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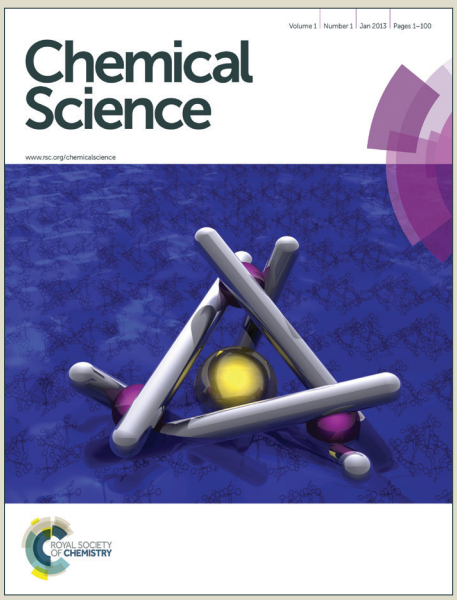

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ARTICLE TYPE

A novel PEG-based solid support enables the synthesis of > 50 amino acids peptide thioesters and the total synthesis of a functional SUMO-1 peptide conjugate

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A *bis*(2-sulfanylethyl)amino PEG-based resin enabled the synthesis of large (~50 Aa) SEA or thioester peptides using Fmoc-SPPS. These peptide segments permitted the first total synthesis of a 97 amino acids long SUMO-1-SEA peptide thioester surrogate and of a functional and reversible SUMO-1 peptide conjugate.

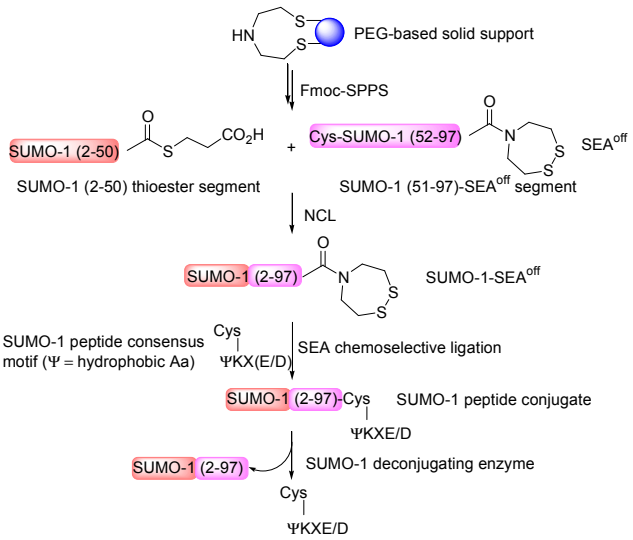
INTRODUCTION

The synthesis of proteins gives access to large peptides or small proteins with an atom-by-atom control of their structure. Today, proteins are usually assembled by the sequential ligation of unprotected peptide segments.[1] Native chemical ligation (NCL)[2, 3] or expressed protein ligation[4, 5] of a C-terminal peptide thioester with an N-terminal cysteinyl peptide is the most popular amide bond forming reaction used in the field, although other complementary methods are emerging.[6-10]

The large diversity of chemical methods available now for producing modified peptides in high purity is increasingly used for the total or semisynthesis of modified proteins which cannot be easily produced using biological methods.[11] Proteins featuring well-defined post-translational modifications at specific sites such as phosphorylation[12] or glycosylation[13] belongs to this family. Another post-translational modification which has attracted a lot of attention recently is ubiquitylation, which has been adressed using total[14-20] or semisynthetic approaches.[21-23] The preparation of ubiquitin conjugates has also been achieved using non native chemoselective ligation methods such as oxime,[24] disulfide,[25] 1,2,3-triazole[26, 27] or thioether[28, 29] bond forming reactions.

Recent work described also the total synthesis of the 85 amino acids-long ubiquitin-fold modifier 1 (UFM1) using sequential α-ketoacid-hydroxylamine ligations.[9] Besides this, the total synthesis and introduction of other protein modifiers is faced with significant challenges. In particular, the total synthesis of small ubiquitin-like modifier (SUMO[30]) proteins and SUMO conjugates remains to be addressed. The C-terminal glycine residue of SUMO modifier is linked to the side chain amino group of a lysine within the target protein through an isopeptidic bond. This reversible modification is implicated in many important physiological functions such as transcription and DNA repair.[31] SUMO modifier is thought to act by modulating the interactions of the target protein with its partners. However, the molecular mechanisms implicated in this regulation are still poorly understood mostly because SUMO conjugates are highly labile within cells, making their isolation highly challenging.[32]

Up to now, SUMO-1[33] or SUMO-2[34] conjugates were prepared by semisynthesis using recombinant SUMO proteins and non-cleavable 1,2,3-triazole linkers. We show for the first time that protein total synthesis can give access to homogeneous, functional and reversible SUMO-1 conjugates.



Scheme 1 Synthesis of long SEA peptide thioester surrogates enabled the assembly of a functional SUMO-1 conjugate.

The work reported here relies on the chemistry of *bis*(2-sulfanylethyl)amido (SEA) thioester surrogate (Scheme 1).[35-38] First, the assembly of SUMO-1 proteins described in this work required to push the limits of current technology by addressing the Fmoc-SPPS synthesis of long (~ 50 Aa) SEA peptides and peptide thioesters. This was achieved by developing novel SEA PEG-based solid supports which are compatible with standard Fmoc-SPPS. We report also the assembly of SUMO-1 isoform modified at the C-terminus by a latent SEA thioester surrogate (Scheme 1). This protein could be isolated or alternately ligated

in situ with a SUMO-1 peptide consensus motif to produce a 12.5 kD SUMO-1-peptide conjugate in a one-pot process. Importantly, the tertiary structure and functionality of SUMO-1 modifier within the conjugate as well as its reversible nature was demonstrated by the specific cleavage of the glycyl-cysteinyl peptide bond with a recombinant SUMO-1 deconjugating enzyme.

RESULTS AND DISCUSSION

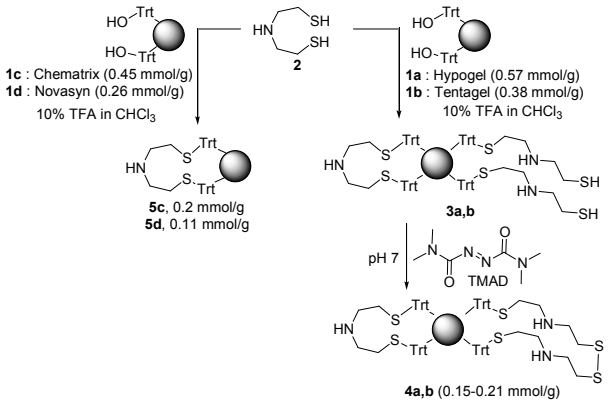
The primary sequence of SUMO-1 protein features only one internal cysteine residue in position 51 (Scheme 1). A Cys residue was appended to the target lysine side-chain within the SUMO-1 peptide consensus motif to enable the assembly of the SUMO-1 peptide conjugate in three pieces using the NCL or related reactions. Lys(Cys) peptides have also been used successfully by other groups for accessing to ubiquitinated proteins.[39, 40] An alternative would be to use a target peptide featuring a γ[41, 42] or δ-mercaptolysine[14, 15, 18]residue. However, the access to a native SUMO-1 conjugate using this latter strategy would require protecting the Cys51 thiol group during the desulfurization of the mercaptolysine residue.

Given the position of the cysteinyl residues in the target SUMO-1 conjugate, the assembly can start with the SUMO-1 peptide consensus motif or alternately with the SUMO-1 (2-50) peptide segment. The latter approach was selected in this work to enable the isolation of SUMO-1 protein featuring a latent thioester surrogate functionality at the C-terminus. We chose the *bis*(2-sulfanylethyl)amido[35-38] (SEA) *N,S*-acyl shift system[43] as latent thioester surrogate, which in its cyclic form called SEA^{off} is compatible with the NCL reaction used for assembling the SUMO-1 protein.[37, 38] This N-to-C assembly strategy resembles to the one developed by Brik and coworkers for the synthesis of an ubiquitin latent thioester.[17] These authors used a C-terminal *S*-protected *N*-methylcysteine as latent thioester,[44] which was deprotected and exchanged by 3-mercaptopropionic acid (MPA) in a subsequent step.

The strategy depicted in Scheme 1 is faced with the challenge of synthesizing 50 amino acids long peptide segments featuring a C-terminal SEA or alkylthioester group. The synthesis of large peptide thioesters using standard Fmoc-SPPS is one of the major bottlenecks for addressing the synthesis of large proteins or complex scaffolds such as SUMO-1 conjugates.[45] C-terminal *bis*(2-sulfanylethyl)amido (SEA) peptides can be synthesized by Fmoc-SPPS[35] and used as precursors for the synthesis of peptide alkylthioesters.[46-48] Up to now, we used a *bis*(2-sulfanylethyl)amino polystyrene (SEA PS) resin for the Fmoc-SPPS of SEA peptides.[35, 49] Although SEA PS resin permitted the synthesis of various SEA peptides composed of up to 35 amino acids, we anticipated that the synthesis of the large SUMO-1 peptide segments would require a modern PEG-based solid support instead which is more adapted for the synthesis of long peptides.

SEA PEG-based solid supports were prepared by reacting the trifluoroacetate salt of *bis*(2-sulfanylethyl)amine **2** with commercially available trityl alcohol PEG-based resins as shown in Scheme 2. We targeted solid supports of type **5** for which both thiol groups are linked to the solid support and protected by a trityl group as for SEA PS resin. However, SEA PS resin was prepared by reacting the *bis*(2-sulfanylethyl)amine **2** with tritylchloride polystyrene resin in *N,N*-dimethylformamide (DMF).[49] In this case, an efficient protection of both thiol groups and the formation of cross-links within the PS beads was facilitated by the large excess and high loading of the starting tritylchloride polystyrene resin (~ 1.4 mmol/g, 10 equiv). The loading of commercially available trityl alcohol PEG-based resins (0.25-0.6 mmol/g) is significantly less than the loading of tritylchloride polystyrene resins. Therefore, partial protection and incomplete cross-linking of the *bis*(2-sulfanylethyl)amine **2** leading to the formation of solid supports of type **3** was a potential issue. Indeed, the reaction of dithiol **2** with PEG-based solid supports **1a,b** in the presence of trifluoroacetic acid yielded solid supports of type **3** as shown by the development of a positive Ellman assay. The presence of free thiols during the Fmoc-SPPS can be troublesome. Therefore, solid supports **3a,b** were treated with *N,N,N′,N′*-tetramethyl-azodicarboxamide (TMAD) at pH 7 to mask the thiol groups as internal disulfides. Indeed, the resulting solid supports **4a,b** furnished a negative Ellman assay, while the chloranil assay was strongly positive. The coupling of Fmoc-Ala-OH using HATU/DIEA activation followed by the quantification of the Fmoc group indicated a loading of 0.15-0.21 mmol/g. The usefulness of solid supports **4a,b** for the Fmoc-SPPS of SEA peptides was verified by the automated synthesis of model peptide ILKEPVHGA-SEA (49-54% crude, 18-21% isolated by HPLC).

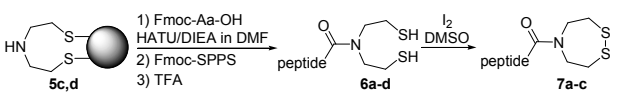
Interestingly, solid supports **1c,d** furnished directly SEA resins of type **5** as shown by the development of a negative Ellman assay and of a strongly positive chloranil assay, with a loading of ~0.11-0.2 mmol/g. Solid supports **5c,d** proved to be useful for the Fmoc-SPPS of long SEA peptides as shown later. Consequently, the study of solid supports **4a,b** was discontinued.



Scheme 2 Synthesis of SEA PEG-based solid supports

Four different peptides were synthesized to illustrate the usefulness of SEA PEG resins **5c,d** for accessing to large SEA peptides (Scheme 3, Table 1). Peptides **7a** and **7b** correspond to SUMO-1 (2-51) and SUMO-1 (52-97) respectively and were used in this work for the assembly of SUMO-1 proteins. Peptides **6d** and **7c** are derived from hepatocyte growth factor (HGF). Peptide **7c** illustrates the compatibility of SEA PEG resins **5d** for the automated batch peptide synthesis, whereas the other peptides including the 52 amino acids peptide **6d** were assembled using an automated column peptide synthesizer. Usually, the oxidation of

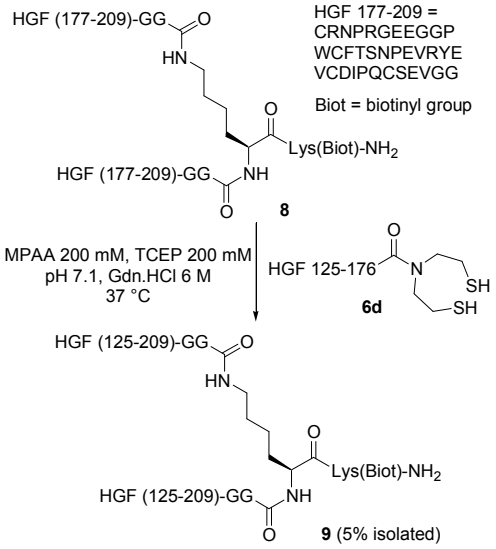
SEA peptides of type **6** (SEA^{on} form) into cyclic disulfides **7** (SEA^{off}) is performed prior to the HPLC purification to avoid the potential *N,S*-acyl shift of SEA^{on} group during the elution step.[49] The oxidation of peptides **6a-c** by iodine yielded successfully peptides **7a-c**. In contrast, this procedure induced the aggregation of peptide **6d**, which was therefore purified directly by HPLC.



a : SDQEAKPSTE DLGDKKEGEY IKLKVIGQDS SEIHFKVKMT THLKKLKESY
b : C(S*t*Bu)QRQGVPMNS LRFLFEGQRI ADNHTPKELG MEEEDVIEVY QEQTGG
c : GQRKRRNTIH EFKKSAKTT LIKIDPALKI KTKKVNTADQ
d : IRNC(S*t*Bu)IIGKGR SYKGTVSITK SGIKC(S*t*Bu)QPWSS MIPHEHSFLP SSYRGKDLQ ENY

Scheme 3 Fmoc-SPPS of SEA peptides

SEA peptide **6d** was further ligated with a 72 amino acids divalent cysteinyl peptide **8** in the presence of 4-mercaptophenylacetic acid (MPAA,[50] 200 mM) and *tris*(2-carboxyethyl)phosphine (TCEP, 200 mM) as shown in Scheme 4 to give the ~ 20 kD branched peptide **9** (Fig. 1). Although the reaction was complicated by the partial aggregation of **6d** and of the ligation product **9** (see Fig. S29), thereby explaining the modest yield for the ligation reaction (5%, 22% per step), this example illustrates the utility of SEA ligation for accessing to large peptide scaffolds



Scheme 4 SEA ligation of peptide **6d** with divalent Cys peptide **8** yielded the ~ 20 kD branched peptide **9**.

The successful SPPS of SUMO-1 peptides **7a,b** set the stage for the assembly of SUMO-1-SEA^{off} protein **11** (Scheme 5). First, SEA^{off} group of peptide **7a** was exchanged by MPA at pH 4 in the presence of TCEP to produce SUMO-1 (2-50)-MPA thioester segment **10**. The exchange proceeded very cleanly (see Fig. S7A). The conversion reached 87% of after 24 h, after which the reaction mixture was purified by HPLC to yield 28% (3.0 mg) of peptide thioester **10**. The modest yield is mainly due to the small scale of synthesis. Gratifyingly, ligation of MPA thioester **10** with SEA^{off} segment **7b** in the presence of MPAA (200 mM) at pH 7 furnished SUMO-1-SEA^{off} protein **11** and the mixed disulfide SUMO-1-SEA^{off}-MPAA **12** as a minor component after HPLC purification (22%, Fig. 2). The presence of the mixed disulfide **12** is not problematic since the reduction of the Cys51-MPAA disulfide bond is expected to occur during the subsequent activation of the C-terminal SEA^{off} group in the presence of TCEP.

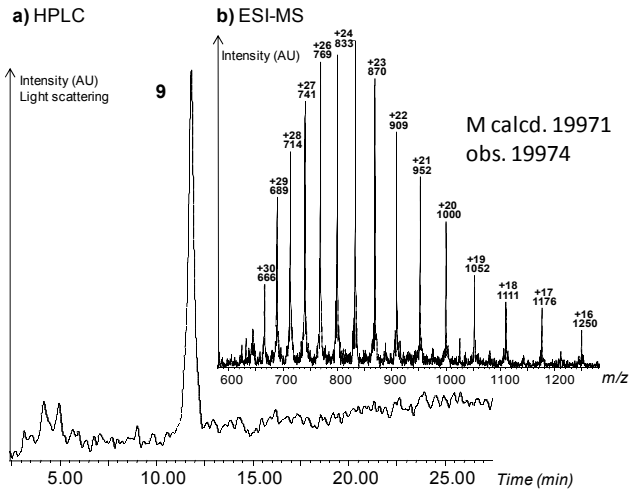
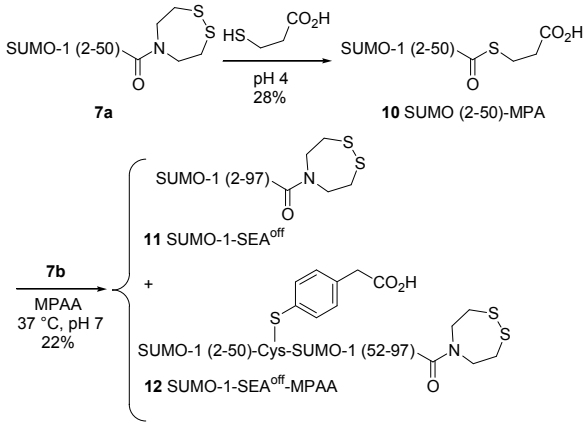


Figure 1 HPLC and ESI characterization of peptide **9**.

Table 1. Fmoc-SPPS of SEA peptides using SEA PEG-based resins.

peptide	protein	type of automated synthesizer	resin	nb of Aa	yield (%)[a]
7a	SUMO-1 (2-51)	column	**5c**	50	6
7b	SUMO-1 (52-97)	column	**5c**	46	3
7c	HGF (31-69)	batch	**5d**	40	7
6d	HGF (125-176)	column	**5c**	52	5

[a] HPLC purified.



Scheme 5 Assembly of SUMO-1-SEA^{off} protein **11**.

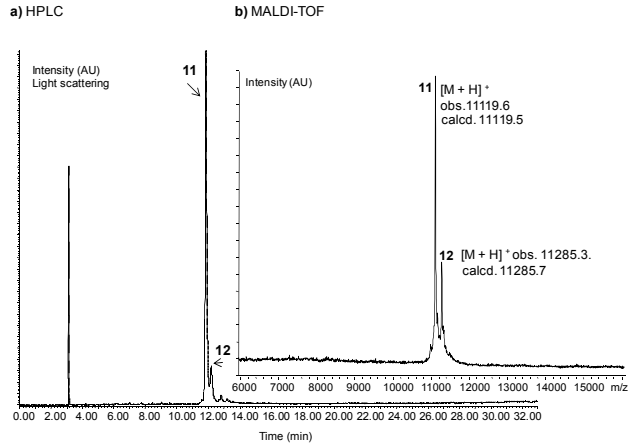
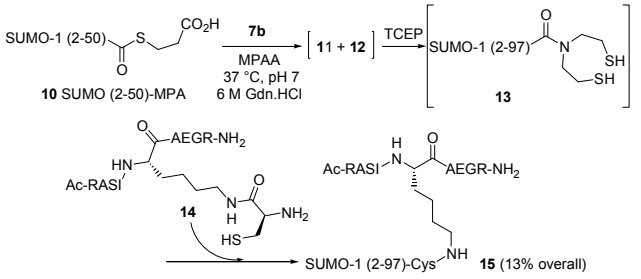


Fig. 2 Characterization of SUMO-1-SEA^{off} protein **11**.

The successful synthesis of SUMO-1-SEA^{off} protein **11** set the stage for the one-pot assembly of SUMO-1 conjugate **15**, for which SUMO-1 modifier is attached to the side-chain of the target lysine residue through a cysteine linker (Scheme 6). Model peptide **14** features a ΨKX(E/D) sumoylation consensus motif where Ψ is a hydrophobic amino acid residue and X any amino acid. These positions are occupied in peptide **14** by Ile and Ala residues respectively. In the one-pot assembly process, the formation of SUMO-1-SEA^{off} protein **11** was followed by the addition of TCEP (200 mM) and of peptide **14** in slight excess. The HPLC trace of the crude reaction mixture (Fig. 3a) shows that the target conjugate **15** is by far the major product, which was purified by HPLC with a 13% overall yield (Fig. 3b). The structure of conjugate **15** was confirmed by ESI (Fig. 3c) and chiefly MALDI-TOF mass spectrometry analysis (Fig. 3d, see also Figs. S21-23 for the in source fragmentation data).



Scheme 6 One-pot assembly of SUMO-1 conjugate **15**

The solution NMR structure of SUMO-1 is available and shows an α-helical content of 10%.[51] Consequently, the α-helical content for conjugate **15** was expected to be ~ 9% if the target peptide is considered to be random coil. The solubilization of conjugate **15** in water furnished the circular dichroism (CD) spectrum shown in Fig. 4a. The ellipticity at 222 nm indicated an α-helix content of 8.6%, that is a value very close to the expected one. To unambigously demonstrate the presence of a folded SUMO-1 domain within conjugate **15**, we used ubiquitin-like specific protease 1 (Ulp1) as a sensor probe. Ulp1 is a yeast cysteine protease which cleaves specifically the isopeptidic bond linking SUMO-1 to its target proteins.[32, 52] The crystal structure of Ulp1 in complex with a yeast SUMO-1 protein analog has

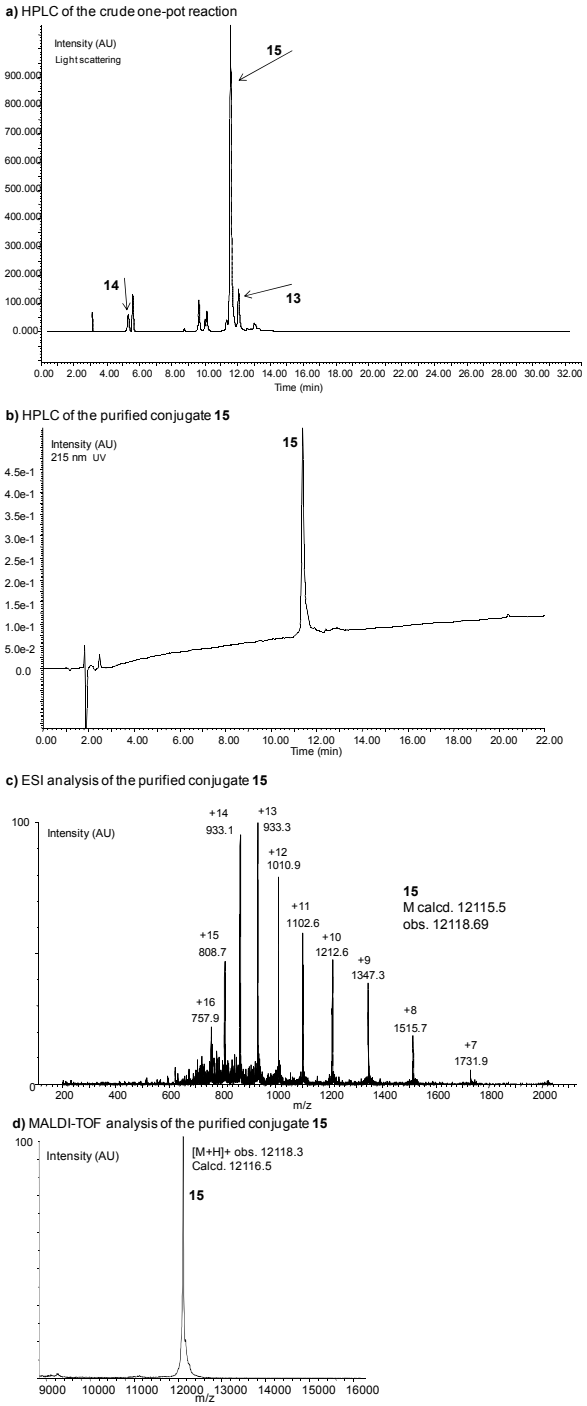


Fig. 3 Characterization of SUMO-1 conjugate **15**

been solved and shows an extensive contact surface area of about 2400 Å^2 between the two proteins.[53] Thus, Ulp1 requires the SUMO-1 domain to be folded to exert its protease activity on SUMO-1 conjugates. Interestingly, incubation of SUMO-1 conjugate **15** with recombinant Ulp1 enzyme resulted in the specific cleavage of the Gly97-Cys bond within the conjugate (Fig. 4b,c). HPLC analysis of the cleavage mixture and MALDI-TOF analysis of the eluted peaks showed the exclusive formation of SUMO-1 protein and of peptide **14** (Fig. 4b).

Taken together, these data show the functionality of the SUMO-1

domain within conjugate **15** and the efficiency of the synthetic strategy depicted in Scheme 1.

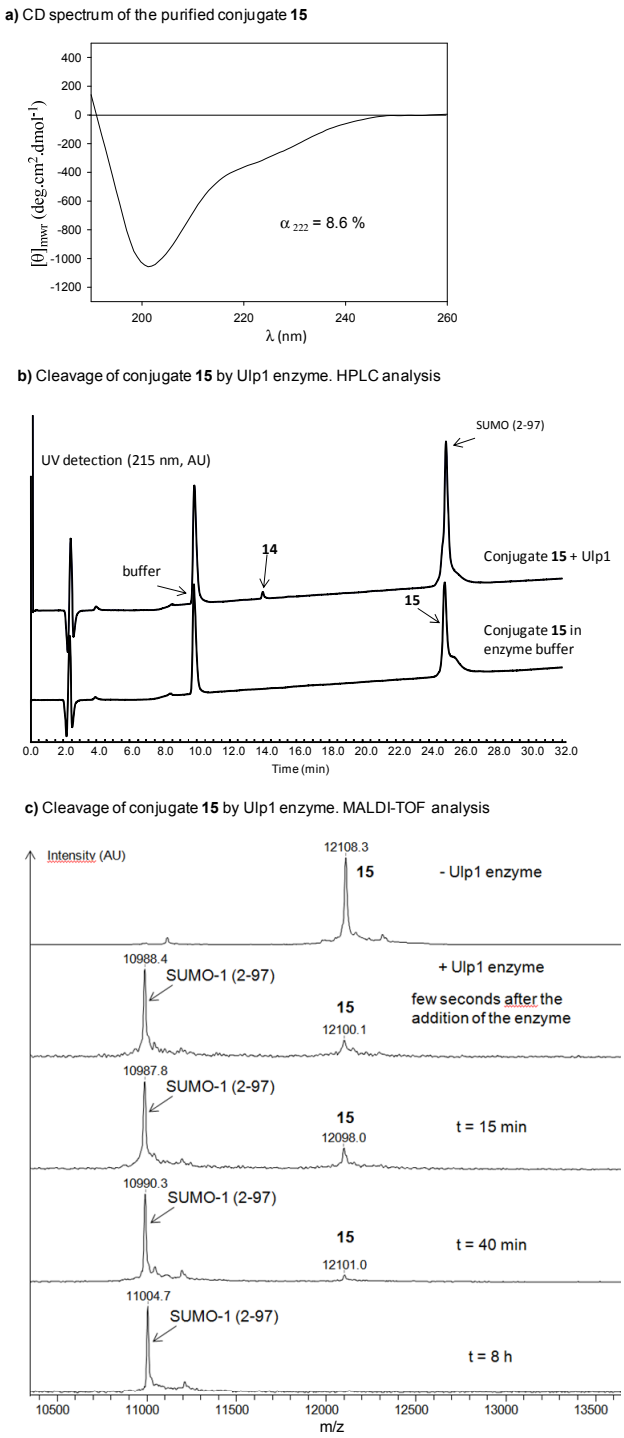


Fig. 4 CD and biochemical characterization of SUMO-1 conjugate **15**. a) CD spectrum in water, b) microLC analysis of the cleavage of **15** by Ulp1 enzyme, c) MALDI-TOF monitoring of the cleavage of **15** by Ulp1 enzyme.

Conclusions

In conclusion, we report a novel *bis*(2-sulfanylethyl)amino (SEA) PEG resin which enables the Fmoc-SPPS of > 50 amino acids long SEA peptide thioester surrogates. The access to such long peptide thioester surrogates enables the synthesis of complex protein targets and extents the limits of protein total synthesis. In particular, we describe for the first time the total synthesis of a folded, functional and reversible SUMO-1 conjugate. Access to SUMO conjugates proteins should facilitate the biochemical and structural implications of this post-translational modification.

Notes and references

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