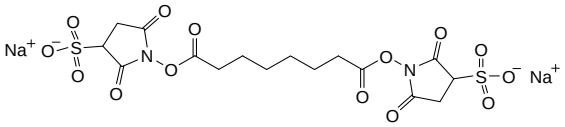
INSTRUCTIONS

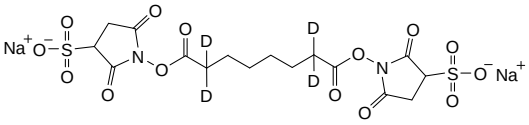
BS^3-d_0/d_4
BS^2G-d_0/d_4

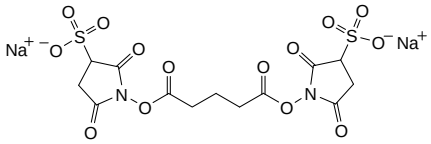
21590 21595 21610 21615

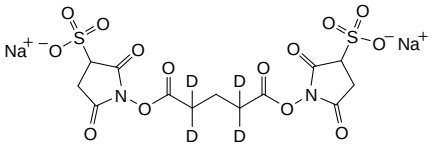
1750.2

Number	Description
21590	**BS^3-d_0** (bis[sulfosuccinimidyl] suberate-d_0), 10 mg Molecular Weight: 572.43 Spacer Arm: 11.4 Å
21595	**BS^3-d_4** (bis[sulfosuccinimidyl] 2,2,7,7 suberate-d_4), 10 mg Molecular Weight: 576.45 Spacer Arm: 11.4 Å
21610	**BS^2G-d_0** (bis[sulfosuccinimidyl] glutarate-d_0), 10 mg Molecular Weight: 530.35 Spacer Arm: 7.7Å
21615	**BS^2G-d_4** (bis[sulfosuccinimidyl] 2,2,4,4 glutarate-d_4), 10 mg Molecular Weight: 534.38 Spacer Arm: 7.7Å









Storage: Upon receipt store desiccated at 4-8°C. Product is shipped at ambient temperature.

Introduction

BS^2G-d_4 and BS^3-d_4 are water-soluble, homobifunctional sulfonated *N*-hydroxysuccinimide (sulfo-NHS) esters with different spacer arm lengths that can act as molecular rulers to estimate spatial relationships in protein structure-function studies. These reagents are deuterated analogs of BS^2G and BS^3. The NHS-ester groups of these reagents react efficiently with primary amino groups (-NH_2) in pH 7-9 buffers to form stable amide bonds. Proteins contain primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide. These reagents are supplied as a sodium salt and are water-soluble up to 10 mM. The charge character of these deuterated reagents, like BS^2G and BS^3, make them useful for cell-surface protein crosslinking applications.

Crosslinking reagents have been used as tools to probe protein structure and gain insight into protein function. A strategy that integrates crosslinking with the power of mass spectrometry (MS) can yield insights into protein tertiary structure and protein complex formation.[1] The strategy uses both hydrogen-containing (light) and discretely substituted deuterium (heavy) analogs of crosslinking agents. Heavy and light analogs are reacted simultaneously with the target protein or protein complex. Incorporating the isotopic label into the crosslinking reagent allows protein crosslinking and labeling to occur in a single step. Using two identical crosslinking agents differing only in the number of deuterium atoms in their composition is a powerful identifier of low-abundant crosslinked peptides. After enzymatic digestion, isotopic MS patterns differing by four mass units identify the crosslinked sequences, and further analysis can yield low-resolution three-dimensional structure information. Intermolecular crosslinking of an interacting protein complex and MS analysis have been used to determine the molecular contact surfaces of binding partners in a protein complex.[2-6]

Important Product Information

- These reagents are moisture-sensitive. Store reagents desiccated at 4-8ºC. Avoid moisture condensation onto the product by equilibrating vials to room temperature before opening.
- Prepare crosslinkers immediately before use. The sulfo-NHS-ester moiety readily hydrolyzes, rendering the reagent non-reactive. Do not prepare stock solutions for storage. Discard any unused reconstituted crosslinker.
- Hydrolysis of the sulfo-NHS ester is a competing reaction with the productive acylation reaction. Hydrolysis will increase with increasing pH values. Hydrolysis typically occurs more readily in dilute protein and peptide solutions. In concentrated protein solutions, the acylation reaction is favored.
- Sulfo-NHS-ester crosslinking reactions are typically performed in phosphate, HEPES or borate buffers. Other buffers also can be used provided they do not contain primary amines such as Tris or glycine. Adding a large excess of neutral-to-basic pH ammonium bicarbonate to the reaction mixture quenches the NHS ester reactive group.

Additional Materials Required

- Dimethylsulfoxide (DMSO, Product No. 20688)
- HEPES Buffer; 20 mM, pH 7.5
- Ammonium bicarbonate (NH_4HCO_3)
- In-Gel or In-Solution Trypsin Digestion Kit (e.g., In-Gel Tryptic Digestion Kit, Product No. 89871 or In-Solution Tryptic Digestion and Guanidination Kit, Product No. 89895)

Intramolecular Crosslinking for Tertiary Protein Structure Analysis

Note: 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 990 µl of 20 mM HEPES buffer (pH 7.5) at 5 µM.
2. Prepare BS^3-d_0/d_4 or BS^2G-d_0/d_4 in DMSO at 5 mM, 25 mM or 50 mM in DMSO for a 10-, 50- or 100-fold molar excess over the protein, respectively. Typically a 1:1 ratio of d_0:d_4 analogs is used in crosslinking reactions. Add 10 µl of the solution to the protein.
3. Prepare a control sample that contains the protein without crosslinking reagent using 10 µl of DMSO in place of the crosslinker.
4. Incubate samples at room temperature. Remove 200 µl aliquots after 5, 15, 30, 60 and 120 minutes.
5. Terminate the reaction by adding NH_4HCO_3 to a final concentration of 20 mM to each aliquot.
6. See the Mass Spectral Analysis of Crosslinked Proteins Section.

Intermolecular Crosslinking for Protein Interaction Analysis

Note: This protocol is designed to result in sufficient crosslinking to facilitate subsequent MS analysis and not disturb the tertiary structures of the interacting proteins caused by excessive crosslinking.

1. Prepare the bait protein at 10 µM in 20 mM HEPES buffer (pH 7.5).
2. Add the prey protein in an equal molar amount to the bait protein to give a final volume of 990 µl.
3. Prepare BS^3-d_0/d_4 or BS^2G-d_0/d_4 at 10 mM, 50 mM or 100 mM in DMSO for a relative molar excess of 10-, 50- or 100-fold over the protein. Add 10 µl of the solution to the protein.
4. Prepare a control solution (990 µl) without the crosslinker present. Add 10 µl of DMSO instead of the crosslinking reagent solution.
5. Incubate the reaction at room temperature. Remove 200 µl aliquots after 5, 15, 30, 60 and 120 minutes.
6. Terminate the reaction by adding NH_4HCO_3 to a final concentration of 20 mM to each aliquot.

Mass Spectral Analysis of Crosslinked Proteins

1. Desalt samples before SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS analysis using a 2 ml Zeba™ Desalt Spin Column (Product No. 89889). This device can process 200-700 µl of sample.
2. Evaluate the extent of crosslinking by 1-D SDS-PAGE and a rapid screen by MALDI-TOF MS. 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 GelCode™ Blue Stain Reagent (Product No. 24590) or Imperial™ Protein Stain (Product No. 24615) or MS-compatible silver stains such as SilverSNAP® II Silver Stain (Product No. 24612) or SilverSNAP® Stain for Mass Spectrometry (Product No. 24600).
3. 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., In-Solution Tryptic Digestion and Guanidination Kit, Product No. 89895).

   Note: Although in-gel digestion produces successful analyses, in-solution digestion is faster, less prone to sample loss and more efficient.
4. Separate and analyze the resulting peptide mixture containing inter- and intra-peptide crosslinking products by liquid chromatography/electrospray ionization (LC/ESI) MS, MALDI-TOF or ESI- Fourier transform ion cyclotron resonance (ESI-FTICR) MS.
5. Assign the resulting inter- and intra-molecularly crosslinked peptides in the mass spectra by their distinctive 1:1 doublet isotopic patterns. Species containing two crosslinker molecules have an isotope pattern of 1:2:1 and a mass difference of 4 units; for species with three crosslinker molecules, the pattern is 1:3:3:1, and so forth.

   Note: Upon crosslinking BS^2G and BS^3 and their deuterated d_4 analogs both form amide-bond coupled peptides that produce characteristic mass shifts in both their fully crosslinked and partially hydrolyzed forms (Table 1).

Table 1. Mass shifts associated with BS^2G and BS^3 crosslinked peptides.

Reagent	**d_0 Analog** bifunctionally cross-linked amide bonds	**d_0 Analog** Single-site amide with monofunctional hydrolysis	**d_4 Analog** bifunctionally cross-linked amide bond	**d_4 Analog** Single-site amide with monofunctional hydrolysis
BS^2G	96.020 amu	114.031 amu	100.045 amu	118.056 amu
BS^3	138.067 amu	156.078 amu	142.092 amu	160.013 amu

Analysis Information

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' (www.expasy.org), the Automated Spectrum Assignment Program (ASAP) (http://roswell.ca.sandia.gov/~mmyoung/asap.html), and the MS2 Assign software.[7] The commercially available program General Protein /Mass Analysis for Windows (GPMAW, http://welcome.to/gpmaw) by Lighthouse Data, Odense, Denmark facilitates the identification of crosslinking products between one or two protein sequences.[8]

References

1. Sinz, A. (2003). Chemical crosslinking and mass spectrometry for mapping three-dimensional structures of proteins and protein complexes. *J. Mass Spectrom.* **38:**1225-37.
2. Dihazi, G.H. and Sinz, A. (2003). Mapping low-resolution three-dimensional protein structure using chemical crosslinking and Fourier transform ion-cyclotron resonance mass spectrometry. *Rapid Commun. Mass Spectrom.* **17:**2005-14.
3. Kalkhof, S., *et al.* (2005). Chemical crosslinking and high-performance Fourier transform ion cyclotron resonance mass spectrometry for protein interaction analysis: application to a calmodulin/target peptide complex. *Anal. Chem.* **77:**495-503.
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7. Schilling, B., *et al*. (2003). MS2 assign, automated assignment and nomenclature of tandem mass spectra of chemically crosslinked peptides. *J. Am. Soc. Mass Spectrom.* **14:**834-50.
8. Peri, S., *et al*. (2001). GPMAW- A software tool for analyzing proteins and peptides. *Trends Biochem. Sci*. **26:**687-9.

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