Supporting Information for:

Modification of proteins with azobenzene crosslinkers using reversible covalent bonds

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Org. Biomol. Chem.

Table of Contents

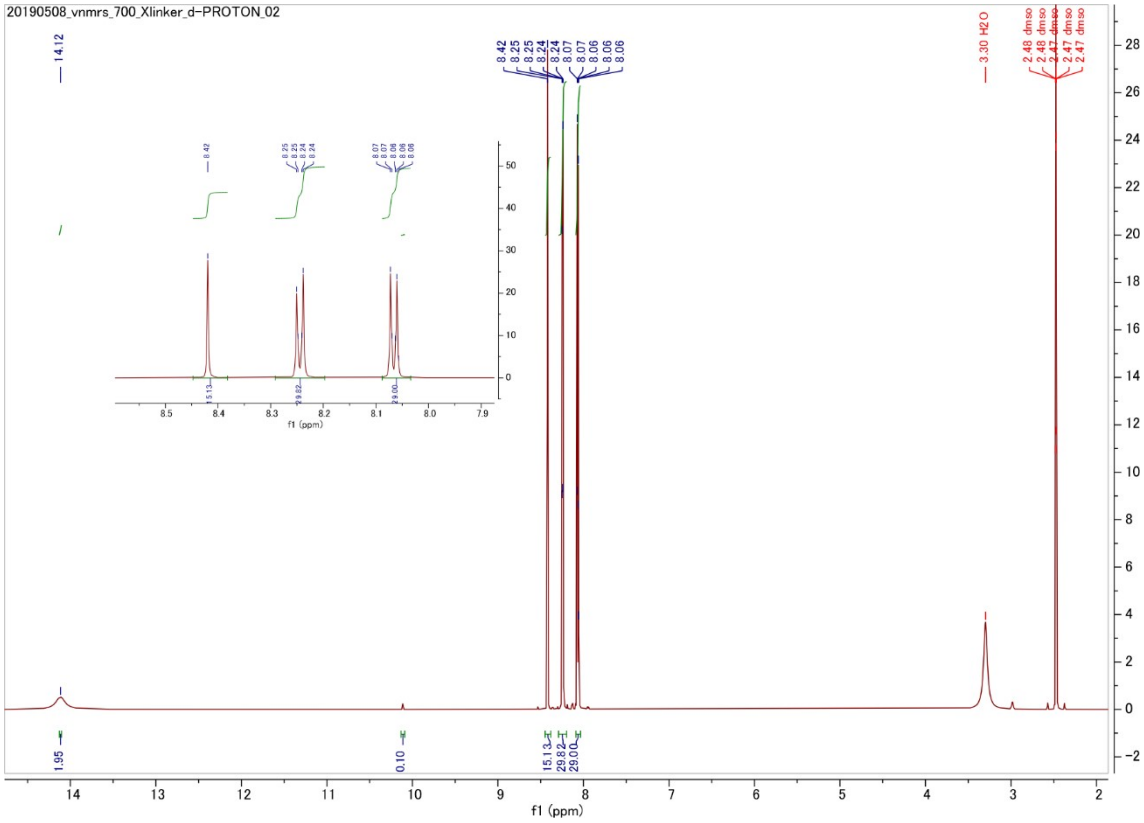
Synthetic Methods

All chemicals were used without further purification. The starting material, azobenzene-4,4'-biscarbaldehyde was synthesized according to previous literature methods.[1, 2]

NMR measurements were performed on either Varian MercuryPlus 400 MHz, Bruker Avance III-400 MHz, Varian Vnmr-S 400 MHz, Agilent DD2-500 MHz, Agilent DD2-600 MHz or Agilent DD2-700 MHz spectrometers. UV spectra were recorded on either a Perkin-Elmer Lambda 35 spectrometer or a diode array. ESI-MS measurements were performed on Agilent Technologies 6538 UHD Accurate-Mass Q-TOF LC/MS.

Synthesis of (2*E*,2'*E*)-3,3'-(((*E*)-diazene-1,2-diyl)bis(4,1-phenylene))bis(2-cyanoacrylic acid) (BCNA)(1)

To a solution of azobenzene-4,4'-biscarbaldehyde (0.052 g, 0.217 mmol) and cyanoacetic acid (0.058 g, 0.679 mmol) in acetonitrile (10 mL) was added a solution of piperidine (0.12 g, 1.42 mmol) in acetonitrile (10 mL). The solution was heated to reflux for 18 h. The resulting dark orange precipitate was collected by suction filtration, washed with acetonitrile (3 × 30 mL), and then dried under vacuum. The solid was then suspended in 10% aqueous HCl 10 mL and stirred for 1 h. The dark orange solid was isolated by filtration, washed with H_2O (5 × 2 mL) and dried *in vacuo*. Yield: 0.06 g, 72%. ^{1}H NMR (700 MHz, DMSO-d_6): δ ppm 8.06 (m, 4H), 8.24 (m, 4H), 8.42 (s, 2H), 14.12 (br, 2H). ^{13}C NMR (125.5 MHz, DMSO-d_6): δ ppm 163.4, 154.1, 153.4, 135.1, 132.4, 124.0, 116. 4, 106.0. ESI-MS: m/z calc'd for: $C_{20}H_{13}N_4O_4^+$ $[M+H]^+$ 373.093681; found: 373.0931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8.42
8.25
8.24
8.07
8.06
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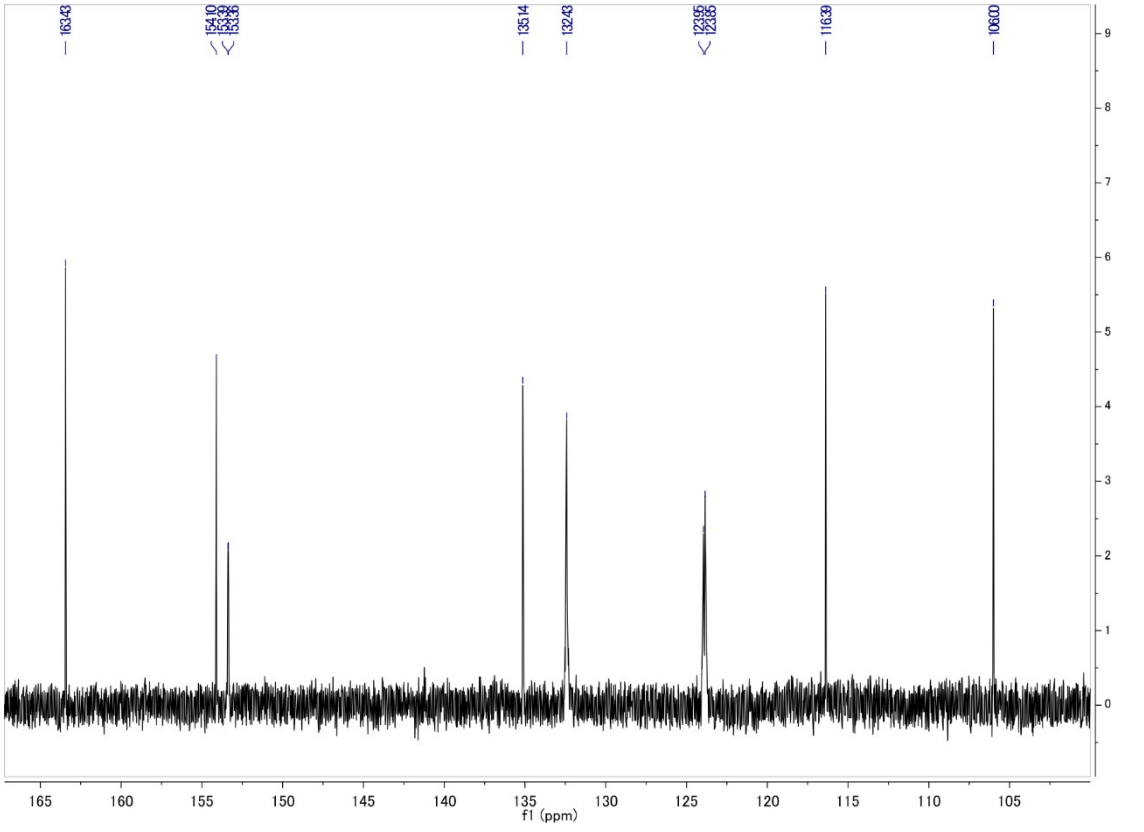
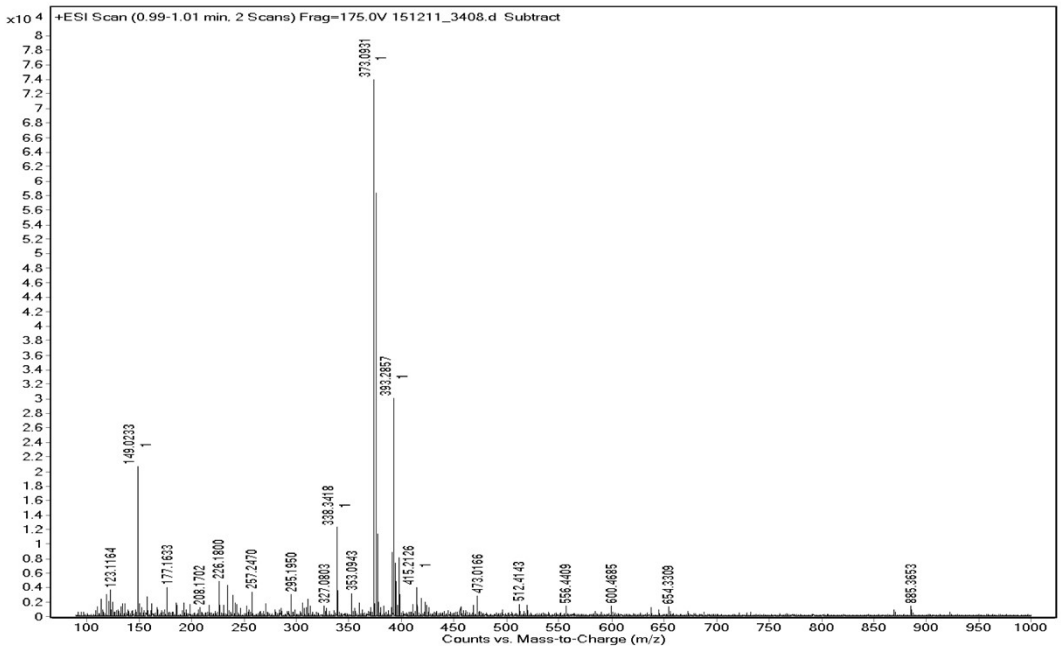
2.47 dmso
1.95
0.10
15.13
29.82
29.00
f1 (ppm)


163.43
154.10
153.36
135.14
132.43
123.95
123.86
116.39
106.00
f1 (ppm)

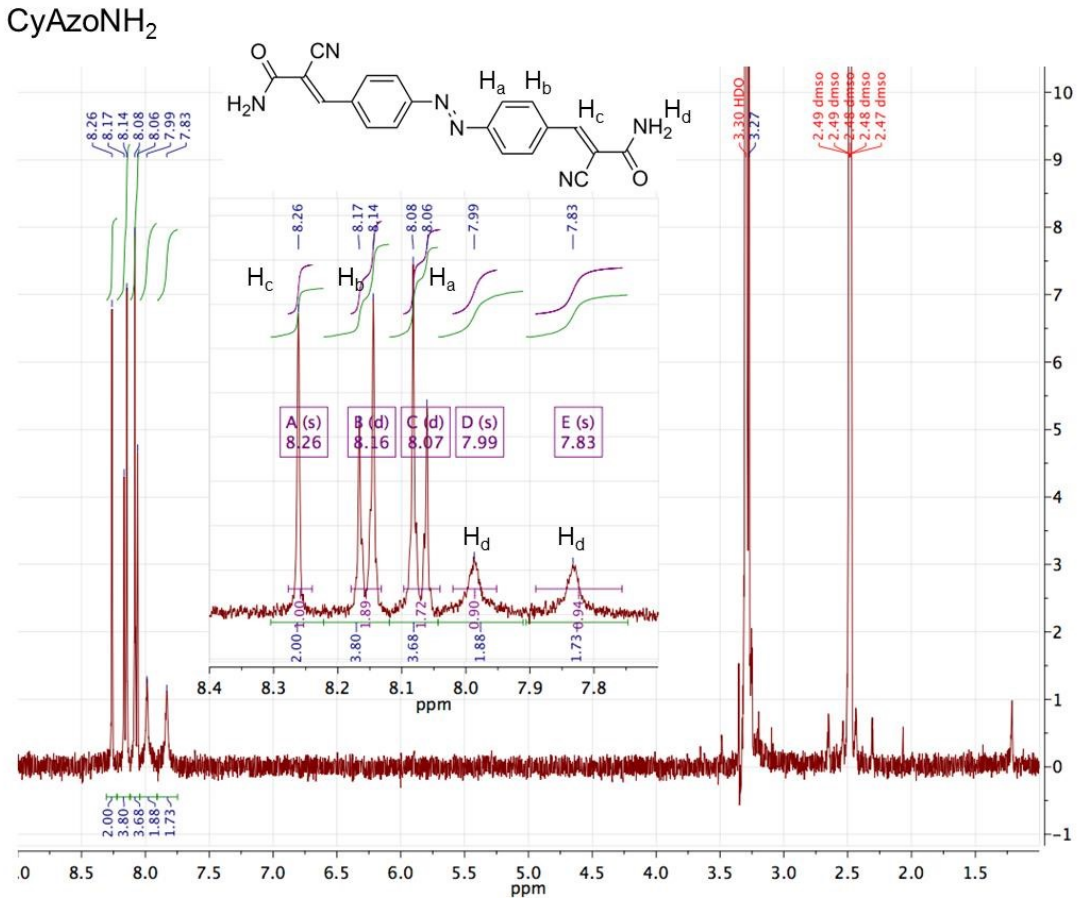
ESI Mass Spectrum

Sample Name	CyAzo-COOH	**Data File**	151211_3408.d	**Acq Method**	AIMS_Default.m
DA Method	AIMS_Accurate_Mass.m	**Instrument**	Agilent 6538 Q-TOF	**Acq Date, Time**	12/11/2015 9:46:13 AM
Comment	ESI+				



Synthesis of (2*E*,2'*E*)-3,3'-(((*E*)-diazene-1,2-diyl)bis(4,1-phenylene))bis(2-cyanoacrylamide) (2)

To a solution of azobenzene-4,4'-biscarbaldehyde (0.04 g, 0.168 mmol) in MeOH (10 mL) was added a solution of 2-cyanoacetamide (0.04 g, 0.458 mmol) and piperidine (0.04 g, 0.456 mmol) in MeOH (10 mL). The resulting mixture was stirred at room temperature for 48 h. The dark orange precipitate was collected by suction filtration, washed with MeOH and dried under vacuum. Yield: 0.05 g (80 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 7.83 (s, 1H), 7.99 (s, 1H), 8.07 (m, 4H), 8.16 (m, 4H), 8.26 (s, 2H). DART-MS: m/z calc'd for $C_{20}H_{15}N_6O_2^+$ $[M+H]^+$: m/z: 371.13 (100.0%), 372.13 (21.9%), 373.13 (3.1%), 372.12 (2.2%); found: 371.1 (100%); 372.1 (20%)



DART Ionization **AIMS Mass Spectrometry Laboratory** **AccuTOF**
Department of Chemistry - U of T

Acq. Data Name: 151106_2859 11/6/2015 3:48:58 PM
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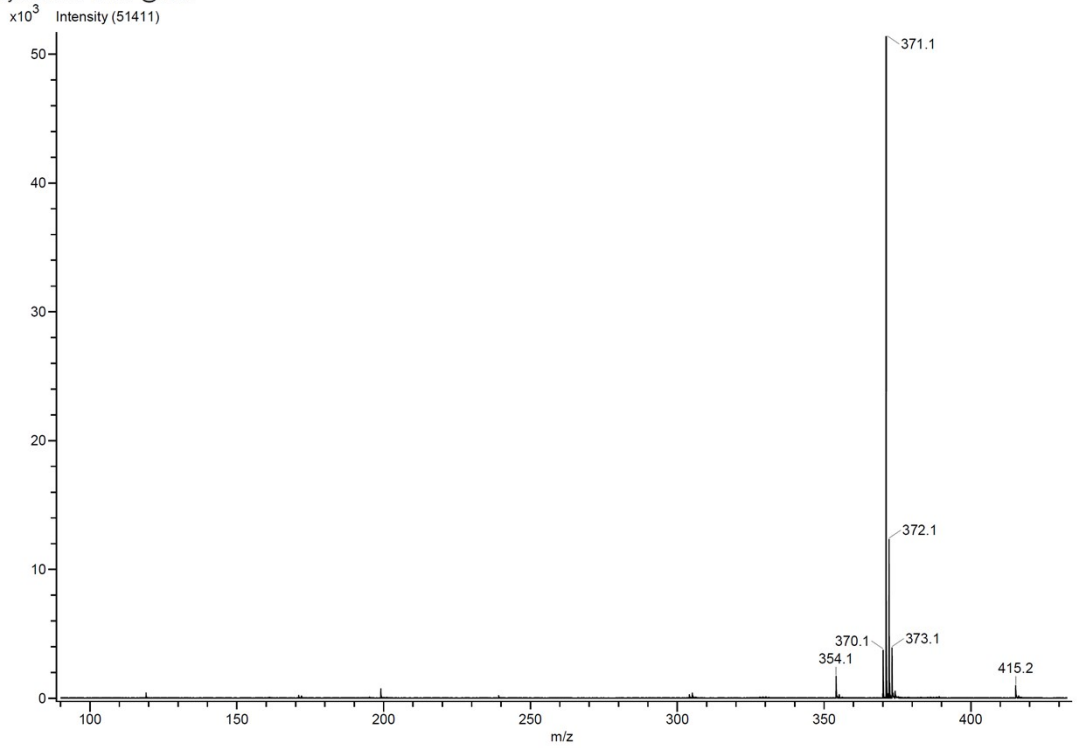
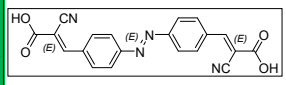
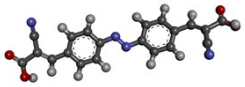
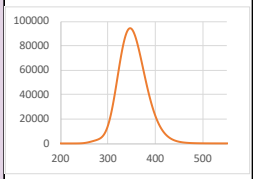
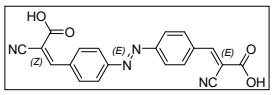
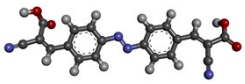
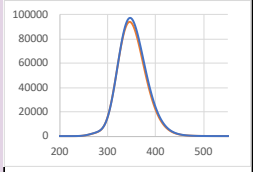
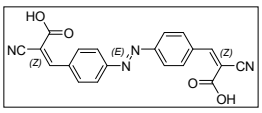
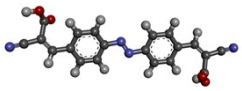
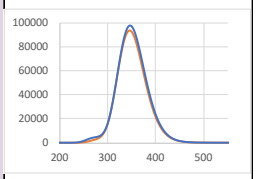
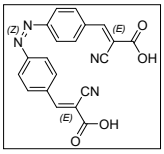
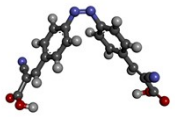
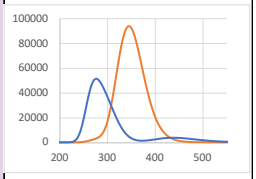
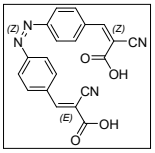
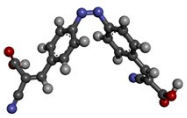
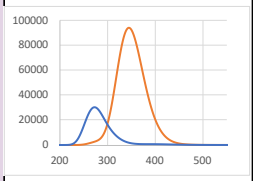
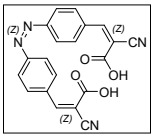
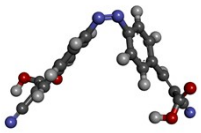
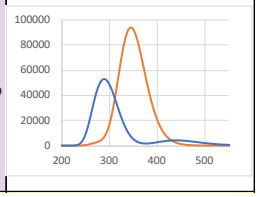
CyanoAzo2 DART @450C

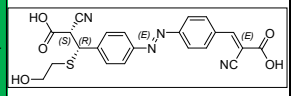
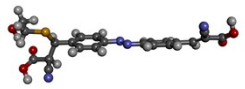
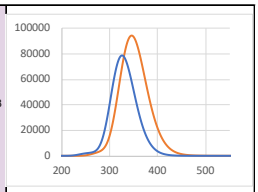
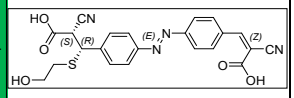
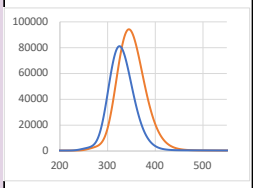
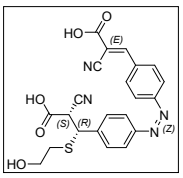
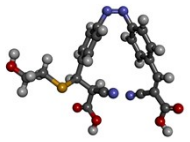
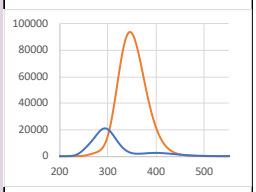
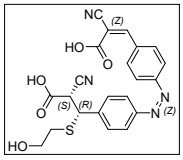
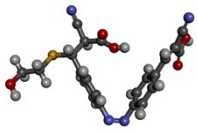
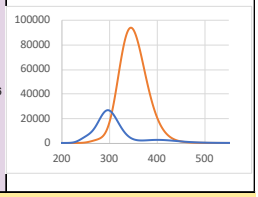
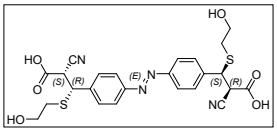
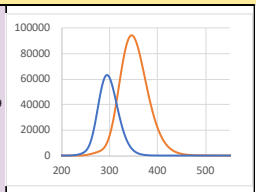
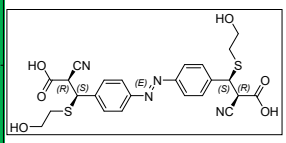
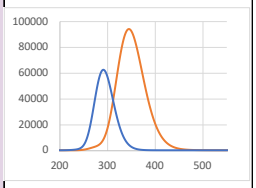
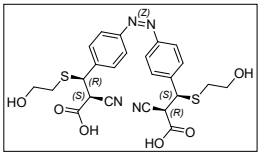
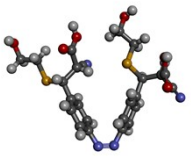
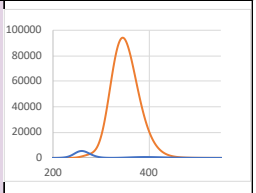
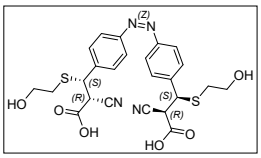
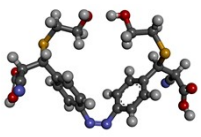
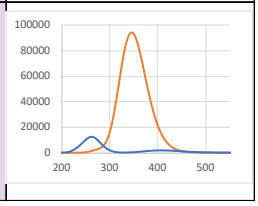


ION MODE: POSITIVE

Table S1

All calculations were carried out using Spartan '18, Version 1.4.1, Jul 26, 2019 (Wavefunction, Inc. Irvine, CA). Methods and basis sets are indicated. Equilibrium geometries were calculated by first sampling conformational space using the Monte Carlo conformer search methods in Spartan using a molecular mechanics (MMFF) forcefield and also by setting a variety of plausible starting structures by hand. Lowest energy conformers were then minimized using DFT methods as detailed in Table S1. In general, we used ωB97X-D methods[3] with default settings (biggrid: 70 shells in the radial direction, 302 Lebedev radial points). A polarizable continuum solvation water model was applied (dielectric=78.30).[4] All minimum structures reported converged normally. The absence of imaginary frequencies confirmed the structures were local minima. TDDFT calculations did not employ solvent. Six states were considered, and spectra were simulated using a sum of gaussians with 0.4 eV line broadening (see: https://gaussian.com/uvvisplot/).

Name:	ChemDraw:	3D:	Relative Energy (kJ/mol):	Eq. Geometry Method:	Energy Method:	Energy (hartrees):	Spectrum: UVVIS=WB97X-D,6-31G* (EEE spectrum shown in orange)
EEE-BCNA			0	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D;6-311+G(2DF, 2P)	-1288.7501	
ZEE-BCNA			23.881548	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D;6-311+G(2DF, 2P)	-1288.741	
ZEZ-BCNA			47.920626	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D;6-311+G(2DF, 2P)	-1288.7319	
EZE-BCNA			48.183176	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D;6-311+G(2DF, 2P)	-1288.7318	
EZZ-BCNA			59.787886	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D;6-311+G(2DF, 2P)	-1288.7274	
ZZZ-BCNA			95.153371	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D;6-311+G(2DF, 2P)	-1288.7139	

EE-**BCNA**-(2-ME)			0	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D/6-31G*	-1841.9268	
EZ-**BCNA**-(2-ME)			23.708265	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D/6-31G*	-1841.9178	
ZE-**BCNA**-(2-ME)			33.29134	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D/6-31G*	-1841.9141	
ZZ-**BCNA**-(2-ME)			76.55958	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D/6-31G*	-1841.8976	
E-**BCNA**-(2-ME)2 (R/S)			0	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D/6-31G*	-2395.1049	
E-**BCNA**-(2-ME)2 (S/S)			-3.806975	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D/6-31G*	-2395.1064	
Z-**BCNA**-(2-ME)2 (R/S)			60.100321	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D/6-31G*	-2395.082	
Z-**BCNA**-(2-ME)2 (S/S)			38.846898	ωB97X-D/6-31G*+CPCM :WATER	ωB97X-D/6-31G*	-2395.0901	

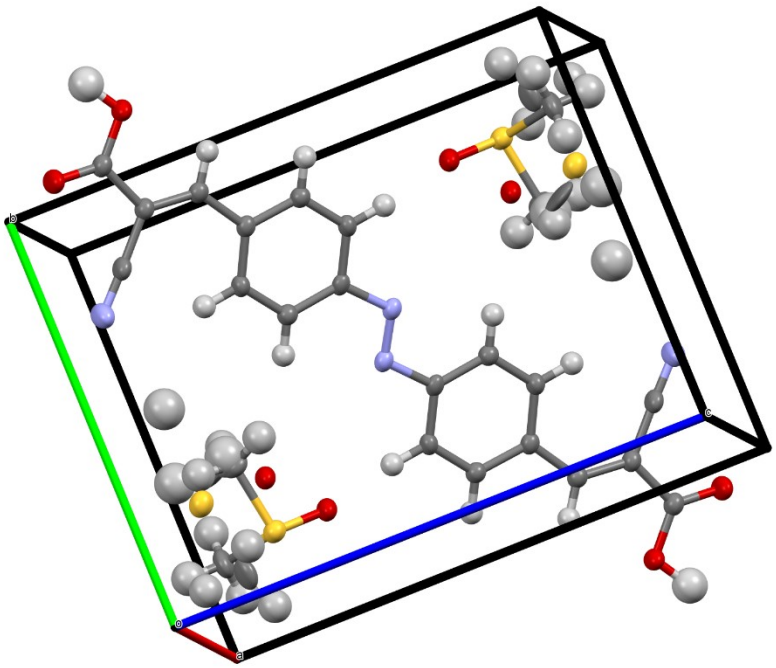
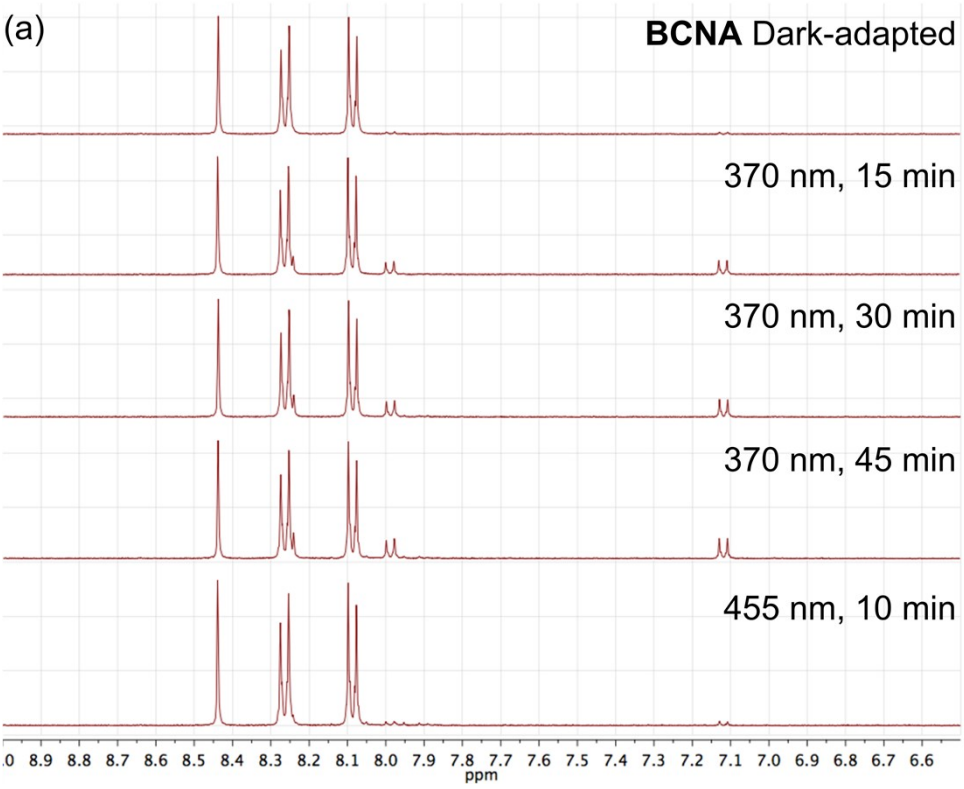
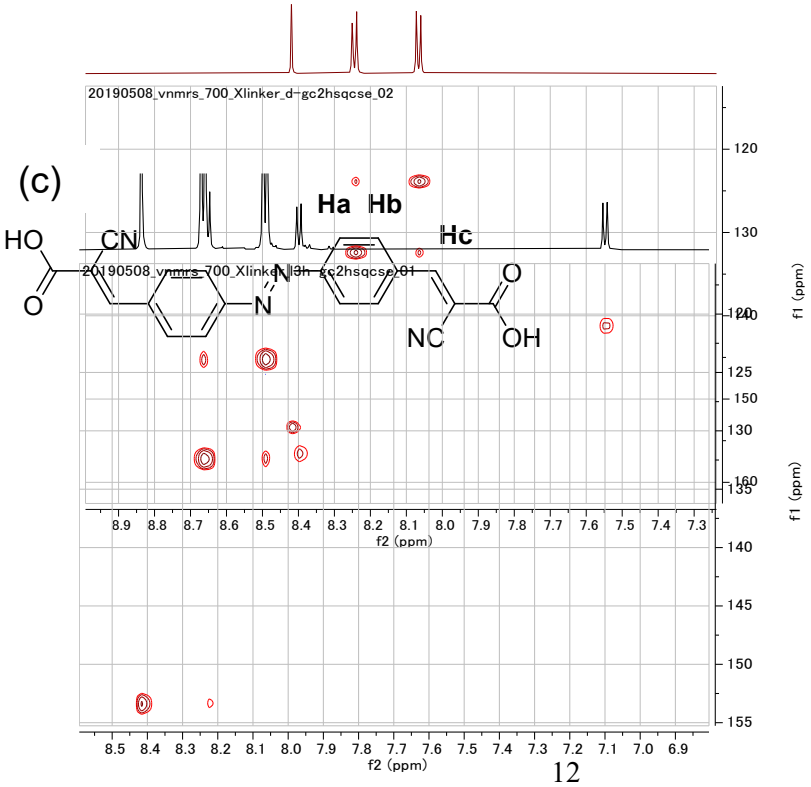


Figure S1. Single crystal X-ray structure analysis of BCNA (solvent is DMSO).

DOI: 10.5517/ccdc.csd.cc238jwt


(a)
BCNA Dark-adapted
370 nm, 15 min
370 nm, 30 min
370 nm, 45 min
455 nm, 10 min
ppm

(b)


(c)
20190508_vnmrs_700_Xlinker_d-gc2hsqcse_02
Ha
Hb
Hc
HO
CN
NC
OH
f2 (ppm)
f1 (ppm)

(d)

	Dark-adapted		UV-irradiated					
	Isomer 1; *EEE*		Isomer 1; *EEE*		Isomer 2; *EZE*		Isomer 3; ?	
Position	^{13}C [ppm]	^{1}H [ppm]	^{13}C [ppm]	^{1}H [ppm]	^{13}C [ppm]	^{1}H [ppm]	^{13}C [ppm]	^{1}H [ppm]
H_a	124	8.07	124	8.07	121	7.10	121	(7.07?)
H_b	132.5	8.24	132.5	8.24	132	7.97	132.3	7.87
H_c	153.5	8.42	153.5	8.42	153.5	8.23	153.5	(8.19?)

Figure S2. Photoisomerization of BCNA detected by NMR in DMSO-d6.

(a) ^{1}H-NMR spectrum of BCNA before irradiation and after irradiation with 370 nm (25 mW/cm^2) and 445 nm (55 mW/cm^2) LEDs for the indicated periods. Irradation was from the outside of the NMR tube.

(b) ^{1}H-^{13}C HSQC spectrum of dark-adapted sample.

(c) ^{1}H-^{13}C HSQC spectrum about 370 nm irradiated **BCNA**. The solution of **BCNA** was irradiated with a 370 nm (25 mW/cm^2) LED, then transferred to an NMR tube and measured immediately.

(d) Chemical shifts of isomers of BCNA. For Isomer 3, chemical shifts are not certain due to overlap of proton peaks.

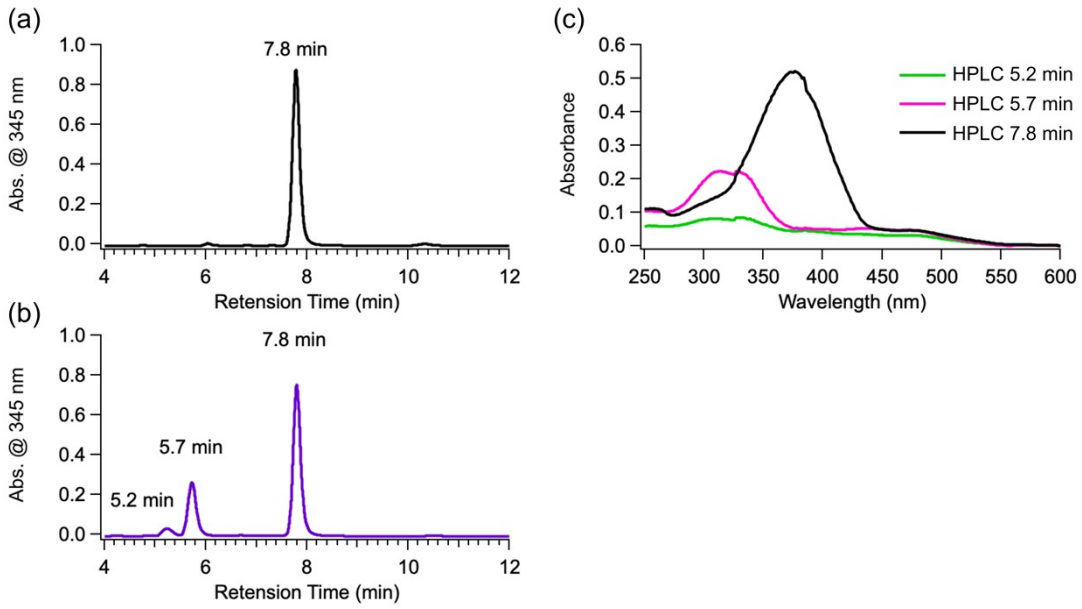


Figure S3. Analysis of BCNA isomers by HPLC and UV-Vis.

(a) HPLC chromatogram of dark-adapted **BCNA**. (b) HPLC chromatogram of UV-irradiated (370 nm) **BCNA**. (c) UV-Vis spectra of HPLC fractions of UV-irradiated **BCNA**. HPLC conditions: Zorbax SB-18 column with a linear gradient of 35–70% acetonitrile/water (containing +0.1% trifluoroacetic acid) over a course of 13.5 min. The peaks with retention times of 5.2 and 5.7 min both have spectra consistent with cis-azo isomers. We attribute the peak with retention time of 5.2 min to a *cis*-azo isomer with one *E* and one *Z* C=C bond (i.e. *EZZ*, or *ZEE*), and the peak with retention time of 5.7 min to a *cis*-azo isomer with two *E* C=C bonds (i.e. *EZE*)

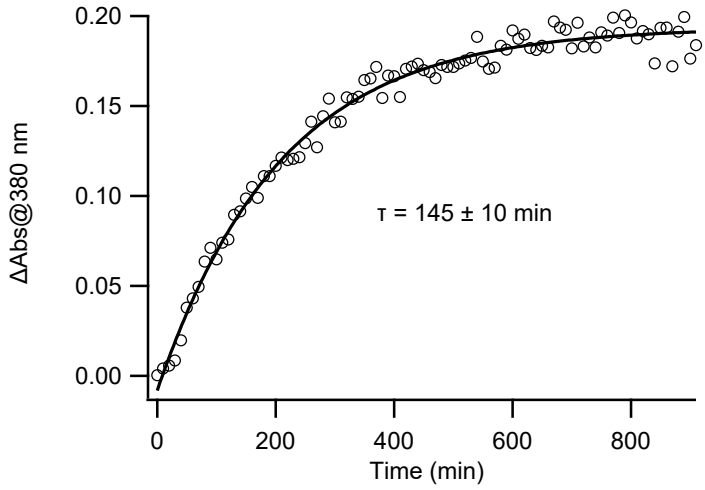


Figure S4. Thermal relaxation of **BCNA** in DMSO

Thermal relaxation of 25 μM **BCNA** in DMSO after irradiation with 370 nm, 15 min at 22°C.

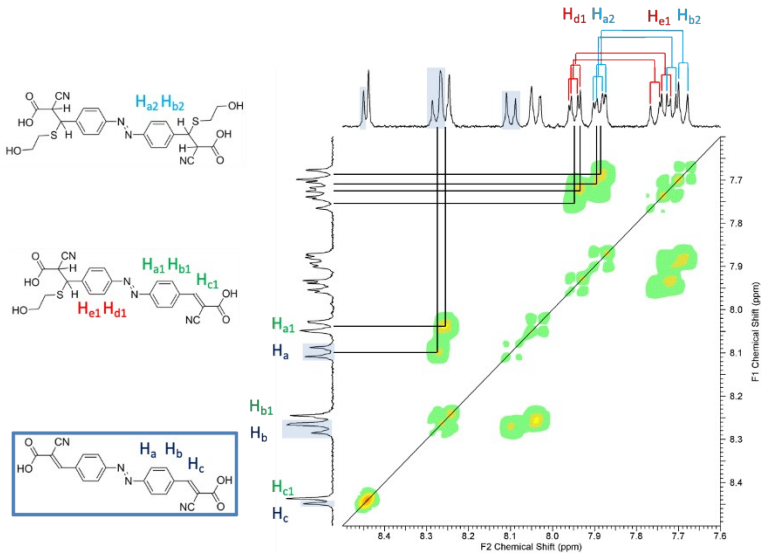


Figure S5. ^{1}H-^{1}H COSY spectrum of 2 mM **BCNA** with 2-ME (8 eq.) in DMSO-d_6.

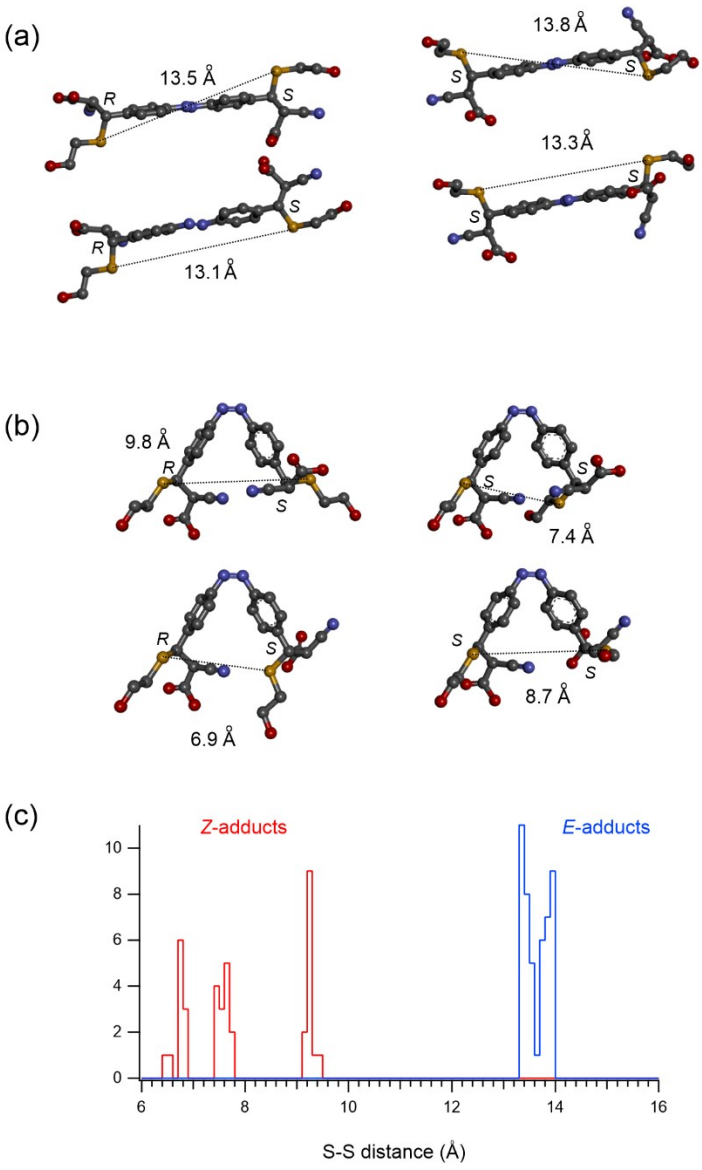


Figure S6. S-S distances in BCNA adducts

See Table S1 for chemical structures. For each of *E*-**BCNA**-(2-ME)$_2$, R/S and *E*-**BCNA**-(2-ME)$_2$, S/S and *Z*-**BCNA**-(2-ME)$_2$, R/S and *Z*-**BCNA**-(2-ME)$_2$, S/S a conformer distribution calculation was carried out using Spartan 2019. The MMFF molecular mechanics force field was used with constraints to maintain the azo unit in DFT optimized *trans* or *cis* geometry. (a) Representative structures of *E*-**BCNA**-(2-ME)$_2$, R/S and *E*-**BCNA**-(2-ME)$_2$, S/S adducts. (b) Representative structures of *Z*-**BCNA**-(2-ME)$_2$, R/S and *Z*-**BCNA**-(2-ME)$_2$, S/S adducts. (c) Histogram showing S-S distances found for *E*-adduct conformers and *Z*-adduct conformers (all conformers within 10 kJ/mol of the lowest calculated energy).

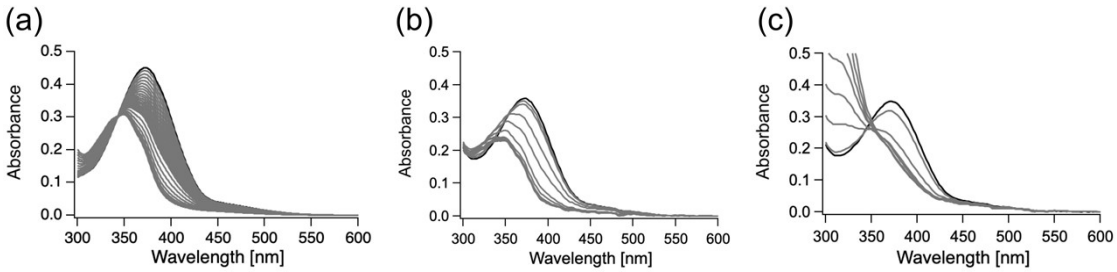


Figure S7. Titrations of BCNA with di-thiols detected by UV-Vis absorption spectra

(a) Spectrum of 10 μM **BCNA** in 20 mM sodium phosphate buffer pH = 7.5 at 20°C, with increasing amounts of SS7L peptide (i,i+7) (black; 0 eq., gray; 0.1 - 6 eq.). (b) Spectrum of 10 μM **BCNA** in 20 mM sodium phosphate buffer pH = 8.0 at 20°C, with increasing amounts of UT386-3 Z-domain (i, i+7) (black; 0 eq., gray; 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 7.5, 10 eq.). (c) Spectrum of 10 μM **BCNA** in 20 mM sodium phosphate buffer pH = 8.0 at 20°C, with increasing amounts of UT386-2 Z-domain (i, i+11) (black; 0 eq., gray; 1, 10, 20, 30, 40, 50, 60 eq.).

K_d = 3 µM

[BCNA-Z7]

BCNA µM	[Z7] total µM 1	5	10	25
1	0.05	0.2	0.35	0.58
2	0.1	0.4	0.7	1.14
5	0.2	0.92	1.6	2.8
10	0.35	1.6	2.8	5.24
25	0.58	2.8	5.2	11
50	0.73	3.6	7.1	16.3
100	0.85	4.2	8.4	20.4

% Z7 bound

BCNA	[Z7] total µM 1	5	10	25
1	5	4	3.5	2.32
2	10	8	7	4.56
5	20	18.4	16	11.2
10	35	32	28	20.96
25	58	56	52	44
50	73	72	71	65.2
100	85	84	84	81.6

K_d = 300 nM

[BCNA-Z7]

BCNA µM	[Z7] total µM 1	5	10	25
1	0.28	0.7	0.84	0.94
2	0.46	1.34	1.64	1.86
5	0.71	2.77	3.9	4.6
10	0.84	3.9	6.6	8.9
25	0.93	4.6	9	19.1
50	0.96	4.8	9.6	23.4
100	0.98	4.9	9.8	24.4

% Z7 bound

BCNA	[Z7] total µM 1	5	10	25
1	28	14	8.4	3.76
2	46	26.8	16.4	7.44
5	71	55.4	39	18.4
10	84	78	66	35.6
25	93	92	90	76.4
50	96	96	96	93.6
100	98	98	98	97.6

K_d = 30 nM

[BCNA-Z7]

BCNA µM	[Z7] total µM 1	5	10	25
1	0.66	0.96	0.98	0.99
2	0.86	1.89	1.96	1.98
5	0.96	4.1	4.83	4.85
10	0.98	4.8	8.74	9.88
25	0.992	4.96	9.88	22.97
50	0.996	4.98	9.96	24.82
100	0.998	4.99	9.98	24.94

% Z7 bound

BCNA	[Z7] total µM 1	5	10	25
1	66	19.2	9.8	3.96
2	86	37.8	19.6	7.92
5	96	82	48.3	19.4
10	98	96	87.4	39.52
25	99.2	99.2	98.8	91.88
50	99.6	99.6	99.6	99.28
100	99.8	99.8	99.8	99.76

K_d = 3 nM	[BCNA-Z7]			
	$[Z7]_{total}$ µM			
BCNA µM	1	5	10	25
1	0.872	0.995	0.998	0.999
2	0.982	1.988	1.99	1.998
5	0.995	4.7	4.98	4.995
10	0.998	4.98	9.58	9.98
25	0.9992	4.99	9.98	24.33
50	0.9996	4.998	9.995	24.98
100	0.9998	4.999	9.998	24.99

	% Z7 bound			
	$[Z7]_{total}$ µM			
BCNA	1	5	10	25
1	87.2	19.9	9.98	3.996
2	98.2	39.76	19.9	7.992
5	99.5	94	49.8	19.98
10	99.8	99.6	95.8	39.92
25	99.92	99.8	99.8	97.32
50	99.96	99.96	99.95	99.92
100	99.98	99.98	99.98	99.96

Table 3. Calculated binding of BCNA to Z (I, i+7) in the presence of GSH.

Assuming a dissociation constant for **BCNA** and GSH of 1 mM, and concentration of GSH of 5 mM, Kintek Explorer was used to numerically solve for equilibrium concentrations of the Z-domain (i, i + 7) adduct using the dissociation constants given (3 µM, 300 nM, 30 nM, 3 nM) and the concentrations shown (all concentrations are given in µM). The left-side Tables show equilibrium concentrations of the Z-domain (i, i + 7) adduct while the right-side Tables give % of Z-domain (i, i + 7) adduct *vs.* total Z-domain (i, i + 7). Values above 95% adduct are coloured red.

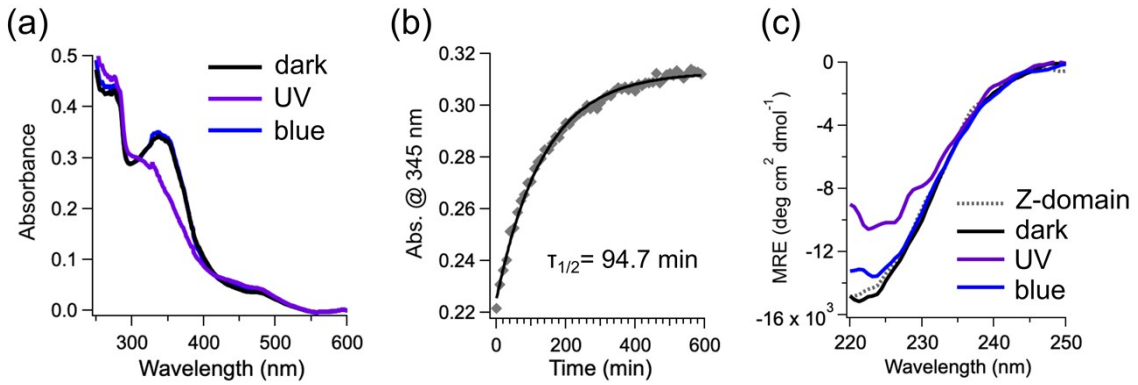


Figure S8. Photoswitching of a BCNA Z-domain (i, i+7) L51S adduct

(a) UV-Vis spectra of 10 µM **BCNA**: Z-domain L51S = 1: 6 eq. in 50 mM sodium phosphate buffer at pH = 8.0 at 20°C, dark-adapted (black), after irradiation with 370 nm light (violet), and after irradiation with 455 nm light (blue). (b)Thermal relaxation of Z-domain adducted **BCNA** after 370 nm irradiation measured at 20°C. (c) CD spectra of 12.5 µM **BCNA**: Z-domain L51S (1 :1 eq) in 10 mM sodium phosphate buffer at pH = 8.0 at 20 °C, including 0.125% DMSO. Dark-adapted (black), after irradiation with 370 nm light (violet), and after irradiation with 455 nm light (blue), Z-domain L51S only (dash).

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