

Supporting Information

Light-Activatable, 2,5-Disubstituted Tetrazoles for the Proteome-wide Profiling of Aspartates and Glutamates in Living Bacteria

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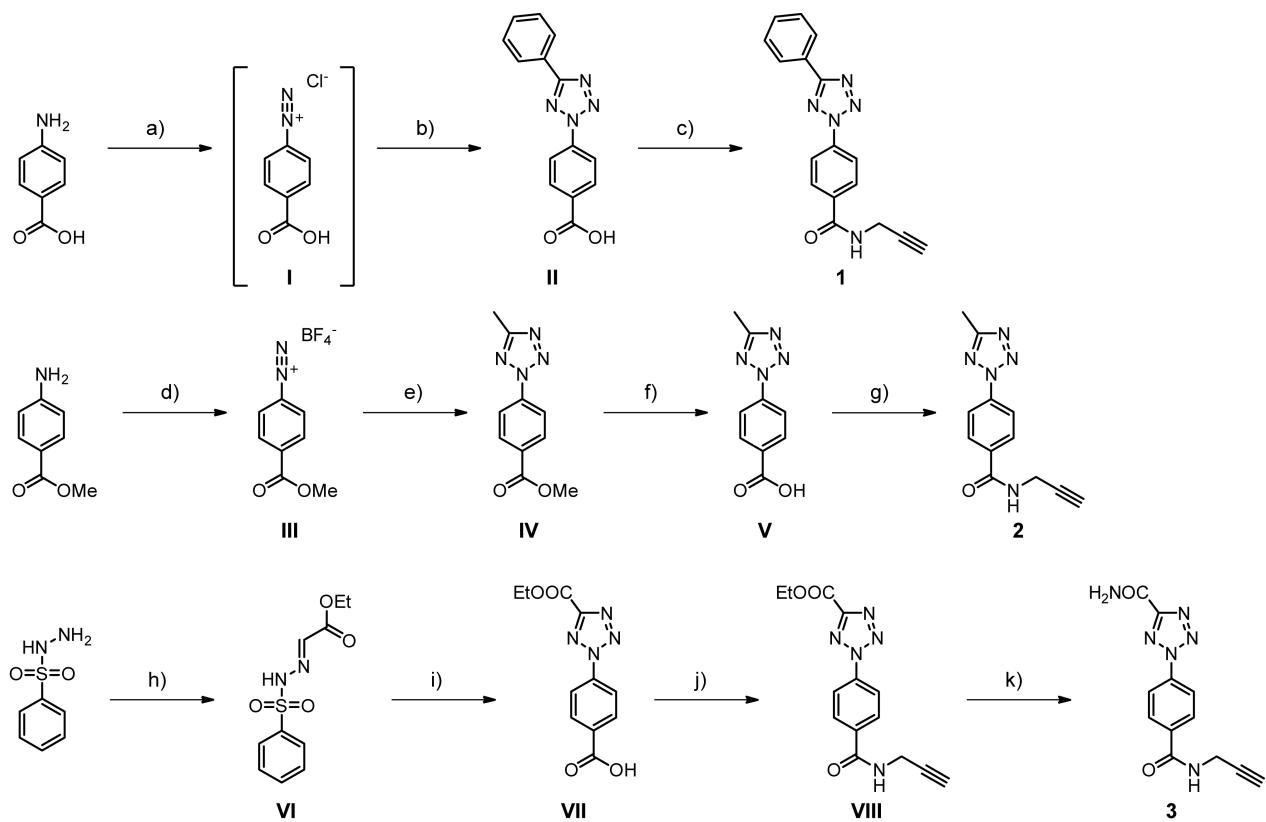
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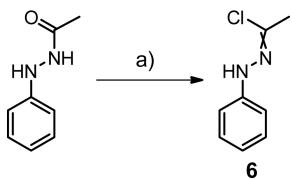
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Supporting Schemes



Scheme S1. Synthesis of probes **1**, **2** and **3**. Conditions: a) HCl, NaNO₂ (H₂O/EtOH). b) Pyridine, PhCH=NNTs (H₂O/EtOH), 55% over two steps. c) NEt₃, EDC·HCl, HOBT·H₂O, propargylamine (DMF), 37%. d) HBF₄, NaNO₂ (H₂O). e) Acetamidine hydrochloride, K₂CO₃, then I₂, KI (DMSO), 46% over two steps. f) NaOH (H₂O/MeOH). g) NEt₃, EDC·HCl, HOBT, propargylamine (DMF), 99% over two steps. h) Ethyl glyoxylate (EtOH/PhMe). i) Pyridine, **I** (H₂O/EtOH), 23% over two steps. j) NEt₃, EDC·HCl, HOBT·H₂O, propargylamine (DMF), 29%. k) NH₄OH (H₂O/THF), 70%.



Scheme S2. Synthesis of compound **6**. Conditions: a) POCl₃ (PhMe), 85%.

Supporting Figures

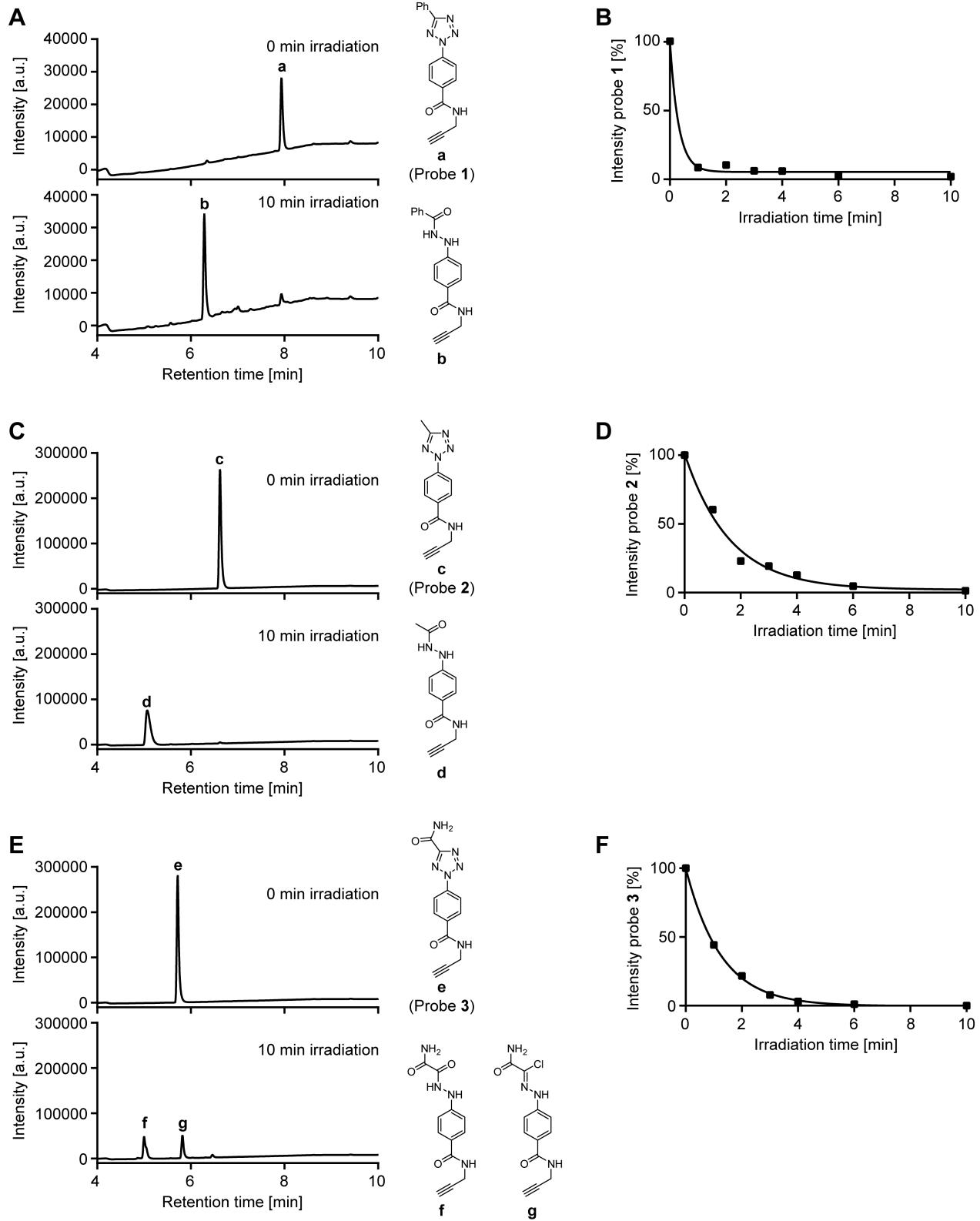


Figure S1. Study of the activation of probes **1**, **2** and **3** by UV light. 100 μ M of the respective probe in PBS were irradiated for the indicated amount of time and the reactions were analyzed by LC-MS. (A,C,E) Chromatograms of the LC-MS analysis monitored with UV absorption at 254 nm. The structures of the respective detected compounds are depicted. The following masses were measured: **a**: detected mass: 304.1194 Da, expected mass: 304.1198 Da; **b**: detected mass: 294.1238 Da, expected mass: 294.1243 Da; **c**: detected mass: 242.1037 Da, expected mass: 242.1042 Da; **d**: detected mass: 232.1081 Da, expected mass: 232.1086 Da; **e**: detected mass: 271.0940 Da, expected mass: 271.0943 Da; **f**: detected mass: 261.0983 Da, expected mass: 261.0988 Da; **g**: detected mass: 279.0644 Da, expected mass: 279.0649 Da. (B,D,F) Quantification of the remaining intensity of the respective probe monitored at 254 nm.

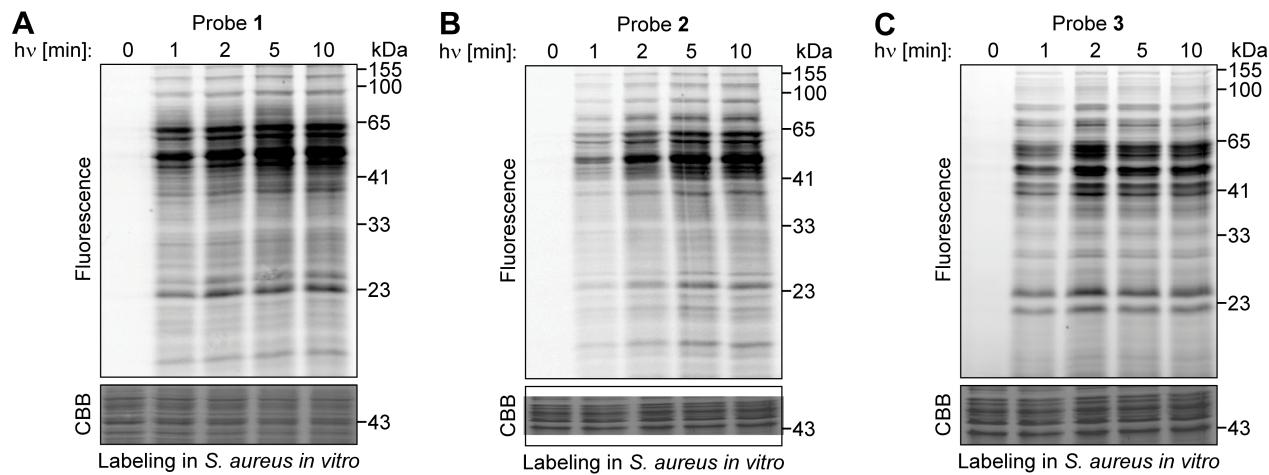


Figure S2. Optimization of the irradiation time for probes **1** (A), **2** (B) or **3** (C) by gel-based analysis. *S. aureus* lysate was treated with 100 μ M of the indicated probe, incubated for 30 min, irradiated with light ($\lambda = 280\text{--}315\text{ nm}$) for the indicated time and labeled with TAMRA-azide using CuAAC. Gel-based analysis was performed with in-gel fluorescence scanning and staining using Coomassie Brilliant Blue (CBB).

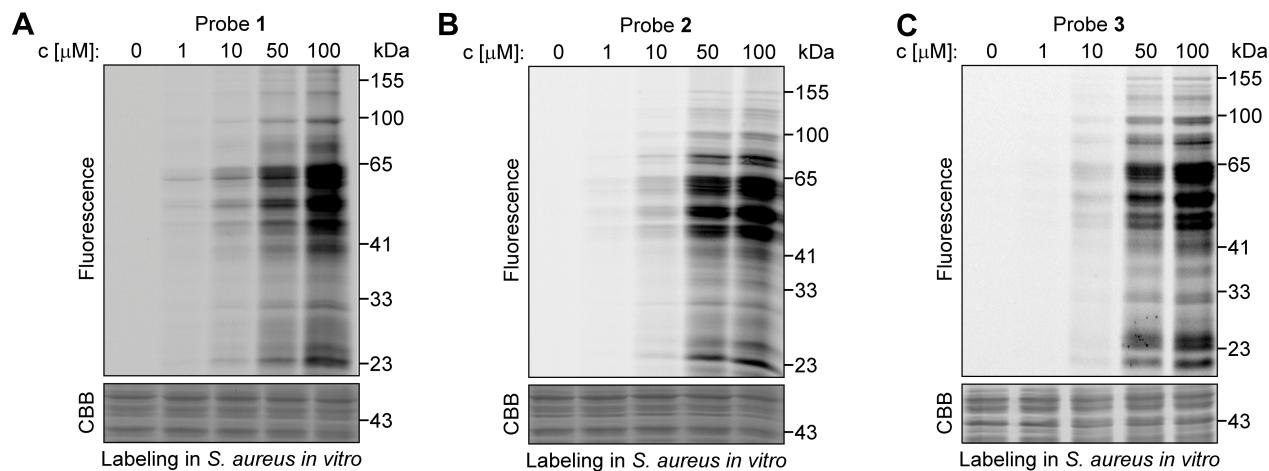


Figure S3. Optimization of the probe concentration for probes **1** (A), **2** (B) or **3** (C) by gel-based analysis. *S. aureus* lysate was treated with the indicated probe at the indicated concentration, incubated for 30 min, irradiated with light ($\lambda = 280\text{--}315\text{ nm}$) for 10 min and labeled with TAMRA-azide using CuAAC. Gel-based analysis was performed with in-gel fluorescence scanning and staining using Coomassie Brilliant Blue (CBB).

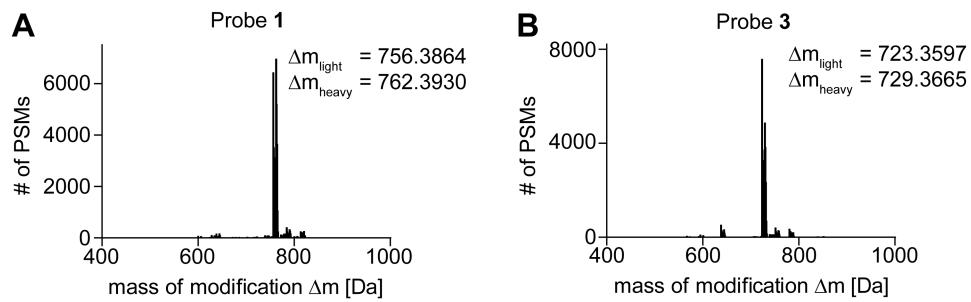


Figure S4. Analysis of the mass of modification on tryptic peptides after labeling of *S. aureus* lysate with 100 μM probe **1** (A) or **3** (B). MSFragger software^[1] was used to determine, which masses of modification occur in the proteomic samples labeled with probe **1** or **3** after light-activation and CuAAC to the light and heavy isoDTB tags. Expected masses of modification for tryptic peptides labeled with **1** according to the reactivity shown in Figure 1C and additionally modified with light or heavy isoDTB tag, respectively, are 756.3820 Da and 762.3895 Da. For **3**, the expected masses are 723.3565 Da and 729.3640 Da. PSM: peptide-spectrum match. Data results from biologically independent duplicates of technical duplicates.

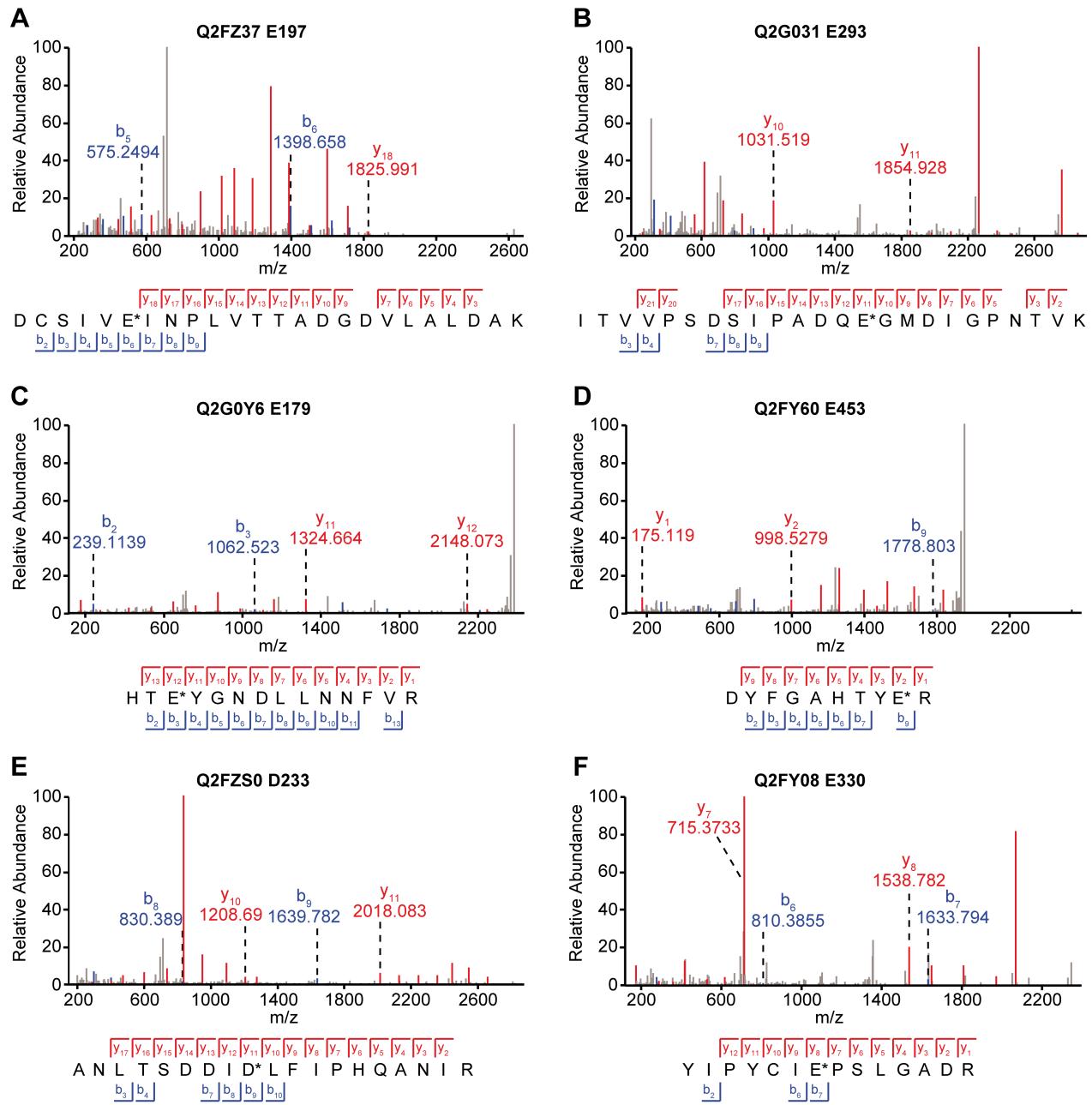


Figure S5. Selected annotated MS2 spectra of peptides modified at aspartates or glutamates that are at functional sites in essential proteins. All assigned b-ions are highlighted in blue and all assigned y-ions are highlighted in red. Fragments that are key to the annotation of the modification site (heaviest expected b- or y-ion without the modification, lightest expected b- or y-ion with the modification) are labeled with the assignment of the respective ion and the mass of the fragment in Da. Peptide sequences are given from *N*- to *C*-terminus with the modified amino acid marked as D* or E*, respectively. All assigned b- and y-ions are indicated above and below the peptide sequence.

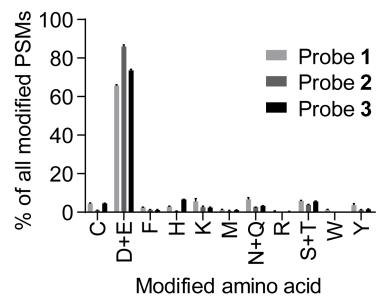


Figure S6. Analysis of the amino acid specificity of the probes. Proteomes labeled with the indicated probe after light-activation and modified by CuAAC with the light and heavy isoDTB tags were analyzed with MSFragger software^[1] allowing the modification on any potentially nucleophilic amino acid. Data shows the mean \pm the standard deviation. PSM: peptide-spectrum match.

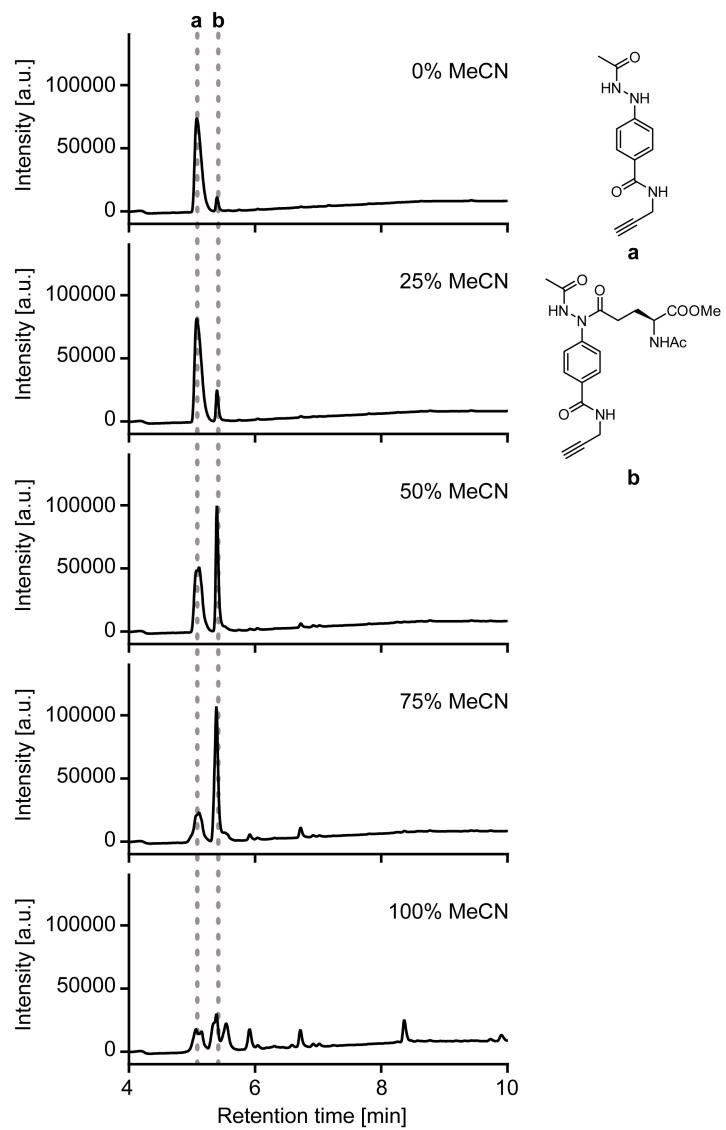


Figure S7. Study of the reaction of probe **2** with $\text{Na}\text{-acetyl}$ and $\text{Ca}\text{-methyl ester}$ protected glutamate after activation by UV light in different mixtures of acetonitrile and PBS. $100 \mu\text{M}$ of the probe **2** and 5 mM of the amino acid were irradiated for 10 min and the reactions were analyzed by LC-MS. Shown are chromatograms of the LC-MS analysis monitored with UV absorption at 254 nm . The structures of the respective detected compounds are depicted. The following masses were measured: **a**: detected mass: 232.1082 Da , expected mass: 232.1086 Da ; **b**: detected mass: 417.1771 Da , expected mass: 417.1774 Da .

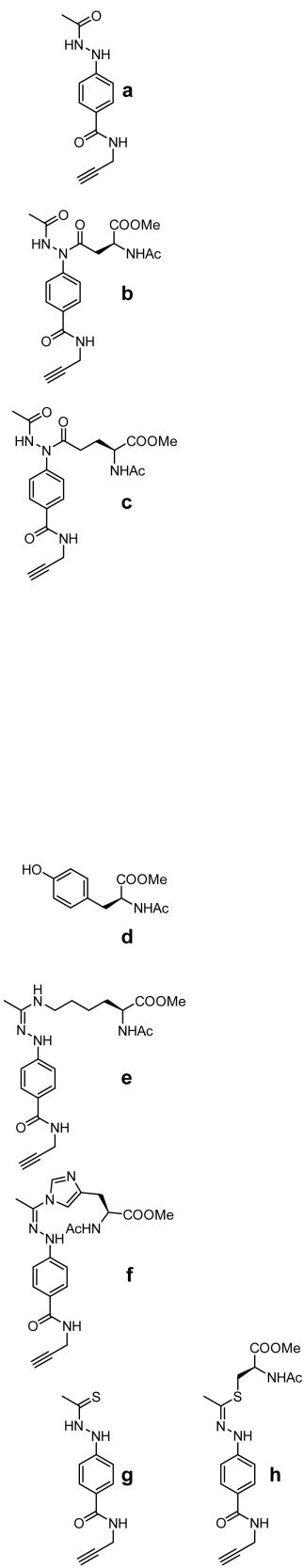
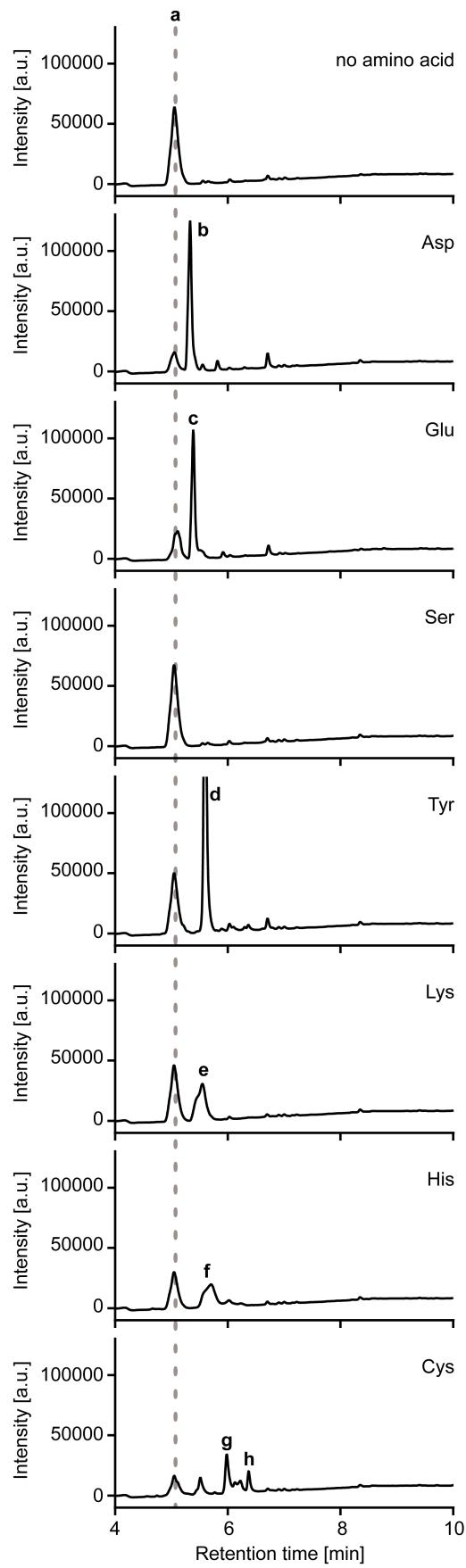


Figure S8. Study of the reaction of probe **2** with different *N* α -acetyl and *C* α -methyl ester protected amino acids after activation by UV light in 75% acetonitrile in PBS. 100 μ M of the probe **2** and 5 mM of the amino acid were irradiated for 10 min and the reactions were analyzed by LC-MS. Shown are chromatograms of the LC-MS analysis monitored with UV absorption at 254 nm. The structures of the respective detected compounds are depicted. The following masses were measured: **a**: detected mass: 232.1082 Da, expected mass: 232.1086 Da; **b**: detected mass: 403.1609 Da, expected mass: 403.1618 Da; **c**: detected mass: 417.1771 Da, expected mass: 417.1774 Da; **d**: detected mass: 238.1075 Da, expected mass: 238.1079 Da; **e**: detected mass: 416.2295 Da, expected mass: 416.2298 Da; **f**: detected mass: 425.1935 Da, expected mass: 425.1937 Da; **g**: detected mass: 248.0854 Da, expected mass: 248.0852 Da; **h**: detected mass: 391.1431 Da, expected mass: 391.1440 Da. Peak **d** for tyrosine-treatment was cut for clarity. It has a total intensity of \sim 300,000.

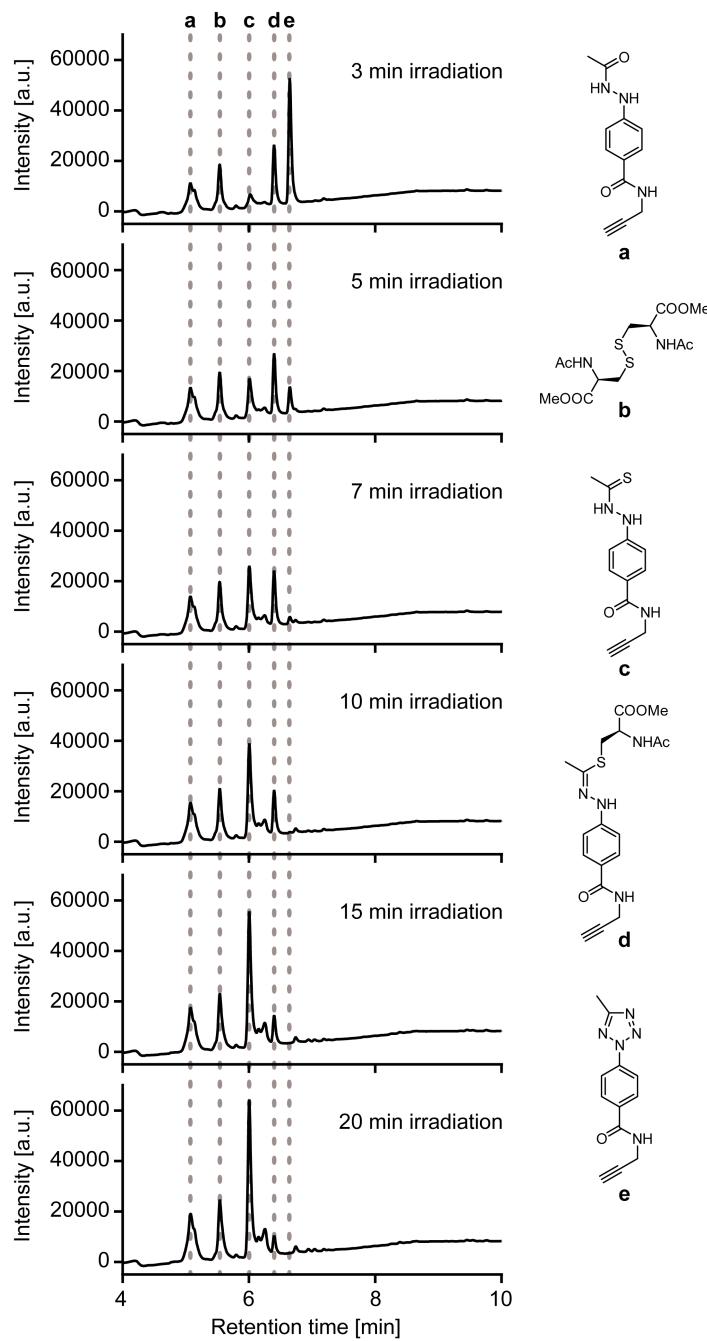


Figure S9. Study of the reaction of probe **2** with *Na*-acetyl and *Ca*-methyl ester protected cysteine after activation by UV light for the indicated times in 75% acetonitrile in PBS. 100 μ M of the probe **2** and 5 mM of the amino acid were irradiated and the reactions were analyzed by LC-MS. Shown are chromatograms of the LC-MS analysis monitored with UV absorption at 254 nm. The structures of the respective detected compounds are depicted. The following masses were measured: **a**: detected mass: 232.1081 Da, expected mass: 232.1086 Da; **b**: detected mass: 353.0838 Da, expected mass: 353.0841 Da; **c**: detected mass: 248.0852 Da, expected mass: 248.0858 Da; **d**: detected mass: 391.1436 Da, expected mass: 391.1440 Da; **e**: detected mass: 242.1037 Da, expected mass: 242.1042 Da.

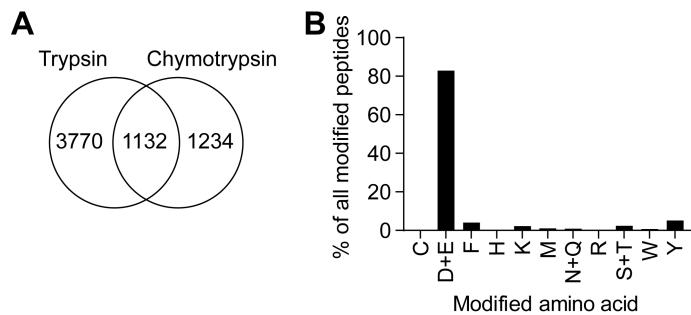


Figure S10. Proteolytic digestion with chymotrypsin can be used to increase coverage of aspartates and glutamates with retained good selectivity. (A) Venn diagram of the number of quantified aspartates and glutamates with probe **2** and proteolytic digestion with trypsin or chymotrypsin. (B) Analysis of the amino acid specificity of probe **2** with proteolytic digestion with chymotrypsin. *S. aureus* lysates labeled with probe **2** after light-activation and modified by CuAAC with the light and heavy isoDTB tags were analyzed with MaxQuant software^[2] allowing the modification on any potentially nucleophilic amino acid. Peptides were included in the analysis if the localization probability for a single residue was more than 75%. A total of 1276 peptides were analyzed for chymotrypsin digestion. Data for trypsin digestion results from biologically independent duplicates of technical duplicates and data for chymotrypsin results from duplicates.

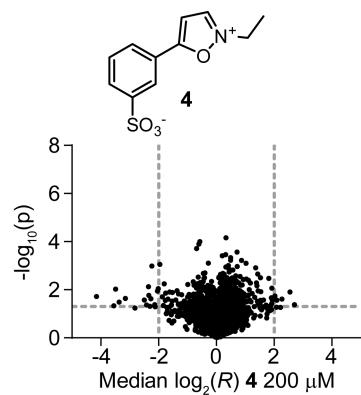


Figure S11. Probe **2** reveals targets of carboxylic acid-directed covalent protein ligands proteome-wide using isoDTB-ABPP *in vitro*. Volcano plot of isoDTB-ABPP experiments comparing samples pretreated with 200 μ M of covalent ligand **4** to a solvent control (1% HCl). Plotted are the ratio ($\log_2(R)$) between the heavy (solvent-treated) and light (compound-treated) labeled samples and the probability in a one-sample t-test that R is equal to one ($-\log_{10}(p)$). Data results from duplicates.

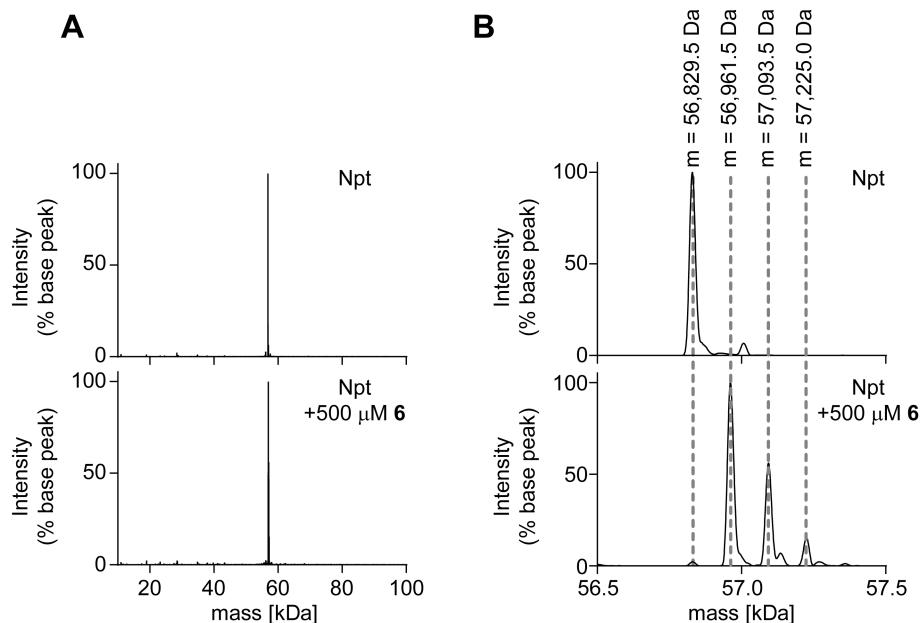


Figure S12. Competitor **6** modifies recombinant nicotinate phosphoribosyltransferase (Npt) identified as competed by isoDTB-ABPP with a mass of modification that is consistent with nitrilimine formation as an intermediate. For this experiment, recombinant nicotinate phosphoribosyl-transferase with and without pretreatment with 500 μ M **6** was analyzed by intact protein MS (IPMS). A) Full deconvoluted spectra. B) Zoom into the region of the base peak. The mass of m=56,829.5 Da corresponds to the mass of the Npt construct with a His-tag without the initial methionine (expected mass: m = 56,829.3 Da). The masses observed after treatment ($\Delta m = 132$ Da, $\Delta m = 264$ Da and $\Delta m = 295.5$ Da) directly correspond to the expected values for single, double and triple modification of Npt by **6** according to the mechanism shown in Figure 1C.

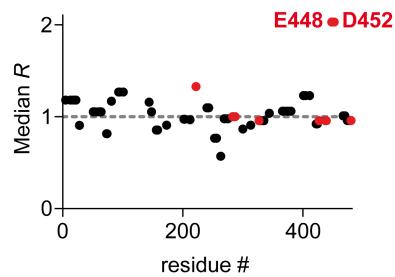
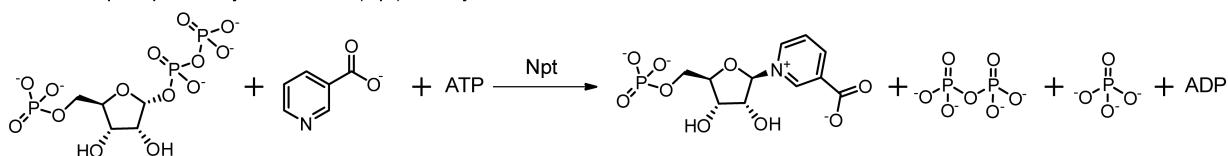
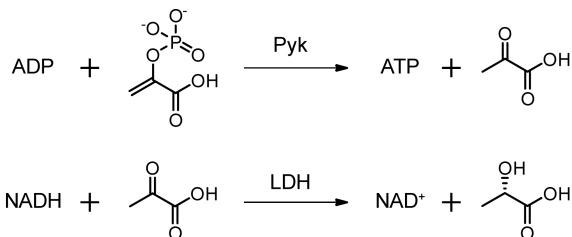


Figure S13. Competitor **6** modifies aspartate D452 of nicotinate phosphoribosyltransferase (Npt) identified as competed by isoDTB-ABPP with a mass of modification that is consistent with nitrilimine formation as an intermediate. For this experiment, recombinant nicotinate phosphoribosyltransferase was pretreated with 500 μ M **6** or DMF as a control, digested, labeled with reductive dimethyl labeling (heavy for the DMF control, light for the compound labeled sample), combined and analyzed by LC-MS/MS.^[3] R values give the ratio between the heavy and the light channel for unmodified peptides. For each residue, the median of all R values for all peptides containing this residue is shown. Only data for aspartate and glutamate residues is shown. All residues, for which we also detect the **6**-modified peptide in the light channel with a mass of modification that is consistent with the mechanism for reactivity after nitrilimine formation shown in Figure 1C are highlighted in red. The residue D452 that is detected as competed by isoDTB-ABPP and the residue E448 that always occurs in all peptides containing D452 and, therefore, has the same ratio R , are additionally labeled. Data results from duplicates.

A Nicotinate phosphoribosyltransferase (Npt) activity



Coupled enzyme reactions for detection (consumption of NADH is monitored by its absorption at 340 nm)



B

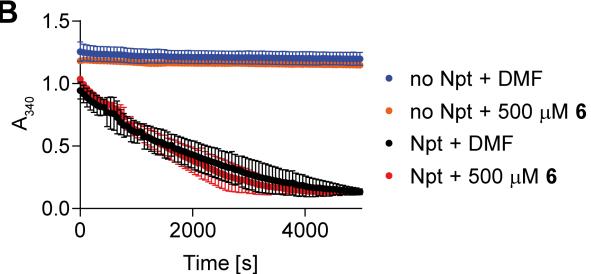


Figure S14. Competitor **6** does not inhibit the enzymatic activity of nicotinate phosphoribosyltransferase (Npt). (A) Concept for the detection of Npt activity. Npt catalyzes the condensation of 5-phospho- α -D-ribose-1-diphosphate with niacin to give nicotinate- β -D-ribonucleotide with simultaneous consumption of adenosine triphosphate (ATP) liberating adenosine diphosphate (ADP), pyrophosphate and phosphate. Liberated ADP is detected by re-phosphorylation with phosphoenolpyruvate catalyzed by pyruvate kinase (Pyk), which results in ATP and pyruvate. Pyruvate is reduced by lactate dehydrogenase (LDH), which utilizes NADH and results in the formation of NAD^+ and lactate. Consumption of NADH is monitored by its absorption at 340 nm. (B) Npt was pretreated with 500 μM **6** or DMF as a control for 3 h at room temperature. For no Npt controls, only the storage buffer of Npt was pretreated with **6** or DMF. The reaction was started by addition of niacin, 5-phospho- α -D-ribose-1-diphosphate and ATP for the Npt reaction as well as phosphoenolpyruvate, pyruvate kinase (from rabbit), NADH and lactate dehydrogenase (from rabbit) for detection. The depletion of NADH is measured by its absorbance at 340 nm (A_{340}). Data shows the mean \pm the standard deviation. Data results from triplicates.

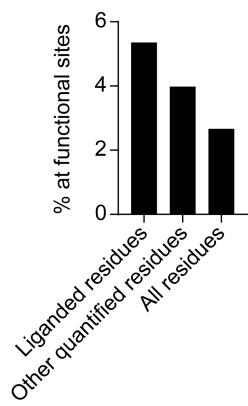


Figure S15. Percentage of aspartates and glutamates that are at functional sites. Aspartates and glutamates are grouped into three categories: liganded residues: aspartates and glutamates that are quantified in our data and engaged ($\log_2(R) > 2$ and $p < 0.05$) by at least one compound; other quantified residues: aspartates and glutamates that are quantified in our data and not engaged by any compound; all residues: all aspartates and glutamates that are present in the *S. aureus* SH1000 genome according to the used FASTA database.

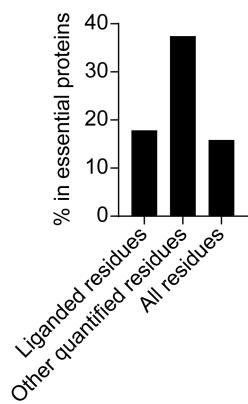


Figure S16. Percentage of aspartates and glutamates that are in essential proteins according to the database of essential genes (DEG database).^[4] Aspartates and glutamates are grouped into three categories: liganded residues: aspartates and glutamates that are quantified in our data and engaged ($\log_2(R) > 2$ and $p < 0.05$) by at least one compound; other quantified residues: aspartates and glutamates that are quantified in our data and not engaged by any compound; all residues: all aspartates and glutamates that are present in the *S. aureus* SH1000 genome according to the used FASTA database.

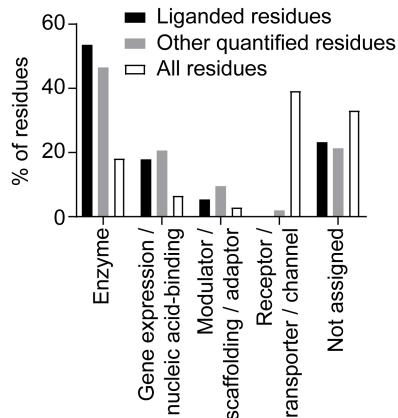


Figure S17. Distribution of aspartates and glutamates into the different functional classes of proteins. Functional classes were derived from Gene Ontology terms^[5] reported in UniProt.^[6] Aspartates and glutamates are grouped into three categories: liganded residues: aspartates and glutamates that are quantified in our data and engaged ($\log_2(R) > 2$ and $p < 0.05$) by at least one compound; other quantified residues: aspartates and glutamates that are quantified in our data and not engaged by any compound; all residues: all aspartates and glutamates that are present in the *S. aureus* SH1000 genome according to the used FASTA database.



Figure S18. The different competitors **4**, **5** and **6** engage diverse aspartates and glutamates. Heat map of the $\log_2(R)$ values for the different competitors at 500 μM concentration for all residues that have $\log_2(R) > 2$ for at least one competitor at 500 μM .

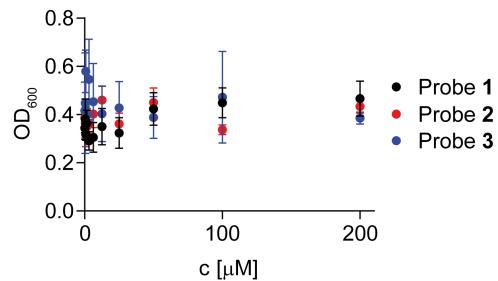


Figure S19. Probes **1**, **2** and **3** show no antibacterial activity in *S. aureus*. MIC experiments were performed by diluting an overnight culture of *S. aureus* SH1000 1:10,000 into fresh B medium and treating this solution with the indicated probe at the indicated concentration overnight at 37 °C with shaking at 200 rpm. Bacterial growth was analyzed by eye and by measuring the optical density at 600 nm (OD₆₀₀) as a measure for turbidity of the bacterial suspension. Data shows mean \pm standard deviation of duplicates.

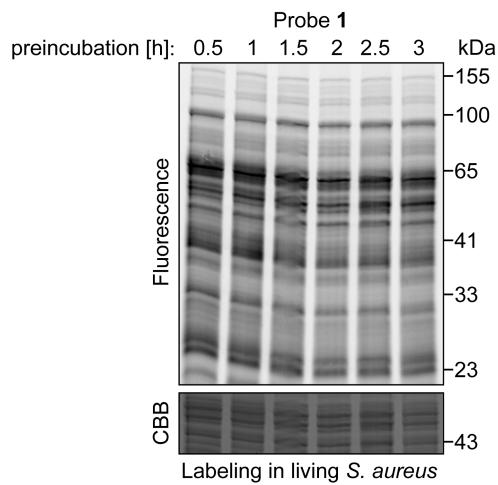


Figure S20. Optimization of the preincubation time for probe **1**, when labeling living *S. aureus*, by gel-based analysis. Living *S. aureus* were treated with 100 μ M probe **1**, incubated for the indicated time at 37 °C with shaking at 200 rpm, irradiated with light ($\lambda = 280\text{--}315$ nm), lysed and labeled with TAMRA-azide using CuAAC. Gel-based analysis was performed with in-gel fluorescence scanning and staining using Coomassie Brilliant Blue (CBB).

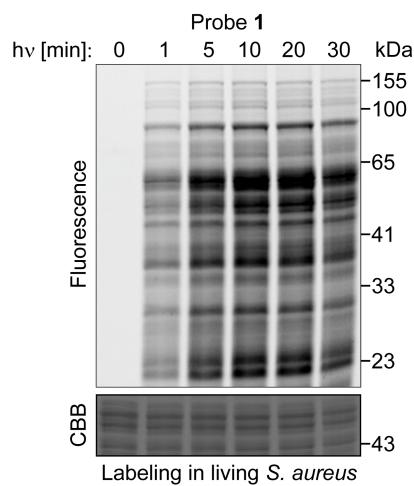


Figure S21. Optimization of the irradiation time for probe **1**, when labeling living *S. aureus*, by gel-based analysis. Living *S. aureus* were treated with 100 μ M probe **1**, incubated for 1 h, irradiated with light ($\lambda = 280\text{--}315\text{ nm}$) for the indicated time, lysed and labeled with TAMRA-azide using CuAAC. Gel-based analysis was performed with in-gel fluorescence scanning and staining using Coomassie Brilliant Blue (CBB).

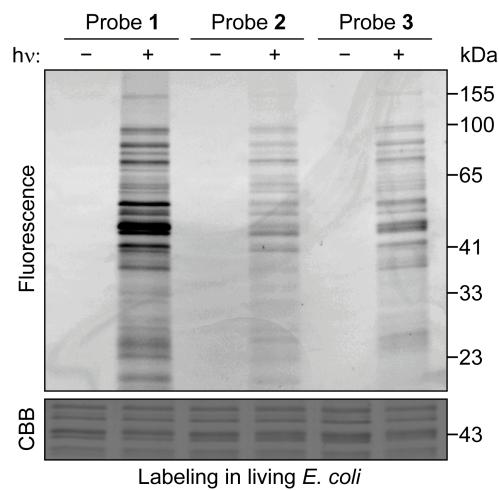


Figure S22. Labeling with the probes **1**, **2** and **3** in living *E. coli* evaluated by gel-based analysis. Living *E. coli* were treated with 100 μ M probe **1**, **2** or **3**, incubated for 1 h, irradiated with light ($\lambda = 280\text{--}315\text{ nm}$), lysed and labeled with TAMRA-azide using CuAAC. Gel-based analysis was performed with in-gel fluorescence scanning and staining using Coomassie Brilliant Blue (CBB).

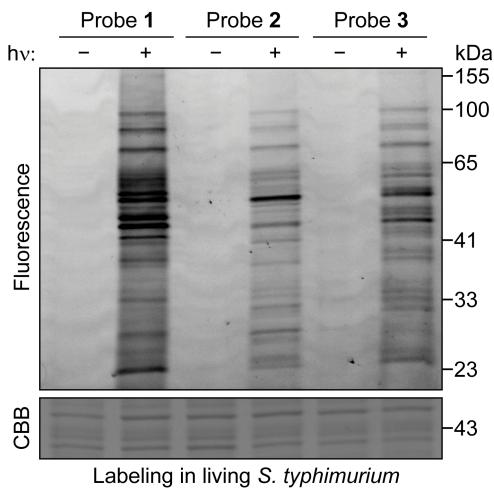


Figure S23. Labeling with the probes **1**, **2** and **3** in living *S. typhimurium* evaluated by gel-based analysis. Living *S. typhimurium* were treated with 100 μ M probe **1**, **2** or **3**, incubated for 1 h, irradiated with light ($\lambda = 280$ –315 nm), lysed and labeled with TAMRA-azide using CuAAC. Gel-based analysis was performed with in-gel fluorescence scanning and staining using Coomassie Brilliant Blue (CBB).

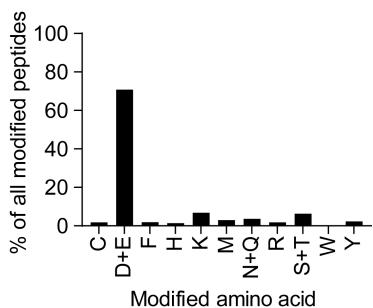


Figure S24. Probe **1** shows good selectivity for aspartates and glutamates in living *E. coli*. Analysis of the amino acid specificity of probe **1**, when living *E. coli* are labeled. Proteomes labeled with probe **1** after light-activation and modified by CuAAC with the light and heavy isoDTB tags were analyzed with MaxQuant software^[2] allowing the modification on any potentially nucleophilic amino acid. Peptides were included in the analysis if the localization probability for a single residue was more than 75%. A total of 1422 peptides were analyzed. Data results from triplicates.

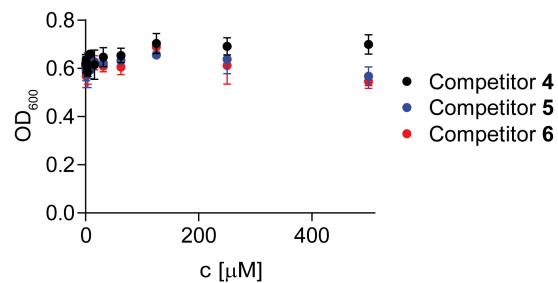


Figure S25. Competitors **4**, **5** and **6** show no antibacterial activity in *S. aureus*. MIC experiments were performed by diluting an overnight culture of *S. aureus* SH1000 1:10,000 into fresh B medium and treating this solution with the indicated competitor at the indicated concentration overnight at 37 °C with shaking at 200 rpm. Bacterial growth was analyzed by eye and by measuring the optical density at 600 nm (OD₆₀₀) as a measure for turbidity of the bacterial suspension. Data shows mean \pm standard deviation of duplicates.

Supporting Tables

Table S1. isoDTB-ABPP data for the evaluation of the mass of modification, selectivity, quantification and sensitivity for probes **1**, **2** and **3** *in vitro* in *S. aureus*, in living *S. aureus* and in living *E. coli*. This data can be found as an additional data file accompanying the manuscript.

Table S2. Competitive isoDTB-ABPP data for the competitors **4**, **5** and **6** *in vitro* in *S. aureus* as well as of reductive dimethyl labeling experiments to establish and quantify the site of labeling on recombinant nicotinate phosphoribosyltransferase. This data can be found as an additional data file accompanying the manuscript.

Table S3. Bacterial strains used in this study

| Strain Name | Systematic Name | Growth Media |
|--|-----------------|-------------------|
| <i>Staphylococcus aureus</i> SH1000 ^[a] | - | B ^[b] |
| <i>Salmonella enterica</i> subsp. <i>enterica</i> Serovar <i>typhimurium</i> LT2 (<i>Salmonella typhimurium</i>) | DSM 17058 | LB ^[c] |
| <i>Escherichia coli</i> | ATCC 25922 | LB ^[c] |

[a] *Staphylococcus aureus* SH1000 was a kind gift from Simon J. Foster, The Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield.^[7] [b] B medium: 10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract, 1 g/L K₂HPO₄. [c] LB medium: 10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract, pH = 7.5.

Table S4. FASTA databases downloaded from uniprot.org^[6] for this study.

| Strain Name | Search at uniprot.org ^[6] | # of Proteins in FASTA | Date |
|--|--|------------------------|------------|
| <i>Staphylococcus aureus</i> SH1000 ^[7] | "93061" as "Taxonomy [OC]" at UniProtKB ^[a] | 2959 | 27.02.2018 |
| <i>Escherichia coli</i> K12 | "UP000000625" in Proteomes | 4436 | 04.02.2019 |
| <i>Escherichia coli</i> ATCC 25922 | "UP000028955" in Proteomes | 4790 | 29.01.2019 |

[a] The downloaded FASTA file corresponds to the *S. aureus* strain NCTC8325. Comparative sequencing of these two strains has been reported^[8] and the respective changes have been made to the FASTA file. These include several point mutations, the deletion of the partial proteins Q2FWJ0 and Q2FWJ1 as well as the addition of the rsbU gene from *S. aureus* Newman (A0A0H3KE27).

Table S5. Unmodified and modified FASTA databases used in this study. All FASTA databases have been deposited to the ProteomeXchange Consortium^[9] via the PRIDE partner repository^[10] with the dataset identifier PXD016659.

| Strain Name | Name of the FASTA Database ^[a] |
|--|---|
| <i>Staphylococcus aureus</i> SH1000 ^[7] | SA59.fasta ^[a] |
| <i>Staphylococcus aureus</i> SH1000 ^[7] | SA59_DO_EU.fasta ^[b] |
| <i>Staphylococcus aureus</i> SH1000 ^[7] | SA59_plus_rev.fasta ^[c] |
| <i>Escherichia coli</i> ATCC 25922 | EC60.fasta ^[a] |
| <i>Escherichia coli</i> ATCC 25922 | EC60_DO_EU.fasta ^[b] |
| <i>Escherichia coli</i> K12 | EC33-2_plus_Q2G235.fasta ^[d] |

[a] Names consist of the first letters of the genus and the species of the respective strain and the number of the strain in our internal database for the unmodified FASTA databases. [b] Names consist of the first letters of the genus and the species of the respective strain and the number of the strain in our internal database. This is followed by “_DO_EU” as an indication that glutamates were individually changed to “U” and aspartates were individually changed to “O” as a placeholder. [c] Names consist of the first letters of the genus and the species of the respective strain and the number of the strain in our internal database for the unmodified FASTA databases. This is followed by “_plus_rev” if the reverse sequences were manually included as needed for MSFragger analysis.^[1] [d] Names consist of the first letters of the genus and the species of the respective strain and the number of the strain in our internal database for the unmodified FASTA databases. In this case, the protein nicotinate phosphoribosyltransferase (UniProt code Q2G235) was manually added to the FASTA database of *E. coli* K12 as it was recombinantly expressed in *E. coli*.

Table S6. All samples analyzed by isoDTB-ABPP or by reductive dimethyl labeling of nicotinate phosphoribosyltransferase in this study. All Data files have been deposited to the ProteomeXchange Consortium^[9] via the PRIDE partner repository^[10] with the dataset identifier PXD016659.

| Name of the Sample | Name of the Data File ^[a] | Description of the Sample |
|--------------------|--------------------------------------|---|
| 190124_P1-1 | 190125_KAB_190124_1_1.raw | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 1 |
| 190124_P1-2 | 190125_KAB_190124_1_2.raw | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 2 |
| 190222_P1-1 | 190301_KAB_190222_P1_1.raw | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 3 ^[b] |
| 190222_P1-2 | 190301_KAB_190222_P1_2.raw | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 4 ^[b] |
| 190124_P2-1 | 190125_KAB_190124_2_1.raw | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 1 |
| 190124_P2-2 | 190125_KAB_190124_2_2.raw | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 2 |
| 190222_P2-1 | 190301_KAB_190222_P2_1.raw | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 3 ^[b] |
| 190222_P2-2 | 190301_KAB_190222_P2_2.raw | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 4 ^[b] |
| 190124_P3-1 | 190125_KAB_190124_3_1.raw | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 1 |
| 190124_P3-2 | 190125_KAB_190124_3_2.raw | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 2 |
| 190222_P3-1 | 190301_KAB_190222_P3_1.raw | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 3 ^[b] |

| | | |
|-------------|----------------------------|--|
| 190222_P3-2 | 190301_KAB_190222_P3_2.raw | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000, replicate 4 ^[b] |
| 190614_P4-1 | 190615_KAB_190614_P4_1.raw | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 with chymotrypsin digestion, replicate 1 |
| 190614_P4-2 | 190615_KAB_190614_P4_2.raw | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 with chymotrypsin digestion, replicate 2 |
| 190614_P4-3 | 190615_KAB_190614_P4_3.raw | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 with chymotrypsin digestion, replicate 3 |
| 190222_PA-1 | 190301_KAB_190222_PA_1.raw | Competitive experiment with 500 μ M 4 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 1 |
| 190222_PA-2 | 190301_KAB_190222_PA_2.raw | Competitive experiment with 500 μ M 4 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 2 |
| 190315_PA-1 | 190405_KAB_190315_PA_1.raw | Competitive experiment with 500 μ M 4 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 3 ^[b] |
| 190315_PA-2 | 190405_KAB_190315_PA_2.raw | Competitive experiment with 500 μ M 4 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 4 ^[b] |
| 190315_PA-5 | 190405_KAB_190315_PA_5.raw | Competitive experiment with 500 μ M 4 (stock in water) in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 5 |
| 190315_PA-6 | 190405_KAB_190315_PA_6.raw | Competitive experiment with 500 μ M 4 (stock in water) in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 6 |

| | | |
|-------------|------------------------------|--|
| 190315_PA-3 | 190405_KAB_190315_PA_3.raw | Competitive experiment with 200 μM 4 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 1 |
| 190315_PA-4 | 190405_KAB_190315_PA_4.raw | Competitive experiment with 200 μM 4 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 2 |
| 190222_PB-1 | 190301_KAB_190222_PB_1.raw | Competitive experiment with 500 μM 5 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 1 |
| 190222_PB-2 | 190301_KAB_190222_PB_2.raw | Competitive experiment with 500 μM 5 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 2 |
| 190315_PB-1 | 190405_KAB_190315_PB_1.raw | Competitive experiment with 500 μM 5 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 3 ^[b] |
| 190315_PB-2 | 190405_KAB_190315_PB_2.raw | Competitive experiment with 500 μM 5 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 4 ^[b] |
| 190614_P2-1 | 190615_KAB_190614_P2_1.raw | Competitive experiment with 500 μM 6 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 1 |
| 190614_P2-2 | 190615_KAB_190614_P2_2.raw | Competitive experiment with 500 μM 6 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 2 |
| 190614_P2-3 | 190615_KAB_190614_P2_3.raw | Competitive experiment with 500 μM 6 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 3 |
| 190719_P1_1 | 20190723_KAB_190719_P1_1.raw | Competitive experiment with 500 μM 6 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 4 ^[b] |
| 190719_P1_2 | 20190723_KAB_190719_P1_2.raw | Competitive experiment with 500 μM 6 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 5 ^[b] |
| 190719_P1_3 | 20190723_KAB_190719_P1_3.raw | Competitive experiment with 500 μM 6 in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 6 ^[b] |

| | | |
|-------------|------------------------------|--|
| 190315_PD-1 | 190405_KAB_190315_PD_1.raw | Competitive experiment with DMSO in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 1 |
| 190315_PD-2 | 190405_KAB_190315_PD_2.raw | Competitive experiment with DMSO in <i>S. aureus</i> SH1000 <i>in vitro</i> , replicate 2 |
| 190311_P1-1 | 190404_KAB_190311_P1_1.raw | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000, replicate 1 |
| 190311_P1-2 | 190404_KAB_190311_P1_2.raw | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000, replicate 2 |
| 190311_P1-3 | 190404_KAB_190311_P1_3.raw | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000, replicate 3 |
| 190311_P2-1 | 190404_KAB_190311_P2_1.raw | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000, replicate 4 ^[b] |
| 190311_P2-2 | 190404_KAB_190311_P2_2.raw | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000, replicate 5 ^[b] |
| 190311_P2-3 | 190404_KAB_190311_P2_3.raw | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000, replicate 6 ^[b] |
| 190506_P4-1 | 190508_KAB_190506_P4_1.raw | Labeling with probe 1 <i>in situ</i> in <i>E. coli</i> , replicate 1 |
| 190506_P4-2 | 190508_KAB_190506_P4_2.raw | Labeling with probe 1 <i>in situ</i> in <i>E. coli</i> , replicate 2 |
| 190506_P4-3 | 190508_KAB_190506_P4_3.raw | Labeling with probe 1 <i>in situ</i> in <i>E. coli</i> , replicate 3 |
| 190724_P1_1 | 20190725_KAB_190724_P1_1.raw | Reductive dimethyl labeling experiment of nicotinate phosphoribosyltransferase with 500 μ M 6 to identify the site of labeling, replicate 1 |
| 190724_P1_2 | 20190725_KAB_190724_P1_2.raw | Reductive dimethyl labeling experiment of nicotinate phosphoribosyltransferase with 500 μ M 6 to identify the site of labeling, replicate 2 |

[a] The sample name gives information on the date when the measurement was set up, the initials of the scientist to set up the experiment and the sample name in that order. [b] These

experiments result from a biologically independent experiment in comparison to the replicates mentioned before.

Table S7. All different MaxQuant analyses performed in this study. All analyses have been deposited to the ProteomeXchange Consortium^[9] via the PRIDE partner repository^[10] with the dataset identifier PXD016659.

| Name of the Analysis | Included Samples ^[a] | Remarks |
|-----------------------------------|---|---|
| 1_in_vitro_S_aureus_quant_1 | 190124_P1-1 190124_P1-2 | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for quantification, biological replicate 1 |
| 1_in_vitro_S_aureus_quant_2 | 190222_P1-1 190222_P1-2 | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for quantification, biological replicate 2 |
| 2_in_vitro_S_aureus_quant_1 | 190124_P2-1 190124_P2-2 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for quantification, biological replicate 1 |
| 2_in_vitro_S_aureus_quant_2 | 190222_P2-1 190222_P2-2 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for quantification, biological replicate 2 |
| 3_in_vitro_S_aureus_quant_1 | 190124_P3-1 190124_P3-2 | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for quantification, biological replicate 1 |
| 3_in_vitro_S_aureus_quant_2 | 190222_P3-1 190222_P3-2 | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for quantification, biological replicate 3 |
| 2_in_vitro_S_aureus_quant_chymo_1 | 190614_P4-1 190614_P4-2 190614_P4-3 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 with chymotrypsin digestion analyzed for quantification |
| 1_in_vitro_S_aureus_light_1 | 190124_P1-1 190124_P1-2 | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the light |

| | | |
|-----------------------------|----------------------------|--|
| | | channel, biological replicate 1 |
| 1_in_vitro_S_aureus_heavy_1 | 190124_P1-1 190124_P1-2 | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the heavy channel, biological replicate 1 |
| 1_in_vitro_S_aureus_light_2 | 190222_P1-1 190222_P1-2 | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the light channel, biological replicate 2 |
| 1_in_vitro_S_aureus_heavy_2 | 190222_P1-1 190222_P1-2 | Labeling with probe 1 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the heavy channel, biological replicate 2 |
| 2_in_vitro_S_aureus_light_1 | 190124_P2-1 190124_P2-2 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the light channel, biological replicate 1 |
| 2_in_vitro_S_aureus_heavy_1 | 190124_P2-1 190124_P2-2 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the heavy channel, biological replicate 1 |
| 2_in_vitro_S_aureus_light_2 | 190222_P2-1 190222_P2-2 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the light channel, biological replicate 2 |
| 2_in_vitro_S_aureus_heavy_2 | 190222_P2-1 190222_P2-2 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid |

| | | |
|-----------------------------------|---|--|
| | | selectivity in the heavy channel, biological replicate 2 |
| 3_in_vitro_S_aureus_light_1 | 190124_P3-1 190124_P3-2 | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the light channel, biological replicate 1 |
| 3_in_vitro_S_aureus_heavy_1 | 190124_P3-1 190124_P3-2 | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the heavy channel, biological replicate 1 |
| 3_in_vitro_S_aureus_light_2 | 190222_P3-1 190222_P3-2 | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the light channel, biological replicate 2 |
| 3_in_vitro_S_aureus_heavy_2 | 190222_P3-1 190222_P3-2 | Labeling with probe 3 <i>in vitro</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the heavy channel, biological replicate 2 |
| 2_in_vitro_S_aureus_light_chymo_1 | 190614_P4-1 190614_P4-2 190614_P4-3 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 with chymotrypsin digestion analyzed for amino acid selectivity in the light channel |
| 2_in_vitro_S_aureus_heavy_chymo_1 | 190614_P4-1 190614_P4-2 190614_P4-3 | Labeling with probe 2 <i>in vitro</i> in <i>S. aureus</i> SH1000 with chymotrypsin digestion analyzed for amino acid selectivity in the heavy channel |

| | | |
|----------------------------|--|---|
| Competitive_data | 190222_PA-1 190222_PA-2 190315_PA-1 190315_PA-2 190315_PA-5 190315_PA-6 190315_PA-3 190315_PA-4 190222_PB-1 190222_PB-2 190315_PB-1 190315_PB-2 190614_P2-1 190614_P2-2 190614_P2-3 190719_P1_1 190719_P1_2 190719_P1_3 190315_PD-1 190315_PD-2 | All competitive data in <i>S. aureus</i> SH1000 <i>in vitro</i> |
| 1_in_situ_S_aureus_quant_1 | 190311_P1-1 190311_P1-2 190311_P1-3 | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000 analyzed for quantification, biological replicate 1 |
| 1_in_situ_S_aureus_quant_2 | 190311_P2-1 190311_P2-2 190311_P2-3 | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000 analyzed for quantification, biological replicate 2 |
| 1_in_situ_S_aureus_light_1 | 190311_P1-1 190311_P1-2 190311_P1-3 | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the light channel, biological replicate 1 |
| 1_in_situ_S_aureus_heavy_1 | 190311_P1-1 190311_P1-2 190311_P1-3 | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the heavy channel, biological replicate 1 |

| | | |
|----------------------------|---|---|
| 1_in_situ_S_aureus_light_2 | 190311_P2-1 190311_P2-2 190311_P2-3 | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the light channel, biological replicate 2 |
| 1_in_situ_S_aureus_heavy_2 | 190311_P2-1 190311_P2-2 190311_P2-3 | Labeling with probe 1 <i>in situ</i> in <i>S. aureus</i> SH1000 analyzed for amino acid selectivity in the heavy channel, biological replicate 2 |
| 1_in_situ_E_coli_quant_1 | 190506_P4-1 190506_P4-2 190506_P4-3 | Labeling with probe 1 <i>in situ</i> in <i>E. coli</i> analyzed for quantification |
| 1_in_situ_E_coli_light_1 | 190506_P4-1 190506_P4-2 190506_P4-3 | Labeling with probe 1 <i>in situ</i> in <i>E. coli</i> analyzed for amino acid selectivity in the light channel |
| 1_in_situ_E_coli_heavy_1 | 190506_P4-1 190506_P4-2 190506_P4-3 | Labeling with probe 1 <i>in situ</i> in <i>E. coli</i> analyzed for amino acid selectivity in the heavy channel |
| Methyl_labeling_Npt | 190724_P1_1 190724_P1_2 | Reductive dimethyl labeling experiment of nicotinate phosphoribosyltransferase with 500 μ M 6 to identify the site of labeling |

[a] As indicated above, some samples were included in various analyses.

Table S8. Annotation database for all aspartates and glutamates in the genome of *S. aureus* SH1000. This data can be found as an additional data file accompanying this article.

Experimental Procedures

General Remarks

All reactions sensitive to air and moisture were carried out in flame-dried glassware under an argon atmosphere using standard Schlenk techniques. Commercially available chemicals were used without further purification unless otherwise mentioned.

Technical solvents for column chromatography were used after distillation. Flash column chromatography was performed on silica gel (VWR, 40-63 μm) with the indicated eluent mixture. Thin Layer Chromatography (TLC) was performed on silica-coated aluminum sheets (Merck, TLC Silicagel 60 F254) with detection by staining with a KMnO_4 solution (1.50 g KMnO_4 , 10.0 g K_2CO_3 and 1.25 mL 10 wt% aqueous NaOH in 200 mL H_2O) followed by heat treatment.

NMR-Spectra were recorded on a Bruker AVHD-300, AVHD-400 or AVHD-500. ^1H -NMR spectra were calibrated to the residual solvent signal ($\delta = 7.26$ ppm for CDCl_3 ; $\delta = 2.50$ ppm for DMSO-d_6 , $\delta = 1.94$ ppm for CD_3CN). ^{13}C -NMR spectra were calibrated to the respective ^{13}C -D multiplets ($\delta = 77.16$ ppm for CDCl_3 ; $\delta = 39.52$ ppm for DMSO-d_6). Exchangeable signal assignments were marked with an asterisk (*). Multiplets are described using following abbreviations: s - singlet, t - triplet, q - quartet, m - multiplet.

HRMS spectra were recorded in the ESI mode with an LTQ-FT Ultra (Thermo Fisher Scientific) coupled to an UltiMate 3000 HPLC system (Thermo Fisher Scientific).

Protein and DNA concentrations were determined in duplicates with a NanoQuant plate (Tecan) by measuring absorbance at $\lambda = 280$ nm or 260 nm, respectively. Primers were custom synthesized (Eurofins) and obtained as lyophilized solids. They were dissolved in ddH_2O at a concentration of 100 μM and diluted with ddH_2O to 10 μM for use in PCR reactions.

No unexpected or unusually high safety hazards were encountered during the performance of this project.

Synthetic Procedures

4-Carboxybenzenediazonium chloride (I):

At -10°C , NaNO_2 (793 mg, 11.5 mmol, 1.00 eq) was added to a solution of 4-aminobenzoic acid (1.58 g, 11.5 mmol, 1.00 eq) in concentrated aqueous HCl (4 mL), H_2O (8 mL) and EtOH (10 mL). The resulting solution of **I** was used without any further purification.

4-(5-Phenyl-2*H*-tetrazol-2-yl)benzoic acid (II):

A freshly prepared solution of **I** (533 mg, 2.00 mmol, 1.00 eq) was added dropwise to a solution of benzaldehyde tosylhydrazone (549 mg, 2.00 mmol, 1.00 eq) in dry pyridine (10 mL) at -10°C . After warming to room temperature, the reaction mixture was diluted with H_2O (30 mL) and extracted with EtOAc (3×20 mL). The combined organic layers were washed with aqueous HCl (2 M, 3×50 mL) and brine (50 mL) before drying over Na_2SO_4 , filtering and concentration *in vacuo*. Purification by column chromatography (Pentane/ EtOAc 3:2 \rightarrow 0:1) yielded **II** as a red solid (292 mg, 1.10 mmol, 55%).

TLC (Pentane/ EtOAc 3:2 + 1% AcOH): $R_f = 0.39$.

$^1\text{H-NMR}$ (500 MHz, DMSO-d_6 , 298 K): δ [ppm] = 7.57-7.65 (m, 3H), 8.16-8.20 (m, 2H), 8.20-8.31 (m, 4H).

$^{13}\text{C-NMR}$ (75.5 MHz, DMSO-d_6 , 300 K): δ [ppm] = 119.8 (s, 2C), 126.2 (s, 1C), 126.7 (s, 2C), 129.4 (s, 2C), 131.1 (s, 1C), 131.2 (s, 2C), 132.3 (s, 1C), 138.7 (s, 1C), 164.7 (s, 1C), 166.3 (s, 1C).

HRMS-ESI (m/z): calc. ($\text{C}_{14}\text{H}_9\text{N}_4\text{O}_2$ [M-H]): 265.0731; found: 265.0731.

4-(5-Phenyl-2*H*-tetrazol-2-yl)-*N*-(prop-2-yn-1-yl)benzamide (1):

II (140 mg, 526 μmol , 1.00 eq) and dry NEt_3 (147 μL , 107 mg, 1.05 mmol, 2.00 eq) were dissolved in dry DMF (3.2 mL) in the dark and $\text{EDC}\cdot\text{HCl}$ (202 mg, 1.05 mmol, 2.00 eq) and $\text{HOEt}\cdot\text{H}_2\text{O}$ (161 mg, 1.05 mmol, 2.00 eq) were added. After 30 min, propargylamine (74.1 μL , 63.8 mg, 1.16 mmol, 2.20 eq) was added and stirring was continued for 21 h. The reaction mixture was then diluted with H_2O (15 mL) and extracted with EtOAc (3×20 mL). The combined organic layers were washed with aqueous HCl (1 M, 20 mL), saturated aqueous NaHCO_3 (20 mL) and brine (20 mL) before drying over Na_2SO_4 , filtering and concentration *in vacuo*. Purification by column chromatography (Pentane/ EtOAc 3:1 \rightarrow 1:1) yielded **1** as a white solid (59.7 mg, 197 μmol , 37%).

TLC (Pentane/ EtOAc 1:1): $R_f = 0.50$.

$^1\text{H-NMR}$ (500 MHz, DMSO-d_6 , 298 K): δ [ppm] = 3.17 (t, $^4J = 2.4$ Hz, 1H, $\text{C}\equiv\text{CH}$), 4.11 (dd, $^3J = 5.3$ Hz, $^4J = 2.4$ Hz, 2H, CH_2N), 7.58-7.67 (m, 3H, H_{Ar}), 8.14-8.18 (m, 2H, H_{Ar}), 8.18-8.22 (m, 2H, H_{Ar}), 8.27-8.31 (m, 2H, H_{Ar}), 9.20 (t, $^3J = 5.3$ Hz, 1H, NH).

¹³C-NMR (75.5 MHz, DMSO-d₆, 300 K): δ [ppm] = 28.9 (s, 1C), 73.1 (s, 1C), 81.0 (s, 1C), 119.7 (s, 2C), 126.3 (s, 1C), 126.7 (s, 2C), 129.3 (s, 2C), 129.3 (s, 2C), 131.0 (s, 1C), 135.0 (s, 1C), 137.9 (s, 1C), 164.7 (s, 1C), 164.7 (s, 1C).

HRMS-ESI (m/z): calc. (C₁₇H₁₄N₅O [M+H]): 304.1198; found: 304.1192.

The analytical data obtained are in agreement with those reported in the literature.^[11]

4-(Methoxycarbonyl)benzenediazonium tetrafluoroborate (III):

Methyl 4-aminobenzoate (1.00 g, 6.62 mmol, 1.00 eq) was added to H₂O (2.65 mL) and HBF₄ (48 wt% in H₂O, 2.32 g, 12.7 mmol, 1.92 eq). At 0 °C a solution of NaNO₂ (456 mg, 6.62 mmol, 1.00 eq) in H₂O (1 mL) was added dropwise over 20 min. Towards the end of the addition H₂O (800 μ L) was added to improve stirring and 15 min after complete addition the reaction mixture was filtered. The resulting yellow solid was dissolved in cool acetone (50 mL) and Et₂O was added until no further precipitation was observed. Filtration and drying *in vacuo* yielded **III** as white solid (1.12 g), which was used without further purification.

¹H-NMR (400 MHz, DMSO-d₆, 298 K): δ [ppm] = 3.96 (s, 3H, CH₃), 8.41-8.48 (m, 2H, H_{Ar}), 8.77-8.83 (m, 2H, H_{Ar}).

HRMS-ESI (m/z): calc. (C₁₁H₉N₄O₄ [M-BF₄]): 163.0502; found: 163.0502.

The analytical data obtained are in agreement with those reported in the literature.^[12]

Methyl 4-(5-methyl-2H-tetrazol-2-yl)benzoate (IV):

To a suspension of acetamidine hydrochloride (142 mg, 1.50 mmol, 1.00 eq), K₂CO₃ (1.04 g, 7.50 mmol, 5.00 eq) in dry DMSO (7.2 mL) was added crude **III** (375 mg, 1.50 mmol, 1.00 eq) in portions over 15 min and the reaction mixture was kept in the dark from thereon. After stirring for 1 h a solution of I₂ (457 mg, 1.80 mmol, 1.20 eq) and KI (374 mg, 2.25 mmol, 1.50 eq) in dry DMSO (5 mL) were added slowly. The mixture was stirred for 1 h before quenching with 5% aqueous Na₂S₂O₃ (35 mL) and, after addition of brine (60 mL), the aqueous layer was extracted with EtOAc (3 \times 45 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo* in the dark. Purification by column chromatography (Pentane/EtOAc 4:1) yielded **IV** as white crystals (223 mg, 1.02 mmol, 46% over two steps).

TLC (Pentane/EtOAc 1:1): R_f = 0.59.

¹H-NMR (500 MHz, CDCl₃, 298 K): δ [ppm] = 2.66 (s, 3H, CCH₃), 3.97 (s, 3H, OCH₃), 8.18-8.25 (m, 4H, H_{Ar}).

¹³C-NMR (75.5 MHz, CDCl₃, 300 K): δ [ppm] = 11.1 (s, 1C), 52.6 (s, 1C), 119.5 (s, 2C), 131.0 (s, 1C), 131.3 (s, 2C), 139.8 (s, 1C), 163.8 (s, 1C), 166.0 (s, 1C).

HRMS-ESI (m/z): calc. (C₁₀H₁₁N₄O₂ [M+H]): 219.0882; found: 219.0877.

The analytical data obtained are in agreement with those reported in the literature.^[13]

4-(5-Methyl-2*H*-tetrazol-2-yl)benzoic acid (V):

Aqueous NaOH (10 wt%, 1.5 mL) was added to a solution of **IV** (215 mg, 985 μ mol, 1.00 eq) in MeOH (2.5 mL) and H₂O (2.5 mL). After heating under reflux in the dark for two hours, the reaction mixture was cooled to room temperature and aqueous HCl (1 M, 3 mL) was added. The formed precipitate was filtered and then dried *in vacuo* to yield a white solid (145 mg), which was used without further purification.

TLC (5% MeOH/CH₂Cl₂ + 1% AcOH): R_f = 0.53.

HRMS-ESI (m/z): calc. (C₉H₈N₄O₂ [M-H]): 203.0569; found: 203.0575.

4-(5-Methyl-2*H*-tetrazol-2-yl)-*N*-(prop-2-yn-1-yl)benzamide (2):

Crude **V** (145 mg, 710 μ mol, 1.00 eq) and dry NEt₃ (198 μ L, 144 mg, 1.42 mmol, 2.00 eq) were dissolved in dry DMF (4 mL) in the dark and EDC·HCl (272 mg, 1.42 mmol, 2.00 eq) and HOBr (192 mg, 1.42 mmol, 2.00 eq) were added. After 30 min, propargylamine (100 μ L, 86.0 mg, 1.56 mmol, 2.20 eq) was added and stirring was continued overnight. The reaction mixture was then diluted with H₂O (20 mL) and extracted with EtOAc (3 \times 25 mL). The combined organic layers were washed with brine (20 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (Pentane/EtOAc 1:1) yielded **2** as a white solid (170 mg, 705 μ mol, 99% over two steps).

TLC (Pentane/EtOAc 1:1): R_f = 0.30.

¹H-NMR (500 MHz, CDCl₃, 298 K): δ [ppm] = 2.31 (t, ⁴*J* = 2.4 Hz, 1H, C≡CH), 2.65 (s, 3H, CH₃), 4.28 (dd, ³*J* = 5.1 Hz, ⁴*J* = 2.4 Hz, 2H, CH₂N), 6.58 (m, 1H, NH), 7.94-8.02 (m, 2H, H_{Ar}), 8.13-8.22 (m, 2H, H_{Ar}).

¹³C-NMR (75.5 MHz, DMSO-d₆, 300 K): δ [ppm] = 10.6 (s, 1C), 28.6 (s, 1C), 73.1 (s, 1C), 81.0 (s, 1C), 119.4 (s, 2C), 129.3 (s, 2C), 134.7 (s, 1C), 137.9 (s, 1C), 163.3 (s, 1C), 164.7 (s, 1C).

HRMS-ESI (m/z): calc. (C₁₂H₁₂N₅O [M+H]): 242.1042; found: 242.1036.

The analytical data obtained are in agreement with those reported in the literature.^[11]

Ethyl-2-(2-(phenylsulfonyl)hydrazono)acetate (VI):

Benzene sulfonyl hydrazide (1.98 g, 11.5 mmol, 1.00 eq) was added to a solution of ethyl glyoxylate (50% in toluene) (2.95 mL, 14.9 mmol, 1.3 eq) in EtOH (60 mL). After stirring for 90 min, the solution was concentrated *in vacuo* to yield crude **VI** as a yellow oil, which was used without further purification.

4-(5-(Ethoxycarbonyl)-2*H*-tetrazol-2-yl)benzoic acid (VII):

A freshly prepared solution of **I** (2.12 g, 11.5 mmol, 1.00 eq) was added dropwise to a solution of crude **VI** (2.95 g, 11.5 mmol, 1.00 eq) in dry pyridine (60 mL) at -10 °C. After warming to room temperature, the reaction mixture was diluted with H₂O (100 mL) and extracted with EtOAc (5 \times 100 mL). The combined organic layers were washed with aqueous HCl (2 M,

8 × 50 mL) and brine (2 × 50 mL) before drying over Na_2SO_4 , filtering and concentration *in vacuo*. Purification by column chromatography (1-4% MeOH/CH₂Cl₂ + 1% AcOH) yielded **VII** as an orange solid (1.82 g, 6.94 mmol, 60%).

TLC (CH₂Cl₂/MeOH 95:5 + 1% AcOH): R_f = 0.45.

¹H-NMR (300 MHz, CD₃CN, 300 K): δ [ppm] = 1.43 (t, 3H, 3J = 7.1 Hz, CH₃), 4.51 (q, 2H, 7.1 Hz, CH₂), 8.23-8.26 (m, 4H, H_{Ar}).

HRMS-ESI (m/z): calc. (C₁₁H₉N₄O₄ [M-H]): 261.0624; found: 261.0630.

The analytical data obtained are in agreement with those reported in the literature.^[11]

Ethyl 2-(4-(prop-2-yn-1-ylcarbamoyl)phenyl)-2H-tetrazole-5-carboxylate (VIII):

VII (1.21 g, 4.61 mmol, 1.00 eq) and dry NEt₃ (1.29 mL, 934 mg, 9.23 mmol, 2.00 eq) were dissolved in dry DMF (50 mL) and EDC·HCl (1.77 g, 9.23 mmol, 2.00 eq) and HOBt (1.25 g, 9.23 mmol, 2.00 eq) were added. After 30 min, propargylamine (650 μ L, 559 mg, 10.2 mmol, 2.20 eq) was added and stirring was continued overnight. Due to incomplete reaction as indicated by TLC, dry NEt₃ (785 μ L, 570 mg, 5.63 mmol, 1.22 eq), EDC·HCl (885 mg, 4.61 mmol, 1.00 eq) and HOBt (624 mg, 4.61 mmol, 1.00 eq) were added, followed by propargylamine (296 μ L, 254 mg, 4.61 mmol, 1.00 eq) after 45 min of stirring. After additional 6 h, starting material was still detected by TLC. Dry NEt₃ (643 μ L, 467 mg, 4.61 mmol, 1.00 eq), EDC·HCl (885 mg, 4.61 mmol, 1.00 eq) and HOBt (624 mg, 4.61 mmol, 1.00 eq) were added. After 30 min, propargylamine (296 μ L, 254 mg, 4.61 mmol, 1.00 eq) was added and stirring was continued overnight. The reaction mixture was then diluted with H₂O (150 mL) and extracted with EtOAc (5 × 50 mL). The combined organic layers were washed with aqueous HCl (1 M, 3 × 50 mL), saturated aqueous NaHCO₃ (3 × 50 mL) and brine (100 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The H₂O and HCl layers were extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with saturated aqueous NaHCO₃ (50 mL) and brine (50 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The residues were combined and purification by column chromatography (1-4% MeOH/CH₂Cl₂ + 1% AcOH) yielded **VIII** as an off-white solid (400 mg, 1.34 mmol, 29%).

TLC (5% MeOH/CH₂Cl₂): R_f = 0.75.

¹H-NMR (500 MHz, DMSO-d₆, 298 K): δ [ppm] = 1.38 (t, 3J = 7.1 Hz, 3H, CH₃), 3.13 (t, 4J = 2.4 Hz, 1H, C≡CH), 4.10 (dd, 3J = 5.4 Hz, 4J = 2.4 Hz, 2H, CH₂N), 4.47 (q, 3J = 7.1 Hz, 2H, CH₂CH₃), 8.10-8.17 (m, 2H, H_{Ar}), 8.18-8.27 (m, 2H, H_{Ar}), 9.19 (t, 3J = 5.4 Hz, 1H, NH).

¹³C-NMR (75.5 MHz, DMSO-d₆, 300 K): δ [ppm] = 13.9 (s, 1C), 28.7 (s, 1C), 62.4 (s, 1C), 73.0 (s, 1C), 81.0 (s, 1C), 120.2 (s, 2C), 129.3 (s, 2C), 135.6 (s, 1C), 137.5 (s, 1C), 157.1 (s, 1C), 157.4 (s, 1C), 164.5 (s, 1C).

HRMS-ESI (m/z): calc. (C₁₅H₁₆N₅O₃ [M+H]): 300.1097; found: 300.1090.

The analytical data obtained are in agreement with those reported in the literature.^[11]

2-(4-(Prop-2-yn-1-ylcarbamoyl)phenyl)-2*H*-tetrazole-5-carboxamide (3):

Concentrated aqueous NH₃ (3 mL) was added to a solution of **VIII** (178 mg, 595 μ mol, 1.00 eq) in dry THF (6 mL). After stirring overnight, the reaction mixture was concentrated *in vacuo* and the resulting residue purified by column chromatography (1-10% MeOH/CH₂Cl₂) yielded **3** as an off-white solid (112 mg, 414 μ mol, 70%).

TLC (5% MeOH/CH₂Cl₂): R_f = 0.66.

¹H-NMR (500 MHz, DMSO-d₆, 298 K): δ [ppm] = 3.15 (t, ⁴*J* = 2.4 Hz, 1H, C≡CH), 4.10 (dd, ³*J* = 5.4 Hz, ⁴*J* = 2.4 Hz, 2H, CH₂N), 8.08-8.20 (m, 3H, H_{Ar}, NHH), 8.20-8.28 (m, 2H, H_{Ar}), 8.51 (br. s, 1H, NHH), 9.20 (t, ³*J* = 5.4 Hz, 1H, CH₂NH).

¹³C-NMR (75.5 MHz, DMSO-d₆, 300 K): δ [ppm] = 29.7 (s, 1C), 73.1 (s, 1C), 81.0 (s, 1C), 120.2 (s, 2C), 129.3 (s, 2C), 135.4 (s, 1C), 137.8 (s, 1C), 157.7 (s, 1C), 160.2 (s, 1C), 164.7 (s, 1C).

HRMS-ESI (m/z): calc. (C₁₂H₁₁N₆O₂ [M+H]): 271.0943; found: 271.0937.

***N*-Phenylacetohydrazonoyl chloride (6):**

A suspension of *N*'-phenylacetohydrazide (300 mg, 2.00 mmol, 1.00 eq) and POCl₃ (280 μ L, 459 mg, 3.00 mmol, 1.50 eq) in dry PhMe (4.2 mL) was stirred for 1 h 50 min at 70 °C. After cooling to room temperature, the resulting pink solution is slowly poured into a solution of NaHCO₃ (800 mg) in ice water (20 mL) and the layers were separated. The aqueous layer was extracted with CH₂Cl₂ (3 \times 20 mL) and the combined organic layers were diluted with pentane (20 mL), washed with brine (40 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (Pentane/EtOAc 15:1) yielded **6** as a light brown solid (190 mg, 1.13 mmol, 85%).

TLC (Pentane/EtOAc 9:1): R_f = 0.56.

¹H-NMR (300 MHz, CDCl₃, 300 K): δ [ppm] = 2.40 (s, 3H, CH₃), 6.89 (tt, *J* = 7.3, 1.2 Hz, 1H), 7.01-7.07 (m, 2H), 7.21-7.31 (m, 2H), 7.55 (br. s, 1H, NH).

¹³C-NMR (75.5 MHz, CDCl₃, 300 K): δ [ppm] = 25.5 (s, 1C), 113.2 (s, 2C), 120.7 (s, 1C), 123.7 (s, 1C), 129.4 (s, 2C), 144.0 (s, 1C).

The analytical data obtained are in agreement with those reported in the literature.^[14]

Bacterial Strains

Bacterial strains and media were used as given in Table S3.

Overnight cultures of bacteria were inoculated with 5 μ L of a corresponding glycerol stock into 5 mL of the indicated medium and grown overnight at 37 °C with shaking at 200 rpm.

For preparation of lysates, the indicated medium was inoculated 1:100 with an overnight culture of the respective bacteria and incubated at 37 °C with shaking at 200 rpm until 1 hit reached the stationary phase. The bacteria were harvested by centrifuging at 8,000 $\times g$ at 4 °C for 10 min, and the pellets were washed two times with PBS prior to the immediate use or storage at -80 °C. PBS was added to the bacterial pellets and the pellets were resuspended and transferred into 7 mL tubes containing 0.1 mm ceramic beads. Cells were lysed in a Precellys 24 bead mill using three 30 s cycles at 6,500 rpm while cooling with an airflow that was pre-cooled with liquid nitrogen. The suspension was transferred into an Eppendorf tube and centrifuged at 20,000 $\times g$ at 4 °C for 30 min. The supernatant of several samples was pooled and filtered through a 0.45 μ m filter. Protein concentration of the lysate was determined using a bicinchoninic acid (BCA) assay and the concentration was adjusted to 1 mg/mL with PBS. The lysates were either used immediately (for all MS experiments) or stored at -20 °C.

For treatment of living bacteria, the indicated medium was inoculated 1:100 with an overnight culture of the respective bacteria and incubated at 37 °C with shaking at 200 rpm until 1 hit reached the stationary phase. The OD₆₀₀ of the bacterial suspension was determined. The bacteria were harvested by centrifuging at 8,000 $\times g$ at 4 °C for 10 min, and the pellets were washed two times with PBS. The bacteria were resuspended to an OD₆₀₀ = 40 and used for experiments immediately.

MIC Determination

Bacterial overnight cultures were diluted 1:10,000. 1 μ L of a 100x stock of the respective compound was added to 100 μ L of this dilution in a sterile 96-well plate. Several growth controls with addition of 1 μ L of the respective solvent of the compound instead of compound to the bacterial suspension and sterile controls with addition of 1 μ L solvent to the used media were included on every plate. The plates were incubated at 37 °C for 20 h with shaking at 200 rpm. The MIC was determined as the lowest concentration, for which no bacterial growth was observed. Bacterial growth was determined by eye and by measurement of the absorbance at 600 nm (OD₆₀₀).

Amino Acid Reactivity

In a 48-well plate, 2 μ L of probe **1**, **2** or **3** (5 mM in DMSO) were added to 100 μ L of the indicated PBS-acetonitrile mixture containing 5 mM of the indicated amino acid. The reaction mixture was irradiated for the indicated time intervals (10 min, if not stated otherwise) with a Luzchem LZC-UVB lamp (280–315 nm) and then analyzed with an UltiMate 3000 HPLC system (Thermo Fisher Scientific) coupled to an LTQ-FT Ultra (Thermo Fisher Scientific) operated in ESI mode in a scan range of m/z = 100-800 Th. The separation was carried out on a Waters XBridge C18 3.5 μ M column (4.6 \times 100 mm) with a flow of 1.10 mL/min and buffer A: 0.1% formic acid in water and B: 0.1% formic acid and 10% water in acetonitrile. Analysis started with washing in 2% B for 4 min followed by a gradient from 2% to 98%

buffer B in 5 min. 98% B was held for 2 min, then decreased to 20% in 0.5 min and held at 20% for another 2 min.

Gel-based ABPP Experiments with probe treatment *in vitro*

Probe **1**, **2** or **3** were added to bacterial lysates (110 μ L, 1 mg/mL) at the indicated concentrations and incubated at room temperature for 30 min. The solutions were transferred into a 48-well plate and irradiated for the indicated time intervals (10 min, if not stated otherwise) with a Luzchem LZC-UVB lamp (280–315 nm). In several experiments, controls were included without irradiation. 50 μ L of each sample was transferred back into an Eppendorf tube. The samples were clicked to TAMRA-azide (Jena bioscience) by adding 6 μ L of a solution consisting of 3 μ L 0.9 mg/mL TBTA ligand in 4:1 *t*BuOH/DMSO, 1 μ L 12.5 mg/mL CuSO₄ in water, 1 μ L 13 mg/ml TCEP in water and 1 μ L 5 mM of TAMRA-azide in DMSO). The reactions were quenched by addition of 50 μ L 2x Laemmli buffer and analyzed by SDS-PAGE. Modification with TAMRA-azide was detected by in-gel fluorescence scanning and protein loading was visualized by Coomassie staining.

Gel-based ABPP Experiments with probe treatment in living bacteria

Bacterial suspensions in PBS (200 μ L, OD₆₀₀ = 40) were treated with probe **1**, **2** or **3** at the indicated concentration (100 μ M final concentration, if not stated otherwise) for the indicated time (1 h, if not stated otherwise) at 37 °C with shaking at 200 rpm. 150 μ L of the suspension were transferred into a 48-well plate and irradiated for the indicated time intervals (10 min, if not stated otherwise) with a Luzchem LZC-UVB lamp (280–315 nm). In several experiments, controls were included without irradiation. 100 μ L were transferred into an Eppendorf tube and harvested by centrifuging at 8,000 \times g at 4 °C for 10 min, and the pellets were washed two times with PBS prior to the immediate use or storage at -80 °C. For *S. aureus* only experiments, the pellets were resuspended in 200 μ L PBS and 1 μ L lysostaphin (10 mg/mL, Sigma) was added. The samples were incubated for 1 h at 37 °C with shaking at 1,000 rpm. 4 μ L 20% SDS in PBS were added and the samples were sonicated for 10 s at 20% of the maximum intensity. Insoluble components were separated by centrifugation at 13,400 \times g for 1 h at room temperature. For the comparison of strains and in general for *E. coli* and *S. typhimurium*, the pellets were resuspended in 200 μ L PBS and transferred into 0.5 mL tubes containing ceramic beads. Cells were lysed in a Precellys 24 bead mill using three 30 s cycles at 6,500 rpm while cooling with an airflow that was pre-cooled with liquid nitrogen. Insoluble components were separated by centrifugation at 13,400 \times g for 30 min at 4 °C. 100 μ L of the supernatant of each sample was transferred into an Eppendorf tube. The samples were clicked to TAMRA-azide (Jena bioscience) by adding 12 μ L of a solution consisting of 6 μ L 0.9 mg/mL TBTA ligand in 4:1 *t*BuOH/DMSO, 2 μ L 12.5 mg/mL CuSO₄ in water, 2 μ L 13 mg/ml TCEP in water and 2 μ L 5 mM of TAMRA-azide in DMSO). The reactions were quenched by addition of 100 μ L 2x Laemmli buffer and analyzed by SDS-PAGE. Modification with TAMRA-azide was detected by in-gel fluorescence scanning and protein loading was visualized by Coomassie staining.

isoDTB-ABPP Experiments *in vitro*

Two samples of 1.10 mL freshly prepared lysate of the indicated bacterial strain were incubated with 22 μ L of probe **1**, **2** or **3** at the indicated concentration, each, and incubated at room temperature for 30 min. In the case of sensitivity experiments, 55 μ L of bovine serum

albumin (BSA) in PBS (20 mM – 20 pM, 0 pM) were additionally added before probe treatment. Samples were transferred into a 6-well plate and irradiated for 10 min with a Luzchem LZC-UVB lamp (280–315 nm). 1.00 mL were transferred back into an Eppendorf tube. One sample was clicked to the heavy and one to the light isoDTB tag by adding 120 μ L of a solution consisting of 60 μ L 0.9 mg/mL TBTA ligand in 4:1 *t*BuOH/DMSO, 20 μ L 12.5 mg/mL CuSO₄ in water, 20 μ L 13 mg/ml TCEP in water and 20 μ L 5 mM of the respective isoDTB tag in DMSO. After incubation of the samples at room temperature for 1 h, the light- and heavy-labeled samples were combined into 8 mL of cold acetone in order to precipitate all proteins. Precipitates were stored at –20 °C overnight.

Competitive isoDTB-ABPP Experiments

Two samples of 1.10 mL fresh lysate of the indicated bacterial strain were prepared. One of the samples was treated with 11 μ L of the respective inhibitor (**4**, **5** or **6**) at the indicated concentration and one sample with 11 μ L of the corresponding solvent as control (1% HCl (two biological replicates at 500 μ M and 200 μ M) or H₂O (one biological replicate at 500 μ M) for **4**, DMSO for **5** and DMF for **6**). The samples were incubated for 1 h at room temperature. Afterwards, probe labeling and precipitation were performed as described for “isoDTB-ABPP Experiments in vitro”, where the solvent-treated control was clicked to the heavy isoDTB tag and the compound-treated sample to the light isoDTB tag.

isoDTB-ABPP Experiments *in situ*

Bacterial suspensions in PBS (1.10 mL, OD₆₀₀ = 40) were treated with probe **1**, **2** or **3** at the indicated concentration (100 μ M, if not stated otherwise) for the indicated time (1 h, if not stated otherwise) at 37 °C with shaking at 200 rpm. The suspension was transferred into a 6-well plate and irradiated for the indicated time intervals (10 min, if not stated otherwise) with a Luzchem LZC-UVB lamp (280–315 nm). 900 μ L were transferred into an Eppendorf tube and harvested by centrifuging at 8,000 \times g at 4 °C for 10 min, and the pellets were washed two times with PBS prior to the immediate use or storage at –80 °C. The pellets were resuspended in 1.00 mL PBS and 5 μ L lysostaphin (10 mg/mL, Sigma) for *S. aureus* or 70 μ L lysozyme (10 mg/mL, Sigma) for *E. coli* were added. The samples were incubated for 1 h at 37 °C with shaking at 1,000 rpm. 20 μ L 20% SDS in PBS were added and the samples were sonicated for 10 s at 20% of the maximum intensity. Insoluble components were separated by centrifugation at 13,400 \times g for 1 h at room temperature. 900 μ L were transferred into a fresh Eppendorf tube. One sample was clicked to the heavy and one to the light isoDTB tag by adding 108 μ L of a solution consisting of 54 μ L 0.9 mg/mL TBTA ligand in 4:1 *t*BuOH/DMSO, 18 μ L 12.5 mg/mL CuSO₄ in water, 18 μ L 13 mg/ml TCEP in water and 18 μ L 5 mM of the respective isoDTB tag in DMSO. After incubation of the samples at room temperature for 1 h, the light- and heavy-labeled samples were combined into 7.2 mL of cold acetone in order to precipitate all proteins. Precipitates were stored at –20 °C overnight.

MS Sample Preparation

For all types of experiments, protein precipitates were centrifuged at 3,500 rpm at 25 °C for 10 min. The supernatant was removed, and precipitates resuspended in 1 mL cold methanol by sonification. After centrifugation at 20,000 \times g at 4 °C for 10 min, the supernatant was removed. This wash step with methanol was repeated one more time. The pellets were dissolved in 300 μ L 8 M urea in 0.1 M triethylammonium bicarbonate (TEAB) by sonification.

900 μ L 0.1 M TEAB were added to obtain a concentration of 2 M urea. This solution was added to 1.2 mL of washed high capacity streptavidin agarose beads (50 μ L initial slurry, Fisher Scientific, 10733315) in 0.2% nonyl phenoxypolyethoxylethanol (NP40 substitute). The samples were rotated at room temperature for 1 h in order to assure binding to the beads.

The beads were centrifuged (1 min, 1,000 $\times g$) and the supernatant was removed. The beads were resuspended in 600 μ L 0.1% NP40 substitute in PBS and transferred to a centrifuge column (Fisher Scientific, 11894131). Beads were washed two times with 0.1% NP40 substitute in PBS, three times with PBS and three times with ddH₂O. The beads were resuspended in 600 μ L 8 M urea in 0.1 M TEAB, transferred to a Protein LoBind tube and centrifuged (1 min, 1,000 $\times g$). The supernatant was removed, and the beads were resuspended in 300 μ L 8 M urea in 0.1 M TEAB. 15 μ L of dithiothreitol (DTT; 31 mg/mL in water) were added and the beads incubated at 37 °C with shaking at 200 rpm for 45 min. Free thiol groups were modified by adding 15 μ L of iodoacetamide (74 mg/mL in water) and incubation at 25 °C with shaking at 200 rpm for 30 min. Remaining iodoacetamide was quenched by adding 15 μ L DTT (31 mg/mL in water) and incubation at 25 °C with shaking at 200 rpm for another 30 min.

For trypsin digestion, 900 μ L TEAB were added to the samples after alkylation to obtain a urea concentration of 2 M. Samples were centrifuged (1 min, 1,000 $\times g$) and the supernatant removed. The beads were resuspended in 200 μ L 2 M urea in 0.1 M TEAB. 4 μ L 0.5 mg/mL trypsin (Promega, V5113) were added and samples incubated at 37 °C with shaking at 200 rpm overnight. For chymotrypsin digestion, samples were washed after alkylation with 600 μ L 100 mM Tris-HCl, 10 mM calcium chloride, 1 M urea (pH = 8) and resuspended in 200 μ L of the same buffer. 2 μ L 1 mg/mL chymotrypsin (Promega, V1062) were added and samples incubated at 25 °C with shaking at 200 rpm overnight.

After the digestion, samples were diluted by adding 400 μ L of 0.1% NP40 substitute in PBS and transferred to a centrifuge column (Fisher Scientific, 11894131). Beads were washed three times with 0.1% NP40 substitute in PBS, three times with PBS (MS grade) and three times with water (MS grade). Peptides were eluted by adding 200 μ L 0.1% formic acid in 50% acetonitrile in water to the column. 100 μ L of the elution buffer were added two times more to the column and peptides eluted into a Protein LoBind tube by centrifuging at 3,000 $\times g$ for 3 min. The solvent was removed in a vacuum concentrator by rotating at 30 °C for approximately 5 h. Samples were dissolved in 30 μ L 1% formic acid in water by sonification for 5 min. Samples were filtered through filters (Merck, UVC30GVNB) washed with the same solvent and transferred into MS sample vials. Samples were stored at -20 °C until measurement.

Sample Analysis by LC-MS/MS

10 μ L of the samples were analyzed using a Q Exactive Plus mass spectrometer (ThermoFisher) coupled to an Ultimate 3000 nano HPLC system (Dionex). Samples were loaded on an Acclaim C18 PepMap100 trap column (75 μ m ID x 2 cm) and washed with 0.1% TFA. The subsequent separation was carried out on an Acclaim C18PepMapRSLC column (75 μ m ID x 50 cm) with a flow of 300 nL/min and buffer A: 0.1% formic acid in water and B:

0.1% formic acid in acetonitrile. Analysis started with washing in 5% B for 7 min followed by a gradient from 5% to 40% buffer B in 105 min, an increase to 60% B in 10 min and another increase to 90% B in 10 min. 90% B was held for 10 min, then decreased to 5% in 0.1 min and held at 5% for another 9.9 min. The Q Exactive Plus mass spectrometer was run in a TOP10 data-dependent mode. In the orbitrap, full MS scans were collected in a scan range of m/z = 300–1500 Th at a resolution of 70,000 and an AGC target of 3e6 with 80 ms maximum injection time. The most intense peaks were selected for MS2 measurement with a minimum AGC target of 1e3 and isotope exclusion and dynamic exclusion (exclusion duration: 60 s) enabled. Peaks with unassigned charge or a charge of +1 were excluded. MS2 spectra were collected at a resolution of 17,500 aiming at an AGC target of 1e5 with a maximum injection time of 100 ms. Isolation was conducted in the quadrupole using a window of 1.6 Th. Fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 27%) and finally detected in the orbitrap.

Adjustment of FASTA Databases for Analysis of isoDTB-ABPP data

For the different bacterial strains and the human cell line, the following FASTA databases were downloaded from uniprot.org^[6] on the indicated days as indicated in Table S4. These were used directly for the experiments evaluating the amino acid selectivity with MaxQuant^[2] and for the experiments evaluating the mass of modification or amino acid selectivity with MSFragger^[1] after addition of the reverse sequences.

The challenge for the quantification of aspartates and glutamates in this project was to quantify the relative abundance of peptides modified at one residue with the probe and the light and heavy isoDTB tags, respectively. While custom-built solutions have been presented for this problem,^[3,15] we wanted to make this data analysis broadly applicable and therefore wanted to apply the broadly used and freely available MaxQuant software.^[2] We have previously reported this procedure for addressing a single modified amino acid, cysteine,^[16] and apply it here for two modified amino acids, aspartate and glutamate. To the best of our knowledge, relative quantification of two “variable modifications” relative to one another is not possible using MaxQuant software at this point. Therefore, we set out to use the “label” function in MaxQuant for quantification. Nevertheless, while this function allows very reliable relative quantification of light- and heavy-labeled peptides, this function assumes every amino acid of a certain type (*e.g.* aspartate and glutamate) to be modified with the label. Therefore, peptides with two or more of these residues are only detected and quantified, if all of these residues have reacted with the probe and the isoDTB tags. Nevertheless, the peptides that are modified at one residue with the probe and the isoDTB tags but are unmodified at the others are not detectable. For this reason, we utilized a workaround in order to achieve this quantification. We utilized “U” respectively “O”, which normally stand for selenocysteine and pyrrolysine, as a placeholder amino acid for the modified residue (“U” used for glutamates and “O” used for aspartates). To do so, we deleted all selenocysteine- or pyrrolysine-containing proteins from the FASTA databases; which were usually very few. We then individually replaced each glutamate in the FASTA database with a “U” and additionally every aspartate individually with an “O” generating n different sequences with a single “U” or “O” for a protein with n aspartates and glutamates. For each individual replacement, we created an entry in the FASTA database, which was named in the format “Uniprot code”_“E”“number of the glutamate” respectively “Uniprot code”_“D”“number of the

aspartate". The unmodified sequence was deleted from the FASTA database, except if the protein did not contain any aspartate or glutamate, in which case the unmodified entry was renamed to "Uniprot Code"_"0" and kept in the database. In this way, for each aspartate and glutamate in the database, we created a unique sequence, in which it is marked as the modified residue (by being replaced by the placeholder "U" or "O") and all other aspartates and glutamates are marked as unmodified (are remaining "D" or "E" in the database). Therefore, we were able to make sure that there is always only one modified residue in each peptide to be detected and quantified. Therefore, this allows us to detect and quantify all peptides that contain several aspartates and glutamates but are only modified with the probe and the isoDTB tags at one of them. During MaxQuant analysis, we define the labels in a way to not only add the modification with the tag but also to transfer the placeholder "U" or "O" back to a glutamate or aspartate.

During downstream data analysis, the "U" or "O" in the sequence is changed back to the indicator for a modified glutamate ("E*") or aspartate ("D*"). Table S5 gives all modified FASTA databases used.

Data evaluation using MSFragger for identification of the mass of modification

The "*.raw"-files of the MS analysis were converted using the "MSConvertGUI (64 bit)" of "ProteoWizard 3.0.19172 (64 bit)"^[17] into the "*.mzML" format with the following settings: "64 bit", "Write index", "use zlib compression" and "TPP compatibility" enabled. The resulting files were analyzed together for technical replicates with MSFragger version 20190628^[1] and Philosopher v1.1.2 using the FragPipe (v9.4) interface. Samples were analyzed with the FASTA database "SA59_plus_rev.fasta" that includes all reverse sequences as decoys. An Open Search was performed with a precursor mass tolerance from -1000 Da to +1000 Da and a fragment mass tolerance of 20 ppm. Digestion with Trypsin (cutting after K and R but not before P) was used with a maximum number of missed cleavages of 1, a peptide length between 7 and 50 amino acids, a peptide mass range between 500 Da and 5000 Da and a maximum fragment charge of 2. No fixed modifications were included and all "Advanced Options" were left at the respective standard values. "PeptideProphet" and "ProteinProphet" were enabled. For downstream analysis the file "modifications.tsv" was used and the "PSMs with Observed Modifications" were further analyzed. Masses of modification of less than 400 Da were excluded from further analysis as the isoDTB tags themselves have masses of 481 Da (light) or 487 Da (heavy) and only peptides modified with the probe and the tags were of interest. These values were plotted in the final graphs.

Data evaluation in MaxQuant for investigation of selectivity

MS raw data were analyzed using MaxQuant software (version 1.6.1.0).^[2] Standard settings were used with the following changes and additions: The normal FASTA databases without manual changes were used ("SA59.fasta" and "EC60.fasta"). Two individual runs were performed for the light and the heavy isoDTB tags. No labels were used. The digestion enzyme was set to Trypsin/P for all regular experiments or Chymotrypsin+ for the experiments with chymotrypsin digestion with maximum number of missed cleavages of 2. Variable modification with the probe and either the light or the heavy isoDTB tag was allowed on all potentially nucleophilic amino acids (C, D, E, F, H, K, M, N, Q, R, S, T, W, Y). The following modifications were used:

- probe **1**, light isoDTB tag: $C_{37}H_{48}N_{12}O_6$
- probe **1**, heavy isoDTB tag: $C_{33}^{13}C_{48}H_{48}N_{10}^{15}N_2O_6$
- probe **2**, light isoDTB tag: $C_{32}H_{46}N_{12}O_6$
- probe **2**, heavy isoDTB tag: $C_{28}^{13}C_{4}H_{46}N_{10}^{15}N_2O_6$
- probe **3**, light isoDTB tag: $C_{32}H_{45}N_{13}O_7$
- probe **3**, heavy isoDTB tag: $C_{28}^{13}C_{4}H_{45}N_{11}^{15}N_2O_7$

Carbamidomethyl (C) was included as a variable modification on cysteine. The max. number of modifications per peptide was 2. The “Re-quantify” option was enabled. Contaminants were included. Peptides were searched with a minimum peptide length of 7 and a maximum peptide mass of 4,600 Da. “Second peptides” and “Dependent peptides” were disabled and the option “Match between run” was enabled with a Match time window of 0.7 min and an alignment window of 20 min. An FDR of 0.01 was used for Protein FDR, PSM FDR and XPSM FDR. Technical replicates were analyzed in the same MaxQuant analysis.

The “Sites.txt” files generated by MaxQuant analysis for modification with the probe and either the light or heavy isoDTB tag were used for further analysis. The information of these two files was combined into one file. All peptides for “reverse” sequences and “potential contamination” were removed. For each peptide, an identifier was generated with the information of the protein, the type of the modified amino acid and the residue number in the format “UniprotCode_””Type of modified amino acid””residue number”. If an identifier occurred in the data more than once, only the peptide with the highest localization probability was kept. The data was filtered to only include peptides with a localization probability of at least 75% for a single amino acid residue. Within these peptides, for each potentially nucleophilic amino acid, the number of sequences was counted that is modified on this amino acid. The sum of all these numbers is the total number of identified modification sites that is reported. To assess specificity, amino acids with the same functional group (D+E, N+Q and S+T) were combined by adding the modified peptides with modification at these amino acids together. The fraction of all identified modification sites that is modified at the respective group of amino acids was calculated and is reported in the graphs. If several biologically independent replicates were performed, the mean and standard deviation are plotted.

Data evaluation using MSFagger for investigation of selectivity

The “*.raw”-files of the MS analysis were converted using the “MSConvertGUI (64 bit)” of “ProteoWizard 3.0.19172 (64 bit)”^[17] into the “*.mzML” format with the following settings: “64 bit”, “Write index”, “use zlib compression” and “TPP compatibility” enabled. The resulting files were analyzed together for technical replicates with MSFagger version 20190628^[1] and Philosopher v1.1.2 using the FragPipe (v9.4) interface. Samples were analyzed with the FASTA database “SA59_plus_rev.fasta” that includes all reverse sequences as decoys. A Closed Search was performed with a precursor mass tolerance from -50 ppm to +50 ppm and a fragment mass tolerance of 20 ppm. Carbamidomethyl at cysteine (57.0215 Da) and the following masses on all potentially nucleophilic amino acids (C, D, E, F, H, K, M, N, Q, R, S, T, W, Y) for the respective probe were allowed as variable modifications:

- probe **1**, light isoDTB tag: 756.3820 Da
- probe **1**, heavy isoDTB tag: 762.3895 Da

- probe **2**, light isoDTB tag: 694.3663 Da
- probe **2**, heavy isoDTB tag: 700.3738 Da
- probe **3**, light isoDTB tag: 723.3565 Da
- probe **3**, heavy isoDTB tag: 729.3640 Da

The maximum number of variable modifications was set to 3. Digestion with Trypsin (cutting after K and R but not before P) was used with a maximum number of missed cleavages of 1, a peptide length between 7 and 50 amino acids, a peptide mass range between 500 Da and 5000 Da and a maximum fragment charge of 2. No fixed modifications were included and all "Advanced Options" were left at the respective standard values. "PeptideProphet" and "ProteinProphet" were enabled. The "psm.tsv" file was further analyzed. PSMs were analyzed if they contained exactly one modification with the light or the heavy isoDTB-tag. For these peptides, the modified amino acid was determined. Within these peptides, for each potentially nucleophilic amino acid, the number of sequences was counted that is modified on this amino acid. The sum of all these numbers is the total number of identified modification sites that is reported. To assess specificity, amino acids with the same functional group (D+E, N+Q and S+T) were combined by adding the modified peptides with modification at these amino acids together. The fraction of all identified modification sites that is modified at the respective group of amino acids was calculated and is reported in the graphs. If several biologically independent replicates were performed, the mean and standard deviation are plotted.

Data evaluation using MaxQuant for quantification

MS raw data were analyzed using MaxQuant software (version 1.6.1.0).^[2] Standard settings were used with the following changes and additions: The modified FASTA databases with individual substitutions of aspartates and glutamates with the placeholder "O" or "U" were used ("SA59_DO_EU.fasta" and "EC60_DO_EU.fasta"). Labels were set on the placeholder amino acids "O" and "U" for the light isoDTB tag as light label and the heavy isoDTB tag as heavy label. The following labels were used:

- probe **1**, light isoDTB tag, on "O" as placeholder for D: C₂₉H₃₄N₁₀O₇
- probe **1**, heavy isoDTB tag, on "O" as placeholder for D: C₂₅¹³C₄H₃₄N₈¹⁵N₂O₇
- probe **1**, light isoDTB tag, on "U" as placeholder for E: C₃₉H₅₀N₁₂O₈Se-1
- probe **1**, heavy isoDTB tag, on "U" as placeholder for E: C₃₅¹³C₄H₅₀N₁₀¹⁵N₂O₈Se-1
- probe **2**, light isoDTB tag, on "O" as placeholder for D: C₂₄H₃₂N₁₀O₇
- probe **2**, heavy isoDTB tag, on "O" as placeholder for D: C₂₀¹³C₄H₃₂N₈¹⁵N₂O₇
- probe **2**, light isoDTB tag, on "U" as placeholder for E: C₃₄H₄₈N₁₂O₈Se-1
- probe **2**, heavy isoDTB tag, on "U" as placeholder for E: C₃₀¹³C₄H₄₈N₁₀¹⁵N₂O₈Se-1
- probe **3**, light isoDTB tag, on "O" as placeholder for D: C₂₄H₃₁N₁₁O₈
- probe **3**, heavy isoDTB tag, on "O" as placeholder for D: C₂₀¹³C₄H₃₁N₉¹⁵N₂O₈
- probe **3**, light isoDTB tag, on "U" as placeholder for E: C₃₄H₄₇N₁₃O₉Se-1
- probe **3**, heavy isoDTB tag, on "U" as placeholder for E: C₃₀¹³C₄H₄₇N₁₁¹⁵N₂O₉Se-1

A multiplicity of 2 was set and a maximum number of labeled amino acids of 1. The digestion enzyme was set to Trypsin/P for all regular experiments or Chymotrypsin+ for the experiments with chymotrypsin digestion with maximum number of missed cleavages of 2. No variable modifications were included. The "Re-quantify" option was enabled.

Carbamidomethyl ($\text{C}_2\text{H}_3\text{NO}$) was used as fixed modification on cysteine. Contaminants were included. Peptides were searched with a minimum peptide length of 7 and a maximum peptide mass of 4,600 Da. “Second peptides” and “Dependent peptides” were disabled and the option “Match between run” was enabled with a Match time window of 0.7 min and an alignment window of 20 min. An FDR of 0.01 was used for Protein FDR, PSM FDR and XPSM FDR. Technical replicates and all competitive data were analyzed in the same MaxQuant analysis.

The “peptides.txt” file of the MaxQuant analysis was used for further analysis. All peptide sequences without a modified aspartate or glutamate (placeholder “O” or “U”) and all reverse sequences were deleted. Only the columns “Sequence”, “Leading Razor Protein”, “Start Position” and the columns for “Ratio H/L” for all experiments were kept. The “Leading Razor Protein” was renamed to the Uniprot Code without the indicator for the number of the aspartate or glutamate. All individual ratios were filtered out if they were “NaN”, and all other values were transformed into the \log_2 -scale. For each peptide in each condition, the data was filtered out, if there were not at least two data points for individual technical replicates or if the standard deviation between the technical replicates exceeded a value of 1.41. For conditions in the competitive data that consist of two or more biologically independent sets of replicates, this was done together for all biological and technical replicates. For each peptide, an identifier was generated in the form “Uniprot Code”_”D””residue number of the modified aspartate” or “Uniprot Code”_”E””residue number of the modified glutamate”. The data for the same experiment for all peptides with the same identifier, and therefore the same modified aspartate or glutamate, were combined. Here, the median of the data was used. The data was filtered out if the standard deviation exceeded a value of 1.41. Each modified aspartate or glutamate was kept in the dataset once with the shortest peptide sequence as the reported sequence. For each modified residue, all values for the same condition were combined, but the individual values are also reported. The values were combined as the median and the data was filtered out, if there were not at least two data points or if the standard deviation exceeded a value of 1.41. For conditions in the competitive data that consist of two or more biologically independent sets of replicates, this was done together for all biological and technical replicates. All modified residues were filtered out if they were not quantified for at least one condition. These are the final ratios $\log_2(R)$ that are reported. The information on the “Gene Name” and “Name” was linked back from the FASTA database. For all comparisons between different MaxQuant runs, the data was combined into one table based on the modified residue.

For the competitive experiments, all conditions were further analyzed using Perseus software.^[18] All individual values for each modified residue for the same condition were loaded into Perseus and analyzed using a one-sample t-test against a value of $\log_2(R) = 0$. Conditions with $p < 0.05$ were considered significant and compounds were deemed to engage a certain residue at a specific condition and called a “hit” or a “liganded residue” if the statistical significance was $p < 0.05$ and the median ratio was $\log_2(R) > 2$.

Analysis of Protein Essentiality

Data on the essentiality of proteins in *S. aureus* SH1000 was derived from the parent strain NCTC8325. The data for the database of essential genes (DEG)^[4] was used. It was downloaded from aureowiki (aureowiki.med.uni-greifswald.de). The information was linked to the information in the FASTA database through the “ordered locus name” that is reported in aureowiki and UniProt.^[6] Assignments for all aspartates and glutamates in the FASTA database for SH1000 can be found in Supporting Information, Table S8.

Analysis of Functional Sites

For all entries in the FASTA database for *S. aureus* SH1000 the information in the categories “Active site”, “Binding Site”, “DNA-binding”, “Nucleotide-binding”, “Site” and “Metal-binding” was downloaded from uniprot.org.^[6] This information gives all amino acid residues that are in the respective functional sites. It was determined for all aspartates and glutamates, whether they are in these functional sites or less than six amino acid residues in the primary sequence away from a residue in a functional site. If this is the case for any kind of functional site, the aspartate or glutamate is considered as being at a functional site. Assignments for all aspartates and glutamates in the FASTA database for SH1000 can be found in Supporting Information, Table S8.

Analysis of Functional Protein Classes

Gene Ontology terms^[5] for all entries in the FASTA database for *S. aureus* SH1000 in the category “GO - Molecular function” were downloaded from uniprot.org.^[6] For each term that was present in the database at least once, a functional class was annotated manually. The list of all manual annotations can be found in Supporting Information, Table S8. In this way, each term for each protein was assigned to a functional class. If a protein was only associated with terms from one functional class, it was assigned to that functional class. If a protein was associated with terms from different functional classes, the functional class of the protein was assigned according to this order of priority: enzyme, modulator / scaffolding / adaptor, receptor / transporter / channel and then gene expression / nucleic acid-binding. If no functional class was assigned to any of the terms, the protein was classified as “not assigned”. Assignments for all aspartates and glutamates in the FASTA database for SH1000 can be found in Supporting Information, Table S8.

Cloning

An *N*-terminal 6xHis-tagged construct of *S. aureus* nicotinate phosphoribosyltransferase (UniProt code Q2G235) was cloned into the pET28a+ expression vector using standard techniques based on polymerase chain reaction (PCR), restriction digest and ligation. Genomic DNA was extracted from 2 mL *S. aureus* SH1000 culture ($OD_{600} = 1$) with a Bacterial DNA Kit (peqlab) and a Nucleo Spin Microbial DNA Kit (Macherey-Nagel). PCRs were carried out in a CFX96 Real-time System in combination with a C1000 Thermal Cycler (BioRad). The PCR mixture contained 10 μ L GC or HF buffer (NEB), 1 μ L dNTP mix (10 mM), 2.5 μ L forward primer (10 μ M, 5'-CTGCAGCATATGTTACATAATGACTTATATCAAATAATATG-3'), 2.5 μ L reverse primer (10 μ M, 5'-TTAGCGGATCCTTACTCATTATCTTCTCCATCTCC-3'), 1 μ L genomic DNA as template (0.1-1 ng), 0.5 μ L Phusion High Fidelity DNA polymerase (NEB) and 32.5 μ L ddH₂O. After initial denaturation (98 °C, 30 s), the mixtures underwent 35 cycles of denaturation (98 °C, 30 s), annealing (66 °C, 30 s) and extension (72 °C, 90 s), before a final

extension (72 °C, 10 min). PCR products were purified by agarose gel electrophoresis on a 1% agarose gel run at 90 V for 50 min. After extraction using a gel extraction kit (VWR, catalog number 101318-972) according to the manufacturer's instructions, 500 ng of the PCR products were digested in Cut Smart buffer (NEB) at 37 °C for 90 min using 5 units of the restriction enzymes BamHI and NdeI, each. pET28a+ vector was extracted from NEB5α *E. coli* using a NucleoSpin Plasmid EasyPure kit. 500 ng vector was digested in Cut Smart buffer (NEB) in a total volume of 50 µL at 37 °C for 1 h using 5 units of BamHI and NdeI, each. In order to dephosphorylate the ends of the restriction sites, 5 units Antarctic phosphatase (1 µL, NEB), 6 µL 10x Antarctic phosphatase buffer (NEB) and 3 µL water were added and digestion was continued for another 30 min. 140 fmol digested pET28a+ vector and 420 fmol digested insert were ligated using 1 µL Quick Ligase (NEB) in Quick Ligase buffer (NEB) in a total volume of 30 µL. The ligation mixture was incubated at room temperature for 10 min and 10-15 ng of the ligation product were subsequently transformed into XL1-blue chemically competent cells (Agilent). For this, 50 µL competent cells were thawed on ice, cautiously mixed with the respective DNA and kept on ice for 30 min. Afterwards, the cells were heat-shocked at 42 °C for 45 s and chilled on ice for 5 min. 500 µL SOC (super optimal broth with catabolite repression) medium were added and the cells incubated at 37 °C, 200 rpm for 1 h. The cells were harvested by centrifugation (6,000 ×g, 2 min), resuspended in SOC medium and plated on LB agar plates containing 250 µg/mL kanamycin. For preparation of the plasmids, 5 mL of LB medium containing 250 µg/mL kanamycin were inoculated with single colonies of the transformation and incubated at 37 °C overnight. Plasmid DNA was prepared using a NucleoSpin Plasmid EasyPure kit (Macherey-Nagel) according to the manufacturer's instructions and sequenced by GeneWiz.

Expression and Purification of Recombinant Nicotinate phosphoribosyltransferase

Chemically competent *E. coli* BL21(DE3) cells were transformed with the respective plasmid following the protocol given in the section "Cloning". For protein overexpression, 20 mL LB medium containing 250 µg/mL kanamycin were inoculated with single colonies or cryostocks of transformed *E. coli* BL21(DE3) cells and incubated at 37 °C, 200 rpm overnight. 1 L LB medium containing 250 µg/mL kanamycin was inoculated with the overnight culture and incubated at 37 °C, 200 rpm. The proteins were expressed overnight at 18 °C after induction at $OD_{600} = 0.5$ with 1 mM isopropyl-β-D-thiogalactoside. Cells were harvested (6,000 rpm, 4 °C, 10 min), washed with 30 mL PBS, resuspended in 20 mL wash buffer 1 (20 mM Tris-HCl, pH = 8.0, 150 mM NaCl, 10 mM imidazole) and lysed by sonication (Bandelin Sonoplus) under constant cooling with ice. Lysate was cleared by centrifugation (18,000 rpm, 30 min, 4 °C) and the supernatant was purified.

Crude soluble lysate was loaded onto 2 mL of Ni-NTA-agarose beads (Qiagen) equilibrated with wash buffer 1 and flow-through was collected. The column was sequentially washed with 10 column volumes of wash buffer 1, 10 column volumes of wash buffer 2 (20 mM Tris-HCl, pH = 8.0, 1 M NaCl, 10 mM imidazole) and 10 column volumes of wash buffer 3 (20 mM Tris-HCl, pH = 8.0, 150 mM NaCl, 20 mM imidazole). The protein was eluted in 5 mL fractions using elution buffer (20 mM Tris-HCl, pH = 8.0, 150 mM NaCl, 300 mM imidazole). 50 µL of each collected fraction were mixed with 50 µL 2x Laemmli buffer and analyzed by SDS-PAGE with Coomassie staining. Fractions that contained the purified protein were concentrated using centrifugal filters (Merck) with a 30 kDa cut-off. During the concentration procedure,

elution buffer was exchanged for storage buffer (20 mM Tris-HCl, pH = 8.0, 150 mM NaCl). Purity of the isolated protein was furthermore analyzed by analytical size-exclusion chromatography, which was performed at 4 °C on an ÄKTA Purifier 10 system (GE Healthcare) using a Superdex 200 10/300 GL column (GE Healthcare) and monitoring at 280 nm. 200 μ L 10 μ M protein samples in storage buffer were loaded into a 500 μ L loop and eluted with storage buffer. Additionally, the protein was analyzed by IPMS.

Intact Protein MS (IPMS) Experiments

High-resolution IPMS was performed to validate the identity of the expressed protein and to measure the degree of covalent modification with **6**. In order to measure the degree of covalent modification with **6**, 120 μ L 1 μ M nicotinate phosphoribosyltransferase were incubated with 500 μ M **6** (6 μ L of a 10 mM stock in DMF) for All samples were measured on a Dionex Ultimate 3000 HPLC system coupled to an LTQ FT Ultra (Thermo) mass spectrometer with an electrospray ionization source (spray voltage 4.0 kV, tube lens 110 V, capillary voltage 48 V, sheath gas 60 a.u., aux gas 10 a.u., sweep gas 0.2 a.u.). Prior to measurement, samples were desalted with a Massprep desalting cartridge (Waters). The mass spectrometer was operated in positive ion mode collecting full scans at high resolution ($R = 200,000$) in a range of $m/z = 600$ -2000 Th. The protein spectra were analyzed using the UniDec software.^[19] The protein spectra were processed ($m/z = 600$ -2000 Th; no subtraction, no smoothing; bin every 0.2) and deconvoluted (charge range 1-100; mass range 10,000-100,000 Da; sample mass every 0.5 Da; Peak FWHM 0.1 Th; Gaussian peak function).

Reductive Dimethyl Labeling Experiments for nicotinate phosphoribosyltransferase

Two samples of 100 μ L recombinant nicotinate phosphoribosyltransferase (1 μ M) were treated with 10 μ L compound **6** (5 mM stock in DMF, 500 μ M final concentration) or DMF as a control for 2 h at room temperature. The samples were precipitated by addition into 800 μ L cold acetone and stored at -20 °C overnight. The precipitates were separated by centrifugation at 13,400 $\times g$ for 10 min at 4 °C and washed twice with 1 mL cold methanol. The pellets were dissolved in 80 μ L 8 M urea in TEAB with sonication. 10 mM DTT (4 μ L of 200 mM stock in water) was added and the reaction was incubated at 37 °C for 45 min following which 20 mM iodoacetamide (4 μ L of 400 mM stock in water) was added and the reaction incubated for 30 min at 25 °C. 10 mM DTT (4 μ L of 200 mM stock in water) was added and the reaction was incubated at 25 °C for 30 min. The samples were then diluted with TEAB (240 μ L) and to this was added trypsin (4 μ L, 0.5 mg/mL) and the samples were allowed to digest over night at 37 °C with shaking. Reductive dimethylation was performed as previously described.^[20] Briefly, DMF-treated samples were labeled with 12 μ L 2% heavy formaldehyde (¹³C, D₂) and compound-treated samples with 12 μ L 2% light formaldehyde (¹²C, H₂) and 12 μ L 0.6 M sodium cyanoborohydride. After 1 h at room temperature with shaking, the reactions were quenched by addition of 48 μ L 1% NH₄OH for 10 min at room temperature followed by acidification with 24 μ L formic acid. The samples were desalted using 50 mg SepPak Cartridges. For this purpose, the columns were washed twice with 1 mL acetonitrile, once with 1 mL elution buffer (80% acetonitrile, 0.5% formic acid), three times with 1 mL 0.1% TFA. The samples were loaded and the columns washed with three times 1 mL 0.1% TFA and once with 500 μ L 0.5% formic acid. Peptides were eluted with three times 250 μ L elution buffer. The solvent was removed in a vacuum concentrator by rotating

at 30 °C for approximately 5 h. Samples were dissolved in 30 μ L 1% formic acid in water by sonification for 5 min. Samples were filtered through filters (Merck, UVC30GVNB) washed with the same solvent and transferred into MS sample vials. Samples were stored at -20 °C until measurement. 3 μ L of the samples were analyzed using a Q Exactive Plus mass spectrometer (ThermoFisher) coupled to an Ultimate 3000 nano HPLC system (Dionex). Samples were loaded on an Acclaim C18 PepMap100 trap column (75 μ m ID x 2 cm) and washed with 0.1% TFA. The subsequent separation was carried out on an Acclaim C18PepMapRSLC column (75 μ m ID x 50 cm) with a flow of 300 nL/min and buffer A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. Analysis started with washing in 5% B for 7 min followed a gradient from 5% to 40% buffer B in 105 min, an increase to 60% B in 10 min and another increase to 90% B in 10 min. 90% B was held for 10 min, then decreased to 5% in 0.1 min and held at 5% for another 9.9 min. The Q Exactive Plus mass spectrometer was run in a TOP10 data-dependent mode. In the orbitrap, full MS scans were collected in a scan range of m/z = 300–1500 Th at a resolution of 70,000 and an AGC target of 3e6 with 80 ms maximum injection time. The most intense peaks were selected for MS2 measurement with a minimum AGC target of 1e3 and isotope exclusion and dynamic exclusion (exclusion duration: 60 s) enabled. Peaks with unassigned charge or a charge of +1 were excluded. MS2 spectra were collected at a resolution of 17,500 aiming at an AGC target of 1e5 with a maximum injection time of 100 ms. Isolation was conducted in the quadrupole using a window of 1.6 Th. Fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 27%) and finally detected in the orbitrap.

MS raw data were analyzed using MaxQuant software (version 1.6.1.0).^[2] Standard settings were used with the following changes and additions: The modified FASTA databases with addition of *S. aureus* nicotinate phosphoribosyltransferase (UniProt code Q2G235) to the FASTA database of *E. coli* K12 was used ("EC33-2_plus_Q2G235.fasta"). Labels for reductive demethylation were set on lysine and the *N*-terminus of each peptide (C₂H₄ for light labels and ¹³C₂D₄ for heavy labels). A multiplicity of 2 was set and a maximum number of labeled amino acids of 4. The digestion enzyme was set to Trypsin/P with maximum number of missed cleavages of 2. Variable modification with **6** was allowed on aspartates and glutamates (C₈H₈N₂). The "Re-quantify" option was enabled. Carbamidomethyl (C₂H₃NO) was used as fixed modification on cysteine. Contaminants were included. Peptides were searched with a minimum peptide length of 7 and a maximum peptide mass of 4,600 Da. "Second peptides" and "Dependent peptides" were disabled and the option "Match between run" was enabled with a Match time window of 0.7 min and an alignment window of 20 min. An FDR of 0.01 was used for Protein FDR, PSM FDR and XPSM FDR. Technical replicates were analyzed in the same MaxQuant analysis.

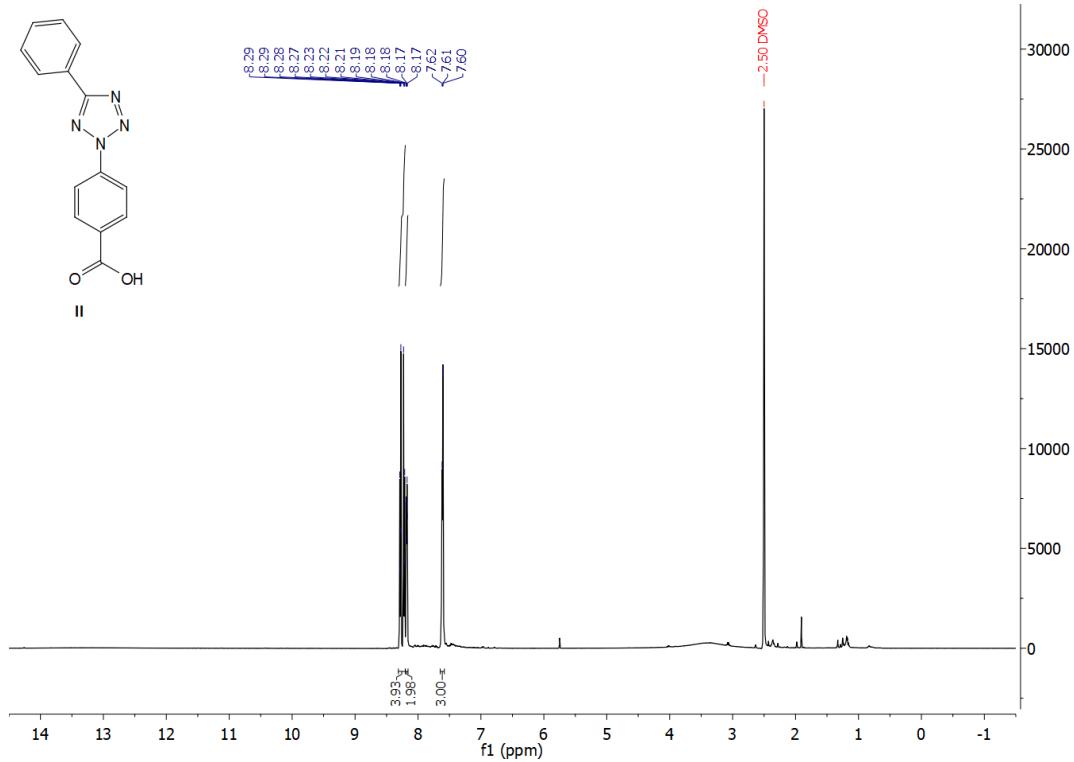
All sites modified with **6** in the protein nicotinate phosphoribosyltransferase were reported. For quantification, the "peptides.txt" file of the MaxQuant analysis was used. Only peptide sequences belonging to nicotinate phosphoribosyltransferase were further analyzed. For these peptides, the value "Ratio H/L" was used as ratio *R*. For residues that occur in more than one peptide, the median of all ratios was used. In the final data, only ratios for aspartate and glutamate residues are reported.

Activity Assay for Nicotinate phosphoribosyltransferase

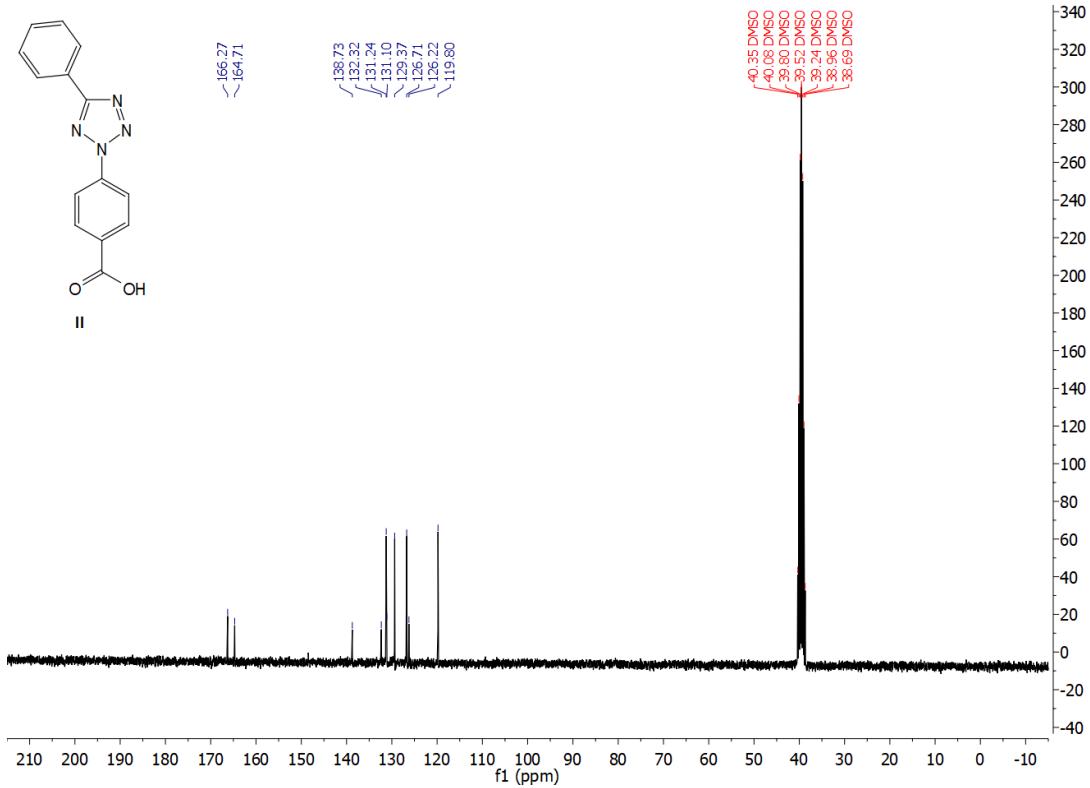
10 μ L recombinant nicotinate phosphoribosyltransferase (2.8 μ M in storage buffer) or only storage buffer were preincubated with 0.5 μ L **6** (10 mM in DMF) or DMF as control for 2 h at room temperature. To 1.8 μ L of this mix were added 50 μ L 2x ATPase mix (2x ATPase mix: 4 mM phosphoenolpyruvate, 200 mM HEPES, 400 mM KCl, 40 mM MgCl₂, 2 mM DTT, 2 mM NADH, 10% glycerol, 100 U/mL lactate dehydrogenase from rabbit and 100 U/mL pyruvate kinase from rabbit), 34.2 μ L water, 2 μ L 10 mM niacin and 2 μ L 10 mM 5-phospho- α -D-ribose-1-diphosphate in a 96 well plate and the enzymatic reaction was started by addition of 10 μ L 2 mM ATP. The absorbance at $\lambda = 340$ nm was measured at $T = 25$ °C over time. Measurements were performed using a Tecan Infinite 200 PRO or Tecan Infinite M Nano plate reader.

NMR Spectra

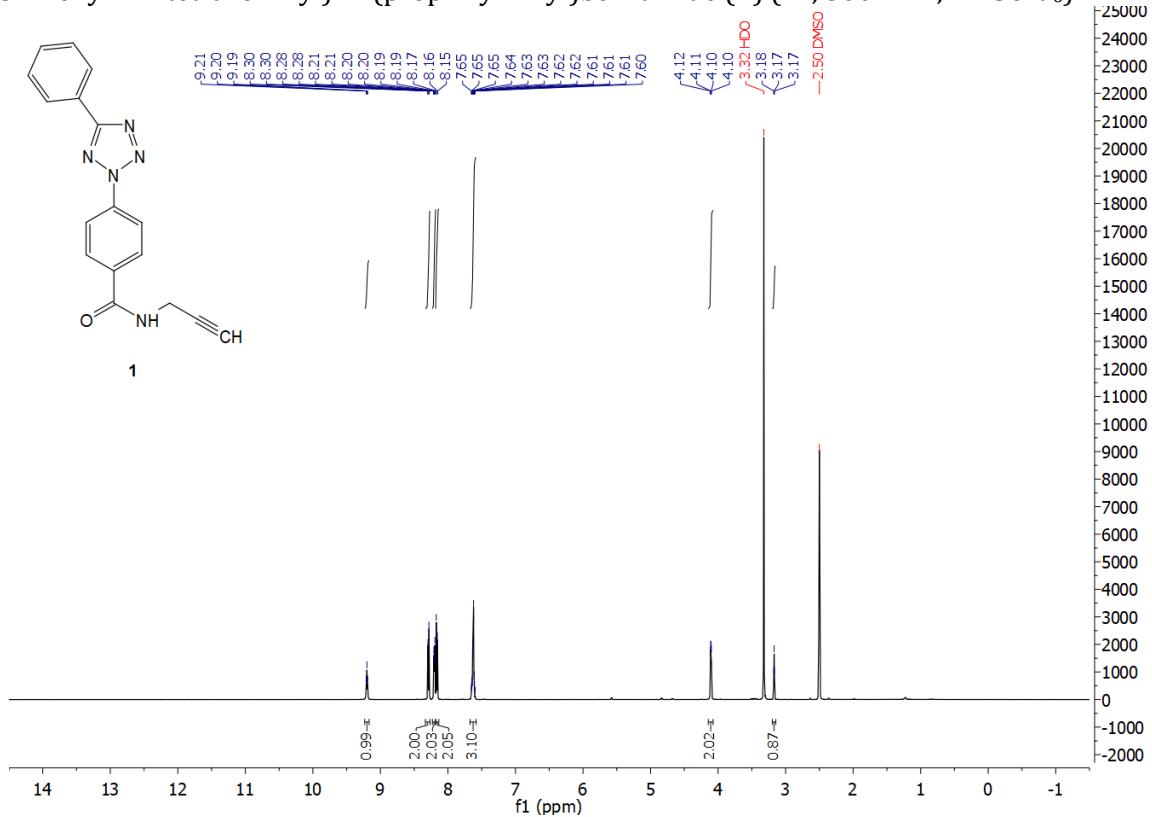
4-(5-Phenyl-2H-tetrazol-2-yl)benzoic acid (**II**) (^1H , 500 MHz, DMSO-d_6):



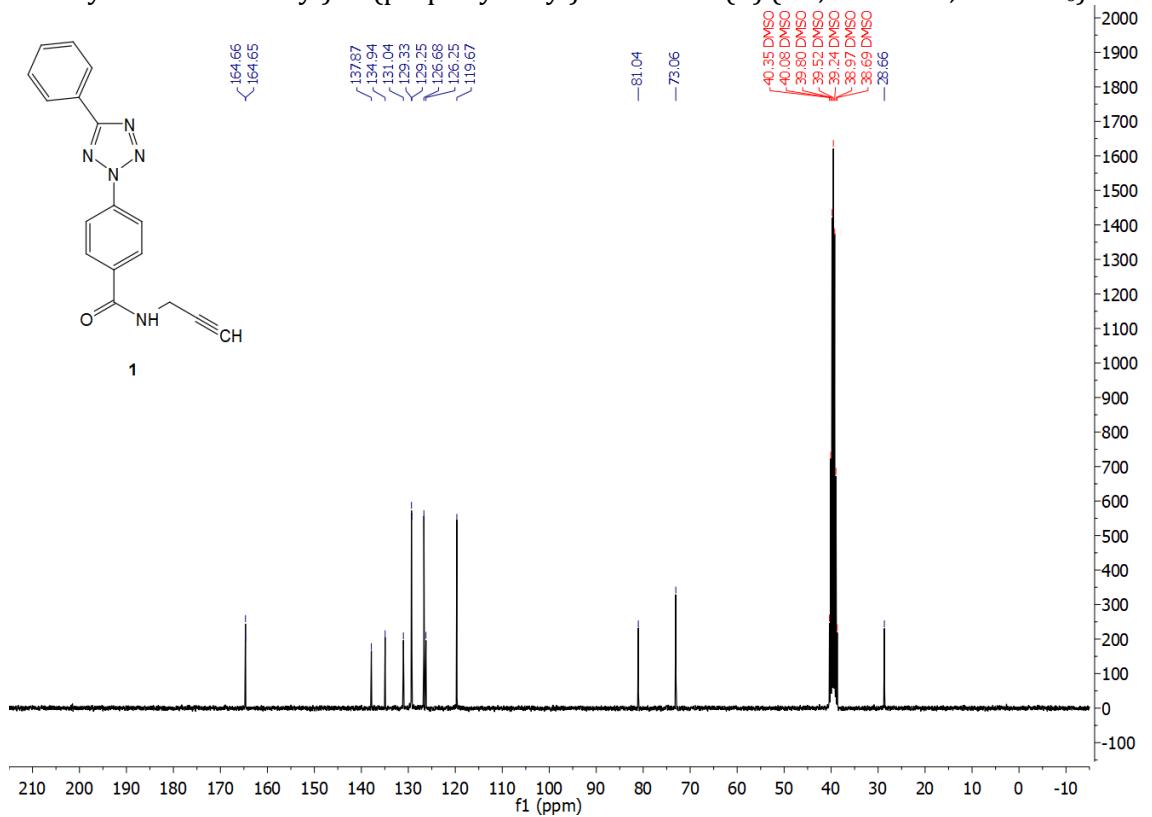
4-(5-Phenyl-2H-tetrazol-2-yl)benzoic acid (**II**) (^{13}C , 75.5 MHz, DMSO-d_6):



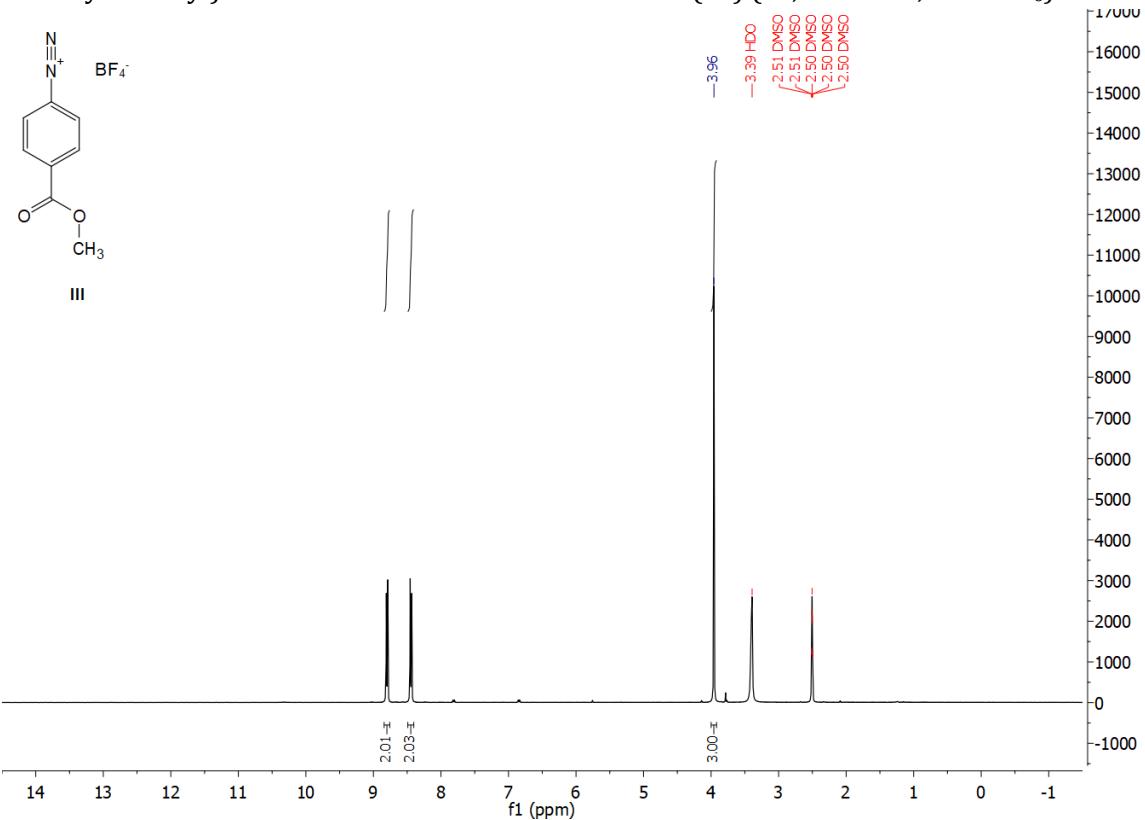
4-(5-Phenyl-2H-tetrazol-2-yl)-N-(prop-2-yn-1-yl)benzamide (**1**) (^1H , 500 MHz, DMSO-d₆):



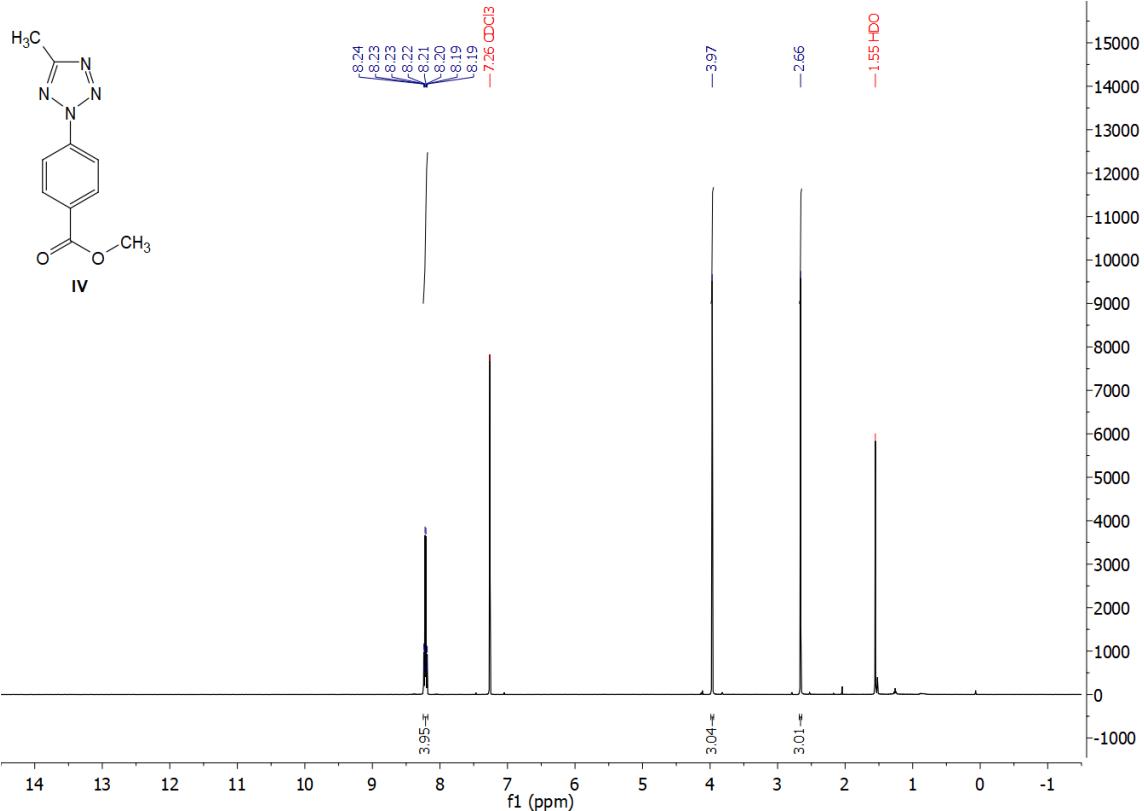
4-(5-Phenyl-2H-tetrazol-2-yl)-N-(prop-2-yn-1-yl)benzamide (**1**) (^{13}C , 75.5 MHz, DMSO-d₆):



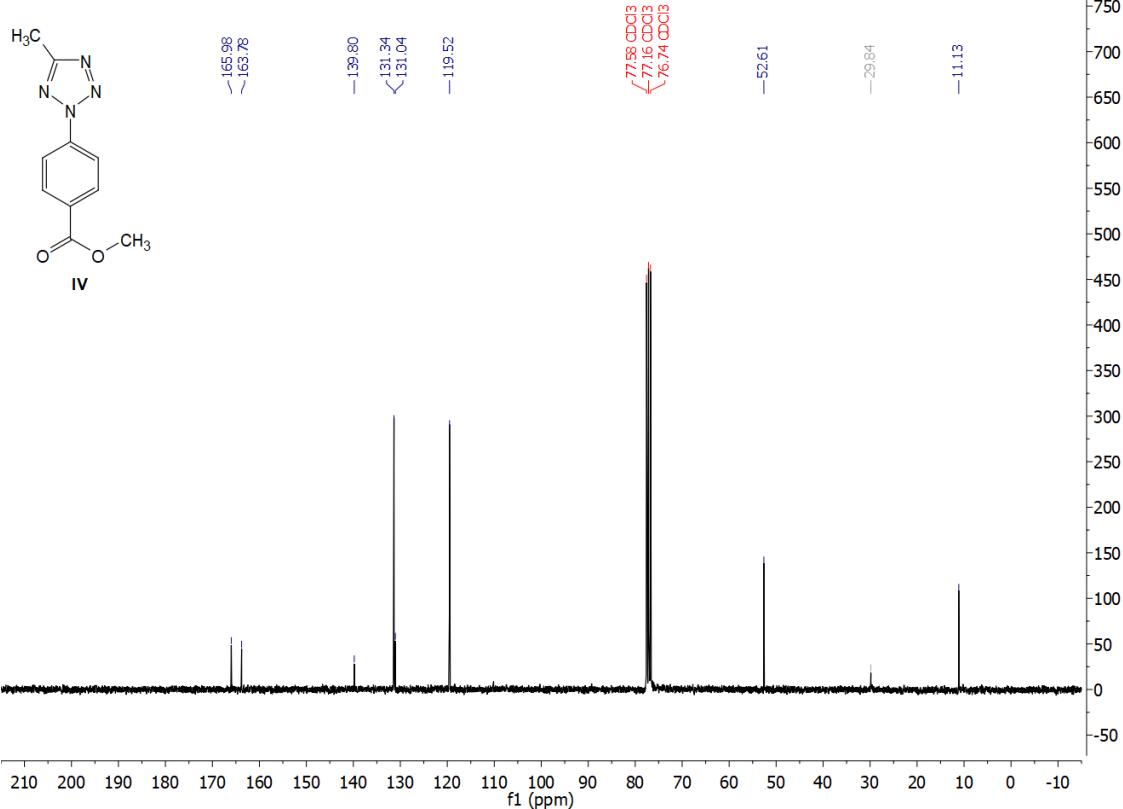
4-(Methoxycarbonyl)benzenediazonium tetrafluoroborate (**III**) (^1H , 400 MHz, DMSO-d_6):



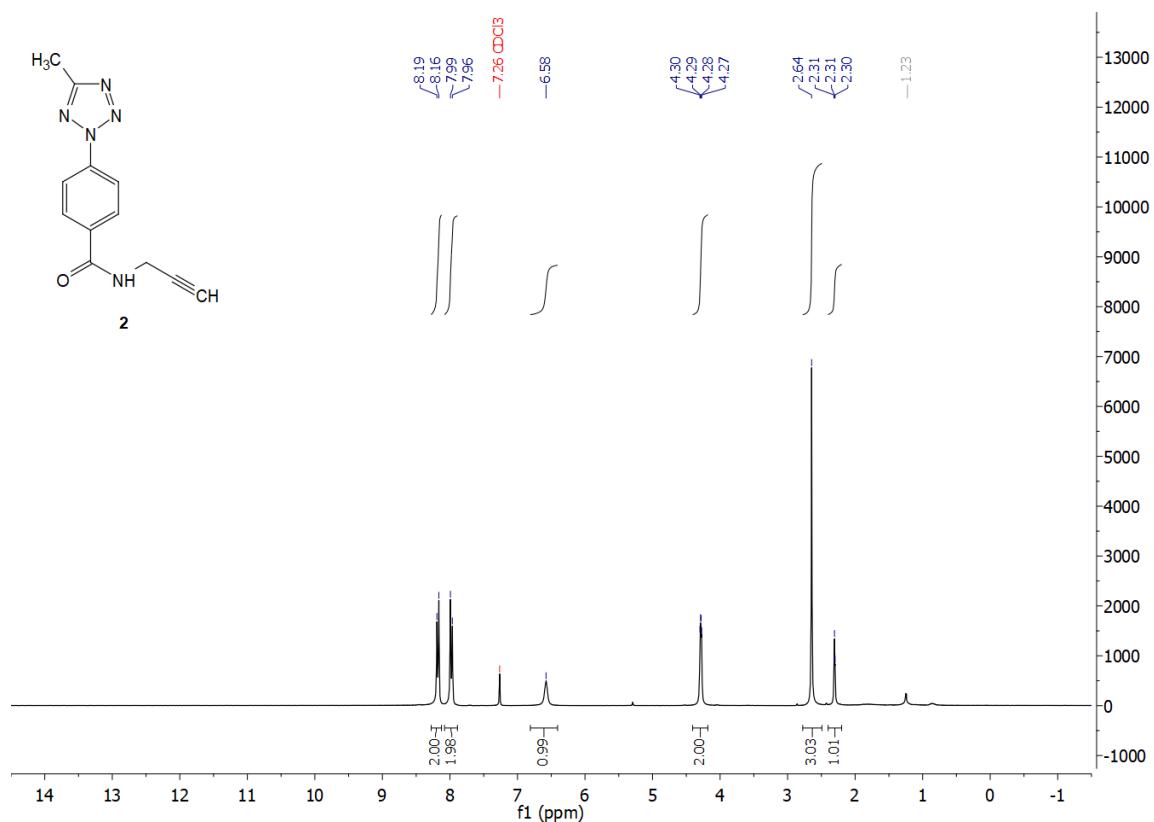
Methyl 4-(5-methyl-2*H*-tetrazol-2-yl)benzoate (**IV**) (^1H , 500 MHz, CDCl_3):



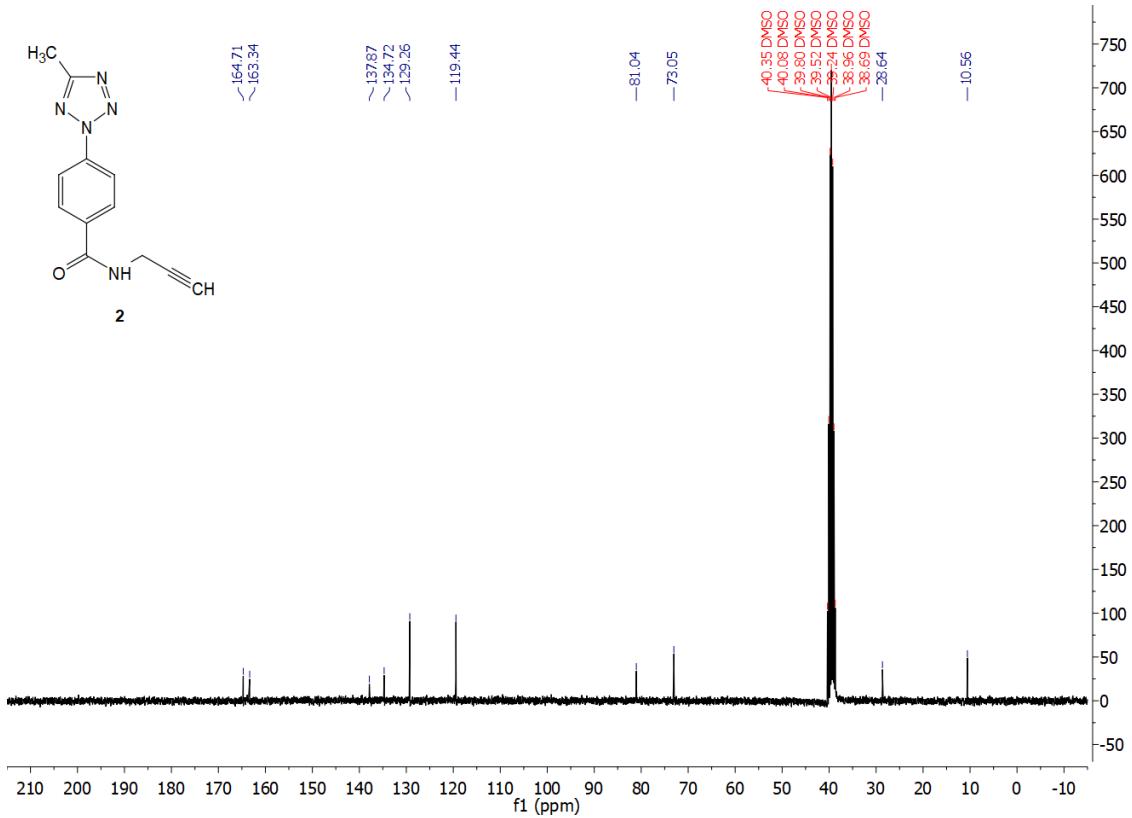
Methyl 4-(5-methyl-2*H*-tetrazol-2-yl)benzoate (**IV**) (^{13}C , 75.5 MHz, CDCl_3):



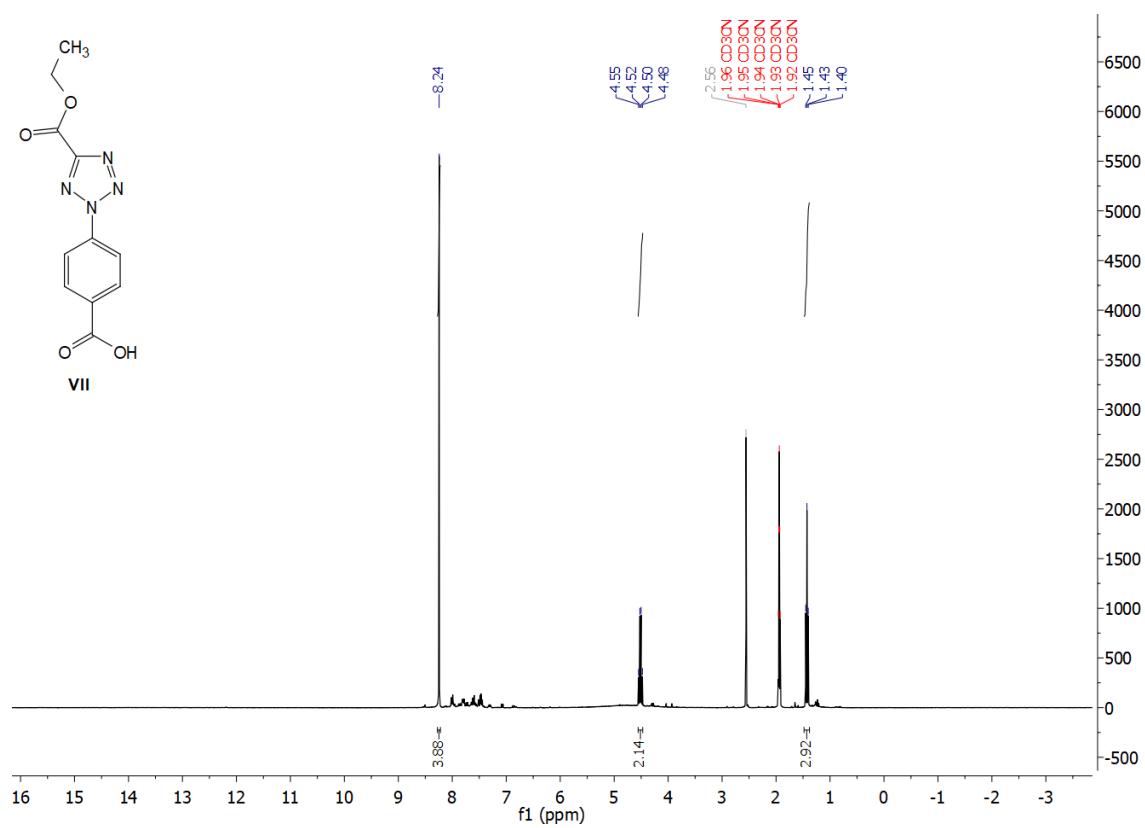
4-(5-Methyl-2*H*-tetrazol-2-yl)-*N*-(prop-2-yn-1-yl)benzamide (**2**) (^1H , 500 MHz, CDCl_3):



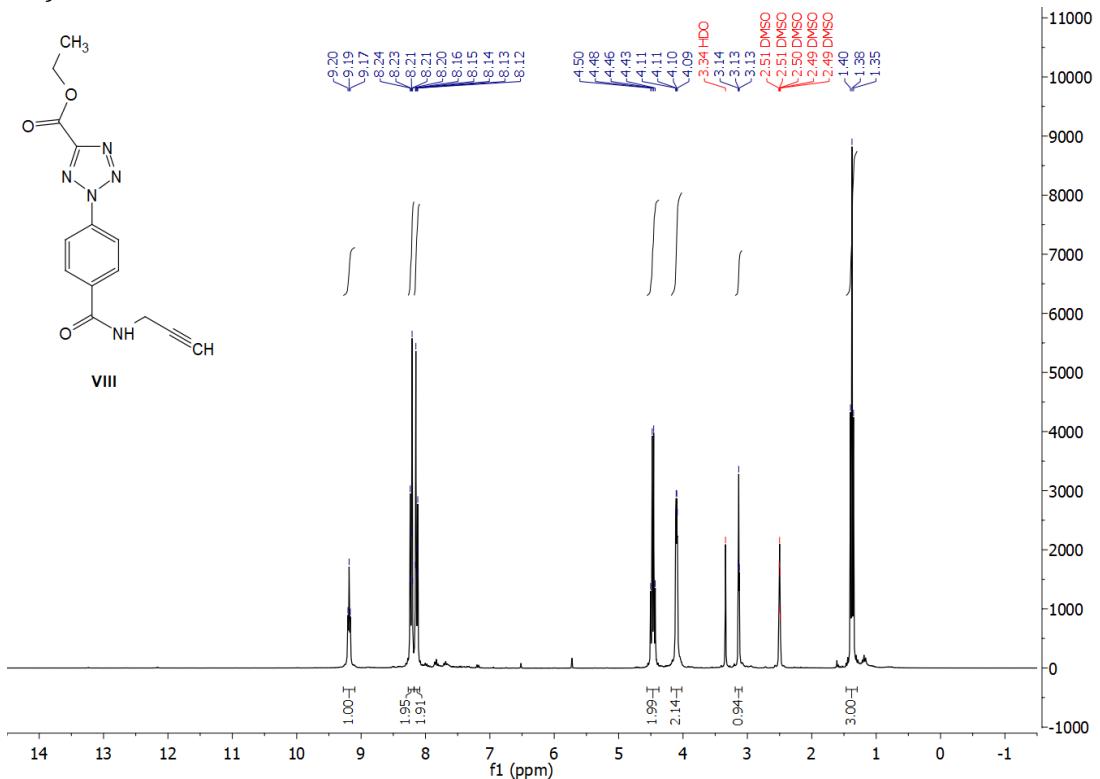
4-(5-Methyl-2*H*-tetrazol-2-yl)-*N*-(prop-2-yn-1-yl)benzamide (**2**) (^{13}C , 75.5 MHz, DMSO-d_6):



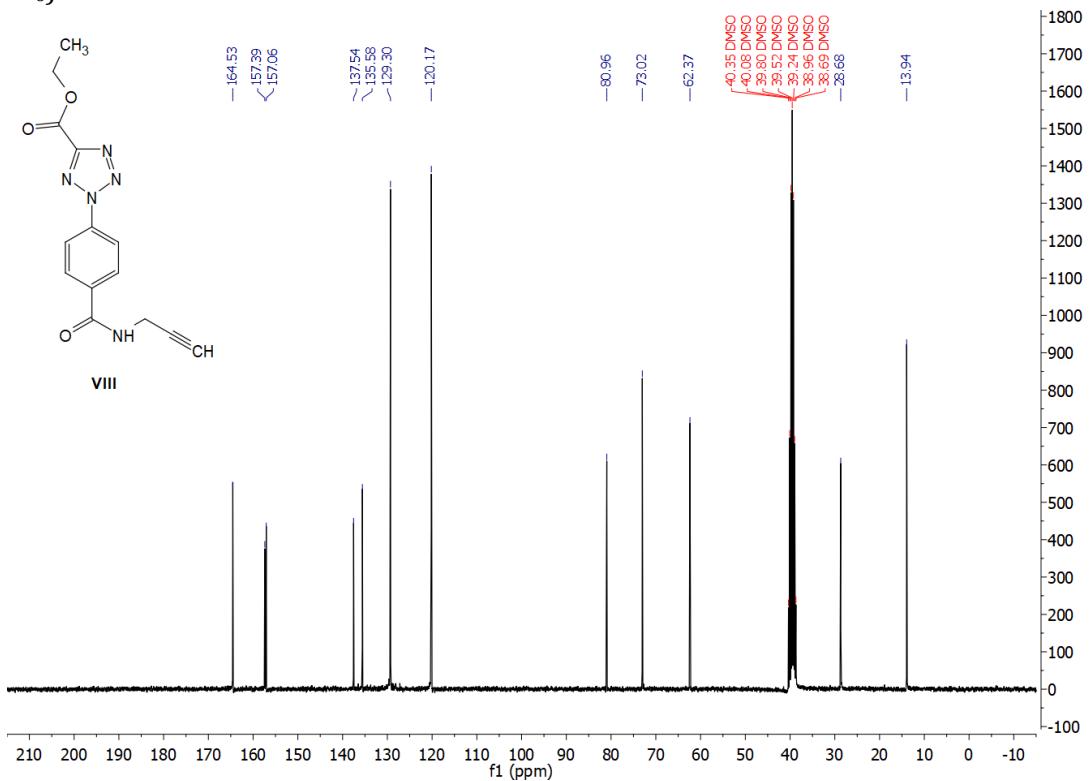
4-(5-(Ethoxycarbonyl)-2H-tetrazol-2-yl)benzoic acid (**VII**) (^1H , 300 MHz, CD_3CN):



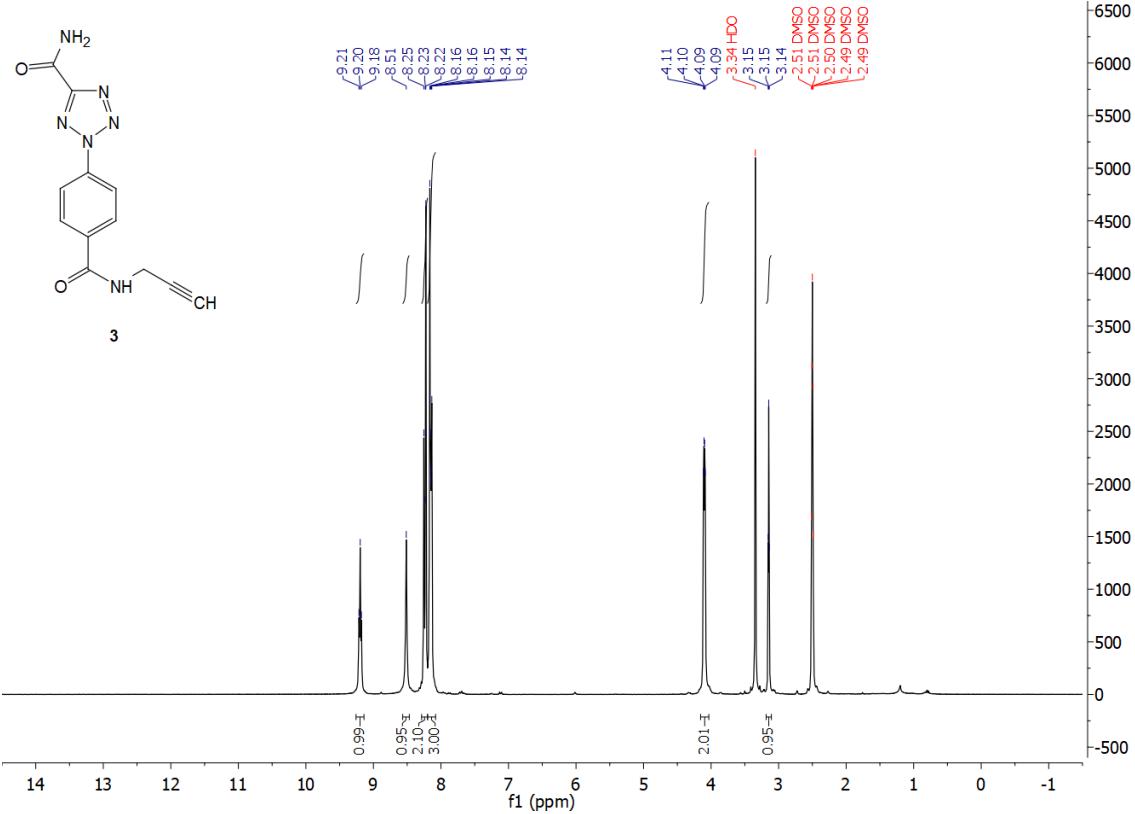
Ethyl 2-(4-(prop-2-yn-1-ylcarbamoyl)phenyl)-2*H*-tetrazole-5-carboxylate (**VIII**) (^1H , 500 MHz, DMSO- d_6)



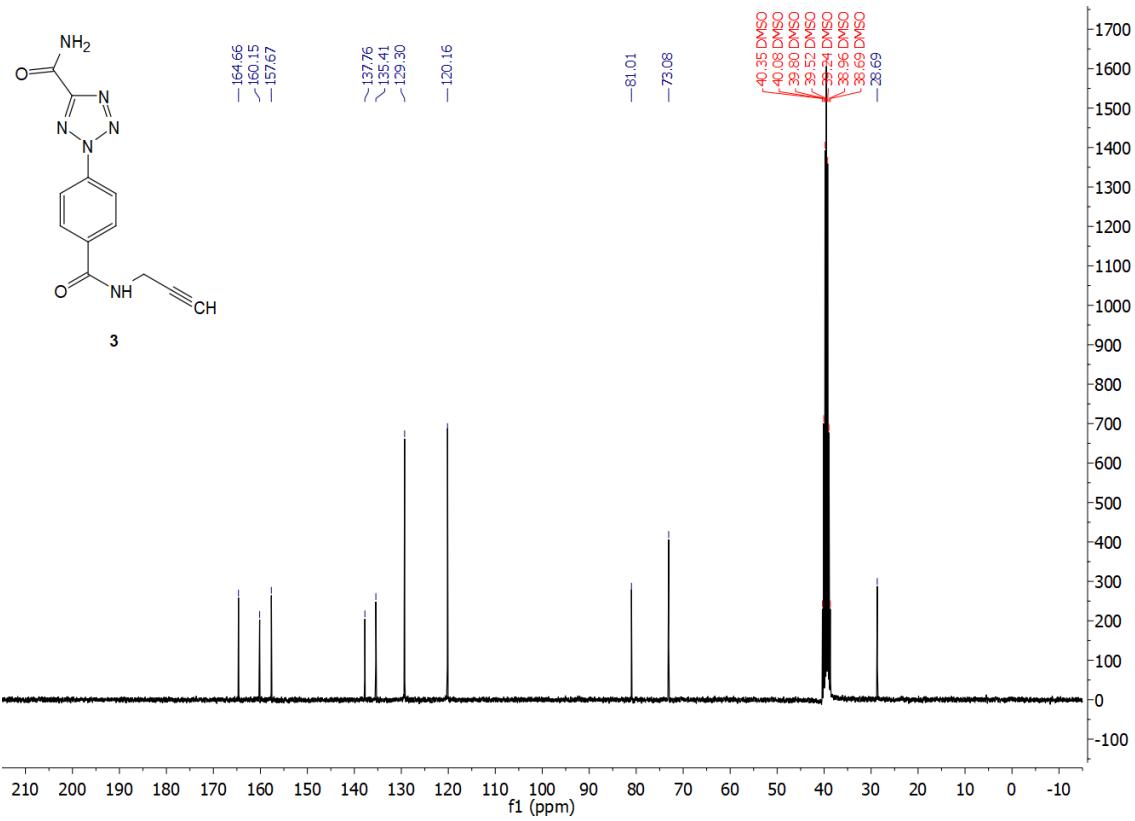
Ethyl 2-(4-(prop-2-yn-1-ylcarbamoyl)phenyl)-2*H*-tetrazole-5-carboxylate (**VIII**): (^{13}C , 75.5 MHz, DMSO- d_6)

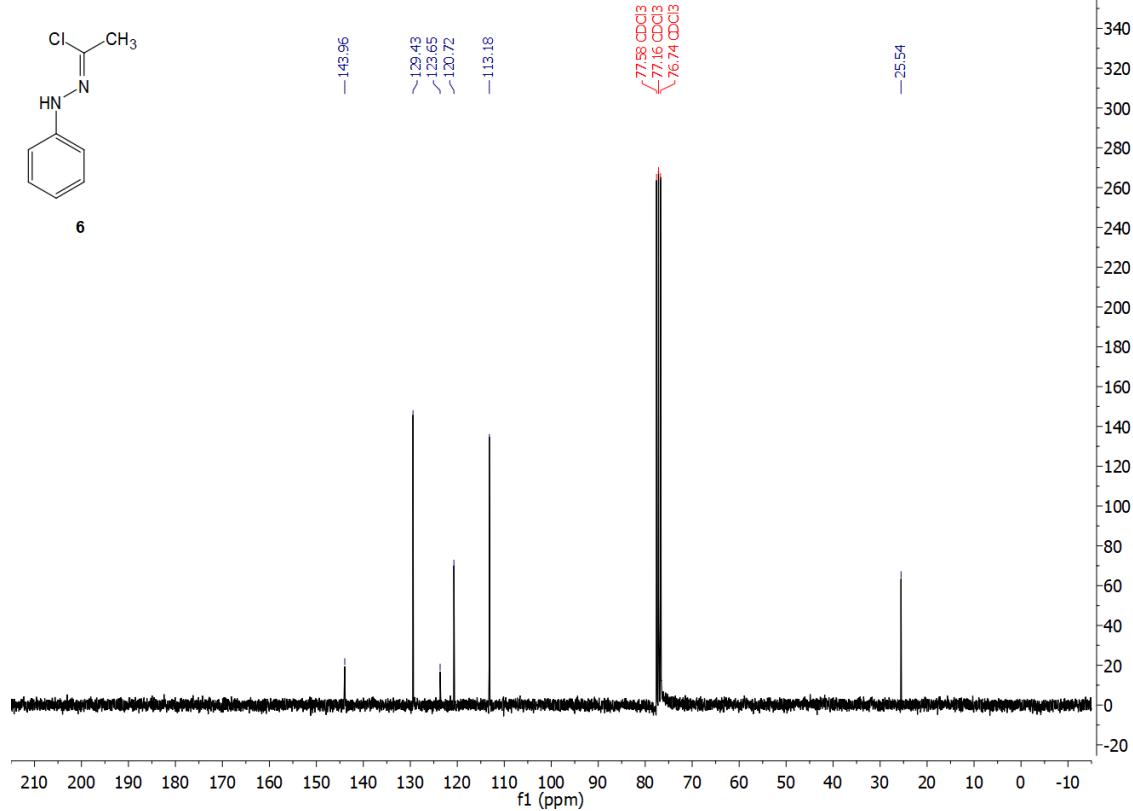
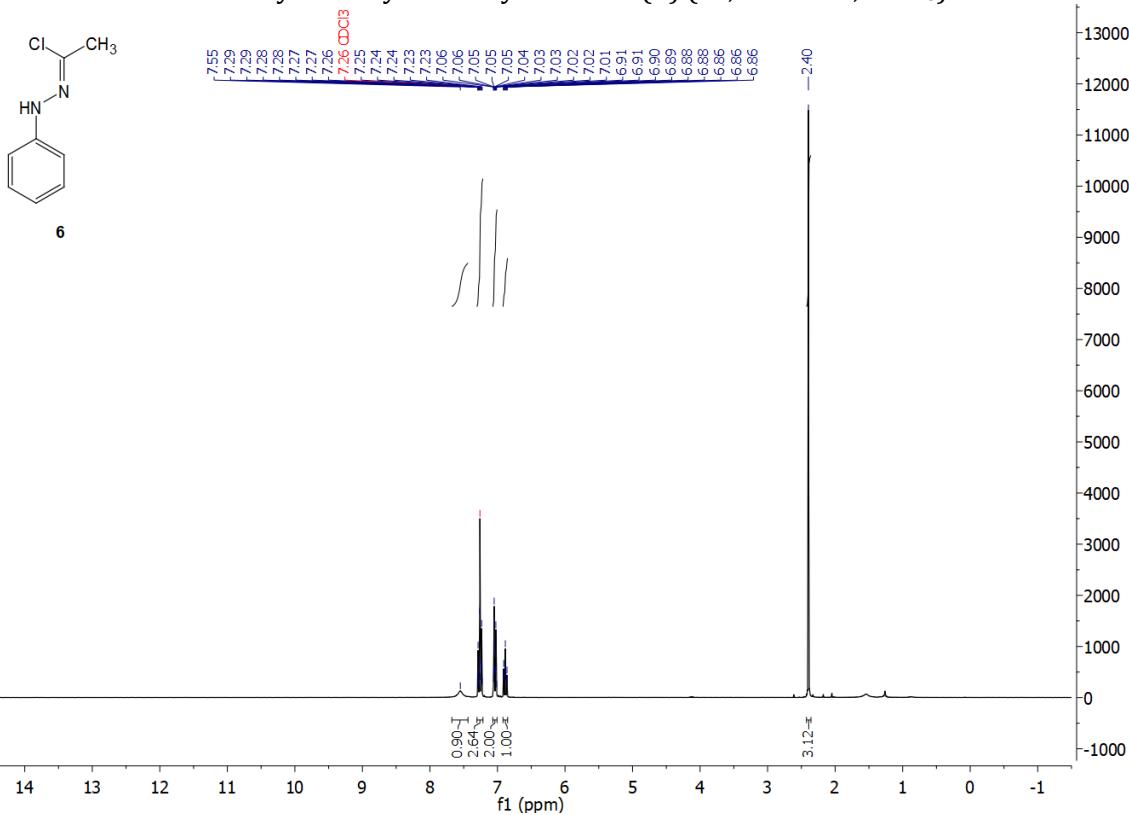


2-(4-(Prop-2-yn-1-ylcarbamoyl)phenyl)-2*H*-tetrazole-5-carboxamide (**3**) (^1H , 500 MHz, DMSO- d_6):



2-(4-(Prop-2-yn-1-ylcarbamoyl)phenyl)-2*H*-tetrazole-5-carboxamide (**3**) (^{13}C , 75.5 MHz, DMSO- d_6):





References

- [1] A. T. Kong, F. V. Leprevost, D. M. Avtonomov, D. Mellacheruvu, A. I. Nesvizhskii, *Nat. Methods* **2017**, *14*, 513-520.
- [2] J. Cox, M. Mann, *Nat. Biotechnol.* **2008**, *26*, 1367-1372.
- [3] S. M. Hacker, K. M. Backus, M. R. Lazear, S. Forli, B. E. Correia, B. F. Cravatt, *Nat. Chem.* **2017**, *9*, 1181-1190.
- [4] R. R. Chaudhuri, A. G. Allen, P. J. Owen, G. Shalom, K. Stone, M. Harrison, T. A. Burgis, M. Lockyer, J. Garcia-Lara, S. J. Foster, S. J. Pleasance, S. E. Peters, D. J. Maskell, I. G. Charles, *BMC Genomics* **2009**, *10*, 291.
- [5] P. Gaudet, M. S. Livstone, S. E. Lewis, P. D. Thomas, *Brief. Bioinform.* **2011**, *12*, 449-462.
- [6] The UniProt Consortium, *Nucleic Acids Res.* **2019**, *47*, D506-D515.
- [7] M. J. Horsburgh, J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, S. J. Foster, *J. Bacteriol.* **2002**, *184*, 5457-5467.
- [8] A. J. O'Neill, *Lett. Appl. Microbiol.* **2010**, *51*, 358-361.
- [9] J. A. Vizcaino *et al.*, *Nat. Biotechnol.* **2014**, *32*, 223-226.
- [10] J. A. Vizcaino, A. Csordas, N. del-Toro, J. A. Dianes, J. Griss, I. Lavidas, G. Mayer, Y. Perez-Riverol, F. Reisinger, T. Ternent, Q. W. Xu, R. Wang, H. Hermjakob, *Nucleic Acids Res.* **2016**, *44*, D447-456.
- [11] K. Cheng, J. S. Lee, P. Hao, S. Q. Yao, K. Ding, Z. Li, *Angew. Chem. Int. Ed.* **2017**, *56*, 15044-15048.
- [12] A. P. Colleville, R. A. J. Horan, N. C. O. Tomkinson, *Org. Process Res. Dev.* **2014**, *18*, 1128-1136.
- [13] M. Ramanathan, Y. H. Wang, S. T. Liu, *Org. Lett.* **2015**, *17*, 5886-5889.
- [14] B. Joseph, P. Rollin, *J. Carbohydr. Chem.* **1993**, *12*, 1127-1138.
- [15] K. M. Backus, B. E. Correia, K. M. Lum, S. Forli, B. D. Horning, G. E. Gonzalez-Paez, S. Chatterjee, B. R. Lanning, J. R. Teijaro, A. J. Olson, D. W. Wolan, B. F. Cravatt, *Nature* **2016**, *534*, 570-574.
- [16] P. R. A. Zanon, L. Lewald, S. M. Hacker, *Angew. Chem. Int. Ed.* **2020**, 2829-2836.
- [17] M. C. Chambers *et al.*, *Nat. Biotechnol.* **2012**, *30*, 918-920.
- [18] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M. Y. Hein, T. Geiger, M. Mann, J. Cox, *Nat. Methods* **2016**, *13*, 731-740.
- [19] M. T. Marty, A. J. Baldwin, E. G. Marklund, G. K. Hochberg, J. L. Benesch, C. V. Robinson, *Anal. Chem.* **2015**, *87*, 4370-4376.
- [20] J. M. Inloes, K. L. Hsu, M. M. Dix, A. Viader, K. Masuda, T. Takei, M. R. Wood, B. F. Cravatt, *Proc. Natl. Acad. Sci.* **2014**, *111*, 14924-14929.