Research Article

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Collision-induced dissociative chemical cross-linking reagent for protein structure characterization: applied Edman chemistry in the gas phase

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Chemical cross-linking combined with a subsequent enzymatic digestion and mass spectrometric analysis of the created cross-linked products presents an alternative approach to assess low-resolution protein structures and to gain insight into protein interfaces. In this contribution, we report the design of an innovative cross-linker based on Edman degradation chemistry, which leads to the formation of indicative mass shifted fragment ions and constant neutral losses (CNLs) in electrospray ionization (ESI)-tandem-mass spectrometry (MS/MS) product ion mass spectra, allowing an unambiguous identification of cross-linked peptides. Moreover, the characteristic neutral loss reactions facilitate automated analysis by multiple reaction monitoring suited for high throughput studies with good sensitivity and selectivity. The functioning of the novel cross-linker relies on the presence of a highly nucleophilic sulfur in a thiourea moiety, safeguarding for effective intramolecular attack leading to predictive and preferred cleavage of a glycyl-prolyl amide bond. Our innovative analytical concept and the versatile applicability of the collision-induced dissociative chemical cross-linking reagent are exemplified for substance P, luteinizing hormone releasing hormone LHRH and lysozyme. The novel cross-linker is expected to have a broad range of applications for probing protein tertiary structures and for investigating protein–protein interactions.

Keywords: chemical cross-linking; peptides; Edman chemistry; collision-induced dissociation; protein structure analysis

Introduction

Chemical cross-linking combined with mass spectrometry (MS) is a viable approach to study the tertiary and quaternary structure of proteins and protein complexes.[1–3] However, an unambiguous, sensitive, reliable and fast identification of the amino acids involved in the covalent derivatization by chemical cross-linking remains analytically challenging. A mass spectrometric identification of the chemically modified amino acids in the respective protein is performed after proteolytically digesting the cross-linking reaction mixtures. This is often hampered by the complexity of the created peptide mixtures, wherein only a relatively small percentage of cross-linked products is present besides a large majority of unmodified peptides. To safeguard for a selective identification of cross-linked peptides using electrospray ionization (ESI)[4–6] combined with collision-induced dissociation (CID)[7] tandem MS (MS/MS),[8] several approaches have been suggested. They include cross-linking reagents that incorporate the use of marker ions resulting from low-energy CID[9,10] or metastable decay,[11] isotope-coding strategies such as proteolytic digestion in ^{18}O-water,[12] isotope coding of the cross-linking reagents[13–16] or isotope coding of the proteins,[17] and enrichment of cross-linked products via affinity tags.[18–20] Identification of cross-linked peptide ions by MS/MS or MS^n is not trivial as product ion spectra of these contain a number of product ions, i.e. b- and y-type ions,[21,22] originating from both peptides involved in the cross-linking product. To enable a more effective analysis of cross-linked peptides by tandem MS analysis, we have developed a novel concept for collision-induced dissociative cross-linking reagents. Thus, our novel cross-linker contains an extremely labile covalent bond located within the linker region, which can be selectively and preferably cleaved by low-energy collision activation in the gas phase. For designing this cross-linker, we chose an approach derived from classic Edman chemistry[23–25] and protein ladder sequencing,[26] which has shown to operate both in the condensed and the gas phase.[27–30]

Our custom-designed cross-linker leads to the formation of indicative fingerprint mass shifted product ions and neutral losses in the product ion mass spectra, which allow an unambiguous

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Journal of MASS SPECTROMETRY

identification and structure elucidation of cross-linked peptides. The concept of this cross-linker relies on the presence of a thiourea moiety (Scheme 1), which is connected to proline via glycine. With the strong nucleophilicity of sulfur in the thiocarbonyl moiety and the pronounced gas-phase basicity of proline[31–33] we combine classic Edman degradation chemistry[23–25] with the extensive knowledge on intramolecular peptide fragmentation reactions,[34] i.e. the preferred cleavage of peptide bonds besides acidic amino acids as aspartic acid, called the *aspartic acid effect*.[34–42]

The *proof-of-principle* of the presented analytical concept (Schemes 2–4) and its versatile applicability is demonstrated for the peptides substance P (amino acid sequence RPKPQQFFGLM-NH_2), luteinizing hormone releasing hormone (LHRH with the amino acid sequence pEHWSYGLRPG) and hen egg lysozyme (14.3 kDa) as model substances.

Experimental Section

Materials

All chemicals and solvents were used as purchased without further purification (Acros Organics, Geel, Belgium; ABCR, Karlsruhe, Germany and Merck KGaA, Darmstadt, Germany). Substance P, LHRH and hen egg lysozyme were used as purchased from Sigma-Aldrich, Taufkirchen, Germany. Toluene and tetrahydrofurane (THF) were dried by distillation from benzophenone and sodium, dichloromethane and pyridine by distillation from calcium hydride. Methanol was distilled from magnesia, whereas all other solvents were distilled over a column prior to use.

Synthesis of the NHS-BuTuGPG-NHS chemical cross-linker

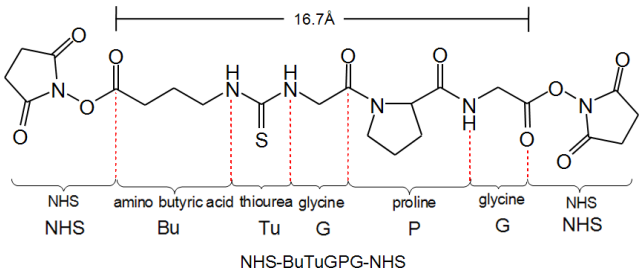
Esterification of 4-amino-butyric acid (**1**) was achieved by reaction at 20 °C with thionyl chloride in methanol for 15 h. After evaporation of the reaction mixture, the crude product was stirred in ethyl acetate. The hydrochloride of 4-amino-butyric acid methyl ester (**2**) was isolated by filtration as white solid at a yield of 98% (Scheme S1 of the Supporting Information).[43]

For the synthesis of 4-isothiocyanato-butyric acid methyl ester (**3**), **2** was suspended in a mixture of dichloromethane and carbon disulfide. After slowly adding triethylamine, the reaction mixture was stirred for 30 min, before methyl chloroformate was added. After refluxing for 4 h, the mixture was cooled to 20 °C and poured into water. The organic layer was washed with diluted hydrochloric acid and brine, dried over magnesium sulfate and concentrated under reduced pressure. The resulting yellowish oil was distilled in high vacuum to give **3** as colorless oil at a yield of 80% (Scheme S1 of the Supporting Information).[44]

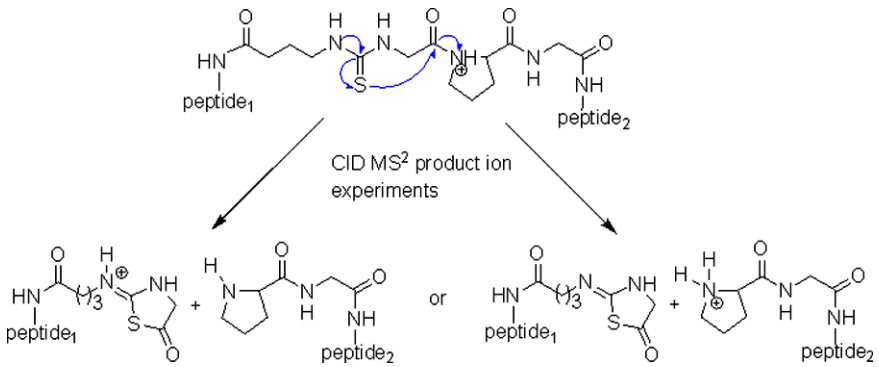
Z-Gly-OSu (**5**) was synthesized in a *N*,*N*′-dicyclohexyl carbodiimide (DCC)-mediated coupling of benzyloxycarbonyl-glycine (**4**) with *N*-hydoxysuccinimide (NHS); **4** was suspended in dichloromethane and NHS and DCC were added at 0 °C. After stirring for 16 h, the solid was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was stirred in ethyl acetate and insoluble solid was removed by filtration. After removal of the solvent under reduced pressure the crude product was recrystallized from *iso*-propanol to give **5** as white solid at 95% yield (Scheme S1 of the Supporting Information).[45]

The synthesis of Z-Gly-Pro-OH (**6**) started with dissolving sodium bicarbonate and proline in water. As soon as gas formation had stopped, a solution of **5** in 1,4-dioxane was added. After letting the reaction proceed at 20 °C for 1 h, most of the solvent was removed under reduced pressure and the residue was acidified to pH 2 with 7_M hydrochloric acid. The solution was extracted three times with dichloromethane, the combined organic layer were washed twice with brine, dried over magnesium sulfate, and the solvent was removed under reduced pressure to give **6** as white solid at a yield of 90% (Scheme S1 of the Supporting Information).[46]

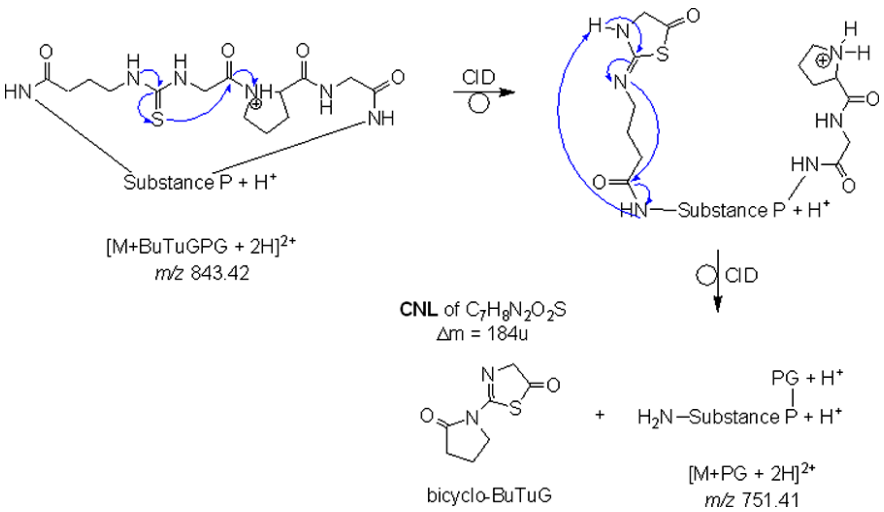
Z-Gly-Pro-Gly-OMe (**7**) was derived from **6** in a DCC-mediated coupling with glycine methyl ester. **6** was dissolved



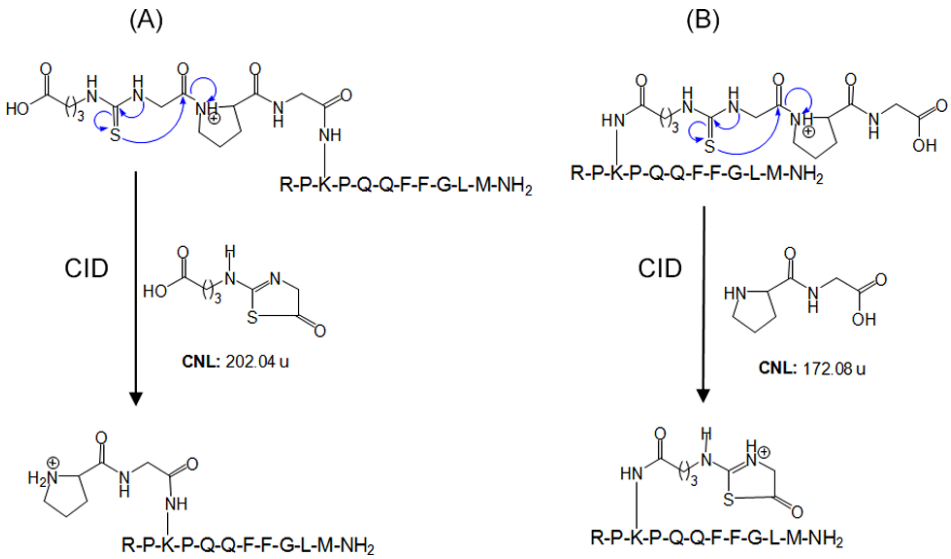
Scheme 1. Structure of the novel homobifunctional chemical cross-linker molecule NHS-BuTuGPG-NHS for structure elucidation of proteins.



Scheme 2. Preferred cleavage of the Gly-Pro amide bond of the linker by selective nucleophilic attack of the sulfur upon CID.



Scheme 3. Characteristic fragmentation reaction of intrapeptidal cross-linked peptides with BuTuGPG upon CID, i.e. formation of the CNL of 184 u.



Scheme 4. Characteristic fragmentation reaction of 'dead-end' cross-linked peptides upon CID. (a) The nucleophilic attack of the sulfur cleaves off the Gly-Pro amide bond and generates the five-membered thiazolone, which is lost as BuTuG (CNL of 202 u). (b) The cross-linker is connected via butyric acid; the analog fragmentation scheme leads to the loss of prolyl-glycin with 172 u.

in dichloromethane, followed by the addition of glycine methyl ester hydrochloride and triethylamine. DCC was added at 0 °C and the reaction mixture was stirred for 14 h. Urea was removed by filtration and the filtrate was evaporated. The residue was stirred in ethyl acetate and insoluble solid was removed by filtration. The filtrate was washed with 0.2 M $NaHCO_3$ solution, 0.5 M hydrochloric acid and again with 0.2 M $NaHCO_3$ solution, dried over magnesium sulfate before the solvent was removed under reduced pressure. The resulting yellowish oil was purified by column chromatography with ethyl acetate/ethanol [20 : 1 (v/v)] to give **7** as colorless oil at a yield of 73% (Scheme S1 of the Supporting Information).[47]

For removing the protecting group, **7** was dissolved in methanol, treated with palladium on charcoal, and stirred for 16 h under hydrogen atmosphere (1 bar). After filtration over celite the filtrate was evaporated to give H-Gly-Pro-Gly-OMe (**8**) as gum-like solid at 94% yield (Scheme S1 of the Supporting Information).[48]

The deprotected tripeptide **8** was suspended in dichloromethane and treated with the thioisocyanate **3** at 0 °C. The reaction mixture was brought to 20 °C and stirred for 14 h before it was washed with saturated $NaHCO_3$ solution and brine. The organic phase was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography with dichloromethane/methanol (10 : 1 (v/v)) to give 4-(3-{2-[2-(methoxycarbonylmethyl-carbamoyl)-pyrrolidin-1-yl]-2-oxo-ethyl}-thioureido)-butyric acid methyl ester (MeO-BuTuGPG-OMe; **9**) as colorless solid at a yield of 77% (Scheme S2 of the Supporting Information).[49,50]

Hydrolysis of the two methyl ester functionalities of **9** was achieved with lithium hydroxide in a mixture of water, methanol, and THF for 15 hrs. at 20 °C. After removing the solvent under reduced pressure, the precipitate was dissolved in methanol and the solvent was evaporated. The crude product was purified by column chromatography with dichloromethane/methanol (2 : 1 (*v/v*)) to give dicarboxylic acid 4-(3-{2-[2-(carboxymethyl-carbamoyl)-pyrrolidin-1-yl]-2-oxo-ethyl}-thioureido)-butyric acid

(HO-BuTuGPG-OH; **10**) as colorless solid at 92% yield (Scheme S2 of the Supporting Information).[51]

10 was suspended in pyridine and treated with *N*-trifluoroacetoxy succinimide – prepared from NHS and trifluoroacetic anhydride[52] – at 0 °C. The mixture was brought to 20 °C within 3 h and pyridine was removed under reduced pressure. The crude product was dissolved in dichloromethane, washed with 1 M $NaHCO_3$ solution and brine. After drying the organic phase over magnesium sulfate, toluene was added and the solvent was removed under reduced pressure to yield 4-[3-(2-{2-[(2,5-dioxo-pyrrolidin-1-yloxycarbonyl-methyl)-carbamoyl]-pyrrolidin-1-yl}-2-oxo-ethyl)-thioureido]-butyric acid 2,5-dioxo-pyrrolidin-1-yl ester (NHS-BuTuGPG-NHS; **11**) as colorless foam with 86% yield (Scheme S2 of the Supporting Information).[53]

Cross-linking reactions

For cross-linking experiments, aqueous stock solutions of substance P (100 µg/ml), LHRH (100 µg/ml) or lysozyme (10 mg/ml) were diluted with 20 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7.4), with 1 ml solution containing the protein/peptide at a concentration of 10 µM per aliquot. The cross-linker [200 mM stock solution in dimethylsulfoxide (DMSO)] was added in 50-, 100- and 200-molar excess over the protein/peptide solution and the reactions were allowed to proceed for 5, 15, 30, 60 and 120 min Reactions were quenched with ammonium bicarbonate (20 mM final concentration). One 200-µl aliquot was taken from each sample and stored at −20 °C before MS analysis. Cross-linked lysozyme was digested with trypsin (1 : 100 (*w*/*w*) enzyme to substrate ratio) overnight at 37 °C according to an existing protocol.[54] The resulting digests were stored at −20 °C before MS analysis.

Linear mode MALDI-TOF MS

Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) MS of intact cross-linked lysozyme was performed in linear and positive ionization mode on an Ultraflex III instrument (Bruker Daltonik, Bremen, Germany) equipped with a Smart beam™ laser using sinapinic acid (SA) as matrix. 5 pmol of each sample was applied onto a ground steel target (Bruker Daltonik, Bremen, Germany) using the double layer method.

Nano-HPLC/MALDI-TOF/TOF-MS

Tryptic peptide mixtures of lysozyme were analyzed by *offline* coupling of a nano-HPLC (high performance liquid chromatography) system (Ultimate 3000, Dionex, Idstein, Germany) to a MALDI-TOF/TOF mass spectrometer (Ultraflex III, Bruker Daltonik, Bremen, Germany). Samples were injected onto a precolumn (Acclaim PepMap, C18, 300 µm × 5 mm, 5 µm, 100 Å, Dionex) and desalted by washing the precolumn for 15 min with 0.1% trifluoroacetic acid (TFA) before the peptides were eluted onto the separation column (Acclaim PepMap, C18, 75 µm × 150 mm, 5 µm, 100 Å, Dionex), which had been equilibrated with 95% solvent A (A: 5% acetonitrile (ACN), 0.05% TFA). Peptides were separated with a 60-min gradient (0–60 min: 5–50% B, 60–62 min: 50–100% B, 62–67 min: 100% B, 67–72 min: 5% B, with solvent B: 80% ACN, 0.04% TFA) at a flow rate of 300 nl/min with UV detection at 214 and 280 nm. Eluates were fractionated into 18 s. fractions using the fraction collector Proteineer fc (Bruker Daltonik), mixed with 1.1 µl of matrix solution (0.8 mg/ml α-cyano-4-hydroxy cinnamic acid (HCCA) in 90% ACN, 0.1% TFA, 1 mM $NH_4H_2PO_4$) and directly prepared onto a 384 MTP 800 µm AnchorChip target (Bruker Daltonik). MALDI-TOF/TOF-MS analyses were conducted in the positive ionization and reflectron mode by accumulating 2000 laser shots in the range *m/z* 700–5000 to one mass spectrum. Mass spectra were externally calibrated using Peptide Calibration Standard II (Bruker Daltonik). Signals with a *signal-to-noise ratio* (S/N) >15 were selected for laser-induced fragmentation. In-source decay (ISD) was performed manually on selected cross-linking product candidates by increasing the laser energy by ca. 10% relative to the usual laser energy. Data acquisition was done automatically by the WarpLC 1.1 software (Bruker Daltonik) coordinating MS data acquisition (FlexControl 1.3) and data processing (FlexAnalysis 3.0) softwares.

Nano-HPLC/nano-ESI-LTQ-Orbitrap-MS

Fractionation of tryptic peptide mixtures (lysozyme) was carried out on an ultimate nano-HPLC system (Dionex Corporation, Idstein, Germany) using reversed phase C18 columns (precolumn: Acclaim PepMap, 300 µm × 5 mm, 5 µm, 100 Å, separation column: Acclaim PepMap, 75 µm × 150 mm, 5 µm, 100 Å, Dionex Corp., Idstein, Germany). After washing the peptides on the precolumn for 15 min with water containing 0.1% TFA, they were eluted and separated using gradients from 0% to 50% B (90 min), 50% to 100% B (1 min), and 100% B (5 min), where solvent A is 5% ACN containing 0.1% formic acid (FA) and solvent B is 80% ACN containing 0.1% FA. The nano-HPLC system was directly coupled to the nano-ESI source (Proxeon, Odense, Denmark) of an linear ion trap LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher Scientific, Bremen, Germany). MS data were acquired over 122 min in data-dependent MS^2 mode: Each high-resolution full scan (*m/z* 300–2000, resolution was set to 60 000) in the orbitrap was followed by three product ion scans in the orbitrap (resolution 15 000) for the three most intense signals in the full-scan mass spectrum (isolation window 1.5 u). MS^3 in the orbitrap was performed on the two most intense signals in the MS^2 spectra with a resolution of 15 000. Dynamic exclusion (exclusion duration 180 s, exclusion window −1 to 2 Th) was enabled to allow detection of less-abundant ions. Data acquisition was controlled via XCalibur 2.0.7 (ThermoFisher) in combination with DCMS link 2.0 (Dionex).

Offline nano-ESI-LTQ-Orbitrap-MS

Cross-linked substance P and LHRH were additionally analyzed by *offline* nano-ESI-LTQ-Orbitrap-MS, with the resolution in the orbitrap set to 100 000. MS/MS and MS^3 experiments were conducted with the relative collision energy in the linear ion trap (LTQ) of 35% and selected product ions were analyzed in the orbitrap analyzer set to a resolution of 100 000.

Analysis of cross-linked products

Cross-linked products were identified by analyzing the MS data using General Protein Mass Analysis for Windows (GPMAW),[55] version 8.10 (Lighthouse Data, Odense, Denmark, http://www.gpmaw.com) and BioTools 3.1.1.36 (Bruker Daltonik, Bremen, Germany). The length of the cross-linker was calculated using the Viewer Light 5.0 software (Accelrys).

Results and Discussion

Cross-linker design and rationale

The structure of the cross-linker features a glycyl-prolyl amide bond that is preferably cleaved by CID. The particular *N*-terminal bond adjacent to proline, which is supposed to be substantially more labile than any other covalent bond in linker-protein condensation products is predestined for cleavage by an intramolecular attack of the sulfur of a thiourea moiety (Scheme 2). This fragmentation reaction results in the formation of a five-membered thiazolone structure and an *N*-terminally truncated peptide (here: proline), i.e. the primary reaction products of the Edman protein degradation reaction (Scheme 2). The design of the linker is tailored for the fragmentation scheme derived from Edman chemistry[23–25] and protein ladder sequencing.[26] The classic Edman degradation reaction proceeds via a nucleophilic attack of the sulfur in an *N*-terminal phenylthiocarbamyl (PTC) peptide derivative, forming a five-membered thiazolone structure. Gaskell and others examined this reaction in the gas phase,[27–30] where collision activated PTC-peptides give rise to analog thiazolone product ions and respective peptide fragment ions with the *N*-terminal amino acid cleaved off. In consideration of the Edman reaction scheme we connected a thiourea moiety via glycine to proline, providing the prerequisites that the Gly-Pro amide bond is substantially weaker than any other covalent bond in either the attached peptides or the linker itself (Scheme 2). This can be assumed as the sulfur in the thiocarbonyl group is a much more effective nucleophile than any carbonyl oxygen.[27,56] Moreover, the very basic amino acid proline (gas-phase basicity: 213.3 kcal/mol[31–33]) is placed in the linker to promote the initial protonation at its amide nitrogen. The protonation at this site is reasonable, especially in multiply-protonated molecular ions of proteins or peptides. In very basic peptides or proteins, in which preferably even more basic amino acids such as Arg and Lys are protonated, a proton shift to the proline in the linker can be assumed to take place upon collision activation according to the mobile proton model.[41,57]

However, the functioning of our Edman chemistry-based collision-induced dissociative chemical cross-linking reagent relies on the correct understanding of intramolecular peptide fragmentation reactions, which are still a matter of scientific debate. Recently, the current status of knowledge on intramolecular peptide fragmentation reactions was exhaustively reviewed by Paizs and Suhai.[34] Especially, the collision-induced cleavage of the Asp–Pro amide bond is extensively examined, for which a charge remote fragmentation mechanism is strongly favored. That reaction scheme starts with the proton transfer from the carboxylic acid of Asp to the very basic backbone amide nitrogen of the adjacent prolyl residue.[35,39,40] This delivers an intermediate zwitterion, which in turn promotes the nucleophilic attack of the carboxylate anion of the aspartyl side chain to the neighbored carbonyl carbon. The intramolecular reaction finally leads to the formation of a cyclic anhydride of the aspartyl residue and cleaves off the proline *N*-terminally.[35,39–42] Based on the experimental observation that the Asp–Pro bond is easily cleaved and the advanced understanding of the mechanism, Soderblom and Goshe presented an aspartyl–prolyl moiety in a chemical cross-linker to selectively dissociate this particular bond upon collision activation.[58,59]

The abbreviation BuTuGPG for the novel linker used throughout the article shown in Scheme 1 is deduced from the respective one letter codes of the amino acids glycine (G) and proline (P) and from thiourea (Tu) and amino-butyric acid (Bu). *N*-hydroxysuccinimidyl active ester is placed on both ends to enable effective condensation with protein amine functionalites.[3] The actual structure BuTuGPG is a result of the extreme nucleophilicity of the sulfur in the liquid phase, that lead to the formation of a number of unwanted cyclized byproducts, i.e. di-keto-piperazines.[60–62] To effectively inhibit these intramolecular condensation reactions an additional glycine is introduced *C*-terminal to proline and the thiourea is connected with butyric acid. Altogether, these two extra parts of the molecule increase the length of the cross-linker to 16.7 Å.[63]

The homobifunctional bisuccinimidyl cross-linker molecule reacts with amine functionalities, e.g. lysine- and *N*-terminus of proteins and peptides.[3] Reactivity of NHS-esters toward functional groups other than amines, e.g. hydroxyl groups in serine, threonine or tyrosine is found to be much lower.[64,65] After adequate separation of cross-linked proteins with subsequent proteolytic digestion ESI-MS delivers protonated molecular ions of cross-linked peptides. Upon low-energy collision activation (QIT, Triple quadrupole-MS) the Gly–Pro amide bond of the linker is preferably cleaved by a selective nucleophilic attack of the sulfur (Scheme 2). The characteristic mass shifted product ions are further examined by MS^n-experiments, in which the primary structure of the peptides and localization of the linker is reliably elucidated by indicative neutral losses as shown for the model peptide substance P in Schemes 3 and 4. This feature is important to improve the capabilities of the cross-linking approach, since characteristic secondary fragmentation reactions allow an elegant identification of chemical cross-linking products with improved sensitivity and selectivity and provide a straightforward access to automated detection by multiple-reaction monitoring (MRM). Mass measurement of the intact cross-linked species, followed by MS^n analysis of each of the peptide chains, will provide both the identities of cross-linked proteins and information regarding the sites of derivatization.

Cross-linking with substance P

To demonstrate the functioning of the analytical concept substance P was chosen as a model peptide that was treated with NHS-BuTuGPG-NHS. The positive electrospray mass spectrum of the reaction products is shown in Fig. 1(a). As characteristic fragment ions evidence, the *N*-terminus of substance P and the side chain amino group of the lysine at position 3 are connected by the cross-linker BuTuGPG. The doubly protonated molecular ion of the intramolecularly cross-linked substance P with BuTuGPG is found at *m/z* 843.42, the singly charged analog is observed at *m/z* 1685.84. Additionally, two reaction products of NHS-BuTuGPG-NHS with substance P are found, in which the cross-linker is partially hydrolyzed and hence only connected via a single amide bond. These 'dead-end' cross-linked ions are found with one (at *m/z* 852.43) and with two linker molecules (at *m/z* 1032.97) attached to substance P.[66] The MS^2 product ion spectrum of the intramolecular cross-linked precursor ion $[M+BuTuGPG + 2H]^{2+}$ at *m/z* 843.42 is presented in Fig. 1(b). Obviously, the vulnerable Gly–Pro bond in the cross-linker is preferably cleaved upon CID. A product ion at *m/z* 751.41 is released, which is formed by the characteristic loss of 184 u. A proposed mechanism for the generation of the neutral loss expelled is shown in Scheme 3. The nucleophilic attack of the sulfur cleaves off the Gly-Pro amide bond and generates the five-membered thiazolone. In the depicted reaction the prolyl moiety is protonated. Subsequently, the thiazolone rearranges and loses a neutral fragment with 184 u either via a charge remote (depicted)

Journal of MASS SPECTROMETRY

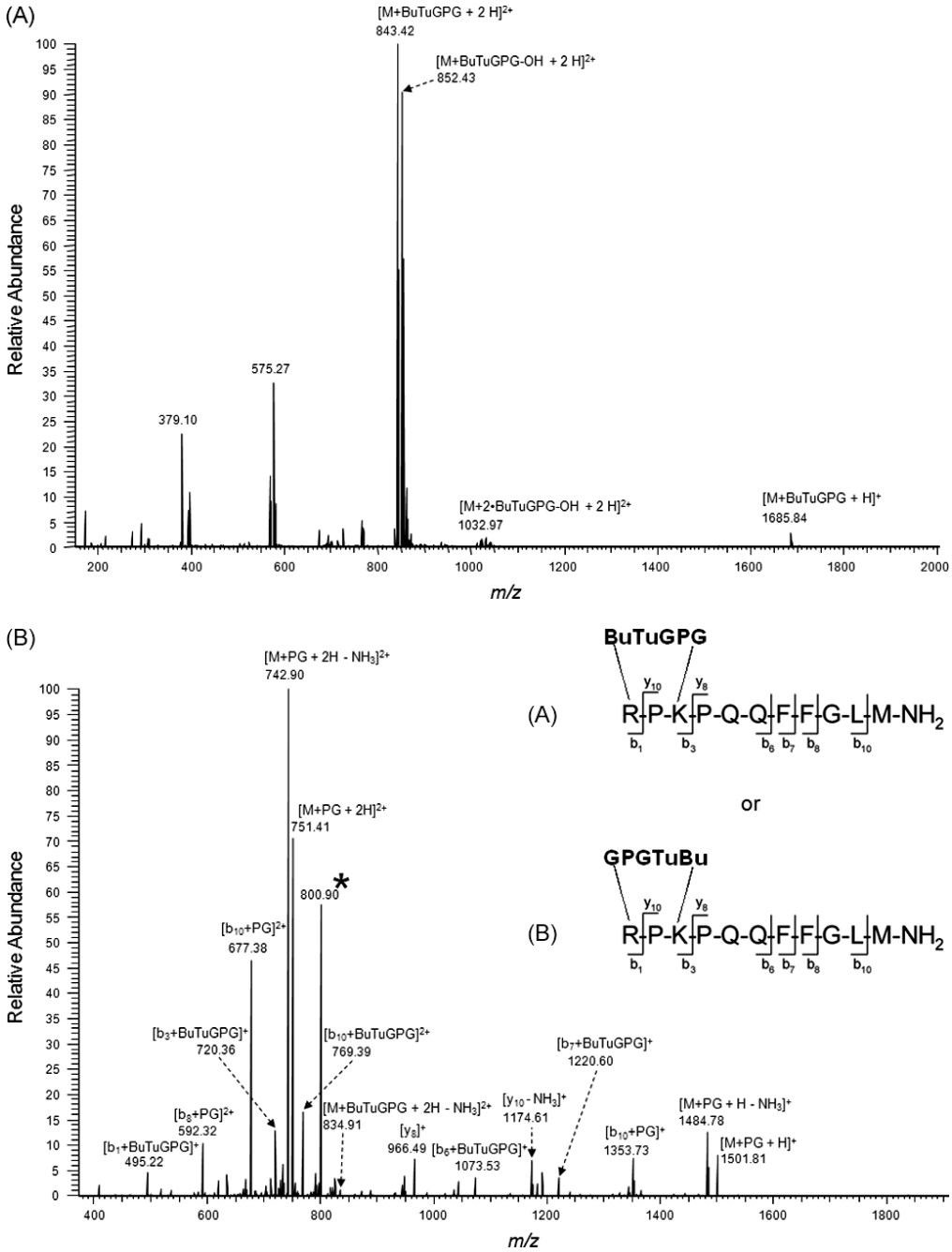


Figure 1. (a) ESI-mass spectrum of substance P modified with BuTuGPG; (b) MS^2-CID-product ion spectrum of $[M+BuTuGPG + 2H]^{2+}$ at *m/z* 843.42 acquired in a linear ion trap, fragment ions were analyzed in the orbitrap. The signal marked with an asterisk (*) belongs to an additional fragmentation of the thiourea group; (c) MS^3-CID-product ion spectrum of $[M+PG + 2H]^{2+}$ at *m/z* 751.41; (d) MS^2-CID-product ion spectrum of $[M+Linker-OH + 2H]^{2+}$ at *m/z* 852.43 acquired in a linear ion trap, fragment ions analyzed in the orbitrap. The signals marked with an asterisk (*) belong to an additional fragmentation of the thiourea group.

or via a charge driven mechanism. Accurate mass measurements of precursor and product ion allowed the determination of the composition of this neutral fragment to be $C_7H_8N_2SO_2$, for which we propose a cyclized BuTuG structure. The fingerprint loss of 184 u is therefore ideally suited for selective and sensitive identification of intra- and intermolecular cross-linked peptides with our new linker. Hence, peptides decorated with the BuTuGPG linker can be tracked down by a CNL experiment for 184 u.[43] After reliable identification of cross-linked peptides by this CNL scan, the primary structure of the modified peptides is determined by successive MS^n product ion experiments. This strategy is demonstrated for respective ions of substance P at *m/z* 843.42 (MS^2; Fig. 1(b)) and *m/z* 751.41 (MS^3; Fig. 1(c)). On the basis of the mass shift of either the complete linker ($\Delta m = 338$ u), here connecting the *N*-terminus with lysine at position 3, or the linker remains on the peptide after CID (Pro-Gly moiety: $\Delta m = 154$ u), the location of the covalent modification can be deduced straightforwardly. The almost complete series of b-fragment ions and additional y-ions observed in the MS^n spectra allow a reliable assignment (Fig. 1(b) and (c)).

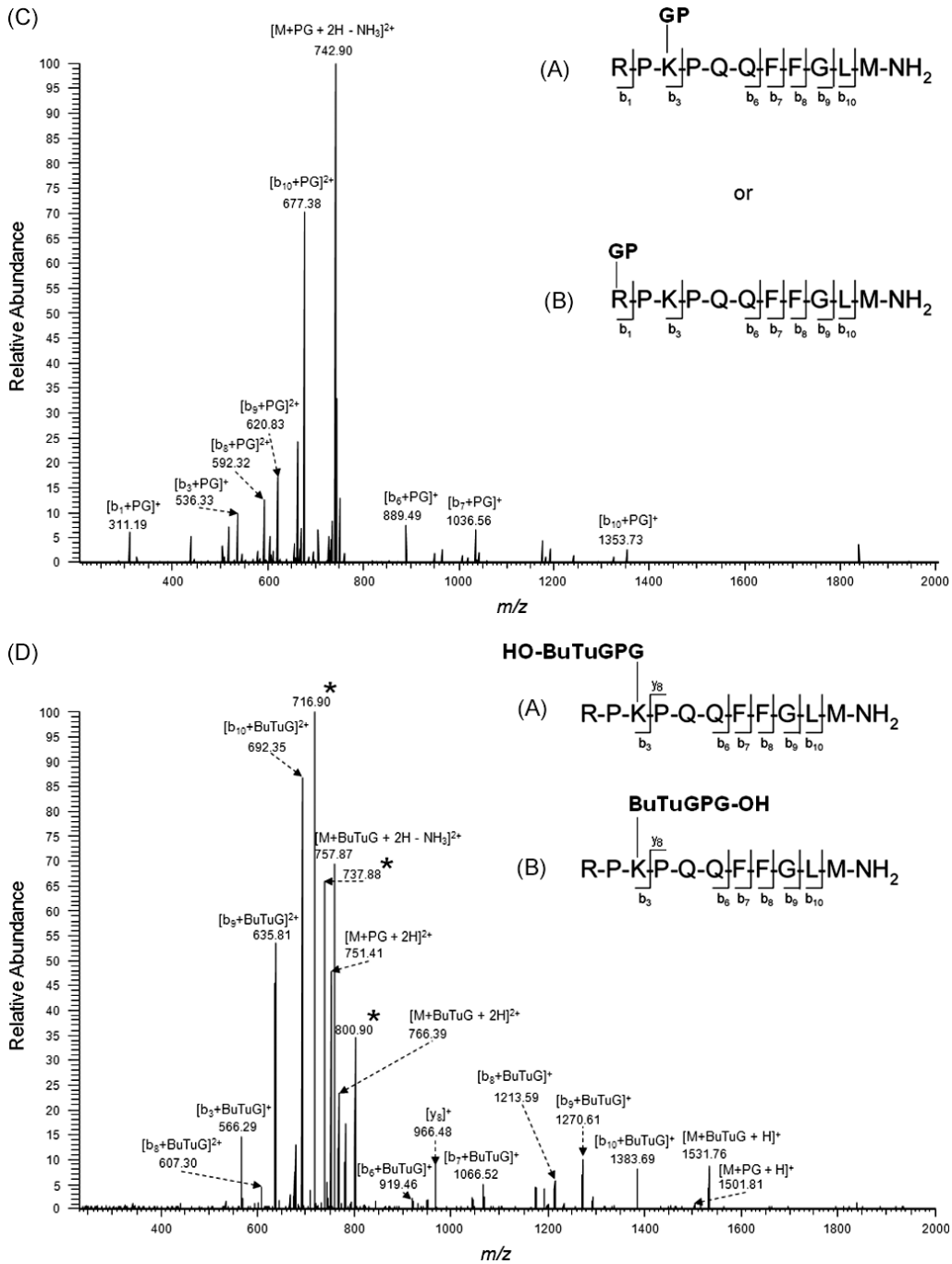


Figure 1. (continued).

It is important to note that the signal at *m/z* 843.42 (Fig. 1(a)) assigned to the doubly protonated molecular ion of intrapeptidal cross-linked substance P could also originate from an isobaric ion of substance P modified with a dehydrated, singly condensed 'dead-end' cross-linker. For the formation of the latter only the free butyric acid part of a partially hydrolyzed cross-linker (see structure A shown in Fig. 1(d)) is considered to be suited for cyclization accompanied with a loss of a water molecule (18 u). The intramolecular condensation reaction is entropically demanding and is somewhat unlikely as the thiourea amide nitrogen with only moderate nucleophilic character has to be responsible for the formation of the δ-lactame derived from the butyric acid moiety. Moreover, all cyclized byproducts found during the linker synthesis in the condensed phase are exclusively generated by intramolecular nucleophilic reactions of the sulfur (see preceding section). Yet, an involvement of the sulfur in the water loss discussed here can be excluded, as the thiocarbonyl sulfur is indispensable for the subsequent formation of the characteristic neutral loss, i.e. 184 u (mechanism A in Scheme 4).

Especially the formation of the y_{10}- and y_{10}-NH_3 fragment ions observed in Fig. 1(b) has to be discussed in the light of these considerations. In the intrapeptidal cross-linking product of substance P the butyric acid of the BuTuGPG linker can be connected either to lysine 3 (see structure B in Fig. 1(b)) or to

Journal of MASS SPECTROMETRY

the *N*-terminus (see structure A in Fig. 1(b)). Both isomers deliver a characteristic loss of 184 u upon CID (Scheme 3), but only the former of the modified peptides (structure B in Fig. 1(b)) can give rise to y_{10}- and y_{10}-NH_3 ions without a respective mass shift related to the linker (see structure B in Fig. 1(c)). Given a dehydrated dead-end linker with a cyclized butyric acid δ-lactame connected to the *N*-terminus is present, a neutral loss of 184 u could also be formed by this ion, as the characteristic neutral loss of 202 u would be shifted by 18 u (H_2O). Hence, from this MS^n-data alone it cannot be excluded that the y_{10}- and y_{10}-NH_3 ions could also be formed by respective fragmentation reactions of an isobaric peptide precursor modified with a dehydrated dead-end linker.

However, we note that a constant neutral loss (CNL) of 154 u (172 u − 18 u = 154 u) is absent in all MS^2 product ion spectra of assigned intramolecular cross-links as shown in Figs 1(b), 3(a) and 4(a), showing that a dehydrated dead-end linked peptide connected via the butyric acid is completely missing. This is reasonable as a formation of a β-lactame as result of a *C*-terminal water loss of glycine is at least sterically strongly hindered.

In Fig. 1(d), the CID MS^2 product ion spectrum of substance P modified with the partially hydrolyzed cross-linker is presented. For this 'dead-end' cross-link peptide modification two indicative neutral losses of either 172 or 202 u are observed upon CID.[43] As illustrated in Scheme 4, these two CNL reactions are characteristic for the directivity of linkage as either the thiazolone-butyric acid (HO-BuTuG) is eliminated (CNL of 202 u) or the prolyl-glycin (PG-OH) is lost (CNL of 172 u). The fact that both CNLs are observed in the product ion spectrum (Fig. 1(d)) of the precursor ion at *m/z* 852.43 can straightforwardly be explained by the presence of both isobaric 'dead-end' linked peptides depicted (structures A and B in Fig. 1(d)). The fragment ions observed in the MS^3 spectrum allow locating the chemical derivatization to Lys-3 of substance P.

Cross-linking with LHRH

In addition to substance P, LHRH was chosen as another model peptide to investigate the properties of the novel cross-linker. ESI-LTQ-Orbitrap-MS and MS/MS data (Figs S1 and S2 of the Supporting Information) were collected confirming the fragmentation behavior that had already been observed for substance P. As the primary structure of LHRH offers no possibility for an intramolecular cross-link, only dead-end cross-linked product ions are found. Tellingly, the two characteristic CNL fragments of 172 and 202 u confirm the presence of both possible dead-end linked isomers (Fig. S2 of the Supporting Information). Moreover, in Fig. S2 of the Supporting Information the dead-end linked peptides of LHRH do not show any water loss (Δm = 18 u) upon CID. This experimental result further supports the assumption that the water loss proceeds via a complicated rearrangement reaction, i.e. the formation of the δ-lactame from linear butyric acid, which is not favored in the gas phase upon CID, making the formation of respective dehydrated species unlikely.

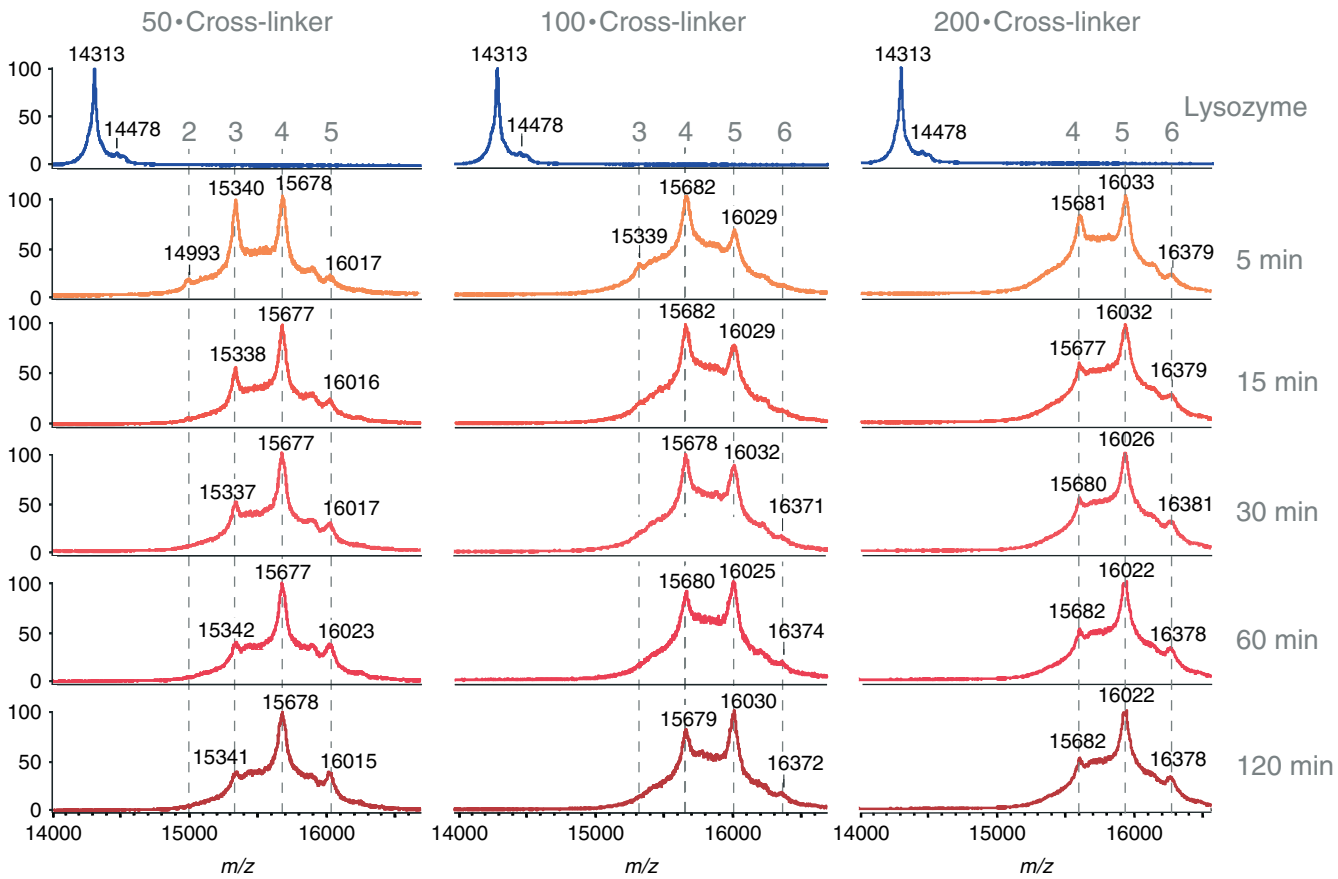


Figure 2. Linear MALDI-TOF-MS of lysozyme; excess of Edman cross-linker (50-, 100- and 200-fold excess) and reaction times (5–120 min) are indicated. The calculated average molecular weight of hen egg lysozyme is 14 313 Da matching perfectly with the signal at *m/z* 14 313. The signal at *m/z* 14 478 probably corresponds to a glycosylated species of lysozyme.

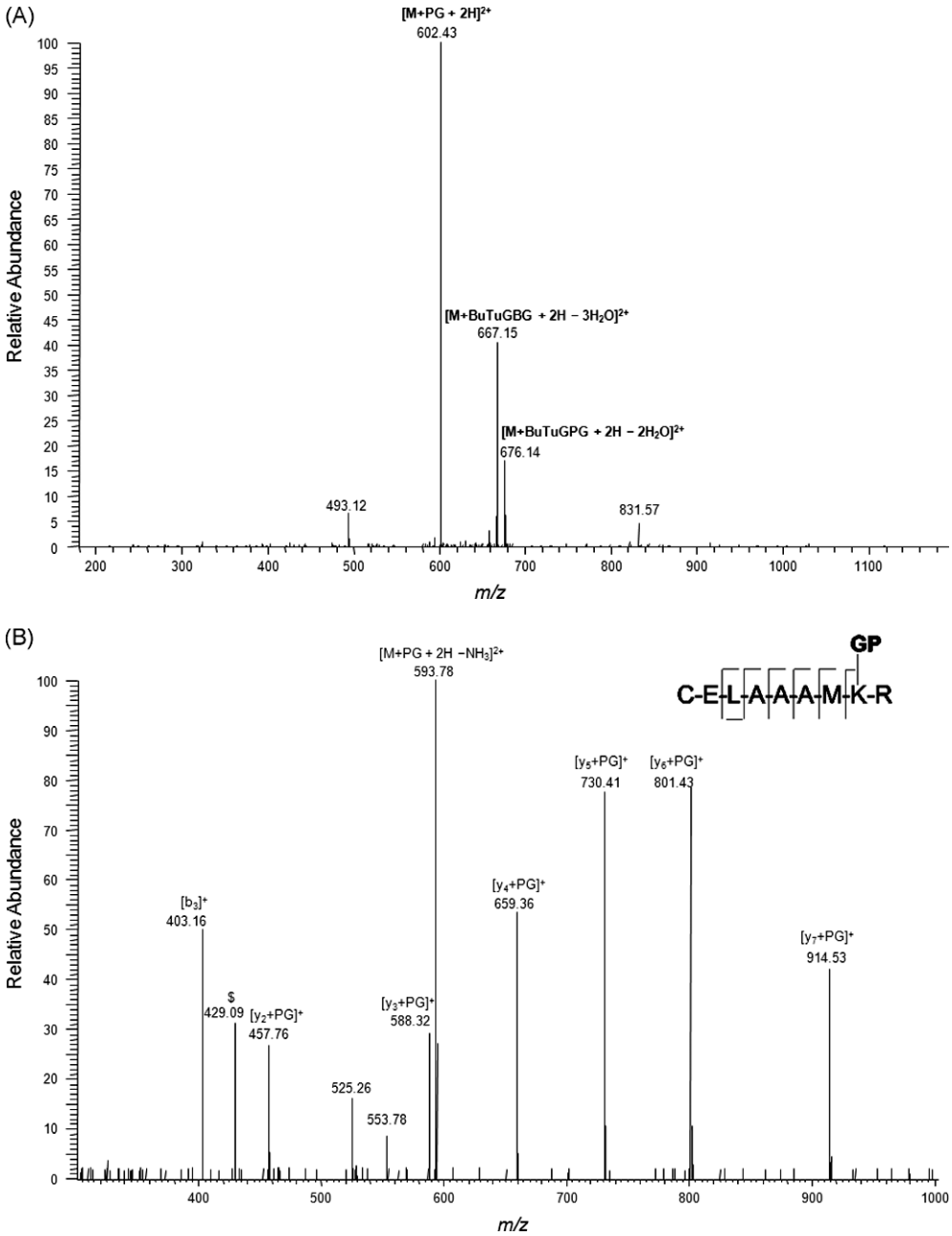


Figure 3. Chemical cross-linking data analysis for lysozyme peptide (amino acids 6–14), modified with the BuTuGPG cross-linker. (a) ESI-LTQ-Orbitrap-MS^2 product ion spectrum of the precursor ion $[M+BuTuGPG + 2H]^{2+}$ at *m/z* 694.15 and (b) ESI-LTQ-Orbitrap-MS^3 product ion spectrum of $[M+PG + 2H]^{2+}$ at *m/z* 602.43.

Cross-linking with lysozyme

In order to evaluate the applicability of our chemical cross-linker for proteins the 14.3 kDa protein lysozyme was derivatized with the novel collision-induced dissociative reagent. Efficient cross-linking was proven by MALDI-TOF mass spectrometric measurements in the linear mode showing the attachment of up to six cross-linker molecules to the protein depending on molar excess (50-, 100- and 200-fold excess) of cross-linker and reaction time (5–120 min) (Fig. 2). Modified lysozyme was *in-solution* digested with trypsin and the resulting peptide mixtures were analyzed by both nano-HPLC/MALDI-TOF/TOF-MS and nano-HPLC/nano-ESI-LTQ-Orbitrap-MS.

In Fig. 3(b) and 3(b), chemical cross-linking data analysis is presented exemplarily for lysozyme peptide (amino acids 6–14), being modified with the BuTuGPG cross-linker. Product ions resulting from up to three water losses are visible in the product ion spectra of derivatized lysozyme peptides (Fig. 3(a)). The multiple loss of water rather originates from unspecific fragmentations of the lysozyme peptide (amino acids 6–14) than from the linker molecule alone. MALDI-MS/MS data confirm the observation that one water molecule is easily lost from the peptide modified with a partially hydrolyzed cross-linker, probably already during the cross-linking reaction (data not shown). The primary fragment ion $[M+PG + 2H]^{2+}$ at *m/z* 602.43 generated in a MS^2 experiment by the loss of 184 u was subjected to a MS^3 product ion experiment

Journal of MASS SPECTROMETRY

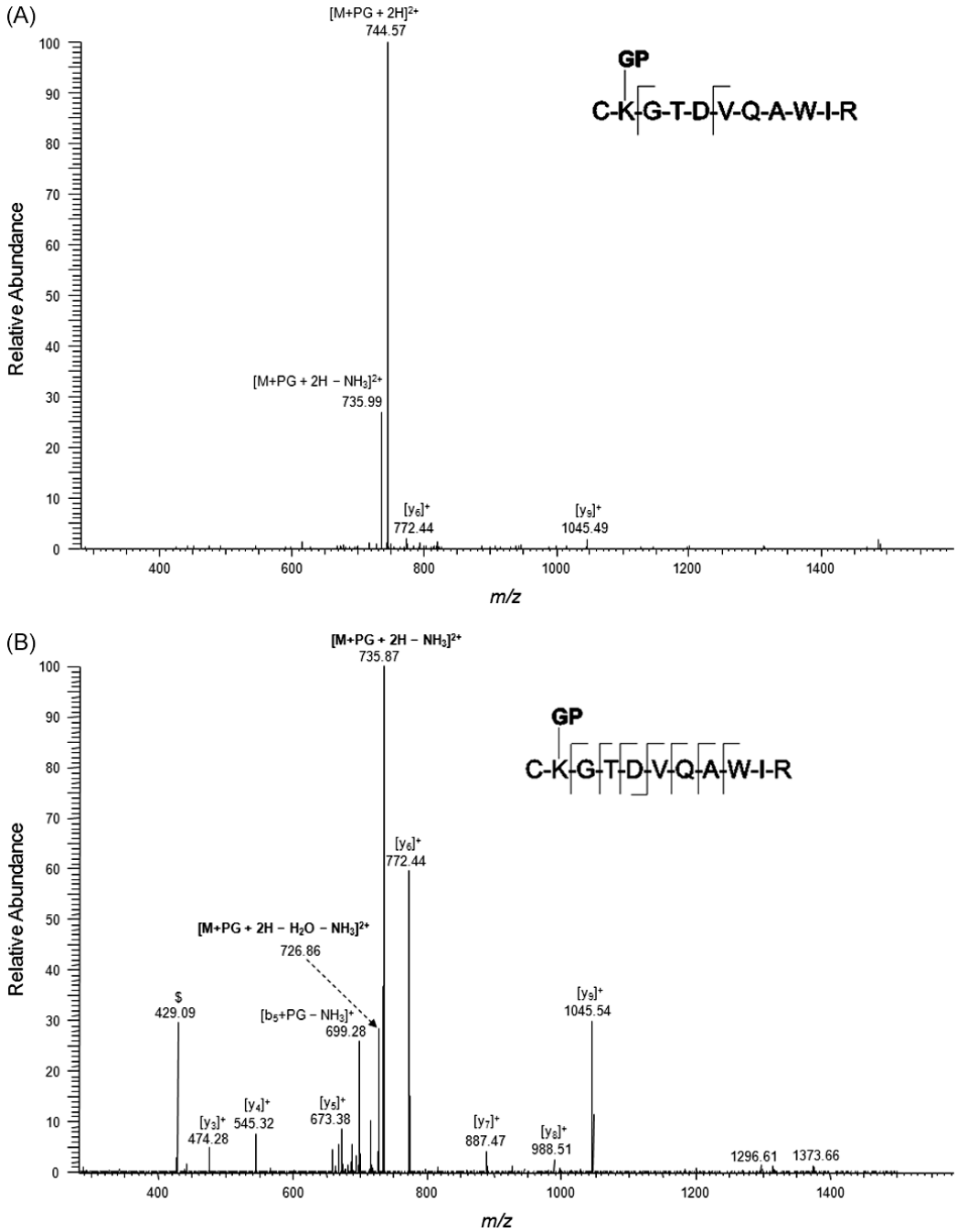


Figure 4. (a) ESI-LTQ-Orbitrap-MS^2 product ion spectrum of $[M+BuTuGPG + 2H]^{2+}$ of intrapeptidal cross-linked (Lys-116 to Thr-118) lysozyme peptide (amino acids 115–125) at *m/z* 836.39 and (b) ESI-LTQ-Orbitrap-MS^3 product ion spectrum of the precursor ion $[M+PG + 2H]^{2+}$ at *m/z* 744.57 formed by CID in a ESI-LTQ-MS^3 experiment.

by CID in the LTQ yielding an almost complete y-type ion series of the modified lysozyme peptide (Fig. 3(a)).

An intriguing case is presented by lysozyme peptide comprising amino acids 115–125 (Fig. 4(a)). In the respective ESI-LTQ-Orbitrap mass spectrum a signal at *m/z* 836.39 was detected, formally corresponding to the doubly charged ion of an intrapeptidal cross-linked species. For this species, the only reactive site in addition to Lys-116 would be the threonine residue at position 118. It has been shown that NHS cross-linkers can also react with serines, threonines and tyrosines in addition to lysines and free N-termini, albeit with lower frequency.[64,65]

The precursor ion at *m/z* 836.39 was further analyzed in an MS^2 experiment (Fig. 4(a)). The $[M+PG + 2H]^{2+}$ ion at *m/z* 744.57 is formed by the characteristic CNL of 184 u (loss of BuTuG). For the same reasons as discussed for substance P we assume that the precursor ion at *m/z* 836.39 is representing the intrapeptidal cross-linked product of the lysozyme peptide containing amino acids 115–125.

An obvious drawback of the presented chemical cross-linker with its pronounced ability for collision-induced cleavage is certainly its sophisticated synthesis, which is time consuming and therefore costly. In addition, the elimination of remaining

ambiguities regarding the formation of dehydrated and partially hydrolyzed cross-linker peptide derivatives besides intrapeptidal cross-linked products motivated us to further refine this approach and to develop a second generation thiourea linker with advanced characteristics. The properties of a simplified cross-linker analogue of the NHS-BuTuGPG-NHS compound are currently under investigation.

Conclusions

The proposed dissociative chemical cross-linker incorporates a thiourea moiety, which contains highly nucleophilic sulfur for respective attack at the adjacent Gly-Pro amide carbonyl, efficiently initiating the cleavage of the linker molecule. The underlying reaction mechanism was examined and the resulting mass shifts and neutral losses were found to provide reliable information on the connectivity of the linker and the location of the respective covalent derivatization. This fundamental study shows that the concept of this dissociative chemical cross-linker has the potential to improve data evaluation of cross-linked proteins and peptides by detailed MS/MS and MS^3 studies. However, the synthesis of the presented cross-linker proved to be more challenging than expected. Therefore, we extended the thiourea-based concept for a dissociative cross-linker to a simplified analog, which is currently under investigation in our laboratories.

The novel dissociative chemical cross-linker safeguards for an effective cleavage in the low (5–100 eV) as well as in the high-energy regime (keV) of CID experiments. The special fragmentation of the linker molecule delivers indicative mass shifts and CNLs, enabling sensitive, selective and unambiguous detection by MRM. Moreover, our approach allows a facile automation for multi-sample unequivocal identification of cross-linked peptides even without the need for high accuracy mass analyzers (e.g. quadruple ion traps and triple quadrupole mass analyzers) as highly selective CNLs are utilized for the identification of cross-linked species. With all requirements of this complex analytical problem addressed the developed technique should raise interest to those who study protein–protein interactions using chemical cross-linking and have access to any mass spectrometer capable of MS/MS analysis.

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