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Total Chemical Synthesis of All SUMO- 2/3 Dimer Combinations

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ABSTRACT

One hallmark of protein chemical synthesis is its capacity to access proteins that living systems can hardly produce. This is typically the case for proteins harboring post-translational modifications such as ubiquitin or ubiquitin-like modifiers. Various methods have been developed for accessing polyubiquitin conjugates by semi or total synthesis. Comparatively, the preparation of small-ubiquitin like modifier (SUMO) conjugates and more particularly of polySUMO scaffolds is much less developed. We describe hereinafter a synthetic strategy for accessing all SUMO-2/3 dimer combinations.

KEY-WORDS

Protein chemical synthesis, convergent, SUMO dimers, SUMO-2, SUMO-3, selective desulfurization.

Introduction

Protein chemical synthesis is a powerful mean for accessing proteins harboring a large variety of post-translational modifications.¹⁻²⁶ Among the recent achievements in the field, the production of polyubiquitin chains^{7-11,26,27} best illustrates the capacity of modern chemical synthesis to address the production of proteins of exceptional size and branching complexity with an atom-by-atom control of their structure.²⁸ Synthetic polyubiquitins open the possibility to investigate precisely how these modifications modulate protein structure and function.²⁹⁻³¹ The chemical synthesis of small ubiquitin-like modifiers (SUMO)¹²⁻¹⁴ and conjugates thereof^{15,16,21-25,32} has been described too but the works in this area are few and far between the reports describing the synthesis of polyubiquitin scaffolds. For example, the chemical synthesis of di-ubiquitin or tetra-ubiquitin chains has been reported by Brik and coworkers in 2011,^{8,9} while the chemical synthesis of homogeneous and branched polySUMO chains remains to be addressed.

Like ubiquitin (Ub), SUMO modifiers are attached by their C-terminal glycine residue to the side-chain of exposed lysine residues (Lys), usually located in SUMOylation consensus motifs within the target proteins. Five SUMO isoforms have been identified so far, among which SUMO-1-3 are constitutively expressed in all eukaryotic cells.^{33,34} SUMO modification

modulates the localization and the activity of the target proteins by changing protein-protein interactions and/or by competing with other lysine modifications such as ubiquitination or acetylation.^{35,36} SUMO-2/3 isoforms differ significantly from SUMO-1 by their sequence and by containing an internal SUMOylation site enabling the formation of polySUMO-2/3 chains *in vivo* (Fig. 1A). Naturally, deciphering the role of polySUMO-2/3 modification stimulates huge efforts worldwide but progress in this area is complicated by the fact that SUMO conjugates and, in particular polySUMO chains, are branched proteins that cannot be precisely programmed using classical recombinant techniques. Therefore, the chemical synthesis of well-defined polySUMO-2/3 scaffolds is a significant goal to pursue.

SUMO-2 and SUMO-3 feature a high sequence similarity and differ by only a few amino acids located in the 13-14 AA N-terminal tail (Fig. 1A). However, we recently showed that SUMO-2 and SUMO-3 have different conformational and biochemical properties.³² Contrary to SUMO-3, the structure of SUMO-2 is stabilized by a weak interaction occurring between the N-terminal tail and the SUMO core domain (Fig. 1B). Since SUMO-2 and SUMO-3 are not equivalent, polySUMO-2/3 chains might have their structure and function modulated by the proportion of each SUMO isoform within the chain. Another point to consider is the presence into the tail of SUMO-2 of the SUMOylation site, Lys11. Although the exact nature of the SUMO-2 tail-core interaction remains to be established, its existence raise the question of the impact of Lys11 modification on the structure and function of polySUMO-2/3 chains. Indeed, the properties of polySUMO-2/3 conjugates might vary not only with the type of SUMO isoforms that they are made of, but also with the order by which they are concatenated. For example, the properties of SUMO-2-SUMO-3 and SUMO-3-SUMO-2 dimers might not be same because the N-terminal tail of SUMO-2 is unmodified in the former case, while it hosts a SUMO modifier in the latter case (Fig. 1C).

An important step for investigating how the composition of polySUMO-2/3 chains impacts their properties is to access these molecules in homogeneous form and substantial amount, i.e., at mg scale. We describe hereinafter a rapid and robust access to all combination of SUMO-2/3 dimers, which, are challenging to produce due to their size (~20 kDa). This strategy gives also access to C-terminal SUMO-2/3 dimer hydrazides. By being useful protein thioester surrogates,³⁷⁻⁴⁰ SUMO-2/3 dimer hydrazides allow to consider in the future the chemical synthesis of polySUMO scaffolds of even higher complexity.

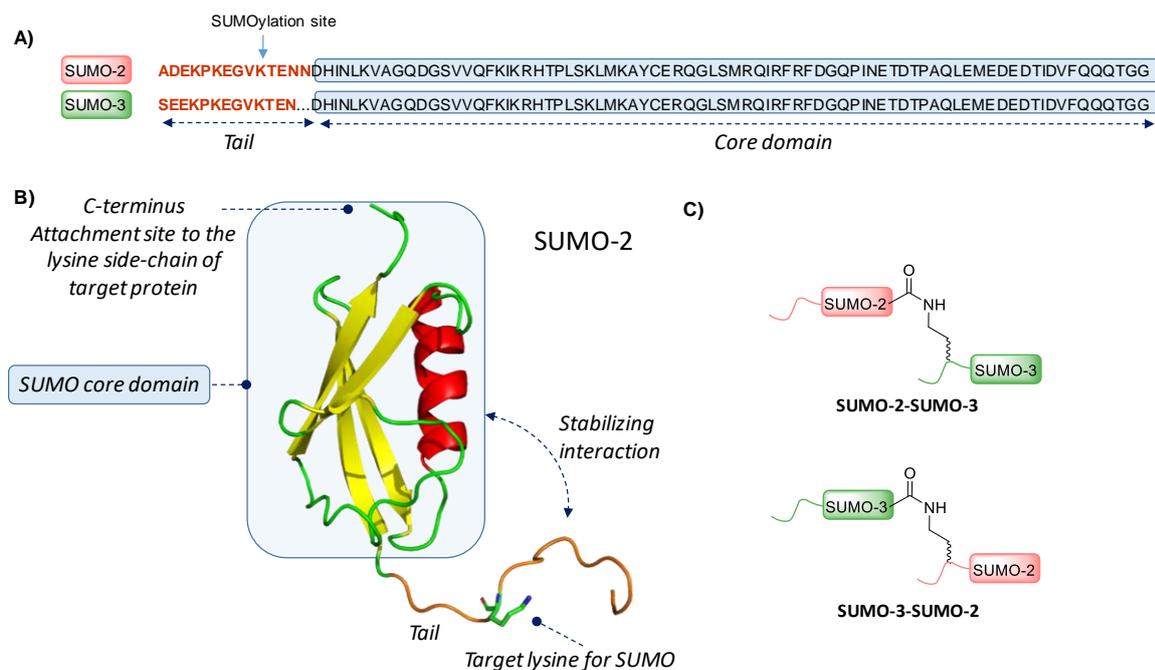


Figure 1. A) Primary structure of SUMO-2 and SUMO-3 proteins showing the tail and core domain. B) Tertiary structure of SUMO-2 (NMR structure, pdb entry 2N1W). C) Schematic representation of SUMO-2-SUMO-3 and SUMO-3-SUMO-2 dimers.

Results and discussion

Synthetic strategy

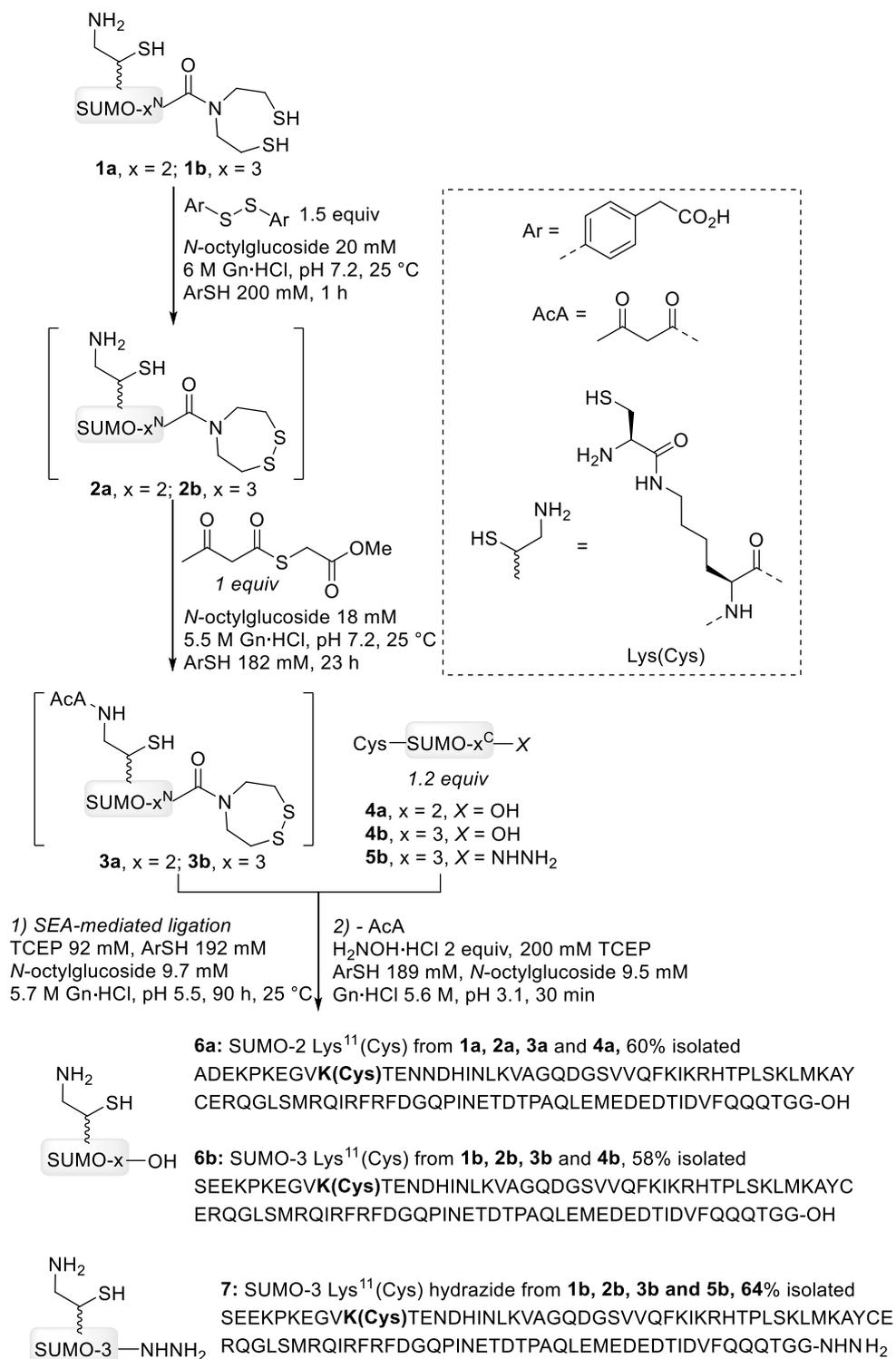
The strategy shown in Fig. 2 was adopted for accessing all four SUMO-2/3 dimers (illustrated with the synthesis of SUMO-2-SUMO-3). According to this strategy, the SUMO-2/3 dimers are assembled in one-pot by ligating three peptide segments. Two of these are produced by solid phase peptide synthesis (SPPS) owing to their length (~45AA) and are used to assemble the left SUMO domain highlighted in pink. The third segment in green used in the one-pot process corresponds to the right SUMO domain. It is produced in one-pot too and features an internal Lys(Cys) residue at position 11 to attach the SUMO domain coming from the first ligation step. Therefore, SUMO-2/3 dimers are accessed through two successive one-pot processes. This strategy enables limiting the number of resolute HPLC purification steps that are known to induce important mass losses.^{41,42}

The SUMO-2/3 dimers produced by this way need to be desulfurized subsequently to remove the thiol function in-between the two SUMO domains.⁴³ Desulfurization is a common post-

ligation treatment applied after assembly of Ub conjugates, with the objective of removing the thiol function utilized for assembling the isopeptidic bond. Usually, any Cys residue present in the polypeptide is concomitantly converted to Ala. This can be useful when Cys residues are introduced intentionally in place of Ala residues to facilitate the assembly of the product by an NCL^{44,45}/desulfurization approach.⁴³ In the case of SUMO-2/3 dimers, a global desulfurization is not recommended because we recently showed that the desulfurization of the central Cys residue and its conversion into Ala results in the destabilization of the SUMO-2/3 domains, SUMO-3 being especially sensitive to this modification.³² Therefore, the synthesis of SUMO-2/3 conjugates requires selectively removing the thiol amino acid used to form the isopeptidic bond by NCL. The performance of a selective desulfurization of SUMO conjugates featuring one SUMO domain has been described in a few studies.^{22,24,32} These reactions are performed in native conditions. The selectivity relies on a difference in accessibility between the thiol to be removed, which is solvent exposed, and the Cys thiol internal to the SUMO domain, which is buried in the hydrophobic core of the folded SUMO domain. However, extending the principle of a selective desulfurization to SUMO dimers for which the isopeptidic bond is located in between two folded domains had to be established.⁴⁶ Note that the burying of isopeptidic bonds in some polyubiquitin conjugates has been observed by Liu and coworkers.¹⁰

Lys[Cys(Trt)]-OH for position 11. Such bifunctional peptide cannot be used as such for the assembly of the SUMO-2/3 Lys(Cys) domains because it can cyclize or oligomerize during ligation with segment **4a,b**. The SEA^{on} group was inactivated *in situ* and prior to ligation by adding 4-mercaptophenylacetic acid (MPAA) disulfide to the reaction vessel. MPAA disulfide oxidizes the SEA^{on} group into the corresponding SEA^{off} disulfide by a thiol-disulfide exchange mechanism. The MPAA arylthiol produced during this step contributes to the catalysis of subsequent ligation reactions.⁴⁷ Then, a thioester derivative of acetoacetic acid (AcA) was added to protect the Lys(Cys) residue through an NCL reaction.⁴⁸ A tight control of the stoichiometry is required during this step as an excess of AcA thioester would inevitably cap the Cys segment introduced in the subsequent step. The AcA-protected segment **3a,b** was not isolated and was ligated directly with segment **4a,b** by adding an excess of MPAA and tris(2-carboxyethyl)phosphine (TCEP). Finally, the AcA group was removed in one-pot by adding two equivalents of hydroxylamine hydrochloride to the ligation mixture. The presence of non-ionic detergent *N*-octylglucoside in the reaction mixture was found to be mandatory to avoid the partial precipitation of the peptides. Peptide segments SUMO-2/3 Lys(Cys) were isolated in good overall yields (58-60% after HPLC purification) and purity (see Supporting Information, Fig. **SX**). The strategy was also applied successfully to the synthesis of a SUMO-3 Lys(Cys) derivative featuring a C-terminal hydrazide group.

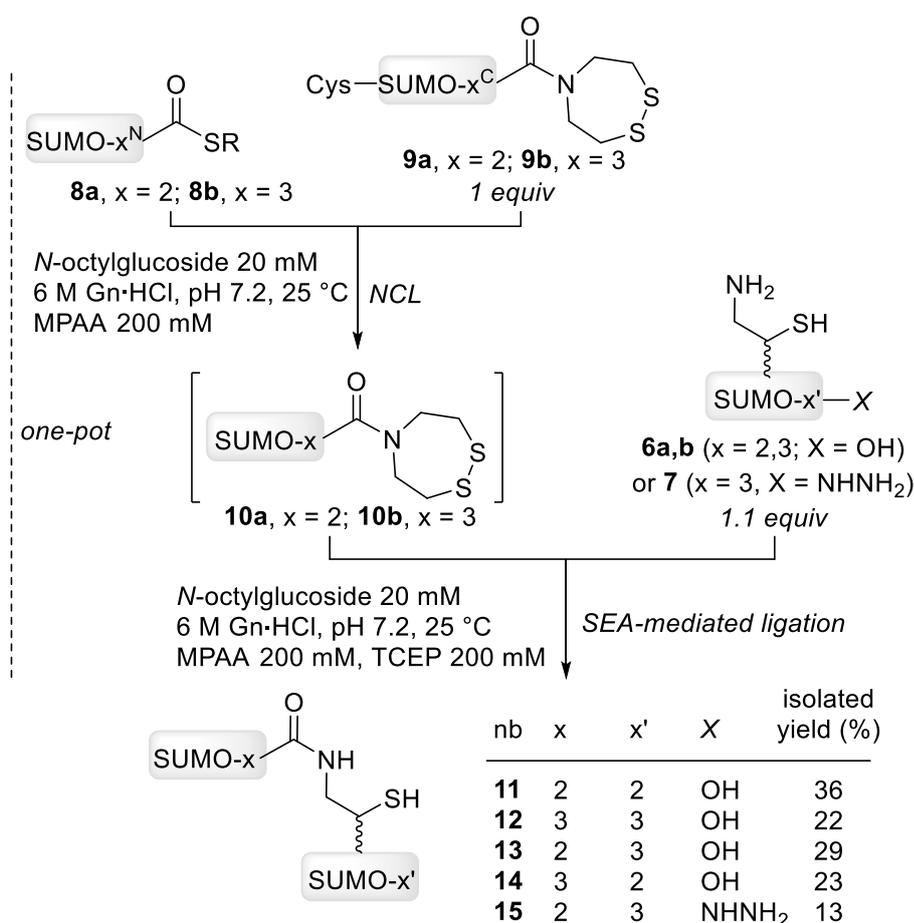
Scheme 1. One-pot synthesis of SUMO-2 or 3 peptide segments equipped with a Lys(Cys) residue at position 11



Assembly of SUMO-2/3 dimers

The successful synthesis of peptide segments **6a,b** and **7** set the stage for the assembly of the SUMO-2/3 dimers according to Scheme 2.⁴⁹ As for the synthesis of SUMO-2/3 Lys(Cys) domains, the use of *N*-octylglucoside and 6 M Gn·HCl as additives ensured the solubilization of starting materials and products throughout the process. The different 21 kDa SUMO-2/3 dimers were produced successfully and isolated by HPLC in excellent purity (Fig. 3). They migrate to an apparent molecular weight of 30 kDa by SDS-PAGE (Fig. 4). SUMO-2/3 dimers are composed of 183-185 amino acid residues and as such represent the largest proteins produced so far using *N,S*-acyl shift thioester surrogates.^{50,51}

Scheme 2. Chemical synthesis of SUMO-2/3 dimers



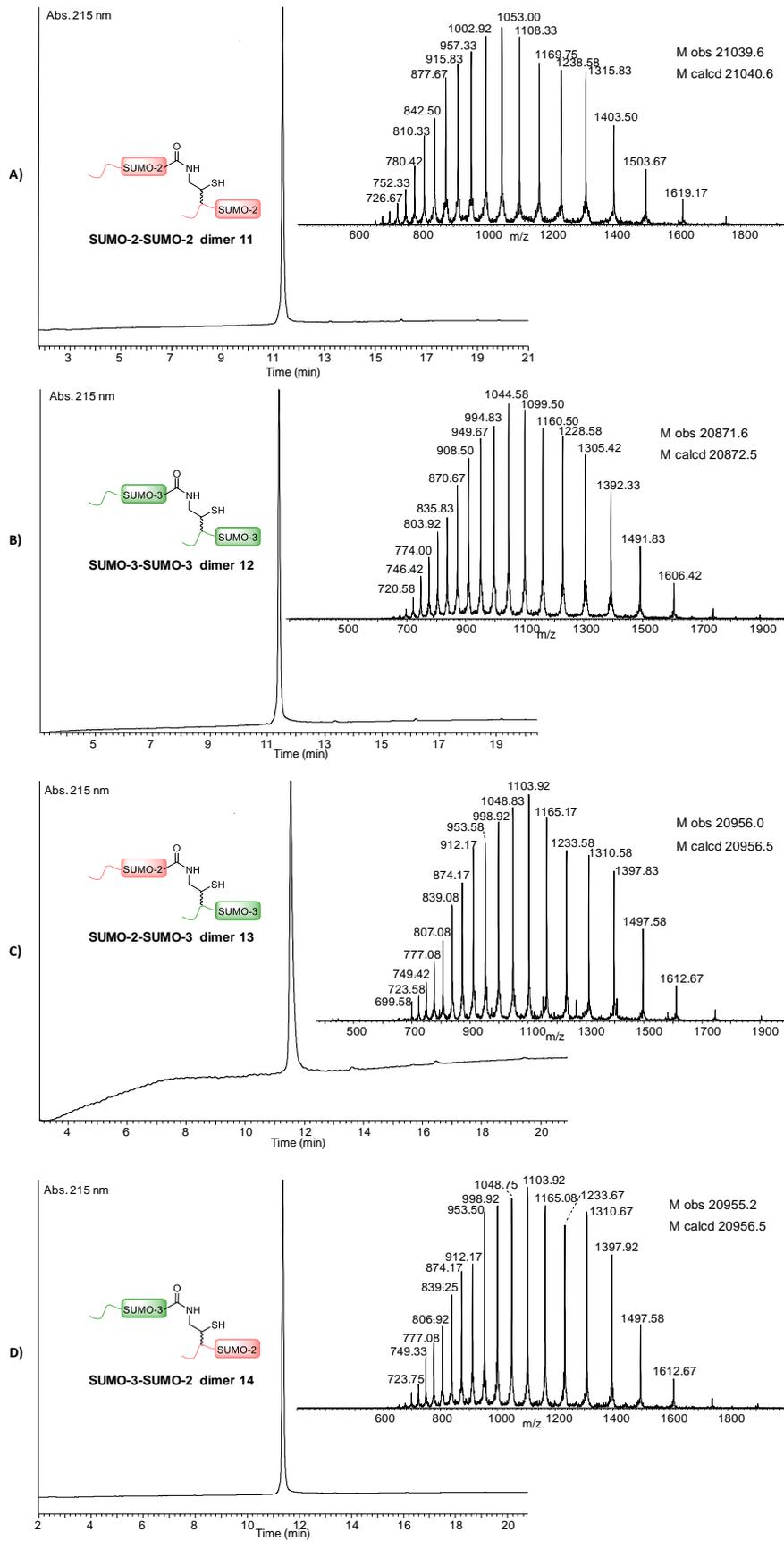


Figure 3. LC-MS analysis of isolated SUMO-2/3 dimers 11-14.

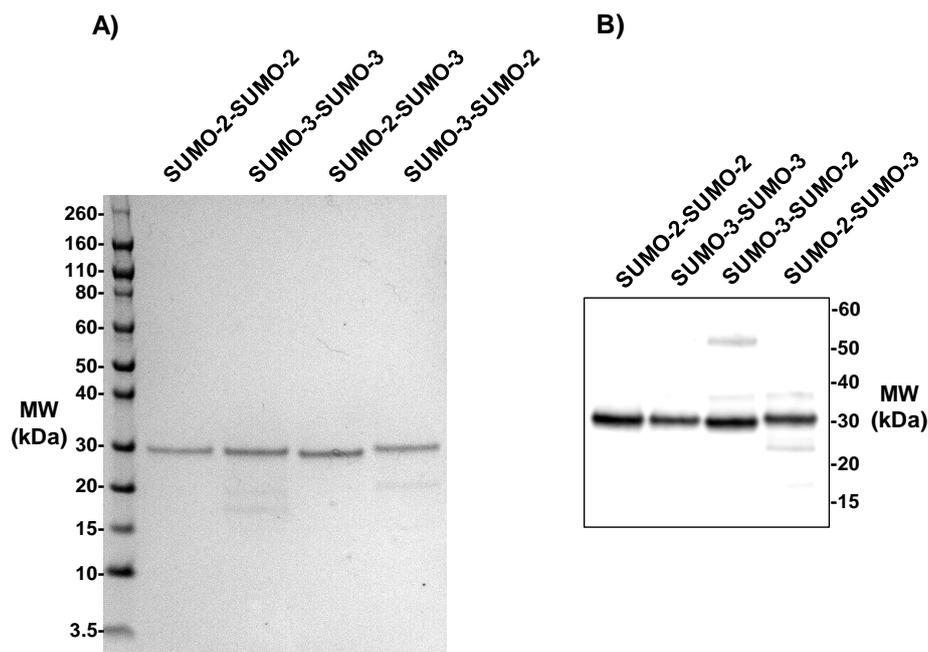
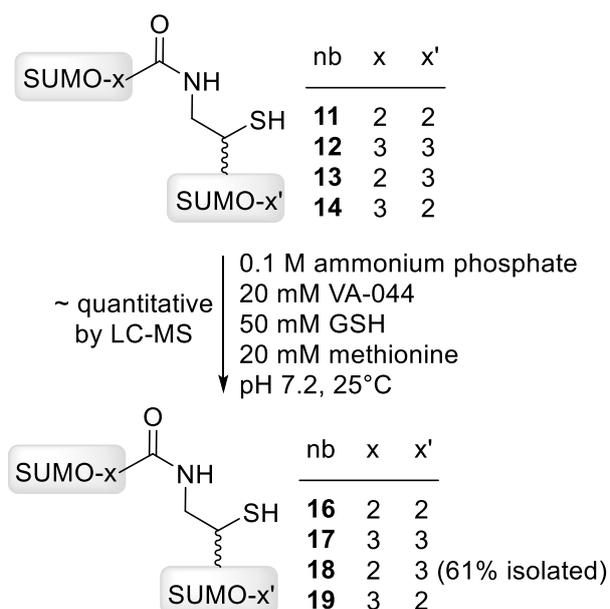


Figure 4. SDS-PAGE analysis of SUMO-2/3 dimers. A) Coomassie staining, 1 μ g of each protein. B) Western-blot analysis using anti-SUMO-2/3 polyclonal antibody, 100 ng of each protein. MW: apparent molecular weight markers.

Selective desulfurization of SUMO-2/3 dimers 11-14

The successful synthesis of SUMO-2/3 dimers set the stage for the desulfurization experiments. The TCEP-induced desulfurization of all four dimers in native conditions proceeded with a total selectivity for the Cys thiol in between the two SUMO domains, as demonstrated by extensive proteomic analysis of the desulfurized products (see Supporting Information). In a control experiment, SUMO 2/3 dimer **13** was desulfurized in 6 M Gn·HCl. In this case, all Cys residues were converted to Ala. Thus under native conditions, the Cys thiol used for linking the SUMO domains remains highly accessible to the solvent whenever the composition of the dimer. The efficient protection of internal Cys residues within SUMO domains under native conditions shows that both SUMO domains are properly folded. SUMO-2-SUMO-3 dimer **13** was desulfurized on a preparative scale to produce the desulfurized analog **18** in 61% isolated yield.

Scheme 3. Selective desulfurization of SUMO-2/3 dimers 11-14



Analysis of SUMO-2-SUMO-3 dimers

SUMO-2-SUMO-3 dimers **13** and **18** were first analysed by circular dichroism (CD) as shown in Fig. 5. The CD analysis indicates that the secondary structure of the dimers and in particular their α -helical content (18%) are highly similar. The α -helical content found for the dimers is consistent with the CD α -helical content of individual SUMO-2 and SUMO-3 domains which is 15%.³² Moreover, the CD spectra of SUMO dimers **13** and **18** are similar to the reconstituted CD spectrum for SUMO-2-SUMO-3 dimer calculated using the experimental CD spectra obtained for individual SUMO-2 and SUMO-3 domains.³²

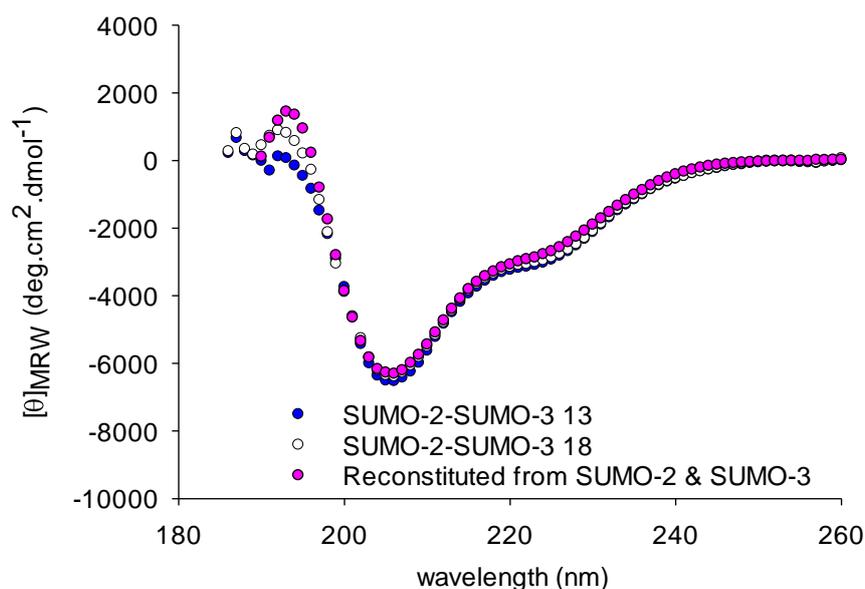


Figure 5. CD analysis of SUMO-2/3 dimers **13** and **18**. Comparison with a reconstituted spectrum calculated using the CD spectra of individual SUMO-2 and SUMO-3 proteins (data taken from ref⁵²).

We further examined the cleavage of SUMO-2-SUMO-3 dimer **18** by Sentrin-specific protease 1 (SEN1⁵²) and 2 (SEN2^{53,54}), which are human SUMO-1, 2 and 3 specific deconjugating enzymes (Fig. 6).^{55,56} SUMO-2-SUMO-3 dimer **18** was cleaved by both SENP enzymes to produce SUMO-2 and SUMO-3 proteins that migrate by SDS-PAGE analysis at an apparent molecular weight of ~15 kDa. The breakdown of SUMO-2-SUMO-3 dimer **18** into individual SUMO-2/3 proteins was confirmed by LC-MS analysis of the cleavage mixture. Taken together, the CD signature of SUMO-2-SUMO-3 dimer **18** and its cleavage by SENP1 and SENP2 shows that the dimer is properly folded and dfunctional.

CONCLUSIONS

We describe in this report a simple and efficient access to SUMO-2/3 dimers which is compatible with the production of SUMO dimers featuring a C-terminal hydrazide functionality. The preparation of SUMO dimer hydrazides opens potentially the possibility to access more elaborated polySUMO conjugates by exploiting the thioester surrogate properties of the hydrazide group. The desulfurization of the thiol amino acid used to produce the

isopeptidic bond has been performed with a high degree of selectivity, regardless the SUMO-2/3 dimer combination examined. The production of SUMO-2/3 dimers at the mg scale is an important step toward further investigations aiming at determining the effect of SUMO dimer composition and order of concatenation on their biophysical and biochemical properties.

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Supporting Information Available

The Supporting Information is available free of charge on the ACS Publications website at DOI: #####.

Experimental procedures and characterization data for all peptides and proteins (LC-MS, MALDI-TOF, CD and proteomic analyses) (PDF).

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