Structure Elucidation

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Facile Disulfide Bond Cleavage in Gaseous Peptide and Protein Cations by Ultraviolet Photodissociation at 157 nm**

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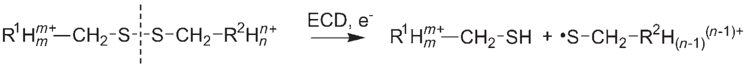
Disulfide bonds formed between cysteine residues stabilize the native structure of many proteins.[1] These covalent cross-linkages are part of the tertiary structure and introduce conformational constraints to the polypeptide architecture, thus improving the thermodynamic stability. Mass spectrometry has become increasingly important in the characterization and identification of proteins as a result of its capability for highly specific measurements on relatively small amounts of analyte. Protein characterization and identification relies on database searches using primary sequence information obtained by tandem mass spectrometry (MS^n or MS/MS). The top-down approach[2] applied to intact proteins ions is an efficient MS/MS method which is potentially suited for the high-throughput characterization of proteins. The problems addressed by the top-down approach include verification of the protein sequence, localization of errors in DNA-predicted sequences, de novo sequencing, as well as the analysis of pre- or posttranslational modifications (PTMs). However, such PTMs as disulfide bonds still remain a challenge for traditional MS/MS techniques that utilize vibrational excitation (VE), as they break peptide bonds preferentially in protonated polypeptides and leave the disulfide bonds intact.[3] As a result, information on the primary sequence is limited to parts of the protein that is not constrained by disulfide bonds. This restriction complicates primary sequence elucidation and limits the utility of automated database searches. The problem of disulfide bonds is solved conventionally by their reduction and alkylation[4] prior to MS/MS measurements. However, the additional step involving solution-phase chemistry, including subsequent purification, greatly increases the analysis time and the risk of sample loss. Another approach is to use an alternative ion-dissociation strategy. Chrisman and McLuckey have shown that VE of deprotonated polypeptides induced by collisionally activated dissociation (CAD)[5] breaks disulfide bonds with high efficiency.[6] However, CAD of peptide anions often produces complicated mass spectra that, together with backbone cleavages, contain significant amounts of neutral mass losses and rearrangement products which make such MS/MS spectra of limited value for sequencing. Electron-capture dissociation (ECD)[7] stands alone in its ability to cleave S–S bonds preferentially in peptide polycations (Scheme 1).[3] A drawback of this technique is that the capture of a low-energy electron by multiply charged polypeptides results in charge reduction prior to bond cleavage, which in turn leads to a charge decrease in one of the two fragments. As species need to contain at least one charge to be detectable in mass spectrometry, the charge reduction in ECD can lead to a loss of information.



$$R^1H_m^{m+}{-}CH_2{-}S{-}{\vdots}{-}S{-}CH_2{-}R^2H_n^{n+} \xrightarrow{ECD,\ e^-} R^1H_m^{m+}{-}CH_2{-}SH + {\bullet}S{-}CH_2{-}R^2H_{(n-1)}^{(n-1)+}$$

Scheme 1. Charge reduction by cleavage of the disulfide bond with ECD.

Previously, when magnetic sector instruments were widely used, high-energy CAD (HE CAD) was utilized to produce cleavage of numerous disulfide bonds in peptides.[8] The abundance of this fragmentation channel compared to low-energy CAD has been explained by electronic excitation. Today, HE CAD can only be found in TOF/TOF instruments that are more suitable for peptides than for proteins, and for which MS^n abilities are limited above $n = 2$. Therefore, a fragmentation technique is needed that is suitable for MS^n of protein cations and that would not involve charge reduction.

We have assumed that if the HE CAD efficiency for cleavage of a disulfide bond is indeed a result of electronic excitation, a similar effect could be expected upon irradiation of polycations with ultraviolet (UV) light. Indeed, irradiation of desorbed cations with light of wavength 337 nm in matrix-assisted laser desorption ionization (MALDI) studies[9] results in partial reduction of the S–S bonds in situ.[10] The

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wavelength of maximum absorption for the S–S bond in, for example, cystine (Cys-S-S-Cys) is approximately 150 nm.[11] Therefore, one may expect an efficient cleavage of the S–S bond upon UV photodissociation (UVPD) at 157 nm. This cleavage would compete with the backbone fragmentation of peptide monocations reported by Reilly and co-workers that resulted in unusual cleavage of the backbone C_α–C bonds (a, x-type fragments).[12] The fragmentation of the peptide backbone in that study was explained as a result of electronic excitation of the polyamide bonds as a consequence of absorption of photons of 7.9 eV.

Herein, we have tested the above assumption and present evidence of a facile cleavage of both intra- and intermolecular disulfide bonds in peptide and protein polycations by UVPD at 157 nm. Figure 1 presents a comparison of the MS/MS spectra obtained with CAD, ECD, and consecutive UVPD and CAD (UVPD/CAD) of a peptide dimer linked by an intermolecular disulfide bridge. The CAD mass spectrum (Figure 1 a) of the $[M{+}3\,H]^{3+}$ ions contains only a few (ten) **b**- and **y**-type fragment ions (in b ions, the charge is retained on the N-terminal side of the peptide, and in y ions on the C-terminal side).[13] However, there was no evidence for cleavage of the disulfide bond. In contrast, ECD (Figure 1 b) produced abundant signals corresponding to free monomers and thus to cleavage of the S–S bond. Charge reduction meant that only singly charged peptide monomers were observed, thus rendering them useless for MS^3 by ECD, as electron capture would neutralize them and their products. UVPD at 157 nm (Figure 1 c) also cleaved the disulfide bond, and led to both singly and doubly charged peptide monomers. The efficiency of the dissociation of the S–S bond (defined as the ratio of the released monomer abundances divided by the total ion abundance) by UVPD at 157 nm was 1.2 % compared to 2.3 % with ECD. However, UVPD at 157 nm was implemented on a linear ion trap that was approximately 100 times more sensitive than the Fourier transform analyzer on which the ECD mass spectrum was obtained. Thus, the twofold loss of efficiency was more than compensated by the huge advantage in sensitivity. Furthermore, the efficiency of the S–S bond cleavage in UVPD increases as the positive charge decreases (data not shown).

Only limited information on the sequence of the peptide dimers was obtained in MS^2 with UVPD at 157 nm, as the S–S bond was cleaved at a higher rate than the peptide backbone. Therefore, the released doubly charged peptide monomers were isolated and subjected to MS^3 by CAD (Figure 1 d and e), which resulted in extensive fragmentation of the backbone (> 60 % sequence coverage). The same procedure was carried

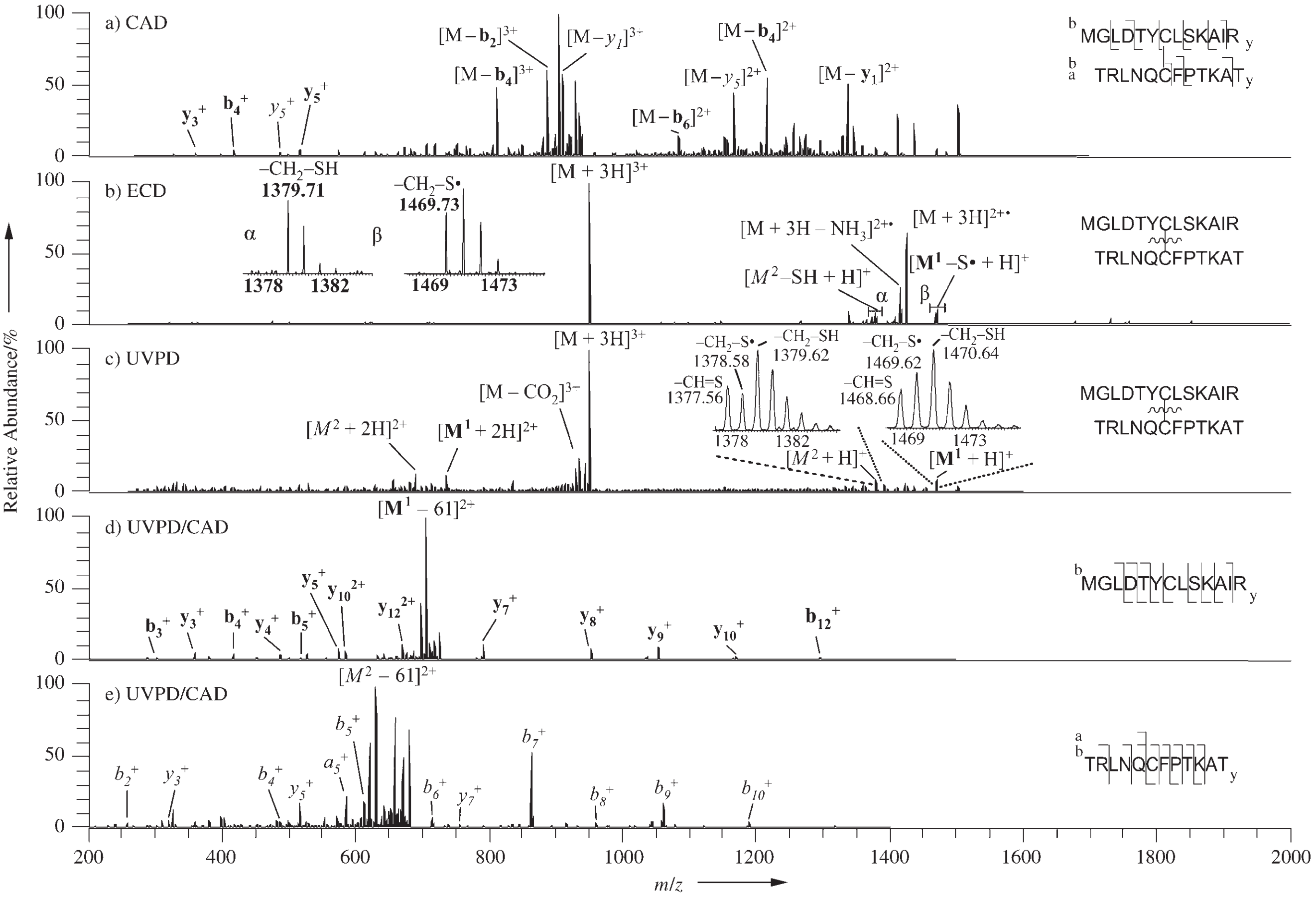


Figure 1. a)–c) Tandem mass spectra of a disulfide-bridged peptide dimer, $\mathbf{M}^1$-S-S-M^2; where $\mathbf{M}^1$ = MGLDTYCLSKAIR and M^2 = TRLNQCFPTKAT. Fragments from $\mathbf{M}^1$ are marked in bold and that from M^2 are marked in italics. The fragmentation techniques were: a) CAD, b) ECD, c) UVPD at 157 nm, d) CAD of isolated $[\mathbf{M}^1{+}2\,H]^{2+}$ after UVPD at 157 nm of $\mathbf{M}^1$-S-S-M^2 trications; e) CAD of isolated $[M^2{+}2\,H]^{2+}$ after UVPD at 157 nm of $\mathbf{M}^1$-S-S-M^2 trications. The identities of the $[M^{1,2}{-}61]^{2+}$ peaks in (d) and (e) are currently under investigation.

out on singly charged peptide monomers, which increased the overall sequence coverage to 96 %, with only one interresidue bond not cleaved. The combined sequence information of all the disulfide-linked peptide dimers studied is summarized in Figure 2.

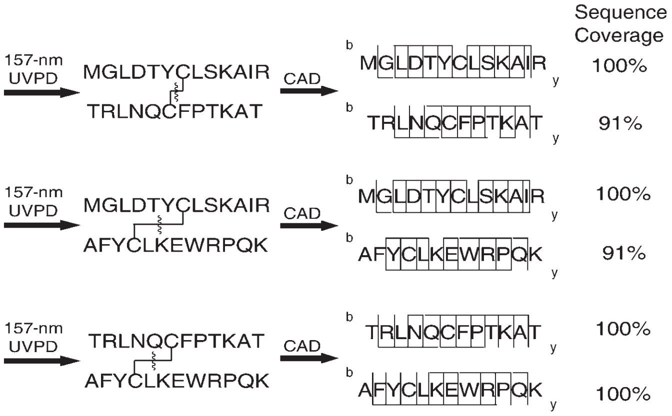


Figure 2. a) Fragmentation schemes of three trications of disulfide-bridged peptide dimers resulting from UVPD/CAD at 157 nm.

We hypothesize that UVPD breaks disulfide bonds by homolytic cleavage induced by electronic excitation. This process should result in two complementary fragments with radicals localized on each of the sulfur atoms ($R^1CH_2S^\bullet$/$R^2CH_2S^\bullet$). However, the isotopic pattern observed for the sulfenyl radical (see inset of Figure 1 c) was much broader than the theoretically calculated distribution. To investigate this effect further we isolated separately two isotope components of the UVPD-released doubly charged TRLNQCFPTKAT peptide monomer and fragmented them by CAD (Figure 3 a and b, respectively). Only the cysteine-containing fragment ions exhibited a mass difference of 1 Da between the two spectra—other fragment ions were of the same mass. This result implies that the mass difference of 1 Da between the two monomeric ions was not exclusively the consequence of stable isotopes (for example, ^{12}C versus ^{13}C), but also the result of the presence of two different species ($R^1CH_2S^\bullet$ and R^1CH_2SH). Similarly, isolation and MS/MS of the isotope 1 Da below the monoisotopic signal confirmed the identity of these ions as $R^1CH{=}S$ (data not shown).

Thus, three mechanisms for the cleavage of disulfide bonds in UVPD at 157 nm were proposed (Scheme 2). Electronic excitation by photon absorption can lead to rapid cleavage of the disulfide bond and formation of two sulfenyl radicals $R^1CH_2S^\bullet$ and $R^2CH_2S^\bullet$ (path a). If fragment separation is delayed because of noncovalent bonds between the monomers, the proximity of the two radical peptide chains can result in intermolecular hydrogen transfer. This process leads to reactions (path b or path c) where either abstraction of a H atom by a peptide monomer from the other monomer forms sulphydryl or thioaldehyde ($-CH_2SH$ or $-CH{=}S$) functional groups. Alternatively, absorption of a photon by a dimer leads to intramolecular hydrogen rearrangement, with an H atom attacking the S–S bond, as has been suggested for ECD.[3,14] Additional evidence for this latter mechanism comes from the observation of abundant loss of H atoms from irradiated precursor ions (data not shown), thus indicating that hydrogen abstraction and rearrangement do play a significant role in UVPD at 157 nm.

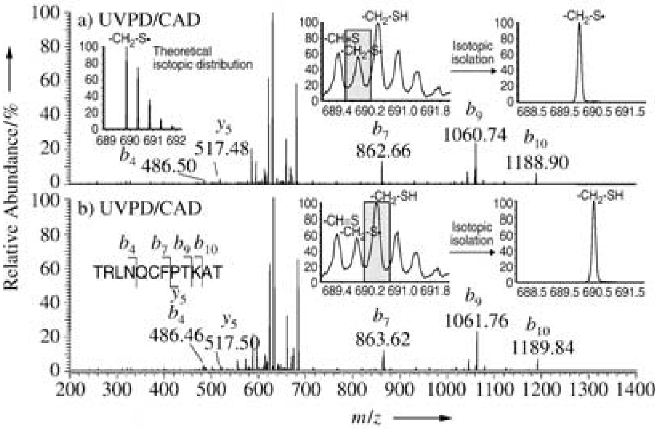
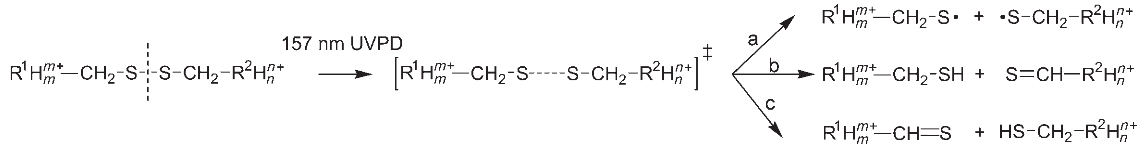


Figure 3. CAD mass spectra of isolated isotopic ions after UVPD at 157 nm of dications of the TRLNQCFPTKAT peptide corresponding to masses of a a) monoisotopic signal and b) the first isotopic signal.

The ability of UVPD at 157 nm to cleave intramolecular disulfide bonds was tested on the peptide RLCADTPICGK with a disulfide bridge between the two cysteine residues. Figure 4 shows the CAD spectrum (Figure 4 a) and the UVPD spectrum (Figure 4 b) for comparison. While CAD yielded only three b and one y ions with no cleavage of the disulfide bond, UVPD generated a series of a-type (a_2–a_{10}) fragment ions (a ions correspond to cleavage of a C_α–C backbone bond with the charge retained on the N-terminal side) and two d-type fragment ions (d_n ions form from radical a_n ions by loss of side-chains from the C-terminal residue), all as a consequence of cleavage of the disulfide bond. The observation of a-type fragment ions in UVPD at 157 nm was in agreement with the results obtained by Reilly and co-workers.[12] Two other peptide ions with intramolecular disulfide bonds gave similar fragmentation (data not shown).

Moving from model peptides to biologically relevant proteins, we applied UVPD at 157 nm to human insulin, a 5.9-kDa molecule containing polypeptide chains A and B linked by two disulfide bonds, and with one S–S bond within the



$$R^1H_m^{m+}{-}CH_2{-}S{-}S{-}CH_2{-}R^2H_n^{n+} \xrightarrow{\text{157 nm UVPD}} \left[R^1H_m^{m+}{-}CH_2{-}S\cdots S{-}CH_2{-}R^2H_n^{n+}\right]^{\ddagger}$$

$$\xrightarrow{a} R^1H_m^{m+}{-}CH_2{-}S^\bullet + {}^\bullet S{-}CH_2{-}R^2H_n^{n+}$$

$$\xrightarrow{b} R^1H_m^{m+}{-}CH_2{-}SH + S{=}CH{-}R^2H_n^{n+}$$

$$\xrightarrow{c} R^1H_m^{m+}{-}CH{=}S + HS{-}CH_2{-}R^2H_n^{n+}$$

Scheme 2. Suggested reaction pathways for the cleavage of the disulfide bond by UVPD at 157 nm.

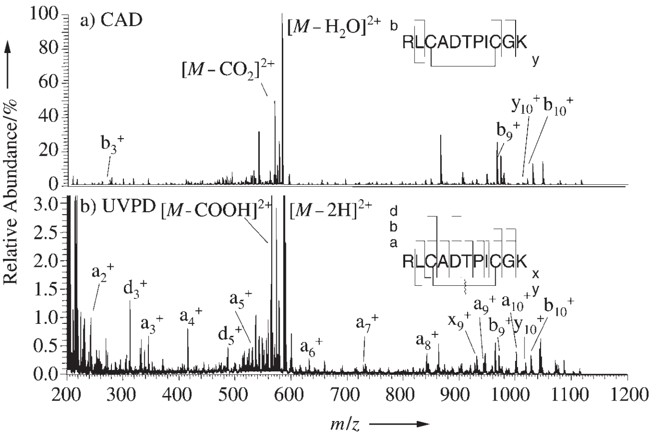


Figure 4. Tandem mass spectra of dications of the peptide RLCADTPICKGK with an intramolecular disulfide bridge, obtained with: a) CAD and b) UVPD at 157 nm.

A chain (Figure 5). Previously, ECD of 6+ ions have released free A and B chains, but only the B chain gave a MS^3 spectrum.[3] Here, UVPD of $[M{+}6\,H]^{6+}$ ions (Figure 5a) yielded predominantly the quadruply charged B chain and doubly charged A chain by simultaneous cleavage of both intermolecular disulfide bonds. CAD on the isolated 4+ charged B chain (Figure 5b) gave abundant *b* and *y* fragment ions covering 59% of the sequence. CAD was performed on 2+ charged A-chain ions (Figure 5c) to determine whether the intramolecular S–S bond in the A chain was also cleaved. This experiment yielded *b* and *y* ions covering 85% of the sequence. Observation of seven fragments arising from backbone cleavages between the two cysteine residues confirmed the breakage of the intramolecular S–S bond. Thus, the UVPD/CAD combination provided the most complete top-down sequence verification of a protein with three S–S bonds to date.

In summary, we have demonstrated the ability of UVPD at 157 nm to cleave both intra- and intermolecular disulfide bonds without charge reduction in peptide and protein cations. This methodology is expected to be useful in de novo sequencing of polypeptides. As a note of caution, competition with backbone cleavages in UVPD complicates identification of complementary fragment pairs arising exclusively from cleavage of the S–S bond in de novo sequencing. This problem can be addressed by first performing CAD of the intact protein to reveal complementary pairs arising from cleavage of the backbone. While classical reduction of the S–S bond in solution may still provide a clearer answer, the faster UVPD approach that avoids time- and sample-consuming wet chemistry will be preferred in many cases. As photons are not affected by electromagnetic irradiation, UVPD can easily be adapted to different types of mass analyzers, which broadens the application range of this technique.

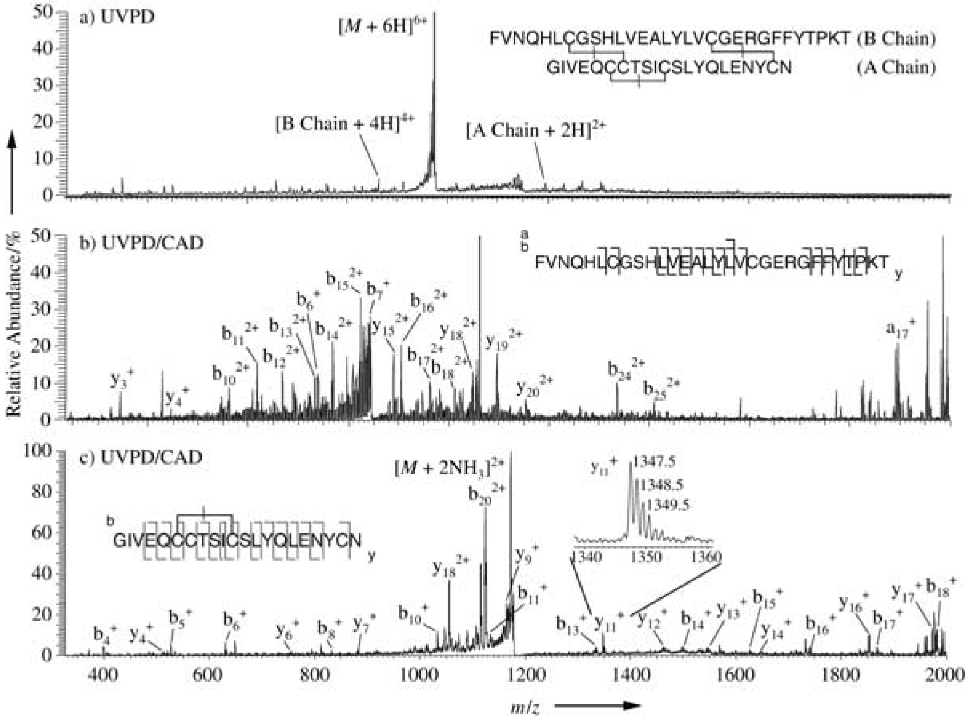


Figure 5. a) Mass spectrum of human insulin 6+ ions obtained by UVPD at 157 nm, b) CAD spectrum of $[M{+}4\,H]^{4+}$ ions of the B chain released in UVPD at 157 nm from 6+ insulin ions, c) CAD spectrum of $[M{+}2\,H]^{2+}$ ions of the A chain released in UVPD at 157 nm from 6+ insulin ions. The inset shows the isotopic distribution of y_{11} ions (Ile–Cys bond cleaved) confirming breakage of the disulfide bond.

Experimental Section

Cysteine-containing peptides were synthesized by automated solid-phase synthesis using the 9-fluorenylmethoxycarbonyl (Fmoc) protection strategy on a research-scale ResPep peptide synthesizer (Intavis AG, Gladbach, Germany). The oxidation of the cysteine residues was performed on 0.5 mg of peptides in a solution of DMSO (20 µL) in 20 mM NH_4HCO_3 (aq, 80 µL) for two days at RT. Human insulin (Sigma, St Louis, MO) was used without further purification. Mass-spectrometric experiments were performed on a linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nanoelectrospray interface operated in the positive ion mode. Oxidized peptides were diluted in a solvent containing acetic acid/methanol/water (2:49:49, v/v/v) to approximately 10^{-6} M and electrosprayed. An excimer F_2 UV laser at 157 nm (Excistar S500, Tuilaser, Germering, Germany) was connected with the mass spectrometer by means of a CaF_2 window on-axis with the ion trap. Unfocused laser pulses (15 ns, 1 mJ) with a repetition rate of 500 Hz were passed through a 1.5-mm collimator installed in front of the window. Isolated precursor ions were irradiated for 3 to 9 ms with UV light. ECD experiments were performed on a 7 Tesla FT mass spectrometer (Thermo Electron AG, Bremen, Germany) equipped with an indirectly heated cathode as an electron source. The electron irradiation in ECD lasted for 70 ms.

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