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**General procedures**

If no further details are given, the reaction was performed under ambient atmosphere and at room temperature. Analytical thin layer chromatography (TLC) was performed on silica gel-coated plates (Merck, 60 F254) with the indicated solvent mixture, visualization was done using ultraviolet (UV) irradiation (λ = 254 nm) and/or staining with aqueous KMnO₄ or ninhydrin. Purification by column chromatography was carried out using silica gel 60 (Merck, 0.040-0.063 mm). ^1H NMR and ^13C NMR spectra were recorded on a Bruker DMX 300 (300 MHz), Bruker Advance III 400 (400 MHz) or 500 (500 MHz) spectrometer. For the ^1H NMR spectra, TMS (δH 0.00) or the NMR solvent residual peak of CDCl₃ ((CHCl₃) δH 7.26 ), CD₂OD ([(CHD₂)O] δH 3.31), or (CD₃)₂SO ((CD₃HD₃SO) δH 2.50) were used as the internal reference. For the ^13C NMR spectra, the central resonance of CDCl₃ (δC 77.2), CD₂OD (δC 49.0) or (CD₃)₂SO (δC 39.5) was used as the internal reference, all ^13C NMR spectra were proton decoupled. The spectra of the THP-protected probes S₄, S₇ and S₁₁ were measured at 50 °C, in order to narrow down the broad peaks in the ^13C NMR. Low-resolution mass spectra (LRMS) of the small molecules were recorded on a Thermo LCQ Advantage Max (Electrospray Ionization (ESI)), high-resolution mass spectra (HRMS) were recorded on a JEOL AccuTOF JMS-T100CS (ESI).

**Caution:** All arylamines should be handled in accordance with NIH Guidelines for the Laboratory use of Chemical Carcinogens.¹
**Scheme S1 – Synthesis of hydroxamic acid 1 and the control probe 2**

\[ \text{O} \begin{array}{c} \text{H} \\ \text{N} \end{array} \text{H} \text{O} \text{N} \text{THP} \xrightarrow{\text{DHP, pTsOH}} \xrightarrow{\text{DMF, 16 h, 89\%}} \text{O} \begin{array}{c} \text{H} \\ \text{N} \end{array} \text{O} \text{THP} \]

**N-((Tetrahydro-2H-pyran-2-yl)oxy)acetamide (S1).** Acetohydroxamic acid (500 mg, 6.66 mmol, 1.0 equiv.) was dissolved in dry DMF (13 mL) and dihydropyran (608 µl, 6.66 mmol, 1.0 equiv.) and p-TsOH (114 mg, 0.67 mmol, 0.1 equiv.) were added. The mixture was stirred overnight. Then brine (100 mL) was added and the solution was extracted with CH₂Cl₂ (20x 20 mL). The combined organic layers were dried with Na₂SO₄, the volatiles were removed under reduced pressure and the crude product was purified with column chromatography (70 % EtOAc in heptane) yielding THP-protected acetohydroxamic acid S1 (750 mg, 71 %) as a white solid. Rf = 0.30 (70 % EtOAc in heptane, v/v). ¹H NMR (300 MHz, CDCl₃) δ 8.41 – 8.14 (m, 1H), 5.00 – 4.84 (m, 1H), 4.10 – 3.79 (m, 1H), 3.62 (dd, J = 11.4, 5.5 Hz, 1H), 2.14 – 1.49 (m, 9H). ¹³C NMR (125 MHz, CDCl₃) δ 167.7, 102.5, 62.6, 28.1, 25.1, 20.0, 18.7. HRMS (ESI) Calculated for C₁₉H₁₃NO₅ [M+Na]⁺ 318.07931, found 318.07842.

**1-((6-Azidohexyl)oxy)-4-iodobenzene (S3).** 1-Azido-6-bromohexane S2 (843 mg, 4.1 mmol, 1.2 equiv.) was dissolved in dry DMF (15 mL) and 4-iodophenol (750 mg, 3.4 mmol, 1.0 equiv.) and K₂CO₃ (1.17 gr, 8.5 mmol, 2.5 equiv.) were added. The mixture was stirred at 70°C overnight, whereupon it was poured into water (100 mL) and extracted with CH₂Cl₂ (3x). The combined organic layers were washed with brine, dried with Na₂SO₄, and the volatiles were removed in vacuo. The crude mixture was purified by column chromatography (1 % EtOAc in heptane) yielding ether S3 (1.15 g, 97 %) as a colourless oil which solidified upon storage in the freezer. Rf = 0.29 (1 % EtOAc in heptane). ¹H NMR (300 MHz, CDCl₃) δ 7.58 – 7.51 (m, 2H), 6.71 – 6.63 (m, 2H), 3.92 (t, J = 6.4 Hz, 2H), 3.28 (t, J = 6.8 Hz, 2H), 1.85 – 1.73 (m, 2H), 1.70 – 1.58 (m, 2H), 1.54 – 1.38 (m, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 159.05, 138.31, 117.05, 82.65, 67.97, 51.51, 29.15, 28.93, 26.63, 25.78. Iodobenzene S3 was not visible on mass spectrometry.
**N-(4-((6-Azidohexyl)oxy)phenyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)acetamide (S4).** Iodobenzene S3 (255 mg, 0.76 mmol, 1.2 equiv.) and THP-protected N-hydroxyacetamide S1 (85 mg, 0.53 mmol, 1.0 equiv.) were dissolved in dry DMF (2.0 mL) and Cul (14 mg, 0.072 mmol, 0.1 equiv.), DMEDA (30 µl, 0.275 mmol, 0.5 equiv.), Cs2CO3 (259 mg, 0.79 mmol, 1.4 equiv.) and molecular sieves (4 Å) were added and the reaction was heated at 80 °C overnight. The mixture was cooled down to room temperature, EtOAc was added and the suspension was filtered over celite. The volatiles were removed under reduced pressure and the crude product was purified with column chromatography (40 % EtOAc in heptane), yielding THP-protected N-hydroxyacetamide S4 (147 mg, 73 %) as a yellow oil. Rf = 0.27 (40 % EtOAc in heptane, v/v). 1H NMR (400 MHz, 50 °C, (CD3)2SO) δ 7.33 – 7.23 (m, 2H), 6.99 – 6.90 (m, 2H), 5.02 – 4.93 (m, 1H), 4.00 (t, J = 6.5 Hz, 2H), 3.66 (ddd, J = 11.5, 8.0, 3.6 Hz, 1H), 3.41 – 3.30 (m, 3H), 2.07 (s, 3H), 1.78 – 1.37 (m, 14H). 13C NMR (100 MHz, 50 °C, (CD3)2SO) δ 169.3, 157.6, 132.9, 127.2, 114.3, 101.3, 67.5, 62.1, 50.4, 28.2, 27.9, 27.8, 25.5, 24.6, 24.1, 21.4, 18.4. HRMS (ESI) Calculated for C31H29N3O5 [M+Na]+ 399.20082, found 399.20057.

**N-(4-((6-Azidohexyl)oxy)phenyl)-N-hydroxyacetamide (1).** THP-protected alcohol S4 (50 mg, 133 µmol, 1.0 equiv.) was dissolved in EtOH (1.0 mL) at ambient atmosphere and pyridinium p-toluenesulfonate (3.3 mg, 13 µmol, 0.1 equiv.) was added and the mixture was stirred at 55 °C overnight. The solvent was evaporated and the solid was purified by column chromatography (60 % EtOAc in heptane) yielding N-hydroxyacetamide 1 (26 mg, 67 %) as a white solid. Rf = 0.28 (60 % EtOAc in heptane, v/v). 1H NMR (500 MHz, CDCl3) δ 7.40 – 7.34 (m, 2H), 7.03 (s, 1H), 6.87 – 6.82 (m, 2H), 3.94 (t, J = 6.3 Hz, 2H), 3.32 – 3.24 (m, 2H), 2.16 (s, 3H), 1.78 (p, J = 6.4 Hz, 2H), 1.64 (p, J = 7.0 Hz, 2H), 1.52 – 1.41 (m, 4H). 13C NMR (125 MHz, CDCl3) δ 165.0, 159.8, 130.6, 128.5, 115.2, 68.2, 51.5, 29.2, 28.9, 26.6, 25.8, 19.6. HRMS (ESI) Calculated for C14H12N2O3 [M+Na]+ 315.14331, found 315.14305.

**N-(4-((6-Azidohexyl)oxy)phenyl)acetamide (2).** Iodobenzene S3 (255 mg, 0.67 mmol, 1.0 equiv.) and acetyl chloride (48 mg, 0.81 mmol, 1.2 equiv.) were dissolved in dry DMF (2.0 mL) and Cul (11 mg, 0.059 mmol, 0.1 equiv.), DMEDA (36 µl, 0.33 mmol, 0.5 equiv.), Cs2CO3 (306 mg, 0.94 mmol, 1.4 equiv.) and molecular sieves (4 Å) were added and the reaction was heated at 80 °C for 2 days. The mixture was cooled down to room temperature, EtOAc was added and the suspension was filtered over celite. The volatiles were removed under reduced pressure and the crude product was purified with column chromatography (60 % EtOAc in heptane), yielding acetamide 2 (101 mg, 75 %) as a white solid. Rf = 0 (60 % EtOAc in heptane, v/v). 1H NMR (500 MHz, CDCl3) δ 7.42 – 7.32 (m, 2H), 7.11 (s, 1H), 6.90 – 6.73 (m, 2H), 3.93 (t, J = 6.4 Hz, 2H), 3.28 (t, J = 6.8 Hz, 2H), 2.15 (s, 3H), 1.85 – 1.72 (m, 2H), 1.70 – 1.57 (m, 2H), 1.56 – 1.37 (m, 4H). 13C NMR (125 MHz, CDCl3) δ 167.6, 155.4, 130.3, 121.4, 114.3, 67.5, 50.9, 28.6, 28.3, 26.0, 25.2, 23.9. HRMS (ESI) Calculated for C14H12N2O2 [M+Na]+ 299.14839, found 299.14814.
6-Azido-N-(4-iodobenzyl)hexanamide (S6). 6-Azidohexanoic acid S5 was synthesized from 6-aminohexanoic acid using imidazole-1-sulfonyl azide as diazotransfer reagent. Then, 4-iodobenzylamine (135 mg, 0.86 mmol, 1.0 equiv.) and 6-azidohexanoic acid S5 (200 mg, 0.86 mmol, 1.0 equiv.) were dissolved in dry CH₂Cl₂ (9 mL) and EDC hydrochloride (197 mg, 1.03 mmol, 1.2 equiv.), HOBr hydrate (158 mg, 1.03 mmol, 1.2 equiv.) and DIPEA (448 µL, 2.57 mmol, 3.0 equiv.) were added. The mixture was stirred overnight, whereupon it was diluted with CH₂Cl₂ and washed with 1 M HCl. The aqueous layer was extracted with CH₂Cl₂ (2x) and the combined organic layers were washed with sat. NaHCO₃ and brine and dried with Na₂SO₄. The mixture was purified using column chromatography (30 to 50 % EtoAc/heptane) yielding azidohexanamide S6 (237 mg, 74 %) as a white solid. Rₐ = 0.40 (EtoAc/heptane 1:1, v/v). ¹H NMR (400 MHz, CDCl₃) δ 7.75 – 7.55 (m, 2H), 7.13 – 6.91 (m, 2H), 5.77 (br. s., 1H), 4.37 (d, J = 5.8 Hz, 2H), 3.27 (t, J = 6.8 Hz, 2H), 2.22 (t, J = 7.5 Hz, 2H), 1.74 – 1.55 (m, 4H), 1.45 – 1.35 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ 172.6, 138.2, 137.9, 129.9, 93.0, 51.4, 43.2, 36.5, 28.8, 26.5, 25.2. HRMS (ESI) m/z calcd. for C₁₃H₁₀N₄O₂ [M+Na]+ 395.03447, found: 395.03431.

6-Azido-N-(4-(N-(tetrahydro-2H-pyran-2-yl)oxy)acetamido)benzyl)hexanamide (S7). Iodobenzene S6 (62 mg, 0.19 mmol, 1.2 equiv.) and THP-protected N-hydroxyacetamide S1 (25 mg, 0.16 mmol, 1.0 equiv.) were dissolved in dry DMF (1.0 mL) and Cul (30 mg, 0.16 mmol, 1.0 equiv.), DMEDA (25 µL, 0.24 mmol, 1.5 equiv.), Cs₂CO₃ (72 mg, 0.22 mmol, 1.4 equiv.) and molecular sieves (4 Å) were added and the reaction was heated at 80 °C overnight. Then the mixture was cooled to room temperature, EtoAc was added and it was filtered over celite. The volatiles were removed under reduced pressure and the crude mixture was purified with column chromatography (50 to 75 % EtoAc in heptane) yielding THP-protected N-hydroxyacetamide S7 (60 mg, 94 %) as a white solid. Rₐ = 0.24 (EtoAc/heptane 3:1, v/v). ¹H NMR (500 MHz, 50 °C, CDCl₃) δ 7.42 – 6.75 (m, 2H), 5.67 (s, 1H), 4.95 (t, J = 4.0 Hz, 1H), 4.45 (d, J = 5.7 Hz, 2H), 3.80 – 3.71 (m, 1H), 3.44 – 3.36 (m, 1H), 3.28 (t, J = 6.8 Hz, 2H), 2.27 – 2.17 (m, 5H), 1.88 – 1.67 (m, 5H), 1.67 – 1.59 (m, 2H), 1.59 – 1.49 (m, 3H), 1.48 – 1.40 (m, 2H). ¹³C NMR (125 MHz, 50 °C, CDCl₃) δ 172.6, 171.0, 139.9, 137.6, 128.4, 125.8, 102.8, 63.8, 51.5, 43.4, 36.6, 29.0, 28.8, 26.6, 25.3, 25.1, 22.3, 19.7. HRMS (ESI) m/z calcd. for C₂₃H₂₁N₄O₂ [M+Na]+ 426.21172, found: 426.21127.
6-Azido-N-(4-(N-hydroxyacetamido)benzyl)hexanamide (3). THP-protected probe S7 (24 mg, 0.059 mmol, 1.0 equiv.) was dissolved in dry CH₂Cl₂ (1.0 mL) and 4 M HCl in dioxane (150 µL, 0.59 mmol, 10.0 equiv.) was added. After the mixture had stirred for 2 h, the volatiles were removed under reduced pressure yielding N-hydroxyacetamide 3 (18 mg, 95 %) as a white solid. ¹H NMR (500 MHz, (CD₃)₂SO) δ 10.58 (s, 1H), 8.30 (t, J = 6.0 Hz, 1H), 7.60 – 7.49 (m, 2H), 7.26 – 7.17 (m, 2H), 4.23 (d, J = 5.9 Hz, 2H), 3.31 (t, J = 6.9 Hz, 2H), 2.18 (s, 3H), 2.14 (t, J = 7.4 Hz, 2H), 1.58 – 1.50 (m, 4H), 1.34 – 1.28 (m, 2H). ¹³C NMR (125 MHz, (CD₃)₂SO) δ 171.9, 140.3, 136.01, 127.16, 120.14, 50.55, 41.53, 35.16, 28.0, 25.8, 24.8, 22.4. HRMS (ESI) m/z calcd. for C₁₅H₂₁N₅O₃ [M+Na⁺] 342.15421, found: 342.15477.

N-(4-Acetimidobenzyl)-6-azidohexanamide (4). Iodobenzene S6 (50 mg, 0.134 mmol, 1.0 equiv.) and acetamide (7.9 mg, 0.134 mmol, 1.0 equiv.) were dissolved in DMF (1.0 mL) and Cul (6.4 mg, 0.034 mmol, 0.25 equiv.), DMEDA (7.2 µL, 0.067 mmol, 0.5 equiv.), Cs₂CO₃ (61 mg, 0.188 mmol, 1.4 equiv.) and molecular sieves (4 Å) were added and the reaction was heated at 80 °C overnight. After the mixture was cooled to room temperature, EtOAc was added and it was filtered over celite. Then the volatiles were removed under reduced pressure and the crude mixture was purified with column chromatography (60 % EtOAc/heptane to 100 % EtOAc) yielding acetamide 4 (20 mg, 49 %) as a slightly yellow solid. Rf = 0.24 (EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 7.48 – 7.42 (m, 2H), 7.25 – 7.18 (m, 3H), 5.69 (s, 1H), 4.39 (d, J = 5.7 Hz, 2H), 3.27 (t, J = 6.9 Hz, 2H), 2.22 (t, J = 7.5 Hz, 2H), 2.17 (s, 3H), 1.74 – 1.64 (m, 2H), 1.65 – 1.58 (m, 2H), 1.46 – 1.36 (m, 2H). ¹³C NMR (125 MHz, CDCl₃) δ 172.6, 168.4, 137.4, 134.4, 128.7, 120.3, 51.4, 43.3, 36.6, 28.8, 26.5, 25.3, 24.8. HRMS (ESI) m/z calcd. for C₁₅H₂₁N₅O₂ [M+Na⁺] 326.15929, found: 326.15962.
N-(6-Azidohexyl)-4-iodobenzamide (S10). 6-Azidohexan-1-amine S9 was synthesized from 1,6-dibromohexane via 1,6-diazidohexane S8. Then, amine S9 (115 mg, 0.81 mmol, 1.0 equiv.) was dissolved in dry CH2Cl2 and 4-iodobenzoic acid (200 mg, 0.81 mmol, 1.0 equiv.), EDC hydrochloride (186 mg, 0.97 mmol, 1.2 equiv.) and DIPEA (421 µL, 2.42 mmol, 3.0 equiv.) were added and the mixture was stirred overnight. The solution was then washed with CH2Cl2 and washed with 1M HCl. The aqueous layer was extracted with CH2Cl2 (2x) and the combined organic layers were washed with sat. NaHCO3 and brine and dried with Na2SO4. The volatiles were removed in vacuo yielding amide S10 (308 mg, quant.) as an off-white solid. Rf = 0.59 (EtOAc/heptane 1:1, v/v). 1H NMR (400 MHz, CDCl3) δ 7.81 – 7.75 (m, 2H), 7.51 – 7.45 (m, 2H), 6.11 (br. t, J = 5.9 Hz, 1H), 3.44 (td, J = 7.2, 5.9 Hz, 2H), 3.27 (t, J = 6.8 Hz, 2H), 1.68 – 1.55 (m, 4H), 1.48 – 1.35 (m, 4H). 13C NMR (100 MHz, CDCl3) δ 166.8, 137.9, 134.3, 128.6, 98.4, 51.5, 40.1, 29.7, 28.9, 26.7, 26.6. HRMS (ESI) m/z calcd for C13H11N4O [M+Na]+: 395.03474, found: 395.03473.

N-(6-Azidohexyl)-4-(N-(tetrahydro-2H-pyran-2-yl)oxy)acetamido)benzamide (S11). Iodobenzene S10 (60 mg, 0.161 mmol, 1.2 equiv.) and THP-protected N-hydroxyacetamide S1 (21 mg, 0.134 mmol, 1.0 equiv.) were dissolved in dry DMF (1.0 mL) and CuI (13 mg, 0.067 mmol, 0.5 equiv.), DMEDA (11 µL, 0.10 mmol, 0.75 equiv.), Cs2CO3 (61 mg, 0.188 mmol, 1.4 equiv.) and molecular sieves (4 Å) were added and the reaction was heated at 80 °C overnight. The mixture was cooled down to room temperature, EtOAc was added and the suspension was filtered over celite. The volatiles were removed under reduced pressure and the crude mixture was purified with column chromatography (50 to 75% EtOAc in heptane) yielding THP-protected N-hydroxyacetamide S11 (34 mg, 63 %) as a white solid. Rf = 0.27 (EtOAc/heptane 3:2, v/v). 1H NMR (500 MHz, 50 °C, CDCl3) δ 7.79 – 7.74 (m, 2H), 7.56 – 7.51 (m, 2H), 6.06 (s, 1H), 4.93 – 4.88 (m, 1H), 3.81 – 3.74 (m, 1H), 3.46 (td, J = 7.2, 5.9 Hz, 2H), 3.42 – 3.36 (m, 1H), 3.27 (t, J = 6.8 Hz, 2H), 2.29 (s, 3H), 1.89 – 1.69 (m, 3H), 1.69 – 1.59 (m, 4H), 1.59 – 1.53 (m, 3H), 1.50 – 1.37 (m, 4H). 13C NMR (125 MHz, 50 °C, CDCl3) δ 171.3, 167.0, 143.1, 133.0, 127.5, 124.0, 103.5, 64.3, 51.6, 40.2, 29.8, 28.99, 29.05, 26.7, 26.6, 25.0, 22.4, 19.9. HRMS (ESI) m/z calcd for C26H22N3O4 [M+Na]+: 426.21172, found: 426.21202.
**N-(6-Azidohexyl)-4-(N-hydroxyacetamido)benzamide (5).** THP-protected probe S11 (30 mg, 0.074 mmol, 1.0 equiv.) was dissolved in dry CH₂Cl₂ (1.0 mL) and 4 M HCl in dioxane (186 µL, 0.74 mmol, 10.0 equiv.) was added. After the mixture had stirred for 2 h, the volatiles were removed under reduced pressure yielding N-hydroxyacetamide 5 (23 mg, 97 %) as a slightly yellow solid. ¹H NMR (500 MHz, (CD₃)₂SO) δ 10.75 (s, 1H), 8.38 (br. t, J = 5.6 Hz, 1H), 7.86 – 7.79 (m, 2H), 7.76 – 7.69 (m, 2H), 3.32 – 3.26 (m, 2H), 3.28 – 3.18 (m, 2H), 2.24 (s, 3H), 1.59 – 1.43 (m, 4H), 1.40 – 1.25 (m, 4H). ¹³C NMR (125 MHz, (CD₃)₂SO) δ 170.3, 165.4, 143.7, 130.1, 127.5, 118.5, 50.6, 39.0, 29.0, 28.2, 26.0, 25.9, 22.8. HRMS (ESI) m/z calcd. for C₁₉H₁₆N₄O₃ [M+Na⁺]⁺ 342.15421, found: 342.15477.

**4-Acetamido-N-(6-azidohexyl)benzamide (6).** Iodobenzene S10 (50 mg, 0.134 mmol, 1.0 equiv.) and acetamide (7.9 mg, 0.134 mmol, 1.0 equiv.) were dissolved in dry DMF (1.0 mL) and CuI (6.4 mg, 0.034 mmol, 0.25 equiv.), DMEDA (7.2 µL, 0.067 mmol, 0.5 equiv.), Cs₂CO₃ (61 mg, 0.188 mmol, 1.4 equiv.) and molecular sieves (4 Å) were added and the mixture was heated at 80 °C overnight. The conversion of S10 was not complete, so again CuI (12.8 mg, 0.067 mmol, 0.5 equiv.) and DMEDA (7.2 µL, 0.067 mmol, 0.5 equiv.) were added and the reaction was stirred again at 80 °C overnight. The mixture was cooled to room temperature, EtOAc was added and the suspension was filtered over celite. The volatiles were removed under reduced pressure and the crude product was purified with column chromatography (60 % EtOAc/heptane to 100 % EtOAc) yielding acetamide 6 (37 mg, 90 %) as a white solid. Rₐ = 0.31 (EtOAc). ¹H NMR (500 MHz, CDCl₃) δ 7.75 – 7.69 (m, 2H), 7.61 – 7.54 (m, 2H), 7.50 (s, 1H), 6.14 (br. t, J = 5.8 Hz, 1H), 3.45 (td, J = 7.2, 5.9 Hz, 2H), 3.27 (t, J = 6.9 Hz, 2H), 2.20 (s, 3H), 1.67 – 1.59 (m, 4H), 1.47 – 1.36 (m, 4H). ¹³C NMR (125 MHz, CDCl₃) δ 168.6, 167.0, 140.9, 130.3, 128.0, 119.3, 51.5, 40.1, 29.8, 28.9, 26.7, 26.6, 24.9. HRMS (ESI) m/z calcd. for C₁₅H₁₂N₂O₂ [M+Na⁺]⁺ 326.15929, found: 326.15979.
Scheme S4 – Synthesis of hydroxamic acid 7 and the control probe 8

Alkynated biotin (S12). Biotin (200 mg, 0.82 mmol, 1.0 equiv.) was dissolved in dry DMF (10 mL) and N-hydroxysuccinimide (104 mg, 0.90 mmol, 1.1 equiv.) and 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (188 mg, 0.98 mmol, 1.2 equiv.) were added. After the mixture was stirred o/n at room temperature, the reaction was concentrated and washed with methanol (3x). The solvent was removed under reduced pressure yielding the crude NHS ester. The solid was directly applied in the next step without further purification. S12 was dissolved in DMF (7.8 mL) and trimethylamine (159 µL, 1.17 mmol, 3.0 equiv.) and propargyl amine (37 µL, 0.88 mmol, 2.3 equiv.) were added. After the mixture was stirred o/n at room temperature, the volatiles were removed under reduced pressure. The crude solid was purified by column chromatography (15 % MeOH in CH₂Cl₂, v/v) yielding the alkynated biotin S12 as a white solid (102 mg, 96 %). Rf = 0.23 (5 % MeOH in CH₂Cl₂, v/v). ¹H NMR (500 MHz, CD₃OD) δ 4.52 – 4.45 (m, 1H), 4.34 – 4.27 (m, 1H), 3.96 – 3.93 (m, 2H), 3.25 – 3.17 (m, 1H), 2.97 – 2.88 (m, 1H), 2.74 – 2.67 (m, 1H), 2.59 – 2.55 (m, 1H), 2.25 – 2.17 (m, 2H), 1.79 – 1.54 (m, 4H), 1.50 – 1.39 (m, 2H). ¹³C NMR (125 MHz, CD₃OD) δ 175.6, 166.1, 80.7, 72.1, 63.4, 61.6, 57.0, 41.0, 36.5, 29.7, 29.5, 29.4, 26.7. LRMS (ESI+ m/z calcd. for C₁₃H₁₉N₃O₈S [M+H]+: 282.1, found: 282.1. The data agrees with the reported literature values.⁵

Biotinylated N-(4-((6-azidohexyloxy)oxy)phenyl)-N-hydroxyacetamide (7). THP protected hydroxamic acid probe S4 (20 mg, 53 µmol, 1.0 equiv.), alkynated biotin S12 (18 mg, 64 µmol, 1.2 equiv.), CuSO₄ (0.33 mg, 1.0 µmol, 0.025 equiv.), and sodium ascorbate (1.1 mg, 5.0 µmol, 0.1 equiv.) were dissolved in H₂O/BuOH (1:1, 1.5 mL). After the mixture was stirred o/n at room temperature, the reaction was diluted with CH₂Cl₂ and washed by H₂O (3× 10 mL), dried over Na₂SO₄ and then concentrated under reduced pressure. The brown solid (S14) was dissolved in EIOH (1.0 mL), pyridinium p-toluenesulfonate (4.0 mg, 16 µmol, 0.3 equiv.) was added and the solution was heated o/n at 55 °C. The solvent was evaporated, and the mixture was purified using column chromatography (0 to 20 % MeOH/CH₂Cl₂, v/v) yielding biotinylated hydroxamic acid 7 as a brown solid (12 mg, 40 %). Rf = 0.38 (10 % MeOH in CH₂Cl₂). ¹H NMR (500 MHz, CD₃OD) δ 7.74 (s, 1H), 7.64 – 7.58 (m, 2H), 7.28 (br. s, 1H), 7.17 – 7.11 (m, 3H), 6.81 (br. s, 1H), 4.38 (dd, J = 7.8, 4.9 Hz, 1H), 4.33 – 4.26 (m, 3H), 4.18 (dd, J = 7.9, 4.4 Hz, 1H), 3.88 (t, J = 5.6 Hz, 2H), 3.10 – 3.05 (m, 1H), 2.81 (dd, J = 12.7, 4.9 Hz, 1H), 2.59 (d, J = 12.7 Hz, 1H), 2.27 (s, 3H), 2.13 (t, J = 7.4 Hz, 3H), 1.83 (p, J = 7.1 Hz, 2H), 1.71 – 1.37 (m, 8H), 1.36 – 1.20 (m, 4H). ¹³C NMR (125 MHz, CD₃OD) δ 176.0, 166.5, 146.3, 143.4, 141.8, 129.8, 127.0, 124.2, 69.1, 63.4, 61.6, 57.0, 51.3, 41.1, 36.5, 35.6, 31.2, 30.1, 29.7, 29.5, 27.2, 26.7, 26.6, 21.3. Peak of quaternary carbon of the acetyl is missing. HRMS (ESI+) m/z calcd. for C₂₇H₃₉N₅O₃S [M+Na]+: 596.26311, found: 596.26359.
Biotinylated $N$-(4-((6-azidoethyl)oxy)phenyl)acetamide (8). Acetamide probe 2 (30.0 mg, 0.11 mmol, 1.0 equiv.) was dissolved in H$_2$O/tBuOH/DMF (1:1:1, 1.5 mL) and alkynated biotin S12 (36.6 mg, 0.13 mmol, 1.2 equiv.), CuSO$_4$ (0.68 mg, 2.7 µmol, 0.025 equiv.), and sodium ascorbate (2.15 mg, 11 µmol, 0.1 equiv.) were added. The mixture was stirred o/n at room temperature, concentrated, and purified by column chromatography (0 to 20 % MeOH in CH$_2$Cl$_2$, v/v) yielding the biotinylated acetamide 8 as a white solid (48 mg, 78 %). $R_f$ = 0.48 (10 % MeOH in CH$_2$Cl$_2$, v/v). $^1$H NMR (500 MHz, CD$_3$OD) δ 7.91 (s, 1H), 7.34 – 7.22 (m, 2H), 6.79 – 6.64 (m, 2H), 4.38 (dd, $J$ = 7.8, 4.8 Hz, 1H), 4.32 (t, $J$ = 6.8 Hz, 3H), 4.19 (dd, $J$ = 7.9, 4.4 Hz, 1H), 3.83 (t, $J$ = 6.3 Hz, 2H), 3.10 (dd, $J$ = 8.1, 3.1 Hz, 1H), 2.82 (dd, $J$ = 12.7, 5.0 Hz, 1H), 2.63 – 2.56 (m, 1H), 2.23 – 2.13 (m, 2H), 1.99 (s, 3H), 1.88 – 1.79 (m, 2H), 1.70 – 1.37 (m, 8H), 1.37 – 1.21 (m, 4H). $^{13}$C NMR (125 MHz, CD$_3$OD) δ 171.4, 157.3, 132.8, 123.1, 115.6, 69.0, 63.4, 61.7, 57.0, 51.6, 41.0, 35.9*, 31.1, 30.2, 29.7, 29.4, 27.2, 26.7, 26.6, 23.5. * Found with HSQC. (4 peaks are missing). HRMS (ESI+) m/z calcd. for C$_{27}$H$_{39}$N$_7$O$_4$S [M+Na]$^+$ 580.26819, found: 580.26861.
Design and cloning of NAT constructs

Genes encoding hNAT1 without or with a targeting sequence were cloned by standard techniques in the pet30A vector or pcDNA3 vector for expression in E.coli or mammalian cells, respectively. Table S1 summarizes the plasmids used for NAT expression in mammalian and bacterial cells.

Oligonucleotide primers and a gBlock fragment encoding the hNAT1 gene were synthesized by Integrated DNA Technologies (Table S2 and S3). PCR reactions were conducted with 1× HF Buffer (New England Biolabs), 1 unit of Phusion polymerase, 20 ng of DNA template, 100 µM dNTP, 10 pmol of each of the primers in a total volume of 50 µL. The reaction mixtures were heated at 98 °C for 30 sec, followed by 25 cycles for 20 sec at 98 °C, 30 sec at 58 °C, and 2 min at 72 °C. To ensure complete extension, the reaction mixture was incubated at 72 °C for 4 min after the 25 cycles. The PCR products were purified with a QiAquick PCR purification kit (Qiagen). Subsequently, the PCR products and the plasmid vector were digested with 2 units of each restriction enzyme for 1 h at 37 °C. The digestion products were isolated with a QiAquick gel extraction kit (Qiagen). The vector DNA (50 ng) was ligated with one of the hNAT1 inserts (30 ng) with T4 Ligase (New England Biolabs) overnight at 16 °C. After heat inactivation of T4 Ligase (20 min at 65 °C), supercompetent Top 10 cells were transformed with the ligation mix (25 ng vector) and selected for the correct antibiotic resistance. The plasmids from positive colonies were isolated with the Miniprep DNA purification system (Qiagen) and sequences were verified by automated DNA sequencing (RadboudUMC sequence facility Nijmegen, the Netherlands).
| Name         | Features                          | Promoter/Vector | Details                                                                 |
|--------------|-----------------------------------|-----------------|-------------------------------------------------------------------------|
| H6-hNAT1     | H6-BamHI-TEVsite-hNAT1-Stop-NotI  | T7/pET30A       | His-tag sequence (H6): HHHHHH                                           |
|              |                                   |                 | TEV cleavage site sequence (TEVsite): ENLYFQSG                           |
| NLS-HA-hNAT1 | BamHI-NLS-HA-hNAT1-Stop-XhoI      | CMV/pcDNA3      | Nuclear localization signal (NLS) sequence: PKKKRKVPDKKRRKVPDKKRRK       |
|              |                                   |                 | HA-tag sequence: YPYPDVPDYA                                              |
| NES-HA-hNAT1 | BamHI-NES-HA-hNAT1-Stop-XhoI      | CMV/pcDNA3      | Nuclear export signal (NES) sequence: LQLPPLERLTLD                       |
**Table S2 - Oligonucleotide sequences used for amplification of the hNAT1 gene, including targeting or tags.**

| Primer name                        | Details                                           | Comments                                                                 |
|-----------------------------------|--------------------------------------------------|--------------------------------------------------------------------------|
| **BamHI-TEVsite-hNAT1**           | GATC GGATCC  
GAGAAGTTCTTTCAAGACCGCG  
GACATTGAAGCATATCTTG       | Forward primer encoding BamHI restriction site, TEV protease cleavage site and overlapping with hNAT1 |
| **hNAT1-STOP-NotI**               | ATATAT GGCCGCG CTA AATA  
GTTAAAATCTATCACCATGTTTG  
GGCAAGA            | Reverse primer encoding NotI restriction site, a STOP codon, and overlapping with hNAT1 |
| **BamHI-HA-hNAT1**               | ATATAT GGATCC GCCACC ATG  
TATCCGTACGACGTACCGGATTAT  
GC  
GACGCGTGACATTGAAGCATAT  | Forward primer encoding BamHI restriction site, Kozak sequence, the HA-tag and overlapping with hNAT1 |
| **BamHI-NLS-HA-hNAT1**            | ATATAT GGATCC GCCACC ATG  
CCGAAAGAAAACCCTAAGGTTGC  
CCGAAAAAGAACCCAGGTTGGAC  
CCAAAGAAAAAADGTAAAGTG  
TATCCGTACGACGTACCGG  | Forward primer encoding BamHI restriction site, Kozak sequence, the NLS-tag and overlapping with the HA-tag |
| **BamHI-NES-HA-hNAT**             | ATATAT GGATCC GCCACC ATG  
CTGCAGCTGCCACTGGAACGT  
CTGACGCTGGAT  
TATCCGTACGACGTACCGG  | Forward primer encoding BamHI restriction site, the NES-tag and overlapping with the HA-tag |
| **hNAT1-STOP-XhoI**               | ATATAT CTGGAG CTA AATAGT  
AAAAATCTATCACCATGTTTG    | Reverse primer encoding XhoI restriction site, a STOP codon, and overlapping with hNAT1 |
Table S3 - gBlock fragment encoding hNAT1 used for cloning of NAT constructs.

| Gene | Amino acid sequence |
|------|---------------------|
| GACATTGAGCATAATCTTGAAAGAATTGGCCTATAAGAAGTCTAGGAACAAATTGGACTTGGAAACATTAACTGACATTCTTCAACA CCAGATCCCCGACGTTCCCTTTGGAAACCTAATCCATAGTGGGGATGCCATGGACTTAGGCTTAGAGGCCATTTTTGATCAAG TTGTGAGAAGAAATCGGGGTGGATGGTGCTCCAGGTCAATCATCTTCTGTACTGGCTCTGACCTATTGGTTTTGAGACCA CGTATGTTGGGAGGGTATGTTTACAGCACTCCAGCCAAAAATACAGCACTGGCATGATTCACCTTCTCCTGCAGGTGACCATTGATGG CAGGAACTACATTGCGATGCTGGGTTTGGACGCTCATACCAGATGTGGCAGCCTCTGGAGTTAATTTCTGGGAAGGATCAGCCTC AGGTGCGCTTGCTCCGTTGACAGGAGAGATGGATTCTGACAAAATCAGAAAGACAGCAAATACCGAAAAATCTACTCCTTTACTCTGAGT GAGGAAGAAATAGAAAAAGTGCTGAAAAATATATTTAATATTTCCTTGCAGAGAAAGCTTGTGCCCAAACATGGTGATAGATTTTT TACTATT |

|       | D I E A Y L E R I G Y K S R N K L D L E T L T D I L Q H Q I R A V P F E N L N I H C G D A M D L G L E A I F D Q V V R R N R G G W C L Q V N H L L Y W A L T I G F E T T M L G G Y V Y S T P A K K Y S T G M I H L Q V T I D G R N Y I V D A G F G R S Y Q M W Q P L E L I S G K D Q P Q V P C V F R L T E E N G F W Y L D Q I R E Q Y I P N E E F L H S D L L E D S K Y R K I Y S F T L K P R T I E D F E S M N T Y L Q T S P S S V F T S K S F C S L Q T P D G V H C L V G F T L T L T H R R F N Y K D N T D L I E F K T L S E E E I E K V L K N I F N I S L Q R K L V P K H G D R F F T I |
Overexpression and purification of H₆-hNat1

The gene encoding hNAT1 was cloned into a pET-30a plasmid containing a hexahistidine (His₆) tag. The pET30A-Nat expression vector was transformed into E. coli BL21 Rosetta(DE3) pLysS cells and selected for chloramphenicol and kanamycin resistance. A single colony was inoculated in LB-medium (100 mL) supplemented with chloramphenicol (25 µg mL⁻¹) and kanamycin (50 µg mL⁻¹) and grown overnight at 37 °C, 200 rpm. 20 mL of overnight culture was used to inoculate TB-medium (1 L) supplemented with kanamycin (50 µg mL⁻¹). Cultures were incubated at 37 °C until the optical density at 600 nm (OD₆₀₀) reached 0.6-0.8 (3-4 h). Protein expression was then induced by addition of 150 µL of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG; Fisher Scientific) for 16 h at 18 °C. Cells were harvested at 5,000 rpm for 20 min at 4 °C and the pellet was resuspended in 30 mL of ice cold lysis buffer (100 mM NaH₂PO₄ pH 7.8, 300 mM NaCl, 0.1 % w/v NP-40, and 1 mM dithiotheitol (DTT) and stored at -80 °C.

After the hNAT1 bacterial lysate was thawed at 37 °C, cells were further lysed by ultrasonic disruption (3× 30 sec) using a Soniprep 150 (MSE) with a 9.5 mm probe. The cell lysate was clarified by centrifugation (4 °C, 30 min, 13,000 rpm) and the supernatant was incubated with 500 µL Ni-NTA agarose beads (50 % suspension, GE Healthcare) for 1 h at 4 °C. The suspension was loaded onto a column and washed three times with 3 mL of wash buffer (100 mM NaH₂PO₄ pH 7.8, 300 mM NaCl, 0.1 % w/v NP-40, 1 mM DTT, and 10 mM imidazole). Subsequently, the protein was eluted in six fractions of 1 mL elution buffer (50 mM NaH₂PO₄ pH 7.8, 300 mM NaCl, 1 mM DTT, and 250 mM imidazole). All fractions of the affinity purification were analysed on SDS-PAGE gel by colloidal staining and western blot (Figure S1A and B). The western blot was stained against anti-His mouse (1:3,000; Amersham Pharmacia Biotech) and anti-hNAT rabbit (1:1,000; Abcam), visualized by goat anti-mouse IRDye 680 LT (1:5,000; Li-Cor) and goat anti-rabbit IRDye800 CW (1:5,000; Li-Cor), and measured on the Odyssey® CLx.

Finally, the elution fractions 1-6 were combined and dialyzed at 4 °C using Amicon® Ultra-15 Centrifugal Filter (10,000 NMWL; Merck Millipore) against storage buffer (20 mM Tris-HCl pH 7.8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT). Finally, the protein concentration was determined at 280 nm (ε = 44,600 M⁻¹ cm⁻¹) and concentrated to 1 mg mL⁻¹ using the centrifugal filters. Fractions of His-tagged hNAT1 were flash frozen at 1 mg mL⁻¹ with 5 % w/v glycerol and stored at -80 °C for maximal two months. The protein yield was about 2 mg hNAT L⁻¹ bacterial culture. The mass of the proteins was confirmed by electron spray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF with Agilent (1100 series HPLC). ESI-TOF: H₆-NAT 40,138 Da, found 40,138 Da (Figure S1C).
Figure S1 - His-tagged hNAT1 (40.1 kDa) was expressed in E. Coli and partly purified using Ni²⁺ affinity chromatography. lys = total lysate, l = insoluble fraction, S = soluble fraction, FT = flow through of Ni²⁺ NTA; b = residual proteins on Ni²⁺ NTA beads; Arrow indicates H₆-tagged hNAT1. A) SDS-PAGE analysis of the purification fractions stained by colloidal. B) Western blot of the purification fractions detected by histidine and NAT antibodies. C) ESI-TOF mass spectrometry of the eluted protein. Top) multiply charged ion series; Bottom) deconvoluted total mass spectrum.
In vitro protein labeling using purified Hs-hNAT1

Time labeling by purified hNAT1 was started by adding 2 µL of 1 mM probe (dissolved in DMSO) to 18 µL of Hs-hNAT1 enzyme (1 mg mL⁻¹). After incubation for different time points at 30 °C, the reaction was stopped by adding 2 µL MQ and 3 µL of blockers, containing 1 µL of 25 mM N-hydroxy-N-phenylacetamide (synthesized using a literature procedure; dissolved in DMSO), 1 µL of 250 mM aniline (diluted in DMSO), and 1 µL of 2,5 M imidazole (for a final concentration of 1 mM N-hydroxy-N-phenylacetamide, 10 mM aniline, and 100 mM imidazole).

Labeled proteins were visualized by the SPAAC reaction using dibenzocyclooctyne-Cyanine5.5 (DBCO-Cy5.5, Jena Bioscience). First, the reduced cysteines were alkylated by adding 3 µL of 500 mM iodoacetamide (prepared fresh, 50 mM final) and 2 µL MQ to the sample (final volume 30 µL) for 30 min on ice. Then, the SPAAC reaction was performed for 2 h on ice by adding 5 µL of 70 µM DBCO-Cy5.5 (dissolved in DMSO; 10 µM final) to the samples (total volume of 35 µL). Finally, 10 µL of 5× sample buffer and 5 µL of MQ was added to all samples for a total volume of 50 µL. Samples (5 µL) were directly loaded and analysed on 10 % SDS-PAGE (Figure 2B and S2). In-gel fluorescence of the SDS-PAGE was measured on the Odyssey® CLx followed by colloidal staining of the proteins.
**Figure S2** - *In vitro* protein labeling by Hs-hNAT1 using azide-functionalized probes 1-6 (100 µM) up to 8 hours at 30 °C followed by the SPAAC reaction using a DBCO-Cy5.5. A) Schematic representation. B) SDS-PAGE analysis of the Cy5.5 signal and stained using colloidal as a loading control. C) Analysis of time response of the three different reactive probes 1, 3 and 5 as shown in B. Error bars indicate standard deviation of three experiments using three purified Hs-hNAT1 protein batches.
Biotinylation of proteins by H$_6$-hNAT1 using probes 7 and 8

**Protein labeling**

Time labeling by purified hNAT1 was started by adding 2 µL of 0.5 mM probe (dissolved in DMSO) to 18 µL of H$_6$-hNAT1 enzyme (1 mg mL$^{-1}$). Protein labeling by purified hNAT1 followed by streptavidin enrichment was performed by adding 2 µL of 0.5, 5 or 50 mM probe (dissolved in DMSO). After incubation for different time points at 30 °C, the reaction was stopped by adding 2 µL MQ and 3 µL of blockers, containing 1 µL of 25 mM N-hydroxy-N-phenylacetamide (synthesized using a literature procedure$^6$; dissolved in DMSO), 1 µL of 250 mM aniline (diluted in DMSO), and 1 µL of 2.5 M imidazole (for a final concentration of 1 mM N-hydroxy-N-phenylacetamide, 10 mM aniline, and 100 mM imidazole).

Labeled proteins were visualized by SDS PAGE by adding 10 µL of 5× sample buffer and 15 µL of MQ to all samples for a total volume of 50 µL. Samples were boiled for 10 min at 98 °C and loaded on 10 % SDS-PAGE (5 µL) followed by western blot transfer. The protein load was visualized by Ponceau S (Bio-Rad) followed by blocking overnight at 4 °C in 3 % w/v BSA (1× PBS). Biotinylated proteins were visualized by streptavidin IRDye 800 CW (1:3,000; Li-Cor), and measured on the Odyssey® CLx (Figure S3B).

**Streptavidin enrichment**

After the biotinylation reaction by H$_6$-hNAT1, the samples were dialyzed to the streptavidin binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl) using amicon ultra spin filters with a cut-off of 10 kDa (Merck Milipore) and concentrated to 50 µL. Of the concentrated sample, 2.5 µL was taken and 2 µL of 5× sample buffer and 5.5 µL MQ was added (input samples on gel). In addition, 10 µL of streptavidin magnetic beads slurry (Thermo Fisher Scientific) were three times washed with the streptavidin binding buffer and diluted sample (300 µL) was added to the beads shaking for 1 h at room temperature. After the flow through was collected, it was concentrated using the amicon ultra spin filters to 50 µL and 10 µL of 5× sample buffer was added. The streptavidin beads were washed three times with 500 µL of streptavidin wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 M urea). To elute the proteins from the beads, 30 µL of 1× sample buffer was added. All samples were boiled for 10 min at 98 °C and loaded on 10 % SDS PAGE (5 µL of the flow through and 2.5 µL of the input and bead fractions) followed by silver staining to visualize the protein loading (Figure S3C).
Figure S3 - *In vitro* biotinylation by H6-hNAT1 using the active probe 7 (100 µM) followed by streptavidin enrichment. A) Schematic representation. B) Western blot analysis of the streptavidin signal and stained using Ponceau S as a loading control. C) SDS-PAGE of the pull down of biotinylated proteins after 1 h reaction by H6-hNAT1 using magnetic streptavidin beads visualized by silver staining. D) The relative amount of proteins binding to the beads compared to the input based on Figure S3C was estimated using ImageJ.
Labeling of the lysate by hNAT

Mammalian cell culture and transfections
The HEK-293T cell line (passage number < 25) was cultured in DMEM supplemented with 10 % v/v heat-inactivated fetal bovine serum (FBS), 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin at 37 °C under 5 % CO₂. Lysate labeling experiments were performed in 6-wells with 2 mL of 80,000 cells mL⁻¹. For imaging experiments, cells were grown on 10 × 10-mm glass coverslips in 48-wells (approximately 300 µL of 80,000 cells mL⁻¹). To improve the adherence of the HEK-293T cells, 6-wells and glass coverslips were pre-treated with poly-L-lysine (0.1 mg mL⁻¹; Sigma-Aldrich) for 5 min and washed three times with 1× PBS. Cells were transfected at 60-80 % confluency using standard TransX-T2® dynamic delivery system protocols (MHrus) with the NES-NA-hNAT1 and the NLS-NA-hNAT1 in a pcDNA3 vector. After 36 h transfection, cells were labeled and/or directly fixed followed by immune-staining.

Protein and lysate labeling by purified hNAT1
Protein labeling by purified hNAT1 was shown using proteins or the lysate: BSA (75 kDa; Sigma-Aldrich), FKBP (15 kDa)⁹, GFP (32 kDa; gift from the Biomolecular Chemistry department, Radboud University), and the protein lysate prepared by lysing HEK-293T cells in RIPA lysis buffer (5 mg mL⁻¹; Thermo Fisher Scientific). Proteins were mixed by adding 5 µL of H±hNAT1 enzyme (1 mg mL⁻¹) with 0.5 µL BSA (20 mg mL⁻¹), 5 µL GFP (10 µM), and 1 µL FKBP (5 mg mL⁻¹), or adding the enzyme to 13.5 µL of lysate (5 mg mL⁻¹). The reaction was started by adding 2 µL of 1 mM probe (dissolved in DMSO) and incubated for one hour at 30 °C. The reaction was stopped by blockers and visualized by the SPAAC reaction using similar conditions as described above (“In vitro protein labeling using purified His-tagged hNAT1” at p. S15) (Figure S4).

Protein labeling in living cells
HEK-293T cells were grown for 24-48 h on a pre-coated 6-well plate and protein labeling was initiated by changing the medium to 2 mL of pre-warmed culture medium containing 100 µM of the probe 1-6 (10 mM stock in DMSO) for a specific time at 37 °C under 5 % CO₂. Labeling was halted by three washes of 2 mL blocking solution, containing 4 µL of 500 mM N-hydroxy-N-phenylacetamide (dissolved in DMSO), 20 µL 1 M aniline (diluted in DMSO), and 200 µL of 1 M imidazole in 1× PBS (for a final concentration of 1 mM N-hydroxy-N-phenylacetamide, 10 mM aniline, and 100 mM imidazole). Next, cells were scraped in ice cold RIPA lysis buffer (Thermo Fisher Scientific), containing 1× protease inhibitor (Roche) and the three blockers (for a final concentration of 1 mM N-hydroxy-N-phenylacetamide, 10 mM aniline, and 100 mM imidazole). The protein load of the lysates was determined on SDS-PAGE (data not shown) by comparing the quantity of the lysates with a sample of HEK-293T cells that was not incubated with a probe and washed with 1× PBS instead of the blocking solution. The concentration of this control sample was measured by the Pierce™ BCA protein assay kit (Thermo Fisher Scientific) and the samples were diluted to 2 mg mL⁻¹.

SPAAC reaction with labeled lysates
Before the SPAAC reaction was started, lysates (20 µL of 2 mg mL⁻¹) were first alkylated for 30 min on ice by adding 5 µL of 250 mM iodoacetamide (prepared fresh, 50 mM final). Subsequently, 5 µL of 40 µM DBCO-Cy5.5 9 (dissolved in DMSO; 10 µM final) was added to perform the SPAAC reaction for 2 h on ice.

CuAAC reaction with labeled lysates
The CuAAC reaction was performed using Cyanine5.5-alkyne (Cy5.5-alkyne 10, Jena Bioscience). To the lysate (20 µL of 2 mg mL⁻¹), 8 µL of a CuAAC mastermix was added, consisting of 2 µL of 20 mM BTAA (Click Chemistry Tools), 1 µL of 10 mM CuSO₄, and 5 µL of 40 µM Cy5.5-alkyne 10 (dissolved in DMSO). The CuAAC reaction was started by adding 2 µL of 100 mM sodium ascorbate (prepared fresh). The final concentrations of the CuAAC reaction components in the lysates were 2 mM BTAA, 1 mM CuSO₄, 10 µM Cy5.5-alkyne 10, and 10 mM sodium ascorbate. The samples were incubated for 2 h on ice and the CuAAC reaction was stopped by adding 5 µL of 350 mM EDTA (50 mM final).
Preparing labeled samples for SDS PAGE

After both bioorthogonal reactions, 5× sample buffer and MQ were added to the samples for a total volume of 50 µL and the samples (15 µL) were directly loaded on SDS-PAGE. In-gel fluorescence of the SDS-PAGE was measured on the Odyssey® CLx followed by western blot. Blots were stained against anti-actin (1:7,500; Sigma-Aldrich), visualized by goat anti-mouse IRDye 800 CW (1:5,000; Li-Cor), and measured on the Odyssey® CLx (Figure 2C, S5, and S6).
**Figure S4** - *In vitro* protein or lysate labeling by H6-hNAT1 using the active probe 1 (100 μM) for 1 h at 30 °C followed by the SPAAC reaction using a DBCO-Cy5.5 9. A) Schematic representation. B) SDS-PAGE analysis of the Cy5.5 signal and stained using colloidal as a loading control.
Figure S5 - HEK-293T cells were incubated for one hour with the probes 1-6 (100 µM), followed by cell lysis and bioorthogonal click chemistry using ± 10 µg (non)-labeled lysate. A) SDS-PAGE analysis of (non)-labeled lysates that were visualized by either the CuAAC or SPAAC reaction using the Cy5.5-alkyne 10 or the DBCO-Cy5.5 9, respectively. B) SDS-PAGE analysis of the comparison of the lysates incubated with probes 1-6 visualized by the CuAAC reaction using Cy5.5-alkyne 10. The fluorescence of the SDS-PAGE of both A and B was measured (top) followed by western blot detected by actin, as a loading control (bottom). C) Line-scan analysis graph of Cy5.5-alkyne 10 fluorescence signal of lysates reacted with the active probes 1, 3, and 5 (from B).
**Figure S6** - Incubation of HEK-293T cells by either no probe (-), or the control probe 2 (100 µM), was performed for one hour, whereas labeling by the reactive probe 1 (100 µM) was performed for several time points. All lysates, non-labelled and labelled, were then clicked with Cy5.5-alkyne 10 using the CuAAC reaction and subsequently analyzed with SDS-PAGE. The fluorescence signal (top) was measured and the loading of the proteins was analyzed by western blot detected against actin (bottom).
HEK-293T immune-staining and confocal microscopy

The conditions for growing and transfecting HEK-293T cells is described above (‘Labeling of the lysate by endogenous hNAT’ at p. S22). (Non)-transfected HEK-293T cells were grown for 48 h on pre-coated coverslips in a 48-well plate (see protocol ‘Mammalian cell culture and transfections’ at p. S23). After discarding the growth medium, cells grown on coverslips were rinsed with 1× PBS followed by labeling (see protocol ‘Subcellular labeling for confocal microscopy’ at p. S23) and/or directly fixed for 15 min at room temperature using 500 µL 3.7 % v/v formaldehyde in 1× PBS. Cells were briefly washed with 1× PBS and permeabilized with 500 µL ice cold methanol for 5 min. After two brief washes with 1× PBS, cells were blocked with 500 µL of 3 % w/v BSA in 1× PBS overnight at 4 °C.

The primary antibodies were diluted in 3 % w/v BSA in 1× PBS, 1:1,000 for anti-HA mouse (Sigma-Aldrich) and 1:500 for anti-hNAT rabbit. The cells were incubated with 300 µl of the antibodies for 1 h at room temperature, followed by three washes with 1× PBS for of at least 5 min each. Secondary Alexa Fluor conjugated antibodies goat anti-mouse-633 and goat anti-rabbit-488 (Invitrogen) were diluted 1:300 in 3 % w/v BSA in 1× PBS. As well, 300 µl of the secondary antibodies was applied on the coverslips for 1 h at room temperature following three washes with 1× PBS of at least 5 min each. Next, cells were stained with 300 µL 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI, 1 µg mL⁻¹; Sigma-Aldrich) for 5 min in 1× PBS. Finally, coverslips were mounted using Mowiol®4-88 mounting medium (Sigma-Aldrich) and the samples were imaged on a Leica SP_8 confocal microscope 63× magnification with 1.0 digital zoom. Images were processed using ImageJ64 software (Figure S7).
HEK-293T cells were either not transfected or transfected with the NES-HA- or the NLS-HA-tagged hNAT1 for 36 hours. Cells were fixed, followed by immunostaining against the HA-tag (in red), against the hNAT enzyme (in green), and nuclear staining by DAPI (in grey). Background labeling by each secondary antibody was determined by single staining with this antibody only. In addition, NES-HA-tagged hNAT1 was as well stained only for HA or the hNAT enzyme to examine photobleaching of the fluorophores in each of the channels. Scale bar = 25 µm.

Figure S7 - HEK-293T cells were either not transfected or transfected with the NES-HA- or the NLS-HA-tagged hNAT1 for 36 hours. Cells were fixed, followed by immunostaining against the HA-tag (in red), against the hNAT enzyme (in green), and nuclear staining by DAPI (in grey). Background labeling by each secondary antibody was determined by single staining with this antibody only. In addition, NES-HA-tagged hNAT1 was as well stained only for HA or the hNAT enzyme to examine photobleaching of the fluorophores in each of the channels. Scale bar = 25 µm.
Subcellular labeling for confocal microscopy

Probe incubation of cells on coverslips
Transfected HEK-293T cells were grown for 48 h on pre-coated coverslips in a 48-well plate (see protocol mammalian cell culture and transfections). Next, the cells were incubated with 300 µL pre-warmed medium containing 100 µM of probe 1-6 (10 mM stock in DMSO) for a specific time at 37 °C under 5 % CO₂. Labeling was halted by three brief washes of 300 µL blocking solution, containing 0.6 µL of 500 mM N-hydroxy-N-phenylacetamide (dissolved in DMSO), 3 µL 1 M aniline (diluted in DMSO), and 30 µL of 1 M imidazole in 1× PBS (for a final concentration of 1 mM N-hydroxy-N-phenylacetamide, 10 mM aniline, and 100 mM imidazole).

Visualizing probe labeling
Then, the cells were fixed and permeabilized (as described below in immune fluorescence and confocal microscopy) and the CuAAC reaction was performed for 2 h at room temperature using 300 µL of CuAAC mix, containing 2 mM BTTAA, 1 mM CuSO₄, 10 µM rhodamine110-PEG₄-alkyne (Fluor488-alkyne 11, dissolved in DMSO, Sigma-Aldrich), and 10 mM sodium ascorbate (added last) in 1× PBS. The cells were washed two times with 1× PBS, once with PBST (1× PBS, 0.1 % w/v Tween-20 (MP Biomedicals), and once with 3 % w/v BSA in 1× PBS for at least 5 min. Finally, the HA-tag of the hNAT1 enzyme and the nucleus were visualized by immune fluorescence (see immune fluorescence and confocal microscopy for details) and samples were imaged on the confocal microscope (Figure 3, S8, S9 and S10).
Figure S8 - HEK-293T cells were transfected with either the NLS-HA- or the NES-HA-hNAT1 for 36 hours (A and B, respectively). Cells were incubated for one hour with no probe (-), reactive probe 1 (100 µM), or control probe 2 (100 µM), followed by fixation and the CuAAC reaction using the Fluor488-alkyne 11 (in green). hNAT1 was visualized by immunostaining against the HA-tag (in red) and the nuclei were stained using DAPI (in grey). Scale bar = 25 µm.
Figure S9 - NLS-HA-hNAT1 transfected HEK-293T cells were incubated with the probes 1-6 (100 µM) for one hour. After the reaction was stopped using blocking solution, cells were fixed and CuAAC was performed using Fluor488-alkyne 11 (shown in green). Finally, NLS-HA-tagged hNAT1 was visualized by immunostaining against the HA-tag (red) and the nuclei were stained using DAPI (grey). Scale bar = 25 µm.
Figure S10 - NLS-HA-hNAT1 transfected HEK-293T cells were incubated for different time points with the active probe 1 (100 µM) or for one hour with the control probe 2 (100 µM). After the reaction was stopped using blocking solution, cells were fixed and CuAAC was performed using Fluor488-alkyne 11 (shown in green). Finally, NLS-HA-tagged hNAT1 was visualized by immunostaining against the HA-tag (red). Scale bar = 25 µm.
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NMR spectra

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (125 MHz, CDCl$_3$)
$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
$^1$H NMR (400 MHz, 50 °C, (CD$_3$)$_2$SO)

$^{13}$C NMR (100 MHz, 50 °C, (CD$_3$)$_2$SO)
$^1$H NMR (500 MHz, CDCl$_3$)

$^{13}$C NMR (125 MHz, CDCl$_3$)
$^1$H NMR (500 MHz, 50 °C, CDCl$_3$)

$^{13}$C NMR (125 MHz, 50 °C, CDCl$_3$)
$^1$H NMR (500 MHz, (CD$_3$)$_2$SO)

$^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO)
$^1$H NMR (400 MHz, CDCl$_3$)

$^{13}$C NMR (100 MHz, CDCl$_3$)
$^1$H NMR (500 MHz, 50 °C, CDCl$_3$)

$^{13}$C NMR (125 MHz, 50 °C, CDCl$_3$)
$^1$H NMR (500 MHz, (CD$_3$)$_2$SO)

$^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO)
$^{1}\text{H NMR (500 MHz, CD$_3$OD)}$

$^{13}\text{C NMR (125 MHz, CD$_3$OD)}$
$^1$H NMR (500 MHz, CD$_3$OD)

$^{13}$C NMR (125 MHz, CD$_3$OD)
