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Brief Article

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Selective Nitrile Inhibitors To Modulate the Proteolytic Synergism of Cathepsins S and F

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Supporting Information

ABSTRACT: A series of dipeptide nitriles with different P3 substituents was designed to explore the S3 binding pocket of cathepsin S. Racemic **7**−**16** and the enantiopure derivative (*R*)-**22** proved to be potent inhibitors of human cathepsin S and exhibited notable selectivity over human cathepsins L, K, and B. Inhibition of cathepsin F, the functional synergist of cathepsin S, was not observed. The azadipeptide analogue of **22**, compound **26**, was highly potent but nonselective.

INTRODUCTION

Major histocompatibility complex class II molecules (MHC-II) are expressed by only few specialized antigen-presenting cell types including macrophages, B cells, and dendritic cells. They are $\alpha\beta$ heterodimers synthesized and assembled in the endoplasmic reticulum. The heterodimerization is assisted by the invariant chain chaperone molecule that additionally blocks the MHC-II peptide-binding groove, preventing premature peptide loading. Furthermore, the invariant chain is important for the transport of assembled MHC class II molecules through the Golgi apparatus.[1,2] Two main proteolytic events occur during MHC-II-mediated antigen presentation: (i) cleavage of the antigens to small antigenic peptides and (ii) degradation of the invariant chain. Cathepsin S was described as the major processing enzyme of the invariant chain in the MHC-II-mediated antigen presentation. Cathepsin S is capable of cleaving the invariant chain, leaving the short class II associated invariant chain peptide (CLIP) which can be replaced by antigenic peptides.[2,3]

The role of cathepsin S in the invariant chain degradation process in cells from cathepsin S deficient mice can be adopted by cathepsin F, which is expressed at high levels in macrophages but not in dendritic and B cells.[4] This proteolytic synergism of cathepsins S and F might be exploited for fine modulation of the immune response, e.g., by taking advantage of selective inhibitors for each of these two enzymes. Cathepsin S deficient mice exhibit defects in immune function but are healthy and normal in most other respects.[2,5] For cathepsin F deficient mice, however, severe neurological disorders were observed.[6]

Cathepsin S was recognized as a promising target for the treatment of autoimmunity. Inhibition of cysteine cathepsins can be attained by low molecular weight compounds that oxidize the active-site cysteine or undergo a nucleophilic addition reaction.[7−9] Thus, several types of peptidomimetic compounds containing electrophilic groups covalently interact with the active-site cysteine. Among them, nitrile-based compounds have received much attention.[9−13] Recently, nitrile-based cathepsin S inhibitors were obtained by a combination of a P2 cysteine sulfone attached to a large group pointing to the S2 pocket, and a small aromatic P3 substituent.[12] Although certain series of inhibitors exhibited remarkable selectivity for cathepsin S over cathepsins L, S, and B,[12,14] it remained unclear whether they are also selective for cathepsin S over the functionally synergistic species cathepsin F.

In this study, we have focused on the P3−S3 interaction of peptidic inhibitors for cathepsin S. To explore the S3 pocket, a small library of dipeptide nitriles, containing a fixed *S*-(isobutyl)cysteine sulfone moiety at the P2 position and different P3 substituents, was synthesized and evaluated on human cathepsins L, S, K, and B. Additionally, the inhibitors were tested on cathepsin F to assess, for the first time, possible differences of their inhibition profiles toward the invariant chain processing enzymes cathepsins S and F.

RESULTS AND DISCUSSION

To obtain dipeptide nitriles with different P3 substituents, the P2 building block **6** containing the *S*-(isobutyl)cysteine sulfone was synthesized (Scheme 1). The thiol group of the Boc-protected cysteine methyl ester **1** was alkylated with isobutyl bromide in the presence of sodium methanolate to obtain **2**.[15] The thioether group of **2** was oxidized with MCPBA, leading to the sulfone **3**. The methyl ester was cleaved under basic conditions, and **4** was obtained as a free acid. The carboxylic group of **4** was coupled with aminoacetonitrile, leading to the Boc-protected dipeptide nitrile **5**. After deprotection with methanesulfonic acid, building block **6** was obtained. Finally, **6** was coupled with various P3 substituents to obtain the targets **7**−**16** (Table 1). This final acylation step was performed with the appropriate acyl chlorides or carboxylic acids. The corresponding P3 building blocks for the preparation of **9**−**12** were synthesized by separate multistep routes (Schemes S1 and S2 in Supporting Information).

The S-alkylation of *N*-acetylcysteine in the presence of sodium alcoholates under elevated temperature without racemization was reported.[15] However, dipeptide nitriles **7**−**16** were obtained in racemic form, as shown for compound **7** using chiral HPLC (Figure S1 in Supporting Information). The

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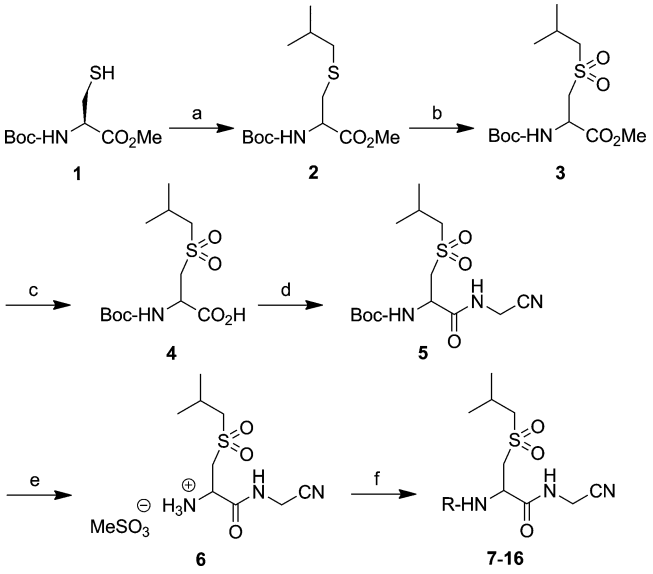
Scheme 1. Synthesis of Dipeptide Nitriles 7−16[a]



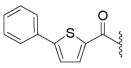
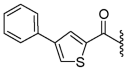
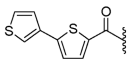
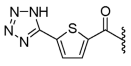
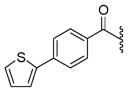
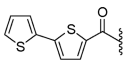
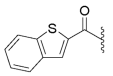
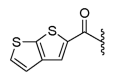
[a]Reagents and conditions: (a) isobutyl bromide, NaOMe, MeOH, Δ; (b) MCPBA, CH_2Cl_2, rt; (c) LiOH, THF, H_2O, rt; (d) (1) NMM, $ClCO_2$-*i*-Bu, THF, −25 °C; (2) $H_2NCH_2CN{\cdot}H_2SO_4$, NaOH, H_2O, −25 °C to rt; (e) $MeSO_3H$, THF, rt; (f) acyl chloride (RCl), Et_3N, THF, rt *or* carboxylic acid (ROH), Et_3N, EDC, DMAP, rt.

synthetic route to the building block **6** (Scheme 1) contains two steps with a risk of racemization: (i) alkylation of the thiol group of the cysteine derivative **1** in the presence of sodium methanolate and (ii) cleavage of the methyl ester **3** with lithium hydroxide. To prevent the racemization, a modified route was applied (Scheme 2). The thiol group of L-cysteine **17** was alkylated under milder conditions and in the presence of the weaker base sodium hydroxide,[16] followed by protection of the amino group using $(Boc)_2O$ to obtain **18**. The thioether derivative **18** was oxidized with $KMnO_4$ instead of MCPBA (Scheme 1), leading to the corresponding sulfone **19**, which was coupled with aminoacetonitrile to form the Boc-protected dipeptide nitrile **20**. After deprotection of **20**, the resulting building block **21** was coupled with benzoyl chloride, leading to the enantiopure dipeptide nitrile **22** whose enantiopurity was confirmed by chiral HPLC (Figure S1, Supporting Information).

The nitrogens of the aza-amino nitrile moiety of azadipeptide nitrile inhibitors are essentially alkylated for reasons of the synthetic access.[17] To synthesize the azadipeptide nitrile **26** (Scheme 2), the aza-analogue of dipeptide nitrile **22**, compound **19** was converted to the corresponding 1,2-dimethylhydrazide **23**. Deprotection of **23** under acidic conditions and the following basic extraction led to **24** which was treated with benzoyl chloride to obtain **25**. The final reaction with cyanogen bromide afforded the azadipeptide nitrile **26**.

All final compounds (**7**−**16**, **22**, and **26**) were evaluated on human cathepsins L, S, K, B, and F in the presence of chromogenic or fluorogenic peptide substrates. In general, these inhibitors showed two different kinetic modes. As expected,[10] the dipeptide nitriles **7**−**16** and **22** were "fast-binding" inhibitors indicated by linear progress curves (Figure S2, Supporting Information). This behavior reflects a relatively fast formation of covalent enzyme−inhibitor thioimidate adducts. The corresponding rates were plotted versus inhibitor concentrations to obtain IC_{50} values by nonlinear regression, which were corrected to zero substrate concentrations leading to inhibition constants K_i. The azadipeptide nitrile **26** exhibited a time-dependent inhibition of cathepsins L, S, K, and B, which implies a relatively slow approach to steady state with an equilibrium between free and inhibitor-bound enzyme (Figure S2, Supporting Information). Besides K_i, the kinetic parameters of association to a covalent isothiosemicarbazide adduct and its dissociation, k_{on} and k_{off}, become accessible.

Table 1. K_i Values of Dipeptide Nitriles 7−16 and 22

cmpd	R	K_i (μM)[a]				
		cat L[b]	cat S	cat K[c]	cat B	cat F[c]
(±)-**7**		> 40	0.040 ± 0.004	> 40	40 ± 2	> 40
(±)-**8**		> 40	0.045 ± 0.002	> 40	24 ± 2	> 40
(±)-**9**		> 4	0.061 ± 0.005	> 4	5.1 ± 0.2	> 4
(±)-**10**		> 4	0.060 ± 0.008	> 4	6.4 ± 0.1	> 4
(±)-**11**		> 4	0.061 ± 0.005	> 4	5.4 ± 0.2	> 4
(±)-**12**		> 4	0.076 ± 0.009	> 4	34 ± 2	> 4
(±)-**13**		> 4	0.20 ± 0.01	> 4	3.9 ± 0.7	> 4
(±)-**14**		> 4	0.094 ± 0.010	1.1 ± 0.1	3.4 ± 0.1	3.2 ± 0.2
(±)-**15**		> 4	0.089 ± 0.012	> 4	6.8 ± 0.1	> 4
(±)-**16**		> 4	0.10 ± 0.01	> 4	7.2 ± 0.2	> 4
(*R*)-**22**		37 ± 1	0.033 ± 0.005	> 40	24 ± 1	> 40

[a]Data were calculated from duplicate experiments with at least five different inhibitor concentrations. Mean values of steady-state rates were used for nonlinear regression to obtain IC_{50}. Standard errors (±SE) of these nonlinear regression analyses are given. Cathepsins L, S, K, and B were assayed as described.[29] For cathepsin F assay, see Supporting Information. [b]For cathepsin L, all limits of >40 μM relate to the maximum possible inhibitor concentrations of >30 μM; all limits of >4 μM relate to maximum possible inhibitor concentrations of ≤30 μM. [c]For cathepsins K and F, all limits of >40 μM relate to the maximum possible inhibitor concentrations of >10 μM; all limits of >4 μM relate to the maximum possible inhibitor concentrations of ≤10 μM.

The K_i values of dipeptide nitriles **7**−**16** and **22** are listed in Table 1. On the one hand, they were all potent inhibitors of human cathepsin S with K_i values between 33 and 200 nM. On

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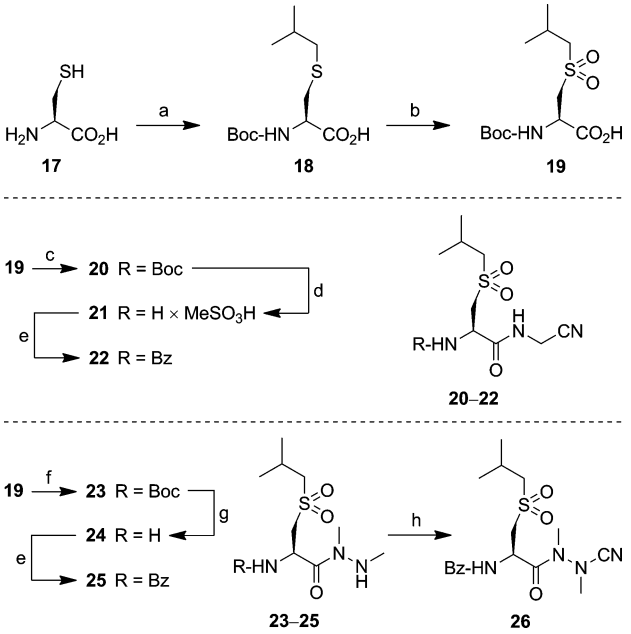
Scheme 2. Synthesis of the Enantiopure Dipeptide Nitrile 22 and the Azadipeptide Nitrile 26[a]



[a]Reagents and conditions: (a) (1) isobutyl bromide, tetrabutylammonium iodide, NaOH, H_2O, EtOH, rt; (2) $(Boc)_2O$, NaOH, H_2O, EtOH, rt; (b) $KMnO_4$, AcOH, H_2O, rt; (c) (1) NMM, $ClCO_2$-*i*-Bu, THF, −25 °C; (2) $H_2NCH_2CN{\cdot}H_2SO_4$, NaOH, H_2O, −25 °C to rt; (d) $MeSO_3H$, THF, rt; (e) benzoyl chloride, DIPEA, THF, rt; (f) (1) NMM, $ClCO_2$-*i*-Bu, THF, −25 °C; (2) $(NHMe)_2{\cdot}2HCl$, NaOH, H_2O, −25 °C to rt; (g) AcCl, EtOH, $MeCO_2Et$, rt, basic extraction; (h) BrCN, NaOAc, MeOH, rt.

the other hand, none of them showed a notable inhibition of cathepsin F, the functional synergist of cathepsin S. Thus, the selected amino acid in P2 position, *S*-(isobutyl)cysteine sulfone, accounts for the affinity to cathepsin S and can favorably be accommodated in its S2 binding pocket. Gauthier et al.[14] demonstrated this interaction by molecular dynamics calculations based on the crystal structure of human cathepsin S.[18] The rather large S2 binding pocket of cathepsin S is lined by the side chain of Met71 and two Gly methylene groups (137 and 165). The pocket is shaped by the side chains of Phe70 and Val162. The S2 subsite is terminated by Phe211. Its side chain is able to perform a conformational switch leading to a remarkable plasticity of the S2 pocket of cathepsin S.[18−20] Therefore, besides the side chains of aliphatic amino acids preferred in P2 position of cathepsin S substrates,[21,22] several hydrophobic moieties of inhibitors can be accommodated in the S2 pocket. Examples include fluorinated phenyl or alkylphenyl,[23,24] cyclohexyl, and 4-methylcyclohexyl groups[20] and the residues of phenylalanine,[18,25] (naphthalen-2-yl)-alanine,[26] leucine,[18,25] cyclohexylalanine[25,26] methyl-branched cycloalkylalanines,[14] (1,2,3,4-tetrahydronaphthalen-2-yl)-alanine,[27] and *S*-(isobutyl)cysteine sulfone.[12] The last amino acid was a particular suitable building block to achieve strong cathepsin S inhibition and selectivity over cathepsins L, K, and B.[12]

Among the amino acid residues that are defining the S2 pocket, Phe70 and Phe211 of cathepsin S are replaced by Leu and Met, respectively, in cathepsin F. The Met residue points toward the Leu residue but does not block the pocket entirely.[28] Thus, medium-sized hydrophobic amino acids at P2 position of peptidic substrates or inhibitors might occupy the S2 pocket. Obviously, the P2 residue of inhibitors **7**−**16** and **22** is too extended for a favorable accommodation within the S2 pocket of cathepsin F.

We introduced structural diversity into the P3 group of the peptide scaffold to perform an active-site mapping with respect to the S3 pocket of cathepsin S. The S3 binding site is defined by three Gly motifs (62, 68, 69) and the backbone structure of Asn63. The sides of this shallow pocket are shaped by the residues Lys64 and Phe70. The flexible nature of Lys64 was considered to allow for a less restricted interaction with a P3 group.[18,20] High affinity for cathepsin S was obtained with peptidic or peptidomimetic ligands containing monocyclic aroyl moieties at P3 position able to interact with Lys64 and Phe70.[12,20] However, the P3 portion could be substantially expanded without a loss of activity.[14]

Thus, we introduced not only monocyclic (hetero)aroyl groups (**7**, **8**) but also acyl substitutents composed of two (hetero)aromatic rings (**9**−**14**) and fused aromatic systems (**15**, **16**). The strongest cathepsin S inhibition was achieved with the nonextended (hetero)aroyl compounds **7** and **8**, exhibiting K_i values of 40 and 45 nM, respectively. Compounds **9**−**14** showed only a somewhat lower activity, provided that the proximal aromatic ring is thiophene; the linear motif as present in **13** was less advantageous. Noteworthy, a distal acidic tetrazole moiety (in **12**) did not produce an effect distinguishable from that of the phenyl or thienyl groups (in **9**−**11** and **14**). In general, the inhibitors listed in Table 1 are selective for cathepsin S over the other cathepsins L, K, B, and F; compounds **7** and **8** are characterized by a selectivity ratio of more than 500. The structure of the benzoyl derivative was chosen to prepare the enantiopure **22**.

The substitution pattern of the azadipeptide nitrile **26** was accordingly derived from **7**/**22**. The replacement of CH by NMe and the concomitant methylation of the P2−P1 peptide bond resulted in an enhanced enzyme inhibition (Table 2), which can be attributed to the formation of stabilized isothiosemicarbazide adducts.[17] However, **26** was no longer selective for cathepsin S. We observed a fast association of cathepsin S and inhibitor, as governed by the second-order rate constant k_{on} of 2 600 000 M^{-1} s^{-1}. This finding indicates that suitable P2 substituents in cathepsin S inhibiting azadipeptide

Table 2. Kinetic Parameters of (*R*)-26[a]

cathepsin	K_i (nM)	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^{-3} s^{-1})
L	15 ± 1[b]	nd[c]	nd[c]
S	0.55 ± 0.03[d]	2600 ± 400	1.4 ± 0.2
K	0.66 ± 0.06	59 ± 2	0.039 ± 0.004
B	5.8 ± 0.2[d]	130 ± 20	0.75 ± 0.12
F	21 ± 3[e]	nd[c]	nd[c]

[a]Data were calculated from duplicate experiments with at least five different inhibitor concentrations. Mean values of steady-state rates were used for nonlinear regression to obtain IC_{50}. Mean values of observed first-order rate constants were used for linear regression to obtain $k_{on}/(1 + [S]/K_m)$ values. The standard errors (±SE) of the linear and nonlinear regression analyses are given. k_{off} was calculated as $k_{off} = K_i k_{on}$. Cathepsins L, S, K, and B were assayed as described.[29] For cathepsin F assay, see Supporting Information. [b]The progress curves were analyzed by linear regression in a time interval between 8 and 16 min. [c]Not determined. [d]The progress curves were analyzed by nonlinear regression in a time interval of 20 min. [e]The progress curves were analyzed by linear regression in a time interval of 30 min.

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nitriles give rise to an accelerated association. For *N*-(benzyloxycarbonyl)cyclohexylalanyl-methylazaalanine-nitrile a likewise high k_{on} value was reported.[29]

CONCLUSION

We have performed a systematic scan to explore the S3 binding pocket of cathepsin S and designed highly potent and selective dipeptide nitrile inhibitors for this protease. Furthermore, the optimized structure (**22**) was applied in the synthesis of an analogous azadipeptide representative (**26**). A substrate specificity profiling of papain-like cysteine proteases with respect to the amino acids at positions P4−P1 revealed a high similarity of the two functionally synergistic proteases cathepsins S and F.[21] Therefore, the selectivity profile of our inhibitors was unexpected. Inhibition of cathepsin F was practically not achieved by the nitrile compounds of this study, with the exception of the unselective azadipeptide nitrile **26**. Thus, it could be demonstrated for the first time that prototype cathepsin S inhibitors may not necessarily affect cathepsin F. Treatment with such cathepsin S inhibitors would only partially reduce antigen presentation, inasmuch as the cathepsin F-catalyzed processing of the invariant chain, particularly in macrophages, would be maintained. Future medicinal chemistry studies will have to focus on selective inhibitors for cathepsin F, as well as dual inhibitors for both proteases, cathepsins S and F.

EXPERIMENTAL SECTION

General methods and materials are given in the Supporting Information. All tested compounds possessed a purity of not less than 95% except for **12**, which had 94% purity.

***N*-(*tert*-Butyloxycarbonyl)-*S*-(isobutyl)cysteine (18).** L-Cysteine (**17**, 5.00 g, 41.3 mmol) was dissolved in a 1:1 EtOH/2 N NaOH mixture (82 mL). Isobutyl bromide (6.22 g, 45.4 mmol) and tetrabutylammonium iodide (0.46 g, 1.25 mmol) were added. The mixture was stirred for 3 days at room temperature. $(Boc)_2O$ (9.91 g, 45.4 mmol) was added, and the mixture was additionally stirred for 1 day at room temperature. EtOH was evaporated under reduced pressure. The aqueous residue was acidified with concentrated HCl (pH ≈ 1) and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% $KHSO_4$ (30 mL) and saturated NaCl (30 mL). The solvent was dried over Na_2SO_4 and evaporated to obtain **18** as an oily yellow product (10.9 g, 95%).

***N*-(*tert*-Butyloxycarbonyl)-*S*-(isobutyl)cysteinylsulfone-glycine-nitrile (20).** Compound **18** (10.8 g, 38.9 mmol) was dissolved in AcOH (80 mL). $KMnO_4$ (12.3 g, 77.8 mmol) was dissolved in H_2O (130 mL) and slowly added to the reaction mixture. It was stirred for 2.5 h, followed by the addition of saturated $KHSO_3$ solution until the mixture became colorless. It was concentrated under reduced pressure, and the aqueous suspension was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were washed with H_2O (30 mL), brine (30 mL) and dried over Na_2SO_4. The solvent was evaporated to obtain **19** as a colorless oily product (11.1 g, 92%). Compound **19** (2.00 g, 6.46 mmol) was dissolved in dry THF (40 mL), and the mixture was cooled to −25 °C. *N*-Methylmorpholine (0.72 g, 7.12 mmol) and isobutyl chloroformate (0.97 g, 7.10 mmol) were added consecutively. Aminoacetonitrile monosulfate (1.47 g, 9.54 mmol) was dissolved in H_2O (2 mL), treated with 2 N NaOH (5 mL), and added to the mixture when the precipitation of *N*-methylmorpholinium chloride occurred. It was allowed to warm to room temperature within 30 min and stirred for additional 90 min. THF was evaporated, and the resulting aqueous suspension was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% $KHSO_4$ (30 mL), saturated $NaHCO_3$ (30 mL), H_2O (30 mL), and saturated NaCl (30 mL). The solvent was dried (Na_2SO_4) and evaporated. The product was recrystallized from ethyl acetate to obtain **20** as a white solid (0.94 g, 42% from **19**).

(*R*)-*N*-(Benzoyl)-*S*-(isobutyl)cysteinylsulfone-glycine-nitrile (22). Compound **20** (0.68 g, 1.96 mmol) was dissolved in dry THF (10 mL). Under ice-cooling, dry methanesulfonic acid (1.14 g, 11.9 mmol) was added, and the resulting mixture was stirred for 14 h at room temperature. The precipitated product was filtered off, washed with petroleum ether, and dried to obtain **21** as a white solid without further purification (0.33 g). Compound **21** (0.33 g, 0.96 mmol) was dissolved in dry THF (30 mL). DIPEA (0.35 g, 2.71 mmol) and benzoyl chloride (0.19 g, 1.35 mmol) were added consecutively. The solution was stirred for 24 h at room temperature. The solvent was evaporated, and the resulting white solid was extracted with ethyl aceate (3 × 30 mL). The combined organic layers were washed with 10% $KHSO_4$ (30 mL) and brine (30 mL). The solvent was dried (Na_2SO_4) and evaporated. The crude product was recrystallized from ethyl acetate to obtain **22** as a white solid (0.28 g, 41% from **20**).

ASSOCIATED CONTENT

Supporting Information

Preparation of compounds, cathepsin F assay, chiral chromatograms, representative kinetic plots, ^1H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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