SUPPLEMENTAL INFORMATION for

Enabling Photoactivated Cross-linking Mass Spectrometric Analysis of Protein Complexes
by Novel MS-cleavable Cross-linkers

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SUPPLEMENTAL METHODS

Materials and Reagents

All chemicals were purchased from Aldrich, Acros Organics, Alfa Aesar, TCI, VWR International or Fisher Scientific and used without further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratories. Solvents were purchased as ACS grade or better and as HPLC-grade and passed through a solvent purification system equipped with activated alumina columns prior to use.

Synthesis and Characterization of SDASO Cross-linkers

All reactions were carried out in oven-dried glassware, under an atmosphere of argon unless otherwise noted. Reactions were monitored by thin layer chromatography (TLC) or electrospray ionization mass spectrometry (ESI-MS). Thin layer chromatography (TLC) was carried out using glass plates coated with a 250 μm layer of 60 Å silica gel. TLC plates were visualized with a UV lamp at 254 nm, or by staining with potassium permanganate, cerium molybdate, or ninhydrin. ESI-MS was analyzed in positive mode with flow injection. Liquid chromatography was performed using a Teledyne ISCO CombiFlash® forced flow with an automated purification system on prepacked silica gel (SiO_2) columns or prepacked C18 columns. Proton NMRs were recorded at 500 MHz or 600 MHz using either a Bruker DRX500 (cryoprobe) or a Bruker AVANCE600 (cryoprobe) NMR, respectively. Carbon NMRs were recorded at 126 MHz or 151 MHz on the Bruker DRX500 or Bruker AVANCE600 NMR, respectively. All NMRs were taken at 25 °C. Chemical shifts (δ) are reported in parts per million (ppm) and referenced to residual solvent peak at 7.26 ppm (^1H) or 77.16 ppm (^13C) for deuterated chloroform (CDCl_3), 2.50 ppm (^1H) or 39.52 ppm (^13C) for deuterated dimethyl sulfoxide (DMSO-d_6), 3.31 ppm (^1H) or 49.00 (^13C) for deuterated methanol (CD_3OD). NMR data are reported as the following: chemical
shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad),
coupling constants (J) in hertz (Hz), and integration. High-resolution mass spectrometry (HRMS)
was performed using a Waters LCT Premier TOF spectrometer with ESI source.

1. SDASO-S

2-(3-Methyl-3\textit{H}-diazirin-3-yl)ethyl 4-methylbenzenesulfonate (S1)

As shown in Fig. S1a, the starting material, 4-hydroxy-2-butanone (3.0 mL, 34.5 mmol, 1.0
equiv), was cooled to 0 °C and then 7N NH\textsubscript{3} in MeOH (35 mL) was added dropwise. After stirring
for 3 h at 0 °C, a solution of hydroxylamine-O-sulfonic acid (H\textsubscript{2}NOSO\textsubscript{3}H; 4.25 g, 37.5 mmol, 1.1
equiv) in MeOH (15 mL) was added dropwise then solution was allowed to warm to rt. After
stirring for 17 h the reaction solution was concentrated \textit{in vacuo}. Next, NEt\textsubscript{3} (7.5 mL, 53.8, 1.6
equiv) was added to a solution of the residue in MeOH (25 mL) at 0 °C. After stirring for 10 min
at 0 °C, iodine was added in aliquots until a dark brown color persisted in the solution. The reaction
solution was diluted in EtOAc, washed with 1.0 M aq. HCl, sat. aq. Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} solution, and brine.
Finally, the combined organic phase was dried over MgSO\textsubscript{4} and concentrated \textit{in vacuo}. The
resulting residue was subject to the next reaction without further purification.

This crude product was dissolved in pyridine (10.0 mL, 124 mmol, 3.6 equiv) and cooled
to 0 °C. Next, \textit{p}-toluenesulfonyl chloride (TsCl; 1.86 g, 10.0 mmol, 0.3 equiv) was added to the
solution. After stirring for 1 h at rt, the reaction solution was diluted with EtOAc, washed with 1N
HCl, sat. aq. NaHCO\textsubscript{3} solution, and brine. The combined organic phase was then dried over MgSO\textsubscript{4}
and concentrated \textit{in vacuo}. The resulting residue was purified by flash chromatography (15% EtOAc in hexanes) to obtain tosylate S1 (971 mg, 11% over two steps). S1: \textsuperscript{1}H NMR (500 MHz,
CDCl\textsubscript{3}): \( \delta \) 7.82 (d, \( J = 8.5 \text{ Hz}, 2\text{H} \)), 7.36 (d, \( J = 8.5 \text{ Hz}, 2\text{H} \)), 3.96 (t, \( J = 6.4 \text{ Hz}, 2\text{H} \)), 2.46 (s, 3H),
1.67 (t, \( J = 6.4 \text{ Hz}, 2\text{H} \)), 1.00 (s, 3H); \textsuperscript{13}C NMR (126 MHz, CDCl\textsubscript{3}): \( \delta \) 145.2, 132.9, 130.1, 128.1,
Spectral data were consistent with those previously reported for the compound (1)

**Methyl 3-((2-(3-methyl-3H-diazirin-3-yl)ethyl)thio)propanoate (S2)**

As shown in Fig. S1a, K$_2$CO$_3$ (477 mg, 3.5 mmol, 1.5 equiv) was added to a solution of tosylate S1 (579 mg, 2.3 mmol, 1.0 equiv) and methyl-3-mercaptopropionate (0.50 mL, 4.6 mmol, 2 equiv) in MeOH (2.3 mL). After stirring for 3 h at rt, the reaction solution was filtered to remove solids. The filtrate was concentrated in vacuo and the resulting residue was purified by flash chromatography (20% EtOAc in hexanes) to obtain ester S2 (408 mg, 88%). S2: $^1$H NMR (500 MHz, CDCl$_3$): δ 3.70 (s, 3H), 2.73 (t, $J = 7.4$ Hz, 2H), 2.56 (t, $J = 7.4$ Hz, 2H), 2.35 (t, $J = 8.0$ Hz, 2H), 1.61 (t, $J = 8.0$ Hz, 2H), 1.02 (s, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): δ 172.3, 51.9, 34.64, 34.61, 27.1, 26.6, 25.3, 19.9; HRMS (ESI, MeOH) $m/z$: [M+Na]$^+$ calcd for C$_8$H$_{14}$N$_2$O$_2$SNa 225.0669, found 225.0677.

**3-((2-(3-Methyl-3H-diazirin-3-yl)ethyl)thio)propanoic acid (S3)**

As shown in Fig. S1a, LiOH·H$_2$O (79 mg, 1.9 mmol, 1.0 equiv) was added to a solution of ester S2 (382 mg, 1.9 mmol, 1.0 equiv) in THF:H$_2$O (4:1, 12.7 mL). After stirring at rt for 1 h, an additional aliquot of LiOH·H$_2$O (79 mg, 1.9 mmol, 1.0 equiv) was added. After stirring for an additional 2 h at rt, the reaction mixture was partitioned between hexanes and H$_2$O. The aqueous phase was acidified to pH = 1 with 1N HCl and extracted with EtOAc five times. The combined organic layers were dried with MgSO$_4$, and concentrated in vacuo. The crude product (acid S3) was subjected to the next step without further purification.

**2,5-Dioxopyrrolidin-1-yl 3-((2-(3-methyl-3H-diazirin-3-yl)ethyl)thio)propanoate (S4)**

As shown in Fig. S1a, N-hydroxysuccinimide (NHS-H; 219 mg, 1.9 mmol, 1.0 equiv) was added to a solution of acid S3 (358 mg, 1.9 mmol, 1.0 equiv) in CH$_2$Cl$_2$ (4.0 mL) followed by an
addition of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl; 401 mg, 2.1 mmol, 1.1 equiv). After stirring at rt for 16 h, the reaction solution was diluted in CH₂Cl₂, washed with H₂O, then brine. The combined organic phase was dried with MgSO₄ and concentrated in vacuo. The resulting residue was purified by flash chromatography (50% EtOAc in hexanes) to obtain NHS ester S₄ (308 mg containing 5 wt% EtOAc by ¹H NMR, 292 mg, 54% over two steps). S₄: ¹H NMR (500 MHz, CDCl₃): δ 2.93–2.78 (m, 8H), 2.42 (t, J = 8.0, 2H), 1.65 (t, J = 8.0, 2H), 1.05 (s, 3H); ¹³C NMR (126 MHz, CDCl₃): δ 169.0, 167.2, 34.6, 32.1, 26.9, 26.6, 25.7, 25.3, 19.9.

2,5-Dioxopyrrolidin-1-yl 3-((2-(3-methyl-3H-diazirin-3-yl)ethyl)sulfinyl)propanoate (SDASO-S)

Finally, as shown in Fig. S1a, 30% aq. H₂O₂ (72 μL, 0.70 mmol, 2.0 equiv) was added to a solution of NHS ester S₄ (100 mg, 0.35 mmol, 1.0 equiv) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; 0.9 mL) was added. After stirring at rt for 20 min, the reaction was quenched with dimethyl sulfide (DMS; 0.15 mL) and the mixture was allowed to stir for an additional 10 min. The reaction mixture was concentrated in vacuo, then partitioned between CHCl₃ and H₂O and the aqueous phase was extracted CHCl₃. The combined organic phase was washed with H₂O and the combined aqueous phase was again extracted with CHCl₃. The combined organic phase was washed with brine, dried with MgSO₄, and concentrated in vacuo to obtain SDASO-S as a white solid (75 mg, 93% purity by ¹H NMR, 70 mg, 66%). SDASO-S: ¹H NMR (500 MHz, CDCl₃): δ 3.21–3.05 (m, 3H), 3.01–2.91 (m, 1H), 2.84 (s, 4H), 2.65–2.56 (m, 1H), 2.56–2.47 (m, 1H), 1.95–1.79 (m, 2H), 1.09 (s, 3H); ¹³C NMR (126 MHz, CDCl₃): δ 168.8, 167.1, 47.0, 46.2, 28.0, 25.7, 24.9, 24.2, 19.8; HRMS (ESI, MeOH) m/z: [M+Na]⁺ calcd for C₁₁H₁₅N₃O₅SNa 324.0625, found 324.0636.

2. SDASO-M
3-(3-Methyl-3H-diazirin-3-yl)propyl 4-methylbenzenesulfonate (S5)

As shown in Fig. S1b, the starting material, 5-hydroxy-2-pentanone (1.5 mL, 14.6 mmol, 1.0 equiv), was cooled to 0 °C and 7N NH₃ in MeOH (15 mL) was added dropwise. After stirring for 3 h at 0 °C, a solution of H₂NOSO₃H (1.90 g, 16.8 mmol, 1.15 equiv) in MeOH (12.4 mL) was added dropwise and the solution was allowed to warm to rt. After stirring for 17.5 h the reaction solution was concentrated *in vacuo*. Next, NEt₃ (3.3 mL, 23.4, 1.6 equiv) was added to a solution of the residue in MeOH (15 mL) at 0 °C. After stirring for 10 min at 0 °C, iodine was added in aliquots until a dark brown color persisted in the solution. The reaction solution was diluted in EtOAc, washed with 1.0 M aq. HCl, sat. aq. Na₂S₂O₃ solution, and brine. The combined organic phase was dried over MgSO₄ and concentrated *in vacuo*. The resulting residue was subject to the next reaction without further purification.

This crude product was dissolved in pyridine (4.2 mL, 52.6 mmol, 3.6 equiv) and cooled to 0 °C. Next, TsCl (835 mg, 4.38 mmol, 0.3 equiv) was added to the solution. After stirring for 1 h at rt, the reaction solution was diluted with EtOAc, washed with 1N HCl, sat. aq. NaHCO₃ solution, and brine. The combined organic phase was dried over MgSO₄ and concentrated *in vacuo*. The resulting residue was purified by flash chromatography (0% to 15% EtOAc in hexanes) to obtain tosylate S5 (102 mg, 3% over two steps). S5: ¹H NMR (500 MHz, CDCl₃): δ 7.75 (d, J = 8.2 Hz, 2H), 7.33 (d, J = 8.0 Hz, 2H), 3.97 (t, J = 6.2 Hz, 2H), 2.43 (s, 3H), 1.55–1.42 (m, 2H), 1.42–1.31 (m, 2H), 0.95 (s, 3H); ¹³C NMR (126 MHz, CDCl₃): δ 145.0, 133.0, 130.0, 127.9, 69.5, 30.4, 25.1, 23.6, 21.7, 19.8.

Methyl 3-((3-(3-methyl-3H-diazirin-3-yl)propyl)thio)propanoate (S6)

As shown in Fig. S1b, K₂CO₃ (79 mg, 0.57 mmol, 1.5 equiv) was added to a solution of tosylate S5 (102 mg, 0.38 mmol, 1.0 equiv,) and methyl-3-mercaptopropionate (84 μL, 0.76 mmol,
2 equiv) in MeOH (0.4 mL). After stirring for 3 h at rt, the reaction mixture was filtered to remove solids. The filtrate was concentrated in vacuo and the resulting residue was purified by flash chromatography (0% to 20% EtOAc in hexanes) to obtain ester S6 (95 mg, containing 6 wt% of EtOAc and 17 wt% methyl-3-mercaptopropionate by $^1$H NMR; 73 mg, 89%). S6: $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 3.69 (s, 3H), 2.81–2.72 (m, 2H), 2.59 (t, $J = 7.3$ Hz, 2H), 2.53–2.45 (m, 2H), 1.51–1.41 (m, 2H), 1.00 (s, 3H).

3-((3-(3-Methyl-3H-diazirin-3-yl)propyl)thio)propanoic acid (S7)

As shown in Fig. S1b, LiOH·H$_2$O (16 mg, 0.38 mmol, 1.05 equiv) was added to a solution of ester S6 (73 mg, 0.36 mmol, 1.0 equiv) in THF:H$_2$O (4:1, 2.5 mL). After stirring at rt for 1 h, an additional aliquot of LiOH·H$_2$O (16 mg, 0.38 mmol, 1.05 equiv) was added. After stirring for an additional 2 h at rt, the reaction mixture was partitioned between hexanes and H$_2$O. The aqueous phase was acidified to pH = 1 with 1N HCl and extracted with EtOAc five times. The combined organic layers were dried with MgSO$_4$, and concentrated in vacuo. The crude product (acid S7) was subjected to the next step without further purification.

2,5-Dioxopyrrolidin-1-yl 3-((3-(3-methyl-3H-diazirin-3-yl)propyl)thio)propanoate (S8)

As shown in Fig. S1b, NHS-H (40 mg, 0.35 mmol, 1.0 equiv) was added to a solution of crude acid S7 (70 mg, 0.35 mmol, 1.0 equiv) in CH$_2$Cl$_2$ (0.7 mL) followed by an addition of EDC·HCl (74 mg, 0.39 mmol, 1.1 equiv). After stirring at rt for 15 h, the reaction solution was diluted in CH$_2$Cl$_2$, washed with H$_2$O, then brine. The combined organic phase was dried with MgSO$_4$ and concentrated in vacuo to obtain crude NHS ester S8 (56 mg). S8: $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 2.95–2.76 (m, 8H), 2.54–2.41 (m, 2H), 1.48–1.37 (m, 4H), 0.99 (s, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 169.1, 167.2, 33.3, 32.1, 31.6, 26.3, 25.7, 25.5, 24.0, 19.9.
2,5-Dioxopyrrolidin-1-yl 3-((3-(3-methyl-3H-diazirin-3-yl)propyl)sulfinyl)propanoate (SDASO-M)

Finally, as shown in Fig. S1b, 30% aq. H$_2$O$_2$ (38 μL, 0.37 mmol, 2.0 equiv) was added to a solution of crude NHS ester S8 (56 mg, 0.19 mmol, 1.0 equiv) in HFIP (0.5 mL). After stirring at rt for 20 min, the reaction was quenched with dimethyl sulfide (DMS; 80 μL) and the solution was allowed to stir for an additional 10 min. The reaction mixture was concentrated in vacuo, then partitioned between CHCl$_3$ and H$_2$O and the aqueous phase was extracted CHCl$_3$. The combined organic phase was washed with H$_2$O and the combined aqueous phase was again extracted with CHCl$_3$. The combined organic phase was washed with brine, dried with MgSO$_4$, and concentrated in vacuo to obtain crude SDASO-M (15 mg). SDASO-M: $^1$H NMR (500 MHz, CDCl$_3$): δ 3.23–2.92 (m, 4H), 2.85 (s, 4H), 2.77–2.57 (m, 2H), 1.82–1.62 (m, 2H), 1.62–1.43 (m, 2H), 1.04 (s, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): δ 168.8, 167.2, 51.7, 46.0, 33.4, 25.72, 25.69, 24.2, 19.7, 17.8.

3. SDASO-L

3-(3-Methyl-3H-diazirin-3-yl)propanoic acid (S9)

As shown in Fig. S1c, 7N NH$_3$ in MeOH (14.8 mL) was added to a solution of levulinic acid (2.0 mL, 19.5 mmol, 1.0 equiv) in MeOH (4.5 mL, 4.30 M) cooled to 0 °C. After stirring for 3 h at 0 °C, a solution of H$_2$NOSO$_3$H (2.54 g, 22.4 mmol, 1.15 equiv) in MeOH (13 mL) was added and the reaction was allowed to warm to rt. The milky white solution was concentrated in vacuo after stirring for 19 h. Next, NEt$_3$ (4.7 mL, 33.6 mmol, 1.5 equiv) was added to a solution of the white oil in MeOH (14 mL) at 0 °C. After stirring for 10 min at 0 °C, iodine was added in aliquots (10.2 g, 40.3 mmol, 1.8 equiv) until a dark brown color persisted in the solution. The brown mixture was diluted in EtOAc, washed with 1.0 M aq. HCl, sat. aq. Na$_2$S$_2$O$_3$ solution, and brine. The aqueous phases were combined and extracted three times with EtOAc. The combined organic
phase was dried over Na₂SO₄ and concentrated in vacuo to obtain a yellow solid which was subject to the next reaction without further purification. Spectral data were consistent with those previously reported for the compound (2).

2,5-Dioxopyrrolidin-1-yl 3-(3-methyl-3H-diazirin-3-yl)propanoate (S10)

As shown in Fig. S1c, trifluoroacetic anhydride (TFAA; 4.1 mL, 29.4 mmol, 2.0 equiv) was added dropwise to a solution of acid S9 (1.88 g, 14.7 mmol, 1.0 equiv), NHS-H (3.38 g, 29.4 mmol, 2.0 equiv), and N,N-diisopropylethylamine (DIPEA; 10.2 mL, 58.7 mmol, 4.0 equiv) in DMF (75.0 mL, 0.20 M) cooled to 0 °C. The orange solution was stirred for 3 h at 0 °C, and then partitioned between EtOAc and brine. The organic phase was washed with brine five times, dried over Na₂SO₄, and concentrated in vacuo to obtain an orange oil. The oil was purified by flash chromatography (20 to 70% EtOAc in hexanes) to obtain NHS ester S10 as a tan powder (1.65 g, 50% over two steps). Spectral data were consistent with those previously reported for the compound (2).

N-(2-mercaptoethyl)-3-(3-methyl-3H-diazirin-3-yl)propenamide (S11)

As shown in Fig. S1c, NHS ester S10 (1.00 g, 4.44 mmol, 1.0 equiv) was added to a solution of 2-aminoethanethiol (343 mg, 4.44 mmol, 1.0 equiv) in CH₂Cl₂ (22.2 mL, 0.20 M). After stirring for 20 min at rt, the reaction solution was concentrated in vacuo. The crude product (thiol S11) was subject to the next step without further purification.

2,5-Dioxopyrrolidin-1-yl acrylate (S12)

As shown in Fig. S1c, NEt₃ (6.30 mL, 45.0 mmol, 1.0 equiv) was added to a solution of NHS-H (5.18 g, 45.0 mmol, 1.0 equiv) in CH₂Cl₂ (90 mL, 0.50 M) cooled to 0 °C followed by an addition of acryloyl chloride (4.0 mL, 49.5 mmol, 1.1 equiv). The reaction was stirred for 3 h at 0 °C, and then vacuum filtered to collect the white precipitate. The filtrate was washed with H₂O
then brine, dried over Na₂SO₄, and concentrated in vacuo to obtain NHS ester S12 as a white solid (5.52 g, 72%). Spectral data were consistent with those previously reported for the compound (3).

2,5-Dioxopyrrolidin-1-yl 3-((2-(3-(3-methyl-3H-diazirin-3-yl)propanamido)ethyl)thio)propanoate (S13)

As shown in Fig. S1c, a solution of thiol S11 (832 mg, 4.44 mmol, 1.0 equiv) in CH₂Cl₂ (22.2 mL, 0.20 M) was added to NHS ester S12 (751 mg, 4.44 mmol, 1.0 equiv) followed by an addition of NEt₃ (0.680 mL, 4.88 mmol, 1.1 equiv). After stirring at rt for 20 min, the reaction solution was washed with H₂O then brine, dried with Na₂SO₄, and concentrated in vacuo. The crude mixture was purified by flash chromatography (20 to 100% EtOAc in hexanes) to obtain sulfide S13 (887 mg, 56% over two steps). S13: ¹H NMR (500 MHz, CDCl₃): δ 6.32 (s, br, 1H), 3.42 (q, J = 6.0 Hz, 2H), 2.96–2.80 (m, 8H), 2.70 (t, J = 6.0 Hz, 2H), 2.00 (t, J = 7.7 Hz, 2H), 1.72 (t, J = 7.7 Hz, 2H), 1.00 (s, 3H); ¹³C NMR (125 MHz, CDCl₃): δ 171.7, 169.2, 167.3, 38.4, 32.4, 32.2, 30.6, 30.1, 26.5, 25.7, 25.6, 20.0; HRMS (ESI, MeOH) m/z calcd for C₁₄H₂₀N₄O₅SNa (M+Na)⁺ 379.1047, found 379.1043.

2,5-Dioxopyrrolidin-1-yl 3-((2-(3-(3-methyl-3H-diazirin-3-yl)propanamido)ethyl)sulfinyl)propanoate (SDASO-L)

Finally, as shown in Fig. S1c, 30% aq. H₂O₂ solution (0.11 mL, 1.11 mmol, 2.0 equiv) was added to a solution of sulfide S12 (200 mg, 0.561 mmol, 1.0 equiv) in HFIP (2.80 mL, 0.20 M). The reaction was stirred for 10 min at rt, and then quenched with DMS (0.20 mL) and the mixture was concentrated in vacuo to obtain the cross linker SDASO (247 mg containing 23 wt% HFIP and 4 wt% DMSO; 181 mg, 87%). SDASO: ¹H NMR (600 MHz, CDCl₃): δ 6.77 (s, br, 1H), 3.85–3.65 (m, 2H), 3.20–3.04 (m, 4H), 2.95–2.80 (m, 4H), 2.63 (s, 4H), 2.04 (t, J = 7.6 Hz, 2H), 1.73 (t, J = 7.6 Hz, 2H), 1.01 (s, 3H); ¹³C NMR (151 MHz, CDCl₃): δ 172.6, 168.9, 167.0, 51.3, 45.9,
34.4, 30.6, 30.0, 25.7, 25.6, 24.3, 19.9; HRMS (ESI, MeOH) \( m/z \) calcd for C\textsubscript{14}H\textsubscript{20}N\textsubscript{4}O\textsubscript{6}Na\textsuperscript{(M+Na)+} 395.0996, found 395.0995.
SUPPLEMENTAL TABLES

Supplemental Table 1A. Detailed Summary of Cross-linked Peptides of BSA Identified for SDASO-L, -M, and -S Cross-linkers

Supplemental Table 1B. Summary of the Unique SDASO K-X Linkages of BSA

Supplemental Table 1C. Distance Mapping of SDASO K-X Linkages onto the BSA Structure (PDB: 4F5S)

Supplemental Table 1D. The Observed Frequency of Diazirine Labeling on Each AAs in BSA

Supplemental Table 2A. Detailed Summary of Cross-linked Peptides of the Yeast 26S Proteasome and its PIPs Identified for SDASO (-L, -M and -S) and DSSO Cross-linkers

Supplemental Table 2B. Summary of the Unique SDASO K-X Linkages of the Yeast 26S Proteasome and PIPs

Supplemental Table 2C. Summary of the Unique DSSO K-K Linkages of the Yeast 26S Proteasome and PIPs

Supplemental Table 3A. Cross-link Distance Mapping onto the Four Structural Models of the Yeast 26S Proteasome (s1-s4)

Supplemental Table 3B. The Observed Frequency of Diazirine Labeling on Each AAs in the Yeast 26S Proteasome

Supplemental Table 3C. XL-PPIs of the Yeast 26S Proteasome Derived from SDASO and DSSO Cross-linking
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Synthesis pathways of the SDASO linkers: (A) SDASO-S. (B) SDASO-M. (C) SDASO-L.

Supplemental Figure 2. MS² fragmentation characteristics of SDASO linkers. (A) The conversion of sulfenic acid modified fragment αₕ to unsaturated thiol modified fragment αₜ. During MS²-CID analysis, the sulfenic acid moiety loses water (-H₂O) to form the more stable unsaturated thiol (T) moiety, which is often detected as the dominant form. (b-c) Predicted MS² fragmentation of SDASO -L, -M and -S dead-end modified peptides (αₔₓ). Two types of dead-end products can be formed for SDASO linkers, depending on which reactive group is hydrolyzed. The products (αₔₓ) with a hydrolyzed NHS ester (B) or a hydrolyzed diazirine end (C) are illustrated. During MS²-CID, the former only generates a thiol modified fragment αₜ, whereas the latter only produces an alkene modified fragment αₐ. (D) Predicted MS² fragmentation of SDASO-L, -M and -S intra-linked peptides (α₁ₙ), yielding one fragment containing both alkene and thiol modifications (αₐₜ).

Supplemental Figure 3. MSⁿ analyses of representative SDASO -L, -M and -S dead-end modified peptides of BSA. MS² spectra of the diazirine dead-end modified peptides αₐₔₓ: (A) SDASO-L: m/z 499.5673³⁺, (B) SDASO-M: m/z 480.2411³⁺, (C) SDASO-S: m/z 475.8982³⁺. MS² spectra of the NHS ester dead-end modified peptides αₐₔₓ of BSA: (D) SDASO-L: m/z 765.0415³⁺, (E) SDASO-M: m/z 745.7052³⁺; (F) SDASO-S: m/z 741.0337³⁺. MS³ spectra of (G) αₐ (m/z 652.32²⁺) detected in (A) and (J) αₜ (m/z 734.70²⁺) detected in (D) from SDASO-L dead-end modified peptides. MS³ spectra of (H) αₐ (m/z 652.32²⁺) detected in (B), and αₜ (m/z 715.69²⁺) detected in (E) from SDASO-M dead-end modified peptides. MS³ spectra of (H) αₐ (m/z 652.32²⁺) detected in (C), and αₜ (m/z 711.02²⁺) detected in (F) from SDASO-S dead-end modified peptides.
The diazirine dead-end modified peptides of BSA were determined as \(^{35}\text{FKDLGEEHFK}\(^{44}\), in which K36 was modified for all SDASO linkers (G-I). The NHS ester dead-end modified peptides of BSA were identified as \(^{168}\text{RHPYFYAPEL}Y\text{YYANK}\(^{183}\) (J), in which E176 was modified for SDASO-L, and P175 or E176 were modified for SDASO-M and SDASO-S (K and L).

**Supplemental Figure 4.** MS\(^n\) analyses of representative SDASO -L, -M and -S intra-linked peptides of BSA. MS\(^2\) spectra of the intra-linked \(\alpha_{\text{intra}}\) peptides (A) SDASO-L: m/z 474.8992\(^{3+}\), (B) SDASO-M: m/z 455.8924\(^{3+}\), (C) SDASO-S: m/z 451.2183\(^{3+}\). MS\(^3\) spectra of: (D) SDASO-L MS\(^2\) fragment ion \(\alpha_{\text{A+T}}\) (m/z 468.89\(^{3+}\)), (E) SDASO-M MS\(^2\) fragment ion \(\alpha_{\text{A+T}}\) (m/z 449.89\(^{3+}\)), (F) SDASO-S MS\(^2\) fragment ion \(\alpha_{\text{A+T}}\) (m/z 445.22\(^{3+}\)). The intra-linked peptides were determined as \(^{25}\text{DTHKSEIAHR}\(^{34}\) with D25 linked to K28 for all three linkers.

**Supplemental Figure 5.** The SDASO XL-MS analysis workflow. SDASO cross-linking involves two steps: 1) lysine labeling by NHS ester; 2) photoactivated diazirine cross-linking of any AAs upon UV irradiation. path I-SDASO-L, path II-SDASO-M, and path III-SDASO-S.

**Supplemental Figure 6.** Reproducibility of SDASO XL-MS data for BSA. Comparisons of SDASO cross-linked peptide sequences among the three biological replicates for (A) SDASO-L, (B) SDASO-M, and (C) SDASO-S. Comparisons of SDASO K-X linkages among the three biological replicates for (D) SDASO-L, (E) SDASO-M, and (F) SDASO-S.

**Supplemental Figure 7.** Circular 2-D XL-maps of BSA. The maps were generated based on cross-links of BSA using (A) DSSO (4), (B) DHSO (5), (C) BMSO (6) and (D) the three SDASO linkers in this work.

**Supplemental Figure 8.** Structure-based quality control for the BSA dataset. Comparison of distance distributions of unique cross-links for (A) SDASO-S, (B) SDASO-M, and (C) SDASO-L with random cross-link distribution of BSA. The random distribution shown in grey was
generated by considering all possible pairwise distances between any K and any residue X (PDB: 4F5S).

**Supplemental Figure 9. Reproducibility of SDASO XL-MS data for the 26S proteasome.**
Comparisons of SDASO cross-linked peptide sequences among the three biological replicates for (A) SDASO-L, (B) SDASO-M, and (C) SDASO-S. Comparisons of SDASO K-X linkages among the three biological replicates for (D) SDASO-L, (E) SDASO-M, and (F) SDASO-S. Note: the XL-MS data were generated from tryptic digests only.

**Supplemental Figure 10. Comparison of SDASO XL-MS data for the 26S proteasome.**
Overlaps of the identified cross-linked peptide sequences using SDASO-L, SDASO-M, and SDASO-S respectively. Note: the XL-MS data were generated from tryptic digests only.

**Supplemental Figure 11. Reproducibility of SDASO-L XL-MS data from chymotryptic digests of the 26S proteasome and comparison of data from trypsin and chymotrypsin digests of the 26S proteasome.** Comparisons of SDASO-L (A) cross-linked peptide sequences and (B) K-X linkages among the three biological replicates of chymotryptic digests. Overlaps of the identified SDASO-L (C) cross-linked peptide sequences and (D) K-X linkages using trypsin and chymotrypsin digestions respectively. SDASO-L: trypsic digests; SDASO-Lc: chymotryptic digests.

**Supplemental Figure 12. Distance distribution plots of SDASO cross-links mapped to the four known states of the yeast 26S proteasome structures (s1-s4).** Respective cross-link distance distribution plots for SDASO-L, -M and -S cross-link data mapped onto (A) s1 (PDB: 4CR2), (B) s2 (PDB:4CR3), (C) s3 (PDB:4CR4) and (D) s4 (PDB:5MPC). Distance satisfaction thresholds are ≤30Å for SDASO-M and −S, and ≤35Å for SDASO-L.
**Supplemental Figure 13.** Structure-based quality control for the 26S proteasome SDASO XL-MS dataset. Comparison of distance distributions of unique cross-links for (A) SDASO-S, (B) SDASO-M, and (C) SDASO-L with random cross-link distribution of the yeast 26S proteasome. The random distribution shown in grey was generated by considering all possible pairwise distances between any K and any residue X (PDB: 4CR2).

**Supplemental Figure 14.** Distance distribution plots of the inter-subunit and intra-subunit K-X linkages mapped to the yeast 26S proteasome structures (s1-s4). (A) Average cross-link distance satisfaction rates across the four state models (s1: 4CR2, s2:4CR3, s3:4CR4, and s4:5MPD) for SDASO -L, -M, and –S, respectively. Respective distance distribution plots of SDASO inter-subunit and intra-subunit cross-links mapped onto (B-D) state s1 (PDB: 4CR2), (E-G) state s2 (PDB: 4CR3), (H-J) state s3 (PDB: 4CR4), and (K-M) state s4 (PDB: 5MPC) based on SDASO-L (B, E, H, K), SDASO-M (C, F, I, L) and SDASO-S (D, G, J, M) XL-MS data. Distance satisfaction thresholds are ≤30Å for SDASO-M and –S, and ≤35Å for SDASO-L.

**Supplemental Figure 15.** Distance distribution plots of the 19S RP and 20S CP K-X linkages mapped to the yeast 26S proteasome structures (s1-s4). (A) Average distance satisfaction rates across the four state models (s1: 4CR2, s2:4CR3, s3:4CR4, and s4:5MPD) for SDASO -L, -M, and -S K-X linkages of the 19S RP and 20S CP, respectively. Respective distance distribution plots of the 19S and 20S cross-links mapped onto (B-D) state s1 (PDB: 4CR2), (E-G) state s2 (PDB: 4CR3), (H-J) state s3 (PDB: 4CR4), and (K-M) state s4 (PDB: 5MPC) based on SDASO-L (B, E, H, K), SDASO-M (C, F, I, L) and SDASO-S (D, G, J, M) XL-MS data. Distance satisfaction thresholds are ≤30Å for SDASO-M and –S, and ≤35Å for SDASO-L.
Supplemental Figure 16. Reproducibility of DSSO XL-MS data for the 26S proteasome. Comparisons of DSSO (A) cross-linked peptide sequences and (B) K-K linkages among the two biological replicates of tryptic digests.

Supplemental Figure 17. Distance distribution analysis of DSSO cross-links of the 26S proteasome. (A) Average distance satisfaction rates of DSSO cross-links across the four state models (s1: 4CR2, s2:4CR3, s3:4CR4, and s4:5MPD) (distance satisfaction threshold for DSSO cross-links ≤ 30Å). (B) Respective distance distribution plots of DSSO cross-links mapped to the four states of the 26S proteasome structures. (C) Distribution of state-specific K-K linkages among the 14 possible state combinations. (D) Average distance satisfaction rates of inter-subunit and intra-subunit K-K linkages across the four state models. (E) Average distance satisfaction rates of the 19S and 20S K-K linkages across the four state models. (F-I) Respective distance distribution plots of inter-subunit and intra-subunit DSSO cross-links mapped to the four state models: (F) s1 (PDB: 4CR2), (G) s2 (PDB: 4CR3), (H) s3 (PDB: 4CR4) and (I) s4 (PDB: 5MPC). (J-M) Respective distance distribution plots of the 19S and 20S cross-links mapped to the four state models: (J) s1 (PDB: 4CR2), (K) s2 (PDB: 4CR3), (L) s3 (PDB: 4CR4) and (M) s4 (PDB: 5MPC).

Supplemental Figure 18. 3-D XL-Maps of SDASO and DSSO intra-subunit linkages of the 26S proteasome. (A) SDASO and (B) DSSO intra-subunit linkages (shown in yellow) mapped to s1 (PDB:4CR2).

Supplemental Figure 19. XL-maps of the Ecm29- and Ubp6-26S interactions. (A) 2-D XL-map describing the interactions of Ecm29 with Rpn1, Rpn6, Rpt1, and Rpt3-6. (B) 2-D XL-map illustrating the interactions of Ubp6 with Rpn1, Rpn2, Rpn8, Rpt1 and Rpt2. (Note: DSSO inter-subunit linkages are shown in blue and SDASO inter-subunit linkages are shown in green.)
**Supplemental Figure 20. Variance in SDASO cross-linked sites.** Distributions of the number of possible cross-linked site locations in the identified SDASO cross-links due to ambiguity in site identification for (A) BSA and (B) 26S Proteasome.

**Supplemental Figure 21. Respective distribution of amino acids targeted by diazirine** based on the identified SDASO cross-links in (A) 26S proteasome and (B) BSA for SDASO -L, -M, and -S linkers.
References


Annotated spectra for cross-link identifications can be viewed through MS-Viewer (https://msviewer.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msviewer) using the provided links:

1. **Spectra for cross-linked peptides of BSA;**
   
   SDASO-S ([https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=x65tqdukzv&search_name=msviewer](https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=x65tqdukzv&search_name=msviewer))  
   
   SDASO-M ([https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=8jj2ya8grp&search_name=msviewer](https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=8jj2ya8grp&search_name=msviewer))  
   
   SDASO-L ([https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=efsepaedxv&search_name=msviewer](https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=efsepaedxv&search_name=msviewer))

2. **Spectra for cross-linked peptides of 26S proteasome;**
   
   SDASO-S ([https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=ood8apmfhr&search_name=msviewer](https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=ood8apmfhr&search_name=msviewer))  
   
   SDASO-M ([https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=lud3axrrfm&search_name=msviewer](https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=lud3axrrfm&search_name=msviewer))  
   
   SDASO-L ([https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=1cegjbxqaf&search_name=msviewer](https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=1cegjbxqaf&search_name=msviewer))  
   
   DSSO ([https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=bbpm76tsng&search_name=msviewer](https://msviewer.ucsf.edu:443/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=bbpm76tsng&search_name=msviewer))
Figure S-1

A  SDASO-S Synthesis

\[ \text{7N NH}_3 \text{ in MeOH, then } \text{H}_2\text{NOSO}_3\text{H, then NEt}_3, I_2 } \rightarrow \text{N=NN} \rightarrow \text{TsCl, pyridine} \rightarrow \text{S1} \]

\[ \text{O} \rightarrow \text{O} \rightarrow \text{S2} \]

\[ \text{K}_2\text{CO}_3, 88\% \rightarrow \text{S3} \]

\[ \text{LIOH-H}_2\text{O} \rightarrow \text{S4} \]

\[ \text{H}_2\text{O}_2, \text{HFIP} 66\% \rightarrow \text{SDASO-S} \]

B  SDASO-M Synthesis

\[ \text{7N NH}_3 \text{ in MeOH, then } \text{H}_2\text{NOSO}_3\text{H, then NEt}_3, I_2 } \rightarrow \text{N=NN} \rightarrow \text{TsCl, pyridine} \rightarrow \text{S5} \]

\[ \text{O} \rightarrow \text{O} \rightarrow \text{S6} \]

\[ \text{LIOH-H}_2\text{O} \rightarrow \text{S7} \]

\[ \text{NHS-H, EDC} 53\% (2 \text{ steps}) \rightarrow \text{SDASO-M} \]

C  SDASO-L Synthesis

\[ \text{7N NH}_3 \text{ in MeOH, then } \text{H}_2\text{NOSO}_3\text{H, then NEt}_3, I_2 } \rightarrow \text{S9} \rightarrow \text{NHS-H, DIPEA, TFAA, DMF} \rightarrow \text{S10} \]

\[ \text{HS-} \text{NH}_2 \text{ CH}_2\text{Cl}_2 \rightarrow \text{S11} \rightarrow \text{S12} \]

\[ \text{NEt}_3, \text{ CH}_2\text{Cl}_2 66\% (2 \text{ steps}) \rightarrow \text{S13} \]

\[ \text{H}_2\text{O}_2, \text{HFIP} 52\% \rightarrow \text{SDASO-L} \]
Figure S-2

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**** MS-cleavable site
Figure S-3

Dead End on Hydrolyzed Diazirine

A. MS^2
B. MS^2
C. MS^2

Dead End on Hydrolyzed NHS Ester

D. MS^2
E. MS^2
F. MS^2

G. SDASO-L
H. SDASO-M
I. SDASO-S

J. SDASO-L
K. SDASO-M
L. SDASO-S
Figure S-4
Figure S-5

Lysine Labeling → UV Activation → Digestion → SEC → LC-MS<sup>n</sup> Analysis

I. K-NH₂ + SDASO-L → + H-X → → →
II. K-NH₂ + SDASO-M → + H-X → → →
III. K-NH₂ + SDASO-S → + H-X → → →

3-D XL-Maps → 2-D XL-Maps → XL PPI Network → Identification of XL Peptides

K K K
X X X
BSA XL-Peptide Triplicate Reproducibility

A  SDASO-L
Rep 1  8 (7.5%)  4 (3.8%)  10 (9.4%)
Rep 2  6 (5.7%)  6 (64.2%)  3 (2.8%)
Rep 3  7 (6.6%)

B  SDASO-M
Rep 1  7 (8.3%)  4 (4.8%)  2 (2.4%)
Rep 2  8 (9.5%)  2 (63.0%)  6 (8.2%)
Rep 3  54 (64.3%)  6 (7.1%)  46 (5.5%)

C  SDASO-S
Rep 1  2 (2.7%)  2 (2.7%)  2 (2.7%)
Rep 2  4 (5.5%)  4 (5.5%)  4 (5.5%)
Rep 3  4 (5.5%)  6 (63.0%)  9 (12.3%)

BSA K-X Linkage Triplicate Reproducibility

D  SDASO-L
Rep 1  48 (11.2%)  39 (9.1%)  44 (10.3%)
Rep 2  213 (49.9%)  16 (3.7%)  44 (10.3%)
Rep 3  23 (5.4%)  16 (3.7%)  44 (10.3%)

E  SDASO-M
Rep 1  39 (9.8%)  31 (9.2%)  142 (42.0%)
Rep 2  46 (13.6%)  21 (6.2%)  26 (7.7%)
Rep 3  33 (9.8%)  21 (6.2%)  142 (42.0%)

F  SDASO-S
Rep 1  32 (10.5%)  16 (5.2%)  31 (10.1%)
Rep 2  130 (42.5%)  14 (4.6%)  17 (5.6%)
Rep 3  16 (5.2%)  14 (4.6%)  66 (21.6%)
Figure S-7

BSA 2-D XL-Maps

A  DSSO  B  DHSO  C  BMSO  D  SDASO (all)
Figure S-8

A

B

C
Figure S-9

26S XL-Peptide Triplicate Reproducibility

A  SDASO-L  B  SDASO-M  C  SDASO-S

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K-X Linkage Triplicate Reproducibility

D  SDASO-L  E  SDASO-M  F  SDASO-S

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<td>(8.6%)</td>
<td>(12.3%)</td>
<td>(12.1%)</td>
<td>(37.0%)</td>
<td>(5.7%)</td>
<td>(8.0%)</td>
<td>(16.3%)</td>
<td>(4.4%)</td>
<td>(8.0%)</td>
</tr>
</tbody>
</table>
26S Comparison of SDASO XL-Peptide Coverage

SDASO-L

SDASO-M

SDASO-S

145 (28.7%)
64 (12.7%)
68 (13.5%)
79 (15.6%)
19 (3.8%)
40 (7.9%)
90 (17.8%)
Reproducibility of Chymotrypsin Digested SDASO-L XL 26S

A  XL-Peptide Triplicate  
Rep 1  Rep 2  Rep 3

B  K-X Linkage Triplicate  
Rep 1  Rep 2  Rep 3

Comparison of Chymotrypsin vs. Trypsin Digested SDASO-L XL 26S

C  XL-Peptides  
Trypsin  Chymotrypsin

D  K-X Linkages  
Trypsin  Chymotrypsin
Figure S-12

A  s1 (PDB:4CR2)  B  s2 (PDB:4CR3)  C  s3 (PDB:4CR4)  D  s4 (PDB:5MPC)

Distance Bins (Å)

% of Unique Linkages

0%  20%  40%  60%

30Å  35Å
Figure S-13

A

B

C

% of Cross-links

Cα-Cα Distance (Å)

SDASO-S

Random

SDASO-M

Random

SDASO-L

Random
Figure S-15

A

B SDASO-L

C SDASO-M

D SDASO-S

E

F

G

H

I

J

K

L

M

PDB: 4CR2

PDB: 4CR3

PDB: 4CR4

PDB: 5MPC
26S DSSO XL Reproducibility

**A**  \( \text{XL-Peptides} \)

<table>
<thead>
<tr>
<th></th>
<th>Rep 1</th>
<th>Rep 2</th>
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<tr>
<td>295</td>
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<td>64.7%</td>
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<tr>
<td>(22.0%)</td>
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<tr>
<td>866</td>
<td>866</td>
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<tr>
<td>(64.7%)</td>
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</tr>
<tr>
<td>178</td>
<td>178</td>
<td>13.3%</td>
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<tr>
<td>(13.3%)</td>
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</tbody>
</table>

**B**  \( \text{K-X Linkages} \)

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<td>143</td>
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<tr>
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<tr>
<td>(65.4%)</td>
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<tr>
<td>143</td>
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<tr>
<td>(12.8%)</td>
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</table>
Figure S-17

A. Overall Summary
   - DSSO >30Å
   - DSSO ≤30Å

B. DSSO Structure Breakdown
   - 5MPC
   - 4CR3
   - 4CR4
   - 5MPC

C. State Analysis
   - Unique Linkages

D. Inter vs Intra Summary
   - Inter-subunit
   - Intra-subunit

E. 19S vs 20S Summary
   - Unique Linkages

F-G-I. Inter vs Intra
   - S1
   - S2
   - S3
   - S4

J-K-L-M. 19S vs 20S
   - S1
   - S2
   - S3
   - S4
Figure S-18

Intra-Subunit 3-D XL-Maps of the 26S

A

SDASO

B

DSSO

s1 (PDB:4CR2)
Figure S-19

A  ECM29

B  Ubp6
Figure S-20

Variance in XL-Sites

A  BSA  B  26S Proteasome

- SDASO-L
- SDASO-M
- SDASO-S

% of K-X Linkages

0.00%  10.00%  20.00%  30.00%  40.00%

# of Possible XL-Site Location

1  2  3  4  5+

% of K-X Linkages

0.00%  10.00%  20.00%  30.00%  40.00%

# of Possible XL-Site Location

1  2  3  4  5+
A

26S Proteasome

% of XL Residues Targeted

Amino Acids

SDASO-L SDASO-M SDASO-S

B

BSA

% of XL Residues Targeted

Amino Acids

SDASO-L SDASO-M SDASO-S