Peptidyl Epoxides: Novel Selective Inactivators of Cysteine Proteases[1]

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Abstract: Peptidyl epoxides were designed as selective pseudo-mechanism-based inactivators of cysteine proteases. Both *threo*- and *erythro*-peptidyl epoxides were synthesized and tested as potential inactivators of serine proteases (chymotrypsin, subtilisin, and elastase) and of cysteine proteases (papain, cathepsin B, and clostripain). Four tripeptidyl epoxides (Cbz-Gly-Leu-Phe-epoxide, Cbz-Ala-Ala-Phe-epoxide, Cbz-Gly-Leu-Ala-epoxide, and Cbz-Ala-Ala-Ala-epoxide), bearing amino acid sequences similar to those of good substrates or known inhibitors of the serine proteases, were tested in this study. Neither the *threo*- nor the *erythro*-peptidyl epoxides exhibited any inhibitory activity toward the serine proteases, even at high concentration and long incubation time. Nor did the *threo*-peptidyl epoxides inhibit the cysteine proteases. On the other hand, the *erythro*-peptidyl epoxides were time- and concentration-dependent inactivators of the cysteine proteases. Furthermore, stereoselectivity toward the natural L-amino acid at the P_1 position was also exhibited upon inhibition of papain. In order to demonstrate selectivity within the cysteine protease family, two other *erythro*-peptidyl epoxides (Cbz-Phe-Ala-epoxide and Cbz-Phe-O-Bn-Thr-epoxide) were synthesized and tested as inhibitors of the three cysteine proteases. These new peptidyl epoxides exhibited selective inactivation of cysteine proteases, with second-order rate constants (k_i/K_i) ranging over 4 orders of magnitude (0.04−330 M^{-1} s^{-1}). Thus, this new family of highly selective cysteine protease inhibitors offers mechanistic implications and may have useful applications.

Proteases play an essential role in all biological systems. Their activity is subjected to tight regulation by endogenous specific inhibitors.[2] This delicate balance may be perturbed by environmental factors or heredity, leading to excessive enzymatic activity associated with different disease states. High blood pressure, cerebral and coronary infarction, inflammation, emphysema, muscular dystrophy, rickets, and rheumatoid arthritis are just a few to mention.[3] Some bacterial and viral diseases are also intimately associated with specific protease activity, when these microorganisms utilize their own proteases for protein procession. Thus, it is clear that selective inhibition of specific proteolytic enzymes could be of high therapeutic interest. Mechanistic studies have also stimulated development of selective protease inhibitors. Peptidyl chloromethanes,[4] peptidyl trifluoromethanes,[5] and peptidyl phosphonates[6] are just a few such examples.

Serine and cysteine proteases share a similar catalytic mechanism, which is based on a nucleophilic attack of the substrate's amide scissile bond by an enzyme residue.[7] On the basis of theoretical calculations, it was recently suggested that protonation of the substrate scissile bond (either on oxygen or on nitrogen) takes place prior to or concerted with the nucleophilic attack of the thiolate of cysteine proteases.[8] In serine proteases, on the other hand, the nucleophilic attack by the serine hydroxide precedes the protonation step, thus leading to a negatively charged tetrahedral intermediate. This constitutes a subtle mechanistic difference between the serine and cysteine proteases. Such a mechanistic difference was earlier depicted as a possible explanation for the selectivity of some inhibitors toward cysteine proteases.[9]

Epoxides are weak electrophiles and are stable in neutral or basic aqueous media.[10] However, they become highly electrophilic upon protonation. This character was exploited in the development of a few protease inhibitors. Epoxides derived from allyl amides and esters exhibited weak inhibition of cathepsin B and papain.[11] A few peptidyl epoxides were demonstrated to inhibit aspartic proteases.[12] There, too, protonation by the active site aspartic acid may activate the epoxide moiety for nucleophilic attack by an enzyme residue. This principle was also utilized for inhibition of carboxypeptidase A, a metalloprotease in which Zn^{2+} can activate an epoxide.[13]

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(1) Abbreviations: amino acids are written in their three-letter codes; Bn, benzyl; Cbz, benzyloxycarbonyl; BAEE, *N*α-benzoyl-L-arginine ethyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; Succ-AAA-pNA, *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide; Succ-AAPF-pNA, *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide; ONp, *o*-nitrophenol; TFA, trifluoroacetic acid; DIBAL, diisobutylaluminum hydride; mCPBA, 3-(chloroperoxy)benzoic acid; Tris, tris(hydroxymethyl)aminomethane.

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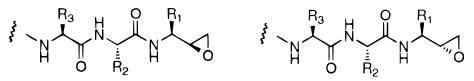
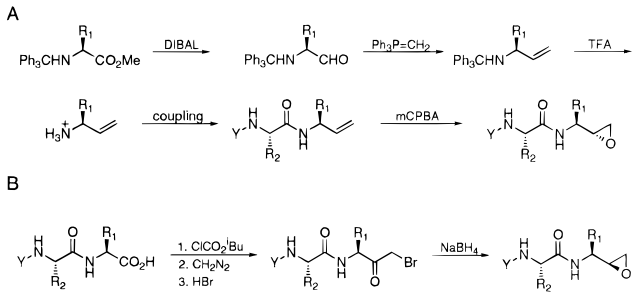


Figure 1. *Erythro* (left) and *threo* (right) peptidyl epoxides.

Scheme 1. Synthetic Approach toward (A) *threo*-Peptidyl Epoxides and (B) *erythro*-Peptidyl Epoxides



As part of our efforts to design selective protease inhibitors, and in order to validate the above-suggested mechanistic difference between serine and cysteine proteases, (2*S*,3*S*)-3-[*N*-(benzyloxycarbonyl)amino]-1,2-epoxy-4-phenylbutane (Cbz-Phe-epoxide) was designed and synthesized as a potential selective inactivator of cysteine proteases. We postulated that it would be activated by protonation in the active site of cysteine proteases, followed by irreversible alkylation of the active site cysteine, but would be unreactive toward the serine proteases. Indeed, it exhibited time- and concentration-dependent inactivation of cysteine proteases, while it did not inhibit serine proteases.[14]

Here, we extended this approach to longer peptidyl epoxides (see Figure 1), in order to generalize the selectivity between the two protease families, to demonstrate selectivity within the family of cysteine proteases, and to gain some insight into the stereoselectivity of the inhibition reaction. We suggest that peptidyl epoxides can be considered as mechanism-based inactivators of cysteine proteases.

Results

Synthesis. *threo*-Peptidyl epoxides were stereoselectively synthesized according to a procedure originally introduced by Luly and co-workers for α-amino epoxides,[15] adapted for peptidyl epoxides by Rich and co-workers,[16] and recently improved by Albeck and Persky.[17] This procedure is described in Scheme 1A. The *threo*-peptidyl epoxides were purified by HPLC, and their isomeric purity was analyzed by HPLC prior to the inhibition studies.[17]

erythro-Peptidyl epoxides were stereoselectively synthesized via a short and efficient route recently introduced by Albeck and Persky (see Scheme 1B).[18] They were utilized in the inhibition studies without being separated from the residual *threo* isomer (see Table 1).

Table 1. *erythro:threo* Ratio of Synthetic Peptidyl Epoxides[a]

peptidyl epoxide	*erythro:threo* ratio
Cbz-Ala-Ala-Ala	2.5
Cbz-Ala-Ala-Phe	13.3
Cbz-Gly-Leu-Ala	6.7[b]
Cbz-Gly-Leu-Phe	4.6
Cbz-Phe-Ala	3.6[b]
Cbz-Phe-O-Bn-Thr	6.1

[a] Synthesized according to the "*erythro*" procedure and crystallized. This product mixture was used for the inhibition studies. [b] Ratio after chromatography.

Interaction with Serine Proteases. The serine proteases chymotrypsin and subtilisin were preincubated with each of the *threo*-peptidyl epoxides Cbz-Gly-Leu-Ala-epoxide, Cbz-Gly-Leu-Phe-epoxide, Cbz-Ala-Ala-Phe-epoxide, and Cbz-Ala-Ala-Ala-epoxide. Inhibitor concentrations were almost at saturation in the 10% (v/v) acetonitrile−buffer preincubation solution (1.0, 0.5, 1.0, 5.0 mM, respectively). No loss of enzymatic activity was detected after 1 h of incubation.

Chymotrypsin and subtilisin were also preincubated with each of the *erythro*-peptidyl epoxides Cbz-Gly-Leu-Ala-epoxide, Cbz-Gly-Leu-Phe-epoxide, and Cbz-Ala-Ala-Phe-epoxide, while the serine protease elastase was preincubated with Cbz-Ala-Ala-Ala-epoxide and Cbz-Ala-Ala-Phe-epoxide. Inhibitor concentrations were almost at saturation in the 10% (v/v) acetonitrile−buffer preincubation solution (ranging from 0.5 to 5.0 mM, as above). Here, too, no loss of enzymatic activity was detected even after long incubation time (up to 2.5 h, except for Cbz-Gly-Leu-Phe-epoxide, which started precipitating after about 1 h).

Interaction with Cysteine Proteases. The activated cysteine proteases papain and cathepsin B were preincubated with each of the *threo*-peptidyl epoxides Cbz-Gly-Leu-Ala-epoxide, Cbz-Gly-Leu-Phe-epoxide, Cbz-Ala-Ala-Phe-epoxide, and Cbz-Ala-Ala-Ala-epoxide. Once again, inhibitor concentrations were almost at saturation. No loss of enzymatic activity was detected after incubation for 1 h.

On the other hand, time- and concentration-dependent inhibition was observed upon incubation of the cysteine proteases papain, cathepsin B, and clostripain with the *erythro* isomer of peptidyl epoxides. One typical graphical presentation of the inactivation process is shown in Figure 2. The corresponding kinetic parameters are summarized in Tables 2−4. It should be noted that various *erythro*/*threo* mixtures were used for this part of the study (see Table 1), and therefore, the actual second-order rate constants for inactivation are somewhat larger then those measured and presented in the tables.

Discussion

Selectivity between Serine and Cysteine Proteases. Both isomers (*erythro* and *threo*) of tripeptidyl epoxides were stereoselectively synthesized and tested as potential inactivators of serine and cysteine proteases. The peptide sequences were chosen to match those of good substrates or known inhibitors of the serine proteases chymotrypsin, subtilisin, and elastase.[19] Therefore, we expected that the enzymes and these specific peptidyl epoxides would form Michaelis-type complexes, placing the epoxide moiety near the active site serine. Nevertheless,

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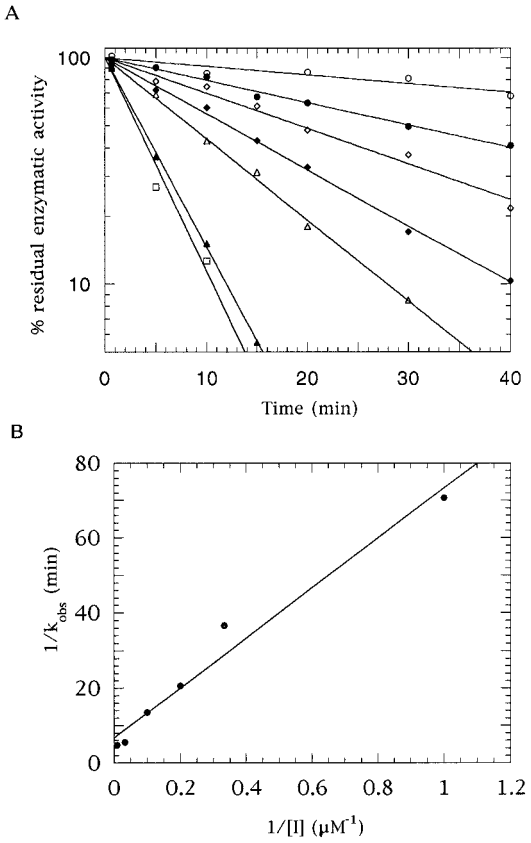

Figure 2. (A) Time course of inactivation of cathepsin B by Cbz-Phe-O-Bn-Thr-epoxide, in 80 mM phosphate buffer, containing 10% (v/v) acetonitrile, 0.5 mM DTT, and 2 mM EDTA, at 25 °C. Inhibitor concentration: (○) 0 μM; (●) 1 μM; (◇) 3 μM; (◆) 5 μM; (△) 10 μM; (▲) 30 μM; (□) 100 μM. (B) Replot of $1/k_{obs}$ vs 1/[I].

Table 2. Kinetic Parameters for the Inactivation of Papain by *erythro*-Peptidyl Epoxides

inhibitor sequence	k_i (min^{-1})	K_i (mM)	k_i/K_i (M^{-1} s^{-1})
Cbz-Ala-Ala-Ala			0.07[a]
Cbz-Ala-Ala-Phe	0.20	1.91	1.75
Cbz-Gly-Leu-Ala	0.02	1.08	0.31
Cbz-Gly-Leu-Phe	0.06	0.64	1.56
Cbz-Gly-Leu-D-Phe			0.04[a,b]
Cbz-Phe[c]	0.21	0.57	6.14
Cbz-Phe-Ala	0.04	0.32	2.08
Cbz-Phe-O-Bn-Thr	0.03	0.03	16.66

[a] Since inactivation was measured in a single inhibitor concentration (1 mM), this value represents k_{obs}/[I]. [b] See Discussion. [c] Taken from Albeck et al.[14]

Table 3. Kinetic Parameters for the Inactivation of Cathepsin B by *erythro*-Peptidyl Epoxides

inhibitor sequence	k_i (min^{-1})	K_i (mM)	k_i/K_i (M^{-1} s^{-1})
Cbz-Ala-Ala-Ala			0.15[a]
Cbz-Gly-Leu-Phe			1.14[b]
Cbz-Phe[c]	0.09	0.37	4.05
Cbz-Phe-Ala	0.07	0.60	1.94
Cbz-Phe-O-Bn-Thr	0.20	0.01	333.33

[a] Since inactivation was measured in a single inhibitor concentration (1 mM), this value represents k_{obs}/[I]. [b] Measurements were limited to the linear range, where [I] $\leq K_i$, due to solubility problems. Therefore, only the second-order rate constant was obtained. [c] Taken from Albeck et al.[14]

no irreversible inactivation was observed with either of the *erythro*- or *threo*-peptidyl epoxides studied. This is in sharp contrast to the formation of covalent adducts between serine proteases and a variety of peptide-based reversible and irreversible inhibitors such as peptidyl aldehydes,[20] peptidyl trifluoromethanes,[21] peptidyl halomethanes,[19b] and peptidyl boronic acids.[22]

Table 4. Kinetic Parameters for the Inactivation of Clostripain by *erythro*-Peptidyl Epoxides

inhibitor sequence	$t_{1/2}$ (min)	[I] (mM)	k_{obs}/[I] (M^{-1} s^{-1})
Cbz-Phe-Ala	270	1.0	0.04
Cbz-Phe-O-Bn-Thr	250	0.1	0.46

The *erythro* isomers of the same peptidyl epoxides were found to be moderate inhibitors of the cysteine proteases papain and cathepsin B. They exhibited time- and concentration-dependent inhibition, in agreement with covalent inactivation. Processing of the data led to the kinetic parameters of the inhibition process, according to the minimal inhibition scheme

$$E + I \overset{K_i}{\rightleftharpoons} E{\cdot}I \xrightarrow{k_i} E{-}I$$

and according to eq 1.[23]

$$1/k_{obs} = (K_i/k_i)(1/[I]) + 1/k_i \quad (1)$$

These results clearly demonstrate the selectivity of *erythro*-peptidyl epoxides as inhibitors of cysteine proteases.

Stereoselectivity. The inactivation of cysteine proteases by peptidyl epoxides exhibited high stereoselectivity with respect to two chiral centers.

The erythro isomer of the four tripeptidyl epoxides (Cbz-Ala-Ala-Ala-epoxide, Cbz-Ala-Ala-Phe-epoxide, Cbz-Gly-Leu-Ala-epoxide, and Cbz-Gly-Leu-Phe-epoxide) inhibited the cysteine proteases papain and cathepsin B, while the corresponding *threo* isomer did not, even upon long incubation time at saturation concentrations.

A second aspect of the stereoselectivity expressed in the inactivation process relates to the preference for the natural L (*S*) isomer of the amino epoxide analog at the P_1 position.[24] In comparing the two peptidyl epoxides *erythro*-Cbz-Gly-Leu-Phe-epoxide and *erythro*-Cbz-Gly-Leu-D-Phe-epoxide, the latter exhibited an apparent second-order rate constant of inactivation only 3% that of the corresponding rate constant of the former L-peptidyl epoxide. It has been previously demonstrated that the synthetic procedure for preparation of *erythro*-peptidyl epoxides involves about 3% epimerization at the Cα of the P_1 amino acid.[18] Therefore, we attribute most of the 3% residual inhibitory activity of the D isomer to contamination by the corresponding L isomer. Thus, we conclude that the inactivation process exhibits >99:1 stereoselectivity, preferring the natural L isomer at the P_1 position of the inhibitor.

These stereoselectivities support an active-site-directed inactivation, in contrast to a nonspecific bimolecular reaction.

Selectivity within the Family of Cysteine Proteases. For most foreseen practical applications of protease inhibition, high selectivity is essential. Therefore, a second set of dipeptidyl

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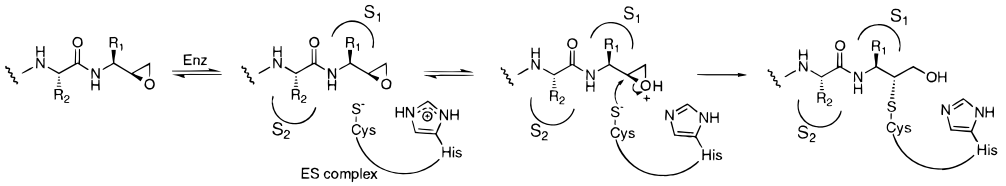
Scheme 2. Proposed Mechanism for the Inactivation of Cysteine Proteases by *erythro*-Peptidyl Epoxides[a]



[a] In this scheme protonation precedes alkylation, but the two chemical steps may be concerted (see text).

epoxides, especially designed to exhibit high selectivity within the family of cysteine proteases, was synthesized. The specific amino acid sequences chosen for these inhibitors were based on reviewing the selectivity of three cysteine proteases—papain, cathepsin B, and clostripain—toward some peptide substrates[25] and other peptide inhibitors.[26]

In general, the second-order rate constants for inhibition of cysteine proteases by peptidyl epoxides are significantly lower than those exhibited by corresponding peptidyl diazomethanes and peptidyl chloromethanes. This is due, at least in part, to lower K_i values of the latter inhibitors. Such K_i values are significantly lower than the actual dissociation constants from the Michaelis-type complex as a result of a fast reversible chemical step (a reversible nucleophilic attack of the active-site thiolate on the inhibitor carbonyl) that precedes the irreversible alkylation step. Such a reversible step is not feasible in the case of peptidyl epoxides, and therefore their K_i values represent the actual dissociation constants.

The results presented in Table 2 show an about 240-fold increase in the second-order rate constant of inactivation of papain upon going from the peptidyl epoxide with a peptide sequence Cbz-Ala-Ala-Ala to the sequence Cbz-Phe-O-Bn-Thr. This increase arises mainly from a decrease of the K_i values, which in the simplest model represent the dissociation constants of the enzyme−inhibitor Michaelis-like complexes. This is in good agreement with the general preference of papain to hydrolyze amide bonds of substrates having a large hydrophobic residue at the P_2 position.[25] The selectivity is much more pronounced in the inactivation of cathepsin B, which exhibits a similar preference for P_2 hydrophobic substrates.[26c,27] Cbz-Phe-O-Bn-Thr-epoxide inhibited the enzyme with a second-order rate constant 170-fold higher than that displayed by Cbz-Phe-Ala-epoxide and 2200-fold higher than that displayed by Cbz-Ala-Ala-Ala-epoxide. Once again, most of the selectivity is manifested in the kinetic parameter K_i.

Comparison of the effectiveness of a given inhibitor toward various cysteine proteases also reveals a high degree of selectivity. The inhibitor Cbz-Phe-O-Bn-Thr-epoxide inactivates cathepsin B 20 times faster than it inactivates papain and 725 times faster than clostripain, while the inhibitor Cbz-Phe-Ala-epoxide inhibits papain and cathepsin B 50 times faster than it inhibits clostripain. The two most extreme cases observed in this study reveal an almost 4 orders of magnitude difference in the second-order rate constant.

Such selectivity and its correlation with the natural selectivity of the enzymes toward synthetic peptides or natural proteins (and with the respective selectivity of other peptide-based inhibitors) further support the suggested active-site-directed inactivation, and lay the grounds for further mechanistic studies.

Mechanistic Implications. Kollman and co-workers suggested that, in serine proteases, the nucleophilic attack of the active site serine on the substrate amide bond precedes protonation. In cysteine proteases, on the other hand, protonation of the scissile amide bond occurs prior to or concomitant with the nucleophilic attack.[8] Such initial protonation is feasible in cysteine proteases due to the existence of a thiolate−imidazolium ion pair in the Michaelis complex and in the free enzyme.[28] On the other hand, in serine proteases the active site configuration does not allow initial protonation since the serine and histidine residues are neutral at the corresponding stages.

A few families of selective cysteine protease inhibitors have been described. However, this selectivity does not have mechanistic implications, as it can be explained on different grounds. E-64 probably places its reactive epoxide away from the active site hydroxyl of serine proteases due to preferential binding of one of its side chains in the enzyme's S_1 site (hydrophobic in chymotrypsin and subtilisin and basic in trypsin). In addition, a "reversed" (retro-amide) mode of binding to serine proteases, analogous to its binding in the active site of papain,[29] may lead to a very high dissociation constant. Similar arguments can explain why the synthetic inhibitor Cbz-Phe-Gly-epoxide inhibited papain and cathepsin B but not chymotrypsin.[11] In fact, we believe that this compound is actually a substrate of chymotrypsin and related serine proteases.

Peptidyl diazomethanes are also selective inhibitors of cysteine proteases. The suggested mechanism of inactivation involves a cyclic sulfonium intermediate.[9] Such a mechanism is possible only in the binding site of cysteine proteases, due to the easy alkylation of sulfides to form sulfonium cations, while the corresponding reaction involving oxonium ion in the binding site of serine proteases would be extremely energetic, and therefore not feasible.

Thus, in principle, the selectivity exhibited by the above two families of inhibitors can be explained in terms of alkylation ability and is unrelated to the timing of the protonation step.

Peptidyl epoxides were designed as inhibitors which utilize the initial (or simultaneous) protonation step in cysteine proteases. The inhibition of papain by Cbz-Phe-epoxide was previously shown to be irreversible, and a good linear correlation was found between the remaining enzymatic activity and the concentration of free thiol in the enzyme active site. A significant rate acceleration in the inactivation process relative to a model reaction in solution was observed.[14] These results as well as the high stereoselectivity and the amino acid sequence selectivity exhibited by the peptidyl epoxide inhibitors toward the cysteine proteases strongly support an active-site-directed, enzyme-catalyzed mechanism of inactivation (see Scheme 2). Taken together with the selectivity toward cysteine proteases as opposed to serine proteases, our results support the suggested

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difference in the catalytic mechanism of these two families of proteases. Thus, peptidyl epoxides can be considered as mechanism-based inhibitors of cysteine proteases.

Conclusions

In the present study peptidyl epoxides were introduced as novel selective cysteine protease inhibitors. High selectivity is manifested in all aspects of this study, starting with stereoselective syntheses of both *erythro* and *threo* isomers of peptidyl epoxides, through selectivity of the inhibition process between serine and cysteine proteases, and finally stereoselectivity and sequence selectivity of inhibition within the family of cysteine proteases. Implications on the catalytic mechanism of cysteine proteases were also considered.

Finally, we believe that besides possible future applications of these cysteine protease inhibitors, peptidyl epoxides could serve as a useful tool to identify cysteine protease activity and a probe for the determination of the spatial arrangement of the active site residues.

Experimental Section

General Procedure. Enzymes, amino acids, protected amino acids, and protected peptides were from Sigma Chemical Co. They were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded at 300 or 200 MHz and 75 or 50 MHz, respectively, in $CDCl_3$, unless otherwise specified. Chemical shifts are reported on the δ scale with TMS resonance in $CDCl_3$ or solvent resonance in other solvents as an internal standard. All ^{1}H NMR assignments were supported by homonuclear decoupling experiments, while ^{13}C NMR assignments were supported by off-resonance heteronuclear decoupling or 2-D hetero-COSY experiments. Mass spectra were recorded in CI mode with either isobutane or ammonia as the reagent gas, unless otherwise indicated. TLC was performed on E. Merck 0.2 mm precoated silica gel F-254 plates, and viewed by either UV light or Cl_2/KI-tolidine.[30] Flash column chromatography[31] was carried out on silica gel 60 (230−400 mesh ASTM, E. Merck).

Synthesis. The synthesis of *threo*- and *erythro*-peptidyl epoxides was previously described.[17,18] New *erythro*-peptidyl epoxides, Cbz-Ala-Ala-Ala-epoxide, Cbz-Phe-Ala-epoxide, and Cbz-Phe-O-Bn-Thr-epoxide, were synthesized according to the same procedure.[18]

Cbz-Ala-Ala-Ala (93% yield): ^{1}H NMR (CD_3OD) δ 1.332 (d, J = 7.2 Hz, 3H, CH_3), 1.358 (d, J = 8 Hz, 3H, CH_3), 1.385 (d, J = 7.3 Hz, 3H, CH_3), 4.134 (q, J = 7.2 Hz, 1H, CHα), 4.352 (m, 2H, CHα), 5.061 (d, J = 12.8 Hz, 1H, CH_2Ph), 5.088 (d, J = 12.8 Hz, 1H, CH_2Ph), 7.27−7.34 (m, 5H, Ph); ^{13}C NMR (CD_3OD) δ 17.66 (CH_3), 18.06 (CH_3), 18.22 (CH_3), 49 (Cα), 50.06 (Cα), 51.99 (Cα), 67.69 (CH_2Ph), 128.80, 128.98, 129.44 (Ph), 174.44, 175.24, 175.72 (CO); HRMS calcd for $C_{17}H_{24}N_3O_6$ (MH^+) 366.1665, found 366.1654; MS *m*/*z* (relative intensity) 348 (10), 277 (16), 258 (84), 240 (18), 212 (20), 187 (22), 169 (28), 141 (31), 116 (69), 91 (100).

Cbz-Ala-Ala-Ala-CH_2Br (53% yield after 1:1 ethyl acetate/hexane → ethyl acetate chromatography): ^{1}H NMR δ 1.37 (d, J = 7 Hz, 3H, CH_3), 1.381 (d, J = 7.2 Hz, 6H, CH_3), 4.05 (d, J = 13 Hz, 1H, CH_2-Br), 4.07 (d, J = 13 Hz, 1H, CH_2Br), 4.242 (quint, J = 6.6 Hz, 1H, CHα), 4.489 (quint, J = 7.2 Hz, CHα), 4.720 (quint, J = 7.1 Hz, CHα), 5.13 (br s, 2H, CH_2Ph), 5.52 (d, J = 6 Hz, 1H, NH), 6.83 (d, J = 6 Hz, 1H, NH), 7.13 (d, J = 6 Hz, 1H, NH), 7.32−7.36 (m, 5H, Ph); ^{13}C NMR δ 16.92 (CH_3), 17.31 (CH_3), 17.76 (CH_3), 31.90 (CH_2Br), 48.67 (Cα), 51.07 (Cα), 52.38 (Cα), 67.28 (CH_2Ph), 128.03, 128.29, 128.56 (Ph), 171.86, 172.25 (CO); HRMS calcd for $C_{18}H_{24}N_3O_5$Br (MH^+) 442.0978, 444.0957, found 442.0747, 444.0714; MS *m*/*z* (relative intensity) 364 (5), 362 (7), 320 (13), 277 (48), 249 (11), 206 (20), 134 (24), 91 (100).

Cbz-Ala-Ala-Ala-epoxide (86% yield after ethyl acetate chromatography, *erythro*:*threo* = 2.3:1) was crystallized from acetone/hexane: ^{1}H NMR (*erythro*) δ 1.141 (d, J = 6.9 Hz, 3H, CH_3), 1.346 (assignment may be inverted) (d, J = 7 Hz, 3H, CH_3), 1.384 (d, J = 7.1 Hz, 3H, CH_3), 2.706 (dd, J = 4.7, 2.7 Hz, 1H, CH_2O), 2.751 (dd, J = 4.7, 3.6 Hz, 1H, CH_2O), 2.937 (ddd, J = 5.3, 3.6, 2.7 Hz, 1H, CHO), 3.987 (sext, J = 7.0 Hz, 1H, CHα), 4.15−4.30 (m, 1H, CHα), 4.488 (quint, J = 7 Hz, 1H, CHα), 5.117 (s, 2H, CH_2Ph), 5.464 (d, J = 6.4 Hz, 1H, NH), 6.64 (d, J = 8 Hz, 1H, NH), 6.71 (d, J = 6.9 Hz, 1H, NH), 7.383 (br s, 5H, Ph); (*threo*) δ 1.257 (d, J = 6.9 Hz, 3H, CH_3), 1.365 (assignment may be inverted) (d, J = 7 Hz, 3H, CH_3), 1.384 (d, J = 7.1 Hz, 3H, CH_3), 2.551 (dd, J = 4.6, 2.5 Hz, 1H, CH_2O), 2.72 (dd, J = 4.6, 4 Hz, 1H, CH_2O), 3.010 (dt, J = 4, 2.5 Hz, 1H, CHO), 4.15−4.30 (m, 2H, CHα), 4.438 (quint, J = 7 Hz, 1H, CHα), 5.117 (s, 2H, CH_2Ph), 5.46 (obscured, 1H, NH), 6.294 (d, J = 8.5 Hz, 1H, NH), 6.71 (obscured, 1H, NH), 7.383 (br s, 5H, Ph); ^{13}C NMR (*erythro*) δ 15.87 (CH_3), 18.21 (CH_3), 18.45 (CH_3), 45.86 (CH_2O), 46.08 (Cα), 48.99 (Cα), 50.99 (Cα), 54.08 (CHO), 67.22 (CH_2Ph), 128.05, 128.55, 136.00 (Ph), 156.07 (OCON), 171.51, 172.19 (CON); (*threo*) δ 18−19 (3C, CH_3), 43.81 (Cα), 44.47 (CH_2O), 49.12 (Cα), 50.77 (Cα), 54.17 (CHO), 67.16 (CH_2Ph), 128.25, 128.30, 136.07 (Ph); HRMS (EI) calcd for $C_{18}H_{25}N_3O_5$ (M^+) 363.1794, found 363.1799; MS (EI) *m*/*z* (relative intensity) 277 (7), 210 (24), 185 (40), 143 (73), 134 (15), 108 (21), 91 (100).

Cbz-Phe-Ala-CH_2Br (45% yield after 1:1 ether/hexane chromatography) was crystallized from ether/hexane: mp 142−143 °C; ^{1}H NMR δ 1.260 (d, J = 7.1 Hz, 3H, CH_3), 3.041 (d, J = 7.0 Hz, 2H, $CH_2\beta$), 3.851 (s, 2H, CH_2Br), 4.478 (q, J = 7.3 Hz, 1H, CHα (Phe)), 4.669 (quint, J = 7.1 Hz, 1H, CHα (Ala)), 5.034 (d, J = 12.4 Hz, 1H, CH_2 (Cbz)), 5.055 (d, J = 12.4 Hz, 1H, CH_2 (Cbz)), 5.615 (d, J = 7.8 Hz, 1H, NH (Phe)), 6.841 (d, J = 5.8 Hz, 1H, NH (Ala)), 7.15−7.35 (m, 10H, Ph); ^{13}C NMR δ 17.15 (CH_3), 31.68 (CH_2Br), 38.42 (Cβ (Phe)), 52.06 (Cα (Phe)), 56.13 (Cα (Ala)), 67.13 (CH_2 (Cbz)), 127.14, 127.90, 128.17, 128.48, 128.67, 129.20, 135.98 (Ph), 155.96 (OCON), 170.92 (CON), 200.17 (CO); HRMS calcd for $C_{21}H_{24}BrN_2O_4$ (MH^+) 447.0919, 449.0899, found 447.0802, 449.0777; MS *m*/*z* (relative intensity) 447, 449 (MH^+, 49, 50), 403 (33), 405 (25), 391 (26), 367 (100), 225 (60), 210 (31), 91 (72).

Cbz-Phe-Ala-epoxide (65% yield after 1:1 ether/hexane chromatography, *erythro*:*threo* = 3.6:1) was crystallized from ether/hexane: mp 136−137 °C; ^{1}H NMR (*erythro*) δ 1.028 (d, J = 6.8 Hz, 3H, CH_3), 2.600 (dd, J = 4.1, 3.2 Hz, 1H, CH_2O), 2.64 (obscured, 1H, CHO), 2.672 (t, J = 4.1 Hz, 1H, CH_2O), 2.995 (dd, J = 13.6, 7.8 Hz, 1H, $CH_2\beta$), 3.121 (dd, J = 13.6, 6.3 Hz, 1H, $CH_2\beta$), 3.898 (m, 1H, CHα (Ala)), 4.369 (q, J = 6.7 Hz, 1H, CHα (Phe)), 5.083 (s, 2H, CH_2 (Cbz)), 5.41 (d, J = 6 Hz, 1H, NH (Phe)), 5.78 (d, J = 5 Hz, 1H, NH (Ala)), 7.15−7.35 (m, 10H, Ph); (*threo*) δ 2.07 (dd, J = 4, 3 Hz, 1H, CH_2O), 2.501 (t, J = 4 Hz, 1H, CH_2O), 2.87 (dt, J = 4, 2.5 Hz, 1H, CHO); ^{13}C NMR (*erythro*) δ 15.90 (CH_3), 38.86 (Cβ (Phe)), 46.00 (CH_2O), 46.40 (Cα (Ala)), 53.89 (CHO), 56.60 (Cα (Phe)), 67.19 (CH_2 (Cbz)), 127.15, 128.05, 128.23, 128.55, 128.77, 129.30, 136.18, 136.38 (Ph), 170.22 (CON); HRMS calcd for $C_{21}H_{25}N_2O_4$ (MH^+) 369.1814, found 369.1795; MS *m*/*z* (relative intensity) 369 (MH^+, 100), 325 (10), 277 (14), 261 (10), 254 (12), 233 (10), 217 (14), 210 (19), 91 (94).

***O*-Benzyl-L-threonine trifluoroacetic acid salt** (1 mmol) was prepared in quantitative yield by stirring *N*-*t*-Boc-*O*-benzyl-L-threonine in 4 mL of TFA/CH_2Cl_2 (1/1 by volume) at room temperature for 2 h, followed by evaporation to dryness: ^{1}H NMR δ 1.171 (d, J = 6.4 Hz, 3H, CH_3), 3.849 (br s, 1H, CHα), 4.027 (dq, J = 3.5, 6.4 Hz, 1H, CHβ), 4.241 (d, J = 11.4 Hz, 1H, CH_2O), 4.443 (d, J = 11.4 Hz, 1H, CH_2O), 7.05−7.20 (m, 5H, Ph), 7.292 (br s, 3H, NH_3), 10.928 (br s, CO_2H); ^{13}C NMR δ 15.99 (CH_3), 58.27 (Cα), 71.09 (Cβ), 71.72 (CH_2O), 128.02, 128.18, 128.50, 136.62 (Ph), 170.67 (CO_2H).

Cbz-Phe-O-benzyl-Thr was prepared by standard coupling (DCC, NHS) of Cbz-Phe and *O*-benzyl-Thr. The dipeptide product was isolated by ether/water extraction, acidification (HCl) of the aqueous phase to pH 1.5, extraction of the aqueous phase with ether, drying of the latter organic phase ($MgSO_4$), filtration, and evaporation to dryness. It was transferred to the next reaction without further purification: ^{1}H NMR δ 1.124 (d, J = 6.1 Hz, 3H, CH_3), 2.951 (dd, J = 13.7, 8.3 Hz, 1H, $CH_2\beta$ (Phe)), 3.17 (dd, J = 13, 5 Hz, 1H, $CH_2\beta$ (Phe)), 4.165 (dq, J = 1.9, 6.1 Hz, 1H, CHβ (Thr)), 4.359 (d, J = 11.7 Hz, 1H, CH_2O

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(Bn)), 4.510 (d, J = 11.7 Hz, 1H, CH_2O (Bn)), 4.60−4.70 (m, 2H, CHα), 4.96 (d, J = 13 Hz, 1H, CH_2O (Cbz)), 5.01 (d, J = 13 Hz, 1H, CH_2O (Cbz)), 5.536 (d, J = 8.1 Hz, 1H, NH), 6.046 (d, J = 7.6 Hz, 1H, NH), 7.10−7.30 (m, 15H, Ph), 10.080 (br s, CO_2H); ^{13}C NMR δ 16.08 (CH_3), 38.21 (Cβ (Phe)), 56.16, 56.70 (Cα), 67.12 (CH_2O (Cbz)), 71.11 (CH_2O (Bn)), 74.03 (Cβ (Thr)), 126.99, 127.71, 127.86, 128.08, 128.34, 128.40, 128.57, 129.26, 135.62, 135.85, 137.33 (Ph), 156.30 (OCON), 172.76 (CO_2H), 173.62 (CON).

Cbz-Phe-*O*-benzyl-Thr-CH_2Br (25% total yield from Cbz-Phe) was crystallized from CH_2Cl_2/hexane: mp 143−144 °C; ^{1}H NMR δ 1.028 (d, J = 6.3 Hz, 3H, CH_3), 3.060 (d, J = 7.0 Hz, 2H, CH_2β (Phe)), 3.706 (d, J = 14.3 Hz, 1H, CH_2Br), 3.808 (d, J = 14.3 Hz, 1H, CH_2-Br), 4.030 (dq, J = 2.5, 6.3 Hz, 1H, CHβ (Thr)), 4.364 (d, J = 11.7 Hz, 1H, CH_2O (Bn)), 4.509 (d, J = 11.7 Hz, 1H, CH_2O (Bn)), 4.550 (dt, J = 8.0, 7.0 Hz, 1H, CHα (Phe)), 4.786 (dd, J = 7.8, 2.5 Hz, 1H, CHα (Thr)), 5.009 (d, J = 12.3 Hz, 1H, CH_2 (Cbz)), 5.063 (d, J = 12.3 Hz, 1H, CH_2 (Cbz)), 5.556 (d, J = 8.0 Hz, 1H, NH (Phe)), 6.877 (d, J = 7.8 Hz, 1H, NH (Thr)), 7.15−7.40 (m, 15H, Ph); ^{13}C NMR δ 15.53 (CH_3), 33.57 (CH_2Br), 38.28 (Cβ (Phe)), 56.29 (Cα (Phe)), 60.64 (Cα (Thr)), 67.00 (CH_2 (Cbz)), 70.96 (CH_2O (Bn)), 73.54 (Cβ (Thr)), 127.05, 127.73, 127.83, 128.01, 128.36, 128.60, 129.12, 135.97, 137.25 (Ph), 155.78 (OCON), 171.40 (CON), 198.56 (CO); HRMS calcd for $C_{29}H_{31}BrN_2O_5$ (MH^+) 567.1495, 569.1474, found 567.1478, 569.1282; MS *m*/*z* (relative intensity) 567, 569 (MH^+, 5, 5), 523 (4), 525 (4), 487 (11), 445 (5), 379 (5), 282 (7), 254 (6), 210 (9), 162 (4), 164 (4), 108 (4), 91 (100).

Cbz-Phe-O-benzyl-Thr-epoxide (95% yield, *erythro*:*threo* = 5.7:1) was crystallized from CH_2Cl_2/hexane: mp 110−112 °C; ^{1}H NMR (*erythro*) δ 1.075 (d, J = 6.3 Hz, 3H, CH_3), 2.680 (m, 2H, CH_2O (epoxide)), 2.715 (obscured, 1H, CHO (epoxide)), 2.948 (dd, J = 13.5, 8.3 Hz, 1H, CH_2β (Phe)), 3.138 (dd, J = 13.5, 5.8 Hz, 1H, CH_2β (Phe)), 3.376 (ddd, J = 8.7, 7.4, 1.5 Hz, 1H, CHα (Thr)), 3.854 (dq, J = 1.5, 6.3 Hz, 1H, CHβ (Thr)), 4.373 (d, J = 11.5 Hz, 1H, CH_2O (Bn)), 4.415 (ddd, J = 5.8, 6.5, 8.3 Hz, 1H, CHα (Phe)), 4.568 (d, J = 11.5 Hz, 1H, CH_2O (Bn)), 5.062 (d, J = 12.3 Hz, 1H, CH_2 (Cbz)), 5.083 (d, J = 12.3 Hz, 1H, CH_2 (Cbz)), 5.452 (d, J = 6.5 Hz, 1H, NH (Phe)), 6.015 (d, J = 8.7 Hz, 1H, NH (Thr)), 7.15−7.40 (m, 15H, Ph); (*threo*) δ 1.103 (d, J = 6 Hz, 3H, CH_3), 2.020 (br s, 1H, CH_2O (epoxide)), 2.471 (t, J = 4.1 Hz, 1H, CH_2O (epoxide)), 3.058 (dd, J = 13.4, 7.2 Hz, 1H, CH_2β (Phe)), 3.088 (m, 1H, CHO (epoxide)), 3.14 (obscured, 1H, CH_2β (Phe)), 3.688 (dq, J = 3.1, 6.3 Hz, 1H, CHβ (Thr)), 4.210 (dt, J = 9.0, 2.4 Hz, 1H, CHα (Thr)), 4.4 (obscured, 1H, CHα (Phe)), 4.480 (d, J = 11.9 Hz, 1H, CH_2O (Bn)), 4.600 (d, J = 11.9 Hz, 1H, CH_2O (Bn)), 5.07 (obscured, 2H, CH_2 (Cbz)), 5.323 (d, J = 6.5 Hz, 1H, NH (Phe)), 6.01 (obscured, 1H, NH (Thr)), 7.15−7.40 (obscured, 15H, Ph); ^{13}C NMR (*erythro*) δ 16.24 (CH_3), 38.98 (Cβ (Phe)), 47.37 (CH_2O (epoxide)), 51.14 (Cα (Thr)), 56.30 (CHO (epoxide)), 56.30 (Cα (Phe)), 67.03 (CH_2 (Cbz)), 71.12 (CH_2O (Bn)), 73.32 (Cβ (Thr)), 127.02, 127.62, 127.73, 127.97, 128.13, 128.34, 128.46, 128.66, 129.18, 136.14, 136.28, 137.92 (Ph), 155.70 (OCON), 170.85 (CON); (*threo*) δ 15.88 (CH_3), 38.56 (Cβ (Phe)), 42.75 (CH_2O (epoxide)), 50.44 (Cα (Thr)), 70.74 (CH_2O (Bn)), 74.56 (Cβ (Thr)); HRMS (EI) calcd for $C_{29}H_{32}N_2O_5$ (M^+) 488.2311, found 488.2290; MS (EI) *m*/*z* (relative intensity) 488 (M^+, 3), 382 (4), 323 (8), 243 (6), 210 (5), 169 (5), 142 (5), 119 (6), 91 (100).

Enzymatic Assays. All enzymatic assays were carried out at 25 °C, by following substrate hydrolysis spectrophotometrically. A 20 μL substrate solution (in the indicated concentration and solvent) was dissolved in 960 μL of buffer. The catalytic reaction was initiated by addition of 20 μL of enzyme solution. The concentration of the enzymes were set such that, under substrate saturation (V_{max}) conditions, the initial velocity of hydrolysis was about 10^{-3} OD/s.

Chymotrypsin (from bovine pancreas, EC 3.4.21.1) was assayed in 100 mM potassium phosphate buffer pH 7.0, by followng the hydrolysis of BTEE (20 mM in DMSO) at 256 nm.[32]

Subtilisin (Sigma protease type XXVII) was assayed in 100 mM potassium phosphate buffer, pH 7.0, by following the hydrolysis of Succ-AAPF-pNA (5 mM in DMSO) at 404 nm.[33]

Elastase (from porcine pancreas, EC 3.4.21.36) was assayed in 100 mM Tris·HCl buffer, pH 8.0, by following the hydrolysis of Succ-AAA-pNA (4.4 mM in the same buffer) at 412 nm.[34]

Papain (EC 3.4.22.2) and cathepsin B (from bovine spleen, EC 3.4.22.1) were activated at 25 °C for 1 h in 80 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM DTT and 2 mM EDTA. They were assayed in 100 mM potassium phosphate buffer, pH 7.0, by following the hydrolysis of Cbz-Gly-ONp (1.25 mM in CH_3CN) at 404 nm.[11]

Clostripain (EC 3.4.22.8) was activated at 25 °C for 2 h in 1 mM $Ca(OAc)_2$ aqueous solution, containing 0.5 mM DTT. It was assayed in 25 mM potassium phosphate buffer, pH 7.6, containing 2.5 mM DTT, by following the hydrolysis of BAEE (12 mM in water) at 254 nm.[35]

Inactivation Studies. The discontinuous method was applied. Typically, the enzyme studied was incubated at 25 °C with the particular inhibitor dissolved in CH_3CN (volume of organic solvent not exceeding 10% of the total volume). Aliquots were removed periodically and diluted into assay solution containing the substrate, and the residual enzymatic activity was measured. A control preincubation solution, containing all of the ingredients except for the inhibitor itself, was run and assayed in parallel. Values of k_{obs} (the apparent inactivation rate constant) were calculated from semilog plots of percent residual enzymatic activity vs time (as (ln 2)/$t_{1/2}$ or by fitting the graphs to the exponential equation % activity$_t$ = 100e$^{-k_{obs}t}$). A replot of 1/k_{obs} vs 1/[I] yielded the inactivation kinetic parameters k_i and K_i.[23]

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of all new compounds (15 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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