Discovery of pyruvylated peptide-metabolizing enzyme using a fluorescent substrate-based protein discovery technique

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Methods

General materials

General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical, Aldrich Chemical Co, Dojindo, Invitrogen and Promega, and were used without further purification. All solvents were used after appropriate distillation or purification. Silica gel column chromatography was performed by using Silica Gel 60 (spherical; Kanto Chemical, Co., Inc., Tokyo, Japan).

General instruments.

^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA300 instrument (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR) or a JEOL JNM-LA400 instrument (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR); δ values are in ppm relative to tetramethylsilane (TMS). Mass spectra (MS) were measured with a JEOL JMS-T100LC AccuTOF (ESI). HPLC analyses were performed on an Inertsil ODS-3 (4.6×250 mm) column (GL Sciences, Inc.) by using an HPLC system composed of a PU-980 pump (Jasco) and MD-2015 or FP-2025 detector (Jasco) or another system composed of a PU-2080 pump (Jasco) and MD-2018 or FP-2025 detector (Jasco). Preparative HPLC was performed on an Inertsil ODS-3 (10×250 mm) column (GL Sciences, Inc.) by using a HPLC system composed of a PU-2080 pump (Jasco) and MD-2015 or FP-2025 detector (Jasco) or another system composed of a PU-2086 pump (Jasco) and MD-2018 detector (Jasco).

Enzymes.

Enzymes were diluted in enzyme reaction buffer (pH 7.4 phosphate-buffered saline (PBS) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) to suitable concentrations to perform assays.

Preparing mouse tissue lysates.

All procedures were approved by the Animal Care and Use Committee of the University of Tokyo. Black mice (C57BL/6JJcl, male, 7-8 weeks old) were sacrificed, and tissues were collected and placed in 1.5 mL plastic tubes. The tubes were freeze-dried in liquid nitrogen immediately after collection, and kept at -80˚C. To prepare the lysate, freeze-dried tissues

(100-200 mg) were placed in a glass homogenizer and 2× volume of phosphate buffer (pH 7.4, containing $CaCl_2$ and $MgCl_2$) was added. The tissues were homogenized, and the homogenate was placed in 1.5 mL plastic tubes and centrifuged (3000 rpm × 10 min at 4˚C). The supernatant was collected as tissue lysate, and the protein concentration was determined with the standard Bradford assay. The lysates were aliquoted and stored at -80˚C.

HeLa cell culture, transfection and lysate preparation.

HeLa cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical) containing 10% fetal bovine serum (FBS; Gibco). Transfection was performed with Lipofectamine LTX and PLUS reagents (Invitrogen). As the standard condition, 1 μg plasmid, 1 μL PLUS reagent, and 3 μL lipofectamine LTX were mixed in 150 μL Opti-MEM (Gibco) and added to 3 mL of cell suspension. Transfection efficiency was confirmed to be 20-40% using Clonetech-YFP (C1) plasmid as a control.

After 1 day, cells were washed twice with PBS. 1 mL PBS was added, and cells were scraped off the plate and collected in a 1.5 mL plastic tube. After centrifugation (1400 rpm×3 min at 4°C), the supernatant was removed, and 200 μL enzyme reaction buffer (pH 7.4 PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) was added. Pellets were homogenized by sonication on ice with 5 pulses (3 W power; 1 pulse = 1 sec) from a Sonicator S-4000 (Misonix). Protein concentration was determined with the standard Bradford assay (Bio-Rad 500-0006; BSA was used for calibration), and the lysates were aliquoted and stored at -80°C. Each assay was performed with a single aliquot to avoid repeated cycles of freeze-thawing.

Enzymatic assay.

Unless otherwise mentioned, enzymatic assay was performed in enzyme reaction buffer (pH 7.4 PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) at 37°C. Half-area 384-well plates (Costar 3694) were used for the assay (16 μL reaction volume). Fluorescence was detected with a plate reader, EnVision 2103 Multilabel Reader (Perkin Elmer), under appropriate filter conditions.

Diced electrophoresis gel assay.

Unless otherwise mentioned, native PAGE was used for one-dimensional diced electrophoresis gel assay, and isoelectric focusing (IEF) and native PAGE were used for two-dimensional diced electrophoresis gel assay[1]. After the electrophoresis, the gel was placed on a 0.5 mm square wire mesh over a 384-well plate (Eppendorf 0030 621.905), and fixed in place with the lid. A specially made plastic frame was placed on top, and the gels were diced by pressing down on the frame. Centrifugation (3000 rpm × 5 min) was performed to load the diced gels into the 384-well plate, and assays were performed by adding 70 μL of probe solution to wells. Fluorescence was read with a plate reader, EnVision 2103 Multilabel

Reader (Perkin Elmer), under appropriate filter conditions until a sufficient signal-to-noise ratio was achieved. In data analysis, spots with signals higher than (average) +3.29 × (S.D.) of background were considered as positive signals (this criterion eliminates 99.9% of false positives).

Two-dimensional diced electrophoresis gel assay to characterize the targets of Pyr-Leu-AMC.

Two-dimensional electrophoresis was performed for mouse liver lysate, and diced electrophoresis gel assay was performed with probes in the enzyme reaction buffer (pH 7.4 phosphate buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$). The well with the highest activity was identified after 18 hr incubation, and gel in the well was collected in a 1.5 mL tube. The collected gel was washed three times with pure water, and kept at -80°C. Gels from 3 independent assays were mixed for a single LC-MS/MS analysis.

Protein digestion and LC-MS/MS analysis (peptide mass fingerprinting (PMF) analysis).

LS-MS/MS-based protein identification was performed as a contract service by APRO Life Science Institute, Inc. Samples were prepared by reductive alkylation and trypsin digestion according to standard protocols.

Peptides were separated on a Paradigm MS2 (Michrom BioResources, Inc.) equipped with an L-column ODS (0.1×50 mm, Chemicals Evaluation and Research Institute) under an acidic solvent condition (0.1% formic acid) with an increasing gradient of acetonitrile. Detection was done with a Q-Tof2 (Waters Micromass) in the positive mode (capillary voltage: 1.8 kV, collision energy: 20-56 ev).

Analysis of LC-MS/MS data.

The acquired LC-MS/MS data was analyzed using MASCOT Server 2.3 (Matrix Science Ltd.) to find the hit proteins. The threshold was set at $P < 0.05$. The database to be searched can include all species. The list of hit proteins may contain multiple proteins in addition to the true target, so available information on all hit proteins in the literature and in enzyme databases (BRENDA; http://www.brenda-enzymes.org/) was scanned to identify those likely to accept the probes as substrates. The selected candidate protein(s) were validated by biochemical methods using overexpressed proteins, inhibitors and antibodies, as described in the main text.

Western blotting.

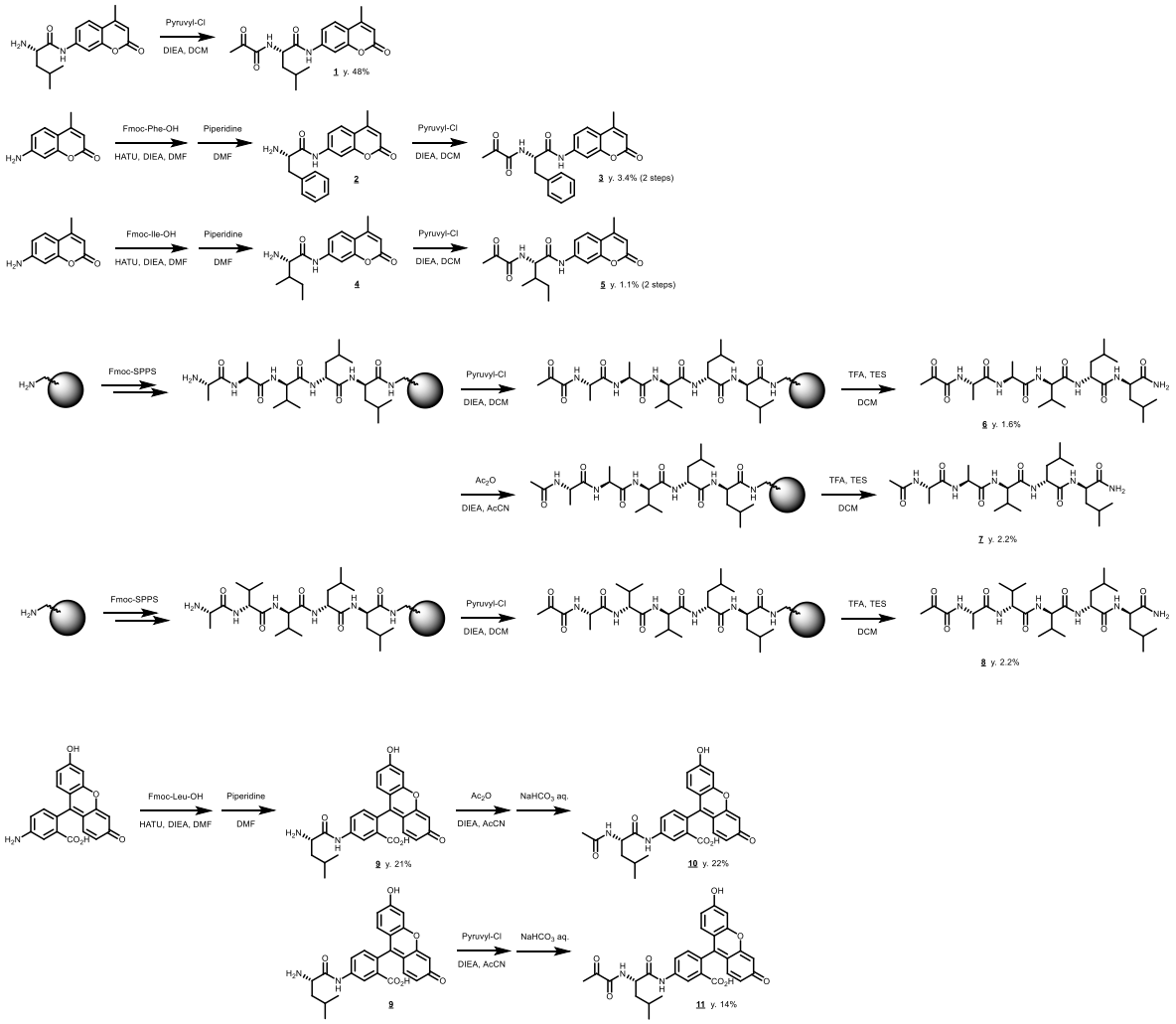
After the SDS-PAGE of mouse liver lysate, proteins were transferred onto PVDF membrane (Bio-Rad) according to the standard western blotting protocol. The membrane was blocked with skim milk, then incubated in Tris-buffered saline with 0.1% Tween-20 (TBS-T)

containing the first antibody (anti APEH) at 4°C for 18 hr, washed three times with TBS-T, and incubated in TBS-T containing the second antibody at room temperature for 2 hr. The membrane was washed three times with TBS-T, and then chemiluminescence reaction was performed with the use of ECL Prime western blotting detection reagent (Amersham). Detection was done with an ImageQuant CAS 4000 mini (GE Healthcare).

LC-MS-based study of metabolism of *N*-pyruvylated peptides.

N-Pyruvated peptides (final 15 μM) and 2-methyl-4-methoxy-TokyoGreen (2-Me-4-OMe-TG) (internal standard; final 1 μM) were incubated with cell lysates for the indicated times. The reaction was terminated by adding a 2-fold excess of 1% formic acid/methanol, and a 40 μL aliquot was injected into an ODS-4 column (GL Sciences) for LC-MS analysis (Agilent 1200 series/6130 Quadrupole LC/MS) under an acidic solvent condition (0.1% formic acid) with an increasing gradient of acetonitrile. Detection was performed in the positive mode, and linearity of the signal was confirmed by the use of a reference standard. The decrease of Pyr-pep or the increase of degraded peptides was determined by analyzing the peak area of chromatograms (*m/z* = value ± 0.5), using that of 2-Me-4-OMe-TG as an internal standard.

Preparation of compounds.



Synthesis of pyruvyl chloride.

Pyruvyl chloride was prepared according to the literature[1].

Synthesis of Pyr-Leu-AMC (1).

L-Leucine 7-amido-4-methylcoumarin (20.4 mg, 70.8 μmol) was dissolved in 3 mL of dichloromethane. After addition of 400 μL DIEA to the solution, 400 μL of pyruvyl chloride was added under Ar at 0 ºC. The solution was stirred for 3 hrs at r.t. Ethyl acetate (20 mL) was added, and the organic layer was washed with brine three times, dried over Na_2SO_4, filtered, and evaporated to give a crude product. Purification was performed with HPLC under the following conditions: A/B = 80/20-0/100 (30 min) (solvent A: H_2O with 0.1% TFA, solvent B: acetonitrile/H_2O (with 0.1% TFA) = 80/20), to afford Pyr-Leu-AMC (**1**, 12.3 mg, 34.3 μmol) as a white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 0.97 (d, 3H, J = 6.3 Hz), 1.00 (d, 3H, J = 6.3 Hz), 1.73 (m, 2H), 1.93 (m, 1H), 2.41 (s, 3H), 2.52 (s, 3H), 6.21 (s, 1H), 7.47 (dd,

1H, J = 8.5, 1.7 Hz), 7.53 (d, 1H, J = 8.3 Hz), 7.63 (d, 1H, J = 2.0 Hz) ^{13}C NMR (75 MHz CD_3CN) δ 18.6, 21.9, 22.9, 24.6, 39.7, 52.9, 107.5, 113.7, 115.7, 125.3, 140.7, 152.1, 160.7, 169.0, 171.7. HRMS (ESI$^+$) Calcd. for $[M+H]^+$,359.1607; Found, 359.1630 (+ 2.3 mmu).

Synthesis of Phe-AMC (2).

7-amino-4-methylcoumarin (200 mg, 1.14 mmol), Fmoc-Phe-OH (440 mg, 1.13 mmol), and 1-[bis(dimethyl-amino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (215 mg, 1.13 mmol) was dissolved in 5 mL of DMF. After addition of 50 μL DIEA, the solution was stirred overnight at r.t. Ethyl acetate (20 mL) was added, and the organic layer was washed with brine three times, dried over Na_2SO_4, filtered, and evaporated to give a crude product. The product was dissolved in 40% piperidine/DMF for 20 min. Ethyl acetate (20 mL) was added, and the organic layer was washed with brine three times, dried over Na_2SO_4, filtered, and evaporated to give a crude **2** (178 mg).

Synthesis of Pyr-Phe-AMC (3).

Crude **2** (118 mg) was dissolved in 10 mL of dichloromethane. After addition of 700 μL DIEA to the solution, 600 μL of **1** was added under Ar at 0 ºC. The solution was stirred for 3 hrs at r.t. Ethyl acetate (20 mL) was added, and the organic layer was washed with brine three times, dried over Na_2SO_4, filtered, and evaporated to give a crude product. Purification was performed with HPLC under the following conditions: A/B = 70/30-0/100 (30 min) (solvent A: H_2O with 0.1% TFA, solvent B: acetonitrile/H_2O (with 0.1% TFA) = 80/20), to afford Pyr-Phe-AMC (**3**, 15.2 mg, 38.7 μmol) as a white solid. ^{1}H NMR (300MHz, DMSO-d_6) μ 10.53 (s, 1H), 8.73 (d, 1H, J = 8.8 Hz), 7.75 (m, 2H), 7.47 (d, 1H, J = 8.8 Hz), 7.28 (s, 2H), 7.27 (s, 2H), 7.22 (m, 1H), 6.29 (s, 1H), 4.69 (m, 1H), 3.13 (m, 2H), 2.41 (s, 3H), 2.29 (s, 3H). LRMS (ESI$^+$) Calcd. for $[M+Na]^+$, 415.1; Found, 415.1.

Synthesis of Ile-AMC (4).

Ile-AMC (**4**) was synthesized by the same procedure as compound **2** from Fmoc--Ile-OH (420 mg, 1.19 mmol), affording a crude product (73 mg).

Synthesis of Pyr-Ile-AMC (5).

Pyr-Ile-AMC (**5**) was synthesized from **4** by the same procedure as compound **3**. Purification was performed with HPLC under the following conditions: A/B = 70/30-0/100 (30 min) (solvent A: H_2O with 0.1% TFA, solvent B: acetonitrile/H_2O (with 0.1% TFA) = 80/20), to afford Pyr-Ile-AMC (**5**) (4.6 mg, 13 μmol) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.62 (s, 1H), 8.45 (d, 1H, J = 8.8 Hz), 7.75 (m, 2H), 7.50 (d, 1H, J = 8.8 Hz), 6.28 (s, 1H), 4.32 (m, 1H), 2.36 (s, 3H), 2.34 (s, 3H), 1.97 (m, 2H), 1.78 (m, 1H), 1.48 (m, 3H), 1.16 (m, 3H). LRMS (ESI$^+$) Calcd. for $[M+Na]^+$, 381.1; Found, 381.1.

General synthesis of *N*-pyruvylated or N-acetylated peptides

All peptides were synthesized on an automatic peptide synthesizer using standard protocols of fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis with 2-chlorotrityl chloride resin or rink amide resin (0.1 mmol reaction site) (Novabiochem, USA). The resin beads were washed 3 times with 3 mL DCM and incubated with excess pyruvyl chloride in 250 μL DIEA/ 2-4 mL DMF. Peptides were cleaved from the resin with 2 mL TFA for 2 hrs. The solution was evaporated and peptides were purified by HPLC.

[Small letters in the peptide indicate *D*-amino acids. Pyr- = *N*-terminally pyruvylated, and Ac- = *N*-terminally acetylated, -NH_2 = *N*-terminally amidated.]

Synthesis of compound 6 (Pyr-AAvll-NH_2).

Purification was performed with HPLC under the following conditions: A/B = 60/40-0/100 (25 min) (solvent A: H_2O with 0.1% TFA, solvent B: acetonitrile/H_2O (with 0.1% TFA) = 80/20), to afford **6** (0.89 mg, 1.6 μmol) as a white powder. HRMS (ESI^+) Calcd. for $[M+Na]^+$, 577.3326; Found, 577.3291 (- 3.5 mmu).

Synthesis of compound 8 (Ac-AAvll-NH_2).

Purification was performed with HPLC under the following conditions: A/B = 60/40-0/100 (25 min) (solvent A: H_2O with 0.1% TFA, solvent B: acetonitrile/H_2O (with 0.1% TFA) = 80/20), to afford **8** (1.3 mg, 2.2 μmol) as a white powder. LRMS (ESI^+) Calcd. for $[M+H]^+$, 527.3; Found, 527.3.

Synthesis of compound 7 (Pyr-Avvll-NH_2).

Purification was performed with HPLC under the following conditions: A/B = 60/40-0/100 (25 min) (solvent A: H_2O with 0.1% TFA, solvent B: acetonitrile/H_2O (with 0.1% TFA) = 80/20), to afford **7** (1.3 mg, 2.2 μmol) as a white powder. HRMS (ESI^+) Calcd. for $[M+Na]^+$, 605.3639; Found, 605.3589 (- 5.0 mmu).

Synthesis of Leu-5-AF (9).

5-Aminofluorescein (346 mg, 1.0 mmol), Fmoc-Leu-OH (423 mg, 1.2 mmol), and 1-[bis(dimethyl-amino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (215 mg, 1.13 mmol) were dissolved in 5 mL of DMF. After addition of 50 μL DIEA, the solution was stirred overnight at r.t.. Ethyl acetate (20 mL) was added, and the organic layer was washed with brine three times, dried over Na_2SO_4, filtered, and evaporated to give a crude product. The product was dissolved in 40% piperidine/DMF for 20 min. After evaporation of the solvent, the crude product was purified by preparative

HPLC under the following conditions: A/B = 70/30-0/100 (30 min) (solvent A: H_2O with 0.1% TFA, solvent B: acetonitrile/H_2O (with 0.1% TFA) = 80/20), to afford Leu-5-AF (**9**) (96 mg, 210 μmol) as an orange solid. ^{1}H NMR (300 MHz, CD_3OD) d 1.06 (d, 2H, J = 6.6 Hz), 1.86 (m, 3H), 4.10 (t, 1H, *J* = 7.5 Hz), 6.61 (dd, 2H, *J* = 9.0, 2.1 Hz), 6.75 (m, 4H), 7.22 (d, 1H, *J* = 9.0 Hz), 7.92 (dd, 1H, *J* = 9.0, 1.8 Hz); 8.45 (d, 1H, J = 1.8 Hz). LRMS (ESI^+) Calcd. for $[M+H]^+$, 460.1; Found, 460.1.

Synthesis of Ac-Leu-5-AF (10).

Leu-5-AF (5 mg, 11 μmol) was dissolved in dry acetonitrile (2 mL) and DIEA (0.5 mL). Acetic anhydride (100 μL) was added dropwise, and the reaction mixture was stirred at room temperature for 30 min. Sat. $NaHCO_3$ aq. 2 mL was added, and the mixture was purified by preparative HPLC to afford **10** (1.2 mg, 2.4 μmol). ^{1}H NMR (300 MHz, CD_3OD) δ 1.02 (m, 6H, 1.72 (m, 3H), 2.05 (s, 3H), 4.60 (m, 1H), 6.68 (m, 4H), 7.23 (m, 3H), 8.00 (d, 1H, *J* = 1.8 Hz), 8.09 (dd, 1H, *J* = 8.7, 1.8 Hz). LRMS (ESI^+) Calcd. for $[M+H]^+$, 503.2; Found, 503.2.

Synthesis of Pyr-Leu-5-AF (11).

Leu-5-AF (5 mg, 11 μmol) was dissolved in dry acetonitrile (2 mL) and DIEA (0.5 mL). Pyruvyl anhydride (100 μL) was added dropwise, and the reaction mixture was stirred at room temperature for 30 min. Sat. $NaHCO_3$ aq. 2 mL was added, and the mixture was purified by preparative HPLC to afford **11** (0.8 mg, 1.5 μmol). (300 MHz, CD_3OD) δ 1.18 (m, 6H), 1.43 (m, 2H), 2.30 (s, 3H), 4.61 (m, 1H), 6.60 (m, 4H), 7.04 (d, 2H, *J* = 8.4 Hz), 7.17 (d, 1H, *J* = 8.1 Hz), 7.90 (dd, 1H, *J* = 8.1, 1.8 Hz), 8.17 (d, 1H, *J* = 1.8 Hz). HRMS (ESI^+) Calcd for $[M+H]^+$, 531.1767; Found, 531.1813 (+ 4.6 mmu).

Supporting Data

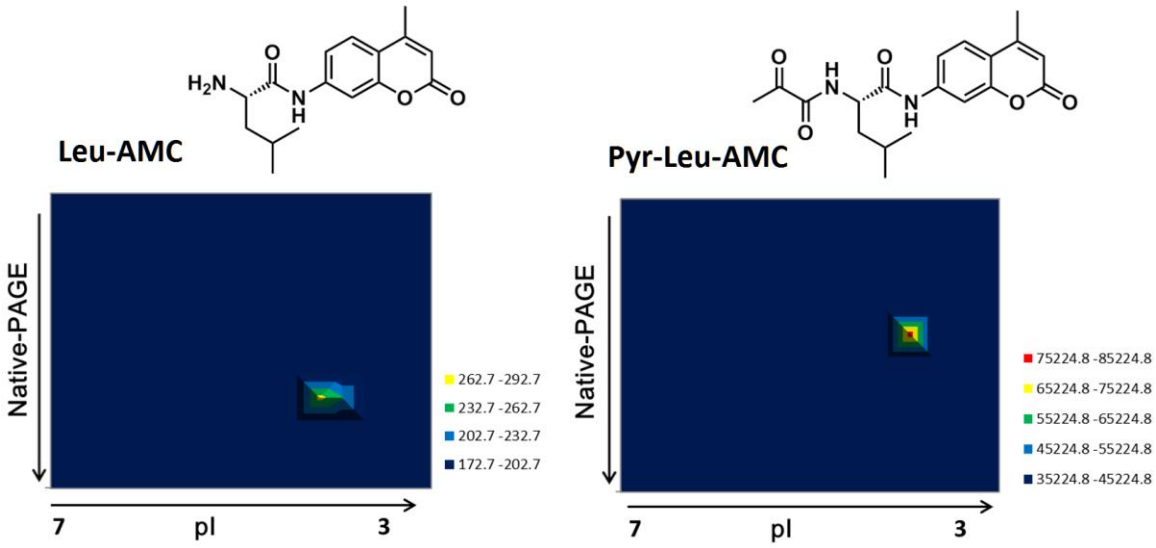


Figure S1. 2D DEG assay of mouse liver lysates (170 μg) with Leu-AMC or Pyr-Leu-AMC (10 μM). Reaction was performed in DPBS at 37°C.

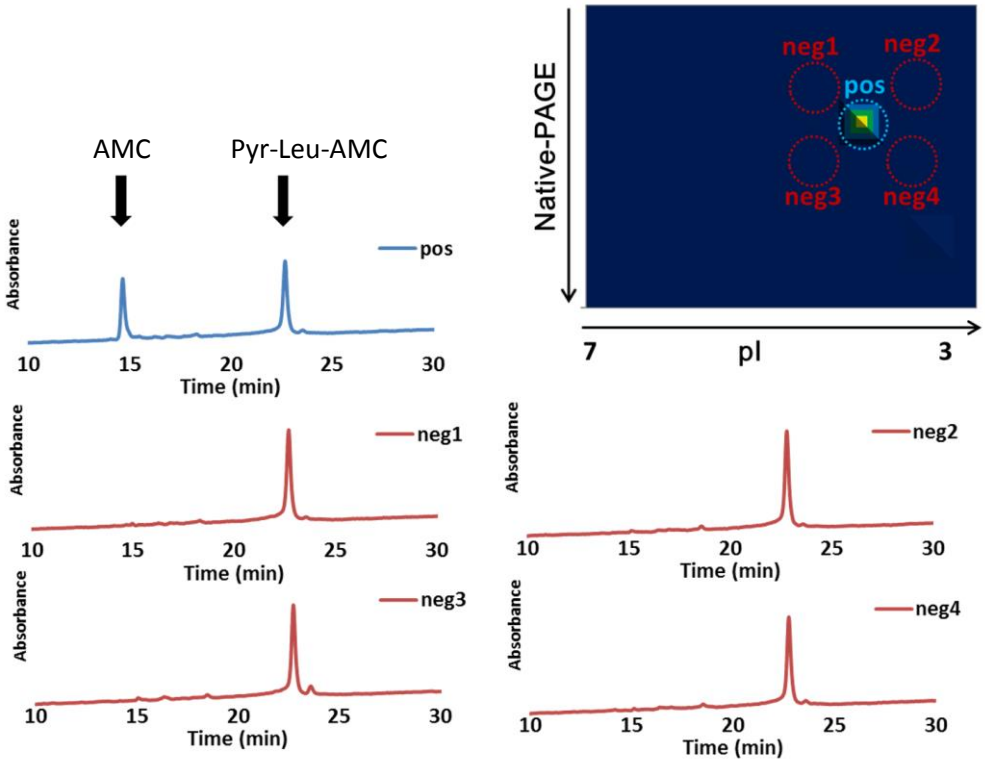


Figure S2. 354 nm absorbance chromatogram (LC-MS) of samples in 2D-DEG assay spots with Pyr-Leu-AMC (10 μM). Reaction was performed in DPBS at 37°C for 12 hr.

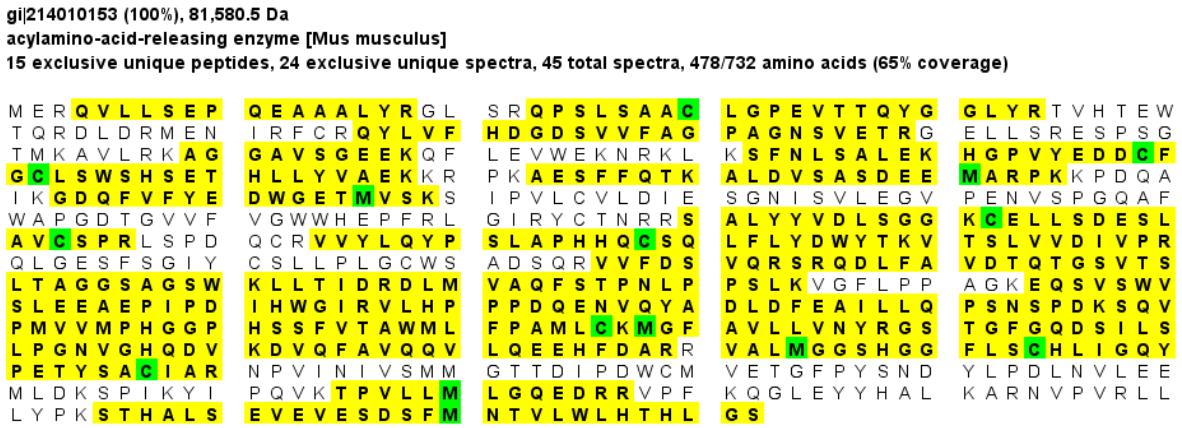


Figure S3. Coverage of mouse acylamino-acid releasing enzyme (APEH; full length) in peptide mass fingerprinting analysis of the spot of Pyr-Leu-AMC-cleaving activity. Yellow = detected peptides. Green = modified amino acids (oxidation for methionine (M) and carbamidomethylation for cysteine (C; modified during the analysis process).

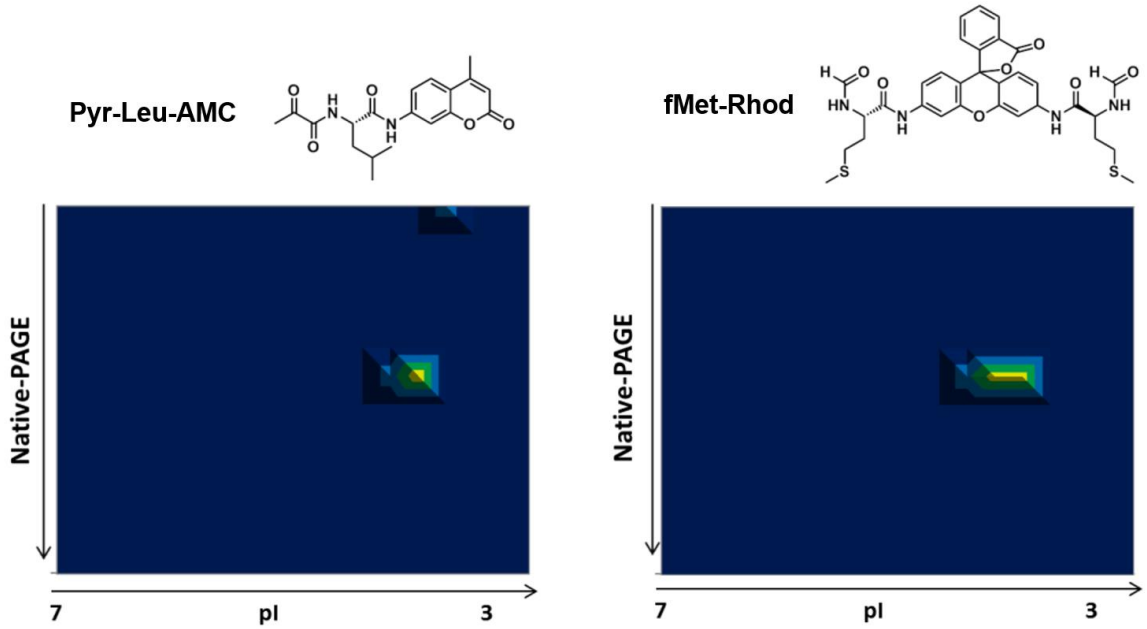


Figure S4. 2-Dimensional DEG assay of mouse liver lysate (170 μg) with Pyr-Leu-AMC (20 μM) and fMet-Rhod[2] (20 μM). Reaction was performed in DPBS at 37°C for 12 hr.

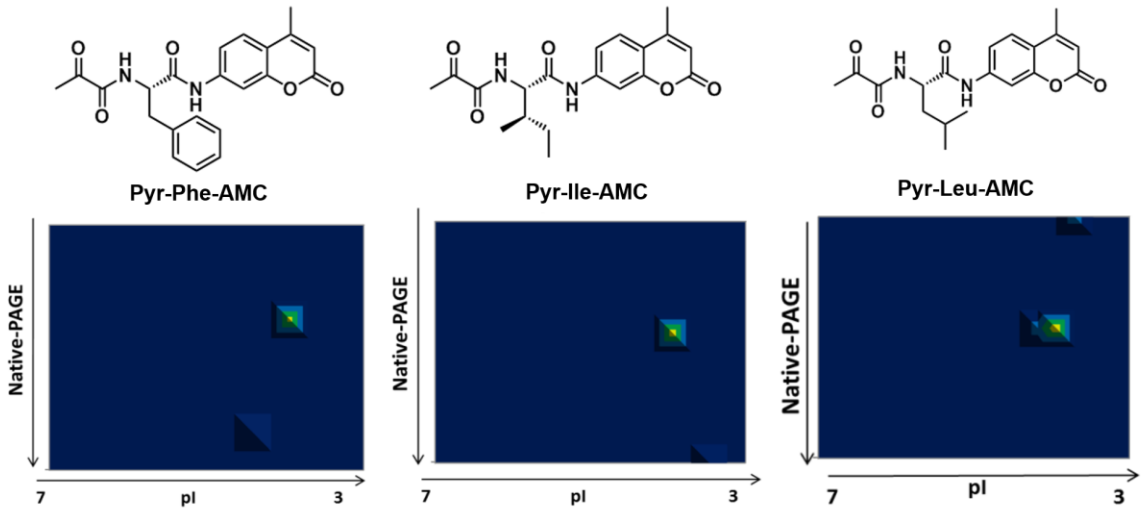


Figure S5. 2D DEG assay of mouse liver lysates (170 μg) with Pyr-Phe-AMC (left), Pyr-Ile-AMC (middle), or Pyr-Leu-AMC (right) (10 μM). Reaction was performed in DPBS at 37°C for 12 hours.

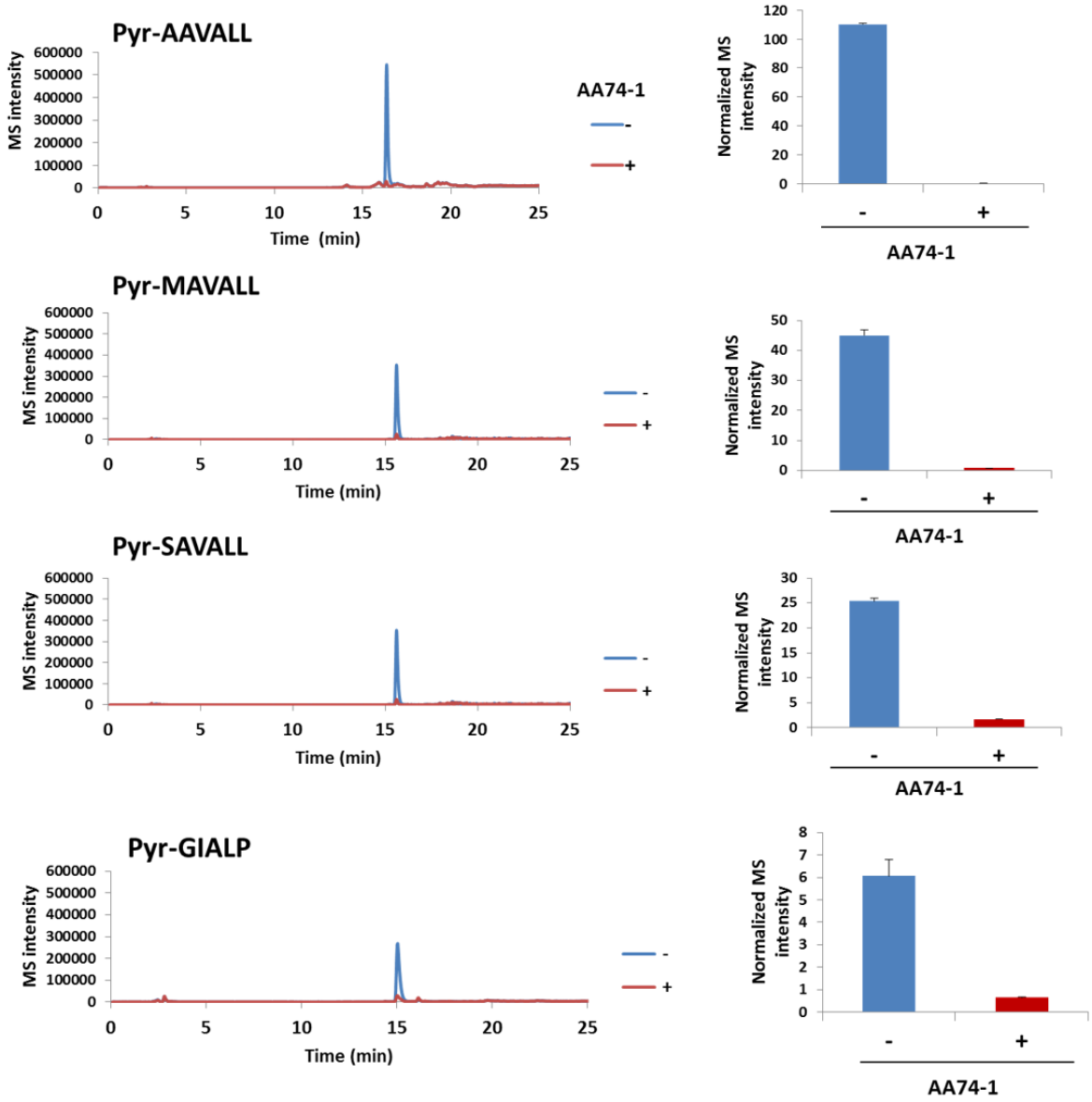


Figure S6. LC-MS chromatograms of predicted peptide substrates after incubation with mouse APEH (5 U/mL) in the presence (blue) or absence (red) of 500 nM AA74-1, a well-known APEH inhibitor. Reaction was performed in DPBS at 37°C for 16 hr.

Supporting References

1. Yoshioka, K., Komatsu, T., Nakada, A., Onagi, J., Kuriki, Y., Kawaguchi, M., Terai, T., Ueno, T., Hanaoka, K., Nagano, T., Urano, Y. *J. Am Chem Soc.* **2015**, *137*, 12187-90.
2. Komatsu, T.; Hanaoka, K.; Adibekian, A.; Yoshioka, K.; Terai, T.; Ueno, T.; Kawaguchi, M.; Cravatt, B. F.; Nagano, T. *J. Am. Chem. Soc.* **2013**, *135*, 6002-6005.