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Synthesis and Evaluation of CE-0266: A New Human Neutrophil Elastase Inhibitor

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The synthesis and evaluation of 4-(methylsulfinyl)phenyl 2-(1-methyl-2-pyrrolyl)butyrate (CE-0266) and the related sulfide and sulfone derivatives, CE-0265 and CE-0267, respectively, are described. The potency of the inhibitors toward human neutrophil elastase increases across the series CE-0265, CE-0266, CE-0267. CE-0266, with a $k_3K_i^*/k_2$ value of 36 nM, exhibited high selectivity for elastase and was chosen for additional *in vivo* studies.

INTRODUCTION

Human neutrophil elastase (HNE) is a proteolytic enzyme released by polymorphonuclear (PMN) leukocytes in response to a variety of inflammatory stimuli. This release of HNE (and its proteolytic activity) is highly regulated and is a normal, beneficial function of PMNs. The degradative capacity of HNE, under normal circumstances, is modulated by relatively high plasma concentrations of α_1-proteinase inhibitor (α_1-PI) (*1*). However, stimulated neutrophils produce a burst of active oxygen metabolites, some of which (hypochlorous acid, for example) are capable of oxidizing a critical methionine residue in α_1-PI (*2*). Oxidized α_1-PI has been shown to have limited potency as an HNE inhibitor (*3*). It is this altered protease/antiprotease balance which permits HNE to perform its degradative functions in a localized and controlled environment (*4*).

Despite this balance of protease/anti-protease activity, there are a number of human disease states in which a breakdown of this control mechanism is implicated in the pathogenesis of the condition. Improper modulation of HNE activity has been suggested as a contributing factor in adult respiratory distress syndrome (ARDS) (*5, 6*), septic shock (*7*), and multiple organ failure (MOF) (*6, 7*). A series of studies have indicated the involvement of PMNs (*8*) and neutrophil elastase (*9*) in myocardial ischemia-reperfusion injury. Humans with below normal levels of α_1-PI (either due to hereditary (*10, 11*) or environmental factors (*12, 13*)) have an

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increased probability of developing emphysema. There is an increasing number of other conditions such as arthritis, periodontal disease, and glomerulonephritis, in which HNE-mediated processes are associated (*5*).

Enzyme inhibitors offer a rational approach toward effective therapeutic intervention and possibly prophylaxis in the conditions described above (*14*). The design and synthesis of potent, specific inhibitors of HNE has been the subject of intense investigation by a number of research groups in recent years (*15*, *16*). We have designed a series of HNE inhibitors based on aromatic esters of 2-(1-methyl-2-pyrrolyl)butyric acid (*17*). We present here the synthesis and characterization of three of these novel compounds CE-0265, CE-0266, and CE-0267.

EXPERIMENTAL PROCEDURES

Synthetic Chemistry

General

Tetrahydrofuran and methylene chloride were obtained as the anhydrous solvents from Aldrich Chemical Co., Inc., and used as received. Hexamethylphosphoramide (HMPA) was distilled from sodium under vacuum and stored over 4 Å molecular sieves prior to use. All other reagents were used without further purification of the commercially available materials. Proton and carbon nuclear magnetic resonance spectra were obtained on a Varian Gemini-300 spectrometer with tetramethylsilane as an internal reference. Melting points were determined on a Fisher–Johns apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN).

Procedure for the Synthesis of CE-0265 (4) and CE-0266 (5)

(±)-*Methyl 2-(1-methyl-2-pyrrolyl)butyrate, 2.* A solution of lithium diisopropylamide (253 mmol) in 600 ml of dry THF was prepared under N_2 and cooled to −78°C. Methyl 2-(1-methyl-2-pyrrolyl)acetate (36.85 g, 241 mmol) was added dropwise with stirring followed by 32 ml of HMPA. The reaction mixture was stirred for 30 min and iodoethane (19.2 ml, 241 mmol) was added dropwise over 15 min. The cooling bath was removed and the reaction mixture was allowed to warm to room temperature. After 2 h the reaction mixture was quenched with H_2O and extracted with ether. The combined organic layers were washed with water, dried over $MgSO_4$, and concentrated. The residue was distilled under vacuum (0.14 mm Hg, 80°C) to afford the product as a pale yellow oil (37.0 g, 85%): ^1H NMR ($CDCl_3$) δ 0.96 (t, 3 H, J = 7.4 Hz), 1.80–1.95 (m, 1 H), 2.05–2.20 (m, 1 H), 3.53 (t, 1 H, J = 7.6 Hz), 3.59 (s, 3 H), 3.67 (s, 3 H), 6.05–6.10 (m, 2 H), 6.55–6.56 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 11.94, 25.01, 33.60, 44.78, 51.75, 106.57, 107.00, 122.25, 130.11, 173.76.

(±)-*2-(1-methyl-2-pyrrolyl)butyric acid, 3.* A mixture of methyl 2-(1-methyl-2-pyrrolyl)butyrate **2** (37.0 g, 204 mmol) and 250 ml of 2.5 N aqueous NaOH was heated under reflux for 3 h. The reaction mixture was cooled to room temperature,

acidified to pH = 1.0, and extracted with ether. The ether layer was dried over anhydrous $MgSO_4$ and concentrated to give 28.9 g (87%) of the desired product: ^1H NMR ($CDCl_3$) δ 0.99 (t, 3 H, J = 7.4 Hz), 1.80–1.95 (m, 1 H), 2.04–2.20 (m, 1 H), 3.52 (t, 1 H, J = 7.6 Hz), 3.60 (s, 3 H), 6.09–6.11 (m, 2 H), 6.56–6.58 (m, 1 H), 12.24 (br s, 1 H, –OH); ^{13}C NMR ($CDCl_3$) δ 11.94, 24.68, 33.63, 44.65, 106.91, 107.20, 122.58, 129.29, 180.19.

(±)-*4-(Methylmercapto)phenyl 2-(1-methyl-2-pyrrolyl)butyrate,* ***4*** *(CE-0265).* Dicyclohexylcarbodiimide (44.0 g, 216 mmol) was added to a stirred solution of 2-(1-methyl-2-pyrrolyl)butyric acid (28.5 g, 176 mmol) and 4-(methylmercapto)phenol (23.7 g, 165 mmol) in 500 ml of dry dichloromethane. After 16 h, acetic acid (120 ml) was added, the solution filtered, and the filtrate concentrated under vacuum. The residue was dissolved in ether and treated with anhydrous potassium carbonate until neutral. The mixture was filtered and the filtrate washed with 5% NaOH, dried over anhydrous $MgSO_4$, and concentrated under vacuum. The residue was distilled under vacuum (0.7 mm Hg, 185°C) to afford 24.6 g (49%) of a cream-colored solid. Recrystallization from cyclohexane afforded white needles: mp 45–45.5°C; ^1H NMR ($CDCl_3$) δ 1.06 (dd, 3 H, J = 7.3, 7.4 Hz), 1.95 (ddt, 1 H, J = 7.3, 7.0, 14 Hz), 2.22 (ddt, 1 H, J = 7.4, 8.2, 14 Hz), 2.44 (s, 3 H), 3.65 (s, 3 H), 3.74 (dd, 1 H, J = 7.0, 8.2 Hz), 6.11 (dd, 1 H, J = 2.8, 3.6 Hz), 6.15 (dd, 1 H, J = 1.9, 3.6 Hz), 6.59 (dd, 1 H, J = 1.9, 2.4 Hz), 6.94 (d, 2 H, J = 8.7 Hz), 7.23 (d, 2 H, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 12.21, 16.50, 25.42, 33.90, 45.08, 106.84, 106.97, 121.82, 122.40, 127.92, 129.24, 135.58, 148.48, 171.38; *Anal.* Calcd for $C_{16}H_{19}NO_2S$: C, 66.41; H, 6.62; N, 4.84; S, 11.08. Found: C, 66.63; H, 6.72; N, 4.79; S, 9.80.

(±)-*4-(Methylsulfinyl)phenyl 2-(1-methyl-2-pyrrolyl)butyrate,* ***5*** *(CE-0266).* Hydrogen peroxide (16.2 ml of a 30% solution) was added to a stirred mixture of 4-(methylmercapto)phenyl 2-(1-methyl-2-pyrrolyl)butyrate (24.6 g, 86.5 mmol) in 120 ml of glacial acetic acid. After 1 h, 120 ml of H_2O was added and the mixture extracted with ether. The organic layer was washed with water and dried overnight over anhydrous potassium carbonate. The solution was filtered and concentrated to afford the product as a white solid. Trituration of the crude solid with ether gave 21.0 g (81%) of the pure product as white crystals: mp 59.5–60.5°C; ^1H NMR ($CDCl_3$) δ 1.07 (dd, 3 H, J = 7.3, 7.4 Hz), 1.98 (ddt, 1 H, J = 7.1, 7.3, 14 Hz), 2.35 (ddt, 1 H, J = 7.4, 8.1, 14 Hz), 2.70, (s, 3 H), 3.67 (s, 3 H), 3.78 (dd, J = 7.1, 8.1 Hz), 6.12 (dd, 1 H, J = 2.7, 3.6 Hz), 6.16 (dd, 1 H, J = 1.8, 3.6 Hz), 6.60 (dd, 1 H, J = 1.8, 2.7 Hz), 7.20 (d, 2 H, J = 8.7 Hz), 7.64 (d, 2 H, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 12.18, 25.42, 33.92, 44.06, 45.07, 107.00, 107.21, 122.54 (2 X), 124.80, 128.86, 142.85, 152.78, 171.01; *Anal.* Calcd for $C_{16}H_{19}NO_3S$: C, 62.93; H, 6.27; N, 4.59; S, 10.50. Found: C, 62.88; N, 6.33; N, 4.43; S, 9.88.

*Procedure for the Synthesis of CE-0267 (**6**)*

(±)-*4-Methylsulfonylphenyl 2-(1-methyl-2-pyrrolyl)butyrate,* ***6*** *(CE-0267).* Trimethylacetyl chloride (1.14 g, 9.5 mmol) was added to a solution of 2-(1-methyl-2-pyrrolyl)butyric acid (1.58 g, 9.5 mmol) and diisopropylethylamine (1.23 g,

9.5 mmol) in 20 ml of dichloromethane. After stirring for 1 h, a solution of 4-(methylsulfonyl)phenol (1.33 g, 9.5 mmol) and diisopropylethylamine (1.23 g, 9.5 mmol) in 15 ml of dichloromethane was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was washed with H_2O (4 × 20 ml), dried over anhydrous $MgSO_4$ and concentrated. The residue was chromatographed on flash silica gel (ethyl acetate/hexane, 1 : 4) to afford 1.30 g (43%) of the product which was recrystallized from ethanol: ^1H NMR ($CDCl_3$) δ 1.07 (dd, 3 H, J = 7.3, 7.4 Hz), 1.98 (ddt, 1 H, J = 7.1, 7.3, 14 Hz), 2.24 (ddt, 1 H, J = 7.4, 8.3, 14 Hz), 3.04 (s, 3 H), 3.68 (s, 3 H), 3.79 (dd, 1 H, J = 7.1, 8.3 Hz), 6.11–6.14 (m, 1 H), 6.15–6.18 (m, 1 H), 6.60–6.62 (m, 1 H), 7.23 (d, 2 H, J = 8.7 Hz), 7.94 (d, 2 H, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 12.20, 25.45, 33.99, 44.59, 45.04, 107.07, 107.31, 122.53, 122.69, 128.62, 129.10, 137.76, 154.79, 170.71; *Anal.* Calcd for $C_{16}H_{19}NO_4S$: C, 59.79; H, 5.96; N, 4.36; S, 9.98. Found: C, 60.02; H, 6.10; N, 4.27; S, 10.00.

4-(Methylsulfonyl)phenol, 8. To a suspension of 4-(methylmercapto)phenol (8.8 g, 63 mmol) in 63 ml of glacial acetic acid was added 63 ml of 30% hydrogen peroxide. The reaction mixture was stirred at room temperature for 72 h. After addition of 30 ml of saturated sodium chloride, the solution was extracted continuously with ether for 48 h. The ether layer was evaporated and the residue dried under vacuum. The resulting solid was chromatographed on flash silica (ether) to provide 6.0 g (55%) of the product as a solid: ^1H NMR ($CDCl_3$) δ 3.06 (s, 3 H), 5.10 (br, –OH, 1 H), 6.98 (d, 2 H, J = 8.7 Hz), 7.82 (d, 2 H, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 44.90, 116.22, 129.69, 131.29, 160.98.

Enzymology

General

All substrates, enzymes, buffers and biochemical reagents were the highest grade available from commercial suppliers and were utilized without further purification. All buffers were prepared and adjusted to the indicated pH at room temperature.

Enzyme Assays

Kinetics of Inhibition

Compounds 4, 5, and 6 are, in fact, alternative substrates which appear to combine with HNE at rates on the order of 10^4 M^{-1} s^{-1} to form covalent intermediates. These intermediates then decompose at rates on the order of 10^{-3} s^{-1} to regenerate active enzyme and hydrolyzed inhibitor. In the presence of a chromogenic peptide substrate for the enzyme, the following overall scheme is likely to apply:

$$\begin{array}{l} E + I \underset{}{\overset{K_i^*}{\rightleftharpoons}} EI^* \underset{L}{\overset{k_2}{\rightarrow}} EI \xrightarrow{k_3} E + P \\ \quad + \\ \quad S \\ K_m \downarrow\uparrow \\ ES \xrightarrow{k_{cat}} E + PA \end{array}$$

In this scheme, K_m and k_{cat} are the Michaelis constant and the catalytic constant for amidolysis of the chromogenic substrate by the enzyme, PA represents the product, *p*-nitroaniline, K_i^* represents the dissociation constant for a reversible complex between the enzyme and the inhibitor (analogous to a classical Michaelis complex), k_2 is the constant for formation of a covalent EI intermediate (apparently an acyl enzyme), and k_3 is the constant for decomposition of this intermediate to liberate free enzyme and 2-(1-methyl-2-pyrrolyl)butyric acid. It can be expected from such a scheme that when the rate of decomposition of the covalent intermediate is equal to the rate of its formation, a steady state can be achieved in which the concentration of enzyme available to combine with a chromogenic peptide substrate, and the rate of amidolysis of that substrate, have reached stable values. In the case of our inhibitors, [EI*] is negligible and, in the absence of competing reactions, the concentration of free inhibitor required to half-saturate the enzyme will be approximated by the expression

$$[I_f]_{50\%} = k_3 K_i^*/k_2 . \qquad [1]$$

For values of $k_3 K_i^*/k_2$ greater than 10 times the concentration of enzyme, this ratio is approximated by the experimentally determined I_{50} for an inhibitor.

If enzyme is added to a mixture of substrate and inhibitor, the rate of amidolysis will decline exponentially to the steady state value. The progress curve which traces this approach to the steady state can be described by the equation

$$[PA]_t = [PA]_0 + v_s t - (v_s - v_0)(1 - e^{-kt})/k, \qquad [2]$$

where PA_0 and PA_t are the concentrations of *p*-nitroaniline at zero time and time t, respectively, v_0 and v_s are the initial and the steady state velocities of amidolysis, respectively, and k is a pseudo-first-order rate constant to describe the exponential approach to the steady state. Equations of this sort have been proposed by Cha (*19*) to describe four types of slow binding reversible inhibition (in which dissociation of the EI complex releases intact inhibitor) and by Baici and Gyger-Marazzi (*20*) to describe slow binding reversible competitive inhibition. We have determined that this relationship can also be applied to the scheme of slow binding pseudo-reversible inhibition illustrated above. Under conditions such that $[S]/K_m \gg [I]/K_i^*$, v_0 is effectively independent of $[I]$, and the term k is given by the relationship

$$k = k_3 + (k_2/K_i^*)[I]/(1 + [S]/K_m). \qquad [3]$$

We have determined the values of k_3 and k_2/K_i^* from plots of k vs $[I]$ for compound 5 with HNE, porcine pancreatic elastase (PPE), and α-chymotrypsin. We have

also computed these values for compounds 4, 5, and 6 with HNE by an alternative method, based on the approach of Baici and Gyger-Marazzi (*20*). When enzyme is added to a mixture of substrate and inhibitor, the following equation holds:

$$[PA] = v_0k_3t/k + v_0k_2[I](1 - e^{-kt})/K_i^*(1 + [S]/K_m)k^2. \quad [4]$$

When the enzyme and inhibitor are preincubated prior to the addition of the chromogenic substrate, another expression holds:

$$[PA] = v_0k_3t/k - v_0k_3(1 - e^{-kt})/k^2 \quad [5]$$

In the case of cathepsin G, the enzyme and inhibitor were diluted into a solution of substrate at a concentration above its K_m, so that the final concentration of inhibitor was at least 10 times below the ratio $k_3K_i^*(1 + S/K_m)/k_2$. Under these conditions, $k \sim k_3$ and Eq. [5] simplifies to

$$[PA] = v_st - v_s(1 - e^{-k_3t})/k_3. \quad [6]$$

Determinations of the kinetic constants for complex formation and decomposition were made by monitoring the release of *p*-nitroaniline at 405 nm in an LKB 4050 Ultrospec spectrophotometer for 40 min after addition of 100 μl of enzyme solution to 900 μl of the appropriate buffer containing substrate and inhibitor. Additional determinations were carried out in a ThermoMax Kinetic Microplate Reader (Molecular Devices Co., Menlo Park, CA), by incubating enzyme and inhibitor in 100 μl of buffer in each well of a 96-well microplate at 37°C for 10 min before adding substrate in 50 μl of buffer to initiate the reaction. Release of *p*-nitroaniline was monitored at 405 nm for 3 min to determine v_0 and for 40 min to determine v_s. Specific conditions are listed below:

Human neutrophil elastase (HNE) (ART Biochemicals, Athens, GA): reactions were measured in Dulbecco's phosphate buffered saline (DPBS), pH 7.2, containing 5% (v/v) DMSO and 0.01% Triton X-100. Substrate was methoxy-succinyl-Ala–Ala–Pro–Val–*p*-nitroanilide. Final concentrations were HNE, 4 nM; substrate, 400 μM; CE-0265, 1–5 μM; CE-0266, 0.1–0.6 μM, and CE-0267, 0.02–0.1 μM.

Human neutrophil cathepsin G (ART Biochemicals): 240 nM, succinyl-Ala–Ala–Pro–Phe–*p*-nitroanilide, 1 mM in 0.1 M Hepes, 0.1 NaCl, 10 mM $CaCl_2$, 0.005% Triton X-100, 5% DMSO buffer, pH 7.5.

Bovine trypsin (Sigma, 3× cryst, TPCK treated): reactions were measured in 0.05 M Tris/0.15 M NaCl, pH 9.0, containing 5% DMSO (*21*) (as modified by Kabi, Franklin, OH). Substrate was benzoyl-Ile–Glu–Gly–Arg-*p*-nitroanilide (S-2222, Kabi). Final concentrations were trypsin, 5 nM; substrate, 100 μM; CE-0266, 2–10 μM.

Bovine α-chymotrypsin (Sigma, 3× cryst.): reactions were measured in 0.1 M Tris/0.15 M NaCl, pH 8.0, containing 5% DMSO (*22*). Substrate was succinyl-Ala–Ala–Pro–Phe–*p*-nitroanilide (Sigma). Final concentrations were chymotrypsin, 1.5 nM; substrate 0.88 μM, CE-0266, 50–300 μM.

Porcine pancreatic elastase (PPE, Elastin Products Co.): reactions were measured in 0.025 M Tris, pH 8.0, containing 0.01% Triton X-100 and 5% DMSO (*23*).

Substrate was succinyl-Ala–Ala–Ala–*p*-nitroanilide (Sigma). Final concentrations were PPE, 15 nM; substrate, 4.5 mM; CE-0266, 0.1–0.5 μM.

Human thrombin (Sigma): reactions were measured in 0.1 M sodium phosphate buffer, pH 7.4, containing 5% DMSO (*24*). Substrate was D-Phe–Pip–Arg–*p*-nitroanilide (S-2238, Kabi). Final concentrations were thrombin, 5 nM; substrate, 100 μM; CE-0266, 1–10 μM.

Human plasmin (Sigma): reactions were measured in 0.05 M Tris/0.15 M NaCl, pH 7.4, containing 5% DMSO (*25*). Substrate was D-Val–Leu–Lys–*p*-nitroanilide (S-2251, Kabi). Final concentrations were plasmin, 10 nM; substrate, 600 μM; CE-0266, 1–10 μM.

Cholinesterase (human erythrocyte, Sigma) and pseudocholinesterase (human serum, Sigma): reactions were measured in DPBS, pH 7.2, containing 5% DMSO. Substrate was propionylthiocholine, with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) added to detect free thiocholine (Sigma). Cholinesterase or pseudocholinesterase (0.01 U/ml) and CE-0266 (20 μM) were incubated at 24°C for 10 min. To 100 μl of this incubated reaction mixture was added 50 μl of substrate (4 mM) and DTNB (0.25 mM). Formation of 5-thio-2-nitrobenzoate was monitored at 405 nm for 3 min.

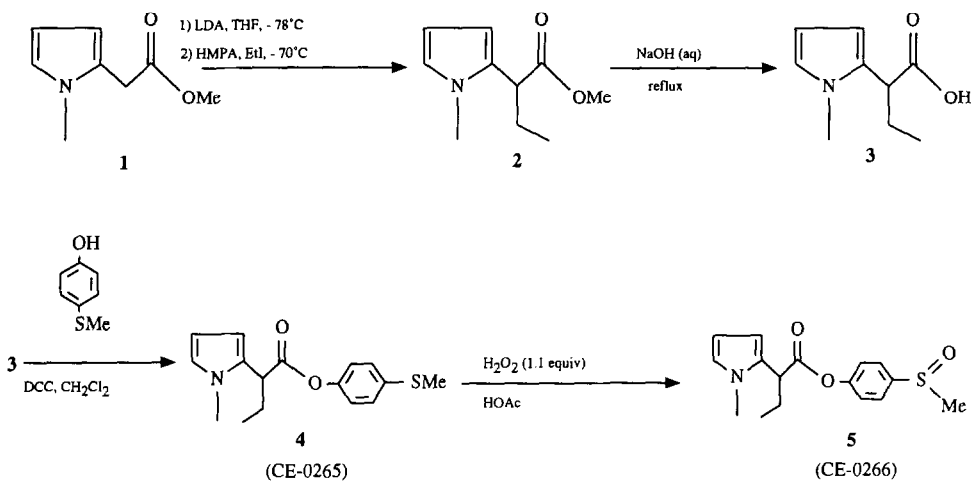
HPLC analysis of reaction of CE-0266 with HNE. HPLC was performed in a similar manner to that described in the stability experiment (vide infra), with a modification of the elution conditions. A shallow 2 → 10% acetonitrile gradient (20 min) was used. CE-0266 (1.6 mM) was dissolved in a 2.6 mM KH_2PO_4, 10.42 mM Na_2HPO_4, 0.145 M NaCl buffer, pH 7.2 (phosphate-saline).

Stability protocol. Equal volumes of plasma and working stock CE-0266 (3 mM) were placed into 1.5-ml Eppendorf microfuge tubes. Tubes were incubated for 0, 5, 10, 15, 30, and 60 min at 37°C. At each time point, samples were rapidly frozen in a dry ice/ethanol bath and stored at 20°C. For HPLC analysis, samples were thawed, filtered through nylon membranes (Rainin), and applied to HPLC (20 μl) using an isocratic 10% acetonitrile in 0.18 M ammonium acetate system, pH 6.8. The half-life ($t_{1/2}$) of CE-0266 was determined by calculation of the compound's area for each time point.

RESULTS AND DISCUSSION

Synthetic Chemistry

The synthetic route utilized in the preparation of the esters **4** and **5** is illustrated in Scheme 1. The pyrrolylacetate derivative **1** is available commercially. Treatment of **1** with lithium diisopropylamide (LDA) generated an insoluble lithium enolate, which was solubilized by addition of HMPA. Addition of iodoethane to the resulting homogeneous solution afforded the alkylated derivative **2** in high yield. Hydrolysis of ester **2** with refluxing aqueous sodium hydroxide provided the key intermediate 2-(1-methyl-2-pyrrolyl)butyric acid (**3**). Esterification of the acid **3** with 4-(methylmercapto)phenol in the presence of dicyclohexylcarbodiimide gave the sulfide **4** (CE-0265). The sulfide **4** was oxidized smoothly to the sulfoxide **5**



SCHEME 1

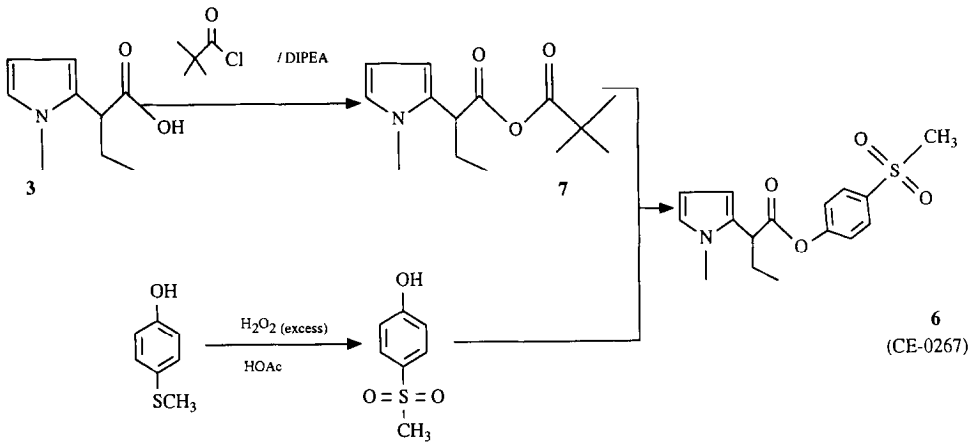
(CE-0266) by hydrogen peroxide in acetic acid. The attempted oxidation of **4** or **5** to the sulfone **6** (CE-0267) with excess hydrogen peroxide resulted in decomposition, presumably by attack on the pyrrole ring.

The synthesis of the sulfone derivative **6** is depicted in Scheme 2. The mixed anhydride **7** was formed by reaction of **3** with pivaloyl chloride in the presence of diisopropylethylamine (DIPEA). Treatment of **7** with 4-(methylsulfonyl)phenol **8** and DIPEA provided the desired sulfonyl derivative **6**. Compound **8** was synthesized from commercially available 4-(methylmercapto)phenol by oxidation with excess hydrogen peroxide.

Enzymology

Since inhibitors **4**, **5**, and **6** are esters, formation of an acyl-enzyme intermediate with liberation of the phenolic leaving group was anticipated. In order to support this mechanism, the following experiment was performed. A solution of HNE (0.1 mM) in 0.05 M ammonium phosphate buffer (pH 7.5) was treated with compound **5** (0.15 mM) and incubated for 2 min at 25°C. The reaction was stopped by the addition of the irreversible HNE inhibitor 3,4-dichloroisocoumarin (3,4-DCI) (0.1 mM) (*26*). A control experiment was carried out in which 3,4-DCI was added prior to compound **5**. The reactants and products were analyzed by HPLC. Approximately the stoichiometric amount of the leaving group, with respect to HNE, was released when compound **5** was mixed with HNE before the addition of 3,4-DCI. Only marginal release of the leaving group was observed in the control experiment.

Inhibition of HNE by compound **5** represents a general situation where catalytic processing of an alternative substrate generates an intermediate (most probably an acyl enzyme, in this case) that turns over catalytically but breaks down so slowly that time-dependent inhibition ensues. The inhibition curves were analyzed as described under Experimental Procedures. The standard kinetic constants, $k_2/$



SCHEME 2

K_i^* and k_3, for **4**, **5**, and **6** are presented in Table 1. Compound 5 (CE-0266) is a potent inhibitor of HNE with a value of k_2/K_i^* of 1.5×10^4 M^{-1} s^{-1} and a ratio $k_3 K_i^*/k_2$ of 36 nM. Compound 6 (CE-0267), with a highly activated leaving group, associates with HNE even more rapidly, with a value of k_2/K_i^* of 8×10^4 M^{-1} s^{-1} and a ratio $k_3K_i^*/k_2$ of 8 nM. Smoke solution (*13*) has no effect on the anti-elastase activity of CE-0266 and CE-0267.

CE-0266 displays good selectivity toward HNE and PPE (see Table 2). CE-0266 exhibited a slight inhibition of α-chymotrypsin, although this interaction is 30–40 times weaker than that observed toward HNE. Interestingly, k_3 values for HNE, PPE, α-chymotrypsin, and Cat G were similar, $0.5–1.3 \times 10^{-3}$ s^{-1}.

Compound **5** showed acceptable stability in 50% human plasma with a $t_{1/2}$ of 50 min. It is hydrolyzed 10 times faster in 50% rat plasma. Compound **6**, with the strongly activating 4-methylsulfonyl substituent, is hydrolyzed rapidly ($t_{1/2}$ = 5 min) in 50% human plasma and was viewed as unacceptable for subsequent *in vivo* experiments.

Low molecular weight, synthetic inhibitors of HNE may become important therapeutic entities for a variety of applications. In particular, compounds that can

TABLE 1

Kinetic Constants of Compounds 4, 5, and 6 for HNE

Compound	k_2/K_i^* ($\times 10^{-3}$ M^{-1} s^{-1})	k_3 ($\times 10^3$ s^{-1})	$k_3K_i^*/k_2$ (nM)
4	2.2 ± 0.2	0.51 ± 0.01	232 ± 1
5	14.8 ± 0.8	0.53 ± 0.03	36 ± 1
6	79.6 ± 10.9	0.59 ± 0.02	8 ± 1

TABLE 2

Specificity of Compound 5 (CE-0266)

Enzyme	$k_3K_i^*/k_2$ (μM)
HNE	0.038
PPE	0.026
α-Chymotrypsin	1.23
Cathepsin G	49
Trypsin	NE[a]
Plasmin	NE[a]
Thrombin	NE[a]
Cholinesterase	NE[a]
Pseudocholinesterase	NE[a]

[a] NE = No effect at $[I] \leq 10$ μM. In the absence of any inhibition at these concentrations one may assume that the value of $k_3 K_i^*/k_2$ for these protease must be greater than 100 μM.

remain active in an oxidant-rich environment offer considerable advantages over endogenous oxidant-sensitive protein antiproteases when the inflammatory process is deemed to be a significant contributor to the morbidity and mortality attendant to the pathophysiology in question. Compounds such as the ones described herein offer such an alternative.

Compound **5** (CE-0266) was evaluated for its ability to reduce myocardial infarct size in a canine model of ischemia/reperfusion injury. The results of those studies are being published elsewhere (*27*).

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