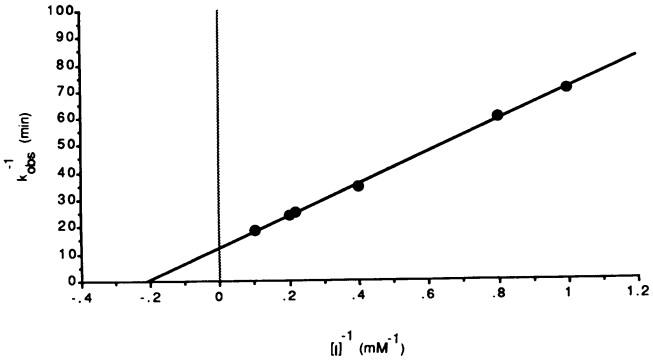


FIG. 1. Double reciprocal plot of the first-order inactivation rate constants of CPA vs. concentration of inactivator **4**.

††We have isolated two products for the enzymic turnover of **4**. The nature of these products will be communicated in a subsequent publication.

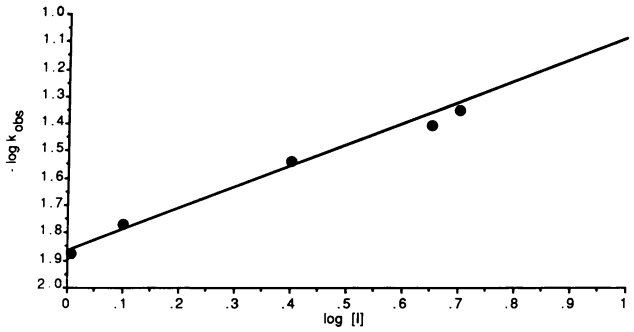


FIG. 2. Plot of the logarithm of the first-order inactivation rate constants of CPA vs. the logarithm of concentrations of compound **4**.

^{14}C was incorporated in the nitrile moiety. After dialysis, 1.17 equivalents of the inactivator were measured to be appended to each molecule of the enzyme. Denaturation of the protein in the presence of 7 M urea, followed by dialysis, revealed that ≈90% of the radioactivity remained associated with the protein, indicative of the covalent nature of CPA modification by compound **4**.

Treatment of Compound 4 As a Reversible Inhibitor of CPA. Compound **4** was studied as a potential competitive inhibitor of ester hydrolysis by the enzyme. Two concentrations of the ester substrate *O*-(*trans*-*p*-chlorocinnamoyl)-L-3-phenyllactate were used to study the mode of inhibition. Analysis according to the method of Dixon (14) showed a pattern consistent with competitive inhibition, with a K_i of 1.8 ± 0.5 μM (Fig. 3).

Determination of the Partition Ratios. The partition ratio was evaluated by the titration method (17). A series of inactivation experiments was carried out where the $[I]_o/[E]_o$ ratio was varied between 1 and ≈2600 (Fig. 4). The deviation from linearity in this kind of plot has been attributed to product inhibition (17). The data points above an $[I]_o/[E]_o$ ratio of 20 deviate from linearity and approach a hyperbola (not all data points are shown in Fig. 4). The linear portion extrapolates to an $[I]_o/[E]_o$ value of 28 ± 3, which equals k_{cat}/k_{inact}, or the partition ratio. The value of k_{inact} was determined to be 0.083 ± 0.003 min^{-1} (see above) and from the partition ratio k_{cat} was evaluated at 1.78 ± 0.06 min^{-1}.

DISCUSSION

In the CPA-catalyzed enolization and α,β-elimination reactions of appropriately designed ketone substrates (compounds **1**, **2**, and **3**), we had observed hydrogen abstraction occurring from the methylene group α to the keto group. It was our intention to exploit this chemical reaction of the enzyme in the design of mechanism-based inactivators. In principle, the most straightforward way to proceed would be

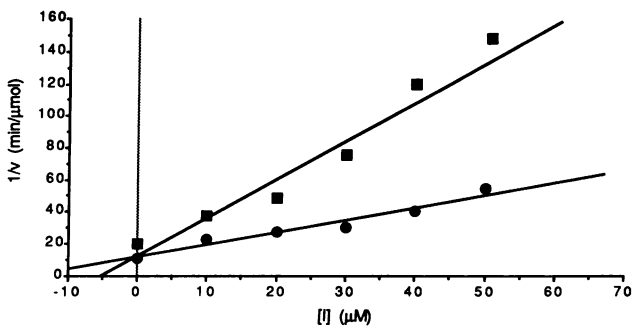


FIG. 3. Plot of the reciprocal of velocity as a function of concentration of compound **4** in the presence of 100 μM (■) and 500 μM (●) substrate.

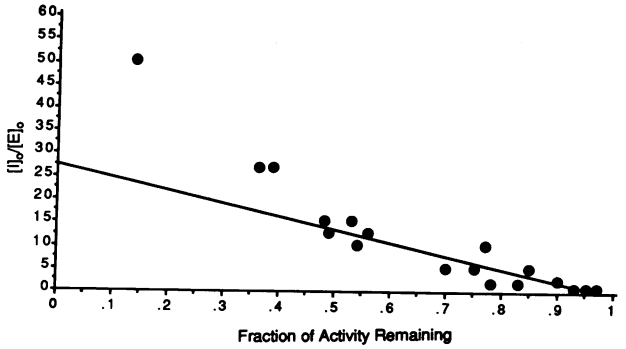


FIG. 4. Plot of $[I]_o/[E]_o$ vs. the fraction of the activity remaining.

to introduce a halomethyl functionality (e.g., trifluoromethyl) on the carbon α to the ketone carbonyl and the benzyl groups. One may expect that α-proton abstraction accompanies β-halide elimination, resulting in a highly electrophilic α,β-unsaturated intermediate. In turn, this reactive species could capture an active site nucleophile (e.g., Glu-270), resulting in enzyme inactivation. However, this approach is unattractive since it is known that introduction of a substituent on the amide nitrogen of peptides—as in N-methylated derivatives—results in very poor substrates (18).

Based on this rationale, our desire was to use the enzymic proton abstraction from an activated methylene group, which was adjacent to the carbonyl, but on the side of the carbonyl away from the terminal carboxyl group. From examination of the CPA crystal structure, we noted that the γ-carboxylate of Glu-270 is oriented in such a manner that it may be able to abstract a hydrogen from the latter type of methylene function (see below). Therefore, compound **4** was designed with the expectation that it may undergo the requisite enzymic proton abstraction by Glu-270—directly or in a general base manner—followed by an isomerization of the nitrile to the corresponding ketenimine compound **5** (Scheme I). Such a rearrangement for nitriles has been exploited in enzyme inactivation (19–22). While this reaction would be obviously thermodynamically uphill, the transient formation of the ketenimine species would lead to the rapid trapping of an enzymic nucleophile such as the γ-carboxylate group of Glu-270. It is noteworthy that because of the conjugation of the ketenimine with the carbonyl group, there might be an increased tendency to form such a reactive intermediate due to stabilization afforded by interaction with an electrophilic center in CPA, such as the active site zinc or Arg-127.

Kinetic Analysis of the Inactivation Process. We attempted to simplify the rate expressions for the processes shown in Eq. **1** to gain insight into a number of kinetic parameters. Initial rate assumptions were made to simplify Eq. **3** to the form given by Eq. **4**. Analysis of initial rates by Eq. **4** gave a k_{inact} of 0.083 ± 0.003 min^{-1} and a K_m of 4.93 ± 0.43 mM (Fig. 1). Treatment of the observed rates of inactivation at different concentrations according to the method of Levy *et al.* (16) gave an order of 0.8 for the inactivator in its reaction with the enzyme (Fig. 2). In addition, it was shown that inactivation by the radioactive form of compound **4** incorporated 1.17 equivalents of the inactivator into the protein, of which ≈90% remained associated with the protein after its denaturation. Thus, it was shown that protein modification was through a covalent linkage and was irreversible under the experimental conditions used. This suggests that the inactivator–enzyme interaction is quite specific and involves an enzyme modification by compound **4** that approaches a 1:1 stoichiometry. Furthermore, the inactivation was active-site directed, as evidenced by the complete protection from inactivation by (*R*,*S*)-benzylsuccinate—a reversible inhibitor of CPA.

Treatment of compound **4** as a competitive inhibitor of CPA hydrolysis of an ester substrate gave a K_i of 1.8 ± 0.5 μM. Insofar as K_i may approximate K_s, the reversible inhibitor binding to the enzyme appears to be with high affinity. We therefore believe that the rate-limiting step in the processes depicted in Eq. **1** must be beyond the formation of EI, reflected by a K_m value that is ≈2000-fold higher than K_i.

A k_{cat}/k_{inact} (i.e., the partition ratio) value of 28 ± 3 was obtained by plotting $[I]_o/[E]_o$ vs. the fraction of activity remaining, indicating that compound **4** is a very effective inactivator for CPA. In turn, we were able to evaluate k_{cat}, which was determined to be 1.78 ± 0.06 min^{-1}.

Mode of Active Site Binding. Currently, the mechanisms by which compound **4** inactivates CPA remains unclear. However, compound **4** was designed rationally based on a number of features of CPA active site and its binding interactions with known substrates and inhibitors that help in proposing a possible mechanism. Compound **4** was prepared as a nonpeptidic isostere of glycyltyrosine, a known substrate—albeit a poor one—for CPA. The carboxylate functionality of compound **4** is assumed to form a salt bridge with the guanidinium group of Arg-145 and the 2-benzyl substituent is expected to be anchored in the hydrophobic pocket in the active site. Crystallographic studies by Lipscomb and colleagues point to two possible binding modes to CPA. Traditionally, the scissile bond of peptidic substrates is understood to be polarized by coordination of the carbonyl group to the zinc ion in the active site prior to hydrolysis. This binding mode has been shown for glycyltyrosine, for instance (23). Crystal structure of the ketonic substrate analogue **1** with CPA at 1.54 Å resolution indicated that the carbonyl oxygen was not coordinated to the zinc ion, but rather formed a bifurcated hydrogen bond with the two nitrogen atoms of the guanidinium group of Arg-127 (24). It is noteworthy that the scissile carbonyl of glycyltyrosine binds similarly to Arg-127 in the apoenzyme (25). It is conceivable that compound **4** may bind to CPA by coordinating to either the zinc ion or Arg-127 as discussed above. Either binding mode may place the γ-carboxylate of Glu-270 in close proximity for proton abstraction from the two methylene groups in compound **4**. Proton abstraction from the methylene group proximal to the benzyl substituent will be "non-productive"; however, such a deprotonation step on the other methylene—either directly or by a general base mechanism—leads to a cyanoenolate intermediate, which may isomerize to the α-ketoketenimine compound **5**. A suitable candidate for trapping by the highly reactive compound **5** can be Glu-270, thereby giving rise to an enzyme modified in the active site. While this modification would be sufficient for the inactivation of the enzyme, it is possible that it may rearrange to the more stable imide form by the intramolecular attack of the nitrogen group on ester carbonyl in the "unraveled" inactivator, a reaction reminiscent of that observed for the rearrangement of *O*-acyl urea intermediates to *N*-acyl urea derivatives (26).

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