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Synthesis of Two New Enrichable and MS-Cleavable Cross-linkers to Define Protein-Protein Interactions by Mass Spectrometry

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KEYWORDS: proteomics, sulfoxide, lysine-reactive, cross-linker, NHS ester, CID-cleavable

ABSTRACT: The cross-linking Mass Spectrometry (XL-MS) technique extracts structural information from protein complexes without requiring highly purified samples, crystallinity, or large amounts of material. However, there are challenges to applying the technique to protein complexes *in vitro*, and those challenges become more daunting with *in vivo* experiments. Issues include effective detection and identification of cross-linked peptides from complex mixtures. While MS-cleavable cross-linkers facilitate the sequencing and identification of cross-linked peptides, enrichable cross-linkers increase their detectability by allowing their separation from non-cross-linked peptides prior to MS analysis. Although a number of cross-linkers with single functionality have been developed in recent years, an ideal reagent would incorporate both capabilities for XL-MS studies. Therefore, two new cross-linkers have been designed and prepared that incorporate an azide (azide-A-DSBSO) or alkyne (alkyne-A-DSBSO) to enable affinity purification strategies based on click chemistry. The integration of an acid cleavage site next to the enrichment handle allows easy recovery of cross-linked products during affinity purification. In addition, these sulfoxide containing cross-linking reagents possess robust MS-cleavable bonds to facilitate fast and easy identification of cross-linked peptides using MS analysis. Optimized, gram-scale syntheses of these cross-linkers have been developed and the azide-A-DSBSO cross-linker has been evaluated with peptides and proteins to demonstrate its utility in XL-MS analysis.

Most proteins act in association with other proteins to form protein complexes stably or transiently in cells, and mapping these interactions is essential to understand their cellular functions. Protein complexes represent functional entities that are often difficult to analyze using conventional structural tools due to their heterogeneous and dynamic nature. Recently, cross-linking Mass Spectrometry (XL-MS) has been recognized as a valuable tool for the structural analysis of protein assemblies,[1] which can be used alone and in combination with other techniques.[1,2] In addition to *in vitro* studies, XL-MS ap-

proaches have been extended to capture protein interactions in living cells.[3] Identification of cross-linked peptides by MS analysis can provide distance constraints to assist computational modeling and yield structural information at amino acid resolution.[4] The advantages of cross-linking studies include small sample size, robust tolerance for size and environment of the protein complex, instrument accessibility, and the speed of handling and data collection. Although successful, inherent limitations in current XL-MS strategies require further developments to enable MS detection and identification of cross-linked peptides with better efficiency, accuracy, sensitivity and speed. Among various approaches to improve existing XL-MS workflow,[5] developing new cross-linking reagents holds the greatest promise towards the ultimate goal of mapping protein-protein interactions in living cells at the systems level. We report the chemical synthesis of two new cross-linking agents whose effectiveness has recently been demonstrated for *in vivo* protein-protein analysis.[6]

Unambiguous identification of cross-linked peptides can be greatly facilitated by the introduction of a MS cleavable bond in a cross-linking reagent, which can fragment during collision induced dissociation (CID) prior to peptide backbone breakage.[7] Previously, we have successfully developed a new class of robust MS-cleavable reagents that contain labile C-S sulfoxide bonds (e.g. DSSO (DiSuccinimidyl-SulfOxide), Figure 1), and thus enables fast and accurate identification of cross-linked peptides using liquid chromatography-multistage tandem mass spectrometry analysis (LC/MS^n).[8,9] With DSSO as an example, this new XL-MS workflow involves protein DSSO cross-linking, trypsin digestion of cross-linked proteins, and LC/MS^n analysis of resulting peptide mixtures. During MS^n analysis, the cross-linked peptides are first detected in MS^1 and selected for subsequent MS^2 analysis. The CID-fragmentation site, i.e. one of the C–S sulfoxide bonds, is selectively fragmented in MS^2, allowing the physical separation of the two DSSO cross-linked peptide constituents for subsequent sequencing. The resulting peptide fragments in MS^2 are then analyzed in MS^3 for unambiguous identification. The integration of these three types of MS data (MS^1, MS^2, MS^3) enables simplified analysis of DSSO cross-linked peptides with improved speed and accuracy. This strategy has been demonstrated to be effective in the structural analysis of purified protein complexes *in vitro*.[4]

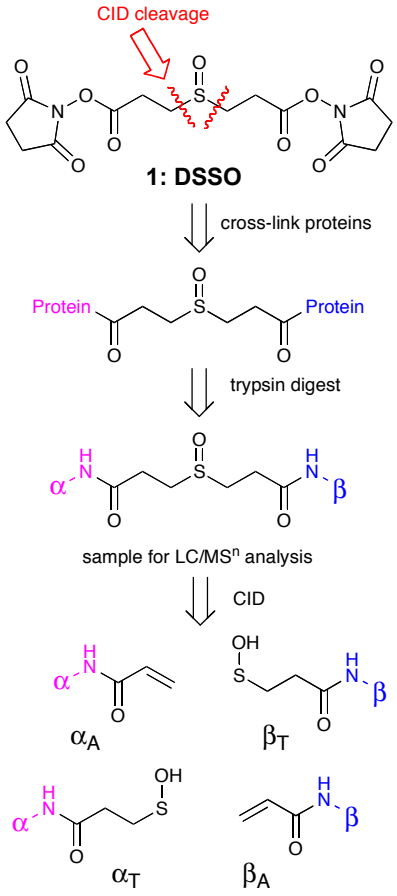
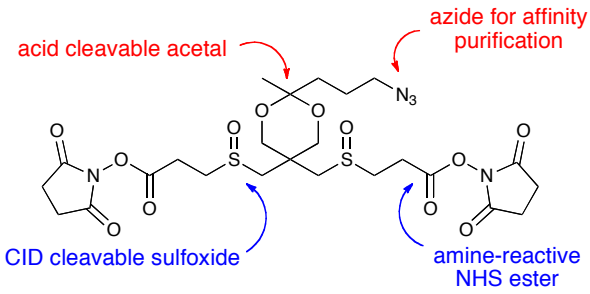


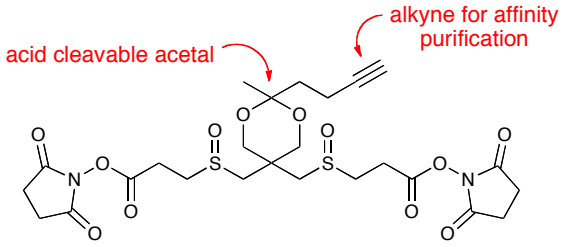
Figure 1. CID cleavable cross-linker DSSO is based on the sulfoxide functional group. The red arrow points to the bond that is broken during the CID process. The workflow for cross-linking proteins is shown. After protein cross-linking, trypsin digest generates the cross-linked peptide for LC/MS^n analysis. CID leads to selective cleavage of the bonds adjacent to the sulfoxide functional group.

The analytical problem with effectively detecting and identifying cross-linked peptides becomes much more daunting with large, complex protein assemblies and especially when studying protein-protein interactions in living cells. A strategy to improve the sensitivity and efficiency of XL-MS analysis is to incorporate an affinity purification handle into the cross-linker itself. To this end, we have developed an azide-tagged cross-linking reagent that allows the incorporation of an affinity purification handle based on click chemistry for enriching cross-linked peptides prior to MS analysis, thus improving their detection and identification.[4a] In comparison to other enrichment handles incorporated in cross-linking reagents,[10] the azide group is advantageous as it is small and bioorthogonal, and click chemistry has been proven effective in enriching biological samples for various proteomic analyses including cross-linking studies.[11] In order to combine these unique features in a multifunctional cross-linking reagent that can advance current XL-MS workflow for studying protein-protein interactions *in vivo* as well as *in vitro*, we

have developed a new class of low molecular weight, membrane permeable, enrichable and MS-cleavable cross-linkers.



3: azide-A-DSBSO



4: alkyne-A-DSBSO

Figure 2. Protein cross-linkers designed with CID cleavable sulfoxide groups, azide (**3**) or alkyne (**4**) groups for clickable enrichment strategies, and an acid labile acetal to facilitate affinity purification.

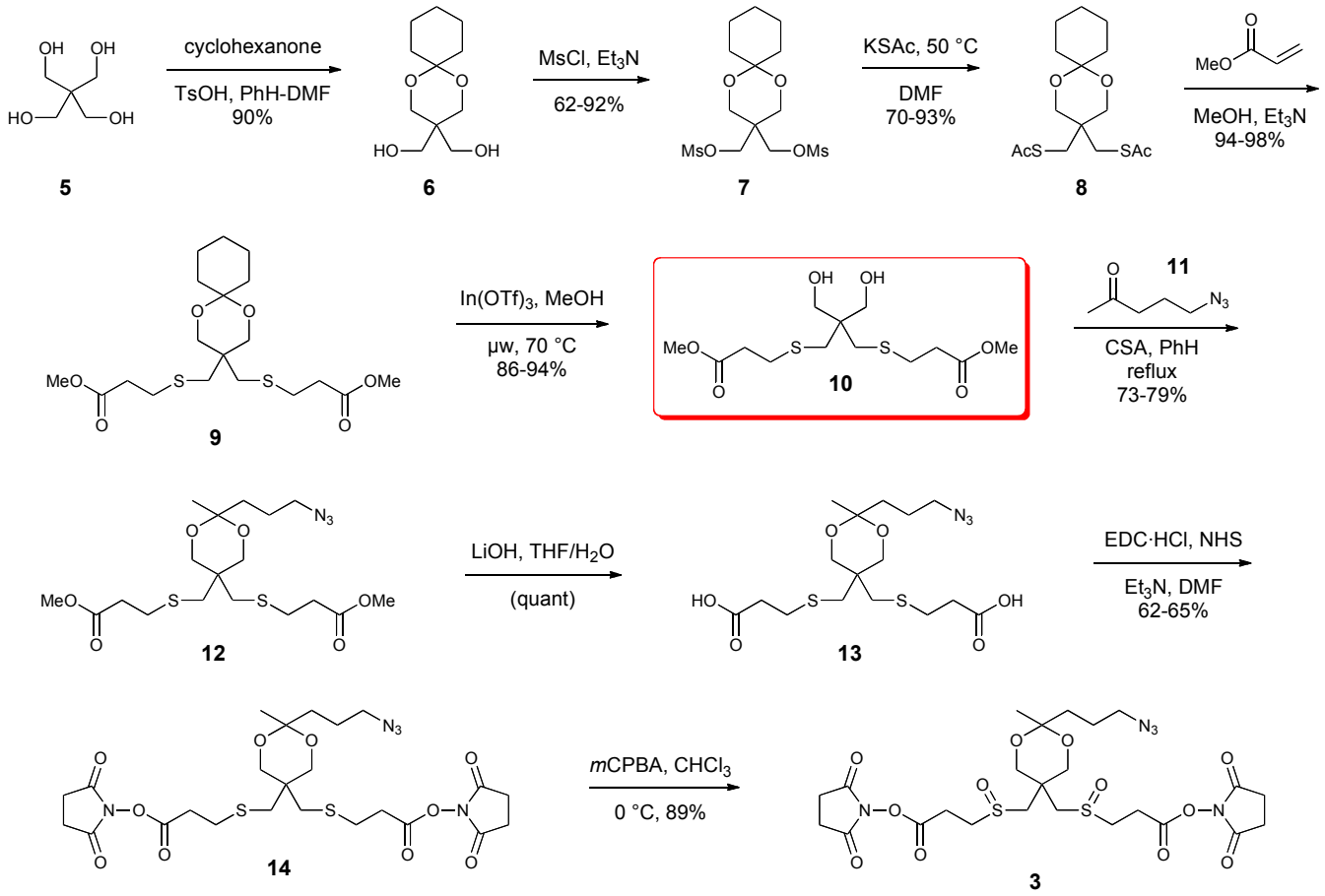
In order to explore the flexibility of using azide-alkyne click chemistry in the XL-MS workflow, we have designed and synthesized two new cross-linkers, i.e. azide-tagged and an alkyne-tagged reagents as presented in Figure 2. The structure of Azide-A-DSBSO (Azide-tagged, Acid-cleavable DiSuccinimidyl-BisSulfOxide) **3** incorporates a number of important design elements. The *N*-hydroxysuccinimidyl (NHS) esters are designed to react with lysine side chains thus cross-linking the proteins in the complex. The sulfoxide groups provide MS-cleavable bonds, and because only one side of each sulfoxide has β-hydrogen atoms, the elimination must take place regioselectively at the outer C–S bond. The design incorporates an azide functional group to be used in click reactions with strained alkynes or in a copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC).[12] The click and CuAAC reactions enable several strategies for affinity purification, including direct coupling with alkyne or azide-functionalized beads or by linking with common affinity ligands such as biotin.[11] Finally, the azide portion of the molecule is joined to the cross-linker with an acid labile acetal bond, which can be cleaved under aqueous acidic conditions to facilitate selective elution from an affinity column. The same elements are incorporated into the alkyne-A-DSBSO (Alkyne-tagged, Acid-cleavable DiSuccinimidyl-BisSulfOxide) **4**, except

that the azide functional group has been exchanged for the complementary alkyne. One other design feature is that both of these cross-linkers, prior to the introduction of the sulfoxides, are achiral and exist as single stereoisomers. This feature offers considerable simplification in the preparation and analysis of the synthetic intermediates, and decreases the chance of any stereoselective behavior in the crosslinking environment. These reagents have been under investigation for several years in our program and their applications in mapping protein-protein interactions at the systems level in living cells were recently described.[6] In this report, the syntheses of these reagents are described in full along with foundational studies on the cross-linking effectiveness and LC/MS^n sequencing.

Results and Discussion

Synthesis of the cross-linkers 3 and 4

The original synthesis of Azide-A-DSBSO **3** began with pentaerythritol (**5**) and is presented in Scheme 1. Selective protection of **5** as cyclohexanone acetal **6** represented an improvement of the literature procedure.[13] Mesylation followed by displacement with potassium thioacetate produced the bis-thioacetate **8** in good overall yield. All three intermediates, **6**-**8**, were crystalline solids that were isolated and purified on multi-gram scale without chromatography. Thioacetate methanolysis and Michael addition of **8** into methyl acrylate was accomplished in a single step in the presence of triethylamine and methanol. The key intermediate **9** was isolated in nearly quantitative yield and used without further purification. Careful hydrolysis of the cyclohexanone acetal using $In(OTf)_3$ catalysis produced 86% of the diol **10** and 8% recovered starting material, which were separated by chromatography. Partial hydrolysis of the acetal was a recurring problem in this step when using Brønsted or Lewis acids in refluxing solvent, which required isolation and recycling of a small amount of the recovered acetal **9**. By carefully optimizing the catalyst quantity, time, and temperature in microwave reaction conditions the conversion to diol **10** could be pushed to completion with isolated yields of ~94%. Diol **10** is a key intermediate in the synthesis, and it is the branch point for the preparation of the azide cross-linker **3** and the alkyne cross-linker **4**. It was prepared in five steps following this route in 64% overall yield with only one chromatographic purification.[14]

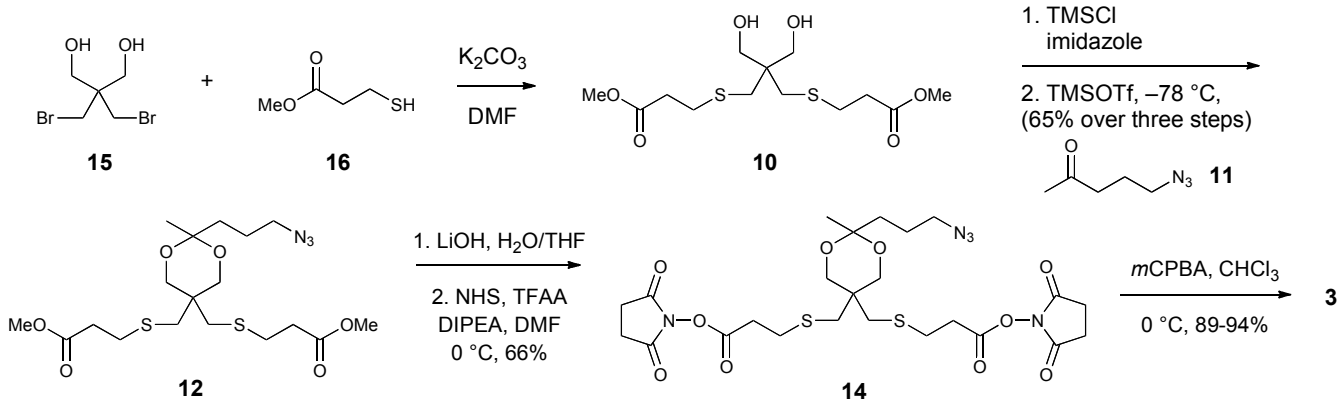


Scheme 1. Synthesis of azide-A-DSBSO (**3**) from pentaerythritol (**5**)

The remainder of the synthesis of azide cross-linker **3** is outlined in Scheme 1. The diol **10** was reacted with 5-azidopentan-2-one (**11**)[15] with acid catalysis using a Dean-Stark trap to remove water. The acetal **12** was isolated in 77% yield by chromatography. The NHS esters were introduced in two steps: hydrolysis of the methyl ester and coupling with *N*-hydroxysuccinimide using EDC•HCl in dimethylformamide (DMF). The overall yield was typically around 60% and the bis-NHS ester **14** was isolated by chromatography. The final oxidation was carried out with *meta*-chloroperoxybenzoic acid (*m*-CPBA); the oxidant was added in aliquots and conversion was monitored by ESI-MS until the starting material and monosulfoxide were no longer present. Azide cross-linker **3** was isolated by extraction. This route produced several grams of the cross-linker **3** in ca. 18% overall yield for the sequence.[14]

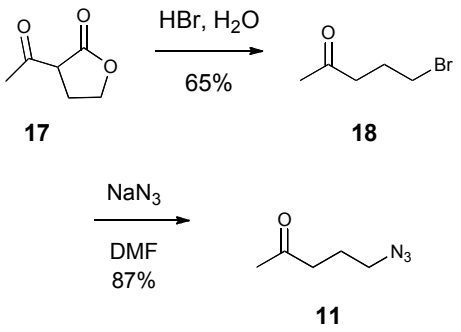
In the course of our *in vivo* studies,[6,11] we found that the azide **3** crossed the membrane and produced cross-links in targeted protein complexes.[6] The studies required a large excess of cross-linker, and led to an ongoing demand for more material. Although the original optimized synthesis in Scheme 1 was effective, it did require nine steps. A shorter route was developed that incorporated several im-

provements in the individual transformations and avoided the use of protecting groups. The new route is presented in Scheme 2.



Scheme 2. Improved synthesis of azide-A-DSBSO (**3**) beginning with 2,2-bis(bromomethyl)propane-1,3-diol (**17**).

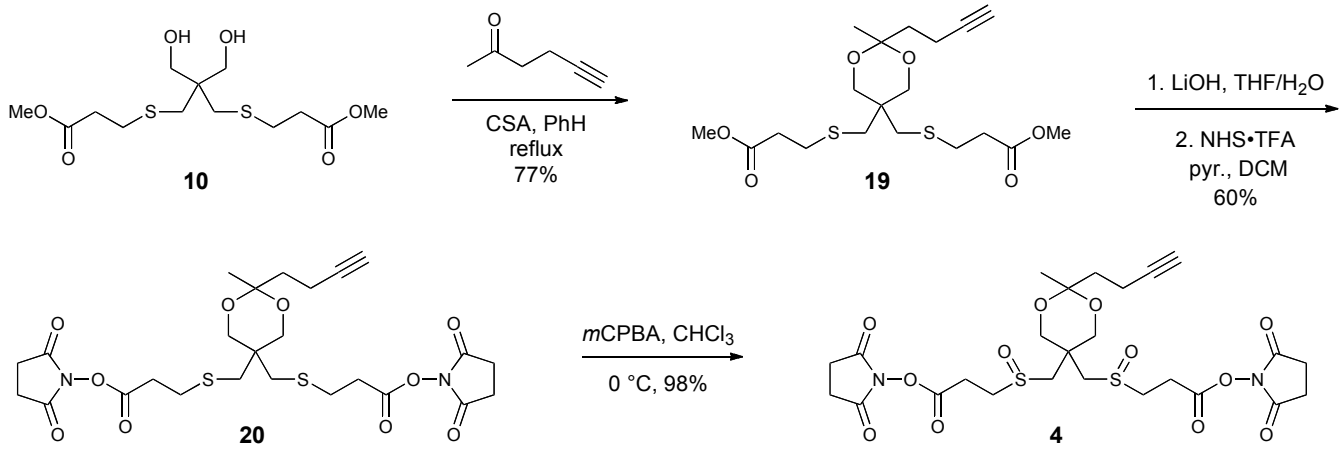
The new route begins with the commercially available and inexpensive dibromide **15** and thiol **16**. Direct alkylation with K_2CO_3 in DMF generated the key intermediate **10** in a single step. Diol **10** could be purified by chromatography on silica gel to produce 75% of pure **10**, but the crude product was carried on in the sequence. By comparison to the original route, this method eliminates four steps in the sequence. The acetal synthesis was carried out using the Noyori protocol,[16] which was found to be more reliable than the original acid-catalyzed method. Diol **10** was silylated and then combined with ketone **11** in the presence of TMSOTf to give acetal **12** in 65% overall yield. Hydrolysis of the dimethyl ester **12** used LiOH as before. The bis-NHS ester **14** was prepared using in situ generated TFA-NHS,[17] which lead to similar overall yields but shorter reaction times, fewer side products, and a more reliable purification. Finally, oxidation to the bis-sulfoxide as previously described gave azide **3**. The new route requires only six steps, three chromatographic purifications, and led to an overall yield of 38%.[14] It is more convenient and reliable than the prior route and has been used to produce multiple grams of azide-A-DSBSO **3**.



Scheme 3. Improved synthesis of 5-azidopentan-2-one (**11**)

The 5-azidoentan-2-one was initially prepared by the alkylation of commercially available bromide **18** with NaN_3.[15] The very high cost of bromide **18** led us to develop a more economical approach starting with lactone **17** (Scheme 3). The lactone **17** was treated with HBr to generate the required 5-bromopentan-2-one (**18**). The standard displacement with sodium azide gave the desired ketone **11** in good overall yield. Scaling up the synthesis of azide-A-DSBSO **3** required a significant quantity of the volatile azide **11**, and the starting with lactone **17** was both effective and economical.

The alkyne **4** was prepared from diol **10** using a very similar route (Scheme 4). The acetal **19** was formed from 5-hexyn-2-one and the diol under Dean-Stark conditions. The dimethyl ester was hydrolyzed to a diacid using LiOH, and the di-NHS ester was prepared using TFA-NHS reagent.[17] Di-NHS ester **20** was isolated in 60% yield using this method. The same compound was also prepared using an EDCI coupling, but the yield was lower and the purification was more difficult. The *m*-CPBA oxidation was conducted as described for the azide substrate to give the Alkyne-A-DSBSO **4**. The route required only four steps from diol **10** and made cross-linker **4** available on gram scale.



Scheme 4. Synthesis of alkyne-A-DSBSO (**4**) from diol **10**

MS^n analysis of Azide-A-DSBSO cross-linked Ac-myelin peptide

Given the similarity of cleavable C–S bonds in azide-A-DSBSO and DSSO, we anticipated that azide-A-DSBSO cross-linked peptides would display comparable fragmentation characteristics to DSSO cross-linked peptides (Figure 3A).[3a] In such experiments, MS^2 produces peptide fragments that are modified with remnant portions of the cross-linking reagents. These remnants are not identical thus producing two products with a separation of 254 Da, the α_A and the α_T fragments. The α_A fragment has an alkene group, while the other half of the cleaved linker results in a terminal thiol group (after hydrolysis of the sulfenic acid intermediate). Although azide-A-DSBSO contains four C–S bonds due to the presence of two sulfoxide groups, the two central C–S bonds cannot undergo fragmentation due to the lack of β-hydrogens. Therefore, only the two C–S bonds closer to cross-linked residues are expected to fragment during MS^2. To examine MS^2 fragmentation patterns of azide-A-DSBSO cross-linked peptides during MS^n analysis, we have first cross-linked and analyzed model peptide Ac-myelin. MS^1 analysis detected azide-A-DSBSO cross-linked Ac-myelin (α-α) homodimer at four different charge states (m/z 501.41^{6+}, 601.45^{5+}, 751.62^{4+}, 1001.82^{3+}) (Figure 3B). MS^2 analyses of inter-linked Ac-myelin homodimer at different charge states yielded the expected fragmentation of two identical inter-linked peptides, i.e. a characteristic fragment pair (α_A/α_T). As an example, the fragment pair α_A/α_T detected in MS/MS spectra of the quadruply (m/z 751.62^{4+}) and sextuply (m/z 501.41^{6+}) charged inter-linked Ac-myelin (α-α) was displayed in Figure 3C and 3D, respectively. The results demonstrate that the MS-cleavable C–S bonds in azide-A-DSBSO cross-linked peptides are preferentially fragmented during MS^2 analysis prior to the breakage of peptide backbones. Similar results were observed with Alkyne-A-DSBSO cross-linked Ac-myelin peptide (data not shown) as Azide-A-DSBSO and Alkene-A-DSBSO are almost identical in structures.

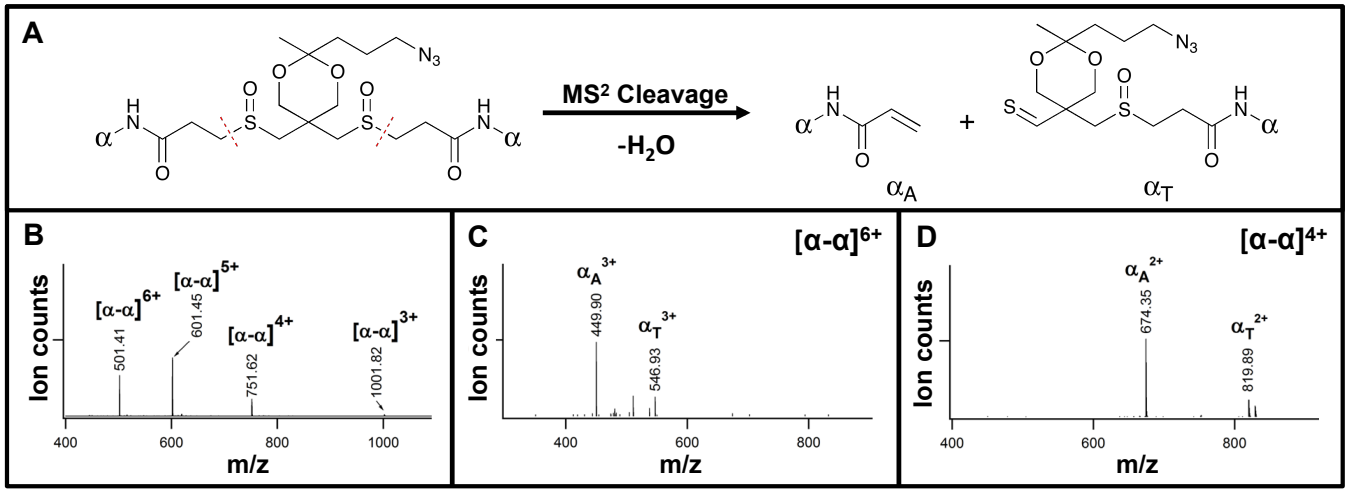


Figure 3. MS^n analysis of Azide-A-DSBSO cross-linked Ac-Myelin synthetic peptide. **(A).** Schematic illustration of MS^2 analysis of a Azide-A-DSBSO interlinked homodimeric peptide (α-α). During collision-induced dissociation in MS^2, cleavage of either of the two symmetric MS-cleavable C-S bonds leads to physical separation of the two inter-linked peptides, thus generating pairs of peptide fragments: i.e. α_A and α_T. **(B)** The interlinked Ac-Myelin was detected as multiple charged ions (m/z 1001.82^{3+}, m/z 751.62^{4+}, m/z 601.45^{5+}, m/z 501.41^{6+}) in MS^1. **(C-D)** MS^2 spectra of interlinked Ac-Myelin at two different charge states: **(C)** [α-α]$^{4+}$ and **(D)** [α-α]$^{6+}$. As shown, two predicted peptide fragment pairs were observed as $\alpha_T^{2+}/\beta_A^{2+}$ (m/z 674.35^{2+}/819.89^{2+}) and $\alpha_T^{3+}/\beta_A^{3+}$ (449.90^{3+}/546.93^{3+}) in (C) and (D) respectively.

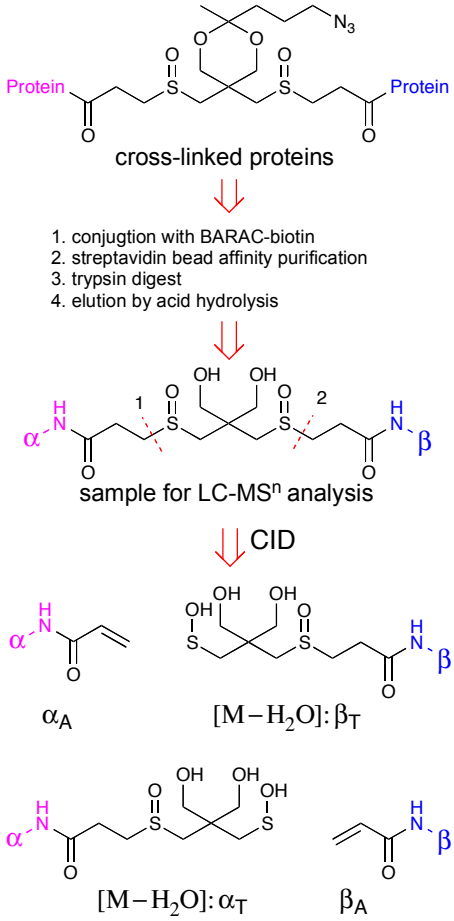


Figure 4. Work flow for affinity purification of cross-linked cytochrome C proteins. The MS^2 fragments resulting from CID cleavage sites are shown.

LC/MS^n analysis of DSBSO cross-linked peptides of cytochrome C after enrichment

To demonstrate the applicability of azide-A-DSBSO for XL-MS studies, we have cross-linked model protein cytochrome C with azide-A-DSBSO. Cytochrome C has been used extensively by us and other groups for evaluating cross-linking reagents because it is a small protein with a high number of lysine residues. Given its success in the past for cross-linking studies,[2d,18] we decided to use it as the model protein for characterizing our new cross-linking reagent. The resulting cross-linked cytochrome C products were conjugated with BARAC-biotin,[19] affinity purified by binding to Streptavidin beads, and digested with trypsin. The cross-linked peptides were eluted from the beads with acid, and thus became acid-cleaved products of azide-A-DSBSO cross-linked peptides, i.e. DSBSO cross-linked peptides, which were then subjected to LC/MS^n analysis. The general workflow and the structure of the cross-linked peptides leading up to LC/MS^n analysis are illustrated in Figure 4. As illustrated, the acid-cleaved products are the final analytes for LC/MS^n analysis. It is noted that the acid-cleaved products of azide-A-DSBSO and alkyne-A-DSBSO cross-linked peptides are the same, because the differentiated group is lost during the acid elution of cross-linked peptides from affinity matrix during enrichment. Since the cleavable C–S bonds in DSBSO are similar to those in DSSO, the general data analysis workflow for the identification of DSBSO cross-linked peptides by LC/MS^n is similar to the analysis of DSSO cross-linked peptides.[4] There are three types of cross-linked peptides, i.e. dead-end, intra-linked, and inter-linked peptides. Among them, inter-linked peptides provide most informative structural details for defining protein-protein interaction interfaces. Therefore, we are most interested in identifying inter-linked peptides between the same and/or different proteins. As an example, Figure 5 describes a representative MS^n analysis of a DSBSO inter-linked cytochrome C peptide (α-β) that was detected as a quadruply charged ion (m/z, 510.0166^{4+}). As shown, MS^2 analysis resulted in two pairs of peptide fragments (i.e. α_A/β_T or α_T/β_A), characteristic fragmentation of inter-linked heterodimeric peptide. Subsequent MS^3 analysis of MS^2 fragment α_A (m/z 430.75^{2+}) and β_A (m/z 489.28^{2+}) determined their sequences as

K_AYIPGTK and M(ox)IFAGIK$_A$K respectively, in which K_A is modified with the alkene moiety. Integration of MS^1, MS^2, and MS^3 results has unambiguously determined this DSBSO cross-linked cytochrome C peptide as [^{74}KYIPGTK^{80} inter-linked to ^{81}M(ox)IFAGIKK^{88}], in which a cross-link was formed between K74 and K87 in cytochrome C.

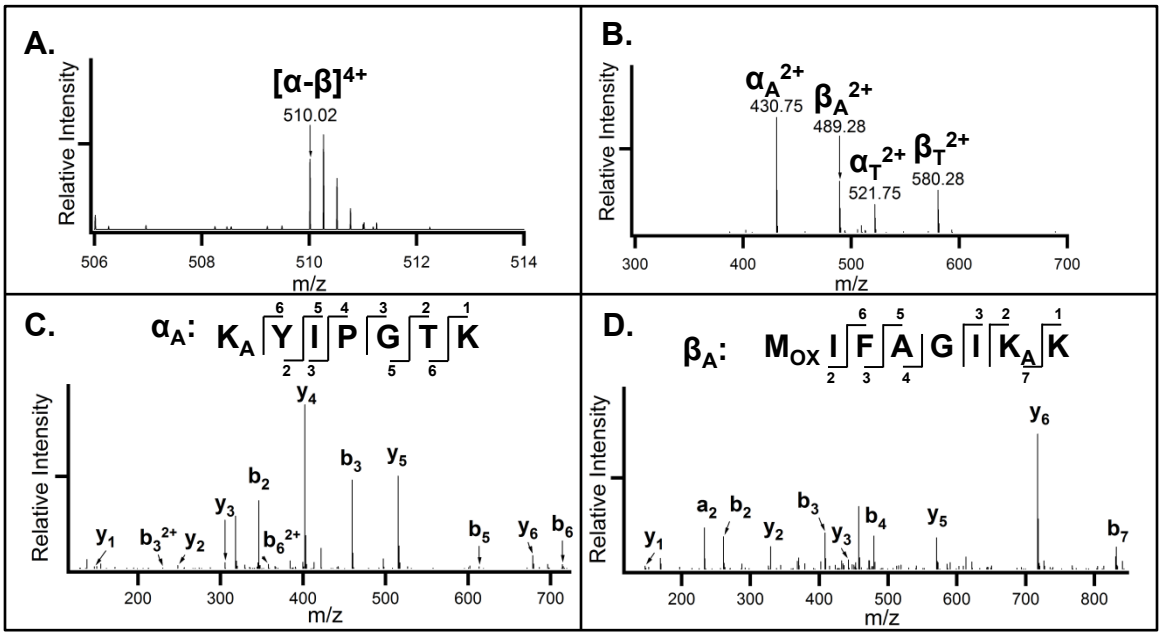


Figure 5. MS^n Analysis of a representative DSBSO **3** Inter-Linked Peptide (α-β) of Cytochrome C (m/z 510.02^{4+}). A) MS^1 and B) MS^2 spectra of the selected peptide. In MS^2 spectrum, two pairs of peptide fragments: i.e. α_A/β_T (m/z 430.75^{2+}/580.28^{2+}) and α_T/β_A(521.75^{2+}/489.28^{2+}) were detected. Note: α_A, β_A, and K_A are alkene modified species (+54 Da); α_T and β_T are unsaturated thiol modified species (+236 Da). C-D) Respective MS^3 spectra of α_A (m/z 430.75^{2+}) and β_A (489.28^{2+}) fragments detected in MS^2. The detection of a series of y and b ions has unambiguously identified their sequences as K_AYIPGTK and MoxIFAGIK$_A$K respectively. Mox: oxidized methionine.

In total, LC/MS^n analysis of enriched cross-linked cytochrome C identified 7 unique inter-linked peptides (Supplemental Table 1). In addition, 11 unique dead-end and 5 unique intra-linked cytochrome C peptides were identified since all types of cross-linked peptides can be selectively enriched (data not shown). The results are comparable to those obtained using DSSO cross-linking,[4] demonstrating the effectiveness of Azide-A-DSBSO based XL-MS strategy. Although it is not necessary to enrich cross-linked peptides for simple proteins like cytochrome C, it is evident that such a process is essential for mapping protein interaction interfaces at the systems level.[6]

Conclusion

Full experimental details are presented for the preparation of two CID-cleavable lysine cross-linkers, an azide (azide-A-DSBSO) and an alkyne (alkyne-A-DSBSO), along with initial characterization using a model peptide and a model protein. The syntheses are not trivial, but the optimized procedures reported herein make these useful compounds available on multigram scale. The azide and alkyne functional groups are suitable for click enrichment strategies. The cross-linkers described here have been utilized in mammalian HEK-293 cells[6] and to facilitate the study of the interaction of subunits in the proteasome complex, which is responsible for degradation of ubiquitin tagged proteins.[6] The importance of developing XL-MS reagents that are applicable for *in vivo* studies is significant because protein-protein interactions are involved in most cell function and are not well understood. We hope that this report detailing the synthesis of these cross-linking agents will enable others to prepare and utilize them to study protein-protein interactions.

Methods

See Supporting Information for experimental details for the synthesis of each cross-linking reagent.

SUPPORTING INFORMATION.

General synthetic methods, experimental data, and spectral data are presented in pdf format. This material is available free of charge via the Internet at http://pubs.acs.org.

ACKNOWLEDGMENT

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ABBREVIATIONS

Ac, acetate; Alkyne-A-DSBSO, Alkyne-tagged, Acid-cleavable DiSuccinimidyl-BisSulfOxide; Azide-A-DSBSO, Azide-tagged, Acid-cleavable DiSuccinimidyl-BisSulfOxide; BARAC, biarylazacyclooctynone; CID, collision-induced dissociation; CSA, camphorsulfonic acid; CuAAC, copper (I) catalyzed azide-alkyne cycloaddition; DIPEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; DSSO, DiSuccinimidylSulfOxide; EDC·HCl, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride; Et_3N, tri-

ethylamine; HB, His-Bio; ESI-MS, electrospray ionization-mass spectrometry; $In(OTf)_3$, indium(III) trifluoromethanesulfonate; $K_AYIPGTK$, lysine(alkene modified)-tyrosine-isoleucine-proline-glycine-threonine-lysine peptide; LC/MS, liquid chromatography-mass spectrometry; MeOH, methanol; $M(ox)IFAGIK_AK$, methionine(oxidized)-isoleucine-phenylalanine-alanine-glycine-isoleucine-lysine(alkene modified)-lysine; *m*-CPBA, *meta*-chloroperoxybenzoic acid; MsCl, methanesulfonyl chloride; NHS, N-hydroxysuccinimidyl; Ph-H, benzene; pyr., pyridine; QTAX, quantitative analysis of tandem affinity purified *in vivo* cross-linked (X) protein complexes; TFAA, trifluoroacetic anhydride; TFA-NHS, *N*-trifluoroacetoxy succinimide; THF, tetrahydrofuran; TMSCl, trimethylsilyl chloride; TMSOTf, trimethylsilyl trifluoromethanesulfonate; TsOH, *para*-toluenesulfonic acid; XL-MS, cross-linking mass spectrometry; μw, microwave.

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