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Native chemical ligation at methionine bioisostere norleucine allows for N-terminal chemical protein ligation†

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The development of γ-thionorleucine (ThioNle) as a handle for native chemical ligation–desulfurization is reported here. ThioNle is a new addition to the expanding thiolated amino acid toolbox and serves as a methionine substitute in NCL with the advantage that it lacks the undesirable oxidation-prone thioether moiety. Its usefulness for N-terminal ubiquitination is demonstrated by efficient preparation of fully synthetic linear diubiquitin with preserved protein folding compared to the expressed material. Interestingly, gel-based deubiquitinating assays revealed that the methionine to norleucine substitution did affect diubiquitin cleavage, which may indicate a more profound role for methionine in the interaction between ubiquitin and the deubiquitinating enzymes than has been known so far.

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Introduction

The development of native chemical ligation (NCL) by Kent and co-workers[1] caused a major improvement in the synthesis of peptides and proteins. This technique allows for the conjugation of two unprotected peptide segments, a C-terminal thioester-containing peptide and a peptide bearing an N-terminal cysteine. Dawson and Yan[2] expanded the scope of NCL beyond the N-terminal cysteine requirement by introducing a catalytic desulfurization step, which effectively turns cysteine into alanine post-NCL (Fig. 1A). The subsequent development of a mild metal-free desulfurization procedure by Wan and Danishefsky[3] opened the way to the application of other proteinogenic amino acids as cysteine surrogates by instalment of a β- or γ-thiol moiety and this has resulted in the expansion of possible ligation sites to Phe,[4] Val,[5] Thr,[6] Leu,[7] Pro,[8] Glu,[9] Arg,[10] Asp,[11] Gln[12] and Trp[13] over the last decade. In addition, the preparation of δ- and γ-thioLys allowed for the formation of isopeptide bonds by NCL, which was applied in chemical ubiquitination.[14] Another development of NCL was the use of thioester surrogates, such as peptide hydrazides,[15] which expanded the scope of thioester formation.

As methionine is encoded by the universal start codon in protein translation and, as a result, each protein is translated with an N-terminal methionine residue, NCL at these sites would allow for the N-terminal modification of proteins.[16] A well-known N-terminal modification is linear ubiquitination which is an important post-translational modification.[17] NCL at internal methionine sites has been performed by applying homocysteine as a thiol donor followed by *S*-methylation

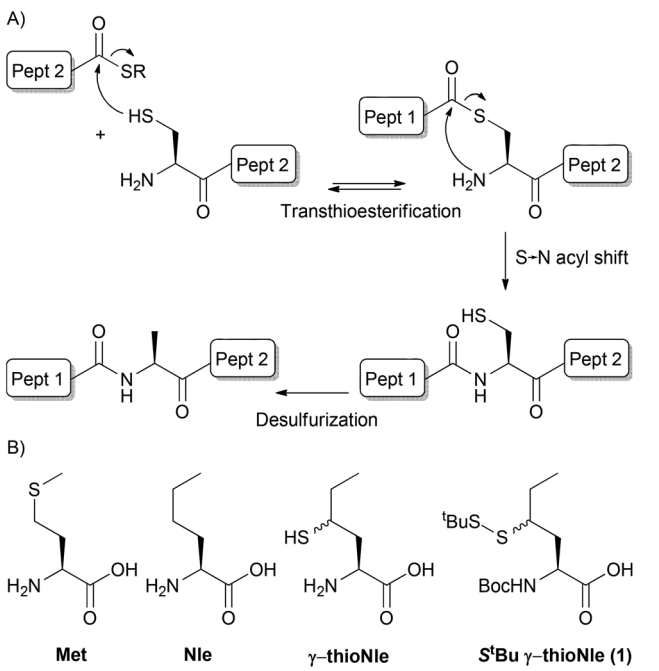

Fig. 1 (A) Native chemical ligation–desulfurization. (B) Structures of methionine, norleucine, γ-thionorleucine and target compound **1**.

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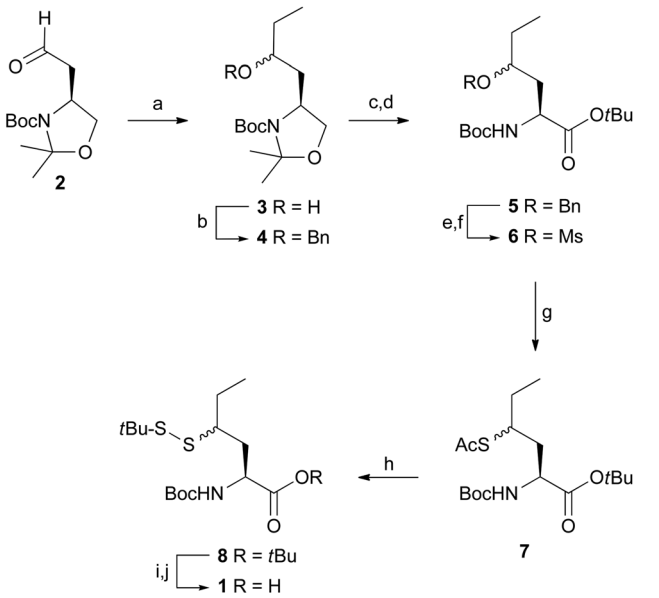
under carefully controlled conditions to prevent over- and undermethylation of homocysteine or mismethylation of other residues.[18] Desulfurization of the homocysteine ligation product has also been reported which effectively leads to the mutation of methionine into 2-aminobutyric acid in the final peptide.[2] Thiomethionine is currently missing from the thiolated amino acid collection; yet it would serve as an attractive handle for the N-terminal modification of proteins by means of NCL. On the other hand, the thioether moiety in methionine is susceptible to oxidation into a sulfoxide or even a sulfone, and this occurs rapidly under aerobic conditions, which often results in a significant loss of bioactivity of the synthesized protein.[19] In addition, the different oxidation states of methionine often lead to a mixture of different molecular weights for a single protein, which complicates the analysis by mass spectrometry. This is especially detrimental to the desulfurization reaction, typically monitored by mass spectrometry, since double oxidation or single oxidation of two methionine residues results in a net mass increase of 32 Da, which is exactly the mass decrease upon effective removal of sulfur during desulfurization. Hence, an overall change in mass is not observed although desulfurization is completed. In order to overcome these limitations, methionine is typically substituted by its closer isostere norleucine (Nle) (Fig. 1B), without substantially affecting the peptide or protein structure and function as described in the literature.[19] We here present the synthesis of γ-thionorleucine (Fig. 1B) and its application in NCL for N-terminal ubiquitination.

Results and discussion

As γ-thionorleucine is installed onto peptides by means of solid-phase peptide synthesis (SPPS) we prepared appropriately protected *N*-Boc, *S*-*tert*-butylsulfide γ-thionorleucine **1** (Fig. 1B), as depicted in Scheme 1. The synthesis commenced with the preparation of *tert*-butyl (*S*)-2,2-dimethyl-4-(2-oxoethyl)oxazolidine-3-carboxylate **2** according to literature procedures.[20] Addition of ethylmagnesium bromide to aldehyde **2** yielded compound **3** as a mixture of two diastereomers and the synthesis was continued with this mixture. The free hydroxyl was protected as benzyl ether (**4**), which was subsequently treated with Jones reagent to hydrolyse the acetonide and concomitantly oxidize the resulting alcohol to a carboxylic acid. This was converted into the corresponding *tert*-butyl ester **5** upon treatment with *O*-*tert*-butyl *N*,*N*′-diisopropylisourea.[21] Palladium-catalysed hydrogenation and subsequent mesylation of the alcohol intermediate resulted in methanesulfonate **6**, which was transformed to acetylated thiol **7** upon treatment with potassium thioacetate. *tert*-Butyl disulfide **8** was obtained after treating compound **7** with *S*-*tert*-butyl methane thiosulfonate, hydroxylamine and Et_3N.[22] TFA treatment and subsequent instalment of a Boc protecting group resulted in target compound **1**.

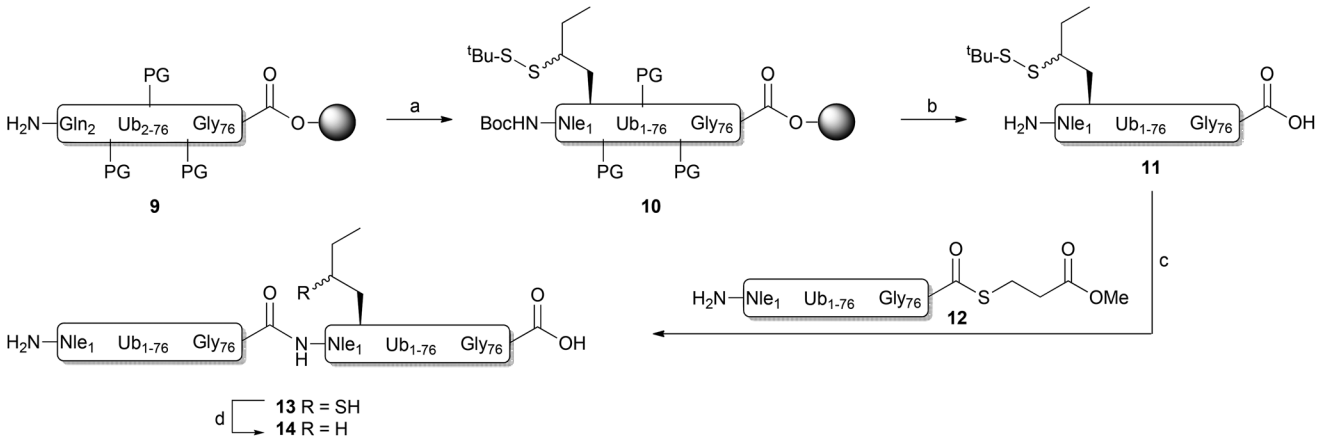
The ability of the thioNle building block to function as a new native chemical ligation handle was assessed by



Scheme 1 Synthesis of γ-thionorleucine. Reagents and conditions: (a) EtMgBr, Et_2O, 93%; (b) BnBr, NaH, *n*-Bu_4NI, DMF, 0 °C, 44%; (c) Jones reagent, acetone; (d) *O*-*tert*-butyl *N*,*N*′-diisopropylisourea, THF, 60 °C, 70%; (e) Pd/C (10% wt), H_2 (4 bar), MeOH, 48 h; (f) MsCl, Et_3N, DCM, 63%; (g) KSAc, 65 °C, 18 h, 60%; (h) *S*-*tert*-butyl methane thiosulfonate, $HONH_2$·HCl, Et_3N, MeOH, 63%; (i) TFA; (j) Boc_2O, K_2CO_3, THF, H_2O, 50%.

N-terminal protein ubiquitination. We chose to ubiquitinate ubiquitin (Ub), which effectively results in a linear diubiquitin (diUb) species. Ubiquitination is a post-translational protein modification that plays an important role in virtually all biological processes.[23] Poly-ubiquitination involves the instalment of multiple successively linked Ubs to a protein and the amino acid residue involved in the linkage between two Ubs (any of its seven lysine residues or the N-terminal Met) determines the eventual biological signal. For example, the canonical polyUb linkage Lys-48 targets the tagged protein for proteasomal degradation. Linear Ub chains (*e.g.* coupled *via* Met-1) on the other hand play a key role in the regulation of NF-κB signalling and cell death.[24] We and others have developed chemical synthesis methods for the generation of all seven isopeptide-linked (*e.g. via* a Lys side chain) Ub linkages using NCL which have led to many new biological insights.[14a,25] However, a method to synthesize the linear Ub linkage has been lacking so far.

Linear diUb was constructed by NCL between a Ub-thioester and γ-thioNle-containing Ub (Scheme 2). The individual Ub proteins were synthesized by Fmoc-based linear SPPS.[14a] Compound **1** was coupled to Ub(2-76) **9** on resin under standard coupling conditions, followed by global deprotection under strong acidic conditions and RP-HPLC purification, which resulted in Ub(1-76, $ThioNle_1$) **11** in multi-milligram amounts. LC-MS analysis of compound **11** resulted in two similar peaks at different retention times but of identical mass (Fig. 2A). We believe that these represent the two diastereomers of compound **11**, having the racemic γ-carbon atom in



Scheme 2 Synthesis of linear diUb. Reagents and conditions: (a) compound **1**, PyBOP, DiPEA, NMP; (b) TFA, H_2O, TIS, phenol, 38%; (c) TCEP, MPAA, 6 M Gnd·HCl, 0.15 M sodium phosphate, pH 7.5, 37 °C, 27%; (d) VA-044, TCEP, GSH, 6 M Gnd·HCl, 0.15 M sodium phosphate, pH 7.0, 37 °C, 93%.

thioNle as indicated in Scheme 2. Ub(1-75) was prepared by SPPS on a hyper-acid-labile trityl resin and protected with a Boc group at the N-terminus. Subsequently, the protein was cleaved from the resin under mild acidic conditions (20 vol% HFIP in DCM) which liberated only the C-terminal carboxylic acid without affecting the other protecting groups. The C-terminus was activated after which methyl-3-(glycylthio)-propionate was coupled, followed by global deprotection and RP-HPLC purification to result in Ub(1-76)-thioester **12**. A first attempt for the NCL between Ub-thioester **12** and γ-thioNle-Ub **11** under previously reported conditions (*e.g.* 50 mg mL^{-1} in 6 M Gnd·HCl/0.15 M sodium phosphate buffer, pH 7.6, 250 mM MPAA)[26] resulted in only trace amounts of the desired dimer **13**, which according to LC-MS analysis was caused by the slow reduction of the *tert*-butyldisulfide moiety in γ-thioNle. Apparently, compared to the reported correspondingly thio-protected γ-thioLys, which is readily reduced by MPAA, the *tert*-butyldisulfide moiety in γ-thioNle is much more stable. A preincubation of **11** with 100 mM TCEP for 90 min at 37 °C readily resulted in the fully liberated thiol moiety as evidenced from LC-MS analysis shown in Fig. 2B. Efficient NCL was achieved using a 40 mg mL^{-1} final Ub concentration and 250 mM MPAA for 2 hours at 37 °C according to LC-MS analysis (Fig. 2C and D), which indicated nearly full consumption of the thioNle-containing Ub and hydrolysis of remaining Ub-thioester excess. Intermediate **13** was obtained after RP-HPLC purification. The desulfurization under standard radical conditions proceeded smoothly and a subsequent purification by RP-HPLC and gel filtration yielded the target linear diUb **14** in a good overall yield (2.5 mg, 25% after NCL-deS) and purity, as confirmed by LC-MS (Fig. 2E and ESI†) and SDS-PAGE analysis (Fig. S1 in the ESI†).

Correct folding of the purified synthetic linear diUb was verified by circular dichroism (CD) spectroscopy (Fig. 3A). The spectra of synthetic and purified expressed recombinant linear diUb were recorded and compared. Similar curves were obtained for both constructs, which indicates correct protein folding of synthetic linear diUb. To verify the biochemical function we compared synthetic and expressed linear diUb by enzymatic cleavage experiments. Deubiquitinase (DUB)-mediated cleavage of synthetic and expressed linear diUb was assessed using OTULIN, USP16, and USP21, three well-studied DUBs from the two largest DUB families, which are known to cleave the linear Ub linkage.[25] Synthetic and expressed diUb were incubated with the three DUBs at 37 °C and the reaction samples were taken and immediately denatured at different time points. All proteins were resolved by SDS-PAGE and visualized by InstantBlue staining. The cleavage of diUb into monoUb is revealed by the disappearance of the diUb protein band and appearance of the monoUb protein band (Fig. 3B). Indeed, synthetic linear diUb is recognized and appropriately processed by all three DUBs, demonstrating proper protein folding and biochemical function.

Interestingly, there appears to be a difference in the cleaving efficiency by the DUBs of synthetic diUb compared to expressed linear diUb, although for USP21 this difference is very small. As proper folding of the synthetic construct was confirmed by CD measurements (Fig. 3A) the observed difference in hydrolysis rates could likely be attributed to the methionine to norleucine substitution. OTULIN is specific for linear Ub chains, and the positioning of the Ub–Ub linkage in the active site was assigned with atomic resolution.[27] From the crystal structures (PDB: 3ZNZ and 5OE7) it becomes apparent that the Ub methionine side chain points outwards from the active site and is probably not directly involved in the binding between enzyme and substrate. Our observation that the methionine to norleucine substitution affects the hydrolysis rate may therefore indicate that the thioether moiety in methionine is important for the interaction between Ub and OTULIN. Except for the finding that Met-1 sulfur can form a hydrogen bond with the Lys-63 backbone amine and that oxidation of this sulfur or Met-1 deletion affects the Ub folding below pH 4,[28] little is known about the contribution of methionine to the biochemical function of Ub. As no structural data on the linear Ub–Ub linkage within the USP16 and USP21 active sites

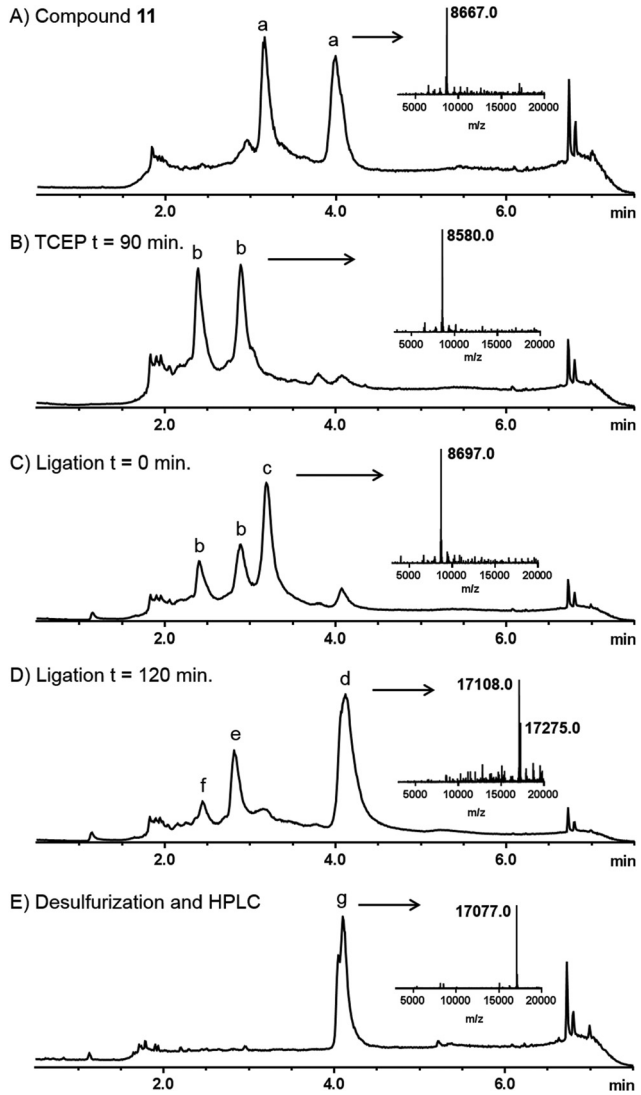


Fig. 2 Analytical LC-MS analysis of the NCL-deS reactions. Mass traces of (A) purified compound **11**, (B) preincubation of **11** with TCEP, (C) ligation reaction between **11** and **12** at t = 0 min, (D) ligation reaction between **11** and **12** at t = 120 min, and (E) product after desulfurization and purification. The insets in each trace represent the deconvoluted mass spectra of the indicated peaks. a = compound **11**, b = reduced disulfide of **11** (free thiol), c = MPAA ester of **12**, d = ligation product (mixture of free thiol and MPAA disulfide), e = hydrolysed Ub-thioester (8547 Da), f = assumed Ub-Gnd. Adduct (8588 Da), g = compound **14** (the small shoulder has a mass identical to the main peak and might indicate a different conformation; see also the ESI†).

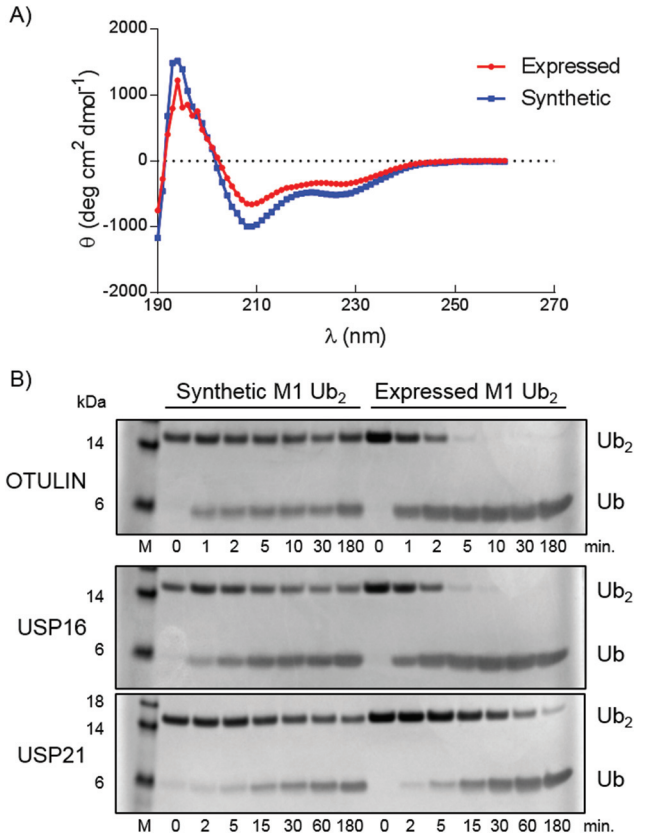


Fig. 3 Characterization of synthetic linear diUb (**14**). (A) CD measurements of synthetic and expressed linear diUb. (B) SDS-PAGE analysis of linear diUb cleavage by OTULIN, USP16 and USP21.

are available, the importance of the methionine residue for these DUBs remains to be investigated.

The development of γ-thionorleucine proved valuable as the NCL-deS construction of linear diUb proceeded efficiently and concomitantly omitted all mass spectrometry disadvantages associated with methionine oxidation. Unexpectedly, the methionine to norleucine substitution did affect the DUB mediated diUb cleavage, which may indicate a more profound role for methionine in the interaction between Ub and DUB than has been known so far.

Conclusions

In summary, we presented thioNle as a new handle for NCL and showed its feasibility for the N-terminal modification of proteins by preparing linear diubiquitin in a fully synthetic way for the first time. ThioNle is a new addition to the expanding thiolated amino acid toolbox and serves as a suitable methionine substitute in NCL with the advantage that it lacks the undesirable oxidation-prone thioether moiety. In addition, the fully synthetic preparation of linear diUb opens the way for the creation of linear diUb-based constructs, such as activity-based probes and assay reagents, which will benefit the field of Ub research.[27b]

Experimental section

General

General reagents were obtained from Sigma Aldrich, Biosolve, Fluka and Acros, and used as received. Solvents were purchased from Biosolve or Sigma Aldrich. Dry THF and DCM were obtained using an Innovative Technology PureSolv Micro Solvent Purification System. Peptide synthesis reagents were purchased from NovaBiochem and Rapp Polymers. Analytical thin layer chromatography (TLC) was performed on Merck alu-

minium sheets (pre-coated with silica gel 60 F_{254}). Compounds were visualized by UV adsorption (254 nm) and/or by using a solution of $KMnO_4$ (7.5 g L^{-1}) and K_2CO_3 (50 g L^{-1}) in water and charring. Column chromatography was carried out on silica gel (40–63 u, 60 Å, Fluorochem). NMR spectra (^1H, ^{13}C) were recorded on a Bruker Ultrashield 300 Spectrometer (^1H: 300.17 MHz, ^{13}C: 75.47 MHz) at 298 K. Peak shapes in NMR spectra are indicated with symbols 'd' (doublet), 's' (singlet), 't' (triplet) and 'm' (multiplet). Chemical shifts (δ) are given in ppm relative to $CDCl_3$ as an internal standard.

LC-MS

LC-MS measurements were performed on an LC-MS system equipped with a Waters 2795 Separation Module (Alliance HT), a Waters 2996 Photodiode Array Detector (190–750 nm), a Waters Xbridge C18 column (2.1 × 30 mm, 3.5 μm) or a Waters Xbridge C18 column (2.1 × 100 mm, 3.5 μm) and a LCT ESI-Orthogonal Acceleration Time of Flight Mass Spectrometer. Samples were run using 2 mobile phases: A = 1% CH_3CN and 0.1% formic acid in water and B = 1% water and 0.1% formic acid in CH_3CN. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1 (deconvolution with Maxent1 function).

Program 1: Waters Xbridge C18 column (2.1 × 30 mm, 3.5 μm); flow rate = 0.8 mL min^{-1}, runtime = 6.2 min, column T = 40 °C, mass detection: 300–2000 Da. Gradient: 0–0.2 min: 5% B; 0.2–3.2 min: 5% → 95% B; 3.2–4.2 min: 95% B; 4.2–4.4 min: 95% → 5% B; 4.4–6.2 min: 5% B.

Program 2: Waters Xbridge C18 column (2.1 × 100 mm, 3.5 μm); flow rate = 0.4 mL min^{-1}, runtime = 13 min, column T = 40 °C, mass detection: 300–2000 Da. Gradient: 0–0.4 min: 5% B; 0.4–9.0 min: 5% → 95% B; 9.0–11.2 min: 95% B; 11.2–11.3 min: 95% → 5% B; 11.3–13.00 min: 5% B.

LC-MS analysis of diUb as well as the TCEP reduction, NCL and desulfurization reactions (as shown in Fig. 2) were recorded on a Waters XEVO-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (capillary voltage: 1.2 kV, desolvation gas flow: 900 L h^{-1}, temperature: 60 °C) with a resolution R = 26 000. Samples were run using 2 mobile phases: A = 0.1% formic acid in water and B = 0.1% formic acid in CH_3CN on a Waters Acquity UPLC Protein BEH C4 column, 300 Å, 1.7 μm (2.1 × 50 mm); flow rate = 0.6 mL min^{-1}, runtime = 10.00 min, column T = 60 °C, mass detection: 50–1500 Da. Gradient: 0–0.80 min: 2% B; 0.80–1.00 min: 2% → 23% B; 1.00–1.50: 23% B; 1.50–3.00 min: 23% → 25.5% B; 3.00–3.30 min: 25.5% B; 3.30–3.50 min: 25.5% → 29% B; 3.50–4.50: 29% → 32% B; 4.50–6.50 min: 32% → 100% B; 6.50–8.00 min: 100% B; 8.00–8.10 min: 100% → 2% B; 8.10–10.00 min: 2% B. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1.

HRMS measurements

High resolution mass spectra were recorded on a Waters XEVO-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (capillary voltage: 3.0 kV, desolvation gas flow: 900 L h^{-1}, temperature: 60 °C) with a resolution R = 22 000 and 200 pg μL^{-1} Leu-Enk (m/z = 556.2771) as a "lock mass". Samples were run using 2 mobile phases: A = 0.1% formic acid in water and B = 0.1% formic acid in CH_3CN on a Waters Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm); flow rate = 0.6 mL min^{-1}, runtime = 3.00 min, column T = 60 °C, mass detection: 50–1500 Da. Gradient: 0–0.15 min: 2% B; 0.15–1.85 min: 2% → 100% B; 1.85–2.05: 100% B; 2.05–2.10 min: 100% → 2% B; 2.10–3.00 min: 100% B. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1.

HPLC purification

HPLC purifications were performed on a Waters HPLC equipped with a Waters 2489 UV/Vis detector, Waters fraction collector III and Waters XBridge prep C18 OBD (30 × 150 mm, 5 μm). Flowrate = 37.5 mL min^{-1}. Mobile phase: A = H_2O, B = CH_3CN and C = 1% TFA in H_2O. Gradient: 0–5 min: 90% A, 5% B, 5% C; 5–7 min: 5 → 20% B, 5% C; 7–18 min: 20 → 45% B, 5% C; 18–18.5 min: 45% → 95% B, 5% C; 18.5–21.6 min: 95% B, 5% C; 21.6–25 min: 95% → 5% B, 5% C.

Alternatively, a Shimadzu LC-20AT equipped with a Shimadzu SPD-20A UV/Vis detector and a Shimadzu FRC-10A fraction collector and a Waters XBridge C18-Prep column (10 × 150 mm, 5 μm) was used. Flowrate = 4.00 or 6.50 mL min^{-1}. Mobile phase: A = 0.05% TFA in H_2O and B = 0.05% TFA in CH_3CN. T = 40 °C. Gradient: 0–8.20 min: 5% B (4.00 mL min^{-1}) and 0–1 min: 5% B; 1 → 2 min 5% → 10% B; 2–17 min: 10% → 70% B; 17–17.10 min: 70% → 95% B; 17.10–19.10 min: 95% B; 19.10–22.10: 5% B.

Synthesis of γ-thionorleucine

***tert*-Butyl (4*S*)-4-(2-hydroxybutyl)-2,2-dimethyloxazolidine-3-carboxylate (3).** Compound **2** (6.1 g, 25.1 mmol), which was synthesized according to literature procedures,[20] was dissolved in dry Et_2O (60 mL) and the reaction mixture was stirred at room temperature under an argon atmosphere, followed by the dropwise addition of ethylmagnesium bromide (2 eq., 5.99 g, 50.2 mmol, 16.7 mL of a 3 M solution in diethyl ether). The stirring was continued for 1 h and TLC analysis showed the complete conversion of the starting material. The reaction was quenched by adding water (5 mL) and the resulting mixture was filtered through a Celite pad. The filtrate was washed with H_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica gel flash column chromatography (EtOAc/heptane 5% → 30%) yielded the title compound (6.4 g, 23.4 mmol, 93%). NMR data were collected based on two pure diastereoisomers and compared with reported data.[29] The first eluting compound: ^1H NMR (300 MHz, $CDCl_3$) δ 4.22–4.16 (m, 1H, αCH), 3.95 (dd, J = 8.7, 5.4 Hz, 1H, OCH_2 in ring), 3.62 (d, J = 8.6 Hz, 1H, OCH_2 in ring), 3.42–3.29 (m, 1H, OCH), 1.74 (ddd, J = 13.6, 11.1, 2.3 Hz, 1H, $\frac{1}{2}$ × βCH_2), 1.51–1.42 (m, 18H, 2 × CH_2, 3 × CH_3, $\frac{1}{2}$ × βCH_2, δCH_2), 0.89 (t, J = 7.4 Hz, 3H, CH_3). The second eluting compound: ^1H NMR (300 MHz, $CDCl_3$) δ 4.16–4.04 (m, 1H, αCH), 3.95 (dd, J = 9.0, 5.7 Hz, 1H, OCH_2),

3.90–3.73 (m, 1H, OCH_2), 3.55–3.49 (m, 1H, OCH), 2.60 (s, 1H, OH), 1.76 (t, J = 6.1 Hz, 1H, $\frac{1}{2} \times \beta CH_2$), 1.60–1.46 (m, 18H, 2 × CH_2, 3 × CH_3, $\frac{1}{2} \times \beta CH_2$, δCH_2), 0.93 (t, J = 7.4 Hz, 3H, CH_3).

***tert*-Butyl (4*S*)-4-(2-(benzyloxy)butyl)-2,2-dimethyloxazolidine-3-carboxylate (4).** Compound **3** (6.4 g, 23.4 mmol) was dissolved in DMF (100 mL) and the reaction solution was cooled to 0 °C, followed by the addition of *n*-Bu_4NI (0.1 eq., 0.85 g, 2.3 mmol) and sodium hydride (1.2 eq., 1.1 g, 28.1 mmol, 60% dispersion in mineral oil). After stirring at 0 °C for another 30 min, benzyl bromide (2.0 eq., 8.00 g, 46.8 mmol, 5.6 mL) was added dropwise. The resulting mixture was stirred for 1.5 h at room temperature. Then saturated aqueous NH_4Cl was added and the mixture was extracted with Et_2O (3×). The combined organic layer was washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica gel flash column chromatography (EtOAc/heptane 2% → 15%) yielded the title compound as a mixture of diastereoisomers (3.7 g, 10.2 mmol, 44%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.40–7.22 (m, 5H, Ph), 4.60–4.35 (m, 2H, OCH_2), 4.22–4.01 (m, 1H, αCH), 3.98–3.74 (m, 2H, CH_2 in ring), 3.56–3.27 (m, 1H, OCH), 2.03–1.76 (m, 1H, βCH_2), 1.72–1.51 (m, 5H, δCH_2, CH_3), 1.50–1.42 (m, 13H, βCH_2, 4 × CH_3), 0.94 (t, J = 7.4 Hz, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 138.90, 128.43, 128.37, 127.77, 127.53, 79.09, 77.50, 70.67, 68.38, 66.98, 56.89, 54.67, 38.16, 37.50, 36.81, 28.62, 28.40, 27.06, 26.83, 26.33, 24.70, 23.38, 14.19 ppm. HRMS: calculated for $C_{21}H_{33}NO_4$ $[M + H]^+$ 364.2488; found 364.2473.

(2*S*)-4-(Benzyloxy)-2-((*tert*-butoxycarbonyl)amino)hexanoic acid. Jones reagent (2.5 eq., 25.5 mmol, 12.8 mL, 2 M in aqueous sulfuric acid) was added to a solution of compound **4** (3.7 g, 10.2 mmol) in acetone (140 mL) at 0 °C. Stirring was continued for 5 h and the reaction was quenched by adding isopropanol (88 mL). The resulting reaction mixture was stirred for 10 min and the pH was adjusted to 4–5 with saturated aqueous $NaHCO_3$. The mixture was extracted with EtOAc (3×) and the combined organic layer was washed with H_2O and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was directly used in the next step without further purification.

***tert*-Butyl (2*S*)-4-(benzyloxy)-2-((*tert*-butoxycarbonyl)amino) hexanoate (5).** The crude product obtained from the previous step was co-evaporated with toluene (3×) and dissolved in dry THF (25 mL), followed by the addition of a solution of *O*-*tert*-butyl *N*,*N*′-diisopropylisourea[21] (1.5 eq., 3.1 g, 15.3 mmol) in THF (15 mL). The reaction mixture was stirred for 3 h at 60 °C. Another portion of *O*-*tert*-butyl *N*,*N*′-diisopropylisourea (1 eq., 2.0 g, 10.2 mmol) in THF (10 mL) was added and the reaction mixture was stirred at 60 °C overnight. The resulting mixture was filtered through a Celite pad and the filter cake was washed with Et_2O. The filtrate was concentrated *in vacuo*. Purification by silica gel flash column chromatography (EtOAc/heptane 5% → 30%) yielded the title compound as a mixture of diastereoisomers (2.8 g, 7.1 mmol, 70%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.39–7.14 (m, 5H, Ph), 5.68–5.00 (m, 1H, NH), 4.52–4.04 (m, 3H, OCH_2, αCH), 3.50–3.33 (m, 1H, OCH), 1.95–1.72 (m, 2H, CH_2), 1.62–1.07 (m, 20H, CH_2, 6 × CH_3), 0.87–0.80 (m, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 172.18, 172.04, 155.72, 155.45, 138.55, 138.46, 138.35, 128.58, 128.45, 128.41, 128.28, 127.84, 127.73, 81.65, 81.55, 79.67, 79.27, 77.16, 76.94, 71.54, 71.04, 52.59, 52.18, 36.11, 35.78, 28.47, 28.19, 28.09, 26.32, 25.95, 9.73, 9.24, 9.00 ppm. HRMS: calculated for $C_{22}H_{35}NO_5$ $[M + H]^+$ 394.2593; found 394.2582.

***tert*-Butyl (2*S*)-2-((*tert*-butoxycarbonyl)amino)-4-((methylsulfonyl)oxy)hexanoate (6).** Compound **5** (2.8 g, 7.1 mmol) was dissolved in methanol (100 mL), followed by the addition of a catalytic amount of palladium on carbon (10% wt). The mixture was placed under H_2 (4 bar, Parr apparatus) for 48 h. It was filtered through a Celite pad and concentrated *in vacuo*. The deprotected intermediate was co-evaporated with toluene (3×) and dissolved in dry DCM (70 mL), followed by the addition of Et_3N (3 eq., 21.3 mmol, 3.0 mL). The reaction solution was cooled to 0 °C and then methanesulfonyl chloride (3 eq., 21.3 mmol, 1.65 mL) was added. After stirring for 3 h at room temperature, saturated aqueous $NaHCO_3$ was added to the reaction mixture. The mixture was then extracted with DCM (3×) and the combined organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica gel flash column chromatography (EtOAc/heptane 5% → 30%) yielded the title compound as a mixture of diastereoisomers (1.7 g, 4.5 mmol, 63%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.35–5.20 (m, 1H, NH), 4.65–4.59 (m, 1H, OCH), 4.07–4.02 (m, 1H, αCH), 2.94–2.90 (m, 3H, SO_3CH_3), 2.07–1.92 (m, 1H, $\frac{1}{2} \times CH_2$), 1.85–1.56 (m, 3H, $1\frac{1}{2} \times CH_2$), 1.40–1.23 (m, 18H, 6 × CH_3), 0.89–0.78 (m, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.03, 170.85, 155.36, 155.26, 82.14, 81.94, 80.80, 80.73, 79.64, 50.95, 50.89, 38.29, 38.19, 36.17, 35.59, 28.10, 27.96, 27.75, 27.18, 8.69, 8.63 ppm. HRMS: calculated for $C_{16}H_{31}NO_7S$ $[M + H]^+$ 382.1899; found 382.1897.

***tert*-Butyl (2*S*)-4-(acetylthio)-2-((*tert*-butoxycarbonyl)amino) hexanoate (7).** Compound **6** (1.7 g, 4.5 mmol) was dissolved in DMF (25 mL) and potassium thioacetate (1.54 g, 13.5 mmol) was added. The resulting reaction mixture was stirred at 65 °C for 18 h. The reaction mixture was allowed to cool to room temperature and was concentrated *in vacuo*. The residue was dissolved in EtOAc and the organic layer was washed with brine until the aqueous layer did not show yellow colour anymore (4×). The aqueous layer was extracted with EtOAc and the combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo.* Purification by silica gel flash column chromatography (EtOAc/heptane 5% → 30%) yielded the title compound as a mixture of diastereoisomers (0.98 g, 2.7 mmol, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.19–4.95 (m, 1H, NH), 4.13–4.06 (m, 1H, αCH), 3.48–3.37 (m, 1H, SCH), 2.17 (s, 3H, CH_3 acetyl), 1.99–1.39 (m, 4H, CH_2), 1.39–1.21 (m, 18H, 6 × CH_3), 0.85–0.79 (m, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 195.49, 194.85, 171.36, 155.36, 155.04, 81.71, 79.45, 52.18, 42.41, 42.23, 37.45, 36.95, 30.60, 30.56, 28.22, 27.85, 27.81, 26.67, 10.97, 10.91 ppm. HRMS: calculated for $C_{17}H_{31}NO_5S$ $[M + H]^+$ 362.2001; found 362.2003.

***tert*-Butyl (2*S*)-2-((*tert*-butoxycarbonyl)amino)-4-(*tert*-butyldisulfanyl)hexanoate (8).** Compound **7** (0.98 g, 2.7 mmol) and

S-tert-butyl methane thiosulfonate[22] (5 eq., 2.27 g, 13.5 mmol) were dissolved in MeOH (25 mL), followed by the addition of hydroxylamine hydrochloride (4 eq., 0.75 g, 10.8 mmol) and Et_3N (4 eq., 10.8 mmol, 1.5 mL). The resulting reaction mixture was stirred at room temperature. After 2 h, additional Et_3N was added (2 eq., 5.4 mmol, 0.75 mL) and the reaction mixture was stirred at room temperature until TLC analysis showed the complete conversion of the starting material. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in EtOAc. The organic layer was washed with H_2O, 1 M $KHSO_4$, sat. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo.* Purification by silica gel flash column chromatography (EtOAc/heptane 1% → 10%) yielded the title compound as a mixture of diastereoisomers (693 mg, 1.7 mmol, 63%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.06 (d, J = 8.7 Hz, 1H, NH), 4.15–4.08 (m, 1H, αCH), 2.70–2.62 (m, 1H, SCH), 1.97–1.70 (m, 3H, $1\frac{1}{2} \times CH_2$), 1.59–1.47 (m, 1H, $\frac{1}{2} \times CH_2$), 1.36–1.32 (m, 18H, $6 \times CH_3$), 1.23–1.20 (m, 9H, $3 \times CH_3$), 0.94–0.87 (m, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.55, 155.31, 155.14, 81.83, 79.44, 52.00, 50.26, 47.58, 38.10, 30.04, 28.25, 27.93, 25.93, 10.75 ppm. HRMS: calculated for $C_{19}H_{37}NO_4S_2$ $[M + H]^+$ 408.2242; found 408.2239.

(2*S*)-2-((*tert*-Butoxycarbonyl)amino)-4-(*tert*-butyldisulfanyl) hexanoic acid (1). Compound **8** (693 mg, 1.7 mmol) was dissolved in trifluoroacetic acid (10 mL) and stirred at room temperature. The reaction progress was followed by LC-MS. Upon completion, the reaction mixture was concentrated *in vacuo* and the resulting residue was co-evaporated with toluene (3×). The deprotected intermediate was dissolved in H_2O (8 mL) and THF (8 mL) and cooled to 0 °C. K_2CO_3 (3 eq., 705 mg, 5.1 mmol) was added, followed by the addition of Boc_2O (2 eq., 742 mg, 3.4 mmol) and the reaction mixture was stirred overnight. The aqueous layer was acidified to pH 3 with 1 M HCl aqueous solution and then extracted with EtOAc (4×). The combined organic phase was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo.* Purification by silica gel flash column chromatography (EtOAc/heptane 10% → 50%) yielded the title compound as a mixture of diastereoisomers (299 mg, 0.85 mmol, 50%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.91 (s, 1H, OH), 5.15 (d, J = 8.6 Hz, 1H, NH), 4.42–4.16 (m, 1H, αCH), 2.88–2.67 (m, 1H, SCH), 2.16–1.94 (m, 2H, βCH_2), 1.91–1.49 (m, 2H, CH_2), 1.43 (s, 9H, CH_3), 1.30 (s, 9H, CH_3), 1.02 (t, J = 7.3 Hz, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 177.19, 155.75, 80.39, 51.71, 50.57, 47.88, 38.04, 30.21, 28.41, 26.32, 11.06 ppm. HRMS: calculated for $C_{15}H_{29}NO_4S_2$ $[M + H]^+$ 352.1616; found 352.1631.

Synthesis of linear diubiquitin

Solid phase peptide synthesis. Ub(1-75, Nle_1) and Ub(2-76) (**9**) polypeptides were synthesized using solid phase peptide synthesis (SPPS). SPPS was performed on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry on 25 μmol scale, using a fourfold excess of amino acids relative to pre-loaded Fmoc amino acid trityl resin, TentaGel® R TRT-Gly Fmoc (Rapp Polymere GmbH; RA1213; 0.2 mmol g^{-1}), following the previously reported procedure.[14a] The resin was washed with NMP and DCM prior to further modifications. The quality and purity of the construct were confirmed by LC-MS analysis (Program 1) of a small amount of the material that was cleaved from the resin using TFA/H_2O/DODT/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 100 μL) for 30 min at 37 °C.

Synthesis of Ub(1-76, Nle_1)-S$(CH_2)_2CO_2$Me (12). Resin-bound H-Ub(1-75, Nle_1) was washed with DCM (3 × 5 mL). Boc_2O (4 eq., 21.83 mg, 100 μmol, 23 μL) and DiPEA (15 eq., 48.47 mg, 375 μmol, 65.3 μL) were dissolved in DCM (1 mL). This solution was added to resin-bound H-Ub(1-75, Nle_1) and the mixture was shaken for 3 hours at room temperature. After 3 hours, the liquid was removed and the resin was washed three times with NMP and DCM alternately followed by three times washing with DCM and MeOH alternately. Boc-Ub(1-75, Nle_1) was cleaved from the trityl resin using a solution of hexafluoroisopropanol (HFIP) in DCM (1/4; v/v; 2.5 mL; 2× 20 min). The resin was rinsed two times with DCM in between HFIP treatments. All combined filtrates were concentrated under reduced pressure. The protected protein was co-evaporated with DCE (3 times, 12 mL), to remove traces of HFIP, and lyophilized overnight. Subsequently, the protected protein was dissolved in DCM (4 mL) and reacted with EDC (3 eq., 14.4 mg, 75 μmol), HOBt (3 eq., 10.1 mg, 75 μmol) and HCl·H-Gly-S$(CH_2)_2CO_2$Me (3 eq., 16 mg, 75 μmol) for 16 hours. To follow the reaction progress a mini deprotection was done. A small amount of the reaction mixture was taken and the protection groups were removed under fast cleavage conditions (TFA/H_2O/DODT/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 100 μL), 30 min at 37 °C). The reaction was checked by LC-MS analysis (Program 1). The reaction mixture was concentrated under reduced pressure and treated with TFA/H_2O/phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 5 mL) for 3.5 hours. The protein was precipitated from ice-cold Et_2O/*n*-pentane (3/1; v/v; 20 mL). The solution was centrifuged and Et_2O/*n*-pentane (supernatant) was removed. The pellet was washed with Et_2O (3 × 20 mL), the solution was vortexed, the suspension was centrifuged and Et_2O was removed. The wash step was repeated twice. The pellet was dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 10 mL) and lyophilized. The protein was subsequently purified using RP-HPLC.

Synthesis of Ub(1-76, ThioNle$_1$) (11). PyBOP (4 eq., 20.9 mg, 40.12 μmol) was dissolved in NMP (100 μL). ThioNle (**1**, 4 eq., ~14 mg, 39.82 μmol) was dissolved in NMP (200 μL). Both solutions were added to Ub(2-76) (**9**) on resin. DiPEA (8 eq., 10.3 mg, 79.79 μmol, 13.9 μL) was dissolved in NMP (60 μL) and this solution was also added to the resin. The reaction mixture was shaken overnight. To follow the reaction progress a mini deprotection was done. A small amount of the reaction mixture was taken and the resin and the protection groups were removed under fast cleavage conditions (*vide supra*). The reaction progress was checked by LC-MS analysis (Program 1). The resin was filtered off, washed three times with DCM and MeOH alternately, three times with DCM and Et_2O alternately and three times with Et_2O.

The polypeptide was deprotected and detached from the resin by treatment with TFA/H_2O/phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 2 mL) for 3 h. The reaction mixture was filtered directly into ice-cold Et_2O/*n*-pentane (3/1; v/v; 15 mL) and the resin was spooled with TFA (2 × 2 mL). The solution was centrifuged and Et_2O/*n*-pentane (supernatant) was removed. The pellet was washed with Et_2O (3 × 15 mL), the solution was vortexed, the suspension was centrifuged and Et_2O was removed. The wash step was repeated twice. The pellet was dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 5 mL) and lyophilized. The protein was subsequently purified using RP-HPLC.

Purification. Crude monoubiquitin was properly dissolved in a minimal amount of DMSO (max. 10 vol% of the final volume) while being heated carefully. The DMSO was added dropwise into H_2O (10 to 20 mL). The pH was checked and it should be below 7. The mixture was centrifuged (3 min @3800 rpm). The supernatant was filtered and purified by RP-HPLC on the Waters HPLC. Pure fractions were pooled and lyophilized. The products were obtained as white solids. LC-MS analysis (Program 2) was done to check the purity.

Yields:

Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ (**12**) = 229.19 mg, 26.44 μmol, 52.88%. LC-MS: R_t: 4.74 min: ESI MS+ (amu) calcd: 8649.0 [M], found 8650.0 (deconv.).

Ub(1-76, $ThioNle_1$) (**11**) = 32.81 mg, 3.79 μmol, 37.9%. LC-MS: R_t: 4.92 min: ESI MS+ (amu) calcd: 8667.1 [M], found 8668.0 (deconv.).

Native chemical ligation of Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ (12) and Ub(1-76, $ThioNle_1$) (11). Ub(1-76, $ThioNle_1$) (**11**, 1 eq., 5.1 mg, 0.58 μmol) was dissolved in 101.2 μL of aqueous buffer containing 8.0 M Gnd·HCl and 0.2 M Na_2HPO_4, pH 7.55. 1 M aqueous TCEP solution at pH 7.0 (12.5 μL) was added. This solution was pre-incubated for 90 min and the disulfide bond cleavage was monitored by LC-MS analysis (XEVO). Ub(1-76, Nle_1)-*S*-$(CH_2)_2CO_2Me$ (**12**, 1.5 eq., 7.64 mg, 0.88 μmol) was dissolved in 151.9 μL of aqueous 8.0 M Gnd·Hcl and 0.2 M Na_2HPO_4 at pH 7.55 and 46.9 μL of 1 M MPAA solution was added. This solution was pre-incubated for 5 minutes. Both solutions were properly mixed and the pH of the reaction mixture was adjusted to 7.45 by the addition of 22 μL of 10% Na_2CO_3 solution. The reaction mixture was shaken for 120 min at 37 °C. The progress of the reaction was checked by LC-MS analysis (XEVO). The diUb formed was purified by RP-HPLC. To prepare the sample, the reaction mixture was added dropwise to 2.5 mL aqueous buffer containing 6.0 M Gnd·HCl and 0.15 M Na_2HPO_4. This solution was diluted with water to 10 mL. 1 M aqueous TCEP solution at pH 7.0 (125 μL) was added. The pH was checked and adjusted below 7. The mixture was centrifuged (5 min @ 3800 rpm), filtered and purified by RP-HPLC on the Shimadzu HPLC. Pure fractions were pooled and lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 15 mL) and lyophilized again. The product was obtained as a white solid. Yield: thiol-containing linear diUb (**13**) = 2.72 mg; 0.159 μmol; 27.2%.

Desulfurization. Thiol-containing linear diUb was dissolved in aqueous buffer containing 6.0 M Gnd·HCl, 0.15 M Na_2HPO_4 and 0.25 M TCEP at pH 7.0 to a concentration of 1 mg mL^{-1} protein. Reduced glutathione (GSH) was added to the solution to a concentration of 100 mM. The pH of the solution was adjusted to 7.20 by the addition of 400 μL of 10% Na_2CO_3 solution. VA-044 was added to the solution to a final concentration of 75 mM. The reaction mixture was flushed with argon and shaken overnight at 37 °C. The progress of the reaction was checked by LC-MS analysis (Program 1). Desulfurized diUb was purified by RP-HPLC using the Shimadzu HPLC. The reaction mixture was therefore diluted with water (same amount as the reaction volume) and 1 M NaOAc/AcOH buffer (40 vol% of the reaction volume). The sample was filled to 10 mL and the pH was checked and adjusted below 7; the sample was centrifuged (5 min @ 3800 rpm) and filtered before it was purified by RP-HPLC. Pure fractions were pooled and lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 15 mL) and lyophilized again.

The product was purified by gel filtration using a Bio-Rad NGC Chromatography system on a size exclusion S75 16/600 superdex PG-GE healthcare column with a volume bed of 120 mL and 3–70 kDa separation range using a filtered aqueous buffer containing 50 mM TRIS·HCl and 100 mM NaCl at pH 7.55 at a flow rate of 1 mL min^{-1}. The sample was prepared by dissolving the product in DMSO (250 μL), and dropwise addition of this solution to MilliQ (2450 μL) and dropwise addition of 10× TRIS buffer (300 μL). The mixture was centrifuged for 5 min @3500 rpm. The fractions were analysed by SDS-PAGE analysis and pure fractions were pooled.

The product was obtained as a colourless solution containing 50 mM TRIS·HCl and 100 mM NaCl buffer at pH 7.55. LC-MS analysis (Program 2 and XEVO) was done to check the purity.

The protein concentration (and synthesis yield) was determined by SDS-PAGE analysis and quantification of band intensities after InstantBlue™ (Expedeon) staining using a GE Healthcare Amersham Imager 600 with ImageQuant TL 8.1 GE Healthcare Life Sciences software. Different amounts monoubiquitin (0.5 μg, 1 μg, 2 μg, and 4 μg) were included on the same gel to calculate the concentration of the final compound. Although quantification of bands from SDS-PAGE analysis is not the most accurate way of quantification, it is the most reliable one due to the relatively low concentration of the solution. Yield: linear diUb (**14**) = 2.54 mg, 0.15 μmol, 93.4%. LC-MS (Program 2): R_t: 5.18 min: ESI MS+ (amu) calcd: 17 075.7 [M], found 17 075.00 (deconv.). LC-MS (XEVO): R_t: 4.10 min: ESI MS+ (amu) calcd: 17 075.7 [M], found 17 077.00 (deconv.)

Expression of linear diubiquitin

Linear diubiquitin was expressed using a pET17b vector by inducing with 250 μM IPTG in BL21 (DE3) cells at an OD of 0.6. Purification was done as described for the yeast ubiquitin proprotein by Larsen *et al.*, 1998.[30] The concentration was determined using a NanoDrop spectrophotometer and estimated as 20.21 mg mL^{-1} (1.18 mM).

Characterization of synthetic and expressed linear diubiquitin

Purity check and concentration normalization were performed using SDS-PAGE gel analysis. Synthetic (**14**) and expressed linear diubiquitin were diluted to ~5.85 μM, ~11.7 μM and ~17.55 μM (~1, ~2 and ~3 μg linear diUb per lane). 10 μL of each sample was diluted with 5 μL sample buffer (3×), containing NUPAGE® LDS sample buffer (4×, Invitrogen) (900 μL), β-mercaptoethanol (90 μL) and water (210 μL), heated at 95 °C for 5 minutes and loaded on 12% NUPAGE® Novex® Bis-Tris Mini Gels (Invitrogen) using MES-SDS running buffer. SeeBlue Pre-stained Plus2 Standard (Invitrogen, LC5925) was used as a marker. InstantBlue™ (Expedeon) stains were scanned using a GE Healthcare Amersham Imager 600. InstantBlue band intensities were determined using ImageQuant TL 8.1 (GE Healthcare Life Sciences).

Circular dichroism (CD) analysis

Sample preparation. Synthetic and recombinant diUb were transferred from the buffer containing 50 mM TRIS, 100 mM NaCl, pH = 7.55 to a buffer containing 100 mM sodium phosphate buffer (pH = 7.41). The concentration in 100 mM sodium phosphate was expected to be the same as that in 50 mM TRIS buffer and diluted to a final concentration of ~75 μM.

The CD measurements were carried out on a JASCO J-815 CD spectrometer fitted with a Peltier temperature controller set to 25 °C. Samples were measured in a quartz cuvette with a 1 mm path length. Spectra were recorded from 260 to 190 nm at 1 nm intervals with a 1 nm bandwidth. The scan speed was 100 nm min^{-1} and the response time was 1 s. Data were obtained by averaging 5 scans. Data were converted to the mean residue molar ellipticity θ (deg cm^2 $dmol^{-1}$) according to the equation:[31]

$$[\theta] = \frac{(\theta)_{\mathrm{obs}}}{c \times n \times l}$$

where $(\theta)_{\mathrm{obs}}$ is the observed ellipticity in mdeg, c is the peptide concentration in M (expressed linear diUb concentration was corrected with the normalization factor determined by SDS-PAGE), n is the number of residues, and l is the path length of the cuvette in mm. The CD signals, which resulted from the buffer, were subtracted from the spectrum of each sample.

DUB cleavage assays

USP16 (human, full length (1-823), produced in-house as previously described[25b]), USP21 (human, cat. domain (196-565), Ubiquigent 64-0037-050) and OTULIN (human, full length (1-352), Ubiquigent 64-0048-050) were diluted to 2× final concentration (150 nM, 740 nM and 13 nM, respectively) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.6, 5 mM DTT and 1 mg mL^{-1} 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS). Subsequently, 40 μL of enzyme was mixed with 40 μL of 2× final concentration of synthetic or recombinant diubiquitin in the same buffer (30.8 μM or 27.3 μM respectively). The samples were incubated at 37 °C for 1, 2, 5, 10, 30 and 180 minutes followed by quenching using a sample buffer containing β-mercaptoethanol and subsequent SDS gel electrophoresis.

A sample of the reaction mixture was diluted with a sample buffer (3×), containing NUPAGE® LDS sample buffer (4×, Invitrogen) (900 μL), β-mercaptoethanol (90 μL) and water (210 μL), heated at 95 °C for 5 minutes and loaded on 12% NUPAGE® Novex® Bis-Tris Mini Gels (Invitrogen) using MES-SDS running buffer. A SeeBlue Pre-stained Standard (Invitrogen, LC5925) was used as a marker. InstantBlue™ (Expedeon) stains were scanned using a GE Healthcare Amersham Imager 600.

Conflicts of interest

HO is a shareholder of UbiQ Bio BV.

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