

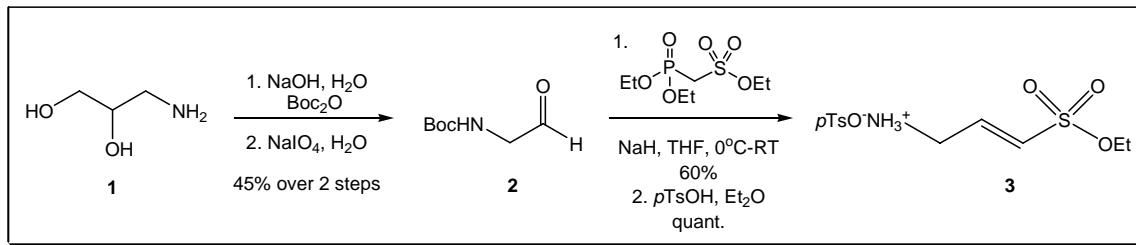
Supporting Information and Methods

Ubiquitin C-terminal electrophiles are activity-based probes for identification and mechanistic study of ubiquitin conjugating machinery

Kerry Routenberg Love, Renuka K. Pandya, Eric Spooner, and Hidde L. Ploegh

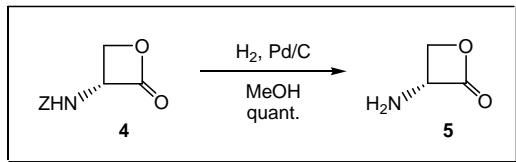
Synthesis and Characterization of Electrophilic Glycine Analogs

General Methods. All chemicals were reagent grade and used as supplied unless otherwise noted. HPLC-grade, low moisture organic solvents (Dri Solv), including *N,N'*-dimethylformamide (DMF), dichloromethane (CH₂Cl₂), tetrahydrofuran (THF), methanol (MeOH), and diethyl ether (Et₂O), were purchased from EMD Chemicals. Analytical thin-layer chromatography was performed on E. Merck silica gel 60 F₂₅₄ plates (0.25 mm). Compounds were visualized by dipping the plates in a cerium sulfate-ammonium molybdate solution or a ninhydrin solution followed by heating. Liquid flash column chromatography was performed using forced flow of the indicated solvent on EMD Chemicals silica (200-400 mesh). ¹H NMR spectra were obtained using a 400 MHz spectrometer and are reported in parts per million (δ) relative to CDCl₃ (7.27 ppm) or MeOD (4.87 ppm, 3.31 ppm).

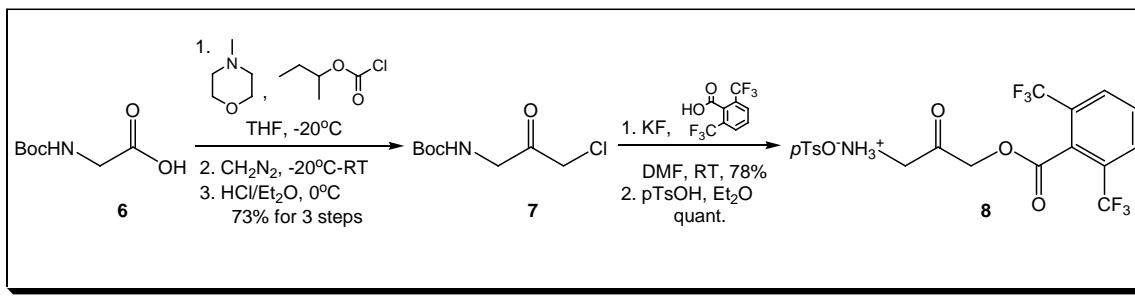


4-amino-but-1-enyl ethoxysulfonate (3). *N*-tert-butyloxycarbonyl (Boc) protected glycinal was prepared as previously reported[1] and was subjected to sodium metaperiodate-mediated oxidative cleavage[2] to obtain *N*-Boc-glycinal (**2**), which was used without further purification. A solution of sodium hydride (258 mg, 10.2 mmol) in THF (60 mL) was cooled to 0°C before the dropwise addition of triethyl α -phosphonylmethanesulfonate (2.66 g, 10.2 mmol)[3], followed by a 5 mL rinse of the phosphonate-source vessel[4]. The reaction mixture was stirred for 20 min at 0°C before adding the above prepared *N*-Boc-glycinal (**2**) as a neat solid. The reaction mixture was allowed to warm to room temperature and was stirred for 6 h before quenching with water (50 mL) and extracting with ethyl acetate (3 \times 50 mL). The combined organic extracts were dried over Na₂SO₄, filtered and concentrated. The resulting crude residue was purified using flash column chromatography by elution with 25% ethyl acetate/hexanes, yielding 0.8 g (60%) pure *N*-Boc-4-amino but-1-enyl ethoxysulfonate. ¹H NMR: 6.78 (dt, 1H), 6.34 (d, 1H), 4.82 (br s, 1H), 4.17 (q, 2H), 3.99 (br s, 2H), 1.45 (s, 9H), 1.39 (t, 3H). Removal of the Boc group was accomplished using three equivalents dry *p*-toluenesulfonic acid as described[5] to yield **3** as the sulfonic acid salt

of the deprotected amine. ESI-MS: Calculated; $[M + H]^+ = 166.05$. Observed; $[M + H]^+ = 166.05$.



Serine-β-lactone (5). *N*-(benzyloxycarbonyl[Z])-D-serine-β-lactone (**4**) was prepared as previously reported[6]. Removal of the Z group was accomplished in quantitative yield by hydrogenation over Pd/C in methanol. ^1H NMR: 4.23 (m, 1H), 4.12 (m, 2H), 3.54 (m, 1H), 1.41 (appd, 1H). ESI-MS: Calculated; $[2M + H]^+ = 177.09$. Observed; $[2M + H]^+ = 177.09$.



2,6-trifluoromethylbenzyloxy glycine methyl ketone (8). Boc-protected glycine (**6**) was converted to the amino acid diazomethyl ketone as previously reported[7] via addition of a cold solution of diazomethane to a mixed anhydride formed by treatment of the protected amino acid with isobutyl chloroformate in the presence of *N*-methyl morpholine. The diazomethyl ketone was easily converted to the chloromethyl ketone (**7**) by addition of a solution of HCl in Et₂O[8]. *N*-Boc-glycine chloromethyl ketone **7** (430 mg, 2.07 mmol) was treated with potassium fluoride (361 mg, 6.21 mmol) in DMF (20 mL), followed by 2,6-trifluorobenzoic acid (587 mg, 2.28 mmol) overnight at room temperature[9]. After 12 h the reaction was diluted with Et₂O and ethyl acetate (50 mL each) and washed with water, aqueous NaHCO₃ (sat.) and brine. The organic layer was dried over Na₂SO₄, filtered and concentrated to yield 0.69 g (78%) of compound **8**, which was used without further purification. ^1H NMR: 7.95 (appd, 2H), 7.76 (appt, 1H), 5.22 (br s, 1H), 4.90 (s, 1H), 4.13 (m, 2H), 1.40 (s, 9H). Removal of the Boc group was accomplished using three equivalents dry *p*-toluenesulfonic acid as described[5] to yield **8** as the sulfonic acid salt of the deprotected amine. ESI-MS: Calculated; $[M + H]^+ = 330.06$. Observed; $[M + H]^+ = 330.06$.

Synthesis and Characterization of HAUb-electrophilic Probes

HAUb-electrophilic probes were made using the electrophilic glycine analogs synthesized above as described in the Experimental Procedures. The FPLC purified HAUb-electrophilic probes were characterized by ESI-LC/MS and multi-charged species were observed for each probe. Probe yields were estimated using traces obtained during FPLC purification. HAUbOEtVS (quant. yield) ESI-MS: Calculated; $[M + 11H]^{11+} =$

937. Observed; $[M + 11H]^{11+} = 936.7266$. HAUbLac (50% yield) ESI-MS: Calculated; $[M + 11H]^{11+} = 931$. Observed; $[M + 11H]^{11+} = 931.4602$. HAUbTF₃BOK (80% yield) ESI-MS: Calculated; $[M + 11H]^{11+} = 952$. Observed; $[M + 11H]^{11+} = 952.0001$.

EL-4 Cell Lysate Labeling and Blocking of Labeling with NEM (N-ethylmaleimide)

30 μ g of protein extract (see Methods section for cell culture and lysate harvesting conditions) treated with NEM (10 mM) for 30 min at room temperature. Following this treatment, 0.2 μ g of HAUb-electrophilic probe (2.0 μ M in each sample) was added and the reactions were incubated for 5 h at room temperature. Control reactions were run for each probe without prior addition of NEM to observe the extent of labeling in 5 h. Reactions were quenched by addition of reducing sample buffer, boiled 10 min and separated by SDS-PAGE (10%). Results were analyzed by immunoblotting with an anti-HA antibody (3F10, Roche).

EL-4 Cell Lysate labeling and Detection of RING Ligase UBAC1 Modification by Gel Shift.

30 μ g EL-4 lysate was incubated with indicated quantities of probe (concentrations are 2.3 μ M, 4.6 μ M, 9.2 μ M, and 18.4 μ M) in buffer containing 50mM Tris pH 8.0, 150mM NaCl for 4 hr at room temperature (24°C). Reactions were quenched by addition of reducing sample buffer, resolved by SDS-PAGE analyzed by immunoblot using either a UBAC1 antibody (Novus Biologicals) or a UCH37 antibody (polyclonal rabbit) for comparison.

Plasmid Construction

The C-terminal residues of ARF-BP1 (amino acids 4012-4374) were subcloned into vector pet28a for expression and purification as an N-terminal His₆ fusion protein using forward primer 5'-ccgcatatgctccggaaagaagacatgg and reverse primer 5'-ccggaattcttaggccagccaaagcc. Single, double, and triple cysteine-to-alanine ARF-BP1 HECT domain mutants at residues C4099, C4341, C4367, were generated using site-directed mutagenesis (Stratagene). A truncated version of ARF-BP1 (ARF-BP1 Δ 4) (residues 4012-4370) was cloned into pet28a for Ub-thioester assays using the forward primer listed above and the reverse primer 5'-cgcggatcccttagcctcagagcactcctg.

The C-terminal residues of Trip12 (amino acids 1629-2040, KIAA0045, Kazusa DNA Research Institute) were subcloned with an N-terminal His₆ tag into the vector pGEX-6P-1 (GE Healthcare) for expression and purification as an N-terminal GST fusion protein using forward primer 5'-cgcggatccggacatcatcatcatcacagcagcggcagagttgcaccttagattgg and reverse primer 5'- cgcgtcgactcaggaaagatggaacgcgt.

The N-terminal residues of Mcl-1 (amino acids 1-327) were cloned with an N-terminal PKA (protein kinase A) recognition sequence into the vector pet16b for expression and purification as an N-terminal His₁₀ fusion protein using forward primer 5'-ccgcatatggactacaaggacgacgacaaggcaggacgtcgcatctgtggatccttgccctcaaagaaacgcgg and reverse primer 5'-cgcggatcccttagccacctctaggtccctctac.

Ubiquitin was cloned with an N-terminal PKA (protein kinase A) recognition sequence into the vector pet28a for expression and purification as an N-terminal His₆ fusion protein using forward primer 5'-ccgcatatgcgtgcacatctgtggatccatgcagatctcgtaagacg and reverse primer 5'-cgccgtatccatccccacgcacgt.

Protein Expression and Purification of ARF-BP1 HECT domain and PKA-Mcl-1

ARF-BP1 HECT domain and FLAG-Mcl-1 containing plasmids were transformed into Rosetta competent cells (DE3(PLysS), Novagen) and cells were plated on LB-agar supplemented with 50 μ g/mL kanamycin (ARF-BP1) or 100 μ g/mL ampicillin (Mcl-1) and 20 μ g/mL chloramphenicol. A single colony for each protein was used to inoculate a 1.5 L liquid culture (LB, Miller) and cultures were grown to late log ($OD_{600} > 0.8$) at 37°C. Cultures were induced with 0.5 mM IPTG at 22°C overnight. Cells were pelleted and washed once with PBS before resuspending in 25 mL lysis buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 10 mM imidazole). DNase was added to a final concentration of 10 μ g/mL and the cells were lysed under pressure (1500 psi) using a French press. Cell debris was pelleted and the supernatant was purified using Ni-NTA beads (Qiagen) for His-tag-based purification. Eluted proteins were further purified by gel filtration (Superdex 75 PC 3.2/30, GE Healthcare) using a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 5 mM MgCl₂, 2 mM DTT, and 5% glycerol.

Protein Expression and Purification of Trip12 HECT domain

Trip 12 HECT domain-containing plasmid was transformed into Rosetta competent cells (DE3(PLysS), Novagen) and cells were plated on LB-agar supplemented with 100 μ g/mL ampicillin and 20 μ g/mL chloramphenicol. A single colony for each protein was used to inoculate a 1.5 L liquid culture (LB, Miller) and cultures were grown to late log ($OD_{600} > 0.8$) at 37°C. Cultures were induced with 0.5 mM IPTG at 22°C overnight. Cells were pelleted and washed once with PBS before resuspending in 25 mL PBS supplemented with DNase (final concentration of 10 μ g/mL) and 1 mM MgCl₂. Cells were lysed under pressure (1500 psi) using a French press. Cell debris was pelleted and the supernatant was purified using Glutathione Sepharose 4B beads (GE Healthcare) for GST-tag-based purification. Eluted proteins were used without further purification.

Protein Expression and Purification of PKA-Ub

PKA-Ub-containing plasmid was transformed into Rosetta competent cells (DE3(PLysS), Novagen) and cells were plated on LB-agar supplemented with 50ug/mL kanamycin and 20ug/mL chloramphenicol. A single colony was used to inoculate 6L liquid culture (LB, Miller) and cultures were growth to late log ($OD_{600} > 0.8$) at 37°C. Cultures were induced with 0.5 mM IPTG at 30°C for 3.5hr. Cells were pelleted and washed once with PBS before resupsending in lysis buffer (50mM Tris pH 8.0, 500mM NaCl, 20mM imidazole, 10ug/mL DNase). Cells were lysed under pressure (1500 psi) using a French press. Cell debris was pelleted and the supernatant purified using Ni-Sepharose (High Performance, GE Healthcare) for His-tag-based purification. Eluted proteins were further purified by gel filtration (Superdex 75 PC 3.2/30, GE Healthcare) using a buffer containing 50mM Tris pH 7.5, 150mM NaCl, and 5% glycerol.

Synthesis of [³²P]-labeled proteins

Recombinant proteins (1 mg Ub or 120 ug Mcl-1) bound to Ni-sepharose (High Performance, GE Healthcare) were washed once in HMK buffer (20mM Tris pH 7.5, 100mM NaCl, 12mM MgCl₂) and incubated with the catalytic subunit of cAMP-dependent Protein Kinase (PKA, New England Biolabs; 50,000 U for Ub or 25,000 U for Mcl-1), and a 10-fold molar excess of ATP in a ratio of 1:100 (Ub) or 1:10 (Mcl-1) γ -[³²P]-ATP (6000 Ci/mmol, 10mCi/mL, PerkinElmer) to unlabeled ATP, in HMK buffer containing 1 mM DTT in a final volume of either 200 ul (Ub) or 100 ul (Mcl-1) for 3 hours at 25°C. The phosphorylation reaction was terminated by incubation of proteins with HMK stop buffer (10mM sodium phosphate pH 8.0, 10mM sodium pyrophosphate, 10mM EDTA, 1mg/ml bovine serum albumin) for 5 min at 25°C. Proteins were washed 5 times with NET buffer and eluted in 50mM Tris pH 8.0, 250mM NaCl, 250mM imidazole.

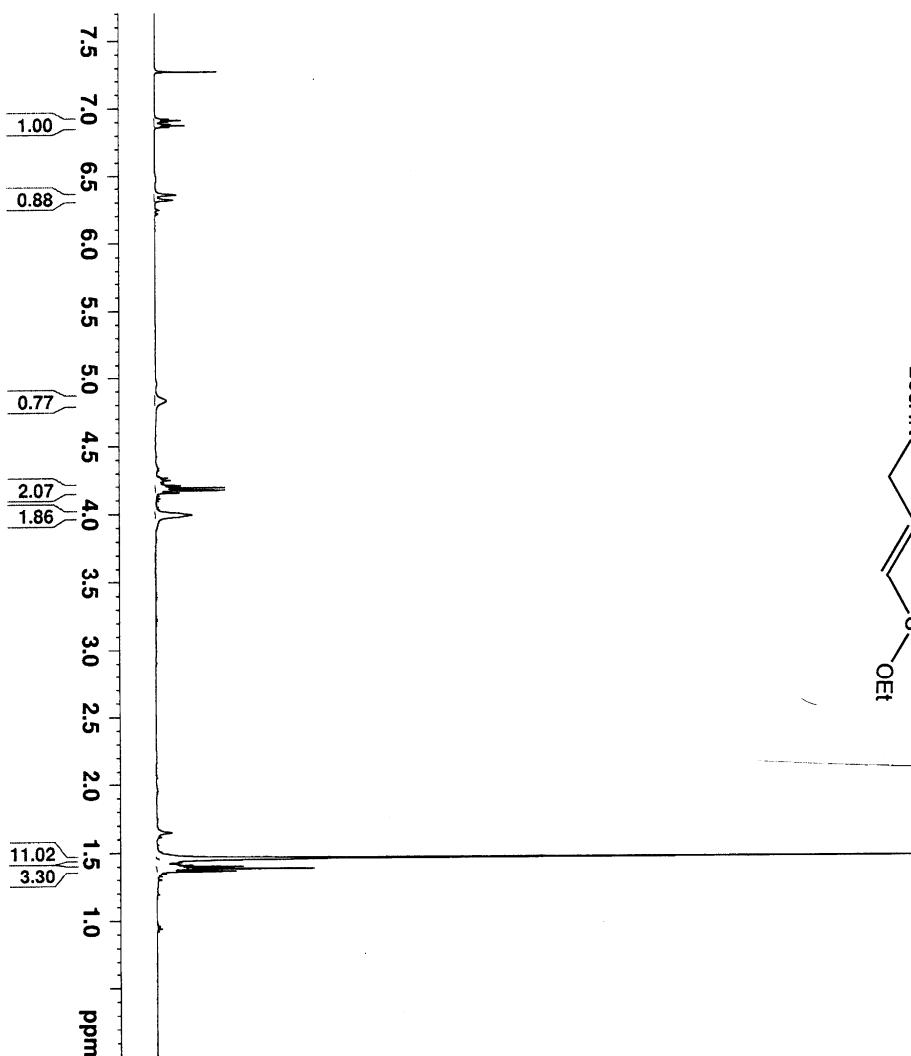
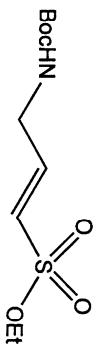
Autoubiquitination Assay with GST-Trip12 HECT Domain

Recombinantly expressed and purified GST-Trip12 HECT domain (10 μ g) (see above for plasmid construction, protein expression and purification conditions) was incubated with 100 ng human E1 (Ube1, Boston Biochem), 1 μ g UbcH7, and 10 μ g Ub with an ATP regenerating system (50 mM Tris [pH 7.6], 5 mM MgCl₂, 5 mM ATP, 10 mM creatine phosphate, 3.5 U/mL creatine kinase) for 10 min at room temperature. After terminating the reactions with reducing SDS-PAGE sample buffer and boiling 10 min, reaction mixtures were separated by SDS-PAGE (8%) and analyzed by immunoblotting using an anti-Ub antibody (Sigma). Control reactions were run without either E1 or E2.

References

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TDRES

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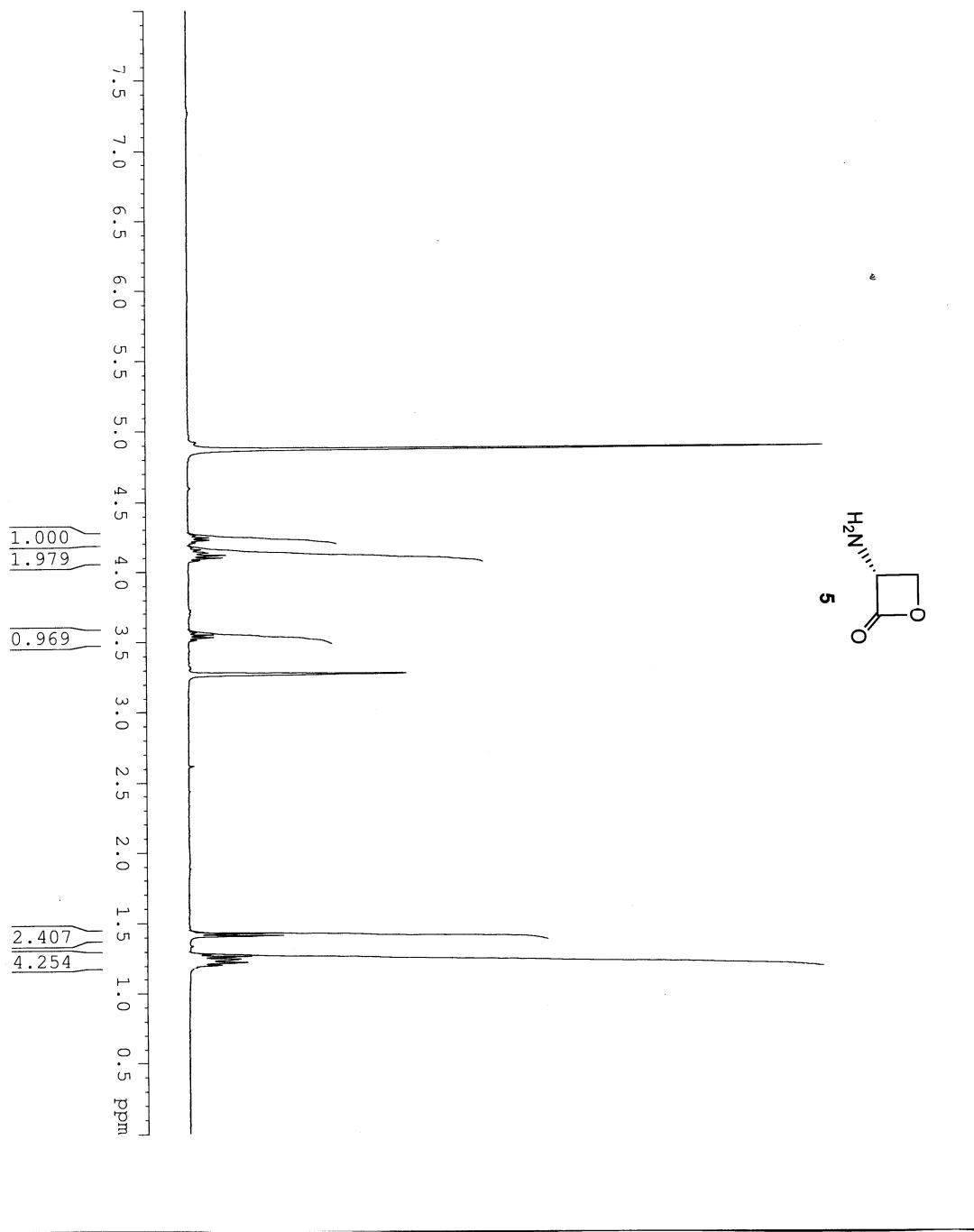
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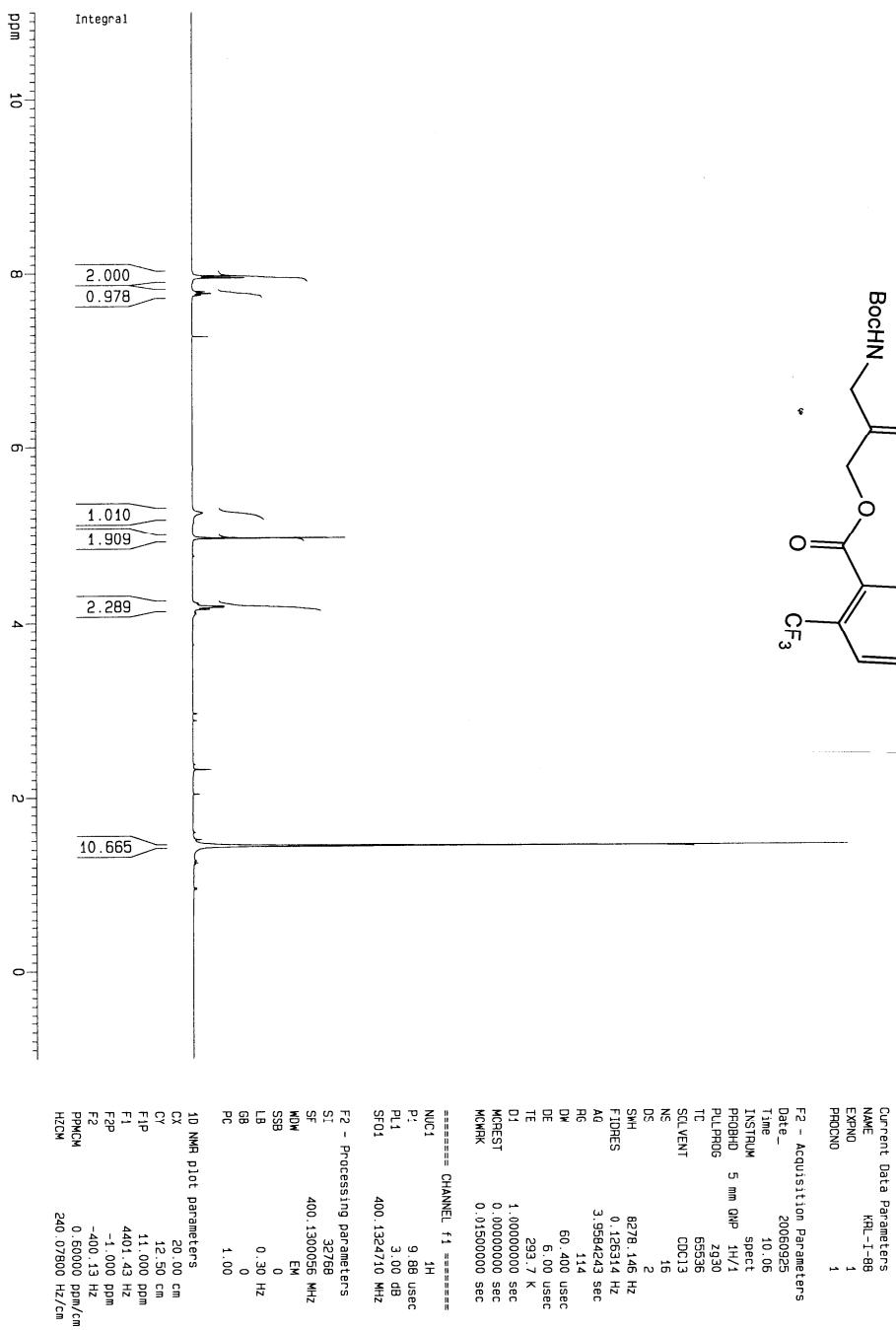
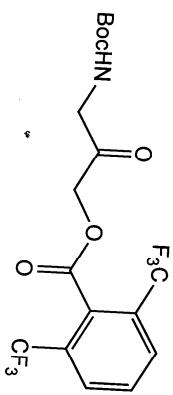
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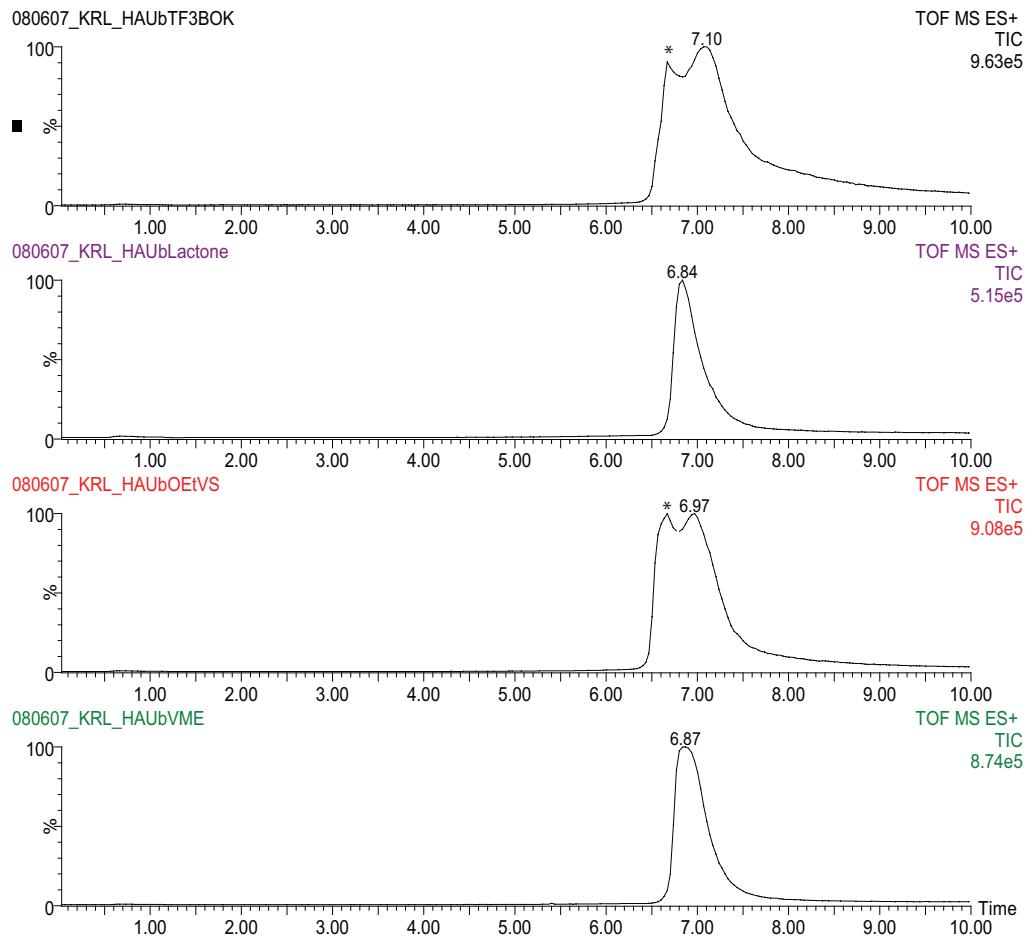
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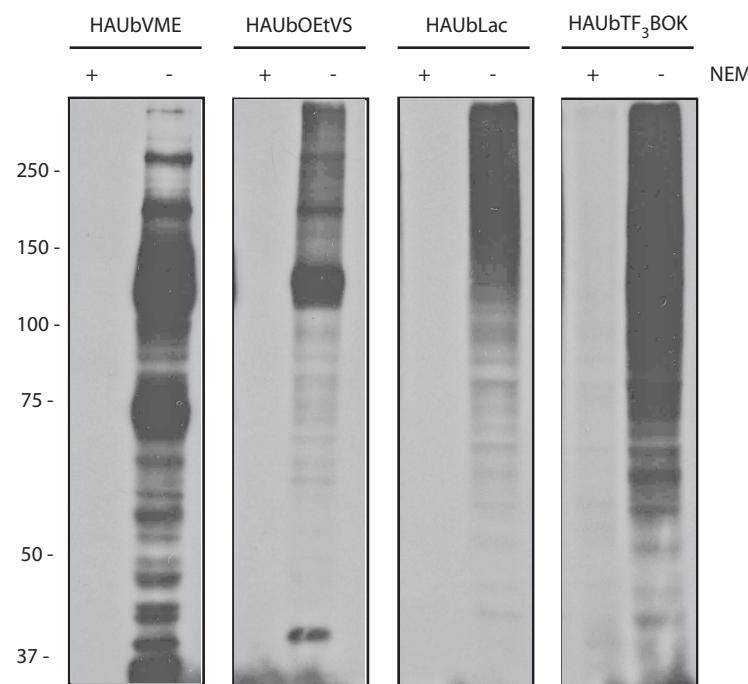
* This peak corresponds to the loss of the electrophilic warhead from the peptide during LC/MS analysis (Calculated: $[M + 11H]^{11+} = 921$; Observed: $[M + 11H]^{11+} = 921.6114$).

Supplementary Figure 1. Labeling of EL-4 cell lysate with Ub electrophilic probes is efficiently blocked by preincubation with NEM (10 mM).

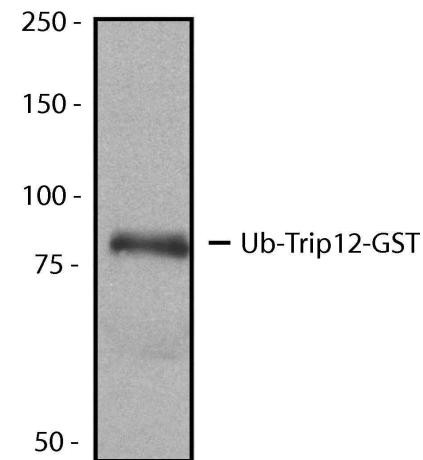
Supplementary Figure 2. Autoubiquitination activity of recombinant HECT domain of GST-Trip12. Ubiquitinated Trip 12 HECT domain was resolved by SDS-PAGE (8%) and visualized by immunoblotting with an anti-Ub antibody.

Supplementary Figure 3. (a) RING ligase UBAC1, present in EL-4 cell lysate, is not directly modified by HAUbVME, suggesting that RING E3s are retrieved in immunoprecipitations of probe-labeled lysate as part of multi-subunit complexes where 1 or more members binds probe directly. (b) UCH37 (UCH-L5), by comparison, is efficiently and quantitatively labeled when lysate containing this DUB is treated with HAUbVME as seen by a ~10 kD increase in its apparent molecular weight.

Supplementary Figure 1



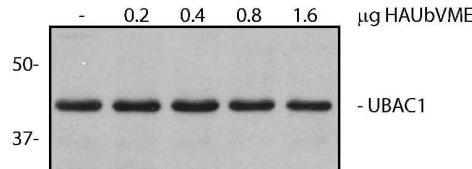
Supplementary Figure 2



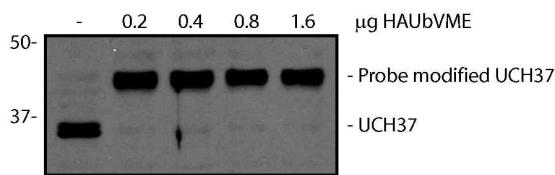
Supplementary Figure 2. Autoubiquitination activity of recombinant HECT domain of GST-Trip12. Ubiquitinated Trip 12 HECT domain was resolved by SDS-PAGE (8%) and visualized by immunoblotting with an anti-Ub antibody.
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Supplementary Figure 3

A



B



Supplementary Figure 3. (a) RING ligase UBAC1, present in EL-4 cell lysate, is not directly modified by HAUbVME, suggesting that RING E3s are retrieved in immunoprecipitations of probe-labeled lysate as part of multi-subunit complexes where 1 or more members binds probe directly. (b) UCH37 (UCH-L5), by comparison, is efficiently and quantitatively labeled when lysate containing this DUB is treated with HAUbVME as seen by a ~10 kD increase in its apparent molecular weight.
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