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# Characterization of Alkali Induced Formation of Lanthionine, Trisulfides, and Tetrasulfides from Peptide Disulfides using Negative Ion Mass Spectrometry

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Peptide disulfides are unstable under alkaline conditions, resulting in the formation of products containing lanthionine and polysulfide linkages. Electrospray ionization mass spectrometry has been used to characterize major species obtained when cyclic and acyclic peptide disulfides are exposed to alkaline media. Studies on a model cyclic peptide disulfide (Boc - Cys - Pro - Leu - Cys - NHMe) and an acyclic peptide, oxidized glutathione, bis ( $\gamma$ Glu - Cys - Gly - COOH), are described. Disulfide cleavage reactions are initiated by the abstraction of C $^{\alpha}$ H or C $^{\beta}$ H protons of Cys residues, with subsequent elimination of H<sub>2</sub>S or H<sub>2</sub>S<sub>2</sub>. The buildup of reactive thiol species which act on intermediates containing dehydroalanine residues, rationalizes the formation of lanthionine and polysulfide products. In the case of the cyclic peptide disulfide, the formation of cyclic products is facilitated by the intramolecular nature of the Michael addition reaction of thiols to the dehydroalanine residue. Mass spectral evidence for the intermediate species is presented by using alkylation of thiol groups as a trapping method. Mass spectral fragmentation in the negative ion mode of the peptides derived from trisulfides and tetrasulfides results in elimination of S<sub>2</sub>. (J Am Soc Mass Spectrom 2009, 20, 783–791) © 2009 American Society for Mass Spectrometry

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Disulfide bonds are widely observed in naturally occurring peptides and proteins. Considerable effort has been spent on mass spectrometry based methods for establishing the presence and assigning the location of cysteine residues involved in the formation of disulfide bridges in natural polypeptides [1–12]. The presence of disulfide bridges in peptide natural products can be established under conditions of negative ion mass spectrometry with neutral loss of H<sub>2</sub>S<sub>2</sub> serving as a diagnostic. Abstraction of the C $^{\alpha}$ -hydrogen results in formation of a peptide enolate at Cys residues, which can subsequently cleave to yield dehydroalanine residue with loss of H<sub>2</sub>S or H<sub>2</sub>S<sub>2</sub>. The use of negative ion mass spectrometry [13–29] for the study of disulfide containing peptide natural products has been established in an extensive series of investigations by Bowie and coworkers [30–35] and other groups [36–38]. During the course of recent attempts to fragment the negative ions of disulfide bridged peptides under mass spectrometric conditions, we noted that the cleavage reactions closely resemble those observed during breakage of disulfide bonds under alkaline conditions [39, 40]. Under basic conditions, both  $\alpha$ - and

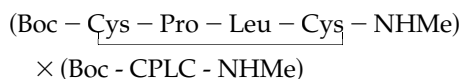
$\beta$ -hydrogens of cysteine residues can be abstracted leading to two distinct modes of cleavage of the disulfide bonds, as noted by Parker and Kharasch in their comprehensive review of the mechanism of scission of sulfur-sulfur bonds [41]. The abstraction of an  $\alpha$ -proton leads to the formation of a dehydroalanine residue and a persulfide, while abstraction of the  $\beta$ -proton results in formation of a thioaldehyde and a free thiol (cysteine). These processes are facile in both basic media and also in the gas-phase, when negative ions are subjected to collision induced dissociation (CID). The species generated during base catalyzed disulfide fragmentation are highly reactive resulting in the formation of several side products.

The formation of lanthionine (Lan) from cystine (Cys-S-S-Cys) was first reported by Horn et al. in 1941 [42], when they isolated a new thioether containing amino acid from wool treated with sodium carbonate. These authors also quickly demonstrated that lanthionine formation could be obtained in several other alkali treated proteins [43]. The chemistry of the base catalyzed degradation of protein disulfides has been the subject of considerable study [44–53]. More recently, Spatola and coworkers demonstrated the formation of lanthionine peptides by alkali treatment of several synthetic disulfide peptides [39, 40]. Interestingly, reports of trisulfide formation when proteins are exposed

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to alkaline treatments have also appeared [54]. The occurrence of a trisulfide moiety in a recombinant human growth hormone has been established by mass spectrometry [55, 56]. The high reactivity of the species formed when disulfides are exposed to basic media prompted us to re-examine the various products generated upon disulfide cleavage, by mass spectrometry. Two model systems were chosen for detailed study: (1) A synthetic 14 membered cyclic disulfide peptide

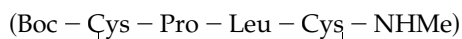


and (2) an acyclic peptide, oxidized glutathione, bis ( $\gamma$ Glu - Cys - Gly - COOH).

## Materials and Methods

### Materials

The synthesis and characterization of the cyclic peptide disulfide



has been described earlier [57]. Oxidized glutathione was obtained from Sigma Chemical Co. (St. Louis, MO).

### Mass Spectrometry

LC-ESI mass spectra were recorded in positive/negative ion mode using an Esquire 3000-plus mass spectrometer (Bruker Daltonics, Bremen, Germany) consisting of two octopole followed by an ion trap. The extract was dissolved in water (MiliQ, Millipore, France) and run through a Phenomenex (Torrance, CA, USA)  $C_{18}$  column (4.6 mm  $\times$  50 mm, 4  $\mu$ m particle size, 90 Å pore size) using acetonitrile/water/0.1% formic acid ( $CH_3CN/H_2O/0.1\% HCOOH$ ) in the positive ion mode. Negative ion mode spectra were recorded using acetonitrile/water/ammonium acetate ( $CH_3CN/H_2O/NH_4COOCH_3$ ) as the eluting solvent. The flow rate was maintained at 0.2 mL  $min^{-1}$ , a gradient of 45 min (30% to 45%  $CH_3CN$  in 10 min, 45% to 75%  $CH_3CN$  in 5 min, 75% to 95%  $CH_3CN$  in 20 min), with detection at 226 nm in both positive and negative ion mode. 10 mM ammonium acetate/water has been used for the negative ion experiments. Helium was used as the collision gas for collision induced dissociation (CID) experiments. The data were processed using Esquire data analysis software, version 3.1.

ESI-MS (direct injection) spectra were also obtained in positive/negative ion mode by direct injection of purified samples into the system using a syringe pump (Cole-Parmer, Vernon Hills, IL) operated at a flow rate of 200 to 240  $\mu$ L  $h^{-1}$ .

### HPLC

HPLC runs were performed on a Shimadzu LC-20AT (Kyoto, Japan) system. The peptide was dissolved in methanol and run through a Phenomenex  $C_{18}$  column (250 mm  $\times$  4.6 mm, 10  $\mu$ m particle size, 90 Å pore size) using methanol/water ( $CH_3OH/H_2O$ ) as the eluting solvent. The flow rate was maintained at 0.5 mL  $min^{-1}$ , a gradient of 45 min (30% to 45%  $CH_3OH$  in 10 min, 45 to 75%  $CH_3OH$  in 5 min, 75% to 95%  $CH_3OH$  in 20 min, 95% to 95%  $CH_3OH$  in 5 min, 95% to 30%  $CH_3OH$  in 5 min), with detection at 226 nm.

### Alkali Treatment

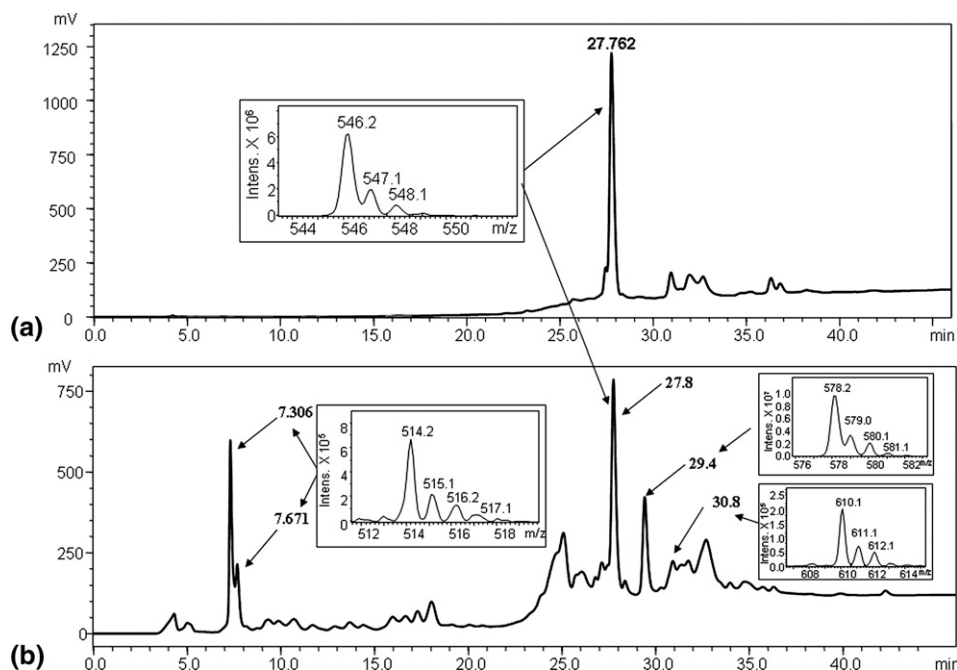
A stock solution (25 mM) of the cyclic peptide disulfide was prepared by dissolving purified lyophilized peptide in methanol/anhydrous methanol. A stock solution of oxidized glutathione (Sigma, St. Louis, MO, USA) was prepared in water to yield a concentration of 250 mM. The reaction mixtures were prepared by taking 50  $\mu$ L of stock peptide/oxidized glutathione solution and adding 40  $\mu$ L of a 25% ammonium hydroxide solution.

## Results and Discussion

### Cyclic Peptide Disulfide

Figure 1 compares the HPLC profile before and after addition of 25%  $NH_4OH$  to peptide solutions. Several additional species appear upon incubation with alkali. The  $[M + H]^+$  ions detected by positive ion electrospray ionization mass spectrometry are shown as insets. Two lanthionine species [ $m/z$  514, ( $M + H^+ - 32 = 514$ )] are detectable at very short retention times (7.3 and 7.7 min), while other species correspond to the trisulfide [ $m/z$  578, ( $M + H^+ + 32 = 578$ )] and tetrasulfide [ $m/z$  610, ( $M + H^+ + 32 + 32 = 610$ )], which elute after the disulfide [ $m/z = 546$ , ( $M + H^+ = 546$ )]. It should be noted that lanthionine formation must occur by intramolecular attack of a nucleophilic cysteine thiol on a dehydroalanine residue (vide infra). This process can, in principle, result in formation of both L and D forms at the newly generated asymmetric carbon atom. The resultant lanthionine peptides are diastereomeric (L, L and meso-lanthionine) resulting in two HPLC peaks, albeit with very similar retention times. Careful examination of the HPLC profile as a function of time suggests that prolonged incubation resulted in a greater concentration of lanthionine containing peptides, while the polysulfides were less evident when the incubation time exceeded 10 days.

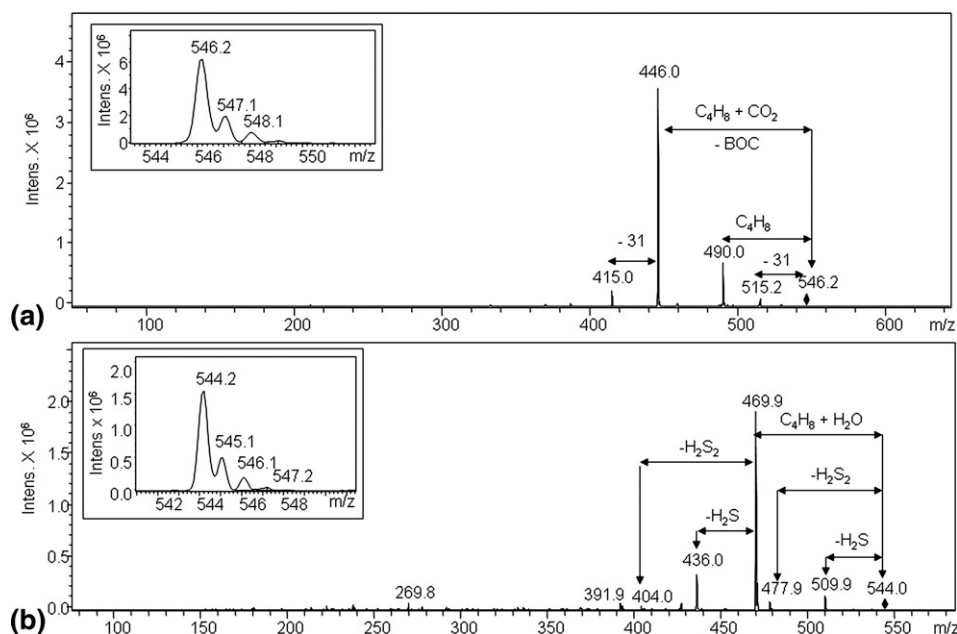
Figures 2 and 3 compare the fragmentation patterns obtained for the cyclic disulfide ( $M = 545$ ) peptide (Figure 2) and the corresponding lanthionine products ( $M = 513$ ) (Figure 3), respectively, under conditions of positive and negative ion mass spectrometry. In the case of fragmentation of positive ions, of the intact



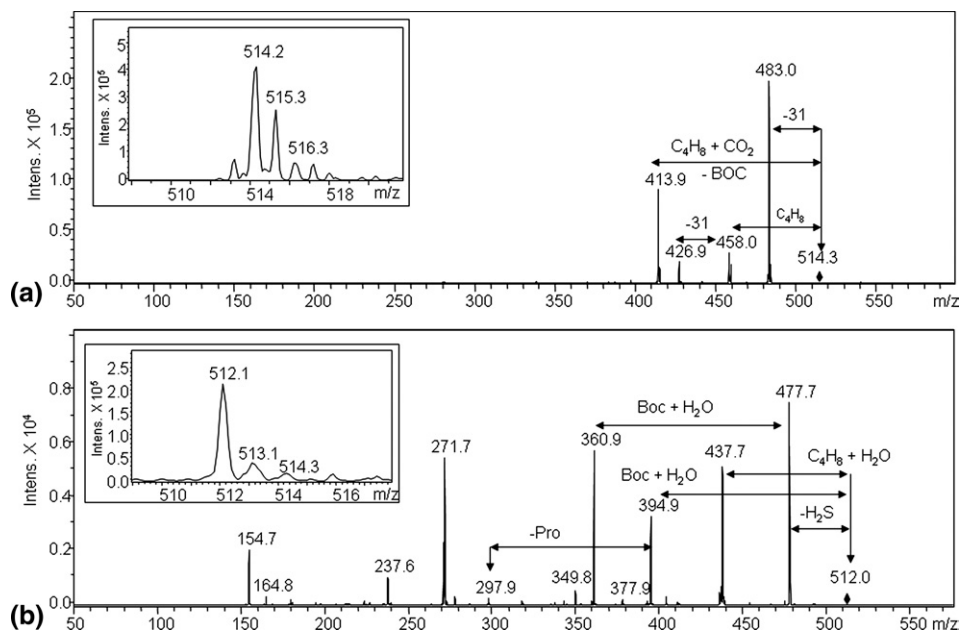
**Figure 1.** HPLC profile of Boc-CPLC-NHMe (a) peptide (b) peptide after alkali treatment (incubation period 7 days). Insets  $[M + H]^+$   $m/z$  546.2, 514.2, 578.2, and 610.1 ion observed by ESI-MS.

cyclic disulfide ( $[M + H]^+ = 546$ ), (Figure 2), the predominant product ion obtained has  $m/z$  446, corresponding to the loss of the Boc (tertiary butyloxycarbonyl) protecting group as isobutylene ( $C_4H_8$ ) and  $CO_2$  (Figure 2a). In sharp contrast, in the case of the negative ion ( $[M - H]^- = 544$ ), the predominant product ion appears at  $m/z$  469.9, arising by the neutral loss of tertiary butanol ( $C_4H_9OH$ ) from the N-terminus protecting group (Figure 2b).

For the lanthionine product (Figure 3), fragmentation of the positive ion ( $M + H$ )<sup>+</sup> results only in observation of loss of the Boc protecting group (Figure 3a). However, fragmentation of the negative ion ( $M - H$ )<sup>-</sup> of the lanthionine product yields a prominent peak at  $m/z$  477.7 corresponding to direct loss of  $H_2S$  (Figure 3b). It is conceivable, that the additional strain inherent in the 13 membered cyclic lanthionine peptide results in making this mode of fragmentation energetically favorable.



**Figure 2.** ESI-MS/MS of Boc-CPLC-NHMe (a)  $[M + H]^+$ ,  $m/z$  546.2 in positive ion mode (b)  $[M - H]^-$ ,  $m/z$  544.0 in negative ion mode. Inset shows parent ions.

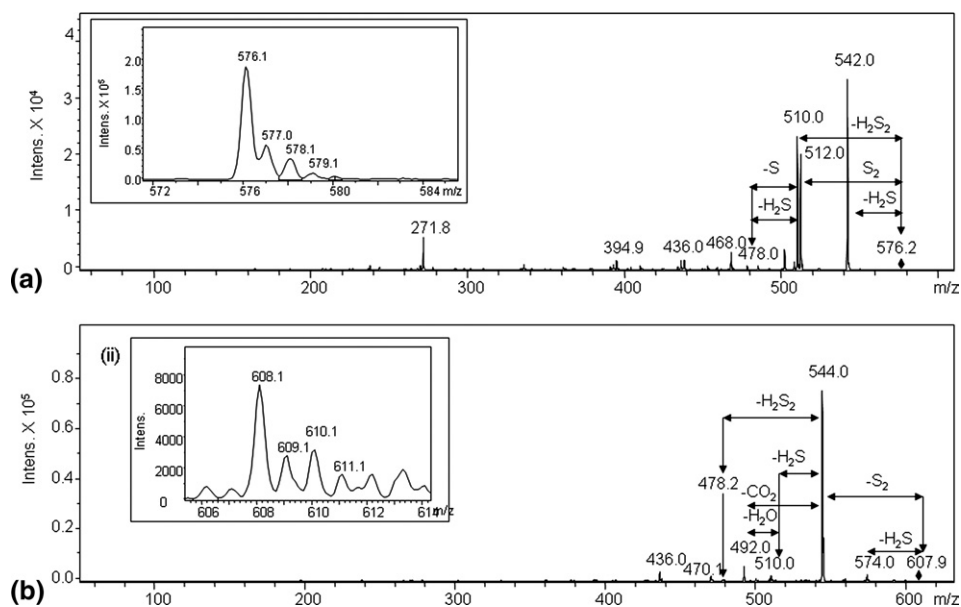


**Figure 3.** ESI-MS/MS of lanthionyl Boc-CPLC-NHMe (a)  $[M + H]^+$ ,  $m/z$  514.2 in positive ion mode (b)  $[M - H]^-$ ,  $m/z$  512.0 in negative ion mode. Inset shows parent ions.

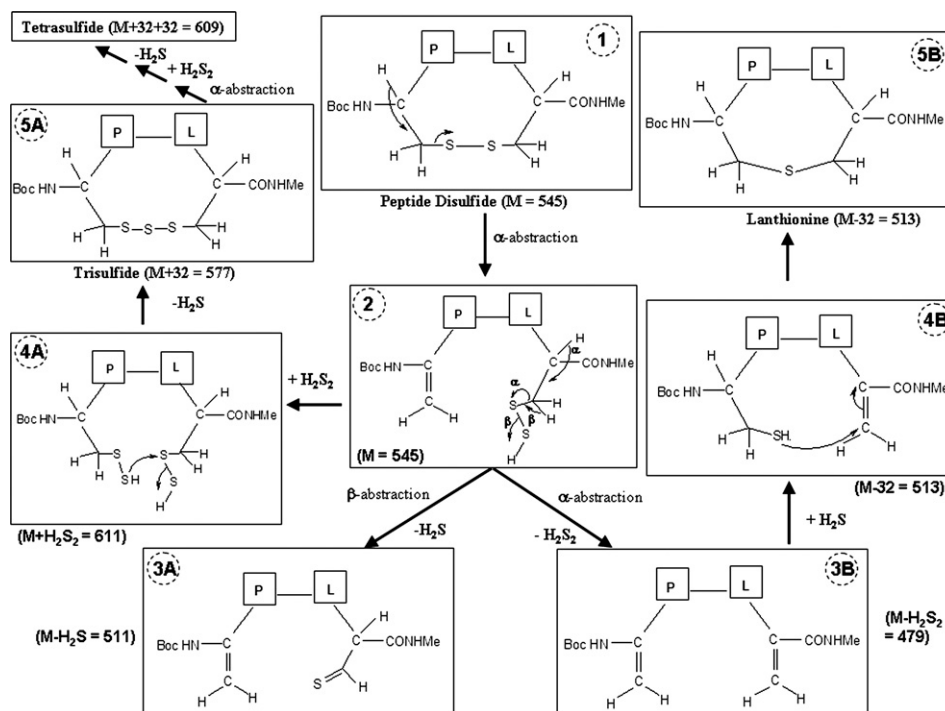
It may be relevant to note that a stable  $\beta$ -turn conformation, stabilized by a  $4 \rightarrow 1$  hydrogen bond has been established for the 14 membered cyclic peptide disulfide [57]. Removal of a sulfur atom to generate the cyclic lanthionine analog will distort the heterodetic peptide backbone. Product ion peaks corresponding to the fragmentation at disulfide or thioether bridges are not detectable under positive ion conditions.

Fragmentation of the positive ion  $(M + H)^+$  obtained from the trisulfide ( $m/z$  578) and tetrasulfide ( $m/z$  610)

products also revealed predominant loss of the Boc protecting group. Figure 4a and b show the fragmentation patterns obtained when the negative ions of the trisulfide ( $m/z$  576,  $(M - H^- + 32 = 576)$ ) and tetrasulfide ( $m/z$  610,  $(M - H^- + 32 + 32 = 608)$ ) products are subjected to collision induced dissociation. Fragments corresponding to loss of  $H_2S$  ( $m/z$  542),  $S_2$  ( $m/z$  512) and  $H_2S_2$  ( $m/z$  510) are observed in the case of trisulfide (Figure 4a). Interestingly, the prominent loss in the case of the tetrasulfide corresponds to the elimination of  $S_2$



**Figure 4.** ESI-MS/MS of trisulfide and tetrasulfide products of Boc-CPLC-NHMe in negative ion mode (a) trisulfide  $[M - H]^-$ ,  $m/z$  576.2 in negative ion mode; (b) tetrasulfide  $[M - H]^-$ ,  $m/z$  607.9 in negative ion mode. Inset shows parent ions.



Scheme 1. Alkali induced fragmentation of the cyclic tetrapeptide.

( $m/z$  545) (Figure 4b). The neutral loss of diatomic sulfur  $S_2$  is considered in the subsequent discussion of the results obtained with glutathione.

Similar results were obtained with a series of peptide disulfide analogs, when the leucine (Leu) residue was replaced by glycine (Gly), alanine (Ala), and phenylalanine (Phe). Interestingly, no significant differences were observed upon neutral replacement of residue 3 in the cyclic peptide disulfide.

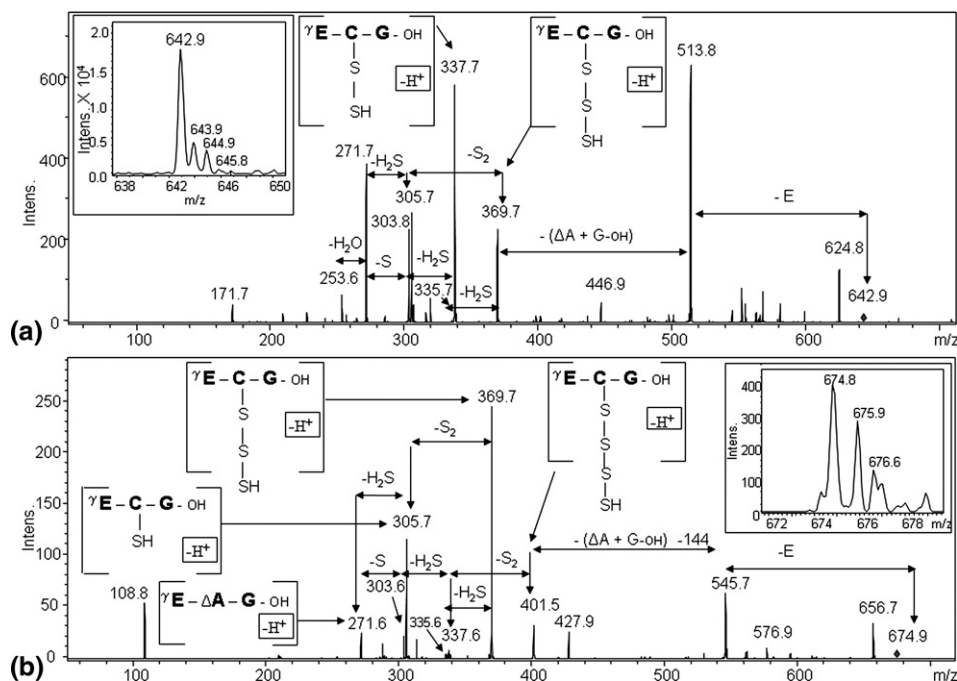
Scheme 1 schematically illustrates the reaction scheme rationalizing the formation of lanthionine and the polysulfides. Initial abstraction of the  $C^\alpha H$  proton from either Cys-1 or Cys-4 can result in the formation of dehydroalanine at one position and the persulfide R-S-S-H at the other position.  $H_2S$  can be generated by abstraction of the  $C^\beta H$  proton resulting in the formation of a thioaldehyde and a free cysteine thiol group [41]. The  $H_2S$  in turn can attack the peptide persulfide resulting in elimination of  $H_2S_2$  and formation of the free thiol group of Cys. Peptide molecules which now contain free Cys and dehydroalanine residues can form the lanthionine peptide by an intramolecular Michael addition. This mechanism invokes formation of  $H_2S$  and requires a significant build up of the reactive species. Nucleophilic attack of  $H_2S_2$  on the persulfide with displacement of  $H_2S$  can yield an S-S-S species, which can then form the higher homolog of the cyclic peptide trisulfide. A similar attack of  $H_2S_2$  on the  $Cys_2S_3$  species results in generation of the  $S_4$  species, which then forms cyclic peptide tetrasulfides. Clearly, under basic conditions the cyclization and cleavage reactions are reversible. The involvement of both  $H_2S$  and  $H_2S_2$  in

mediating sulfur addition to the original peptide disulfide necessarily limits the extent of conversion.

Evidence for some of the species shown in Scheme 1 has been obtained in a trapping experiment, where the reactive thiol intermediates are captured by reaction with iodoacetamide (Supplementary Figure S1, which can be found in the electronic version of this article). In this process, the addition of a carboxamido group to a free thiol results in a mass increase of 57 Da. The ESI-MS positive ion spectrum obtained from a solution of the peptide, incubated in 25%  $NH_4OH$  for 72 h followed by addition of an excess amount of iodoacetamide contains prominent peaks at  $m/z$  514.2 and 571.3, confirming the intermediacy of the species 4B (Scheme 1), which has been alkylated at the single free thiol. Further fragmentation of alkylated lanthionine precursor species at  $m/z$  571.3 in the positive ion mode revealed the anticipated loss of the Boc group ( $m/z$  471.1). Loss of Boc and  $NH_3$  ( $m/z$  454.1) and again further loss of  $NH_3$  ( $m/z$  437.2) confirms two sites for ammonia loss. The loss of  $NH_3$  confirms the presence of both the terminal amino group and the carboxyamidomethyl moiety at the Cys residue. The identity of the  $m/z$  571.3 species is further confirmed by the neutral loss of the  $SH-CH_2CONH_2$  fragment, (-91 Da), in the negative ion mode (Supplementary Figure S2).

The peak observed at  $m/z$  588.2 may be assigned to the ammonium adduct of the alkylated product from 4B (Scheme 1). The intense peaks at  $m/z$  471.2 and 488.2 correspond to species derived by the loss of Boc group under the acidic condition for infusion into the electrospray source ( $\Delta M = 101$ ). Interestingly, the ion at  $m/z$





**Figure 5.** ESI-MS/MS of trisulfide and tetrasulfide products of glutathione in negative ion mode (a) trisulfide  $[M - H]^-$ ,  $m/z$  642.9 (b) tetrasulfide  $[M - H]^-$ ,  $m/z$  674.9. Inset shows parent ions.

662.2 corresponds to reduction of the parent peptide followed by the alkylation of the dithiol. This may be anticipated in view of the number of reactive thiols in the basic medium, which can act as reducing agents. A peak of very low intensity is observed at  $m/z$  480, which may be assigned to the didehydroalanine peptide 3B (Scheme 1).

### Oxidized Glutathione

Incubation of oxidized glutathione under alkaline conditions also leads to the detection of lanthionine, trisulfide, and tetrasulfide species (Supplementary Figure S3). It is observed that the extent of formation of these species is considerably lower in the case of glutathione as compared to the synthetic cyclic disulfide peptide. This is undoubtedly a consequence of the fact that the lanthionine and polysulfide products are formed by nucleophilic attack of precursor  $(S_n)^-$  species on the dehydroalanine residues. For the cyclic peptide disulfide, this reaction is intramolecular. On the contrary, in the case of glutathione, this reaction becomes an intermolecular process. Despite the 10-fold increase in the concentration of glutathione used, the extent of product formation is limited.

Fragmentation of the  $[M + H]^+$  species ( $m/z$  581) of lanthionyl glutathione results in predominant product ions generated by the successive loss of N-terminal glutamyl residues (Supplementary Figure S4).

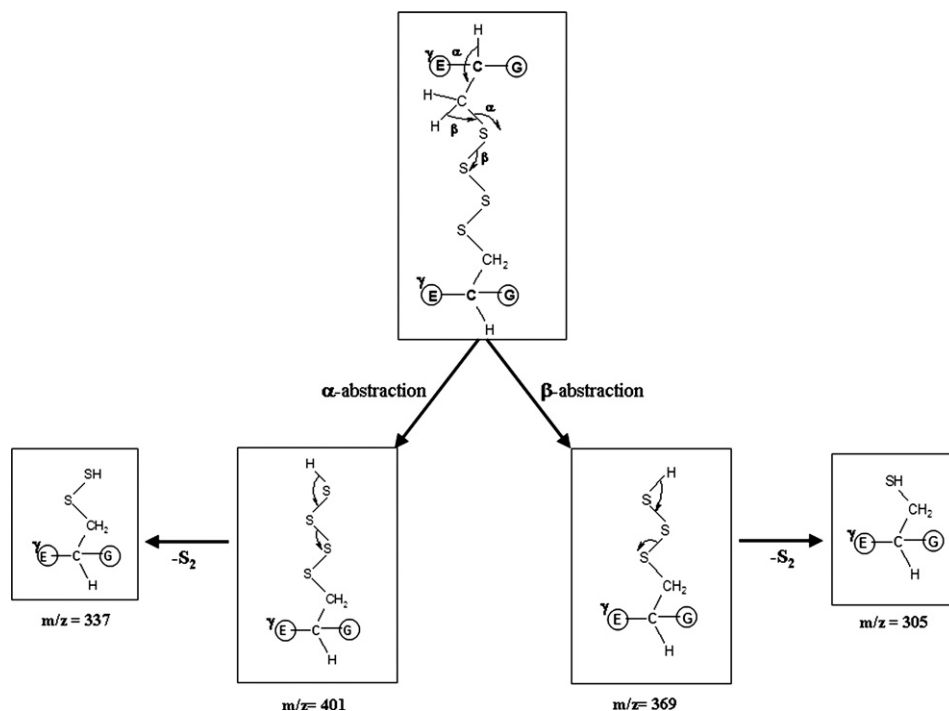
In the case of the negative ion  $[M - H]^-$  ( $m/z$  579) of lanthionyl glutathione, elimination of a glutamyl residue yields a prominent  $m/z$  449.9 peak (Supplementary Figure S3). For oxidized glutathione which has been

previously described, this fragmentation has been rationalized by invoking the neutral loss of a pyroglutamyl moiety [24]. The abstraction of a  $C^\alpha$  proton, followed by the cleavage at the thioether bridge, results in generation of the peak corresponding to reduced glutathione ( $m/z$  305.8)  $[M - H]^-$ . The neutral loss of 144 Da corresponds to elimination of the dipeptide fragment  $\Delta A-G$  (dehydroalanyl-glycine).

Figure 5 illustrates the fragmentation of the negative ions generated from the trisulfide and tetrasulfide products of glutathione obtained under alkaline condition. Fragmentation of the species at  $m/z$  642.9, corresponding to the trisulfide analog of glutathione (Figure 5a, top panel), yields prominent product ions at  $m/z$  513.8, 369.7, and 337.7, with the loss of a  $\gamma$  glutamyl residue resulting in the peak with  $m/z$  513.8. The peak at 369.7 corresponds to the thiol form of the  $S_3$  species, while the  $m/z$  337.7 peak, corresponds to the thiol form of the  $S_2$  species. Inspection of the fragmentation pattern of the tetrasulfide analog (Figure 5b, bottom panel) reveals an intense peak at  $m/z$  369.7, which is assigned to the thiol form of the  $S_3$  species, while the peak at  $m/z$  401.5 is assigned to the thiol form of the  $S_4$  species.

Scheme 2, provides a mechanistic rationalization for the generation of these product ions. The abstraction of the  $\alpha$ -proton leads to the breakdown of the polysulfide analog of glutathione to yield the dehydroalanyl fragment and glutathionyl  $S_n$  thiol species. The abstraction of the  $\beta$ -proton results in fragmentation of the thioaldehyde derived from glutathione  $m/z$  401 and glutathione  $S_{n-1}$  thiol species.

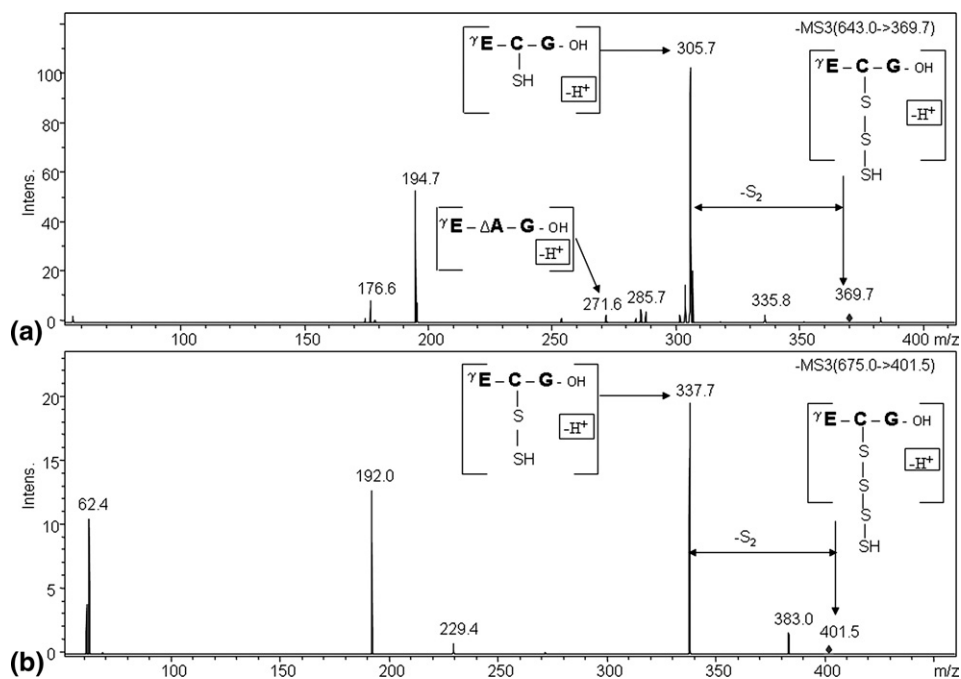
Figure 6 compares the further fragmentation of the  $S_n$  thiol species. The selection of the  $m/z$  369.7 species ( $S_3$ )



**Scheme 2.** Generation of product ions by mass spectral fragmentation of peptide polysulfides.

yields an intense product ion  $m/z$  305.7, corresponding to the negative ion of reduced glutathione. This must arise by elimination of the sulfur diatomic ( $S_2$ ) species as indicated in **Scheme 2**. Interestingly, selection of the peak at  $m/z$  401.5 corresponding to the  $S_4$  thiol species yields an intense product ion at  $m/z$  337.7, which corresponds to the

persulfide generated from glutathione. This is once again a consequence of loss of  $S_2$ . Notably, the diatomic sulfur  $S_2$  species has been extensively investigated over the last two decades. The work of Harpp and coworkers has established dialkyl disulfides as precursors for the diatomic  $S_2$  species at elevated temperatures [58, 59].



**Figure 6.** Fragmentation of selected product ions obtained in the ESI- $MS_3$  spectra of tri and tetrasulfides obtained from glutathione. (a)  $m/z$  369.2 derived from trisulfide  $[M - H]^-$ ,  $m/z$  642.9 (from Figure 5a) (b)  $m/z$  401.2 derived from tetrasulfide  $[M - H]^-$ ,  $m/z$  674.9 (from Figure 5b).

The neutral loss of 64 Da observed in Figure 6 is fully consistent with the extrusion of S<sub>2</sub>. Notably, S<sub>2</sub> loss is most prominent in the negative ion fragmentation of tetrasulfide spectra derived from the cyclic peptide disulfide. This is readily rationalized by the initial fragmentation to a S<sub>4</sub>-thiol species at one Cys position and a dehydroalanyl residue at the other. Subsequent S<sub>2</sub> elimination results in a persulfide at the other Cys position.

## Conclusions

The base catalyzed chemical transformations of peptide disulfides are mediated by abstraction of protons from the C<sup>α</sup> and C<sup>β</sup> positions of cystine residues. The subsequent cleavage processes in solution resemble those obtained under conditions of negative ion mass spectrometry in the gas phase. The reactions of reactive thiol species and dehydroalanine residues generated by chemical cleavage at disulfide bonds results in the formation of lanthionine, trisulfide and tetrasulfide products from both cyclic and linear peptides exposed to alkaline media. Polysulfide products are generated by bimolecular reaction involving H<sub>2</sub>S and H<sub>2</sub>S<sub>2</sub> generated during the disulfide breakdown process and are necessarily concentration dependent. Lanthionine formation is more facile in the case of cyclic disulfide, since the Michael addition of the thiol to the dehydroalanine residue is an intramolecular process. The present study provides an unambiguous characterization of lanthionine, trisulfide, and tetrasulfide products using negative ion mass spectrometry, which permits facile fragmentation at thioether and polysulfide linkages.

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## Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2008.12.019.

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