Identification of a Potent Peptide Deformylase Inhibitor from a Rationally Designed Combinatorial Library

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Received May 1, 2000

Peptide deformylase catalyzes the removal of the N-terminal formyl group from nascent polypeptides during prokaryotic protein synthesis and maturation and is essential for bacterial survival. Its apparent absence from mammalian organisms makes it an attractive target for designing novel antibacterial agents. Based on the substrate specificity of peptide deformylase from *Escherichia coli*, a focused library of peptide thiols was synthesized on TentaGel resin using a disulfide linkage. Screening of the library against the purified deformylase was carried out in solution phase after the inhibitors were released from the resin with a reducing agent. A potent deformylase inhibitor was obtained from a 750-member library and was further optimized through rational modification into a low nanomolar inhibitor (K_I = 15 nM against *E. coli* deformylase).

Introduction

Combinatorial chemistry has become a powerful method for the discovery and optimization of ligands (peptides, oligonucleotides, and small organic molecules) for a variety of enzymes and protein receptors.[1] A major challenge in designing a small-molecule library is how to choose a set of building blocks so that the resulting library has maximal structural diversity and yet a practically manageable size. An increasingly popular approach, which has proven to be highly successful, is to model the inhibitor structures based upon the substrate (ligand) specificity of the target protein, if such information is already available.[2] Our laboratory has been pursuing a two-step approach to inhibitor design, which is particularly suitable for enzymes and receptors whose physiological substrates/ligands are peptides or proteins, in the absence of prior knowledge about the specificity of a target. During the first stage, a peptide substrate (or ligand) library is constructed and screened against the protein target to identify the most preferred peptide sequence(s). During the second stage, a focused small-molecule library is constructed with a set of building blocks that have been carefully chosen based on the substrate/ligand specificity of the target protein. Here we demonstrate the validity of this approach by the rapid identification of potent peptide deformylase (PDF) inhibitors from a small "rationally" designed library (<1000).

PDF is essential for protein maturation in bacteria by hydrolytically cleaving the N-terminal formyl group from nascent polypeptides.[3] Deletion of the PDF gene from a bacterial genome is lethal.[4] This conserved enzyme in bacteria is, however, apparently absent in mammalian organisms,[5] providing an attractive target for designing novel antibacterial agents. Indeed, we and others have recently shown that PDF inhibitors exhibit potent antibacterial activity against a wide spectrum of Gram-positive and Gram-negative bacteria.[6,7] Therefore, in recent years, there has been a growing interest in developing PDF inhibitors as novel antibiotics.[6−8] In this work, we have designed and synthesized a small focused library of peptide thiols, based on the substrate specificity of *Escherichia coli* PDF. A potent PDF inhibitor has been identified from this library.

Results and Discussion

PDF is a metalloenzyme, which utilizes an Fe^{2+} ion as the catalytic metal.[5,9] The metal ion is tetrahedrally coordinated by the two histidines of a conserved HEXXH motif, the cysteine of a conserved EGCLS motif, and a water molecule.[10,11] The proposed mechanism involves a nucleophilic attack on the substrate formyl group by a metal-bound hydroxide ion to generate a tetrahedral intermediate, which is stabilized by the metal ion and the side chains of PDF active-site residues.[10−12] Although PDF has evolved as a broad-specificity enzyme capable of deformylating polypeptides of diverse N-terminal sequences, it does so at drastically different rates depending on the identity of the four N-terminal amino acid residues.[13] Screening of an N-formylated tetrapeptide library against *E. coli* PDF has revealed a consensus sequence of f-MX(F/Y)Y for efficient substrate deformylation (X = any amino acid).[14] In an attempt to identify specific inhibitors for PDF, an oriented combinatorial library was designed with a 3-mercaptopropionyl core (Scheme 1). We anticipated that the thiol group would occupy the formyl-binding subsite in the PDF active site and provide a high-affinity ligand for the catalytic Fe^{2+} ion.[15] Because PDF strongly prefers a methionine or norleucine at the P_1' position, the side chains of norleucine (**1**), leucine (**2**), and phenylalanine (**3**) were incorporated into the 2-position of the 3-mercaptopropionyl core. The side chains of glycine (**4**) and alanine (**5**) were also incorporated into

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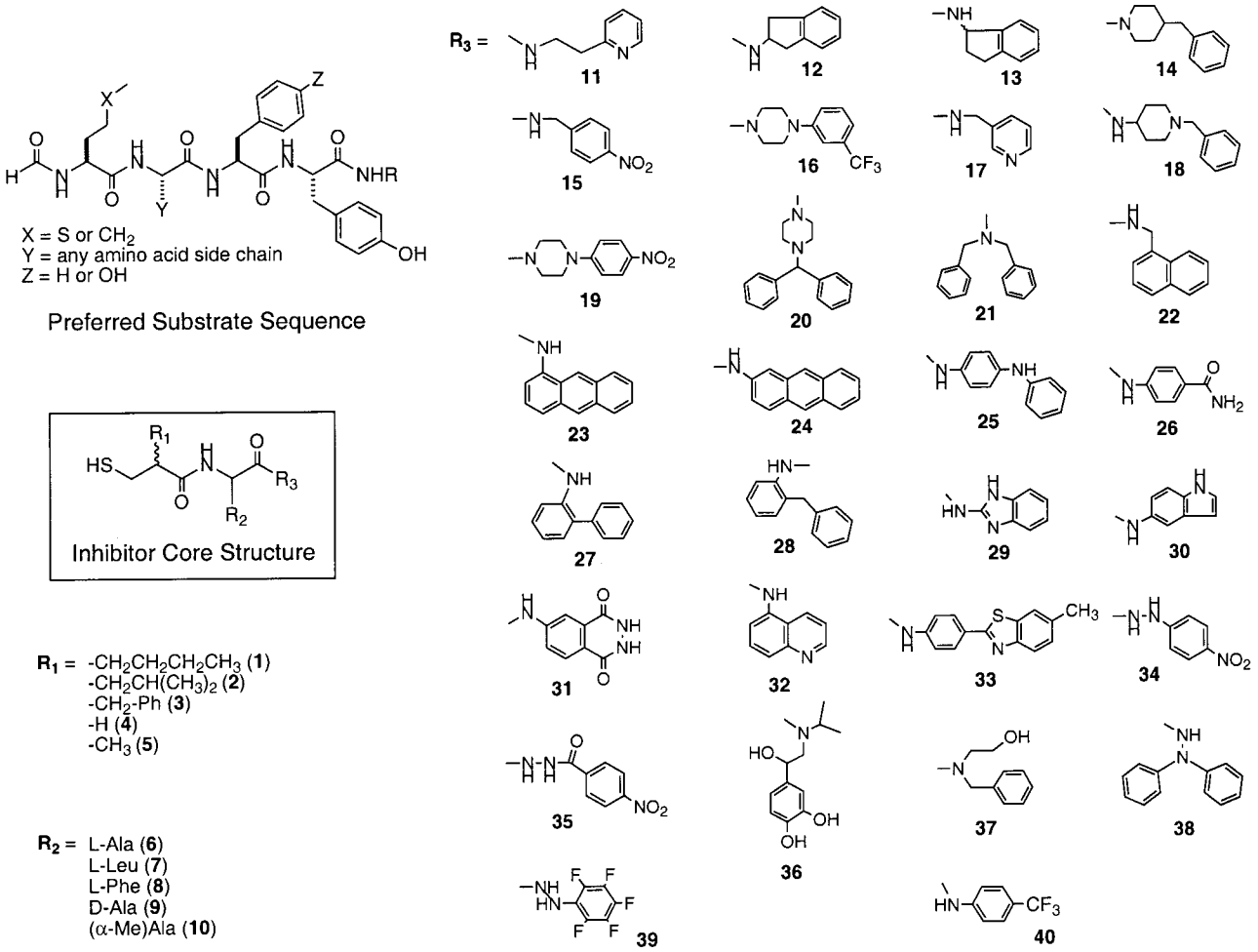
10.1021/cc000036n CCC: $19.00
Published on Web 09/16/2000

Scheme 1

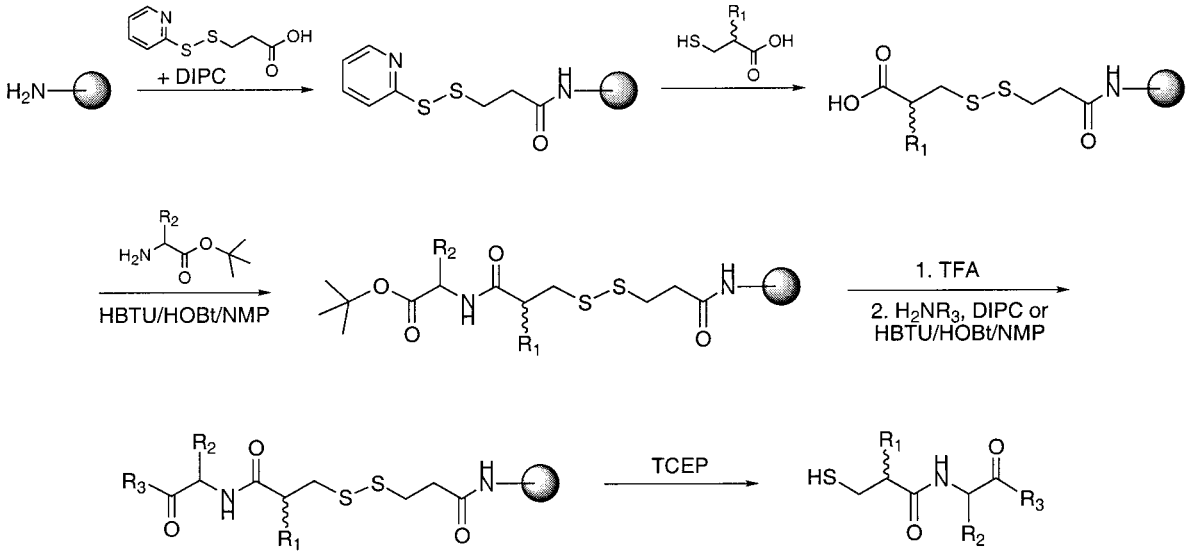


this position as negative controls to demonstrate the validity of the screening method. At the P_2' position, since PDF does not have strong preference for any particular amino acid, L-Ala (**6**), L-Leu (**7**), and L-Phe (**8**) were chosen to simplify the synthetic operations. D-Ala (**9**) and α-methylalanine (**10**) were also added into the library to make the corresponding inhibitors more resistant to proteolytic degradation. At the P_3' and P_4' positions, PDF prefers aromatic side chains presumably for hydrophobic interactions.[14] However, amino acids such as phenylalanine and tyrosine were avoided because the resulting peptides would be susceptible to proteolysis. Instead, 30 commercial aromatic amines of diverse structures (**11**−**40**) were used to mimic the side chains of phenylalanine and tyrosine. This resulted in a library of $5 \times 5 \times 30 = 750$ unique structures, not counting the other set of diastereomers which have the opposite stereochemistry at the P_1' α-position.

The split-pool synthesis method was employed to construct the library on TentaGel S resin.[16] We chose to attach the inhibitors to the resin through a disulfide bond for several reasons. First, the only common feature for all 750 members of the inhibitor library is the presence of a free thiol group which, therefore, serves as the most convenient site of attachment. Second, the disulfide bond is stable to a variety of chemical reagents including all of the reagents used in this work, but is easily converted into the desired free thiol form by reducing agents such as tris(carboxyethyl)phosphine (TCEP). This allows for convenient release of the inhibitors from the solid phase under exceptionally mild conditions. The resulting inhibitor solution (in aqueous methanol) can be directly used in solution-phase assays without further treatment. An added advantage is that TCEP actually acts as a powerful stabilizing agent for PDF, which has a half-life of ∼1 min if without the presence of TCEP or other stabilizing agents.[9,13] Third, attachment at the free thiol group eliminates the need for thiol protection during library synthesis. To this end, a linker, 3-mercaptopropionic acid, was first attached to the resin-bound amine through an amide bond using diisopropylcarbodiimide (DIPC) as the coupling agent (Scheme 2). The resin was split into five equal portions, and five different 3-mercapto-2-alkylpropionic acids were coupled to the resin via a disulfide exchange reaction. Five carboxyl-protected amino acids (as *tert*-butyl esters) were next coupled to the resin using standard peptide chemistry (HBTU/HOBt). Finally, the carboxyl group was deprotected with trifluoroacetic acid (TFA)/anisole (1:1 v/v), and 30 aromatic amines were added to the C-termini using either DIPC or HBTU as the coupling reagent. Double couplings were performed for the addition of the amino acid esters, and the aromatic amines to ensure complete reactions.

The library was screened for PDF inhibition using an iterative strategy for active sequence identification.[17] After cleavage from the resin at the disulfide linkage with TCEP, each of the 30 pools of compounds contained a specific P_3'

Scheme 2



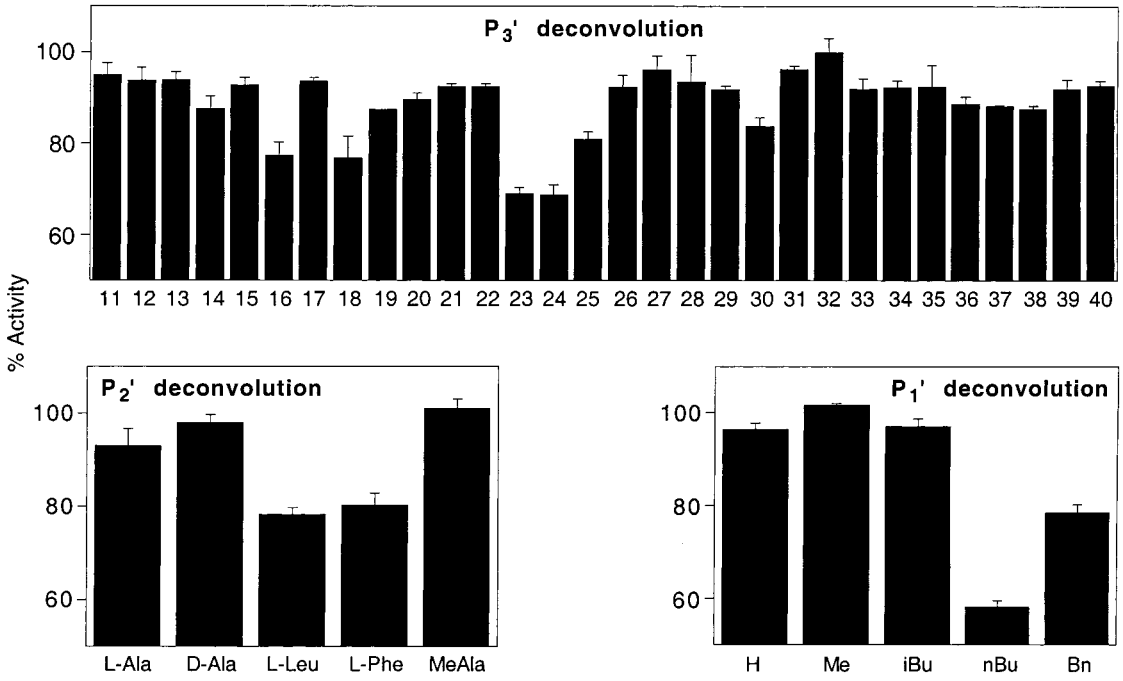
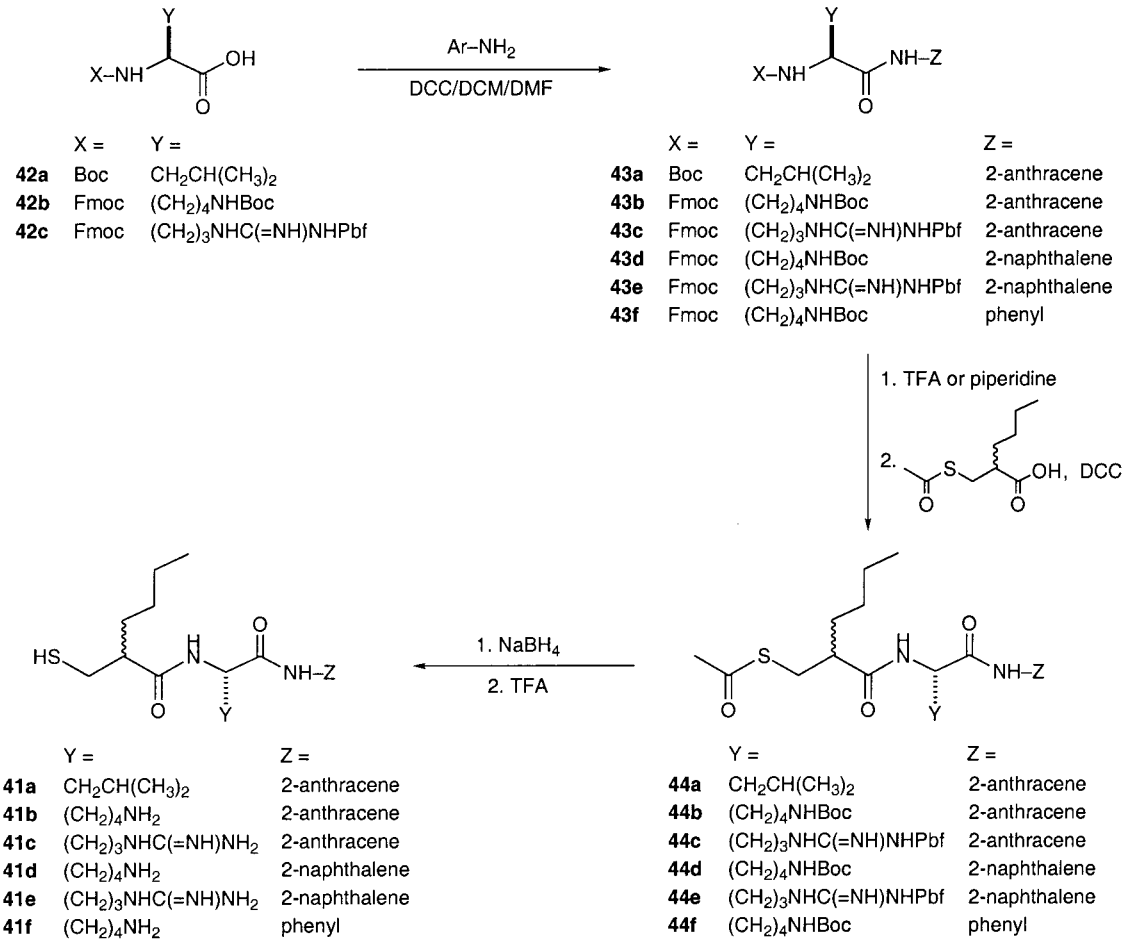


Figure 1. Inhibition of *E. coli* PDF activity by inhibitor pools. The *y*-axis shows the percentage rate for the hydrolysis of f-ML-*p*NA in the presence of each pool (100% in the absence of inhibitor), whereas the *x*-axis indicates the identity of the building blocks. The error bars indicate standard deviation (from a minimum of three measurements). H, hydrogen; Me, methyl; ^{i}Bu, isobutyl; ^{n}Bu, *n*-butyl; Bn, Benzyl.

residue but had a random mixture of the P_1' and P_2' residues (a total of 25). Each pool was individually assayed against *E. coli* PDF at several concentrations using *N*-formylmethionylleucyl-*p*-nitroanilide (f-ML-*p*NA)[18] as substrate. While many of the 30 pools exhibited some inhibition, an order of preference for the P_3' residue was observed: **23**, **24** > **16**, **18** > **25** > **30** > others (Figure 1). The pool containing **24** (2-aminoanthracene) as the P_3' residue was chosen for further deconvolution. Thus, a sublibrary of five separate pools was constructed in which all members contained 2-aminoanthracene (**24**) as the P_3' residue, but each of the five pools had a different P_2' residue and a mixture of five P_1' residues. Evaluation of the five inhibitor pools revealed that L-leucine is most preferred at the P_2' position followed by L-phenylalanine, whereas inhibitors containing other amino acids at this position showed significantly less inhibition. Finally, five inhibitors were synthesized with L-leucine and 2-aminoanthracene as the P_2' and P_3' residues, respectively, but each with a different 3-mercaptopropionyl moiety. Activity assay of the five inhibitors showed that an *n*-butyl group at the 2-position of 3-mercaptopropionyl moiety produced the most potent inhibitor (Scheme 3, compound **41a**), followed by the benzyl group. This is in keeping with the known substrate specificity of this enzyme.[13,14] Similar preferences at the P_1' and P_2' positions were observed when 1-aminoanthracene (**23**) was fixed as the P_3' residue (data not shown).

The most potent inhibitor identified from the combinatorial library, *N*-[(3-mercapto-2-*n*-butyl)propionyl]-L-leucyl-2-anthramide (**41a**), was resynthesized on a large scale via

Scheme 3



	X =	Y =
42a	Boc	$CH_2CH(CH_3)_2$
42b	Fmoc	$(CH_2)_4NHBoc$
42c	Fmoc	$(CH_2)_3NHC(=NH)NHPbf$

	X =	Y =	Z =
43a	Boc	$CH_2CH(CH_3)_2$	2-anthracene
43b	Fmoc	$(CH_2)_4NHBoc$	2-anthracene
43c	Fmoc	$(CH_2)_3NHC(=NH)NHPbf$	2-anthracene
43d	Fmoc	$(CH_2)_4NHBoc$	2-naphthalene
43e	Fmoc	$(CH_2)_3NHC(=NH)NHPbf$	2-naphthalene
43f	Fmoc	$(CH_2)_4NHBoc$	phenyl

	Y =	Z =
41a	$CH_2CH(CH_3)_2$	2-anthracene
41b	$(CH_2)_4NH_2$	2-anthracene
41c	$(CH_2)_3NHC(=NH)NH_2$	2-anthracene
41d	$(CH_2)_4NH_2$	2-naphthalene
41e	$(CH_2)_3NHC(=NH)NH_2$	2-naphthalene
41f	$(CH_2)_4NH_2$	phenyl

	Y =	Z =
44a	$CH_2CH(CH_3)_2$	2-anthracene
44b	$(CH_2)_4NHBoc$	2-anthracene
44c	$(CH_2)_3NHC(=NH)NHPbf$	2-anthracene
44d	$(CH_2)_4NHBoc$	2-naphthalene
44e	$(CH_2)_3NHC(=NH)NHPbf$	2-naphthalene
44f	$(CH_2)_4NHBoc$	phenyl

solution-phase chemistry (Scheme 3). Boc-L-leucine was coupled to 2-aminoanthracene in 1:1 (v/v) dichloromethane/DMF with dicyclohexylcarbodiimide (DCC). After removal of the Boc group with TFA, the resulting amine was acylated with 3-(*S*-acetyl)mercapto-2-(*n*-butyl)propionic acid, followed by reduction of the thioester with sodium borohydride, to afford the desired inhibitor **41a** as a mixture of two diastereomers (which differ at the stereochemistry at the P_1' site). The diastereomeric mixture was assayed for its ability to inhibit *E. coli* PDF using a coupled assay involving f-ML-*p*NA as substrate.[18] Unfortunately, due to the very poor solubility of **41a** in aqueous solutions, detailed kinetic assays were not possible. Based on assays conducted at low inhibitor concentrations, a K_I value of $\sim$1 μM was estimated for the mixture. HPLC analysis of the mixture indicated an approximately 1:1 ratio for the two stereoisomers (not shown). Therefore, the more potent isomer, which presumably has its P_1' residue in the *S* configuration, has an estimated K_I value of $\sim$500 nM (the other isomer is much less potent) (vide infra).

To improve the aqueous solubility as well as potency of **41a**, we have undertaken rational modification of its structure. Our earlier work has shown that an *n*-butyl side at the P_1' site is crucial for substrate/inhibitor binding, whereas the P_2' side chain is much more tolerant to modifications.[6,13,14] X-ray crystal structure of PDF bound to a substrate analogue indicates that the P_2' side chain is mostly solvent exposed.[11d] Thus, the hydrophobic side chain of leucine at the P_2' position was replaced by the more polar side chains of lysine and arginine to give inhibitors **41b** and **41c**, respectively (Scheme 3). Compounds **41b** and **41c** were synthesized in a fashion similar to **41a**, from N^{α}-Fmoc-N^{ϵ}-Boc-L-Lys-OH and N^{α}-Fmoc-N^{γ}-2,2,5,7,8-pentamethyldihydrobenzofuran-5-sulfonyl-L-Arg-OH, respectively. They indeed have much greater solubility in water; it was therefore possible to separate the diastereomeric mixture into pure stereoisomers by preparative HPLC.

Compounds **41b** and **41c** exhibited competitive inhibition against *E. coli* PDF (Figure 2), with K_I values of 110 and 80 nM, respectively, for the more potent diastereomers (Table 1). The other diastereomers of **41b** and **41c** have K_I values of 1050 and 270 nM, respectively. Thus, in addition to improving the aqueous solubility, substitution of polar side chains at the P_2' site also fortuitously increased the inhibitor potency, the reason of which is not yet clear. Meinnel et al. also reported that inclusion of a positively charged side chain at the P_2' position significantly increases the potency of peptide thiol inhibitors against *E. coli* PDF.[8c] We tentatively designate the more potent diastereomers as L-**41a** and L-**41b**, in which the P_1' side chain is in the (*S*) configuration, and the less potent ones as the D-isomers. This assignment is based on our previous observation that (1) PDF does not accept *N*-formyl-D-methionyl peptides as substrate[13] and (2) a phosphonate inhibitor with its P_1' side chain (*n*-butyl) in the L configuration is more potent than the corresponding D-isomer.[8b]

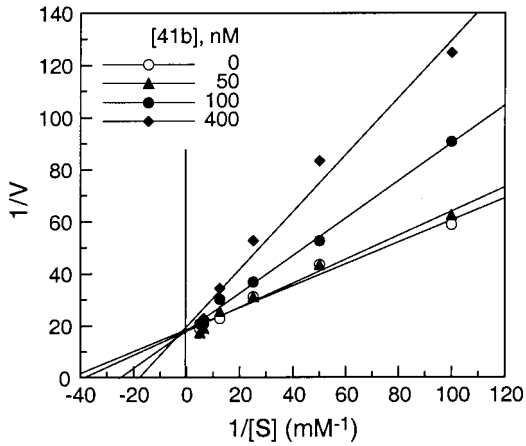


Figure 2. Lineweaver−Burk plot of hydrolysis of f-ML-*p*NA by *E. coli* Fe-PDF in the presence of indicated amounts of inhibitor **41b**. Data were fitted against the Michaelis−Menten equation.

Table 1. Inhibition Constants against *E. coli* PDF

	K_I (nM)	
inhibitor	L-isomer (*S, S*)	D-isomer (*R, S*)
41a	∼500	ND
41b	110 ± 10	1050 ± 110
41c	80 ± 5	270 ± 27
41d	38 ± 2	640 ± 55
41e	15 ± 2	410 ± 68
41f	49 ± 3	490 ± 36

To gain insight into the structure−activity relationship regarding the contribution of the aromatic amine to the overall binding affinity, the anthracene moiety was replaced by the smaller 2-naphthalene (**41d** and **41e**) and phenyl rings (**41f**); the resulting compounds have further improved aqueous solubility. Surprisingly, reduction of the size of the aromatic amine by one ring actually increased the inhibitor potency by 3−5-fold (K_I = 38 and 15 nM for L-**41d** and L-**41e**, respectively) (Table 1). Further truncation of the aromatic moiety to just one ring, however, slightly decreased the inhibitor potency (K_I = 49 nM for L-**41f**). Therefore, an aromatic amine containing two rings appears to be best accommodated by the PDF active site.[8c] Note that the precise nature of the aromatic rings is also critical for inhibitor binding. For instance, the inhibitor pool containing 5-aminoindole (**30**), a close analogue of 2-aminonaphthalene, as the P_3' residue has significantly lower inhibitory activity than either the 1- or 2-aminoanthracene pools (Figure 1). Also, while substitution of *p*-nitroaniline for the 2-aminonaphthalene of **41d** resulted in a more potent inhibitor (K_I = 19 nM against *E. coli* PDF),[6] the inhibitor pools containing *p*-nitrobenzylamine (**15**) or *p*-nitrophenylhydrazine (**34**) as the P_3' residue showed much weaker inhibition than 2-aminoanthracene pool (**24**).

Conclusion

We have demonstrated the validity of a two-stage combinatorial approach to enzyme/receptor inhibitor design. During the first stage, the substrate specificity of the target enzyme/receptor is systematically evaluated by building and screening a combinatorial peptide library. During the second stage, a focused small-molecule library (peptidometics in this case) is constructed using a relatively small set of building blocks, carefully chosen based on the structure of the most preferred substrate(s). This strategy permits one to construct a very small library (750 in this work) of compounds that have high probability of inhibiting the target protein, without any prior knowledge of the three-dimensional structure of the target protein. From a first-generation library of 750 members, we were able to identify an inhibitor that has a K_I value of ∼500 nM against *E. coli* PDF; with minimal modifications, this inhibitor was further optimized to have K_I values in the low nanomolar range (K_I = 15 nM for the most potent variant). These are among the most potent PDF inhibitors that have been reported. Since PDF has been shown to be a promising target for designing novel antibacterial drugs,[4,6,7] these inhibitors may provide useful leads for further development into therapeutic agents. In addition, we have demonstrated that a disulfide bond serves as a convenient linkage for solid-phase synthesis of combinatorial libraries, especially for the synthesis of thiol-containing inhibitors.

Experimental Section

General. TentaGel S NH_2 resin (100 μm), all protected amino acids, and peptide synthesis reagents were purchased from Advanced ChemTech (Louisville, KY). All other chemicals were from Aldrich Chemical (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO). PDF was purified as previously described.[13,19] ^{1}H NMR spectra were recorded on Bruker spectrometers at the indicated field strength, and chemical shifts are given as parts per million (ppm) relative to tetramethylsilane. Mass spectrometric analysis was performed at The Ohio State University Campus Chemical Instrument Center.

Synthesis of 3-(*S*-Acetyl)mercapto-2-alkylpropionic Acids (1−5). These compounds were prepared as described earlier by this laboratory.[6]

Library Synthesis. Linker Attachment. TentaGel S NH_2 resin (100 μm, 0.30 mmol/g loading) was used as the solid support for the inhibitor libraries. Synthesis was carried out on a 2.0 g scale on a homemade library synthesis apparatus. Three equivalents of 3-(2-pyridyldithio) propionic acid[20] and DIPC were added to the resin suspended in 10 mL of dichloromethane. The reaction was complete after being shaken at room temperature for 1 h, as indicated by negative ninhydrin test. After washing with DMF (5 × 5 mL), the resin was manually divided into five equal portions and placed into five separate reaction vessels.

Addition of P_1' Residue. 3-(*S*-Acetyl)mercapto-2-alkylpropionic acids (**1**−**5**) (0.6 mmol) were treated with 2 N NH_3/MeOH (5 mL) at room temperature for 2 h to remove the acetyl group, followed by evaporation to dryness. The residue from each reaction was dissolved in 100 mM sodium phosphate buffer (pH 7.0)/acetonitrile (4.5 mL/0.5 mL) and added to the resin in a different reaction vessel. After 2 h at room temperature, the resin was drained and the amount of 2-thiopyridone released during the disulfide exchange reaction was measured at 343 nm. The results showed quantitative disulfide exchange reaction in all vessels. After washing with acetic acid/dichloromethane/methanol (5:4:1 v/v), the

resin was incubated in the same mixture solvents with shaking for 1 h to protonate the carboxylate group. The beads were then washed exhaustively with dichloromethane to remove any residual acetic acid and methanol. The beads were dried under vacuum, and 50 mg from each vessel was set aside for later use. The rest of the beads from all five reaction vessels were combined, thoroughly mixed, and divided again into five equal portions.

Addition of P_2' Residue. Five amino acid *tert*-butyl esters (**6**−**10**) were coupled to the resin in the five vessels using standard peptide chemistry with HOBt/HBTU/NMP in DMF for 1 h. All reagents were used in 3-fold excess, and the coupling reactions were repeated once to ensure complete reaction. The resin was then washed with DMF and dichloromethane and treated with 5 mL of TFA/anisole (1:1 v/v) for 1 h to deprotect the *tert*-butyl ester. TFA was removed by washing exhaustively with methanol and dichloromethane. After drying under vacuum, a 50 mg portion of resin was removed from each vessel and saved for later use. The remaining resin from all five vessels was combined, thoroughly mixed, and redistributed evenly into 30 reaction vessels (50 mg each).

Addition of P_3' Residue. All arylamines (3 equiv) were coupled to the resin using 3 equiv of DIPC and 0.3 equiv of (dimethylamino)pyridine in 1 mL of dichloromethane/DMF (1:1 v/v) for 1 h. All other amines (3 equiv) were coupled to the resin with 3 equiv of HBTU, HOBt, and NMP in DMF (1 mL). All coupling reactions were repeated once to ensure complete reaction. The resin was washed exhaustively with DMF.

Cleavage of Inhibitors from Solid Phase. The resin from each of the 30 reaction vessels (50 mg, 15 μmol) was suspended in 1.0 mL of MeOH/100 mM sodium phosphate buffer (pH 7.0) (8:1, v/v), and 3 equiv of TCEP was added. After incubation for 1 h at room temperature, the reaction solution was drained into a clean microcentrifuge tube and stored at −20 °C until use.

Library Screening. Since some of the library members also inhibit *Aeromonas* aminopeptidase, the coupling enzyme used in our PDF assay, the assay was carried out in a discontinuous fashion.[18] Briefly, assay reactions (total volume of 1.0 mL) were performed at room temperature in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM NaCl, 1.0 mM TCEP, 50 μM f-ML-*p*NA as substrate, 0.60 μg/mL *E. coli* Fe−PDF, and a total concentration of 10−50 μM inhibitor mixture. The reaction was allowed to proceed for 30 s before being quenched with 10 mM hydrogen peroxide, which inactivates both PDF and the inhibitors (by converting them into inactive disulfide dimers). Any remaining thiols were quenched by the addition of 100 μM *p*-chloromercuribenzoic acid (final concentration) and incubation for 5 min at room temperature. *Aeromonas* aminopeptidase (0.4 unit) was then added to the reaction mixture, and the reaction was incubated for an additional 5 min, followed by absorbance measurement at 405 nm. The percentage of activity remaining was calculated by dividing the absorbance derived from a PDF reaction in the presence of inhibitors by that of the control reaction (no inhibitor). All reactions were performed in triplicates.

After each round of screening, the inhibitor pool that showed the most inhibition (lowest remaining activity) was selected for the next round of deconvolution. Following the coupling of 30 amines at the P_3' position and TCEP cleavage, the 30 individual inhibitor pools were assayed for PDF inhibition (round 1). The amine that gave the most potent inhibitor pool was coupled to the five 50 mg portions of resin that had been saved during the addition of the P_2' residue. The resulting 5 inhibitor pools were again cleaved from the resin and tested for inhibitory activity to identify the most optimal residue at the P_2' position (round 2). Finally, the five 50 mg portions of resin that had been saved during the addition of the P_1' residue were derivatized with the selected, most preferred amino acid and amine at the P_2' and P_3' positions, respectively, to give five individual inhibitors. The five inhibitors were cleaved from the resin and assayed for PDF inhibition to determine the identity of the most preferred P_1' residue (round 3).

***N*-[(3-Mercapto-2-*n*-butyl)propionyl]-L-leucyl-2-anthramide (41a).** t-Boc-Leucine (0.12 g, 0.50 mmol) was reacted with 2-aminoanthracene (0.097 g, 0.50 mmol) using DCC (0.55 mmol) in 8 mL of dichloromethane/DMF (1:1 v/v) for 1 h at room temperature. The precipitate formed was removed by filtration, and the filtrate was diluted with diethyl ether (20 mL) and washed with H_2O (2 × 20 mL). The organic phase was concentrated to dryness, and the product was purified by silica gel chromatography (eluted with 1:2 ethyl acetate/hexane, v/v) to give 0.15 g of an off-white solid (**43a**) (75% yield). Compound **43a** (0.11 g, 0.25 mmol) was dissolved in 3 mL of 1:1 (v/v) TFA/anisole, and the solution was stirred for 1 h at room temperature. The reaction mixture was concentrated in vacuo, and the residue was triturated with hexane (10 mL). Upon standing overnight, an off-white solid was formed. The solid was filtered, washed with hexane (2 × 10 mL), and coupled to 3-(*S*-acetyl)mercapto-2-(*n*-butyl)propionic acid (0.052 g, 0.25 mmol) with HBTU (0.095 g, 0.25 mmol) and triethylamine (0.075 g, 0.75 mmol) in 3 mL of DMF. After 1 h, the reaction was diluted with 10 mL of diethyl ether and washed with H_2O (3 × 10 mL). The organic phase was concentrated to dryness, and the solid was chromatographed on a silica gel column eluted with 1:2 (v/v) ethyl acetate/hexane to give 0.12 g of an off-white solid (**44a**) (93% yield for two steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.05−9.25 (m, 1H), 8.33 (d, J = 12 Hz, 1H), 8.07−8.20 (m, 2H), 7.70−7.90 (m, 3H), 7.35−7.45 (m, 3H), 6.50 (m, 1H), 4.82 (m, 1H), 3.00−3.15 (m, 2H), 2.42−2.52 (m, 1H), 2.13 & 2.32 (s, 3H), 1.45−2.00 (m, 5H), 1.10−1.45 (m, 4H), 0.65−1.10 (m, 9H).

Compound **44a** (20 mg, 0.04 mmol) in 15 mL of anhydrous methanol was treated with $NaBH_4$ (20 mg) for 5 h at room temperature. The reaction was quenched by the addition of 5% HCl and extracted with ethyl acetate (20 mL). The organic phase was dried over $MgSO_4$, and the solvent was evaporated under vacuum to give 15 mg of a white solid (82% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.86 & 8.95 (br s, 1H), 8.20−8.35 (m, 3H), 7.80−8.00 (m, 3H), 7.30−7.45 (m, 3H), 6.27 (m, 1H), 4.74−4.82 (m, 1H), 2.75−2.90 (m, 1H), 2.55−2.70 (m, 1H), 2.30−2.40 (m, 1H), 1.50−2.05 (m, 6H), 1.15−1.40 (m, 4H), 0.65−1.10 (m, 9H). ESI−HRMS

calcd for $C_{54}H_{69}N_4O_4S_2^+$ (disulfide dimer + H^+), 901.4754; found, 901.4825.

***N*-[(3-Mercapto-2-*n*-butyl)propionyl]-L-lysyl-2-naphthylamide (41d).** DCC (100 mg, 0.49 mmol) was added to a solution of N^{α}-Fmoc-L-Lys(Boc)-OH (200 mg, 0.42 mmol) and 2-aminonaphthalene (60 mg, 0.42 mmol) in 5 mL of anhydrous DMF. After stirring for 4 h at room temperature, the mixture was diluted into 40 mL of ethyl acetate, extracted with water (4 × 20 mL) and brine (20 mL), and dried over $MgSO_4$. Evaporation of solvent and silica gel chromatography gave 250 mg of a white solid (**43d**) (98% yield). ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.55 (br s, 1H), 8.10 (s, 1H), 7.10−7.70 (m, 14H), 5.55 (br s, 1H), 4.50 (br s, 1H), 4.30 (d, J = 7.1 Hz, 2H), 3.95−4.15 (m, 2H), 3.06−3.20 (m, 2H), 1.32 (s, 9H), 0.90−1.90 (m, 6H).

Compound **43d** (300 mg, 0.51 mmol) was dissolved in 10 mL of 20% piperidine in DMF, and the mixture was stirred for 3 h at room temperature. Evaporation of solvent and silica gel chromatography gave 100 mg of a yellow solid. The yellow solid was dissolved in 20 mL of dichloromethane, and DCC (100 mg, 0.48 mmol) and 3-(*S*-acetyl)mercapto-2-(*n*-butyl)propionic acid (100 mg, 0.44 mmol) were added to the solution. After being stirred for 4 h at room temperature, the mixture was filtered to remove the white precipitate formed. The filtrate was concentrated and purified by silica gel chromatography to give 110 mg of a white solid (**44d**) (69% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.13 & 9.09 (s, 1H), 8.20 (d, J = 12.0 Hz, 1H), 7.71 (m, 3H), 7.35−7.50 (m, 3H), 6.81 (m, 1H), 4.49 (br s, 1H), 3.89 (m, 1H), 3.00−3.20 (m, 4H), 2.60−2.40 (m, 1H), 2.16 & 2.32 (s, 3H), 1.43 (s, 9H), 1.10−1.90 (m, 12H), 0.88 (t, J = 6.9 Hz, 3H).

Compound **44d** (110 mg, 0.20 mmol) was dissolved in 15 mL of ethanol and treated with sodium borohydride (50 mg, 1.36 mmol) for 6 h at room temperature. The reaction was quenched by the addition of 1 mL of water and several drops of 5% HCl solution. After removal of the solvent, the residue was dissolved in 20 mL of ethyl acetate, washed with brine, dried over $MgSO_4$, and concentrated under vacuum to give a yellowish solid. The yellow solid was dissolved in 20 mL of dichloromethane plus 3 mL of TFA, and the mixture was stirred for 5 h. After removal of the solvent, the residue was triturated with diethyl ether (10 mL) to give 50 mg of a white solid (59% yield in two steps). ^{1}H NMR (250 MHz, CD_3COCD_3) δ 8.27 (br s, 1H), 7.75−7.60 (m, 4H), 7.26−7.33 (m, 3H), 4.50 (br s, 1H), 3.83 (m, 1H), 3.69 (t, J = 7.08 Hz, 2H), 2.50−2.90 (m, 2H), 2.15−2.40 (m, 1H), 1.85−1.65 (m, 4H), 1.60−1.30 (m, 5H), 1.25−1.04 (m, 4H), 0.71 (t, J = 6.75 Hz, 3H). ESI−HRMS calcd for $C_{23}H_{34}N_3O_2S^+$, 416.2366; found, 416.2393.

***N*-[(3-Mercapto-2-*n*-butyl)propionyl]-L-arginyl-2-naphthylamide (41e).** This compound was prepared in a manner similar to **41d**. ^{1}H NMR (250 MHz, CD_3COCD_3) δ 8.22 (br s, 1H), 7.67−7.47 (m, 4H), 7.35−7.23 (m, 3H), 4.63 (m, 1H), 3.56−3.20 (m, 4H), 2.90−2.60 (m, 1H), 1.80−1.65 (m, 4H), 1.60−1.10 (m, 6H), 0.74 (m, 3H). ESI−HRMS calcd for $C_{23}H_{34}N_5O_2S^+$, 444.2428; found, 444.2462.

***N*-[(3-Mercapto-2-*n*-butyl)propionyl]-L-lysyl-2-anthramide (41b).** This compound was prepared in a manner similar to **41d**. ^{1}H NMR (250 MHz, CD_3COCD_3) δ 8.64 (br s, 1H), 8.48−8.43 (m, 2H), 8.12−8.03 (m, 4H), 7.72 (m, 1H), 7.55−7.47 (m, 3H), 4.70−4.45 (m, 1H), 3.90−3.90 (m, 2H), 3.10−2.70 (m, 2H), 2.57 (m, 1H), 2.00−1.80 (m, 4H), 1.70−1.30 (m, 9H), 0.81 (m, 3H). FAB-MS calcd for $C_{27}H_{36}N_3O_2S^+$, 466.2523; found, *m*/*z* (relative intensity) 391.23 (100), 466.25 (3), 929.55 (6) (dimer).

***N*-[(3-Mercapto-2-*n*-butyl)propionyl]-L-arginyl-2-anthramide (41c).** This compound was prepared in a manner similar to **41d**. ^{1}H NMR (250 MHz, CD_3COCD_3) δ 10.19 (m, 1H), 8.36−8.25 (m, 3H), 7.89−7.81 (m, 3H), 7.47 (d, 1H), 7.33−7.09 (m, 3H), 4.39 (m, 1H), 3.64−3.50 (m, 2H), 3.03−2.20 (m, 3H), 1.75−1.25 (m, 6H), 1.20−1.01 (m, 4H), 0.69 (t, J = 7.0 Hz, 3H). FAB-MS calcd for $C_{27}H_{36}N_5O_2S^+$, 494.2584; found, *m*/*z* (relative intensity) 494.24 (66), 736.32 (100), 985.52 (40) (dimer).

***N*-[(3-Mercapto-2-*n*-butyl)propionyl]-L-lysyl-anilide (41f).** This compound was prepared as described earlier by this laboratory.[6]

Resolution of Inhibitor Isomers. Each of the inhibitors (**41a**−**f**) was synthesized as an approximately 1:1 mixture of two diastereomers, as judged by analytical HPLC analysis. These inhibitors were separated into their pure diastereomers by reversed-phase HPLC equipped with a semipreparative C_{18} column, which was eluted with a linear gradient of acetonitrile (20−50% in 60 min) in water plus 0.05% TFA (flow rate 5 mL/min). Baseline resolution was achieved in each case, and each of the resulting pure isomers was essentially free of the other isomer. Inhibitor stock solutions were prepared in methanol, and their concentrations were determined by measuring absorbencies at 365 (for **41a**−**c**) or 280 nm (for **41d**−**e**) and comparing with standard lines generated with 2-acetamidoanthracene or 2-acetamidonaphthalene. The concentration of **41f** was determined based on the sample mass and methanol volume used to prepare the stock solution.

Enzyme Inhibition Assays. Assay reactions (total volume of 1.0 mL) were carried out at room temperature in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM NaCl, 1.0 mM TCEP, 0−200 μM f-ML-*p*NA, 20−200 ng/mL *E. coli* Fe-PDF, and varying concentrations of inhibitor **41a**−**f**. The reaction was allowed to proceed for 10 min at room temperature before being quenched with H_2O_2, and the reaction product was quantitated as described above. K_I values were calculated by the equation

$$V'/V = (K_M + [S])/\{(1 + [I]/K_I)K_M + [S]\}$$

where V' and V are PDF reaction rates in the presence and absence of inhibitors, respectively, whereas [S] and [I] are substrate and inhibitor concentrations used, respectively.

Acknowledgment. This work was supported by the National Institutes of Health (AI40575).

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