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A Coumarin-Labeled Vinyl Sulfone as Tripeptidomimetic Activity-Based Probe for Cysteine Cathepsins

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A coumarin-tetrahydroquinoline hydride **8** was synthesized as a chemical tool for fluorescent labeling. The rigidified tricyclic coumarin structure was chosen for its suitable fluorescence properties. The connection of **8** with a vinyl sulfone building block was accomplished by convergent synthesis thereby leading to the coumarin-based, tripeptidomimetic activity-based probe **10**, containing a Gly-Phe-Gly motif. Probe **10** was evaluated as inactivator of the therapeutically relevant human cysteine cathepsins S, L, K, and B: it showed particularly strong inactivation of cathepsin S. The detection of recombinant and native cathepsin S was demonstrated by applying **10** to in-gel fluorescence imaging.

Activity-based probes (ABPs) have been widely used for protein identification and profiling.[1] Owing to their covalent mechanism of catalysis, serine and cysteine hydrolases are particularly well suited to be addressed by ABPs.[2] Most ABPs consist of three main elements: a "warhead" for covalent interaction, a tag (reporter or affinity label) that allows the detection or isolation of the target, and a linker, which can additionally control the probe's selectivity for the protein of interest. Among the target enzymes for ABPs, human cysteine cathepsins have attracted considerable interest.[3–5] Much of this attention arises from the importance of these proteases in a variety of (patho)physiological processes and disease states, such as cancer progression, degradation of the extracellular matrix, angiogenesis, bone remodeling, prohormone processing, aneurysm formation, atherosclerosis, and rheumatoid arthritis.[6] Human cathepsin S is the main processing enzyme of the major histocompatibility complex class II-associated invariant chain in antigen-presenting cells.[7] Thus, cathepsin S is a target enzyme for the development of inhibitors as potential therapeutics against autoimmune diseases, and for the design of ABPs as pharmacological tools to better understand its role in the processes of adaptive immunity.[5,8] Several electrophilic warheads to interact with the active-site cysteine have been incorporated into cysteine protease inhibitors. Among these, peptidyl vinyl sulfones are known to efficiently inactivate their targets by a Michael-type irreversible reaction.[9] Recent reports have illustrated the successful development of peptidyl vinyl sulfones as inactivators of human cathepsins B, L, and K,[10–12] caspase-3,[13] cathepsin B from *Schistosoma mansoni*,[14] as well as falcipain-2 and -3, cruzain, and rhodesain.[10,15] Moreover, an ABP with a vinyl sulfone substructure was developed for cathepsin C,[4] and a panel of vinyl sulfone probes was used in a cysteine protease microarray.[16]

Fluorescence labeling is of growing interest in the life sciences. Various classes of fluorophores are synthetically accessible and commercially available, and these are widely employed for manifold purposes. In addition to BODIPY and fluorescein derivatives, coumarins constitute a commonly used class of fluorescent label; these are valued for their large Stokes shifts.[17–19] The coumarins' small molecular size makes them attractive, in particular, for incorporation into peptides and for assembling artificial protease substrates and peptidic protease inhibitors.[20–23] Several derivatization reagents bearing a coumarin moiety as a fluorophore have been reported.[24] Structure–fluorescence relationships of the coumarin chemotype are well understood. Among the coumarin derivatives bearing a combination of an electron-withdrawing group at the 3-position and an electron-donating substituent at the 7-position, 7-methoxy- or 7-aminocoumarins produce high fluorescence quantum yields. The latter possess a typical red shift of absorption and emission, a favorable feature with regard to background fluorescence. For example, 7-diethylaminocoumarin-3-carboxylic acid (DEAC) is an established fluorophore,[18,21,22] but its fluorescence is quenched in aqueous environment.[22,23] Rigidization of the amino group in one ring (e.g., tetrahydropyrido-coumarins ATTO390 and ATTO425) or two rings (julolidine-type coumarin 343) significantly restores fluorescence in aqueous media.[17,23,25]

Herein, we describe the synthesis of a coumarin-tetrahydroquinoline hydride molecule (**8**, Scheme 1), a new member of the 3-acceptor-substituted 7-aminocoumarins. Its rigid structure was expected to allow the desired bathochromic shift and sufficient fluorescence in aqueous media. The incorporated nitrogen can be considered as part of a glycine linker for peptide bond formation.

The seven-step synthesis of **8** started from 7-hydroxy-3,4-dihydroquinolin-2(1*H*)-one (**1**, Scheme 1). After benzylation to

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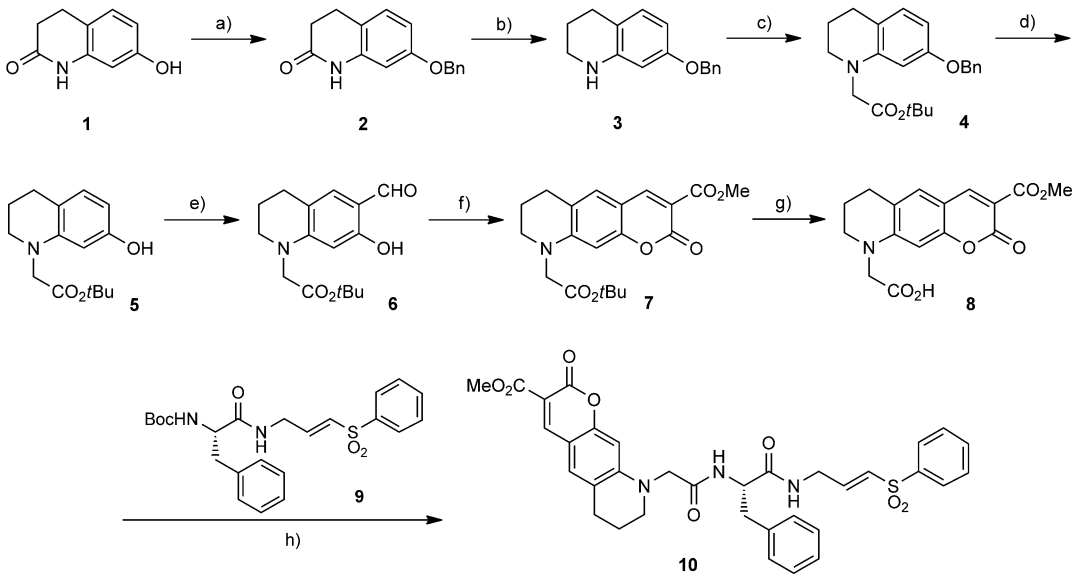

Scheme 1. Synthesis of coumarin-labeled activity-based probe **10**. Reagents and conditions: a) BnBr, K_2CO_3, DMF, 16 h, 84%; b) BH_3·THF, THF, 0 °C, 99%; c) $BrCH_2CO_2tBu$, K_2CO_3, DMF, 80 °C, 16 h, 84%; d) H_2, Pd/C, MeOH, 3 h, 93%; e) $POCl_3$, DMF, 0 °C, 30 min, 90 °C, 30 min, 92%; f) dimethyl malonate, piperidine, MeOH, 2 h, 56 °C, 81%; g) CF_3CO_2H, CH_2Cl_2, 2 h, 97%; h) i: MeOH, AcCl, EtOAc, **9**, 30 min; ii: **8**, HATU, DIPEA, DMF, 2 h, 18% over two steps.

protect the phenolic group, the lactam moiety of **2** was reduced with borane to furnish the 1,2,3,4-tetrahydroquinoline derivate **3**. Alkylation with *tert*-butyl bromoacetate afforded **4**, which was catalytically hydrogenated to deprotect the phenol. The salicylaldehyde derivative **6** was obtained by formylation of **5** under Vilsmeier conditions. Subsequent Knoevenagel condensation with dimethyl malonate yielded coumarin **7**, with two orthogonally protected esters. The *tert*-butyl ester of **7** was cleaved under acidic conditions to give **8** (overall yield 47%). Building block **9**, which was separately synthesized in five steps according to a reported method,[12] was labeled with fluorophore **8**. This final step of the convergent synthesis included Boc-deprotection of **9** and HATU-mediated amide coupling. The activity-based probe **10** was designed as a tripeptide whose terminal nitrogen is part of the tricyclic fluorophore and whose carboxylic group is replaced by the vinyl unit. If there is a preference, **10** would be expected to favor cathepsin S rather than the other cathepsins included in this study, considering the structure of a related vinyl sulfone inhibitor.[12]

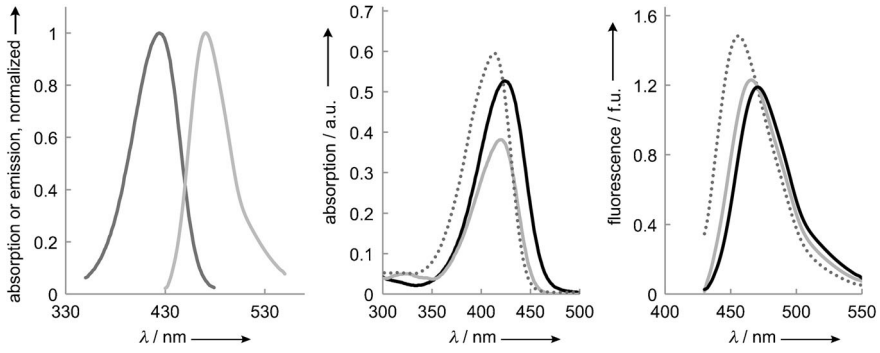

Figure 1. Absorption and emission spectra of **10**. Left: normalized absorption and emission spectra of **10** (in H_2O). Center: absorption spectra of **10** (10 μM, 1% DMSO) in CH_2Cl_2 (dotted), MeOH (gray), and H_2O (black). Right: emission spectra of **10** (1 μM, 1% DMSO) in CH_2Cl_2, MeOH, and H_2O.

The activity-based probe **10** was characterized for its spectroscopic properties in three solvents of different polarity (Figure 1). The spectra show slight bathochromic shifts in polar solvents for both absorption maxima (413 nm in CH_2Cl_2, 420 nm in MeOH, 424 nm in H_2O) and emission maxima (456 nm in CH_2Cl_2, 466 nm in MeOH, 470 nm in H_2O), thus resulting in Stokes shifts of 43–46 nm. Noteworthy, the fluorescence of **10** was similarly intense in each solvent; no quenching was observed in methanol or water (in contrast to DEAC derivatives).[22,23] The $\log D_{7.4}$ value, a reliable predictor of cell permeability, was determined by an HPLC-based procedure.[26] The $\log D_{7.4}$ value of 1.93 indicates the ability of the fluorophore-labeled probe **10** to cross membranes and penetrate into cells.

The inhibitory potency of **10** against four human cysteine cathepsins (S, L, K, and B) was evaluated by activity assays with chromogenic or fluorogenic peptide substrates. Time-dependent inactivation of the cathepsins was monitored over 60 min (Figure 2). The probe was able to inactivate all tested cathepsins, with cathepsin S being most efficiently affected (second-order rate constant 49000 $M^{-1}s^{-1}$; Table 1).

AutoDock 4.2 (Scripps Research Institute, La Jolla, CA) was used to predict the binding mode of **10** at the active site of human cathepsin S (Figure 3). The covalent sulfur–carbon bond was manually constructed involving the active-site cys-

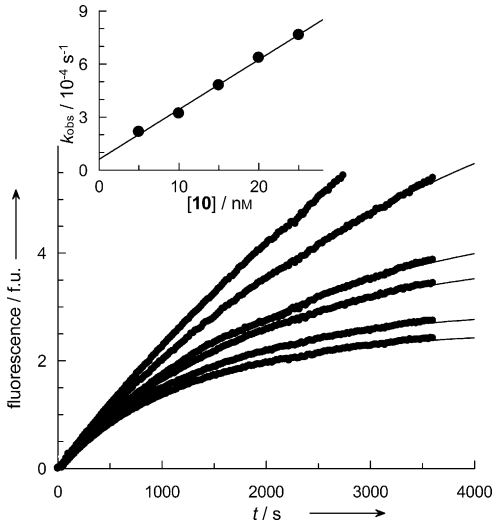

Figure 2. Inhibition of human cathepsin S by **10**. Cathepsin S-catalyzed hydrolysis of Z-Phe-Arg-AMC (40 μM) in the presence of **10** (top to bottom: 0 nM, 5 nM, 10 nM, 15 nM, 20 nM, and 25 nM). Inset: plot of k_{obs} against concentration of **10**.

Table 1. Inhibition of human cysteine cathepsins by **10**.

	Cat S	Cat L	Cat K	Cat B
$k_{obs}/[I]$ [M^{-1} s^{-1}][a]	22820 ± 1100	2450 ± 160	61.7 ± 8.6	128 ± 12
k_{inac}/K_i [M^{-1} s^{-1}][b]	49000 ± 1900	16900 ± 1100	884 ± 123	186 ± 17

[a] Data $k_{obs}/[I] \pm$ SEM are from duplicate measurements with five different inhibitor concentrations. The k_{obs} values were obtained by nonlinear regression of the progress curves. The $k_{obs}/[I]$ values were obtained by linear regression from plots of k_{obs} against [I]. [b] The k_{inac}/K_i values were calculated by using the equation $k_{inac}/K_i = (1 + [S]/K_m) \times k_{obs}/[I]$.

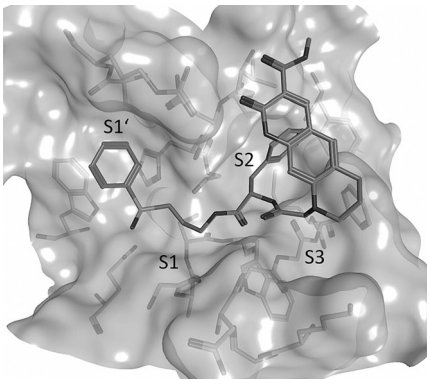

Figure 3. Predicted binding mode of compound **10** (dark gray) within the active site of human cathepsin S. AutoDock 4.2 was used for covalent ligand docking. Active-site residues are depicted in light gray; the surface is rendered as transparent.

teine and the prochiral electrophilic β-carbon of the inactivator. The preceding noncovalent complex gives rise to nucleophilic attack of the Cys25 thiolate from the *si* face, thereby leading to the *S*-configuration of the resulting chiral center (in accordance with the X-ray crystal structure of another vinyl sulfone inactivator in complex with human cathepsin S).[27] Systematic exploration of the conformational space available to covalently linked **10** at the active site revealed that the tripeptide backbone is oriented in a substrate-like manner, thus forming multiple hydrogen bond interactions with Gly69 and Asn163 (see Figure S1 in the Supporting Information).

An overlay of the putative binding mode of **10** with the crystal structure revealed a similar orientation (Figure S2).[27] The S2 subsite of cathepsin S (shaped by residues Met71, Gly137, Gly165, Phe70, Val162, and Phe211)[27] provides a hydrophobic environment that well accommodates the side chain of phenylalanine at the P2 position of **10**. The coumarin-tetrahydroquinoline moiety is directed towards the S3 pocket but orientated away from the enzyme, and is thus solvent exposed. However, its position might be stabilized by hydrogen bond interactions between its two carbonyl groups and the side chain of Arg141 and the backbone NH of Val162 (Figure S1). The S1′ subsite is occupied by the phenyl sulfone group of the inhibitor, thus enabling formation of π–π interactions with the side chains of His164 and Trp186. The SO_2 group is potentially in hydrogen bonding distance from Gln19 and the indole nitrogen of Trp186.

The feasibility of **10** for direct in-gel fluorescence detection of human cysteine cathepsins was demonstrated by using cathepsin S and a common imaging tool. Cathepsin S (500 ng) was treated with 5 μM **10** for 30 min and subjected to SDS-PAGE; a strong fluorescent band at 25 kDa was clearly detected (Figure 4). The binding mode of **10** was confirmed by a

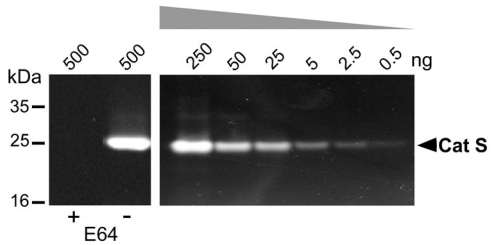

Figure 4. Imaging human cathepsin S with the fluorescent probe **10**. Left: purified recombinant cathepsin S (500 ng) was labeled with **10** (5 μM). In the control experiment, the enzyme was treated with E64 (5 μM) prior to **10** (5 μM). Right: cathepsin S (250 to 0.5 ng) was treated with **10** (5 μM). Labeling reactions were performed for 1 h at pH 6.5. The mixtures were resolved by SDS-PAGE and visualized with a Typhoon 9410 fluorescence imager.

competition experiment with the active-site-directed broad-spectrum cysteine protease inhibitor E64 to protect cathepsin S from inactivation by **10**. First, cathepsin S (500 ng) was treated with E64 (5 μM), then incubated with **10** (5 μM). No fluorescence at 25 kDa was visible, as the active-site cysteine of cathepsin S was blocked by covalently bound E64 (Figure 4, left). These findings confirmed the covalent interaction between the active-site cysteine and inactivator **10**, and suggest that the surface nucleophiles of the enzyme were not affected by this probe.

Next, the sensitivity of the probe **10** was tested. The amount of cathepsin S in the labeling reaction was decreased to 0.5 ng. In each lane, a clear band was observed thus indicating the

probe's suitability for sub-nanogram detection of human cathepsin S (Figure 4, right).

For studying its selectivity, **10** was applied to label cathepsin S in the presence of a complex proteome. Lysate from HEK293 cells was prepared, spiked with cathepsin S, and treated with **10**. As controls, cathepsin S and HEK293 lysate were separately incubated with **10**. The incubation mixtures were subjected to SDS-PAGE and visualized by protein staining and fluorescence imaging (Figure 5). This analysis revealed selective

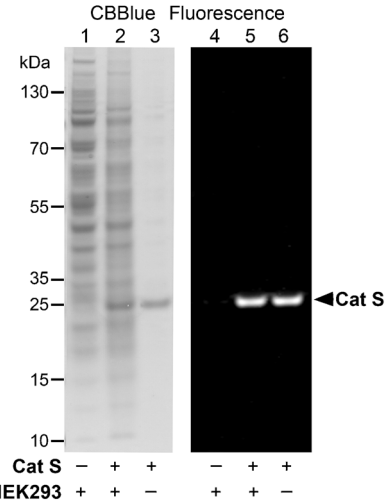

Figure 5. Imaging of human cathepsin S with the fluorescent probe **10** in the presence of cell lysate. Purified recombinant cathepsin S (250 ng) was mixed with HEK293 cell lysate (25 μg) and treated with **10** (5 μM) for 1 h at pH 6.5. The reaction mixture was resolved by SDS-PAGE and visualized by fluorescence in a Typhoon 9410 imager (right) followed by protein staining with Coomassie Brilliant Blue (left).

labeling of the target cathepsin S in the presence of a large excess of lysate proteins, without detectable nonspecific interactions of **10** (Figure 5, lanes 2 and 5).

Furthermore, we labeled native cathepsin S in the protein extract from human placenta and demonstrated high selectivity of the labeling reaction (Figure 6, lanes 5–7). The experiment

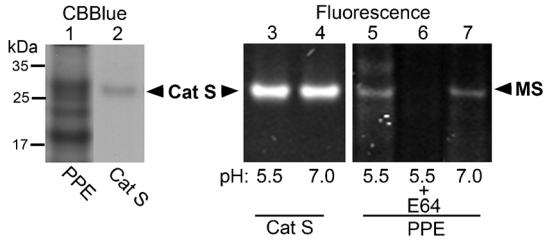

Figure 6. Imaging of cathepsin S with the fluorescent probe **10** in human placenta extract. Protein extract from human placenta (PPE; 10 μg) or recombinant human cathepsin S (Cat S, 250 ng) was treated with **10** (1 μM) for 1 h at pH 5.5 or 7.0. In the control experiment, PPE was pretreated with E64 (5 μM) prior to **10**. The reaction mixture was resolved by SDS-PAGE and fluorescence was visualized with a Typhoon 9410 imager (right) followed by protein staining with Coomassie Brilliant Blue (left). Human cathepsin S was verified in the labeled band of PPE ("MS") by LC-MS/MS analysis.

was carried out at mild acidic pH as well as neutral pH (at which cathepsin S is active and stable, in contrast to other cysteine cathepsins).[28] It can therefore be concluded that the probe is suitable for selectively detecting human cathepsin S in complex protein mixtures.

In summary, we have introduced a straightforward synthesis of building block **8** with a tailored tricyclic structure accounting for suitable fluorescent properties. This coumarin-tetrahydroquinoline hydride is considered to be of great value for versatile applications in the design of fluorescently tagged molecules. One implementation was realized in this study by incorporating **8** in the tripeptidic activity-based probe **10**, the first coumarin-based ABP. The Gly-Phe-Gly motif was particularly favorable for recognition and inactivation of the therapeutically relevant cysteine protease cathepsin S. A plausible binding mode of **10** in the active site of cathepsin S is proposed. Finally, we successfully employed **10** for direct in-gel fluorescence imaging of recombinant and native cathepsin S with high sensitivity and specificity. In future studies, the activity-based probe **10** will be examined for its feasibility in the determination of active cathepsin S by HPLC in combination with a fluorescence detector.

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