MS-Cleavable Crosslinkers

Catalog Number A33545, A35459

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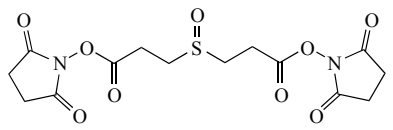
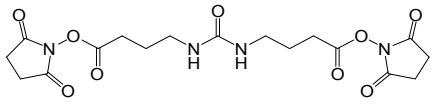
Product description

Disuccinimidyl sulfoxide (DSSO) and disuccinimidyl dibutyric acid (DSBU or BuUrBu) are MS-cleavable crosslinkers containing an amine-reactive *N*-hydroxysuccinimide (NHS) ester at each end of a 7-atom and 11-atom carbon spacer arm, respectively (Kao, 2011; Müller, 2010). NHS esters react efficiently with primary amine groups (-NH_2) in pH 7-9 buffers to form stable amide bonds. DSSO and DSBU have similar reactivity compared to DSS, but contain linkers (e.g., sulfoxide or urea) that can be cleaved in the gas phase during tandem mass spectrometry (MS/MS) using collision-induced dissociation (CID). The ability to specifically cleave crosslinked peptides during MS/MS enables targeted MS3 acquisition methods, which facilitate peptide sequencing using traditional database search engines. The cleavage of DSSO and DSBU during MS/MS also generates distinguishing ion doublets that enable searching using database search engines such as MeroX™ or XlinkX™ (Liu, 2015; Müller, 2010).

Chemical crosslinking, in combination with MS, is a powerful method to determine protein-protein interactions (Kao, 2011; Müller, 2010; Arlt, 2016). This method has been applied to recombinant and native protein complexes and to whole cell lysates or intact unicellular organisms in efforts to identify protein-protein interactions on a global scale. Both traditional non-cleavable and MS-cleavable crosslinkers can be used for identification of protein-protein interaction sites, but MS-cleavable crosslinkers are advantageous because of their ability to generate distinguishing ions using different types of gas phase fragmentation methods (e.g., CID, HCD, ETD and EThcD) and levels of tandem MS (e.g., MS2 and MS3) to improve identification of crosslinked peptides.

Contents

Contents	Amount	Storage
DSSO (disuccinimidyl sulfoxide) MW = 388.35 Spacer Arm = 10.3 Å 	10 × 1 mg	Store at 4°C protected from moisture.
DSBU (disuccinimidyl dibutyric urea, BuUrBu) MW = 426.38 Spacer Arm = 12.5 Å 		

Additional information

- Crosslinkers are moisture-sensitive. To avoid moisture condensation onto the product, equilibrate vial to room temperature before opening. Prepare these crosslinkers immediately before use. The NHS-ester moiety readily hydrolyzes to become non-reactive; therefore, do not prepare stock solutions for storage. Discard any unused reconstituted crosslinker.
- Hydrolysis of the NHS ester competes with crosslinking primary amines of proteins/peptides (acylation). Acylation is favored near neutral pH (pH 6–9) and with concentrated protein solutions.
- Use amine-free buffers at pH 7-9 such as PBS (100 mM sodium phosphate, 150 mM NaCl, pH 7.4, Product No. 28372); 20 mM HEPES; 100 mM carbonate/biocarbonate; or 50 mM borate. Do not use buffers that contain primary amines such as Tris or glycine, which compete with acylation.
- Dissolve DSSO and DSBU in a dry, water-miscible organic solvent, such as DMSO and DMF, before diluting to < 10% solvent in final aqueous reaction buffer. DSSO and DSBU may precipitate if added directly to aqueous buffer at concentrations > 30 mM.

Materials required but not supplied

- Dimethylsulfoxide (DMSO, Product No. 20688)
- HEPES Buffer, 20 mM, pH 7.5
- Ammonium bicarbonate (NH_4HCO_3) or Tris Buffer, 1 M, pH 8.0
- Thermo Scientific™ Pierce™ Mass Spec Sample Prep Kit for Cultured Cells (Product No. 84840) or In-Gel Trypsin Digestion Kit (Product No. 89871)

Crosslink *in vitro* proteins

This protocol is designed to result in sufficient crosslinking to facilitate subsequent MS analysis, but not disturb the tertiary structure of the protein from excessive crosslinking. Maintaining a protein concentration in the micromolar range during the reaction reduces unwanted intermolecular crosslinking between proteins.

1. Dissolve the protein in 490 µL of 20 mM HEPES buffer (pH 7.5) at 10 µM.
2. For DSSO, prepare a 50 mM stock solution of crosslinker by dissolving 1 mg DSSO in 51.5 µL of DMSO or DMF. For DSBU, prepare a 50 mM stock solution of crosslinker by dissolving 1 mg DSBU in 46.9 µL of DMSO or DMF.
3. Add 10 µL of crosslinker solution to the protein for a 100-fold molar excess of crosslinker over the protein concentration.

   Note: It may be necessary to titrate the final crosslinker concentration to determine the optimal molar excess for protein crosslinking. Typically, a 20-fold molar excess is required to observe minimal crosslinking, but up to 300-fold may be necessary to fully crosslink some protein complexes.
4. Prepare a control sample containing the protein without crosslinking reagent using 10 µL of DMSO in place of the crosslinker.
5. Incubate samples at room temperature for 30-60 minutes.
6. Terminate the reaction by adding 1 M NH_4HCO_3 or Tris Buffer to a final concentration of 20 mM to each sample.
7. Proceed to "Analyze crosslinked proteins using mass spectrometry" on page 2.

Crosslink intra- and extra-cellular proteins

Crosslinking can be performed on cells in suspension or on adherent cells in culture plates. In the latter situation, diffusion of the crosslinking reagent to all cell surfaces will be limited and will occur predominantly on the exposed surface. Culture media must be washed from the cells otherwise amine-containing components will quench the reaction. Using a more concentrated cell suspension is most effective as less reagent will be required in the reaction.

1. Remove media from 10^7 cells and wash twice with ice cold PBS.
2. Prepare a 50 mM stock solution of DSSO or DSBU by dissolving 1 mg in 51.5 µL or 46.9 µL of DMSO, respectively.
3. Add a final crosslinker concentration (diluted in PBS) of 5-10 mM to the cells.
4. Incubate the reaction at room temperature for 10 minutes or on ice for 30 minutes.
5. Stop the reaction by adding 1 M NH_4HCO_3 or Tris Buffer to a final concentration of 20 mM.
6. Incubate the reaction at room temperature for 5 minutes or on ice for 15 minutes.
7. Remove excess non-reacted crosslinker from cells and rinse twice with PBS.
8. Harvest and lyse cells for analysis of crosslinked proteins.

Analyze crosslinked proteins using mass spectrometry

It is recommended to evaluate the extent of crosslinking by 1-D SDS-PAGE. The occurrence of crosslinking identified at this stage establishes the optimal condition for the different crosslinking reagents. Stain gels with MS-compatible coomassie stains such as Thermo Scientific™ GelCode™ Blue Stain Reagent (Product No. 24590) or Imperial™ Protein Stain (Product No. 24615) or MS-compatible silver stains such as Pierce™ Silver Stain for Mass Spectrometry (Product No. 24600).

1. For crosslinked protein complexes separated by SDS-PAGE, excise the gel band relating to the crosslinked complex and prepare it for in-gel tryptic digestion (In-Gel Trypsin Digestion Kit, Product No. 89817). Alternatively, separate the reaction mixture by size-exclusion chromatography and digest the peak corresponding to the crosslinked complex using an in-solution method (e.g., Pierce™ Mass Spec Sample Prep Kit for Cultured Cells (Product No. 84840).

   Note: Although in-gel digestion produces successful analyses, in-solution digestion is faster, less prone to sample loss, and more efficient. Crosslinked protein fractionation and in-solution digestion is highly recommended for analysis of crosslinked peptides from complex samples derived from cells and tissues.
2. Separate and analyze the resulting peptide mixture containing inter- and intra-peptide crosslinking products by liquid chromatography-mass spectrometry (LC-MS).
3. Detect the resulting inter- and intra-molecularly crosslinked peptides in the mass spectra by their distinct doublet pattern observed during CID MS/MS and peptide mass difference.

   Note: Peptides will have mass shifts in both their fully crosslinked (Mip, Mi) (Table 1) and partially hydrolyzed forms (MOH, MNH_2, MTris) (Table 2). After fragmentation, each peptide will have one or more mass shifts based on the site of crosslinker cleavage (Table 3).

Table 1 Mass modifications associated with crosslinked peptides.

Reagent	Modification	Inter-peptide crosslink (Mip)	Intra-peptide crosslink (Mi)
DSSO	C6 H6 O3 S	158.0038 amu	158.0038 amu
DSBU	C9 H14 O3	196.0848 amu	196.0848 amu

Table 2 Mass modifications associated with crosslinker monoadducts.

Reagent	Monolink water quench (MOH)	Monolink ammonia quench (MNH_2)	Monolink Tris quench (MTris)
DSSO	176.0143 amu	175.0303 amu	279.0777 amu
DSBU	214.0954 amu	213.1113 amu	317.1587 amu

Table 3 Mass modifications associated with crosslinked peptide cleavage products.

Reagent	Cleavage mode	Modification	Mass addition
DSSO	CID/HCD	C3 H2 O	54.01056 amu
		C3 H4 O2 S	103.9932 amu
		C3 H2 O S	85.982635 amu
DSBU	CID/HCD	C4 H7 N O	85.05276 amu
		C5 H5 N O2	111.0320 amu

Analyze crosslinked protein data

Commercially available or free bioinformatics software packages can analyze complex mixtures of crosslinked peptides. Public domain packages include:

- ExPASy Proteomics Tool in the Swiss-Prot Database 'FindPept' at **expasy.org**
- Protein Prospector at **prospector.ucsf.edu/prospector**
- StavroX and MeroX at **stavrox.com** are free software tools for analysis of spectra from traditional and MS-cleavable crosslinkers, respectively. (Müller, 2010; Arlt, 2016).
- XlinkX at **xlinkx.hecklab.com** is a dedicated algorithm that searches disulfide-bridged and crosslinked peptides. XlinkX software is also available as a node in Thermo Scientific™ Proteome Discoverer™ 2.2 Software. XlinkX operates in two searching modes, enumeration mode and linear-peptide mode. In enumeration mode, the crosslink is searched by considering all possible peptide-peptide combinations for the crosslink precursor mass. As the database size enormously increases with the number of peptides in the database, it is recommended to use a database containing less than 50 proteins. In the linear-peptide mode, the crosslinked peptide is searched by obtaining the precursor mass of each linked peptide based on the signature fragmentation pattern of the crosslink; therefore able to search against proteome databases (Liu, 2015).

Related products

Product	Cat. no.
BupH™ Phosphate Buffered Saline Packs	28372
DSS (disuccinimidyl suberate)	21658
BS3 (bis[sulfosuccinimidyl] suberate)	21585
BS^3-d_0 (bis[sulfosuccinimidyl] suberate-d_0	21590
BS^3-d_4 (bis[sulfosuccinimidyl] 2,2,7,7 suberate-d_4	21595
DSG (disuccinimidyl glutarate)	20593
BS^2G-d_0 (bis[sulfosuccinimidyl] glutarate-d_0)	21610
BS^2G-d_4 (bis[sulfosuccinimidyl] 2,2,4,4 glutarate-d_4)	21615
Slide-A-Lyzer™ Dialysis Cassettes Kit	66382
Zeba™ Spin Desalting Columns, 2 mL	89889
In-Gel Trypsin Digestion Kit	89871
Pierce™ Mass Spec Sample Prep Kit for Cultured Cells	84840

Troubleshooting

Observation	Possible cause	Recommended action
Minimal or no crosslinking observed.	NHS ester hydrolyzed.	Allow product to equilibrate to room temperature before opening.
		Ensure DMSO (or DMF) used to prepare stock solution is dry (e.g., anhydrous, molecular-sieves treated).
	Used inappropriate conjugation buffer.	Avoid buffers that contain primary amines such as Tris or glycine.
	Used inappropriate molar excess of product to target.	Optimize product-to-target ratio. Use 20-400 molar excess for 2 mg/mL protein or 10-15 molar excess for 10 mg/mL protein.
	Lysines were not within optimal crosslinking distance.	Use a crosslinker with a different linker length or reactivity (e.g., sulfhydryl or carboxylic acid).

Limited product warranty

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References

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Revision history: Pub. No. MAN0016303

Revision	Date	Description
B.0	04 May 2017	Adding DSBU product
A.0	06 November 2016	New manual

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