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Abstract

Cathepsin D, a lysosomal aspartic protease, has been suggested to play a role in the metastatic potential of several types of cancer A high activated cathepsin D level in breast tumor tissue has been associated with an increased incidence of relapse and metastasis. High levels of active cathepsin D have also been found in colon cancer, prostate cancer, uterine cancer, and ovarian cancer. Hydroxyethyl isosteres with cyclic tertiary amine have proven to be clinically useful as inhibitors of aspartyl proteases, such as cathepsin D and the HIV-1 aspartyl protease. Also cathepsin D has recently been associated with the development of Alzheimer's disease. Specific proteinase inhibitors, useful in investigations of the mechanisms and pathways of intracellular protein degradation, could lead to the development of therapeutic agents for treatment of many types of carcinomas as well as Alzheimer's disease. The design and the synthesis of (hydroxyethyl)amine isostere inhibitors with the cyclic tertiary amines is described. The IC_{50} and apparent Ki values for several cathepsin D inhibitors are reported.

Introduction

A number of drugs currently in clinical use exert their action by inhibiting a specific enzyme, the target enzyme, present either in the tissues of the individual under treatment, or those in the invading organism. Proteolytic enzymes (proteases) are involved in many biological functions and deteriorative diseases (Handsley and Edwards, 2005; Zamorano et al., 2005). Among the most biologically important proteases are aspartyl proteases. Many serious medical problems, such as cardiac disease (Soylu et al., 2004), acquired immunodeficiency syndrome (AIDS); (Bonhoeffer et al., 2004), Alzheimer's disease (Bornebroek and Kumar-Singh, 2004), malaria (Bjelic and Aqvist, 2004), as well as colorectal and breast cancer (Wiedswang et al., 2004), and pancreatic cancer (Shen et al., 2004), either result directly from, or are characterized by, uncontrolled aspartyl protease activity. For example, the HIV-1 aspartyl protease, which is responsible for the maturation of HIV into the infectious viral particles (Darke and Huff, 1994), has become an important therapeutic target for treatment of AIDS (Johnson et al., 2004; Harrigan et al., 2005).

Cathepsin D is an aspartyl protease that is very similar to the HIV-1 aspartyl protease in substrate specificity. Cathepsin D is normally restricted to the lysosomes where it is involved in normal protein degradation. However, high levels of active serum cathepsin D are often found in many cancer patients (Vetvicka, 2004), and also in patients with advanced Alzheimer's disease (Li et al., 2004). Cathepsin D is clearly involved in the process of tumor invasion and metastasis (Wang and Lin, 2004). In fact, blood tests are given to many cancer patients where cathepsin D is measured as a prognostic indicator in several cancers, including breast cancer (Fan et al., 2004), bladder cancer (Gontero et al., 2004), and lung cancer (Vetvicka, 2004).

The first step in the development of a new drug is the discovery or synthesis of a library of compounds with a desirable biological activity (Berwowitz and Katzung, 2001). A large number of these compounds are selected for cell studies. Many are then eliminated at this point due to poor cell permeability. Factors such as shape, size, polarity, solubility, lipophilicity, and pKa of the compounds effect cell permeability. Only those compounds that prove most effective in cell studies are carried on to animal studies. In animal studies poor bioavailability, pharmacokinetics, or pharmacodynamics eliminate many other compounds as potential drug candidates. Therefore, the larger the initial library of compounds with the desirable biological activity, the more likely a viable drug candidate will be found. Also, occasionally, compounds reported in the literature to be good inhibitors of one enzyme constitute important lead compounds for the development of inhibitors of a different but similar enzyme. So, the structure of reported cathepsin D inhibitors can be lead to the design of anti-HIV-1 or anti-malarial agents. We have, therefore, undertaken the development of a library of cathepsin D inhibitors with varying physical properties (solubility, pKa, etc.). The compounds reported in this article are important additions to our earlier work (McConnell, et. al., 2003).

The use of hydroxyethyl isosteres with cyclic tertiary amines has lead to compounds with enhanced oral

bsorption (Smith et al., 1997). The (R)-hydroxyethylamine isert is incorporated as a key component of many clinically sed, highly potent, HIV-1 protease inhibitors. Initially everal compounds that contain hydroxyethyl amine sosteres with flexible alkyl amines were developed Beaulieu et al., 1997), but they suffered limited in vivo half-ives and were not therapeutically useful. Molecular modeling (HYPERCHEM) has shown that a six-member ring forming the tertiary amine is able to orient the backbone of the inhibitor toward a bioactive conformation. This also provides more of a non-peptide functionality which may greatly improve the half-life of the inhibitor in vivo. We have shown by molecular modeling that the phenyl group of a phenyalanine-type hydroxyethylene or hydroxyethyl amine is easily positioned in the S1 site of the HIV-1 aspartyl protease (McConnell et al., 1991, 2003). Other studies show that a bulky amine or amide might fit reasonably well into the S_2 and S_3 sites (Paul et al., 1995). Therefore, we decided to synthesize compounds that contain a peptide portion to accommodate the S_1, S_2, and S_3 subsites and a non-peptide hydroxyethyl isostere portion with a cyclic tertiary amine to accommodate the S_1, S_2, and S_3 enzyme subsites. The general structure of our synthetic target is shown in Fig. 1.

Materials and Methods

All reagents were purchased from either Aldrich, Sigma, or Bachem Chemical Company. Anhydrous solvents were "anhydrous grade" from Aldrich Chemical Company. Dry solvents were distilled from sodium just prior to use. All other solvents were HPLC grade. Thin layer chromatography (TLC) was run on Whatman PE SIL G/UV 250µm silica gel plates. Column chromatography was run on either Aldrich TLC grade silica gel 2-25µm particle size with average pore diameter 60D or Sigma Sephadex LH-20, lipophilic, bead size 20-100µm. The ^{1}H NMR spectra were collected either on a Bruker 200 MHz AC 200 superconducting spectrometer or on a Hitachi 60 MHz R1200 RS NMR spectrometer. ^{1}H NMR of final compounds and major intermediates were collected on the 200 MHz spectrometer, while the spectra of minor intermediates were collected on the 60 MHz NMR spectrometer. The spectral data were processed by NTNMR software produced by TeleMag.

3-(*S*)-BOCamino-4-phenyl–1-*N*-piperazine-2(*S*)-butanol (1a). A solution of 3-(*S*)-*t*-butoxycarbonyl (BOC) amino-4-phenyl-2-(*R*)-oxirane (McConnell et al., 2003); (1.0 g, 3.1 mmol) in 100 mL dry THF was treated with 0.861 g (10 mmol) piperazine. The solution was refluxed for 48 hrs. The mixture was then cooled to room temperature, concentrated under reduced pressure to about one half its volume, and partitioned between ethyl acetate (200 mL) and 5% aqueous sodium potassium tartarate (200 mL) containing 1.0 g NaCl. The organic layer was washed with distilled water (100 mL) and dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure to give a white solid (1.127 g). The crude product was purified by silica gel column chromatography (2.5 cm x 60 cm length) using 60% ethyl acetate/hexanes as the mobile phase to give 0.784 g. TLC (60% ethyl acetate/hexanes) R_f=0.59. ^{1}H NMR (CDCl3/TMS, 200 MHz) ß 0.9895 (9H, s), 1.653 (2H, t), 2.255 (8H, t), 2.445 (2H, d), 3.845 (1H, d of t), 4.3821 (1H, m), 5.015 (1H, m, exchangable), 7.115 (5H,s).

3-(*S*)-BOC-amino-4-phenyl–1-*N*-phenylpiperazine-2-(*S*)-butanol (1b). A solution of 3-(*S*)-*t*-butoxycarbonyl (BOC) amino-4-phenyl-2-(*R*)-oxirane (McConnell et al., 2003); (1.0 g, 3.1 mmol) in 100 mL dry THF was treated with 3.25 g (20 mmol) N-phenylpiperazine. The solution was refluxed for 48 hrs. The mixture was then cooled to room temperature, concentrated under reduced pressure to about one half its volume, and partitioned between ethyl acetate (200 mL) and 5% aqueous sodium potassium tartarate (200 mL) containing 1.0 g NaCl. The organic layer was washed with distilled water (100 mL) and dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure to give a white solid (0.837 g). The crude product was purified by silica gel column chromatography (2.5 cm x 60 cm length) using 60% ethyl acetate/hexanes as the mobile phase to give 0.663 g. TLC (50% ethyl acetate/hexanes) R_f = 0.45. ^{1}H NMR (CDCl3/TMS, 200 MHz) ß 1.003 (9H, s), 1.685 (2H, t), 2.338 (4H, t), 2.498 (2H, d), 2.691 (4 H, t), 3.892 (1H, d of t), 4.491 (1H, m), 4.925 (1H, m, exchangable), 7.135 (5H,s), 7.367 (5 H, s).

3-(*S*)-BOC-amino-4-phenyl–1-*N*-(p-nitrophenyl) piperazine-2-(*S*)-butanol (1c). A solution of 3-(*S*)-*t*-butoxycarbonyl(BOC) amino-4-phenyl-2-(*R*)-oxirane (McConnell et al., 2003); (1.0 g, 3.1 mmol) in 125mL dry THF was treated with 5.18 g (25 mmol) 1-(4-nitro-phenyl)piperazine. The solution was refluxed for 48 hrs. The mixture was then cooled to room temperature, concentrated under reduced pressure to about one half its volume, and partitioned between ethyl acetate (200 mL) and 5% aqueous sodium potassium tartarate (200 mL) containing 1.0 g NaCl. The organic layer was washed with distilled water (100 mL) and dried over anhydrous magnesium sulfate. The solvent was evaporated under reduced pressure to give a white solid (0.717 g). The crude product was purified by silica gel column chromatography (2.5 cm x 60 cm length) using 60% ethyl acetate/hexanes as the mobile phase to give 0.588 g. TLC (60% ethyl acetate/hexanes) R_f = 0.53. ^{1}H NMR (CDCl3/TMS, 200MHz)ß1.002 (9H, s), 1.639 (2H, t), 2.445 (4H, t), 2.501 (2H, d), 3.081 (4 H, t), 3.892 (1H, m), 4.531 (1H, m), 5.035 (1H, m, exchangable), 7.105 (5H,s), 7.557 (2 H, d), 8.015 (2H, d).

3-(*S*)-amino-4-phenyl–1-*N*-piperazine-2-(*S*)-butanol

dihydrochloride (2a). 0.50 g of 1a was dissolved in 100 mL cold (0°C) 4 M HCl in chloroform. The mixture was stirred at 0°C for 1 hour. Cold diethyl ether (300 mL) was added to induce precipitation of the product. The liquid was decanted, and the precipitant was washed twice with cold ether (150 mL). The crude solid was dissolved in 25 mL methanol and then recrystallized by the addition of 300 mL cold ether. The white solid was again washed twice with cold ether (150 mL) and dried under reduced pressure (0.44 g). TLC (10% ethanol/ethyl acetate) $R_f = 0.38$. ^{1}H NMR (D2O, 60MHz) ß 1.9 (2H, t), 2.9 (8H, m), 3.2 (2H, d), 3.7 (1H, m), 4.4 (1H, m), 7.1 (5H,s).

3-(*S*)-amino-4-phenyl–1-*N*-phenylpiperazine-2-(*S*)-butanol dihydrochloride (2b). 0.55 g of 1b was dissolved in 100 mL cold (0°C) 4 M HCl in chloroform. The mixture was stirred at 0°C for 1 hour. Cold diethyl ether (300 mL) was added to induce precipitation of the product. The liquid was decanted, and the precipitant was washed twice with cold ether (150 mL). The crude solid was dissolved in 20 mL methanol and then recrystallized by the addition of 300 mL cold ether. The white solid was again washed twice with cold ether (150 mL) and dried under reduced pressure (0.43 g). TLC (10% ethanol/ethyl acetate) $R_f = 0.43$. ^{1}H NMR (D_2O, 60MHz) ß 2.0 (4H, t), 2.7 (8H, m), 3.0 (2H, d), 3.8 (1H, m), 4.4 (1H, m), 7.1 (5H,s).

3-(*S*)-amino-4-phenyl–1-*N*-(*p*-nitrophenyl) piperazine-2-(*S*)-butanol dihydrochloride (2c). 0.53 g of 1c was dissolved in 100 mL cold (0°C) 4 M HCl in chloroform. The mixture was stirred at 0°C for 1 hour. Cold diethyl ether (300 mL) was added to induce precipitation of the product. The liquid was decanted, and the precipitant was washed twice with cold ether (150 mL). The crude solid was dissolved in 20 mL methanol and then recrystallized by the addition of 300 mL cold ether. The white solid was again washed twice with cold ether (150 mL) and dried under reduced pressure (0.45 g). TLC (10% ethanol/ethyl acetate) $R_f = 0.26$. ^{1}H NMR (D_2O, 60MHz) ß 2.0 (4H, t), 2.8 (8H, m), 3.1 (2H, d), 3.8 (1H, m), 4.4 (1H, m), 7.1 (5H,s), 7.6 (2H, d), 8.1 (2H, d).

General Procedure for Coupling Cbz-dipeptide to 3-(*S*)-amino-4-phenyl-1-*N*-piperazine (*N*-phenyl piperazine or *N*-*p*-nitrophenylpiperazine)-2-(*S*)-butanol (3ak). A precooled solution (-150°C) of the appropriate carbobenzoxy-dipeptide (Sigma) (0.35 mmol) in 10 mL anhydrous DMF was treated with 56 µL (0.40 mmol) triethyl amine. The mixture was allowed to react at -150°C for 30 minutes and was then treated with 34 µL (0.35 mmol) ethyl chloroformate. The mixture was stirred under N_2 atmosphere for 1 hour at -150°C. A precooled (0°C) solution containing 0.32 mmole of either 2a, 2b, or 2c in 25 mL anhydrous DMF and 125µL (1.0 mmole) triethyl amine was then added to the mixed anhydride of the Cbz-dipeptide. The combined mixture was stirred under N_2 at 0°C for 4 hours, allowed to warm to room temperature, and stirred overnight at room temperature. The mixture was partitioned between the layers of ethyl acetate (250 mL) and 0.01 M aqueous NaOH. The organic layer was removed and saved. The aqueous layer was extracted again with another 250 mL ethyl acetate. The organic layers were pooled, washed with distilled water (100 mL), dried over anhydrous magnesium sulfate, and evaporated under reduced pressure.

3-(*S*)-[Cbz-L-alanyl-L-phenylalanylamino]-4-phenyl-1-*N*-piperazine-2-(*S*)-butanol hydrochloride (3a). 0.162 g. TLC (15% ethanol/ethyl acetate) $R_f = 0.19$. ^{1}H NMR (DMSO-d_6 60 MHz) ß 1.9 (4H, m), 2.5 (2H, d), 2.9 (8 H, m), 3.1 (2H, d), 3.7 (1H, m), 4.6 (3H, m), 5.1 (1H, m), 5.4 (2H, s), 6.8 (5H, s), 7.1 (5H,s), 7.4 (5H, s).

3-(*S*)-[Cbz-L-valyl-L-phenylalanylamino]-4-phenyl-1-*N*-piperazine-2-(*S*)-butanol hydrochloride (3b). 0.125 g. TLC (15% ethanol/ethyl acetate) $R_f = 0.27$. ^{1}H NMR (DMSO-d_6 60 MHz) ß 1.3 (6H, d), 1.9 (4H, t), 2.5 (3, m), 2.7 (2H, d), 2.9 (8H, m), 3.2 (2H, d), 3.7 (1H, m), 4.6 (3H, m), 5.0 (1H, m), 5.4 (2H, s), 6.8 (5H, s), 7.1 (5H,s), 7.4 (5H, s).

3-(*S*)-[Cbz-L-leucyl-L-phenylalanylamino]-4-phenyl-1-*N*-piperazine-2-(*S*)-butanol hydrochloride (3c). 0.131 g. TLC (15% ethanol/ethyl acetate) $R_f = 0.34$. ^{1}H NMR (DMSO-d_6 60 MHz) ß 1.3 (6H, d), 1.8 (4H, m), 1.9 (3H, m), 2.7 (2H, d), 3.0 (8H, m), 3.2 (2H, d), 3.7 (1H, m), 4.6 (3H, m), 5.0 (1H, m), 5.4 (2H, s), 6.9 (5H, s), 7.3 (5H,s), 7.5 (5H, s).

3-(*S*)-[Cbz-L-leucyl-L-leucyl]-4-phenyl-1-*N*-piperazine-2-(*S*)-butanol hydrochloride (3d). 0.145 g. TLC (15% ethanol/ethyl acetate) $R_f = 0.43$. ^{1}H NMR (DMSO-d_6 60 MHz)ß 1.4 (12H, d), 1.7 (6H, m), 1.9 (2H, d), 2.9 (8H, m), 3.1 (2H, d), 3.7 (1H, m), 4.5 (3H, m), 4.8 (1H, m), 5.3 (2H, s), 7.0 (5H, s), 7.3 (5H, s).

3-(*S*)-[Cbz-L-alanyl-L-phenylalanylamino]-4-phenyl-1-*N*-phenylpiperazine-2-(*S*)-butanol hydrochloride (3e). 0.122 g. TLC (18% ethanol/ethyl acetate) R_f=0.21. ^{1}H NMR (DMSO-d_6 60 MHz)ß 1.9 (4H, m), 2.3 (3H, d), 3.0 (10H, d), 3.7 (1H, m), 4.5 (3H, m), 4.8 (1H, m), 5.4 (2H, s), 6.8 (5H, s), 7.1 (5H,s), 7.5 (5H, s), 7.9 (5H, s).

3-(*S*)-[Cbz-L-valyl-L-phenylalanylamino]-4-phenyl-1-*N*-phenylpiperazine-2-(*S*)-butanol hydrochloride (3f). 0.115 g. TLC (18% ethanol/ethyl acetate) R_f=0.25. ^{1}H NMR (DMSO-d_6 60 MHz)ß 1.3 (6H, d), 2.0 (5H,mt), 3.1 (10H, m), 3.8 (1H, m), 4.7 (3H, m), 5.0 (1H, m), 5.3 (2H, s), 6.9 (5H, s), 7.2 (5H,s), 7.6 (5H, s).

3-(*S*)-[Cbz-L-leucyl-L-phenylalanylamino]-4-phenyl-1-*N*-phenylpiperazine-2-(*S*)-butanol hydrochloride (3g). 0.112 g. TLC (18% ethanol/ethyl acetate) R_f=0.31. ^{1}H NMR (DMSO-d_6 60 MHz)ß 1.1 (6H, d), 1.5 (3H, m), 2.0 (4H, d), 3.1 (6H, m), 3.3 (4H, t), 3.8 (1H, m), 4.7 (3H, m), 5.0 (1H, m), 5.3 (2H, s), 6.8 (5H, s), 7.2 (5H,s), 7.6 (5H, s).

3-(*S*)-[Cbz-L-leucyl-L-leucyl]-4-phenyl-1-*N*-phenylpiperazine-2-(*S*)-butanol hydrochloride (3h). 0.112 g. TLC (18% ethanol/ethyl acetate) R_f=0.31. ^{1}H NMR (DMSO-d_6 60 MHz) ß 1.3 (12H, d), 1.6 (6H, m), 2.0 (2H, d),

0 (6H, m), 3.3 (4H, t), 3.7 (1H, m), 4.7 (3H, m), 5.0 (1H, ı), 5.3 (2H, s), 6.9 (5H, s), 7.3 (5H,s), 7.6 (5H, s).

3-(*S*)-[Cbz-L-alanyl-L-phenylalanylamino]-4-phenyl--*N*-(*p*-nitrophenyl)piperazine-2-(*S*)-butanol ydrochloride (3i). 0.102 g. TLC (20% ethanol/ethyl cetate) $R_f = 0.26$. ^{1}H NMR (DMSO-d6 60 MHz)ß 2.0 (4H, n), 2.2 (3H, d), 3.0 (6H, d), 3.5 (4H, t), 3.7 (1H, m), 4.5 (3H, n), 4.8 (1H, m), 5.4 (2H, s), 6.8 (5H, s), 7.1 (5H,s), 7.5 (5H, s), 8.0 (2H, d), 8.4 (2H, d).

3-(*S*)-[Cbz-L-valyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*p*-nitrophenyl)piperazine-2-(*S*)-butanol hydrochloride (3j). 0.122 g. TLC (20% ethanol/ethyl acetate) $R_f = 0.29$. ^{1}H NMR (DMSO-d6 60 MHz) ß 1.3 (6H, d), 2.0 (5H, m), 3.0 (6H, m), 3.5 (4H, t), 3.8 (1H, m), 4.7 (3H, m), 5.0 (1H, m), 5.3 (2H, s), 6.9 (5H, s), 7.2 (5H,s), 7.6 (5H, s), 8.0 (2H, d), 8.4 (2H, d).

3-(*S*)-[Cbz-L-leucyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*p*-nitrophenyl)piperazine-2-(*S*)-butanol hydrochloride (3k). 0.108 g. TLC (20% ethanol/ethyl acetate) $R_f = 0.33$. ^{1}H NMR (DMSO-d6 60 MHz) ß 1.1 (6H, d), 1.5 (3H, m), 2.0 (4H, d), 3.1 (6H, m), 3.4 (4H, t), 3.8 (1H, m), 4.7 (3H, m), 5.0 (1H, m), 5.3 (2H, s), 6.8 (5H, s), 7.2 (5H,s), 7.5 (5H, s), 8.1 (2H, d), 8.5 (2H, d).

3-(*S*)-[Cbz-L-leucyl-L-leucyl]-4-phenyl-1-*N*-(*p*-nitrophenyl)piperazine-2-(*S*)-butanol hydrochloride (3l). 0.912 g. TLC (20% ethanol/ethyl acetate) $R_f = 0.35$. ^{1}H NMR (DMSO-d6 60 MHz) ß 1.3 (12H, d), 1.6 (6H, m), 2.0 (2H, d), 3.0 (6H, m), 3.5 (4H, t), 3.7 (1H, m), 4.7 (3H, m), 5.0 (1H, m), 5.3 (2H, s), 6.9 (5H, s), 7.3 (5H,s), 7.6 (5H, s), 8.1 (2H, d), 8.5 (2H, d).

General Procedure for Preparation of 3-(*S*)-[Acetyldipeptide-amino]-4-phenyl-1-*N*-piperazine (or phenylpiper azine or *p*-nitrophenylpiperazine)-2-(*S*)-butanol hydro chloride (4a-l). A solution of the carbobenzy protected compound (3a-l); (0.20 mmol) in 250 mL methanol and 1 mL 0.01 M aqueous HCl was treated with 0.050 g pre-moistened 10% Pd-C to form a slurry in a 3 neck flask. H_2 gas was bubbled (1 atm) through the rapidly stirring mixture at room temperature for 3 hours. The mixture was then filtered to remove the catalyst, and the solvent was evaporated under reduced pressure. The crude amine hydrochloride was dissolved in 15 mL dimethyl sulfoxide (DMSO) and treated with 125 µL (0.10 mole) triethyl amine. The mixture was stirred at room temperature for 30 minutes. Acetic anhydride (95 µL, 1.0 mmol) was added, and the mixture was stirred overnight at room temperature. Cold diethyl ether (250 mL) was added to precipitate the product. The liquid was decanted, and the white solid was washed three times with cold ether (200 mL). The crude product was purified by Sephadex LH-20 column chromatography (column size 5 cm dia. x 80 cm) using methanol as the mobile phase.

3-(*S*)-[Acetyl-L-alanyl-L-phenylalanylamino]-4-phenyl-1-*N*-piperazine-2-(*S*)-butanol hydrochloride (4a). 0.56 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) R_f=0.46. ^{1}H NMR (methanol-d4 200 MHz)ß 2.071 (4H, d), 2.165 (3H, d), 2.279 (3H, s), 2.462 (4H, m), 2.992 (4H, t), 3.189 (2H, d), 3.465 (4H, t), 3.716 (1H, m), 4.610 (3H, m), 5.081 (1H, m), 6.949 (5H, s), 7.210 (5H,s).

3-(*S*)-[Acetyl-L-valyl-L-phenylalanylamino]-4-phenyl-1-N-piperazine-2-(*S*)-butanol hydrochloride (4b). 0.49 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) R_f= 0.51. ^{1}H NMR (methanol-d_4 200 MHz)ß 1.689 (6H, d), 1.989 (1H, m), 2.087 (4H, d), 2.266 (3H, s), 2.458 (4H, m), 3.099 (2H, d), 3.477 (4H, t), 3.720 (1H, m), 4.613 (3H, m), 4.999 (1H, m), 6.959 (5H, s), 7.203 (5H,s).

3-(*S*)-[Acetyl-L-leucyl-L-phenylalanylamino]-4-phenyl-1-N-piperazine-2-(*S*)-butanol hydrochloride (4c). 0.46 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) R_f = 0.66. ^{1}H NMR (methanol-d_4 200 MHz)ß 1.298 (6H, d), 1.690 (2H, m), 1.990 (1H, m), 2.077 (4H, t), 2.199 (3H, s), 2.459 (4H, m), 3.102 (2H, d), 3.479 (4H, t), 3.721 (1H, m), 4.614 (3H, m), 4.993 (3H, m), 6.960 (5H, s), 7.213 (5H,s).

3-(*S*)-[Acetyl-L-leucyl-L-leucyl]-4-phenyl-1-N-piperazine-2-(*S*)-butanol hydrochloride (4d). 0.49 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) R_f = 0.71. ^{1}H NMR (methanol-d_4 200 MHz)ß 1.308 (12H, d), 1.694 (4H, m), 1.990 (2H, m), 2.077 (2H, t), 2.201 (3H, s), 2.461 (4H, m), 3.109 (2H, d), 3.519 (4H, t), 3.724 (1H, m), 4.634 (3H, m), 5.013 (3H, m), 6.977 (5H, s), 7.243 (5H,s).

3-(*S*)-[Acetyl-L-alanyl-L-phenylalanylamino]-4-phenyl-1-N-phenylpiperazine-2-(*S*)-butanol hydrochloride(4e). 0.61 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) R_f = 0.58. ^{1}H NMR (methanol-d_4 200 MHz)ß 1.999 (4H, d), 2.155 (3H, d), 2.315 (3H, s), 3.156 (2H, d), 3.257 (4H, d), 3.431 (4H, t), 3.715 (1H, m), 4.615 (3H, m), 4.998 (3H, m), 6.999 (5H, s), 7.242 (5H,s), 8.045 (5H, s).

3-(*S*)-[Acetyl-L-valyl-L-phenylalanylamino]-4-phenyl-1-N-phenylpiperazine-2-(*S*)-butanol hydrochloride (4f). 0.74 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) R_f = 0.63. ^{1}H NMR (methanol-d_4 200 MHz)ß 1.652 (6H, d), 2.009 (4H, d), 2.160 (1H, m), 2.396 (3H, s), 3.180 (2H, d), 3.264 (4H, d), 3.401 (4H, t), 3.722 (1H, m), 4.625 (3H, m), 4.999 (3H, m), 6.989 (5H, s), 7.301 (5H,s), 8.066 (5H, s).

3-(*S*)-[Acetyl-L-leucyl-L-phenylalanylamino]-4-phenyl-1-N-phenylpiperazine-2-(*S*)-butanol hydrochloride (4g). 0.78 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) R_f = 0.69. ^{1}H NMR (methanol-d_4 200 MHz)ß 1.409 (12H, d), 1.650 (6H, m), 2.020 (4H, d), 2.297 (3H, s), 3.162 (2H, d), 3.255 (4H, d), 3.411 (4H, t), 3.725 (1H, m), 4.622 (3H, m), 4.997 (3H, m), 6.996 (5H, s), 7.300 (5H,s), 8.088 (5H, s).

3-(*S*)-[Acetyl-L-leucyl-L-leucyl]-4-phenyl-1-N-phenylpiperazine-2-(*S*)-butanol hydrochloride (4h). 0.69 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) R_f = 0.72. ^{1}H NMR (methanol-d_4 200 MHz)ß 1.308 (12H, d), 1.694 (4H, m), 1.990 (2H, m), 2.077 (2H, t), 2.300 (3H, s), 3.261 (2H, d),

3.375 (4H, d), 3.509 (4H, t), 3.724 (1H, m), 4.634 (3H, m), 5.013 (3H, m), 6.977 (5H, s), 7.243 (5H,s), 8.097 (5H, s).

3-(*S*)-[Acetyl-L-alanyl-L-phenylalanylamino] -4-phenyl-1-*N*-(p-nitrophenyl)piperazine-2-(*S*)-butanol hydrochloride (4i). 0.45 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) $R_f = 0.42$. ^1H NMR (methanol-d_4 200 MHz)ß 1.999 (4H, d), 2.155 (3H, d), 2.315 (3H, s), 3.156 (2H, d), 3.257 (4H, d), 3.491 (4H, t), 3.715 (1H, m), 4.615 (3H, m), 4.998 (3H, m), 6.999 (5H, s), 7.242 (5H,s), 8.055 (2H, d), 8.444 (2H, d).

3-(*S*)-[Acetyl-L-valyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*p*-nitrophenyl)piperazine-2-(*S*)-butanol hydrochloride (4j). 0.55 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) $R_f = 0.43$. ^1H NMR (methanol-d_4 200 MHz)ß 1.652 (6H, d), 2.009 (4H, d), 2.160 (1H, m), 2.396 (3H, s), 3.180 (2H, d), 3.264 (4H, d), 3.500 (4H, t), 3.722 (1H, m), 4.625 (3H, m), 4.999 (3H, m), 6.989 (5H, s), 7.301 (5H,s), 8.077 (2H, d), 8.467 (2H, d) .

3-(*S*)-[Acetyl-L-leucyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*p*-nitrophenyl)piperazine-2-(*S*)-butanol hydrochloride (4k). 0.56 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) $R_f = 0.45$. ^1H NMR (methanol-d_4 200 MHz)ß 1.409 (12H, d), 1.650 (6H, m), 2.020 (4H, d), 2.297 (3H, s), 3.162 (2H, d), 3.255 (4H, d), 3.521 (4H, t), 3.725 (1H, m), 4.622 (3H, m), 4.997 (3H, m), 6.996 (5H, s), 7.300 (5H,s), 8.091 (2H, d), 8.444 (2H, d).

3-(*S*)-[Acetyl-L-leucyl-L-leucyl]-4-phenyl-1-*N*-(*p*-nitrophenyl)piperazine-2-(*S*)-butanol hydrochloride (4l). 0.39 g. TLC (1-butanol/H_2O/acetic acid, 15/2/1) $R_f = 0.54$. ^1H NMR (methanol-d_4 200 MHz) ß 1.309 (12H, d), 1.695 (4H, m), 1.991 (2H, m), 2.078 (2H, t), 2.302 (3H, s), 3.261 (2H, d), 3.344 (4H, d), 3.619 (4H, t), 3.725 (1H, m), 4.635 (3H, m), 5.011 (3H, m), 6.987 (5H, s), 7.244 (5H,s), 8.099 (2H, d), 8.455 (2H, d).

Cathepsin D Assay. The potency of compounds 4a-l was measured as inhibitors of the cathepsin D hydrolysis of human hemoglobin (Sigma), and the results are presented in Table 1. Inhibition of cathepsin D was measured (Yasuda et al., 1999) by the following method: 225 µL of the inhibitor of appropriate concentration in sodium formate-formic acid buffer (0.50 M, pH 3.2) and 250 µL of a 0.5% hemoglobin solution were mixed and incubated at 450°C for 20 minutes. Human cathepsin D (Sigma), 25 µL of a 1.0 µg/mL solution, was added and mixed for a total enzyme concentration of 1.1×10^{-9} M. The mixture was incubated at 450°C for 1.5 hours. The reaction was quenched by the addition of 1.0 mL cold 0.3 M trichloroacetic acid. The solution was mixed thoroughly and then chilled in ice for 30 min to allow separation of precipitated protein. The mixture was centrifuged and warmed to 250°C. The liquid was decanted into a quartz cuvette and the absorbance measured at 280 nm. The absorbance of a blank containing no enzyme was subtracted from the reading. The inhibition of the enzyme activity was measured 4 times at 5 or more inhibitor concentrations. The average absorbance of each inhibitor concentration was utilized in the calculations of the IC_{50} values. All absorbances were within #0.002 standard deviations from the mean for a given inhibitor concentration. The standard error for the linear regression plots was in each case less than 3%. A plot of percent inhibition versus the log of the inhibitor concentration provided a value for the 50% inhibition concentration (IC_{50}). All plots were linear through the 50% inhibition value and have slopes ranging from 22 to 40. The apparent inhibition constants, $Ki_{(app)}$, were calculated (Evans et al., 1985; McConnell et al., 1991) as $Ki_{(app)} = IC_{50} - 0.5[E_0]$, where $[E_0]$ is the enzyme concentration.

Results and Discussion

Our synthetic plan of the potential cathepsin D inhibitors involved two phases: (a) preparation of the protected hydroxyethyl amine isostere portion and (b) condensation and deblocking of the peptide and non-peptide portions. The hydroxyethyl amine isosteres were prepared from a tert-butoxycarbonyl-(BOC) chiral amino aminoalkyl epoxide reported earlier (McConnell et al., 2003). Similar chiral aminoalkyl epoxides (with opposite stereochemistry) have been used successfully in the preparation of several HIV-1 aspartyl protease inhibitors with hydroxyethyl amine isosteres (Fassler et al., 1996; Barrish et al., 1994). The 2S,3S epoxide is utilized to prepare HIV-1 protease inhibitors with the desired *R*-hydroxyethyl amine isostere (Fassler et al., 1996; Barrish et al., 1994). However, since the *S*-hydroxyethyl amine isostere is reported to be the more active isomer for cathepsin D inhibition (Kick et al., 1997), we utilized the 2*R*,3*S* protected amino epoxide in our synthesis (Scheme 1). Either piperazine, *N*-phenyl piperazine, or *N*-(*p*-nitrophenyl) piperazine was used as a nucleophile in the preparation of the cyclized tertiary amines. The BOC protecting group was removed from the primary amine with non-aqueous acid (4 M HCl in chloroform).

In the second phase of our synthesis, the Cbz-protected dipeptide was condensed with ethyl chloro formate and then reacted with the basified primary amine of the hydroxyethyl amine isostere portion (Scheme 2). The Cbz protecting group of the resulting compound was then removed and replaced with an acetyl group. The final product was purified by sephadex HP chromatography and characterized by TLC and ^1H NMR.

The twelve synthetic compounds were screened for their cathepsin D inhibition by a spectrophotometric assay (Yasuda et al., 1999) of hemoglobin hydrolysis (Table 1). Modifications in the ring of the hydroxy ethyl tertiary amine appears to have affected the potency of the inhibitors. Those compounds with a phenyl piperazine or *N*-(*p*-

nitrophenyl) piperazine ring (4e-l) show a slightly better cathepsin D inhibition than the compounds without a phenyl group attached to the piperazine (4a-d). Also, the variation in the *N*-terminal amino acid side appears to affect the cathepsin D inhibition. Compounds with an alanine in the P_3 position, were somewhat less effective inhibitors than those compounds with a valine or leucine in the P_3 position. These are general trends observed in the initial screening.

Conclusions

Our synthetic route shows a great deal of promise for the future synthesis of similar hydroxyethyl amine isosteres. The initial screening shows our synthetic compounds to be potent inhibitors of cathepsin D activity. Since a major method for developing new drug candidates is through random screening of large libraries of compounds previously shown to have desirable biological activity (Berwowitz and Katzung, 2001), the inhibitors described in this paper, along with our earlier work, are important contributions to the development of a library of cathepsin D inhibitors. Although many of our cathepsin D inhibitors will be limited in their therapeutic usefulness, due to potential limitations in bioavailability, with a large enough pool of active compounds it is possible that a few of these inhibitors may someday prove to be promising drug candidates for the treatment of cancer. Detailed kinetic data of the synthetic inhibitors will be determined by more sensitive fluorometric techniques (Pimenta et al., 2001) to determine the inhibition mechanism.

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Table 1. Synthetic Inhibitors

No.	Compound Name	IC_{50}, M	$Ki_{(app)}$, M
4a	3(*S*)-[Acetyl-L-alanyl-L-phenylalanylamino]-4-phenyl-1-*N*-piperazine-2(*S*)-butanol	5.0×10^{-7}	4.28×10^{-7}
4b	3(*S*)-[Acetyl-L-valyl-L-phenylalanylamino]-4-phenyl-1-*N*-piperazine-2(*S*)-butanol	3.0×10^{-7}	3.38×10^{-7}
4c	3(*S*)-[Acetyl-L-leucyl-L-phenylalanylamino]-4-phenyl-1-*N*-piperazine-2(*S*)-butanol	1.8×10^{-7}	1.68×10^{-7}
4d	3(*S*)-[Acetyl-L-leucyl-L-leucylamino]-4-phenyl-1-*N*-piperazine)-2(*S*)-butanol	9.5×10^{-7}	8.28×10^{-7}
4e	3(*S*)-[Acetyl-L-alanyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*N*-phenylpiperazine)-2(*S*)-butanol	1.3×10^{-7}	1.28×10^{-7}
4f	3(*S*)-[Acetyl-L-valyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*N*-phenylpiperazine)-2(*S*)-butanol	3.5×10^{-7}	3.38×10^{-7}
4g	3(*S*)-[Acetyl-L-leucyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*N*-phenylpiperazine)-2(*S*)-butanol	1.8×10^{-7}	1.68×10^{-7}
4h	3(*S*)-[Acetyl-L-leucyl-L-leucylamino]-4-phenyl-1-*N*-(*N*-phenylpiperazine)-2(*S*)-butanol	3.5×10^{-7}	1.28×10^{-7}
4i	3(*S*)-[Acetyl-L-alanyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*N*-*p*-NO_2-phenylpiperazine)-2(*S*)-butanol	5.5×10^{-7}	5.49×10^{-7}
4j	3(*S*)-[Acetyl-L-valyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*N*-*p*-NO_2-phenylpiperazine)-2(*S*)-butanol	4.5×10^{-8}	4.38×10^{-8}
4k	3(*S*)-[Acetyl-L-leucyl-L-phenylalanylamino]-4-phenyl-1-*N*-(*N*-*p*-NO_2-phenylpiperazine)-2(*S*)-butanol	4.6×10^{-8}	4.48×10^{-8}
4l	3(*S*)-[Acetyl-L-leucyl-L-leucylamino]-4-phenyl-1-*N*-(*N*-p-NO_2-phenylpiperazine)-2(*S*)-butanol	7.7×10^{-7}	7.69×10^{-7}

Spectrophotometric assays (A_{280}) at pH 3.2 of cathepsin D hydrolysis of hemoglobin. Apparent inhibition constants, $K_{i\ (app)}$, were calculated [29] as $K_{i\ (app)} = IC_{50} - 0.5[E_0]$, where $[E_0]$ is the initial enzyme concentration.

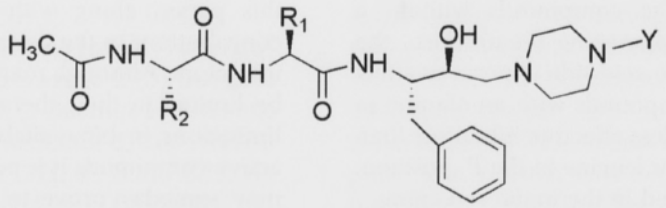
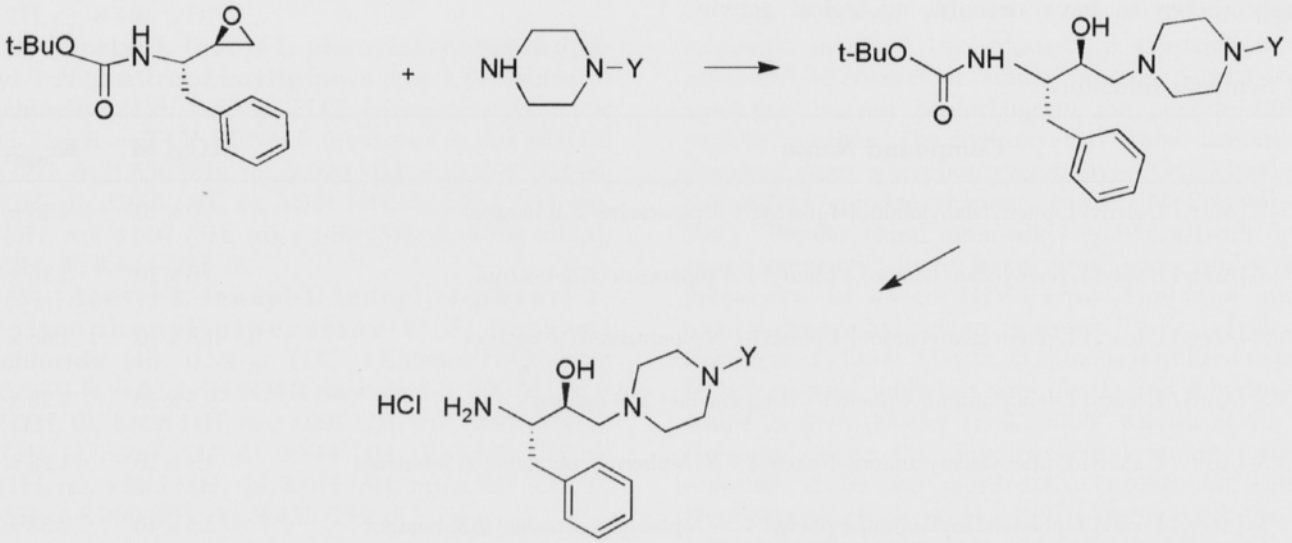
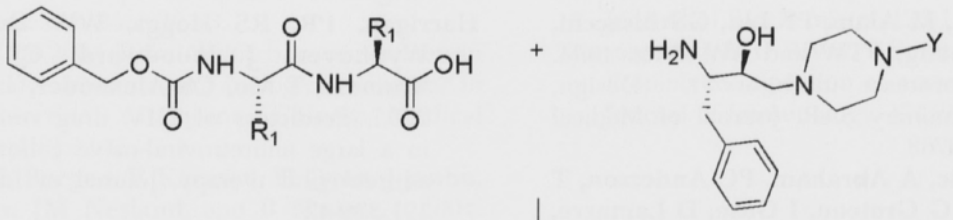


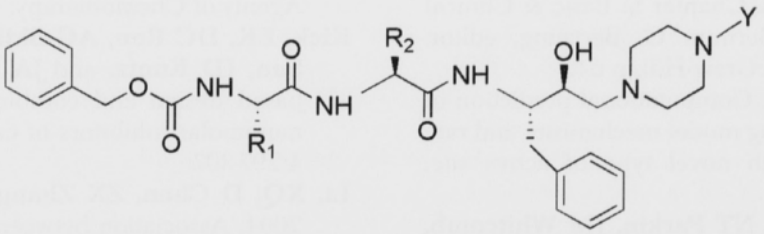
Fig. 1.



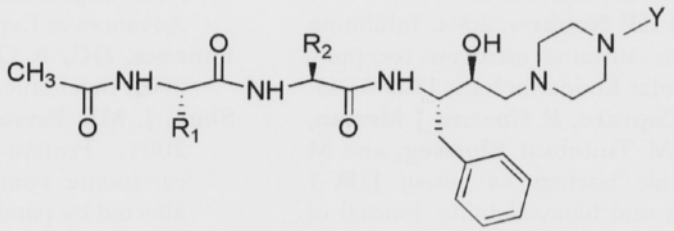
Sch. 1.



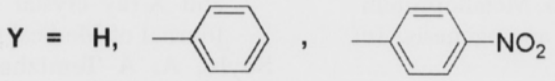
2 a-c



3 a-l



4 a-l



Sch. 1.

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