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Fluorescent nitrile-based inhibitors of cysteine cathepsins

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ABSTRACT

Cysteine cathepsins play an important role in many (patho)physiological conditions. Among them, cathepsins L, S, K and B are subjects of several drug discovery programs. Besides their role as drug targets, cysteine cathepsins are additionally considered to be possible biomarkers for inflammation and cancer. Herein, we describe the design, synthesis, biological evaluation and spectral properties of fluorescently labeled dipeptide- and azadipeptide nitriles.

Cysteine cathepsins belong to the subfamily of papain-like proteases exhibiting a high degree of homology. Eleven human cysteine cathepsins have been described. They are involved in many (patho)physiological processes such as bone remodeling, antigen presentation, osteoporosis, autoimmune disorders, and cancer.[1] Therefore, cathepsins represent important targets for new therapeutic strategies. Among them, cathepsins L, S, K and B are subjects of several drug discovery programs.[2]

Inhibitors of cathepsins are mainly peptidic or peptidomimetic structures containing electrophilic groups for covalent interactions with the active-site cysteine. Nitrile-based inhibitors have received the most attention in the current research.[2,3] Recently, we have reported on the development of azadipeptide nitriles as highly potent and non-selective inhibitors of cysteine cathepsins with K_i values in picomolar range, which additionally exhibited antimalarial activities.[4,5] Moreover, using the example of cathepsin K, an approach towards developing selective azadipeptide nitriles by structural optimization of the inhibitor scaffold was demonstrated.[6,7] Loh et al. described peptide conjugates of azadipeptide nitriles to achieve organelle-specific delivery.[8]

Besides their role as possible drug targets, cysteine cathepsins are considered to be possible biomarkers for certain pathological conditions including inflammation and cancer.[9–14] Therefore, there is a need for sensitive methods to label cathepsins for diagnostic and research proposals.[15–20] Recently, 'activity-based' probes, containing an azadipeptide nitrile scaffold, were developed for rhodesain from *Trypanosoma brucei*.[21]

Herein we describe the synthesis, biological evaluation and spectral properties of novel fluorescent dipeptide- and azadipeptide nitriles which can prospectively be used to label cysteine cathepsins. The design was focused on cathepsin K as the target protease, as this enzyme is of particular therapeutic and diagnostic importance.[3]

First, the coumarin derivative **2** was synthesized as a fluorescent reporter by the reaction of 4-diethylamino-2-hydroxy-benzaldehyde with isopropylidene malonate in the presence of piperidinium acetate (Scheme 1).[22] Compound **1** and dansyl chloride **3** were commercially available.

Next, the homocycloleucyl-glycine nitrile scaffold **6** was synthesized as shown in Scheme 2. The Cbz-protected homocycloleucine **4** was reacted with oxalyl chloride in the presence of a catalytic amount of DMF to obtain homocycloleucine-NCA **5** in high yield and purity. Compound **5** was heated with aminoacetonitrile leading to the dipeptide nitrile **6**. Finally, the free amino group of **6** was converted with dansyl chloride **3** and the coumarin derivative **2** to obtain the fluorescently labeled dipeptide nitriles **7** and **8**, respectively (Scheme 2). As expected, the amino group of **6** showed a decreased reactivity due to the *gem*-dialkyl effect operative in homocycloleucine derivatives.[7,23] Accordingly, the target compounds **7** and **8** were isolated by chromatographic separation only in low yields of 36% and 20%, respectively.

The fluorescently labeled compound **8** exhibited better kinetic properties than **7** with respect to cathepsin K inhibition. In order to extend the residence time of the inhibitor, **8** was chosen for a Cα/N exchange in the P1 amino acid. From previous studies, it could be expected that the resulting azadipeptide nitrile forms a slowly dissociating enzyme-inhibitor complex.[4,6] The design of the corresponding azadipeptide nitrile **12** is shown in Scheme 3.

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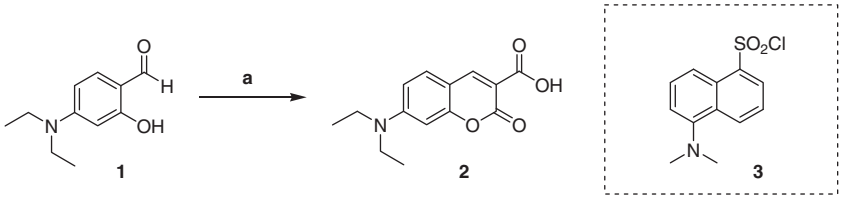
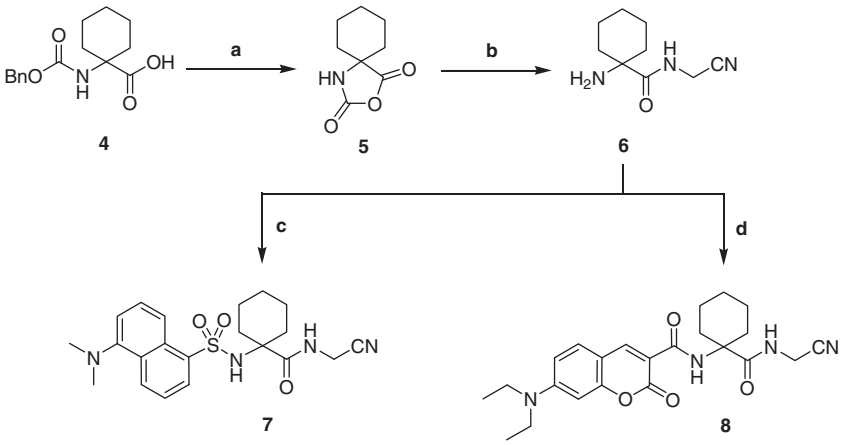
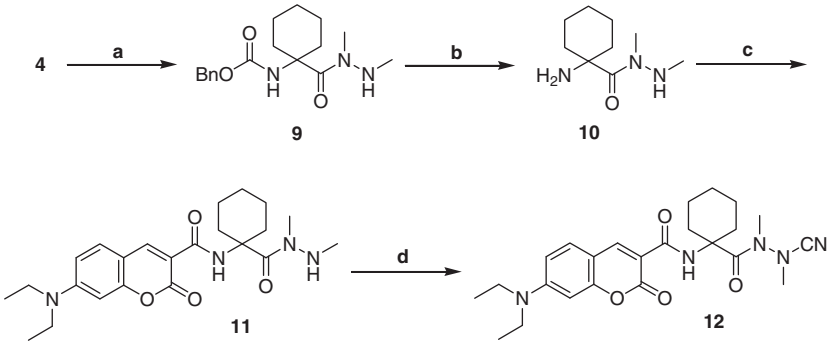


Scheme 1. Synthesis of the coumarin derivative **2**. (a) isopropylidene malonate, piperidinium acetate, EtOH, rt to Δ.



Scheme 2. Synthesis of fluorescently labeled dipeptide nitriles **7** and **8**. (a) $(COCl)_2$, CH_2Cl_2, DMF, rt; (b) $H_2NCH_2CN \times H_2SO_4$, THF, DIPEA, 100 °C; (c) compound **3**, Et_3 N, THF, rt then Δ; (d) compound **2**, EDC, DIPEA, THF, rt.



Scheme 3. Synthesis of the fluorescently labeled azadipeptide nitrile **12**. (a) 1. EDC, DMAP, THF, rt; 2. $(NHMe)_2 \times 2HCl$, Et_3N, rt; (b) Pd/C, H_2, MeOH, rt; (c) compound **2**, EDC, DMAP, THF, rt; (d) BrCN, NaOAc, MeOH, rt.

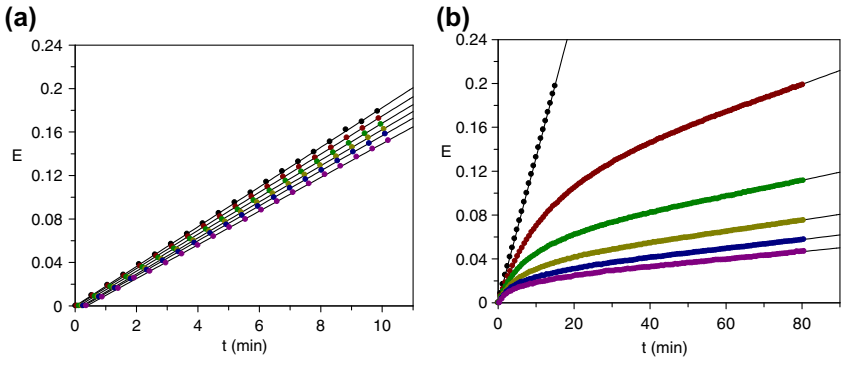


Figure 1. (a) Monitoring of the human cathepsin B-catalyzed hydrolysis of Z-Arg-Arg-pNA (500 μM) in the presence of increasing concentrations of compound **7** (●, 0; ●, 2 μM; ●, 4 μM; ●, 6 μM; ●, 8 μM; ●, 10 μM). The linear progress curves indicate a fast-binding behavior of the inhibitor; (b) Monitoring of the human cathepsin B-catalyzed hydrolysis of Z-Arg-Arg-pNA (500 μM) in the presence of increasing concentrations of the slow-binding inhibitor **12** (●, 0; ●, 2 μM; ●, 4 μM; ●, 6 μM; ●, 8 μM; ●, 10 μM).

Table 1
K_i values of compounds **7**, **8**, and **12**

Compd	K_i (μM)[a]			
	Cat L	Cat S	Cat K	Cat B
7	>40	6.6 ± 1.3	0.88 ± 0.18	>40
8	18 ± 2	8.4 ± 1.5	0.23 ± 0.02	>40
12	0.76 ± 0.08[b]	0.75 ± 0.03	0.019 ± 0.002	0.14 ± 0.01

[a] For cathepsin assays, see the Supplementary data. Compounds **7**, **8** and **12** were tested in duplicate measurements with five different inhibitor concentrations. The reactions were followed over 10 min for fast-binding inhibitors **7** and **8**. Reactions with compound **12** displaying a slow-binding behavior were followed over 80 min.
[b] The enzymatic reaction was started by addition of chromogenic substrate after 30 min preincubation of cathepsin L with compound **12**. The progress curves were analyzed by linear regression over 10 min.

Table 2
k_{on} and k_{off} values of **12**

	k_{on} ($\times 10^3$ M^{-1} s^{-1})	k_{off} ($\times 10^{-3}$ s^{-1})
Cat L	n.d.[a,b]	n.d.
Cat S	0.49 ± 0.09	0.37 ± 0.08
Cat K	9.3 ± 1.7	0.18 ± 0.04
Cat B	0.32 ± 0.03	0.045 ± 0.005

[a] Not determined.
[b] For [**12**] = 30 μM, a k_{obs} value could not be obtained by non-linear regression. Therefore, a limit $k_{obs}(1+[S]/K_m)/[I] < 1.0 \times 10^3$ M^{-1} s^{-1} was estimated.

Table 3
λ_{ex} and λ_{em} of compounds **7**, **8** and **12** (10 μM, 1% DMSO)

Compd	$\lambda_{ex}(\lambda_{em})$ (nm)		
	CH_2Cl_2	MeOH	H_2O
7	343(494, 522)	340(534)	328(566)
8	423(458)	422(470)	433(486)[a]
12	423(458)	423(468)	434(482)

[a] The absorption and emission spectra of **8** in H_2O + 1% DMSO were recorded with a final concentration of 1 μM (see the Supplementary data).

The Cbz-protected homocycloleucine **4** was activated with EDC and DMAP and reacted with 1,2-dimethylhydrazine to obtain the dimethylhydrazide **9**. The Cbz protecting group was hydrogenolytically removed producing the building block **10** which was coupled with the fluorescent reporter **2** using an EDC/DMAP procedure. In the final step, the obtained 1,2-dimethylhydrazide derivative **11** was converted to the azadipeptide nitrile **12** by the reaction with cyanogen bromide. Compound **12** was obtained in 19% yield after column chromatography.

The fluorescent probes **7**, **8** and **12** were evaluated on human cathepsins L, S, K and B using chromogenic or fluorogenic peptides as substrates. While the dipeptide nitriles **7** and **8** exhibited a fast-binding behavior, the aza-analogous counterpart **12** was a slow-binding inhibitor (Fig. 1). In general, compounds **7**, **8** and **12** showed a slight preference for cathepsin K over cathepsins L, S and B (Table 1). Such a profile was expected, since homocycloleucine was established to be an optimized P2 moiety prone to favorable interaction with the S2 binding pocket of cathepsin K.[2,3,24]

The K_i values of dipeptide nitriles **7** and **8** on cathepsin K were in the nanomolar range. Compound **7** was ca. 8–45-fold selective for cathepsin K over cathepsins L, S and B. Compound **8** showed a better selectivity (ca. 40–170-fold). Furthermore, the replacement of the α-carbon in compound **8** by a nitrogen atom, leading to the corresponding azadipeptide nitrile **12**, resulted in a ca. 10-fold improvement of the inhibitory activity toward cathepsin K (0.23 μM versus 0.019 μM). The fluorescent probe **12** exhibited an approximately 40-fold selectivity for cathepsin K over cathepsins L and S and was only moderately selective for cathepsin K over cathepsin B. Due to the slow-binding behavior of **12**, k_{on} and k_{off} values could be additionally calculated for this compound (Table 2). From the k_{off} values, half life periods of the corresponding covalent isothiosemicarbazide adducts of **12** with cathepsins S, K und B were calculated to be in the range of 0.5–4 h.

The absorption and emission spectra of the fluorescently labeled dipeptide nitriles **7** and **8** and the corresponding azadipeptide nitrile **12** were recorded in different solvents to study their spectral properties (Table 3). The fluorescence of compounds **7**, **8** and **12** was quenched by methanol and water, as it is exemplarily

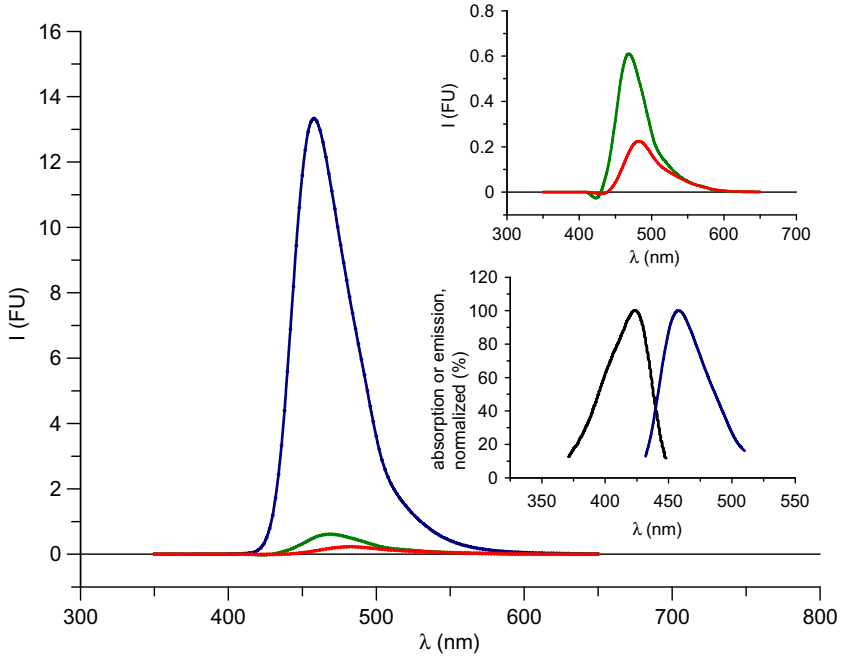


Figure 2. Emission spectra of **12** (10 μM, 1% DMSO) in CH_2Cl_2 (●, λ_{ex} = 423 nm), MeOH (●, λ_{ex} = 423 nm) and H_2O (●, λ_{ex} = 434 nm). The insert above shows the magnified emission spectra in MeOH and H_2O. In the insert below, normalized excitation (●) and emission (●) spectra of **12** (10 μM, 1% DMSO) in CH_2Cl_2 are depicted.

shown for the azadipeptide nitrile **12** (Fig. 2). Compound **7**, containing the well-established dansyl fluorophore, showed a remarkable Stoke shift but weaker fluorescence intensities than **8** and **12** in the three solvents (for the absorption and emission spectra of **7** and **8**, see the Supplementary data). The derivatives **8** and **12** are equipped with the 7-(diethylamino)coumarin-3-carboxamide moiety. This coumarin represents an established fluorescent reporter which was utilized in several 'activity-based' probes and sensors. Such molecules have been applied to study the cellular distribution of drugs,[25–27] or the transport of antimycotic compounds through the fungus.[28] This coumarin was also employed as the fluorescent component of a target-directed probe for the molecular chaperone Hsc70,[29] and of sensors for cellular caspase-3 and protein kinase activities.[30,31] Moreover, *N*-(2-(1-maleimidyl)ethyl)-7-(diethylamino) coumarin-3-carboxamide (MDCC) serves as a common thiol reactive agent for protein labeling.[32,33]

In summary, the fluorescently labeled dipeptide nitriles **7** and **8** were synthesized and their inhibitory activities were evaluated on human cysteine cathepsins. Compounds **7** and **8** exhibited a fast-binding kinetic type and were active against cathepsin K with K_i values in the nanomolar range. Furthermore, they showed a slight selectivity for cathepsin K over cathepsins L, S and B. Compound **8** was converted in the corresponding azadipeptide nitrile **12** by Cα/N exchange of the P1 amino acid. The highly potent, slow-binding inhibitor **12** was ca. 10-fold more active on cathepsin K than the dipeptide nitrile **8**. The spectral properties of **12** were studied in different solvents. In future studies, the developed fluorescent probe **12** will be examined for its suitability to label cathepsin K for diagnostic and research purposes.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.09.086.

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