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Peptidic 1-Cyanopyrrolidines: Synthesis and SAR of a Series of Potent, Selective Cathepsin Inhibitors

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Abstract—1-Cyanopyrrolidines have previously been reported to inhibit cysteinyl cathepsins (Falgueyret, J.-P. et al., *J. Med. Chem.* 2001, *44*, 94). In order to optimize binding interactions for a given cathepsin and simultaneously reduce interactions with the other closely related enzymes, small peptidic substituents were introduced to the 1-cyanopyrrolidine scaffold, either at the 2-position starting with proline or at the 3-position of aminopyrrolidines. The resulting novel compounds proved to be micromolar inhibitors of cathepsin B (Cat B) but nanomolar to picomolar inhibitors of cathepsins K, L, and S (Cat K, Cat L, Cat S). Several of the compounds were >20-fold selective versus the other three cathepsins. SAR trends were observed, most notably the remarkable potency of Cat L inhibitors based on the 1-cyano-D-proline scaffold. The selectivity of one such compound, the 94 picomolar Cat L inhibitor **12**, was demonstrated at higher concentrations in DLD-1 cells. Although none of the compounds in the proline series that was tested proved to be submicromolar in the in vitro bone resorption assay, two Cat K inhibitors in the 3-substituted pyrrolidine series, **24** and **25** were relatively potent in that assay.

Introduction

Cathepsins that contain active site nucleophilic cysteine residues play key roles in a variety of biological processes.[1,2] Both mouse knockout experiments[3] and a primate model of bone resorption[4] have demonstrated the importance of Cat K in that process. Similarly, the roles of Cat S in MHC Class II antigen presentation[5] and of Cat B in cancer metastasis[6,7] as well as apoptosis[8] are the subjects of much ongoing research. For these and other efforts, potent and selective cathepsin inhibitors would be useful to induce 'chemical knockouts' for target validation in cellular and animal studies. Such tool compounds, which ideally should have physical properties and metabolic stability suitable for in vivo testing, are rarely available. Consequently efforts to make such compounds are of great interest.

Endogenous inhibitors of cathepsins include high molecular weight propeptide segments and cystatins.[9] Small molecule inhibitor design typically involves a di- or tripeptide covalently attached to an electrophilic 'warhead'. The backbone hydrogen bonding and usually hydrophobic sidechain interactions of the peptidic portion provide binding interactions with the enzyme and afford selectivity against closely related enzymes, while the electrophilic segment reacts covalently with the active site thiol, either in a reversible manner as with aldehydes[10] and ketones[11] or irreversibly as with vinyl sulfones.[12]

It was recently reported that 1-cyanopyrrolidines such as **1** (Fig. 1) and the closely related 1-cyanoazetidines react covalently with the active site thiol group of papain via reversible isothiourea formation.[13,14] The reported compounds displayed nanomolar activity for Cat K and modest selectivity versus other cathepsins. We decided to investigate in an empirical fashion whether or not additional potency and selectivity could be gained through interactions provided by amino acid substitution at various positions in this scaffold. This would

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allow us to quickly explore SAR using a variety of readily available, chirally pure starting materials. Peptidic substituents were also of interest due to their inherent conformational constraints and hydrogen bonding potential. Information thus obtained might translate into peptidomimetics less susceptible to metabolic cleavage, should this prove to be a problem. Here we report the results of these efforts.

Chemistry

No X-ray crystal structure of a cyanamide bound at the active site of a cysteine protease was available and attempts to dock these compounds into the active site of the enzymes did not provide definitive conformations consistent with observed SAR. We decided to base our initial series of compounds on the readily available 1-cyanoprolines. To the best of our knowledge compounds of this type have not been previously reported.

As shown in Scheme 1, standard peptide coupling of α-aminoesters with Boc protected-proline was followed by N-terminal deprotection to afford the dipeptide **3**. Reaction with cyanogen bromide was carried out under biphasic buffered conditions.[15] This afforded a good yield of the 1-cyano dipeptide **4**, a colorless oil that was stable for weeks at ambient temperature. Since no model of inhibitor binding was available it was impossible to predict which stereoisomer would be more active. Therefore both D- and L-amino acids were used at each position. Table 1 (entries **4** and **7**–**18**) lists the compounds prepared in this general way. D-Prolylleucine benzyl ester, prepared by similar succinate ester coupling, was reacted with ethyl formimidate to afford the corresponding amidine **5** and with ethyl formate to give formamide **6**. *N*-Cyanation of peptides that were either commercially available or prepared through standard methods gave compounds **19**–**22**.

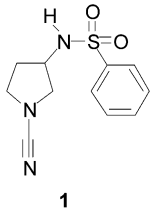

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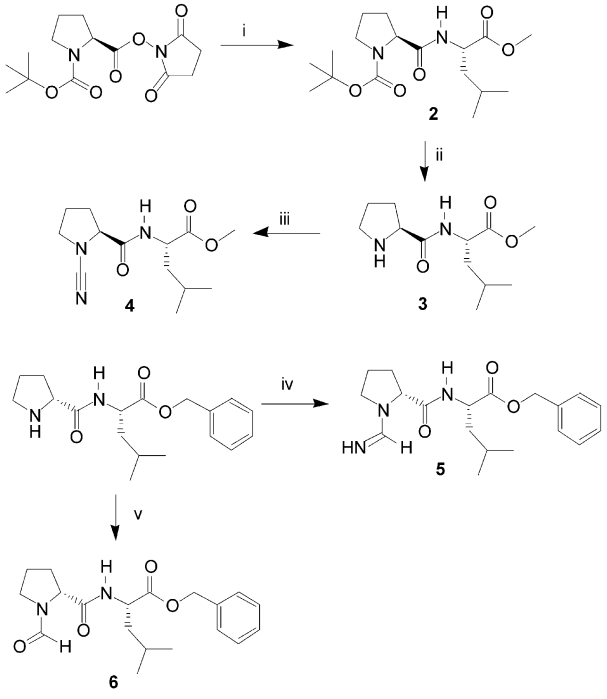
Figure 1.

Compounds substituted in the 3-position of the pyrrolidine ring, hence peptidic analogues of **1**, were prepared as outlined in Scheme 2. Coupling of the commercially



Scheme 1. Synthesis of 1-substituted proline dipeptides. Reagents: (i) H-Leu-OMe, DIPEA; (ii) TFA; (iii) BrCN, $MgCO_3$; (iv) EtOCH(NH)·HCl; (v) EtOC(O)H.

Table 1. Cathepsin inhibitory activities (K_i apparents) for peptidic cyanamides

Compd	Structure	Cat B K (μM)	Cat K K_i (μM)	Cat L K_i (μM)	Cat S K_i (μM)
4	NC-Pro-Leu-OMe	3.3±0.36	0.067±.008	1.2±0.13	0.052±0.0052
5	See Scheme 1	>150	27±7.6	19±6.1	>150
6	See Scheme 1	>150	>150	>150	>150
7	NC-D-Pro-Leu-OMe	94±15	0.13±0.012	0.010±0.0011	0.54±0.081
8	NC-Pro-D-Leu-OMe	20±1.6	0.65±0.046	4.7±1.2	1.1±0.15
9	NC-D-Pro-D-Leu-OMe	>150	0.51±0.02	0.53±0.12	26±6.8
10	NC-hyPro(OBn)-Leu-OMe	0.78±0.078	0.027±0.003	0.36±0.061	0.03±0.0051
11	NC-Pro-Leu-OBn	1.1±0.13	0.015±0.0017	0.17±0.017	0.014±0.0029
12	NC-D-Pro-Leu-OBn	4.7±0.47	0.0078±0.00062	0.000094±0.0000085	0.012±0.00072
13	NC-Pro-Phe-OMe	0.96±0.096	1.9±0.27	2.5±0.22	0.24±0.029
14	NC-Pro-Ala-OMe	5.3±1.6	0.16±0.027	2.7±0.92	0.53±0.042
15	NC-hyPro-Leu-OMe	9.8±1.8	0.055±0.0072	2.1±0.32	0.054±0.0081
16	NC-D-Pro-Ala-OMe	>150	2.8±0.2	0.41±0.09	54±8.6
17	NC-D-Pro-Leu-NMe_2	110±12	0.063±0.012	0.0052±0.00073	0.15±0.026
18	NC-D-Pro-Ala-NHBn	>150	22±3.5	1.3±0.17	30±7.8
19	NC-Pro-Leu-Gly-OEt	10.6±1.8	1.7±0.17	8.0±0.8	0.035±0.0053
20	NC-Pro-Leu-Leu-Leu-OMe	2±0.36	0.94±0.11	0.14±0.021	0.0067±0.0011
21	NC-(3,4-Didehydro)Pro-LeuOMe	1.2±0.28	0.057±0.0074	1.5±0.11	0.033±0.005
22	NC-Pro-NH(isoamyl)	3.2±0.99	0.055±0.0055	0.26±0.016	0.28±0.039
24	See Scheme 2	1.5±0.14	0.012±0.0011	0.25±0.042	0.31±0.078
25	See Scheme 2	13±0.91	0.018±0.0009	0.010±0.0005	0.97±0.11

available, resolved (3*R*)- or (3*S*)-1-benzyl-3-aminopyrrolidine with CBZ-leucine succinate ester gave either (3*R*)- or (3*S*)-**23**. Von Braun reaction[16] of **23** with cyanogen bromide gave the corresponding epimers **24** or **25**.

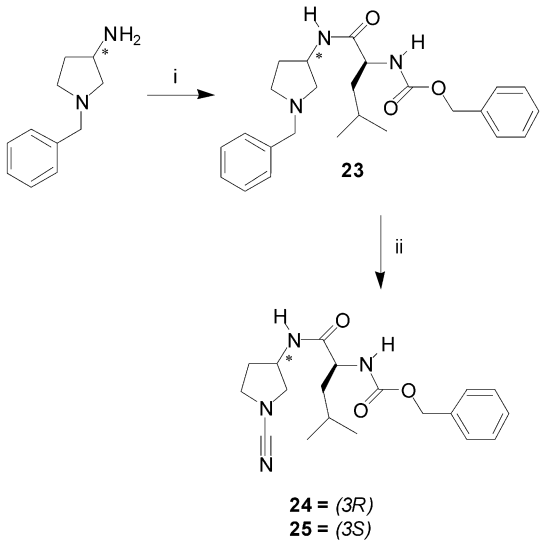
Results

The synthesis of a series of analogous inhibitors with single structural 'point mutations' and subsequent screening against a 'panel' of cathepsins (B, K, L, and S) allowed for a wealth of SAR information to be determined. The question of the importance of covalent interaction with the N-terminal nitrile group to inhibitory potency was first addressed through the synthesis of amidine 5 and the formamide **6** (Scheme 1). These were made through routes which did not involve possible contamination by traces of cyanamides that might provide spurious activity. Both compounds lacking the *N*-cyano group were essentially inactive compared to cyanamides such as **12**. For example, formamidine **5** proved to be 200,000-fold less active as a Cat L inhibitor than the corresponding cyanamide **12**. Free amino dipeptide **3** had a $K_i > 150$ μM versus all four cathepsins. Clearly the covalent interaction of the enzymes' Cys-29 sulfhydryl groups (using the papain numbering scheme) with the cyanamide 'warhead' were necessary for submicromolar potency and attempts to prepare noncovalent inhibitors lacking such interactions would prove difficult at best.

Table 1 shows that the peptidic cyanamides prepared were micromolar to picomolar inhibitors of cathepsins B, K, L, and S. Potency against CatB was usually weakest, in agreement with a trend previously observed for nonpeptidic cyanamides.[13] The most remarkable SAR observed involved the apparent preference of Cat L for D-versus L-prolines. For example, the L-proline containing **4** was a 1200 nM inhibitor of Cat L, while the analogous D-proline compound **7** was a 10 nM inhibitor. In compounds terminated by benzyl, as opposed to methyl esters, the D-proline compound **12** proved to be an extraordinary 94 picomolar Cat L inhibitor, while the L-proline analogue **11** was 170 nM versus CatL. In the absence of crystallographic data or knowledge of the basic binding mode (prime side or unprimed side) no structural explanation for this subtle but important difference can be offered. Empirically the effect imparted >80-fold Cat L selectivity for compound **12**.

To confirm this selectivity in a cellular environment, experiments were carried out using inhibitor **12** in DLD-1 cells, which express both Cat L and Cat B (see Fig. 2). After incubating the cells with inhibitor at various concentrations, the cells were treated with ^{125}I-labelled Tyr-Ala DMK, a cell permeable, irreversible inhibitor of both cathepsins.[17,18] As shown in Figure 2, SDS-PAGE gel electrophoresis revealed that at a concentration of 100 nM, **12** caused the disappearance of nearly all of the Cat L bands at ~20 and ~24 kD with much less reduction in the ~22 kD Cat B band. At a high enough (micromolar) concentration both enzymes were inhibited. The epimer of **12**, compound **11**, which was a far less potent Cat L inhibitor, seemed to be a much poorer inhibitor of cellular Cat L as well. Although the concentration of **12** required to inhibit cellular Cat L was much higher than the inhibitor's K_i for that enzyme, most likely due to cell permeability and/or stability issues, selectivity was borne out for this D-proline containing compound.

SAR analysis of compounds containing a D-amino acid at the second position was less fruitful. A 4- to 50-fold loss of inhibitory potency versus all four cathepsins (comparing the potencies of **8** to those of **4,** for example) was observed. Leucine, a hydrophobic residue originally used because it binds well in the S2 pocket of Cat K,[19] was varied to alanine in **14** and phenylalanine in **13**. Both modifications resulted in a loss of potency versus cathepsins K, L, and S relative to the leucine analogue **4**. Also, substitutions at the 3-position of the proline by the hydroxy (**15**) or benzyloxy (**10**) groups had little effect on potencies which probably indicates a



Scheme 2. Synthesis of 3-amino substituted pyrrolidines. Reagents: (i) CBZ-Leu-OSu; (ii) BrCN.

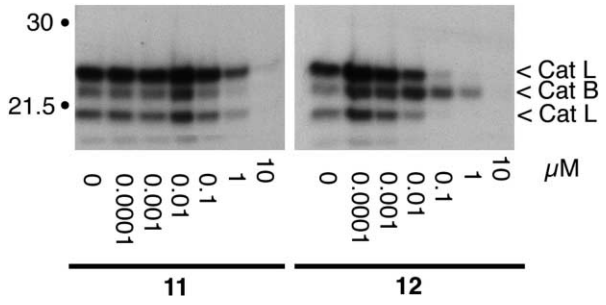


Figure 2. Effect of compounds on intracellular Cat B and Cat L activity. DLD-1 cells were treated with the indicated concentration of compound for 45 min before adding 2 μCi of ^{125}I-Tyr-Ala-DMK. Cells were further incubated for 2 h at 37 °C. Samples were collected and analyzed by SDS-PAGE and autoradiography. The position of active Cat B and Cat L are indicated on the right.

lack of binding interactions at this position within this series.

The simple addition of a phenyl ring to the C-terminus of the 1-cyano dipeptides (benzyl vs methyl esters) caused an increase in inhibitory potency towards all four cathepsins. Benzyl ester **11**, for example, was a 15 nM Cat K inhibitor and 14 nM versus Cat S while the corresponding methyl ester **4** had K_i's of 67 and 52 nM, respectively. The Cat L activity of benzyl ester **12** was 100-fold greater than that of the corresponding methyl ester **7**. Extending the C-terminus via PLG-containing **19** had only small effects on cathepsin activities while extending out to tetrapeptide **20** increased potency versus Cat S (6.7 nM) while decreasing potencies versus Cat K and Cat L, resulting in the most Cat S selective, highest molecular weight compound in this series. Shortening the inhibitor to the simple capped monopeptide **22** resulted in a slight increase in Cat K and Cat L activity and loss of Cat S activity relative to **4** thus indicating that smaller, less peptidic *N*-cyanoprolines should be feasible. This effect, along with the general levels of activity and selectivity observed with the dipeptide cyanamides, was somewhat surprising in light of the much more modest potencies of several 2-substituted 1-cyanopyrrolidines reported earlier.[13] One explanation may lie in the conformational constraints and/or hydrogen bonding interactions provided by the amide bond at C-2, which were not present in the previously described compounds.

In order to examine the effects of peptide substitution on the 3-substituted pyrrolidine cyanamide scaffold described previously,[13] a second series of compounds was prepared starting with commercially available N-1 protected, chirally pure 3-aminopyrrolidines. The compound resulting from the coupling of the *R*-enantiomer with CBZ-leucine, followed by *N*-cyanation, **24**, proved to be a 12 nM, 20-fold selective Cat K inhibitor. The corresponding *S*-epimer, **25** retained similar (18 nM) Cat K potency but was much more active (10 nM versus 250 nM) than **24** as a Cat L inhibitor, and thus could be thought of as a mixed Cat K/L inhibitor.

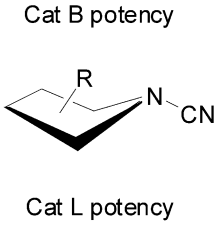


Figure 3. Potency for Cat B is higher when substituent R is on the top face as drawn while potency for Cat L increases with substitution on the bottom face as drawn.

Table 2. In vitro bone resorption IC_{50} values for peptidic cyanamides[a]

Compd	Bone resorption IC_{50} (μM)
7	>1.0
11	>1.0
12	>1.0
15	>1.0
21	>1.0
24	0.103
25	0.072

[a]Inhibition of bone resorption as described in ref 13.

Examination of cathepsin inhibitory data for matched pairs of stereoisomers (**4**/**7**, **8**/**9**, **11**/**12**, **14**/**16**, and **24**/**25**) revealed consistent trends in the stereochemistry required for Cat B and Cat L potency as shown in Fig. 3. Compounds substituted on the top face of the pyrrolidine ring as drawn, which is the *Si* face in the proline series and the *Re* face in the 3-aminopyrrolidine series, are more potent Cat B inhibitors than their corresponding epimers, while the opposite is true for Cat L. Cat K and Cat S did not display consistent trends in this regard. The structural basis for these preferences is at present unknown.

As shown in Table 2, seven of the compounds synthesized were examined for their effects on bone resorption in the functional assay previously described.[13] In the proline series even the most potent inhibitor of Cat K and Cat L, compound **12**, did not display a submicromolar IC_{50}. In contrast, both 3-aminopyrrolidine epimers **24**, a selective Cat K inhibitor, and **25**, which is a mixed inhibitor of both Cat K and Cat L, had similar submicromolar IC_{50}s (103 nM for **24** and 72 nM for **25**) in the bone resorption assay. These results again must be interpreted in light of the additional factors of cell permeability and inhibitor stability. Interestingly, **24** and **25** were nearly equipotent in the bone resorption assay despite their 25-fold difference in Cat L potencies. A recent publication[20] as well as an earlier report of a Cat L knockout mouse, where no bone abnormalities were observed,[21] suggest that unlike Cat K, Cat L is not crucial to bone resorption.[3]

Conclusions

Peptidic substitution at two different points on the 1-cyanopyrrolidine scaffold made structurally novel compounds with major differences in inhibitory potencies and selectivities towards four different cathepsins (B, K, L, and S). The complex, unpredictable, and differential effects of structural changes on potency for these four cathepsins should serve as a caveat against using 'selective' cathepsin inhibitors in proof of concept studies without full screening against a panel of related enzymes. Potencies against all four cathepsins were shown to be dependent on covalent binding to the cyanamide 'warhead'. Although no binding model was available, empirical variations in the peptide substituents allowed for the synthesis of nanomolar, at least 20-fold selective inhibitors of Cat K, Cat L, and Cat S. Two of the Cat K inhibitors displayed submicromolar activity in an in vitro functional assay. Thus, substituted 1-cyano-pyrrolidines may prove useful as tools for future studies addressing the roles of cysteine proteases in normal and pathophysiological conditions.

Experimental

General

Melting points were obtained using a Mel-Temp 3.0 melting point apparatus and are uncorrected. NMR spectra were recorded on either a GE QE300 (300 MHz) or a Jeol Eclipse (270 MHz) instrument, with chemical shifts shown in δ values (ppm) with tetramethylsilane (TMS) as internal reference. APT ^{13}C spectra note even (e) or odd (o) peak multiplicity. All assayed compounds were at least 95% pure by HPLC analysis. HPLC was carried out using an Agilent 1100 HPLC equipped with a 5-μ Phenomenex C-18 column running a 10–100% gradient elution of methanol in 0.1% acetic acid. This was attached to a Sciex ABI 150EX single quadrupole mass spectrometer. Electrospray mass spectra obtained without LC analyses were done using a Finnegan TSQ7000 triple quadrupole instrument. Chemical ionization mass spectra were obtained using a Hewlett Packard 5989A mass spectrometer. Resolved 1-benzyl-3-aminopyrrolidines were purchased from TCI America. Cathepsin B (Calbiochem, San Diego, CA, USA) and cathepsin L (Athens Research and Technology, Athens, GA, USA) were purified from human liver and purchased from the indicated commercial sources. Human cathepsins S and K were cloned and expressed as described.[22,23] The enzyme active site concentrations were measured by titration with either E-64 or the vinyl-sulfone APC-3316, Me-Pip-Phe-HphVSPh.[24]

(*tert*-Butoxycarbonyl)-prolylleucine methyl ester (2). *N*-(*tert*-Butoxycarbonly)-(L)-proline *N*-hydroxysuccinimide ester (1.25 g, 4.0 mmol), (L)-leucine methyl ester hydrochloride (0.727 g, 4.0 mmol), and diisopropylethylamine (1.4 mL, 8.0 mmol) were stirred at ambient temperature in a solution of 14 mL acetonitrile and 1 mL *N*,*N*-dimethylformamide for 20 h. The reaction mixture was filtered and the filtrate rotary evaporated. The residue was partitioned between ethyl acetate and 1 N HCl in a separatory funnel. The organic phase was separated, washed with 1 N HCl, water, saturated sodium bicarbonate, brine, and then dried over anhydrous $MgSO_4$. Filtration and solvent evaporation afforded 874 mg of **2** as a white solid, a 64% yield. Proton NMR analysis at 20 °C showed two sets of NH doublets due to rotamers. At 60 °C, these peaks coalesced. ^1H NMR (DMSO, 60 °C, 300 MHz) δ 7.98 (bs, 1H, N*H*), 4.24 (m, 1H, leucine NC*H*CO), 4.10 (m, 1H, proline 2-C*H*), 3.59 (s, 3H, OCH_3), 3.2–3.4 (m, 2H, proline 5-CH_2), 1.40–2.18 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 1.35 (s, 9H, *tert*-butyl), 0.84 [6H, apparent dd, $J = 6.4$, 13.3 Hz, CH(CH_3)$_2$].

Prolylleucine methyl ester (3). Compound **15** (0.700 g, 2.04 mmol) was dissolved in 7 mL dichloromethane and 3 mL TFA was added. After 1 h at ambient temperature, the solvent was rotary evaporated and the residue was partitioned between ethyl acetate and a 1:1 solution of saturated aqueous sodium bicarbonate and saturated aqueous sodium carbonate in a separatory funnel. The organic phase was separated, washed with brine, and dried over anhydrous $MgSO_4$. Filtration and solvent evaporation gave 430 mg of **3** as a semisolid (an 88% yield). ^1H NMR (DMSO, 300 MHz) δ 8.17 (d, $J = 8.6$ Hz, 1H, N*H*), 4.34 (m, 1H, leucine NC*H*CO), 3.62 (s, 3H, OCH_3). 3.55 (m, 1H, proline 2-C*H*), 2.77 (m, 2H, proline 5-CH_2), 1.40–2.00 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.84 [6H, apparent dd, $J = 5.1$, 12.0 Hz, CH(CH_3)$_2$].

1-Cyanoprolylleucine methyl ester (4). A mixture of 5 mL diethyl ether, 1 mL water, and 100 mg $MgCO_3$ was vigourously stirred in an ice-bath. 0.23 mL of a 5.0 M solution of cyanogen bromide in acetonitrile (1.13 mmol) was added, followed by a solution of **3** (0.183 g, 0.755 mmol) in 2.5 mL diethyl ether. The ice-bath was removed and the mixture was stirred vigorously for 1 h. The reaction mixture was partitioned between diethyl ether and water in a separatory funnel. The organic phase was separated, washed with brine, and dried over anhydrous $MgSO_4$. Filtration and solvent evaporation gave a residue that was flash chromatographed on silica gel using 3% methanol in dichloromethane. Compound **4** (176 mg) was obtained as a colorless oil (an 87% yield). ^1H NMR (DMSO, 300 MHz) δ 8.52 (d, $J = 7.8$ Hz, 1H, N*H*), 4.29 (m, 1H, leucine NC*H*CO), 4.16 (m, 1H, proline 2-C*H*), 3.62 (s, 3H, OCH_3), 3.45 (m, 2H, proline 5-CH_2), 1.44–2.20 [m, 7H, proline 3-CH_2, 4- CH_2, CH_2C*H*(CH$_3$)$_2$], 0.84 [6H, apparent dd, $J = 6.2$, 13.5 Hz, CH(CH_3)$_2$]; ^{13}C APT NMR (67.5 MHz, $CDCl_3$) δ 172.8 (e, proline *C*O), 169.9 (e, leucine *C*O), 116.3 (e, N*C*N), 64.8 (o, proline 2-*C*H), 52.6 (o, leucine N*C*HCO), 52.5 (e, proline 5-*C*H_2), 51.0 (o, O*C*H_3), 41.4 [e, *C*H_2CH(CH$_3$)$_2$], 30.9 (e, proline 3-*C*H_2), 25.3 [o, *C*H(CH$_3$)$_2$], 24.7 (e, proline 4-*C*H_2), 22.8 (o, CH*C*H$_3$), 21.4 (o, CH*C*H$_3$). MS (electrospray): mH^+ 267.9 (100), (m + Na)$^+$ 289.9 (46).

1-Iminomethyl-(D)-prolyl leucine benzyl ester (5). (D)-Prolyl leucine benzyl ester had previosly been prepared through the coupling of Boc-(D)-proline succinate ester with leucine benzyl ester followed by deprotection with TFA in this same manner as **3**. Ethyl formimidate hydrochloride (83 mg, 0.76 mmol) and (D)-prolylleucine benzyl ester (191 mg, 0.60 mmol) were dissolved in 2 mL ethanol and stirred at room temperature overnight. The solvent was rotary evaporated and the residue was flash chromatographed on silica gel, eluting with 85/13/2 dichloromethane/methanol/HOAc. 78.2 mg of **5** was obtained as a yellow gum. ^1H NMR (DMSO + D_2O, 60 °C, 300 MHz) δ 7.96 (s, 1H, N*H*), 7.34 (s, 5H, aryl C*H*), 5.12 (s, 2H, PhCH_2), 4.58 (m, 1H, proline 2-C*H*), 4.35 (m, 1H, leucine NC*H*CO), 3.46 (m, 2H, proline 5-CH_2), 2.88 (s, 1/2H, amidine C*H*), 2.72 (s, 1/2H, amidine C*H*), 1.45–2.30 [m, 7H, NCH$_2$CH_2 CH_2, CH_2C*H*(CH$_3$)$_2$], 0.84 [6H, apparent dd, $J = 5.4$, 14.7 Hz, CH(CH_3)$_2$]; ^{13}C APT NMR (67.5 MHz, CD_3OD) δ 172.5 (e, proline *C*O), 170.9 (e, leucine *C*O), 154.7 (o, amidine *C*H), 135.8 (e, 1-phenyl *C*), 128.3,128.1, 128.0 (o, aryl *C*Hs), 66.8 (e, Ph*C*H$_2$), 64.3 (o, proline 2-*C*H), 51.8 (o, leucine N*C*HCO), 47.9 (e, proline 5-*C*H_2), 39.7 [e, *C*H_2CH(CH$_3$)$_2$], 30.5 (e, proline 3-*C*H_2), 24.8 [o, *C*H(CH$_3$)$_2$], 23.1 (e, proline 4-*C*H_2), 21.9 (o, CH*C*H$_3$), 20.4 [o, CH(*C*H$_3$)]. MS (electrospray): mH^+ 346.0 (100).

1-Formyl-(D)-prolylleucine benzyl ester (6). (D)-Prolylleucine benzyl ester (124 mg, 0.389 mmol) was dissolved in 5 mL of ethyl formate. The solution was heated to reflux for 2 h, then cooled to room temperature and rotary evaporated. The residue was flash chromatographed on silica gel, eluting with 1% methanol in dichloromethane. 41 mg of **6** (a 30% yield) was obtained as white crystals, mp 117.5–118.5 °C. ^{1}H NMR (DMSO, 270 MHz) δ 8.47 (d, $J=7.9$ Hz, 1/2H, formamide C*H* rotamer), 8.16 (bs, 1H, N*H*), 7.99 (s, 1/2H, formamide C*H* rotamer), 7.36 (bs, 5H, Ph), 5.10 (s, 2H, CH_2Ph), 4.2–4.45 (m, 2H, proline 2-C*H*, leucine NC*H*CO), 3.52 (m, 2H, proline 5-CH_2), 1.35–2.20 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.83 [m, 6H, CH(CH_3)$_2$]. MS (electrospray): mH^+ 347.0 (100), (m + Na)$^+$ 369.1 (54).

The following compounds were prepared using the method described for **4.**

1-Cyano-(D)-prolylleucine methyl ester (7). Colorless oil. ^{1}H NMR (DMSO, 300 MHz) δ 8.47 (d, $J=8.1$ Hz, 1H, N*H*), 4.28 (m, 1H, leucine NC*H*CO), 4.12 (m, 1H, proline 2-C*H*), 3.59 (s, 3H, OCH_3), 3.46 (m, 2H, proline 5-CH_2), 1.40–2.20 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.81 [6H, apparent dd, $J=6.0$, 18.9 Hz, CH(CH_3)$_2$]. MS (electrospray): mH^+ 268.0 (100), (m + Na)$^+$ 290.0 (57).

1-Cyanoprolyl-(D)-leucine methyl ester (8). Colorless oil. ^{1}H NMR (DMSO, 270 MHz) δ 8.49 (d, $J=7.9$ Hz, 1H, N*H*), 4.34 (m, 1H, leucine NC*H*CO), 4.15 (m, 1H, proline 2-C*H*), 3.63 (s, 3H, OCH_3), 3.43 (m, 2H, proline 5-CH_2), 1.45–2.25 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.84 [6H, apparent dd, $J=6.2$, 16.8 Hz, CH(CH_3)$_2$].

1-Cyano-(D)-prolyl-(D)-leucine methyl ester (9). Colorless oil. ^{1}H NMR (DMSO, 270 MHz) δ 8.52 (d, $J=7.9$ Hz, 1H, N*H*), 4.33 (m, 1H, leucine NC*H*CO), 4.15 (m, 1H, proline 2-C*H*), 3.62 (s, 3H, OCH_3), 3.42 (m, 2H, proline 5-CH_2), 1.45–2.25 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.87 [6H, apparent dd, $J=6.2$, 12.9 Hz, CH(CH_3)$_2$].

1-Cyano-(*trans*-benzyloxy)prolylleucine methyl ester (10). Colorless oil. ^{1}H NMR (DMSO, 270 MHz) δ 8.63 (d, $J=7.7$ Hz, 1H, N*H*), 7.32 (bs, 5H, Ph), 4.52 (s, 2H, CH_2Ph), 4.28 (m, 3H, hyPro 2-C*H*, hyPro 4-C*H*, leucine NC*H*CO), 3.62 (s, 3H, OCH_3), 3.58 (m, 2H, hyPro 5-CH_2), 2.29 (m, 1H, hyPro 3-C*H*), 1.95 (m, 1H, hypro 3-C*H*), 1.44–1.72 [m, 3H, CHCH_2C*H*(CH$_3$)$_2$], 0.88 [6H, apparent dd, $J=6.4$, 12.1 Hz, CH(CH_3)$_2$]. MS (PCI): mH^+ 374.

1-Cyano-prolylleucine benzyl ester (11). Colorless oil. ^{1}H NMR (DMSO, 300 MHz) δ 8.53 (d, $J=7.5$ Hz, 1H, N*H*), 7.32 (s, 5H, Ph), 5.08 (ABq, $J=12.6$ Hz, 2H, CH_2Ph), 4.31 (m, 1H, leucine NC*H*CO), 4.13 (m, 1H, proline 2-C*H*), 3.40 (m, 2H, proline 5-CH_2), 1.44–2.18 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.81 [6H, apparent dd, $J=6.0$, 15.3 Hz, CH(CH_3)$_2$]. MS (electrospray): mH^+ 344.0 (100).

1-Cyano-(D)-prolylleucine benzyl ester (12). Viscous, colorless oil. ^{1}H NMR (DMSO, 300 MHz) δ 8.52 (d, $J=8.1$ Hz, 1H, N*H*), 7.35 (s, 5H, Ph), 5.11 (s, 2H, CH_2Ph), 4.34 (m, 1H, leucine NC*H*CO), 4.15 (m, 1H, proline 2-C*H*), 3.44 (m, 2H, proline 5-CH_2), 1.45–2.20 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.84 [6H, apparent dd, $J=6.0$, 19.8 Hz, CH(CH_3)$_2$]. ^{13}C APT NMR (67.5 MHz, CDCl$_3$) δ 172.4 (e, proline *C*O), 170.0 (e, leucine *C*O), 135.2 (e, 1-phenyl *C*), 128.7, 128.6, 128.4 (o, aryl *C*Hs), 116.4 (e, N*C*N), 67.3 (e, Ph*C*H$_2$), 65.3 (o, proline 2-*C*H), 52.7 (e, proline 5-*C*H$_2$), 50.9 (o, leucine N*C*HCO), 41.1 [e, *C*H$_2$CH(CH$_3$)$_2$], 31.2 (e, proline 3-*C*H$_2$), 24.9 [o, *C*H(CH$_3$)$_2$], 24.5 (e, proline 4-*C*H$_2$), 22.9 (o, CH*C*H$_3$), 21.7 (o, CH*C*H$_3$). MS (electrospray): mH^+ 344.0 (100), (m + Na)$^+$ 366.0 (18).

1-Cyanoprolylphenylalanine methyl ester (13). White solid. ^{1}H NMR (DMSO, 270 MHz) δ 8.55 (d, $J=7.9$ Hz, 1H, N*H*), 7.25 (m, 5H, Ph), 4.52 (m, 1H, phenylalanine NC*H*CO), 4.1 (dd $J=4.7$, 6.4 Hz, 1H, proline 2-C*H*), 3.62 (s, 3H, OCH_3), 3.34 (m, 2H, proline 5-CH_2), 3.02 (m, 2H, *C*H$_2$Ph), 2.07 (m, 1H, proline 4-C*H*), 1.75 (m, 1H, proline 4-C*H*), 1.65 (m, 2H, proline 3-CH_2). ^{13}C APT NMR (67.5 MHz, DMSO) δ 172.2 (e, proline *C*O), 171.0 (e, phenylalanine *C*O), 137.7 (e, 1-phenyl C*H*), 129.6, 128.8, 127.1 (o, phenyl *C*H), 116.8 (e, N*C*N), 63.2 (o, proline 2-*C*H), 54.0 (o, phenylalanine N*C*HCO), 52.5 (e, proline 5-*C*H$_2$), 51.7 (o, O*C*H$_3$), 36.8 (e, *C*H$_2$Ph), 31.2 (e, proline 3-*C*H$_2$), 24.4 (e, proline 4-*C*H$_2$).

1-Cyanoprolylalanine methyl ester (14). Colorless oil. ^{1}H NMR (CDCl$_3$, 300 MHz) δ 6.92 (d, 1H, N*H*), 4.51 (m, 1H, alanine NC*H*CO), 4.10 (dd, $J=4.0$, 8.4 Hz, 1H, proline 2-C*H*), 3.70 (s, 3H, OCH_3), 3.59 (m, 1H, proline 5-C*H*), 3.44 (m, 1H, proline 5-C*H*), 1.80–2.29 (m, 4H, proline 3-CH_2, 4-CH_2), 1.38 (d, $J=7.2$ Hz, 3H, CH_3). MS (electrospray): mH^+ 225.8 (100).

1-Cyano-(*trans*-hydroxy)prolylleucine methyl ester (15). Colorless oil. ^{1}H NMR (DMSO, 300 MHz) δ 8.62 (d, $J=7.9$ Hz, 1H, N*H*), 5.25 (d, $J=3.5$ Hz, 1H, exchangeable, O*H*), *4.29* (m, 3H, leucine NC*H*CO, proline 2-C*H*, proline 4-C*H*), 3.62 (s, 3H, OCH_3), 3.54 (dd, $J=3.7$, 9.9 Hz, 1H, proline 5-C*H*), 3.22 (d, $J=9.9$ Hz,1H, proline 5-C*H*), 2.16 (m, 1H, proline 3-C*H*), 1.85 (m, 1H, proline 3-C*H*), 1.45–1.76 [m, 3H, CH_2C*H*(CH$_3$)$_2$], 0.88 [apparent dd, $J=6.4$, 12.1 Hz, 6H, CH(CH_3)$_2$].

1-Cyano-(D)-prolylalanine methyl ester (16). Colorless oil. ^{1}H NMR (DMSO, 300 MHz) δ 8.55 (d, $J=7.4$ Hz, 1H, N*H*), 4.28 (m, 1H, alanine NC*H*CO), 4.15 (m, 1H, proline 2-C*H*), 3.62 (s, 3H, OCH_3), 3.39 (m, 2H, proline 5-CH_2), 2.13 (m, 1H, proline 4-C*H*), 1.82 (m, 3H, proline 3-CH_2, 4-C*H*), 1.28 (d, $J=7.4$ Hz, 3H, CH_3).

1-Cyano-(D)-prolylleucine dimethylamide (17). Viscous, colorless oil. ^{1}H NMR (DMSO, 300 MHz) δ 8.35 (d, $J=8.4$ Hz, 1H, N*H*), 4.74 (m, 1H, leucine NC*H*CO), 4.14 (m, 1H, proline 2-C*H*), 3.41 (m, 2H, proline 5-CH_2), 2.99 (s, 3H, NCH_3), 2.80 (s, 3H, NCH_3), 1.45–2.20 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.82 [m, 6H, CH(CH_3)$_2$]. MS (electrospray): mH^+ 281.0 (100).

1-Cyano-(D)-prolylleucine benzylamide (18). White solid. ^{1}H NMR (DMSO, 270 MHz) δ 8.55 (bs, 1H, amide N*H*), 7.24 (s, 5H, Ph), 6.79 (bs, 1H, carbamate N*H*), 4.66 (m, 1H, leucine NC*H*CO), 4.27 (d, $J=5.9$ Hz, 2H, NCH_2Ph), 3.98 (m, 1H, proline 2-C*H*), 3.32 (m, 1H, proline 5-C*H*), 3.14 (m, 1H, proline 5-C*H*), 2.29 (m, 1H, proline 4-*CH*), 2.07 (m, 1H, proline 4-*CH*), 1.39–1.90 [m, 5H, proline 3-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.86 [apparent d, $J=6.9$ Hz, 6H, CH(CH_3)$_2$]. MS (PCI): mH$^+$ 343.

1-Cyanoprolylleucinylglycine ethyl ester (19). White solid. ^{1}H NMR (DMSO, 270 MHz) δ 8.40 (bt, 1H, glycine N*H*), 8.29 (d, $J=8.2$ Hz, 1H, leucine N*H*), 4.36 (apparent q, $J=9.2$ Hz,1H, leucine C*H*CO), 4.20 (apparent dd, 1H, proline 2-C*H*), 4.07 (q, $J=7.6$ Hz, 2H, OCH_2), 3.80 (m, 2H, glycine CH_2), 3.45 (m, 2H, proline 5-CH_2), 1.54–2.20 [m, 7H, proline 3-CH_2, 4-CH_2, CH_2C*H*(CH$_3$)$_2$], 1.16 (t, $J=7$, 6 Hz, 3H, OCH$_2$CH_3), 0.86 [apparent dd, $J=3.7$, 10.4 Hz, 6H, CH(CH_3)$_2$]. MS (PCI): mH$^+$ 339.

1-Cyanoprolylleucylleucylleucine methyl ester (20). White solid, mp 172–174 °C. ^{1}H NMR (DMSO, 270 MHz) δ 8.28 (d, $J=8.1$ Hz, 1H, N*H*), 8.19 (d, $J=7.9$ Hz, 1H, N*H*), 7.97 (d, $J=8.1$ Hz, 1H, N*H*), 4.23–4.39 (m, 3H, 3×leucine NC*H*CO), 4.18 (m, 1H, proline 2-C*H*), 3.60 (s, 3H, OCH_3), 3.43 (m, 2H, proline 5-CH_2), 1.40–2.20 [m, 13H, proline 3-CH_2, 4-CH_2, 3CH_2C*H*(CH$_3$)$_2$], 0.87 [m, 18H, 3CH(CH_3)$_2$]. LC–MS (electrospray): mH$^+$ 494.4.

1-Cyano-(3,4-didehydro)prolylleucine methyl ester (21). Colorless oil. ^{1}H NMR (CDCl$_3$, 270 MHz) δ 6.73 (bd, 1H, N*H*), 5.86 (m, 2H, proline 3-C*H*, 4-C*H*), 4.95 (m, 1H, proline 2-C*H*), 4.60 (m, 1H, leucine C*H*CO), 4.35 (m, 2H, proline 5-CH_2), 3.69 (s, 3H, OCH_3), 1.46–1.70 [m, 3H, CH_2C*H*(CH$_3$)$_2$], 0.88 [apparent d, $J=5.9$ Hz, 6H, CH(CH_3)$_2$]. MS (PCI): mH$^+$ 266.

1-Cyanoproline isoamylamide (22). Colorless oil. ^{1}H NMR (CDCl$_3$, 270 MHz) δ 6.61 (bs, 1H, N*H*), 4.04 (m, 1H, proline C*H*), 3.55 [m, 1H, proline 5-C*H*], 3.36 (m, 1H, proline 5-C*H*), 3.19 (m, 2H, HNCH_2), 2.15 (m, 2H, proline 3-CH_2), 1.87 (m, 2H, proline 4-CH_2), 1.53 (m, 1H, C*H*(CH$_3$)$_2$), 1.35 (m, 2H, CH$_2$CH_2CH), 0.83 [apparent d, $J=6.4$ Hz, 6H, CH(CH_3)$_2$].

(*S*)-[1-(1-Benzyl-pyrrolidin-3(*R*)-ylcarbamoyl)-3-methyl-butyl]-carbamic acid benzyl ester (*R*-23). (3*R*)-(−)-1-Benzyl-3-aminopyrrolidine (0.882 g, 5.0 mmol) and CBZ-leucine NHS ester (1.81 g, 5.0 mmol) were dissolved in 15 mL anhydrous acetonitrile and stirred at ambient temperature for 6 h. The precipitate was filtered and washed with a few mL of acetonitrile. 435 mg of (*R*)-**23** was obtained as a white solid, mp 130.5–132 °C, a 21% yield of analytically pure material. Additional material could be obtained upon workup of the mother liquor. ^{1}H NMR (DMSO, 300 MHz) δ 8.02 (d, $J=7.2$ Hz, 1H, pyrrolidine N*H*), 7.30 (m, 11H, 2 Ph, N*H*COO), 5.00 (s, 2H, COOCH_2Ph), 4.11 (m, 1H, pyrrolidine 3-C*H*), 4.00 (m, 1H, leucine NC*H*CO), 3.55 (s, 2H, NCH_2Ph), 2.25–2.75 (m, 4H, pyrrolidine 2,5-CH_2), 1.27–2.15 [m, 5H, pyrrolidine 3-CH_2, CH_2C*H*(CH$_3$)$_2$], 0.84 [apparent t, $J=6.3$ Hz, 6H, CH(CH_3)$_2$].

(*S*)-[1-(1-Cyano-pyrrolidin-3(*R*)-ylcarbamoyl)-3-methyl-butyl]-carbamic acid benzyl ester (24). Compound ***R*-23** (0.297 g, 0.70 mmol) was dissolved in 8 mL dry benzene and 2 mL diethyl ether and stirred. Over the course of 10 min, a solution of 0.98 mmol cyanogen bromide in 2 mL dry benzene was added. After 1 h, 1 N HCl was added and the mixture was stirred vigorously, then transferred to a separatory funnel. The organic phase was separated, the aqueous phase was washed with diethyl ether, and the combined organic phase was washed with brine, then dried over anhydrous MgSO$_4$. Filtration and solvent evaporation gave an oily residue. This was flash chromatographed on silica gel, eluting with 1/6/93 methanol/acetone/dichloromethane. 80.0 mg (a 32% yield) of **(*R*-24)** was obtained as a white solid, mp 109–110 °C. ^{1}H NMR (DMSO, 300 MHz) δ 8.24 (d, $J=6.9$ Hz, 1H, pyrrolidine N*H*), 7.32 (bs, 6H, Ph, N*H*COO), 4.99 (s, 2H, CH_2Ph), 4.20 (m, 1H, pyrrolidine 3-C*H*), 3.98 (m, 1H, leucine NC*H*CO), 3.51 (dd, $J=3.5$, 9.6 Hz, 1H, pyrrolidine 2-C*H*), 3.38 (m, 2H, pyrrolidine 5-CH_2), 3.09 (dd, $J=3.5$, 9.6 Hz, 1H, pyrrolidine 2-C*H*), 2.00 (m, 1H, pyrrolidine 4-C*H*), 1.71 (m, 1H, pyrrolidine 4-C*H*), 1.28–1.76 [m, 3H, CH_2C*H*(CH$_3$)$_2$], 0.83 [6H, apparent t, $J=6.0$ Hz, CH(CH_3)$_2$]. MS (electrospray): mH$^+$ 359.1 (100), (m + Na)$^+$ 381.0 (18).

(*S*)-[1-(1-Benzyl-pyrrolidin-3(*S*)-ylcarbamoyl)-3-methyl-butyl]-carbamic acid benzyl ester (*S*-23). Beginning with (3*S*)-(+)-1-benzyl-3-aminopyrrolidine and using the same procedure as for the preparation of ***R*-23** above, a 54% yield of ***S*-23** was obtained as a white solid. ^{1}H NMR (DMSO, 300 MHz) δ 7.99 (d, $J=6.9$ Hz, 1H, pyrrolidine N*H*), 7.27 (m, 11H, 2 Ph, N*H*COO), 4.98 (s, 2H, COOCH_2Ph), 4.08 (m, 1H, pyrrolidine 3-C*H*), 3.97 (m, 1H, leucine NC*H*CO), 3.52 (2H, ABq, $J=13.2$ Hz, 2H, NCH_2Ph), 1.93–2.62 (m, 5H, pyrrolidine 2-CH_2, 4-C*H*, 5-CH_2), 1.22–1.62 [m, 4H, CH_2C*H*(CH$_3$)$_2$, 4-C*H*], 0.82 [6H, apparent t, $J=6.9$ Hz, CH(CH_3)$_2$]. MS (electrospray): mH$^+$ 424.2 (100).

(*S*)-[1-(1-Cyano-pyrrolidin-3(*S*)-ylcarbamoyl)-3-methyl-butyl]-carbamic acid benzyl ester (25). Starting with ***S*-23**, using the same procedure detailed above for the synthesis of **24**, a 40% yield of **25** was obtained as a white solid, mp 88–89 °C. ^{1}H NMR (DMSO, 300 MHz) δ 8.23 (d, $J=6.3$ Hz, 1H, pyrrolidine N*H*), 7.32 (bs, 6H, Ph, N*H*COO), 4.98 (s, 2H, CH_2Ph), 4.16 (m, 1H, pyrrolidine 3-C*H*), 3.96 (m, 1H, leucine NC*H*CO), 3.36–3.51 (m, 3H, pyrrolidine 2-C*H*, 5-CH_2), 3.08 (dd, $J=3.6$, 9.6 Hz, 1H, pyrrolidine 2-C*H*), 1.99 (m, 1H, pyrrolidine 4-C*H*), 1.77 (m, 1H, pyrrolidine 4-C*H*), 1.27–1.68 [m, 3H, CH_2C*H*(CH$_3$)$_2$], 0.84 [apparent t, $J=6.3$ Hz, 6H, CH(CH_3)$_2$]. MS (electrospray): mH$^+$ 359.1 (100).

Enzyme inhibition assays. Inhibitor potency measurements were performed at 23 °C using 96-well kinetic-plate readers. Reaction velocities were monitored at varying inhibitor concentrations by following the hydrolysis of aminomethylcoumarin substrates (ex$_{355}$

and em_{460}) as indicated. All substrates were added at a concentration equal to their K_m. Control reactions in the absence of inhibitor were performed in parallel. The $K_{iapparent}$ (K_i') values were determined by a non-linear least squares regression fit of the experimentally derived data to the Morrison equation for tight-binding inhibitors as described[25] or by least squared regression fit of the Henderson equation for tight-binding inhibitors.[26] Enzyme and inhibitor were incubated 30 min prior to initiation of reaction by the addition of substrate. *Cathepsin B:* Enzyme (5.0 nM) was mixed with inhibitor in 50 mM MES or BES (pH 6.0), 2.5 mM DTT, 2.5 mM EDTA, 0.05% Tween 20 and 10% DMSO. The substrate was Z-Phe-Arg-AMC (300 μM). *Cathepsin K*: Enzyme (3.6 nM) was mixed with inhibitor in 50 mM MES (pH 5.5), 2.5 mM DTT, 2.5 mM EDTA, 0.05% Tween-20 and 10% DMSO. The substrate was Z-Phe-Arg-AMC (40 μM). *Cathepsin L:* Enzyme (1.3 nM) was mixed with inhibitor in 50 mM MES (pH 5.5), 2.0 mM EDTA, 2 mM DTT, 0.05% Tween-20 and 10% DMSO. The substrate was Z-Phe-Arg-AMC (10 μM). *Cathepsin S:* Enzyme (1.0 nM) was mixed with inhibitor in 50 mM MES (pH 6.5), 100 mM NaCl, 2.5 mM EDTA, 2.5 mM 2-mercaptoenthanol, 0.001% bovine serum albumin and 10% DMSO. The substrate was Z-Val-Val-AMC (10 μM).

Cellular inhibition assay. The following method is a modification of Mason et al.[17,18] Z-Tyr-Ala-diazomethylketone (DMK) was purchased from ESP (Livermore, CA, USA) and commercially iodinated with ^{125}I by Amersham Pharmacia (Piscataway, NJ, USA) to a specific activity of 2000 Ci/mmol. Dried material was stored at 4 °C. 100 μCi of material was reconstituted with 200 μL of serum-free RPMI medium (Gibco BRL, Rockville, MD, USA). For labeling studies, DLD-1 cells (ATCC, Manassas, VA, USA) were seeded at 50,000 cells per well in a 96-well tissue culture plate and grown overnight. The following day, the indicated concentration of compounds were diluted from 1000X DMSO stock solutions into DMEM supplemented with 2% Nutridoma (Roche Molecular Biochemicals, Indianapolis, IN, USA) and applied to the cells for 45 min. Following this incubation, 2 μCi ^{125}I-labeled DMK were added to each well and incubated at 37 °C for 2 h. The medium containing compound and ^{125}I-DMK were removed and the cells resuspended in 100 μL of 2 reducing sample buffer. 20 μL of sample was applied to a 12% SDS-polyacrylamide gel and the samples resolved. The gel was dried down and exposed to film for 6 h.

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