Identification of Potent and Selective Mechanism-Based Inhibitors of the Cysteine Protease Cruzain Using Solid-Phase Parallel Synthesis

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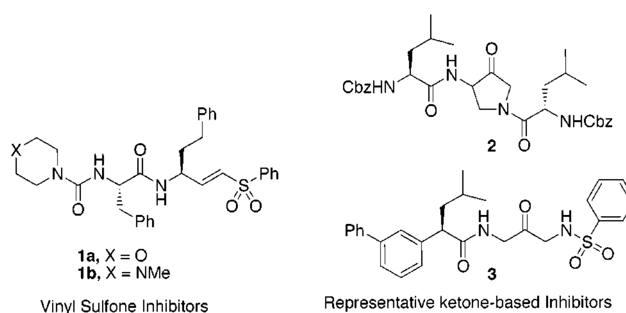
Targeted libraries of ketone-based cysteine protease inhibitors were synthesized and screened against cruzain, a cysteine protease implicated in Chagas' disease. A number of single digit nanomolar, low molecular weight inhibitors were identified and optimized for solubility and potency. Specifically, the best inhibitors identified have K_i values of 0.9−10 nM and molecular weights between 499 and 609 Da. The most effective inhibitor was also found to be greater than 1000-fold selective for cruzain relative to cathepsin B and 100-fold selective for cruzain relative to cathepsin L.

Introduction

Chagas' disease, the leading cause of heart disease and inflammation in South America, is caused by the protozoan parasite *Trypanosoma cruzi*.[1] The only currently available treatment is unsatisfactory due to significant toxicity.[2] The lack of effective treatments has stimulated efforts to identify novel drug targets against this parasite. One target that has received considerable attention is cruzain, the major cysteine protease found in *T. cruzi*. Recently, McKerrow and co-workers have shown that irreversible inhibitors of cruzain can cure parasitic infections in mouse models.[3] These results demonstrate the therapeutic promise of inhibitors of cruzain for the treatment of Chagas' disease.

Previous design of inhibitors of cruzain has exclusively focused on irreversible inhibitors such as fluoromethyl ketones and vinyl sulfones. Although animal studies have established that vinyl sulfone inhibitors (**1a** and **1b**) (Chart 1) are not toxic at therapeutic doses, the poor selectivity of these irreversible inhibitors for cruzain over human cysteine proteases[3] remains a significant concern. Highly potent and selective *reversible* inhibitors have been identified for other cysteine proteases, with the ketone carbonyl acting as one of the most promising pharmacophores.[4] In particular, Veber and co-workers have identified potent and highly selective reversible ketone-based inhibitors of cathepsin K, such as **2** and **3**, that are efficacious in the treatment of osteoperosis in animal models of this disease.[5] Herein we report the application of targeted library synthesis to identify potent, ketone-based inhibitors of cruzain (K_i ≤ 0.9−10 nM), the most promising of which shows greater than 1000-fold selectivity for cruzain over human cathepsin B and 100-fold selectivity for cruzain over human cathepsin L.

Chart 1



Results

Synthetic Strategy. We have previously reported the development of a solid-phase synthesis approach to prepare ketone-based inhibitors.[6] An allyloxycarbonyl (Alloc)-protected chloromethyl ketone, prepared in a one-pot procedure[7] from the corresponding *N*-Alloc amino acid, introduces the R_1 side chain and provides sites for further functionalization on both sides of the ketone carbonyl (Scheme 1). The chloromethyl ketone scaffold is attached to support using a hydrazine linker **4** to provide support-bound hydrazone **5**. The hydrazone not only provides the site of attachment to the solid support but also protects the carbonyl from nucleophilic attack as well as racemization at the α-stereocenter. Displacement of the chlorine with nucleophiles, such as amines and thiols, introduces functionality on one side of the ketone. Removal of the Alloc group under Pd(0)-mediated conditions, followed by acylation and cleavage from support, gives the fully functionalized ketone-based inhibitors. This synthetic strategy allows the rapid parallel synthesis of mercaptomethyl (**7**) and amidomethyl (**9**) classes of inhibitors in moderate to high yields.

Design of Inhibitors. Our initial design was based upon previously developed diazomethyl ketone[8] and vinyl sulfone[9] inhibitors. These inhibitors established effective binding interactions *N*-terminal to the site of peptide cleavage (P side) but provided little information regarding binding interactions C-terminal to the site of peptide cleavage (P′ side). We therefore chose to display diverse functionality at the P′ side[10] of the ketone carbonyl (Figure 1) while fixing the P side as *N*-Cbz Phe-Phe, which is present in a number of known potent irreversible inhibitors of cruzain.[8,9] A 192-compound library of amidomethyl ketones (**9**) was first synthesized using a diverse set of 12 amines and 16 carboxylic acids

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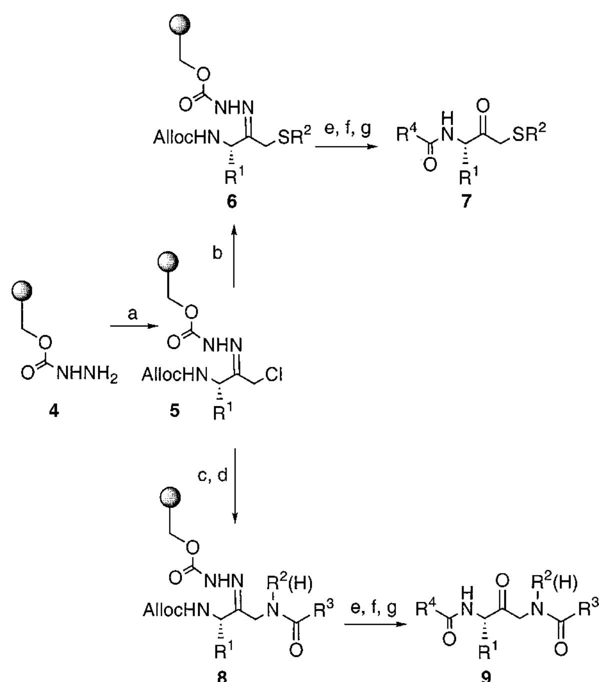
Scheme 1[a]



[a] (a) Chloromethyl ketone derivative of *N*-Alloc amino acid, THF, 4 h, rt; (b) R^2SH, *i*-Pr_2EtN, DMF; (c) NH_2R^2, DMF or (1) NaN_3, MeOH (2) $SnCl_2$, PhSH, *i*-Pr_2EtN, THF; (d) R^3CO_2H, PyBOP, HOAt, *i*-Pr_2EtN, DMF; (e) $Pd(PPh_3)_4$, CH_2Cl_2, $TMSN_3$, TBAF·$3H_2O$; (f) R^4CO_2H, PyBOP, HOAt, *i*-Pr_2EtN, DMF; (g) 1:4:15 TFA/H_2O/trifluoroethanol.

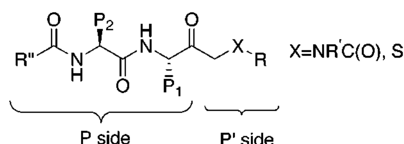


Figure 1. Standard nomenclature for protease cleavage.

(not shown). The most active amidomethyl inhibitors had IC_{50} values between 500 nM and 1 μM against cruzain.

More promising was a second library of 20 mercaptomethyl ketones (**10a**–**s**), which showed improved potency over the amidomethyl ketones with most inhibitors having K_i values below 50 nM (Table 1). Evaluation of the SAR data suggests that cruzain can accommodate large bulky substituents or long hydrophobic hydrocarbon chains in the P′ side of the inhibitor structure. The most potent compounds contained isopropyl mercaptan (**10o**), phenethyl mercaptan (**10p**), ethyl 3-mercaptopropionate (**10q**), *tert*-butyl mercaptan (**10r**), and 3-phenyl propyl mercaptan (**10s**).

To reduce the number of aromatic rings present in the inhibitor structure while concomitantly reducing the molecular weight and improving the solubility,[12] we explored replacing phenylalanine with leucine at the P_2 position, affording compounds **11a**–**d.** Both aromatic and aliphatic hydrophobic amino acids are known to be accommodated by the enzyme at this site.[13] As shown in Table 2, replacement of Phe with Leu at the P_2 position resulted in comparable or improved K_i values.

Optimization of Solubility. The Cbz inhibitors proved to be poorly soluble in aqueous solution due to the number of hydrophobic phenyl rings in the inhibitor structures. The Cbz group was therefore replaced with

Table 1. Cbz-Phe-Phe Mercaptomethyl Ketones

10a-s

inhibitor	R	% yield[a]	K_i (nM)[b]
10a	$-CH_2$(4-Cl Ph)	62	82.7 ± 6.4
10b	$-CH_2Ph$	89	44.0 ± 2.6
10c	$-CH(CH_3)CO_2Et$	67	37.0 ± 6.6
10d	$-CH_2CH_2NHCOCH_3$	37	34.3 ± 1.6
10e	$-c-(C_5H_9)$	42	34.3 ± 1.5
10f	$-CH_2(4-OCH_3$ Ph)	90	32.4 ± 2.3
10g	$-CH_2CO_2Et$	48	31.2 ± 1.5
10h	$-(CH_2)_2CH(CH_3)_2$	75	30.1 ± 3.2
10i	$-CH_2(4-C(CH_3)_3$ Ph)	97	22.0 ± 1.6
10j	$-CH_2CONHCH_3$	96	15.0 ± 0.9
10k	$-C(CH_3)_2CH_2C(CH_3)_3$	46	10.4 ± 0.6
10l	$-c-(C_6H_{11})$	60	7.5 ± 0.5
10m	$-CH(CH_3)CH_2CH_3$	63	5.8 ± 0.5
10n	$-(C(CH_3)(CH_2C(CH_3)_3)_2$	50	5.0 ± 0.7
10o	$-CH(CH_3)_2$	51	4.6 ± 0.4
10p	$-CH_2CH_2Ph$	64	4.2 ± 0.2
10q	$-(CH_2)_2CO_2Et$	82	2.9 ± 0.3
10r	$-C(CH_3)_3$	49	2.5 ± 0.2
10s	$-(CH_2)_3Ph$	63	2.0 ± 0.2

[a] Overall yield of purified product for five steps. [b] K_i values determined at four to eight concentrations in duplicate using the linear regression analysis of Williams and Morrison.[11]

Table 2. Cbz-Leu-Phe Mercaptomethyl Ketones

11a-d

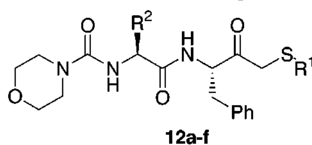
inhibitor	R	% yield[a]	K_i (nM)[b]
11a	$-(CH_2)_2CO_2Et$	44	8.0 ± 0.8
11b	$-CH(CH_3)_2$	52	7.8 ± 0.6
11c	$-C(CH_3)_3$	51	1.3 ± 0.1
11d	$-(CH_2)_3Ph$	44	1.0 ± 0.1

[a] Overall yield of purified product for five steps. [b] K_i values determined at four to eight concentrations in duplicate using the linear regression analysis of Williams and Morrison.[11]

a morpholinecarbonyl group. Bromme and co-workers demonstrated the use of this functionality as an alternative to Cbz in several vinyl sulfone inhibitors targeting cruzain.[9] Incorporation of the morpholinecarbonyl group was achieved on support via acylation with the appropriate *N*-morpholinecarbonyl amino acid prior to cleavage from resin. The *N*-morpholinecarbonyl amino acids were easily synthesized by addition of 4-morpholine carbonyl chloride to the desired α-amino acid using Schotten–Bauman conditions. With the three mercaptyl side chains that provided the highest activity, six morpholinecarbonyl dipeptide mercaptomethyl ketones were synthesized, with Phe or Leu in the P_2 position. While the previously reported morpholine-derived vinyl sulfones were very potent inhibitors of cruzain, our morpholine-derived ketones (**12a**–**f**) were poor to modest inhibitors (Table 3).

To improve the solubility of the inhibitors while still maintaining potency, we next replaced the Cbz group with a pyridinylmethylcarbonyl group. Kempf and co-workers had previously found that pyridinyl analogues of Cbz promote higher bioavailability and increased

Table 3. Replacement of Cbz with Morpholinecarbonyl



inhibitor	R[1]	R[2]	% yield[a]	K_i (nM)[b]
12a	$-C(CH_3)_3$	$-CH_2CH(CH_3)_2$	70	59.7 ± 6.4
12b	$-C(CH_3)_3$	$-CH_2Ph$	55	54.6 ± 6.3
12c	$-(CH_2)_2CO_2Et$	$-CH_2CH(CH_3)_2$	76	69.6 ± 5.6
12d	$-(CH_2)_2CO_2Et$	$-CH_2Ph$	75	59.1 ± 6.5
12e	$-(CH_2)_3Ph$	$-CH_2CH(CH_3)_2$	54	130.7 ± 13.3
12f	$-(CH_2)_3Ph$	$-CH_2Ph$	70	216.3 ± 18.9

[a] Overall yield of purified product for five steps. [b] K_i values determined at four to eight concentrations in duplicate using the linear regression analysis of Williams and Morrison.[11]

Scheme 2

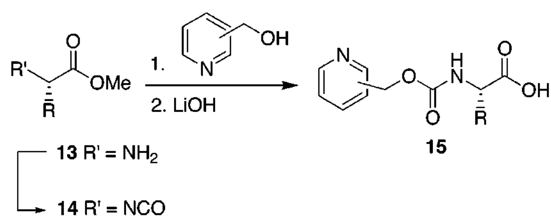
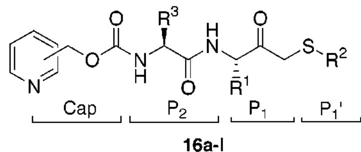


Table 4. Pyridinylmethyl Carbamate Mercaptomethyl Ketones



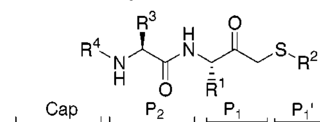
inhibitor	Cap	P[2]	P[1]	R[2]	% yield[a]	K_i (nM)[b]
16a	2-Pyr	Phe	Phe	$(CH_2)_2CO_2Et$	65	30.4 ± 2.4
16b	3 Pyr	Phe	Phe	$(CH_2)_2CO_2Et$	64	22.7 ± 2.0
16c	3 Pyr	Phe	Phe	$(CH_2)_3Ph$	50	5.5 ± 0.4
16d	3 Pyr	Leu	Phe	$(CH_2)_3Ph$	63	5.4 ± 0.2
16e	3 Pyr	Phe	hPhe	$(CH_2)_3Ph$	73	0.9 ± 0.1
16f	3 Pyr	Leu	hPhe	$(CH_2)_3Ph$	64	1.1 ± 0.1
16g	2 Pyr	Leu	Phe	$C(CH_3)_3$	52	13.8 ± 0.8
16h	4-Pyr	Leu	Phe	$C(CH_3)_3$	65	14.1 ± 0.9
16i	3-Pyr	Phe	Phe	$C(CH_3)_3$	58	4.6 ± 0.2
16j	3-Pyr	Leu	Phe	$C(CH_3)_3$	60	4.8 ± 0.2
16k	3-Pyr	Phe	hPhe	$C(CH_3)_3$	51	1.4 ± 0.1
16l	3-Pyr	Leu	hPhe	$C(CH_3)_3$	50	2.0 ± 0.1

[a] Overall yield of purified product for five steps. [b] K_i values determined at four to eight concentrations in duplicate using the linear regression analysis of Williams and Morrison.[11]

aqueous solubility for a series of HIV protease inhibitors.[14] The pyridinyl capped inhibitors were synthesized on support by acylation with the *N*-pyridinylmethyloxycarbonyl-protected α-amino acids **15**. Acids **15** were prepared by reaction of the 2-, 3-, or 4-substituted hydroxymethylpyridine with an amino acid ester isocyanate **14** followed by ester hydrolysis (Scheme 2).

The pyridinyl inhibitors (**16a–l**) showed improved solubility while maintaining the potency observed for the Cbz-protected mercaptomethyl ketones (Table 4). Incorporation of the 3-pyridinyl group typically resulted in more potent inhibitors than incorporation of the 2- and 4-substituted pyridinyl groups. For example, 3-pyridinyl inhibitor **16j** (K_i = 4.8 ± 0.2) is 3 times more potent than either the corresponding 2-pyridinyl inhibitor **16g** (K_i = 13.8 ± 0.8) or the 4-pyridinyl inhibitor **16h** (K_i = 14.1 ± 0.9). To quantify the increase in solubility, aqueous solubility measurements were determined for the lead compound **10r** and the more soluble pyridinyl analogues **16i** and **16j** (Table 5). Replacement of the Cbz group with the pyridinylmethyl carbamate resulted in a 4-fold increase in aqueous solubility, and further replacement of Phe in the P_2 position with Leu resulted in a modest increase in solubility.

Table 5. Aqueous Solubility Measurements



inhibitor	Cap	P[2]	P[1]	R[2]	aqueous solubility (mM)[a]
10r	Cbz	Phe	Phe	$C(CH_3)_3$	0.070
16i	3-Pyr	Phe	Phe	$C(CH_3)_3$	0.288
16j	3-Pyr	Leu	Phe	$C(CH_3)_3$	0.378

[a] Solubility determined using UV analysis. Inhibitor was preincubated for 2 h at 37 °C.

Table 6. Selectivity against Other Cysteine Proteases

inhibitor	cruzain K_i (nM)[a]	cathepsin B K_i (nM)[a]	cathepsin L K_i (nM)[a]
10q	2.9 ± 0.3	42.7 ± 7.1	1.5 ± 0.1
10r	2.5 ± 0.2	45.0 ± 7.1	1.7 ± 0.1
10s	2.0 ± 0.2	45.4 ± 10.5	2.8 ± 0.4
11c	1.3 ± 0.1	111.8 ± 12.8	7.9 ± 1.5
11d	1.0 ± 0.1	135.9 ± 18.2	4.7 ± 1.1
16e	0.9 ± 0.1	700 ± 100	28.8 ± 2.3
16f	1.1 ± 0.1	1700 ± 300	144.4 ± 16.0
16h	14.1 ± 0.9	4800 ± 300	28.8 ± 2.3
16k	1.4 ± 0.1	600 ± 200	9.6 ± 0.8
16l	2.0 ± 0.1	1300 ± 400	44.0 ± 5.0

[a] K_i values determined at four to eight concentrations in duplicate using the linear regression analysis of Williams and Morrison.[11]

To further enhance the potency and to reduce the potential for proteolytic degradation of the inhibitors, the homophenylalanine side chain was used in place of the phenylalanine side chain at the P_1 position. For irreversible inhibitors, incorporation of the homophenylalanine side chain at P_1 has resulted in inhibitors that are not degraded by human proteases, in contrast to the corresponding inhibitors that incorporate natural amino acid side chains at this position.[15] The inhibitors (**16e**, **16f**, **16k**, and **16l**) with homophenylalanine at the P_1 position showed a 2- to 4-fold increase in potency.

Specificity of Inhibitors. To probe the selectivity of these inhibitors for cruzain over human cysteine proteases, the 10 best inhibitors against cruzain were screened against human cathepsin B and human cathepsin L. These compounds are very poor inhibitors of cathepsin B (Table 6), especially the inhibitors incorporating the pyridinylmethyl carbamate. For cathepsin L, the Cbz-protected inhibitors share similar K_i values with cruzain. However, the inhibitors incorporating the pyridinylmethyl carbamate show a 5- to 100-fold selectivity for cruzain over cathepsin L. The data demonstrates that both the P_2 side chain and the mercaptyl functionality are also important for conferring selectivity with Phe at P_2 and the 3-phenyl propyl mercaptan providing the greatest selectivity. This level of selectivity is quite promising, since cruzain is classified as a

cathepsin L-like enzyme with the central domain of cruzain showing a 56.8% homology with cathepsin L.[13]

Time Dependence of Inhibitors. In the enzyme inhibition assays, some of the identified inhibitors showed a very modest time dependence. Specifically, the enzyme and inhibitor were preincubated for 5 min, 1 h, and 3 h, followed by addition of the substrate to begin the reaction. The Cbz-protected inhibitors exhibited the same IC_{50} values for each preincubation time. However, the pyridinyl inhibitors exhibited some time-dependent behavior, with a 2- to 4-fold increase in potency after a 3 h incubation.

For the most potent compounds, time-dependent behavior was also evaluated for activity against cathepsin B and cathepsin L. The substrate was added only after a 5 min and 1 h preincubation, due to instability of these enzymes over prolonged incubation times. For cathepsin B, none of the inhibitors showed any change in IC_{50}, except for **16j**. Interestingly, for cathepsin L, the IC_{50}s of the Cbz-protected inhibitors showed a 2- to 3-fold time dependence after 1 h, while the IC_{50}s of the pyridinyl inhibitors were unchanged, which is the opposite trend as observed for cruzain.

The modest time dependence suggests that the inhibitors are either slow binding or very slowly inactivating. Alkyl mercaptomethyl ketones have previously been reported to show time-dependent inhibition of cathepsin K[16] and the caspases.[17] There are no reports of alkylmercaptomethyl ketone inhibitors that are irreversible inactivators.[18]

Conclusions

We have described a series of potent mercaptomethyl ketones against cruzain ($K_i \leq 1$ nM) that are selective for cruzain over cathepsin B and cathepsin L. These inhibitors were synthesized rapidly using a solid-phase combinatorial strategy. Substititution at the P_1' position has provided insight into the size of the binding pocket, showing a preference for large bulky substituents and long hydrophobic side chains.

Studies to elucidate the binding mode of these inhibitors are currently being undertaken by analyzing the crystal structure of cruzain bound to the ketone inhibitors. Knowledge of the binding interactions between the enzyme and inhibitor should provide valuable insight toward the rational design of future inhibitors.

Experimental Section

General Methods. Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. THF was distilled under N_2 from sodium/benzophenone, and CH_2Cl_2 and *i*-Pr_2EtN were distilled over CaH_2 immediately prior to use. $Pd(PPh_3)_4$ was prepared according to literature procedure.[19] ArgoGel-OH resin was purchased from Argonaut Technologies (San Carlos, CA). Diazomethane was generated in situ using the following literature procedure.[20] To a 0.7 M solution of *p*-toluenesulfonylmethylnitrosamide (Diazald) in absolute ethanol was added small aliquots of 1.5 N 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 the stirring THF solution of the mixed anhydride.

Reaction progress was monitored through thin-layer chromatography on Merck 60 F_{254} 0.25 mm 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.[21] Infrared spectra were recorded with a Perkin-Elmer 1600 series Fourier transform spectrometer as thin films on NaCl plates or as KBr pellets, and only partial data are listed. ^1H and ^{13}C NMR spectra were obtained with Bruker AMX-300, AMX 400, AM-400, and DRX 500 spectrometers. Unless otherwise specified, all spectra were obtained in $CDCl_3$; chemical shifts are reported in parts per million relative to TMS, and coupling constants are reported in hertz. Melting points were determined on a MelTemp apparatus and are reported uncorrected. Solubility measurements were performed by Robertson Microlit Laboriatories (Madison, NJ). Solubility was quantitated using UV analysis ($\lambda = 211$ nm), and inhibitors were preincubated at pH 7.4 aqueous buffer for 2 h at 37 °C. M-H-W Laboratories (Phoenix, AZ) performed elemental analyses. For most compounds, combustion analysis data are reported. For the remaining compounds, purity was evaluated by HPLC analysis using two different solvent systems. The first solvent system (I) was acetonitrile/water with a gradient of 40% to 80% in 15 min. The second solvent system (II) was methanol/water with a gradient of 40% to 80% in 10 min. The first system was recorded on a Rainin Microsorb C^{18} column (100 Å, 5 μM, 25 cm). The second system was recorded on an LCMS (Hewlett-Packard, C^{18} column).

General Methods for Solid-Phase Synthesis. Solvents were distilled for resin reactions. Unless otherwise stated, reactions were conducted in 12 mL polypropylene cartridges with 70 mm PE frits attached to Teflon stopcocks. Cartridges and stopcocks were obtained from Applied Separations (Allentown, PA). Syringe plungers from 10 mL disposable syringes were used as stoppers for the cartridges. Capped syringes were gently rocked on an orbital shaker table during solid-phase reactions. Solvents were removed by taking off the syringe barrel and opening the stopcock. Resin was incubated with wash solvent for 2−5 min before filtration.

General Methods for Cleavage of Compounds from Resin and for Yield Quantitation. To 0.2 g of derivatized resin was added a 5 mL solution of 1:4:15 TFA/H_2O/trifluoroethanol. After the mixture sat at room temperature for 4 h, the solution was removed. The resin was washed with THF (3 × 5 mL), and the washings were combined and concentrated. Toluene was added to form an azeotrope with the residual water and TFA. After concentration, the resultant crude oil or solid was quantitated for yield calculation by ^1H NMR calibration with *p*-xylene (0.02 mmol). Analytically pure samples were obtained by purification by column chromatography or recrystallization.

General Procedure for Synthesis of Mercaptomethyl Ketones. A solution of *N*-allyloxycarbonyl chloromethyl ketone (4 equiv) in 0.2 M of THF was added to carbazate linker **4** presolvated in THF. After 4 h of gentle rocking on a shaker table, the solution was removed, and the resin was rinsed with THF (5 × 5 mL). The support-bound ketone was used immediately in order to prevent decomposition through azodiene formation. A solution of the thiol (4 mmol, 50 equiv) and *i*-Pr_2EtN (4.8 mmol, 60 equiv) in a 2 M solution of DMF was added to the resin. The cartridge was gently rocked on the shaker table for 12 h. After filtration of the solution, the resin was rinsed with DMF (5 × 5 mL) and CH_2Cl_2 (5 × 5 mL). A solution of trimethylsilyl azide (0.09 mL, 0.7 mmol, 8 equiv), tetrabutylammonium fluoride (0.063 g, 0.24 mmol, 3 equiv), and $Pd(PPh_3)_4$ (0.018 g, 0.16 mmol, 0.2 equiv) in 5 mL of CH_2Cl_2 was added to the resin in a glovebag under N_2 atmosphere. The resin was gently rocked for 4 h and was protected from light. After removal of the solution, the resin was rinsed with CH_2Cl_2 (5 × 5 mL) and DMF (5 × 5 mL).

An acylation stock solution of the desired carboxylic acid (0.4 mmol, 5 equiv), *i*-Pr_2EtN (0.8 mmol, 10 equiv), HOAt (0.4 mmol, 5 equiv), and PyBOP (0.4 mmol, 5 equiv) in 0.2 M of DMF was prepared immediately before addition. The acylation solution was added to the resin, and the cartridge was gently rocked for 4 h. Upon removal of the solution, the resin was rinsed with DMF (5 × 5 mL) and the acylation reaction

sequence was repeated. After excess reagents were filtered, the resin was rinsed with DMF (5 × 5 mL) and THF (5 × 5 mL) and dried overnight in vacuo. The material was cleaved and quantified as previously described.

Synthesis of *N*-Cbz-Phe-Phe Ketone Inhibitors. Loading, displacement, and deprotection of the *N*-allyloxycarbonyl phenylalanine chloromethyl ketone were performed according to the general procedure. Acylation using *N*-Cbz phenylalanine as the desired carboxylic acid resulted in the formation of the desired inhibitor in 39–97% yields, as determined by NMR calibration with *p*-xylene (0.02 mmol).

10a: 62% yield. IR: 1654, 1688, 1715 cm^{-1}. ^{1}H NMR (500 MHz): δ 2.87–3.02 (m, 6H), 3.45 (d, J = 13.6, 1H), 3.49 (d, J = 13.6 Hz, 1H), 4.38 (m, 1H), 4.98 (m, 1H), 5.06 (d, J = 12.3 Hz, 1H), 5.09 (d, J = 12.3 Hz, 1H,), 5.17 (m, 1H), 6.38 (d, J = 6.8 Hz, 1H), 7.01–7.34 (m, 19H). ^{13}C NMR (125 MHz): δ 35.0, 37.3, 37.6, 38.1, 56.1, 57.2, 67.2, 127.1, 127.2, 128.0, 128.3, 128.5, 128.65, 128.67, 128.8, 129.1, 129.2, 130.5, 133.1, 135.4, 135.7, 135.9, 136.0, 155.8, 170.4, 202.0. Anal. ($C_{34}H_{33}ClN_2O_4S$) C, H, N.

10b: 89% yield. IR: 1658, 1691, 1709 cm^{-1}. ^{1}H NMR (500 MHz): δ 2.89–3.07 (m, 6H), 3.50 (d, J = 13.5 Hz, 1H), 3.55 (d, J = 13.5 Hz, 1H), 4.38 (m, 1H), 4.97 (m, 1H), 5.06 (s, 2H), 5.17 (m, 1H), 6.38 (d, J = 7.2 Hz, 1H), 7.02–7.33 (m, 20H). ^{13}C NMR (125 MHz): δ 35.8, 37.5, 37.6, 38.1, 56.1, 57.3, 67.2, 127.1, 127.2, 127.3, 128.1, 128.2, 128.5, 128.6, 128.8, 129.15, 129.17, 129.23, 135.8, 136.0, 136.0, 155.8, 170.4, 202.1. Anal. ($C_{34}H_{34}N_2O_4S$) C, H, N.

10c: Since racemic mercaptan was used, an approximate 1:1 mixture of diastereomers was produced. 67% yield. IR: 1658, 1686, 1714, 1730 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.27 (t, J = 7.1 Hz, 3H), 1.35 (d, J = 7.2 Hz, 3H), 2.90–3.08 (m, 4H), 3.20–3.43 (m, 3H), 4.16 (q, J = 7.1 Hz, 2H,), 4.38 (m, 1H), 4.89–4.97 (m, 1H), 5.07 (s, 2H), 5.20 (m, 1H), 6.46–6.41 (m, 1H), 7.05–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 14.1, 16.7, 16.8, 37.3, 37.4, 38.1, 38.75, 38.76, 40.97, 41.04, 56.1, 57.6, 57.9, 61.3, 67.1, 67.9, 127.11, 127.15, 128.0, 128.22, 128.23, 128.5, 128.66, 128.67, 128.74, 128.8, 129.13, 129.14, 129.2, 155.8, 170.50, 170.52, 172.3, 172.4, 202.3, 202.5. Anal. ($C_{32}H_{36}N_2O_6S$) C, H, N.

10d: 37% yield. IR: 1653, 1700, 1716 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.97 (d, J = 5.9 Hz, 3H,), 2.47–2.56 (m, 2H), 2.85–3.16 (m, 6H), 3.21–3.33 (m, 2H), 4.37 (m, 1H), 5.01–5.10 (m, 3H), 5.19 (m, 1H), 6.18 (m, 1H), 6.47 (m, 1H), 6.98–7.34 (m, 15H). ^{13}C NMR (125 MHz): δ 30.3, 32.2, 36.0, 37.4, 38.0, 38.9, 56.0, 57.2, 67.2, 127.3, 128.0, 128.3, 128.6, 128.77, 128.79, 128.9, 129.1, 129.2, 135.6, 135.9, 155.8, 170.4, 170.7, 203.1. Anal. Calcd for $C_{31}H_{35}N_3O_5S$: C, 66.29; H, 6.28; N, 7.48. LCMS (EI): m/z 562.3 $(M + 1)^+$; t_r = 10.78 min (95% purity) using solvent system I, t_r = 9.08 min (99% purity) using solvent system II.

10e: 42% yield. IR: 1658, 1691, 1714 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.34–1.42 (m, 2H), 1.55 (m, 2H), 1.70 (m, 2H), 1.86–1.93 (m, 2H), 2.86 (app quintet, J = 7.0 Hz, 1H), 2.94–3.11 (m, 6H), 4.38 (m, 1H), 5.04–5.17 (m, 3H), 5.18 (m, 1H), 6.42 (m, 1H), 7.04–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 24.6, 24.7, 33.1, 33.4, 37.8, 38.1, 39.2, 43.7, 56.1, 57.1, 67.1, 127.0, 127.1, 128.1, 128.2, 128.5, 128.6, 128.8, 129.17, 129.23, 135.9, 136.0, 155.8, 170.3, 202.6. Anal. ($C_{32}H_{36}N_2O_4S$) C, H, N.

10f: 90% yield. IR: 1655, 1689, 1715 cm^{-1}. ^{1}H NMR (500 MHz): δ 2.89–3.05 (m, 6H), 3.46 (d, J = 13.4 Hz, 1H), 3.50 (d, J = 13.4 Hz, 1H), 3.78 (s, 3H), 4.38 (m, 1H), 4.97 (m, 1H), 5.07 (s, 2H), 5.19 (m, 1H), 6.39 (d, J = 6.7 Hz, 1H), 6.83 (d, J = 8.7 Hz, 2H), 7.01 (d, J = 6.2 Hz, 2H), 7.14–7.34 (m, 15H). ^{13}C NMR (125 MHz): δ 35.3, 37.4, 37.7, 38.2, 55.3, 56.1, 57.3, 67.2, 114.0, 127.1, 127.2, 128.1, 128.3, 128.6, 128.7, 128.78, 128.83, 129.2, 129.3, 130.4, 135.9, 136.1, 155.8, 158.9, 170.4, 202.2. Anal. ($C_{35}H_{36}N_2O_5S$) C, H, N.

10g: 48% yield. IR: 1655, 1701, 1717 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.27 (t, J = 7.1 Hz, 3H), 2.90–3.13 (m, 6H), 3.25 (m, 2H), 4.17 (q, J = 7.1 Hz, 2H), 4.37 (m, 1H), 4.94 (m, 1H), 5.07 (s, 2H), 5.17 (m, 1H), 6.40 (d, J = 7.5 Hz, 1H), 7.04–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 14.1, 33.1, 37.3, 38.1, 38.9, 56.1, 57.6, 61.5, 67.1, 127.10, 127.14, 128.0, 128.2, 128.5, 128.7, 128.8, 129.1, 129.2, 135.8, 136.1, 155.8, 169.6, 170.5, 202.0. Anal. ($C_{31}H_{34}N_2O_6S$) C, H, N.

10h: 75% yield. IR: 1658, 1687, 1717 cm^{-1}. ^{1}H NMR (500 MHz): δ 0.86 (d, J = 3.0 Hz, 3H), 0.87 (d, J = 3.0 Hz, 3H), 1.34–1.39 (m, 2H), 1.60 (septet, J = 6.6 Hz, 1H), 2.32 (t, J = 7.8 Hz, 2H), 2.93–3.08 (m, 6H), 4.38 (m, 1H), 5.02 (m, 1H), 5.08 (s, 2H), 5.18 (m, 1H), 6.42 (d, J = 7.3 Hz, 1H), 7.04–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 22.1, 22.2, 27.3, 30.0, 37.6, 37.8, 38.1, 38.7, 56.1, 57.1, 67.2, 127.06, 127.14, 128.1, 128.2, 128.5, 128.6, 128.8, 129.16, 129.23, 135.9, 136.0, 155.8, 170.4, 202.1. Anal. ($C_{32}H_{38}N_2O_4S$) C, H, N.

10i: 97% yield. IR: 1652, 1700, 1715 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.43 (s, 9H), 2.88–3.09 (m, 6H), 3.48 (d, J = 13.4 Hz, 1H), 3.53 (d, J = 13.4 Hz, 1H), 4.38 (m, 1H), 4.98 (m, 1H), 5.06 (s, 2H), 5.20 (m, 1H), 6.40 (d, J = 7.2 Hz, 1H), 6.98–7.33 (m, 19H). ^{13}C NMR (125 MHz): δ 30.3, 31.3, 34.5, 35.4, 37.6, 37.6, 38.2, 56.1, 57.3, 67.1, 125.5, 127.07, 127.14, 128.1, 128.2, 128.5, 128.6, 128.78, 128.84, 129.16, 129.23, 133.7, 135.7, 135.9, 136.0, 150.3, 155.8, 170.4, 202.1. Anal. ($C_{38}H_{42}N_2O_4S$) C, H, N.

10j: 96% yield. IR: 1655, 1702, 1714 cm^{-1}. ^{1}H NMR (300 MHz): δ 2.75–3.10 (m, 9H), 3.23 (d, J = 15.5 Hz, 1H), 4.38 (m, 1H), 4.86 (app q, J = 7.2 Hz, 1H), 5.06 (s, 2H), 5.21 (d, J = 7.5 Hz, 1H), 6.54 (d, J = 7.3 Hz, 1H), 6.98–7.38 (m, 15H). ^{13}C NMR (125 MHz): δ 26.5, 35.6, 37.2, 37.9, 39.7, 56.1, 57.8, 67.2, 127.2, 127.2, 127.3, 128.0, 128.3, 128.6, 128.8, 129.1, 129.2, 135.5, 135.8, 136.0, 155.8, 168.6, 170.8, 202.6. LCMS (EI): m/z 548.7 $(M + 1)^+$; t_r = 4.44 min (95% purity) using solvent system I, t_r = 2.70 min (95% purity) using solvent system II.

10k: 46% yield. IR 1653, 1699, 1715 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.01 (s, 9H), 1.31 (s, 6H), 1.56 (s, 2H), 2.93–3.07 (m, 4H), 3.18 (d, J = 15.1 Hz, 1H), 3.24 (d, J = 15.0 Hz, 1H), 4.40 (m, 1H), 5.02–5.09 (m, 3H), 5.23 (d, J = 7.1 Hz, 1H), 6.41 (d, J = 7.0 Hz, 1H), 7.05–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 30.1, 31.6, 32.7, 37.6, 37.9, 38.2, 48.2, 54.6, 56.0, 57.5, 67.1, 127.10, 127.13, 128.0, 128.2, 128.5, 128.7, 129.1, 129.3, 135.8, 136.1, 155.8, 170.3, 204.4. Anal. ($C_{35}H_{44}N_2O_4S$) C, H, N.

10l: 60% yield. IR: 1653, 1699, 1715 cm^{-1}. ^{1}H NMR (300 MHz): δ 1.17–1.43 (m, 5H), 1.60–1.88 (m, 5H), 2.43 (m, 1H), 2.92–3.16 (m, 6H), 4.38 (m, 1H), 5.01–5.16 (m, 4H), 6.43 (d, J = 7.2 Hz, 1H), 7.05–7.34 (m, 15H). ^{13}C NMR (125 MHz): δ 25.6. 25.8, 32.8, 37.3, 37.8, 43.3, 56.1, 57.1, 67.2, 127.0, 127.1, 128.1, 128.2, 128.5, 128.6, 128.8, 129.18, 129.22, 135.93, 135.99, 136.01, 155.8, 170.4, 202.7. Anal. ($C_{33}H_{38}N_2O_4S$) C, H, N.

10m: Since racemic *sec*-butyl mercaptan was used, an approximate 1:1 mixture of diastereomers was produced. 63% yield. IR: 1654, 1701, 1716 cm^{-1}. ^{1}H NMR (500 MHz): δ 0.92 (m, 3H), 1.14–1.26 (m, 3H), 1.41–1.55 (m, 2H), 2.53 (m, 1H), 2.94–3.14 (m, 6H), 4.38 (m, 1H), 5.03–5.13 (m, 3H), 5.17 (m, 1H), 6.40 (m, 1H), 6.98–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 10.9, 11.2, 19.9, 20.1, 28.9, 29.1, 37.6, 37.78, 37.88, 37.9, 38.1, 41.6, 41.7, 56.1, 57.1, 67.1, 127.06, 127.13, 128.1, 128.2, 128.5, 128.6, 128.8, 129.15, 129.23, 135.9, 136.0, 155.8, 170.3, 202.8. Anal. ($C_{31}H_{36}N_2O_4S$) C, H, N.

10n: 50% yield. IR 1653, 1699, 1716 cm^{-1}. ^{1}H NMR (500 MHz): δ 0.81–1.44 (m, 25H), 2.92–3.20 (m, 6H), 4.40 (m, 1H), 5.07 (m, 3H), 5.23 (m, 1H), 6.41 (m, 1H), 6.99–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 28.4, 29.4, 30.3, 37.1, 37.8, 38.2, 56.0, 57.5, 67.1, 127.1, 128.01, 128.02, 128.2, 128.5, 128.68, 128.73, 129.1, 129.2, 135.8, 136.0, 155.8, 170.4, 204.2. Anal. ($C_{39}H_{52}N_2O_4S$) C, H, N.

10o: 51% yield. IR: 1653, 1699, 1716 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.16 (d, J = 6.7 Hz, 3H), 1.19 (d, J = 6.6 Hz, 3H), 2.70 (septet, J = 6.7 Hz, 1H), 2.93–3.15 (m, 6H), 4.38 (m, 1H), 5.05–5.13 (m, 1H), 5.08 (s, 2H), 5.18 (m, 1H), 6.41 (m, 1H), 6.92–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 22.65, 22.71, 35.0, 37.8, 37.9, 38.1, 56.1, 57.1, 67.2, 127.06, 127.13, 128.1, 128.2, 128.5, 128.6, 128.8, 129.15, 129.22, 135.9, 136.0, 155.8, 170.4, 202.6. Anal. ($C_{30}H_{34}N_2O_4S$) C, H, N.

10p: 64% yield. IR: 1655, 1700, 1715 cm^{-1}. ^{1}H NMR (500 MHz): δ 2.59 (m, 2H), 2.79 (m, 2H), 2.90–3.11 (m, 6H), 4.38 (m, 1H), 5.00 (m, 1H), 5.07 (s, 2H), 5.19 (m, 1H), 6.40 (d, J =

7.0 Hz, 1H,), 6.99–7.34 (m, 20H). ^{13}C NMR (125 MHz): δ 33.2, 35.3, 37.7, 38.1, 38.7, 56.1, 57.1, 67.2, 126.4, 127.1, 127.2, 128.0, 128.2, 128.5, 128.5, 128.5, 128.6, 128.8, 129.15, 129.20, 135.8, 136.0, 138.2, 139.8, 155.8, 170.4, 202.0. Anal. ($C_{35}H_{36}N_2O_4S$) C, H, N.

10q: 82% yield. IR: 1653, 1699, 1715, 1732 cm^{-1}. ^{1}H NMR (300 MHz): δ 1.26 (t, J = 7.1 Hz, 3H), 2.47–2.63 (m, 4H), 2.93–3.10 (m, 6H), 4.14 (q, J = 7.1 Hz, 2H,), 4.37 (m, 1H), 4.97 (m, 1H), 5.07 (s, 2H), 5.16 (m, 1H), 6.38 (d, J = 7.9 Hz, 1H,), 7.03–7.36 (m, 15H). ^{13}C NMR (125 MHz): δ 14.1, 26.7, 34.0, 37.7, 38.7, 56.1, 57.2, 60.7, 61.2, 127.1, 127.2, 128.1, 128.2, 128.5, 128.7, 128.8, 129.1, 129.2, 135.8, 136.0, 155.8, 170.4, 171.4, 202.0. Anal. ($C_{32}H_{36}N_2O_6S$) C, H, N.

10r: 49% yield. IR: 1655, 1701 cm^{-1}. ^{1}H NMR (300 MHz): δ 1.24 (s, 9H), 2.90–3.08 (m, 4H), 3.16 (d, J = 15.2 Hz, 1H), 3.22 (d, J = 15.3 Hz, 1H), 4.38 (m, 1H), 5.02–5.09 (m, 3H), 5.21 (d, J = 7.9 Hz, 1H), 6.40 (d, J = 7.5 Hz, 1H), 6.98–7.34 (m, 15H). ^{13}C NMR (125 MHz): δ 30.6, 37.7, 37.9, 38.2, 56.0, 57.3, 67.1, 127.1, 128.0, 128.2, 128.5, 128.69, 128.74, 129.1, 129.2, 135.7, 136.0, 155.8, 170.4, 204.5. Anal.($C_{31}H_{36}N_2O_4S$) C, H, N.

10s: 63% yield. IR: 1653, 1700, 1716 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.81 (m, 2H), 2.34 (t, J = 7.3 Hz, 2H), 2.65 (t, J = 7.5 Hz, 2H), 2.92–3.09 (m, 6H), 4.37 (m, 1H), 5.01 (m, 1H), 5.08 (s, 2H), 5.15 (m, 1H), 6.36 (m, 1H), 7.03–7.34 (m, 20H). ^{13}C NMR (125 MHz): δ 30.2, 31.3, 34.6, 37.8, 38.1, 38.7, 56.1, 57.1, 67.2, 126.0, 127.08, 127.10, 128.0, 128.2, 128.4, 128.5, 128.6, 128.8, 129.15, 129.22, 131.8, 135.8, 136.0, 141.1, 155.8, 170.4, 202.1. Anal. ($C_{36}H_{38}N_2O_4S$) C, H, N.

Synthesis of *N*-Cbz-Leu-Phe Ketone Inhibitors. Loading, displacement, and deprotection of the *N*-allyloxycarbonyl phenylalanine chloromethyl ketone were performed according to the general procedure. Acylation using *N*-Cbz leucine as the desired carboxylic acid resulted in the formation of the desired inhibitor in 44–52% yields, as determined by ^{1}H NMR calibration with *p*-xylene (0.02 mmol).

11a: 44% yield. IR: 1655, 1708 cm^{-1}. ^{1}H NMR (500 MHz): δ 0.90 (d, J = 6.2 Hz, 6H), 1.25 (t, J = 7.1 Hz, 3H), 1.39–1.43 (m, 1H), 1.56–1.62 (m, 2H), 2.51–2.53 (m, 2H), 2.65 (m, 2H), 2.98–3.01 (m, 1H), 3.12–3.26 (m, 3H), 4.14 (m, 3H), 5.00–5.09 (m, 4H), 6.65 (d, J = 7.1 Hz, 1H), 7.14–7.36 (m, 10H). ^{13}C NMR (125 MHz): δ 14.2, 22.8, 24.6, 26.8, 34.0, 37.6, 38.9, 41.0, 53.5, 57.2, 60.7, 67.2, 127.1, 128.1, 128.3, 128.5, 128.7, 129.2, 135.95, 136.0, 156.1, 171.5, 171.8, 202.6. Anal. ($C_{29}H_{38}N_2O_6S$) C, H, N.

11b: 52% yield. IR: 1656, 1701 cm^{-1}. ^{1}H NMR (500 MHz): δ 0.90 (d, J = 6.2 Hz, 6H), 1.19 (d, J = 6.8 Hz, 3H), 1.21 (d, J = 6.6 Hz, 3H), 1.38–1.43 (m, 1H), 1.56–1.66 (m, 2H), 2.76 (septet, J = 6.7 Hz, 1H,), 2.99–3.03 (m, 1H), 3.15 (d, J = 6.7 Hz, 13.9, 1H), 3.22 (s, 2H), 4.13 (m, 1H), 5.04 (d, J = 7.2 Hz, 1H), 5.08–5.16 (m, 3H), 6.61 (d, J = 7.2 Hz, 1H), 7.05–7.37 (m, 10H). ^{13}C NMR (125 MHz): δ 22.7, 22.8, 24.6, 35.1, 37.9, 38.1, 41.1, 53.5, 57.1, 67.2, 127.1, 128.1, 128.2, 128.5, 128.7, 129.2, 136.0, 156.0, 171.7, 203.2. Anal. ($C_{27}H_{36}N_2O_4S$) C, H, N.

11c: 51% yield. IR: 1657, 1705 cm^{-1}. ^{1}H NMR (500 MHz): δ 0.90 (d, J = 6.1 Hz, 6H), 1.23–1.61 (m, 12H), 2.99–3.03 (m, 1H), 3.10–3.15 (m, 1H), 3.29–3.30 (m, 2H), 4.14 (m, 1H), 5.04 (d, J = 7.2 Hz, 1H,), 5.09–5.15 (m, 3H), 6.58 (d, J = 7.3 Hz, 1H,), 6.91–7.37 (m, 10H). ^{13}C NMR (125 MHz): δ 22.8, 24.6, 30.6, 37.7, 37.9, 41.2, 43.3, 53.4, 57.3, 67.1, 127.1, 128.1, 128.2, 128.5, 128.7, 129.2, 135.9, 136.0, 156.0, 171.7, 204.9. Anal. ($C_{28}H_{38}N_2O_4S$) C, H, N.

11d: 44% yield. IR: 1657, 1702 cm^{-1}. ^{1}H NMR (500 MHz): δ 0.90 (d, J = 6.2 Hz, 6H), 1.39–1.43 (m, 1H), 1.56–1.63 (m, 2H), 1.80–1.88 (m, 2H), 2.40 (t, J = 7.2 Hz, 2H), 2.66 (t, J = 7.6 Hz, 2H), 2.98–3.02 (m, 1H), 3.12–3.22 (m, 3H), 4.13 (m, 1H), 5.02 (d, J = 7.1 Hz, 1H), 5.05–5.10 (m, 3H), 6.61 (d, J = 7.4 Hz, 1H,), 7.14–7.35 (m, 15H). ^{13}C NMR (125 MHz): δ 22.8, 24.6, 30.3, 31.3, 34.6, 37.8, 38.9, 41.1, 53.5, 57.1, 67.2, 126.0, 127.1, 128.1, 128.3, 128.37, 128.39, 128.5, 128.7, 129.2, 136.0, 141.1, 156.0, 171.7, 202.6. Anal. ($C_{33}H_{40}N_2O_4S$) C, H, N.

Synthesis of Ketone Inhibitors That Incorporate the *N*-Morpholine Urea. Loading, displacement, and deprotection of the *N*-allyloxycarbonyl phenylalanine chloromethyl ketone were performed according to the general procedure. Acylation using *N*-morpholinecarbonyl phenylalanine or *N*-morpholinecarbonyl leucine as the desired carboxylic acid resulted in the formation of the desired inhibitor in 54–76% yields, as determined by ^{1}H NMR calibration with *p*-xylene (0.02 mmol).

12a: 70% yield. IR: 1622, 1654, 1719 cm^{-1}. ^{1}H NMR (300 MHz): δ 0.89 (d, J = 6.2 Hz, 3H,), 0.91 (d, J = 6.3 Hz, 3H), 1.25 (s, 9H), 1.27–1.68 (m, 3H), 3.02 (dd, J = 7.3 Hz, 14.0, 1H), 3.13 (dd, J = 6.7 Hz, 14.0, 1H), 3.27–3.34 (m, 6H), 3.67 (app t, J = 4.9 Hz, 4H), 4.27–4.34 (m, 1H), 4.75 (d, J = 7.7 Hz, 1H), 5.09 (app q, J = 7.2 Hz, 1H), 6.74 (d, J = 7.5 Hz, 1H), 7.16–7.32 (m, 5H). ^{13}C NMR (125 MHz): δ 22.2, 22.8, 24.8, 30.6, 37.8, 37.8, 41.4, 43.2, 43.9, 52.8, 57.5, 66.4, 127.0, 128.6, 129.2, 136.2, 157.2, 172.9, 205.0. Anal. ($C_{25}H_{39}N_3O_4S$) C, H, N.

12b: 55% yield. IR: 1621, 1654, 1718 cm^{-1}. ^{1}H NMR (300 MHz): δ 1.25 (s, 9H), 2.89–3.10 (m, 4H), 3.18–3.38 (m, 6H), 3.61–3.64 (m, 4H), 4.49–4.56 (m, 1H), 4.86 (d, J = 7.1 Hz, 1H), 4.99–5.06 (m, 1H), 6.49 (d, J = 7.5 Hz, 1H), 7.02–7.32 (m, 10H). ^{13}C NMR (125 MHz): δ 30.7, 37.65, 37.68, 38.3, 43.3, 43.9, 55.3, 57.4, 66.3, 126.99, 127.03, 128.6, 128.7, 129.1, 129.3, 136.0, 136.6, 156.8, 171.3, 204.5. Anal. ($C_{28}H_{37}N_3O_4S$) C, H, N.

12c: 76% yield. IR: 1626, 1660, 1713, 1729 cm^{-1}. ^{1}H NMR (400 MHz): δ 0.87–0.90 (m, 6H), 1.24 (t, J = 7.2 Hz, 3H), 1.40–1.44 (m, 1H), 1.51–1.59 (m, 2H), 2.49–2.52 (m, 2H), 2.62–2.66 (m, 2H), 3.00 (dd, J = 7.5 Hz, 13.9, 1H), 3.10–3.34 (m, 7H), 3.65 (m, 4H), 4.12 (q, J = 7.1 Hz, 2H), 4.28 (m, 1H), 4.83 (d, J = 7.5 Hz, 1H), 4.96 (m, 1H), 6.91 (d, J = 7.6 Hz, 1H), 7.15–7.28 (m, 5H). ^{13}C NMR (101 MHz): δ 14.2, 22.2, 22.8, 24.8, 26.9, 34.1, 37.6, 39.0, 41.2, 44.0, 52.8, 57.4, 60.8, 66.4, 127.1, 128.7, 129.2, 136.2, 157.3, 171.6, 173.0, 202.8. Anal. ($C_{26}H_{39}N_3O_6S$) C, H, N.

12d: 75% yield. IR: 1624, 1659, 1729 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.17 (t, J = 7.1 Hz, 3H), 2.40–2.47 (m, 2H), 2.50–2.52 (m, 2H), 2.82–3.04 (m, 6H), 3.14–3.17 (m, 4H), 3.51–3.53 (m, 4H), 4.05 (q, J = 7.1 Hz, 2H), 4.44 (m, 1H), 4.80–4.94 (m, 2H), 6.60 (d, J = 7.6 Hz, 1H), 6.94 (m, 2H), 6.96–7.20 (m, 8H). ^{13}C NMR (125 MHz): δ 14.1, 26.7, 33.9, 37.4, 38.1, 38.6, 43.8, 55.4, 57.2, 60.6, 66.2, 126.9, 127.0, 128.5, 128.6, 129.1, 129.2, 136.0, 136.5, 156.7, 171.4, 171.4, 202.1. Anal. ($C_{29}H_{37}N_3O_6S$) C, H, N.

12e: 54% yield. IR: 1621, 1658, 1713 cm^{-1}. ^{1}H NMR (500 MHz): δ 0.90 (m, 6H), 1.41–1.47 (m, 1H), 1.55–1.65 (m, 2H), 1.78–1.87 (m, 2H), 2.39 (t, J = 7.3 Hz, 2H), 2.66 (t, J = 7.6 Hz, 2H), 3.01 (dd, J = 7.4, 14.0 Hz, 1H), 3.12–3.21 (m, 3H), 3.27–3.36 (m, 4H), 3.67 (app t, J = 4.9 Hz, 4H), 4.28–4.33 (m, 1H), 4.78 (d, J = 7.6 Hz, 1H), 5.03 (app q, J = 7.2 Hz, 1H), 6.87 (d, J = 7.7 Hz, 1H), 7.15–7.29 (m, 10H). ^{13}C NMR (125 MHz): δ 22.2, 22.8, 24.8, 30.3, 31.4, 34.6, 37.7, 39.0, 41.3, 43.9, 52.8, 57.1, 66.3, 126.0, 127.0, 128.36, 128.37, 128.6, 129.2, 136.2, 141.1, 157.2, 172.8, 202.8. Anal. ($C_{30}H_{41}N_3O_4S$) C, H, N.

12f: 70% yield. IR: 1623, 1657, 1712 cm^{-1}. ^{1}H NMR (500 MHz): δ 1.77–1.88 (m, 2H), 2.36 (t, J = 7.3 Hz, 2H), 2.66 (t, J = 7.6 Hz, 2H), 2.91–3.19 (m, 6H), 3.20–3.29 (m, 4H), 3.58–3.65 (m, 4H), 4.53 (m, 1H), 4.86 (d, J = 7.1 Hz, 1H), 4.98 (m, 1H), 6.57 (d, J = 7.7 Hz, 1H), 7.02–7.04 (m, 2H), 7.15–7.29 (m, 13H). ^{13}C NMR (125 MHz): δ 30.2, 31.3, 34.6, 37.6, 38.2, 38.7, 43.9, 55.4, 57.1, 66.3, 126.0, 127.0, 127.1, 128.4, 128.5, 128.7, 129.2, 129.3, 136.1, 136.6, 141.1, 156.8, 171.3, 202.2. Anal. ($C_{33}H_{39}N_3O_4S$) C, H, N.

Synthesis of *N*-[(*n*-Pyridinyl)methoxy]carbonyl] Ketone Inhibitors. Loading, displacement, and deprotection of the *N*-allyloxycarbonyl-protected phenylalanine or homophenylalanine chloromethyl ketones were performed according to the general procedure. Acylation using *N*-[(*n*-pyridinyl)-methoxy] carbonyl] phenylalanine or leucine as the desired carboxylic acid resulted in the formation of the desired inhibitor in 50–73% yields, as determined by ^{1}H NMR calibration with *p*-xylene (0.02 mmol).

16a: 65% yield. IR: 1670, 1726 cm^{-1}. ^1H NMR (300 MHz): δ 1.25 (m, 3H), 2.45−2.57 (m, 4H), 2.87−3.11 (m, 6H), 4.13 (q, J = 7.1 Hz, 2H), 4.34−4.36 (m, 1H), 4.95−5.03 (m, 1H), 5.20 (d, J = 14.3 Hz, 1H), 5.37 (d, J = 14.2 Hz, 1H), 5.84 (m, 1H), 6.79 (d, J = 7.5 Hz, 1H), 7.09−7.30 (m, 10H), 7.44−7.49 (m, 2H), 7.96 (m, 1H), 8.74 (m, 1H). ^{13}C NMR (125 MHz): δ 14.2, 26.7, 34.0, 37.4, 38.0, 38.8, 56.6, 57.2, 60.7, 64.1, 122.7, 123.9, 127.0, 127.1, 128.6, 128.7, 129.2, 136.00, 136.01, 146.8, 154.4, 155.1, 170.6, 171.5, 202.3. LCMS (EI): m/z 578.3 (M + 1)$^+$; t_r = 10.10 min (94% purity) using solvent system I, t_r = 9.31 min (97% purity) using solvent system II.

16b: 64% yield. IR: 1670, 1722 cm^{-1}. ^1H NMR (500 MHz): δ 1.25 (t, J = 7.1 Hz, 3H), 2.48−2.51 (m, 2H), 2.55−2.61 (m, 2H), 2.93−3.09 (m, 6H), 4.14 (q, J = 7.1 Hz, 2H), 4.38 (m, 1H), 5.00 (m, 1H), 5.15 (s, 2H), 5.56 (s, 1H), 6.51 (d, J = 5.5 Hz, 1H), 7.05−7.30 (m, 10H), 7.60 (s, 1H), 7.98 (d, J = 7.9 Hz, 1H), 8.67 (s, 1H), 8.73 (s, 1H). ^{13}C NMR (125 MHz): δ 14.1, 26.8, 34.0, 37.6, 38.3, 38.8, 56.3, 57.2, 60.8, 63.3, 125.2, 127.1, 127.2, 128.7, 128.8, 129.1, 129.2, 134.6, 135.7, 135.9, 140.1, 144.8, 155.1, 170.3, 174.5, 202.1. LCMS (EI): m/z 578.3 (M + 1)$^+$; t_r = 9.88 min (>99% purity) using solvent system I, t_r = 9.04 min (98% purity) using solvent system II.

16c: 50% yield. IR: 1670, 1719 cm^{-1}. ^1H NMR (500 MHz): δ 1.78−1.84 (m, 2H), 2.34 (m, 2H), 2.65 (t, J = 7.7 Hz, 2H), 3.06−2.93 (m, 6H), 4.37 (m, 1H), 5.03 (m, 1H), 5.15 (s, 2H), 5.48 (m, 1H), 6.43 (d, J = 7.2 Hz, 1H), 7.05−7.29 (m, 15H), 7.59 (br s, 1H), 7.96 (d, J = 7.7 Hz, 1H), 8.67−8.73 (m, 2H). ^{13}C NMR (125 MHz): δ 30.2, 31.3, 34.5, 38.1, 38.3, 38.8, 56.2, 57.1, 63.3, 125.9, 126.0, 127.1, 127.2, 128.38, 128.4, 128.6, 128.8, 129.1, 129.2, 135.8, 135.9, 140.0, 141.0, 144.8, 155.1, 170.2, 202.1. LCMS (EI): m/z 596.3 (M + 1)$^+$; t_r = 14.09 min (>99% purity) using solvent system I, t_r = 11.95 min (>99% purity) using solvent system II.

16d: 63% yield. IR: 1669, 1716 cm^{-1}. ^1H NMR (300 MHz): δ 0.86−0.90 (m, 6H), 1.43−1.67 (m, 3H), 1.75−1.85 (m, 2H), 2.38 (t, J = 7.3 Hz, 2H), 2.63 (t, J = 7.6 Hz, 2H), 2.96−3.03 (m, 1H), 3.09−3.22 (m, 3H), 4.10−4.18 (m, 1H), 5.07 (app q, J = 7.3 Hz, 1H), 5.20 (s, 2H), 5.59 (d, J = 7.8 Hz, 1H), 6.79 (d, J = 7.7 Hz, 1H), 7.13−7.30 (m, 10H), 7.65−7.69 (m, 1H), 8.11 (d, J = 7.9 Hz, 1H), 8.68 (br s, 1H), 8.83 (br s, 1H). ^{13}C NMR (125 MHz): δ 21.8, 22.7, 24.6, 30.3, 31.4, 34.5, 37.6, 39.0, 41.2, 53.8, 57.1, 63.2, 125.3, 126.0, 127.10, 127.11, 128.4, 128.6, 129.2, 135.0, 135.9, 140.5, 140.5, 141.1, 144.3, 155.3, 171.7, 202.7. LCMS (EI): m/z 562.3 (M + 1)$^+$; t_r = 13.58 min (>99% purity) using solvent system I, t_r = 11.69 min (>99% purity) using solvent system II.

16e: 73% yield. IR: 1670, 1719 cm^{-1}. ^1H NMR (500 MHz): δ 1.83−1.89 (m, 3H), 2.2 (m, 1H), 2.44 (t, J = 7.2 Hz, 2H), 2.52 (m, 2H), 2.68 (t, J = 7.5 Hz, 2H), 3.00−3.19 (m, 4H), 4.44 (m, 1H), 4.83 (m, 1H), 5.16 (s, 2H), 5.63 (m, 1H), 6.66 (d, J = 6.4 Hz, 1H), 7.08−7.28 (m, 15H), 7.56 (s, 1H), 8.00 (d, J = 7.6 Hz, 1H), 8.26 (s, 1H), 8.75 (s, 1H). ^{13}C NMR (125 MHz): δ 30.3, 31.5, 33.1, 34.5, 37.9, 38.3, 56.4, 56.4, 63.1, 125.4, 126.0, 126.3, 127.2, 128.3, 128.37, 128.38, 128.5, 128.8, 129.2, 135.1, 135.9, 140.3, 140.8, 141.1, 144.0, 155.2, 162.4, 170.6, 202.7. LCMS (EI): m/z 610.3 (M + 1)$^+$; t_r = 14.95 min (>99% purity) using solvent system I, t_r = 12.34 min (>99% purity) using solvent system II.

16f: 64% yield. IR: 1668, 1713 cm^{-1}. ^1H NMR (500 MHz): δ 0.92−0.95 (m, 6H), 1.51−1.54 (m, 1H), 1.60−1.65 (m, 2H), 1.85−1.93 (m, 3H), 2.24 (m, 1H), 2.47 (t, J = 7.2 Hz, 2H), 2.59 (t, J = 7.6 Hz, 2H), 2.67 (t, J = 7.4 Hz, 2H), 3.25 (d, J = 14.5 Hz, 1H), 3.32 (d, J = 14.5 Hz, 1H), 4.19 (m, 1H), 4.87 (m, 1H), 5.18−5.30 (m, 2H), 5.54 (s, 1H), 6.76 (d, J = 6.1 Hz, 1H), 7.11−7.28 (m, 10H), 7.59 (s, 1H), 8.09 (d, J = 7.4 Hz, 1H), 8.62 (s, 1H), 8.82 (s, 1H). ^{13}C NMR (125 MHz): δ 21.8, 22.8, 24.7, 30.3, 31.53, 31.54, 33.2, 34.5, 38.0, 41.2, 53.9, 56.4, 63.1, 126.0, 126.3, 128.4, 128.6, 132.6, 135.4, 140.6, 140.9, 141.1, 143.6, 143.7, 155.4, 171.9, 203.1. LCMS (EI): m/z 476.3 (M + 1)$^+$; t_r = 14.62 min (>99% purity) using solvent system I, t_r = 12.15 min (>99% purity) using solvent system II.

16g: 52% yield. IR: 1670, 1721 cm^{-1}. ^1H NMR (300 MHz): δ 0.86 (d, J = 6.3 Hz, 3H), 0.89 (d, J = 6.4 Hz, 3H), 1.21 (s, 9H), 1.26−1.61 (m, 3H), 2.96 (dd, J = 7.6, 14.0 Hz, 1H), 3.12 (dd, J = 6.7, 14.0 Hz, 1H), 3.25 (s, 2H), 4.10−4.15 (m, 1H), 5.09 (m, 1H), 5.26 (d, J = 14.7 Hz, 1H), 5.46 (d, J = 14.7 Hz, 1H), 6.01 (d, J = 7.8 Hz, 1H), 7.03 (d, J = 7.8 Hz, 1H), 7.16−7.33 (m, 5H), 7.59 (d, J = 7.5 Hz, 2H), 8.07 (t, J = 7.6 Hz, 1H), 8.84 (m, 1H). ^{13}C NMR (125 MHz): δ 21.6, 22.7, 24.6, 30.6, 37.6, 37.8, 41.0, 43.1, 54.2, 57.3, 64.1, 123.1, 124.4, 127.0, 128.6, 129.2, 136.1, 141.3, 145.8, 153.9, 155.0, 171.9, 204.9. LCMS (EI): m/z 500.3 (M + 1)$^+$; t_r = 11.20 min (96% purity) using solvent system I, t_r = 9.87 min (96% purity) using solvent system II.

16h: 65% yield. IR: 1668, 1720 cm^{-1}. ^1H NMR (500 MHz): δ 0.90 (d, J = 6.7 Hz, 3H), 0.92 (d, J = 6.6 Hz, 3H), 1.24 (s, 9H), 1.45−1.65 (m, 3H), 3.02−3.06 (m, 1H), 3.09−3.14 (m, 1H), 3.26 (d, J = 15.4 Hz, 1H), 3.31 (d, J = 15.0 Hz, 1H), 4.16 (m, 1H), 5.16−5.31 (m, 3H), 5.66 (m, 1H), 6.68 (d, J = 6.9 Hz, 1H), 7.14−7.28 (m, 5H), 7.67 (app s, 2H), 8.76 (app s, 2H). ^{13}C NMR (125 MHz): δ 21.8, 22.7, 24.7, 30.6, 37.8, 37.8, 41.5, 43.3, 53.8, 57.4, 64.0, 123.3, 127.2, 128.7, 129.1, 135.7, 143.6, 155.0, 155.2, 171.5, 205.0. LCMS (EI): m/z 500.3 (M + 1)$^+$; t_r = 10.64 min (>99% purity) using solvent system I, t_r = 9.72 min (96% purity) using solvent system II.

16i: 58% yield. IR: 1668, 1720 cm^{-1}. ^1H NMR (500 MHz): δ 1.26 (s, 9H), 2.93−3.07 (m, 4H), 3.01−3.07 (m, 1H), 3.20 (d, J = 15.1 Hz, 1H), 3.24 (d, J = 15.0 Hz, 1H), 4.38 (m, 1H), 5.10 (app q, J = 7.1 Hz, 1H), 5.15 (s, 2H), 5.58 (d, J = 7.3 Hz, 1H), 6.50 (d, J = 6.8 Hz, 1H), 7.07−7.66 (m, 10H), 7.66 (m, 1H), 8.04 (d, J = 7.3 Hz, 1H), 8.69 (br s, 1H), 8.76 (br s, 1H). ^{13}C NMR (125 MHz): δ 30.6, 37.7, 37.8, 38.4, 43.3, 56.2, 57.4, 63.1, 125.3, 127.1, 127.2, 128.67, 128.73, 129.1, 129.2, 134.9, 135.7, 135.9, 140.5, 144.31, 144.34, 155.0, 170.1, 204.5. LCMS (EI): m/z 534.2 (M + 1)$^+$; t_r = 11.25 min (>99% purity) using solvent system I, t_r = 9.99 min (96% purity) using solvent system II.

16j: 60% yield. IR: 1663, 1715 cm^{-1}. ^1H NMR (500 MHz): δ 0.88−0.91 (m, 6H), 1.24 (s, 9H), 1.43−1.61 (m, 3H), 3.00−3.04 (m, 1H), 3.09−3.13 (m, 1H), 3.25 (d, J = 15.4 Hz, 1H), 3.30 (d, J = 15.3 Hz, 1H), 4.10−4.16 (m, 1H), 5.15 (m, 1H), 5.20 (app s, 2H), 5.45 (d, J = 7.7 Hz, 1H), 6.67 (d, J = 7.1 Hz, 1H), 7.15−7.30 (m, 5H), 7.67 (m, 1H), 8.10 (d, J = 7.9 Hz, 1H), 8.69 (s, 1H), 8.81 (s, 1H). ^{13}C NMR (125 MHz): δ 21.8, 22.7, 24.6, 30.6, 37.77, 37.8, 41.4, 43.3, 53.7, 57.3, 63.1, 125.4, 127.1, 128.7, 129.1, 135.8, 140.8, 144.00, 144.01, 155.2, 171.1, 205.0. LCMS (EI): m/z 500.3 (M + 1)$^+$; t_r = 10.49 min (>99% purity) using solvent system I, t_r = 8.48 min (95% purity) using solvent system II.

16k: 51% yield. IR: 1667, 1717 cm^{-1}. ^1H NMR (500 MHz): δ 1.26 (s, 9H), 1.85 (m, 1H), 2.24 (m, 1H), 2.52 (m, 2H), 3.00−3.11 (m, 2H), 3.29 (d, J = 15.1 Hz, 1H), 3.33 (d, J = 14.9 Hz, 1H), 4.42 (m, 1H), 4.94 (m, 1H), 5.14 (d, J = 13.3 Hz, 1H), 5.18 (d, J = 13.7 Hz, 1H), 5.54 (m, 1), 6.58 (d, J = 6.8 Hz, 1H), 7.09−7.30 (m, 10H), 7.58 (m, 1H), 7.99 (d, J = 7.4 Hz, 1H), 8.64 (s, 1H), 8.74 (s, 1H). ^{13}C NMR (125 MHz): δ 30.6, 31.3, 33.0, 36.5, 38.3, 43.5, 56.3, 56.5, 63.2, 125.2, 126.3, 127.2, 128.3, 128.5, 128.8, 129.2, 134.9, 135.9, 140.4, 140.6, 144.4, 155.1, 170.4, 171.2, 204.8. LCMS (EI): m/z 548.3 (M + 1)$^+$; t_r = 12.25 min (>99% purity) using solvent system I, t_r =10.60 min (95% purity) using solvent system II.

16l: 50% yield. IR: 1668, 1720 cm^{-1}. ^1H NMR (500 MHz): δ 0.93 (m, 6H), 1.25 (s, 9H), 1.50−1.53 (m, 1H), 1.60−1.64 (m, 2H), 1.89 (m, 1H), 2.25 (m, 1H), 2.59 (m, 2H), 3.39 (m, 2H), 4.18 (m, 1H), 4.96 (m, 1H), 5.18 (d, J = 13.4 Hz, 1H), 5.24 (d, J = 13.4 Hz, 1H), 5.52 (m, 1), 6.73 (d, J = 7.2 Hz, 1H), 7.12−7.33 (m, 5H), 7.59 (m, 1H), 8.07 (d, J = 7.7 Hz, 1H), 8.63 (d, J = 4.2 Hz, 1H), 8.80 (s, 1H). ^{13}C NMR (125 MHz): δ 21.8, 22.8, 24.7, 30.6, 31.5, 33.2, 36.6, 41.3, 43.5, 53.8, 56.5, 63.1, 126.3, 128.4, 128.6, 129.9, 135.2, 140.6, 140.7, 143.9, 155.4, 171.8, 205.2. LCMS (EI): m/z 514.2 (M + 1)$^+$; t_r = 11.69 min (>99% purity) using solvent system I, t_r = 10.38 min (96% purity) using solvent system II.

General Procedure for Formation of *N*-[[(*n*-Pyridinyl)methoxy]carbonyl]amino Acids. In a 250 mL round-bottomed flask, 50 mL of CH_2Cl_2 and 50 mL of saturated sodium bicarbonate were added to 5 mmol of the amino acid ester hydrochloride. This suspension or solution was stirred at 0 °C for 10 min. A solution of phosgene (1.93 M in toluene,

5.2 mL, 10 mmol) was added by syringe to the organic layer. The solution was stirred an additional 20 min. After the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL), the extracts were dried over $MgSO_4$, filtered, and concentrated in vacuo to afford the crude isocyanate as a milky-white oil. A solution of the hydroxymethylpyridine in 0.2 M of CH_2Cl_2 was added to the crude isocyanate and stirred overnight. The methyl ester was purified by silica gel chromatography to give 66−100% of the product ester. To hydrolyze the ester, a solution of the phenylalanine methyl ester (2.5 g, 7.96 mmol) in dioxane (29 mL, 0.27 M) was treated with aqueous LiOH (32 mL, 0.5 M). After being stirred for 2h, the solution was quenched with HCl (14 mL, 1.0 M). The solution was extracted with EtOAc (3 × 25 mL), dried over $MgSO_4$, and concentrated to provide 55−69% of the desired N-protected amino acid as a white solid.

15a (2-PyrPheOH): 68% yield. IR: 1660, 3436 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 2.81−2.86 (m, 1H), 3.06−3.09 (m, 1H). 4.16−4.20 (m, 1H), 5.00 (s, 2H), 7.17−7.30 (m, 7H), 7.74−7.78 (m, 2H), 8.49 (d, J = 4.1 Hz, 1H). ^{13}C NMR (125 MHz, DMSO): δ 36.0, 56.0, 66.4, 121.3, 123.2, 126.8, 128.6, 129.5, 137.2, 138.3, 149.1, 156.2, 157.0, 173.4. LCMS (EI): *m*/*z* 301.1 $(M + 1)^+$; t_r = 4.04 min (>99% purity) using solvent system I, t_r = 0.84 min (>99% purity) using solvent system II.

15b (3-PyrPheOH): 60% yield. IR: 1655, 3425 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 2.79−2.84 (m, 1H), 3.02−3.08 (m, 1H), 4.14−4.18 (m, 1H), 4.98 (d, J = 12.9 Hz, 1H), 5.02 (d, J = 12.8 Hz, 1H), 7.19−7.37 (m, 6H), 7.64−7.69 (m, 2H), 8.50 (s, 2H). ^{13}C NMR (125 MHz, DMSO): δ 36.9, 56.0, 63.4, 123.9, 126.8, 128.6, 129.5, 133.0, 135.9, 138.3, 149.35, 149.43, 156.2, 173.7. Anal. ($C_{16}H_{16}N_2O_4S$) C, H, N.

15c (4-PyrPheOH): 68% yield. IR: 1661, 1713, 3436 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 2.84 (m, 1H), 3.10 (dd, J = 4.0, 14.0 Hz, 1H), 4.18−4.22 (m, 1H), 5.01 (s, 2H), 7.10−7.29 (m, 7H), 7.83 (d, J = 8.6 Hz, 1H), 8.49−8.50 (m, 2H). ^{13}C NMR (125 MHz, DMSO): δ 36.9, 56.0, 66.8, 121.7, 126.8, 128.6, 129.5, 138.3, 146.8, 149.9, 156.1, 173.7. LCMS (EI): *m*/*z* 301.1 $(M + 1)^+$; t_r = 3.91 min (95% purity) using solvent system I, t_r = 0.68 min (>99% purity) using solvent system II.

15d (2-PyrLeuOH): 69% yield. IR: 1655, 3425 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 0.84 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H), 1.44−1.58 (m, 2H), 1.62−1.69 (m, 1H), 3.95−4.00 (m, 1H), 5.07 (s, 2H), 7.29−7.31 (m, 1H), 7.37 (d, J = 7.8 Hz, 1H), 7.69 (d, J = 8.2 Hz, 1H), 7.80 (td, J = 7.7, 1.8 Hz, 1H), 8.52 (m, 1H). ^{13}C NMR (125 MHz, DMSO): δ 21.5, 23.3, 24.7, 52.7, 66.6, 121.5, 123.2, 137.3, 149.4, 156.4, 157.0, 174.8. LCMS (EI): *m*/*z* 267.1 $(M + 1)^+$; t_r = 3.88 min (>99% purity) using solvent system I, t_r = 0.82 min (>99% purity) using solvent system II.

15e (3-PyrLeuOH): 55% yield. IR: 1651, 1712 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 0.83 (d, J = 6.5 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H), 1.42−1.55 (m, 2H), 1.58−1.65 (m, 1H), 3.93−3.98 (m, 1H), 5.05 (d, J = 12.7 Hz, 1H), 5.08 (d, J = 12.7 Hz, 1H), 7.38 (dd, J = 4.8, 7.8 Hz, 1H), 7.62 (d, 1, J = 8.2), 7.76 (d, J = 7.8 Hz, 1H), 8.51−8.56 (m, 2H). ^{13}C NMR (125 MHz, DMSO): δ 21.5, 23.3, 24.7, 52.6, 63.6, 123.9, 133.0, 136.0, 149.5, 149.5, 156.4, 174.7. LCMS (EI): *m*/*z* 267.1 $(M + 1)^+$; t_r = 3.75 min (>99% purity) using solvent system I, t_r = 0.71 min (>99% purity) using solvent system II.

15f (4-PyrLeuOH): 56% yield. IR: 1649, 3401 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 0.84 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H), 1.45−1.49 (m, 1H), 1.52−1.58 (m, 1H), 1.64−1.67 (m, 1H), 3.94−4.00 (m, 1H), 5.05 (d, J = 14.3 Hz, 1H), 5.10 (d, J = 14.3 Hz, 1H), 7.26−7.35 (m, 2H), 7.73 (d, J = 8.2 Hz, 1H), 8.53−8.54 (m, 2H). ^{13}C NMR (125 MHz, DMSO): δ 21.5, 23.3, 24.7, 52.7, 64.0, 121.9, 146.7, 150.0, 156.3, 174.7. LCMS (EI): *m*/*z* 267.1 $(M + 1)^+$; t_r = 3.80 min (97% purity) using solvent system I, t_r = 0.70 min (>99% purity) using solvent system II.

General Procedure for Formation of *N*-Morpholinecarbonyl Amino Acid. These N-protected amino acids were synthesized using a modified procedure by Stevens and Watanabe.[19] 4-Morpholinecarbonyl chloride was substituted for Alloc chloroformate. These amino acids were used without purification in the solid-phase synthesis.

17a (MuLeuOH): 24% yield. IR: 1643, 1662 cm^{-1}. ^1H NMR (500 MHz, DMSO): δ 0.82 (d, J = 6.5 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H), 1.43−1.62 (m, 3H), 3.11 (m, 2H), 3.21−3.30 (m, 2H), 3.48−3.54 (m, 4H), 4.00 (m, 1H), 6.50 (m, 1H). ^{13}C NMR (125 MHz, DMSO): δ 21.9, 23.5, 24.8, 44.4, 47.2, 66.30, 66.35, 158.1, 163.5. Anal. ($C_{11}H_{20}N_2O_4$) C, H, N.

17b (MuPheOH): 56% yield. IR: 1630, 1727 cm^{-1}. ^1H NMR (300 MHz, DMSO): δ 2.83−2.91 (m, 1H), 3.00 (m, 1H), 3.09−3.21 (m, 4H), 3.45 (m, 4H), 4.15−4.22 (m, 1H), 6.73 (d, J = 8.2 Hz, 1H), 7.14−7.38 (m, 5H). ^{13}C NMR (125 MHz, DMSO): δ 37.0, 44.3, 47.2, 55.8, 66.26, 66.29, 126.7, 128.5, 129.5, 138.8, 157.8, 174.6. Anal. ($C_{14}H_{18}N_2O_4$) C, H, N.

High Throughput Cruzain Assay. A fluorometric high throughput assay for activity toward cruzain was performed in 96-well microtiter plates. The assay was performed in DYNATECH Microfluor fluorescence microtiter plates (opaque white plates), and readings were taken on a Perkin-Elmer LS-50B with an attached 96-well plate reader. The excitation wavelength was 355 nm, and the emission wavelength was 450 nm. A 430 nm cutoff filter for emission was used. The peptide substrate Z-Phe-Arg-AMC (Bachem, K_m = 1 μM) concentration was 2.5 μM, and the cruzain concentration was 0.1 nM. The buffer consisted of a 100 mM solution of pH 5.5 sodium acetate buffer and 1 mM of DTT.

The fluorescent unit readings were taken at four time points within the linear region of the substrate cleavage, and percentage activity of the enzyme was determined by comparing the change of fluorescent units (FU) for each well against the average change in FU for eight control wells without inhibitor. All compounds were assayed in duplicate.

Sample procedure: In each well was placed 25 μL of enzyme solution, 125 μL of buffer solution, and 10 μL of the inhibitor in DMSO. The compounds were placed only in rows 2−11, with row 12 used for the control wells. Following a 5 min preincubation of the enzyme and inhibitor, 50 μL of the substrate solution was added to each well. The plate was immediately placed into the plate reader, and fluorescent readings were taken. The data were analyzed by transferring each plate's readings from the spectrophotometer to EXCEL spreadsheets. An EXCEL macro was written to obtain the slope for each well compared to the slope of the controls. Inhibitors were screened at 1 μM, 330 nM, 110 nM, and 55 nM. Potent inhibitors were screened in 2-fold dilutions from 55 nM to 1.7 nM.

Inhibition Assay for Cathepsin B and Cathepsin L. Continuous fluorometric inhibition assays for cathepsin B and cathepsin L were performed as described in the literature.[22] Cathepsin B (10 nM) and cathepsin L (6.9 nM) were assayed at room temperature with the substrate Z-Phe-Arg-AMC (for cathepsin B 10 μM, K_m = 220 μM; for cathepsin L 4 μM, K_m = 2.9 μM). The assay is the same as performed above for cruzain, except that the buffer was composed of 0.25 M solution of pH 5.5 sodium acetate buffer containing 2 mM EDTA, 0.015% Brij-35, and 1 mM DTT.

K_i Determination. For potent inhibitors, the data was fit by nonlinear regression analysis to the equation derived by Williams and Morrison:[11]

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

For cruzain, the K_m for the substrate was determined to be 1 μM by using a Lineweaver Burke Plot and Eadie−Hofstee Plot. The variables S, E_t, and I_t are the concentrations of substrate, active enzyme, and inhibitor, respectively.

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