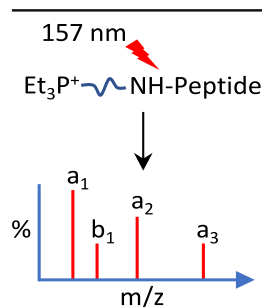ASMS

RESEARCH ARTICLE

A Novel Triethylphosphonium Charge Tag on Peptides: Synthesis, Derivatization, and Fragmentation

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Abstract. Charge tagging is a peptide derivatization process that commonly localizes a positive charge on the N-terminus. Upon low energy activation (e.g., collision-induced dissociation or post-source decay) of charge tagged peptides, relatively few fragment ions are produced due to the absence of mobile protons. In contrast, high energy fragmentation, such as 157 nm photodissociation, typically leads to a series of a-type ions. Disadvantages of existing charge tags are that they can produce mobile protons or that they are undesirably large and bulky. Here, we investigate a small triethylphosphonium charge tag with two different linkages: amide (158 Da) and amidine bonds (157 Da). Activation of peptides labeled with a triethylphosphonium charge tag through an amide bond can lead to loss of the charge tag and the production of protonated peptides. This enables low intensity fragment ions from both the protonated and charge tagged peptides to be observed. Triethylphosphonium charge tagged peptides linked through an amidine bond are more stable. Post-source decay and photodissociation yield product ions that primarily contain the charge tag. Certain amidine induced fragments are also observed. The previously reported *tris*(trimethoxyphenyl) phosphonium acetic acid *N*-hydroxysuccinimidyl ester charge tag shows a similar fragment ion distribution, but the mass of the triethylphosphonium tag label is 415 Da smaller.

Keywords: Charge tag, Photodissociation, PSD, Phosphonium ion, Peptide fragmentation

Introduction

Tandem mass spectrometry is a well-known tool to identify peptides and investigate their sequences [1–4]. In low-energy fragmentation, such as collision-induced dissociation (CID) and post-source decay (PSD), peptides generally exhibit cleavage at the backbone amide bonds because mobilization of a proton to this functional group significantly weakens the bond [5, 6]. This results in N-terminal b-type fragments and C-terminal y-type ions [1, 2, 7]. The observation of a series of b- or y-type ions can facilitate peptide sequencing [8–10].

The presence of a charge sequestering site in peptides can have an impact on fragmentation. This occurs, for example, when the number of arginines present is greater than or equal to the number of protons [5, 11]. In this case, backbone cleavages resulting from aspartic or glutamic acid side chain or C-terminal carboxylic acid interactions induce bond cleavages [5, 12–14]. Observation of b- and y-type ions when a charge is sequestered can also be explained by the formation of salt-bridges [15]. Likewise, a fixed charge on a peptide is by definition not mobile. Applying MALDI ionization to fixed charged peptides generates singly charged ions [16]. If the charge remains localized, activation produces less fragmentation because product ions are formed through the charge sequestered processes and only those that contain the charge tag are detected [16–20].

Many charge tags have been shown to affect peptide ionization and fragmentation. One such tag is trimethylammonium butyric acid *N*-hydroxysuccinimidyl ester (TMAB), which contains a quaternary nitrogen atom [19, 21–24]. Unfortunately, activation of peptides derivatized with TMAB results in unusual fragmentation pathways, including cyclization of the charge tag to generate a mobile proton and rearrangement of methyl groups from the quaternary ammonium ion [25]. Another popular label is *tris*(trimethoxyphenyl) phosphonium acetic acid *N*-hydroxysuccinimidyl ester (TMPP), which includes a quaternary phosphonium ion with three bulky trimethoxyphenyl groups [16, 20, 24, 26]. This stable tag has been reported to be more

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hydrophilic than the somewhat simpler triphenylphosphonium label due to its trimethoxy groups [16]. TMPP enables the detection of low concentration peptides [20] and is useful for de novo sequencing [27, 28]. The application of high energy fragmentation, such as 157 nm photodissociation (PD) in a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometer, has been shown to produce contiguous series of a-type ions, with some b- and d-type ions [26]. Unfortunately, TMPP-derivatized peptides tend to lose the charge tag during fragmentation [26] and the bulky 572 Da label creates relatively high mass fragment ions for which detection sensitivity is lower [29].

Surprisingly, smaller trialkyl groups on a phosphonium ion have not been reported. In the present work, we synthesized a triethylphosphonium (TEP) charge tag in two ways. The first contains a succinimidyl ester that reacts with a primary amine producing an amide bond linkage. The second contains a thioimidate ester that also reacts with primary amines but yields an amidine linkage. Charge tagged peptides were subsequently fragmented with PSD and 157 nm photodissociation in a MALDI-TOF/TOF mass spectrometer. Results are compared with the commercially available TMPP. The TEP charge tag would be advantageous for peptide sequencing because the label creates an adduct less than 160 Da, which is approximately the mass of a single residue.

Experimental

Materials

A variety of peptides, either commercially available, synthesized, or from protein digests, were labeled with the TEP charge tag to investigate the effects that sequence composition have on fragmentation. Angiotensin I (DRVYIHPFHL), bradykinin 2–9 (PPGFSPFR), bradykinin 1–8 (RPPGFSPF), Glu-fibrinopeptide B (EGVNDNEEGFFSAR), hemoglobin, trypsin, α-cyano-4-hydroxycinnamic acid (CHCA), chloroacetonitrile, ethanethiol, N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, triethylphosphine, and iodoacetamide were purchased from Sigma (St. Louis, MO, USA). Other peptides included in this work were synthesized in-house. The monoclonal antibody, rituximab, was provided by Genentech (South San Francisco, CA, USA). Concentrated sulfuric acid was obtained from Avantor Performance Materials (Center Valley, CA, USA), and p-dioxane was from J.T. Baker (Phillipsburg, NJ, USA). Sodium chloride, anhydrous diethyl ether, and dichloromethane were from VWR Analytical (Radnor, PA, USA). Bromoacetic acid was acquired from Acros Organics (Morris Plains, NJ, USA), and toluene was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). Dithiothreitol was from Bio-Rad (Hercules, CA, USA).

Synthesis of (2-((2,5-Dioxopyrrolidin-1-yl)Oxy)-2-Oxoethyl)Triethylphosphonium Bromide (NHS-TEP$^+$)

Scheme 1 illustrates the procedure for this synthesis that was adapted from previous work [30, 31]. Bromoacetic acid (1.2 g, 8.6 mmol), N-hydroxysuccinimide (1.2 g, 10 mmol, 1.2 equiv), and N,N'-dicyclohexylcarbodiimide (2.0 g, 9.5 mmol, 1.1 equiv.) were dissolved in p-dioxane (66 mL). After stirring for 1 h at 20 °C, the dicyclohexylurea was removed by filtration, and the filtrate was concentrated in vacuo. The filtrate was crystallized from dichloromethane to yield a white crystalline solid (1, 0.23g, 81%). ^{1}H NMR (400MHz, CDCl$_3$) δ = 4.12 (s, 2H), 2.88 (s, 4H).

Bromoacetoxysuccinimide (1, 0.49 g, 2.1 mmol) and triethylphosphine (0.31 mL, 2.1 mmol, 1 equiv.) were dissolved in toluene (8.4 mL) and dichloromethane (8.4 mL). After stirring for 30 min at 20 °C, the product was isolated through filtration to yield a white solid (2, 0.30 g, 40%). ^{1}H NMR (600 MHz, DMSO-d^6) δ = 4.42 (d, J = 15.0 Hz, 2H), 2.83 (s, 4H), 2.39 (dq, J = 15.1, 7.6 Hz, 6H), 1.17 (dt, J = 19.5, 7.6 Hz, 9H).

Synthesis of Ethyl 2-Chloroethanimidothioate Hydrochloride Salt (EtS(CNH)-Cl)

The mechanism for the synthesis of EtS(CNH)-Cl is shown in Scheme 2. Chloroacetonitrile (0.19 mL, 3 mmol) and ethanethiol (2.2 mL, 30 mmol, 10 equiv.) were dissolved in dry dichloromethane (1.2 mL). Dry hydrogen chloride gas was generated by adding concentrated sulfuric acid (10 mL, 184 mmol, 61 mmol) to sodium chloride (40 g, 684 mmol, 228 equiv.) dropwise. At 0 °C, the hydrogen chloride gas was passed through the stirred solution for 1 h. The solution stood at −20 °C for 16 h. Excess dry diethyl ether was added and shaken to form a white precipitate. Following storage at −20 °C for 24 h, the precipitate was filtered off and dried in vacuo to yield a white crystalline solid (4, 0.51 g, 98%). ^{1}H NMR
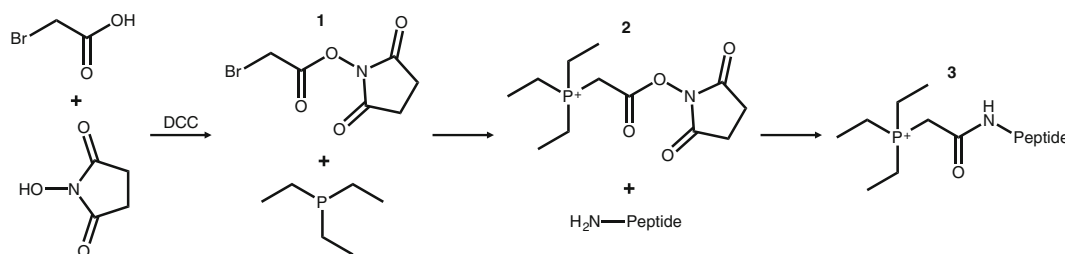
Scheme 1. Synthesis of NHS-TEP$^+$ (**2**) and its reaction with peptides
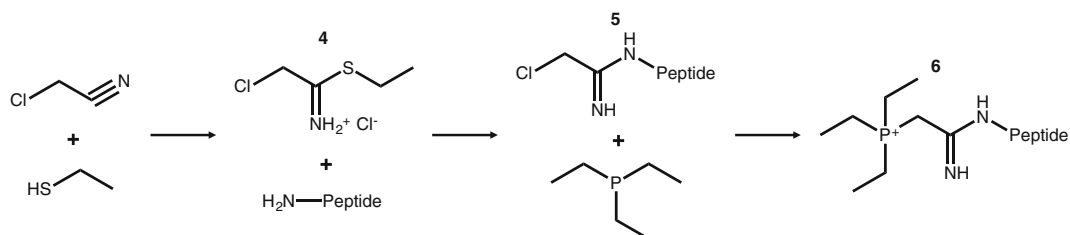
Scheme 2. Synthesis of EtS(CNH)-Cl (**4**) and its reaction with peptides

(600MHz, CDCl$_3$) δ = 4.75 (s, 2H), 3.51 (q, J = 7.5 Hz, 2H), 1.49 (t, J = 7.5 Hz, 3H).

Protein Digest

Sixty µg of rituximab were first reduced in water with 5 mM dithiothreitol at 37 °C for 1 h and alkylated in 100 mM ammonium bicarbonate with 14 mM iodoacetamide in the dark at room temperature for 1 h. A 3 kDa spin filter was used to remove reagents by adding 100 mM ammonium bicarbonate. The final volume of protein was approximately 40 µL. Subsequently, 20–40 µg of protein was combined with 1 µg of trypsin in a 50 µL solution with 90% ammonium bicarbonate and 10% acetonitrile. An accelerated digestion at 37 °C was performed with a Pressure BioSciences Inc. barocycler (South Easton, MA, USA) for 20 cycles (50 s at 20 kpsi and 10 s at atmosphere) for hemoglobin or 99 cycles for rituximab.

Charge Tag Derivatizations

Five nmol (10 µL) of commercial or synthesized peptides and 10 µL of protein digested were used for reactions with the charge tagging reagents. NHS-TEP$^+$ reacted with peptides for 1 h at room temperature at a ratio of 300:1 in anhydrous dimethylsulfoxide (50 µL). The solution was dried down under vacuum and the labeled peptides (structure 3 from Scheme 1) were resolubilized in water.

The reaction of EtS(CNH)-Cl and peptides occurred at a ratio of 300:1 in 50 mM HEPES buffer (pH 7.4, 50 µL). After 30–60 min at room temperature, 10 µL of triethylphosphine in 10 µL of acetonitrile were added to the reaction that proceeded for another 30–60 min. These labeled peptides contain a TEP tag with an amidine bond, as illustrated by structure 6 in Scheme 2.

Mass Spectrometry

Before depositing peptides onto a MALDI plate, excess reagents and buffers were removed with either 10 µL C18 micropipette tips or a C18 reversed-phase column (10 µL injection) on an Eksigent 2D Nano LC (Framingham, MA, USA) coupled to an Eksigent Ekspot MALDI spotter. Single peptides were primarily cleaned with the C18 pipette tips and eluted using matrix solution (10 mg/mL CHCA in 50:50 water and acetonitrile with 0.1% trifluoroacetic acid). To reduce ionization competition, peptides from protein digests were separated with the Eksigent 2D Nano LC instrument. CHCA

was subsequently added to the dried peptide spots on a MALDI sample plate.

An ABI 4800 MALDI-TOF/TOF mass spectrometer (Framingham, MA, USA) was used to obtain singly charged positive peptide ions and fragment them by PSD and photodissociation. The modification of this mass spectrometer to allow 157 nm photodissociation with a Coherent F$_2$ Laser (Santa Clara, CA, USA) was described previously [32]. Peptide ions traveling through the instrument absorb a single 157 nm photon from an unfocused 2–4 mJ pulse with a 5 × 10 mm rectangular beam profile. Peptides observed in MS1 were selected for activation by PSD and PD and spectra are the average of 500–1000 MALDI laser pulses. In this configuration, between 25%–80% of the ions irradiated by a single light pulse were fragmented.

Multiply charged ions were collisionally fragmented in a Thermo LTQ linear ion trap mass spectrometer (Waltham, MA, USA) with an electrospray ionization (ESI) source. Peptides were first trapped on a C18 column with an Eksigent 2D Nano LC to remove excess buffer and reagents and the eluent was coupled to the ESI source. Specific masses were included in the method for isolation and collisional activation. An isolation width of 3 Da, 35 eV collision energy, 0.250 activation Q, and 30 ms activation time were utilized.

Data Analysis

Two-point mass internal calibrations were performed in ABI software Data Explorer 4.6 as described by He and co-workers [33]. Accurate monoisotopic masses of the known 158 Da and b$_1$ ions were used for a reference file.

Results and Discussion

TEP$^+$-CH$_2$-CO-Peptides

The peptide AFVDSLYR was derivatized at the N-terminal amine with the reagent NHS-TEP$^+$ to form TEP$^+$-CH$_2$-CO-AFVDSYLR (Scheme 1, structure 3) leading to a mass increase of 158 Da. This peptide was charge tagged through an amide bond and MALDI yielded singly charged ions. The PSD mass spectrum is displayed in Figure 1a. Most mass spectra presented in this work contain a gray shaded region, in which the scale is expanded to enhance low intensity fragment ions. In this PSD spectrum, fragment ions with the charge tag are labeled in black and those that do not contain the charge tag are labeled in red. Two abundant fragment ions outside of the
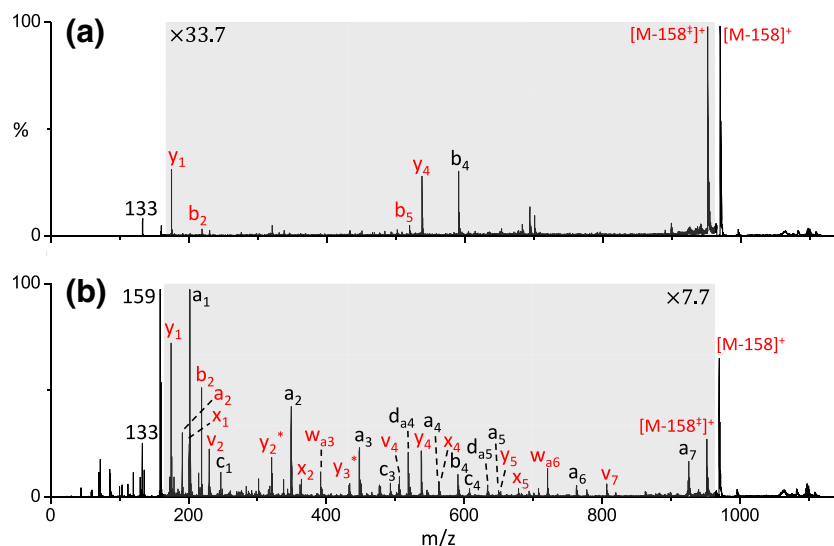
Figure 1. (a) PSD and (b) PD spectra of TEP$^+$-CH$_2$-CO-AFVDSLYR (M$^+$ = 1128.5 Da). Ions in black are from TEP$^+$-CH$_2$-CO-AFVDSLYR and those in red are secondary fragments from AFVDSLYR without a charge tag. The shaded area indicates an expanded region in the mass spectrum; * and ‡ signify ammonia and water losses, respectively

expanded region in this PSD spectrum correspond to a 133 Da charge tag fragment and to loss of the charge tag from the peptide ([M-158]$^+$). Supplementary Scheme S1 illustrates a proposed mechanism for forming the 133 Da charge tag fragment. The alpha carbon to the phosphonium ion can form a stable ylide after bond cleavage between the carbon and carbonyl group [34–36]. Subsequently, it abstracts a proton from the adjacent nitrogen to form a 133 Da ion. The [M-158]$^+$ ion represents the protonated peptide and its high intensity indicates that the modification is very labile. A possible rationalization for its appearance is that the protonated peptide is formed by abstraction of a proton and a hydrogen atom from the charge tag fragment, which yields a neutral ylide structure (Supplementary Scheme S2). In the x33.7 gray expanded region, low intensity fragments are identified with and without the charge tag. The b$_4$ ion contains the charge tag, whereas y$_1$, b$_2$, b$_5$, y$_4$, and [M-158-H$_2$O]$^+$ fragments do not. The presence of fragments without a charge tag indicates that these ions are formed by a double cleavage process, in which the charge tag is lost to form the protonated peptide and the protonated peptide then fragments. Both b$_4$ and y$_4$ ions are formed by an enhanced cleavage on the C-terminal side of aspartic acid [5, 12]. The production of ions that do not contain the charge tag is undesirable since this complicates the resulting spectrum.

TEP$^+$-CH$_2$-CO-AFVDSYLR was also photofragmented with 157 nm light and the resulting spectrum is in Figure 1b. High-energy ions with and without the charge tag are identified. The fragments labeled in black that contain the charge tag are mainly N-terminal a-, d-, and b-type ions. Those in red are mainly C-terminal x, y, v, and w ions probably from photodissociation of the protonated peptide that had already been formed by PSD. Likewise, a$_2$ and b$_2$ ions that have lost the charge tag also appear. The 159 Da ion is the charge tag, TEP$^+$-CH=C=O, and is much more intense in photodissociation than

in the PSD spectrum. Analogously, PSD and PD of TMPP labeled peptides commonly produce a 573 Da peak, which is the charge tag by itself [26].

This same experiment was performed with the peptide AFVDSLYK. Substituting arginine for lysine provides another primary amine that is capable of reacting with the charge tag reagent. However, reaction at both the N-terminus and lysine was not commonly observed. After derivatizing the peptide with a single TEP, PSD and PD mass spectra were acquired. These fragmentation spectra are displayed in Supplementary Figure S1 and contain three types of fragments: those with the charge tag at the N-terminus (in black), those with the charge tag at lysine (in green), and those without a charge tag (in red). The presence of ion fragments that do not contain the tag is undesirable and limits the utility of this derivatization approach.

TEP$^+$-CH$_2$-CNH-Peptides Derivatization Process

As an alternative to the TEP charge tag with the amide linkage, a TEP charge tag coupled to the peptide through an amidine bond was investigated to see if TEP retention could be improved. To attach a TEP tag with an amidine linkage to the N-terminal amine of a peptide, a different approach is necessary. A two-step reaction with the peptide is illustrated in Scheme 2. The first step involved reacting the peptide with EtS(CNH)-Cl (**4**) producing an amidine linkage to the chlorinated reagent. In the second step this modified peptide (**5**) reacted with triethylphosphine to form a quaternary phosphonium labeled peptide, the mass of which is now shifted by 157 Da. As an example of this procedure, AFVDSLYR was charge tagged as displayed in Figure S2a. The labeling procedure is not complete as evident from the unmodified peptide signal. The first step of adding EtS(CNH)-Cl to the peptide yielded an incomplete reaction; after addition of triethylphosphine, there is no

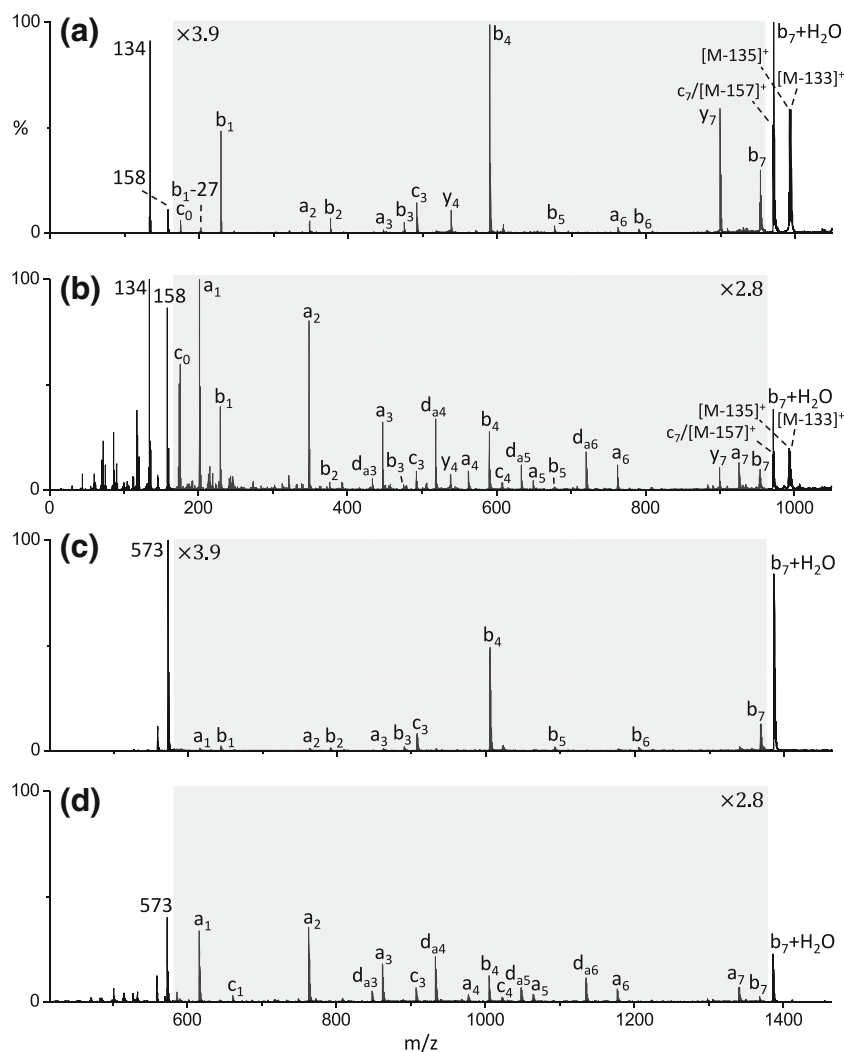**Figure 2.** (**a**) PSD and (**b**) PD spectra of TEP$^+$-CH$_2$-CNH-AFVDSLYR (M$^+$ = 1127.5 Da). (**c**) PSD and (**d**) PD spectra of TMPP-AFVDSLYR (M$^+$ = 1542.5 Da)

unreacted chlorinated peptide in the mass spectrum. The peak labeled with an asterisk is actually a byproduct of the peptide synthesis with a mass adduct of 96 Da, suggesting that the peptide is trifluoroacetylated. The b-type ions from PSD of this peptide are mass shifted by 96 Da, while the y-type ions are unmodified. This indicates that the N-terminal amine is most likely blocked and cannot participate in this charge-tagging reaction.

In an attempt to avoid this two-step reaction process, a complete TEP reagent with an ethane thiol group was synthesized analogous to the NHS-TEP$^+$ reagent. Unfortunately, it did not react with peptides. A possible reason for this is that the positively charged phosphorous may interfere with the thioimidate group. If the latter were deprotonated as a result of this interaction, it would be less inclined to react with primary amines [37].

TEP$^+$-CH$_2$-CNH-AFVDSLYR

The PSD mass spectrum of TEP$^+$-CH$_2$-CNH-AFVDSLYR is shown in Figure 2a. The low mass ions, 134

and 158 Da, associated with the charge tag are discussed below. Several b-type ions that contain the charge tag, including b_1, b_4, and $b_7 + H_2O$, are typical charge remote fragmentation products. The presence of a b_1 ion would be unusual for a standard protonated peptide, since a stabilizing oxazolone structure is not possible [38, 39]. However, amidine linkages are known to produce b_1 ions due to formation of five-membered rings [40]. The b_4 ion is the result of an enhanced cleavage at the amide bond adjacent to aspartic acid, in which the carboxyl side chain interacts with the peptide backbone to initiate fragmentation [5, 12]. Similarly, the $b_7 + H_2O$ ion is formed from the carboxylic acid at the C-terminus initiating loss of the C-terminal residue and transferring an OH group to the leaving b-type ion. This results in a b + H$_2$O ion that contains a standard C-terminus [14, 15]. Other unusual fragment ions include y-type ions and losses of 133 and 135 Da from the precursor. These are discussed below. The photodissociation spectrum for TEP$^+$-CH$_2$-CNH-AFVDSLYR is displayed in Figure 2b. High energy photofragments result from cleavage of C$_\alpha$–CO bonds [41]. As

a result, a much richer spectrum is obtained; several a-type ions are identified, as well as some b-, c-, and d-type ions.

AFVDSLYR was also derivatized with the commercially available TMPP for comparison with TEP results. The corresponding PSD spectrum in Figure 2c is very similar to that displayed in Figure 2a. Both spectra contain regions expanded by the same scale; fragment ions from TEP tagged peptides tend to be somewhat more intense. However, the TMPP labeled peptide does not yield a b_1 ion because this modification does not create a cyclic structure that stabilizes this small ion. Although these spectra are very similar, the mass of the fragment ions in the TMPP spectrum are much greater. The PD spectrum for TMPP-AFVDSLYR in Figure 2d also indicates a very similar distribution of ions except for c_0, b_1, y-type, and charge tag loss ions for TEP. The c_0 structure is simply TEP^+-CH_2-CNH-NH_2. Additionally, the scaling for the TMPP PD spectrum is unusual because although not shown, a low mass ion at 181 Da is the most intense peak in the spectrum [42, 43]. Earlier published work showed that low abundance peptides can be detected after derivatization with TMPP [20]. This experiment was repeated with TEP using 10 pmol of AFVDSLYR, but only 2 pmol of reacted peptide were spotted. The results shown in Supplementary Figure S2b are comparable to those obtained with 125 pmol of peptide (Supplementary Figure S2a), except that signals are more intense for the larger sample.

The charge tagged peptide TEP^+-CH_2-CNH-AFVDSLYR was also electrosprayed into an ion trap mass spectrometer. Singly and doubly charged peptide ions were observed and both were collisionally fragmented. The singly charged TEP^+-CH_2-CNH-AFVDSLYR mass spectrum (Figure 3a) produced a somewhat different result than the PSD spectrum (Figure 2a). Low mass 134 and 158 Da ions are below the low mass cutoff of the ion trap. The $[M-133]^+$ fragment is the dominant peak, as opposed to b_7 + H_2O. The MS/MS spectrum of the $[M-133]^+$ ion is shown in Supplementary Figure S3a and consists of

many b- and y-type ions whose masses confirm that the 133 Da loss had arisen from the N-terminal charge tag. The CID mass spectrum for the doubly charged peptide (Figure 3b) yielded a mixture of b- and y-type ions. The b-type ions contain the charge tag and the y-type ions contain an arginine that sequesters the second charge. Intense y_4 and b_4 ions are attributable to the aspartic acid side chain effect [5, 12]. An $[M-157]^+$ peak was also observed. Collisional activation of this fragment ion (Supplementary Figure S3b) yielded mainly y-type ions formed from the peptide without the charge tag. The CID mass spectrum of the unmodified doubly charged AFVDSLYR in Supplementary Figure S3c displays a different distribution of fragments. In particular, the aspartic acid effect is inhibited due to the availability of a mobile proton. These results suggest that MALDI is the preferred ionization method because singly charged precursor ions primarily yield N-terminal fragments and charge tag loss peaks are less abundant.

TEP^+-CH_2-CNH-AFVDSLYK

To investigate whether lysines would also be derivatized, AFVDSLYK was labeled with TEP. PSD and PD spectra appear in Supplementary Figure S4a and b. PSD produces relatively few fragments, similar to the arginine analog TEP^+-CH_2-CNH-AFVDSLYR in Figure 2. An unusual fragment ion, b_1-27, will be discussed below. PD produces a more complete fragmentation, including a series of a-type ions and some d- and b-type ions. As displayed in Supplementary Figure S4c and d, TMPP-AFVDSLYK yields similar ion distributions. In the present experiments, no fragment ions that contain a modified lysine were observed. Amidination of peptides is known to label both N-termini and lysines [44]. TEP labeling occurs at the N-terminus because the pH of the solution used to perform the reaction is at 7.4, which may inhibit reaction at lysines, the pKas of which are much higher than those of N-terminal amines [37]. This can ensure that even when a peptide contains
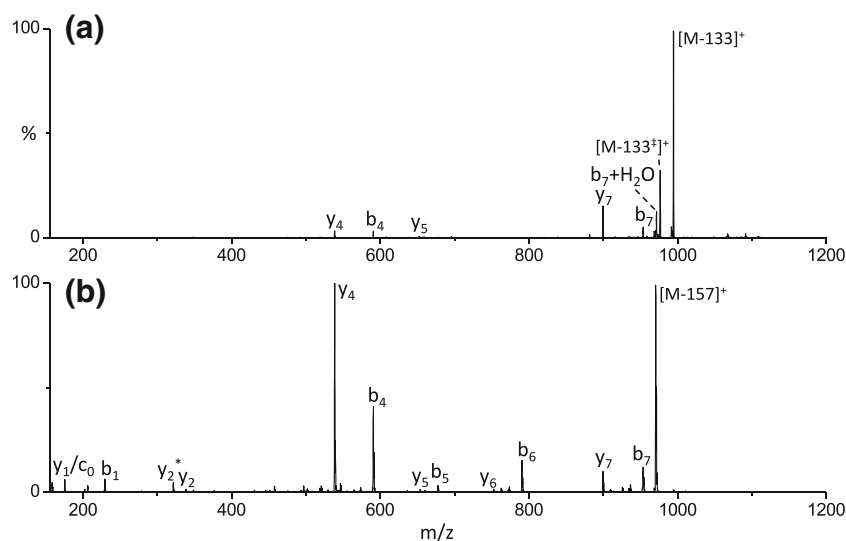

Figure 3. CID mass spectra of (**a**) singly (M^+ = 1127.5 Da) and (**b**) doubly charged ($[M + H]^{2+}$ = 564.3 Da) TEP^+-CH_2-CNH-AFVDSLYR; * signifies ammonia loss
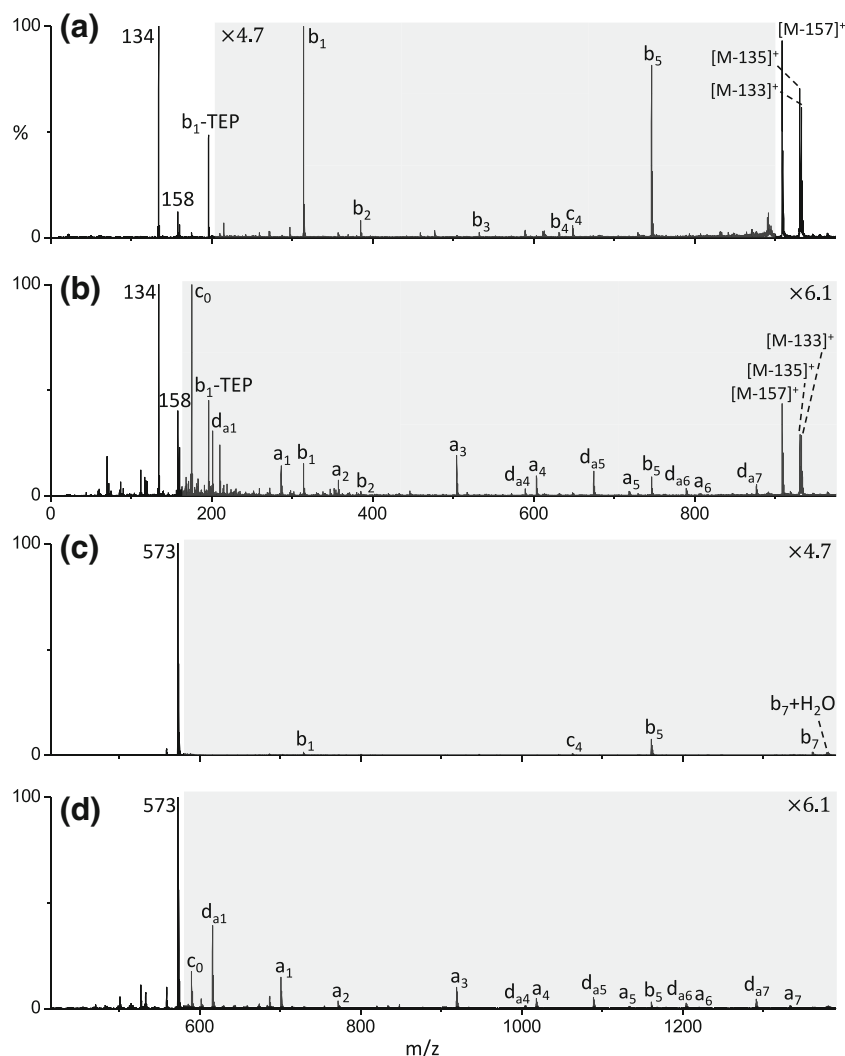
Figure 4. (a) PSD and (b) PD spectra of TEP$^+$-CH$_2$-CNH-RAFVDSLT (M$^+$ = 1065.5 Da). (c) PSD and (d) PD spectra of TMPP-RAFVDSLT (M$^+$ = 1480.5 Da)

a lysine at the C-terminus, labeling will occur predominately at the N-terminus.

TEP$^+$-CH$_2$-CNH-RAFVDSLT

The PSD mass spectrum for the peptide TEP$^+$-CH$_2$-CNH-RAFVDSLT is in Figure 4a. The b_1 and b_5 ions attributable to peptide backbone interactions with the amidine bond and aspartic acid side chain are observed with significant intensity. An unusual fragment, b_1-TEP, results from loss of triethylphosphine from the rest of the b_1 ion. Its origin is discussed below. Photodissociation of the peptide TEP$^+$-CH$_2$-CNH-RAFVDSLT produces high energy fragments as shown in Figure 4b. As in the previous examples, several a-type ions along with some b- and d-type ions are observed. Figure 4c contains the PSD spectrum for the TMPP labeled RAFVDSLT. One of the previously reported issues with TMPP derivatized peptides is the lack of fragmentation in PSD and photodissociation when an arginine was located at the N-terminus with a TMPP label. This has been ascribed to an interaction between

TMPP and the arginine side chain that prevented formation of fragment ions [26]. In this PSD spectrum, the intensities of fragment ions are less than 2% of the 573 Da base peak. In contrast, TEP$^+$-CH$_2$-CNH-RAFVDSLT displays rather intense PSD fragments. The photodissociation spectrum of the TMPP labeled peptide in Figure 4d reveals ion fragments that are similar to those found in the analogous TEP spectrum. However, they are somewhat less intense.

TEP$^+$-CH$_2$-CNH-EGVNDNEEGFFSAR

TEP derivatized peptides tend to form more abundant sequence ions than TMPP peptides. An example that shows this improvement is in Supplementary Figure S5. The PSD and PD spectra for TEP$^+$-CH$_2$-CNH-EGVNDNEEGFFSAR are in Supplementary Figure S5a and b, while those for TMPP-EGVNDNEEGFFSAR are in Supplementary Figure S5c and d. In these spectra, fragment ions from TEP and TMPP derivatized peptides show similar distributions, but the signal for ions with a TEP label is larger than for those with TMPP.

This signal improvement is summarized by the bar graph in Supplementary Figure S6. The blue and orange bars represent the intensities of fragment ions from TEP^+-CH_2-CNH-EGVNDNEEGFFSAR and TMPP-EGVNDNEEGFFSAR, respectively. The trend reveals that TEP produces more intense b- and a-type ions than TMPP. In order to perform this experiment, the same amount of peptide was used in each reaction and both were LC separated. However, after analysis, peptide ion signals were about five times greater for TEP, although differences in sample preparation may affect the final concentration of these two charge tagged peptides. For example, the charge tag derivatizations were performed in different buffers with a different solution volume. In order to create the same peptide concentrations, a C18 spin column was implemented to isolate TEP derivatized peptides and decrease the solution volumes that were equal. Alternatively, there could be effects that impact the production of TMPP-peptide ions. MALDI ionization of a TMPP peptide could be impeded by the additional 415 Da mass. Fragmentation of TMPP peptides may have two disadvantages. First, three trimethoxyphenyl rings absorb UV light better than ethyl groups. Instead of the peptide forming sequence ions as Vasicek and Brodbelt have reported with other aromatic chromophores such as 4-sulfophenyl

isothiocyanate and 4-methylphosphonophenyl isothiocyanate [45], TMPP could be absorbing light and preferentially fragmenting the charge tag. Alternatively, fragment ions from TMPP-peptides are of larger mass and the signal in PD spectra may inherently be lower than those from TEP-peptides. Lastly, the crystallization of these spots could be slightly different because these samples were LC-MALDI spotted separately. Despite these ambiguities, TEP tends to produce somewhat better results.

Unique Fragmentation of TEP^+-CH_2-CNH-Peptides

The TEP^+-CH_2-CNH-peptides above exhibited some unique fragment ions in PSD. To see if these were commonly observed, a few dozen peptides were labeled with a TEP charge tag and fragmented with PSD. These peptides were either commercially available, synthesized, or from protein digests with trypsin. Results are summarized in Table 1. All spectra were inspected for the unusual fragment ions b_1-TEP, b_1-27, b_1, y_{N-1}, $[M-157]^+$, $[M-135]^+$, and $[M-133]^+$. The relative heights of the 134 and 158 Da peaks were also compared for each peptide.

Table 1. PSD Observed Fragment Ions for TEP Tagged Peptides

Peptide	134/158	b_1-TEP	b_1-27	b_1	y_{N-1}	$[M-157]^+$	$[M-135]^+$	$[M-133]^+$
AFVDSLYR	>>	N	W	M	M	S	S	S
AFVDSLYK	<	N	M	M	N	M	N	N
RAFVDSLT	>>	M	N	W	W	S	S	S
KAFVDSLT	<<	N	W	M	N	M	W	W
VAAF	<<	N	N	W	N	N	N	N
LCPATNNAIET	<<	N	W	W	N	W	N	N
DRVYIHPFHL	>>	N	N	M	S	S	S	S
PPGFSPFR	<<	N	N	M	M	S	N	N
RPPGFSPF	>>	M	N	M	M	S	W	M
EGVNDNEEGFFSAR	>>	N	W	M	M	M	M	S
Hemoglobin Digest								
VVAGVANALAHK	>>	N	S	M	N	M	N	W
LRVDPVNFK	>>	N	W	M	M	M	M	M
VVAGVANALAHKYH	>>	N	S	M	W	M	N	N
EFTPPVQAAYQK	<	N	M	M	N	M	N	N
MFLSFPTTK	<	N	M	M	N	M	N	N
FLASVSTVLTSK	>	N	M	M	N	W	N	N
LLVVYPWTQR	>>	N	M	M	M	M	S	S
FFESFGDLSTPDAVMGNPK	>	N	M	M	W	M	W	W
Rituximab Digest								
ALPAPIEK	>	N	M	M	N	M	N	N
ALPAPIEKT	>>	N	M	M	N	M	W	W
GPSVFPLAPSSK	<<	N	W	M	N	M	W	W
VYACEVTHQGLSSPVTK	>>	N	S	M	N	M	W	W
VYAC*EVTHQGLSSPVTK	>>	N	S	M	N	W	W	W
HKVYAC*EVTHQGLSSPVTK	>>	N	S	M	N	M	M	M
FSGSGSGTSYSLTISR	>>	N	W	M	M	M	S	S
FNW*VDGVEVHNAK	>>	N	M	S	N	M	W	W
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	>>	N	M	S	N	N	N	N
GLEW*IGAIYPGNGDTSYNQK	<	N	M	M	N	W	N	N
RTVAAPSVFIFPPSDEQLK	>>	M	N	M	M	M	M	S
VVSVLTVLHQDW*LNGK	>>	N	S	M	N	W	N	N
VVSVLTVLHQDWLNGKEYK	>>	N	S	M	N	M	W	W
ASSSVSYIHW*FQQKPGSSPK	>>	N	M	M	N	M	M	M
GFYPSDIAVEW*ESN*GQPENNYK	<	N	M	M	N	M	N	N

S = intense peak, M = medium intensity peak, W = low abundance peak, N = not observed
W* is oxidized tryptophan, C* is carbomidomethyl cysteine, and N* is deamidated asparagine
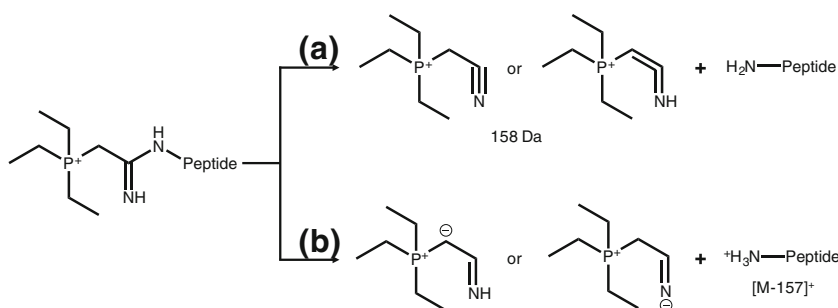
**Scheme 3.** Mechanisms to form (**a**) 158 Da and (**b**) [M-157]$^+$ ions

Some of the unusual fragments are due to loss of the charge tag. The peak at 158 Da is the intact charge tag, the structure of which is shown in Scheme 3a. [M-157]$^+$ is loss of the charge tag modification from the peptide, as presented in Scheme 3b. The hydrogen on the carbon adjacent to the positively charged phosphorus is most likely removed to produce a negative charge that is stabilized by the formation of an ylide and resonance. The 134 Da peak is not formed by cleavage between the carbon and amidine group of the charge tag. A possible explanation for this ion involves a rearrangement of the quaternary phosphonium ion in a Wittig-like reaction, as illustrated in Scheme 4 [35, 36]. The double bonded nitrogen in the amidine can form a four-membered ring to the phosphorous and rearrange electrons releasing two unusual products. The position of the second hydrogen in the amidine may determine which product is charged. In Scheme 4a, one hydrogen is on each of the nitrogens in the amidine and the [M-133]$^+$ ion would be charged and the TEP=NH would be neutral. The charge in this product is no longer fixed and is able to initiate fragmentation, as demonstrated in the ESI study above. If both hydrogens on the nitrogen are transferred to TEP as in Scheme 4b, the TEP$^+$-NH$_2$ product is charged and has a mass of 134 Da. Another fragment, [M-135]$^+$ is two hydrogens less than [M-133]$^+$ requiring double bond formation somewhere. It should be noted that this reaction is not exactly the same as a standard Wittig reaction, in which a double bonded oxygen, not an amidine nitrogen, would make a four-membered ring and the phosphonium ion is typically an ylide [35, 36]. It is also possible that the resulting structure to form either [M-133]$^+$ or 134 Da is a proton bound dimer, in which the proton remains on the more basic fragment. This mechanism is similar to the a$_1$-y$_x$ pathway for peptides described previously [6]. The 134 and 158 Da intensity comparison in Table 1 also indicates that

the 134 Da ion is more abundant when arginine or multiple basic residues are present. A possible explanation is that there is some interaction between these amino acids and the tag that promotes the formation of this ion.

A b$_1$ ion is identified in all TEP tagged peptides. This is a characteristic fragment of amidinated peptides [40]. The nitrogen in the amidine can form a bond to the next amide carbon, forming a five-membered ring. A hydrogen is transferred to the leaving nitrogen and a stable b$_1$ ion is produced, as shown in Scheme 5. Unlike ordinary amidinated peptides, TEP labeled peptides have only one available hydrogen on the double bonded nitrogen in this transfer process, which would suggest that y$_{N-1}$ ions, which are abundantly formed from amidinated peptides, should not be observed from charge tagged peptides since they do not contain the fixed charge. However, y$_{N-1}$ ions are in fact identified in some of the peptides fragmented. Upon inspection of Table 1, it can be deduced that peptides containing arginine tend to form more intense y$_{N-1}$, [M-157]$^+$, [M-135]$^+$, and [M-133]$^+$ peaks than those that do not. As the most basic amino acid, arginine is likely capable of removing a proton from the carbon adjacent to the phosphorus to generate a singly charged C-terminal fragment ion and a stable ylide. Peptides containing less basic residues such as lysine tend to produce these ions with low intensity. Formation of a salt-bridge may facilitate the production of these C-terminal fragments [15]. Although TEP$^+$-CH$_2$-CO-peptides generate significant [M-157]$^+$ ions that were subsequently fragmented to produce sequence ions (Figure 1 and Supplementary Figure S1), this double cleavage process is not dominant in TEP$^+$-CH$_2$-CNH-peptides. For example, Supplementary Figure S7 contains PSD and PD spectra for TEP tagged angiotensin I (DRVYIHPFHL). Because of the arginine near the N-terminus intense y$_9$, [M-157]$^+$, [M-135]$^+$, and [M-133]$^+$ ions
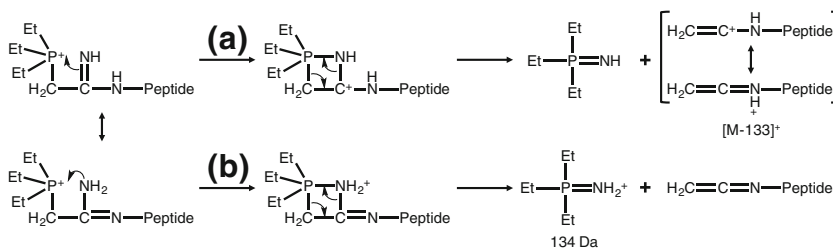
Scheme 4. Mechanisms to form (**a**) [M-133]$^+$ and (**b**) 134 Da ions
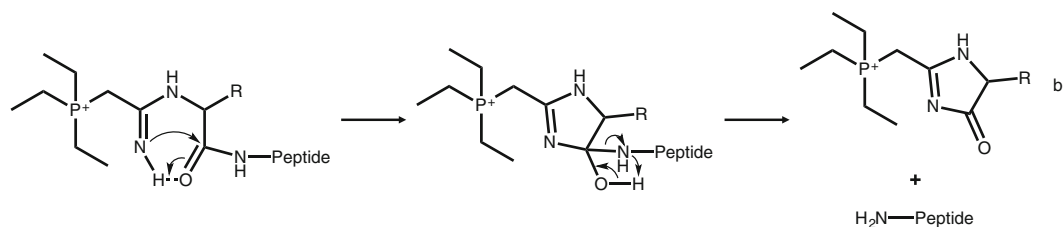
Scheme 5. Mechanism to form a b_1 ion in which R is an amino acid side chain

are observed. Other PSD fragments include b-type ions and an abundant c_0 ion, which is most likely attributed to the propensity to form a c-type ion from cleavage on the N-terminal side of aspartic acid [13]. All c_0 ions for TEP tagged peptides have the same TEP^+-CH_2-CNH-NH_2 structure with a mass of 175 Da because the amino acid side chain is not included in this fragment. In photodissociation, a series of a-type ions, along with some d- and b-type ions are identified. However, neither of these spectra contains abundant N-terminal sequencing fragments that do not contain a charge tag, suggesting that fragmentation of peptides labeled with TEP through amidine linkages rarely involve double cleavages.

Loss of $(C_2H_5)_3P$ from the b_1 ion is commonly observed when arginine is at the N-terminus. Three peptides in Table 1 contain an arginine at the N-terminus and produce this ion. Other amino acids at this position do not form this fragment. A possible rationalization for this result is that arginine acts as a nucleophile to attack the carbon adjacent to the positive phosphonium ion and releases triethylphosphine. A similar mechanism is exhibited by lysine at the N-terminus of an unmodified peptide, in which loss of ammonia occurs by nucleophilic attack of the N-terminal amine [46].

PSD of TEP charge tagged peptides typically shows a b_1-27 ion. Loss of 28 Da would be more expected because this would yield an a-type ion. Loss of 27 Da is unusual because it may indicate that the ion forms by rearrangement to lose CNH from a b_1 ion or an odd-electron a_1+1 ion, which has been observed in ion trap photodissociation studies [47]. However, low energy fragmentation typically only produces even electron a-type ions with weak intensity; these ions are formed from b-type ions [2, 48]. In the present experiments, this unusual b_1-27 or a_1+1 ion is observed with a significant intensity, such as from the peptide VVAGVANALAHK as shown in Supplementary Figure S8a. With 157 nm photodissociation, both an even electron a_1 ion and the b_1-27/a_1+1 ion are both observed, as indicated in Supplementary Figure S8b. An accurate mass of this PSD fragment can distinguish a b_1-CNH ion from a b_1-CO+H (i.e., a_1+1) ion enabling its molecular formula to be identified. A two-point calibration was performed utilizing two identified low mass fragments, TEP^+ and the b_1 ion of five peptides listed in Supplementary Table S1. These fragments surround the b_1-27/a_1+1 mass, providing accurate calibrants. For all five cases, the b_1-CNH assignment more accurately matches the experimentally measured mass. These results suggest that a rearrangement is occurring to release CNH; however, the exact mechanism for this process is unknown. b_1-27 ions have not been previously reported [40], suggesting that the

combination of the quaternary phosphonium ion and the amidine linkage may promote their formation.

The peptide PPGFSPFR contains a secondary amine at the N-terminus, attributable to the ring structure of proline. Thioimidate chemistry is known to be predominately reactive with primary amines and not reactive with secondary amines [44]. As a result, the charge tagging efficiency was limited as shown in Supplementary Figure S9a. This MS^1 spectrum contains a highly abundant unmodified peptide and a low intensity TEP^+-CH_2-CNH-PPGFSPFR. Thus, a low intensity PSD spectrum of the tagged peptide was obtained. Supplementary Figure S9b displays some b-type ions that provide limited sequence information. This derivatization indicates that it is possible to label peptides with proline at the N-terminus, but with lower efficiency.

Conclusions

A small triethylphosphonium charge tag was synthesized and derivatized to peptides. With an amidine linkage to the N-terminus of peptides, the triethylphosphonium ion promoted charge remote fragmentation in MALDI PSD and 157 nm photodissociation and produced similar fragmentation patterns to the larger commercially available TMPP. Unique PSD fragments were identified and rationalized; however, these ions did not interfere with 157 nm photodissociation experiments. In addition to the small size of the label, the benefit of TEP is that fragments are notably more intense in both PSD and PD than those obtained from TMPP labeled peptides. The unique presence of a b_1 ion from PSD of TEP peptide provides information about the first residue that is not commonly observed for TMPP. Additionally, production of fragment ions with a TEP label is not impeded by the location of arginine as is typical with TMPP. A future study will be to apply the photodissociation data from charge tagged peptides to de novo sequencing because a contiguous series of a-type ions can facilitate sequence characterization.

Acknowledgments

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