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Bioorganic & Medicinal Chemistry Letters 15 (2005) 3039–3043
Bioorganic & Medicinal Chemistry Letters

Acyclic cyanamide-based inhibitors of cathepsin K

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Received 24 February 2005; revised 12 April 2005; accepted 18 April 2005
Available online 17 May 2005

Abstract—Conversion of the proline-derived cyanamide lead to an acyclic cyanamide capable of forming an additional hydrogen bond with cathepsin K resulted in a large increase in inhibitory activity. An X-ray structure of a co-crystal of a cyanamide with cathepsin K confirmed the enzyme interaction. Furthermore, a representative acyclic cyanamide inhibitor **6r** was able to attenuate bone resorption in the rat calvarial model.

Osteoporosis results from an imbalance in the normally tightly coupled processes of bone formation and bone resorption that maintain the adult skeleton.[1] This skeletal fragility, resulting from the loss of bone mineral density and integrity, is the leading cause of non-traumatic fracture. In light of the risks associated with hormone replacement therapy, there is continued interest in the development of new agents to treat osteoporosis.[2] One target receiving considerable attention from the pharmaceutical industry is the cysteine protease cathepsin K.[3]

Cells of hematopoietic origin called osteoclasts resorb bone by adhering to the bone surface and secreting acid and proteases that degrade the mineral and protein components of bone. The cysteine protease cathepsin K is highly expressed in osteoclasts, and, in complex with glycosaminoglycans, rapidly hydrolyzes type I collagen, the major component of bone matrix.[4] Cathepsin K inhibitors have proven effective in protecting skeletal integrity in animal models of bone resorption, and several have entered clinical trials for the treatment of postmenopausal osteoporosis.[5–7]

As part of a sustained effort to develop novel cathepsin K inhibitors, researchers from these laboratories recently reported the discovery of reversible, competitive, cyanamide-based cathepsin K inhibitors.[8] Starting from a high throughput screening hit, a key pharmacophore **1** (K_i = 2100 nM) was identified. Addition of P^2–P^3 substituents to this pyrrolidine cyanamide led to the development of nanomolar cathepsin K inhibitors such as **2** (K_i = 60 nM) and **3** (K_i = 1.8 nM). An X-ray crystal structure of a cyanamide-based inhibitor bound to cathepsin K showed that the active site thiol of cathepsin K adds to the carbon nitrogen triple bond of the cyanamide to form an isothiourea, confirming the conclusions of researchers at Merck, who applied ^{13}C NMR studies to determine the mode of inhibition of papain by cyanamides.[9] The crystal structure also revealed that the proposed P^2 substituent did indeed occupy the S^2 pocket, although this interaction was not optimal. Peptide aldehyde-based inhibitors from these laboratories form a key hydrogen bond between their carbamate NH and ^{161}Asn of cathepsin K,[10,11] whereas cyanamides such as **1**–**3** cannot undergo such interactions. Surmising that additional potency could be achieved by introducing a hydrogen bond donor into the cyanamide-based

Keywords: Cyanamide; Cathepsin K; Cysteine protease inhibitor.
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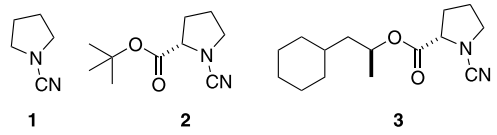
doi:10.1016/j.bmcl.2005.04.032

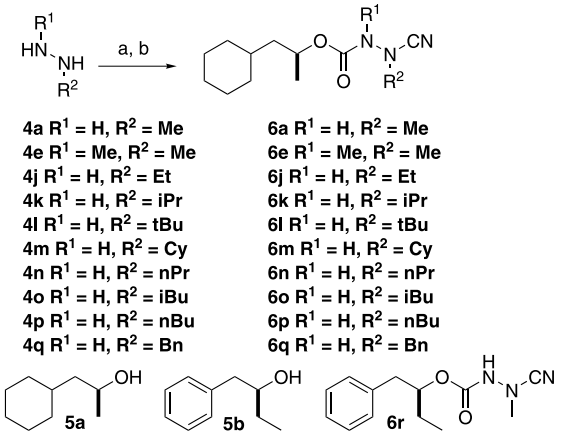
inhibitors, it was decided to break open the pyrrolidine ring and replace the resulting sp^3 carbon atom adjacent to the nitrogen with a second nitrogen atom.



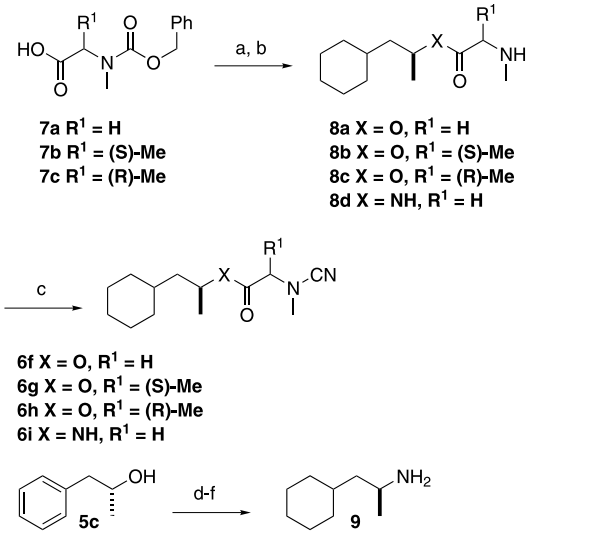
As shown in Scheme 1, the acyclic cyanamides could be synthesized from commercially available hydrazines **4a**,**e**, and **j**–**q**. Treatment of the hydrazines with cyanogen bromide in the presence of a base produced the azacyanamides, which were then coupled to the chloroformates derived from known alcohols **5a**[11] and **5b**[11] to afford the desired cyanamides **6a**,**e**, and **j**–**r**.

The cyanamides **6f**–**i** were synthesized as depicted in Scheme 2. The commercially available carboxylic acids **7a**–**c** were esterified with alcohol **5a**. Then, the benzyl carbamates were cleaved by hydrogenolysis to give the amines **8a**–**c**. Subsequent treatment with cyanogen bromide in the presence of a base provided the cyanamides **6f**–**h**. Cyanamide **6i** was derived from acid **7a** and amine **9**, which was synthesized from commercially available alcohol **5c**. Mitsunobu reaction of phthalimide with alcohol **5c**, followed by hydrazine cleavage of the imide protecting group provided the phenethylamine. Then, exhaustive catalytic hydrogenation of the aryl ring yielded the amine **9**. Amine **9** was coupled to the acid **7a** employing the carbodiimide method. This amide was unmasked, as before, to give the amine **8d**. Once again, reaction of the amine **8d** with cyanogen bromide provided the cyanamide **6i**.

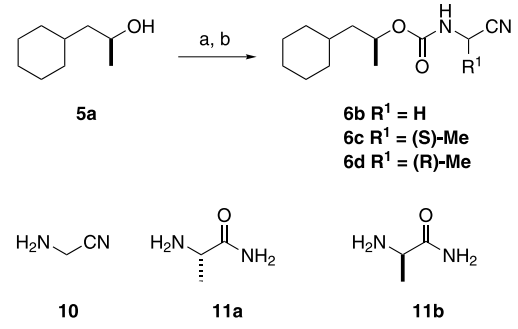
The nitriles **6b**–**d** were prepared as illustrated in Scheme 3. Reaction of the commercially available amine **10** with the chloroformate derived from alcohol **5a** produced the nitrile **6b**. Alternatively, treatment of this chloroformate with commercially available primary amides **11a** or **b**, followed by dehydration utilizing trifluoroacetic anhydride gave the nitriles **6c** and **d**.



Scheme 1. Reagents and conditions: (a) BrCN, Na_2CO_3, H_2O, CH_2Cl_2, 67–96%; (b) **5a** or **b**, 1.93 M $COCl_2$ in PhMe, pyridine, CH_2Cl_2, −20 °C to rt; cyanamide, *i*-Pr_2NEt, THF, 52–92%.



Scheme 2. Reagents and conditions: (a) **7a**–**c**, **5a**, TsOH, PhH, ↑↓, 81–94%; or **9**, **7a**, EDC, HOBt, *i*-Pr_2NEt, THF, 60%; (b) H_2/Pd–C, MeOH, 77–97%; (c) BrCN, K_2CO_3, MeCN, 94–99%; (d) phthalimide, DEAD, Ph_3P, THF, 93%; (e) NH_2NH_2, MeOH, Δ, 84%; (f) H_2/PtO_2, AcOH, 89%.

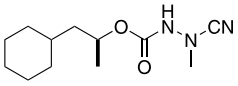
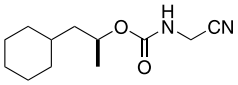
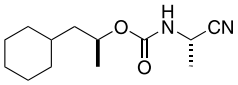
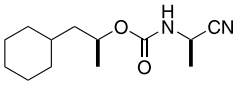
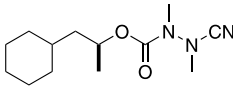
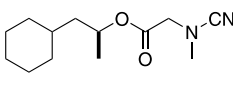
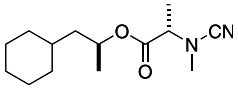
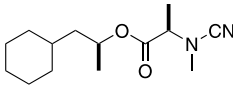
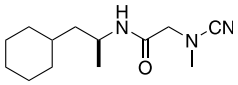


Scheme 3. Reagents and conditions: (a) **5a**, 1.93 M $COCl_2$ in PhMe, pyridine, CH_2Cl_2, −20 °C to rt; **10**, **11a** or **b**, *i*-Pr_2NEt, THF, 73–97%; (b) $(CF_3CO)_2O$, CH_2Cl_2, 33–42%.

As shown in Table 1, the acyclic azacyanamide **6a** (K_i = 0.009 nM) was over two orders of magnitude more potent than the proline-derived cyanamide **3** (K_i = 1.8 nM), in agreement with the original hypothesis. This is in sharp contrast to results from Merck researchers, wherein the acyclic cyanamide diethylamino-*N*-carbonitrile was >100-fold less active than *N*-pyrrolidine carbonitrile.[9]

Attempting to confirm the design hypothesis for the synthesis of acyclic cyanamide **6a**, the *N*-methyl derivative **6e** (K_i = 62 nM) was prepared. The large decrease in activity relative to analog **6a** supported the hypothesis that the carbamate NH forms a hydrogen bond to ^{161}Asn of the enzyme. However, the loss in activity could also be the result of unfavorable steric interactions between the methyl substituent and the protein. To distinguish between these two possibilities and to obtain a more direct comparison of an acyclic azacyanamide and an acyclic cyanamide, cyanamides **6f**–**h** were prepared. The potency of the methylene analog **6f** (K_i = 26 nM)

Table 1. Inhibition of human cathepsin K

#	Inhibitor	K_i (nM)[a]
6a		0.009
6b		7900
6c		>13,000
6d		>13,000
6e		62
6f		26
6g		2300
6h		>13,000
6i		830

[a] Inhibition of recombinant human cathepsin K activity in a fluorescence assay using 400 pM cathepsin K with 10 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, 120 mM NaCl, pH = 5.5. The K_i values are the mean of two or three inhibition assays, individual data points in each experiment were within a 3-fold range of each other.

is similar to that of the *N*-methyl derivative **6e**, whereas the (*S*)-methyl analog **6g** (K_i = 2300 nM) is substantially less potent and the (*R*)-methyl analog **6h** is inactive at concentrations up to 13,000 nM. Thus, the enzyme may not be able to fully accommodate a methyl group projecting in these directions at this site. The enhanced potency of **6a** relative to **6e**–**h** probably arises from the formation of a key hydrogen bond interaction with ^{161}Asn as well as better enzyme fit. This better fit may be the result of not only decreased steric repulsion of hydrogen versus methyl substituents (compare **6a to 6e**,**g**, and **h**), but also of the sp^2 character of the carbamate nitrogen, which may allow an energetically more favorable interaction between inhibitor and enzyme than does the sp^3 α-carbon of the ester (compare **6a to 6f**).

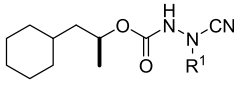
Due to the presence of the electronegative nitrogen attached to the carbon nitrogen triple bond, cyanamides are stronger electrophiles than are nitriles. To explore how important this enhanced electrophilicity was to the inhibitory activity of the azacyanamides, nitrile analogs of **6a** were studied. Nitrile **6b** (K_i = 7900 nM) is a micromolar inhibitor of cathepsin K, whereas the conformationally biased alanine-derived nitriles **6c** (K_i > 13,000 nM) and **6d** (K_i > 13,000 nM) were even poorer inhibitors. This million-fold difference in inhibitory potency was surprising, and may be due to more than the increased electrophilicity of the cyanamide warhead. As stated above, the conformation afforded by the sp^2 character of the cyanamide nitrogen might allow a better enzyme fit than the sp^3 carbon of the nitrile inhibitors.

Replacing the ester oxygen of analog **6f** with nitrogen might still allow a hydrogen bond interaction with ^{161}Asn, but from a different angle, perhaps leading to an increase in potency relative to **6f**. However, cyanamide **6i** (K_i = 830 nM) proved significantly less active than **6f** or **6a**, suggesting that the amide NH is unable to form a similar hydrogen bond as the carbamate NH of azacyanamide **6a**. The loss in potency of **6i** versus **6f** is presumably the result of the higher entropic cost of desolvating an amide versus an ester.

The inhibitory activity of P^1 cyanamide analogs is shown in Table 2. These results parallel the P^1 SAR of aldehyde-based cathepsin K inhibitors recently reported from this laboratory.[10] A comparison of the *iso*-propyl analog **6k** (K_i = 0.019 nM), cyclohexyl analog **6m** (K_i = 0.016 nM), and *tert*-butyl analog **6l** (K_i = 1500 nM) with the ethyl analog **6j** (K_i = 0.003 nM) reveals that increased steric bulk adjacent to the cyanamide nitrogen decreases enzyme inhibition. In contrast, a comparison of the *iso*-butyl analog **6o** (K_i = 0.004 nM) and benzyl analogs **6q** (K_i = 0.006 nM) with the *n*-propyl analog **6n** (K_i = 0.004 nM) shows that branching at the β-position from the cyanamide nitrogen has little effect on potency.

As shown in Table 3, the azacyanamide analogs **6a**,**k**, and **r** exhibit moderate selectivity versus cathepsins B (B/K = 8–70), H (H/K = 20–160), and L (L/K = 1–40). In contrast, although cyanamide **6f** is a less potent cathepsin K inhibitor, it is more selective versus cathepsins B (B/K = 100), H (H/K = >500), and L (L/K = 60). The ability of these cyanamides to inhibit cathepsin S, another closely related endopeptidase, was not studied.

Table 2. Inhibition of human cathepsin K by P^1 analogs



#	R^1	K_i (nM)[a]
6a	Me	0.009
6j	Et	0.003
6k	*i*-Pr	0.019
6l	*t*-Bu	1500
6m	Cy	0.016
6n	*n*-Pr	0.004
6o	*i*-Bu	0.004
6p	*n*-Bu	0.007
6q	Bn	0.006

[a] Inhibition of recombinant human cathepsin K activity in a fluorescence assay using 400 pM cathepsin K with 10 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, 120 mM NaCl, pH = 5.5. The K_i values are the mean of two or three inhibition assays, individual data points in each experiment were within a 3-fold range of each other.

Table 3. Cathepsin B, H, and L inhibition and selectivity

#	Cat K K_i (nM)	Cat B K_i (nM)[a]	Cat H K_i (nM)[b]	Cat L K_i (nM)[c]
6a	0.009	0.077	1.4	0.025
6f	26	2600	>13,000	1500
6k	0.019	1.3	0.46	0.79
6r	0.032	0.25	—	0.036

[a] Inhibition of recombinant human cathepsin B activity in a fluorescence assay using 220 pM cathepsin B with 10 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, 120 mM NaCl, pH = 5.5. The K_i values are the mean of two or three inhibition assays, individual data points in each experiment were within a 2-fold range of each other.

[b] Inhibition of recombinant human cathepsin H activity in a fluorescence assay using 107 nM cathepsin H with 50 μM L-Arg-β-naphthalamide as substrate in 100 mM NaOAc, 10 mM DTT, 120 mM NaCl, pH = 5.5.

[c] Inhibition of recombinant human cathepsin L activity in a fluorescence assay using 300 pM cathepsin L with 5 μM Cbz-Phe-Arg-AMC as substrate in 100 mM NaOAc, 10 mM DTT, 120 mM NaCl, pH = 5.5.

In contrast to proline-based cyanamides, the acyclic azacyanamides were rapidly degraded by S9 liver slices (<10% remaining after 1 h of incubation),[12] and, with the exception of the *tert*-butyl analog **6l** ($t_{1/2}$ > 12 h), they were also short-lived in plasma (e.g., **6a** $t_{1/2}$ < 1 h). In addition to potential lability to plasma enzymes such as amidases, the azacyanamides are probably more reactive than the proline-based cyanamides to free thiols. At pH = 5.5 in the presence of glutathione, the K_is of the azacyanamides were not significantly different from their K_is determined in its absence. In contrast, in the presence of glutathione at pH = 7.0, the azacyanamides were substantially poorer inhibitors of cathepsin K. This increased reactivity is similar to that seen by Merck researchers with azetidine-based cyanamides.[9] These liabilities will need to be overcome or they may limit the in vivo utility of these azacyanamides. Despite these shortcomings, cyanamide **6r** (human K_i = 0.032 nM, rat K_i = 0.072 nM) was profiled in an ex vivo rat calvarial resorption assay.[13,14] As measured by deoxypyridinoline crosslink release from type I collagen, treatment of rat calvaria with parathyroid hormone (PTH) enhanced bone resorption (see Table 4). Analog **6r** dose-dependently attenuated the release of this marker of bone resorption by PTH. This efficacy reached statistical significance at the 3000 nM and 10,000 nM doses. There is a large discrepancy between the rat ex vivo assay and the in vitro human and rat inhibition values. The poor stability of **6r** (plasma $t_{1/2}$ < 30 min) may partially account for this difference.

Table 4. Rat calvarial assay

	Media	PTH	**6r** 1000 nM	**6r** 3000 nM	**6r** 10,000 nM
Av	10.00[a]	14.41[a]	12.53[a,b]	9.45[a,c]	8.41[a,d]
SD	0.21	1.35	1.23	0.91	1.23

[a] Deoxypyridinoline crosslink release from type I collagen.
[b] Dunnett's multiple comparison test, $p = 0.293$.
[c] Dunnett's multiple comparison test, $p = 0.003$.
[d] Dunnett's multiple comparison test, $p = 0.0008$.

An X-ray crystal structure of cathepsin K and *t*-BuO(C=O)NHN(Me)CN is shown in Figure 1.[15] The *tert*-butyl P^2 group occupies the S^2 pocket formed by residues ^{67}Tyr, ^{68}Met, ^{134}Ala, ^{163}Ala, and ^{209}Leu. This is the deepest, most hydrophobic binding region of the cathepsin K active site. Also, the carbonyl oxygen of the carbamate forms a hydrogen bond to the backbone NH of ^{66}Gly. Furthermore, as hypothesized, the carbamate NH also donates its hydrogen to the backbone carbonyl oxygen of ^{161}Asn. This interaction accounts for some of the increased potency of the acyclic cyanamide inhibitor class compared with the cyclic cyanamide analogs. The P^1 methyl group has limited interaction with the S^1 groove formed from ^{23}Gly, ^{24}Ser, ^{64}Gly, and ^{65}Gly. However, it is clear that there is limited space available for larger groups at the S^1 subsite, helping explain the steric effects of the P^1 substituents shown in Table 2.

A covalent isothiourea intermediate is formed by the cyanamide moiety of the inhibitor and the active site ^{25}Cys of the enzyme. Moreover, the NH of the isothiourea is stabilized by two hydrogen bonds to the side chain carbonyl oxygen of ^{19}Gln and the backbone NH of ^{25}Cys. This mode of binding is similar to the reaction of cysteine proteases with aldehydes to form hemithioacetals.[10] In fact, this cyanamide structure is virtually superimposable on the *t*-BuO(C=O)NHCH(Cy)CHO structure with cathepsin K previously disclosed by these researchers.[10] Direct comparison of cyanamide **6p** with its corresponding aldehyde (K_i = 2.7 nM) reveals that the cyanamide is >100-fold more potent inhibitor of cathepsin K. In addition to differences in the electrophilicity of the warheads, this discrepancy could be partially explained by hybridization differences between the warheads. The sp^2 hybridized aldehyde requires a limited Dunitz–Burgi attack angle by the active site thiol

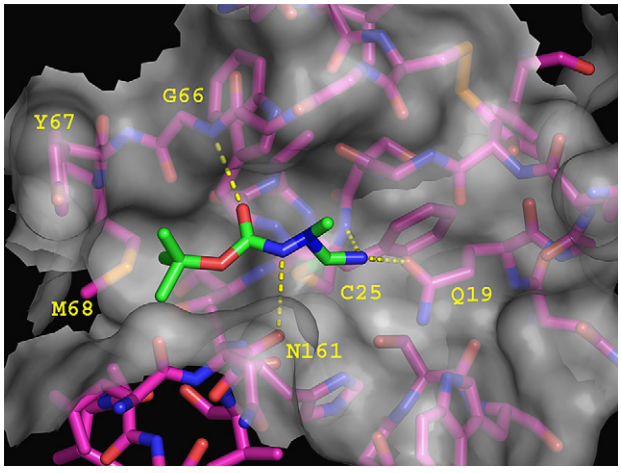


Figure 1. Active site of the X-ray co-crystal structure of *t*-BuO(C=O)NHN(Me)CN complexed with cathepsin K. The cathepsin K carbons are colored magenta with the inhibitor carbons colored green. The semi-transparent white surface represents the molecular surface, while hydrogen bonds are depicted as yellow dashed lines. The coordinates have been deposited in the Brookhaven Protein Data Bank, accession number 1YK8. This figure was generated using PYMOL version 0.97 (Delano Scientific, www.pymol.org).

on the carbonyl carbon electrophile; whereas the sp hybridized cyanamide carbon accommodates less optimal trajectories of assault. This azacyanamide contains no P^3 group, thus no interaction is realized with the S^3 subsite. Additional binding energy is obviously obtained via incorporation of a P^3 substituent as in cyanamides **6a** and **r**.

In summary, very potent acyclic cyanamides such as **6a** were designed and synthesized, starting from the proline derived cyanamide **3**. Verifying the design hypothesis, an X-ray crystal structure of a cathepsin K/azacyanamide complex confirmed that these inhibitors form a key hydrogen bond with ^{161}Asn of the protein. Furthermore, a representative cyanamide inhibitor **6r** is capable of attenuating bone resorption in a rat calvarial assay. These promising results warrant additional efforts to improve the metabolic stability and other drug properties of these novel inhibitors.

Acknowledgments

The authors would like to thank James G. Conway for statistical analyses.

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15. (t-BuO(C=O))$_2$NN(Me)CN was synthesized in a similar manner to Scheme 1. Methyl hydrazine was treated with cyanogen bromide and the resulting product was then reacted with di-*tert*-butyl dicarbonate and triethylamine to provide (t-BuO(C=O))$_2$NN(Me)CN. Co-crystallization of (t-BuO(C=O))$_2$NN(Me)CN with cathepsin K at acidic pH caused the loss of one *tert*-butyloxycarbonyl protecting group, resulting in a inhibitor/enzyme co-crystal containing t-BuO(C=O)NHN(Me)CN with cathepsin K. The parent (t-BuO(C=O))$_2$NN(Me)CN inhibited cathepsin K with an K_i = 20 nM. In hindsight, it is probably hydrolyzed under the acidic conditions of the enzyme assay (pH = 5.5) to produce small quantities of t-BuO(C=O)NHN(Me)CN. Therefore, the inhibitory activity of t-BuO(C=O)NHN(Me)CN is likely below 20 nM. Since this X-ray crystal structure was solved several months after resources were shifted to higher priority targets, t-BuO(C=O)NHN(Me)CN was never prepared and tested.