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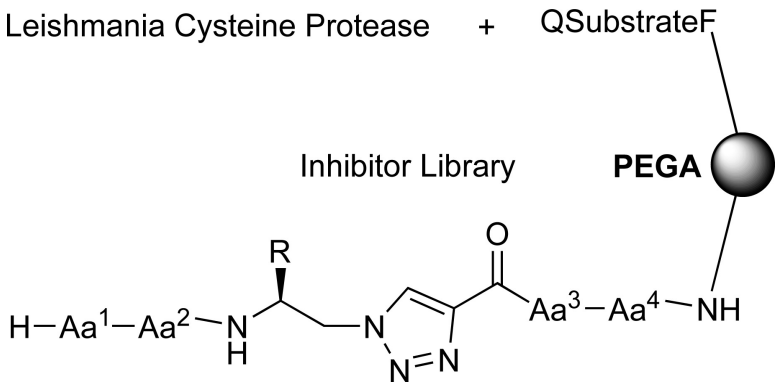

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Articles

Combinatorial Library of Peptidotriazoles: Identification of [1,2,3]-Triazole Inhibitors against a Recombinant *Leishmania mexicana* Cysteine Protease

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A library consisting of about half of 800 000 possible peptidotriazoles on 450 000 beads was prepared by solid-phase peptide synthesis combined with a regiospecific copper(I)-catalyzed 1,3-dipolar cycloaddition between a resin-bound alkyne and a protected amino azide. The central [1,2,3]-triazole was flanked on each side by two randomized amino acids introduced in a combinatorial approach. Importantly, the formation of the triazole could be performed quantitatively in a randomized fashion. The library was screened on solid phase for inhibitory effect against a recombinant cysteine protease, *Leishmania mexicana* CPB2.8ΔCTE and sorted by a high-throughput instrument, COPAS beadsorter (up to 200 000 beads/h). Forty-eight hits were analyzed by MALDI-TOF MS providing structural information about the protease specificity, and 23 peptidotriazoles were resynthesized and evaluated in solution, with the best inhibitor displaying a K_i value of 76 nM. A one-pot procedure was used to convert Fmoc-amino azides into their corresponding Boc derivatives. The crucial influence of weak interactions with a spacer used for detection by MALDI-TOF MS on screening results was observed.

Introduction

Many cysteine proteases are essential in regulation of physiological processes and disease propagation, and the proteases play important roles in treatment of cardiovascular diseases, oncology, osteoporosis and arthritis.[1] Control of dysfunctional protease activity is one way of controlling diseases, and many protease inhibitors have been developed in the past.[1−3]

More than 12 million people worldwide are infected with leishmaniasis, caused by the protozoal parasite *Leishmania*, and many more are exposed with the risk of infection. The parasite causes cutaneous or visceral lesions, many of which are difficult to treat.[4] A tandem array of 19 genes express a cysteine protease, CPB, which has been identified as an important virulence factor in *Leishmania mexicana*.[5] Thus, CPB is a good target for inhibition in order to prevent disease.[5] The protease, designated *Leishmania mexicana* CPB2.8ΔCTE, has been cloned and isolated in a recombinant form lacking the C-terminal extension[6] and used for substrate specificity studies both in solution[7] and on solid phase.[8] Protease inhibitors for this enzyme have also been identified with intramolecularly quenched fluorogenic peptides[9] and by screening of combinatorial bicyclic ketone and reduced peptide bond inhibitor libraries on solid support.[10,11]

Successful protease inhibitors must have a good binding affinity but also display high selectivity among the numerous proteases present in biological systems to avoid serious side effects. This can be achieved by screening many putative inhibitors to find a potent yet selective compound. Combinatorial libraries offer a large number of compounds that may or may not display biological activity; however, screening of a library and selecting the best hits can provide new lead structures. Several reviews of combinatorial libraries with biological effect have recently been presented.[12−14]

The strategy for discovery and development of protease inhibitors can be based on prior knowledge of the protease, such as X-ray crystal structure, substrate specificity, SAR studies, and natural inhibitors. If little information is available or new drug leads are desired, then combinatorial libraries can be very valuable because they contain a large number of putative inhibitors. With the development of one-bead-two-compounds libraries,[15] it became possible to screen

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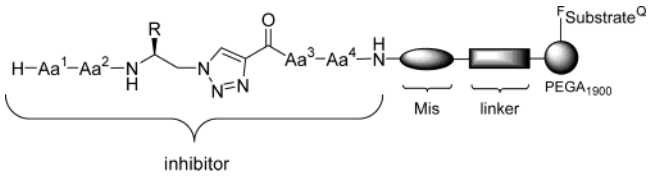
Scheme 1. Basic Construct of the Peptidotriazole Library[a]



[a] [Mis], mass/ionization spacer; F, fluorophore; Q, quencher.

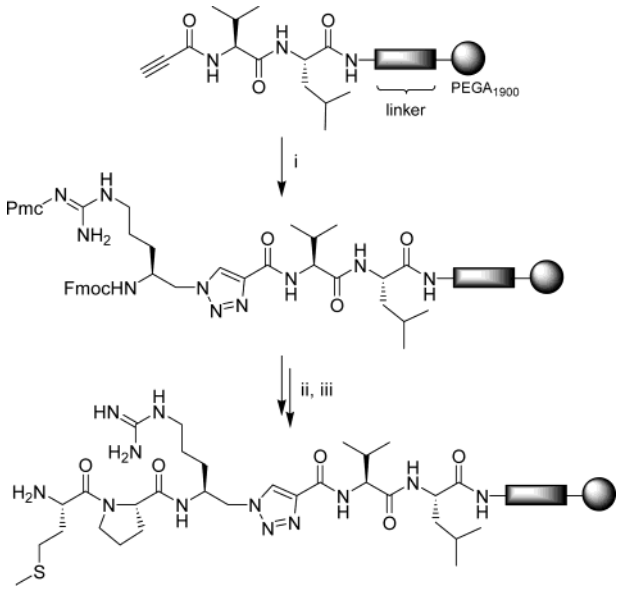
millions of compounds in a competitive fashion in which each inhibitor in a single bead competes with a fluorogenic quenched substrate for binding to the protease. Since the synthetic strategy (the split-and-mix method[16]) initially produce a unique structure on each bead to which a common fluorescence quenched substrate is then attached, the library can be viewed as a large collection of microreactors (the volume of each bead is ~50 nL) that will illuminate upon cleavage of the substrate when containing poor inhibitors. Potent inhibitors, in turn, can be identified by selecting the darkest beads where the substrate is intact due to high protease affinity for the inhibitor. This strategy has previously been used to identify potent inhibitors of Subtilisin Carlsberg, Cruzipain, Cathepsin B, Cathepsin L, *L. mexicana* CPB2.8ΔCTE, and MMP-12.[10,15,17,18]

Peptidomimetics prepared by solid-phase peptide synthesis combined with various organic reactions can provide compounds that bind to proteases as a natural peptide substrate but is more resistant to hydrolysis because of a structural mimetic within the peptide backbone. [1,2,3]-Triazoles have previously been used as anti-HIV agents,[19,20] selective β_3 adrenergic receptor agonists,[21] and antiinflammatory agents[22] and have shown antimicrobial activity.[23] In a recent report the regiospecific copper(I)-catalyzed synthesis of 1*H*-[1,2,3]-triazoles on solid support has been described.[24] The small, rigid, and aromatic structure of triazoles combined with its hydrogen-bonding capabilities and resistance to enzymatic hydrolysis renders it as a candidate for incorporation into peptidomimetics. Here, the synthesis and screening of a combinatorial library of peptidotriazoles is reported (Scheme 1), and novel peptides containing a 1*H*-[1,2,3]-triazole moiety in the backbone inhibited *L. mexicana* CPB2.8Δ*C*TE.

Results and Discussion

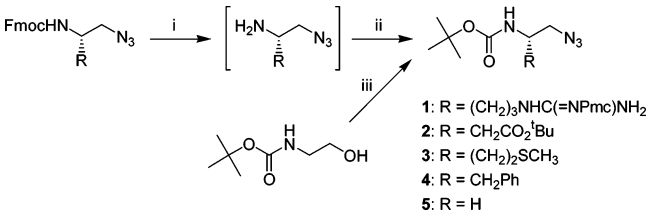
1,3-Dipolar Cycloadditions on Solid Phase. An efficient copper(I)-catalyzed 1,3-dipolar cycloaddition between a terminal alkyne and an azide was investigated on solid support, because of the mild reaction conditions, compatibility with solid-phase peptide chemistry and quantitative conversion.[24] Terminal alkynes react with copper(I) to form polarized copper acetylides, generally catalyzing the cycloaddition to azides, including primary, secondary, and tertiary alkyl azides, aryl azides, and azido sugars. The reaction is very versatile and works with most azides in a wide range of solvents with high conversion and purity. The quantitative nature of the reaction is essential for solid-phase reactions and particularly for library synthesis. The reaction conditions for the cycloaddition (0.1 equiv CuI in pyridine at 25 °C) are compatible with both Fmoc and Boc chemistry and, therefore, suitable for incorporation into a one-bead-two-compounds library encoded by ladder[25] synthesis. By coupling propargylic acid to an N-terminal amino group and subsequent copper(I)-catalyzed cycloaddition with a Fmoc-protected β-amino azide, the peptide synthesis can be continued after the triazole formation, as seen in Scheme 2.

Scheme 2. Synthesis of a Model Peptidotriazole, H-Met-Pro-RTr-Val-Leu-NH_2[a]



[a] (i) Fmoc-Arg(Pmc)-ψ[CH_2N_3], CuI; (ii) 20% piperidine/DMF; (iii) SPPS and deprotection.

Scheme 3. Preparation of Boc-β-amino Azides by Protecting Group Exchange or Mitsunobu Reaction[a]

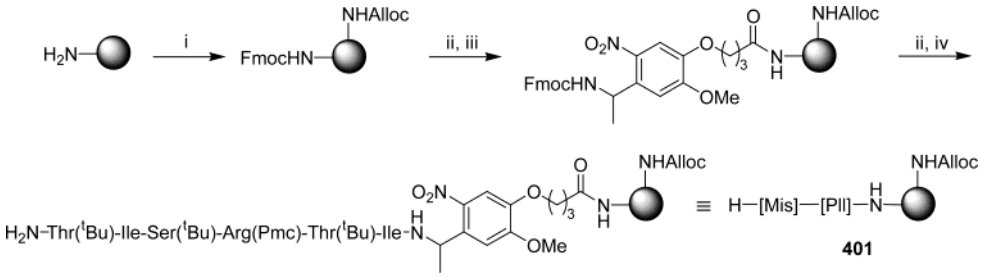


[a] (i) 20% piperidine/DMF; (ii) Boc_2O, Et_3N; (iii) PPh_3, DIAD, HN_3.

Previous communications have described the solid phase synthesis of [1,2,3]-triazoles by diazotransfer using tosyl azide and thermal cycloaddition of alkynes and azides; however, only few examples were given, and the scope of the reactions and yields were very limited.[26,27] Superpositions of more than 250 protease-inhibitor crystal structures have shown that substrates and inhibitors generally bind in an extended β-strand conformation, and preorganization of inhibitors can lead to increased binding.[28] Thus, the 1,4-substituted [1,2,3]-triazole has a favorable geometry for inducing an extended peptide conformation when incorporated into the peptide backbone (see Scheme 2). Furthermore, it is small, aromatic, and cannot be hydrolyzed by proteases.

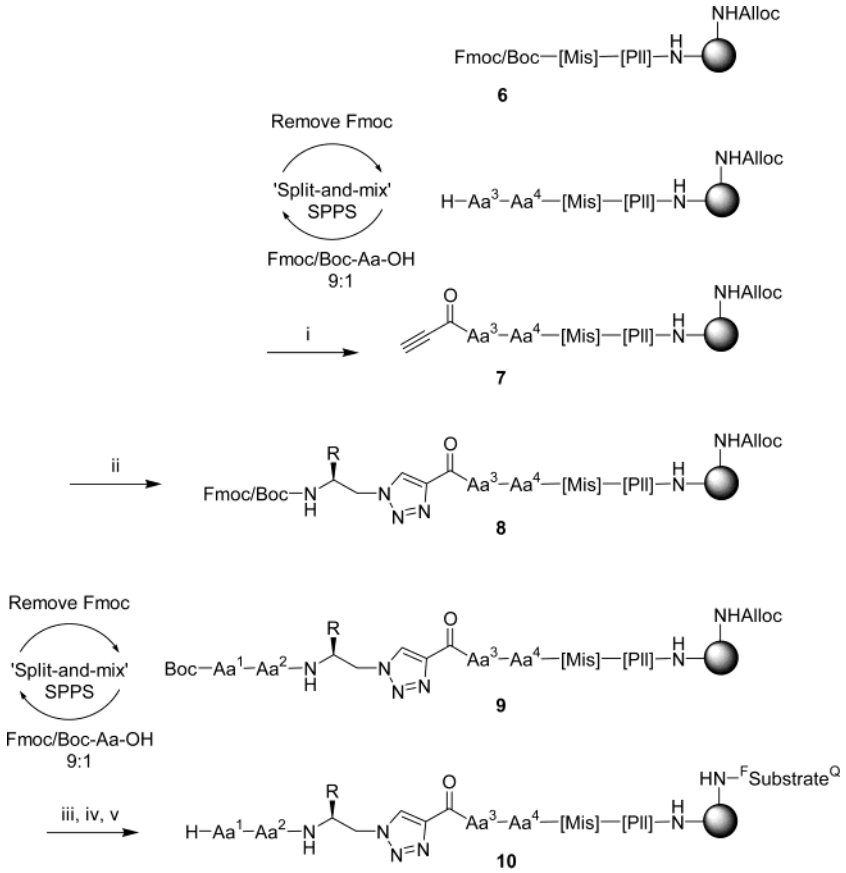
Preparation of Boc-β-amino Azides. As a result of the synthetic strategy (vide infra), both Fmoc- and Boc-β-amino azides were required for the cycloaddition reaction. Five Fmoc-protected amino azides derived from Fmoc-amino alcohols were prepared by the Mitsunobu reaction, as previously described.[24] These Fmoc-protected azide analogues of Pmc-protected arginine, *tert*-butyl-protected aspartic acid, glycine, methionine, and phenylalanine were

Scheme 4. Preparation of the One-Bead-Two-Compounds Library Construct **6**[a]



[a] (i) Fmoc-Gly-OH/Alloc-Gly-OH, TBTU, NEM; (ii) 20% piperidine/DMF; (iii) Fmoc-[Pll]-OH, TBTU, NEM; (iv) SPPS.

Scheme 5. Library Synthesis Using the Split-and-Mix Protocol and Coupling of a Fluorescence-Quenched Substrate To Give the Fully Deprotected Library **10**[a]



[a] F, fluorophore; Q, quencher; [Mis], mass/ionization spacer; [Pll], photolabile linker. (i) Propargylic acid, EEDQ; (ii) Fmoc/Boc-Aa-ψ[CH_2N_3], CuI; (iii) $Pd(PPh_3)_4$, NEM, HOAc; (iv) Protected ^{F}substrate^Q, TBTU, NEM; (v) deprotection with TFA and scavengers.

deprotected with 20% piperidine in DMF and then reprotected in situ with Boc_2O, affording Boc-amino azides **1**−**4** in 61−74% yield. The glycine derivative (**5**) however, had to be prepared directly from *N*-Boc-ethanolamine under Mitsunobu conditions (DIAD, Ph_3P and HN_3, 76% yield) because deprotection of Fmoc-Gly-ψ[CH_2N_3] gave the volatile 2-azido-ethylamine, and excess piperidine could not be removed selectively by concentration in vacuo.

Library Design and Synthesis. The synthesis of the peptidotriazole library is outlined in Schemes 4 and 5. The resin was orthogonally derivatized to give Fmoc- and Alloc-protected amines in a 2:1 ratio, thus facilitating inhibitor synthesis on one amine and coupling of substrate on the other. The combinatorial inhibitor library was generated by the split-and-mix method by coupling to the amines released upon Fmoc cleavage. A photolabile linker[29] (Pll) was attached for easy and mild UV-release of the inhibitor followed by assembly of the mass/ionization spacer (Mis). The [Mis]-sequence was selected not to be a substrate for the enzyme, the molecular weight of the peptide (m/z > 600 Da) was outside the peaks of the matrix-region, and the residues selected increased the desorption and flight properties in MALDI-TOF MS, as described by Valero et al.[30]

Implementing ladder synthesis, first described by Youngquist et al.[25] and later modified by St. Hilaire et al.,[31] a small amount of compound was capped in each reaction cycle by using a 9:1 mixture of, for example, Fmoc- and Boc-amino acids, allowing deprotection of only the Fmoc protected amines for continued synthesis and leaving truncated fragments as well as affording the full sequence. These fragments served as a record of the synthetic history because the mass difference of adjacent peaks in the MALDI spectrum

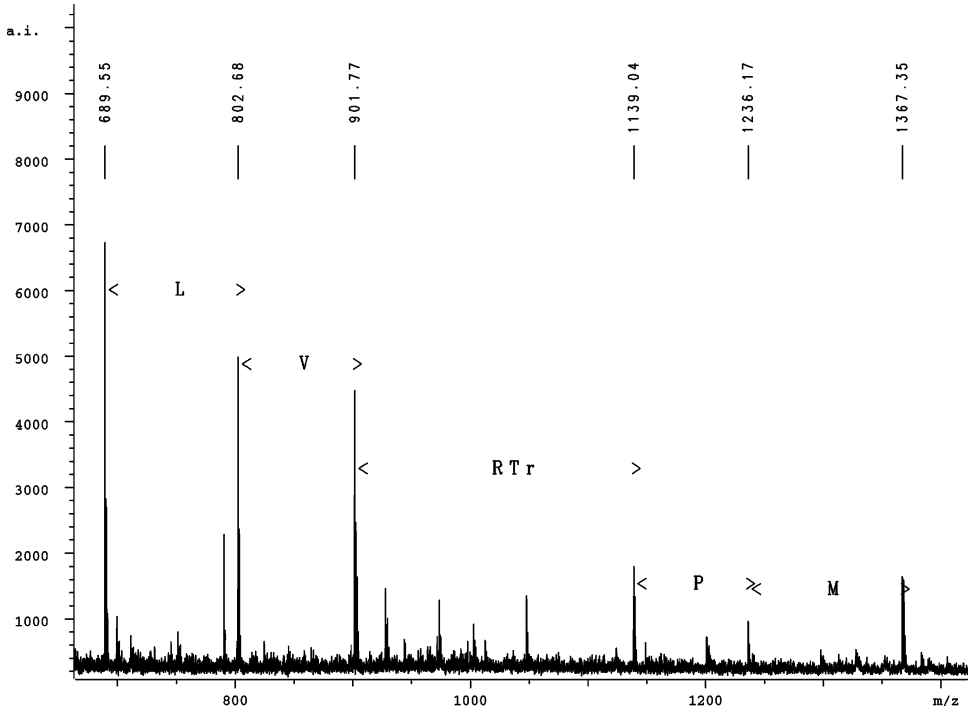


Figure 1. MALDI-TOF mass spectrum of one single bead. The mass difference between two major peaks corresponds to one amino acid, and by calculating all the differences, the full sequence was assigned to Met-Pro-RTr-Val-Leu.

corresponded to the molecular weight of a specific amino acid. Upon photolytic cleavage and extraction from the bead on target, a MALDI-TOF spectrum was recorded, and masses were assigned to significant peaks. A sequence assignment program (Bruker) was used to calculate mass differences and unambiguously assign the structure on the bead (e.g., Met-Pro-RTr-Val-Leu in Figure 1). The triazole moiety was identified because a 9:1 mixture of Fmoc- and Boc-Aa-ψ-[CH_2N_3] was used in the cycloaddition reaction to afford a small amount of capped triazole.

The ultimate [Mis]-amino acid was coupled as a 9:1 mixture of Fmoc/Boc-Thr(^{t}Bu)-OH to provide a terminated sequence corresponding to the mass/ionization spacer. Resin **6** was transferred to a multiple-column peptide synthesizer[32] (MCPS) and distributed evenly in the 20 wells. Eighteen genetically encoded amino acids, 3-pyridylalanine, and *p*-chlorophenylalanine were used in the library and coupled using a 9:1 mixture of Fmoc- and Boc-amino acids activated by TBTU to generate the ladder. Isoleucine and glutamine were omitted because of their isobaric molecular weight with leucine and lysine, respectively. From previous studies,[24] it was known that sensitive amino acids, such as cysteine, methionine and tryptophan were not affected by the copper-(I)-catalyzed cycloaddition, so these amino acids were also included in the library. To gain maximum diversity, the β-amino azides used for the cycloaddition were selected in order to vary in size, polarity, aromaticity, and hydrophobicity. So the glycine, methionine, phenylalanine, aspartic acid, and arginine analogues represented small, hydrophobic, aromatic, anionic, and cationic residues, respectively.

Between each coupling step, the synthesis block (MCPS) was closed and mixed thoroughly upside down, and then the resin was redistributed evenly. After the first two couplings, the liberated amine was acylated with propargylic acid, affording resin **7** that was reacted with five Fmoc/Boc-protected β-amino azides at 25 °C catalyzed by copper(I) iodide to give 1,4-substituted triazoles. Upon removal of Fmoc groups, two more randomized amino acid positions were coupled to resin **8**, affording the complete inhibitor library **9**. The library was analyzed before attachment of substrate by selecting 24 beads, cleaving all the protecting groups, releasing the peptides from the resin under UV-light, and analyzing the products by MALDI-TOF MS. From the mass spectra, it appeared that the library synthesis had performed well without any deletion inhibitor sequences or side reactions. Five triazoles, DTr, FTr, GTr, MTr, and RTr, were identified among the selected beads as were all 20 amino acids (data not shown).

A substrate for *L. mexicana* CPB2.8ΔCTE was coupled by TBTU activation after the Alloc group had been removed by $Pd(PPh_3)_4$ to yield the fully protected one-bead-two-compounds library (**10**). Cleavage of all side-chain protecting groups was achieved using a cocktail consisting of TFA and scavengers affording the deprotected library ready for screening with enzyme. Since the enzyme-specific substrate was coupled after inhibitor assembly, small portions of the library could alternatively be screened against other types of proteases by attachment of different fluorogenic quenched substrates specific for these other proteases, thereby increasing the versatility of the library.

After completion of the library synthesis, each bead thus displayed both a unique putative inhibitor and a fluorescence quenched 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 CPB2.8ΔCTE from *L. mexicana*. In theory, two situations can occur: (i) a weak inhibitor cannot compete with the substrate, which then is hydrolyzed, and due to release and removal of the quencher, the bead is

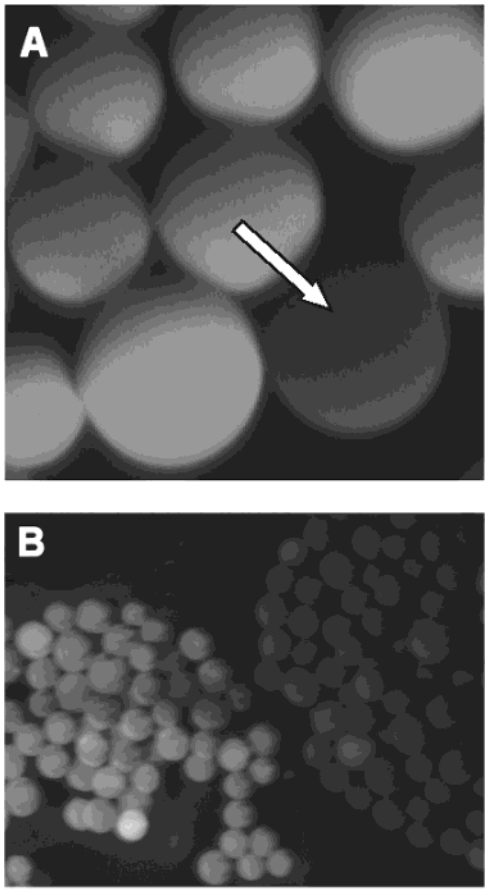


Figure 2. (a) Only few beads remained dark after 24 h of incubation with *L. mexicana* CPB2.8ΔCTE. (b) Bright beads are separated from dark ones after automated sorting with the COPAS beadsorter.

illuminated by Abz-fluorescence under a UV-microscope; and (ii) the inhibitor binds the enzyme, tightly prohibiting substrate hydrolysis, and the bead remains dark. The one-bead-two-compounds construct gives a direct visualization of the ongoing inhibition assay and is a powerful technique for identifying novel inhibitors.

Finally, upon UV irradiation of individual beads with a Hg lamp, both the full-length inhibitor and truncated sequences (arising from ladder synthesis) were released from the photolabile linker and extraction with a 1:1 mixture of acetonitrile/water, enabling full sequence assignment from the acquirement of a single MALDI-TOF mass spectrum.

Screening of Library. After complete deprotection of both the substrate and inhibitor, a portion of the library (750 mg, ∼450 000 beads) was incubated with *L. mexicana* CPB2.8ΔCTE at 37 °C. The fluorescence intensity was monitored frequently, and after 24 h, only a few beads remained dark (Figure 2a), so the enzyme was quenched by addition of 2% TFA. The long exposure to enzyme ensured that only the most potent inhibitors were selected by the sorting procedure. The library was carefully washed and resuspended in neutral detergent sheath fluid that prevented beads from aggregation and allowed individual analysis. They were sorted on a custom-made high-throughput COPAS beadsorter developed in collaboration with Union Biometrica by modification of a nematode sorter.[33] The instrument facilitated sorting of large combinatorial libraries and has reduced the time used for a large library from weeks to hours; thus, it was feasible to analyze more than a million beads a day. The outline of the instrument was previously described.[34] All the 450 000 beads of the library were sorted initially at high speed (40 beads/s) and after each sorting all the fluorescent beads were resorted for complete collection of dark beads. The sorting process was repeated on the sorted beads at a lower rate (5−10 beads/s) for improved accuracy until only 48 very dark beads remained (0.11‰ of the library).

Analysis of Hits. The dark beads from the sorting procedure were washed with water before being transferred to steel MALDI targets and irradiated under a Hg UV-lamp. The peptides released from the photolabile linker were extracted onto the target and analyzed by MALDI-TOF MS. In most cases, full sequence assignment was possible (36 out of 48 beads, 75% readability); however, 12 spectra did not give any meaningful sequence. No incomplete sequences were detected due to the efficiency of the peptide couplings and, particularly, the cycloaddition. The 36 full sequences are available in the Supporting Information, and the results are illustrated in Figures 3 and 4.

A preference for the arginine-derived triazole (RTr, 44%) was observed with a contribution from the hydrophobic methionine- and phenylalanine-triazoles (MTr, 25% and FTr, 17%). The last two triazoles (DTr, 8%, and GTr, 6%) were present but not well-tolerated by the enzyme. The selectivity in Aa^1 and Aa^2 was low, whereas the Aa^3 and Aa^4 positions showed strong preferences for arginine (24%) and leucine (49%), respectively. Hydrophobic residues were present in 58% of the sequences in Aa^1, and tryptophan was slightly favored. Alanine was found in Aa^2 in 11% of all sequences, and hydrophobic residues predominated. Cationic (arginine, 24%) as well as hydrophobic amino acids were tolerated in Aa^3, and an unusually high selectivity for leucine in Aa^4 was evident from Figure 3, where Leu was present in half of all the sequences. In conclusion, Arg and Leu were preferred on the C-terminal side of the triazole, whereas no significant selectivity was found on the N-terminal side.

Evaluation of Hits. Excellent agreement between solution- and solid-phase screening for inhibitors have been reported,[18,35] but it is always important to evaluate selected hits in solution. Ten consensus sequences (**11**−**20**) were derived from statistical considerations of the hits, 10 sequences (**21**−**30**) were selected out of the 36 analyzed, and **31**−**33** contained part of the mass/ionization spacer. The 23 peptides were prepared by solid-phase peptide synthesis combined with the copper(I)-catalyzed 1,3-dipolar cycloaddition, purified by RP-HPLC, isolated in 30−89% yield, and analyzed by ^{1}H NMR (both 1D and 2D), MALDI-TOF MS and high-resolution MS. All inhibitors were synthesized as C-terminal carboxamides with a free N terminus to mimic the inhibitors present in the solid-phase library. The selected sequences were presumed to be responsible for the inhibition, even though the truncated fragments from the ladder synthesis or the mass/ionization spacer could in theory have an effect. Stock solutions in DMF were prepared, and the peptides were evaluated for inhibitory effect against *L. mexicana* CPB2.8ΔCTE (results shown in Table 1). Five

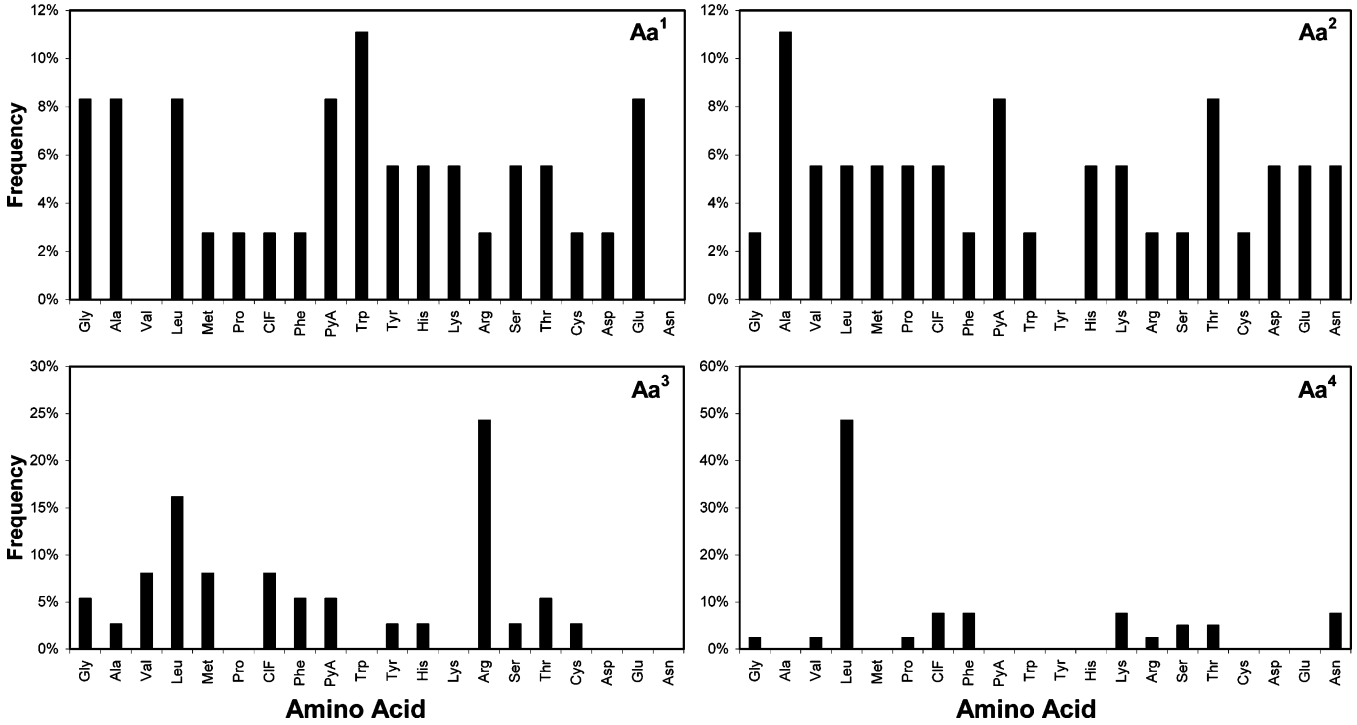


Figure 3. Distribution of amino acids found in positions 1−4 based on the analysis of 36 hits. Full peptide sequences are reported in Supporting Information.

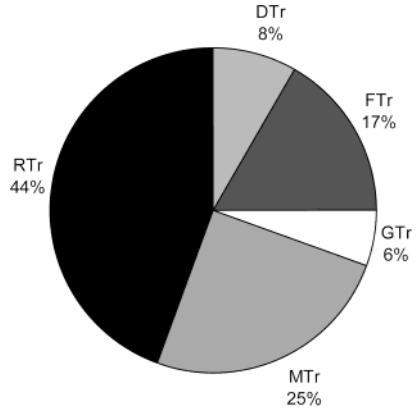


Figure 4. Distribution of triazoles found on the 36 darkest beads. DTr (Asp-triazole), FTr (Phe-triazole), GTr (Gly-triazole), MTr (Met-triazole), RTr (Arg-triazole).

sequences (**19**−**22** and **29**) displayed low micromolar inhibition, whereas **31**−**33** had K_i values between 76 and 240 nM. The rest showed either poor or no inhibition of the cysteine protease.

Previous substrate studies[7,8] have shown that arginine and lysine were much preferred for substrates in the S_3, S_1, and S_3' pockets, and hydrophobic residues were found in S_2. These findings correlated well to the observed selectivity in the peptidotriazole library for cationic residues (RTr as the triazole and Arg in Aa^3) and leucine in Aa^4. However, since inhibitors **11**−**30** did not show very potent inhibition ($K_i \geq 1\ \mu M$), it was proposed that although the enzyme could not cleave the mass/ionization spacer (Mis) it might have recognized the N-terminal part, including the arginine. Compounds **31**−**33** contained four amino acids from the [Mis]-sequence, Thr-Ile-Ser-Arg, and the nanomolar inhibition constants of **31**−**33** confirmed the hypothesis that the protease did bind to this part of the mass/ionization spacer. The lack of selectivity for Aa^1 and Aa^2 substantiated the proposed binding mode where only the mass/ionization spacer, the positions Aa^3 and Aa^4 with high consensus and the triazole were recognized, with the triazole moiety being displaced two subsites to S_3 rather than S_1 of the protease binding site. Furthermore, the position of the arginines and arginine-triazole in **31**−**33** corresponded well with the substrate study.[8]

Previously, the specific [Mis]-sequence has been used successfully with matrix metallo proteases[18] affording transition-state inhibitors with low nanomolar K_i values and [Mis]-recognition of the enzyme was not observed. Thus, for future reference, it is important to design a mass/ionization spacer that does not interact even weakly with the given enzyme by, for example, including oligo prolines or by use of nonpeptidic spacers.

Comparing the three triazoles in sequences **11**−**13** (where only the substitution of the triazole is different), **12** (FTr) was a stronger inhibitor than **11** (Mtr) and **13** (RTr) by a factor of 1.2 and 2, respectively. However, none of these sequences was potent, and much emphasis should not be placed on this result. Isoleucine was omitted from the library and, because of the high preference for leucine in Aa^4. peptide **14** was synthesized to discriminate between these two amino acids. The K_i value of **14** was decreased 3-fold compared to **13**, so leucine was accommodated better in the binding pocket than isoleucine. Sequence **20** with lysine in position Aa^4 showed low micromolar inhibition, but compared with **29**, it was 2-fold less potent. Cationic residues in Aa^1 were not well-tolerated (**15** and **17** had no inhibitory effect), and substitutions in Aa^2 did not seem to change affinity much. Most of the sequences with Arg in Aa^3 were poor inhibitors ($K_i > 150\ \mu M$), even though statistics indicated otherwise (Figure 3). This discrepancy was ex-

Table 1. Characterization of the Resynthesized Inhibitors **11**−**33**, Their Sequences, K_i Values for Their Inhibition of *L. mexicana* CPB2.8ΔCTE, Analytical Data, and Yield

sequence	Aa[1]	Aa[2]	triazole	Aa[3]	Aa[4]	K_i/μM	yield (%)
11	Trp	Ala	MTr	Arg	Leu	178	57
12	Trp	Ala	FTr	Arg	Leu	151	47
13	Trp	Ala	RTr	Arg	Leu	306	88
14	Trp	Ala	RTr	Arg	Ile	930	74
15	Lys	Ala	RTr	Arg	Leu	>1000	79
16	Glu	Ala	RTr	Arg	Leu	458	84
17	His	Thr	RTr	Arg	Leu	739	57
18	Leu	PyA	RTr	Arg	Leu	185	89
19	Trp	Ala	RTr	Leu	Phe	3.42	49
20	Trp	Ala	RTr	ClF	Lys	1.53	65
21	Ala	Thr	FTr	Leu	Leu	1.93	59
22	Trp	Thr	FTr	Arg	Phe	33	30
23	PyA	Asp	FTr	Arg	Leu	199	82
24	Ala	Glu	MTr	Arg	Leu	240	61
25	Thr	PyA	MTr	Arg	Leu	237	82
26	Leu	Ser	MTr	Arg	Leu	254	78
27	Pro	Ala	RTr	Arg	Leu	248	49
28	Asp	PyA	RTr	Arg	Leu	296	68
29	Gly	Leu	RTr	ClF	Leu	0.87	52
30	Gly	Lys	RTr	Met	Asn	312	70
31	H-Gly-RTr-Arg-Leu-Thr-Ile-Ser-Arg-Gly-NH_2					0.14	74
32	H-Gly-FTr-Arg-Phe-Thr-Ile-Ser-Arg-Gly-NH_2					0.24	81
33	H-Gly-RTr-ClF-Leu-Thr-Ile-Ser-Arg-Gly-NH_2					0.076	51

plained by sequences **31**−**33**, in which the [Mis]-sequence was included, and then Arg in Aa^3 gave potent inhibitors (76−240 nM).

These results show the importance of evaluating selected sequences in solution after screening a solid-phase library because the results obtained from screening can be misinterpreted (i.e., which sequences should be resynthesized).

Perspective. These peptidotriazoles could be used against other enzymes, for example, Cathepsin B/L and Cruzipain, because of their structural resemblance to *L. mexicana* CPB2.8ΔCTE.[6] More sequences that include amino acids from the mass/ionization spacer may reveal selective subnanomolar inhibitors, which is the subject for future work.

Conclusion. The copper(I)-catalyzed 1,3-dipolar cycloaddition was used to generate a library of peptidotriazoles that was screened against a recombinant cysteine protease, *L. mexicana* CPB2.8ΔCTE, affording novel inhibitors with K_i values in the low nanomolar to high micromolar range. Sequences **31**−**33**, which included amino acids from the mass/ionization spacer, were the best inhibitors, with inhibition constants between 76 and 240 nM. The library was screened and sorted in a high-throughput fashion with the COPAS beadsorter, and selected beads were analyzed by MALDI-TOF MS, allowing assignment of the full inhibitor sequence from a single bead. Only one-half of the possible 800 000 library members were synthesized, since it has previously been found that there is a large redundancy in the ligand protein interaction. Resynthesis and kinetic evaluation in solution of 23 peptidotriazoles validated the library and the screening/sorting process and underlined the importance of selecting the proper masss/ionization spacer.

Experimental Section

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX250 (250 MHz) spectrometer. MALDI spectra were acquired using α-cyano-4-hydroxycinnamic acid on a Bruker Reflex III MALDI-TOF mass spectrometer, and sequence assignment was automated using the Aura macro LabelDelta. UV inspection of the library was performed with a Leica UV fluorescence microscope with an external UV source, a 320-nm band-pass filter for excitation, and a 410-nm low-pass filter for detection attached to a Leica CCD camera. Beadsorting was performed on a custom-made COPAS beadsorter from Union Biometrica (Massachusetts). Analytical and preparative reversed-phase HPLC separations were performed on a Waters HPLC system using analytical Zorbax 300SB-C_{18} (4.5 × 50 mm) and Delta PAK (25 × 300 mm) C_{18} columns with a flow rate of 1 cm^3 min^{-1} and 10 cm^3 min^{-1}, respectively. Detection was at 215 nm on a multiwavelength detector (Waters 490E) for analytical purposes, and a photodiode array detector (Waters M991) was used for preparative separations. A solvent system consisting of A, 0.1% TFA in water, and B, 0.1% TFA in 90:10 acetonitrile/water, was used. IR spectra were recorded on a Perkin-Elmer 1600 FTIR instrument as neat liquids or as KBr pellets. Optical rotations were measured on a Perkin-Elmer 241 Polarimeter at 25 °C. Solution-phase kinetics of inhibition was carried out on a Perkin-Elmer Lambda 50 luminescence spectrometer.

General Procedures. Coupling of Fmoc- and Boc-amino acids to amino groups was performed with Fmoc-/Boc-AA-OH (3 equiv), mixed with NEM (4 equiv) in DMF and addition of TBTU (2.88 equiv) and preactivation for 8 min. Fmoc-Aa-OPfp esters (3 equiv) were coupled with Dhbt-OH (1 equiv) in DMF. Amino acid couplings were followed by the Kaiser test.[36] Fmoc deprotection was effected with 20% piperidine in DMF for 2 + 18 min, followed by washing of the resin with DMF (×6). The resin was washed with the appropriate solvent (×6) between each reaction step. Preparation of the Fmoc-β-amino azides has previously been described.[24] All amino acids were L-amino acids. The

following commercially available chemicals were used as purchased without further purification: CuI, Dhbt-OH, DIAD, DIPEA, EEDQ, Fmoc- and Boc-Aa-OH, Fmoc-Aa-OPfp, *N*-ethylmorpholine, $Pd(PPh_3)_4$, piperidine, propargylic acid, TBTU, and triphenylphosphine.

General Procedure for Conversion of Fmoc-amino Azides to Boc-amino Azides (1−4). The Fmoc-amino azide (1 equiv, 0.2 mmol) was dissolved in 20% piperidine/DMF and stirred at 25 °C for 20 min. It was concentrated in vacuo and twice with toluene. The residue was redissolved in dry DMF (4 mL) under argon, and Et_3N (2.5 equiv, 0.5 mmol) and Boc_2O (1.25 equiv, 0.25 mmol) were added and stirred at 25 °C overnight. The reaction mixture was concentrated in vacuo and once with toluene. The product was purified by flash chromatography.

(4-[(Amino-(2,2,5,6,8-pentamethyl-chroman-7-sulfonylimino)-methyl)-amino]-(*S*)-1-azidomethyl-butyl)-carbamic Acid *tert*-Butyl Ester, Boc-Arg(Pmc)-ψ[CH_2N_3] (1). Fmoc-Arg(Pmc)-ψ[CH_2N_3] (0.077 mmol) afforded **1** (26 mg, 61%) after flash chromatography (PE/EA 1:2). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.30 (s, 6H, $C_q(CH_3)_2$), 1.42 (s, 9H, Boc group), 1.51 (m, 4H, CH_2^{β} and CH_2^{γ}), 1.80 (t, J = 7 Hz, 2H, Ar−CH_2CH_2), 2.11, 2.55 and 2.57 (s, 9H, 3 × Ar−CH_3), 2.63 (t, J = 7 Hz, 2H, Ar−CH_2), 3.20 (m, 2H, CH_2^{δ}), 3.29 (m, 2H, CH_2N_3), 3.67 (m, 1H, CH^{α}), 4.86 (d, J = 8 Hz, 1H, NH^{α}), 6.19 (br s, 3H, NH and NH_2 of guanidino group). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 10.5, 15.8 and 16.9 (3 × Ar−CH_3), 19.8 (Ar−CH_2), 23.9 (CH_2^{γ}), 25.1 ($C_q(CH_3)_2$), 26.7 (*tert*-butyl CH_3), 28.2 (Ar−CH_2CH_2), 31.2 (CH_2^{β}), 39.3 (CH_2^{δ}), 48.1 (CH^{α}), 53.4 (CH_2N_3), 72.0 (Ar−OC_q), 78.3 (C_qCH_3), 116.3−152.0 (aromatic carbons), 154.3, and 154.5 (C = N and Boc−CO). IR: 1701, 2104 cm^{-1}. $[\alpha]^{25}_D$ = −16° (c = 1.0, $CHCl_3$). HR-MS: calcd ($MNa^+ = C_{25}H_{41}N_7O_5SNa^+$), 574.2782; found ($MNa^+$), m/z 574.2780.

4-Azido-(S)-3-*tert*-butoxycarbonylamino-butyric Acid *tert*-Butyl Ester, Boc-Asp(^{t}Bu)-ψ[CH_2N_3] (2). Fmoc-Asp(^{t}Bu)-ψ[CH_2N_3] (0.12 mmol) afforded **2** (27 mg, 74%) after flash chromatography (PE/EA 11:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.44 and 1.45 (s, 18H, *tert*-butyl and Boc group), 2.48 (d, 2H, J = 6 Hz, CH_2CO), 3.46 (m, 2H, CH_2N_3), 4.05 (m, 1H, CH^{α}), 5.11 (m, 1H, NH). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 28.4 and 28.7 (*tert*-butyl CH_3), 37.9 (CH_2CO), 47.9 (CH^{α}), 54.2 (CH_2N_3), 80.2 and 81.9 (C_qCH_3), 155.3 (Boc-CO), 170.6 (Asp−CO). IR: 1711, 2106 cm^{-1}. $[\alpha]^{25}_D$ = −10° (c = 1.0, $CHCl_3$). HR-MS: calcd ($MNa^+ = C_{13}H_{24}N_4O_4Na^+$), 323.1690; found ($MNa^+$), m/z 323.1696.

(*S*)-(1-Azidomethyl-3-methylsulfanyl-propyl)-carbamic Acid *tert*-Butyl Ester, Boc-Met-ψ[CH_2N_3] (3). Note: After Fmoc-removal, the mixture was not concentrated with toluene, but only excess piperidine was removed in vacuo (to avoid loss of the volatile H-Met-ψ[CH_2N_3]), and excess Boc_2O (10 equiv) was used. Fmoc-Met-ψ[CH_2N_3] (0.08 mmol) afforded **3** (13 mg, 62%) after flash chromatography (PE/EA 13:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.45 (s, 9H, *tert*-butyl CH_3), 1.79 (m, 2H, CH_2^{β}), 2.11 (s, 3H, SCH_3), 2.50 (m, 2H, CH_2^{γ}), 3.44 (m, 2H, CH_2N_3), 3.83 (m, 1H, CH^{α}), 4.60 (m, 1H, NH). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 16.0 (SCH_3), 28.7 (*tert*-butyl CH_3), 31.0 (CH_2^{γ}), 32.3 (CH_2^{β}), 50.1 (CH^{α}), 55.1 (CH_2N_3), 80.3 (C_qCH_3), 155.6 (Boc-CO). IR: 1706, 2105 cm^{-1}. $[\alpha]^{25}_D$ = −30° (c = 1.0, $CHCl_3$). ES-MS: calcd ($MK^+ = C_{10}H_{20}KN_4O_2S^+$), 299.09; found ($MK^+$), m/z 299.01. Found: C, 45.56%; H, 7.59%; N, 21.07%. Calculated for $C_{10}H_{20}N_4O_2S$: C, 46.13%; H, 7.74%; N, 21.52%.

(1-Azidomethyl-(*S*)-2-phenyl-ethyl)-carbamic Acid *tert*-Butyl Ester, Boc-Phe-ψ[CH_2N_3] (4). Fmoc-Phe-ψ[CH_2N_3] (0.23 mmol) afforded **4** (47 mg, 73%) after flash chromatography (PE/EA 13:1). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.44 (s, 9H, *tert*-butyl CH_3), 2.84 (m, 2 H, CH_2N_3), 3.37 (m, 2H, CH_2^{β}), 3.96 (m, 1 H, CH^{α}), 4.65 (m, 1 H, NH), 7.20−7.33 (5 H, aromatic protons). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 28.7 (*tert*-butyl CH_3), 38.6 (CH_2N_3), 51.8 (CH^{α}), 53.6 (CH_2^{β}), 80.2 (C_qCH_3), 120.0−137.5 (aromatic carbons), 155.5 (Boc−CO). $[\alpha]^{25}_D$ = −9° (c = 1.0, $CHCl_3$). IR: 1706 and 2105 cm^{-1}. HR-MS: calcd ($MNa^+ = C_{14}H_{20}N_4O_2Na^+$), 299.1478; found ($MNa^+$), m/z 299.1481. The data are in agreement with literature values.[37]

(1-Azido-ethyl)-carbamic Acid *tert*-Butyl Ester, Boc-Gly-ψ[CH_2N_3] (5). *N*-Boc-ethanolamine (1 equiv, 0.96 mmol), Ph_3P (1.5 equiv), and HN_3 in toluene[38] (5 equiv, 1.5 M) were dissolved in dry THF (7 mL) under argon and cooled to 0 °C. DIAD (1.6 equiv) was added dropwise, and the reaction stirred at 25 °C for 0.5 h. The mixture was concentrated in vacuo and purified by flash chromatography (PE/EA 5:1) affording **5** (136 mg, 76%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.41 (s, 9H, *tert*-butyl CH_3), 3.24 (m, 2H, CH_2^{α}), 3.37 (m, 2H, CH_2N_3), 4.94 (m, 1H, NH). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 28.7 (*tert*-butyl CH_3), 40.4 (CH^{α}), 51.6 (CH_2N_3), 80.1 (C_qCH_3), 156.1 (Boc-CO). IR: 1708, 2104 cm^{-1}. The data are in agreement with literature values.[38]

Triazole Library Synthesis. $PEGA_{1900}$ resin (1.50 g) was prewashed with methanol and DMF. Fmoc-Gly-OH (3 equiv)/NEM (4 equiv)/TBTU (2.88 equiv) was coupled overnight, and the resin was treated with Ac_2O (10 equiv) for 10 min (×2) in DMF. The loading was measured to 0.133 mmol/g, and Fmoc groups were removed. Fmoc-Gly-OH (2.0 equiv)/Alloc-Gly-OH (1.0 equiv)/NEM (4 equiv)/TBTU (2.88 equiv) was coupled overnigh, and the loading was measured to 0.080 mmol/g (Fmoc) and 0.041 mmol/g (Alloc). Fmoc groups were removed, and Fmoc-Pll-OH (1.5 equiv toward Fmoc-group)/NEM (2.3 equiv)/TBTU (1.4 equiv) was coupled overnight. The following amino acids were coupled (3 equiv Fmoc-Aa-OH, 4 equiv NEM, 2.88 equiv TBTU) successively, followed by Fmoc-removal: Ile, Thr(^{t}Bu), Arg(Pmc), Ser(^{t}Bu), and Ile. Fmoc-Thr(^{t}Bu)-OH (2.7 equiv)/Boc-Thr(^{t}Bu)-OH (0.3 equiv)/NEM (4 equiv)/TBTU (2.88 equiv) coupling overnight gave resin **6**. The resin was transferred to a MCPS and distributed evenly in the 20 wells, and the Fmoc groups were removed.

Twenty different amino acids were used in the following couplings:

First randomized position: Fmoc-Aa-OH (2.7 equiv)/Boc-Aa-OH (0.3 equiv)/NEM (4 equiv)/TBTU (2.88 equiv) was coupled (with 8 min preactivation) for 5 h, the resin washed with DMF (×2), and the coupling was repeated overnight. The resin was washed with DMF (×6), and excess DMF was added to the MCPS, which was closed and shaken

thoroughly upside down for 1 h, followed by removal of Fmoc-groups.

Second randomized position: The procedure for the first randomized position was repeated.

Formation of triazoles: The resin was washed with DCM (×6). Propargylic acid (3.0 equiv) and EEDQ (3.1 equiv) were added to each reaction vessel and allowed to react for 5 h and washed with DCM (×2), and the coupling was repeated overnight, affording **7**. The resin was washed with DCM (×6) and THF (×6). Five different β-amino azides (**1**−**5**, derived from arginine, aspartic acid, glycine, methionine, and phenylalanine) were used (each in four wells) in the cycloaddition: Fmoc-Aa-ψ[CH_2N_3] (1.7 equiv)/Boc-Aa-ψ[CH_2N_3] (0.3 equiv)/CuI (0.1 equiv, 0.1 M in pyridine)/DIPEA (10 equiv) were added and reacted in THF for 5 h and washed with THF (×2), and the coupling was repeated overnight. The resin was washed with THF (×6), DMF (×3), 0.5% $Et_2NCSSNa$/0.5% DIPEA in DMF (×3), and DMF (×6). Excess DMF was added to the MCPS, which was closed and shaken thoroughly upside down for 1 h. Removal of Fmoc-groups afforded resin **8**.

Third randomized position: The procedure for the first randomized position was repeated.

Fourth randomized position: Boc-Aa-OH (3.0 equiv)/NEM (4 equiv)/TBTU (2.88 equiv) was coupled (with 8 min preactivation) for 5 h, the resin washed with DMF (×2), and the coupling was repeated overnight. The resin was washed with DMF (×6), and excess DMF was added to the MCPS, which was closed and shaken thoroughly upside down for 1 h. The resin was transferred to a syringe, washed with DCM (×10), and lyophilized to give resin **9**.

Analysis of library: A small aliquot of resin was deprotected with TFA/DCM/H_2O/$PhSCH_3$/$(CH_2SH)_2$/TIPS (66.5:20:5:5:2.5:1) for 2.5 h and washed with 95% HOAc (×3), DCM (×3), DMF (×3), 5% DIPEA in DMF (×3), DMF (×3), DCM (×3), THF (×3), MeOH (×3), and H_2O (×10). It was treated with 0.1 M NaOH (aq) for 1 h and then washed with H_2O (×3), 0.1% aqueous TFA (×3), and H_2O (×10). Twenty-four beads were randomly selected, placed on a MALDI target, irradiated for 2 h, and analyzed by MALDI-TOF MS (full sequence could be assigned for 83% of the beads).

Coupling of substrate: A 750-mg portion of resin was transferred to a new syringe and purged with argon. The resin was treated with $Pd(PPh_3)_4$ (3 equiv) in a degassed solution of $CHCl_3$/HOAc/NEM (92.5:5:2.5) for 2 h and then washed with $CHCl_3$ (×2). The Alloc deprotection was repeated, and the resulting resin was washed with $CHCl_3$ (×3), DMF (×3), 0.5% $Et_2NCSSNa$/0.5% DIPEA in DMF (×3), and DMF (×6). Boc-Y(NO_2)-E(^{t}Bu)-K(Boc)-F-R(Pmc)-R(Pmc)-G-K(Boc)-K(Abz-Boc)-G-OH (substrate for *L. mexicana* CPB2.8ΔCTE, 1.5 equiv)/NEM (2 equiv)/TBTU (1.4 equiv) was coupled (with 5 min preactivation) for 5 h and washed with DMF (×2), and the coupling was repeated overnight. It was washed with DMF (×3) and DCM (×6) and lyophilized. The library was deprotected with TFA/DCM/H_2O/$PhSCH_3$/$(CH_2SH)_2$/TIPS (66.5:20:5:5:2.5:1) for 2.5 h and washed with 95% HOAc (×3), DCM (×3), DMF (×3), 5% DIPEA in DMF (×3), DMF (×3), DCM (×3), THF (×3), MeOH (×3), and H_2O (×10). Treatment with 0.1 M NaOH (aq) for 1 h and then washing with H_2O (×10) gave resin **10**.

Incubation of Library with *L. mexicana* CPB2.8ΔCTE and Sorting of the Active Hits. The deprotected library (**10**) was washed with PBS buffer (×10, 100 mM PBS, 2 mM EDTA, 200 mM NaCl, 10 mM DTT, pH = 6.0) and incubated with 200 nM of the recombinant protease *L. mexicana* CPB2.8ΔCTE for 24 h at 37 °C. The resin was washed with H_2O (×3), 2% aqueous TFA (×3), H_2O (×3), 2% aqueous $NaHCO_3$ (×3), and H_2O (×6).

Sorting and Analysis. The beads were diluted to a concentration of ∼200 beads/mL with sheath fluid and initially sorted at ∼40 beads/s and later resorted at a lower rate (5−10 beads/s) to ensure isolation of the persistent dark beads (48 in total, 0.11‰ of the screened beads). The beads were washed with H_2O (×3), 0.1% aqueous TFA (×3), H_2O (×10); placed on MALDI targets; and irradiated for 1.5 h under a Hg UV lamp. The inhibitor sequences were extracted onto the target with 1:1 H_2O/CH_3CN (0.2 μL) and matrix solution (0.1 μL), and MALDI spectra were acquired in the positive ion mode for each bead on a Bruker Reflex III high-resolution MALDI-TOF mass spectrometer. Spectra were obtained using the lowest power required to facilitate desorption and ionization. Each significant peak was assigned a mass, and a program calculated all the mass differences and compared these with given values for each amino acid. In this way, each peak corresponding to a ladder fragment could be assigned and the whole sequence elucidated. Minor peaks were present in many spectra, but when included in the automatic assignment procedure, they did not give rise to any meaningful sequence.

Synthesis of Inhibitor Sequences 11−30. $PEGA_{800}$ resin (1.51 g, 0.344 mmol/g) was derivatized with the Rink amide linker (3 equiv) activated by TBTU and NEM. The resin was then distributed equally in the 20 wells of a MCPS, and Fmoc groups were removed. The peptides were synthesized using Fmoc-Aa-OPfp (3 equiv)/Dhbt-OH (1 equiv), or Fmoc-Aa-OH (only ClF and PyA, 3 equiv) with TBTU activation. Coupling of propargylic acid (3 equiv) was effected with EEDQ (3.1 equiv) and for the cycloaddition CuI (0.1 equiv, 0.1 M in pyridine) and 2 equiv of Fmoc-Arg(Pmc)-ψ-[CH_2N_3], Fmoc-Met-ψ[CH_2N_3] or Fmoc-Phe-ψ[CH_2N_3] were used. At the end of the synthesis, Fmoc groups were removed, and cleavage from the resin and side-chain protecting groups was effected with a mixture of TFA/H_2O/TIPS (92.5:5:2.5) for 0.5 h (×5). The peptides were purified by RP-HPLC, affording peptides **11**−**30** in 30−89% yield. They were analyzed by MALDI-TOF MS, HR-MS, and ^{1}H NMR (1D and COSY).

Inhibitors **31**−**33** were synthesized in a similar fashion, but amino acids from the mass/ionization spacer were included in the sequences.

H-Trp-Ala-MTr-Arg-Leu-NH_2 (11). Purification by RP-HPLC afforded 11.1 mg of **11** (57% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.79 and 0.83 (d, 6H, J = 6 Hz, Leu $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.07 (d, 3H, J = 7 Hz, Ala CH_3^{β}), 1.53 (m, 7H, Arg CH_2^{γ}, Leu CH_2^{β}, Leu CH^{γ} and MTr CH_2^{β}), 1.81 (m, 2H, Arg CH_2^{β}), 2.00 (s, 3H, MTr CH_3^{ϵ}), 2.45 (m,

2H, MTr CH_2^{γ}), 3.10 (t, 2H, $J = 7$ Hz, Arg CH_2^{δ}), 3.16 and 3.31 (dd, 2H, $J = 6$ Hz, $J' = 8$ Hz, Trp CH_2^{β}), 4.06 (m, 1H, Ala CH^{α}), 4.12 (t, 1H, $J = 8$ Hz, Trp CH^{α}), 4.24 (m, 1H, Leu CH^{α}), 4.26 (m, 1H, MTr CH^{α}), 4.27 (m, 2H, MTr CH_2-triazole), 4.45 (m, 1H, Arg CH^{α}), 7.03−7.53 (5H, Trp aromatic protons), 8.24 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{34}H_{54}N_{13}O_5S^+$), 756.41; found ($MH^+$), *m/z* 756.64. HR-MS: calcd (MH^+), 756.4086; found (MH^+), *m/z* 756.4084.

H-Trp-Ala-FTr-Arg-Leu-NH_2 (12). Purification by RP-HPLC afforded 9.4 mg of **12** (47% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.78 and 0.83 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 0.95 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.52 (m, 5H, Arg CH_2^{γ}, Leu CH_2^{β} and Leu CH^{γ}), 1.76 (m, 2H, Arg CH_2^{β}), 2.67 and 2.86 (d, 2H, $J = 6$ Hz, $J' = 14$ Hz, FTr CH_2^{β}), 3.09 (t, 2H, $J = 7$ Hz, Arg CH_2^{δ}), 3.16 (m, 2H, Trp CH_2^{β}), 3.25 (m, 2H, FTr CH_2-triazole), 4.01 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.08 (m, 1H, Trp CH^{α}), 4.21 (m, 1H, Leu CH^{α}), 4.38 (1H, FTr CH^{α}), 4.43 (m, 1H, Arg CH^{α}), 7.13−7.69 (10H, Phe and Trp aromatic protons), 8.17 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{38}H_{54}N_{13}O_5^+$), 772.44; found ($MH^+$), *m/z* 772.64. HR-MS: calcd (MH^+), 772.4365; found (MH^+), *m/z* 772.4362.

H-Trp-Ala-RTr-Arg-Leu-NH_2 (13). Purification by RP-HPLC afforded 17.8 mg of **13** (88% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.77 and 0.82 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.07 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.54 (m, 9H, Arg CH_2^{β}, Arg CH_2^{γ}, Leu CH_2^{β}, Leu CH^{γ}, RTr CH_2^{β} and RTr CH_2^{γ}), 3.07 (m, 4H, Arg CH_2^{δ} and RTr CH_2^{δ}), 3.16 and 3.33 (d, 2H, $J = 6$ Hz, $J' = 15$ Hz, Trp CH_2^{β}), 4.05 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.11 (t, 1H, $J = 6$ Hz, Trp CH^{α}), 4.22 (m, 1H, Leu CH^{α}), 4.27 (m, 1H, RTr CH^{α}), 4.44 (m, 1H, Arg CH^{α}), 4.46 (m, 2H, RTr CH_2-triazole), 7.00−7.56 (5H, Trp aromatic protons), 8.24 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{35}H_{57}N_{16}O_5^+$), 781.47; found ($MH^+$), *m/z* 781.64. HR-MS: calcd (MH^+), 781.4692; found (MH^+), *m/z* 781.4715.

H-Trp-Ala-RTr-Arg-Ile-NH_2 (14). Purification by RP-HPLC afforded 14.9 mg of **14** (74% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.77 (t, 3H, $J = 7$ Hz, Ile CH_3^{δ}), 0.81 (d, 3H, $J = 7$ Hz, Ile $CH_3^{\gamma 2}$), 1.06 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.52 (m, 9H, Ile CH^{β}, Ile $CH_2^{\gamma 1}$, Arg CH_2^{γ}, RTr CH_2^{β}, and RTr CH_2^{γ}), 1.77 (m, 2H, Arg CH_2^{β}), 3.07 (m, 4H, Arg CH_2^{δ}, and RTr CH_2^{δ}), 3.15 and 3.32 (d, 2H, $J = 6$ Hz, $J' = 15$ Hz, Trp CH_2^{β}), 4.06 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.11 (m, 1H, Trp CH^{α}), 4.17 (m, 1H, Ile CH^{α}), 4.27 (m, 2H, RTr CH_2-triazole), 4.48 (m, 1H, Arg CH^{α}), 4.51 (m, 1H, RTr CH^{α}), 7.00−7.56 (5H, Trp aromatic protons), 8.23 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{35}H_{57}N_{16}O_5^+$), 781.47; found ($MH^+$), *m/z* 781.49. HR-MS: calcd (MH^+), 781.4692; found (MH^+), *m/z* 781.4722.

H-Lys-Ala-RTr-Arg-Leu-NH_2 (15). Purification by RP-HPLC afforded 14.8 mg of **15** (79% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.79 and 0.83 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.06 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.32 (m, 2H, Lys CH_2^{γ}), 1.57 (m, 11H, Arg CH_2^{γ}, Leu CH_2^{β}, Leu CH^{γ}, Lys CH_2^{δ}, RTr CH_2^{β}, and RTr CH_2^{γ}), 1.77 (m, 4H, Arg CH_2^{β}, and Lys CH_2^{β}), 2.86 (t, 2H, $J = 8$ Hz, Lys CH_2^{ϵ}), 3.08 (m, 4H, Arg CH_2^{δ}, and RTr CH_2^{δ}), 3.81 (t, 1H, $J = 7$ Hz, Lys CH^{α}), 4.07 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.25 (m, 1H, Leu CH^{α}), 4.45 (m, 1H, Arg CH^{α}), 4.48 (m, 1H, RTr CH^{α}), 4.52 (m, 2H, RTr CH_2-triazole), 8.29 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{30}H_{59}N_{16}O_5^+$), 723.49; found ($MH^+$), *m/z* 723.45. HR-MS: calcd (MH^+), 723.4849; found (MNa^+), *m/z* 723.4872.

H-Glu-Ala-RTr-Arg-Leu-NH_2 (16). Purification by RP-HPLC afforded 15.6 mg of **16** (84% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.78 and 0.86 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.07 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.54 (m, 9H, Arg CH_2^{γ}, Leu CH_2^{β}, Leu CH^{γ}, RTr CH_2^{β} and RTr CH_2^{γ}), 1.80 (m, 2H, Arg CH_2^{β}), 2.02 (m, 2H, Glu CH_2^{β}), 2.41 (t, 2H, $J = 8$ Hz, Glu CH_2^{γ}), 3.08 (m, 4H, Arg CH_2^{δ} and RTr CH_2^{δ}), 3.88 (t, 1H, $J = 7$ Hz, Glu CH^{α}), 4.08 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.26 (m, 1H, Leu CH^{α}), 4.43 (m, 1H, RTr CH^{α}), 4.44 (m, 1H, Arg CH^{α}), 4.53 (m, 2H, RTr CH_2-triazole), 8.29 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{29}H_{54}N_{15}O_7^+$), 724.43; found ($MH^+$), *m/z* 724.46. HR-MS: calcd (MH^+), 724.4325; found (MH^+), *m/z* 724.4350.

H-His-Thr-RTr-Arg-Leu-NH_2 (17). Purification by RP-HPLC afforded 11.2 mg of **17** (57% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.80 and 0.83 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$, and $CH_3^{\delta 2}$), 0.93 (d, 3H, $J = 6$ Hz, Thr CH_3^{γ}), 1.57 (m, 9H, Arg CH_2^{γ}, Leu CH_2^{β}, Leu CH^{γ}, RTr CH_2^{β}, and RTr CH_2^{γ}), 1.80 (m, 2H, Arg CH_2^{β}), 3.10 (m, 4H, Arg CH_2^{δ}, and RTr CH_2^{δ}), 3.27 (d, 2H, $J = 7$ Hz, His CH_2^{β}), 3.90 (m, 1H, Thr CH^{β}), 4.10 (d, 1H, $J = 5$ Hz, Thr CH^{α}), 4.24 (m, 1H, His CH^{α}), 4.26 (m, 1H, Leu CH^{α}), 4.42 (m, 1H, RTr CH^{α}), 4.44 (m, 1H, Arg CH^{α}), 4.50 (m, 2H, RTr CH_2-triazole), 7.33 (s, 1H, His $CH^{\delta 2}$), 8.34 (s, 1H, triazole-H^4), 8.57 (s, 1H, His $CH^{\epsilon 1}$). MALDI-TOF MS: calcd ($MH^+ = C_{31}H_{56}N_{17}O_6^+$), 762.46; found ($MH^+$), *m/z* 762.61. HR-MS: calcd (MH^+), 762.4594; found (MH^+), *m/z* 762.4620.

H-Leu-PyA-RTr-Arg-Leu-NH_2 (18). Purification by RP-HPLC afforded 18.1 mg of **18** (89% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.78 and 0.83 (d, 12H, $J = 6$ Hz, Leu/Leu′ $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.52 (m, 12H, Arg CH_2^{γ}, Leu/Leu′ CH_2^{β}, Leu/Leu′ CH^{γ}, RTr CH_2^{β}, and RTr CH_2^{γ}), 1.81 (m, 2H, Arg CH_2^{β}), 3.00 (m, 2H, PyA CH_2^{β}), 3.10 (m, 4H, Arg CH_2^{δ}, and RTr CH_2^{δ}), 3.77 and 4.25 (m, 2H, Leu/Leu′ CH^{α}), 4.28 (m, 1H, RTr CH^{α}), 4.43 (m, 2H, RTr CH_2-triazole), 4.45 (t, 1H, $J = 6$ Hz, Arg CH^{α}), 4.58 (m, 1H, PyA CH^{α}), 7.89−8.62 (4H, PyA aromatic protons), 8.31 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{35}H_{61}N_{16}O_5^+$), 785.50; found ($MH^+$), *m/z* 785.69. HR-MS: calcd (MH^+), 785.5005; found (MH^+), *m/z* 785.5028.

H-Trp-Ala-RTr-Leu-Phe-NH_2 (19). Purification by RP-HPLC afforded 9.7 mg of **19** (49% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.75 and 0.80 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$, and $CH_3^{\delta 2}$), 1.05 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.51 (m, 7H, Leu CH_2^{β}, Leu CH^{γ}, RTr CH_2^{β}, and RTr CH_2^{γ}), 2.96 (m, 2H, Phe CH_2^{β}), 3.08 (m, 2H, RTr CH_2^{δ}), 3.15 and 3.33 (d, 2H, $J = 6$ Hz, $J' = 15$ Hz, Trp CH_2^{β}), 4.06 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.11 (m, 1H, Trp CH^{α}), 4.35 (m, 1H, Leu CH^{α}), 4.37 (m, 2H, RTr CH_2-triazole), 4.49 (m, 1H, Phe CH^{α}), 4.50 (m, 1H, RTr CH^{α}), 6.99−7.56 (10H, Phe and Trp aromatic protons), 8.23 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{38}H_{54}N_{13}O_5^+$), 772.44; found

(MH^+), *m/z* 772.67. HR-MS: calcd (MNa^+), 794.4185; found (MNa^+), *m/z* 794.4189.

H-Trp-Ala-RTr-ClF-Lys-NH_2 (20). Purification by RP-HPLC afforded 13.8 mg of **20** (65% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 1.02 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.27 (m, 2H, Lys CH_2^{γ}), 1.54 (m, 6H, Lys CH_2^{δ}, RTr CH_2^{β}, and RTr CH_2^{γ}), 1.70 (m, 2H, Lys CH_2^{β}), 2.83 (t, 2H, $J = 8$ Hz, Lys CH_2^{ϵ}), 3.04 (m, 2H, ClF CH_2^{β}), 3.07 (t, 2H, $J = 6$ Hz, RTr CH_2^{δ}), 3.17 and 3.33 (d, 2H, $J = 6$ Hz, $J' = 15$ Hz, Trp CH_2^{β}), 4.04 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.12 (m, 1H, Trp CH^{α}), 4.18 (m, 1H, Lys CH^{α}), 4.29 (d, 2H, $J = 9$ Hz, RTr CH_2-triazole), 4.49 (m, 1H, RTr CH^{α}), 4.69 (dd, 1H, $J = 6$ Hz, ClF CH^{α}), 7.00−7.57 (9H, ClF and Trp aromatic protons), 8.19 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{38}H_{54}ClN_{14}O_5^+$), 821.41; found ($MH^+$), *m/z* 821.57. HR-MS: calcd (MH^+), 821.4085; found (MH^+), *m/z* 821.4092.

H-Ala-Thr-FTr-Leu-Leu-NH_2 (21). Purification by RP-HPLC afforded 9.8 mg of **21** (59% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.76 (d, 3H, $J = 6$ Hz, Thr CH_3^{γ}), 0.85 (m, 12H, Leu/Leu′ $CH_3^{\delta 1}$, and $CH_3^{\delta 2}$), 1.33 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.58 (m, 6H, Leu/Leu′ CH_2^{β}, and CH^{γ}), 2.73 and 2.90 (dd, 2H, $J = 4$ Hz, $J' = 13$ Hz, FTr CH_2^{β}), 3.72 (m, 1H, Thr CH^{β}), 3.93 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.00 (d, 1H, $J = 6$ Hz, Thr CH^{α}), 4.23 and 4.47 (m, 2H, Leu/Leu′ CH^{α}), 4.45 (m, 2H, FTr CH_2-triazole), 4.52 (m, 1H, FTr CH^{α}), 7.15−7.28 (5H, FTr aromatic protons), 8.28 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{31}H_{50}N_9O_6^+$), 664.39; found ($MH^+$), *m/z* 644.50. HR-MS: calcd (MNa^+), 666.3698; found (MNa^+), *m/z* 666.3685.

H-Trp-Thr-FTr-Arg-Phe-NH_2 (22). Purification by RP-HPLC afforded 6.5 mg of **22** (30% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.76 (d, 3H, $J = 6$ Hz, Thr CH_3^{γ}), 1.40 (m, 2H, Arg CH_2^{γ}), 1.66 (m, 2H, Arg CH_2^{β}), 2.85 (m, 2H, FTr CH_2^{β}), 3.02 (t, 2H, $J = 7$ Hz, Arg CH_2^{δ}), 3.07 (m, 2H, Phe CH_2^{β}), 3.16 (m, 2H, Trp CH_2^{β}), 3.74 (m, 1H, Thr CH^{β}), 4.03 (t, 1H, $J = 6$ Hz, Thr CH^{α}), 4.14 (m, 1H, Trp CH^{α}), 4.33 (m, 2H, FTr CH_2-triazole), 4.36 (t, 1H, $J = 8$ Hz, Arg CH^{α}), 4.53 (m, 1H, FTr CH^{α}), 4.54 (m, 1H, Phe CH^{α}), 6.94−7.53 (15H, FTr, Phe and Trp aromatic protons), 8.22 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{42}H_{54}N_{13}O_6^+$), 836.43; found ($MH^+$), *m/z* 836.65. HR-MS: calcd (MNa^+), 858.4134; found (MNa^+), *m/z* 858.4168.

H-PyA-Asp-FTr-Arg-Leu-NH_2 (23). Purification by RP-HPLC afforded 16.5 mg of **23** (82% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.81 and 0.83 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.53 (m, 2H, Arg CH_2^{γ}), 1.54 (m, 3H, Leu CH_2^{β} and CH^{γ}), 1.81 (m, 2H, Arg CH_2^{β}), 2.41 (m, 2H, Asp CH_2^{β}), 2.83 (m, 2H, FTr CH_2^{β}), 3.10 (t, 2H, $J = 7$ Hz, Arg CH_2^{δ}), 3.15 (m, 2H, PyA CH_2^{β}), 4.04 (m, 1H, PyA CH^{α}), 4.42 (m, 1H, Asp CH^{α}), 4.45 (m, 1H, Arg CH^{α}), 4.45 (m, 1H, Leu CH^{α}), 4.48 (m, 1H, FTr CH^{α}), 4.57 (m, 2H, FTr CH_2-triazole), 7.10−7.28 (5H, FTr aromatic protons), 7.75−8.60 (4H, PyA aromatic protons), 8.31 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{36}H_{52}N_{13}O_7^+$), 778.41; found ($MH^+$), *m/z* 778.37. HR-MS: calcd (MH^+), 778.4107; found (MH^+), *m/z* 778.4122.

H-Ala-Glu-MTr-Arg-Leu-NH_2 (24). Purification by RP-HPLC afforded 11.0 mg of **24** (61% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.78 and 0.86 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$, and $CH_3^{\delta 2}$), 1.36 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.54 (m, 5H, Leu CH_2^{β}, Leu CH^{γ}, and MTr CH_2^{β}), 1.57 (m, 2H, Arg CH_2^{γ}), 1.73 (m, 2H, Glu CH_2^{β}), 1.77 (m, 2H, Arg CH_2^{β}), 2.00 (s, 3H, MTr CH_3^{ϵ}), 2.16 (t, 2H, $J = 8$ Hz, Glu CH_2^{γ}), 2.46 (m, 2H, MTr CH_2^{γ}), 3.11 (t, 2H, $J = 7$ Hz, Arg CH_2^{δ}), 3.92 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.14 (t, 1H, $J = 6$ Hz, Glu CH^{α}), 4.26 (m, 1H, Leu CH^{α}), 4.30 (m, 1H, MTr CH^{α}), 4.43 (m, 1H, Arg CH^{α}), 4.52 (m, 2H, MTr CH_2-triazole), 8.31 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{28}H_{51}N_{12}O_7S^+$), 699.37; found ($MH^+$), *m/z* 699.45. HR-MS: calcd (MH^+), 699.3719; found (MH^+), *m/z* 699.3740.

H-Thr-PyA-MTr-Arg-Leu-NH_2 (25). Purification by RP-HPLC afforded 15.8 mg of **25** (82% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.78 and 0.83 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.14 (d, 3H, $J = 6$ Hz, Thr CH_3^{γ}), 1.56 (m, 7H, Arg CH_2^{γ}, Leu CH_2^{β}, Leu CH^{γ}, and MTr CH_2^{β}), 1.80 (m, 2H, Arg CH_2^{β}), 1.99 (s, 3H, MTr CH_3^{ϵ}), 2.41 (m, 2H, MTr CH_2^{γ}), 2.98 and 3.18 (d, 2H, $J = 6$ Hz, $J' = 14$ Hz, PyA CH_2^{β}), 3.11 (t, 2H, $J = 7$ Hz, Arg CH_2^{δ}), 3.70 (d, 1H, $J = 6$ Hz, Thr CH^{α}), 4.00 (m, 1H, Thr CH^{β}), 4.27 (m, 1H, Leu CH^{α}), 4.32 (m, 1H, MTr CH^{α}), 4.39 (m, 2H, MTr CH_2-triazole), 4.46 (m, 1H, Arg CH^{α}), 4.60 (m, 1H, PyA CH^{α}), 7.89−8.61 (4H, PyA aromatic protons), 8.30 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{32}H_{54}N_{13}O_6S^+$), 748.40; found ($MH^+$), *m/z* 748.51. HR-MS: calcd (MH^+), 748.4035; found (MH^+), *m/z* 748.4058.

H-Leu-Ser-MTr-Arg-Leu-NH_2 (26). Purification by RP-HPLC afforded 14.1 mg of **26** (78% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.81 and 0.84 (d, 12H, $J = 6$ Hz, Leu/Leu′ $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.58 (m, 10H, Arg CH_2^{γ}, Leu/Leu′ CH_2^{β}, Leu/Leu′ CH^{γ} and MTr CH_2^{β}), 1.80 (m, 2H, Arg CH_2^{β}), 1.99 (s, 3H, MTr CH_3^{ϵ}), 2.45 (m, 2H, MTr CH_2^{γ}), 3.11 (t, 2H, $J = 7$ Hz, Arg CH_2^{δ}), 3.53 (m, 2H, Ser CH_2^{β}), 3.87 (t, 1H, $J = 7$ Hz, Leu CH^{α}), 4.26 (m, 1H, Leu′ CH^{α}), 4.30 (m, 1H, Ser CH^{α}), 4.33 (m, 1H, MTr CH^{α}), 4.45 (m, 1H, Arg CH^{α}), 4.53 (d, 2H, MTr CH_2-triazole), 8.31 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{29}H_{55}N_{12}O_6S^+$), 699.41; found ($MH^+$), *m/z* 699.55. HR-MS: calcd (MH^+), 699.4083; found (MH^+), *m/z* 699.4112.

H-Pro-Ala-RTr-Arg-Leu-NH_2 (27). Purification by RP-HPLC afforded 8.8 mg of **27** (49% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.79 and 0.84 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.06 (d, 3H, $J = 7$ Hz, Ala CH_3^{β}), 1.54 (m, 9H, Arg CH_2^{γ}, Leu CH_2^{β}, Leu CH^{γ}, RTr CH_2^{β}, and RTr CH_2^{γ}), 1.82 (m, 2H, Arg CH_2^{β}), 1.88 (m, 2H, Pro CH_2^{γ}), 2.30 (m, 2H, Pro CH_2^{β}), 3.10 (m, 4H, Arg CH_2^{δ}, and RTr CH_2^{δ}), 3.27 (m, 2H, Pro CH_2^{δ}), 4.06 (q, 1H, $J = 7$ Hz, Ala CH^{α}), 4.23 (m, 1H, Leu CH^{α}), 4.26 (m, 1H, RTr CH^{α}), 4.28 (m, 1H, Pro CH^{α}), 4.45 (m, 1H, Arg CH^{α}), 4.54 (m, 2H, RTr CH_2-triazole), 8.28 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{29}H_{54}N_{15}O_5^+$), 692.44; found ($MH^+$), *m/z* 692.64. HR-MS: calcd (MNa^+), 714.4263; found (MNa^+), *m/z* 714.4266.

H-Asp-PyA-RTr-Arg-Leu-NH_2 (28). Purification by RP-HPLC afforded 13.8 mg of **28** (68% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.79 and 0.85 (d, 6H, $J = 6$ Hz,

Leu $CH_3^{\delta 1}$, and $CH_3^{\delta 2}$), 1.54 (m, 9H, Arg CH_2^{γ}, Leu CH_2^{β}, Leu CH^{γ}, RTr CH_2^{β}, and RTr CH_2^{γ}), 1.81 (m, 2H, Arg CH_2^{β}), 2.89 (d, 2H, $J = 6$ Hz, Asp CH_2^{β}), 3.08 (m, 4H, Arg CH_2^{δ}, and RTr CH_2^{δ}), 3.09 (m, 2H, PyA CH_2^{β}), 4.09 (t, 1H, $J = 6$ Hz, Asp CH^{α}), 4.20 (m, 1H, Leu CH^{α}), 4.25 (m, 1H, RTr CH^{α}), 4.41 (m, 2H, RTr CH_2-triazole), 4.45 (m, 1H, Arg CH^{α}), 4.56 (m, 1H, PyA CH^{α}), 7.89−8.61 (4H, PyA aromatic protons), 8.29 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{33}H_{55}N_{16}O_7^+$), 787.44; found ($MH^+$), *m*/*z* 787.60. HR-MS: calcd (MNa^+), 809.4236; found (MNa^+), *m*/*z* 809.4257.

H-Gly-Leu-RTr-ClF-Leu-NH_2 (29). Purification by RP-HPLC afforded 9.6 mg of **29** (52% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.64 and 0.68 (d, 6H, $J = 6$ Hz, Leu $CH_3^{\delta 1}$, and $CH_3^{\delta 2}$), 0.76 and 0.81 (d, 6H, $J = 6$ Hz, Leu′ $CH_3^{\delta 1}$ and $CH_3^{\delta 2}$), 1.12 (m, 3H, Leu CH_2^{β}, and Leu CH^{γ}), 1.45 (m, 3H, Leu′ CH_2^{β}, and Leu′ CH^{γ}), 1.46 (m, 4H, RTr CH_2^{β}, and RTr CH_2^{γ}), 3.09 (m, 4H, ClF CH_2^{β}, and RTr CH_2^{δ}), 3.66 (s, 2H, Gly CH_2^{α}), 4.01 (t, 1H, $J = 6$ Hz, Leu CH^{α}), 4.18 (m, 1H, Leu′ CH^{α}), 4.28 (m, 1H, RTr CH^{α}), 4.47 (m, 2H, RTr CH_2-triazole), 4.71 (m, 1H, ClF CH^{α}), 7.19−7.27 (4H, ClF aromatic protons), 8.21 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{32}H_{53}ClN_{12}O_5^+$), 719.39; found ($MH^+$), *m*/*z* 719.66. HR-MS: calcd (MH^+), 719.3867; found (MH^+), *m*/*z* 719.3853.

H-Gly-Lys-RTr-Met-Asn-NH_2 (30). Purification by RP-HPLC afforded 12.3 mg of **30** (70% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 1.08 (m, 2H, Lys CH_2^{γ}), 1.41 (m, 2H, Met CH_2^{β}), 1.48 (m, 2H, Lys CH_2^{δ}), 1.54 (m, 2H, RTr CH_2^{γ}), 1.59 (m, 2H, RTr CH_2^{β}), 2.03 (s, 3H, Met CH_3^{ϵ}), 2.08 (m, 2H, Lys CH_2^{β}), 2.55 (m, 2H, Met CH_2^{γ}), 2.64 (m, 2H, Asn CH_2^{β}), 2.83 (t, 2H, $J = 7$ Hz, Lys CH_2^{ϵ}), 3.08 (t, 2H, $J = 6$ Hz, RTr CH_2^{δ}), 3.68 (s, 2H, Gly CH_2^{α}), 4.02 (m, 1H, Met CH^{α}), 4.21 (m, 1H, RTr CH^{α}), 4.41 (m, 2H, RTr CH_2-triazole), 4.58 (m, 1H, Asn CH^{α}), 4.59 (m, 1H, Lys CH^{α}), 8.31 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{26}H_{49}N_{14}O_6S^+$), 685.37; found ($MH^+$), *m*/*z* 685.49. HR-MS: calcd (MH^+), 685.3680; found (MH^+), *m*/*z* 685.3684.

H-Gly-RTr-Arg-Leu-Thr-Ile-Ser-Arg-Gly-NH_2 (31). Purification by RP-HPLC afforded 26.4 mg of **31** (74% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.76−0.86 (m, 12H, Ile CH_3^{δ}, Ile $CH_3^{\gamma 2}$, Leu $CH_3^{\delta 1}$, and $CH_3^{\delta 2}$), 1.06 (d, 3H, $J = 6$ Hz, Thr CH_3^{γ}), 1.56 (m, 14H, Arg/Arg′ CH_2^{γ}, Ile CH^{β}, Ile $CH_2^{\gamma 1}$, Leu CH_2^{β}, Leu CH^{γ}, RTr CH_2^{β}, RTr CH_2^{γ}), 1.79 (m, 4H, Arg/Arg′ CH_2^{β}), 3.08 (m, 6H, Arg/Arg′ CH_2^{δ}, RTr CH_2^{δ}), 3.61 (m, 2H, Ser CH_2^{β}), 3.71 (m, 1H, Thr CH^{β}), 3.77 and 3.78 (s, 4H, Gly/Gly′ CH_2^{α}), 4.12 and 4.46 (m, 2H, Arg/Arg′ CH^{α}), 4.16 (m, 1H, Ile CH^{α}), 4.19 (m, 1H, Ser CH^{α}), 4.26 (m, 1H, Leu CH^{α}), 4.34 (m, 1H, RTr CH^{α}), 4.35 (m, 1H, Thr CH^{α}), 4.52 (m, 2H, RTr CH_2-triazole), 8.32 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($M + 2H^+ = C_{44}H_{84}N_{22}O_{11}^+$), 1096.6690; found ($M + 2H^+$), 2*m*/*z* 1096.6832.

H-Gly-FTr-Arg-Phe-Thr-Ile-Ser-Arg-Gly-NH_2 (32). Purification by RP-HPLC afforded 29.5 mg of **32** (81% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.81 (m, 6H, Ile CH_3^{δ}, Ile $CH_3^{\gamma 2}$), 1.05 (d, 3H, $J = 6$ Hz, Thr CH_3^{γ}), 1.44−1.80 (m, 11H, Arg/Arg′ CH_2^{γ}, Ile CH^{β}, Ile $CH_2^{\gamma 1}$, Arg/Arg′ CH_2^{β}), 2.74 (m, 2H, FTr CH_2^{β}), 2.90 (m, 2H, Phe CH_2^{β}), 3.06 (m, 4H, Arg/Arg′ CH_2^{δ}), 3.72 (m, 2H, Ser CH_2^{β}), 3.76 and 3.77 (s, 4H, Gly/Gly′ CH_2^{α}), 4.05 (m, 1H, Thr CH^{β}), 4.12 and 4.37 (m, 2H, Arg/Arg′ CH^{α}), 4.25 (m, 1H, Ile CH^{α}), 4.26 (m, 1H, Thr CH^{α}), 4.35 (m, 1H, Ser CH^{α}), 4.54 (m, 1H, FTr CH^{α}), 4.60 (m, 2H, FTr CH_2-triazole), 4.63 (m, 1H, Phe CH^{α}), 7.21−7.31 (m, 10H, FTr and Phe aromatic protons), 8.29 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{50}H_{78}N_{19}O_{11}^+$), 1120.61; found ($MH^+$), *m*/*z* 1120.75. HR-MS: calcd (MH^+), 1120.6128; found (MH^+), *m*/*z* 1120.6137.

H-Gly-RTr-ClF-Leu-Thr-Ile-Ser-Arg-Gly-NH_2 (33). Purification by RP-HPLC afforded 18.6 mg of **33** (51% yield). ^{1}H NMR (250 MHz, D_2O/CD_3CN): δ 0.75−0.83 (m, 12H, Ile CH_3^{δ}, Ile $CH_3^{\gamma 2}$, Leu $CH_3^{\delta 1}$, and $CH_3^{\delta 2}$), 1.07 (d, 3H, $J = 6$ Hz, Thr CH_3^{γ}), 1.37−1.67 (m, 12H, Arg CH_2^{γ}, Ile CH^{β}, Ile $CH_2^{\gamma 1}$, Leu CH_2^{β}, Leu CH^{γ}, RTr CH_2^{β}, RTr CH_2^{γ}), 1.79 (m, 2H, Arg CH_2^{β}), 3.07 (m, 4H, Arg CH_2^{δ}, RTr CH_2^{δ}), 3.12 (m, 2H, ClF CH_2^{β}), 3.60 (m, 2H, Ser CH_2^{β}), 3.71 (m, 1H, Thr CH^{β}), 3.76 and 3.78 (s, 4H, Gly/Gly′ CH_2^{α}), 4.11 (m, 1H, Arg CH^{α}), 4.19 (m, 1H, RTr CH^{α}), 4.22 (m, 1H, Ser CH^{α}), 4.26 (m, 1H, Ile CH^{α}), 4.33 (m, 1H, Leu CH^{α}), 4.34 (m, 1H, Thr CH^{α}), 4.45 (m, 2H, RTr CH_2-triazole), 4.73 (m, 1H, ClF CH^{α}), 7.23 (4H, ClF aromatic protons), 8.26 (s, 1H, triazole-H^4). MALDI-TOF MS: calcd ($MH^+ = C_{47}H_{79}ClN_{19}O_{11}^+$), 1120.58; found ($MH^+$), *m*/*z* 1120.71. HR-MS: calcd (MH^+), 1120.5888; found (MH^+), *m*/*z* 1120.5885.

Kinetic Evaluation of Inhibitor Sequences 11−33. Equilibrium constants (K_i) were determined from 12 experiments for each inhibitor (six different concentrations with double determination that varied by <5%) at 37 °C in PBS buffer (pH 6) augmented with 10 mM DTT as described by Nicklin and Barrett.[39] Enzyme concentration was 20 nM, $[S_0] = 0.5\ \mu$M, and Cbz-Phe-Arg-AMC ($K_M = 0.7\ \mu$M) was used as the substrate.

Abbreviations. Abz, 2-aminobenzoyl; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; ClF, *p*-chlorophenylalanine; CPB, cysteine protease B; CTE, C-terminal extension; DCM, dichloromethane; Dhbt-OH, 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine; DIAD, diisopropyl azodicarboxylate; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DTr, Asp-triazole; DTT, dithiothreitol; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; F, fluorophore; Fmoc, 9-fluorenylmethoxycarbonyl; FRET, fluorescence resonance energy transfer; FTr, Phe-triazole; GTr, Gly triazole; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCPS, multiple column peptide synthesizer; MeOH, methanol; Mis, mass/ionization spacer; MTr, Met-triazole; NEM, *N*-ethylmorpholine; PBS, phosphate buffered saline; PEGA, poly(ethylene glycol)-polydimethyl acrylamide resin; Pfp, pentafluorophenyl; Pll, photolabile linker; Pmc, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PyA, 3-pyridylalanine; Q, quencher; RP-HPLC, reverse-phase high-pressure liquid chromatography; RTr, Arg-triazole; SPPS, solid-phase peptide synthesis; TBTU, *N*-[(1*H*-benzotriazol-1-yl)−(dimethylamino)-methylene]-*N*-methylmethan-aminium tetrafluoroborate *N*-oxide; ^{t}Bu, *tert*-butyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIPS, triisopropylsilane; Y(NO_2), 3-nitrotyrosine.

One- and three-letter codes are used for the amino acids according to IUPAC.

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Supporting Information Available. All 36 sequences from the darkest beads, crude HPLC diagrams of peptides **11**−**33**, and ^{1}H NMR of the purified peptides **11**−**33**. This material is available free of charge via the Internet at http://pubs.acs.org.

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