**Supporting Information**

# NHS-Esters as Versatile Reactivity-Based Probes for Mapping Proteome-Wide Ligandable Hotspots

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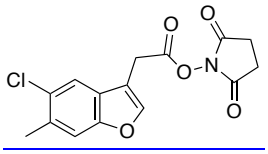
**Supporting Methods**

**Synthesis of CW 1-26 and CW 1-33**

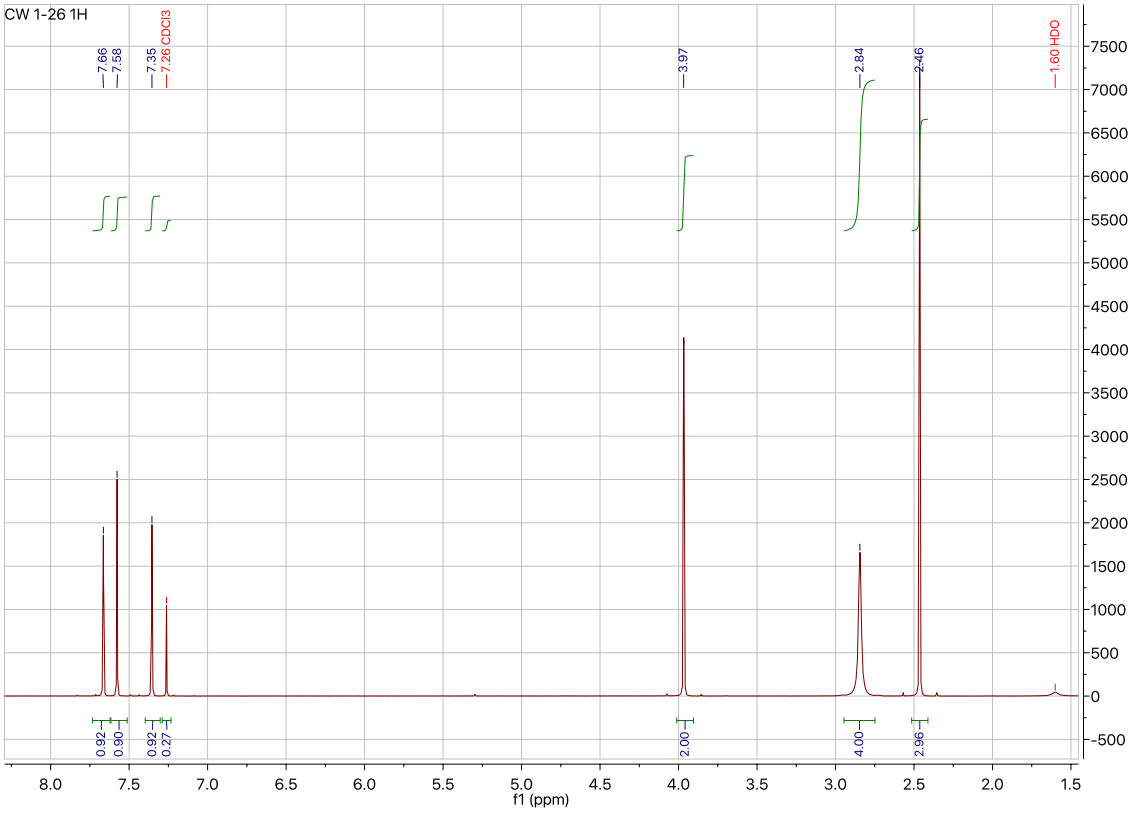


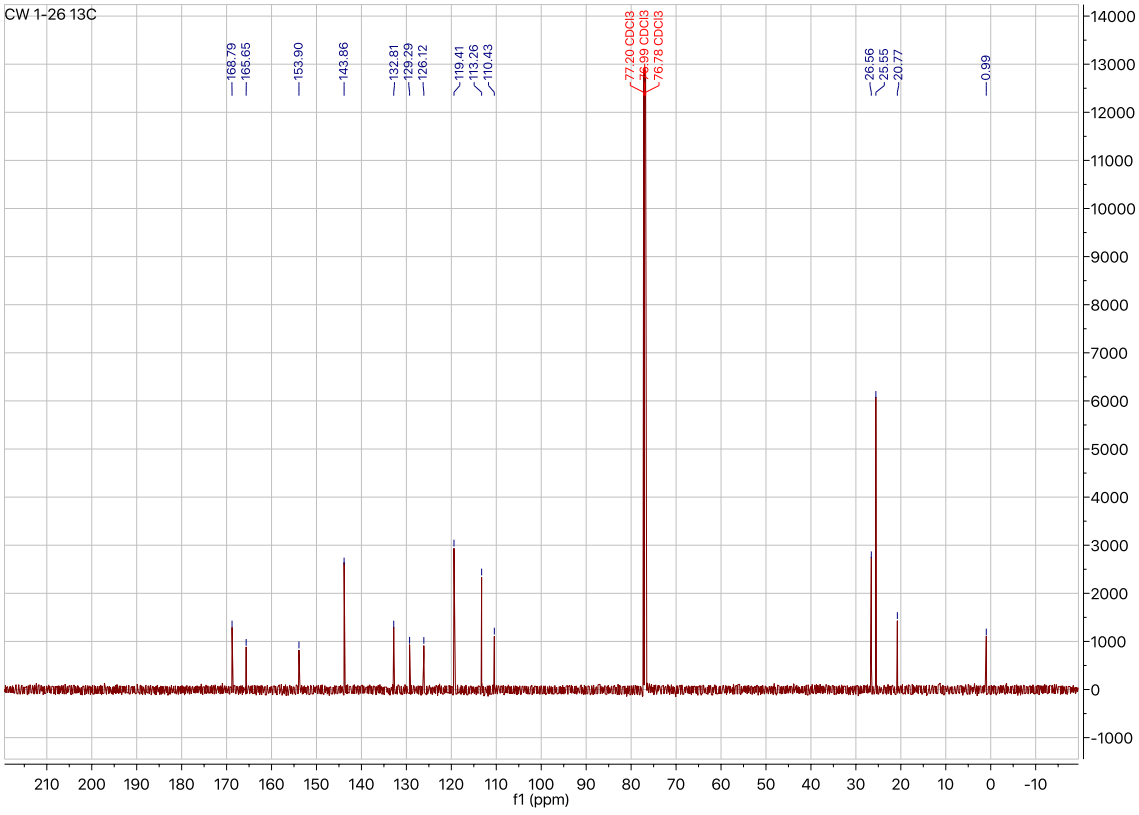

**Synthesis of 2,5-dioxopyrrolidin-1-yl 2-(5-chloro-6-methylbenzofuran-3-yl)acetate, CW 1-26**: 35.6 mg (160 µmol) of (5-chloro-6-methyl-1-benzofuran-3-yl)acetic acid (Sigma) were dissolved in anhydrous DCM. 1.3 equivalents of NHS (23.9 mg, 208 µmol, Sigma) and EDC-HCl (39.7 mg, 208 µmol, Acros) were added and the mixture was stirred overnight under nitrogen. TLC (70% EtOAc/Hex) showed > 90% conversion. The crude mixture was filtered, concentrated and applied directly to a silica gel column and purified with a mobile phase of 50% EtOAc/Hex, which yielded 28.7 mg of product (56% yield).

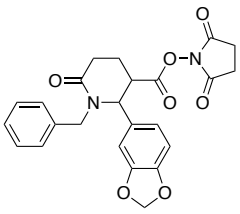
**$^{1}$H NMR (600MHz, $CDCl_3$)**: δ 7.66 (s, 1H), 7.58 (s, 1H), 7.35 (s, 1H), 3.97 (s, 1H), 2.00 (s, 2H), 2.84 (s, 4H), 2.46 (s, 3H).

**$^{13}$C NMR (125MHz, $CDCl_3$)**: δ 168.8, 165.7, 153.9, 143.9, 132.8, 129.3, 126.1, 119.4, 113.3, 110.4, 26.6, 25.6, 20.8

**HRMS (-ESI):** Calculated: 320.0331 ($C_{15}H_{11}ClNO_5$). Observed: 320.0326


CW 1-26 1H
7.66
7.58
7.35
7.26 CDCl3
3.97
2.84
2.46
1.60 HDO
0.92
0.90
0.92
0.27
2.00
4.00
2.96
f1 (ppm)


CW 1-26 13C
168.79
165.65
153.90
143.86
132.81
129.29
126.12
119.41
113.26
110.43
77.20 CDCl3
76.99 CDCl3
76.78 CDCl3
26.56
25.55
20.77
0.99
f1 (ppm)

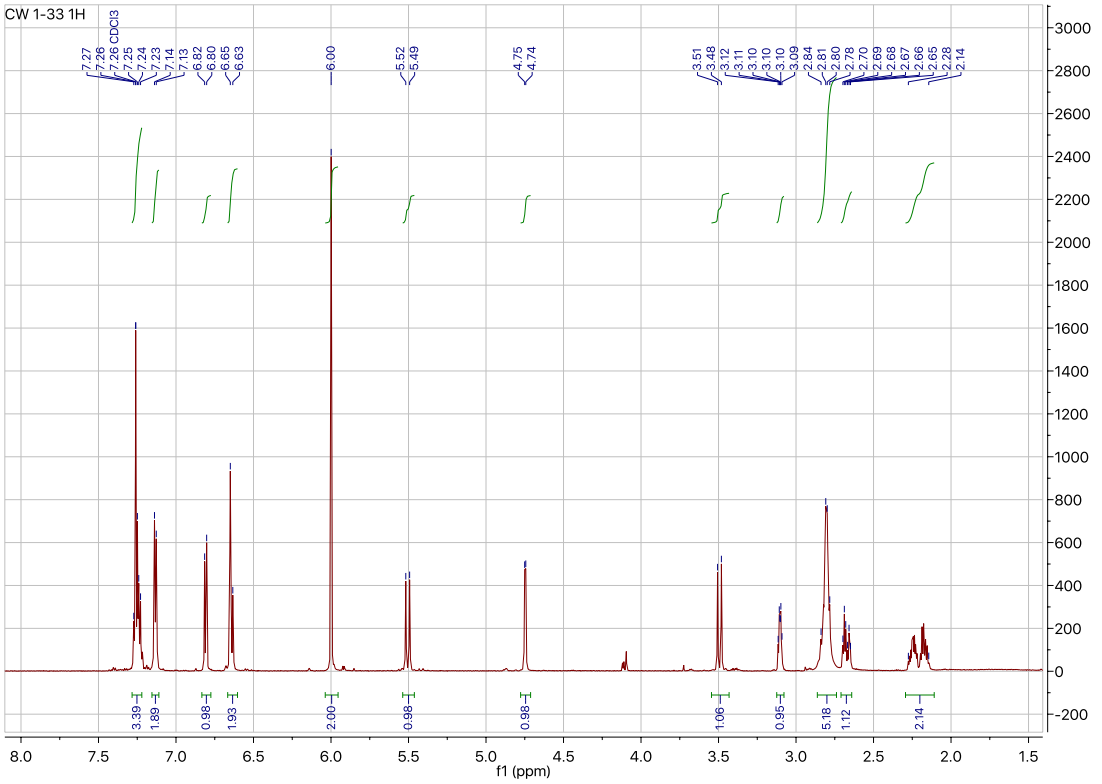


**Synthesis of 2,5-dioxopyrrolidin-1-yl 2-(benzo[*d*][1,3]dioxol-5-yl)-1-benzyl-6-oxopiperidine-3-carboxylate, CW 1-33:** 51.5 mg of 2-(1,3-Benzodioxol-5-yl)-1-benzyl-6-oxo-3-piperidinecarboxylic acid (146 µmol, Matrix scientific) were dissolved in 7.5 mL of anhydrous DCM. 1.4 equivalents of NHS (23.5 mg, 204 µM, Sigma) and EDC-HCl (39.1 mg, 204 µM, Acros) were added and the mixture stirred overnight under nitrogen. TLC (EtOAc) of the reaction showed > 90% conversion after 14 hours. The mixture was filtered, concentrated and applied directly to a silica gel column and purified with a mobile phase of EtOAc. 31.0 mg were recovered (47%).

**$^{1}$H NMR (600MHz, $CDCl_3$)**: δ 7.27-7.23 (m, 3H), 7.13 (d, *J* = 6.9 Hz, 2H), 6.81 (d, *J* = 7.8 Hz, 1H), 6.67-6.21 (m, 2H), 6.00 (s, 2H), 5.51 (d, *J* = 14.9 Hz, 1H), 4.75 (d, *J* = 4.4 Hz, 1H), 3.49 (d, *J* = 14.8 Hz, 1H), 3.10 (dt, *J* = 6.4, 4.2 Hz, 1H), 2.84-2.78 (m, 5H), 2.67 (dt, *J* = 18.4, 5.7 Hz, 1H), 2.28-2.14 (m, 2H)

**$^{13}$C NMR (125MHz, $CDCl_3$)**: δ 169.3, 168.7, 167.7, 148.7, 147.9, 136.3, 132.7, 128.8, 128.5, 127.5, 120.7, 108.8, 107.1, 101.6, 60.2, 47.9, 45.1, 29.8, 25.7, 20.7

**HRMS (+ESI):** Calculated: 451.1500 ($C_{24}H_{23}O_7N_2$). Observed: 451.1500


CW 1-33 1H
7.27
7.26
7.26 CDCl3
7.25
7.24
7.23
7.14
7.13
6.82
6.80
6.65
6.63
6.00
5.52
5.49
4.75
4.74
3.51
3.48
3.12
3.11
3.10
3.10
3.10
3.09
2.84
2.81
2.80
2.78
2.70
2.69
2.68
2.67
2.66
2.55
2.28
2.14
3.39
1.89
0.98
1.93
2.00
0.98
0.98
1.06
0.95
5.18
1.12
2.14
f1 (ppm)

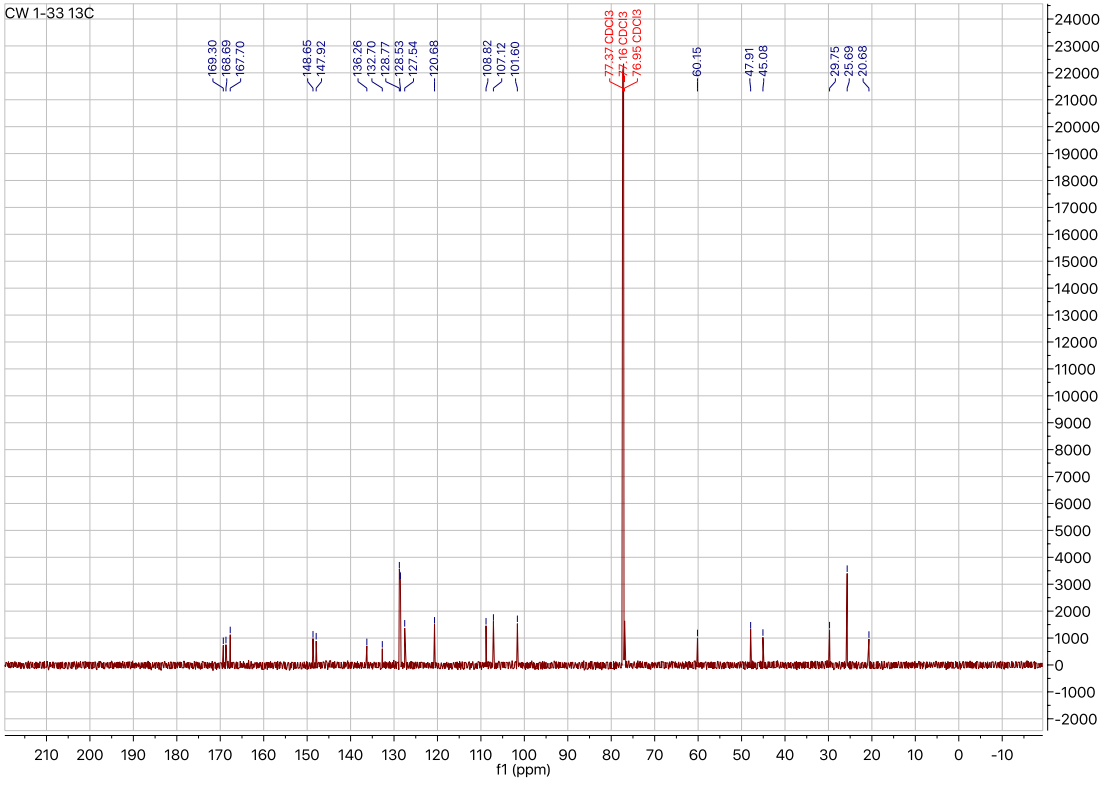

CW 1-33 13C
169.30
168.69
167.70
148.65
147.92
136.26
132.70
128.77
128.53
127.54
120.68
108.82
107.12
101.60
77.37 CDCl3
77.16 CDCl3
76.95 CDCl3
60.15
47.91
45.08
29.75
25.69
20.68
f1 (ppm)

**Table Legends**

**Table S1. IsoTOP-ABPP proteomic data**

**Tabs 1-9.** Amino acid reactivity of NHS-ester-alkyne in mouse liver proteome. Mouse liver proteomes were labeled with NHS-ester-alkyne (500 or 100 μM), followed by appendage of biotin-azide tags bearing an isotopically light (for 500 μM) or heavy (for 100 μM) tags and a TEV recognition sequence, followed by mixing heavy or light proteomes in 1:1 ratio, avidin-enrichment of probe-labeled proteins, digestion of enriched proteins by trypsin, and isolation of release of probe-modified tryptic peptides by TEV protease for subsequent LC-LC/MS/MS analysis. Reported in **Tabs 1, 3, 5, 7,** and **9** are probe-modified peptides separated by amino acid reactivity, the specific amino acids labeled, the light to heavy isotopic ratios, Uniprot identification numbers, and protein designations. Only peptides that were present in 2 out of 4 biological replicates are reported here. Ratios shown are average ratios from the biological replicates. Reported in **Tabs 2, 4, 6, 8,** and **10** are known annotations from probe-modified peptides based on the Uniprot database.

**Tab 10.** Lysine-reactivity of the Dichlorotriazine-alkyne probe in mouse liver proteomes. Mouse liver proteomes were labeled with dichlorotriazine-alkyne (500 or 100 μM), followed by appendage of biotin-azide tags bearing an isotopically light (for 500 μM) or heavy (for 100 μM) tags and a TEV recognition sequence, followed by mixing heavy or light proteomes in 1:1 ratio, avidin-enrichment of probe-labeled proteins, digestion of enriched proteins by trypsin, and isolation of release of probe-modified tryptic peptides by TEV protease for subsequent LC-LC/MS/MS analysis. Only peptides that were present in 2 out of 3 biological replicates are reported here. Ratios shown are average ratios from the biological replicates.

**Tabs 11 and 12.** IsoTOP-ABPP analysis of CW 1-26 and CW 1-33 in mouse liver proteomes. DMSO vehicle or CW 1-26 (100 μM) or CW 1-33 (100 μM) were pre-treated in mouse liver proteomes for 30 min prior to labeling of proteomes with NHS-ester-alkyne (500 μM) for 1 h at room temperature, followed by appendage of light (for vehicle-treated control) or heavy (for fragment-treated) biotin-TEV-azide tags, followed by the isoTOP-ABPP procedure. Shown are the probe-modified peptides identified, the isotopic light to heavy ratios, sites of probe-modification, uniprot IDs, and protein designations. Only peptides that were present in 2 out of 3 biological replicates are reported here. Ratios shown are average ratios from the biological replicates.

**Figure S1**

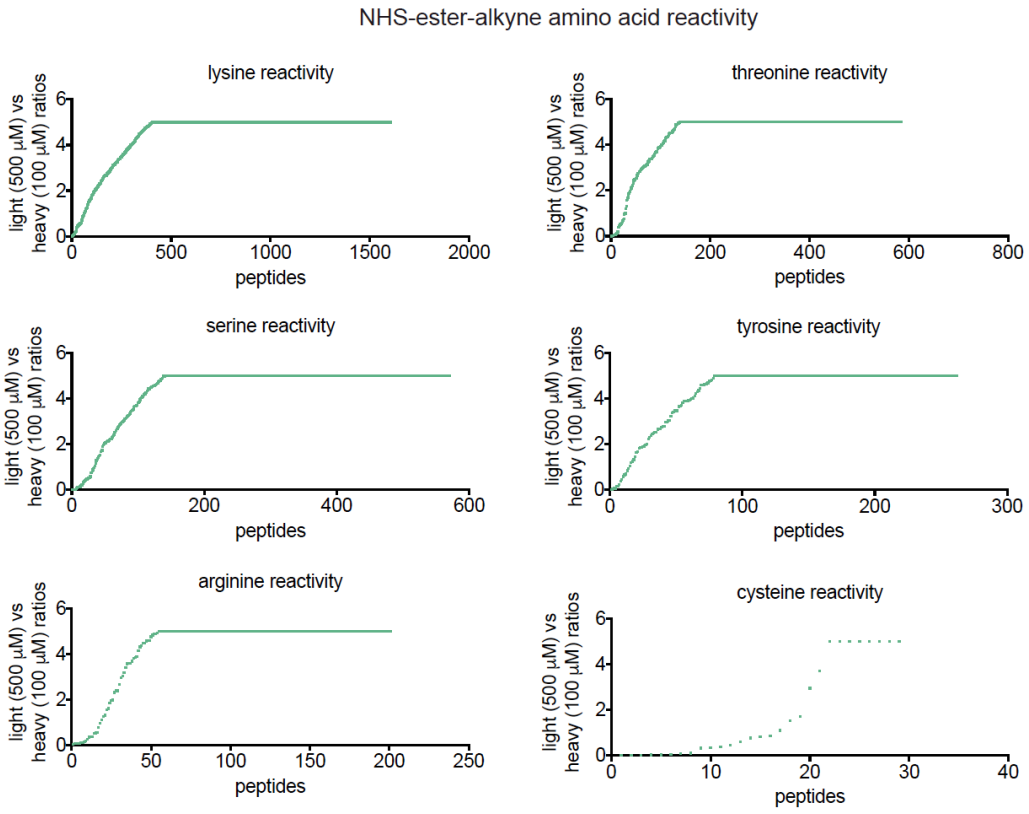



**Figure S1. NHS-ester-alkyne amino acid reactivity**. We labeled mouse liver proteomes with 500 versus 100 μM NHS-ester-alkyne probe, followed by CuAAC-mediated conjugation of an isotopically light (for 500 μM) or heavy (for 100 μM) biotin-azide handles bearing a TEV protease recognition sequence, followed by isoTOP-ABPP analysis of isolated probe-modified tryptic peptides. Shown are the ratios for the individual peptides identified categorized by amino acid reactivity. A light to heavy isotopic ratio of 5 indicates a site that is not hyper-reactive, versus an isotopic ratio of <2 which can be considered hyper-reactive. Data shown are average ratios for probe-modified peptides that were found in at least two out of three biological replicates. Those ratios that were >5 are just shown as having a ratio of 5. Raw data for these plots can be found in **Table S1.**

**Figure S2.**

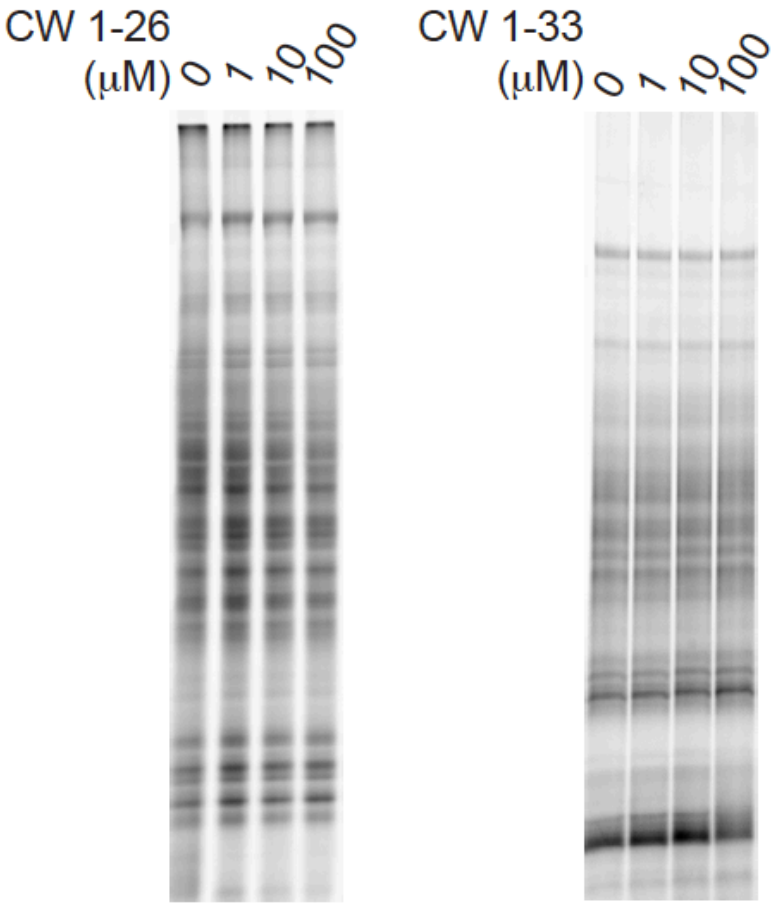



**Figure S2. CW 1-26 and CW 1-33 competition against NHS-ester-alkyne reactivity in mouse liver proteomes.** DMSO vehicle or CW 1-26 or CW 1-33 were pre-incubated for 30 min with mouse liver proteomes prior to labeling with NHS-ester-alkyne (100 μM) for 30 min. Rhodamine-azide was then appended to probe-labeled proteins and proteins were separated by SDS/PAGE and NHS-ester-alkyne reactivity was assessed by in-gel fluorescence. Shown is a representative grey-scale gel.