Supporting Information

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SI Materials and Methods

Purification of $C_{12}H_{15}NO_4$ from Culture Medium. Approximately 1 L of HEK 293/ABCC5 culture medium was deproteinized by adding 3 L of methanol, followed by centrifugation, rotary evaporation at reduced pressure, and freeze-drying. Purification was performed in four consecutive steps. The first separation was performed on a Sepacore Flash chromatograph (Buchi) using a 40-gauge C_{18} Flash column (Screening Devices) and mobile phases consisting of 5 mM ammonium acetate in water and in acetonitrile/water [97.5:2.5 (vol/vol)]. Fractions were collected and analyzed on LC/MS for the presence of $C_{12}H_{15}NO_4$, as described in *Materials and Methods*, *Untargeted Metabolomics*.

Further separations were performed on an Ultimate 3000 LC System (Dionex). First, ion-pair fractionation was performed on a Luna 3u C_{18} 100A (150 × 10 mm, 3 μm) column (Phenomenex) with mobile phases previously described (1). Collected fractions were dried under vacuum and analyzed on LC/MS. The third fractionation step was performed on the same Luna 3u C_{18} 100A (150 × 10 mm, 3 μm) column, but with the reversed-phase mobile phases used for the flash chromatography. The fractions containing $C_{12}H_{15}NO_4$ were finally purified on a ReprolSil-Pur Phenyl column (150 × 4.6 mm, 3 μm; Dr. Maisch) with mobile phases consisting of 5 mM ammonium acetate in water and methanol. Fractions containing $C_{12}H_{15}NO_4$ were pooled, freeze-dried three times to remove ammonium acetate and water residues, and dissolved in D_2O for NMR analysis.

Chemical *N*-L-Lac-L-Phe Synthesis. General reagents were obtained from Sigma–Aldrich and used as received. The L-Phe ethyl ester hydrochloride was purchased from Bachem and (−)-ethyl-L-lactate was obtained from Aldrich. Solvents were purchased from Biosolve. Analytical TLC was performed on aluminum sheets precoated with silica gel 60 F254 (Merck), and spots were visualized using 20% (wt/vol) ninhydrin in ethanol and heating by a heat gun. NMR spectra [^{1}H-NMR, ^{13}C-NMR (attached proton test), and ^{1}H-COSY] were determined in D_2O as described in *Materials and Methods*, *Structure Elucidation*. Peak shapes in NMR spectra are indicated with the symbols "d" (doublet), "dd" (double doublet), "s" (singlet), "q" (quartet), and "m" (multiplet). Chemical shifts (δ) are given in parts per million, and coupling constants (*J*) are given in hertz. The accurate mass and MS^2 fragmentation spectrum were acquired using the same LC/MS conditions as described in *Materials and Methods*, *Untargeted Metabolomics*, at a normalized collision energy of 35.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (815 mg, 5.25 mmol, 1.05 eq) was added to a cold mixture (ice bath) of (−)-ethyl-L-lactate (1.15 g, 5 mmol, 1 eq) and 1-hydroxybenzotriazole (1.35 g, 10 mmol, 2 eq) in dichloromethane (DCM; 15 mL). Stirring was continued for 10 min before a mixture of L-Phe ethyl ester hydrochloride (450.4 mg, 5 mmol) and *N*,*N*-diisopropylethylamine (871 μL, 5 mmol, 1 eq) in DCM (15 mL) was added. The ice bath was removed, and the reaction mixture was stirred for 16 h at room temperature. After removal of the solvent, the residue was taken up in ethylacetate (EtOAc); washed with $KHSO_4$, brine, $NaHCO_3$, and brine; dried (Na_2SO_4); and then concentrated. The crude *N*-L-lac-L-Phe ethyl ester was dissolved in tetrahydrofuran (10 mL), and 2 N of LiOH (5 mL) was added. The resulting mixture was stirred at room temperature for 1 h. After hydrolysis of the ester was complete, as indicated by TLC analysis [eluent: EtOAC/heptane, 1:1 (vol/vol)], the reaction was quenched by the addition of 6 N of HCl (2 mL) and diluted with water (50 mL). Organic solvents were removed under reduced pressure, and the aqueous residue was extracted with EtOAC (150 mL). The organic layer was dried (Na_2SO_4), concentrated, and coevaporated with DCM (three times), and then dried under high vacuum to give *N*-L-lac-L-Phe as a white foam (783 mg, 66%): ^{1}H-NMR (300 MHz, D_2O) δ 7.49–7.05 (m, Ph CH, 5H), 4.43 [dd, J = 8.3, 4.9 Hz, αCH (Lac), 1H], 4.10 [q, J = 6.9 Hz, αCH (Phe), 1H], 3.17 [dd, J = 13.8, 4.9 Hz, βCH_2 (Phe), 1H], 2.93 [dd, J = 13.8, 8.3 Hz, βCH_2 (Phe), 1H], 1.14 [d, J = 6.9 Hz, CH_3 (Lac), 3H]; ^{13}C-NMR (75 MHz, D_2O) δ 177.7 (C = O), 176.5 (C = O), 137.5 (Ph qCH), 129.5 (Ph CH), 128.6 (Ph CH), 126.9 (Ph CH), 67.7 [αCH (Lac)], 55.4 [αCH (Phe)], 37.8 [βCH_2 (Phe)], 19.5 [CH_3 (Lac)]; LC/MS (*m*/*z*): 236.093 $[M\text{-}H]^-$.

Proteomics. Equal aliquots (30 μL) of the selected fractions were diluted with SDS sample loading buffer and cleared by short, partial SDS/PAGE separation. The gel was stained with GelCode Blue stain reagent (Thermo Scientific), followed by excision of gel bands, reduction of the proteins with DTT, and alkylation with iodoacetamide. Proteins were digested with trypsin (3 ng/μL) overnight at 37 °C. After digestion, peptides were extracted with acetonitrile and dried in a speedvac. Before MS analysis, the peptides were reconstituted in 10% formic acid.

Peptides were separated using the Proxeon nLC 1000 system (Thermo Scientific) fitted with a trapping column (ReproSil-Pur 120 C18-AQ, 3 μm, 100 μm × 200 mm; Dr. Maisch) and an analytical column (Agilent Poroshell EC-C18 120, 2.7 μm, 50 μm × 300 mm), both packed in-house. The outlet of the analytical column was coupled directly to a Thermo Orbitrap Fusion (Q-OT-qIT; Thermo Scientific) using a Proxeon nanoflex source. Nanospray was achieved using a distally coated, fused silica tip emitter (generated in-house: o.d. = 375 μm, i.d. = 20 μm) operated at 2.0 kV. Solvent A was 0.1% formic acid/water, and solvent B was 0.1% formic acid/acetonitrile. Samples (50% of the resuspended volume) were eluted from the analytical column at a constant flow of 150 nL·min^{-1} in a 55-min gradient containing a 36-min linear increase from 5 to 24% solvent B, followed by a 2-min wash at 80% solvent B. Survey scans of peptide precursors from *m*/*z* 375–1,500 were performed at a resolution of 120 K with a 4×10^5 ion count target. Tandem MS was performed by quadrupole isolation at 1.6 Da, followed by collision-induced dissociation fragmentation with normalized collision energy of 35 and ion trap MS^2 fragment detection. The MS^2 ion count target was set to 10^4, and the maximum injection time was set to 35 ms. Only precursors with a charge state of 2–6 were sampled for MS^2. Monoisotopic precursor selection was turned on; the dynamic exclusion duration was set to 10 s with a 10-ppm tolerance around the selected precursor and its isotopes. The instrument was run in top speed mode with 3-s cycles.

Raw data files were processed using Proteome Discoverer (version 1.4.0.288; Thermo Fisher Scientific). MS^2 spectra were searched against the UniProt database (release 2013_12, 541954 entries) using Mascot (version 2.4.1; Matrix Science) and *Homo sapiens* as a taxonomy filter. Carbamidomethylation of Cys was set as a fixed modification, and oxidation of Met was set as a variable modification. Trypsin was specified as the enzyme, and up to two miscleavages were allowed. Data filtering was performed using the percolator algorithm (2), resulting in a 1% false discovery rate. Additional filters were search engine rank 1 peptides and an ion score >20.

Determination of the Equilibrium Constant of *N*-Lac-Phe Formation in Vitro. Recombinant human CNDP2 (1 μg, C-terminal HIS-tagged; ProSpec) was incubated with different concentrations of

lactate and Phe in 25 mM Tris·HCl (pH 7.4) containing 0.1 mM $MnCl_2$ in a total volume of 1 mL. At the indicated time points, 100-μL samples were taken, which were immediately quenched in 900 μL of 10% (vol/vol) acetic acid and applied to a Strata-X solid phase extraction column (33 u polymeric reversed phase, 30 mg/1 mL; Phenomenex) preconditioned with 1 mL of methanol and 1 mL of water. After washing the column with 1 mL of 2% (vol/vol) acetic acid in 5% (vol/vol) methanol, the *N*-lac-Phe was eluted with 1 mL of 2% (vol/vol) NH_4OH in 92% (vol/vol) methanol. The eluates were dried in a speedvac and reconstituted in ion-pairing mobile phase A. *N*-lac-Phe was quantified by LC/MS using the following gradient: 0–3 min: 50% B, 3–8 min: 50–100% B, 8–12 min: 100% B, 12–13 min: 100–50% B, 13–16 min: 50% B. Mobile phases and MS conditions were as described in *Materials and Methods*, *Untargeted Metabolomics*.

Formation of *N*-Lactoyl-Amino Acids in Whole Blood and Plasma. Whole blood was collected in lithium heparin tubes (BD) and placed on ice. One part was centrifuged (10 min, 3,000 × *g*, 4 °C), after which the plasma layer was collected. Three aliquots of the plasma were stored on ice and processed as such. Additional aliquots of 0.5 mL of plasma and 1.4 mL of whole blood were spiked with lactate or Phe to a final concentration of 10 mM and 500 μM, respectively. After incubating the samples for 30 min at 37 °C, the whole-blood samples were centrifuged (10 min, 3,000 × *g*, 4 °C) and 0.5 mL of the plasma phase was collected. All plasma samples were finally processed as described for the human exercise plasma samples.

***N*-Lac-Phe Formation from Methyl-Lactate.** Recombinant human CNDP2 (C-terminal HIS-tagged; R&D Systems) was incubated (8 h, 37 °C) with combinations of lactate, (−)-methyl L-lactate, and Phe in 25 mM Tris·HCl (pH 7.4) containing 0.1 mM $MnCl_2$. The reactions were quenched by the addition of 50 μL of acetic acid and processed as described in the main text.

***N*-Lac-Phe Hydrolysis by CNDP2.** Recombinant human CNDP2 (1 μg, C-terminal HIS-tagged; ProSpec) was incubated (37 °C) with *N*-lac-Phe (2.5 μM) in 1 mL of 25 mM Tris·HCl (pH 7.4) containing 0.1 mM $MnCl_2$. At the indicated time points, a sample of 100 μL was collected, deproteinated with 300 μL of cold methanol, and centrifuged (10 min, 21,800 × *g*, 4 °C). The supernatant was evaporated to dryness in a speedvac, reconstituted in 50 μL of mobile phase A, and analyzed by LC/MS as described in *SI Materials and Methods*, *Determination of the Equilibrium Constant of N-Lac-Phe Formation in Vitro*.

CNDP2-Mediated Cys-Gly Hydrolysis. CNDP2-mediated Cys-Gly hydrolysis was determined essentially as described by Kaur et al. (3). In short, 100 μL of reaction buffer [25 mM Tris·HCl (pH 7.4), 0.1 mM $MnCl_2$, 1 mM DTT, 2 mM Cys-Gly with or without 0.1 μg of CNDP2] was incubated for 60 min at 37 °C. The reaction was quenched by adding 100 μL of glacial acetic acid. Cys release was determined by adding 100 μL of ninhydrin reagent (250 mg of ninhydrin dissolved in 3 mL of acetic acid and 2 mL of concentrated HCl) and incubation for 10 min at 100 °C. After cooling on ice, samples were diluted with 700 μL of ethanol and the A_{560} was determined in 200 μL of the diluted samples using an M200 Pro (TECAN). The concentration of Cys was determined using a standard curve containing known amounts of Cys.

1. Jansen RS, et al. (2013) ABCC6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. *Proc Natl Acad Sci USA* 110(50):20206–20211.
2. Käll L, Canterbury JD, Weston J, Noble WS, MacCoss MJ (2007) Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat Methods* 4(11):923–925.
3. Kaur H, Kumar C, Junot C, Toledano MB, Bachhawat AK (2009) Dug1p Is a Cys-Gly peptidase of the gamma-glutamyl cycle of Saccharomyces cerevisiae and represents a novel family of Cys-Gly peptidases. *J Biol Chem* 284(21):14493–14502.

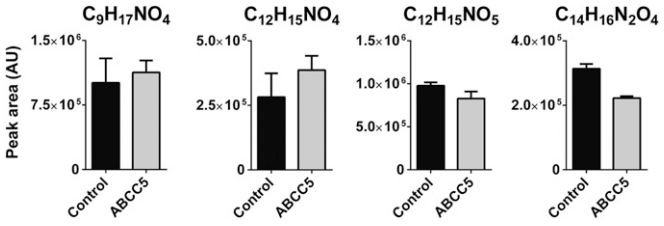

Fig. S1. Relates to Fig. 1. Intracellular levels of the unknown metabolites that accumulate in HEK 293/ABCC5 culture medium do not differ substantially between HEK 293 and HEK 293/ABCC5. HEK 293 control and HEK 293/ABCC5 cells were grown to confluence in six-well plates and cultured for an additional 3 d. Cell lysates were analyzed using untargeted LC/MS metabolomics. Data are presented as mean plus SD (n = 3). AU, arbitrary units.

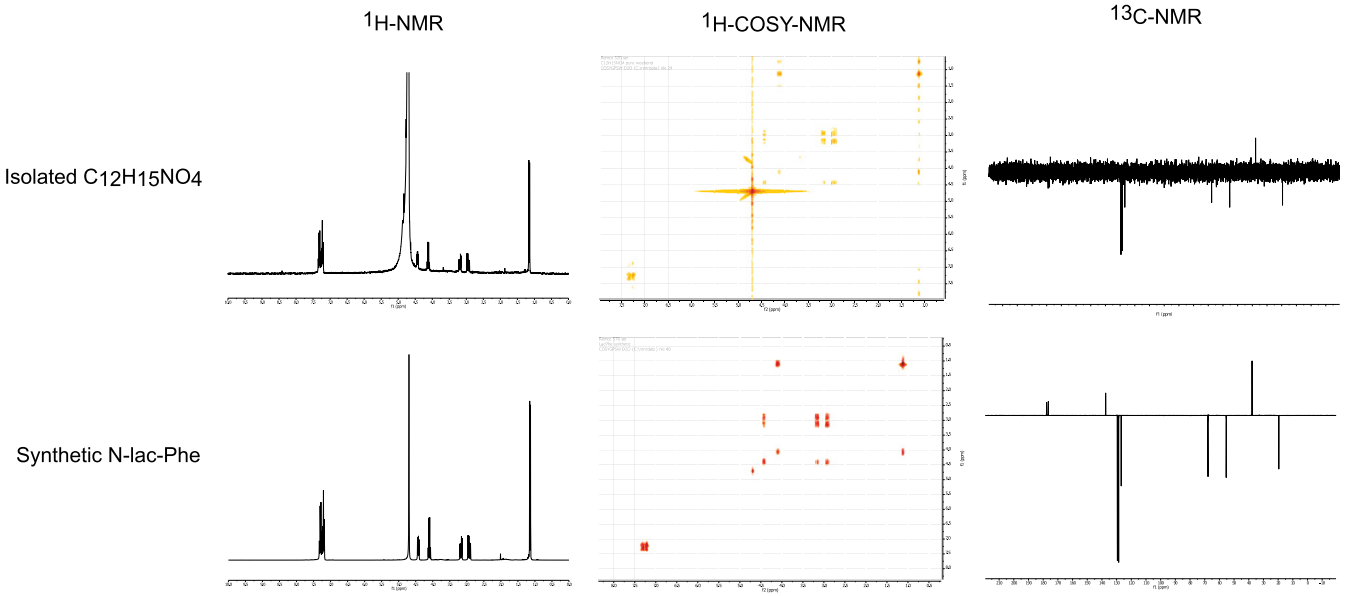

Fig. S2. Relates to Fig. 2. NMR and MS^2 spectra of unknown $C_{12}H_{15}NO_4$ purified from culture medium match those NMR and MS^2 spectra of chemically synthesized *N*-L-lac-L-Phe. Differences in intensities can be attributed to the lower concentration of isolated $C_{12}H_{15}NO_4$ compared with synthetic *N*-L-lac-L-Phe.

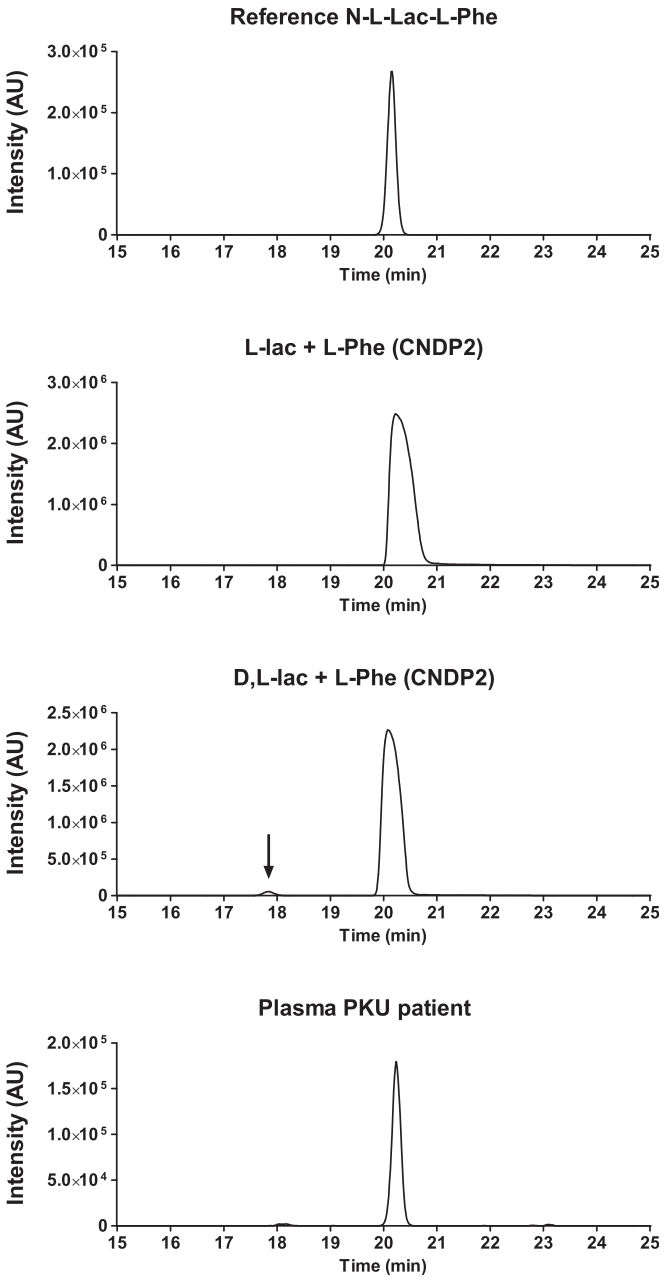

Fig. S3. Relates to Fig. 2. Endogenous *N*-lac-Phe consists of *N*-L-lac-L-Phe. Synthesized *N*-L-lac-L-Phe elutes at the same time as the product formed by incubation of L-Lac and L-Phe with CNDP2. Incubation of CNDP2 with racemic D,L-Lac and L-Phe yields a minor (1.5%) secondary peak with the same mass and fragmentation spectrum, which we consider to be the diastereomer *N*-D-lac-L-Phe. Plasma from a patient with PKU almost exclusively contained *N*-L-lac-L-Phe. The differences in retention time compared with Fig. 2 are due to differences in column age.

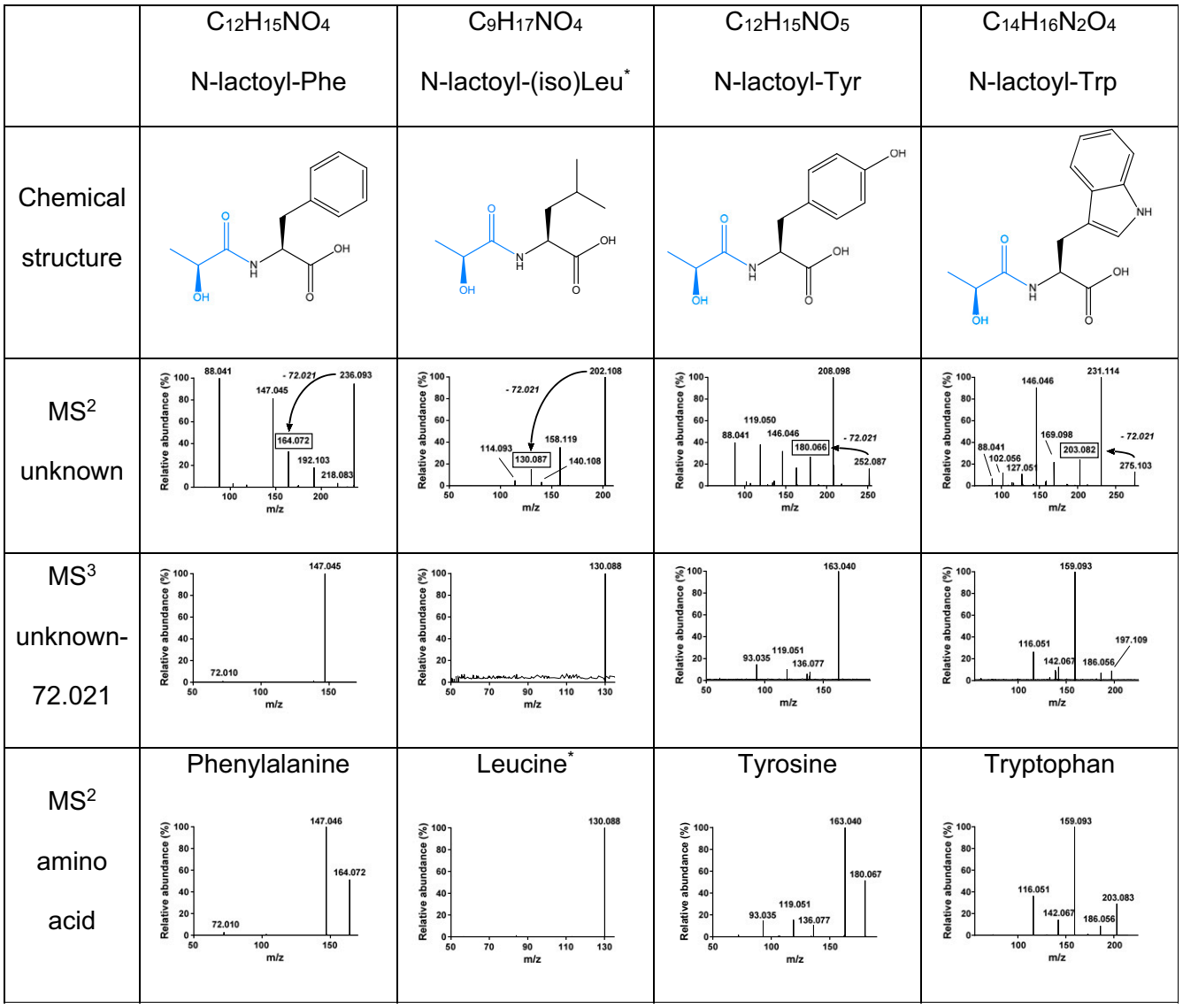

Fig. S4. Relates to Fig. 2. MS fragmentation spectra of the unknowns and amino acids indicate that the unknowns are pseudopeptides of lactate and amino acids. The MS^2 fragmentation spectra of the identified *N*-lac-Phe and the remaining unknowns show common fragments, such as *m/z* 88.041 and *m/z* 146.046. Additionally, the MS^2 spectra all show a neutral loss of 72.021 Da (lactic acid – H_2O) that leads to a fragment with the mass of an amino acid (highlighted in the boxes in row 2). The MS^3 fragmentation spectra of these MS^2 fragments match the MS^2 spectra of reference amino acids. *The MS^2 spectra of Ile and Leu were identical, preventing further specification. The lactate moiety in the identified metabolites is highlighted in blue.

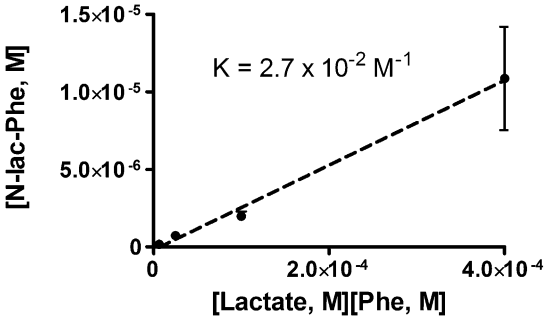

Fig. S5. Relates to Fig. 4. Determination of the equilibrium constant. Recombinant human CNDP2 (1 μg) was incubated (24 h, 37 °C) with different concentrations of lactate and Phe in 25 mM Tris·HCl (pH 7.4) containing 0.1 mM $MnCl_2$. The *N*-lac-Phe levels were determined by LC/MS. Data are presented as mean (filled circles; $n = 3$) plus SD. A linear curve was fitted through the data and is represented by the dashed line.

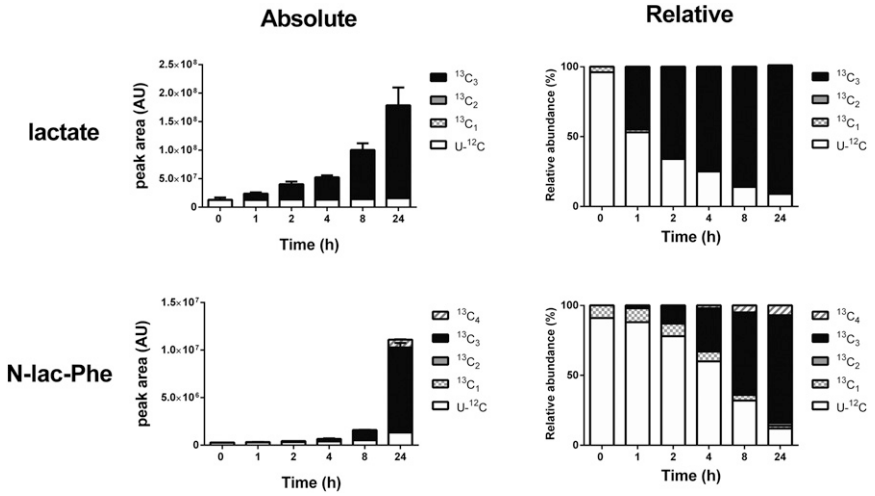

Fig. S6. Relates to Fig. 5. ^{13}C-Metabolic labeling of lactate and *N*-lac-Phe in culture medium from HEK 293 cells. HEK 293 cells were grown to confluence in six-well plates, at which point the medium was replaced with medium containing $^{13}C_6$-glucose. At several time points, the amount of lactate and *N*-lac-Phe containing only ^{12}C (U-^{12}C; unlabeled) or one to four ^{13}C atoms per molecule ($^{13}C_{1-4}$) were determined in lysate by accurate mass LC/MS. Fully ^{13}C-labeled lactate contains three ^{13}C-atoms, which is reflected in the $^{13}C_3$-labeling of *N*-lac-Phe. The minor presence of $^{13}C_1$, $^{13}C_2$, and $^{13}C_4$ isotopologs can be explained by the natural occurrence of the ^{13}C isotope (~1%). Levels are expressed as absolute (AU) and relative values (percentage of isotope total). Data are presented as mean ($n = 3$) and SD (only for absolute values).

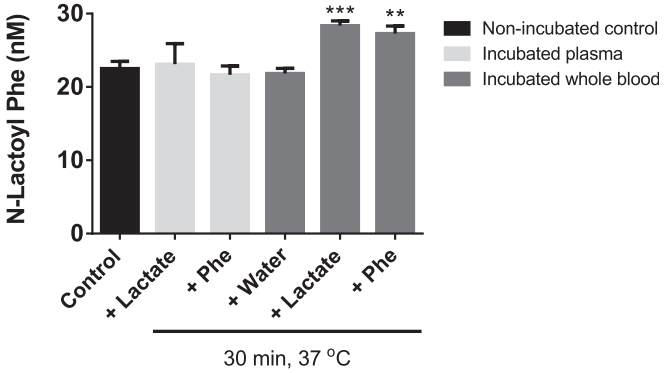

Fig. S7. Relates to Fig. 6. *N*-lac-Phe is not formed in plasma and is formed only slowly in whole blood. Control plasma or whole blood was spiked with 10 mM lactate or 500 μM Phe and incubated for 30 min at 37 °C. Aliquots of the whole blood were immediately processed to plasma and analyzed without incubation. After centrifugation of the whole-blood samples and collection of the plasma layer, all plasma samples were analyzed for *N*-lac-Phe by LC/MS. *N*-lac-Phe levels were modestly increased when whole blood was incubated with either lactate or Phe. This increase was not due to lysis of erythrocytes because the addition of an identical volume of water did not affect *N*-lac-Phe levels. Significance is calculated using a two-sided Student's *t* test, utilizing the water control as a reference. Data are expressed as mean plus SD ($n = 3$). $^{**}P < 0.01$; $^{***}P < 0.001$.

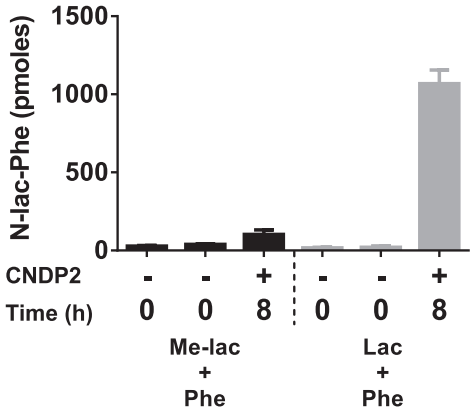

Fig. S8. Relates to Fig. 4. CNDP2 prefers lactate over methyl-lactate. Recombinant human CNDP2 (1 μg) was incubated (8 h, 37 °C) with methyl-lactate plus Phe (5 μmol) or lactate plus Phe (5 μmol) in 25 mM Tris·HCl (pH 7.4) containing 0.1 mM $MnCl_2$. The *N*-lac-Phe levels were determined by LC/MS. Data are presented as mean (n = 3) plus SD.

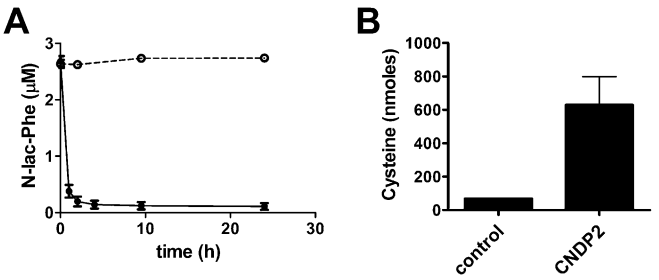

Fig. S9. Relates to Fig. 4. CNDP2 hydrolyzes *N*-lac-Phe and the classical substrate Cys-Gly. (*A*) *N*-lac-Phe (2.5 μM) was incubated (37 °C) in the presence (●) or absence (○) of recombinant human CNDP2 [1 μg in 1 mL of 25 mM Tris·HCl (pH 7.4) containing 0.1 mM $MnCl_2$]. At several time points, samples corresponding to 0.1 μg of CNDP2 were collected, deproteinated, and analyzed by LC/MS. (*B*) Cys-Gly (2 mM) was incubated (37 °C) in the presence and absence of recombinant human CNDP2 [1 μg in 1 mL of 25 mM Tris·HCl (pH 7.4) containing 0.1 mM $MnCl_2$ and 1 mM DTT] for 1 h. Cys-Gly hydrolysis was assessed by measuring the release of Cys, using ninhydrin.