Design and Synthesis of Dipeptide Nitriles as Reversible and Potent Cathepsin S Inhibitors

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The specificity of the immune response relies on processing of foreign proteins and presentation of antigenic peptides at the cell surface. Inhibition of antigen presentation, and the subsequent activation of T-cells, should, in theory, modulate the immune response. The cysteine protease Cathepsin S performs a fundamental step in antigen presentation and therefore represents an attractive target for inhibition. Herein, we report a series of potent and reversible Cathepsin S inhibitors based on dipeptide nitriles. These inhibitors show *nano*molar inhibition of the target enzyme as well as cellular potency in a human B cell line. The first X-ray crystal structure of a reversible inhibitor cocrystallized with Cathepsin S is also reported.

Introduction

Autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS) occur when a sustained, adaptive immune response is mounted against self-antigens resulting in chronic inflammation and injury to the tissues. The modulation of antigen-specific immune responses has long been a desirable approach for the treatment of autoimmune diseases. In autoimmune diseases, self-proteins are internalized by antigen presenting cells, degraded into peptides, and then delivered to the cell surface in association with the Major Histocompatibility Complex (MHC) Class II molecules. The MHC Class II/peptide complex engages CD4+ T cells, which precipitates an immune response. Although the biochemical events involved in antigen processing and presentation are complex,[1−5] clear targets exist within the pathway that are amenable to therapeutic intervention. MHC Class II heterodimers have been shown to associate intracellularly with a molecule designated Invariant Chain (Ii).[6] Invariant Chain interacts directly with the MHC Class II binding groove and must be proteolyzed prior to antigen loading and presentation. Current research suggests that Invariant Chain is selectively proteolyzed by a series of aspartyl and cysteine proteases within the endosomal compartments of the cell.[4] In the final step of this proteolysis Cathepsin S, a 24 kD cysteine protease from the papain superfamily degrades the remaining Invariant Chain oligomer (Iip10) to a small peptide termed CLIP. CLIP is subsequently released from the MHC Class II binding groove by association with a third protein termed HLA-DM.[7,8] Once CLIP is released, the free MHC Class II binding groove loads with antigenic peptides. The MHC Class II peptide complexes are then transported to the cell surface for presentation to T-cells, and the immune response is initiated. Cathepsin S, through proteolysis of Invariant Chain, provides a fundamental step in the generation of the immune response and therefore is an attractive target for immunoregulation. Recent work has shown that splenocytes from mice deficient in Cathepsin S have impaired Invariant Chain processing and peptide loading.[9,10]

Cathepsin S acts catalytically through the nucleophilic addition of the thiolate anion of cysteine-25 (formed as an ion-pair with histidine-164) to the carbonyl carbon of the peptide bond followed by hydrolysis to yield the peptide products. Most of the inhibitors of cysteine proteases reported in the literature depend on the chemical interaction of an electrophilic "warhead" with the cysteine thiolate anion of the active site and, in principle, the enzyme can be inactivated either irreversibly or reversibly by such inhibitors.[11−17] Irreversible inhibitors, such as the dipeptide-vinyl sulfone LHVS[12−14] or the epoxide E64,[15] have been shown to be effective inhibitors of Cathepsin S. Since irreversible inhibitors have the potential drawback of haptenization which could cause immunogenicity, we chose to focus on the design of reversible inhibitors. Reversible inhibitors of Cathepsin S and related enzymes, both with and without an electrophilic warhead, have been reported in the patent literature.[17−20] Reversible inhibitors with electrophilic warheads, such as α,α′-bis-amino ketones, have also been shown to inhibit the closely related cysteine protease Cathepsin K.[17] Palmer et al.[18] described the use of peptidyl sulfones as reversible inhibitors of Cathepsin S, while Klaus et al.[19,20] have reported that peptidyl ethylenediamines inhibit Cathepsin S reversibly, though, in each case no covalent bond to Cys-25 is formed. Nitriles are known to be inhibitors of

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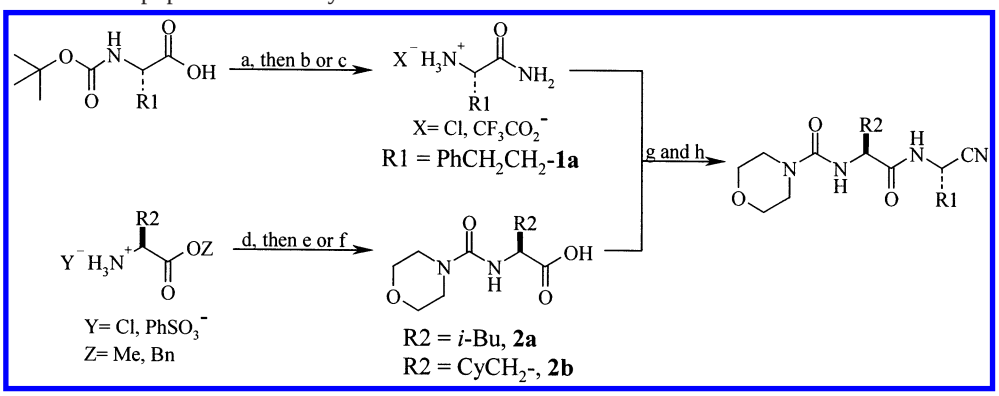
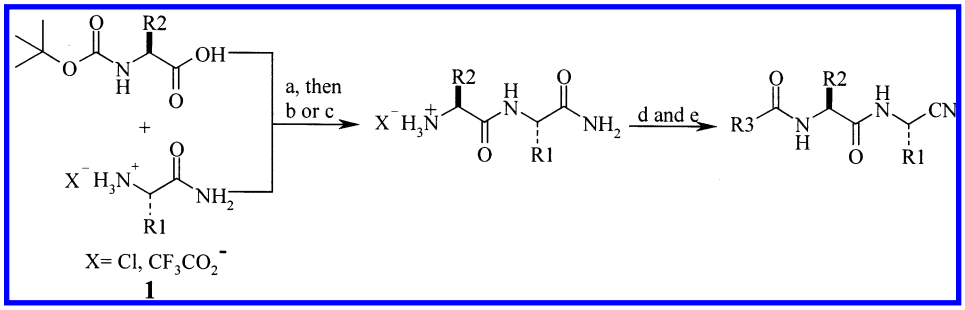
Scheme 1. Method A of Dipeptide Nitrile Synthesis[a]



[a] Conditions: (a) EDC, HOBt, DMF, 0 °C, 15 min, then NH_4OH; (b) 4 N HCl/dioxane; (c) 50% TFA/CH_2Cl_2; (d) 4-morpholinecarbonyl chloride, Hunig's base, DMF, 22 °C; (e) when Z= Bn then Pearlman's catalyst, cyclohexene, EtOH, reflux, 45 min; (f) when Z = Me then 0.5 N LiOH, water/MeOH (1 to 1), 22 °C, 16 h; (g) activate the carboxylic acid with EDC and HOBt in DMF, DMF, 0 °C, 15 min, then add the amino-amide salt and 4 equiv of Hunig's base, 22 °C, 16 h; (h) cyanuric chloride, DMF, 0 °C, 1 h.

Scheme 2. Method B of Dipeptide Nitrile Synthesis[a]



[a] Conditions: (a) activate the *N*-Boc-amino acid with EDC and HOBt, DMF, 0 °C, 15 min, then add the amino-amide salt and Hunig's base, 22 °C, 16 h; (b) 4 N HCl/dioxane; (c) 50% TFA/CH_2Cl_2; (d) $R3CO_2H$, EDC, HOBt, Hunig's base, DMF, 22 °C, 16 h; (e) cyanuric chloride, DMF, 0 °C, 1 h.

cysteine proteases and were first reported by Hanzlik as inhibitors of the plant protease papain.[21] These peptide nitriles were shown by NMR to form covalent bonds with the active site cysteine, and this bond formation was shown to be reversible. At the time we began our work there were no reports of peptide nitriles as inhibitors of Cathepsins, and we chose to explore the feasibility of this approach.[22−32]

Synthetic Chemistry. The nitrile inhibitors of this study were prepared by three different general methods. In Method A, as described in Scheme 1, treatment of the commercially available *N*-Boc amino acids with EDC, HOBt, and NH_4OH followed by Boc-deprotection with either HCl/dioxane or TFA/CH_2Cl_2 led to amino amide salts **1**. Reaction of the commercially available tosylate or hydrochloride salts of either the benzyl or methyl esters of amino acids with commercially available 4-morpholinecarbonyl chloride in DMF with Hunig's base followed by de-esterification of the esters led to carboxylic acids **2**. Coupling of carboxylic acids **2** and amino amide salts **1** mediated by EDC/HOBt followed by dehydration of the terminal primary amide with a combination of cyanuric chloride in DMF provided the desired dipeptide nitriles.

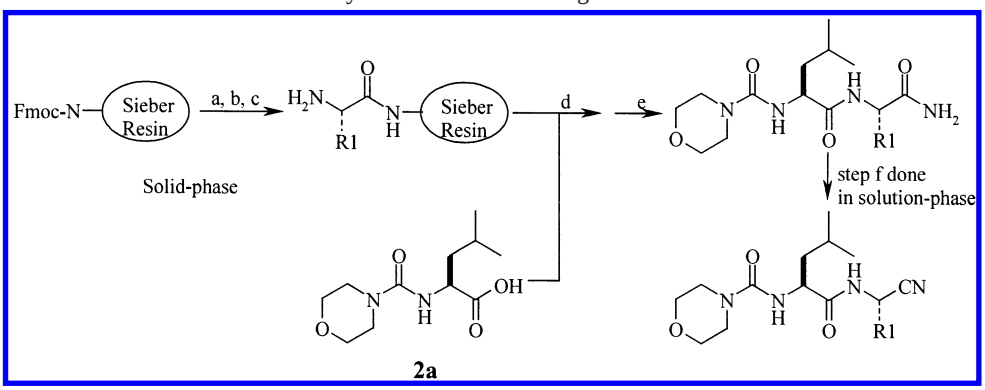
In Method B, as described in Scheme 2, coupling of amino amide salts **1** to commercially available *N*-Boc amino acids, Boc-deprotection, coupling with carboxylic acids, and dehydration of the terminal amide provided the desired dipeptide nitriles.

In addition, a number of the analogues discussed below were prepared via a combination of a parallel synthesis of the dipeptide amides on solid-phase followed by a solution-phase dehydration as outlined for Method C in Scheme 3.[33] *N*-Fmoc protected Sieber-amide resin was deprotected with piperidine in DMF followed by coupling with commercially available *N*-Fmoc amino acids. Fmoc-deprotection was followed by EDC/HOBt-mediated coupling with 4-methyl-2-[(morpholine-4-carbonyl)-amino]-pentanoic acid **2a**. Cleavage of the amide from the resin was accomplished with 10% TFA/CH_2Cl_2. The dehydration with cyanuric chloride/DMF was performed in a parallel solution-phase operation. The final products were purified by a combination of solid-phase extraction followed by purity-analysis by reverse-phase HPLC.

The methylene homologue of inhibitor **6** was prepared in the manner outlined in Scheme 4. *N*-Boc-L-*homo*phenylalanine was converted to a mixed anhydride using isobutyl chloroformate and *N*-methylmorpholine in THF. The mixed anhydride was reduced with $NaBH_4$ in MeOH/H_2O/THF to give alcohol **3**. Mesylation with the sulfonyl chloride was followed by displacement of the mesylate with cyanide anion in DMSO to give Boc-protected nitrile **4**. Deprotection of the nitrile with 50% TFA/CH_2Cl_2 followed by coupling of the deprotected nitrile to carboxylic acid **2a** afforded the homologous nitrile **5**.

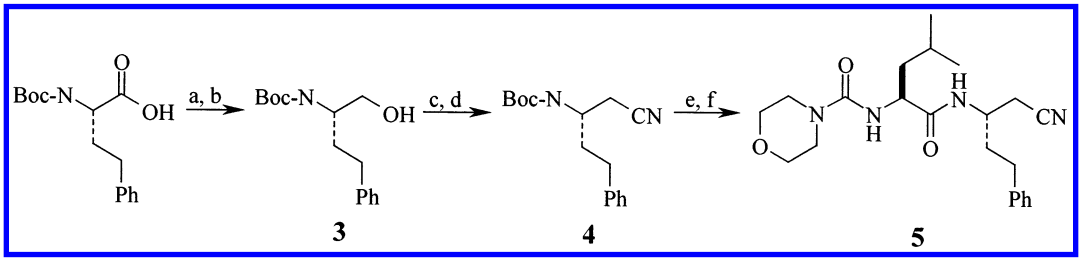
Biochemistry. Human recombinant Cathepsin S expressed in baculovirus is used at a final concentration of 10 nM in buffer at pH 6.5. Cathepsin S is incubated with inhibitor for 10 min at 37 °C, and then the fluorescent substrate (7-amino-4-methylcoumarin-CBZ-

Scheme 3. Method C: Solid/Solution-Phase Synthesis of P1 Analogues[a]



[a] Conditions: (a) piperidine, DMF, 22 °C, 30 min; (b) Fmoc-NCH(R1)CO_2H was preactivated with EDC and HOBt in DMF and then mixed with the deprotected amine resin followed by addition of Hunig's base, DMF, 22 °C, 16 h; (c) piperidine, DMF, 22 °C, 30 min; (d) **2a** is preactivated with EDC and HOBt in DMF and then mixed with the resin-bound amino amide followed by addition of Hunig's base, DMF, 22 °C 16 h; (e) 10% TFA/CH_2Cl_2, 22 °C, 30 min; (f) cyanuric chloride, DMF, 0 °C, 1 h.

Scheme 4. Synthesis of Homologous Nitrile 5[a]



[a] Conditions: (a) *N*-methylmorpholine, isobutyl chloroformate, THF, −10 °C, 15 min; (b) $NaBH_4$, water/MeOH/THF, 0 °C, 30 min; (c) Et_3N, $MeSO_2Cl$, CH_2Cl_2, 0 °C, 1 h; (d) NaCN, DMSO, 90 °C, 2 h; (e) 50% TFA/CH_2Cl_2; (f) **2a**, TBTU, Hunig's base, CH_2Cl_2, 22 °C, 4 h.

L-valyl-L-valyl-L-arginineamide) (custom synthesis by Molecular Probes) is added to assay (substrate concentration 5 μM) and incubated for an additional 10 min at 37 °C. Inhibitor activity is measured by diminished fluorescence compared to DMSO control when read at 360 nm excitation and 460 nm emission.

The K_d values were determined by a fluorescence-based equilibrium competitive binding assay using either of the dansylated compounds **11a**,**b** as the fluorescent probe (see Experimental Section for details).

Kinetic parameters k_{on} and k_{off} were determined using a ligand-exchange assay as described in the Experimental Section.

Results and Discussion

The goal for this work was to answer three fundamental questions: (1) can nitriles act as potent, reversible inhibitors of Cathepsin S; (2) what are the optimal residues for P1, P2, and P3;[34] and (3) can dipeptide nitriles afford inhibition of Cathepsin S in a cellular system? To address these questions, dipeptide nitrile **6** was synthesized as the first member of the series (Figure 1).

Compound **6** was found to be a potent and reversible inhibitor of Cathepsin S (IC_{50} = 42 nM, K_d = 7 ± 2 nM) (Table 1). Reversibility of complexation of **6** with Cathepsin S was demonstrated by three different methods (see Experimental Section for details): (1) incubation of **6** with Cathepsin S and analysis of the sample solution via HPLC/MS demonstrated that the inhibitor does not irreversibly modify the enzyme; (2) kinetic constants (k_{on} and k_{off}) could be measured for a number of the nitrile inhibitors by a ligand-exchange assay; and (3) size-exclusion-chromatography of a sample of excess **6**

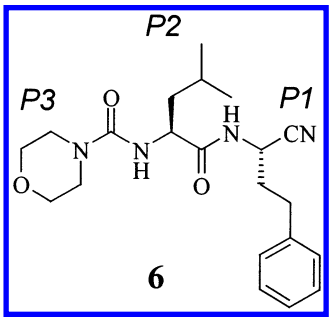
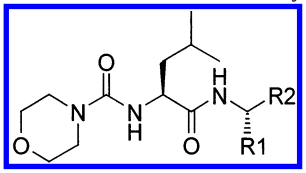


Figure 1. Lead structure **6**.

Table 1. Importance of the Nitrile for Activity



compd	R1	R2	IC_{50} (nM)	K_d (nM)
6	CH_2CH_2Ph	-CN	42	7 ± 2
7	CH_2CH_2Ph	H	>28000	n.d.
8	CH_2CH_2Ph	Me	>339000	n.d.
9	H	CN	124	n.d.
10	H	CCH	97000	n.d.
5	CH_2CH_2Ph	CH_2CN	>13000	>20000

incubated with Cathepsin S restored catalytic activity to the enzyme. With these results in hand, the necessity of the nitrile for potency was further explored. Replacement of the nitrile with a hydrogen atom resulted in a dramatic loss in potency (compound **7**, IC_{50} > 28000 nM, Table 1), lending support to the theory that the nitrile functions as an electrophilic warhead. However, one

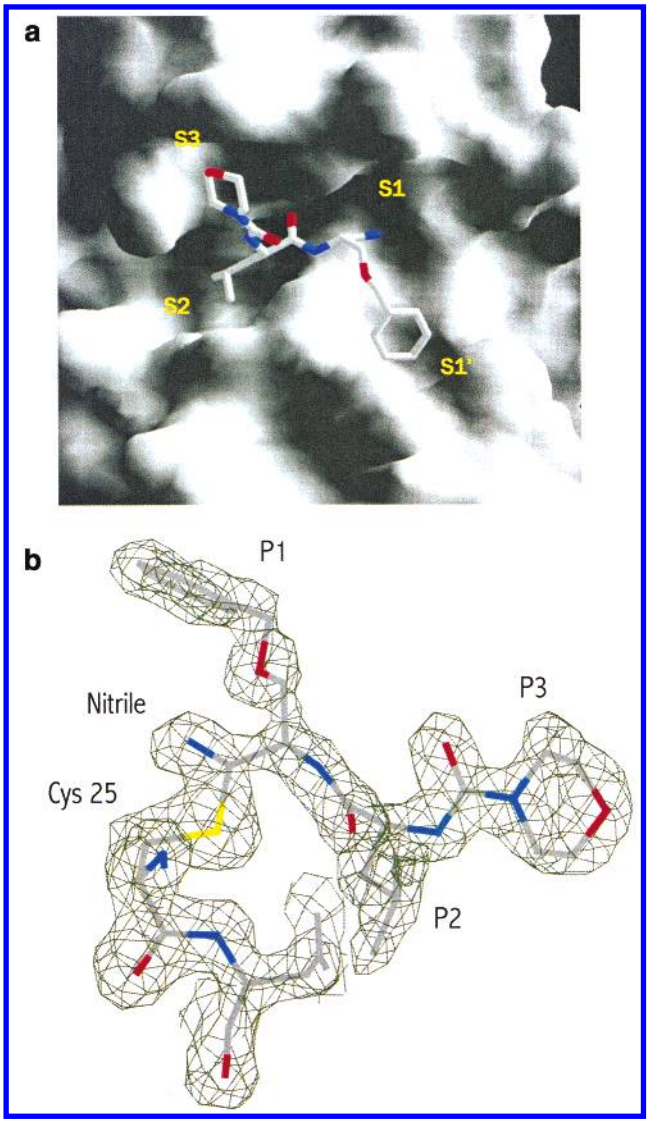
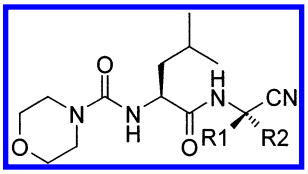


Figure 2. Two views of X-ray structure of inhibitor **22** (a and b).

could still argue that the nitrile was acting as a lipophilic group and its replacement with a hydrogen results in a loss of some favorable hydrophobic interaction with the enzyme. To test this theory, we replaced the nitrile of inhibitor **6** with a methyl group, which also gave an inactive compound (compound **8**, IC_{50} > 339000 nM). Comparison of dipeptide nitrile **9** (IC_{50} = 124 nM) with compound **10** (IC_{50} = 97000 nM) demonstrates that the dramatic loss in activity is also observed when the nitrile is replaced by the similarly hybridized alkyne. To assess the importance of the nitrile's location on the inhibitor backbone, we prepared a methylene analogue of inhibitor **6** (compound **5**, IC_{50} > 13000 nM) which also resulted in a great loss in potency. Whether the loss of activity for compound **5** is electronic in nature or is due to a geometric restraint is unknown. We concluded from these experiments that the nitrile is critical for potency against Cathepsin S and is not simply acting as a lipophilic substituent. This conclusion was later supported by solution of the X-ray structure of compound **22** cocrystallized with Cathepsin S (Figure 2b) which clearly shows electron density between the active-site cysteine residue and the nitrile carbon, indicating the formation of a reversible thio-imidate bond.

Table 2. SAR of the P1 Residue



compd	R1	R2	IC_{50} (nM)	K_d (nM)
6	H	CH_2CH_2Ph	47[a]	7 ± 2
9	H	H	124	n.d.
12	H	Me	142	n.d.
13	H	*n*-Pr	82	n.d.
14	H	*n*-Bu	56	n.d.
15	H	*i*-Pr	1400	n.d.
16	H	*t*-Bu	107000	n.d.
17	H	Ph	51	4 ± 2
18	H	CH_2Ph	130	63 ± 10
19	H	CH_2(3,4-diCl)Ph	56	7 ± 3
20	CH_2CH_2Ph	H	387[b]	28 ± 6
21	Me	Me	114	n.d.
22	H	CH_2OCH_2Ph	19[c]	0.3 ± 0.2
23	H	CH_2OCH_2(*o*-Cl)Ph	9	0.3 ± 0.2
24	H	CH_2OCH_2(*m*-Cl)Ph	9	0.5 ± 0.2
25	H	CH_2OCH_2(*p*-Cl)Ph	27	2 ± 1

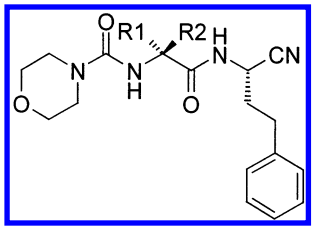
[a] Mean of eight assays between 14 and 88 nM. [b] Mean of four assays between 213 and 627 nM. [c] Mean of three assays between 9 and 25 nM.

After establishing the importance of the nitrile, a systematic investigation of the SAR of the P1 residue was begun. Unbranched alkyl side chains showed a general trend toward somewhat increased potency with length (compounds **12**−**14**, Table 2). However, branching at the β-carbon of the amino acid residue gave less potent inhibitors (compounds **15**, IC_{50} = 1400 nM and **16**, IC_{50} = 107000 nM). The single exception to this trend is the phenylglycine derivative **17** which displays excellent potency (IC_{50} = 51 nM and K_d = 4 ± 2 nM). Phenylalanine at P1 proved to be less potent than *homo*phenylalanine (compare inhibitors **6** and **18**, Table 2), however, the loss in binding could be recovered by substituting on the phenylalanine ring with a halogen such as chlorine (compound **19**, IC_{50} = 56 nM).

To our surprise, D-*homo*phenylalanine derivative **20** (IC_{50} = 387 nM, K_d = 28 ± 6 nM) retained significant potency against Cathepsin S. While this result could possibly be explained by presence of a small amount of P1-epimer **6**, such epimerization could not be detected by ^{1}H NMR of a sample of **20**. At an earlier stage of our investigation, we had established the configurational stability of inhibitor **6** at pH from 4.5 to 8. Only at pH = 2 did inhibitor **6** show any formation of epimer **20** (4% of **6** had epimerized to **20** after 7 days). In addition, the formation of diastereomeric mixtures during inhibitor synthesis was observed only once from the many analogues synthesize (see experimental for P1-phenylglycine analogue **17**). These results lead us to investigate an α,α-disubstituted amino acid as the P1-residue resulting in α,α-dimethylglycine analogue **21** which retained comparatively good potency (IC_{50} = 114 nM).

A breakthrough in the molecular potency occurred when the P1 position was substituted by *O*-benzyl serine analogues (compounds **22**−**25**, Table 2) which resulted in picomolar binding constants to Cathepsin S. *Ortho*- and *meta*-substitution of the benzyl group were found to be slightly more favorable than *para*-substitution (compare inhibitors **23** and **24** with inhibitor **25**, Table 2).

Table 3. SAR of P2 Residue



compd	R1	R2	IC_{50} (nM)	K_d (nM)
6	H	CH_2(*i*-Pr)	47	7 ± 2
26	CH_2(*i*-Pr)	H	11000	n.d.
27	CH_3[a]	CH_2(*i*-Pr)	76000	n.d.
28	H	CH_3	134000	n.d.
29	H	*n*-Bu	41	9 ± 1
30	H	*i*-Pr	6000	n.d.
31	H	Cy	84	5 ± 3
32	H	CH_2Ph	173	60 ± 4
33	H	CH_2*t*-Bu	23	0.9 ± 0.1
34	H	CH_2Cy	5	0.8 ± 0.5

[a] 50/50 mixture of diastereomers where R1 = CH_2(*i*-Pr) and R2 = CH_3.

Next we sought to optimize the P2 residue. Inverting the stereochemistry of the leucine residue of dipeptide nitrile **6** resulted in a weakly active inhibitor (compound **26**, IC_{50} = 11000 nM, Table 3). In addition, α-methylation of the P2 residue of inhibitor **6** resulted in nearly complete loss of activity (compound **27**, IC_{50} = 76000 nM). As with the P1 side chain, increasing the length of straight-chain alkyls at P2 improved the binding of the inhibitors to the enzyme; for example, changing from alanine to *n*-butylglycine, improves the IC_{50} from 134000 nM to 41 nM (compare inhibitors **28** and **29**, Table 3). Mono-branching from the *β*-carbon of the P2 residue results in some loss in potency unless the substituent more completely fills the S2 pocket (compare inhibitors **30** and **31**, Table 3). Phenylalanine proved to be an unsatisfactory residue for the P2 position (inhibitor **32**, K_d = 60 ± 4 nM). Two optimal P2 residues were found to be L-*β*-*tert*-butylalanine (inhibitor **33**, K_d = 0.9 ± 0.1 nM), and L-*β*-cyclohexylalanine (inhibitor **34**, K_d = 0.8 ± 0.5 nM).

With new P1 and P2 residues in hand, we began to combine the best of both groups in an attempt to gain synergistic improvement of our inhibitors (Table 4). For inhibitors bearing homophenylalanine and substituted or unsubstituted O-benzylserines as the P1 residue, the effect of changing the P2 residue from L-leucine to L-*β*-*t*-butylalanine or L-*β*-cyclohexylalanine provided an improvement of between 3- and 20-fold based on comparison of K_ds, providing a number of very potent picomolar inhibitors of Cathepsin S (inhibitors **33**−**36** and **38**−**40**, Table 4). Replacing L-leucine with L-*β*-cyclohexylalanine at P2 resulted in a 4-fold improvement in the potency of the P1 phenylglycine analogues (compare inhibitors **17** and **41**, Table 4). Replacing L-leucine with L-*β*-cyclohexylalanine as the P2 residue resulted in an approximate 6-fold improvement in the potency of the inhibitor with α,α-dimethylglycine as the P1 residue (compare IC_{50}s of inhibitors **21** and **42**, Table 4).

Solution of the crystal structure of inhibitor **22** complexed with Cathepsin S revealed that the *O*-benzylserine binds in the S1′ pocket of the enzyme rather than in the S1 (Figure 2a).[34] Examination of the structure reveals that the lipophilic S2 pocket is large enough to accommodate P2 side chains larger than *i*-butyl which can explain the enhancement of potency seen with the larger side-chains such as *neo*pentyl and *cyclo*hexylmethyl.

A number of acceptable P3 residues were also identified which were essentially equipotent to the morpholin-4-yl series (inhibitors **36** and **43**−**46**, Table 5).

With potent inhibitors of the Cathepsin S enzyme in hand, we next investigated their activity in a cellular assay. The primary cellular assay was the same as developed by Riese et al.,[5a] in which HOM2 cells (human B cell line) are starved of methionine for 30 min, pulsed with ^{35}S methionine and chased for 4 h in the presence of compound. After lysing, the MHC Class II and associated Invariant Chain is precipitated with mouse anti-human Class II monoclonal antibody Tu 36. Antibody/Class II complexes are subjected to SDS−PAGE, and the presence or absence of p10 Invariant Chain fragment (the natural Cathepsin S substrate) is determined by exposure of the gel to a PhosphorImager. The numbers reported in Table 4 are the *lowest* doses of compound at which the p10 band can be detected and are called the minimal inhibitory concentrations (MICs). For each gel, the inactive nitrile **5** is used as a negative control at 1 μM for background subtraction. In general, L-*β*-cyclohexylalanine as the P2 residue provided the most potent compounds in the cellular assay followed by L-*β*-*tert*-butylalanine and L-leucine, the same order of potency based on the IC_{50}s and K_ds (compare inhibitors **6** and **33**−**34**, **22** and **35**−**36**, plus **37**−**39**, Table 4). The most active compounds in cells were **34, 36**, **41**, and **42** with p10 MICs of 100 nM. Thus, the dipeptide nitriles afford inhibition of Cathepsin S in a cellular system.

Study of the kinetics (k_{on} and k_{off}) of several of the more potent inhibitors revealed an interesting observation: inhibitor **42**, with the dimethylglycine residue at P1, had on- *and* off-rates 2 to 3 orders of magnitude slower than the P1-monosubstituted inhibitors such as **6**, **33**, **22**, **35**, and **36** (Table 4). The explanation for this difference in behavior is not yet clear; however, the increased steric bulk about the electrophilic nitrile could account for the slower on-rate.

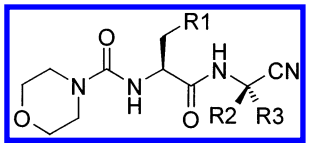
Conclusion

In the present report we have described the design of potent and reversible dipeptide nitrile inhibitors of the Cathepsin S enzyme. Systematic investigation of the SAR of the P1, P2, and P3 positions has provided picomolar inhibitors of Cathepsin S. We have presented the first published X-ray crystal structure of dipeptide nitriles cocrystallized with Cathepsin S and have shown that binding in the S1′ selectivity site plays an important role in molecular potency. In addition, we have demonstrated that our dipeptide nitriles exhibit potency in a cellular assay. Finally, the detection of the p10 Invariant Chain fragment (the substrate for Cathepsin S) in the cellular assay demonstrates that we are inhibiting the molecular target in the cellular system.

Experimental Section

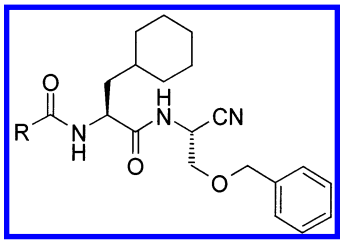
General Synthetic Procedures. ^1H NMR spectra were recorded on a Bruker AM-400 MHz or AM-270 MHz NMR in either DMSO-d_6 or $CDCl_3$. Electrospray ionization mass

Table 4. In Vitro Activities of the Most Potent Inhibitors



compd	R1	R2	R3	IC_{50} (nM)	K_d (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d calcd (k_{off}/k_{on}) (nM)	p10 MIC (nM)
6	*i*-Pr	H	CH_2CH_2Ph	47	7 ± 2	4.2×10^7	2.6×10^{-1}	6.2	5000
33	*t*-Bu	H	CH_2CH_2Ph	23	0.9 ± 0.1	4.7×10^7	6.3×10^{-2}	1.0	5000
34	c-Hex	H	CH_2CH_2Ph	5	0.8 ± 0.5	n.d.	n.d.	n.d.	100
22	*i*-Pr	H	CH_2OCH_2Ph	19	0.3 ± 0.2	1.7×10^8	2.2×10^{-2}	0.13	500
35	*t*-Bu	H	CH_2OCH_2Ph	8	0.6 ± 0.4	6.9×10^7	2.1×10^{-2}	0.30	500
36	c-Hex	H	CH_2OCH_2Ph	6	0.4 ± 0.2	2.0×10^8	8.5×10^{-2}	0.43	100
37	*i*-Pr	H	CH_2OCH_2(*o*-Me)Ph	19	3 ± 1	n.d.	n.d.	n.d.	5000
38	*t*-Bu	H	CH_2OCH_2(*o*-Me)Ph	12	0.3 ± 0.1	n.d.	n.d.	n.d.	1000
39	c-Hex	H	CH_2OCH_2(*o*-Me)Ph	7	0.1 ± 0.05	n.d.	n.d.	n.d.	500
23	*i*-Pr	H	CH_2OCH_2(*o*-Cl)Ph	9	1 ± 1	n.d.	n.d.	n.d.	500
40	c-Hex	H	CH_2OCH_2(*o*-Cl)Ph	8	0.4 ±	n.d.	n.d.	n.d.	n.d.
17	*i*-Pr	H	Ph	51	4 ± 2	n.d.	n.d.	n.d.	5000
41	c-Hex	H	Ph	9	1 ± 0.3	n.d.	n.d.	n.d.	100
21	*i*-Pr	Me	Me	114	n.d.	n.d.	n.d.	n.d.	n.d.
42	c-Hex	Me	Me	17	4 ± 3	2.6×10^5	8.5×10^{-4}	3.3	100

Table 5. SAR of P3 Residue



compd	R	IC_{50} (nM)	K_d (nM)
36	morpholin-4-yl	6	0.4 ± 0.2
43	pyridin-4-yl	6	0.7 ± 0.1
44	furan-2-yl	21	0.4 ± 0.2
45	thien-2-yl	5	0.1 ± 0.05
46	pyrazinyl	9	0.6 ± 0.2

spectra were obtained on a Micromass Platform LCZ. Purity of compounds was verified by elemental analysis (C, H, N) unless otherwise noted. Reactions were monitored by TLC on precoated silica gel 60 F_{254} plates purchased from EM-Science. Protected amino acids were purchased from Bachem, Sigma, or Novobiochem and used as received. All other reagents were purchased from Aldrich, Lancaster, or Fluka and used without further purification. Anhydrous solvents were purchased from Aldrich, and all other solvents were purchased from EM-Science and used as received. Silica gel chromatography was performed with Silca Gel 60 (mesh 230−400) purchased from EM-Science using either hexanes/EtOAc or MeOH/CH_2Cl_2 as the mobile-phase. Analytical HPLC was performed on analytical C18 columns (Hypersil) using acetonitrile/water with 0.1% trifluoroacetic acid as the mobile phase.

Preparation of Morpholine-4-carboxylic Acid [1*S*-(2-Cyano-1*S*-phenethyl-ethylcarbamoyl)-3-methylbutyl]-amide (5). (a) Preparation of (1*S*-Hydroxymethyl-3-phenylpropyl)carbamic Acid *tert*-Butyl Ester (3). *N*-Boc-L-homophenylalanine (1 g, 3.58 mmol) was mixed in dry THF (50 mL). *N*-Methylmorpholine (362 mg, 3.58 mmol) was added, and the resulting clear solution was cooled to −10 °C with a methanol/ice bath. Isobutyl chloroformate (464 μL, 489 mg, 3.58 mmol) was added dropwise from a syringe over a 1 min period during which time the *N*-methylmorpholine HCl salt precipitated. Stirring was continued for 15 min at which time the supernatant was transferred via filter-tipped cannula into a rapidly stirred solution of $NaBH_4$ (271 mg, 7.16 mmol) in 50 mL of ice water followed by 2 × 5 mL washes of dry THF. The resulting mixture was stirred for 30 min at which time the reaction mixture was poured into 10% aqueous bicarbonate solution (100 mL) and the product extracted with 2 × 100 mL of CH_2Cl_2. The organic layers were combined, dried over Na_2SO_4, decanted, and concentrated to give alcohol **3** (961 mg) as a thick oil which was used without further purification.

(b) Preparation of (1*S*-Cyanomethyl-3-phenylpropyl)-carbamic Acid *tert*-Butyl Ester (4). Alcohol **3** (950 mg, 3.58 mmol) was dissolved in 25 mL of CH_2Cl_2 along with Et_3N (1.5 mL, 1.1 g, 10.7 mmol). The solution was cooled to 0 °C. Methanesulfonyl chloride (673 μL, 996 mg, 8.77 mmol) was added dropwise via syringe over a 1 min period followed by stirring for 1 h. The reaction solution was poured into 50 mL of 10% aqueous bicarbonate solution. The product was extracted with 50 mL of CH_2Cl_2. The layers were separated, and the organic layer was dried over Na_2SO_4, decanted, and concentrated to yield the desired crude mesylate as clear oil (1.2 g, 97%). The crude mesylate (1 g, 2.91 mmol) was dissolved in 15 mL of dry DMSO. Sodium cyanide (799 mg, 2.91 mmol) was added, and the resulting mixture was heated at 90 °C for 2 h. The reaction mixture was cooled and poured into 100 mL of water. The product was extracted with 100 mL of EtOAc. The organic layer was washed with 3 × 100 mL of brine, dried over Na_2SO_4, decanted, and concentrated to yield (1S-cyanomethyl-3-phenylpropyl)carbamic acid *tert*-butyl ester (**4**) as a yellow oil (780 mg) which was used without further purification.

(c) Preparation of Morpholine-4-carboxylic Acid [1*S*-(2-Cyano-1*S*-phenethyl-ethylcarbamoyl)-3-methylbutyl]-amide (5). Nitrile **4** (780 mg) was dissolved in 5 mL of 50% TFA in CH_2Cl_2, and the solution was stirred for 30 min and then concentrated. The residue was redissolved in 50 mL of CH_2Cl_2, and the organic solution was washed with 50 mL of 10% aqueous bicarbonate. The organic layer was dried over Na_2SO_4, decanted, and concentrated to yield the desired amino-nitrile as an oil (451 mg). Acid **2a** (500 mg, 2.05 mmol) and TBTU (789 mg, 2.46 mmol) were dissolved in 20 mL of CH_2Cl_2 followed by addition of Hunig's base (1.42 mL, 8.19 mmol). After stirring for 15 min, the amino-nitrile (392 mg, 2.26 mmol) was added as a solution in 5 mL of CH_2Cl_2. After 4 h, the reaction solution was poured into 100 mL of 10% bicarbonate solution. The product was extracted with 100 mL of EtOAc. The organic layer was washed with 100 mL 1 N aqueous HCl, 100 mL saturated aqueous bicarbonate, and 100 mL of water, sequentially. The organic layer was concentrated to a thick gum. The crude product was purified by flash chromatography on SiO_2 using 10% 2-propanol in CH_2Cl_2 to yield nitrile **5** (650 mg) as a white glass. ^1H NMR (400 MHz, $CDCl_3$): δ 7.31−7.26 (m, 2H), 7.21 (tt, J = 7.3, 1.3 Hz, 1H), 7.14 (d, J = 6.8 Hz, 2H), 6.77 (d, J = 7.4 Hz, 1H), 4.77 (d, J = 7.9 Hz, 1H), 4.32 (dt, J = 8.3, 6.0 Hz, 1H), 4.07−3.99 (m, 1H), 3.69−3.61 (m, 4H), 3.40−3.30 (m, 4H), 2.74 (dd, J = 16.8, 5.5 Hz, 1H), 2.70−2.60 (m, 2H), 2.53 (dd, J = 16.8, 4.5 Hz, 1H), 2.00−1.92 (m, 2H), 1.73−1.64 (m, 2H), 1.55−1.48 (m, 1H), 0.97−0.94 (m, 6H); MS (ESI) m/z 401 (M + H). Anal. ($C_{22}H_{32}N_4O_3$) C, H, N.

Preparation of Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-3-phenylpropylcarbamoyl)-3-methylbutyl}amide 6. (a) Preparation of 4-Methyl-2*S*-[(morpholine-4-carbonyl)amino]pentanoic Acid (2a). Benzyl L-leucine *p*-toluenesulfonate salt (8.00 g, 20.3 mmol) was dissolved in 20 mL of DMF followed by addition of *N*,*N*-diisopropylethylamine (10.61 mL, 60.9 mmol) and stirring under Ar for 15 min. 4-Morpholinecarbonyl chloride (4.55 g, 30.4 mmol) was added, and stirring was continued overnight (16 h). The solution was diluted with 500 mL of EtOAc and washed with 3 × 500 mL of water. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give 8.01 g of the crude product. The product was purified by flash chromatography (SiO_2, 40% EtOAc/hexane) resulting in a thick oil. The oil (18 g, 53.8 mmol, combination of two separate preparations) was dissolved in ethanol (500 mL). $Pd(OH)_2$ (642 mg) was added followed by cyclohexene (100 mL). The mixture was refluxed for 45 min at which time TLC indicated consumption of the benzyl ester. The reaction was cooled and filtered through diatomaceous earth and evaporated to dryness to give 4-methyl-2*S*-[(morpholine-4-carbonyl)amino]pentanoic acid (**2a**) as a very thick oil (13 g, 99%) that was used without further purification.

(b) Preparation of 1*S*-Carbamoyl-3-phenylpropylammonium Trifluoroacetate (1a). *N*-Boc-L-homophenylalanine (0.50 g, 1.79 mmol) was dissolved in 20 mL of DMF which was cooled with an ice−water bath. 1-Hydroxybenzotriazole (HOBt) (0.29 g, 2.14 mmol) and EDC (0.41 g, 2.00 mmol) were added followed by stirring for 20 min. Ammonium hydroxide (0.5 mL) was added, and stirring was continued overnight (16 h). The reaction mixture was diluted with 50 mL of methylene chloride to give a white precipitate. The mixture was filtered, and the filtrate was washed with brine (100 mL) followed by saturated bicarbonate (100 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give the corresponding amide (0.45 g, 90%). The amide was dissolved in 10 mL of CH_2Cl_2 and 10 mL of trifluoroacetic acid (TFA) was added. Stirring was continued for 30 min at which time the reaction mixture was evaporated to dryness giving 1*S*-carbamoyl-3-phenylpropylammonium trifluoroacetate (**1a**) as an off-white paste that was used without further purification.

(c) Preparation of Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-3-phenylpropylcarbamoyl)-3-methylbutyl}-amide (6). 4-Methyl-2*S*-[(morpholine-4-carbonyl)amino]pentanoic acid (**2a**) (100 mg, 0.41 mmol), HOBt (72 mg, 0.53 mmol), and EDC (102 mg, 0.53 mmol) were dissolved in 10 mL of DMF, and the resulting solution was stirred at 0 °C for 15 min. To the cold solution was added 1*S*-carbamoyl-3-phenylpropylammonium trifluoroacetate (**1a**) (118 mg, 0.41 mmol) as a solution in 5 mL of DMF, followed by addition of *N*-methylmorpholine (94 μL, 0.86 mmol). The ice bath was removed, and the reaction was stirred at ambient temperature overnight (16 h). The reaction was diluted with 50 mL of CH_2Cl_2 to give a white precipitate. The mixture was filtered and the solid washed with an additional 50 mL of CH_2Cl_2. The filtrates were combined and washed with saturated bicarbonate (100 mL), 1 N HCl (100 mL), and brine (2 × 100 mL). The organic layer was dried over $MgSO_4$ and concentrated by rotary evaporation to give an oily residue. The residue was chromatographed (SiO_2, 5% MeOH in CH_2Cl_2) to give the dipeptide amide intermediate as a white solid (130 mg, 78%). The amide intermediate (130 mg, 0.32 mmol) was dissolved in 2 mL of DMF and cooled to 0 °C with an ice−water bath. To the solution was added cyanuric chloride (40 mg, 0.32 mmol). The ice bath was removed and the reaction stirred to ambient temperature over the next 1 h. During the course of the reaction a white precipitate formed. The reaction was diluted with 100 mL of EtOAc and washed with 100 mL of water (3×). The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give the crude product as an oily residue. The residue was purified by chromatography (SiO_2, 40% hexane in EtOAc) to give **6** as a hard white glass (103 mg, 84%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56 (d, J = 8.0 Hz, 1H), 7.31−7.20 (m, 3H), 7.13 (d, J = 8.3 Hz, 2H), 4.85 (d, J = 7.6 Hz, 1H), 4.73 (q, J = 7.6 Hz, 1H), 4.32 (dt, J = 8.1, 5.9 Hz, 1H), 3.69−3.60 (m, 4H), 3.39−3.29 (m, 4H), 2.79−2.67 (m, 2H), 2.13−1.98 (m, 2H), 1.69−1.62 (m, 2H), 1.57−1.48 (m, 1H), 0.94 (t, J = 9.8 Hz, 3H), 0.93 (d, J = 9.8 Hz, 3H); MS (ESI) m/z 387 (M + H). Anal. ($C_{21}H_{30}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [3-Methyl-1*S*-(3-phenylpropylcarbamoyl)butyl]amide (7). Amide **7** was prepared by EDC-mediated coupling of acid **2a** with commercially available 1-amino-3-phenylpropane. ^{1}H NMR 270 MHz ($CDCl_3$) δ 7.33−7.24 (m, 2H), 7.23−7.13 (m, 3H), 6.37−6.27 (m, 1H), 4.99 (d, J = 8.5 Hz, 1H), 4.26 (ddd, J = 8.5, 8.5, 6.5 Hz, 1H), 3.65 (t, J = 5.0 Hz, 4H), 3.40−3.30 (m, 4H), 3.30−3.15 (m, 2H), 2.63 (dd, J = 7.5, 7.5 Hz, 2H), 1.82 (d, J = 7.5 Hz, 2H), 1.70−1.44 (m, 3H), 0.93 (d, J = 6.0 Hz, 3H), 0.92 (d, J = 6.0 Hz, 3H); MS (ESI) m/z 362 (M + H). Anal. ($C_{20}H_{31}N_3O_3$) C, H, N.

Morpholine-4-carboxylic Acid [3-Methyl-1*S*-(1*R*-methyl-3-phenylpropylcarbamoyl)butyl]amide (8). Amide **8** was prepared by EDC/HOBt mediated coupling of acid **2a** with commercially available (2*R*)-2-amino-4-phenylbutane. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.29−7.25 (m, 2H), 7.19−7.15 (m, 3H), 6.02 (d, J = 8.5 Hz, 1H), 4.89 (d, J = 8.1 Hz, 1H), 4.27 (dt, J = 8.2, 6.1 Hz, 1H), 4.04−3.97 (m, 1H), 3.69−3.60 (m, 4H), 3.37−3.60 (m, 4H), 2.67−2.55 (m, 2H), 1.78−1.74 (m, 2H), 1.69−1.62 (m, 1H), 1.55−1.47 (m, 1H), 1.17 (d, J = 6.6 Hz, 3H), 0.96−0.94 (m, 6H); MS (ESI) m/z 376 (M + H). Anal. ($C_{21}H_{33}N_3O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(cyanomethylcarbamoyl)-3-methylbutyl]amide (9). Inhibitor **9** was prepared by EDC/HOBt-mediated coupling of acid **2a** with commercially available aminoacetonitrile hydrochloride. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.53 (t, J = 5.7 Hz, 1H), 4.79 (d, J = 7.7 Hz, 1H), 4.33 (dt, J = 8.7, 5.7 Hz, 1H), 4.17 (dd, J = 17.4, 5.8 Hz, 1H), 4.10 (dd, J = 17.4 Hz, 5.8 Hz), 3.73−3.66 (m, 4H), 3.41−3.31 (m, 4H), 1.75−1.63 (m, 2H), 1.59−1.50 (m, 1H), 1.00 (d, J = 6.5 Hz, 3H), 0.90 (d, J = 6.5 Hz, 3H); MS (ESI) m/z 283 (M + H). Anal. ($C_{13}H_{22}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [3-Methyl-1*S*-(1-prop-2-ynylcarbamoyl)butyl]amide (10). Amide **10** was prepared by EDC/HOBt-mediated coupling of acid **2a** with commercially available propargylamine. ^{1}H NMR 400 MHz ($CDCl_3$) δ 6.47 (t, J = 5.3 Hz, 1H), 4.82 (d, J = 8.0 Hz, 1H), 4.32 (dt, J = 8.0, 5.7 Hz, 1H), 4.08 (ddd, J = 17.6, 5.6, 2.6 Hz, 1H), 3.99 (ddd, J = 17.6, 5.1, 2.6 Hz, 1H), 3.70−3.68 (m, 4H), 3.37−3.34 (m, 4H), 2.22 (t, J = 2.6 Hz, 1H), 1.73−1.62 (m, 2H), 1.57−1.49 (m, 1H), 0.97−0.80 (m, 6H); MS (ESI) m/z 282 (M + H). Anal. ($C_{14}H_{23}N_3O_3$) C, H, N.

2*S*-(5-Dimethylaminonaphthalene-1-sulfonylamino)-4-methylpentanoic Acid (1*S*-Cyano-3-phenylpropyl)amide (11a). Amide **11a** was prepared by an appropriate modification of Method C. ^{1}H NMR 270 MHz ($CDCl_3$) δ 8.58 (d, J = 8.5 Hz, 1H), 8.28−8.22 (m, 2H), 7.62 (t, J = 9.1 Hz, 1H), 7.54 (dd, J = 8.4, 7.4 Hz, 1H), 7.35−7.13 (m, 6H), 6.48 (d, J = 8.4 Hz, 1H), 4.91 (d, J = 7.3 Hz, 1H), 4.53 (q, J = 7.6 Hz, 1H), 3.59 (m, 1H), 2.88 (s, 6H), 2.69 (m, 2H), 1.87 (m, 2H), 1.28−1.19 (m, 3H), 0.65 (d, J = 6.3 Hz, 3H), 0.37 (d, J = 6.3 Hz, 3H); MS (ESI) m/z 507 (M + H). Anal. ($C_{28}H_{34}N_4O_3S$) C, H, N.

2*S*-(5-Dimethylaminonaphthalene-1-sulfonylamino)-4-methylpentanoic Acid (*R*-Benzyloxymethylcyanomethyl)amide (11b). Amide **11b** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 8.60 (d, J = 8.5 Hz, 1H), 8.29 (m, 2H), 7.63 (t, J = 8.8 Hz, 1H), 7.53 (dd, J = 8.5, 7.5 Hz, 1H), 7.47−7.38 (m, 5H), 7.24 (d, J = 7.5 Hz, 1H), 6.79 (d, J = 8.8 Hz, 1H), 5.08 (d, J = 7.5 Hz, 1H), 4.82 (ddd, J = 8.0, 4.0, 4.0 Hz, 1H), 4.63 (m, 1H), 3.69−3.64 (m, 1H), 3.61 (dd, J = 10.0, 4.0 Hz, 1H), 3.46 (dd, J = 10.0, 4.0 Hz, 1H), 2.92 (s, 6H), 1.54−1.52 (m, 1H), 1.36−1.27 (m, 2H), 0.70 (d, J = 6.5 Hz, 3H), 0.42 (d, J = 6.5 Hz, 3H); MS (ESI) m/z 523 (M + H). Anal. ($C_{28}H_{34}N_4O_4S$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyanoethylcarbamoyl)-3-methylbutyl]amide (12). Amide **12** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.92 (d, J = 8.1 Hz, 1H), 5.03 (d, J = 7.6 Hz, 1H), 4.83 (p, J = 8.0 Hz, 1H), 4.33 (q, J = 7.6 Hz, 1H), 3.70−3.63

(m, 4H), 3.41−3.30 (m, 4H), 1.69−1.51 (m, 3H), 1.50 (d, J = 8.0 Hz, 3H), 0.97−0.93 (m, 6H); MS (ESI) m/z 297 (M + H). Anal. ($C_{14}H_{24}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyanobutylcarbamoyl)-3-methylbutyl]amide (13). Amide **13** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 8.11 (d, J = 8.1 Hz, 1H), 5.31 (d, J = 7.8 Hz, 1H), 4.80 (q, J = 8.0 Hz, 1H), 4.41 (q, J = 7.8 Hz, 1H), 3.70−3.62 (m, 4H), 3.41−3.31 (m, 4H), 1.87−1.40 (m, 7H), 0.97−0.89 (m, 9H); MS (ESI) m/z 325 (M + H). Anal. ($C_{16}H_{28}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyanopentylcarbamoyl)-3-methylbutyl]amide (14). Amide **14** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.90 (d, J = 8.1 Hz, 1H), 5.22 (d, J = 7.8 Hz, 1H), 4.77 (q, J = 8.1 Hz, 1H), 4.35 (dt, J = 7.8, 5.9 Hz, 1H), 3.75−3.68 (m, 4H), 3.45−3.32 (m, 4H), 1.89−1.55 (m, 5H), 1.50−1.40 (m, 4H), 0.96−0.89 (m, 9H); MS (ESI) m/z 339 (M + H). Anal. ($C_{17}H_{30}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-2-methyl-propylcarbamoyl)-3-methylbutyl]amide (15). Amide **15** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 8.10 (d, J = 8.2 Hz, 1H), 5.20 (d, J = 8.0 Hz, 1H), 4.75 (dd, J = 8.5, 8.1 Hz, 1H), 4.45 (q, J = 8.0 Hz, 1H), 3.80−3.65 (m, 4H), 3.40−3.29 (m, 4H), 2.01−1.96 (m, 1H), 1.72−1.53 (m, 3H), 1.00 (t, J = 7.0 Hz, 6H), 0.97−0.91 (m, 6H); MS (ESI) m/z 325 (M + H). Anal. ($C_{16}H_{28}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-2,2-dimethylpropylcarbamoyl)-3-methylbutyl]amide (16). Amide **16** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.95 (d, J = 8.2 Hz, 1H), 4.99 (d, J = 8.1 Hz, 1H), 4.75 (d, J = 8.2 Hz, 1H), 4.45 (q, J = 8.0 Hz, 1H), 3.80−3.60 (m, 4H), 3.45−3.35 (m, 4H), 1.80−1.55 (m, 3H), 1.10 (s, 9H), 0.93−0.91 (m, 6H); MS (ESI) m/z 339 (M + H). Anal. ($C_{17}H_{30}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[(Cyano-phenyl-*S*-methyl)carbamoyl]-3-methylbutyl}amide (major isomer) and Morpholine-4-carboxylic Acid {1*S*-[(Cyanophenyl-*R*-methyl)carbamoyl]-3-methylbutyl}amide (minor isomer) (17). Amide **17** was prepared by an appropriate modification of Method A in which the phenylglycine residue epimerized to an inseparable 2 to 1 ratio of diastereomers. ^{1}H NMR (major isomer) 400 MHz ($CDCl_3$) δ 7.66 (d, J = 8.0 Hz, 1H), 7.47−7.38 (m, 5H, overlapped with minor isomer), 6.06 (d, J = 8.2 Hz, 1H), 4.77 (d, J = 8.3 Hz, 1H), 4.44−4.37 (m, 1H, overlapped with minor isomer), 3.63−3.60 (m, 4H), 3.28−3.24 (m, 4H), 1.76−1.69 (m, 2H, overlapped with minor isomer), 1.63−1.52 (m, 1H, overlapped with minor isomer), 0.99−0.77 (m, 6H, overlapped with minor isomer); ^{1}H NMR (minor isomer) 400 MHz ($CDCl_3$) δ 7.74 (d, J = 8.4 Hz, 1H), 7.47−7.38 (m, 5H, overlapped with major isomer), 6.03 (d, J = 8.4 Hz, 1H), 4.75 (d, J = 8.5 Hz, 1H), 4.44−4.37 (m, 1H, overlapped with major isomer), 3.70−3.66 (m, 4H), 3.34−3.31 (m, 4H), 1.76−1.69 (m, 2H, overlapped with major isomer), 1.63−1.52 (m, 1H, overlapped with major isomer), 0.99−0.77 (m, 6H, overlapped with major isomer); MS (ESI) m/z 359 (M + H). Anal. ($C_{19}H_{26}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-2-phenylethylcarbamoyl)-3-methylbutyl]amide (18). Amide **18** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 8.32 (d, J = 8.0 Hz, 1H), 7.33−7.18 (m, 5H), 5.32 (d, J = 8.1 Hz, 1H), 4.95 (q, J = 8.0 Hz, 1H), 4.32 (q, J = 8.1 Hz, 1H), 3.69−3.61 (m, 4H), 3.39−3.30 (m, 4H), 3.15−3.05 (m, 2H), 1.80−1.60 (m, 3H), 0.95 (d, J = 9.5 Hz, 3H), 0.92 (d, J = 9.5 Hz, 3H); MS (ESI) m/z 373 (M + H). Anal. ($C_{20}H_{28}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[1*S*-Cyano-2-(3,4-dichlorophenyl)ethylcarbamoyl]-3-methylbutyl}amide (19). Amide **19** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.45 (d, J = 8.1 Hz, 1H), 7.41 (d, J = 8.2 Hz, 1H), 7.36 (d, J = 2.1 Hz), 7.13 (dd, J = 8.2, 2.1 Hz, 1H), 5.04 (ddd, J = 8.3, 7.0, 7.0 Hz, 1H), 4.61 (d, J = 7.6 Hz, 1H), 4.21 (ddd, J = 8.5, 8.5, 6.0 Hz, 1H), 3.72−3.65 (m, 4H), 3.39−3.26 (m, 4H), 3.09−3.03 (m, 2H), 1.72−1.47 (m, 3H), 0.95−0.88 (m, 6H); MS (ESI) m/z 441 (M + H). Anal. ($C_{20}H_{26}N_4O_3Cl_2$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*R*-Cyano-3-phenylpropylcarbamoyl)-3-methylbutyl}amide (20). Amide **20** was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.44 (d, J = 8.5 Hz, 1H), 7.31 (t, J = 7.3 Hz, 2H), 7.23 (t, J = 7.5 Hz, 1H), 7.19 (d, J = 7.0 Hz, 2H), 4.77−4.69 (m, 2H), 4.33−4.27 (m, 1H), 3.70−3.68 (m, 4H), 3.38−3.35 (m, 4H), 2.86−2.77 (m, 2H), 2.19−2.11 (m, 2H), 1.74−1.64 (m, 2H), 1.55−1.48 (m, 1H), 0.95 (d, J = 6.0 Hz, 3H), 0.92 (d, J = 6.5 Hz, 3H); MS (ESI) m/z 387 (M + H). Anal. ($C_{21}H_{30}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[(1-Cyano-1-methyl)ethylcarbamoyl]-3-methylbutyl}amide (21). Amide **21** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.91 (s, 1H), 5.82 (d, J = 8.1 Hz, 1H), 4.32 (ddd, J = 8.1, 8.1, 6.0 Hz, 1H), 3.69−3.60 (m, 4H), 3.38−3.30 (m, 4H), 1.70−1.50 (m, 9H), 0.95 (d, J = 9.8 Hz, 3H), 0.92 (d, J = 9.8 Hz, 3H); MS (ESI) m/z 311 (M + H). Anal. ($C_{15}H_{26}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(2-Benzyloxy-1*R*-cyano-ethylcarbamoyl)-3-methylbutyl]amide (22). Amide **22** was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.40−7.31 (m, 5H), 7.09 (d, J = 8.0 Hz, 1H), 5.03−4.99 (m, 1H), 4.72 (d, J = 7.5 Hz, 1H), 4.65 (d, J = 11.8 Hz, 1H), 4.60 (d, J = 11.8 Hz, 1H), 4.34−4.29 (m, 1H), 3.74 (dd, J = 10.0, 4.0 Hz, 1H), 3.67−3.63 (m, 5H), 3.37−3.26 (m, 4H), 1.73−1.61 (m, 2H), 1.56−1.51 (m, 1H), 0.95 (t, J = 5.5 Hz, 6H); MS (ESI) m/z 403 (M + H). Anal. ($C_{21}H_{30}N_4O_4$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[2-(2-Chlorobenzyloxy)-1*R*-cyanoethylcarbamoyl]-3-methylbutyl}amide (23). Amide **23** was prepared by Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.50 (dd, J = 7.0, 2.0 Hz, 1H), 7.39 (dd, J = 7.3, 1.8 Hz, 1H), 7.32−7.26 (m, 3H), 7.18 (d, J = 8.5 Hz, 1H), 5.07−5.03 (m, 1H), 4.75−4.69 (m, 3H), 4.37−4.31 (m, 1H), 3.84 (dd, J = 9.5, 4.0 Hz, 1H), 3.73 (dd, J = 9.5, 4.5 Hz, 1H), 3.69−3.62 (m, 4H), 3.36−3.26 (m, 4H), 1.72−1.64 (m, 2H), 1.57−1.52 (m, 1H), 0.95 (d, J = 6.0 Hz, 3H), 0.94 (d, J = 5.5 Hz, 3H); MS (ESI) m/z 437 (M + H). Anal. ($C_{21}H_{29}N_4O_4Cl$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[2-(3-Chlorobenzyloxy)-1*R*-cyanoethylcarbamoyl]-3-methylbutyl}amide (24). Amide **24** was prepared by Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.68 (d, J = 8.0 Hz, 1H), 7.35−7.21 (m, 4H), 5.06−5.01 (m, 1H), 4.75 (d, J = 7.5 Hz, 1H), 4.61 (d, J = 19.5 Hz, 1H), 4.56 (d, J = 19.5 Hz, 1H), 4.37−4.30 (m, 1H), 3.76−3.73 (m, 1H), 3.68−3.64 (m, 5H), 3.36−3.30 (m, 4H), 1.75−1.52 (m, 3H), 0.95 (t, J = 6.3 Hz, 6H); MS (ESI) m/z 437 (M + H). Anal. ($C_{21}H_{29}N_4O_4Cl$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[2-(4-Chlorobenzyloxy)-1*R*-cyano-ethylcarbamoyl]-3-methylbutyl}amide (25). 25 was prepared by Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.34 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 6.0 Hz, 1H), 7.29 (d, J = 8.0 Hz, 2H), 5.04−5.00 (m, 1H), 4.78 (d, J = 7.5 Hz, 1H), 4.60 (d, J = 19.3 Hz, 1H), 4.54 (d, J = 19.3 Hz, 1H), 4.36−4.30 (m, 1H), 3.72 (dd, J = 9.5, 4.0 Hz, 1H), 3.66−3.61 (m, 5H), 3.35−3.28 (m, 4H), 1.71−1.51 (m, 3H), 0.95 (t, J = 6.5 Hz, 6H); MS (ESI) m/z 437 (M + H). Anal. ($C_{21}H_{29}N_4O_4Cl$) C, H, N.

Morpholine-4-carboxylic Acid [1*R*−(1*S*-Cyano-3-phenyl-propylcarbamoyl)-3-methylbutyl}amide (26). 26 was prepared by an appropriate modification of Method B.

^{1}H NMR 400 MHz ($CDCl_3$) δ 7.37 (d, J = 8.1 Hz, 1H), 7.34−7.19 (m, 5H), 4.76−4.70 (m, 2H), 4.29 (dt, J = 8.7, 4.9 Hz, 1H), 3.74−3.65 (m, 4H), 3.41−3.32 (m, 4H), 2.87−2.75 (m 2H), 2.19−2.10 (m, 2H), 1.75−1.48 (m, 3H), 0.95 (d, J = 6.5 Hz, 3H), 0.92 (d, J = 6.5 Hz, 3H); MS (ESI) m/z 387 (M + H). Anal. ($C_{21}H_{30}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*R*−(1*S*-Cyano-3-phenyl-propylcarbamoyl)-1,3-dimethyl-butyl]-amide and morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-3-phenyl-propylcarbamoyl)-1,3-dimethyl-butyl]-amide (27). 27 was prepared as an inseparable mixture of isomers by an appropriate variation of *Method B* using (D,L)-*N*-Boc-α-methyl-leucine.

^{1}H NMR 400 MHz ($CDCl_3$) δ 7.63 and 7.58 (two d, J = 7.1 and 7.2 Hz, 1H), 7.32−7.20 (m, 5H), 5.09 and 5.01 (two s, 1H), 4.83−4.67 (two overlapping q, J = 7.1 and 7.1 Hz, 1H), 3.69−3.67 (m, 4H), 3.38−3.30 (m, 4H), 2.84 (*pseudo* q, J = 7.4 Hz, 2H), 2.20−1.98 (m, 3H), 1.70−1.60 (m, 2H), 1.50 and 1.46 (two s, 3H), 0.96−0.83 (m, 6H); MS (ESI) *m*/*z* 401 (M + H). Anal. ($C_{22}H_{32}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-cyano-3-phenylpropylcarbamoyl)-ethyl]-amide (28). 28 was prepared by an appropriate variation of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.76 (d, J = 8.0 Hz, 1H), 7.31−7.22 (m, 3H), 7.15−7.13 (m, 2H), 5.03 (d, J = 7.4 Hz, 1H), 4.73 (q, J = 7.6 Hz, 1H), 4.43 (p, J = 7.1 Hz, 1H), 3.68−3.62 (m, 4H), 3.39−3.31 (m, 4H), 2.78−2.71 (m, 2H), 2.18−2.01 (m, 2H), 1.39 (d, J = 7.1 Hz, 3H); MS (ESI) *m*/*z* 345 (M + H). Anal. ($C_{18}H_{24}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-cyano-3-phenylpropylcarbamoyl)-pentyl]-amide (29). 29 was prepared by an appropriate modification of *Method B*. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.30−7.14 (m, 4H), 7.15 (d, J = 8.3 Hz, 2H), 4.87 (d, J = 7.7 Hz, 1H), 4.76 (q, J = 7.6 Hz, 1H), 4.24 (q, J = 7.3 Hz, 1H), 3.69−3.63 (m, 4H), 3.38−3.31 (m, 4H), 2.82−2.70 (m, 2H), 2.17−2.01 (m, 2H), 1.88−1.79 (m, 1H), 1.66−1.60 (m, 1H), 1.39−1.31 (m, 4H), 0.91 (t, J = 7.0 Hz, 3H); MS (ESI) *m*/*z* 387 (M + H). Anal. ($C_{21}H_{30}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-3-phenyl-propylcarbamoyl)-2-methyl-propyl]-amide (30). 30 was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 8.13 (d, J = 8.0 Hz, 1H), 7.28−7.18 (m, 3H), 7.07−7.05 (m, 2H), 5.22 (d, J = 8.7 Hz, 1H), 4.71 (apparent q, J = 7.0 Hz, 1H), 4.24 (apparent t, J = 8.2 Hz, 1H), 3.70−3.59 (m, 4H), 3.41−3.30 (m, 4H), 2.73−2.60 (m, 2H), 2.07−1.89 (m, 2H), 0.98 (apparent t, J = 7.0 Hz, 6H); MS (ESI) *m*/*z* 373 (M + H). Anal. ($C_{20}H_{28}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [(1*S*-Cyano-3-phenylpropylcarbamoyl)-cyclohexyl-R-methyl]-amide (31). 31 was prepared by an appropriate variation of *Method B*. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.30−7.23 (m, 3H), 7.15 (d, J = 6.9 Hz, 2H), 6.89 (d, J = 8.3 Hz, 1H), 4.94 (d, J = 8.4 Hz, 1H), 4.76 (q, J = 7.6 Hz, 1H), 4.04 (t, J = 8.0 Hz, 1H), 3.74−3.62 (m, 4H), 3.38−3.27 (m, 4H), 2.80−2.72 (m, 2H), 2.15−2.03 (m, 2H), 1.81−1.68 (m, 5H), 1.43−0.85 (m, 6H); MS (ESI) *m*/*z* 413 (M + H). Anal. ($C_{23}H_{32}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-3-phenyl-propylcarbamoyl)-2-phenyl-ethyl]-amide (32). 32 was prepared by an appropriate variation of *Method B*. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.33 (m, 8H), 7.11 (d, J = 7.1 Hz, 2H), 6.90 (d, J = 7.9 Hz, 1H), 4.93 (d, J = 7.4 Hz, 1H), 4.68 (q, J = 7.5 Hz, 1H), 4.47 (q, J = 7.4 Hz, 1H), 3.63−3.60 (m, 4H), 3.31−3.22 (m, 4H), 3.10−3.04 (m, 2H), 2.71−2.67 (*pseudo* t, J = 7.6 Hz, 2H), 2.10−1.94 (m, 2H); MS (ESI) *m*/*z* 421 (M + H). Anal. ($C_{24}H_{28}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-3-phenyl-propylcarbamoyl)-3,3-dimethyl-butyl]-amide (33). 33 was prepared by an appropriate modification of Method B. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.53 (d, J = 7.5 Hz, 1H), 7.35−7.24 (m, 3H), 7.18 (d, J = 7.5 Hz, 2H), 4.87 (d, J = 7.5 Hz, 1H), 4.74 (ddd, J = 7.5, 7.5, 7.5 Hz, 1H), 4.37 (m, 1H), 3.68 (m, 4H), 3.37 (m, 4H), 2.79 (m, 2H), 1.92 (dd, J = 4.5, 14.5 Hz, 1H), 1.52 (dd, J = 8.0, 14.5 Hz, 1H), 1.00 (s, 9H); MS (ESI) *m*/*z* 401 (M + H). Anal. ($C_{22}H_{32}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(1*S*-Cyano-3-phenyl-propylcarbamoyl)-2-cyclohexyl-ethyl]-amide (34). 34 was prepared by an appropriate variation of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.39 (d, J = 8.1 Hz, 1H), 7.31−7.22 (m, 3H), 7.14 (d, J = 7.0 Hz, 2H), 4.80−4.72 (m, 2H), 4.31 (m, 1H), 3.65 (m, 4H), 3.33 (m, 4H), 2.80−2.69 (m, 2H), 2.15−2.01 (m, 2H), 1.73−1.59 (m, 6H), 1.54−1.47 (m, 1H), 1.33−1.13 (m, 4H), 0.97−0.87 (m, 2H); ^{13}C NMR 100 MHz ($CDCl_3$) δ 173.9, 157.9, 139.6, 129.2, 128.8, 127.1, 118.8, 66.7, 52.7, 44.5, 40.3, 34.9, 34.6, 33.8, 33.4, 31.9, 26.7, 26.6, 26.5; MS (ESI) *m*/*z* 427 (M + H). Anal. ($C_{24}H_{34}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(2-benzyloxy)-1*R*-cyanoethylcarbamoyl)-3,3-dimethyl-butyl]-amide (35).

(a) NH_4OH (10 mL) was added to a premixed (15 min) solution of *N*-(*t*-butoxycarbonyl)-L-(*O*-benzyl)serine (10.0 g, 33.9 mmol), EDC (7.80 g, 40.7 mmol), and HOBt (5.50 g, 40.7 mmol) in DMF (120 mL) at room temperature. After 16 h the reaction mixture was diluted with CH_2Cl_2 and filtered, washed sequentially with 10% aq. HCl, satd. aqueous $NaHCO_3$, H_2O, brine, then dried over Na_2SO_4 and concentrated giving *N*-(*t*-butoxycarbonyl)-L-(*O*-benzyl)serinamide (11.5 g) as a white solid.

(b) TFA (5 mL) was added to a solution of *N*-(*t*-butoxycarbonyl)-L-(*O*-benzyl)serinamide (1.28 g, 4.35 mmol) in CH_2Cl_2 (20 mL) at room temperature. After stirring for 0.5 h the reaction mixture was concentrated giving a colorless oil. The oil was diluted with CH_2Cl_2 (10 mL) and Hunig's base (2.80 g, 21.7 mmol) and added to a premixed (15 min) solution of *N*-(benzyloxyoxycarbonyl)-L-(4-methyl)leucine (2.0 g, 4.34 mmol), EDC (933 mg, 5.21 mmol), HOBt (704 mg, 5.21 mmol) in CH_2Cl_2 (15 mL) at room temperature. After stirring for 16 h the reaction was quenched by the addition of H_2O, diluted with EtOAc, washed sequentially with 10% aq. HCl, satd. aqueous $NaHCO_3$, H_2O, brine, then dried over Na_2SO_4 and concentrated giving *N*-[*N*-(benzyloxyoxycarbonyl)-L-(4-methyl)leucinyl]-L-(*O*-benzyl)serinamide (2.0 g, quantitative) as a white solid.

(c) Cyclohexene (271 mg, 3.30 mmol) was added to a suspension of Pd/C (30 mg, 10% by wt of substrate) and *N*-[*N*-(benzyloxyoxycarbonyl)-L-(4-methyl)leucinyl]-L-(*O*-benzyl)serinamide (300 mg, 0.659 mmol) in EtOH (5 mL) at room temperature. The mixture was heated at 75 °C for 1 h, cooled to room temperature, filtered through Celite and concentrated giving *N*-(L-(4-methyl)leucinyl)-L-(*O*-benzyl)serinamide (200 mg, 94%) as a colorless oil.

(d) 4-Morpholinecarbonyl chloride (100 mg, 0.668 mmol) was added to a solution of *N*-(L-(4-methyl)leucinyl)-L-(*O*-benzyl)-serinamide (195 mg, 0.607 mmol), DIPEA (118 mg, 0.912 mmol), and DMAP (10 mg, 0.082 mmol) in CH_2Cl_2 (5 mL) at room temperature. After stirring for 16 h the reaction was quenched by the addition of H_2O, diluted with EtOAc, washed sequentially with 10% aq. HCl, satd. $NaHCO_3$, H_2O (×3), brine, dried over Na_2SO_4, and concentrated giving *N*-[*N*-(4-morpholinecarbonyl)-L-(4-methyl)leucinyl]-L-(*O*-benzyl)serinamide (150 mg) as a white solid.

(e) Cyanuric chloride (59 mg, 0.322 mmol) was added to a solution of *N*-[*N*-(4-morpholinecarbonyl)-L-(4-methyl)leucinyl]-L-(*O*-benzyl)serinamide (140 mg, 0.322 mmol) in DMF (1.5 mL) at 0 °C. After stirring for 2 h, the reaction was quenched by addition of satd. $NaHCO_3$, filtered, diluted with EtOAc, washed sequentially with H_2O (×5), brine, dried over Na_2SO_4, and concentrated giving 150 mg of a yellow foam. A portion of the crude foam was purified by preparative HPLC (65% AcCN/H_2O) giving nitrile **35** (4 mg) as a white solid: ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.39−7.31 (m, 6H), 7.39 (dd, J = 7.3, 1.8 Hz, 1H), 4.99 (ddd, J = 8.5, 4.0, 4.0 Hz, 1H), 4.69 (d, J = 8.5 Hz, 1H), 4.63 (d, J = 12.0 Hz, 1H), 4.59 (d, J = 12.0 Hz, 1H), 4.35 (ddd, J = 8.0, 8.0, 4.5 Hz, 1H), 3.72 (dd, J = 10.0, 4.0 Hz, 1H), 3.66−3.60 (m, 5H), 3.35−3.25 (m, 4H), 1.88 (dd, J = 14.5, 4.5 Hz, 1H), 1.47 (dd, J = 14.5, 8.0 Hz, 1H), 0.97 (s, 9H); MS (ESI) *m*/*z* 417 (M + H). Anal. ($C_{22}H_{32}N_4O_4$) C, H, N.

Morpholine-4-carboxylic Acid [1*S*-(2-Benzyloxy-1*R*-cyanoethylcarbamoyl)-2-cyclohexylethyl]amide (36). Amide **36** was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.37−7.27 (m, 6H), 5.02 (ddd, J = 8.3, 4.2, 4.2 Hz, 1H), 4.73 (d, J = 8.0 Hz, 1H), 4.62 (d, J = 11.9 Hz, $\Delta\nu$ = 13.9 Hz, 1H), 4.58 (d, J = 11.9 Hz, $\Delta\nu$ = 13.9 Hz, 1H), 4.37 (ddd, J = 8.3, 8.3, 6.3 Hz, 1H), 3.71 (dd, J = 9.8, 4.1 Hz, 1H), 3.66−3.60 (m, 5H), 3.36−3.26 (m, 4H), 1.71−1.65 (m, 6H), 1.52 (ddd, J = 14.7, 8.7, 6.2 Hz, 1H), 1.36−1.10 (m, 4H), 1.01−0.86 (m, 2H); MS (ESI) *m*/*z* 443 (M + H). Anal. ($C_{24}H_{34}N_4O_4$) C, H, N.

Morpholine-4-carboxylic Acid {1-[1-Cyano-2-(2-methyl-benzyloxy)ethylcarbamoyl]-3-methylbutyl}amide (37). Amide **37** was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.36 (d, J = 6.0 Hz, 1H), 7.30 (d, J = 6.5 Hz, 1H), 7.26−7.17 (m, 3H), 5.01−4.96 (m, 1H), 4.68 (d, J = 7.5 Hz, 1H), 4.63 (s, 2H), 4.38−4.32 (m,

1H), 3.74 (dd, J = 9.5, 4.0 Hz, 1H), 3.67−3.62 (m, 5H), 3.38−3.29 (m, 4H), 2.37 (s, 3H), 1.73−1.60 (m, 2H), 1.56−1.47 (m, 1H), 0.92 (t, J = 6.5 Hz, 6H); MS (ESI) m/z 417 (M + H). Anal. ($C_{22}H_{32}N_4O_4$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[1*R*-Cyano-2-(2-methyl-benzyloxy)ethylcarbamoyl]-3,3-dimethylbutyl}-amide (38). Amide **38** was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.34 (d, J = 7.0 Hz, 1H), 7.28−7.23 (m, 4H), 5.03 (ddd, J = 8.0, 4.0, 4.0 Hz, 1H), 4.72 (br s, 1H), 4.68 (d, J = 12.0 Hz, 1H), 4.64 (d, J = 12.0 Hz, 1H), 4.37 (br s, 1H), 3.77 (dd, J = 10.0, 4.5 Hz, 1H), 3.72−3.68 (m, 5H), 3.44−3.33 (m, 4H), 2.41 (s, 3H), 1.91 (dd, J = 14.5, 4.0 Hz, 1H), 1.55−1.43 (m, 1H), 1.01 (s, 9H); MS (ESI) m/z 431 (M + H). Anal. ($C_{23}H_{34}N_4O_4$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[1*R*-Cyano-2-(2-methylbenzyloxy)ethylcarbamoyl]-2-cyclohexylethyl}-amide (39). Amide **39** was prepared by an appropriate variation of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.35−7.21 (m, 4H), 7.10 (bs, 1H), 5.05 (ddd, J = 8.0, 4.0 4.0 Hz, 1H), 4.75 (bs, 1H), 4.67 (d, J = 11.5 Hz, 1H), 4.63 (d, J = 11.5 Hz, 1H), 4.41−4.34 (m, 1H), 3.77 (dd, J = 10.0, 4.0 Hz, 1H), 3.70−3.68 (m, 5H), 3.39−3.32 (m, 4H), 2.41 (s, 3H), 1.85−1.52 (m, 7H), 1.40−1.32 (m 1H), 1.29−1.13 (m, 3H), 1.05−0.90 (m, 2H); MS (ESI) m/z 457 (M + H). Anal. ($C_{25}H_{36}N_4O_4 \cdot 0.25H_2O$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[2-(2-Chlorobenzyloxy)-1*R*-cyanoethylcarbamoyl]-2-cyclohexylethyl}amide (40). Amide **40** was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.52−7.47 (m, 2H), 7.39−7.37 (m, 1H), 7.32−7.25 (m, 2H), 5.03 (ddd, J = 8.3, 4.2, 4.2 Hz, 1H), 4.76−4.69 (m, 3H), 4.39 (ddd, J = 8.8, 7.8, 6.0 Hz, 1H), 3.84 (dd, J = 9.7, 3.8 Hz, 1H), 3.73 (dd, J = 9.7, 4.4 Hz, 1H), 3.69−3.64 (m, 4H), 3.36−3.34 (m, 4H), 1.77−1.61 (m, 6H), 1.50 (ddd, J = 14.7, 8.9, 6.0 Hz, 1H), 1.32−1.11 (m, 4H), 0.99−0.85 (m, 2H); MS (ESI) m/z 477 (M + H). Anal. ($C_{24}H_{33}N_4O_4Cl$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[(Cyanophenyl-*S*-methyl)carbamoyl]-2-cyclohexylethyl}amide (41). Amide **41** was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 8.47 (d, J = 8.2 Hz, 1H), 7.37−7.28 (m, 5H), 6.04 (d, J = 8.2 Hz, 1H), 4.98 (d, J = 8.3 Hz, 1H), 4.57 (ddd, J = 8.3, 8.3, 6.5 Hz, 1H), 3.64−3.51 (m, 4H), 3.27−3.10 (m, 4H), 1.76−1.65 (m, 6H), 1.55 (ddd, J = 14.5, 8.4, 6.3 Hz, 1H), 1.40−1.12 (m, 4H), 0.99−0.86 (m 2H); MS (ESI) m/z 399 (M + H). Anal. ($C_{22}H_{30}N_4O_3$) C, H, N.

Morpholine-4-carboxylic Acid {1*S*-[(Cyanodimethylmethyl)carbamoyl]-2-cyclohexylethyl}amide (42). Amide **42** was prepared by an appropriate modification of Method A. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.49 (s, 1H), 5.18 (d, J = 8.0 Hz, 1H), 4.29 (ddd, J = 8.7, 8.7, 6.0 Hz, 1H), 3.73−3.66 (m, 4H), 3.42−3.31 (m, 4H), 1.77−1.63 (m, 6H, overlapped with methyl singlets), 1.66 (s, 3H), 1.63 (s, 3H), 1.55 (ddd, J = 14.8, 9.2, 5.8 Hz, 1H), 1.35−1.12 (m, 4H), 1.00−0.80 (m, 2H); MS (ESI) m/z 351 (M + H). Anal. ($C_{18}H_{30}N_4O_3$) C, H, N.

***N*-[1*S*-(2-Benzyloxy-1*R*-cyanoethylcarbamoyl)-2-cyclohexylethyl]isonicotinamide (43).** Amide **43** was prepared by an appropriate variation of Method C. ^{1}H NMR 400 MHz (DMSO-d_6) δ 10.51 (s, 1H), 10.01 (d, J = 7.2 Hz, 2H), 9.56 (d, J = 7.8 Hz, 1H), 9.09 (d, J = 7.7 Hz, 1H), 8.50 (d, J = 7.2 Hz, 2H), 7.34−7.25 (m, 5H), 5.04 (*pseudo* q, J = 6.5 Hz, 1H), 4.65−4.55 (m, 3H), 3.68 (d, J = 5.9 Hz, 2H), 1.78−1.56 (m, 7H), 1.41−1.29 (m, 1H), 1.17−1.08 (m, 3H), 0.96−0.88 (m, 2H); MS (ESI) m/z 435 (M − Cl). Anal. ($C_{25}H_{30}N_4O_3$) C, H, N.

Furan-2-carboxylic Acid [1*S*-(2-Benzyloxy-1*R*-cyanoethylcarbamoyl)-2-cyclohexylethyl]amide (44). Amide **44** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.47 (dd, J = 1.7, 0.8 Hz, 1H), 7.34−7.31 (m, 5H), 7.14 (dd, J = 4.3, 0.8 Hz, 1H), 7.11 (d, J = 8.2 Hz, 1H), 6.62 (d, J = 8.1 Hz, 1H), 6.52 (dd, J = 3.5, 1.7 Hz, 1H), 5.02 (ddd, J = 8.3, 4.1, 4.1 Hz, 1H), 4.64 (ddd, J = 8.8, 8.8, 5.6 Hz, 1H), 4.60 (d, J = 12.0 Hz, $\Delta\nu$ = 7.2 Hz, 1H), 4.56 (d, J = 12.0 Hz, $\Delta\nu$ = 7.2 Hz, 1H), 3.70 (dd, J = 9.8, 3.9 Hz, 1H), 3.59 (dd, J = 9.8, 4.4 Hz, 1H), 1.84−1.77 (m, 2H), 1.70−1.62 (m, 5H), 1.40−1.35 (m, 1H), 1.26−1.12 (m, 3H), 0.98−0.87 (m, 2H); MS (ESI) m/z 424 (M + H). Anal. ($C_{24}H_{29}N_3O_4$) C, H, N.

Thiophene-2-carboxylic Acid [1*S*-(2-Benzyloxy-1*R*-cyanoethylcarbamoyl)-2-cyclohexylethyl]amide (45). Amide **45** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 7.53−7.51 (m, 2H), 7.34−7.31 (m, 5H), 7.09 (dd, J = 5.0, 3.8 Hz, 1H), 7.01 (d, J = 8.3 Hz, 1H), 6.30 (d, J = 8.1 Hz, 1H), 5.02 (ddd, J = 8.2, 4.1, 4.1 Hz, 1H), 4.67−4.56 (m, 3H), 3.72 (dd, J = 9.8, 3.9 Hz, 1H), 3.61 (dd, J = 9.8, 4.2 Hz, 1H), 1.82−1.77 (m, 2H), 1.71−1.63 (m, 5H), 1.43−1.34 (m, 1H), 1.24−1.17 (m, 3H), 1.09−0.89 (m, 2H); MS (ESI) m/z 440 (M + H). Anal. ($C_{24}H_{29}N_3O_3S$) C, H, N.

Pyrazine-2-carboxylic Acid [1*S*-(2-Benzyloxy-1*R*-cyanoethylcarbamoyl)-2-cyclohexylethyl]amide (46). Amide **46** was prepared by an appropriate modification of Method C. ^{1}H NMR 400 MHz ($CDCl_3$) δ 9.37 (d, J = 1.4 Hz, 1H), 8.78 (d, J = 2.5 Hz, 1H), 8.54 (dd, J = 2.5, 1.4 Hz, 1H), 8.04 (d, J = 8.3 Hz, 1H), 7.34−7.26 (m, 5H), 6.97 (d, J = 8.2 Hz), 5.04 (ddd, J = 8.1, 4.0 4.0 Hz, 1H), 4.68−4.56 (m, 3H), 3.72 (dd, J = 9.8, 3.9 Hz, 1H), 3.62 (dd, J = 9.8, 4.2 Hz, 1H), 1.89−1.63 (m, 7H), 1.40−1.35 (m, 1H), 1.25−0.86 (m, 3H), 0.99−0.86 (m, 2H); MS (ESI) m/z 436 (M + H). Anal. ($C_{24}H_{29}N_5O_3$) C, H, N.

Reversibility Studies. Cathepsin S was combined with 1 μL of a 5 mg/mL solution of inhibitor **6** in DMSO for a final ratio of 23 μM enzyme to 368 μM inhibitor. The sample was incubated 1 h at room temperature before injection onto a reverse-phase HPLC column (Vydac C18 column 2.0 × 250 mm). This was eluted with a gradient of 0.06% aqueous TFA to 0.052% TFA in acetonitrile over 25 min. UV detection at 210, 260, and 280 nm was used to identify and collect the Cathepsin S peak. The retention time of this peak was identical to that of the Cathepsin S peak from a control sample of Cathepsin S/DMSO. The collected fractions from the sample and the control sample were frozen in liquid nitrogen and stored at −80 °C until analyzed by electrospray mass spectrometry (Autospec OATOF MS (Micromass, Manchester UK). The mass spectrometer was scanned from m/z = 1000−2000, and the resulting multiply charged spectra were deconvoluted with the Maxent software (Micromass) to calculate molecular weight. Known irreversible inhibitors such as E-64 and vinyl sulfones produce an adduct protein that is separated by the HPLC column from unaltered Cathepsin S and has a mass increased by the molecular weight of adduct. Only the mass for Cathepsin S (MW = 24654) was detected with no adduct peak detected for Cathepsin S/**6**.

In addition, samples Cathespin S incubated with 1000-fold concentrations of **6** and subjected to size-exclusion chromatography [Sephadex G-25 (NAP-5 Pharmacia) columns (0.5 mL)] followed by desalting show recovered enzymatic activity. Known irreversible compounds were not removed from the enzyme, and after desalting, the Cathepsin S enzyme activity was not restored.

Competitive Binding Assay (K_d determination). The equilibrium dissociation constants (K_d) for inhibitors were determined by a fluorescence-based equilibrium competitive binding assay. The reactants used in the competition assay were the more active inhibitors as determined by measuring IC_{50}s, dansylated probes **11a** and **11b** and Cathepsin S. The K_d's for probes **11a** and **11b** were determined to be 10 and 8 nM, respectively, by methods previously described.[35a] Competition assays were performed in the following buffer: 100 mM sodium acetate, pH 4.5, 2.5 mM EDTA, 10% glycerol, and 1 mM DTT. All pipet tips (Rainin Instrument Company, Inc.), Dynatech 96-well "U" bottom black microtiter plates (Dynex Technologies, Inc.), centrifuge tubes, and reagent reservoirs (Matrix Technologies Corp) were siliconized with Sigmacote (Sigma SL2). The inhibitors and probes were solubilized in DMSO. Competition reactions were initiated by adding a preincubated mixture of 2 μM dansylated probe (**11a** or **11b**) and 600 nM of Cathepsin S to microtiter plate wells containing serially diluted inhibitors. Final concentrations of Cathepsin S and dansylated probe were 300 nM and 1 μM, respectively. The reactions were allowed to come to equilibrium at room

temperature for at least 1 h. Fluorescence intensities were measured on a Polarstar Fluostar 403 μ_BG Lab Technologies Platereader equipped with a 290 nm-excitation filter and 520−535-nm emission filter. The fluorescence intensity of the dansylated probe is dependent upon resonance energy transfer between tryptophan residues of Cathepsin S and bound probe. The raw fluorescence data were converted to SAS data sets that included reactant concentrations. The data sets were fit for inhibitor K_d by applying ordinary nonlinear least-squares regression techniques to a competitive binding model using the Marquardt−Levenberg minimization method.[35a]

Kinetic Binding Assay for the Determination of Rate Constants and K_d. Kinetic constants (k_{on}, k_{off}) for all compounds binding to Cathepsin S were determined using a ligand-exchange assay.[35b] The reactants for each experiment consisted of a dansylated probe (inhibitors **11a** or **11b**), inhibitor, and Cathepsin S. Assay buffer for all ligand-exchange assays consisted of 50 mM sodium acetate pH 4.5, 2.5 mM EDTA, 1 mM DTT, and 2% v/v DMSO. Fluorescence changes with respect to time were measured using a KinTek stopped-flow instrument model SF-2001. Rate constants were determined by performing the kinetic assay as two half reactions. In the first reaction, Cathepsin S (600 nM) was preincubated with the dansylated probe (800 nM) in a final volume of 1 mL and loaded into one of the stopped-flow syringes. The other syringe was loaded with 800 nM compound in a final volume of 1 mL. Compounds were diluted from a DMSO stock to their final concentration in the assay buffer described above. The exchange reaction was then initiated by injecting 40 μL from each syringe at a flow rate of 10 mL/s into a quartz observation cell. The sample was excited at a wavelength of 290 nm from a 100-W mercury short arc lamp and fluorescence emission was detected through a PMT with an attached 505 nm long wave pass filter. The fluorescence intensity of the dansylated probe increases when bound to Cathepsin S. Data acquisition began at the start of the injection with a dead time of approximately 2 ms and continued until the reaction had reached equilibrium. The reaction temperature was held at 23 °C using an external water bath. The second reaction was carried out as the reciprocal of the first reaction. Cathepsin S (600 nM) was preincubated with compound (800 nM) and loaded into one of the stopped-flow syringes. The other syringe was loaded with 800 nM dansylated probe in a final volume of 1 mL. The exchange reaction was initiated and fluorescence was followed as described above. The above two reactions together constitute a paired receptor−ligand exchange reaction. The complementary data from these two reactions can be combined into a single data set and then modeled simultaneously for the rate constants. The derivation of the integrated rate equation describing the overall ligand exchange reaction has been described elsewhere.[35b] All of the data were analyzed using the SAS statistical software system (version 6.12, SAS Institute Incorporated, Cary, NC). Typically, two ASCII data files containing fluorescence measurements from a pair of ligand-exchange reactions were converted into a single SAS data set, with the respective subsets labeled as 'association' or 'dissociation' (referring to the disposition of the probe). Parameter estimates (k_{on}, k_{off}) were obtained by applying ordinary nonlinear least-squares regression techniques to a ligand exchange model using the Marquardt−Levenberg minimization method.[35b]

Crystallization and Structure Determination. Purified Cathepsin S was mixed with compound **22** at a 5:1 ratio of compound:protein on ice and then concentrated to 10 mg/mL at 4 °C. Crystals were grown by vapor diffusion at room temperature in 2 μL hanging drops that contained an equal volume of protein complex and reservoir solution consisting of 1.6−2.0 M $(NH_4)_2SO_4$. Crystals were briefly (about 10 s) soaked in 100 mM Na acetate pH 4.5, 2 M $(NH_4)_2SO_4$, and 20% glycerol and flash-frozen under a cool −170 °C nitrogen stream for the diffraction experiments. The diffraction experiment was carried out using an R-AXIS-IIc image-plate system mounted on a Rigaku RU200 X-ray generator powered at 100 mA and 50 kV and equipped with Osmic focusing mirrors.

Table 6. Crystallographic Data Summary

Data Collection	
space group	$P4_122$
$a = b$ (Å	85.29
c (Å	150.07
molecules per asymmetric unit	2
resolution range (å)	50−1.90
measurements	545688
unique reflections[a]	42565
R_{sym} [b]	0.146
completeness (%)	95.9
Refinement Statistics	
resolution range (Å)	50−1.90
R-factor[c]	0.169
R_{free}	0.197
protein atoms	3366
water molecules	466
inhibitor atoms	58
average B-factor for all atoms (Å^2)	11.3
cross-validated estimated coordinate error (Å)[d]	0.21
rms deviation from expected geometry [e]:	
bond length (Å)	0.010
bond angles (deg.)	1.33
dihedral angles (deg.)	24.8
improper angles	0.77
Ramachandran Statistics	
most favored and allowed regions	99.7%
generously allowed region	0.3%

[a] A control set of 5% of these reflections, chosen randomly, were used to calculate an R_{free} to monitor the progress of refinement. [b] $R_{sym} = \Sigma \mid I_i - <I> \mid / \Sigma I_i$, where I_i is the scaled intensity of the ith measurement and $<I>$ is the mean intensity of that reflection. [c] R-factor $= \Sigma \mid\mid F_{obs}\mid - \mid F_{calc}\mid\mid / \Sigma \mid F_{obs}\mid$ where $\mid F_{obs}\mid$ and $\mid F_{calc}\mid$ are the calculated and observed structure factor amplitudes, respectively. [d] Luzzati[36] method was used along with the control set of reflections used to calculate R_{free}. [e] Engh and Huber[37] stereochemical parameters were used in the refinement. [f] No angle pair falls in the disallowed region.

Data were measured on a monocrystal of tetragonal symmetry to a resolution of 1.9 Å (Table 6) with two molecules occupying the asymmetric unit and reduced with DENZO and SCALEPACK[38] that yielded an average redundancy on measurements of 12.8 and a R_{sym} of 0.146.

The structure was solved by molecular replacement using AMORE.[39] Compound **22** was incorporated between rounds of model building using O[40] and refinement using X-PLOR 98 (MSI, Inc.).[41] The last round of refinement included simulated annealing carried out with CNS[42] using maximum likelyhood target and bulk solvent correction but without constraints on noncrystallographic symmetry and then followed by individual temperature factor refinement. The refinement results are summarized in Table 6. The coordinates may be found in the Protein Data Bank under ID code 1MS6.

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Supporting Information Available: HPLC analyses data. This material is available free of charge via the Internet at http://pubs.acs.org.

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