Amino Acids
DOI 10.1007/s00726-012-1397-5

ORIGINAL ARTICLE

Supported oligomethionine sulfoxide and Ellman's reagent for cysteine bridges formation

Luisa Ronga · Pascal Verdié · Pierre Sanchez · Christine Enjabal · Amélie Maurras · Magalie Jullian · Karine Puget · Jean Martinez · Gilles Subra

Received: 28 June 2012 / Accepted: 30 August 2012

Abstract A large number of bioactive peptides are cyclized through a disulfide bridge. This structural feature is very important for both bioactivity and stability. The oxidation of cysteine side chains is challenging not only to avoid intermolecular reaction leading to oligomers and oxidation of other residues but also to remove solvents and oxidant such as dimethyl sulfoxide. Supported reagents advantageously simplify the work-up of such disulfide bond formation, but may lead to a significant decrease in yield of the oxidized product. In this study, two resins working through different mechanisms were evaluated: Clear-Ox, a supported version of Ellman's reagent and Oxyfold, consisting in a series of oxidized methionine residues. The choice of the supported reagent is discussed on the light of reaction speed, side-products formation and yield considerations.

Keywords Oxidation · Supported reagent · Disulfide bond · Cyclic peptide · Methionine · Trisulfide · Oxyfold · Clear-Ox

L. Ronga and P. Verdié are equal contributors to the study.

Electronic supplementary material The online version of this article (doi:10.1007/s00726-012-1397-5) contains supplementary material, which is available to authorized users.

L. Ronga · A. Maurras · M. Jullian · K. Puget
Genepep SA, Les Coteaux Saint Roch, 12 Rue du Fer à Cheval, 34430 St Jean de Védas, France

P. Verdié · P. Sanchez · C. Enjabal · J. Martinez · G. Subra (✉)
Institut des Biomolécules Max Mousseron IBMM, UMR 5247, Université Montpellier 1, Université Montpellier 2, CNRS, 15 avenue Charles Flahault, 34000 Montpellier, France
e-mail: gilles.subra@univ-montp1.fr

Introduction

Peptides are attracting, increasing attention as therapeutics due to their role as mediators of key biological functions associated with their low toxicity and high specificity. In 2011, more than 60 peptide drugs were on the market (four of them have reached global sales over US$ one billion), and nearly, ten times more are still in clinical development (Reichert et al. 2010). A significant number of them are peptides cyclized through a disulfide bridge (Fong et al. 1964; Bauer et al. 1982; Ducreux et al. 2000; Williams et al. 2008; Hruby et al. 1990). Part of the success in developing peptides as drug candidates is due to facile automation of solid phase peptide synthesis (SPPS) protocols. Compared to solution phase synthesis, SPPS improves the speed and increases the crude purity, using excess of reactants that can be easily removed by filtration. The real bottleneck of peptide synthesis lays in post-cleavage work-up including purification and treatments carried out in solution such as disulfide bridge formation. Disulfide bonds are very common in natural cysteine containing peptides. They are key structural elements, they prevent enzymatic degradation and modulate the activity of the target peptide. Their synthesis is challenging, regarding the formation of oxidation by-products and intermolecular reactions leading to dimers or oligomers. Diluted conditions ([peptide] <0.1 mM) are routinely used to minimize the unwanted intermolecular bridges. It implies the removal of a large amount of aqueous buffer and eventually additional oxidant such as dimethyl sulfoxide (DMSO) when the atmospheric oxygen is not efficient enough to promote disulfide bond formation. This constitutes a bottleneck for large-scale peptide production as well as for library syntheses.

The use of solid-supported reagents is now accepted as a highly valuable tool for the organic chemist. It constitutes

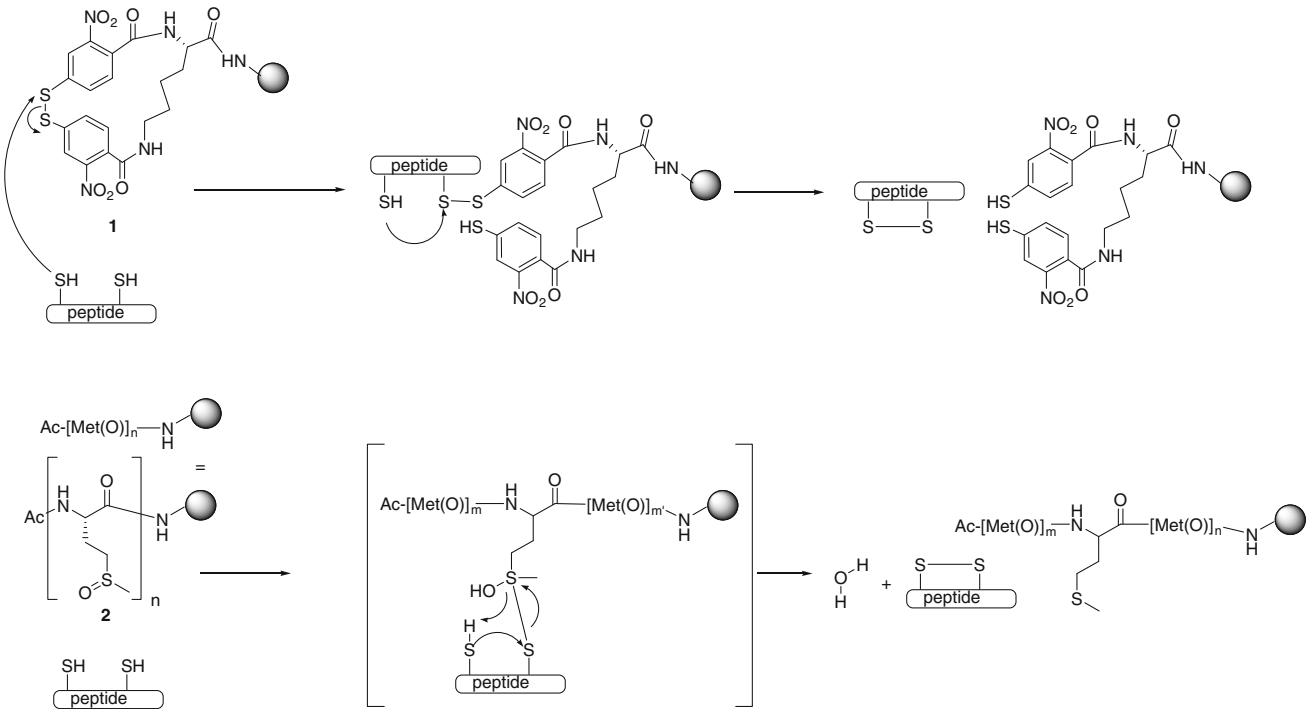
Published online: 12 September 2012

also an attractive alternative to reactions requiring high dilution. Indeed, the pseudo-dilution phenomenon proceeds through immobilization of an intermediate on the support that is advantageously released by intramolecular reaction. In this context, two supported reagents were designed to promote disulfide bond formation in cysteine containing peptides. The first is a polymer-bound version of 5,5′-dithiobis(2-nitrobenzoic) acid (Ellman's reagent) (Ellman 1959), linked to a polymer matrix through a lysine residue (Annis et al. 1998; Hargittai et al. 2000). This oxidant reagent linked to a PEG-based cross-linked polymer is commercially available as Clear-Ox (**1**, Scheme 1) (Darlak et al. 2004). More recently, a series of methionine sulfoxide immobilized on a solid support were successfully used as a supported substitute of DMSO. Oxyfold reagent (Verdie et al. 2011) (**2**, Scheme 1) is obtained by methionine *N*-carboxyanhydride polymerization initiated by free amine groups on PEGA resin. It is worth noting that these two reagents were prepared on hydrophilic polymer [polyethylene glycol-polyamide (Meldal et al. 1994) and ethoxy acrylate (Kempe and Barany 1996)] whose swelling is good even in polar solvents and aqueous buffers used to solubilize unprotected reduced peptides.

Disulfide bridge formation proceeds through different oxidation mechanisms (Scheme 1) that are pH dependent. Indeed, we postulate that Oxyfold-mediated oxidation is comparable to DMSO oxidation in which the protonation of sulfoxide yields an unstable adduct that is rapidly consumed by a thiolate ion to give the disulfide. Protonation favored by acidic pH is counterbalanced by the decrease in thiolate formation leading to an overall pH-independent oxidation. On the contrary, it has been described that Ellman's-mediated oxidation is more efficient at high pH values (pH $\geq$ 6) due to the stabilization of thiolate nitrobenzoic leaving group formed during disulfide exchange (Annis et al. 1997, 1998). Depending on peptide sequence, different buffers are preferred for solubilization, implying a defined pH range. Beside the speed of reaction, special attention should be paid to the recovered yield of crude and purified oxidized peptide as well. Indeed, resin beads can release side-products that will contaminate the crude mixture or even entrap desired materials (covalently or not), resulting in a decrease in yield and purity. In this study, these issues were examined through the disulfide bond formation of two model peptides using Clear-Ox, Oxyfold, and DMSO in solution, in a both qualitative and quantitative analyses.

Materials and methods

The following abbreviations were used: BTC, bis(trichloromethyl)carbonate; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; TFA, trifluoroacetic acid; HBTU, *O*-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium



Scheme 1 Proposed mechanism for intramolecular disulfide formation mediated by supported methionine sulfoxide and Ellman's reagent

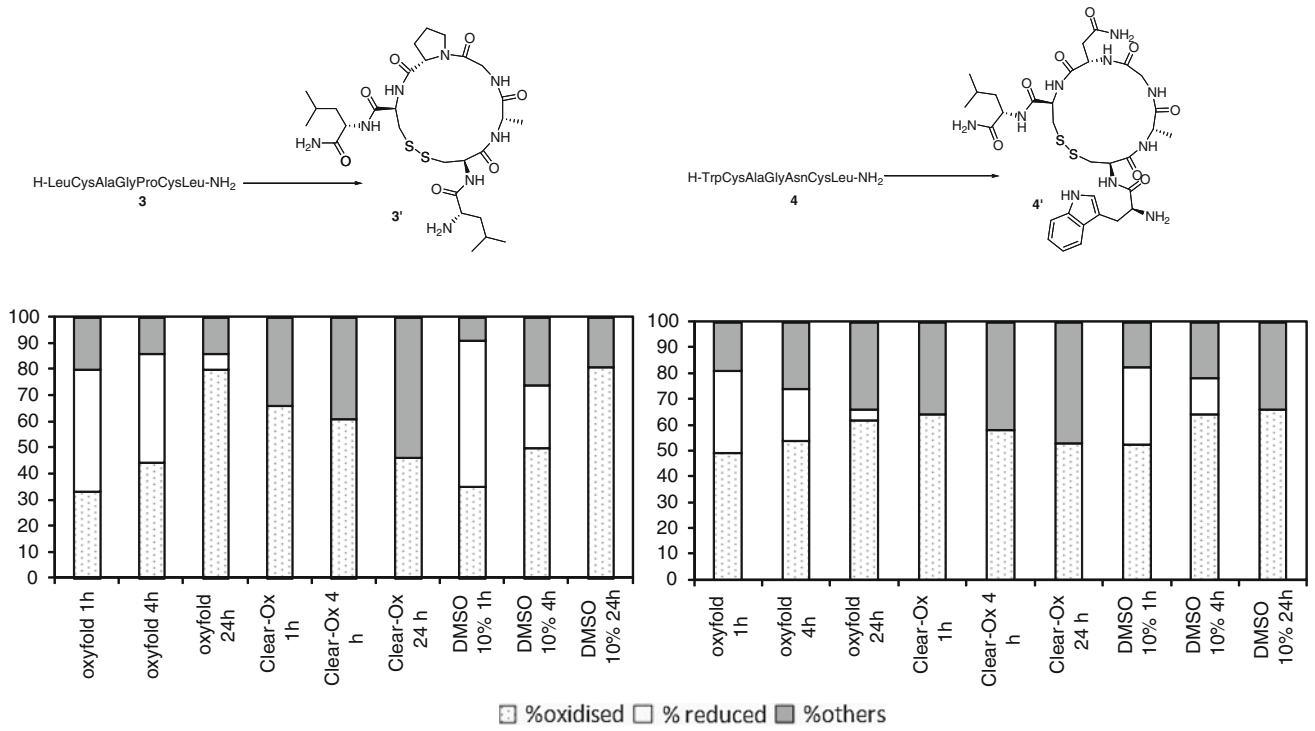


Fig. 1 Oxidation of peptides **3** and **4** with DMSO, Clear-Ox and Oxyfold. Proportions are determined by integration of peaks during LC analysis at 214 nm

hexafluorophosphate; HOBt, hydroxybenzotriazole. Other abbreviations used are those recommended by the IUPAC-IUB Commission.

Materials

All the solvents were obtained from Carlo Erba and Sigma Aldrich, and were used without purification. Protected amino acids, HBTU were purchased from Iris Biotech GmbH. Clear-Ox resin was purchased from Peptides International Inc. Other reagents were purchased from Aldrich and Lancaster.

Peptide synthesis

Peptide chains were elongated by means of a Liberty™ Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC, USA), an additional module of Discover™ (CEM Corporation, Matthews, NC, USA) that combines microwave energy at 2,450 MHz to SPPS following the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (tBu) strategy.

Syntheses were conducted on a 0.25-mmol scale on Fmoc-Rink-Amide PS resin (0.640 mmol/g). All coupling reactions were performed with 5 equiv. amino acid in DMF (0.2 M), 5 equiv. of HBTU in DMF (0.5 M), and 10 equiv. of DIPEA in NMP solution (2 M).

Fmoc deprotections were performed with a 20 % piperidine DMF solution.

Each deprotection and coupling reaction was performed with microwave energy and nitrogen bubbling. Microwave cycle was characterized by two deprotection steps: the first one was for 30 s, the second one for 180 s. All coupling reactions were for 300 s.

Washing steps were performed between coupling and deprotection steps. Three washes of DMF were used between steps.

Cleavage of Rink amide-PS resin

Peptides were cleaved from the resin using 25 mL of cleavage cocktail (trifluoroacetic acid/water/triisopropylsilane 95/2.5/2.5 v/v/v) per well for 3 h. After removal of the resin by filtration, the cleavage cocktail was concentrated by evaporation.

Compounds were precipitated three times in diethyl ether, dissolved in an acetonitrile/water 50/50 solution containing 0.1 % TFA and freeze dried.

Oxidation of peptides **3** and **4** with DMSO, Clear-Ox and Oxyfold

Peptides were dissolved at 3 mM concentration in 1.5 mL phosphate buffer (pH 7.5)/acetonitrile (70/30, v/v). Supported oxidation reactants were used at 5 equiv. relative to the amount of peptide to be oxidized, and for DMSO oxidation 10 % v/v of this solvent was used. The reactors

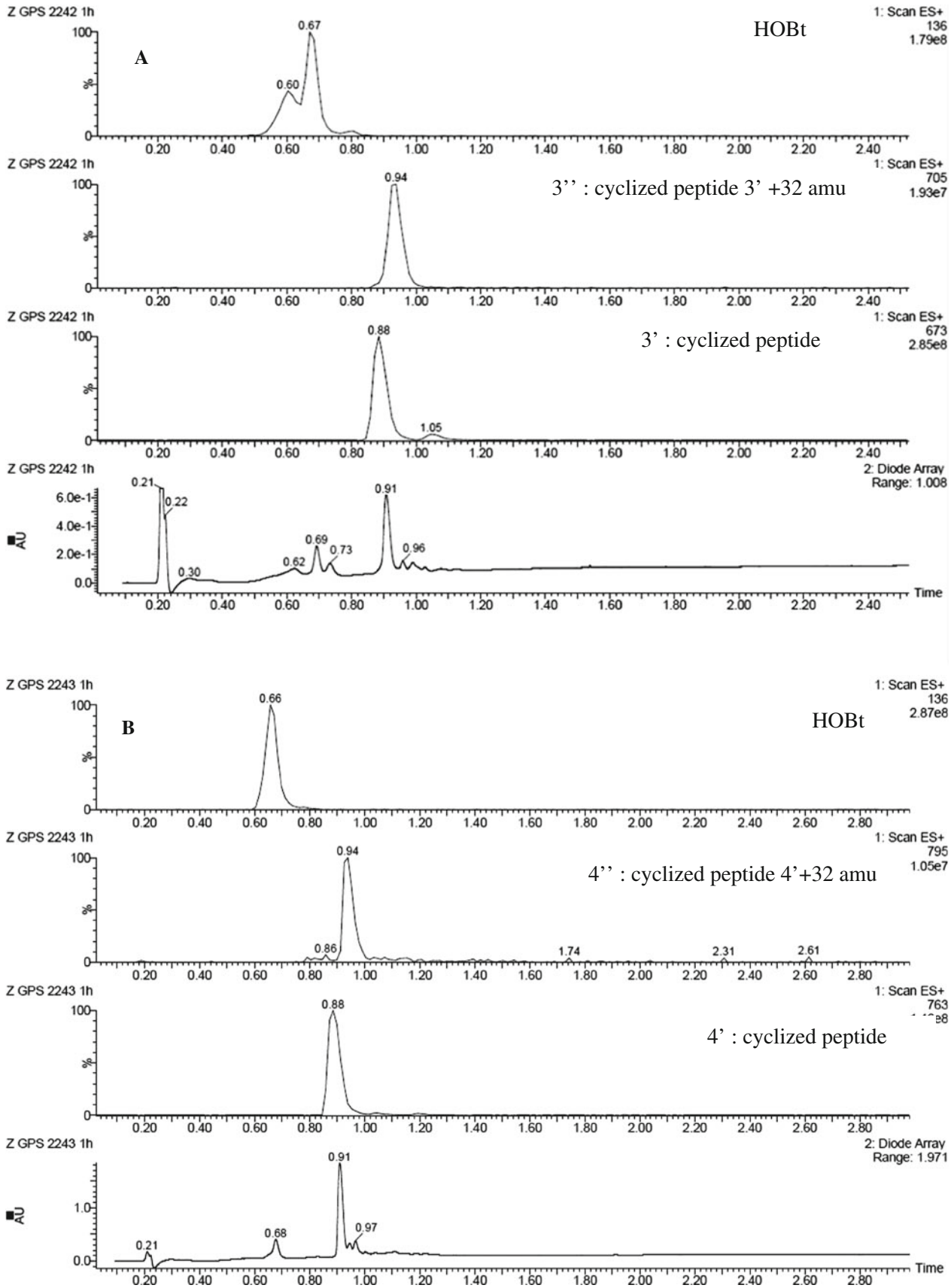


Fig. 2 LC/MS analysis of Clear-Ox-mediated oxidation of peptide **3** (*A*) and **4** (*B*)

were placed on an orbital shaker (600 rpm) at room temperature for 24 h and aliquots of 20 µL diluted to 200 µL with a CH_3CN/H_2O (50/50, v/v) solution were then filtered were immediately subjected to HPLC analyses at $t = 1$, 4, and 24 h. The percentage of oxidized peptide was calculated as a function of the area of the chromatographic peaks obtained at 214 nm (excluding DMSO peak but taking into account the possible presence of by-products) and reported as a function of time (Fig. 1).

Products were analyzed by analytical RP-HPLC (Sample Manager 2700, Pump 1525, equipped with a diode array detector, Waters) using a Chromolith Speed Rod RP-18 (50 × 4.6 mm) column (Merck) at 5 mL/min with a 0–100 % linear gradient *B* for 3 min. The solvent system used was *A* (0.1 % TFA in H_2O) and *B* (0.1 % TFA in CH_3CN). t_R of reduced peptide **3** = 1.03 min, t_R of oxidized peptide **3′** = 1.00 min, t_R of reduced peptide **4** = 1.08 min, t_R of oxidized peptide **4′** = 1.01 min.

Preparation of Oxyfold supported oxidation reagent (Verdie et al. 2011)

Methionine (2.4 g, 16.1 mmol) was dissolved in anhydrous THF (57 mL) and the mixture was kept under argon in a three-neck round-bottom flask flushed with argon. BTC (1.59 g, 5.4 mmol) was added under argon and the reaction vessel was washed with dry THF (5 mL). The reaction mixture was stirred under reflux for 2.5 h, while the medium turned into a limpid solution. The reaction medium was evaporated and clear oil (Met-NCA) was obtained and used without further treatment for polymerization experiments. It was solubilized in anhydrous THF (20 mL) and transferred on freeze-dried amino PEGA resin (0.4 mmol/g, 800 mg); a few drops of DIEA were added until basic pH. The reaction was stirred for 1 h. After filtration, the resin was washed with THF, DMF, MeOH, and DCM and treated with acetic anhydride in DCM. The resin was then oxidized by treatment with H_2O_2 (35 % w) for 1.5 h and washed with H_2O and DCM. Elemental analysis performed on an aliquot of resin indicated 6.2 % of sulfur content. This value corresponds to an average length of $n = 6.9$ methionine sulfoxide residues per amino function of the starting resin. It corresponds to a loading (related to sulfoxide) of 1.94 mmol/g (see Supporting Information for calculation).

Mass spectrometry

Aliquots of 20 µL diluted to 200 µL with a CH_3CN/H_2O (50/50, v/v) solution and then filtered were immediately subjected to LC/MS analyses at $t = 1$, 4, and 24 h. The LC/MS system consisted of a Waters Alliance 2695 HPLC, coupled to a ZQ spectrometer (Waters-Micromass, Manchester, UK) fitted with an electrospray ionization source (ESI) and a quadrupole mass analyzer. All the analyses were carried out using a Chromolith Flash, 25 × 4.6 mm column. *A* flow rate of 3 mL/min and a gradient of (0–100) % *B* for 2.5 min was used (eluent A: H_2O/0.1 % HCO_2H; eluent B: acetonitrile/0.1 % HCO_2H). Positive ion electrospray mass spectra were acquired at a solvent flow rate of 0.2 mL/min (eluent from LC splitted prior to ESI source entrance). Nitrogen was used for both the nebulizing and the drying gas. Data were obtained in a scan mode ranging from 100 to 1,000 *m/z* at 0.1 s intervals; ten scans were summed up to get the final spectrum.

High-resolution measurements and MS/MS experiments were conducted on a Q-Tof I mass spectrometer (Waters, Manchester, UK) fitted with an electrospray ion source. The mass spectrometer was calibrated in the positive ion mode using 1 % phosphoric acid in H_2O/CH_3CN solution (50/50, v/v). Data were acquired by the Tof analyzer at 1 acquisition/s from *m/z* 50 to *m/z* 3,000 with a resolution of 7,500. Ten acquisitions were summed to produce the final spectrum. Samples were dissolved in H_2O/CH_3CN (50/50, v/v) and infused into the ESI source at a flow rate of 10 µL/min. Voltages were set at +3.5 kV for the capillary and adjusted for the sampling cone. The source was heated at 80 °C. Nitrogen constituted both nebulizing and desolvation gas. The latter was heated at 150 °C. In MS/MS experiments, the second quadrupole analyzer was set in the rf mode using argon as collision gas and the collision energy varied depending on the samples to give optimal fragmentation.

Purification by preparative HPLC

Samples were prepared in an acetonitrile/water (50/50, v/v) mixture containing 0.1 % TFA, and purified by preparative RP-HPLC (Waters Prep LC 4000 System) using a Delta-Pak C18 column 15 µm 100 Å (100 × 25 mm) at 20 mL/min using first isocratic conditions at 100 % *A* for 3 min then a linear gradient of 0–20 % *B* for 10 min. The solvent systems used were *A* (0.1 % TFA in H_2O) and *B* (0.1 % TFA in CH_3CN). Detection was performed at 214 nm.

Results and discussion

Qualitative study

Model heptapeptides **3** and **4** (H-LCAGPCL-NH_2 and H-WCAGNCL-NH_2) already described in our previous paper (Verdie et al. 2011) were chosen for this study. Reduced and oxidized forms (**3′**, **4′**) of these peptides were easily separated within 3 min HPLC gradient on RP C18 column allowing a quick and straight-forward monitoring

of parallel oxidation experiments. Both peptides were synthesized by microwave-assisted Fmoc SPPS (Amblard et al. 2006) on Rink amide-PS resin (0.64 mmol/g). Peptides **3** and **4** were cleaved from the support by TFA cocktail treatment, precipitated in diethyl ether and filtered. (85 and 82 % purity, respectively, determined by HPLC at 214 nm; see Supporting Information Figures S1 and S6).

Oxyfold resin **2** was prepared by polymerization of methionine NCA initiated by amino PEGA resin, as described previously ("Materials and methods", Verdie et al. 2011).

Peptides **3** (3.4 mg) and **4** (3.0 mg) were dissolved in phosphate buffer (pH 7.5)/acetonitrile (70/30, v/v) to yield a 3 mM solution. 1.5 mL of each solution was dispensed in Eppendorf flasks containing 5 equiv.of oxidant: 132 mg of Clear-Ox resin (0.17 mmol/g) or 13 mg of Oxyfold resin (1.9 mmol/g). It is worth noting that the quantity of Oxyfold resin used was more than ten times lower than Clear-Ox, thanks to the high loading of Oxyfold resulting from the number of methionine sulfoxide units introduced in each oligomer.

In addition, peptides were dissolved at the same concentration (3 mM) in a 10 % DMSO solution [phosphate buffer (pH 7.5)/Acetonitrile/DMSO, 70/20/10, v/v/v]. The solutions were shaken for 24 h. Aliquots of each solution (20 µL) were submitted to LC analysis at $t = 1$, 4, and 24 h to determine the proportion of reduced and oxidized species. The parallel oxidation experiments were started with a gap of 6 min in a way that the sampling could be done sequentially. Results are presented in Fig. 1 and HPLC analyses are presented in Supporting Information.

As expected, the oxidized peptides **3′** and **4′** were detected in all experiments. Oxyfold-mediated oxidation of peptide **3** and **4** yielded a ratio of oxidized/reduced peptides **3′** and **4′** of 93 and 97 %, respectively, within 24 h. DMSO and Clear-Ox oxidation resulted in complete oxidation of peptides **3** and **4** in the same reaction conditions. These results were similar to what was already observed (Verdie et al. 2011). On the other hand, oxidation mediated by Clear-Ox proceeded more quickly. Indeed, no remaining reduced peptide was observed after 1 h oxidation.

Clear-Ox side-products identification

Nevertheless, along with the disappearance of the starting reduced peptide, new major side-products appeared in the HPLC chromatogram of Clear-Ox-mediated oxidation during the first hour stirring. LC/MS analyses were performed to identify these side-products (Fig. 2). Traces of cyclic dimer were detected for peptide **3** (Fig. 2, *A* $t_R = 1.05$). This was already discussed in the pioneer publication of Annis et al. (1998) showing that the pseudo-dilution advantage was not absolute in this case. Indeed, the resin bound *S*-Sthionitrobenzoic peptidyl intermediate can be displaced by another sulfhydryl peptide. It was shown that dimer formation can be lowered by decreasing the concentration below 0.5 mM. This implies that the volume of buffer to be removed after oxidation will increase significantly. As expected, insignificant amounts of dimer were observed with Oxyfold. This could be explained by the unstable sulfoxide intermediate that is quickly displaced by the sulfhydryl group followed by subsequent dehydration.

Surprisingly, HOBt was detected in both oxidized peptides **3′** and **4′** using Clear-Ox (Fig. 2, *A* and *B* 16 %, $t_R = 0.68$, $[M+H]^+$ *m/z* 136.1; Supporting Information Figures S11 B. and S12 A.). The HOBt side-product probably resulted from non-covalently entrapped HOBt existing within the polymer matrix and coming from preparation of the supported reagent. Indeed, Fmoc-Lys(Fmoc)-OH is coupled on amino group of Clear resin using BOP/HOBt/DIEA activation. This HOBt bleaching is probably one of the reasons, manufacturers recommend to extensively wash Clear-Ox resin before use.

Finally, another type of side-product was detected in the oxidation of both peptides **3** (Fig. 2, *A* 3 %, $t_R = 0.94$, $[M + H]^+$ *m/z* 705.3; Supporting Information Figures S11 C and S12 B) and **4** (Fig. 2, *B* 6 %, $t_R = 0.94$, $[M+H]^+$ *m/z* 795.3; Supporting Information) using Clear-Ox. These compounds named **3″** and **4″** were characterized by a difference of +32 amu compared to the mass of oxidized peptide. We suspected that these compounds were the trisulfide analogues of the desired peptides, formed by sulfur insertion.

HRMS analysis of the side-product obtained along with peptide **3** showed that the molecular formula $C_{28}H_{49}N_8O_7S_3$, corresponding to an extra sulfur atom in the formula of the oxidized peptide **3′**, was the most compatible with the mass 705.2863. (Supporting Information Figure S13). MS^2 and MS^3 experiments were performed and confirmed that a tri-sulfur containing peptide was obtained.

The expected oxidized peptide **3′** at *m/z* 673 and the extra sulfur containing side-product **3″** at *m/z* 705 were both dissociated upon low energy dissociation conditions (CID) (Paizs and Suhai 2005; Mouls et al. 2007). Comparison of both fragmentation patterns for these amidated peptides **3′** and **3″** (Mouls et al. 2007) helped to assign the position of the added sulfur. The nomenclature of the sequence fragment ions, a/b and y ions refers to N-terminal and C-terminal part of the peptide sequences, respectively (Roepstorff and Fohlman 1984). As expected, MS^2 experiment allowed fragmentation of the peptide backbone outside the cyclic disulfide ring by the consecutive loss of two terminal leucine residues (Fig. 3). To gain insight into the pentapeptide cyclic ring, a second round of dissociation

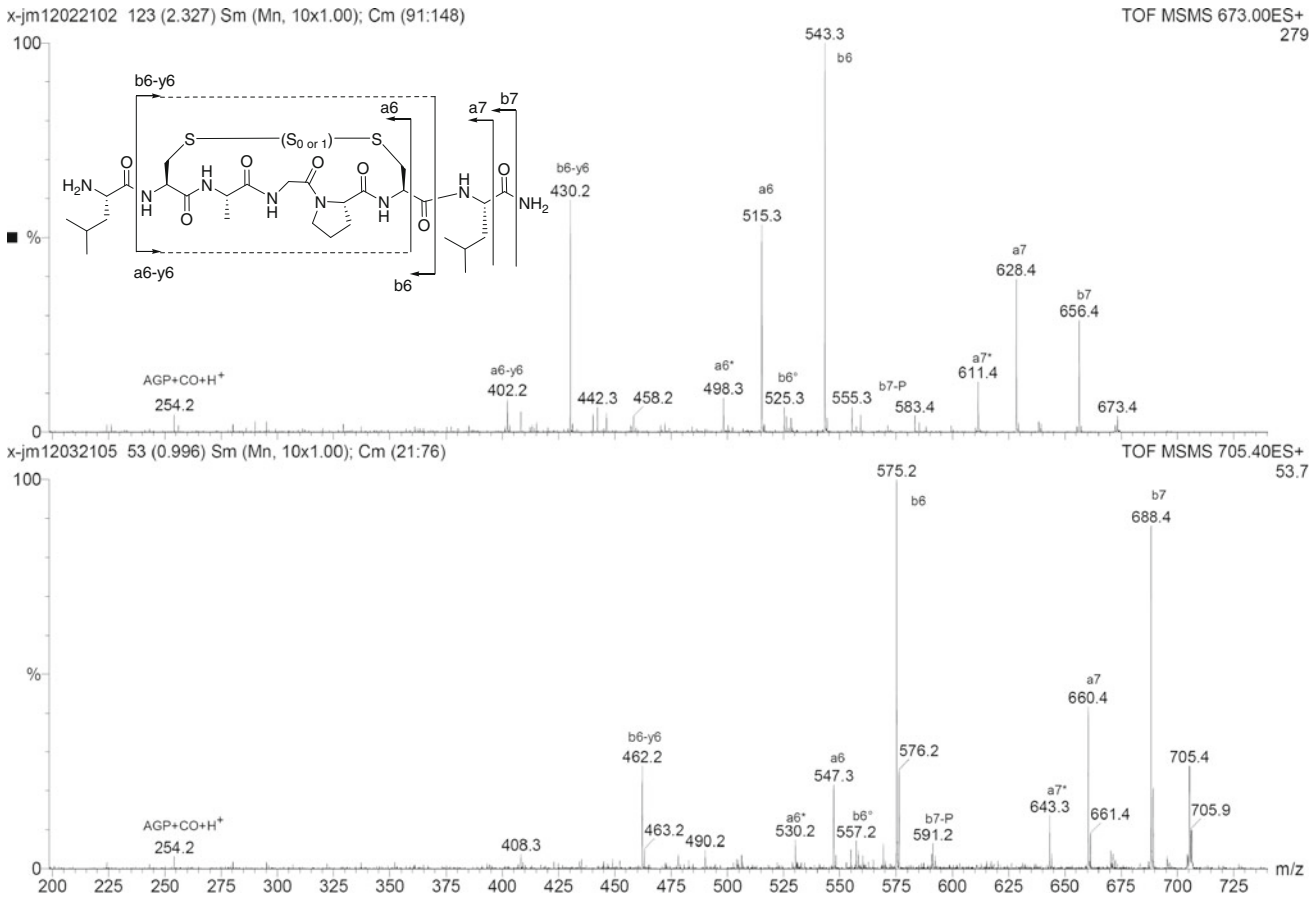


Fig. 3 MS^2 *m/z* 673 and *m/z* 705 (oxidized peptide **3** with Clear-Ox). Cone voltage 20 V; collision energy 25 eV

was performed by selecting a fragment ion produced in the ESI source, upon high source voltage and activation upon CID in the collision cell (Fig. 4). The b6–y6 internal fragment ions detected at *m/z* 430 and 462 for peptide **3** and its oxidized counterpart peptide **3′**, respectively, were selected for MS/MS experiments due to their cyclic structure devoid of pendant amino acids. Both resulting MS^3 spectra showed fragment ions with low *m/z* values that were related to the inner dipeptide part (Gly-Pro) such as *m/z* 155 corresponding to an internal b2-type ion (H-Gly-Pro$^+$) associated with the a2 ion at *m/z* 127 obtained by decarbonylation, together with c-type ions at *m/z* 115 (H-Pro-NH_2 + H^+) and *m/z* 172 (H-Gly-Pro-NH_2 + H^+). Furthermore, ions produced by the cleavage of one cysteine side-chain were also observed for both compounds. Notably, the ions at *m/z* 167 and 224 could be depicted as b2 and b3 ions from the Gly-Pro-Cys part of peptides **3** and **3′**, the cysteine side-chain having lost the SH_2 molecule to give a dehydroalanine residue (*m/z* 167: H-Pro-Cys$^+$–SH_2 = H-Pro-ΔAla$^+$ and *m/z* 224: H-Gly-Pro-Cys$^+$–SH_2 = H-Gly-Pro-ΔAla$^+$). Although other intense signals were observed in both spectra (i.e., ions at *m/z* 187 and 289 with relative abundances above 50 %), none of these fragments provided information on the localization of the additional sulfur atom. The only ions that allowed differentiating the two peptides were recorded at *m/z* 231/203 and 263/235 in the MS^3 spectra of peptide **3** and its disulfide counterpart peptide **3′**, respectively. It should be noticed that each set of these fragment ions is exclusively detected in one MS^3 spectrum. The loss of CO from 321 and 263 led to 203 and 235 for both compounds. Thus, these two couples of b/a ions were separated by 32 mass units corresponding to the mass of the sulfur atom indicating a fragment bearing the di- or tri-sulfur containing bridge moiety. Indeed, the ion at *m/z* 231 formerly corresponded to the ion at *m/z* 167 plus the mass of S_2 (64 g/mol). This ion could be depicted as an ion with the Cβ of the dehydroalanine residue being substituted by the SSH group (H-Pro-ΔAla-S-SH$^+$). Similarly, the signal observed at *m/z* 263 could be related to the ion at *m/z* 167 present in the MS^3 data of peptide **3′** by the addition of three sulfur atoms leading to the b2 ion (H-Pro-ΔAla-S-S-SH$^+$).

The presence of trisulfide bond in peptides was first reported in the mid 1990s (Jespersen et al. 1994). Very few

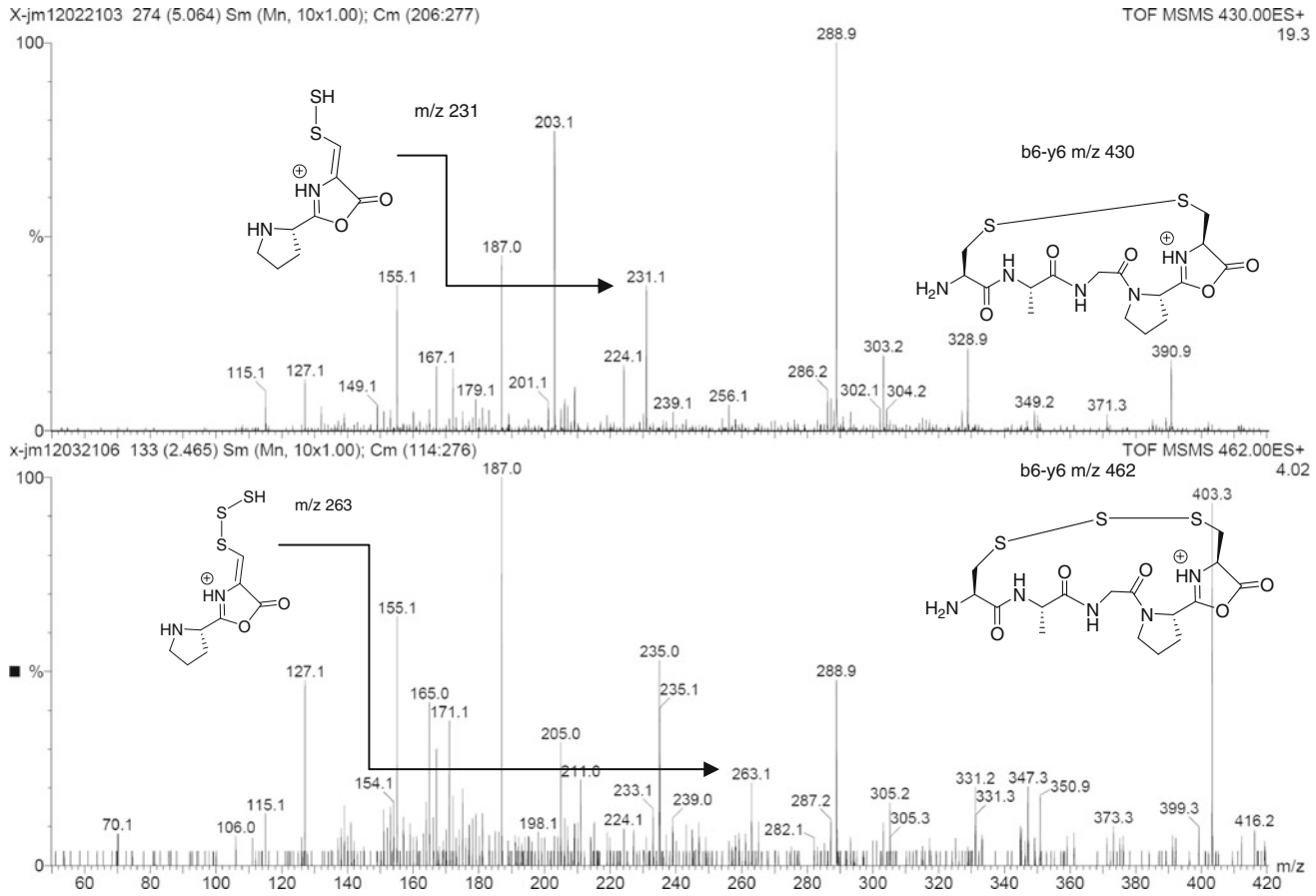


Fig. 4 MS^3 *m/z* 430 and *m/z* 462 (oxidized peptide **3** with Clear-Ox). Cone voltage 30 V; collision energy 17 eV

proteins are known to display this post-translational modification. Due to their low abundances, their detection remains challenging (Nielsen et al. 2011). The few methods reported for their synthesis relies on sulfur-transfer reagent such as *N*,*N*′-thiobisphtalimide (Lundin et al. 1994). However, the formation of trisulfide form could be explained by sulfur exchange with a trisulfide analog of supported DTNB present in the Clear-Ox resin.

Quantitative study

The oxidation of peptides **3** and **4** mediated by the two different Clear-Ox and Oxyfold resins proceeded very differently. Even though unwanted side-products were observed while using Clear-Ox, the reduced peptide was consumed more rapidly than when Oxyfold was used. Although the purity of the crude oxidized compound is important, it is not the only factor to take into account for the efficient synthesis of disulfide containing peptides. The overall yield of post-synthesis procedures, including purification, is of high importance. Deep examination of published studies surprisingly witness low purification yields (Annis et al. 1997; Darlak et al. 2004; Green and Bulaj 2006). These results cannot be fully explained by a poor purity of the oxidized crude-product, which would have result in a troublesome purification. Likely, some amounts of oxidized peptide could remain trapped, non-covalently or covalently, within the polymer matrix. This is probably the case of the supported Ellman's reagent from which an *S*-Sthionitrobenzoic peptidyl intermediate is formed. It normally undergoes an intramolecular attack by the free sulfhydryl to yield the disulfide bond. However, if the remaining free sulfhydryl reacts with another DTNB, the peptidyl intramolecular disulfide bond formation cannot occur (Supporting Information Scheme S1). Hopefully, the pseudo-dilution conditions imposed by the solid support lower the probability of this intermolecular side reaction. Annis et al. (1998) already discussed this issue. This intermolecular reaction was easily observed during the solution phase oxidation of peptide **3** (5 mg) with 5 equiv. of DTNB (14 mg) in 5 mL of phosphate buffer pH 7.4. After 1 h stirring, LC/MS analysis clearly showed the presence of *S*-Sthionitrobenzoic double adduct on peptide **3** H-LC (TNB)AGPC(TNB)L-NH_2 (see Supporting Information Figure S14).

To evaluate the impact on undesired trapping and side-product formation on the overall yield, peptide **3** was oxidized in higher quantity (samples of 30 mg) with both supported reagents, purified by preparative RP-HPLC to precisely determine the recovery yield of **3′**.

Peptide **3** (30 mg, 44.5 μmol) was solubilized in 14.8 mL of phosphate buffer pH 7.5/acetonitrile mixture (70/30, v/v) to obtain a 3 mM peptide solution that was shaken with 5 equiv. of Oxyfold resin 1 (118 mg). After 48 h the oxidation was complete and the oxidized peptide filtered from the resin and purified by RP-HPLC. After lyophilization, 17 mg of pure oxidized peptide were recovered (yield = 57 %).

The same experiment was repeated with Clear-Ox resin. Having identified the bleaching of HOBt, extensive washings (2 × DCM, 3 × DMF, 3 × MeOH, 3 × H_2O and 3 × ACN/H_2O, 1/1 v/v) were performed on Clear-Ox prior use. Resin was then freeze-dried and oxidation of peptide 3 was performed (633 mg Clear-Ox) using the same protocol than with Oxyfold. After RP-HPLC purification and freeze-drying, 10 mg of pure oxidized peptide were recovered (yield = 33 %). It is worth noting that during the purification process, two fractions of about 4 and 6 mg were isolated, containing in majority the trisulfide and the dimer. As expected, HOBt was not detected, witnessing that washings were necessary and efficient to remove this contaminant from Clear-Ox matrix.

Conclusion

Clear-Ox oxidation offered a quick and straightforward tool to obtain oxidized sulfur containing peptides. The results obtained on two model peptides are consistent with literature data showing the disappearance of the reduced peptide forms within 1 h. This can be a great advantage when small amount of several disulfide peptides have to be prepared. However, in our hands, the yield of recovery was significantly lower than when using Oxyfold, which proceeded with a mechanism that did not allow covalent trapping within the resin. When longer reaction time (48 h) is used, Oxyfold oxidation led to quantitative formation of the disulfide bridge with no dimer formation and good overall yield of recovery after purification. On the contrary, the Clear-Ox resin induced formation of dimer but also of a trisulfide analog in significant amounts whose formation is still under investigation in our laboratory. If the reaction time is not an issue, Oxyfold has to be preferred especially to oxidize more complex or valuable peptide sequences for which maximizing the recovery is the most important parameter.

Conflict of interest The authors declare that they have no conflict of interest.

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