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Convenient Synthesis of Multifunctional EDTA-Based Chiral Metal Chelates Substituted with an *S*-Mesylcysteine

Andrei Leonov,[a] Brigitte Voigt,[a] Fernando Rodriguez-Castañeda,[a] Peyman Sakhaii,[b] and Christian Griesinger*[a]

Abstract: We describe the synthetic route to ethylenediaminetetraacetic acid (EDTA) derivatives that can be attached to surface-exposed thiol functional groups of cysteine residues in proteins, via a methylthiosulfonate moiety that is connected in a stereochemically unique way to the C-1 carbon atom of EDTA. Such compounds can be used to align proteins in solution without the need to add liquid crystalline media, and are, therefore, of great interest for the NMR spectroscopic analysis of biomolecules. The binding constant for the paramagnetic tag to lanthanide ions was determined by measuring luminescence. For the Tb^{+3}–ligand complex, a K_b value of $6.5\times10^{17}\,M^{-1}$ was obtained. This value is in excellent agreement with literature values for the related EDTA compound. In addition, it could be shown that there is no significant reduction in the luminescence intensity upon addition of a 10^4 excess of Ca^{2+} ions, indicating that this paramagnetic tag is compatible with buffers containing high concentrations of divalent alkaline earth ions.

Keywords: EDTA · lanthanides · NMR spectroscopy · protein tagging · synthetic methods

Introduction

The ability of ethylenediaminetetraacetic acid (EDTA) and its derivatives to form stable complexes with a variety of metal ions allows for their use as probes in various areas of chemistry, biology, and medicine. For example, derivatives of EDTA–Ce^{IV} are effective cleavage reagents for oligonucleotides[1] and are, therefore, used to investigate oligonucleotide structure and function. Protein and nucleic acid cleavage experiments have been used to probe the tertiary structure of biomolecules and drug binding sites, as well as to identify folding intermediates and to investigate interactions with other macromolecules.[2,3,4] Alternatively, complexation of radioactive metals by EDTA,[5,6] as well as DOTA, DTPA, and their derivatives,[7] has found extensive use in medicine.

One of the recently applied new tools of structural chemistry and biology is the use of residual dipolar couplings (RDC) as structural restraints.[8,9,10] Although dipolar couplings assume values of several kHz in the solid state, they are normally completely averaged out in isotropic solution. They can be recovered by orienting the molecule anisotropically in solution. In principle, there are two ways to align proteins; either by putting them into an anisotropic solution, such as dilute liquid crystals,[7] or by changing the molecule such that it aligns by itself in the external magnetic field.[8] Anisotropic media can be made from a variety of sources, as recently reviewed.[11] However, these alignment media are sometimes not compatible with the compound under investigation, or they may even bind to the molecule of interest, thereby precluding its study. Consequently, alignment by attaching a paramagnetic tag to the molecule becomes attractive. The tag must host a paramagnetic species that has a fast-relaxing electron spin, is highly anisotropic, does not interfere with the protein, and has a short linker to minimize averaging of the anisotropy of the alignment tensor.

Lanthanides have been used successfully in aqueous solutions for the alignment of proteins that have a metal binding site.[12,13] The clear limitation of this concept is that it can be applied only to metal-binding proteins; however, due to the

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rigidity of the binding site with respect to the remainder of the protein, strong alignment with large dipolar couplings could be achieved, and coupling constants of up to 40 Hz were observed.[13] Lanthanide binding sites have been introduced into proteins by attaching either a calmodulin half-domain[14] or the EDTA compound **1** (Figure 1).[15,16] A lanthanide complex, named CLaNP (caged lanthanide NMR probe), has been developed recently for the characterization of proteins by paramagnetic NMR spectroscopy;[17] however, it yields up to five sets of signals due to uncontrolled stereochemistry. The feasibility of the tag proposed in the present study, which permits alignment yielding only one set of signals, has been recently shown.[18]

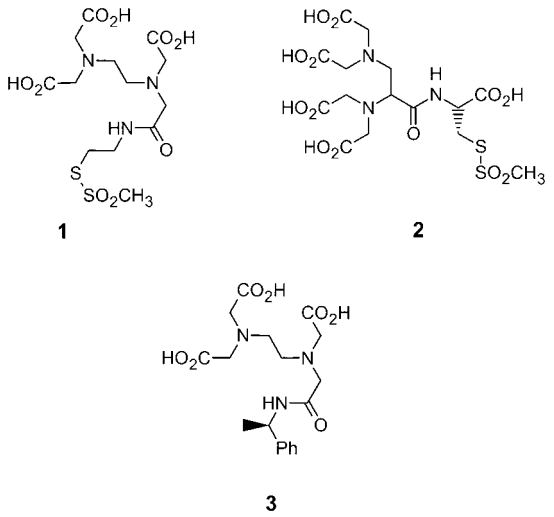


Figure 1. Structures of the EDTA-based metal chelates.

The present work describes the synthesis and binding affinities of a novel, stereochemically unique EDTA-based tag that can be attached to a broad range of different proteins containing one accessible cysteine residue. If no cysteine residue is present in the native protein, it can be introduced by performing site-directed mutagenesis. The proposed tag has a high affinity to lanthanides. In addition, it reacts readily with cysteine side chains in proteins, because of the mesyl leaving group of the methanethiosulfonyl (MTS) moiety, this approach has been used before to attach fluorescent dyes or spin labels to proteins for electron paramagnetic resonance (EPR) or relaxation studies. We used EDTA to retain the high affinity to lanthanides,[19,20,21] and we designed the tag so that no chirality is introduced upon complexation of the tag by the lanthanide. In addition, the linker between the cysteine residue and the tag should be as short as possible to minimize conformational averaging and, therefore, to maximize dipolar couplings.

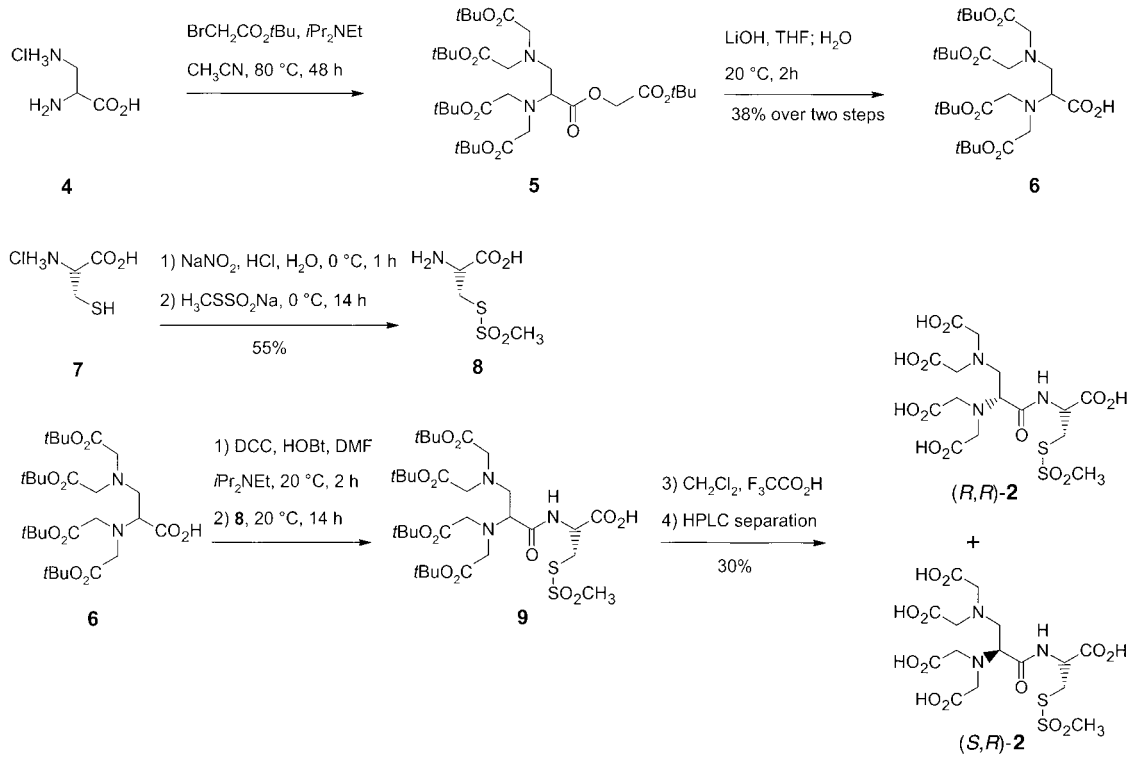
The most difficult step is obtaining tags that do not assume different stereoisomers upon complexation of the lanthanide.[18] This was overcome by attaching the linker with defined stereochemistry to the C-1 position of the EDTA, as shown for compounds **2** (Figure 1). Indeed, compound **1** does not have this property and, therefore, shows different alignment tensors when attached to a protein. Attachment of compound **1** (Figure 1) to a cysteine mutant of the protein trigger factor[22] yields a doubling of the protein resonances upon addition of dysprosium metal ions in a J_{HN}-coupled $^{1}H,^{15}N$-heteronuclear single quantum coherence (HSQC) spectrum, which is due to the epimeric lanthanide complexes, as described in detail in reference [18]. This problem has been demonstrated especially clearly for the complex of compound **3** with a lanthanide metal,[18] for which the chiral nitrogen could be identified as the source of the chirality of the lanthanide complex.

Results and Discussion

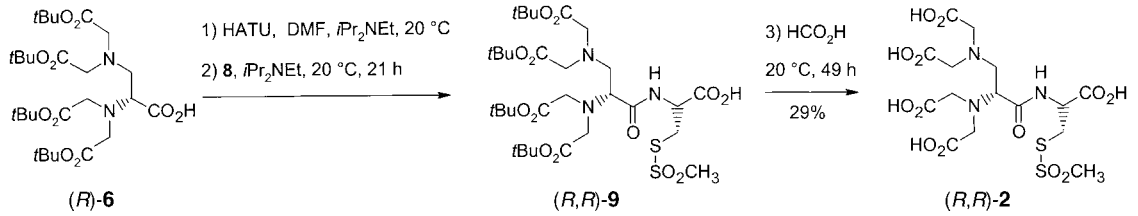
Compound **2** was synthesized in five steps (Scheme 1). The acid **6** was prepared according to a modification of the method of Arya et al.,[23] both in the racemic as well as in the enantiomerically pure way. In the first step, commercially available racemic 2,3-diaminopropionic acid hydrochloride **4** was exhaustively alkylated with *tert*-butyl bromoacetate in the presence of *N*-ethyldiisopropylamine to provide the pentaester **5**. The literature conditions[23] for preparing compound **6** were the incubation of **5** at 100 °C in dimethylformamide in the presence of a 1 M equivalent of sodium thiophenoxide. Under these reaction conditions, we obtained only traces of compound **6** in the mixture, and this was very difficult to purify. Therefore, we changed the deprotecting agent. The pentaester **5** was selectively saponified without purification by using lithium hydroxide to provide the carboxylic acid **6** in 38 % yield for the two steps.

The preparation of the second building block **8** (Scheme 1) has been described by Block and Weidner.[24] However, the synthetic route utilizes the commercially unavailable reactant methanethiosulfonic acid *S*-trifluoromethyl ester. This ester can be synthesized using the toxic and commercially unavailable reactant trifluoromethanesulfenyl chloride. To avoid usage of toxic compounds, a reliable procedure on a gram-scale for the synthesis of (*R*)-*S*-mesylcysteine **8** was found by applying the Hart protocol.[25] Treatment of (*R*)-cysteine hydrochloride hydrate **7** in water with sodium nitrite and hydrochloric acid afforded the unstable *S*-nitrosocysteine in situ. The crude reaction mixture was treated with sodium methanesulfinate and the target compound **8** was isolated in 55 % yield by simple filtration. The racemic acid **6** was then coupled to (*R*)-*S*-mesylcysteine **8** in dimethylformamide (DMF) by using standard HOBt/DCC (*N*-hydroxybenzotriazole/*N*,*N*′-dicyclohexylcarbodiimide) techniques,[26] followed by deprotection of the diastereomeric mixture **9** by trifluoroacetic acid without purification. The mixture of diastereomeric compounds **2** was subjected to chromatographic separation (HPLC) to afford pure diastereomers as two peaks with retention times of 9.87 min for peak 1 and 10.53 min for peak 2, in 30 % yield.

The absolute configurations of the 2,3-diaminopropionic fragments of the two diastereomeric compounds **2** were determined as follows (Scheme 2). The optically active (*R*)-



Scheme 1. Synthesis of the EDTA-based metal chelates.



Scheme 2. Synthesis of (*R*,*R*)-**2** for the stereochemical determination of peaks 1 and 2.

2,3-bis[di(*tert*-butyloxycarbonylmethyl)amino]propionic acid (*R*)-**6** was synthesized from commercially available (*R*)-2,3-diaminopropionic acid hydrochloride by the procedure used for the synthesis of racemic acid **6**. The resulting product was then coupled to (*R*)-*S*-mesylcysteine **8** by using HATU (*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide) as the condensation reagent, to give the diastereomer (*R*,*R*)-**2** in the same yield after deprotection with formic acid followed by HPLC purification. This compound with known configuration of both stereogenic centers was used as a standard for the HPLC analysis of the two diastereomers (*R*,*R*)-**2** and (*S*,*R*)-**2** synthesized from racemic acid **6**. By comparing the HPLC traces we can conclude that peak 1 has the same retention time as (*R*,*R*)-**2**. The $^{1}H,^{13}C$ NMR spectroscopy and MS data of peak 1 were in agreement with data from (*R*,*R*)-**2**. Thus, peak 2 is for (*S*,*R*)-**2**.

Both the (*R*,*R*)-**2** (*epi*-**3** in reference [18]) and the (*S*,*R*)-**2** (**3** in reference[18]) were used for the tagging reaction of the cysteine-mutant trigger factor protein and, due to the stereochemical purity, yielded only one set of signals in the NMR spectrum. The alignment tensors were rotated relative to each other, thus inducing linearly independent dipolar couplings, as well as pseudocontact shifts.

The novel tag molecules, (*R*,*R*)-**2** and (*S*,*R*)-**2**, provide only one set of signals after complexation with lanthanide. This is because strong binding of the lanthanide does not induce additional chirality in the molecule, due to the nitrogen becoming tetrahedral. The two nitrogen atoms have two identical ligands, namely the carboxymethyl groups. Even when the nitrogen becomes locked in the tetrahedral configuration upon binding of the metal, it does not become chiral, because the two carboxymethyl groups remain as two identical ligands. This is the key difference to compound **1** (Figure 1), which becomes chiral at the nitrogen to which the linker is attached, because there are four different ligands: the carboxymethyl, the aminocarbonyl with the sulfur linker attached, the lanthanide, and the 2-[di(carboxymethyl)amino]ethyl. The two chiral conformations of **1** are shown in Figure 2.

The feasibility of the tag for various biomolecular systems could be established by using ubiquitin and calmodulin. An

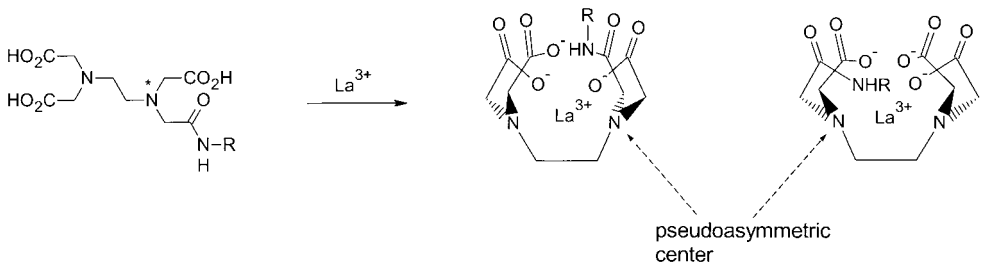


Figure 2. The two chiral conformations of compound **1** upon complexation of the EDTA part with a lanthanide. If the residue R has, for example, the chirality *R*, two diastereomeric compounds are formed upon complexation of the lanthanide.

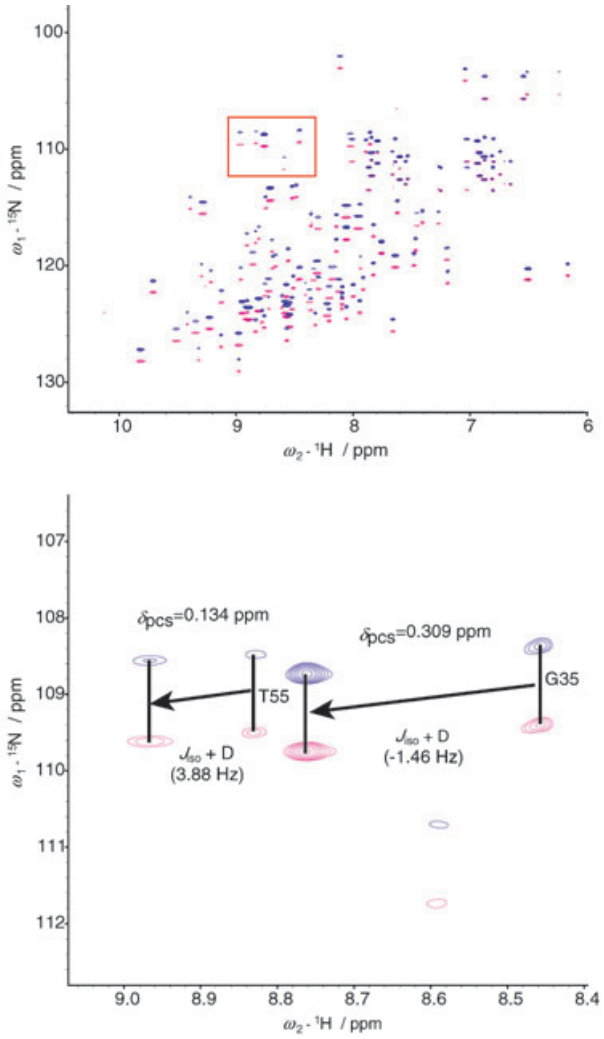


Figure 3. Superimposition of the properly combined IPAP spectra of the T12C mutant of ubiquitin tagged with (*S*,*R*)-**2** and complexed with Tb^{3+}. The coupling constants and the pseudocontact shifts range between −6 and 8 Hz, and −0.93 and 0.549 ppm, respectively.

in-phase/anti-phase (IPAP) HSQC spectrum of the ubiquitin T12C mutant attached to (*S*,*R*)-**2** (**3** in reference[18]) complexed with Tb^{3+} is shown in Figure 3. The doublets in ω_1 for the tagged species are clean peaks, so that there is only one alignment tensor induced for this molecule through the paramagnetic tagging by either of the two diastereomeric

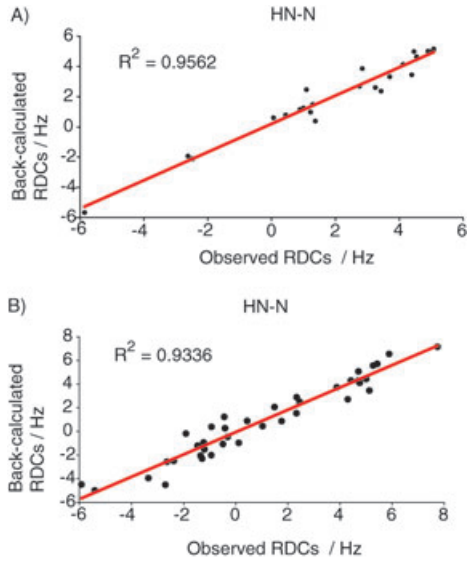
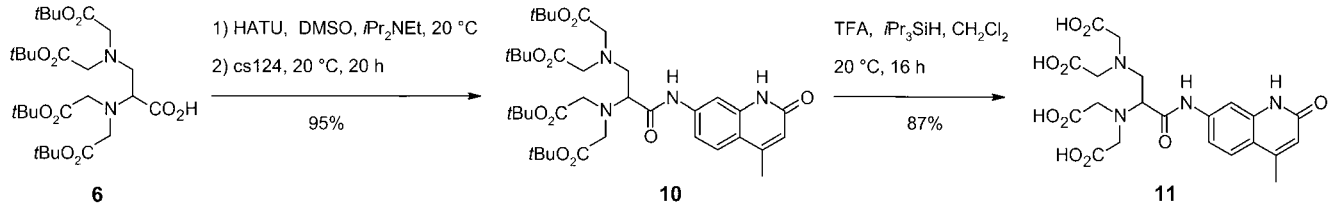


Figure 4. Correlation plots of the observed residual dipolar couplings (RDC) versus the calculated RDC values from the NMR data of human ubiquitin (PDB 1D3Z); A) ubiquitin T12C–*R*,*R*-**2**–Tb^{3+} and B) ubiquitin T12C–*S*,*R*-**2**–Tb^{3+}.

tags. A total of 38 and 24 residual dipolar couplings ranging from −6 to 8 Hz were measured, and a correlation plot of the measured versus expected dipolar couplings for this mutant is shown in Figure 4. The pseudocontact shifts assumed values ranging from −0.93 to 0.549 ppm. The two tensors have an angle in five dimensional space of 65°, which is similar to the angle measured in the case of trigger factor.[18]

The behavior of tag **2** is different to that of the commercially available compound **1**. The latter showed doubling of the resonances upon complexation with the lanthanide[18] due to formation of the new chiral center, as shown in Figure 2. Together with the chiral protein, two diastereomeric compounds are formed that show different physicochemical properties and, therefore, also different alignment tensors.

To check the versatility of the tag, its binding constant to lanthanide ions was determined by measuring luminescence based on the energy transfer of the laser dye cs124 (7-amino-4-methylquinolin-2(1*H*)-one)[27] to a proximal lanthanide. For this purpose, the racemic acid **6** was attached to



Scheme 3. Synthesis of complexes **11** for the determination of stability constants.

the laser dye cs124 by using HATU as coupling reagent to give amide **10**. The subsequent deprotection under acidic conditions and HPLC purification afforded **11** in 83% total yield (Scheme 3).

The luminescence was measured by using a 1:1 stoichiometry of lanthanide ion and tag **11**. The binding constant K_b for the tag was determined relative to the binding affinity of EDTA to terbium ($K_b = 2\times10^{17}\,\text{M}^{-1}$) by titrating the complex with increasing amounts of EDTA according to Oser's protocol.[28] A 14 μM solution of the **11**–Tb^{3+} complex gave a fluorescence value of 68 units at the highest emission line at 546 nm in the buffer used for the NMR spectroscopy measurements (50 mM MOPS (3-(*N*-morpholino)propanesulfonic acid), 50 mM NaCl, pH 6.8). Upon increasing the EDTA concentrations, a linear reduction in the luminescence was observed. The K_b value obtained from the **11**–Tb^{3+} complex was $6.5\times10^{17}\,\text{M}^{-1}$, confirming that the binding of terbium and other similar lanthanide ions is of the same order of magnitude as the binding to EDTA. We suggest that the binding affinity of tag **11** to the Tb^{3+} ion is three times higher than the affinity to EDTA, because of the stabilizing effect of the additional amide group. In addition, no significant reduction in the luminescence intensity was seen upon addition of a 10^4 excess of Ca^{2+} ions, indicating that this paramagnetic tag is compatible with buffers that contain high concentrations of divalent alkaline earth ions.

Conclusion

We have described an efficient synthesis of two diastereomeric EDTA-based tag molecules that can be attached to the cysteine residue of a protein. The tags are stereochemically pure, so that they induce only one set of signals once loaded with a lanthanide. The two diastereomers induce linearly independent alignment tensors, which is advantageous for the determination of protein structure. The binding affinity is six times higher than that for EDTA, which facilitates the use of the tag even for metal-binding proteins, such as calmodulin.

Experimental Section

General: Starting materials and solvents were commercially available unless otherwise noted. All reactions were conducted under an atmosphere of argon. Melting points were determined by using a Stuart Scientific (BIBBY, UK) capillary apparatus and are uncorrected. Thin layer chromatography (TLC): Macherey–Nagel precoated sheets, 0.25 mm Polygram SIL G/UV_{254} plates, detection with UV and/or by charring with 10 wt% ethanolic phosphomolybdic acid reagent followed by heating at 200 °C. Flash column chromatography was performed by using Merck silica gel 60 (0.015–0.040 mm). Analytical and preparative high performance liquid chromatography (HPLC) were performed by using a Waters HPLC system with a Waters 996 Photodiode Array Detector. All separations involved a mobile phase of 0.1% trifluoroacetic acid (TFA) (v/v) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). HPLC was performed by using a reversed-phase (RP) column Eurospher RP 18, 100 Å, 5 μm, 250×4.6 mm (analytical) and 250×16 mm (preparative) at flow rates of 1 mL min^{-1} (analytical) and 7 mL min^{-1} (preparative). Electrospray ionization mass spectrometry (ESI-MS) and liquid chromatography/mass spectrometry (LC/MS) analyses were obtained by using a Waters Micromass ZQ 4000 mass spectrometer in conjunction with the Waters HPLC apparatus described above. High-resolution mass spectra (HRMS) were recorded by using an MS Finnigan LCQ (Ion-Trapp) mass spectrometer and are reported in *m*/*z*. Optical rotations were recorded by using a Perkin–Elmer 241 digital polarimeter 1 dm cell and are given in units of 10^{-1} deg cm^3 g^{-1} dm^{-1}.The terbium luminescence measurements were obtained by using a Varian Cary Eclipse spectrofluorimeter. Elemental analyses were performed at the Microanalytical Laboratory of the Institute of Organic Chemistry, Göttingen, Germany. NMR spectra were recorded at a temperature of 298 K by using a 400 MHz Bruker Avance spectrometer (Bruker AG, Rheinstetten, Germany) equipped with a TXI HCN z-gradient probes header. The IPAP ^{1}H,^{15}N NMR spectra were recorded at a temperature of 303 K by using a 900 MHz Bruker Avance spectrometer. The lyophilized ^{15}N-tagged ((*R*,*R*)-**2** or (*S*,*R*)-**2**) ubiquitin was dissolved in the appropriate buffer (50 mM MOPS buffer, 50 mM NaCl, pH 6.8) with 10% (v/v) D_2O for the lock signal. For NMR spectroscopy measurements, the 280 μl sample was introduced into a Shigemi NMR spectroscopy sample tube of 5 mm diameter with an adaptor-piston inserted upward (BMS-3). The ^{1}H,^{15}N NMR spectra were obtained with spectral widths of 15 ppm (^{1}H) and 30 ppm (^{15}N). The carrier positions were 115 ppm for ^{15}N and 4.7 ppm for ^{1}H. The IPAP-HSQC experiments were acquired with 2048 and 512 complex points in t_2 and t_1, respectively. The processing was performed by using NMR Pipe.[29] Zero filling in the indirect dimension to 8192 points led to a digital resolution of 0.33 Hz/point. All other spectra were processed by using XWINNMR 3.1 (Bruker AG, Karlsruhe, Germany). ^{1}H NMR chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) ($\delta = 0.00$ in $CDCl_3$), [D_5]DMSO ($\delta = 2.49$ in [D_6]DMSO), and 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt (DSS) ($\delta = 0.00$ in D_2O) as internal standards. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, br = broadened, m = multiplet), coupling constants (*J*, given in Hz), integration. ^{13}C NMR chemical shifts (δ) are reported in parts per million (ppm) relative to $CDCl_3$ ($\delta = 77.0$), [D_6]DMSO ($\delta = 39.7$), and DSS ($\delta = 0.00$ in D_2O) as internal standards. The following two dimensional (2D) experiments were used for resonance assignments: 2D-[^{13}C,^{1}H]-HSQC (heteronuclear single quantum coherence),[30] 2D-[^{13}C,^{1}H]-HMBC (heteronuclear multiple bond correlation).[31]

2,3-Bis[di(*tert*-butoxycarbonylmethyl)amino] propionic acid (6): *tert*-Butyl bromoacetate (6.83 mL, 9.02 g, 46.2 mmol) was added to a suspension of 2,3-diaminopropionic acid hydrochloride **4** (1.00 g, 7.1 mmol) and *N*-ethyldiisopropylamine (DIPEA) (8.52 mL, 6.44 g, 49.8 mmol) in aceto-

nitrile (50 mL). After stirring at reflux for 16 h, additional *tert*-butyl bromoacetate (1.05 mL, 1.39 g, 7.1 mmol), and DIPEA (1.22 mL, 0.92 g, 7.1 mmol) were added. The reaction mixture was refluxed for 32 h, cooled, and the solvent was removed under reduced pressure. The residue was mixed with diethyl ether (100 mL), refluxed for 1 h, cooled, and filtered. The filtrate was washed with 0.1 M phosphate buffer pH 2.0 (3× 40 mL), dried (molecular sieve 0.4 nm powder), and concentrated under reduced pressure. The residue was dissolved in tetrahydrofuran (60 mL), and lithium hydroxide (1 M solution in water, 7.1 mL, 7.1 mmol) was added. After stirring at room temperature for 3 h, additional LiOH (1 M solution in water, 3.6 mL, 3.6 mmol) was added, and stirring was continued for 2 h. Acetic acid (0.63 mL, 0.66 g, 11 mmol) was added and the solvent was removed under reduced pressure. Phosphate buffer (0.1 M, pH 2.0, 40 mL) was added and the mixture was extracted with chloroform (3×10 mL). The organic phase was dried (molecular sieve 0.4 nm powder) and concentrated under reduced pressure. The residue was purified by performing silica gel chromatography (10:1 chloroform/methanol, $R_f = 0.4$ (streak)) to afford **6** (1.52 g, 2.7 mmol, 38%) as a slightly yellow oil. ^{1}H NMR and MS data were in agreement with literature data.[22,25] ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 28.47$ (12×C, 4×(CH_3)$_3$C), 55.04 (2× C, CH_2CO_2tBu), 55.28 (C-3), 57.03 (2×C, CH_2CO_2tBu), 63.45 (C-2), 82.11 (4×C, (CH_3)$_3C$), 170.43 (2×C, CH_2CO_2tBu), 171.85 (2×C, CH_2CO_2tBu), 173.62 ppm (C-1).

(*R*)-2,3-Bis[di(*tert*-butoxycarbonylmethyl)amino] propionic acid ((*R*)-6): In a similar manner, (*R*)-2,3-diaminopropionic acid hydrochloride (2.00 g, 14.2 mmol) gave *R*-**6** (3.31 g, 5.9 mmol, 41%) as a white solid. M.p. 66–69 °C; $[\alpha]_D^{20} = +15.7$ ($c = 1.00$ in methanol); ^{1}H NMR (400 MHz, $CDCl_3$, HSQC, HMBC): $\delta = 1.45$ (s, 36H; 4×OtBu), 3.12 (d, $J = 7.4$ Hz, 2H; H-3), 3.45 (d, $J = 17.1$ Hz, 2H; CH_2CO_2tBu), 3.50 (d, $J = 17.6$ Hz, 2H; CH_2CO_2tBu), 3.56 (d, $J = 17.1$ Hz, 2H; CH_2CO_2tBu), 3.59 (d, $J = 17.6$ Hz, 2H; CH_2CO_2tBu), 3.72 (t, $J = 7.4$ Hz, 1H; H-2), 12.20 ppm (br s, 1H; CO_2H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 28.45$ (6×C, 2×(CH_3)$_3$C), 28.50 (6×C, 2×(CH_3)$_3$C), 55.04 (2×C, CH_2CO_2tBu), 55.28 (C-3), 57.03 (2×C, CH_2CO_2tBu), 63.45 (C-2), 82.08 (2×C, (CH_3)$_3C$), 82.16 (2×C, (CH_3)$_3C$), 170.43 (2×C, CH_2CO_2tBu), 171.85 (2×C, CH_2CO_2tBu), 173.62 ppm (C-1); ESI-MS m/z (MeOH, positive mode): calcd for $C_{27}H_{49}N_2O_{10}$ $[M+H]^+$: 561.68; found: 561.30; elemental analysis calcd (%) for $C_{27}H_{48}N_2O_{10}\cdot 0.5\,CHCl_3$ (620.37): C 53.24, H 7.88; found: C 53.32, H 8.02.

(*R*)-*S*-Mesylcysteine (8): An ice-cold solution of sodium nitrite (1.38 g, 20.0 mmol) in water (10 mL) was slowly added dropwise at 5 °C to a stirred solution of (*R*)-cysteine hydrochloride monohydrate **7** (3.51 g, 20.0 mmol) in 2 N HCl (20 mL). After 1 h, the deep red solution was treated with sodium methanesulfinate (4.08 g, 40.0 mmol). After stirring at 5 °C for 2 h, additional sodium methanesulfinate (1.09 g, 10.0 mmol) was added and the reaction mixture was stirred for 12 h at 5 °C. The resulting precipitate was filtered off, then washed with ice-cold water (2× 5 mL) and diethyl ether (2×5 mL). Drying in vacuo at 20 °C afforded pure (*R*)-*S*-mesylcysteine **8** (2.21 g, 11.0 mmol, 55%) as a white solid. M.p. 146–149 °C (decomp); $[\alpha]_D^{20} = -61.7$ ($c = 1.03$ in water); ^{1}H NMR (400 MHz, D_2O, HSQC, HMBC): $\delta = 3.56$ (s, 3H; CH_3), 3.71 (dd, $J =$ 15.8, 7.1 Hz, 1H; H_A-3), 3.79 (dd, $J = 15.8$, 4.7 Hz, 1H; H_B-3), 4.18 ppm (dd, $J = 7.1$, 4.7 Hz, 1H; H-2); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 35.81$ (C-3), 50.73 (CH_3), 53.44 (C-2), 170.07 ppm (C-1); ESI-HRMS (MeOH, positive mode): calcd for $C_4H_{10}NO_4S_2$ $[M+H]^+$: 200.00458; found: 200.00469; elemental analysis calcd (%) for $C_4H_9NO_4S_2$ (199.25): C 24.11, H 4.55; found: C 24.14, H 4.36.

***N*-[(*R*)-2,3-Bis[di(carboxymethyl)amino]propionyl]-*S*-mesyl-(*R*)-cysteine ((*R*,*R*)-2) and *N*-[(*S*)-2,3-bis[di(carboxymethyl)amino]propionyl]-*S*-mesyl-(*R*)-cysteine ((*S*,*R*)-2)**: DCC (1 M solution in dichloromethane, 500 μL, 500 μmol) was added at 5 °C to a stirred solution of racemic acid **6** (280 mg, 500 μmol) and HOBt·H_2O (77 mg, 500 μmol) in dichloromethane (3 mL) and DMF (1.5 mL). The reaction mixture was stirred at 5 °C for 1 h and at 20 °C for 2 h, followed by filtration. The filtrate was evaporated to dryness, and the residue was redissolved in dichloromethane (2 mL) and DMF (5 mL). (*R*)-*S*-Mesylcysteine **8** (141 mg, 707 μmol) and DIPEA (513 μL, 388 mg, 3.0 mmol) were added and the reaction mixture was stirred 14 h at 20 °C. The solvent was removed under reduced pressure, the residue redissolved in ethyl acetate (10 mL), washed with 0.1 M phosphate buffer pH 2.0 (2×5 mL), dried (molecular sieve 0.4 nm powder), and concentrated under reduced pressure. The residue was dissolved in a deprotection mixture (CH_2Cl_2/TFA, 1:1, v/v, 20 mL). After stirring for 20 h the solvent was removed by evaporation under reduced pressure. The oily residue was redissolved in acetonitrile/water (1:1, 30 mL), filtered (0.45 μm nylon filter), and lyophilized to give a colorless foam. The crude diastereomeric mixture was separated by RP-HPLC (gradient 0–10% solvent B in 30 min, RT ~10–12 min) to afford both diasteromers. The combined fractions showing pure material were lyophilized, affording peak 1 (*R*,*R*-**2**) (38 mg, 74 μmol, 15%; analytical HPLC 0–10% solvent B in 30 min, retention time (RT) 9.87 min) and peak 2 (*S*,*R*-**2**) (39 mg, 75 μmol, 15%; analytical HPLC 0–10% solvent B in 30 min, RT 10.53 min).

N-*[(*R*)-2,3-Bis[di(carboxymethyl)amino]propionyl]*-S-*mesyl-(*R*)-cysteine (peak 1,* (R,R)-*2)*: ^{1}H NMR (400 MHz, D_2O, HSQC, HMBC): $\delta = 3.44$ (s, 3H; CH_3), 3.56 (dd, $J = 14.6$, 7.0 Hz, 1H; Cys-H_A-3), 3.62 (m, 1H; H_A-3′), 3.65 (dd, $J = 14.6$, 4.7 Hz, 1H; Cys-H_B-3), 3.69 (m, 1H; H_B-3′), 3.70 (s, 4H; 2×CH_2CO_2H), 4.04 (dd, $J = 10.2$, 4.9 Hz, 1H; H-2′), 4.18 (d, $J =$ 17.3 Hz, 2H; CH_2CO_2H), 4.21 (d, $J = 17.3$ Hz, 2H; CH_2CO_2H), 4.78 ppm (dd, $J = 7.0$, 4.7 Hz, 1H; Cys-H-2); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 35.94$ (Cys-C-3), 49.28 (CH_3), 51.66 (2×C, CH_2CO_2H), 51.82 (Cys-C-2), 54.01 (C-3′), 55.62 (2×C, CH_2CO_2H), 59.29 (C-2′), 168.38 (2×C, CH_2CO_2H), 169.67 (C-1′), 171.64 (Cys-C-1), 174.99 ppm (2×C, CH_2CO_2H); ESI-HRMS (MeOH, positive mode): calcd for $C_{15}H_{24}N_3O_{13}S_2$ $[M+H]^+$: 518.07451; found: 518.07434.

N-*[(*S*)-2,3-Bis[di(carboxymethyl)amino]propionyl]*-S-*mesyl-(*R*)-cysteine (peak 2,* (S,R)-*2)*: ^{1}H NMR (400 MHz, D_2O, HSQC, HMBC): $\delta = 3.47$ (s, 3H; CH_3), 3.57 (dd, $J = 14.7$, 7.6 Hz, 1H; Cys-H_A-3), 3.64 (dd, $J = 10.0$, 3.8 Hz, 1H; H_A-3′), 3.67 (d, $J = 18.1$ Hz, 2H; CH_2CO_2H), 3.71 (dd, $J =$ 10.0, 5.2 Hz, 1H; H_B-3′), 3.72 (d, $J = 18.1$ Hz, 2H; CH_2CO_2H), 3.73 (dd, $J = 14.7$, 4.4 Hz, 1H; Cys-H_B-3), 4.05 (dd, $J = 10.0$, 5.2 Hz, 1H; H-2′), 4.12 (d, $J = 17.1$ Hz, 2H; CH_2CO_2H), 4.16 (d, $J = 17.1$ Hz, 2H; CH_2CO_2H), 4.76 ppm (dd, $J = 7.6$, 4.4 Hz, 1H; Cys-H-2); ^{13}C NMR (100.6 MHz, D_2O): $\delta = 36.44$ (Cys-C-3), 49.83 (CH_3), 52.25 (2×C, CH_2CO_2H), 52.34 (Cys-C-2), 54.37 (C-3′), 56.28 (2×C, CH_2CO_2H), 59.73 (C-2′), 169.12 (2× C, CH_2CO_2H), 170.29 (C-1′), 172.41 (Cys-C-1), 175.29 ppm (2×C, CH_2CO_2H); ESI-HRMS (MeOH, positive mode): calcd for $C_{15}H_{24}N_3O_{13}S_2$ $[M+H]^+$: 518.07451; found: 518.07468.

***N*-[(*R*)-2,3-Bis[di(carboxymethyl)amino]propionyl]-*S*-mesyl-(*R*)-cysteine ((*R*,*R*)-2)**: Compound (*R*)-**6** (280 mg, 500 μmol), HATU (190 mg, 500 μmol), and DIPEA (86 μL, 65 mg, 500 μmol) were preincubated in DMF (5 mL) and dichloromethane (2 mL). After 10 min, **7** (120 mg, 600 μmol) and DIPEA (120 μL, 90 mg, 700 μmol) dissolved in DMF (5 mL) were added, and stirring was continued for 21 h. The solvent was removed under reduced pressure, the residue redissolved in ethyl acetate (30 mL), washed with 0.1 M phosphate buffer pH 2.0 (2×10 mL), dried (molecular sieve 0.4 nm powder), and concentrated under reduced pressure. The residue was dissolved in formic acid (10 mL). After stirring for 49 h, toluene (10 mL) was added, and the solvent was removed by evaporation under reduced pressure. The oily residue was redissolved in acetonitrile/water (20:1, 21 mL), filtered (0.45 μm nylon filter), and lyophilized to give a colorless foam. The crude product was purified by performing RP-HPLC (gradient 0–30% solvent B in 30 min, RT ~10–12 min) to afford the pure compound. The combined fractions showing pure material were lyophilized, affording *R*,*R*-**2** (76 mg, 147 μmol, 29%) as a white powder. Analytical HPLC was performed (0–10% solvent B in 30 min, RT 9.87 min). ^{1}H NMR, ^{13}C NMR and MS data were in agreement with data for *R*,*R*-**2** synthesized using racemic acid **6**.

2,3-Bis[di(*tert*-butoxycarbonylmethyl)amino]-*N*-(4-methyl-2-oxo-1,2-dihydroquinolin-7-yl)propanamide (10): Compound **6** (667 mg, 1.19 mmol), HATU (494 mg, 1.30 mmol), and DIPEA (204 μL, 154 mg, 1.19 mmol) were preincubated in dimethyl sulfoxide (5 mL). After 10 min, cs124 (174 mg, 1.00 mmol) was added, and stirring was continued for 17 h. Ethyl acetate (50 mL) was added and the mixture was washed with 0.1 M phosphate buffer pH 2.0 (2×20 mL), dried (molecular sieve 0.4 nm powder), and concentrated under reduced pressure. The residue was purified by performing silica gel chromatography (10:1 chloroform/methanol,

$R_f = 0.64$) to afford **10** (680 mg, 0.95 mmol, 95%) as a white solid. M.p. 80–83 °C. ^{1}H NMR (400 MHz, $CDCl_3$, HSQC, HMBC): $\delta = 1.45$ (s, 36H; 4×O*t*Bu), 2.44 (s, 3H; CH_3-C-4′), 3.00 (dd, $J = 14.2$, 7.7 Hz, 1H; H_A-3), 3.38 (dd, $J = 14.2$, 5.6 Hz, 1H; H_B-3), 3.43 (d, $J = 17.6$ Hz, 2H; CH_2CO$_2$*t*-Bu), 3.590 (d, $J = 17.6$ Hz, 2H; CH_2CO$_2$*t*Bu), 3.593 (s, 4H; 2×CH_2CO$_2$*t*-Bu), 3.76 (t, $J = 7.3$ Hz, 1H; H-2), 6.40 (s, 1H; H-3′), 7.39 (d, $J = 8.6$ Hz, 1H; H-6′), 7.57 (d, $J = 8.6$ Hz, 1H; H-5′), 7.85 (s, 1H; H-8′), 9,50 (brs, 1H; *H*N-C-2′), 10.92 ppm (brs, 1H; *H*N-C-1); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 19.01$ (*C*H$_3$-C-4′), 28.12 (12×C, 4×(*C*H$_3$)$_3$C), 54.25 (C-3), 54.43 (2×C, *C*H$_2$CO$_2$*t*Bu), 56.53 (2×C, *C*H$_2$CO$_2$*t*Bu), 65.10 (C-2), 81.15 (2×C, (CH$_3$)$_3$*C*), 81.47 (2×C, (CH$_3$)$_3$*C*), 104.83 (C-8′), 114.54 (C-6′), 116.52 (C-4a′), 119.08 (C-3′), 125.15 (C-5′), 138.80 (C-8a′), 140.86 (C-7′), 148.42 (C-4′), 163.03 (C=O, C-2′), 170.65 (2×C, *C*O$_2$*t*Bu), 171.74 (2×C, *C*O$_2$*t*Bu), 171.99 ppm (C=O, C-1); ESI-MS (MeOH, positive mode): calcd for $C_{37}H_{57}N_4O_{10}$ $[M+H]^+$: 717.8; found: 717.4; elemental analysis calcd (%) for $C_{37}H_{56}N_4O_{10}\cdot 0.1CHCl_3$ (728.82): C 61.14, H 7.76, N 7.69; found: C 61.01, H 7.51, N 7.30.

2,3-Bis[di(carboxymethyl)amino]-*N*-(4-methyl-2-oxo-1,2-dihydroquinolin-7-yl)propanamide (11): Compound **10** (773 mg, 1.07 mmol) was dissolved in a deprotection mixture (TFA/CH_2Cl_2/*i*Pr$_3$SiH, 5:5:1.8, v/v, 11.8 mL). After stirring for 16 h, toluene (10 mL) was added, and the solvent was removed by evaporation under reduced pressure. Ethyl acetate (5 mL) was added and the heterogeneous mixture was stirred for 1 h at 40 °C. The resulting precipitate was filtered off, washed with ethyl acetate (2× 5 mL), and mixed with water (20 mL). Lyophilization afforded the pure compound **11** (492 mg, 0.93 mmol, 87%) as a white solid. M.p. 240 °C (decomp). ^{1}H NMR (400 MHz, $CDCl_3$, HSQC, HMBC): $\delta = 2.36$ (s, 3H; CH_3-C-4′), 3.09 (dd, $J = 13.6$, 8.3 Hz, 1H; H_A-3), 3.22 (dd, $J = 13.6$, 6.0 Hz, 1H; H_B-3), 3.56 (d, $J = 17.9$ Hz, 2H; CH_2CO$_2$H), 3.625 (s, 4H; 2× CH_2CO$_2$H), 3.631 (d, $J = 17.9$ Hz, 2H; CH_2CO$_2$H), 3.78 (t, $J = 7.2$ Hz, 1H; H-2), 6.25 (s, 1H; H-3′), 7.24 (d, $J = 8.7$ Hz, 1H; H-6′), 7.62 (d, $J = 8.7$ Hz, 1H; H-5′), 7.82 (s, 1H; H-8′), 10,65 (brs, 1H; *H*N-C-1), 11.53 (brs, 1H; *H*N-C-2′), 12.40 ppm (brs, 4H; CO$_2$*H*); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 18.84$ (*C*H$_3$-C-4′), 53.58 (2×C, *C*H$_2$CO$_2$H), 54.35 (C-3), 54.85 (2×C, *C*H$_2$CO$_2$H), 63.36 (C-2), 104.96 (C-8′), 114.06 (C-6′), 116.06 (C-4a′), 119.48 (C-3′), 125.69 (C-5′), 139.94 (C-8a′), 140.82 (C-7′), 148.02 (C-4′), 162.43 (2×C, *C*O$_2$H), 170.96 (C=O, C-1), 172.26 (C=O, C-2′), 173.95 ppm (2×C, *C*O$_2$H); ESI-MS (MeOH, positive mode): calcd for $C_{21}H_{25}N_4O_{10}$ $[M+H]^+$: 493.4; found: 493.0; elemental analysis calcd (%) for $C_{21}H_{24}N_4O_{10}\cdot 2H_2O$ (528.47): C 47.73, H 5.34; found: C 47.88, H 5.18.

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