Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary Information

A tailored phosphoaspartate probe unravels CprR as a response regulator in *Pseudomonas aeruginosa* interkingdom signaling

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Supplementary Figures

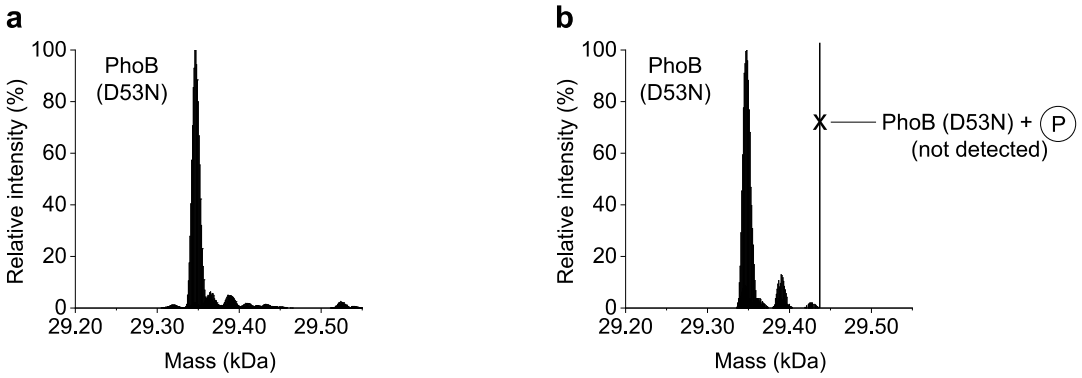

Fig. S1 Confirmation of site-specific *in vitro* phosphorylation of wt *E. coli* PhoB at D53. IPMS analysis of PhoB D53N mutant before (a) and after (b) treatment with acetyl phosphate (AcP). No phosphorylation was observed for PhoB D53N.

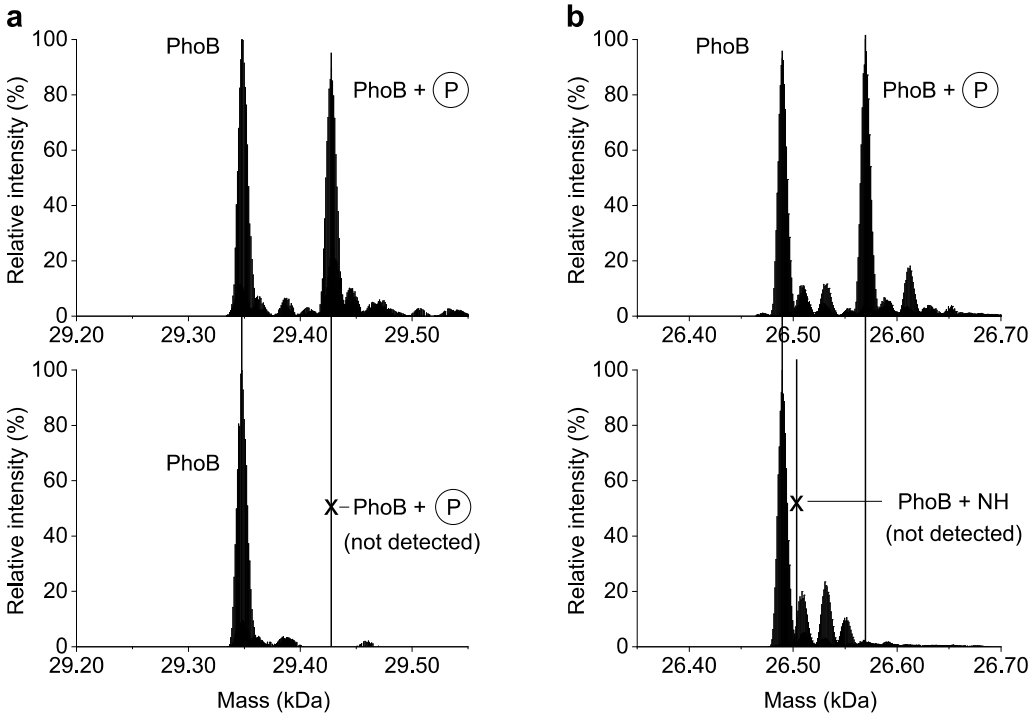

Fig. S2 Hydrolysis of phosphorylated wt PhoB without and with hydroxylamine treatment at pH 7. IPMS analysis of phosphorylated wt PhoB incubated without (a) and with 500 mM hydroxylamine (b).

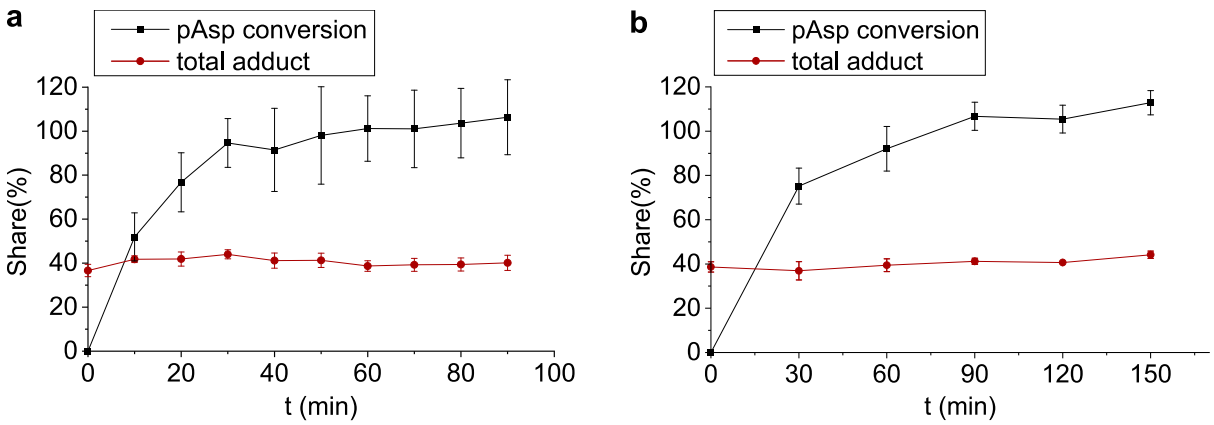

Fig. S3 Kinetics of the reaction of phosphorylated wt PhoB with 500 mM Hydroxylamine (a) or **HA-yne** (b) at pH = 4.

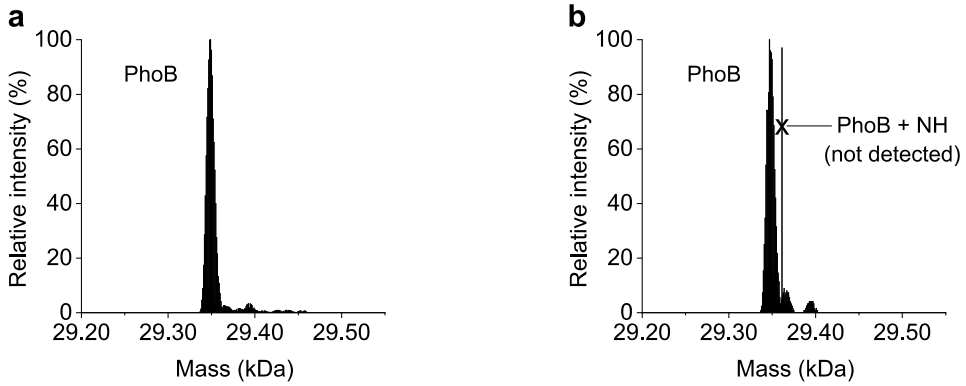

Fig. S4 Control reaction, that unphosphorylated wt PhoB does not react with hydroxylamine. IPMS analysis of unphosphorylated wt PhoB before (a) and after (b) treatment with 500 mM Hydroxylamine. No formation of *N*-hydroxyasparagine was observed.

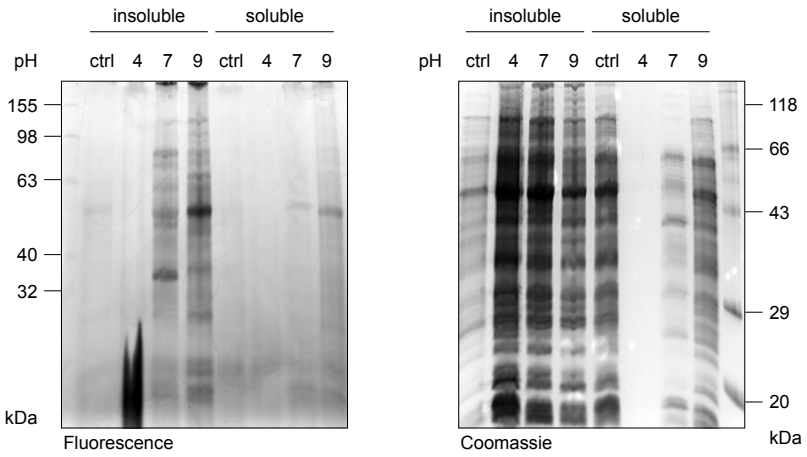

Fig. S5 Optimization of labeling conditions by pH-dependent labeling. Exponentially growing *B. subtilis* were lyzed in the presence of 500 mM **HA-yne** at different pH-values and labeled proteins were clicked to rhodamine azide (ctrl: no **HA-yne**, 20 mM HEPES, pH = 7). SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue. The labeling pattern and protein solubility turned out to be strongly pH dependent.

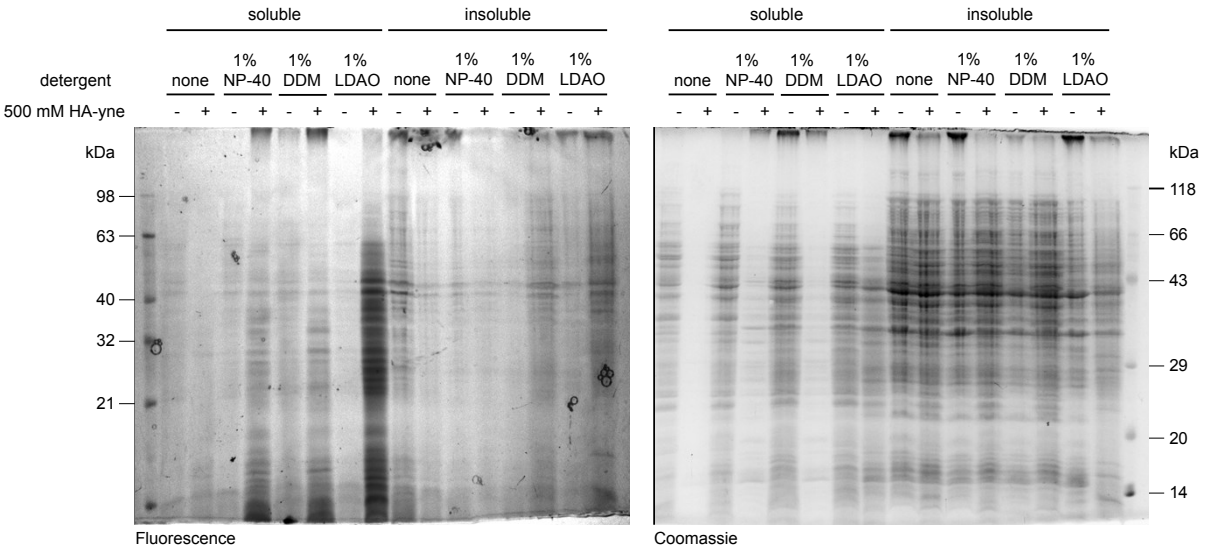

Fig. S6 Optimization of labeling conditions by assessment of detergents. Exponentially growing *B. subtilis* were lyzed in the presence of 500 mM **HA-yne** at pH = 4 in labeling buffer containing different detergents. Labeled proteins were clicked to rhodamine azide and SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue. Addition of 1% (w/v) LDAO revealed the most efficient solubilization of the proteome and the most pronounced labeling pattern.

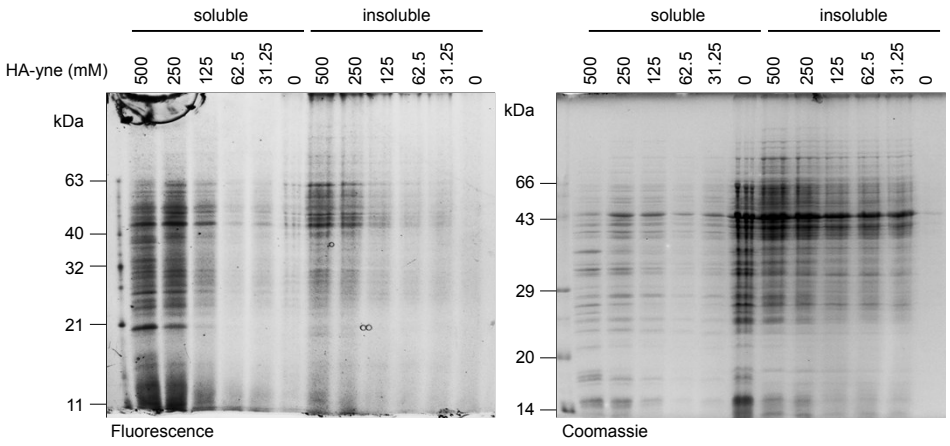

Fig. S7 Optimization of labeling conditions by dose-dependent labeling. Exponentially growing *B. subtilis* were lyzed in the presence of different concentrations of **HA-yne** at pH = 4 in HEPES buffer containing 1% (w/v) LDAO. Labeled proteins were clicked to rhodamine azide and SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue. A probe concentration of 125 mM was chosen for RP-ABPP experiments.

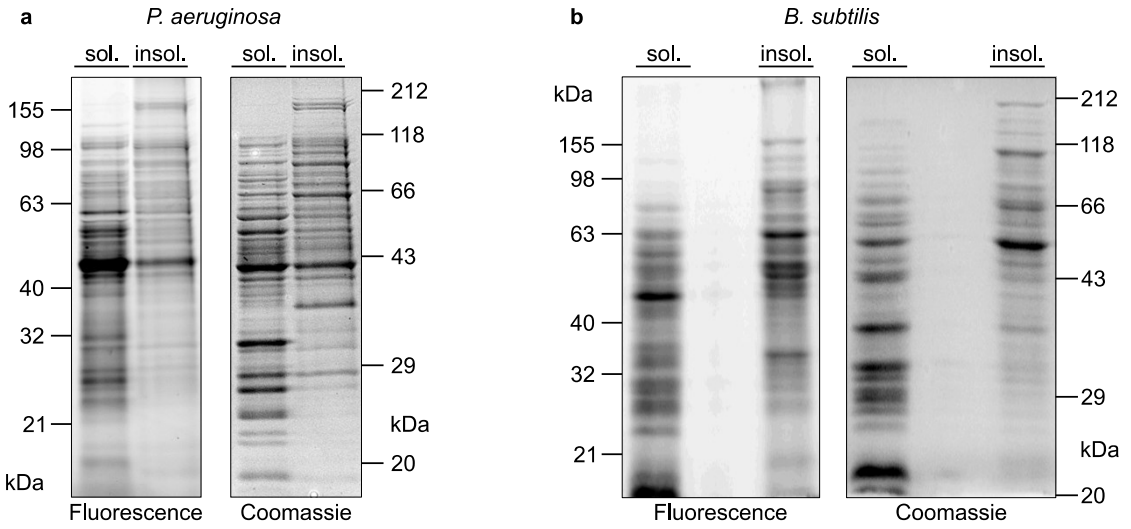

Fig. S8 Labeling of bacterial lysates under optimized conditions. Exponentially growing *P. aeruginosa* (a) and *B. subtilis* (b) were lyzed in the presence of 125 mM **HA-yne** at pH = 4 and labeled proteins were clicked to rhodamine azide. SDS-PAGE analysis was performed by in-gel fluorescence scanning and staining using Coomassie Brilliant Blue.

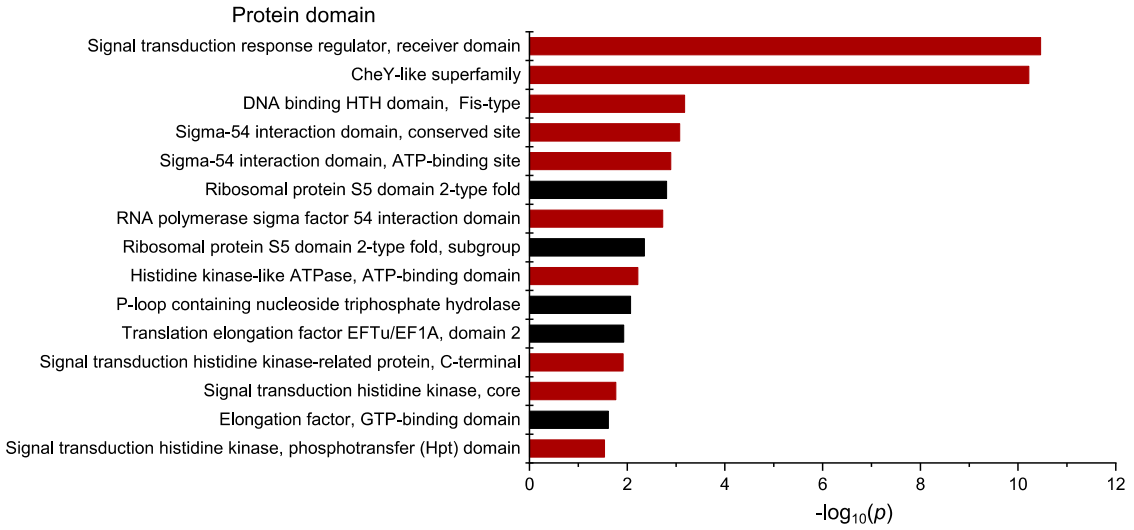

Fig. S9 Enriched protein domains among **HA-yne** modified proteins in *P. aeruginosa* using DAVID[1, 2]. Statistically most significant categories are shown. Phosphoaspartate related protein domains are indicated in red.

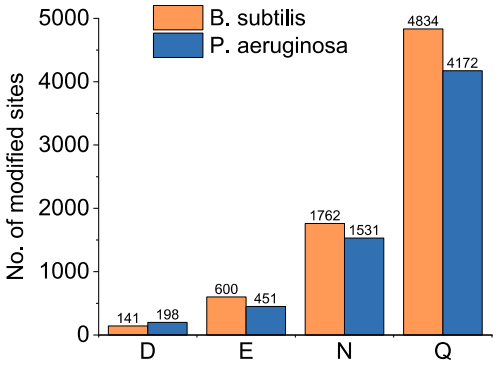

Fig. S10 Distribution of **HA-yne** modified residues. Analysis of the amino acid specificity of **HA-yne** labeled proteomes was performed using MaxQuant software[3] allowing the modification to be either on Asp/Glu (+ 509.33 Da) or Asn/Gln (+ 510.32 Da) residues, respectively. Peptides were only included in the analysis if the Andromeda localization probability for a specific residue exceeded 75%.

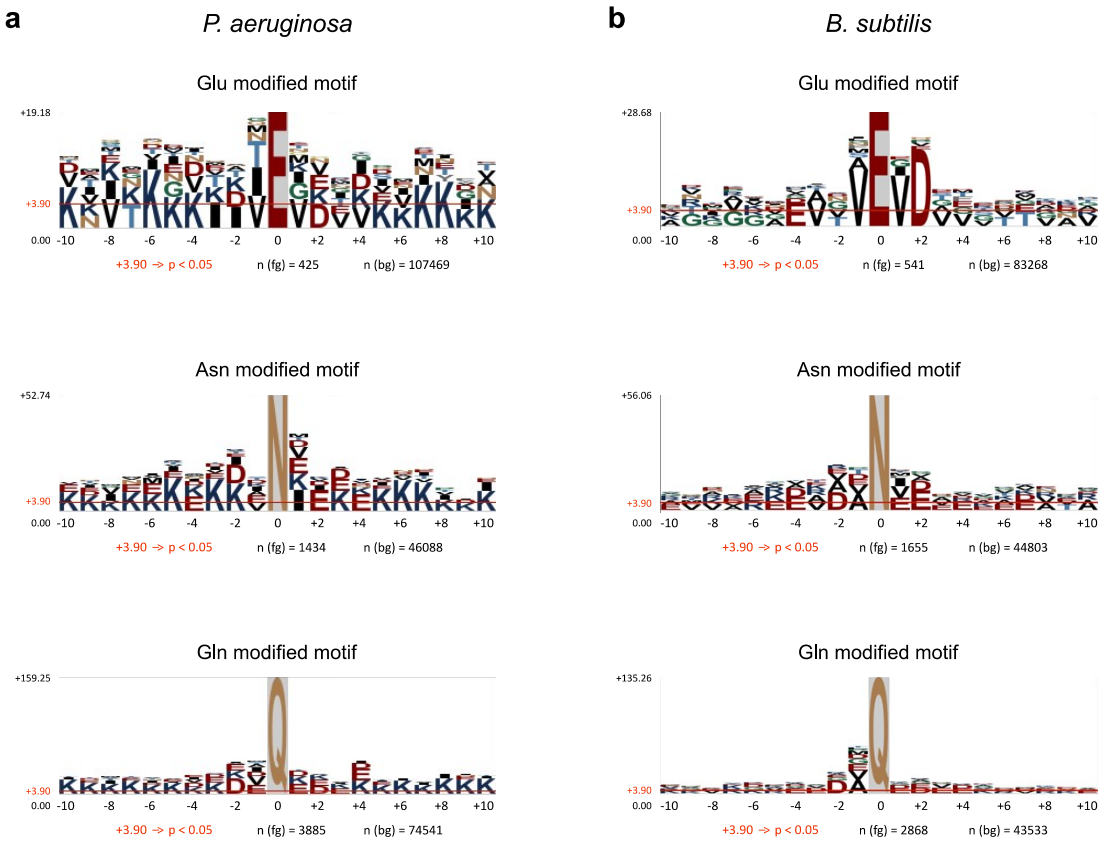

Fig. S11 Comparison of **HA-yne** modified (at Glu, Asn, Gln) sequence motifs in *P. aeruginosa* (a) and *B. subtilis* (b) using pLogo.[4] Residues at positions ranging from -10 to +10 next to the modification site were included in the analysis. **HA-yne** modified sequences (fg) were compared with the complete proteomic background (bg) in *P. aeruginosa* and *B. subtilis* from the UniProt database.[5] Red horizontal bars indicate the Bonferroni-corrected statistical significance (p = 0.05).

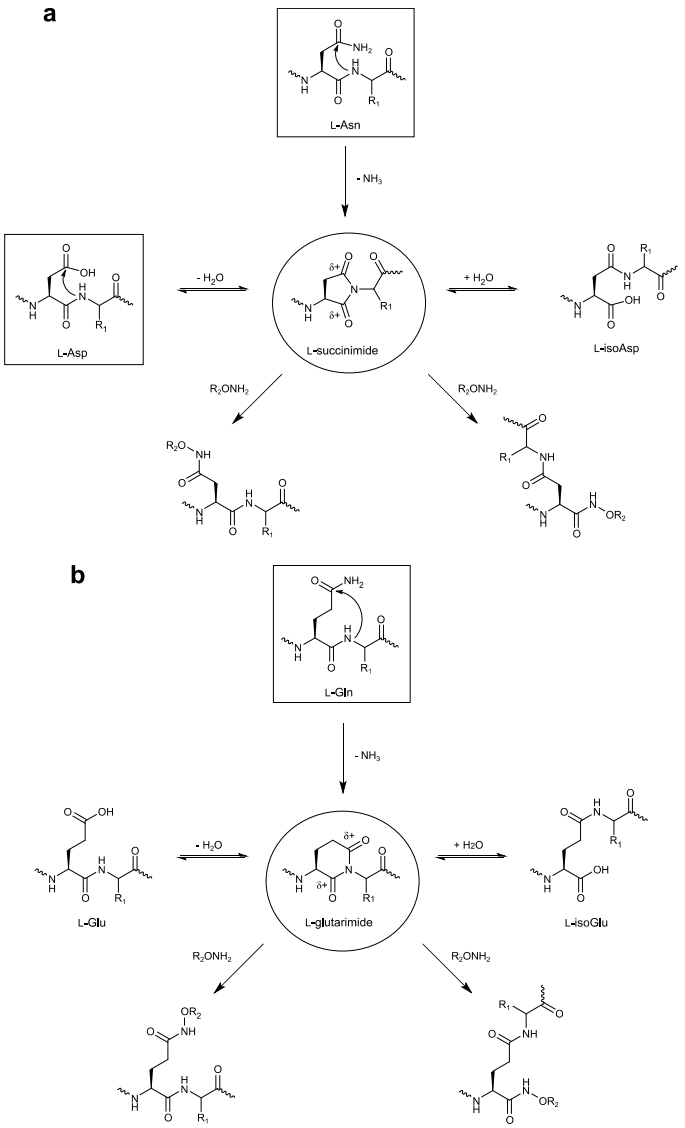

Fig. S12 Asn (a) and Gln (b) deamidation, isomerization and possible electrophilic sites for nucleophilic attack by hydroxylamines. The Figure was adapted from Geiger and Clarke.[6]

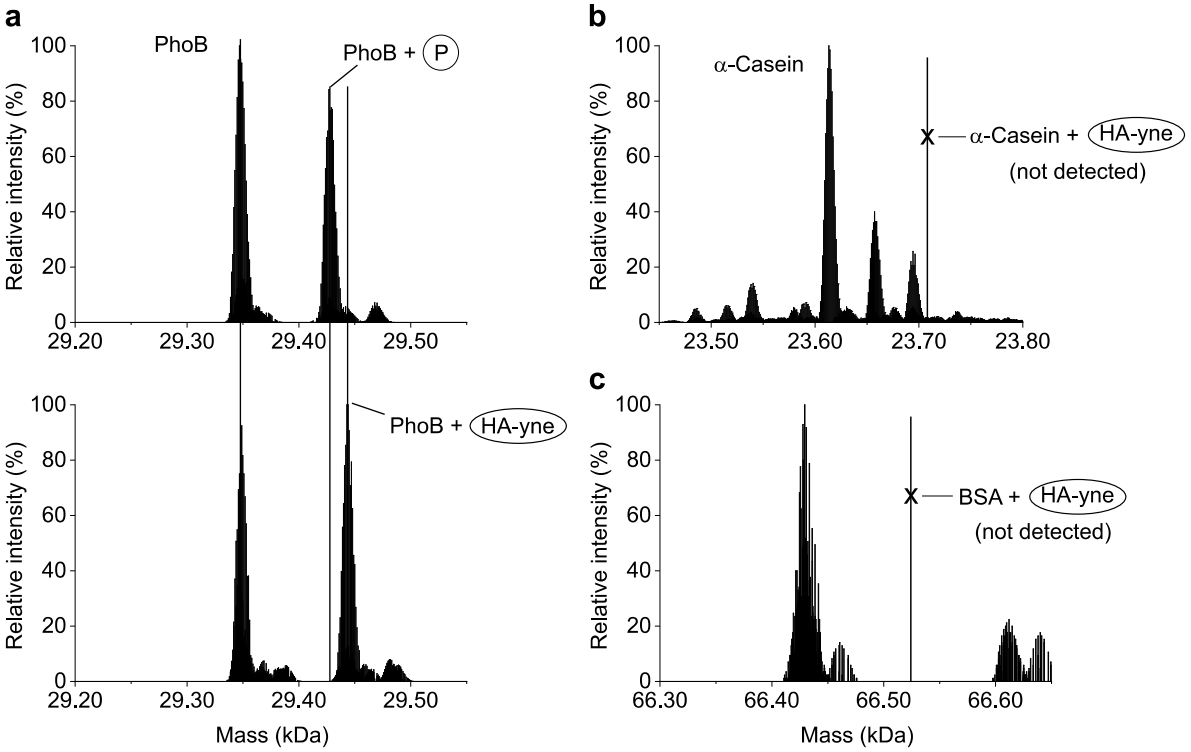

Fig. S13 Analysis of background probe reactivity by IPMS analysis of phosphorylated PhoB, α-Casein and BSA with 125 mM **HA-yne** at pH = 4. (a) PhoB was *in vitro* phosphorylated with acetyl phosphate and converted with **HA-yne**. α-Casein (b) and BSA (c) were treated with **HA-yne** without prior phosphorylation. No background reactions could be observed under the applied conditions.

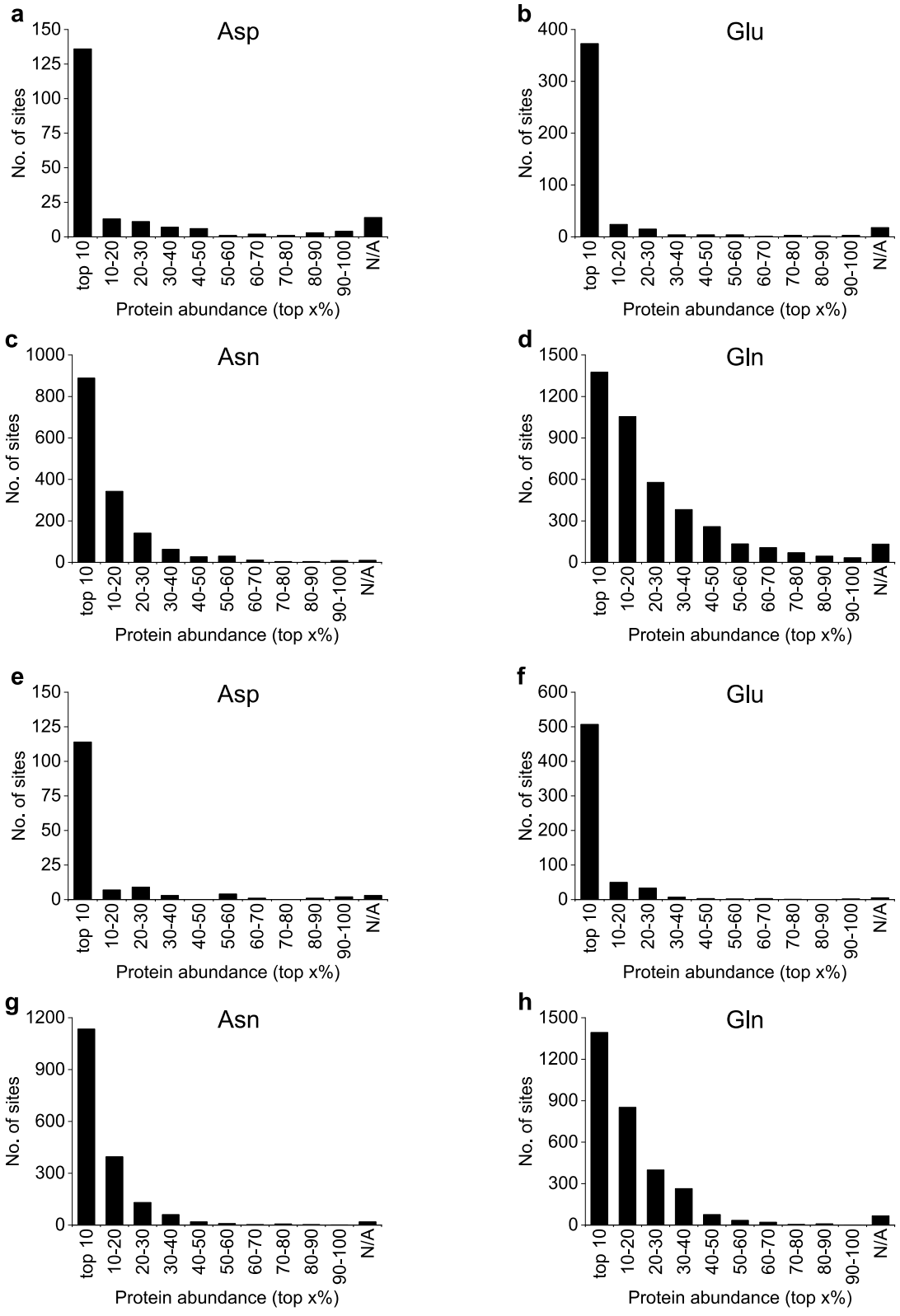

Fig. S14 Abundance of **HA-yne** modified proteins in proteomes of *P. aeruginosa* (a-d) and *B. subtilis* (e-h). Proteins were ranked according to their abundance in the relative organism and grouped in 10% steps. The last group indicates proteins, for which no abundance data is available (N/A). The number of **HA-yne** modified peptides (Table S1, ESI†) was assigned to the relative category. Modification of mainly high abundance proteins suggests some background reactivity. Protein abundance data were obtained using the PaxDb database.[7]

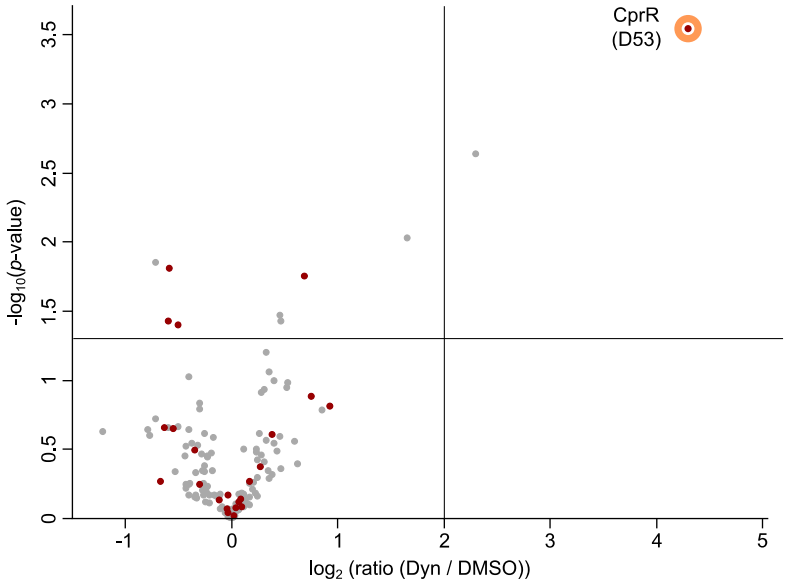

Fig. S15 Volcano plot of the isoDTB-ABPP experiment comparing the **HA-yne** modified sites of DMSO (heavy) and dynorphin (Dyn, light) pretreated samples. Plots show the $\log_2$-fold enrichment of the ratio between light and heavy labeled samples and the probability in a one-sample *t*-test that the ratio is equal to one ($-\log_{10}$ (*p*)). Red and gray indicate proteins annotated as "phosphoaspartate" in UniProt and all other proteins, respectively. Data were visualized using Perseus software.[8]

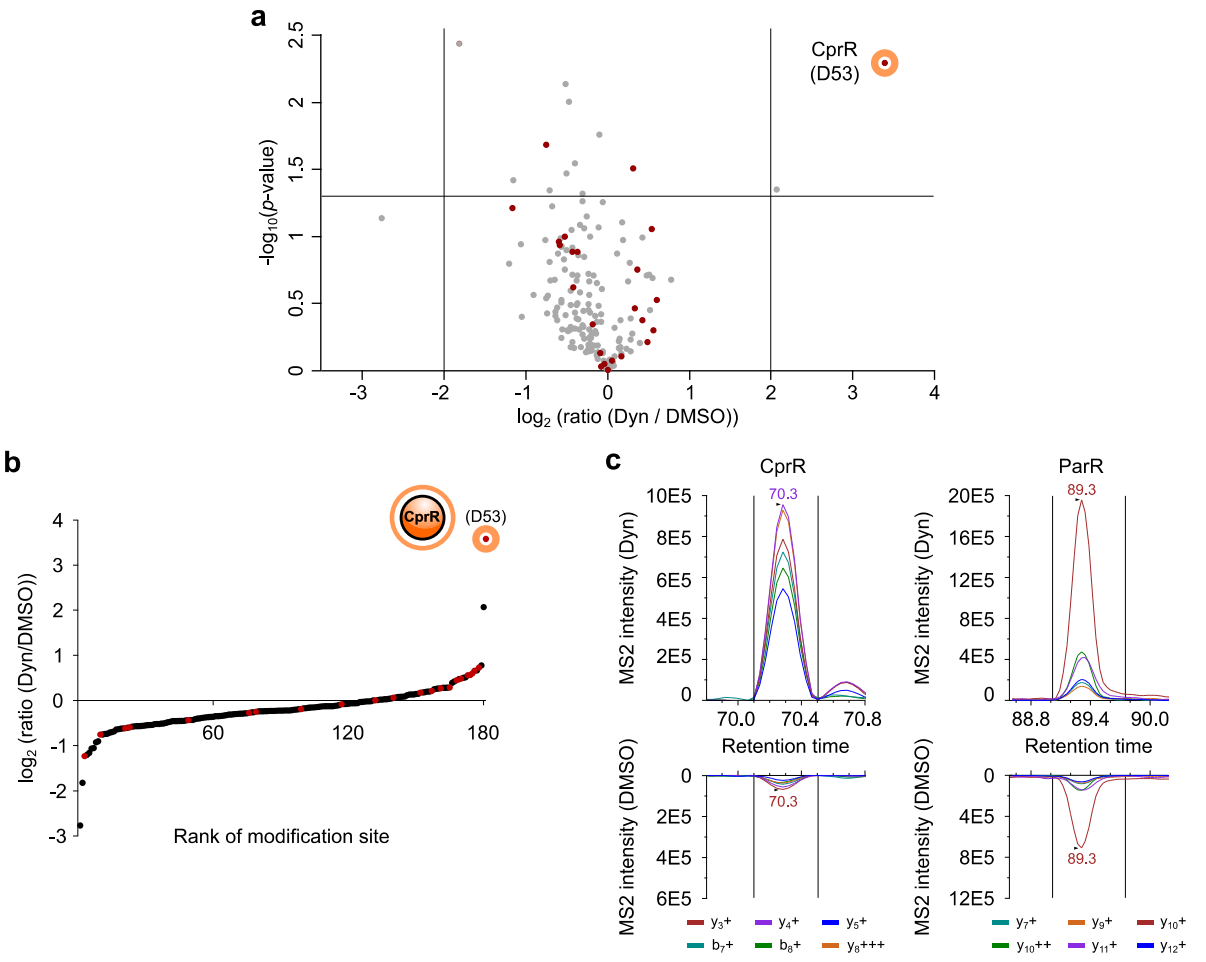

Fig. S16 Quantitative MS1 and MS2 (PRM) analysis for 30 min dynorphin A vs. DMSO treatment. (a) Volcano plot of the isoDTB-ABPP experiment comparing the **HA-yne** modified sites of DMSO (heavy) and dynorphin (Dyn, light) pretreated samples. Plots show the $\log_2$-fold enrichment of the ratio between light and heavy labeled samples and the probability in a one-sample *t*-test that the ratio is equal to one ($-\log_{10}(p)$). Red and gray indicate proteins annotated as "phosphoaspartate" in UniProt and all other proteins, respectively. Data were visualized using Perseus software.[8] (b) Waterfall plot representing the ratio between dynorphin A (light) and DMSO (heavy) treated **HA-yne** modified Asp and Glu residues. Red dots indicate sites, that are also annotated as pAsp sites in UniProt. (c) PRM transitions (Dyn/light vs. DMSO/heavy) of pAsp annotated and **HA-yne** modified peptides of response regulators CprR and ParR. Data was analyzed using the Skyline software.[9] MS2 ratios of 19.4 and 2.8 were obtained for CprR and ParR, respectively, unraveling CprR as the only protein with highly enhanced modification.

Supplementary Tables

Table S1. RP-ABPP data (DDA) for the evaluation of the selectivity and quantification of the sites of modification with **HA-yne** and either DTB or the isoDTB tags using MaxQuant software.[3] The data can be found as an additional data file accompanying the manuscript.

Table S2. Precursor information for the establishment of the PRM method and RP-ABPP data (PRM) for the analysis of PROCAL peptides and the quantification of the sites of modification with **HA-yne** and the isoDTB tags using Skyline.[9] The data can be found as an additional data file accompanying the manuscript.

Table S3. Assignment of all raw files to the corresponding RP-ABPP experiment samples in this study. All Data files have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) *via* the PRIDE partner repository[10] with the dataset identifier PXD022426.

Description of the RP-ABPP sample	Raw file name
B.subtilis_spike_in_E.coli_PhoB	
soluble + insoluble fraction	181103_SMH_181031_PA3
B.subtilis_DTB	
biol. repl. 1, techn. repl. 1, soluble fraction	190421_PA_190411_1
biol. repl. 1, techn. repl. 2, insoluble fraction	190421_PA_190411_2
biol. repl. 1, techn. repl. 3, soluble + insoluble fraction	190505_PA_190503_1
biol. repl. 2, techn. repl. 4, soluble fraction	190421_PA_190411_3
biol. repl. 2, techn. repl. 5, insoluble fraction	190421_PA_190411_4
biol. repl. 2, techn. repl. 6, soluble + insoluble fraction	190505_PA_190503_2
PAO1_DTB	
biol. repl. 1, techn. repl. 1, soluble fraction	190521_PA_190520_1
biol. repl. 1, techn. repl. 2, insoluble fraction	190523_PA_190520_1
biol. repl. 2, techn. repl. 3, soluble fraction	190523_PA_190520_2
biol. repl. 2, techn. repl. 4, insoluble fraction	190523_PA_190520_3
PAO1_DynA_DTB	
1 min DynA, biol. repl. 1, techn. repl. 1, soluble fraction	190826_PA_190826_1_DL1
1 min DynA, biol. repl. 1, techn. repl. 2, insoluble fraction	190826_PA_190826_3_DL2
5 min DynA, biol. repl. 2, techn. repl. 3, soluble fraction	190826_PA_190826_9_DL3
5 min DynA, biol. repl. 2, techn. repl. 4, insoluble fraction	190826_PA_190826_11_DL4
15 min DynA, biol. repl. 2, techn. repl. 5, soluble fraction	190826_PA_190826_17_DL5
15 min DynA, biol. repl. 2, techn. repl. 6, insoluble fraction	190826_PA_190826_19_DL6
1 min DMSO, biol. repl. 1, techn. repl. 1, soluble fraction	190826_PA_190826_5_L1
1 min DMSO, biol. repl. 1, techn. repl. 2, insoluble fraction	190826_PA_190826_7_L2
5 min DMSO, biol. repl. 2, techn. repl. 3, soluble fraction	190826_PA_190826_13_L3
5 min DMSO, biol. repl. 2, techn. repl. 4, insoluble fraction	190826_PA_190826_15_L4
15 min DMSO, biol. repl. 2, techn. repl. 5, soluble fraction	190826_PA_190826_21_L5
15 min DMSO, biol. repl. 2, techn. repl. 6, insoluble fr.	190826_PA_190826_23_L6

PAO1_DynA_isoDTB and PAO1_DynA_isoDTB_DDA_quant	
biol. repl. 1, techn. repl. 1, soluble fraction	200409_PA_200318_3
biol. repl. 1, techn. repl. 2, insoluble fraction	200409_PA_200318_6
biol. repl. 2, techn. repl. 3, soluble fraction	200409_PA_200318_9
biol. repl. 2, techn. repl. 4, insoluble fraction	200409_PA_200318_12
biol. repl. 3, techn. repl. 5, soluble fraction	200303_PA_200224_3_HC1
biol. repl. 3, techn. repl. 6, insoluble fraction	200303_PA_200224_6_HM1
biol. repl. 4, techn. repl. 7, soluble fraction	200303_PA_200224_9_HC2
biol. repl. 4, techn. repl. 8, insoluble fraction	200303_PA_200224_12_HM2

PAO1_DynA_isoDTB_PROCAL_PRM_quant	
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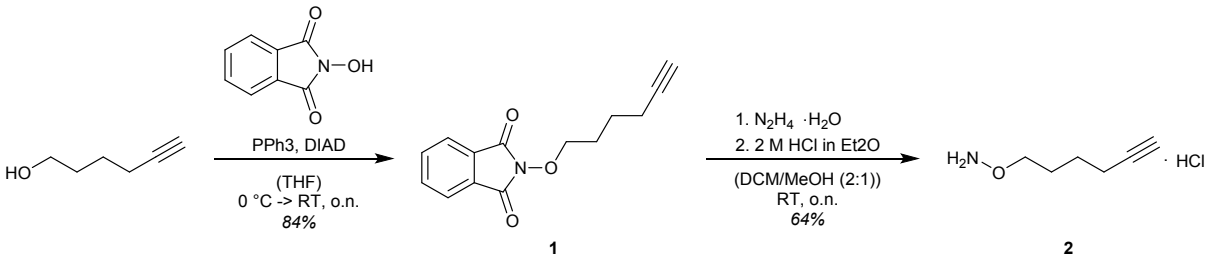
biol. repl. 2, techn. repl. 3, soluble fraction	200731_PA_200318_9_PA3
biol. repl. 2, techn. repl. 4, insoluble fraction	200731_PA_200318_12_PA4
biol. repl. 3, techn. repl. 5, soluble fraction	200731_PA_200224_HC1_PA5
biol. repl. 3, techn. repl. 6, insoluble fraction	200731_PA_200224_HM1_PA6
biol. repl. 4, techn. repl. 7, soluble fraction	200731_PA_200224_HC2_PA7
biol. repl. 4, techn. repl. 8, insoluble fraction	200731_PA_200224_HM2_PA8

Experimental Procedures

General Remarks

All reactions sensitive to air and moisture were carried out under argon atmosphere in oven-dried flasks. Chemicals were purchased from *Acros Organics*, *Alfa Aesar*, *Fisher Chemical* and *Sigma-Aldrich* and were used without further purification. Solvents for column chromatography were distilled prior to use. Analytical thin layer chromatography was carried out on silica-coated aluminum plates (Silica gel 60 F_{254}, *Merck*) with detection by UV-absorption (λ = 254 and/or 366 nm) and/or by coloration using a potassium permanganate ($KMnO_4$) staining solution with subsequent heat treatment. Flash column chromatography was performed on silica gel (40-63 µM, *VWR*) with solvent compositions reported as volume/volume (v/v) ratios. ^{1}H- and ^{13}C-NMR spectra were recorded on *Bruker* Avance III HD (400 MHz and 500 MHz) instruments and referenced to the residual solvent signal (δ_H = 7.26 ppm and δ_C = 77.16 ppm for $CDCl_3$; δ_H = 2.50 ppm and δ_C = 39.52 ppm for DMSO-d_6). Signal assignment was reported using following abbreviations: s - singlet, d - doublet, t - triplet, q - quartet, m - multiplet. High-resolution mass spectrometry (HR-MS) spectra were recorded in the ESI mode on an LTQ-FT Ultra (*Thermo Fisher Scientific*) coupled to an UltiMate 3000 HPLC system (*Thermo Fisher Scientific*). DNA and Protein concentrations were determined in duplicates with a NanoQuant plate on an Infinite F200 PRO reader (*Tecan*) by measuring the absorbance at λ = 260 nm or 280 nm, respectively. Primers were purchased as custom synthesized and lyophilized solids (*Eurofins*). Dynorphin A (1-13) was obtained from *Bachem*.

Synthetic Procedures



2-(Hexyloxy)isoindoline-1,3-dione (1):

5-Hexyn-1-ol (3.07 g, 30.0 mmol, 1.00 eq.) was dissolved in dry THF (120 mL) and *N*-hydroxyphthalimide (6.36 g, 39.0 mmol, 1.30 eq.) and triphenylphosphine (PPh_3) (11.8 g, 45.0 mmol, 1.50 eq.) were added. Upon cooling to 0 °C, a solution of diisopropyl azodicarboxylate (DIAD) (8.83 mL, 45.0 mmol, 1.50 eq.) in dry THF (30 mL) was added over 30 min at 0 °C. The mixture was stirred overnight at room temperature. After all volatiles were removed under reduced pressure, the residue was filtered and washed with hexane (3 × 15 mL). Purification by column chromatography (dry loading) (hexane/EtOAc 4:1) yielded **1** as a white solid (6.23 g, 25.2 mmol, 84%).

^{1}H-NMR (400 MHz, $CDCl_3$, 298 K): δ [ppm] = 7.86-7.79 (m, 2H, H_{Ar}), 7.77-7.70 (m, 2H, H_{Ar}), 4.22 (t, ^{3}J = 6.3 Hz, 2H, OCH_2), 2.37-2.22 (m, 2H, CCH_2), 1.95 (t, ^{4}J = 2.7 Hz, 1H, CH), 1.94-1.86 (m, 2H, $OCH_2\underline{CH_2}$), 1.84-1.67 (m, 2H, $CCH_2\underline{CH_2}$).

^{13}C-NMR (101 MHz, $CDCl_3$, 300 K): δ [ppm] = 163.73 (s, 2C), 134.58 (s, 2C), 129.09 (s, 2C), 123.62 (s, 2C), 83.97 (s, 1C), 77.94 (s, 1C), 68.93 (s, 1C), 27.24 (s, 1C), 24.62 (s, 1C), 18.12 (s, 1C).

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

***O*-(Hex-5-yn-1-yl)hydroxylamine (2):**

1 (2.47 g, 10.0 mmol, 1.00 eq.) was dissolved in DCM/MeOH (2:1) and hydrazine monohydrate (509 µL, 10.5 mmol, 1.05 eq.) was added dropwise. The solution was stirred overnight at room temperature and completion was indicated by TLC. After solvent removal under reduced pressure, the residue was resuspended in H_2O and the pH adjusted to 12. The mixture was extracted with DCM (3 × 20 mL) and the combined organic layers were washed with brine (20 mL), dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure and the residue was dissolved in Et_2O, followed by addition of HCl in Et_2O (2 M). The formed precipitate was cooled to -20 °C overnight, filtered and dried under high vacuum to yield **2** as a light yellowish solid (962 mg, 6.43 mmol, 64%).

^{1}H-NMR (500 MHz, DMSO-d_6, 298 K): δ [ppm] = 10.84 (s, 3H, NH_3), 3.99 (t, ^{3}J = 6.3 Hz, 2H, OCH_2), 2.80 (t, ^{4}J = 2.7 Hz, 1H, CH), 2.20 (td, ^{3}J = 7.1, ^{4}J = 2.6 Hz, 2H, CCH_2), 1.71-1.61 (m, 2H, $OCH_2\underline{CH_2}$), 1.54-1.45 (m, 2H, $CCH_2\underline{CH_2}$).

^{13}C-NMR (101 MHz, DMSO-d_6, 300 K): δ [ppm] = 84.09 (s, 1C), 73.41 (s, 1C), 71.55 (s, 1C), 26.23 (s, 1C), 24.24 (s, 1C), 17.27 (s, 1C).

HRMS-ESI (m/z): calc. ($C_6H_{12}NO$ $[M+H]^+$): 114.0919; found: 114.0914.

Bacterial Strains and Media

Unless stated otherwise, *E. coli* BL21 (DE3), *B. subtilis 168* and *P. aeruginosa (PAO1)* were cultivated in LB medium (10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract, pH 7.5). For the growth of *E. coli* BL21 (DE3) bearing the pET300 expression vector, LB medium was supplemented with ampicillin (100 mg/L). Overnight cultures of bacteria were inoculated with a pipette tip of the corresponding glycerol stock in 5 mL of the corresponding medium and cells were grown overnight at 37 °C with shaking at 220 rpm.

Cloning, Expression and Purification of *E. coli* PhoB

N-terminal His_6-tagged *E. coli* PhoB with a TEV-cleavage site between the His_6-tag and the protein sequence was cloned in a pET300 vector in *E. coli* BL21 (DE3) competent cells via Gateway cloning (*Life Technologies*). For PhoB expression, LB medium was inoculated (1:100) with *E. coli* overnight cultures (37 °C, 220 rpm) and incubated at 37 °C, 220 rpm. After induction at OD_{600} = 0.6 with 0.5 mM IPTG, PhoB was expressed overnight at 25 °C with shaking at 220 rpm. Cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with PBS, resuspended in lysis buffer (20 mM Trizma, pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole, 0.4% (v/v) NP-40) and lyzed by sonication (2 x (7 min, 30% int.; 3 min, 80% int.); *Bandelin* Sonopuls HD 2070) under constant cooling with ice. The lysate was centrifugued (38,000 g, 4 °C, 30 min) to remove cellular debris and by using an ÄKTA Purifier 10 system (*GE Healthcare*), the supernatant was loaded on a 5 mL HisTrap HP column (*GE Healthcare*) equilibrated with wash buffer 1 (20 mM Trizma, pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole). The column was washed with wash buffer 1 (8 CV), wash buffer 2 (20 mM Trizma, pH 8.0, 1 M NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole; 8 CV) and wash buffer 3 (20 mM Trizma, pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol, 40 mM imidazole; 8 CV). Elution was performed with elution buffer (20 mM Trizma, pH 8.0, 1 M NaCl, 2 mM β-mercaptoethanol, 500 mM imidazole; 4 CV) and PhoB containing fractions were pooled, concentrated using a 50 kDa MWCO centrifugal filter (Merck) and purified by size-exclusion chromatography with a HiLoad 16/60 Superdex 76 pg column (*GE Healthcare*) equilibrated in PhoB storage buffer (20 mM HEPES, pH 7.0, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT). Fractions containing PhoB were pooled, concentrated and stored at -80 °C after addition of 10% (v/v) glycerol. Purity of the protein was verified by SDS-PAGE and intact-protein mass spectrometry (IPMS).

A point mutant of PhoB (D53N) was generated using the Quikchange Site-Directed Mutagenesis Kit (*Stratagene*) with the pET300 PhoB expression vector as template. Sequences of PhoB and the D53N point mutant were verified by Sanger sequencing (*GATC Biotech AG*). Expression and purification of the mutant was performed as described above.

Unless stated otherwise, tagged PhoB was used for further experiments since it behaved identical to tag-free PhoB. For the generation of tag-free PhoB, the protein was dialyzed after His-affinity purification in PhoB storage buffer at 4 °C overnight. PhoB was incubated with 1:3 (w/w) TEV protease at 10 °C overnight without shaking and complete cleavage was verified by IPMS. Tag-free PhoB was concentrated and purified by size-exclusion chromatography as described above.

Intact Protein Mass Spectrometry

High-resolution IPMS measurements were performed on an UltiMate 3000 HPLC system (*Thermo Fisher Scientific*) coupled to an LTQ-FT Ultra (*Thermo Fisher Scientific*) 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.). Protein samples (1-10 pmol) were desalted on-line with a Massprep desalting cartridge (*Waters*) prior to measurement. The mass spectrometer was operated in positive ion mode and full scans were recorded at high resolution (200,000) in a range of m/z = 600-2000 Th. Protein spectra were deconvoluted using the Xcalibur Xtract algorithm (*Thermo Fisher Scientific*).

***In vitro* phosphorylation and phosphoaspartate conversion of PhoB**

In vitro phosphorylation of PhoB was initiated by addition of $MgCl_2$ and lithium potassium acetyl phosphate to PhoB in reaction buffer (20 mM HEPES, pH 7.0, 0.1 mM DTT; final concentrations: 12.5 µM PhoB, 10 µM $MgCl_2$ and 20 µM lithium potassium acetyl phosphate). The reaction mixture was incubated for 1 h at 37 °C without shaking. Meanwhile, Bio-Spin 6 Columns (*Bio-Rad*) for gel filtration were equilibrated four times by addition of 500 µL reaction buffer, centrifugation (1,000 g, 1 min, 4 °C) and removal of the supernatant. Acetyl phosphate was then removed from the sample by application of the sample to the column and centrifugation (1,000 g, 4 min, 4 °C). An aliquot of the sample was taken and 1-10 pmol protein were subjected to IPMS analysis to assess the degree of phosphorylation. The remaining sample was treated with either hydroxylamine or **HA-yne** at the indicated concentrations. Both preceding steps (IPMS and nucleophile addition) were conducted immediately in order to minimize loss of phosphorylation. Unless stated otherwise, the reaction proceeded at pH = 4 and was checked with pH-indicator strips (*Merck*). The reaction was allowed to stand at room temperature for 1 h before IPMS measurement and assessment of phosphoaspartate conversion. For time-course experiments, aliquots of the samples were subjected to IPMS analysis at the indicated time points and performed in triplicates.

Several control experiments were conducted in order to prove the selectivity of the conversion with hydroxylamine and **HA-yne** at the optimized conditions with exclusively phosphoaspartate modified proteins. Control experiments were conducted analogously to the procedure described above. For reactions at different pH values, the pH value of the solution containing the nucleophile was adjusted with 0.5 M KOH or HCl prior to the reaction with the indicated protein. Proteins α-Casein and BSA were used as 200 µg/mL solutions and treated with **HA-yne** without prior phosphorylation.

Gel-based RP-ABPP Experiments for HA-yne Labeling Optimization

For the development of an RP-ABPP workflow, exponentially growing *B. subtilis* were labeled with **HA-yne** on analytical scale. LB medium was inoculated (1:100) with *B. subtilis* overnight cultures (37 °C, 220 rpm) and incubated at 37 °C with shaking at 220 rpm. At OD_{600} = 0.5-0.6, cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD_{600} = 40 in 100 µL lysis buffer (basis: 20 mM HEPES, pH 7.0, 500 mM **HA-yne**; pH value, detergent content and **HA-yne** were adjusted as indicated) for each condition to be tested. Cells were lyzed by sonication (3 x 15 s, 80% int.) under constant cooling with ice and fractions were separated by centrifugation (21,000 g, 4 °C, 30 min). The

insoluble fraction was washed twice with 100 µL ice-cold PBS and stored at -20 °C until subjection to click chemistry. To remove excess **HA-yne**, the soluble fraction was precipitated in 400 µL of cold acetone (-80 °C) and stored overnight at -20 °C. The precipitate was centrifuged (9,000 g, 4 °C, 10 min) and washed twice by respuspension in 100 µL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant.

Soluble and insoluble fractions were resuspended in 100 µL 0.8% SDS in PBS by sonication (10 s, 10% int.) and clicked to rhodamine azide by addition of 6 µL TBTA ligand (0.9 mL/mL in 4:1 *t*BuOH/DMSO), 2 µL rhodamine azide (5 mM stock in DMSO; final concentration: 100 µM), 2 µL TCEP (13 mg/mL in water) and 2 µL $CuSO_4$ (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature in the dark, quenched by addition of 112 µL 2× Laemmli buffer and analyzed by SDS-PAGE. Rhodamine azide modified proteins were detected by in-gel fluorescence scanning and protein loading was visualized by Coomassie Brilliant Blue staining.

E. coli* PhoB spike-in RP-ABPP Experiments in *B. subtilis

For PhoB spike-in experiments, PhoB was phosphorylated and converted with 500 mM **HA-yne** as described in section "in vitro phosphorylation and phosphoaspartate conversion of PhoB." 5 µg of **HA-yne** modified PhoB was precipitated in 400 µL of cold acetone (-80 °C) and stored at -20 °C until further processing.

LB medium was inoculated (1:100) with a *B. subtilis* overnight culture (37 °C, 220 rpm) and incubated at 37 °C with shaking at 220 rpm. At OD_{600} = 0.5-0.6, cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD_{600} = 40 in 1 mL of **HA-yne** buffer (20 mM HEPES, pH 4.0, 125 mM **HA-yne**, 1% (w/v) LDAO). Cells were lyzed by sonication (4 x 15 s, 80% int.) under constant cooling with ice. The reaction proceeded for 1 h at 37 °C without shaking. Fractions were separated by centrifugation (21,000 g, 4 °C, 30 min). The insoluble fraction was washed twice with 1 mL ice-cold PBS and stored at -20 °C until subjection to click chemistry. The soluble fraction was precipitated in 4 mL of cold acetone (-80 °C) and incubated overnight at -20 °C. The precipitate was centrifuged (9,000 g, 4 °C, 10 min) and washed twice by respuspension in 1 mL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant.

The pellet of PhoB, the soluble and insoluble fraction were resuspended in 0.8% SDS in PBS by sonication (10 s, 10% int.) and combined (final volume: 1 mL). 1 mL of each sample was clicked to desthiobiotin azide (*Jena Bioscience*) by addition of 60 µL TBTA ligand (0.9 mL/mL in 4:1 *t*BuOH/DMSO), 20 µL desthiobiotin azide (5 mM stock in DMSO, final concentration: 100 µM), 20 µL TCEP (13 mg/mL in water) and 20 µL $CuSO_4$ (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature and quenched by addition of 4 mL of cold acetone (-80 °C) and stored overnight at -20 °C.

RP-ABPP Experiments in *B. subtilis* and *P. aeruginosa*

LB medium was inoculated (1:100) with *B. subtilis* or *P. aeruginosa* overnight cultures (37 °C, 220 rpm) and incubated at 37 °C with shaking at 220 rpm. At OD_{600} = 0.5-0.6, cells were

harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD_{600} = 40 in 1 mL of **HA-yne** buffer (20 mM HEPES, pH 4.0, 125 mM **HA-yne**, 1% (w/v) LDAO). Cells were lyzed by sonication (4 x 15 s, 80% int.) under constant cooling with ice. The reaction proceeded for 1 h at 37 °C without shaking. Fractions were separated by centrifugation (21,000 g, 4 °C, 30 min). The insoluble fraction was washed twice with 1 mL ice-cold PBS and stored at -20 °C until subjection to click chemistry. The soluble fraction was precipitated in 4 mL of cold acetone (-80 °C) and incubated overnight at -20 °C. The precipitate was centrifuged (9,000 g, 4 °C, 10 min) and washed twice by respuspension in 1 mL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant. Soluble and insoluble fractions were resuspended in 1 mL 0.8% SDS in PBS by sonication (10 s, 10% int.) and protein concentration of both fractions was determined using a bicinchoninic acid (BCA) assay and adjusted to 1 mg/mL with 0.8% SDS in PBS. 1 mL of each sample was clicked to desthiobiotin azide by addition of 60 µL TBTA ligand (0.9 mL/mL in 4:1 *t*BuOH/DMSO), 20 µL desthiobiotin azide (5 mM in DMSO), 20 µL TCEP (13 mg/mL in water) and 20 µL $CuSO_4$ (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature and quenched by addition of 4 mL of cold acetone (-80 °C) and stored overnight at -20 °C.

For gel-based analysis, 100 µL of all samples (1 mg/mL) were additionally clicked to rhodamine azide and visualized as described in the previous section.

Moreover, one additional sample was prepared for *B. subtilis* replicates, for which the soluble and insoluble fraction (500 µL each) of the lysate was combined before the click reaction and further processing.

RP-ABPP Experiments in dynorphin A treated *P. aeruginosa*

MOPS medium (50 mM MOPS, pH 7.2, 20 mM NH_4Cl, 20 mM di-sodium succinate, 1 mM $MgSO_4$, 10 mM KCl, 4 mM K_2HPO_4 and 3.5 µM $FeSO_4$) was inoculated (1:100) with a *P. aeruginosa* overnight culture (37 °C, 220 rpm) in LB medium and incubated at 37 °C with shaking at 220 rpm. At OD_{600} = 0.8-1.0, cells were harvested by centrifugation (6,000 g, 4 °C, 10 min) and washed with ice-cold PBS. Cells were resuspended to OD_{600} = 1 in 40 mL PBS and incubated at 37 °C with shaking at 220 rpm. 4 µL of Dynorphin A (1-13) (100 mM stock in DMSO; final concentration: 10 µM) or DMSO were added and incubated for either 1, 5 or 15 min at 37 °C with shaking at 220 rpm. The cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD_{600} = 40 in 1 mL of **HA-yne** buffer (20 mM HEPES, pH 4.0, 125 mM **HA-yne**, 1% (w/v) LDAO). The samples were lyzed, separated and washed as described above. Soluble and insoluble fractions were resuspended in 1 mL 0.8% SDS in PBS by sonication (10 s, 10% int.) and protein concentration of both fractions was determined by BCA assay and adjusted to 0.5 mg/mL with 0.8% SDS in PBS. 1 mL of each sample was clicked to desthiobiotin azide by addition of 60 µL TBTA ligand (0.9 mL/mL in 4:1 *t*BuOH/DMSO), 20 µL desthiobiotin azide (5 mM stock in DMSO, final concentration: 100 µM), 20 µL TCEP (13 mg/mL in water) and 20 µL $CuSO_4$ (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature and quenched by addition of 4 mL of cold acetone (-80 °C) and stored overnight at -20 °C.

isoDTB-ABPP Experiments in dynorphin A treated *P. aeruginosa*

MOPS medium (50 mM MOPS, pH 7.2, 20 mM NH_4Cl, 20 mM di-sodium succinate, 1 mM $MgSO_4$, 10 mM KCl, 4 mM K_2HPO_4 and 3.5 µM $FeSO_4$) was inoculated (1:100) with a *P. aeruginosa* overnight culture (37 °C, 220 rpm) in LB medium and incubated at 37 °C with shaking at 220 rpm. At OD_{600} = 0.8-1.0, cells were harvested by centrifugation (6,000 g, 4 °C, 10 min) and washed with ice-cold PBS. Cells were resuspended to OD_{600} = 1 in 40 mL PBS and incubated at 37 °C with shaking at 220 rpm. 4 µL of Dynorphin A (1-13) (100 mM in DMSO; final concentration: 10 µM) or DMSO were added and incubated for 1 min or 30 min at 37 °C with shaking at 220 rpm. The cells were harvested by centrifugation (6,000 g, 4 °C, 10 min), washed with ice-cold PBS and resuspended to OD_{600} = 40 in 1 mL of **HA-yne** buffer (20 mM HEPES, pH 4.0, 125 mM **HA-yne**, 1% (w/v) LDAO). The samples were lyzed, washed and separated as described above. Soluble and insoluble fractions were resuspended in 1 mL 0.8% SDS in PBS by sonication (10 s, 10% int.) and protein concentration of both fractions was determined by BCA assay and adjusted to 0.5 mg/mL with 0.8% SDS in PBS. Dynorphin A treated samples were clicked to the light isoDTB tag and DMSO treated samples were clicked to the heavy isoDTB tag by addition of 60 µL TBTA ligand (0.9 mL/mL in 4:1 *t*BuOH/DMSO), 20 µL of the respective isoDTB tag (5 mM stock in DMSO; final concentration: 100 µM), 20 µL TCEP (13 mg/mL in water) and 20 µL $CuSO_4$ (12.5 mg/mL in water). The click reaction was incubated for 1 h at room temperature and quenched by combination of light and heavy isoDTB-tagged samples into 8 mL of cold acetone (-80 °C). Precipitated samples were stored overnight at -20 °C.

MS Sample Preparation of PhoB spike-in RP-ABPP Experiments (Protein Enrichment)

Precipitates were centrifuged (9,000 g, 4 °C, 10 min) and washed twice by respuspension in 1 mL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant. Pellets were dissolved in 300 µL 8 M urea in 0.1 M triethylammonium bicarbonate (TEAB) by sonication (10 s, 10% int.). 900 µL 0.1 M TEAB were added to obtain a urea concentration of 2 M. This solution was added to 1.2 mL of washed streptavidin agarose beads (50 µL initial slurry; A9207, *Sigma Aldrich*) in 0.2% nonyl phenoxypolyethoxylethanol (NP-40 alternative), which were previously washed by addition of 0.2% NP-40 alternative in PBS (4 × 1 mL), centrifugation (400 rpm, 2 min) and removal of the supernatant. The samples were incubated by rotation at room temperature for 1 h.

To remove unbound proteins, the beads were centrifuged (1,000 g, 2 min) and the supernatant was removed. The beads were resuspended in 600 µL 0.1% NP-40 alternative in PBS and transferred to a centrifuge column (11894131, *Fischer Scientific*). The beads were washed with 0.1% NP-40 alternative (2 × 600 µL), PBS (3 × 600 µL) and ddH_2O (3 × 600 µL) and then resuspended in 600 µL 8 M urea in 0.1 M TEAB. After transfer to a Protein LoBind tube, centrifugation (1,000 g, 2 min) and removal of the supernatant, the beads were resuspended in 300 µL 8 M urea in 0.1 M TEAB.

After reduction of disulfides by addition of 15 µL dithiothreitol (DTT; 31 mg/mL) and incubation at 37 °C with shaking at 850 rpm for 45 min, free thiols were alkylated by adding 15 µL iodoacetamide (IAA; 74 mg/mL) and incubation in the dark at 25 °C with shaking at 850 rpm for 30 min. Remaining IAA was quenched by addition of 15 µL DTT (31 mg/mL) and

incubation at 25 °C with shaking at 850 rpm for 30 min. 900 µL 0.1 M TEAB were added to obtain a urea concentration of 2 M for trypsin digestion. 2 µL of 0.5 mg/mL sequencing grade modified trypsin (1 µg; *Promega*) were added and samples were incubated at 37 °C with shaking at 220 rpm overnight. After centrifugation (400 rpm, 2 min) and removal of the supernatant, the beads were washed three times by addition of 50 µL Tris-HCl buffer (50 mM, pH 7.5), centrifugation (1,000 g, 2 min) and removal of the supernatant. The beads were reuspended in 100 µL Tris-HCl buffer, followed by addition of 16 µL of 0.04 mg/mL sequencing grade AspN (0.64 µg; *Promega*) and incubation at 37 °C with shaking at 220 rpm for 7 h.

The beads were resuspended in 500 µL 0.1% NP-40 alternative in PBS and transferred to a centrifuge column (11894131, *Fischer Scientific*). The beads were washed with 0.1% NP-40 alternative (2 × 600 µL), PBS (3 × 600 µL) and ddH_2O (3 × 600 µL). The peptides were eluted by addition of 200 µL elution buffer (0.1% formic acid (FA) in 1:1 acetonitrile (ACN)/H_2O) and two more elution steps with 100 µL elution buffer, followed by centrifugation (5,000 g, 3 min). The solvent was removed using a vacuum centrifuge and samples were stored at -80°C until further processing. The samples were dissolved by addition of 30 µL 1% FA in H_2O and sonication for 3 min. The samples were filtered through pre-equilibrated 0.22 µm PVDF filters (UVC30GVNB, *Merck*) and transferred into MS vials for LC-MS/MS analysis.

MS Sample Preparation for RP-ABPP and isoDTB-ABPP Experiments (Peptide Enrichment)

Precipitates were centrifuged (9,000 g, 4 °C, 10 min) and washed twice by respuspension in 1 mL MeOH (-80 °C) by sonication (10 s, 10% int.), centrifugation (9,000 g, 4 °C, 10 min) and removal of the supernatant. Pellets were dissolved in 300 µL 8 M urea in 0.1 M triethylammonium bicarbonate (TEAB) by sonication (10 s, 10% int.). After reduction of disulfides by addition of 15 µL dithiothreitol (DTT; 31 mg/mL) and incubation at 37 °C with shaking at 850 rpm for 45 min, free thiols were alkylated by adding 15 µL iodoacetamide (IAA; 74 mg/mL) and incubation in the dark at 25 °C with shaking at 850 rpm for 30 min. Remaining IAA was quenched by addition of 15 µL DTT (31 mg/mL) and incubation at 25 °C with shaking at 850 rpm for 30 min. 900 µL 0.1 M TEAB were added to obtain a urea concentration of 2 M for trypsin digestion. 20 µL of 0.5 mg/mL sequencing grade modified trypsin (10 µg; *Promega*) were added and samples were incubated at 37 °C with shaking at 220 rpm overnight. This solution was added to 1.2 mL of washed streptavidin agarose beads (50 µL initial slurry; A9207, *Sigma Aldrich*) in 0.2% nonyl phenoxypolyethoxylethanol (NP-40 alternative), which were previously washed by addition of 0.2% NP-40 alternative in PBS (4 × 1 mL), centrifugation (400 rpm, 2 min) and removal of the supernatant. The samples were incubated by rotation at room temperature for 1 h.

To remove unbound peptides, the beads were centrifuged (1,000 g, 2 min) and the supernatant was removed. The beads were resuspended in 600 µL 0.1% NP-40 alternative in PBS and transferred to a centrifuge column (11894131, *Fischer Scientific*). The beads were washed with 0.1% NP-40 alternative (2 × 600 µL), PBS (3 × 600 µL) and ddH_2O (3 × 600 µL). The peptides were eluted by addition of 200 µL elution buffer (0.1% formic acid (FA) in 1:1 ACN/H_2O) and two more elution steps with 100 µL elution buffer, followed by centrifugation (5,000 g, 3 min). The solvent was removed using a vacuum centrifuge and samples were stored

at -80°C until further processing. The samples were dissolved by addition of 30 µL 1% FA in H_2O and sonication for 3 min. The samples were filtered through pre-equilibrated 0.22 µm PVDF filters (UVC30GVNB, *Merck*) and transferred into MS vials for LC-MS/MS analysis.

LC-MS/MS Analysis

Samples (injection volume: 5 µL) were analyzed with an Ultimate 3000 nano HPLC system (*Dionex*) coupled to a Q Exactive Plus mass spectrometer (*Thermo Fisher Scientific*). Samples were loaded on an Acclaim C18 PepMap100 trap column (75 µm ID x 2 cm), washed with 0.1% TFA and separated on an Acclaim C18PepMapRSLC column (75 µm ID x 50 cm) with a flow of 300 nL/min using buffer A (0.1% FA in H_2O) and buffer B (0.1% FA in ACN): 5% B for 7 min, 5-40% B in 105 min, 40-60% B in 10 min, 60-90% B in 10 min, 90% B for 10 min, 90-5% B in 0.1 min, 5% B for 9.9 min. The Q Exactive Plus mass spectrometer was operated in a TOP10 data dependent acquisition mode (DDA). Full MS (MS1) scans were acquired at a resolution of 70,000, a scan range of m/z = 300-1500 Th, an automomatic gain control (AGC) target of 3e6, and a maximum injection time of 80 ms. The ten most intense precursors (Top10) were selected for MS2 scan acquisition at a resolution of 17,500, an AGC target of 1e5, and a maximum injection time of 100 ms. Precursors with unassigned charge or a charge of +1 were excluded and dynamic exclusion was set to 60 s. Quadrupole isolation of the precursor was set to a window of 1.6 Th. Fragment ions were generated using higher-energy dissociation (HCD) with a normalized collision energy (NCE) of 27% and detected in the orbitrap.

RP-ABPP Data Analysis

MS raw data were analyzed using MaxQuant software (version 1.6.2.10).[3] Standard settings were used with the following changes and additions: The normal FASTA databases without manual changes were downloaded from UniProt[5] (*B. subtilis* 168 taxon identifier: 224308, date of download: 20.09.2018; *P. aeruginosa* PAO1 taxon identifier: 208964, date of download: 22.05.2019). No labels were used. The proteolytic enzyme was set to Trypsin/P with up to three missed cleavages. Variable modifications with **HA-yne** and desthiobiotin azide were allowed on Asp, Glu; Asn and Gln:

- **HA-yne** and desthiobiotin azide on Asp or Glu: $C_{24}H_{43}N_7O_5$ (509.3326 Da)

- **HA-yne** and desthiobiotin azide on Asn or Gln: $C_{24}H_{42}N_6O_6$ (510.3166 Da)

N-terminal acetylation and oxidation of methionine were selected as further variable modifications and carbamidomethylated cysteine as fixed modification. The maximum number of modifications per peptide was 5. The "Re-quantify" option was enabled. Contaminants were included. Peptides were searched with a minimum peptide length of 6 and a maximum peptide mass of 4,600 Da. "Second peptides" was enabled and "Dependent peptides" was disabled. "Match between runs" was enabled with a Match time window of 0.7 min and an alignment window of 20 min. A minimal Andromeda score[12] of 40 and a delta score of 6 was set for modified peptides. 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 "DTB-PEG-N3-Sites.txt" files generated by MaxQuant analysis for modification with **HA-yne** and the desthiobiotin azide tag were used for further analysis. All peptides for "reverse" sequences and "potential contamination" were removed. The data were 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 electrophilic amino acid, the number of sequences was counted that is modified on one specific residue. Identical sequences with **HA-yne** modification at different positions are possible and these are counted as separate sites, if they exceed the 75% localization probability cutoff. The sum of all modified sites within our cutoff from biologically independent replicates was reported.

isoDTB Data Analysis

MS raw data were analyzed using MaxQuant software (version 1.6.2.10).[3] Standard settings were used with the following changes and additions: The normal FASTA databases without manual changes were downloaded from UniProt[5] (*P. aeruginosa* PAO1 taxon identifier: 208964, date of download: 22.05.2019). No labels were used. The proteolytic enzyme was set to Trypsin/P with up to three missed cleavages. Variable modifications with **HA-yne** and either the light or heavy isoDTB tag were allowed on Asp and Glu:

- **HA-yne** and light isoDTB tag on Asp or Glu: $C_{26}H_{44}N_{10}O_5$ (576.3496 Da)

- **HA-yne** and heavy isoDTB tag on Asp or Glu: $C_{22}{}^{13}C_4H_{44}N_8{}^{15}N_2O_5$ (582.3571 Da)

N-terminal acetylation and oxidation of methionine were selected as further variable modifications and carbamidomethylated cysteine as fixed modification. The maximum number of modifications per peptide was 5. 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” was enabled and “Dependent peptides” was disabled. “Match between runs” was enabled with a Match time window of 0.7 min and an alignment window of 20 min. A minimal Andromeda score[12] of 40 and a delta score of 6 was set for modified peptides. 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 “isoDTB light HA-yne (DE)Sites.txt” and ”isoDTB heavy HA-yne (DE)Sites.txt” files generated by MaxQuant analysis for modification with **HA-yne** and isoDTB tags were used for further analysis. All peptides for “reverse” sequences and “potential contamination” were removed. The data were 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 electrophilic amino acid, the number of sequences was counted that is modified on one specific residue. Identical sequences with **HA-yne** modification at different positions are possible and these are counted as separate sites, if they exceed the 75% localization probability cutoff. The sum of all modified sites within our cutoff from biologically independent replicates was reported.

Adjustment of FASTA Databases for quantitative isoDTB-ABPP Data Analysis[13]

The challenge for the quantification of modified aspartates and glutamates in this project was to quantify the relative abundance of peptides modified at one residue with **HA-yne** and the light and heavy isoDTB tags, respectively. We have previously reported this procedure for addressing a single modified amino acid, cysteine,[14] and also applied it for two modified amino

acids, aspartate and glutamate.[13] 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. However, 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 our workaround as described previously,[13] 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 database, which were very few or nonexistent, respectively. 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*").

isoDTB-ABPP Data Analysis for quantification

MS raw data were analyzed using MaxQuant software (version 1.6.2.10).[3] Standard settings were used with the following changes and additions analogous to our previous study:[13] The modified FASTA database with individual substitutions of aspartates and glutamates with the placeholder "O" or "U" was used ("PA8_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:

- **HA-yne** and light isoDTB tag on "O" as placeholder for D: $C_{18}H_{30}N_8O_6$

- **HA-yne** and heavy isoDTB tag on "O" as placeholder for D: $C_{14}{}^{13}C_4H_{30}N_6{}^{15}N_2O_6$

- **HA-yne** and light isoDTB tag on "U" as placeholder for E: $C_{28}H_{46}N_{10}O_7Se_{-1}$

- **HA-yne** and heavy isoDTB tag on “U” as placeholder for E: $C_{24}{}^{13}C_4H_{46}N_8{}^{15}N_2O_7Se_{-1}$

Multiplicity of 2 and maximum number of labeled amino acids of 1 was set. The proteolytic enzyme was set to Trypsin/P with up to three missed cleavages. The “Re-quantify” option was enabled. *N*-terminal acetylation and oxidation of methionine were selected as further variable modifications and carbamidomethylated cysteine as fixed modification. Contaminants were included. Peptides were searched with a minimum peptide length of 7 and a maximum peptide mass of 4,600 Da. “Second peptides” was enabled and “Dependent peptides” was disabled. “Match between runs” was enabled with a Match time window of 0.7 min and an alignment window of 20 min. A minimal Andromeda score[12] of 40 and a delta score of 6 was set for modified peptides. 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 with an Andromeda Score[12] below 40 were deleted. Also all peptides for “reverse” sequences and “potential contamination” were removed. 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, the data were 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 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 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 were 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 of replicates were combined, but the individual values are also reported. The values were combined as the median and the data were filtered out, if there were not at least two data points or if the standard deviation exceeded a value of 1.41. These are the final ratios $\log_2$ (ratio L/H) that are reported. For all comparisons between different MaxQuant runs, the data were combined into one table based on the modified residue.

All individual values (4 biological replicates: soluble and insoluble) for each modified residue were loaded into Perseus (version 1.6.5.0)[8] and analyzed using a one-sample *t*-test against a value of $\log_2$ (ratio L/H) = 0. Sites were considered as significantly regulated, if the statistical significance was $p < 0.05$ and the median ratio was $\log_2$ (ratio L/H) > 2.

Ratios and *p*-values of the modified peptides were matched with the corresponding UniProt data (*P. aeruginosa* PAO1 taxon identifier: 208964) and the corresponding categorized protein abundance data obtained from PaxDb[7] and listed in Table S1.

PRM method development

Based on the results of the quantitative data dependent acquisition (DDA) isoDTB experiments, the most interesting **HA-yne** modified peptides with the highest light to heavy MS1 ratios (L/H) were chosen for PRM measurements. The corresponding peptides from the response regulators CprR and ParR were selected for fragmentation, showing the highest or a so far uncharacterized MS1 ratio (light to heavy), respectively. Additionally, two peptides (from response regulators GacA and PhoP) with an MS1 ratio of roughly one, were chosen as controls. Precursors for fragmentation were selected based on their respective most intense charge state from the DDA measurements analyzed by MS1 Filtering using Skyline (version 20.2.1.286).[9] Experimental spectral libraries were built within Skyline using DDA and PRM isoDTB data processed with MaxQuant and are available for download from Panorama Public[15] (https://panoramaweb.org/pAsp-isoDTB-PAO1.url). For retention time comparison, PROCAL retention time peptides (*JPT Peptide Technologies*) were used, consisting of 40 non-naturally occurring peptides. PROCAL peptides were spiked into the samples (final quantity: 100 fmol/peptide). For 34 PROCAL peptides only MS1-chromatogram information was acquired in PRM mode, while five PROCAL peptides were also selected for fragmentation. For further information see Table S2 and https://panoramaweb.org/pAsp-isoDTB-PAO1.url.

PRM LC-MS/MS Analysis

For PRM measurements, the same samples from the isoDTB experiments were used. Additionally, PROCAL retention time peptides were spiked into the samples (v/v 1:6) directly before measurement. 6 µL of sample were injected in order to obtain similar intensities as in previous DDA measurements and 100 fmol/peptide of the PROCAL retention time peptides.

PRM measurements were performed using the same instruments and LC-setup as described in section "LC-MS/MS Analysis", but the Q Exactive Plus (*Thermo Fisher Scientific*) was operated in PRM mode. Full MS (MS1) scans were acquired at a resolution of 70,000, a scan range of m/z = 300-1500 Th, an automatic gain control (AGC) target of 3e6, and a maximum injection time of 80 ms. Targeted MS2 scans were acquired at a resolution of 17,500, an AGC target of 1e5, and a maximum injection time of 100 ms. The number of targeted precursors was adjusted to maintain a maximum cycle time of 2 s for at least 8 points across the peak in a non-scheduled PRM measurement. In total, 4 different **HA-yne**-modified peptides from proteins CprR, ParR, GacA and PhoP (light/heavy isoDTB version) and 5 PROCAL peptides were targeted (Table S2). Quadrupole isolation of the precursor was set to a window of 1.6 Th. Fragment ions were generated using higher-energy dissociation (HCD) with a normalized collision energy (NCE) of 27% and detected in the orbitrap.

PRM Data Analysis

PRM data analysis was performed using the Skyline-daily (64-bit) software (version 20.2.1.286).[9] For all target peptides, the 6 most intense fragment ions (top6) were automatically picked by Skyline using the generated experimental spectral library. Raw PRM data were also processed by MaxQuant in order to visualize in Skyline the exact time point of successful peptide identification for any given MS2 spectrum. Peak picking, peak integration and transition interferences were reviewed and integration boundaries were adjusted

manually in Skyline, if necessary. Mass accuracy information ("average mass error [ppm]"), correlation of fragment ion intensities between the detected light and heavy peptides ("dot product L/H") and correlation of fragment ion intensities between the detected peptides measured by PRM and the experimental library spectrum from Skyline ("library dot product" separately for light and heavy) were exported from Skyline. Peptide identifications with a dot product L/H > 0.9 and a library dot product > 0.85 were included for the overall ratio (L/H) calculation. The ratio of the respective MS2 peak areas ("total area fragment" L/H) was used for the ratio (L/H) calculation. For further information see Table S2 and https://panoramaweb.org/pAsp-isoDTB-PAO1.url.

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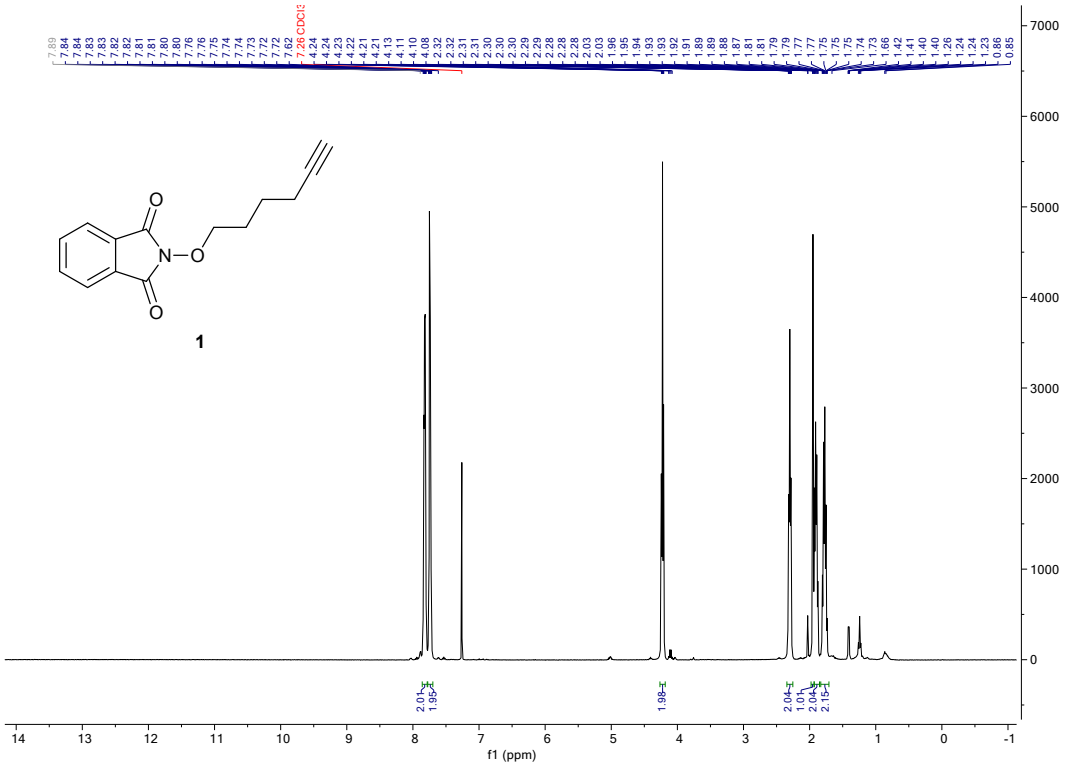
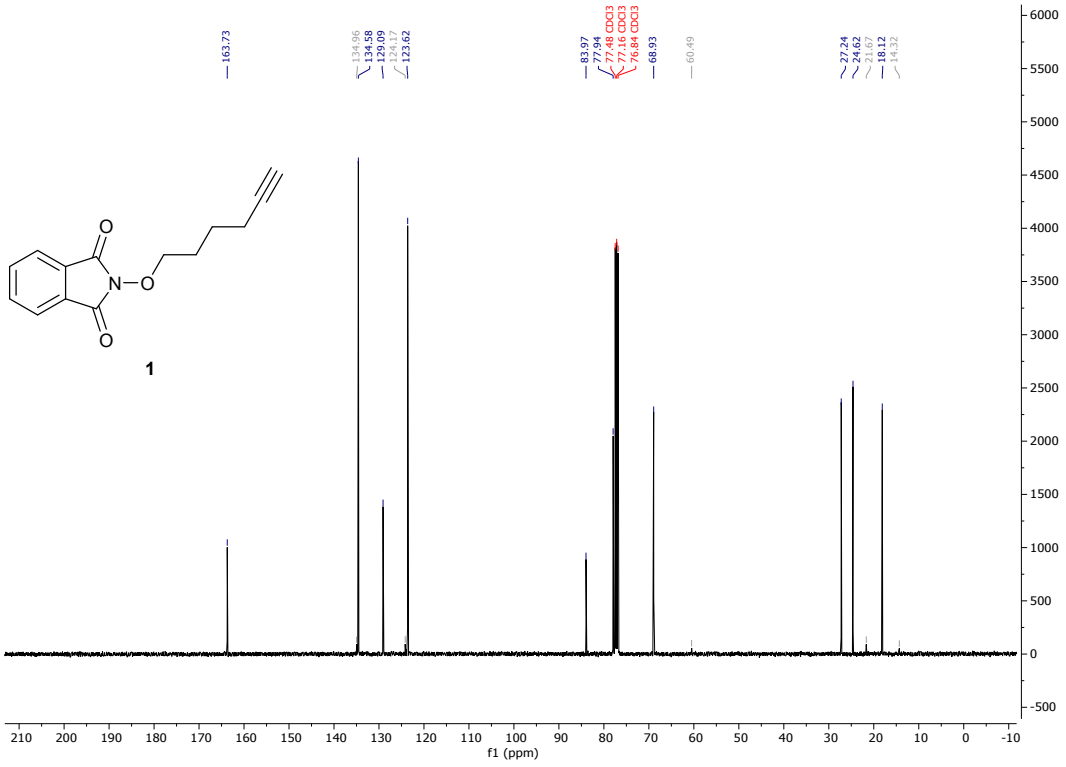
NMR Spectra

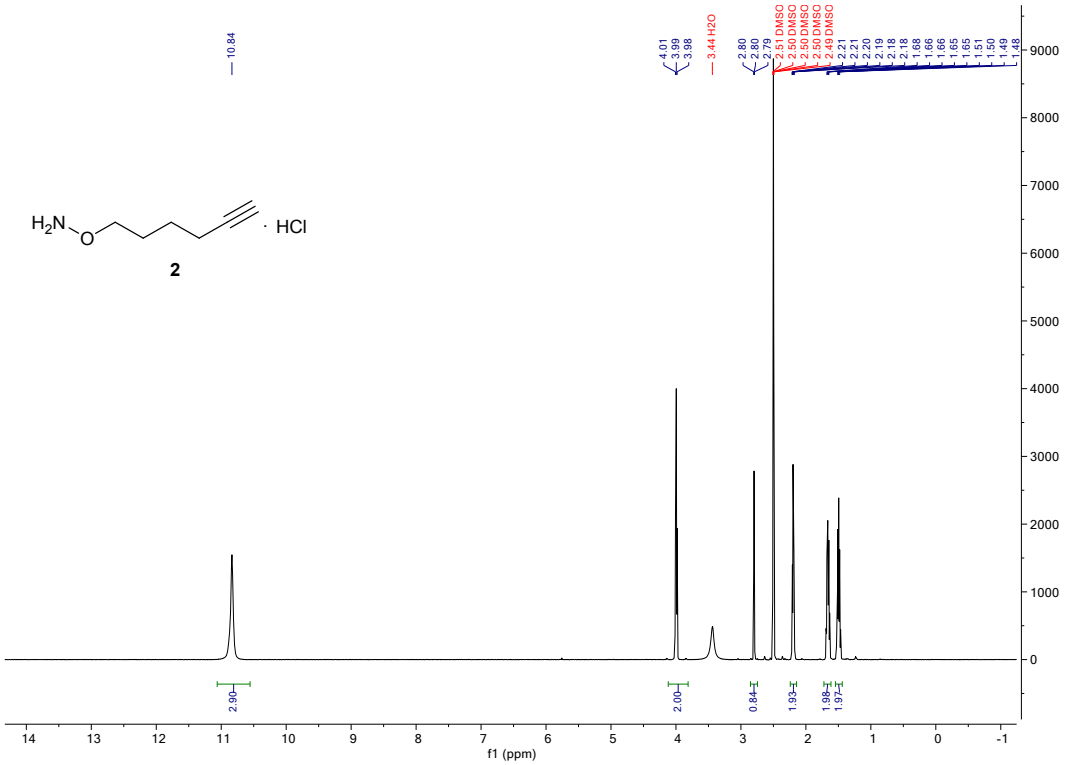
2-(Hexyloxy)isoindoline-1,3-dione (**1**) (^{1}H, 400 MHz, DMSO-d_6):



2-(Hexyloxy)isoindoline-1,3-dione (**1**) (^{13}C, 101 MHz, $CDCl_3$):



O-(Hex-5-yn-1-yl)hydroxylamine (**2**) (^{1}H, 500 MHz, DMSO-d$_6$):



O-(Hex-5-yn-1-yl)hydroxylamine (**2**) (^{13}C, 101 MHz, DMSO-d$_6$):