Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Electronic Supplementary Information

Development of Stable Phosphoarginine Analog for Producing Phosphoargining Antibodies

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Table of Content

Supplementary Note 1

General Materials

All buffering salts, inorganic salts, organic solvent and imidazole were purchased from Sinopharm Chemical Reagent (Shanghai). LC-MS grade methanol (MeOH), acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Tedia (Fairfield, OH, USA). LC-MS grade formic acid (FA, as eluent additive for HPLC-MS), LC-MS grade ammonium acetate (as eluent additive for HPLC-MS), LC-MS grade ammonium formate (as eluent additive for HPLC-MS), O-Phospho-L-serine (pSer), O-Phospho-L-threonine (pThr), O-Phospho-L-tyrosine (pTyr), Freund's complete and incomplete adjuvant, Bovine serum albumin (BSA, as carrier protein) and Keyhole limpet hemocyanin (KLH) (cat. No. H8283) were purchased from Sigma-Aldrich. Bovine serum albumin (BSA, as blocking agent in ELISA and WB), Isopropyl-β-D-thiogalactopyranoside (IPTG), Phenylmethanesulfonyl fluoride (PMSF), β-mercaptoethanol (BME), Coomassie brilliant blue G250, adenosine triphosphate (ATP), glutaraldehyde, glycine, Tris base, Acrylamide/Bis-acrylamide (29:1, 30% solution), *N*,*N*,*N'*,*N'*-Tetramethylethylenediamine (TEMED) and dithiothreitol (DTT) were purchased from Sangon Biotech (Shanghai, China). Complete protease inhibitor cocktail tablet and phosphatase inhibitor cocktail tablet were purchased from Roche Diagnostics (Mannheim, Germany). PageRuler pre-stained protein ladder (cat. No. 26616) was purchased from Thermo Fisher. Cation exchange chromatography AG 50W-X8 resin was purchased from BioRad (Hercules, CA). Restore Western Blot Striping Buffer, Protein G Agarose Resin, Goat anti mouse HRP secondary antibody were purchased from ProteinTech Group (Wuhan, China). Amicon Ultra centrifugal filter units (0.5 mL, 4 mL or 15 mL, MWCO 3500), nitrocellulose membrane (0.45 μm), PVDF membrane (0.45 μm) were purchased from Millipore. Goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM-HRP were purchased from AbD Serotec (UK). DH5α strain, BL21 (DE3) strain and BL21 (DE3) pLys strain were maintain in our lab and used to generate "in-house" high competency cell lines. Ni-NTA resin and potent ECL substrate were purchased from Smart-Lifesciences (Wuxi, China). 96-well ELISA plate, TMB ELISA substrate were provided as generous gifts by INNOVAX (Xiamen, China)

General Equipment

Size-exclusion chromatography was carried out on an ÄKTA FPLC system from GE Healthcare on a Superdex 75 10/300 column. Analytical RP-HPLC was performed on Agilent 1260 series instruments equipped with an Agilent TC-C18 column (5 μm, 4.6 x 150 mm) at a flow rate of 1 mL/min. Electrospray ionization mass spectrometric analysis (ESI-MS) was performed on pAIE, pAIE precursors by direct infusion on a Bruker Dalton Amazon mass spectrometer. Coomassie blue or Silver stained gels were imaged on an EPSON PERFECTION V10 scanner. ELISA absorbance measurements at 450 nm

were recorded on a BioRad micro plate reader. Western blot membranes and Dot blots were imaged using the GE Amersham Imager 600. All plasmids used in this study were sequenced by Sangon Biotech (Shanghai, China) to verify the correct DNA sequence. Cells were lysed using a Sonics & Materials (NewTown, CT) Sonifier. Samples were lyophilized on a CHRIST (Gemany) lyophilizer. High resolution mass spectra of organic compounds were recorded on a Bruker FT-MS. Nano-LC-MS/MS analyses were performed on high-mass-accuracy, reversed-phase Nano-UPLC-MS platforms，consisting of an Easy nLC Ultra 1000 nano-UPLC system (Thermo Fisher Scientific) coupled to a Q-Extractive Orbitrap mass spectrometer (Thermo Fisher Scientific, USA).

Supplementary Note 2

Computational Modeling of the Side-chains in pHis and pAIE

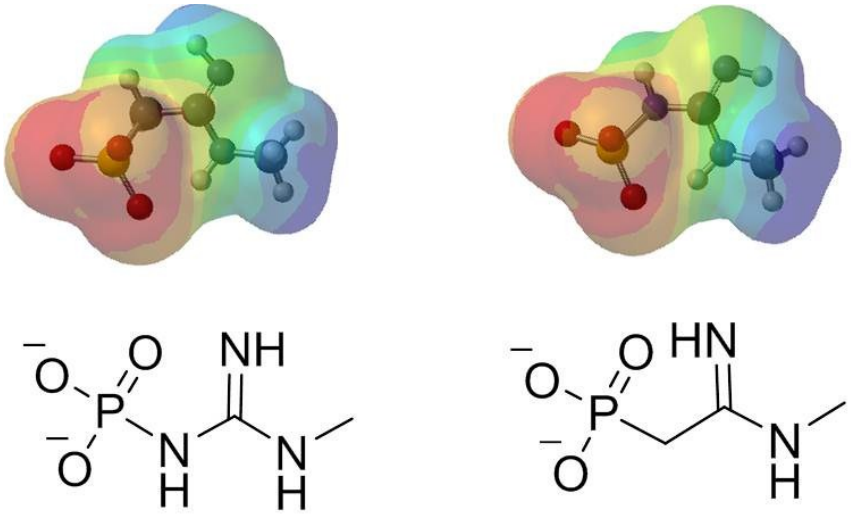


Figure S1. Computational modeling of the sidechains in ω-pArg (left) and pAIE (right). The optimum geometry and the electrostatic potential surface were obtained by DFT calculation (6-31* basis set) using the Spartan '10® package.[1]

Synthesis of pAIE

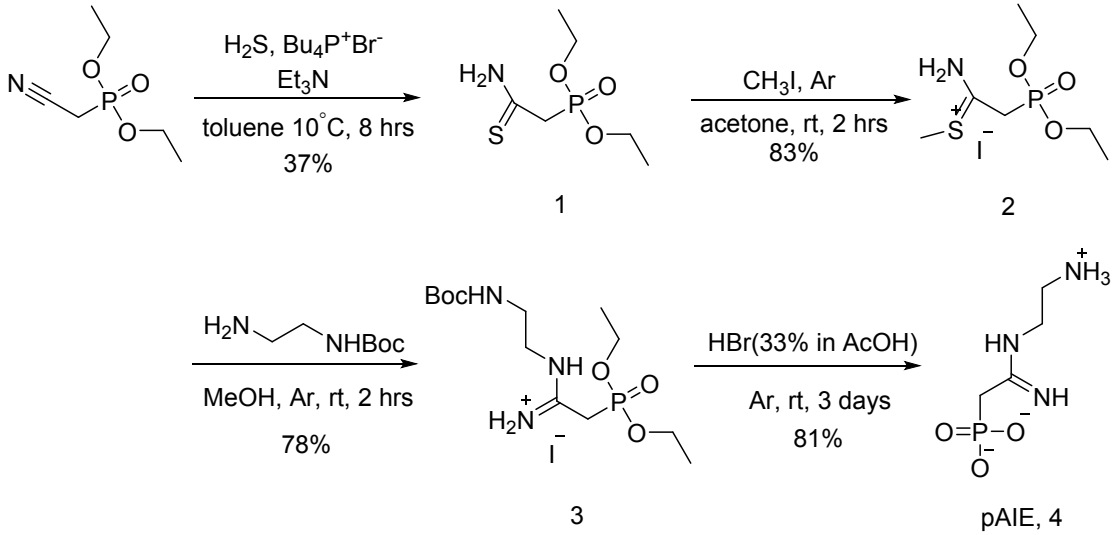


Figure S2. Synthetic scheme for pAIE.

General Synthetic Materials and Methods

All chemicals for the synthesis of pAIE were obtained from Sigma-Aldrich unless otherwise noted and used without further purification. All solvents were obtained from Sinopharm Chemical Reagent (Shanghai) unless otherwise noted. Silica gel (200-400 mesh) for flash column chromatograph (FCC) and glass-backed thin-layer chromatography (TLC) plates were purchased from Sinopharm Chemical Reagent. $CDCl_3$ and D_2O NMR solvents were purchased from Cambridge Isotope Laboratories. Anhydrous solvents were prepared according to <Purification of Laboratory Chemicals> (by D.D.Perrin and W.L.F. Armarego, Seventh Edition). Unless otherwise noted, all reactions were carried out in oven-dried (>100 °C) round-bottom flask equipped with a Teflon coated magnetic stir bar under a positive pressure of argon. Rotary evaporation was performed using a Heidolph rotary evaporator equipped with a circulating water vacuum pump. Compounds were visualized on TLC plates by UV light irradiation, or by treatment with a solution of ninhydrin in ethanol followed by heating in oven, or by treatment with a solution of phosphomolybdic acid and ceric sulfate (CAM developer) followed by heating on electric hot plate, or directly developed in iodine vapor. Product yields were calculated by weight and refer to pure compounds, unless otherwise indicated.

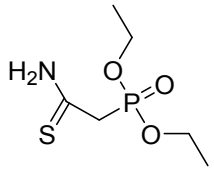
Ninhydrin developer: 0.2 g ninhydrin dissolved in 100 mL EtOH supplied with 0.5 mL acetic acid

CAM developer: 5 g ammonium molybdate tetrahydrate, 2 g ceric sulfate, dissolved in 200 mL 10% (w/w) Sulfuric acid

NMR Spectroscopy

^{1}H-NMR spectra were recorded on a Bruker 400 Avance-III (Bruker BioSpin, Billerica, MA). ^{1}H NMR chemical shifts are reported in parts per million (ppm) and are referenced relative to the proton signal of tetramethylsilane (TMS) in $CDCl_3$ at 0 ppm or the residual solvent proton signal for D_2O at 4.79 ppm.^{1}H-NMR data are tabulated in the following format: chemical shift, multiplicity [singlet (s), broad singlet (brs), doublet (d), triplet (t), multiplet (m)], coupling constant [Hz], number of protons, and structural assignments. ^{13}C-NMR spectra were recorded on a Bruker 400 Avance-III (Bruker BioSpin, Billerica, MA).^{13}C-NMR chemical shifts are reported in ppm relative to carbon signals for CDCl3 at 77.16 ppm; in D_2O, they are reported relative to the solvent deuterium signal[2]. ^{13}C-NMR data are tabulated in the following format: chemical shift, multiplicity [doublet (d)] (if applicable), coupling constant [Hz], and structural assignments.^{31}P-NMR spectra were recorded on a Bruker 400 Avance-III. ^{31}P-NMR chemical shifts are reported relative to ortho-phosphoric acid as an external standard.

Synthesis of diethyl (2-amino-2-thioxoethyl)phosphonate



In a round bottom flask equipped with a magnetic stir bar, diethyl (cyanomethyl)phosphonate (5.30 g, 30.0 mmol) was dissolved in 25 mL toluene, followed by addition of tetrabutyl phosphonium bromide ($Bu_4P^+Br^-$, 125 mg) and triethylamine (TEA, 6.05 g, 60.0 mmol). Dry hydrogen sulfide (H_2S) was bubbled through the mixture for 5 h with stirring at 10 °C. The reaction mixture was stirred overnight at room temperature, followed by purged with dried nitrogen and then cooled at 4 °C for 1 hour. After filtration, the resultant yellow solid was repeatedly washed with toluene in the atmosphere of Argon to give the product as yellow solid (**Compound 1**) (2.37 g, 37% yield).

Dry hydrogen sulfide (H_2S) was prepared according to the standard procedure. Briefly, ferrous sulfide (FeS) was reacted with hydrochloric acid (HCl) in a Kipp's

apparatus, and the generated H_2S was dried by passing through solid phosphorus pentoxide (P_2O_5).

^{1}H NMR (500MHz, $CDCl_3$): δ 8.45 (s, 1H, **NH** protons) and 7.73 (s, 1H, **NH** protons), 4.19 (dq, *J* = 14.2, 7.1 Hz, 4H, -O**CH_2**CH_3), 3.44 (d, *J* = 20.7 Hz, 2H, -P**CH_2**), 1.38 (dt, *J* = 7.1 Hz, 6H, -OCH_2**CH_3**) ppm.

^{13}C NMR (126 MHz, $CDCl_3$): δ 198.07 (d, *J* = 6.5 Hz, -**C**=S), 63.24 (d, *J* = 6.7 Hz, -O**CH_2**CH_3), 43.05 (d, *J* = 125.4 Hz, -P**CH_2**), 16.30 (d, *J* = 6.1 Hz, -OCH_2**CH_3**) ppm.

^{31}P NMR (200 MHz, $CDCl_3$): δ 21.03 ppm.

ESI-MS: 234.2 (calculated for $[MW+Na]^+$), 234.3 (observed).

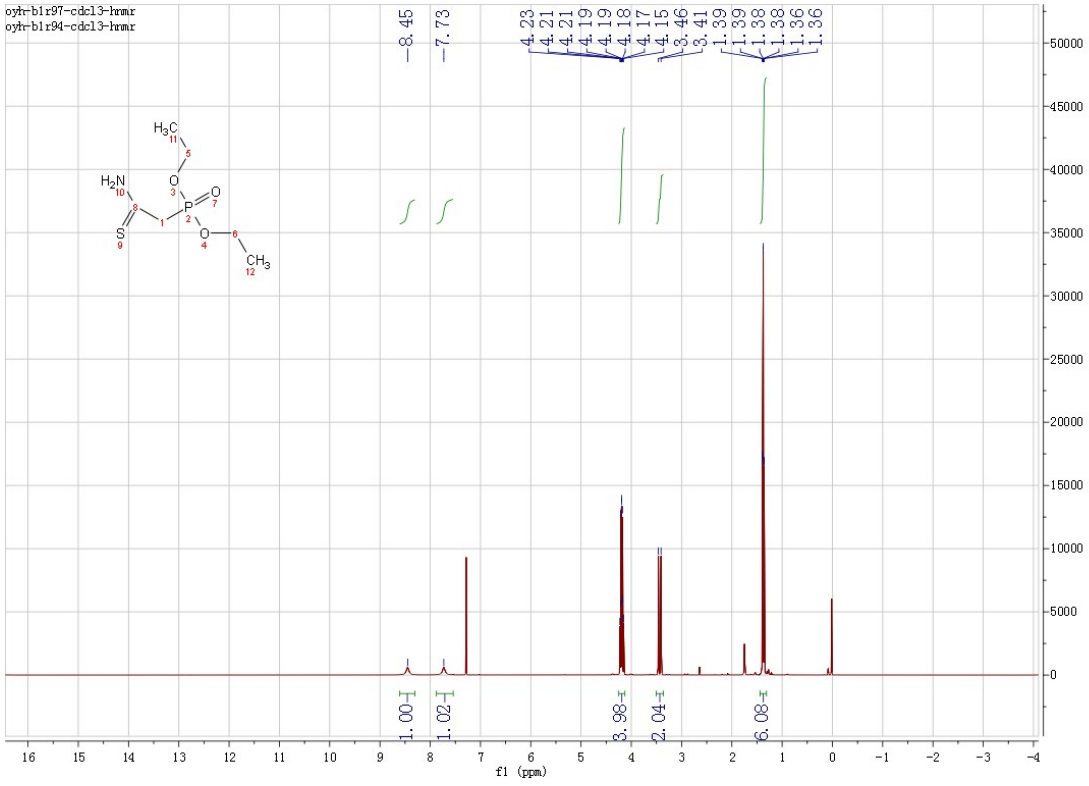


Figure S3. ^{1}H NMR spectrum of compound 1 (in $CDCl_3$)

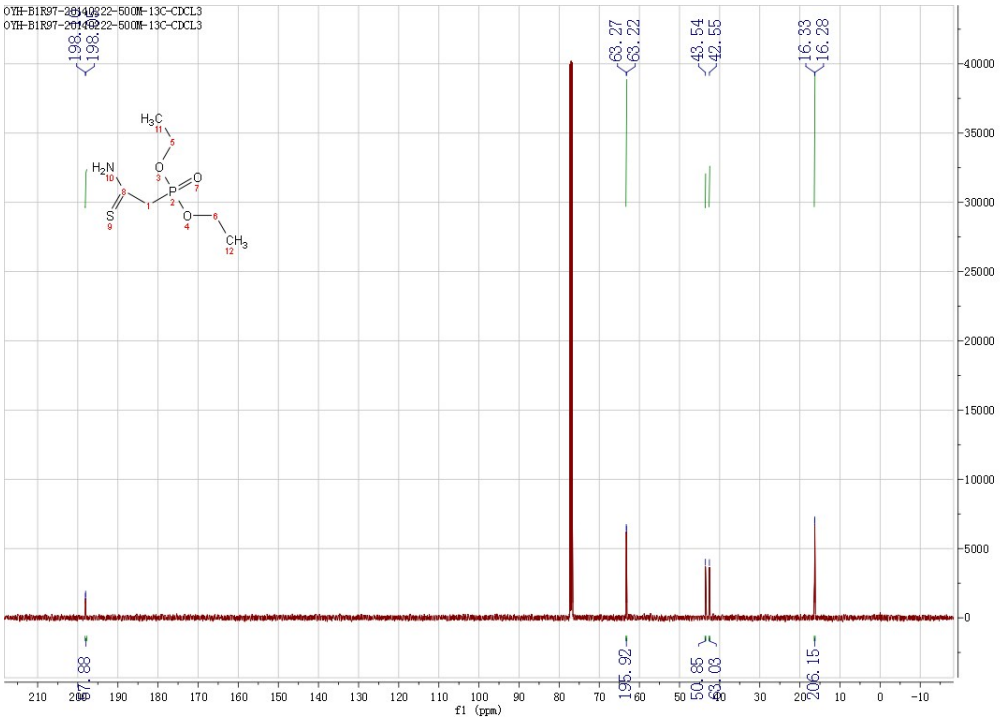


Figure S4. ^{13}C NMR spectrum of compound 1 (in $CDCl_3$)

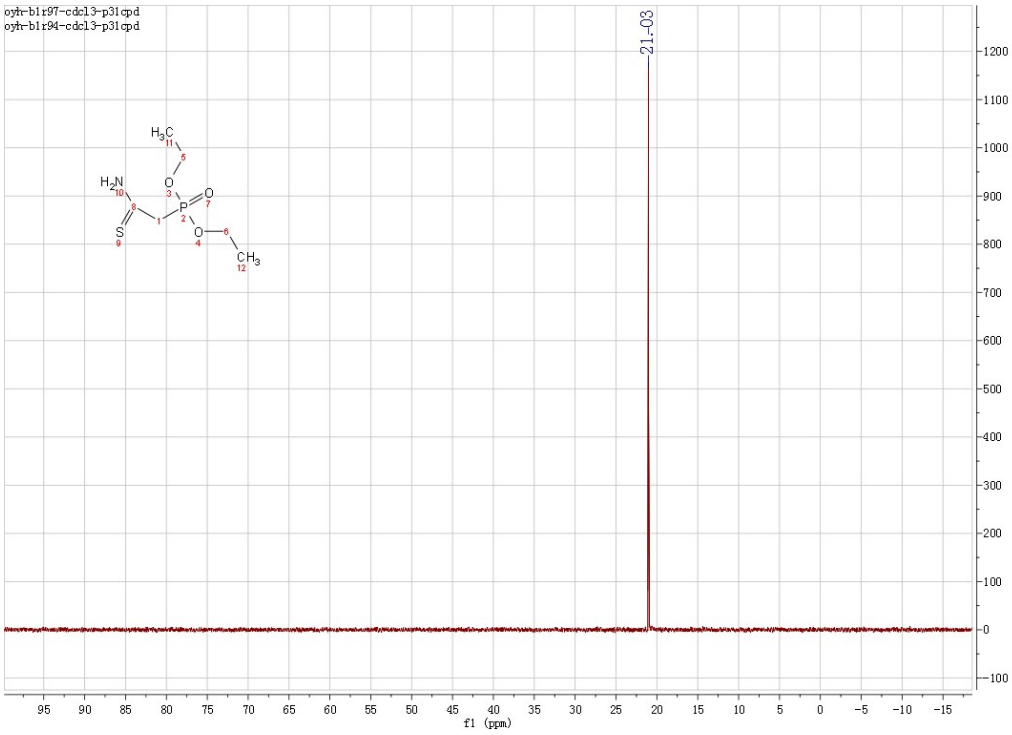
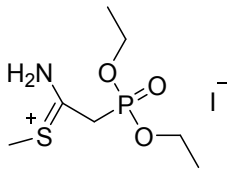


Figure S5. ^{31}P NMR spectrum of compound 1 (in $CDCl_3$)

Synthesis of 2-(Diethylphosphono)-S-methylthioacetamidium Iodide



In a round bottom flask equipped with a magnetic stir bar, diethyl (2-amino-2-thioxoethyl)phosphonate (**Compound 1)** (470 mg, 2.23 mmol) was dissolved in 2.5 mL acetone, followed by addition of methyl iodide (1.26 g, 8.91 mmol) dropwise at room temperature under Argon atmosphere. The reaction mixture was then stirred for 1 hour at room temperature until the white solid was precipitated from the mixture. The resultant white solid was filtered out, washed with 5 mL anhydrous ether twice, and then dried under vacuum to give the air-sensitive white solid (**Compound 2**) (666 mg, 83% yield).

^{1}H NMR (500MHz, $CDCl_3$): δ 4.50-4.16 (m, 4H, -O**CH_2**CH_3), 3.96 (d, *J* = 22.5 Hz, 2H, -P**CH_2**), 3.04 (d, *J* = 0.9 Hz, 3H, -S**CH_3**), 2.67 (s, 2H, **-NH_2**), 1.41 (dt, *J* = 7.0 Hz, 6H, -OCH_2**CH_3**) ppm.

^{31}P NMR (200 MHz, $CDCl_3$): δ 15.81 ppm.

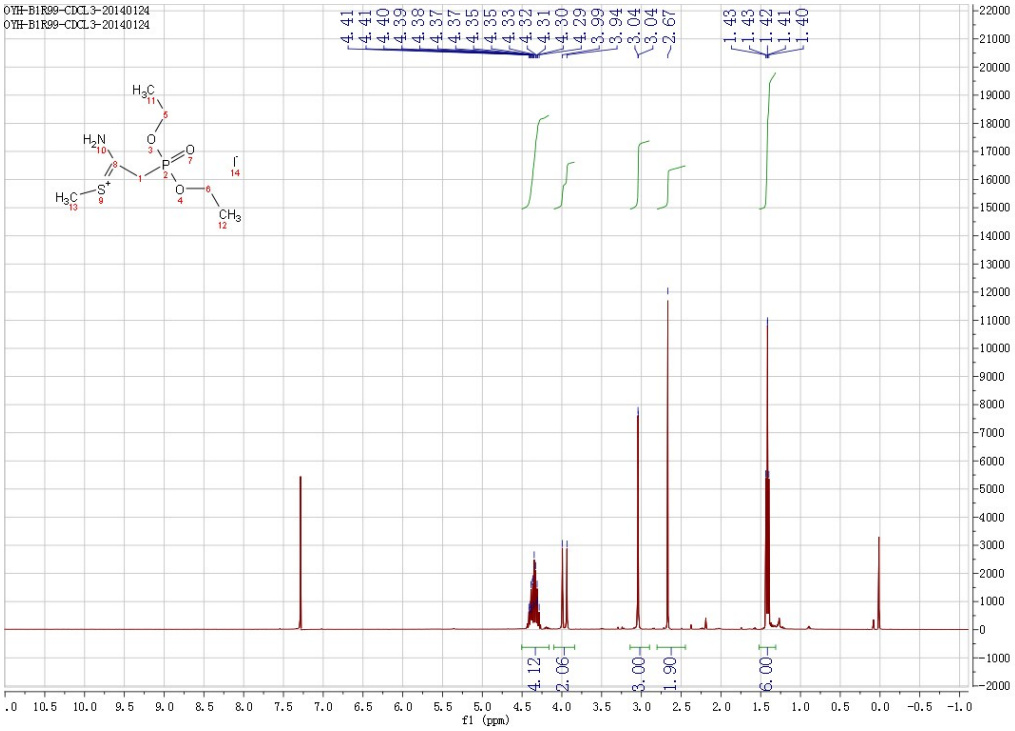


Figure S6. ^{1}H NMR spectrum of compound 2 (in $CDCl_3$)

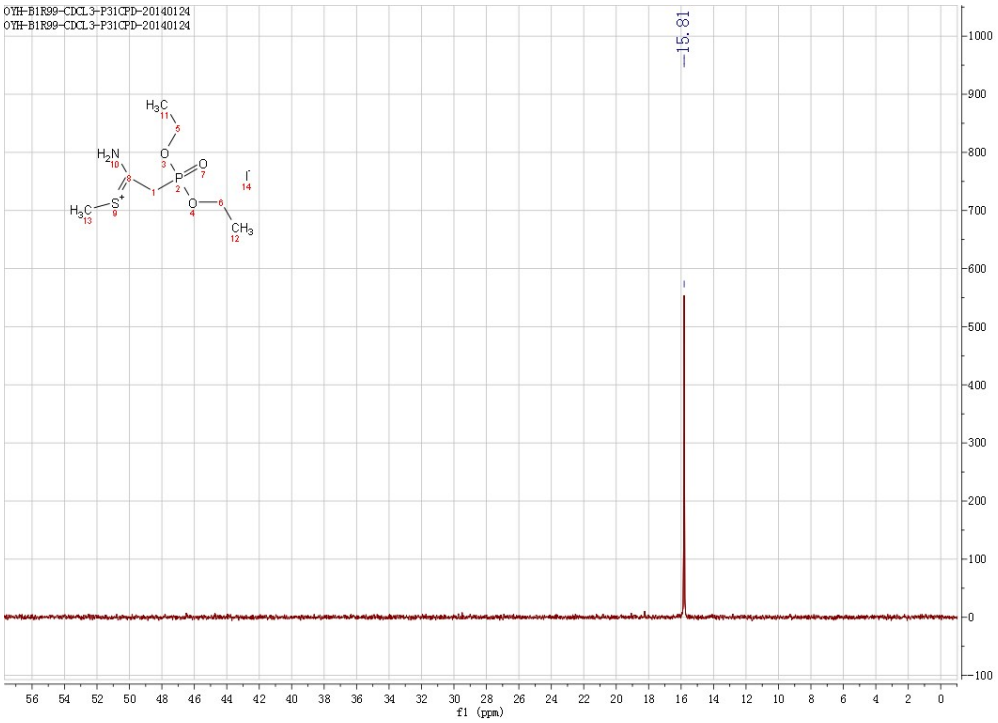
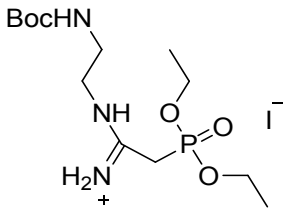


Figure S7. ^{31}P NMR spectrum of compound 2 (in $CDCl_3$)

Synthesis of 1-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-2-(diethoxyphosphoryl)ethaniminium iodide



In a round bottom flask equipped with a magnetic stir bar, 2-(Diethylphosphono)-S-methylthioacetamidium Iodide (**Compound 2**) (611 mg, 1.73 mmol) and N-Boc-ethylenediamine (277 mg, 1.73 mmol) were dissolved in 3 mL methane and stirred for 3 h until TLC indicated the consumption of compound 2 at room temperature under Argon atmosphere. The reaction mixture was concentrated and the solvent was removed by rotary evaporation to give a white crude solid. The crude solid was washed with diethyl ether to give the pure product as a white solid (**Compound 3**) (628 mg, 78% yield).

^{1}H NMR (400 MHz, D_2O): δ 4.16 (p, *J* = 7.2 Hz, 4H, -O**CH_2**CH_3), 3.39 (d, *J* = 5.8 Hz, 2H, -C(O)NH**CH_2**CH_2), 3.28 (d, *J* = 5.0 Hz, -C(O)NHCH_2**CH_2**), 1.35 (s, 9H, -(**CH_3**)$_3$C), 1.28 (t, *J* = 7.0 Hz, 6H, -OCH_2**CH_3**) ppm. (-**CH_2**P, exchange in D_2O)

^{13}C NMR (101 MHz, D_2O): δ 160.21 (d, *J* = 6.1 Hz, -**C**=NH), 158.15 (s, -**C**=O), 81.42 (s, -(CH_3)$_3$**C**), 65.01 (d, *J* = 6.9 Hz, -O**CH_2**CH_3), 42.39 (s, -C(O)NH**CH_2**CH_2), 37.86 (s, -C(O)NHCH_2**CH_2**), 27.70 (s, -(**CH_3**)$_3$C), 15.69 (d, *J* = 5.7 Hz, -OCH_2**CH_3**) ppm.

^{31}P NMR (162 MHz, D_2O): δ 20.51 ppm.

ESI-MS: 339.2 (calculated for $[MW+H]^+$), 339.3 (observed).

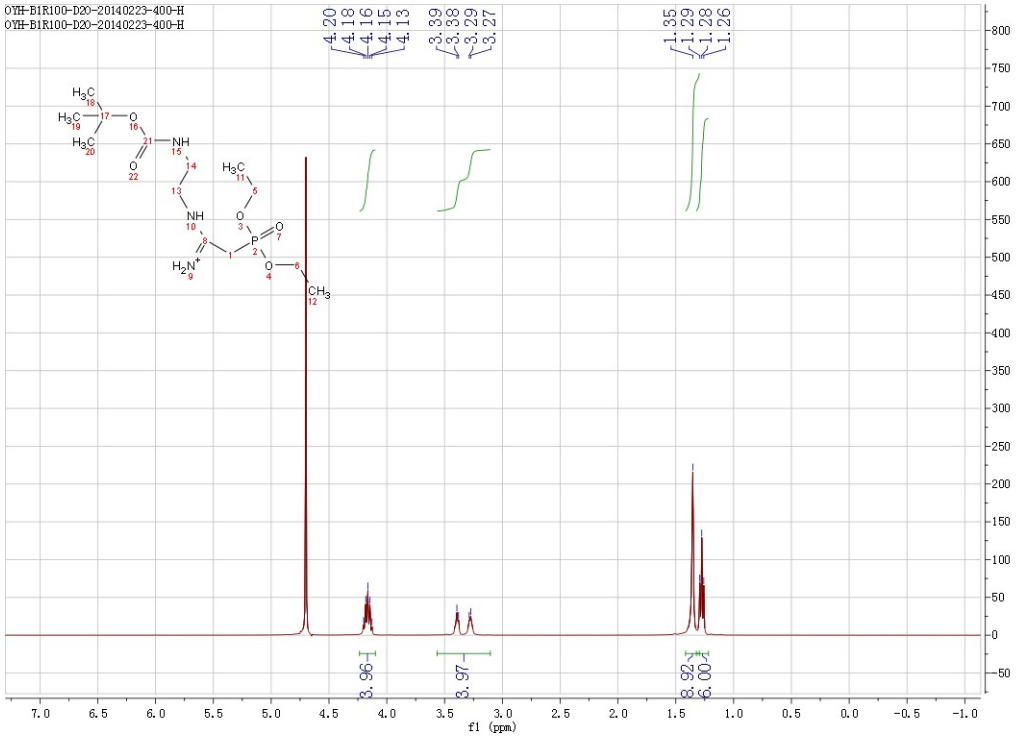


Figure S8. ^{1}H NMR spectrum of compound 3 (in D_2O)

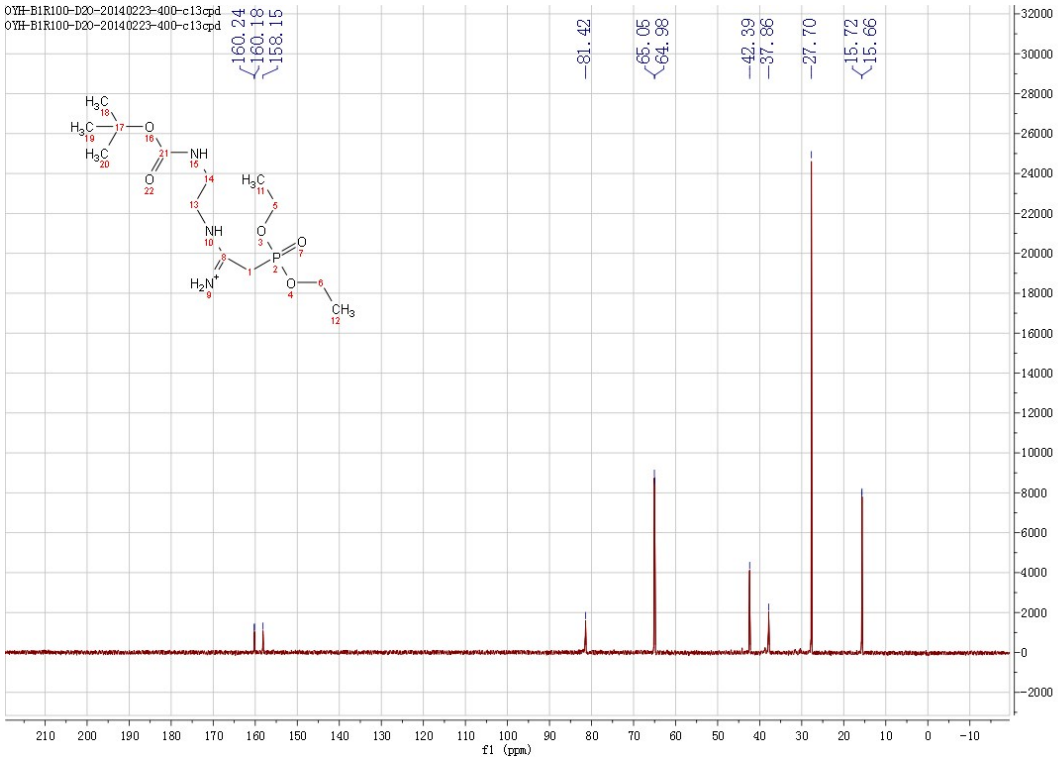


Figure S9. ^{13}C NMR spectrum of compound 3 (in D_2O)

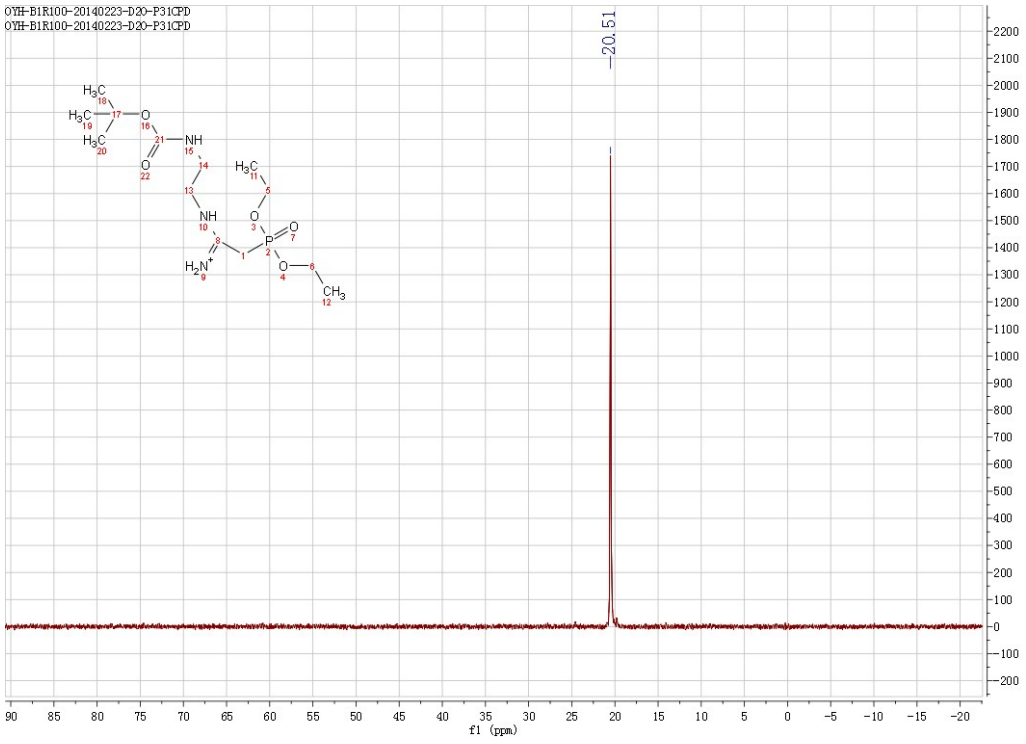
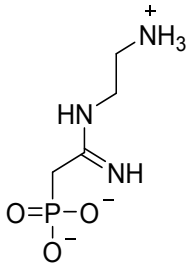


Figure S10. ^{31}P NMR spectrum of compound 3 (in D_2O)

Synthesis of (2-((2-ammonioethyl)amino)-2-iminoethyl)phosphonic acid (pAIE)



In a round bottom flask equipped with a magnetic stir bar, 1-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-2-(diethoxyphosphoryl)ethaniminium iodide (**Compound 3**) (370 mg, 0.80 mmol) was dissolved in 10 mL 33% HBr in acetic acid. The reaction mixture was then capped with a glass stopper and stirred at room temperature for 48-72 hours under Argon atmosphere until ESI-MS indicated complete loss of Boc- and ethyl protecting groups. The reaction process was further verified with ^{31}P NMR which indicated the consumption of Compound 3 and in which a new peak appeared in the upfield region. The reaction mixture was then concentrated to a solid by rotary evaporation. HBr was removed by freeze-drying and repeating addition and evaporation of methanol. Residual HBr was removed by repeating dissolve-lyophilization cycle with ddH_2O The crude solid was then dissolved in 2 mL of 0.1% AcOH and purified by cation exchange chromatography. This was done by loading the crude product onto 6 mL of strongly acidic cation exchange resin pre-equilibrated with 0.1% AcOH. The column was then washed with 12 mL 0.1% AcOH, followed by elution with 12 mL 500 mM ammonium acetate. All fractions including flow-through and eluent were collected in 1 mL fractions. Fractions containing product were identified by spotting on TLC plate and visualized with ninhydrin. Further verification was performed by ESI-MS if necessary. Fractions containing product were combined and then flash frozen in liquid nitrogen and lyophilized to dryness to give pAIE as a white solid (**Compound 4, pAIE**) (117 mg, 81% yield).

^{1}H NMR (500 MHz, D_2O): δ 3.64 (t, *J* = 5.8 Hz, 2H, -C(NH)NH**CH_2**CH_2), 3.28 (t, *J* = 5.9 Hz, 2H, -CNHCH_2**CH_2**), 2.90 (dd, *J* = 20.0, 6.7 Hz, 2H, -**CH_2**P) ppm.

^{13}C NMR (126 MHz, D_2O): δ 164.39 (d, *J* = 4.2 Hz, -**C**=NH), 39.73 (s, -C(NH)NHCH_2**CH_2**), 37.15 (s, -C(NH)NH**CH_2**CH_2), 34.57 (d, *J* = 117.4 Hz, -**CH_2**P) ppm.

^{31}P NMR (200 MHz, D_2O): δ 10.53 ppm.

ESI-MS: 182.1 (calculated for $[MW+H]^+$), 182.0 (observed).

HRMS: 182.0700 (expected), 182.0704 (observed)

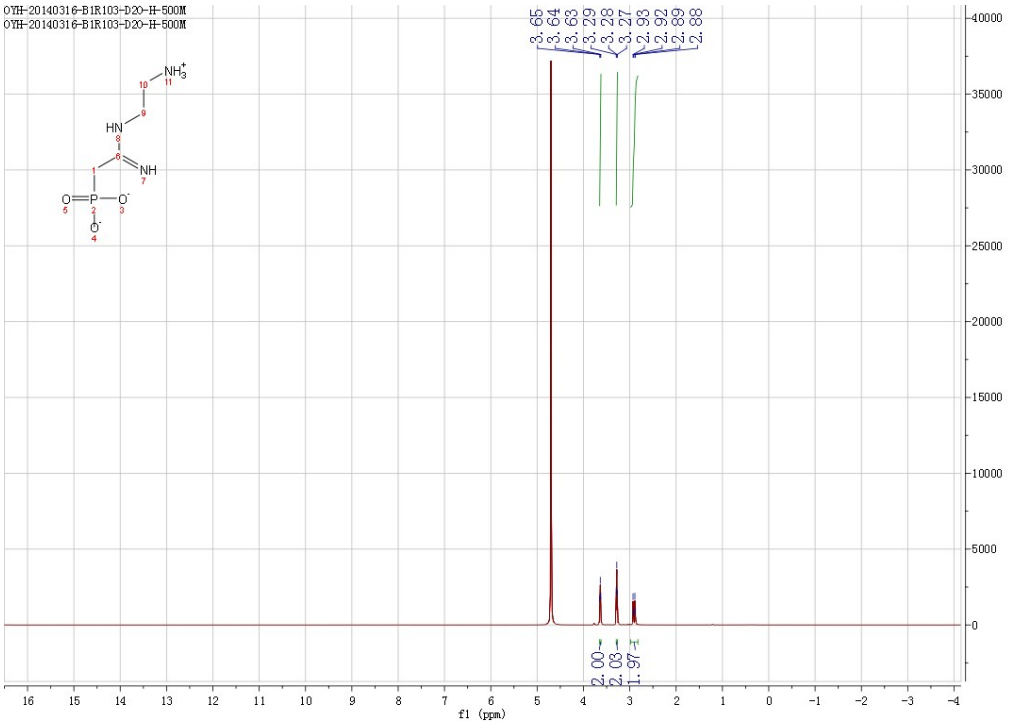


Figure S11. ^{1}H NMR spectrum of compound 4 (pAIE) (in D_2O)

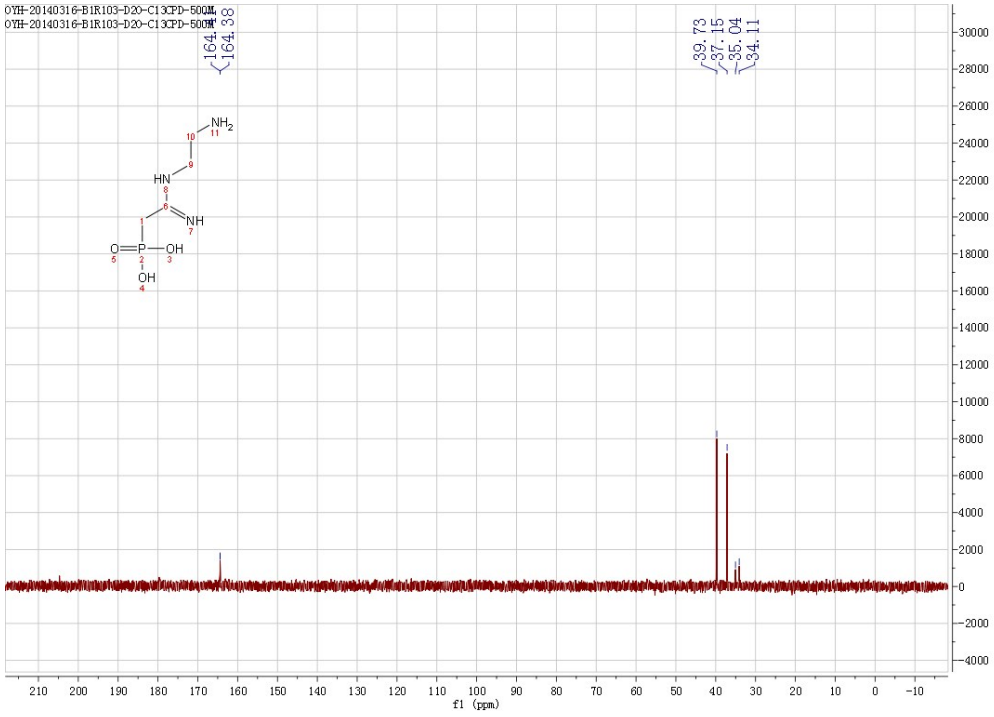


Figure S12. ^{13}C NMR spectrum of compound 4 (pAIE) (in D2O)

Supplementary Note 3

Conjugation of pAIE to KLH

pAIE-KLH (5) was prepared employing glutaradehyde as a coupling reagent. Briefly, pAIE and keyhole limpet hemocyanin (KLH) were dissolved in coupling buffer (0.1 M Na_2CO_3, 0.15 M NaCl, pH 8.5) respectively and then mixed. The final concentration of pAIE is15 mM and that of KLH is 2 mg/mL. Glutaraldehyde (25% v/v) was slowly added to the mixture to get the final concentration of 0.5% (v/v). The mixture was incubated for two hours at RT, followed with the addition of solid $NaBH_4$ to a final concentration of 10 mg/mL and incubated for 2 hours at RT. Excessive and unreacted pAIE, Glutaraldehyde and $NaBH_4$ were removed by dialysis against PBS (pH 7.4) overnight. The concentration of protein was determined by Bradford method using BSA as a reference.

Conjugation of pArg and other phosphoamino acids to BSA

pArg-BSA (6), pSer-BSA (12), pThr-BSA (13) and pTyr-BSA (14) were prepared employing glutaradehyde as a coupling reagent. Briefly, pAIE and keyhole limpet hemocyanin (KLH) were dissolved in coupling buffer (0.1 M Na_2CO_3, 0.15 M NaCl, pH 8.5) respectively and then mixed. The final concentration of pAIE is15 mM and that of KLH is 2 mg/mL. Glutaraldehyde (25% v/v) was slowly added to the mixture to get the final concentration of 0.5% (v/v). The mixture was incubated for two hours at RT, followed with the addition of solid $NaBH_4$ to a final concentration of 10 mg/mL and incubated for 2 hours at RT. Un-reacted pAIE, excessive glutaraldehyde and $NaBH_4$ were removed by dialysis against PBS (pH 7.4) overnight. The concentration of protein was determined by Bradford method using BSA as a reference.

Generation of Mouse Polyclonal pArg-Antibody

The mouse polyclonal antiserum was raised against pAIE-KLH (5). Four mice were subcutaneously injected with pAIE-KLH conjugate using a standard immunization protocol. Briefly, each mouse was injected with 200 µg pAIE-KLH conjugate per shot combined with equal volume of Fraud's complete adjuvant for primary immunization and with equal volume of Fraud's incomplete adjuvant for the subsequent injection. The interval time between two successive injections was around 2-3 weeks. The individual bleeds were screened for detection of the phosphor-arginine modification (pArg-BSA or Peptide 8) by ELISA using BSA or Peptide 7 as a control, respectively. All animal experiments were performed in keeping with AAALAC regulations and in accordance with animal ethical standards.

ELISA of crude anti-pArg antiserum

Buffers used in ELISA Protocol

Coating Buffer: 0.032 M Na_2CO_3, 0.068 M $NaHCO_3$, pH 9.6

Wash Buffer: 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% v/v Tween 20

Pep 7 and pArg-Pep (8) in 1 mg/mL stock solutions were diluted 500-fold into coating buffer and 50 µL of each sample was added to a Nunc-Immuno Maxisorp 96-well plate to give 100 ng peptide/well. The plate was incubated for 2 hours at room temperature on a nutator. The wells were then washed three times with wash buffer. The wells were then blocked by adding 1% BSA in wash buffer at 200 µL/well and incubated for 1 hours at room temperature on a nutator. After removing blocking solution, mouse crude antiserum diluted in wash buffer at a indicated ratio was added to each well to give 100 µL/well and incubated for 45 minutes at room temperature on a nutator. The wells were then emptied and washed three times with wash buffer. Goat-anti rabbit-HRP secondary antibody was diluted 5000-fold into wash buffer and added to the wells at 50 µL/well and incubated for 45 minutes at room temperature on a nutator. The wells were emptied and washed four times with wash buffer. 100 µL of Ultra TMB ELISA substrate was added to each well and incubated for 15 minutes at room temperature followed by addition of 50 µL of 2 N H_2SO_4 to quench the reaction. The absorbance at 450 nm was measured on a BioRad plate reader.

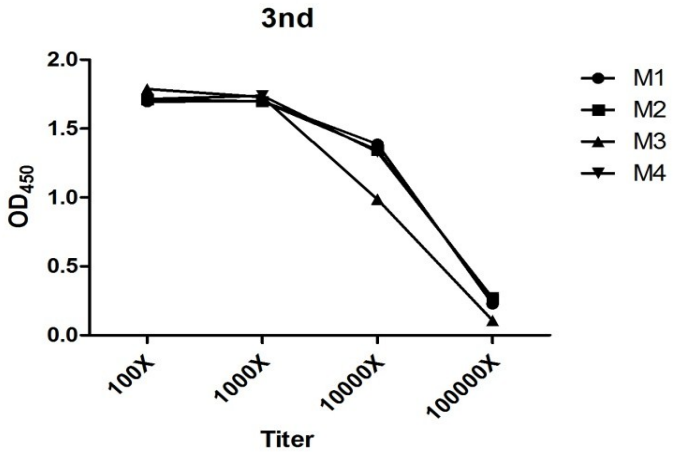


Figure S13. Measuring of Anti-serum titer against pArg-BSA after the 3nd injection.

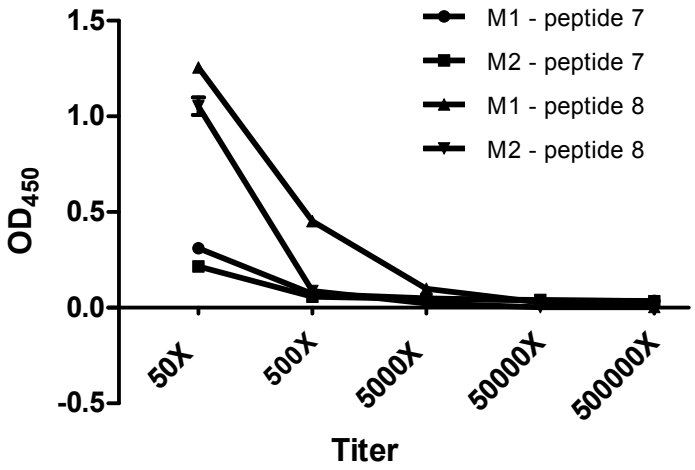


Figure S14. Measuring of Anti-serum titer against peptide 8 after the 3nd injection, using peptide 7 as a control.

Protein A purification of anti-pHis polyclonal antiserum

Equilibration/Wash Buffer: 20 mM Na_2HPO_4, 0.15 M NaCl, pH 8.0

Elution Buffer: 100 mM glycine, pH 2.5

Neutralization Buffer: 1 M Tris, pH 8.5

10 µL Protein A agarose resin was packed in a in-house made micro-column and washed with four 5 column volumes of TBS Equilibration buffer and then loaded with 50 µL 1:10 diluted mouse anti-serum in Equilibration buffer. The flow-through was collected, and the resin was washed with 25 µL TBS for 5 times. Antibodies were eluted from the column in 25 µL fractions with elution buffer. After elution off of the column, the elution fractions were immediately neutralized by adding 2.5 µL of 1 M Tris (pH8.0). Fractions containing pArg-Ab were determined by SDS-PAGE and ELISA.

SDS-PAGE of pArg-Ab Fractions

0.5 µL of the input and flow through fractions, and 0.5 µL of the wash and elution fractions were mixed 1:1 with 2× loading buffer (80 mM Tris pH 6.8, 20% v/v Glycerol, 2% SDS, 0.04% w/v Bromophenol Blue, 4% v/v 2-mercaptoethanol) and boiled at 95 °C for 5 min. The samples were loaded onto in-house made 12% Bis-Tris polyacrylamide gel and the proteins were resolved by electrophoresis by running at 180 V for 1 hour. The gel was stained by conventional Silver stain method and imaged on a GE Amersham Imager 600.

Supplementary Note 4

Expression and Purification of His_6-tagged McsB

Plasmid Encoded McsB gene is a generous gift from Dr. Tim Clausen at Research Institute of Molecular Pathology, Austria[3]. The plasmid was transformed to *E. coli* BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with Ampicillin (50 µg/mL) over night at 37 °C. 1 L media was inoculated with 5 mL overnight cultures and grown until the OD600 reached 0.8. The bacteria was induced by 0.5 mM IPTG at 18 °C, 200rpm for additional 12 h and then harvested by centrifugation (8000g, 10 min, 4 °C). Cell pellet was re-suspended in Lysis buffer containing 50 mM KH_2PO_4 (pH 7.5), 500 mM KCl, 5% glycerol, 1 mM β-mercaptoethanol, 1 mM PMSF and pH 7.5 and disrupted by sonication. His_6-McsB was purified using standard His-tag purification protocol according to manufacturer's recommendation, followed by SEC chromatograph on superdex G-75 column equilibrated with 20 mM Tris (pH8.0), 150 mM KCl. Purified His_6-McsB was analyzed by SDS-PAGE and the concentration was determined with Bradford method.

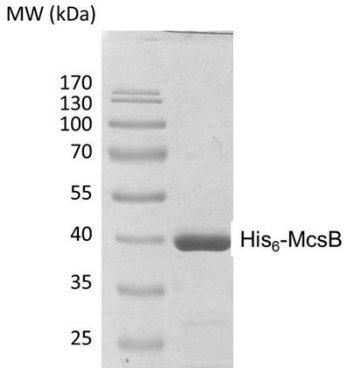


Figure S15. SDS-PAGE analysis of purified His_6-McsB

Sequence of His_6-McsB

DNA:

ATGTCGTTCGGGAAGTTTTTCAACACAGCGGTCAGCGCTTGGATGAGCCAGGAGGG
GCCGAATTCGGACATCGTGTTGAGCAGCCGCATCCGGCTGGCGCGCAATATTGTTGA
TTTTCGTTTCCCAACGCTGTTTAGCAGCGAAGAGGCGAAACAAATCGTTGCCTTGTT
TGAGCGCGCGTTCGTCCATCGTCCGTATGGGGAAGCGGGACGGTTTGAACTGTTGAA
AATGTCGGAGCTTCAACCGATTGAAAAACGGGTATTGGTGGAAAAGCACTTAATCA
GCCCGCATTTGGCTGAAGATTCGCCGTTTGGCGCCTGCCTGCTTTCGGAAAATGAGG
AAATCAGCATTATGATCAACGAGGAAGACCATATCCGCATTCAGTGTCTGTTTCCAG
GGCTTCAGCTTGCCGAGGCGCTTGAGGCGGCGAGCGAGTTGGATGATTGGATTGAA
GGGCATGTCAACTATGCGTTCGATGAACGGCTTGGTTACTTAACGAGCTGTCCGACG

AACGTCGGAACGGGGTTGCGGGCGTCGGTGATGATGCATCTTCCGGCGCTGGTGCTG
ACGCAGCAGATCAACCGCATCATCCCTGCCATCAACCAGTTGGGACTCGTCGTCCGC
GGCACGTACGGGGAAGGAAGTGAGGCTTTAGGGAACATTTTCCAAATTTCGAACCA
AATTACGCTCGGAAAATCAGAAGAAGATATTGTCGCTGATTTGCATACGATCGTTGA
GCAGCTCATCGCCCAAGAAAGGGCGGCTCGCCAGGCATTAGTGAAAACGTTGGGCA
TACAATTAGAAGACAAGGTATTCCGTTCATACGGCATATTGGCCAACTGCCGCGTCA
TCGATTCGAAGGAGGCGGCGCAATGCCTGTCGGATGTGCGTTTAGGGATCGATTTAG
GATATATCAAAAACGTCTCGCGCAACATTTTAAATGAGCTGATGATTTTGACGCAGC
CAGGATTTTTGCAACAATATGCCGGAGGGGTGCTTCGCCCTGAGGAACGGGATGTCC
GCCGGGCGGCGTTGATTCGGGAACGCTTAAGGATGGAAACACGAAGAAAGATGGA
GGGTGATGAACGAGTCGAGCACCACCACCACCACCAC

Protein:

MSFGKFFNTAVSAW**M**SQEGPNSDIVLSSRIRLARNIVDFRFPTLFSSEEAKQIVALF
ERAFVHRPYGEAGRFELLK**M**SELQPIEKRVLVEKHLISPHLAEDSPFGACLLSENEEI
SI**M**INEEDHIRIQCLFPGLQLAEALEAASELDDWIEGHVNYAFDERLGYLTSCPTNV
GTGLRASV**M M**HLPALVLTQQINRIIPAINQLGLVVRGTYGEGSEALGNIFQISNQI
TLGKSEEDIVADLHTIVEQLIAQERAARQALVKTLGIQLEDKVFRSYGILANCRVIDS
KEAAQCLSDVRLGIDLGYIKNVSRNILNEL**M**ILTQPGFLQQYAGGVLRPEERDVRR
AALIRERLR**M**ETRRK**M**EGDERVEHHHHHH

Expression and Purification of His_6-tagged CtsR

The DNA fragment encoded His_6-CtsR was chemically synthesized and ligated into pET pET 21a by using NdeI and XhoI sites. The plasmid was transformed to *E. coli* BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with Ampicillin (50 μg/mL) over night at 37 °C. 1 L media was inoculated with 5 mL overnight cultures and grown until the OD600 reached 0.8. The bacteria was induced by 0.5 mM IPTG at 37 °C, 250rpm for additional 2 h and then harvested by centrifugation (8000g, 10 min, 4 °C). Cell pellet was re-suspended in Lysis buffer containing 25 mM Tris (pH 8.0), 200 mM NaCl, 1 mM β-mercaptoethanol, 1 mM PMSF and disrupted by sonication. His_6-CtsR was purified using standard His-tag purification protocol according to manufacturer's recommendation. Briefly, the lysate was cleared by centrifugation at 14000g for 10 min at 4 °C, and the supernatant was loaded onto the 1 mL Ni-NTA column pre-equilibrated with 25 mM Tris pH 8.0, 200 mM NaCl, 20 mM imidazole and then eluted according to a standard protocol. Fractions containing objective His_6-CtsR were pooled. Excessive imidazole was removed by using Ultla Centrigugal Unit (Millipore, MWCO 3K), and the buffer was changed to500mM KCl,15Mm KH_2PO_4, 2mM β -Mercaptoethanol Ph7.5. . Purified His_6-CtsR was analyzed by SDS-PAGE and the concentration was determined with Bradford method.

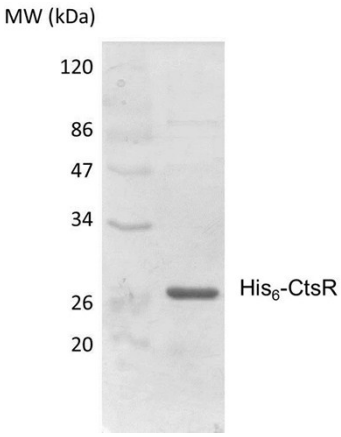


Figure S16. SDS-PAGE analysis of purified His_6-CtsR

Sequence of His_6-CtsR

DNA:

ATGCCGAACATTTCCGACATCATTGAGCAATATTTGAAGCAAGTACTCAATATGAGC
GACCAAGACATCGTTGAAATTAAACGAAGTGAAATTGCTAATAAATTCCGATGTGTT
CCCTCGCAAATCAACTATGTCATCAACACGAGATTCACGCTCGAGCGCGGATATATT
GTTGAAAGCAAGCGCGGTGGCGGCGGCTATATCCGCATTATGAAAGTAAAGACGAA
AAGCGAGGCGCAGCTGATTGACCAGTTGCTCGAGCTGATCGACCACCGCATCAGCC
AGTCGAGCGCCGAAGATGTGATAAAACGACTTATGGAAGAAAAGGTGATTTCGGAG
CGGGAAGCGAAAATGATGTTGAGCGTGATGGATCGCTCCGTTTTATATATCGATTTG
CCCGAGCGGGATGAACTGCGGGCGCGCATGTTAAAGGCGATGCTGACGTCGCTGAA
ATACAAACTCGAGCACCACCACCACCACCAC

Protein:

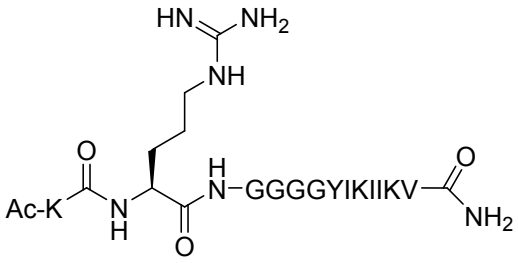
M P N I S D I I E Q Y L K Q V L N **M** S D Q D I V E I K R S E I A N K F R C V P S Q I N Y V I N T R F T L E R G Y I V
E S K R G G G G Y I R I **M** K V K T K S E A Q L I D Q L L E L I D H R I S Q S S A E D V I K R L **M** E E K V I S E R E A
K **M** **M** L S V **M** D R S V L Y I D L P E R D E L R A R **M** L K A **M** L T S L K Y K L E H H H H H H

Supplementary Note 5

Peptide Synthesis

Peptide 7 and 9-11 were chemically synthesized on a Rink-amide resin by an automated peptide synthesizer via Fmoc chemistry. For the phosphoamino acid residues, monobenzyl-protected phosphoamino acids were used. The peptide was cleaved from the resin with a mixture of trifluoroacetic acid, triisopropylsilane, water (95:2.5:2.5). The crude peptide was then purified by preparative RP-HPLC. All chemically synthesized peptides were quantified by weight.

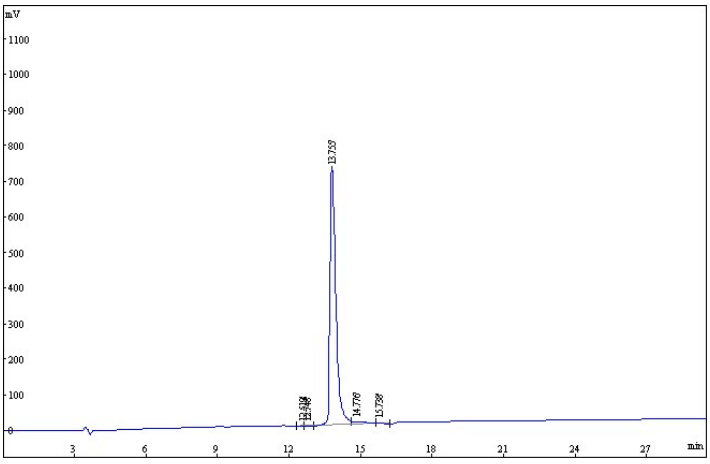
Peptide 7

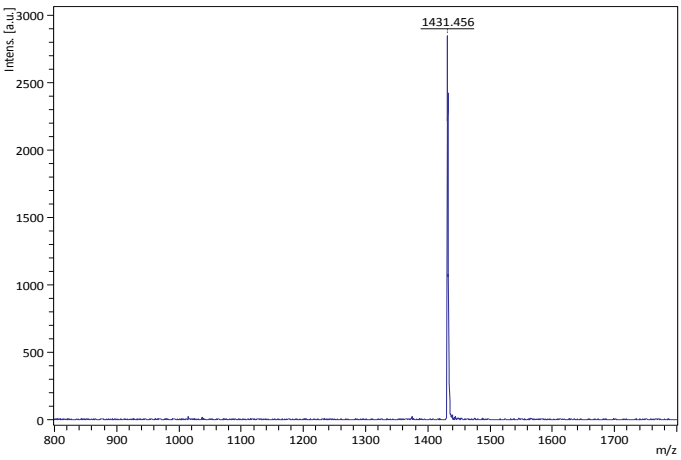


7

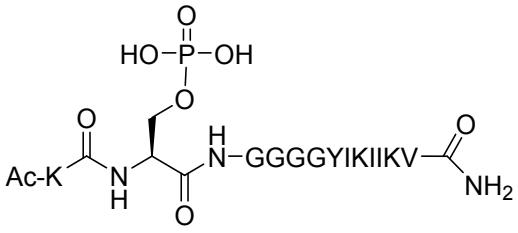
MALDI-TOF-MS: 1429.8 (calculated); 1431.5 ±0.5 (observed, $[M+H]^+$).

HPLC retention time: 13.74 min (Kromasil C18 column, 250×4.6 mm, 5 μm, 15-55% solution B over 30 min, 1 mL/min)





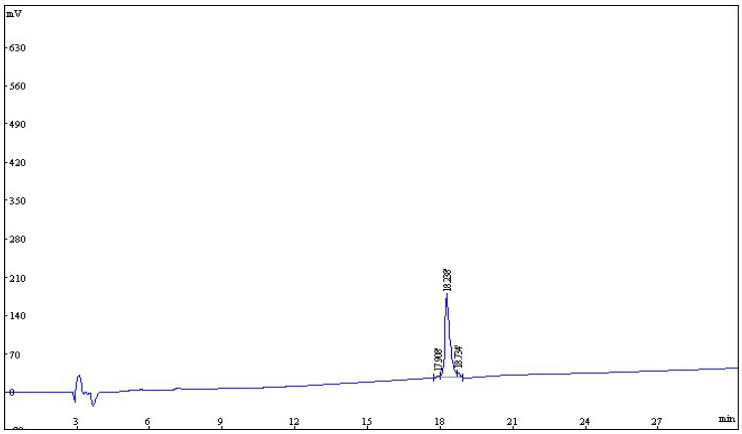
Peptide 9

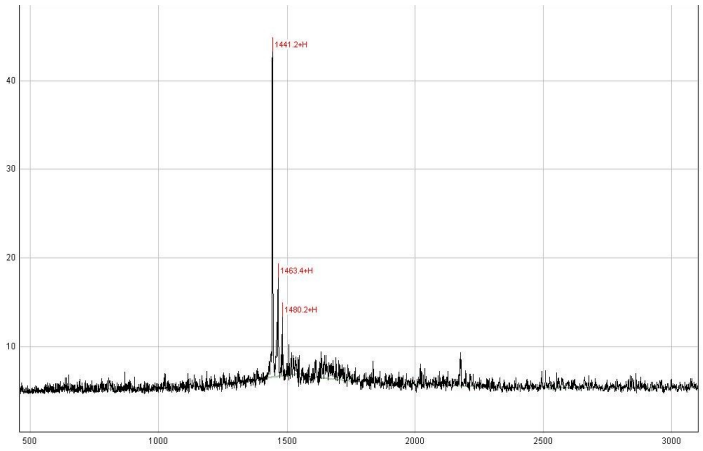


9

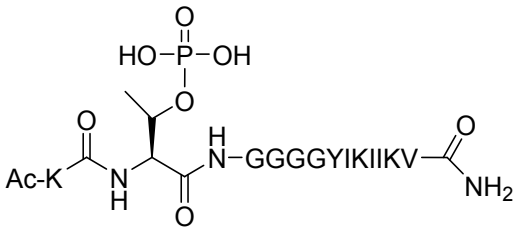
MALDI-TOF-MS: 1440.6 (calculated); 1441.2 ±0.5 (observed, $[M+H]^+$).

HPLC retention time: 18.24 min (Kromasil C18 column, 250×4.6 mm, 5 μm, 15-55% solution B over 30 min, 1 mL/min)





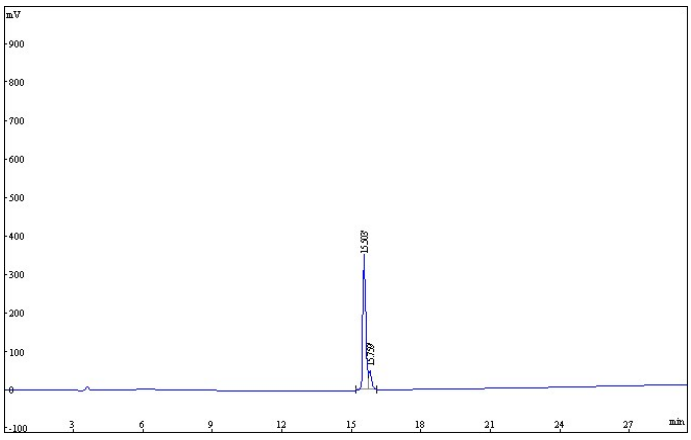
Peptide 10

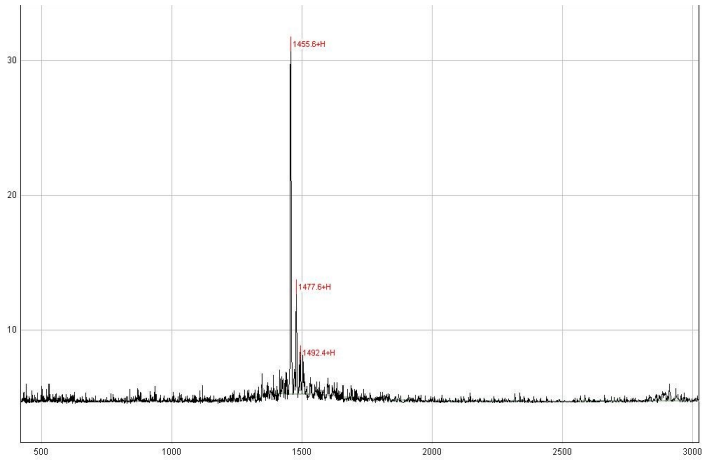


10

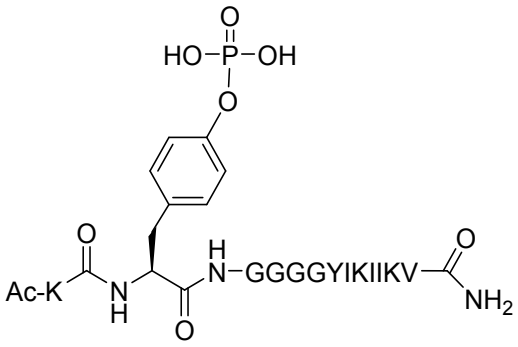
MALDI-TOF-MS: 1454.6 (calculated); 1455.6 ±0.5 (observed, $[M+H]^+$).

HPLC retention time: 15.50 min (Kromasil C18 column, 250×4.6 mm, 5 μm, 15-55% solution B over 30 min, 1 mL/min)





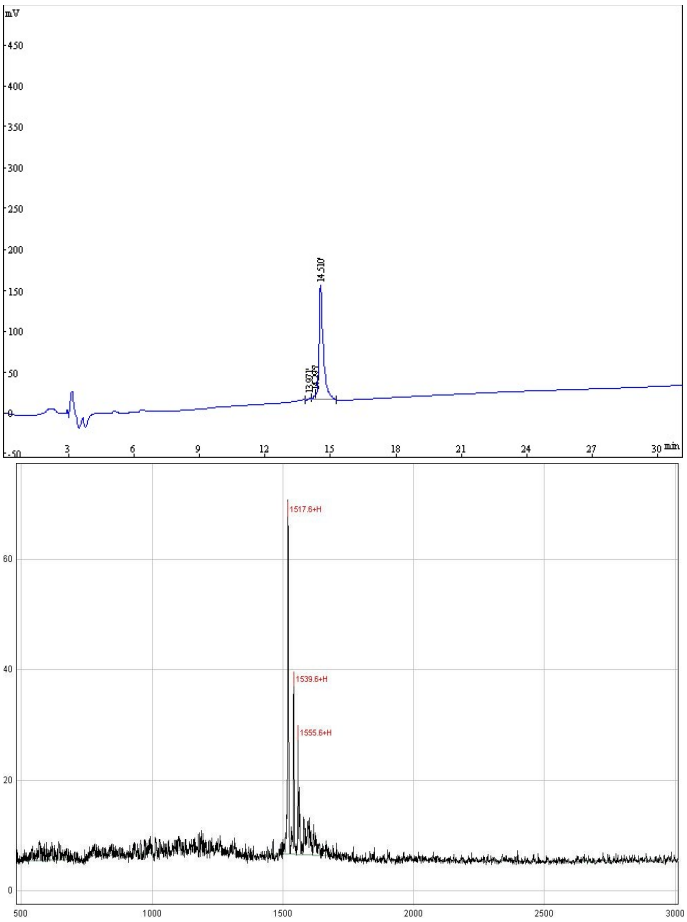
Peptide 11



11

ESI-MS: 1416.7 (calculated); 1417.6 ±0.5 (observed, $[M+H]^+$).

HPLC retention time: 14.51 min (Kromasil C18 column, 250×4.6 mm, 5 μm, 15-55% solution B over 30 min, 1 mL/min)



In vitro Phosphorylation of Peptide 7 with McsB

Peptide 7 was enzymatically phosphorylated with recombinant protein arginine kinase McsB according to the literature with modification.[3] The kinase reaction was carried out in the presence of 40 μM Peptide 7, 15 μM McsB, 1mM ATP and 5mM $MgCl_2$ in 100 μL 20 mM Tris (pH8.0) at 30 °C for 1h. The sample was flash frozen and then stored at -80 °C or directly injected for LC separation. The product peak in LC chromatogram was identified by using coupled ESI-MS detector. When performing large-scale preparation, the sample could be freezing-dry first, to reduce injection volume.

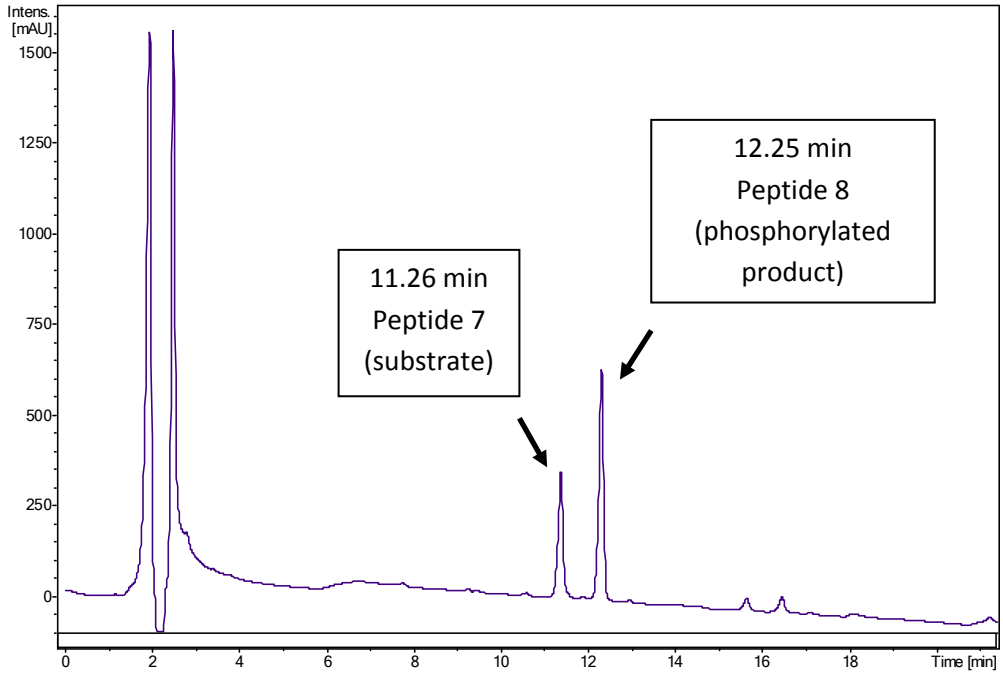
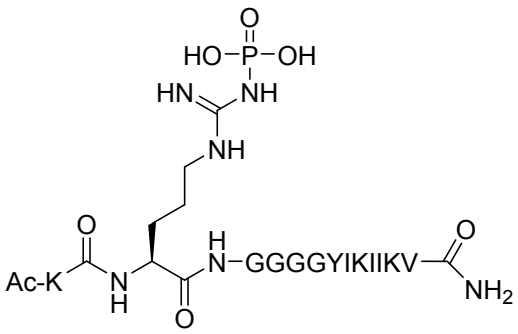


Figure S17. LC separation of kinase reaction mixture

Preparation of pArg-Peptide 8

Peptide 8 was enzymatically synthesized from Peptide7 with protein arginine kinase McsB via *in vitro* kinase reaction. The reaction mixture was separated on Analytical RP-HPLC equipped with TC-C18 column (Agilent, 15cm×4.6mm) at a flow rate of 1 mL/min. The elution was carried out employing gradients of solvent A (0.1% formic acid in water) and solvent B (95% acetonitrile in water with 0.1% formic acid). For preparation, a 27-minute linear gradient with increasing solvent B from 5% to 70% was adopted. All fractions containing the phosphorylated product were collected followed by neutralization to pH=8 with concentrated ammonia (25%). The organic solvent consisted of HPLC mobile phase was firstly removed by Speed-Vacuum at room temperature. Residual water in the fractions, excessive ammonia and other volatile substances were further removed by freeze-drying. Purified Peptide 8 was finally stored frozen at -80 ºC for further use. Peptide 8 could also be re-dissolved in PBS, and quantified by measuring its UV absorption at 275 nm with Nano-drop (Thermal Scientific) using Peptide 7 stock solution as reference.

Peptide 8



8

Isolated yield: 68%

ESI-MS: 1509.70 (calculated); 756.36 (observed $[M+2H]^{2+}$).

HPLC retention time: 12.25 min (Agilent TC-C18 column, 0-70% solution B over 27 min, 1 mL/min)

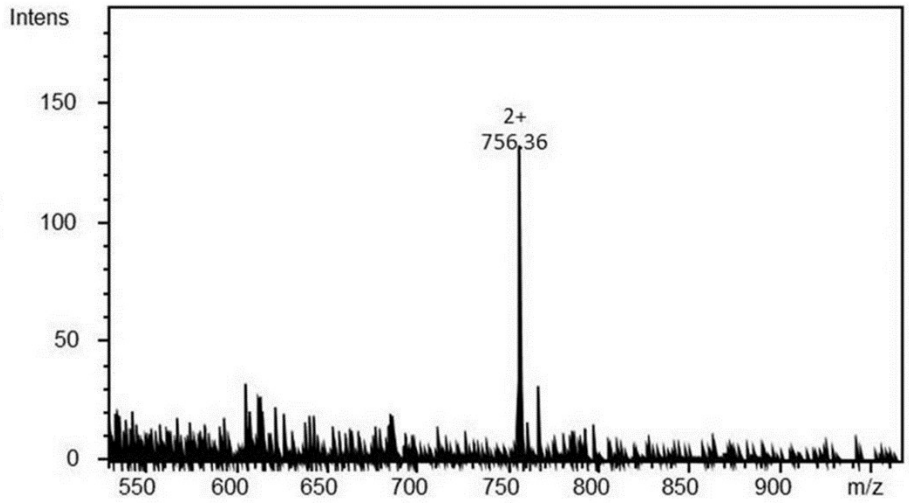


Figure S18. ESI-MS spectrum of purified peptide 8

Nano-LC-MS analysis of Peptide 8

For Nano-LC-MS/MS analyses, 100 ng purified peptide 8 was separated on nanoC18 column (Acclaim, 15 cm x 50 μm x 2 μm, Dionex) at a flow of 350nL/min, under a linear gradient of A and B solution with increasing solvent B from 8%-35% over 30min (solvent A: water with 0.1% formic acid; solvent B: 95% acetonitrile with 0.1% formic acid).

Alternatively, direct infusion with a sprayer tip was used. Full-scan mass spectra were acquired in positive-ion mode over the m/z range of 350–1800 at a resolution of 70,000. MS/MS spectra were simultaneously acquired using HCD for the 20 most abundant multiply charged species in the full-scan spectrum having signal intensities of > 1000. For protein identification experiments, dynamic exclusion was set such that MS/MS was acquired only once for each species over a period of 60 s. All spectra were acquired in profile mode.

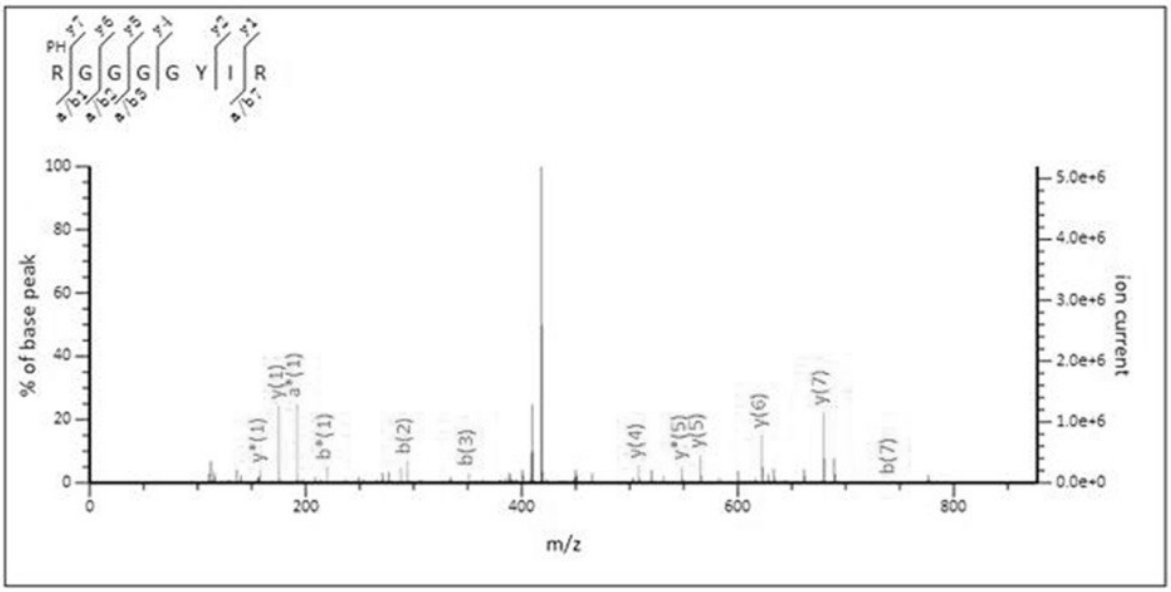
In vitro Phosphorylation of CtsR

CtsR was phosphorylated with recombinant protein arginine kinase McsB according to the literature with modification[3]. The kinase reaction was carried out in the presence of 10 μM purified CtsR, 5 μM McsB, 1mM ATP and 5mM $MgCl_2$ in 100 μL 20 mM Tris (pH8.0) at 30 °C for 1h. The reaction was quenched with equal volume of 4 ×Basic Loading Buffer and stored at -80 °C for further use.

Nano-LC-MS analysis of phosphorylated CtsR

After *in vitro* phosphorylation, the McsB/CtsR reaction mixture was 5-fold diluted with 50 mM NH_4HCO_3, reducing the concentration of CtsR to 2 μM CtsR. The mixture was treated with 25 mM DTT for 30 minutes at RT and then 50 mM iodoacetamide for 30 minutes in dark, followed by addition of DTT to the concentration of 100 mM and incubation at RT for another 30 minutes in dark. Excessive DTT and iodoacetamide were removed by using Ultra Centrifigual Unit (Millipore, MWCO 3K) and the buffer was changed to 50 mM NH_4HCO_3. Trypsin digestion was perform at 37 °C for 8 hours with a ratio of 100:1 (by weight, protein : trypsin) and the final concentration of protein was 0.1 mg/mL. Resulted enzymatic hydrolysate was freezing-dry for further analysis.

For Nano-LC-MS/MS analyses, 1 μg enzymatic hydrolysate were separated on nanoC18 column (Acclaim, 15 cm x 50 μm x 2 μm, Dionex) at a flow of 350nL/min, under a linear gradient of A and B solution with increasing solvent B from 8%-35% over 30min (solvent A: water with 0.1% formic acid；solvent B: 95% acetonitrile with 0.1% formic acid). Full-scan mass spectra were acquired in positive-ion mode over the m/z range of 350–1800 at a resolution of 70,000. MS/MS spectra were simultaneously acquired using HCD for the 10 most abundant multiply charged species in the full-scan spectrum having signal intensities of > 1000. For protein identification experiments, dynamic exclusion was set such that MS/MS was acquired only once for each species over a period of 60 s. All spectra were acquired in profile mode.



b	b^{++}	b*	b^{*++}	Seq.	y	y^{++}	y*	y^{*++}	#
237.0747	119.0410	220.0482	110.5277	R					8
294.0962	147.5517	277.0696	139.0385	G	*679.3522*	340.1797	662.3257	331.6665	7
351.1176	176.0625	334.0911	167.5492	G	*622.3307*	311.6690	605.3042	303.1557	6
408.1391	204.5732	391.1126	196.0599	G	*565.3093*	283.1583	548.2827	274.6450	5
465.1606	233.0839	448.1340	224.5707	G	*508.2878*	254.6475	491.2613	246.1343	4
628.2239	314.6156	611.1974	306.1023	Y	451.2663	226.1368	434.2398	217.6235	3
741.3080	371.1576	724.2814	362.6443	I	*288.2030*	144.6051	271.1765	136.0919	2
				R	*175.1190*	88.0631	158.0924	79.5498	1

Figure S19. Detection of arginine phosphorylation on CtsR by MS.

Supplementary Note 6

General Notes for Assays with anti-pArg antibody

Purified pArg-Ab and anti-serum were stored at 4 °C for at least three months without any detected loss in binding affinity. Tris buffered saline pH 8.0 was used in all pArg antibody assays. A basic gel-loading buffer with a pH of 8.5 was selected for WB and SDS-PAGE and we did NOT boil the samples after mixing with loading buffer prior to loading them onto the gel. Tris-HCl gels were preferred over Bis-Tris gels due to its more basic running buffer (pH 8.3 vs. pH 7.3) according to literatures[4]. 3% BSA but not conventional skim milk was selected as a blocking agent in ELISAs and Western Blots due to the possible casein contamination in skim milk.

Comparison on the Coating Efficiency of Peptide 7 and 8

In view of the possible problem in ELISA using short peptides as antigen, a pre-experiment was conducted to determine the coating efficiency of Peptide 7 and 8 (~1.5 kDa), a pair of positive and negative control used in ELISA for evaluation of anti-pArg serum. Briefly, Peptide 7 or Peptide 8 in 1 mM stock solutions were diluted 1000-fold into coating buffer, and 1 mL of each sample was added to an INNOVAX 96-well plate to give 50 µL/well. The plate was incubated for 2 hours at RT on a nutator, and the coating solution of each peptide was recovered from each well, pooled and lyophilized. The residues were re-dissolved in 20 µL ddH_2O, and quantified by measuring its UV-absorption at 275 nm relative to 1 mL Peptide solution or 1 mL Coating buffer processed with the same procedures except for “coating step”. The coating efficiency of Pep 7 and Pep 8 were 88.3 % vs. 86.5 % (3 individual experiment), which is comparable without obvious difference.

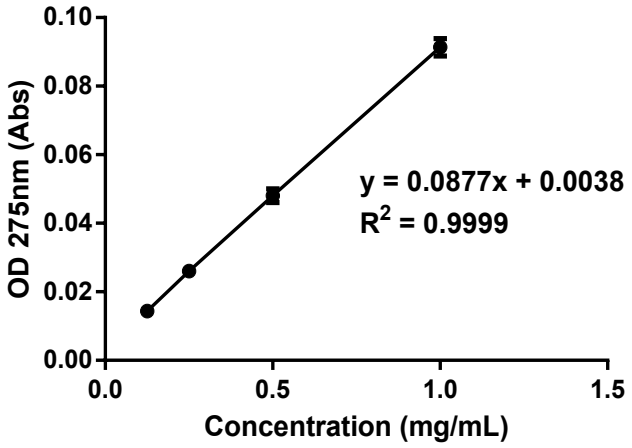


Figure S20. Standard curve for peptide quantification.

ELISA of crude anti-pArg antiserum

Peptide 7 and Peptide 8 in 1 mM stock solutions or BSA, pArg-BSA were diluted into coating buffer to give the concentration of 1 μM and 2 μg/mL for peptide and protein samples, respectively. The ELISA was then carried out in an analogous manner to the procedure described above. Briefly, after adding the coating samples, the plate was incubated for 2 hours at RT and then washed with wash buffer. The wells were then blocked with 200 μL 1 % BSA in wash buffer. The blocking solution was then removed from each well and a serial of 10-fold diluted crude mouse anti-pArg antiserum or 1:100 diluted Protein A purified antibody in wash buffer was added to the wells and incubated for 45 mins at RT. The wells were then washed with wash buffer for 3 times. Goat-anti mouse HRP secondary antibody diluted 5000-fold into wash buffer was the added to the wells and incubated for 45 mins at RT. The wells were washed with wash buffer and treated with 100 μL Ultra TMB ELISA substrate. 50 μL 2 N H_2SO_4 was then added to each well and the absorbance at 450 nm was measured on a Bio-Rad plate reader.

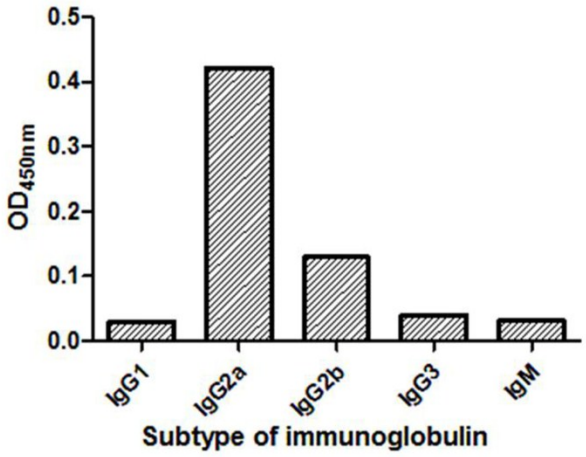


Figure S21. Detection of polyclonal pArg-Ab subtype in mouse serum. The ELISA plate was coated with peptide 8 as a capture antibody and peptide 7 was used as a control for background deduction

General Procedure for Dot Blot Assay

Peptides 7-11 were diluted to a final concentration of 500 μM in ddH_2O buffer and further serially diluted three-fold with ddH_2O in a siliconized Eppendorf tube. 0.2 μL of each solution was spotted onto nitrocellulose membrane and the membrane was air dried followed by blocking with 3% BSA in TBST (10 mM Tris, pH 8.0, 150 mM NaCl,

0.05% v/v Tween 20) for 1 h and subsequent incubation with purified pArg-Ab (1:1000 diluted in TBST) for 45 min at RT. The membrane was then washed with TBST for 3 times (5 min, 10 min, 15 min) at RT followed by incubation with goat anti-mouse-HRP conjugate secondary antibody (1:5000 diluted in 1% BSA with TBST) for 45 min at RT. The membranes were again washed with TBST for 3 times (5 min, 10 min, 15 min) at RT, drained, and incubated with ECL chemiluminescence substrate reagent for1 min at RT. The membranes were imaged using GE Amersham Imager 600.

General procedure for Western blot of pArg-containing proteins

Buffers used in SDS-PAGE and Western blots

5 x Tris-Glycine running buffer: 125 M Tris, 960 mM glycine, 0.5% (v/v) SDS, pH 8.3

5 x Towbin buffer: 125 M Tris, 960 mM glycine, pH 8.3

Wash Buffer: 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% v/v Tween 20

4×Basic loading buffer: 160 mM Tris, pH 8.5, 40% (v/v) glycerol, 4% (w/v) SDS, 0.08% (w/v) bromophenol blue, and 8% (v/v) BME

Samples were dilutied into 4×Basic loading buffer and resolved by SDS-PAGE. Samples were loaded onto a Tris-HCL gel (12% or 15%) and run for 1 hour at 180 V (max current is limited to <40 mA/gel).

The resolved proteins were electroblotted onto a PVDF membrane in 0.5 x Towbin buffer supplied with 20% MeOH at 100 V for 60-90 minutes. The membrane was blocked with 3% BSA in wash buffer for 1 h at RT, followed by incubation with Protein A purified pArg-Ab diluted 1:100 in wash buffer for 1 h at RT. After washing with wash buffer (3 ×5 min), incubation with goat anti-mouse IgG-HRP (1:5000 diluted in wash buffer) for 1 h at RT, the membrane was washed with wash buffer (3 ×5 min), drained and incubated with ELC chemiluminescence solution for 1 min at RT. The membranes were imaged using GE Amersham Imager 600.

Reference

1. Y. Shao, L. F. Molnar, Y. Jung, J. Kussmann, C. Ochsenfeld, S. T. Brown, A. T. B. Gilbert, L. V. Slipchenko, S. V. Levchenko, D. P. O'Neill, R. A. DiStasio, Jr., R. C. Lochan, T. Wang, G. J. O. Beran, N. A. Besley, J. M. Herbert, C. Y. Lin, T. Van Voorhis, S. H. Chien, A. Sodt, R. P. Steele, V. A. Rassolov, P. E. Maslen, P. P. Korambath, R. D. Adamson, B. Austin, J. Baker, E. F. C. Byrd, H. Dachsel, R. J. Doerksen, A. Dreuw, B. D. Dunietz, A. D. Dutoi, T. R. Furlani, S. R. Gwaltney, A. Heyden, S. Hirata, C.-P. Hsu, G. Kedziora, R. Z. Khalliulin, P. Klunzinger, A. M. Lee, M. S. Lee, W. Liang, I. Lotan, N. Nair, B. Peters, E. I. Proynov, P. A. Pieniazek, Y. M. Rhee, J. Ritchie, E. Rosta, C. D. Sherrill, A. C. Simmonett, J. E. Subotnik, H. L. Woodcock, III, W. Zhang, A. T. Bell, A. K. Chakraborty, D. M. Chipman, F. J. Keil, A. Warshel, W. J. Hehre, H. F. Schaefer, III, J. Kong, A. I. Krylov, P. M. W. Gill and M. Head-Gordon, *Physical Chemistry Chemical Physics*, 2006, **8**, 3172-3191.
2. A. Schmidt, D. B. Trentini, S. Spiess, J. Fuhrmann, G. Ammerer, K. Mechtler and T. Clausen, *Molecular & Cellular Proteomics*, 2014, **13**, 537-550.
3. J. Fuhrmann, A. Schmidt, S. Spiess, A. Lehner, K. Turgay, K. Mechtler, E. Charpentier and T. Clausen, *Science*, 2009, **324**, 1323-1327.
4. J.-M. Kee, R. C. Oslund, D. H. Perlman and T. W. Muir, *Nat Chem Biol*, 2013, **9**, 416-421.