Enzyme Inhibitors

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Azadipeptide Nitriles: Highly Potent and Proteolytically Stable Inhibitors of Papain-Like Cysteine Proteases**

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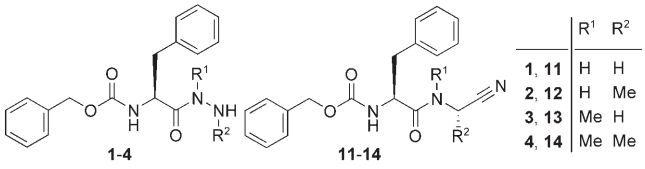
Cysteine proteases of the clan CA, the papain-like cysteine proteases, are of outstanding medical interest owing to their importance as virulence factors in different human pathogenic parasites and their involvement in a number of different systemic diseases. Apart from the active-site thiol, these proteases share pronounced substrate specificity towards the amino acid in the P^2 position. Eleven human papain-like lysosomal cysteine proteases with a high degree of homology, the cathepsins, have been described.[1] Among them, the cathepsins L, S, and K are involved in several pathological conditions, such as tumor growth and invasion, autoimmune diseases, and osteoporosis,[2] and are currently in the focus of many drug discovery programs.[3] Inhibitors for papain-like cysteine proteases are mainly derived from peptides and contain electrophilic entities prone to covalent interactions with the thiol at the active site. Among this generic type of inhibitor, peptide nitriles have received much attention in recent research.[4] Nitriles inhibit cysteine proteases by the formation of a thioimidate adduct resulting from the attack of the active-site thiol at the C–N-triple bond,[5] and, owing to a soft–soft relationship according to the HSAB principle, they are therefore potent and selective inhibitors for cysteine proteases. Peptidic inhibitors however, generally exhibit low bioavailability because of their susceptibility to degradation by other proteases.

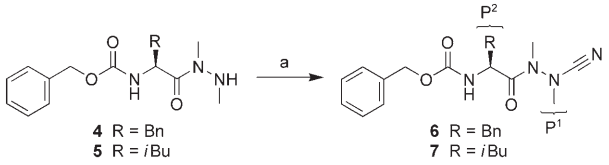
The isoelectronic replacement of the $C_\alpha H$ group by a nitrogen atom to give azapeptides is a common structural modification in the chemistry of peptides and peptidomimetics.[6] In contrast to other protease inhibitors,[7] this structural modification has never been applied to the P^1 position of peptide nitriles. Nonpeptidic cyanamides, which are chemically closely related to nitriles, have been described as powerful cathepsin K inhibitors.[8] We have recently found that the replacement of the $C_\alpha H$ group by a nitrogen atom in the P^2 position of a cathepsin-inhibiting dipeptide nitrile led to the loss of inhibitory capacity.[9] As part of our ongoing interest in cysteine protease inhibition, it was intended to explore the impact of the CH/N exchange in the P^1 position of dipeptide nitriles on the enzyme–inhibitor interaction. Herein we describe a synthetic entry to azadipeptide nitriles and their inhibition kinetics and binding affinity towards papain and the therapeutically important cathepsins L, S, and K.

It was envisaged that the synthesis of the aza-analogous nitriles could be achieved by reaction of the corresponding amino acid-derived hydrazides (Scheme 1) with cyanogen bromide. This approach was initially unsuccessful: in the case



Scheme 1. Hydrazides **1**–**4** and carbadipeptide nitriles **11**–**14**.

of the unsubstituted and monomethylated hydrazides **1**–**3**, the formation of various cyclization products was observed. We therefore considered N^1,N^2-dimethylation to prevent cyclization and, indeed, treatment of the hydrazide **4** with cyanogen bromide led to the desired open-chain azadipeptide nitrile **6** (Scheme 2). Compound **6**, the first representative of azadipeptide nitriles resulting from a CH/N replacement in the P^1 position, showed inhibitory activity towards papain in the low nanomolar range, and was even more potent towards the therapeutically important cathepsins L, S, and K, with picomolar K_i values (Table 1). This unexpectedly strong inhibition was time-dependent in terms of slow-binding inhibition,[10] as shown in Figure 1. The linear plots of the pseudo-first-order rate constants versus inhibitor concentration suggest that the enzyme–inhibitor interaction follows a one-step mechanism (see the Supporting Information). The second-order rate



Scheme 2. Synthesis of azadipeptide nitriles **6** and **7**. a) BrCN, NaOAc, MeOH, RT. Bn = benzyl.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

Table 1: Inhibition of cysteine proteases by azadipeptide nitriles **6–10** and dipeptide nitriles **11–14**.

Compd.	Cathepsin L			Cathepsin S			Cathepsin K			Papain	
	K_i [nM]	k_{on} [10^3 M^{-1} s^{-1}]	k_{off} [10^{-3} s^{-1}]	K_i [nM]	k_{on} [10^3 M^{-1} s^{-1}]	k_{off} [10^{-3} s^{-1}]	K_i [nM]	k_{on} [10^3 M^{-1} s^{-1}]	k_{off} [10^{-3} s^{-1}]	K_i [nM]	k_{on} [10^3 M^{-1} s^{-1}]	k_{off} [10^{-3} s^{-1}]
6	0.074 ± 0.010	7300 ± 100	0.54 ± 0.07	0.22 ± 0.01	2700 ± 100	0.60 ± 0.03	0.022 ± 0.001	5800 ± 100	0.13 ± 0.01	3.9 ± 0.4	100 ± 10	0.40 ± 0.04
7	0.69 ± 0.07	1400 ± 100	0.99 ± 0.09	0.38 ± 0.05	3700 ± 200	1.4 ± 0.2	0.029 ± 0.004	8900 ± 100	0.26 ± 0.04	8.2 ± 1.1	9.6 ± 0.1	0.079 ± 0.011
8	0.92 ± 0.03	1400 ± 100	1.3 ± 0.1	1.5 ± 0.1	1200 ± 100	1.9 ± 0.1	0.36 ± 0.03	950 ± 70	0.34 ± 0.04	42 ± 3	13 ± 1	0.55 ± 0.05
9	0.84 ± 0.12	2600 ± 100	2.2 ± 0.2	0.46 ± 0.04	1400 ± 100	0.66 ± 0.05	0.26 ± 0.02	1900 ± 100	0.48 ± 0.05	6.3 ± 0.8	9.4 ± 0.3	0.060 ± 0.008
10	0.63 ± 0.07	2300 ± 100	1.4 ± 0.2	0.58 ± 0.04	1300 ± 100	0.75 ± 0.05	0.30 ± 0.03	1500 ± 100	0.45 ± 0.05	10 ± 1	12 ± 2	0.12 ± 0.03
11[a]	230 ± 20			510 ± 50			260 ± 10			360 ± 10		
12	280 ± 20			1900 ± 200			1400 ± 100			2200 ± 100		
13	46000 ± 6000			>100000[b]			7700 ± 1000			610000 ± 20000		
14	57000 ± 7000			>100000[b]			12000 ± 4000			750000 ± 80000		

[a] Data from ref. [9]. [b] Estimation owing to limited solubility under conditions of the cathepsin S assay with 1% v/v DMSO.

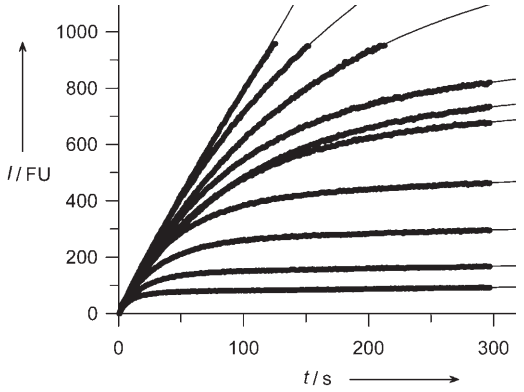
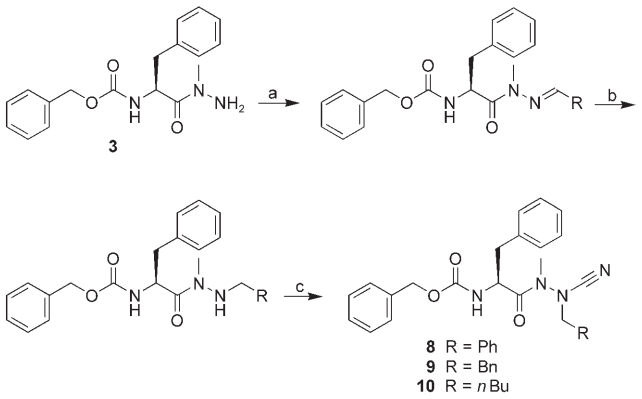


Figure 1. Monitoring of the human cathepsin L-catalyzed hydrolysis of Z-Phe-Arg-NHMec (10 μM; NHMec = 4-methylcoumarin-7-yl) in the presence of increasing concentrations of compound **6** (from top to bottom: 0, 3, 4, 8, 9, 10, 20, 30, 50, 100 nM). The reaction (100 mM sodium phosphate pH 6.0, 100 mM NaCl, 5 mM ethylenediamine tetraacetate, 0.01% v/v polyoxyethylene(23) lauryl ether (Brij 35), 25 μM 1,4-dithiothreitol, 1% DMSO, 37°C) was initiated by addition of the enzyme (I = fluorescence intensity, FU = fluorescence units).

constants k_{on} for the approach to the enzyme–inhibitor equilibrium were in the range of 10^5–10^7 M^{-1} s^{-1}, indicating a rather fast association of the enzyme with the inhibitor (Table 1). In spite of the *N*-methylated P^2–P^1 amide bond, which is generally disadvantageous for the interaction of proteases with substrates or inhibitors,[11] the azadipeptide nitrile **6** showed affinities to the papain-like enzymes that are higher by at least two orders of magnitude than those of the corresponding carba-analogue **11**[9] (Scheme 1, Table 1). Although having high affinities to the enzymes, compound **6** is clearly a reversible inhibitor, as demonstrated by the reactivation of cathepsin L and papain (see the Supporting Information).

To comparatively assess the activity of the azadipeptide nitrile **6** more precisely, the carba-analogues **12**, **13**, and **14** (Scheme 1) were synthesized by standard methods (see the Supporting Information). The nitriles **12** and **14** were prepared from the corresponding dipeptide amides by dehydration with cyanuric chloride in DMF.[12] As shown in Table 1, the *N*-methylated carba-analogues **13** and **14** exhibited activities in the high micromolar range. Comparing the direct analogues **6** and **14**, it becomes obvious that the isoelectronic CH/N replacement accounts for an increase in the inhibitory activities by more than five orders of magnitude.

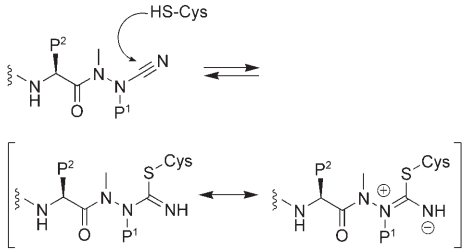
By applying the same synthetic methodology as for the azadipeptide nitrile **6**, the leucine derivative **7** was obtained (Scheme 2), which showed selectivity for cathepsin K with a K_i value of 29 pM (Table 1). To introduce substituents other than methyl into the P^1 position, it was intended to convert the N^1-methylated hydrazide **3** by reductive alkylation into N^1,N^2-dialkylated hydrazides, followed by treatment with cyanogen bromide (Scheme 3). By reacting **3** with different aldehydes, hydrazones were obtained in good yields. To reduce the C–N double bond of these intermediates, several established reducing agents were tried: sodium triacetoxyborohydride,[13] sodium cyanoborohydride,[14] sodium borohy-



Scheme 3. Synthesis of azadipeptide nitriles **8**–**10**. a) RCHO, THF, RT; b) 1. $(CH_3)_2NH \times BH_3$, *p*-toluenesulfonic acid, CH_2Cl_2, 4 °C; 2. 1.5 M NaOH, RT; c) BrCN, NaOAc, MeOH, RT.

dride, and sodium borohydride in the presence of Ni^{2+} as activating azaphilic Lewis acid.[15] All these attempts failed, mostly leading to isolation of the unconverted hydrazones. By applying an alternative reducing agent, dimethylamine–borane complex, recently described by Casarini et al. for the 1,2-reduction of α,β-unsaturated hydrazones,[16] an almost quantitative and smooth reduction of the hydrazones to the N^1-methyl-N^2-alkylhydrazides was achieved. Upon treatment with cyanogen bromide, the phenylalanine-derived N^1-methyl-N^2-alkylhydrazides could be easily converted into the desired azadipeptide nitriles **8**–**10** bearing different residues in the P^1 position. With the exception of inhibition of cathepsin S by compound **8**, all cathepsins were inhibited by **8**–**10** with subnanomolar affinities, although these compounds were less active than **6**. The introduction of residues other than methyl into the P^1 position of the azadipeptide nitriles did not lead to selective inhibition among the cathepsins, and especially not between cathepsin L and S. However, to draw conclusions regarding the impact of the P^1 substituent on selectivity, a greater number of analogues need to be made in future investigations. This approach is very promising, as a large variety of aldehydes available by different synthetic procedures can be applied.

The azadipeptide nitriles showed a time-dependent slow-binding inhibition towards all investigated cysteine proteases (Table 1, Figure 1). Unexpectedly, their binding affinity was higher by five orders of magnitude in comparison to their carba-analogues. This phenomenon can be understood from the time-dependency of the inhibition by the azadipeptide nitriles. Generally, slow-binding inhibition allows the determination of the kinetic constants k_{on} and k_{off}.[10] The second-order rate constant k_{on} governs the association of the enzyme and the inhibitor to the enzyme–inhibitor complex, and k_{off} the decay of that complex. As the carba-analogous peptide nitriles showed a time-independent inhibition (see the Supporting Information), it can be concluded that they exhibit higher second-order rate constants k_{on} than their nitrogen counterparts. In spite of this, the azadipeptide nitriles had lower inhibition constants than the carbadipeptide nitriles. Because of the relation $K_i = k_{off}/k_{on}$, the first-order rate constants k_{off} of the azadipeptide nitriles have to be dramatically reduced in comparison to the corresponding values of their carba-analogues. This reduction can be understood by considering that in the azadipeptide nitriles, the cyano group is attached to a nitrogen atom having an electron lone pair. Upon attack of the active-site thiol, a trigonal-planar isothiosemicarbazide adduct is formed from the cyanamide-like carbon atom. The enhanced stability of such a covalent enzyme–inhibitor complex may arise from the resonance of the nitrogen atom lone pair and the sp^2-hybridized carbon atom derived from the cyano group (Scheme 4).



Scheme 4. Reversible formation of isothiosemicarbazides from azadipeptide nitriles and cysteine proteases.

As the amide bond in these novel dipeptide derivatives is *N*-methylated, an enhanced stability towards degradation by proteolytic enzymes was assumed.[17] To test the stability, the cleavage of the Phe-derived azadipeptide nitrile **6** and its carba-analogues **12** and **14** catalyzed by chymotrypsin, a serine protease which shows distinct specificity for phenylalanine in the P^1 position, was studied. Compounds **6**, **12**, and **14** were initially tested for inhibition of the chymotrypsin-catalyzed conversion of a chromogenic peptide using an enzyme concentration of 10 ng mL^{-1} (see the Supporting Information). The azadipeptide nitrile **6** showed only poor inhibition ($IC_{50} = 1300$ μM), and **12** and **14**, at a concentration of 600 μM, did not inhibit chymotrypsin at all. Next, the stability of **6**, **12**, and **14** towards chymotrypsin was examined by HPLC analysis (Figure 2). The nonmethylated carba-analogue **12** was degraded with a half-life of 45 min at a rather high chymotrypsin concentration of 100 μg mL^{-1}. A similar concentration of chymotrypsin is present in the human small intestine.[18] At the same enzyme concentration, the *N*-methylated carba-analogue **14** was cleaved at only a very low rate. The azadipeptide nitrile **6** showed a similar stability towards chymotryptic cleavage as the carba-analogue **14**. The degradation of the three dipeptide derivatives was due to a chymotrypsin-catalyzed cleavage of the P^2–P^1 amide bond. This was confirmed by comparing the rates of Z-Phe-OH formation (Z = benzyloxycarbonyl) with the decay of the inhibitors (see the Supporting Information). These results lead to the conclusion that the azadipeptide nitriles exhibit a considerable resistance to protease-catalyzed degradation. Herein we have revealed azadipeptide nitriles to be novel, highly potent, and proteolytically stable inhibitors for papain-like cysteine proteases. Furthermore, the established synthetic entry will allow the introduction of high structural

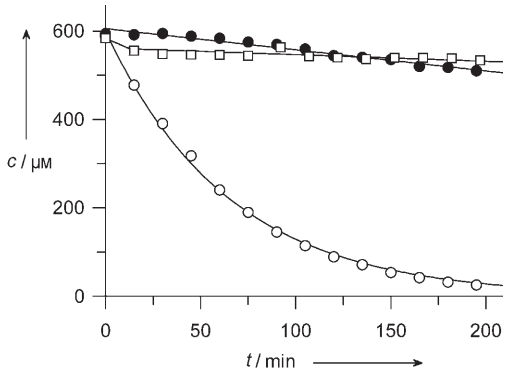


Figure 2. Time course of the chymotrypsin-catalyzed degradation of the azadipeptide nitrile **6** (□) and the dipeptide nitriles **12** (○) and **14** (●). Mixtures of the corresponding nitrile (600 μM) and chymotrypsin (100 $\mu g\,mL^{-1}$) in 20 mM Tris-HCl pH 8.4 (Tris = tris(hydroxymethyl)-aminomethane), 150 mM NaCl, 10% v/v acetonitrile were kept at 25 °C, and 20 μL aliquots were injected into the HPLC apparatus. The first-order rate constant for the decay of **12** was $(0.015 \pm 0.001)\ min^{-1}$, which corresponds to a half-life of 45 min. Linear regression of the data points for the hydrolysis of **14** gave an initial rate of $(0.48 \pm 0.03)\ \mu M\,min^{-1}$, which corresponds to a half-life of 14 h, assuming a first-order kinetics. The hydrolysis of **6** was also slow but followed more complex kinetics (see the Supporting Information).

diversity into the P^1 position of these peptide-derived inhibitors, which provides a strategy to develop stable and selective inhibitors for this important class of proteases.

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