Articles

Solid-Phase Library Synthesis, Screening, and Selection of Tight-Binding Reduced Peptide Bond Inhibitors of a Recombinant *Leishmania mexicana* Cysteine Protease B

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A one-bead−two-compound inhibitor library was synthesized by the split−mix method for the identification of inhibitors of a recombinant cysteine protease from *Leishmania mexicana*, CPB2.8ΔCTE. The inhibitor library was composed of octapeptides with a centrally located reduced bond introduced by reductive amination of the resin-bound amines with Fmoc amino aldehydes. The library was screened on solid phase, and less than 1% of the library contained active compounds. The inhibitors displayed great specificity in the subsites flanking the enzyme catalytic triad with Cha and Ile/Leu preferred in P_2, Phe in P_1, Cha and Ile/Leu in P_1', and Ile/Leu in P_2'. Some of the inhibitors were resynthesized, and the kinetics of inhibition were determined in solution-phase assays. Most of the inhibitors had micromolar K_i values, and a few inhibited the enzyme at nanomolar concentrations. One inhibitor, DKHF(CH_2NH)LLVK ($K_i = 1$ μM), was tested for antiparasite efficacy and shown to affect parasite survival with an IC_{50} of approximately 50 μM.

Introduction

The parasite *Leishmania mexicana*, a causative agent of cutaneous leishmaniasis, expresses in a stage-regulated manner high levels of several classes of cysteine proteases (CPs) belonging to the papain family. In *Leishmania mexicana*, one group of cysteine proteases, CPBs (denoting cysteine protease group B),[1] is expressed from a single tandem array comprising 19 gene copies (CPB1−CPB19), with individual genes encoding subtly different isoenzymes.[2] CPBs are thought to be crucial for the survival and infectivity of the parasite in its human host and have been implicated in a number of processes including the successful invasion of host macrophages by promastigotes, the transformation of one parasitic form to another, parasite nutrition, and evasion of the host's immune system.[3−6] Inhibitors of the CPB isoenzymes have been shown to reduce the infectivity of wild-type parasites both in vitro toward macrophages[6,7] and in vivo toward BALB/c mice,[8] thus providing further evidence that these CPB isoenzymes are virulence factors.

Because of their tremendous importance in the etiology of leishmaniasis, the parasite's CPs are attractive targets for therapeutic agents against leishmaniasis. Traditionally, this disease has been treated using three major drug classes: pentavalent antimonials (e.g., sodium stibogluconate, which inhibits the protozoal enzymes required for glycolytic and fatty acid oxidation), aromatic diamidines (e.g., pentamidine, which interferes with nuclear metabolism), and polyene microlide antibiotics (e.g., amphotericin B, which acts on cell membrane sterols and phospholipids). These chemotherapeutic modalities are generally unsatisfactory because of high toxicity[9] and the development of drug-resistant parasites.[10,11]

While several strategies can be invoked for the design and selection of protease inhibitors, the development of cysteine protease inhibitors as drug candidates is greatly facilitated by a combinatorial library approach. Consequently, the one-bead−two-compound fluorescence inhibitor assay for the rapid selection and identification of protease inhibitors was first reported from our laboratory.[12] This assay has been used for the successful identification of inhibitors of serine proteases,[12] cysteine proteases,[13,14] and matrix metalloproteases.[15] In this report, we describe the synthesis and

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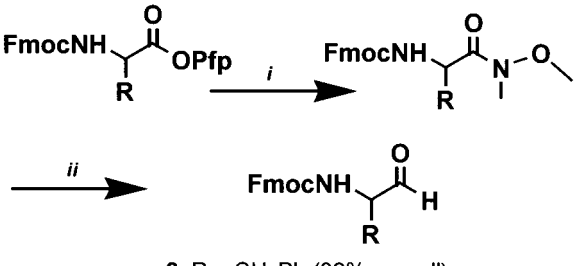
Scheme 1[a]



2: R = CH_2Ph (89% overall)
3: R = $(CH_2)_4NH(Boc)$ (55 % overall)
4: R = $(CH_2)_3NHC(NH)NH(Pmc)$ (52 % overall)

[a] (i) $HN(OCH_3)CH_3 \cdot HCl$, DhbtOH, DIPEA, CH_2Cl_2; (ii) $LiAlH_4$, THF, −78 °C.

screening of a library of potential cysteine protease inhibitors comprising octapeptides with the simplest modification of the scissile amide bond, the reduced peptide bond, at the putative P_1-P_1' bond. Reduced bond peptides are, for the most part, ideal as aspartic protease inhibitors[16−20] because the proteolytically stable secondary amine does not introduce any major structural changes in the peptide, and the resulting peptides tend to bind linearly in the active site such that the tetrahedral intermediate of a good substrate is mimicked. However, this class of compounds has not been utilized as reversible cysteine and serine protease inhibitors, in contrast to aldehydes and ketones (see ref 35 for a review of various classes of protease inhibitors). It is hoped that for cysteine proteases, reduced bond peptides could be inhibitory if the side chains of the amino acids flanking the nonhydrolyzable secondary amine encouraged tight binding of the inhibitor to the enzyme subsites, thus sterically preventing any substrate access to the reactive thiol. Furthermore, the increased flexibility of the secondary amine compared to the amide bond may be advantageous when an induced fit is required for tight binding. We present herein the results from the solid-phase screening of a "one-bead−two-compound" reduced bond octapeptide inhibitor library with CPB2.8ΔCTE,[21] an isoform of CPB cysteine protease from *Leishmania mexicana* lacking the C-terminal domain.

Results and Discussion

Synthesis of Fmoc Amino Aldehydes. Fmoc amino aldehydes were synthesized by a modification of the method of Fehrentz and Castro[22] wherein the acid was first converted to the Weinreb amide by reaction with *O*,*N*-dimethylhydroxylamine and subsequently reduced to generate the aldehyde (Scheme 1). In our case, we elected to synthesize the amide intermediate from the pentafluorophenyl ester, thereby circumventing the potential problems and reduced yields associated with the separation of the product from the byproducts of the carbodiimide, uronium, or phosphonium salts typically used for activation of the acid. The yields for the formation of the amide were 95% for Lys, quantitative for Phe, and somewhat lower for Arg (75%). The amide was then reduced by treatment with $LiAlH_4$ at −78 °C according to literature procedure.[23] The yield for the reduction of the Phe analogue was 90%, whereas the yields for Arg and Lys were 69% and 58%, respectively. The reduced yield was due to unreacted starting material and loss of side chain protecting groups.

Optimization of Library Reductive Amination Conditions. (Experimental details are in Supporting Information). Reductive alkylation of resin-bound amines using Fmoc- or Boc-protected amino aldehydes has been successfully carried out on solid phase by several groups since the seminal work of Coy and co-workers.[24,25] Typically, a single aldehyde is reacted to a single amine nucleophile and libraries of reduced-bond-containing compounds are synthesized through parallel arrays of compounds that may be cleaved such that mixtures of compounds result, for example, in the synthesis of linear and cyclic peptides libraries of enkephalin surrogates.[26] In the case of "true" libraries synthesized by the split−mix method,[27,28] for the introduction of the reduced bond, each Fmoc amino aldehyde is reacted simultaneously, in separate vessels or compartments, with several thousand different amine nucleophiles that are each attached to a resin particle (see ref 29 for an early example). While solid-phase synthesis has the advantage of driving a reaction to completion, in the case of reductive alkylation, a balance must be sought between reaction completeness and overalkylation. It was therefore decided to first optimize the conditions for reductive amination before synthesizing the library. For the optimization process, three different minilibraries of resin-bound amines were initially synthesized: (Ala, Arg, Leu, Gln, Cys, Pro, His)-IMP-Pll-K(Alloc)-PEGA resin; (Asn, Met, Val, Lys, Ser, Trp)-IMP-Pll-K(Alloc)-PEGA resin, and (Thr, Ile, Asp, Glu, Phe, Gly, Tyr)-IMP-Pll-K(Alloc)-PEGA resin. Reductive alkylation of these minilibraries with Fmoc-protected Phe, Arg, and Lys aldehydes was conducted under different conditions where the number of equivalents of both the aldehyde and the borohydride, the reaction time, temperature, and solvent were varied. Reaction completeness was assessed by the Kaiser test and by MALDI-TOF mass spectrometry. Mass spectrometry proved to be a useful tool in expediting the assessment of reaction completeness by comparison of the peak heights of the starting material, products, and byproducts. Since both the reactant and the product have identical peptide spacers, their ionization and flight properties are similar. Therefore, the relative product-to-reactant signal intensities represent a pseudoquantitative measure of reaction progress. In obtaining pseudoquantitative measurements from MALDI-TOF MS spectra, signal intensities are thought to be as accurate as peak areas for gauging the extent of reaction,[30] and these were used in the assessment of reaction completeness. However, as additional proof, we compared the determination of reaction completeness using both MS and HPLC of the cleaved products (data not shown). The results were similar for both methods. Analysis of the three minilibraries indicated that some amino acids, particularly, Val, Ile, Cys and Pro, were difficult to reductively alkylate completely. There was also evidence for the double alkylation of Gly. The conditions that eventually gave the most satisfactory results were the use of 7.0 equiv of the Fmoc aldehyde reacting first for 45 min at 50 °C and followed by the addition of 10.5 equiv of $NaCNBH_3$ for 150 min at 50 °C. In our hands, the best solvent for the reaction was a mixture of DMF/MeOH/TEOF (1:1:1) containing 1% acetic acid, since it was effective in properly solvating the peptide and in remov-

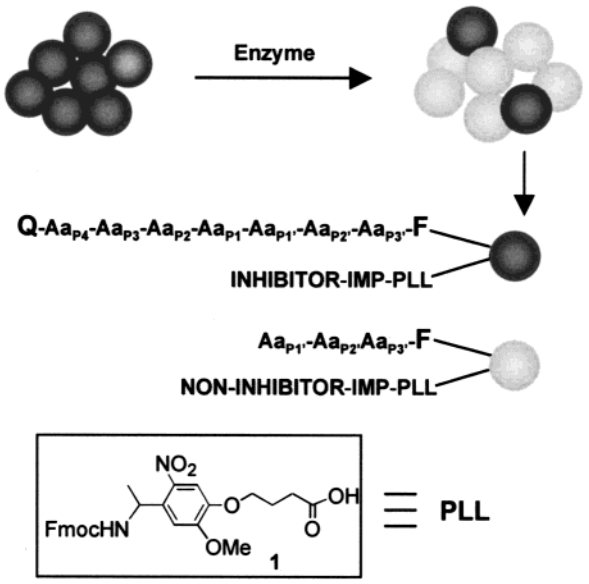
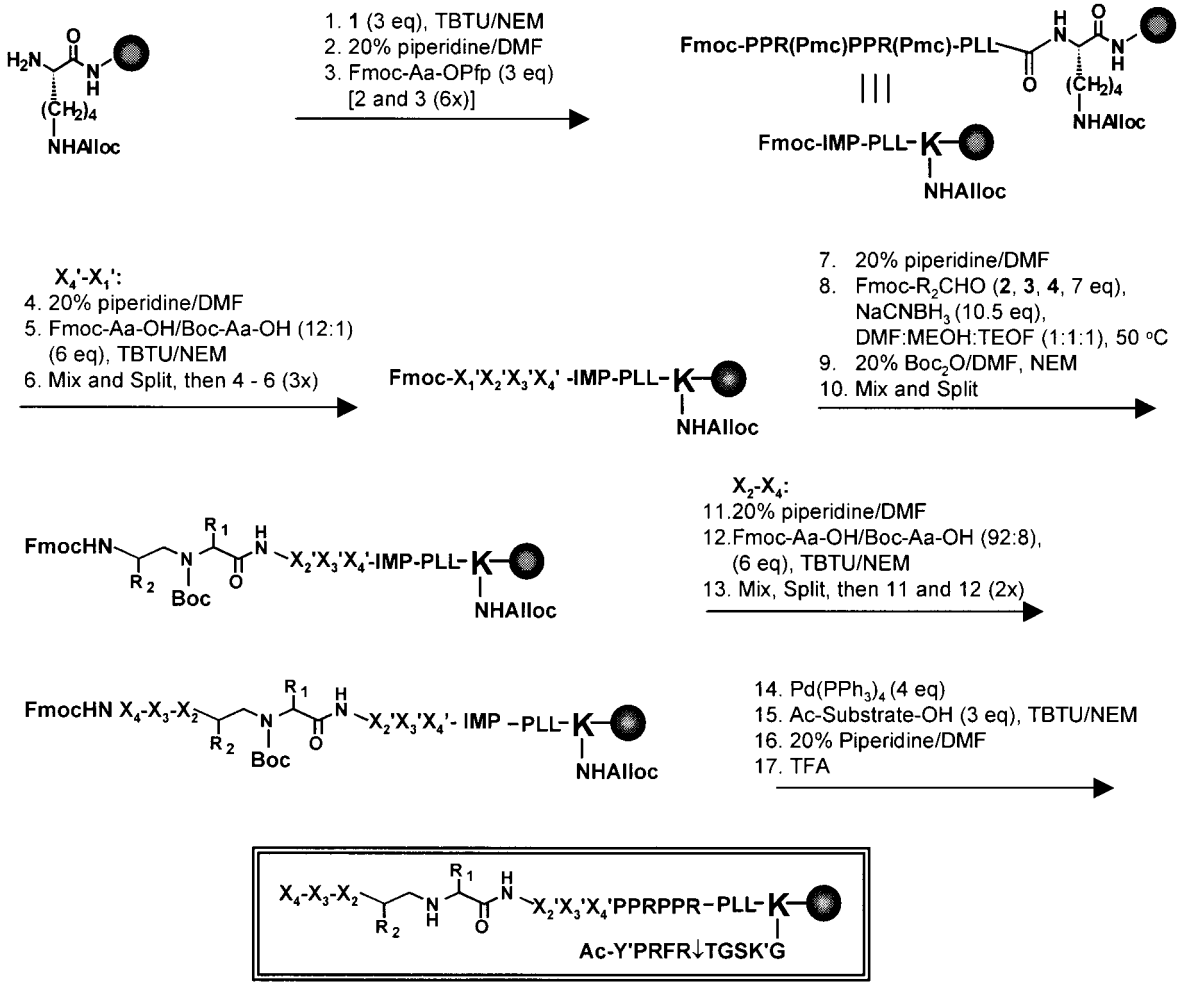


Figure 1. Principle of the one-bead−two-compound fluorescence-quenched assay. If the enzyme is strongly inhibited, then the substrate is not cleaved and beads remain dark. If the substrate is hydrolyzed, the beads become fluorescent.

ing the water byproduct. The extent of diastereomer formation in the library was not investigated; however, on the basis of the results of the resynthesis of hits from the library, it is highly likely that it did occur to varying degrees. Finally, we were concerned that the Alloc protecting group of Lys could be reduced during the reductive amination step in light of evidence that cyclopentenone is reduced to cyclopentanol upon treatment with $NaBH_3CN$ at pH 3.[31] In our case, the Alloc group could be quantitatively removed after the reductive amination step.

Inhibitor Library Synthesis. The selection of inhibitors by the enzyme from a library was monitored by the implementation of the one-bead−two-compound assay developed in our laboratory (Figure 1).[12] In this assay, each bead serves as a microreactor wherein a resin-bound substrate and inhibitor compete for binding to the enzyme. Such an assay requires the introduction of two orthogonally protected functional groups on each bead. Several approaches to the synthesis of these orthogonally protected bifunctional arms have been explored.[12−15] In the present case, a simple approach wherein the library is synthesized on the N-terminus of Lys while the presynthesized protected substrate is attached to the amino group on the side chain of Lys after completion of the library synthesis has been chosen. The synthesis of the one-bead−two-compound reduced bond inhibitor library is shown in Scheme 2. In this synthesis, 750 mg of $PEGA_{4000}$ resin (300−800 μm, ca. 130 000 beads) was derivatized with N^{α}-Fmoc-Lys(Alloc)-OPfp, and after removal of the Fmoc group, a photolabile linker[32] and an ionization mass spacer,

Scheme 2



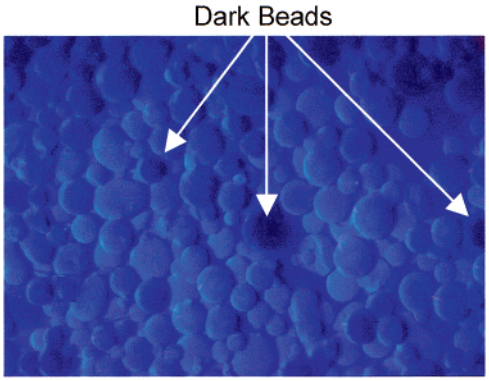


Figure 2. One-bead−two-compound fluorescence-quenched assay in real time. Dark beads were isolated, washed, and then analyzed by MALDI-TOF MS.

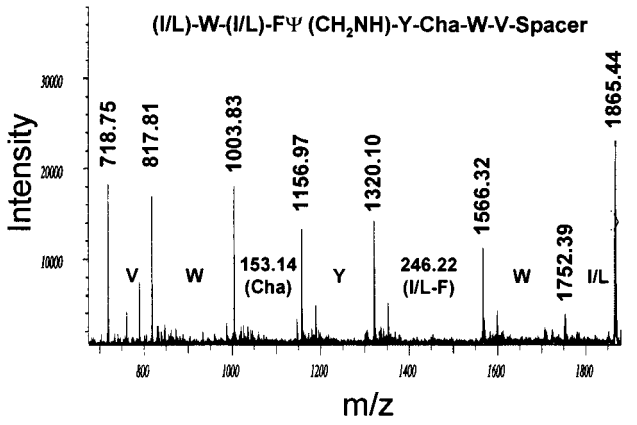


Figure 3. Representative MALDI-TOF mass spectrum of dark bead from the inhibitor library. Each peak in the spectrum represents the mass of a capped truncated oligomer. The mass difference between each peak directly indicates the amino acid introduced at that position.

PPR(Pmc)PPR(Pmc), were introduced to facilitate rapid analysis of active compounds from the library. An eight-residue peptide was synthesized because modeling studies (vide infra) with fluorescent-quenched substrates for this enzyme indicate that eight residues are optimal for filling the active site. Randomized positions $X_4'-X_1'$ and X_2-X_4 were generated using the portion mixing method, and in situ capping was achieved by coupling a mixture of Fmoc- and Boc-protected amino acids (92:8) after TBTU/NEM preactivation.[33] Nineteen genetically encoded amino acids were used in the library at each coupling step; Cys was replaced by Cha. The reduced bond was formed on solid phase by reaction of the resin-bound amines with 7 equiv of the N^{α}-Fmoc-protected amino aldehyde analogues of Arg, Lys, and Phe and 10.5 equiv of $NaCNBH_3$ in DMF/TEOF/MeOH (1:1:1) for 3.25 h at 50 °C. After completion of the laddered inhibitor library, the Alloc group was removed by treatment with $Pd(PPh_3)_4$, and a protected fluorescent quenched substrate for *L. mexicana* CPB2.8ΔCTE, AcY(NO_2)PR(Pmc)FR(Pmc)T(tBu)GS(tBu)K(Abz)G-OH ($K_m = 1.13\ \mu M$; $k_{cat}/K_m = 1300\ mM\ s^{-1}$ for the non-acetylated analogue),[34] was coupled using TBTU/NEM activation. After complete deprotection, some library beads were removed and irradiated with UV light, and the structures attached were analyzed by MS. From the mass spectra, it appeared that the synthesis had proceeded well.

Library Screening. After deprotection of the amino acid side chains, a portion of the library (200 mg) was incubated with *L. mexicana* CPB2.8ΔCTE. The fluorescence intensity of the beads was monitored with a fluorescent microscope every hour for indications of hydrolysis. After 3 h, most of the beads showed a fluorescent "ring" indicating hydrolysis of the substrate. The reaction mixture was incubated for 7 h, and after the beads were washed, the fluorescence intensity of the beads was assessed by inspection with a fluorescence microscope (Figure 2). Out of a possible 34 000 compounds (not including possible diastereomers), 70 dark beads (0.21% of library) were collected and transferred to stainless steel MALDI-TOF targets and irradiated for sequence identification. Beads containing both putative inhibitor and substrate but not treated with enzyme remained completely nonfluorescent. Beads with substrate only attached were also incubated with the enzyme and were brightly fluorescent within 30 min. It should be noted that some degree of quenching of the aminobenzoyl fluorescence in cleaved substrates occurred because of the proximity of the *o*-nitrobenzyl photolabile linker to the fluorescent probe in the current library construct. This low degree of quenching, however, did not affect the selection of dark beads containing inhibitors of the enzyme.

Analysis of Library Hits. The structure of the inhibitors attached to the beads was analyzed by MALDI-TOF MS (Figure 3). It was possible to determine the full structure of 41 of the inhibitors (58.6%) and four to six amino acids in an additional 15 inhibitors (21.4%) (Table 1). From the mass spectrum it was, of course, impossible to distinguish between isobaric amino acids such as Leu and Ile and Lys and Gln. The identity of the reduced bond dipeptide was determined by comparing the mass difference in the spectrum corresponding to the dipeptide with the possible masses from the combination of aldehyde and the 20 amino acids in position 4 of the library. For example, in Figure 3, the mass difference of the dipeptide is 246.22, and that corresponds to the reduced bond dipeptide Fψ(CH_2-NH)I/L of mass 246.31. Certain dipeptide combinations were also isobaric because the same amino acids were used as both the amine nucleophile and the aldehyde, e.g., Rψ(CH_2NH)K and Kψ(CH_2NH)R, or coincidentally such as in Rψ(CH_2NH)T and Kψ(CH_2NH)E.

The inhibitor specificity of CPB2.8ΔCTE was examined by analyzing the frequency of the amino acid in the enzyme subsite assuming that the inhibitor peptides were bound such that the reduced amide bond replaced the scissile bond of a substrate (Figure 4). From the frequency diagram, it is clear that primarily the subsites flanking the catalytic residues, S_2, S_1, S_1', and S_2', determine the specificity of the inhibitors. A similar conclusion was reached upon examination of the crystal structure of the Rous Sarcoma virus protease S9 variant and the HIV-1 protease with a reduced bond inhibitor based on the HIV-1 protease CA-p2 cleavage site, RVL-(CH_2NH)FEANle.[17] In that study, it was observed that the CA-p2 analogue inactivated both aspartic proteases to a similar extent despite having differences in the conformation and interaction in the distal inhibitor residues at P_3/P_4 and P_3'/P_4'. Additionally, recent work examining the substrate specificity of *L. mexicana* CPB2.8ΔCTE, the enzyme in this study, also suggested

Table 1. *Leishmania mexicana* CPB2.8ΔCTE Reduced Peptide Bond Inhibitor Sequences:[a]
$X_4X_3X_2X_1\Psi(CH_2NH)X_1'X_2'X_3'X_4'$-PPRPPR-PLL-PEGA$_{4000}$ Resin

bd[b]	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	inhibitor[c]
A1	D	K/Q	K/F	F/K	A	Cha	G	K/Q	**6**
A2	L/I	T	G	F	Y	L/I	V	A	**8**
A3	L/I	W	L/I	F	Y	Cha	W	V	
A4a	M	L/I	Cha	K	M	N	D	T	
A4b	M	P	Cha	F	L/I	N	D	T	
A5	V	T	D	F	M	L/I	L/I	A	
A6	D	M	R	F	M	P	N	N	
A7a	P	P	M	K	P	L/I	Cha	E	
A7b	P	P	L	F	P	L/I	V	K/Q	
A8	Y	P	P	F	Y	D	A	K/Q	
A9	D	K/Q	H/K/R	F/R/K	L/I	L/I	V	K/Q	**5**
A10	V	S	S	K	Y	L/I	Cha	K/Q	
A12	W	W	S	F	M	L/I	L/I	A	
A13	K/Q	A	E/T	K/R	P	G	E	E	
A14	M	A	D/S	K/R	S	R	R	P	
A15	Y	L/I	L/I	F	Cha	A	Cha	Y	
A17	V	Cha	G	R	L/I	L/I	H	D	**18**
A19	S	K/Q	L/I	R	P	H	M	V	
A20	Cha	A	E/K	F	H	E	D	H	
A23	T	P	H	K	L/I	Cha	K/Q	L/I	
A24	Cha	Cha	Cha	K	F	N	L/I	K/Q	**17**
B3	P	W	G	F	W	W	F	W	**12**
B4	M	W	R/F	F/R	A	L/I	L/I	T	**10**
B5	L/I	V	T	K	A	L/I	Cha	T	**16**
B10	D	S	Cha	F	Cha	S	M	L/I	**7**
B12	Cha	L/I	Cha	F	T	Y	L/I	Cha	
B15	Cha	F	L/I	F	Cha	G	N	Cha	
B22	L/I	D	G	K	L/I	Y	A	P	**15**
B23	E	L/I	F/K	K/F	Cha	Cha	F	F	
C1	L/I	M	F	F	L/I	L/I	L/I	A	**14**
C2	P	P	H/R/K	F/K/R	F	L/I	Cha	V	**11**
C3	Cha	N	L/I	F	K/Q	N	W	K/Q	
C4	Cha	A	L/I	F	Y	M	Y	Y	**13**
C5	W	D	Cha/R	F/R	Y	L/I	W	V	
C6	M	G	N	R	G	E	K/Q	L/I	
C8	K/Q	P	V/K/Q	F/K/K	F	L/I	Cha	K/Q	**9**
C10	Cha	F	V/A	K/R	P	L/I	Cha	T	
C11	W	S	W/H/R	K/R/R	Cha	Cha	Cha	Cha	
C26	F	Cha	Cha	K	L/I	S	F	F	
B9	T		L/I	K	R	L/I	L/I	K/Q	
A11			R/F	F/R	Y	L/I	K/Q	L/I	
A16			E/T	K/R	Cha	L/I	K/Q	V	
A25			L/I/S	K/R	L/I	W	M	L/I	
B24			R	F	Cha	G	S	L/I	
C14			D/L/I	F	F	L/I	Cha	F	
A18					Cha	L/I	W	L/I	
A22					M	W	M	A	
A26					L/I	Y	K/Q	K/Q	
B21					L/I	Cha	F	K/Q	
C9					Cha	L/I	M	L/I	
C12					Cha	L/I	M	N	
C13					Cha	V	N	Cha	
C17					L/I	L/I	N	L/I	
C19					L/I	F	M	L/I	
C21					G	V	F	F	
C18							F	Cha	
C15							F	Cha	

[a] All possibilities for isobaric amino acids and reduced bond dipeptide are listed. [b] Three sets of beads, A−C were analyzed with set A having the darkest beads. The a,b designation denotes the two possible sequences from MS data that were not due to isobaric dipeptides or amino acids. [c] Inhibitors that were resynthesized for kinetic analysis (see Table 2 for results).

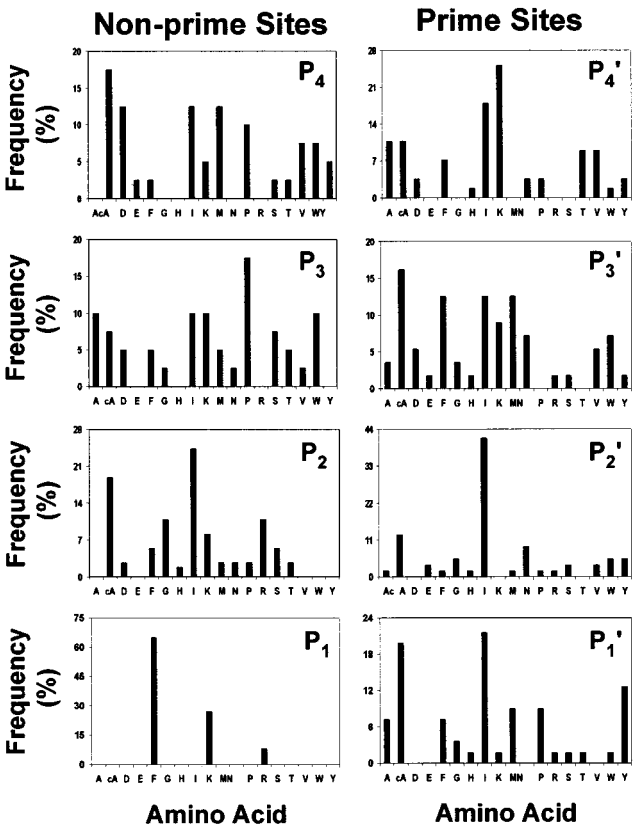


Figure 4. Frequency of inhibitor peptide amino acids in enzyme subsites based on the analysis of 56 hits. Peptide sequences are shown in Table 1. cA denotes cyclohexylalanine.

that the S_2 and S_1' subsites are the most crucial for specificity.[34] This observation has also formed the basis of the general design of small di-, tri-, and tetrapeptide inhibitors of several classes of proteases.[35] These small inhibitors, however, typically interact only with one-half of the enzyme active site and furthermore generally suffer from poor selectivity among related enzymes, bringing to debate the evolutionary purpose/advantage of the extended enzyme active site for the majority of cysteine proteases as well as what constitutes the most effective strategy for designing selective protease inhibitors.

More recently, inhibitors spanning parts of both the acyl and amino sides of the active site have demonstrated higher selectivity. For example, a series of potent, active-site spanning inhibitors of cathepsin K, which demonstrated selectivity over human cathepsin B, L, and S, were developed.[36−39] Later, epoxysuccinate inhibitors with some degree of interaction with both the primed and nonprimed sides of the active site demonstrated in vitro and in vivo selectivity for cathepsin L over cathepsins B, C, S, and K.[40] Additionally, vinyl sulfonate esters and vinyl sulfones[41] as well as epoxysuccinate inhibitors[42] showed selectivity for cruzain over bovine cathepsin B, leishmanial cathepsin B, and papain. The combinatorial methodology was used to generate libraries of various active-site spanning inhibitors, resulting in potent, selective inhibitors of matrix metalloproteases,[15,43] the malaria aspartyl protease plasmepsin II,[44] and plasmin,[45] albeit less successfully in the last two instances.

In the S_2 subsite, there is a preference for hydrophobic amino acids, Cha and Ile/Leu, in keeping with the substrate specificity of the enzyme as determined using a combinatorial library approach[34] and from systematic modification of the P_2 residues of substrate Abz-KL-RFSQ-EDDnp,[46] in which both demonstrated a preference for Leu in that position. On the other hand, there was a surprising and overwhelming preference for the hydrophobic Phe in the S_1 subsite. It was expected that the enzyme would maintain it's substrate-like prefer-

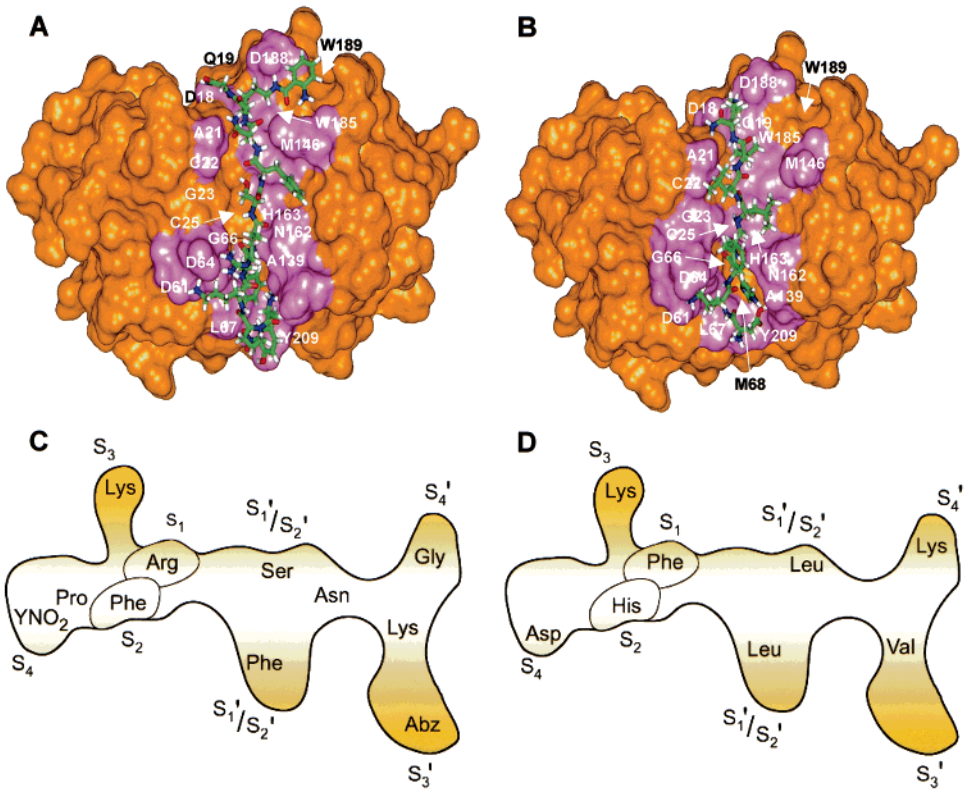


Figure 5. Molecular dynamics calculations of the enzyme−substrate (A) and enzyme−inhibitor (B) complexes using a homology-built model of *L. mexicana* CPB2.8ΔCTE. Both substrate Y(NO_2)PKFR↓SNFK(Abz)G and inhibitor DKHF(CH_2NH)LLVK bind in an extended conformation. (C, D) Schematic representation of the enzyme's subsites and the relative modes of binding of the substrate and inhibitor.

ence for a basic amino acid (Arg or Lys) in the S_1 subsite.[34] From a model of a good substrate, Y(NO_2)-PKFRSNFK(Abz)G, to a model of *L. mexicana* CPB2.8ΔCTE (parts A and C of Figure 5), it is suggested that the guanidino side chain of Arg interacts with D64 of the enzyme, forming a salt bridge. It is possible that the reduced bond secondary amine, which is protonated at physiological (and assay) pH, can also interact with D64. In the docking of the lead inhibitor obtained from the library screen, DKHF(CH_2NH)LLVK, into a model of the CPB2.8ΔCTE active site, the aromatic ring of the Phe at P_1 lies in the narrow cavity above Cys25 formed by the bridge residues D64, G65, and G66 on one side and N162 and H163 on the other side (parts B and D of Figure 5). Therefore, a hydrophobic amino acid in P_1 of the reduced bond inhibitor could confer the advantage of both strong hydrophobic interactions in addition to ionic/hydrogen bonding interactions with the reduced bond amine. It is possible that having Arg or Lys in P_1 is necessary for catalytic activity such that the ionic interaction of the amine or guanidino group with D64 aids in the positioning of the substrate without excessively tight binding, since the substrate must be released after hydrolysis. Alternatively, peptides containing a hydrophobic residue in P_1 may bind tightly to the enzyme by utilizing the cavity at S_1, thus inducing a peptide conformation not conducive to catalysis. Indeed, peptides containing hydrophobic residues in P_2 and P_1 have been shown to be nanomolar inhibitors of this enzyme.[46,47] Furthermore, it is known that the inhibitory proregions of several cysteine proteases possess a basic−hydrophobic−hydrophobic motif in the S_3−S_1 subsites of the enzyme.[46] In the absence of a crystal structure of CPB2.8, it is not certain what residues of the proregion interact with which subsites of the enzyme. However, studies using short peptides indicate that the presence of a basic−hydrophobic−hydrophobic motif is important for inhibitory activity.[46] Unlike substrates, the S_1' subsite also showed a preference for Cha and Ile/Leu while the S_2' subsite was specific for Ile/Leu.

The subsites further away from the catalytic triad demonstrated less specificity. In the S_4 subsite, the amino acids occurring with the highest frequency were Cha, Asp, Ile/Leu, Met, and Pro. Pro was somewhat preferred over other amino acids in the S_3 subsite, in keeping with the substrate specificity of the enzyme.[34] On the primed sites, Cha, Phe, Ile/Leu, and Met were most frequent in S_3' while Ile/Leu and Lys/Gln were preferred in S_4' subsite. Although the subsite frequency diagram suggests a general preference for peptides with hydrophobic residues in every subsite, the individual inhibitors were primarily hydrophobic, polar, or a combination of both (Table 1).

Inhibition of CPB2.8ΔCTE by Reduced Bond Inhibitors. Inhibitors were selected for resynthesis and testing in solution-phase assays on the basis of their darkness in the library screening and the number of preferred amino acids they contained. Some inhibitors containing isobaric amino acids (Table 2, **5** and **13**) were resynthesized using both amino acids. Inhibitors were synthesized on solid phase using PEGA synthesis resin ($PEGA_{800}$). The synthesis generally proceeded in good yield except for inhibitor **12**, which contained several tryptophans. In that case, several products were obtained including alkylated tryptophans despite the use

Table 2. Characterization of Reduced Bond Inhibitor Peptides the K_i Values for Their Inhibition of *L. mexicana* CPB2.8ΔCTE

no.[a]	inhibitor[b]	mass, exptl	mass, obsd	ratio[c]	yield[d] (%)	K_i[e] (μM)
5a	DKHF*LLVK	984.25	984.92	1.16/1	100	1.0
5b	DKHF*IIVK	984.25	984.44	1.25/1	56.6	20.0
5c	DKGF*LIVK	904.16	904.35	3.13/1	83.2	18.6[f]
5d	DQHF*IIVQ	984.16	985.34	1/1	75.5	30.0
5e	DQHF*LLVQ	984.16	984.73	1/1	82.4	0.5
6a	DKKF*AChaGK	931.09	931.59	1.26/1	100	31.1
6b	DKFK*AChaGK	931.09	931.23	1/1.41	100	0.6
7	DSChaF*ChaSMI	990.08	990.57	1/1.3	55.0	10.1
8	ITGF*YIVA	868.08	868.85	one peak	85.4	53.3
9	KPVF*FICha	1016.28	1016.95	1.13/1	93.6	13.0[f]
10	MWRF*AIIT	1022.32	1022.55	1/1.25	63.5	6.5
11	PPHF*FIChaV	994.19	994.38	1/1.5	69.8	6.6
12	PWGF*WWFW	1196.42	1197.25	ND	19.5	0.2
13a	ChaALF*YMYY	1108.3	1108.64	2.7/1	34.3	1.1
13b	ChaAIF*YMYY	1108.3	1108.40	1.6/1	44.7	0.1
14	IMFF*IIIA	952.31	952.38	1.3/1	96.7	0.05
15	IDGK*LYAP	861.05	861.06	6.6/1	58.2	5.3
16	IVTK*AIChaT	883.09	883.80	one peak	100	100.0[f]
17	ChaChaChaK*FNIK	1093.22	1093.56	1/2.19	81.9	6.3[f]
18	VChaGR*LIHD	947.19	947.53	5.6/1	59.2	71.6
19	PKFR*SFNK[g]	1008.23	1008.42	1.2/1	59.2	8.8
20	AIWrYWAAV[h]	1062.16	1062.96		84.7	44.7

[a] Inhibitor sequences derived from variation of amino acids in the active hit are denoted a, b, c, etc. [b] Position of reduced bond is denoted by *. [c] Ratio of first eluted diastereomer to second eluted determined from analytical HPLC of crude product. [d] Yield of both diastereomers after HPLC purification based on the assumption that 40 mg of resin (0.34 mmol/g) was evenly distributed in all the wells. [e] Inhibitors assayed as an approximately 1:1 mixture of diasteroemers except for **6a**, **8**, **12**, **14**, **16**, and **17**, which were assayed as mixtures of >90% single diastereomer. Assay conditions: 0.1 M Na phosphate, 10.0 mM DTT, pH 6.0, 37 °C, 10 min of enzyme activation. [S] = 0. 7 μM Z-FR-AMC; K_m = 0.7 μM. [E] = 1.1 nM. [f] Assay conditions: 0.1 M Na acetate, 2.0 mM EDTA, 200.0 mM NaCl, 10.0 mM DTT, pH 5.5, 37 °C, 15 min of enzyme activation. [S] = 18.5 μM Ac-FR-AMC; K_m = 15.0 μM. [E] = 1.1 nM. [g] Sequence obtained from substrate; scissile bond is replaced by a reduced peptide bond. [h] D-Amino acid inhibitor of cruzain.[13]

of scavengers in the cleavage cocktail. Most of the inhibitors were obtained as a mixture of two diastereomers (Table 2) because of racemization during the reductive amination step, and most were assayed as mixtures.

The potency of selected inhibitors against the enzyme was examined in solution-phase assays, and the results are listed in Table 2. On average, the hydrophobic inhibitors (**12**, **13a**, and **14)** were more potent (K_i = 50−200 nM) than the more polar ones, although **5a**, **5e**, and **6b** also displayed good potency (K_i = 0.5−1 μM). Since it was impossible to differentiate between Leu and Ile and between Lys and Gln, by mass spectrometry, some inhibitors were synthesized in the possible combinations (e.g., **5a**−**5e**). In the P_4 and P_4' positions, there is little difference between a Lys and Gln, since they can establish similar hydrogen bonding patterns and the enzyme active site is wide in those positions. However, there is significant difference between a Leu and Ile in the P_2' and P_3' positions with Leu giving rise to superior inhibitors (inhibitors **5a**−**5e**). Conversely, Ile in P_2 made **13b** a 10-fold better inhibitor than the corresponding analogue **13a**, with Leu in P_2. The reduced peptide bond inhibitors were generally more effective than a D-amino acid containing peptide inhibitor **20**, which was an efficient inhibitor of the related enzyme cruzain.[13]

Modification of the scissile bond and side chains of a good substrate sometimes leads to the design of inhibitors. We therefore tried to compare the inhibitory potency of substrate Y(NO_2)PRFR↓TGSK(Abz)G-OH (K_m = 1.13 μM; k_{cat}/K_m = 1300 mM s^{-1}) with its reduced bond analogue PRFR(CH_2NH)TGSK (**19**). The reduced bond peptide inhibited the activity of CPB2.8ΔCTE toward Z-FR-AMC with an inhibition constant of 8.8 μM (Table 2). The moderately decreased interaction could be attributed to an increased entropic penalty for the flexibility introduced by the secondary amine but was most likely due to the loss of hydrophobic interaction from the aminobenzoyl and possible nitrotyrosyl groups with the enzyme. It has been observed in other studies that the fluorescence donor and quencher groups can greatly increase binding affinity, particularly when placed in the P_3 and P_3'/P_4' positions.[46,48] This increase in affinity occurs presumably by interactions (stacking, aromatic π interactions, hydrophobic, or otherwise) with the conserved aromatic and hydrophobic residues in or near the active site of the enzyme: Leu 67 for the nonprimed sites and Trp 177 and Trp 181 for the primed sites (papain numbering).[36,48]

We then examined the interaction of substrate Y(NO_2)PKFRSNFK(Abz)G and inhibitor DKHF(CH_2-NH)LLVK, with a homology-built model of *L. mexicana* CPB2.8ΔCTE to investigate their modes of binding. From Figure 5, it can be seen that both substrate and inhibitor occupy the enzyme active site in a virtually extended conformation. According to the modeling, the substrate interacts with the enzyme through key interactions of Lys3 with the negative charge of D61, of Phe4 with the hydrophobic S_2 pocket lined by L67, M68, and A139, of the guanidino group of Arg5 with D64, and of Phe8 with the extended S_1'/S_2' pocket lined by M146, H163, and N162. Ser6 is positioned in S_1'/S_2' to give a tight fit under the protuberance formed by C63, G23, and A21, while the Gly10 extends to interact with D18. The Lys9(Abz) extends over the shallow groove containing W185 and W189. By comparison, the reduced bond inhibitor binds in a similar mode in which the amino-terminal Asp1 occupies the S_4 subsite of the enzyme previously occupied by YNO_2 while Lys2 has a charge−charge interaction with D61 and D64. His3 occupies the hydrophobic S_2 pocket, while Phe4 of the reduced bond protrudes from the narrow S_1 subsite above the catalytic

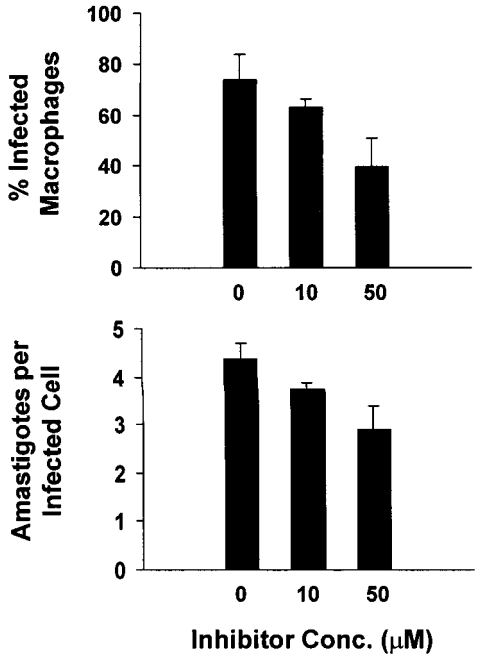


Figure 6. Effect of reduced bond inhibitor DKHF(CH_2NH)-LLVK on *Leishmania brasiliensis* infection of mouse macrophages. The percent of infected macrophages (top) and the number of parasites per infected cell (bottom) 24 h postinfection are given as the mean ± SD from three experiments.

triad. Leu5 and Leu6 occupy the S_1' and S_2' subsites, respectively; Leu5 interacting with M146 and H163 and Leu6 interacting with C22 and A21. Val7 interacts with W185 in S_3', while the side chain of C-terminal Lys8 extends out to give a charge−charge interaction with D18.

Inhibition of Parasite Infectivity. The effect of reduced bond inhibitors on parasite survival in macrophages was investigated using *Leishmania braziliensis*, LC2177 strain. This strain expresses a cysteine protease sharing 66% sequence identity with *Leishmania mexicana* CPB (Jorge Arevalo, unpublished data). *L. braziliensis* promastigotes were treated with varying concentrations of inhibitor for 50 min at 27 °C before challenging mouse peritoneal macrophages. After incubation of the cells for 24 h at 34 °C, the number of infected macrophages and the number of amastigotes per infected macrophage were determined. The most potent inhibitor, **14** (K_i = 50 nM against CPB2.8ΔCTE itself), was not tested because of its high hydrophobic index, hence its insolubility. Inhibitors **5a** and **13b** were selected because of their relatively high potency, K_i values of 1.0 and 0.1 μM, respectively, against the enzyme. Unfortunately, inhibitor **13b** precipitated from the assay medium at the concentrations used in the experiment (10 and 50 μM). Inhibitor **5a**, DKHF(CH_2-NH)LLVK, was effective in decreasing both the number of macrophages infected and the number of amastigotes per infected cell (Figure 6). There was no apparent adverse effect on the macrophages for the duration of the experiment. These results provide proof of concept that treatment with cysteine protease inhibitors impairs the parasites' ability to successfully invade its host cell. The results are similar to those obtained using genetically manipulated parasites devoid of CPB,[6] suggesting that the inhibitor's effect is mediated through inhibition of this enzyme.

Conclusion

The development of protease inhibitors draws on a variety of approaches including computer-assisted structure-based design, de novo drug synthesis, natural product isolation and screening (ethnobotanical approaches), and combinatorial chemistry. Usually, it is an iterative process combining many of the above that leads to successful drugs. In the present work, we have used the combinatorial approach as the starting point, and through application of the one-bead−two-compound library method, we have allowed the enzyme to select potential inhibitors from thousands of possibilities. The library was efficiently synthesized using a combination of the building block approach and on-resin reactions. The use of PEG-based resins and a photolabile linker facilitated rapid on-bead screening and identification of hits. Surprisingly, the reduced bond peptides identified from the library have proven to be effective inhibitors of a recombinant enzyme from *Leishmania mexicana* and of *Leishmania* parasite invasion and survival in infected cells. Through modeling, we have gained insight into the contacts that are important for the binding of the inhibitor to the enzyme active site. This information will be utilized for the synthesis of second-generation inhibitor libraries that better conform to the criteria for an effective drug.

Experimental Section

Abbreviations. Abz, 2-aminobenzoyl; AMC, 7-amino-4-methylcoumarin; CHC, α-cyano-4-hydroxycinnamic acid; CPB2.8ΔCTE, cysteine protease group B isoenzyme lacking C-terminal extension; Dhbt-OH, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; EDT, ethanedithiol; HMBA, hydroxymethylbenzoic acid; HOAc, acetic acid; MeIm, *N*-methylimidazole; MSNT, 1-(mesitylene-2-sulfonyl)-3-nitro-*1H*-1,2,4-triazole; MALDI-TOF MS, matrix-assisted laser desorption ionization mass spectrometry; NEM, *N*-ethylmorpholine; PEGA, poly(ethylene glycol) acrylamide copolymer; Pfp, pentafluorophenyl; Pmc, 2,2,5,7,8-pentamethyl chroman-6-sulfonyl; PNA, *p*-nitroanilide; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TEOF, triethylorthoformate; TFA, trifluoroacetic acid; TIS, trisisopropylsilane.

Materials and Methods. All solvents were of HPLC grade and used without further purification with the exception of dichloromethane and DMF, which were dried, distilled, and stored over 3 Å molecular sieves under inert gas. Flash chromatography was performed on Silica Gel 60 (Merck, Darmstadt, Germany). Solid-phase peptide coupling reactions were monitored using the Kaiser test,[49] and solution-phase reactions were monitored by thin-layer chromatography (TLC) performed on Merck silica gel 60 F_{254} aluminum-backed sheets with detection by UV light followed by ammonium molybdate solution. $PEGA_{4000}$ resin for library use (0.12 mmol/g, 300−800 μm) was custom-synthesized,[50] while $PEGA_{800}$ resin for peptide synthesis (0.4 mmol/g, 150−300 μm) was obtained from Polymer Laboratories (Amherst, MA). Fluoren-9-ylmethoxycarbonyl (Fmoc) amino acids and their pentafluorophenyl (Pfp) ester derivatives were purchased from Bachem and NovaBiochem. Fmoc-Lys(Boc-Abz)-OH and Fmoc-Tyr(NO_2)-OH were prepared as previously described.[51] The substitution of the resins was determined by spectrophotometric analysis (Perkin-Elmer Lambda 7 UV/vis spectrophotometer) at 290 nm of the dibenzofulvene−piperidine adduct formed upon deprotection of the amino terminal Fmoc group. Purification of inhibitor peptides was performed by preparative reverse-phase HPLC on a Waters HPLC system with a Delta pak C-18 column (200 mm × 25 mm) and a linear gradient of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 90% aqueous MeCN) at a flow rate of 20 mL min^{-1}. MALDI-TOF MS of resin-bound inhibitors and some soluble peptides was performed on a

Bruker Reflex III with a matrix of α-cyano-4-hydroxycinnamic acid. The mass of other soluble peptides was determined by ES-MS recorded in the positive mode on a Fisons VG Quattro instrument. NMR spectra were recorded on a Bruker AMX-250 spectrometer. Solution-phase kinetics of inhibition was carried out on a Perkin-Elmer Lambda 50 fluorimeter.

Enzyme. *L. mexicana* CPB2.8ΔCTE was expressed, purified, and activated as previously described.[21] The concentration of the enzyme stock solution (11.4 μm) was determined by active site titration with human cystatin (a generous gift from Dr. Magnus Abrahamson (University of Lund, Sweden)) using Z-Phe-Arg-AMC as the substrate.

Solution-Phase Inhibition. Peptide inhibitors were assayed as according to Nicklin and Barrett[52] using either Z-Phe-Arg-AMC (K_m = 0.7 μM) or 18.5 μM Ac-Phe-Arg-AMC (K_m = 15.0 μM) as substrate. Assay conditions were as follows: 0.1 M sodium phosphate, 10.0 mM DTT, pH 6.0, 37 °C, with 10 min of enzyme activation before use. [S] = 0. 7 μM while [E] ranged from 1.1 to 4.5 nM. For the latter substrate, the conditions used were as follows: 0.1 M sodium acetate, 2.0 mM EDTA, 200.0 mM NaCl, 10.0 mM DTT, pH 5.5, 37 °C, with 15 min of enzyme activation before use. [S] = 18.5 μM while [E] = ranged from 0.5 to 1.1 nM. Inhibitors were made up as 2 mM stock solutions in DMF/H_2O (1:1), and concentrations ranged from 0.01 to 50 μM. Precise inhibitor concentrations were obtained from the integration of HPLC peaks of the inhibitor after calibration of the HPLC at 210 nm using two peptides: Y(NO_2)PKFRSFNK(Abz)G-OH and AFMV-OH. $K_{i(app)}$ values were obtained from the gradient of the plotting of $[(v_o/v_i) - 1]$ vs [I], where v_o and v_i are the velocity of less than 2% substrate hydrolysis in the absence and in the presence of different inhibitor concentrations [I], respectively. The K_i parameters were obtained from the equation $K_i = K_{i(app)}/(1 + [S]/K_m)$. Results are the mean of two experiments that varied by less than 5%.

Effect of Inhibitor DKHF(CH_2NH)LLVK on the Survival of *Leishmania*-Infected Macrophages. LC2177 *Leishmania braziliensis* parasites were grown in blood agar biphasic medium at 27 °C. Promastigotes from the stationary phase (day 4) were harvested by centrifugation at 2500 rpm for 10 min at 4 °C. The pellet was washed twice with M 199 medium under the same centrifugation conditions and resuspended in 1 mL of medium for subsequent in vitro infection studies. Resident macrophages were harvested from the peritoneal cavities of 6 week old Balb/c mice in ice-cold M199 medium supplemented with 20% (v/v) heat-inactivated fetal calf serum, plated onto microcoverslips (3 mm × 3 mm) laid in 96-well plates, and incubated at 37 °C under an atmosphere of 5% CO_2. After 2 h the wells were washed with M199 medium (3×). Predominant macrophages were kept under the same conditions of incubation overnight.

Parasites were incubated for 50 min at different concentrations of DKHF(CH_2NH)LLVK inhibitor and then were washed twice by centrifugation with M199 medium as described above. The pellet was resuspended in complete M199 medium. These parasites were added at a 20:1 parasite/macrophage ratio to the wells containing attached macrophages for 1 h. Non-attached parasites were washed (3×), and the infection was pursued at 34 °C for 24 h. At the end of this period, cells were stained with Giemsa and May Grünwald solutions. The percentages of infected macrophages and amastigotes per infected cell of four microcoverslips were recorded for each time point. Each experiment was repeated three times.

Synthesis of Fmoc Amino Aldehydes 2−4. Fmoc amino aldehydes were synthesized according to the procedure of Wen and Crews,[23] and the NMR data were in agreement with previously reported assignments of Phe,[23] Lys, and Arg.[53] Yields: Fmoc-Phe-N(Me)OMe, 99%; Fmoc-Phe-H, 90%; Fmoc-Lys(Boc)-N(Me)OMe, 95%; Fmoc-Lys(Boc)-H, 58%; Fmoc-Arg-(Pmc)-N(Me)OMe, 75%; Fmoc-Arg(Pmc)-H, 69%.

General Solid-Phase Peptide Synthesis. Synthesis of the inhibitor library and lead inhibitor peptides was carried out manually in a custom-made 20-well Teflon synthesis block[54] as previously described.[55] The spacer peptide, PPR-(Pmc)PPR(Pmc), was synthesized manually in a syringe using Fmoc-Aa-OPfp esters (3 equiv). Reaction completion was assessed by using Dhbt-OH (1 equiv), which served as both an acylation catalyst and an indicator of the reaction completeness,[56] or by using the Kaiser test.[49] For coupling to amines, free acids were activated for 6−8 min using TBTU (0.1 equiv less than the acid equivalent) and NEM (1.5 times the equivalent of acid). For coupling to the HMBA linker, free acids were activated in situ by equivalent quantities of MSNT and *N*-MeIm.[57] Coupling times ranged from 3 h to overnight. The photolabile linker was synthesized as previously described.[32] All manipulations (synthesis and screening) of peptides linked to the resin via the photolabile linker were carried out in subdued light (protected from UV radiation).

Deprotection Protocols. After each acylation, the resin was washed with DMF (6×), and then the Fmoc protecting groups were removed by treating the resin with a 20% piperidine/DMF (2 + 18 min) solution. The resin was then washed with DMF (6×). For the removal of Boc and other side chain protecting groups, the resin was first washed with CH_2Cl_2 (8×) and then treated with a cocktail consisting of TFA/thioanisole/ethanedithiol/water (87.5:5:2.5:5) initially for 10 min and then for 2.5 h. The resin was then washed with 95% acetic acid (4×), DMF (2×), 5% DIPEA (2×), DMF (2×), and CH_2Cl_2 (6×) and then was dried in vacuo. In the case of inhibitor peptides, the TFA filtrate was collected, the resin was washed with 95% acetic acid (2×), and the combined filtrates were concentrated.

Synthesis of Reduced Peptide Bond Library. The inhibitor library was synthesized on custom-made $PEGA_{4000}$ resin[50] (750 mg, 0.12 mmol/g, 300−800 μm). In a 20 mL syringe, the resin was first washed with DMF (5×), N^{α}-FmocLys(Alloc)-OPfp (4 equiv) and DhbtOH (1 equiv) in DMF were added, and the mixture was allowed to react for 4 h. The Fmoc group was removed, and the photolabile linker (**1**) was coupled using TBTU/NEM methodology. An ionization mass spacer, FmocPPR(Pmc)PPR(Pmc), was then synthesized using the Fmoc/OPfp method. The resin was then transferred to a 20-well custom-made (2.0 mL/well capacity) library generator. The first four randomized positions in the library were generated by split−mix synthesis using a mixture of Fmoc and Boc amino acids (92:8, 6 equiv) in DMF (700 μL) after preactivation by TBTU/NEM in DMF (100 μL). After each coupling, the resin was pooled, mixed, and divided before Fmoc removal. The usual washing protocol followed each coupling and deprotection step. After the 4th cycle, the resin in two of the wells was redistributed into the remaining 18 wells and the resin was washed with solution A (TEOF containing 1% HOAc) (6×). Aldehydes **2**−**4**, (7 equiv) dissolved in solution B (DMF/TEOF/MeOH (1:1:1) containing 1% HOAc (800 μL) were added such that each aldehyde was added to six wells. After 45 min at 50 °C, $NaCNBH_3$ (10.5 equiv) in solution B (100 μL) was added and the mixture was reacted for an additional 2.5 h. After completion of coupling, the resin was washed with DMF (2×) MeOH (2×), DMF (2×), and Boc_2O (20% in DMF, 900 μL), and excess NEM (100μL) was added for 20 min. After deprotection of the N-terminal Fmoc, the remaining three positions were coupled in a randomized manner as described above. After completion of the laddered inhibitor library, 200 mg of the resin was transferred to a syringe and treated with $Pd(PPh_3)_4$ (4 equiv) in a degassed mixture of $CHCl_3$/AcOH/NEM (92.5:5:2.5) for a period of 4.5 h and then was washed with $CHCl_3$ (3×), DMF (3×), 0.5% Et_2NCS_2Na in DMF (1×), and DMF (4×). Substrate AcY(NO_2)PR(Pmc)FR(Pmc)T(tBu)-GS(tBu)K(Abz)G-OH (3 equiv) in DMF was activated with TBTU/NEM and was added to the resin, and the mixture reacted for 5 h. The substrate and inhibitor library were deprotected as described in the general methods section.

Synthesis of Lead Inhibitor Peptides. $PEGA_{800}$ resin (800 mg, 0.34 mmol/g, 150−300 μm beads) was derivatized with the Rink amide linker (3 equiv) via TBTU/NRM activation. The resin was then apportioned into the 20 wells (40 mg/well), and peptides were synthesized using Fmoc-Aa-OPfp (3 equiv). Reductive amination was carried out as described for

inhibitor library synthesis. At the end of the synthesis, the Fmoc group was removed and the peptides were simultaneously cleaved and deprotected by treatment with the following TFA mixtures for 45 min and 2.5 h if Arg was present: mixture 1 (93% TFA, 5% H_2O, 2% TIS) for peptides **5a**−**e**, **6a**,**b**, **9**, **11**, **14**, **17**, and **18**; mixture 2 (90% TFA, 5% H_2O, 5% thiocresol) for peptides **8**, **15**, **16**, and **19**; mixture 3 (82.5% TFA, 5% H_2O, 5% thiocresol, 2.5% EDT) for peptides **7**, **10**, **12**, and **13a**,**b**. The peptides were purified by preparative HPLC and analyzed by MALDI-TOF MS and analytical HPLC (Table 2).

Solid-Phase Library Screening. The library (200 mg; ca. 34 000 beads) was washed (2 × 10 min) with assay buffer (100 mM phosphate buffer, pH 6.0, augmented with 10 mm DTT) and then incubated with activated CPB2.8ΔCTE (30 nmol) at 37 °C in 5 mL of assay buffer. The fluorescence intensity of the beads was monitored with a fluorescent microscope every hour for indications of hydrolysis. After 3 h, most of the beads showed a fluorescent "ring" indicating hydrolysis of the substrate. The reaction mixture was incubated for 7 h and then was treated with 2% aqueous TFA solution (2 × 5 min), water (2×), 2% $NaHCO_3$ (2×), and then water (3×). The fluorescence intensity of the beads was assessed by inspection with a fluorescence microscope, and the 70 darkest beads were collected and transferred to a stainless steel MALDI-TOF target for sequence identification.

MALDI-TOF Mass Spectrometry. Library Hits. Beads were irradiated on stainless steel targets with a UV lamp for 30 min. The analyte was extracted on the target from the beads using CH_3CN/H_2O (3:2, 0.5 μL) containing 1% TFA, matrix α-cyano-4-hydroxycinnamic acid (CHC, 10 mg in 1 mL of 70% acetonitrile) was added (0.5 μL), and the sample was dried with a hair dryer (ca. 50 °C). The spectrum was recorded in the positive-ion mode on a Bruker Reflex III MALDI-TOF mass spectrometer. Spectra were obtained (80−300 pulses) using the lowest power required to facilitate desorption and ionization.

Soluble Peptides. HPLC solutions containing the peptides were mixed with an equal volume of CHC matrix (0.5 μL) and were analyzed in the positive mode. Bradykinin (1060.2 mu), renin (1759.0 mu), and mellitin (2846.5 mu) were used as the standards for internal calibration of the mass.

Modeling. Molecular dynamics calculations were carried out on a Silicon Graphics Octane workstation using the InsightII/Discover program. A homology-based protein model of a *L. major* cathepsin L-like cysteine protease (GeneBank locus U43706, PDB identification code 1bmj)[58] was built using InsightII software (Biosym Technologies) and the crystal structures of papain[59] and cruzain[60] as reference proteins. The model of the *L. mexicana* CPB2.8ΔCTE isoform was obtained by changing the amino acid residues that were different from those of *L. major* and then shortly minimizing the protein. The calculations were performed with the substrate Y(NO_2)-PKFRSNFGK(Abz) and the inhibitor DKHF(CH_2NH)LLVK to examine the differences in binding modes of substrate and inhibitors. During all calculations, the amide backbone of CPB2.8ΔCTE was fixed, while all the side chains in contact with the substrate/inhibitor were allowed to move. Each calculation was carried out as an annealing at decreasing temperatures, 650 and then 500 and 300 K. At 500 and 300 K, the CPB2.8ΔCTE residues in contact with the ligand were allowed to move freely including backbone atoms in sequence ranges of five successive amino acids or less. The ligands were initially energy-minimized from the extended structures and were added in the resulting conformations approximately 15 Å away from the binding site. A weak constraint of ∼3 kcal/mol (boundaries 3 and 4 Å) was added between Cys 25 thiol and the scissile bond carbonyl carbon of the substrate or to the analogous CH_2 of the inhibitor. These constraints were maintained throughout all calculations. Additional constraints of ∼3 kcal/mol (boundaries 2 and 3 Å) were applied for distances between the C25 sulfhydryl hydrogen and the π-nitrogen of H163 and between the τ-NH of the H163 and the N175 side chain carbonyl. The calculations were initiated with 10 steps of minimization, and then 30 000−60 000 steps were calculated at each temperature at 1 fs intervals. The development of each calculation was monitored, and when persistent obstructions to the progress (i.e., distance greater than 5 Å between the sulfur atom and the scissile carbonyl carbon) of the calculation were observed, a new starting substrate conformation was used. This was obtained by modification of one or two peptide torsion angles in the extended peptide followed by a short MD calculation alone. Only the $\phi-\psi$ angles allowed in the ϕ/ψ space were used as starting conformations. As many as five independent successful calculations were performed for each ligand, and when the calculations were allowed to reach equilibrium, the results obtained with different starting conformations converged to one or two related bound conformations. Constraints were completely removed from the final structure, and the complex was subjected to extended calculations to determine whether the ligand remained bound to the CPB2.8ΔCTE molecule in a stable conformation.

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Supporting Information Available: Details of reductive amination optimization and synthesis of protected substrate. This material is available free of charge via the Internet at http://pubs.acs.org.

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