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Scalable synthesis of γ-thiolysine starting from lysine and a side by side comparison with δ-thiolysine in non-enzymatic ubiquitination†

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We developed a scalable synthesis of γ-thiolysine starting straight from lysine. The application of γ-thiolysine was compared to δ-thiolysine in the chemical synthesis of K48 and K33 linked diubiquitin conjugates. Both γ- and δ-thiolysine were found to perform equally efficiently as handles for non-enzymatic ubiquitination.

Introduction

Ubiquitination is recognized as one of the most important posttranslational modifications and plays a role in a wide variety of processes ranging from proteolysis by the proteasome and transcription to the control of intracellular trafficking.[1] The attachment of ubiquitin (Ub) most commonly occurs through an isopeptide bond between the C-terminal carboxylate of Ub and the ε-NH_2 lysine side chain in the target protein or through another Ub molecule, although N-terminal ubiquitination is also possible. *In vivo*, Ub conjugation is initiated by the activity of ATP-dependent Ub-activating enzymes (E1s). They transfer Ub onto one of several Ub-conjugating enzymes (termed E2s) to form a thioester-charged E2 complex. Finally, Ub–protein conjugates are generated with the help of Ub ligases (E3s), which interact physically with the target substrates. The specific combination of E2 and E3 ligases determines the ubiquitination pattern of the substrate.[2] In addition, Ub conjugates and Ub polymers can be disassembled by any of approximately 100 deubiquitinating enzymes (DUBs) known today.[3] This results in a large number of possible (poly)Ub topoisomers, the shape, overall charge and size of which determine how they interact with the proteome.[4] Malfunction of Ub ligation or Ub removal contributes to the pathology of human disease.

To investigate the Ub code, sufficient amounts of well-defined Ub conjugates are needed that are difficult to obtain *via* biological methods alone, *i.e.* the characteristic isopeptide linkage between Ub and its target protein cannot be introduced using standard recombinant expression techniques. In addition, specific E2/E3 combinations for defined substrate ubiquitination are not available. Alternatively, non-enzymatic strategies have been developed for Ub conjugation *via* a native isopeptide bond that offer full control over ubiquitin chain topology. Typically these methods make use of the Chemoselective native chemical ligation (NCL) reaction between a Ub C-terminal thioester and a target peptide which contains a thiol ligation handle. Several non-proteogenic thiol-containing amino acid building blocks and their application in the synthesis of Ub conjugates have been described in the recent literature.[5] The first successful example of non-enzymatic Ub ligation makes use of a photolabile thiol-containing auxiliary group as the ligation handle for ubiquitination of a histone H2B derived peptide.[6] The method enabled the regioselective coupling of Ub *via* a native isopeptide bond, however, the slow reaction kinetics limit its wider applicability. This limitation can be overcome by the use of δ- or γ-thiolysine, lysine analogs that contain a thiol group at the δ- or of γ-carbon (Fig. 1), that can be used to effect non-enzymatic ubiquitination.

The γ- and δ-thiolysine ligation handles were independently reported by the groups of Liu,[7] Brik[8] and us.[9] However, a direct comparison between the use of both γ- and δ-thiolysine building blocks in Ub conjugation efficiency has not been described. Moreover, we have a need for a large scale synthesis of the ligation handle which is currently not available. The synthetic routes for γ- and δ-thiolysine building blocks as described by Liu and Brik, start from L-glutamic acid and L-aspartic acid respectively. Alternatively, we have recently published an efficient synthesis of δ-thiolysine starting from commercial δ-hydroxylysine, and used it to construct the complete set of lysine-linked diUb topoisomers.[10] Despite the straightforward route which could be scaled up to start with

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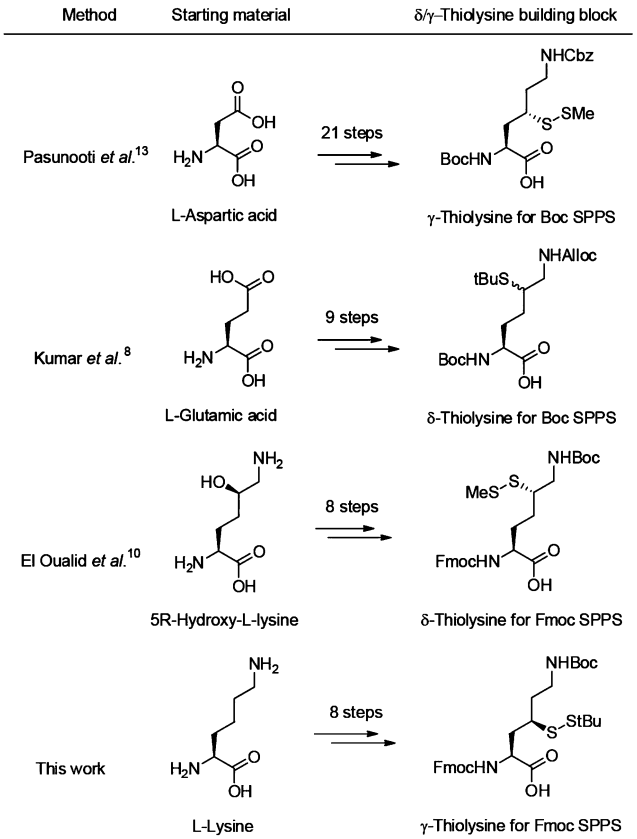
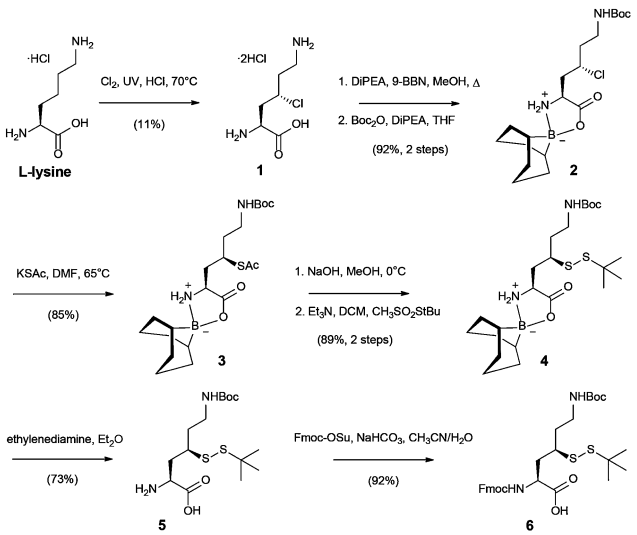


Fig. 1 δ/γ-Thiolysine building blocks for non-enzymatic ubiquitination.



Scheme 1 Synthesis of γ-thiolysine starting from lysine.

10 grams of δ-hydroxylysine, further scale-up is prohibitive because of the relatively high price of the starting material.

As the research aimed at unravelling the Ub code requires robust synthetic methods to produce substantial amounts of well-defined Ub-conjugates, we decided to investigate the large scale synthesis of γ-thiolysine and to compare its relative efficiency to δ-thiolysine in non-enzymatic ubiquitination.

Results and discussion

γ-Thiolysine synthesis

Interestingly, lysine could be selectively converted into γ-chlorolysine in just one synthetic step through direct free-radical chlorination with chlorine gas in concentrated acidic solution at elevated temperatures under continuous UV-irradiation (Scheme 1) following the procedure of Kollonitsch *et al.*[11] The chlorination reaction proved very reproducible and crude chloride **1** was isolated by precipitation directly from the reaction mixture in 21–25% yield.

NMR and LC-MS analysis showed that the crude product mainly consisted of **1**. We did not find evidence for the formation of substantial amounts of a second diastereomer, however, we did find a mass that indicated the formation of a doubly chlorinated lysine side product (see the ESI†). The final product (**1**) could be obtained in high purity as a single isomer in 11% yield after recrystallization from concentrated HCl. The expected *erythro* configuration[12] of the γ-chlorinated product was confirmed by X-ray crystallography. Suitable crystals for structure determination were obtained after conversion of **1** to the bis-Boc protected methylester of γ-chlorolysine (**7**) (see Fig. 2 and the ESI†). The convenience of the straightforward one-step procedure and the extremely low costs of both the starting material (lysine) and reagents (HCl, Cl_2) outweigh the modest yield for this remarkably selective chlorination reaction.

Chloride **1** was reacted with 9-borabicyclo[3.3.1]nonane (9-BBN) to selectively and simultaneously protect the α-amino and carboxylate groups. Subsequently, the ε-amino group was protected with a Boc group to yield **2** in 92% over 2 steps. The γ-mercapto group was introduced *via* substitution of the chloride with sodium thioacetate in DMF at 65 °C affording thioacetate **3** in near quantitative yield. Next, the thioacetate was hydrolyzed with 1 N NaOH in methanol and the free thiol was protected as the *S-tert*-butyl disulfide using *S-tert*-butyl methanesulfonothioate. Lastly, the borane protecting group was removed with ethylene diamine and the α-amino group was reacted with *N*-(9-fluorenylmethoxycarbonyloxy) succinimide to produce the suitably protected thiolysine building block **6**, which is fully compatible with standard Fmoc SPPS methods. The synthetic route as described here could be scaled up to produce 17.3 g of **6** starting from lysine in eight straightforward steps.

δ/γ-Thiolysine mediated diUb synthesis

The application of δ- and γ-thiolysine building blocks in Ub ligation has been well described in the literature[7,8,10,13–28] and both δ- and γ-thiolysine mediated ligations were found to proceed efficiently with rates comparable to unhindered junction sites in cysteine mediated NCL reactions.[29] A direct comparison between both δ/γ-thiolysine building blocks in the synthesis of Ub conjugates, however, is not available. We decided to look into this and performed a side by side comparison of γ- and δ-thiolysine in the synthesis of diUb conjugates. First, we used K48γ-thiolysine and K48δ-thiolysine Ub mutants for ligation to UbMESNa thioester[10] (Fig. 3). The

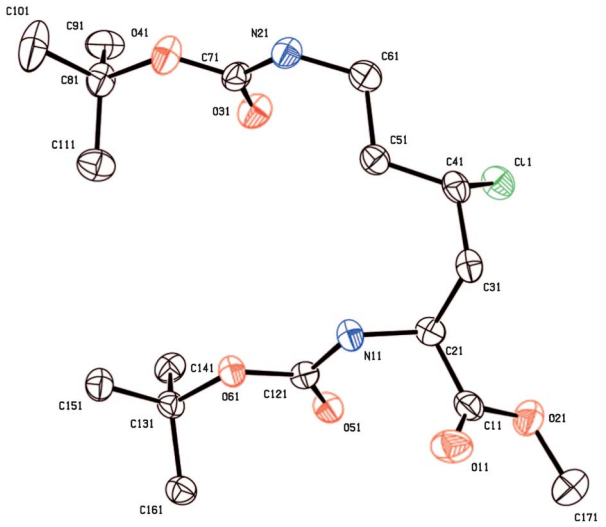


Fig. 2 ORTEP representation of the structure of bis-Boc protected γ-chlorolysine methylester (**7**), drawn at the 50% probability level. Only one of three independent molecules is shown. Hydrogen atoms are omitted for clarity.

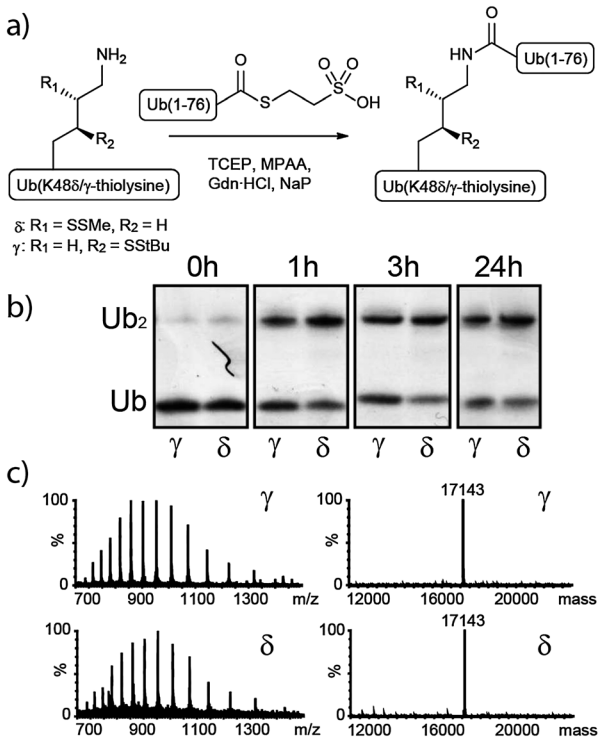


Fig. 3 Comparison of δ- and γ-thiolysine mediated synthesis of K48 linked diUb. (a) Reaction conditions, (b) gel analysis of the ligation reaction (both the K48δ/γ-thiolysine Ub mutant and hydrolysed UbMESNa run at the same hight) and (c) MS (ESI+) analysis of the reaction product.

ligation reactions were performed in parallel which enabled us to compare the performance of both δ/γ-thiolysine building blocks side by side. In two separate reaction vessels, the ubiquitin mutants were dissolved in sodium phosphate buffer (pH 7) containing 6 M Gdn·HCl and incubated with TCEP at 37 °C for 40 minutes. Next, a solution of UbMESNa thioester (1.25 equiv.) in 0.2 M sodium phosphate buffer (pH 7) containing 6 M Gdn·HCl and MPAA (100 mM) was added to the δ/γ-thiolysine mutant solutions. The ligation reaction mixtures were sealed under a nitrogen atmosphere and incubated at 37 °C. During the progress of the reaction, samples were taken for LC-MS and SDS-PAGE analysis. After 4 h all UbMESNa thioester was consumed. However, conversion to the desired diUb product was incomplete for both samples as was indicated by LC-MS analysis. Part of the Ub thioester was hydrolyzed and to ensure full consumption of the δ/γ-thiolysine Ub mutant starting materials, an additional amount of UbMESNa (1 equiv.) was added to each of the ligation mixtures after 6 h and the reactions were continued overnight. Although initial reaction rates seemed higher for the K48δ-thiolysine Ub mutant (see ESI†), both ligations went to completion within 24 h and both reactions performed equally efficiently in producing the diUb conjugate. The diUb products were isolated by preparative HPLC in almost equivalent yields of 48% for the γ-thiolysine diUb conjugate and 53% for the δ-thiolysine diUb conjugate.

The diUb synthesis was repeated using K33γ-thiolysine and K33δ-thiolysine Ub mutants which were ligated in parallel to UbMESNa under conditions as described above for the ligation of the K48δ/γ-thiolysine Ub mutants. Again, no clear difference in overall ligation efficiency between the K33γ-thiolysine and δ-thiolysine Ub mutants could be observed (see ESI†).

Free-radical desulfurization of diUb conjugates

After δ/γ-thiolysine mediated Ub ligation, the diUb products still contained an unnatural thiol moiety which can be removed *via* radical-mediated desulfurization.[30,31] Previously we have successfully applied V-50 (200 mM) as a radical initiator at 60 °C in the presence of glutathione (40 mM), and TCEP (250 mM) for complete desulfurization of diUbs after incubation overnight where desulfurizations using VA-044 at ambient temperature proceeded less efficiently.[10] Although desulfurization reactions with V-50 were efficient, the conditions used are not compatible with all reagents. For instance, we found that the commonly used fluorescent label 5-carboxytetramethylrhodamine (TAMRA) underwent demethylation during V-50 mediated desulfurization (see ESI†).

Therefore we reinvestigated the free-radical desulfurization of diUb ligation products using VA-044 under mild conditions. We found that synthetic diUb conjugates could be efficiently desulfurized at 37 °C using VA-044 (40 mM) in the presence of glutathione (40 mM) and TCEP (200 mM).

Unexpectedly, it was also found that when desulfurization reactions were performed using low GSH concentrations a minor side product was detected by MS analysis of the reaction mixture. The structure of this side product remains unknown but its mass was indicative for a Ub-deconjugation side reaction of the desulfurized diUb. Notably, the side reaction was only observed for δ-thiolysine modified diubiquitin and did not occur during the desulfurization of γ-thiolysine modified diubiquitin. The observed side reaction differs from the previously proposed retroligation side reaction which takes place under acidic conditions at elevated temperatures.[18]

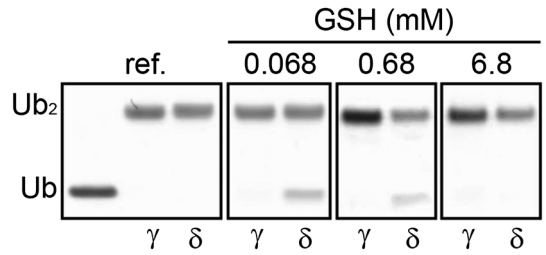


Fig. 4 Deconjugation side reaction of δ-thiolysine modified diUb during free-radical desulfurization. *Conditions*: K6δ/γ-thiolysine linked diUb (2 mg mL^{-1}), 0.2 M sodium phosphate (pH 6.5), 6 M Gdn·HCl, GSH (0.068–6.8 mM), TCEP (200 mM) and VA-044 (40 mM).

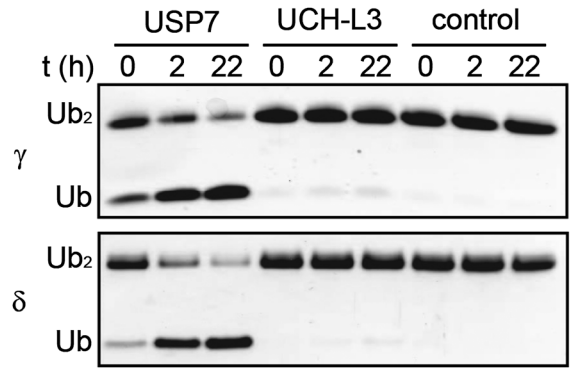


Fig. 5 DUB treatment of synthetic diUbs. *Conditions*: 40 nM DUB, 15 μM diUb, 20 mM Tris (pH = 7.5), 50 mM NaCl, 5 mM DTT.

It was decided to further investigate the deconjugation side reaction by SDS-page and LCMS analysis of the reaction mixture during free-radical desulfurization of K6γ-thiolysine and K6δ-thiolysine linked diUb in the presence of different concentrations of GSH. In the case of K6δ-thiolysine linked diUb desulfurizations at low GSH concentrations (<1 mM), LCMS analysis of the reaction mixture after 5 h showed a mass that was indicative for the hypothesized deconjugation side reaction. Additionally, SDS-PAGE gel analysis of K6δ-thiolysine linked diUb desulfurizations at low GSH concentrations (<1 mM) showed the appearance of a band corresponding in size to mono-Ub (Fig. 4). This result is in line with the deconjugation hypothesis. Apparently, the side reaction is radical initiated and presumable takes place when the lysine radical is not scavenged sufficiently fast.

Enzymatic hydrolysis of synthetic diUb by DUBs

To further verify their correct synthesis and biochemical function, the diUb conjugates were investigated by circular dichroism (CD) spectroscopy (see the see ESI†) and treated with deubiquitinating enzymes (DUBs). Two DUBs were used: HAUSP/USP7, which is known to deubiquitinate Ub chains and UCH-L3, which should not be able to hydrolyse diUb conjugates. Enzymatic deubiquitination was visualized using SDS-PAGE (Fig. 5). Efficient turnover of synthetic Ub conjugates was confirmed upon incubation with HAUSP/USP7. Conversely, treatment with UCH-L3 did not lead to substrate hydrolysis as expected. UCH-L3 activity was confirmed in a control experiment using UbAMC as substrate (see ESI†). No significant differences between the turnover of diUb conjugates that were obtained *via* either γ-thiolysine or δ-thiolysine mediated ubiquitination could be observed. These results demonstrate the high biochemical integrity of the synthetic diUb conjugates.

Conclusions

We have developed a new synthetic route for γ-thiolysine starting straight from lysine. The eight straightforward steps could be scaled up to produce substantial amounts of a suitably protected γ-thiolysine building block which is fully compatible with standard Fmoc SPPS methods. Non-enzymatic ubiquitinations using the γ-thiolysine handle were found to proceed as efficiently as δ-thiolysine mediated ligations in the synthesis of diUb conjugates.

The increased availability of γ-thiolysine will enable the efficient production of well-defined Ub-modified peptides and diUb conjugates. Access to this type of reagents, which could so far not be routinely produced (bio)chemically, is of paramount importance for study of the Ub code.

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