Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

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Substrate mimicry in an activity-based probe that targets the nitrilase family of enzymes

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Experimental Procedures

Synthesis of Probes

General

All chemicals were obtained from Aldrich, Acros, Novabiochem, or Molecular Probes, and were used without further purification, except where noted. Solvents and triethylamine were used as received or passed over an activated alumina column (CH_2Cl_2). All reactions were carried out under an argon atmosphere using oven-baked glassware cooled in a desiccator. Purification was performed on a Hitachi D-700 HPLC system. ^1H spectra were recorded on a Bruker AMX-400 MHz spectrometer. Chemical shifts are reported in δ values relative to tetramethylsilane, and coupling constants (*J*) are reported. Mass spectra were obtained on an Agilent 1100 Series LC-MSD (ESI) or an IonSpec Ultima FTMS (MALDI-FTMS).

Solid-Phase Synthesis

Peptides were synthesized on Sieber amide resin (Novabiochem) using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis. For the X=H library (Figure 2, example given for R_1-R_2 = Leu-Ala), Fmoc protected resin (15 mg, 0.008 mmol) was deprotected with 20% piperidine/dimethylformamide (DMF, 2 x 2mL, 8 min) and washed (3 x DMF, 3 x CH_2Cl_2, 3 x DMF) to remove excess reagents. For the X=H library (Figure 2), Fmoc-Lys(N-ε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl)-COOH [Fmoc-Lys(ivDde)-OH (Novabiochem) 5 equiv, 0.04 mmol, 23 mg) was coupled to the resin (30 min) using 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU; 5 equiv, 0.04 mmol, 15 mg), N-hydroxybenzotriazole (HOBt; 5 equiv, 0.04 mmol, 5.4 mg), and diisopropylethylamine (DIEA; 8 equiv, 0.08 mmol, 13 μL) in NMP (1 mL). Complete coupling was confirmed using a Kaiser test. The resin was washed and deprotected as described above, and Fmoc-Leu-OH (5 equiv, 0.04 mmol, 14 mg) was coupled as described above. After a second deprotection, Fmoc-Ala-OH (5 equiv, 0.04 mmol, 12 mg) was coupled as described above. For other probes, fully protected amino acids R_1 and R_2 were coupled in place of Leu and Ala (all 5 equiv, 0.04 mmol) as described above. Protecting groups were chosen to facilitate deprotection in 2% TFA. The following protected amino acids were used: Fmoc-Leu-OH, Fmoc-His(N-im-methyltrityl)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-Ala-OH, Fmoc-Tyr(O-2-chlorotrityl)-OH, Fmoc-Ser(O-trityl)-OH, Fmoc-Glu(γ-2-phenylisopropyl)-OH, Fmoc-Asp(γ-2-phenylisopropyl)-OH, Fmoc-D-Leu-OH, Fmoc-Pro-OH, Fmoc-D-Pro-OH, Fmoc-Trp-OH, Fmoc-Asn-OH, Fmoc-D-Asp(*O*-t-Bu)-OH, Fmoc-phosphoSer(O-benzyl)-OH. Following the final deprotection, capping was affected using 1 M chloroacetylanhydride in N-methylpyrrolidinone (NMP,

1 mL). The C-terminal lysine(ivDde) residue was then deprotected on-resin with 2% hydrazine/DMF (2 x 2 min, 2 mL each). Note—longer deprotection times caused hydrazine displacement of the chloroacetamide functionality. The free lysine was then coupled to 5-(and-6)-Carboxytetramethylrhodamine succinimidyl ester (5 mg, 1.2 equiv, 0.04 mmol) dissolved in NMP (1 mL), and DIEA (8 μL, 6 equiv, 0.08 mmol) overnight. The resin was washed extensively with CH_2Cl_2 (3 x 5 mL), MeOH (3 x 5 mL), NMP (3 x 5 mL), and CH_2Cl_2 (3 x 5 mL). Cleavage and deprotection were affected using 2% trifluoroacetic acid (TFA) in CH_2Cl_2 (5 x 5 mL, 20 min each), and the resin rinsed again with 5 mL CH_2Cl_2. 10 mL toluene was added to form a TFA azeotrope and solvent was removed under vacuum. Peptides containing D-Asp(O^{t}Bu)-OH were re-dissolved in 1 mL 50% TFA/ CH_2Cl_2 with 1% triethylsilane ($SiHEt_3$) and stirred for 1 hour for complete deprotection, and then dried again under vacuum after addition of 1 mL toluene. The crude products were purified by HPLC chromatography (C18-reverse phase column, 5%-100% CH_3CN in H_2O with 0.05% TFA). Removal of CH_3CN under reduced pressure and lyophilization of the H_2O gave the final product as a dark red solid.

For trifunctional (X = lysine(biotin) (Figure 2)), the first residue coupled on the resin was Fmoc-Lys(Biotin)-COOH (Novabiochem, 5 equiv) using the same conditions as above, and the rest of the synthesis followed as described above. For alkyne-probe synthesis, the first residue was Fmoc-aspartate-alkyne (see solution phase chemistry section), and the rest of the synthesis followed as described above.

The alkyne probe was synthesized on solid phase as described above, with Fmoc-Asp(alkyne)-COOH being the first residue coupled, followed by Fmoc-Leu-OH, Fmoc-Asp(γ-2-phenylisopropyl)-OH Asp, and chloroacetamide capping

Compound Characterization

R_1	R_2	Molecular Formula	Expected Mass	Found Mass
Leu	His	$C_{45}H_{54}ClN_9O_8$	884.4	884.4
His	Leu	$C_{45}H_{54}ClN_9O_8$	884.4	884.8
Leu	Gly	$C_{41}H_{54}ClN_9O_8$	804.3	804.3
Gly	Leu	$C_{41}H_{54}ClN_9O_8$	804.3	804.5
Leu	Ile	$C_{45}H_{58}ClN_7O_8$	860.4	860.4
Leu	Leu	$C_{45}H_{58}ClN_7O_8$	860.4	860.5
Leu	Phe	$C_{48}H_{56}ClN_7O_8$	894.4	894.8
Leu	Ala	$C_{42}H_{52}ClN_7O_8$	818.4	818.7
Ala	Leu	$C_{42}H_{52}ClN_7O_8$	818.4	818.7
Leu	Tyr	$C_{48}H_{56}ClN_7O_9$	910.4	910.7
Tyr	Leu	$C_{48}H_{56}ClN_7O_9$	910.4	910.7
Leu	Ser	$C_{42}H_{52}ClN_7O_9$	834.4	834.7
Ser	Leu	$C_{42}H_{52}ClN_7O_9$	834.4	834.7
Leu	Glu	$C_{44}H_{54}ClN_7O_{10}$	876.7	876.4
Glu	Leu	$C_{44}H_{54}ClN_7O_{10}$	876.8	876.4
Leu	D-Leu	$C_{45}H_{58}ClN_7O_8$	860.4	860.8
D-Leu	Leu	$C_{45}H_{58}ClN_7O_8$	860.4	860.7
Leu	Trp	$C_{50}H_{57}ClN_8O_8$	933.4	933.8
Trp	Leu	$C_{50}H_{57}ClN_8O_8$	933.4	933.8
Leu	Asn	$C_{43}H_{53}ClN_8O_9$	861.4	861.8
Asn	Leu	$C_{43}H_{53}ClN_8O_9$	861.4	861.8
Leu	Pro	$C_{44}H_{54}ClN_8O_8$	844.4	844.7
Leu	D-Pro	$C_{44}H_{54}ClN_8O_8$	844.4	844.8
D-Pro	Leu	$C_{44}H_{54}ClN_8O_8$	844.4	844.8
Leu	phosphoSer	$C_{42}H_{51}ClN_7O_{12}P$	914.4	914.7
phosphoSer	Leu	$C_{42}H_{51}ClN_7O_{12}P$	914.4	914.7
Leu	D-Asp	$C_{43}H_{52}ClN_7O_{10}$	862.7	862.4
D-Leu	Asp	$C_{43}H_{52}ClN_7O_{10}$	862.5	862.4
D-Leu	D-Asp	$C_{43}H_{52}ClN_7O_{10}$	862.5	862.4
Asp	D-Leu	$C_{43}H_{52}ClN_7O_{10}$	862.3	862.4
D-Asp	Leu	$C_{43}H_{52}ClN_7O_{10}$	862.7	862.4
D-Asp	D-Leu	$C_{43}H_{52}ClN_7O_{10}$	862.5	862.4

The following compounds were described in an earlier publication[2] (Listed as R_1-R_2) Ile-Leu, Leu-Met, Met-Leu, Pro-Leu, Phe-Leu, Leu-Asp, Asp-Leu, Leu-Arg, Arg-Leu, Leu-phosphoTyr, phosphoTyr-Leu.

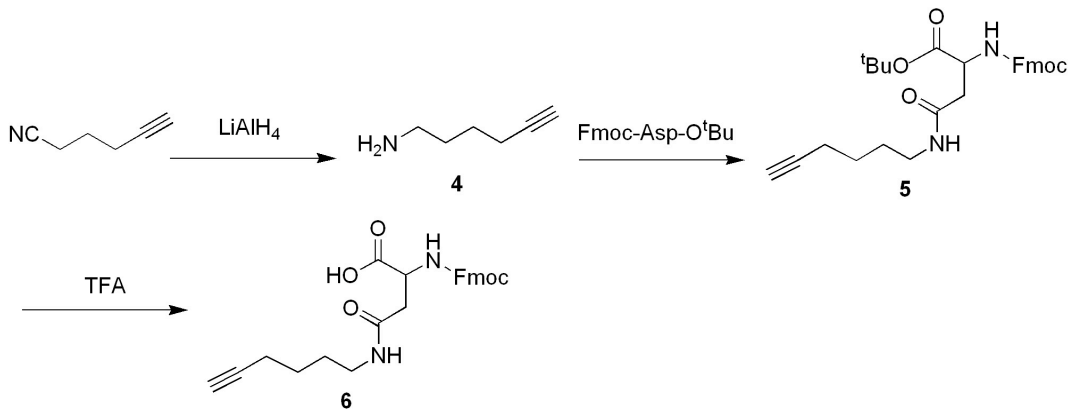
Alkyne and rhodamine containing Leu-Asp probe (used interchangeably with rhodamine-containing Leu-Asp probe[2]): ESI 1056.3 ($C_{53}H_{68}ClN_{10}O_{11} + H^+$ requires 1056.5)

Biotin and rhodamine containing Leu-Asp probe (X = lysine biotin (Figure 2), used for protein identification): ESI 1216.4 ($C_{59}H_{78}ClN_{11}O_{13}S + H^+$ requires 1216.5)

(*N*-2-chloroacetamide)-Asp-Leu-Asn(5-hexynyl)-NH_2 (**3**). MALDI-FTMS 516.2213 ($C_{22}H_{34}ClN_5O_7 + H^+$ requires 516.2219)

(*N*-2-chloroacetamide)-Asp-Leu-NH_2 (**2**). MALDI-FTMS 322.1163 ($C_{12}H_{20}ClN_3O_5 + H^+$ requires 322.1164) ^{1}H NMR (MeOD, 400 MHz), δ 4.74 (t, J = 5.4 Hz, 1H, $C_\alpha H$), 4.36 (m, 1H, $C_\alpha H$), 4.11 (s, 2H, $ClCH_2CO$), 2.82 (m, 2H, $HOC(O)CH_2$), 1.68 (m, 1H, $(CH_3)_2CHCH_2$), 1.59 (m, 2H, $(CH_3)_2CHCH_2$), 0.95 (d, J = 6.4 Hz, 3H, $(CH_3)_2CH$), 0.91 (d, J = 6.4 Hz, 3H, $(CH_3)_2CH$).

Solution-Phase Synthesis

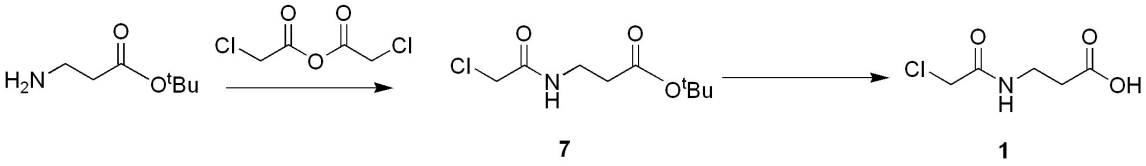


6-Amino-hexyne (**4**). To a 200 mL roundbottom flask precooled to 0 ºC was added dry ether (40 mL) and lithium aluminum hydride (LAH, 1 M solution in ether, 22 mL, 22 mmol, 1.05 equiv). The solution was allowed to cool to 0 ºC with stirring for 20 min. 6-cyano-hexyne (2.2 mL, 21.5 mmol, 1 equiv), was dissolved in dry ether (10 mL), and added dropwise, with stirring, to the LAH solution over 1 hour. The reaction was stirred at 0 ºC for 15 min, then allowed to warm to rt and stirred for 30 min, then refluxed overnight at 42 ºC. The reaction formed a white slurry. The reaction was cooled to 0 ºC and 100 mL water was then added and the solution stirred for 20 min to give 2 layers and a white solid, which was filtered through sintered glass/sand/celite (1 cm) to give a clear, 2-layer solution. The aqueous layer was removed, leaving a yellow ether layer, which was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. 1 M hydrogen chloride in ether (20 mL) was added to the crude oil, and the product precipitated as a yellow solid. The solvent was removed by filtration over a small-pore sintered glass funnel. The solid was washed with ether (3 x 10 mL), and acetone (2 x 10 mL), and dried

for 30 min, leaving the white solid **1** (500 mg, 25%, which was carried on without further purification. ESI 98.2 ($C_6H_{11}N + H^+$ requires 98.1). ^{1}H NMR (DMSO-d6, 400 MHz), δ 8.06 (s, 3H, N*H*$_3$), 2.83 (t, *J* = 2.6 Hz, 1H, ≡C*H*), 2.77 (t, *J* = 7.5 Hz, NH_3CH_2, 2 H), 2.19 (t of d, *J* = 7.0 Hz, 2.7 Hz, 2H, HC≡C-C*H*$_2$), 1.65 (m, 2H, $NH_3CH_2CH_2$), 1.5 (m, 2H, HC≡C-CH_2-C*H*$_2$)

Fmoc-Asn(5-hexynyl)-*O*-t-Bu (**5**). To a dry 25 mL roundbottom flask was added Fmoc-Asp-(COOH)-*O*-t-Bu (100 mg, 0.24 mmol, 1.2 equiv), **4** (27 mg, 0.20 mmol, 1 equiv), 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC, 46 mg, 0.24 mmol, 1.2 equiv), and hydroxybenzotriazole (HOBt, 32 mg, 0.24 mmol, 1.2 equiv), dry CH_2Cl_2 (5 mL), and dry NEt_3 (72 μL, 0.56 mmol, 2.8 equiv). The reaction was stirred under argon overnight, then quenched with CH_2Cl_2 (5 mL)/ sat. NaCl (5 mL). The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (10 mL), then the organic layers were combined and washed with sat. $NaHCO_3$ (2 x 20 mL), sat. NaCl (1 x 20 mL), 10% HCl (2 x 20 mL), and sat. NaCl (1 x 20 mL). The organic layer was dried over $MgSO_4$, filtered, and solvent removed under reduced pressure to give **5**, which was carried on without further purification (103 mg, 87%). ^{1}H NMR ($CDCl_3$, 400 MHz), δ 7.76 (m, 2H, FmocAr*H*), 7.60 (m, 2H, FmocAr*H*), 7.40 (m, 2H, FmocAr*H*), 7.30 (m, 2H, FmocAr*H*), 4.46 (m, 1H, Fmoc*H*), 4.35 (m, 2H, FmocH-C*H*$_2$), 4.22 (t, *J* = 7 Hz, 1H, $C_\alpha H$), 3.26 (m, 2H, C_α-C*H*$_2$-CONH), 2.80 (m, 1H, ≡C*H*), 2.18 (m, 2H, HC≡C-C*H*$_2$), 1.59 (m, 2H, $CONH_2CH_2CH_2$), 1.53 (m, 2H, HC≡C-CH_2-C*H*$_2$), 1.47 (s, 9H, O(C*H*$_3$)$_3$)

Fmoc-Asn(5-hexynyl)-OH (**6**). To a 25 mL round bottom flask was added **5** (103 mg, 0.21 mmol, 1 equiv) dry CH_2Cl_2 (4 mL), Et_3SiH (100 μL) and TFA (3 mL). The reaction was allowed to stir at room temperature for 4 h, and solvent was removed under reduced pressure to yield a white powder **3** (90 mg, quant), which was carried on without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz), δ 7.76 (m, 2H, FmocAr*H*), 7.58 (m, 2 H, FmocAr*H*), 7.40 (m, 2 H, FmocAr*H*), 7.31 (m, 2H, FmocAr*H*), 4.46 (m, 1H, Fmoc*H*), 4.36 (m, 2H, FmocH-CH_2), 4.20 (t, J = 7 Hz, 1H, $C_\alpha H$), 3.32 (m, 2H, C_α-CH_2-CONH), 2.85 (m, 1H, ≡C*H*), 2.21 (m, 2H, HC≡C-CH_2), 1.65 (m, 2H, $CONH_2CH_2CH_2$), 1.55 (m, 2H, HC≡C-CH_2-CH_2).



7 **1**

3-(2-chloroacetamido)propanoic acid, t-butyl ester (**7**). To a stirred solution in 5 mL dry CH_2Cl_2 of O-t-Bu-β-Ala (Novabiochem, 100 mg, 0.69 mmol, 1 equiv) was added chloroacetic anhydride (118 mg, 0.828 mmol, 1.2 equiv) pre-dissolved in dry CH_2Cl_2 (0.5 mL) and pyridine (54 μL, 0.69 mmol, 1 equiv), and the reaction was stirred under argon for 1 hour. CH_2Cl_2 (15 mL) were added, and the reaction quenched with 10% citric acid (15 mL). The layers were separated, and the organic layer was washed with sat. citric acid (2 x 15 mL), sat. NaCl (1 x 15 mL), sat. $NaHCO_3$, (2 x 15 mL), sat. NaCl (1 x 15 mL) and sat. citric acid (1 x 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed under reduced pressure to give **4** (67 mg, 66%), ^{1}H NMR

($CDCl_3$, 400 MHz), δ 7.19 (br s, 1H, N*H*) 4.04 (s, 2H, ClCH_2), 3.54 (d t, *J* = 6 Hz, 6 Hz, 2H, $ClCH_2NHCH_2$), 2.49 (t, *J* = 5.9 Hz, 2H, COCH_2), 1.47 (s, 9H, s, 9H, O(CH_3)$_3$)

3-(2-chloroacetamido)propanoic acid (**1**). To a solution of **4** (35 mg, 0.158 mmol, 1 equiv) in CH_2Cl_2 (0.5 mL) was added TFA (0.5 mL) and Et_3SiH (20 μL). The solution was stirred for 2 h, then toluene (1 mL) was added and solvent removed under reduced pressure. Toluene (1 mL) was added again and removed under reduced pressure to remove the TFA as an azeotrope. Drying overnight under reduced pressure gave **5** (27 mg, quant.) and used without further purification. MALDI-FTMS 164.0118 ($C_5H_8ClNO_3$ - H^+ requires 164.0114). ^{1}H NMR (MeOD, 400 MHz), δ 4.07 (s, 2H, ClCH_2), 3.51 (t, *J* = 6.2 Hz, 2H, $ClCH_2NHCH_2$), 2.49 (t, *J* = 6.0 Hz, 2H, COCH_2).

Proteome sample preparation and probe labeling

Mouse tissues were flash frozen (liquid N_2) immediately upon removal, then dounce-homogenized in phosphate-buffered saline buffer (50 mM Tris-HCl, pH 8.0) and sonicated. Soluble fractions (supernatant) were isolated by 100,000 x *g* (45 min) to remove membrane fractions (pellet). Total protein concentration was taken using a protein assay kit (Bio-Rad). Samples were diluted to 2 mg/mL and stored at –80 °C until use. Probe solutions were made up in DMSO (1 mM) and stored at –20 °C until use. Probe-proteome reactions (50 μL volume) were run at 20 μM final probe concentration at rt. Reactions were gently vortexed and allowed to sit for 1 h, then quenched with equal volume 2 x SDS-PAGE loading buffer (reducing, with protease inhibitor). Quenched

reactions were immediately placed on ice and run on 1D SDS-PAGE gels (30 μg protein/lane) or stored at –80 °C. Samples were visualized in-gel using a Hitachi FMBio Ile Flatbed laser-induced fluorescence scanner (MiraiBio, Alameda, CA). Labeled proteins were quantified by measuring integrated band intensity and averaged over n=3 samples.

Protein enrichment and identification

For protein identification, 1 mL of proteome at 2.5 mg/ml concentration (prepared as above) was labeled with rhodamine and biotin containing trifunctional probe **8** at room temperature for 2 hours. Labeled proteomes were then applied to a PD-10 size exclusion column (Bio-Rad) to remove excess probe. Samples were diluted to 8 mL and incubated with avidin-agarose beads (Sigma) for 1 h. Beads were washed 3x with 1% SDS, 3x with 6 M Urea, and 3 x with 50 mM tris buffer, pH 8.0. At this stage, beads were either subjected to ABPP-MudPIT[1] or used for gel-band IDs. For gel-band IDs, affinity-enriched proteins were separated on SDS-PAGE gels, visualized by in-gel fluorescence scanning, and bands cut using the fluorescence scan as a template as previously described.[2] Gel slices were washed 3 x with 50% methanol/50% water, then subjected to in-gel reduction, alkylation, and trypic digestion. Digested peptides were acidified to 5% formic acid and loaded on a 1D reverse-phase nanospray column according to established protocols.

ABPP-MudPIT analysis was carried out in soluble mouse liver proteome according to published protocols.[1] Briefly, after labeling with a suite of trifunctional probes (Leu-Asp

α-CA, D-Leu-D-Asp α-CA, and Leu-D-Asp α-CA) and enrichment with avidin-agarose as above, samples were subject to on-bead reduction [10 mM tris(2-carboxylethyl) phosphine (TCEP)], alkylation (12 mM iodoacetamide), and digest for 16 hours at 37 ^{o}C with trypsin in 2 M Urea containing 2 mM $CaCl_2$. The digested peptides were filtered from the avidin beads, acidified to 5% formic acid, and loaded on a nanospray 2D-LC column as described previously.

In both cases (gel-band and MudPIT), tryptic peptides were analyzed by nanoLC-MS/MS (1D for gel spots, analysis on a ThermoFinnegan LTQ set in a data-dependent acquisition mode with dynamic exclusion turned on (60 s). Specifically, one full MS survey scan was followed by 7 MS/MS scans and results searched against public databases of mouse proteins using the SEQUEST search algorithm.[1]

Recombinant protein expression and assays

Transient transfection

Ureidopropionase and nitrilase 1 were expressed by transient transfection in COS-7 cells. Full-length constructs (based on PubMed database sequences) were obtained by PCR from a 4-mouse liver cDNA library, topo-cloned into pcDNA3.1/V5-His-Topo (Invitrogen) and screened for directionality. Active site mutants (C233A) of ureidopropionase were generated using standard quik-change protocols (Stratagene), and C-terminal myc tags were introduced via PCR. All clones were fully sequenced from both ends and transiently transfected using the Fugene 6 reagent (Roche) using

established protocols. Transfected cells were harvested after 48 hours by scraping, then were homogenized in phosphate-buffered saline (PBS), dounce-homogenized, sonicated, and spun at 100,000 x *g* for 45 min. Soluble protein (supernatant) was diluted to 1 mg/mL and labeled with probe as described above.

Competitive ABPP screens

Gel-based screens

Competitive agent (10-400 μM) was pre-incubated with 50 μL of 1 mg/mL COS-7 proteome transiently transfected with full-length ureidopropionase for 5-60 minutes, then 50 μM rhodamine-Leu-Asp α-CA probe was added for 10 minutes. Reactions were quenched by the addition of 2 x SDS-PAGE loading buffer (reducing, with protease inhibitor), and run on 1D SDS-PAGE gels (30 μg protein/lane). Samples (n = 3) were visualized and quantified in-gel as described above, and % inhibition was quantified by comparison to a zero-time point (competitive agent and probe added at the same time) control. k_{obs}/[I] values were averaged from at least 3 inhibitor concentrations and at least 5 time points.

Click Chemistry with alkyne-Leu-Asp α-CA probe

Click chemistry with rhodamine-azide was carried out on alkyne-Leu-Asp α-CA probe labeled proteome according to published protocols.[3]

Site of Labeling

Site of labeling experiments were carried out by peptide capture of rhodamine-probe labeled proteome according to published protocols.[4]

References

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Supplementary Table 1. Identification of Upβ and Nit2 as targets of the Leu-Asp α-CA probe in mouse liver proteome by ABPP-MudPIT*.

Enzyme	Spectral counts†	% Sequence coverage
Upβ	188 ± 40	78.4%
Nit2	59 ± 13	86.6%

*Mouse liver proteome was treated with a trifunctional variant of the Leu-Asp α-CA probe containing both a biotin and rhodamine reporter groups. The probe-labeled proteomes were then enriched with avidin chromatography, digested on-bead with trypsin, and analyzed by multidimensional LC-MS analysis, as described previously (Jessani et al. *Nat Methods* **2005**, , 691).

†Data represent the average spectral counts ± standard error for three independent experiments. No spectral counts were observed for either Upb or Nit2 in no probe control reactions in which the trifunctional Leu-Asp α-CA probe was not added to the proteome.

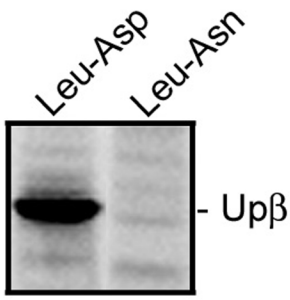
Supplementary Figure Legends

Supplementary Figure 1. The liver 44 kDa protein identified as Upβ reacts with Leu-Asp α-CA, but not Leu-Asn α-CA.

Supplementary Figure 2. Identification of the 44-kDa Leu-Asp α-CA reactive band from mouse liver as ureidopropionase-β (Upβ). After enrichment with avidin beads and separation by SDS-PAGE, the protein band was excised from the gel and subjected to in-gel reduction, alkylation, and tryptic digest, and analyzed by nano-LC-MS/MS. Shown in bold are the peptides identified and assigned to Upβ) by Sequest. 59 total spectral counts were observed that collectively covered ~60% of the protein sequence. No spectral counts were observed in a control sample where the Leu-Asp α-CA probe was not added to the liver proteome.

Supplementary Figure 3. Click chemistry with a rhodamine (Rh)-azide reporter group confirms that alkyne-Leu-Asp α-CA covalently labels Upβ. Flurorescent gel image shown in grayscale. Lane 1, labeling of Upβ with the rhodamine probe. Lane 2, the alkyne agent **3** competes labeling of the rhodamine probe. Lane 3, addition of rhodamine-N_3 via click chemistry restores fluorescent labeling to Upβ. Lane 4, rhodamine-N_3 alone does not label Upβ.

Supplementary Figure 1



Supplementary Figure 2

Upβ protein sequence

M**AGPEWQSLEQCLEK**HLPPDDLAQVKRILYGKQTRNLDLPREALKAASER
NFELK**GYAFGAAK**EQQRCPQIVRVGLVQNR**IPLPTSAPVAEQVSALHK**S
IEEIAEVAAMCGVNIICFQ**EAWNMPFAFCTREKLPWTEFAESAEDGLT**
TRFCQKLAK**KHNMVVVSPILER**DREHGGVLWN**TAVVISNSGLVMGK**T
RKNHIPR**VGDFNESTYYMEGNLGHPVFQTQFGR**IAVNICYGRHHPLNW
LMYSINGAEIIFNPS**ATIGELSESLWPIEARNAAIANHCFTCALNRVGQ**
EHFPNEFTSGDGKKAHHDLGYFYGSSYVAAPDGSRTPGLSRNQDGL
LVTELNLN**LCQQINDFWTFK**MTGRLEMYAR**ELAEAVKPNYSPNIVKE**
DLVLAP SSG

58.8% sequence coverage, 59 spectral counts

Supplementary Figure 3