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FURTHER EVIDENCE FOR THE IMPORTANCE OF FREE CARBOXYLATE IN EPOXYSUCCINATE INHIBITORS OF THIOL PROTEASES

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Analogs of Ep-475 (**2a**), designed to explore the role played by the carboxylate in epoxysuccinate thiol protease inhibitors, have been synthesized and tested as inhibitors of papain and cathepsin B. Papain and cathepsin B are rapidly inactivated by carboxylates **2a** and **6a**, but are inactivated much more slowly by **2b–2f**, **6c**, and **6f**, in which the carboxylate is absent or replaced by an amide, ester, or ketone. This order of reactivity contrasts with the inherent reactivity of substituted epoxides toward a non-enzymatic thiolate, previously shown to decrease in the order: $COCH_3 > CO_2CH_3 > CONH_2 > H > CO_2H$. The results suggest that electrostatic attraction between the carboxylate of the inhibitor and protonated His^{159} of papain facilitates docking of the inhibitor in the active site of the enzyme, a conclusion reached previously from X-ray crystallographic structures of epoxysuccinates bound to papain. The most reactive isoleucine analog, **6a**, was significantly less reactive than leucine-containing Ep-475 (**2a**), while the less reactive isoleucine derivatives, **6c** and **6f**, were similar in reactivity to the corresponding leucine derivatives, **2c** and **2f**, respectively.

KEY WORDS: Epoxysuccinate inhibitors, thiol proteases, Ep-475, papain, cathepsin B.

INTRODUCTION

The naturally occurring epoxysuccinate E-64 (**1**), isolated from *Aspergillus japonicus*, is a rapid and potent irreversible inhibitor of many thiol proteases including papain, ficin, bromelain, calpain, and cathepsin B, H, and L.[1–4] Estatin A and estatin B, inhibitors isolated from *Myceliophthora thermophila*, are analogs of E-64 in which Leu is replaced by Phe and Tyr, respectively.[5] A large number of epoxysuccinate analogs including Ep-475 (E-64c, **2a**) have been synthesized as inhibitors of potentially pathophysiological thiol proteases such as calpain and cathepsin B.[6,7]

E-64 and its analogs function by S-alkylating the active-site cysteine of thiol proteases. Spectroscopic[8] and X-ray crystallographic[9–11] evidence has demonstrated that the Cys^{25} anion of papain exclusively attacks the epoxide α to the carboxylate in E-64 and Ep-475 to afford covalent adducts **3a** and **3b**, respectively.

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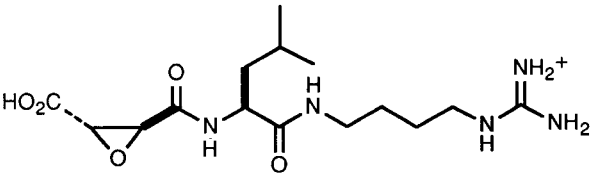
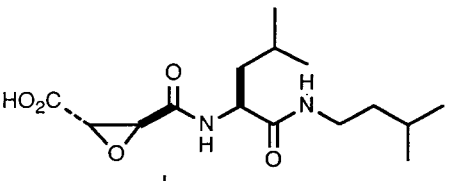
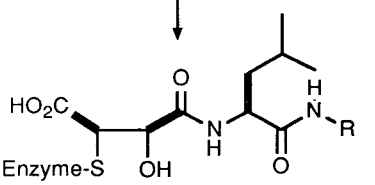


E-64 (**1**)



Ep-475 (**2a**)

Enzyme-SH



3a; R = $(CH_2)_4NHC(=NH_2)NH_2^+$
b; R = $CH_2CH_2CH(CH_3)_2$

We have now prepared a series of epoxides designed to explore the role which the carboxylate plays in epoxysuccinate thiol protease inhibitors. Previous investigators have reported that esterification of the carboxylate decreases reactivity toward papain.[1,6,12,13,14] However most of these studies are only qualitative since they report IC_{50} values after a fixed time, inappropriate for quantification of rapid irreversible inhibition,[4] rather than reaction rates. Recent reports demonstrated that cathepsin B and calpain were inhibited by certain amides of epoxysuccinates, but again only IC_{50} or K_i values were reported.[7,13,15,16] For electronic reasons, one would expect the carboxylate, deprotonated under physiological conditions, to deactivate the epoxide toward attack by the thiol anion of cysteine. Deactivation was in fact observed in the nonenzymatic reactions of a series of simple epoxides with methanethiolate: The presence of a carboxylate α to the epoxide decreases its reaction rate by an order of magnitude relative to the unsubstituted epoxide. The reactivity of α-substituted epoxides decreases in the order $COCH_3 > CO_2CH_3 > CONH_2 > H > CO_2H$.[17] In order to study the electronic and steric effects of various substituents on inhibitory activity, we have prepared epoxides **2b–2f**, **6c**, and **6f**, in which the carboxylate is deleted or replaced by an amide, ester, or ketone.

EXPERIMENTAL

Chemistry

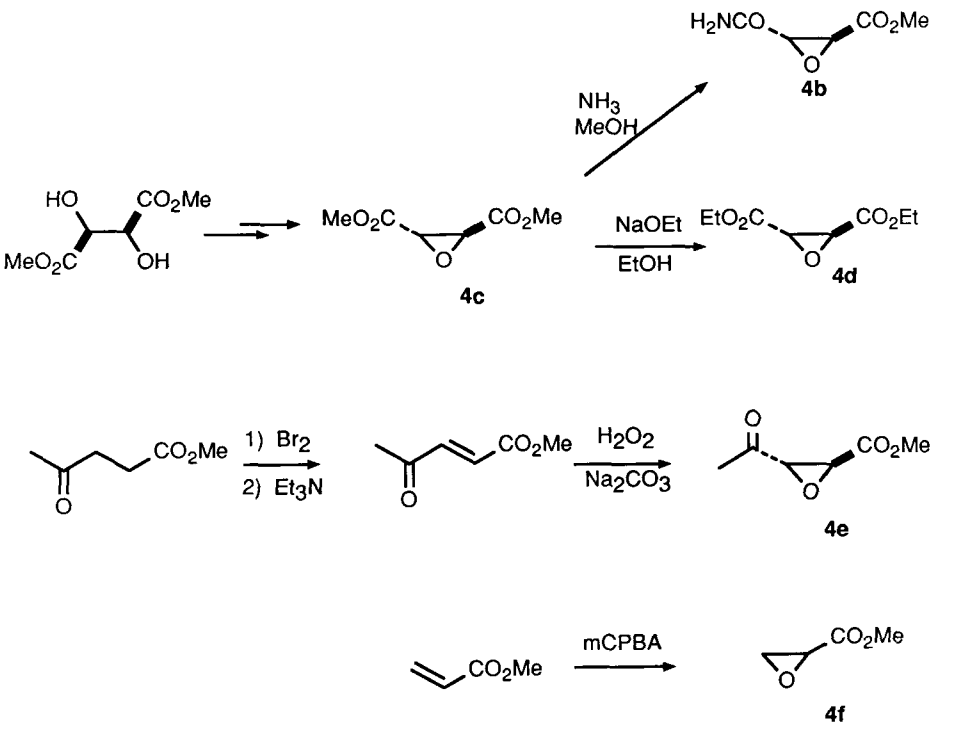
Methyl (*S,S*)-*trans*-3-(aminocarbonyl)oxirane-2-carboxylate (**4b**). A solution of ammonia in MeOH (2.1 mL, 3.2 M, 6.72 mmol, 105 mol %) was added to the dimethyl ester **4c** (1.02 g, 6.38 mmol) suspended in MeOH (6 mL). All of the solid dissolved after 10 min, and a new precipitate formed after 18 h. The The MeOH was evaporated, and the residue was triturated with ether and filtered. The filtrate contained starting material (150 mg, 15% recovery). The precipitate was extracted with chloroform (5 mL, hot), MeOH (2 × 5 mL), and chloroform-MeOH (80:20, 5 mL). The combined chloroform-MeOH filtrates were concentrated to afford **4b** contaminated with diamide (890 mg). Chromatography on silica gel (dichloromethane-MeOH, 100:0 to 95:5) afforded **4b** (680 mg, 74% yield) as a colorless solid: mp 120–122°C. NMR (DMSO-d_6) δ 3.54 (1H, d, J = 3 Hz), 3.63 (1H, d, J = 3 Hz), 3.72 (3H, s), 7.58 (1H, br.s), 7.77 (1H, br.s); $[\alpha]_D^{25} + 33.8°$ (c = 1.0, H_2O). Anal. Calc. for $C_5H_7NO_4$: C, 41.38; H, 4.86; N, 9.65. Found: C, 41.13; H, 4.70; N, 9.39%.

trans-(*S,S*)-oxirane-2,3-dicarboxamide. The white precipitate insoluble after extraction with chloroform and MeOH consisted of diamide (150 mg, 14%): mp 205–208°C. NMR (DMSO-d_6) δ 3.39 (2H, s), 7.48 (2H, s), 7.73 (2H, br.s); $[\alpha]_D^{25}57.7°$ (c = 1.0, H_2O). Calc. for $C_4H_6N_2O_3$: C, 36.93; H, 4.65; N, 21.53. Found: C, 36.54; H, 4.48; N, 21.19%.

Dimethyl (*S,S*)-*trans*-oxirane-2,3-dicarboxylate (**4c**). This compound was prepared from dimethyl (*S,S*)-D-tartrate as described for the diethyl ester,[18] and purified by kugelrohr distillation (110–145°C, 3 mm). mp 72–76°C; lit. mp 70–73°C; $[\alpha]_D^{25} = +125°$ (c = 1.3, MeOH); lit. $[\alpha]_D^{25} = 137°$.[19] NMR ($CDCl_3$) δ 3.70 (2H, s), 3.82 (6H, s).

Diethyl (*S,S*)-*trans*-oxirane-2,3-dicarboxylate (**4d**). Dimethyl ester **4c** (990 mg, 6.19 mmol) was added to EtOH (12 mL) containing NaOEt (0.57 mmol, 9 mol %). The solvent was evaporated from the pale yellow solution after 20 h at 20°C, and the residue was treated with EtOH (12 mL) containing NaOEt (0.43 mmol, 7 mol %). The solvent was evaporated after 20 h at 20°C. The residue was treated with water and extracted into ether. The ether extract was dried and the solvent was evaporated to afford crude product (1.00 g, 86% yield), which kugelrohr distilled (135–145°C, 1 mmHg) to afford the product (700 mg, 60% yield) as a clear oil. $[\alpha]_D^{25} + 104°$ (c = 1.2, EtOH); lit. $[\alpha]_D^{25} + 109.3°$ (c = 1.0, EtOH).[12]

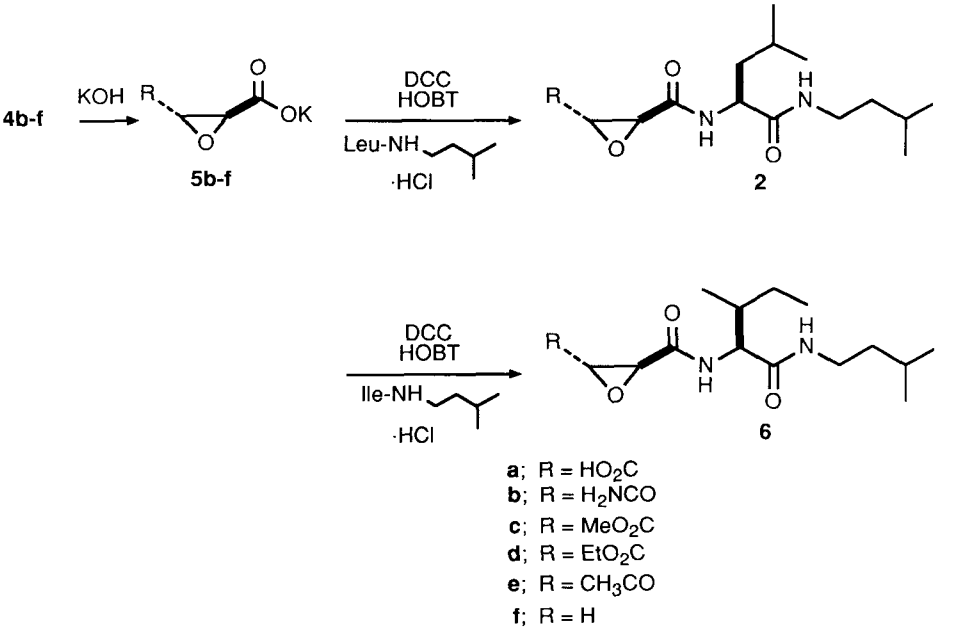
Methyl (±)-*trans*-3-(1-oxoethyl)oxirane-2-carboxylate (**4e**). Methyl *E*-4-oxo-2-pentenoate was prepared by a modification of the literature procedure.[20,21] A chloroform solution of methyl 4-oxopentanoate was treated with bromine (110 mol %, added over 1 h), cooled to 0°C and treated with triethylamine (250 mol %, added over 1 h). After 16 h at 0°C, the mixture was extracted with water and saturated NaCl, then dried. Evaporation of the solvent followed by kugelrohr distillation (100–140°C, 2.3 mmHg) and recrystallization from ethanol gave methyl 4-oxo-2-pentenoate (51% yield): mp 55–59°C; lit. mp 59°C. Methyl *E*-4-Oxo-2-pentenoate (1.0 g, 7.80 mmol) was added to a mixture of water (20 mL) and hydrogen peroxide (0.70 mL, 50% soln, 12.7 mmol, 163 mol %). Sodium carbonate (420 mg, 3.96 mmol, 51 mol %) was added, and the mixture was stirred for 30 min, then extracted three times with dichloromethane. The organic extract was dried and concentrated to yield product (600 mg, 53% yield) contaminated with 10% starting material. Flash chromatography on silica gel (ether-hexanes 70:30) afforded **4e**. NMR ($CDCl_3$) δ 2.16 (3H, s), 3.66 (2H, AB-q), 3.84 (3H, s).



Methyl (±)-oxiranecarboxylate (**4f**). Methyl acrylate (40 g, 465 mmol) and 2-chloroperbenzoic acid (88 g, 510 mmol, 110 mol %) were refluxed in dichloromethane (200 mL) for 18 days. The precipitate was removed by filtration and rinsed with dichloromethane (100 mL). The solvent was rotary evaporated from the combined filtrate (45°C, 200 mmHg) to give crude product (33.6 g) which was kugelrohr distilled (75–130°C, 40 mmHg) to give pure product (18 g, 38% yield) as an oil.[22]

Potassium *trans*-(*S*,*S*)-3-(aminocarbonyl)-2-oxiranecarboxylate (**5b**). Methyl ester **4b** (324 mg, 2.23 mmol) suspended in MeOH (2.5 mL) was treated with KOH (147 mg, 2.62 mmol, 118 mol %) in MeOH (1 mL). All of the starting material dissolved after 1 min, and a new precipitate formed, which was collected after 20 min by vacuum filtration, then rinsed with MeOH and ether to afford the product (170 mg, 45% yield) as an off-white solid: mp 125–130°C (decomp). Evaporation of the filtrate gave additional product (140 mg, 37% yield). NMR (D_2O) δ 3.47 (1H, d, J = 2.1 Hz), 3.53 (1H, d, J = 2.1 Hz); $[\alpha]_D^{25}$ +87.95° (c = 1.0, H_2O). Calc. for $C_4H_4NO_4K$-0.1MeOH-0.65H_2O: C, 26.75; H, 3.12; N, 7.61; K, 21.24. Found: C, 26.98; H, 3.08; N, 7.37; K, 21.22%.

Monomethyl potassium *trans*-(*S*,*S*)-2,3-oxiranedicarboxylate (**5c**). This compound was prepared as described for the racemate[1,2] and isolated by trituration with ether (81% yield). The free acid was prepared by acidification with aqueous HCl, extracted into EtOAc, dried, and kugelrohr distilled (145–160°C, 2 mmHg) to give a clear oil. NMR ($CDCl_3$) δ 3.74 (2H, s), 3.84 (3H, s), 9.52 (1H, br.s); $[\alpha]_D^{25}$ +118° (c = 1.6, H_2O). Calc. for $C_5H_6O_5$: C, 41.11; H, 4.14. Found: C, 40.72; H, 4.18%.



Monoethyl potassium *trans*-(*S*,*S*)-2,3-oxiranedicarboxylate (**5d**). This compound was prepared as described[2,12] and isolated by trituration with ether (81% yield).

Potassium (±)-*trans*-3-(1-oxoethyl)oxirane-2-carboxylate (**5e**). Methyl ester **4e** (1.53 g, 10.6 mmol) in methanol (7.65 mL) was added to a solution of KOH (696 mg, 12.4 mmol, 117 mol %) in methanol (7.65 mL). After 2 min, 1 M HCl was added to pH 5.7. The solvent was evaporated to afford a mixture containing crude product and KCl (1.62 g), which was utilized directly in the next step. NMR (D_2O) δ 2.30 (3H, s), 3.52 (1H, d, J = 1 Hz), 3.76 (1H, d. J = 1 Hz).

Potassium (±)-oxiranecarboxylate (**5f**).[23] Methyl (±)-Oxiranecarboxylate **4f** (8.90 g, 87.3 mmol) was added over 2 min to a solution of KOH (5.40 g, 96.4 mmol, 110 mol %) in MeOH (75 mL) while cooling to maintain the temperature below 25°C. After stirring for 2 h at 20°C, ether (200 mL) was added. The mixture was cooled to 0°C and filtered. The precipitate was rinsed with ether and dried in vacuo to give a very hygroscopic white solid (9.73 g, 89% yield): mp 130–135°C. NMR (D_2O) δ 2.799 (1H, dd), 2.96 (1H, t), 3.39 (1H, dd); Calc. for $C_3H_3O_3K$-0.5H_2O: C, 26.66; H, 2.98. Found: C, 26.63; H, 2.98%.

(2*S*,3*S*)-3-[[[(*S*)-3-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]amino]-carbonyl]oxiranecarboxylic acid (**2a**, Ep-475, E-64c). KOH (21 mg, 0.37 mmol, 123 mol %) in MeOH (1 mL) was added to a solution of methyl ester **2c** (100 mg, 0.30 mmol) in MeOH (0.1 mL). After 2.5 h, the solvent was evaporated from the homogeneous mixture. Water and 1 M HCl were added to pH 1, and the aqueous mixture was extracted three times with EtOAc. The organic phase was dried and concentrated to give **2a** (90 mg). Recrystallization from EtOAc-hexanes gave **2a** as a white solid (67 mg, 70% yield): mp 153–156°C.; lit. mp 157–160°C. $[\alpha]_D^{25}$ + 39.0° (c = 0.26, EtOH); lit. $[\alpha]_D$ 39.0; 45.5° (c = 1, EtOH).[4]

General procedure for synthesis of **2b–2f**. L-Leucine isopentylamide-HCl (prepared from BOC-L-leucine isopentylamide[12]) (944 mg, 3.99 mmol) was dissolved in dichloromethane (35 mL). Finely ground potassium salt **5** was added, and the mixture was stirred and sonicated, then cooled to 0°C. HOBT-H_2O (640 mg, 4.18 mmol) and dicyclohexylcarbodiimide (860 mg, 4.17 mmol) were added. The mixture was stirred at 0°C for 1–2 h and 20°C for 14–18 h, then filtered. The filtrate was rinsed with water, 1 M HCl, saturated Na_2CO_3, and saturated NaCl solutions, dried, and refiltered. The product was purified by chromatography or recrystallization.

(2*S*,3*S*)-3-[[[(*S*)-3-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]amino]-carbonyl]oxiranecarboxamide (**2b**). The crude product (35% yield) was purified by flash chromatography on silica gel (chloroform-MeOH 95:5) (13% yield) to give a white solid, mp 109–113°C, which forms viscous gels in dichloromethane and chloroform. NMR (CD_3OD) δ 0.94 (12H, m), 1.38 (2H, q), 1.60 (4H, m), 3.19 (2H, dt), 3.51 (1H, d, J = 1.9 Hz), 3.60 (1H, d. J = 1.9), 4.41 (1H, dd); $[\alpha]_D^{25} + 34.0°$ (c = 2.3, MeOH). Calc. for $C_{15}H_{27}N_3O_4$: C, 57.49; H, 8.68; N, 13.41. Found: C, 57.22; H, 8.74; N, 13.12%.

Alternatively, **2b** was isolated in 10% yield after DCC/HOBT coupling between **2a** and ammonia in dichloromethane. Again, isolation was complicated by gel formation.

Methyl (2*S*,3*S*)-3-[[[(*S*)-3-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]-amino]carbonyl]oxiranecarboxylate (**2c**). The crude product (91% yield) was purified by recrystallization from hot hexanes-EtOAc (10:1) (74% yield) to give a white solid, mp 96–100°C. Recrystallization from EtOH-water gave mp 107–111°C. NMR ($CDCl_3$) δ 0.93 (12H, m), 1.40 (2H, m), 1.65 (4H, m), 3.26 (2H, m), 3.49 (1H, d, J = 2 Hz), 3.69 (1H, d, J = 2 Hz) 3.82 (3H, s), 4.40 (1H, q), 6.1 (1H, br.t), 6.7 (1H, br.d); $[\alpha]_D^{25} + 39.6°$ (c = 1.26, MeOH). Calc. for $C_{16}H_{28}N_2O_5$-0.25H_2O: C, 57.73; H, 8.63; N, 8.41. Found: C, 57.77; H, 8.57; N, 8.60%.

Ethyl (2*S*,3*S*)-3-[[[(*S*)-3-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]-amino]carbonyl]oxiranecarboxylate (**2d**, E-64d; EST; Aloxistatin). The crude product (87% yield) was purified by recrystallization from hot EtOH (53% yield) to give a white solid, mp 191–194°C; lit. mp 126.2°C. $[\alpha]_D^{25} + 46.7°$ (c = 1.1, EtOH); lit $[\alpha]_D^{20} + 51.7°$ (c = 1.0, EtOH).[12]

(2*RS*,3*RS*)-3-[[[(*S*)-3-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]amino]-carbonyl]oxiranyl-1-ethanone (**2e**). The crude product (92% yield) was purified by flash chromatography on silica gel (chloroform-EtOAc 75:25) (16% yield) to give a white solid, mp 99–109°C. NMR ($CDCl_3$) δ 0.90 (12H, m), 1.40 (2H, m), 1.60 (3H, m), 2.12 (1.5H, s), 2.14 (1.5H, s), 3.25 (2H, m), 3.43 (0.5H, d, J = 2 Hz), 3.50 (0.5H, d, J = 2 Hz), 3.59 (0.5H, d, J = 2 Hz), 3.60 (0.5H, d, J = 2), 4.34 (1H, m), 5.85 (0.5H, t), 5.90 (0.5H, t), 6.50 (0.5H, d), 6.59 (0.5H, d); $[\alpha]_D^{25} - 38.4°$ (c = 0.26, $CHCl_3$). Calc. for $C_{16}H_{28}N_2O_4$-0.25H_2O: C, 60.64; H, 9.06; N, 8.84. Found: C, 60.64; H, 9.10; N, 8.62%.

(2*RS*,)-2-[[[(*S*)-3-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]amino]-carbonyl]oxirane (**2f**). The crude product (100% yield) was purified by recrystallization from hot EtOAc-hexanes (52% yield) to give a white solid, mp 109–113°C. NMR ($CDCl_3$) δ 0.91 (12H, m), 1.39 (2H, q), 1.6 (4H, m), 2.75 (0.5H, dd), 2.81 (0.5H, dd), 3.01 (1H, q), 3.26 (2H, m), 3.45 (1H, m), 4.38 (1H, dd), 6.1 (1H, m), 6.5 (1H, m); $[\alpha]_D^{25} - 58.1°$ (c = 0.5, $CHCl_3$). Calc. for $C_{14}H_{26}N_2O_3$: C, 62.19; H, 9.69; N, 10.36. Found: C, 61.91; H, 9.62; N, 10.32%.

(2*S*,3*S*)-3-[[[(*S*)-2-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]amino]-carbonyl]oxiranecarboxylic acid (**6a**). This was prepared by hydrolysis of **6c** as

described for **2a** and had mp 95–120°C. NMR ($CDCl_3$) δ 0.92 (12H, m), 1.20 (1H, m), 1.40 (2H, dd), 1.60 (1H, septet), 1.9 (1H, m), 3.22 (1H, m), 3.37 (1H, m), 3.55 (1H, d, J = 1.7 Hz), 3.59 (1H, d, J = 1.7 Hz), 4.38 (1H, t), 6.6 (1H, t), 8.3 (1H, d); $[\alpha]_D^{25} + 34.3°$ (c = 0.4, EtOH). Calc. for $C_{15}H_{26}N_2O_5$: C, 57.31; H, 8.34; N, 8.91. Found: C, 57.58; H, 8.52; N, 9.24%.

Methyl (2*S*,3*S*)-3-[[[(*S*)-2-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]-amino]carbonyl]oxiranecarboxylate (**6c**). This was prepared from isoleucene isopentylamide-HCl and **5c** as described for **2c** and had mp 191–194°C. NMR ($CDCl_3$) δ 0.91 (12H, m), 1.10 (1H, m), 1.40 (2H, dd), 1.60 (1H, septet), 1.9 (1H, m), 3.23 (1H, m), 3.32 (1H, m), 3.50 (1H, d, J = 1.7 Hz), 3.69 (1H, d, J = 1.7 Hz), 3.82 (3H, s), 4.17 (1H, t), 5.9 (1H, br.t), 6.8 (1H, d); $[\alpha]_D^{25} + 46.8°$ (c = 1.0, MeOH). Calc. for $C_{16}H_{28}N_2O_5$: C, 58.52; H, 8.59; N, 8.53. Found: C, 58.29; H, 8.58; N, 8.77%.

(2*RS*)-2-[[[(*S*)-2-Methyl-1-[[(3-methylbutyl)amino]carbonyl]butyl]amino]-carbonyl]oxirane (**6f**). This was prepared from isoleucene isopentylamide-HCl and **5f** as described for **2f** and had mp 137–144°C. NMR ($CDCl_3$) δ 0.90 (12H, m), 1.40 (2H, q), 1.6 (2H, m), 1.85 (1H, m), 2.77 (0.5H, dd), 2.80 (0.5H, dd), 3.00 (1H, 2q), 3.3 (2H, m), 3.44 (1H, m), 4.15 (1H, t), 6.0 (1H, m), 6.7 (1H, m); $[\alpha]_D^{25} - 26.0°$ (c = 0.5, $CHCl_3$). Calc. for $C_{14}H_{26}N_2O_3$: C, 62.19; H, 9.69; N, 10.36. Found: C, 62.09; H, 9.83; N, 10.41%.

Inhibition of Papain and Cathepsin B

The irreversible inactivation method was used to measure the inhibition of papain and cathepsin B by epoxysuccinate inhibitors. An aliquot of inhibitor in DMSO (50–100 μL) was added to 0.50 mL of a buffered enzyme solution (0.08–0.045 μM) to initiate the inactivation reaction. Aliquots (50–100 μL) were withdrawn at various intervals and the residual enzymatic activity was measured. Papain was assayed with Bz-Arg-AMC in 50 mM Tris-HCl, 2 mM EDTA, 5 mM cysteine (freshly prepared), pH 7.5 buffer at 25°C,[24] and cathepsin B was assayed with Z-Arg-Arg-AMC in 50 mM KH_2PO_4, 1.33 mM EDTA, 2.7 mM cysteine (freshly prepared), pH 6.0 buffer at 25°C.[25] The AMC (7-amino-4-methylcoumarin) release was followed fluorometrically (excitation at 380 nm, emission at 440 nm). The DMSO concentration in the reaction mixtures was 8%, and the inhibitor concentrations are shown in Table 1. Pseudo

Table 1 Inhibition rate constants of papain and cathepsin by inhibitors **2** and **6**

Inhibitor	Papain [I] (μM)	Papain $k_{obs}/[I]$ ($M^{-1}\,s^{-1}$)	Cathepsin B [I] (μM)	Cathepsin B $k_{obs}/[I]$ ($M^{-1}\,s^{-1}$)
2a	0.30	329,000[a]	0.55	175,000[a]
2b	3.7	4,500	15	150
2c	54	320	530	30
2d	130	250	500	10.5
2e[c]	280	160	28	260
2f[c]	580	3	580	NI[b]
6a	3.5	30,000	1.7	75,000
6c[c]	440	110	440	67
6f[c]	320	3.4	640	NI[b]

[a]Second order inhibition constant (k_{2nd}) was obtained at equimolar concentrations of inhibitor and enzyme.
[b]No inhibition observed. [c]Mixture of diastereomers, see text.

first-order inactivation rate constants were obtained from plots of ln v_i/v_o vs time, and the correlation coefficients were greater than 0.98. For rapid inhibition of papain and cathepsin B by **2a**, equimolar concentrations of inhibitor and enzyme were used and the second-order inhibition rate constants (k_{2nd}) were obtained.

RESULTS AND DISCUSSION

Synthesis

Synthesis of the target Ep-475 analogs (**2**) commenced with preparation of epoxyesters **4**. Dimethyl (*S,S*)-*trans*-oxirane-2,3-dicarboxylate (**4c**) was prepared from (*S,S*)-D-dimethyl tartrate[18,19] and transesterified to afford diethyl ester (**4d**). Dimethyl ester **4c** reacted with ammonia in methanol to afford monoamide **4b** along with a small amount of the diamide. Epoxyketoester **4e** was prepared by epoxidation of methyl *trans*-4-oxo-2-pentenoate[20,21] with aqueous hydrogen peroxide and sodium carbonate. Oxidation with hydrogen peroxide and sodium hydroxide gave an inferior yield. Methyl oxiranecarboxylate (**4f**) was prepared from methyl acrylate by prolonged oxidation with MCPBA.[22] Attempted preparation of oxiranecarboxylate (**5f**) by oxidation of acrolein with alkaline hydrogen peroxide[23] gave a very low yield in our hands.

Esters **4b–4f** were hydrolyzed with potassium hydroxide to afford potassium oxiranecarboxylates **5b–5f**. Hydrolysis of **4e** had to be conducted with care: decomposition of **5e** occurred upon continued contact with base. Without conversion to free acids, potassium salts **5b–5f** were suspended in dichloromethane and directly coupled with leucyl isopentylamide hydrochloride in the presence of DCC and HOBT. Ep-475 (**2a**) was prepared by hydrolysis of **2c**. It should be noted that **2e** and **2f** were obtained as diastereomeric mixtures, since the precursors, **5e** and **5f**, were racemates.

Three analogs of Ep-475, **6a**, **6c**, and **6f**, in which Leu was replaced by Ile were synthesized as above from isoleucine isopentylamide hydrochloride and the appropriate oxiranecarboxylates.

Inhibitory Activity

Compounds **2** and **6** were examined for their ability to inhibit papain and cathepsin B catalyzed hydrolysis of the fluorogenic substrates Bz-Arg-AMC and Z-Arg-Arg-AMC, respectively.[24,25] Inhibition constants for **2a** (Ep-475) agreed well with reported data of 357,000 and 298,000 $m^{-1} s^{-1}$ for papain and cathepsin B, respectively.[4]

Rates of inactivation of papain and cathepsin B by inhibitors **2** and **6** are shown in Table 1. The most reactive isoleucine analog. **6a**, was significantly less reactive than leucine derivative Ep-475 (**2a**), while the less reactive isoleucine derivatives, **6c** and **6f**, were similar in reactivity to the corresponding leucine derivatives, **2c** and **2f**, respectively. Papain and cathepsin B are inactivated rapidly by carboxylates **2a** and **6a**, but inactivated much more slowly by **2b–2f**, **6c**, and **6f**, in which the carboxylate is absent or replaced by an amide, ester, or ketone. The rate constant of **2d** is significantly lower than previously reported.[12] Rates of inhibition of papain by **2a–2f** follow the order $CO_2H > CONH_2 > CO_2R > COCH_3 > H$, a result independently confirmed subsequent to the completion of our investigation.[26] This order of reactivity cannot be attributed to a steric inability of the inhibitors to fit in the active site of the enzymes. The observed order of reactivity is at variance with the inherent

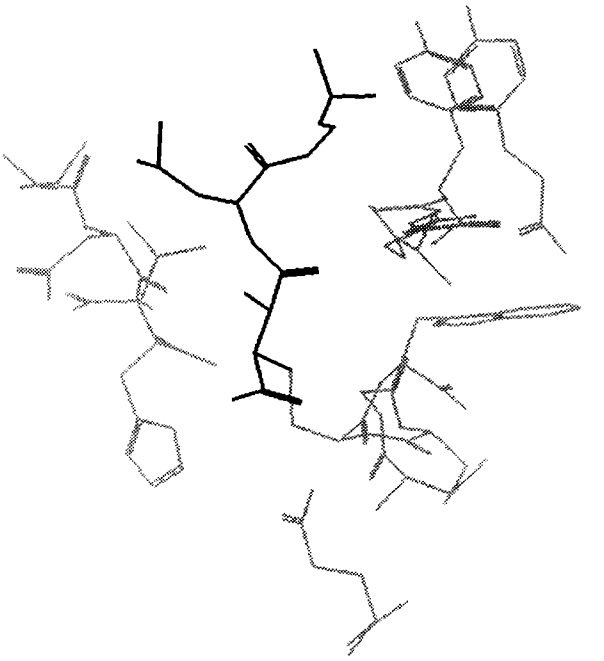


Figure 1 Ep-475 (**2a**, black) bound to active site of papain (grey). The carboxylate of Ep-475 is attracted to His^{159} (lower left) of papain.

reactivity of non-enzymatic thiolate with substituted epoxides, which has been shown to decrease in the order $COCH_3 > CO_2CH_3 > CONH_2 > H > CO_2H$.[17]

The data confirm that the carboxylate of **2a**, deprotonated at physiological pH, is important for rapid inactivation of papain and cathepsin B. This had previously been observed in related esters of epoxysuccinyl-Leu-OH derivatives (measuring IC_{50}s), and was attributed to dissociation of the Cys^{25}-His^{159} ion pair in papain.[6,14] Similarly, short-chain amides and esters of epoxysuccinyl-Leu-Pro-OH are less active inhibitors of papain, cathepsin L, and cathepsin H than the corresponding carboxylates.[13,16] Examination of the covalently bound complex (**3b**) between papain and Ep-475 (**2a**), determined by X-ray crystallography[10,11] and illustrated in Figure 1, reveals a likely explanation for the importance of the carboxylate in Ep-475. As previously revealed by the crystallographic structures of epoxysuccinates bound to papain,[9,10,11] the carboxylate anion of Ep-475 is electrostatically attracted to the positively charged protonated His^{159} of papain. *Therefore the rate-limiting step in the enzyme inactivation is not the inherent reactivity of the epoxide, but rather appears to be the rate with which the inhibitor docks in the active site prior to formation of the covalent complex.* Thus, as previously suggested, His^{159} has a binding role during inactivation by epoxysuccinates in contrast to its catalytic role in proteolysis.[9]

Although the free carboxylate of epoxysuccinates **2** and **6** is important for inhibition of papain and cathepsin B, certain esters and amides of epoxysuccinyl-Ile-Pro-OH are actually better inhibitors of cathepsin B (but not of papain, cathepsin L, or cathepsin H) than the corresponding carboxylate.[7,13,16] Molecular modelling suggests

that in these inhibitors the carboxylate of proline, instead of the carboxylate of epoxysuccinate, binds to His of cathepsin B.[13] This model would explain why a free epoxysuccinate carboxylate is not mandatory in this class of cathepsin B inhibitors.

The crystal structure of Ep-475 bound to papain reveals many interactions between the enzyme and the inhibitor.[10,11] Besides the covalent bond to Cys^{25}, and several hydrogen bonds, weak interactions were also observed between the leucyl side chain of Ep-475 and the main chains of Val^{157} and Asp^{158} of papain. The fact that the isoleucyl analog **6a** is ten fold less active toward papain than leucine-containing Ep-475 indicates that these hydrophobic interactions may be disturbed in the complex of papain with **6a**.

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