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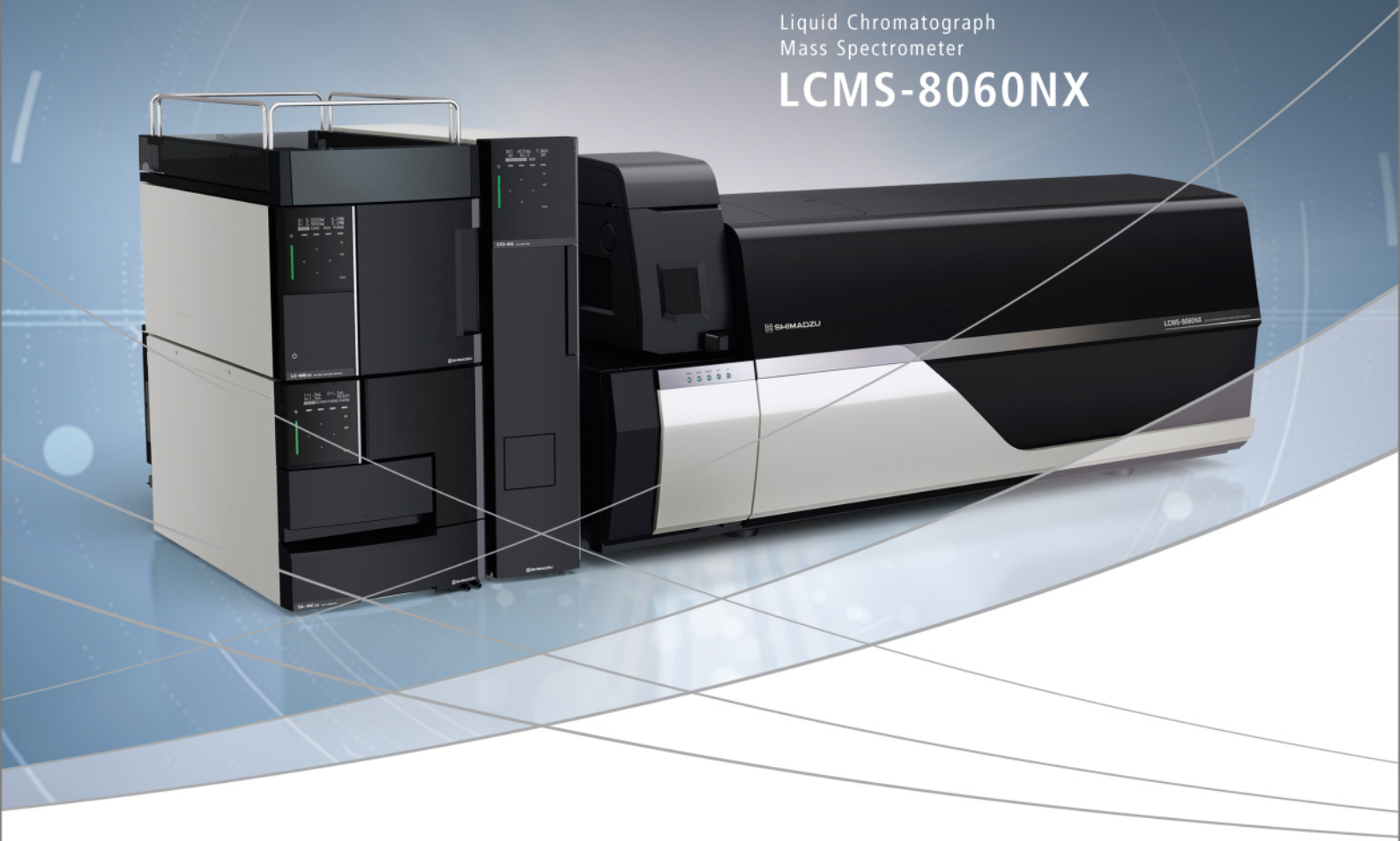

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APPLICATION NOTE

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A biuret-derived, MS-cleavable cross-linking reagent for protein structural analysis: A proof-of-principle study

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Abstract

Chemical cross-linking combined with mass spectrometry (XL-MS) and computational modeling has evolved as an alternative method to derive protein 3D structures and to map protein interaction networks. Special focus has been laid recently on the development and application of cross-linkers that are cleavable by collisional activation as they yield distinct signatures in tandem mass spectra. Building on our experiences with cross-linkers containing an MS-labile urea group, we now present the biuret-based, CID-MS/MS-cleavable cross-linker imidodicarbonyl diimidazole (IDDI) and demonstrate its applicability for protein cross-linking studies based on the four model peptides angiotensin II, MRFA, substance P, and thymopentin.

KEYWORDS

chemical cross-linking, cleavable cross-linkers, mass spectrometry, peptides, protein conformation

1 | INTRODUCTION

Chemical cross-linkers that are cleavable by collisional activation have impressively demonstrated their potential not only for determining protein 3D structures but also for mapping protein interaction networks in cell lysates, intact cells, organelles, and tissue.[1-4] Among the most prominent CID-MS/MS-cleavable cross-linkers are disuccinimidyl sulfoxide (DSSO)[5] and disuccinimidyl dibutyric urea (DSBU).[6] We have laid our focus on establishing a family of cross-linkers harboring a MS-labile urea group, namely, DSBU, diallyl urea (DAU),[7] and 1,1′-carbonyldiimidazole (CDI)[8] (Figure 1). These cross-linkers all exhibit distinct patterns upon collisional activation that greatly aid in the correct identification of cross-linked species, especially in in system-wide cross-linking experiments. The three homobifuncional cross-linkers differ in their reactivities as well as in their distance they are able to bridge. As such, they can yield complementary structural information on the 3D structures of proteins and protein assemblies. DSBU possesses two *N*-hydroxysuccinimide (NHS) esters as active sites that will

Abbreviations: CDI, 1,1′-carbonyldiimidazole; CID, collision-induced dissociation; DAU, diallyl urea; DSBU, disuccinimidyl dibutyric urea; DSSO, disuccinimidyl sulfoxide; ESI, electrospray ionization; HCD, higher-energy collision-induced dissociation; IDDI, 2-imidodicarbonyl diimidazole; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NHS, *N*-hydroxysuccinimide; THF, tetrahydrofuran; TRIS, tris (hydroxymethyl)methylamine; XL-MS, cross-linking/mass spectrometry

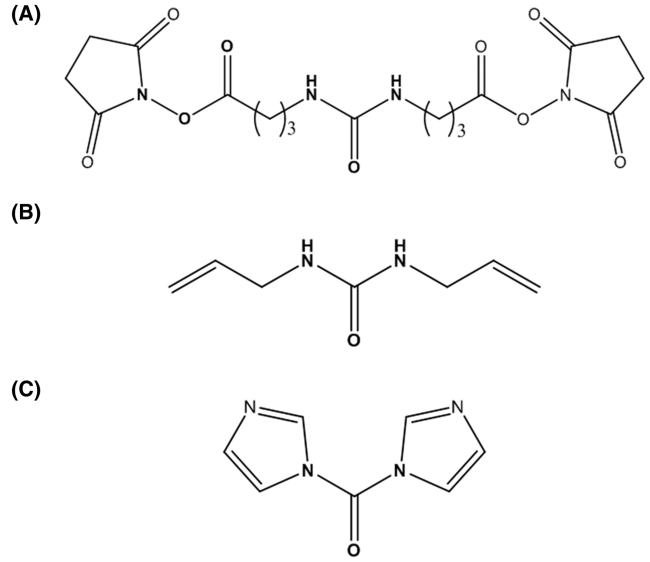

FIGURE 1 Comparison of three urea-based, MS-cleavable cross-linkers. (A) DSBU; (B) DAU; (C) CDI. The three cross-linkers exhibit complementary reactivities and distances (for details see text)

preferably react with amine groups in proteins, such as lysine residues or the proteins' N-termini. The maximum Cα-Cα distance to be bridged by DSBU is between approximately 26 to 30 Å.[9] In addition to DSBU, also shorter analogons with only two or three carbon atoms in the spacer connecting the two NHS ester groups are now commercially available.[10]

The DAU cross-linker reacts specifically with sulfhydryl SH groups in cysteine residues via a thiol-ene photo-reaction after a radical starter, such as benzophenone, has been applied.[7] The Cα-Cα distance DAU will brigde is ~9 Å. CDI is the shortest MS-cleavable cross-linker that can be perceived that will react via azolide-based coupling reactions with amines in lysine residues and with hydroxy groups in serines, threonines, and tyrosines.[8] One of the major advantages of CDI is that so-called "dead-end" cross-linked products are not stable and will therefore not further complicate the cross-linking mixture. In these "dead-end" cross-links, one site of the cross-linker has reacted with the protein, while the other reactive site has been hydrolyzed.

In this work, we present a novel, biuret-type cross-linker, termed imidodicarbonyl diimidazole (IDDI), that is cleavable upon collisional activation. In a proof-of-principle study, we demonstrate the successful application of the IDDI linker to four selected peptides.

2 | EXPERIMENTAL

2.1 | Chemicals and peptides

All chemicals and the peptides angiotensin II, the four-amino acid peptide composed of the amino acids methionine, arginine, phenylalanine, and alanine (MRFA), as well as substance P and thymopentin were purchased from Sigma-Aldrich.

2.2 | Synthesis of the IDDI linker

The IDDI linker was synthesized according to a described protocol[11] from imidazole and potassium cyanate (KNCO). The second reaction step (Figure 2A) was performed by adding 1.1 Eq of CDI in anhydrous pyridine and keeping the reaction mixture at 80°C for 5 hours. The educt dissolved slowly within approximately 3 hours. After removing nonreacted educt by filtration at room temperature, IDDI was precipitated with 10 Eq of neat THF within 24 hours. After washing three times with THF, IDDI was obtained as colorless, crystalline solid (yield approximately 50%). The identity of the compound was confirmed by HR-ESI-MS, and IDDI was used without further purification for the cross-linking reactions.

2.3 | Cross-linking reaction and MS/MS

The IDDI linker was dissolved in neat DMSO and added at 10M excess to a 50μM peptide solution in 20mM HEPES buffer, pH 8.0. The cross-linking reaction was quenched after 30 minutes with TRIS buffer (final concentration, 45mM). Peptide samples were desalted with C18-ZipTip pipette tips (Millipore). MS and MS/MS analysis was performed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific), equipped with nano-ESI source (Nanospray Flex Ion Source, Thermo Fisher Scientific). Gold-coated capillaries were prepared in-house. The capillary voltage was set to −1.1 kV, and the source temperature was kept at 275°C. MS data were collected from *m/z* 300 to 1500, or higher if necessary. For MS/MS measurements, ions were isolated in the quadrupole (isolation window, 2 Th), fragmented with higher-energy collision-induced dissociation (HCD; 20% normalized collision energy), and analyzed in the orbitrap mass analyzer (R = 120 000 at *m/z* 200) with external calibration (experimental error < 1 ppm).

2.4 | Identification of cross-linked products

Spectra were automatically annotated with MeroX, version 1.6.6.6.[12,13] All cross-links were manually validated. Mass deviations were set to 3 ppm (MS) and 10 ppm (MS/MS), the signal-to-noise ratio was ≥2. Lysines, serines, threonines, tyrosines, and the peptides' N-termini were considered as potential cross-linking sites.

3 | RESULTS AND DISCUSSION

The biuret-type IDDI linker is accessible at low costs from imidazole, CDI (0.30 €/g) and KNCO, and reacts with amine and hydroxy groups in amino acid side chains (Figure 2). Upon collisional activation, carbonyl-NH bonds are preferably cleaved resulting in distinct mass modifications of the connected peptides, as indicated by four characteristic mass doublets of ~43 u and ~26 u, respectively.

We sought to demonstrate the concept of our novel IDDI linker using the four model peptides angiotensin II, MRFA, substance P, and thymopentin. Cross-linked dipeptides were generated with the

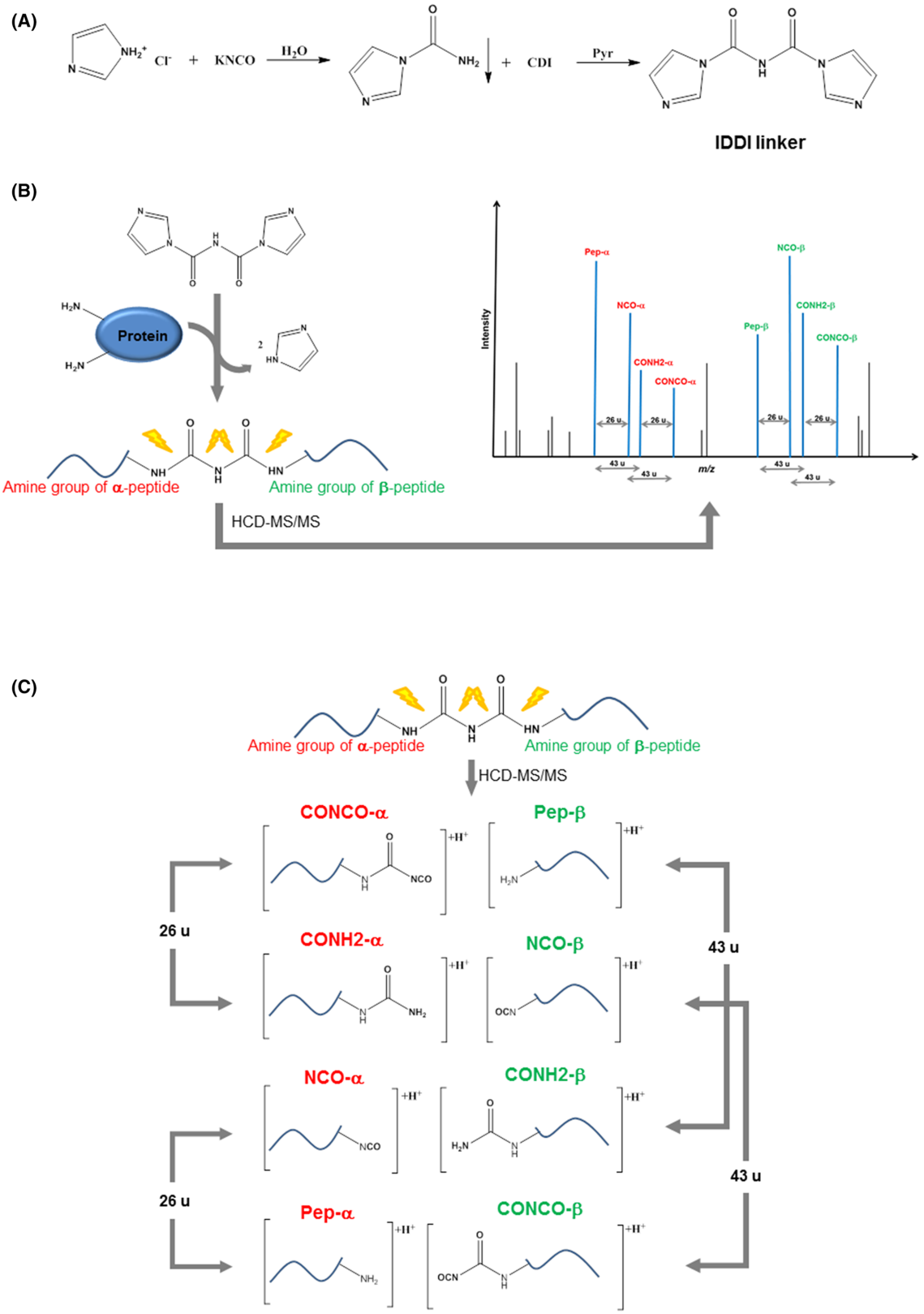

FIGURE 2 Synthesis and cleavage upon collisional activation of the biuret-type IDDI linker. (A) The IDDI linker is synthesized from imidazole, CDI, and KNCO. (B) Cross-linked products of IDDI with amine and hydroxy groups in proteins can be discriminated based on their characteristic mass doublets of approximately 43 u and approximately 26 u; cleavage sites are indicated with arrows. The cross-linking reaction is exemplified for lysine side chains containing an ε-amine group. (C) Upon collisional activation, four characteristic fragment ions are created from each of the two cross-linked peptides [Colour figure can be viewed at wileyonlinelibrary.com]

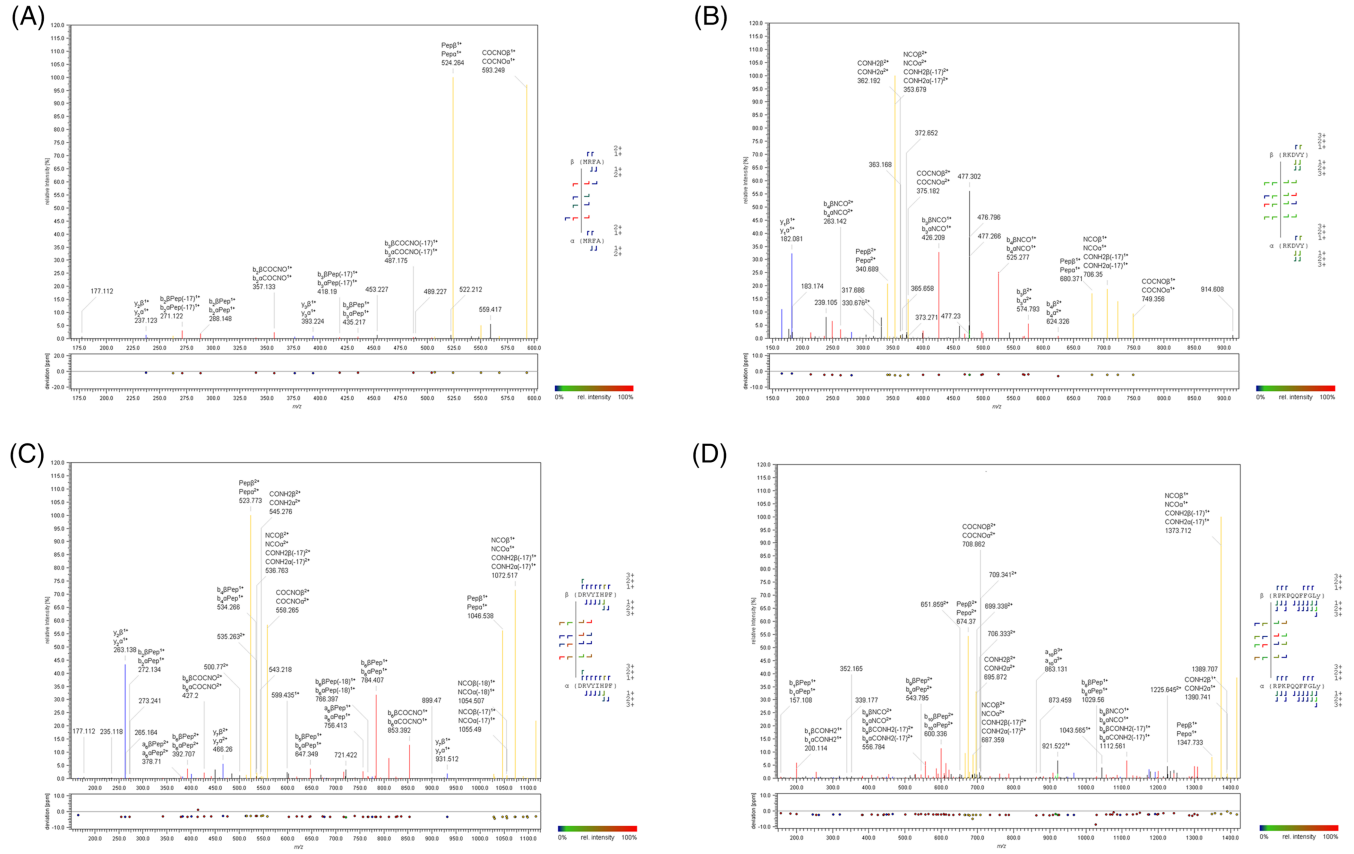

FIGURE 3 Product ion mass spectra of cross-linked products from the peptides (A) MRFA, (B) thymopentin, (C) angiotensin II, and (D) substance P. Cross-linked products were fragmented by HCD-MS/MS (normalized collision energy 20) and automatically annotated by the MeroX software. (A) Interpeptide cross-link of MRFA, $[M+2H]^{2+}$ at *m/z* 558.75. (B) Interpeptide cross-link of thymopentin, $[M+3H]^{3+}$ at *m/z* 476.9. (C) Interpeptide cross-link of angiotensin II, $[M+3H]^{3+}$ at *m/z* 721.0. (D) Interpeptide cross-link of substance P, $[M+3H]^{3+}$ at *m/z* 921.8. The amidated C-terminus of substance P is annotated as "y." The characteristic fragment ions of the IDDI linker are shown in yellow, b-type ions are shown in blue, y-type ions are presented in red. The IDDI linker fragments (see Figure 2C) are annotated in MeroX as "Pep", "NCO", "CONH2", and "COCNO" [Colour figure can be viewed at wileyonlinelibrary.com]

N-terminus being the preferred reaction site for all four peptides, as shown in Figure 3.

Data analysis can easily be performed with the MeroX software [12, 13, 14] to guarantee a fully automated assignment of the characteristic doublet fragmentation patterns of IDDI in the product ion mass spectra. An automated analysis of cross-linked products is a prerequisite for a future application of the IDDI linker to more complex samples, such as large protein assemblies or even whole cell lysates.

Our initial results demonstrate the feasibility of our approach: The four cross-linked peptides used in this study fragmented under collisional activation as predicted (Figure 3). HCD-MS/MS data reveal the presence of the 43-u and 26-u doublets as well as fragmentation of the peptide backbone (b- and y-type ions). This not only allows deriving information about the amino acid sequences of the connected peptides but can also be used pinpointing the connected amino acids. This information is readily available from tandem MS experiments, eliminating the need to perform MS^3 experiments. MS/MS data show the complete fragmentation patterns of the IDDI linker, with all four labile bonds being cleaved upon collisional activation as predicted (Figure 2B). The MeroX software annotates fragment ions characteristic of the cross-linker (Figure 3, signals marked in yellow) directly in the product ion mass spectra. On top of that, MeroX gives a schematic representation of fragmentation sites, detailed information on the fragment ions' charge states, their intensities, and mass deviations (Figure 3, right hand side and bottom panel).[12] This confirms that the MeroX software can handle the fragmentation patterns generated by the IDDI linker, which will form the basis of a fully automated analysis of more complex protein samples.

4 | CONCLUSIONS

In a proof-of-concept study, we demonstrate the concept of the novel, biuret-type MS-cleavable IDDI linker. The IDDI linker potentially reacts with amine and hydroxy groups in amino acids side chains and bridges Cα-Cα distances of approximately 18 Å, making it an ideal tool for protein 3D-structural studies. The cleavability of the IDDI linker, resulting in the appearance of four characteristic signals upon collisional activation, is the key to its future application for complex protein systems. Data analysis can readily be performed with the

MeroX software, which will enable the design of fully automated workflows based on the unique properties of the IDDI linker.

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