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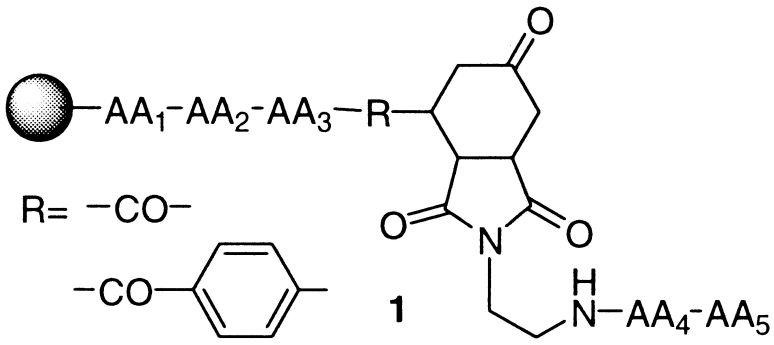

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Combinatorial Library of Peptide Isosters Based on Diels−Alder Reactions: Identification of Novel Inhibitors against a Recombinant Cysteine Protease from *Leishmania mexicana*

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A combinatorial split-and-mix library of peptide isosters based on a Diels−Alder reaction was synthesized as a "one-bead−two-compounds" library and encoded by ladder synthesis for facile analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. In the "one-bead−two-compounds" library approach, each bead contains a library member as a putative protease inhibitor along with a fluorescence-quenched substrate for the protease. When the library was screened with CPB2.8 ΔCTE, a recombinant cysteine protease from *L. mexicana*, several beads containing compounds with inhibitory activity could be selected from the library and analyzed by MALDI-TOF MS for structure elucidation. Two types of inhibitors were revealed. One novel class of inhibitors had the bicyclic Diels−Alder product isosteric element incorporated internally in a peptide, while the other type was an N-terminal α,β-unsaturated ketone Michael acceptor used as starting material for the Diels−Alder reaction. Selected hit sequences and constructed consensus sequences based on the observed frequencies of amino acids in different subsites were resynthesized and assayed in solution for inhibitor activity and were shown to have IC_{50} values in the high nanomolar to low micromolar range.

Introduction

Proteases selectively catalyze the cleavage of polypeptide bonds and are involved in a multitude of physiological processes, ranging from digestion to highly specific functions such as control of growth, cell differentiation, signaling, or host invasion by infective organisms. Protozoan parasites of the genus *Leishmania* are the causative agents of leishmaniasis, a debilitating disease affecting millions of people worldwide. The species *Leishmania mexicana* expresses from a tandem array of 19 genes a group of cysteine proteases belonging to the papain family, which are assumed to be vital for the ability of the parasite to survive and propagate in the host organism.[1,2] The substrate specificity of a recombinant protease from this group, CPB2.8 ΔCTE, has recently been characterized[3] using a fluorescence-quenched combinatorial peptide library approach.[4] Proteases specifically recognize their substrates, and traditionally, knowledge about substrate specificity has aided the development of inhibitors through trial and error processes. Recently, combinatorial chemistry has also been applied to the discovery or optimization process for drug leads.[5−10] When screening split-and-mix combinatorial libraries, mixtures are encountered. However, if the screening is conducted on-bead, the mixture of beads will display one unique structure as a result of the synthetic strategy. Hence, on-bead screening of a combinatorial library can be regarded as individual screenings of each of the library members, with the volume of the bead playing the role of a microreactor. A fluorescence-quenched assay for endo-protease inhibitors based on this principle has been developed.[5] In this assay "one-bead−two-compounds" combinatorial libraries were used to identify inhibitors of Subtilisin Carlsberg, Cruzipain, Cathepsin B, Cathepsin L, and MMP-12.[5−7] Currently, work is in progress to expand the concept of "one-bead−two-compounds" combinatorial libraries to more organic peptidomimetics by preparation of peptide isoster libraries using various organic reactions on the solid phase. These are then used for on-bead screening for endo-protease inhibitors.[7,11,12] The assay requires active enzyme to migrate into the interior of the solid support, and PEGA resin has been shown to be the superior choice.[13,14] Here, the synthesis and screening of a split-and-mix putative protease inhibitor library of peptide isosters formed via a solid-phase Diels−Alder reaction are reported. The library was screened for inhibitors against a recombinant parasitic cysteine protease to identify two novel classes of inhibitors with IC_{50} values in the high nanomolar to low micromolar range, using a fluorescence-quenched assay.

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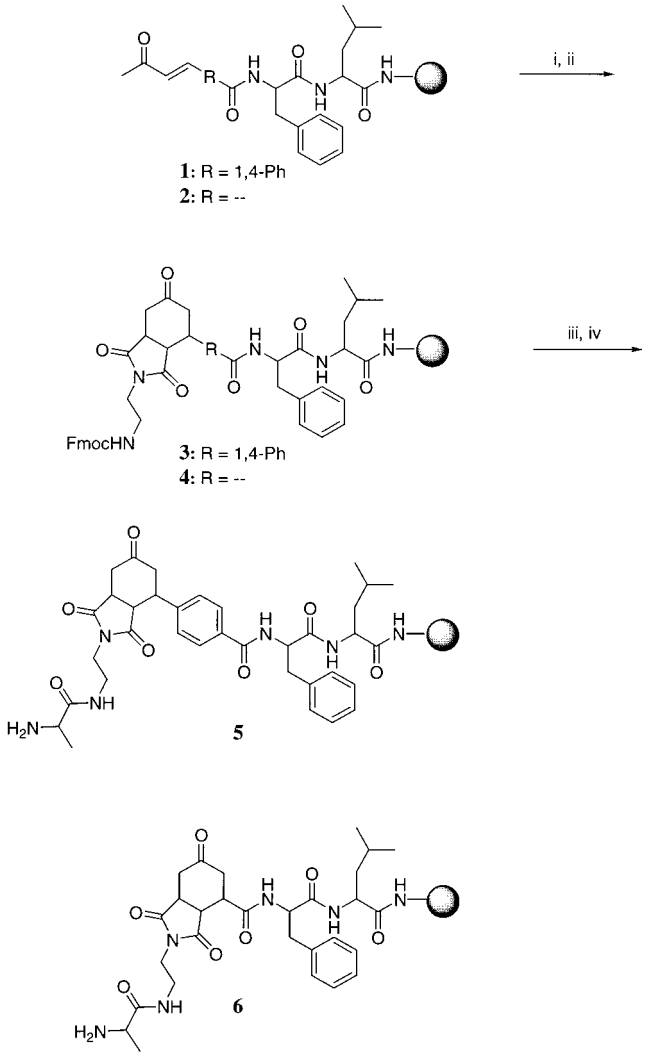
Scheme 1. Solid Phase Diels−Alder Reactions of Activated N-Terminal 1-Peptidyl-3-silyloxy Dienes Generated in Situ Followed by Continued Peptide Synthesis[a]



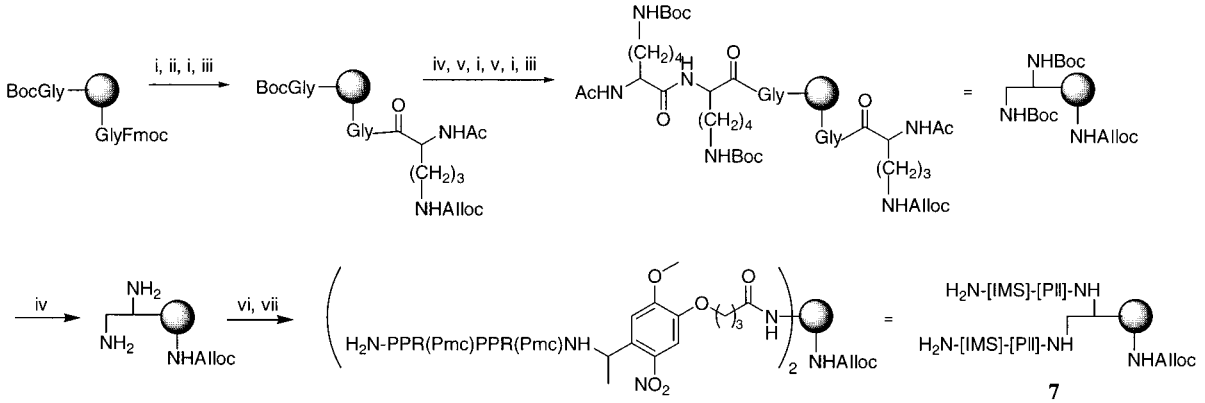
[a] (i) NEt_3, TBDMSOTf; (ii) *N*-(Fmoc-amino)ethylmaleimide; (iii) 20%piperidine/DMF; (iv) Fmoc-Ala-OPfp.

Results and Discussion

Solid-Phase Diels−Alder Reactions. Several solid-phase reactions incorporating non-peptidic elements into a peptide chain on PEG-based resins have previously been described.[11,15−17] For the incorporation of an organic isostere placed at an internal position of a peptide chain, thereby allowing continued peptide synthesis, the Diels−Alder reaction of an N-terminal 1-dipeptidyl-3-silyloxy-1,3-butadiene and *N*-(2-((*N*-fluoren-9-ylmethoxycarbonyl)amino)-ethylmaleimide was established (Scheme 1).[12] Also, the compatibility of standard Fmoc-based peptide synthesis with the Diels−Alder reaction conditions was determined, including the compatibility of the Diels−Alder reaction with the presence of functionalities and protection groups encountered in peptide synthesis. Thus, in a study conducted with a series of N-terminal 1-dipeptidyl-3-silyloxy-1,3-butadienes containing various amino acids, and *N*-(2-((*N*-fluoren-9-ylmethoxycarbonyl)amino)ethylmaleimide, it was determined that the Diels−Alder reaction was generally compatible with the types of side-chain functionalities found in amino acids except for histidine and cysteine. When these residues were present, the Diels−Alder reactions resulted in complex mixtures. It was also found that the Boc protection group was incompatible with the Diels−Alder reaction conditions. This was not surprising because Boc is known to be heat-labile.[18] The problem was solved by reaction with Boc_2O after the Diels−Alder reaction. Furthermore, in the presence of ^{t}Bu-protected aspartic acid a possible loss of the protecting group was indicated with formation of a rearranged product.[19] When taking these considerations into account, the Diels−Alder reaction shown in Scheme 1 was deemed suitable for application to a library format because the reactions studied were quantitative and clean. Upon further elongation with amino acids, the reactions yielded a bicyclic fragment internally in a peptide. Bicyclic structures of this type have been shown by Kahn et al. to be β-strand secondary structure mimetics,[20,21] and potent inhibitors of thrombin based on a similar structure at a terminal position have been identified. It is generally believed that proteolytic enzymes bind and possibly recognize substrates or inhibitors in the extended conformation through the formation of a β-sheet between the compound and the active-site cleft.[22,23] Hence, to identify inhibitors of a recombinant cysteine protease from *L. mexicana*, a combinatorial library of putative protease inhibitors based on these Diels−Alder reactions was prepared.

Library Design. The library was designed as a "one-bead−two-compounds" split-and-mix library prepared according to the principle of ladder synthesis[24] for facile analysis by MALDI-TOF mass spectrometry. Screening of the library according to the principle of "one-bead−two-compounds" involves the attachment of a preformed quenched fluorogenic protease substrate to reactive sites on each bead after the synthesis of the putative inhibitor library has been completed, followed by incubation with enzyme and selection of beads showing no substrate cleavage.[5] The preparation of the basic construct necessary for the "one-bead−two-compounds" library is illustrated in Scheme 2. The "one-bead−two-compounds" library relies on the utilization of three orthogonal protecting groups. In inhibitor library assembly, two orthogonal sets of protecting groups are used. The third orthogonal group is required to reserve sites throughout the library synthesis for the attachment of a fluorescence-quenched substrate in the last step.[25] Initially, separate sites for library synthesis and substrate attachment on the resin were introduced on PEGA resin with a 1:1 mixture of Boc- and Fmoc-protected glycine using TBTU activation, thus forming orthogonal sites for further derivatization at random positions throughout the resin. At the Boc-protected sites, the putative protease inhibitor library was synthesized following the attachment of a photolabile linker [Pll][26] and synthesis of an ionization-mass spacer [IMS] sequence PPRPPR. The sequence of the IMS was chosen because the two arginine residues facilitated ionization in the MALDI-TOF mass spectrometric analysis,[27] and the high preponderance of proline residues greatly reduced the risk of protease cleavage in this region. Also, the IMS had a mass of greater than 600, which placed the mass of the attached peptide fragments outside the region containing peaks from

Scheme 2. Preparation of the Basic Construct Necessary for the Synthesis of the One-Bead−Two-Compounds Library[a]



[a] (i) 20%piperidine/DMF; (ii) Fmoc-Orn(Aloc)-OH, TBTU, NEM; (iii) 50% Ac_2O/pyridine (1:1)/DMF; (iv) 95% TFA; (v) Fmoc-Lys(Boc)-OPfp; (vi) Fmoc-Pll-OH, TBTU, NEM; (vii) SPPS.

matrix adducts. The initial Fmoc-protected sites were converted into Alloc sites to retain orthogonality during the Fmoc-based library synthesis. After completion of the library, the Alloc sites were deprotected to release an amino functionality for the attachment of a preformed, quenched fluorogenic substrate Y(NO_2)EKFR- - -RGKK(Abz)G (k_{cat}/k_M = 4298 mM^{-1} s^{-1}; - - - indicates cleavage site) for the recombinant cysteine protease CPB2.8 ΔCTE from *L. mexicana*.[3]

This library design deviates from the "biantenna" design used earlier,[7] in which the library members were placed in proximity to a substrate molecule at the two ends of a functional biantenna. Instead, the design of this library is analogous to that originally reported in that the putative protease inhibitor library and the quenched fluorogenic substrate are placed at random positions on the solid support, i.e., at spatially separated positions.[5] It was preferable to spatially separate the two types of compounds present on each bead because initial experiments had revealed some levels of quenching of Abz fluorescence by the [Pll] when the biantenna approach was taken.[28] This contribution of the [Pll] to fluorescence quenching would result in reduced sensitivity. However, the problem was easily solved by the attachment of substrate and inhibitor to separate sites, as demonstrated by derivatizing a portion of the library with Abz at the substrate sites, resulting in brightly fluorescent beads when viewed under a fluorescence microscope. These beads also served as a reference to beads with a completely cleaved substrate after incubation. Conversely, a nonincubated portion of the library derivatized with fluorescence-quenched substrate was used to mark the level of inherent fluorescence of the library beads.

Theoretically, because each bead contained a unique putative inhibitor molecule, some of the beads would yield poor while others would yield potent inhibitors, giving rise to different levels of substrate cleavage upon incubation with the protease. Indeed, the brightness of the beads after incubation with the enzyme was highly differentiated, and only the very darkest beads were selected for sequence analysis. The use of conventional Edman degradation for sequence analysis was not feasible in this case because the inhibitory sequences contained a non-peptidic fragment. Instead, the inhibitor library was synthesized using the ladder

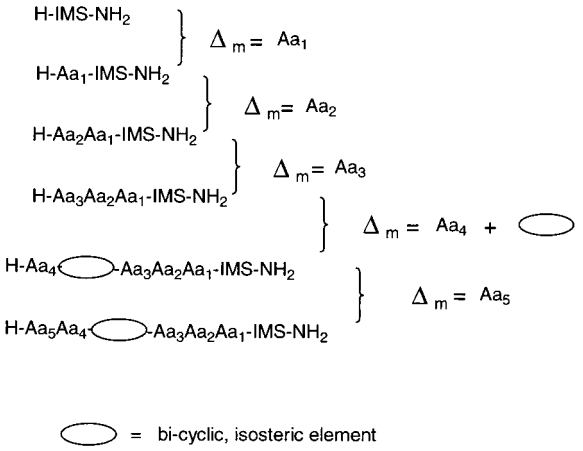
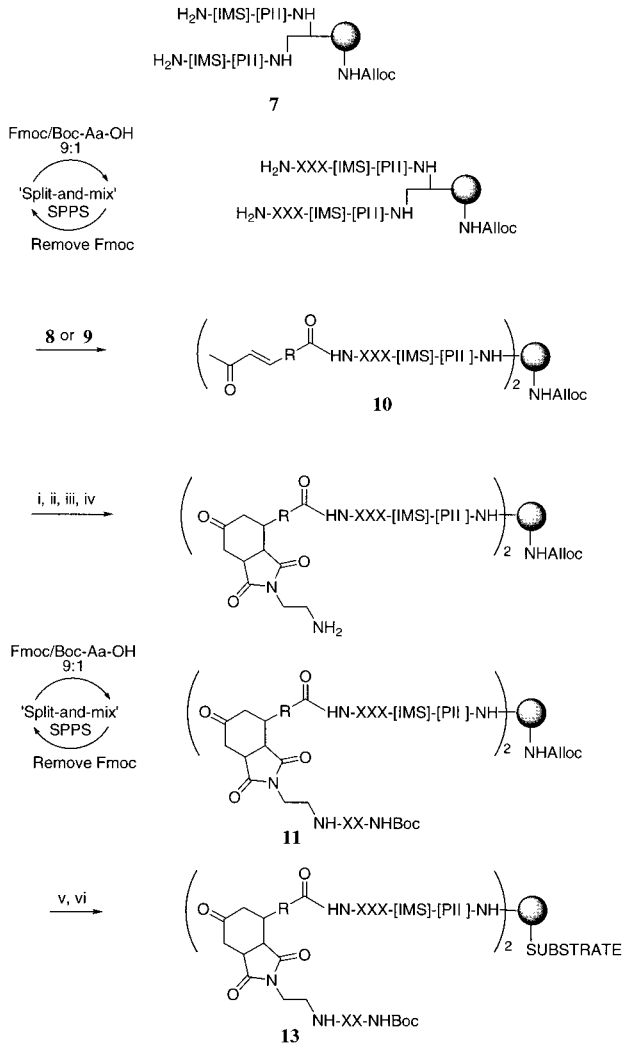


Figure 1. Fragments present on each bead as a result of ladder synthesis. Upon release of all fragments, the sequence can be read in a single mass spectrum from the mass difference between the fragments.

synthesis approach.[24] This implies the use of a capping agent in each step of the library assembly, leading to a series of truncated fragments with distinct masses from which the amino acid sequence can be read (Figure 1). For this approach to work successfully, it is important that accurately measured quantities of amino acid and capping agent are added simultaneously and that complete coupling and capping occur in each step. Matching of the reactivity of the two agents is conveniently obtained by using the Boc-protected analogue of the relevant amino acid component as the capping agent in a 1:9 mixture with the Fmoc-protected derivative.[29] Hence, in each step of the synthesis, only 90% of the amino functionalities were released for further couplings upon treatment with 20% piperidine in DMF. The non-peptidic inhibitory fragment present in the sequence was not encoded in this way. Instead, because the two possible non-peptidic fragments gave rise to distinct masses, the amino acids included in the following step were chosen to ensure that the sum of the masses of either non-peptidic fragment and any of the amino acids employed was unique. Thus, it was possible to determine the identity of the non-peptidic fragment and the following amino acid constituent unambiguously. Upon irradiation of the individual beads with a Hg lamp (UV light), a mixture of full-length inhibitor and

Scheme 3. Ladder Synthesis of a Combinatorial Split-and-mix One-Bead−Two-Compound Library[a]



[a] (i) NEt_3, TBDMSOTf; (ii) *N*-(Fmoc-amino)ethylmaleimide; (iii) Boc_2O, NEM; (iv) 20% piperidine/DMF; (v) $Pd(PPh_3)_4$, NEM, HOAc; (vi) **12**, TBTU, NEM. R = − or $-C_6H_4-$.

truncated fragments were released from the [Pll] and a single MALDI-TOF mass spectrum afforded the sequence of the library member present on each selected bead in a fraction of the time required for Edman sequencing.

Library Synthesis. The synthesis of the library is illustrated in Scheme 3. First, $PEGA_{1900}$ resin (loading: 0.16 mmol/g) was derivatized with a 1:1 mixture of Boc- and Fmoc-protected glycine. The Fmoc group was removed next, and the released amines coupled to N^{α}-Fmoc protected ornithine carrying the Pd-labile N^{δ}-Alloc protecting group on the side chain. The Fmoc group was removed (loading: 0.08 mmol/g) and replaced with acetyl using Ac_2O and pyridine in DMF (1:1:2). In the next step, Boc was removed under acidic conditions (95% TFA in H_2O) and the released amines were coupled to Fmoc-Lys(Boc) Pfp ester. At this point, the Fmoc loading was measured to be 0.07 mmol/g. Again, Fmoc was removed and the loading doubled through the coupling of another Fmoc-Lys(Boc) Pfp ester. After exchange of Fmoc for acetyl, Boc was removed and the photolabile linker was attached using TBTU activation followed by the amino acids of the ionization-mass spacer sequence using Fmoc amino acid Pfp esters to yield resin **7** bearing a basic skeleton containing distinct sites for the combinatorial library attached to the resin via a linker, and sites for attachment of substrate. To perform the combinatorial split-and-mix[30] part of the library synthesis, resin **7** was transferred to a custom-made multiple column peptide synthesis block.[4] The following peptide couplings were performed using mixtures of Fmoc- and Boc- protected amino acids in a molar ratio of 9:1 with TBTU activation to generate the ladder, with mixing and redistribution of the resin into the 20 wells of the synthesizer between each coupling with one activated Fmoc/Boc amino acid mixture in each well. To unambiguously read the ladder sequence, Ile and Gln were omitted throughout the library because their masses are isobaric with those of Leu and Lys, respectively. Also, the sulfur-containing amino acids Cys and Met were omitted. In the amino acid couplings prior to the Diels−Alder reaction forming the non-peptidic inhibitory fragment, His and the acidic residues Asp and Glu were omitted because of incompatibility with the chemistry used in the Diels−Alder reaction. Cyclohexylalanine was included in duplicate in all couplings and Phe, Lys, Arg, Val, and Leu were doubly represented in the first three couplings and in the first coupling after the Diels−Alder reaction. In the last coupling, only Boc-protected amino acids were employed because this reaction generated the full-length fragment and hence obviated the need for a ladder coding. In this step, Cha, Lys, and Leu were included in duplicate. The amino acids present in each coupling reaction are summarized in Table 1. Amino acids doubly represented were chosen to bias the library toward basic residues, which were highly selected in the substrate library for the CPB2.8 ΔCTE protease, and toward bulky, hydrophobic residues selected for by the protease in a reduced bond inhibitor library.[31]

After three amino acid couplings to **7** using split-and-mix methodology, the resin was split into two equal portions in syringes and coupled to **8** or **9** after Fmoc deprotection to yield resin **10**. When both reactions were complete, the resin portions were recombined and transferred to a flask after thorough washing and drying. Silyl enolization to generate an activated diene was performed using TBDMSOTf/NEt_3 under inert conditions. The resin was washed carefully and dried before it was submitted to Diels−Alder reaction with *N*-(2-((N-fluoren-9-ylmethoxycarbonyl)amino)ethylmaleimid in toluene overnight at 80−85 °C. After transfer to a syringe the resin was carefully washed with CH_2Cl_2 and DMF. Previous experiments had revealed that the Boc protecting group was unstable under the Diels−Alder reaction conditions; hence, it was necessary to reintroduce this group prior to any further peptide couplings. This was easily accomplished using Boc_2O (40 equiv) and NEM (80 equiv) in DMF overnight at ambient temperature. After this, the resin was redistributed in the 20 wells of the MCPS block and Fmoc removed for continued SPPS. After the final coupling, the inhibitor part of the library was completed to yield resin **11**. A sample was removed and fully deprotected with a TFA/scavenger cocktail, and 72 beads were analyzed by MALDI-TOF MS on a high-resolution spectrometer to assess the quality of the library synthesis. The results of the

Table 1. Amino Acids Included in Respective Positions (Multiplicity)

first position X_3'	second position X_2'	third position X_1'	fourth position X_1	fifth position X_2
Ala, Cha(×2), Phe(×2), Gly, Lys(×2), Leu(×2), Asn, Pro, Arg(×2), Ser, Thr, Val(×2), Trp, Tyr	Ala, Cha(×2), Phe(×2), Gly, Lys(×2), Leu(×2), Asn, Pro, Arg(×2), Ser, Thr, Val(×2), Trp, Tyr	Ala, Cha(×2), Phe(×2), Gly, Lys(×2), Leu(×2), Asn, Pro, Arg(×2), Ser, Thr, Val(×2), Trp, Tyr	Asp, Glu, Cha(×2), Phe(×2), Gly, His, Lys(×2), Leu(×2), Asn, Pro, Arg(×2), Thr, Val, Trp, Tyr	Ala, Asp, Glu, Cha(×2), Phe, Gly, His, Lys(×2), Leu(×2), Asn, Pro, Arg, Ser, Thr, Val, Trp, Tyr

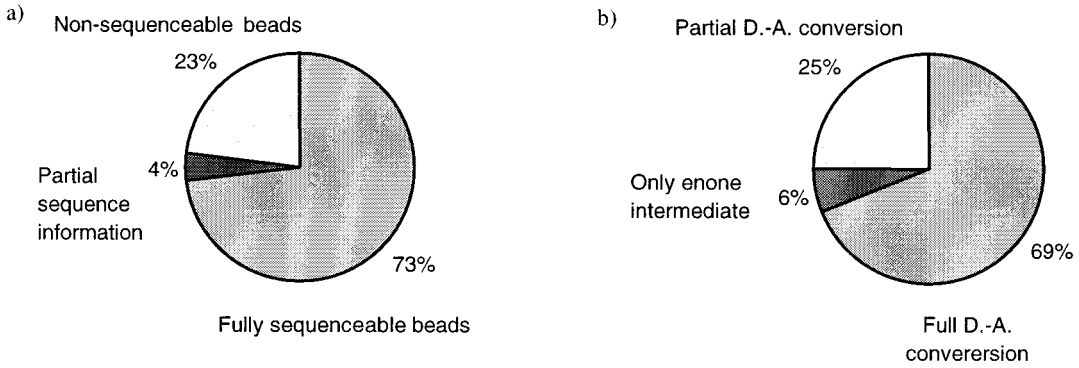
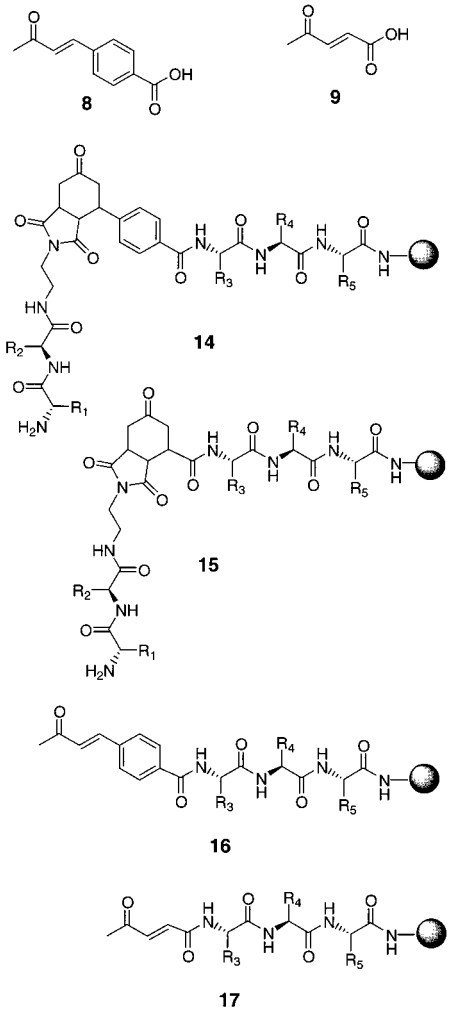


Figure 2. (a) Result of library analysis. (b) Result of the library synthesis with respect to the Diels−Alder reaction.

library synthesis are illustrated in Figure 2. Overall, 73% of the beads analyzed gave a full, unambiguous sequence assignment. Moreover, 4% of the beads gave rise to a partial sequence assignment. The remaining 23% of the beads failed to produce any meaningful sequences or gave more than one possible sequence. Among the 73% readable beads, 69% contained only the full-length product; i.e., the Diels−Alder reactions were complete in these cases because no fragments corresponding to unreacted enone **10** were detected. In 25% of the beads, the Diels−Alder reaction had not gone to completion, since fragments corresponding to unreacted enone were detected. Finally, 6% of the beads had failed to react in the Diels−Alder reaction and displayed as the heaviest fragment the mass corresponding to unreacted enone. The distribution between incorporated **8** and **9** was 1:1 within each group, indicating that the Diels−Alder reactions were equally efficient using either diene. The occurrence of incomplete Diels−Alder reactions was unexpected because the preliminary investigations of the Diels−Alder reactions with dipeptidic substrates containing various combinations of amino acids included in the library had not indicated incomplete reactions as a problem.[12] In fact, in these studies, the Diels−Alder reactions were quantitative, as judged by LC−MS. However, because a split-and-mix combinatorial library displays a high degree of order in an on-bead screening protocol, it was decided that the screening of the library would proceed because beads containing unreacted material would not interfere with the screening of completely reacted beads.

Screening of the Library. From a 300 mg portion of resin **11** (approximately 150 000 beads) Alloc was removed[32] and a fully protected, quenched fluorogenic substrate Boc-Y(NO_2)E(^{t}Bu)K(Boc)FR(Pmc)- - -R(Pmc)GK(Boc)K((Boc)-Abz)G-OH (**12**) was coupled to the free amines using TBTU as a coupling reagent to yield resin **13**. The substrate was prepared by standard SPPS procedures[33] on PEGA resin using the base-labile 4-hydroxymethylbenzoic acid linker and purified by flash chromatography. After attachment of the substrate, **13** was fully deprotected of all acid-labile protecting groups using TFA containing appropriate scavengers followed by washings and neutralization with 2% aqueous $NaHCO_3$. Finally, the resin was washed three times with assay buffer. Incubation of the resin was performed with a 200 nM solution of the CPB2.8 ΔCTE cysteine protease at 37 °C for 30 h. The incubation time was determined by removing small samples of resin at intervals for inspection under the fluorescence microscope. Substrate cleavage was rather slow, since many dark beads remained even after 24 h. To select only the best inhibitors from the library, it was important that only the darkest few beads were picked. After incubation, the enzyme was deactivated with acid and the resin neutralized with 2% aqueous $NaHCO_3$ and washed several times with H_2O before viewing under a fluorescence microscope. The fluorescence intensity of the bead varied from extremely bright ones to relatively dark ones, and the darkness of these beads was most reliably evaluated when they were removed from the bright beads to ensure that only the very darkest were collected. Collection of beads was performed manually and beads were directly placed onto a MALDI-TOF MS target for rapid analysis. From the screened beads, 77 were selected corresponding to approximately 0.05% of the library; these gave rise to 66 unambiguously determined sequences. The spectra obtained were of a good quality, and sequencing was fast. Peaks were assigned a mass, and a sequence assignment program was used to calculate all possible mass differences and compare them with a list of molecular masses of the amino acids. Hence, the program was able to automatically assign the inhibitor sequence. In most cases, more peaks were present in a spectrum than could be accounted for by the amino acid

Scheme 4. Diene Precursors and General Structures of Active Hits from the Library



sequence. However, these peaks were included in the calculation routine but most frequently did not give rise to any meaningful sequence and were consequently assigned as impurities.

Library Hits. The results of the library screen are illustrated in Figure 3. Most notably, the library hits fall into three categories: category A comprising 21 beads (32% of all hits) that contain full-length inhibitors only and no fragments from enone Diels−Alder starting material; category B comprising 20 (30%) beads containing *only* enone Diels−Alder starting material and no full-length fragment; category C of 25 (38%) beads containing both full-length compound and unreacted enone starting material. In other words, two distinct classes of compounds, the bicyclic full-length structures of category A and the half-length α,β-unsaturated ketone Michael acceptors of category B, were found to inhibit the protease. The frequencies of amino acids found at various positions in active inhibitors obtained from the library are illustrated in Figures 4−6. In the following, a substrate-like orientation of the inhibitors is assumed and the amino acid subsites are assigned as X_2, X_1, X_1', X_2', and X_3', in analogy to the standard $P_2\cdots P3'$ nomenclature of protease substrates and inhibitors.[34] In this assignment, the isoster is placed between X_1 and X_1'. However, at present the binding mode and orientation of the identified inhibitors

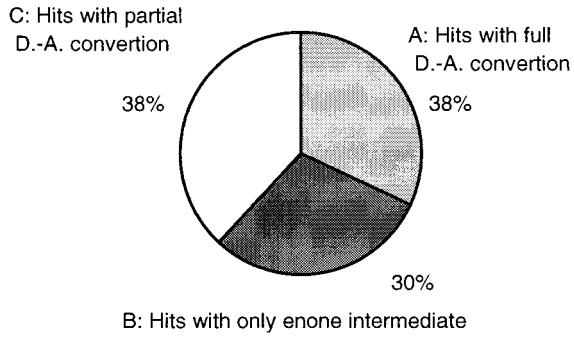


Figure 3. Distribution of hits in categories A, B, and C.

in the enzyme active site is unknown. Within category A, 95% (19 of 21) of the inhibitors were of the general structure **14**; i.e., they contained the isoster derived from aromatic acid **8**. Compared with the library as such, this is a very high selectivity because isosters from either **8** or **9** occurred with equal frequency within the group of fully reacted beads. Also, a consensus of amino acid occurrences in different subsites was observed. Figure 4a) shows the frequencies of amino acids in all inhibitors of general structure **14**. In X_2, aromatic residues were highly preferred, with Trp as the most frequently occurring residue, and in X_1', β-branched aliphatic residues were preferred with high occurrences of Val or Cha. Subsites X_1 and X_2' were unspecific, whereas the situation for X_3' was more complex. The hits of category A can be further subdivided into groups depending on the nature of the residue in subsite X_3'. Figure 4b shows the subsite specificities of six hits characterized by having a basic residue in X_3' (subgroup A1). It is seen that apart from the imposed selectivity in X_3', the selectivities of X_2 and X_1' were still preserved within this subgroup. Likewise, the other five hits were characterized by having either of the aromatic residues Phe or Tyr in X_3', as illustrated in Figure 4c (subgroup A2). Again, the subsite selectivities in X_2 and X_1' were preserved within this subgroup. Curiously, two hits containing Trp in X_3' had completely different amino acid occurrences in X_2 and X_1.

It was evident that the beads of category B containing only the enone starting material were strongly selected by the protease because the occurrence of this group was only 6% in the library as such, yet 30% among the hits. This could indicate a less specific, possibly irreversible interaction. Irreversible Michael acceptor type inhibitors of cysteine proteases have been previously described.[35,36] The subsites of inhibitors of category B were labeled $X_1'-X_3'$ to maintain analogy with the labeling of inhibitors of category A, hence assuming interaction with only half of the active site of the enzyme. Interaction with only half of the active site is well established for many cysteine protease inhibitors developed to date, and most interact with the nonprime site of the enzyme, terminating in an electrophilic moiety.[35] The frequencies of amino acids found in individual subsites are illustrated in Figure 5. For inhibitors of general structure **15** (Figure 5a), Cha was the most abundantly occurring amino acid in all subsites, along with other hydrophobic residues such as Ala, Val, and Leu. Subsite X_3' seemed to be the least specific because aromatic (Trp), basic (Lys), and amide (Asn) residues also occurred in this subsite. For general

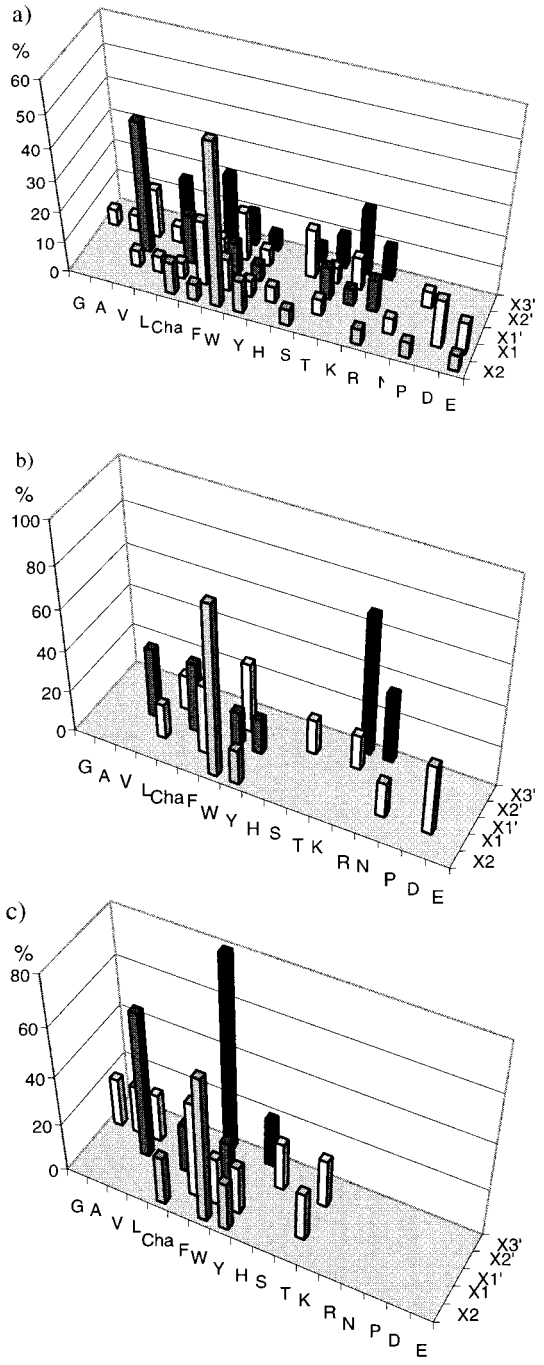


Figure 4. (a) Distribution of amino acids in subsites of category A hits (general structure **14**, total). (b) Distribution of amino acids in subsites of category A1 hits. (c) Category A2 hits.

structure **16** (Figure 5b), Leu was highly preferred in subsite X_1', whereas subsites X_2' and X_3' were more unspecific, albeit with a preference toward hydrophobic residues. The strong preference for Leu in X_1' indicates similarity to other well-known general inhibitors of cysteine proteases of the papain family such as E-64[37] and leupeptin analogues,[35] where Leu is the amino acid preceding the electrophilic isoster. The generally high predominance of hydrophobic residues in the inhibitors of category B may be a result of the "grease effect", i.e., the tendency of hydrophobic entities to pack together in an aqueous environment because cysteine proteases tend to have relatively shallow, solvent-exposed active sites.[35]

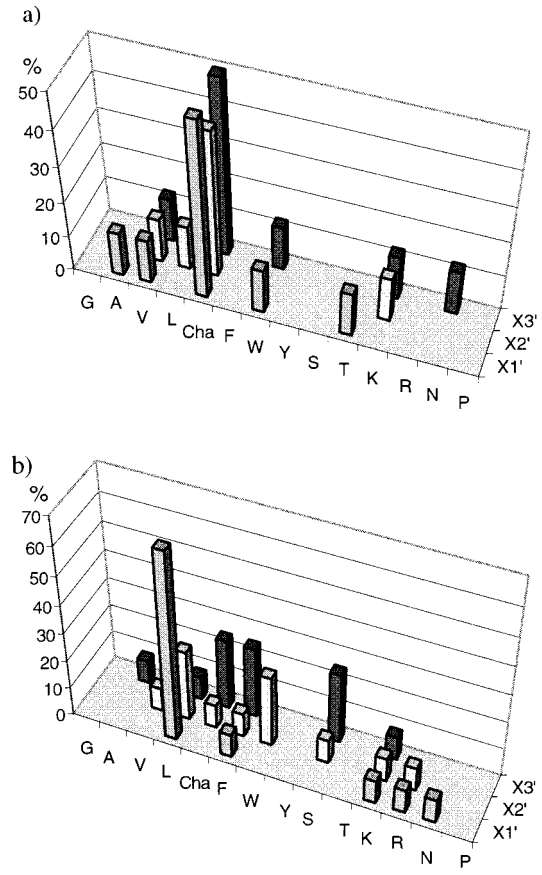


Figure 5. (a) Distribution of amino acids in subsites of category B hits of general structure **15**. (b) Distribution of amino acids in subsites of category B hits of general structure **16**.

The beads of category C containing full-length product as well as Diels−Alder starting material comprised 38% of the hits, corresponding to a slight over-representation. This was unexpected considering the 25% frequency in the library as such and the strong selection of beads of category B by the protease. This could indicate that the inhibiting species was the full-length products in most of these cases. However, structures based on acid **9** were selected in category C (16 out of 25 (64%)) in contrast to the 5% inhibitors based on **9** in category A. In the library as such, structures derived from **8** or **9** were equally abundant among beads of the category C type. When the frequencies of amino acids in the subsites of inhibitors of general structure **14** + **15** (illustrated in Figure 6a) are examined, a completely different pattern from those of category A hits was observed. Hence, Trp was no longer predominant in X_2, but in X_1, an unspecific site in category A hits. The primed subsites were all unspecific in category C hits of type **14** + **15**. From examination of the sequences from individual beads, however, there are three displayed amino acid residues that would fit the selectivity pattern of category A hits. The amino acid frequencies of hits of general structure **16** + **17** are displayed in Figure 6b. Hits of this type displayed a certain preference for Trp in X_2 and a certain preference for Phe or acidic residues Asp and Glu in X_1. The greatest selectivity was found in X_1' with Leu and Phe as the most abundant residues, in accordance with the selectivity observed for structure **16** hits of category B. In subsite X_2' a preference for aromatic residues Phe, Tyr,

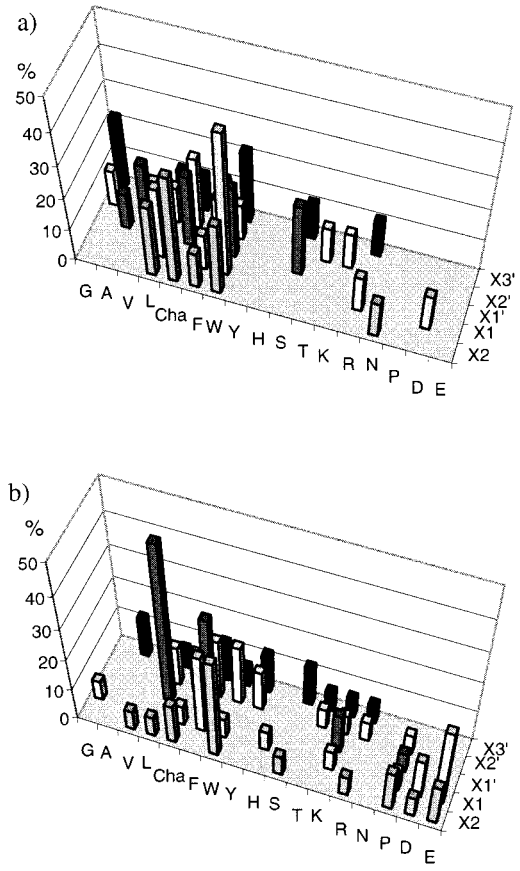


Figure 6. (a) Distribution of amino acids in subsites of category B hits of general structure **14** + **15**. (b) Distribution of amino acids in subsites of category B hits of general structure **16** + **17**.

and Trp or bulky aliphatics Leu and Cha was observed, whereas X_3' was unspecific. Thus, it appeared that category C hits of type **16** + **17** generally resembled category B hits of type **16**, whereas the situation was less clear for category C hits of structure **14** + **15**.

Evaluation of Hits. To verify that the library was indeed yielding inhibitors of the CPB2.8 ΔCTE protease, a number of sequences were selected and resynthesized for solution-phase assaying as inhibitors of CBP2.8 ΔCTE (Table 2). All sequences were synthesized as carboxamides to mimic the polymer-bound situation better and to avoid the negative charge of a free terminal carboxylic acid. In the selection of sequences for resynthesis, it was assumed that none of the fragments present on a bead as a result of the ladder synthesis was responsible for inhibition, although this is theoretically possible. Of the synthesized sequences, **20**, **21**, **23**, **26**, and **30** were "consensus sequences", i.e., constructed on the basis of frequently occurring residues in the respective subsites. IC_{50} values of the inhibitors were determined against the substrate Cbz-Phe-Arg-Amc and used to rank their potencies (Table 2). With a few exceptions, the IC_{50} values were in the high nanomolar to low micromolar range. Interestingly, it was possible to design relatively potent inhibitors on the basis of frequently occurring amino acids in the respective subsites. In fact, one of the most potent inhibitors identified was a constructed category A2 consensus sequence (**23**). A category A hit not following the amino acid selectivity pattern was slightly less potent (**25**). Most surprisingly, the constructed sequence **26** following the amino acid selectivity of a category A2 hit but with the isosteric element based on acid **9** turned out as one of the most potent inhibitors, indicating that the pronounced preference for the isoster derived from **8** in category A hits is not an absolute requirement. A category C hit (**27**) synthesized as a full-length structure was a poor inhibitor, indicating that the inhibitory species present on the bead was probably the Michael acceptor Diels−Alder starting material. This assumption was supported by the fact that a category B hit (**28**) strongly resembling the primed sites of **27** was identified as the most potent inhibitor. However, other category B sequences, especially **30**, were less potent.

Table 2. Sequences, Analytical Data, and IC_{50} Values of Resynthesized Inhibitors

	H−X_2X_1 isoster[a] $X_1'X_2'X_3'$-NH_2	MW	ES-MS, found[b]	purity,[c] %	IC_{50}, μM
18	W D ar.i. V W R	1070.9	1072.4	96	>30
19	Y L ar.i. Cha L K	999.2	1000.7	98	0.5
20	W F ar.i. V W K	1075.5	1076.6	100	nd
21	W D ar.i. V S K	943.7	945.5	93	1.7
22	W F ar.i. V K F	1035.9	1037.6	95	2.5
23	Cha W ar.i. V V F	1013.2	1014.5	98	0.45
24	F Cha ar.i. V V L	940.1	941.5	90	1.5
25	R E ar.i. T P L	924.7	926.5	97	8
26	W F al. i. L Y Y	1024.9	1026.4	95	0.5
27	L D ar.i. Cha Cha G	920.3	921.5	100	>50
28	ar.e. Cha Cha Cha	648.9	650.5	90	0.23
29	ar.e. Cha L K	583.9	584.3	95	10
30	al.e. L W S	499.5	500.3	95	>50
31	al.e. L L Cha	492.8	493.4	90	4

[a] ar.i. = aromatic isoster (general structure **14**), al.i. = alifatic isoster (general structure **17**), ar.e.= aromatic enone (general structure **15**), al.e. = alifatic enone (general structure **16**). [b] $[M + H]^+$. [c] RP-HPLC at 215 nm.

The results from the resynthesized sequences demonstrate that the correlation between inhibitor activity on the solid phase and in solution is generally good. Most of the resynthesized inhibitors had IC_{50} values in the high nanomolar to low micromolar range, with just a few exceptions. It is also worth noting that constructed consensus sequences and inhibitors identified from the library had IC_{50} values in the same range. The results indicate that the detection limit of the library was in the 0.2−2 μM range; i.e., it was impossible to detect beads containing inhibitors with 0.2 μM IC_{50} values and below. The detection limit of the assay can be altered by lowering the ratio of inhibitor sites to substrate sites on the resin, by using a substrate with higher affinity for the protease, by using higher enzyme concentrations in the screening, or by performing repeated, sequential incubations. However, for the first screen, these factors were well balanced in the present work because the detection limit was sufficient to reveal inhibitors with 0.2−2 μM IC_{50} values and to reveal subsite specificity trends.

Conclusion

The present study describes the preparation of a combinatorial library of peptide isosters formed in a solid-phase reaction, placing the isoster at an internal position. The isoster was formed in a solid-phase Diels−Alder reaction, yielding a bicyclic, non-peptidic element. The library was synthesized

according to the ladder synthesis approach, allowing the sequence of individual beads to be read in a single MALDI-TOF mass spectrum.

When a quenched, fluorogenic substrate was attached, the library was screened for inhibitors against the recombinant cysteine protease CPB2.8 ΔCTE from the parasite *L. mexicana*. Several hits were identified upon incubation with the protease, and their structures revealed that the library contained two distinct types of inhibitors: the expected full-length peptide isoster and a "half-length" inhibitor terminating in a Michael acceptor α,β-unsaturated ketone used as starting material for the Diels−Alder reaction. Also, the hits obtained from the solid-phase screening revealed a strong preference for certain amino acids in some of the respective subsites of the peptide isosters. Solution-phase evaluation of selected hit sequences identified from the library as well as constructed sequences based on the subsite frequencies of amino acids revealed inhibitors with IC_{50} values in the high nanomolar to low micromolar range. When structures exhibiting inhibitory activity against the CPB2.8 ΔCTE protease on the solid phase as well as in solution are provided, the solid-phase screening approach of the "one-bead−two-compounds" library is validated. A full kinetic characterization of the identified inhibitors and a specificity study of related cysteine proteases are subject to future work.

Experimental Section

All solvents were HPLC grade and were purchased from LabScan (Dublin, Ireland). DCM was dried by distillation from $CaCl_2$ and stored over 4 Å molecular sieves under inert gas. PEGA library resin with a PEG_{1900} cross linker ($PEGA_{1900}$) and PEGA resin with a PEG_{800} cross linker ($PEGA_{800}$) were purchased from Polymer Laboratories. Fmoc-amino acids and their Pfp ester derivatives were obtained from Bachem or Novabiochem. Fmoc-Lys((Boc)-Abz)-OH and Fmoc-Tyr(NO_2)-OH were prepared as previously described.[25] MSNT, TBTU, and *N*-(Fmoc)ethylendiamine hydrochloride were purchased from Novabiochem. *N*-Methylimidazole, NEM, DIPEA, 4-carboxybenzaldehyde, *N*-(methoxycarbonyl)maleimid, and TBDMSOTf were purchased from Fluka. HATU, TIS, and triethylamine were obtained from Aldrich. 3-Acetylacrylic acid was from Lancaster. Cbz-Phe-Arg-Amc was obtained from Sigma. Trifluoroacetic acid was purchased from Merch-Schuchardt, and piperidine was purchased from Riedel-de-Häen. All reagents and solvents were used as received without further purification. Analytical RP-HPLC was performed on a Waters system (490E detector, two 510 pumps with a gradient controller, and an 8 mm i.d. RCM C_{18} column). Semipreparative RP-HPLC was carried out on a Waters system (991 photodiode array detector and 600 E system controller) fitted with a Waters 25 mm i.d. RCM C_{18} column. All RP-HPLC procedures were carried out using a linear gradient at a flow rate of 10 mL/min. Buffers were (A) 0.1% TFA in H_2O and (B) 0.1% TFA in CH_3CN/H_2O (9:1). Electrospray mass spectra were acquired on a Hewlett-Packard HP1100-MSD mass spectrometer (mobile phase 0.3% acetic acid in water/0.03% acetic acid in acetonitrile (1:1); flow rate 100 μL/min). MALDI-TOF mass spectra were recorded on a Bruker Reflex III high-resolution spectrometer, and sequencing was facilitated using the Aura macro DeltaLabel supplied with the software. Library beads were examined under an Optical Star fluorescence microscope with a 320 nm band-pass filter for excitation (20 nm wide) and a 410 nm low-pass filter for detection.

Deprotection of N^{α}-Fmoc was done using 20% piperidine in DMF for 4 and 16 min. Washing volumes were always 1−2 times the volume necessary to swell the resin, and washings were generally repeated six times unless otherwise specified. Minimum reaction volumes were used in all coupling reactions. Amino group loadings were measured by determining spectrophotometrically at 190 nm, using a standard curve, the amount of released piperidine/dibenzofulvene adduct from a known sample. Amino acid couplings were followed using the Kaiser test[38] (primary amines) or the chloranil test[39] (secondary amines).

Products **5** and **6** were prepared as summarized in Scheme 1 and characterized by NMR and ES-MS after cleavage from the resin.[12]

Substrate for Recombinant *L. mexicana* Cysteine Protease CPB2.8 ΔCTE (12). The substrate was synthesized in a syringe using $PEGA_{800}$ resin (3.16 g; loading, 0.4 mmol/g) derivatized with a preactivated solution of the base-labile 4-hydroxymethylbenzoic acid linker (576 mg, 3.79 mmol), NEM (800 μL, 6.32 mmol), and TBTU (1136 mg, 3.54 mmol) in DMF. When the coupling was complete as judged by the Kaiser test, the resin was reacted with a solution of Fmoc-Gly-OH (1126 mg, 3.79 mmol), *N*-methylimidazole (300 μL, 3.79 mmol), and MSNT (1126 mg, 3.79 mmol) in DCM for 45 min at room temperature. The coupling was repeated and the resin washed with DCM (×5) and DMF (×3). Loading was measured at 0.35 mmol/g. Fmoc was removed, and the resin reacted with a preactivated solution of Fmoc-Lys((Boc)Abz)-OH (1116 mg, 1.9 mmol), NEM (400 μL, 3.16 mmol), and TBTU (586 mg, 1.77 mmol) in DMF. Following Fmoc deprotection, the sequence Fmoc-Glu(^{t}Bu)-Lys(Boc)-Phe-Arg(Pmc)-Arg(Pmc)-Gly-Lys-(Boc) was coupled using solutions of Fmoc amino acid OPfp esters (1.9 mmol amounts: Lys(Boc) 1206 mg; Gly 878 mg; Arg(Pmc) 1575 mg; Phe 1051 mg; Glu(^{t}Bu) 1124 mg) and DhbtOH (205 mg, 1.26 mmol) in DMF. Fmoc was removed, and the resin reacted with a preactivated solution of Fmoc-Tyr(NO_2)-OH (1633 mg, 3.79 mmol), NEM (800 μL, 6.32 mmol), and TBTU (1136 mg, 3.54 mmol) in DMF. When the Kaiser test was negative, the resin was washed and the Fmoc removed and replaced by Boc by reacting with Boc_2O (2755 mg, 12.64 mmol) and NEM (2.4 mL, 19 mmol) in DMF for 16 h. The resin was washed with DMF (×6) and MeOH (×6), then transferred to a sintered funnel for cleavage of the peptide product; the resin was treated with a 1:1 mixture of 1 M aqueous NaOH and MeOH for 30 min at room temperature, then drained and washed with THF (×3). All washings were collected and combined. The cleavage was repeated, this time without the organic cosolvent, followed by washing with THF, CH_3CN, and MeOH. When no more of the bright-orange product eluted from the resin, the combined washings and cleavage mixtures were cooled to 0 °C and neutralized with 1 M aqueous HCl. The

organics were removed under reduced pressure, and the remaining aqueous solution was freeze-dried to yield an orange powder. This was dissolved in MeOH, filtered to remove NaCl, and then purified by flash chromatography on silica using $CH_3CN/EtOAc/H_2O$ as eluents with a gradient from 9:9:1 to 9:9:2. Combined pure fractions were dried and evaporated. Yield: 1290 mg (42%). ESI-MS: calcd *m*/*z* 2420.9 (M); found *m*/*z* (%) 2422.9 (5) ($M + H^+$), 1211.7 (100) ($M + 2H^+$).

Ac-Lys(Fmoc-[IMS]-[Pll])Lys(Fmoc-[IMS]-[Pll])/Ac-Orn(Alloc)-PEGA (7). A mixture of Fmoc-glycine (114 mg, 0.38 mmol) and Boc-glycine (67.4 mg, 0.38 mmol) was dissolved in DMF, and NEM (162 μL, 1.28 mmol) was added followed by TBTU (230 mg, 0.72 mmol). The mixture was preactivated for 5 min before it was added to $PEGA_{1900}$ resin (1.6 g; loading, 0.16 mmol/g). The reaction was followed by the Kaiser test, and when the reaction was complete, the resin was drained and washed with DMF. Fmoc was removed, and the resin was washed with DMF. A solution of Fmoc-Orn(Alloc)-OH (168.4 mg, 0.38 mmol) in DMF was added to NEM (81 μL, 0.64 mmol) and TBTU (115 mg, 0.36 mmol). After a preactivation time of 5 min the mixture was added to the resin. When the reaction was complete, the resin was drained and washed with DMF, Fmoc was removed (loading: 0.08 mmol/g), and the resin was washed. A mixture of Ac_2O (5 mL), pyridine (5 mL), and DMF (10 mL) was prepared and added to the resin. After 30 min, the resin was washed with DMF (×5) and H_2O (×5). Boc was removed by deprotection with 95% TFA/H_2O for 30 min, then the resin was washed AcOH (×3), DMF (×3), 5% DIPEA/DMF (×3), and DMF (×6). A solution of Fmoc-Lys(Boc)-OPfp (241 mg, 0.38 mmol) and DhbtOH (20 mg, 0.13 mmol) in DMF was added to the resin, and after complete coupling and deprotection this step was repeated. After the second Fmoc removal (loading: 0.07 mmol/g) and after the resin was washed, the resin was acetylated as described above and Boc was removed as described above. The photolabile linker (400 mg, 0.77 mmol) was mixed with NEM (162 μL, 1.28 mmol) and TBTU (230 mg, 0.72 mmol) in DMF and added to the resin. After this point the reaction vessel was wrapped in aluminum foil at all times to prevent exposure to light. When the reaction was complete according to the Kaiser test, the resin was drained and washed and Fmoc was removed. The sequence Fmoc-Pro-Pro-Arg(Pmc)-Pro-Pro-Arg(Pmc) was coupled using Fmoc-protected Pfp esters of the amino acids (0.77 mmol amounts: Pro 386 mg; Arg(Pmc) 638 mg) and DhbtOH (42 mg, 0.26 mmol); amino acid and DhbtOH were dissolved in DMF and added to the resin. Couplings were followed by the Kaiser test (coupling to a primary amine) or the chloranil test (coupling to a secondary amine). After completion of the sequence the resin was washed with DMF (×5) and DCM (×5) and lyophilized. A resin sample was irradiated for 2 h with an Hg lamp to cleave the IMP, and a MALDI-TOF mass spectrum was acquired. MALDI-MS: calcd *m*/*z* 717.9 (M); found *m*/*z* 718.7 ($M + H^+$).

Library Synthesis. Ac-Lys(Fmoc-[IMS]-[Pll])Lys(Fmoc-[IMS]-[Pll]-)/Ac−Orn(Alloc)-PEGA **7** (1.6 g, 0.26 mmol) was placed in a 20-column MCPS reactor[4] and distributed equally in the columns. This was easily done by filling the reactor block with DMF above the level of the columns, closing it with a lid, shaking it upside down, and then gently turning it upright. NOTE: it is important to perform all mixing and splitting steps while the resin is in the protected state of the reaction cycles because it is otherwise sticky and will adhere to the sides of reaction vessel, making all manipulations very difficult.

After distribution into the columns, Fmoc was removed and the resin washed with DMF. The split-and-mix ladder synthesis was carried out by a repetitive protocol. (1) The resin was coupled with 9:1 mixtures of the Fmoc- and Boc-protected amino acids specified for each coupling in Table 1 with one specific Fmoc/Boc amino acid added to each column. Stock solutions were prepared in DMF with each Fmoc/Boc amino acid mixture as well as NEM and TBTU, calculating the amounts of reagents for performing double couplings in each step to ensure complete reactions. The stoichiometries used were 3 equiv of Fmoc/Boc amino acid, 5 equiv of NEM, and 2.8 equiv of TBTU. Thus, for each coupling 140 μL of Fmoc/Boc amino acid solution, 140 μL of NEM solution, and 140 μL of TBTU solution were mixed in Eppendorf tubes and preactivated for 5 min before they were added to the individual columns. All couplings were performed as double couplings with the first coupling proceeding for 6 h and the second overnight. (2) The resin was washed with DMF (×6). (3) The reactor block was filled with DMF above the level of the columns, the lid placed on top, and the reactor block containing the sample gently agitated upside down for 15 min. The synthesizer was turned upright in a gentle movement, and it was ensured that the resin was equally redistributed in the columns. (4) The resin was drained, and Fmoc was removed. (5) The resin was washed with DMF (×6). The steps were then repeated starting from step 1.

After of three amino acids were coupled, the resin was removed from the reactor and distributed equally between two syringes. In both syringes, Fmoc was removed and the resin washed with DMF. To one syringe, a preactivated solution of 4-(3-oxo-but-1-enyl)benzoic acid **8** (73 mg, 0.38 mmol), NEM (81 μL, 0.64 mmol), and TBTU (115 mg, 0.36 mmol) was added. To the other syringe, a preactivated solution of 3-acetyl acrylic acid **9** (44 mg, 0.38 mmol), NEM (81 μL, 0.64 mmol), and TBTU (115 mg, 0.36 mmol) was added. Both reactions were allowed to proceed for 6 h and were then repeated and allowed to run overnight. Both resin portions were drained and washed with DMF and DCM and then recombined, mixed, and lyophilized to yield resin **10**.

Resin **10** was placed in an argon atmosphere in a flame-dried flask and swollen in dry DCM (22 mL), then cooled to 0 °C. NEt_3 (1.34 mL, 9.63 mmol) was added, and the resin was stirred for 10 min. Then TBDMSOTf (1.65 mL, 7.19 mmol) was added dropwise with stirring. The resin mixture was kept at 0 °C for 1 h, then at room temperature for 2.5 h before it was transferred to a syringe and washed with DCM (×6) and dried. *N*-(2-(Fmoc-amino)ethyl)maleimide (1.27 g, 3.52 mmol) was dissolved with heating in toluene (15 mL) in a flask, and the resin was added to the warm solution and kept at 85 °C overnight. Then it was

drained and washed with toluene (×2), DCM (×5), and DMF (×5). Next, a solution of Boc_2O (2.0 g, 9.2 mmol) and NEM (2.27 mL, 18 mmol) in DMF (10 mL) was prepared and added to the resin. The reaction was allowed to run overnight, then the reagents were removed by suction, and the resin was washed with DMF (×8). The resin was then redistributed in the multiple-column peptide synthesizer, and one amino acid was coupled following steps 1−5 of the ladder synthesis protocol as described above. After Fmoc deprotection, only Boc-protected amino acids were coupled in the last step to form library **11**.

A sample of **11** (50 mg) was removed for analysis: The resin was treated with TFA/H_2O/TIS (95:2.5:2.5) for 2 h, then washed with TFA/H_2O (×1), AcOH (×3), DMF (×3), 5% DIPEA/DMF (×3), and DMF (×5). For MALDI-TOF analysis, individual beads were placed on a target and irradiated for 2 h with an Hg lamp. H_2O (0.2 μL), CH_3CN (0.2 μL), and a matrix solution (0.2 μL, 10 mg of α-cyano-4-hydroxy-cinnamic acid in 1 mL of CH_3CN/H_2O (7:3)) were added to each bead and allowed to dry before acquisition of the spectrum. Of the analyzed beads, 73% gave a fully readable, unambiguous sequence. A total of 4% gave rise to a partial sequence, whereas 23% of the beads were not readable or gave rise to more than one possible sequence.

One-fifth of resin **11** corresponding to 300 mg of underivatized resin or approximately 150 000 beads (approximately 500 000 beads/g of resin) was removed and treated in an argon atmosphere with $Pd(PPh_3)_4$ (83 mg, 0.072 mmol) dissolved in degassed $CHCl_3$ containing NEM (2.5%) and AcOH (5%) for 2 h at ambient temperature. The resin was drained and washed with DCM (×3), DMF (×5), 0.5% Et_2NCS_2Na/DMF (×1), and DMF (×5), then treated with a preactivated mixture of substrate **12** (174 mg, 0.072 mmol), NEM (15 μL, 0.12 mmol), HOAt (3.2 mg, 0.024 mmol), and HATU (26 mg, 0.067 mmol) in DMF at room temperature overnight. The resin was washed with DMF (×5) and DCM (×5). All protecting groups were removed with TFA/H_2O/TIS (95:2.5:2.5) as described above, and after deprotection the resin was washed with HOAc (×3), DMF (×3), 5% DIPEA (×3), DMF (×3), H_2O (×3), and assay buffer (×3) prior to incubation with the protease.

Incubation of Library with CPB2.8 ΔCTE and Selection of Positive Beads. A 200 nM solution of the recombinant protease CPB2.8 ΔCTE in phosphate buffer (10 mM, pH = 6.1) augmented with cysteine (5 mM) was added to the substrate as a derivatized, fully deprotected library and incubated at 37 °C for 24 h to ensure that only a few beads would remain dark. The resin was drained and washed with H_2O (×2), 2% aqueous TFA (×2), H_2O (×3), 2% aqueous $NaHCO_3$ (×2), and H_2O (×5). Small portions of resin were spread in a single layer on a microscope slide and kept slightly wetted with H_2O while viewed under a fluorescence microscope. By use of a long needle, the beads could be moved around on the slide. When a dark bead was found, it was isolated from other beads and its darkness reevaluated in the absence of bright beads. Beads deemed dark even after evaluation in isolation were transferred to a target for MALDI-TOF MS by means of a bent closed-glass capillary by simply adhering the bead to the glass surface. In this manner, 77 dark beads were selected, corresponding to approximately 0.05% of the incubated beads. The beads were irradiated, extracted, and analyzed by MALDI-TOF mass spectrometry as described in the previous section (see text for results).

Synthesis of Inhibitor Sequences 18−31. MCPS of sequences **18−31** was carried out with $PEGA_{800}$ resin derivatized with Rink amide linker. For each compound, 75 mg of resin (0.4 mmol/g, 0.03 mmol) was used. Amino acid couplings were performed with Fmoc amino acid OPfp esters (3 equiv, 0.9 mmol) with addition of DhbtOH (5 mg, 0.03 mmol). Washing and deprotection were as described in previous sections. The products were cleaved from the support using TFA/H_2O (95:5), purified by semipreparative RP-HPLC, and analyzed by analytical RP-HPLC/ES-MS (Table 2).

Kinetic Evaluation of Inhibitor Sequences 18−31. A 10 mM stock solution in DMF was prepared for each of the compounds. IC_{50} values for inhibition were determined using CBZ-Phe-Arg-Amc as the substrate and inhibitor concentrations between 50 μM and 250 nM, according to the method of Nicklin and Barrett[40] (Table 2).

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Supporting Information Available. A total of 20 assigned MALDI-TOF spectra of library members or hits, and a full list of identified hit sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations

Abz: 2-aminobenzoyl
Alloc: allyloxycarbonyl
Amc: 7-amido-4-methylcoumarin
Boc: *tert*-butoxycarbonyl
^{t}Bu: *tert*-butyl
Cbz: benzyloxycarbonyl
Cha: β-cyclohexylalanine
DCM: dichloromethane
DhbtOH: 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine
DIPEA: *N*,*N*-diisopropyl-*N*-ethylamine
DMF: dimethylformamide
Fmoc: *N*-fluoren-9-ylmethoxycarbonyl
ES-MS: electrospray ionization mass spectrometry
HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HOAt: 1-hydroxy-7-azabenzotriazole
IMS: ionization-mass spacer
LC−MS: liquid chromatography−mass spectrometry
MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MCPS: multiple-column peptide synthesis
MSNT: mesitylenesulfon-1-yl-3-nitro-1,2,4-triazole
NEM: *N*-ethylmorpholine
NMI: *N*-methylimidazole
PEGA: poly(ethylene glycol)−polyacrylamide copolymer
Pfp: pentafluorophenyl
Pll: photolabile linker
Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl
RP-HPLC: reversed-phase high-pressure liquid chromatography
SPPS: solid-phase peptide synthesis

TBDMSOTf: *tert*-butyldimethylsilyl trifluoromethansulfonate
TBTU: *N*-(1*H*-benzotriazol-1-yl)-(dimethylamino)methylene-*N*-methylmethanaminium tetrafluoroborate *N*-oxide
THF: tetrahydrofuran
TFA: trifluoroacetic acid
$Y(NO_2)$: 3-nitrotyrosine

One- and three-letter abbreviations for amino acids were used according to the recommendations of IUPAC.

References and Notes

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