An Electrophoretic Mobility Shift Assay Identifies a Mechanistically Unique Inhibitor of Protein Sumoylation

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Supporting Information

General Information

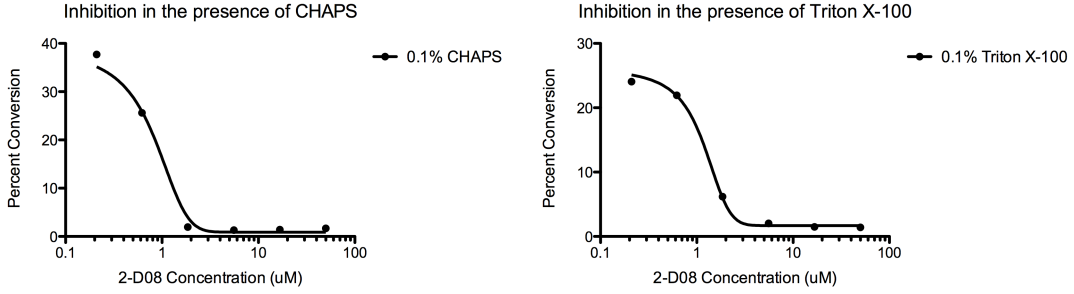
Sumoylation assays were performed as described in the experimental procedures section of the article, except when described differently below.

2-D08 inhibits sumoylation in the presence of detergents

Detergents may be used to break up aggregates as a method of eliminating nonspecific inhibitors in assays. We assayed 2-D08 in the presence of 0.1% CHAPS and 0.1%Triton X-100 to determine whether or not they had any effect on the inhibitory activity. In both cases, the IC_{50} value did not shift substantially from the value in the absence of detergents.

The in vitro SUMOylation assay was performed as described in the general procedure, but in the presence of 0.1% Triton X-100 (Sigma-Aldrich) or 0.1% CHAPS (Sigma-Aldrich) in 384-well plate format. After incubation for 90 min at room temperature, EDTA (0.25 M, 10 μL) was added to each well in order to quench the reaction. Samples were analyzed using a LabChip EZ Reader II (Caliper Life Sciences, Inc.).

Figure SI-1. 2-D08 Inhibits Sumoylation in the Presence of Detergents

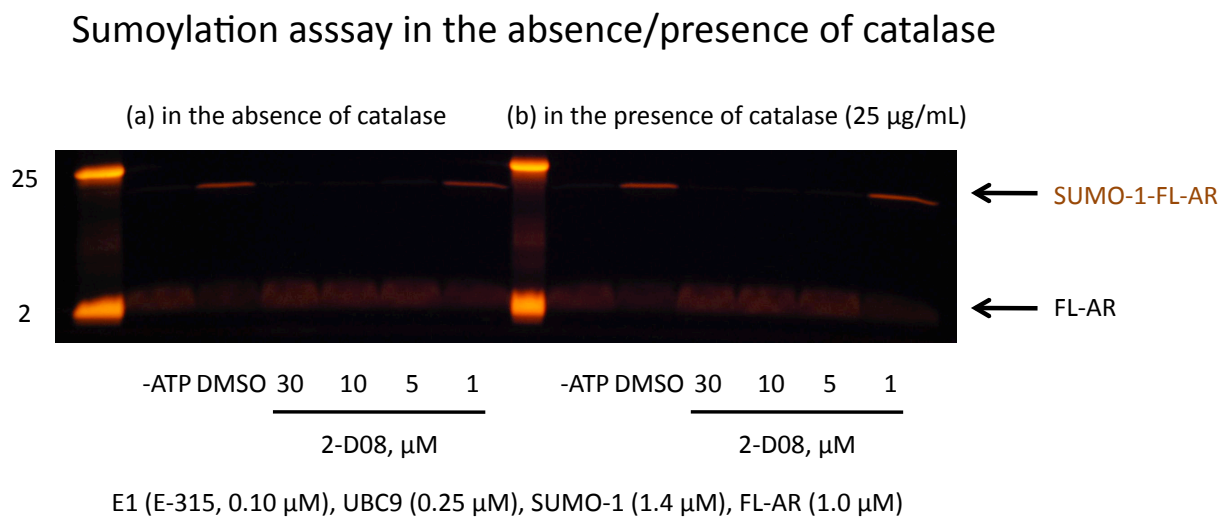


2-D08 inhibits sumoylation in the presence of catalase.

Addition of catalase to biochemical assays has previously been shown to suppress the nonspecific reaction of small molecules with molecular oxygen to generate hydrogen peroxide or hydroperoxide adducts (see Tjernberg, *et al., Bioorg. Med. Chem. Lett*. **2004**, *14*, 891-895.). In this case, we performed the sumoylation assay in the presence of 25 μg/mL catalase, and still observed inhibition with 2-D08 by in-gel fluorescence.

The in vitro SUMOylation assay was performed as described in the general procedure above, but in the absence/presence of catalase (25 μg/mL, Sigma-Aldrich). After incubation for 90 min at room temperature, samples were mixed with sample buffer and then SUMOylation level was measured by in-gel fluorescence imaging.

Figure SI-2. 2-D08 Inhibits Sumoylation in the Presence of Catalase



Dynamic Light Scattering

Dynamic light scattering (DLS) measurement was performed on a DynaPro Titan instrument (Wyatt Technology Corporation) in Sumoylation assay buffer in the presence of 0.1% Triton X-100.

Figure SI-3. Dynamic Light Scattering of 2-D08 in Water

Annotations

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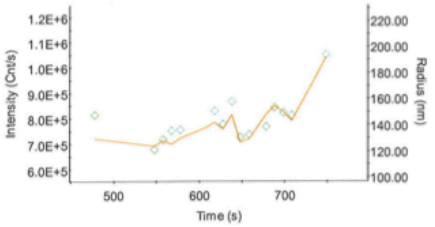
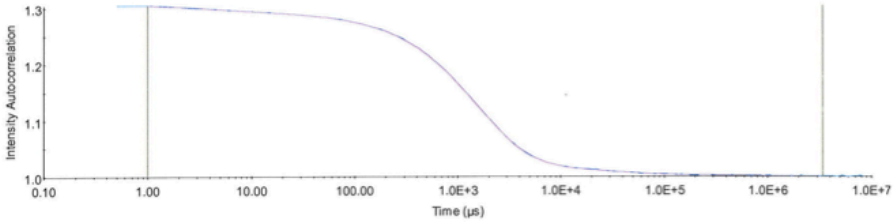
Last Saved By: 7.1.1.3
Collected By: 7.1.1.3
Last Modified: 13 Jul 2012 14:44:33
Exp Time Stamp: 13 Jul 2012 09:10:15
Comments:

① 2D08. 100μM

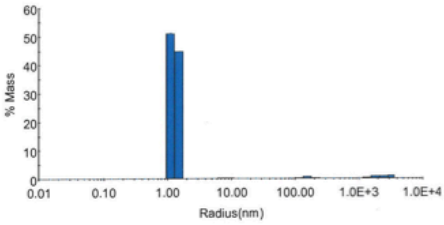
Datalog Graph: 1/2D08 100



Correlation Function: 1/2D08 100



Regularization Results: 1/2D08 100



	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	1.300	15.5	6	0.4	95.7
Peak 2	8.716	14.4	534	0.3	0.2
Peak 3	153.327	20.9	437614	83.9	1.0
Peak 4	2550.420	29.3	314752000	15.3	3.1

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Figure SI-4. Dynamic Light Scattering of 2-D08 in the Presence of Triton-X100

Annotations

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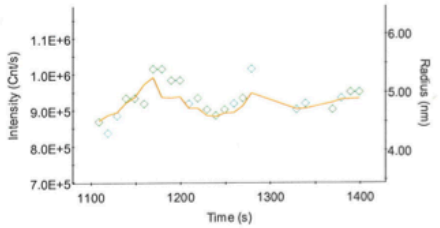
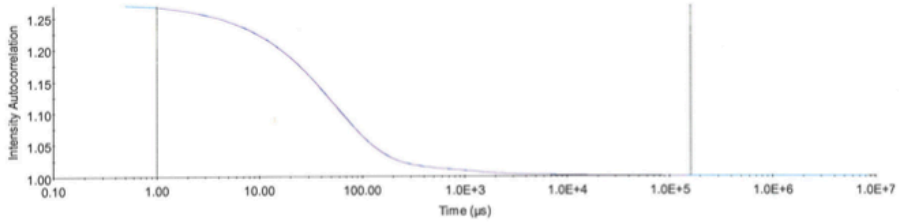
Collected By: 7.1.1.3
Last Modified: 13 Jul 2012 14:44:33
Exp Time Stamp: 13 Jul 2012 09:10:15
Comments:

0.1% triton. X100
(5) 2D08. 100μM

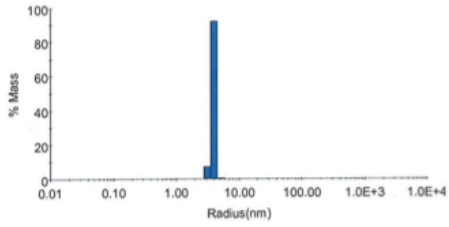
Datalog Graph: 5/2D08 100



Correlation Function: 5/2D08 100



Regularization Results: 5/2D08 100



	Radius (nm)	%Pd	Mw-R (kDa)	%Intensity	%Mass
Peak 1	4.0	6.5	86	75.6	99.9
Peak 2	58.1	11.9	45105	19.1	0.0
Peak 3	631.4	18.3	12003700	5.3	0.0

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Chemistry

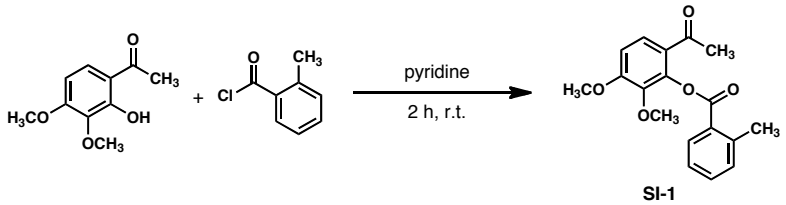
General Procedures

Reactions were magnetically stirred in oven-dried single neck round bottom flasks under an atmosphere of argon. Solvents were purchased from commercial sources and purified by passage through an aluminum oxide column. Other reagents were purchased from commercial sources and used without further purification unless otherwise noted. Air- and moisture-sensitive reagents were added to reaction mixtures by stainless steel syringe or cannula. Analytical TLC was performed using Analtech Uniplate GHLF TLC Plates with 250 micron silica thickness, followed by submersion in aqueous basic potassium permanganate solution ($KMnO_4$), followed by brief heating with a heat gun. Flash column chromatography was performed using a Teledyne ISCO CombiFlash R_f automated chromatography system. Organic solvents were removed by rotary evaporation. NMR spectra were collected on Oxford 400MHz or 500MHz magnets with Varian INOVA consoles. Low-resolution mass spectra were collected on an Agilent HPLC/MS 1100 series LC/MS system.

Synthesis of 6 and 7.

Note: Chloro and methyl derivatives **6** and **7** were prepared following routes analogous to those reported by Liu and colleagues *J. Med. Chem.* **2010**, *53*, 8274-8286. Other flavonoids were purchased from Indofine or TimTec.

*Preparation of **SI-1***

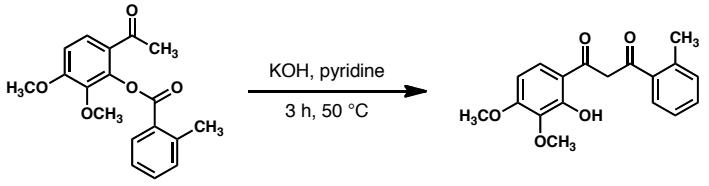


SI-1

Galleacetophenone 3,4-dimethyl ether (1.00 g, 5.10 mmol, 1.0 equiv) was dissolved in 5 mL pyridine. To the resultant solution was added 2-methylbenzoyl chloride (1.178 g, 0.994 mL, 7.65 mmol, 1.5 equiv) dropwise by syringe. The solution was stirred for 2 h, after which it was diluted with 1 N aqueous HCl (20 mL). The aqueous solution was extracted with ethyl acetate (2 x 30 mL), and the organic layer was washed with water (2 x 30 mL) and with brine (1 x 30 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated by rotary evaporation. The residue was purified by column chromatography (ethyl

acetate/hexanes) to afford **SI-1** (1.581 g, 5.03 mmol, 98%) as a pale yellow, amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.23 (d, *J* = 8.0 Hz, 1H), 7.69 (d, *J* = 9.0 Hz, 1H), 7.49 (t, *J* = 8.2 Hz, 1H), 7.34 (m, 2H), 6.89 (d, *J* = 8.9 Hz, 1H), 3.96 (s, 3H), 3.84 (s, 3H), 2.68 (s, 3H), 2.50 (s, 3H).

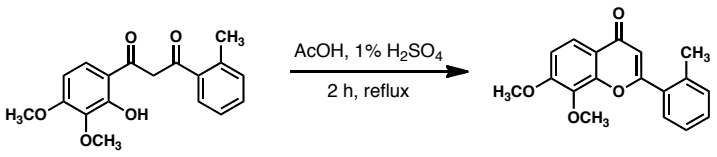
*Preparation of **SI-2***



SI-1 **SI-2**

SI-1 (500 mg, 1.59 mmol, 1 equiv) was dissolved in 3 mL pyridine. To the solution was added KOH (119 mg, 2.12 mmol, 1.33 equiv). The solution was heated to 50 ºC for 3 h. After cooling to room temperature, the solution was diluted with 2 N HCl (20 mL). The aqueous layer was extracted with ethyl acetate (2 x 30 mL), and the organic layer was washed with water (2 x 30 mL) and with brine (1 x 30 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated by rotary evaporation. The residue was purified by column chromatography (ethyl acetate/hexanes) to afford **SI-2** (449 mg, 1.42 mmol, 90%) as an amorphous pale yellow solid which was carried forward without further purification.

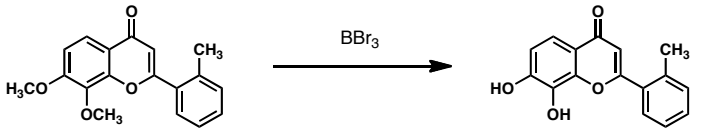
*Preparation of **SI-3***



SI-2 **SI-3**

SI-2 (100 mg, 0.32 mmol, 1.0 equiv) was dissolved in 8 mL AcOH. To the solution was added 0.08 mL concentrated H_2SO_4 and the reaction mixture was heated to reflux for 2 h. After cooling to room temperature, the reaction mixture was poured onto ice. The resultant aqueous solution was extracted with ethyl acetate (2 x 20 mL), and the combined organic layers were washed with brine (1 x 20 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated by rotary evaporation. The residue was purified by flash chromatography (ethyl acetate/hexanes) to afford **SI-2** (64 mg, 0.22 mmol, 68%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.01 (d, *J* = 9.2 Hz, 1H), 7.55 (m, 2H), 7.44 (t, *J* = 6.9 Hz), 7.35 (d, *J* = 7.35 Hz, 1H), 7.08 (d, *J* = 9.2 Hz, 1H), 6.47 (s, 1H), 4.02 (s, 3H), 3.97 (s, 3H), 2.55 (s, 3H).

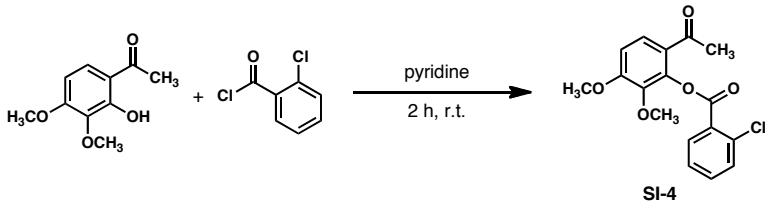
Preparation of ***6***



SI-3 **6**

SI-3 (27 mg, 0.09 mmol, 1.0 equiv) was dissolved in 845 μL dichloromethane. To the solution was added BBr_3 (80 μL, 0.845 mmol, 10 equiv) dropwise by syringe. The solution was stirred for 48 h, after which the reaction mixture was poured over ice and diluted with 2 N HCl (20 mL). The precipitate was collected and purified by flash chromatography (hexanes/ethyl acetate) to afford **6** (11 mg, 0.041 mmol, 46%) as a pale yellow, amorphous solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.30 (brs, 1H), 9.30 (brs, 1H), 7.56 (d, *J* = 7.7 Hz, 1H), 7.28-7.44 (m, 3H), 6.91 (d, *J* = 8.6 Hz, 1H), 6.30 (s, 1H), 2.41 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 177.7, 165.6, 151.2, 147.8, 137.5, 134.2, 133.5, 132.0, 131.6, 130.3, 127.1, 117.8, 116.0, 115.0, 111.2, 21.1. HRMS: ESI $[M + H]^+$ Calcd for $C_{15}H_{12}O_4$: 269.0808. Found: 269.0791.

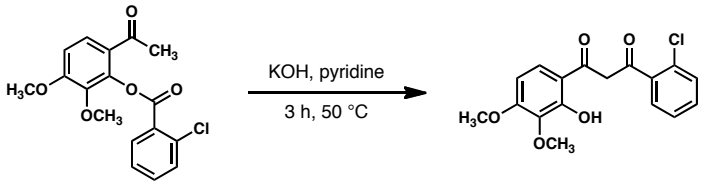
Preparation of ***SI-4***



SI-4

The reaction conditions for the preparation of **SI-4** were analogous to **SI-1**. Product was isolated as a white, amorphous powder in 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.23 (dd, *J* = 1.9, 7.8 Hz, 1H), 7.68 (d, *J* = 9.2 Hz, 1H), 7.51 (m, 2H), 7.42 (m, 1H), 6.90 (d, *J* = 9.2 Hz, 1H), 3.96 (s, 3H), 3.87 (s, 3H), 2.53 (s, 3H).

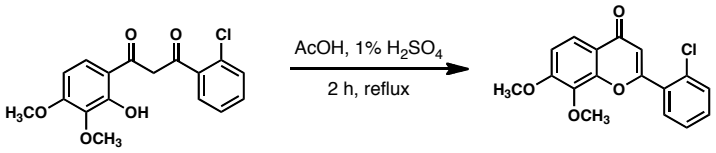
Preparation of ***SI-5***



SI-4 **SI-5**

The reaction conditions for the preparation of **SI-5** were analogous to **SI-2**. Product was isolated as a pale yellow, amorphous solid in 51% yield and carried on without further purification.

*Preparation of **SI-6***



SI-5 **SI-6**

The reaction conditions for the preparation of **SI-5** were analogous to **SI-3**. Product was isolated as a pale pink solid in 72% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.99 (d, *J* = 8.9 Hz, 1H), 7.65 (dd, *J* = 2.4, 8.1 Hz, 1H), 7.4-7.5 (m, 2H), 7.08 (d, *J* = 8.8 Hz), 6.60 (s, 1H), 4.01 (s, 3H), 3.99 (s, 3H).

*Preparation of **7***

SI-6 **7**

The reaction conditions for the preparation of **7** were analogous to **6**. Product was isolated as a light tan, amorphous solid in 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.35 (brs, 1H), 9.32 (brs, 1H), 7.76 (dd, *J* = 2.2, 8.0 Hz, 1H), 7.63 (dd, *J* = 1.8, 8.2 Hz, 1H), 7.47-7.58 (m, 2H), 7.38 (d, *J* = 9.0 Hz, 1H), 6.93 (d, *J* = 9.1 Hz, 1H), 6.40 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 178.9, 162.4, 150.3, 147.1, 132.7, 132.5, 131.6, 131.5, 130.6, 130.3, 126.8, 116.6, 115.8, 114.0, 111.4. HRMS: ESI $[M + H]^+$ Calcd for $C_{15}H_9O_4Cl$: 289.0262. Found: 289.0251.

Peptide Synthesis

Materials

PL-Rink amide resin and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were purchased from Polymer Laboratories. Fmoc-protected amino acids, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, and Fmoc-His(Trt)-OH were purchased from EMD Chemicals. 5-Carboxyfluorescein succinimidyl ester was purchased through AnaSpec, Inc. Triisopropylsilane, methanol, acetic anhydride, diisopropylethylamine, piperidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and trifluoroacetic acid (TFA) were purchased through Sigma-Aldrich. N-methylpyrolidinone, acetonitrile,

dimethylformamide (DMF), and diethyl ether were purchased through Fisher.

Peptide Synthesis and Purification

Resin bound peptide (sequence: HARIKLENPL) was synthesized using standard Fmoc solid phase peptide synthesis with HCTU activation on a Tribute automated peptide synthesizer. To confirm if the desired peptide was synthesized small amount of resin bound peptide was cleaved and side-chain deprotected using TFA/water/triisopropylsilane (38:1:1) for two hours under argon. Crude peptide was purified with semi-preparative HPLC (Agilent, C18 column) and characterized by ESI-MS (see Fig SI-5). After soaking with N-methylpyrolidinone for an hour resin bound peptide was mixed with 5-carboxyfluorescein succinimidyl ester and diisopropylethylamine in DMF. After shaking overnight at room temperature, resin bound peptide was filtered, washed with dichloromethane, cleaved and side-chain deprotected using TFA/water/triisopropylsilane (38:1:1) for 1.5 hours under argon. After filtration, crude peptide was precipitated with cold diethyl ether, purified by RP-HPLC (Vydac C18 Column), and characterized by ESI-MS (see Fig SI-5 and Fig SI-6).

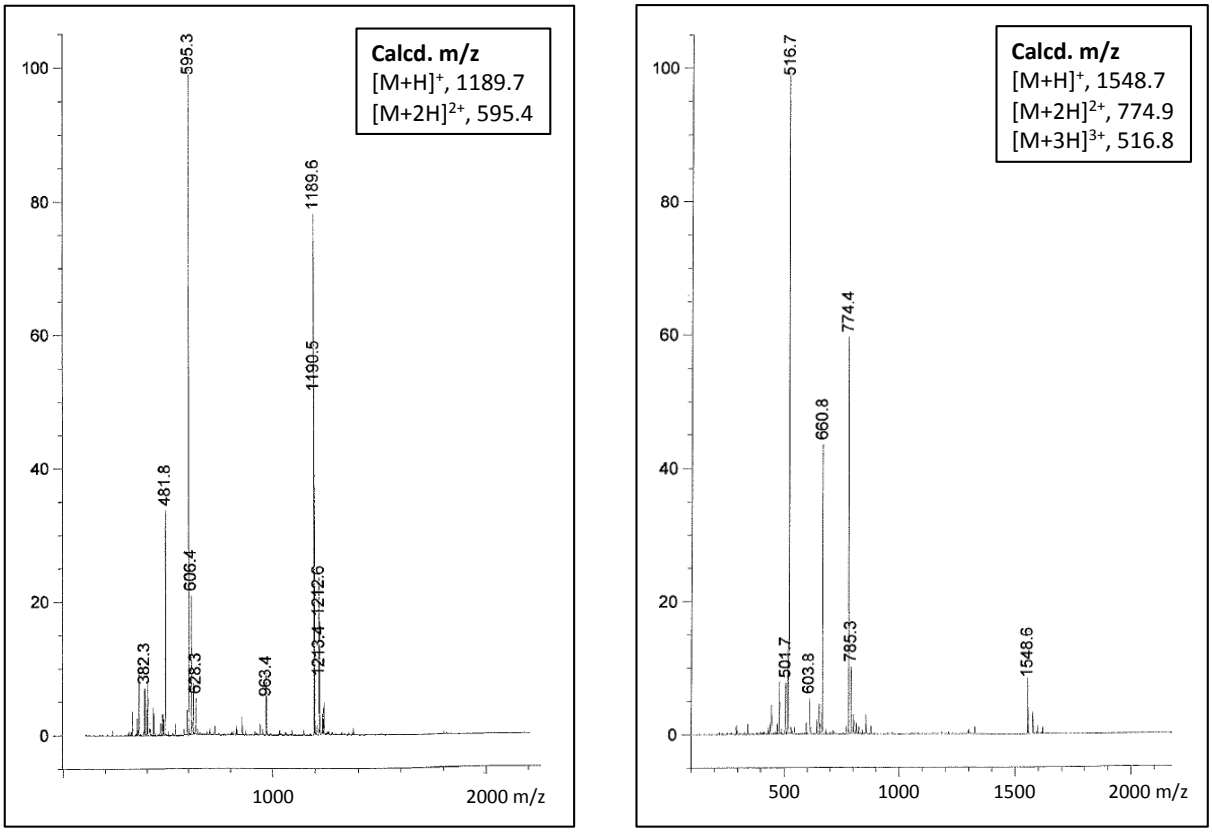


Fig SI-5. ESI-MS spectra of unlabeled peptide (HARIKLENPL, left) and fluorescently labeled peptide (FL-AR, right).

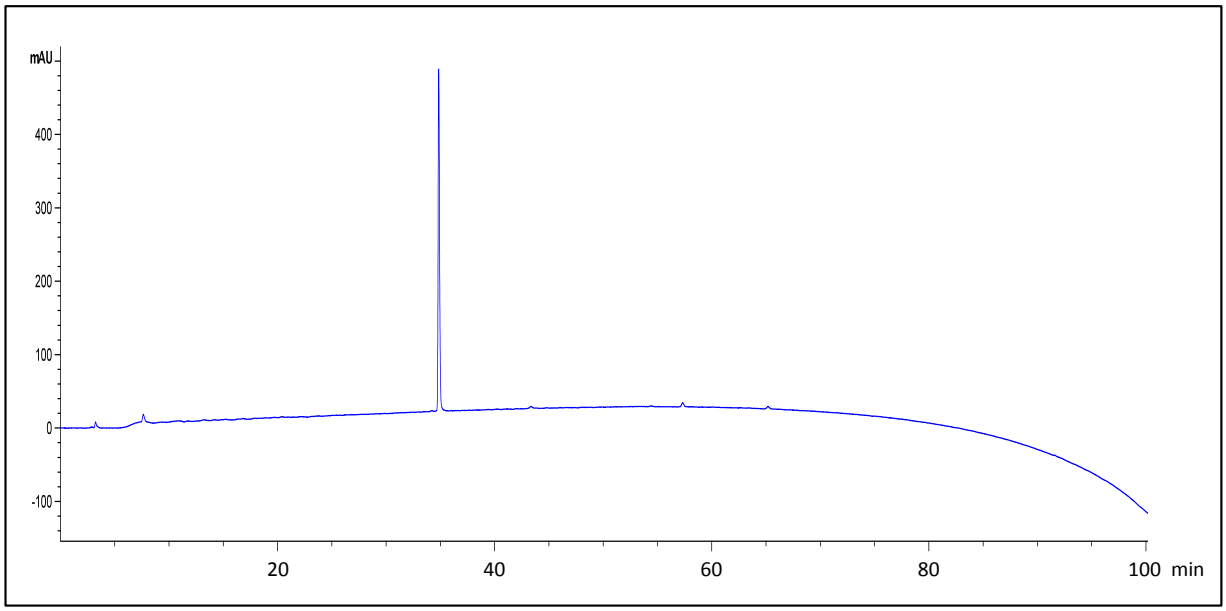


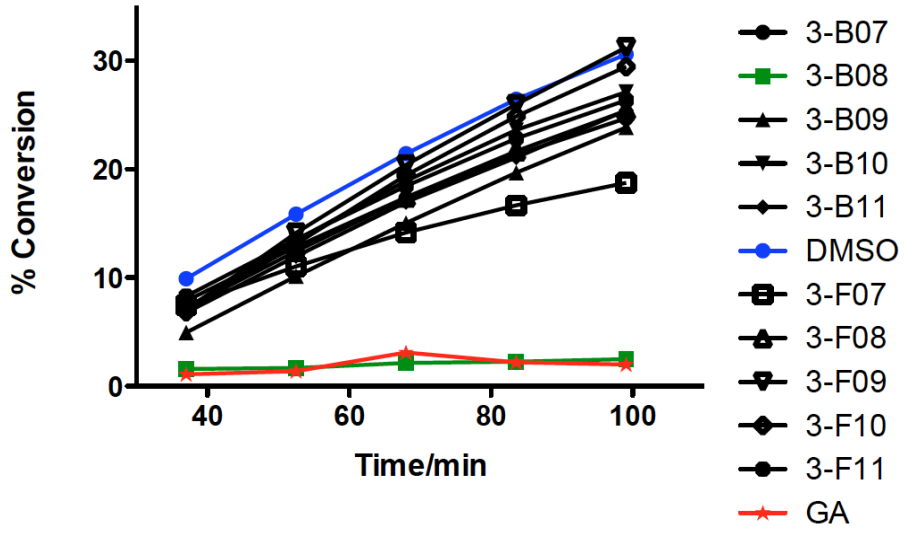
Fig SI-6. HPLC trace of pure peptide FL-AR (C18 column, 0.1% TFA-water).

Screening Data

Representative Kinetic Data

A subset of kinetic screening data is depicted below. Included are the negative control (DMSO), positive control (ginkgolic acid 30 μM, GA), inactive screening compounds and an active hit (3-B08). Kinetic data were collected on all 500 compounds screened. Inactive and active compounds shown are arbitrarily selected from the full screening set.

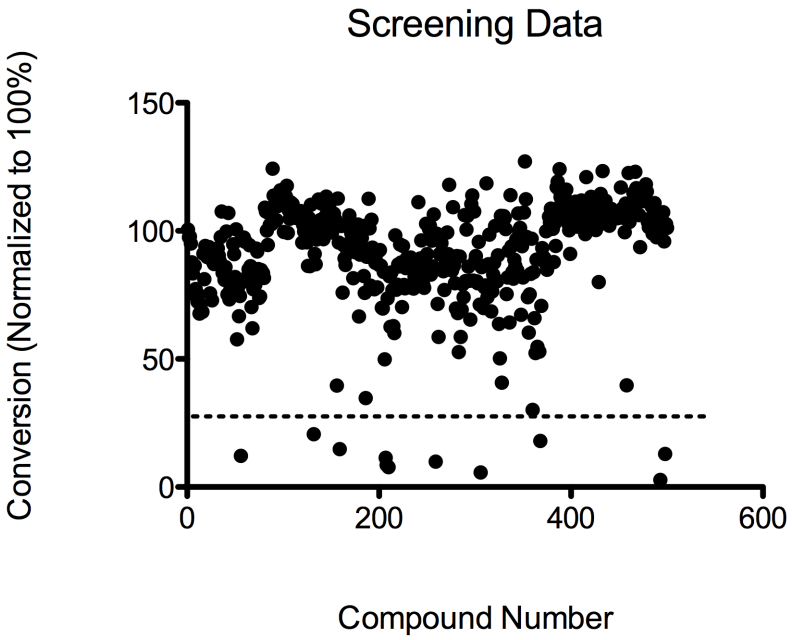
Figure SI-7. Representative Kintetic Data from Screening Compounds. DMSO = Negative control, GA = Positive control (ginkgolic acid, 30 μM), 3-B08 = active compound.



Full Screening Data

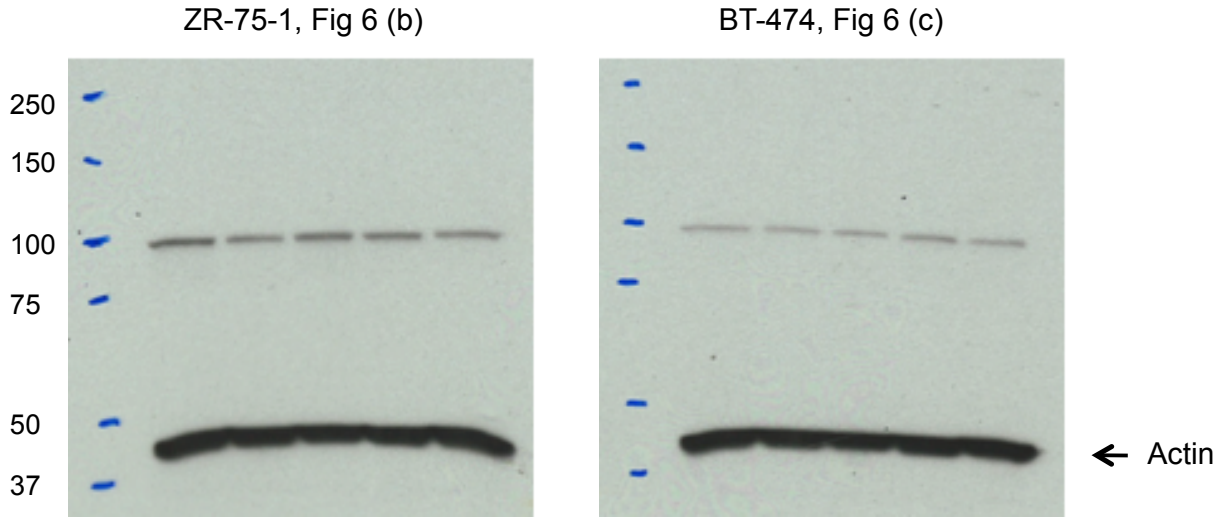
Complete screening data is reported below. Conversion is normalized to 100% (relative to DMSO controls). Dotted line represents hit cutoff, defined as three standard deviations from the mean conversion. The data reported are for EDTA quenched samples at the two-hour timepoint. Mean conversion was 92.0%, with a standard deviation of 19.9%. Total hit rate was 2.2%.

Figure SI-8. Full Screening Data from the Two Hour Timepoint (EDTA Quenched)



Western Blot

Figure SI-9. The same blots in Figures 6 (b) and 6 (c) were probed with mouse monoclonal anti-actin (C4) antibody to show equal loading.



Thioester Bond Formation Assay

Figures SI-10 and SI-11 show thioester bond formation assay under both non-reducing and reducing conditions (Figures 5 (a) and 5 (b) show in-gel fluorescence images for only non-reducing condition).

Figure SI-10. E1-SUMO-1 Thioester Bond Formation

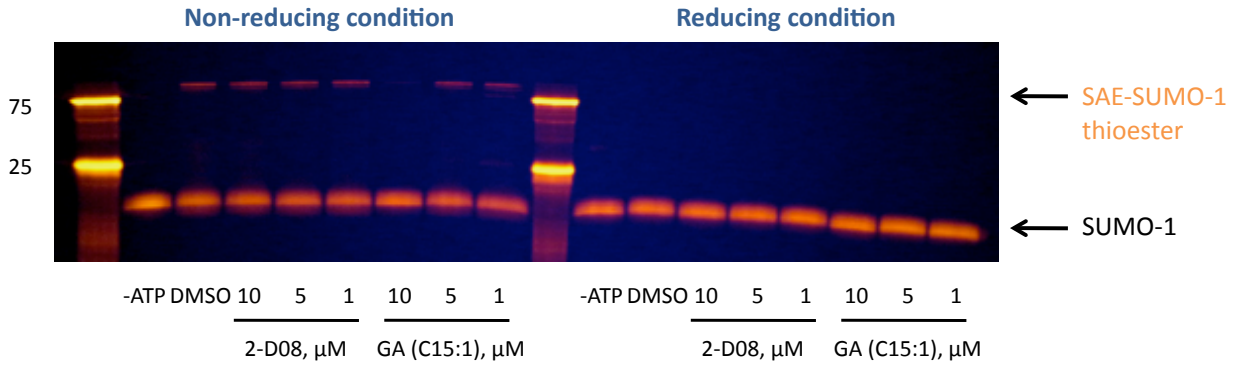


Figure SI-11. UBC9-SUMO-1 Thioester Bond Formation