O'-(Epoxyalkyl)tyrosines and (Epoxyalkyl)phenylalanine as Irreversible Inactivators of Serine Proteases: Synthesis and Inhibition Mechanism

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A series of O'-(epoxyalkyl)tyrosines and a carboxy terminal (epoxyalkyl)tyrosine and -phenylalanine were synthesized as potential serine protease inhibitors. N-Acetyl derivatives showed irreversible inactivation vis-a-vis subtilisin, while the N-benzoyl ones were specific toward chymotrypsin. The most potent inactivation of chymotrypsin was achieved by a O'-(3,4-epoxybutyl)-L-tyrosine derivative. The inactivation was shown to be stereospecific since a D derivative led to no irreversible inactivation. Placement of the epoxyalkyl group at the carboxy terminus led to potent rapid inactivation. Under these conditions some of the activity was later recovered. The two classes of inactivators (O'-epoxyalkyl and carboxy-epoxyalkyl) appear to operate by different mechanisms. Most importantly, it was found that irreversible inactivation by O'-(epoxyalkyl)-L-tyrosine only resulted if the carboxy terminus was a substrate (i.e. a compound with free carboxy terminus did not lead to inactivation). The ultimate activity kinetic assay (Daniels, S. B.; et al. J. Biol. Chem. 1983, 258, 15046–15053.) indicated that the epoxyalkyl group on the phenolic oxygen had an optimal length of four carbons with respect to the turnover ratio (the ratio of molecules undergoing turnover compared to those that inactivate the enzyme) for chymotrypsin. A different kinetic assay (Ashani, Y.; Wins, P.; Wilson, I. B. Biochim. Biophys. Acta 1972, 284, 427–434.) demonstrated that substratelike turnover was proceeding at considerably slower rates than for the corresponding true substrates and with rate-limiting deacylation of the acyl–enzyme. Amino acid analysis subsequent to acid hydrolysis demonstrated that Met had been selectively alkylated by the O'-(epoxyalkyl)tyrosine derivative. By contrast, α-chymotrypsin inactivated with N-benzoyl-L-Phe-2,3-epoxypropyl ester then subjected to amino acid analysis showed no change in the content of any amino acid that would serve as a potential nucleophile to the inhibitor. Yet, the L-Phe content increased, indicating that a covalent bond had been formed between the inhibitor and the enzyme. Either the bond between the inhibitor and the enzyme did not withstand the hydrolytic conditions and/or there was less than 10% decrease in the amino acids with nucleophilic side chains upon inactivation. Finally, two tripeptides containing O'-(epoxyalkyl)-L-tyrosines were synthesized [N-(tert-butoxycarbonyl)-L-alanyl-L-alanyl-O'-(2,3-epoxypropyl)-L-tyrosine ethyl ester and N-(trifluoroacetyl)-L-valyl-L-valyl-O'-(2,3-epoxypropyl)-L-tyrosyl-L-valine methyl ester] as potential elastase inhibitors and were found to reversibly and competitively inhibit porcine pancreatic elastase.

L-Tyrosine is an important component of many biologically active peptides. It is also near the scissile bond of many synthetic peptide inhibitors of a variety of proteases. On the basis of the documented ability of some simple epoxides to inhibit serine[2] and acid proteases[3,4] and of the naturally occurring E-64 to inhibit thiol proteases,[5] we have undertaken a synthetic program aimed at the construction of potential serine protease inhibitors that carry the electrophilic epoxyalkyl moiety. We here report our results on two classes of such potential inhibitors: one carrying the epoxyalkyl group on the O'-tyrosyl position, the other on the carboxy terminus. We have determined the kinetics of interaction of these compounds with subtilisin and α-chymotrypsin; several were found to be potent and specific irreversible inhibitors.
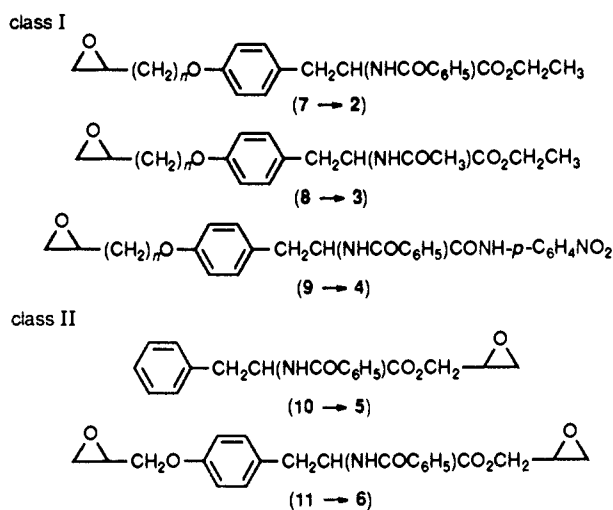
All compounds had the L configuration except where noted; in any series compound **a** corresponds to n = 1, compound **b** to n = 2, and compound **c** to n = 3.

Amino acid analysis of the inactivated enzymes and the kinetic evidence suggest that the two classes of inactivators react with different nucleophiles at the active center of α-chymotrypsin.

Guided by the above results, two tripeptides possessing the O'-(epoxypropyl)-L-tyrosine moiety were synthesized as potential elastase inhibitors. Their kinetics of interaction with porcine pancreatic elastase is reported.

Results

Synthesis. The monomeric compounds synthesized are listed above. The general scheme of epoxide synthesis follows.

class I

(7 → 2)

(8 → 3)

(9 → 4)

class II

(10 → 5)

(11 → 6)
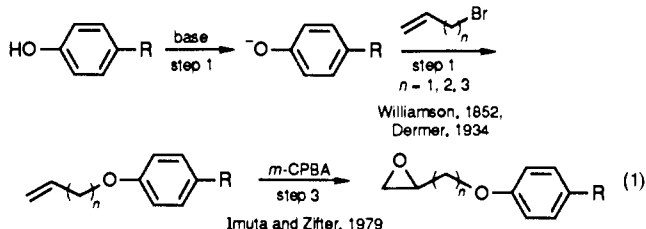
The yields of step 2 were much higher (80–90%)[6,7] than those of step 3 (20–40%).[8] For n = 1 an alternative method was also employed as shown in eq 2 that led to overall yields of 50–65%.[9] Finally, the sequences in eqs

(1) Abbreviations: CHT, α-chymotrypsin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; m-CPBA, m-chloroperbenzoic acid.
(2) Hartley, B. Biochem. J. 1970, 119, 805–806.
(3) Tang, J. J. Biol. Chem. 1971, 246, 4510–4517.
(4) Misono, K. S.; Inagami, T. Biochemistry 1980, 19, 2616–2622.
(5) Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. Biochem. J. 1982, 201, 189–198.

(6) Williamson, A. W. J. Chem. Soc. 1852, 279.
(7) Dermer, O. C. Chem. Rev. 1934, 14, 385–430.
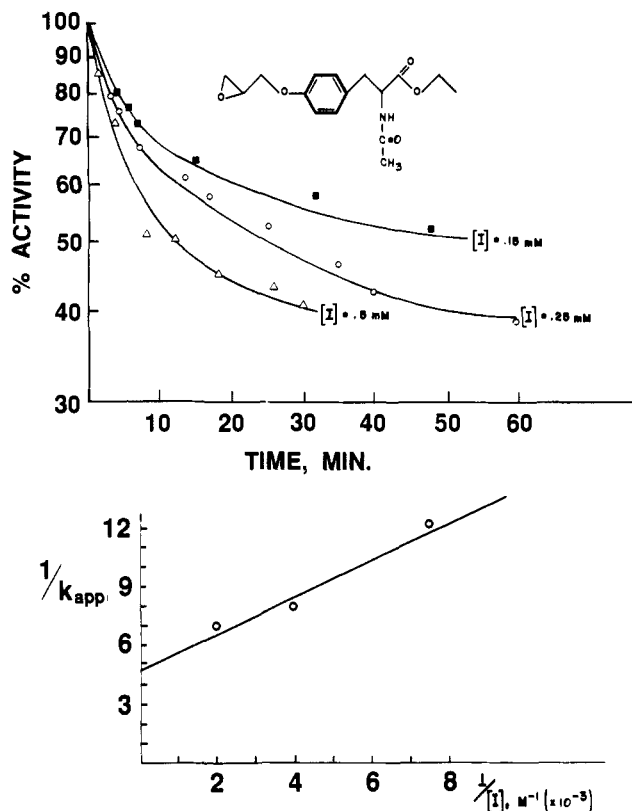(8) Imuta, M.; Zifter, H. J. Org. Chem. 1979, 44, 1351–1352.

Figure 1. (A) The time course of inactivation of subtilisin (1.6 × 10^{-8} M, 0.02 M trizma base, pH 7.8, at 19 °C by N-acetyl-O-(2,3-epoxypropyl)-L-tyrosine ethyl ester (substrate: N-carbobenzoxyglycine-p-nitroanilide, 2 × 10^{-4} M, dissolved in p-dioxane); (B) Kitz-Wilson plot of data from Figure 1A.

1 and 2 were applied in some cases to carboxylate functionalities as well.

Zandersons and Tardenaka, 1983
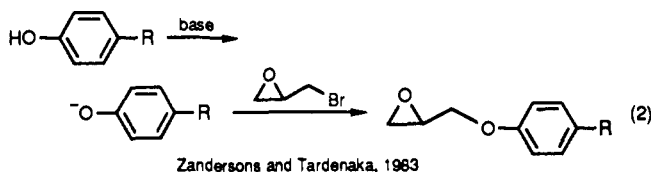
Two tripeptides containing the O'-(2,3-epoxypropyl)-L-tyrosyl residue were synthesized [N-(tert-butoxycarbonyl)-L-alanyl-L-alanyl-O'-(2,3-epoxypropyl)-L-tyrosine ethyl ester and N-(trifluoroacetyl)-L-valyl-O'-(2,3-epoxypropyl)-L-tyrosyl-L-valine methyl ester] as potential elastase inhibitors by using standard peptide synthesis techniques (see the Experimental Section). The reactions proceeded in respectable yields until the final epoxidation stage. Elemental analyses, spectroscopic data (^{1}H NMR and IR), and mass spectral evidence (especially FAB) was convincing in favor of the assigned structures.

The Kinetic Behavior of O'-(Epoxyalkyl)tyrosine Derivatives vis-a-vis Subtilisin and Chymotrypsin. Subtilisin was inhibited in a time-dependent manner by N-acetyl-O'-(2,3-epoxypropyl)-L-tyrosine ethyl ester (3a) (Figure 1A) and N-acetyl-O'-(3,4-epoxybutyl)-L-tyrosine ethyl ester (3b) (Figure 2A). The inhibitor solutions were always freshly prepared since the epoxy compounds could undergo slow ring opening upon prolonged standing in aqueous buffer solutions. To facilitate dissolution of the inhibitor, 1–3% spectroscopic-grade p-dioxane was employed. A pH of 7.8 was used throughout. In all experi-
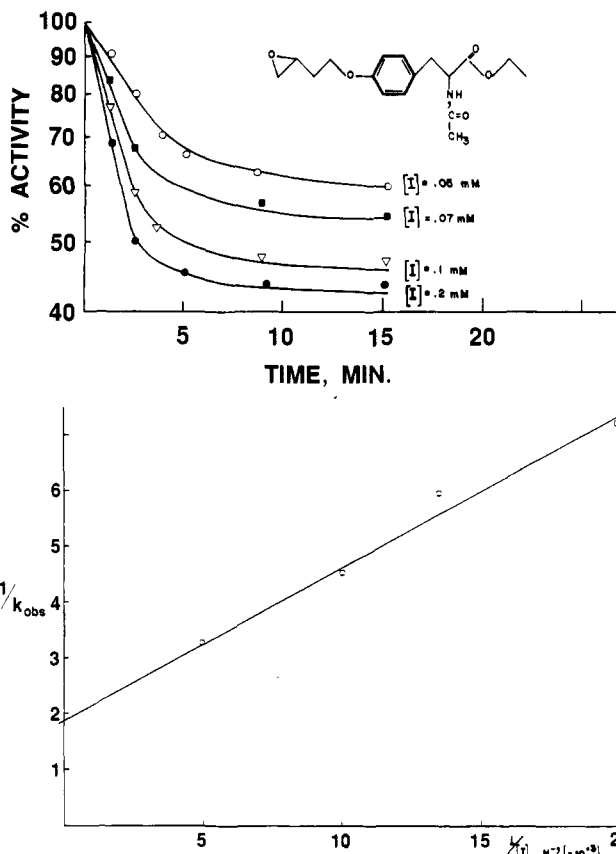

Figure 2. (A) The time course of inactivation of subtilisin (1.6 × 10^{-8} M) in 0.02 M trizma base, pH 7.8, at 19 °C by N-acetyl-O-(3,4-epoxybutyl)-L-tyrosine ethyl ester (substrate: N-carbobenzoxyglycine-p-nitroanilide, 2 × 10^{-4} M, dissolved in p-dioxane); (B) Kitz-Wilson plot of data from Figure 2A.
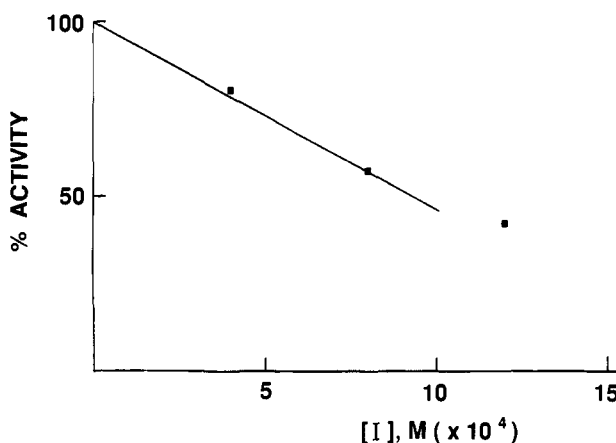

Figure 3. The activity of subtilisin (1.6 × 10^{-8} M) in 0.02 M trizma base, pH 7.8, at 19 °C in the presence of the time-independent inhibitor N-acetyl-O-(2,3-epoxypropyl)-D-tyrosine ethyl ester (substrate: N-carbobenzoxyglycine-p-nitroanilide, 2 × 10^{-4} M, dissolved in p-dioxane).

ments the reaction was allowed to proceed for several hours. Extrapolation of the initial linear portions of the semilog plots in Figures 1A and 2A allowed estimation of the $t_{1/2}$ for inactivation. Such $t_{1/2}$'s were then employed in the construction of Kitz-Wilson plots (see Figures 1B and 2B) assuming rapid reversible E·I complex formation prior to the irreversible step.[10] The known competitive inhibitor N-acetyl-L-tryptophan at 1 mM concentration afforded significant protection of subtilisin from time-

(9) Zandersons, J. G.; Tardenaka, A. T. Khim. Drev. 1983, 30–33; Chem. Abstr. 1983, 99, 141775n.

(10) Kitz, R.; Wilson, I. B. J. Biol. Chem. 1962, 237, 3245–3249.

Table I. Kinetic Constants for O'-(Epoxyalkyl)tyrosyl Inhibitors[a]

enzyme inhibited	inhibitor	$K_i,$[b] mM	$k_i \times 10^3,$[b] s^{-1}	$k_i/K_i,$ M^{-1} min^{-1}	$\frac{k_{obs} \times}{10^{3,c}}$ s^{-1}	$k_H,$[d] s^{-1}	k_H/k_i[e]	turnover ratio[f]
α-chymotrypsin	**2a**	0.38	1.7	260	0.55	5.2	300	68
	2b	0.057	13.8	14600	0.27	0.38	28	13
	2c	0.035	1.4	2400	0.17	0.15	109	26
	4a	0.066	3.3	3000	0.37	0.61	184	80
	4b	0.059	13.8	14000	0.56	0.83	60	20
subtilisin	**3a**	0.2	3.5	1000	1.1	13.8	3900	2000
	3b	0.14	9.2	3900	1.4	11.8	1300	500

[a] All experiments performed at 19 °C, except with **2a** at 24 °C. [b] Calculated from Kitz–Wilson plots.[10] [c] Calculated according to eq 5. [d] Calculated according to eq 6. [e] The ratio of rate constants obtained from eq 6 and the Kitz–Wison plot. [f] Calculated from the intercept on the abscissa of the extrapolation of the initial linear portions of the ultimate activity assay curves.[11,12]
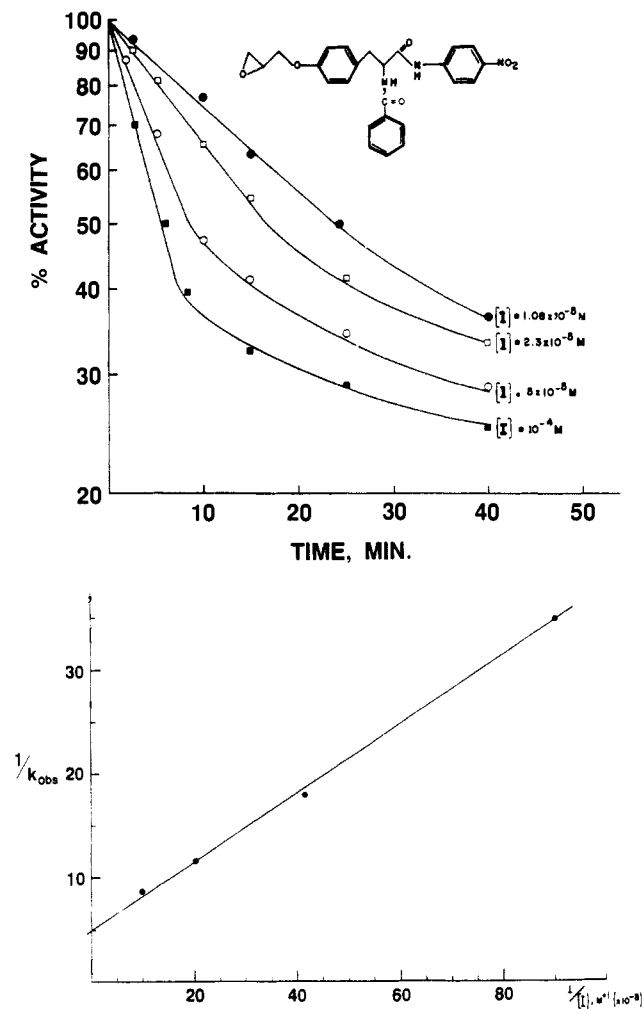

Figure 4. (A) The time course of inactivation of chymotrypsin (4 × 10^{-8} M) in 0.02 M trizma base, pH 7.8, at 19 °C by N-benzoyl-O-(2,3-epoxypropyl)-L-tyrosine-p-nitroanilide (substrate: N-benzoyl-L-tyrosine ethyl ester, 2 × 10^{-4} M, dissolved in p-dioxane); (B) Kitz–Wilson plot of data from Figure 4A.

dependent inactivation by compound **3a** (not shown). Two further control experiments are important. Subtilisin was only reversibly inhibited by N-acetyl-O'-(2,3-epoxypropyl)-D-tyrosine ethyl ester (Figure 3) with a K_i near 1 mM. Subtilisin was not inhibited (either in a time-dependent or time-independent manner) by N-benzoyl-O'-(2,3-epoxypropyl)-L-tyrosine ethyl ester at 1 mM concentration.

Chymotrypsin was inhibited in a time-dependent manner by compounds **2a–c** and **4a,b** (for examples with accompanying Kitz–Wilson plots see Figures 4 and 5), strongly suggesting irreversible inactivation. When the epoxide side chain is at the carboxyl ester position, very fast initial inactivation results that is followed by a sub-

Figure 5. (A) The time course of inactivation of chymotrypsin (4 × 10^{-8} M) in 0.02 M trizma base, pH 7.8, at 19 °C by N-benzoyl-O'-(3,4-epoxybutyl)-L-tyrosine-p-nitroanilide (substrate: N-benzoyl-L-tyrosine ethyl ester, 2 × 10^{-4} M, dissolved in p-dioxane); (B) Kitz–Wilson plot of data from Figure 5A.

sequent slower recovery of activity (see results with compounds **5** and **6** in Figures 6 and 7). An important control experiment showed that α-chymotrypsin was not inhibited by 1 mM N-acetyl derivative **3a**, just as subtilisin was not
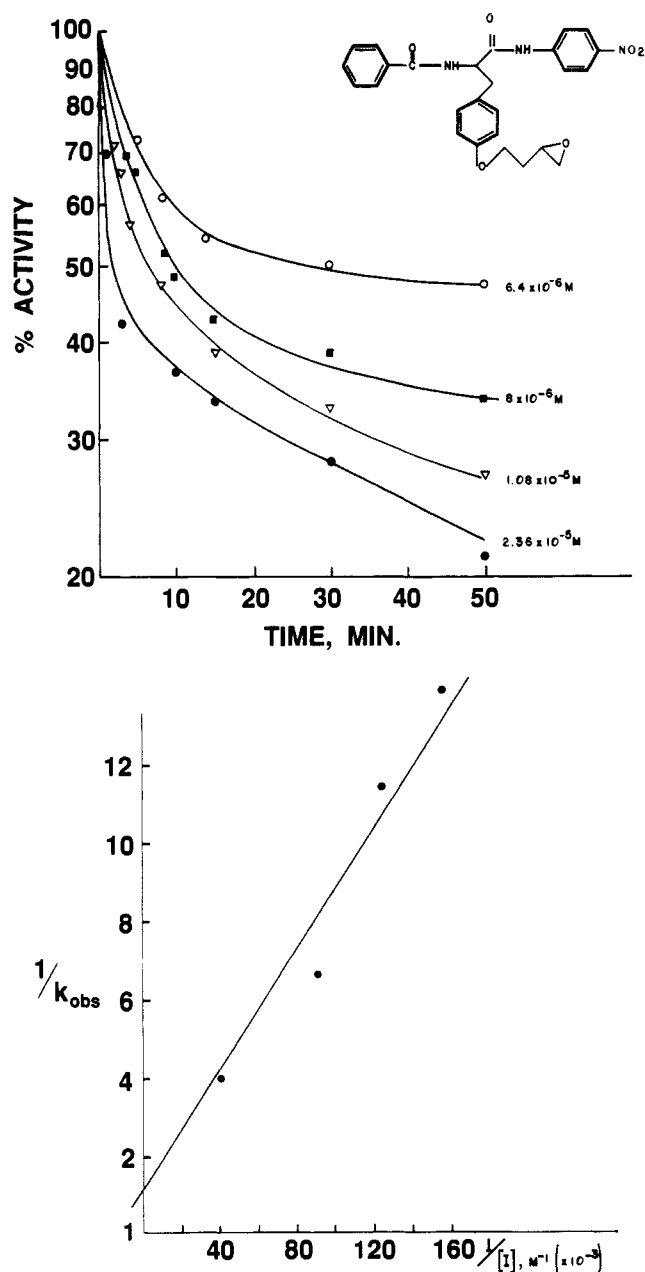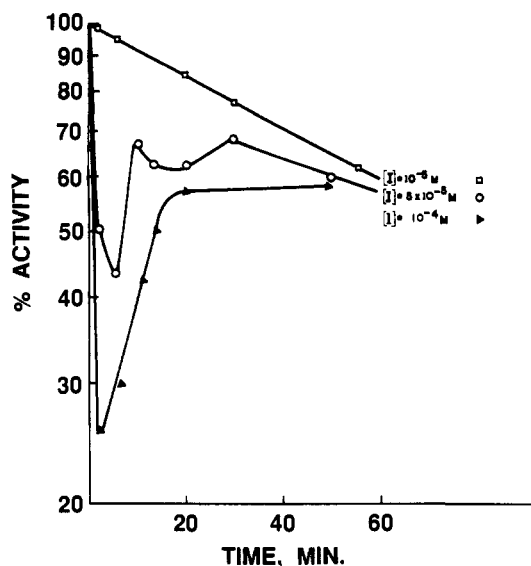
Figure 6. The time course of inactivation of chymotrypsin (2 × 10⁻⁸ M) in 0.02 M trizma base, pH 7.8, at 19 °C by *N*-benzoyl-*O'*-(2,3-epoxypropyl)-L-tyrosine 2,3-epoxypropyl ester (substrate: *N*-benzoyl-L-tyrosine ethyl ester, 2 × 10⁻⁴ M, dissolved in *p*-dioxane).

Figure 8. Ultimate activity assay of *N*-benzoyl-*O'*-(2,3-epoxypropyl)-L-tyrosine ethyl ester (1), *N*-benzoyl-*O'*-(4,5-epoxypentyl)-L-tyrosine ethyl ester (2), and *N*-benzoyl-*O'*-(3,4-epoxybutyl)-L-tyrosine ethyl ester (3), vis-a-vis α-chymotrypsin (4 × 10⁻⁸ M) in 0.02 M Tris, pH 7.8, at 19 °C. The activity was measured with 2.4 × 10⁻⁴ M BTEE (initially dissolved in *p*-dioxane) as substrate.
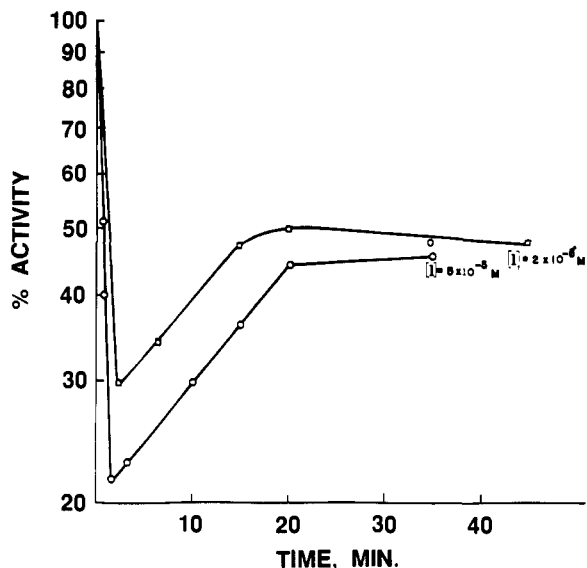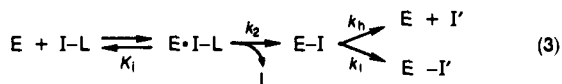

Figure 7. The time course of inactivation of chymotrypsin (2 × 10⁻⁸ M) in 0.02 M trizma base, pH 7.8, at 19 °C by *N*-benzoyl-L-phenylalanine 2,3-epoxypropyl ester (substrate: *N*-benzoyl-L-tyrosine ethyl ester, 2 × 10⁻⁴ M, dissolved in *p*-dioxane).

inhibited by 1 mM *N*-benzoyl derivative **2a**. Table I summarizes the kinetic constants calculated from Kitz–Wilson plots.

Turnover Ratio: Ultimate Activity Assay. The efficiency of the inactivators diminishes in a time-dependent manner due to enzyme-catalyzed hydrolysis (depicted by the rate constant k_h) of the leaving group at the carboxy terminus. A plausible scheme for the mechanism of action of compounds in class I is given in eq 3, where

$$E + I\text{-}L \underset{K_i}{\rightleftharpoons} E\bullet I\text{-}L \xrightarrow[L]{k_2} E\text{-}I \begin{array}{c} \xrightarrow{k_h} E + I' \\ \xrightarrow{k_i} E -I' \end{array} \qquad (3)$$

E·I–L is the Michaelis complex, E–I is the acyl–enzyme, I' is inhibitor that underwent substratelike turnover, and E–I' is irreversibly inactivated enzyme; i.e. only the rate constant k_i leads to reaction of the enzyme with the epoxide. Without detailed kinetic studies, the first two steps
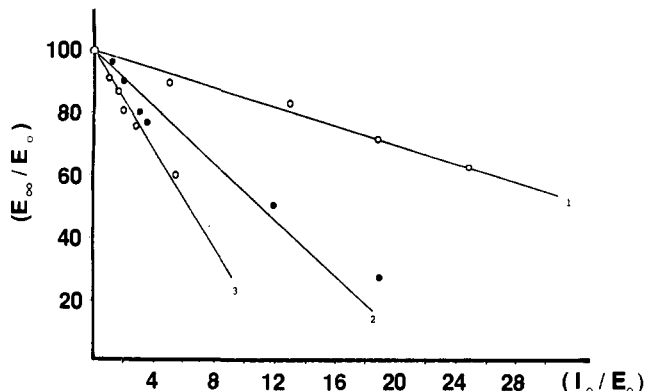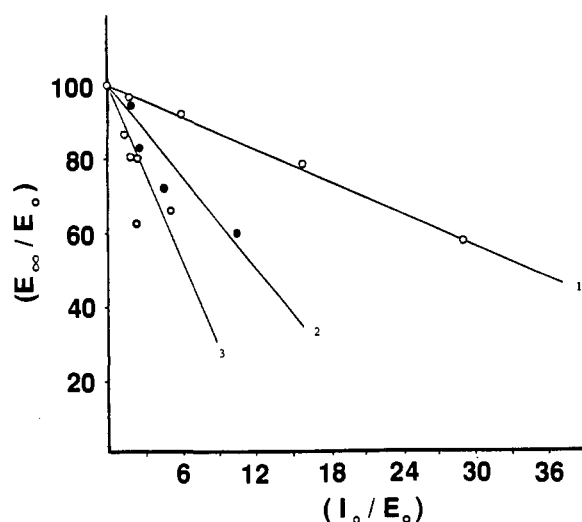

Figure 9. Ultimate activity assay of *N*-benzoyl-*O'*-(2,3-epoxypropyl)-L-tyrosine-*p*-nitroanilide (*1*), *N*-benzoyl-*O'*-(3,4-epoxybutyl)-L-tyrosine-*p*-nitroanilide (2), and *N*-benzoyl-*O'*-(3,4-epoxybutyl)-L-tyrosine ethyl ester (3) vis-a-vis α-chymotrypsin (4 × 10⁻⁸ M) in 0.02 M Tris, pH 7.8, at 19 °C. The activity was measured with 2.4 × 10⁻⁴ M BTEE (initially dissolved in dioxane) as substrate.

may be combined to give the simplified expression shown in eq 4.

$$E + I\text{-}L \underset{K_i'}{\rightleftharpoons} E\text{-}I \begin{array}{c} \xrightarrow{k_h} E + I' \\ \xrightarrow{k_i} E -I' \end{array} \qquad (4)$$
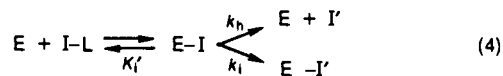
The ratio $(k_i + k_h)/k_i$ is usually termed the turnover ratio, and a kinetic expression relating the ratio to the initial enzyme and inhibitor concentrations has been derived by others.[11,12] In the present case, spontaneous hydrolysis of the epoxide on the inhibitor during the time of inactivation is unimportant.[13] Under these conditions

$$I_0/E_0 = A(1 + E/E_0)$$

where A is the turnover ratio.

(11) Daniels, S. B.; Cooney, E.; Sofia, M. J.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1983**, *258*, 15046–15053.
(12) Daniels, S. B.; Katzenellenbogen, J. A. *Biochemistry* **1986**, *25*, 1436–1444.
(13) Pocker, Y.; Ronald, B. P.; Anderson, K. W. *J. Am. Chem. Soc.* **1988**, *110*, 6492–6497.

Figure 10. Ultimate activity assay of N-acetyl-O'-(2,3-epoxy-propyl)-L-tyrosine ethyl ester ($\square$) and N-acetyl-O'-(3,4-epoxy-butyl)-L-tyrosine ethyl ester ($\triangle$) vis-a-vis subtilisin (1.6 × 10⁻⁸ M) in 0.02 M Tris, pH 7.8, at 19 °C. The activity was measured with 2.4 × 10⁻⁴ M N-CBZ-glycine-p-nitroanilide (initially dissolved in p-dioxane) as substrate.
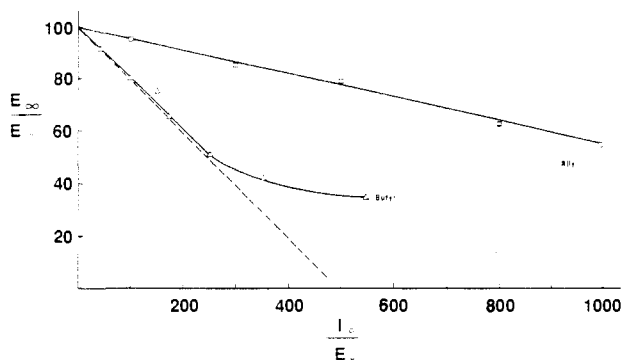
Figure 8 demonstrates the application of the ultimate activity assay to three O'-(epoxyalkyl)-L-tyrosine derivatives directed against α-chymotrypsin and varying the alkyl chain length. Figure 9 demonstrates the application of the ultimate activity assay to α-chymotrypsin-specific inhibitors with different leaving groups, i.e. ester vs p-nitroanilide. Figure 10 demonstrates the application of the ultimate activity assay to two subtilisin-specific inhibitors. Table I summarizes the turnover ratios derived from the ultimate activity assays.

Determination of the Rate Constant for Hydrolysis. The catalytic rate constant for a substrate k_{cat} is normally determined by measuring the initial rate of substrate consumption or product formation. In the present case there are two kinetic events that proceed concurrently, the mechanism-based inactivator undergoes turnover and is absorbed during inactivation. A method developed by Wilson and co-workers[14] was adapted[15] to the mechanism depicted in eq 4 and gave the following:

$$\ln \{\ln ([\epsilon_t]/[\epsilon_0]) - \ln ([\epsilon_\infty]/[\epsilon_0])\} =$$
$$\ln \{I_0 k_i / K_i' k_{obs}\} - k_{obs} t \quad (5)$$

where

$$k_{obs} = k_h [\epsilon_0] / K_i' \quad (6)$$

The symbols have the following meaning: ϵ_0 is the initial active enzyme concentration (4 × 10⁻⁸ M for chymotrypsin and 1.6 × 10⁻⁸ M for subtilisin), ϵ_t and ϵ_∞ are the active enzyme remaining at time t and at time infinity (for purposes of our experiments these readings were taken to be at 8 h), all obtained from activity remaining vs time plots; I_0 is the stoichiometric inhibitor concentration. A plot of the left-hand side of eq 5 vs time provides k_{obs} from the slope. From the Kitz–Wilson parameter K_i' and k_{obs} one can estimate k_h, the hydrolytic rate constant, according to eq 6. Also the ratio of k_h to k_i can then be compared to the turnover ratio determined above from the "ultimate activity assay". Thereby one can obtain an independent check on the consistency of the kinetic data. Such comparisons indicate that the two estimates for k_h/k_i agree with each other within factors of 2–5, not unreasonable considering all of the assumptions. Table I summarizes all of the kinetic information.

To determine k_{obs} we performed four or five experiments for each inhibitor in which the enzyme activity was mea-
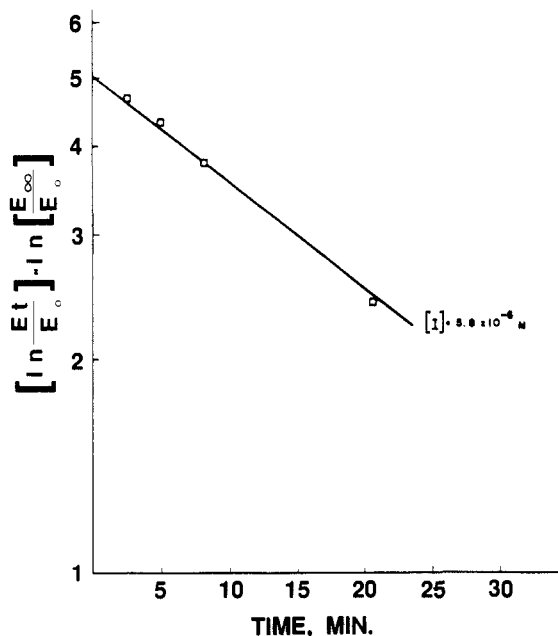
(14) Ashani, I.; Wins, P.; Wilson, I. B. *Biochim. Biophys. Acta* **1972**, *282*, 427–434.

(15) Tous, G. I. Ph.D. Dissertation, Rutgers University, Graduate Faculty at Newark, NJ, 1986.

Figure 11. Plot to evaluate k_{obs} from eq 5 for the chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine ethyl ester and inhibited by compound **4b**. The reaction was performed at 19 °C, pH 7.8, in 0.02 M Tris with the following initial concentrations: chymotrypsin, 4 × 10⁻⁸ M; substrate, 2 × 10⁻⁴ M; inhibitor **4b**, 5.8 × 10⁻⁶ M.
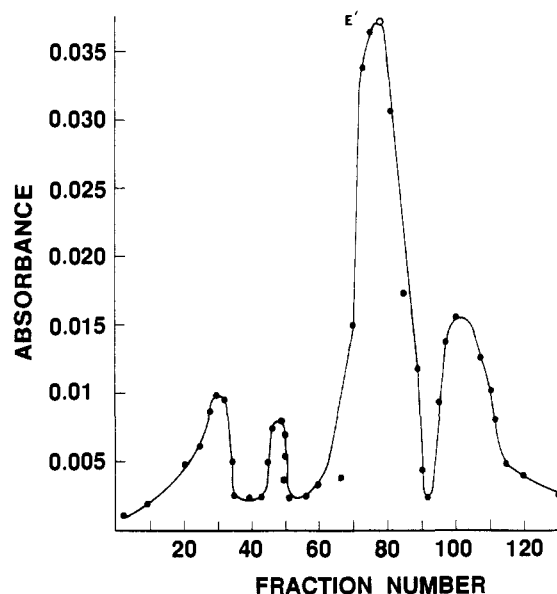
Figure 12. Separation and purification of chymotrypsin modified with **2b** on Amberlite G-50, see details in the Experimental Section; E' represents modified enzyme.

sured as a function of time following the injection of inhibitor into the solution of chymotrypsin or subtilisin. After ca. 20 min, practically all of the inhibitor was hydrolyzed, rendering it ineffective as demonstrated by the fact that the enzyme activity reached a constant and stable value. A typical data set is shown in Figure 11.

Site of Modification of α-Chymotrypsin by a Class I Type Inhibitor. To perform this experiment we employed the best α-chymotrypsin-directed inhibitor at our disposal, N-benzoyl-O'-(3,4-epoxybutyl)-L-tyrosine ethyl ester. After the enzyme was inactivated, a Sephadex G-25 (medium) column was used to separate the active enzyme remaining and the modified enzyme from small proteolytic fragments. Next the protein fraction was subjected to chromatography on Amberlite G-50 (Figure 12). After the

Table II. Amino Acid Analysis[a] of Native α-Chymotrypsin and Subsequent to Modification with **2b** and **5**

amino acid	no. in sequence	no. determined in native enzyme	no. determined in enzyme modified by	
			2b	**5**
Ala	22	24	22.3	24
Arg	3	3.3	3.0	2.6
Asn/Asp	25	25	22.8	24.3
Cys	10	nd	nd	nd
Gln/Glu	15	18	17.1	18
Gly	23	26	24.1	nd
His	2	2.2	2.1	2.2
Ile	10	11.0	9.8	10
Leu	19	20.0	18.5	22
Lys	14	14.1	13.1	13
Met	2	1.7	0.4	2.0
Phe	6	7.1	5.8	9
Pro	9	nd	nd	nd
Ser	27	25	24	18.5
Thr	22	21	18	17
Trp	8	nd	nd	nd
Tyr	4	3.3	5.5	4.5
Val	23	25	22	18

[a] All determinations in triplicates. nd = not determined; see specific methods employed under the Experimental Section. The enzyme modified with **2b** was subjected to two different hydrolytic conditions, giving essentially identical results. Bovine serum albumin was subjected to identical hydrolytic conditions and amino acid analysis to serve as a control.

desired fraction was separated, its SDS polyacrylamide slab gel pattern showed the modified protein to be ca. 500–1000 Da heavier than the native enzyme. This implied that either one or two molecules of inactivator were covalently bonded to each chymotrypsin molecule during the inactivation. Table II summarizes the results of amino acid analysis on the modified chymotrypsin and on the native enzyme. Amino acid analysis was performed on two different samples by two different methods, each time in triplicates. For the second analysis gas-phase hydrolysis was used.[16] No discernible differences were found between the two different sets of data. The method of Meltzer reports a standard deviation of 6–12%.[16] To ensure complete hydrolysis, two controls were run, one on native chymotrypsin (Table II), the other on bovine serum albumin (not shown). The recovery and composition (within 6–12%) of each control was satisfactory. In the enzyme modified by **2b** there appears to be an increase of 1.5 (from four) tyrosines (37%) and a decrease of 1.6 (from two) methionines (80%) upon modification. No other amino acids changed more than 10%.

Site of Modification of α-Chymotrypsin by a Class II Type Inhibitor. N-Benzoyl-L-phenylalanine 2,3-epoxypropyl ester was used to inactivate the enzyme to an extent of ca. 50%. After reverse-phase HPLC separation and purification on a Supelco C-8 column (Figure 13), the modified enzyme was subjected to amino acid analysis. As Table II indicates there was a ca. 50% increase in the Phe content of the modified enzyme (from six to nine amino acids), indicating that as many as three inhibitor molecules per enzyme were covalently bound in this particular inactivation experiment. Most importantly, the content of His and Met remained unchanged, indicating no alkylation of these side chains. The 66% recovery of Ser and Thr content is somewhat lower than the usual 80–90% (but nothing unusual). The serum albumin control also only showed 72% recovery of Ser and 75% of Thr. The amino
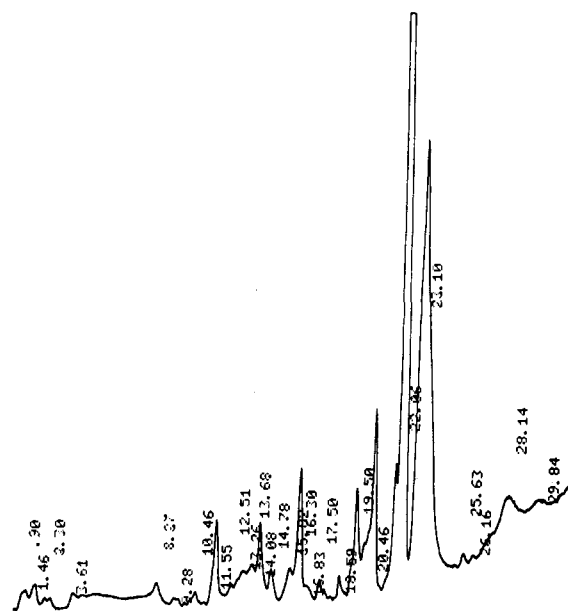
Figure 13. HPLC separation of native chymotrypsin from enzyme modified with compound **5**; the retention time of native enzyme was 22.06 min and that of modified enzyme was 23.10 min. The fractions were monitored by fluorescence (excitation at 280 nm, emission at 340 nm); for other details see the Experimental Section.
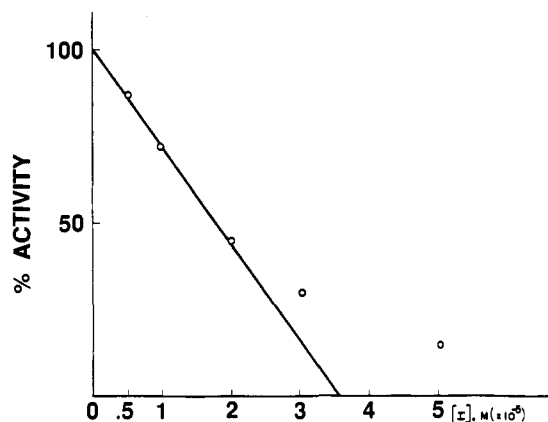
Figure 14. Inhibition of porcine pancreatic elastase with N-(trifluoroacetyl)-L-valyl-O'-(2,3-epoxypropyl)-L-tyrosyl-L-valine methyl ester (see the Experimental Section for experimental details).

acids Asp, Asn, Glu, and Gln remained unchanged during the hydrolytic procedure.

Inhibition of Porcine Pancreatic Elastase by 20 and 29. Elastase from porcine pancreas was reversibly inhibited by the two O'-(2,3-epoxypropyl)-L-tyrosyl tripeptides **20** and **29**. Inhibitor solutions contained 10% dimethyl sulfoxide to aid in dissolving these sparingly soluble compounds. As Figures 14 and 15 demonstrate ca. 1.6×10^{-5} M compound **29** and 8×10^{-5} M compound **20** were required to produce 50% inhibition on 1.2×10^{-7} M porcine pancreatic elastase. The inhibitors were competitive; the extent of inhibition decreased with increased concentration of substrate N-succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide.[17]

Discussion

Inhibition by Monomeric (Epoxyalkyl)Tyrosyl Derivatives. In the past, it was reported that epoxide-

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(17) Bieth, J.; Spiess, B.; Wermuth, C. G. *Biochem. Med.* **1974**, *11*, 350–357.
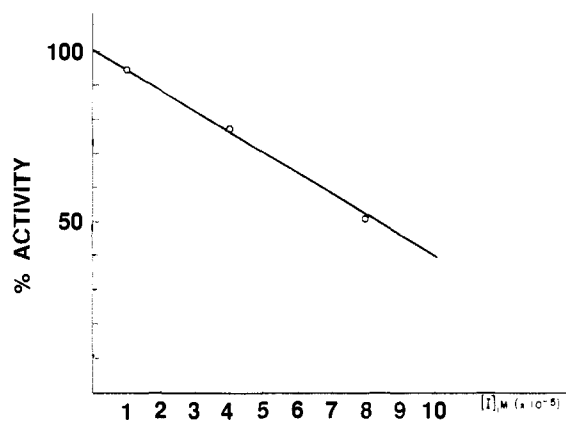
Figure 15. Inhibition of porcine pancreatic elastase with *N*-(*tert*-butoxycarbonyl)-L-alanyl-L-alanyl-*O'*-(2,3-epoxypropyl)-L-tyrosine ethyl ester (see the Experimental Section for details).

containing compounds could effectively inhibit cysteine[5] and acid proteases.[3,4] It was found that the compound E-64 rapidly inactivated the cysteine proteinases cathepsin B, H, and L, as well as papain but not the serine protease elastase.[5] On the other hand, Tang[3] and Hartley[2] reported that the simple epoxide 1,2-epoxy-3-(*p*-(nitrophenoxy)-propane could inhibit papain and chymotrypsin, respectively, but without any selectivity.

Among the compounds here reported all but five had L-Tyr as the starting material. L-Tyrosine was selected because of the synthetic opportunities it provides and because a number of serine proteases (as well as acid proteases) will accept substrates with L-Tyr on their scissile peptide bond. Our results allow us to conclude the following.

1. To assure that the compounds were recognized as active-site directed, the carboxy terminus always carried a hydrolyzable leaving group L (OR or NH-*p*-C$_6$H$_4$NO$_2$). That the unhydrolyzed material was the true inhibitor at pH 7.8 was demonstrated by the synthesis of compound 12 [(*N*-acetyl-*O'*-(2,3-epoxypropyl)-L-tyrosine] that gave no time-dependent inactivation of subtilisin even at 1 mM concentration, conditions under which **3a** gave readily detectable inactivation. Furthermore, it was also demonstrated that the inactivators also underwent substratelike behavior by monitoring their hydrolysis (**2a–c** at 237 nm, **3a–b** at 236 nm, **4a,b** at 400 nm) catalyzed by the enzymes. On the basis of these data one can conclude that the inactivation is taking place during catalytic turnover. Because of the nature of the leaving group, one can further postulate on the basis of much literature precedent[18] that the mechanism of turnover of such compounds proceeds with rate-limiting deacylation of the acyl–enzyme intermediate; i.e. there is a relatively long-lived acyl–enzyme. Further affirmation of the catalytic center-directed nature of these novel compounds is evident from the fact the *N*-acetyl-L-tryptophan, a relatively weak competitive inhibitor of subtilisin, protected the enzyme from inactivation by **3a**. An *O'*-(epoxyalkyl)tyrosine devoid of a carboxy terminal leaving group failed to show inhibition.

2. Selectivity of the compounds synthesized based on the *N*-acyl group was impressive. *N*-Acetyl compounds **3a** and **3b** did not inhibit chymotrypsin at 1 mM concentration, whereas *N*-benzoyl compounds **2a–c** did not inhibit subtilisin at 1 mM concentrations. The preference for the *N*-acetyl group by subtilisin is consistent with the much lower K_M exhibited by subtilisin than by chymo-

trypsin toward *N*-acetyl-Tyr ethyl ester as substrate.[19]

3. Stereoselectivity of inhibition was demonstrated. Compound **3a** was synthesized in both D and L configurations with respect to the C$_\alpha$ atom. The D-**3a** derivative did not lead to irreversible inactivation of subtilisin even at 1 mM concentration, rather it gave weak, reversible inhibition with a K_i of approximately 1 mM, while the L-**3a** compound was an inactivator presumably proceeding via the acyl–enzyme intermediate. This is consistent with the known preference of the enzyme for the L configuration of substrates.[20]

4. For the α-chymotrypsin-specific inhibitors the inhibiting efficiency represented by the ratio k_i/K_i is optimized for a four-carbon *O*-alkyl chain length. The results of the ultimate activity assay experiments confirm this since the turnover ratio is also optimized for a butyl group. These experiments suggest that the distance between the electrophilic epoxide and the scissile carboxy terminus is important.

5. Further confirmation of the substratelike behavior of the inhibitor is evident from a comparison of the k_i/K_i ratio for compounds **2b** and **4b** vis-a-vis α-chymotrypsin. The great similarity of these ratios (Table I) suggests that epoxide-based inactivation originates from the acyl–enzyme complex since the k_i/K_i ratio is essentially the same irrespective of the leaving group L.

6. While the *O'*-epoxyalkyl-based inhibitors all lead to monotonic time-dependent inactivation, many of the log (activity remaining) vs time plots are curved at later times. Several plausible explanations were ruled out. Hydrolysis by buffers was tested by preincubation of inhibitor with the buffer for 1 h. The extents of inactivation by this "preincubated" inhibitor compared to freshly prepared material were essentially the same. Similarly, preincubation of the inhibitor for 1 h with 1 mg/mL bovine serum albumin prior to kinetic assay also led to an extent of inactivation identical with that obtained in the absence of the "nonspecific" protein. This experiment tends to rule out the possibility that the inhibitor is consumed by functional groups of chymotrypsin or subtilisin outside the active center. Inhibition of the reaction by the product of hydrolysis was ruled out as mentioned in point 1 above. Apparently, the lifetime of the bound *O'*-(epoxyalkyl)-tyrosine derivative that cannot form an acyl–enzyme (is missing the leaving group as in **12**) is too short at the active site to undergo reaction with an enzymic nucleophile. The inhibitor undergoes substratelike turnover, hence its concentration is continuously being depleted, which probably accounts for most of the curvature observed in the log (activity remaining) vs time plots. This statement is also supported by experiments in which additional inhibitor was added to partially inactivated enzyme that then proceeded on to much lower levels of activity (data not shown).

7. The kinetic experiments based on eqs 5 and 6 provide a measure of the actual hydrolytic rate constant characteristic of substratelike turnover. Data from Berezin et al.[18] can provide direct comparison with some of the data quoted in Table I. The K_s of chymotrypsin for *N*-benzoyltyrosine ethyl ester is 0.0638 mM, essentially identical with the K_i's quoted for **2b, 2c, 4a**, and **4b** (data for **2a** were collected under different conditions, hence are not directly comparable). On the other hand, the rate constants for the same substrate were the following: k_{cat} = 86 s^{-1}, $k_{acylation}$ = 250 s^{-1}, and $k_{deacylation}$ = 131 s^{-1}.[18] Even

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(19) Bosshard, H. R.; Berger, A. *Biochemistry* **1974**, *13*, 266–277.
(20) Ingles, D. W.; Knowles, J. R. *Biochem. J.* **1967**, *104*, 369–377.

accounting for the lower temperature used in our study (19 °C compared to 25 °C in ref 18), our estimated values for k_h are 2–3 orders of magnitude slower than that of the corresponding substrate devoid of the O'-epoxyalkyl group. The conclusion from the kinetic analysis then is that it is not the binding constants but rather the hydrolytic rate constants that are affected by modification of the substrate by the epoxyalkyl group. On the basis of the similarity of the kinetic results for two different "good" leaving groups, *p*-nitroaniline and primary alcohol, deacylation should be rate-limiting. It is during the slow deacylation step that periodically (as demonstrated by the turnover ratio) a nucleophilic attack on the epoxide by the enzyme takes place, rendering the enzyme inactive to subsequent turnover.

8. Compounds **5** and **6** appear to inactivate α-chymotrypsin differently than do **2a–c** and **4a,b**. In the former two compounds there is an epoxide functionality on the carboxy terminus (in **5** exclusively at this position). As shown in Figures 6 and 7, compounds **5** and **6** give the most potent initial inactivation (greater than 75% inactivation in a few minutes at 20–50 μM concentration). However, with time, some of the activity was regained. We suspect that the rapid initial inactivation by the epoxide of these compounds is at or very near the active center triad. On the basis of the minor changes in amino acid analysis subsequent to inactivation by the class II inactivator *N*-benzoyl-L-phenylalanine 2,3-epoxypropyl ester (**5**), one can assume that this class inactivates by forming either ester (from Asp or Glu) or ether bonds (from Ser to Thr). Some of these bonds may be formed reversibly, and all would be hydrolyzed by the 6 N HCl during the protocol preceding amino acid analysis. It is important that no changes in His or Met content were observed, only an increase in Phe, indicating that covalent modification had taken place.

9. The increase in tyrosine content (due to covalent bonding of inhibitor) on inactivation of chymotrypsin with **2b** is in excellent quantitative agreement with the decrease in methionine content. No other potentially nucleophilic side chains appear to have been lost (it is especially important to point out that there was no change in His content upon modification). On the basis of these results, the class I inhibitor tested must have reacted with either Met 180 or Met 192. There are only two Met, four Tyr, and two His residues in native α-chymotrypsin, making such conclusions firm. The nucleophilic reactivity of Met 192 had been reported by others,[2,21,22] in fact refs 2 and 21 demonstrated such alkylation specifically by epoxides, hence precedent exists for the reactivity here found. On the basis of the distance between the scissile bond and the terminal epoxy carbon (ca. 12.8 Å), interaction with Met 180 is more likely, but not yet proven.

Inhibition of Porcine Pancreatic Elastase by Compounds 20 and 29. *N*-(Trifluoroacetyl)-L-Val-*O'*-(2,3-epoxypropyl)-L-Tyr-L-Val methyl ester (**29**) and *N*-(*tert*-butoxycarbonyl)-L-Ala-L-ala-*O'*-(2,3-epoxypropyl)-L-Tyr ethyl ester (**20**) were found to be reversible, competitive inhibitors of porcine pancreatic elastase. The affinity of **29** was ca. 4-fold greater for the enzyme than that of **20**. The former compound was synthesized on the basis of the observation by Dimicoli et al.[23] that *N*-(trifluoroacetyl)-Val-Tyr-Val is a reversible inhibitor (K_i = 0.56 mM) of

porcine pancreatic elastase and a much better inhibitor of human leukocyte elastase (K_i = 1.3 μM). The rationale for Val at the P_1 site is the smaller S_1 binding pocket in elastase compared to those in subtilisin and chymotrypsin. Synthesis of compound **20** was inspired by the known preference of elastase for L-Ala[24] and our desire to place an *O'*-(epoxyalkyl)-Tyr near the scissile bond. While both inhibitors were found to the active center, apparently the electrophilic epoxide was too distant from any enzymic nucleophile to induce irreversible inactivation.

The failure of compound **29** to act as an irreversible inactivator may be due to (a) the NCF_3CO group pulling the tripeptide out of the binding pocket $S_3S_2S_1S_1'$ and (b) the preference of elastase for an alkyl hydrophobic side chain at the S_2 subsite.[25] The failure of compound **20** to act as an irreversible inactivator may be due to a shift of the *O'*-(epoxyalkyl)-Tyr into the S_2' subsite, known to have an affinity for Phe, whereas the cleavage of peptides that have a Tyr or Phe in the P_1 position is probably very sluggish.[24]

Conclusions

In summary, two novel classes of time-dependent serine protease inactivators were synthesized that took advantage of the binding affinity of *N*-acyl-(or -aroyl)tyrosine alkyl esters (or anilides) for subtilisin and chymotrypsin and incorporated the electrophilic epoxyalkyl side chain either at the phenolic oxygen or at the carboxy terminus. By simple manipulation of the amino substituent, selectivity for subtilisin or chymotrypsin could be achieved. Variation of the *O'*-epoxyalkyl chain length indicated that the *n*-butyl derivative gave the most effective inhibition. It was also demonstrated that these substrate-like inhibitors underwent normal catalytic turnover simultaneously with the time-dependent inactivation of the enzymes. While the best inactivators were shown to have K_i's near 30 μM and relatively favorable turnover ratios, the efficacy of the inhibitors could, in principle, be greatly enhanced and the specificity increased by elaboration of the peptide side chain toward the S_2, etc., subsites. The *O'*-epoxyalkyl type inhibitor appears to react with Met in chymotrypsin (180 or 192), whereas the carboxy terminally labeled inhibitors probably react either with Asp/Glu or Ser/Thr near the active center. Both types clearly form a covalent bond with chymotrypsin according to amino acid analysis.

In view of the recent report demonstrating that such alkyl epoxides possess excellent stability in aqueous medium (half-lives nearly 100 h at 35 °C between pH 7 and 12),[13] such alkylepoxides are appealing potential electrophiles for a variety of protease classes, including the acid proteases, in addition to the serine proteases here explored.

Experimental Section

General Procedures. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. ¹H NMR spectra were recorded on an IBM WP-200 SY instrument at 200.13 MHz. All tyrosine-based starting materials were purchased from Sigma. All reagents were of the highest purity commercially available. Column chromatography was performed on silica (Davison Chemical, 60–200 mesh) or alumina (Fisher Scientific Basic Brockman Activity IV). Thin-layer chromatography was performed on silica (Kodak Chromatogram Sheet) or alumina (Eastman Chromatogram Sheet) (13252 alumina). IR spectra were recorded on a Nicolet-10-MX or Beckman Acculab 6 instrument.

Enzyme Kinetics. All kinetic data were collected on a Cary 219 UV–vis spectrophotometer. The progress of the reaction was monitored at 237, 256, or 400 nm, depending on the substrate.[26]

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The kinetic data were corrected for spontaneous hydrolysis of substrates at the scissile bond (rather than at the epoxide) by the buffer (Tris, pH 7.8). The epoxide groups were always found to be stable for the entire time course of inactivation.

Initial Rate Studies on Subtilisin. An enzyme solution was prepared by dissolving 0.4 mg (1.6 × 10⁻⁷ M) subtilisin Carlsberg or BPN' in 100 mL of 0.02 M Tris, pH 7.8, at 0–5 °C in an ice bath. The enzyme stock solution was prepared by mixing 5 mL of this solution with 45 mL of 0.02 M Tris that also contained 5% (v/v) spectral-grade p-dioxane. The final concentration of enzyme was 1.6 × 10⁻⁸ M. A stock solution of substrate was prepared by dissolving N-Cbz-glycine-p-nitroanilide in p-dioxane at a concentration of 0.02 M. The reaction was initiated by injecting 30 μL of substrate solution into 3.0 mL of the enzyme solution that had been preincubated at 19 °C and the release of p-nitroaniline was monitored at 400 nm. Initial rate studies in the presence of inhibitor were performed by adding appropriate aliquots of inhibitor (dissolved in p-dioxane) to the enzyme stock solution and incubating this mixture at 19 °C. Periodically, the remaining activity was assayed by adding substrates to a 3-mL portion of the enzyme–inhibitor solution and monitoring the rate of release of p-nitroaniline. In a typical experiment six enzyme solutions were prepared at 45-min intervals. All the reaction flasks were incubated at 19 °C; the temperature was maintained throughout the kinetic assay. One of the enzyme solutions was kept as a control. In the other five flasks, two or three controls were run per flask. An aliquot of the inhibitor stock solution was added to the second flask to provide an inhibitor concentration of 10⁻³–10⁻⁵ M. A kinetic assay was performed immediately. Data were collected every 3 min for approximately 20 min, then every 10 min for the remainder of 1 h. This protocol was repeated for the other flasks at different inhibitor concentrations.

Initial Rate Studies on α-Chymotrypsin. A stock solution of α-chymotrypsin (twice recrystallized from bovine pancreas; Sigma, St. Louis, MO) was prepared by dissolving 0.8 mg of enzyme in 100 mL of 0.001 N HCl (3.2 × 10⁻⁷ M). This solution was further diluted 25-fold into 0.02 M Tris, pH 7.8, that contained 5% (v/v) spectral-grade p-dioxane (final concentration of enzyme was 1.3 × 10⁻⁸ M). N-Benzoyl-L-tyrosine ethyl ester was dissolved in p-dioxane to a concentration of 4 × 10⁻² M. Assays were initiated by injecting 30 μL of the BTEE solution onto 3.0 mL of the diluted enzyme solution that had been preincubated at 19 °C, the ester hydrolysis was monitored at 256 nm. Initial rate studies in the presence of inhibitor were performed similarly to those outlined for subtilisin except that inhibitor concentrations ranged from 10⁻⁴ to 10⁻⁶ M.

Initial Rate Studies on Elastase. Porcine pancreatic elastase (2.4 mg, twice recrystallized, Sigma, St. Louis, MO) was dissolved in 10 mL of 0.2 M trizma base, pH 7.8, containing 10% (v/v) dimethyl sulfoxide. The final concentration of enzyme in this stock solution was 1 × 10⁻⁷ M. An appropriate amount of N-succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide (Sigma) was dissolved in 0.2 M trizma base, pH 7.8, containing 10% dimethyl sulfoxide to give a concentration of 5 × 10⁻² M. Assays were performed by injecting 30 μL of substrate solution into 3 mL of enzyme stock solution and monitoring the release of p-nitroaniline at 400 nm at 19 °C. Studies in the presence of inhibitor were performed as outlined above for subtilisin.

Ultimate Activity Assay: Determination of Turnover Ratios. The experiments followed the protocol outlined above in the presence of inhibitor. The concentration of inhibitor was 10⁻⁵–10⁻⁷ for subtilisin and 10⁻⁵–10⁻⁸ M for α-chymotrypsin. Time infinity was assumed to be 8 h. The change in enzyme activity due to autolysis during this time was estimated[27] and also determined from the controls incubated in the absence of inhibitor.

Hydrolysis Constants for Chymotrypsin and Subtilisin. The experiments followed the protocol outlined above for enzyme assay in the presence of inhibitor. The concentration of inhibitors was 5 × 10⁻⁵–10⁻⁶ M for chymotrypsin and 10⁻⁴–10⁻⁶ M for subtilisin. Time infinity was assumed to be 8 h, and the data recorded were corrected for the 10–15% decrease in activity (due to au-

tolysis) during this time by an appropriate control run for the same time period and under the same conditions but in the absence of inhibitor.

Protein Chemistry. Protein content was determined according to the method of Bradford.[28] Electrophoresis was performed in a slab-gel apparatus in sodium dodecyl sulfate and employed a Sigma molecular weight standard set (bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, and lactalbumin) in 12% of acrylamide.

Characterization of Modified α-Chymotrypsin. Modification with Compound 2b. After inactivation of the enzyme, the protein mixture was first chromatographed on a Sephadex G-S-25 medium column, then on Amberlite G-50. Samples for amino acid analysis were prepared as follows. A 1.0-mg sample of modified (or native) α-chymotrypsin was dissolved in 0.2 mL of double-distilled water, 0.1 mL of concentrated HCl, and 2% (w/v) thioglycolic acid. An alternating cycle of freezing and thawing of the sample (while keeping it under vacuo) was repeated three times before the sample was hermetically sealed in a Pyrex test tube. Next, the tube was placed in a 110 °C oven for 24 h, then the sample was removed from the tube, lyophilized, redissolved in doubly distilled water, and lyophilized again. The freeze-dried sample was then dissolved in 1 mL of sample buffer (Pierce, Rockford, IL), pH 2.2, and subjected to amino acid analysis. Amino acid analysis was performed by gradient high-performance liquid chromatography [mobile phases: (A) 0.07% decyl sulfate in 0.025% phosphoric acid; (B) 0.12% heptane sulfonate in water/CH₃CN (70/30) with 0.05% phosphoric acid] and postcolumn o-phthaldialdehyde derivatization and fluorescence detection. The column was from ES Industries (3 μm MC-18 particles packed in a 10 cm × 4.6 mm stainless steel column). A second set of samples were hydrolyzed and analyzed according to the method of Meltzer et al.[16]

Modification with Compound 5. A solution of α-chymotrypsin (1 mg/mL) was prepared in 0.001 M HCl. A 15-μL aliquot of the solution was added to 10 mL of Tris 0.02 M, pH 7.8. To this latter solution was added 5 mL of a solution that contained 5 mg of N-benzoyl-L-Phe 2,3-epoxypropyl ester dissolved in 250 μL of dioxane (in the final solution 600 pmol of enzyme and 200 nmol of inhibitor were present) and the reaction was allowed to proceed for 2 min. After 2 min the pH was readjusted to 7.8 with 1.5 M Tris, pH 9.0, and two more 5-μL aliquots of inhibitor were added, the pH being readjusted after each aliquot. After the third addition the enzyme activity was assayed, showing 50% decrease in activity. The reaction mixture was divided into two portions and the solutions were evaporated on a Speed Vac. The protein was purified by HPLC on a Supelco 300 A 5-μm C-8 column (0.46 cm × 2 cm) at room temperature with a flow rate of 1 mL/min. The solvents were all HPLC grade. Chromatography was carried out with the following gradient: 100% phase A for 5 min and then a shallow linear gradient ranging from 100 to 30% A in 25 min and then a steeper linear gradient from 30 to 10% A in 5 min. The flow rate was 1 mL/min. Phase A consisted of 0.1% trifluoroacetic acid in water. The second phase (B) consisted of 0.078% trifluoroacetic acid in 70% acetonitrile. The UV absorbance of the effluent was monitored at 217 nm; the fluorescence emission of tryptophan was monitored at 340 nm (excitation at 280 nm). The chromatogram of the HPLC separation is shown in Figure 5: 80% of the applied protein was recovered in three peaks [autolysis products, unreacted chymotrypsin (22.0 min), and derivatized protein (23.0 min)]. Amino acid analysis was performed according to Meltzer et al.[16]

Synthesis. N-Benzoyl-O'-(prop-2-enyl)-L-tyrosine Ethyl Ester (7a). To a solution of N-benzoyl-L-tyrosine ethyl ester (2.2 g, 7.0 mmol) in acetone (10 mL) was added K₂CO₃ (0.97 g, 7.0 mmol) followed by allyl bromide (0.6 mL, 7.0 mmol). The mixture was refluxed for 6 h and then cooled and the solvent was removed on a rotary evaporator to yield a yellow oil. The oil was redissolved in 5% NaOH/ether (1:1 v/v; 50 mL) and the aqueous layer was extracted with ether (4 × 20 mL). The combined organic layers were dried (MgSO₄); the solution was concentrated and chromatographed on silica gel to give 7a (2.3 g, 93.7%): mp 75 °C;

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R_f (silica, CHCl$_3$) = 0.60; IR (CHCl$_3$) 3430, 3000, 1740, 1665, 1510, 1210 cm^{-1}; ^{1}H NMR (CDCl$_3$/TMS, 200 MHz) δ 7.73 (dd, 2 H, J = 1.3, 8.0 Hz), 7.44 (m, 3 H), 7.05 (d, 2 H, J = 8.6 Hz), 6.83 (d, 2 H, J = 8.7 Hz), 6.60 (d, 1 H, J = 7.5 Hz, exchanged in D$_2$O), 6.02 (m, 1 H, J = 4 Hz), 5.39 (dd, 1 H, J = 1.5, 17.2 Hz), 5.26 (dd, 1 H, J = 1.4, 10.4 Hz), 5.02 (dt, 1 H, J = 2.0, 7.5 Hz), 4.50 (dd, 2 H, J = 1.5, 6.6 Hz), 4.21 (q, 2 H, J = 7.2 Hz), 3.20 (dd, 2 H, J = 5.3, 5.7 Hz), 1.28 (t, 3 H, J = 7.2 Hz). mass spectrum (chemical ionizations) m/e = 354 (M + 1); Anal. (C$_{21}$H$_{23}$NO$_4$) C, H, N.

N-Benzoyl-O'-(2,3-epoxypropyl)-L-tyrosine Ethyl Ester (2a). To a solution of **7a** (1.7 g, 4.6 mmol) in CH$_2$Cl$_2$/0.1 M K$_2$HPO$_4$, pH 8.0 (50/50 v/v; 100 mL), was added *m*-CPBA (0.90 g, 5.1 mmol) in small portions over 10 min at 4 °C. After stirring the mixture for 5 h at room temperature, an additional 0.90 g of *m*-CPBA was added over 10 min at 4 °C. The mixture was reacted for a further 30 h, the buffer layer was changed, 2 more equiv (1.8 g) of *m*-CPBA was added, and the reaction was continued for another 60 h. Next the aqueous layer was extracted with CH$_2$Cl$_2$ (4 × 60 mL). The combined organic layers were dried (MgSO$_4$), concentrated on a rotary evaporator, and chromatographed on alumina (CH$_2$Cl$_2$) to yield **2a** (0.70 g, 42%): mp 72 °C; R_f (alumina, CHCl$_3$) = 0.6; IR (CHCl$_3$) 3700, 3640, 3010, 1740, 1670, 1510, 1220 cm^{-1}; ^{1}H NMR (CDCl$_3$/TMS, 200 MHz) δ 7.73 (dd, 2 H, J = 1.4, 8.0 Hz), 7.44 (m, 3 H), 7.08 (dd, 2 H, J = 2.0, 6.6 Hz), 6.83 (dd, 2 H, J = 2.0, 6.6 Hz), 6.63 (d, 1 H, J = 7.5 Hz), 5.02 (dt, 1 H, J = 5.7 Hz), 4.22 (m, 3 H), 3.92 (dd, 1 H, J = 5.7, 11.0 Hz), 3.33 (m, 1 H), 3.20 (dd, 2 H, J = 5.3, 5.7 Hz), 2.89 (m, 1 H), 2.74 (m, 1 H), 1.28 (t, 3 H, J = 7.1 Hz). Anal. (C$_{21}$H$_{23}$NO$_5$) C, H, N.

Synthesis of Compound 2a Using Epibromohydrin.[9] A mixture of K$_2$CO$_3$ (0.90 g, 6.4 mmol), *N*-benzoyl-L-tyrosine ethyl ester (2.0 g, 6.3 mmol), and epibromohydrin (20 mL) was stirred for 8 h at room temperature. Next the epibromohydrin was removed under a high vacuum to yield 4.5 g of residue. The residue was partitioned between CH$_2$Cl$_2$ and water (100 mL of each). Next, the water layer was extracted with CH$_2$Cl$_2$. The combined organic layers were dried (MgSO$_4$), the solvent was removed at reduced pressure to give 2.1 g of residue, and the residue was chromatographed on alumina yielding 1.2 g (50% yield) of **2a** that according to all spectroscopic comparisons was identical with the compound prepared by the previous method.

N-Benzoyl-O'-(but-3-enyl)-L-tyrosine Ethyl Ester (7b). To a solution of *N*-benzoyl-L-tyrosine ethyl ester (4.0 g, 12.7 mmol) in acetone (15 mL) was added K$_2$CO$_3$ (1.8 g, 13 mmol) followed by 4-bromo-1-butene (1.2 mL, 14 mmol). The solution was heated at reflux for 48 h, at which time 1.2 mL of additional 4-bromo-1-butene was added. The reaction was continued at reflux for another 48 h, then a third portion of 1.2 mL of 4-bromo-1-butene was added and the reflux was continued for a final 48 h. After cooling, the solvent was evaporated on a rotary evaporator, yielding a yellow oil. This oil was redissolved in 5% NaOH/ether (1/1 v/v; 250 mL). The aqueous layer was extracted with ether (4 × 50 mL), the combined organic layers were dried (MgSO$_4$), and the solution was concentrated and chromatographed on silica gel (CHCl$_3$) to provide **7b** (2.6 g, 55%): mp 75 °C; R_f (silica, CHCl$_3$/ethanol 97/3) = 0.75; IR (CH$_2$Cl$_2$) 3780, 3660, 3440, 3000, 1740, 1660, 1510, 1240 cm^{-1}; ^{1}H NMR (CDCl$_3$/TMS, 200 MHz) δ 7.73 (dd, 2 H, J = 1.7, 6.5 Hz), 7.45 (m, 3 H), 7.05 (dd, 2 H, J = 2.2, 8.5 Hz), 6.83 (dd, 2 H, J = 2.2, 8.5 Hz), 6.59 (d, 1 H, J = 6.7 Hz), 5.90 (m, 1 H), 5.23–4.98 (m, 2 H), 4.21 (q, 2 H, J = 7.1 Hz), 3.98 (t, 2 H, J = 6.7 Hz), 3.18 (m, 2 H), 2.52 (t, 2 H, J = 6.6 Hz), 1.29 (t, 3 H, J = 7.0 Hz). Anal. (C$_{22}$H$_{25}$NO$_4$) C, H, N.

N-Benzoyl-O'-(3,4-epoxybutyl)-L-tyrosine Ethyl Ester (2b). To a solution of **7b** (3.68 g, 10 mmol) in CH$_2$Cl$_2$/0.1 M Na$_2$HPO$_4$, pH 8.0 (1/1 v/v; 200 mL), was added *m*-CPBA (1.74 g, 10 mmol) in small portions at 4 °C over 10 min. After 24 h of reaction at room temperature, another equivalent of *m*-CPBA was added at 0 °C while the aqueous buffer layer was changed. This routine was repeated for 10 days. Next, the aqueous layer was extracted with CH$_2$Cl$_2$ (4 × 100 mL). The combined organic layers were concentrated on a rotary evaporator and chromatographed on alumina (CH$_2$Cl$_2$), yielding a white solid (**2b**, 1.6 g, 42%): mp 75 °C; R_f (alumina, CHCl$_3$) = 0.40; IR (CH$_2$Cl$_2$) 3700, 2460, 3010, 1740, 1670, 1510, 1220 cm^{-1}; ^{1}H NMR (CDCl$_3$/TMS, 200 MHz) δ 7.72 (dd, 2 H, J = 1.8, 7.3 Hz), 7.46 (m, 3 H), 7.05 (d, 2 H, J = 8.8 Hz), 6.80 (d, 2 H, J = 8.6 Hz), 6.50 (d, 1 H, J = 6.9 Hz), 5.01 (dt, 1 H, J = 7.0 Hz), 4.22 (q, 2 H, J = 7.0 Hz), 4.05 (t, 2 H,

J = 6.1 Hz), 3.18 (m, 3 H), 2.82 (m, 1 H), 2.57 (m, 1 H), 2.04 (m, 2 H), 1.28 (t, 3 H, J = 7.1 Hz). Anal. (C$_{22}$H$_{25}$NO$_5$) C, H, N.

N-Benzoyl-O'-(pent-4-enyl)-L-tyrosine Ethyl Ester (7c). To a solution of *N*-benzoyl-L-tyrosine ethyl ester (2.2 g, 7.0 mmol) in acetone (15 mL) was added K$_2$CO$_3$ (0.97 g, 7.0 mmol), followed by 5-bromo-*n*-pent-1-ene (1.1 mL, 8.0 mmol). The mixture was refluxed for 44 h, then the solvent was removed on a rotary evaporator, yielding an oily residue. The residue was redissolved in 5% NaOH/ether (1/1 v/v, 50 mL) and the aqueous layer was extracted with ether (4 × 20 mL). The combined organic layers were dried (MgSO$_4$), the solvent was removed at reduced pressure, and the residue was chromatographed on silica (CH$_2$Cl$_2$) to yield **7c** (2.44 g, 91.2%): mp 92 °C; R_f (silica, CH$_2$Cl$_2$) = 0.45; IR (CHCl$_3$) 3445, 3020, 1740, 1640, 1510, 1230, 1060, 1000, 910, 850, 800 cm^{-1}; ^{1}H NMR (CDCl$_3$/TMS, 200 MHz) δ 7.73 (dd, 2 H, J = 1.4, 8.0 Hz), 7.44 (m, 3 H), 7.04 (d, 2 H, J = 8.6 Hz), 6.81 (d, 2 H, J = 8.6 Hz), 6.58 (d, 1 H, J = 7.2 Hz), 5.85 (m, 1 H), 5.04 (m, 3 H), 4.22 (q, 2 H, J = 7.1 Hz), 3.93 (t, 2 H, J = 6.5 Hz), 3.20 (dd, 2 H, J = 2.7, 5.4 Hz), 2.21 (q, 2 H, J = 7.6 Hz), 1.86 (m, 2 H), 1.29 (t, 3 H, J = 7.1 Hz). Anal. (C$_{23}$H$_{27}$NO$_4$) C, H, N.

N-Benzoyl-O'-(4,5-epoxypentyl)-L-tyrosine Ethyl Ester (2c). To a solution of **7c** (1.0 g, 2.5 mmol) in CH$_2$Cl$_2$/0.1 M K$_2$HPO$_4$, pH 8.0 (1/1 v/v, 100 mL), was added *m*-CPBA (0.90 g, 5.1 mmol) over 10 min at 4 °C. After stirring of the mixture for 24 h at room temperature, an additional 0.45 g (2.5 mmol) of *m*-CPBA was added at 4 °C over a 10-min period and the reaction was continued for 72 h longer at room temperature. Next, the buffer layer was changed, 2 equiv of *m*-CPBA was added (0.90 g) at 4 °C, and the reaction was continued for a final 72 h. The aqueous layer was then extracted with CH$_2$Cl$_2$ (3 × 50 mL), the combined organic layers were dried (MgSO$_4$), the solution was concentrated on a rotary evaporator, and the residue was chromatographed (alumina, CH$_2$Cl$_2$) to yield **2c** (0.39 g, 39% yield): mp 79 °C; R_f (alumina, CHCl$_3$) = 0.65; IR (CH$_2$Cl$_2$) 3680, 3440, 3030, 3000, 1740, 1665, 1510, 1230, 1110, 1020, 930, 840 cm^{-1}; ^{1}H NMR (CDCl$_3$/TMS, 200 MHz) δ 7.73 (dd, 2 H, J = 1.4, 8.0 Hz), 7.44 (m, 3 H), 7.04 (d, 2 H, J = 8.6 Hz), 6.81 (d, 2 H, J = 8.6 Hz), 6.59 (d, 1 H, J = 7.5 Hz), 5.02 (dd, 1 H, J = 5.4, 13.0 Hz), 4.14 (q, 2 H, J = 7.1 Hz), 3.97 (m, 2 H), 3.2 (dd, 2 H, J = 3.4, 5.4 Hz), 3.0 (m, 1 H), 2.76 (m, 1 H), 2.5 (dd, 1 H, J = 2.7, 4.9 Hz), 1.8 (m, 4 H), 1.29 (t, 3 H, J = 7.1 Hz). Anal. (C$_{23}$H$_{27}$NO$_5$) C, H, N.

N-Acetyl-O'-(prop-2-enyl)-L-tyrosine Ethyl Ester (8a). To a solution of *N*-acetyl-L-tyrosine ethyl ester (1.0 g, 4.3 mmol) in acetone was added K$_2$CO$_3$ (0.6 g, 4.3 mmol) followed by allyl bromide (0.37 mL, 4.1 mmol). The mixture was refluxed for 4 h and then cooled and the solvent was removed on a rotary evaporator to give a white residue. The residue was redissolved in 5% NaOH/ether (1/1 v/v; 50 mL) and the aqueous layer was extracted with ether (4 × 20 mL). The combined organic layers were dried (MgSO$_4$); the solution was concentrated and chromatographed on silica gel to give **8a** as a white solid (0.99 g, 85%): mp 69.5 °C; R_f (silica, 97/3 CHCl$_3$/ethanol) = 0.70; IR (CH$_2$Cl$_2$) 3740, 3420, 3000, 1735, 1660, 1505, 1210 cm^{-1}; ^{1}H NMR (CDCl$_3$/TMS, 200 MHz) δ 6.94 (d, 2 H, J = 8.8 Hz), 6.77 (d, 2 H, J = 8.8 Hz), 5.91 (m, 1 H, J = 5.0 Hz), 5.86 (d, 1 H, J = 7.5 Hz), 5.33 (dq, 1 H, J = 1.5, 17.2 Hz), 5.21 (dq, 1 H, J = 1.6, 10 Hz), 4.75 (dq, 1 H, J = 2.1, 7.8 Hz), 4.44 (dt, 2 H, J = 1.4, 5.3 Hz), 4.10 (q, 2 H, J = 7.2 Hz), 2.99 (d, 2 H, J = 5.5 Hz), 1.92 (s, 3 H), 1.18 (t, 3 H, J = 7.1 Hz). Anal. (C$_{16}$H$_{21}$NO$_4$) C, H, N.

N-Acetyl-O'-(2,3-epoxypropyl)-L-tyrosine Ethyl Ester (3a). To a solution of **8a** (1 g, 5.1 mmol) in CH$_2$Cl$_2$/0.1 M Na$_2$HPO$_4$, pH 8.0 (1/1, 100 mL), was added *m*-CPBA (0.974 g, 5.6 mmol) in small portions at 4 °C over a 10-min period. The reaction mixture was then stirred at room temperature for 5 h, another portion (1.575 g) of *m*-CPBA was added at 4 °C, and the reaction was stirred at room temperature for 18 h longer. Next, the aqueous buffer was replaced and 2 more equiv of *m*-CPBA was added followed by 30 h further reaction. The aqueous layer was extracted with CH$_2$Cl$_2$ (3 × 30 mL). The combined organic layers were dried over MgSO$_4$, concentrated on a rotary evaporator, and chromatographed on alumina (CH$_2$Cl$_2$) to yield a white solid (**3a**, 0.50 g, 32%): mp 73 °C; R_f (alumina, CH$_2$Cl$_2$/ether, 50/50) = 0.30; IR (CH$_2$Cl$_2$) 3680, 3600, 3420, 3000, 1730, 1665, 1500, 1200 cm^{-1}; ^{1}H NMR (CDCl$_3$/TMS, 200 MHz) δ 6.92 (dd, 2 H, J = 1.9, 6.8 Hz), 6.73 (dd, 2 H, J = 1.9, 6.5 Hz), 6.05 (d, 1 H, J = 7.8 Hz), 4.71 (dt, 1 H, J = 1.8, 7.8 Hz), 4.09 (m, 3 H), 3.81 (dd, 1 H, J = 5.7,

11 Hz), 3.25 (m, 1 H), 2.95 (dd, 2 H, J = 2.6, 5.7 Hz), 2.80 (t, 1 H, J = 4.5 Hz), 2.65 (dd, 1 H, J = 2.6, 4.8 Hz), 1.88 (s, 3 H), 1.16 (t, 3 H, J = 7.1 Hz). Anal. ($C_{16}H_{21}NO_5$) C, H, N.

Synthesis of Compound 3a Using Epibromohydrin. A mixture of K_2CO_3 (0.9 g, 6.4 mmol), N-acetyl-L-tyrosine ethyl ester (1.6 g, 6.4 mmol), and epibromohydrin (20 mL) was stirred for 19 h at room temperature. Next the epibromohydrin was removed under high vacuum, yielding 4.2 g of a residue that was partitioned between water and CH_2Cl_2 (3 × 50 mL), and the combined organic layers were dried ($MgSO_4$). The solvent was removed at reduced pressure and yielded 1.9 g of residue that was chromatographed on alumina, giving **3a** (1.1 g, 54% yield) identical, according to all spectroscopic comparisons, with the compound prepared by the previous method.

N-Acetyl-O'-(prop-2-enyl)-D-tyrosine Ethyl Ester (D-8a). To a solution of N-acetyl-D-tyrosine ethyl ester (0.20 g, 0.79 mmol) in acetone (10 mL) was added K_2CO_3 (0.11 g, 0.8 mmol) followed by allyl bromide (0.07 mL, 0.8 mmol). The mixture was heated at reflux for 16 h. After cooling, the solvent was removed on a rotary evaporator, yielding a white residue. The solid was re-dissolved in 5% $NaOH/CH_2Cl_2$ (1/1 v/v; 30 mL) and the aqueous layer was dried over $MgSO_4$, concentrated, and chromatographed on silica gel (CH_2Cl_2) to yield a white solid (D-8a, 0.205 g, 89%): mp 70 °C; R_f (silica, 50/50 v/v ether/CH_2Cl_2) = 0.70; IR (CH_2Cl_2) 3680, 3620, 3440, 3000, 1735, 1670, 1505, 1210 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 6.94 (dd, 2 H, J = 2.1, 8.8 Hz), 6.76 (dd, 2 H, J = 2.0, 8.8 Hz), 5.94 (m, 1 H, J = 5.1 Hz), 5.90 (d, 1 H, J = 6 Hz), 5.34 (dq, 1 H, 1.6, 17.2 Hz), 5.21 (dq, 1 H, J = 1.5, 10.5 Hz), 4.77 (t, $^1/_2$ H, J = 7 Hz), 4.73 (t, $^1/_2$ H, J = 7.7 Hz), 4.43 (dt, 2 H, J = 1.4, 5.3 Hz), 4.10 (q, 2 H, J = 7.1 Hz), 2.98 (d, 2 H, J = 5.7 Hz), 1.92 (s, 3 H), 1.18 (t, 3 H, J = 7.1 Hz). Anal. ($C_{16}H_{21}NO_4$) C, H, N.

N-Acetyl-O'-(2,3-epoxypropyl)-D-tyrosine Ethyl Ester (D-3a). To a solution of D-8a (0.180 g, 0.61 mmol) in CH_2Cl_2/Na_2HPO_4 0.1 M, pH 8.0 (1/1 v/v; 40 mL) was added m-CPBA (0.106 g, 0.61 mmol) in small portions over 10 min at 4 °C. After stirring for 24 h at room temperature, the aqueous buffer was changed and 2 equiv of m-CPBA was added (0.212 g) at 0 °C. This routine was repeated for 3 more consecutive days. Next the aqueous layer was extracted with CH_2Cl_2 (4 × 20 mL). The combined organic layers were dried ($MgSO_4$), concentrated on a rotary evaporator, and chromatographed on alumina to yield D-3a (0.090 g, 48%): mp 74 °C; R_f (alumina, CH_2Cl_2/ether 50/50) = 0.30; IR (CH_2Cl_2) 3680, 3610, 3420, 3000, 1730, 1670, 1610, 1500, 1200, 740 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 6.94 (dd, 2 H, J = 2.1, 6.8 Hz), 6.75 (dd, 2 H, J = 2.1, 6.8 Hz), 6.05 (d, 1 H, J = 7.7 Hz), 4.75 (dt, 1 H, J = 2.0, 5.6 Hz), 4.10 (q, 2 H, J = 7.1 Hz) superimposed on 4.11 (m, 1 H), 3.85 (dd, 1 H, J = 5.6 Hz), 3.27 (m, 1 H), 2.99 (dd, 2 H, J = 1.5, 5.8 Hz), 2.83 (t, 1 H, J = 4.2 Hz), 2.68 (dd, 1 H, J = 1.5, 5.8 Hz), 1.92 (s, 3 H), 1.18 (t, 3 H, J = 7.1 Hz). Anal. ($C_{16}H_{21}NO_5$) C, H, N.

N-Acetyl-O'-(but-3-enyl)-L-tyrosine Ethyl Ester (8b). To a solution of N-acetyl-L-tyrosine ethyl ester (6 g, 23 mmol) in acetone (25 mL) was added K_2CO_3 (3.84 g, 23 mmol) followed by 4-bromo-1-butene (2.4 mL, 23 mmol). The mixture was heated at reflux for 16 h and allowed to cool, and the solvent was removed on a rotary evaporator to yield a white solid mixture. This mixture was dissolved in 5% $NaOH$/ether (1/1 v/v; 250 mL). The aqueous layer was extracted with ether (3 × 75 mL). The combined organic layers were dried ($MgSO_4$), concentrated, and chromatographed on silica gel (CH_2Cl_2) to provide a white solid (8b, 4.7 g, 66%): mp 82.5 °C; R_f (silica, $CHCl_3$) = 0.80; IR (CH_2Cl_2) 3700, 3430, 3000, 1745, 1670, 1570, 1230 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 6.98 (d, 2 H, J = 8.7 Hz), 6.8 (d, 2 H, J = 8.6 Hz), 5.95 (m, 2 H), 5.19 (m, 2 H), 4.80 (q, 1 H, J = 7.8 Hz), 4.20 (q, 2 H, J = 7.0 Hz), 3.95 (t, 2 H, J = 6.6 Hz), 3.15 (d, 2 H, J = 5.1 Hz), 2.50 (q, 2 H, J = 6.6 Hz), 1.95 (s, 3 H), 1.25 (t, 3 H, J = 7.1 Hz). Anal. ($C_{17}H_{23}NO_4$) C, H, N.

N-Acetyl-O'-(3,4-epoxybutyl)-L-tyrosine Ethyl Ester (3b). To a solution of 8b (3.0 g, 11 mmol) in CH_2Cl_2/Na_2HPO_4 0.1 M, pH 8.0 (1/1 v/v; 50 mL), was added m-CPBA (1.7 g, 10 mmol) in small portions over 10 min at 4 °C. The mixture was reacted at room temperature for 24 h, then another 2 equiv of m-CPBA was added. After 48 h of further reaction, the buffer layer was changed and 2 more equiv of m-CPBA was added. After 48 h of further reaction, the buffer layer was changed, 2 more equiv

of m-CPBA acid was added, and the reaction was allowed to proceed for 24 h longer. Next, the aqueous layer was extracted with CH_2Cl_2 (4 × 25 mL). The combined organic layers were dried ($MgSO_4$), concentrated on a rotary evaporator, and flash chromatographed on alumina ($CHCl_3$) to yield a white solid (**3b**, 1.8 g, 52%): mp 104 °C; R_f (alumina, $CHCl_3$) = 0.42; IR (CH_2Cl_2) 3760, 3700, 3440, 3000, 1740, 1675, 1510, 1260 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.02 (dd, 2 H, J = 2.0, 6.5 Hz), 6.82 (dd, 2 H, J = 2.0, 6.6 Hz), 5.95 (d, 1 H, J = 7.9 Hz), 4.81 (dt, 1 H, J = 5.8, 7.8 Hz), 4.17 (q, 2 H, J = 7.2 Hz), 4.08 (t, 2 H, J = 5.6 Hz), 3.13 (m, 1 H), 3.06 (dd, 2 H, J = 1.8, 5.6 Hz), 2.82 (t, 1 H, J = 4.5 Hz), 2.58 (dd, 1 H, J = 2.7, 4.9 Hz), 1.99 (m, 2 H) superimposed on 1.98 (s, 3 H), 1.26 (t, 3 H, J = 7 Hz). Anal. ($C_{17}H_{23}NO_5 \cdot 0.2H_2O$) C, H, N.

N-Benzoyl-O'-(prop-2-enyl)-L-tyrosine-p-nitroanilide (9a). To a solution of N-benzoyl-L-tyrosine-p-nitroanilide (1.0 g, 2.4 mmol) in acetone (10 mL) was added K_2CO_3 (0.32 g, 2.2 mmol). The mixture was heated under reflux for 10 min to give a yellow solution. After cooling, allyl bromide was added (0.2 mL, 2.2 mmol) and the mixture was heated under reflux for another 6 h. After cooling, the solvent was removed on a rotary evaporator, yielding a yellow oil. The oily residue was redissolved in H_2O/ether (1/1 v/v; 50 mL) and the aqueous layer was extracted with ether (4 × 20 mL). The combined organic layers were dried ($MgSO_4$), concentrated, and chromatographed on silica gel ($CHCl_3$) to yield a yellowish compound (9a, 0.78 g, 78%): mp 202 °C; R_f (silica, 97/3 $CHCl_3$/ethanol) = 0.6; IR ($CHCl_3$) 3680, 3620, 3400, 3280, 3000, 1710, 1650, 1510, 1340, 1210 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS 200 MHz) δ 9.36 (s, 1 H), 8.12 (d, 2 H, J = 9.2 Hz), 7.73 (d, 2 H, J = 6.9 Hz), 7.57 (d, 2 H, J = 9.2 Hz), 7.44 (m, 3 H, J = 7.3 Hz), 7.17 (d, 2 H, J = 8.6 Hz), 6.95 (d, 1 H, J = 7.5 Hz), 6.82 (d, 2 H, J = 8.6 Hz), 6.01 (m, 1 H, J = 4.5 Hz), 5.36 (dd, 1 H, J = 1.5, 17.2 Hz), 5.25 (dd, 1 H, J = 1.5, 10.4 Hz), 5.13 (q, 1 H, J = 7.5 Hz), 4.45 (dd, 2 H, J = 1.3, 5.2 Hz), 3.25 (d, 2 H, J = 7.0 Hz). Anal. ($C_{25}H_{23}N_3O_5$) C, H, N.

N-Benzoyl-O'-(2,3-epoxypropyl)-L-tyrosine-p-nitroanilide (4a). To a solution of N-benzoyl-O-prop-2-enyl-L-tyrosine-p-nitroanilide (9a, 0.5 g, 1.1 mmol) in CH_2Cl_2/0.1 M K_2HPO_4, pH 8.0 (1/1 v/v; 50 mL), stirred at 4 °C was added m-CPBA (0.39 g, 2.2 mmol) in small portions over a 10-min period. After 24 h of reaction at room temperature another 2 equiv of m-CPBA was added. After 2 more days of reaction the buffer layer was changed and 2 more equiv of m-CPBA was added and the reaction was terminated after a final 12 h. The aqueous layer was extracted with CH_2Cl_2 (4 × 25 mL). The combined organic layers were dried ($MgSO_4$), concentrated on a rotary evaporator and chromatographed on alumina (80–200 mesh; $CHCl_3$) and yielded a yellowish solid (4a, 0.20 g, 38%): mp 182 °C; R_f (alumina, $CHCl_3$/ethanol 97/3) = 0.3; IR (CH_2Cl_2) 3680, 3620, 3410, 3000, 1710, 1650, 1510, 1200, 750 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 9.61 (s, 1 H), 8.12 (d, 2 H, J = 9.1 Hz), 7.72 (d, 2 H, J = 7.2 Hz), 7.55 (m, 3 H), 7.45 (m, 2 H), 7.17 (d, 2 H, J = 8.5 Hz), 6.90 (d, 1 H, J = 7.6 Hz), 6.78 (d, 2 H, J = 8.5 Hz), 5.10 (q, 1 H, J = 7.5 Hz), 4.14 (dd, 1 H, J = 2.9, 11.0 Hz), 3.84 (m, 1 H), 3.27 (m, 3 H), 2.87 (m, 1 H), 2.71 (m, 1 H). Anal. ($C_{25}H_{23}N_3O_6$) C, H, N.

N-Benzoyl-O'-(but-3-enyl)-L-tyrosine-p-nitroanilide (9b). To a solution of N-benzoyl-L-tyrosine-p-nitroanilide (3 g, 6.5 mmol) in acetone (15 mL) was added K_2CO_3 (0.95 g, 6.8 mmol) followed by 4-bromo-1-butene (0.8 mL, 7.0 mmol). The solution was heated at reflux for 48 h, 0.8 mL more of 4-bromo-1-butene was added, reflux was continued for 48 h longer, a final 0.8 mL of 4-bromo-1-butene was added, and the reflux was continued for 3 more days (a total reaction time of 1 week at reflux). After cooling, the solvent was evaporated on a rotary evaporator, yielding a yellow oil. This oil was dissolved in 5% $NaOH$/ether (1/1 v/v; 150 mL). The aqueous layer was extracted with ether (4 × 50 mL). The combined organic layers were dried ($MgSO_4$); the solution was concentrated and chromatographed on silica gel ($CHCl_3$/ethanol 99/1) to provide a yellowish solid (9b, 2.2 g, 73%): mp 203 °C; R_f (silica, $CHCl_3$/ethanol 97/3) = 0.80; IR (CH_2Cl_2) 3700, 3640, 3420, 3000, 1720, 1655, 1520, 1220 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 9.34 (d, 1 H, J = 5.8 Hz), 8.14 (dd, 2 H, J = 1.9, 9.6 Hz), 7.73 (dd, 2 H, J = 1.9, 6.8 Hz), 7.58 (dd, 2 H, J = 1.4, 9.5 Hz), 7.47 (m, 3 H), 7.14 (d, 2 H, J = 8.4 Hz), 6.95 (dd, 1 H, J = 3.0, 7.6 Hz), 6.74 (dd, 2 H, J = 1.7, 8.5 Hz), 5.84 (m, 1 H), 5.10 (m, 2 H), 3.81 (t, 2 H, J = 6.7 Hz), 3.20 (dd, 2 H,

J = 2.4, 7.2 Hz), 2.43 (q, 2 H, J = 6.7 Hz). Anal. ($C_{26}H_{25}N_3O_5$) C, H, N.

N-Benzoyl-O'-(3,4-epoxybutyl)-L-tyrosine-p-nitroanilide (4b). To a solution of **9b** (1.2 g, 2.6 mmol) in CH_2Cl_2/0.1 M K_2HPO_4, pH 8.0 (1/1 v/v, 100 mL), stirred at 4 °C (cold room) was added m-CPBA (0.45 g, 2.6 mmol). The reaction was allowed to proceed for 4 weeks. Each week the aqueous layer, including the m-CPBA, was replaced by fresh solution. After the 4 weeks the aqueous layer was separated and extracted with CH_2Cl_2 (3 × 40 mL). The combined organic layers were dried ($MgSO_4$), concentrated on a rotary evaporator, and chromatographed on alumina (Basic Brockman Activity IV, 80–200 mesh, $CHCl_3$ solvent) to yield a yellowish solid (**4b**, 0.28 g, 22%): mp 195.5 °C; R_f (alumina, $CHCl_3$) = 0.15; IR (CH_2Cl_2) 3705, 3460, 3000, 1720, 1660, 1520, 1220 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 9.74 (s, 1 H), 8.04 (d, 2 H, J = 9.0 Hz), 7.74 (d, 2 H, J = 7.3 Hz), 7.52 (m, 2 H), 7.41 (m, 3 H), 7.17 (d, 1 H, J = 7.0 Hz), 7.13 (d, 2 H, J = 7.0 Hz), 6.98 (d, 2 H, J = 6.7 Hz), 5.25 (q, 1 H, J = 6.0 Hz), 3.75 (t, 2 H, J = 6.4 Hz), 3.11 (m, 3 H), 2.71 (t, 1 H, J = 4.4 Hz), 2.44 (dd, 1 H, J = 2.6, 4.8 Hz), 1.91 (m, 1 H), 1.72 (m, 2 H). Anal. ($C_{26}H_{25}N_3O_6$) C, H, N.

N-Benzoyl-L-phenylalanine Allyl Ester (10). To a solution of N-benzoyl-L-phenylalanine (1.0 g, 3.7 mmol) in acetone (10 mL) was added K_2CO_3 (0.56 g, 3.8 mmol) and the mixture was heated under reflux for 10 min. After cooling, allyl bromide (0.36 mL, 3.8 mmol) was added and the mixture was heated under reflux for another 20 h. After cooling, the solvent was removed on a rotary evaporator, yielding a yellow solid. The solid was redissolved in saturated $NaHCO_3$/ether (1/1 v/v; 50 mL). The aqueous layer was extracted first with ether (4 × 20 mL) then with $CHCl_3$ (25 mL). The combined organic layers were dried ($MgSO_4$), concentrated, and chromatographed on silica gel ($CHCl_3$) to yield **10** as a white solid (0.60 g, 53%): mp 90 °C; R_f (silica, $CHCl_3$) = 0.75; IR (CH_2Cl_2) 3710, 3460, 3040, 1750, 1680, 1520, 1680, 1520, 1225 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.66 (d, 2 H, J = 6.9 Hz), 7.37 (m, 3 H, J = 7.3 Hz), 7.19 (m, 3 H), 7.08 (m, 2 H), 6.53 (d, 1 H, J = 5.9 Hz), 5.84 (m, 1 H, J = 2.3, 5.3 Hz), 5.31 (d, 1 H, J = 9.0 Hz), 5.23 (d, 1 H, J = 6.0 Hz), 5.05 (q, 1 H, J = 5.4 Hz), 4.59 (d, 2 H, J = 5.5 Hz), 3.21 (d, 2 H, J = 5.1 Hz). Anal. ($C_{19}H_{19}NO_3$·0.2H_2O) C, H, N.

N-Benzoyl-O'-(prop-2-enyl)-L-tyrosine Allyl Ester (11). To a solution of N-benzoyl-L-tyrosine (1.0 g, 3.6 mmol) in acetone (14 mL) was added K_2CO_3 (0.90 g, 3.6 mmol) followed by allyl bromide (0.8 mL, 7.4 mmol). The mixture was heated at reflux for 16 h. After cooling, the solvent was removed on a rotary evaporator, yielding a tan oil. The oil was redissolved in H_2O/$CHCl_3$ (1/1 v/v, 50 mL) and the aqueous layer was extracted with $CHCl_3$ (3 × 70 mL). The combined organic layers were dried ($MgSO_4$), concentrated, and chromatographed on silica gel ($CHCl_3$) to yield a white solid (**11**, 0.868 g, 65%): R_f (silica, 50/50 $CHCl_3$/ether) = 0.50; IR (CH_2Cl_2) 3400, 3000, 1725, 1650, 1490, 1190 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.73 (dd, 2 H, J = 2.1, 6.5 Hz), 7.46 (m, 3 H, J = 2.0, 7.2 Hz), 7.05 (dd, 2 H, J = 1.7, 8.6 Hz), 6.83 (dd, 2 H, J = 2.1, 8.6 Hz), 6.57 (d, 1 H, J = 7.2 Hz), 6.03 (m, 1 H, J = 5.3 Hz), 5.89 (m, 1 H, J = 3.7 Hz), 5.47 (dd, 2 H, J = 1.7, 15.0 Hz), 5.32 (m, 2 H), 5.07 (q, 1 H, J = 7.5 Hz), 4.65 (dd, 2 H, J = 1.2, 5.8 Hz), 4.50 (dd, 2 H, J = 1.4, 6.6 Hz), 3.22 (m, 2 H). Anal. ($C_{22}H_{23}NO$) C, H, N.

N-Benzoyl-L-phenylalanine 2,3-Epoxypropyl Ester (5). To a solution of **10** (0.6 g, 1.9 mmol) in CH_2Cl_2/0.1 M K_2HPO_4, pH 8.0 (50/50 v/v; 100 mL), was added m-CPBA (0.3 g, 1.7 mmol) in small portions over a 10-min period at 0 °C. After stirring for 24 h at room temperature a second equivalent of m-CPBA was added (0.3 g, 1.7 mmol) and stirring was continued for 24 h longer. Next the aqueous layer was changed and 2 further equiv (0.6 g, 3.4 mmol) of m-CPBA was added at 0 °C. The reaction was completed after 48 h of stirring. The organic layer was separated, the aqueous layer was extracted with CH_2Cl_2 (4 × 50 mL), and the combined organic layers were dried ($MgSO_4$). Next, the solvent was evaporated and the residue was chromatographed on alumina (CH_2Cl_2) to yield **5** (0.19 g, 30% yield): mp 109 °C; IR (KBr pellet) 3300, 3080, 3060, 3025, 1745, 1645, 1545, 1360, 1270, 1220, 1190, 1100, 900, 700 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.72 (dd, 2 H, J = 1.2, 8.0 Hz), 7.48 (m, 3 H), 7.35 (m, 3 H), 7.21 (m, 2 H), 6.57 (d, 1 H, J = 7.1 Hz), 5.12 (dd, 1 H, J = 1.2, 7.4 Hz), 4.45 (m, 1 H), 4.01 (m, 1 H), 3.28 (dd, 2 H, J = 2.0, 5.6 Hz), 3.19

(m, 1 H), 2.84 (m, 1 H), 2.62 (m, 1 H). Anal. ($C_{19}H_{19}NO_4$·$^1/_5H_2O$) C, H, N.

N-Benzoyl-O'-(2,3-epoxypropyl)-L-tyrosine 2,3-Epoxypropyl Ester (6). To a solution of **11** (0.35 g, 0.96 mmol) in CH_2Cl_2/0.1 M Na_2HPO_4, pH 8.0 (1/1 v/v, 150 mL), was added m-CPBA (0.36 g, 2.0 mmol) over a 10-min period at 0 °C. After 45 min, the reaction mixture was brought to room temperature and stirred for another 5 h. Next, another portion of m-CPBA was added at 0 °C over a 10-min period. After 24 h of stirring at room temperature, the buffer layer was changed and the routine was repeated. After a total of 96 h the aqueous layer was separated and extracted with $CHCl_3$ (3 × 75 mL). The combined organic layers were dried ($MgSO_4$), the solvent was removed, and the residue was chromatographed on alumina (CH_2Cl_2) to give **6** (0.034 mmol, 13 mg, 4.2% yield): mp 132 °C; R_f (alumina, CH_2Cl_2/ether 1/1) = 0.15; IR (KBr pellet) 3410, 3300, 3075, 3015, 1740, 1635, 1540, 1520, 1440, 1360, 1220, 1180, 1030, 1020, 980, 900, 700 cm^{-1}; ^{1}H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.73 (dd, 2 H, J = 1.4, 8.0 Hz), 7.45 (m, 3 H), 7.03 (d, 2 H, J = 8.5 Hz), 6.75 (d, 2 H, J = 8.5 Hz), 6.56 (d, 1 H, J = 7.0 Hz), 5.09 (dt, 1 H, J = 2.0, 6.3 Hz), 4.47 (m, 2 H), 4.05 (m, 2 H), 3.14 (m, 4 H), 2.75 (m, 4 H); MS (FAB, m/e) 398 (M + 1), 342 (–$CH_2CH(O)CH_2$), 296 (–2 × CH_2CH-(O)CH_2). Anal. ($C_{22}H_{23}NO_6$·$^1/_2H_2O$) C, H, N.

An Alternative Route to 6.[9,29] To an aqueous (200 mL) $(NH_4)_2CO_3$ (0.2 M, pH 7.7) solution was added α-chymotrypsin (Sigma, St. Louis, MO, Typel-S, crystallized three times from bovine pancreas, 10 mg, 0.4 μmol) and N-benzoyl-L-tyrosine ethyl ester (200 mg, 0.64 mmol) previously dissolved in 10 mL of dioxane. The pH was maintained at 7.7 by the addition of $(NH_4)_2CO_3$ (1 M). After 20 min the reaction was completed, the solution was extracted with CH_2Cl_2 (2 × 75 mL) and the aqueous layer was lyophilized overnight. The residue was redissolved in 2 mL of water and applied to a Sephadex G-25 medium column (1 × 12 cm) using water as an eluant. The small molecule containing fractions were pooled and lyophilized to yield N-benzoyl-L-tyrosine diammonium salt (190 mg, 82.7% yield). To a solution of dimethyl formamide (50 mL) and epibromohydrin (10 mL) was added N-benzoyl-L-tyrosine diammonium salt (180 mg, 0.49 mmol) at –70 °C. With stirring, the solution was allowed to warm to room temperature overnight. The solution was concentrated under high vacuum and the residue was redissolved in ethyl acetate (50 mL). The ethyl acetate was extracted with water (3 × 25 mL) then with brine (25 mL). The organic layers were dried ($MgSO_4$), and the solvent was removed under high vacuum to yield 200 mg of a mixture that was chromatographed (alumina), yielding **6** (150 mg, 71% yield). The material produced was identical with the compound synthesized by the previous route according to all spectroscopic criteria employed.

N-Acetyl-O'-(2,3-epoxypropyl)-L-tyrosine (12).[29] To a solution of N-acetyl-O'-(2,3-epoxypropyl)-L-tyrosine ethyl ester (**3a**, 150 mg, 0.54 mmol) in 0.1 M $(NH_4)_2CO_3$, pH 7.8/dioxane (95:5, 50 mL) was added subtilisin Carlsberg (20 mg). The pH of the solution during the reaction was maintained at 7.8 by the addition of 1 M $(NH_4)_2CO_3$. The reaction ceased after approximately 50 min (no further drop in pH). The solution was extracted with CH_2Cl_2 (2 × 50 mL) and the aqueous layer was lyophilized to yield a white mixture. The mixture was applied to a Sephadex G-25 (medium) column (20 × 1 cm) and eluted with water, yielding compound **12** as a white solid (the protein eluted in the void volume) (130 mg, 92% yield); mp 200 °C with decomposition; IR (Nujol) 3280, 3180, 2900, 2840, 2200, 1960, 1730, 1630, 1580, 1240, 1035, 690 cm^{-1}; ^{1}H NMR (DMSO-d_6/TMS, 200 MHz) δ 7.47 (d, 1 H, J = 7.7 Hz), 7.02 (d, 2 H, J = 8.6 Hz), 4.24 (dd, 1 H, J = 2.6, 11.4 Hz), 4.09 (m, 1 H), 3.76 (m, 1 H), 2.98 (dd, 1 H, J = 5.0, 13.3 Hz), 2.75 (m, 4 H), 1.75 (s, 3 H); MS (FAB, m/e) 302 (M + Na^+), 280 (M + 1), 238 (–$COCH_3$ + 1), 207 (–CH_2OCHCH_2O + 1). Anal. ($C_{14}H_{17}NO_5$·NH_4HCO_3) C, H, N.

N-(tert-Butoxycarbonyl)-L-tyrosine Ethyl Ester (13).[30] To a solution of L-tyrosine ethyl ester (5.0 g, 24 mmol) dissolved in tetrahydrofuran/methanol (100 mL/30 mL), was added $NaHCO_3$ (1.97 g, 23.8 mmol) and the reaction mixture was stirred

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for 5 min. Next di-*tert*-butyl dicarbonate (4.37 g, 23.8 mmol) dissolved in tetrahydrofuran (10 mL) was added and the mixture was allowed to react at room temperature for 20 h. The solution was concentrated in vacuo and then partitioned between H_2O and $CHCl_3$ (100 mL of each). The organic layer was dried ($MgSO_4$), and the solvent was removed at a vacuum to yield the product (5.89 g, 19 mmol, 82.6%) as a white solid: mp 84–85 °C (lit.[30] mp 83 °C); R_f (CH_2Cl_2, silica gel) = 0.20; R_f (95/5, CH_2Cl_2/CH_3OH) = 0.85.

N-(*tert*-Butoxycarbonyl)-O-benzyl-L-tyrosine Ethyl Ester (14). To a solution of N-(*tert*-butoxycarbonyl)-L-tyrosine ethyl ester (5.89 g, 19 mmol) dissolved in acetone (20 mL) was added K_2CO_3 (2.91 g, 21 mmol) followed by benzyl bromide[31] (3.75 g, 21 mmol). The mixture was heated at reflux for 6 h and then cooled and the solvent was removed on a rotary evaporator yielding a brownish oil. The oil was redissolved in 5% $NaOH/CHCl_3$ (100 mL of each) and the aqueous layer was extracted with $CHCl_3$ (4 × 50 mL). The combined organic layers were dried ($MgSO_4$); the solvent was removed on a rotary evaporator, yielding the product (6.4 g, 16 mmol, 84%) as a colorless oil: $[\alpha]^{26} = -21.8°$ (MeOH); IR (neat film) 3400, 3000, 1730, 1700, 1610 cm^{-1}; ^1H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.45–7.28 (m, 4 H), 7.06 (d, 2 H, J = 8.8 Hz), 6.90 (d, 2 H, J = 8.8 Hz), on which is superimposed (d, 1 H, J = 6.5 Hz) that exchanged with D_2O, 6.66 (d, 1 H, J = 8.5 Hz), 5.04 (s, 2 H), 5.00 (m, 1 H), 4.14 (q, 2 H, J = 7.1 Hz), 3.02 (m, 2 H), 1.42 (s, 9 H), 1.23 (t, 3 H, J = 7.1 Hz); MS (FAB, m/e + 1) 399, 344 (-C_4H_9), 300 (-$CO_2C_4H_9$).

O'-Benzyl-L-tyrosine Ethyl Ester Hydrochloride (15).[32] A solution of N-(*tert*-butoxycarbonyl)-O-benzyl-L-tyrosine ethyl ester (6.35 g, 15 mmol) dissolved in dioxane (100 mL, previously saturated with HCl at 0–5 °C) was reacted at room temperature for 2 h, then the solution volume was reduced under vacuum to about 10 mL. The resulting yellowish oil was triturated with ether (300 mL) to yield a white precipitate. After 1 h the ether was decanted; the white precipitate was resuspended in ether (300 mL) and was allowed to precipitate again. After 1 h the ether was decanted and the white precipitate was concentrated under vacuum to yield the desired compound as a white solid (4.02 g, 12 mmol, 80%): mp 189–190 °C (lit.[32] mp 191 °C).

N-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-O'-benzyl-L-tyrosine Ethyl Ester (18). A 100-mL flask while flushed with N_2 was charged with O-benzyl-L-tyrosine ethyl ester hydrochloride (4.0 g, 12 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride[33] (2.3 g, 12 mmol), and 1-hydroxybenzotriazole[34] (1.83 g, 12 mmol). Next, N-(*tert*-butoxycarbonyl)alanine (3.36 g, 18 mmol) dissolved in dimethylformamide (10 mL) was added, followed by N-methylmorpholine (2.4 g, 24 mmol) under stirring. The mixture was stirred for 20 h at room temperature, then the solvent was removed under high vacuum, yielding a yellow oil. The oil was partitioned between H_2O and ethyl acetate (100 mL of each). The aqueous layer was next extracted with ethyl acetate (2 × 75 mL); the combined organic layers were dried ($MgSO_4$) and then concentrated on a rotary evaporator, yielding N-(*tert*-butoxycarbonyl)-L-alanyl-O'-benzyl-L-tyrosine ethyl ester (16). Without further purification this intermediate was redissolved in dioxane (100 mL, previously saturated with HCl gas at 0–5 °C; ref 35). The solution was allowed to react for 2 h at room temperature, the solution volume was reduced to about 10 mL, and the resulting yellowish oil was triturated with ether (300 mL), yielding a white precipitate. After 1 h the ether was decanted, the white precipitate was resuspended in ether (300 mL) and allowed to precipitate again and to settle for 1 h. The ether solution was decanted and concentrated under high vacuum, yielding the desired product L-alanyl-O'-benzyl-L-tyrosine ethyl ester hydrochloride salt (17, 3.2 g, 8.5 mmol, 71.5%) as a white solid. Without further purification, this intermediate was charged into a flask under a stream of N_2, along with 1-(3-(dimethyl-

amino)propyl)-3-ethylcarbodiimide hydrochloride (1.62 g, 8.5 mmol) and 1-hydroxybenzotriazole (1.3 g, 8.5 mmol). Next N-(*tert*-butoxycarbonyl)-L-alanine (2.37 g, 12.7 mmol) dissolved in dimethylformamide (10 mL) was added, followed by N-methylmorpholine (1.7 g, 17 mmol) with stirring. The solution was stirred for 20 h at room temperature then concentrated under high vacuum to yield a yellow oil. The oil was partitioned between H_2O and ethyl acetate (100 mL of each) and the aqueous layer was extracted with ethyl acetate (2 × 75 mL). The combined ethyl acetate layer was dried ($MgSO_4$) and concentrated, giving a yellow oil. The oil was chromatographed on silica gel (2 × Prep 500-A HPLC cartridges on a Waters Instrument) eluting with $CHCl_3/CH_3OH$ (97/3) (R_f = 0.2) and yielded the title compound as white, crystalline needles (3.0 g, 5.5 mmol, 65.3%); IR (Nujol) 3310, 3000, 1735, 1720, 1700, 1650, 1600, 1250, 1170, 750 cm^{-1}; ^1H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.45–7.20 (m, 5 H), 7.02 (d, 2 H, J = 8.7 Hz), 6.89 (d, 2 H, J = 8.7 Hz), 6.67 (d, 1 H, J = 7.4 Hz), 6.47 (d, 1 H, J = 7.3 Hz), 5.03 (s, 2 H), 4.96 (d, 1 H, J = 6.9 Hz), 4.74 (m, 1 H), 4.42 (m, 1 H), 4.16 (m, 1 H) superimposed on 4.16 (q, 2 H, J = 7.2 Hz), 3.05 (dd, 2 H, J = 1.0, 5.9 Hz), 1.44 (s, 9 H), 1.41–1.20 (m, 9 H); MS (FAB, m/e + 1) 542, 486 (-CH_2C-HCH_2O), 442 (-$CO_2C_4H_9$), 371 (-Ala $CO_2C_4H_9$), 300 (-Ala-Ala $CO_2C_4H_9$); $[\alpha]^{26}_D = -32.8°$ (CH_3OH). Anal. ($C_{29}H_{39}N_3O_7$) C, H, N.

N-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-L-tyrosine Ethyl Ester (19). To a Parr bomb containing 1.0 g of Pd/C catalyst in 70 mL of absolute ethanol was added N-(*tert*-butoxycarbonyl)-L-alanyl-L-alanyl-O-benzyl-L-tyrosine ethyl ester (3.0 g, 5.5 mmol) dissolved in absolute ethanol (70 mL) and 1–2 drops of 1 N HCl.[31,36] The hydrogenation was run at 20 atm pressure for 30 min. Next the solution was filtered by gravity and the filtrate was concentrated under high vacuum, yielding the desired product as a white solid (1.9 g, 4.2 mmol, 76.6%): mp 180 °C; $[\alpha]^{26}_D = -40.1$ (CH_3OH); IR (Nujol) 3400, 3200, 3000, 1740, 1720, 1700, 1660, 1610 cm^{-1}; ^1H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.35 (d, 1 H, J = 8.0 Hz), 7.1 (d, 1 H, J = 8.0 Hz), 6.94 (d, 2 H, J = 8.5 Hz), 6.74 (m, 1 H) superimposed on 6.74 (d, 2 H, J = 8.4 Hz), 4.76 (m, 1 H), 4.45 (m, 1 H), 4.18 (m, 1 H) superimposed on 4.18 (q, 2 H, J = 7.1 Hz), 3.05 (m, 2 H), 1.44 (s, 9 H), 1.41–1.21 (m, 9 H); MS (FAB, m/e + 1) 452, 352 (-$CO_2C_4H_9$), 281 (-Ala $CO_2C_4H_9$), 210 (-Ala-Ala $CO_2C_4H_9$). Anal. ($C_{22}H_{33}N_3O_7 \cdot H_2O \cdot {}^1/_2CH_3CH_2OH$) C, H, N.

N-(*tert*-Butoxycarbonyl)-L-alanyl-L-alanyl-O'-(2,3-epoxypropyl)-L-tyrosine Ethyl Ester (20). To a mixture of dimethylformamide (25 mL) and epibromohydrin[9] (5 mL) was added N-(*tert*-butoxycarbonyl)-L-alanyl-L-alanyl-L-tyrosine ethyl ester (0.95 g, 2.1 mmol) at –70 °C followed by K_2CO_3 (0.3 g, 2.1 mmol). With stirring the solution was allowed to warm to room temperature and to react further for a total of 16 h. The solution was concentrated under high vacuum and the residue was redissolved in ethyl acetate (50 mL). The ethyl acetate solution was extracted with water (2 × 25 mL) and then with brine (25 mL). The organic layer was dried ($MgSO_4$) and the solvent was removed under vacuum, yielding a mixture that on chromatography gave the desired compound (20 mg, 1.9%): mp 168 °C; IR (evaporated film from $CHCl_3$) 3320, 3000, 1735, 1715, 1690, 1650, 1600, 1500, 1250, 1160, 1030, 850 cm^{-1}; ^1H NMR ($CDCl_3$/TMS, 200 MHz) δ 7.35 (m, 1 H), 7.02 (d, 2 H, J = 8.6 Hz), 6.84 (d, 2 H, J = 8.6 Hz), 6.62 (d, 1 H, J = 8.0 Hz), 6.59 (d, 1 H, J = 8.0 Hz), 4.91 (m, 1 H), 4.76 (m, 1 H), 4.4 (m, 1 H), 4.19 (m, 1 H) superimposed on 4.18 (q, 2 H, J = 7.0 Hz), 4.00 (m, 1 H), 3.34 (m, 1 H), 3.05 (m, 2 H), 2.90 (m, 1 H), 2.75 (m, 1 H), 1.45 (s, 9 H), 1.416–1.214 (m, 9 H); MS (FAB m/e + 1) 508, 452 (-CH_2O-$CHCH_2$), 408 (-$CO_2C_4H_9$), 337 (-Ala $CO_2C_4H_9$) 266 (-Ala-Ala $CO_2C_4H_9$). Anal. ($C_{25}H_{37}N_3O_8 \cdot {}^1/_3$hexane) C, H, N.

N-(*tert*-Butoxycarbonyl)-O'-(prop-2-enyl)-L-tyrosine Ethyl Ester (21).[31,37] To a solution of N-(*tert*-butoxycarbonyl)-L-tyrosine ethyl ester (13, 8 g, 25 mmol) dissolved in acetone (15 mL) was added K_2CO_3 (3.8 g, 27 mmol) followed by allyl bromide (2.1 mL, 25 mmol). The mixture was stirred at room temperature for 24 h. The solution was concentrated in vacuo,

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yielding a viscous brownish oil. The oil was redissolved in 5% NaOH/CHCl₃ (50 mL/150 mL). The organic layer was dried (MgSO₄); the solvent was removed on a rotary evaporator, yielding the product (8.0 g, 23 mmol, 92%) as a colorless oil: R_f (CHCl₃, silica) = 0.35; R_f (CHCl₃/ethanol, 97/3) = 0.90.

N-(tert-Butoxycarbonyl)-O'-(prop-2-enyl)-L-tyrosine (22). A solution of N-(tert-butoxycarbonyl)-O-(prop-2-enyl)-L-tyrosine ethyl ester (8.0 g, 23 mmol) dissolved in ethanol (20 mL) was cooled in an ice bath (0–5 °C) for 10 min. Next NaOH (40 mL, 46 mmol) was added. The solution was allowed to warm to room temperature and was left to stir overnight. The solution volume was reduced in vacuo to 35 mL and washed with CHCl₃ (50 mL). The aqueous layer was acidified with NaHSO₄, pH 2–3, and extracted with CHCl₃ (3 × 50 mL). The combined organic layers were dried (MgSO₄), and the solvent was removed on a rotary evaporator to yield the product (7.4 g, 23 mmol, 99%).

N-(tert-Butoxycarbonyl)-O'-(prop-2-enyl)-L-tyrosylvaline Methyl Ester (23). A 200-mL flask while flushed with Ar was charged with L-valine methyl ester (4.5 g, 34 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (5.0 g, 28 mmol), and 1-hydroxybenzotriazole (3.5 g, 23 mmol). Next N-(tert-butoxycarbonyl)-O'-(prop-2-enyl)-L-tyrosine (7.4 g, 23 mmol) dissolved in dimethylformamide (20 mL) was added, followed by triethylamine (4.6 mL, 46 mmol). The mixture was stirred for 20 h at room temperature, then the solvent was removed under high vacuum, yielding a yellow oil. The oil was partitioned between H₂O and ethyl acetate (100 mL of each). The aqueous layer was next extracted with ethyl acetate (2 × 100 mL), the combined organic layers were dried (MgSO₄) and concentrated on a rotary evaporator, yielding 10 g of an oily mixture. The oil was chromatographed on silica gel, eluting with CHCl₃/CH₃CH₂OH (98/2) (R_f = 0.45), and yielded the desired compound as white, crystalline needles (7.6 g, 17 mmol, 73.9%): mp 79–81 °C; IR (Nujol) 3320, 3300, 2990, 1740, 1685, 1660, 1610, 1580, 1540, 1170 cm⁻¹; ¹H NMR (CDCl₃/TMS, 200 MHz) δ 7.11 (m, 1 H) superimposed on 7.11 (d, 2 H, J = 8.5 Hz), 6.80 (d, 2 H, J = 8.5 Hz), 5.97 (m, 1 H), 5.55 (m, 1 H), 5.37 (dd, 1 H, J = 1.5, 17.2 Hz), 5.23 (dd, 1 H, J = 1.4, 10.4 Hz), 4.47 (m, 4 H), 3.68 (s, 3 H), 3.01 (m, 2 H), 2.04 (m, 1 H), 1.39 (s, 9 H), 0.88 (m, 6 H); MS (FAB, m/e + 1) 435, 379 (–C₄H₉), 335 (–CO₂C₄H₉). Anal. (C₂₃H₃₄N₂O₆) C, H, N.

N-(tert-Butoxycarbonyl)-L-valine (24).[38] To a solution of L-valine (14.85 g, 127 mmol) dissolved in tetrahydrofuran/methanol (200 mL/60 mL) was added triethylamine (27.0 mL, 244 mmol) and the reaction mixture was stirred for 5 min. Next di-tert-butyl dicarbonate (27.7 g, 127 mmol) dissolved in tetrahydrofuran (50 mL) was added and the mixture was allowed to react at room temperature for 20 h. The solution was concentrated in vacuo, then partitioned between NaOH and CHCl₃ (25 mL/50 mL). The water layer was acidified with KHSO₄ to pH 2–3 and extracted with CHCl₃ (4 × 40 mL). The combined organic layer was dried (MgSO₄) and the solvent was removed on a rotary evaporator to yield the product (23.3 g, 107 mmol, 85%): mp 76–78 °C (lit.[38] mp 78 °C).

O'-(Prop-2-enyl)-L-tyrosyl-L-valine Methyl Ester Hydrochloride (25). A solution of 23 (7.3 g, 16.8 mmol) dissolved in dioxane (200 mL, previously saturated with HCl at 0–5 °C) was reacted at room temperature for 2 h. Next, the solution was reduced under vacuum to about 15 mL. The resulting yellowish oil was triturated with ether (300 mL) to yield a white precipitate. After 1 h the ether was decanted; the white precipitate was resuspended in ether (300 mL) and was allowed to precipitate again. After 1 h the ether was decanted and the white precipitate was concentrated under vacuum to yield the desired compound as a white solid (5.1 g, 15 mmol, 91%): ¹H NMR (CDCl₃/TMS, 200 MHz) δ 8.40 (m, 1 H), 7.20 (d, 2 H, J = 8.5 Hz), 6.85 (d, 2 H, J = 8.5 Hz), 5.96 (m, 1 H), 5.38 (dd, 1 H, J = 1.2, 17.3 Hz), 5.25 (dd, 1 H, J = 1.0, 9.9 Hz), 4.66 (m, 1 H), 4.52 (m, 2 H), 4.18 (m, 1 H), 3.61 (s, 3 H), 3.00 (m, 2 H), 2.08 (m, 1 H), 0.96 (m, 6 H).

N-(tert-Butoxycarbonyl)-L-valyl-O'-(prop-2-enyl)-L-tyrosyl-L-valine Methyl Ester (26). A 200-mL flask while flushed with Ar was charged with 25 (5.10 g, 15 mmol), 1-[3-(dimethyl-

amino)propyl]-3-ethylcarbodiimide hydrochloride (2.86 g, 15 mmol), and 1-hydroxybenzotriazole (2.3 g, 15 mmol). Next, N-(tert-butoxycarbonyl)valine (4.88 g, 22 mmol) dissolved in dimethylformamide (15 mL) was added, followed by triethylamine (3.2 mL, 30 mmol). The mixture was stirred for 24 h at room temperature, then the solvent was removed under high vacuum, yielding a yellow oil. The oil was partitioned between H₂O and ethyl acetate (100 mL of each). The aqueous layer was next extracted with ethyl acetate (2 × 100 mL); the combined organic layers were dried (MgSO₄) and concentrated on a rotary evaporator, yielding a yellow oil. The oil was chromatographed on silica gel (500 mL) an eluted first with methylene chloride (1000 mL) and then with a mixture of CH₂Cl₂/CH₃CH₂OH (97/3, 2000 mL) to give a white solid (5.80 g, 10.8 mmol, 72%): mp 152–154 °C; IR (Nujol) 3320, 3300, 3075, 2990, 1745, 1690, 1650, 1620, 1585, 1540, 1250, 1170, 1020, 925, 830, 800 cm⁻¹; ¹H NMR (CDCl₃/TMS, 200 MHz) δ 7.13 (d, 2 H, J = 8.6 Hz), 6.83 (d, 2 H, J = 8.6 Hz), 6.54 (d, 1 H, J = 7.2 Hz), 6.24 (d, 1 H, J = 8.0 Hz), 6.05 (m, 1 H), 5.39 (dd, 1 H, J = 1.5, 17.3 Hz), 5.27 (dd, 1 H, J = 1.4, 10.4 Hz), 4.89 (d, 1 H, J = 8.2 Hz), 4.61 (m, 1 H), 4.5 (m, 2 H), 4.39 (m, 1 H), 3.91 (m, 1 H), 3.69 (s, 3 H), 2.97 (m, 2 H), 2.10 (m, 2 H), 1.43 (s, 9 H), 0.86 (m, 12 H); MS (FAB, m/e + 1) 534, 478 (–C₄H₉), 434 (–CO₂C₄H₉), 335 (–Val CO₂C₄H₉). Anal. (C₂₈H₄₃N₃O₇·¹/₂H₂O) C, H, N.

L-Valyl-O'-(prop-2-enyl)-L-tyrosyl-L-valine Methyl Ester Hydrochloride (27). A solution of 26 (5.0 g, 9.3 mmol) dissolved in dioxane (125 mL, previously saturated with HCl at 0–5 °C) was reacted at room temperature for 2 h. Next the solution volume was reduced under vacuum to about 10 mL. The resulting yellowish oil was triturated with ether (300 mL) to yield a white precipitate. After 1 h the ether was decanted; the white precipitate was resuspended in ether (300 mL) and was allowed to precipitate again. After 1 h the ether solution was decanted and the white precipitate was concentrated under vacuum to yield the desired compound as a white solid (4.0 g, 9.2 mmol, 96%): mp 237–239 °C; IR (Nujol) 3300, 3280, 3100, 2990, 1740, 1730, 1650, 1560, 1250, 840, 800, 730 cm⁻¹. Anal. (C₂₃H₃₄N₃O₅·HCl·¹/₂H₂O) C, H, N.

N-(Trifluoroacetyl)-L-valyl-O'-(prop-2-enyl)-L-tyrosyl-L-valine Methyl Ester (28). To a solution of 27 (2.0 g, 4.2 mmol) dissolved in CH₂Cl₂ (25 mL) at –60 °C was added trifluoroacetic anhydride (20 mL) and triethylamine (0.8 mL, 7.5 mmol).[39,40] Next, the solution was stirred for 30 min at –60 °C, then it was allowed to react at 10 °C for 1 h. Next, the solution volume was reduced under vacuum to about 10 mL. The resulting yellowish oil was redissolved in CH₂Cl₂ (50 mL) and concentrated to an amber oil. The oil was chromatographed on silica gel (500 mL) eluting first with a hexane/ethyl acetate mixture (1000 mL, 85/15) and then with 2000 mL (60/40) to give the desired product as an oil (0.3 g, 0.56 mmol, 13.5%); IR (neat film) 3320, 3000, 1745, 1735, 1680, 1640, 1580, 1270, 1210, 1180, 1150, 950, 780, 740 cm⁻¹; ¹H NMR (CDCl₃/TMS, 200 MHz) δ 7.26 (m, 1 H), 7.10 (m, 1 H), 6.98 (d, 2 H, J = 8.5 Hz), 6.72 (d, 2 H, J = 8.5 Hz), 6.0 (m, 1 H), 5.39–5.27 (m, 2 H), 4.43 (m, 4 H), 3.70 (s, 3 H), 3.0 (m, 2 H), 2.10 (m, 2 H), 0.924 (m, 12 H). Anal. (C₂₅F₃H₃₃N₃O₆) C, H, F, N.

N-(Trifluoroacetyl)-L-valyl-O'-(2,3-epoxypropyl)-L-tyrosyl-L-valine Methyl Ester (29). To a solution of 28 (0.285 g, 0.55 mmol) dissolved in CH₂Cl₂ (50 mL) at 0–5 °C was added m-chloroperbenzoic acid (0.196 g, 1.12 mmol) during a 10-min period.[41] Next, the mixture was allowed to react at room temperature for 24 h, then m-chloroperbenzoic acid (0.196 g, 1.12 mmol) was added at 0–5 °C during a 10-min period and the mixture was allowed to react at room temperature for a further 24 h. The solution was washed with NaHCO₃ (5%, 25 mL) and dried (MgSO₄). The organic layer was concentrated under high vacuum to give 0.40 g of a mixture that was chromatographed on alumina (Brockman Activity IV) eluting first with hexane/ethyl acetate (750 mL, 80/20) and then with hexane/ethyl acetate (1500 mL, 50/50) to yield the desired product (45 mg, 0.082 mmol, 14%) as a tan solid: mp 171 °C; ¹H NMR (CDCl₃/TMS, 200 MHz) δ 7.41 (m, 1 H), 7.08 (m, 2 H), 6.80 (m, 3 H), 6.59 (m, 1 H), 4.80 (m, 1 H), 4.43 (m, 2 H), 4.01 (m, 2 H), 3.69 (s, 3 H), 3.32 (m, 1

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H), 2.87 (m, 4 H), 2.04 (m, 2 H), 0.92 (m, 12 H); MS (FAB, *m/e* + 1) 546, 450 (–CF₃CO), 349 (–CF₃CO Val), 333 (–CF₃CO Val, Me). Anal. ($C_{25}H_{33}F_3N_3O_7$) C, H, F, N.

Structure–Activity Relationships of the Lissoclinamides: Cytotoxic Cyclic Peptides from the Ascidian *Lissoclinum patella*

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Two new lissoclinamides (lissoclinamides 7 and 8) have been isolated from the aplousobranch ascidian *Lissoclinum patella*. These lissoclinamides are cyclic heptapeptides with the same structural features as lissoclinamides 4 and 5 reported earlier, containing an oxazoline ring, one proline, one valine, two phenylalanine residues, and thiazole and/or thiazoline rings. All four peptides have the same sequence of amino acids around the ring and differ from one another only in their stereochemistry or the number of thiazole and thiazoline rings. The cytotoxicities of the compounds were tested with human fibroblast and bladder carcinoma cell lines and normal lymphocytes. Slight changes in structure resulted in marked differences in the cytotoxicities of these compounds. The most potent is lissoclinamide 7, containing two thiazoline rings, which rivals didemnin B in cytotoxicity in vitro.

Cytotoxic cyclic peptides from marine organisms are showing great promise as potential antineoplastic agents. Didemnin B from the ascidian *Trididemnum solidum* is now in phase II clinical trials.[1,2] In a recent paper we described the isolation and characterization of three new cyclic peptides from *L. patella*: patellamide D, lissoclinamide 4, and lissoclinamide 5.[3] We now report the structures of two new lissoclinamides from the same species, lissoclinamides 7 and 8. The four lissoclinamides are made up of the same amino acids, in the same sequence, yet display dramatic differences in their cytotoxicity in vitro. In the present study structural-functional correlations are investigated.
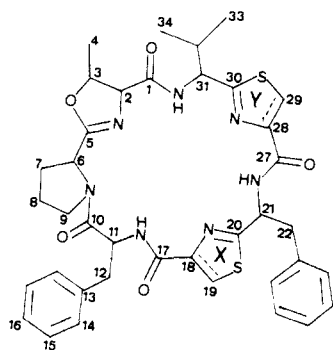
	X	Y
lissoclinamide 4	thiazole	thiazoline
lissoclinamide 5	thiazole	thiazole
lissoclinamide 7	thiazoline	thiazoline
lissoclinamide 8	thiazole	thiazoline

Results and Discussion

Isolation and Structure Determination. *L. patella* was extracted with methanol/toluene and the extract was chromatographed on a preparative reverse-phase column as previously described.[3] The elution profile is shown in Figure 1. The peaks corresponding to lissoclinamides 7 and 8 have been marked L7 and L8. The fractions corresponding to these compounds were pooled and rechro-matographed on the reverse-phase column or further purified by Sephadex LH-20 chromatography with methanol/water (80:20). The structures of the pure compounds were determined by a combination of high-resolution electron-impact mass spectrometry (HREIMS), acid hydrolysis followed by chiral gas chromatography, and two-dimensional NMR techniques.

HREIMS of lissoclinamide 7 ($C_{38}H_{45}N_7O_5S_2$) gave a molecular ion peak of m/z 743.2935 (calcd m/z 743.2927). Chiral gas chromatography of the *N*-pentafluoropropionyl isopropyl esters yielded L-threonine, L-proline, DL-phenylalanine, DL-valine, and L-cysteine (double the peak observed in lissoclinamides 4 and 8). The NMR data for this compound are presented in Table I. The assignments were determined by ¹H–¹H COSY 45 and ¹H–¹³C COSY experiments. The sequence was established with COLOC and COSY 45 correlations namely C-1 to NH-1 and H-31 to NH-1. This places the valine residue at position 31; thus the sequence is the same as in lissoclinamide 4.[3]

As described in our previous paper,[3] we are unable to determine the stereochemistry of amino acids adjacent to thiazole rings, and hydrolysis of amino acids adjacent to thiazoline rings leads to racemization.

Due to racemization of the amino acids adjacent to the thiazoline rings, we can make only a tentative statement about the stereochemistry at these positions. The large proportion of D-phenylalanine obtained (D/L = 0.64) would suggest this configuration at position 21. (The phenylalanine at C-11 is always of the L configuration, so if the phenylalanine at C-21 is completely racemized, one would expect a D/L ratio of 0.25.)

D- and L-valine were obtained in equal proportions, so it is not possible to comment on the stereochemistry at position 31 for this compound.

HREIMS of lissoclinamide 8 ($C_{38}H_{43}N_7O_5S_2$) gave a molecular ion peak of m/z 741.2785 (calcd m/z 741.2767). Chiral gas chromatography of the *N*-pentafluoropropionyl

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