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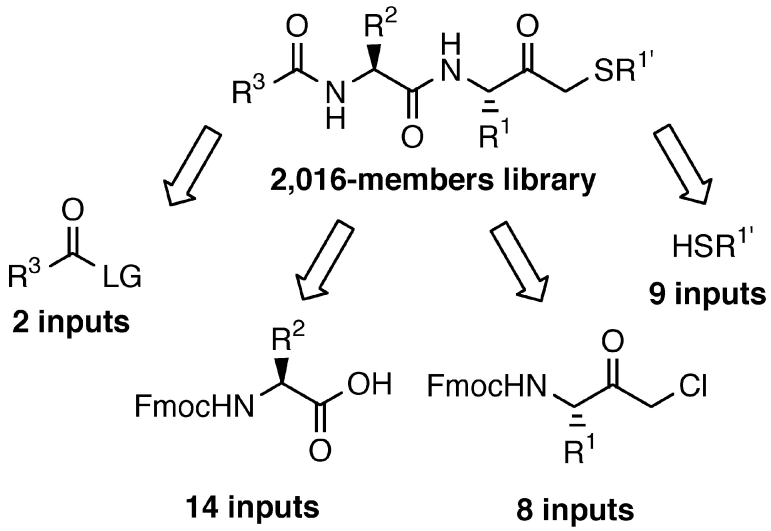

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Synthesis of a Diverse Library of Mechanism-Based Cysteine Protease Inhibitors

Warren J. L. Wood, Lily Huang, and Jonathan A. Ellman*

Center for New Directions in Organic Synthesis, Department of Chemistry, University of California, Berkeley, California 94720

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We report improvements of our method for the solid-phase synthesis of mechanism-based mercaptomethyl ketone inhibitors of cysteine proteases (Lee, A.; Huang, L.; Ellman, J. A. *J. Am. Chem. Soc.* **1999**, *121*, 9907−9914). Specifically, Fmoc-protected chloromethyl ketones were used, rather than the Alloc-protected counterparts. In addition, we further demonstrated that diverse polar functionality can be incorporated at the $R^{1'}$, R^1, and R^2 sites, in contrast to our previous efforts, where primarily hydrophobic groups were incorporated at these positions. On the basis of these results, a 2016-membered library of potential mercaptomethyl ketone inhibitors was prepared that incorporated diverse functionality. The library was screened against cathepsin B, which is implicated in cancer, resulting in the identification of single-digit nanomolar inhibitors. Because of the diverse functionality incorporated in this library, it should be a rich source of potent inhibitors against many other cysteine proteases.

Introduction

Cysteine proteases are important therapeutic targets because of their roles in many diseases,[2] including cathepsin B in tumor growth,[3] cathepsin K in osteoporosis,[4,5] calpain I in neurodegenerative diseases such as Alzheimer's disease,[6] caspase-1 in diseases caused by the misregulation of apoptosis,[7,8] rhodesain in African sleeping sickness,[9] and cruzain in Chagas' disease.[10]

Cysteine proteases catalyze the hydrolysis of amide bonds in peptides and proteins through nucleophilic attack by the active-site cysteine thiol on the amide carbonyl (Figure 1). Most inhibitors of cysteine proteases have exploited this mechanism of amide bond hydrolysis (Scheme 1) and contain an electrophilic functionality, such as a carbonyl or Michael acceptor that reacts with the active-site cysteine residue.[2] The first class of reversible inhibitors to be reported was peptidyl aldehydes.[2] However, the aldehyde pharmacophore is inherently reactive to nucleophilic attack and oxidation, which are liabilities for obtaining good pharmacokinetics. Thus, more chemically stable ketone-based inhibitors have been reported. With the ketone pharmacophore, diverse functionality can be displayed on both sides of the carbonyl, allowing more sites of recognition for achieving enhanced selectivity and affinity.

Selective and potent ketone-based reversible inhibitors have been identified for a number of cysteine proteases. In one recent example, Veber and co-workers identified potent, selective, and bioavailable inhibitors, such as **1** (Figure 2), which has a K_i of 0.16 nM against human cathepsin K and 42% oral bioavailability in rat pharmacokinetic studies.[11] Previously, we reported the synthesis of a library (~200 compounds) of amidomethyl and mercaptomethyl ketones that were tested against cruzain, rhodesain, cathepsin B, and cathepsin L.[12] In general, the mercaptomethyl ketones were more potent than the amidomethyl ketones (nanomolar versus micromolar inhibition). Selective nanomolar inhibitors of the targeted proteases included compounds such as **2** (Figure 2), which is a low nanomolar inhibitor of cruzain and is selective for this protease over cathepsins B and L. The 130-fold selectivity between cruzain and cathepsin L is encouraging, as the central domain of cruzain, which comprises a portion of the catalytic site, is similar to cathepsin L (57% sequence homology).[13]

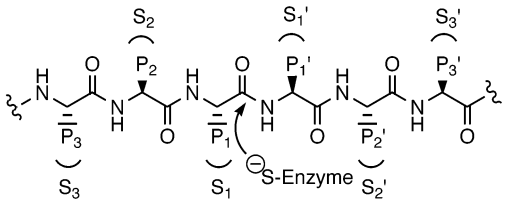
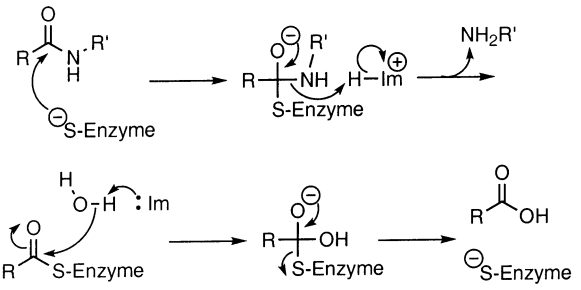


Figure 1. Standard nomenclature for protease substrate cleavage. P_n, P_3, P_2, P_1, P_1', P_2', P_3', P_n', designate the amino acid side chains of a peptide substrate. Cleavage occurs between the P_1 and P_1' residues. The corresponding binding sites in the protease active-site are designated as S_n, S_3, S_2, S_1, S_1', S_2', S_3', S_n'.

Scheme 1. Mechanism of Amide Bond Hydrolysis for Cysteine Proteases



* Corresponding author. Fax: +1-510-642-8369. E-mail: jellman@uclink.berkeley.edu.

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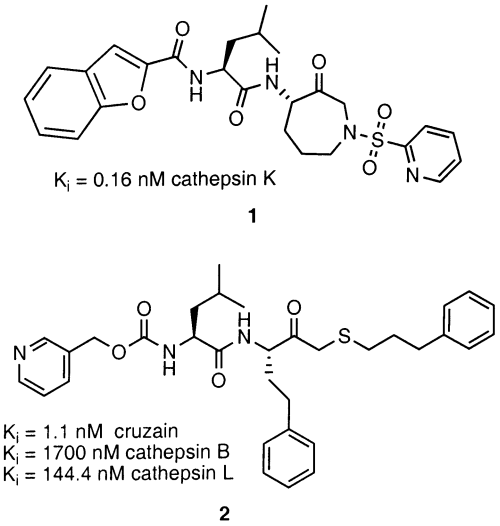
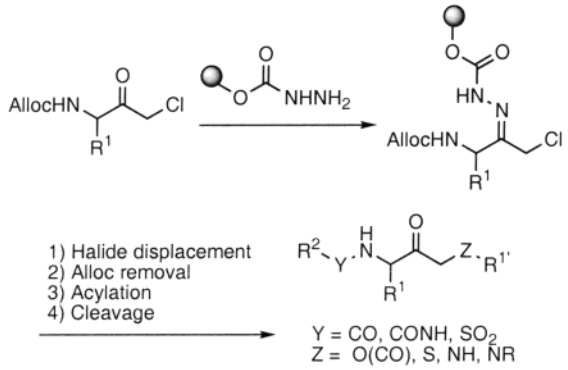


Figure 2. Examples of potent ketone-based cysteine protease inhibitors.

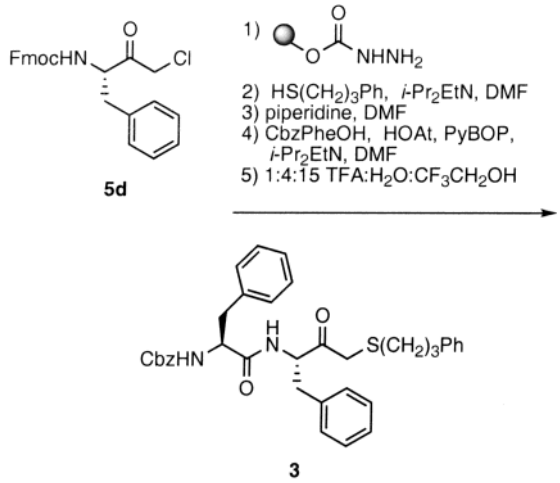
Scheme 2. General Solid-Phase Method for the Synthesis of Ketone-Based Inhibitors



Scheme 2 shows the general solid-phase method we developed for the parallel synthesis of mechanism-based inhibitors.[1] This procedure was used for the synthesis of focused inhibitor libraries targeting cruzain, which culminated in the development of inhibitor **2**. In this method, diverse functionality is displayed about the ketone pharmacophore. The first step in the synthesis is the loading of chloromethyl ketones onto a solid support via a hydrazone linkage. The linker prevents racemization at the α-stereocenter and protects the ketone from nucleophilic attack. Functionality on one side of the ketone carbonyl is introduced by displacement of the halogen. Removal of the *N*-allyloxycarbonyl (Alloc) protecting group allows the introduction of functionality on the other side of the carbonyl. Cleavage from the support provides acyloxymethyl, mercaptomethyl, and amidomethyl ketones in moderate to high yields. All of these classes of compounds have previously been reported to be cysteine protease inhibitors. [8,14−17]

Herein, we report a more expeditious method for the parallel synthesis of cysteine protease inhibitors by employing Fmoc-protected chloromethyl ketones, rather than the Alloc-protected counterparts. In addition to this useful protecting group change, we further demonstrate that diverse polar functionality can be incorporated at the $R^{1'}$, R^1, and R^2 sites, in contrast to our previous efforts, where primarily hydrophobic groups were incorporated at these positions.[1]

Scheme 3. Fmoc-protected Chloromethyl Ketone Used in Solid-Phase Sequence



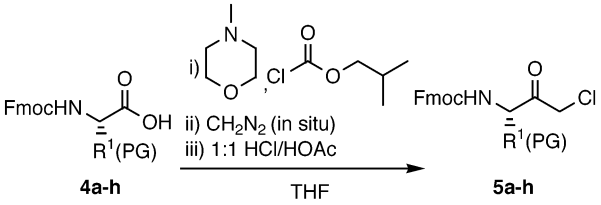
On the basis of these results, a 2016-membered library of potential mercaptomethyl ketone inhibitors was prepared that incorporated diverse functionality at the $R^{1'}$, R^1, and R^2 sites. The utility of this library was then demonstrated by screening it against the cysteine protease cathepsin B, which is implicated in cancer, resulting in the identification of single-digit nanomolar inhibitors.

Results

Synthetic Strategy. The previously reported methodology was expanded to enable the synthesis of compounds with greater functional group diversity. First, for ease of synthesis, replacement of the Alloc group was desired because few Alloc-protected amino acids are commercially available and because removal of this group is carried out via Pd(0)-mediated conditions under an inert atmosphere. The compounds are cleaved from the support under acidic conditions, and therefore a base-labile protecting group was desired. The fluorenylmethyloxycarbonyl (Fmoc) protecting group was an ideal replacement for the Alloc group because it can be removed under basic conditions and because many Fmoc amino acids are commercially available. Second, further development was necessary to incorporate polar functionality. This required the synthesis of chloromethyl ketone derivatives of amino acids with side-chain functionality protected with acid-labile groups to install the R^1 side chain. The introduction of acid-labile protecting groups at the $R^{1'}$, R^1, and R^2 sites also required the development of acidic conditions to simultaneously cleave the compounds from the support and remove all of the protecting groups.

Synthesis and Loading of Chloromethyl Ketones. In initial studies, Fmoc-protected chloromethyl ketones were determined to be effective starting materials for mercaptomethyl ketone inhibitor synthesis. As shown in Scheme 3, high yields could be obtained by loading of the Fmoc-chloromethyl ketone **5d** onto the solid support, followed by the sequence of halide displacement, Fmoc-deprotection, acylation, and cleavage. Indeed, the yield observed for product **3** (66%) is comparable to that observed when the corresponding Alloc-protected chloromethyl ketone was employed (63%), as determined by *p*-xylene calibration in NMR analysis.

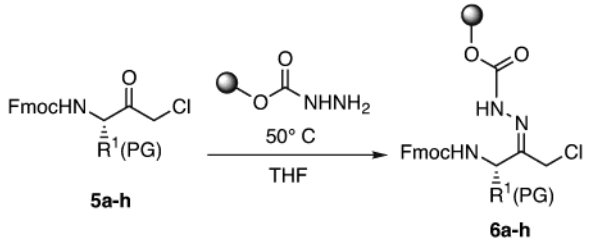
Table 1. Chloromethyl Ketone Synthesis



ketone	R^1 (PG)	yield (%)[a]
5a	−H	69
5b	$-CH_3$	89
5c	$-CH_2CH(CH_3)_2$	73
5d	$-CH_2Ph$	68
5e	$-(CH_2)_3NHC(NH)NH(Pbf)$	65
5f	$-(CH_2)_4NH(Boc)$	78
5g	$-CH_2CO_2(t\text{-Bu})$	67
5h	$-CH_2(C_6H_4)$-*p*-O(*t*-Bu)	68

[a] Isolated yield.

Table 2. Chloromethyl Ketone Loading Conditions

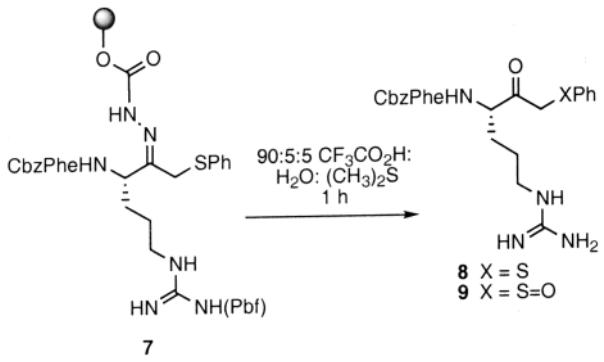


ketone	R^1 (PG)	time	yield (%)[a]
5a	−H	10 min	100
5b	$-CH_3$	1 h	100
5c	$-CH_2CH(CH_3)_2$	3 h	99
5d	$-CH_2Ph$	3 h	95
5e	$-(CH_2)_3NHC(NH)NH(Pbf)$	3 h	99
5f	$-(CH_2)_4NH(Boc)$	3 h	100
5g	$-CH_2CO_2(t\text{-Bu})$	3 h	95
5h	$-CH_2(C_6H_4)$-*p*-O(*t*-Bu)	3 h	100

[a] Based on Fmoc quantitation.

To target diverse cysteine protease inhibitors, it is desirable to incorporate not only hydrophobic but also polar amino acid side chains at the R^1 position (see Scheme 2). For example, the caspases require an aspartic acid side chain at this position,[18] cathepsin B accepts an arginine side chain,[19] and cruzain prefers basic functionality but can tolerate hydrophobic side chains.[20] The desired chloromethyl ketones (**5a**−**h**) were synthesized in good yield by a one-pot procedure from the corresponding commercially available *N*-Fmoc-protected amino acids (**4a**−**h**) (Table 1).[16] Polar side-chain functionality was protected with acid-labile groups (**4a**−**h**). Loading of the chloromethyl ketones **5a**−**h** onto the solid support to provide **6a**−**h** proceeded in excellent yields (as determined by Fmoc quantitation[21]) (Table 2). Heating at 50 °C resulted in near quantitative loading of the *N*-Fmoc-protected chloromethyl ketones, although the time required for reaction completion varied depending on the sterics of the R^1 group. Most of the chloromethyl ketones (**5c**−**h**) loaded in 3 h. The alanine chloromethyl ketone derivative (**5b**) loaded in excellent yield in 1 h, and the chloromethyl ketone derivative of glycine (**5a**) loaded in 10 min. Extended loading times for **5a** caused decomposition.

Scheme 4. Cleavage of the Pbf Group and the Solid-Phase Linker

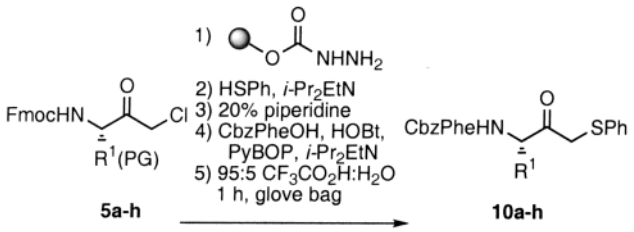


Optimization of Cleavage Conditions. The hydrazone linker can be cleaved with a 1:4:15 solution of trifluoroacetic acid (TFA)/water/2,2,2-trifluoroethanol. However, to cleave the protecting groups, more strongly acidic conditions were required. To develop universal cleavage conditions that would both remove the compounds from the solid support and cleave all of the protecting groups, we examined the cleavage of the support-bound compound **7** derived from **5e** (Scheme 4) because the Pbf group (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) is the least acid-labile protecting group employed. The Pbf group could be completely cleaved with a 90:5:5 solution of TFA/water/dimethyl sulfide. Unfortunately, the strongly acidic conditions caused a significant amount of oxidation of the thioether (**8**) to the sulfoxide (**9**). Attempts to prevent the oxidation by replacing dimethyl sulfide with ethanedithiol in the cleavage solution led to ethanedithiol-derived thioacetal products. Thus, to prevent oxidation of the desired mercaptomethyl ketones, the cleavage step was conducted in an inert atmosphere using a solution of 95:5 TFA/water. With these conditions, oxidized product was not observed.

Ketone products **10a**−**h** were prepared to test the compatibility of each of the *N*-Fmoc chloromethyl ketones **5a**−**h** with the solid-phase synthesis sequence and the new cleavage conditions. Chloromethyl ketones **5a**−**h** were first loaded onto the solid support according to the conditions described previously (Table 2). Thiophenol displacement of the chloride was followed by Fmoc deprotection under standard conditions. The resulting free amine was then acylated with CbzPheOH, and subsequent cleavage from the support using the new acidic conditions provided ketone products **10b**−**h** in 56−75% yields and **10a** in 34% yield (Table 3). The lower yield for **10a** could be due to decomposition of **5a** during the loading step. However, even with milder conditions for loading **5a** onto the support, higher yields in the full solid-phase sequence were not observed.

Library Design. With a more efficient and general procedure in hand for the solid-phase synthesis of mercaptomethyl ketone-based cysteine protease inhibitors, a library of discrete ketone inhibitors (**15**) designed to target many cysteine proteases was prepared. Diverse inputs were selected for the four sites of variability: chloromethyl ketones, which are derived from *N*-Fmoc amino acids (**11**); *N*-Fmoc amino acids (**12**); acylating agents (**13**); and thiols (**14**) (Figure 3). The ketone inhibitor products (**15**) are designed to interact

Table 3. Evaluation of **5a**−**h** in a Solid-Phase Reaction Sequence



compound	R^1	overall yield (%)[a]
10a	−H	34
10b	$-CH_3$	56
10c	$-CH_2CH(CH_3)_2$	61
10d	$-CH_2Ph$	69
10e	$-(CH_2)_3NHC(NH)NH_2$	75
10f	$-(CH_2)_4NH_2$	71
10g	$-CH_2CO_2H$	59
10h	$-CH_2(C_6H_4)$-*p*-OH	70

[a] Based on NMR calibration with *p*-xylene.

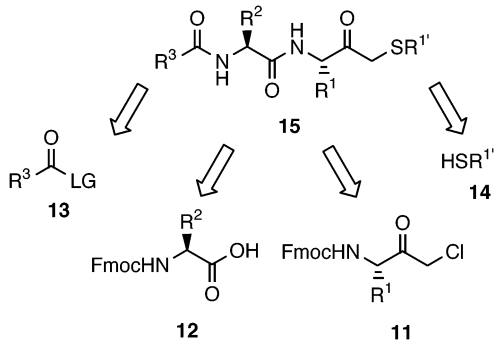


Figure 3. Four inputs for the library of inhibitors.

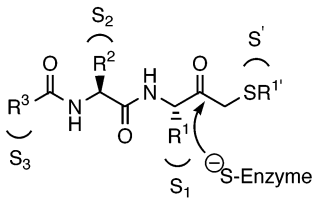


Figure 4. Designed mode of inhibition of cysteine proteases.

with the enzyme in the manner shown in Figure 4. The R^1, R^2, R^3, and $R^{1'}$ substituents should generally fit into the S_1, S_2, S_3, and S′ binding sites of the enzyme, respectively.

In selecting library inputs, we relied on the substrate specificity requirements of known therapeutically relevant cysteine proteases. As indicated previously, at the P_1 position, caspases require an aspartic acid residue, cathepsin B accepts an arginine side chain, and cruzain prefers basic functionality but can tolerate hydrophobic side chains. To prepare inhibitors that would be active against a diverse range of cysteine proteases, we prepared chloromethyl ketones from glycine, alanine, leucine, phenylalanine, arginine, lysine, aspartic acid, and tyrosine (**5a**−**h**) (Figure 5).

The R^2 group of the inhibitors is designed to fit into the S_2 binding site. Because there is a high degree of specificity at the S_2 binding site for many cysteine proteases, we incorporated a diverse group of amino acids at this position. Fourteen Fmoc-protected amino acids were selected: glycine (**16**), alanine (**17**), leucine (**18**), valine (**19**), methionine (**20**), phenylalanine (**21**), aspartic acid (**22**), glutamic acid (**23**), serine (**24**), tyrosine (**25**), lysine (**26**), arginine (**27**), histidine (**28**), and proline (**29**) (Figure 5).

Cysteine proteases make multiple hydrogen-bonding interactions with the peptide backbone of peptide substrates. Thus, the acetyl group, to be installed with acetic anhydride (**30**), was employed as an R^3 input to provide potential amide bond contacts while minimizing inhibitor size. The carbonyloxybenzyl (Cbz) group, to be installed with **31**, was also employed to incorporate a hydrophobic group at the R^3 position (Figure 5).

For the input designed to interact with the S′ side of the enzyme, both polar and nonpolar thiols displaying a variety of functional groups were desired. To select the thiols, Reagent Selector (MDL Co., San Leandro, CA) was used to form 11 clusters of the commercially available thiols with molecular weights of <200 mass units, excluding thiols with isotopes, primary and secondary amines, and costs of greater than $100/g. A molecular weight cutoff of 200 was chosen to keep the molecular weights of the potential inhibitors near 500, and primary and secondary amines were excluded to minimize the need for protection in the reaction sequence. Clusters of undesirable thiols, such as alkyl chains with numerous rotatable bonds and reactive functionalities, were excluded. Eight thiols were selected from the five remaining clusters of 177 compounds (Figure 5). Thiols **32**−**35** were chosen from the hydrophobic clusters. Thiol **36** was chosen because it incorporates an amide functional group, thiol **37** was selected to introduce basic functionality, and thiol **38** was chosen to introduce carboxylic acid functionality. Thiol **38** could readily be prepared according to literature procedures.[22] Unfortunately, additional desirable polar functionality was not present in the commercially available thiols.

For the treatment of diseases, reversible inhibitors are desirable; however, irreversible inhibitors are useful tools for identifying cysteine proteases and determining their biological function.[23] Therefore, the acidic thiophenol derivative **39** was also selected because it could serve as a leaving group in a displacement reaction with the active-site cysteine thiol. The more acidic thiol **40** (Figure 5) was also selected despite a molecular weight of >200 mass units because it should serve as an even better leaving group.

Evaluating Library Inputs. Although the compatibility of the chloromethyl ketone inputs with the reaction sequence and the new cleavage conditions had previously been established (Table 3), all of the other inputs still required evaluation to establish compatibility. Therefore, 65 compounds were synthesized using the optimized sequence shown in Scheme 5, employing every input in at least three compounds. By LCMS, the desired molecular ions for 64 of the 65 compounds were observed, indicating that every input was compatible with the reaction sequence.

Library Synthesis and Characterization. The spatially separated 2016-member library was synthesized as shown in Scheme 5. A chloromethyl ketone input (**5a**−**h**) was loaded onto the solid phase via the hydrazone linkage to yield intermediate **41**. The resin was divided for the nine thiols inputs (**32**−**40**), and after chlorine displacement, the resin from each cartridge was transferred into the columns of four

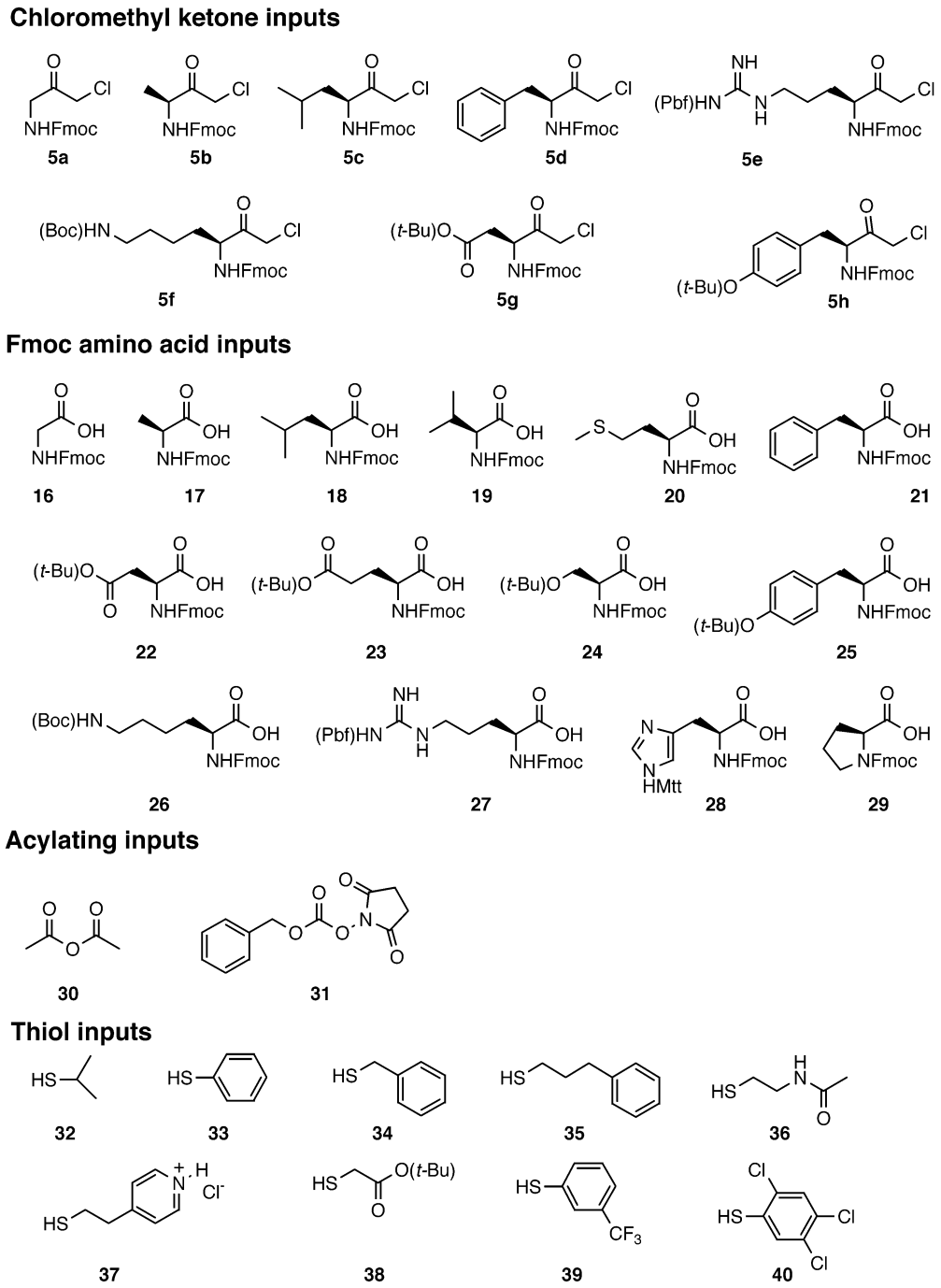


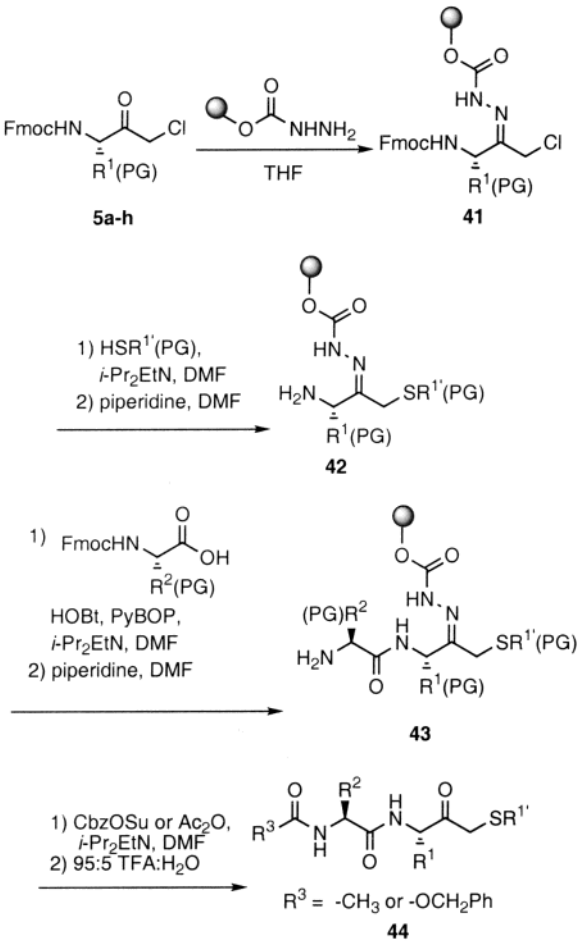
Figure 5. Library inputs.

Robbins blocks. After the Fmoc group had been removed, the 14 Fmoc amino acid inputs (**16**−**29**) were placed into rows of the blocks and coupled onto the resin-bound intermediate **42** using standard coupling conditions. After Fmoc deprotection, the free amines of intermediate **43** were reacted with acetic anhydride (**30**) or CbzOSu (**31**). The 2016 library members (**44**) were then cleaved from the support using a solution of 95:5 TFA/water in an atmosphere of nitrogen. Twenty milligrams of resin (9.8 μmol) was used in the synthesis of each library member, except for library members derived from **5a** for which the amount of resin was doubled, to obtain similar amounts of each library member.

Of the 2016 library members, 134 (6.6%) were randomly analyzed by LCMS, and 119 (88%) of these library members had chemical purities of >80% as determined by UV detection at 220 nm. Twenty-one library members were randomly selected and analyzed by ^{1}H NMR spectroscopy with an internal standard of hexamethyldisiloxane (see Supporting Information for LCMS data and spectra). The average yield was 6.5 μmol (∼65% for compounds derived from **5b**−**h** and ∼32% for compounds derived from **5a**).

Library Screening against Cathepsin B. The complete 2016-member library was screened against cathepsin B at 1 μM, based on the average yield. Of the compounds screened, 110 library members inhibited >50% of the enzyme activity

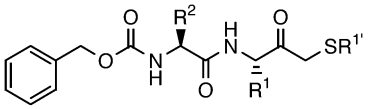
Scheme 5. Synthesis of a Library of Mercaptomethyl Ketone Inhibitors



upon a 5-min incubation with cathepsin B (as determined by rates of cleavage of the fluorescent substrate Cbz-Phe-Arg-AMC). Enzyme activity was monitored by the release of the fluorescent aminomethylcoumarin (AMC) group. Structure−activity relationship (SAR) data from this screening revealed that the side chains of glycine, lysine, and arginine are preferred at R^1. At the R^2 position, leucine, valine, phenylalanine, and tyrosine side chains are preferred. In general, library members with the Cbz group are more potent than library members with an acetyl group. In general, these data agree well with the known specificity of cathepsin B.[19,24]

These 110 library members were screened in the same manner at 333 nM, and 18 library members were found to cause >50% inhibition of the enzyme. The 18 library members can be divided into three different categories according to the R^1 side chain: compounds with the basic side chain of lysine or arginine (11 library members), those with a proton at R^1 (six library members), and those with a leucine side chain (one library member). Nine diverse compounds of the 11 most potent library members incorporating the side chain of lysine or arginine at R^1 were synthesized and purified (**45**−**53**) (Table 4). The remaining two library members were similar in structure to **52** and **53**, incorporating the side chains of valine or leucine at R^2, and were not synthesized. Compounds **45**−**53** were assayed against cathepsin B and determined to be reversible inhibitors with K_i values ranging between 2.0 and 140 nM. The K_i values for compounds **45**−**51** were determined from Dixon plots.[25] Compounds **52** and **53** were tight-binding inhibitors under the assay conditions (enzyme concentration of 1.4 nM, as determined by E-64 titration[26]), and the K_i values were determined using the linear regression analysis method of Williams and Morrison.[27] For all of these compounds, except **52**, time-dependent inhibition was observed. Assays of percent of enzyme inhibition versus time demonstrated that equilibrium between inhibitors **45**−**48** and **53** and cathepsin B were established between 15 and 90 min, with a ∼30% increase in enzyme inhibition relative to the percent activity observed after a 5-min incubation. For compounds **49**−**51**, time dependence was not observed over 30 min; however, after this time, a decrease in enzyme inhibition was observable (∼20% over 120 min). For compound **52**, no time dependence was observed, even after a 120-min incubation. Return of enzyme activity was observed for compounds **45**−**53** after dialysis when the enzyme was incubated with an inhibitor concentration of $>100K_i$, demonstrating that the compounds are reversible inhibitors. We were also interested in the compounds that had a nonbasic side chain at R^1. Three of the six library members that showed inhibition at 333 nM and that had a proton at R^1 were synthesized, purified, and found to be false positives. Thus, the other three library members with a proton at R^1 that showed inhibition in the library screen were not pursued. The observed enzyme inhibition for these library members derived from **5a** must be due to a minor impurity that might have resulted from

Table 4. Synthesized and Purified Mercaptomethyl Ketone Inhibitors of Cathepsin B



	R^2	R^1	$R^{1'}$	K_i (nM)
45	$-CH_2Ph$	$-(CH_2)_3NHC(NH)NH_2$	$-CH_2CH_2$-4-Pyr	13 ± 8^a
46	$-CH_2(C_6H_4)$-*p*-OH	$-(CH_2)_3NHC(NH)NH_2$	$-CH_2CH_2$-4-Pyr	110 ± 10^a
47	$-CH_2(C_6H_4)$-*p*-OH	$-(CH_2)_3NHC(NH)NH_2$	$-(C_6H_2)$-2,4,5-Cl	50 ± 20^a
48	$-CH_2CH(CH_3)_2$	$-(CH_2)_3NHC(NH)NH_2$	$-CH_2CH_2CH_2Ph$	140 ± 30^a
49	$-CH_2CH(CH_3)_2$	$-(CH_2)_4NH_2$	$-CH_2CH_2CH_2Ph$	130 ± 20^a
50	$-CH_2CH(CH_3)_2$	$-(CH_2)_4NH_2$	$-CH_2Ph$	111 ± 8^a
51	$-CH_2CH(CH_3)_2$	$-(CH_2)_4NH_2$	$-CH_2CH_2$-4-Pyr	50 ± 30^a
52	$-CH_2Ph$	$-(CH_2)_4NH_2$	$-CH_2CO_2H$	4.7 ± 0.7^b
53	$-CH_2(C_6H_4)$-*p*-OH	$-(CH_2)_4NH_2$	$-CH_2CO_2H$	2.0 ± 0.3^b

[a] K_i determined from a Dixon plot. [b] K_i determined using linear regression analysis method of Williams and Morrison.[27]

the decomposition of **5a** during the loading of this chloromethyl ketone onto the solid support.

Judging from the K_i values of **48** (140 nM) and **49** (130 nM), there is no significant difference in inhibition due to the arginine or lysine side chain with these compounds. The 8-fold difference in inhibition between **45** and **46**, 13 and 110 nM, respectively, indicates that the phenylalanine side chain is preferred over the tyrosine side chain at R^2 when R^1 is the arginine side chain. As represented by compounds **48**−**51**, which incorporate a leucine side chain at R^2, the introduction of aliphatic groups at R^2 can also provide effective inhibition. Collectively, the compounds in Table 4 indicate that a variety of functionalities can be accommodated at the $R^{1'}$ position. However, the most potent compounds, **52** (4.7 nM) and **53** (2.0 nM), contain a carboxylic group at this site that can hydrogen bond or form an ionic interaction with the enzyme.

Conclusions

A highly general solid-phase method was developed to incorporate diverse functionality at every position in mercaptomethyl ketone inhibitors of cysteine proteases. A library of 2016 diverse mercaptomethyl ketones was synthesized in high yields and purity. Screening this library against cathepsin B resulted in the identification of new, potent nanomolar inhibitors. This library should be a rich source of potent inhibitors against many other cysteine proteases because of the diverse functionality incorporated.

Experimental Section

General Methods. Unless otherwise noted, all reagents were obtained from commercial suppliers and used without purification. THF was distilled under N_2 from sodium/benzophenone, and *i*-Pr_2EtN and CH_2Cl_2 were distilled over CaH_2 immediately prior to use. ArgoGel-OH resin was purchased from Argonaut Technologies (San Carlos, CA). *N*-Fmoc amino acids, *N*-hydroxybenzotriazole (HOBt), and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) were purchased from Novabiochem (San Diego, CA).

Reaction progress was monitored using thin-layer chromatography on Merck 60 F_{254} 0.25-μm silica plates. Unless otherwise specified, extracts were dried over $MgSO_4$, and solvents were removed with a rotary evaporator at aspirator pressure. Flash chromatography was carried out with Merck 60 230−400 mesh silica gel according to the procedure described by Still.[28] Reverse-phase HPLC purification was conducted with an Agilent 1100 series instrument. Infrared spectra were recorded with a Nicolet Avatar 360 Fourier transform spectrometer as thin films on NaCl plates or as solids. Liquid chromatography−mass spectrometry data were obtained using a Hewlett-Packard 1100 series liquid chromatograph instrument and mass-selective detector. ^1H and ^{13}C NMR spectra were obtained with Bruker AMX-300 and DRX-500 spectrometers. Unless otherwise specified, all spectra were obtained in $CDCl_3$, and chemical shifts are reported in parts per million (ppm) relative to internal $CHCl_3$. Coupling constants are reported in Hertz. Elemental analyses and high-resolution mass spectrometry analysis were performed by the University of California at Berkeley Micro Analysis and Mass Spectrometry Facilities.

General Method for Chloromethyl Ketone Synthesis of *N*-α-Fmoc-Protected Amino Acids. Chloromethyl ketones **5a**−**d** were synthesized according to the literature procedure.[16] Specifically, a 0.2 M THF solution of the *N*-α-Fmoc-protected amino acid was stirred in a dry ice/acetonitrile bath at a temperature of approximately −25 °C. To this solution were added 1.4 equiv of *N*-methylmorpholine and then 1.3 equiv of isobutyl chloroformate. After the addition of the latter compound, a white solid formed. The reaction mixture was stirred vigorously at −25 °C. After 1 h, the reaction mixture was filtered, and the solid was rinsed with 3−4 mL of ice-cold THF. The reaction mixture was stirred at −25 °C until filtration resulted in a clear, colorless solution. Diazomethane was introduced in situ, according to a literature procedure,[29] by using 3.1 equiv of *p*-toluenesulfonylmethylnitrosamide (Diazald) while the flask was maintained at −25 °C. To generate diazomethane in situ, a 0.7 M solution of Diazald in absolute ethanol was added in small aliquots of 1.5 M KOH until the Diazald solution became white. Diazomethane gas was produced and transferred by cannula (two fire-polished pipets connected by rubber tubing) under positive N_2 flow into a stirred solution of the mixed anhydride. After the addition of diazomethane, the solution was stirred at −25 °C for 0.5 h and then at room temperature for 0.5 h. In an ice/water bath, a 1:1 solution of concentrated hydrochloric acid and glacial acetic acid was added dropwise to the solution until the evolution of N_2 was no longer observed. The solution was concentrated and then diluted with ethyl acetate and transferred to a separatory funnel. The solution was washed with water and neutralized to pH 7 with saturated potassium carbonate. The organic layer was dried, and the solvent was removed under reduced pressure. To obtain chloromethyl ketones **5e**−**h**, the workup procedure was modified to prevent cleavage of acid-labile protecting groups. For these compounds, the same procedure was followed except that, after the addition of a 1:1 solution of concentrated hydrochloric acid and glacial acetic acid, the solution was diluted with ethyl acetate and transferred directly to a separatory funnel. For all chloromethyl ketones, carbamate amide bond rotamers (less than 15%) were observed. Heating to 50 °C resulted in coalescence. Only the major rotamers are reported.

Chloromethyl Ketone Derivative of *N*-α-Fmoc-L-glycine (5a). Compound **5a** (9.2 g, 28 mmol, 69%) was prepared from 12.0 g (40 mmol) of *N*-α-Fmoc-L-glycine (**4a**) and purified by recrystallization from ethyl acetate to obtain a white solid, mp 137.1−137.7 °C. IR: 1699, 1730 cm^{-1}. ^1H NMR (500 MHz): δ 4.14 (s, 2), 4.24 (t, 1, J = 7.0 Hz), 4.32 (d, 2, J = 7.0 Hz), 4.43 (d, 2, J = 5.1 Hz), 5.40 (bs, 1), 7.33−7.34 (m, 2), 7.40−7.43 (m, 2), 7.59−7.61 (m, 2), 7.77−7.78 (m, 2). ^{13}C NMR (125 MHz): δ 45.9, 47.1, 48.6, 67.2, 120.0, 125.0, 127.0, 127.7, 141.3, 143.6, 156.1, 198.5. Anal. Calcd for $C_{18}H_{16}ClNO_3$: C, 65.56; H; 4.89, N, 4.25. Found: C, 65.68; H, 4.92; N, 4.17.

Chloromethyl Ketone Derivative of *N*-α-Fmoc-L-alanine (5b). Compound **5b** (9.5 g, 28 mmol, 89%) was

prepared from 10.0 g (31 mmol) of *N*-α-Fmoc-L-alanine (**4b**) and purified by recrystallization from ethyl acetate as a white solid, mp 135.4−136.2 °C. IR: 1682, 1743 cm^{-1}. ^1H NMR (500 MHz): δ 1.39 (d, 3, $J = 7.1$ Hz), 4.19−4.22 (m, 3), 4.39−4.50 (m, 2), 5.48 (d, 2, $J = 6.8$ Hz), 7.32 (t, 2, $J = 7.4$ Hz), 7.41 (t, 2, $J = 7.4$ Hz), 7.59 (t, 2, $J = 6.8$ Hz), 7.77 (d, 2, $J = 7.5$ Hz). ^{13}C NMR (125 MHz): δ 17.4, 46.1, 47.2, 53.5, 66.9, 120.0, 124.9, 125.0, 127.1, 127.8, 141.3, 143.6, 155.7, 201.6. Anal. Calcd for $C_{18}H_{16}ClNO_3$: C, 66.38; H; 5.28, N, 4.07. Found: C, 66.42; H, 5.49; N, 4.12.

Chloromethyl Ketone Derivative of *N*-α-Fmoc-L-leucine (5c). Compound **5c** (11.9 g, 31 mmol, 73%) was prepared from 15.0 g (42 mmol) of *N*-α-Fmoc-L-leucine (**4c**) and purified by recrystallization from ethyl acetate as a white solid, mp 134.7−135.3 °C. IR: 1704, 1739 cm^{-1}. ^1H NMR (500 MHz): δ 0.95 (d, 3, $J = 6.5$ Hz), 0.97 (d, 3, $J = 6.5$ Hz), 1.40−1.46 (m, 1), 1.58−1.63 (m, 1), 1.65−1.73 (m, 1), 4.17−4.25 (m, 3), 4.42−4.51 (m, 2), 4.57−4.61 (m, 1), 5.27 (d, 1, $J = 8.2$ Hz), 7.31 (t, 2, $J = 7.4$ Hz), 7.41 (t, 2, $J = 7.3$ Hz), 7.60 (d, 2, $J = 7.2$ Hz), 7.77 (t, 2, $J = 7.5$ Hz). ^{13}C NMR (125 MHz): δ 21.4, 23.2, 24.8, 40.2, 46.6 47.2, 56.2, 66.8, 120.0, 124.9, 125.0, 127.0, 127.1, 127.7, 127.8, 141.3, 143.6, 156.1, 202.0. Anal. Calcd for $C_{22}H_{24}ClNO_3$: C, 68.48; H, 6.27; N, 3.63. Found: C, 68.68; H, 6.50; N, 3.71.

Chloromethyl Ketone Derivative of *N*-α-Fmoc-L-phenylalanine (5d). Compound **5d** (7.4 g, 18 mmol, 68%) was prepared from 10.0 g (26 mmol) of *N*-α-Fmoc-L-phenylalanine (**4d**) and purified by recrystallization from ethyl acetate as a white solid, mp 145.3−146.5 °C. IR: 1692, 1738 cm^{-1}. ^1H NMR (500 MHz): δ 3.01 (dd, 1, $J = 7.1$, 13.9 Hz), 3.12 (dd, 1, $J = 6.7$, 13.9 Hz), 3.96 (d, 1, $J = 16.2$ Hz), 4.12 (d, 1, $J = 16.2$ Hz), 4.18 (t, 1, $J = 6.7$ Hz), 4.44 (d, 2, $J = 6.7$ Hz), 4.74 (ap q, 1, $J = 7.2$ Hz), 5.37 (d, 1, $J = 7.5$ Hz), 7.14 (d, 2, $J = 7.1$ Hz), 7.28−7.33 (m, 5), 7.40−7.43 (m, 2), 7.53−7.57 (m, 2), 7.78 (d, 2, $J = 7.5$ Hz). ^{13}C NMR (125 MHz): δ 37.5, 47.2, 47.3, 58.7, 66.9, 120.0, 125.0, 125.0, 127.1, 127.4, 127.8, 127.8, 128.9, 129.1, 135.4, 141.3, 143.5, 143.6, 155.7, 200.9. Anal. Calcd for $C_{25}H_{22}ClNO_3$: C, 71.51; H, 5.28; N, 3.34. Found: C, 71.63; H, 5.26; N, 3.23.

Chloromethyl Ketone Derivative of *N*-α-Fmoc-N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl-L-arginine (5e). Compound **5e** (6.8 g, 10 mmol, 65%) was prepared from 10.0 g (15 mmol) of *N*-α-Fmoc-N^G-2, 2, 4, 6, 7-pentamethyldihydrobenzofuran-5-sulfonyl-L-arginine (**4e**) and purified by flash chromatography (67:33:0 to 0:0:100 hexane/ethyl acetate/dichloromethane) as a white solid, mp decomposed at >100 °C. IR: 1712, 1720 cm^{-1}. ^1H NMR (500 MHz, CD_3OD): δ 1.38−1.50 (m, 9), 1.74−1.81 (m, 1), 2.05 (s, 3), 2.50 (s, 3), 2.56 (s, 3), 2.94 (s, 2), 3.10−3.25 (m, 2), 4.16−4.29 (m, 4), 4.00−4.43 (m, 1), 4.47−4.51 (m, 1), 7.28 (t, 2, $J = 7.4$ Hz), 7.34−7.37 (m, 2), 7.62 (t, 2, $J = 6.7$ Hz), 7.76 (d, 2, $J = 7.6$ Hz). ^{13}C NMR (125 MHz, $(CD_3)_2SO$): δ 12.7, 14.5, 18.0, 19.4, 21.1, 27.1, 28.7, 42.9, 47.2, 48.0, 58.3, 60.2, 65.9, 86.7, 116.7, 120.5, 124.7, 125.5, 125.6, 127.4, 128.0, 131.8, 134.6, 137.7, 141.2, 144.1, 156.5, 157.9, 201.6. Anal. Calcd for $C_{35}H_{41}ClN_4O_6S$: C, 61.71; H, 6.07; N, 8.22. Found: C, 61.79; H, 6.30; N, 8.12.

Chloromethyl Ketone Derivative of *N*-α-Fmoc-*N*-ϵ-*tert*-butyl-L-lysine (5f). Compound **5f** (12.5 g, 25 mmol, 78%) was prepared from 15.0 g (32 mmol) of *N*-α-Fmoc-*N*-ϵ-*tert*-butyl-L-lysine (**4f**) and purified by recrystallization from ethyl acetate as a white solid, mp 93.6−94.7 °C. IR: 1698, 1715, 1737 cm^{-1}. ^1H NMR (500 MHz): δ 1.26−1.55 (m, 13), −1.66−1.67 (m, 1), 1.86−1.89 (m, 1), 3.10−3.12 (m, 2), 4.17−4.24 (m, 3), 4.38−4.42 (m, 1), 4.48−4.65 (m, 3), 5.50−5.60 (m, 1), 7.32 (t, 2, $J = 7.4$ Hz), 7.41 (t, 2, $J = 7.5$ Hz), 7.59 (d, 2, $J = 6.7$ Hz), 7.77 (d, 2, $J = 7.5$ Hz). ^{13}C NMR (125 MHz): δ 22.1, 28.4, 29.7, 30.6, 39.6, 46.4, 47.2, 57.7, 66.9, 79.3, 120.0, 124.9, 125.0, 127.1, 127.1, 127.7, 127.7, 141.3, 143.6, 143.6, 143.5, 156.1, 159.2, 201.4. Anal. Calcd for $C_{27}H_{33}ClN_2O_5$: C, 64.73; H, 6.64; N, 5.59. Found: C, 65.10; H, 6.50; N, 5.27.

Chloromethyl Ketone Derivative of *N*-α-Fmoc-L-aspartic acid-*β*-*tert*-butyl ester (5g). Compound **5g** (10.9 g, 25 mmol, 67%) was prepared from 15.0 g (36 mmol) of *N*-α-Fmoc-L-aspartic acid-*β*-*tert*-butyl ester (**4g**) and purified by recrystallization from ethyl acetate as a white solid, mp 119.6−120.8 °C. IR: 1715, 1736 cm^{-1}. ^1H NMR (500 MHz): δ 2.73 (dd, 1, $J = 7.9$, 17.2 Hz), 2.96 (dd, 1, $J = 4.6$, 17.2 Hz), 4.07−4.34 (3, m), 4.43−4.46 (1, m), 5.81 (d, 1, $J = 8.7$ Hz), 7.33−7.35 (2, m), 7.42 (t, 2, $J = 7.5$ Hz), 7.58−7.60 (2, m), 7.78 (d, 2, 7.6). ^{13}C NMR (125 MHz): δ 28.0, 36.8, 47.00, 47.2, 54.9, 66.9, 82.3, 120.0, 120.0, 124.8, 125.0, 127.0, 127.1, 127.8, 127.9, 141.3, 141.4, 143.4, 143.6, 155.9, 170.4, 200.0. Anal. Calcd for $C_{24}H_{26}ClNO_5$: C, 64.93; H, 5.90; N, 3.16. Found: C, 64.90; H, 6.10; N, 3.21.

Chloromethyl Ketone Derivative of *N*-α-Fmoc-O-*tert*-butyl-L-tyrosine (5h). Compound **5h** (5.1 g, 10 mmol, 68%) was prepared from 7.0 g (15 mmol) of *N*-α-Fmoc-O-*tert*-butyl-L-tyrosine (**4h**) and purified by recrystallization from hexane/ethyl acetate as a white solid, mp decomposed at >95 °C. IR: 1687, 1742 cm^{-1}. ^1H NMR (500 MHz): δ 1.32 (s, 9), 3.00−3.07 (m, 2), 3.86 (d, 1, $J = 16.3$ Hz), 4.07 (d, 1, $J = 16.$ Hz), 4.18 (t, 1, $J = 6.4$ Hz), 4.39−4.44 (m, 2), 4.69 (dd, 1, $J = 7.2$, 14.4 Hz), 5.33 (d, 1, $J = 7.5$ Hz), 6.93 (d, 2, $J = 8.2$ Hz), 7.02 (d, 2, $J = 8.2$ Hz), 7.32 (t, 2, $J = 7.4$ Hz), 7.41 (t, 2, $J = 7.4$ Hz), 7.56 (t, 2, $J = 7.1$ Hz), 7.77 (d, 2). ^{13}C NMR (125 MHz): δ 28.8, 37.1, 47.1, 47.5, 58.1, 66.9, 78.6, 120.0, 124.6, 125.0, 127.1, 127.8, 129.6, 129.9, 141.3, 143.5, 143.6, 154.7, 155.7, 201.1. Anal. Calcd for $C_{29}H_{30}ClNO_4$: C, 70.79; H, 6.15; N, 2.85. Found: C, 70.87; H, 6.34; N, 2.88.

General Methods for Solid-Phase Synthesis. For reactions involving resin, all solvents were distilled according to the methods described in the General Methods section above. Unless otherwise noted, reactions were conducted in 12-mL polypropylene cartridges equipped with 70-mm PE frits (obtained from Applied Separations, Allentown, PA) and Teflon stopcocks. Syringe plungers from 10-mL disposable syringes were used as stoppers for the cartridges. Reactions were gently rocked on an orbital shaker table during the solid-phase reactions. The resin was washed with the indicated solvent for a duration of 2−5 min unless otherwise stated. The carbazate linker was prepared according to the method previously described by Lee et al.[1]

Loading of the Chloromethyl Ketone Derivative 5a−h. A 0.2 M solution of chloromethyl ketone **5a**−**h** (4 equiv) in THF was added to the carbazate-derivatized resin presolvated with THF. The capped vessel was placed in a 50 °C oil bath and heated for various time periods depending on the chloromethyl ketone. Compound **5a** was heated for 10 min, compound **5b** was heated for 1 h, and compounds **5c**−**h** were heated for 3 h. The resin was transferred as a slurry into a polypropylene cartridge and subsequently rinsed with five portions of THF. The support-bound ketones were used immediately in the chloride displacement step.

General Method for Cleaving Compounds from the Solid Support. To 0.2 g of derivatized resin was added 2 mL of a solution of 95:5 trifluoroacetic acid/water (purged with N_2 for 5 min) in a N_2 glovebag. The resin was gently rocked for 1 h, and after removal of the solution, the resin was washed with five portions of THF, and the washings were combined and concentrated. Toluene was added to form an azeotrope with the cleavage solution.

General Procedure for the Synthesis of Compounds 10a−h. Loading of the chloromethyl ketone onto the solid support was performed as previously described. To the support-bound chloromethyl ketone, a 0.5 M solution of thiophenol (10 equiv) and *i*-Pr_2EtN (12 equiv) in DMF was added. The cartridge was placed on the shaker table overnight. After removal of the thiol solution, the resin was rinsed with five portions of DMF. To remove the Fmoc group, a 20% solution of piperidine in DMF was added to the cartridge, and the reaction mixture was shaken for 1 min. The solution was removed, and the resin was washed with DMF. The deprotection sequence was then repeated. Following removal of the solution, the resin was washed exhaustively with DMF until a bromophenol blue test of a THF wash indicated that no base was present. The resin was rinsed with two portions of DMF. A 0.2 M solution of CbzPheOH (5 equiv), *i*-Pr_2EtN (10 equiv), PyBOP (5 equiv), and HOBt (5 equiv) in DMF was prepared and added to the cartridge. The cartridge was shaken for 4 h. After removal of the acylation solution, the resin was rinsed with one portion of DMF, and the acylation reaction was repeated. The resin was then rinsed with five portions of DMF and five portions of THF. The resin was dried overnight in vacuo and then subjected to the cleavage conditions previously described. The resulting product was analyzed by ^{1}H NMR spectroscopy, and the yield was determined by calibration with *p*-xylene as the internal standard.

Procedure for the Synthesis and Evaluation of the Library. Library synthesis was conducted using the FlexChem Synthesis/Filtration Blocks and Covers system. Specifically, 96-well FlexChem reactor blocks and FlexChem natural rubber gaskets were used. All washes and piperidine solutions were added to the wells of the Robbins block using a trigger dispenser with an eight-needle stream splitter and removed using the FlexChem solvent removal manifold. This equipment was purchased from Robbins Scientific Co. (Sunnyvalle, CA). Solutions were added using multichannel pipets. A footprint for the library is shown in Figure 6.

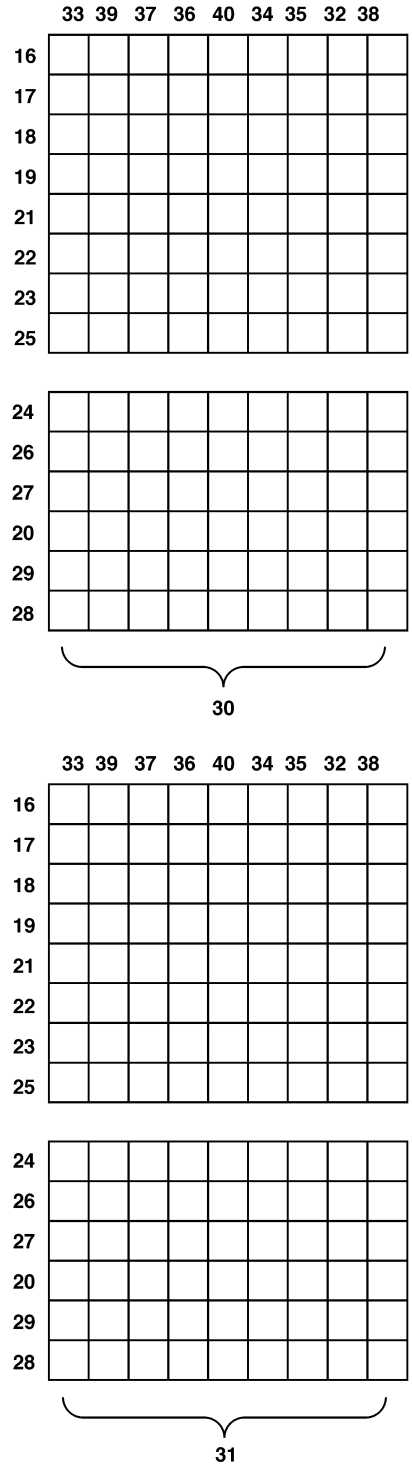


Figure 6. Library footprint for each chloromethyl ketone.

Chloromethyl ketones **5a**−**h** were loaded onto 2.5 mmol (5.0 g) of solid support as previously described. The chloromethyl ketone of glycine (**5a**) was loaded onto 5.0 mmol (10.0 g) of solid support as previously described. The resin was transferred as a slurry into nine polypropylene cartridges and subsequently was rinsed with five portions of THF. To the support-bound chloromethyl ketone (0.28 mmol, 0.56 g of resin) in separate cartridges was added a 0.5 M solution of thiols **32**−**36** and **38**−**40** (10 equiv, 2.7 mmol) and *i*-Pr_2EtN (12 equiv, 3.3 mmol, 0.58 mL) in DMF. For thiol **37**, a 0.5 M solution of thiol **37** (10 equiv, 2.7

mmol) and *i*-Pr_2EtN (24 equiv, 1.16 mL) in DMF was added. The cartridges were placed on the shaker table overnight. The resin was rinsed with three portions of DMF.

The resin from each cartridge was transferred into columns of four Robbins blocks (Figure 6) [28 wells, approximately 9.8 μmol (~20 mg of resin) per well, except for the resin initially loaded with **5a,** for which approximately 19.6 μmol (~40 mg of resin) were used per well]. Transfer of the resin was performed using an isopycnic slurry of 5.9 mL of DMF and 1.1 mL of 1,2-dichloroethane. The resin was washed with three portions of DMF. To remove the Fmoc group, 0.3 mL of a 20% solution of piperidine in DMF was added to the wells of the Robbins blocks for at least 1 min. The solution was removed, and the resin was washed with DMF. The 1-min deprotection sequence was then repeated. The resin was washed with two portions of DMF and three portions of THF. A bromophenol blue test of the third THF wash indicated that no base was present. A 0.2 M solution of 14 Fmoc amino acids (**16**−**29**) (5 equiv, 1.5 mmol), *i*-Pr_2-EtN (10 equiv, 3.1 mmol, 0.53 mL), PyBOP (5 equiv, 1.5 mmol, 0.80 g), and HOBt (5 equiv, 1.5 mmol, 0.21 g) in DMF was prepared and added to the rows (18 wells) of the Robbins blocks (Figure 6). The blocks were shaken for 4 h, and the acylating solution was then removed. The resin was rinsed with one portion of DMF, a second acylation solution was added, and the blocks were shaken overnight. The resin was rinsed with three portions of DMF, and then the Fmoc deprotection procedure was repeated. To the wells in two of the Robbins block was added a 0.2 M solution of acetic anhydride (**30**) (5 equiv, 12.3 mmol, 1.2 mL) and *i*-Pr_2EtN (10 equiv, 24.6 mmol, 4.3 mL) in DMF. To the other two blocks was added a 0.2 M solution of **31** (5 equiv, 12.3 mmol, 3.1 g) and *i*-Pr_2EtN (10 equiv, 24.6 mmol, 4.3 mL) in DMF. The blocks were gently shaken overnight. The resin was rinsed with two portions of DMF and three portions of THF. Following the last THF wash, each block was left on the vacuum manifold for at least 5 min.

For the cleavage step, the top and bottom gaskets were covered with Teflon tape. To each well was added 400 μL of a 95:5 trifluoroacetic acid/water solution (purged with N_2 for 5 min) in a N_2 glovebag. The resin was gently rocked for 1 h, and the blocks were placed in a −78 °C freezer for at least 2 h. In a N_2 glovebag, the frozen blocks were opened and placed on top of a 2-mL 96-well square plate (Beckman Instruments, Fullerton, CA). After the cleavage solution had drained into the 96-well square plate, the resin was washed with one portion of THF, and the solution was expelled from each well of the Robbins block into the 96-well square plate. Toluene was added to each well to form an azeotrope, and the solutions in the square plates were concentrated using a Savant SC210A Speedvac Plus concentrator (Holbrook, NY).

Of the 2016 library members, 134 (6.6%) were randomly selected and evaluated for purity using an LCMS with a mobile phase of acetonitrile/water with 0.1% TFA on a C18 column. The library members were evaluated with the following gradient: 5% acetonitrile to 95% acetonitrile over 15 min, 95% acetonitrile for 2 min, 95% acetonitrile to 5% over 2 min, and then 3 min at 5% (0.4 mL/min). Of the 134 library members analyzed, 119 (88%) had chemical purities of >80% as determined by UV detection at 220 nm. For library members with the arginine side chain, the UV absorbance for the cleaved Pbf group was excluded for determining chemical purity. Twenty-one library members were randomly selected and analyzed by ^{1}H NMR spectroscopywith an internal standard of hexamethyldisiloxane. The average yield was 6.5 μmol (~65% for compounds derived from **5b**−**h** and ~32% for compounds derived from **5a**).

Library Assay against Cathespin B. A fluorometric high-throughput assay for activity against cathepsin B was performed in 96-well microtiter plates. The assays were performed in Dynatech Microfluor fluorescence microtiter plates (opaque white plates), and readings were taken on a Molecular Devices Spectra Max Gemini XS instrument. The excitation wavelength was 355 nm, and the emission wavelength was 450 nm. The concentration of the peptide substrate Cbz-Phe-Arg-AMC (Bachem California, Torrance, CA) was 10 μM, and the cathespin B concentration was 1.4 nM. The assay buffer consisted of a 0.25 M solution of pH 5.5 sodium acetate buffer with 1 mM of DTT, 2 mM of EDTA, and 0.1% Brij 35. The relative fluorescent unit (RFU) readings were taken at seven time points within the linear region of substrate cleavage. The percent activity of the enzyme was determined by comparing the change in RFU for each well against the average change in RFU for eight control wells without inhibitor. Another eight wells with substrate, buffer, and DMSO revealed that there was no background fluorescence for the buffer or uncleaved substrate. All library members were screened at 1 μM, and the 110 library members that caused greater than 50% inhibition were assayed at 333 nM. All library members were assayed in duplicate.

Sample Procedure: In each well was placed 30 μL of enzyme solution, 150 μL of buffer solution, and 10 μL of library member solution in DMSO. The library members were placed into columns 2−10, with row 11 used for the control wells and row 12 used for buffer and substrate. Following a 5-min enzyme and inhibitor incubation period, 10 μL of substrate solution was added to each well. The plate was immediately placed into the plate reader and analyzed. The slope reading for each well was transferred from the spectrophotometer to an Excel spreadsheet, and the percent activity for each library member was determined from the average of the duplicate percent activity values.

Synthesis and Purification of Compounds 45−53. Compounds **45**−**53** were synthesized in cartridges in a manner analogous to the library synthesis. These compounds were purified by reverse-phase HPLC using a preparatory column.

45. ^{1}H NMR (300 MHz, CD_3OD): δ 1.50−1.54 (m, 3), 1.88−1.90 (m, 1), 2.82−3.36 (m, 10), 4.35−4.40 (m, 1), 4.54−4.57 (m, 1), 4.99−5.05 (m, 2), 7.17−7.30 (m, 10), 7.93 (d, 2, J = 6.0 Hz), 8.70 (d, 2, J = 6.3 Hz). HRMS (FAB+) m/z: 591.2752 (MH^+ $C_{31}H_{39}N_6O_4S$ requires 591.2754).

46. ^{1}H NMR (300 MHz, CD_3OD): δ 1.52−1.60 (m, 3), 1.88−1.92 (m, 1), 2.81−2.85 (m, 3), 2.97 (dd, 1, J = 7.4, 13.7 Hz), 3.11−3.19 (m, 4), 3.24−3.27 (m, 2), 4.31 (t, 1, J = 7.7 Hz), 4.52−4.55 (m, 1), 5.03−5.06 (m, 2), 6.68 (d, 2,

$J = 8.4$ Hz), 7.05 (d, 2, $J = 8.4$ Hz), 7.28−7.31 (m, 5), 7.96 (d, 2, $J = 6.5$ Hz), 8.70 (d, 2, $J = 6.4$ Hz). HRMS (FAB+) *m/z*: 607.2712 (MH^+ $C_{31}H_{39}N_6O_5S$ requires 607.2703).

47. ^{1}H NMR (300 MHz, CD_3OD): δ 1.45−1.60 (m, 3), 1.85−1.91 (m, 1), 2.85 (dd, 1, $J = 7.7$, 13.6 Hz), 2.96 (dd, 1, $J = 7.6$, 13.7 Hz), 3.10 (t, 2, $J = 6.8$ Hz), 3.73 (d, 1, $J = 16.8$ Hz), 3.82 (d, 1, $J = 16.8$ Hz), 4.31 (t, 1, $J = 7.7$ Hz), 4.39−4.43 (m, 1), 5.02−5.04 (m, 2), 6.68 (d, 2, $J = 8.5$ Hz), 7.07 (d, 2, $J = 8.4$ Hz), 7.24−7.34 (m, 6), 7.56 (s, 1). HRMS (FAB+) *m/z*: 680.1250 (MH^+ $C_{30}H_{33}Cl_3N_5O_5S$ requires 680.1268).

48. ^{1}H NMR (500 MHz, CD_3OD): δ 0.93 (d, 3, $J = 6.6$ Hz), 0.96 (d, 3, $J = 6.6$ Hz), 1.50−1.74 (m, 6), 1.85 (quintet, 2, $J = 7.4$ Hz), 1.92−1.98 (m, 1), 2.48 (t, 2, $J = 7.2$ Hz), 2.69 (t, 2, $J = 7.5$ Hz), 3.14−3.18 (m, 2), 3.31−3.41 (m, 2), 4.15 (dd, 1, $J = 5.7$, 9.5 Hz), 4.71−4.73 (m, 1), 5.04−5.12 (m, 2), 7.13−7.34 (m, 10). HRMS (FAB+) *m/z*: 570.3092 (MH^+ $C_{30}H_{44}N_5O_4S$ requires 570.3109).

49. ^{1}H NMR (500 MHz, CD_3OD): δ 0.93 (d, 3, $J = 6.6$ Hz), 0.95 (d, 3, $J = 6.6$ Hz), 1.41−1.46 (m, 2), 1.58−1.73 (m, 6), 1.84 (quintet, 2, $J = 7.3$ Hz), 1.90−1.94 (m, 1), 2.47 (t, 2, $J = 7.2$ Hz), 2.67 (t, 2, $J = 7.5$ Hz), 2.87 (t, 2, $J = 7.5$ Hz), 3.36 (d, 1, $J = 14.7$ Hz), 3.40 (d, 1, $J = 14.7$ Hz), 4.16 (dd, 1, $J = 5.8$, 9.8 Hz), 4.71 (dd, 1, $J = 4.0$, 10.0 Hz), 5.08 (m, 2), 7.14−7.34 (m, 10). HRMS (FAB+) *m/z*: 542.3037 (MH^+ $C_{30}H_{44}N_3O_4S$ requires 542.3053).

50. ^{1}H NMR (300 MHz, CD_3OD): δ 0.92 (d, 3, $J = 6.6$ Hz), 0.95 (d, 3, $J = 6.6$ Hz), 1.38−1.73 (m, 8), 1.83−1.90 (m, 1), 2.86 (t, 2, $J = 7.5$ Hz), 3.19−3.27 (m, 2), 3.67 (s, 2), 4.13 (dd, 1, $J = 5.7$, 9.5 Hz), 4.67 (dd, 1, $J = 4.1$, 9.9 Hz), 5.07 (s, 2), 7.22−7.34 (m, 10). HRMS (FAB+) *m/z*: 514.2748 (MH^+ $C_{28}H_{40}N_3O_4S$ requires 514.2740).

51. ^{1}H NMR (500 MHz, CD_3CN): δ 0.89 (d, 3, $J = 6.5$ Hz), 0.92 (d, 3, $J = 6.6$ Hz), 1.35−1.40 (m, 2), 1.48−1.71 (m, 6), 1.78−1.81 (m, 1), 2.85 (t, 2, $J = 7.1$ Hz), 2.90 (bs, 2), 3.11 (t, 2, $J = 7.0$ Hz), 3.41 (d, 1, $J = 15.5$ Hz), 3.48 (d, 1, $J = 15.4$ Hz), 4.08−4.12 (m, 2), 4.44−4.49 (m, 1), 5.04 (d, 1, $J = 12.5$ Hz), 5.08 (d, 1, $J = 12.6$ Hz), 6.32 (d, 1, $J = 7.3$ Hz), 7.28−7.42 (m, 9), 7.81 (d, 2, $J = 5.7$ Hz), 8.67 (d, 2, $J = 5.3$ Hz). HRMS (FAB+) *m/z*: 529.2849 (MH^+ $C_{28}H_{41}N_4O_4S$ requires 529.2848).

52. ^{1}H NMR (300 MHz, CD_3OD): δ 1.27−1.44 (m, 2), 1.51−1.70 (m, 3), 1.82−1.93 (m, 1), 2.83−2.95 (m, 3), 3.07 (dd, 1, $J = 6.9$, 13.7 Hz), 3.16 (s, 2), 4.37 (t, 1, $J = 7.6$ Hz), 4.58 (dd, 1, $J = 4.5$, 9.5 Hz), 5.00−5.07 (m, 2), 7.21−7.32 (m, 10). OC−CH_2−S exchanged. HRMS (FAB+) *m/z*: 516.2168 (MH^+ $C_{26}H_{34}N_3O_6S$ requires 516.2176).

53. ^{1}H NMR (300 MHz, CD_3OD): δ 1.28−1.38 (m, 2), 1.50−1.64 (m, 3), 1.81−1.92 (m, 1), 2.78−2.88 (m, 3), 2.97 (dd, 1, $J = 7.0$, 13.7 Hz), 3.22 (s, 2), 4.29 (t, 1, $J = 7.5$ Hz), 4.55 (dd, 1, $J = 4.4$, 9.4 Hz), 5.01 (d, 1, $J = 12.6$ Hz), 5.06 (d, 1, $J = 12.6$ Hz), 6.70 (d, 2, $J = 8.5$ Hz), 7.05 (d, 2, $J = 8.4$ Hz), 7.27−7.35 (m, 5). OC−CH_2−S exchanged HRMS (FAB+) *m/z*: 532.2136 (MH^+ $C_{26}H_{34}N_3O_7S$ requires 532.2117).

Cathepsin B Assays of Compounds 45−53. The assays were performed in Dynatech Microfluor fluorescence microtiter plates (opaque black plates). Compounds **45**−**53** were assayed, as described above, to confirm the results from the library assay. Time-dependent inhibition assays of compounds **45**−**53** were conducted as described above, except that the enzyme and inhibitor incubation time varied from 5 to 120 min. For all K_i determinations, the assays were conducted at a time when equilibrium between the enzyme and inhibitor had been established, as determined by the time-dependent inhibition assays. The K_i values for compounds **45**−**51** were determined from Dixon plots in which the reciprocal of the slope vs inhibitor concentration was plotted at two substrate concentrations (10 and 30 μM). The X value where the two slopes intercept is equal to $-K_i$ for reversible inhibitors. At least four inhibitor concentrations were used with percent activity values of 30−100% at each substrate concentration for the Dixon plots. Assays were conducted in triplicate.

For potent inhibitors **52** and **53**, the data were fit by nonlinear regression analysis to the equation derived by Williams and Morrison[27]

$$v = \frac{v_o}{2E_t}\left\{\sqrt{\left[\left(K_I\left(1+\frac{S}{K_m}\right)+I_t-E_t\right)^2+4K_I\left(1+\frac{S}{K_m}\right)E_t\right]}-\left[K_I\left(1+\frac{S}{K_m}\right)+I_t-E_t\right]\right\}$$

The K_m value for the substrate was determined to be 140 μM by using a Lineweaver−Burke plot. The variables S, I_t, and E_t are the concentrations of the substrate, inhibitor, and active enzyme, respectively. The active enzyme concentration was determined by titrating the enzyme with known concentrations of irreversible E-64. The symbols v and v_o represent the velocity of substrate cleavage with and without inhibitor, respectively. Six inhibitor concentrations in triplicate were used for these assays.

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Supporting Information Available. LCMS and ^{1}H NMR data for the library. This material is available free of charge via the Internet at http:/pubs.acs.org.

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