Experimental Procedures

Materials for synthesis.

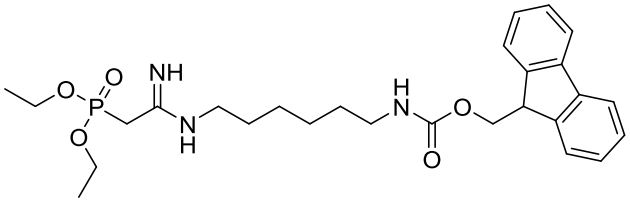
All chemicals were of reagent grade quality or better, typically purchased from Sigma-Aldrich and used without further purifications. Diethyl-(cyanomethyl)phosphonate was purchased from Combi-Blocks, Fmoc-1,6-diaminohexane was purchased from Santa Cruz Biotechnology.

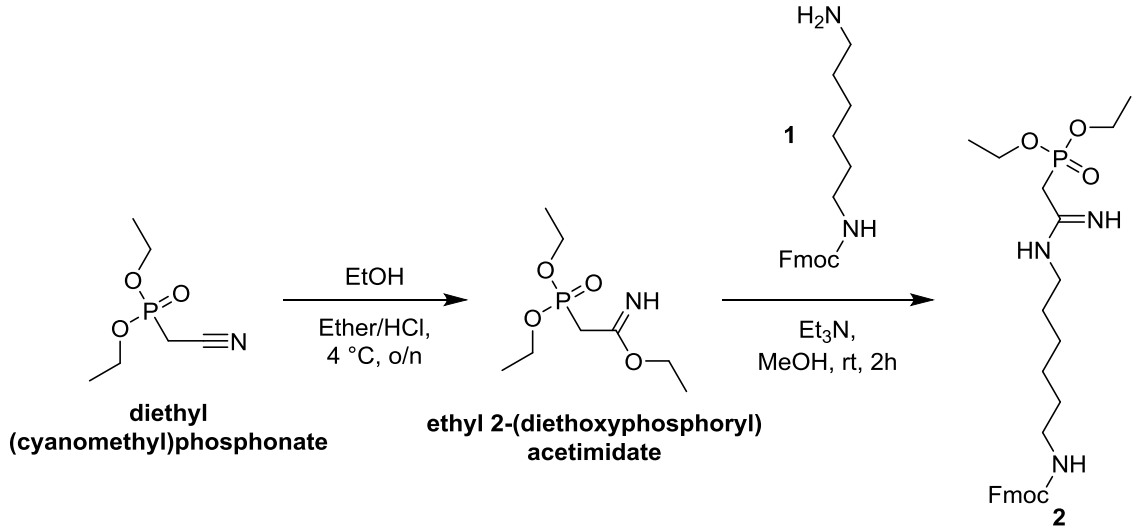
General synthetic methods and instrumentation.

Synthetic reactions were carried out in an oven-dried (>100 °C) round-bottom flask equipped with a Teflon coated magnetic stir bar under nitrogen atmosphere. Rotary evaporation was performed using a Büchi Rotavapor R-210 equipped with a vacuum pump to concentrate the sample and remove solvents and volatiles. Microwave reactions were carried out using the Initiator Microwave Synthesizer (Biotage) under constant stirring at 60 °C employing normal absorption level. Semi-preparative reverse phase HPLC (RP-HPLC) was conducted on a 1260 LC instrument (Agilent) using a C18 Zobrax column (5 μm, 21.2 x 150 mm) at 10 mL/min. Low resolution mass spectra were recorded on a 6120 quadrupole LC/MS system (Agilent) connected to a 1220 Infinity LC instrument (Agilent) equipped with a C18 Poroshell 120 column (2.7 μm, 3.0 x 50 mm) to monitor the progression of reactions and initial compound characterization. High resolution mass spectrometry (HR-MS) was performed on a 6230 TOF LC/MS (Agilent) mass spectrometer in the positive ion mode. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded in deuterated solvents on a Bruker AM 400 spectrometer. The chemical shifts, δ, are relative to internal TMS (^{1}H: δ 0.00 ppm) or were calibrated relative to solvent peaks and are reported in ppm (parts per million), whereas the coupling constants (*J*) are reported in Hertz (Hz). The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), t (triplet), q (quartet), dt (doublet of triplets), m (multiplet) and br (broad). ^{13}C and ^{31}P NMR spectra were recorded using proton decoupled methods.

Chemical synthesis.

(9H-fluoren-9-yl)methyl (6-(2-(diethoxyphosphoryl)acetimidamido)hexyl)carbamate (**2**):





In a round bottom flask equipped with a magnetic stir bar, absolute ethanol (0.43 mL, 7.32 mmol) and diethyl-(cyanomethyl)phosphonate (1 mL, 6.1 mmol) were dissolved in 5 mL of 2 M HCl/diethyl ether at 4 °C under nitrogen atmosphere. The solution was stirred vigorously overnight and subsequently concentrated under a stream of nitrogen. Notably, the desired ethyl 2-(diethoxyphosphoryl)acetimidate (HCl) did not precipitate out of solution. The resultant oily liquid comprises a mixture of ethyl 2-(diethoxyphosphoryl)acetimidate (HCl), unreacted diethyl-(cyanomethyl)phosphonate and the hydrolyzed ethyl 2-(diethoxyphosphoryl)acetate ester, and was used without further purification. Neither the parent compound, diethyl-(cyanomethyl)phosphonate, nor the ester are able to react with free amines, therefore only the acetimidate was able to couple to a free amine in the next step. Fmoc-1,6-diaminohexane (**1**) (74.98 mg, 0.2 mmol) was reacted with the mixture containing ethyl 2-(diethoxyphosphoryl)acetimidate (HCl) (~0.206 mL, 0.8 mmol) and triethylamine (0.14 mL, 1.0 mmol) in 2 mL of anhydrous methanol, and the suspension was stirred for 2 h at ambient temperature under nitrogen atmosphere. The reaction was quenched with water (5 mL) and acidified with TFA. The resulting compound, (9H-fluoren-9-yl)methyl (6-(2-(diethoxyphosphoryl)acetimidamido)hexyl)carbamate (**2**), was purified by semi-preparative RP-

HPLC using a water/acetonitrile gradient supplemented with 0.05% trifluoroacetic acid to afford the product as an off-white hygroscopic powder (24.7 mg, 24% yield). The identity and purity of the resulting compound was validated by HR-MS and NMR.

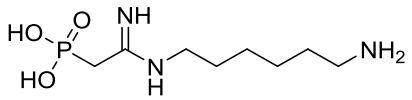
Expected mass for $C_{27}H_{38}N_3O_5P$ $(M + H)^+$: 516.2622 Da, observed mass: 516.2624 Da.

^{1}H NMR (methanol-D4, 400 MHz), δ(ppm): 7.70 (d, *J* = 7.5 Hz 2H), 7.54 (d, *J* = 7.4 Hz, 2H), 7.29 (t, *J* = 7.4 Hz, 2H), 7.21 (t, *J* = 7.5 Hz, 2H), 4.25 (d, *J* = 6.8 Hz, 2H) 4.15-4.04 (m, 5H), 3.18 (dt, *J* = 7.0, 1.5 Hz, 2H), 3.07 (d, *J* = 22.2 Hz, 2H), 3.01 (t, *J* = 6.9 Hz, 2H), 1.61-1.48 (m, 2H), 1.47-1.37 (m, 2H), 1.36-1.20 (m, 10H);

^{13}C NMR (methanol-D4, 100 MHz), δ(ppm): 161.30 (d, *J* = 6.1 Hz), 158.98, 145.35, 142.63, 128.80, 128.14, 126.14, 120.96, 67.57, 64.96 (d, *J* = 6.8 Hz), 48.51, 43.94, 41.48, 32.40 (d, *J* = 135 Hz), 30.74, 28.57, 27.34, 27.25, 16.69 (d, *J* = 5.9 Hz);

^{31}P NMR (methanol-D4, 162 MHz), δ(ppm): 19.13.

(2-((6-aminohexyl)amino)-2-iminoethyl)phosphonic acid, PO_3-amidine (**3**):



The Fmoc protected diethyl (2-((6-aminohexyl)amino)-2-iminoethyl)phosphonic acid (**2**) (20.61 mg, 0.04 mmol), was incubated with bromotrimethylsilane (TMSBr) (0.052 mL, 0.4 mmol) in 5 mL acetonitrile in a microwave reactor at 60 °C for 5 h, by which time the complete removal of the ethyl protecting groups was observed by LC-MS. The solution was further concentrated using a rotary evaporator to remove the volatile unreacted TMSBr and solvent. The readily cleavable TMS protected phosphonate was incubated in 10 mL water/methanol mixture (90/10) yielding the unprotected phosphonate. The solution was concentrated under reduced pressure and removal of the Fmoc protecting group was effected with 20% piperidine in DMF (0.5 mL) for 20 min. The fully deprotected compound **3** was precipitated with 20 mL diethyl ether and purified by semi-preparative RP-HPLC using a water/acetonitrile gradient supplemented with 0.05% trifluoroacetic acid to afford the final product as an off-white hygroscopic powder (7.4 mg, 78% yield). The identity and purity of the resulting compound, PO_3-amidine (**3**), was validated by HR-MS and NMR .

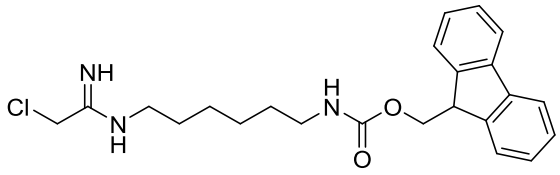
Expected mass for $C_8H_{20}N_3O_3P$ $(M + H)^+$: 238.1315 Da, observed mass: 238.1320 Da.

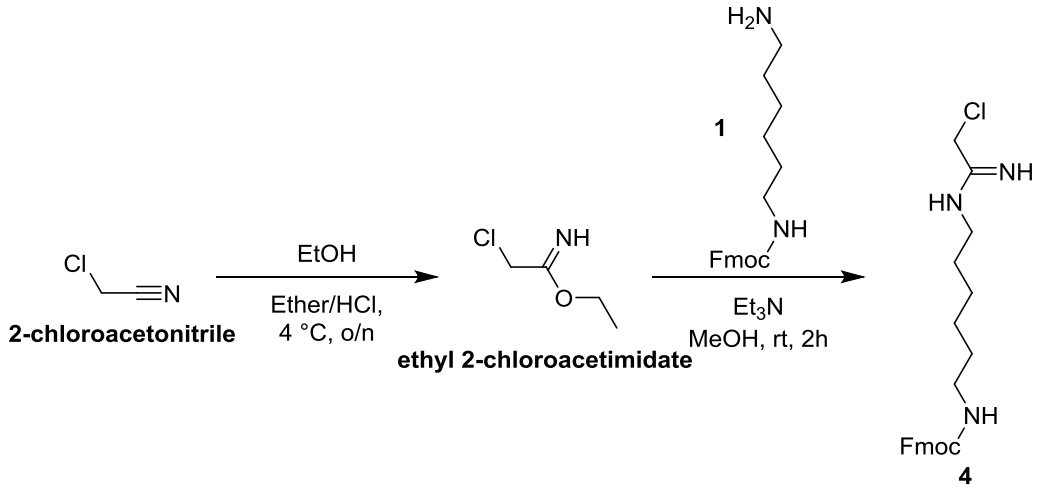
^{1}H NMR (D_2O, 400 MHz), δ(ppm): 3.18 (dt, *J* = 6.9, 1.5 Hz, 2H), 2.88 (t, *J* = 7.5 Hz, 2H), 2.78 (d, *J* = 20.2 Hz, 2H), 1.651-1.61 (m, 4H), 1.26-1.35 (m, 4H);

^{13}C NMR (D_2O, 100 MHz), δ(ppm): 162.38 (d, *J* = 5.5 Hz), 42.17, 39.26, 33.83 (d, *J* = 120.0 Hz), 26.45, 26.36, 25.29, 25.04 ;

^{31}P NMR (D_2O, 162 MHz), δ(ppm): 10.81.

(9H-fluoren-9-yl)methyl (6-(2-chloroacetimidamido)hexyl)carbamate (**4**):



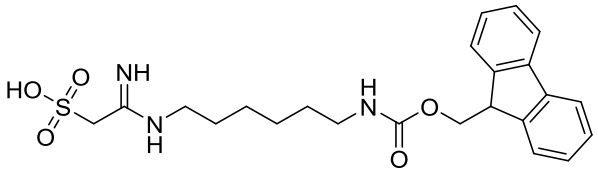


Ethyl 2-chloroacetimidate was synthesized starting from 2-chloroacetonitrile in a one-step reaction with ethanol in acidified ether using the Pinner reaction as described in Luo et al.[1] Fmoc protected 1,6-diaminohexane (37.49 mg, 0.1 mmol) (**1**) was dissolved in methanol (2 mL). Then, ethyl 2-chloroacetimidate-HCl (31.4 mg, 0.2 mmol) and triethylamine (0.042 mL, 0.3 mmol) were added and the reaction was stirred for 2 h. The reaction was quenched with water (5 mL) and acidified with TFA. The resulting compound, (9H-fluoren-9-yl)methyl (6-(2-chloroacetimidamido)hexyl)carbamate (**4**), was purified by semi-preparative RP-HPLC using a water/acetonitrile gradient supplemented with 0.1% formic acid to afford the product as an off-white hygroscopic powder following lyophilization (34 mg, 82% yield). The identity and purity of the resulting compound was validated by HR-MS and NMR. Expected mass for $C_{23}H_{28}ClN_3O_2$ $(M + H)^+$: 414.9535 Da, observed mass: 414.9538 Da.

^{1}H NMR (methanol-d_4, 400 MHz), δ(ppm): 7.66 (d, *J* = 7.5 Hz 2H), 7.52 (d, *J* = 7.4 Hz, 2H), 7.27 (t, *J* = 7.4 Hz, 2H), 7.18 (t, *J* = 7.4 Hz, 2H), 4.26 (s, 2H), 4.21 (d, *J* = 6.9 Hz, 2H), 4.06 (t, *J* = 6.8 Hz, 1H) 3.16 (t, *J* = 7.1 Hz, 2H), 2.99 (t, *J* = 6.9 Hz, 2H), 1.58-1.48 (m, 2H), 1.45-1.34 (m, 2H), 1.33-1.18 (m, 4H);

^{13}C NMR (methanol-D4, 100 MHz), δ(ppm): 164.42, 158.94, 145.35, 142.61, 128.83, 128.18, 126.20, 121.00, 67.59, 48.51, 44.00, 41.55, 40.12, 30.71, 28.40, 27.38, 27.29.

2-((6-(((((9H-fluoren-9-yl)methoxy)carbonyl)amino)hexyl)amino)-2-iminoethane-1-sulfonic acid (**5**):



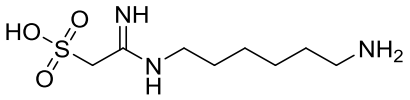
30 mg (0.072 mmol) (9H-fluoren-9-yl)methyl (6-(2-chloroacetimidamido)hexyl)carbamate (**4**) and Na_2SO_3 (27.7 mg, 0.22 mmol) were dissolved in ethanol/water (50/50). The mixture was stirred for 3 h at ambient temperature to yield compound **5**. The product was isolated by RP-HPLC using a water/acetonitrile gradient supplemented with 0.1% formic acid to afford the product as a white hygroscopic powder following lyophilization (28 mg, 85% yield). The identity and purity of the resulting compound, 2-((6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)hexyl)amino)-2-iminoethane-1-sulfonic acid (**5**), was validated by HR-MS and NMR.

Expected mass for $C_{23}H_{29}N_3O_5S$ $(M + H)^+$: 460.1901 Da, observed mass: 460.1906 Da.

^{1}H NMR (DMSO-d_6, 400 MHz), δ(ppm): 8.83 (brs, 3H), 7.82 (d, *J* = 7.5 Hz, 2H), 7.62 (d, *J* = 7.4 Hz 2H), 7.35 (t, *J* = 7.4 Hz, 2H), 7.26 (t, *J* = 7.5 Hz, 2H), 7.21 (t, *J* = 5.6 Hz, 1H), 4.23 (d, *J* = 6.9 Hz, 2H), 4.14 (t, *J* = 6.8 Hz, 1H), 3.54 (s, 2H), 3.11 (t, *J* = 7.0 Hz, 2H), 2.90 (q, *J* = 6.4 Hz, 2H), 1.48-1.38 (m, 2H), 1.37-1.27 (m, 2H), 1.27-1.13 (m, 4H);

^{13}C NMR (DMSO-D6, 100 MHz), δ(ppm): 160.62, 156.05, 143.90, 140.70, 127.56, 127.01, 125.12, 120.08, 65.09, 53.52, 46.74, 41.79, 39.72, 29.19, 27.17, 25.75, 25.66.

2-((6-aminohexyl)amino)-2-iminoethane-1-sulfonic acid, SO_3-amidine (**6**):



2-((6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)hexyl)amino)-2-iminoethane-1-sulfonic acid (**5**) (20 mg, 0.044 mmol) was dissolved 20% piperidine in DMF (0.5 mL) and reacted at ambient temperature for 20 min. After complete Fmoc deprotection, the final compound, 2-((6-aminohexyl)amino)-2-iminoethane-1-sulfonic acid (**6**) was precipitated in 20 mL diethyl ether before being purified by semi-preparative RP-HPLC using a water/acetonitrile gradient supplemented with 0.05% trifluoroacetic acid to afford the final product as an off-white hygroscopic powder following lyophilization (10 mg, 95% yield). The identity and purity of the resulting compound, SO_3-amidine (**6**) was validated by HR-MS and NMR.

Expected mass for $C_8H_{19}N_3O_3S$ $(M + H)^+$: 238.1220 Da, observed mass: 238.1225 Da.

^{1}H NMR (D_2O, 400 MHz), δ(ppm): 3.91 (s, 2H), 3.23 (t, J = 6.8 Hz, 2H), 2.88 (t, J = 7.5 Hz, 2H), 1.63-1.51 (m, 4H), 1.36-1.26 (m, 4H);

^{13}C NMR (D_2O, 100 MHz), δ(ppm): 158.90, 53.30, 42.36, 39.24, 26.43, 26.23, 25.20, 24.99.

^{31}P NMR pH titration experiment for p*K*a dtermination.

pH dependent ^{31}P NMR spectra of PO3-amidine (**3**) (0.4 mM) in water were acquired at various pH increments (in steps of ~0.5 pH units) by addition of NaOH. The proton decoupled ^{31}P NMR spectra consist of 100 scans at each pH. The adjusted pH was measured using an InLab® Ultra-Micro pH electrode (Mettler Toledo) before and after the NMR measurement at each step to ensure reliable pH recordings. The pK_{a2} value of the phosphonate group was derived by sigmoidal nonlinear regression analysis (Prism software) by plotting the chemical shift of ^{31}P versus pH value.

Protein expression and purification.

The protein arginine kinase McsB and the arginine phosphatase YwlE were overexpressed as C-terminal 6x His-tagged proteins in *Escherichia coli* BL21 (DE3) cells. Cells were grown at 37 °C in LB medium in the presence of ampicillin (100 μg/mL) and expression was induced with 0.5

mM IPTG for 2 h at an OD_{600nm} of 0.8. After centrifugation at 8600 g, the cell pellet was resuspended in 300 mM NaCl, 50 mM Tris/HCl pH 8.0 and disrupted by sonication. The cleared lysate was loaded on a Ni^{2+}-nitrilotriacetate (Ni-NTA) column (Qiagen), and the His-tagged proteins were eluted by applying a step-wise imidazole gradient to 250 mM imidazole. The enzyme containing fraction was directly applied to a Superdex-75 column (prep grade, GE Healthcare) equilibrated with 20 mM Tris/HCl pH 7.6, 100 mM NaCl. Fractions containing the respective enzymes were pooled and concentrated using a Centricon concentrator (Millipore Corp.) with a 10 kDa nominal molecular mass cutoff. The concentrated protein was flash frozen in liquid nitrogen, and stored at -80 °C.

Pan-phosphoarginine antibody generation and purification.

Polyclonal antibody sera were raised in rabbits against the phosphoarginine mimicking haptens **3** and **6**. For this purpose, the haptens were conjugated to KLH (keyhole limpet hemocyanine) carrier protein via glutaraldehyde crosslinking. Two rabbits were injected with each hapten/KLH conjugate using standard immunization protocols. These experiments were performed by New England Peptide Inc (NEP). Individual bleeds were tested for recognition of phosphoarginine by dot blot or Western blot experiments. The resulting serum with the highest pArg binding reactivity was purified by affinity chromatography using arginine phosphorylated lysozyme crosslinked to N-hydroxysuccinimide (NHS)-activated agarose beads (Pierce) to afford the pure polyclonal anti-pArg antibody. Briefly, arginine phosphorylated lysozyme was obtained by reaction with McsB in the presence of 2 mM ATP in 20 mM Tris pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$ (**see Figure S16**). Then, the phosphorylated lysozyme was separated by Heparin chromatography using a 50 mM to 1 M NaCl gradient in 50 mM Tris, pH 7.5. The resulting arginine phosphorylated lysozyme was validated by electrospray ionization mass spectrometry analysis (see below). Prior to agarose coupling, the phosphorylated lysozyme sample was desalted using a PD-10 column in coupling buffer (20 mM NaH_2PO_4 pH 7.5) and concentrated. 5 mg of the phospho-lysozyme sample was crosslinked to a slurry of 2 mL NHS-activated Agarose beads for 2 h at ambient temperature. After washing with coupling buffer, the remaining unreacted NHS was blocked using 1 M ethanolamine, pH 7.4. The pArg lysozyme coupled agarose beads were washed extensively with coupling buffer and TBS and stored at 4 °C. Subsequently the serum was diluted 1:5 in TBS and applied onto the pArg lysozyme resin. The mixture was incubated for 6 h at 4 °C with end-over-end rotation. The beads were washed using TBS and the bound anti-pArg antibodies were eluted by applying 4x 100 mM glycine, pH 2.4.

The resulting eluates were immediately neutralized with 1 M Tris, pH 8.0, desalted into PBS and stored at -80 °C.

Dot blot experiments using anti-pArg antibody.

Phosphotyrosine BSA, phosphoserine and phosphothreonine BSA were all purchased from Sigma-Aldrich. Phosphoarginine BSA, phosphohistidine and phospholysine BSA were generated as described below. Protein samples were directly spotted onto the nitrocellulose membrane and dried for ~10 minutes. The membrane was blocked with either 5% milk powder in TBS (25 mM Tris pH 7.6, 150 mM NaCl) for 1 h. The membrane was then incubated for 1 h with anti-pArg antibody (1:7,000) diluted in 1% milk powder in TBS. The membrane was washed 4 times in TBST (TBS supplemented with 0.05% TWEEN) and the secondary HRP coupled goat anti-rabbit antibody ab97051 (1:20,000, AbCam) was incubated for 1 h in 1% milk in TBS. After washing the membrane 4 times in TBST, the HRP signal was detected by Pierce ECL Western Blotting substrate.

Generation of phosphoarginine BSA, phosphohistidine BSA and phospholysine BSA.

Phosphoarginine BSA was synthesized enzymatically by treatment of BSA with the protein arginine kinase McsB. Briefly, 5 µM McsB was incubated with 10 µM heat denatured BSA in 10 mM $MgCl_2$, 2 mM ATP, 20 mM Tris pH 8.0 for 1 h. The His-tagged McsB kinase was removed by addition of equilibrated Ni-NTA beads (Qiagen) followed by incubation for 30 minutes and centrifugation at 2,000 rpm. The supernatant was collected and the procedure was repeated two times to ensure complete removal of the His-tagged kinase. The resulting arginine phosphorylated BSA sample was desalted using a PD-10 column (GE Life Sciences) in TBS and concentrated using Amicon Ultra-0.5 centrifugal filter devices (Millipore).

Phosphohistidine BSA was synthesized chemically by incubation of BSA with potassium phosphoramidate according to the procedure by Kee et al.[2] Potassium phosphoramidate was synthesized from $POCl_3$ and ammonia as described by Wei and Matthews. [3] The freshly prepared potassium phosphoramidate (100 mM) was added to 1 mg/mL BSA and incubated overnight at ambient temperature under slight agitation. The resulting histidine phosphorylated BSA sample was desalted using a PD-10 column (GE Life Sciences) in TBS and concentrated using Amicon Ultra-0.5 centrifugal filter devices (Millipore).

Phospholysine BSA was prepared chemically by incubation of BSA with phosphorous oxychloride according to the procedure by Mayer et al. and Zetterqvist et al.[4] Briefly,

phosphorous oxychloride (10 mM) was added dropwise to 1 mg/mL BSA at 4 °C, while the pH was adjusted with NaOH to ~pH 9. The reaction mixture was incubated overnight at 4 °C. The resulting lysine phosphorylated BSA sample was desalted using a PD-10 column (GE Life Sciences) in TBS and concentrated using Amicon Ultra-0.5 centrifugal filter devices (Millipore). To verify the presence of acid labile phosphoramidate bonds in the generated N-phosphorylated BSA proteins, the proteins were incubated in 1 M HCl for 1 h to hydrolyze the acid labile phosphoryl groups. Analysis of the acid-labile phosphate content was performed using the malachite green phosphate assay kit (Cayman), recording the absorbance of the resulting molybdophosphoric acid complex at 620 nm on a SpectraMax M5 (Molecular Devices) microplate reader. This assay revealed that ~3, ~5 and ~15 acid labile phosphoryl groups are attached per pArg, pHis and pLys modified BSA protein, respectively.

Western blotting using anti-pArg antibody.

Protein samples were mixed with 6x SDS loading dye and directly separated by 15% SDS-PAGE. Separated proteins were then transferred to a nitrocellulose membrane at 15 V for 35 min using semi-dry blotting. To minimize non-specific antibody binding, the membrane was blocked with either 5% milk powder in TBS (25 mM Tris pH 7.6, 150 mM NaCl) or protein-free blocking reagent in TBS (cat., 37570, Pierce) for 1 h. The membrane was then incubated for 1 h with anti-pArg antibody (1:3,000) diluted in protein-free blocking reagent in TBS. The membrane was washed 4 times in TBST (TBS supplemented with 0.05% TWEEN) and the secondary HRP coupled goat anti-rabbit antibody ab97051 (1:20,000, AbCam) was incubated for 1 h in 1% milk in TBS. After washing the membrane 4 times in TBST, the HRP signal was detected by Pierce ECL Western Blotting substrate.

ELISA using anti-Arg antibody.

To test the anti-pArg antibody for recognizing arginine phosphorylated McsB we developed a highly specific ELISA screen. For this purpose McsB (10 μM) was autophosphorylated for 30 minutes in a solution containing 20 mM Tris pH 8.0, 50 mM NaCl, 2 mM ATP, 10 mM $MgCl_2$. Moreover, to validate the presence of arginine phosphorylation the McsB sample was treated with 1 μM YwlE or 10% acetic acid as indicated. The 6x His-tagged McsB was then diluted in TBS to 2 μg/mL, bound onto Ni-NTA coated pre-blocked microplates (Qiagen) and incubated for 1 h at 4 °C. The plates were washed five times with TBST (TBS supplemented with 0.05% Tween-20) and treated with 0.5 μg/mL anti-pArg antibody for 1 h. In addition, as shown in **figure S15,** free phosphoarginine (Toronto Research Chemicals) was added simultaneously

with the primary antibody to test the effect of blocking the anti-pArg antibody. The wells were washed four times using TBST. Anti-rabbit-HRP secondary antibody (AbCam) was added at 1:20,000 dilution and incubated for 45 min. After extensive washing (5 times with TBST), chromogenic reaction was initiated by addition of TMB (Pierce) and stopped with 2 M H_2SO_4 to quench the reaction. The absorbance of the resulting signal was recorded on a SpectraMax M5 (Molecular Devices) microplate reader at 450 nm.

Electrospray ionization mass spectrometry (ESI-MS).

The molecular mass of phosphorylated lysozyme was measured on a LTQ XL linear Ion trap mass spectrometer (Thermo Scientific) connected to a liquid chromatography (LC) system. Phosphorylated lysozyme sample was separated and desalted onto a C8 column and subsequently analyzed in the mass spectrometer. Spectra were acquired in the positive ion mode. The deconvolution program MaqTran was used to obtain the mass spectra. Theoretical average molecular mass of lysozyme and its phosphorylated form(s) were calculated using online tools (http://web.expasy.org/compute_pi/ and http://www.peptidesynthetics.co.uk/tools/).

Immunoprecipitation of arginine phosphorylated proteins.

Immunoprecipitation experiments were initiated by incubating ProteinA/G Plus-agarose beads (Santa Cruz Biotechnology) with purified anti-pArg antibody, according to the manufacturer's instructions. Stressed (500 μM diamide) and non-stressed *B. subtilis* and *B. subtilis ΔywlE* cells were grown in 500 mL LB media at 37 °C to an OD_{600} of 0.5. Cells were harvested by centrifugation at 8,600 g at 4 °C, washed and resuspended in TBS buffer and lysed by sonication. The cleared cell lysate was then incubated with 50 μL of the anti-Arg antibody protein A/G bead slurry for 1 h. The beads were centrifuged at 13,400 g for 60 s, the supernatant discarded and the beads washed 4 times with 1 mL of 20 mM Tris pH 7.6, 100 mM NaCl, 0.5% Triton X-100. The beads were resuspended in 20 μL 6x SDS-PAGE loading buffer and boiled for 5 min. The samples were loaded on to a 15% SDS-PAGE gel, blotted onto nitrocellulose membranes and the resulting protein signals were detected as described above. For ClpC detection the anti-ClpC antibody (kindly provided by Dr. Tim Clausen) was diluted 1:7,000 and for GroEL identification the monoclonal anti-GroEL antibody LS-C15687 (LifeSpan BioSciences, Inc.) was diluted 1:1,000. The Clean-Blot IP detection reagent (HRP) antibody

(Life Technologies), recognizing native primary antibodies, was used at 1:2,000 to detect the primary antibodies.

Figure S1. ^{1}H (400 MHz) NMR spectrum of (9H-fluoren-9-yl)methyl (6-(2-(diethoxyphosphoryl)acetimidamido)hexyl)carbamate (**2**) in methanol-D4.

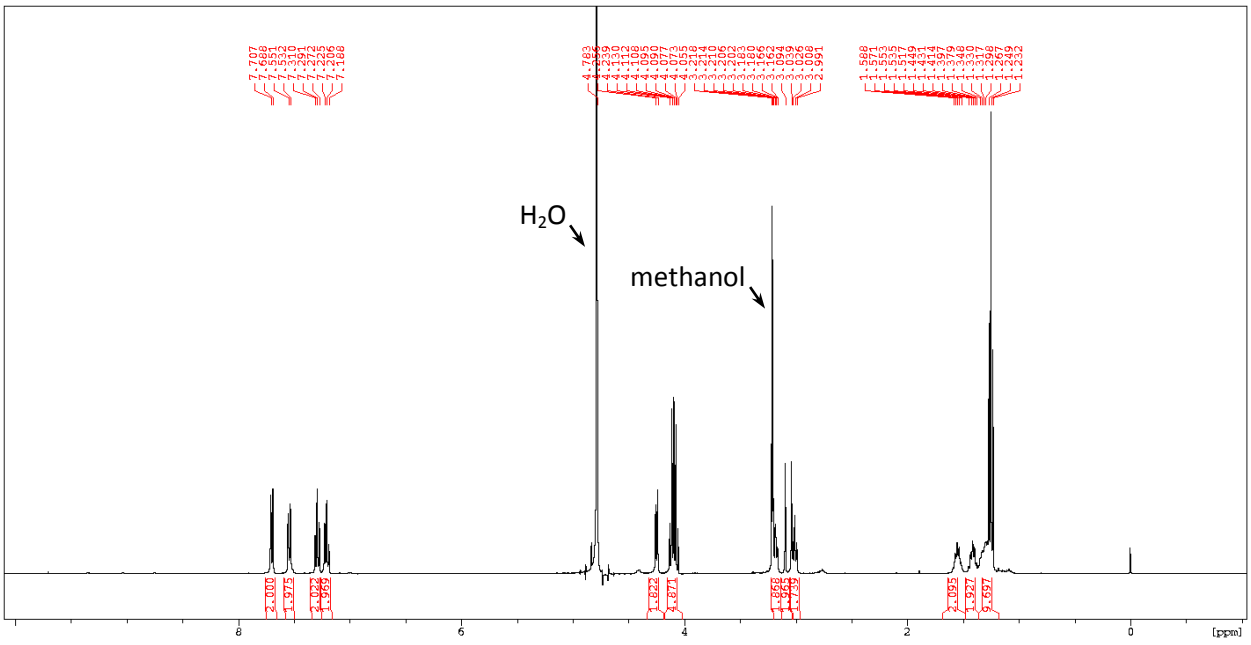


Figure S2. ^{13}C (100 MHz) NMR spectrum of (9H-fluoren-9-yl)methyl (6-(2-(diethoxyphosphoryl)acetimidamido)hexyl)carbamate (**2**) in methanol-D4.

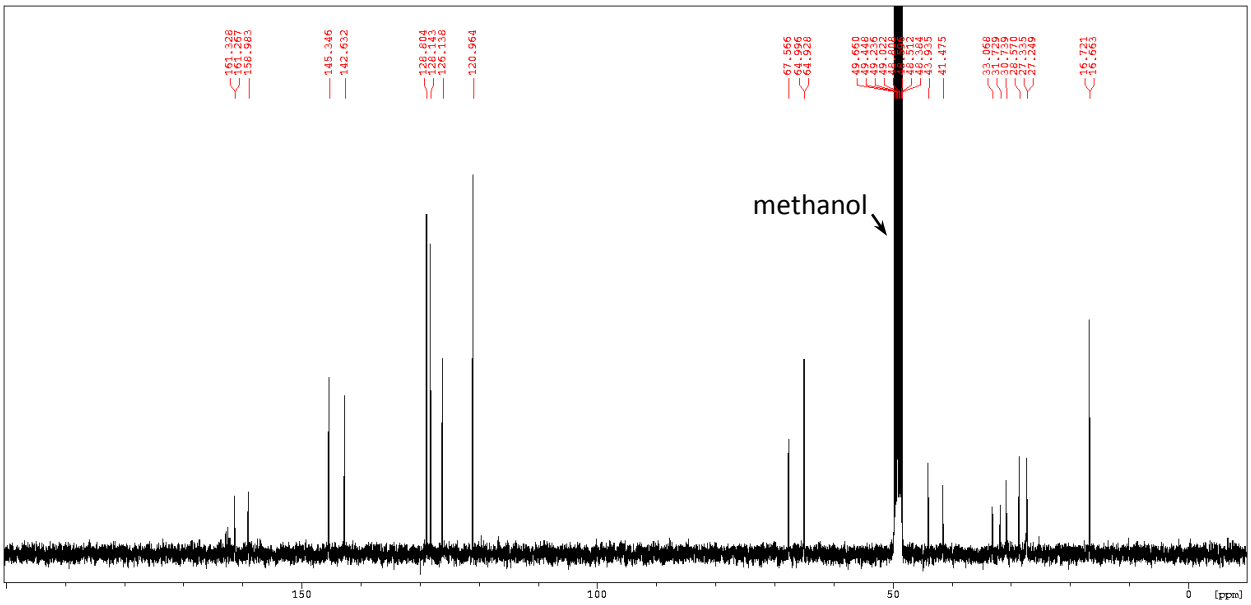


Figure S3. ^{31}P (162 MHz) NMR spectrum of (9H-fluoren-9-yl)methyl (6-(2-(diethoxyphosphoryl)acetimidamido)hexyl)carbamate (**2**) in methanol-D4.

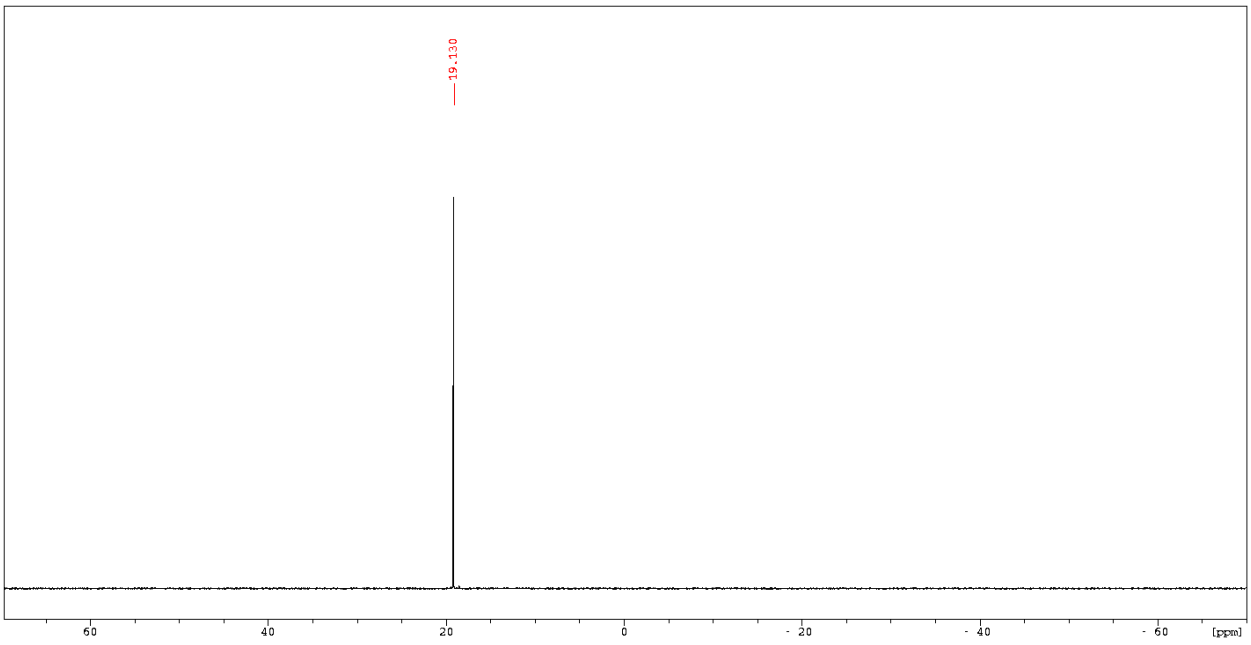
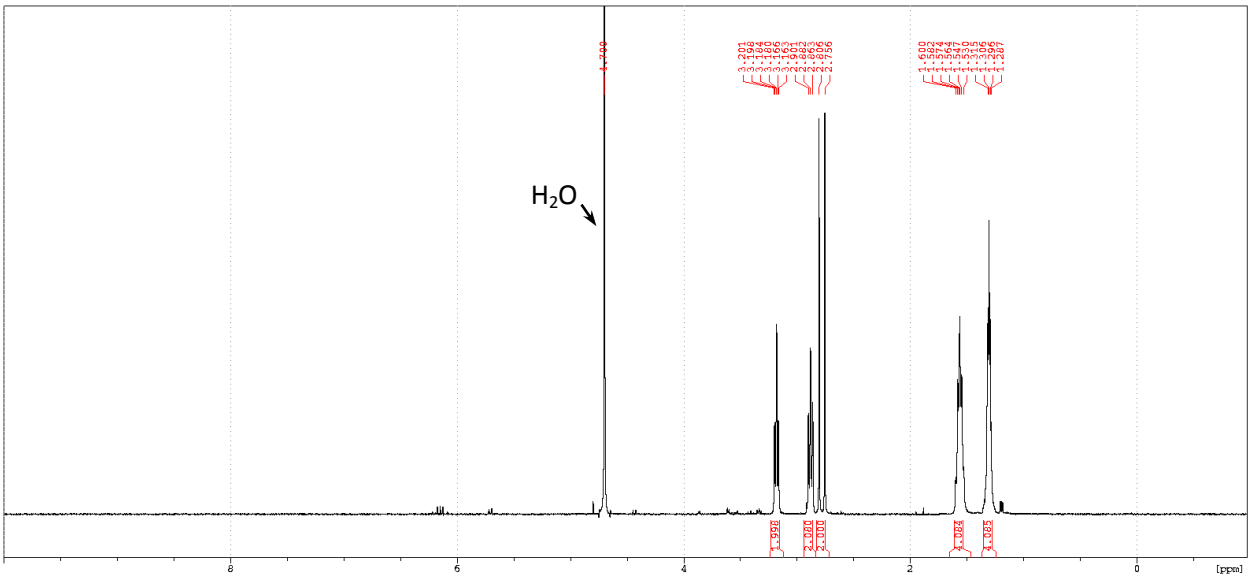


Figure S4. a) ^{1}H (400 MHz) NMR spectrum of (2-((6-aminohexyl)amino)-2-iminoethyl)phosphonic acid (**3**) in D_2O.



b) ^{1}H (400 MHz) NMR spectrum of (2-((6-aminohexyl)amino)-2-iminoethyl)phosphonic acid (**3**) in D_2O using a water suppression program.

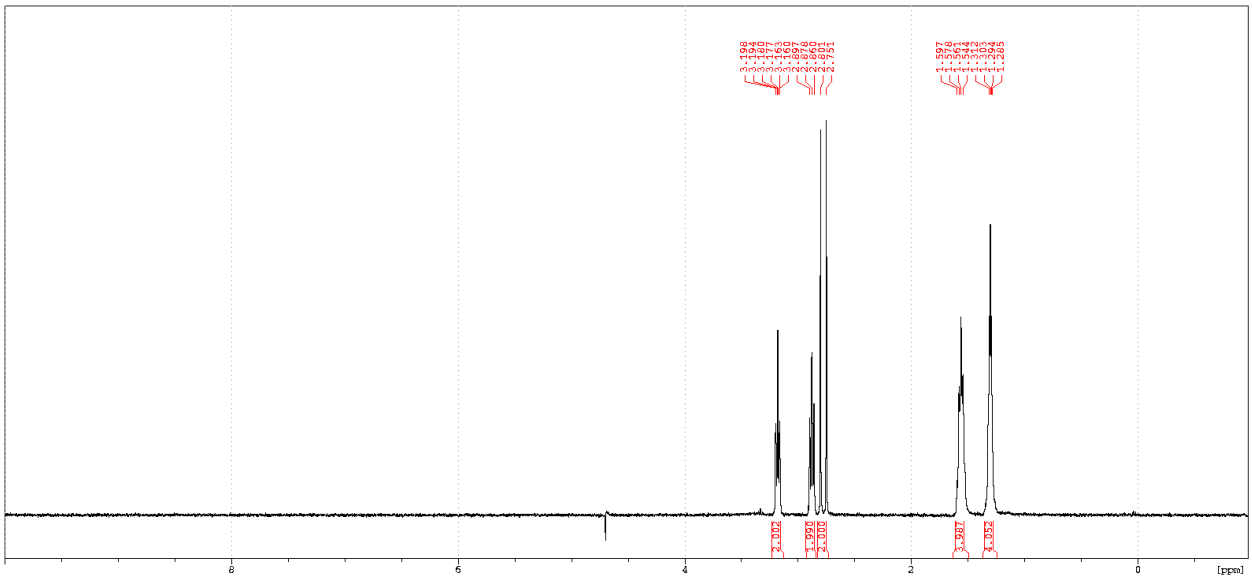


Figure S5. ^{13}C (100 MHz) NMR spectrum of (2-((6-aminohexyl)amino)-2-iminoethyl)phosphonic acid (**3**) in D_2O.

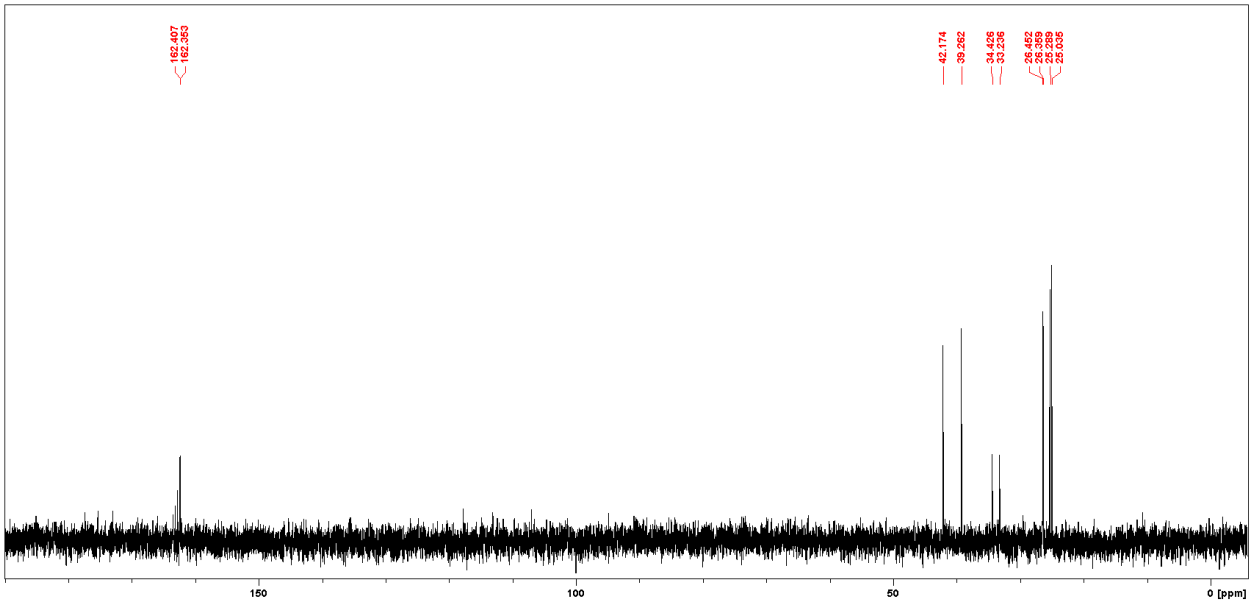


Figure S6. ^{31}P (162 MHz) NMR spectrum of (2-((6-aminohexyl)amino)-2-iminoethyl)phosphonic acid (**3**) in D_2O.

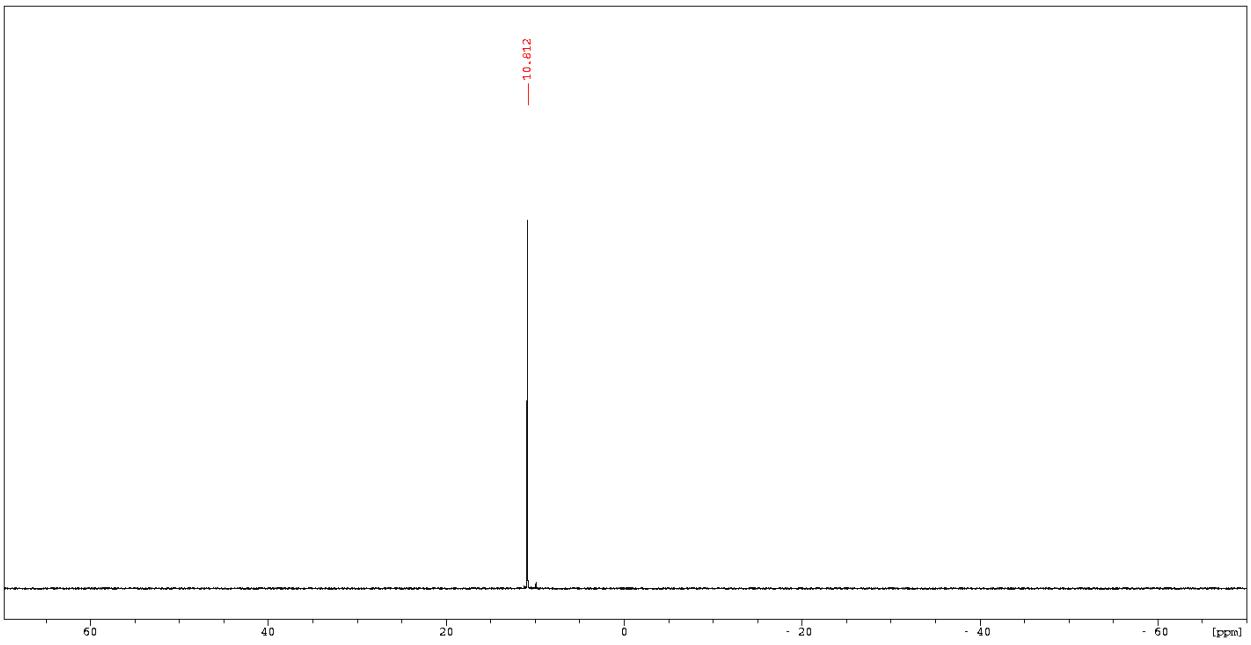


Figure S7. ^{1}H (400 MHz) NMR spectrum of (9H-fluoren-9-yl)methyl (6-(2-chloroacetimidamido)hexyl)carbamate (**4**) in methanol-D4.

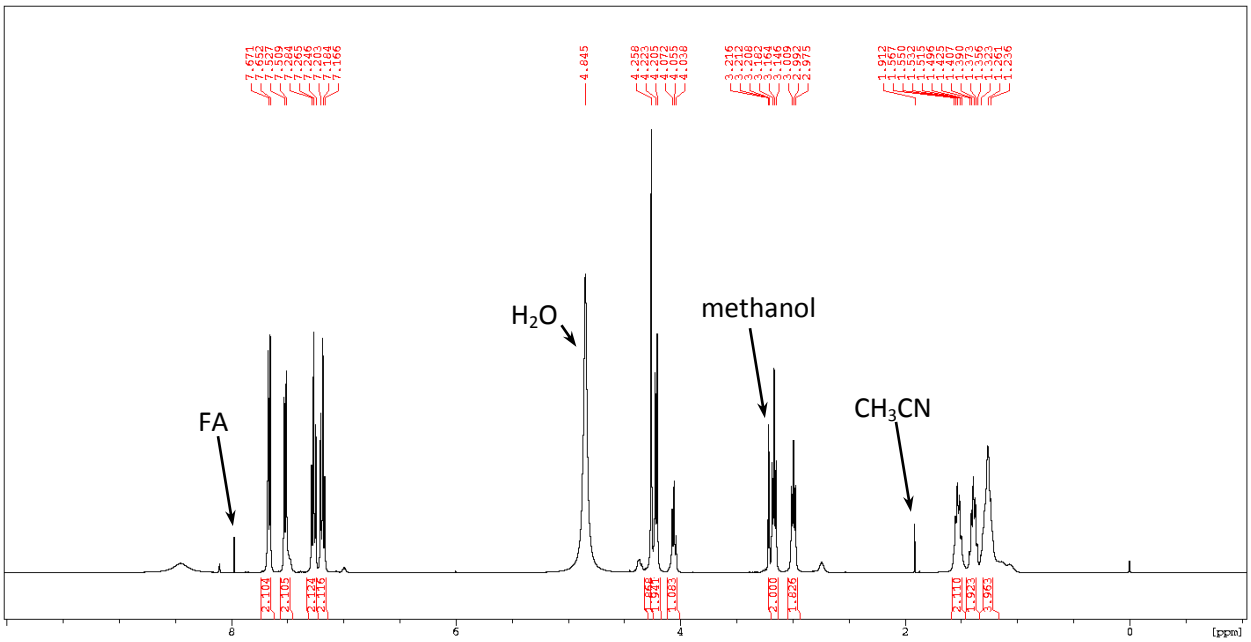


Figure S8. ^{13}C (100 MHz) NMR spectrum of (9H-fluoren-9-yl)methyl (6-(2-chloroacetimidamido)hexyl)carbamate (**4**) in methanol-D4.

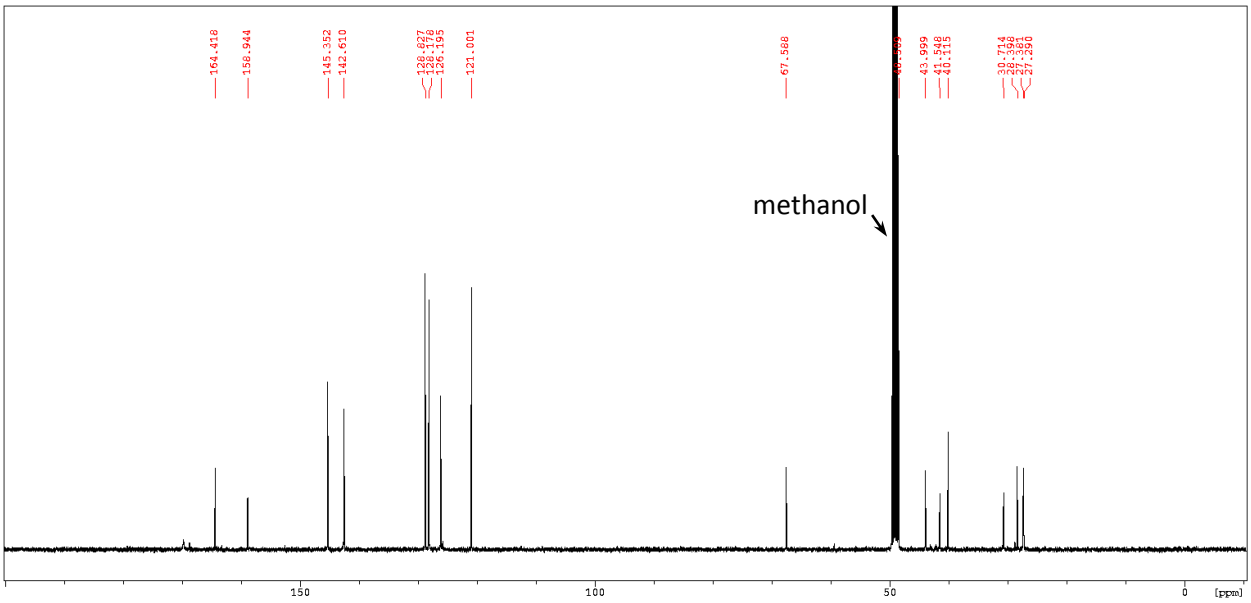


Figure S9. ^{1}H (400 MHz) NMR spectrum of 2-((6-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)hexyl)amino)-2-iminoethane-1-sulfonic acid (**5**) in DMSO-D6.

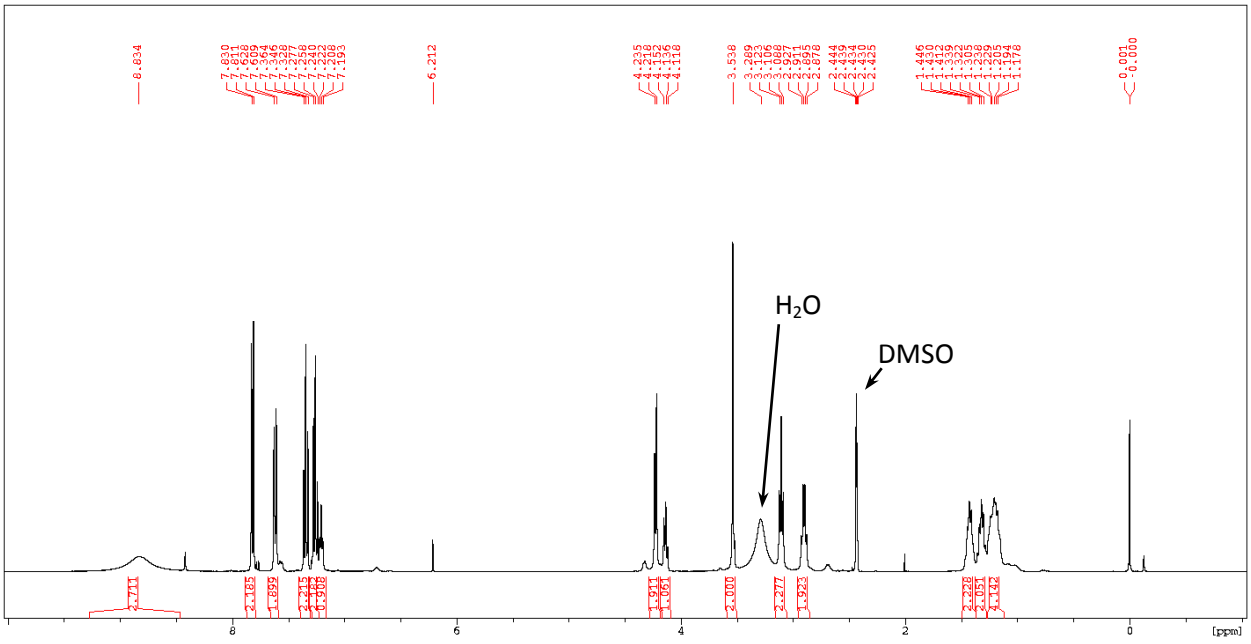


Figure S10. The Fmoc protected SO3-amidine (**5**) becomes deprotected in DMSO.

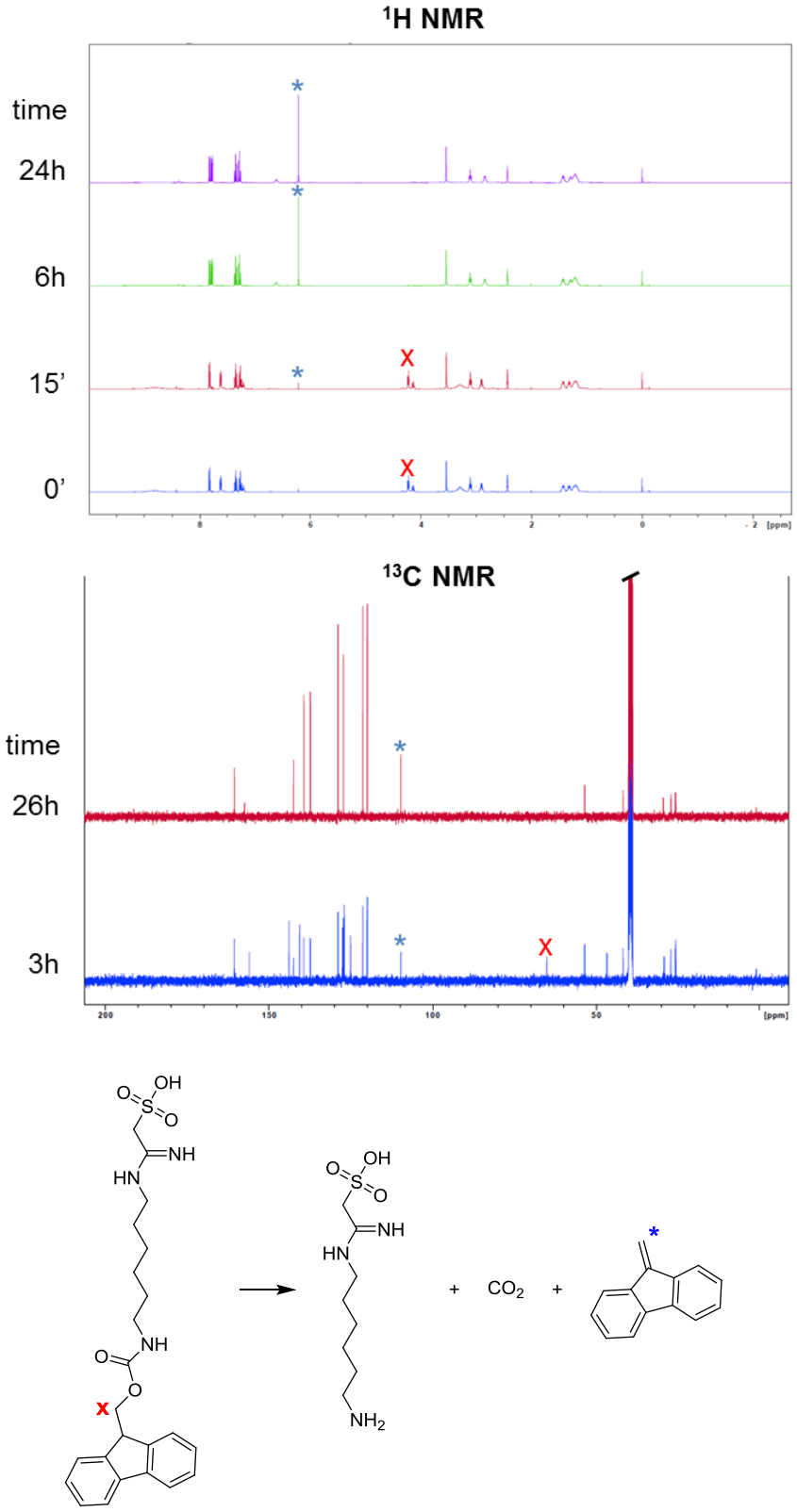


Figure S11. ^{1}H (400 MHz) NMR spectrum of 2-((6-aminohexyl)amino)-2-iminoethane-1-sulfonic acid (**6**) in D_2O.

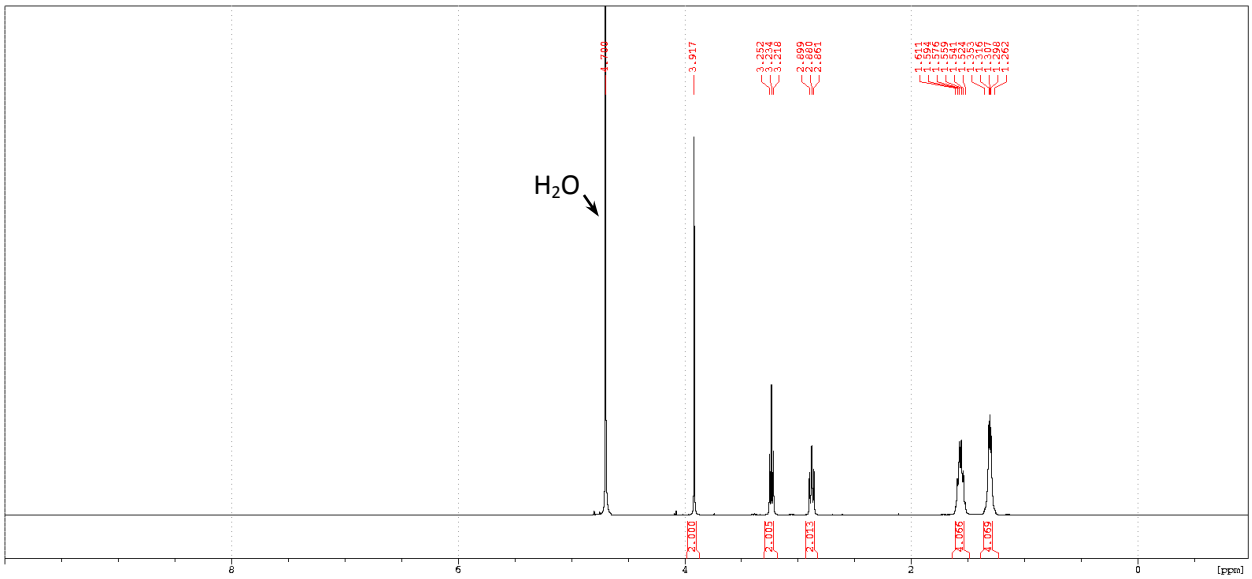


Figure S12. ^{13}C (100 MHz) NMR spectrum of 2-((6-aminohexyl)amino)-2-iminoethane-1-sulfonic acid (**6**) in D_2O.

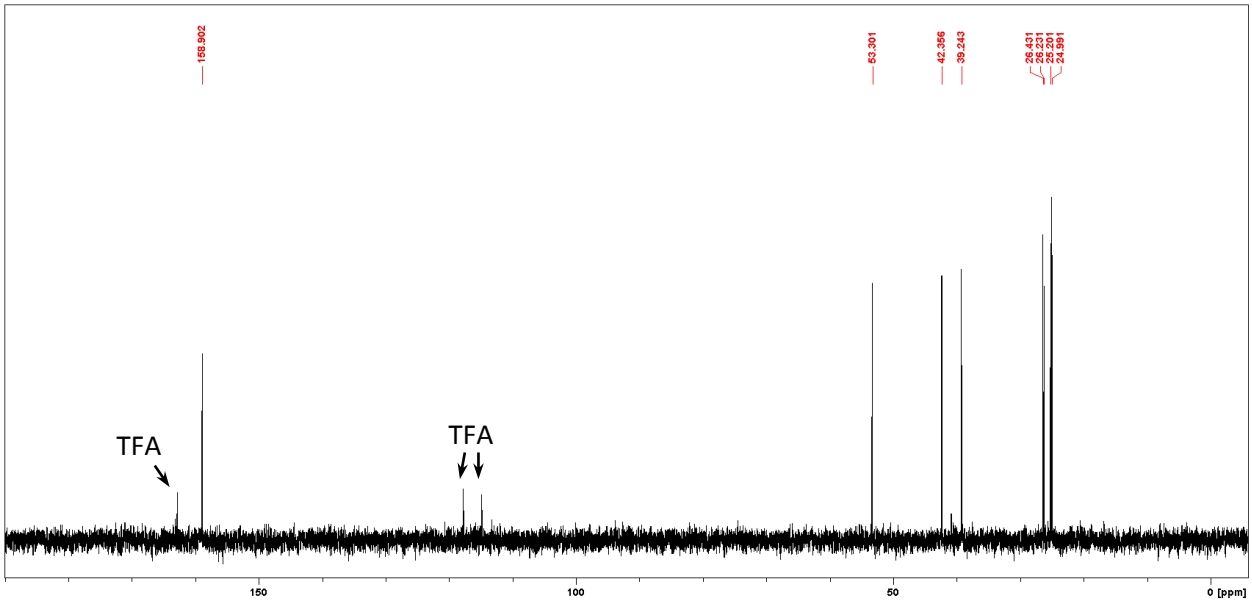


Figure S13. ^{31}P NMR pH titration experiment of PO3-amidine (**3**). ^{31}P NMR spectra (top) of PO3-amidine (**3**) were measured in various pH intervals. The fitted chemical shift perturbations indicate that the second p*K*a of the phosphonate group is around pH 6.0.

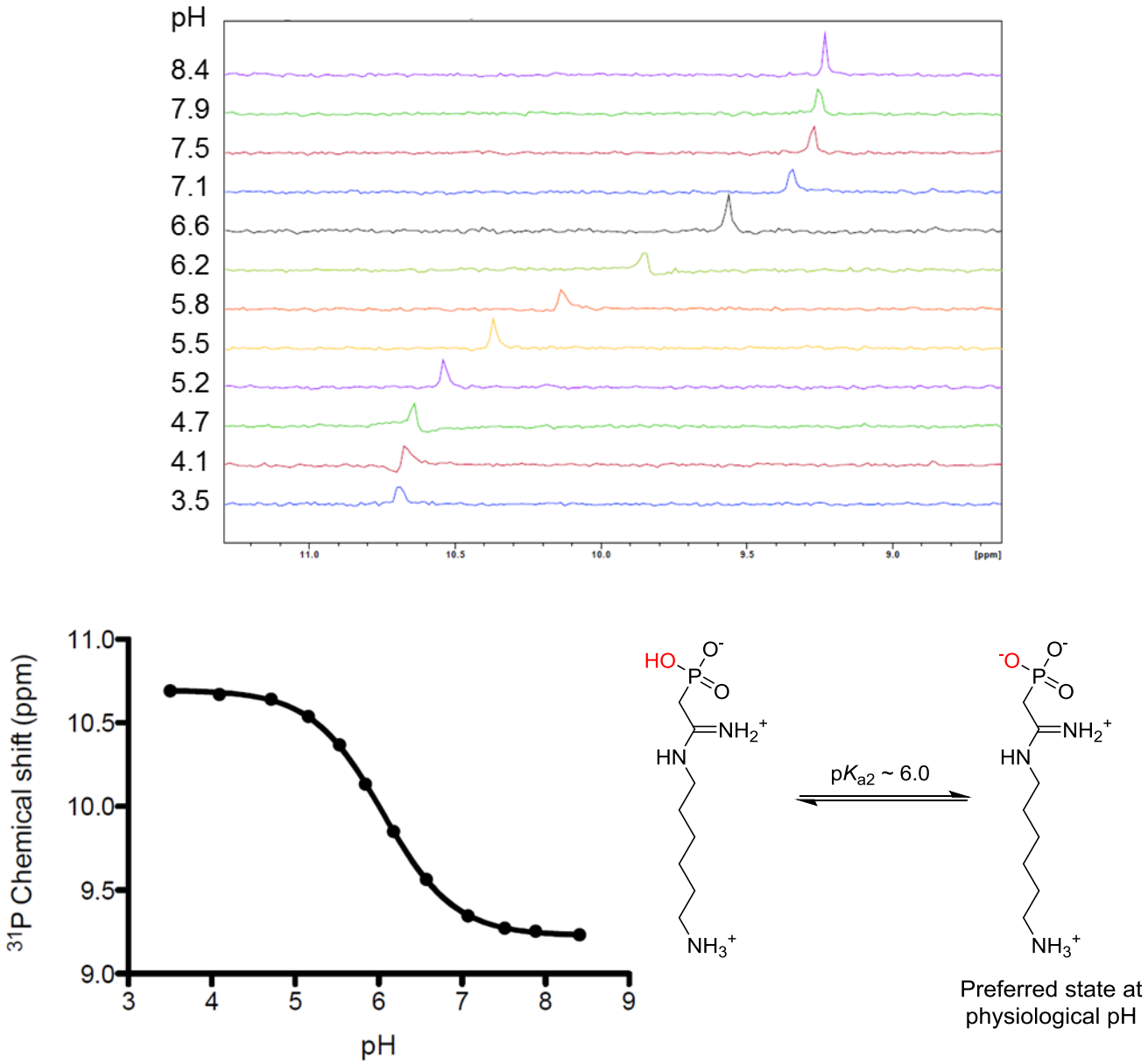


Figure S14. Characterization of the SO_3-amidine raised antibody. a) Western Blot analysis shows the lack of detection of arginine phosphorylated McsB using the SO3-amidine raised antibody. b) Dot blot analysis of SO_3-amidine antibody towards the conjugated SO3-amidine hapten and arginine phosphorylated and non-phosphorylated McsB.

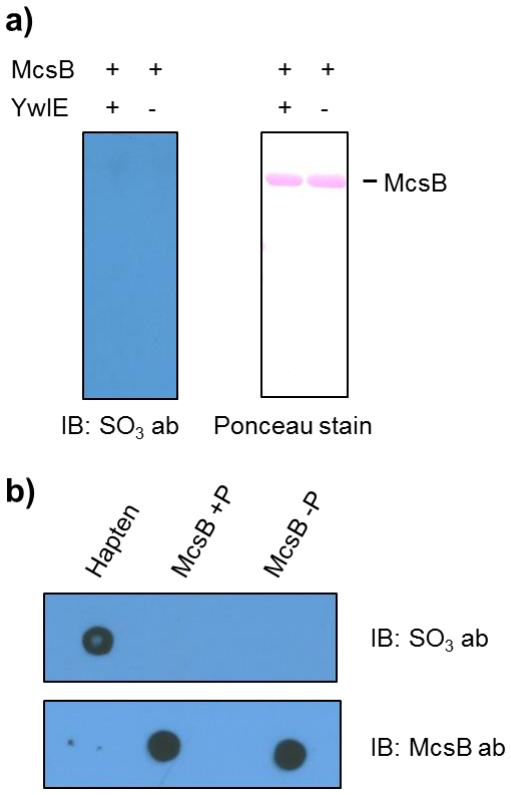


Figure S15. ELISA analysis showing the specificity of the anti-pArg antibody for phosphoarginine using recombinant arginine phosphorylated McsB in the absence (control) and presence of the arginine phosphatase YwlE and free phosphoarginine (L-pArg) as well as neutralized pArg McsB pretreated with acetic acid (n = 3, mean value ± SD).

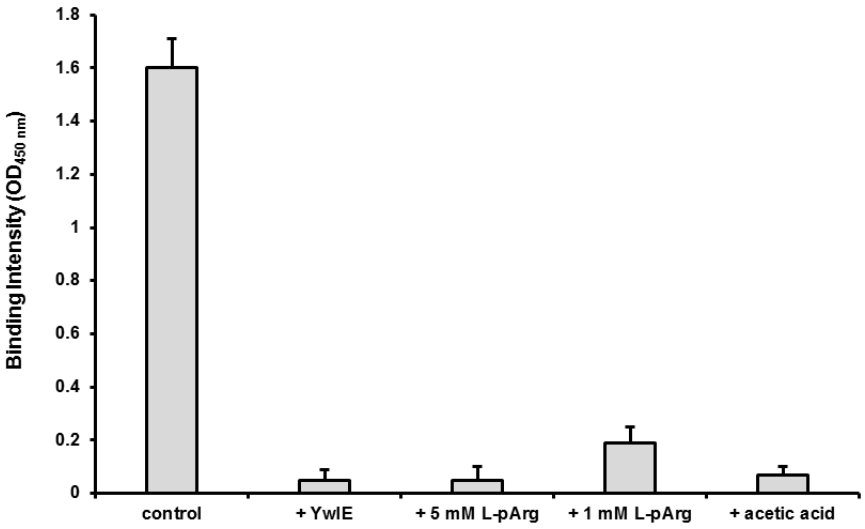
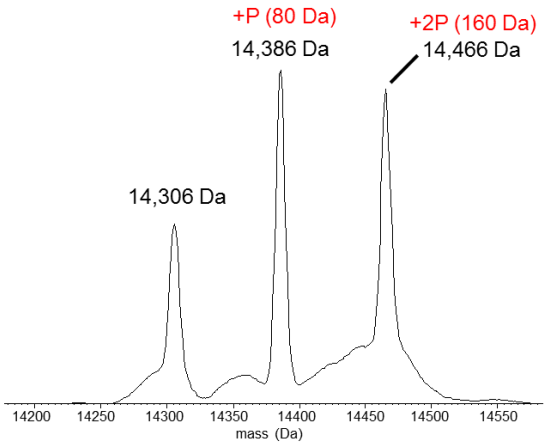


Figure S16. Intact mass analysis of McsB phosphorylated lysozyme using ESI-MS. The mass of the deconvoluted spectra confirmed the phosphorylation of lysozyme represented by + 80 Da shifts.



	Calculated mass (Da)	Measured mass (Da)
Lysozyme:	14,305.1	14,306
Lysozyme +1P:	14,385.1	14,386
Lysozyme +2P:	14,465.1	14,466

Figure S17. Purification of PO3-amidine raised phosphoarginine antibodies. Dot blot analysis of input serum (S), flow through (FT), wash (W) and elution fractions (E) from the affinity purification. Abbreviations: ab HC, antibody heavy chain; ab LC, antibody light chain.

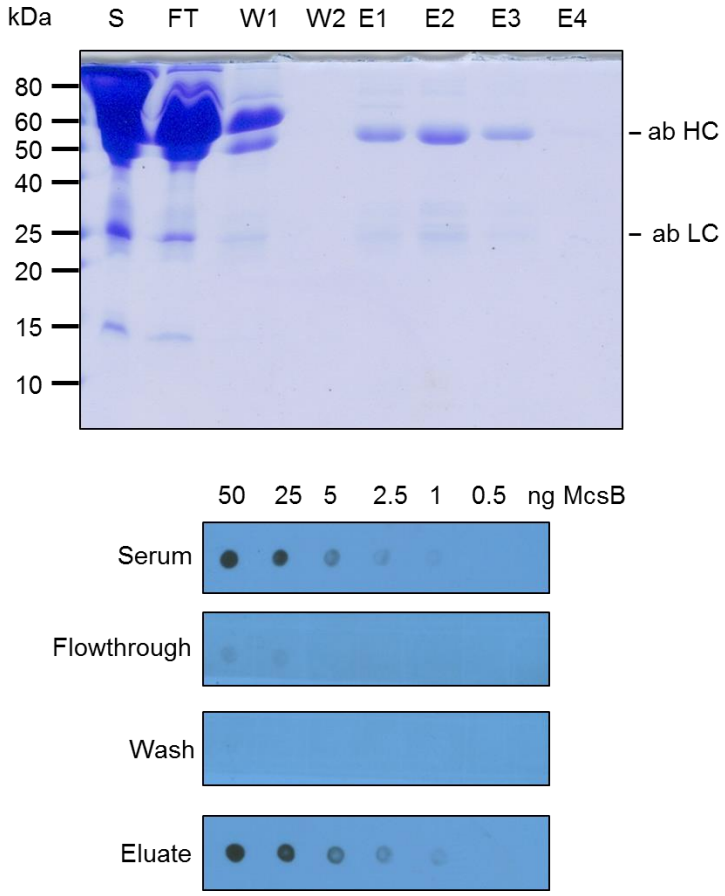


Figure S18. Comparing the sensitivity of anti-pArg antibodies generated using a hapten and phage display approach. The anti-McsB antibody was used as control.

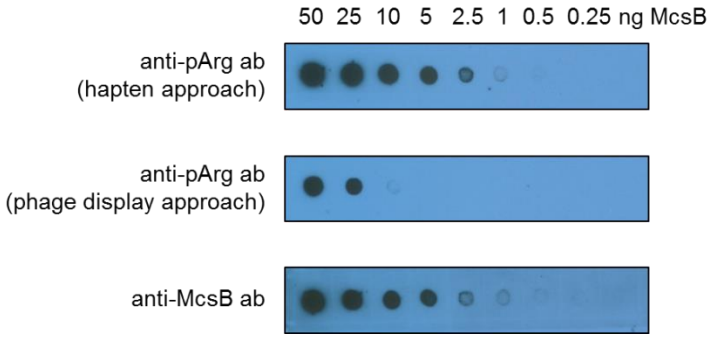


Figure S19. Western blot analysis of arginine phosphorylated lysozyme and McsB using the generated anti-pArg antibody.

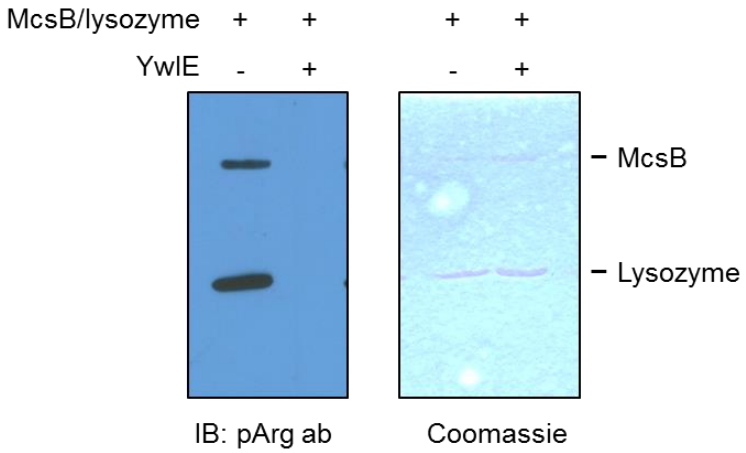
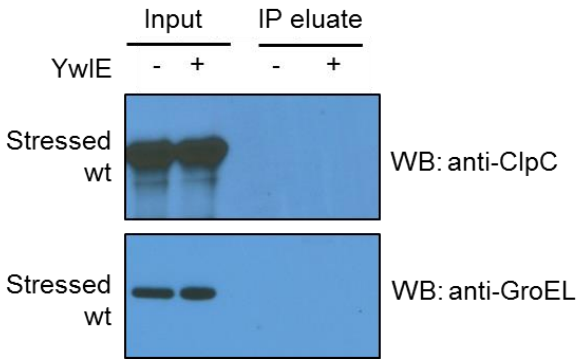


Figure S20. Immunoprecipitation experiments using the previously described, phage-display generated anti-pArg antibody.[5] The data show that this antibody is not able to enrich arginine phosphorylated ClpC and GroEL from stressed wild type cell samples, presumably due to its low affinity. Abbreviations: wt, *B. subtilis* wild type strain.



Supporting references:

[1] Y. Luo, K. Arita, M. Bhatia, B. Knuckley, Y. H. Lee, M. R. Stallcup, M. Sato, P. R. Thompson, *Biochemistry* 2006, *45*, 11727-11736.

[2] J. M. Kee, R. C. Oslund, D. H. Perlman, T. W. Muir, *Nat. Chem. Biol.* 2013, *9*, 416-U428.

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[4] a) M. Mayer, M. Heidelberger, *J. Am. Chem. Soc.* 1946, *68*, 18-25; b) O. Zetterqvist, L. Engstrom, *Biochim. Biophys. Acta* 1967, *141*, 523-532.

[5] J. Fuhrmann, B. Mierzwa, D. B. Trentini, S. Spiess, A. Lehner, E. Charpentier, T. Clausen, *Cell Rep.* 2013, *3*, 1832-1839.