

Ionic Reagent for Controlling the Gas-Phase Fragmentation Reactions of Cross-Linked Peptides

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Chemical cross-linking combined with proteolytic digestion and mass spectrometry (MS) is a promising approach to provide inter- and intramolecular distance constraints for the structural characterization of protein topologies and functional multiprotein complexes. Despite the relative straightforwardness of these methodologies, the identification and characterization of cross-linked proteins presents a significant analytical challenge, due to the complexity of the resultant peptide mixtures, as well as the array of inter-, intra-, or “dead-end”-cross-linked peptides that may be generated from a single cross-linking experiment. To address these issues, we describe here the synthesis, characterization, and initial evaluation of a novel “fixed charge” sulfonium ion-containing cross-linking reagent, S-methyl 5,5'-thiodipentanoylhydroxysuccinimide. The peptide products obtained by reaction with this reagent are all shown to fragment exclusively via facile cleavage of the C–S bond directly adjacent to the fixed charge during CID-MS/MS, resulting in the formation of characteristic product ions that enable the presence and type (i.e., inter, intra, or dead-end) of the cross-linked products to be readily determined, independently of the “proton mobility” of the precursor ion. Subsequent isolation and dissociation of these products by MS³ provides additional structural information required for identification of the peptide sequences involved in the cross-linking reactions, as well as for characterization of the specific site(s) at which cross-linking has occurred. The specificity of these gas-phase fragmentation reactions, as well as the solubility and stability of the cross-linking reagent under aqueous conditions, suggests that this strategy holds great promise for use in future studies aimed at the structural analysis of large proteins or multiprotein assemblies.

The mapping of protein structures and protein–protein interactions is central to understanding protein structure–function relationships. Recently, chemical cross-linking followed by proteolytic digestion and subsequent characterization by mass spectrometry (MS) has shown great promise in providing distance constraints, albeit at relatively low resolution, for assigning protein

topologies and protein interactions.^{1,2} Despite the relative straightforwardness of these methodologies, a significant challenge associated with the identification and characterization of proteolytically derived cross-linked peptides arises due to the enormous complexity of the peptide mixtures that result from digestion of cross-linked proteins. Further complications arise due to the array of intermolecular, intramolecular, or dead-end cross-linked peptides that may be generated from a single cross-linking experiment.³

In recent years, a range of cross-linking reagents have been developed for use in specific strategies to facilitate the selective identification of cross-linked peptides.^{1,4} These include those incorporating specific affinity tags such as biotin into the cross-linking reagent, which enable the enrichment of cross-linked peptides with avidin-containing reagents prior to MS analysis.^{5–9} The use of cross-linking reagents containing solution-phase cleavage sites has also been demonstrated to result in an improved ability to identify the presence of cross-linked peptides from within complex mixtures.^{10–13} These cleavage reactions may be performed by hydrolysis¹⁰ or by the use of reducing agents in the case of disulfide-containing cross-linking reagents,^{11–13} thereby allowing cross-linked peptides to be identified during subsequent MS analysis via the mass shifts observed before and after the cleavage reaction. Alternatively, the incorporation of differential isotope labels has been employed to enable the detection of cross-

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linked peptides via identification of their distinct isotope patterns during MS analysis.^{14–20} This can be realized by incorporation of the isotope label within the protein or peptide^{14–17} or within the cross-linker itself.^{18–20} Examples of the former approach include the introduction of ¹⁸O to the C-terminal carboxyl group(s) of the polypeptide chain(s) formed during proteolytic digestion.^{14,15} Intermolecular cross-linked peptides are readily distinguished by a characteristic 8-Da mass shift compared to peptides formed by proteolysis using naturally abundant water. However, nonmodified, dead-end, and intramolecular cross-linked peptides are not able to be distinguished, as they will all exhibit a common 4-Da mass increment. In contrast, incorporation of the isotope label within the cross-linking reagent potentially allows all cross-linked peptides to be detected, via identification of their distinct isotope patterns upon reaction with 1:1 mixtures of stable isotope-labeled and nonlabeled cross-linking reagents.^{18,19} Recently, Seebacher et al. have combined these two strategies in order to facilitate the improved discrimination of intermolecular, intramolecular, and dead-end cross-linked reaction products.²⁰ However, limitations to these approaches may be encountered when the *m/z* values of differentially labeled cross-linked peptide products overlap with unlabeled peptides also present in the mixture, thereby precluding identification of the characteristic isotopic multiplets.

In recent years, therefore, several groups have initiated the development and application of novel classes of gas-phase cleavable cross-linking reagents, whereby cross-linked reaction products are identified and characterized based on their characteristic fragmentation behavior observed during tandem mass spectrometry.^{21–26} These gas-phase cleavage sites may be incorporated into a side chain on the cross-linking reagent,²¹ resulting in formation of a stable “reporter” ion (thereby maintaining the cross-linked peptide linkages), or incorporated directly into the cross-linker spacer chain,^{22–25} thereby resulting in cleavage of the cross-link upon MS/MS. In each case, further structural interrogation of the peptide product ions formed following the initial cleavage reaction can be achieved by MSⁿ analysis. However, consistent with the current state of knowledge regarding the mechanisms and other factors that influence the fragmentation reactions of

protonated peptide ions in the gas phase,²⁷ the mechanisms responsible for the fragmentation of protonated cross-linked peptides are expected to be highly dependent on the charge state and amino acid composition (i.e., proton mobility) of the mass selected precursor ion, such that selective cleavage of the desired bond within the cross-link may only be observed for a subset of the peptides selected for MS/MS or observed as only one of many dissociation channels. The inability to control the selectivity of these fragmentation reactions thereby potentially limits the sensitivity and widespread applicability of these approaches for comprehensive cross-linked peptide identification and characterization.

Here, based on results from our recent studies aimed at the development of fixed charge sulfonium ion chemical derivatization strategies for “targeted” MS/MS-based identification, characterization, and quantitative analysis of peptides containing specific functional groups (e.g., the side chains of methionine or cysteine),^{28–32} and as a first step toward the development of an improved MS/MS-based approach for the comprehensive analysis of protein–protein interactions using chemical cross-linking and multistage tandem mass spectrometry, we describe the synthesis, characterization, and initial demonstration of the selective gas-phase fragmentation behavior of cross-linked peptide ions formed by reaction with a novel amine reactive, sulfonium ion containing cross-linking reagent, *S*-methyl 5,5'-thiodipentanyloxyhydroxysuccinimide (**1**).

MATERIALS AND METHODS

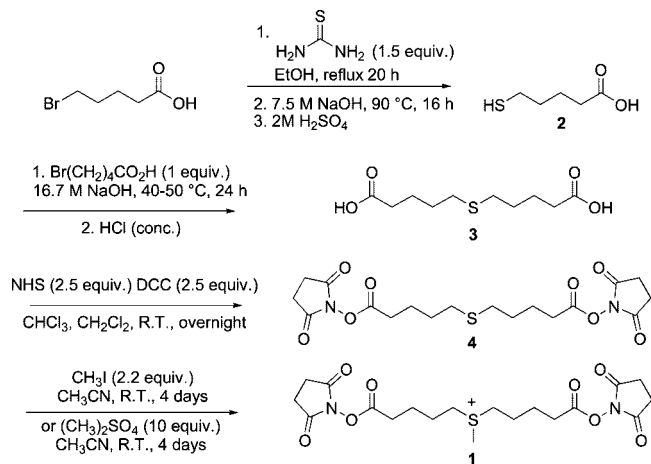
Materials. All chemicals were analytical reagent (AR), or of a comparable or higher grade, and used without further purification. Dicyclohexylcarbodiimide (DCC) was purchased from Fluka. 5-Bromovaleric acid, thiourea, neurotensin, angiotensin II, and [Glu¹]-fibrinopeptide B were from Sigma-Aldrich (St. Louis, MO). Substance P was obtained from Bachem (Torrance, CA). *N*-Hydroxysuccinimide (NHS) was purchased from Pierce (Rockford, IL). The synthetic peptide VTMAHFWNFGK (pep_{VWK}) and GAILDGAILR (pep_{GDR}) were obtained from Auspep (Parkville, Australia). The phosphoserine containing peptide LSVPTpS-DEEDEVPAKPR (pep_{LPSR}) was synthesized by Sigma-Genosys (The Woodlands, TX) and used without further purification. Deionized water was obtained from a Barnstead nanopure diamond purification system (Dubuque, IA). Dimethylformamide (DMF) was dried over 3-Å molecular sieves (Spectrum Chemicals) and filtered prior to use. All reactions were performed in oven-dried glassware.

Synthesis of Cross-Linking Reagent. Synthesis of the ionic cross-linking reagent **1** (*S*-methyl 5,5'-thiodipentanyloxyhydroxysuccinimide iodide **1'** or *S*-methyl 5,5'-thiodipentanyloxyhydroxysuccinimide methylsulfate **1''**) was achieved by initial preparation of

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Scheme 1. Synthesis of Ionic Cross-Linking Reagent S-Methyl 5,5'-Thiodipentanoylhydroxysuccinimide (1)



5-mercaptopentanoic acid (**2**), followed by alkylation with 5-bromovaleric acid. Then, esterification of the resultant 5,5'-thiodipentanoic acid (**3**) using NHS yielded 5,5'-thiodipentanoylhydroxysuccinimide (**4**). Finally, alkylation of **4** with either iodomethane or dimethyl sulfate provided the target molecule (Scheme 1). A detailed description of the synthesis of **1'** and **1''** are provided below.

Synthesis of 5-Mercaptopentanoic Acid (2). Using a procedure adapted from Jessing et al.,³³ 5-bromovaleric acid (2.2 g, 12.1 mmol) and thiourea (1.4 g, 18.4 mmol) were dissolved in ethanol (25 mL) and refluxed for 20 h. The solvent was removed under reduced pressure, and 7.5 M NaOH (aq) (25 mL, 187 mmol) was added. The mixture was stirred for an additional 16 h at 90 °C. Then, with cooling on an ice bath, 2 M H_2SO_4 (aq) was added slowly with stirring until the pH 1 and the product extracted twice with dichloromethane (2 × 100 mL). The combined extracts were then dried with anhydrous Na_2SO_4 and concentrated by rotary evaporation to give the title acid **2** as a colorless oil in quantitative yield. The product was used without further purification. ^1H NMR (300 MHz, CDCl_3): δ 1.32 (t, 1H, $J = 7.8$), 1.59–1.73 (m, 4H), 2.32 (t, 2H, $J = 7.5$), 2.49 (q, 2H, $J = 6.9$), 8.95 (s, broad, 1H). ^{13}C NMR (75 MHz, CDCl_3): δ 23.21, 24.06, 33.07, 33.35, and 179.49.

Synthesis of 5,5'-Thiodipentanoic Acid (3). Following the method of Rabinovich et al.,³⁴ a freshly prepared solution of 5-bromovaleric acid (3.4 g, 18.8 mmol) in 16.7 M NaOH (8 mL) was added dropwise to an ice bath cold solution of freshly prepared **2** (2.5 g, 18.7 mmol) dissolved in 16.7 M NaOH (8 mL). The resulting reaction mixture was stirred at 40–50 °C for 24 h. After the heating was terminated, the product mixture was acidified with concentrated hydrochloric acid to pH 1 and repeatedly extracted with dichloromethane (5 × 50 mL). Extracts were combined, dried over anhydrous Na_2SO_4 , filtered, and evaporated under reduced pressure to give **3** as a white solid in 78% (3.4 g) yield. ^1H NMR (300 MHz, CDCl_3): δ 1.49–1.67 (m, 8H), 2.26 (t, 4H, $J = 7.2$), 2.44 (t, 4H, $J = 7.2$). ^{13}C NMR (75 MHz, CDCl_3): δ 23.84, 28.80, 31.42, 33.39, and 176.79.

Synthesis of 5,5'-Thiodipentanoylhydroxysuccinimide (4). Compound **3** (1.17 g, 5.0 mmol) and NHS (1.44 g, 12.5 mmol) were dissolved in 10:1 v/v mixture of chloroform and dichloromethane (55 mL) and stirred for 5 min at room temperature. DCC (2.58 g, 12.5 mmol) was then added, and the mixture was stirred overnight. After filtration of dicyclohexylurea (DCU) precipitate and solvent removal under reduced pressure, the oily residue was dissolved in a minimum amount of ethyl acetate. The remaining DCU was precipitated and removed by filtration. Following rotary evaporation of the ethyl acetate, the residue was dissolved in DCM, washed with 1 M NaOH and H_2O , and then evaporated to near dryness. The residue was then recrystallized with ethyl ether containing a trace amount of acetone to give a light yellow solid in 84% (1.8 g) yield. ^1H NMR (300 MHz, CDCl_3): δ 1.63–1.71 (m, 4H), 1.76–1.84 (m, 4H), 2.50 (t, 4H, $J = 7.5$), 2.60 (t, 4H, $J = 7.5$), 2.78 (s, 8H). ^{13}C NMR (75 MHz, CDCl_3): δ 23.60, 25.50, 28.35, 30.42, 31.03, 168.32, and 169.14.

Synthesis of S-Methyl 5,5'-Thiodipentanoylhydroxysuccinimide Iodide (1'). A mixture of **4** (214 mg, 0.5 mmol) and methyl iodide (160 mg, 1.1 mmol) in acetonitrile (1.5 mL) was allowed to react at room temperature for 4 days. Following removal of the solvent under vacuum, a pale yellow solid was obtained, which was used for cross-linking reactions without further purification. ^1H NMR (500 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$): δ 1.89–1.91 (m, 8H), 2.68 (t, 4H, $J = 6.5$), 2.78 (s, 8H), 3.02 (s, 3H), 3.53 (t, 4H, $J = 7.5$). ^{13}C NMR (125 MHz, $\text{CDCl}_3 + \text{CD}_3\text{OD}$): δ 22.51, 22.66, 23.00, 25.53, 29.99, 40.34, 168.21, and 169.73.

Synthesis of S-Methyl 5,5'-Thiodipentanoylhydroxysuccinimide Methylsulfate (1''). A mixture of **4** (214 mg, 0.5 mmol) and dimethyl sulfate (0.63 g, 5 mmol) in acetonitrile (2 mL) was allowed to react at room temperature for 4 days. Following freeze-drying, a dark brown oily residue was obtained, which was used for cross-linking reactions without further purification. ^1H NMR (500 MHz, CD_3CN): same as for **1'** above.

NMR. ^1H NMR spectra were obtained on Varian Inova 300- or 500-MHz instruments and are reported in parts per million (ppm) relative to the solvents resonances (δ), with coupling constants (J) in hertz (Hz).

Peptide Cross-Linking. For single peptide cross-linking reactions, peptides were dissolved in phosphate-buffered saline (800 mg of NaCl, 217 mg of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg of KCl, and 20 mg of KH_2PO_4 per 100 mL, pH 7.5) to a concentration of 0.5 mM. Cross-linking reagent **1'** or **1''** was dissolved in DMF and then immediately added to the peptide solution to a concentration of 0.75 mM (the final concentration of DMF was 1%), and the reaction allowed to proceed at room temperature for 30–120 min. Multipeptide cross-linking reactions (5.0 mM each in phosphate-buffered saline) were carried out using **1'** or **1''** at a peptides/cross-linking reagent molar ratio of 1.6:1 and allowed to proceed at room temperature for 2 h. As the primary goal of the current study was to examine the utility of a sulfonium ion containing cross-linking reagent for controlling the specificity of the gas-phase fragmentation reactions of inter-, intra-, or dead-end cross-linked peptides, as well as to examine the potential competition between cleavage of the cross-linker and cleavage of facile bonds within the peptide sequence (e.g., enhanced cleavage at the C-terminal side of aspartic acid residues, at the N-terminal side of proline residues, or at the side chain of post-translational modified

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residues such as loss of H_3PO_4 from phosphoserine or phosphothreonine), a series of synthetic peptides were employed. Thus, only limited evaluation of the reaction conditions required to optimize the abundances of specific types of cross-linking reaction products was carried out. Furthermore, the concentrations of the peptide solutions subjected to cross-linking reactions in this study were ~ 1 order of magnitude higher than that used in prior protein cross-linking studies (in native protein structures or protein complexes, lower concentrations may be employed as the "local concentration" of the reactive groups subjected to cross-linking are defined by the structural fold of the protein or protein complex of interest). Note, however, that the other conditions employed for the peptide cross-linking reactions (i.e., the use of a biologically relevant solution composition, pH, peptide/reagent molar ratio, etc.) are similar to those employed in previous protein cross-linking studies, thereby allowing the current methods to be readily applied to future studies involving the analysis of intact proteins. All reaction products were desalted by Sep-pak (Waters, Milford, MA) purification, with elution in 40, 60, and 80% acetonitrile (aq) containing 0.05% formic acid prior to mass spectrometry analysis.

Mass Spectrometry Analysis. Mass spectrometry analysis of cross-linked peptide ions was performed using Thermo Scientific model LCQ Deca 3D quadrupole ion trap or Thermo Scientific model LTQ linear quadrupole ion trap mass spectrometers (San Jose, CA), each equipped with nESI sources. Samples ($\sim 10 \mu\text{M}$) were introduced at a flow rate of $0.5 \mu\text{L min}^{-1}$. The spray voltage was maintained at 2.0–2.5 kV, while the capillary temperature was in the range 150–200 °C. Collision-induced dissociation (CID)-MS/MS and MS^n experiments were performed using standard isolation and activation conditions. The spectra shown are typically the average of 20–50 scans. Repeated analysis of individual samples was found to result in less than 5% variation in relative product ion abundances.

RESULTS AND DISCUSSION

Cross-Linker Design and Rationale. A cross-linking reagent that demonstrates preferential gas-phase fragmentation reactions under low-energy CID-MS/MS conditions must satisfy a number of criteria. The reagent must be soluble and stable when subjected to cross-linking reaction conditions in aqueous solutions at mild pH values. The cross-linker should contain an appropriate linker length for detecting informative protein–protein interaction sites within protein complexes (typical commercially available cross-linking reagents have a spacer arm less than 20 Å). The structure of the cross-linker (or a functional group within the cross-linker) should facilitate enrichment of the cross-linked reaction products in solution, in order to allow their observation during MS and to enable subsequent selection of the precursor ions for MS/MS analysis. Finally, a requirement that is specific to a gas-phase cleavable cross-linking reagent is that fragmentation of the cross-linker during MS/MS should occur exclusively and independently of the proton mobility of the cross-linked peptide precursor ion; i.e., the energy required for cleavage within the linker region should be lower than that for cleavage within the peptide backbone or side chains, regardless of the number of protons, the amino acid composition, or the sequence of the peptide.

NHS esters are commonly employed as the reactive groups of cross-linking reagents in order to target primary amino groups, (i.e., the ϵ -amino groups of lysine amino acid side chains or the

α -amino group of the N-terminus), located within the protein or proteins of interest.^{35,36} However, the application of NHS esters to the analysis of large protein complexes is typically limited by their poor solubility under "native" aqueous conditions, thereby requiring the use of sulfo-NHS esters. However, simple members of ionic sulfonium salts typically exhibit high solubilities in water.³⁷ Thus, a sulfonium ion containing cross-linker would be expected to offer an advantage for the mapping of large protein complexes due to its substantial solubility under aqueous conditions. The presence of the sulfonium ion would also facilitate enrichment of cross-linked peptides using strong cation-exchange chromatography prior to MS and MS/MS analysis.

Previous studies carried out in our laboratory have demonstrated that peptide ions containing fixed-charge sulfonium ion derivatives introduced to the side chains of certain amino acid residues (e.g., methionine and cysteine) undergo exclusive loss of a dialkylsulfide moiety during the time scale of ion activation in either ion trap or quadrupole mass spectrometers, via selective cleavage at the site of the fixed charge.^{28–32} Using a combination of experimental data and theoretical calculations, the mechanism for this loss has been proposed to proceed via neighboring group participation reactions involving nucleophilic attack from an adjacent amide bond, resulting in the formation of 6-membered oxazoline or 5-membered iminohydrofuran product ions.^{29,32} Importantly, these selective fragmentation reactions have been demonstrated to occur independently of the proton mobility of the peptide precursor ions containing the sulfonium ion derivatives. These results suggest that the incorporation of a fixed charge sulfonium ion into the backbone of a chemical cross-linking reagent, containing an NHS ester (that subsequently reacts with a primary amino group within the protein of interest to form a nucleophilic amide bond) and that has an appropriate alkyl chain (propyl or butyl) linker attached to the electrophilic sulfonium ion, would result in selective fragmentation of the cross-linker upon CID-MS/MS.

Synthesis of **1** was carried out as described above. ESI-MS analysis in a quadrupole ion trap of this reagent revealed a single ion at m/z 443.3, corresponding to the M^+ precursor ion. CID-MS/MS of the m/z 443.3 precursor ion resulted in the formation of a dominant product ion at m/z 198.1, via the neutral loss of 5-(methylthio)pentanoylhydroxysuccinimide (data not shown).

Potential mechanisms for the gas-phase fragmentation reactions of the various types of peptide products formed by reaction with cross-linking reagent **1** (i.e., intermolecular, dead-end, and intramolecular) are shown in Scheme 2. For intermolecular peptide cross-links (Scheme 2A), the two peptide chains are connected by a cross-linker arm (designated here as **I-S**) between two amide nitrogens. Fragmentation of a labile C–S bond on either side of the fixed charge upon low-energy CID MS/MS would result in the formation of two separated peptide chains containing unique modifications, one containing a protonated six-membered iminotetrahydropyran (**I**) with a mass increment of 83 Da, and the other containing a neutral 5-methylthiopentanoyl (**S**) group with a mass increment of 130 Da. Note that fragmentation of the

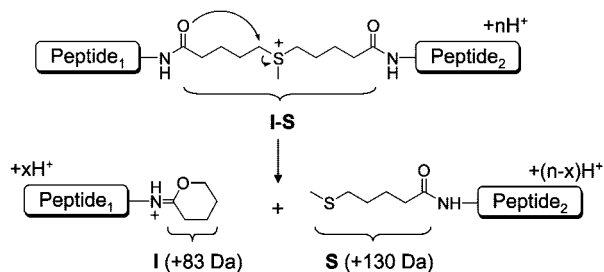
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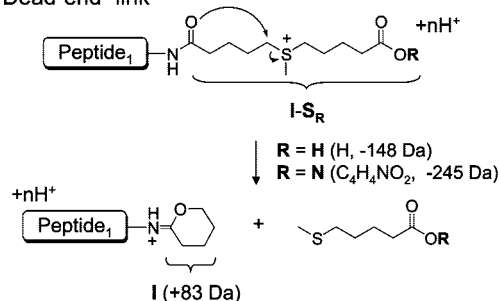
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Scheme 2. Selective Gas-Phase Fragmentation Reactions of Intermolecular, Dead-End, and Intramolecular Cross-Linked Peptide Products Formed by Reaction with Ionic Cross-Linking Reagent 1^a

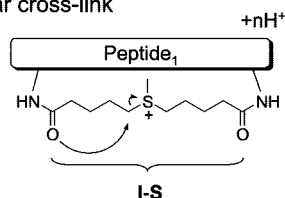
(A) Intermolecular cross-link



(B) "Dead-end" link



(C) Intramolecular cross-link



^a The linker formed upon cross-linking is denoted as **I-S**. Cleavage of the C-S bond within the linker region results in two specific peptide modifications, one corresponding to a 6-membered iminotetrahydropyran (**I**) and the other corresponding to S-methylthiopentanoyl (**S**) group. A "dead-end" modification (**I-S_R**) on an intact peptide is labeled **I-S_N** when an unreacted NHS functional group is retained, and **I-S_H** when a carboxyl group is formed via hydrolysis of the NHS ester.

cross-linker in Scheme 2A may occur on either side of the sulfonium ion linkage. Therefore, due to the symmetrical structure of the cross-linking reagent, the dissociation of a homodimeric intermolecular cross-linked precursor ion (i.e., when peptide₁ and peptide₂ are the same) would result in the formation of a single pair of **I** and **S** modified peptide product ions. In contrast, dissociation of a heterodimeric cross-linked precursor would yield two pairs of **I** and **S** modified products. This difference in fragmentation therefore allows these two types of reaction products to be readily differentiated. For "dead-end" cross-links (designated here as **I-S_R**), where one reactive NHS group has undergone a cross-linking reaction and where the other ester remains intact (**I-S_N**) or has undergone hydrolysis (**I-S_H**) (Scheme 2B), CID-MS/MS would result in the neutral loss of 5-(methylthio)pentanoic acid (148 Da, where R = H) or the loss of 5-(methylthio)pentanoylhydroxysuccinimide (245 Da, where R = C₄H₄NO₂). In both cases, it is predicted that the cleavage would preferentially take place at the C-S bond closest to the peptide chain, since an amide nitrogen is expected to be a better

nucleophile compared to either an ester or acid oxygen. For both intermolecular and dead-end cross-linked peptide products, subsequent isolation and MS³ dissociation of the initial MS/MS product ions can then be used to provide additional structural information required for identification of the peptide sequences involved in the cross-linking reactions, as well as for characterization of the specific site(s) at which cross-linking has occurred. For an intramolecular peptide cross-link (Scheme 2C), the desired C-S bond cleavages are also expected to be the energetically favored process. However, as no change in *m/z* will be observed upon initial dissociation of these bonds, it is expected that immediate further fragmentation would occur during MS/MS to yield b- and y-type sequence ions. The symmetrical structure of the cross-linking reagent **1**, would again be expected to result in similar fragmentation efficiencies at the two possible C-S bond cleavage sites. Thus, a given b- or y-type product ion may be observed with either **I** or **S** modifications.

Multistage Tandem Mass Spectrometry Analysis of Sulfonium Ion Containing Cross-Linked Peptide Ions. *Intermolecular Cross-Linked Reaction Products.* In order to initially examine the multistage CID-MS/MS gas-phase fragmentation behavior of the peptide products formed by reaction with cross-linking reagent **1'** or **1''**, the model synthetic peptide VTMAHFVNFVWGK (pep_{VWVK}) was subjected to cross-linking as described above. The ESI mass spectrum obtained following a 30-min cross-linking reaction with reagent **1'** is shown in Figure 1. Reaction with reagent **1''** was found to proceed more slowly, with similar yields of the various cross-linked peptide products obtained only after 90 min using 2.5 equiv of cross-linker (data not shown).

The complexity of the mass spectrum in Figure 1 arises due to the formation of multiple reaction products, including intermolecular (**a** in Figure 1) and intramolecular (**b** in Figure 1) cross-links (labeled [2M + nH + (I-S)]⁽ⁿ⁺¹⁾⁺ and [M + nH + (I-S)]⁽ⁿ⁺¹⁾⁺, respectively), monolinked unhydrolyzed (**c** in Figure 1) dead-end links ([M + nH + (I-S_N)]⁽ⁿ⁺¹⁾⁺), and mono- (**d** in Figure 1) and dilinked (**e** in Figure 1) hydrolyzed dead-end links ([M + nH + (I-S_H)]⁽ⁿ⁺¹⁾⁺ and [M + nH + 2(I-S_H)]⁽ⁿ⁺²⁾⁺, respectively), as well as due to the presence of residual unreacted cross-linking reagent (I-S)_{NN}⁺, hydrolyzed cross-linking reagent (I-S)_{NH}⁺, and doubly hydrolyzed cross-linking reagent (I-S)_{HH}⁺. Despite this complexity, the identification and characterization of each of these products was readily achieved by examination of the characteristic fragmentation behavior of each of their respective precursor ions.

Dissociation of the +5 charge state of the homodimeric intermolecular cross-linked precursor ion of pep_{VWVK} ([2M + 4H + (I-S)]⁵⁺ in Figure 1) resulted in exclusive fragmentation of the cross-link to yield a single pair of product ions containing iminotetrahydropyran (**I**) and S-methylthiopentanoyl (**S**) modifications ([M + 2H + **I**]³⁺ and [M + 2H + **S**]²⁺ in Figure 2A, respectively), consistent with that outlined in Scheme 2A. Importantly, the specificity of this fragmentation was found to be independent of the precursor ion charge state (see the product ion spectra obtained from the +4 and +3 charge states of the homodimeric intermolecular cross-linked precursor ion of pep_{VWVK} ([2M + 3H + (I-S)]⁴⁺ and ([2M + 2H + (I-S)]³⁺ in Figure 2B and C, respectively). Due to the presence of a single peptide in this reaction, and the symmetrical structure of the cross-linking

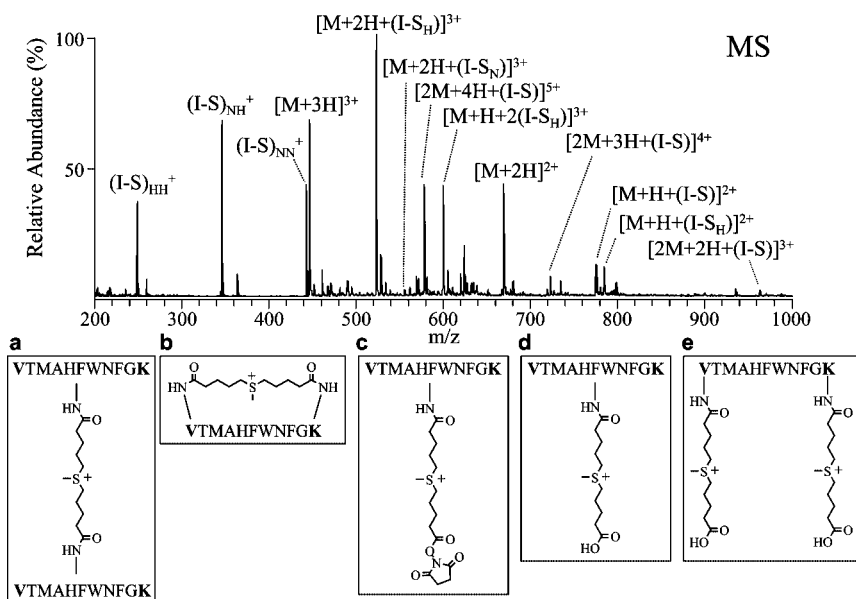


Figure 1. ESI-mass spectrometry analysis of cross-linked VTMAHFWNFGK (pep_{VWVK}) formed by reaction with reagent **1'**. Intermolecular cross-linked ions (structure **a**) are indicated as $[2M + nH + (I-S)]^{(n+1)+}$. Intramolecular cross-linked ions (structure **b**) are indicated as $[M + nH + (I-S)]^{(n+1)+}$. Unhydrolyzed monolinked ions (structure **c**) are indicated as $[M + nH + (I-S_N)]^{(n+1)+}$. Hydrolyzed monolinked ions (structure **d**) are indicated as $[M + nH + (I-S_H)]^{(n+1)+}$. Hydrolyzed dilinked ions (structure **e**) are indicated as $[M + nH + 2(I-S_H)]^{(n+2)+}$. The residual unreacted cross-linking reagent is labeled $(I-S)_{NN}^+$. Hydrolyzed cross-linking reagent is labeled $(I-S)_{NH}^+$. Doubly hydrolyzed cross-linking reagent is labeled $(I-S)_{HH}^+$.

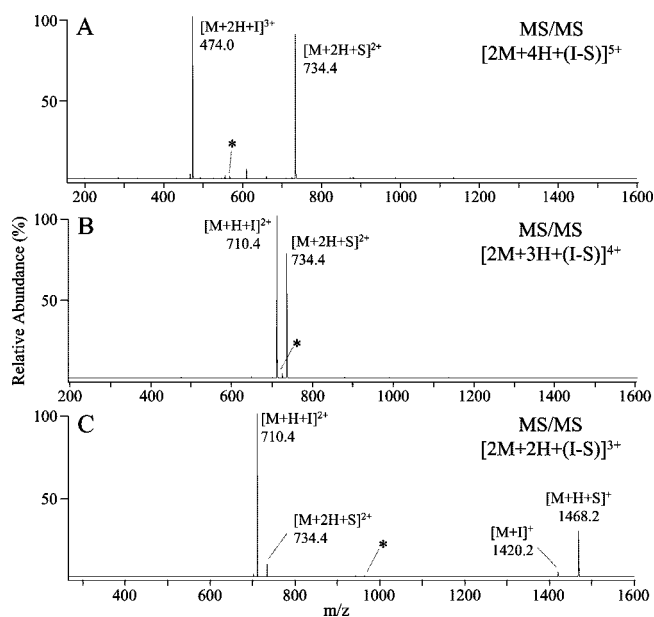


Figure 2. CID-MS/MS of intermolecular cross-linked ions formed from the model peptide (pep_{VWVK}) (A) $[2M + 4H + (I-S)]^{5+}$, (B) $[2M + 3H + (I-S)]^{4+}$, and (C) $[2M + 2H + (I-S)]^{3+}$ from Figure 1. The m/z of the precursor ions selected for dissociation in each spectrum are indicated by an asterisk.

reagent **1**, dissociation of the homodimeric intermolecular cross-linked precursor ions resulted in the formation of a single pair of **I** and **S** modified product ions.

A result similar to that described above was also observed upon CID-MS/MS analysis of the homodimeric reaction products formed by cross-linking of the aspartic acid containing peptide pep_{GDR} (GAILDGAILR). Panels A and B in Figure S1, Supporting Information (SI), show the product ion spectra obtained from the $[2M + 2H + (I-S)]^{3+}$ and $[2M + H + (I-S)]^{2+}$ precursor ions.

Importantly, essentially exclusive cleavage of the cross-linker was observed for these ions, even though an aspartic acid residue that was observed to undergo preferential cleavage giving rise to a single dominant product ion upon dissociation of its unmodified protonated precursor ion (Figure S1C, SI) was present within the peptide sequences. Previous studies to examine the global factors influencing the fragmentation reactions of protonated peptide ions have demonstrated that cleavage at the C-terminal side of aspartic acid residues is significantly enhanced under “partially mobile” and “nonmobile” proton conditions.²⁷ The +3 and +2 precursor ion charge states of the cross-linked sulfonium ion containing pep_{GDR} homodimer would both be classified as nonmobile, as would the +1 precursor ion charge state of the monomeric pep_{GDR} peptide. Thus, the observation of only low-abundance product ions corresponding to cleavage at the C-terminal side of an aspartic acid residue in the homodimeric cross-linked peptide in Figure S1B (SI) is consistent with our previous studies demonstrating that fragmentation of sulfonium ion derivatives is an energetically favorable process compared to those for amide bond cleavage reactions.^{29,32}

The mass spectrum obtained by ESI-MS analysis following cross-linking of a 1:1 mixture of neurotensin (α , pELYENK-PRRPYL) and angiotensin II (β , DRVYIHPF) with reagent **1'** is shown in Figure 3A. CID-MS/MS analysis of the homodimeric cross-linked precursor ions from neurotensin ($[2\alpha + 4H + (I-S)]^{5+}$) and angiotensin II ($[2\beta + 4H + (I-S)]^{5+}$) are shown in Figure 3B and C, respectively. Similar to that discussed above, a single pair of product ions containing **I** and **S** modifications were observed as the dominant fragmentation pathway in each case. In contrast, dissociation of the heterodimeric intermolecular cross-linked peptide product ($[\alpha + \beta + 3H + (I-S)]^{4+}$) resulted in the formation of two pairs of characteristic **I** and **S** modified product

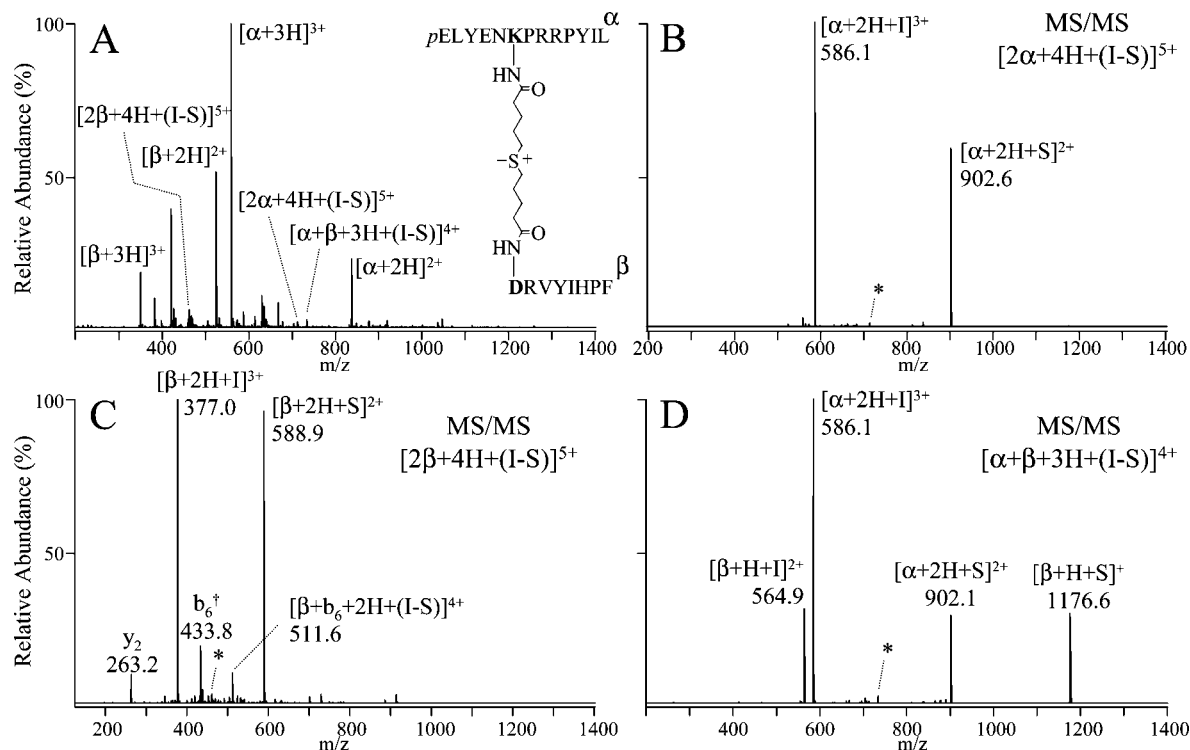


Figure 3. (A) ESI-mass spectrometry analysis of cross-linked neurotensin (α) and angiotensin II (β) formed by reaction with reagent **1'**. CID-MS/MS of (B) the $[2\alpha + 4H + (I-S)]^{5+}$ precursor ion of neurotensin containing a homodimeric intermolecular cross-link, (C) the $[2\beta + 4H + (I-S)]^{5+}$ precursor ion of angiotensin II containing a homodimeric intermolecular cross-link, and (D) the $[\alpha + \beta + 3H + (I-S)]^{4+}$ precursor ion of neurotensin and angiotensin II containing a heterodimeric intermolecular cross-link. The m/z of the precursor ions selected for dissociation in each spectrum are indicated by an asterisk. A † indicates product ions containing an **I** modification.

ions (Figure 3D). Therefore, the characteristic products formed from the dissociation of homo- and heterodimeric cross-linking reactions allow these products to be readily differentiated. A similar result was also obtained upon CID-MS/MS of the intermolecular heterodimeric cross-linked peptide product formed by reaction between $[Glu^1]$ -fibrinopeptide B and substance P (Figure S2, SI).

Note that in addition to cleavage of the cross-linker for the homodimeric cross-linked angiotensin II peptide reaction product in Figure 3C, several minor product ions formed by cleavage at the His_6-Pro_7 amide bond within the angiotensin II peptide sequence ($[\beta + b_6 + 2H + (I-S)]^{4+}$ and y_2) as well as by sequential fragmentation of the cross-linker and the His_6-Pro_7 amide bond (b_6^\dagger , where † indicates the presence of an **I** modification) were also observed (Figure 3C). Previous studies to examine the global factors influencing the fragmentation reactions of protonated peptide ions have demonstrated that cleavage at the N-terminal side of proline residues is significantly enhanced under “mobile” and “partially mobile” proton conditions²⁷ (the +5 charge state of the sulfonium ion containing cross-linked angiotensin II homodimer would be classified as partially mobile). Again, therefore, the observation of these ions at only low abundance in Figure 3C is consistent with fragmentation of sulfonium ion derivatives being an energetically favorable process.^{29,32}

Neurotensin and angiotensin II each contain only a single amino group amenable for reaction with the NHS ester of the cross-linking reagent. Thus, it was not necessary to obtain further evidence to assign the sites at which the cross-linking reactions

had occurred for the reaction products in Figure 3. In contrast, the model peptide pep_{VWK} could undergo reaction at either the α -amino or ϵ -amino groups to yield an isomeric mixture of intermolecular cross-linked homodimeric products (i.e., α -amino to α -amino, ϵ -amino to ϵ -amino, or α -amino to ϵ -amino). Therefore, in order to determine the modification site(s) at which the homodimeric cross-linking reaction had occurred, as well as to estimate the extent of reaction occurring at each site, both of the MS/MS peptide products shown in Figure 2A were isolated then subjected to further dissociation by MS³, followed by analysis of the resultant b- and y-type product ions (Figure 4A and B). The CID-MS³ spectra from the $[M + H + I]^{2+}$ product ions from Figure 2B and C and the $[M + H + S]^+$ product ion from Figure 2C are shown in Figure S3 (SI). In all cases, both b- and y-type product ions containing **I** (labeled with a † in Figure 4A and Figure S3A, SI) or **S** (labeled with a ‡ in Figure 4B and Figure S3B, SI) modifications were observed, indicating that cross-linking had occurred on both α -amino and ϵ -amino functional groups.

An attempt to estimate the extent to which the cross-linking reactions had occurred at the α - or ϵ -amino groups of the pep_{VWK} peptide was made by measuring the ratio of the summed relative abundances of the modified b-type ions and unmodified y-type ions (i.e., α -amino modified), to the summed relative abundances of the unmodified b-type ions and modified y-type ions (i.e., ϵ -amino modified) in Figure 4 and Figure S3 (SI). However, as ion detection efficiency in ion trap mass spectrometers is affected by both the charge state and product ion mass (some product ions fall below the low-mass cutoff of the instrument so are not observed), some variability was observed (63:37 for the $[M + 2H$

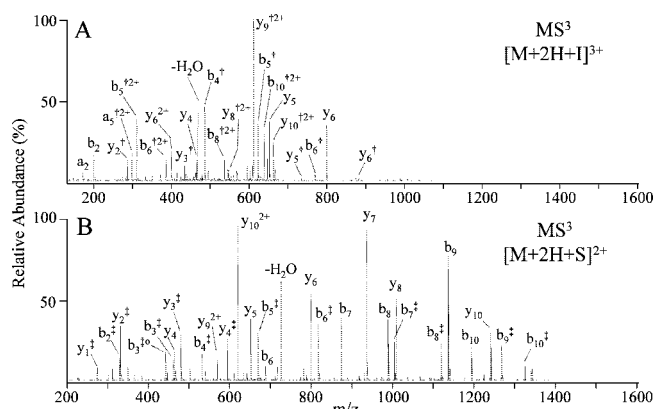


Figure 4. CID-MS³ spectra of (A) the [M + 2H + I]³⁺ product ion and (B) the [M + 2H + S]²⁺ product ions from Figure 2A. A † indicates product ions containing an **I** modification. A ‡ indicates product ions containing an **S** modification. A superscript “o” indicates the neutral loss of H₂O.

+ I]³⁺ ion in Figure 4A, 68:32 for the [M + 2H + S]²⁺ ion in Figure 4B, 25:75 for the [M + H + I]²⁺ ion in Figure S3A (SI), and 79:21 for the [M + H + S]⁺ ion in Figure S3B (SI), depending on the specific product ions that were observed in each case. Overall, however, there was a preference for reaction at the α-amino position, consistent with its lower pK_a compared to that of the ε-amino group.

Importantly, in all cases, as the individual peptide chains involved in intermolecular cross-linking reactions are separated upon cleavage of the sulfonium ion linker upon performing MS/MS, characterization of the peptide sequences by MS³ is greatly simplified (i.e., no product ions are generated that contain amino acid residues from both peptides). Furthermore, the presence of **I** or **S** modifications was found to have only a minimal influence on the appearance of the product ions produced following MS³, compared to their unmodified peptides (data not shown).

Dead-End Cross-Linked Reaction Products. Dissociation of the triply charged precursor ions from the intact (i.e., unhydrolyzed) and hydrolyzed monolinked products formed by reaction of the model peptide pep_{VWK} with the sulfonium ion containing cross-linking reagent **1'** ([M + 2H + (I-S_N)]³⁺ and [M + 2H + (I-S_H)]³⁺ from Figure 1, respectively) each resulted in essentially exclusive neutral losses of 5-(methylthio)pentanoylhydroxysuccinimide (245 Da) or 5-(methylthio)pentanoic acid (148 Da), respectively (Figure 5A and B). This is consistent with the mechanism proposed in Scheme 2B where the major cleavage was expected to take place on the C–S bond closest to the peptide chain due to the greater nucleophilic strength of the amide nitrogen compared to either the acid or ester oxygen. However, a minor product ion corresponding to cleavage on the “outer” C–S bond ([M + 2H + S]²⁺ in Figure 5B), involving nucleophilic attack from the acid oxygen, was also observed from the hydrolyzed monolinked ion, indicating that some, albeit minor, competition between these cleavage sites is possible for this reaction product. Similar fragmentation behavior was also observed upon MS/MS dissociation of the doubly charged precursor ions from the intact and hydrolyzed monolinked reaction product from the pep_{VWK} peptide (data not shown), as well as for the doubly and singly charged precursor ions from the hydrolyzed monolinked reaction

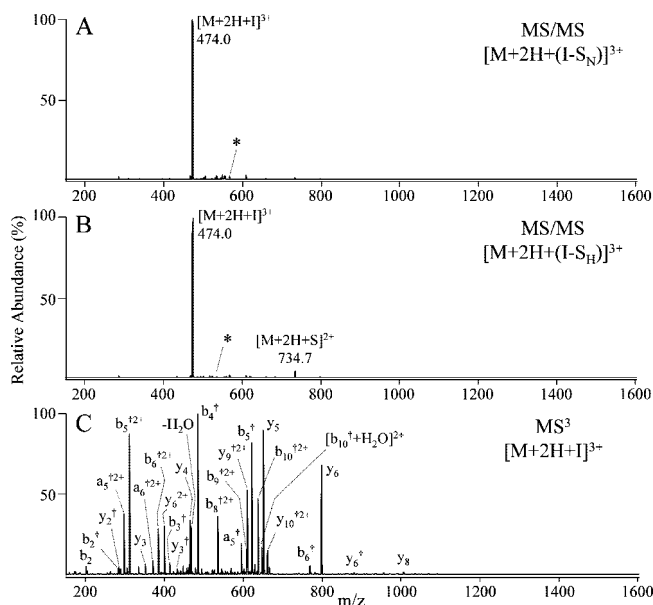


Figure 5. CID-MS/MS and MS³ of pep_{VWK} containing unhydrolyzed (I-S_N) and hydrolyzed (I-S_H) monolinks. (A) MS/MS of the [M + 2H + (I-S_N)]³⁺ precursor ion, and (B) MS/MS of the [M + 2H + (I-S_H)]³⁺ precursor ion from Figure 1. The *m/z* of the precursor ions selected for dissociation in each spectrum are indicated by an asterisk. (C) MS³ of the [M + 2H + I]³⁺ product ion from panel A. Note that an identical spectrum was obtained by MS³ of the [M + 2H + I]³⁺ product ion from panel B. A † indicates product ions containing an **I** modification.

product from the pep_{GDR} peptide (Figure S4, SI). Notably, the specificity associated with fragmentation of the cross-linker from the triply and doubly charged precursor ions of the intact monolinked reaction product from the phosphoserine containing pep_{LpSR} peptide was found not to be significantly affected by the potentially competing loss of H₃PO₄ (Figure S5, SI). Interestingly, comparison of the spectra obtained by CID-MS³ of the [M + 2H + I]³⁺ product ions (shown in Figure 5C) from Figure 5A and B with that observed from the intermolecular cross-linked product ion in Figure 4A revealed a significantly higher abundance of b-type ions containing the **I** modification for the monolinked products (labeled in the spectra with a †), suggesting a somewhat greater specificity for modification of the α-amino functional group.

Although the cross-linking reactions were carried out at a low cross-linker to peptide ratio, a relatively abundant hydrolyzed dilinked peptide reaction product was also observed in Figure 1 ([M + H + 2(I-S_H)]³⁺). A particular feature of this dilinked product, which distinguishes it from monolinked modifications, is its multistage MS/MS behavior. As predicted, a major neutral loss of 148 Da was observed from MS/MS of the [M + H + 2(I-S_H)]³⁺ precursor ion (Figure 6A). However, the sequential fragmentation of a second 148-Da neutral species was also observed in this spectrum, indicating the presence of a second **I-S_H** modification in this ion. MS³ of the [M + H + I + (I-S_H)]³⁺ product ion from Figure 6A confirmed the presence of this additional modification (Figure 6B), while MS⁴ of the [M + H + 2I]³⁺ ion (Figure 6C) was employed to confirm the sites of modification to the N-terminal α-amino and lysine side chain ε-amino functional groups. Note that while peptide ions containing mono- or di- dead-end links do not provide information regarding the spatial relationships of target functional groups within the

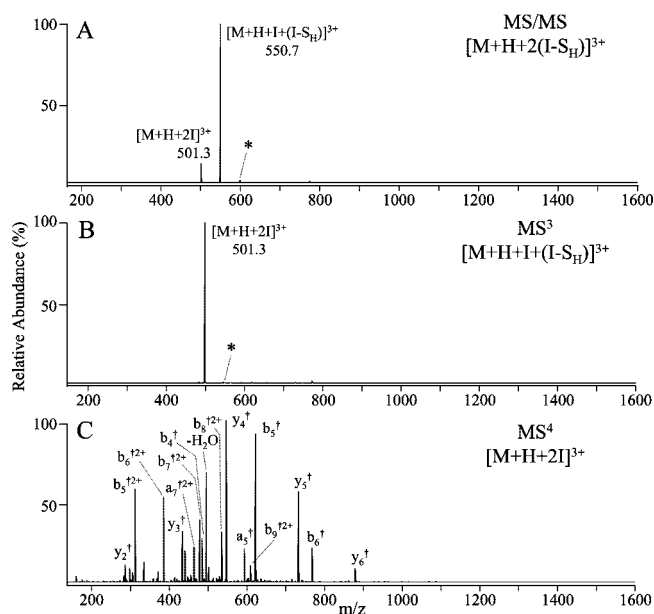


Figure 6. (A) CID MS/MS of the $[M + H + 2(I-S_H)]^{3+}$ precursor ion of pep_{VWK} containing a hydrolyzed (I-S_H) dilink from Figure 1. (B) MS³ of the $[M + H + I + (I-S_H)]^{3+}$ product ion from panel A. The *m/z* of the precursor ions selected for dissociation in each spectrum are indicated by an asterisk. (C) MS⁴ of the $[M + H + 2I]^{3+}$ product ion from panel B. A † indicates product ions containing an **I** modification.

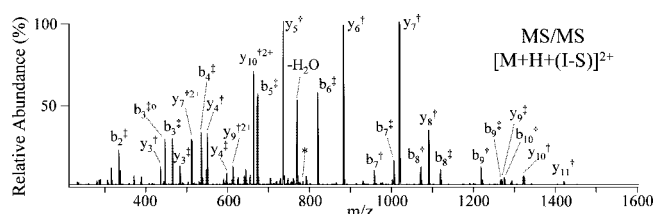


Figure 7. CID-MS/MS of the $[M + H + (I-S)]^{2+}$ precursor ion of pep_{VWK} containing an intramolecular cross-link from Figure 1. The *m/z* of the precursor ions selected for dissociation in the spectrum is indicated by an asterisk. A † indicates product ions containing an **I** modification. A ‡ indicates product ions containing an **S** modification.

protein of interest, the identification of these products can yield useful information regarding the surface accessibility of these functional groups.^{3,38}

Intramolecular Cross-Linked Reaction Products. Consistent with the proposal outlined in Scheme 2C, MS/MS of the intramolecular cross-linked $([M + H + (I-S)]^{2+})$ precursor ion from the pep_{VWK} peptide from Figure 1 resulted in initial cleavage of the cross-linker, followed by immediate further fragmentation within the peptide backbone, thereby allowing detailed structural information to be obtained by direct analysis of the MS/MS product ion spectrum (Figure 7). Similar results were also obtained following CID-MS/MS of the intramolecular cross-linked $([M + H + (I-S)]^{2+})$ precursor ions observed following reaction of **1'** with either substance P (Figure S6A, SI) or the phosphoserine

containing pep_{LpSR} peptide (Figure S6B, SI). From analysis of the spectrum in Figure 7, it appeared that **I** modifications (labeled in the spectra with a †) were mainly located on *y*-type product ions while **S** modifications (labeled in the spectra with a ‡) were mainly located on the *b*-type product ions. These data indicate that the two C–S bonds do not have identical cleavage efficiencies. We hypothesize that this is due to the individual intramolecular “solvation” environment of the nucleophilic α -amide bond and ϵ -amide bond that are responsible for the sulfonium ion bond cleavage reactions, that subsequently affects the nucleophilic reactivity of these groups. Further studies are currently underway to obtain more detailed insights into this observation.

CONCLUSIONS

The results presented here illustrate that CID-MS/MS of cross-linked peptides containing a fixed charge sulfonium ion can be employed to effectively identify cross-linked peptides from unmodified peptides, via recognition of their distinct fragmentation patterns, as well as to distinguish between the various types of cross-linked products (i.e., intra, inter, and dead-end) that may be formed from a cross-linking reaction. Homodimeric intermolecular cross-linked peptides are readily identified by the formation of a single pair of characteristic product ions, while heterodimeric intermolecular cross-linked peptides are identified by the formation of two pairs of product ions. Peptides containing intact or hydrolyzed dead-end cross-links are identified based on the observation of their characteristic neutral losses. MS³ of these initial products can be used to provide information required for characterization of the peptide sequences and localization of the modification site(s) involved in the cross-linking reactions. MS/MS of intramolecular cross-linked peptides results in initial cleavage of the cross-linker, followed by immediate further fragmentation of the peptide backbone, thereby allowing detailed structural information to be obtained by direct analysis of the MS/MS product ion spectrum. The specificity of these gas-phase fragmentation reactions, along with the solubility and stability of the sulfonium ion containing cross-linking reagent under aqueous conditions, suggests that this reagent holds great promise for the mass spectrometry based structural analysis of large proteins or multiprotein assemblies in future studies.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Figures S1–6, containing MS/MS and MS³ product ion spectra. Raw data files for each of the CID-MS/MS, -MS³ or -MS⁴ spectra shown in Figures 1–7 and SI Figures S1–S6 may be obtained from the authors upon request. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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