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# Chemical introduction of the green fluorescence: imaging of cysteine cathepsins by an irreversibly locked GFP fluorophore†

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An activity-based probe, containing an irreversibly locked GFP-like fluorophore, was synthesized and evaluated as an inhibitor of human cathepsins and, as exemplified with cathepsin K, it proved to be suitable for *ex vivo* imaging and quantification of cysteine cathepsins by SDS-PAGE.

## Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and related fluorescent proteins are widely used as universal genetically encoded fluorescent labels and have revolutionized the field of live cell imaging. Following protein translation, the fluorophore, *i.e.* 4-(4-hydroxybenzylidene)-1*H*-imidazol-5(4*H*)-one (**I**, Fig. 1), is formed autocatalytically by cyclization of the protein backbone at positions 65–67 (Ser-Tyr-Gly) and dehydrogenation of the Tyr side chain.[1,2] Inspired by the GFP fluorophore, several studies have targeted its synthetic analogues.[3–10] The simple dimethyl compound **II**, identical to the native GFP core, does however not possess applicable fluorescence properties. This is predominantly due to *cis–trans* isomerization and/or free rotation of the phenyl ring resulting in a decay of the fluorophore's excited state.[11] However, when buried within the protective β-barrel structure of GFP, this internal conversion is suppressed. In the absence of the rigid H-bonding protein environment, the radiationless decay can be sufficiently prevented upon complexation with $Zn^{2+}$ as realized in **III**,[12] or by introducing the $BF_2$ entity leading to irreversible locked analogues **IV**.[13] Among the irreversibly locked analogues, the phenol derivative **V** is structurally closest to the native GFP fluorophore.[14] Therefore, we have selected the known fluorophore **V** to design a probe to exogenously label native proteins and to demonstrate the chemical introduction of the green fluorescence. For this purpose, the concept of 'activity-based' probes (ABPs) was applied to specifically address the active site of enzymes, using the example of the therapeutically relevant cysteine cathepsins.

Human cysteine cathepsins (cat) belong to the subfamily of papain-like cysteine proteases and are involved in a variety of different (patho)physiological processes. In particular, cathepsins L, S, K and B are important subjects of several drug discovery programs.[15–18] Besides their importance in drug development, cysteine cathepsins are considered to be possible biomarkers for certain pathological conditions including inflammation and cancer.[19–24] Recently, remarkable scientific efforts have been made to develop sensitive methods for the detection of cysteine proteases using labeled inhibitors and fluorescently quenched substrates.[24–33] Human cathepsin K, playing a crucial role in bone remodeling and osteoporosis, was chosen as a model enzyme in this study to demonstrate a useful procedure for the visualization of cathepsin activities by the introduction of a green GFP-like label.

## Results and discussion

The synthesis of the new fluorescent reporter **5**, an irreversibly locked analogue of the GFP fluorophore, is shown in Fig. 2. The oxazolone derivative **1**[4] was reacted with GABA under basic conditions, followed by heating with $Cs_2CO_3$ in DMF. The resulting compound **2** was treated with *tert*-butyldiphenylchlorosilane (TBDPSCl) in the presence of *N*,*N*-diisopropylethylamine (DIPEA) to protect both the phenolic and the carboxylic group as *tert*-butyldiphenylsilyl ether/ester moieties. Compound **3** was subsequently converted to the highly fluorescent organoborane derivative **4** by consecutive reactions with $BBr_3$, EtOH and HF. The ethyl ester of **4** was finally saponified leading to the green fluorophore **5**, bearing a flexible linker with a distal carboxyl group.

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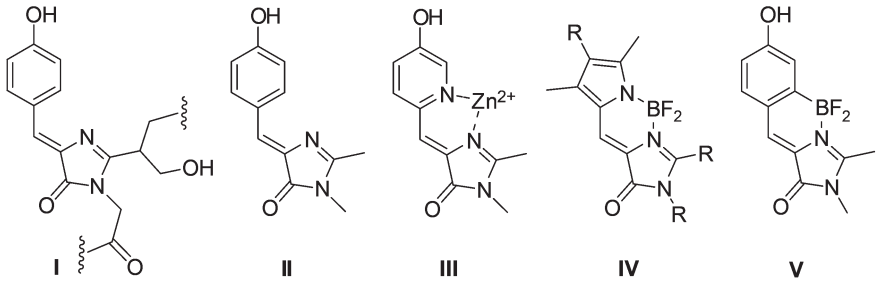


**Fig. 1** The native GFP fluorophore and its synthetic analogues.

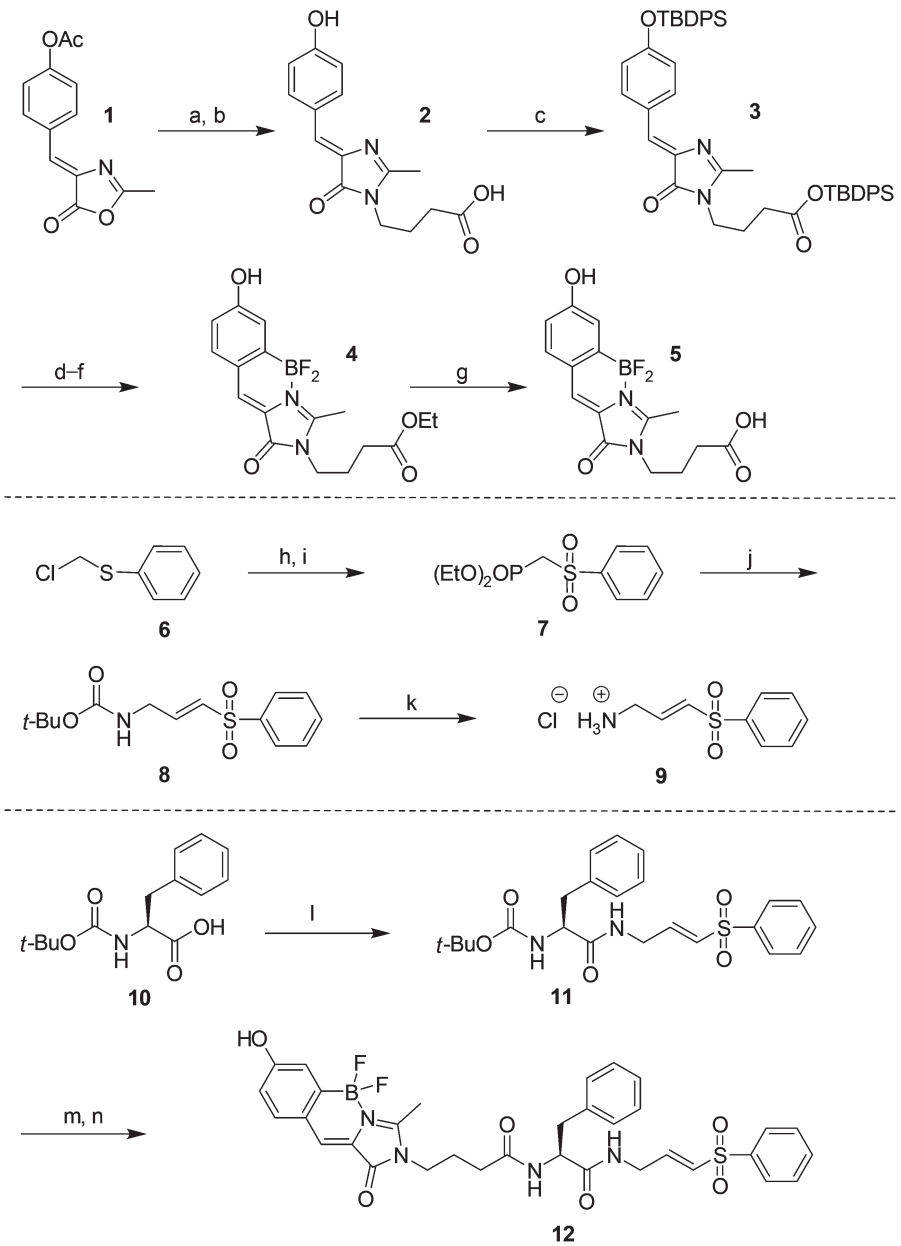


**Fig. 2** Synthesis of the probe **12**. (a) GABA, $Et_3N$, EtOH, $H_2O$, rt; (b) $Cs_2CO_3$, DMF, Δ; (c) TBDPSCl, DIPEA, imidazole, THF, rt; (d) $BBr_3$, molecular sieves, $CH_2Cl_2$, rt; (e) EtOH, THF, rt; (f) HF, THF, rt; (g) NaOH, $H_2O$, rt; (h) $P(OEt)_3$, 130 °C, sealed tube; (i) $KMnO_4$, AcOH, $H_2O$, rt; (j) Boc-Gly-H, NaH, THF, rt; (k) AcCl, MeOH, AcOEt; (l) i. NMM, $ClCO_2i$-Bu, THF, −25 °C, ii. **9**, NaOH, $H_2O$, −25 °C to rt; (m) AcCl, MeOH, AcOEt, rt, basic extraction; (n) **5**, NMM, $ClCO_2i$-Bu, THF, −25 °C to rt.

Separately, a vinyl sulfone 'warhead' for irreversible interactions with the active-site cysteine of human cathepsins was synthesized, since peptidyl vinyl sulfones have been established as potent irreversible inhibitors of cysteine cathepsins.[34–39] Chloromethyl phenyl sulfide **6** was heated with triethyl phosphite in a sealed tube, and the resulting Arbuzov product was oxidized with $KMnO_4$ leading to **7** which was reacted with Boc-protected glycinal in the presence of NaH to obtain the corresponding (*E*)-vinyl sulfone **8** *via* Wittig–Horner reaction. Hydrogen chloride-promoted deprotection of **8** provided the Michael acceptor **9** as a hydrochloride salt.[35]

For the final synthetic steps of the convergent route to the envisaged ABP **12**, we chose phenylalanine as a suitable building block for the P2 position. Boc-protected phenylalanine **10** was activated *via* a mixed anhydride and coupled under basic conditions with the vinyl sulfone **9** to obtain compound **11**. This was deprotected upon treatment with hydrogen chloride, and the resulting product was coupled, after a previous basic extraction, with the fluorescent reporter **5** leading to compound **12**.

Next, the probe **12** was evaluated in activity assays with human cathepsins L, S, K and B in the presence of chromogenic or fluorogenic peptide substrates. The kinetic analyses confirmed an irreversible, slow-binding mode of interaction (Fig. 3). The progress curves were analyzed by non-linear regression using the equation $E/I = v_i/k_{obs} \times (1 - \exp(-k_{obs} \times t)) + d$ where $E/I$ is the extinction/fluorescence intensity, $v_i$ is the initial rate, $k_{obs}$ is the observed first-order rate constant, and $d$ is the offset. The plots of the $k_{obs}$ values *versus* [**12**] proved to be hyperbolic. Therefore, $k_{inac}$ and $K_i'$ constants were obtained by non-linear regression of the data pairs ($k_{obs}$, [I]) using the equation $k_{obs} = k_{inac}[I]/(K_i' + [I])$ where $k_{inac}$ is the first-order inactivation rate constant, $K_i'$ is the apparent inhibition constant and [I] is the concentration of **12**. The apparent inhibition constant was corrected to the zero substrate concentration, and the second-order rate constant $k_{2nd}$ was calculated using the equation $k_{2nd} = k_{inac}/K_i$ where $K_i$ is the true inhibition constant.

**Table 1** Kinetic parameters of **12**. Data were calculated from duplicate experiments with at least five different inhibitor concentrations

| | $k_{2nd}$ ($M^{-1}$ $s^{-1}$) | $k_{inac}$ ($s^{-1}$) | $K_i$ (μM) |
|---|---|---|---|
| Cat L | 2200 ± 200 | 0.0031 ± 0.0001 | 1.4 ± 0.1 |
| Cat S | 18 000 ± 2000 | 0.0020 ± 0.0001 | 0.11 ± 0.01 |
| Cat K | 4600 ± 900 | 0.0011 ± 0.0001 | 0.24 ± 0.04 |
| Cat B | 290 ± 70 | 0.0029 ± 0.0003 | 10 ± 2 |

The kinetic parameters of **12** are listed in Table 1. In general, the ABP **12** was active against all tested cathepsins. The second-order rate constants ($k_{2nd}$), representing the main characteristic of irreversible inhibitors, were at least 290 $M^{-1}$ $s^{-1}$. Particularly high $k_{2nd}$ values were calculated for cathepsin S ($k_{2nd}$ = 18 000 $M^{-1}$ $s^{-1}$) and cathepsin K ($k_{2nd}$ = 4600 $M^{-1}$ $s^{-1}$).

In the case of irreversible inhibition, the true inhibition constants ($K_i$) describe the formation of non-covalent enzyme–inhibitor complexes, prior to the subsequent inactivation step. The first-order inactivation rate constants ($k_{inac}$) were in the same range. These findings are in agreement with the expected common modification reaction, *i.e.* the nucleophilic attack of the active-site cysteine at the Michael acceptor site of **12**, governed by $k_{inac}$. In contrast to this parameter, the corresponding $K_i$ values varied between 0.11 μM and 10 μM, reflecting the distinct affinity of **12** to the four target enzymes, determining its inhibitory activity ($k_{2nd} = k_{inac}/K_i$) and slight preference for cathepsins S and K.

The photochemical properties make compound **12** well suited as an ABP. Due to the excited-state proton transfer, the GFP-like fluorophore of **12** partly dissociates already at neutral pH and thus exists as two different species in water.[14] As shown in Fig. 4, two emission maxima, *i.e.* at 486 nm (neutral form) and at 528 nm (anionic form), were observed for **12** in

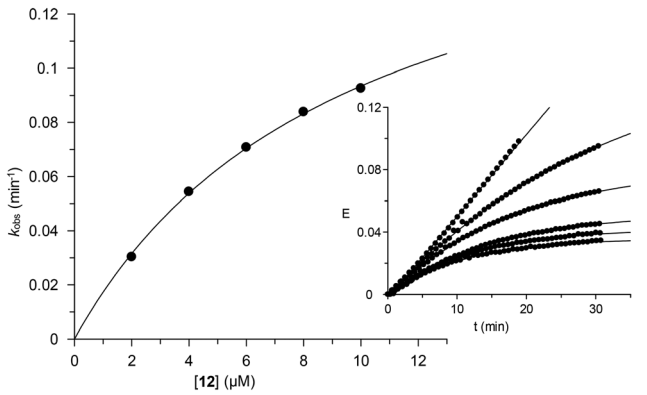


**Fig. 3** Kinetic behavior of **12**. Inset: monitoring of the human cathepsin L-catalyzed hydrolysis of *Z*-Phe-Arg-pNA (100 μM) in the presence of **12** (from top to bottom: 0 μM, 2 μM, 4 μM, 6 μM, 8 μM and 10 μM). Graph: plot of the $k_{obs}$ values *versus* the increasing concentration of **12**. Non-linear regression of the resulting data pairs ($k_{obs}$, [**12**]) gave an apparent inhibition constant $K_i' = (1 + [S]/K_m)K_i = 9.6 \pm 0.7$ μM. The corresponding $k_{inac}$ value was calculated to be 0.18 ± 0.01 min$^{-1}$.

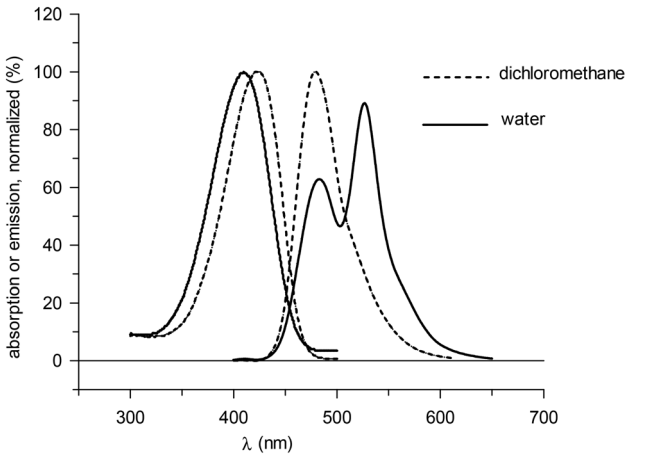


**Fig. 4** Representative normalized spectra of **12** (10 μM). $CH_2Cl_2$: $\lambda_{ex}$ = 423 nm, $\lambda_{em}$ = 480 nm; $H_2O$: $\lambda_{ex}$ = 410 nm, $\lambda_{em}$ = 486/528 nm.

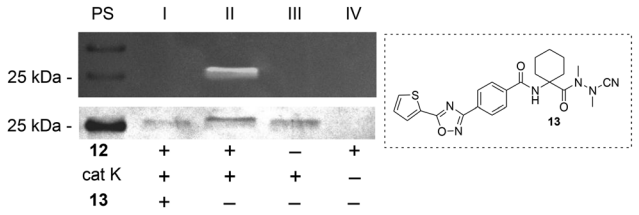


**Fig. 5** Imaging and competition experiments. Top: fluorescence analysis. Bottom: Coomassie staining. PS – protein standard; (I) 130 ng of **12**, 280 ng cat K, 930 ng of **13**; (II) 130 ng of **12**, 280 ng cat K; (III) 280 ng cat K; (IV) 130 ng of **12**.

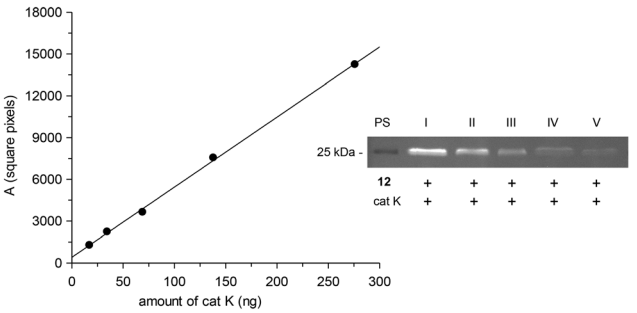


**Fig. 6** Plot of calculated areas of the fluorescent bands *versus* increasing amounts of the labeled cathepsin K. PS – protein standard; (I) 130 ng of **12**, 280 ng cat K; (II) 66 ng of **12**, 140 ng cat K; (III) 33 ng of **12**, 69 ng cat K; (IV) 99 ng of **12**, 35 ng cat K; (V) 50 ng of **12**, 17 ng cat K. Slope = 50 ± 1 square pixels $ng^{-1}$, correlation coefficient ~1. For quantification, the ImageJ freeware was used.

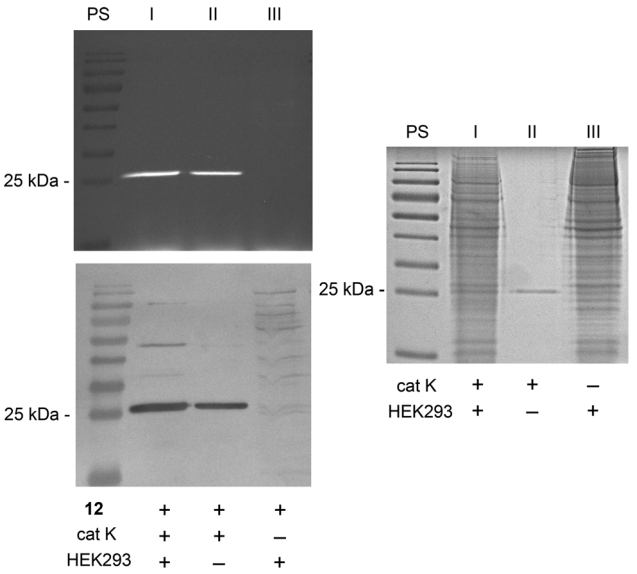


**Fig. 7** Selectivity of labeling. Top: fluorescence analysis. Bottom: western blot using cathepsin K antibodies. PS – protein standard; (I) 260 ng of **12**, 370 ng cat K, 24 µg protein (HEK293 cell lysate); (II) 260 ng of **12**, 370 ng cat K; (III) 260 ng of **12**, 24 µg protein (HEK293 cell lysate). Right: Coomassie staining of the gel, which was performed with the same amounts of cathepsin K and proteins from the HEK293 cell line as in the case of the fluorescence and western blot analyses, but in the absence of **12**. PS – protein standard; (I) 370 ng cat K, 24 µg protein (HEK293 cell lysate); (II) 370 ng cat K; (III) 24 µg protein (HEK293 cell lysate).

an aqueous environment. The corresponding absorption maximum in water was at 410 nm allowing the excitation of **12** using a VIS transilluminator.

Next, it was intended to demonstrate the possibility of direct in-gel fluorescence detection of cathepsins using human cathepsin K, the fluorescent probe **12** and a common gel documentation device. Moreover, the binding mode of **12** was investigated in a competition experiment (Fig. 5). Activated cathepsin K was incubated with the probe **12**. Simultaneously, the same amount of activated cathepsin K was treated with the selective, active-site directed cathepsin K inhibitor **13**,[40] prior to incubation with **12**, in order to protect cathepsin K from inactivation by **12**. In the control experiments, solutions of activated cathepsin K and **12** were separately prepared. Certain aliquots of each incubation mixture were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions and analyzed using a gel documentation device. No fluorescence was detectable at ~26 kDa when cathepsin K was pre-incubated with compound **13** (lane I, Fig. 5, top). In contrast, a strong fluorescent signal of the labeled cathepsin K was observed in the absence of the competitor (lane II, Fig. 5, top). These findings indicated a covalent interaction between **12** and the active-site cysteine of cathepsin K, while the surface nucleophiles of the enzyme were obviously not affected by the probe **12**. To state the presence of cathepsin K in the labeling, competition and control experiments, the gel was additionally stained with Coomassie blue (Fig. 5, bottom).

In a further experiment, activated cathepsin K was incubated with compound **12**. Different aliquots of the incubation mixtures were subjected to reducing SDS-PAGE, and the resulting gel was imaged and subsequently quantified. The calculated areas of the fluorescent bands were plotted against the increasing amounts of cathepsin K showing a strong linear dependence (Fig. 6).

The selectivity of labeling was investigated as follows. Activated cathepsin K was mixed with a lysate from HEK293 cells and treated with **12**. Simultaneously, activated cathepsin K and the HEK293 lysate were separately incubated with **12** as positive and negative controls, respectively. Certain aliquots of each incubation mixture were subjected to reducing SDS-PAGE. The fluorescence analysis of the resulting gel showed that the probe **12** selectively imaged cathepsin K under the conditions used, but not the proteins from the HEK293 cell lysate (lane I, Fig. 7, top). The sensitivity of the fluorescence analysis was comparable with that of the corresponding western blot (lane I, Fig. 7, bottom), and the ABP-based detection appeared to be even more selective. A further gel electrophoresis was performed under the same conditions, but in the absence of **12**. The following Coomassie staining indicated that the ratio between cathepsin K and the proteins from the HEK293 cell lysate, which had been used for the fluorescence and western blot analyses, was appropriate to show the

 

selectivity of labeling by **12**. The cathepsin K band at ~26 kDa did not appear to be stronger than the bands of the proteins from the HEK293 cell lysate (Fig. 7, right).

To summarize, the newly synthesized ABP **12** caused an irreversible, slow-binding inhibition of human cathepsins with a slight preference for cathepsins S and K. Using the example of cathepsin K, we demonstrated the possibility of cathepsins' direct in-gel fluorescence imaging by **12**. The fluorescence properties of the GFP-like fluorophore of **12** turned out to be optimal for a detection using a common gel documentation device, equipped with a commercially available VIS transilluminator containing a light source for excitation between about 420 and 500 nm. As an ABP, **12** allows for the detection of only the active enzyme fraction. In contrast, other visualization techniques such as Coomassie or silver staining as well as the western blot analysis provide, by a different degree of selectivity, the imaging of the whole protein including both, active and inactive forms of cathepsins. We demonstrated the strong selectivity, but also a notable sensitivity of the cathepsin detection by ABP **12**, comparable to that of the western blot protocol. ABP **12** proved to be appropriate for imaging and quantification of cathepsin activities *ex vivo*. The suitability of the probe **12** to detect cysteine cathepsins in human materials should be investigated in further studies.

## Conclusion

The highly fluorescent probe **12**, containing an irreversibly locked GFP-like fluorophore, was designed, synthesized and evaluated as an inhibitor of human cathepsins L, S, K and B. While the biotechnologically introduced GFP domain becomes an integral part of GFP-tagged fluorescent proteins *ab initio*, fluorescent labeling by **12** arises from a specific protein–ligand recognition process. Nevertheless, such probes also lead to a permanent labeling of the target protein, owing to the covalent, active-site directed modification. Thus, **12** can be considered as a new tool for the chemical introduction of green fluorescence.

## Experimental

### General methods and materials

Compounds **2–5**: NMR spectra were recorded using Bruker Avance III-600 MHz and Bruker DRX-500 MHz instruments. HRMS spectra were recorded using a Bruker Daltonics micrOTOF II mass spectrometer using electrospray ionization. Thin layer chromatography was performed using Merck aluminum sheets. Preparative column chromatography was performed using silica gel 60, 0.060–0.200 mm. Compounds **7–12**: thin layer chromatography was performed using Merck aluminum sheets. Preparative column chromatography was performed using silica gel 60, 0.060–0.200 mm. $^{13}$C NMR (125 MHz) and $^{1}$H NMR (500 MHz) spectra were recorded using a Bruker Avance DRX 500 spectrometer. Elemental analyses were performed using a Vario EL apparatus. LC-DAD chromatograms and ESI-MS spectra were recorded using an Agilent 1100 HPLC system with an Applied Biosystems API-2000 mass spectrometer. For compound **12**, the MS (ESI) spectra were recorded using a Bruker Daltonics micrOTOF-Q spectrometer and the corresponding MS (EI) spectrum with an A.E.I. MS-50 spectrometer. To study the spectral properties of compound **12** as well as its inhibitory activities on human cathepsins, a Varian Cary 50 Bio spectrophotometer and a Monaco Safas spectrofluorometer flx were used. Human cathepsins L and K were obtained from Enzo Life Science, Lörrach, Germany and human cathepsins S and B from Calbiochem, Darmstadt, Germany. The kinetic parameters were calculated using the software GraFit 5. SDS-PAGE (14%, w/v) was performed under reducing conditions using a Power Supply EV243 Consort. Direct in-gel fluorescence detection of cathepsin K was carried out using a Gel iX Imager from Intas, equipped with a dark reader from MoBiTec (excitation wavelength about 420–500 nm), a supersensitive 'scientific grade' CCD-camera and an ethidium bromide filter. For quantification, the ImageJ freeware was used. Coomassie staining was carried out with the 'Page Blue' protein staining solution from Thermo Scientific, St. Leon-Rot, Germany. For western blotting, a polyclonal rabbit anti-cathepsin K antibody from Abcam, Cambridge, UK was used.

**(*Z*)-4-(4-(4-Hydroxybenzylidene)-2-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-1-yl)butanoic acid (2).** Compound **1**[4] (7.35 g, 30.0 mmol) was suspended in ethanol (150 mL), and a mixture of GABA (9.27 g, 89.9 mmol), $Et_3N$ (7.58 g, 74.9 mmol) and water (10 mL) was added. The reaction mixture was stirred for 3 h at room temperature and evaporated. The residue was dissolved in dry DMF (80 mL), $Cs_2CO_3$ (3.26 g, 10.0 mmol) was added, and the mixture was refluxed for 15 min. The solvent was removed *in vacuo*, and the mixture was dissolved in water (100 mL), acidified to pH ~ 5 and extracted with EtOAc (3 × 300 mL). The extract was washed with water (50 mL) and brine (5 × 50 mL) and dried over $Na_2SO_4$ (in the case of the precipitate formation on this or previous step, the precipitates should be collected by filtration and combined with the residues of the subsequent evaporation). The solution was removed *in vacuo*, the product was washed with a cold EtOH–$Et_2O$ (1 : 2) mixture and dry $Et_2O$ to obtain **2** as a yellowish solid (7.60 g, 88% from **1**). mp: 240–243 °C. $^{1}$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 12.10 (bs, 1H), 10.10 (bs, 1H), 8.07 (d, $^{3}J$ = 8.77 Hz, 2H), 6.88 (s, 1H), 6.83 (d, $^{3}J$ = 8.77 Hz, 2H), 3.58 (t, $^{3}J$ = 7.23 Hz, 2H), 2.35 (s, 3H), 2.26 (t, $^{3}J$ = 7.02 Hz, 2H), 1.78 (m, 2H); $^{13}$C NMR (151 MHz, DMSO-$d_6$) $\delta$ 15.14, 23.93, 30.67, 39.23, 115.71, 125.28, 125.60, 134.06, 159.57, 161.74, 169.82, 173.71; HRMS (ESI) $m/z$ = 289.1193 $[M + H]^+$, calcd for $C_{15}H_{16}N_2O_4$ $m/z$ = 289.1188 $[M + H]^+$.

**(*Z*)-*tert*-Butyldiphenylsilyl 4-(4-(4-((*tert*-butyldiphenylsilyl)-oxy)benzylidene)-2-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-1-yl)-butanoate (3).** A solution of compound **2** (7.20 g, 25.0 mmol), *tert*-butyl-diphenyl-silyl chloride (16.5 g, 60.0 mmol), diisopropyl-ethylamine (9.68 g, 74.9 mmol) and imidazole (170 mg,

2.50 mmol) was stirred for 30 h in dry THF (250 mL). The solvent was evaporated, and EtOAc (500 mL) was added. The solution was washed with aqueous HCl (5%, 150 mL), water (2 × 100 mL) and brine (2 × 100 mL) and dried over $Na_2SO_4$. The solvent was evaporated, and the product was purified by column chromatography using EtOAc–heptane (1 : 10) as an eluent to obtain **3** as a yellowish oil (15.3 g, 80%) which was used in the next step without further purification. HRMS (ESI) $m/z$ = 765.3525 $[M + H]^+$, calcd for $C_{47}H_{52}N_2O_4Si_2$ $m/z$ = 765.3544 $[M + H]^+$.

**(*Z*)-Ethyl 4-(4-(2-(difluoroboryl)-4-hydroxybenzylidene)-2-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-1-yl)butanoate (4).** Compound **3** (15.3 g, 20.0 mmol) was dissolved in dry dichloromethane (150 mL). A solution of boron tribromide in dichloromethane (1 M, 100 mL) and molecular sieves (20 g. 4A, 20 g. 3A) was added. The mixture was stirred for 120 h at room temperature, filtered, the sieves were washed with dry dichloromethane (3 × 100 mL), and the solvent was removed *in vacuo*. The residue was dissolved in dry THF (200 mL) and dry ethanol (100 mL) was added. The mixture was stirred for 20 min, and saturated aqueous HF (20 mL) was added. After 30 min, the solvent was evaporated to 1/3 of the initial volume, and the residue was dissolved in EtOAc (400 mL), washed with water (5 × 100 mL), aqueous $K_2CO_3$ (5%, 2 × 100 mL) and brine (2 × 100 mL) and dried over $Na_2SO_4$. The solvent was evaporated, and the product was purified by column chromatography using $CHCl_3$–EtOH (20 : 1) as an eluent to obtain **4** as a yellow solid (2.62 g, 36% from **3**). mp 185–187 °C; $^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 10.22 (bs, 1H), 7.55 (s, 1H), 7.49 (d, $^3J$ = 8.33 Hz, 1H), 7.01 (d, $^4J$ = 2.19 Hz, 1H), 6.74 (dd, $^3J$ = 8.33 Hz, $^4J$ = 2.19 Hz, 1H), 4.02 (q, $^3J$ = 7.02 Hz, 2H), 3.75 (t, $^3J$ = 7.23 Hz, 2H), 2.74 (s, 3H), 2.40 (t, $^3J$ = 7.02 Hz, 2H), 1.89 (m, 2H), 1.16 (t, $^3J$ = 7.02 Hz, 3H); $^{13}$C NMR (151 MHz, DMSO-$d_6$) $\delta$ 12.61, 13.97, 23.02, 30.56, 40.42, 59.91, 115.14, 118.50, 123.87, 124.84, 129.41, 134.33, 161.47, 162.86, 164.11, 172.19; HRMS (ESI) $m/z$ = 403.0984 $[M + K]^+$, calcd for $C_{17}H_{19}BF_2N_2O_4$ $m/z$ = 403.1041 $[M + K]^+$.

**(*Z*)-4-(4-(2-(Difluoroboryl)-4-hydroxybenzylidene)-2-methyl-5-oxo-4,5-dihydro-1*H*-imid-azol-1-yl)butanoic acid (5).** Compound **4** (1.82 g, 5.00 mmol) was dissolved in water (200 mL), and ethanol (50 mL) and NaOH (400 mg, 10.0 mmol) were added. The mixture was stirred for 120 min at room temperature, acidified by aqueous HF, extracted with EtOAc (3 × 100 mL), washed with water (50 mL), brine (2 × 50 mL) and dried over $Na_2SO_4$. The solvent was evaporated, and the product was purified by column chromatography using $CHCl_3$–EtOH (5 : 1) as an eluent to obtain **5** as a yellow solid (1.29 g, 77%). mp: >200 °C under decomposition. $^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 11.88 (bs, 1H), 10.30 (bs, 1H), 7.55 (s, 1H), 7.49 (d, $^3J$ = 8.2 Hz, 1H), 7.00 (d, $^4J$ = 2.2 Hz, 1H), 6.74 (dd, $^3J$ = 8.2 Hz, $^4J$ = 2.2 Hz, 1H), 3.75 (t, $^3J$ = 7.23 Hz, 2H), 2.754 (s, 3H), 2.32 (t, $^3J$ = 7.02 Hz, 2H), 1.85 (m, 2H); $^{13}$C NMR (151 MHz, DMSO-$d_6$) $\delta$ 12.61, 23.13, 30.67, 40.42, 115.12, 118.48, 123.86, 124.83, 129.38, 134.29, 161.47, 162.85, 164.14, 173.71; HRMS (ESI) $m/z$ = 359.0977 $[M + Na]^+$, calcd for $C_{15}H_{15}BF_2N_2O_4$ $m/z$ = 359.0988 $[M + Na]^+$.

**Diethyl phenylsulfonylmethylphosphonate (7).** A mixture of chloromethyl phenyl sulfide (**6**, 5.00 g, 31.5 mmol) and triethyl phosphite (5.23 g, 31.5 mmol) was heated at 130 °C in a sealed tube for 16 h. The resulting solution was distilled under reduced pressure to remove the starting materials. The oily residue was diethyl phenylthiomethylphosphonate (7.69 g, 94%). Diethyl phenylthiomethylphosphonate (7.68 g, 29.5 mmol) was dissolved in ice acetic acid (20 mL). $KMnO_4$ (9.34 g, 59.1 mmol) was dissolved in $H_2O$ (50 mL) and slowly dropped into the reaction mixture. It was stirred for 1.5 h at room temperature, followed by the addition of a sat. $KHSO_3$ solution until the reaction mixture became colorless. The colorless aqueous suspension was extracted with ethyl acetate (3 × 30 mL), washed with brine (30 mL) and evaporated *in vacuo* to obtain **7** as an oily colorless product (6.80 g, 79%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.14 (t, $^3J$ = 7.1 Hz, 6H), 3.93–4.02 (m, 4H), 4.41 (d, $^2J_{PH}$ = 17 Hz, 2H), 7.62–7.95 (m, 5H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 16.07, 16.11, 51.68 (d, $^1J_{PC}$ = 131.3 Hz), 62.45, 62.50, 127.95, 129.16, 133.92, 140.65.

**(*E*)-*tert*-Butyl 3-(phenylsulfonyl)allylcarbamate (8).** Compound **7** (3.20 g, 10.9 mmol) was dissolved in dry THF (20 mL), treated with 60% NaH in mineral oil (0.52 g, 13.1 mmol) and stirred for 30 min at room temperature. *N*-Boc-glycinal (1.74 g, 10.9 mmol) was added, and the resulting reaction mixture was stirred for 2 h at rt. THF was evaporated under reduced pressure, and the oily residue was suspended in $H_2O$. The aqueous suspension was extracted with ethyl acetate (3 × 30 mL), washed with brine (30 mL), dried over $Na_2SO_4$ and evaporated *in vacuo*. The crude product was purified by column chromatography using ethyl acetate–petroleum ether (1 : 1) to obtain **8** as a white solid (1.68 g, 52%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.35 (s, 9H), 3.78 (bs, 2H), 6.65 (d, $^3J$ = 15.4 Hz, 1H), 6.82 (dt, $^3J$ = 15.1 Hz, $^3J$ = 4.6 Hz, 1H), 7.15 (bs, 1H), 7.62–7.85 (m, 5H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 28.24, 78.36, 127.25, 129.71, 130.00, 133.76, 140.47, 144.80, 155.57; MS (ESI) $m/z$ = 315.3 ($[M + NH_4]^+$); Anal. $C_{14}H_{19}NO_4S$ (297.37 g $mol^{-1}$) calcd C 56.55, H 6.44, N 4.71; found C 56.47, H 6.38, N 4.75.

**(*E*)-3-(Phenylsulfonyl)prop-2-en-1-amine hydrochloride (9).** Compound **8** (0.90 g, 3.03 mmol) was dissolved in ethyl acetate (10 mL). AcCl (11.0 g, 140 mmol) was slowly dropped into MeOH (10 mL) under ice-cooling and stirred for 10 min. The solutions were combined and stirred for 10 min at rt. The precipitated white solid was filtered off, and the filtrate was stirred additionally for 30 min at room temperature. The precipitated white solid was again filtered off. The precipitates were combined, washed with *n*-hexane and dried *in vacuo* to obtain **9** as a white solid (0.55 g, 78%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 3.71 (dd, $^3J$ = 5.4 Hz, $^4J$ = 1.6 Hz, 2H), 6.90 (dt, $^3J$ = 15.4 Hz, $^3J$ = 5.4 Hz, 1H), 7.10 (dt, $^3J$ = 15.4 Hz, $^4J$ = 1.6 Hz, 1H), 7.65–7.88 (m, 5H), 8.50 (s, 3H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 38.61, 127.42, 129.84, 133.15, 134.08, 138.88, 139.86.

**(*S*,*E*)-*tert*-Butyl 1-oxo-3-phenyl-1-(3-(phenylsulfonyl)allylamino)-propan-2-ylcarbamate (11).** *N*-Boc-phenylalanine (**10**, 1.21 g, 4.56 mmol) was dissolved in dry THF (20 mL) and cooled to −25 °C. *N*-Methylmorpholine (0.51 g, 5.04 mmol) and isobutyl

chloroformate (0.69 g, 5.05 mmol) were added consecutively. Compound **9** (1.07 g, 4.58 mmol) was dissolved in $H_2O$ (2 mL), treated with 2 N NaOH (3 mL) and added to the reaction mixture when the precipitation of *N*-methylmorpholinium chloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for an additional 90 min. The solvent was evaporated, and the resulting white solid was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% $KHSO_4$ (30 mL) and brine (30 mL). The solvent was dried ($Na_2SO_4$) and evaporated. The oily product was purified by column chromatography on silica gel using ethyl acetate–petroleum ether (1 : 1) as an eluent to obtain **11** as a white solid (1.05 g, 52%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.28 (s, 9H), 2.74 (dd, $^2J$ = 13.6 Hz, $^3J$ = 9.5 Hz, 2H), 2.90 (dd, $^2J$ = 13.7 Hz, $^3J$ = 5.2 Hz, 2H) 3.82–4.02 (m, 2H), 4.06–411 (m, 1H), 6.59 (d, $^3J$ = 15.1 Hz, 1H), 6.85 (dt, $^3J$ = 15.1 Hz, $^3J$ = 4.1 Hz, 1H), 6.99 (d, $^3J$ = 7.9 Hz, 1H), 7.14–7.24 (m, 5H), 7.62–7.83 (m, 5H), 8.22 (t, $^3J$ = 5.7 Hz, 1H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 28.27, 37.24, 56.15, 78.26, 126.36, 127.19, 128.16, 129.25, 129.69, 129.72, 133.70, 138.15, 140.56, 144.65, 155.48, 171.97.

**4-((*Z*)-4-(2-(Difluoroboryl)-4-hydroxybenzylidene)-2-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-1-yl)-*N*-((*S*)-1-oxo-3-phenyl-1-((*E*)-3-(phenylsulfonyl)allylamino)propan-2-yl)butanamide (12).** Compound **11** (1.00 g, 2.25 mmol) was dissolved in ethyl acetate (20 mL). Acetyl chloride (10 mL) was slowly added to MeOH (9 mL) under ice-cooling. The solutions were combined and stirred for 30 min at room temperature. The solvent was evaporated under reduced pressure, and the resulting residue was dissolved in $H_2O$ and adjusted to a pH of ~9 with sat. $NaHCO_3$. It was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with brine (30 mL). The solvent was dried ($Na_2SO_4$) and evaporated to obtain (*S*,*E*)-2-amino-3-phenyl-*N*-(3-(phenylsulfonyl)allyl)propanamide as a colourless oil (0.63 g, 81%). Compound **5** (0.10 g, 0.30 mmol) was dissolved in dry THF (20 mL) and cooled to −25 °C. *N*-Methylmorpholine (30.0 mg, 0.30 mmol) and isobutyl chloroformate (41.0 mg, 0.30 mmol) were added consecutively. (*S*,*E*)-2-Amino-3-phenyl-*N*-(3-(phenylsulfonyl)allyl)propanamide (0.19 g, 0.55 mmol) in dry THF (10 mL) was added to the reaction mixture when the precipitation of *N*-methylmorpholinium chloride occurred. It was allowed to warm to room temperature within 30 min, and stirred for an additional 3.5 h. The solvent was evaporated, and the resulting white solid was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with 10% $KHSO_4$ (30 mL), sat. $NaHCO_3$ (fast with 30 mL) and brine (30 mL). The solvent was dried ($Na_2SO_4$) and evaporated. The brown solid was purified by column chromatography on silica gel using ethyl acetate (twelve 100 mL fractions) and then ethyl acetate–MeOH (5 : 1) as eluents to obtain **12** as a yellow green solid (0.10 g, 50% from **5**). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 1.67–1.77 (m, 2H), 2.08–2.15 (m, 2H), 2.67 (s, 3H), 2.74 (dd, $^2J$ = 13.7 Hz, $^3J$ = 9.3 Hz, 2H), 2.94 (dd, $^2J$ = 13.9 Hz, $^3J$ = 5.7 Hz, 2H), 3.55–3.67 (m, 2H), 3.84–3.95 (m, 2H), 4.40–4.44 (m, 1H), 6.53 (dt, $^3J$ = 15.1 Hz, $^4J$ = 1.9 H, 1H), 6.73 (dd, $^3J$ = 8.2 Hz, $^4J$ = 2.5 Hz, 1H), 6.82 (dt, $^3J$ = 15.3 Hz, $^3J$ = 4.2 Hz, 1H), 7.00 (d, $^4J$ = 2.3 Hz, 1H), 7.12–7.22 (m, 5H), 7.48 (d, $^3J$ = 8.5 Hz, 1H), 7.55 (s, 1H), 7.61–7.82 (m, 5H), 8.27 (t, $^3J$ = 7.9 Hz, 1H), (t, $^3J$ = 5.7 Hz, 1H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 12.76, 14.21, 23.87, 31.97, 37.47, 39.00, 54.42, 115.34, 118.68, 124.00, 125.02, 126.43, 127.19, 128.19, 129.17, 129.67, 129.79, 133.70, 134.56, 137.89, 140.50, 144.37, 161.71, 162.93, 164.18, 171.45; LC-DAD (90% $H_2O$ to 100% MeOH in 20 min, then 100% MeOH to 30 min, DAD 220.0–500.0 nm) $t_r$ = 8.59, 94% purity; MS (ESI) (pos.) $m/z$ = 685.2 ($[M + Na]^+$); MS (ESI) (neg.) 661.2 ($[M - H]^-$); MS (EI) $m/z$ = 662.4 ($M^{\cdot +}$).

### Enzyme kinetics

Compound **12** was tested on human cathepsins L, S, K and B using the conditions described,[41] at five different inhibitor concentrations and in duplicate experiments. The enzymatic reactions were followed for 30 min, see also S2 of the ESI.†

### Spectral properties

To study the spectral properties of the probe **12**, 10 μL of the compound stock solution in DMSO (1 mM) were added to 990 μL of the corresponding solvent (dichloromethane, methanol and water). The absorption spectra were recorded using a Cary 50 Bio spectrophotometer in a range between 800 nm and 200 nm after baseline correction. The emission spectra were performed using a Monaco Safas spektrofluorometer flx in a range between 800 nm and 200 nm after baseline correction, see also S2 of the ESI.†

### Imaging and competition experiments

Thirty μL of a human recombinant cathepsin K (Enzo Life Sciences) stock solution (23 μg mL$^{-1}$ in 50 mM sodium acetate, pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT) were treated with 19 μL of the cathepsin K assay buffer (100 mM sodium citrate, pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS) containing 5 mM DTT and incubated for 30 min at 37 °C. Activated cathepsin K was treated with 0.5 μL of the probe **12** (1 mM) and 0.5 μL of DMSO, and the resulting solution (13.8 μg mL$^{-1}$ of cathepsin K, 10 μM of **12**, 2% DMSO) was incubated for 40 min at 37 °C. Simultaneously, the same amount of activated cathepsin K was preincubated for 10 min with 0.5 μL of the selective, active-site directed cathepsin K inhibitor **13** (10 mM)[40] treated subsequently with 0.5 μL of **12** (1 mM) and incubated for 40 min at 37 °C. The final concentrations were 13.8 μg mL$^{-1}$ of cathepsin K, 100 μM of **13**, 10 μM of **12**, and 2% DMSO. In the control experiments, activated cathepsin K (13.8 μg mL$^{-1}$) and **12** (10 μM) were separately shaken in the presence of 2% DMSO for 40 min at 37 °C in the assay buffer containing 5 mM DTT. Certain amounts (20 μL) of each incubation medium were treated with the reducing running buffer, heated for 4 min at 98 °C and filled into the sample wells of a 14% (w/v) polyacrylamide gel. After separation, the resulting gel was analyzed using a Gel iX Imager from Intas equipped with a transilluminator from MoBiTec (excitation wavelength about 420–500 nm). Subsequently, Coomassie staining was performed.

### Quantification experiment

Incubation medium (a): A volume of 30 μL of a human recombinant cathepsin K (Enzo Life Sciences) stock solution (23 μg $mL^{-1}$ in 50 mM sodium acetate, pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT) was treated with 19 μL of the cathepsin K assay buffer (100 mM sodium citrate, pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS) containing 5 mM DTT and incubated for 30 min at 37 °C. 0.5 μL of the probe **12** (1 mM) and 0.5 μL of DMSO were added, and the resulting solution (13.8 μg $mL^{-1}$ of cathepsin K, 10 μM of **12**, 2% DMSO) was incubated for 40 min at 37 °C. Incubation medium (b): A volume of 5 μL of a human recombinant cathepsin K (Enzo Life Sciences) stock solution (23 μg $mL^{-1}$ in 50 mM sodium acetate, pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT) was treated with 44 μL of the cathepsin K assay buffer (100 mM sodium citrate, pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS) containing 5 mM DTT and incubated for 30 min at 37 °C. A volume of 0.5 μL of the probe **12** (1 mM) and 0.5 μL of DMSO were added, and the resulting solution (2.3 μg $mL^{-1}$ of cathepsin K, 10 μM of **12**, 2% DMSO) was incubated for 40 min at 37 °C. Volumes of 20 μL, 10 μL, and 5 μL of the incubation medium (a) and 15 μL and 7.5 μL of the incubation medium (b) were treated with the reducing running buffer, heated for 4 min at 98 °C and filled into the sample wells of a 14% (w/v) polyacrylamide gel. Electrophoresis was performed as described above.

### Preparation of the cell lysate

Human embryonic kidney cells (HEK) 293 were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin (100 U $mL^{-1}$), streptomycin (100 μg $mL^{-1}$), glutamine (2 mM; all substances from Invitrogen, Karlsruhe, Germany) and fetal bovine serum (10% (v/v); PAA, Linz, Austria) under a humidified atmosphere of 5% $CO_2$ at 37 °C. For the preparation of the cell lysate, confluent HEK293 cells seeded on a 5 $cm^2$ growth area were washed three times with PBS and scraped off with 1 mL of ice-cold PBS buffer followed by homogenization *via* ten aspirations through a 23-gauge needle. Intact cells and nuclei were removed by centrifugation (2000*g*, 5 min, 4 °C). The resulting supernatants were subjected to protein quantification with a Roti-Nanoquant (Carl Roth, Karlsruhe, Germany).

### SDS-PAGE and fluorescence analysis

Incubation medium (a): A volume of 20 μL of a human recombinant cathepsin K (Enzo Life Sciences) stock solution (23 μg $mL^{-1}$ in 50 mM sodium acetate, pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT) were treated with 10 μL of HEK293 cell lysate (3 mg $mL^{-1}$) and 19 μL of the cathepsin K assay buffer (100 mM sodium citrate, pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS) containing 5 mM DTT and incubated for 30 min at 37 °C. A volume of 0.5 μL of the probe **12** (1 mM) and 0.5 μL of DMSO were added, and the resulting solution (9.2 μg $mL^{-1}$ of cathepsin K, 600 μg $mL^{-1}$ of the HEK293 cell lysate, 10 μM of **12**, 2% DMSO) was incubated for 40 min at 37 °C. Incubation medium (b): A volume of 20 μL of a human recombinant cathepsin K (Enzo Life Sciences) stock solution (23 μg $mL^{-1}$ in 50 mM sodium acetate, pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT) was treated with 29 μL of the cathepsin K assay buffer (100 mM sodium citrate, pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS) containing 5 mM DTT, and incubated for 30 min at 37 °C. A volume of 0.5 μL of the probe **12** (1 mM) and 0.5 μL of DMSO were added, and the resulting solution (9.2 μg $mL^{-1}$ of cathepsin K, 10 μM of **12**, 2% DMSO) was incubated for 40 min at 37 °C. Incubation medium (c): A volume of 10 μL of HEK293 cell lysate (3 mg $mL^{-1}$) was treated with 39 μL of the cathepsin K assay buffer (100 mM sodium citrate, pH 5.0, 100 mM NaCl, 1 mM EDTA, 0.01% CHAPS) containing 5 mM DTT and incubated for 30 min at 37 °C. A volume of 0.5 μL of the probe **12** (1 mM) and 0.5 μL of DMSO were added, and the resulting solution (600 μg $mL^{-1}$ of the HEK293 cell lysate, 10 μM of **12**, 2% DMSO) was incubated for 40 min at 37 °C. Certain volumes (40 μL) of the incubation media (a), (b) and (c) were treated with the reducing loading buffer, heated for 4 min at 98 °C and filled into the sample wells of a 14% (w/v) polyacrylamide gel. Electrophoresis was performed as described above.

### Western blotting

After SDS-PAGE, separated proteins (see above) were transferred onto the nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Nitrocellulose membranes were blocked by incubation with 5% (w/v) non-fat dried milk (Carl Roth) in PBST (PBS with 0.2% (v/v) Tween-20) for 1 h at room temperature. Antigens were detected by incubation with polyclonal rabbit anti-cathepsin K antibody (Abcam ab19027; 1 : 66) for 1 h at room temperature. After three washing steps with PBST, primary antibody was incubated with species specific alkaline phosphatase-conjugated secondary antibody diluted to 1 : 5000 in PBST (goat anti-rabbit IgG; Calbiochem) followed by three washing steps with PBST and one washing step with PBS. The resulting antigen–antibody complexes were detected with NBT/BCIP (nitro tetrazolium blue chloride/5-bromo-4-chloro-3-indolyl phosphate, Sigma-Aldrich, Saint Louis, USA).

### Coomassie staining

Solution (a): A volume of 20 μL of a human recombinant cathepsin K (Enzo Life Sciences) stock solution (23 μg $mL^{-1}$ in 50 mM sodium acetate, pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT) was treated with 10 μL of a cell lysate from the HEK293 cell line (3 mg $mL^{-1}$) and 20 μL of demineralized water to obtain a solution containing 9.2 μg $mL^{-1}$ of cathepsin K and 600 μg $mL^{-1}$ of the HEK293 cell lysate. Solution (b): A volume of 20 μL of a human recombinant cathepsin K (Enzo Life Sciences) stock solution (23 μg $mL^{-1}$ in 50 mM sodium acetate, pH 5.5, 50 mM NaCl, 0.5 mM EDTA, 5 mM DTT) was treated with 30 μL of demineralized water to obtain a solution containing 9.2 μg $mL^{-1}$ of cathepsin K. Solution (c): A volume of 10 μL of a lysate from the HEK293 cell line (3 mg $mL^{-1}$) was treated with 40 μL of demineralized water to obtain a solution containing 600 μg $mL^{-1}$ of the HEK293 cell lysate. Certain

volumes (40 μL) of solutions (a), (b) and (c) were treated with the reducing loading buffer, heated for 4 min at 98 °C and filled into the sample wells of a 14% (w/v) polyacrylamide gel. Electrophoresis was performed, and the resulting gel was stained using the protein staining solution 'Page Blue' from Thermo Scientific.

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