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Inhibitors of Bacterial *N*-succinyl-L,L-diaminopimelic Acid Desuccinylase (DapE) and Demonstration of in vitro Antimicrobial Activity

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Inhibitors of bacterial *N*-succinyl-l,l-diaminopimelic acid desuccinylase (DapE) and demonstration of in vitro antimicrobial activity

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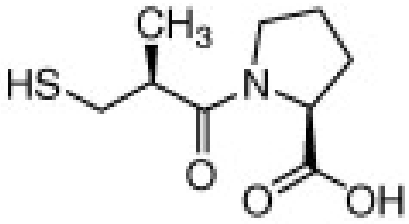
Abstract

The dapE-encoded *N*-succinyl-l,l-diaminopimelic acid desuccinylase (DapE) is a critical bacterial enzyme for the construction of the bacterial cell wall. A screen biased toward compounds containing zinc-binding groups (ZBG's) including thiols, carboxylic acids, boronic acids, phosphonates and hydroxamates has delivered a number of

micromolar inhibitors of DapE from *Haemophilus influenzae*, including the low micromolar inhibitor l-captopril (IC_{50} = 3.3 μM, K_i = 1.8 μM). In vitro antimicrobial activity was demonstrated for l-captopril against *Escherichia coli*.

Graphical abstract

A screen biased toward compounds containing zinc-binding groups (ZBG's) delivered a number of micromolar inhibitors of DapE from *Haemophilus influenzae*, including l-captopril (IC_{50} = 3.3 μM, K_i = 1.8 μM). In vitro antimicrobial activity was demonstrated for l-captopril against *Escherichia coli*.



IC50 = 3.3 μM vs. DapE
Ki =1.82±0.09 μM (competitive)

Keywords

Enzyme inhibition, *N*-Succinyl-l,l-diaminopimelic acid desuccinylase, DapE, l-Captopril, ZBG (zinc-binding group), Antimicrobial

Article

Bacterial infections are a significant and growing medical problem in both the United States and around the world.[1] The CDC recently reported that there are now several strains of *Staphylococcus aureus* that are resistant to all known antibiotics including vancomycin.[2] These cases underscore the fact that limited numbers of drugs are available to prevent a simple *Staph* infections from becoming deadly.[3] At least four other strains of common bacterial species capable of causing life-threatening illnesses (*Enterococcus faecalis*, *Mycobacterium tuberculosis*, *Escherichia coli* O157:H7, and *Pseudomonas aeruginosa*) are already resistant to nearly all drugs in clinicians, arsenal comprised of more than 100 drugs.[4, 5] Thus, the search for new antibiotics that target enzymes in unexplored bacterial biosynthetic pathways is critically important, as confirmed in Supuran's excellent review of bacterial protease inhibitors.[6]

The dapE-encoded *N*-succinyl-l,l-diaminopimelic acid desuccinylase (DapE) enzyme is a member of the *meso*-diaminopimelate (mDAP)/lysine biosynthetic pathway.[7] The amino acids mDAP and/or lysine are essential components of the peptidoglycan cell wall for Gram-negative and most Gram-positive bacteria, providing a link between polysaccharide strands.[7] Therefore, enzymes involved in the mDAP/lysine biosynthetic pathway, including DapE, are potential antibiotic targets.

DapE's are small, dimeric enzymes (41.6 kDa/subunit) that require two Zn(II) ions per mole of polypeptide for full enzymatic activity.[8, 9] On the basis of sequence alignments with other aminopeptidases[10] and several DapE gene sequences, all of the residues that function as ligands in the dinuclear active site of those enzymes are strictly conserved in DapE from *Haemophilus influenzae*. Studies on the E134A- and E134D-altered DapE revealed that E134 acts as the general acid/base in the hydrolysis of the substrate and is absolutely required for catalytic activity.[11] Investigations on H67A- and H349A-altered enzymes together with construction of a three-dimensional homology structure of DapE from *Haemophilus influenzae* (generated using the X-ray crystal structure of the Apo-DapE from *Neisseria meningitidis* as a template with superposition on the structure of the

aminopeptidase from *Aeromonas proteolytica* (AAP)) confirmed the identification of active site histidine zinc ligands.[12] Based on this homology model, the active site of DapE contains two Zn(II) ions at a distance of ~3.30 Å (Fig. 1).

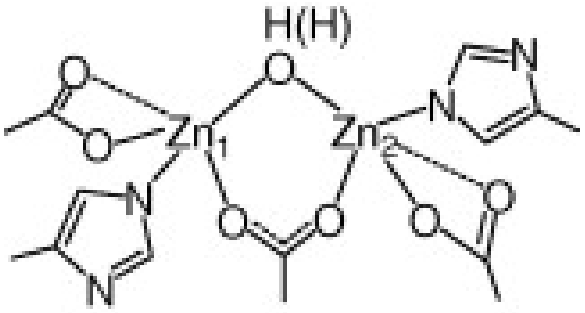
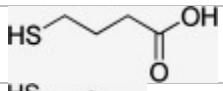
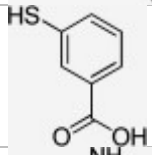
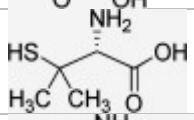
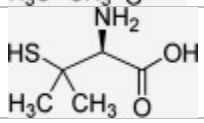


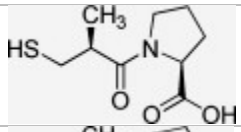
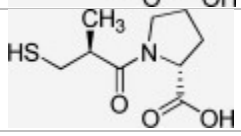
Figure 1. Proposed active site of DapE enzymes.

Each of the Zn(II) ions adopts a distorted tetrahedral geometry and is coordinated by one imidazole group (H67 for Zn1 and H349 for Zn2) and one carboxylate group (E163 for Zn1 and E135 for Zn2). Both Zn(II) ions are bridged by an additional carboxylate groups (D100) on one side and water/hydroxide on the opposite side, forming a (μ-aquo)(μ-carboxylato)dizinc(II) core with one terminal carboxylate and one histidine residue at each metal site.

In order to identify appropriate lead molecules for the inhibition of DapE, we have screened[13] over thirty molecules representing various structural classes and containing different zinc-binding groups (ZBG's) using *N*-succinyl l,l-diaminopimelic acid (l,l-SDAP) as the substrate.[14] These ZBG's include thiols, hydroxamates, carboxylic acids, boronic acids, and phosphates, and this fruitful initial screen has led to the identification of a number of low micromolar inhibitors (Table 1). We biased our initial screen to include bifunctional molecules that contained, in addition to the ZBG, a carboxylate moiety that could interact with the positively-charged lysine and/or arginine side chains that purportedly reside near the active site. Table 1 shows carboxylic acid-containing thiols that were found to inhibit DapE fairly potently. Even delta-mercaptobutyric acid has an IC_{50} of 43 μM versus DapE, and *meta*-mercaptobenzoic acid has a measured IC_{50} of 34 μM. l-Penicillamine gave an IC_{50} of 13.7 μM, and a measured K_i of 4.6 μM. DapE is stereoselective with respect to recognition of inhibitors, as d-penicillamine gave an IC_{50} of 50 μM. Given the success with these carboxylic acid-containing thiols, we turned our attention to captopril, which contains the requisite ZBG and carboxylate functionalities. l-Captopril exhibited an IC_{50} of 3.3 μM and a measured K_i of 1.8 μM (competitive). Again, the binding is stereoselective, as d-captopril[15] was an order of magnitude less potent, with an IC_{50} of 42.0 μM.

Table 1. Thiols tested as inhibitors of DapE

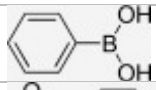
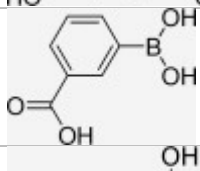
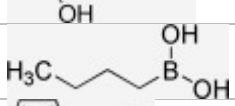
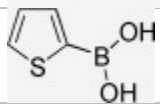
Compound	Structure	IC_{50} (μM)[a]
delta-Mercapto-butyric acid		43 ± 4
3-Mercapto-benzoic acid		34
l-Penicillamine		13.7 K_i = 4.6 ± 0.6 μM (competitive)
d-Penicillamine		50

l-Captopril		3.3 *K*i = 1.82 ± 0.09 µM (competitive)
d-Captopril		42.0 ± 2.5

a. Values are means of three experiments.

Table 2 shows boronic acid derivatives that were tested as inhibitors of DapE. Phenylboronic acid itself was encouraging with an IC_{50} of 107 µM and a measured K_i of 56.9 µM (competitive). Incorporation of a carboxylic acid along with the boronic acid was not productive, as both 4-carboxyphenylboronic acid and 3-carboxyphenylboronic acid did not inhibit DapE. It is hypothesized that geometric constraints may have precluded a productive Coulombic interaction of the carboxylate with positively-charged residues in the active site. Butylboronic acid was a weak inhibitor of DapE (IC_{50} ~10 mM). 2-Thiopheneboronic acid was comparable to phenylboronic acid, with an IC_{50} of 92 µM and a measured K_i of 67 µM, but the inhibitor was noncompetitive. Surprisingly, 1-butaneboronic is only a very weak inhibitor of DapE (IC_{50} ~ 10,000 µM) even though it is a potent inhibitor of AAP (K_i = 10 µM).[16], [17]

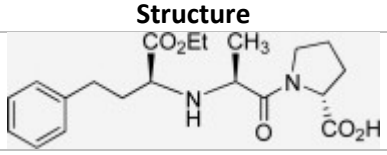
Table 2. Boronic acid tested as inhibitors of DapE

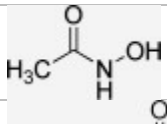
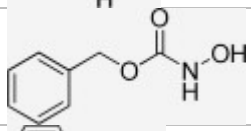
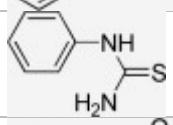
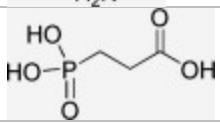
Compound	Structure	IC_{50} (µM)[a]
Phenylboronic acid		107 K_i = 56.9 ± 3.6 µM (competitive)
4-Carboxyphenyl-boronic acid		>10,000
3-Carboxyphenyl-boronic acid		>10,000
Butylboronic acid		~10,000
2-Thiopheneboronic acid		92 K_i = 67 ± 3.8 µM (non-competitive)

a. Values are means of three experiments.

Table 3 shows several other compounds that were screened versus the DapE enzyme. Given the very good potency of the ACE inhibitor l-captopril noted above (IC_{50} = 3.3 µM), the ACE inhibitor enalapril was screened, but did not show any potency versus DapE. Two simple hydroxamate compounds, acetohydroxamic acid and *N*-(benzyloxycarbonyl)hydroxylamine (actually an *N*-hydroxy carbamate) were also screened but were too weak to measure the inhibitory potency. *N*-Phenylthiourea could potentially function as a zinc-binding compound and showed some inhibition of DapE, but the IC_{50} is greater than 100 µM. Phosphonic acids can also inhibit metalloproteases, and (2-carboxyethyl)phosphonic acid was shown to have a very weak IC_{50} of 1620 µM.

Table 3. Other potential inhibitors explored as potential inhibitors of the DapE enzyme

Compound	Structure	IC_{50} (µM)[a]
Enalapril (maleate salt)		>1000

Aceto-hydroxamic acid		>1000
N-(Benzyloxy-carbonyl)hydro xylamine		>1000
N-Phenyl-thiourea		>100
(2-Carboxyethyl)-phosphonic acid		1620 K_i = 800

a. Values are means of three experiments.

Several alkyl and aryl phosphates have also been tested in addition to diaminopimelic acid amides and ornithine amides (structures not shown). In all cases, these molecules exhibited little or no inhibitory potency (IC_{50} >1000 µM to >10,000 µM). Finally, (d,l)-(2-phosphonomethyl)-pentanedioic acid did not inhibit DapE at concentrations up to 10 mM. 2-Carboxyethyl phosphonic acid is a significantly better inhibitor (K_i = 800 µM) than phosphonoacetic acid, 3-phospho-propanoic acid, or *N*-(phosphonomethyl)glycine, thus presenting an optimal chain length for interactions with DapE. We expect an optimal distance between thiol and carboxylate in the thiol series as well, depending on conformational mobility of the series.

We have confirmed antibiotic activity with the DapE inhibitor, l-captopril, in an antibiotic plate assay as illustrated in the following photos (Fig. 2). Application of l-captopril directly to plates cultured with *Escherichia coli* showed a dose–responsive antibiotic activity for this DapE inhibitor. Very little inhibition is observed for 1 mg of l-captopril, but 5 mg demonstrates a clear positive antibiotic result, and the zone of inhibition is even greater for 20 mg of l-captopril. Furthermore, this confirms that the enzyme inhibitor is crossing the bacterial cell membrane and reaching the desired target. The positive antibiotic control ampicillin is shown as well showing its zone of inhibition.

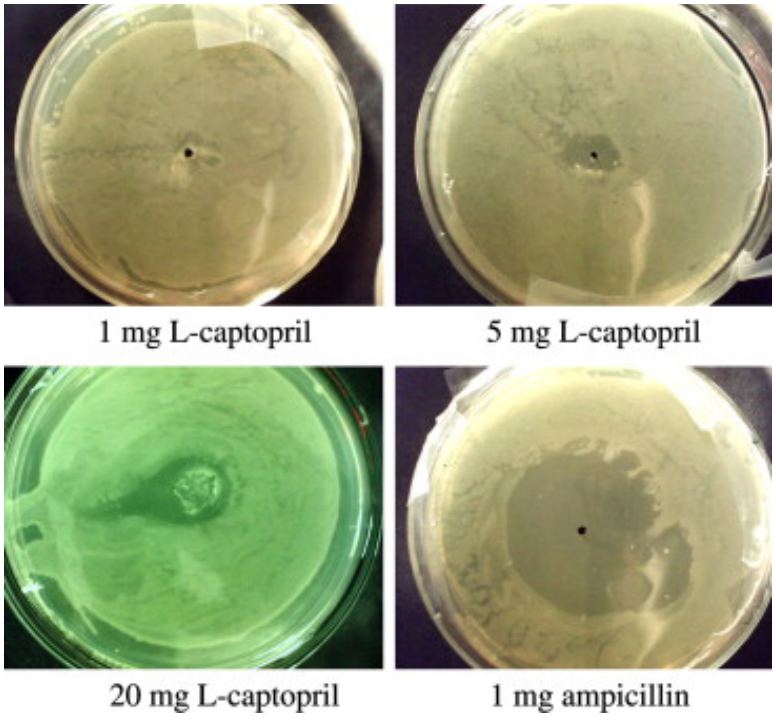


Figure 2. Antibiotic activity of l-captopril against *E. coli.*

In summary, a screen of compounds containing ZBG's against DapE from *Haemophilus influenzae* has delivered a number of micromolar inhibitors including captopril, functioning as a competitive, reversible inhibitor with an IC_{50} of 3.3 μM (K_i = 1.8 μM). Furthermore, antibiotic activity has now been demonstrated for the DapE inhibitor l-captopril. Captopril is an excellent lead for medicinal chemistry optimization in consideration of its low molecular weight (217) and low measured log *P* of 0.34[18, 19] following the rules of Lipinski[20] and containing few rotatable bonds,[21] consistent with its known oral bioavailability.

Acknowledgments

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Supplementary data

Materials and Methods

Materials. D,L-α,ε-DAP (98% pure), succinic anhydride and ion exchange resin (Dowex 50WX8-200, H^+ form) were purchased from Sigma, 2-naphthalenesulfonic acid 99% was from Acros Organics. Cellulose for column chromatography was purchased from Fluka. L-penicillamine, D-penicillamine, 4-mercaptobutyric acid were purchased from Chem-Impex International, (Wood Dale, IL)and L-captopril from MP Biomedicals Inc. (Solon, OH). D-Captopril was prepared according to the method of Smith.{{1595 Smith,Elizabeth M. 1988; }} All other chemicals used in this study were purchased from Sigma and were of the highest quality available.

Synthesis of N-succinyl-diaminopimelic acid (L,L-SDAP). The D,D- and L,L-isoforms of DAP were separated from the D,L- isoform using the method described by Bergmann and Stein (*16*). SDAP was synthesized using the procedure described by Lin *et al*. (*17*) providing an overall yield of 41% (1.84g; 5.7mmol). NMR (D_2O, 270MHz), δ (ppm) 1.25-1.45 (m, 2H), 1.55-1.90 (m, 4H), 2.29-2.52 (m, 4H), 3.64 (dd, 1H), 4.05 (dd, 1H), 4.76 (bs, H_2O + ammonium ion). The L,L- and D,D- SDAP isoforms were separated using an HPLC (Shimadzu SCL-10A VP) with a Chirobiotic T column (250 x 10 mm; Alltec). The isocratic mixture of 20% methanol in water (adjusted to pH 4) was used as the eluting solvent. The two isoforms were present in an approximate 1:1 ratio.

Protein Expression and Purification. The recombinant DapE from *H. influenzae* was expressed and purified, as previously described (Timothy L. Born, Renjian Zheng, and John S. Blanchard (1998) Biochemistry 37:10478–10487) with minor modifications, from a stock culture kindly provided by Professor John Blanchard. Briefly, ~12 grams of cell paste were suspended in 50 mL of 10 mM Tricine buffer, pH 7.8, containing one protease inhibitor tablet (complete protease inhibitor cocktail tablets, EDTA-free, Roche Diagnostic GmbH, Mannheim, Germany). Lysozyme (10 mg) was added to the mixture and stirred for 30 minutes at 4 °C. Cells were lysed by sonication (Sonicator 3000, Misonix, Farmingdale, NY) at 30s intervals, eight repetitions (27W), with 45s break after each sonication. The cell debris was removed by centrifugation for 40 minutes at 12,000 G. DNase (2 mg) was added and after ~20 min. of incubation it was spun again for 15 min. at 18000 RPM. The solution was loaded onto a fast-flow Q-sepharose anion exchange column (XK50, GE Healthcare, Amersham Biosciences Corp., Piscataway, NJ, USA) that had been pre-equilibrated with 10 mM Tricine, pH 7.8. A flow rate of 3 mL per minute was used and a 600-minute linear gradient of NaCl (0.2 to 0.5 M) was used to elute DapE. DapE activity was detected between 0.25 and 0.4 M NaCl. The active fractions were concentrated using an Amicon YM-10 membrane. Purified DapE from *H. influenzae* exhibited a single band on SDS-PAGE indicating M_r = 41,500. Protein concentrations were determined from the absorbance at 280 nm using a molar absorptivity calculated using the method developed by Gill and Hippel (Gill, Stanley C.; Von Hippel, Peter H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. Anal Biochem 182:319–326) (ε_{280} = 36,040 $M^{-1}cm^{-1}$). The protein concentration determined using this molar absorptivity was in close agreement to that obtained using a Bradford assay. Individual aliquots of purified DapE were stored in liquid nitrogen until needed.

Metal removal from altered DapE enzymes. Apo-DapE enzyme was prepared by extensive dialysis for 3 to 4 days against 10 mM EDTA in 50 mM HEPES, pH 7.5. DapE enzyme was then exhaustively dialyzed against metal-free (Chelex-100 treated) 50 mM HEPES, pH 7.5. The presence of any remaining metal ions in solution or bound by DapE were estimated by comparing the activity of the apo-enzyme with a sample that had been reconstituted with Zn(II). DapE enzyme samples incubated with EDTA typically had less than 5% residual activity after dialysis.

Enzymatic assay of DapE. The specific activity, V_{max} (velocity) and K_m (Michaelis constant) of the Zn reconstituted DapE enzyme was determined in triplicate by monitoring amide bond cleavage of L,L-SDAP at 225 nm in Chelex-100 treated 50 mM potassium phosphate buffer, pH 7.5 (DD,LL-SDAP was used as a substrate; ε_{225nm}= 304 M^{-1} cm^{-1}). Enzyme activities are expressed as units/mg where one unit is defined as the amount of enzyme that cleaves 1 mol of substrate at 25 ˚C in 1 min. Catalytic activities were determined with an error of ± 10 %. Initial rates were fit directly to the Michaelis-Menten equation to obtain the catalytic constants K_m=1.75mM and V_{max}=0,027mM/min.

Inhibition Studies. All experiments were performed in 50 mM chelexed potassium phosphate buffer pH 7.6. 3mM solution of DD,LL-SDAP in the same buffer was used for IC50 determination. K_i was determined using 0,5-4,5mM substrate concentrations and different inhibitor concentrations (depending on the inhibitor). Total volume of the mixture was 1ml.

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