Epoxysuccinyl Dipeptides as Selective Inhibitors of Cathepsin B†

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Epoxysuccinyl dipeptide analogs of E-64 (R-EpsLeuPro-R′) (Figure 1) have been synthesized with the carboxylate group on the epoxide ring either free (R = OH) or converted to an ester or an amide (R = EtO or i-BuNH) and with the C-terminal amino acid proline either blocked (R′ = OBzl) or free (R′ = OH). These compounds were used to investigate the recently reported selectivity of this type of inhibitor for the lysosomal cysteine protease cathespin B.[1] It was shown that derivatization of the carboxylate on the epoxide ring confers selectivity for cathepsin B over papain only when it is combined to a dipeptidyl moiety with a free negatively charged C-terminal residue. It is proposed that this selectivity reflects interactions with histidine residues on a loop located in the primed subsites of cathepsin B which provides a positively charged anchor for the C-terminal carboxylate group of the inhibitor. The primed subsite loop of cathepsin B is not found in other cysteine proteases of the papain family and offers a unique template for designing selectivity in cysteine protease inhibitors.

Introduction

A variety of cysteine proteases have been shown to be present in mammalian tissues.[2,3] The most notable of these proteases are the lysosomal cathepsins (cathepsins B, H, S, and L) and the cytoplasmic Ca^{2+}-dependent enzymes, the calpains. These enzymes are members of the papain family of cysteine proteases, a group of enzymes from various sources that possess closely related amino acid sequences and overall folding structures[4] and utilize an identical catalytic group, the thiolate–imidazolium ion pair for their activity. Under normal conditions the mammalian cysteine proteases function in a variety of biological processes including cell differentiation,[5] platelet aggregation,[6] cell invasiveness,[7] postribosomal processing of proteins,[8] and protein turnover.[2,9] However, under conditions where they become deregulated, cysteine proteases have also been implicated in a variety of disease states, particularly several diseases that involve aberrant protein turnover such as muscular dystrophy,[10] bone resorption,[11] myocardial infarction,[12] malignancy,[13] and pulmonary emphysema.[14] These enzymes are, therefore, excellent targets for the development of specific inhibitors as possible therapeutic agents.

A number of inhibitors can inactivate cysteine proteases without significantly inhibiting other classes of proteases. Peptidyl diazomethanes,[15] fluoromethyl ketones,[16] and epoxysuccinyl derivatives[17] are good examples of such compounds. These reagents allow one to differentiate, to some degree, between the cysteine proteases. Such selectivity must be enhanced, however, if these inhibitors are to be put to general use in characterizing the various cysteine proteases or as drug prototypes.

Recently, analogs of E-64 have been reported that show a strong selectivity toward cathepsin B over cathepsin H and L.[1] E-64 is an epoxysuccinyl peptide ([1-[N-[[(L-3-*trans*-carboxyoxiran-2-yl)carbonyl]-L-leucyl]amino]-4-guanidinobutane) isolated from a culture of *Aspergillus japonicus* that irreversibly inhibits cysteine proteases by alkylating the active site cysteine residue.[17] The analogs reported to show selectivity for cathepsin B are derivatives of *N*-[(L-3-*trans*-carboxyoxiran-2-yl)carbonyl]-L-isoleucyl-L-proline (R-EpsIlePro-OH) with the terminal carboxylic acid of the epoxide either esterified (R = EtO, i-PrO, i-BuO, c-HexO) or converted to an amide functionality (R = EtNH, iPrNH, n-PrNH, i-BuNH, *n*-BuNH, i-AmNH, *n*-AmNH, *n*-HexNH, $PhCH_2NH$, c-HexNH). The mode of action of these compounds remains obscure due to the lack of quantitative kinetic analysis and structural characterization. In particular, the authors have assumed that the E-64 analogs designed must bind in the S subsites of cysteine proteases,[18] by analogy to the binding mode of E-64 to papain reported from the crystal structure of a papain–E-64 complex.[19] This assumption causes problems when one looks at the inhibitors where the epoxide ring carboxylate is derivatized with large groups such as $PhCH_2$-NH_2. The carboxylate group in the papain–E-64 complex is involved in a number of interactions in the oxyanion hole of the enzyme. It would be very difficult for cathepsin B to accept large substitutions at this position of the inhibitor, unless the oxyanion hole region of the enzyme would be very different from that of papain. This does not seem to be the case if one compares the immediate vicinity of the oxyanion hole in the crystal structures of both papain and cathepsin B.[20]

In order to identify the structural features of these E-64 analogs responsible for their cathepsin B selectivity, a series of (epoxysuccinyl)leucylproline derivatives (R-EpsLeuPro-R′) were synthesized (Figure 1). The R and R′ groups have been systematically varied to obtain esterified and amidated derivatives of the carboxylate residue with the C-terminal proline either free (i.e., bearing a negatively charged carboxylate) or blocked by a benzyl ester function. To characterize these inhibitors, the second-order rate constant was measured for inactivation of recombinant rat cathespin B and papain, the latter enzyme being used as a representative of the cysteine proteases that show less susceptibility to inactivation by compounds of the type R-Eps-dipeptide-OH. In addition, the nature of the enzyme adducts formed was analyzed by mass spectrometry to verify that these compounds alkylate cysteine proteases in the same manner. A model is proposed for binding of the inhibitors at the active site of cathespin B

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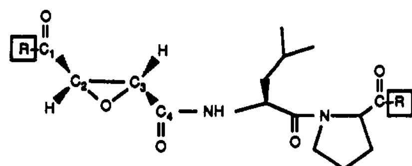

R	R'	
OH	OBzl	(HO-EpsLeuPro-OBzl)
OH	OH	(HO-EpsLeuPro-OH)
OEt	OBzl	(EtO-EpsLeuPro-OBzl)
OEt	OH	(EtO-EpsLeuPro-OH)
i-BuNH	OBzl	(i-BuNH-EpsLeuPro-OBzl)
i-BuNH	OH	(i-BuNH-EpsLeuPro-OH)

Figure 1.

Table I. Molecular Mass (Da) of Enzyme–Inhibitor Adducts by Ion-Spray Mass Spectrometry

inhibitors	molecular mass of inhibitor	molecular mass of E–I adduct expected	molecular mass of E–I adduct observed
HO-EpsLeuPro-OBzl	432	23854	23855
HO-EpsLeuPro-OH	342	23764	nd[a]
EtO-EpsLeuPro-OBzl	460	23882	23883
EtO-EpsLeuPro-OH	370	23792	23792
i-BuNH-EpsLeuPro-OBzl	488	23910	23911
i-BuNH-EpsLeuPro-OH	398	23820	23820

[a] Not determined.

that can account for the preferential potency of these inhibitors against cathepsin B over papain.

Results and Discussion

N-[[(2*S*,3*S*)-3-*trans*-(Ethoxycarbonyl)oxiran-2-yl]carbonyl]-L-leucyl-L-prolinebenzyl ester (EtO-EpsLeuPro-OBzl) was synthesized by coupling leucylprolinebenzyl ester with the monoethyl ester of the epoxysuccinate as synthesized by the method of Mori.[21] The ethyl ester was then cleaved using pig liver esterase[22] to give the acid derivative (HO-EpsLeuPro-OBzl) and also the diacid (HO-EpsLeuPro-OH). Removal of the benzyl ester in the presence of the ethyl ester was accomplished to catalytic hydrogenation as described by Anwer and Spatola.[23] The isobutyl amide derivative (i-BuNH-EpsLeuPro-OBzl) was synthesized by coupling isobutylamine to the corresponding acid (HO-EpsLeuPro-OBzl). The benzyl ester was then removed by catalytic hydrogenation to give the corresponding proline acid (i-BuNH-EpsLeuPro-OH).

The synthesized inhibitors have been fully characterized by NMR, IR, and FAB mass spectrometry (see Experimental Section). However, to ensure that it was not a modified (i.e., hydrolyzed) form of the inhibitor that reacted with the enzyme, enzyme–inhibitor adducts were also analyzed by ion spray mass spectrometry (Table I). The increase in molecular mass observed for the inactivated enzyme reflects the covalent addition of the inhibitor to the enzyme. The molecular masses of the adducts correspond to those expected for reaction of the intact inhibitors, which indicates that no side reaction from contaminating compounds or from a modified form of the inhibitors contribute to enzyme inactivation. Small amounts of HO-EpsLeuPro-OH and HO-EpsLeuPro-OBzl were detected in solutions of EtO-EpsLeuPro-OH and

Table II. Second-Order Rate Constants for Inactivation of Papain and Cathepsin B by R-EpsLeuPro-R′

inhibitors	(k_{inac}/K_i (10^3 M^{-1} s^{-1})) papain	cathepsin B	ratio cat. B/papain
HO-EpsLeuPro-OBzl	176 ± 2	8.7 ± 0.4	0.05
HO-EpsLeuPro-OH	30.0 ± 0.8	2.2 ± 0.1	0.07
EtO-EpsLeuPro-OBzl	0.11 ± 0.01	0.030 ± 0.001	0.3
EtO-EpsLeuPro-OH	0.76 ± 0.02	13.8 ± 0.9	18
i-BuNH-EpsLeuPro-OBzl	2.07 ± 0.05	0.206 ± 0.003	0.1
i-BuNH-EpsLeuPro-OH	0.558 ± 0.004	52 ± 6	93

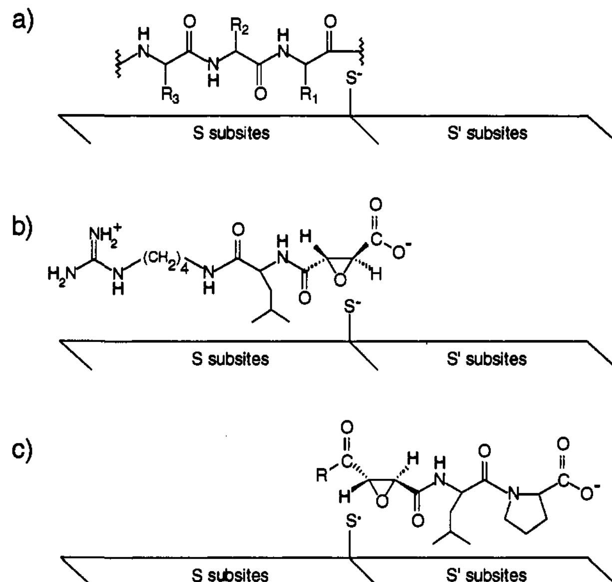

Figure 2. Binding modes for substrates and E-64-like inhibitors to cysteine proteases. (a) Binding of a substrate in the S subsites. (b) Binding of E-64 in the S subsites. It must be noted that in this case, the direction of the peptide bond is opposite of that found for the substrate. (c) Proposed binding mode of E-64 analogs showing selectivity for cathepsin B in the S′ subsites of the enzyme. In this mode, the direction of the peptide bond is the same as with a substrate.

EtO-EpsLeuPro-OBzl, respectively, but did not affect significantly the rates of enzyme inactivation.

The second-order rate constants (k_{inac}/K_i) for the irreversible inhibition of rat cathepsin B and papain by the epoxysuccinyl dipeptide analogs are presented in Table II. The best inhibitor in terms of cathepsin B selectivity is i-BuNH-EpsLeuPro-OH with a k_{inac}/K_i value of 52 × 10^3 M^{-1} s^{-1} for cathepsin B corresponding to a 93-fold preference over papain (k_{inac}/K_i = 0.558 × 10^3 M^{-1} s^{-1}). The ethyl ester derivative EtO-EpsLeuPro-OH also displays cathepsin B selectivity but at a lower level. In both cases this selectivity disappears if the C-terminal carboxylate of the proline residue is blocked by forming the benzyl ester. Both i-BuNH-EpsLeuPro-OBzl and EtO-EpsLeuPro-OBzl are better inhibitors of papain than cathepsin B, and this is mainly due to a decrease in k_{inac}/K_i values for cathepsin B, which are much lower than the values obtained when the C-terminal proline is not blocked. The inactivation rates for papain with HO-EpsLeuPro-OH and HO-EpsLeuPro-OBzl are relatively high (30.0 × 10^3 and 176 × 10^3 M^{-1} s^{-1}, respectively), and these compounds are much better inhibitors of papain than cathepsin B.

Unquestionably, the single most helpful piece of information needed to interpret these results would be the three-dimensional structures of the enzyme–inhibitor adducts or of related complexes (Figure 2). One structure that is available and that can give insight into the possible mode

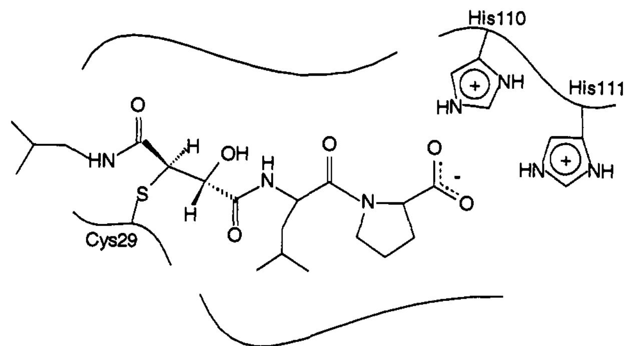

Figure 3. Proposed binding of the (epoxysuccinyl)leucylproline derivatives (R-EpsLeuPro-OH, R = OEt or i-BuNH) in the S′ subsite of cathepsin B. The complex is shown with the sulfur atom of the active site cysteine residue bound to the C_2 carbon of the epoxide as observed in the papain–E-64 structure.[19]

of binding of the E-64 analogs is that of a papain–E-64 complex.[19] In this structure the inhibitor binds in the S subsites (Figure 2b), and the carboxylic acid funtionality on the epoxide ring is hydrogen-bonded via a water molecule to Gln 19 in the oxyanion hole of papain. When this acid functionality on the epoxide ring is converted to an ester or an amide, most of the activity of the inhibitor is lost with papain, suggesting that there is not enough room in the oxyanion hole to accommodate the larger derivatives of the terminal carboxylic acid group. If one assumes a similar situation for cathepsin B, it is difficult to envision how such substituents as EtO and i-BuNH could fit into the oxyanion hole of the enzyme, and accordingly, the inhibitors EtO-EpsLeuPro-OBzl and i-BuNH-EpsLeuPro-OBzl are poor inhibitors of cathepsin B. However, our data cannot rule out the possibility that the differences in the second-order rate of inactivation between the inhibitors bearing a free carboxylate on the epoxide ring and the ester or amide derivatives could be due to nonspecific electrostatic effects rather than steric hindrance in the oxyanion hole. When the benzyl ester is removed, the compounds EtO-EpsLeuPro-OH and i-BuNH-EpsLeuPro-OH become good selective inhibitors of cathepsin B. Examination of the recently determined crystal structure of human liver cathepsin B[20] can offer a possible explanation for this observation. This structure was shown to be very similar to that of papain except for the presence of several insertion loops located on the surface of cathepsin B. In particular, one of the insertion loops occludes part of the S′ subsites of cathepsin B. Two histidine residues (His 110 and His 111) located on this loop likely confer to cathepsin B its well-known dipeptidyl carboxypeptidase activity by providing positively charged anchors for the C-terminal carboxylate group of peptidyl substrates. The existence of a similar interaction between cathepsin B and the inhibitors R-EpsLeuPro-OH can possibly explain the selectively observed for cathepsin B with these compounds. If the proline is not esterified, the terminal negatively charged carboxylate can interact with the positive charges in the S_2' subsite provided by the His 110 and His 111 residues on the occluding loop (Figure 3). This interaction would force the R-EpsLeuPro-OH inhibitors to bind in the S′ subsites of cathepsin B and therefore "relieve" any possible strain in the oxyanion hole from the substituted epoxide ring leading to the relatively high values of k_{inac}/K_i obtained for EtO-EpsLeuPro-OH and i-BuNH-EpsLeuPro-OH with cathepsin B. It must be noted that in Figure 3, the interaction in the S′ subsites is shown with the inhibitor tethered to the enzyme. The kinetic data suggests that such an interaction is present in the transition state of the reaction and contributes to increase the rate of inactivation by stabilizing the transition state. However, in the alkylated enzyme the inhibitor could possibly be positioned differently. In addition, as pointed out by one reviewer, in the case of i-BuNH-EpsLeuPro-OH both C_2 and C_3 carbon atoms of the epoxide are α-epoxy amides, their reactivities are probably very similar. The relative reactivity of the C_2 and C_3 carbon atoms is also likely to be affected in the EtO derivatives. Therefore, the site of alkylation could conceivably be different from that where the carboxylate group on the epoxide ring is free. This might help to accommodate the i-BuNH or EtO substituents in that region of the enzyme and might also facilitate the positioning of the proline-carboxylate to interact with the histidine residues in the S_2' subsite of cathepsin B.

In conclusion, two factors seem to be important to obtain cathepsin B selectivity with dipeptidyl epoxysuccinyl analogs of E-64: the carboxylate group on the epoxide ring must be derivatized and the C-terminal carboxylate on the dipeptidyl portion of the inhibitor must be free, possibly to interact with the positively charged histidines in the S_2' subsite of cathepsin B. This work shows that in spite of the great similarities between various cysteine proteases, differences do exist that can be exploited to obtain selective inhibitors. In particular, the primed subsite of cathepsin B contains a surface loop that is not found in other cysteine proteases of the papain family and which therefore offers a unique template for designing inhibitors selective to cathepsin B. This has been done using E-64 as a lead compound, but other types of inhibitors could be modified to make use of this specific interaction.

Experimental Section

All compounds were checked for purity either by HPLC or TLC, and all new compounds were then subjected to high-resolution exact mass analysis to confirm their identities. All ^1H NMR spectra were recorded at 298 K using a Bruker AM 500 spectrometer at 500 MHz. Tetramethylsilane was used as an internal standard. All FAB and EI (70 eV) mass spectrometric measurements were conducted using a VG Analytical ZAB-EQ tandem hybrid mass spectrometer equipped with a VG 11-250J data system. Chemical ionization (CI) analyses using isobutane reagent gas were performed on a Finnigan 4500 quadrupole mass spectrometer equipped with and INCOS data system. Sample sizes in the range of 100–300 nmol were dissolved in 1–2 μL of glycerol or 2-hydroxyethyl sulfide and deposited on a gold probe tip prior to their FAB analyses. An 8-keV Xe^0 beam (Ion Tech FAB gun 1-mA discharge current) was used. Conventional FAB mass spectra were obtained at a resolving power (10% valley definition) of 1000, scanning the range m/z 50–1000 Da with a cycle time of approximately 10 s. Mass assignments were reliable to within 0.1 Da. In CI analyses approximately 0.1 mg of material was deposited in a glass capillary prior to its thermal desorption. The probe was heated from 25 to 300 °C at a rate of 20 °C/min, and the quadrupole mass analysor was scanned from 50 to 700 with a cycle time of 3 s. Accurate mass measurements of ions formed in FAB ionization were performed by peak matching using a matrix cluster (m/z 185, 277, 369, 461) as reference ion. The resolving power was typically 8000 in all mass measurements.

Kinetic Measurements. Papain (crystallized suspension in sodium acetate) was obtained from Sigma Chemical Co. and purified by the method of Sluyterman and Wijdenes.[24] It was activated from its mercurated form on the day of the experiment with β-mercaptoethanol and the concentration of active papain determined by DTNB (5,5′-dithiobis(2-nitrobenzoic acid) titration.[25] Recombinant rat cathepsin B was expressed in yeast, purified, and stored as described by Fox et al.[26] On the day of

the experiment it was activated for 15 min on ice, by diluting stock enzyme into buffer containing 1 mM DTT. The kinetic assay was carried out at pH 6.5 for papain and pH 6.0 for cathepsin B in 50 mM phosphate buffer, 200 mM NaCl, and 5 mM EDTA. Acetonitrile (10% v/v) was used to increase inhibitor solubility and DTT (0.5 mM) was added to cathepsin B assays. The rate of cysteine protease inactivation by the irreversible inhibitors was determined using the continuous assay described by Tian and Tsou.[27] The substrate used was CbzPheArgMCA (carbobenzoxyphenylalanylarginylaminomethylcoumarin), and the first-order rate constant for inactivation (k_{obs}) was obtained by monitoring the product released from hydrolysis of the substrate (measured by the variation in fluorescence: λ_{exc} = 380 nm, λ_{em} = 440 nm) in presence of the inhibitor as a function of time until complete inactivation of the enzyme (typically 5–30 min). This was done at various concentrations of inhibitor, and the second-order rate for inactivation, k_{inac}/K_i was obtained from eq 1. Since saturating conditions of inhibitor could not be obtained, the individual values of k_{inac} and K_i could not be determined.

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

Mass Spectral Data. Mass spectrometric analysis was performed on a triple quadrupole mass spectrometer (the API III LC/MS/MS syste, Sciex, Thornhill, Ontario, Canada) as described previously.[28] The samples typically contained 20 μM papain and approximately 40 μM of inhibitor (2-fold excess) in the presence of 20% acetonitrile. No buffer was used in these experiments to avoid interferences with the ionization process. The pH of the solution was kept between 5.5 and 6.5. Prior to injection in the mass spectrometer, acetic acid was added to the sample to a final concentration of 20%. The spectra were acquired after a few minutes incubation of the enzyme with the inhibitor (where the reaction is incomplete) or after more than 1 h where the reaction has gone to completion. The spectrum obtained after a few minutes incubation allows visualization of trace amounts of contaminating compounds that can react much faster than the analog under study. The molecular mass values were calculated from the m/z peaks in the charge distribution profiles of the multiply charged ions.[29]

(*tert*-Butoxycarbonyl)arginylproline Benzyl Ester (BocLeuPro-OBzl). *N*-Methylmorpholine (0.92 mL, 19.54 mmol) was added to a suspension of the hydrochloride salt of proline benzyl ester (4.72 g, 19.54 mmol) in DMF (10 mL). This suspension was added to a cooled solution (0 °C) of (*tert*-butoxycarbonyl)leucine (4.52 g, 19.54 mmol) in DMF (20 mL). DCC (4.03 g, 19.54 mmol) was added, and after 0.5 h of stirring of 0 °C, HOBt (2.64 g, 19.54 mmol) was added. The solution was stirred for 1 h at 0 °C and overnight at room temperature. The DCU was filtered off and the DMF removed in vacuo. The product was purified by flash chromatography (hexane/ethyl acetate) to give 4.3 g (14.1 mmol, 72% yield) of a colorless oil. $[\alpha]^{25}_D$: −80.8 (c 1.48, MeOH). NMR (CD_3OD): δ .90 (dd, J = 7.5 Hz, J = 13.9 Hz, 6 H, CH_3), 1.35 (m, 2 H, CH_2), 1.4 (s, 9 H, Boc), 1.68 (m, 1 H, CH), 1.92 (m, 1 H, CH), 1.96 (m, 3 H, CH_2CH), 2.25 (m, 1 H, CHC(O)), 3.56 (m, 1 H, CHN), 3.81 (m, 1 H, CHN), 4.33 (dd, J = 4.2 Hz, J = 10.3 Hz, 1 H, α-CH), 4.50 (dd, J = 4.65 Hz, J = 8.6 Hz, 1 H, α-CH), 5.10 (dd, J = 12.2 Hz, J = 30.0 Hz), 7.31 (m, 5 H, ArH). IR ($CHCl_3$): 3000, 1750, 1710, 1650, 1500, 1430 cm^{-1}. MS (FAB): 418.2451, calcd 418.2468.

Leucylproline Benzyl Ester (LeuPro-OBzl). *tert*-Butoxycarbonylleucylproline benzyl ester (BocLeuPro-OBzl) (1.6 g, 3.82 mmol) was dissolved in 98% formic acid (6 mL) and stirred at room temperature overnight. The formic acid was removed in vacuo to give the formic acid salt of leucylproline benzyl ester (1.20 g, 3.03 mmol, 86.4% yield) which was used without further purification. $[\alpha]^{25}_D$: −50.64 (c 1.154, MeOH). NMR (CD_3OD): δ .95 (dd, J = 6.5 Hz, J = 10.3 Hz, 6 H, CH_3), 1.58 (m, 2 H, CH_2), 1.73 (m, 1 H, CH), 1.98 (m, 4 H, CH_2CH_2), 2.29 (m, 1 H, CHC(O)), 3.53 (m, 1 H, CHN), 3.69 (m, 1 H, CHN), 4.15 (m, 1 H, α-CH), 4.55 (dd, J = 4.92 Hz, J = 8.53 Hz, 1 H, α-CH), 5.1 (dd, J = 12.2 Hz, J = 29.1 Hz, 2 H, CH_2Ar), 7.32 (m, 5 H, ArH), 8.10 (s, NH). IR ($CHCl_3$): 3000, 1650, 1450, 1220 cm^{-1}. MS (FAB): 318.1906, calcd 318.1943.

***N*-[[(2*S*,3*S*)-3-*trans*-(Ethoxycarbonyl)oxiran-2-yl]carbonyl]-L-leucyl-L-proline Benzyl Ester (EtO-EpsLeuPro-OBzl).** *N*-Methylmorpholine (0.28 mL, 5.95 mmol) was added dropwise to a suspension of the formic acid salt of leucineproline benzyl ester (1.9 g, 5.95 mmol) in THF (10 mL). This suspension was added to a solution of the monoethyl ester of the epoxide (0.95 g, 5.95 mmol) in THF (10 mL) at 0 °C. DCC (1.23 g, 5.95 mmol) was added, and after 0.5 h of stirring HOBt (0.804 g, 5.95 mmol) was added. The solution was stirred for 1 h at 0 °C and for 36 h at room temperature. The DCU was filtered off and the THF removed in vacuo. The residue was dissolved in CH_2Cl_2 and refrigerated overnight to precipitate the remaining DCU. The solvent was removed, and the remaining oil was purified by flash chromatography to yield 495 mg of a colorless oil (1.07 mmol, 18% yield). $[\alpha]^{25}_D$: −23.6 (c 4.93, MeOH). NMR (CD_3OD): δ 0.90 (dd, J = 5.7 Hz, J = 6.7 Hz, 6 H, CH_3), 1.26 (t, J = 3.8 Hz, 3 H, CH_3), 1.44 (m, 1 H, CH), 1.51 (m, 1 H, CH), 1.64 (m, 1 H, CH), 1.96 (m, 1 H, CH), 2.01 (m, 2 H, CH_2), 2.25 (m, 1 H, CH), 3.53 (d, J = 1.85 Hz, 1 H, EpoxH), 3.58 (m, 1 H, CHN), 3.64 (d, J = 1.85 Hz, 1 H, EpoxH), 3.82 (m, 1 H, CHN), 4.20 (m, 2 H, CH_2), 4.48 (dd, J = 4.8 Hz, J = 4.4 Hz, α-CH), 4.65 (dd, J = 4.4 Hz, J = 5.0 Hz, α-CH), 5.11 (J = 12.4 Hz, J = 30.2 Hz, 2 H, CH_2Ar), 7.32 (m, 5 H, ArH). IR ($CHCl_3$): 3020, 1740, 1685, 1645, 1520, 1440, 1210 cm^{-1}. MS (FAB): 460.2195, calcd 460.2209.

***N*-[[(2*S*,3*S*)-3-*trans*-(Hydroxycarbonyl)oxiran-2-yl]carbonyl]-L-leucyl-L-proline Benzyl Ester (HO-EpsLeuPro-OBzl).** The ethyl ester was hydrolyzed using pig liver esterase as per the method of Jones and Sabbioni[22] to give a 63.5% yield of the desired acid after reverse-phase chromatography. $[\alpha]^{23}_D$: −25.09 (c 0.80, MeOH). NMR (CD_3OD): δ .90 (dd, J = 6.5 Hz, J = 12.43 Hz, 6 H, CH_3), 1.43 (m, 1 H, CH), 1.52 (m, 1 H, CH), 1.64 (m, 1 H, CH), 1.94 (m, 1 H, CH), 2.02 (m, 2 H, CH_2), 2.25 (m, 1 H, CH), 3.48 (d, J = 1.45 Hz, 1 H, EpoxH), 3.57 (m, 1 H, CHN), 3.62 (d, J = 1.45 Hz, EpoxH), 3.83 (m, 1 H, CHN), 4.49 (dd, J = 4.8 Hz, J = 8.54 Hz, 1 H, α-CH), 4.66 (dd, J = 4.35 Hz, J = 10.1 Hz, 1 H, α-CH), 5.12 (dd, J = 12.3 Hz, J = 30.8 Hz, 2 H, CH_2Ar), 7.33 (m, 5 H, ArH). IR ($CHCl_3$): 2960, 1745, 1630, 1455, 1170 cm^{-1}. MS (FAB) 432.1897, calcd 432.1896.

***N*-[[(2*S*,3*S*)-3-*trans*-(Ethoxycarbonyl)oxiran-2-yl]carbonyl]-L-leucyl-L-Proline (EtO-EpsLeuPro-OH).** The benzyl group was removed by catalytic hydrogenation as described by Anwer and Spatola.[23] The ester (146 mg, 0.317 mmol) was dissolved in DMF (6 mL), and Pd/C was added. A solution of ammonium formate (146 mg) in H_2O was added dropwise to the ester/DMF solution, and the reaction mixture was stirred at room temperature for 24 h. The carbon was filtered off, the DMF was removed in vacuo, and the residue was purified by reverse-phase chromatography to give 60 mg (0.16 mmol, 51.3% yield) of product. $[\alpha]^{22}_D$: −6.62 (c 0.6, MeOH). NMR (CD_3OD): δ 0.94 (dd, J = 6.4 Hz, J = 6.6 Hz, 6 H, CH_3), 1.27 (t, J = 7.0 Hz, CH_3), 1.58 (m, 2 H, CH_2), 1.70 (m, 1 H, CH), 1.98 (m, 1 H, CH), 2.00 (m, 2 H, CH_2), 2.24 (m, 1 H, CH), 3.54 (d, J = 1.56 Hz, 1 H, EpoxH), 3.62 (m, 1 H, CHN), 3.65 (d, J = 1.57 Hz, 1 H, EpoxH), 3.82 (m, 1 H, CHN), 4.2 (m, 2 H, CH_2), 4.42 (dd, J = 4.5 Hz, J = 8.7 Hz, 1 H, α-CH), 4.70 (dd, J = 5.0 Hz, J = 9.5 Hz, 1 H, α-CH). IR ($CHCl_3$): 3020, 1740, 1630, 1450, 1210, 750 cm^{-1}. MS (FAB): 370.1749, calcd 370.1740.

***N*-[[(2*S*,3*S*)-3-*trans*-(Hydroxycarbonyl)oxiran-2-yl]carbonyl]-L-leucyl-L-proline (HO-EpsLeuPro-OH).** *N*-[[(2*S*,3*S*)-3-*trans*-(Ethoxycarbonyl)oxiran-2-yl]carbonyl]-L-leucine-L-proline was hydrolyzed using pig liver esterase at pH 6.5 by the method described above to give a 15% yield of the desired product after reverse phase chromatography. $[\alpha]^{23}_D$: −11.87 (c 0.56, MeOH). NMR (CD_3OD): δ 0.96 (t, J = 6.71 Hz, 6 H, CH_3), 1.59 (m, 2 H, CH_2), 1.71 (m, 1 H, CH), 1.98 (m, 1 H, CH), 2.04 (m, 2 H, CH_2), 2.26 (m, 1 H, CH), 3.57 (d, J = 1.65 Hz, EpoxH), 3.61 (m, 1 H, CHN) 3.65 (d, J = 1.65 Hz, EpoxH), 3.83 (m, 1 H, CHN), 4.42 (dd, J = 4.5 Hz, J = 8.75 Hz, 1 H, α-CH), 4.70 (dd, J = 5.0 Hz, J = 9.5 Hz, 1 H, α-CH). IR (CH_3CN): 3020, 1740, 1630, 1445, 1200 cm^{-1}. MS (FAB): 342.1447, calcd 342.1427.

***N*-[[(2*S*,3*S*)-3-*trans*-[(isobutylamino)carbonyl]oxiran-2-yl]carbonyl]-L-leucyl-L-proline Benzyl Ester (i-BuNH-EpsLeuPro-OBzl).** Isobutylamine (58.3 mL, 0.59 mmol) was added to a solution of *N*-[[(2*S*,3*S*)-3-*trans*-(hydroxycarbonyl)oxiran-2-yl]carbonyl]-L-leucyl-L-proline benzyl ester (254 mg, 0.59 mmol) in THF (10 mL) at 0 °C. DCC (121 mg, 0.59 mmol)

was added, and after 0.5 h HOBt (79.3 mg, 0.59 mmol) was added, and the reaction mixture was stirred for 5 days. The DCU was filtered off and the THF removed in vacuo. The residue was dissolved in EtOAc, washed with H_2O, and dried, and the solvent removed in vacuo to yield a yellow oil which was purified by reverse-phase chromatography to give 35% of the desired product (0.021 mmol, 100 mg). $[\alpha]^{21}_D$: −20.14 (c 0.54, MeOH). NMR (CD_3OD): δ 0.89 (d, J = 6.6 Hz, 6 H, CH_3), 0.91 (dd, J = 6.6 Hz, J = 18 Hz, 6 H, CH_3), 1.43 (m, 1 H, CH), 1.53 (m, 1 H, CH), 1.65 (m, 1 H, CH), 1.77 (m, 1 H, CH), 1.95 (m, 1 H, CH), 2.01 (m, 2 H, CH_2), 2.26 (m, 1 H, CH), 3.01 (m, 2 H, CH_2N), 3.50 (d, J = 1.8 Hz, 1 H, EpoxH), 3.57 (d, J = 1.8 Hz, EpoxH), 3.58 (m, 1 H, CHN), 3.83 (m, 1 H, CHN), 4.48 (dd, J = 4.9 Hz, J = 8.63 Hz, 1 H, α-CH), 4.66 (m, 1 H, α-CH), 5.11 (dd, J = 12.2 Hz, J = 31.2 Hz, 2 H, CH_2), 7.28–7.35 (m, 5 H, ArH). IR ($CHCl_3$): 3020, 1745, 1680, 1645, 1530, 1450 cm^{-1}. MS (FAB): 487.2652, calcd 487.2682.

N-[[(2S,3S)-3-*trans*-[(isobutylamino)carbonyl]oxiran-2-yl]carbonyl]-L-leucyl-L-proline (i-BuNH-EpsLeuPro-OH). N-[[(2S,3S)-3-*trans*-[(isobutylamino)carbonyl]oxiran-2-yl]carbonyl]-L-leucyl-L-proline benzyl ester (49.3 mg, 0.101 mmol) was dissolved in a solution of HCOOH/MeOH 4.4% (15 mL), and a suspension of palladium (50 mg) in HCOOH/MeOH (4.4%) (10 mL) was added. The reaction mixture was stirred at room temperature for 24 h, filtered, and evaporated to dryness, and the product was purified by reverse-phase chromatography to give a 48% yield of the desired product (0.048 mmol, 19 mg). $[\alpha]^{22}_D$: −24.21 (c 0.81, CH_3CN). NMR ($(CD_3)_2CO$): δ 0.88 (dd, J = 1.6 Hz, J = 6.7 Hz, 6 H, CH_3), 0.95 (dd, J = 6.5 Hz, J = 18.1 Hz, 6 H, CH_3), 1.55 (m, 2 H, CH_2), 1.71 (m, 1 H, CH), 1.79 (m, 1 H, CH), 2.02 (m, 1 H, CH), 2.04 (m, 2 H, CH_2), 2.25 (m, 1 H, CH), 3.05 (m, 2 H, CH_2N), 3.54 (d, J = 2.0 Hz, 1 H, EpoxH), 3.56 (d, J = 2.0 Hz, 1 H, EpoxH), 3.64 (m, 1 H, CHN), 3.81 (m, 1 H, CHN), 4.44 (m, 1 H, α-CH), 4.79 (m, 1 H, α-CH), 7.37 (m, 1 H, NH), 7.45 (d, J = 8.5 Hz, 1 H, NH). IR (CH_3Cl): 2900, 1725, 1680, 1640, 1540, 1460 cm^{-1}. MS (FAB): 397.2216, calcd 397.2213.

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