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.

Introduction

The development of native chemical ligation (NCL) by Kent and co-workers1 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 Yan2 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 Danishefsky3 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, Val, Thr, Leu, Pro, Glu, Arg, Asp, Gln and Trp 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.4 Another development of NCL was the use of thioester surrogates, such as peptide hydrazides,5 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.6 A well-known N-terminal modification is linear ubiquitination which is an important post-translational modification.7 NCL at internal methionine sites has been performed by applying homocysteine as a thiol donor followed by S-methylation.

![Fig. 1](image_url)
under carefully controlled conditions to prevent over- and undermethylation of homocysteine or mismethylation of other residues. 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. 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. 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. We here present the synthesis of \( \gamma \)-thionorleucine (Fig. 1B) and its application in NCL for N-terminal ubiquitination.

## Results and discussion

As \( \gamma \)-thionorleucine is installed onto peptides by means of solid-phase peptide synthesis (SPPS) we prepared appropriately protected \( \text{N-Boc, } \gamma \)-tert-butylsulfide \( \gamma \)-thionorleucine 1 (Fig. 1B), as depicted in Scheme 1. The synthesis commenced with the preparation of \( \text{tert-butyl } (S)-(\text{2-2,3-dimethyl-4-(2-oxoethyl)}\text{)oxazolidine-3-carboxylate } 2 \) according to literature procedures. 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 hydroxylation 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 \( \text{tert-butyl ester } 5 \) upon treatment with \( \text{O}-\text{tert-butyl } N,N'-\text{diisopropylcarbodiimide} \). 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. \( \text{tert-Butyl disulfide } 8 \) was obtained after treating compound 7 with \( \text{S}-\text{tert-butyl methane thiosulfonate, hydroxylamine and Et}_3\text{N} \), 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 N-terminal protein ubiquitination. We chose to ubiquitate 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. 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 \( \text{(e.g. coupled via Met-1) on the other hand play a key role in the regulation of NF-κB signalling and cell death.} \) We and others have developed chemical synthesis methods for the generation of all seven isopeptide-linked \( \text{(e.g. via a Lys side chain) Ub linkages using NCL which have led to many new biological insights.} \) 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 \( \gamma \)-thioNle-containing Ub (Scheme 2). The individual Ub proteins were synthesized by Fmoc-based linear SPPS. 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)- 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 \( \gamma \)-carbon atom in

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**Scheme 1** Synthesis of \( \gamma \)-thionorleucine. Reagents and conditions: (a) Et3MgBr, Et2O, 93%; (b) BnBr, NaH, \( \text{Bn}_2\text{N} \), DMF, 0 °C, 44%; (c) Jones reagent, acetone; (d) \( \text{O}-\text{tert-butyl } N,N'-\text{diisopropylcarbodiimide} \), THF, 60 °C, 70%; (e) Pd/C (10% wt), H2 (4 bar), MeOH, 48 h; (f) MsCl, Et3N, DCM, 63%; (g) KSAc, 65 °C, 18 h, 60%; (h) \( \text{S}-\text{tert-butyl methane thiosulfonate, HONH}_2\text{-HCl, Et}_3\text{N, MeOH, } 63\% \); (i) TFA; (j) \( \text{Boc}_2\text{O, K}_2\text{CO}_2\text{P, THF, H}_2\text{O, } 50\% \).
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. 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\(^{[38]}\)) 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-butyl disulfide moiety in γ-thioNle. Apparently, compared to the reported correspondingly thio-protected γ-thioLys, which is readily reduced by MPAA, the tert-butyl disulfide moiety in γ-thioNle is 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 ESIf) and SDS-PAGE analysis (Fig. S1 in the ESIf).

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...
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.

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-
High resolution mass spectra were recorded on a Waters XEVO-G2 Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (capillary voltage: 3.0 kV, desorption 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 CH₃CN on a Waters Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm); flow rate = 0.6 mL min⁻¹, runtime = 30.00 min, column T = 60 °C, mass detection: 50–150 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, 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 H₂O and B = 0.05% TFA in CH₃CN. 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 (4S)-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 Et₂O (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 H₂O and brine, dried over MgSO₄ and concentrated in vacuo. Purification by silica gel flash column chromatography (EtOAc/heptane 5%) yielded the title compound (6.4 g, 22.10 min: 5% B; 22.10: 5% B).

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, desorption 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 CH₃CN on a Waters Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm); flow rate = 0.6 mL min⁻¹, runtime = 30.00 min, column T = 60 °C, mass detection: 50–150 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.

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% CH₃CN and 0.1% formic acid in water and B = 1% water and 0.1% formic acid in CH₃CN. 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 dTub as well as the TCEP reduction, NCL and desulfurization reactions (as shown in Fig. 2) was 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, desorption 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 CH₃CN 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: 50–150 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.
NaHCO₃. The mixture was extracted with EtOAc (3×) and the reaction was quenched by adding isopropanol (0.85 g, 2.3 mmol) in acetone (140 mL) at 0 °C. Stirring was continued for 5 h and the reaction was quenched by adding isopropanol (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, 28.40, 27.06, 26.83, 26.33, 24.70, 23.38, 14.19 ppm. HRMS: calculated for C₁₇H₃₁NO₅ [M + H]+ 364.2488; found 364.2473.

Another portion of the compound (0.98 g, 2.7 mmol) and sodium hydride (1.2 eq., 1.1 g, 28.1 mmol, 60% dispersion in mineral oil) was added. The resulting mixture was stirred for 1.5 h at room temperature. Then saturated aqueous NH₄Cl was added and the mixture was extracted with Et₂O. The filtrate was concentrated in vacuo. The deprotected intermediate was co-evaporated with toluene (3×) and dissolved in dry DCM (70 mL), followed by the addition of Et₃N (3 eq., 21.3 mmol, 3.0 mL). The reaction solution was cooled to 0 °C and then methanesulfonfyl chloride (3 eq., 21.3 mmol, 1.65 mL) was added. After stirring for 3 h at room temperature, saturated aqueous NaHCO₃ was added to the reaction mixture. The mixture was then extracted with DCM (3×) and the combined organic layer was dried over MgSO₄ and concentrated in vacuo. Purification by silica gel flash column chromatography (EtOAc/heptane 5%) yielded the title compound as a mixture of diastereoisomers (1.7 g, 4.5 mmol, 63%). ¹H NMR (300 MHz, CDCl₃) δ = 1.35–1.60 (m, 2H, CH₂), 0.80–0.85 (m, 3H, CH₃) ppm. ¹³C NMR (75 MHz, CDCl₃) δ = 138.90, 128.43, 128.37, 127.77, 127.53, 79.09, 77.50, 76.07, 68.38, 66.98, 56.89, 38.16, 37.30, 36.81, 28.62, 28.40, 27.06, 26.83, 26.33, 24.70, 23.38, 14.19 ppm. HRMS: calculated for C₁₆H₃₁NO₇S [M+H]+ 382.1899; found 382.1897.

Jones reagent (2.5 eq., 25.5 mmol, 12.8 mL, 2 M in aqueous THF (25 mL), followed by the addition of a solution of diastereoisomers (4.04, 3.55 ppm. ¹³C NMR (75 MHz, CDCl₃) 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 C₁₄H₂₁NO₅S [M+H]+ 332.1899; found 332.1897.

tert-Butyl (2S)-4-(4-methylsulfonylloxoy)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 H₂ (4 bar, Parr apparatus) for 48 h. It was filtered through a Celite pad and concentrated in vacuo. The crude product was directly used in the next step without further purification.

tert-Butyl (2S)-4-(acetyltithio)-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 (3×) and the combined organic layer was washed with H₂O and brine, dried over MgSO₄ and concentrated in vacuo. The crude product was then 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-diisopropylisourea²¹ (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 Et₂O. 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 (0.98 g, 2.7 mmol) 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 then extracted with EtOAc (3×) and the combined organic layer was washed with H₂O and brine, dried over MgSO₄ and concentrated in vacuo. The crude product was used in the next step without further purification

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S-tert-butyl methane thiosulphonate (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 Et3N (4 eq., 10.8 mmol, 1.5 mL). The resulting reaction mixture was stirred at room temperature. After 2 h, additional Et3N 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 H2O, 1 M KHSO4, sat. NaHCO3 and brine. The organic layer was dried over MgSO4 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%). 1H NMR (300 MHz, CDCl3) 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, 12 × CH3), 1.59–1.47 (m, 1H, 2 × CH3), 1.36–1.32 (m, 18H, 6 × CH3), 1.23–1.20 (m, 9H, 3 × CH3), 0.94–0.87 (m, 3H, CH3) ppm. 13C NMR (75 MHz, CDCl3) δ = 171.55, 155.31, 155.14, 81.83, 79.44, 52.00, 50.26, 45.37, 38.10, 30.04, 28.25, 27.93, 25.93, 10.75 ppm. HRMS: calculated for C19H37NO4S2 [M + H]+ 408.2242; found 408.2239.

(2S)-2-[(tert-Butyloxycarbonyl)amino]-(4-tert-butylsulfinyl) 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 H2O (8 mL) and THF (8 mL) and cooled to 0 °C. K2CO3 (3 eq., 705 mg, 5.1 mmol) was added, followed by the addition of Boc2O (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 MgSO4 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%). 1H NMR (300 MHz, CDCl3) 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, βCH2), 1.91–1.49 (m, 2H, CH2), 1.43 (s, 9H, CH3), 1.30 (s, 9H, CH3), 1.02 (t, J = 7.3 Hz, 3H, CH3) ppm. 13C NMR (75 MHz, CDCl3) 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 C15H34NO8S2 [M + H]+ 352.1616; found 352.1631.

Synthesis of linear diubiquitin

Solid phase peptide synthesis. Ub(1-75, Nle1) 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/H2O/DODT/iPr3SiH (90.5/2.5/2; v/v/v; 100 µL) for 30 min at 37 °C.

Synthesis of Ub(1-76, Nle1)-S(CH2)2CO2Me (12). Resin-bound H-Ub(1-75, Nle1) was washed with DCM (3 × 5 mL). Boc2O (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, Nle1) 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, Nle1) 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(CH2)2CO2Me (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/H2O/DODT/iPr3SiH (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/H2O/phenol/iPr3SiH (90/5/2.5/2; v/v/v/v; 5 mL) for 3.5 hours. The protein was precipitated from ice-cold Et2O/n-pentane (3/1; v/v; 20 mL). The solution was centrifuged and Et2O/n-pentane (supernatant) was removed. The pellet was washed with Et2O (3 × 20 mL), the solution was vortexed, the suspension was centrifuged and Et2O was removed. The wash step was repeated twice. The pellet was dissolved in H2O/CH3CN/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, ThioNle1) (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 Et2O alternately and three times with Et2O.
The polypeptide was deprotected and detached from the resin by treatment with TFA/H2O/phenol/iPr3SiH (90.5/5/2.5/2; v/v/v/v; 2 mL) for 3 h. The reaction mixture was filtered directly into ice-cold Et2O/n-pentane (3/1; v/v; 15 mL) and the resin was spooled with TFA (2 x 2 mL). The solution was centrifuged and Et2O/n-pentane (supernatant) was removed. The pellet was washed with Et2O (3 x 15 mL), the solution was vortexed, the suspension was centrifuged and Et2O was removed. The wash step was repeated twice. The pellet was dissolved in H2O/CH3CN/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 H2O (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, Nle1)-S(CH2)2CO2Me (12) = 229.19 mg, 26.44 µmol, 52.88%. LC-MS: Rf: 4.74 min: ESI MS+ (amu) calcd: 8649.0 [M], found 8650.0 (deconv.).

Ub(1-76, ThioNle1) (11) = 32.81 mg, 3.79 µmol, 37.9%. LC-MS: Rf: 4.92 min: ESI MS+ (amu) calcd: 8676.1 [M], found 8686.0 (deconv.).

**Native chemical ligation of Ub(1-76, Nle1)-S(CH2)2CO2Me (12) and Ub(1-76, ThioNle1) (11).** Ub(1-76, ThioNle1) (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 Na2HPO4, 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, Nle1)-S-(CH2)2CO2Me (12, 1.5 eq., 7.64 mg, 0.88 µmol) was dissolved in 151.9 µL of aqueous buffer containing 8.0 M Gnd-HCl and 0.2 M Na2HPO4 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% Na2CO3 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. The reaction 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 at pH 7.55. LC-MS analysis (Program 2 and XEVO) was done to check the purity.

**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. 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 50 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 cm² dmol⁻¹) according to the equation:

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

where \((\theta)_{\text{obs}}\) 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[25]), 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] dimethyl-ammonio] 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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