Gas-Phase Ion Chemistry

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Nuclear Magnetic Resonance Structure Elucidation of Peptide b_2 Ions**

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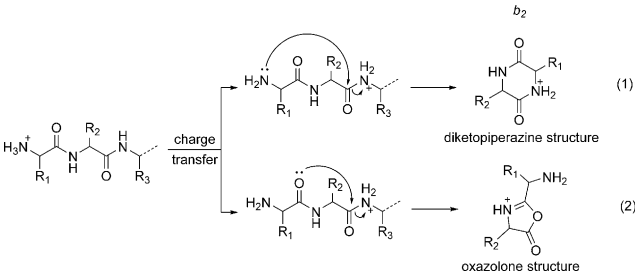
Dedicated to Professor Richard N. Zare on the occasion of his 75th birthday

Abstract: *Tandem mass spectrometry (MS/MS) is powerful for chemical identification but it is still insufficient for explicit ion structure determination. A strategy is introduced to elucidate MS fragment ion structures using NMR spectroscopy for the first time. In our experiments, precursor ions are dissociated at atmospheric pressure and the resulting fragment ions are identified by mass spectrometry but collected outside the mass spectrometer, making the subsequent NMR measurements possible. This new strategy has been applied to determine the chemical structure of the characteristic b_2 fragment ion, a subject of longstanding debate in MS-based proteomics.*



Scheme 1. Proposed b_2 ion mechanisms.

Although MS/MS is powerful for chemical identification based on ion dissociation patterns,[1] it is not ideal for explicit structure determination. The reason for this is simply that information is not sufficient to detail the atomic connectivity in the ions. Here we introduce a strategy to tackle this challenge, by elucidating fragment ion structures using NMR spectroscopy. In our experiments, precursor ions are dissociated at atmospheric pressure and the resulting fragment ions are identified in mass spectra but condensed and collected outside the mass spectrometer. This approach is applied to determine chemical structure of the characteristic b_2 fragment ion, formed from proteins and peptides by collision-induced dissociation (CID).

Upon low-energy CID, peptide ions fragment preferentially via amide bond cleavage to produce *N*-terminal *b* ions and *C*-terminal *y* ions (using the Roepstorff/Biemann nomenclature).[2] However, *b* ion structures, even for the smallest b_2 ion, have been the focus of long debate.[3] Biemann proposed the acylium ion structure in the early days of peptide MS/MS.[2] Later studies revealed that the acylium structure is unstable and spontaneously loses CO.[3a] Subsequent work has focused on two possible structures, the six-membered diketopiperazine[4] and the five-membered oxazolone[5] (Scheme 1). The mechanisms of formation of these two isomers are similar, except that the former involves attacking with *N*-terminal amino nitrogen, rather than the adjacent carbonyl oxygen atom. Because structural elucidation of b_2 ions using only MS was challenging, additional structural methods such as infrared multiple photon dissociation (IRMPD) spectroscopy,[5a,6] H/D exchange (HDX),[5b,7] and theoretical calculations[6a,8] were used. These recent investigations of b_2 ions revealed that not all b_2 ions share a common structure and these structures are highly dependent on the constituent amino acids. For example, the b_2 ions of Gly-Gly[9] and Ala-Ala[5c] were suggested to be oxazolones and Polfer and co-workers demonstrated that peptides with acidic side-chain amino acids such as Glu tend to produce oxazolone b_2 ions.[10] Wysocki and co-workers observed proline-containing b_2 ions to have a diketopiperazine structure.[4a,11] Furthermore, with special amino acids, such as Gln and Asn, special b_2 ion structures were observed, which were neither diketopiperazines nor oxazolones.[6b,12] The complexity of the b_2 ion structural problem and its importance for understanding protein/peptide ion dissociation chemistry, justifies further effort to develop a method of explicit structure elucidation. In this study, we adopted atmospheric pressure thermal dissociation (APTD)[13] to dissociate precursor peptide ions at atmospheric pressure so that the resulting b_2 ions could be condensed and collected outside the mass spectrometer for structural elucidation by NMR spectroscopy, a "gold standard" for chemical structural characterization.

In APTD experiments, precursor ions generated by electrosonic spray ionization (ESSI)[14] were directed into a heated coiled stainless steel tube to undergo dissociation outside a mass spectrometer while passing through the hot

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tube (see Figure 1S in the Supporting Information). Ionic fragments can be detected directly by MS, while neutral fragments arising from the same dissociation event can be detected via online re-ionization to gain increased structural information on the peptide/protein examined.[13] Likewise, as the ion dissociation in APTD occurs outside the mass spectrometer, it should be possible to collect the fragmentation products for subsequent structure elucidation. Indeed, in our experiments, abundant fragments generated from APTD were deposited on the inside wall of the tube and could simply be rinsed with solvent for product collection (see the discussion in the Supporting Information).

As a proof-of-principle showing the possibility of performing ion dissociation and ion collection at atmospheric pressure, lucigenin, a bisacridinium diquaternary salt, was used. When 0.1 mg mL^{-1} lucigenin in MeOH/H_2O (1:1 *v*/*v*) was ionized by ESSI and passed through a coiled tube at room temperature, the mass spectrum recorded online (Figure 2S a) showed the dication at *m*/*z* 193, the singly charged nitric salt of the dication at *m*/*z* 448, and the monodeprotonated ion at *m*/*z* 385. When the coiled tube was heated to 350 °C, a major fragment ion of *m*/*z* 371 appeared, corresponding to the monomethyl-biacridinium cation (Figure 2S b). Another peak at *m*/*z* 386 was observed, probably due to charge reduction during APTD.[13a] These fragment ion products were collected by rinsing the heated coiled tube with MeOH/H_2O. ESSI-MS analysis of the rinsed solution is shown in Figure 2S c. Evidently, the demethylated fragment ion at *m*/*z* 371 is the most significant product, with a yield determined as 34 % (see the Supporting Information). This yield is much higher than the yields (< 1 %) typical of traditional preparative MS techniques based on ion soft landing.[15] This result shows the feasibility of carrying out ion dissociation and ion collection at atmospheric pressure.

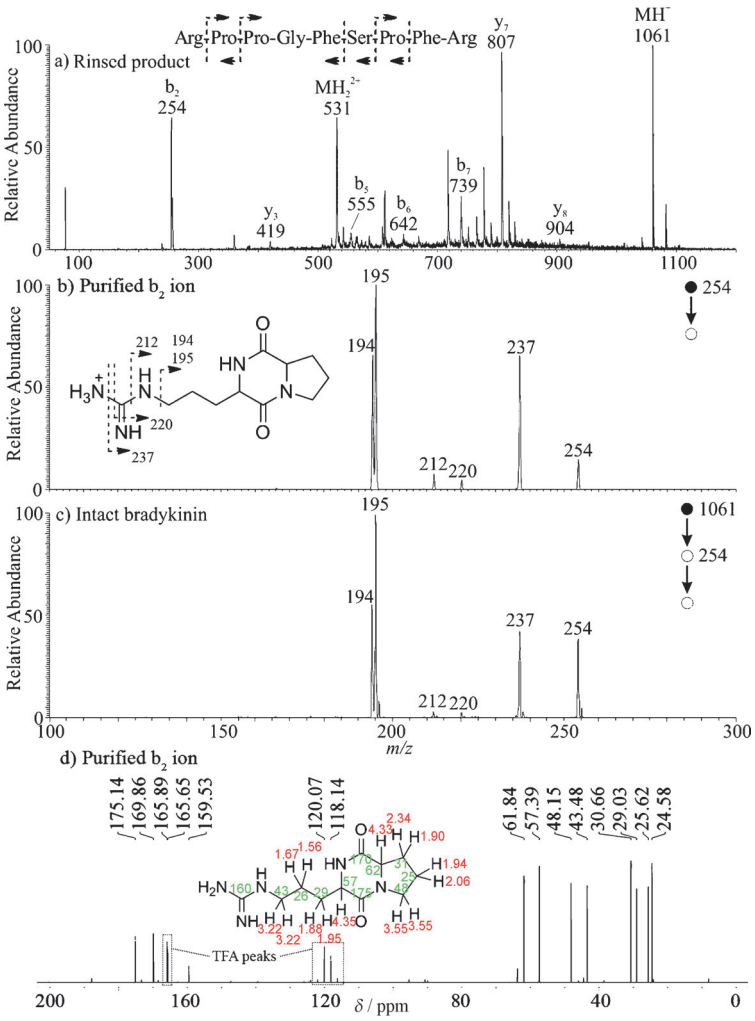

Figure 1. a) MS spectrum of the collected products from the APTD of bradykinin; b) MS/MS spectrum of ions from the purified b_2 product; c) MS^3 spectrum of intact bradykinin b_2 ion; and d) ^{13}C NMR spectrum of the purified b_2 product.

Next, APTD-based ion dissociation and collection was used to prepare b_2 ions for explicit structure elucidation by NMR spectroscopy. The ions are prepared and captured but the collected material could be the deprotonated neutral compound or the ion (depending on the solution pH and b_2 acidity) and structural changes during this step are not automatically excluded. However, such changes are indeed excluded in this case by the identity of the collected product and authentic b_2 ion fragmentation patterns.

As a demonstration, 5.0 mg mL^{-1} bradykinin in MeOH/H_2O/HOAc (50:50:1 *v*/*v*/*v*) was sprayed by ESSI and dissociated using APTD at 350 °C. The online APTD-MS experiment showed b_2 ions as well as other fragment ions (Figure 4S a). The rinsed product was analyzed by ESSI-MS. As shown in Figure 1 a, the b_2 ion was observed at *m*/*z* 254 as a major product. In a control experiment where the peptide was directly heated with N_2 protection but without pre-ionization, no b_2 ion product was detected, suggesting that the produced b_2 ion was produced from the APTD process rather than by mechanisms like pyrolysis. The APTD and subsequent collection of bradykinin was repeated to collect enough products for NMR structural analysis. The collected b_2-containing mixtures were combined and purified by HPLC to a final mass of 1.1 mg (see the Supporting Information for HPLC methods and results). The purified b_2 product was identified by MS and NMR spectroscopy.

CID-MS/MS analysis of the purified b_2 showed that characteristic fragment ions included *m*/*z* values 237, 220, 212, 195, and 194 by losses of NH_3, $2NH_3$, CH_2N_2, CH_5N_3, and CH_6N_3, as a result of cleavages/rearrangements from the arginine side chain (indicated in the inset of Figure 1 b). Significantly, this MS/MS spectrum is virtually identical to the MS^3 spectrum of the bradykinin b_2 ion (Figure 1 c), confirming that the collected ion was structurally the same as the b_2 ion generated during the CID-MS/MS process. Also, it is the same as the MS/MS spectrum of b_2 generated from online APTD (Figure 4S-b, Supporting Information), suggesting the successful condensation and collection of b_2 in our experiment. Note that, besides b_2 ions, some other ions such as b_5, b_6, b_7, y_3, y_7, and y_8 are also seen in Figure 1 a and in the online

APTD mass spectrum (Figure 4S a), suggesting that they also survive the post-APTD condensation and collection processes.

NMR was used to identify the structure of the purified b_2 sample from bradykinin using D_2O solvent by recording a series of ^1H, ^{13}C, and two-dimensional spectra, which allowed the assignment of the b_2 structure as a diketopiperazine (inset in Figure 1 d). Specifically, 14 protons were observed in the ^1H spectrum (Figure 6S), including eight alkane protons without nearby heteroatoms (1.5–2.4 ppm), 4 heteroatom-linked alkane protons (3.2–3.6 ppm), such as -O-CH_n and -*N*-CH_n, and 2 alkane protons surrounded by multiple heteroatoms (4.3 ppm). In addition, 11 carbons were observed in the ^{13}C spectrum (Figure 1 d), counting 4 alkane carbon atoms (24–31 ppm), 2 mono-heteroatom-linked alkane carbon atoms (43–49 ppm), 2 multiple-heteroatom-linked alkane carbon atoms (57–62 ppm), and 3 downfield C=O/C=N carbon atoms (159–176 ppm). Importantly, besides the guanidine carbon (160 ppm), the other two downfield carbons were observed at around 170 ppm, correlating to the two carbonyl groups in the diketopiperazine structure. In contrast, the oxygenated sp^2 carbon in oxazolone has a chemical shift of around 150 ppm,[16] which was not observed in the NMR data; this result excludes the oxazolone possibility which would have one carbonyl and one oxygenated sp^2 carbon [Scheme 1, Eq. (2)]. A search of standard compound ^{13}C NMR shift databases also led to the same conclusion (Figure 16S). In the ^{13}C NMR spectrum, trifluoroacetate peaks were observed at 120 and 165 ppm. Since TFA was used in the b_2 purification process, the existence of the trifluoroacetate peaks in the NMR spectrum indicates that the b_2 exists in ionic form and trifluoroacetate serves as the counter ion. This is reasonable as b_2 from bradykinin carries the very basic arginine residue and no basic solvent was used for deprotonation during APTD, solvent rinsing and separation steps. Furthermore, the ^{13}C DEPT-135 spectrum and the two-dimensional spectra (Figures 7S–15S) confirmed two CH-CH_2-CH_2-CH_2 spin systems in the structure, corresponding to the arginine side chain and the proline side chain, respectively, and thus confirmed the b_2 structure (see the Supporting Information for detailed spectral interpretation). This result, the first use of NMR spectroscopy in solving a gas-phase ion structure problem, matches a previous assignment made by gas-phase ion spectroscopy of a proline-containing b_2 ion as having a diketopiperazine structure.[4a]

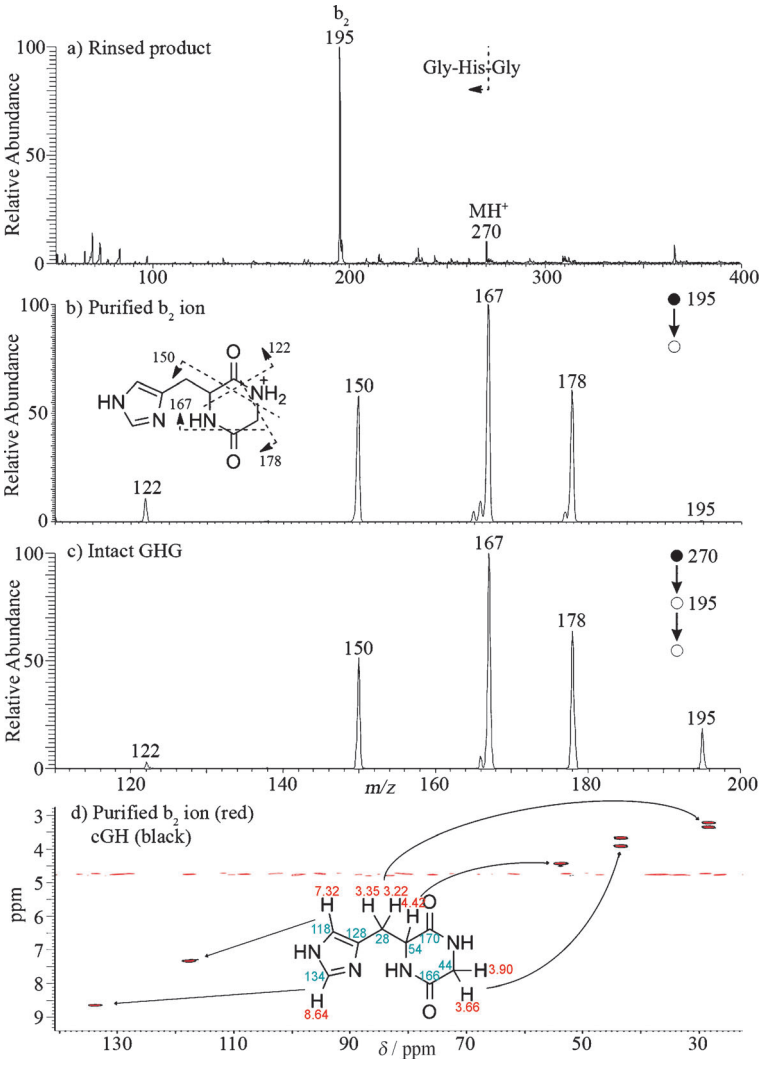

Figure 2. a) MS spectrum of the collected products from the APTD of GHG; b) MS/MS spectrum of ions from the purified b_2 product; c) MS^3 spectrum of intact GHG b_2 ion; and d) ^1H-^{13}C HSQC NMR spectra of the purified b_2 product (red color) and standard cGH (black color).

Another peptide, Gly-His-Gly (GHG), was also tested. The APTD experiment of GHG was performed under the same conditions used for bradykinin. The online APTD-MS experiment showed b_2 ion a major fragment (Figure 17S a). The ESSI-MS spectrum of the rinsed product is shown in Figure 2 a. In the spectrum, the b_2 ion at m/z 195 is observed as the dominant peak along with the intact protonated peptide at m/z 270. The produced b_2 ion was further purified by HPLC and examined by multiple analytical techniques including NMR spectroscopy (see details in the Supporting Information). CID-MS/MS was also used to identify the purified b_2 ion. As shown in the MS/MS spectrum (Figure 2 b), fragment ions were observed at m/z 178, 167, 150, and 122 by losses of NH_3, CO, CH_3NO, and $C_2H_5N_2O$, respectively, which is identical with the MS^3 spectrum of the b_2 ion produced from intact protonated GHG at m/z 270. This result suggests that the APTD produced b_2 ion has the same structure with that produced in vacuum by CID.

Furthermore, a standard compound cyclo-(Gly-His) (cGH), a synthesized diketopiperazine consisting of one glycine and one histidine, was used for comparison with the b_2 ion produced by APTD. At first, both standard cGH and APTD b_2 products were analyzed by NMR spectroscopy. The 2D ^1H-^{13}C HSQC and HMBC spectra were recorded and are shown in Figure 2 d and Figure 19S, respectively. In these

spectra, the data for cGH (marked black) and the APTD-derived b_2 product (marked red) are identical, which provides strong evidence that the APTD b_2 product has the same diketopiperazine structure as cGH. In detail, a total of seven protons were observed in the NMR spectra, including five upfield protons (3.2–4.5 ppm) and two downfield protons (7.3–8.3 ppm). In addition, eight carbon signals were observed, including two CH_2 carbon atoms, three CH carbon atoms, and three carbon atoms without attached protons. These observations also confirm the diketopiperazine structure.

Moreover, the secondary and tertiary carbon atoms and their attached protons could be accurately assigned based on the HSQC spectra (Figure 2d). According to the structure of cGH, the CH_2 in the diketopiperazine could be assigned as C(44 ppm)-H(3.66, 3.90 ppm); CH_2 between the imidazole and the deketopiperazine could be assigned as C(28 ppm)-H(3.22, 3.35 ppm); the CH in the diketopiperazine could be assigned as C(54 ppm)-H(4.42 ppm); and the two CH in the imidazole could be assigned as C(118 ppm)-H(7.32 ppm) and C(134 ppm)-H(8.64 ppm). Furthermore, three carbon atoms without attached protons were also observed in the HMBC spectra (Figure 19S), at 128, 166, and 170 ppm, respectively. Among these three carbon atoms, those at 166 and 170 ppm were assigned to the two carbonyl groups in the deketopiperazine ring, which also agrees with the NMR spectra interpretation of the APTD-produced b_2 ion from bradykinin. In addition to the HSQC spectra, the observed correlations in the HMBC spectra also confirmed the structure (see detail elucidation in the Supporting Information). The chemical structure with assigned carbon/proton shifts is shown in the inset of Figure 2d. Besides NMR spectroscopy, the standard cGH and the APTD b_2 product were also analyzed by FT-IR spectroscopy and showed a high similarity by comparison (Figure 20S).

In conclusion, we have demonstrated the utility of APTD-based ion dissociation and collection to solve the critical problem of structural determination of b_2 species in combination with NMR spectroscopy. Peptides bradykinin and GHG were found to produce b_2 ions with diketopiperazine structures, as confirmed by NMR results. It is expected that this strategy would be applicable to many difficult fragment ion structures in both fundamental ion chemistry studies and in applied proteomics research.

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