Ionization, dissociation, and the investigation of structure in small molecular models to develop a broader understanding of gas phase ion chemistry

DISSertation

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By

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Abstract

This dissertation focuses on molecular systems in the low-mass range to determine how chemical and structural changes can affect subsequent fragmentation chemistry and protonation site. Each system was investigated using MS analysis and gas-phase ion structural techniques selected from tandem MS (MSMS), hydrogen-deuterium exchange (HDX), ion-mobility (IM), and action infra-red multi-photon dissociation (IRMPD).

In Chapter 3, the non-standard amino acid gamma-aminobutyric acid (GABA) was placed into a peptide system to test a mechanism which explained the lack of a$_3$ ions in standard peptide fragmentations. GABA extends the peptide backbone by two methylene units and its insertion into the second position of larger peptides increases the intensity of a$_3$ ion. Using MSMS, it was found that this was a result of blocking common favorable fragmentation pathways. The results demonstrated the use of modified peptides for revealing reasons for how peptides fragment.

Chapter 4 focused on a unique set of non-canonical amino acids and their ability to affect the trans/cis nature of adjacent amide bonds in peptide sequences. In solution, 4-R-Flouroproline (R-Flp) is found to favor the trans peptide bond and 4-S-fluoroproline (S-flp) favors the cis bond. IRMPD and IM-MS were employed to investigate the fragment ions containing these two prolyl-ring substitutions as the structures of the b$_2$ ions might indicate the prevalence of cis vs. trans peptide bond in the gas-phase. In experiments, the residues favoring trans vs. cis bonds, respectively, formed greater proportions of the
trans/cis fragment ions, showing a correlation between solution and gas-phase structural trends of peptides.

The two cancer drugs pomalidomide (Pom) and lenalidomide (Len) differ by the change of a single carbonyl functional group. Because these two drugs behave quite differently in vivo, the objective of Ch 5 to investigate their gas phase behavior using IRMPD, IM, and HDX, as differences in their gas-phase ion structures might shed light on their unique biological activities. From these techniques, it was determined that Pom alone possessed a clear amide-iminol tautomerization and the presence of multiple structures in its precursor and fragment ion populations.

The carotenoids α-carotene, β-carotene, and lycopene are isobaric compounds, which differ in their ability to serve as precursors to vitamin A in vivo. Additionally their solution-state double-bond conformations influence their provitamin activity. The objective of Chapter 6 was to use IMS and MSMS to separate their radical ions to both quantify them without solution-phase separation and also to determine their pre-ionization trans/cis conformations. IM-MS provided reasonable relative quantification. Although the isomers gave different IM drift time distributions, even after activation, trans/cis conformation retention was not confidently ascertained.

In Chapter 7, high work-function metal oxides, ReO₃ and WO₃ microparticles (µP) were tested as matrices for a number of small molecules encompassing protonation, sodiation, radical ionization, and pre-charged ablation. The particles' ability to ionize these compounds was compared with the standard matrix CCA. WO₃ µP performed well for sodiated compounds while ReO3 µP worked well for protonated and radical species simultaneously. Moreover, ReO₃ was less destructive of fragile ions compared to CCA at equivalent laser flux.
Dedication

This body of work is dedicated to my parents, Robert and Patricia Bernier.
Acknowledgements

There is a long list of people I cannot thank enough for all the support, encouragement, and assistance that got me through the last five years of my graduate school career. On the top of that list is my advisor, Dr. Vicki Wysocki, who provided an invaluable opportunity for me to grow as a scientist and professional in her lab. Not only did Vicki offer support as a research advisor but also as a mentor in life outside of science and I greatly appreciate my experiences as a member of her group.

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I got to experience an incredible set of people as a member of the Department of Chemistry at the University of Arizona and me some of my best friends in Tucson, AZ, who taught me the appropriate balance of work and fun in my first few years of graduate school. I am equally glad and undoubtedly my life has been enriched from the
opportunity to come to Columbus, OH to become a member of another Department of Chemistry at The Ohio State University. Undoubtedly, I have met another set of great people and scientists who have also become supportive and close friends while here. Likewise, my friends in Virginia Beach, VA have been nothing but sources of encouragement to me during my years from home. Lastly, my family is the world to me and I could never have done this without them. Thank you Mom, Dad, Tim, Meaghan, Dan, Maurika, Becky, Washington, Dinah, Eugene, Olivia, and little Piet.
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- Bernier, M.C., S. Dagan, V.H. Wysocki, Laser Desorption Ionization of small
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  Preparation

- Bernier, M.C., J. Chamot-Rooke, V.H. Wysocki, Proline ring substitution and
  its effect on the formation of b2 ions in gas-phase peptide fragmentation. In
  Preparation


Fields of Study

Major Field: Chemistry
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<td>AC</td>
<td>alternating current</td>
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<td>CCS</td>
<td>collision cross section</td>
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<td>CID</td>
<td>collision induced dissociation</td>
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<td>direct current</td>
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<td>ERMS</td>
<td>energy-resolved mass spectrometry</td>
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<td>ESI</td>
<td>electrospray ionization</td>
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<td>FMOC</td>
<td>fluorenlymethyloxycarbonyl chloride</td>
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<td>HBTU</td>
<td>O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate</td>
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<td>IRMPD</td>
<td>Infrared multi-photon dissociation</td>
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<td>RF</td>
<td>radio frequency</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight mass spectrometer</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
</tbody>
</table>
1.1. Overview

The work presented here details the use of gas-phase techniques to analyze several types of small molecular systems with the aim of exploring their structures following ionization as well as changes to structure upon variations to functional group chemistry. Analytes studied included peptides, both standard protein and non-protein substituted form of amino acids, drugs used for cancer treatment and pain, as well as carotenoid species necessary for photosynthesis in fruits and vegetables. The systems investigated all possess biological significance; peptides are the building block of proteins, drugs selected for study have been FDA approved to treat multiple myeloma, and carotenoids are believed to assist in the prevention of cancer and are strong anti-oxidants that reduce reactive oxygen species while some are precursors to the formation of vitamin A.

While their biological activity is of importance and a strong supporting reason of why they were chosen for study, this fact was only a small part of why they were studied here. Each system offered unique examples of fundamental chemistry that could be probed using mass spectrometry (MS) in conjunction with other gas-phase ion structural elucidation techniques. In Chapters 3 and 4, unique non-peptide amino acid substitutions were investigated, to better understand specific trends in the fragmentation of peptides, fragmentation mechanisms, and the formation of b and a-ion structures. The specifics of formation of these ions will be described in detail below in section 1.4.1. In Chapter 5, two cancer drugs, pomalidomide and lenalidomide, which derive from
thalidomide, were studied by MS to determine how their functional differences, discriminated by the presence of one carbonyl group, could affect their ion structure. This difference included the possibility of tautomer forms of each species as well as the site of protonation. In Chapter 6, the molecular system under study was a set of three isobaric carotenoids, α-carotene, β-carotene, and lycopene, which are all conjugated hydrocarbons terminating as either a fully linear chain (lycopene) or as six-membered rings with β-carotene remaining fully conjugated at the ring and α-carotene shifting the position of one double bond. These molecules were differentiated by their fragmentation patterns as well as their ionic size and shape using ion mobility, which is discussed in section 1.5.2. Additionally, to supplement the fundamental studies from the first four data chapters, a method development study is discussed in Chapter 7 which focuses on detecting small molecular ions using an alternative method to MALDI.

As an introduction, a synopsis of the ionization and gas-phase structural techniques used in this study with detailed outlines of the mass analyzers utilized is provided along with a summary of the key small molecular systems studied up to this point in time.

1.2. Ionization methods

The realm of small molecule ionization has had a long history in the field of mass spectrometric analysis. At the outset of mass spectrometry development, systems under investigation were mainly small molecules, as this regime of compounds are more readily ionized and volatile.\(^1,2\) compared to the emerging field of protein ionization (i.e. protein non-covalent complexes).\(^3,4\) Initially, ionization processes were simplistic, destructive, and mainly involved directing highly energetic species (i.e. electrons or ions) at the neutrals to promote electron transfer and subsequent ionization.\(^5,6\) These early techniques included electron impact ionization (EI),\(^7\) a technique typically coupled to
gas-chromatography and involving collisions of electrons with analytes of interest introduced as neutral gas molecules. Two other early techniques included chemical ionization (CI), a proton transfer mechanism also often coupled to GC that can retain the precursor ion with minimal formation of fragments upon ionization, and fast-atom bombardment (FAB), developed in 1981 by Barber et al., a secondary ionization process where a stream of charged atoms are trained onto a solid sample to induce ionization. These ionization methods are crude and often destructive in that they result in fragmentation of the precursor ion of interest due to energy transfer from the respective charged species to the neutral with EI imparting the most energy.

Moving on from the development of these highly energetic techniques, small molecule analysis has benefited from the development of so-called “soft” ionization techniques, which could retain precursor ion intensity in MS mode. Starting with the discovery of electrospray ionization (ESI) in 1984 by John Fenn et al. inspired by the work of Dole et al. the field of non-destructive ionization techniques has expanded dramatically and increased the ability of probing ion structure and fragmentation chemistry with their development. Following ESI, matrix-assisted laser desorption ionization (MALDI), and several atmospheric ionization methods including desorption electrospray ionization (DESI), direct analysis in real-time (DART), as well as atmospheric pressure chemical ionization (APCI) expanded the field to further increase the tools in both the applications of small analyte discovery (i.e. metabolomics, lipidomics, and pharmaceuticals).

Below are brief descriptions of the soft ionization techniques performed for the experiments in this dissertation, their applications and advantages in small molecule ionization.
1.2.1. Electrospray Ionization techniques and sub 1000 Dalton analysis

Electrospray ionization (ESI) has become the most widely used ionization technique in mass spectrometry due to its ability to produce multiply-charged ions for a wide variety of analyte types.\textsuperscript{18} It can be used to ionize not volatiles with virtually no unwanted fragmentation of the precursor, and also allows biomolecules up to MDa size ranges to be transmitted with full quaternary structure intact.\textsuperscript{2,6,13} Figure 1.1 below shows the basic design of the ESI device coupled to an MS instrument, developed by John Fenn. The device is distinguished by the use of a stainless steel capillary needle with a high voltage of 3-6 kV applied to the tip, to induce droplets containing the analytes of interest which then evaporate to form desolvated, singly to multiply charged, analytes that move into the ion optics of the MS instrumentation set in place downstream of the source.

Figure 1.1 Original schematic of the ESI-MS coupled device reprinted with permission from John Wiley and Sons.\textsuperscript{6} This plan outlined the first working ESI to MS coupling and was developed by the group of John Fenn at Yale in 1982.

The physical processes involved in forming charged and desolvated analyte ions is shown in a schematic representation below (Figure 1.2). A high positive voltage (the polarity is switched to a high negative ESI voltage to induce negative mode ions,
however, the process remains similar) is applied at the tip of the metal needle. A voltage applied here results in a build-up of positive charges and so decreases the surface tension of the layer of solvent at the tip of the ESI needle to form what is called a “Taylor cone.” The Taylor cone sprays out a constant series of charged droplets and following droplet formation, the formation of ions is dependent on “desolvating” the ions from the droplets made up of charged particles, solvent, and analytes of interest. Following rapid solvent evaporation the droplets experience an increase in charge density, until they reach the “Rayleigh limit” and at this point they undergo fission, discussed in more detail in the mechanistic paragraph below, to produce the smaller charged particles. This is the repeated evaporation and fission events that produce the desolvated ions. For this reason, a bath gas of nitrogen is added to the spray needle to focus the molecular beam and effectively “dry” the droplets in the spray plume. Upon the addition of this gas source, it is also necessary to have variable stages of pumping in order to lower the presence of background of neutrals travelling in the MS instrument along with a cone (nozzle) set to a filter out neutrals, focus the beam of ions, allow for greater vacuum pumping, and provide a voltage to induce movement of the ions into the instrument. Not shown in this first design, is the other common addition of a heated glass capillary following the skimmer that is used in some ESI-MS set-ups in order to promote better desolvation of droplets before entering the MS region.
There are two major theories on the mechanism of forming ions from the droplets that are induced via electrospray. The first ESI ion formation theory is the Charged Residue Mechanism (CRM) and the process is illustrated in Figure 1.3 by the top arrow following “droplet fission.” In this theory, it is established that the charged droplet formed by the nebulizing Taylor cone via ESI contains a number of analyte molecules and charged particles. Upon the evaporation of solvent, the droplet splits into smaller droplets until there is only one analyte and a portion of charged particles left in the droplet before entrance into the mass spectrometer. The second mechanism for ion formation is the Ion Evaporation Model (IEM), depicted in the bottom arrow of Figure 1.3. This mechanism also starts with the formation of the charged droplet after leaving the ESI Taylor cone. Upon the evaporation of the charged droplet down to a specific “critical dimension”, however, it is postulated that the buildup of charge on the surface of the droplet induces “Coulombic explosion” to eject the analyte along with the charged...
molecules surrounding it, to form the gas-phase analytes of varying charge immediately from this larger droplet. The main difference in these two mechanisms is the point at which the molecular ion is isolated from the rest of the droplet and left with only the charged particles attached to it.\textsuperscript{20,22,24}

In addition to the mechanisms discussed in detail above, a third theory regarding the ionization and desolvation of ions has also been put forward which is thought to be pertinent in the ionization of disordered/denatured proteins and polymers. This theory put forward by Konermann et al. in 2013, is called the “Chain Ejection Mechanism.”\textsuperscript{25} As these species can form hydrophobic linear chains they migrate to the surface of the droplet, this mechanism suggest that upon evaporation, portions of an extended molecule, beginning with a termini, could be ejected from a droplet in steps and as the droplet gets smaller, more of the species could be removed leading to the very high charge states often seen for these species. While not a likely method of ESI ion formation for the systems studied in this dissertation it is discussed in brief here, for completeness. While not a likely method of ESI ion formation for the systems studied in this dissertation it shares features of that of the IEM model and further confirms that the ESI process starts with the formation of charged droplets from which the process of final analyte isolation is debated.
Figure 1.3  Comparison of the two main electrospray models, the charged residue model (CRM) and the ion evaporation model (IEM). Reprinted per open access from Crotti et al.\textsuperscript{26}

The development of ESI opened up a new field of experimentation on large supramolecules, however, its development, and the development of other “soft” ionization techniques that followed after, also enhanced and created new fields of research on small sub 1000 Dalton analytes.\textsuperscript{18,27-29} This ionization method allows for low energy input both large and small analytes alike, and this allows for the mass spectrometer to probe gas-phase ion chemistry with structural and fragmentation techniques which will be discussed in more detail below.

1.2.2. Matrix-Assisted Laser Desorption Ionization and the challenge of small molecular analysis

The technique of Matrix-assisted laser desorption ionization (MALDI) was developed in the work of Karas and Hillenkamp,\textsuperscript{30} and of Tanaka\textsuperscript{31}, in 1985 and 1988, respectively. Like ESI described above in section 1.2.1, MALDI offers another “soft” ionization technique which can retain the fully intact precursor analytes up to hundreds of KDa biomolecules and polymers.\textsuperscript{32} Unlike ESI, however, MALDI typically forms only singly and some doubly charged species upon laser ablation.\textsuperscript{33,34} A simplified cartoon
schematic of the MALDI process, and the basic set up for its introduction into the mass analyzer is shown in Figure 1.4. This process consists of co-spotting solutions of analyte and matrix onto a metal plate and allowing this spot to dry resulting in a solid region of analyte imbedded in a high ratio of matrix molecules. To introduce the analytes to MS analysis, the plate is typically placed in a vacuum region after drying with focusing elements just above the plate to direct the analyte ions into the mass analyzer as seen in the top region of Figure 1.4.

To produce ions, a laser (typically of UV wavelength) is focused on the spot which produces a plume of analyte, matrix, and cations directly above the plate. After formation of the analyte ions, the plate acts as a pusher with a high voltage of ~10 kV applied to move the ions to the mass analyzer through the focusing elements immediately above the plate. Additionally, an extraction grid is also added before the MS focusing elements to apply a gradient voltage to the region. This gradient allows for application of varying kinetic energies to ions, which significantly improve the resolution of mass-to-charge when coupling the MALDI source to a time-of-flight (TOF) mass analyzer. Although MALDI can be coupled to different instrumental set-ups and can be utilized with many mass analyzers, the TOF is most often coupled to MALDI. MALDI-TOF instrumentation was used for all MALDI based studies and this coupling will be discussed in further detail below in section 1.3.3.
Standard matrixes are small aromatic organic acids, such as 2, 5-dihydroxy benzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CCA). The use of these types of molecules offers efficient absorption of impinging photon energy and an acidic environment for proton transfer. The mechanism of MALDI ionization is still not completely understood, but mainly involves the absorption of the laser by the matrix and subsequent proton transfer to the analyte via a high energy process.

With respect to small molecule analysis, MALDI offers simple sample preparation with co-spotting of analyte and matrix and also the ability to spot hundreds of samples at once and routine analysis of each. There is a significantly disadvantage with standard MALDI, however, as typical matrices are usually between 100 and 300 Da. This affects their usability in the low mass region as they are easily ionized and can form monomers, dimers, and adducts to produce a spectra in which the low m/z region is cluttered with matrix derived background peaks. This is a problem since the MALDI matrix is in a high
ratio relative to analytes and being a strong absorber of laser radiation matrix-derived interferences can be significant. Further in this dissertation in Chapter 7, this problem of matrix-derived interference will be further discussed and methods of alleviating this issue will be addressed.

1.3. Mass analyzers utilized

The research described in this dissertation focuses on the use of two ionization methods, described above in section 1.2, to introduce molecular systems of small molecular weight to the gas-phase, but makes use of several different types of mass analyzers post-ionization. The basics of each of these mass analyzers are described within this section.

1.3.1. Quadrupole Mass Analyzers

The standard quadrupole mass analyzer serves as a robust yet low resolving power mass filtering device commonly used to isolate particular ions of interest. By applying an electric field in two orthogonal planes of space, a “quad” allows a single m/z, typically within 1 amu, to remain stable throughout the length of the device and therefore separate it from a series of ions and allow down-field analysis. In the context of this dissertation, this piece of equipment serves as an isolating region for both the FT-ICR used in Chapter 3 and the Q-TOF type Synapt G2/G2S instruments used for ion mobility experiments in Chapters 4, 5, and 6.

The device is composed of four metal rod electrodes placed in parallel as shown in the top half of Figure 1.5, with AC and DC fields applied to each pair of rod. To isolate a single mass-to-charge of interest, one set of parallel rods will act as a low-mass filter, removing all ions below that ion’s m/z, and one will serve as a high-mass filter, making unstable all ions of m/z above that small region, leaving only the desired ion of interest
by the end of the quadrupole region. For the purposes of this description, the low-mass filter will be the X-Z plane, and the Y-Z plane will represent the high-mass filter. The equations describing the application of the electric fields can be expressed by the partial derivatives of the potential distributions (φ) as a function of time (t) in all three dimensions, as follows:

\[ E_x = -\frac{\delta \phi}{\delta x} = \frac{U + V \cos(\omega t)}{r_o^2} \frac{x}{r_o^2} \]

\[ E_y = -\frac{\delta \phi}{\delta y} = -\frac{U + V \cos(\omega t)}{r_o^2} \frac{y}{r_o^2} \]

\[ E_z = -\frac{\delta \phi}{\delta z} = 0 \]

These expressions define the nature of the electric potentials with the following variables, where x and y are the distance from the rods in each dimension, U is the DC potential applied, V is the radio-frequency (rf) or AC potential applied, t is the time, \( \omega \) is the angular frequency of the rf field, and \( r_o \) is the radius the quadrupole device measured by the distance of the center axis (z-axis) of the quadrupole to the closest surface of the rods, which should all be equidistant. As can be seen in the z direction, there is no potential field applied, and so in each plane, the field is stronger in the XZ plane as an ion moves towards the rod (larger x) and the field is stronger in the YZ direction as an ion displaces and moves closer to the rod in the y direction.
As the x-axis rods apply a constant positive DC potential, positive mode ions are repulsed and kept towards the z-axis of the quadrupole device. With the modulation of the AC potential, however, cause the set of quads to be negatively charged for a portion of their waveform and since ions of low m/z respond quickly to the variations of AC are more likely to be made be attracted to the rods and hit them, quenching the ion. In this way, a specific DC voltage on top of the AC potential creates and off-set that cuts-off low mass ions at a certain values dependent on the amplitude of the AC and DC voltages and effectively makes this set of rod a low-mass filter. In the case of the y-axis rods, the polarity is switched to a constant negative DC potential with an AC potential applied on top of that. Since high m/z ions will not respond quickly to the change back to positive charge induced by the AC portion of the potential, the y-axis rods will attract larger ions to the rods, subsequently quenching them and in this way act as a high-mass filter.
Combining both sets of rods together will serve to allow only one narrow band of ions above the low mass cut-off and below the high mass cut-off to pass.

The mathematical way of showing the given stability for ions in the quadrupole mass filter is with the Mathieu diagram shown in Figure 1.6. At the exactly ratio of AC and DC voltage, a narrow range of masses (about 1 amu) are stable in the shaded region. This ratio can be scanned to change the value of m/z stabilities and by this way a quad can act as an MS scanner, allowing each every m/z collection of ions to pass the quad and be collected and measured with a detector. It is also important to note that increasing “a”, is necessary for creating This also explains the way in which a quadrupole may act as an ion focusing element, given all ions the ability to pass. If no DC potential is applied, and therefore no “a” value applied, then all mass-to-charge values will be stable up to the upper limit of the “q” value of the quadrupole and therefore m/z differences will not affect stability within the quad and all ions will oscillate under the rf applied and eventually pass through the quad. This is why a quadrupole operating without any isolation is referred to as being in “rf-only” mode.
1.3.2. Linear and 3-D Ion Traps

An ion trap follows very similar physical characteristics as that of the standard quadrupole mass filter. In the case of the 3D ion trap, shown in Figure 1.7a, the process of ion manipulation can be likened to a quad wrapped in on itself. The device consists of one large “donut” shaped center electrode and two end-caps on either end with the only way in and out of the trap region through the end caps. The linear ion trap, shown in Figure 1.7b is a more modern design allowing for a higher amount of ions in the trap cell and with off axis detectors to allow. In Chapters 4, 5, and 6, the linear ion trap was utilized in a dual cell design in the Velos Pro, which uses one cell as a low pressure ejection/detection cell and the other as a high pressure fragmentation/isolation cell.43
Figure 1.7 a) A cut-away section schematic of the basic 3-D ion trap design. The design employs a single cylindrical electrode with two end-caps that act to collect and cool ions in the cavity and then with changing of the AC voltage applied, eject mass-to-charge ions of interest from the end-cap. This portion of the figure was reprinted with permission from the American Chemical Society.41 b) A cartoon schematic detailing the end cap and inner cell of a linear ion trap design. The cylindrical pieces on the top and bottom are detectors used for off-axis detection. Ions can also be ejected down the axis to be submitted to more chemical analysis or alternative MS analysis after isolation and fragmentation.

These devices are able to collect all ions from the ionization source and then subsequently isolated one mass in particular, fragment it, and then isolate the fragments of that and continue this process and in so doing allow for an MSⁿ analysis of an analyte of interest. To perform this trapping and isolation, ion traps employ an rf-only method of MS detection which can be explained in the Matheiu stability diagram in Figure 1.8 below. In this device since there is no DC potential applied, the ions made stable in the Matheiu diagram are ejected from the trap by applying AC waveforms that are able eject mass both above and below the ion of interest to be isolated. The only issue with this waveform application is that it shifts the q value, or maximum rf amplitude applied to the device, and results in minimum measurement of the fragments collected in an MSMS experiment by a percentage of the mass of the ion selected (~1/3). Even so, ion traps offer highly stable and sensitive mass spectrometers that are able to probe the activation chemistry of small molecular systems over several iterations using MSⁿ and are
invaluable in performing MS-based structural techniques (the details of which are discussed below in section 1.5), due to their effective trapping ability.

![Extended Mathieu diagram](image)

**Figure 1.8** An extended Mathieu diagram showing the stability region in which ion traps operate. This figure was used with permission from the American Chemical Society.

1.3.3. Time-of-Fight mass analyzer

The time-of-flight mass analyzer makes use of the differences in velocity of ions of different mass-to-charge after being accelerated by a uniform electric field. In simple terms, a set of ions will travel at a constant speed after experiencing an accelerating force as long as they are in a field-free region for the duration of flight. The velocity of each ion is dependent on the initial accelerating voltage the ions experience as shown by the following equation:

\[ Kinetic\ Energy = \frac{mv^2}{2} = qE \]
where $m$ is the mass of the ion, $v$ is its velocity, $q$ is the charge, and $V$ is the voltage applied to induce acceleration. When this is simplified, taking into account that $v$ is distance/time the following expression is used, directly relating $m/z$ with time:

$$t = L \sqrt{\frac{m}{q}} \cdot \frac{1}{2eV}$$

This expression provides a direct relationship between the flight time of an ion and its mass-to-charge, with the only other terms necessary to account for are the accelerating voltage ($V$) and the tube length ($L$).\textsuperscript{44}

This device was dramatically improved, especially for ions of low $m/z$, with the introduction of the reflectron, developed in 1973 by Mamyrin et al and improved and applied to the TOF MS device by the work of Cotter et al.\textsuperscript{45} An illustration of this device implemented into a TOF analyzer is shown in Figure 1.9.\textsuperscript{33} The reflectron mode adds a second stage of electrodes placed directly in the path of the initial pulse of accelerated ions and turns them towards a secondary detector. Ideally, ions of the same $m/z$ should all experience the same initial accelerating voltage and therefore have the same kinetic energy at the start of a TOF pulse. In practical terms, however, this is never the case as ions may sit further away or closer to the plate and for those reason ions of the same mass will possess a range of velocities (i.e. internal kinetic energies) and reach the detector at different times, causing a poor mass resolution. The reflectron corrects for the spread in energies and so improves mass resolution by manipulating the ion path field so that all ions of the same $m/z$, regardless of kinetic energy, will reach the detector at the same time.\textsuperscript{45-47}
As the use of a reflectron improves ion resolution by allowing slower, less energetic ions, to “catch-up” to ions of higher kinetic energy, another method, delayed extraction, is a process that reduces the amount of kinetic method spread the ions experience before entering the drift tube. In delayed extraction, an initial pulse of the laser is separated at a set amount of time from the application of the accelerating potential in order to allow ions to equilibrate above the acceleration plate and compensate for kinetic energy variations. Furthermore, by applying a gradient voltage upon acceleration (i.e. a 10 kV acceleration voltage at the plate but a 7 kV voltage above the ion cloud at the gating optics), the variation between ions is even further reduced and the resolution of TOF analyzer has been dramatically improved using these features.
1.3.4. Fourier Transform Ion Cyclotron Resonance mass analyzer

The Fourier transform ion cyclotron resonance (ICR) mass analyzer is another common ion trapping type device allowing for very high resolution but requiring a high magnetic field for function. Unlike the quadrupole and ion traps which are mass filters and require a spatially distinct detector, and time-of-flight mass analyzers which use pulsed time information for m/z analysis and require an ion destroying detector as part of that process, the ICR uses the frequencies of the ions trapped in the cell itself by measuring a non-destructive “image current” after an excitation pulse induced the ions into “cyclotron” motion. A depiction of ion movement and a basic schematic of the simplest design of this ICR cell is shown in Figure 1.10.

Figure 1.10  Diagram of the motion of ions in excitation and detection modes (top) and basic instrumentation schematic (bottom) of the Fourier Transform ion cyclotron resonance (FT-ICR). This figure was reprinted with permission from John Wiley and Sons.48
To trap ions in an ICR device, the same focusing optics and use of end cap electrodes are employed as that used for 3D and linear ion traps. The detection scheme, however, relies on the physical description of Lorentzian force induced by the magnetic field surrounding the cell, which can be explained by the following equation:

\[ m \frac{v_c^2}{r} = qv_cB_0 \]

where force is mass x acceleration, \( r \) is the radius of the circular motion induced in the magnetic field, \( v_c \) is the velocity of the motion, \( q \) the charge of the ion, and \( B_0 \) the strength of the magnetic field around the cell. If we put this expression into the terms of angular momentum, which define the frequency of motion, where

\[ w_c = \frac{v_c}{r} \]

then the previous Lorentz force expression can be simplified to

\[ w_c = \frac{qB_0}{m} \]

where \( m/z \) is defined as the following:

\[ \frac{m}{q} = \frac{B_0}{\omega_c} \]

In an ICR cell, therefore, each \( m/z \) ion will have its own angular frequency and in a collection of ions, the signal for each frequency can be determined by Fourier transforming the image currency collected from the detection plate.

Since the cloud of ions induced into cyclotron motion ion inside the ICR trap, there is no ion destruction, and additionally, no theoretical limit to how long the ions can travel in this detection motion. The limitation on how long a set of ion can be excited and detected in a cell is only one part of the resolution issues in Fourier transform ICR experiments, as the magnetic field strength also influences the ability to distinguish ion flights, but even in the early stage low-magnetic field (~5.6 Tesla) technology, ICR cells were able to achieve resolutions of 100,000.\(^{48}\) New designs making use of optimized cell
structure and magnetic fields of up to 21 T have increased this MS resolution to over 60,000 for up to 2000 m/z in practical experiments.\textsuperscript{49,50}

1.4. Gas-phase Collision-induced dissociation of small molecular analytes

After ionization, the ability to input additional energy into the ion cloud is of great importance in order for complete identification of an analyte of interest. An unknown sample can be ionized and its precursor mass-to-charge ratio determined via a mass analyzer, but without the ability to also break a precursor into its most basic parts the full structural character of a sample will be impossible to determine by mass spectrometry. As discussed above, the use of early ionization techniques, in particular with EI, provided the needed energy at source for this fragmentation “fingerprint” of a small analyte of interest, but with gentler methods, the energy input needed to be introduced within the vacuum region.

Collision-induced dissociation is the process by which ions are subjected to a cloud of neutral gas molecules (N\textsubscript{2}, He, Ar, and Xe) and is the most common method of dissociation in MS studies.\textsuperscript{11,29,51,52} It was also an actively used process to unravel the fragmentation chemistry of peptide ions, cancer drug ions, and carotenoid molecules in the work presented here. A description of how this method of dissociation applies to these systems is described here.

1.4.1. CID of peptides

The gas-phase fragmentation of peptide fragments has been a topic of extensive research for several years and it of crucial importance in the field of proteomics, as this one of the most common methods of identifying proteins in biological samples.\textsuperscript{53-55} For that reason, a great deal of work has gone into studying how and for what reasons peptides fragment the way they do in the gas phase. Figure 1.11 shows an I-form
pentapeptide and details the nomenclature used for the fragmentation of a peptide along its backbone. As CID is the most common method for dissociation of peptides in standard proteomics studies the highest proportion of fragments observed in these experiments are b and y ions, which as the figure shows are labeled in red and are formed from the breaking of the backbone at the peptide bond.\textsuperscript{53,56,57} The denotation of b and y describe whether the charge is retained on the N-terminal side of the fragmented bond (b-ions) or whether it is retained on the C-terminal side (y-ions). The subscript after the denoted ion type is a number that describes the number of amino acid residues belonging to that particular fragment, as for example, in the case of the third peptide bond dissociation in Figure 1.11, the b-ion formed will be the “b\textsubscript{3}” since the fragment will retain amino acid residues R1, R2 and R3, while the y-ion will be the “y\textsubscript{2}” ion of this model peptide since it only retains the R4 and R5 amino acids.

Figure 1.11  A standard l-form pentapeptide labeled at the backbone covalent bonds for the formation of a, b, and c-ions (where charge is retained on the N-terminus) and the x, y, and z-ions (where charge is retained on the C-terminus). The number subscripted at each ion refers to the number of R-groups retained on the product ion, as for example, at the third peptide bond of a pentapeptide the b ion will retain three residues (b\textsubscript{3}) and the y ion will only have two residues (y\textsubscript{2}), as shown in the red label above.
A great deal of work has been devoted to understanding the mechanism of formation in b and y-ion formation with emphasis on the structures formed for the b-ion.\textsuperscript{58-61} This is of particular importance in the field of peptide fragmentation studies as the b-ion must adopt a structure to compensate for the loss of the OH group of standard peptide ions. In the case of the y-ion, the fragment structure will resemble that of a truncated form of the precursor peptide it was formed from while b-ions will form the most likely structures inherent in the mechanism with which the fragment formed. For example, in Scheme 1.1, the $b_3$ and $y_2$ ions are shown as being formed from the nucleophilic attack of the carbonyl oxygen of the second amino acid residue on the carbonyl carbon of the third residue. This is a very common mechanism of formation in CID to form either the truncated $y_2$ or the five-member oxazolone ring $b_3$, the most common structure of b-ions observed in the gas phase. There is the alternative however, not shown in this scheme, of the nucleophilic attack taking place by the N-terminal amine, which would still result in the same truncated $y$-ion but result in a 9-membered macrocyclic ring $b_3$ ion. Therefore, by identifying the structure of the resulting b-ions in peptide fragmentations can typical provide direct information as the mechanism that produced fragmentation.
Scheme 1.1  Formation of the $b_3$ ion and $y_2$ neutral upon CID and cleavage of the third peptide bond of a pentapeptide. The $y_2$-ion can subsequently be formed via the formation of a proton transfer from the oxazolone ring of the $b_3$ to the N-terminus of the $y_2$.\textsuperscript{62}

Originally, b ions were postulated to be acylium ion structures, until in 1995 use tandem MS results, model system substitution, and theoretical calculation, it was concluded that the most stable b-ion species would be the oxazolone.\textsuperscript{58} Since then, cases have arisen where the most stable species have been other than the oxazolone and in each of these cases the chemical nature of the amino acid residues have been found to alter the stability or accessibility of the oxazolone pathway.\textsuperscript{63-67} This is most commonly the case of the $b_2$ ion, where carbonyl attack from the N-terminal amine will result in a six-membered diketopiperazinone structure, of absolute relative stability lower
than that of the corresponding oxazolone, yet more difficult to access in pathways due to the higher barrier of activation in forming the starting conformations (i.e. cis peptide bond formation).\textsuperscript{68,69}

The ability to gain more information about the gas-phase mechanisms of peptides using the structure of the b-ion, and its subsequent fragment, the a-ion, formed from the loss of a C=O from the b-ion \textsuperscript{70} is the central focus in the work presented in Chapters 3 and 4.

1.4.2. CID of biologically active small molecules

The CID of non-peptide small molecules including drugs and their metabolites, hydrocarbons and radical ions was an important focus in the work presented in Chapter 5 and 6, respectively. It is for this reason necessary to outline how previous studies have played a role in informing the work in this dissertation with regard to fragmentation of these species.

While not as well established as peptide fragmentation via CID, the metabolic pathways of drugs and biologically important natural products are an important part of pharmokinetics and metabolomics.\textsuperscript{71} The difficulties with generalizing this aspect of CID fragmentation is that unlike peptides, which have a uniform chemistry pattern being essentially a polymeric chain of amides, non-peptide drugs and natural products are incredibly diverse and are not set to particular patterns in MSMS patterns. The main method of analysis in metabolomics and investigations of small biologically relevant molecules is LC separation followed by fragmentation to determine trace analysis quantification of species of interest. Each biologically relevant species is dissociated using CID to determine the best transitions with which an ion precursor can be quantified.\textsuperscript{72-74} For this reason, there is no well characterized pattern by which either the cancer drugs or the carotenoids could be modeled that match the fragmentation pattern
work of peptides as described above in section . There has, however, been previous work in identifying the common fragments in some of these systems. In work by Thevis et al. similar drug compounds to the thalidomide drug compounds (Chapter 5) were studied by ESI and their fragmentation schemes proposed.\textsuperscript{75} Likewise, the carotenoids carotene lycopene (Chapter 6) have been previously studied and their MSMS fragmentations been determined by the work of VanBreeman et al.\textsuperscript{76}

1.5. Techniques coupled to MS for structural probing

Mass determinations and formation of principal fragments will not allow for complete characterization of any ion of interest. Ions can adopt different protonation sites, dissociate into fragments of different stabilities, and possess various conformations. To better determine the types of structures, both lower and higher order, a number of chemical and physical techniques have been developed. The set of ion probing methods utilized in the studies of this dissertation are described here.

1.5.1. Action infrared multi-photon dissociation (IRMPD) for vibrational structure information

Action IRMPD spectroscopy is a powerful technique that makes use of MS detection in order to probe the vibrational chemistry of fragment ions post ionization. The method, as is diagramed in Figure 1.12, is carried out by first ionizing and trapping analyte ions of interest through some trap MS device (3-D/linear ion trap or ICR cell). A variable wavelength laser is then focused into the cavity occupied by the ions.\textsuperscript{77} Often, optical parametric oscillator/amplifier (OPO/OPA)\textsuperscript{78,79} lasers are used for gas-phase spectroscopy,\textsuperscript{80,81} but in the case of IRMPD, free-electron lasers (FEL) are the most best choice of equipment as high photon density in the 1000-2000 cm\textsuperscript{-1} region is ideal and these a complex and high energy photon sources offer the best option given the low
cross-section and density of ions within ion trapping devices. Free-electron lasers (FEL) are synchrotron based light emitters making use of high field magnets and a high energy electron gun to induce laser wavelengths that provide a wide range of wavelengths with fine-tuning and the specifics of which are outside the scope of this introduction.\textsuperscript{82,83}

To achieve vibrational information, the laser is focused in the ion cloud region of the trapping device and is scanned incrementally within a set time period.\textsuperscript{84-86} Through manipulation of pulse timing, the trapped precursor ion being probed is collected in the cell, the laser focused onto the cell for an extended period of time (~5ms), and the ions in the cell after firing the laser scanned and detected.\textsuperscript{79} As the laser energy varies, the photons are either absorbed, if the wavelength matches a vibrational mode of the ion molecule, or not if the wavelength does not excite a vibrational mode of the molecule. If vibrational absorption occurs, intramolecular vibrational relaxation (IVR) redistributes the energy immediately, bringing up the total internal energy of the molecule and this process of photon absorption occurs multiple times until the internal energy reaches a pathway of dissociation and fragmentation occurs.\textsuperscript{79,87} The process of IVR means that the vibrational mode being excited at any time will be independent of the pathways to form fragment ions and that as the laser is tuned, a plot can be generated of incident light wavelength and fragments formed.\textsuperscript{79} The fragment ion signal produced is normalized into fragmentation efficiency which follows the equation,

\[
\text{frag. eff.} = \frac{\Sigma(I_{\text{fragments}})}{\Sigma(I_{\text{fragments+precursor}})}
\]

which becomes the y-axis of the vibrational spectrum for ions probed via this method.\textsuperscript{79}
Figure 1.12 Cartoon flowchart depicting the procedure of producing vibrational spectra via action-IRMPD.

This technique has been used for a number of years to determine the chemistry of precursors and fragment ions after by collision-induced dissociation. Especially noteworthy is the use of this process in the exploration of peptide fragmentation identities performed in the Wysocki group.\textsuperscript{68,88-90} There has also been work on other organic molecules other than peptide fragments using action IRMPD spectroscopy, such as work by Crampton et al. that has provided ion structures of a series of halouracils.\textsuperscript{91} The IRMPD data showed that each halouracil existed in unexpected tautomeric forms in the gas phase. The authors concluded that these were non-canonical nucleosides and that these forms may also exist in solution. This would lead to a primary cause for disruption of DNA transcription and replication by these molecules.\textsuperscript{91} All of these studies make use of the 1000-2000 cm\textsuperscript{-1} region, which has an advantage in identifying the C=O and C=N amide stretches prevalent in peptide derived structures, nucleic acids,
and many biologically active molecules. This technique is therefore an option for investigating the ion chemistry and a wide field of small molecular structures and their fragments.

The work in this dissertation has made use of action IRMPD on peptide fragment ions in Chapter 4 and also on precursor cancer drug molecules in Chapter 5, which was performed at the Centre Laser Infrarouge d’Orsay (CLIO), a facility possessing a free electron laser (FEL) there tuned to the energy range of 1000 cm\(^{-1}\) to 2000 cm\(^{-1}\) and paired to a Bruker Esquire Ion Trap 3000+.

1.5.2. Fundamentals of Ion Mobility for shape and size of small molecular systems

Ion mobility can be described as a “gas-phase electrophoresis”, where ions are drawn through a drift cell with a high pressure of neutral bath gas under the force of an electric field to separate them by their size, shape, and charge. The simplest expression of a standard linear drift time, which defines the time is takes for an ion to travel the full length \(L\) of the cell is define by the following:

\[
t = \frac{L}{v} = \frac{L}{KE}
\]

where ions travelling at velocity, \(v\), will travel to travel the length \(L\) of the drift cell in time \(t\), while the second equivalent expression show that in order words the time is inversely dependent on the mobility of the ion, \(K\), and the strength of the electric field, \(E\).\(^{92}\) This is an expression, however, that only holds true in a low field strength where the \(E/N\) (where \(N = \text{gasser gas density}\) is less than \(6\times10^{-7}\) V\(\) cm\(^{-2}\) for singly charged ions in a helium drift cell at 300 K since at higher electric fields, the ions will become more dependent on \(E\), complicating this simple expression and reducing the usability of this expression for exactly calculating mobility.\(^{92,93}\) The mobility value \(K\) from the previous expression can also be expressed with the following formula:
where $z$ is the charge state of the ion, $e$ is the elementary charge, $N$ is Avogadro’s number, $\mu$ is the reduced mass of the ion-drift gas pair, $k_B$ is Boltzmann’s constant, $T$ is the temperature of the cell, and $\Omega$ is the collision-cross section (CCS). The collision cross-section value $\Omega$ is at an inverse relationship with mobility meaning that ions of larger size or shape will travel slower in the drift cell. Additionally, molecules of double and triple charge will move at directly increased speeds. Likewise, changing the bath gas (i.e. from He to N$_2$) will affect the reduced mass value changing the mobility of the ion and additionally, changing of the gas will also affect CCS, as the area of an ion will increase relative to the size of the gas molecules it collides with in the cell.

To carry out ion mobility spectroscopy, a number of designs have been employed, and Figure 1.13 highlights the basic designs for the three most common forms of ion mobility cells. All three have been coupled with MS analysis to provide a second dimension of analysis of ions. The first labeled “LDT IM-MS” is the standard linear drift tube where a stacked series of ring electrodes are used to form the drift cell and each electrode ring has a set DC value that guides the ions from the front of the cell to the end. In this design, since the electrodes are well defined and constant, the previous expression ($t=L/v$) can be used to directly determine the mobility if all physical parameters are known, and from that the CCS value can be determined for each ion, which is a direct area measurement inherent in all ions travelling in space, no matter what the conditions. The standard drift tube has the advantage of providing the best resolution of the IMS cells and as the design is simple and uniform, the cells can be stacked to very long lengths of up to one meter or even set up into a circular cell to allow multiple passes with up to ~200 meter drifts of IM separation.
The second type of IMS cell, the transfer wave ion guide (TWIG), was used in the ion mobility experiments of this dissertation in Chapters 4, 5, and 6. Unlike the standard drift cell, which uses constant DC applied to the set of ring electrodes in series, the TWIG cell induces a voltage “wave” with the ability of applying DC voltage to individual electrodes in the ion guide. A wave is produced by sequentially increasing than decreasing each ring of the ion guide, and the “wave” can be controlled by allowing both the maximum amplitude of the voltage and a velocity with which the voltage changes over time to be set. Ions are pushed through the cell by “riding” this wave of DC potential and with fast wave speed ions of low mobility will be pushed over the wave and be thus separated from higher mobility ions. The advantage of the TWIG design is that if provides higher sensitivity than the linear drift cell. By using a variable electric field, however, the initial expression of ion mobility may not be used to directly determine CCS values and therefore all CCS values must be derived from known CCS calibration protocols.
1.5.3. Hydrogen-deuterium exchange in the gas-phase for small molecule structural probing

The process of hydrogen-deuterium exchange (HDX) consists of incubating a target analyte with a solvent or gas that is “deuterated” in that all of its available hydrogens are replaced with deuterium, and allowing the compound to freely exchange its’ hydrogens with the available deuterium in a controlled way so as to examine chemical nature of its hydrogen system and from inference determine information about the structure of the analyte.\textsuperscript{100,101} In this dissertation, H-D exchange was performed with deuterated ND\textsubscript{3} and as such, all HDX experiments were performed in the gas phase within an ion trap cell.

In solution-phase HDX, exchange can happen very rapidly for small molecules, and typically this phase of exchange is performed before MS analysis of proteins in order to
determine solvent accessible regions of a protein or protein-ligand bound complex. This process consists of placing a solution of analyte in a solution with D₂O, CD₃OD, or other liquid deuterating reagent for a set period of time (seconds up to hours) and then quenching the reaction by lowering the pH from 7 to ~2 and bringing the temperature down to 0 C. Only after HDX and quenching has taken place would the deuterated compound be sprayed and MS is carried out in order to determine the extent of deuteration. Gas-phase HDX requires the ability to leak a deuterated gas (ND₃, CD₃OD, D₂O) into an ion trap or ICR cell and letting the analyte ions accumulate and remain with the gas for set periods of time of milliseconds up to the order of minutes. With different deuterating reagents, the process by which exchange occurs are guided by different mechanisms.¹⁰²,¹⁰³ These mechanisms, determined by observations of exchange patterns in polypeptides, help explain the variable strengths of exchange between deuterating reagents (with D₂O being one of the weakest). In short, the mechanisms include involvement of the N-terminus basic amine and C-terminal carboxylic acid hydrogen-bond donator to create complex and proton transfers between function groups and include the “relay” mechanism, salt-bridge mechanism, “flip-flop” mechanism, and “tautomer” mechanism. Additionally, direct exchange can occur with better deuterating reagents such as ND₃, which explains the better exchange characteristics of this reagent.¹⁰³

The opposing mechanisms of exchange detail the way in which H-D exchange occurs, but in principle the nature of available hydrogens does not shift dramatically from changing the type of reagent. In general, available hydrogens are those that provide the charge by protonation, available basic sites, and hydrogens that are readily exposed if not quite basic. The available exchangeable hydrogens on a standard peptide for example, would be the protonating hydrogen, the three hydrogens on the N-terminal amine, the readily accessible C-terminal carboxylic acid hydrogen, and the hydrogens of
any basic residues (i.e. Lysine, Arginine, Histidine, etc.). The possibility of carbon hydrogen exchange has also been shown, however, and work by Morrison et al. detailed a case in peptide exchange where this exchange was present.¹⁰⁴

What is significant in the monitoring of exchange for these and other functional groups is that each type of group of hydrogens will have a different rate of exchange. For this reason if two isobaric structures have different positions for their hydrogen-containing functional groups, they will end up separating into a “faster” exchanging and “slower” exchanging population and therefore mixtures of structures can be discerned using gas-phase HDX. Differentiation of small molecule ions has been shown in several studies and in each variations in exchange time have led to separation of conformations and also determination of functional group chemistry structure.¹⁰⁵,¹⁰⁶
2.1. Peptide preparation methods (Chapters 3 and 4)
2.1.1. Fmoc Solid-Phase Peptide Synthesis

All peptides were synthesized using standard Fmoc-solid phase synthesis described in detail elsewhere. Briefly, this process involves using a Wang resin-attached attached to amino acid residue on its C-terminal OH link which is protected by Fmoc (fluorenylmethyloxycarbonyl chloride) on its N-terminus that is deprotected in a piperidine (Sigma, MO) solution to remove the N-linked Fmoc group followed by coupling to the next Fmoc-protected amino acid residue in the sequence along with the coupling catalysts O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIEPA) (Sigma-Aldrich, MO). This process is repeated until all the desired amino acids are attached in order from C to N terminus, following which the entire peptide is cleaved from the resin bead using trifluoroacetic acid. After cleavage from the resin, diethyl ether (Sigma-Aldrich, MO) was added to the peptide solution and water added to extract the peptides from the ether. Each peptide was diluted to approximately 1:100 into H$_2$O: ACN with 0.1% formic acid to have a final concentration of approximately 10 to 50 μM. All solvents were purchased from Sigma-Aldrich and used without any further purification.

2.1.2. N-acetylation protocol

Acetylation of the N-terminus of synthesized and purchased peptides was performed in the work conducted in Chapter 3 and was based on a protocol developed through previous work in the Wysocki group. This method involved spinning down peptide
solution with a SpeedVac into a solid and dissolving 100 µg of the peptide into a solution of 50 µl methanol with 10 % acetic anhydride. The reaction was allowed to proceed at ambient temperature for 2 hrs and then the solution was diluted with electrospray solvents before MS analysis.

2.2. Drug and hydrocarbon analyte preparations
2.2.1. Pomalidomide and Lenalidomide preparation

Pomalidomide and lenalidomide were supplied in solid form by Dr. Ching-Shih Chen and Dr. Michael Phelps of the College of Pharmacy at the Ohio State University (Columbus, OH). Both molecules had low solubility in nano-pure water and were prepared in solutions of 50:50 H₂O:MeOH. Solutions were prepared at 1 mg/mL and diluted to 10 to 50 µM for further mass spectrometric and spectroscopic analysis.

2.2.2. Carotenoid sample preparation

Lycopene and α-carotene were provided by the food science research lab of Dr. Steven Schwartz at the Ohio State University (Columbus, OH). The third tetraterpenoid studied here, β-carotene, was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Solutions of 50 µM were prepared for each carotenoid with 50:50 MeOH:CHCl₃ for all mass spectrometry experiments detailed below.

2.3. Metal oxide experimental conditions
2.3.1. Preparation of CCA, Rhenium Oxide, and Tungsten Oxide solutions

WO₃ nanoparticles (nP) (< 100 nm), WO₃ microparticles (μP) (<20 µm), WO₂ μP (<150 µm), and ReO₃ powders were purchased from Sigma Aldrich. To make particle suspensions, 10 mg of each inorganic matrix was dispersed via sonication for approximately 60 minutes into 1 mL of nanopure H₂O purified in a Sartorius water
purifier (18.3 Ω•cm). Standards of pure cocaine, ametryn, glucose, choline, and a polyaromatic hydrocarbon (PAH) standard mix were purchased from Sigma-Aldrich and each serially diluted to final concentrations of 1 ng/μL (3.3 μM) in MeOH for cocaine, 1 ng/μL (4.4 μM) in H$_2$O for ametryn, 100 ng/μL (556 μM) glucose in H$_2$O, and 500 pg/μL (1.7 μM) of PAH standard. Some of these solutions were further diluted in H$_2$O to lower concentrations as noted below. To spot each sample, a 1:1 mixture of each inorganic particle solution and analyte solution was produced by mixing 4 μL of each and, after vortexing the suspension mixture, spotting 1 μL on a standard stainless steel MALDI plate.

2.3.2. Synthesis of the 3-methyl benzylpyridinium thermometer ion

The 3-methyl benzylpyridinium (BP) thermometer ions were synthesized using methods described previously.$^{109}$ 3-methyl benzyl chloride and pyridine, purchased from Sigma-Aldrich (St. Louis, MO), were mixed together at a 1 to 1.2 molar equivalent ratio for 12 hours at room temperature in dry acetonitrile (Fischer Scientific). The resulting benzylpyridinium salt was crashed out of the ACN solution with the addition of cold diethyl ether, filtered, and then washed with hexane. Stock solutions of 1 mg/mL in nanopure H$_2$O were made from the solid salt and diluted to a concentration of 100 ng/μL (543 μM) in H$_2$O, for spotting onto the MALDI plate.

2.4. Instrument Conditions

2.4.1. Velos Pro coupled with HDX and Kinetic Method Experiments

To perform H-D exchange experiments, the Velos Pro ion trap was modified with a deuterated ammonia line at a T-valve that allowed the ND$_3$ gas to mix with the helium going into the trap and to leak directly into the high pressure region of the trap. After a full MS scan for each spectrum, the intact precursor was isolated in MSMS mode and
the accumulation time of the MSMS scan could be adjusted to allow for varying amounts of exchange with the ND₃ gas in the high pressure region of the dual linear ion trap cell. Precursor ions as well as fragment ions were incubated from period of 10 ms, the shortest accumulation time which would result in no exchange, up to 10 seconds for a large degree of H-D exchange. In order to perform HDX on the fragments, MS³ was necessary, with accumulation times increased for exchange and an appropriate collision energy (approximately 10-20 % collision energy) applied in the MSMS mode to produce the fragment before exchange. Thermo uses % collision energy rather than a simple collision energy defined by acceleration vs charge state because they make a correction/adjustment for m/z of precursor, which provides only a maximum CE for each isolation that must be normalized to 100 %.

Kinetic method experiments, designed to estimate proton affinities (PA) of compounds of unknown PA, were also performed with the Velos Pro dual linear ion trap. The reference compounds chosen for this particular experiment were a set of amino acids with a range of proton affinities from 905.9 kJ/mol to 933.3 kJ/mol. Those chosen included serine, isoleucine, threonine, tyrosine, proline, and asparagine; their corresponding proton affinities are shown in Table 5.1, as calculated by Afonso et al. Reference bases were dissolved in solutions of 50:50 H₂O:MeOH with 0.1% formic acid to solutions of ~10 μM and mixed 1:1 with pomalidomide and lenalidomide prepared at matching concentration and solution conditions. The 1:1 analyte:reference base solutions were ionized using standard electrospray utilizing a capillary voltage of 4.5 kV and the proton bound heterodimers (A---H+-B) isolated in MSMS mode. For example, in the case of (Len+Ser)⁺, with MW’s of 259 g/mol for lenalidomide (Len) and 105 g/mol for serine, the heterodimer mass isolated was 105+259+1 at m/z 365. To collect the necessary data, the heterodimers isolated at a window of m/z 1 in MSMS mode were then dissociated at incrementally increasing activation amplitudes starting at 0 % and
going up to 50 % at steps of 0.5 to 1 %. At all collision energies, the intensities of the heterodimer precursor, protonated drug analyte, and protonated reference base were monitored and recorded and the ratios of these values used to produce the kinetic method plots below in section 5.3.5.

2.4.2. Bruker Apex ICR SORI CID

Additional experiments were performed in a Bruker APEX Qe 9.4 T FTICR (Bremen, Germany). MS$^3$ was achieved through fragmentation of the peptide ions in source, isolation of the investigated fragment ion in the ICR cell, and inducing further fragmentation by SORI-CID with no collision cell CID beforehand. All precursor peptides were ionized via electrospray ionization at 4.8 kV. SORI-CID was performed in the ICR cell after source fragmentation, where activation percentages of SORI stayed at 1.2 - 1.5 % of their maximum excitation with the length of each SORI pulse set at 0.3 sec. Quadrupole isolation was not performed on precursors or to form fragments, as all precursors and fragments were isolated in the ICR after in-source CID. Source CID was performed due to low sensitivity of some fragment ions, despite the fact that selectivity in quad selection, followed by CID before the cell, would have resulted in more selectivity of the fragment ions collected.

2.4.3. Bruker Esquire 3000+ with action IRMPD

Action IRMPD was performed at the Centre Laser Infrarouge d'Orsay (CLIO) using a free electron laser set up and a Bruker Esquire 3000+ 3-D ion trap with an electrospray ionization source (need to find). Maitre, Ortega, and co-workers have described the process in detail.$^{84-86}$ Briefly, the ion trap was used to first isolate precursors and either carry out IRMPD on those or perform i CID in the ion trap to produce selected fragment ions, before IRMPD. Subsequently, isolation of the $b_2$ ion was performed followed by irradiation of the trapped $b_2$ fragment ion by the FEL at 4-10 cm$^{-1}$ steps from 1000-2000
cm$^{-1}$ with averaging of the fragmentation spectra at each energy step of the laser. The IRMPD spectra were produced by plotting the intensity of all $b_2$ ion fragments over the intensity of residual $b_2$ ion precursor at each wavenumber. The laser output consists of macropulses of 9 $\mu$s each at a repetition rate of 25 Hz. These macropulses consist of approximately 600 micropulses at 0.5-3 ps in length, spaced out at gaps of 16 ns. With laser powers of 0.5-1 W per micropulse, the total amount of energy in each pulse comes out to about 30-100 $\mu$J with dependence on the wavelength and exact power of the laser.$^{115}$

2.4.4. Waters Synapt G2/G2S with Ion mobility and CCS Calibration

Ion mobility experiments were performed on a Waters Synapt G2S QTOF mass spectrometer (Figure 2.1) and samples were introduced with the aqueous solutions described previously in Sections 2.1 and 2.2, unless otherwise stated for peptide solutions. Standard ESI was performed at a capillary voltage of 2.5 kV. Fragment ions were produced in the trap region (TWIG type as described in section 1.5.2) at collision energies of 5 to 25 eV in order to generate structures that represent a range of possible activation barriers for each tripeptide system. Helium gas flow and ion mobility gas flow ($N_2$) were set to 180 and 90 mL/min, respectively. In order to calibrate the drift time region, a mixture of drug calibrants including ondansetron, clozapine N-oxide, colchicine, verapamil, and reserpine, defined specifically for $N_2$ gas IMS experiments in the Waters G2S by the Campunzano et al. was used.$^{116}$ The precursor and fragment ions optimized as described in the paragraph below were taken for MOBCAL algorithm described below in Section 2.5.2 to determine theoretical collisional cross sections to compare to those found from Synapt IM-MS.

For the cancer drug molecules ion mobility experiments were performed with standard ESI at a capillary voltage of 3.0 kV. Fragment ions were produced in the trap
region at trap collision energies of 5 to 20 eV. Helium gas flow and ion mobility gas flow, which was changed to CO$_2$ to improve the ion mobility separations of such small molecules, were set to 120 and 75 mL/min, while the wave height was set to 35.0 V and wave velocity to 1000 m/s. CCS MOBCAL calculations were not performed nor were polyalanine or drug calibration as it is difficult to compare MOBCAL to CO$_2$ based drift time calibrations.

Figure 2.1 Schematic of the Waters Synapt G2 mass spectrometer with ion mobility cell in the center of the Tri-Wave region. Image is provided courtesy Mowei Zhou.\textsuperscript{117}

Ion mobility-mass spectrometry (IM-MS) of the carotenoids experiments were performed with standard ESI at a capillary voltage of 3.0-4.0 kV with high voltages required for the MeOH: CHCl$_3$ spray solvents used and the high ionization energy of the carotenoids under study. Unlike most analytes investigated in this dissertation, the carotenoids are not protonated in ESI and instead radical cations are produced, which changes the experimental considerations regarding the MS conditions. Fragmentation was produced in the trap CID region at collision energies of 5 to 20 eV. Helium gas flow and ion mobility gas flow (N$_2$) were set to 120 and 75 mL/min, while the wave height was set to 35.0 V and wave velocity to 1000 m/s. Helium CCS are reported and compared to
the experimental values found by calibrating with a mixture of d/l-polyalanine and using the CCS values found for this mixture by the Clemmer group who used a linear drift cell.\textsuperscript{118,119} The drug calibrant mixture used to determine N\textsubscript{2} derived CCS for Chapter 4 was not used in this case as the range of CCS for all drug molecules was too low to cover the extended (larger CCS) linear carotenoid molecules. Polyalanine, however, fits this range adequately and was therefore used for all CCS values presented for carotenoids.

In order to attain the excitation conditions in the high pressure StepWave region of the instrument (as applied in Figure 6.4), the RF control was set to automatic settings, with the initial StepWave section’s wave velocity set to 300.0 m/s, entering wave height set to 10.0 V, ending height set to 1.0 V, and final StepWave section set to a wave velocity of 300.0 m/s and 0.0 V. The StepWave Transfer offset was set to 25 V, the first differential aperture set to 3.0 V, second to 0.0 V and the StepWave RF offset 1 set to 300.0 V and StepWave RF offset 2 set to 350.0 V. To achieve “softer” StepWave conditions, the StepWave was set to equivalent wave heights and velocities, but the StepWave Transfer offset was set to 10 V and the two differential apertures to 5.0 V each.

2.4.5. Bruker UltraFlextreme MALDI-TOF/TOF LDI conditions

Spectra were collected on a Bruker “ultrafleXtreme” MALDI-MS operated in reflectron mode with a mass range set from m/z 20 to 900. This system uses a Smartbeam\textsuperscript{TM} laser which produces a focused beam at 355 nm. The laser settings were maintained at a frequency of 20 Hz and in each run 500 shots were collected. Within this subrange of 20 to 45% of the total laser flux, relative percentages of 20 to 45% were selected to produce all spectra.[flexControl 3.0 User Manual] The laser was manually rastered
along the area of the spot, in each case, to sample for any potential hot spots and test whether the edge or middle of the spot was a better source of analyte ions.

2.5. DFT and MOBCAL Calculations
2.5.1. DFT Calculations: optimization, relative energy determination, and frequency

To calculate energies of structures for the peptides, drugs, and hydrocarbons requiring theoretical approaches, the B3LYP/6-31+G(d,p) level was used (unless otherwise stated) with the Gaussian 09 program.\textsuperscript{120} Stepwise calculations going from lower theoretical levels to higher allowed for faster calculation speeds at each theoretical level and the removal of redundant structures in Chapter 3, where many iterations of calculations were necessary given the large number of structure variations tested. The final reported energy values were determined at the B3LYP/6-31+G(d,p) level, zero-point energy and other thermochemical corrections were obtained from vibrational frequencies calculated at the B3LYP/6-31G(d) level. Relative Gibbs free energies were calculated at 298K.

Maestro (version 9.1) was used to build the starting structures for all fragment ions.\textsuperscript{121} In order to generate a number of starting structures for further quantum chemical calculations, a conformation search was performed with a Monte Carlo mixture model (MCMM) using the Merck molecular force field MMFF with the torsional sampling option from the Schrodinger software program MacroModel.\textsuperscript{122} The MCMM conformational method is an algorithm that explores geometries generated by randomly changing torsion angles which are then energy optimized and either considered a valid structure or not based on a number of parameters.\textsuperscript{123} This process is repeated a specified number of times based on user preference. Here, 10000 structures were generated with 2500 steps per rotatable bond. Of the 10000 structures generated, a cut-off of 1.0-2.0 Å atom deviation was set up and redundant structures were removed.
Between 10 and 20 starting structures, selected based on representative hydrogen bonding and torsional features, were chosen from the 100-200 minimized structures to be run at the PM3 and higher optimizations

2.5.2. MOBCAL and collision cross section approximations

MOBCAL is a program developed by Jarrold et al. that uses the coordinate files of a molecular ion to calculate the CCS. It is used to directly compare the experiment CCS found from the TWIG IM results described previous (Section…). Three separate approaches are performed when with each coordinate submission: the projection approximation (PA), the exact hard-sphere (EHS), and the trajectory method (TJM). The PA approach provides the quickest and simplest method for calculating CCS in that it considering the absolute area that a molecule and can be treated like the 2-D “shadow” or projection that the molecule would cast if it was rotationally averaged in all possible orientations of space. The downside of the PA approach is that it underestimates CCS for molecules with cavities and ignores the physical interactions that analyte and bath gas molecules would experience in collisions during an actual experiment. The EHS method does consider these interactions, but does so by simulating the colliding ions and neutral gas as spheres with two concrete options during a calculation, collision or no collision. The slowest method, but most accurate of the three is the trajectory method, which also simulates actual collisions, but instead of using an algorithm of hit-or-miss, the method also considers the Lennard-Jones interaction potentials so that bath gas and ion molecules can experience attractive and repulsive forces without even colliding. As TJM is most computational expensive, it works most effectively for small molecules, which were fortunately the focus of all the studies in this dissertation.

MOBCAL input files were collected using the optimized structures produced with conformational searching and Gaussian DFT. DFT was needed in order to input
Mulliken partial charge values into the .mfc files needed for the MOBCAL CCS determinations. MOBCAL conditions were used matching the standard helium CCS inputs produced by the Jarrold group\textsuperscript{125,127} as well as N\textsubscript{2} conditions developed by Campuzano \textit{et al}.\textsuperscript{116} as experimental drift times were calculated in the Synapt G2/G2S using N\textsubscript{2}. In the current iteration of the software both N\textsubscript{2} and He bath gas options can be calculated, and in the Chapters using MOBCAL, the trajectory method in both gas options were used.
3.1. Introduction

A great deal of research has focused on understanding how gas-phase peptide ions fragment upon activation in collision induced dissociation (CID). Studies on the structures and reactivities of peptide fragments are of great importance due to the widespread use of tandem mass spectrometry (MS/MS) for the identification of proteins in proteomics. In proteomic analyses, proteins are first broken down into peptides by enzymatic digestion generating a mixture of peptides. This mixture is then separated by HPLC and subsequently analyzed by automated MS/MS. Peptide ions are typically subjected to CID, where energy is imparted into the ion by collisions with a neutral gas and the ion then fragments producing product ion spectra. CID predominantly forms fragments by breaking the peptide bonds, with the resulting fragments denoted as b ions (when the N-terminal half is observed) or y ions (complementary C-terminal half), with the former often fragmenting further to a ion fragments. The information encoded in the observed mass-to-charge ratios is then used to decipher sequences, frequently by automated bioinformatics tools. Most software assumes an even distribution of cleavage probabilities for the amide bonds and will compare such theoretical spectra, or even simple m/z lists with little or no intensity information, to experimental fragmentation patterns to identify the peptide sequence. As actual spectra are influenced by the placement of certain amino acid residues that will shift the likelihood of peptide bond cleavages, there is the possibility of misidentification of
peptides when this change in peptide bond cleavage probability is not considered. Recent research efforts in the gas-phase ion chemistry community are aimed at the greater understanding of why certain peptide fragments are formed with greater frequency, and elucidation of the mechanisms which guide this fragmentation. It has been proposed that this information can be used to improve the current sequencing/identification software and therefore could lead to improved data-processing in large-scale proteomics projects.

While recent studies have shed light on the fine details of the formation, structures, and reactivities of b-ion fragments, less is known about a-ion fragments. It is generally accepted that a_n ions are produced via CO loss from the oxazolone isomer of the b_n fragment ions, as proposed by Harrison and co-workers. The resulting imine isomer (\(-\text{CO-HN+=CH-R}_n\)) has a linear backbone; e.g., CO is eliminated from the C-terminal oxazolone ring of the b_n ions in the b_n \(\rightarrow\) a_n reaction. The imine isomer a_n ions are reactive and can undergo a variety of rearrangement and fragmentation reactions. In order to determine their most stable structures, recent studies employed infrared multi-photon dissociation (IRMPD) spectroscopy and ion mobility spectrometry both combined with extensive molecular modeling. These investigations indicated that the linear imine isomer of a_n ions can undergo head-to-tail cyclization forming a macrocyclic isomer with a backbone composed of a secondary amine and amide groups. This macrocyclic isomer can open up to reform the original linear imine or, after proton transfer to the amide nitrogen adjacent to the secondary amine, to a rearranged ‘imine-amide’ isomer. These a_3 pathways as well as the formation of a_3 from b_3 are shown in Scheme 3.1. Theoretical studies indicate the macrocyclic isomer is energetically favored over the linear imine and the rearranged ‘imine-amide’ structure is highly favored in comparison to both other structures. The actual abundance ratios of these structures depend on the experimental conditions.
under which the $a_n$ ions are formed and probed. For example, ion mobility data on the $a_4$ of protonated YGGFL indicate the presence of all three structures while a recent IR spectroscopy study performed in an ion trap instrument on the same ion indicates dominance of the ‘imine-amide’ isomer.

Two major dissociation reactions of $a_n$ ions are described in the literature including elimination of ammonia to form the $a_n$-$\text{NH}_3$ ($a_n^*$) ion and fragmentation to the next-
lower b ion, the b$_n$-1 ion.$^{145}$ A mechanism proposed previously, which is shown on pathway b of Scheme 3.1, suggests the b$_n$-1 can be formed via attack of the closest N-terminal carbonyl oxygen on the carbonyl carbon N-terminal to the protonated imine$^{145}$ leading to loss of the C-terminal imine and formation of Harrison’s stable linear oxazolone b$_n$-1.$^{139}$ There is greater debate about what mechanism leads to a$_n^*$ formation.$^{70,143,144}$ It was previously proposed by Glish and coworkers$^{141}$ that this ion was formed after proton transfer to the N-terminus of the linear form by elimination of the N-terminal –NH$_3^+$ as ammonia upon attack of the C-terminal imine nitrogen by an SN2 reaction that forms a macrocycle. This macrocycle has a fixed charge at the original imine nitrogen that could then in principle unfold to an acylium (or rather oxazolone terminated) a$_n^*$ ion,$^{141}$ as shown in Scheme 3.1 pathway a. More recently, Cooper et al.$^{144}$ proposed an alternative mechanism, described in Scheme 3.1 pathway c, that leads to elimination of the imine nitrogen (e.g. not the N-terminal amine nitrogen) in the departing ammonia. The process is initiated via nucleophilic attack by the N-terminal adjacent carbonyl oxygen on the carbonyl carbon adjacent to the C-terminal imine, e.g. via the same reaction that leads to the b$_n$-1 ion. Before complete loss of the imine to form b$_n$-1, however, a proton-bound dimer (PBD) of the two fragments is formed. This PBD can rearrange bringing the imine close to the N-terminus of the b$_n$-1 fragment where it covalently binds via a C-N bond. This new re-associated structure can then undergo a proton transfer to give an NH$_3$ group that is easily lost.$^{144}$ Site-specific $^{15}$N labeling of the FGGFL peptide was used to probe the SN2 and PBD ammonia elimination reactions; only the latter was compatible with the labeling data indicating it is the dominant pathway.$^{147}$ An additional mechanism for ammonia elimination was recently proposed$^{151}$ from the rearranged ‘imine-amide’ a$_n$ structures (see above). Formally, these have the R$_n^*$-CH=NH$^+$-CHR$_1$–…-CO-NH$_2$ backbone, if the ionizing proton
is transferred to the C-terminal amide nitrogen $R_n$-CH=N-CHR$_1$-...-CO-NH$_3^+$ is formed from which NH$_3$ is eliminated by attack of the N-terminal adjacent carbonyl oxygen.

One unusual trend in the chemistry of $a_n$ ions is the low intensity of the $a_3$ fragment ions compared to that of other $a_n$ fragments. Glish and co-workers$^{70}$ analyzed 29 peptides and discovered that the CID spectra of the majority of these did not feature the $a_3$ fragment while $a_2$ and $a_4$ were observed under the same CID conditions. Using a theoretical approach, Glish and coworkers investigated the $b_4 \rightarrow a_4 \rightarrow b_3 \rightarrow a_3 \rightarrow b_2 \rightarrow a_2 \rightarrow a_1$ reaction cascade and concluded that the barrier to the $a_3 \rightarrow b_2$ reaction was significantly lower than to those of $a_4 \rightarrow b_3$ and $a_2 \rightarrow a_1$, making the $a_3$ structure easily convertible to the more stable $b_2$. Concurrently, Zubarev and co-workers$^{152}$ reached the same conclusion in a statistical analysis of a large spectral database collected in studies on human and *E. Coli* cell lysates. Two trends were observed in these data: one that showed the high frequency of the $b_2$ ions in the $b_n$ series and one that showed significantly reduced frequency of $a_3$ compared to the rest of the $a_n$ ion series. Zubarev and coworkers explained the latter by formation of alternative (cyclic peptide isomer) $b_2$ ions and their increased stability compared to other $b_n$ fragments. Moreover, a subsequent IR spectroscopy study performed in an ion trap did observe a stable $a_3$ ion from the GGGG peptide$^{150}$. It was established to be a seven-membered cyclic isomer formed from the linear imine $a$-ion structure. It was produced by the attack of the first-residue amide oxygen on the carbon center of the C-terminal imine. It is worth noting here that the metastable ion spectra of the $b_3$ ions of protonated AAAAA and YGGFL feature abundant $a_3$ fragments that are likely very fragile because their abundance becomes very low even under mild CID conditions$^{139}$.

Previous studies of this `$a_3$ phenomenon' have focused on peptides composed exclusively of alpha amino acids. Here is presented experimental and theoretical data on peptides containing gamma-amino butyric acid (Gaba, NH$_2$-(CH$_2$)$_3$-COOH); AGabaAIG
and GabaAAIG. The Gaba amino acid increases the length of the backbone by two methylenes, and was chosen to create an artificial, less kinetically favored, seven-membered ring b\textsubscript{2} ion instead of the five-membered oxazolone ring of the b\textsubscript{2} ion, potentially encouraging retention of the a\textsubscript{3} fragment. In fact, in the product ion spectra of protonated AGabaAI\textsubscript{G} the a\textsubscript{3} ion is present as one of the most abundant fragments, while the GabaAA\textsubscript{I}G does not form abundant a\textsubscript{3} ions upon CID. The peptides were terminated with -IG since it was believed (based on unpublished statistical analysis of the likelihood of b\textsubscript{3} ion cleavage by residue; work by Wenzhou Li in the Wysocki group) that this ending sequence could provide high b\textsubscript{3} ion intensity with the potential to provide high a\textsubscript{3} ion intensity. Here the formation, structure and reactivity of the a\textsubscript{3} ions of AGabaA and GabaAA were studied with the aim of better understanding the chemistry of a\textsubscript{3} ions in general.

3.2. Experimental
3.2.1. Peptide synthesis and mass spectrometer conditions

All peptides were synthesized using standard Fmoc Solid-Phase synthesis\textsuperscript{153} with all unlabeled, protected amino acids purchased from EMD Biosciences (Danvers, MA) or ChemPep (Wellington, FL). Fmoc-\textsuperscript{15}N-Ala-OH was purchased from Sigma-Aldrich (St. Louis, MO) and used in the same solid phase synthesis method to produce (\textsuperscript{15}N-A)GabaAI\textsubscript{G} and AGaba(\textsuperscript{15}N-A)IG. All peptides were synthesized and then diluted in 50:50 H\textsubscript{2}O:ACN solution with 0.1% formic acid to concentrations of approximately 10-50 µM.

The mass spectra were collected on a modified Waters Synapt G2 (Manchester, UK) in positive ion mode using electrospray ionization. The source voltage was set between 3-4 kV with cone and extractor voltages set to ~30 V and ~5 V, respectively. Ions were selected in the initial quadrupole and fragmentation performed with argon gas in the trap.
stacked ring ion guide located immediately before the IMS cell of the instrument. Although not used in this study, the instrument has been modified with a surface-induced dissociation (SID) device placed between the IMS cell and a shortened transfer cell. To perform pseudo MS\textsuperscript{3} fragmentation, the sampling cone voltage was raised 20-30 V and resulting fragments isolated by the quadrupole with trap CID occurring afterwards.

Additional experiments were performed in a Bruker APEX Qe 9.4 T FTICR (Bremen, Germany). MS\textsuperscript{3} was achieved through fragmentation of the peptide ions in source, isolation of the investigated fragment in the ICR and inducing further fragmentation by SORI-CID. All precursor peptides were ionized via electrospray ionization at 4.8 kV. SORI-CID was performed in the ICR cell after source fragmentation, where power percentages stayed at 1.2 - 1.5 % of their maximum excitation with the length of each SORI pulse set at 0.3 sec. Quadrupole isolation was unnecessary for precursor or fragments, since there was sufficient source energy to result in each CID ion, which was then isolated in the cell.

3.2.2. Computational Details

Maestro (version 9.1) was used to build the starting structures for all fragments. In order to generate a number of starting structures for further quantum chemical calculations, a conformation search was performed with a Monte Carlo mixture model (MCMM) using the Merck molecular force field MMFF with the torsional sampling option from the Schrodinger software program MacroModel. The MCMM conformational method is an algorithm that explores geometries generated by randomly changing torsion angles which are then energy optimized and either considered a valid structure or not based on a number of parameters. This process is repeated a specified number of times based on user preference. Here, 10000 structures were generated with 2500 steps per rotatable bond. Of the 10000 structures generated, a cut-off of 1.0-2.0 Å
atom deviation was set up and redundant structures were removed. Between 10 and 20 starting structures, selected based on representative hydrogen bonding and torsional features, were chosen from the 100-200 minimized structures to be run at the PM3 and higher optimizations. To calculate energies of each structure at the PM3, HF/3-21G, B3LYP/6-31G(d), and B3LYP/6-31+G(d,p) theoretical levels, the Gaussian 09 program was used\textsuperscript{120}. Stepwise calculations going from lower theoretical levels to higher allowed for faster calculation speeds at each theoretical level and the removal of redundant structures. The final reported energy values were determined at the B3LYP/6-31+G(d,p) level, zero-point energy and other thermochemical corrections were obtained from vibrational frequencies calculated at the B3LYP/6-31G(d) level. Relative Gibbs free energies were calculated at 298K.

3.3. Results and Discussion
3.3.1. CID of protonated AGabaAIG, GabaAAIG, and AAAIG

The CID spectra of protonated AGabaAIG, GabaAAIG, and AAAIG are shown in Figure 3.1. Both Gaba precursor ions (\(m/z\) at 416.25) give a variety of \(b_n\) and \(a_n\) fragments indicating facile dissociation at the backbone amide bonds. For AGabaAIG (Figure 3.1a) the base peak at 22 eV collision energy is the \(a_3\) ion. Therefore, CID of protonated AGabaAIG is ‘unusual’ in terms of the ‘\(a_3\) phenomenon’. The fragmentation pattern of protonated GabaAAIG (Figure 3.1b) significantly differs from that of protonated GabaAAIG. The product ion spectrum of protonated GabaAAIG (Figure 3.1b) is dominated by the \(b_2\) ion at 22 eV collision energy (laboratory frame) with the \(b_4\), \(a_4\), \(b_5\), and \(a_3^*\) fragments being less abundant. The CID behavior of this peptide is ‘usual’ in terms of the ‘\(a_3\) phenomenon’ because no significant peak is observed at the expected \(m/z\) (200) of the \(a_3\) ion. Furthermore, the \(a_3^*\) ion is more abundant than it is in CID of protonated GabaAAIG and fragments are observed at \(m/z\) 140.1, 139.09, and 112.1,
which were not present in the GabaAAIG case. The MSMS spectrum of AAAIG (Figure 3.1c), is shown to highlight that the standard Ala containing model peptide does not show any significant $a_3$ ion intensity. At the same conditions (22 eV collision energy in the Waters Synapt G2), this ‘normal’ peptide has significant ion intensity at the $b_3$ followed in abundance by the $b_2$.

Figure 3.2 shows the energy resolved mass spectra (ERMS) graphs for both Gaba peptides. As expected from the MSMS studies these are different, with that of protonated GabaAAIG similar to ERMS graphs observed for similar peptides with alpha amino acids and with that of protonated AGabaAIG significantly different from those literature cases. Again, the ERMS of the AAAIG (not shown) shows no significant $a_3$ intensity throughout the range of collision energies. The most striking difference between the two plots in Figure 3.2, besides the lack of $a_3$ in the GabaAAIG case and dominance of the $a_3$ species in the AGabaAIG case, is the prevalence of the $b_2$ ion in the former. This suggests that the $b_2$ ion with the GabaA sequence is kinetically favored or very stable, or both. It is also worth noting that $a_3^*$ is substantially less abundant in the GabaAAIG case than in AGabaAIG for the whole collision energy range studied. These observations indicate that the fragmentation chemistries of protonated AGabaAIG and GabaAAIG are different. Substitution of an alanine residue by Gaba in the AAA sequence and therefore expansion of the backbone by two methylene groups produces a rarely seen $a_3$ fragment when on the second position, but gives instead a very stable $b_2$ when on the first position. In order to further understand the effects this backbone extension can have on fragment ion formation, $MS^3$ studies were performed on selected fragments of protonated AGabaAIG.
Figure 3.1  Product ion mass spectra of protonated (a) AGabaAIG, (b) GabaAAIG, and (c) AAAIG recorded on the G2 Synapt instrument at 22 eV collision energy (laboratory frame).
3.3.2. CID of the b<sub>3</sub>, a<sub>3</sub>, a<sub>3</sub>*, and b<sub>2</sub> fragments of protonated AGabaAIG

Figure 3.3 displays the results of CID experiments performed on b<sub>3</sub>, a<sub>3</sub>, a<sub>3</sub>*, and b<sub>2</sub> of protonated AGabaAIG. Both the b<sub>3</sub> and a<sub>3</sub> spectra show abundant formation of a<sub>3</sub>* and the fragment at m/z 140.07. CID of a<sub>3</sub> features only a marginally abundant b<sub>2</sub> peak at m/z 157.10 while CID of b<sub>3</sub> has a moderately abundant peak at this position. CID of a<sub>3</sub>*
results in two major peaks, at \( m/z \) 140.07 and 112.08, respectively, indicating that these ions appear to be part of the \( a_3 \rightarrow a_3^* \rightarrow m/z \) 140.07 cascade. An \( m/z \) 140.07 fragment is, however, also observed in CID of \( b_2 \) and it is not clear without additional data whether this is a structurally different isomer or exists in the same structure as \( m/z \) 140.07 in CID of \( a_3^* \). Additionally, the \( b_3 \) MS\(^3\) spectrum in Figure 3.3d shows a peak at \( m/z \) 139.09 not present in the \( a_3 \), \( a_3^* \) or \( b_2 \) spectra and a peak at \( m/z \) 111.09 that is observed only in CID of \( b_2 \). This indicates the \( m/z \) 139.09 is formed on a pathway specific to the \( b_3 \) ion or that the energetics for each starting fragment ion can drastically affect the intensity of the ions within a particular cascade. The latter is a likely possibility since the MS\(^3\) of the \( m/z \) 139.09 fragment contains the \( m/z \) 111.09 ion as well (spectrum not shown).

Figure 3.3  MS\(^3\) tandem mass spectra (recorded using SORI-CID after source fragmentation) of a) the \( b_2 \), b) the \( a_3^* \), c) the \( a_3 \) and d) the \( b_3 \) fragment ions of protonated AGabaAIG.
3.3.3. CID of fragments of protonated ($^{15}$N-A)GabaAIG, AGaba($^{15}$N-A)AIG, and N-terminally acetylated AGabaAIG

In previous reports, CID of isotopically labeled peptides has been successfully applied to probe the dissociation pathways of a few b$_n$ and a$_n$ ions $^{144,147}$. For example, the formation of a$_3^*$ ion of protonated GGGG was probed by labeling the N-terminal Gly with $^{15}$N and performing CID. These experiments demonstrated that it is not the N-terminal amino nitrogen, but rather the nitrogen of the third Gly that is eliminated as NH$_3$ in the a$_3$ → a$_3^*$ reaction. CID of protonated GGGG is ‘usual’ in terms of the ‘a$_3$ phenomenon’ because the a$_3$ signal is observed only under very mild CID conditions $^{148}$. In order to further probe the chemistry behind the ‘unusual’ ‘a$_3$ phenomenon’ featured in CID of protonated AGabaAIG, the N-terminus of AGabaAIG was acetylated and AGaba($^{15}$N-A)IG and ($^{15}$N-A)GabaAIG were synthesized and their dissociation pathways were investigated by CID. The main goal of these experiments was to determine which nitrogen was lost as ammonia in CID of the AGabaAIG peptide and whether the nitrogen could be lost if the N-terminus was blocked. Figure 3.4, Figure 3.5, and Figure 3.6 show the CID spectra of the b$_3$, a$_3$ and b$_2$ ions of the two isotopically labeled peptides, while Figure 3.7 shows the product ion spectrum of the acetylated unlabeled peptide.

As expected, the m/z values of the a$_3$ and b$_3$ ions are the same for the ($^{15}$N-A)GabaAIG and AGaba($^{15}$N-A)IG cases (Figure 3.4) as both fragments will contain $^{15}$N. The fragments located at lower m/z, however, show significant differences between the two spectra. For example, a$_3^*$ appears dominantly at m/z 184.1 for ($^{15}$N-A)GabaAIG and as a doublet at m/z 183.1 and 184.1 dominated by the former when generated from b$_3$ of AGaba($^{15}$N-A)IG. Similarly, a splitting of the formal b$_2$ peak is observed in both cases. Most interestingly, the peak pairs 111.09 & 112.08 and 139.09 & 140.07, observed for the unlabeled AGabaAIG and for ($^{15}$N-A)GabaAIG merge in the AGaba($^{15}$N-A)IG case.
under unit resolution but appear as separate peaks if high resolution spectra are acquired (Figure 3.4b inset).

![Figure 3.4](image_url)

Figure 3.4 MS³ tandem mass spectra (recorded using SORI-CID) of (a) the b₃ of protonated (1⁵N-A)GabaAIG and (b) the b₃ of protonated AGaba(1⁵N-A)IG. Insets in (b) are expanded ranges of the m/z 140 and m/z 180-187 regions.

Isolation and CID of the a₃ ions of protonated (1⁵N-A)GabaAIG and AGaba(1⁵N-A)IG (Figure 3.5) provide unambiguous information on the ammonia elimination reaction; ¹⁴NH₃ is eliminated from the former and ¹⁵NH₃ is eliminated from the latter. This clearly indicates that the nitrogen of the third Ala residue is eliminated as ammonia from stable (e.g. isolable) a₃ ions. The formal b₂ fragment of AGabaAIG appears as a doublet in CID of the b₃ ions of protonated (1⁵N-A)GabaAIG and AGaba(1⁵N-A)IG (Figure 3.4). Figure 3.6 shows CID of the m/z 157.10 component of this doublet for (1⁵N-A)GabaAIG and AGaba(1⁵N-A)IG. For (1⁵N-A)GabaAIG one observes only loss of ammonia while for
AGaba\(^{15}\text{N-A})\text{IG} only formation of \(m/z\) 111.09 is present. This indicates the two \(m/z\) 157.10 ions have different structures and the \(m/z\) 157.10 ion of protonated unlabeled AGabaAlG is composed of two structural isomers.

Figure 3.5  
\(\text{MS}^3\) tandem mass spectra (recorded using SORI-CID) of \(m/z\) 201.13 (a) the \(a_3\) of protonated \((^{15}\text{N-A})\text{GabaAlG}\) and (b) the \(a_3\) of protonated AGaba\(^{15}\text{N-A})\text{IG}.\)
Figure 3.6  MS$^3$ tandem mass spectra (recorded using SORI-CID) of the m/z 157.1 fragment of a) protonated ($^{15}$N-A)Gaba$\epsilon$G and b) protonated AGaba($^{15}$N-A)$\epsilon$G.
Figure 3.7  Product ion mass spectra of protonated acetylated-AGabaAlG recorded on the G2 Synapt instrument at 20 eV collision energy (laboratory frame).

Comparing the CID spectrum of AGabaAlG (Figure 3.1a) to its N-terminally acetylated form (Figure 3.7) also shows distinct differences. The CID of protonated acetyl-AGabaAlG (m/z 458.28) recorded at 20 eV collision energy (laboratory frame) has a base peak at the b1 fragment, which is only possible due to the acetyl group on the N-terminus. The next most intense peak is the a3 ion and the spectrum also features b2, b3, a4, b4, m/z 86.1, m/z 129.1, and m/z 157.1 peaks. Interestingly, in this spectrum there is no significant a3* ion intensity observed. With the addition of an acetyl group blocking the N-terminus, the ‘a3 phenomenon’ is still entirely possible, but the ammonia loss from the a3 fragment ion becomes very unfavorable.
3.3.4. Formation, structure, and fragmentation pathways of the \( a_3 \) of protonated AGabaAIG

The product ion spectra of protonated AGabaAIG and acetyl-AGabaAIG and the \( b_3, a_3, a_3^* \) and \( b_2 \) ions of protonated AGabaAIG, \((^{15}\text{N-A})\text{GabaAIG, and AGaba}^{(15}\text{N-A)}\) provide detailed information on the related dissociation and rearrangement reactions, summarized in Scheme 3.2. In order to obtain candidate structures of the fragment ions theoretical studies were undertaken, performing molecular modeling and quantum chemical calculations.

Dissociation of protonated AGabaAIG at the A-I amide bond leads to the oxazolone isomer \( b_3 \) ion with the AGabaA sequence (AGabaAox), which is expected to fragment further to form the unusually stable \( a_3 \) fragment. The \( b_3 \) ion either undergoes dissociation to form \( a_3 \) on the \( b_3 \to a_3 \) pathway or head-to-tail cyclization \(^{155}\) to form the corresponding protonated cyclo-(AGabaA) isomer. The latter reaction is energetically not
favored for the $b_3$ ion with the AAA sequence, however, the increased flexibility of the AGabaA $b_3$ due to the two extra backbone methylenes allows formation of the eleven-membered macro-ring. Theoretical calculations indicate the macrocyclic $b_3$ ion isomer (Structure B in Chart 3.1) is only slightly less favored (2.4 kcal/mol) than the most energetically favored linear form (Structure A, C-terminal oxazolone protonation). This is in clear contrast to the AAA case where the linear vs. macrocyclic energy gap is more than 20.0 kcal/mol. The isomeric seven-membered ring terminated AAGaba form (Structure D in Chart 3.1) is energetically much less favored than the oxazolone terminated AGabaA species; this demonstrates the superior stability of Harrison’s oxazolone ring over seven-membered ring structures. Formation of a seven-membered ring is also kinetically less favorable than formation of a five-membered ring; because CID is a kinetic process, this must also be considered. On the other hand, the amino protonated GabaAA form (Structure C in Chart 3.1) is energetically nearly as favored as AGabaAox. These theoretical data indicate that while AAGaba is particularly less favored as a 7-membered ring terminated structure, one should consider both the AGabaAox and GabaAAox sequence isomers when considering dissociation products of $b_3$.

Experimental evidence for the head-to-tail cyclization and ring-opening to form the GabaAAox $b_3$ ion is obtained from CID of the $b_3$ and $b_2$ ions of the isotopically labeled peptides. In Figure 3.4, one observes splitting of the formal $b_2$ peak into $m/z$ 157.1 and 158.1 in both panels. The fate of the labeled Ala nitrogen is explicitly followed in Schemes Scheme 3.3 and Scheme 3.4; briefly CID of $b_3$ with the ($^{15}$N-A)GabaA sequence gives ($^{15}$N-A)Gaba $b_2$ ($m/z$ 158.1) and after scrambling the GabaAox internal ion ($m/z$ 157.1). The peak at $m/z$ 157.1 is more abundant than the peak at $m/z$ 158.1, e.g. the internal ion GabaAox is formed preferentially. Similar conclusions can be drawn from analysis of Figure 3.4b based on Scheme 3.4. Furthermore, CID data presented in
Figure 3.6 for the m/z 157 ions from protonated ($^{15}$N-A)GabaAIG and AGaba($^{15}$N-A)IG clearly indicate two isomeric structures. Exclusive loss of ammonia is in line with the AGaba isomer while formation of m/z 111.09 can be explained considering the GabaA sequence. Most likely this loss of m/z 46 is a combination of a water and carbonyl group, as makes sense given the exact mass work performed on the ICR. Furthermore, the DFT calculations performed on the relative energies of each possible b$_2$ ion structures shown in Chart 3.2 provide an explanation for the preferential formation of GabaAox over other GabaA b$_2$ isomers. That is, GabaAox is about 9 kcal/mol lower in energy than the AGaba b$_2$ isomer and 8 kcal/mol lower than the protonated AGaba head-to-tail cyclized structure. This energetic preference for the GabaAox b$_2$ ion explains the dominance of this fragment in the CID of protonated GabaAAIG (Figure 3.1b and Figure 3.2) where this ion can directly be formed from the parent peptide and higher b ions without structural rearrangements.
Scheme 3.3  Step-wise diagram tracking the location of the isotopic label of $^{15}$N-A)GabaAlG from $b_3$ to its possible fragment ions
Scheme 3.4  Step-wise diagram tracking the location of the isotopic label of AGaba(^{15}N-A)IG from $b_3$ to its possible fragment ions.
Chart 3.1 Various structures for the $b_3$ fragment of protonated AGabaALG: A) linear AGabaA isomer terminated by the oxazolone ring; B) protonated cyclo-(AGabaA); C) linear GabaAA isomer terminated by the oxazolone ring; D) linear AAGaba isomer terminated by a seven-membered ring. Characterized protonation sites are indicated by arrows with the corresponding relative energies (kcal/mol) and relative Gibbs free energies (in parentheses, kcal/mol). The relative energies are calculated with respect to the oxazolone protonated AGabaAox species A.
Chart 3.2  Structures for the b\textsubscript{2} fragment of protonated AGabaAlG and GabaAAIg: A) linear AGaba isomer terminated by the seven-membered ring; B) protonated eight-membered cyclo-(AGaba); C) linear GabaA isomer terminated by the oxazolone ring. As with previous charts, protonation sites are indicated by arrows with the corresponding relative energies (kcal/mol) and relative Gibbs free energies (in parentheses, kcal/mol). The relative energies are calculated with respect to the ring nitrogen protonated AGaba species.

Theoretical studies on the a\textsubscript{3} ion of protonated AGabaAlG focused on the AGabaA sequence formed from the AGabaAox b\textsubscript{3} isomer. No detailed calculations were performed for the a\textsubscript{3} isomer with the GabaAA sequence because the GabaAAIG was shown to feature ‘usual’ behavior in terms of the ‘a\textsubscript{3} phenomenon’, e.g. negligible abundance. CID of protonated GabaAAIG does not lead to a prominent a\textsubscript{3} ion, and b\textsubscript{2} is much more abundant then a\textsubscript{3}\textsuperscript{*} indicating that the GabaAA a\textsubscript{3} ion dissociates preferentially to form the b\textsubscript{2}. Therefore, there was no expected deviation from the ion structures and mechanisms previously discussed for a\textsubscript{3} ion chemistry\textsuperscript{70,144,152}. The a\textsubscript{3} ion with the AGabaA sequence can be formed from the AGabaAox b\textsubscript{3} isomer via the b\textsubscript{3}→ a\textsubscript{3} CO loss TS at 35.2 kcal/mol (Table 3.1) which is relatively comparable in energy to the
reported AAA value of 31.7 kcal/mol for the same transition \(^{70}\). This indicates \(b_3\) can easily convert to \(a_3\) upon excitation and the Gaba residue at position two does not hinder this reaction channel.

<table>
<thead>
<tr>
<th>Structure</th>
<th>(E_{ZPE})</th>
<th>(E_{Rel_b3})</th>
<th>(E_{Rel_a3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(b_3)-(a_3) (TS)</td>
<td>-781.359857</td>
<td>35.2</td>
<td>-</td>
</tr>
<tr>
<td>(a_3)-(b_2) imine loss (TS)</td>
<td>-781.351990</td>
<td>39.6</td>
<td>24.8</td>
</tr>
<tr>
<td>(a_3) macrocycle formation</td>
<td>-781.354096</td>
<td>33.6</td>
<td>18.9</td>
</tr>
<tr>
<td>PT on the macrocycle</td>
<td>-781.355771</td>
<td>38.2</td>
<td>23.5</td>
</tr>
<tr>
<td>Ammonia Loss (structure E)</td>
<td>-781.352666</td>
<td>37.2</td>
<td>22.5</td>
</tr>
<tr>
<td>Ammonia Loss (structure K)</td>
<td>-781.361477</td>
<td>39.1</td>
<td>24.4</td>
</tr>
</tbody>
</table>

Table 3.1 ZPE-corrected total (\(E_{ZPE}\), Hartree) and relative energies (\(E_{Rel\_b3}\) and \(E_{Rel\_a3}\), kcal/mol) of selected transition structures on the PES of the \(b_3\) fragment of protonated AGabaA. Two relative energies are given for the TSs related to the \(a_3\) ion which are calculated using the oxazolone protonated AGabaA_{ox} \(b_3\) and either the imine protonated AGabaA_{im} \(a_3\) as reference structures, respectively. The \(E_{ZPE}\)s of the \(a_3\) structures presented contain the ZPE-corrected total energy of CO (-113.304422 Hartrees). All energies were determined at the B3LYP/6-31+G(d,p) level of theory.

The calculations in this study considered those from the structures (Chart 3.3) proposed by Bythell et al. \(^{148}\) for the \(a_3\) ion of protonated GGGG. IR studies on that ion indicate it forms a cyclic structure generated by nucleophilic attack of the N-terminal amide oxygen on the carbon of the C-terminal imine. The macrocyclic isomer (C in Chart 3.3) is energetically as favored as the linear imine terminated isomer (A in Chart 3.3) and the structure formed by proton transfer to the adjacent amide nitrogen (D in Chart 3.3) is unstable and opens up to the rearranged imine-amide isomer (E in Chart 3.3) which is the energetically most favored AGabaA \(a_3\) species at ~ -13 kcal/mol relative energy. On the other hand, the corresponding AGabaA structures (G and H in Chart
3.3) are energetically disfavored. Similarly, the structures (I, J, and K in Chart 3.3) formed by re-association of the formal b2 and imine fragments in the PBD formed by elimination of the C-terminal imine are energetically disfavored at 18-20 kcal/mol relative energy.

Chart 3.3 Various a3 (AGabaA sequence) ion structures: A & B) linear form protonated at the imine or N-terminal amino groups, respectively; C and D) macrocyclic (11-membered ring) isomer protonated at the secondary amine or the adjacent amide nitrogen, respectively; E) and F) rearranged linear form protonated at the N-terminal imine group or the C-terminal amide nitrogen, respectively; G and H) nine-membered ring isomer protonated at the N-terminal amine or ring imine nitrogen, respectively; I, J, and K) seven-membered ring terminated re-associated linear form protonated at the secondary amine, N-terminal amine, or ring nitrogen, respectively. The respective protonation sites are indicated by arrows. Relative ZPE-corrected energies (Gibbs free energies at 298 K) are given.
Scheme 3.5 shows the dissociation pathways of the \( a_3 \) ion with the AGabaA sequence (those structures for which energies were presented in Chart 3.3 are labeled with the appropriate letter code). Three major reactions are considered here: i) imine loss from structure A to form \( b_2 \); ii) macrocycle formation, and reopening the macro-ring after proton transfer to produce the linear reordered \( a_3 \) structure \( E \) and subsequent ammonia loss; and iii) formation of a proton bound dimer of \( b_2 \) and the eliminated imine, reassociation of the fragments at the N-terminus of the \( b_2 \), and elimination of ammonia, e.g. the PBD \( a_3 \rightarrow a_3^* \) pathway.

Elimination of the C-terminal imine from \( a_3 \) requires passing a transition state at 24.8 kcal/mol relative energy (calculated using the imine protonated AGabaA \(_{im} \) as reference structure). This threshold energy is significantly higher than literature \( a_3 \rightarrow b_2 \) values, for example Cooper et al. reported 13.8 kcal/mol for the GGG \( a_3 \rightarrow b_2 \) reaction, whereas Glish and coworkers reported 16.7 kcal/mol barriers for both the AAAA \( a_4 \rightarrow b_3 \) and the AAA \( a_3 \rightarrow b_2 \) reactions. The relatively high barrier is explained by formation of the seven-membered C-terminal ring for the AGaba \( b_2 \) ion; this structure is energetically much less favored than the isomeric GabaAox structure (see above in Chart 3.2) featuring Harrison’s oxazolone ring. The relative energy of the separated products (\( b_2 \) and Ala imine) is 32.5 kcal/mol, again much higher that that calculated for the AAA case (21.4 kcal/mol). These energetics mean that the AGabaA \( a_3 \) ion is significantly, kinetically more stable than the AAA \( a_3 \) ion offering a reasonable explanation for the abundant \( a_3 \) peak in CID of protonated AGabaAlG (Figure 3.1a).

The barrier to formation of the macrocyclic \( a_n \) isomers is 10-20 kcal/mol as reported for literature cases, so this is not likely to be the highest energy transition on the ii) pathway of Scheme 3.5. The 18.9 kcal/mol value for the macrocycle formation of the AGabaA \( a_3 \) falls in-line with these literature cases. The proton transfer to form structure D from C is, however, expected to be one of the rate limiting steps for this pathway due
to the formal 4-atom transition structure involved. The lowest energy proton transfer TS, from the transfer of the proton in the macrocycle to promote unfolding calculated here, is at 23.5 kcal/mol, just slightly below the threshold energy of the $a_3 \rightarrow b_2$ reaction. On the product branch of such TSs one does not locate stable D structures protonated at the amide nitrogen, rather these species spontaneously transfer to the rearranged E structure. As the energetically most favored species on the $a_3$ potential energy surface (PES), structure E can in principle undergo proton transfers to form the C-terminal amide nitrogen protonated form (structure F) at 14.1 kcal/mol relative energy. Such structures can eliminate ammonia by nucleophilic attack of the N-terminal adjacent carbonyl oxygen on the carbon of the protonated terminal amide bond. The relative energy of this $a_3 \rightarrow a_3^*$ TS is 22.5 kcal/mol, which is about 2 kcal/mol lower in energy than the $a_3 \rightarrow b_2$ transition state structure.

The second channel to form $a_3^*$ is the PBD pathway that is initiated by elimination of the C-terminal imine at 24.8 kcal/mol threshold energy. It is assumed here that subsequent rearrangements, reassociation, and PT all involve TS’s at lower energies. The relative energy of the resulting K structure that has the N-terminal NH$_3^+$ moiety is 19.6 kcal/mol and the corresponding ammonia loss TS is at 24.4 kcal/mol relative energy. This TS is lower in energy than the $a_3 \rightarrow b_2$ TS by only 0.4 kcal/mol and is about 2 kcal/mol higher in energy than the other ammonia loss from structure F at 22.5 kcal/mol.

The $a_3 \rightarrow b_2$ and the critical $a_3 \rightarrow a_3^*$ TSs are within 3-4 kcal/mol relative to each other for the AGabaA sequence. All these critical energies are higher than the $a_3 \rightarrow b_2$ value calculated for the AAA sequence. This observation provides a reasonable explanation for the stability of the AGabaA $a_3$ ion; one needs to impart much more energy into this species to induce fragmentation than is necessary to dissociate the corresponding AAA $a_3$ ion. Furthermore, the macrocyclic AGabaA $a_3$ ion can undergo cyclization and
possible ring-opening to form the energetically favored imine-amide isomer. Such a reaction is not possible for the AAA $a_3$ ion because of the energetically disfavored cyclization process. Formation of the low-energy imine-amide structure for AGabaA can be another reason for the unusually stable $a_3$ ion. Additional studies on the AGabaA $a_3$ ion using IRMPD have the potential to shed light on this phenomenon, but were however, outside of the scope of this study.
Scheme 3.5  Fragmentation pathways of AGabaA a\textsubscript{3} ion. Relevant transition state barriers reported relative to the a\textsubscript{3} structure A ion in kcal/mol.
3.4. Conclusions

With an extended backbone at the second position amino acid, the intensity of the $a_3$ ion can be significantly increased compared to standard alpha amino acid containing peptides. Through isotopic labeling and tandem mass spectrometry experiments, the fragments of protonated AGabaAlG were analyzed and a reaction scheme defined for the production of $a_3$ and its consecutive products. Modeling and DFT calculations have revealed that the $a_3$ ion is most likely stabilized for two distinct reasons. i) The barrier to produce a macrocyclic structure is relative low and results in a macrocycle that is energetically as favored as the trans imine $a_3$. After proton transfer this macrocycle can unfold into an imine-amide $a_3$ 13 kcal/mol lower in energy than the original imine isomer. ii) The AGaba $b_2$ ion is energetically noticeably disfavored due to the seven-membered C-terminal ring and the transition state to form this $b_2$ ion via an imine loss is higher in energy than the similar TS calculated for the AAA sequence. Another important feature of the two methylene backbone extension was the ease with which the fragments could cyclize. In the case of the $b_3$, the AGabaAox structure can be converted to GabaAAox while this chemistry is prohibited for the AAA sequence due to the energetically disfavored macrocyclic structure. In future work, the energetics and kinetics of $a_3$ formation for systems with varying backbone length, such as peptides with the A(βAla)A motif, will be studied.
<table>
<thead>
<tr>
<th>Transition/Fragment Structure</th>
<th>Total energy</th>
<th>ZPE correction</th>
<th>Gibbs Free Energy correction</th>
</tr>
</thead>
<tbody>
<tr>
<td>b₃-a₃ (TS)</td>
<td>-781.647381</td>
<td>0.288424</td>
<td>0.241716</td>
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<td>a₃-b₂ Imine Loss (TS)</td>
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<td>0.278976</td>
<td>0.234851</td>
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<td>Macro PT (TS)</td>
<td>-668.321462</td>
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<td>0.241031</td>
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<td>Ammonia Loss Structure E (TS)</td>
<td>-668.321233</td>
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<td>Ammonia Loss Structure K (TS)</td>
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<tr>
<td>a₃ macrocycle formation (TS)</td>
<td>-668.329416</td>
<td>0.280230</td>
<td>0.238481</td>
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</tbody>
</table>

Table 3.2  Total energies (Hartree) and zero-point energy and Gibbs free energy corrections (Hartree) of various transition structures on the PES of b₃ of protonated AGabaAlG. Total energies were calculated at the B3LYP/6-31+G(d,p) level of theory and zero-point energy and Gibbs free energy corrections were calculated using vibrational frequencies determined at the B3LYP/6-31G(d) level.
Chapter 4.  Fluorine substituted proline analogues and their effect on ion structure

4.1. Introduction

In the field of peptide fragmentation, the b ion structures formed via collision induced dissociation (CID) have been well characterized for several different types of sequences. This includes model peptides of repeating units (i.e. Gly and Ala repeats), standard well-known sequences like leu-enkephalin, and also studies on isolated residue effects like that of the proline and aspartic/glutamic acid residues.\textsuperscript{69,135,139,156,157} CID induces fragmentation along the peptide backbone at the C-N peptide bond, and when this bond is cleaved charge can be retained either C-terminally to the dissociated peptide bond to form a y-ion or N-terminally of this bond to form a b-ion. While y-ions are truncated peptides, b-ions can adopt several different structures to adapt to a C-terminus lacking the hydroxyl group. In the case of the b\textsubscript{2} ion, there is the chance of forming either a five-membered oxazolone ring, in which the peptide bond retains the trans conformation, or a six-membered head-to-tail cyclic diketopiperazine structure (Scheme 4.1).\textsuperscript{138,139} To form the diketopiperazine, the peptide bond must undergo a trans/cis isomerization. Despite calculations that show the diketopiperazine is typically the more stable ion, the formation of the kinetically-favored oxazolone fragment is more frequently observed.\textsuperscript{158} This is believed to be, at least in part, due to the unfavorable cis conformation necessary for diketopiperazine formation, but has also been proposed to result from the relative barriers of ring closure between the oxazolone and diketopiperazine structures.\textsuperscript{159}
Scheme 4.1 Depiction of the formation of oxazolone and diketopiperazine structures for the $b_2$ ion. The oxazolone $b_2$ ions retain the *trans* amide bond conformation of the original peptide precursor while the diketopiperazine $b_2$ ions must form via a *cis* amide bond.\textsuperscript{128}

The amino acid proline favors the formation of the *cis* peptide bond more than any other natural amino acid, although the trans amide bond is still favored even in Pro.\textsuperscript{160,161} On average for protein amino acid residues, the barrier to *trans/cis* isomerization of the peptide bond adjacent to the N-terminus is approximately 20 kcal/mol, while for proline, this barrier is only 13 kcal/mol.\textsuperscript{162} Additionally, the relative energies between the *trans* and *cis* isomers is about 2 kcal/mol less for proline in comparison to other residues. The lower barrier to isomerization allows $b$ ions with a proline residue to form the diketopiperazine with greater frequency, as demonstrated by previous work from the Wysocki group.\textsuperscript{68,69,163} Gucinski *et al.* showed that the $b_2$ ion structures of Val-Pro, Ala-Pro, and Ile-Pro are all mixtures of oxazolone and diketopiperazine, while His-Pro formed exclusively diketopiperazine ions.\textsuperscript{69,163} The work concluded that the *trans/cis* the presence of Pro in the second position was an influential factor in the formation of the diketopiperazine ions for these systems. The exclusive diketopiperazine, furthermore,
for His-Pro, was determined to be a result of the basic His residue in the first position, providing a site for proton bridging, along with the second position proline contributing to cis stability of the peptide.

These earlier studies inspired us to explore the influence of substitution of the Pro ring on oxazolone/diketopiperazine formation. The effect of substitution of the prolyl ring on structure has been examined predominantly in the solution-phase. For example, it is well known that collagen contains 4-hydroxyproline (Hyp) and, significantly, this amino acid modification in the repeating collagen unit provides stability to the collagen triple helix.\textsuperscript{161,164-166} The group of Ronald Raines has shown that inserting other substituents (i.e. fluorine and methyl) onto the 4\textsuperscript{th}-position of prolyl rings in the Pro-Hyp-Gly motif can increase the melting temperature of the construct by over 20 °C compared to standard collagen.\textsuperscript{165,167} Further research by this group has also considered the isolated effects of proline substitution on trans/cis isomerization. In the case of a single acetylated and O-methylated (Ac-Xxx-OMe) form where Xxx is a 4-substituted Pro, the $K_{\text{trans/cis}}$ value of the acetyl-Xxx peptide bond was shown to change significantly with different substituents placed in the $R$ or $S$ position. Using 1-D proton NMR, the $K_{\text{trans/cis}}$ of Pro was found to be 4.6. This ratio increased to 6.7 when fluorine was placed in the $R$ position and decreased to 2.5 with fluorine in the $S$ position.

Previous studies have suggested that the trans/cis character of the N-terminally adjacent peptide bond is a result of the influence of the puckering of the prolyl ring following prolyl substitution. H, OH, or F in the $S$ position have been shown to pucker the ring toward the adjacent peptide bond to produce the C\textsuperscript{I}-endo conformation while in the $R$ position OH or F causes the ring to pucker away from the peptide bond into the C\textsuperscript{I}-exo conformation.\textsuperscript{164,166,168,169} This effect is summarized in Figure 4.1, highlighting the nine peptides studied in the experiments detailed below in this chapter. For Tyr-flp-Ala, Val-flp-Ala, and His-flp-Ala (Figure 4.1a, Figure 4.1b, and Figure 4.1c), where fluorine is
in the S position, the ring is puckered into the Cγ-endo conformation while for the Hyp 2nd position and Flp 2nd position tripeptides Tyr-Hyp-Ala, Val-Hyp-Ala, His-Hyp-Ala, Tyr-Flp-Ala, Val-Flp-Ala, and His-Flp-Ala (Figure 4.1d-i), the fluorine is in the R position and the prolyl ring is puckered downward into the Cγ-exo position. The relationship of ring pucker to trans isomer favorability has been explained by the gauche effect of the downwards Cγ-exo puckering resulting in a torsional strain in the peptide backbone, which locks the trans conformation into place. A study by Crestoni et al. demonstrated that the ions of the R and S 4-hydroxyproline amino acid (Hyp and hyp) could be distinguished using gas-phase vibrational spectroscopy, marked by a blue shift of the carbonyl stretching mode for the S configuration. Along with this discovery they also noted that the Cγ ring puckering for these gas-phase molecules followed the same trend as in the previous solution-phase collagen studies discussed above.
Figure 4.1  Structures and sequences of the model peptides studied in this chapter and their proposed ring puckering positions: a) Tyr-flp-Ala (Cγ-endo), b) Val-flp-Ala (Cγ-endo), c) His-flp-Ala (Cγ-endo), d) Tyr-Hyp-Ala (Cγ-exo), e) Val-Hyp-Ala (Cγ-exo), f) His-Hyp-Ala (Cγ-exo), g) Tyr-Flp-Ala (Cγ-exo), h) Val-Flp-Ala (Cγ-exo), and i) His-Flp-Ala (Cγ-exo).

In this chapter a series of nine model peptides of sequence Tyr-xxx-Ala, Val-xxx-Ala and His-xxx-Ala were studied (where xxx represents Flp, flp or Hyp). The aim of these studies was to determine the effect of the modified second amino acid in the gas-phase
ionic structures. The model systems were designed to have either Val, His or Tyr in the first amino acid position. Valine was chosen as it has been observed to produce a mixture of $b_2$ oxazolone and diketopiperazine structures in the Val-Pro case, while histidine was observed to only form diketopiperazine structures for the His-Pro peptide studied previously, but a mixture in His-Ala.69 Tyrosine was also studied as it offers a non-basic phenyl group with very different properties from the imidazole side chain of histidine, whose effects on $b$-ion formation result in an overwhelming proportion of diketopiperazine. Furthermore, Tyr has previously been shown to influence $trans/cis$ character when N-terminally adjacent to proline.172 As peptide bond isomerization is necessary for producing the diketopiperazine fragment, the $trans$ to $cis$ tendency of each fluoro or hydroxyl substituted peptide may correlate with the population of oxazolone and diketopiperazine products for each $b_2$ ion population. This section explores whether loss or enhancement of the diketopiperazine character for each substitution is consistent with the system’s gas-phase peptide $trans/cis$ bond preference. Identification of the $b_2$ ion structures is achieved via gas-phase action IRMPD spectroscopy supported by theoretical calculations, enabling chemical information about each fragment ion to be obtained. Additionally, MS$^3$ and ion mobility of the $b_2$ ions was performed to determine how the $R$ to $S$ change of the fluorine on the prolyl ring influences the overall stability and structure of the $b_2$ ion generated in each case.

4.2. Experimental:
4.2.1. Peptide synthesis and preparation for MS analysis

All peptides were synthesized using standard Fmoc-solid phase synthesis described in detail elsewhere.107 Briefly, this process involves using a Wang resin-attached Fmoc-protected C-terminal residue that is deprotected in a piperidine (Sigma, MO) solution to remove the N-linked Fmoc group followed by coupling to the next Fmoc-protected amino
acid residue in the sequence along with the coupling catalysts O-(Benzotriazol-1-yl)-
N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine
(Sigma-Aldrich, MO). This process is repeated until all the desired amino acids are
attached in order from C to N terminus, following which the entire peptide is cleaved
from the resin bead using trifluoroacetic acid. Substituted Fmoc-protected R-
Fluoroproline (Flp) and S-fluoroproline (flp) were purchased from Bachem while all other
amino acids and synthesis reagents were purchased from EMD Biosciences, unless
otherwise stated. After cleavage from the resin, diethyl ether (Sigma-Aldrich, MO) was
added to the peptide solution and water added to extract the peptides from the ether.
Each peptide was diluted to approximately 1:100 into H$_2$O: ACN with 0.1% formic acid to
have a final concentration of approximately 10 to 50 μM. All solvents were purchased
from Sigma-Aldrich and used without any further purification.

4.2.2. Action IRMPD Conditions and Bruker Esquire 3000 S Ion trap Parameters

Action IRMPD was performed at the Centre Laser Infrarouge d'Orsay (CLIO) using a
free electron laser set up and a Bruker Esquire 3000$^\text{S}$ 3-D ion trap with an electrospray
ionization source. Maitre, Ortega, and co-workers have described the process in
detail.$^{84-86}$ Briefly, the ion trap was used to first isolate each precursor tripeptide and
perform in trap CID to produce the b$_2$ ion. Subsequently, isolation of the b$_2$ ion was
performed followed by irradiation of the trapped b$_2$ fragment ion by the FEL at 4-10 cm$^{-1}$
steps from 1000-2000 cm$^{-1}$ with averaging of the fragmentation spectra at each energy
step of the laser. The IRMPD spectra were produced by plotting the intensity of all b$_2$ ion
fragments over the intensity of residual b$_2$ ion precursor at each wavenumber. The
laser output consists of macropulses of 9 μs each at a repetition rate of 25 Hz. These
macropulses consist of approximately 600 micropulses at 0.5-3 ps in length, spaced out
at gaps of 16 ns. With laser powers of 0.5-1 W for each micropulse, the total amount of
energy in each pulse comes out to about 30-100 μJ with dependence on the wavelength and exact power of the laser.

4.2.3. MS³ fragmentation and Ion Mobility-MS analysis conditions

MS³ CID fragmentation spectra were produced for each b₂ ion on a Thermo Scientific Velos Pro dual linear ion trap at the Ohio State University. The precursor tripeptide of each system was isolated in the low pressure cell and brought into the high pressure cell for collision induced dissociation. Normalized collision energies of 20 to 23 % (relative to the maximum collision energy of the trap) were used to form the fragments which were moved back into the low pressure cell for isolation of the b₂ ions at an isolation width of m/z 1.0. To produce the MS³ spectra the isolated b₂ ion for each tripeptide fragment was dissociated via CID at 20 to 30 % normalized collision energy.

Ion mobility experiments were performed on a Waters Synapt G2S and the tripeptides were introduced with the same aqueous solution used for both the MS³ and IRMPD experiments. Standard ESI was performed at a capillary voltage of 2.5 kV. Fragment ions were produced in the trap region at collision energies of 5 to 25 eV in order to generate structures that represent a range of possible activation barriers for each tripeptide system. Helium gas flow and ion mobility gas flow (N₂) were set to 180 and 90 mL/min, respectively. In order to calibrate the drift time region, a mixture of drug calibrants including ondansetron, clozapine N-oxide, colchicine, verapamil, and reserpine, defined specifically for N₂ gas IMS experiments in the Waters G2S was used.¹¹⁶ The tripeptides and fragment ions optimized as described in the paragraph below were taken for MOBCAL calculations to determine theoretical collisional cross sections to compare to those found from Synapt IM-MS.
4.2.4. Theoretical Conditions

Theoretical $b_2$ ion structures were calculated using hybrid density functional theory at the B3LYP/6-311+G** basis set. First a conformational search of possible structures was produced using an energy optimization with the Merck Molecular Force Field (MMFF) and torsional sampling of the structures using the Monte Carlo Multiple Minimum (MCMM). From the list of generated structures, the 30-50 most stable unique structures of each sequence were submitted to Gaussian 09 for both optimization and frequency calculations. The frequencies of these structures were scaled at 0.975 and used for comparison to the experimental IRMPD spectra produced at CLIO with artificial peak widths of 10 cm$^{-1}$ used. For comparison of starting precursor peptides, tripeptide ion conformations for Tyr-$\text{Xxx}$-$\text{Ala}$, Val-$\text{Xxx}$-$\text{Ala}$, and His-$\text{Xxx}$-$\text{Ala}$ were found and optimized using the same methods as was performed for all $b_2$ ions with a lower basis set, B3LYP/6-31+G**.

4.3. Results and Discussion
4.3.1. Action IRMPD Spectra of Tyr-flp/Hyp/Flp, Val-flp/Hyp/Flp, and His-flp/Hyp/Flp

In order to determine the structures of the $b_2$ ions of the prolyl ring substituted Tyr-$\text{Xxx}$-$\text{Ala}$, Val-$\text{Xxx}$-$\text{Ala}$, and His-$\text{Xxx}$-$\text{Ala}$, the gas-phase vibrational spectra were collected via action IRMPD, where Xxx was flp, Hyp, or Flp. The blue traces in the top panel of Figure 4.2a-c are the experimental action IRMPD produced at CLIO for the $b_2$ ions of Tyr-flp-Ala, Tyr-Hyp-Ala, and Tyr-Flp-Ala, respectively (in order of increasing $K_{\text{trans/cis}}$ character of the second position prolyl residue). In order to identify the experimental vibrational stretches of the $b_2$ ion structures, theoretical spectra were produced from the respective Gaussian 09 optimized diketopiperazine and oxazolone structures of $b_2$ ions of each sequence. The red traces below each experimental spectrum are theoretical spectra of the calculated lowest energy diketopiperazine (middle panel) and oxazolone
structures with corresponding models inset into the spectra. For the respective Gaussian optimized b$_2$ ion structures, the diketopiperazine is the structure of lowest relative energy (0.0 kcal/mol). Each oxazolone structure was found to be at least 11 kcal/mol higher in energy than the diketopiperazine structure and in the case of Tyr-Flp, is 21 kcal/mol higher in energy as indicated in Figure 4.2c. In the experimental spectra obtained for all three b$_2$ ions a stretch is observed in the 1950 cm$^{-1}$ region, with particular dominance of this region for Tyr-Flp, and two broad band features observed within the region between 1580 and 1620 cm$^{-1}$, respectively, possibly suggesting multiple, overlapping peaks. The 1950 cm$^{-1}$ stretch is indicative of an oxazolone structure, corresponding to the amide I carbonyl stretch observed for each theoretical oxazolone spectrum (red trace in the bottom panel Figure 4.2). The theoretical spectra of the oxazolone ions also show multiple bands in the region $\sim$1600 cm$^{-1}$ suggesting the broad feature observed for Tyr-Flp in particular could be due to this structure. For Tyr-Hyp and Tyr-Flp (Figure 4.2b and c), this oxazolone band region is the prominent feature of the spectrum above 1200 cm$^{-1}$ suggesting a dominant structure for the Flp and Hyp containing b$_2$ ions are the oxazolone structures, while for Tyr-flp there are a unique set of bands at approximately 1670 and 1750 cm$^{-1}$ that make up a major proportion of the fragmentation efficiency signal. For Tyr-Hyp, however, when zoomed in x10, the similarly placed peaks at 1680 and 1750 cm$^{-1}$ become clear. The calculated diketopiperazines (red trace in the middle panel Figure 4.2) match these two peaks and correspond to the ring carbonyl stretching (1750 cm$^{-1}$) and the C=N in-ring stretching (1670 cm$^{-1}$). Interestingly, there are no high intensity, readily observable peaks for the Tyr-Flp (Figure 4.2c) b$_2$ ion that match the diketopiperazine stretching region, though the stretch at $\sim$1735 cm$^{-1}$ shown in the green zoom-in may suggest a faint band in this case. Considering the ratio of the diketopiperazine bands to the oxazolone band between the three systems it is clear that the prevalence of the diketopiperazine grows in proportion
as the identity of the prolyl ring changes from one with the highest $K_{trans/cis}$ (Flp), to slightly lower $K_{trans/cis}$ (Hyp, to the lowest $K_{trans/cis}$ (Flp).

Figure 4.2 Experimental IR spectra (blue) of a) Tyr-flp-Ala, b) Tyr-Hyp-Ala, and c) Tyr-Flp-Ala, (green line insets are x10 zoom) compared to the theoretical diketopiperazine and oxazolone structures of each, red traces in the middle and bottom panels respectively. The relative energies of each pair of calculated structures as well as ball and stick models of each structure are shown in the insets of each calculated spectrum.

Figure 4.3a-c contains the action IRMPD experimental spectra produced at CLIO for the $b_2$ ions of Val-flp-Ala, Val-Hyp-Ala, and Val-Flp-Ala, with matching oxazolone and diketopiperazine models shown below in red for each case. For Val-flp, there is a clear peak at 1760 cm$^{-1}$ and a smaller peak at 1690 cm$^{-1}$ adjacent to a broad feature at $\sim$1600 cm$^{-1}$. These bands correspond to the ring carbonyl stretching (1750 cm$^{-1}$) and the C=N in-ring stretching (1670 cm$^{-1}$). The carbonyl stretch is in excellent agreement with the experimental band at 1760 cm$^{-1}$ whereas the C=N stretch is significantly blue shifted in the action IRMPD spectrum. The lower diketopiperazine calculated “fingerprint” band at 1645 cm$^{-1}$ for Val-flp is about 40 cm$^{-1}$ red-shifted from the 1690 cm$^{-1}$ experimental peak. In examining the theoretical spectra of the higher energy diketopiperazines, no pair of diketopiperazine stretches was found from the list of computed structures to match
completely the two experimental peaks at 1690 and 1750 cm$^{-1}$. In the case of Val-Flp, the green enhancement line is set to x10, however, bands at 1690 and 1750 cm$^{-1}$ are still not discernible. There is, however, a small band at 1650 cm$^{-1}$ adjacent and contributing to broad feature centered at about 1615 cm$^{-1}$. This 1650 cm$^{-1}$ band matches well with the C=N in-ring stretch of the calculated diketopiperazine, but the lack of a corresponding ~1700 cm$^{-1}$ feature strongly suggests that this is the oxazolone band that corresponds to the smaller 1640 cm$^{-1}$ band of the two oxazolone peaks at 1590 cm$^{-1}$ and 1640 cm$^{-1}$.

Figure 4.3  Experimental IR spectra (blue) of a) Val-flp-Ala, b) Val-Hyp-Ala, and c) Val-Flp-Ala, (green line insets are x10 zoom) compared to the theoretical diketopiperazine and oxazolone structures (red) of each. The relative energies of each pair of calculated structures as well as ball and stick models of each structure are shown in the insets of each calculated spectrum.

Like all other oxazolone models, the His-containing oxazolones have a distinguishing oxazolone C=O stretch at ~1930 cm$^{-1}$. The experimental spectra for His-flp (Figure 4.4a and His-Hyp (Figure 4.4b) show no peaks in the ~1900 cm$^{-1}$ region suggesting that there is no oxazolone population for either. The His-Flp spectrum (Figure 4.4c) shows a low intensity peak at 1920 cm$^{-1}$ that is red shifted from the theoretical oxazolone at 1950 cm$^{-1}$. This peak may, however, be an artifact from the low amount of the b$_2$ ion.
population for His-Flp, which is about 5 times lower in intensity than for the \( b_2 \) ion produced at equivalent collision energies for His-flp. All three experimental spectra show dominant peaks at 1600, 1650, and 1750 cm\(^{-1}\), which match extremely well to the corresponding imidazole protonated diketopiperazine models for each substituted prolyl ring system. The peaks at 1600 and 1650 cm\(^{-1}\) correspond to the amide C=O stretching hydrogen bonded to the protonated imidazole while the 1750 cm\(^{-1}\) peak is the C=O stretch of the other free amide of the six-membered ring. Without imidazole protonation, the theoretical diketopiperazine vibrational spectra change significantly, as seen in the red calculated spectra labeled “diketo O-prot calc.” for each system. From this data it is clear that the major structure for all three His-Xxx systems is the diketopiperazine and that the protonation site must be on the side chain of the Histidine.

From the action IRMPD spectra, it is clear that there is little to no formation of diketopiperazine when substituting the 2\(^{\text{nd}}\) position proline with a 4\(^{\text{R}}\)-fluorine (Flp - high \( K_{\text{trans/cis}} \)), but a promoting of the diketopiperazine when placing the fluorine in the S position (flp - low \( K_{\text{trans/cis}} \)). The S-substitution retains the oxazolone bands in Tyr-flp (Figure 4.2a) shown by the presence of a weak amide I carbonyl vibrational band intensity at \( \sim 1950 \) cm\(^{-1}\), however, the diketopiperazine structure becomes more favorable. This is in contrast to the Val-Xxx systems (Figure 4.3), in which all three second position prolyl substitutes retain large oxazolone bands from 1930-1950 cm\(^{-1}\) with only the expected diketopiperazine peaks at 1700 and 1760 cm\(^{-1}\) observed in the Val-flp case. This suggests that even if the S-fluoro substitution creates greater cis favorability to promote diketopiperazine structure, there is still a substantial proportion of ions that retain a trans peptide bond, inducing formation of the oxazolone structure when Val is in the first position. Conversely, the IRMPD spectra in Figure 4.4 of a) His-flp, b) His-Hyp, and c) His-Flp shows how the first residue can overcome the usual nature of
*trans* peptide bond character and any effects of the prolyl ring substitution in the second position to enhance one form of the peptide bond over the other.

![Figure 4.4](image)

Figure 4.4  Experimental IR spectra (blue) of a) His-flp-Ala, b) His-Hyp-Ala, and c) His-Flp-Ala, (green line insets are x10 zoom) compared to the theoretical diketopiperazine and oxazolone structures (red) of each. The relative energies of each pair of calculated structures as well as ball and stick models of each structure are shown in the insets of each calculated spectrum.

4.3.2. MS^3 b_2 ion fragmentation of Tyr-flp/Flp, Val-flp/Flp, and His-flp/Flp

In determining the differences in b_2 ion identity, the fragmentation chemistry can confirm the structural identifications elucidated by the IRMPD vibrational spectra above in section 4.3.1. From this point on, because the Hyp substitution showed structural information in between of the two extreme cases of flp and Flp second position, the MS^3 and subsequent analytical sections will focuses on only flp and Flp as the second position prolyl substitution, for Tyr-Xxx-Ala, Val-Xxx-Ala, and His-Xxx-Ala.
Figure 4.5  MS$^3$ of $b_2$ ions of a) Tyr-flp-Ala and b) Tyr-Flp-Ala, acquired on a Velos Pro linear ion trap. Precursor tripeptides were isolated over a 1 Da window and fragmented at 30% HCD collision energy followed by isolation and fragmentation of each $b_2$ at $m/z$ 279 at the same isolation window and 25% CID collision energy. The -Tyr$_{imm}$, -flp$_{imm}$, and -Flp$_{imm}$ labels indicate losses of the neutral tyrosine, S-fluoroproline, and R-Fluoroproline imines while Tyr$_{imm}$, flp$_{imm}$, and Flp$_{imm}$ labels indicate the immonium ions of Tyrosine, S-fluoroproline, and R-Fluoroproline, respectively.

Figure 4.5 presents the fragmentation comparison of the $b_2$ ions formed when Flp and flp are in the second position with Tyr in the first position with MSMS at 30% HCD and MS$^3$ performed at 25% CID. The IRMPD spectra above showed diketopiperazine dominated clearly for Tyr-flp while only oxazolone was readily observed for Tyr-Flp, and significant differences in MS$^3$ of the two $b_2$ ions are observed. A distinct feature of the Tyr-flp $b_2$ ion fragmentation is that its most intense peak is the Tyr immonium ion and not the $a_2$ ion as is the case for the MS$^3$ spectra obtained from Tyr-Flp. Because it is most facile to form the $a_2$ through the oxazolone structure via a carbonyl loss, the fact that this fragment is less intense for the Tyr-flp $b_2$ correlates with the action IRMPD.
results in which the oxazolone band stretches were less intense for Tyr-flp. Another important difference between the ions is that the Tyr-flp b_2 loses the HF group with some regularity from the b_2 and from the a_2 while there is no HF loss intensity from the b_2 or any subsequent fragment for Tyr-Flp. Likewise, there is a CO_2 loss from the b_2 ion only observed for Tyr-Flp and a greater proportion of the Tyrosine-imine loss for Tyr-Flp. On the other hand, for Tyr-flp, the flp-imine neutral loss is prevalent, based on the presence of several fragments corresponding to its neutral loss. This was made clear by the proportion of b_2-flp_{im}-H_2O and b_2-flp_{im}-CO-NH_3 for Tyr-flp (10% intensity for each of these fragments compared to 2% and less than 1%, respectively for Tyr-Flp) and greater b_2-Tyr_{imi} (at about 10% versus 2% for Tyr-flp) for Tyr-Flp.

Figure 4.6 shows the b_2 MS^3 fragmentation spectra of Val-flp-Ala (Figure 4.6a) and Val-Flp-Ala (Figure 4.6b). Both fragmentation spectra were collected using the same 23% CID collision energy for the precursor and b_2 ion. The top spectrum, Val-flp-Ala contains a residual precursor b_2 ion at approx 23% normalized CID intensity and the a_2 ion (loss of C=O) as the most intense peak. The HF loss from the a_2 and the [HF+CO] loss from a_2 also produce dominant peaks at this fragmentation energy. The b_2 ion undergoes NH_3 loss, HF loss, a Valine-imine loss to produce m/z 144, as well as combinations of these losses (a_2-HF-CO-NH_3 and b_2-imine-CO). Additionally, both the flp and Val immonium ion fragments are observed. In the case of the Val-Flp b_2 ion, significantly fewer high intensity fragments are observed. Again, the most intense peak in the spectrum is the a_2 ion at m/z 187, followed by the imine loss fragment. Other fragments present include the b_2-H_2O, a peak at m/z 171 believed to be a CO_2 loss from the b_2, a CO loss from the b_2-imine fragment, and the two peptide immonium ion fragments Val_{im} and Flp_{im}. Significantly, for the Val-Flp b_2 ion, no peak of reasonable intensity which corresponds to a HF loss is observed. This may be directly due to the C^\gamma-exo ring conformation of the Val-Flp b_2 ion, in which the substituent is positioned in the
proline ring such that it is inaccessible to any other functional groups within the small, inflexible fragment ion. Conversely, the Cγ-endo conformation of the Val-flp b2 ion makes it readily accessible to H-bonding with the other functional groups, allowing many possible fluorine interactions to occur resulting in HF loss, as observed (b2-HF, a2-HF, a2-HF-CO, a2-HF-CO-NH3), a point which is shared by the Tyr case above (Figure 4.5 and is discussed in more detail below.

Figure 4.6 MS^3 of b2 ions of a) Val-flp-Ala and b) Val-flp-Ala on Velos Pro linear ion trap. Precursor tripeptides were isolated at one m/z windows and fragmented at 23% CID collision energy followed by isolation and fragmentation of each b2 at m/z 215 at the same isolation window and collision energy. The Valimm label indicates the loss of the Valine neutral imine while Valimm, flpimm, and Flpimm labels indicate the immonium ions of Valine, S-fluoroproline, and R-Fluoroproline, respectively.
Significantly, it is clear that there is more residual $b_2$ in the spectrum of Val-flp than the corresponding Flp-containing ion precursor, where the $b_2$ ion intensity has almost disappeared. This suggests that the $S$ position fluorine of flp ($C^\gamma$-endo) is adding stability to the $b_2$ ion, compared to $R$ position fluorine of Flp ($C^\gamma$-exo). It may also be that the ring puckering is retained in the oxazolone structure and is contributing to the significant differences observed in the MS$^3$ spectra of the tripeptides containing the two substituted prolyl ring residues. The stability of these fragment ions could in part be due to the ability of the fluoryl functional group to participate in further fragmentation pathways of the molecule, making the fragment’s inherent stability dependent on the ring substituents stereochemistry. This would provide an explanation for the significant differences in $b_2$ precursor signal intensity observed, at the same fragmentation energy. Despite the fact that the $b_2$ ion appears more stable for Val-flp there are, however, also a significantly greater number of different fragments for this $b_2$ ion. Interestingly, this is less of a feature in the tyrosine fragmentation comparison (Figure 4.5), where the two precursors are both almost completely gone, there is, however, approximately double the intensity of the Tyr-flp $b_2$ ion precursor relative to the Tyr-Flp precursor suggesting there is also a stabilizing influence to the $S$-position fluoryl group in the Tyr-flp.

Figure 4.4 presents vibrational data of the His-Xxx-Ala peptides, which form strictly diketopiperazine structures for the $b_2$ ions. In order to further investigate this further MS$^3$ experiments were performed. Figure 4.7 shows a comparison the $b_2$ fragmentation for the His-flp and His-Flp sequences. As expected, the major fragments and their corresponding relative ratios are extremely similar for His-flp (Figure 4.7a) and His-Flp (Figure 4.7b), in which the $a_2$-CO-NH$_3$ is the most intense ion in both cases. Additionally, the histidine immonium ion is also prevalent in each case. There are, however, a few interesting differences between the $b_2$ ions where presumably there is only a difference in the stereochemical location of the F of the prolyl ring in the second position ring. One
significant difference is in the relative ratio of the $a_2$ and related ions, with a high population of C=O loss ion (i.e. $a_2$ ion) for the His-Flp $b_2$ case at an almost equivalent proportion to the highest fragment population of $a_2$-CO-NH$_3$, whereas the His-flp $a_2$ forms at about 20 % of the relative abundance. This was also observed for Tyr-Xxx, but since the His-flp and His-Flp were both observed to be diketopiperazine, the differences in relative ratios of this ions for the His case cannot be explained on the basis of oxazolone versus diketopiperazine population and instead must be due to the conformation of the prolyl ring induced by the position of the fluorine substitution. Additionally, the HF loss from the direct $b_2$ ion is only observed for the His-flp case, as was the case for both Val-Flp and Tyr-Flp. Unlike the Val and Tyr first position cases, however, for the His first-position both systems had an HF loss from the $a_2$ ion along with the additional losses for the fragments ($a_2$-HF-NH$_3$ and $a_2$-CO-NH$_3$-HF). This observation suggests the possibility that the formation of the $a_2$ of the His-Flp case enables the fluorine atom to undergo intramolecular reactions that it could not access in the $b_2$ ion and that it is the diketopiperazine derived $a_2$ that is unique for this accessibility. Likewise, it is important to note that the histidine imidazole function group could also have interacted with the His-Flp fluorine atom itself causing an observation of this functional group loss not observed for Tyrosine or Valine. This MS$^3$ comparison also shows how two mostly similar ions, known to be diketopiperazine in structure, can still be distinguished based on the stereochemistry of a relatively remote part of the molecule and hence the power of MS to distinguish even slight alterations in structures of molecules.
Figure 4.7  MS$^3$ of b$_2$ ions of a) His-flp-Ala and b) His-Flp-Ala on Velos Pro linear ion trap. Precursor tripeptides were isolated at one m/z windows and fragmented at 20% CID collision energy followed by isolation and fragmentation of each b$_2$ at m/z 253 at the same isolation window and 30% CID collision energy. The -Flp$_{imi}$ and -flp$_{imi}$ labels indicates the loss of the two fluoroproline neutral imines while His$_{imi}$, flp$_{imi}$, and Flp$_{imi}$ labels indicate the immonium ions of Histidine, S-fluoroproline, and R-Fluoroproline, respectively.

4.3.3. Ion Mobility Comparisons

Up to this point, both the vibrationally active regions and the resulting dissociation products of the b$_2$ ions for Tyr-, Val-, and His-containing prolyl ring substituted tripeptides have been presented and discussed. In order to further probe these gas-phase structures and the and the relative sizes they adopt, ion mobility experiments were also performed, in which ions are separated by their size, shape and charge. The Tyr-F/flp
drifttime populations are shown at multiple collision energies in Figure 4.8. For the Tyr-flp case, all three collision energies show identical spectra at a CCS of 161 Å² with a peak width of about 10-12 Å. In the Tyr-Flp case, there are two drift times, a lower one at 157 Å² and a higher one at 161 Å² matching the one drift time population of Tyr-flp. At 5 eV (Figure 4.8d), the 161 Å² peak is only a small shoulder next to a dominant 157 Å² all with peak widths very similar to that of the Tyr-flp 161 Å² peak. As Trap collision energy increases, however, the 161 peak goes from a small, barely resolved shoulder, in 5 eV, to about 23 % of the relative intensity at 15 eV (Figure 4.8e), and to 85 % relative intensity with a defined maximum at 25 eV (Figure 4.8f) matching the peak position of Tyr-flp exactly. From the IRMPD spectra in section 4.3.1, Tyr-Flp was found to be dominant by oxazolone stretches with no sign of any diketopiperazine contributing to the spectrum (Figure 4.2c). Yet the IRMPD was only performed at one standard energy, and it is possible that the slow-heating of the 3-D ion trap provided energies unable to access the diketopiperazine structure for Tyr-Flp. Likewise, the only peak seen for Tyr-flp, which had a dominant diketopiperazine structure from IRMPD (Figure 4.2a) is presumably the diketopiperazine distribution, and due to the high energy deposition of the QTOF instrumentation, the oxazolone structure may not be part of the ion population at higher energies, as the Tyr-Flp seems to form a drift time species matching exactly with Tyr-flp’s at 161 Å². The diketopiperazine and oxazolone CCS calculated using MOBCAL were found to be , which are much higher than this so direct comparison of these experiment and theoretical results is problematic. While absolute determinations of CCS using the MOBCAL calculations are not verifiable, the relative CCS differences between calculated diketopiperazine and oxazolone can be used to help interpret the IM-MS results in Figure 4.8. For Tyr-Flp, the lowest energy calculated oxazolone was found to be 166.7 Å² and lowest diketo 167.7 Å². It would make sense being that the 161 Å² is diketo and that the lower drift time for Tyr-Flp is oxazolone at 157 Å² but since there
were no diketo bands for Tyr-Flp from IRMPD and no sign of two structures for Tyr-flp in IM-MS, yet a confirmation of two in IRMPD, the issue could be a result of different instrumental conditions but this needs further investigation to reconcile the incongruence of the two techniques.

Figure 4.8 Ion Mobilograms of the b2 ions of Tyr-flp-Ala at a) 5 eV, b) 15 eV, and c) 25 eV and Tyr-Flp-Ala and the same energies in d), e), and f), respectively.

The IMS distributions of both Val-flp and Val-Flp in Figure 4.9 show one drift time at 145 Å², with no shifting of upon increasing collision energy. The first possibility for explaining this feature, since IRMPD suggested a mixture of oxazolone and
diketopiperazine for all three prolyl substitution structures (Figure 4.3), was that the change in drift time between the oxazolone and diketopiperazine may be too small to be observed with the IMS resolution provided by the Waters Synapt G2S TWIG drift cell. For MOBCAL comparison, the N₂ calculated results provided CCS much better in agreement to those experimentally determined than those found for the Tyr case with theoretical diketopiperazines of 144.8 Å² for Flp and 144.4 Å² for flp and oxazolones of 145.4 Å² for Flp and 145.8 Å² for flp. With possible shifts between experimental and calculated data still possible, it is not reasonable to assume the 145 Å² for each Val b2 comes from the oxazolone. With a less than 1 Å² shift for each MOBCAL result, it is too close to tell and it may well be the case that both structures are present in the wide peaks of each Val b₂ distribution in Figure 4.9. As with Tyr-flp/Flp, the peaks are slightly broad at the base, with about 9-10 Å² spanning each peak about the 10 % intensity mark, and a noticeable tailing moving upwards in drift time as well suggesting the present of a mixture of peaks. Even though IRMPD suggested that Val-Flp was only populated by an oxazolone structure, given the energy difference for the two instruments discussed in the Tyr case above, it is reasonable to assume that this may be possible for Val as well.
The IM-MS results of His-Flp and His-flp shown in Figure 4.10 show energy independent drift time distributions that differ by 1 Å$^2$ in CCS. For the His-flp IMS distributions at 5 eV (Figure 4.10a), 15 eV (Figure 4.10b), and 25 eV (Figure 4.10c), the peak is centered at 154 Å$^2$ and for His-Flp at the same three collision energies (Figure 4.10d-f) the peak is at 155 Å$^2$. One drift time presumably equates to one structure which from IRMPD and MS$^3$ results suggest that both are still diketopiperazine with the R versus S fluoroproline position causing the shift. It is important to note that the width of all six peak distributions was about 6 Å$^2$ at the ~10% height with no tailing observed.
Comparing this to the larger peak widths of Tyr (10-12 Å²) and Val (8-10 Å²) the thinness of the His case presents another compelling reason to postulate that both b₂ ions form diketopiperazine even in this separate instrument and conditions, which for Val and Tyr showed some significant differences than the instruments used for IRMPD and MS³. Likewise, calculations support this conclusion despite a shift observed in absolute CCS. For His-flp and His-Flp the diketopiperazine protonated on the imidazole ring are 169.3 Å² and 169.8 Å², respectively. The relative shift in MOBCAL CCS is almost an Å² with His-Flp being the larger drift time as is exactly the case for the experimental condition, over several trials and different energies.

![Ion Mobilograms of the b₂ ions of His-flp-Ala at a) 5 eV, b) 15 eV, and c) 25 eV and His-Flp-Ala and the same energies in d), e), and f), respectively.](image)

Figure 4.10 Ion Mobilograms of the b₂ ions of His-flp-Ala at a) 5 eV, b) 15 eV, and c) 25 eV and His-Flp-Ala and the same energies in d), e), and f), respectively.
4.3.4. Comparison to solution-phase trends via gas-phase theoretical optimization:

The experimental action IRMPD, MS³, and IM-MS spectra shown above define a clear trend for oxazolone/diketopiperazine ratios for S-substituted flp that agree with the $K_{\text{trans/cis}}$ trend found by Raines et al. using solution-phase 1-D proton NMR. This trend is that for the S-substituted flp second position tripeptides of Tyr-flp and Val-flp the cis peptide bond is more likely in solution and the experiments above follow that the diketopiperazine in present in greater proportions for these substations. As the $K_{\text{trans/cis}}$ trend observed previously in solution correlates with the peptide bond conformation necessary for diketopiperazine formation, it is desirable to also determine whether the prolyl ring puckering observed in the solution-phase is preserved the gas-phase. The lowest energy N-protonated tripeptides (a) Tyr-flp-Ala, (b) Val-flp-Ala, (c) Tyr-Flp-Ala, and (d) Val-Flp-Ala, calculated with DFT, are shown in Figure 4.11. The His-Xxx-Ala tripeptides were not considered in this section because the IRMPD data presented Figure 4.4 shows that regardless of the identity of the Xxx prolyl ring substitute, the Histidine in the first position locked the peptide in position for formation of only the diketopiperazine $b_2$ ion upon dissociation and hence prolyl ring substitution in the Histidine case is practically irrelevant.

As shown in the boxed region in Figure 4.11 on the prolyl ring section of the molecule, the fluorine substitution on the 4th position of the prolyl ring causes the ring to pucker towards the side on which the fluorine is added. This was observed previously by Crestoni et al. for Hyp and hyp in silico in addition to Raines et al. in solution-phase studies of collagen and Ac-Xxx-OMe where Xxx = Pro, flp, Flp, hyp, or Hyp. For Tyr-flp-Ala and Val-flp-Ala in Figure 4.11a and c, the ring orients into a C-endo position and the fluorine is involved in a hydrogen bond with the 3rd amide nitrogen (alanine). For Tyr-Flp-Ala and Val-Flp-Ala, the ring orients itself into the C'-exo conformation, away from the majority of the other functional groups. Likewise, it appears that the strain of
this ring results in less hydrogen bonding for the N-terminus and an overall lack of stability for the tripeptide (3 kcal/mol higher in energy). Val-flp-Ala is approximately 2.6 kcal/mol lower in energy than Val-Flp-Ala and Tyr-flp-Ala is 2.4 kcal/mol lower than Tyr-Flp-Ala.

Figure 4.11  a) Tyr-flp-Ala, b) Tyr-Flp-Ala, c) Val-flp-Ala, and d) Val-Flp-Ala tripeptide models calculated at the B3LYP/6-31+G** with Gaussian 09, with fluorine indicated in gold. Using gas-phase conditions the ring puckering of the R and S fluoroproline structures form the Cγ-endo and Cγ-exo conformations that are present in solution. Prolyl rings for all fluoroprolines are oriented to show a full side view to most clearly display the ring puckering.

Previous studies on the ring puckering of proline only considered whole peptides and not the fragments corresponding to the b2 ion, hence, Gaussian structures of the
oxazolone and diketopiperazine fragments were also studied here to determine if ring puckering would be retained in their gas-phase formation. Figure 4.12 shows DFT optimized lowest energy structures of the oxazolones (Figure 4.12a-d) and diketopiperazines (Figure 4.12e-h) of Tyr-flp, Tyr-Flp, Val-flp and Val-Flp. As shown in the MS$^3$ data above, the R and S position of the fluoro-substitution has a large effect on not only the precursor peptide which forms the b$_2$ but on the b$_2$ ion itself. Here the theoretical structures show that the ring puckering is retained independent of whether the ion is oxazolone or diketopiperazine. Furthermore, the models containing flp in the second position explain the HF loss observed for Tyr-flp and Val-flp and the absence of these fragments for the Flp analogues in MS$^3$ experiments (Figure 4.5b and Figure 4.6b, respectively). For Flp containing oxazolone or diketopiperazine (Figure 4.12b, d, f, and h), no other functional group can participate in an internal reaction with the fluoryl group on the prolyl ring without a significant strain on the molecule, resulting in little to no reactivity of this substituent after formation of the b$_2$ product ion. In contrast for flp (Figure 4.12a, c, e, and g), H-bonding to F is possible and allows for reactions resulting in the HF-loss, as seen in the flp-containing b$_2$ ions above in the MS$^3$ section 4.3.2 (Figure 4.5a, Figure 4.6a, & Figure 4.7a)

The potential of theoretical calculations coupled with the data from the IRMPD and MS$^3$ allows for a confirmation of the not only the structural differences of the R/S prolyl substitutions but also helps elucidate the reason for why these differences occur in the gas-phase.
Figure 4.12 Oxazolone $b_2$ models (top row) of a) Tyr-flp, b) Tyr-Flp, c) Val-flp, d) Val-Flp and their corresponding diketopiperazine $b_2$ ion models directly below each oxazolone model (e-h) calculated at the B3LYP/6-311+G** level with Gaussian 09. Even in these two fragment structures, the ring pucker conformations of the R and S fluoropropoline structures from the tripeptide precursor state are retained.

4.4. Conclusions:

The action IRMPD results above reveal that the substitution of the prolyl ring in the second position of tripeptides has a significant effect on relative populations of diketopiperazine and oxazolone for the $b_2$ ion fragment. From IRMPD spectra in section 4.3.1, distinct trends were found for Tyr, Val, and His with Flp or flp in the second position. For Tyr-Flp versus Tyr-flp, there is a stark contrast in stretches with oxazolone the only structure observed for Tyr-Flp and the diketopiperazine the dominant structure for Tyr-flp, with only a tiny peak corresponding to oxazolone present. For Val, the dominant structure remained the oxazolone in both case but with a diketopiperazine population present for Val-flp. For His, the identity of the $b_2$ ion population was independent of the stereochemistry of the 4\textsuperscript{th} position of the prolyl ring and both His-flp and His-Flp were exclusively diketopiperazine. These trends carried over into the Velos Pro instrument where MS3 of the b2 ions was performed. For Tyr, the difference
between the fragmentation spectra was considerable, implying that the two populations were truly difference structures. Val showed less difference between the two while His showed very little, as matched the differences in IRMPD spectra for these two cases. Another trend was also observed between all three first position cases, where the flp residue underwent fluorine atom loss by HF consistently whereas this loss was blocked or limited for the Flp substitution.

This ion’s structure dependence is correlated to the $K_{\text{trans/cis}}$ character previously found in solution-phase studies of these prolyl substituted residues. From the data presented it seems clear that first peptide bond directly influences the presence of the diketopiperazine in gas-phase fragmentation. There is the possibility, however, that the lack of diketopiperazine ion for Flp and the residues high in solution trans peptide bond prevalence are parallel processes independent of each other, but the correlation strongly suggests that they are linked. Second position substitutions of flp and Flp, the most extreme in solution trans/cis character systems previously studied by Ronald Raines, produced a primarily cis or trans $b_2$ ion matching the previously observed trend. Additionally, the $b_2$ ions of $R$ and $S$ fluoroproline experience different degrees of MS$^3$ fragmentation. By moving the fluorine on the gamma carbon of the prolyl ring from the $S$ to the $R$ conformation there is a significant change to the both the stability of the $b_2$ ion and identity of its subsequent MS$^3$ fragments. As confirmation of density functional theory that ring puckering is in fact retained in the gas-phase, the behavior of the gas-phase precursors and $b_2$ ions (whether oxazolone or diketo) could be ascribed to the same ring puckering trend as observed in previous gas and solution-phase studies.
Chapter 5. Pomalidomide and Lenalidomide Gas-phase Structural Studies

5.1. Introduction

A number of anti-tumor drugs are being actively researched using a variety of techniques investing in research determining both their structure and function as it pertains to biological activity. Among these are pomalidomide (Figure 5.1a) and lenalidomide (Figure 5.1b) and a series of derivatives of lenalidomide, which are derivatives of the molecule thalidomide and are part of a class of drugs known as the immunomodulatory drugs (IMiDs). \(^{174-176}\) Both drugs are currently used in combination with the glucocorticoid drug dexamethasone to treat multiple myeloma, an incurable cancer of human plasma. \(^{177-180}\) Current research on these drugs is directed at elucidating the mechanisms for inducing apoptosis, identifying the ways in which the drugs interact with their target proteins, and determining how each drug is metabolized and expelled from treated organisms. \(^{180}\) The current approach used to address these functional questions assays specific biomolecules (proteins, hormones, etc.) and evaluates any change in regulation upon addition or removal of a specific drug. \(^{71}\) In order to further understand the differences in function of these molecules supplementary structural studies are required. In this chapter a combination of gas-phase techniques are used to provide insight into the structures of these medically important compounds. Techniques used include action infrared multi-photon dissociation (IRMPD) spectroscopy, a method that combines a tunable wavelength laser and a mass spectrometer to gather chemical information on each compound in the gas phase, ion mobility to monitor the shape and size of ions in vacuum, and H-D exchange to observe
the chemical nature of functional groups on the ions. Application of these techniques enables a direct determination of the chemical structure of the drug molecules without the confounding variables associated with in vivo studies. During gas-phase ionization molecules travel from a polar (solvent) environment to a more non-polar environment (in vacuo), this can be thought of as simulating the travel across membranes of cells, as well as the environment on the interior of globular proteins. Therefore, the analysis of the charge state distributions of the molecules, lenalidomide and its derivative pomalidomide, could provide insight into why only one can cross the blood-brain barrier. Additionally, by electrospraying from solution into the gas-phase, the solution conditions which influence the amide-iminol tautomerization of thalidomides can be assessed, this is an important point of consideration as it is known to alter their effectiveness.\textsuperscript{180-182}

![Figure 5.1 Structures of two anti-cancer drugs\textsuperscript{180} that are part of the thalidomide class: a) Pomalidomide,\textsuperscript{178} b) Lenalidomide.\textsuperscript{179}](image)

The underlying structural chemistry of drugs must be determined in order to understand how they can adapt their structure as they react to various biological environments. If a therapeutic is to be of benefit, it must successfully reach and subsequently interact with its target and in order to do so often have to negotiate through complex and varying environments. During this process the molecule can transition through blood, membranes, intercellular fluid, and possibly acidic stomach fluid;
environments of different pHs and different solution polarities.\textsuperscript{183,184} Moreover, certain anti-tumor molecules must take part in intracellular protein activity and their chemistry in that stage must allow them to fit into binding motifs. Isomerization of the drug molecule is likely to play a large role in the efficiency with which the molecule migrates through these different environments and takes part in specified biological functions.\textsuperscript{181,185,186} Action IRMPD spectroscopy offers a direct determination of the structure and protonation site(s) of the active molecules involved in cancer treatment. Structural interrogation by this technique will provide valuable information that may help solve several still undetermined questions about these drugs: 1) how can a derivative of one drug (pomalidomide - Figure 5.1a) molecule pass through the blood-brain barrier and the other cannot\textsuperscript{181} when they differ only by the presence or absence of a single carbonyl group (lenalidomide versus its derivative pomalidomide), 2) are there significant differences in the chemistry of the fragment ions which may also help understand the precursors’ metabolism \textit{in vivo}? The field of pharmacokinetics is largely concerned with addressing these questions and gas-phase structural assignments for each candidate cancer drug could provide insight into the understanding how the chemical structure is related to the efficacy of anti-tumor drugs.

Pharmacy research labs like that of Phelps \textit{et al.} are conducting research to better understand the pharmacokinetics of these two drugs with the ultimate goal of improving the efficiency with which they are absorbed by the body as well as their effectiveness in treatments.\textsuperscript{187-189} One of the fundamental objectives of this research is to understand how pomalidomide, which differs from lenalidomide by the absence of a single carboxyl group (C=O) at pomalidomide’s five-membered phthaloyl ring, is able to pass through the blood-brain barrier, while lenalidomide, which is believed to be pH sensitive, cannot.\textsuperscript{190} It is additionally possible that lenalidomide and pomalidomide hydrolyze while in the body as has also been a reported problem with thalidomide.\textsuperscript{184} Lenalidomide
sensitivity to hydrolysis is believed to be related to its sensitivity to changes in pH that it experiences when moving between different biological environments and there is interest in probing the pH levels at which lenalidomide is hydrolyzed.\textsuperscript{183} This could naturally be observed by examining changes to its overall structure even before hydrolysis.

A drug’s ability to transport to a target cell and the mechanisms guiding apoptosis are critical to understanding why certain drug regime treatments work better than others. An understanding of these pathways will additionally provide the means for improving drug delivery to target cells and act as a guideline for synthesizing more potent or efficient analog drugs. The current evaluation methods of these treatments utilize indirect methods of measurement to study \textit{in vivo} effects of the drugs. For example, the up and down regulation of proteins and metabolic levels following drug treatment is currently probed using LC-MS, UV-Vis spectroscopy, and gel electrophoresis.\textsuperscript{182,191} While mass spectrometry and some spectroscopic techniques are used to observe these drug molecules and understand their pharmacokinetics and dynamics, these techniques are only for detection and do not probe the changes to chemical structure that may occur during biological interaction.

The primary goal of the research described in this chapter is to directly observe the protonated structures of lenalidomide and its close derivative pomalidomide and to determine whether they possess unique protonation features that help explain their increased potency and selectivity in anti-tumor growth. For lenalidomide alone, there are a total of six protonation sites and three tautomerizations that can occur. A tautomerization is the shift of a proton and a double bond to convert from an amide structure to that of an iminol structure. Pomalidomide, with its fourth carbonyl, can be protonated at seven sites and possess four tautomerization sites. With protonation sites and amide-iminol forms, these two drugs can possess more than a dozen structures
each. Moreover, if hydrolysis readily occurs for lenalidomide and pomalidomide, the structures of the hydrolysis products will also be explored. In the long term, this project could help direct further study on new derivatives of both of these classes of molecules and also provide a method of study for other anti-tumor molecules being researched today.

For these gas-phase structures (produced by electrospraying from solution), information can be gained to help to develop an understanding of in vivo biology. Recent work has shown that the gas-phase structures can be relevant to solution-phase conditions. Therefore ion structures of the precursor drugs, and, because derivatives and metabolites of these drugs can form in vivo, the characterization of the ion structures of each drug’s fragments was performed. This approach could help direct ongoing research by revealing the ways in which these drugs adapt to different environments. Furthermore, relating these studies to previous and on-going research may shed light on whether the adaptable forms play a role in transport and their in vivo activity.

5.2. Experimental
5.2.1. Pomalidomide and lenalidomide preparation

Pomalidomide and lenalidomide were supplied in solid-form by Dr. Ching-Shih Chen and Dr. Mitchell Phelps of the College of Pharmacy at The Ohio State University (Columbus, OH). Both molecules have low solubility in nano-pure water and were prepared in solutions of 50:50 H₂O:MeOH. Solutions were prepared at 1 mg/mL and diluted down to 10 to 50 μM for further mass spectrometric and spectroscopic analysis.
5.2.2. Action IRMPD Conditions and Bruker Esquire 3000+ Ion trap Parameters

Action IRMPD was performed as described in the previous chapter (Chapter 4 Section 4.2.1) in the Bruker Esquire 3000+, with the electrospray ionization set-up described previously. In this set of experiments, the linear ion trap was used only to isolate the pomalidomide or lenalidomide protonated molecules in MSMS mode and followed by laser irradiation, with 5-10 cm$^{-1}$ steps from 1000-2000 cm$^{-1}$ as with the previously described b ions.

5.2.3. Tandem MS, HDX, and Kinetic Method analysis

Isolation and efficient fragmentation for both MSMS and MS$^3$ CID fragmentation spectra was performed on a Velos Pro mass spectrometer as detailed in Chapter 4 Section 4.2.1. Normalized collision energies in the region of 20 % were used to form the fragments which were moved back into the low pressure cell for isolation of each fragment to perform MS$^3$ with an isolation width of m/z 1.0.

To perform H-D exchange experiments, the Velos Pro ion trap was fixed with a deuterated ammonia line at a T-valve that allowed the ND$_3$ exchange gas to mix with the helium going into the trap and be leaked directly into the high pressure region of the trap. After a full MS scan was performed and a spectrum acquired, the protonated molecule (precursor ion) was isolated in MSMS mode. The accumulation time of the MSMS scan was adjusted to allow for varying amounts of exchange with the ND$_3$ gas in the high pressure region of the dual linear cell.

In order to perform HDX on the fragments, MS$^3$ was necessary, with accumulation times raised for exchange and collision energy percentage applied in the MSMS mode to produce the fragment before exchange. Precursor ions as well as fragment ions were incubated from period of 10 ms, the shortest accumulation time which would result in no exchange, up to 10 seconds for a large degree of H-D exchange.
Kinetic method experiments were also performed with the Velos Pro dual linear ion trap. The reference compounds chosen for this particular experiment were a set of amino acids ranging in proton affinities from 905.9 kJ/mol to 933.3 kJ/mol. Those chosen included serine, isoleucine, threonine, tyrosine, proline, and asparagine and their matching proton affinities are shown in Table 5.1 calculated by Afonso et al.\textsuperscript{112} These compounds were chosen to be used to determine the semi-quantitative proton affinities of Pom and Len has previous studies have done previously.\textsuperscript{111,113,114} References bases were dissolved in solutions of 50:50 H\textsubscript{2}O:MeOH with 0.1% formic acid to solutions of \textasciitilde10 μM and mixed 1:1 with pomalidomide and lenalidomide prepared at matching concentration and solution conditions. The 1:1 analyte:reference base solutions were ionized using standard electrospray utilizing a capillary voltage of 4.5 kV and the proton bound heterodimers (A---H+---B) isolated in MSMS mode. For example, in the case of (Len+Ser)\textsuperscript{H+}, with MW's of 259 g/mol for lenalidomide and 105 g/mol for serine, the heterodimer mass isolated was 105+259+1 at m/z 365. To collect the necessary data, the heterodimers isolated at a window of m/z 1 in MSMS mode were then dissociated at incrementally increasing activation amplitudes starting at 0 % and going up to 50 % at steps of 0.5 to 1 %. At all collision energies, the intensities of the heterodimer precursor, protonated drug analyte, and reference base were monitored and recorded and the ratios of these values used to produce the kinetic method plots below in section 5.3.5.
Table 5.1 Table of reference bases used for extended kinetic method plot determination of proton affinity. All proton affinities calculated by Afonso et al.\textsuperscript{112}

<table>
<thead>
<tr>
<th>Reference Base</th>
<th>PA\textsuperscript{a} (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>905.9</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>913.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>917.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>921.6</td>
</tr>
<tr>
<td>Proline</td>
<td>936.6</td>
</tr>
<tr>
<td>Asparagine</td>
<td>933.3</td>
</tr>
<tr>
<td>Average</td>
<td>921.5</td>
</tr>
</tbody>
</table>

5.2.4. Ion Mobility-Mass Spectrometry conditions

Ion mobility experiments were performed on a Waters Synapt G2S with standard ESI at a capillary voltage of 3.0 kV. Fragment ions were produced in the trap region at trap collision energies of 5 to 20 eV. Helium gas flow and ion mobility gas flow, which was changed to CO\textsubscript{2} to improve the ion mobility separations of such small molecules, were set to 120 and 75 mL/min, while the wave height was set to 35.0 V and wave velocity to 1000 m/s. CCS MOBCAL calculations were not performed nor were polyalanine or drug calibration as it is difficult to compare MOBCAL to CO\textsubscript{2} based drift time calibrations.

5.2.5. Computational Details

All ionic structures were calculated using a hybrid density functional theory at the B3LYP/6-311+G** with Gaussian 09 and starting structures collected using the MacroModel conformational search with all parameters and theory levels identical to those described in Chapter 4 section 4.2.2.\textsuperscript{122,173} As the DFT used was B3LYP/6-
the calculated vibrational spectra were scaled to 0.975 for experimental IRMPD comparison.\textsuperscript{195,196}

5.3. Results
5.3.1. Action IRMPD Spectra of pomalidomide and lenalidomide

Figure 5.2  Action IRMPD spectra of a) pomalidomide and b) lenalidomide.

The spectra in Figure 5.2 show the gas-phase vibration spectra of the positively charged ions for pomalidomide (Figure 5.2a) and lenalidomide (Figure 5.2b), with the structures for each drug molecule inset into each spectrum. It is important to note that ionization of these two drugs results in varying amounts of precursor ion intensity before the isolation and irradiation. Direct comparison shows how the removal of one of the carbonyl function groups from the phthaloyl ring of pomalidomide to produce the
isoindolinone ring of lenalidomide can result in significant differences in several vibrational regions for the two ions. Both spectra have rather complex and broad regions that are similarly located in several cases. Notably, both have features in the 1720-1820 cm\(^{-1}\) range with at least two peaks in each case. Furthermore, there are an intense set of peaks at \(\sim 1650\) cm\(^{-1}\), which for pomalidomide is the second most intense region of the spectrum. In this region, the more intense peak for pomalidomide is centered at 1647 cm\(^{-1}\), about 25 cm\(^{-1}\) blue shifted from the highest lenalidomide peak in the matching region, at 1620 cm\(^{-1}\). Additionally, there are more shoulder peaks just below 1600 cm\(^{-1}\) for pomalidomide centered at 1580 cm\(^{-1}\).

Considering next the very broad region between 1300 and 1500 cm\(^{-1}\), important observations are made. First, there is a region of “dead” vibrational activity for both drug ions at \(\sim 1530\) cm\(^{-1}\), which for pomalidomide is actually an area of relatively diminished vibrational activity with clear fragmentation efficiency but which dips dramatically below the other adjacent regions of the spectrum. Then below that at \(\sim 1470\) cm\(^{-1}\) both pomalidomide and lenalidomide have very intense peaks, which for lenalidomide exhibits a clear shoulder at 1430 cm\(^{-1}\) and then fragment efficiency signal drops off in the 1380-1400 cm\(^{-1}\) region and returns for a small peak at 1350 cm\(^{-1}\). For pomalidomide, however, the broad feature continues with irresolvable signal in the same region and then another very intense peak at the same 1350 cm\(^{-1}\) region.

While direct comparisons between the two drugs highlights significant differences, comparisons with computational models are required to assess the identity of the vibrational bands. Such studies would hence allow location of the protonation sites for each drug and illustrate how the two rings (the phthaloyl/isoindolinone and glutarimide ring) are positioned relative to each other. Likewise, theoretical comparisons should reveal if there exist any stable and gas-phase observable amide-iminol tautomerization sites and whether these features are actually different between the two drugs. In Figure
5.3 the experimental pomalidomide IRMPD spectrum in Figure 5.3a (top blue panel) is compared to three distinctly different gas-phase protonated pomalidomide models. The first model is shown in Figure 5.3b labeled as “Pomcal1” and is at a high energy (28.03 kcal/mol higher than the lowest calculated structure) but was the best fit of the 61 starting structures calculated. In region 1 (~1800 cm$^{-1}$), matching well with the experimental spectrum, Pomcal1 has two distinct but unresolved stretches at 1789 cm$^{-1}$ and 1774 cm$^{-1}$ (Figure 5.3b) marked in blue. The structure of this calculated model is inset immediately right of the spectrum with these two stretches labeled. The stretch at 1789 cm$^{-1}$ corresponds to the unprotonated carbonyl of the phthaloyl ring and is very close, but slightly red-shifted, to the highest energy stretch of the experimental spectrum at 1804 cm$^{-1}$. The stretch labeled “2” in Figure 5.3b matches the stretch for the carbonyl amide of the glutarimide ring farthest from the phthaloyl ring (O$_{2nd}$). Pomcal2 and 3 also show the same doublet but in slightly different positions with the stretches in Pomcal2 (Figure 5.3c) being at 1770 and 1809 cm$^{-1}$ and the stretches of Pomcal3 (Figure 5.3c) at 1794 and 1814 cm$^{-1}$. These calculated stretches also correspond to the same unprotonated phthaloyl carbonyl (“1”) and the second glutarimide carbonyl (“2”), suggesting, with three unique calculated models that all have the same doublet of carbonyls stretches that match extremely well with the experimental region, that these are the clear identities of the two pomalidomide experimental stretches ~1800 cm$^{-1}$, which was expected considering that with two carbonyls at each ring, both should presumably have at least one unprotonated (or non-tautomerized) carbonyl.

The second important element of the pomalidomide experimental spectrum is the broad feature between 1550 and 1700 cm$^{-1}$ (Region 2). Pomcal1 has two features that match well with the broad and intense peak centered at 1647 cm$^{-1}$. The first stretch, labeled “3” in Figure 5.3b at 1652 cm$^{-1}$, corresponds to the C=N stretch of the HO-C=N iminol tautomer of the glutarimide ring while the adjacent stretch, labeled “4” in Figure
5.3b at 1643 cm$^{-1}$, is the first of three distinct peaks corresponding to the NH$_2$ primary amine on the phthaloyl ring. This and the other two stretches labeled “4” (in green at 1619 and 1599 cm$^{-1}$) for the Pomcal1 structure explain both the intensity of the first feature and the presence of the smaller shoulder. Additionally, the broad nature of the highest peak is expected considering the proximity of stretch “3” with that of stretch “4”. This feature is corroborated with an identical stretch of 1643 cm$^{-1}$ for Pomcal3 (Figure 5.3d) that also corresponds to the unprotonated free amine on the aromatic portion of the phthaloyl ring. Pomcal2 (Figure 5.3c), however, shows no stretches in this broad region and taking a close look at the structure possesses no iminol tautomer on the glutarimide ring and is protonated at the primary phthaloyl amine. Instead these two features are blue shifted of Region 2, at 1740 cm$^{-1}$ (Figure 5.3c), corresponding to a standard amide carbonyl stretch on the glutarimide ring, and 1692 cm$^{-1}$, corresponding to a protonated NH$_3^+$ amine on the phthaloyl ring, where there is no evidence of peaks for the experimental spectrum. Likewise, Pomcal3 has a C=O glutarimide “X1” feature at 1770 cm$^{-1}$ which when paired with stretching features “1” and “2”, would show a triplet feature in the ~1800 cm$^{-1}$ region, and suggests that this is not the case for the pomalidomide ion.

Region 3 between 1250 and 1550 cm$^{-1}$ show lower correlation between the experimental spectrum and the three calculated structures. The only clear agreement is for the stretch labeled “5” for Pomcalc1 (Figure 5.3b), which corresponds to the “breathing” of the bicyclic phthaloyl ring. Furthermore, none of the other stretches observed in this region are clear signs of any “fingerprint” vibrations that would indicate one ionic structure over another for pomalidomide and also for the lenalidomide IRMPD spectra below. The remaining indicative feature of note is the presence of a stretch at 1545 cm$^{-1}$ for Pomcal3 (Figure 5.3d) at the upper part of Region 3. This region contains no definitive peaks for the experimental spectrum and while this stretch corresponds to a
protonated carbonyl on the phthaloyl, it is the carbonyl opposite the one protonated on Pomcal1 and also is hydrogen bonded to the amide nitrogen of the glutarimide ring. Neither of these features is postulated to be a part of the pomalidomide ion population considering the much better matches observed for the experimental spectrum and Pomcal1. Pomcal1 provides a great match due to the presence of the iminol tautomer and protonation site on the carbonyl oxygen opposite the amine side of the phthaloyl ring while Pomcal2 and Pomcal3 are poor matches because of Pomcal2’s amine protonation and both lacking the amide-iminol tautomerization.
Figure 5.3 Comparison of a) experimental IRMPD of pomalidomide to three calculated pomalidomide structures; b) Pomcal1 - phthaloyl carbonyl ring protonated with HO-C=N iminol tautomer (28.03 kcal/mol relative energy), c) Pomcal2 - phthaloyl ring primary amine protonated with standard glutarimide ring (5.78 kcal/mol relative energy), d) Pomcal3 - phthaloyl carbonyl ring protonated H-bonded to standard glutarimide ring nitrogen (13.19 kcal/mol). The dotted black line present for b), c), and d) is an overlay of the experimental pomalidomide spectrum onto each calculated structure’s spectrum.

The IRPMD spectrum of lenalidomide is shown in Figure 5.4 compared to three distinct computed structures in order to determine the most likely nature of the lenalidomide ionic population. Again, for simplicity, the experimental spectrum can be broken up into three separate regions for identification purposes. As with pomalidomide, Region 1 of lenalidomide possesses two peaks, which for lenalidomide are present at
1744 and 1787 cm$^{-1}$. Comparisons are first made to Lencal1, a model calculated to be only 1.57 kcal/mol higher in energy than the lowest energy structure of the 90 candidate structures collected from conformation searches of every protonation and tautomer possible for the molecular ion. Of the 90 structures, this model has the best matching stretches considering all three regions and is comprised of an isoindolinone ring protonated on the one carbonyl of this ring and an on-axis glutarimide ring with no iminol tautomerization for either carbonyl, on contrast to the observations made for the most likely pomalidomide structure. For region 1, Lencal1 possesses stretches at 1775 cm$^{-1}$ and 1736 cm$^{-1}$ (Figure 5.4b), corresponding to the two carbonyls of the glutarimide rings with “1” matching the carbonyl furthest from and “2” closest to, the isoindolinone ring. Additionally, both Lencal2 and Lencal3 have “1” stretches at the most isolated carbonyl on the glutarimide at 1794 cm$^{-1}$ and 1775 cm$^{-1}$, respectively. Neither Lencal2 nor Lencal3 has a stretch at “2” and instead Lencal2 displays a stretch for the adjacent carbonyl on the glutarimide ring, which is hydrogen-bonded to the isoindolinone ring protonated carbonyl, while the corresponding stretch for Lencal3 is the adjacent oxygen taking the iminol form. Both theoretical stretches are not observed in the experimental spectrum (Figure 5.4c and Figure 5.4d).

Region 2 contains a small peak at 1667 cm$^{-1}$, a vibrationally inactive region at ~1650 cm$^{-1}$, and a larger and broad region with a peak centered at 1628 cm$^{-1}$ with a decreasing shoulder indicative of possible smaller peaks overlapping. Like the pomalidomide Region 2, this region, is dominated by the stretches corresponding to the free amine on the aromatic ring of lenalidomide. There are three matching peaks for Lencal1 for stretch “3” at 1638 cm$^{-1}$, 1623 cm$^{-1}$, and 1594 cm$^{-1}$ that fit extremely well with the broad feature at 1628 cm$^{-1}$ in Region 2. Likewise, both Lencal2 and 3 have features corresponding to stretch “3”, since they also have an unprotonated free amine on the aromatic region of the isoindolinone ring. With at least three separate stretches possible
for this unprotonated functional group, it is not surprising that the region would possess a broad feature and like pomalidomide, it seems clear that the primary amine here is very unlikely to be protonated considering the good agreement of the experimental spectrum and all three calculated structures in Figure 5.4.

Finally, like Region 3 of pomalidomide, there are no clearly indicative peaks in Region 3 of lenalidomide to help identify the structures of this drug in the gas-phase. From Region 1 and 2, however, there is strong evidence that for lenalidomide, the ion population possesses little amide-iminol tautomerization and rather exists as a standard O=C-NH-C=O glutarimide ring, with a protonation site on the one carbonyl of the isoindolinone ring and correspondingly no sign of any protonation of the free amine. In comparison to the pomalidomide, there are some similarities in that both seem to have an unprotonated NH₂ primary amine and a protonation of the carbonyl on the five-membered ring of this bicyclic feature, with the pomalidomide carbonyl protonation on the same carbonyl that remained for the lenalidomide (the opposite side of the ring from the amine). The most obvious difference between the IRMPD determined structures of pomalidomide and lenalidomide, if considering the best matched theoretical structures, is the presence of an iminol-tautomerization for the glutarimide ring of pomalidomide while lenalidomide has no evidence of any iminol-forms for the molecule. Additionally, while the on/off-axis trends of the theoretical structures do not directly translate as to the vibrational spectra matched to the experimental spectra, the appropriate shifts in functional group vibrations necessitate the proper positions of the function groups relative to each other (i.e. position of each ring to allow H-bonding or not). Therefore, it is reasonable to argue that the most likely conformation for both pomalidomide and lenalidomide is an out-of-plane relationship between their respective sets of two rings.
Figure 5.4  Comparison of a) experimental IRMPD of lenalidomide to three calculated lenalidomide structures; b) Lencal1 - isoindolinone ring carbonyl protonated with standard glutarimide ring and on-axis of isoindolinone (1.57 kcal/mol relative energy), c) Lencal2 - isoindolinone ring carbonyl protonated H-bonded to on-axis standard glutarimide ring carbonyl, (0.00 kcal/mol relative energy), d) Lencal3 - isoindolinone ring carbonyl protonated with off-axis HO-C=N iminol glutarimide ring (23.45 kcal/mol).  The dotted black line present for b), c), and d) is an overlay of the experimental lenalidomide spectrum onto each calculated structure’s spectrum.

It is important to note here that these IRMPD comparisons provide the most accurate chemical stretching information of the ionic structure that is either most populated in a mixture of structures, or one that provides the most intense bands or most labile fragments. That said, it is possible that these experimental spectra could be from a series of possible structures or that minor structures may not be readily observable due
to signal suppression from more abundant structures. It is therefore necessary to further probe these important drugs molecules using several other analytical perspectives including their possible fragments, their exchange populations upon introduction to a deuterating reagent, and also their collisional cross-sections determined by their movement in a bath gas, which combined will provide detailed insight into these drugs.

5.3.2. MS/MS fragmentation spectra of pomalidomide and lenalidomide

To supplement the vibrational analysis above, it is necessary to determine the fundamental chemistry of each of these drugs while in a gas-phase ionic state. Figure 5.5 below shows the fragmentation spectra for both pomalidomide (Figure 5.5a) and lenalidomide (Figure 5.5b) with the same collision energy of 35 % HCD in a linear ion trap. Structures of the drugs are inset into each spectrum with the subsequent fragments labeled and the corresponding covalent bond breakages marked for each fragment ion produced. For pomalidomide, the precursor at m/z 274 can lose both water and a carbonyl, as well as undergo the loss of an amide group. Considering the structure, it would seem probable that the amide group loss would occur on the glutarimide ring and the loss would occur after carbonyl loss from the same glutarimide ring. The most intense peak in the MSMS spectrum is the remaining positive ion after the loss of the full O=C-NH-C=O group of the glutarimide ring at m/z 201. The other major fragments include the loss of the full glutarimide ring to leave the protonated phthaloyl ring at m/z 163 (C₈H₇N₂O₂), the loss of water from this ion at m/z 145 (C₈H₅N₂O), and the C₄H₆NO ion at m/z 84.

For lenalidomide (Figure 5.5b), the 35 % HCD results in a low remaining intensity of the precursor at m/z 260. As with pomalidomide, there are neutral losses of a carbonyl and amide group, as well as the loss of the full O=C-NH-C=O glutarimide functional group to form the m/z 187 peak. Furthermore, lenalidomide forms the m/z 84 fragment,
which is identical to the C₄H₆NO fragment formed by pomalidomide. In contrast to pomalidomide, however, this fragment is not the most intense peak for the lenalidomide MSMS spectrum, but rather the full glutarimide ring loss to form the protonated isoindolinone at m/z 149 (C₈H₉N₂O) is the dominant peak for this drug ion. Moreover, lenalidomide can fragment into the m/z 106 ion (C₇H₈N), formed as the 6-membered aromatic ring retaining the methylene from the second ring portion of the isoindolinone.
There are a few significant differences in the MSMS spectra observed between pomalidomide and lenalidomide that can be explained by their obvious difference in starting structure as well as key observations based on their proposed ion structures determined by action IRMPD and theoretical comparisons. The first main difference is...
the relative intensity of the remaining precursor. At identical mass-adjusted energies of 35 %, with the same gas conditions and experiments performed on the same day the precursor of pomalidomide was found to remain at about 30 % of the total relative abundance while for lenalidomide remaining precursor was only at 2 % of the relative abundance of the fragmentation spectrum. This suggests that the pomalidomide precursor is more stable which suggests that additional carbonyl oxygen must provide added avenues for possible H-bonding with the glutarimide ring, even if this H-bonding was not explicitly observed by action IRMPD spectroscopy. Another major difference is the presence of the [MH-H₂O]⁺ fragment for pomalidomide, which is absent in the case of lenalidomide. Since the free amine adjacent carbonyl is not present for lenalidomide there is one fewer oxygen atom to lose as a water and, therefore, this fragment is absent, and since this carbonyl location serves as the most probable site for an H₂O loss to occur, the absence of any signal for this ion is predictable. The final major difference between the two drugs, as discussed above, is the identity of the most intense fragment ion, which for lenalidomide is the complete glutarimide loss to leave the isoindolinone, while for pomalidomide is not the full phthaloyl ring (which forms from its complete glutarimide ring loss), but instead the loss of only the NH₂CHO neutral. This is interesting because from the IRMPD analysis, pomalidomide’s most likely ionic structure encompasses an iminol-tautomer on the glutarimide rather than the standard amide-form as with lenalidomide, which could result in a higher prevalence of this dissociation event over the loss of the complete glutarimide ring observed for lenalidomide. A more thorough investigation of the energy pathways and transition state structures, however, is required in order to confirm this, which is outside of the scope of these studies. In addition, these two fragments being the most intense confirms the IRMPD determined location of the protonation site for both drug ions. As each was postulated to be positively charged by protonation of the carbonyl oxygen opposite the primary amine of
the bicyclic ring, it is not surprising that for both fragmentation spectra all major fragments comprise that charge containing carbonyl.

Scheme 5.1 Possible ion structures for pomalidomide and lenalidomide fragments.

5.3.3. H-D Exchange data of pomalidomide, lenalidomide, and their fragments

Using the same instrumentation in which HCD fragmentation spectra were produced (Figure 5.5), H-D exchange experiments were performed, during which deuterated ammonia was leaked into the trap and allowed to equilibrate and settle for a short period of time (5 minutes) prior to studies commencing. Ions are then introduced into the instrument and isolated in the trap with the deuterated ammonia for an extended period of time (typically 1-10 seconds). Figure 5.6 shows the precursor pomalidomide (Figure 5.6a) and lenalidomide (Figure 5.6b) after a 10 second exchange time. The exchanges show that for pomalidomide, there are at least three possible exchange sites; however, the d$_0$ peak remains the most intense peak of the distribution indicating that the sites are not fully exchanged. While this single distribution suggests only one structure this is not to say that others are not also possible and may either be overshadowed by this one, not observable given the H-D exchange conditions or exchange gas, or possess similar exchange properties.
For lenalidomide, there are instead only two readily available exchange sites given that the maximum exchange peak at 10 second is the \( d_2 \). Additionally, this ion appears to give a faster exchange rate than pomalidomide with the 10 second exchange producing a distribution with the \( d_1 \) peak at 100% relative abundance and the \( d_0 \) peak at \( m/z \) 260 and the \( d_2 \) peak at 50%, each. The reason for the faster exchange of lenalidomide in comparison to pomalidomide could be that without an adjacent carbonyl, the primary amine of the isoindolinone exchanges rapidly, and so two quick exchanges are observed for this 10 second exchange time. This may also explain why there is no third exchange observed for this ion. If the \( \text{NH}_2 \) exchanges rapidly, the time needed for the slower amide nitrogen on the glutarimide ring to exchange could be overtaken by the easily accessed and back exchanged primary amine. This theory, however, does not account for the carbonyl on the isoindolinone ring, which should exchange with at least moderate speed, being the presumed protonation site for the ion. The primary amine may also dominate this exchange as well though. For the number of exchanges of pomalidomide, two exchanges can be accounted for from the same \( \text{NH}_2 \) function group possibly accounting for the exchanges of lenalidomide. The third exchange may either be on the glutarimide ring or from the protonation site location (believed to be on the same carbonyl of lenalidomide). It would make sense that if the \( \text{NH}_2 \) of pomalidomide were a slower exchanging species due to the presence of the adjacent carbonyl, that either the protonation site or glutarimide ring would be able to be protonated. The other possible explanation for the additional exchanges (with even tiny populations of \( d_4 \) and \( d_5 \) barely visible in Figure 5.6), is the presence of the stable and well matched iminol form observed with IRMPD seen in (Figure 5.3). With the occurrence of amide-iminol tautomerization, there is an additional method of exchange for the glutarimide ring. Instead of only direct exchange on the nitrogen portion of the amide, the OH functionality
of the iminol is also open to exchange which may happen faster and even allow for trapping of the deuterium by tautomerization back to the amide form.

Figure 5.6 Hydrogen-deuterium exchange of the protonated precursor a) pomalidomide and b) lenalidomide. H-D exchange was performed in a Velos Pro with leaking in of deuterated ammonia at an exchange time of 10 seconds.

Comparison of the H-D exchange profiles of the major fragment ions for each drug ion is important in order to better understand the intrinsic chemistry of these slightly varied functional groups. They will also help to confirm, or possibly refute theories as to the differences in ion structure between pomalidomide and lenalidomide, discussed above. Figure 5.7 shows the three major fragments observed for both drugs with a) and b) matching the O=C-NH-C=O loss for pomalidomide and lenalidomide, respectively, c) and d) matching the complete glutarimide ring loss for each, and finally e) and f)
compare the shared $\text{C}_2\text{H}_6\text{NCO}$ $m/z$ 84 fragment. The O=C-NH-C=O losses in a) and b) are almost identical for the two drug ions with a dominant $d_0$ at $m/z$ 201 for pomalidomide and $m/z$ 187 for lenalidomide and a very small $d_1$ for pomalidomide in a) and even lower intensity $d_1$ for lenalidomide in b). The much slower exchange and the fact that there appears to be only one exchange observed after the loss of the exchangeable functional groups on the glutarimide ring suggests that this region of the molecule is more involved in H-D exchange than was suggested by the H-D exchange data above in Figure 5.6. In describing Figure 5.6, it was suggested that it was the primary amine most involved in exchange, however, with a rotatable bond between the two rings, it is not unreasonable to assume that the additional carbonyl on pomalidomide could also limit the exchange rate of the glutarimide ring, just as was proposed above for the primary amine on the phthaloyl ring. Another possibility is that $m/z$ 201 and 187 rearrange after the O=C-NH-C=O loss into a structure that inhibits exchange on the remaining amine group and can only slowly exchange at the protonation site of the carbonyl.

The H-D exchange spectra of c) and d) are even more interesting. With the loss of the full glutarimide ring, and having only the protonated phthaloyl ring for pomalidomide (Figure 5.7a) and protonated isoindolinone ring for lenalidomide (Figure 5.7b) a drastic shift in the deuterated populations is observed. In c) there is the shift to two separate exchange populations with a slowly exchanging one, observed by the remaining height of the $d_0$ $m/z$ 163, which is difficult to determine the extent of exchange due to the overlap of the faster exchanging population. This faster exchanging population centers at $d_3$ but appears to exchange out to $d_4$. That this $m/z$ 163 can exchange out to $d_4$ is not surprising, considering that with the glutarimide ring removed the amide functionality of the phthaloyl is capable of rearranging to also exchange deuteriums. This would explain there being one slow and limited exchanging population, or “ring-retained” population,
and another extended and fast exchanging population, where the five-membered functionality of the ring has rearranged with the amide nitrogen free for more accessible exchange. The lenalidomide spectrum for m/z 149 in Figure 5.7d is significantly different in that with only the isoindolinone, there is what appears at first glance to be only one population with a moderate exchange, a dominating d₁ exchange, and a small d₂ observed. There also appears though to also be tiny d₃ and d₄ peaks in similar proportion to d₂, which suggests that without the second carbonyl, the chemistry promoting the opening of the five-membered ring is less favorable, but still possible, be it at a much lower intensity than the “ring opening” population of pomalidomide.

The final H-D exchanged fragment ion observed was that of the m/z 84, a peak that has the formula C₃H₄CONH₂. With this formula, the species should have a double bond equivalent of 3 with a carbonyl moiety, one double bond, and either a second double bond or has some sort of cyclic feature. Scheme 5.1 above shows two of the possible structures. Since the functional groups are identical for pomalidomide and lenalidomide, it is not surprising that both have the same core features for their 84 exchange populations. Both have a dominating exchange peak at d₂ and up to 4 exchanges, with the possibility of two separate structures based on what appear to be two distributions. The most important thing about these spectra, however, is that their good match shows that the H-D conditions are well matched between the two trials, since they should presumably be the same structure not considering possible kinetic shifts in fragmentation due to the presence of a phthaloyl ring instead or isoindolinone ring. Therefore, the comparisons in ion structure should be valid and the reasoning for differences be sound enough for further investigation of described differences.
Figure 5.7  Hydrogen-deuterium exchange of a) the $m/z$ 201 of pomalidomide, b) the $m/z$ 187 of lenalidomide, c) the $m/z$ 163 of pomalidomide, d) the $m/z$ 149 of lenalidomide, e) the $m/z$ 84 of pomalidomide, and f) the $m/z$ 84 of lenalidomide. H-D exchange was performed in a Velos Pro with deuterated ammonia at an exchange time of 10 seconds and activation amplitudes of 20% CID.

5.3.4. Ion mobility of pomalidomide, lenalidomide, and their fragments ions

Figure 5.8 shows arrival time distribution (ATD) of the two precursor ions a) pomalidomide and b) lenalidomide in a Waters Synapt G2S. For the $m/z$ 274 precursor of pomalidomide, there is a large peak at a drift time 8.05 ms and a small shoulder to this at 8.60 ms. This is clear evidence for pomalidomide of the presence of two separate arrival time distributions (ATDs) and therefore two clear ionic structures that were not observed from either the IRMPD data or from the H-D exchange spectra for this precursor ion. For lenalidomide, however, there is instead only one drift time distribution at 8.12 ms, which is in between the ATD’s of the two drift populations from pomalidomide. Additionally, the width of the peaks for the lower drift time pomalidomide
and that of lenalidomide are close but there is a slight broadness to the lenalidomide peak suggesting that there may be ion populations of very similar nature to the (i.e. on/off-axis) that exist in the distribution. There is not a way unfortunately, given the resolution of this instrument to determine this, so it must with this equipment be assumed that the lenalidomide is indeed providing one conformational family from this one drift time distribution and pomalidomide presenting two separate structural families; a larger proportion small CCS population and a less abundant and more extended population.

Unequivocally for pomalidomide, there must be a sharp change between the two populations to provide the drift time difference of ~0.55 ms. The identity of this difference could not be discerned from the previous two ionic analyses but it is possible that the precursor is rearranging its structure via a ring opening. A ring opening is nonetheless unlikely given the low energy conditions used when this spectrum was collected (5 eV) and likewise there is no shift in the proportion of the higher ATD distribution when applying higher energy trap CID. It is also surprising that the lowest drift time peak for pomalidomide is lower in collision cross section than the single peak observed for lenalidomide. With the presence of an additional carbonyl in the phthaloyl ring, it would be expected for this functional group to create more cross-sectional space and while this might be the case in the longer drift time species at 8.60, somehow the pomalidomide is maintaining a small CCS for its major population. The most likely possibility for conformation is that the out-of-plane ring structure for lenalidomide is frozen in place and there is not an in-plane conformation to provide a second drift-time population. Comparison to the IRMPD derived structures sheds more light on these conformations and from these studies it could be suggested that the iminol form for pomalidomide may stabilize an in-plane conformation and result in a bulkier, less flexible ion that results in the more extended albeit less intense population observed in Figure
5.8. The more flexible population appears to be more prevalent in the Synapt as this is a QTOF and the amount of energy deposition here may result in a higher proportion of the more flexible form as greater amount of energy allows for breaking any stabilizing hydrogen bonds.

Figure 5.8  Ion mobility of the precursors of a) pomalidomide and b) lenalidomide. IM-MS was performed with a bath gas of CO\textsubscript{2} with identical IMS conditions and drift time scales used for both spectra and trap collision energy set to 5 eV.

Matching the selected ions from the H-D exchange Figure 5.7, Figure 5.9 shows the ion mobility spectra of the pomalidomide and lenalidomide fragments (i.e. O=C-NH-C=O function group loss, glutarimide ring loss, and m/z 84 fragment) compared with the top panels a), c), and e) containing the pomalidomide-derived fragments, and the bottom
panels b), d), and f) the lenalidomide-derived fragments. For the O=C-NH-C=O loss in Figure 5.9 a and b there is a narrow peak at 6.05 ms for m/z 201 of pomalidomide and a shorter drift time and slightly broader peak at 5.75 for m/z 187. Upon loss of the O=C-NH-C=O group, the phthaloyl ring of m/z 201 for pomalidomide is a more extended population by 0.3 ms than the corresponding isoindolinone ion (m/z 187), this is in contrast to the precursors (Figure 5.8) for which pomalidomide is more extended.

The most important feature of Figure 5.9 is the comparison of c) and d), the two spectra representing the drift time distributions of the full phthaloyl ring of pomalidomide (m/z 163) and the isoindolinone ring. Here are two dramatically different ATDs with the phthaloyl ring fragment showing almost baseline resolved peaks 0.73 ms (5.90 ms to 6.63 ms) apart in drift time while a more compact but single drift time distribution indicated by the ATDs of the isoindolinone fragment (5.48 ms) for lenalidomide in Figure 5.9d. These two spectra matched extremely well with the H-D exchange shown in section 5.3.3, Figure 5.7 c and d, where again the phthaloyl fragment exchange profile of c) clearly showed two distributions while only one was observed for the isoindolinone fragment of d). The possibility that the phthaloyl ring is opened upon fragmentation is a reinforced theory given the large difference in mobilities between the two drift time distributions in c). It would be necessary to determine whether this shift in drift time could be matched using theoretical models and MOBCAL calculations in order to fully establish an understanding of the gas-phase ion chemistry occurring for this molecule.

The m/z 84 fragment ion mobility comparison (Figure 5.9 e and f) shows two ion populations that are extremely similar in conformation. Likewise, the width of each peak matched well and the value of each suggested that again there could be at least two possible structures (Scheme 5.1) for the fragment, yet despite this the two gave very good agreement thus again suggesting that the proportion of the multiple structures was not influenced by identity of the bicyclic ring. There is a lack of uniformity for the m/z 84
peak of lenalidomide in f), with some dramatic shifts in the slope of the peak, but this is attributed to the lack of adequate signal of the \( m/z \) 84 peak for the IMS conditions used rather than any possible shoulder peaks present for the lenalidomide ion population over the ion population of pomalidomide. What is most important to note between these two ATDs, and their clear similarity, is that the two separate fragmentation/Ion mobility experiments matched up well in instrument conditions and since drift time relationships for the same fragment (\( m/z \) 84) were successfully equivalent, it could be concluded that comparisons of the other fragments could be carried out without issue.

Figure 5.9  Ion mobility of a) the \( m/z \) 201 of pomalidomide, b) the \( m/z \) 187 of lenalidomide, c) the \( m/z \) 163 of pomalidomide, d) the \( m/z \) 149 of lenalidomide, e) the \( m/z \) 84 of pomalidomide, and f) the \( m/z \) 84 of lenalidomide. IM-MS was performed with a bath gas of \( \text{N}_2 \) with identical IMS conditions and drift time scales used for both spectra. To produce fragments trap collision energies of 18-20 eV were used.
5.3.5. Kinetic method of lenalidomide and examining of the issues for performing the Kinetic method for pomalidomide

There are ways in which the physical characteristics of ions such as pomalidomide and lenalidomide can be measured directly in the gas-phase. The Kinetic method, developed by Cooks et al. is such a technique that allows for the determination of the proton affinity of ionic species. Figure 5.10 shows the extended kinetic method plot of $\Delta H(Base)i-\Delta H(average)$ versus $\ln[(I(Base)i+/I(Len))$ at four separate energies of 10 % (blue diamonds), 15 % (red squares), 25 % (green triangles), and 35 % (orange circles). Essentially, this graph is a correlation of the relative value for the proton affinity of each reference base plotted against the relative ratio of the amount of reference base and lenalidomide formed upon CID of their heterodimer. Since each is bound to the other in the heterodimer, the one with a greater proton affinity should retain the proton after dissociation and therefore have a greater ion intensity relative to the other. From these plots of all collision energies a line of best fit was derived with the slope of each corresponding directly to $1/RT_{eff}$ and the y-intercept to the negative value of $[\Delta H_{Len}-\Delta H_{avg}]-T_{eff}\Delta S]/RT_{eff}$. Plotting these two values with $x$ as the $1/RT_{eff}$ value versus $[\Delta H_{Len}-\Delta H_{avg}]-T_{eff}\Delta S]/RT_{eff}$ in the y-axis at several collision energies produces a plot such as the one in Figure 5.11 where the slope of the line provides the deviation of the proton affinity of lenalidomide from the average value of the collection of reference bases. With this entropy-corrected extended kinetic method analysis of lenalidomide, the value of its proton affinity was found to be 889 kJ/mol. This value on its own is useful in studying the ionic properties of lenalidomide, but without a direct comparison of a similar value to pomalidomide the proposed goal of examining their differences is impossible.

For pomalidomide, several attempts were made to produce proton-bound heterodimers of the drug in the ion trap with this set of reference bases, but however, were all unsuccessful. Dimers could be observed at small intensities with spraying the
1:1 mixtures of Pom with each reference base, but signal upon MSMS was erratic and did not show populations of the Pom+ and Base+ with any regularity. While this is of course a setback to studying the gas phase properties of these two species side-by-side, there is a qualitative lesson to be had from the lack of pomalidomide kinetic method data. The issues with the kinetic method plots of pomalidomide suggest that the most stable ionic structure of pomalidomide is not allowing it to form hydrogen bound complexes with other species in solution. The proton affinity should not drop enough for there to be absolutely no sign of dimer and as dimer did appear to form, in small quantities, their inability to fragment into Pom and Base suggests that these were either artifact peaks or the dimers were atypical in their form and so did not dissociate in the expected manner of forming respective monomers of protonated reference Base and Pom.

Figure 5.10   Extended kinetic method plot of lenalidomide with reference bases serine, Isoleucine, threonine, tyrosine, proline, and asparagine. Plot was produced using relative collision energies of 10 % (blue diamonds), 15 % (red squares), 20% (green triangles), and 35% (orange circles).
5.4. Conclusions

In this chapter, the ionic structures of two cancer drugs have been analyzed chemically, physically, and qualitatively, with the differences in fragmentation as well as the fragment ions chemical features considered. For the two drugs, gas-phase ionization induces unique properties, and although differing by only a carbonyl group, the drugs’ ionic chemistries are significantly different. From IRMPD, the structure of pomalidomide could be observed to have an iminol tautomer on the glutarimide ring while no tautomerization was observed for lenalidomide. Additionally, spectroscopy could rule out any odd protonation sites in either molecule with both showing a predominant site on the carbonyl oxygen of the five-membered region of the bicyclic ring moiety.

HDX and IMS show that there are obviously different chemistries for the two but that pomalidomide can also undergo a significant change resulting in a drastically different
drift time and intriguing drift time distribution. Most notably, the precursor ATD with two distinct drift times and the unique exchange distribution and ATD of the phthaloyl ring show that there is more than one structure that pomalidomide and its major fragment can adopt in the gas-phase. Additionally, from the kinetic method studies, lenalidomide could be found to form stable proton bound heterodimer complexes with reference bases and provided a reasonable 888 kJ/mol proton affinity. Pomalidomide, however, seemed to resist any complex formation with these basic amino acid biomolecules, which doesn't appear to be necessarily a result from any possible shift in its proton affinity relative to that of lenalidomide has both have the same protonation site and can be observed independently quite easily upon standard ESI.

Pomalidomide is an interesting ionic compound both for its precursor structure and resulting fragments, which are important in the continuing studies of its metabolic pathway investigations. Due to pH sensitive, lenalidomide is not able to pass the blood brain barrier, yet pomalidomide can. Upon ionization/protonation lenalidomide appears to arrange into a single form (IMS/HDX) and does not appear to rearrange or deviate from the expected starting structure, yet pomalidomide does. Could this variability in the chemistry of pomalidomide and also its inability to form expected heterodimers be a reason for its unique bioavailability? With multiple techniques this study has come to the conclusion that pomalidomide has a higher stability and greater flexibility in the gas phase than that of lenalidomide.
Chapter 6. Lycopene and α/β-carotene: Radical ionization, fragmentation, and ion mobility analysis

6.1. Introduction

Carotenoids are tetraterpenoid molecules involved in the processes of all photosynthetic species. They are essential in this process as the energy of impinging UV light induces important structural changes to these conjugated hydrocarbons which directly influences their role in photosynthesis.\textsuperscript{199,200} All carotenoids are important in for normal functioning with some, like α-carotene, β-carotene, and β-cryptoxanthin being provitamin A, meaning that they can be ingested and metabolized into retinoids which are in turn used for various essential biological functions, such as the growth of epithelial cells and closely related to that the proper function of vision and limiting of tumor growth.\textsuperscript{201} Other carotenoids which are not pro-vitamin A, such as lutein and lycopene serve as scavengers and are excellent anti-oxidants, species which assist in removal of reactive oxygen species.\textsuperscript{202} Furthermore, both pro-vitamin A and non-provitamin A carotenoids have been implicated in the prevention of diseases, including cancer and vascular disease.\textsuperscript{202-204} In nature, carotenoids primarily exist in the all-trans form, but they can be isomerized to cis forms through exposure to heat or light. Isomerization plays a significant role in the biological activity of several carotenoids. For example, the cis isoforms of pro-vitamin A are only 50% active compared to the all-trans form.\textsuperscript{205} Metabolism of the pro-vitamin A carotenoid β-carotene to vitamin A requires the all-trans form, while in vitro studies of metabolism of lycopene show preference for cis-isomers.\textsuperscript{202,204,206,207}
Figure 6.1 shows the all-\textit{trans} forms of the carotenoid lycopene (Figure 6.1a), a non-provitamin A carotenoid, as well as the provitamin A carotenoids \(\alpha\)-carotene (Figure 6.1b) and \(\beta\)-carotene (Figure 6.1c). Previous work on the separation and quantification of these carotenoid molecules has been routinely performed by high performance liquid chromatography (HPLC).\textsuperscript{205,207-210} Simplification of real-world food based samples involves saponification to separate the non-polar carotenoids in Figure 6.1 from interference by polar carotenoids (xanthophylls) as well as fatty acids.\textsuperscript{208,211} In order to achieve accurate quantitation of these compounds, however, saponification is discouraged as it could influence the distribution of all-\textit{trans} versus \textit{cis} isoforms of each carotenoid.\textsuperscript{212}

Figure 6.1  The structures of all-\textit{trans} a) lycopene, b) \(\alpha\)-carotene, and c) \(\beta\)-carotene

Ion mobility mass spectrometry (IM-MS) offers the potential of differentiating isobaric carotenoid species without up-front HPLC separation and operating without the necessity of any extraction or modification steps before analysis. Radical ionization and subsequent MS analysis has been well studied with regard to site and products formed.\textsuperscript{213-215} Previous work, however, from the van Breeman group on the IM-MS of lycopene and \(\beta\)-carotene, individually, has shown that standard electrospray ionization
and possibly the instrument conditions after ionization are not sensitive to the populations of \textit{trans} and \textit{cis} isoforms in solution and cause isomerization of the precursors as observed by the IM analysis.\textsuperscript{216} Instead, sprayed from source and instrument conditions of a first-generation Waters G1 instrument, a more compact population was found to be equivalent to or even higher in proportion to the most extended more-\textit{trans} population of the \(\beta\)-carotene and lycopene carotenoids studied by the researchers. Furthermore, the work showed that the populations of extended and compact can be influenced by instrument conditions including source temperature and from unwanted energy deposition.

In this work, the potential of analyzing lycopene, \(\beta\)-carotene, and the closely related \(\alpha\)-carotene with IM-MS alone was further explored with the intent of determining if any \textit{cis}/\textit{trans} information could still be gleaned from IM-MS despite the harsh nature of ionization. Additionally, the possibilities of determining the relative amount of lycopene, \(\alpha\)- and \(\beta\), regardless of geometrical isomer, were explored.

6.2. Experimental

6.2.1. Carotenoid synthesis and preparation

Lycopene and \(\alpha\)-carotene were provided by the food science research lab of Dr. Steven Schwartz at the Ohio State University (Columbus, OH). The third tetraterpenoid studied, \(\beta\)-carotene, was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Solutions of 50 \(\mu\)M were prepared for each carotenoid with 50:50 MeOH:CHCl\(_3\) for all mass spectrometry experiments detailed below.

6.2.2. Ion mobility-mass spectrometry studies

Ion mobility-mass spectrometry (IM-MS) experiments were performed on a Waters Synapt G2S with standard ESI at a capillary voltage of 3.0-4.0 kV with high voltages
required for the MeOH:CHCl₃ spray solvents used and the electrochemical nature of the carotenoids under study. Fragmentation was produced in the trap region at trap collision energies of 5 to 20 eV. Helium gas flow and ion mobility gas flow (N₂) were set to 120 and 75 mL/min, while the wave height was set to 35.0 V and wave velocity to 1000 m/s. Helium CCS's were reported below in section and the experimental values were found by calibrating with a mixture of d/l-polyalanine and using the CCS values found for this mixture by the Clemmer group.¹¹⁸,¹¹⁹ The drug calibrant mixture used to determine N₂ derived CCS used in Chapter 4 was not used in this case as the range of CCS for all drug molecules was too low to cover the extensive linear carotenoid molecules. Polyalanine, however, fits this range adequately and was therefore used for all CCS values presented below.

In order to attain the excitation conditions in the high pressure StepWave region of the instrument (as applied in Figure 6.4), the RF control was set to automatic settings, with the initial StepWave sections wave velocity set to 300.0 m/s, enter wave height set to 10.0 V, ending height set to 1.0 V, and final StepWave section set to a wave velocity of 300.0 m/s and 0.0 V. The StepWave Transfer offset set to 25 V, the first differential aperture set to 3.0 V, second to 0.0 V and the StepWave RF offset 1 set to 300.0 V and StepWave RF offset 2 set to 350.0 V. To achieve “softer” StepWave conditions, the StepWave was set to equivalent wave heights and velocities, but the StepWave Transfer offset was set to 10 V and the two differential apertures to 5.0 V each.

6.2.3. Theoretical Analysis and MOBCAL Calculations

All ionic structures were calculated using a hybrid density function theory (DFT) at the B3LYP/6-311+G** with Gaussian 09 and starting structures collected using the MacroModel conformational search with all parameters and theory levels identical to those described in Chapter 4 section 4.2.2.¹²²,¹⁷³
MOBCAL input files were collected using the optimized structures produced with conformational searching and Gaussian DFT. DFT was needed in order for Mulliken partial charge values used in the input files of the MOBCAL CCS determinations. MOBCAL conditions were used matching the standard Helium CCS inputs produced by the Jarrold group and more detail on MOBAL and DFT is provided above in section 2.5.

6.3. Results and Discussion
6.3.1. Conformational analysis by IM-MS for α-carotene, β-carotene and lycopene

The drift time distributions of the three isobaric carotenoid species, α-carotene, β-carotene and lycopene, subjected to collision energies of 0 eV, 10 eV, and 20 eV in the Trap region are shown in Figure 6.2 a-c, d-f and g-i, respectively. All samples were freshly prepared standards of all-trans α-carotene, β-carotene, and lycopene and each possesses two main drift-time populations with increasing populations of the shorter drift time, more compact, population upon higher collision energy. As these species were all in solid form and synthesized as the all-trans form conformations, it was surmised that with no collision energy applied the major conformation of each ion would correspond to a large population of the all-trans carotenoid structure. In Figure 6.2a it is clear that α-carotene does in fact show a single major conformational family with an estimated collision cross section (CCS) of 217 Å², with a barely visible second peak before it, with no distinct central peak profile but estimated to be ~170 Å². Similarly for β-carotene at 0 eV, there is one large conformational family with a barely visible secondary more “compact” population, with a central maximum at about ~175 Å². The more extended population of β-carotene is very close in drift time to α, but is about 2 Å² smaller in CCS at 215 Å², as is seen in the slight shift of the peak from the dotted line beginning at the center of the extended population peak in Figure 6.2a. Lycopene at 0 eV collision
energy (Figure 6.2g) also has two distinct peaks, with the more extended longer drift time population several Å$^2$ larger at 226 Å$^2$, as is not surprising given the fully linear structure of lycopene in contrast to the two carotene isomers which each contain a six-membered ring at each termini of the molecule. Additionally, the smaller CCS population for lycopene, at about 172 Å$^2$, possesses a greater intensity at 0 eV relative to α and β (174 Å$^2$ and 177 Å$^2$ respectively), suggesting that this ion population is easier to form in the gas-phase than the more compact populations for the two carotenoids.

Considering next the higher Trap region collision energies at 10 eV and 20 eV there are clear differences in the behavior of α-carotene, β-carotene, and lycopene as the energy increases as shown in Figure 6.2. For α-carotene the relative intensity of the more compact species increases from 0.7% at 0 eV to 1.5% at 10 eV and finally 5.6% at 20 eV. For β, the relative ratio of the more compact population starts at 1.1% and moves to 3 and 15% for 10 eV and 20 eV respectively (Figure 6.2d, e, and f) while for lycopene the more compact species is significantly more intense observed at 1.6% relative intensity for 0 eV, for 5.1% 10 eV, and 34.0% for 20 eV (Figure 6.2g, h, and i). There is notably no significant shift with respect to CCS for either conformation for α, β, or lycopene in each case. Instead, taking only Trap collision energy into account for standard instrument conditions, the two populations change in relative intensity as higher CE’s are used.
Figure 6.2 Collision cross section distributions at trap collision energies of 0 eV for a), d), and g), 10 eV for b), e), and h), and 20 eV for c), g), and i) under gentle StepWave conditions for the M•+ of α-carotene (a, b, and c), β-carotene (d, e, and f), and lycopene (g, h, and i). The x-axis was converted from drift time to CCS (Å²) using a polyalanine standard.²¹⁷ The CCS range selected for all spectra were set from 124 to 251 Å².

In order to further probe the conformations of these carotenoids the CCS distributions obtained at 20 eV (Figure 6.2c, g, and i) were further considered and the experimental CCS obtained compared to theoretical CCS obtained from MOBCAL (Figure 6.3). As discussed above, the polyalanine calibrated CCS for the two carotenes and lycopene were found to be 217 Å², 215 Å², and 226 Å², respectively. At the 20 eV CE conditions shown here these extended populations dominate for all three in
comparison to the more compact distributions of 174 Å² for α-carotene, 177 Å² for β-
carotene, and 172 Å² for lycopene. For comparison, the conformations of the
carotenoids were also studied with no Trap collision energy and instead monitored
following activation upon RF excitation in the higher pressure region in the StepWave
section of the instrument. Following this high pressure excitation, the more compact
populations dramatically increase in intensity becoming equal to or greater than the most
extended populations. Most important to note is that β-carotene produces two new
distinct compact populations at 167 and 182 Å², while α-carotene retains only the
centered peak observed in Figure 6.4a at 173 Å², only at a higher intensity, making it
about 60 % of the total relative intensity compared to the more extended conformation.
Figure 6.3  CCS distributions of the M$^+$ of a) α-carotene, b) β-carotene, and c) lycopene under gentle StepWave conditions and at trap collision energies of 20 eV. The x-axis was converted from drift time to CCS (Å$^2$) using a polyalanine calibration standard. The red lines signify the centered collision cross section of each peak and blue dashed lines show the MOBCAL calculated CCS’s of all-trans and selected cis conformers of the respective structures (inset).

The more compact conformations observed following RF excitation were not accessible using higher collision energies in the Trap region of the instrument as the higher energies only resulted in fragmentation of the precursor, significantly, this experiment followed the same trend observed for Trap CID highlighted in Figure 6.2 with regards to the relative stability of the extended precursor population, in which it was observed that lycopene was most susceptible to excitation into its most compact population, with a 100 % relative intensity for the drift time population at 172 Å$^2$ and a remaining more extended population at 226 Å$^2$ at 18 % (Figure 6.4c). While β-carotene retained its higher CCS species at 215 Å$^2$ at 47 % of the relative intensity (Figure 6.4b),
α-carotene (Figure 6.4a), the more compact species, was still the less dominant population at 44 % the extended population’s intensity.

![Diagram showing CCS distributions of α-carotene, β-carotene, and lycopene](image)

Figure 6.4 CCS distributions of the M⁺ of a) α-carotene, b) β-carotene, and c) lycopene under harsh StepWave conditions and at 0 eV trap collision energies. The x-axis was converted from drift time to CCS (Å²) using a polyalanine calibration standard. Red lines signify the centered collision cross section of each peak and blue dashed lines show the MOBCAL calculated CCS’s of all-trans and selected cis conformers of the respective structures (inset).

The other important issue detailed in both Figure 6.4a and Figure 6.4b is that while their experimental CCS’s are consistent between each other (exemplified by the solid red lines), the MOBCAL calculated CCS for the all-trans form of α-carotene, β-carotene, and lycopene, as well as the 9-cis of α and β-carotene and 10-cis of lycopene, which are shown as dashed blue lines in both Figure 6.3 and Figure 6.4, do not agree with either the extended or compact populations observed here. The theoretical CCS determined for the all-trans forms of α and β-carotene was 225 Å² while the extended population of
each of these experimentally was found to be 217 and 215 Å², respectively. This would suggest that the instrument conditions and ionization method chosen do retain the all-trans conformation of each, while the disagreement between these experimental CCS and the ones calculated by MOBCAL suggests that the extended structure may not be all-trans at all. Likewise, the relative shifts between the very common double bond isomerizations for 9-cis and 10-cis for the computed CCS and the all-trans forms only amount to about 10 Å². This is in high contrast to the shifts from the extended and compact center peaks for the experimental distributions, with close to 40 Å² for the carotenes and 50 Å² for lycopene. Therefore, even if the extended populations observed in Figure 6.3 and Figure 6.4 are in fact all-trans conformers, the lower populations must undergo several trans-to-cis isomerizations to make such a dramatic change in ionic size.

6.3.2. Ion mobility of a known cis/trans pair: all-trans retinal and 9-cis retinal

In order to determine whether conjugated hydrocarbon chains of a set conformation can in fact retain their structure upon ionization, a secondary set of retinoid compounds, all-trans retinal and 9-cis retinal, were studied by IM-MS, in the same manner as discussed above for the carotenoids (see section 6.3.1). Additionally, the structures of retinal all-trans and 9-cis were calculated and MOBCAL determinations performed to compare the experimental values as shown for α-carotene, β-carotene, and lycopene in Figure 6.3 and Figure 6.4. These studies were performed, on well characterized systems, in order to determine if the synthesized carotenoid isobaric molecules are unique with respect to their significant experimentally observed shift from theoretical CCS values and ultimately whether a possible issue exists for either the calculations used in the comparison or the assignment of the experimental IMS distributions.
Figure 6.5 below shows the drift time distributions converted into CCS distributions using the polyalanine calibration of the protonated precursor ions all-trans retinal (Figure 6.5a) and 9-cis retinal (Figure 6.5b). Peak centers are labeled as red dashed lines with the CCS value at that point and for both all-trans retinal and 9-cis retinal, interestingly it is observed that under exactly the same instrumental conditions the drift time distributions are essentially identical. Despite a change in the double bond character for 9-cis retinal, both retinoids form two distinct drift time populations which present at exactly the same CCS values and ratios for both compounds. The more extended population is centered at 118 Å² and this is the dominant peak for both spectra. The more compact population is centered at 105 Å² for both species, but is at 40 % relative intensity compared for all-trans retinal, however, for 9-cis retinal this population is at lower intensity presenting at ~30 % relative intensity. This slight difference in the ratio of compact/extended peak is a minor point; yet, it is interesting as it appears to be counter-intuitive since the 9-cis retinal should presumably have a more compact ion population than the all-trans species if there is retention of structural double information carried over from the solution phase to the protonated ion. However, despite the differences in the solution-phase structures both the 9-cis retinal and the all-trans retinal are observed to populate two identical, with respect to CCS, gas phase populations. In fact, all-trans retinal, the more extended in solution, is observed to have a higher population of the more compact conformation. Additionally, the widths of the extended and compact peaks for both retinoids are equal and this conveys the assumptions that any unresolved conformations present in the distributions are equivalent.

It is also important to comment on the calculated CCS value for the all-trans conformation as determined by MOBCAL, which are represented by the dashed blue line in Figure 6.5. The theoretical CCS as derived from MOBCAL for all-trans retinal is 127 Å² while the experimental CCS centered at 118 Å² for the “extended” population and 105
Å² for the more “compact”. The discrepancies here are extremely helpful in better understanding the disagreement between the theory and experimental values of the carotenoids shown in Section 6.3.1. As with those values, the MOBCAL theoretical CCS determinations are substantially higher than the extended population. The IM-MS analysis of the model retinoids in conjunction with the studies on the carotenoids enables trends to be discerned. In both cases the theoretical CCS determined from MOBCAL for the all-trans species was larger than the most extended species observed experimentally. Furthermore, the two retinoids have major differences in their solution phase structure, but as discussed above, are indistinguishable with respect to their CCS distributions. Taken together, these observations suggest that the most extended peaks present for all five molecules presented in this chapter are not exclusively all-trans in structure. For both the M•+ and MH•+ ions formed from the carotenoids and retinoids, respectively, there appears to be a rearrangement upon entrance into the gas-phase that erases much of the original structural information, as least with regard to the location of double bond cis/trans isomerizations and assuming the products were completely all-trans in their solid form.
Figure 6.5 CCS distributions of the MH\textsuperscript{+} precursor ions of a) all-\textit{trans} retinal and b) 9-\textit{cis} retinal under gentle StepWave conditions and at trap collision energies of 0 eV. The x-axis was converted from drift time to CCS (Å\textsuperscript{2}) using a polyalanine calibration standard.\textsuperscript{218} The red lines signify the centered collision cross section of each peak and blue dashed line shows the MOBCAL calculated CCS of the all-\textit{trans} retinal structure (inset).

6.3.3. MSMS and IM-MSMS of the isomeric carotenoid species α-carotene, β-carotene, and lycopene

After determining that the CCS distributions of the retinoid molecules and whether the double bond conformations could be retained upon ionization of this retinoid, it is now important to assess the differences in the fragmentation spectra of α-carotene, β-carotene, and lycopene. It is also necessary here to determine whether the radical ionization that appears to form compact and extended conformations for each precursor in IM-MS can result in the same variations in the resulting fragment ions of each carotenoid upon MSMS followed by IMS. Specifically, this centers on understanding
whether the type of fragmentation (even or odd electron loss), as well as where the fragmentation occurs, can elicit information which can be used to comprehend the nature of the smaller and larger populations observed for each respective precursor molecule.

Figure 6.6 shows the comparison of Trap CID fragmentation of α-carotene at 25 eV (Figure 6.6a), β-carotene at 25 eV (Figure 6.6b), and lycopene at 20 eV (Figure 6.6c). These collision energies were chosen as they provide the best proportion of fragments and which clearly highlight the differences between the dissociation pathways for the three carotenoids. At very high energies, all three carotenoids breakdown into fragments indistinguishable between each carotenoid in the lower m/z region while at low energies the three show high intensity of the common toluene fragment loss at m/z 444 which is discussed in more detail below. Since the collision energies shown in Figure 6.6, for the three molecules are indeed different discussing any quantitative differences in the fragmentation is not appropriate and instead discussion will focus on the qualitative differences. Not surprisingly, all three spectra show a set of hydrocarbon peaks in the region of m/z 100-300, which show that after the main fragment losses, the molecules are broken into individual methylene subunits different only in saturations. Another set of features all three carotenoids share is the loss of the 15 g/mol neutral methyl radical shown at m/z 521 labeled -CH₃. Additionally, all three molecules undergo the equivalent neutral loss of toluene (labeled -C₇H₈) at m/z 444, which for α and β is the dominant fragment formed at a collision energy of 25 eV, and for lycopene is the second major at 20 eV collision energy. Beyond these three similarities in the MSMS spectra, the fragment identities diverge in several ways allowing these isobaric compounds to be identified by their key fragments.

For α-carotene (Figure 6.6a), there are three distinct peaks at m/z 480, m/z 388, and m/z 321, that are not readily observed for the other two compounds, and which have
been assigned as neutral losses. All three of these neutral loss structures, in addition to the toluene loss at $m/z$ 444, are labeled in Figure 6.6a. The first unique fragment which presents at $m/z$ 480 is the isobutylene of formula C$_4$H$_8$. Isobutylene is a fragment unique to α-carotene since the change in double bond position of the one terminal ring opens up the dimethyl portion of the ring for the fragmentation to occur there. The $m/z$ 388 (-C$_{16}$H$_{23}$) and $m/z$ 321 (-C$_{11}$H$_{16}$) fragment ions are less easy to explain considering the double bond positions of α-carotene. There is a commonality between the neutral losses forming the $m/z$ 388 and 321 fragments in that both are radical (odd electron) neutral losses that retain the double bond equivalency observed in the full carotenoid. It is most likely that cleavage will occur in between the first and second double bond of the linear chain for $m/z$ 321 and the third and fourth double bond of the chain for $m/z$ 388 alternatively the fragment observed at $m/z$ 388 could also be formed from loss of the isobutylene neutral from the $m/z$ 444 fragment. Since both α-carotene and β-carotene lose toluene to form the $m/z$ 444 fragment, it is possible that the $m/z$ 388 fragment is a result of a second isobutylene loss, which will only occur on the α-carotene unique terminal ring and not the terminal ring commonly shared between α-carotene and β-carotene that remains. The $m/z$ 388 fragment could form from β-carotene as well if the corresponding neutral loss occurred in one step at the linear double bond segment itself, but since only α-carotene shows this fragment the neutral loss at this point must not be stable enough to take place. There is a great deal of stability imparted by the full conjugation of β-carotene that isn’t present with the change in the unique ring’s double bond position of α-carotene, even after the toluene natural loss and formation of the $m/z$ 444 fragment.

In Figure 6.6, β-carotene is interesting for its lack of unique fragments compared to α-carotene and lycopene (discussed in detail below). All three lose toluene, which is the most intense fragment ion observed for β-carotene. Furthermore, the other high intensity
fragment ions observed for β-carotene, at $m/z$ 429 and $m/z$ 323 (labeled with the structures of the corresponding losses in Figure 6.6b) are also present for α-carotene, but at diminished relative intensities. Half of α-carotene’s structure is identical to β-carotene so it is expected that the two should in fact share many similar fragments. As α-carotene and β-carotene share a terminal ring the fragment ions corresponding to cleavage in this ring will be shared between these two molecules. Furthermore, as β-carotene is a structural mirror image containing this ring there can be no unique fragments, in comparison to α-carotene, resulting in no unique fragment ions in comparison to α-carotene. So while an interesting observation, the lack of any distinguishable β-carotene fragments is expected given the structures for each carotenoid. These labeled fragments, however, are interesting given their very different neutral loss structures, in comparison to the α-carotene neutral losses. With toluene as the exception, the fragments are odd electron radical losses. They clearly show rearrangements to form new rings, however, given the double bond equivalencies. The fragment observed at $m/z$ 323 corresponding to the $-\text{C}_{16}\text{H}_{21}$ loss is reduced by two hydrogens, compared to the $m/z$ 321 $-\text{C}_{16}\text{H}_{23}$ loss of α-carotene, and the resulting reaction would have the neutral undergoing formation of a bicyclic ring. Presumably this would involve the first methyl carbon attacking the second position carbon of the ring where the other mono-methyl sits, via a radical intermediary state, however, the determination of the mechanism of this reaction is beyond the scope of this dissertation. Considering next the species at $m/z$ 429, this fragment ion could occur via a ring opening but this is less of a oddity as it could also easily form from the $-\text{CH}_{3}\bullet$ radical loss of $m/z$ 15 from the toluene loss fragment at $m/z$ 444. It is important to note that the discussions of the other ring fragments, especially $m/z$ 321 and $m/z$ 323, assumed fragmentation occurred directly from the precursor carotenoid. Taking $m/z$ 429 into account, this was not necessarily the case for the other fragments, as some more
complicated losses could be formed via step-wise mechanisms where fragments form from subsequent fragments. In the studies presented here, however, attention is placed on the differences between these three isobaric hydrocarbons with respect to the qualitative trends observed and fragments formed as opposed to the intricacies related to their formation.

The last fragmentation spectrum discussed here is that of lycopene (Figure 6.6c) which shows two unique fragments at $m/z$ 467 and $m/z$ 375. The $m/z$ 467 corresponds to the loss of isoprene, a neutral loss only possible due to the fully linear structure of lycopene. The other unique fragment at $m/z$ 375 is comes also from the isoprene neutral loss, but in this case is lost from the $m/z$ 444 fragment. That the isoprene neutral loss fragment is only observed for lycopene is not surprising. Being fully linear it should be easy to lose from the terminal section of the carotenoid molecule and in the case of $m/z$ 375, the isoprene fragment can be formed on one end of lycopene and the toluene formed at the other. Lycopene is also interesting in that it does not share the specific fragments observed for both $\alpha$-carotene and $\beta$-carotene, including the isobutylene loss at $m/z$ 480 or the two fragments at $m/z$ 321 and 323. In one case, the $m/z$ 444 fragment does not lose a radical methyl group to form the $m/z$ 429 peak. After toluene loss there is seemingly no stable way to undergo a secondary methyl loss. It may be that this pathway is accessible but not at the energy selected here. Additionally, the two $\alpha$-carotene unique peaks at $m/z$ 388 and 321 are not present for lycopene. If in fact the $\alpha$-carotene was able to form these loses in a single step following cleavage between the 1$^{st}$ and 2$^{nd}$ double bond or 3$^{rd}$ and 4$^{th}$ double bond, then it would then also be possible to form a single step cleavage at these same sites in lycopene. The fact that these fragments are not observed for lycopene suggests that the $\alpha$-carotene unique terminal ring is necessary for their formation and that the formation must involve the loss isobutylene as has been shown only this ring can produce.
Figure 6.6 MSMS fragmentation spectra of a) α-carotene, b) β-carotene, and c) lycopene, taken with Trap collision energies of 25 eV for α-carotene and β-carotene and 20 eV for lycopene. The major neutral losses for each carotenoid are labeled and associated with the corresponding fragment by a black arrow.

Now that the each unique and shared fragment ions have been identified and discussed, it is interesting to next consider the drift time distributions of each in order to better understand the ways in which the fragments associate with the conformations of the precursors. Figure 6.7 shows a zoomed in region of the α-carotene MSMS in the top panel and four CCS distributions corresponding to the labeled fragments at m/z 521 (Figure 6.7a), m/z 480 (Figure 6.7b), m/z 444 (Figure 6.7c), m/z 388 (Figure 6.7d), and m/z 321 (Figure 6.7e). As discussed above, m/z 321, m/z 388, and m/z 480 are the fragments unique to α-carotene in comparison to β-carotene and lycopene, while m/z 444 and m/z 521 were identified as fragments of the toluene methyl radical losses shared by all three molecules. For the m/z 521 peak, there is one major peak at 165 Å²
and what may be a shoulder of longer drift time at 176 Å². The signal for this peak, however, was rather low so this shoulder may be an artifact of having not enough averages to smooth out this side peak. Moving to the first unique peak at m/z 480 in Figure 6.7b and corresponding to the isobutylene neutral fragment loss from the precursor carotenoid, there are two distinct peaks observed. The larger peak is the more compact form at a CCS of 165 Å² while the other main feature is at 195 Å². The precursor m/z 536 peak also showed two major drift time populations at 215 Å² and 173 Å² and the loss of isobutylene produces fragment ions with a CCS difference between compact and extended conformer of 31 Å², compared to the difference of 42 Å² for the two precursor ion populations. It is also interesting to note that the more extended m/z 480 population is actually larger in CCS than the more compact precursor population at 173Å². This conveys the idea that the 195 Å² conformation population comes directly from the 215 Å² precursor population, which retains its extended form, while the 165 Å² m/z 480 fragment population may come directly from the more compact 173 Å² precursor population. It is interesting to note that this m/z 480 fragment, unique to α-carotene, can also retain some of the conformational information of the precursor. The fact that the more compact population is approximately double the intensity of the more extended population suggests that either the reaction pathway results in conformational rearrangement of the α-carotene, or that it is more likely to form the isobutylene loss from the more compact precursor population. For the fragment observed at m/z 444 (Figure 6.7), which corresponds to the toluene loss shared by all three carotenoids, there is only a single main peak at 175 Å² with a small tail at lower CCS. Again, this CCS appears to be well extended and the fact that it is 2 Å² above to the most compact peak of the precursor at 173 Å² is telling in that the fragment m/z 444 must need to “unravel” the precursor in order to be formed, otherwise the toluene loss fragment should have a major population below 173 Å² taking into account the loss of the C₇H₆ group. For the
second unique α-carotene fragment of m/z 388 (Figure 6.7), which results from the loss of isobutylene from m/z 444, the CCS distribution is very similar to that of m/z 444, as could be expected. As with m/z 444, there is an extended peak centered at 156 Å² and a tail at a lower CCS, but in the case of m/z 388 this tail is slightly larger in relative intensity compared to the major extended peak. Additionally, the width of the extended peak is similar to the fragment at m/z 444 but at the lower portion of the peak there is a width increase due to the more compact tail's overall intensity. It could be possible that the m/z 444 compact tail is more likely to undergo isobutylene loss as with m/z 480 (Figure 6.7b) the small CCS population is more intense and this also suggested that the more compact the carotene the more likely the isobutylene loss. The final unique peak of α-carotene at m/z 321 (Figure 6.7e) shows a single peak distribution. The fact that these is no tail or second minor distribution either more or less extended than single peak at 120 Å² is an interesting feature. At greater loss of the length of the carotenoid, eventually the minor changes to conformation can be removed until only one conformation is possible as seems to be the case for m/z 321.
Figure 6.7 Collision cross section distributions of the major fragments of α-carotene a) \( m/z \ 521 \), b) \( m/z \ 480 \), c) \( m/z \ 444 \), d) \( m/z \ 388 \), and e) \( m/z \ 321 \). Top panel shows the fragmentation spectrum of α-carotene at trap collision energy of 25 eV with the fragments CCS distributions in the panel below labeled and the neutral losses corresponding to each fragment depicted. The x-axis was converted from drift time to CCS (\( \text{Å}^2 \)) using a polyalanine calibration standard.\(^{217,218} \)

In Figure 6.8 the β-carotene MSMS is shown for reference with the fragment CCS distributions of a) \( m/z \ 521 \), b) \( m/z \ 444 \), c) \( m/z \ 429 \), and d) \( m/z \ 323 \). The fragments at \( m/z \ 323 \) and 429 were also observed for α-carotene but were present at greater intensity for β-carotene and, therefore, should be assessed by IMS due to their significant proportions upon CID fragmentation. Due to their presence in the MSMS spectra of all three carotenoids it is of interest to compare the fragments at \( m/z \ 444 \) and 521 to
determine any conformational similarities or differences. As was seen in α-carotene (Figure 6.7a), the m/z 521 methyl radical loss fragment for β-carotene presents in a single major conformational family, in this case mainly centered around 167 Å², as compared to 165 Å² for α-carotene. The width of the peak is much larger for β-carotene than for α-carotene, but this may be that the shoulder peak observed for α-carotene is less resolved here but still adding to the higher CCS side of the central 167 Å² peak, which is supported by the tailing in that direction. In Figure 6.8b, the toluene loss at m/z 444 is essentially identical to the α-carotene m/z 444 CCS distribution (Figure 6.7c) where the dominant extended conformation is at 174 Å² compared to 175 Å² for α-carotene. The two CCS distributions are so similar that it was surmised that the toluene loss goes through the same pathway to result in products of the same conformation. Whether this means that the toluene loss comes from the β-carotene terminal ring and the α-carotene unique terminal ring for α-carotene is difficult to determine. That the m/z 444 peak of α-carotene also loses isobutylene, however, whereas no isobutylene is lost in this case at of β-carotene means that the toluene fragment is mostly likely a result of the fully conjugated terminal ring shared by both molecules. Most likely, both sides of the α-carotene terminal rings can lose this species as even lycopene below has this as a dominant fragment.

For the fragment at m/z 429 (Figure 6.8c), which corresponds to the loss of the methyl radical from m/z 444, the CCS distribution shows there are two peaks very close to one another at 149 Å² and 164 Å². Since this fragment comes from the m/z 444 peak, it is interesting to compare one to the other. The fragmentation into m/z 429 changes the CCS distribution dramatically, in that what once was an ion population with a large peak at 174 Å² and a small tail off in the 152 Å² region, then transitions by the loss of CH₃⁺ into a more dominant compact peak with a large shoulder ~15 Å² larger in CCS. As the m/z 521 fragment is also a methyl radical loss, it is interesting to note that the two
are broad peaks with a tail or shoulder in the region of larger CCS than the peak center. The last fragment under consideration in Figure 6.8d is the m/z 323 fragment, not unique but a peak used for comparison to the unique m/z 321 peak of α-carotene. Like the m/z 321 peak of α-carotene, the m/z 323 only has one narrow distribution at 120 Å² while that of α-carotene above has a CCS value at 120 Å² as well. Clearly these fragments have very different neutral losses and possible very different pathways of formation, but after losing so much from fragmentation, the two appear to arrange into incredibly similar structures as there is little room for differentiation at this size of fragment.
Figure 6.8  CCS distributions of the major fragments of β-carotene a) m/z 323, b) m/z 429, c) m/z 444, and d) m/z 521. Top panel shows the fragmentation spectrum of β-carotene at trap collision energy of 25 eV with the fragments CCS distributions in the panel below labeled and the neutral losses corresponding to each fragment depicted. The x-axis was converted from drift time to CCS (Å²) using a polyalanine calibration standard.$^{217,218}$

The lycopene major fragment IMS analysis in Figure 6.9 show the CCS distributions of the fragment ions presenting at m/z 521, m/z 467, m/z 444, and m/z 375 in Figure 6.9 respectively. For unique fragments in this carotenoid, the m/z 375 and m/z 467 represent the two separate isoprene neutral losses from m/z 444 and the M⁺, respectively, as were discussed previously in this section. The m/z 521 of the methyl radical loss in Figure 6.9a and m/z 444 toluene loss in Figure 6.9c show two CCS
distributions that they emulate the peak shapes (i.e. width and tailing) of α-carotene and β-carotene. The peak positions are extended to 170 Å² for m/z 521 and 183 Å² for m/z 444, compared to 165 Å² and 175 Å² for α-carotene and 167 Å² and 174 Å² for β-carotene. Because the remaining peak after the neutral loss will still contain a fully extended terminal opposite the side of the loss, the lycopene fragments should naturally be observed at larger CCS to account for this extension, relative to the two carotene molecules. The two unique peaks for lycopene, however, are very interesting in their retention of two separate drift time populations. In the case of m/z 467 in Figure 6.9b, the isoprene loss results in a large compact conformation peak at 153 Å² and a less intense much more extended peak at 195 Å². As with the m/z 480 peak of α-carotene (Figure 6.7b), the single loss of isoprene appears to occur in a pathway either more favorable for the more compact population of the precursor lycopene or that upon loss of isoprene the remaining fragment is perhaps stabilized by the isomerization into the 153 Å² distribution population. Moving to fragment at m/z 375, there are again two distinct peaks, but they are less resolved in that they possess a smaller gap with the more extended conformation centered at 154 Å² and the more compact at 134 Å². This peak is formed from the loss of isobutylene from the m/z 444 fragment and comparing the CCS distribution of m/z 444 (Figure 6.9c) to this peak raises some important questions. As with the m/z 429 methyl radical loss seen from β-carotene in Figure 6.8c, the distribution of m/z 444 is significantly affected by the radical neutral fragment dissociation. Instead of a compact peak with a larger tail/shoulder formed as those of the methyl radical loss, the isoprene loss from m/z 444 can be seen to form almost equivalent populations with a CCS shift of 20 Å². As the CCS distribution m/z 444 of lycopene in Figure 6.9c had a small tail at ~152 Å² compared to the 30 Å² shift to the major peak at 183 Å², the fact that the isoprene dissociation would produce a similarly spaced group of conformers is not surprising. Again, however, the more compact
population forms in much greater intensity for the fragment at $m/z$ 375, which suggests a stability to the more compact structure or also likely the ability to form the isoprene loss from this smaller CCS population of the $m/z$ 444 fragment starting structure.

Figure 6.9  CCS distributions of the major fragments of lycopene a) $m/z$ 521, b) $m/z$ 467, c) $m/z$ 444, and d) $m/z$ 375. Top panel shows the fragmentation spectrum of lycopene at trap collision energy of 20 eV with the fragments CCS distributions in the panel below labeled and the neutral losses corresponding to each fragment depicted. The x-axis was converted from drift time to CCS ($\text{A}^2$) using a polyalanine calibration standard.\textsuperscript{217}
The fragments of these three carotenoids are varied and complicated to interpret when considering their independent conformational populations. What they appear to show, however, is a trend in the formation of radical neutral losses which produce a larger proportion of compact structures, which is attributed to trans-cis isomerization, than the starting structure from which they were derived. Even after the loss of toluene, an even electron loss which produced mainly one extended fragment ion population with a smaller compact tail for all three carotenoids (Figure 6.7c, Figure 6.8b, and Figure 6.9c), the loss of isoprene for lycopene reformed the ion population into divergent conformations in Figure 6.9d. Additionally, the loss of the same isoprene and also isobutylene radicals from the precursor M•⁺, were observed with large gaps between a clearly isomerized compact structure and an extended feature, which in both cases (α-carotene and lycopene in Figure 6.7b and Figure 6.9c, respectively) were higher in CCS than that of the compact precursor peak. Clearly, while the even electron loss of toluene resulted in conformationally equivalent structures, the odd electron radical neutral losses resulted in varied and interesting changes to carotenoid structure. After the losses leading to m/z 321 and 323, however, the degree of conformational specificity was reduced, presumably due to the loss of degrees of freedom from the size of the chain upon the formation of these fragments. These fragment CCS distributions are a source of interest in the investigation of radical ionization and the fundamental physical chemistry guiding their dissociation pathways. Further work in this topic requires detailed reaction pathway investigation, which is beyond the scope of this work but nonetheless, the fragment CCS distribution allow insight into the relative differences between the carotenoids α-carotene, β-carotene, and lycopene and could therefore be used in the future to allow for better differentiation of the isobaric compounds. The next section outlines the beginning of this separation process using IMS as the main tool in
the quantification of each in mixture and the CCS distributions of each fragment were critical to study in order to better understand and carry out the quantitation utilized.

6.3.4. Quantitation of α:β and α:β:lycopene ratios via ion mobility measurements

Relative quantification of α, β, and lycopene can be performed by exploiting the combination of a consistent CID energy and collection of IM peaks for all the resulting fragment ions. Upon comparison of the MSMS of α, β, and lycopene, as detailed above in section 6.3.3, unique fragments were found for α-carotene and lycopene. Notably, for α-carotene fragment at m/z 480 corresponding to the loss of isobutylene and m/z 321 were observed (Figure 6.6a), while for lycopene, it was the fragment at m/z 467, corresponding to loss of isoprene (Figure 6.6c). The mixtures were fragmented and the drift times of specific fragments processed to determine ratios of peak area for these fragments. At each concentration the intensity of the unique fragment m/z 480 or 467, was compared to the intensity of m/z 521, a methyl radical loss present for all three carotenoids. IM-MS was particularly useful for this method of ratiometric analysis since it allowed for a further isolation of the fragment peak via its drift time distribution. This made it much easier to determine the intensity of various fragment peaks, as the area under the curve for the IM distributions of each could be used to relate their relative ratios. Calibration curves were produced for the amount of β relative to lycopene and α by combining standards of each at varying concentrations by exploiting the differences in fragmentation spectra of the two species.

Starting with an isolated case in Figure 6.10, the application of this technique to identify the relative ratios of α-carotene to β-carotene was assessed using mixtures of α:β at 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10 along with solutions of 100% α and β and collision energies of 15, 20 and 25 eV. The first observation from these experiments is that at a Trap collision energy of 15 eV (Figure 6.10a), the CCS distribution peak area
ratio of \(m/z\) 321 and the precursor at \(m/z\) 536 has an excellent linearity with an \(R^2\) value of 0.9954. Since \(m/z\) 321 is a fragment unique to \(\alpha\)-carotene is can be used to provide well correlated calibrations quantifying the percentage of \(\alpha\)-carotene in a mixture of the two compounds. It is important though to consider the other two plots which are also \(m/z\) 321 CCS distribution peak ratios to the precursor at \(m/z\) 536, which were derived following an incremental increase of Trap collision energy to 20 eV in Figure 6.10b and 25 eV in Figure 6.10c. For 20 eV, the \(R^2\) value drops from 0.9954 seen at 15 eV to 0.9841, while at 25 eV the linearity \(R^2\) value drops down to 0.8934. Since all three were carried out with identical conditions on the same day, it is suggested that in order to achieve suitable calibration curves using the area under the peak of the CCS distributions produced in the drift cell, the two peak areas being compared must both have decent signal. At the 20 eV and 25 eV trials, the remaining precursor which served as the IMS peak to compare to the \(\alpha\)-carotene peak \(m/z\) 321 was reduced in intensity so that upon trials of 10:1 to 1:10 \(\alpha:\beta\), a lack of consistency was observed in the relative amount of precursor observed. At 15 eV, however, the balance between fragment and precursor was balanced enough to result in measurements where only \(m/z\) 321 would change significantly sample by sample and therefore \%\(\alpha\)-carotene scaled well at this energy to the peak ratios of \(m/z\) 321 and precursor.
Figure 6.10  Relative quantitation calibration curves for percentage of α-carotene in α-carotene + β-carotene mixtures using the m/z 321 and 536 peaks at a) 15 eV, b) 20 eV, and c) 25 eV trap collision energy. Curves were produced with ratio mixtures of fresh α:β of 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, and solutions of 100 % α and 100 % β.

In Figure 6.11, α to β quantitation is shown using a different set of fragment ions for ratiometric determination. The y-axis in this case is the ratio of the CCS distribution peak areas under the m/z 480 fragment, the unique isobutylene loss from the α-carotene precursor, and the m/z 521 peak, the radical methyl neutral loss of the precursor observed for both α and β at similar intensities. Here the three plots also show an
important point of this ratiometric process in that Figure 6.11a uses the α:β mixtures of 5:1, 2:1, 1:1, 1:2, and 1:5, Figure 6.11b uses another set of α:β mixtures at 7:1, 3:1, 1:3, 1:7, while Figure 6.11c shows a combine data set where these two trials performed at separate days are added to the same calibration curve. As the $R^2$ values indicate, with all fragments formed at 20 eV, the $m/z$ 480 to $m/z$ 521 works very well with a high degree of linearity observed. In day one, the five point calibration curve provides linearity at an $R^2$ value of 0.9959, day two with the four-point calibration at a whole other set of mixtures results in linearity corresponding to an $R^2$ of 0.9976. While the separate trials produced curves of very high correlation, trying to combine the two into one set in Figure 6.11c results in a much lower $R^2$ value at 0.9233. There appears to be a significant effect on the experimental data from slight changes to the condition of the instrument from day-to-day, resulting in no reproducibility between trials one day apart. As long as a full calibration curve can be produced within the same run, an unknown sample’s % of α-carotene can be measured in a mixture of β-carotene and α-carotene.

It is also important to compare the success in using the $m/z$ 480 to $m/z$ 521 CCS distribution peak ratios as compared to the $m/z$ 321 to $m/z$ 536 used in Figure 6.10. The highest $R^2$ value for the previous set of calibrations was at 15 eV CE with an $R^2$ of 0.9954, but both trials in this set of calibrations achieved at least an $R^2$ of 0.9959 and this could be due to several factors. The $m/z$ 321 fragment is unique to α-carotene but the area is populated with other ions that both carotenes share (i.e. $m/z$ 323), which could result in overlap skewing the peak ratios at each mixture. The region around $m/z$ 480, however, is very clean as the only major loss directly from the precursor is toluene, which is separated by 36 mass units from $m/z$ 480. Additionally, it would be reasonable to pick fragments that come directly from the precursor as the $m/z$ 321 fragment forms from the $m/z$ 444. This means that the fragment is more energy dependent to form as the precursor α-carotene must form $m/z$ 444 which still must have enough energy to
form m/z 321 while the route to form the m/z 480 is direct and changes to absolute amount of α-carotene will be less influenced in the formation of fragment m/z 480 than the of m/z 321. In fact, using the exact same data from these two trials in Figure 6.11, the m/z 321 in place of the m/z 480 measured against m/z 521 in the same method provides R² values that are below 0.990, which contributes to the idea that the m/z 480 is a more robust fragment ion to use for this particular mixture ratio determination for the possible reasons detailed here.
Figure 6.11 Two separate trials of relative quantitation calibration curves for percentage of α-carotene in α-carotene + β-carotene mixtures using the m/z 480 and 521 peaks at 20 eV trap collision energy. Curves a) and b) were produced a day apart with ratio mixtures of fresh α:β, which for a) was 5:1, 2:1, 1:1, 1:2, and 1:5 for b) was 7:1, 3:1, 1:3, and 1:7. Plot c) is the combination calibration plot of the two days, showing that this method is not reproducible unless performed on same day.

The last set of calibration curves presented here are shown in Figure 6.12 and were compiled using standards containing α-carotene, β-carotene, and lycopene, with the goal to calibrate the % of α-carotene (Figure 6.12a) and % of lycopene (Figure 6.12b)
with respect to β-carotene and between each other (Figure 6.12c), all in one calibration run. The mixtures selected for calibration were α:β:lycopene ratios of 5:1:1, 3:1:1, 1:5:1, 1:5:1, 1:1:5, 1:1:3, and 1:1:1. Even though there were 7 calibration mixtures, in order to produce the calibration curve correlations shown, only 5 could be selected for each. This is because for each trial, the % of one species could only be compared to the change in one of the other two analytes in order to get the most consistent calibration plots, and so for lycopene and α-carotene changes in concentration were only compared to β-carotene. For example, for the % α-carotene in Figure 6.12a, the ratios of α:β:lycopene, only the 1:5:1, 1:3:1, 1:1:1, 3:1:1, and 5:1:1 were used as this reduced variation of the third ion population lycopene, allowing a monitoring the change in fragment ion ratios for primarily α-carotene and β-carotene throughout the whole trial. For this reason, the comparison in Figure 6.12 of the m/z 467 and m/z 480 was used to directly compare lycopene and α-carotene so that a complete relative relationship for all three species could be performed with one set of standards. Since the fragments chosen were unique to α-carotene and lycopene, the x-axis was plotted for only the ratio of α-to-lycopene and so the mixtures prepared at ratios of 5:1, 3:1, 1:1, 1:3, and 1:5 provided the R² value of 0.9817.

The multiplexed study here highlights another important point. Upon the addition of a third carotenoid the % α-carotene can still be monitored in Figure 6.12a with m/z 480 and m/z 521, but this relationship becomes logarithmic, which is attributed to the added intensity of the m/z 521 peak from the fragmentation of the lycopene present in the mixture. This was also the case of Figure 6.12b, where the % of lycopene can be monitored with the peak ratio of the fragment ions at m/z 444 and m/z 467. This relationship is logarithmic because the α-carotene contribution to the fragment at m/z 444 which results in an exponential rather than linear shift to the ratio. This calibration, however, is at an R² value 0.9694, which is the lowest of the correlation of the curves
shown. The reason for this lack of linearity is unknown and therefore the fragment CCS
distribution analysis of lycopene should be investigated further to determine if there are
any better options for ion mobility distribution ratios to determine lycopene percentage in
a mixture of α-carotene and β-carotene. It may be better to determine the ratio of α-
carotene relative to lycopene as seen in Figure 6.12c and indirectly relate the ratio of β-
carotene to lycopene from the ratio of α-carotene to β-carotene. All of these options for
ratiometric determination would be incredibly valuable in a spiking experiment with a real
world mixture as known proportions of each addition could be corrected for to determine
initial quantities of all three carotenoids in such a sample.
Figure 6.12  Relative quantitation calibration curves for percentage of a) α-carotene and b) lycopene in a α-carotene + β-carotene + lycopene mixture and c) a curve comparing α versus lycopene using in a) the \(m/z\) 480 and 521 peaks, in b) the \(m/z\) 444 and 467 peaks, and in c) the \(m/z\) 467 and \(m/z\) 480 fragment peaks, with all data collected in the same trial at 20 eV trap collision energy. Ratio mixtures of fresh α:β:lycopene of 1:1:1, 3:1:1, 1:3:1, 1:1:3, 3:1:1, 5:1:1, 1:5:1, and 1:1:5 were used with only the change in β-carotene used to compare to % α-carotene in a) and % lycopene in b).
6.4. Conclusions

The radical ionization of the three isobaric carotenoid species α-carotene, β-carotene, and lycopene results in species with many significant differences in gas-phase ion chemistry. With the conjugated double bond structure a key feature, the trans/cis isomerization nature of these species can easily be assessed upon ionization by ion mobility spectroscopy coupled to mass spectrometry. Two and up to three drift time populations are observed with large gaps in CCS between the most extended (217 Å$^2$ for α-carotene, 215 Å$^2$ for β-carotene, and 226 Å$^2$ for lycopene) and compact (174 Å$^2$ for α, 177 Å$^2$ for β, and 172 Å$^2$ for lycopene) with the instrument conditions of the Waters Synapt G2S. With disagreement between theoretical CCS results, which place the trans carotenoids at least 10 Å$^2$ higher than the extended populations, there is some question as to the amount of trans-cis isomerization during the ionization process and as the ions travel through the instrument.

The differences in CCS distributions and MSMS of the α-carotene and β-carotene and lycopene, however, show that the isobaric compounds can be discriminated using either high pressure StepWave excitation (α versus β) or by fragmented to observe the isobutylene neutral losses (α) or isoprene losses (lycopene). Using MSMS coupled with IMS analysis to find the relative ratios of fragments from α and lycopene, calibration curves were obtained that were able to achieve R$^2$ values above 0.996 in several trials. Optimal calibration curves were obtained for fresh samples and it was determined that each mixture could be tuned with collision energies between 15 eV and 20 eV, depending on instrument conditions and the fragments being measured for IMS peak area. Linearity of the curve could shift, as was seen between the three plots in Figure 6.10. The use of this method could potentially allow for an LC-free quantitation of several carotenoids in a mixture, even for three carotenoids of the exact same mass.
Chapter 7. Metal and Iron Oxide Particles as Matrices in LDI

7.1. Introduction

One of the most robust and rapid mass spectrometry ionization techniques for large molecule analysis is matrix-assisted laser desorption ionization (MALDI).\textsuperscript{32,220,221} This technique, developed in the work of Karas and Hillenkamp\textsuperscript{222,223} and Tanaka et al.\textsuperscript{224}, relies on the properties of matrix molecules coprecipitated on a plate with a target analyte, to absorb laser irradiation and subsequently desorb protonated molecules. Typically, the matrix is a small (100-300 Da) organic aromatic molecule with either proton donating or abstracting features. The conjugated aromatic property allows for efficient absorption of an incident UV laser beam to excite and transfer energy to the analyte, the process by which this occurs, however, is still under debate\textsuperscript{35-40}. By having an acidic or basic character, the matrix is believed to assist in the protonation or deprotonation of analytes in either positive or negative mode mass spectrometry, respectively. When using this technique for smaller analytes, one major difficulty that arises is that, since the MALDI matrix is so abundant and a strong absorber of laser radiation, spectra can be plagued by matrix-derived interferences in the low mass range. This is true for many common matrices, such as 2,4-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnaminic acid (CCA), and sinapinic acid, which exhibit protonated molecules at \textit{m/z} 154, 189, and 224, respectively. Furthermore, these matrices can also produce proton-bound dimers, fragments, and other interfering matrix peaks in the LDI process.
Several techniques have been developed in recent years to eliminate complications in the low mass range, and produce high ionization efficiency of small target analytes without background peaks resulting from the chemical matrix.\textsuperscript{225,226} In lieu of standard organic matrices, many of these methods involve the use of inorganic substrates or particles so that there is no “contamination” of spectra by either a small molecule matrix or the inorganic substrate itself. One of the well-known techniques based on the use of inorganic substrates is desorption/ionization on porous silicon (DIOS), developed by Siuzdak \textit{et al.}\textsuperscript{227,228} In this process, the analyte is deposited onto a layer of porous silicon, which is generated \textit{via} galvanostatic etching. The energy transferred to desorb the analyte is due to the UV absorption properties of the silicon wafer\textsuperscript{228}. With this method small molecules, such as drugs and short peptides, can be detected at a femto/attomole level of detection with highly reduced matrix-derived background in the mass spectra. Earlier work in surface-assisted laser desorption ionization (SALDI) utilized graphite as the surface and was applied to the analysis of small peptides, identifying several peptides from a tryptic digest, with no detrimental effects of matrix-derived interference in most cases. This technique was, however, limited by the production of carbon ion clusters at higher laser power irradiation.\textsuperscript{226,229} In recent years, silicon nanowire surfaces have been optimized as a commercial substrate under the name of nano-assisted laser desorption ionization (NALDI).\textsuperscript{230-236} Results have been promising for a wide array of analyte types and it has been proposed that NALDI is a more sensitive technique than other current methods of matrix free LDI \cite{20}. Additionally, Siuzdak \textit{et al} have recently developed a technique known as nanostructure initiator mass spectrometry.\textsuperscript{237-240} This technique involved the use of etched silicon wafers with fluorinated and hydrocarbon polymeric “initiator” molecules co-imbedded in the Si nanostructures with the ions of interest.
Nano and microparticles have also been reported as a stage for matrix-free small molecule desorption/ionization. Previous work from our group in this area has focused on optimizing detection with 5-50 nm Si particles. Like the porous surface used in DIOS, the level of background from nanoparticles of Si is low and an enhanced ionization effect can be observed. Low level detection of small drug molecules with almost no matrix peaks as interference has been readily achieved using this method. This technique, called SPALDI (silicon-nanoparticle-assisted laser desorption ionization) is part of a continuing effort to use inorganic nanoparticles for matrix free LDI of small molecule analytes. Other attempts have made use of metal oxides such as ZnO, MnO, CoO, WO$_3$, TiO$_2$, SnO$_2$, Fe$_3$O$_4$, and bare metals such as Au, Ag, Sn, and Al.

SPALDI, along with the other alternative, successful LDI techniques (NALDI, DIOS, NIMS), relies on the use of silicon as the main platform for ionization. In all of these techniques, however, chemical modification of the surface with a non-interacting species such as a perfluorinated compound is required to prevent oxidation and reduce the analyte-surface interactions that would suppress the analyte ion intensity in the mass spectra produced.

In addition to the ability to efficiently absorb UV incident laser radiation and then transfer that energy to desorb and ionize a target analyte without any unintended ions formed, there are other characteristics of inorganic particles to consider that make them successful in assisting LDI of a target analyte. One of these is the interaction of the inorganic matrix with the analyte molecules themselves; i.e. whether the LDI environment induces unintended interactions between the analyte and the particle/surface. This would be an issue with any metal oxide which can covalently bind to functional groups of an analyte or that may induce strong analyte-particle electrostatic forces, reducing the intensity of signal during laser irradiation. Additionally, while the presence/transfer of protons is a necessity for many analyte molecules, the presence
of strong electron donators or acceptors could also influence the ionization efficiency of small molecules, inducing electron transfers that could either enhance or decrease efficiency in the formation of cations and anions in an LDI environment.

In this work we focused specifically on the performance of several high work-function metal oxide particles, exploring their potential in matrix-free LDI of several small molecule analytes. These included microparticle species of WO$_3$ and ReO$_3$, in addition to nanoparticle size WO$_3$. In previous studies, the work-functions of Rhenium and Tungsten metals in oxygen-rich environments were found to be very high, in the range of 6.4 and 6.0 eV respectively $^{261,262}$, and surfaces of those metals provided promising platforms for ionization due to these work-functions. $^{263,264}$ These higher work-functions are also conducive to photodetachment. Tungsten oxide microstructured surfaces have previously been explored for LDI and seem to be able to promote ionization and enhancement of both protonation and electron transfer events. Rhenium oxide has been studied for its charge transfer properties while doped in layers of organic light-emitting diodes (OLEDs). $^{262,265,266}$ Using these metal oxide particles as bare metal surfaces for LDI, we expect an enhancement in positive mode ionization without the need for any modification step to passivate the surface. The analytes chosen in this work encompassed a range of compounds with varying affinities for protonation, precharged bio-related species, and molecules that could form radical ions via photodetachment. They included the readily protonatable drugs cocaine and ametryn, the relatively difficult to protonate sugar glucose, the precharged species choline, and a standard mixture of polyaromatic hydrocarbons described below, known to form radical ions upon laser desorption. Each metal oxide particle was compared to alpha-cyano-4-hydroxycinnamic acid (CCA) as a standard MALDI matrix, whose wide use and performance with protonatable small-molecule species is well documented. $^{267}$ Additionally, the
fragmentation survival yields of 3-methyl benzylpyridine and cocaine were measured for all four matrices to assess the energy deposited upon LDI.

7.2. Experimental
7.2.1. Preparation and spotting of unaltered tungsten and rhenium particles

WO$_3$ nanoparticles (nP) (<100 nm), WO$_3$ microparticles (μP) (<20 μm), WO$_2$ μP (<150 μm), and ReO$_3$ powders were purchased from Sigma Aldrich. To make particle suspensions, 10 mg of each inorganic matrix was dispersed via sonication for approximately 60 minutes into 1 mL of nanopure H$_2$O purified in a Sartorius water purifier (18.3 Ω•cm). Standards of pure cocaine, ametryn, glucose, choline, and a polyaromatic hydrocarbon (PAH) standard mix were purchased from Sigma-Aldrich and each serially diluted to final concentrations of 1 ng/μL (3.3 μM) in MeOH for cocaine, 1 ng/μL (4.4 μM) in H$_2$O for ametryn, 100 ng/μL (556 μM) glucose in H$_2$O, and 500 pg/μL (1.7 μM) of PAH standard. Some of these solutions were further diluted in H$_2$O to lower concentrations as noted below. To spot each sample, a 1:1 mixture of each inorganic particle solution and analyte solution was produced by mixing 4 μL of each and, after vortexing the suspension mixture, spotting 1 μL on a standard stainless steel MALDI plate

7.2.2. Preparation of thermometer ion 3-methyl benzylpyridinium

The 3-methyl benzylpyridinium (BP) thermometer ions were synthesized using methods described previously.$^{109}$ 3-methyl benzyl chloride and pyridine, purchased from Sigma-Aldrich (St. Louis, MO), were mixed together at a 1 to 1.2 molar equivalent ratio for 12 hours at room temperature in dry acetonitrile (Fischer Scientific). The resulting benzylpyridinium salt was crashed out of the ACN solution with the addition of cold diethyl ether, filtered, and then washed with hexane. Stock solutions of 1 mg/mL in
nanopure H$_2$O were made from the solid salt and diluted to a concentration of 100 ng/μL (543 μM) in H$_2$O, for spotting onto the MALDI plate.

7.2.3. MALDI instrument conditions

Spectra were collected on a Bruker “ultrafleXtreme” MALDI-MS operated in reflectron mode with a mass range set from m/z 20 to 900. This system uses a Smartbeam™ laser which combines both nitrogen and Nd: YAG technology to produce focused beams at 355 nm. The laser settings were maintained at a frequency of 20 Hz and in each run 500 shots were collected. The laser attenuator was set to an offset, or lower limit of half the full laser power, of 66 % with a range, or upper limit of 10% this value. Within this range, the percentages of 20 to 45 % were selectively scanned for all spectra in this work. Within this subrange of 20 to 45 % of the total laser flux, relative percentages of 20 to 45% were selected to produce all spectra.[flexControl 3.0 User Manual] The laser was manually rastered along the area of the spot, in each case, to sample for any potential hot spots and test whether the edge or middle of the spot was a better source of analyte ions.

7.3. Results and Discussion
7.3.1. Performance of detection for protonated, sodiated, and radically ionized small molecule species

Figure 7.1 shows a collection of low molecular weight analytes under LDI analysis with ReO$_3$ powder (Figure 7.1a-c) and WO$_3$ μP (Figure 7.1d and e) as the matrix. The analyte: matrix pairings shown were selected as the best performing matches between all four inorganic matrices (WO$_3$ nP, WO$_3$ μP, WO$_2$ μP, and ReO$_3$ μP), as well as CCA and a blank spot (no matrix). The criteria for selection included the overall intensity of the precursor at equivalent laser shots and flux, the suppression of background, the survivability of the precursor ion with respect to possible fragment ions in the case of
cocaine, and the ability to detect the widest range of analytes in the standard mixture of polyaromatic hydrocarbons.

In the case of cocaine co-spotted with ReO$_3$ μP (Figure 7.1a), the dominant peak in the spectrum was the $m/z$ 304.1 precursor, protonated via the single tertiary amine. At a concentration of 1 ng/μL (1 ng on spot), an additional distinct peak is observed at $m/z$ 182, identified to be a cocaine fragment ion formed from the loss of benzoic acid, which has been commonly observed in both MALDI and ESI-MS investigations of cocaine.$^{268,269}$ Both the precursor cocaine structure and the observed fragment are shown in the inset of the spectrum. The other protonated analyte, ametryn, was also spotted with ReO$_3$ and, as with cocaine, no ReO$_3$ background was observed in the spectra (Figure 7.1b). A background was, however, observed due to small impurities on the MALDI plate which could be detected at the low ametryn concentration of 1 ng/μL (1 ng on spot). As for other background, there was some signal, which was apparently a result of small impurities on the plate that could be detected at the low ametryn concentration of 1 ng/μL. For cocaine, the limit of detection was at 100 pg/μL (100 pg on spot) and that for ametryn was slightly higher at 100 pg/μL (100 pg on spot).

In addition to the protonated analytes cocaine and ametryn, ReO$_3$ proved useful in detecting a mixture of polyaromatic hydrocarbons (PAHs), as shown in Figure 7.1. The mixture was spotted at a concentration of 500 pg/μL (500 pg on spot). As each of the PAH analytes could directly absorb the laser and therefore not necessarily require any assistance in ionization, the concentration was lowered to determine the degree of enhancement with the inorganic particles studied. The four observed peaks at $m/z$ 228, 252, 276, and 278 correspond to chrysene, benzo [k] fluoranthene, benzo [ghi] perylene, and dibenz [a,h] anthracene, respectively, with chrysene and benzo [ghi] perylene existing as radical ions. The ReO$_3$ provided a much cleaner signal than the CCA matrix and provided better detection of the lower mass peaks ($m/z$ 228 and 252), as well as
matched the signal of a blank LDI with only water mixed 1:1 with the PAH standard solution (Figure 7.2). This supports the ability of ReO3 to detect both protonated and self-ionizing radicals at the same time without any interference and ion suppression observed for a standard matrix like CCA. On the whole, ReO3 μP provided the best LDI of both the two protonated drugs as well as the radical ions of the PAH mixture. This may be due to the higher work-function of rhenium trioxide compared to the other particles, where both radical and protonated ions should keep their charge and not be neutralized on the particle surface, a hypothesis which can, in the future, be explored with additional compounds.

The analyte requiring the highest concentration on spot (100 ng/μL or 100 ng on spot) was glucose shown in Figure 7.1 and it was detected with minimal background with WO3 μP matrix, as the sodiated m/z 203 peak. The signal at this concentration provided the cleanest spectrum but this analyte could even be detected as low as 1 ng/μL (not shown). Glucose is a difficult molecule to detect regardless of the ionization method chosen and its detection by WO3 μP is likely due, in part, to the “edge” effect present for this particle at its 10 mg/mL concentration. The “edge” effect is believed to be a spatial concentration of the analyte on the boundary of the spot as it moves with the solvent upon spotting, which results in an increased intensity of analyte at this area. This “edge” effect (enhancement of analyte signal at the edge), however, did not appear to be a factor for the other three inorganic oxide particles. At the same analyte concentration, the other oxides tested produced a substantially weaker signal in comparison to that obtained with WO3 microparticle, either on the edge or within the boundaries of the spot. Furthermore, sodium iodide was added to all the matrices to determine if a possible impurity of Na+ was contributing to the elevated intensity of [glucose+Na]+ for WO3 μP. Interestingly, with the addition of this salt at 200 ng/μL (200 ng on spot), the WO3 μP
provided a significantly stronger signal of the sodiated glucose analyte, while no improvement to the [glucose+Na]⁺ signal was observed in any other matrix.

A pre-charged tertiary amine, choline (m/z 104), was next considered with WO₃ μP, which, because it does not require protonation, provides an interesting test for this matrix. While this matrix did not outperform the CCA matrix in the intensity of analyte (Figure 7.3), it did provide limited background interference, much lower than with the matrix derived peaks of CCA.

The WO₃ nanoparticles and WO₂ microparticles did not show the best signal-to-noise or intensity for any of the analytes chosen. Results of the nano versus micro-size particles will be discussed in more detail below with consideration of each particle’s “softness” on both cocaine and the thermometer ion, 3-methyl BP. The results with the WO₂ microparticle exhibited relatively high background and some instability upon increase of laser flux (not shown). This μP did not give improved results over standard CCA matrix, and therefore was not included in the thermometer ion comparison.
Figure 7.1  Performance of Tungsten and Rhenium oxide particles on a variety of small molecule analytes in positive LDI mode at a laser power of 40 % of the available laser flux. The first three spectra were produced using the ReO$_3$ microparticle as matrix: a) 1 ng/μL (1 ng on spot) protonated cocaine with its major fragment, b) 1 ng/μL (1 ng on spot) protonated ametryn, and c) radical ions of a polyaromatic hydrocarbon (PAH) standard mixture with the four hydrocarbons chrysene (m/z 228), benzo [k] fluoranthene (m/z 252), benzo [ghi] perylene (m/z 276), and dibenz [a,h] anthracene (m/z 279) at 500 pg/μL each (500 pg on spot). The bottom two spectra are from using WO$_3$ microparticle as matrix: d) 100 ng/μL (100 ng on spot) glucose (sodiated) with 200 ng/μL NaI and e) 1 ng/μL (1 ng on spot) of the pre-charged choline analyte. Each case above provides the highest and cleanest signal among all particles explored with each analyte. The metal oxide particle suspensions were spotted and mixed 1:1 with the analytes.
Figure 7.2  Radical ions of a polyaromatic hydrocarbon (PAH) standard mixture with the four hydrocarbons chrysene ($m/z$ 228), benzo [k] fluoranthene ($m/z$ 252), benzo [ghi] perylene ($m/z$ 276), and dibenz [a,h] anthracene ($m/z$ 279) mixed 1 to 1 for a final concentration of 250 pg/μL (250 pg on spot) with a) nanopure water, b) ReO$_3$ μP, and c) CCA. All three spectra are set to the same arbitrary intensity of 13000, for comparison.
Figure 7.3 Pre-charged choline analyte mixed 1 to 1 for a final concentration of 500 pg/μL (500 pg on spot) with a) nanopure water, b) WO$_3$ μP, and c) CCA. All three spectra are set to the same arbitrary intensity of 13000 for comparison. All three spectra are set to the same arbitrary intensity of 2700, for comparison.

7.3.2. Survival yields of cocaine and 3-methyl benzylpyridine compared for matrices CCA, ReO$_3$ μP, WO$_3$ μP, and WO$_3$ nP

In addition to the signal intensity and background, another interesting aspect of LDI is the amount of internal energy deposited into the analyte, reflecting on the “softness” of the LDI method. Figure 7.4 summarizes the LDI survivability of the thermometer ions 3-methyl BP (Figure 7.4a and b) and cocaine (Figure 7.4c and d). Both cocaine and 3-methyl BP have an easily formed fragment ion and the relative ratio of the major fragment peak to precursor was shown to vary significantly with the laser flux when
either CCA or the three trioxide particles (ReO₃, WO₃ μP, and WO₃ nP) were co-spotted. For the monitoring of one fragmentation pathway, the survival yield (SY) is determined as the peak intensity ratio between the precursor and the (precursor + fragments) and can be used to determine the overall energy deposition for the ionization method being used. In the case of 3-methyl BP, the precursor at m/z 184 is fragmented by the loss of the neutral pyridine to form the m/z 105 fragment, where SY = I [184]/ (I [184] + I [105]) so that the higher the survival yield, the less fragmentation and the “softer” the resulting ionization of the sample being laser desorbed with the matrix. The same process was carried out for cocaine with the precursor at m/z 304 and major fragment at m/z 182, as shown in Figure 7.4.

The matrices shown in Figure 7.4 include CCA in blue (diamonds), ReO₃ in red (squares), WO₃ μP (circles) in black, and WO₃ nP (triangles) in green. In Figure 7.4a, the survival yield breakdown of 3-methyl BP clearly demonstrates the “softest” ionization coming from ReO₃, with a small standard deviation between trials run on separate days. It is, however, evident that when the laser flux percentage was below 35%, SY with CCA improved dramatically and that below the lowest attenuation shown for both the 3-methyl BP (Figure 7.4a) and cocaine (Figure 7.4c), the CCA survival yield exceeds that of ReO₃. As laser flux increases, the SY with ReO₃ μP is retained and is higher than CCA, for both the pre-charged 3-methyl BP and the protonated cocaine analytes, even though in the case of cocaine the signal intensity is lower than CCA.

It is important to comment on the advantage of the WO₃ μP over the WO₃ nP. It might have been presumed that since the nanoparticle is <100 nm, it would absorb better in the region of the 355 nm wavelength and impart a greater amount of energy into the analytes, increasing the amount of LDI relative to the micro-sized particle. This is not the case, however, and throughout the 3-methyl BP and cocaine intensity plots (Figure 7.4b and Figure 7.4d, respectively) the WO₃ μP provides larger signal and better
SY (i.e. “softer”) for the μP for all cases, except for the 35% laser flux of 3-methyl BP, where the two are equivalent within error. Particle analysis with scanning electron microscopy (SEM) or transmission electron microscopy (TEM) was not performed to determine whether aggregation was a factor, resulting in the differences observed between the two particles. Whether it is the case that the 355 nm laser flux absorbance did not impart enough energy to overcome the advantage in crystallization and uniformity of the higher size particles provided or that the “edge” effect, where more analyte concentrated in the edge of the spot, could have been better facilitated by the greater space between particles of micro-particle size, is unclear. More investigation is required to adequately determine the main cause for this observation. It is, however, clear that there is a pronounced difference in “edge” effect between the smaller nano and larger micro-sized particles of WO$_3$ with a greater enhancement of signal for the micro-size and little advantage for nano.
Figure 7.4  Plots of survival yields versus laser flux percentage of a) 3-methyl benzylpyridinium ion at 100 ng/μL and for c) cocaine at 1 ng/μL for CCA matrix (blue diamonds), ReO₃ μP (red squares), WO₃ microparticle (black circles), and WO₃ nanoparticle (green triangles). The overall intensity for 500 laser shots for b) 3-Methyl BP and d) cocaine are shown. In plots a) and b) error bars represent the standard deviation for an average of at least three runs taken at different days.

7.4. Conclusions

It is the overall conclusion that two metal oxides, ReO₃ and WO₃ μP, without any treatment but rather procured and spotted as is, can be used as matrices, providing clean signal for trace amounts of analytes in the low mass range under LDI. Not only did the ReO₃ and WO₃ microparticles show clean and significant ionization for the analytes chosen, rhenium oxide either outperformed or matched CCA in intensity and survival yield of cocaine and 3-methyl BP at comparable laser flux, while tungsten oxide was
able to detect sodiated glucose where no such peaks could be seen for CCA. The ReO$_3$ microparticle had a particular advantage over CCA at higher laser fluxes because even as laser flux increased, the inorganic particle was not observed to produce any matrix derived peaks cluttering the low mass regions, unlike CCA.

What is most important to conclude from this work is that the ionization of different classes of small molecules could be influenced by the presence of two high work function metal oxides upon laser desorption but that these classes of analytes responded in different ways to the conditions established by the presence of each class of matrix. In previous chapters, this dissertation focused on different forms of analytes chemistry after ionization and introduction to the gas phase, while the work above develops an idea of how and what factors are necessary for ionization to occur. Whether these specific analytes are influenced by the size factor of the ReO$_3$ and WO$_3$ particles, that the differing work functions of each species either allowed or disallowed electron transfer to induce the mechanisms important in laser ablation (ReO$_3$ for protonated species), or the ability to preconcentrate low ionizing ability analytes allowed for enhancement of signal for non-protonatable species (WO$_3$ for sodiated glucose) is difficult to completely characterize. There is, however, the ability to begin to discern these differences and showcase the variable nature of ionization, even for analytes low in mass and seemingly simple with regards to its ionization chemistry.
Chapter 8. Conclusions and Future Directions

8.1. Summing up the structural, chemical, and laser ionization investigations of various small molecular systems

The molecular systems presented in the preceding chapters are all unique systems that offer the opportunity to gain insight into, and hence better understand, their gas-phase ionic properties. To conclude, the main messages of each project are briefly summarized and the future directions outlined. In addition, a brief discussion of how all of these small molecule projects can be viewed within one overarching scope, defined by the goal of better understanding gas phase ionization and ion chemistry, is also given.

In Chapter 3, a peptide fragmentation pathway explaining the consistent lack of $a_3$ ion intensity observed for peptides, compared to the higher intensity of other $a_n$ fragments, was confirmed by the use of a non-standard extended backbone amino acid (gamma aminobutyric acid) at the second position residue of a pentapeptide. Through isotopic labeling and tandem mass spectrometry experiments, the fragments of protonated $\text{A(Gaba)}\text{AlG}$ were analyzed and a reaction scheme defined for the production of $a_3$ and its product ions. Density functional theory (DFT) calculations provided corroborating evidence that the extended backbone inherent to these Gaba-containing $a_3$ ions could be stabilized by rearrangement into a macrocyclic structure via a low energy barrier pathway. Additionally, theoretical studies and mass spectrometry (MS) fragmentation of the $b_2$ and $b_3$ ions from the model peptide studied here confirmed
the stability of the typically unobserved $a_3$ ion on the reaction diagram, thus allowing it to be measured by MS and observed.

As with Chapter 3, Chapter 4 showcased the ability to probe intrinsic gas-phase organic chemistry of a peptide system. In this case, the stereochemical effect of prolyl ring substitution on the cis vs. trans tendencies of the adjacent peptide bond was considered to determine whether R vs S substitution influences the structure of the ring-closure product ion formed by intramolecular attack by the N-terminus vs the first carbonyl oxygen. For this study, MS-based action infrared multiphoton dissociation (IRMPD) revealed chemical information validating the role that R and S substitution of the prolyl ring can have on the populations of diketopiperazine and oxazolone for gas phase $b_2$ ion fragments. In the case of R-fluoroproline substitution, the fragment structure necessitating a trans nature, the oxazolone was formed with greater frequency, while for S-fluoroproline substitution an enhanced diketopiperazine, requiring the peptide bond to undergo an isomerization to cis, was seen. These results confirmed that R and S substitutions on a prolyl ring can have dramatic effects on the identity of the resulting intramolecular ring closure fragment ions. MS$^3$ and ion mobility-mass spectrometry experiments confirmed these observations, and their application in this study also highlighted an energy dependence for the oxazolone vs. diketopiperazine ratios of these particular systems that could not be determined by the IRMPD experiments alone. This study, furthermore, presented clear evidence of a correlation between solution-phase studies previously conducted on the cis vs. trans character of these proline-ring substitutions and the gas phase ion structures determined here.$^{165,167,270}$

Moreover, both Chapter 3 and 4 showcased the advantage that MS techniques including action IRMPD, tandem MS, H-D exchange, and ion mobility have on answering the fundamental questions of how the gas phase environment influences the chemistry of biologically relevant peptide ion molecules.
Chapter 5 presented another set of critically important biologically relevant compounds - two drugs, pomalidomide (Pom) and lenalidomide (Len), used to treat cancer and with known variations in their ability to access specific biological environments. The studies presented here unequivocally determined that Pom and Len have differences in their gas-phase structures and protonation sites that could contribute to the explanation of their dramatically different responses in vivo. Their ionic structures were able to be analyzed chemically, physically, and qualitatively. As with the studies in Chapter 4, IRMPD revealed chemical information of the two ionic populations that would have been impossible to determine otherwise. Pomalidomide was shown to possess a unique tautomerization in its glutarimide ring while no tautomerization sites were present for lenalidomide. Spectroscopy also confirmed a single protonation site shared by both compounds; the carbonyl oxygen present on bicyclic ring feature shared by the molecules. Both H-D exchange MS and ion mobility added information that further highlighted the differences between pomalidomide and lenalidomide, with pomalidomide possessing unexpected multiple drift times, and also unique H/D exchange distributions highlighting multiple structures for its fragments. Additionally, work would be needed to confirm the exact structures of the structures found for Pom in IM and HDX and whether there may be additional structures in each population that are of similar mobilities and would therefore be poorly resolved. Furthermore, pomalidomide was unable to form successful gas-phase proton-bound complexes with any of the amino acids chosen as reference bases in the gas-phase kinetic method experiments conducted so future studies should be performed with additional reference bases including single ring cyclic amides. Upon ionization through protonation, lenalidomide produced a single drift time distribution and successfully formed complexes via a proton with all amino acids in the kinetic method approach. The gas-phase studies presented here reveal that pomalidomide, has higher stability and greater flexibility that
lenalidomide, which differs by only a single functional group. These observed differences could help explain their different activities as cancer therapeutics.

Chapter 6 explored another small molecular biologically relevant system in the radical ionization of the three isobaric carotenoid species α-carotene, β-carotene, and lycopene. The trans/cis isomerization of the conjugated double-bonds of each compound upon ionization was under investigation here and, hence, ion mobility mass spectrometry was used as a conformational probe. Multiple drift time populations were observed with corresponding large gaps in CCS between the most extended (217 Å² for α-carotene, 215 Å² for β-carotene, and 226 Å² for lycopene) and compact (174 Å² for α, 177 Å² for β, and 172 Å² for lycopene) structures in these studies. The differences in CCS distributions and coupling of this data to tandem MS fragmentation of the α-carotene, β-carotene, and lycopene allowed for their differentiation and even allowed relative ion amounts to be determined without any liquid chromatographic separation. The use of this method could potentially allow for an LC-free quantitation of several carotenoids in a mixture, even for three carotenoids of the same exact mass, and thus drastically speed up the investigation and determination of carotenoid character in real world samples. To validate this relative quantification method, it would be necessary in to directly compare LC-derived quantities of each isobaric species in a mixture with values derived from this IM-MS method and compare them. Additionally, this study highlighted the issues involved in radical ionization, as it appeared that the solution-phase double bond conformations of the molecules could be altered upon formation of the radical ions by ESI. This observation raises the need in the future of determining a way to retain pure conformation of conjugated hydrocarbons upon ionization and transfer from solution to the gas-phase.

Finally Chapter 7 presented a shift in the focus, from systems of study where the work dealt with the ion after formation, to the ionization process itself, particularly laser
desorption ionization. Untreated ReO$_3$ and WO$_3$ microparticles showed clean and significant ionization for analytes chosen as model compounds, with analytes chosen to represent protonation, sodiation, radical ionization, and pre-charged ions. Additionally, rhenium oxide either outperformed or matched CCA, a standard MALDI matrix, in intensity and survival yield of two more fragile analytes, cocaine and 3-methyl BP at comparable laser flux. Significantly, tungsten oxide was able to detect sodiated glucose when no such peaks could be seen using CCA, demonstrating a clear advantage of using alternative matrices. Differing work functions of ReO$_3$ and WO$_3$ could be responsible for permitting electron transfer to induce ionization via laser ablation and the ability to pre-concentrate could be enhancing signal for some of the analytes chosen. Future work would require the use of other high-work function metal oxides and comparison of these to low work-function metal oxides to confirm the physical reasons for the variable ionization ability of these matrices. Additionally, more small compounds of protonation, sodiation, radical ionization, and pre-charge should be tested to establish the trends observed in this study.

The research results presented here helped elucidate the intrinsic gas-phase chemistry of small molecular ions, across different structural classes, and utilized mass spectrometry based techniques to do so. In addition to answering fundamental questions regarding ion structure, the work progressed to consider the ionization process itself, in the applications-based work of Chapter 7 where alternative “matrices” for laser desorption ionization were investigated.
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