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Synthesis of CID-cleavable protein crosslinking agents containing quaternary amines for structural mass spectrometry†

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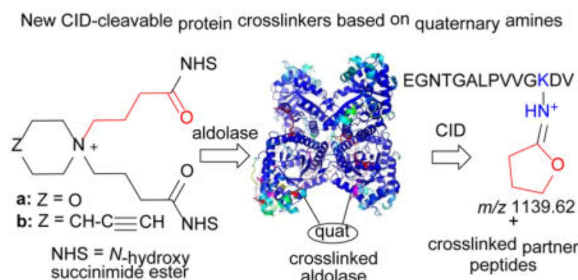
Abstract

Two novel cyclic quaternary amine crosslinking probes are synthesized for structural mass spectrometry of protein complexes in solution and for analysis of protein interactions in organellar and whole cell extracts. Each exhibits high aqueous solubility, excellent protein crosslinking efficiencies, low collision induced dissociation (CID) energy fragmentation efficiencies, high stoichiometries of reaction, increased charges of crosslinked peptide ions, and maintenance of overall surface charge balance of crosslinked proteins.

Graphical abstract

New CID-cleavable protein crosslinkers based on quaternary amines



Chemical crosslinking coupled with tandem mass spectrometry (CXL-MS) has been increasingly applied to structural analysis of protein complexes in solution[1–13] and to global analysis of protein interactions in organelles and whole cells[14–17]. This is because in the

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Conflicts of interest
There are no conflicts to declare.

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former case, CXL-MS can provide distance constraints and other information that aids determination of the structures of proteins recalcitrant to classical methods such as X-ray crystallography, NMR, and other techniques. Computational tools are available to support these efforts, which integrate disparate sources of structural information[2, 4, 5, 18–22]. In the latter case, crosslinking represents an effective way to globally monitor proteins in intact organelles and in living cells with the potential to directly probe changes in protein interactions *in vivo.*

Classical "bottom-up" approaches to CXL-MS using non-fragmenting crosslinkers, where crosslinked proteins are digested to smaller peptides for MS analysis, suffer from two disadvantages (Fig. 1) arising from the resistance of the crosslink bonds to collision-induced dissociation (CID). The first is that the larger size of the crosslinked analytes and the increased degrees of freedom result in lower fragmentation efficiencies, particularly at lower energies. The second is that the two crosslinked peptides fragment simultaneously, leading to a complex mixture of fragment ions whose search space is N^2, where N is the total number of calculated proteolytic peptides[23, 24]. This large search space increases computational time and has a negative impact on the false discovery rate (FDR) making them challenging for complex mixtures.

An effective approach to alleviating these limitations has been the development of crosslinkers that have lower fragmentation energies than the peptide bonds[25–36]. In this approach, collision induced dissociation (CID) of the crosslinked peptides in tandem mass spectrometry (MS2 in Fig. 1) results in scission of the crosslinker, generating a characteristic marker for the crosslinked species that can be used to trigger selection and CID of each component peptide for subsequent identification (MS3 in Fig. 1). Alternative data acquisition schema using CID-cleavable crosslinkers can also be employed (e.g., alternating low and high collision energies, higher-energy collision dissociation (HCD) in an Orbitrap, etc.). This uncoupling of the identification of crosslinked ions from the identification of the component peptides reduces the search space dramatically to 2N from N^2, with consequential improvements in the FDR and the complexity of samples that can be readily analyzed. Incorporation of affinity tags into cleavable crosslinkers provides enrichment capabilities advantageous for complex mixtures[21, 37].

We report the synthesis and initial characterization of two novel CID-cleavable protein crosslinkers exhibiting excellent protein crosslinking and low energy fragmentation efficiencies (Fig. 2A). The first, compound **1a**, possesses a central morpholino group and is shorter than our previous crosslinkers[33], having an extended conformation length of approximately 13 angstroms. The second, compound **1b**[§], has a central 4-(ethynyl)piperidinyl moiety that is amenable to enrichment by Click chemistry[38–40]. Crosslinkers **1a** and **1b** are also referred to by the abbreviated names MC4 and PAC4, respectively. Alkyne **1b** has some structural similarity to alkyne-A-DSBSO[41] (Fig. 2B), but is shorter and designed to be highly water soluble. Additionally, it undergoes MS-cleavage by a completely different mechanism.

The synthesis of each crosslinker is delineated in Scheme 1. Key structural features of each probe include a cyclic nitrogen heterocycle core flanked by two identical side chains, each

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made up of a 3-carbon methylene spacer connecting the quaternary nitrogen of the core to an *N*-hydroxysuccinimide (NHS) ester "warhead". The route developed for **1a** commenced with the alkylation of morpholine (**2a**), using a variation of a patent procedure[42], with ethyl 4-bromobutyrate in refluxing toluene to provide **3a**. Attempted tandem alkylation with excess bromo ester was sluggish and necessitated that installation of the second arm be carried out in a separate step. After considerable experimentation, we found that reaction of **3a** with the more reactive ethyl 4-iodobutyrate[43] occurred slowly at 115 °C under neat conditions to provide the key bis-quaternary ammonium salt ester **4a**. Subsequent acid hydrolysis with concentrated HCl in acetic acid proceeded slowly, but cleanly, at 100 °C to provide diacid **5a** as a mixed chloride/iodide salt. Activation of this to the bis-NHS ester was accomplished by mild heating of **5a** with excess *N,N′*-disuccinimidyl carbonate and pyridine in acetonitrile following a literature procedure[44], whereas reaction in neat pyridine left only starting material. Unreacted starting diacid **5a** was filtered away from **1a**, which was then precipitated by slow addition of crude product, dissolved in minimal acetonitrile, into a large excess of cold ethyl acetate. The overall yield of **1a** was 31% in a four-step sequence of reactions.

Having completed the synthesis of **1a**, we then set out to apply our reaction methodologies to the construction of congeneric crosslinker **1b**. However, the substitution of morpholine for 4-ethynylpiperidine as the core heterocycle had a profound impact on the chemistry, necessitating a partial redesign of our route (Scheme 1). The major modification required swapping the ethyl ester of **4a** with the *tert*-butyl ester of **4b** due to the instability of the alkyne moiety to the vigorous acid hydrolysis conditions of **4a**. Second, we found that the piperidine nitrogen of **2c** was less reactive than that of morpholine, necessitating the use of *tert*-butyl-4-iodobutanoate[45] for both alkylation reactions. Thus, room temperature reaction of 4-ethynylpiperidine hydrochloride[46] (**2c**) with a slight excess of *tert*-butyl 4-iodobutyrate in acetonitrile under basic conditions over a three-day period gave **3b** in good yield. Further transformation to **4b** with excess iodo ester proved problematic, due to an unexpected putative fragmentation of the nascent bis-ester product during its formation in refluxing acetonitrile. We believe this is driven by the presence of the quaternary center as a control run shows that **3b** is stable under these conditions. After investigation of several alternative conditions (temperature, solvent), we eventually were able to generate **4b** in modest yield as a sharp-melting white solid by stirring **3b** and the iodo ester at room temperature as a very concentrated solution in acetonitrile over a 13-day period. Mild, non-aqueous hydrolysis of **4b** then proceeded uneventfully with anhydrous HCl in *p*-dioxane at room temperature to cleanly give diacid **5b** as a mixed chloride/iodide salt. Hydrolysis with excess trifluoroacetic acid in dichloromethane[47] worked also, but resulted in an oily, less pure product. Further conversion of **5b** to target crosslinker **1b** was effected under similar conditions described for the synthesis of **1a**. The overall yield of **1b** was 10% in a four-step sequence of reactions, with the lower yield reflecting problematic reactions relative to **1a**. We feel that the overall yield of both crosslinkers can be substantially improved through a modest route optimization campaign.

All compounds were rigorously purified by flash chromatography or crystallization, and their structural assignments are supported by diagnostic peaks in the ^{1}H NMR spectra and

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by mass spectrometry. Digital copies of these along with full experimental procedures can be found in Supplementary Information.

Both **1a** and **1b** are efficient crosslinkers, exhibiting essentially stoichiometric crosslinking of the tetramer (Fig. 3) at a 1:5 molar ratio of lysyl residue: crosslinker with no significant increase in higher order complexes up to 1:100. Low energy CID of peptides crosslinked with **1a** or **1b** results in fragmentation on either side of the central cyclic amine (morpholino or 4-(alkynyl)piperidinyl) giving characteristic pairs of doublets, where each doublet corresponds to one of the crosslinked peptides (Fig. 4). Fragmentation efficiencies are excellent but the relative intensities of the doublets can vary depending on peptide sequence and the charge state of the precursor. Identification of the peptides can be accomplished by various acquisition protocols, including mass tag-triggered MS3, alternating low and high energy MS2, and HCD.

In summary, we have synthesized two novel quaternary cyclic amine probes that exhibit high aqueous solubility and which, in initial studies coupled with tandem mass spectrometry, exhibit excellent protein crosslinking and low energy fragmentation efficiencies. Future studies will be directed to the application of these crosslinkers to structural mass spectrometry of protein complexes and the dynamics of protein interactions *in vitro* and *in vivo*. We consider the core structure common to **1a** and **1b**, which confers the major properties listed above, to be a useful template for the development of additional crosslinking and tagging reagents having a range of functionality at the Z-position of the cyclic moiety. Our establishment of precise synthetic methodologies applicable to these will facilitate these studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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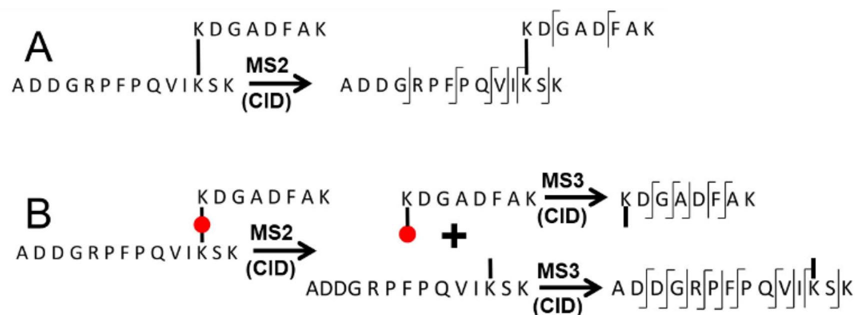

Figure 1.

Fragmentation schema for Lys-directed non-cleavable (A) and cleavable (B) crosslinkers. Tryptic peptide examples are for illustration only. Increased backbone fragmentation typically observed for linear peptides is indicated. Left spurs on hashes represent observed “B-ions” and right spurs are “Y-ions”. The red circle represents the CID-cleavable crosslinking moiety. Only two of the four possible products are shown for MS3 in B.

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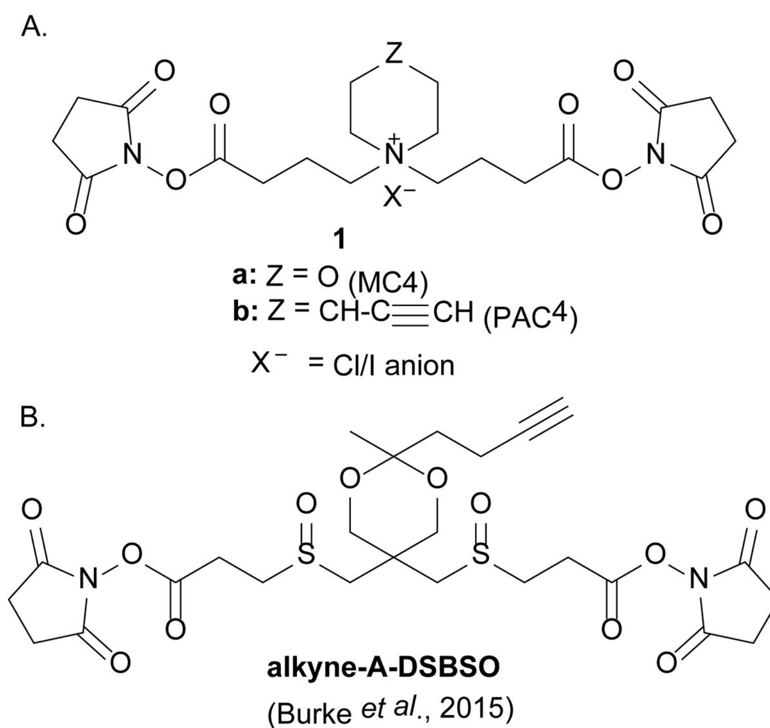

Figure 2.

A. Structures of quaternary amine crosslinkers **1a** (MC4) and **1b** (PAC4); **B.** Structure of sulfoxide crosslinker alkyne-A-DSBSO.

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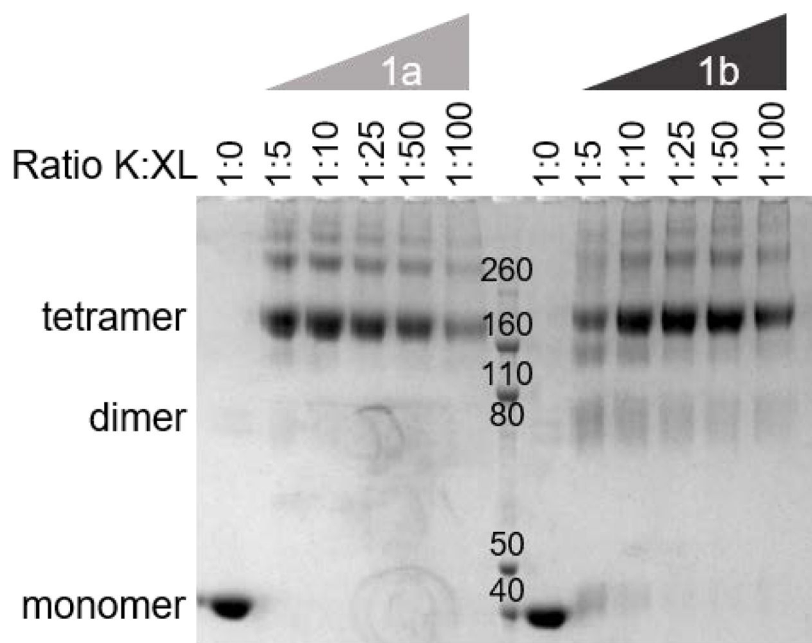

Figure 3.

Titration of CID cleavable crosslinkers **1a** and **1b**for aldolase. Coomassie Brilliant Blue stained NuPAGE Bis-Tris (4% – 12%) gel of crosslinked samples after 15 min of reaction at room temperature (25 C) with an increasing ratio of Lys-residue/crosslinker (K:XL). Peptide standards are indicated in the center lane.

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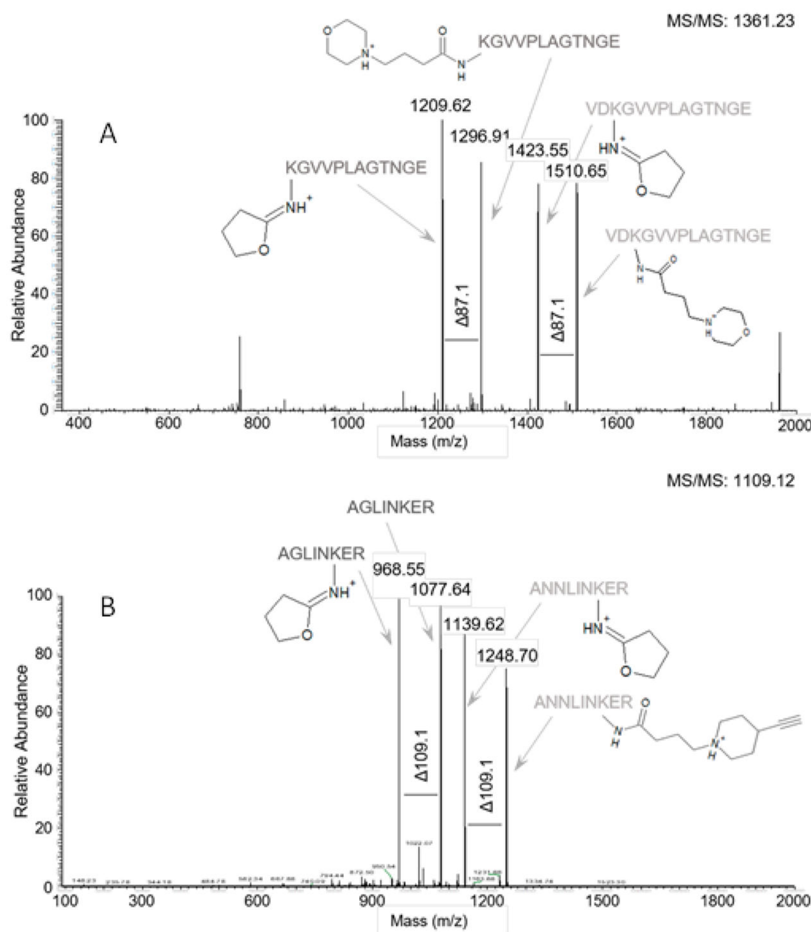

Figure 4.

CID fragmentation of a proteolytic digest product of (A) **1a** crosslinked inter subunit peptides from aldolase (KGVVPLAGTNGE-XL-VDKGVVPLAGTNGE showing the mass difference marker of 87.11), (B) **1b** crosslinked synthetic peptide (AGLINKER-XL-ANNLINKER with the mass difference marker of 109.15 indicated). XL is the crosslinker. The lower mass ion in each doublet is typically selected for MS3.

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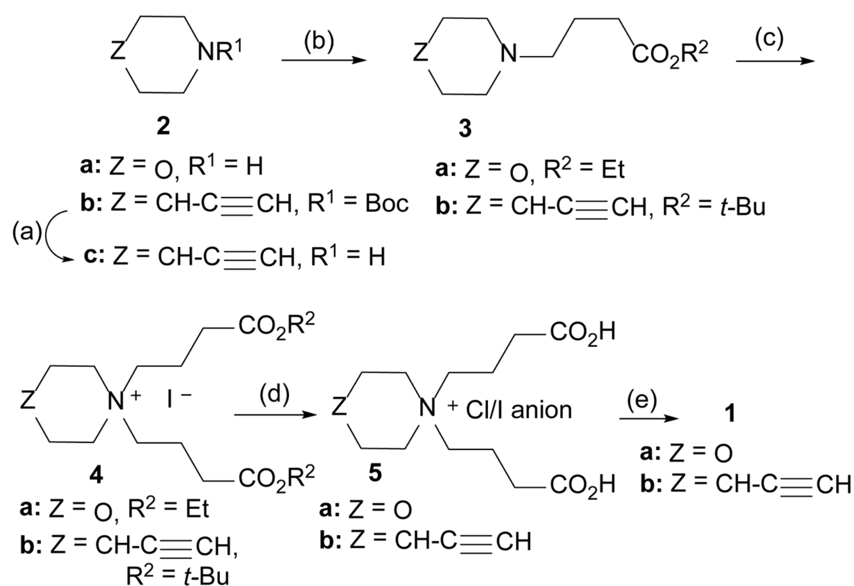

Scheme 1.

Reagents and conditions: (a) HCl in *p*-dioxane, RT, 30 min, ~100%. (b) for **3a**: ethyl 4-bromobutanoate, toluene, 120°C, 2 h, 65%; for **3b**: *t*-Bu 4-iodobutanoate, K2CO3, ACN, RT, 3 d, 80%. (c) for **4a**: neat ethyl 4-iodobutanoate, 115°C, 16 h, 95%; for **4b**: *t*-Bu 4-iodobutanoate, ACN, RT, 13 d, 50%. (d) for **5a**: conc. HCl, gla. HOAc, 100°C, 2 d, 91%; for **5b**: HCl in *p*-dioxane, DCM, RT, 41 h, 67%. (e) *N*, *N*′-disuccinimidyl carbonate, pyridine, ACN, 50 – 60°C, 16 – 64 h, 37 – 64%.