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**STUDIES ON THE FRAGMENTATION REACTIONS OF FIXED CHARGE
SULFONIUM ION CONTAINING PEPTIDES BY TANDEM MASS
SPECTROMETRY**

BY

MAHASILU AMUNUGAMA

A THESIS

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ABSTRACT

STUDIES ON THE FRAGMENTATION REACTIONS OF FIXED CHARGE

SULFONIUM ION CONTAINING PEPTIDES BY TANDEM MASS

SPECTROMETRY

BY

MAHASILU AMUNUGAMA

In order to enable the development of improved tandem mass spectrometry based methods for selective proteome analysis, the mechanisms, product ion structures and other factors influencing the gas-phase fragmentation reactions of methionine side chain derivatized 'fixed-charge' phenacyl sulfonium ion containing peptide ions have been examined. Dissociation of these peptide ions results in exclusive loss of the derivatized side chain, thereby enabling their selective identification. The resultant product ion(s) are then subjected to further dissociation to obtain sequence information for subsequent protein identification. Molecular orbital calculations (at the B3LYP/6-31+G** level of theory) performed on a simple peptide model, together with experimental evidence obtained by multistage dissociation of a regioselectively deuterated methionine derivatized sulfonium ion containing tryptic peptide, indicate that the initial fragmentation occurs via S_N2 reactions resulting in the formation of cyclic five- and six-membered hydrofuran and oxazine product ions. Further, molecular orbital calculations and experimental evidence obtained from various *para*-substituted phenacyl sulfonium ion peptide ions have determined that the ratio of neutral versus charged losses of the derivatized side chain exhibits a linear dependence on the proton affinity of the side chain fragmentation product, as well as the proton mobility of the peptide product ions.

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CHAPTER ONE

INTRODUCTION

1.1 Proteomics

The rapid completion of the human genome sequence [1] has enabled research efforts aimed at determining the unknown functional roles of the proteins encoded by these genes. However, in contrast to the static genome, the proteome is dynamic. Proteins are continuously synthesized and degraded in order to carry out the functions of the cell. Proteins can often be post-translationally modified, and interact with other proteins in such a way that they can function as a part of a large protein complex. The studies of proteins are important because it is believed that a disease can arise due to altered protein expression. Thus, changes in protein expression or modification state may be used in medicine as specific biomarkers for the early diagnosis of disease. Therefore, the systematic large scale analysis of protein expression under normal and perturbed cellular conditions, such as stressed, diseased, or drugged states may lead to the development of novel therapeutic drugs [2]. The major goal of proteomic studies is the comprehensive separation, identification, characterization and quantitative analysis of all the gene products, i.e., proteins, expressed by a particular cell or tissue type at a given time, together with the systematic characterization of their specific localization, modification state and functional interactions.

1.2 Mass Spectrometry

Mass spectrometry, which measures an intrinsic property of a molecule (i.e., mass to charge) is one of the most important analytical techniques whose beginning dates back to the start of the last century. Figure 1.1 shows the main components of a mass spectrometer. The mass spectrometer consists of an ion source from which the charged ions are generated, a mass analyzer that measures the mass to charge ratio (m/z) of the ionized analytes and a detector that convert the ion flux into a proportional electric current. A data system then records the magnitude of the electrical signal as a function of m/z which is then shown as a mass spectrum. In the early 1980s, fast atom bombardment (FAB) mass spectrometry was employed to identify and characterize biological molecules [3, 4, 5]. Since the late 1980's, the development of the electrospray ionization (ESI) [6] and matrix-assisted laser desorption ionization (MALDI) [7] soft ionization techniques, coupled with the development of improved mass analyzers yielding better sensitivity and higher resolution, has led to mass spectrometry becoming an essential tool for proteomics research.

1.2.1 Electrospray Ionization (ESI)

Fenn, *et. al.* first introduced the electrospray ionization (ESI) technique in the late 1980's [6]. A liquid containing the analyte is pumped through a small diameter needle to which a high voltage is applied to produce an electrospray containing small micrometer sized droplets. These charged droplets rapidly evaporate in the presence of high temperature or a sheath gas, and undergo a series of coulombic fission events to yield gas-phase ions according to the charge residue [8] or the ion evaporation models [9, 10]. The most significant advantage of electrospray ionization technique is that it is operated

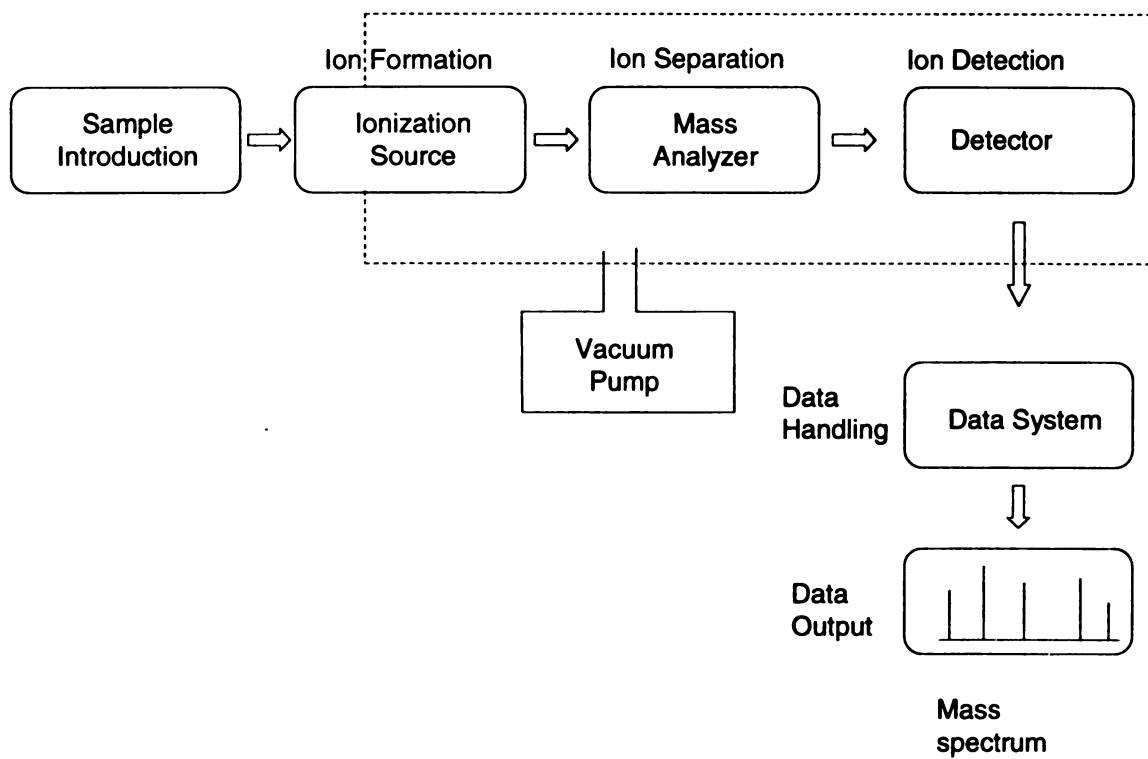


Figure 1.1 Components of a mass spectrometer (adapted from “What is Mass Spectrometry”. www.asms.org).

under atmospheric pressure, enabling a wide range of compounds including proteins and peptides that are sufficiently polar to allow attachment of a charge to be analyzed. ESI can be operated in either an infusion mode or in combination with high performance liquid chromatography (HPLC). In the infusion mode, the analyte is dissolved in a suitable solvent (typically 1:1 methanol:water) and introduced to the ESI capillary via an injection pump at flow rates as low as few hundred nanoliters per minute. When on-line HPLC is employed, the analytes are introduced to the ESI source as they elute from the chromatographic column. Hence, sample clean up, concentration and separation of the analytes can be achieved in a single step. A striking feature of ESI is the ability to form a series of pseudomolecular ion charge states, including both singly and multiply charged ions. The extent of multiple charging is influenced by factors such as the composition and pH of the electrospray solvent, as well as the chemical nature of the analyte. These multiple charge states can be used to provide independent verification of the mass, and allow large molecules such as proteins and peptides to be observed using mass analysers with limited m/z charge ranges.

1.2.2 Matrix-assisted laser desorption ionization (MALDI)

Karas and Hillenkamp developed matrix-assisted laser desorption ionization (MALDI) technique in the late 1980s [7]. MALDI is achieved in two steps. First the analyte is mixed with a solvent containing small organic molecules (matrix) that has a strong absorption at the wavelength of the laser employed for ionization, then allowed to dry on a metal substrate. Second, the resulting solid is placed under vacuum and irradiated by nanosecond laser pulses for a short duration. This generates a rapid heating

of the matrix crystals and sublimation of matrix and analyte molecules into the gas phase. Ionization can occur during any time of this process, however, the exact mechanism by which ionization takes place is not yet fully understood [11]. Typically, singly charged pseudomolecular ions are observed by MALDI.

1.2.3 Mass Analyzers

The mass analyzer is one of the most important components of the mass spectrometer. Once the ions have been produced, the mass analyzer separates ions according to their mass to charge (m/z) ratio. Key analytical figures of merit in proteomics include resolution, sensitivity and mass accuracy, which are all dependant on the performance of the mass analyzer. There are four basic types of mass analyzers currently used in proteomics. They are (i) quadrupole (ii) quadrupole ion trap, (iii) time of flight (TOF) and (iv) Fourier transform ion cyclotron resonance (FT- ICR) analyzers. The quadrupole and quadrupole ion trap mass analyzers are described in more detail below.

1.2.3.1 Quadrupole Mass Analyzers

Quadrupole mass analyzers are made using four parallel hyperbolic or cylindrical rods, upon which is imposed a variable amplitude oscillating AC (RF) voltage superimposed with a variable DC voltage to separate ions according to their m/z ratios. The potentials applied to opposite sets of rods (Φ_0) are given by the following equations.

$$\Phi_0 = + (U - V \cos \omega t) \quad \text{and} \quad - \Phi_0 = - (U - V \cos \omega t)$$

where U is the amplitude of the DC potential, V is the amplitude of the AC potential, $\omega = 2\pi\nu$ is the angular frequency (ν is the frequency of the radio frequency) and t is time.

The force on the ions in the x and y directions of the quadrupole rods are given by

$$F_x = m \frac{d^2 x}{dt^2} = -ze \frac{\partial \Phi}{\partial x} \dots\dots\dots (1)$$

$$F_y = m \frac{d^2 y}{dt^2} = -ze \frac{\partial \Phi}{\partial y} \dots\dots\dots (2)$$

Where Φ is a function of Φ_0

$$\Phi_{xy} = \Phi_0 (x^2 - y^2) / r_0^2 = \frac{(x^2 - y^2) * (U - V \cos \omega t)}{r_0^2} \dots\dots\dots (3)$$

After differentiating and rearranging this leads to the following equations

$$\frac{d^2 x}{dt^2} + \frac{2ze}{mr_0^2} (U - V \cos \omega t) x = 0 \dots\dots\dots (4)$$

$$\frac{d^2 y}{dt^2} - \frac{2ze}{mr_0^2} (U - V \cos \omega t) y = 0 \dots\dots\dots (5)$$

In this equation, x and y are the distances of the ion from the center of the quadrupoles in the x and y directions, respectively. U and V are variables. As long as the values of x and y do not reach r_0 , the distance between the center and the quadrupole, the trajectories of the ions are stable. These two differential equations are similar to the Mathieu equation written below

$$\frac{d^2u}{d\xi^2} + (a_u - 2q_u \cos 2\xi)u = 0 \dots\dots\dots (6)$$

In equation (6), 'u' is similar to either x or y in equation (4) and (5), where $\xi = \frac{\omega x}{2}$

Therefore, the previously determined solutions for a_u and q_u from the Mathieu equation can be used to obtain solutions for U and V.

$$a_u = a_x = -a_y = \frac{8zeU}{m\omega^2 r_0^2}$$

$$q_u = q_x = -q_y = \frac{4zeV}{m\omega^2 r_0^2}$$

After rearranging these equations, we obtain the following equations used to derive the operating parameters for the quadrupole mass analyzer.

$$U = a_u \frac{m\omega^2 r_0^2}{z8e} \dots\dots\dots (7)$$

$$V = q_u \frac{m\omega^2 r_0^2}{z4e} \dots\dots\dots (8)$$

In these solutions, r_0 and ω are constants for a given quadrupole. Therefore, for a given m/z , r_0 , e and ω are constant. A plot of U and V gives the stability areas for different masses where ions have a stable trajectory through the quadrupole. Figure 1.2 shows the stability areas for positive ions with increasing m/z values from m_1 to m_4 . To obtain a mass spectrum, we can scan the different ions by changing U linearly as a function of V at a constant U/V ratio along a straight line near the apex of the stability diagram. When a given U/V ratio is selected near the apex, only one m/z ion corresponding to that U/V ratio is stable, whereas other ions are unstable and are lost. Thus, the higher the slope of scanning line, the higher the resolution. If the slope of the scanning line is low, a range of m/z ions will be stable at a given U/V value, resulting in lower resolution since the stability areas overlap each other [12].

Quadrupole mass spectrometers have been constructed to perform tandem mass spectrometry (MS/MS) by coupling three quadrupole mass analyzers together in series. In a triple quadrupole mass spectrometer, the first and the third quadrupoles are used to scan ions while the second is used as a collision cell. A product ion scan mode tandem mass spectrum can be obtained by selecting a precursor ion of interest using the first quadrupole and by fragmenting it in the second quadrupole while scanning the third

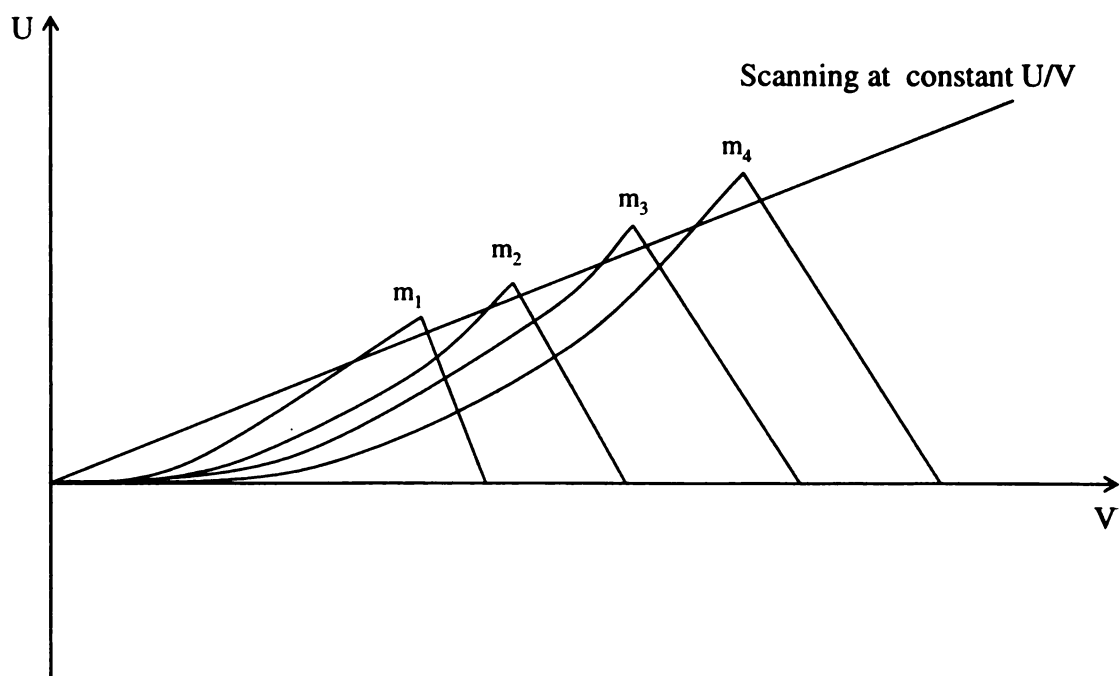


Figure 1.2 Stability areas as a function of U and V for positive ions with different masses where $m_1 < m_2 < m_3 < m_4$. We can scan all ions by changing U linearly as a function of V along a straight operating line. (Reproduced and modified from the reference [12]).

quadrupole for product ions. Similarly, precursor ion scan, neutral loss scan and selected reaction monitoring experiments can be performed [12].

1.2.3.2 The Quadrupole Ion Trap Mass Analyzer

In the three dimensional ion trap, a variable amplitude radio frequency is applied to the ring electrode to create a three dimensional quadrupole electric field to trap ions at the center of the trap. Typically, the ion trap is filled by an inert gas (such as helium) to a pressure of about 1 mTorr. Ion collisions with the inert gas decreases the kinetic energy of the ions and help to contract their trajectories quickly toward the center of the trap under the influence of the oscillating radio frequency field. The solution of the Mathieu equation can also be used to describe the theory of ion motion in three dimensional ion traps. Since a DC potential is not required for ion storage, the solution for a_u of Mathieu equation is equal to zero whereas q_z is given by following equations,

$$q_u = \frac{4zeV}{mr_0^2\omega^2} \dots\dots\dots (9)$$

$$q_u = -2q_r = \frac{8zeV}{m(r_0^2 + 2z_0^2)\omega^2} \dots\dots\dots (10)$$

where r_0 and z_0 which are constant for a given ion trap, are the distances from the centre of the trap to the ring electrode and end cap electrodes, respectively, V is the amplitude of the RF potential, ω is the angular velocity of the RF potential which is a constant at a fixed frequency. Equation (9) represents the q value for an ‘ideal’ quadrupole ion trap,

whereas equation (10) represents the stretched quadrupole ion trap geometry from which the well characterized problem of mass shift can be overcome [13]. Ions are stable in the ion trap as long as their trajectories do not reach the distances r_0 and z_0 . Figure 1.3 shows the stability diagram for the 3D quadrupole ion trap. According to the equation (9) and (10), q is inversely proportional to the m/z value and directly proportional to V . In this figure, larger balls represent high m/z ions at low q values and small balls represent low m/z ions at high q values. Since there is no DC voltage applied, the ion trap is operated along the $V(q)$ axis. When V is increased, ions move towards higher q values. Once ions exceed a q value of 0.908 at the boundary of the Mathieu stability diagram, the ions trajectory becomes unstable and is ejected. Some percentage of these ions reaches the detector. However, in order to obtain improved mass analysis performance, a supplementary (auxiliary) radio frequency is typically applied to the end cap electrodes to enable resonance ejection. In this mode, when V is increased, ions again move towards higher q values. However, when the ions reach a q value corresponding to the applied resonance ejection RF value below the normal instability q value as shown in Figure 1.3, ions are rapidly destabilized and ejected from the ion trap. The supplementary RF may also be used for ion excitation during MS/MS.

The main advantage of the ion trap is the ability to perform multistage tandem mass spectrometry (MS^n) experiments. This can be achieved by isolation of an ion of interest by applying a high amplitude broadband resonance ejection supplementary RF signal to the end caps to eject all ions except the ion of interest. Then the isolated ion is subjected to a low amplitude single frequency supplementary RF signal. The ion undergoes energetic collisions with the inert gas present in the trap, and subsequently

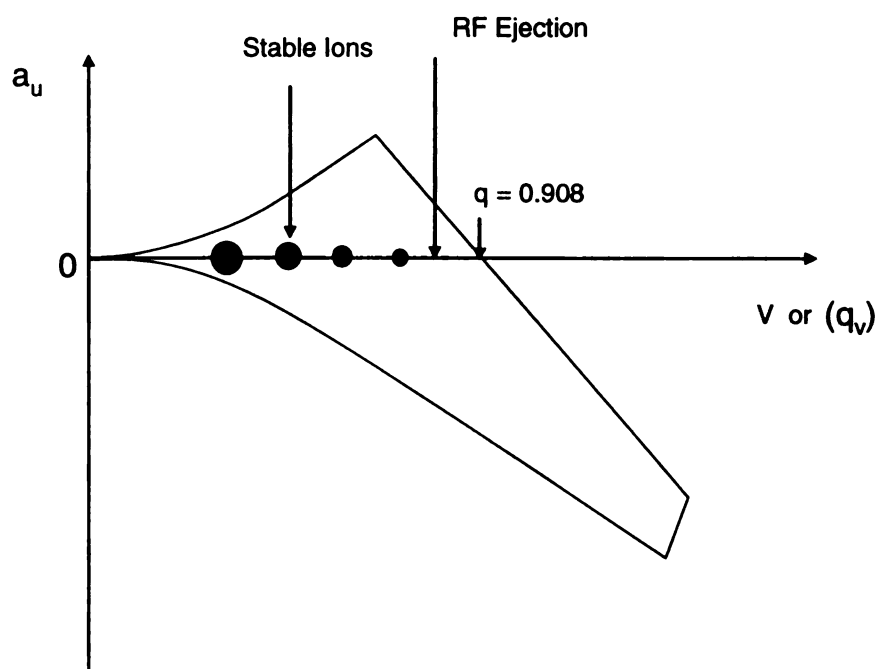


Figure 1.3 Typical stability diagram for quadrupole ion trap. The larger balls represent high mass ions whereas smaller balls represent the low mass ions. (Reproduced and modified from reference [12])

undergoes dissociation. This fragmentation is performed at a relatively low $V(q)$ value to reduce the low mass cut off. However, if the V value is too low during fragmentation, it may be difficult to apply enough energy for ion fragmentation to occur prior to ion ejection, due to the lower Dehmelt potentials at low V . The Dehmelt potential curve is shown in Figure 1.4. The energy E_{RE} in the Figure 1.4 represents the energy that should be provided for that ion by resonant excitation to eject the ion from the trap. Other advantages of ion traps are sensitivity, robustness and their relative inexpensive. The main disadvantage of the ion trap is the low mass accuracy due to the limited number of ions that can be accumulated before space charge effects distort the ion motions. The recently developed linear two-dimensional (2D) quadrupole ion trap can be used to overcome most of these disadvantages. The linear ion trap generates a two dimensional electric field and thus ions can enter the trap more easily. Ions are trapped by a combination of RF applied to the quadrupole rods, and a static DC potential applied to the electrodes situated at both end of quadrupoles. The linear ion trap also has higher ion storage capacity and exhibits less space charge effects [14,15]. Alexander Makarov *et al* very recently developed a new orbitrap mass analyser from which high mass accuracy up to parts per million and high mass resolution can be obtained [16,17,18,19].

1.3 Current Approaches Employed for Protein Identification and Characterization by Mass Spectrometry

Prior to mass spectrometry, protein mixtures are typically partially resolved by column chromatography, affinity chromatography or one- or two-dimensional gel electrophoresis.

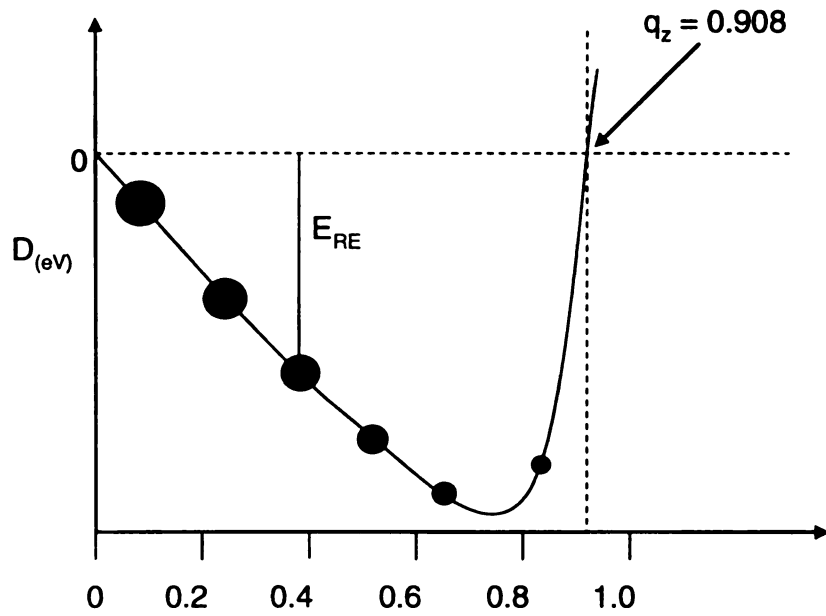


Figure 1.4 Dehmelt pseudo well potential D (eV) as a function q_z . Higher mass ions for a given V value have lower Dehmelt trapping energies. (Reproduced and modified from reference [12]).

Once a protein of interest is adequately purified, it is then enzymatically digested (usually with trypsin) to form a series of peptides. These peptides are then introduced into the mass spectrometer to obtain mass spectra. Although these masses may provide a 'peptide mass fingerprint' that can be compared to the theoretically expected peptides in a database to identify the protein of interest, the intact masses alone may not be sufficient for unambiguous protein identification or characterization. This is a particular issue for the identification and characterization of post translationally modified proteins, or for the identification of proteins present in more complex unresolved mixtures. Therefore, tandem mass spectrometry (MS/MS) experiments are commonly performed to obtain information regarding the amino acid. Protein identification is then achieved by database analysis as described below.

1.3.1 Database Search Algorithms for Protein Identification

Several database search algorithms, such as Sequest [20] and Mascot [21], have been developed for protein identification using mass spectrometry derived data. These algorithms calculate a score for how well an experimental product ion tandem mass spectrum agrees with a theoretical product ion spectrum generated by the search engine. The algorithms first theoretically cleave all the proteins in the database into peptides according to the experimental enzyme cleavage specificity, then extract all the peptides from the database whose masses correspond to the mass of the experimentally observed peptide, within a given mass tolerance. The presence of post translational or chemical modification to certain amino acids may be accounted for in this step. Then, the matching peptides are theoretically fragmented to give the masses of the b- and y- type ions, and

compared to the experimentally observed product ion masses. Peptides are accepted as genuine hits if they fulfill criteria such correct enzymatic cleavage specificity, a score above a pre-defined cut off value, and the absence of other peptides that match with similar scores [22, 23].

The success of the database search approaches outlined above requires that the experimental peptide sequence must be contained within the sequence database. Thus, when the protein of interest is not in the database, it is necessary to determine its identity by *de novo* peptide sequence analysis [24]. Furthermore, the success of the algorithms also depends on their ability to accurately predict the types and abundances of the product ions that will be generated by MS/MS. The database search algorithms typically only consider product ions formed by amide bond cleavage. However, most spectra also contain product ion formed via the loss of water, ammonia, carbon monoxide, cleavage of side chains, or internal fragmentations. Furthermore, some amino acid residues within a peptide sequence lead to unusual cleavage pathways which are not adequately accounted for by current algorithms. Phosphorylation, oxidation, acetylation, methylation, glycosylation, ubiquitination and lipid modifications are some of the most common post translational modifications (PTMs) [25]. Post-translational modifications change the mass of the attached amino acid within the peptide. Although the Sequest and Mascot algorithms have been extended to account for the changes in peptide mass occurring as a result of these post translational modifications [23, 26], they typically do not account for changes in the product ion spectra induced by these modifications. Oxidation of methionine containing proteins, as a result of post translational modification or sample handling, may result in the characteristic 'non-sequence' neutral loss of methane sulfenic

acid (CH_3SOH , 64 Da) [27]. Reid et. al. have recently studied the fragmentation reactions of a series of methionine sulfoxide containing peptides. Under mobile-proton conditions (the mobile proton model is described in section 1.4.1), fragmentation of the protonated precursor ions resulted in non-specific fragmentations along the peptide backbone, whereas dissociation of the same peptides under non-mobile proton conditions resulted in exclusive loss of CH_3SOH at the methionine sulfoxide side chain via a cis 1,2 elimination mechanism (a “charge remote” mechanism). Phosphorylation of serine and threonine also often leads to ‘non-sequence’ fragmentation reactions involving the neutral loss of phosphoric acid (H_3PO_4 , 98 Da) [28] under ‘non-mobile proton’ conditions. In these cases, the non-sequence derived product ions may dominate the spectrum, suppressing the formation of sequence ions [28, 29]. Therefore, as the dominant ‘non-sequence’ product ion fragmentation pathways are not taken into consideration by the search algorithms, when the required sequence ions are in low abundance or absent, low database search scores may result.

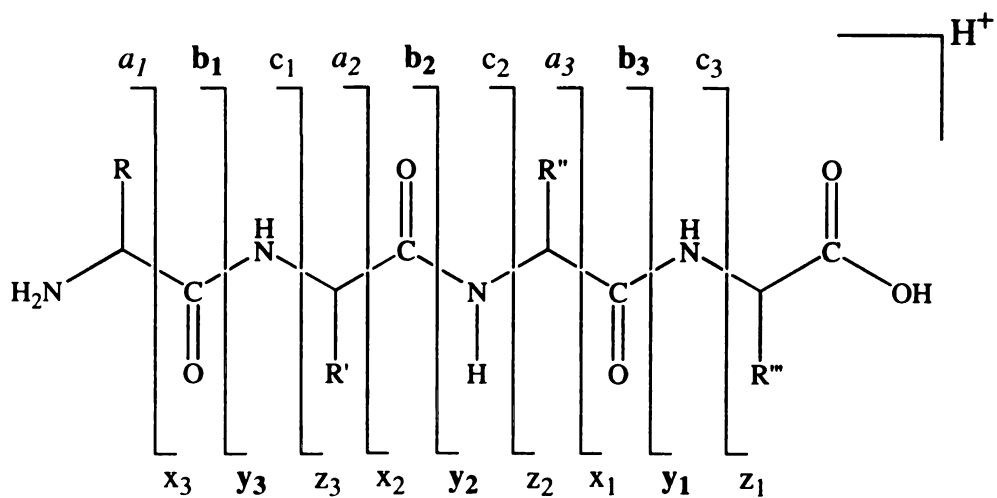
1.4. Understanding and Controlling Peptide Fragmentation Reactions

The limitations of the search algorithms described above are mainly due to the lack of understanding of the factors that effect the gas phase fragmentation reactions of peptide ions upon MS/MS. Therefore, determining the mechanisms and other factors influencing the dissociation behavior of peptide ions is a key to increasing the accuracy of search algorithms employed for protein identification, or for the development of methods for controlling and directing the fragmentation of peptide ions toward the formation of analytically useful product ions.

1.4.1 Mechanisms for the Fragmentation of Protonated Peptide Ions

Scheme 1.1 shows the generally accepted peptide fragment ion nomenclature. The ions a, b, and c represent products retaining the charge on the N-terminal, whereas ions x, y and z denote products retaining the charge on the C-terminal. b- and y-type ions originating via the cleavage of C-N amide bonds along the peptide backbone are typically the dominant product ions produced under the low energy collision induced dissociation conditions employed in triple quadrupole and quadrupole ion trap mass spectrometers.

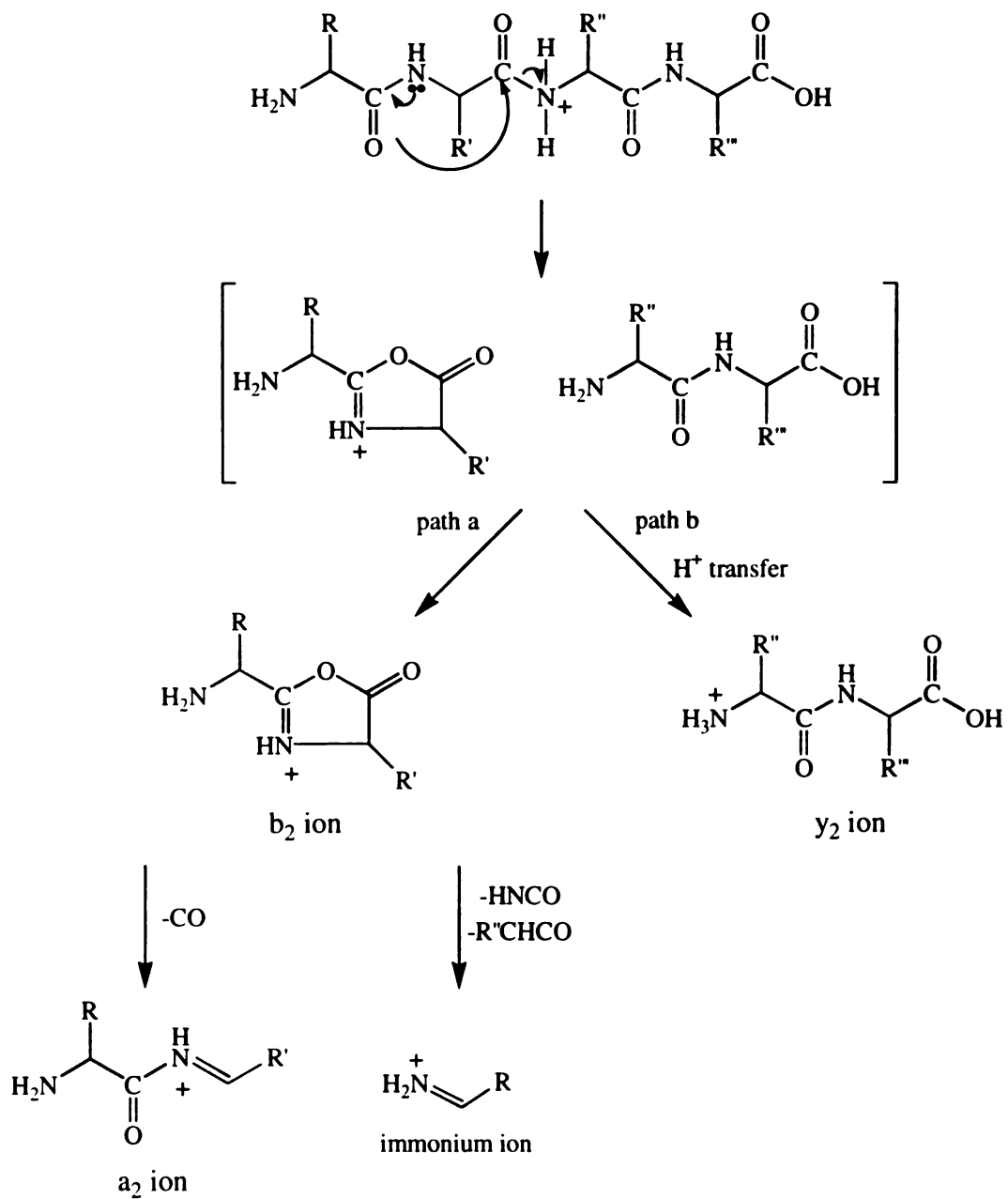
The fragmentation of amide bonds under low energy activation conditions are influenced by the ability of an ionizing proton to migrate from the initial site of protonation (the side chains of basic amino acids, or the N-terminal amino group) along the peptide backbone to an amide bond, where localization of the proton promotes bond cleavage. This has been called the ‘mobile proton model’ by Dongre *et. al.* [30]. The initial site of protonation is determined by the relative proton affinities of the various functional groups contained within the peptide. Experimental mass spectrometry approaches to determining proton affinities have been described [31,32]. Alternatively, proton affinities may be determined by theoretical molecular orbital modeling calculations [33, 34]. Proton mobility can be divided into 3 categories; “non mobile” when the total number of protons $\leq$ the number of Arg residues, “mobile” when the total number of protons $>$ than the total number of basic residues (i.e. combined number of Arg, Lys and His), or “partially mobile” when the total number of basic residues $\geq$ the total number of protons $\geq$ number of Arg residues. [35, 27]. Under ‘mobile’ or partially-mobile’ proton conditions, migration of an ionizing proton may result in localization at an amide nitrogen along the peptide backbone.



Scheme 1.1 Nomenclature for peptide fragment ions.

It is generally considered that fragmentation of the amide bond then occurs by nucleophilic attack from an adjacent amide carbonyl to yield complementary b- and/or y-type product ions, as shown in Scheme 1.2. Oxazolone b-type product ions may then lose CO to form a-type ions [36]. The losses of small neutral molecule such as water or ammonia, via similar mechanisms can also be observed [37]. Note that although Scheme 1.2 shows the amide bond cleavage reaction proceeding via an amide nitrogen protonated precursor, it has been shown using theoretical *ab initio* and semi-empirical molecular orbital calculations that protonation of the carbonyl oxygen of the amide bond is thermodynamically preferred over protonation of the amide nitrogen in peptide ions [38,39,40,41,42]. Thus, fragmentation via an amide carbonyl oxygen protonation precursor should also be considered as a viable dissociation pathway.

The presence of additional basic residues in peptide ions can lead to localization of the ionizing protons at their side chains (i.e., resulting in 'non-mobile' proton conditions). Under these conditions, higher energies are required for proton mobilization, and as a result, alternative dissociation reactions driven by "charged remote" fragmentation mechanisms may predominate, such as those involving enhanced 'sequence' cleavages at the C-terminal of aspartic acid residues [35,43] or enhanced 'non-sequence' cleavages at the side chains of methionine sulfoxide residues [27], suppressing other 'sequence' ion formation.



Scheme 1.2 Mechanisms for the formation of b-type, y-type, a-type and immonium ions.

1.4.2 Controlling Peptide Fragmentation Reactions

Understanding the mechanisms for peptide fragmentation has been a key step toward developing methods for controlling or directing peptide fragmentation towards the formation of analytically useful product ions from which peptides or proteins may be more readily identified and/or characterized. A number of different methods for the control of peptide fragmentation, involving the introduction of a fixed charge to the peptide, have been developed.

1.4.2.1 N-terminal Fixed Charge Derivatization and Fragmentation

Fixed charge derivatization strategies for peptides have been used previously in conjunction with MS/MS for sequencing applications. However, most prior work has been limited to derivatization of the N- and C-termini, and has largely been focused on directing fragmentation toward formation of a series of amide backbone cleavage ‘sequence’ ions [44,45]. Watson and co-workers have demonstrated that N-terminal peptide derivatization with tris(2,4,6-trimethoxyphenyl) phosphonium (TMPP)⁺-Ac_SC₆F₅ bromide results in the formation of a well controlled series of N-terminal a-type product ion series under high energy fragmentation conditions [46]. Adamczyk *et. al.* have used a similar reagent [tris(2,4,6-trimethoxyphenyl) phosphonium] acetic acid N-hydroxysuccinimide ester (TMPP⁺-Ac_OSU) to generate a series of N-terminal b-type ions accompanied by a less intense a-type product ion series under low energy fragmentation conditions in an ion trap mass spectrometer [47]. It is believed that the formation of N-terminal b- and a-type ions are due to a ‘charge remote’ mechanism involving a shift of the amide hydrogen to the C-terminal carbonyl carbon of the amide

bond [48]. Wysocki and co-workers have demonstrated that the selective gas phase cleavage of the amide bond C-terminal to Aspartic acid residues maybe controlled using a $\Phi_3\text{P}^+\text{CH}_2\text{C}(=\text{O})-$ [$\Phi=2,4,6$ -trimethoxyphenyl) reagent introduced to the N-terminal of the peptide [49].

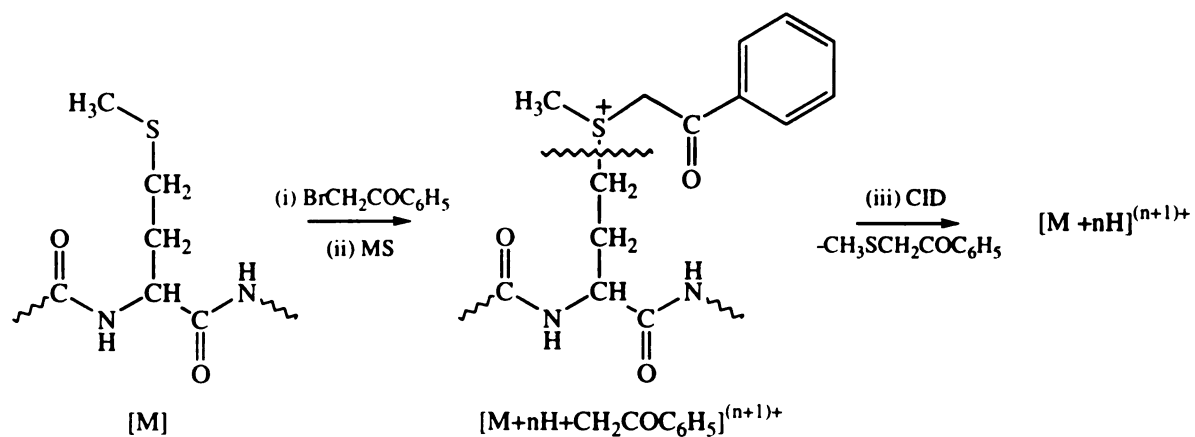
1.5 Side Chain Fixed Charge Derivatization and Fragmentation

Reid *et. al.* have recently described a novel method for the selective identification of peptide ions, via the derivatization and selective fragmentation of peptide ions containing certain side-chain functional groups [50,51]. In contrast to the fixed charge derivatization methods described above, the MS/MS dissociation of peptide ions containing these fixed charge derivatives are directed toward exclusive formation of a single characteristic side chain cleavage product ion at the site of the fixed charge. Thus, the fixed charge containing peptide ions may be readily identified from complex mixtures for example, by selective neutral loss scan mode MS/MS methods. Incorporation of 'light' and 'heavy' isotopically encoded labels into the fixed-charge derivatives also facilitates the application of this MS/MS method to the quantitative analysis of differential protein expression. This approach thereby offers the potential for significantly improved sensitivity and selectivity for the identification and quantitative analysis of peptides or proteins containing certain structural features, without the requirement for extensive fractionation or otherwise enrichment from a complex mixture prior to analysis.

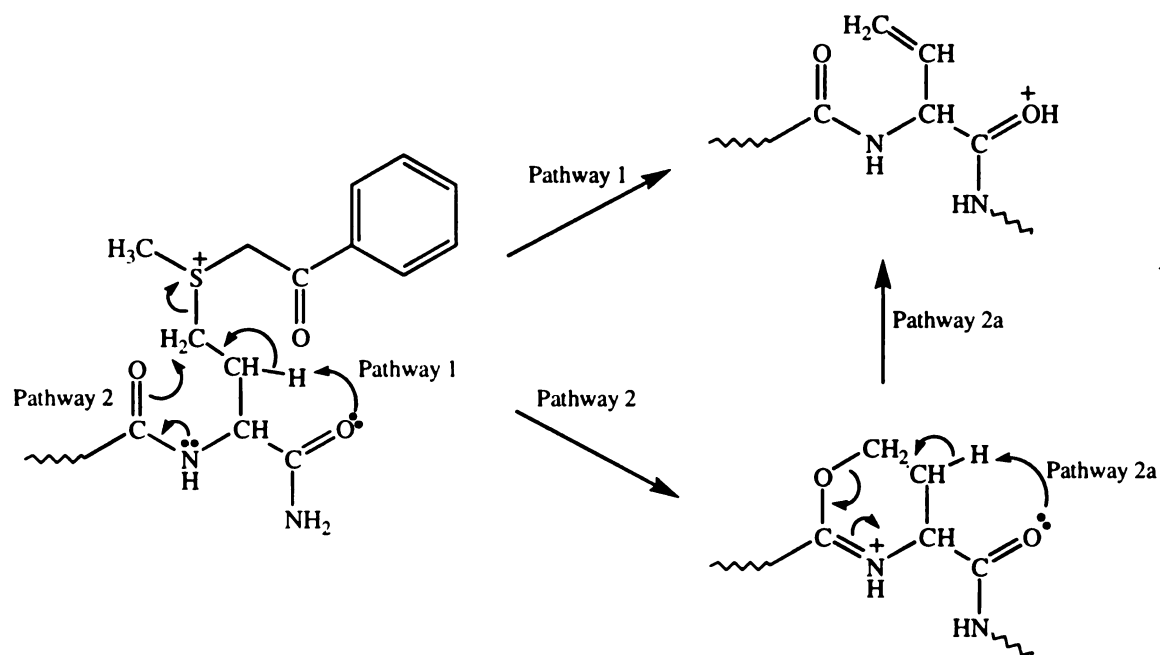
The fixed charge derivatization and selective fragmentation reactions of peptides containing methionine were described in the above mentioned work, via the initial formation of a side chain phenacylsulfonium ion (Scheme 1.3). Mechanisms to account

for the loss of $\text{CH}_3\text{SCH}_2\text{COC}_6\text{H}_5$ from the methionine containing peptide ions following low energy CID MS/MS were proposed (Scheme 1.4), involving either of the amide carbonyl groups adjacent to the side chain in intramolecular E2 proton transfer elimination (pathway 1) or $\text{S}_{\text{N}}2$ elimination (pathway 2) reactions. These mechanisms would result in elimination of the side chain to yield acyclic 3-amino-1-butenic acid (vinyl glycine) or five or six membered cyclic containing peptide product ions, respectively. It was further proposed that ring opening of the cyclic product ions via an intramolecular proton transfer reaction (pathway 2a), could occur to yield the acyclic vinyl glycine containing product. However, extensive studies to determine the operating mechanisms, and the influence of the resultant product ion structures on subsequent fragmentation reactions, were not performed. Thus, given that further characterization of the sequence of the identified methionine containing peptides may be achieved by subjecting the initial MS/MS product ions to multistage MS/MS (MS^3) in a quadrupole ion trap mass spectrometer, or by energy resolved 'pseudo' MS^3 in a triple quadrupole mass spectrometer, it is important to determine the mechanisms by which the neutral loss occurs and to determine the structures of the resultant product ions.

It was also noted in the above mentioned work that while the fragmentation of side chain fixed charge methionine containing peptides were observed to undergo exclusive dissociation at the amino acid side chain, independently of the amino acid composition and precursor ion charge state (i.e., proton mobility), the loss of the side chain fragment as a neutral versus protonated species was observed to vary depending on the proton mobility of the precursor ion.



Scheme 1.3 Fixed charge derivatization and selective fragmentation of methionine sulfonium ion derivatized peptide ions.



Scheme 1.4 Potential mechanisms for the selective fragmentation of methionine sulfonium ion derivatized peptide ions.

Thus, if neutral loss or precursor ion scan mode MS/MS and MS³ methods are to be employed for the comprehensive identification of side chain fixed charge peptide ions, the factors influencing this loss must be more fully understood.

1.6 Aims of this Thesis

The aims of this thesis are:

1. to identify the mechanisms and product ion structures associated with the neutral loss of CH₃SR from the side chains of methionine fixed charge sulfonium ion containing peptides, and;
2. to determine the influence of para substituents on the ratio of charged versus neutral loss product ions formed by dissociation of phenacylbromide alkylated fixed charge sulfonium ion containing methionine peptides.

CHAPTER TWO

Experimental

2.1 Materials

Unless stated otherwise all reagents were analytical reagent (AR) grade and used as supplied without further purification. The synthetic tryptic peptides GAILMGAILK and GAILAGAILK were obtained from Auspep (Melbourne, Vic.). Poly(4-vinylpyridinium tribromide) and Phenacylbromide (Compound **1** in scheme 2.1) were purchased from Fluka (Switzerland). Fmoc-chloride, methanol (HPLC grade), 4-Acetylbenzoic acid, Ethylacetate, Acetyl Chloride, 4-Acetylbenzoic acid, D,L-Methionine and D-Methionine were purchased from Sigma-Aldrich (St. Louis, MO, USA). d₄-D,L-Methionine was purchased from CDN Isotopes (Canada). Glacial acetic acid (ACS grade), P-Dioxane, Magnesium Sulfate, Sodium Carbonate and Sodium bicarbonate were purchased from Spectrum Chemicals (Gardena, CA, USA). Acetonitrile (HPLC grade) was purchased from OmniSolv (Gibbstown, NJ, USA). Hydrochloric acid and hexane (ACS grade) were purchased from Columbus Chemical Industries (Columbus, WI, USA). Diethyl Ether and Bromine were purchased from Jade Scientific (Canton, Michigan, USA). Ethyl acetate was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). 4-Ethylacetophenone and 4-Methylacetophenone were purchased from Acros Organics (New Jersey, USA). All solutions were prepared using deionized water purified by a Barnstead nanopure diamond purification system (Dubuque, Iowa, USA).

2.2 Synthesis of (D, L)-, (D) - and (d₄-D, L)-Fmoc-Methionine

D-Methionine (100 mg), D,L-methionine (100 mg), or d₄-D,L-Methionine (100 mg), were dissolved in 5 ml of 10% Na₂CO₃. Then 2.5 mL of P-Dioxane was added and finally, 170 mg of Fmoc-chloride dissolved in 5 mL of P-Dioxane was added dropwise while stirring at 0°C. The reaction was allowed to proceed for one hour at 0°C and then continued for 24 hours at room temperature. The resulting mixture was poured onto 75 ml of ice-water and chloroformate was removed by extracting twice with diethyl-ether (40 mL). The pH of the resulted aqueous layer was adjusted to 2 by adding conc. HCl at 0°C. Fmoc-Methionine was extracted twice into diethyl-ether (40mL) and dried with MgSO₄. The solvent was evaporated under reduced pressure and Fmoc-Methionine was recrystallized with EtOAc-hexane. The GAIL-(D)-MGAILK, GAIL-(DL)-MGAILK and GAIL-(d₄-DL)-MGAILK peptides were then synthesized by Syn Pep Company (Dublin, CA, USA).

2.3 Synthesis of 4-Bromoacetylbenzoic acid (Compound 2 in Scheme 2.1)

4-Acetylbenzoic acid (503 µl, 3.37 mmol) was dissolved in acetic acid (20 mL) and the reaction mixture was heated at 70 °C until dissolution was achieved. The reaction solution was then cooled to room temperature and Bromine (190 µL, 3.7 mmol) was added in one portion with stirring which was continued over night at room temperature. The reaction mixture was then filtered, the precipitate washed with water and dissolved in Ethylacetate (20 mL). The insoluble material was filtered and the filtrate washed with water (20 mL x 1). The Ethylacetate was then removed under a gentle stream of N₂, then completely dried under high vacuum to yield a white solid.

2.4 Synthesis of Methyl-4-bromoacetylbenzoate (Compound 3 in Scheme 2.1)

Acetyl chloride (1.6 mL, 2 mol) was added drop wise to Methanol (10 mL) with cooling and stirring in an ice-bath. 4'-Acetylbenzoic acid (328 mg, 2 mmol) was then added in one portion and the reaction mixture was left to stir at room temperature for 3 days. The solvent was allowed to evaporate off overnight at room temperature to yield a white/yellow solid, which was then collected and dried under vacuum. The solid was then dissolved in Acetic acid (10 mL) and Bromine (190 μ L, 3.7 mmol) was added in one portion with stirring at room temperature, which was continued over night. The reaction solution was then diluted with H₂O (50 mL) and extracted with Diethyl ether (3 x 20 mL). The combined organic extracts were washed with 0.1M NaHCO₃ (2 x 20 mL) and H₂O (1 x 20 mL), then dried over MgSO₄ and concentrated *invacuo* to give a white solid which was dried under high vacuum.

2.5 Synthesis of 4-Methylphenacylbromide (Compound 4 in Scheme 2.1)

4'-Methylacetophenone (0.5g, 3.7 mmol) was added to poly(4-vinylpyridinium tribromide) (1.86 g , ~3 meq Br₃⁻ /g resin) in 20 mL Methanol. The reaction mixture was then stirred at room temperature for 4 hours. The reaction mixture was filtered, and the methanol was evaporated off under a gentle stream of N₂ to yield a white solid.

2.6 Synthesis of 4-Ethylphenacylbromide (Compound 5 in Scheme 2.1)

4-Ethylacetophenone was dissolved in acetic acid and bromine was added in one portion with stirring at room temperature which was continued over night. The reaction

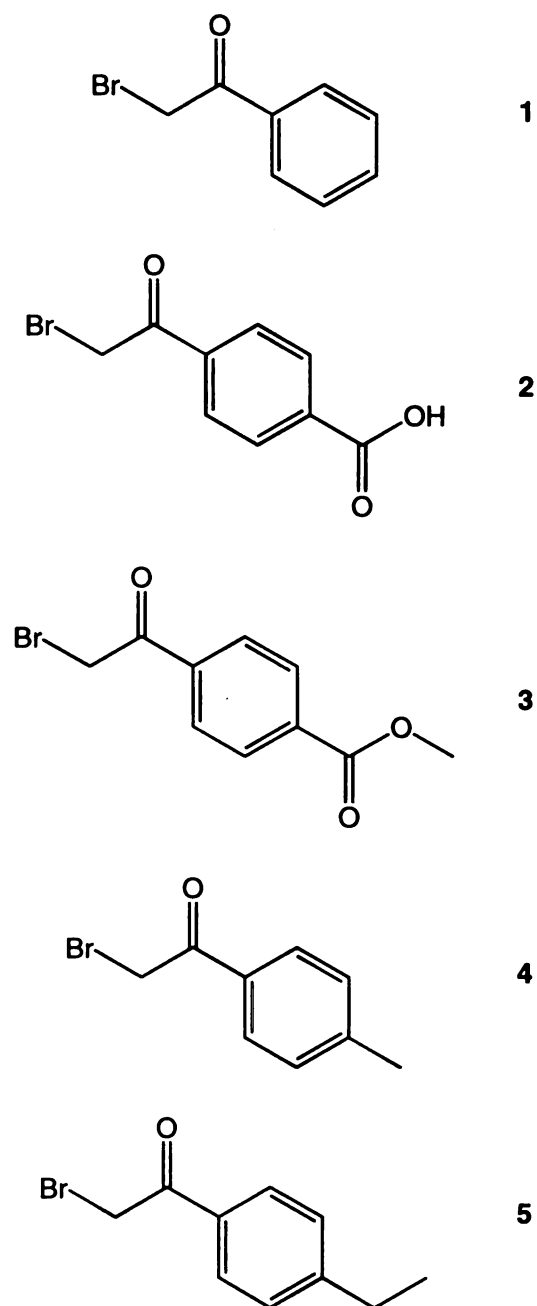
solution went from a dark brown/orange color to a light yellow color over this time. The reaction solution was diluted with H₂O (50mL) and extracted with Diethyl ether (3 x 20mL) then H₂O (1 x 20mL) then dried over MgSO₄ and concentrated under reduced pressure to give a dark yellow liquid.

2.7 Side Chain Fixed-Charge Derivatization of Methionine-Containing Peptides

Side chain fixed-charge sulfonium ion derivatives of synthetic methionine-containing model “tryptic” peptides were produced by the addition of 10 µL of a 1M solution of alkylating reagent (Compounds **1-5** in Scheme 2.1) to 100 µg of either GAILMGAILK, GAIL-(D)-MGAILK, GAIL-(D,L)-MGAILK, GAIL-(d₄-D,L)-MGAILK, VTMAHFWNFGK or VTMGHFDNFGK dissolved in 100 µL of aqueous 20% HOAc containing 30% CH₃CN. The reactions were allowed to proceed for 24 h at room temperature after which the samples were diluted and introduced to the mass spectrometer without further purification.

2.8 Mass Spectrometry

Peptides were introduced to a linear quadrupole ion trap mass spectrometer (Thermo model LTQ, San Jose, CA) by electrospray ionization (ESI). Samples, (0.02 mg/mL) dissolved in 50% MeOH, 1% AcOH were introduced into the mass spectrometer at 0.5 µL/min. The spray voltage was set at 1.8 kV. The heated capillary temperature was 200°C. CID MS/MS and MS³ experiments were performed on monoisotopically mass selected ions using standard isolation and excitation procedures.



Scheme 2.1 The structures of sulfonium ion derivatization reagents.

2.9 Computational Methods

2.9.1 Computational Methods Employed for Mechanistic Studies

The model peptide N-acetyl methionine (S,S-dimethyl sulfonium)-N-methyl amide ($\text{CH}_3\text{CONHCH}(\text{CH}_2\text{CH}_2\text{S}^+(\text{CH}_3)_2)\text{CONHCH}_3$) was used for computational calculations. Low energy transition state structures related to the possible mechanisms for dissociation of the methionine sulfonium ion side chain, were initially found at the PM3 semi empirical level of theory, followed by further optimization at the B3LYP level of theory using the 6-31+G(d,p) basis set using the GAUSSIAN 98 (version 5.2) molecular modeling package. Intrinsic reaction coordinate (IRC) searches were then performed, followed by geometry optimizations to locate the appropriate reactant and product ion structures associated with each transition state. All optimized structures were subjected to harmonic vibrational frequency analysis and visualized using the computer package Gauss View 2.1 to determine the nature of the stationary points. Zero point energies were obtained from harmonic frequency calculations without scaling.

2.9.2 Computational Methods Employed for Proton Affinity Calculations

Full conformational searches on neutral and protonated para-substituted S-methyl phenacyl derivatives (Compounds 6-15 in Scheme 2.2) were initially performed at the PM3 semi empirical level of theory, followed by further optimization of low energy structures at the B3LYP level of theory using the 6-31 + G (d,p) basis set using the GAUSSIAN 98 (version 5.2) molecular modeling package. All optimized structures were subjected to harmonic vibrational frequency analysis and visualized using the computer

package Gauss View 2.1 to determine the nature of the stationary points. Zero-points energies were obtained from harmonic frequency calculations without scaling.

2.9.3 Proton Affinity Calculations

Consider the following equation for the molecule M and its protonated structure, MH^+ .



The proton affinity (PA) of a molecule M can be calculated according to the negative of the enthalpy (ΔH) of Eq. 1 via the equations below, where $\Delta_R H$ is the enthalpy of the reaction.

$$PA = -\Delta_R H_{298}^\circ \quad (\text{Eq. 2})$$

The enthalpy of the reaction can be written in terms of the translational ($\Delta_t E$), rotational ($\Delta_r E$), and vibrational ($\Delta_v E$) energy of the species,

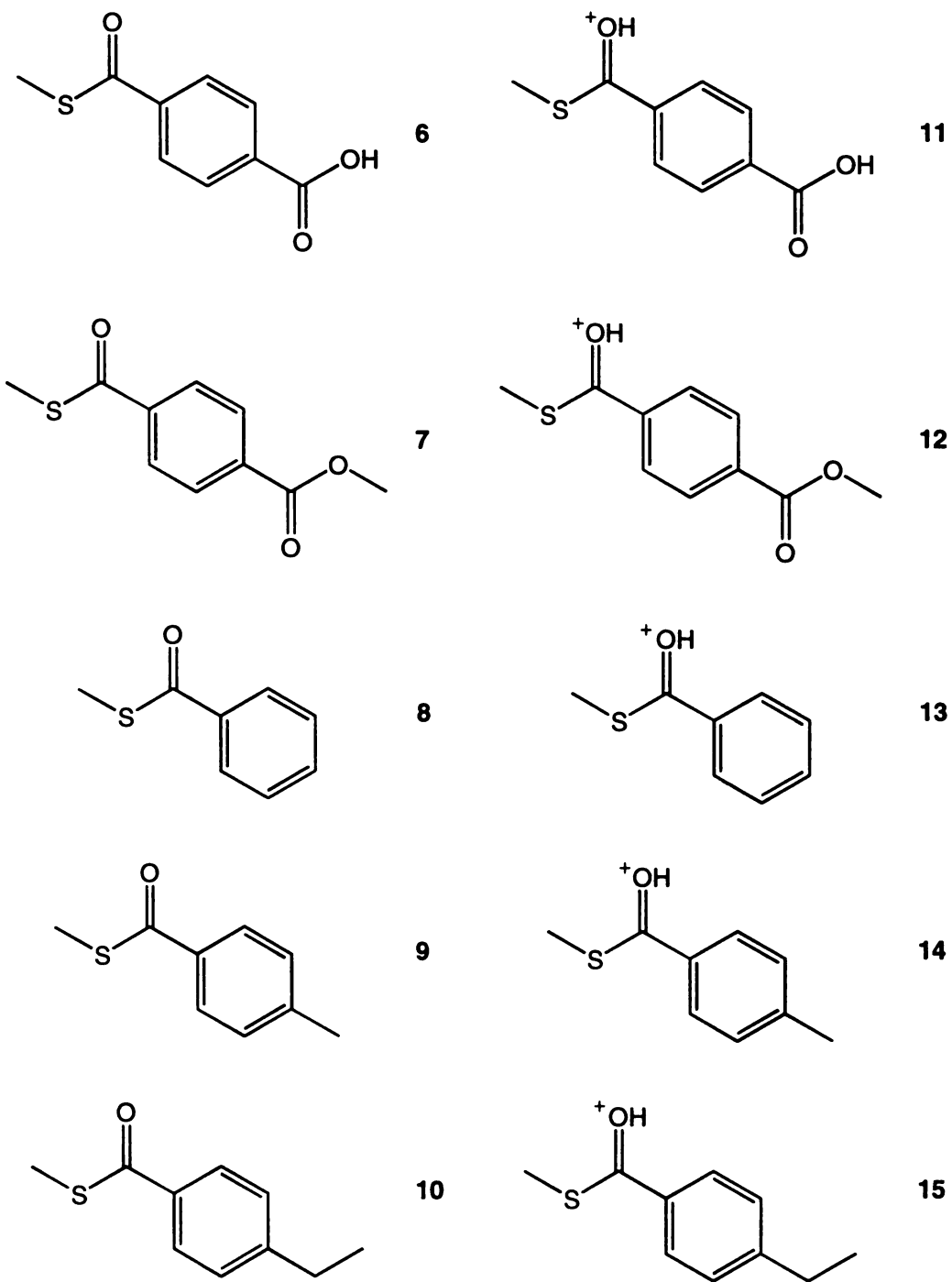
$$\Delta_R H_{298}^\circ = -\Delta_x E + \Delta(\Delta E_{t,298}) + \Delta(\Delta E_{r,298}) + \Delta(\Delta E_{v,298}) + \Delta PV \quad (\text{Eq. 3})$$

$$\Delta_x E = E_{\text{elec}}(MH^+) - E_{\text{elec}}(M) + ZPVE(MH^+) - ZPVE(M) \quad (\text{Eq. 4})$$

, where $\Delta PV = -RT$, E_{elec} is the electronic energy, and ZPVE is the zero point vibrational energy correction (i.e., the difference in translational, rotational and vibrational energy

from 0K to 298K). The value of the translational energy $\Delta(\Delta E_{t,298}) = 3/2RT$ and the rotational energy $\Delta(\Delta E_{r,298}) = 0$. $E_{\text{elec}}(\text{MH}^+)$, $E_{\text{elec}}(\text{M})$, $\Delta E_{v,298}(\text{MH}^+)$, $E_{v,298}(\text{M})$ and ZPVE values may all be obtained from the output of the structural optimizations described above. Thus, the proton affinity of a molecule can be calculated using the final equation below.

$$\Delta_{\text{R}}\overset{\circ}{\text{H}}_{298} = E_{\text{elec}}(\text{MH}^+) - E_{\text{elec}}(\text{M}) + \text{ZPVE}(\text{MH}^+) - \text{ZPVE}(\text{M}) + \Delta E_{v,298}(\text{MH}^+) - E_{v,298}(\text{M}) - 5/2RT \quad (\text{Eq. 4})$$



Scheme 2.2 The structures of neutral and protonated *para*-substituted S-methyl phenacyl derivatives.

CHAPTER THREE

Mechanisms for the Selective Gas-phase Fragmentation Reactions of Methionine

Side Chain Fixed Charge Sulfonium Ion Containing Peptides

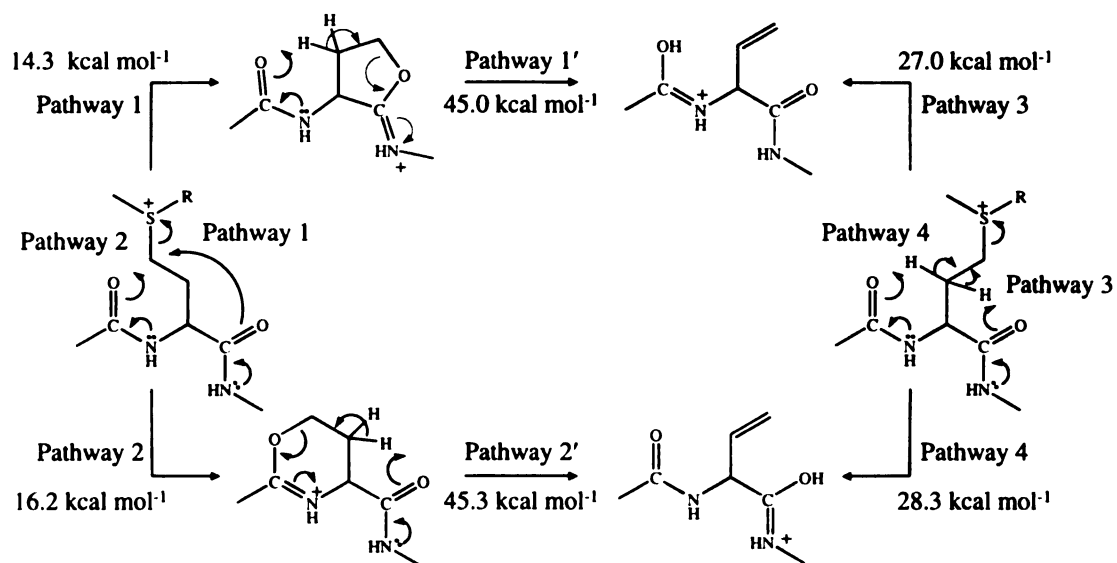
3.1 Introduction

The gas-phase fragmentation reactions of peptide ions containing a phenacylsulfonium fixed charge on the side chain of methionine residues have recently been examined in order to develop a novel strategy to selectively identify methionine containing proteins by tandem mass spectrometry [50]. It was shown that dissociation of these fixed charge derivatives under low energy CID MS/MS conditions results in exclusive loss of the derivatized side chain, with the formation of a single characteristic side chain cleavage product ion (Scheme 1.3 in Chapter 1). It was further demonstrated that the characteristic loss of the fixed charge sulfonium ion methionine side chain could be used to selectively identify methionine containing peptides from within complex mixtures by selective neutral loss scan mode MS/MS methods. Although several mechanistic possibilities involving intramolecular E2 or S_N2 elimination reactions (Scheme 1.4 in Chapter 1) were proposed for these selective fragmentation reactions in the above mentioned work, extensive studies to determine the operating mechanisms, and the influence of the resultant product ion structures on subsequent fragmentation reactions, were not performed. Determining these mechanisms and product ion structures is of particular importance, as characterization of the amino acid sequence of the methionine containing peptides identified via the diagnostic loss of the side chain may potentially be achieved by further subjecting the initial MS/MS product ions to multistage

MS/MS (MS^3) in a quadrupole ion trap mass spectrometer, or by energy resolved 'pseudo' MS^3 in a triple quadrupole mass spectrometer. Here, a combination of transition state calculations at the B3LYP/6-31+G** level of theory performed on a simple peptide model, as well as experimental studies based on the multistage dissociation of a regioselectively deuterated methionine derivatized sulfonium ion containing tryptic peptide, have been carried out to obtain evidence to support the proposed S_N2 reaction mechanism.

3.2 Molecular Orbital Calculations to Examine Mechanisms for the Loss of CH_3SR from Methionine Fixed Charge Sulfonium Ion Containing Peptides.

As shown in Scheme 3.1, the neutral loss of CH_3SR from the side chains of methionine fixed charge containing peptide ions could occur via several different mechanistic pathways. Pathways 1 and 2 of Scheme 3.1 shows neighboring group participation reactions involving either nucleophilic attack from the carbonyl group of the C-terminal amide bond, or the carbonyl group of the N-terminal amide bond, respectively. These reactions pathways would result in the formation of protonated five membered hydrofuran or six membered oxazine product ions, respectively. Alternatively, pathways 3 and 4 of Scheme 3.1 show E2 elimination reactions involving intramolecular proton transfer from the β methyl group of the methionine fixed charge containing side chain to the N or C-terminal amide carbonyl oxygens to yield protonated acyclic 3-amino-1-butenic acid (vinyl glycine) product ions. Furthermore, the five membered hydrofuran and the six membered oxazine product ions formed from Pathways 1 and 2 in Scheme 3.1 could potentially undergo intramolecular proton transfer ring opening



Scheme 3.1. Possible reaction mechanisms to account for the neutral loss of CH₃SR from methionine side chain fixed charge containing peptide ions.

reactions to yield the vinyl glycine product ions (Pathways 1' and 2' in Scheme 3.1, respectively).

To determine which of the mechanistic possibilities described in Scheme 3.1 are energetically feasible, a series of molecular orbital calculations were performed to determine the transition state barriers associated with each reaction pathway. Potential transition state structures for each of the possible mechanisms shown in Scheme 3.1 were initially examined at the PM3 semi empirical level of theory, using a simple peptide model, N-acetyl methionine-N-methyl amide, containing a fixed charge on the thioether side chain, $\text{CH}_3\text{CONHCH}(\text{CH}_2\text{CH}_2\text{S}^+(\text{CH}_3)_2)\text{CONHCH}_3$. Low energy conformers were then re-optimized at the B3LYP/6-31+G** density functional level of theory. Vibrational frequency analysis was then performed to determine the nature of the optimized stationary point structure. Then, intrinsic reaction coordinate searches were performed, followed by geometry optimization at the same level of theory to locate the appropriate precursor and product ion structures associated with each transition state structure. The total energies, zero point vibrational energies (ZPVE) and relative energies (total energies + ZPVE, relative to structure (A)) obtained for each of the optimized low energy transition state structures, as well as those of the optimized precursor, intermediate and product ion structures are given in Table 3.1.

Structure TS1 in Figure 3.1(A) shows the predicted low energy transition state structure for the loss of CH_3SCH_3 to form a protonated five membered hydrofuran product ion via an $\text{S}_{\text{N}}2$ neighboring group participation reaction involving the amide bond C-terminal to the methionine residue. After optimizing this structure, IRC calculations followed by geometry optimizations were performed to locate the precursor ion (A) and

Table 3.1 Total energies (E_{total}), zero point vibrational energies (ZPVE) and relative energies (E_{rel}) computed for the precursor ions, transition states, intermediate product ions and product ion structures associated with each reaction pathway at the B3LYP/6-31+G** level of theory.

Structure	E_{total} (Hartree)	ZPVE (Kcal mol ⁻¹)	E_{rel} (kcal mol ⁻¹) ^{a,b}
A	-1012.103755	178.01581	0.0
TS1	-1012.080944	176.84406	+14.3
B	-1012.112208	178.01406	-5.3
C	-534.1521621	177.46909	+0.9
TS2	-534.081918	124.64946	+45.0
D	-534.1330484	128.06278	+12.9
E	-1012.100463	177.68801	+2.1
TS3	-1012.07791	176.74105	+16.2
F	-1012.114764	178.32422	-6.9
G	-534.150836	130.07242	+1.7
TS4	-534.0813041	124.88532	+45.3
H	-534.1325096	127.84273	+13.2
I	-1012.096112	177.93925	+4.8
TS5	-1012.060713	173.96721	+27.0
J	-1012.092994	176.09255	+6.7
K	-534.1324999	127.8488	+13.2

Table 3.1 (continued)

L	-1012.101371	177.91885	+1.5
TS6	-1012.058067	173.63621	+28.7
M	-1012.09511	175.66895	+5.4
N	-534.1330465	128.06403	+12.9
O	-1012.107568	178.4326	-2.3
TS7	-1012.058023	173.90797	+28.7
P	-1012.09213	175.65843	+7.3
Q	-534.1306281	128.0657	+14.4
R	-1012.09593	178.13605	+4.9
TS8	-1012.058689	173.865	+28.3
S	-1012.088737	175.95845	+9.4
T	-534.1281226	127.75565	+16.0
U	-534.1478423	129.97671	+3.6
TS9	-534.0731113	124.95592	+50.5
V	-534.1404751	127.10571	+8.2
TS10	-534.0641064	124.99521	+56.1
W	-534.1249397	127.93632	+18.0
S(CH ₃) ₂	-477.9502026	47.51316	

^aE_{rel}= total energy + (ZPVE). ^bEnergy relative to structure A.

the intermediate product ion (B) associated with this reaction coordinate. The relative energies of the transition state and the product ion were then calculated with respect to the energy of the precursor ion. The product ion (C) was then obtained by geometry optimization following the removal of CH_3SCH_3 from structure (B). The relative energy of (C) was obtained by summing the energy of the product ion (C) and the energy of the geometry optimized CH_3SCH_3 . Structure (TS3) in Figure 3.1(B) represents the low energy transition state structure for the neutral loss of CH_3SCH_3 via a neighboring group participation reaction involving the amide bond N-terminal to the methionine residue to form a protonated six membered oxazine product ion. After performing IRC calculation on transition state (TS3), the optimized precursor ion, (E) the intermediate (F) and the product ion (G) were obtained as mentioned above. The energies of all these structures are given with respect to the energy of structure (A).

According to Figure 3.1(A), the predicted transition state barrier for pathway 1, to yield the cyclic protonated five membered hydrofuran ring, is $14.3 \text{ kcal mol}^{-1}$ with respect to the energy of structure (A), whereas the transition state barrier to form the six membered oxazine ring via pathway 2 is predicted to be $16.2 \text{ kcal mol}^{-1}$ relative to the energy of the structure (A). The predicted transition state barriers for pathways 1 and 2 (structures (TS1) and (TS3) in Figure 3.1) are therefore relatively comparable. The slightly lower energy of transition state (TS1) is likely due to better hydrogen bonding between the carbonyl oxygen and the amide nitrogen on the N- and C-terminal amide bonds ($1.93 \text{ \AA}$ in (TS1) versus $2.08 \text{ \AA}$ in (TS3)). The predicted energy difference between these transition states is not considered significant enough to suggest the formation of one

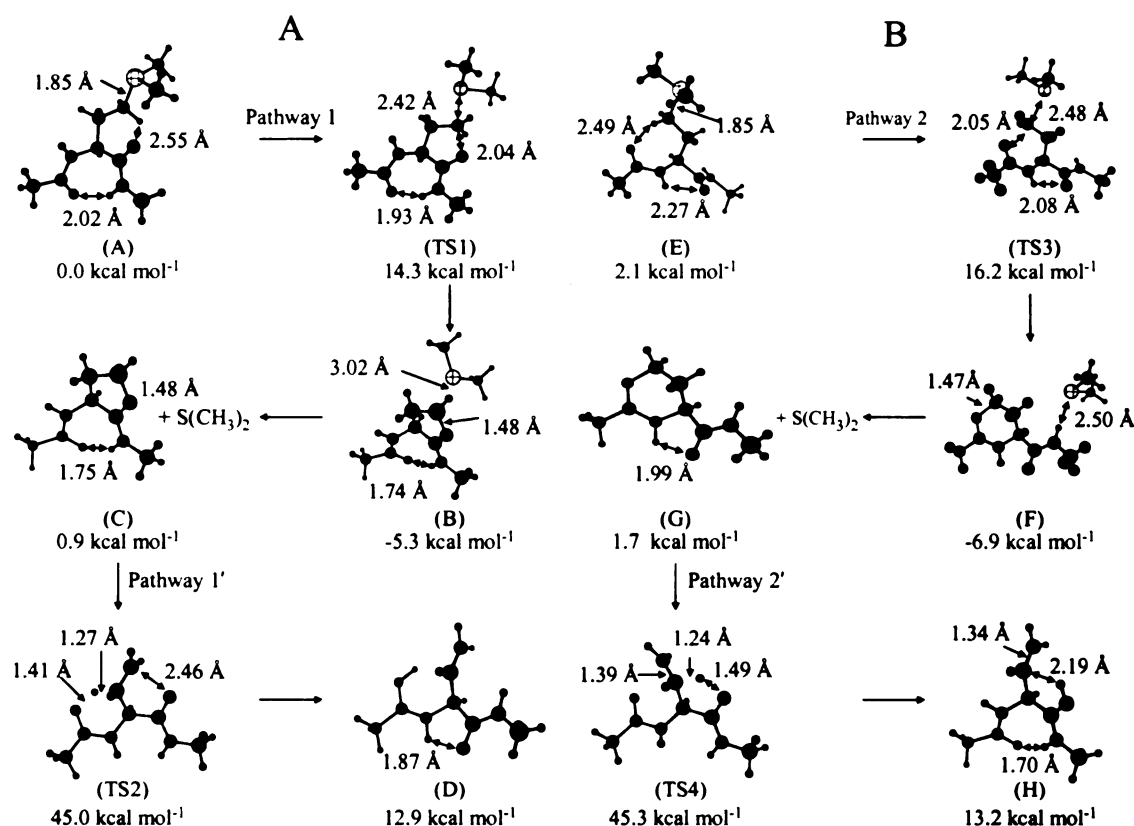


Figure 3.1 Optimized precursor, transition state and product ion structures (at the B3LYP/6-31+G** + ZPVE level of theory) for the loss of CH₃SCH₃ from the simple model peptide N-acetyl methionine-N-methyl amide, due to a neighboring group participation reaction involving (A) the C-terminal carbonyl group to form a 5 membered hydrofuran product ion (Pathway 1), or (B) the N-terminal carbonyl group to form a 6 membered oxazine product ion (Pathway 2). The transition state and product ion structures for ring opening of the product ions formed from Pathways 1 and 2, involving intramolecular proton transfer (β_1 -H) to either the N-terminal carbonyl group (Pathway 1' in A) or the C-terminal carbonyl group (Pathway 2' in B) to yield protonated vinyl glycine product ions, are also shown. The energies of all molecules are given with respect to structure A.

Images in this thesis/dissertation are presented in color

particular product over another for the loss of CH_3SCH_3 via either reaction pathways 1 or 2. Thus, both the five and six membered rings could potentially be formed.

Figure 3.2(A) shows the predicted low energy transition state structure (TS5) found for the E2 elimination reaction involving intramolecular proton transfer of one of the two hydrogens from the β methyl group of the methionine side chain to the C-terminal carbonyl group to subsequently yield a vinyl glycine product ion. IRC calculations were again performed to obtain the appropriate precursor and intermediate product ions (I) and (J), respectively. The structure of product ion (K) was obtained by optimization of intermediate structure (J) after removal of CH_3SCH_3 , as mentioned above. The energies of these structures are given with respect to the energy of structure (A) in Figure 3.1. Figure 3.2(B) shows the predicted low energy transition state structure (TS6) associated with the E2 elimination reaction involving intramolecular proton transfer of one of the two hydrogens from the β methyl group of the methionine side chain to the N-terminal carbonyl group. The energies of this transition state relative to structure (A) in Figure 3.1 was predicted to be $28.7 \text{ kcal mol}^{-1}$. Furthermore, as there are two hydrogens on the β methyl group in the methionine side chain, transition state calculations predicting the barriers to the E2 elimination reactions involving the alternate hydrogens to those shown in Figure 3.2 were also determined. The structures associated with these transition states (structure (TS7) in pathway 3-2 and structure (TS8) in pathway 4-2) are shown in Figures 3.3. The relative energies of these transition states were predicted to be 28.7 and $28.3 \text{ kcal mol}^{-1}$, respectively. Thus, the four E2 elimination reaction pathways all have comparable activation barriers.

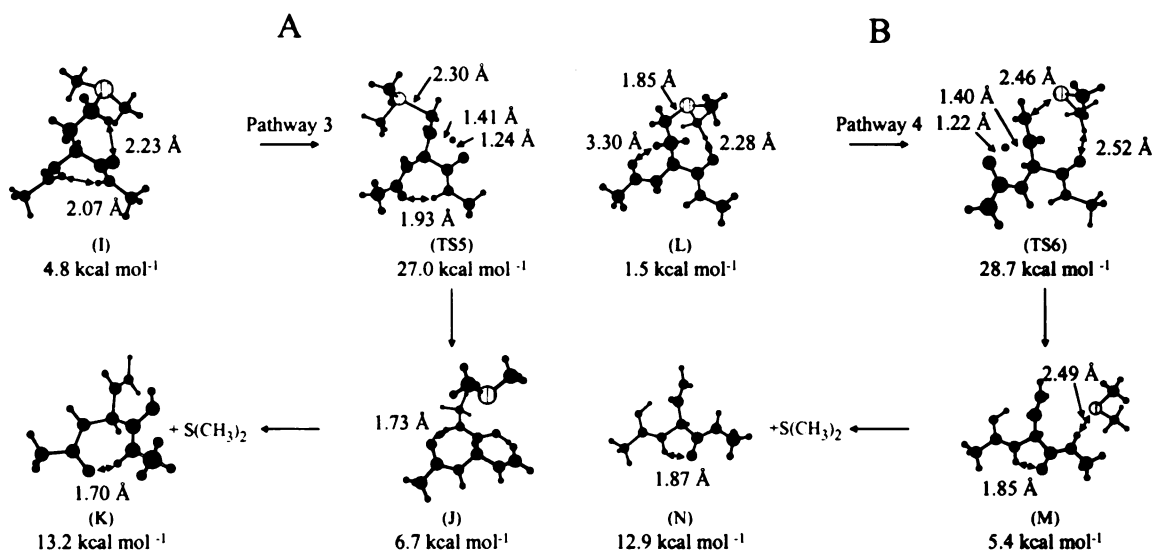


Figure 3.2 Optimized precursor, transition state and product ion structures (at the B3LYP/6-31+G** + ZPVE level of theory) for the loss of CH₃SCH₃ from the simple model peptide N-acetyl methionine-N-methyl amide due to intramolecular proton (β_1 -H) transfer to (A) the C-terminal carbonyl group (Pathway 3) or (B) the N-terminal carbonyl group (Pathway 4) to yield protonated vinyl glycine. The energies of all molecules are given with respect to structure A in Figure 3.1.

Pathways 1 and 2 in Figure 3.1 can be compared with pathways 3, 3-2, 4 and 4-2 in Figures 3.2 and 3.3 to predict the favorable mechanistic pathway for the neutral loss of CH_3SCH_3 . The transition states associated with the neighboring group participation processes (TS1) and (TS3) have energies about 10 kcal mol^{-1} lower than those for the E2 elimination reactions (TS5), (TS6), (TS7) and (TS8). Thus, given that the significant difference in transition state barriers between the neighboring group and E2 elimination reaction pathways suggest that pathways 1 and 2 are likely to be more favorable, it is expected that the loss of CH_3SR from the side chain of the methionine fixed charge sulfonium ion peptides will result in the formation of the five membered hydrofuran and/or six membered oxazine product ions.

3.3 Potential Ring Opening of the Five and Six Membered Cyclic Product Ions via Intramolecular Proton Transfer Reactions.

Having established above that the initial loss of CH_3SR from the side chain fixed charge sulfonium ion containing peptides following MS/MS is likely to occur via neighboring group participation mechanisms involving pathways 1 or 2 in Scheme 3.1, it was then considered whether the resultant cyclic product ions could undergo ring opening to yield the acyclic vinyl glycine containing product, via intramolecular proton transfer reactions according to pathways 1' and 2' in Scheme 3.1, prior to their further dissociation by MS^3 .

Structure (TS2) in Figure 3.1(A) shows the predicted low energy transition state for the ring opening reaction of the five membered hydrofuran product ion via pathway 1' in Scheme 3.1. The activation barrier for pathway 1' was predicted to be $45.0 \text{ kcal mol}^{-1}$

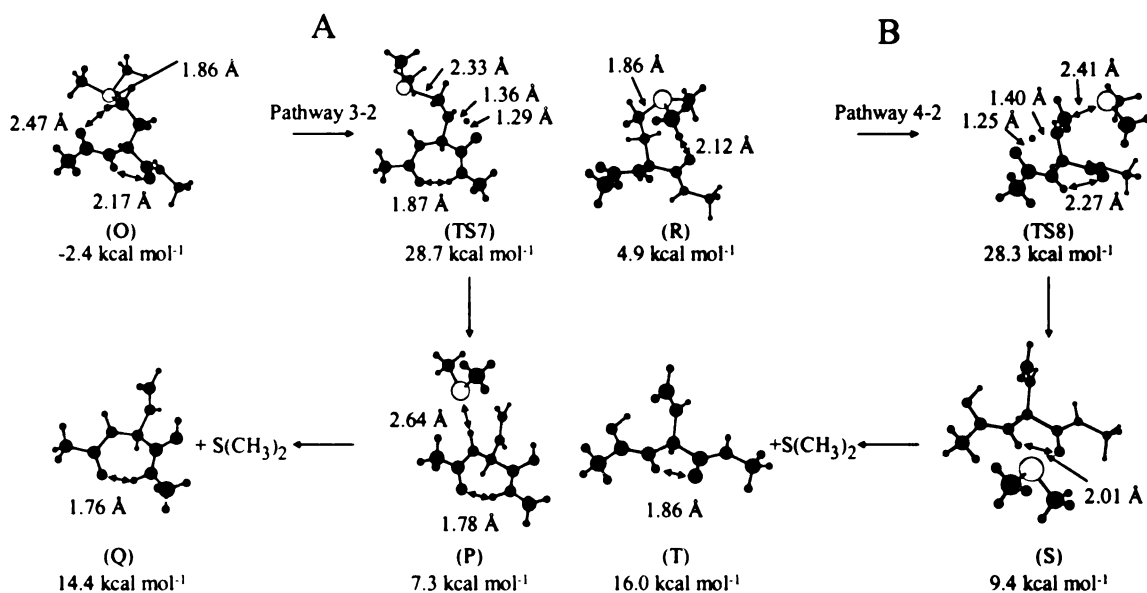


Figure 3.3 Optimized precursor, transition state and product ion structures (at the B3LYP/6-31+G** + ZPVE level of theory) for the loss of CH₃SCH₃ from the simple model peptide N-acetyl methionine-N-methyl amide involving the alternate intramolecular proton (β_2 -H) transfer to (A) the C-terminal carbonyl group (Pathway 3-2) or (B) the N-terminal carbonyl group (Pathway 4-2) to yield protonated vinyl glycine. The energies of all molecules are given with respect to structure A in Figure 3.1.

with respect to the energy of structure (A). The structure of transition state (TS2) associated with pathway 1' is similar to the structure of transition state (TS6) associated with pathway 4, and result in the formation of identical product ions (compare structure (D) in Figure 3.1A and structure (N) in Figure 3.2B). However, it can be seen from Figures 3.1 and 3.2 that transition state (TS2) is predicted to have a significantly higher energy ($45.0 \text{ kcal mol}^{-1}$) compared to transition state (TS6) ($28.7 \text{ kcal mol}^{-1}$). Justification for this difference may be obtained by consideration of the bonds being cleaved in each case i.e., transition state (TS2) involves cleavage of a strong carbon oxygen bond, whereas transition state (TS6) involves cleavage of a weak carbon sulfur bond.

The predicted transition state barrier for pathway 2' (transition state (TS4) in Figure 3.1), involving ring opening of the six membered oxazine was predicted to be $45.3 \text{ kcal mol}^{-1}$ with respect to the energy of structure (A), which is comparable to that predicted for ring opening of the five membered ring in Pathway 1'. Similar to that discussed above, the structure of transition state (TS4) is similar to the structure of transition state (TS5) associated with pathway 3, and again result in the formation of identical product ions (compare structure (H) in Figure 3.1B and structure (K) in Figure 3.2A). However, the relative energy of transition state (TS5) was predicted to be significantly lower than that for transition state (TS4).

The transition state barriers for ring opening of the five membered hydrofuran product ion via intramolecular proton transfer pathways 1'-2 and 2'-2, involving the alternate hydrogens to those shown in Figure 3.1 were also determined (structures (TS9) and (TS10) in Figure 3.4). The predicted barriers relative to structure (A) for these

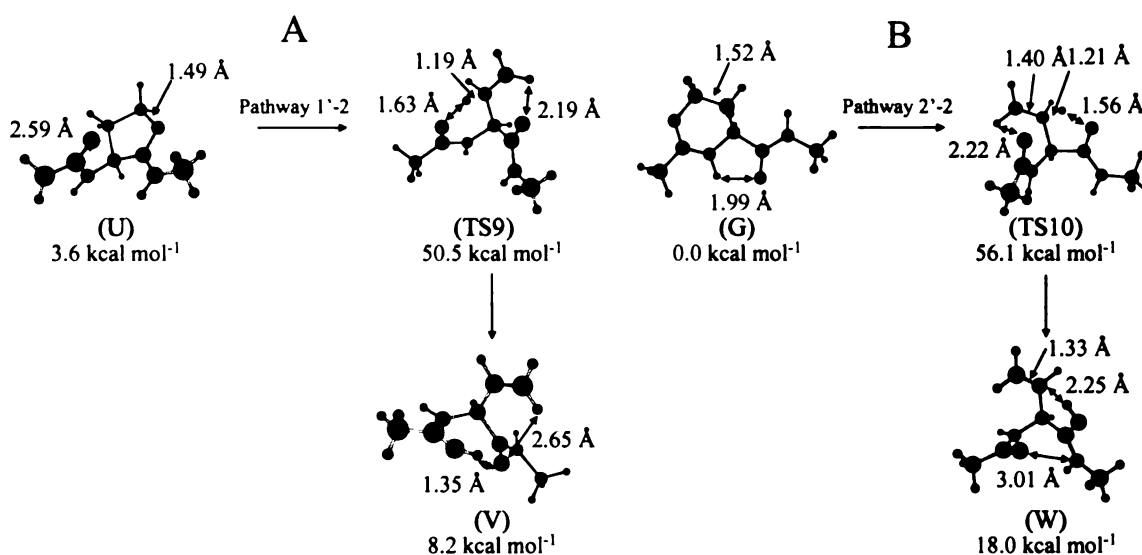


Figure 3.4. Optimized precursor, transition state and product ion structures (at the B3LYP/ 6-31+G** + ZPVE level of theory) for ring opening of the product ions formed from Pathways 1 and 2, involving the alternate intramolecular proton (β_2 -H) transfer to either the N-terminal carbonyl group (Pathway 1' in A) or the C-terminal carbonyl group (Pathway 2' in B) to yield protonated vinyl glycine product ions. The energies of all molecules are given with respect to structure A.

pathways were found to be 50.5 and 56.1 kcal mol⁻¹, respectively. Thus, each of the transition state barriers predicted for pathways 1', 1'-2, 2' and 2'-2 are all significantly higher in energy than those associated with pathways 1, 2, 3, and 4.

Recently, Paizs *et. al.* have predicted that the transition state barriers for amide bond cleavage reactions range from 29 to 33 kcal mol⁻¹, depending on the proton mobility [52]. Thus, the predicted low energy transition states (TS1) and (TS3) for the neutral loss of CH₃SR via the neighboring group participation reactions discussed above are around 15 kcal mol⁻¹ lower than these predicted amide bond fragmentation barriers. Thus, it is expected that fragmentation of the methionine fixed charge sulfonium ion containing peptide precursors under the 'slow heating' collision induced dissociation methods commonly employed for tandem mass spectrometry [53], would preferentially result in formation of the five membered hydrofuran and six membered oxazine product ions. Furthermore, given that the barriers to amide bond cleavage are expected to be much lower than the barriers predicted for the ring opening reactions of these initial cyclic product ions, it is expected that these ring opening reactions are unlikely to occur, and that further dissociation of the initial product ions would result in fragmentations along the peptide backbone. A summary of the predicted activation barriers associated with the various mechanistic pathways for dissociation of methionine side chain fixed charge containing sulfonium ion peptides discussed above is given in Figure 3.5.

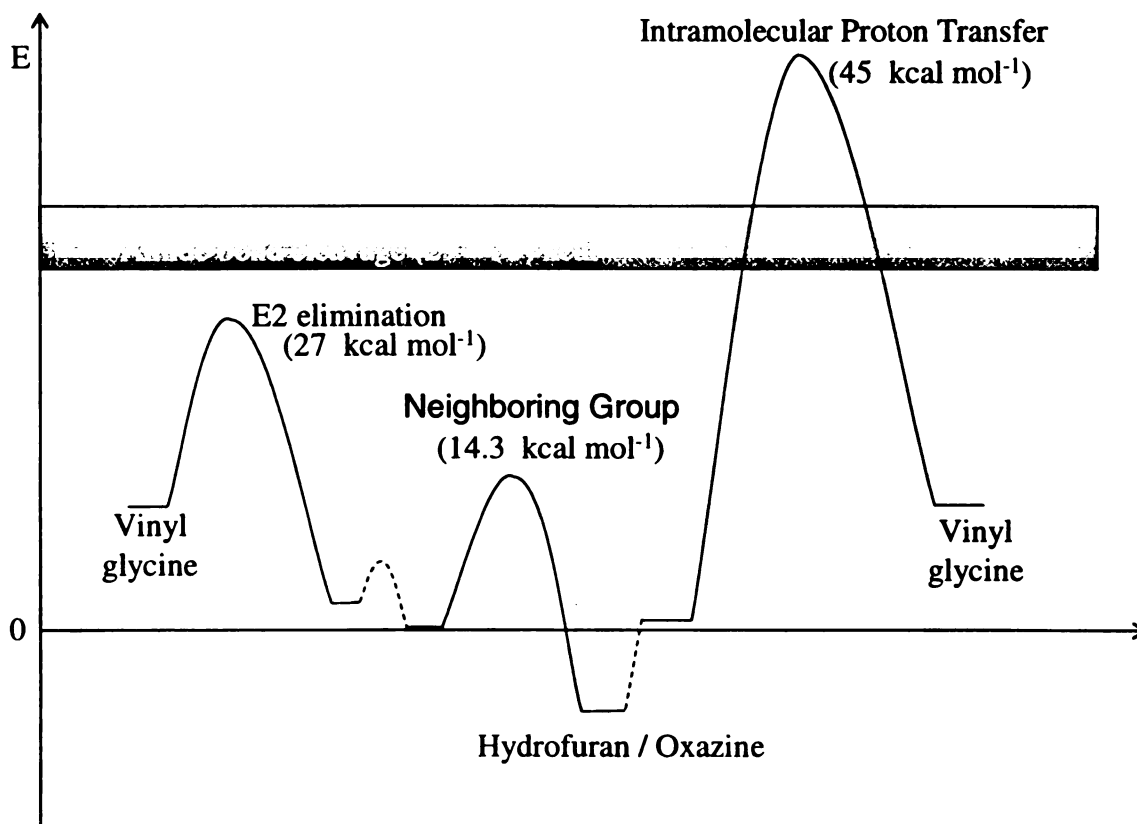


Figure 3.5. Summary of the predicted low energy transition state barriers (at the B3LYP/6-31+G** +ZPVE level of theory) for the dissociation of methionine side chain fixed charge containing sulfonium ion peptides.

3.4 Obtaining Experimental Evidence for the Mechanisms Responsible for the Loss of CH₃SR from Methionine Fixed Charge Sulfonium Ion Containing Peptides.

In order to obtain experimental evidence to support the results from the theoretical calculations discussed above, the multistage tandem mass spectrometry (MS/MS and MS³) fragmentation reactions of a sulfonium ion derivatized regioselectively deuterated methionine containing peptide, as well as those of a simpler alanine containing model peptide, have been examined in a linear quadrupole ion trap. It can be seen from Scheme 3.1 that loss of the methionine fixed charge containing side chain via either an E2 elimination reaction would involve transfer of one of the β-methyl hydrogens in the methionine side chain, resulting in ‘mobilization’ of this hydrogen along the peptide backbone. This ‘mobilization’ would also occur if either of the β-methyl hydrogens were involved in intramolecular proton transfer ring opening reactions of the cyclic five or six membered hydrofuran or oxazine product ions initially formed via the neighboring group participation reactions. In either case, facile migration or ‘scrambling’ of this hydrogen (proton) along the peptide backbone would be expected prior to or during further dissociation of the side chain cleavage product ion by MS³ [54].

3.4.1 Regioselective Deuterium Labeling Experiments to Examine Mechanisms for the Side Chain Fragmentation Reactions of Methionine Fixed Charge Containing Peptide Ions.

To determine whether or not facile scrambling of the methionine side chain β-methyl hydrogens does occur prior to or during MS³ dissociation, the MS/MS and MS³ fragmentation reactions of the synthetic peptide GAIL(d₄-M)GAILK, containing a

methionine side chain fixed charged phenacylsulfonium ion derivative, and where the hydrogens at the β and γ carbon positions of the methionine side chain were regioselectively exchanged for deuterium, were examined.

Figures 3.6A and B show the CID MS/MS product ion spectra of the doubly $[M^+ + H]^{2+}$ and triply $[M^+ + 2H]^{3+}$ charged precursor ions of GAIL(d_4 -M(R))GAILK, where $R = CH_2COC_6H_5$. From the doubly charged precursor ion product spectrum in Figure 3.6A, exclusive neutral loss of the fixed charge methionine side chain CH_3SR was observed. By examination of the product ion spectra obtained from the triply charged precursor in Figure 3.6B, it can be seen that the neutral loss of CH_3SR is also accompanied by ‘charged’ loss of the fixed charge methionine side chain, giving rise to complementary $-(CH_3SR + H^+)$ and $CH_3SR + H^+$ product ions. The formation of these protonated ‘charged loss’ product ions indicate that the transfer of a deuterium atom via an intermolecular deuterium transfer from the β methyl group of the methionine side chain similar to pathways 1’ or 2’ in Scheme 3.1 (see Scheme 3.2) does not occur. Therefore, this result provides evidence to support the contention that fragmentation of the fixed charge methionine side chain does not result in the formation of a vinyl glycine containing product ion.

Figure 3.6C shows the MS^3 product ion spectrum obtained by dissociation of the neutral loss $[M^+ + H - CH_3SR]^{2+}$ product ion of the $[M^+ + H]^{2+}$ precursor of GAIL(d_4 -M)GAILK from Figure 3.6A, from which a clear series of b- and y-type ions are observed. As mentioned above, if dissociation of the fixed charge containing precursor ion occurs via an E2 elimination reaction, or if ring opening of the initial cyclic product ion occurs via intramolecular proton transfer, a deuterium atom from the β methyl group

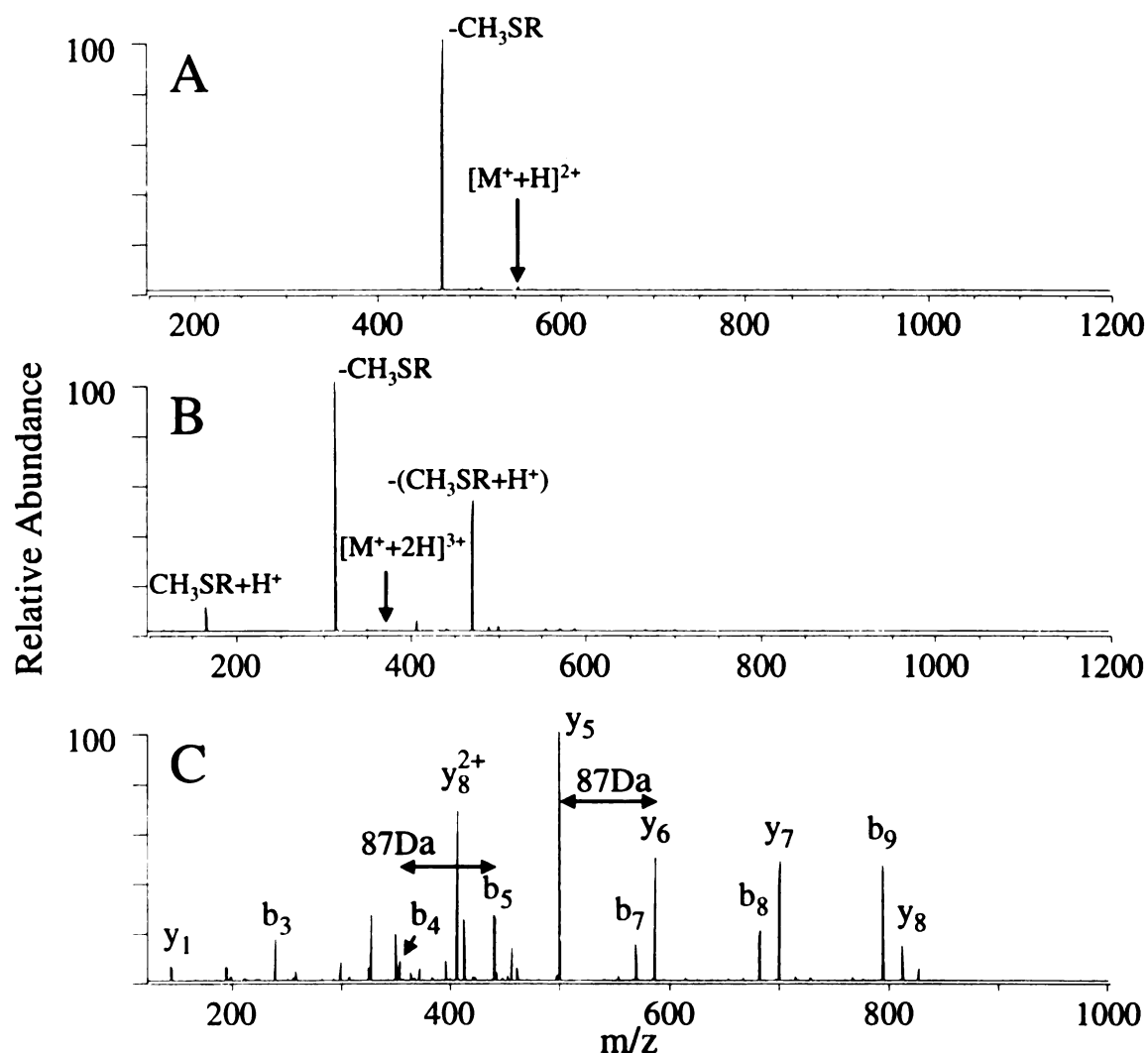
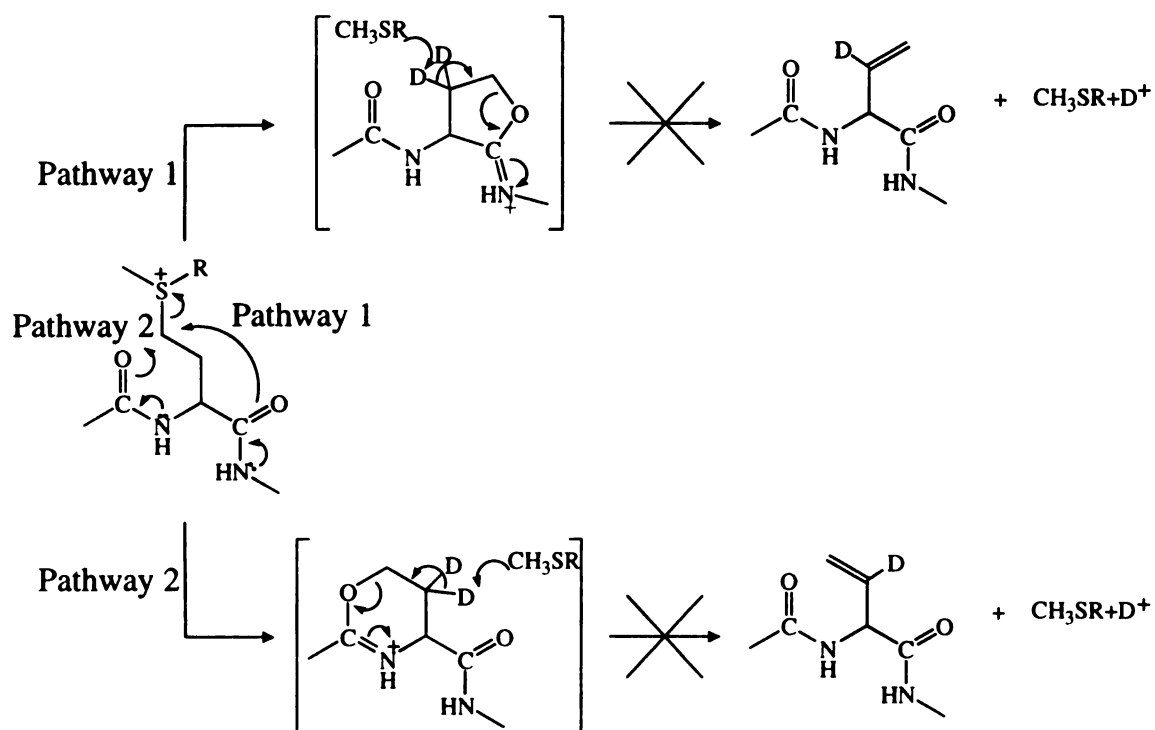


Figure 3.6 Linear ion trap CID MSⁿ analysis of a methionine side chain phenacyl sulfonium ion fixed charge derivative of GAIL(d₄-M)GAILK. (A) CID MS/MS product ion spectrum of the $[M^++H]^{2+}$ ion. (B) CID MS/MS product ion spectrum of the $[M^++2H]^{3+}$ ion. (C) CID MS³ product ion spectrum of $[M^++H-CH_3SR]^{2+}$ ion from Figure 3.6A.



Scheme 3.2. Potential intermolecular deuterium transfer reactions for the charged loss of $\text{CH}_3\text{SR} + \text{D}^+$ from methionine side chain fixed charge containing peptide ions.

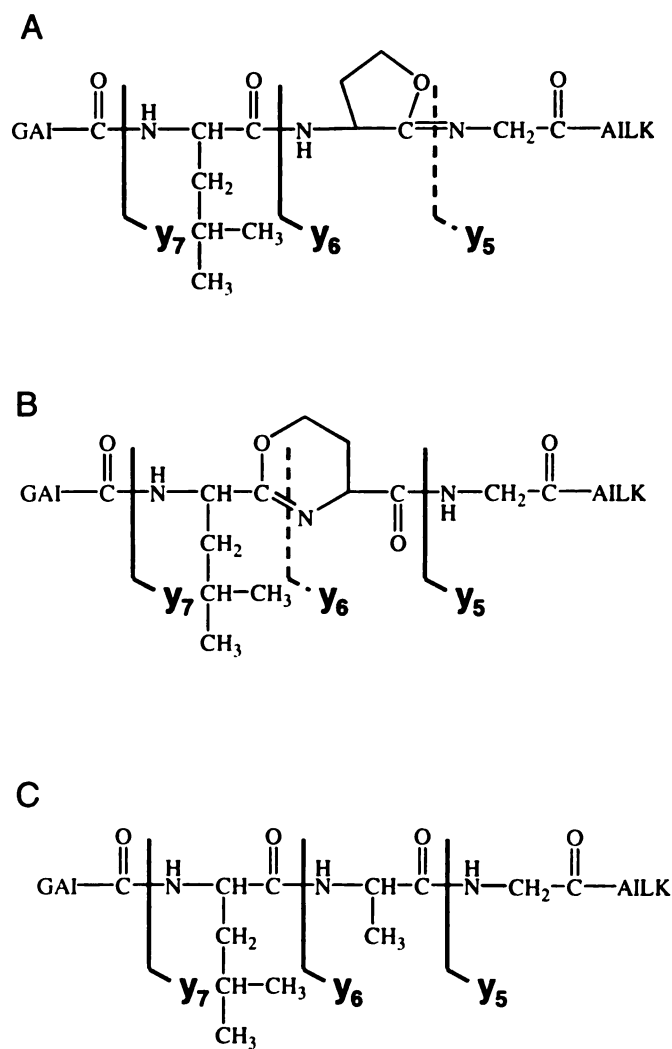
of the methionine side chain would be mobilized in the resultant product ion to a position along the peptide backbone. As a result, statistical ‘scrambling’ of this deuterium along the peptide backbone would be expected. However, it can be seen from Figure 3.6C that a mass difference of 87 Da is observed between the y_5 and y_6 product ions, as well as between the b_4 and b_5 product ions. These ions represent product ions formed by fragmentation of the amide bonds on the N and C-terminal sides of the methionine residue. This mass difference, as well as the lack of evidence for deuterium incorporation into any of the other product ions formed, indicates that all four deuterium atoms are located in the side chain of the product ion, and are therefore not involved in the initial fragmentation reaction. This data further indicates that the E2 elimination and ring opening reactions are not involved in fragmentation of the fixed charged methionine side chain sulfonium ion peptides, and is therefore strongly suggestive of the formation of cyclic hydrofuran and oxazine product ions.

3.4.2 Multistage Tandem Mass Spectrometry Experiments to Examine the Structure(s) of the Product Ions formed by Side Chain Fragmentation of Methionine Fixed Charge Containing Peptides.

The MS/MS and MS³ data obtained from the deuterium labeling experiments above indicate that fragmentation of methionine side chain fixed charge sulfonium ion containing peptides results in the formation of cyclic five and/or six membered hydrofuran or oxazine containing product ions via neighboring group participation reaction mechanisms, and that these product ions do not undergo ring opening via intramolecular proton transfer reactions prior to further dissociation.

Scheme 3.3 shows the proposed structures of the five and six membered product ion formed via the loss of CH_3SR from GAILM(R)GAILK (Structures A and B, respectively) and the structure of a 'control' peptide GAILAGAILK (structure C). The fragmentation of product ions containing these cyclic structures (e.g., the y_6 and y_7 ions from structure A, and the y_7 ion from structure B) should differ from the fragmentation of the same product ions formed from a 'control' peptide GAILAGAILK lacking these cyclic structures (structure C in Scheme 3.3). Therefore, in order to examine the structure(s) of the initial product ions formed by side chain fragmentation of methionine fixed charge containing peptides, the multistage (MS^3 and MS^4) product ion spectra of the doubly charged neutral loss product ion from the GAILM(R)GAILK peptide, as well as the MS/MS and MS^3 product ion spectra of the doubly charged 'control' peptide GAILAGAILK peptide have been obtained.

Figures 3.7A shows the mass spectra obtained by MS^3 of the neutral loss $[\text{M}^+ + \text{H} - \text{CH}_3\text{SR}]^{2+}$ product ion from the doubly charged precursor of GAILM(R)GAILK , while Figures 3.7B and 3.7C show the mass spectra obtained by MS^4 of the y_6 and y_7 product ions from Figure 3.7A, respectively. Figures 3.7D-F show the MS/MS spectrum obtained from the $[\text{M} + 2\text{H}]^{2+}$ precursor ion, as well as the MS^3 spectra obtained from the resultant y_6 and y_7 ions. Clearly, the spectra obtained by dissociation of the $[\text{M}^+ + \text{H} - \text{CH}_3\text{SR}]^{2+}$ ion from GAILM(R)GAILK and the $[\text{M} + 2\text{H}]^{2+}$ ion of GAILAGAILK are quite similar, with a complete series of b- and y-type ions observed in each case. Although y_5 and y_6 product ions corresponding to fragmentation on either side of the methionine side chain in the GAILM(R)GAILK peptide are both observed, fragmentation to yield the y_5 ion is unlikely for the cyclic five membered hydrofuran containing product ion,



Scheme 3.3 Structures of (A) the five membered hydrofuran product ion formed via the loss of CH_3SR from GAILM(R)GAILK , (B) the six membered oxazine product ion formed via the loss of CH_3SR from GAILM(R)GAILK , and (C) GAILAGAILK . The amide bond cleavage sites for formation of the y_5 , y_6 and y_7 product ions in each case are indicated.

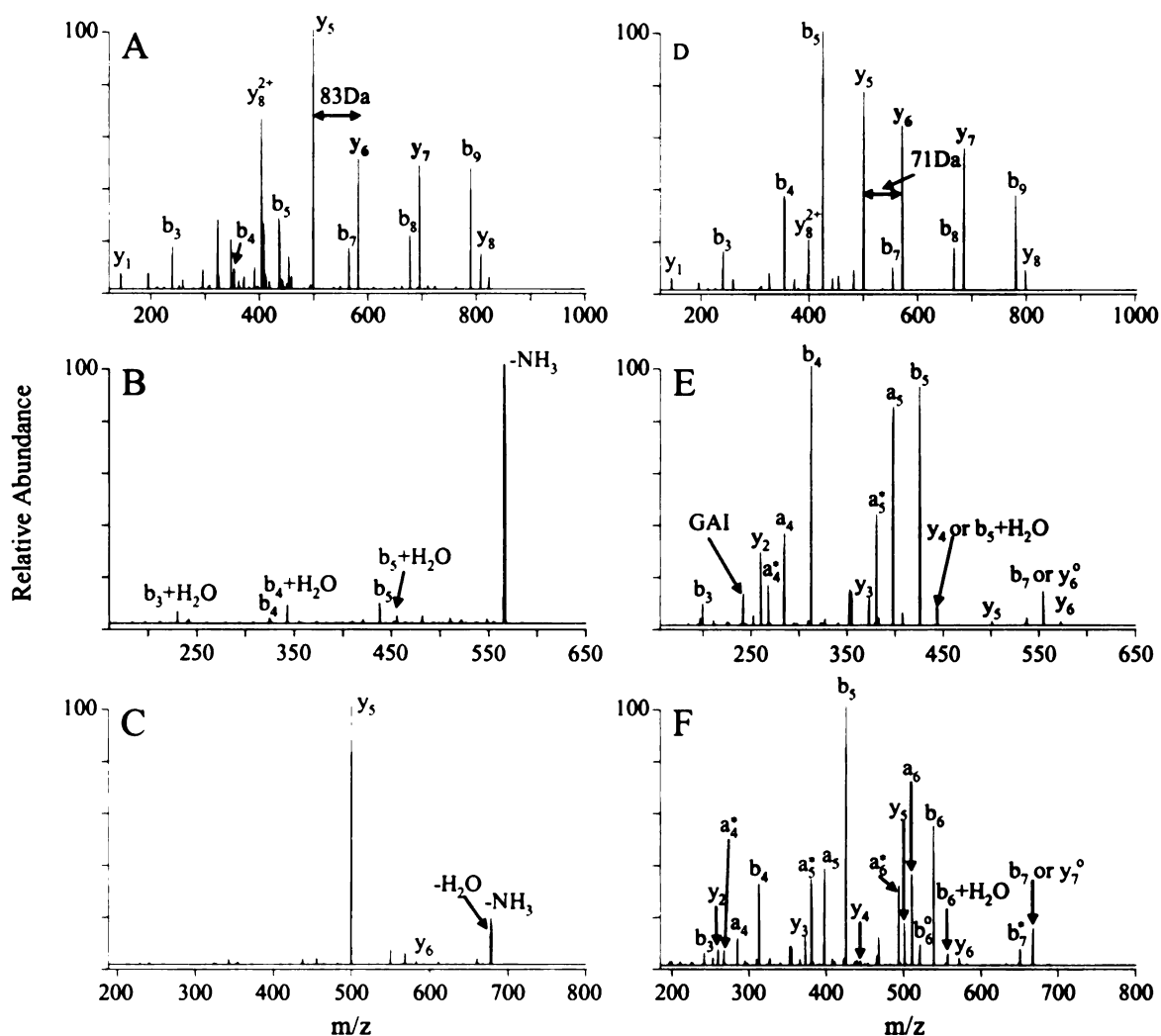


Figure 3.7 Multistage (MS/MS, MS³ and MS⁴) tandem mass spectrometry analysis of GAILM(R)GAILK (where R=CH₂COC₆H₅) and GAILAGAILK. (A) CID MS³ product ion spectrum of the $[M^+ + H - CH_3SR]^{2+}$ ion from GAILM(R)GAILK. (B) CID MS⁴ product ion spectrum of the y₆ product ion from Figure 3.7A. (C) CID MS⁴ product ion spectrum of the y₇ product ion from Figure 3.7A. (D) CID MS/MS product ion spectrum of the $[M + 2H]^{2+}$ ion of GAILAGAILK (E) CID MS³ product ion spectrum of the y₆ product ion from Figure 3.7D. (F) CID MS³ product ion spectrum of the y₇ product ion from Figure 3.7D.

due to the double bond character of the amide bond, whereas fragmentation to yield the y_6 ion is not likely to occur in the cyclic six membered oxazine containing product ion due to the presence of the oxazine ring. Thus, the fact that both ions are observed in Figure 3.7A suggests that both cyclic products may be formed, consistent with the results from the theoretical transition state calculations described earlier.

It can be seen by comparison of Figure 3.7B with 3.7E and Figure 3.7C with 3.7F that the product ion spectra obtained by dissociation of the y_6 and y_7 ions from the two peptides are very different. It is expected that these differences are due to the presence of the cyclic structures in the GAILM(R)GAILK peptide upon loss of the side chain. The series of product ions observed in Figure 3.7E and F by dissociation of the y_6 and y_7 ions from the $[M+2H]^{2+}$ ion of the GAILAGAILK peptide correspond to a relatively complete series of a-, b-, and y-type ions. In contrast, the y_6 ion from the $[M^+ + H - CH_3SR]^{2+}$ ion of the GAILM(R)GAILK peptide may contain only the five membered hydrofuran ring, whereas the y_7 product ion may contain either the five membered hydrofuran or six membered oxazine rings. The dominant product ions observed by dissociation of these y_6 and y_7 ions (Figure 3.7B and 3.7C, respectively) correspond to the loss of NH_3 and/or the formation of a y_5 ion. The loss of NH_3 in Figures 3.7B is consistent with fragmentation of the five membered hydrofuran ring containing y_6 precursor ion, while the formation of the y_5 ion in Figure 3.7C is consistent with fragmentation of the six membered oxazine ring containing y_7 product ion.

3.5 Conclusion

Taken together, the results from the theoretical calculations and experimental data presented here indicate that the loss of CH_3SR from methionine side chain fixed charge sulfonium ion containing peptide ions occurs via neighboring group participation reactions, resulting in the formation of a mixture of both five membered hydrofuran and six membered oxazine product ions. The data also suggests that these cyclic product ions do not rearrange by intermolecular proton transfer reactions prior to or during further dissociation. However, the resultant product ion spectra obtained by MS^3 are not significantly different from the spectra obtained from 'control' acyclic peptides, particularly with respect to the formation of product ions resulting from cleavage of amide bonds either side of the site of the loss of the fixed charge, and is likely due to the presence of a mixture of both cyclic structures formed from the initial neutral loss.

CHAPTER FOUR

Substituent Effects on the Fragmentation Reactions of Methionine Side Chain Fixed Charge Phenacylsulfonium Ion Containing Peptides.

4.1 Introduction

It was observed in Chapter 3 that fragmentation of the triply charged phenacylsulfonium ion derivative of GAILM(R)GAILK resulted in the formation of protonated 'charged loss' product ions in addition to the expected neutral loss product (Figure 3.6 B). These charged loss products are formed via intermolecular proton transfer from the triply protonated peptide product ion to the methylphenacylsulfide side chain fragment following the initial fragmentation reaction. Control over the formation of the characteristic low m/z charged loss product ion could potentially be used to selectively identify the presence of fixed charge methionine containing peptides from within complex mixtures by using a precursor ion scan mode MS/MS experiment. Alternatively, the complementary high m/z charged loss peptide product ion could be used for selective identification of the fixed charge methionine containing peptides by using a 'neutral gain' MS/MS scan [55]. It is expected that the ability for proton transfer from the peptide product ion to the side chain fragment will vary according to differences in their respective proton affinities. The proton affinity of the neutral side chain fragment may potentially be modulated as a function of the identity of the para-substituent of the alkylating reagent employed for the initial fixed charge derivatization. Substituents that result in the formation of a more acidic neutral molecule should result in more abundant neutral loss, while more basic substituents should result in more abundant charged losses.

However, charged losses are only expected when the proton affinity of the resultant peptide product ion is relatively low i.e., when the number of total charges on the resultant peptide product ion exceeds that of the number of basic sites within the ion. Here, the theoretical proton affinities of a series of para-substituted (-COOH (structure 6 in Figure 4.1), -COOCH₃ (structure 7 in Figure 4.1), -H (structure 8 in Figure 4.1), -CH₃ (structure 9 in Figure 4.1) and -CH₂CH₃ (structure 10 in Figure 4.1)) side chain fragments have been calculated by using high level density functional theory calculations of the neutral species and protonated ions at the B3LYP/6-31+G** +ZPVE level of theory. Then, the influence of these side chain fragment proton affinities on the relative abundance of neutral versus charged side chain losses resulting from the dissociation of methionine fixed charge containing peptide ions have been determined by examination of the fragmentation reactions of a series of para-substituted phenacylsulfonium ion containing peptides.

4.2 Proton Affinity Calculations

The structures of the various para-substituted neutral side chain fragments formed via cleavage of the side chain methionine fixed charge sulfonium ion peptides, as well as their protonated ions, were initially subjected to full conformational searches at the PM3 level of theory. Then, low energy conformers were re-optimized at the B3LYP/6-31+G** density functional level. Vibrational frequency analysis was then performed to determine the nature of the optimized stationary point structure, and to obtain values for the zero point vibrational energies (ZPVE) for subsequent proton affinity calculations. Figure 4.1 shows the optimized low energy structures for each of the neutral species and protonated

ions determined from these calculations. The proton affinities of the neutral structures were then calculated according to the method described in section 2.9.3 of Chapter 2. The proton affinities derived from these calculations are given in Table 4.1. According to these results, the proton affinity of the neutral fragment increases from 210.6 – 219.0 kcal mol⁻¹ in the order of structures 6, 7, 8, 9 and 10. These results are consistent as being due to the expected changes in the electron density of the fragments, as determined from the Hammett equation σ -values for the substituents (Table 4.1) [56,57,58].

4.3 Determining the Influence of Side Chain Fragment Proton Affinities on the Relative Abundance of Neutral versus Charged Side Chain Losses from Methionine Fixed Charge Containing Peptide Ions.

Figure 4.2A-C shows the CID MS/MS product ion spectra obtained by dissociation of the singly $[M]^+$, doubly $[M^+ + H]^{2+}$ and triply $[M^+ + 2H]^{3+}$ charged methionine side chain fixed charge phenacylsulfonium ion derivative of the synthetic peptide GAILMGAILK, respectively. It can be seen from these figures that dissociation of the singly and doubly charged precursor ions results in exclusive neutral loss of the side chain (labeled as $-CH_3SR$). In contrast, dissociation of the triply charged precursor ion results in the formation of both neutral loss ($-CH_3SR$) and charged loss (labeled as $-(CH_3SR + H^+)$ and $CH_3SR + H^+$) product ions. This result is consistent with the expected proton affinities of the resultant peptide product ions. The proton affinity of the peptide product is expected to decrease with increasing charge state. The ionizing proton on the singly charged peptide product ion is likely to reside at either the ϵ -amino group of the lysine side chain or along the peptide backbone, while the two ionizing protons on the

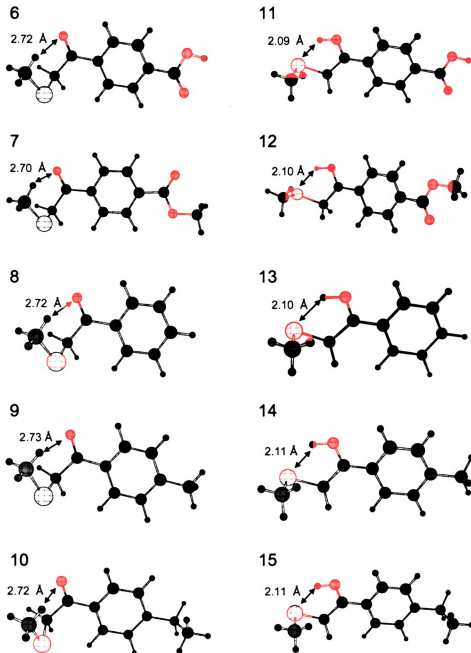


Figure 4.1 Optimized low energy structures at the B3LYP/ 6-31+G** + ZPVE level of theory for the neutral and protonated products of the para-substituted methylphenacysulfide derivatives employed for proton affinity calculations.

Table 4.1 Total energies (E_{total}), zero point vibrational energies (ZPVE), vibrational energies ($E_{\text{vibrational}}$) and proton affinities computed at the B3LYP/6-31+G** level of theory for the para-substituted methylphenacylsulfide derivatives.

Para-substituent	Structure	E_{Total} (Hartree)	ZPVE (kcal mol ⁻¹)	$E_{\text{vibrational}}$ (kcal mol ⁻¹)	Proton Affinity (kcal mol ⁻¹)
-COOH	Neutral (6)	-1011.009419	114.375630	121.249000	210.6
	Protonated (11)	-1011.354606	121.810810	128.692000	
-COOCH ₃	Neutral (7)	-1050.31664	131.888080	139.810000	212.8
	Protonated (12)	-1050.662343	139.323310	147.298000	
-H	Neutral (8)	-822.424349	105.043650	110.119000	215.0
	Protonated (13)	-822.776703	112.701460	117.755000	
-CH ₃	Neutral (9)	-861.746192	122.103150	128.38900	218.5
	Protonated (14)	-862.104304	129.788560	136.04300	
-CH ₂ CH ₃	Neutral (10)	-901.063164	140.288790	147.256000	219.0
	Protonated (15)	-901.422005	147.935160	154.93900	

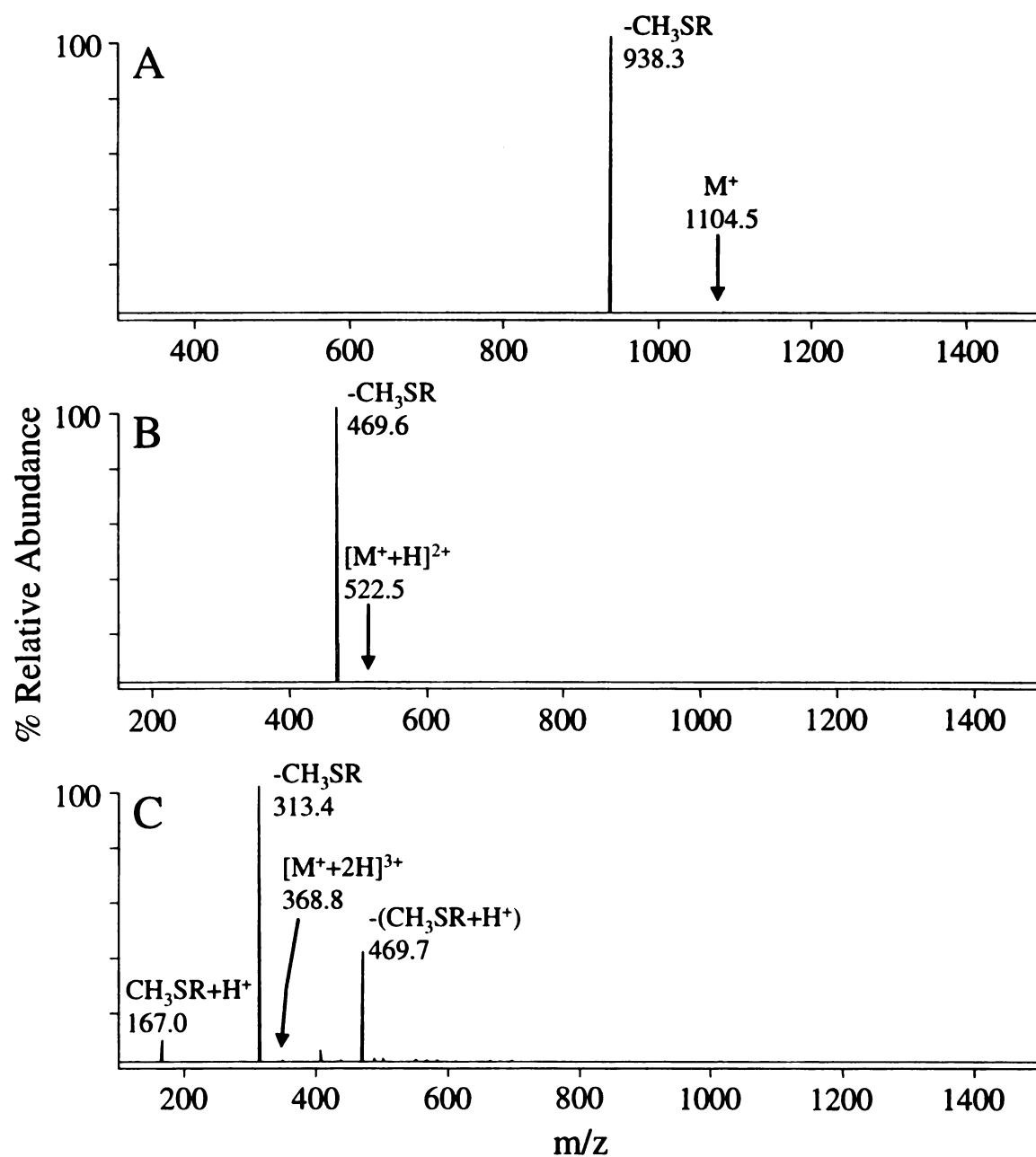


Figure 4.2 Linear ion trap CID MS/MS analysis of methionine side chain fixed charge phenacylsulfonium ions of GAILMGAILK. (A) CID MS/MS product ion spectrum of the $[M^+]$ ion. (B) CID MS/MS product ion spectrum of the $[M^+ + H]^{2+}$ ion. (C) CID MS/MS product ion spectrum of the $[M^+ + 2H]^{3+}$ ion.

doubly charged peptide product ion are likely to reside at both side chain and backbone positions. In the case of the triply charged peptide product ion however, increased columbic repulsion, as well as a decrease in the local proton affinities of the remaining sites potentially available for protonation, are likely to result in intermolecular proton transfer from the peptide fragment to the side chain fragment following bond cleavage, but prior to separation of the initial ion-neutral complex.

The fragmentation reactions of the multiply charged precursor ions of the methionine side chain fixed charge phenacylsulfonium ion containing synthetic peptides VTMGHFDNFGR and VTMAHFWNFGK were also examined (Figure 4.3 and Figure 4.4, respectively). Similar to that discussed above for dissociation of the GAILMGAILK peptide ions, dissociation of VTMGHFDNFGR and VTMAHFWNFGK resulted in the exclusive neutral loss of the side chain from the low charge state precursor ions ($[M^+ + H]^{2+}$ and $[M^+ + 3H]^{3+}$), where the ionizing protons in the resultant peptide product ions could be accommodated at the side chains of the lysine, arginine and histidine amino acids within the sequence or along the peptide backbone. For the quadruply charged ($[M^+ + 4H]^{4+}$) precursor ions however, extensive charged losses are observed. Interestingly, when the CID MS/MS product ion spectra of the triply charged GAILMGAILK, and the quadruply charged VTMGHFDNFGR and VTMAHFWNFGK peptide ions are examined, it can be seen that the abundance of the neutral versus charged loss product ions formed are highly dependant on the charge state and amino acid sequence of the peptide precursor. The relative abundance of neutral versus charged loss product ions observed from the fixed charge containing peptides is expected to vary according to the differences in proton

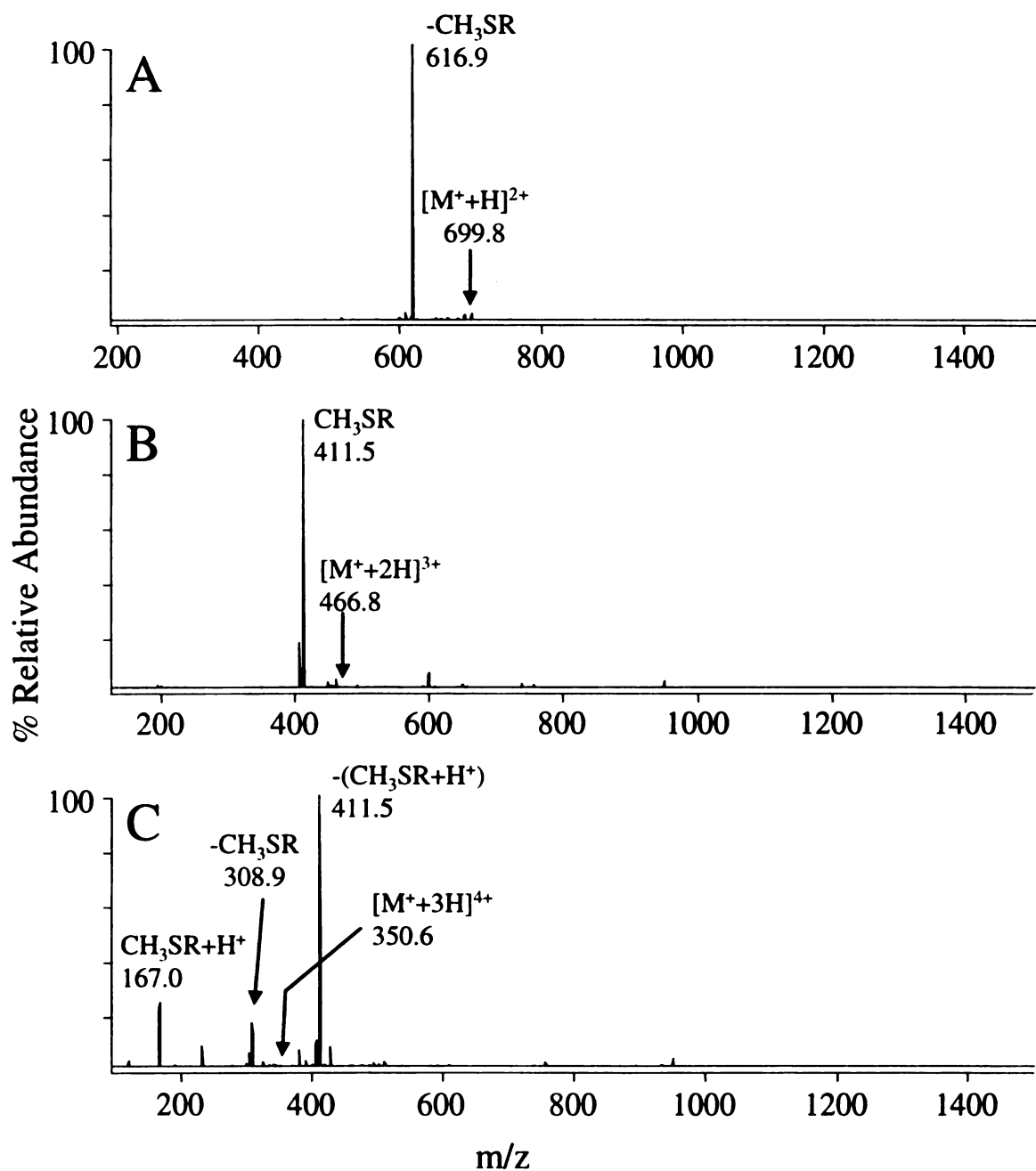


Figure 4.3 Linear ion trap CID MS/MS analysis of methionine side chain fixed charge phenacylsulfonium ions of VTMGHFDNFR. (A) CID MS/MS product ion spectrum of the $[M^+ + H]^{2+}$ ion. (B) CID MS/MS product ion spectrum of the $[M^+ + 2H]^{3+}$ ion. (C) CID MS/MS product ion spectrum of the $[M^+ + 3H]^{4+}$ ion.

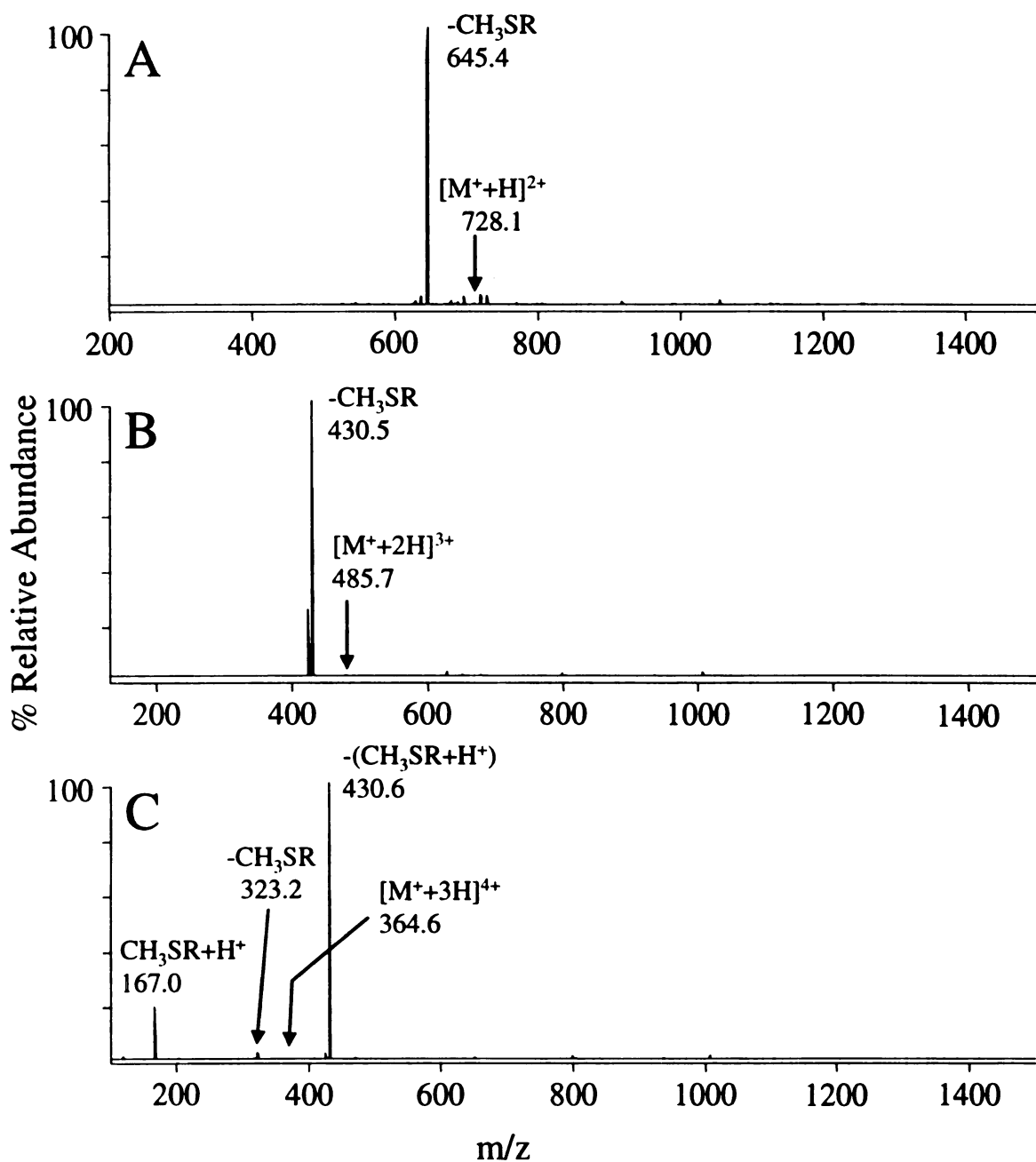


Figure 4.4 Linear ion trap CID MS/MS analysis of methionine side chain fixed charge phenacylsulfonium ions of VTMAHFWNFGK. (A) CID MS/MS product ion spectrum of the $[\text{M}^+ + \text{H}]^{2+}$ ion. (B) CID MS/MS product ion spectrum of the $[\text{M}^+ + 2\text{H}]^{3+}$ ion. (C) CID MS/MS product ion spectrum of the $[\text{M}^+ + 3\text{H}]^{4+}$ ion.

affinity between the peptide product ions and the neutral side chain fragment. Thus, from the results observed above, it can be determined that the proton affinities of the triply charged VTMGHFDNFGR and VTMAHFWNFGK product ions are both lower than that of the doubly charged GAILMGAILK product ion, and that the proton affinity of the triply charged VTMAHFWNFGK product ion is lower than that of the triply charged VTMGHFDNFGR product ion.

Previous studies carried out to examine the effect of proton affinity on the ratios of product ion abundances formed by CID have observed a linear relationship between the log of the ratio of the b_2 and y_1 type product ion abundances and the proton affinities of the C-terminal amino acid for a series of protonated tripeptides Gly-Gly-Xxx (where Xxx represents various amino acid residues) [59]. Here, to determine the effect of the leaving group proton affinities on the ratio of neutral versus charged loss from the methionine side chain fixed charge sulfonium ion containing peptides, the fragmentation of a series of para-substituted phenacylsulfonium ion containing peptides have been examined. Figures 4.5A-D show the CID MS/MS product ion spectra obtained from the triply charged $[M+2H^+]^{3+}$ precursor ions of GAILMGAILK derivatized at the methionine side chains using the para-benzoic acid (Figure 4.5A), para-methylbenzoate (Figure 4.5A), para-methyl (Figure 4.5A) and para-ethyl (Figure 4.5A) substituted phenacylbromide reagents (Compounds 2-5 in Chapter 2, respectively). Figures 4.6 and 4.7 show the CID MS/MS spectra obtained by dissociation of the quadruply charged $[M+3H^+]^{4+}$ precursor ions of VTMGHFDNFGR and VTMAHFWNFGK, respectively, each formed by derivatization at their methionine side chains using the same reagents as above.

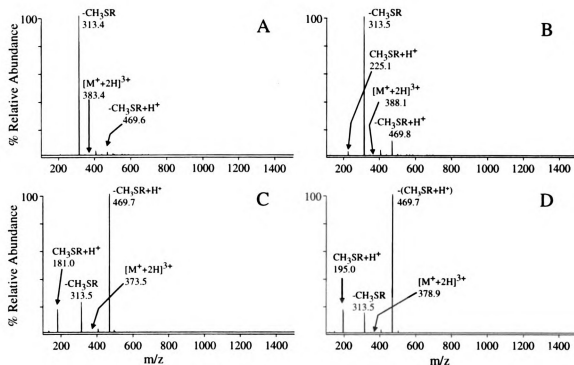


Figure 4.5 Linear ion trap CID MS/MS analysis of methionine side chain fixed charge para-substituted phenacylsulfonium ions of GAILMGAILK. (A) CID MS/MS product ion spectrum of the para-benzoic acid phenacylsulfonium $[M^{+}+2H]^{3+}$ ion. (B) CID MS/MS product ion spectrum of the para-methylbenzoate phenacylsulfonium $[M^{+}+2H]^{3+}$ ion. (C) CID MS/MS product ion spectrum of the para-methyl phenacylsulfonium $[M^{+}+2H]^{3+}$ ion, and (D) CID MS/MS product ion spectrum of the para-ethyl phenacylsulfonium $[M^{+}+2H]^{3+}$ ion.

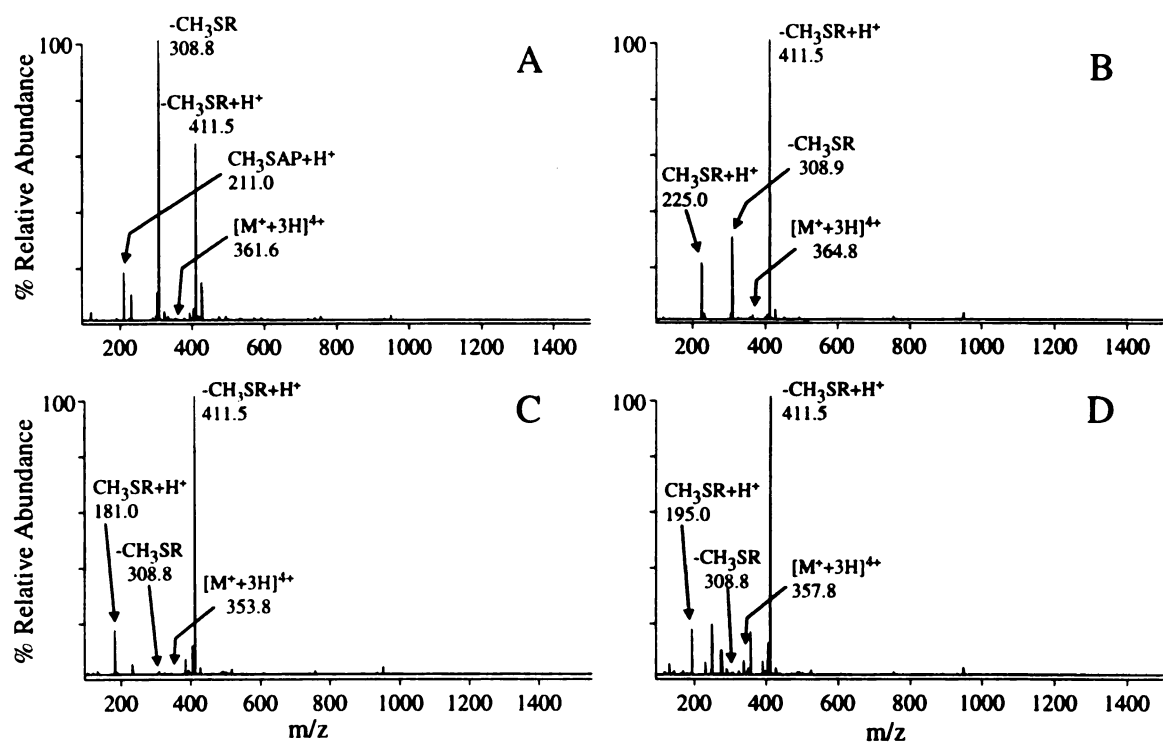


Figure 4.6 Linear ion trap CID MS/MS analysis of methionine side chain fixed charge para-substituted phenacylsulfonium ions of VTMGHFDNFR. (A) CID MS/MS product ion spectrum of the para-benzoic acid phenacylsulfonium $[M^+ + 3H]^{4+}$ ion. (B) CID MS/MS product ion spectrum of the para-methylbenzoate phenacylsulfonium $[M^+ + 3H]^{4+}$ ion. (C) CID MS/MS product ion spectrum of the para-methyl phenacylsulfonium $[M^+ + 3H]^{4+}$ ion, and (D) CID MS/MS product ion spectrum of the para-ethyl phenacylsulfonium $[M^+ + 3H]^{4+}$ ion.

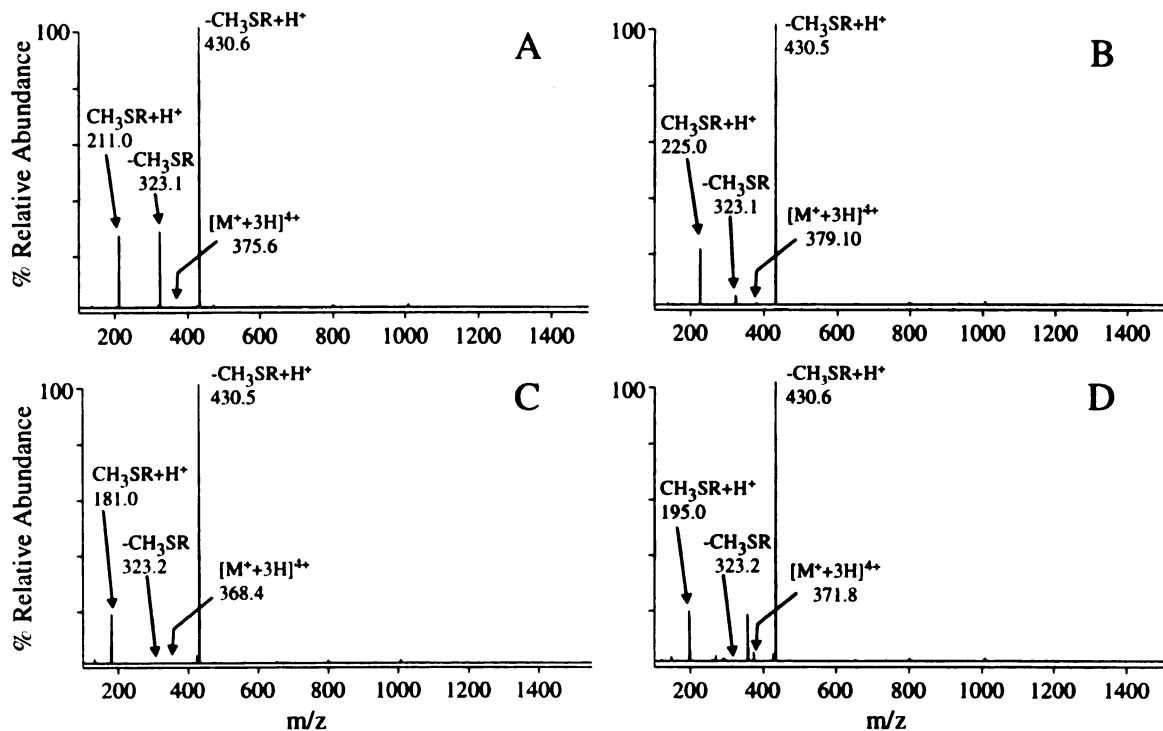


Figure 4.7 Linear ion trap CID MS/MS analysis of methionine side chain fixed charge para-substituted phenacylsulfonium ions of VTMAHFWNFGK. (A) CID MS/MS product ion spectrum of the para-benzoic acid phenacylsulfonium $[M^++3H]^{4+}$ ion. (B) CID MS/MS product ion spectrum of the para-methylbenzoate phenacylsulfonium $[M^++3H]^{4+}$ ion. (C) CID MS/MS product ion spectrum of the para-methyl phenacylsulfonium $[M^++3H]^{4+}$ ion, and (D) CID MS/MS product ion spectrum of the para-ethyl phenacylsulfonium $[M^++3H]^{4+}$ ion.

It can be seen from Figures 4.5, 4.6 and 4.7 that the ratio of neutral versus charged losses from the methionine side chain of these precursor ions varies as a function of the identity of the neutral side chain fragment, and that the extent of charged loss product ions increases with increasing proton affinity of the neutral side chain fragment. For example, when the triply charged para-benzoic acid substituted phenacylsulfonium ion derivative of GAILMGAIK was subjected to CID-MS/MS (Figure 4.5A), the neutral loss of the side chain was observed as the dominant fragmentation pathway. This result indicates that the proton affinity of the neutral side chain fragment (calculated as 210.6 kcal mol⁻¹, see structure 6 in Table 4.1) is lower than that of the doubly protonated peptide product ion. In contrast, CID-MS/MS of the triply charged para-ethyl phenacylsulfonium ion derivative of the same peptide gave rise to almost exclusive charged loss of the side chain. Thus, in this case, the proton affinity of the neutral side chain fragment (calculated as 219.0 kcal mol⁻¹, see structure 10 in Table 4.1) must have a higher proton affinity compared to the doubly protonated peptide product ion. These data therefore provide a means to 'bracket' the proton affinity of the doubly charged peptide product ion to between these two values. Similar proton affinity bracketing of the triply charged product ions of VTMGHFDNFGR and VTMAHFWNFGK was obtained by dissociation of the quadruply charged substituted phenacylsulfonium ion derivatives.

The plots shown in Figure 4.8 show a linear correlation between the log of the experimentally observed ratios of the charged versus neutral loss product ion abundances and the theoretically calculated proton affinities for the para-substituted methylphenacylsulfide side chain fragments of GAILMGAILK (Figure 4.8A), VTMGHFDNFGR (Figure 4.8B) and VTMAHFWNFGK (Figure 4.8C).

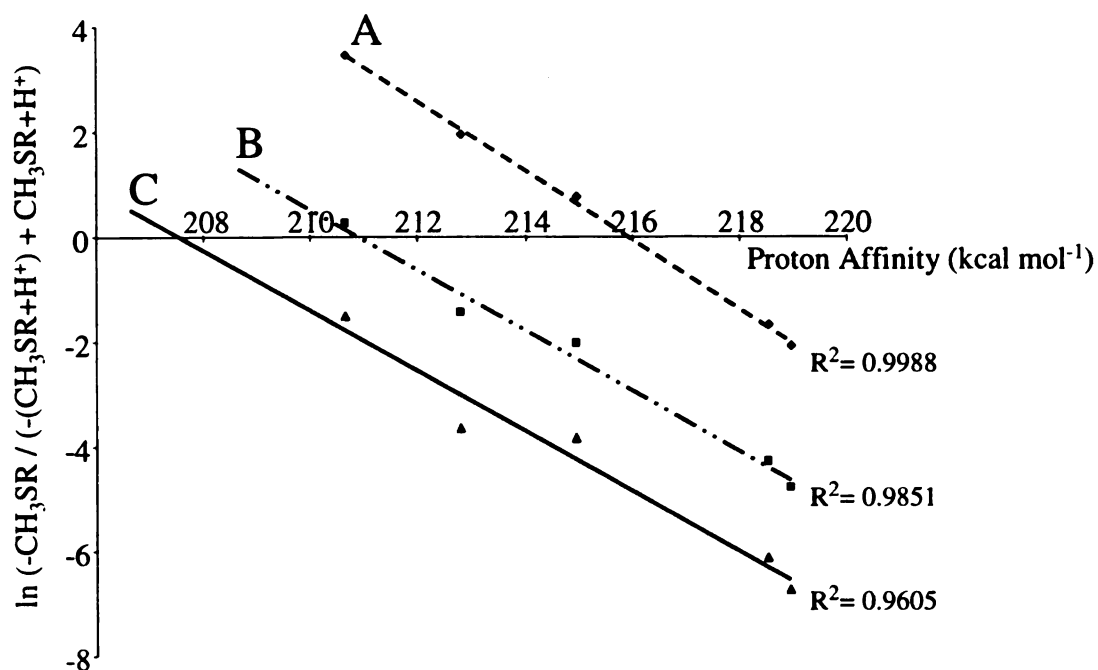


Figure 4.8. Plot of $\ln[-\text{CH}_3\text{SR} / (-(\text{CH}_3\text{SR}+\text{H}^+) + \text{CH}_3\text{SR}+\text{H}^+)]$ versus calculated proton affinity for the phenacylsulfide derivatives of (A) the $[\text{M}+2\text{H}]^{2+}$ side chain cleavage product ion of GAILMGAILK (B) the $[\text{M}+3\text{H}]^{3+}$ side chain cleavage product ion of VTMGHFDNFGR and (C) the $[\text{M}+3\text{H}]^{3+}$ side chain cleavage product ion of VTMAHFWNFGK.

In order to account for any charge state dependant detector response, and therefore to obtain a closer approximation of the actual abundances of the neutral and charged loss side chain cleavage product ions formed, the product ion abundances were first normalized with respect to charge state (i.e., intensity divided by charge), assuming mass detection response to be linear with respect to the charge state of the ion. The x-axis intercept of these plots may be used to estimate the proton affinities of the doubly charged product ion from GAILMGAILK ($216 \text{ kcal mol}^{-1}$), and the triply charged product ions of VTMGHFDNFGR ($211 \text{ kcal mol}^{-1}$) and VTMAHFWNFGK ($207.5 \text{ kcal mol}^{-1}$). The proton affinity of the amide bonds along the peptide backbone may be estimated from a simple model of the peptide amide bond, N-methyl acetamide, which has a proton affinity of $212.4 \text{ kcal mol}^{-1}$ [60]. Thus, each of the values estimated from the data above are consistent with the ionizing proton being located at a position along the peptide backbone.

4.4 Conclusions and Future Directions

These data described above indicates that the ratio of neutral loss to charged loss product ion abundances from fixed charge containing peptide ions varies according to the differences between the proton affinities of the neutral side chain fragment and the peptide product ion. Given that the ratio of charged versus neutral loss observed for a given peptide is observed to change as a function of peptide ion charge state, specific information regarding the number of basic sites contained within the peptide may potentially be obtained by having control over this reaction. In the future, the potential for utilizing this information, along with knowledge regarding the presence of a specific

amino acid residue (e.g., methionine), proteolytic enzyme cleavage specificity, chromatographic retention time, or accurate peptide mass, will be examined for directly identifying peptides from the sequence databases, without need for further structural information. Several precedents supporting the concept of improving the specificity of database searches for protein identification, by promoting the formation of specific product ions through chemical derivatization e.g., enhanced C-terminal aspartic acid cleavage from singly-charged peptides by conversion of lysine to homoarginine, or enhanced N-terminal amino acid cleavage by phenylthiocarbamyl modification have recently appeared in the literature [61].

REFERENCES

1. Stein, L.D. End of the beginning. *Nature*, **2004**, 431, 915-916.
2. Hanash, S. Disease proteomics. *Nature*, **2003**, 422, 226-232.
3. Barber, M.; Bordoli, R.S.; Sedgwick, D.; Tyler, A.N. Fast Atom Bombardment of Solids (F.A.B.): A New Ion Source for Mass Spectrometry. *J. Chem. Soc., Chem. Commun.* **1981**, 7, 325-327.
4. Rinehart, K.L. Fast Atom Bombardment Mass Spectrometry. *Science*, **1982**, 218, 254-260.
5. Fisher, D.L.L.; Gross, M.L. Fast Atom Bombardment and Tandem Mass Spectrometry for Sequencing Peptides and Polyamino Alcohols. *Anal. Chem.* **1985**, 57, 1174-1180.
6. Fenn, J.B.; Mann, M.; Meng, C.K.; Wong, S.F.; Whitehouse, C.M.; Electrospray Ionization for Mass Spectrometer of Large Biomolecules. *Science*, **1989**, 246, 64-71.
7. Karas, M.; Hillenkamp, F. Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10 000 Daltons. *Anal. Chem.* **1988**, 60, 2299-2301.
8. Gomez, A.; Tang, K. Charge and fission of droplets in electro static sprays. *Phys. Fluids* **1994**, 6, 404-414.
9. Thomson, B.T.; Iribarne, J.V. Field induced ion evaporation from liquid surfaces at atmospheric pressure. *J. Chem. Phys.* **1979**, 71, 4451-4463.
10. Iribarne, J.V.; Thomson, B.T. On the evaporation of small ions from charged droplets. *J. Chem. Phys.* **1976**, 64, 2287-2294.
11. Zenobi, R.; Knochenmuss, R. Ion Formation in MAIDI Mass Spectrometry. *Mass Spectrom. Rev.* **1998**, 17, 337-366.
12. Hoffmann, E.D.; Stroobant, V. *Mass Spectrometry Principles and Applications*, 2nd edition, John Wiley and Sons, New York, Page 69.

13. March, R.E. Quadrupole Ion Trap Mass Spectrometry: Theory, Simulation, Recent Developments and Applications. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1543-1554.
14. Hager, J.W. A new linear ion trap mass spectrometer. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 512-526.
15. Schwartz, J.C.; Senko, M.W.; Syka, J.E. A two-Dimensional Quadrupole Ion Trap Mass Spectrometer. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 659-669.
16. Makarov, A. Electrostatic axially harmonic orbital trapping: a high-performance technique of mass analysis. *Anal. Chem.* **2000**, *72*, 1156-1162.
17. Hardman, M.; Markarov, A. Interfacing the orbitrap mass analyzer to an electrospray ion source. *Anal. Chem.* **2003**, *75*, 1699-1705.
18. Hu, Q.; Noll, R.J.; Li, H.; Makarov, A.; Hardman, M.; Cooks, R.G. The Orbitrap: a new mass spectrometer. *J. Mass Spectrom.* **2005**, *40*, 430-443.
19. Olsen, J.V.; de Godoy, L.M.F.; Li, G.; Macek, B.; Mortensen, P.; Pesch, R.; Makarov, A.; Lange, O.; Horning, S.; Mann, M. Parts per Million Mass Accuracy on an Orbitrap Mass Spectrometer via Lock Mass Injection into a c-trap. *Mol. Cell. Proteomics.* **2005**, *4*, 2010-2021.
20. Eng, J.K.; McCormack, A.L.; Yates, J.R. An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino acid Sequences in a Protein Database. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 976-989.
21. Perkins, D.N.; Pappin, D.J.C.; Creasy, D.M.; Cottrell, J.S. Probability-base protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20*, 3551-3567.
22. Yates, J.R.; Eng, J.K.; McCormack, A.L.; Schieltz, D. Method to Correlate Tandem Mass Spectra of Modified Peptides to Amino Acid Sequences in the Protein Database. *Anal. Chem.* **1995**, *67*, 1426-1436.
23. MacCoss, M.J.; Wu, C.C.; Yates, J.R. Probability-Based Validation of Protein Identifications Using a Modified SEQUEST Algorithm. *Anal. Chem.* **2002**, *74*, 5593-5599.

24. Hunt, D.F.; Yates, J.R.; Shabanowitz, J.; Winston, S.; Hauer, C.R. Protein sequencing by tandem mass spectrometry. *Proc. Natl. Acad. Sci.* **1986**, *83*, 6233-6237.
25. de Hoog, C.L.; Mann, M. Proteomics. *Genomics Hum. Genet.* **2004**, *5*, 267-293.
26. Creasy, D. M.; Cottrell, J.S. Error Tolerant searching of uninterpreted tandem mass spectrometry data. *Anal. Chem. Proteomics* **2002**, *2*, 1426-1434.
27. Reid, G. E.; Roberts, K. D.; Kapp, E. A.; Simpson, R. J Statistical and Mechanistic Approaches to Understanding the Gas-Phase Fragmentation Behavior of Methionine Sulfoxide Containing Peptides. *J. Proteome Res.* **2004**, *3*, 751-759.
28. Annan, R.; Carr, S. A. Phosphopeptide Analysis by Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry. *Anal. Chem.* **1996**, *68*, 3413-3421.
29. Mann, M.; Jensen, O.N. Proteomics analysis of post-translational modifications. *Nature Biotechnology*, **2003**, *21*, 255-261.
30. Dongre, R.A.; Jones, J.L.; Somogyi, A.; Wysocki, V.H. Influence of Peptide Composition, Gas-Phase Basicity, and Chemical Modification on Fragmentation Efficiency: Evidence for the Mobile Proton Model. *J. Am. Chem. Soc.* **1996**, *118*, 8365-8374.
31. Cooks, R.G.; Kruger, T.L Intrinsic basicity Determination Using Metastable Ions. *J. Am. Chem. Soc.* **1977**, *99* 1279-1281.
32. Armentrout, P.B. Entropy Measurements and the Kinetic Method: A Statistically Meaningful Approach. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 371-379.
33. Kuntz, A.E.; Boynton, A.W.; David, G.A.; Colyer, K.E.; Poutsma, J. C. The Proton Affinity of Proline Analogs Using the Kinetic Method with Full entropy Analysis. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 72-81.
34. Schroeder, O.E.; Andriole, E.J.; Carver, K.L.; Colyer, K.E.; Poutsma, J.C. Proton Affinity of Lysine Homologues from the Extended Kinetic Method. *J. Phys. Chem. A*, **2004**, *108*, 326-332.

35. Kapp, E.A.; Schultz, F.; Reid, G.E.; Eddes, J.S.; Moritz, R.L.; O'Hair, R.A.J.; Speed, T.P.; Simpson, R.J. Mining a Tandem Mass Spectrometry Database To Determine the Trends and Global Factors Influencing Peptide Fragmentation. *J Anal. Chem.* **2003**, *75*, 6251-6264.
36. Schlosser, A.; Lehmann, W.D. Five-membered ring formation in unimolecular reactions of peptides; a key structural element controlling low-energy collision-induced dissociation of peptides. *J. Mass Spectrom.* **2000**, *35*, 1382-1390.
37. Paizs, B.; Suhai, S. Fragmentation Pathways of Protonated peptides. *Mass Spectrom. Rev.* **2005**, *24*, 508-548.
38. McCormack, A.L.; Somogyi, A. Dongre, A.R.; Wysocki, V.H. Fragmentation of Protonated Peptides: Surface-Induced Dissociation in Conjunction with a Quantum Mechanical Approach. *Anal. Chem.* **1993**, *65*, 2859-2872.
39. Vekey, V.; Gomory, A. Theoretical Modeling of Mass Spectrometric Behavior of Peptides: Singly and Doubly Protonated Tetraglycine. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1485-1496.
40. Somogyi, A.; Wysocki, V.H.; Mayer, I. The Effect of Protonation Site on Bond Strengths in Simple Peptides: Application of Ab Initio and Modified Neglect of Differential Overlap Bond Order and Modified Neglect of Differential Energy Partitioning. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 704-717.
41. Wu, J.; Lebrilla, C.B. Gas-Phase Basicities and Sites of Protonation of Glycine Oligomers. *J. Am. Chem. Soc.* **1993**, *115*, 3270-3275.
42. Zhang, K.; Cassady, C.J.; Phillips, C.A. Ab Initio Studies of Neutral and Protonated Triglycines: Comparison of Calculated and Experimental Gas-Phase Basicity. *J. Am. Chem. Soc.* **1994**, *116*, 11512-11521.
43. Lee, S.W.; Kim, H.S.; Beauchamp, J.L. Salt Bridge Chemistry Applied to Gas-Phase Peptide Ions Adjacent to Aspartic Acid Residues. *J. Am. Chem. Soc.* **1998**, *120*, 3188-3195.
44. Roth, K.D.W., Huang, Z, H., Sadagopan N.; Watson J.T. Charge derivatization of peptides for analysis by mass spectrometry. *Mass Spectrom. Rev.* **1998**, *17*, 255-274.

45. Keough, T.; Youngquist, R.S.; Lacey, M.P. Sulfonic acid derivatives for peptide sequencing by MALDI MS *Anal. Chem.* **2003**, *75*, 156A-165A.
46. Huang, Z.; Wu, J.; Roth, K.D.W.; Gage, D.A.; Watson, J.T. A Picomole-Scale Method for Charge Derivatization of Peptides for Sequence Analysis by Mass Spectrometry. *Anal. Chem.* **1997**, *69*, 137-144.
47. Adamczyk, M.; Gebler, J.C.; Wu, J. Charge Derivatization of Peptides to Simplify their Sequencing with an Ion Trap Mass Spectrometer. *Rapid Commun. Mass spectrum.* **1999**, *13*, 1413-1422.
48. Sadagopan, N.; Watson, J.T. Mass spectrometric evidence for mechanisms of fragmentation of charge-derivatized peptides. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 399-409.
49. Gu, C.; Tsaprailis, G.; Brechi, L.; Wysocki, V.H. Selective Gas-Phase Cleavage at the Peptide Bond C-Terminal to Aspartic Acid in Fixed-Charge Derivatives of Asp-Containing Peptides. *Anal. Chem.* **2000**, *72*, 5804-5813.
50. Reid, G.E.; Roberts, K.D.; Simpson, R.J.; O'Hair, R.A.J. Selective Identification and Quantitative Analysis of Methionine Containing Peptides by Charge Derivatization and Tandem Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1131-1150.
51. Reid, G.E.; Simpson, R.J.; O'Hair, R.A.J. Method for Analyzing Amino Acids, Peptides and Proteins. *PCT Int. Appl.* **2004**, WO 2004046731.
52. Paizs, B.; Suhai, S. Towards Understanding the Tandem Mass Spectra of Protonated Oligopeptides. 1: Mechanism of Amide Bond Cleavage. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 103-113.
53. Mcluckey, S.A.; Goeringer, D.E. Slow Heating Methods in Tandem Mass Spectrometry. *J. Mass Spectrom.* **1997**, *32*, 461-474.
54. Johnson, R.S.; Krylov, D.; Walsh, K.A. Proton mobility within Electrosprayed Peptide ions. *J. Mass Spectrom.* **1995**, *30*, 386-387.
55. Averbukh, M.E.; Pipkorn, R.; Lehmann, W.D. Phosphate Group-Driven Fragmentation of Multiply Charged Phosphopeptide Anions. Improved Recognition of

Peptides Phosphorylated at Serine, Threonine, or Tyrosine by Negative Ion Electrospray Tandem Mass Spectrometry. *Anal. Chem.* **2006**, 78, 1249-1256.

56. Hammett, L.P. The Effect of Structure upon the Reactions of Organic Compounds, benzene Derivatives. *J. Am. Chem. Soc.* **1937**, 59, 96-103.

57. Jaffe, H. A re-examination of the Hammett Equation. *Chem. Rev.* **1953**, 53, 191-261.

58. Hansch, C., Leo, A.; Taft, R.W. A Survey of Hammett Substituent Constants and Resonance and Field Parameters. *Chem. Rev.* **1991**, 97, 165-195.

59. Morgan, D.G.; Bursey, M.M. A Linear Free-energy Correlation in the Low-energy Tandem Mass Spectra of Protonated Tripeptides Gly-Gly-Xxx. *Org. Mass Spectrom.* **1994**, 29, 354-359.

60. Hunter, E.P.L.; Lias, S.G. Evaluated Gas Phase Basicities and Proton Affinities of Molecules: An Update, *J. Phys. Chem. Ref. Data*, **1998**, 27, 413-656.

61. Sidhu, K.S.; Sangvanich, P.; Brancia, F.L.; Sullivan, A.G.; Gaskell, S.J.; Wolkenhauer, O.; Oliver, S.G.; Hubbard, S.J. Bioinformatic assessment of mass spectrometric chemical derivatisation techniques for proteome database searching. *Proteomics*, **2001**, 1, 1368-1377.

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