Supporting Information for

Harnessing the Intrinsic Photochemistry of Isoxazoles for the Development of Chemoproteomic Crosslinking Methods

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Associated Files: Data sets for all proteomic experiments are supplied as separate Microsoft Excel spreadsheets in **SFX_Proteomic_Data.xlsx** (**SFX-1**-only experiments). Python scripts utilized for data analysis are deposited on GitHub (https://github.com/ejp-lab/EJPLab_Computational_Projects/tree/master/PhotoCrosslinking).

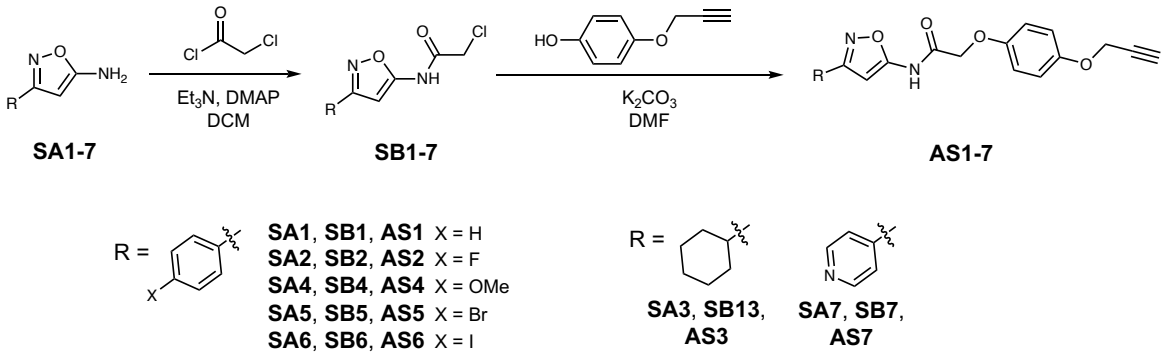
General Information

All cell culture was conducted in a HEPA-filtered cell culture hood under sterile conditions. Buffers were made with MilliQ filtered (18 MΩ) water (Millipore; Billerica, MA, USA). DC protein assay kits were purchased from Bio-Rad (Bio-Rad; Hercules, CA, USA). Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) data were collected with a Bruker Ultraflex III MALDI-TOF/TOF mass spectrometer (Billerica, MA, USA). UV/Vis absorbance spectra were obtained with a Thermo Fisher Scientific GENESYS 150 UV-Vis spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). DC assay protein quantitation was conducted using a Tecan Spark microplate reader (Tecan; Mannedorf, Switzerland). SDS-PAGE gels were run using a Bio-Rad PowerPac Basic Power Supply. Gel images were obtained with a Bio-Rad ChemiDoc MP instrument. Protein constructs were purified on an Äkta pure FPLC (Cytiva; Marlborough, MA, USA). NMR spectra were obtained on a Bruker NEO 400 spectrometer. Electrospray ionization (ESI) mass spectra were obtained on a Waters Acquity Ultra Performance LC connected to a single quadrupole detector (SQD) mass spectrometer (Waters Corp.; Milford, MA, USA). High resolution electrospray ionization mass spectra (ESI-HRMS) were obtained on a Waters LCT Premier XE liquid chromatograph/mass spectrometer. Orbitrap liquid chromatography MS/MS (LC-MS/MS) data were acquired on a Thermo Fisher Scientific QE-HF instrument. (Thermo Fisher Scientific; Waltham, MA, USA). Python version 3.7.5 was used for data analysis scripts. MaxQuant (version 1.6.17.0) was downloaded from https://www.maxquant.org/download_asset/maxquant/latest. ProteomeDiscoverer version used was 2.4. GraphPad Prism version used was 9.0.2.

Synthetic Chemistry Methods

Chemical Reagents and Instruments. Chemicals were obtained from commercial sources and used without further purification. Solvents were purchased from commercial sources and used as received unless stated otherwise. Reactions were performed at room temperature unless stated otherwise. Reactions were monitored by thin layer chromatography (TLC) on pre-coated silica 60 F254 aluminum plates (MilliporeSigma, Burlington, MA, USA), spots were visualized by ultraviolet (UV) light. Evaporation of solvents was performed under reduced pressure at 40 °C using a rotary evaporator. Flash column chromatography was performed on a Biotage® (Charlotte, NC, USA) Isolera One system equipped with Biotage® SNAP KP-Sil cartridges. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker (Billerica, MA, USA) Avance Neo 400 (400.17 MHz for ^{1}H and 100.63 MHz for ^{13}C) with chemical shifts (δ) reported in parts per million (ppm) relative to the solvent ($CDCl_3$, ^{1}H 7.26 ppm, ^{13}C 77.16 ppm; dimethyl sulfoxide (DMSO)-*d6*, ^{1}H 2.50 ppm, ^{13}C 39.52 ppm). Low resolution Liquid Chromatography Mass Spectrometry (LCMS) was carried out using a Waters (Milford, MA, USA) SQD equipped with an Acquity UPLC instrument in positive ion mode. High Resolution Mass Spectrometry (HRMS) for small molecules were obtained on a Waters LCT Premier XE LC/MS system.

Scheme S1. Synthesis of **AS1-7** compounds

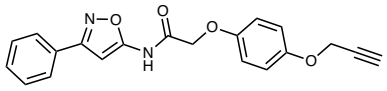


General Procedure A. An aryl isoxazol-5-amine (**SA1-7**, 0.40 mmol, 1.0 equiv.) and 4-dimethylamino-pyridine (DMAP, 5 mol%) were charged to an oven-dried round bottom flask with a stir bar. The flask was sealed with a rubber septum and sparged with argon. Dry CH_2Cl_2 (3.0

mL) and trimethylamine (3.0 equiv.) were then added to the flask, and the solution was cooled on ice for approximately 5 min. 2-chloroacetyl chloride (3 equiv.) was then added dropwise. The reaction was allowed to stir for 20 min before the ice was removed and the reaction was stirred overnight at 32 °C. The reaction was quenched with water (10 mL) and the reaction mixture was further diluted with water (30 mL) and CH_2Cl_2 (40 mL) and then layers were separated. The aqueous layer was further extracted 2 times with CH_2Cl_2 (40 mL). The combined organic layer was washed with saturated aqueous $NaHCO_3$ (50 mL) and saturated brine solution (50 mL), the organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude compound, **SB1-7**, was purified by flash column chromatography (gradient of 30-50% EtOAc/hexanes) to obtain the desired product in 60-80% yield.

General Procedure B. 4-(prop-2-yn-1-yloxy)phenol (0.35 mmol, 2.0 equiv.) and K_2CO_3 (3.0 equiv.) were charged to an oven-dried round bottom flask with a stir bar. The flask was then sealed with a rubber septum and sparged with argon and dry DMF (3.0 mL) was added to flask. The respective acetyl chloride **SB1-7** (1.0 equiv.) was solubilized in DMF (1 mL) and added slowly to the solution. The flask was then heated at 65 °C overnight. The reaction mixture was diluted with water (50 mL) and extracted with EtOAc (2x 50 mL). The combined organic layer was washed with saturated brine solution (50 mL), the organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude compound, **AS1-7**, was purified by flash column chromatography (gradient of 40-70% EtOAC/hexanes) to obtain desired product in 60-80% yield.

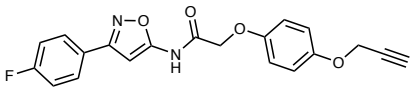
***N*-(3-phenylisoxazol-5-yl)-2-(4-(prop-2-yn-1-yloxy)phenoxy)acetamide (AS1)**



TLC (Hexanes:EtOAc, 65:35 v/v): R_f = 0.35; Off White Solid; yield 74%; **^{1}H NMR (600 MHz, $CDCl_3$):** δ 9.20 (s, 1H), 7.82 (dd, *J* = 7.4, 2.2 Hz, 2H), 7.46 (d, *J* = 1.0 Hz, 1H), 7.45 (d, *J* = 2.4 Hz, 2H), 6.98 (d, *J* = 9.2 Hz, 2H), 6.94 (d, *J* = 9.2 Hz, 2H), 6.82 (s, 1H), 4.66 (d, *J* = 2.4 Hz, 2H), 4.65 (s, 2H), 2.52 (t, *J* = 2.4 Hz, 1H); **^{13}C NMR (150 MHz, $CDCl_3$):** δ 163.7, 162.8, 158.5, 152.1,

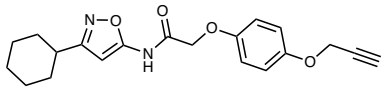
150.3, 129.2, 127.9, 127.8, 125.8, 115.4, 114.9, 86.6, 77.4, 74.6, 66.9, 55.4; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{20}H_{16}N_2O_4$ $[M+Na]^+$ 371.1008; found 371.1008.

***N*-(3-(4-fluorophenyl)isoxazol-5-yl)-2-(4-(prop-2-yn-1-yloxy)phenoxy)acetamide (AS2)**



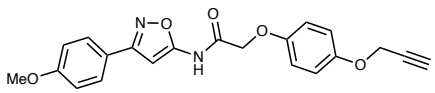
TLC (Hexanes:EtOAc, 70:30 v/v): R_f = 0.25; Off White Solid; yield 52%; **^{1}H NMR (600 MHz, $CDCl_3$):** δ 9.18 (s, 1H), 7.82 (dd, *J* = 8.4, 5.3 Hz, 2H), 7.15 (t, *J* = 8.6 Hz, 2H), 6.98 (d, *J* = 9.2 Hz, 2H), 6.94 (d, *J* = 9.2Hz, 2H), 6.78 (s, 1H), 4.67 (d, *J* = 2.4 Hz, 2H), 4.65 (s, 2H), 2.52 (t, *J* = 2.4 Hz, 1H); **^{13}C NMR (150 MHz, $CDCl_3$):** δ 164.8, 164.7, 163.1, 162.9, 159.6, 152.3 (d, *J* = 276.9 Hz), 128.8 (d, *J* = 8.2 Hz), 125.1 (d, *J* = 3.2 Hz), 116.4, 116.0 (d, *J* = 22.0 Hz), 115.9, 87.5, 78.5, 75.6, 67.9, 56.4; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{20}H_{15}FN_2O_4$ $[M+H]^+$ 367.1094; found 367.1094.

***N*-(3-cyclohexylisoxazol-5-yl)-2-(4-(prop-2-yn-1-yloxy)phenoxy)acetamide (AS3)**



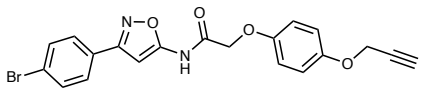
TLC (Hexanes:EtOAc, 65:35 v/v): R_f = 0.30; White Solid; yield 57%; **^{1}H NMR (600 MHz, $CDCl_3$):** δ 9.06 (s, 1H), 6.96 (d, *J* = 9.1 Hz, 2H), 6.91 (d, *J* = 9.1Hz, 2H), 6.33 (s, 1H), 4.66 (d, *J* = 2.4 Hz, 2H), 4.60 (s, 2H), 2.72-2.69 (m, 1H), 2.51 (t, *J* = 2.4 Hz, 1H), 1.98-1.95 (m, 2H), 1.83-1.80 (m, 2H), 1.74-1.71 (m, 1H), 1.50-1.43 (m, 2H), 1.41-1.34 (m, 2H), 1.29-1.25 (m, 1H); **^{13}C NMR (150 MHz, $CDCl_3$):** δ 170.2, 164.6, 158.5, 153.1, 151.4, 116.4, 115.9, 87.9, 78.5, 75.6, 67.8, 56.4, 36.3, 31.8, 25.9, 25.8; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{20}H_{22}N_2O_4$ $[M+H]^+$ 355.1658; found 355.1657.

***N*-(3-(4-methoxyphenyl)isoxazol-5-yl)-2-(4-(prop-2-yn-1-yloxy)phenoxy)acetamide (AS4)**



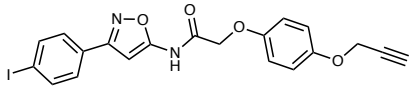
TLC (Hexanes:EtOAc, 60:40 v/v): R_f = 0.30; Off White Solid; yield 68%; **^{1}H NMR (600 MHz, $CDCl_3$):** δ 9.15 (s, 1H), 7.76 (d, *J* = 8.8 Hz, 2H), 6.99-6.96 (m, 4H), 6.94 (d, *J* = 9.2 Hz, 2H), 6.76 (s, 1H), 4.66 (d, *J* = 2.4 Hz, 2H), 4.64 (s, 2H), 3.85 (s, 3H), 2.52 (t, *J* = 2.4 Hz, 1H); **^{13}C NMR (150 MHz, $CDCl_3$):** δ 164.7, 163.4, 161.2, 159.2, 153.1, 151.4, 128.2, 121.4, 116.4, 115.8, 114.3, 87.4, 78.5, 75.6, 67.9, 56.4, 55.4; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{21}H_{18}N_2O_5$ $[M+H]^+$ 379.1294; found 379.1291.

***N*-(3-(4-bromophenyl)isoxazol-5-yl)-2-(4-(prop-2-yn-1-yloxy)phenoxy)acetamide (AS5)**



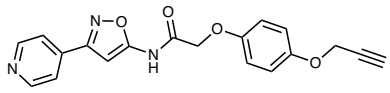
TLC (Hexanes:EtOAc, 70:30 v/v): R_f = 0.25; White Solid; yield 55%; **^{1}H NMR (600 MHz, $CDCl_3$):** δ 9.17 (s, 1H), 7.70 (d, *J* = 8.4 Hz, 2H), 7.60 (d, *J* = 8.4 Hz, 2H), 6.98 (d, *J* = 9.1Hz, 2H), 6.94 (d, *J* = 9.1Hz, 2H), 6.79 (s, 1H), 4.67 (d, *J* = 2.2 Hz, 2H), 4.65 (s, 2H), 2.52 (t, *J* = 2.2 Hz, 1H); **^{13}C NMR (150 MHz, $CDCl_3$):** δ 164.7, 162.9, 159.7, 153.2, 151.3, 132.2, 128.3, 127.4, 124.6, 116.4, 115.9, 87.4, 78.5, 75.6, 67.9, 56.4; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{20}H_{15}BrN_2O_4$ $[M+H]^+$ 427.0293; found 427.0291.

***N*-(3-(4-iodophenyl)isoxazol-5-yl)-2-(4-(prop-2-yn-1-yloxy)phenoxy)acetamide (AS6)**



TLC (Hexanes:EtOAc, 70:30 v/v): R_f = 0.25; Off White Solid; yield 51%; **^{1}H NMR (600 MHz, $CDCl_3$):** δ 9.18 (s, 1H), 7.80 (d, *J* = 8.4 Hz, 2H), 7.56 (d, *J* = 8.4 Hz, 2H), 6.98 (d, *J* = 9.1Hz, 2H), 6.94 (d, *J* = 9.1Hz, 2H), 6.79 (s, 1H), 4.67 (d, *J* = 2.2 Hz, 2H), 4.65 (s, 2H), 2.52 (t, *J* = 2.3 Hz, 1H); **^{13}C NMR (150 MHz, $CDCl_3$):** δ 164.7, 163.0, 159.7, 153.2, 151.3, 138.1, 128.4, 128.3, 116.4, 115.9, 96.5, 87.4, 78.5, 75.7, 67.9, 56.4; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{20}H_{15}IN_2O_4$ $[M+H]^+$ 475.0155; found 475.0147.

2-(4-(prop-2-yn-1-yloxy)phenoxy)-*N*-(3-(pyridin-4-yl)isoxazol-5-yl)acetamide (AS7)

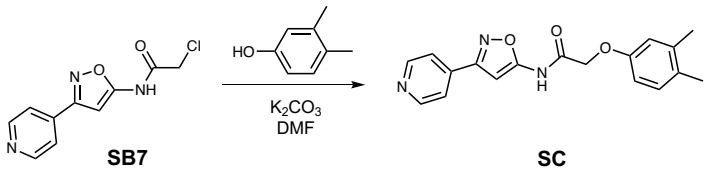


TLC (Hexanes:EtOAc, 35:65 v/v): R_f = 0.35; Light brown Solid; yield 45%; **^{1}H NMR (600 MHz, DMSO-d$_6$):** δ 12.0 (s, 1H), 8.72 (d, *J* = 5.4 Hz, 2H), 7.85 (d, *J* = 6.0 Hz, 2H), 6.95 (s, 4H), 9.93 (s, 1H), 4.78 (s, 2H), 4.73 (d, *J* = 2.3 Hz, 2H), 3.53 (t, *J* = 2.3 Hz, 1H); **^{13}C NMR (150 MHz, DMSO-d$_6$):** δ 166.5, 163.0, 161.5, 152.7, 152.2, 151.0, 136.4, 121.2, 116.3, 116.0, 87.2, 79.9, 78.5, 67.5, 56.3; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{19}H_{15}N_3O_4$ $[M+H]^+$ 350.1141; found 350.1126.

4-(prop-2-yn-1-yloxy)phenol

A flask of dihydroquinone (90.818 mmol, 3.0 eq.), DMF (60 ml), and K_2CO_3 (93.846 mmol, 3.1 eq.) under argon was charged with propargyl bromide (30.273 mmol, 1.0 eq.) dropwise with stirring. Immediately following addition, the flask was heated to 60 °C and the reaction was allowed to stir overnight. The crude reaction mixture was then filtered through Celite, and the filter cake was washed three times with EtOAc (25 ml). The resulting solution was then acidified with 1M HCl (200 ml) and extracted three times with EtOAc (50 ml). The organic phase was then washed twice with brine (200 ml), dried over Na_2SO_4, and adsorbed onto silica gel. The crude product was then purified by Biotage flash column chromatography (100 g column, gradient 0-40% EtOAc/hexanes, 30 column volumes) to obtain 4-(prop-2-yn-1-yloxy)phenol in 60% yield. TLC (Hexanes:EtOAc, 50:50 v/v): R_f = 0.56; off-white solid; **^{1}H NMR (600 MHz, CDCl$_3$):** δ 6.88 (d, J = 9.0 Hz, 2H), 6.78 (d, J = 9.0 Hz, 2H), 4.85 (s, 1H), 4.63 (d, J = 2.4 Hz, 2H), 2.51 (d, J = 4.8 Hz, 1H); **^{13}C NMR (151 MHz, CDCl$_3$):** δ 151.84, 150.31, 116.54, 116.20, 78.95, 75.55, 56.84; **LRMS-(ESI-TOF) (*m/z*):** calcd for $C_9H_8O_2$ $[M+H]^+$ 149.060; found 149.028.

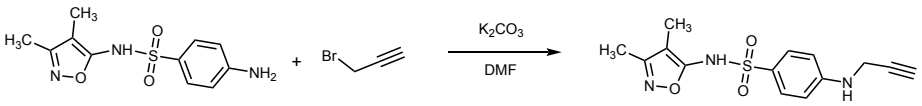
2-(3,4-dimethylphenoxy)-*N*-(3-(pyridin-4-yl)isoxazol-5-yl)acetamide (SC)



SB7 **SC**

SC was synthesized from **SB7** following General Procedure B, substituting 3,4-dimethylphenol for 4-(prop-2-yn-1-yloxy)phenol in the final step. TLC (CH_2Cl_2:MeOH, 95:05 v/v): R_f = 0.30; Brown Solid; yield 68%; **^{1}H NMR (400 MHz, DMSO-d$_6$):** δ 12.05 (s, 1H), 8.71 (d, *J* = 5.4Hz, 2H), 7.84 (d, *J* = 5.8 Hz, 2H), 7.04 (d, *J* = 8.3 Hz, 1H), 6.92 (s, 1H), 6.80 (d, *J* = 2.3 Hz, 1H), 6.69 (dd, *J* = 8.3, 2.5 Hz, 1H), 4.77 (s, 2H), 2.18 (s, 3H), 2.13 (s, 3H); **^{13}C NMR (100 MHz, DMSO-d$_6$):** δ 164.4, 162.8, 161.5, 156.2, 151.1, 137.8, 136.3, 130.6, 129.3, 121.2, 116.5, 111.9, 87.3, 67.0, 20.1, 18.9; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{18}H_{17}N_3O_3$ $[M+H]^+$ 324.1348; found 324.1390.

***N*-(3,4-dimethylisoxazol-5-yl)-4-(prop-2-yn-1-ylamino)benzenesulfonamide (SFX-1)**

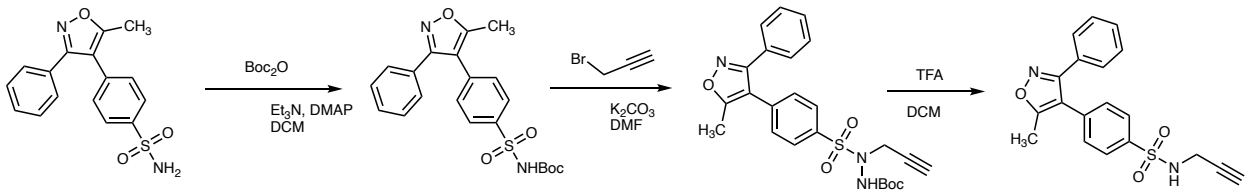


SFX **SFX-1**

N-(3,4-dimethylisoxazol-5-yl)-4-(prop-2-yn-1-ylamino)benzenesulfonamide (**SFX**, 0.40 mmol, 1.0 equiv.) and K_2CO_3 (0.40 mmol, 0.75 equiv.) were charged to an oven-dried round bottom flask with a stir bar. The flask was sealed with a rubber septum and sparged with argon. Dry DMF (2.0 mL) was then added to flask, and the solution was cooled on ice for approximately 5 min. Propargyl bromide solution, 80 wt.% in toluene (0.40 mmol, 0.5 equiv.), was then added dropwise. The reaction was allowed to stir for 20 min before the ice was removed and the reaction was stirred for 5 h at room temperature. The reaction was quenched with water (25 mL) and ethyl acetate (25 mL) and then layers were separated. The aqueous layer was further extracted 2 times with ethyl acetate (25 mL). The combined organic layer was washed with saturated brine solution (50 mL), the organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude compound was purified by flash column chromatography (gradient of 30-70% EtOAC/hexanes) to obtain the desired product **SFX-1** in 48% yield. TLC (Hexanes:EtOAc, 50:50 v/v): R_f = 0.35; Off White

Solid; yield 48%; **^{1}H NMR (600 MHz, $CDCl_3$):** δ 7.52 (d, *J* = 8.7 Hz, 2H), 6.64 (d, *J* = 8.7 Hz, 2H), 4.25 (br, 2H), 4.23 (d, *J* = 2.5 Hz, 2H), 2.23 (s, 3H), 2.18 (t, *J* = 2.5 Hz, 1H), 1.97 (s, 3H); **^{13}C NMR (150 MHz, CDCl3):** δ 161.9, 157.4, 151.5, 130.1, 125.4, 114.0, 111.8, 76.6, 73.9, 39.6, 11.0, 7.0; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{14}H_{15}N_3O_3S$ $[M+H]^+$ 305.0834; found 306.0897.

4-(5-methyl-3-phenylisoxazol-4-yl)-*N*-(prop-2-yn-1-yl)benzenesulfonamide (VCOX-1)



4-(5-methyl-3-phenylisoxazol-4-yl)benzenesulfonamide (**VCOX**, 0.40 mmol, 1.0 equiv.) and DMAP (2.5 mol%) were charged to an oven-dried round bottom flask with a stir bar. The flask was then sealed with a rubber septum, sparged with argon, and dry CH_2Cl_2 (5.0 mL) was added to flask. Triethyl amine (0.40 mmol, 1.5 equiv.) was added and then di-*tert*-butyl dicarbonate (0.40 mmol, 1.1 equiv.) was solubilized in CH_2Cl_2 (3 mL) and added slowly to the solution. The flask was then stirred at room temperature for 3h. The reaction was quenched with water (50 mL) and EtOAc (50 mL) and then layers were separated. The aqueous layer was further extracted with EtOAc (50 mL). The combined organic layer was washed with water (50 mL) and saturated brine solution (50 mL), then the organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude compound, *t*ert-butyl ((4-(5-methyl-3-phenylisoxazol-4-yl)phenyl)sulfonyl)carbamate (**SD),** was subjected to the next step without further purification.

SD (0.40 mmol, 1.0 equiv.) and K_2CO_3 (0.40 mmol, 2 equiv.) were charged to an oven-dried round bottom flask with a stir bar. The flask was sealed with a rubber septum and sparged with argon. Dry DMF (2.0 mL) was then added to flask, and a propargyl bromide solution, 80 wt.% in toluene (0.40 mmol, 1.1 equiv.), was then added dropwise. The reaction was stirred for 20 h at room temperature. The reaction was quenched with water (50 mL) and ethyl acetate (50 mL) and then layers were separated. The aqueous layer was further extracted 2 times with EtOAc (50 mL). The combined organic layer was washed 2 times with water (50 mL) and saturated brine solution (100 mL), then the organic layer was dried over Na_2SO_4 and concentrated under vacuum.

The crude compound, *t*ert-butyl 2-((4-(5-methyl-3-phenylisoxazol-4-yl)phenyl)sulfonyl)-2-(prop-2-yn-1-yl)hydrazine-1-carboxylate (**SE**), was subjected to the next step without further purification.

SE (0.40 mmol, 1.0 equiv.) was charged to an oven-dried round bottom flask with a stir bar. The flask was then sealed with a rubber septum, sparged with argon, and dry CH_2Cl_2 (5.0 mL) was added to flask. Trifluoroacetic acid (0.40 mmol, 5 equiv.) was added slowly to the solution at room temperature and reaction mixture stirred further for 3 h. The reaction mixture was concentrated under vacuum and the crude compound was dissolved in CH_2Cl_2 (100 mL). A 1 M NaOH solution was added to adjust the pH to 7. Water was added and then layers were separated. The aqueous layer was further extracted with CH_2Cl_2 (50 mL). The combined organic layer was washed with saturated brine solution (100 mL), then the organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude compound was purified by flash column chromatography (gradient of 40-70% EtOAC/hexanes) to obtain desired product **VCOX-1** in 68 % yield over 3 steps. TLC (CH_2Cl_2:MeOH, 95:05 v/v): R_f = 0.40; White Solid; yield 68%; **^{1}H NMR (600 MHz, $CDCl_3$):** δ 7.88 (d, *J* = 8.4 Hz, 2H), 7.38 (t, *J* = 6.8 Hz, 3H), 7.32 (t, *J* = 8.3 Hz, 4H), 4.81 (t, *J* = 4.4 Hz, 1H), 3.90 (dd, *J* = 4.4, 2.5 Hz, 2H), 2.48 (s, 3H), 2.07 (t, *J* = 2.5 Hz, 1H); **^{13}C NMR (150 MHz, $CDCl_3$):** δ 167.3, 161.1, 138.8, 135.6, 130.3, 129.7, 128.7, 128.6, 128.5, 127.8, 114.5, 77.9, 73.0, 32.9, 11.7; **HRMS-(ESI-TOF) (*m/z*):** calcd for $C_{19}H_{16}N_2O_3S$ $[M+H]^+$ 353.0960; found 353.0955.

Protein Expression and Purification

α-Synuclein Production. α-Synuclein (αS) was expressed, purified, and aggregated as previously described, with slight modifications.[1] Human αS with a C-terminal intein-His_6 fusion was transformed into *Escherichia coli* (*E. coli*) BL21 cells and plated on ampicillin plates (100 μg/ml). A single colony was then inoculated into a 5 ml primary culture containing ampicillin (100 μg/ml) in Luria-Bertain (LB) media and grown for 5-6 h with shaking (250 rpm) at 37 °C. The primary culture was then transferred to 1 L LB containing ampicillin (100 μg/ml) and grown as previously described until reaching an optical density (OD_{600}) of 0.8-1.0. At this stage, protein production was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to 1 mM final concentration, and the culture was grown with shaking (250 rpm) overnight at 18 °C. The following day, cells were harvested by centrifugation at 4 °C for 20 min at 4000 rpm (Sorvall GS3 rotor). Pellets were then re-suspended in 20 ml/L culture of 40 mM Tris, pH 8.3 supplemented with EDTA-free protease inhibitor tablets (Pierce Biotechnology; Waltham, MA, USA) and transferred to a metal cup for sonication. Cells were lysed by sonication on ice with a Q700 probe sonicator (QSonica LLC; Newtown, CT, USA) with the following settings: Amplitude 50, Process Time 2-3 min, Pulse-ON Time 1 s, Pulse-OFF Time 1 s. Crude lysate was then transferred to 50 ml centrifugation tubes and clarified via centrifugation at 14,000 rpm for 45 min (Sorvall SS34 rotor). Following centrifugation, supernatant was removed and transferred to a 50 ml Falcon tube. 5 ml of nickel agarose resin (GoldBio; St. Louis, MO, USA) was added, and the lysate-nickel mixture was incubated with nutation at 4 °C for 1-2 h. Lysate-nickel mixture was then poured into a 20 mL fritted column, and the flowthrough was saved. The remaining resin was then washed with ~20 mL Wash Buffer 1 (50 mM HEPES buffer, pH 7.5), ~20 ml Wash Buffer 2 (50 mM HEPES, 5 mM imidazole, pH 7.5), and eluted with 12 mL Elution Buffer (50 mM HEPES, 300 mM imidazole, pH 7.5). 2-mercaptoethanol (Bio-Rad Laboratories; Hercules, CA, USA) was then added to crude lysate (200 mM final concentration), and the mixture was allowed to incubate with nutation at room temperature overnight. The resulting cleaved protein was then dialyzed against 20 mM Tris pH 8.0 for 8-10 h. The resulting dialysate was then treated with 5 mL nickel agarose resin (GoldBio) and incubated with nutation at 4 °C for 1-2 h. The mixture was then applied to a 20 mL fritted column and flowthrough containing αS was collected in a 15 ml Falcon tube. The resulting enriched protein mixture was then dialyzed against 20 mM Tris, pH 8.0 overnight and

purified via FPLC using a 5 ml HiTrap Q-HP column (Cytiva; Marlborough, MA, USA) using the following method: Buffer A: 20 mM Tris, pH 8.0; Buffer B: 20 mM Tris, 1 M NaCl, pH 8.0; Gradient: 0% Buffer B – 5 column volumes, 0-10% Buffer B – 5 column volumes, 20-30% Buffer B – 20 column volumes, 30-100% Buffer B – 10 column volumes; flowrate 3 mL/min. The resulting fractions were then assessed for purity via MALDI-TOF MS, and pure fractions were combined. Protein was then concentrated, and buffer exchanged into phosphate-buffered saline (PBS, NaCl 0.137 M, KCl 0.0027 M, Na_2HPO_4 0.01 M, KH_2HPO_4 0.0018 M) to a final concentration of 100-200 µM via Amicon 3 kDa MWCO filters (Millipore Sigma; St. Louis, MO, USA). Purified protein was aliquoted into 1.5 mL tubes and stored at -80 °C until further use.

Calmodulin Production. Calmodulin (CaM) was prepared as described previously.[2] Briefly, CaM with a C-terminal intein-His_6 fusion was transformed into *E. coli* BL21 cells and plated on ampicillin plates (100 µg/mL). A single colony was then inoculated into a 5 ml primary culture containing ampicillin (100 µg/mL) in non-inducing media (NIM, prepare as described previously[3]) and grown for 5-6 h with shaking (250 rpm) at 37 °C. 12.5 ml of primary culture was then transferred to 250 ml secondary culture containing auto-inducing media (AIM, prepare as described previously[3]) supplemented with ampicillin (100 µg/mL) and grown at 37 °C with shaking at 250 rpm for four h. The temperature and shaking speed were then decreased to 30 °C and 200 rpm respectively, and culture was allowed to incubate for 18-22 h. The following day, cells were harvested by centrifugation at 4 °C for 20 min at 4000 rpm (Sorvall GS3 rotor). Pellets were then re-suspended in 20 mL/L culture of 40 mM Tris, pH 8.3 supplemented with EDTA-free protease inhibitor tablets (Pierce Biotechnology) and transferred to a metal cup for sonication. Cells were lysed by sonication on ice with a Q700 probe sonicator with the following settings: Amplitude 50, Process Time 2-3 min, Pulse-ON Time 1 s, Pulse-OFF Time 1 s. Crude lysate was then transferred to 50 ml centrifugation tubes and clarified via centrifugation at 14,000 rpm for 45 min (Sorvall SS34 rotor). Following centrifugation, supernatant was removed and transferred to a 50 mL Falcon tube. 5 ml of nickel agarose resin (GoldBio) was added, and the lysate-nickel mixture was incubated with nutation at 4 °C for 1-2 h. Lysate-nickel mixture was then poured into a 20 mL fritted column, and the flowthrough was saved. The remaining resin was then washed with ~20 mL Wash Buffer 1 (50 mM HEPES buffer, pH 7.5), ~20 ml Wash Buffer 2 (50 mM HEPES, 5 mM imidazole, pH 7.5), and eluted with 12 mL Elution Buffer (50 mM HEPES, 300

mM imidazole, pH 7.5). 2-mercaptoethanol (Bio-Rad Laboratories) was then added to crude lysate (200 mM final concentration), and the mixture was allowed to incubate with nutation at room temperature overnight. The resulting cleaved protein was then dialyzed against 20 mM Tris pH 8.0 for 8-10 h. The resulting dialysate was then treated with 5 mL nickel agarose resin (GoldBio) and incubated with nutation at 4 °C for 1-2 h. The mixture was then applied to a 20 mL fritted column and flowthrough containing CaM was collected in a 15 mL Falcon tube. The resulting enriched protein mixture was then dialyzed against 20 mM Tris, pH 8.0 overnight and purified via FPLC using a 5 ml HiTrap Q-HP column (Cytiva) using the following method: Buffer A: 20 mM Tris, pH 8.0; Buffer B: 20 mM Tris, 0.5 M NaCl, pH 8.0; Gradient: 0-100% Buffer B – 100 minutes; flowrate 3 mL/min. The resulting fractions were then assessed for purity via MALDI-TOF MS, and pure fractions were combined. Protein was then concentrated to a final concentration of 100-200 µM via Amicon 3 kDa MWCO filters (Millipore Sigma). Purified protein was aliquoted into 1.5 mL tubes and stored at -80 °C until further use.

α-Synuclein Fibril Preparation. Fibrils were prepared by first diluting monomeric αS to 100 µM in PBS in a 1.5 mL tube (500 µL final volume). Tubes were then sealed with both Teflon tape and parafilm and were shaken for 7 days at 37 °C in an Ika MS 3 control orbital shaker (Wilmington, NC, USA) at 1300 rpm.

Radioligand Binding Competition Assay. Total binding is measured with the absence of competitors and non-specific binding is defined by the presence of 100 nM unlabeled BF2846 in the working buffer, 50 mM Tris-HCl, 0.01% BSA. The mixture with a total reaction volume of 150 µL is incubated for 1.5 hours at 37 °C in a non-binding 96 well plate (Corning, 3605). Bound and free radioligand is harvested with a Unifilter-96 harvesting system. (Perkin Elmer), followed by 3X washing with 250 µL ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 15 mM NaCl and 20 % EtOH. Filters containing bound ligands are added with 50 µL scintillation cocktail (MicroScint-20, PerkinElmer) and counted on the Microbeta system (Perkin Elmer). [^{3}H]-BF2846 (~4 nM) was incubated with fixed concentrations of 50 nM αS fibrils and ten increasing concentrations of competitor **SC** (0.05 nM-1000 nM). All data points are collected in triplicate. The equilibrium inhibition constant (K_i) was obtained from the IC_{50} using the K_d of BF2846 (3 nM) by non-linear regression in GraphPad Prism v.9.3.1 similarly to our previous report.[4]

Photo-reactivity Studies

Probe Photophysical Characterization. All probes (**AS1-7**, **SFX**) were diluted from a 10 mM DMSO stock to 100 μM final concentration in 50/50 MeOH/H_2O. The probe solution was then transferred to a quartz cuvette and analyzed from 210-400 nm using a UV-Vis spectrophotometer (Thermo Fisher Scientific; Waltham, MA) blanked against the diluent. Raw spectra were plotted in GraphPad Prism 9. These data are shown in **Fig. S2.**

Photo-crosslinking and Fluorescence SDS-PAGE Analysis. Fibrils (50-100 μM) prepared as described above were treated with 100 μM **AS1-7** or **SC** probe molecule from a DMSO stock (50 μL total volume, 1% DMSO) in a 1.5 mL tube. The sample was incubated at 37 °C for 1 hour. Following incubation, the lid was opened, and the sample was irradiated with shaking (500-750 rpm) for 10 min, with the lamp (UVP Multiple Ray Lamp, 254 nm, 8 watt; Analytik Jena; Jena, Germany) positioned approximately 1-2 cm from the top of the tube. Following irradiation, fibrils were directly subjected to copper-catalyzed click chemistry. Briefly, 16.5 μL of PBS was added to each sample to pre-adjust for the final reaction volume. 1.0 μL of Atto488-azide (Millipore Sigma) or TAMRA-azide (Lumiprobe; Cockeysville, MD, USA) was then added to each sample. Click-mix was made by pre-mixing 1.875 μL $CuSO_4$ (25 mM) with 1.875 μL *tris*(3-hydroxypropyltriazolylmethyl)amine (THPTA, 50 mM) per reaction/sample (i.e. 10 samples = 18.75 μL of each). 3.75 μL of this Click-mix was then charged to each sample ($CuSO_4$ final concentration: 500 μM, THPTA final concentration: 1.25 mM). Finally, 3.75 μL of sodium ascorbate (40 mM) was added (final concentration: 2 mM). Reactions were allowed to react with gentle agitation at room temperature for 1.5-2 h. Reactions were then quenched via the addition 25 μL 4xLDS loading buffer (Thermo Fisher; Waltham, MA, USA) with dithiothreitol (DTT, 200 mM) and were boiled for 30 min to disaggregate fibrillar species. 20 μL of the resulting solution was then separated via SDS-PAGE and imaged for fluorescence on a Chemidoc gel imager (Bio-Rad).

Optimal Wavelength and Irradiation Time Determination. Fibrils were treated as described above with varying wavelengths of irradiation (UVP Multiple Ray Lamp, 254 nm, 365 nm, and 427 nm; 8 watt; Analytik Jena) for 10 min or at 254 nm, for varying amounts of time. Click

chemistry with Atto488-azide and analysis via fluorescence SDS-PAGE were conducted as described above. Wavelength data are shown in **Fig. S3.**

Crosslinking Visualization via Enhanced Chemiluminescence. αS fibrils were treated as described above with irradiation at 254 nm for varying amounts of time. Click chemistry with biotin azide (Click Chemistry Tools; Scottsdale, AZ, USA) was then conducted as described for fluorophore azides. Proteins were then transferred to 0.2 μm nitrocellulose paper via wet transfer using an XCell blot II module (Invitrogen) for 1.5 h, using 1xNuPage transfer buffer. The blot was then blocked overnight using 3% bovine serum albumin (BSA; Millipore Sigma; St. Louis, MO, USA) in PBS with 0.1% Tween-20 (PBST). The following day, the blot was treated with streptavidin-horse radish peroxidase (strep-HRP; Thermo Fisher; Waltham, MA, USA) at 1:50000 dilution of 1 mg/mL stock for 1 h. The sample was then washed 5 times with PBST for 5 min each and exposed to ECL substrate (Pierce Biotechnology). After 5 min, the blot was imaged using a ChemiDoc MP gel imager. These data are shown in **Fig. S4**.

Desthiobiotin Azide Enrichment Procedures. αS fibrils were treated as described above with irradiation at 254 nm for varying amounts of time. Click chemistry with desthiobiotin azide (Click Chemistry Tools; Scottsdale, AZ, USA) was then conducted as described for fluorophore azides. Samples were then directly applied to 200 μL of streptavidin agarose resin (GoldBio; St. Louis, MO, USA) and allowed to incubate at 4°C overnight. After incubation, the resin was washed 5 times with water, and bound proteins were eluted via treatment with 10 mM biotin (MilliporeSigma, Burlington, MA, USA) for 2 hours at room temperature. Resulting monomeric crosslinked DTBA-labeled αS samples were subjected to a chloroform/methanol/water precipitation (4:3:1), as previously described.[5] The resulting protein pellet was then re-solubilized in 10 μL H_2O, 0.1%TFA. The sample was prepared for MALDI-TOF by spotting 1 μL of analyte in 1 μL of sinapic acid matrix (saturated solution in 50:50 CH_3CN:H_2O, 0.1% TFA) on a ground steel MALDI plate. The sample was then analyzed using linear positive mode with scanning between 5 and 20 kDa at 23% laser power on a Bruker UltraFlex III instrument. These data are shown in **Fig. S5**.

Mass Spectrometry Analysis of α-Synuclein Crosslinking

Intact Protein Crosslinking and MALDI-TOF Analysis. Fibrils were prepared and irradiated in a 1.5 mL tube as described above. After irradiation, 50 µL of 250 mM aqueous SDS solution (250 mM) was added, for a final SDS concentration of 125 mM. Samples were then boiled for 30 min. Resulting monomeric crosslinked αS samples were subjected to a chloroform/methanol/water precipitation (4:3:1), as previously described.[5] The resulting protein pellet was then re-solubilized in 25 µL H_2O, 0.1%TFA. The sample was prepared for MALDI-TOF by spotting 1 µL of analyte in 1 µL of sinapic acid matrix (saturated solution in 50:50 CH_3CN:H_2O, 0.1% TFA) on a ground steel MALDI plate. The sample was then analyzed using linear positive mode with scanning between 5 and 20 kDa at 23% laser power on a Bruker UltraFlex III instrument. These data are shown in **Fig. 1**, **Fig. S1** and **Fig. S5**.

LC-MS/MS Crosslinking Site Determination. αS crosslinked peptide samples were prepared as described above for MALDI-TOF analysis. Monomeric αS was re-solubilized in 100 µL ammonium bicarbonate buffer (50 mM NH_4HCO_3, pH 7.4) and digested overnight at 37°C via the addition of 1 µg of trypsin (Promega; Madison, WI, USA). Digestion was quenched by adding 1.0 µL of formic acid, and the resulting peptide mixture was cleaned up using custom C18 stage tips.[6] The eluted mixture from the stage tip was then concentrated via Speedvac (Savant), and re-solubilized in 20 µL of H_2O with 0.1% TFA. 2 µL of this sample was then analyzed directly via Orbitrap mass spectrometry on a Thermo QE-HF instrument (see MS/MS Data Acquisition and Analysis section below). These data are shown in **Fig. 1** and **Fig. S6**.

Sulfisoxazole Crosslinking

Cell Culture Procedures. For routine cultures, MDA-MB-231 or MCF7 cells were grown on 100 mm or 150 mm dishes in sterile-filtered Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) (Corning), and 1% pen/strep (Mediatech, 10 mcg/ml P – 10 mg/ml S). All cells for stable isotope labeling by amino acids in cell culture (SILAC) experiments were passaged 5 times in SILAC DMEM supplemented with 10% dialyzed FBS and heavy or light lysine ($^{13}C_6{}^{15}N_2$) and arginine ($^{13}C_6{}^{15}N_4$) from a SILAC protein quantitation kit (Thermo Fisher, cat. no. A33972). Cells were then washed with PBS and harvested in 5 ml PBS by scraping. Cells were pelleted by centrifugation at 1000 rpm for 5 min, and the supernatant was removed. The resulting cell pellet was re-suspended in 300-500 µL PBS with 0.1% Triton X-100 (Bio-Rad) and 1x HALT protease inhibitor cocktail without EDTA (Thermo Fisher), then lysed by sonication for two 30 s cycles (cycle 1: 2s on 2s off, amp 50; cycle 2: 2s on 2s off, amp 55) using a QSonica Q700 fitted with a microtip. Following sonication, lysate was centrifuged at 13,200 rpm for 60 min to separate membrane and cytosolic protein fractions. The resulting lysate cytosolic or membrane fraction was then used immediately for photo-crosslinking studies following concentration determination by DC protein assay (Bio-Rad).

Photo-crosslinking Fluorescence SDS-PAGE Analysis. Cell lysates were prepared as described above. Samples were then treated with 100 µM probe (**SFX-1** or **VCOX-1**), incubated for 1 h at 37 °C, and irradiated with either 254 nm or 365 nm light (UVP Multiple Ray Lamp, 8 watt) for varying amounts of time. Following irradiation, click chemistry and SDS-PAGE analysis was conducted as described above for αS. These data are shown in **Fig. S9** and **S32**.

Probe Treatments and Enrichment. MDA-MB-231 or MCF7 lysates were prepared as described above and the concentrations were adjusted to 7.5 mg/mL. Then, 50 µL of the SILAC sample was treated with either 250 µM probe (Heavy "H" condition) or 250 µM probe and 1 mM competitor (Light "L" condition) with a fixed final DMSO concentration of 2%. In each experiment the probe is **SFX-1** and the competitor is the corresponding unmodified compound (**SFX**). The sample was then incubated at 37 °C for 1 h and irradiated with 254 nm light for 10 min (Analytik Jena UVP Multiple Ray Lamp, 8 watt). Following irradiation, samples were conjugated to a UV-cleavable

biotin azide probe (Click Chemistry Tools) via copper-catalyzed click chemistry as described above for αS. Heavy and light lysates were then diluted with 400 µL PBS + 0.05% tween20, mixed, and applied directly to 200 µL of streptavidin-agarose resin (GoldBio). Samples were allowed to rotate at 4 °C overnight. The samples were then centrifuged at 2000 rpm for 3 min, and the flowthrough was saved. The resulting slurry was washed 5 times with PBS + 0.05% tween20, and 3 times with LC-MS grade water (Pierce Biotechnology). The washed resin was then resuspended in 500 µL of LC-MS grade water and irradiated at 365 nm for 30-45 min with shaking to fully cleave the enriched proteins from the resin. Note that in our studies of isoxazole irradiation under different wavelengths of light, reactions were negligible with 365 nm irradiation. The eluate was then harvested via centrifugation, and the resin was washed again with 500 µL of water and collected. The eluates were then combined and concentrated via speed-vacuum (Savant).

MS/MS Data Acquisition and Analysis

General Procedure for LC-MS/MS Experiments. Dried samples were resuspended in 50 µL of triethylammonium bicarbonate (TEAB) resuspension buffer (2.5% SDS and 50 mM TEAB final concentrations) and reduced with final 10 mM DTT (US Biological; Salem, MA, USA) for 30 min at 30 °C, followed by alkylation with final 50 mM iodoacetamide (Sigma Aldrich) for 30 min at 30 °C. The proteins were captured in an S-TrapTM mini column (C02-mini, Protifi; Farmingdale, NY, USA) to remove contaminants, salts, and detergents and concentrate the proteins in the column for efficient digestion then digested with trypsin (Thermo Fisher Scientific) in 1:10 (w/w) enzyme/protein ratio for 1 h at 47 °C. Peptides eluted from this column, with 50 mM TEAB, 0.2% formic acid, and 60% acetonitrile in order, were vacuum-dried and resuspended with 0.1% (v/v) TFA in LC-MS grade water for mass spectrometry analysis.

Digested samples were analyzed by a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 UHPLC system (Thermo Fischer Scientific) equipped with an in-house made 15 cm long fused silica capillary column (75 µm ID), packed with reversed phase Repro-Sil Pure C18-AQ 2.4 µm resin (Dr. Maisch GmbH, Ammerbuch, Germany). Elution was performed by the following method: a linear gradient from 4 to 38% buffer B (90 min), followed by 95% buffer B (5 min), and re-equilibration from 95 to 4% buffer B (5 min) with a flow rate of 300 nL/min (buffer A: 0.1% formic acid in water; buffer B: 80% acetonitrile with 0.1% formic acid). Data were acquired in data-dependent MS/MS mode. Full scan MS settings were as follows: mass range 200–1600 m/z, resolution 120,000; MS1 AGC target 3E6; MS1 Maximum IT 100. MS/MS settings were: resolution 30,000; AGC target 5E5; MS2 Maximum IT 100 ms; fragmentation was enforced by higher-energy collisional dissociation with stepped collision energy of 25, 27, 30; loop count top 20; isolation window 1.4; MS2 Minimum AGC target 800; charge exclusion: unassigned, 1, 8 and >8; peptide match preferred; exclude isotope on; dynamic exclusion 45 s.

MS/MS Data Analysis. AS3 αS crosslinking samples were searched using Proteome Discoverer (Thermo Fisher) with Sequest HT against the *E. coli* proteome with αS sequence information added in. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance was set to 0.02 Da. **AS3** crosslinker was specified as a dynamic modification of +354.158 Da on all amino acids.

Search parameters were set to full trypsin digestion, with maxima of four missed cleavages and three modifications per peptide. Acetylation, Met-loss, and Met-loss+acetylation were also specified as dynamic modifications. A strict false discovery rate (FDR) of 0.01 was used for the identification of high-confidence targets and a relaxed FDR of 0.05 was used for differentiation of medium and low-confidence targets. Following database searching, peptides identified as belonging to αS were filtered for containing the **AS3** modification as well as not being present in the DMSO control sample. The peptides were then sorted by descending raw abundance values, and spectra were each manually inspected to confirm crosslinking. Skyline was used for the generation of all extracted ion chromatograms, precursor/fragment spectra, and histograms shown in **Fig. S12-S31**.[7]

SFX-1/SFX-1 SILAC samples were searched using MaxQuant (Version 1.6.17.0) against the reviewed human proteome (accessed 11-15-2021). A multiplicity of 2 was used with Arg10 ("Heavy" Arginine, $^{13}C_6{}^{15}N_4$) and Lys8 ("Heavy" Lysine, $^{13}C_6{}^{15}N_2$) specified for quantification, with a maximum labeling setting of 3. Met oxidation, N-terminal acetylation, and carbamido-methylation were specified as dynamic modifications. All other settings were default using the "orbitrap" instrument type. The first search peptide tolerance was set to 20 ppm and the main search peptide tolerance was set to 4.5 ppm. Under "*miscellaneous"*, re-quantify was checked to bolster quantification of peptides identified containing only one labeling pair. Under protein quantification in global parameters, the label minimum ratio count for quantification was set to 1.

Following identification and quantification, several python scripts were used to analyze data. Briefly, minimalist filtering was conducted to remove contaminants, reverse hits, and proteins with quantification in less than 70% of replicates. A k-Nearest Neighbor (kNN) imputation approach was then adopted using the sklearn module in Python, with optimization of k against a dummy dataframe constructed by arbitrarily removing values from proteins with quantification across all replicates to match the percentage of missing data in the real dataset. The root mean square error (RMSE) of the k-imputed dummy dataset can then be cross-validated against the real dataset from which it was derived to optimize k. After this, a one-sample t-test against a theoretical population mean of 1 (no enrichment in SILAC experiment) was conducted to gauge confidence in enrichment of the given proteins, and the Benjamini-Hochberg correction was implemented with an FDR of 0.01. More stringent filtering was then conducted to select for

proteins with a computed H/L ratio of greater than or equal to 1.5, and an andromeda score of greater than 15 (just above medium confidence). This filtered protein list is available as a supplement to this manuscript. The highly enriched protein dataset was then annotated according to families and domains present in the Uniprot database, and the annotated domains were amalgamated to reduce redundancies. The domain list was then one-hot encoded and filtered for the top-10 most abundant domains by number of proteins associated. The resulting protein list was then clustered using k-means with optimization of k using the silhouette coefficient. The resulting clusters were then analyzed for the domains present. These data are shown in **Fig. 2**. Filtered and unfiltered spreadsheets along with quantification and clustering of all **SFX** protein targets can be found in the associated file **SFX_Proteomics_Data.xlsx**.

Gene ontology analysis was conducted using A GO Tool (https://agotool.org/) by feeding in the filtered gene list of proteins identified with a fold change of greater than or equal to 1.5, a Benjamini-Hochberg corrected p-value with a FDR of 0.01, and an Andromeda score of greater than or equal to 20 (200 total genes). A GO Tool was conducted against the entire Homo sapiens genome screening for overrepresented GO categories (process, function, and cellular compartment). The corresponding report was generated with a p-value cutoff of 0.01 and a corrected p-value (FDR) cutoff of 0.05. Foreground count was filtered for one-hit terms, and redundant parent terms were filtered. These proteins are shown in **Fig. 2** and **Table S1.**

Calmodulin Crosslinking Verification. CaM was crosslinked and analyzed by SDS-PAGE in-gel fluorescence similarly to αS. CaM (100 μM) prepared as described above was treated with 100 μM **SFX** probe molecule from a DMSO stock (50 μL total volume, 1% DMSO) in a 1.5 mL tube. The sample was incubated at 37 °C for 1 h. Following incubation, the lid was opened, and the sample was irradiated with shaking (500-750 rpm) for 10 min, with the lamp (UVP Multiple Ray Lamp, 254 nm, 8 watt) positioned approximately 1-2 cm from the top of the tube. Following irradiation, CaM was directly subjected to copper-catalyzed click chemistry. Briefly, 16.5 μL of PBS was added to each sample to pre-adjust for the final reaction volume. 1.0 μL of TAMRA-azide (Lumiprobe) was then added to each sample. Click-mix was made by pre-mixing 1.875 μL $CuSO_4$ (25 mM) with 1.875 μL THPTA (50 mM) per reaction/sample (i.e. 10 samples = 18.75 μL of each). 3.75 μL of this Click-mix was then charged to each sample ($CuSO_4$ final concentration: 500 μM, THPTA final concentration: 1.25 mM). Finally, 3.75 μL of sodium ascorbate (40 mM)

was added (final concentration: 2 mM). Reactions were allowed to react with gentle agitation at room temperature for 1.5-2 h. Reactions were then quenched via the addition 25 µL 4xLDS loading buffer (Thermo Fisher) with dithiothreitol (DTT, 200 mM) and were heated at 75 °C for 10 min. 20 µL of the resulting solution was then separated via SDS-PAGE and imaged for fluorescence on a Chemidoc gel imager (Bio-Rad). These data are shown in **Fig. S11**.

Thioredoxin Crosslinking Verification. Human thioredoxin-1 was obtained commercially from Sigma Aldrich (MilliporeSigma, Burlington, MA, USA). Thioredoxin (20 µM) was incubated for 1 hour at 37 °C in the presence of **SFX-1** (5 µM) probe molecule, and with varying concentrations of **SFX** (competitor dose response: 500 µM, 50 µM, 5 µM, 0.5 µM, 0.05 µM) in a 1.5 ml tube with 20 µL final volume. Following equilibration, the lid was opened, and all samples were irradiated with 254 nm light for 10 minutes with the lamp (UVP Multiple Ray Lamp, 254 nm, 8 watt) positioned approximately 1-2 cm from the top of the tube. Samples were then directly subjected to copper catalyzed click chemistry. Briefly, 6.7 µL of PBS was added to each sample to pre-adjust for the final reaction volume. 0.3 µL of TAMRA-azide (50 µM final conc.) (Lumiprobe) was then added to each sample. Click-mix was made by pre-mixing 0.75 µL $CuSO_4$ (25 mM) with 0.75 µL THPTA (50 mM) per reaction/sample (i.e. 7 samples = 5.25 µL of each). 1.5 µL of this Click-mix was then charged to each sample ($CuSO_4$ final concentration: 500 µM, THPTA final concentration: 1.25 mM). Finally, 1.5 µL of sodium ascorbate (40 mM) was added (final concentration: 2 mM). Reactions were quenched by addition of 10 µL 4xLDS loading buffer (Thermo Fisher) with dithiothreitol (DTT, 200 mM) and were heated at 75 °C for 10 min. 10 µL of the resulting solution was then separated via SDS-PAGE and imaged for fluorescence on a Chemidoc gel imager (Bio-Rad). These data are shown in **Fig. S11**.

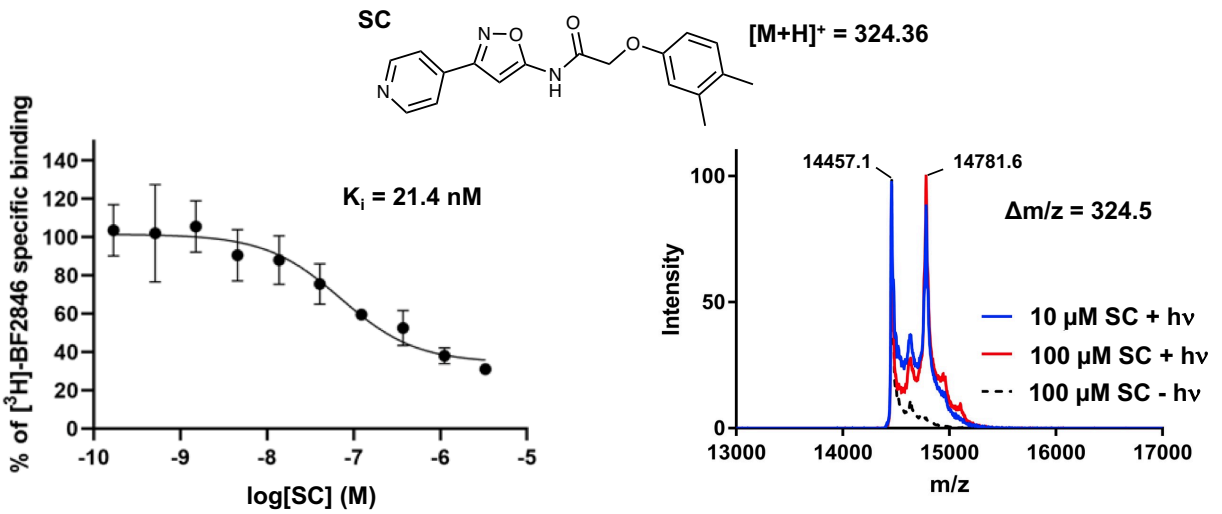


Fig. S1. SC is a high affinity αS fibril binder with intrinsic photo-crosslinking reactivity. Left: Radioligand competition binding of varying concentrations of **SC** with 4 nM [^{3}H]-BF2846. Right: Whole protein MALDI MS analysis of **SC** photo-crosslinking reactions.

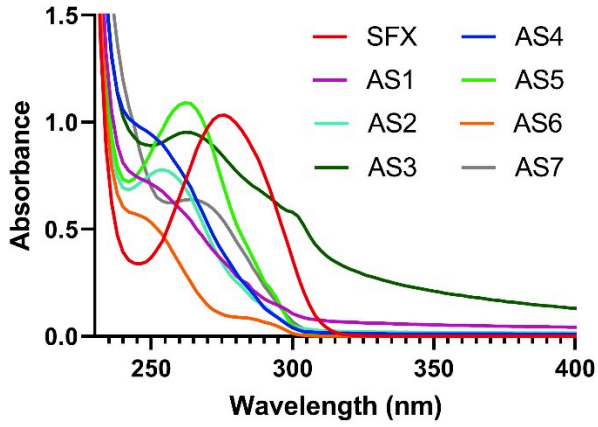


Fig. S2. UV-Vis spectra of all photo-crosslinking probes at 100 µM in 50/50 MeOH:Water obtained on a Thermo Fisher Scientific GENESYS 150 UV-Vis spectrophotometer.

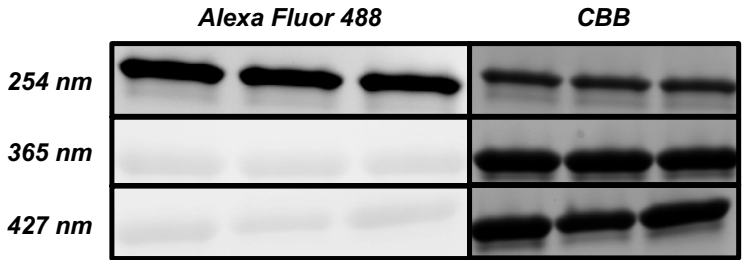


Fig. S3. Photo-crosslinking of αS with **AS3** under irradiation by varying wavelengths of light.

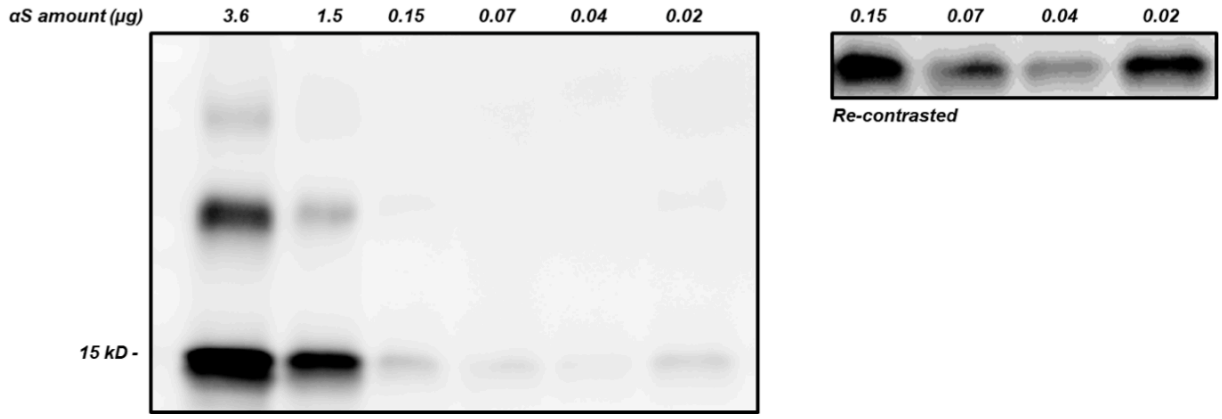


Fig. S4. Assessment of the sensitivity of probe **AS3** in identifying various amounts of αS via enhanced chemiluminescence (ECL) blotting.

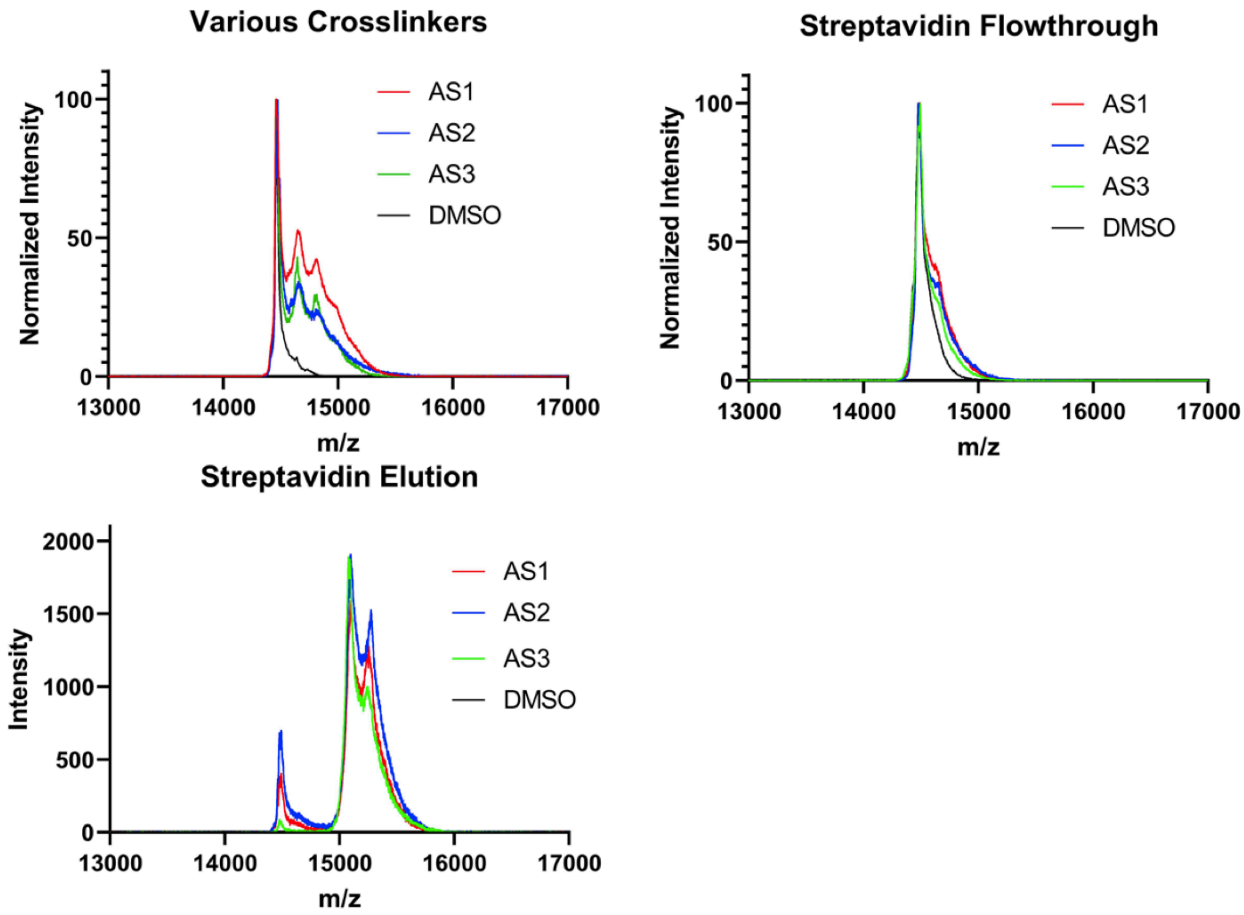
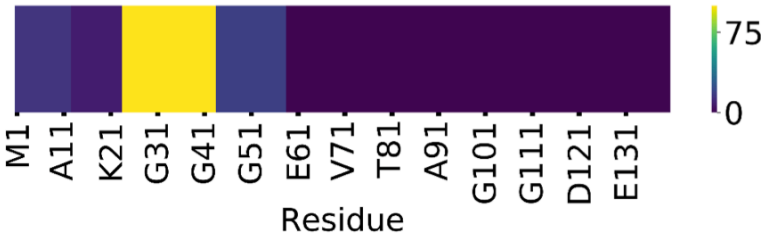


Fig. S5. Validation of **A**. crosslinking **B**. depletion by streptavidin and **C**. elution from streptavidin of various photo-crosslinkers via click chemistry with desthiobiotin azide and subsequent enrichment.



Annotated Sequence	*Positions in Master Proteins*	*Normalized Abundance*
[K].QGVAEAAGKTKEGVLYVGSK.[T]	[24-43]	100.00
[K].EGVLYVGSKTKEGVVHGVATVAEK.[T]	[35-58]	18.45
[-].MDVFMKGLSKAK.[E]	[1-12]	15.16
[K].AKEGVVAAAEKTKQGVAEAAGK.[T]	[11-32]	7.59
[K].TKEGVLYVGSK.[T]	[33-43]	4.20

Fig. S6. AS3 photo-crosslink enabled binding site mapping in αS.

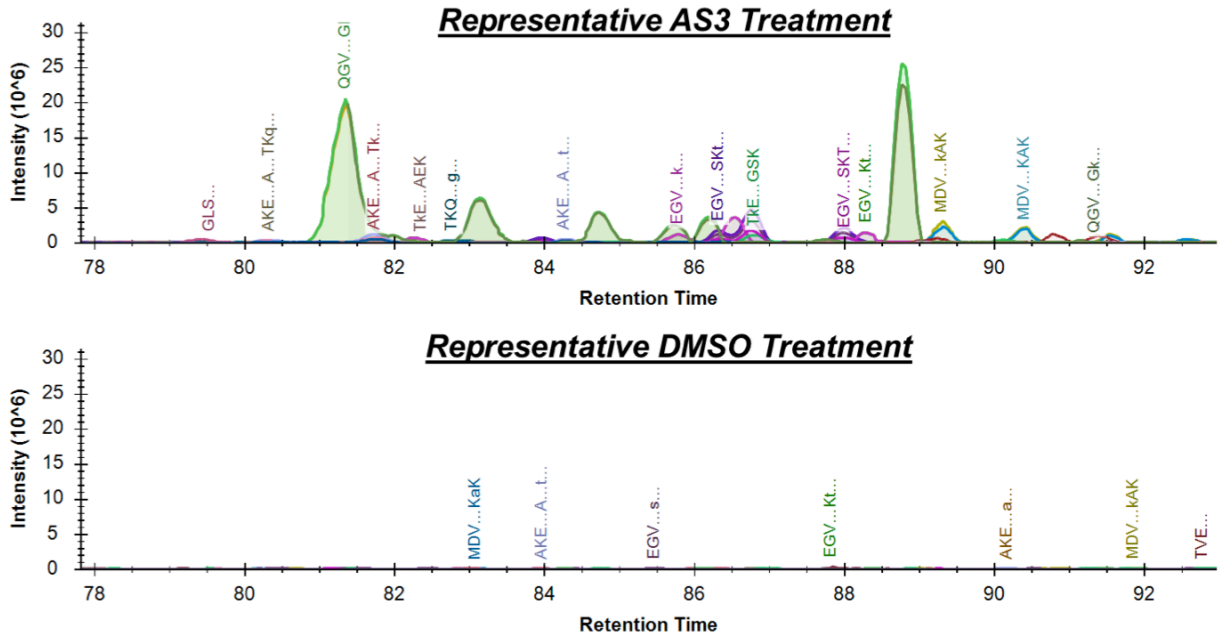


Fig. S7. Comparison of the extracted ion chromatograms of all αS crosslinks identified to **AS3** versus those identified in a DMSO negative control.

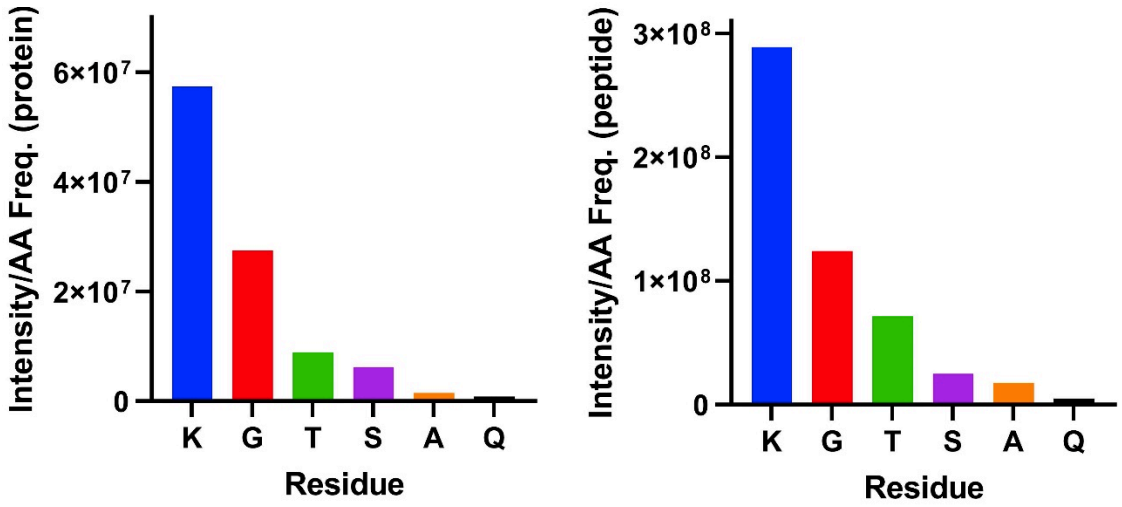


Fig. S8. Frequency of high confidence identified **AS3** crosslinks observed to various amino acids within αS normalized to the corresponding amino acid frequency (AA Freq.) in either the entire protein sequence (Left) or the peptide fragments containing the crosslinks (Right).

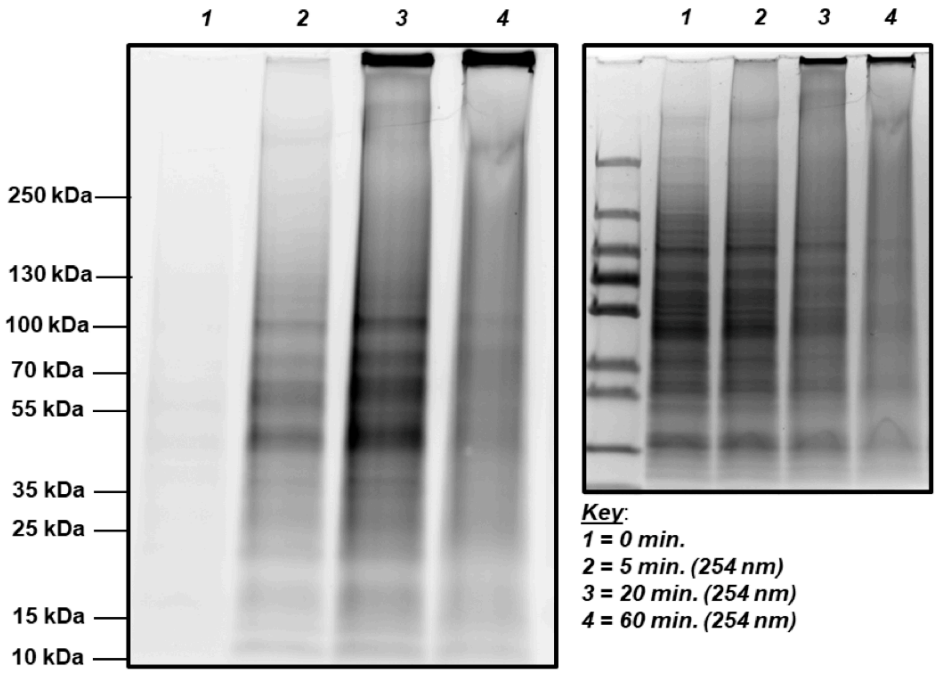


Fig. S9. Time-dependent light response of top - sulfisoxazole probe (**SFX-1**) in the presence of HEK cell lysate (2 mg/mL) Substantial aggregation can be observed at the top of the gel following irradiation times longer than 20 min.

Table S1. All 122 proteins included in domain-enrichment studies, ordered by Andromeda score.

Gene	*Fold Change*	*P-Value*	*Score*	*Domains*
IQGAP1	1.67	4.34E-05	323.31	Ras-GAP, Calponin-homology (CH), IQ, WW
FLNC	0.64	6.82E-05	323.31	Calponin-homology (CH)
ACTN4	0.99	7.67E-06	323.31	Calponin-homology (CH), EF-hand
PLEC	1.61	5.04E-06	323.31	SH3, Calponin-homology (CH)
CNN3	1.07	1.58E-04	46.093	Calponin-homology (CH)
MAPRE1	1.23	3.48E-05	88.61	Calponin-homology (CH), EB1 C-terminal
TAGLN2	1.16	3.27E-05	144.6	Calponin-homology (CH)
FLNB	0.71	9.60E-05	323.31	Calponin-homology (CH)
CNN2	1.00	5.92E-05	16.493	Calponin-homology (CH)
ACTN1	0.83	3.88E-05	323.31	Calponin-homology (CH), EF-hand
PARVA	1.18	2.20E-05	44.896	Calponin-homology (CH)
PLS3	0.92	5.94E-05	200.99	Calponin-homology (CH), EF-hand
SPTBN1	1.19	1.24E-05	323.31	PH, Calponin-homology (CH)
FLNA	0.84	6.55E-05	323.31	Calponin-homology (CH)
HNRNPA3	2.17	1.07E-03	91.12	RRM
MATR3	1.97	2.43E-04	134.54	RRM
FUS	1.69	6.16E-04	20.774	RRM
HNRNPH1	2.23	8.95E-06	176.79	RRM
HNRNPM	2.17	1.16E-04	109.37	RRM
HNRNPH3	1.62	2.82E-03	27.184	RRM
PTBP1	2.01	7.25E-05	208.2	RRM
U2AF2	1.24	3.04E-05	51.15	RRM
HNRNPA2B1	2.23	2.59E-05	323.31	RRM
RBMX	1.40	2.58E-06	32.797	RRM
HNRNPF	1.87	1.36E-05	111.76	RRM
TARDBP	1.56	1.05E-03	28.718	RRM
SRSF3	2.04	2.02E-04	16.078	RRM
SRSF1	1.78	6.63E-06	24.014	RRM
HNRNPA0	2.42	5.55E-05	66.482	RRM
G3BP1	1.42	5.83E-04	31.485	NTF2, RRM
PABPC4	1.35	8.58E-05	17.754	PABC, RRM
HNRNPD	1.39	2.86E-05	161.18	RRM
EIF4H	1.23	1.79E-03	58.641	RRM
ELAVL1	2.16	4.15E-04	36.674	RRM
PABPN1	1.14	1.34E-05	21.208	RRM
HNRNPAB	1.78	9.89E-06	21.031	RRM
SLIRP	1.62	4.47E-04	15.246	RRM
PUF60	0.74	1.99E-03	43.646	RRM 3; atypical, RRM
RALY	2.28	6.32E-05	32.622	RRM
EIF3B	1.43	9.90E-05	84.953	RRM
NCL	1.96	4.61E-05	159.34	RRM
RBM8A	0.97	1.27E-03	16.499	RRM
HNRNPR	2.17	1.96E-04	78.424	RRM
HNRNPA1	2.18	6.70E-05	323.31	RRM
SSB	0.95	4.51E-04	91.833	HTH La-type RNA-binding, xRRM, RRM
EIF3G	1.58	1.07E-04	32.448	RRM
PABPC1	1.76	2.27E-05	139.42	PABC, RRM
SYNCRIP	2.05	9.92E-05	117.71	RRM
HNRNPDL	1.89	1.97E-05	15.903	RRM
HNRNPC	2.22	5.48E-05	72.91	RRM
HNRNPL	2.13	1.20E-04	164.22	RRM
GSTP1	1.11	1.71E-04	323.31	GST N-terminal, GST C-terminal
GSTM3	1.62	1.29E-05	27.764	GST N-terminal, GST C-terminal
CLIC1	1.03	5.48E-05	226.13	GST C-terminal
GSTZ1	0.80	1.01E-03	23.44	GST N-terminal, GST C-terminal
EEF1B2	1.03	2.10E-05	142	GST C-terminal
EEF1G	1.33	2.68E-05	248.17	GST N- & C-terminal, EF-1-gamma C-terminal
VARS1	1.72	2.56E-05	86.362	GST C-terminal
GSTO1	1.09	6.10E-05	71.226	GST N-terminal, GST C-terminal
TXNDC5	0.95	9.65E-05	97.378	Thioredoxin

P4HB	0.92	6.54E-05	323.31	Thioredoxin
TXNDC17	1.35	2.27E-05	18.096	Thioredoxin
GLRX3	0.89	1.85E-04	34.222	Thioredoxin, Glutaredoxin
ERP44	0.62	1.93E-04	27.433	Thioredoxin
PDIA6	1.07	4.12E-05	128.98	Thioredoxin
TXNL1	0.69	3.36E-05	27.584	PITH, Thioredoxin
PRDX1	0.95	1.96E-04	81.513	Thioredoxin
TMX1	0.97	7.19E-04	17.461	Thioredoxin
PDIA4	1.49	6.56E-05	62.078	Thioredoxin
PRDX6	1.32	3.48E-05	102.75	Thioredoxin
PRDX3	1.38	1.85E-05	100.69	Thioredoxin
PRDX2	1.23	3.84E-05	74.763	Thioredoxin
PDIA3	1.20	1.70E-05	323.31	Thioredoxin
TXN	1.25	1.72E-05	43.505	Thioredoxin
EIF3M	1.55	2.42E-05	135.65	PCI
PSMD3	1.58	6.69E-06	79.745	PCI
EIF3A	1.82	1.94E-04	155.53	PCI
EIF3C	1.96	5.51E-05	108.49	PCI
PSMD6	1.38	3.73E-05	55.742	PCI
PSMD12	1.54	1.96E-05	80.25	PCI
EIF3E	2.09	4.84E-05	46.592	PCI
EIF3L	2.04	3.22E-05	195.16	PCI
COPS4	1.14	1.96E-04	32.138	PCI
COPS3	1.19	2.50E-04	20.408	PCI
PSMD13	1.34	5.57E-05	131.5	PCI
PSMD11	1.55	1.42E-05	104.63	PCI
DDX6	1.31	5.48E-03	30.724	Helicase ATP-binding, Helicase C-terminal
DDX39B	1.33	3.38E-05	164.68	Helicase ATP-binding, Helicase C-terminal
EIF4A3	1.81	1.22E-06	24.862	Helicase ATP-binding, Helicase C-terminal
DDX5	2.13	2.19E-03	68.334	Helicase ATP-binding, Helicase C-terminal
DDX3X	2.46	4.74E-05	71.302	Helicase ATP-binding, Helicase C-terminal
EIF4A1	2.23	3.20E-05	273.45	Helicase ATP-binding, Helicase C-terminal
DHX15	2.18	3.12E-05	46.339	Helicase ATP-binding, Helicase C-terminal
DHX9	2.41	1.70E-04	150.25	Helicase ATP-binding, DRBM, Helicase C-terminal
EHD1	2.04	2.89E-05	135.43	Dynamin-type G, EH, EF-hand
MYL12A	1.76	1.24E-05	215.36	EF-hand
RCN1	1.63	1.92E-05	65.921	EF-hand
EHD2	2.23	8.38E-04	54.405	Dynamin-type G, EH, EF-hand
CAPN2	1.69	2.33E-05	323.31	Calpain catalytic, EF-hand
CALU	1.73	9.45E-04	99.975	EF-hand
SPTAN1	1.14	2.35E-05	323.31	SH3, EF-hand
PRKCSH	1.65	3.20E-04	71.618	MRH, LDL-receptor class A, EF-hand
CALM3	0.89	1.74E-04	19.063	EF-hand
S100A11	1.01	1.94E-04	30.32	EF-hand
CAPNS1	1.65	1.15E-05	148.73	EF-hand 1; atypical, EF-hand
HPCAL1	0.86	3.14E-05	36.403	EF-hand
MYL6	1.54	2.97E-05	68.304	EF-hand
CAPN1	1.48	1.09E-04	58.506	Calpain catalytic, EF-hand
S100A6	1.13	6.28E-05	33.982	EF-hand
EPHA2	1.31	1.34E-04	21.98	SAM, Fibronectin type-III, Eph LBD, Protein kinase
MAP2K2	1.82	9.26E-05	46.446	Protein kinase
CDK1	1.54	5.31E-03	22.993	Protein kinase
CSNK2A1	1.38	2.65E-05	57.815	Protein kinase
EGFR	1.23	4.48E-04	24.608	Protein kinase
PAK2	0.92	1.07E-03	64.151	CRIB, Protein kinase
OXSR1	1.78	4.12E-03	23.7	Protein kinase
KPNB1	1.13	3.85E-05	323.31	Importin N-terminal
CSE1L	1.82	3.82E-05	268.44	Importin N-terminal
TNPO1	1.70	4.30E-04	49.239	Importin N-terminal
IPO7	1.94	5.34E-05	80.164	Importin N-terminal
XPO1	2.20	2.14E-05	93.247	Importin N-terminal
IPO5	1.14	3.89E-05	137.11	Importin N-terminal

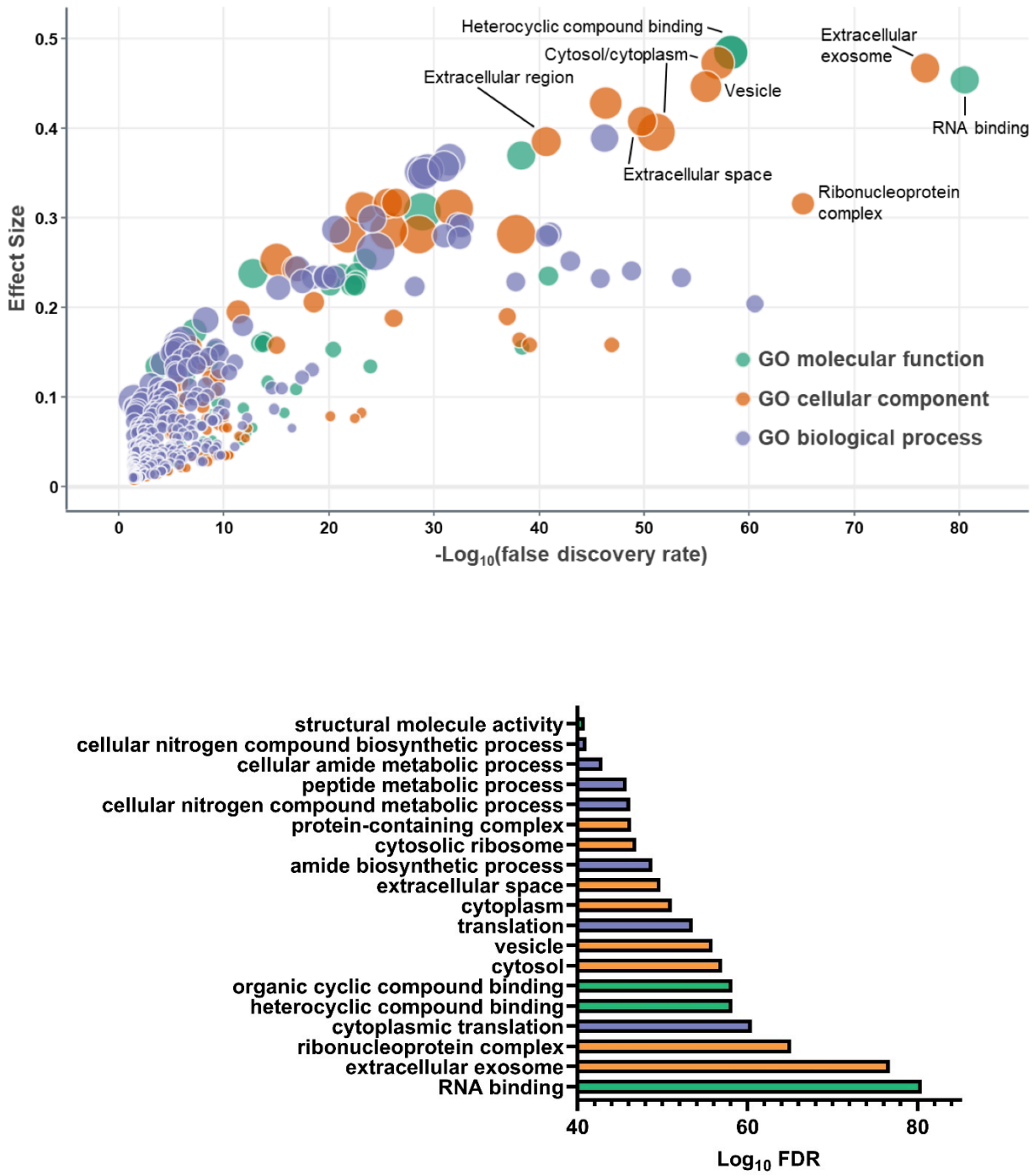


Fig. S10. Top - gene set enrichment analysis conducted in A GO Tools using the preliminary filtered protein set. Bottom – histogram representation of the top 19 GO terms enriched by FDR.

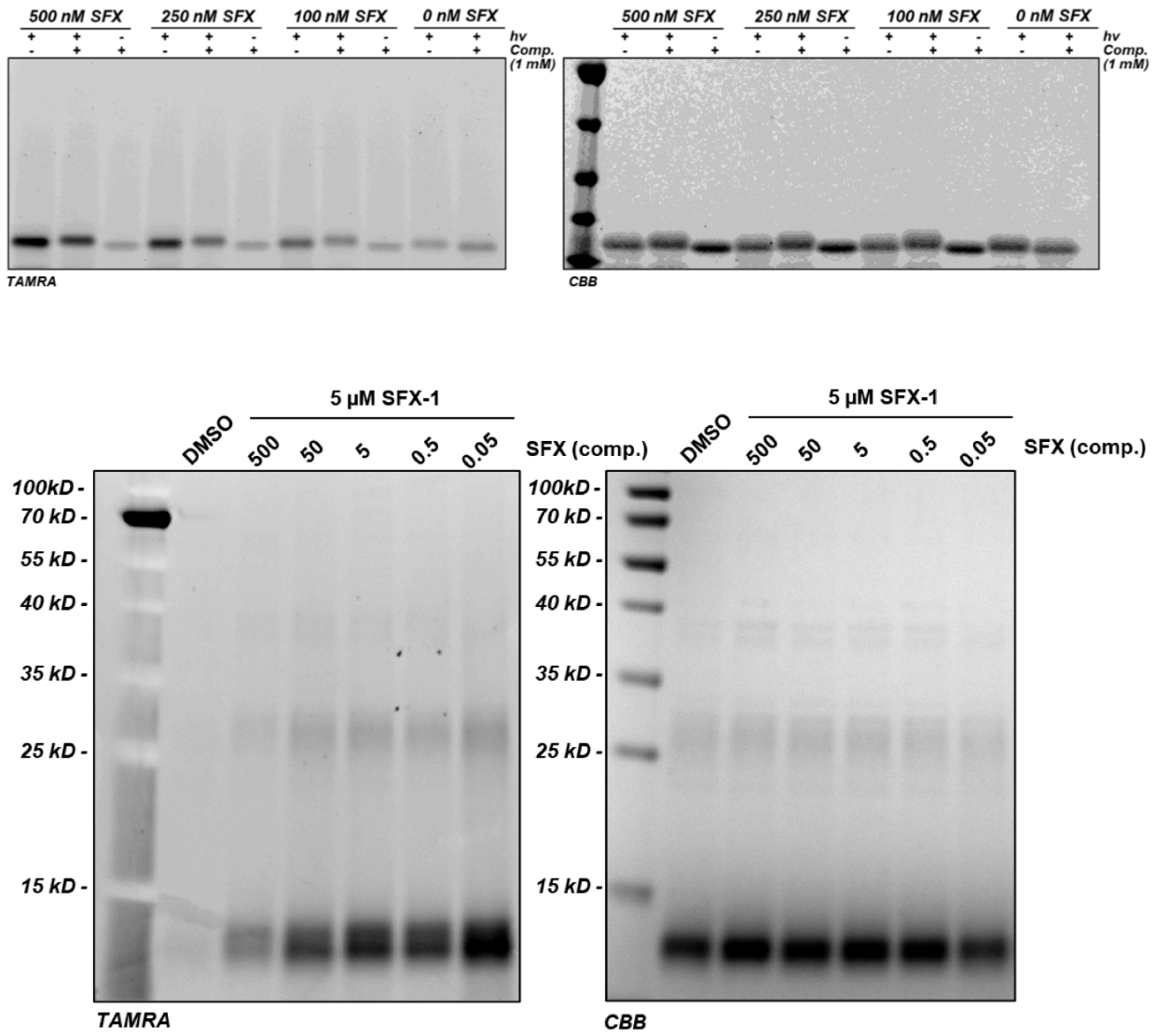


Fig. S11. Top: Photo-crosslinking to recombinant calmodulin (CaM) under varying concentration of **SFX-1** probe molecule with or without light and **SFX** competitor (1 mM). Bottom: Dose response of **SFX** competitor with thioredoxin in the presence of 5 µM **SFX-1** probe.

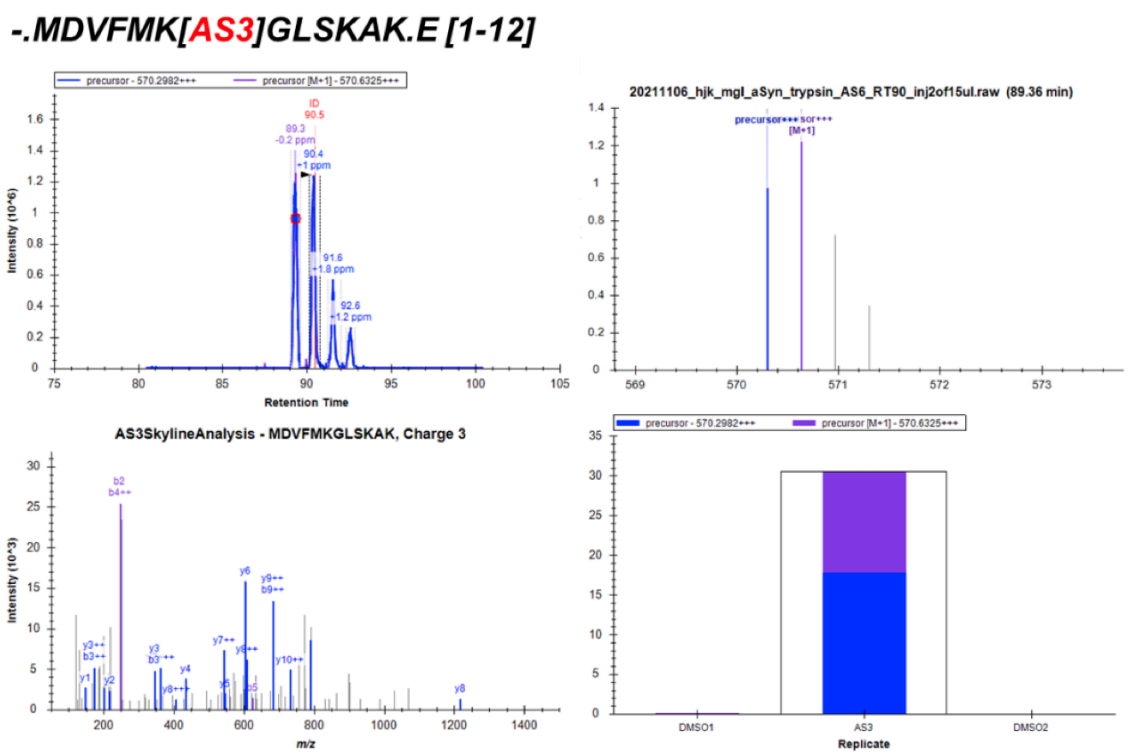


Fig. S12. Crosslinking MS/MS data

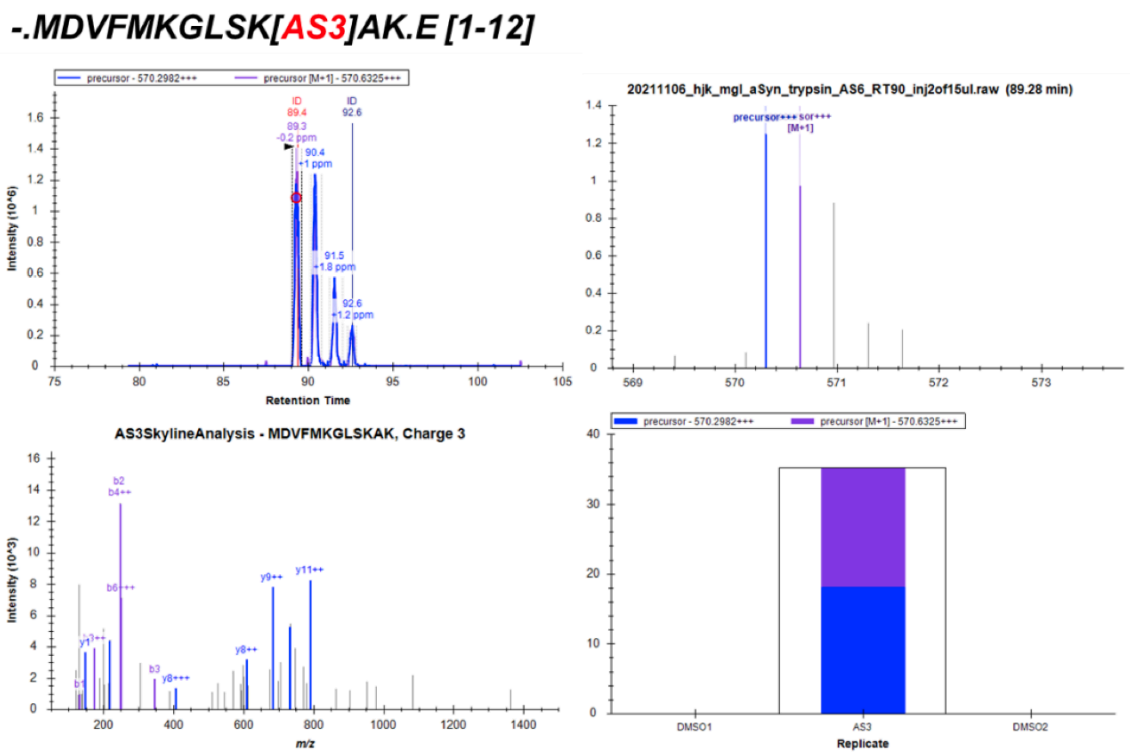


Fig. S13. Crosslinking MS/MS data

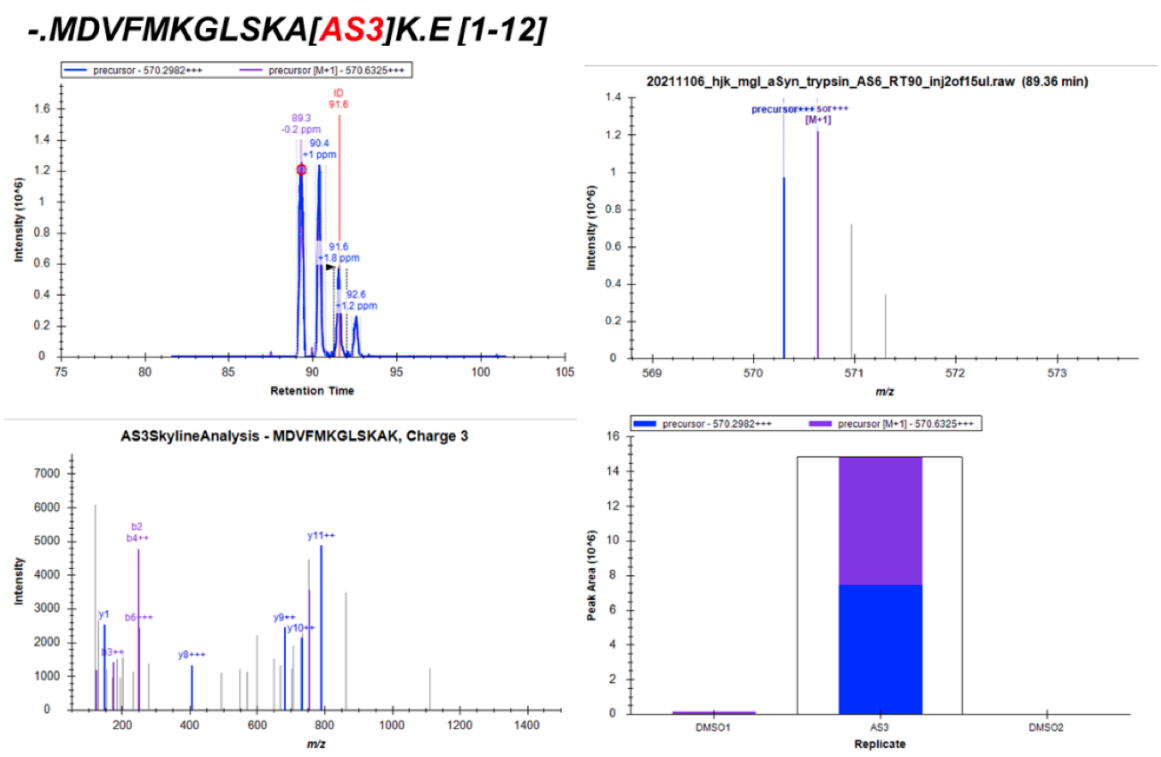


Fig. S14. Crosslinking MS/MS data

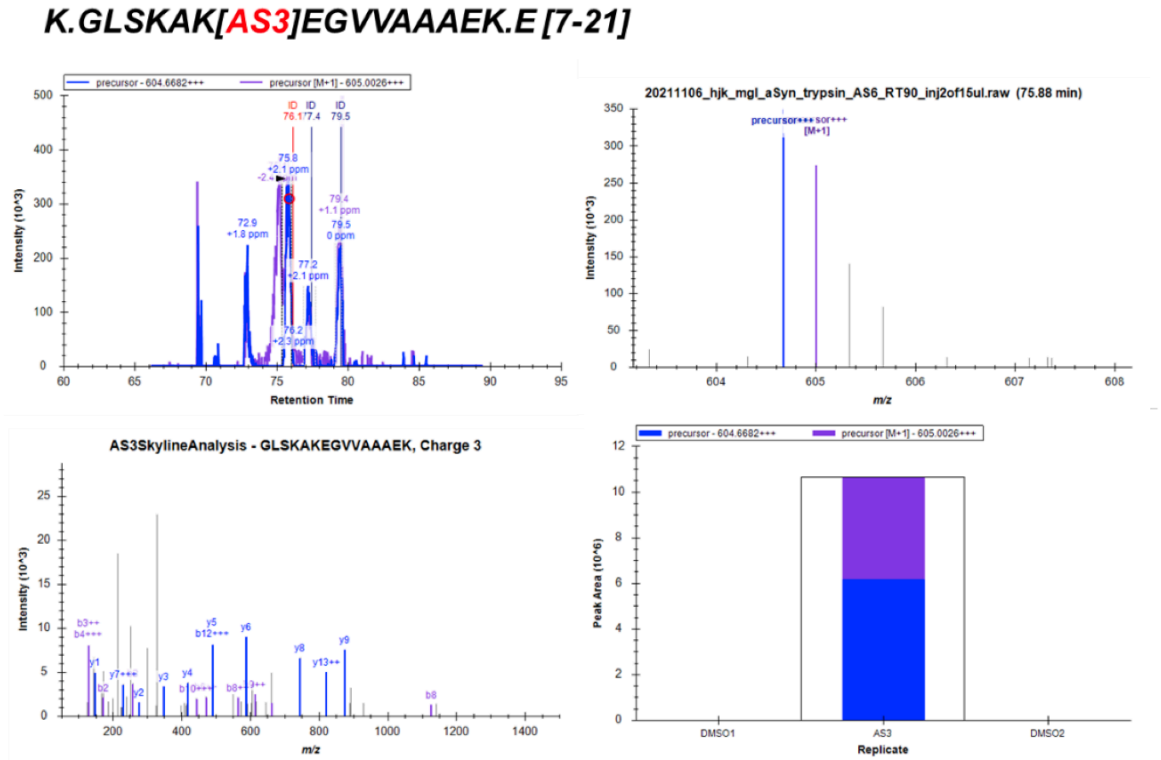


Fig. S15. Crosslinking MS/MS data

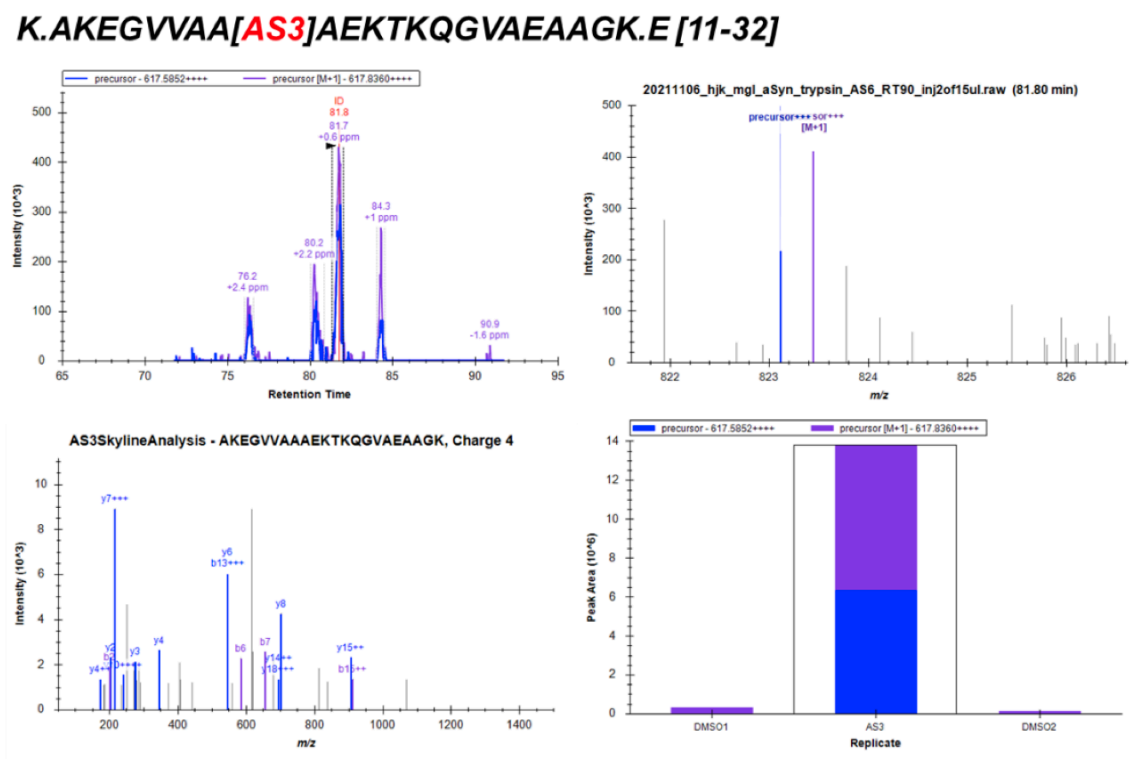


Fig. S16. Crosslinking MS/MS data

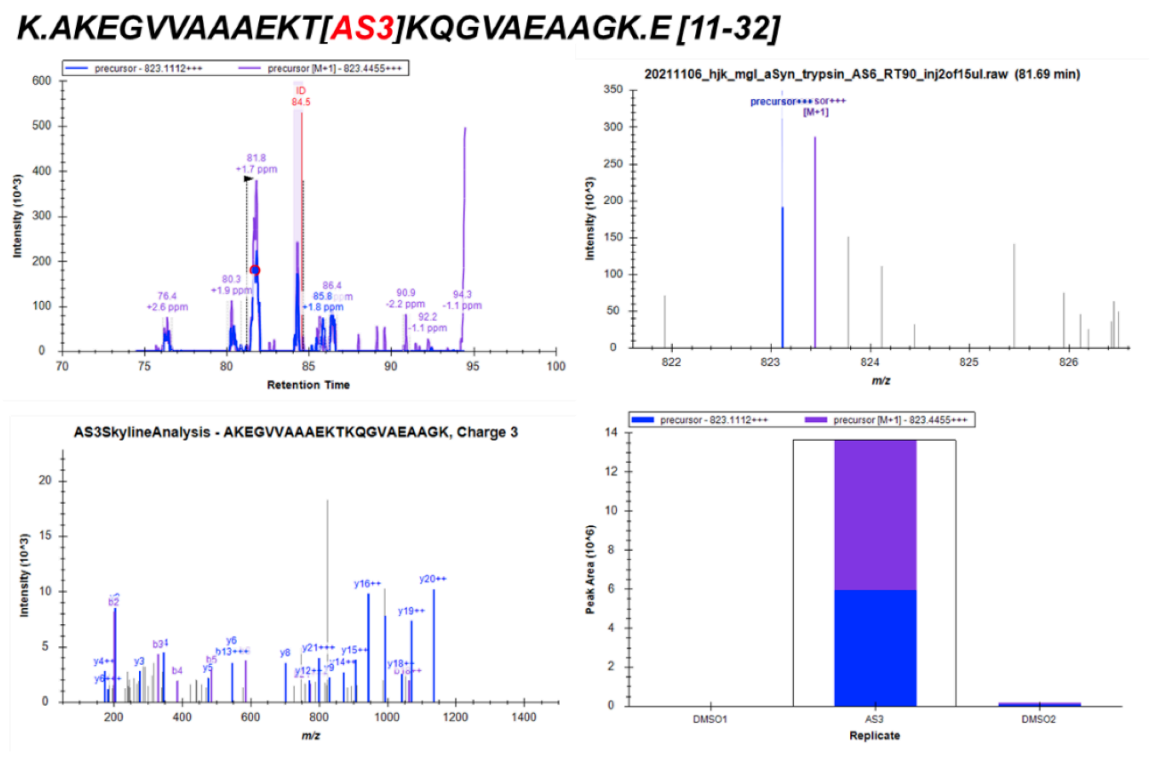


Fig. S17. Crosslinking MS/MS data

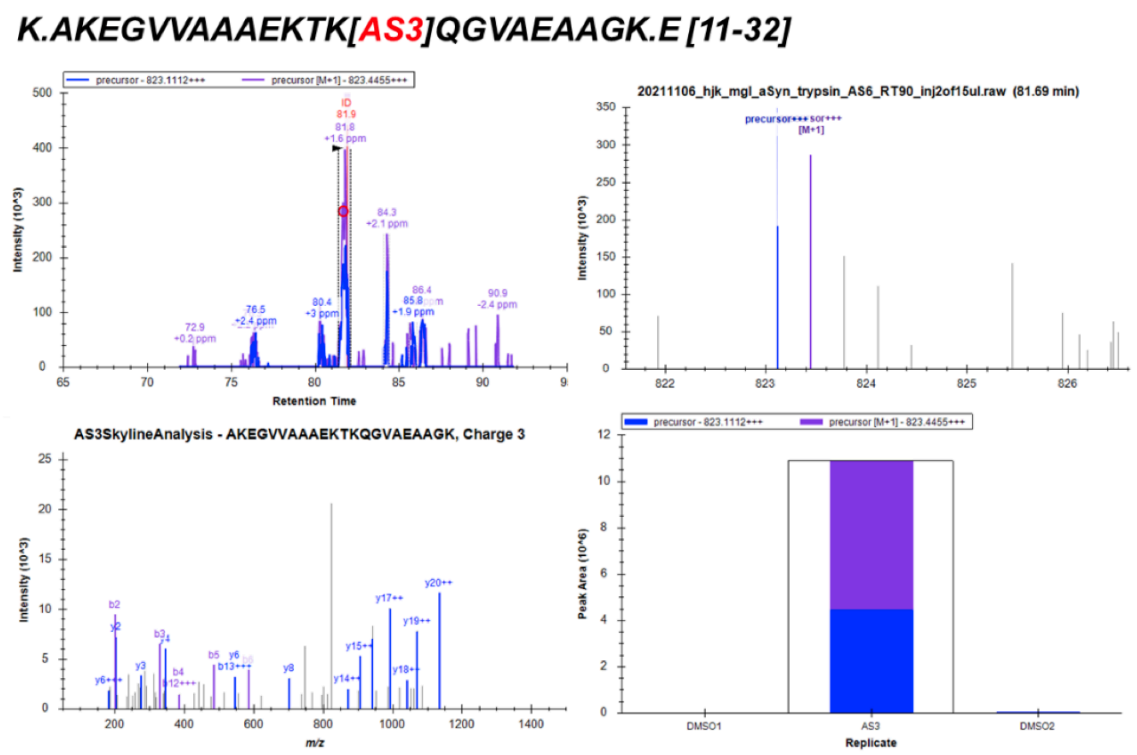
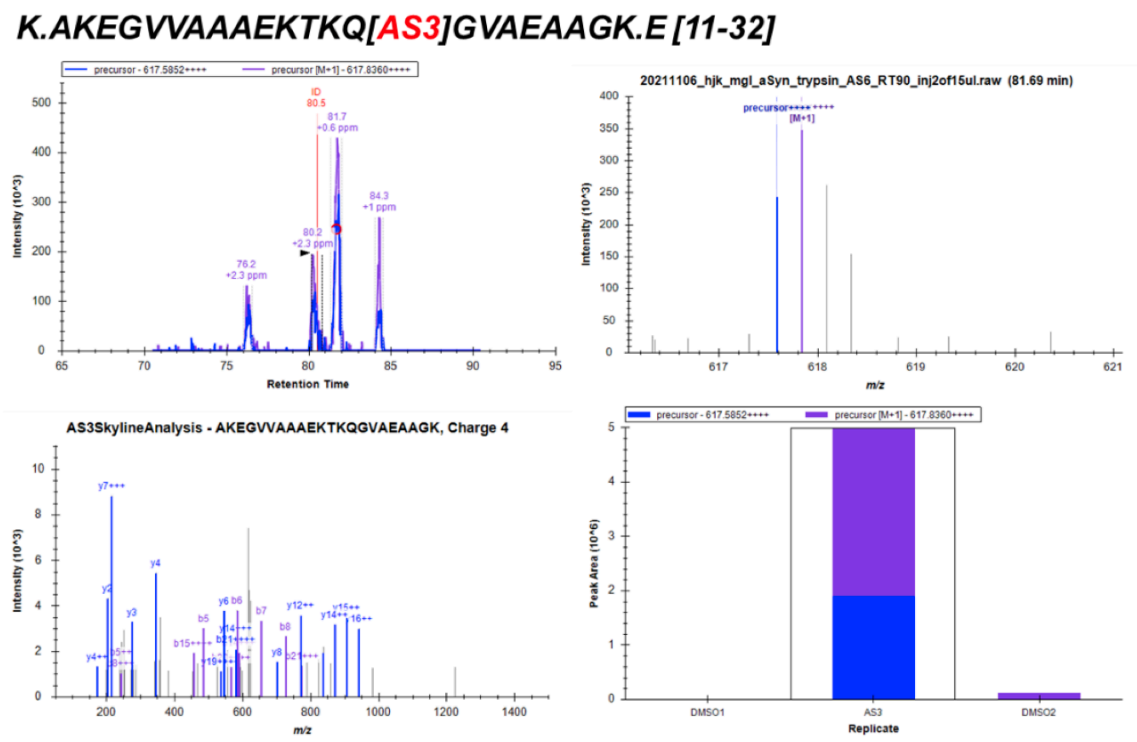


Fig. S18. Crosslinking MS/MS data



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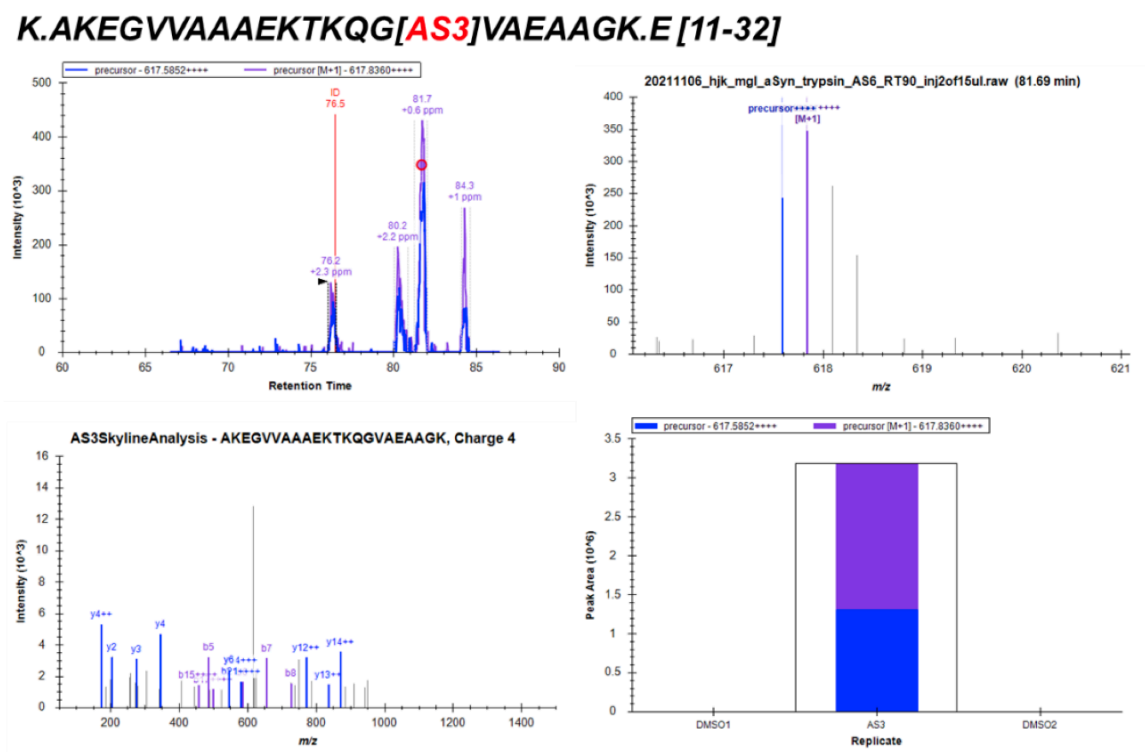


Fig. S20. Crosslinking MS/MS data

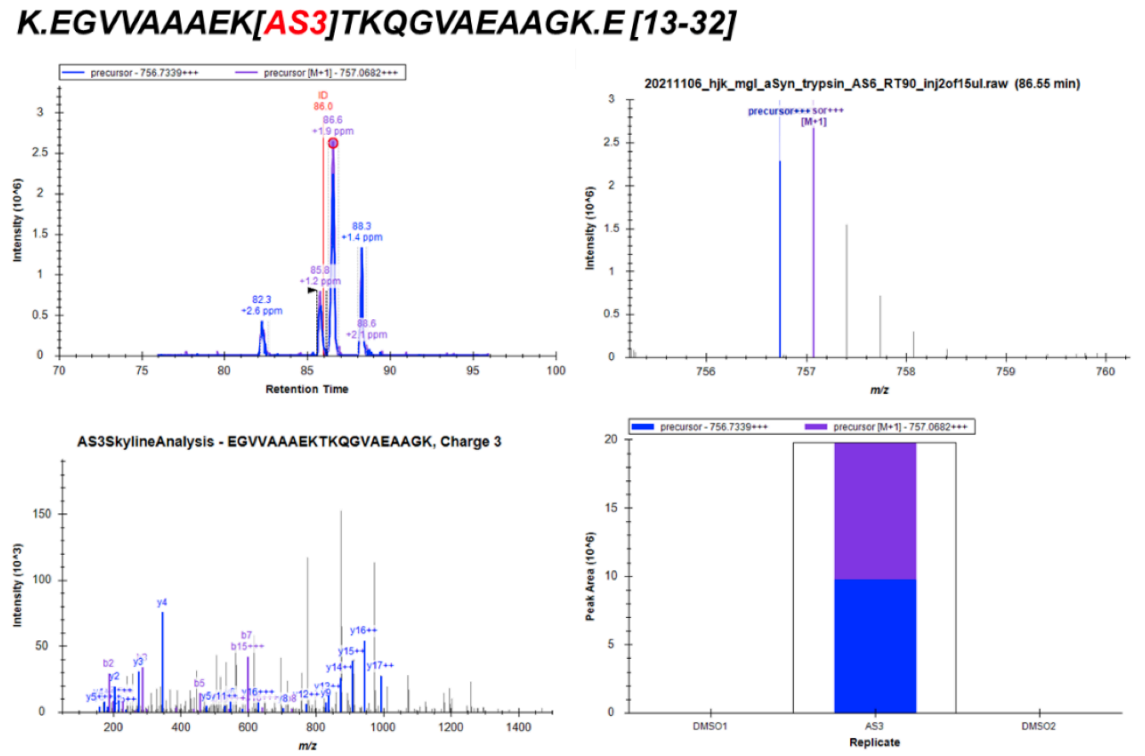


Fig. S21. Crosslinking MS/MS data

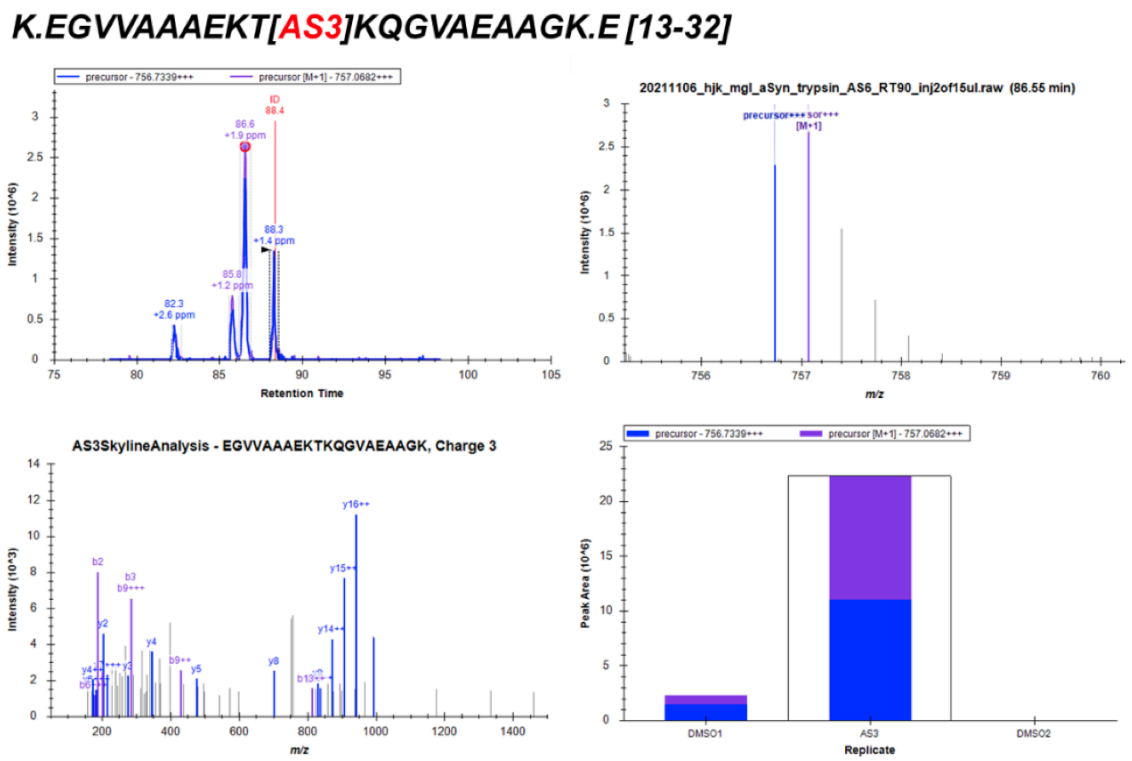


Fig. S22. Crosslinking MS/MS data

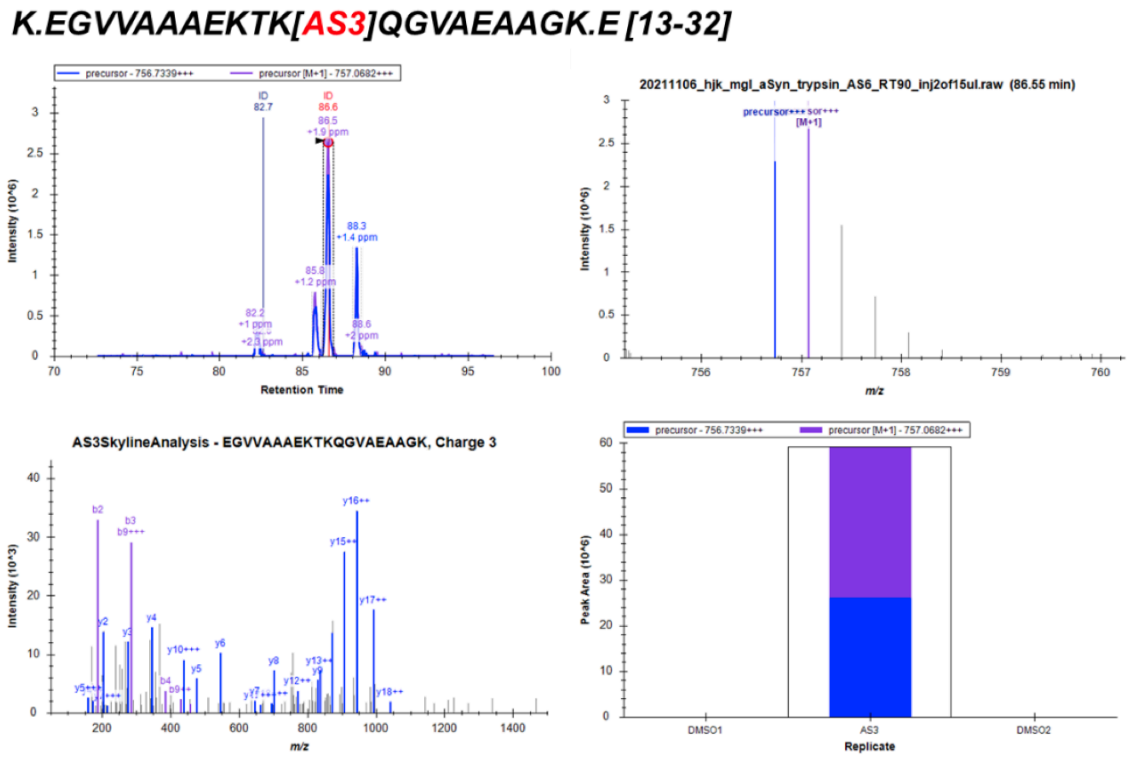


Fig. S23. Crosslinking MS/MS data

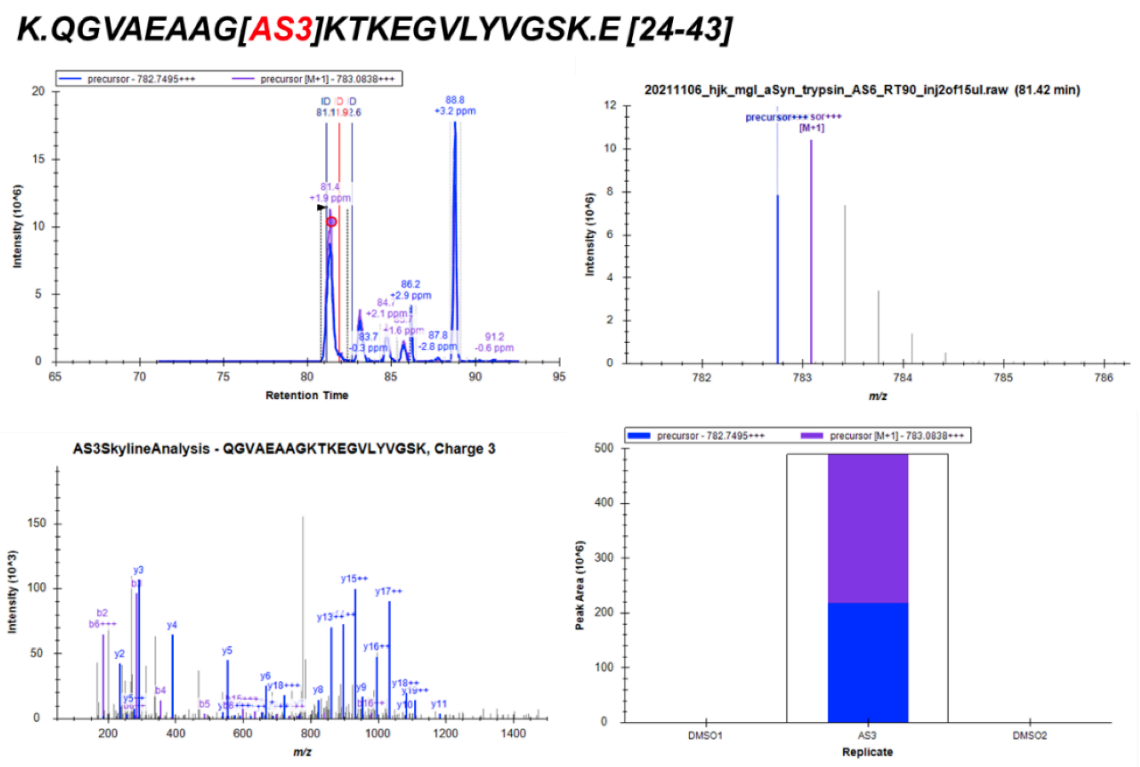
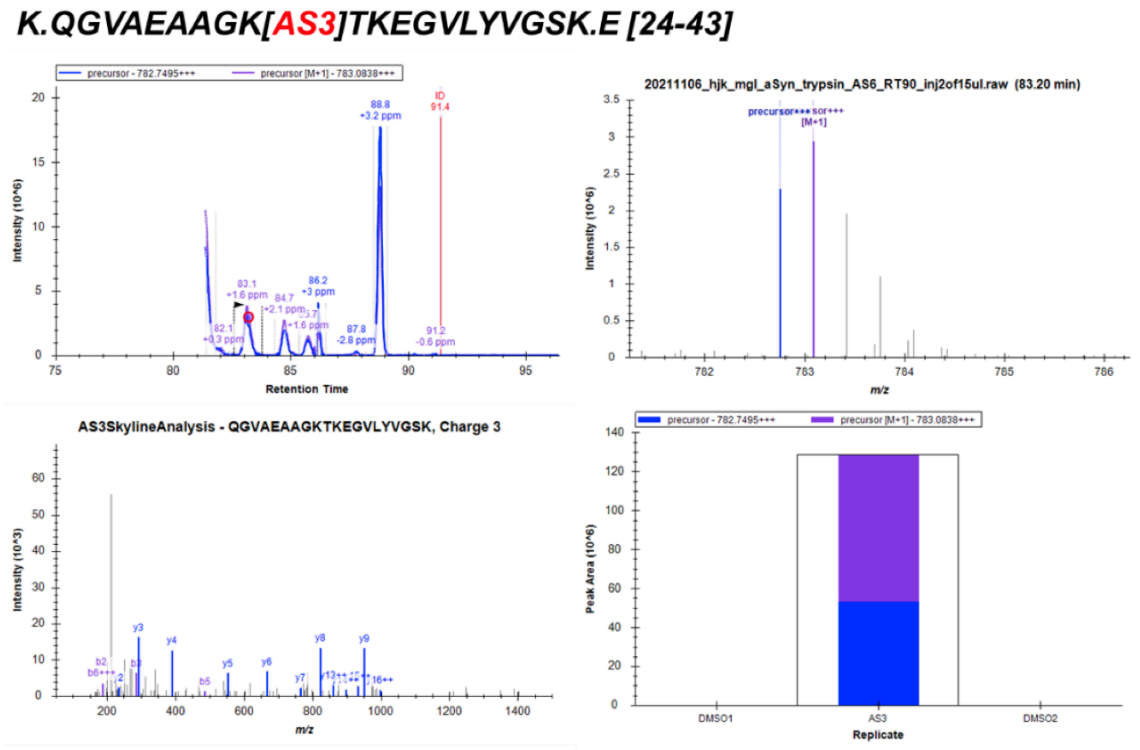


Fig. S24. Crosslinking MS/MS data



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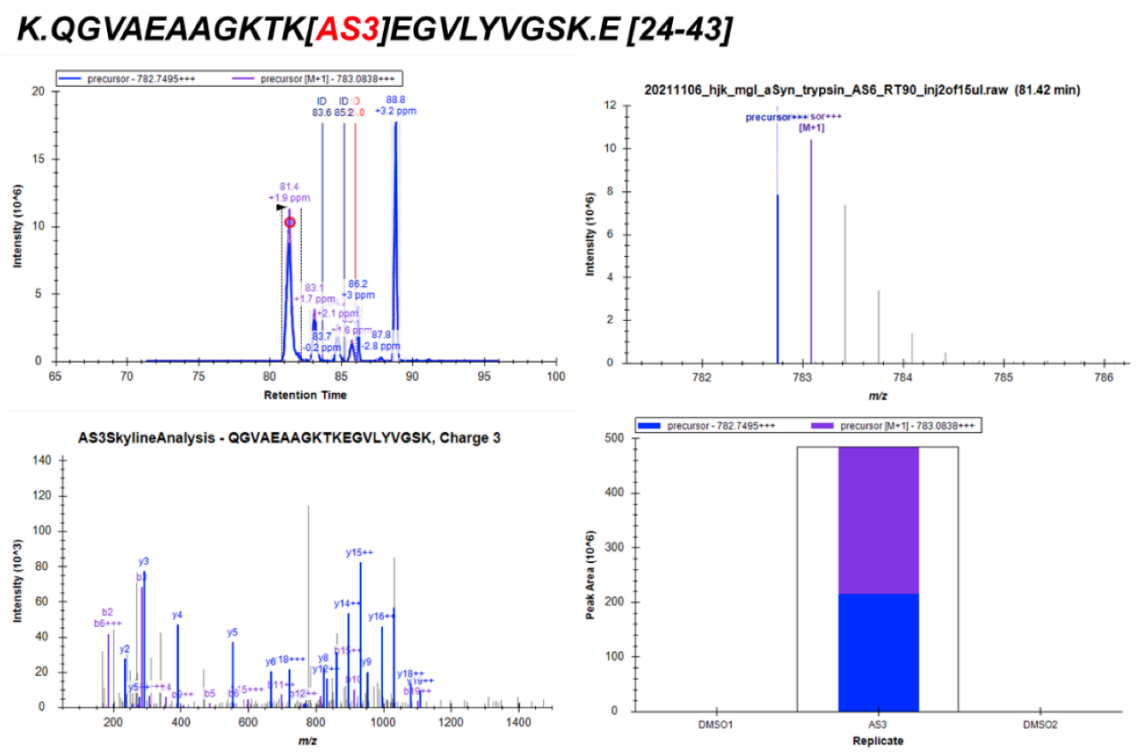

Fig. S26. Crosslinking MS/MS data

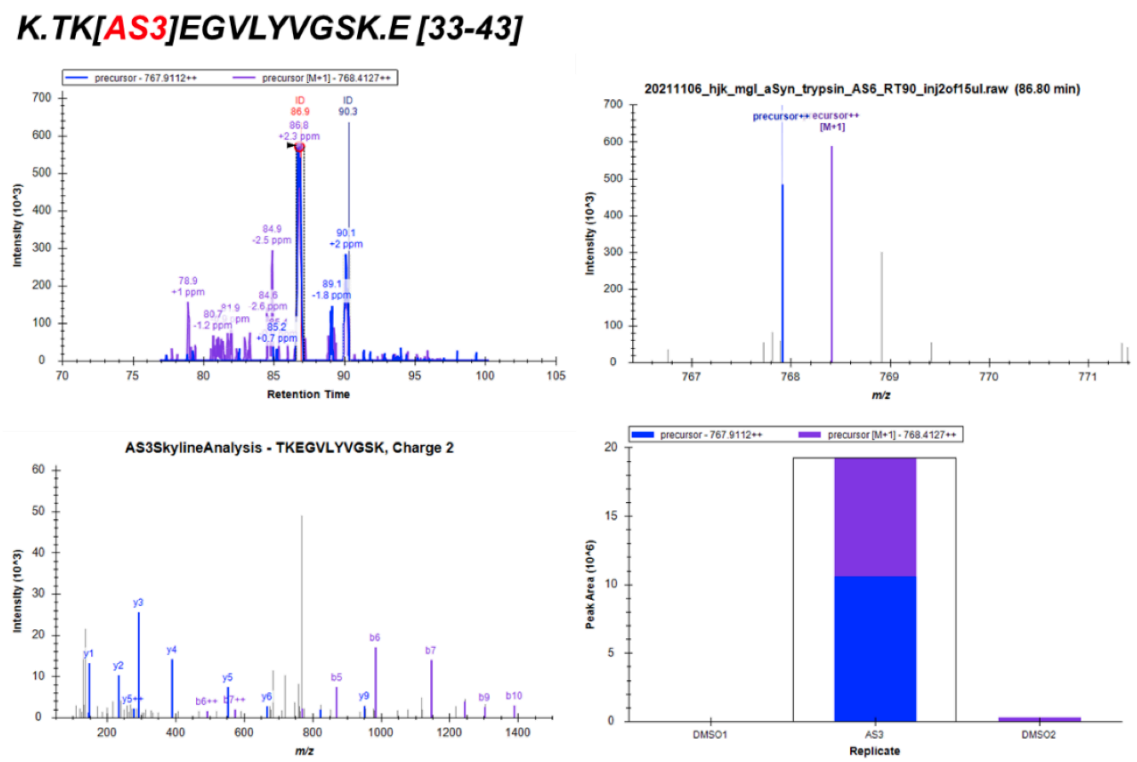

Fig. S27. Crosslinking MS/MS data

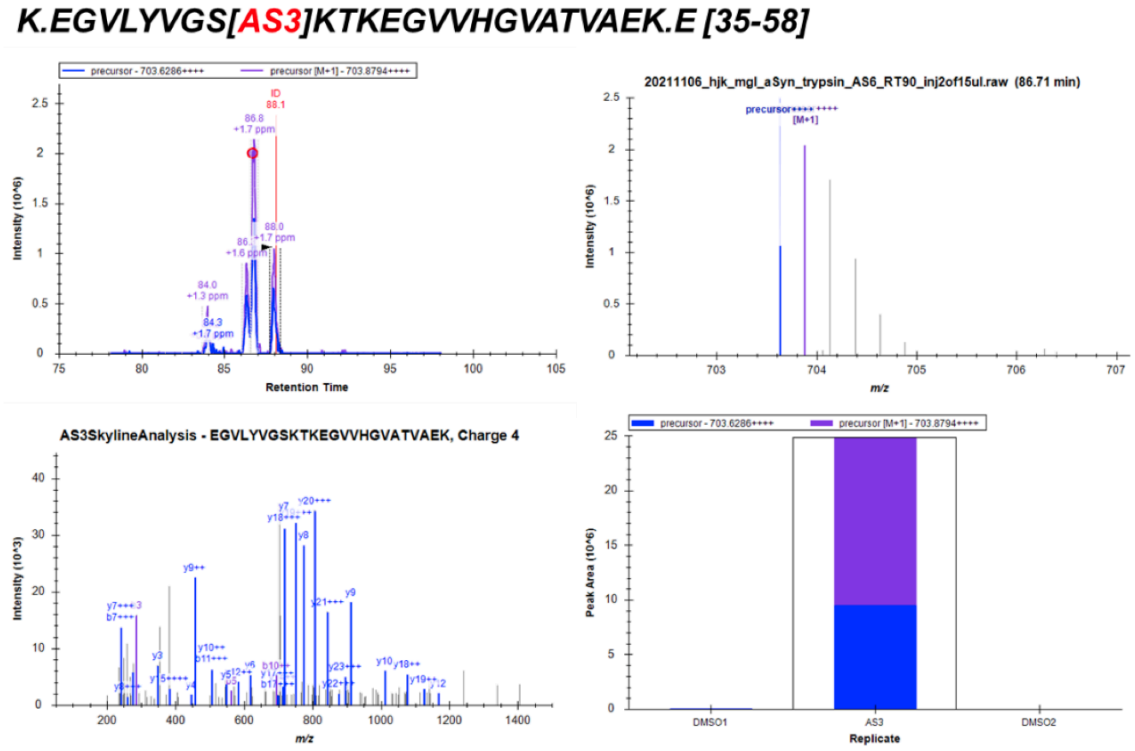

Fig. S28. Crosslinking MS/MS data

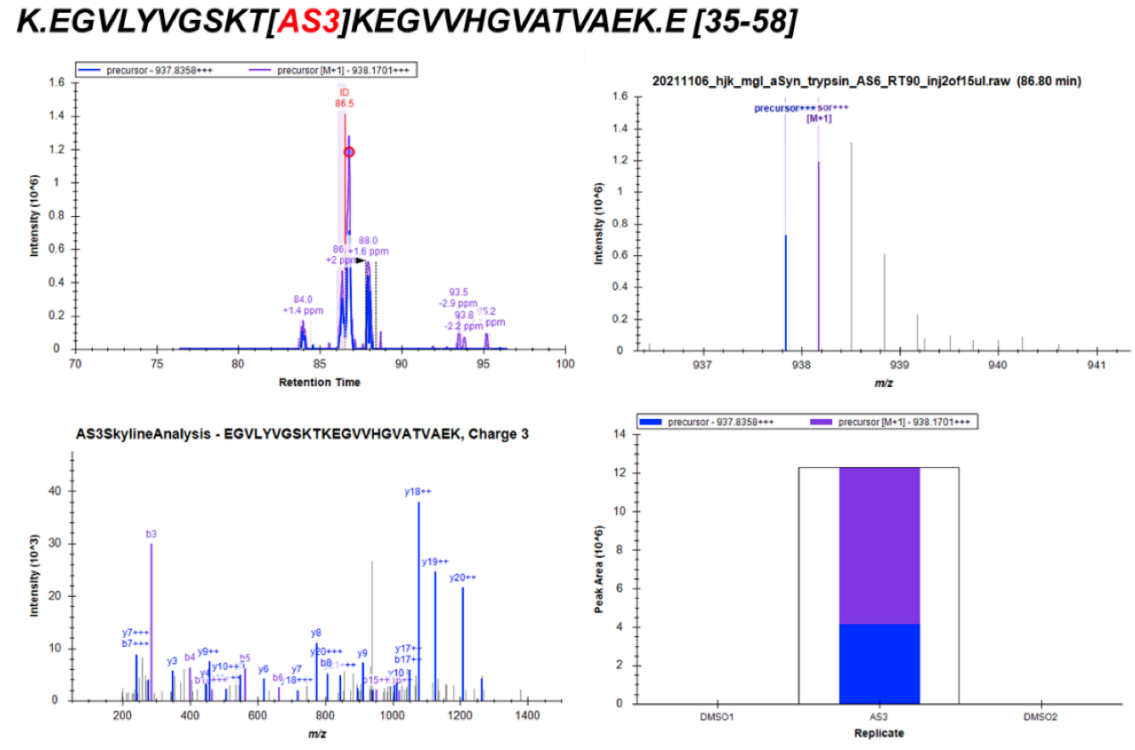

Fig. S29. Crosslinking MS/MS data

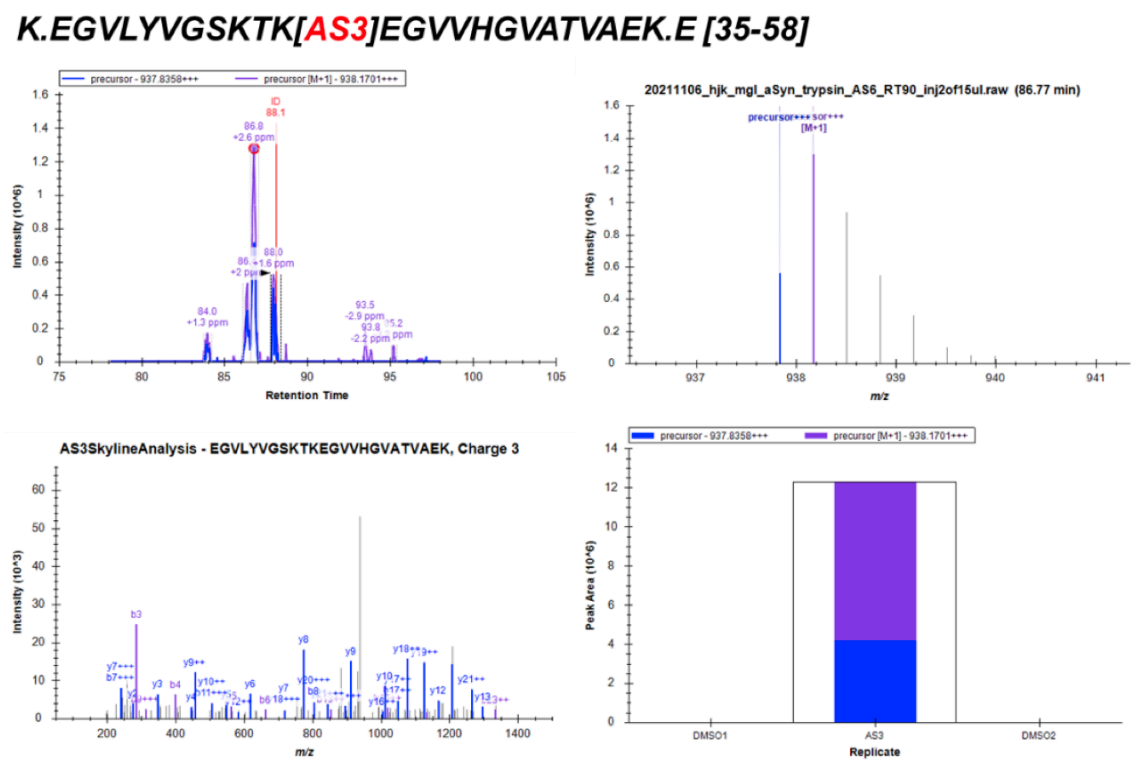

Fig. S30. Crosslinking MS/MS data

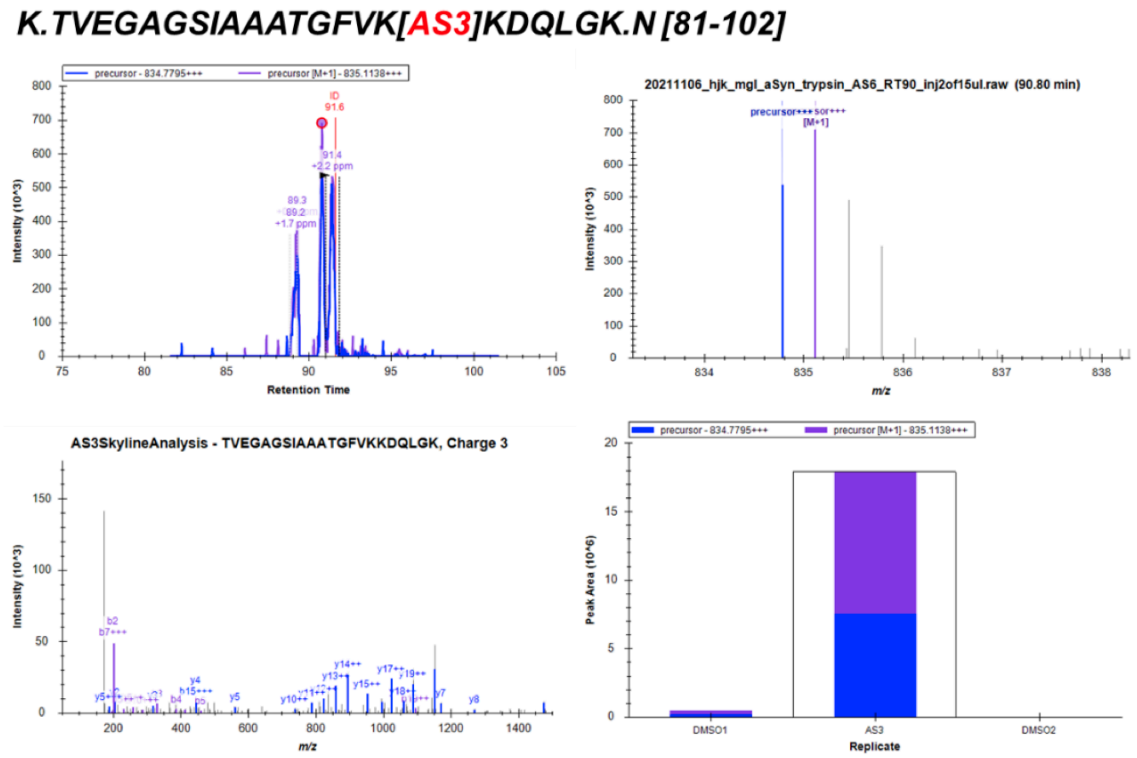

Fig. S31. Crosslinking MS/MS data

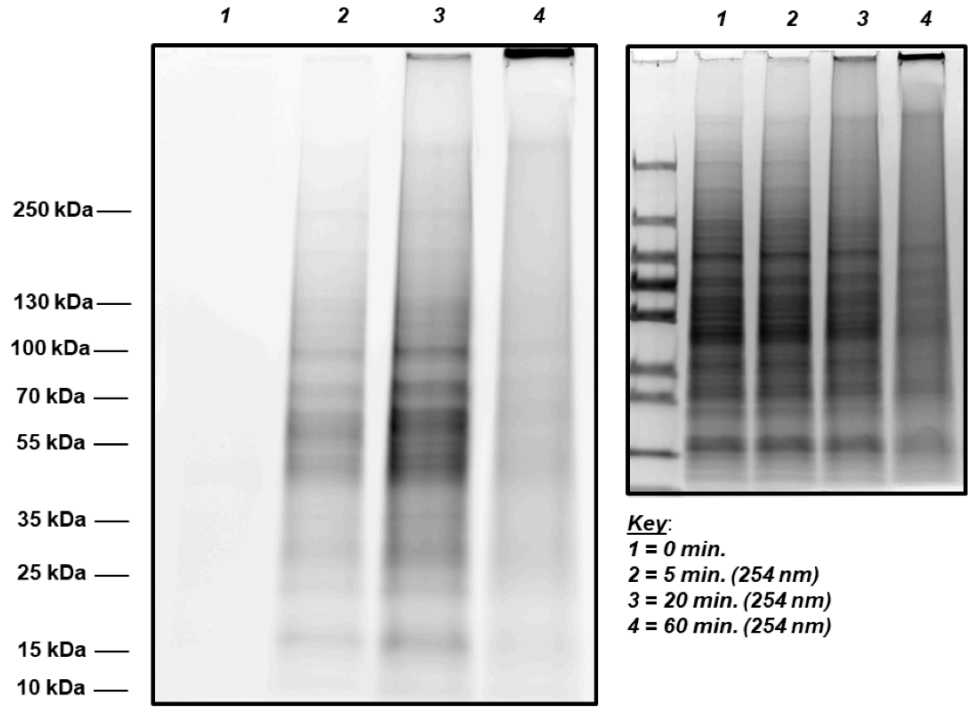

Fig. S32. Time-dependent crosslinking of Valdecoxib in the presence of HEK cell lysate (2 mg/ml)

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