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Supplementary Information

Distinct phosphorylation and dephosphorylation dynamics of a protein arginine kinase revealed by fluorescent activity probes

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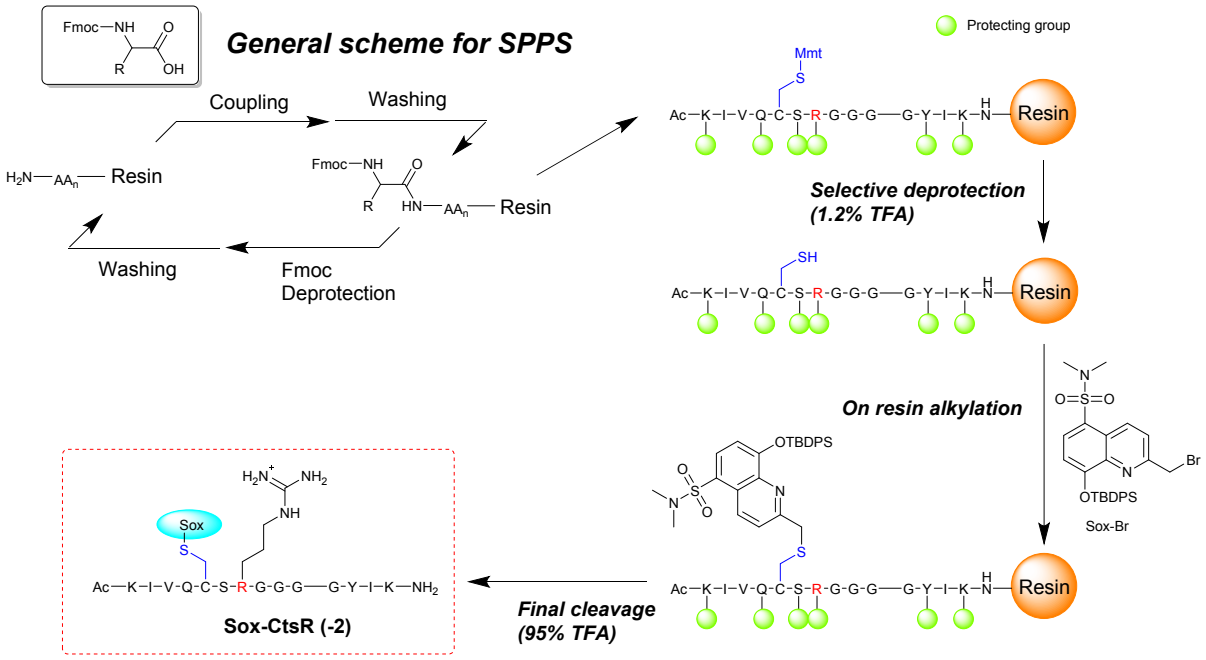


Fig. S 1. Preparation of fluorescent probes for Arg phosphorylation.

Table S 1. Sequence of Sox-CtsR probes

Entry	Compound	Peptide sequence
(−3)	Sox-CtsR(−3)	Ac-KVI(CSox)SKRGGGGYIK-NH_2
(−2)	Sox-CtsR(−2)	Ac-KVIQ(CSox)KRGGGGYIK-NH_2
(−1)	Sox-CtsR(−1)	Ac-KVIQS(CSox)RGGGGYIK-NH_2
(+1)	Sox-CtsR(+1)	Ac-KVIQSKR(CSox)GGGYIK-NH_2
(+2)	Sox-CtsR(+2)	Ac-KVIQSKRG(CSox)GGYIK-NH_2
(+3)	Sox-CtsR(+3)	Ac-KVIQSKRGG(CSox)GYIK-NH_2

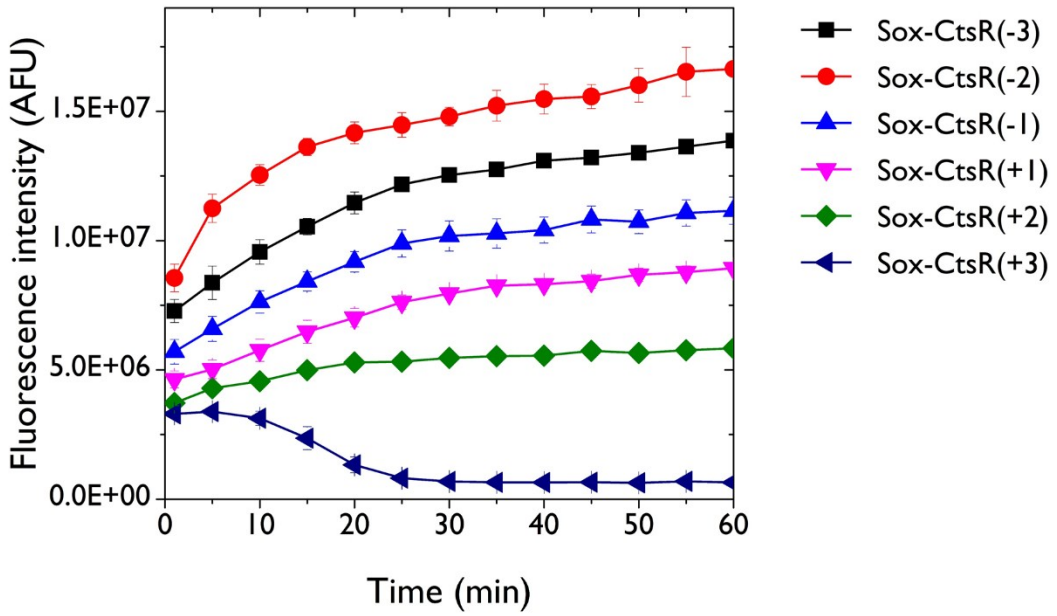


Fig. S 2. Increment in the fluorescence signal of probes upon phosphorylation by McsB.

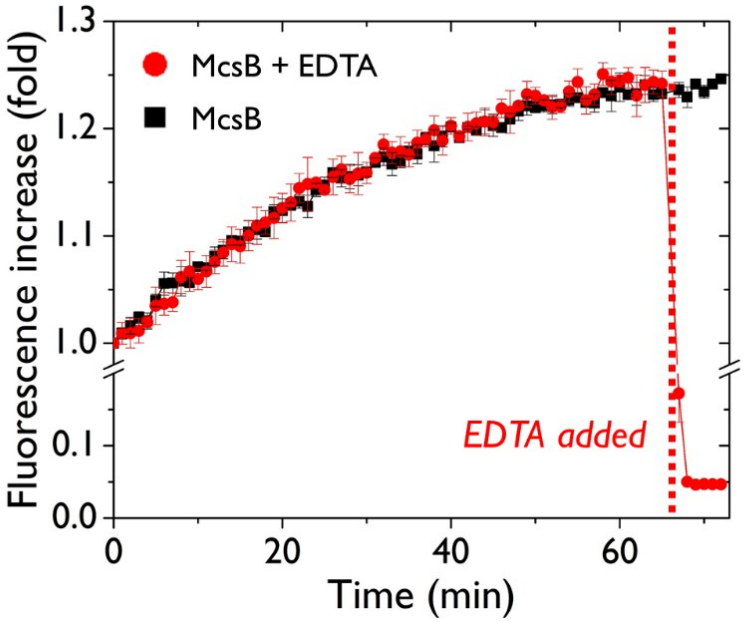


Fig. S 3. Effect of EDTA treatment to the probe fluorescence.

For the kinase reaction, two separate batches of 5 μM McsB, 50 μM Sox-CtsR(-2), 1.5 mM ATP, and 20 mM $MgCl_2$ were incubated at for phosphorylation. After 65 min, 20 mM EDTA was added to one batch (red dots) to competitively chelate Mg^{2+}, thereby reducing the CHEF effect. Despite the dramatic fluorescence decrease in the EDTA-treated sample, both showed a comparable degree of Arg-phosphorylation (20.8% for control vs. 18.6% for EDTA-treated), as quantified by independent HPLC measurements.

Table S 2. Quantification of nucleotides, pArg and Arg probe (Fig 4).

	ADP only			ADP + McsB			pArg-Sox-CtsR(-2)	
	AMP	ADP	ATP	AMP	ADP	ATP	Arg	pArg
pmol	242.0	3030.0	103.0	428.0	1763.0	231.0	352.0	1355.0
%	7.2	89.8	3.1	17.7	72.8	9.5	20.6	79.4

	ADP + pArg-Sox-CtsR(-2)				
	AMP	ADP	ATP	Arg	pArg
pmol	200.0	2585.0	171.0	423.0	1189.0
%	6.8	87.4	5.8	26.2	73.8

	ADP + McsB_E122A + pArg-Sox-CtsR(-2)				
	AMP	ADP	ATP	Arg	pArg
pmol	445.7	2575.9	121.1	181.5	1273.8
%	14.2	82.0	3.8	12.5	87.5

	ADP + McsB + pArg-Sox-CtsR(-2)				
	AMP	ADP	ATP	Arg	pArg
pmol	309.0	1596.0	1286.0	1228.0	261.0
%	9.7	50.0	40.3	82.5	17.5

Table S 3. Estimation of equilibrium constant for McsB reverse reaction

Equilibrium constant equation	Entry	[ATP]/[ADP]	[Arg]/[pArg]	$K_{eq}{}'$
$K_{eq}{}' = \frac{[ATP][Arg]}{[ADP][pArg]}$	1	1.71	3.96	6.77
	2	4.14	1.25	5.20
	3	2.48	3.77	9.34
	4	2.91	1.73	5.04
	5	3.53	1.22	4.29

General Materials

All reagents were purchased from Alfa Aesar and TCI without further purification unless otherwise noted. All biological reagents (unless other noted) were purchased from Bio-rad (Hercules, CA). All buffering salts and organic solvents were purchased from Daejung (Siheung, Republic of Korea). Tris/HCl salt, Tris base, B-PER™ Bacterial Protein Extraction Reagent and HisPur Ni-NTA Resin were purchased from Fisher Scientific (Pittsburgh, PA). Bovine serum albumin (BSA), dithiothreitol (DTT), adenosine 5'-triphosphate disodium salt (ATP), 5'-diphosphate disodium salt (ADP), 5'-monophosphate disodium salt (AMP), P^1, P^5-Di(adenosine-5′) pentaphosphate pentasodium salt (Ap_5A), Protease Inhibitor Cocktail powder for bacterial extracts and Pyruvate Kinase from rabbit muscle were purchased from Sigma-Aldrich (St. Louis, MO). Coomassie brilliant blue, glycerol and magnesium chloride hexahydrate ($MgCl_2$) were purchased from Biosesang (Seongnam, Republic of Korea). HBTU and Rink-amide resin were purchased from Matrix innovation (Quebec, Canada). All amino acids used in peptide synthesis (unless otherwise noted) were purchased from GL Biochem (Shanghai, China). Fmoc-Cys(Mmt)-OH was purchased from Bepharm (Shanghai, China). LB Broth and LB Agar were purchased from Affymetrix (Santa Clara, CA). Phenylmethylsulfonyl fluoride (PMSF, protease inhibitor) was purchased from Alfa Aesar (Ward Hill, MA). Ampicillin sodium salt and isopropyl β-D-1-thiogalactopyranoside (IPTG) were from MP Biomedicals (Santa Ana, CA).

General Methods

Analytical RP-HPLC was performed on Agilent 1200 series instruments equipped with a C18 inertSustain (5 μm, 4.6 x 150 mm) column at a flow rate of 1 mL/min employing gradients of solvent A (0.1% trifluoroacetic acid (TFA) in water) and solvent B (90% acetonitrile in water with 0.1% TFA). For all analytical RP-HPLC runs a two-minute isocratic period in initial conditions was followed by 8 or 20-minute linear gradient with increasing solvent B concentration. The solvent gradients are specified in each of HPLC experiment (see below). We performed semi-preparative RP-HPLC with a C18 InertSustain (5 μm, 10 x 250 mm) at a flow rate of 2.5 mL/min. All semi-preparative RP-HPLC runs were carried out employing gradients of solvent A (0.1% TFA in water) and solvent B (90% acetonitrile in water with 0.1% TFA). For all semi-preparative RP-HPLC runs, a five-minute isocratic period in initial conditions was followed by a 20 or 40-minute linear gradient with increasing solvent B concentration. Cells were lysed using a Branson Ultrasonic Corp 250 DIG SONIFIER 115V (Fisher Scientific, Pittsburgh, PA). Samples were lyophilized on a Scanvac CoolSafe Freeze Dryer. The pH of buffers was measured with a pH meter, Orion Star A211 (Thermo Scientific, Waltham, MA). Mass spectra were obtained on a Ultraflex III-MALDI-TOF/TOF (Bruker BioSpin, Billerica, MA), a Q Exactive™ Hybrid Quadrupole-Orbitrap™, or a Q Exactive™ plus Hybrid Quadrupole-Orbitrap™ (Thermo Scientific, Waltham, MA). Absorbance and fluorescence were measured with a SpectraMax i3x (Molecular devices, Sunnyvale, CA). Chemiluminescence from western blot analysis was measured with a ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA).

General Protocols of Solid Phase Peptide Synthesis (SPPS)

The peptides were synthesized on Rink amide resin (Matrix) on a 0.1 mmol scale. The resin was swollen by incubation in DMF with gentle agitation (by bubbling with N_2). After 30 min, the resin was washed with two flow washes and one final batch wash with DMF. All the subsequent resin washing steps were carried out using this method. Fmoc deprotections were carried out using a 20% piperidine in DMF in two steps (1 min incubation and 20 min deprotection). Then, the resin was washed. Amino acids were coupled using HBTU/ DIPEA as the activating agents. Desired amino acid (0.5 mmol) was weighed and dissolved in 1 mL of 0.49 M HBTU solution (in DMF) and DIPEA (1 mmol) was added immediately. The mixture was thoroughly mixed and allowed to be activated for 3~5 min, and the solution was poured into the resin. The reaction mixture was agitated bybubbling N_2 for 30 minutes. Then, the resin was washed. With the last N-terminal residue, the acetylation was performed by incubating the resin with 20 mol equiv. of Ac_2O and 40 mol equiv. of DIPEA in DMF for 10 min (repeat 2 times). The peptide was cleaved from the resin by treating with a mixture of 95% TFA, 2.5% triisopropylsilane (TIPS) and 2.5% water for 1.5 hr at room temperature. The cleaved peptide was precipitated in ice-cold ethyl ether. The peptide precipitate was collected by filtration or centrifugation, and then dissolved in 50% HPLC solvent B to separate from the resin. The crude peptide was purified by preparative RP-HPLC.

Western Blotting Using Anti-pArg Antibody

The pan anti-pArg antibody was generously donated by Prof. Paul Thompson (University of Massachusetts Medical School). Protein samples were diluted into 4× basic loading buffer and incubated for 5 min at RT and resolved by SDS-PAGE. The resolved proteins were electroblotted onto a PVDF membrane (Bio-Rad; cat. no.: 162-0177) in Towbin buffer with 20% MeOH and 0.02% SDS at 75 V (constant 210 mA) for 1.5 hr at 4°C. The membrane was blocked with 3% BSA in TBS-T for 1 hr at RT. The membrane was then incubated with anti-pArg antibody (1:3,000) diluted in the blocking solution (3% BSA in TBS-T) for 1 hr at RT or 12 hr at 4°C. The membrane was washed with TBS-T (3 × 5 min) and then incubated with goat anti-rabbit IgG-HRP conjugate (Bio-Rad; cat. no. 170-6515; diluted 1:5,000 in TBS-T) for 30 min at RT. The membrane was washed with TBS-T (3 × 5 min), drained and incubated with ECL chemiluminescence substrate solution (Dongin LS; cat. no. ECL-PS100) for 3 min at RT. The chemiluminescence from the membrane was imaged using Biorad ChemiDoc™ XRS+ system.

4× loading buffer: 250 mM Tris–HCl (pH 6.8), 0.2% (w/v) bromophenol blue, 40% (v/v) glycerol, 10 mM DTT, 8% (w/v) SDS

5× gel running buffer: 125 mM Tris, 960 mM Glycine, 0.5% (w/v) SDS, pH 8.3

5× Towbin buffer: 125 mM Tris, 960 mM Glycine, pH 8.3

TBS-T: 50 mM Tris, 150 mM NaCl, pH 8.5 with 0.1% (v/v) Tween 20

Plasmid Construction for Recombinant Protein

Several plasmids were prepared by PCR-based restriction-free cloning method. Primers were designed considering each vector and template to reach the appropriated melting temperature. Primers were synthesized at Macrogen (Seoul, Republic of Korea). PCR-amplified megaprimers were incorporated into a pET21a(+) vector by overlap-extension PCR. For overlap-extension PCRs, Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific, Cat. No.: F531S) was used. MAX Efficiency® DH5α TM Chemically Competent Cells (Invitrogen, Cat. No.: 18258-012) was used as the transformation host. All plasmid sequences were confirmed by DNA sequencing (Macrogen, Korea).

Overlap-extension PCR

PCRs were performed according to the literature.[1] Primers were diluted into 50 μM with distilled water. For megaprimer synthesis, 10 μL of 2 X Phusion High-Fidelity PCR Master Mix was added and 2 μL of forward and reverse primer respectively. Then, 1 μL of the respective template was added (100 ng/μL). Distilled water was added to the final volume of 20 μL. PCRs were carried out following the manufacturer's instruction. Finally, the megaprimer was purified by 0.8 % agarose gel electrophoresis followed by extraction with AccuPrep® Gel Purification Kit (Bioneer, Cat. No.: K-3035). The product megaprimer concentration was measured by Nanodrop®.

For overlap-extension PCR, to a 10 μL of 2 X Phusion High-Fidelity PCR Master Mix, 20~40 ng of vector and 100 ng of megaprimer were added. Final volume was adjusted to 20 μL with distilled water. Overlap-extension PCRs were run using the following parameters. Final product was treated with FastDigest DpnI (Thermo Scientific, Cat. No.: FD1703) for 5 min at 37°C according to the manufacturer's instruction to remove the original vector template, followed by transformation to DH5α and Mini-prep for DNA sequencing.

Table S 4. Method of overlap-extension PCR

Step		Temperature	Time
Initial denaturation		96°C	30 sec
Cycles (25~30 repeats)	Denaturation	96°C	30 sec
	Annealing	55°C to 65°C	60 sec
	Extension	72°C	2-3 min
Final extension		72°C	7 min

General Procedures for Fluorescent Real-Time Enzyme Activity with Probes

In vitro phosphorylation and dephosphorylation assays were performed in 25 mM HEPES, 300 mM KCl, pH 7.5 buffer with Gs McsB or in 25 mM Tris, 300 mM KCl, pH 7.5 with Bs McsB. Enzyme activities were monitored with fluorescence measurements ($\lambda_{ex}/\lambda_{em}$ =360/500 nm) at 40°C. Reaction volume was 200 µL and the experiments were performed in triplicates on black 96 well plate. Sox-CtsR(-2) and McsB were premixed and activated at 40°C for 5 min. Typically, the reaction was started by adding ATP solution.

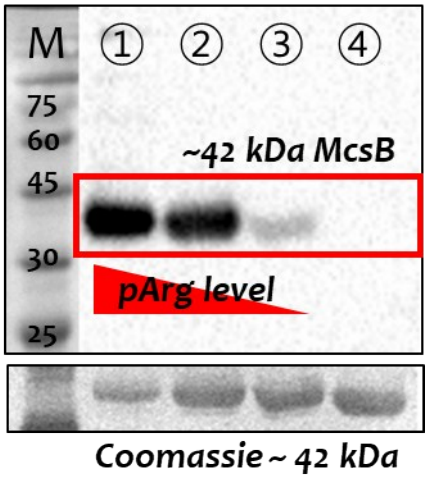
Table S 5. Reagents for fluorescent phosphorylation and dephosphorylation assays

Reagents	Stock concentration	Final concentration
Sox-CtsR(-2)	500 µM	50 µM
McsB	15 µM	5 µM
McsA	25 µM	5 µM
YwlE	66 µM	3 µM
Pyruvate kinase (PK)	14 µM	700 nM
ATP	30 mM	1.5 mM
$MgCl_2$	200 mM	20 mM
PEP	20 mM	0.5 mM

Autophosphorylation effects on McsB kinase activity

To test the effect of McsB autophosphorylation on its kinase activity, batches of McsB with various pArg levels were prepared (Table S 6). Excess nuclotides or enzymes were removed by size exclusion chromatography to yield pure McsB. Fluorescence assays with Sox-CtsR(-2) probe were conducted in same conditions with Table S 5.

Table S 6. Preparation of McsB with various pArg level

Entry		Reaction condition	pArg quantification
①	pArg rich McsB	Gs McsB + PK + PEP + ATP → Size exclusion chromatoraphy	
②	Wild type McsB	Gs McsB, no reaction → Size exclusion chromatoraphy	
③	pArg poor McsB	Gs McsB + YwlE → Size exclusion chromatoraphy	
④	Mutant McsB	Gs McsB^{E122A}, no reaction → Size exclusion chromatoraphy	

ATP/ADP Quantification with IPRP-HPLC

ATP and ADP were quantified by ion-pairing reverse phase HPLC (IPRP-HPLC) following the literature protocols with slight modifications.[2] The modified analysis condition is given below.

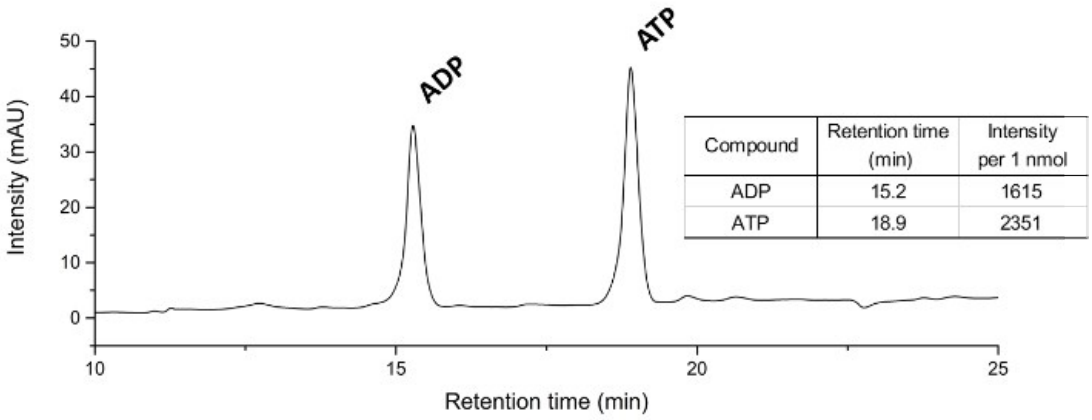
Column: C18 InertSustain (5 μm, 10 x 250 mm)

Buffer A: 5 mM tetrabutylammonium hydroxide(TBA-OH), 30 mM KH_2PO_4, pH 6

Buffer B: 35% buffer A + 65% acetonitrile

Gradient: 10-55B, 1 ml/min over 20 min linear gradient

Standard nucleotides mixture was quantified (**Fig. S4**).



Compound	Retention time (min)	Intensity per 1 nmol
ADP	15.2	1615
ATP	18.9	2351

Fig. S 4. Quantification of ATP and ADP with IPRP-HPLC

Validation of McsB reversibility

pArg-Sox-CtsR(-2) peptide was prepared through *in vitro* phosphorylation in presence of PK and PEP for higher efficiency. Peptide phosphorylation was monitored through HPLC. Phosphorylated peptide, pArg-Sox-CtsR(-2) was purified by semi preparative RP-HPLC.

Semi preparative RP-HPLC method: 25-35B, 2.5 ml/min over 20 min

pArg-Sox-CtsR(-2) was incubated with McsB, $MgCl_2$ and ADP at 40°C with same buffer with fluorescence assay. By time, remaining ADP and newly generated ATP amount were quantified with IPRP-HPLC, as well as pArg and Arg-Sox-CtsR(-2) peptides through RP-HPLC. All experiments were triplicated.

Table S 7. Reagents for McsB reversibility tests with the pArg-peptide probe

Reagents	Stock concentration	Final concentration
pArg-Sox-CtsR(-2)	350 μM	50 μM
Gs McsB	12 μM	5 μM
Bs McsB	7 μM	3.5 μM
Bs McsA	22 μM	3.5 ~ 7 μM
ADP	2 mM	100 μM
$MgCl_2$	200 mM	5 mM

Ap_5A effect test on McsB reactivities

pArg-Sox-CtsR(-2) or Sox-CtsR(-2) was incubated with McsB, $MgCl_2$ and ADP at 40°C with the same buffer for fluorescence assays in the presence of Ap_5A. Then, fluorescence change was monitored with plate reader. Over time, pArg peptide was dephosphorylated into Arg, decreasing the fluorescence intensity. Furthermore, remaining ADP and newly generated ATP amount were quantified with IPRP-HPLC, as well as pArg and Arg-Sox-CtsR(-2) peptides through RP-HPLC. All experiments were triplicated.

Table S 8. Reagents for Ap5A effect test on McsB reactivities

Reagents	Stock concentration	Final concentration
pArg-Sox-CtsR(-2)	350 μM	50 μM
Gs McsB	12 μM	5 μM
ATP	30 mM	1.5 mM
ADP	2 mM	100 μM
$MgCl_2$	500 mM	5 mM
Ap_5A	50 μM	1 ~10 μM

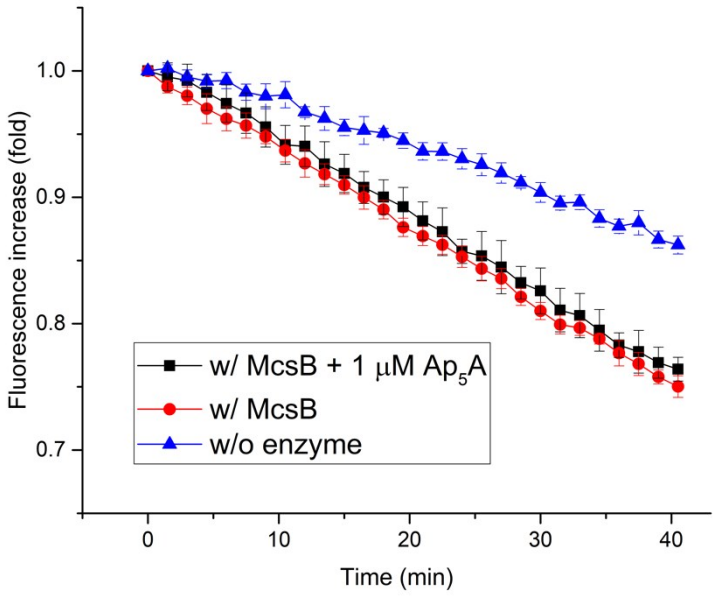


Fig. S 5. Ap_5A effect on McsB reverse pArg dephosphorylation reaction

Table S 9. Quantification of nucleotides, pArg and Arg probe (Fig. S5 and Fig. S6)

Condition	Reverse reaction_w/ McsB + 1 μM Ap_5A				
Compound	AMP	ADP	ATP	Arg	pArg
pmol	116.8	1331.0	179.0	455.9	305.3

Condition	Reverse reaction_ w/ McsB				
Compound	AMP	ADP	ATP	Arg	pArg
pmol	119.7	1311.7	167.3	450.7	326.6

Condition	Reverse reaction_w/o enzyme				
Compound	AMP	ADP	ATP	Arg	pArg
pmol	90.8	1477.3	101.8	218.1	491.6

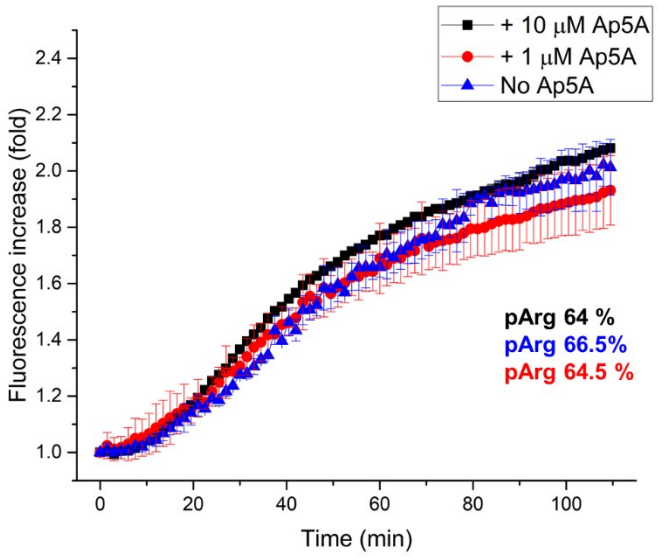


Fig. S 6. Ap_5A effect on McsB forward Arg phosphorylation reaction

Validation of [ATP]/[ADP] effect on protein pArg level by Western blot

As [ADP] increased, pArg levels of both McsB and CtsR decreased (lane 1 to 4). However, with western blots using the pan-pArg antibody, it was difficult to observe the McsB-mediated pArg dephosphorylation by excess [ADP] (comparing lanes 5 and 6, lane 5; pArg dephosphorylation caused by McsB and excess ADP, lane 6; natural decomposition of pArg).

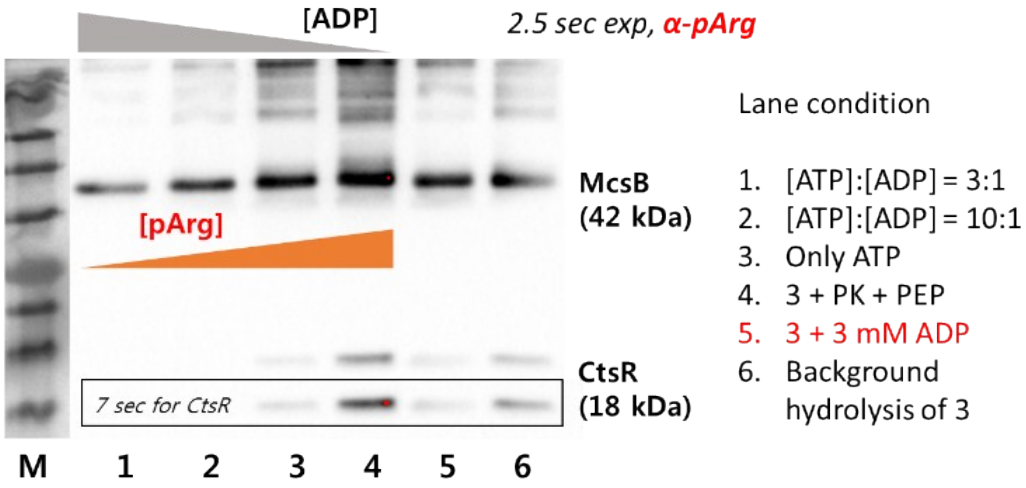


Fig. S 7. ADP effect on Arg phosphorylation in protein level checked by western blots with a pan-pArg antibody

5 μM McsB, 10 μM CtsR, 0.7 μM PK, 1.5 mM ATP, 2 mM PEP, 5 mM $MgCl_2$, 2 hr for phosphorylation, 45 min for dephosphorylation at 40°C.

Preparation of Plasmids

The following plasmids were generously donated by Dr. Tim Clausen at Research Institute of Molecular Pathology, Austria.

McsB-His_6: pET21a(+) derivative containing McsB from *G. stearothermophilus*

CtsR-His_6: pET21a(+) derivative containing CtsR from *G. stearothermophilus*

YwlE-His_6: pET21a(+) derivative containing YwlE from *G. stearothermophilus*

Site-directed mutagenesis

Vector and template: pET21a(+) dervative containing McsB or CtsR from *G. stearothermophilus*

Gene	Foward	Reverse
Gs $McsB^{E122A}$-His_6	GATCAACGAGGCAGACCATATCCG	CGGATATGGTCTGCCTCGTTGATC
Gs $YwlE^{C9S}$-His_6	GTTCGTTAGCACGGGCAATAC	GTATTGCCCGTGCTAACGAAC

Table S 10. Primers for Site-directed mutagenesis for inactive mutants

For the single mutagenesis of the animo acid sequence, site-directed mutagenesis was proceeded with each forward and reverse primers by using Phusion polymerase according to the manufacturer's instruction.

Overlap extension PCR

Vector: pET21a(+)

Template: pTOP Blunt V2 derivative containing McsA from *B. subtilis* (synthesized from Macrogen)

Table S 11. Primers for overlap extension PCR of *Bs* McsA

Gene	Foward	Reverse
Bs McsA-His_6	GTTTAACTTTAAGAAGGAGATATACA T ATGCACCATCATCATCATCATGGC	CGCCTTTAACATGCGTTA CTCCTGTTCCTCCTCAC

The plasmid Bs McsA-His_6 was prepared in two steps by overlap-extension PCR. First, the Bs McsA-His_6 gene was amplified from its template by Phusion polymerase using forward and reverse primers that contain 5' and 3' sequences that overlap with regions of a modified pET21a(+) plasmid. The resulting product DNA was purified by agarose gel electrophoresis followed by gel extraction using Bioneer gel extraction kit according to the manufacturer's instructions.

In the second step, *Bs* McsA-His_6 gene product was inserted into a modified pET21a(+) plasmid using Phusion polymerase. Then the template plasmid was globally digested using DpnI at 37˚C for 5 min.

Cloning of *B. subtilis* proteins

Template: Genomic DNA of *Bacillus subtilis* (Strain 168, KCTC 2217, the biological resources used in this research were distributed from KCTC)

Vector: pET21a(+)

Gene	Foward	Reverse
Bs McsB-His_6	CATTCCCTCTAGAATAATTTTGTTTAA CTTTAAGAAGGAGATATACATATGTCG CTAAAGCATTTTATTCAGGACG	TCAGTGGTGGTGGTGGTGGTGTAT CGATTCATCCTCCTGTCTTTTCCC
Bs CtsR-His_6	CATTCCCTCTAGAATAATTTTGTTTAA CTTTAAGAAGGAGATATACATATGGGA CATAATATTTCTGACATCATTGAAC	TCAGTGGTGGTGGTGGTGGTGTTT TAATTTTAAAGAAGTCAGCATTGC CTTCATC

Table S 12. Primers for PCR

The plasmid *Bs* McsB-His_6 and *Bs* CtsR-His_6 were prepared in two steps by overlap-extension PCR. First, the *Bs* McsB and *Bs* CtsR gene were amplified from *B. subtilis* DNA by Phusion polymerase using forward and reverse primers that contain 5' and 3' sequences that overlap with regions of a modified pET21a(+) plasmid. In the second step, each amplified gene product was inserted into a modified pET21a(+) plasmid using Phusion polymerase. Then the template plasmid was globally digested using DpnI at 37˚C for 5 min.

Finally, the resulting product plasmids were used to transform *E. coli* DH5α cells by heat shock (42˚C for 45 sec). The transformed bacteria were grown in LB media (no antibiotics) for 1 hr at 37˚C and plated onto an ampicillin-containing LB-agar plate. The plate was incubated at 37˚C for overnight, and the colonies were individually picked and inoculated into 5 mL LB (with ampicillin 50 μg/mL). The bacteria were grown overnight at 37˚C in a shaker and the plasmid DNA was extracted using a Bioneer Miniprep kit according to the manufacturer's instructions. The obtained plasmids were sequenced and used to transform *E. coli* BL21(DE3) cells.

Expression and Purification of Recombinant Proteins

Expression and purification of recombinant proteins were carried out following the literature[3,4].

Geobacillus stearothermophilus McsB-His_6

The plasmid was transformed into *E. coli* BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with ampicillin (50 μg/mL) overnight at 37°C. 200 mL of the media was inoculated with 2 mL the overnight culture and grown until its O.D.600 reached 0.8. The bacteria was induced by 0.5 mM IPTG at 37°C, 200 rpm for 3 hr and then harvested by centrifugation (4000 g, 30 min at 4°C). The cell pellet was re-suspended in the lysis buffer (25 mM HEPES, 300 mM KCl, 2.5 mM PMSF, pH 7.5) and disrupted by sonication (40% amplitude, 5 sec pulse followed by a 10 sec pause, total 2 min). *Gs* McsB-His_6 was purified using the standard His-tag purification protocol according to manufacturer's recommendation, followed by SEC chromatography on a HiLoad 16/600 Superdex 75 pg column equilibrated with 25 mM HEPES, 300 mM KCl, pH 7.5 buffer. Purified *Gs* McsB-His_6 was analyzed by SDS-PAGE and the concentration was determined with the Bradford method.

Geobacillus stearothermophilus $McsB^{E122A}$-His_6

The plasmid was transformed into E. coli BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with ampicillin (50 μg/mL) overnight at 37°C. 200 mL of the media was inoculated with 2 mL overnight cultures and grown until its O.D.600 reached 0.6. The bacteria was induced by 0.5 mM IPTG at 18°C, 200 rpm for 12 hr and then harvested by centrifugation (4000 g, 30 min at 4°C). The cell pellet was re-suspended in the lysis buffer (25 mM Tris, 50 mM NaCl, 2.5 mM PMSF, pH 7.5) and disrupted by sonication (40% amplitude, 5 sec pulse followed by 10 sec pause, total 2 min). *Gs* $McsB^{E122A}$-His_6 was purified using the standard His-tag purification protocol according to manufacturer's recommendation, followed by SEC chromatograph on a HiLoad 16/600 Superdex 75 pg column equilibrated with 25 mM Tris, 50 mM NaCl, pH 7.5 buffer. Purified *Gs* $McsB^{E122A}$-His_6 was analyzed by SDS-PAGE and the concentration was determined with the Bradford method.

Geobacillus stearothermophilus CtsR-His_6

The plasmid was transformed into *E. coli* BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with ampicillin (50 μg/mL) overnight at 37°C. 200 mL of the media was inoculated with the 2 mL overnight cultures and grown until its O.D.600 reached 0.6. The bacteria was induced by 0.5 mM IPTG at 37°C, 200 rpm for 3 hr and then harvested by centrifugation (4000 g, 30 min at 4°C). The cell pellet was re-suspended in the lysis buffer (25 mM Tris, 300 mM KCl, 2.5 mM PMSF, pH 7.5) and disrupted by sonication (40% amplitude, 5 sec pulse followed by 10 sec pause, total 2 min). *Gs* CtsR-His_6 was purified using the standard His-tag purification protocol according to manufacturer's recommendation. Purified *Gs* CtsR-His_6 was analyzed by SDS-PAGE and the concentration was determined with the Bradford method.

Geobacillus stearothermophilus YwlE-His_6 and YwlE^{C9S}-His_6

Each plasmids were transformed into E. coli BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with ampicillin (50 μg/mL) overnight at 37°C. 200 mL media was inoculated with the 2 mL overnight cultures and grown until its O.D.600 reached 0.8. The bacteria was induced by 0.5 mM IPTG at 37°C, 200 rpm for 3 hr and then harvested by centrifugation (4000 g, 30 min at 4°C). Cell pellet was re-suspended in the B-PER complete with 2.5 mM PMSF and incubated at RT until it became clear. *Gs* YwlE-His_6 and YwlE^{C9S}-His_6 were purified using the standard His-tag purification protocol according to manufacturer's recommendation. Purified *Gs* YwlE-His_6 and YwlE^{C9S}-His_6 were analyzed by SDS-PAGE and the concentration was determined with the Bradford method.

Bacillus subtilis McsB-His_6

The plasmid was transformed into *E. coli* BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with ampicillin (50 μg/mL) overnight at 37°C. 200 mL media was inoculated with the 2 mL overnight cultures and grown until its O.D.600 reached 0.6. The bacteria was induced by 0.5 mM IPTG at 18°C, 200 rpm for 12 hr and then harvested by centrifugation (4000 g, 30 min at 4°C). The cell pellet was re-suspended in the lysis buffer (25 mM Tris, 300 mM KCl, 2.5 mM PMSF, pH 7.5) and disrupted by sonication (40% amplitude, 5 sec pulse followed by 10 sec pause, total 2 min). *Bs* McsB-His_6 was purified using the standard His-tag purification protocol according to manufacturer's recommendation, followed by SEC chromatograph on a HiLoad 16/600 Superdex 75 pg column equilibrated with 25 mM Tris, 300 mM KCl, pH 7.5 buffer. Purified *Bs* McsB-His_6 was analyzed by SDS-PAGE and the concentration was determined with the Bradford method.

Bacillus subtilis McsA-His_6

The plasmid was transformed into *E. coli* BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with ampicillin (50 μg/mL) overnight at 37°C. 200 mL media was inoculated with 2 mL overnight cultures and grown until its O.D.600 reached 0.6. The bacteria was induced by 0.5 mM IPTG at 20°C, 200 rpm for 5 hr and then harvested by centrifugation (4000 g, 30 min at 4°C). Cell pellet was re-suspended in the lysis buffer (25 mM Tris, 300 mM KCl, 1 mM TCEP, 2.5 mM PMSF, pH 7.5) and disrupted by sonication (40% amplitude, 5 sec pulse followed by 10 sec pause, total 2 min). *Bs* McsA-His_6 was purified using standard His-tag purification protocol according to manufacturer's recommendation, followed by SEC chromatograph on a HiLoad 16/600 Superdex 75 pg column equilibrated with 25 mM Tris, 300 mM KCl, 1 mM TCEP, pH 7.5 buffer. Purified *Bs* McsA-His_6 was analyzed by SDS-PAGE and the concentration was determined with the Bradford method.

Bacillus subtilis CtsR-His_6

The plasmid was transformed into E. coli BL21(DE3)-pLys strain. One picked up clone was grown in 5 mL LB media supplemented with ampicillin (50 μg/mL) overnight at 37°C. 200 mL media was inoculated with 2 mL overnight cultures and grown until its O.D.600 reached 0.6. The bacteria was induced by 0.5 mM IPTG at 37°C, 200 rpm for 3 hr and then harvested by centrifugation (4000 g, 30 min, 4°C). Cell pellet was re-suspended in the lysis buffer (25 mM Tris, 300 mM KCl, 2.5 mM PMSF, pH 7.5) and disrupted by sonication (40% amplitude, 5 sec pulse followed by 10 sec pause, total 2 min). *Bs* CtsR-His_6 was purified using the standard His-tag purification protocol according to manufacturer's recommendation. Purified *Bs* CtsR-His_6 was analyzed by SDS-PAGE and the concentration was determined with the Bradford method.

Summary of the recombinant protein overexpression and cell lysis conditions

Table S 13. Recombinant protein overexpression and cell lysis conditions

Protein	Induction condition	Lysis buffer
Gs McsB-His_6	O.D. 600 = 0.8, 0.5 mM IPTG, 3 hr at 37˚C	25 mM HEPES, 300 mM KCl, pH 7.5
Gs $McsB^{E122A}$-His_6	O.D. 600 = 0.6, 0.5 mM IPTG, O/N at 18˚C	25 mM Tris, 50 mM NaCl, pH 7.5
Gs CtsR- His_6	O.D. 600 = 0.6, 0.5 mM IPTG, 3 hr at 37˚C	25 mM Tris, 300 mM KCl, pH 7.5
Gs YwlE-His_6	O.D. 600 = 0.6, 0.5 mM IPTG, 3 hr at 37˚C	B-PER buffer (Fisher Scientific, Catalog no.: 90084)
Gs $YwlE^{C9S}$-His_6	O.D. 600 = 0.6, 0.5 mM IPTG, 3 hr at 37˚C	B-PER buffer (Fisher Scientific, Catalog no.: 90084)
Bs McsB- His_6	O.D. 600 = 0.6, 0.5 mM IPTG, O/N at 18˚C	25 mM Tris, 300 mM KCl, pH 7.5
Bs McsA- His_6	O.D. 600 = 0.6, 0.5 mM IPTG, 5 hr at 25˚C	25 mM Tris, 300 mM KCl, 1 mM TCEP, pH 7.5
Bs CtsR- His_6	O.D. 600 = 0.6, 0.5 mM IPTG, 3 hr at 37˚C	25 mM Tris, 300 mM KCl, pH 7.5

In vitro Phosphorylation of a Synthetic Peptide to Monitor McsB Activity

For the activity test of the recombinant McsB-His_6, a wild-type CtsR peptide was phosphorylated *in vitro* following the literature protocol with slight modifications.[5] The reaction was carried out in 500 μL of 20 mM Tris pH 8.0 buffer with 5 mM $MgCl_2$, 1 mM ATP, 13.5 μM *Gs* McsB-His_6 and 50 μM peptide. All reactions were carried out at 40°C for 1.5 hr. For the dephosphorylation reaction, the reaction mixture was treated with YwlE-His_6 and incubated for an additional 30 min. Reactions were monitored with analytical reverse-phase HPLC (Inertsil C18, 05-70B, 1 mL/min, over 20 min) and newly formed peaks were characterized by matrix-assisted laser desorption/ionization (MALDI) MS using the matrix containing 2',4'-Dihydroxyacetophenone (DHAP) and diammomium citrate (DAC).

Table S 14. HPLC retention time profiles of components

Compounds	Retention time
ATP	2.9 min
ADP	3.6 min
WT-CtsR peptide	10.4 min
Gs McsB-His_6	19.0 min
Gs YwlE-His_6	16.5 min

In the absence of McsB, only the nonphosphorylated peptide was observed. However, with McsB, 50% of the WT-CtsR peptide was phosphorylated within 1.5 hour. When phosphoarginine phosphatase YwlE was added to 50% phosphorylated peptide, the phosphopeptide peak disappeared, suggesting phosphorylation had been on the Arg residue by McsB.

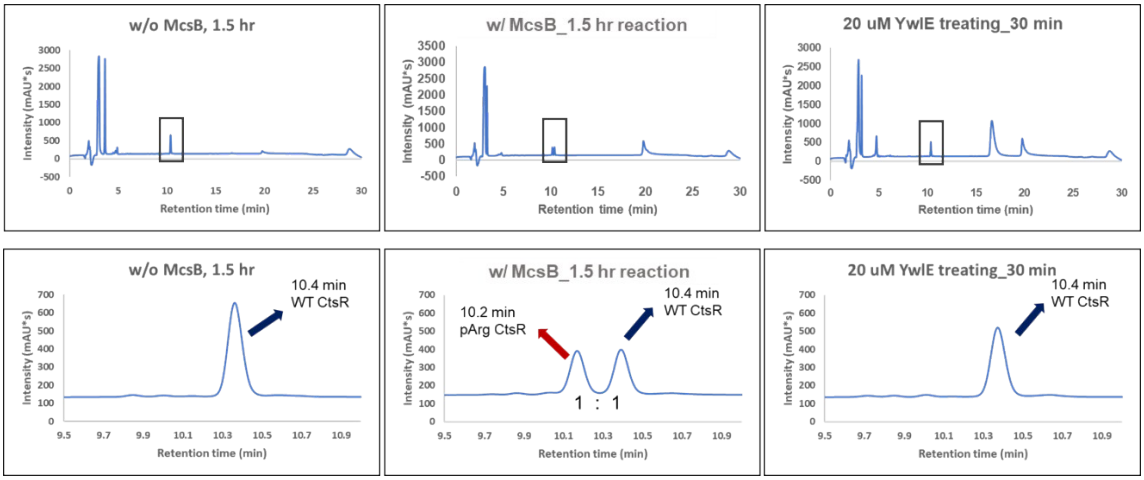


Fig. S 8. *In vitro* phosphorylation of WT-CtsR peptide with recombinant McsB

RP-HPLC, C18, 1 mL/min, 05-70B over 20 min, Sig=254 nm, Ref=360 nm

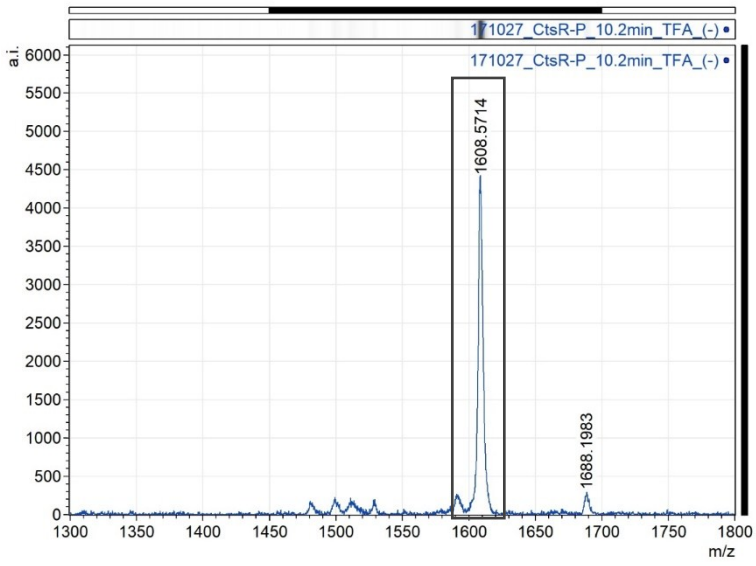


Fig. S 9. MALDI-TOF (Linear negative mode) of 10.2 min peak

[M]= WT CtsR peptide, Calculated [pM-H]⁻: 1609.8585 m/z, Observed [pM-H]⁻: 1608.5714 m/z

Experimental Procedure and Spectroscopic Data

On-Resin Alkylation of Peptides with Sox-Br

Preparation of Sox-Br and Sox alkylation to Cys was carried out following the procedure in the literature[5,6] with slight modifications. The modified alkylation procedure is given below.

The resin with fully protected peptide (20 μmol) was swollen in CH_2Cl_2 (5 × 5 min × 2 mL) with N_2 bubbling and drained. To the resin-bound peptide was added 2 mL of 1.2% trifluoroacetic acid (TFA) and 5% TIPS in CH_2Cl_2 for the selective Mmt deprotection, and the mixture was agitated by bubbling with N_2 for 20 min. Yellow color appeared immediately. The Mmt deprotection was repeated (usually more than 6 times) until no more yellow color was observed. Following the deprotection, the resin was washed (5 × CH_2Cl_2 then 5 × anhydrous DMF). To activate the thiol on Cys, 17.3 μL (100 μmol) of diisopropylethylamine (DIPEA) dissolved in 430 μL of anhydrous DMF was added to the resin. After 2 min, Sox-Br (11.6 mg, 20 μmol) in 210 μL of anhydrous DMF was added to the resin and it was agitated by bubbling with N_2 for 1 hr in the dark. After washing with DMF, Sox-Br (11.6 mg, 20 μmol) in 210 μL of anhydrous DMF was added again for complete alkylation. Following the alkylation, the resin was washed (5 × DMF, 5 × CH_2Cl_2, 5 × MeOH) and subjected to the final cleavage with 95% TFA, 2.5% TIPS and 2.5% water, followed by the purification via semi-preparative HPLC.

Analytical data of WT CtsR and Sox-CtsR(-3) to (+3)

Table S 15. Complete list of synthesized WT-CtsR and Sox-CtsR

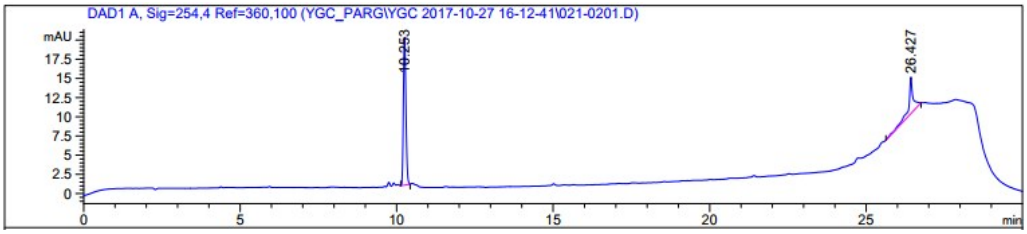
ENTRY	COMPOUND	PEPTIDE SEQUENCE	CHARACTERIZATION DATA (HPLC, MS)
WT	WT-CtsR	Ac-KVIQSKRGGGGYIK-NH_2	HPLC, MALDI-TOF
	WT-CtsRP	Ac-KVIQSKpRGGGGYIK-NH2	MALDI-TOF
(-3)	Sox-CtsR(-3)	Ac-KVI(CSox)SKRGGGGYIK-NH_2	HPLC, ESI-MS (HR-MS)
	Sox-CtsR(-3)P	Ac-KVI(CSox)SKpRGGGGYIK-NH_2	LC-MS/MS (ETD)
(-2)	Sox-CtsR(-2)	Ac-KVIQ(CSox)KRGGGGYIK-NH_2	HPLC, ESI-MS (HR-MS)
	Sox-CtsR(-2)P	Ac-KVIQ(CSox)KpRGGGGYIK-NH_2	LC-MS/MS (ETD)
(-1)	Sox-CtsR(-1)	Ac-KVIQS(CSox)RGGGGYIK-NH_2	HPLC, ESI-MS (HR-MS)
	Sox-CtsR(-1)P	Ac-KVIQS(CSox)pRGGGGYIK-NH_2	LC-MS/MS (ETD)
(+1)	Sox-CtsR(+1)	Ac-KVIQSKR(CSox)GGGYIK-NH_2	HPLC, ESI-MS (HR-MS)
	Sox-CtsR(+1)P	Ac-KVIQSKpR(CSox)GGGYIK-NH_2	LC-MS/MS (ETD)
(+2)	Sox-CtsR(+2)	Ac-KVIQSKRG(CSox)GGYIK-NH_2	HPLC, ESI-MS (HR-MS)
	Sox-CtsR(+2)P	Ac-KVIQSKpRG(CSox)GGYIK-NH_2	HPLC, MALDI-TOF, LC-MS/MS (ETD)
(+3)	Sox-CtsR(+3)	Ac-KVIQSKRGG(CSox)GYIK-NH_2	HPLC, ESI-MS (HR-MS)
	Sox-CtsR(+3)P	Ac-KVIQSKpRGG(CSox)GYIK-NH_2	LC-MS/MS (ETD)

Table S 16. Analytical methods for the CtsR peptides

Analytical method	Details
RP-HPLC	1 mL/min, 5-70% B over 20 min, Sig=254 nm, Ref=360 nm
MALDI-TOF	Reflectron-Positive mode unless otherwise noted, DHAP and DAC
ESI-MS	Direct injection, Positive mode, (Resolution 70000)
LC-MS/MS	Positive, ETD mode

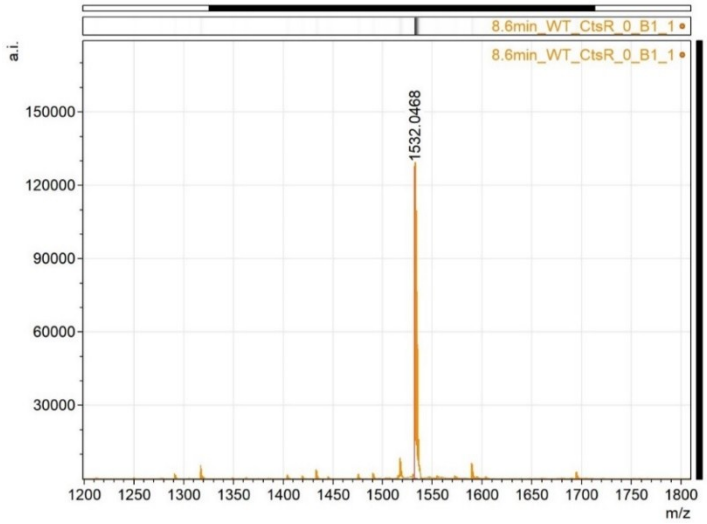
WT-CtsR: Ac-KIVQSKRGGGGYIK-NH_2

HPLC



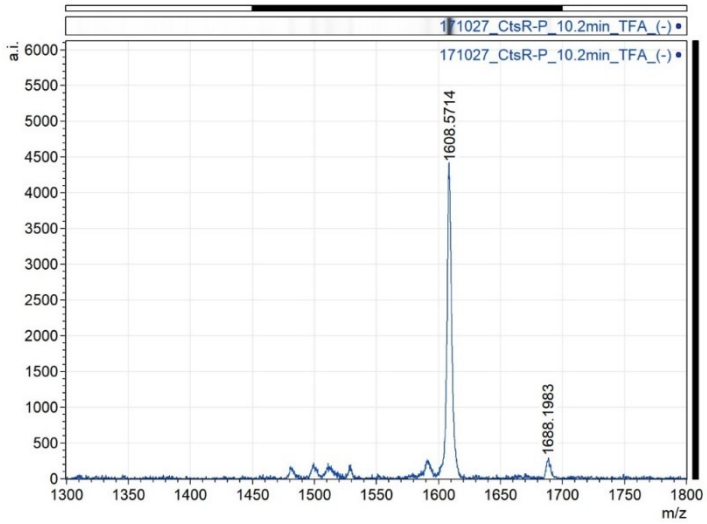
Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	10.253	0.0857	105.15366	19.26990	68.8808
2	26.427	0.1350	47.50678	4.73697	31.1192

MALDI-TOF: Calculated $[M+H]^+$: 1531.9067 m/z, Observed $[M+H]^+$: 1532.0468 m/z



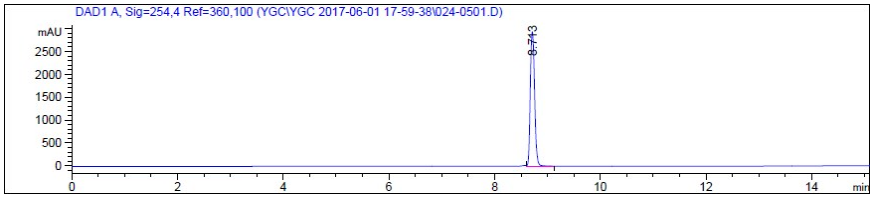
WT-CtsR: Ac-KIVQSKpRGGGGYIK-NH_2

MALDI-TOF (Linear-Negative mode): Calculated $[pM-H]^-$: 1609.8585 m/z, Observed $[pM-H]^-$: 1608.5714 m/z



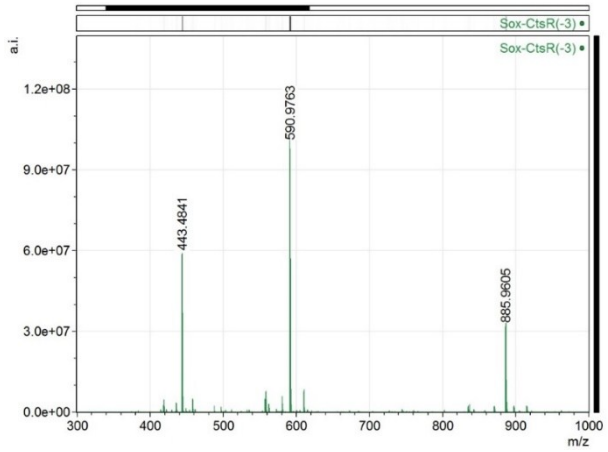
Sox-CtsR(-3): Ac-KIVC(Sox)SKRGGGGYIK-NH_2

HPLC



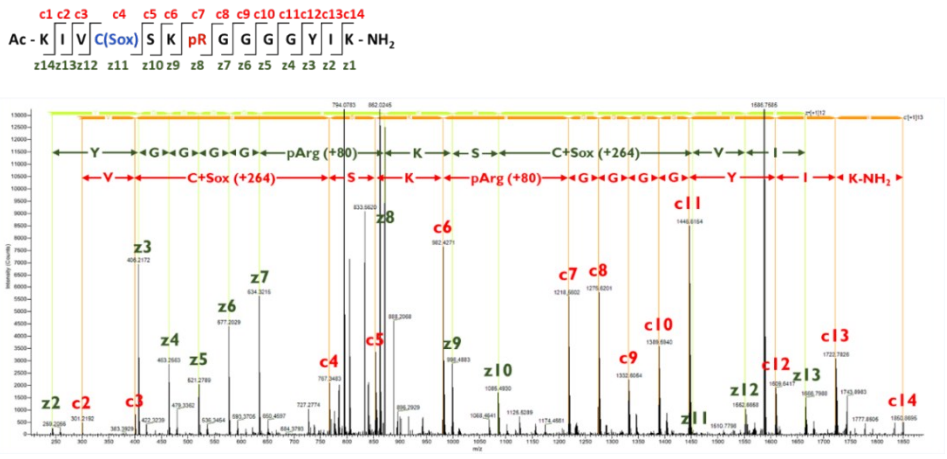
Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.713	0.0922	1.66805e4	2940.28271	100.0000

ESI-MS (HR-MS): a. Calculated $[M+2H]^{+2}$: 885.9607 m/z, Observed $[M+2H]^{+2}$: 885.9605 m/z/ b. Calculated $[M+3H]^{+3}$: 590.9762 m/z, Observed $[M+3H]^{+3}$: 590.9763 m/z/ c. Calculated $[M+4H]^{+4}$: 443.4840 m/z, Observed $[M+4H]^{+4}$: 443.4841 m/z



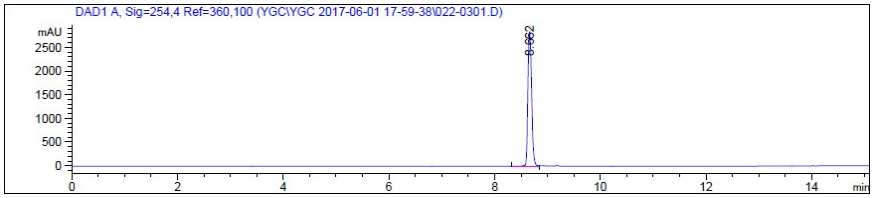
Sox-CtsR(-3)P: Ac-KIVC(Sox)SKpRGGGGYIK-NH_2

LC-MS/MS (ETD)



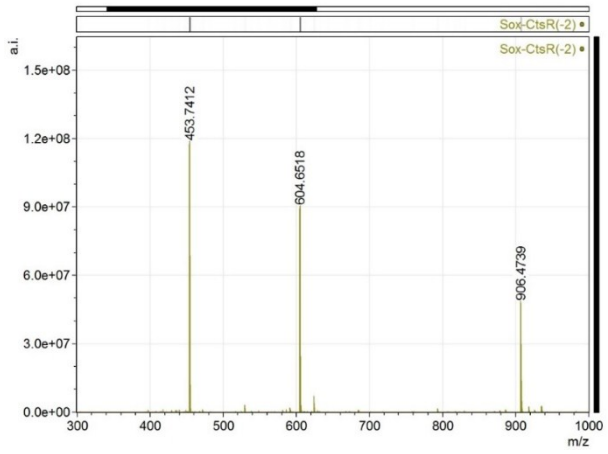
Sox-CtsR(-2): Ac-KIVQC(Sox)KRGGGGYIK-NH_2

HPLC



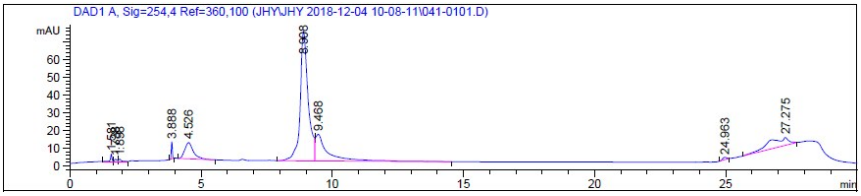
Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.662	0.0769	1.38599e4	2844.07178	100.0000

ESI-MS (HR-MS): a. Calculated $[M+2H]^{+2}$: 906.4740 m/z, Observed $[M+2H]^{+2}$: 906.4739 m/z / b. Calculated $[M+3H]^{+3}$: 604.6518 m/z, Observed $[M+3H]^{+3}$: 604.6518 m/z / c. Calculated $[M+4H]^{+4}$: 453.7406 m/z, Observed $[M+4H]^{+4}$: 453.7412 m/z



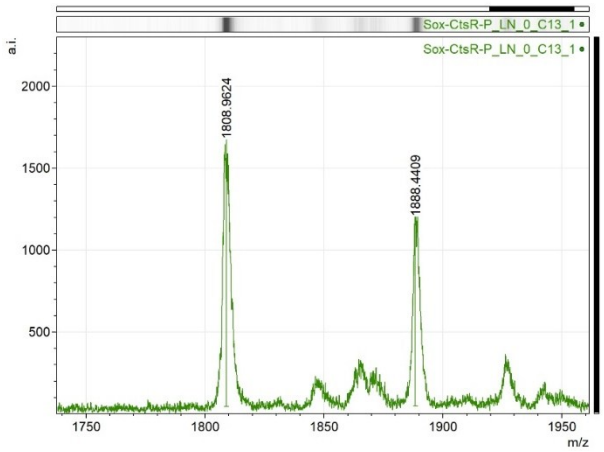
Sox-CtsR(-2)P: Ac-KIVQC(Sox)KpRGGGGYIK-NH_2

HPLC

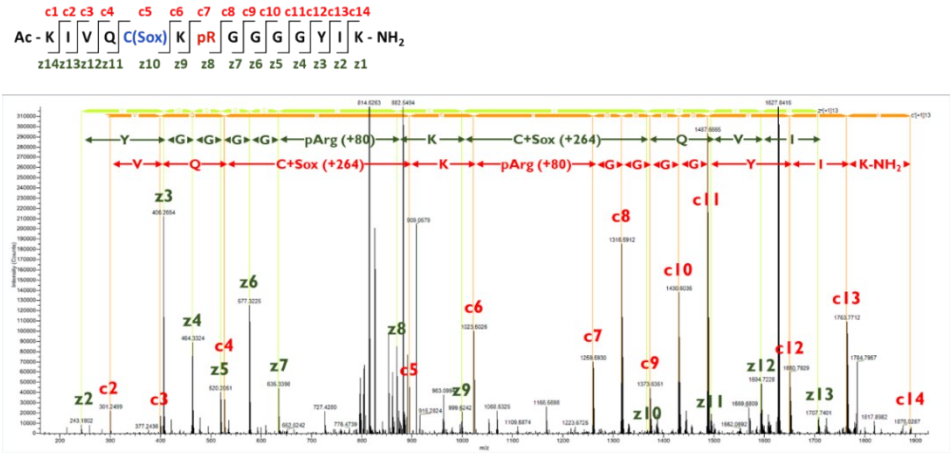


Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.581	0.0614	16.82093	4.14264	0.6449
2	1.738	0.0893	7.84892	1.28475	0.3009
3	1.898	0.1163	11.03064	1.30892	0.4229
4	3.888	0.0469	27.09406	9.60934	1.0387
5	4.526	0.3385	211.37035	9.18468	8.1036
6	8.908	0.3049	1553.35803	73.40982	59.5529
7	9.468	0.4396	500.21930	15.05800	19.1775
8	24.963	0.1717	13.43851	1.26728	0.5152
9	27.275	0.7438	267.18454	4.39140	10.2434

MALDI-TOF (Linear-Negative mode): Calculated $[M-H]^-$: 1809.9262 m/z, Observed $[M-H]^-$: 1808.9624 m/z, Calculated $[pM-H]^-$: 1889.8925 m/z, Observed $[pM-H]^-$: 1888.4409 m/z

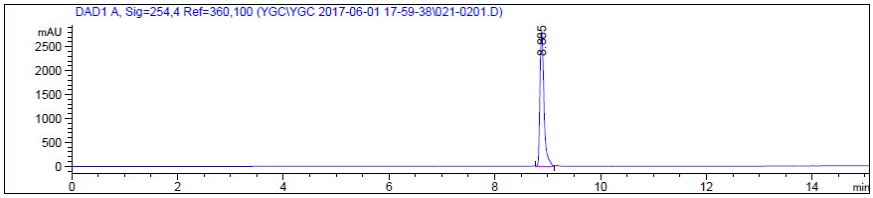


LC-MS/MS (ETD)


c1 c2 c3 c4 c5 c6 c7 c8 c9 c10 c11 c12 c13 c14
Ac - K I V Q C(Sox) K pR G G G G Y I K - NH2
z14 z13 z12 z11 z10 z9 z8 z7 z6 z5 z4 z3 z2 z1
Y G G G G pArg (+80) K C+Sox (+264) Q V I
V Q C+Sox (+264) K pArg (+80) G G G G Y I K-NH2
z3
c8
c11
c10
c13
z6
c6
z4
z8
c4
c7
c12
z5
z7
c5
c9
z12
z2
c2
z9
z11
z13
c3
z10
c14

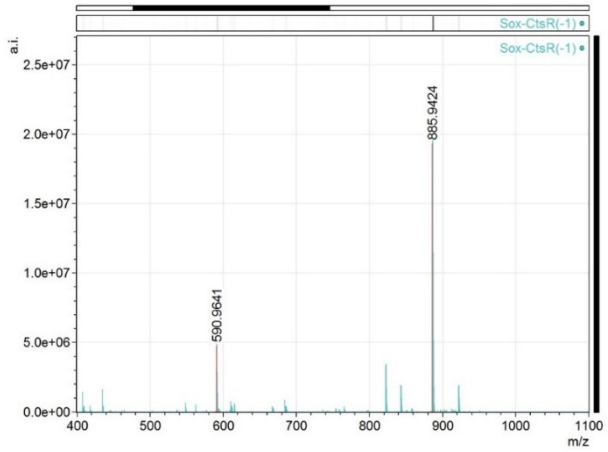
Sox-CtsR(-1): Ac-KIVQSC(Sox)RGGGGYIK-NH_2

HPLC



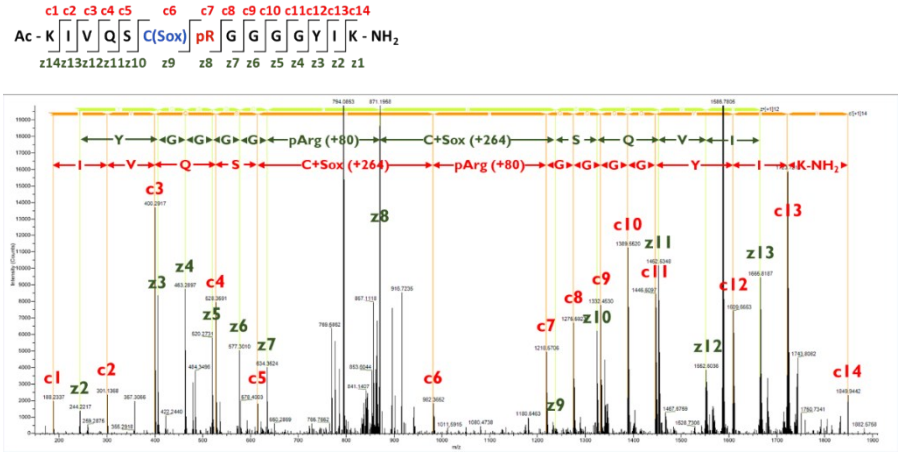
Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.885	0.0816	1.48744e4	2815.06128	100.0000

ESI-MS (HR-MS): a. Calculated $[M+2H]^{+2}$: 885.9425 m/z, Observed $[M+2H]^{+2}$: 885.9424 m/z / b. Calculated $[M+3H]^{+3}$: 590.9641 m/z, Observed $[M+3H]^{+3}$: 590.9641 m/z



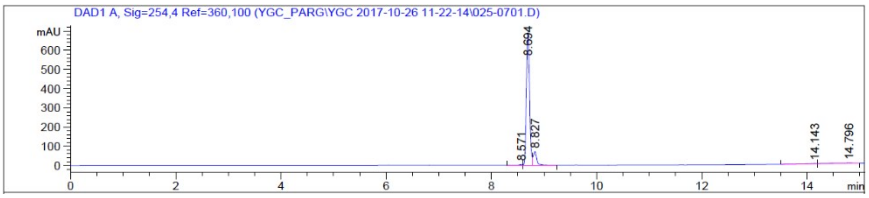
Sox-CtsR(-1)P: Ac-KIVQSC(Sox)pRGGGGYIK-NH_2

LC-MS/MS (ETD)



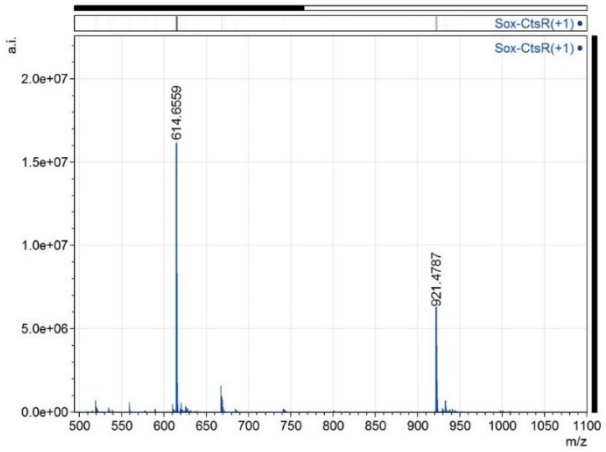
Sox-CtsR(+1): Ac-KIVQSKRC(Sox)GGGYIK-NH_2

HPLC



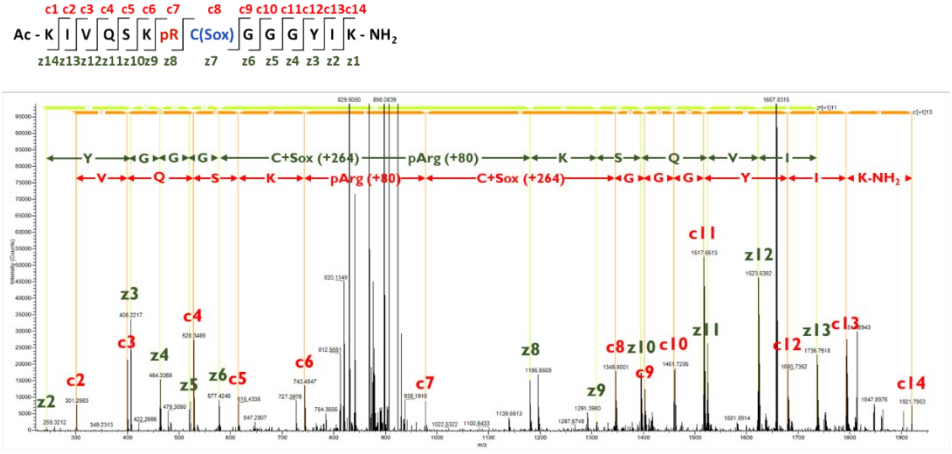
Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.571	0.0621	15.14617	3.67534	0.4646
2	8.694	0.0639	2846.69702	693.35034	87.3151
3	8.827	0.0686	325.24374	72.15897	9.9760
4	14.143	0.1324	16.17807	1.62144	0.4962
5	14.796	0.3543	56.99352	1.97729	1.7481

ESI-MS (HR-MS): a. Calculated $[M+2H]^{+2}$: 921.4793 m/z, Observed $[M+2H]^{+2}$: 921.4787 m/z / b. Calculated $[M+3H]^{+3}$: 614.6553 m/z, Observed $[M+3H]^{+3}$: 614.6559 m/z



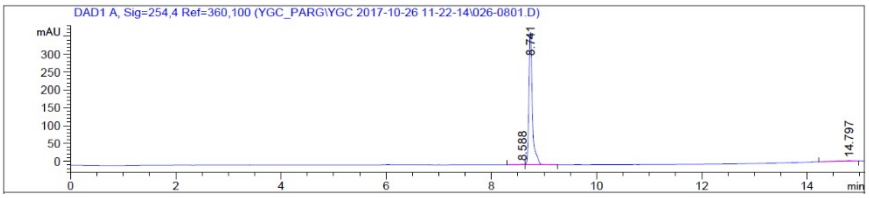
Sox-CtsR(+1)P: Ac-KIVQSKpRC(Sox)GGGYIK-NH_2

LC-MS/MS (ETD)



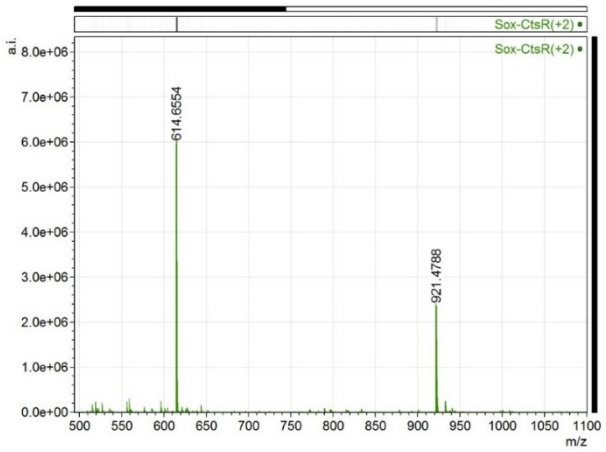
Sox-CtsR(+2): Ac-KIVQSKRGC(Sox)GGYIK-NH_2

HPLC



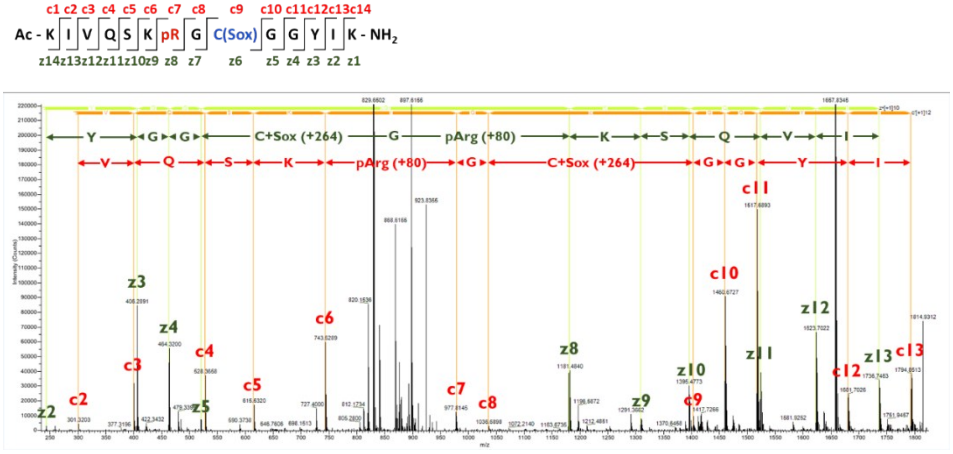
Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.588	0.0706	7.31772	1.56339	0.4193
2	8.741	0.0698	1715.91870	371.89633	98.3145
3	14.797	0.1832	22.09989	1.57982	1.2662

ESI-MS (HR-MS): a. Calculated $[M+2H]^{+2}$: 921.4793 m/z, Observed $[M+2H]^{+2}$: 921.4788 m/z / b. Calculated $[M+3H]^{+3}$: 614.6553 m/z, Observed $[M+3H]^{+3}$: 614.6554 m/z



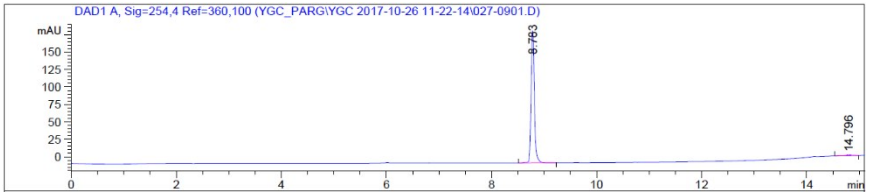
Sox-CtsR(+2)P: Ac-KIVQSKpRGC(Sox)GGYIK-NH_2

LC-MS/MS (ETD)



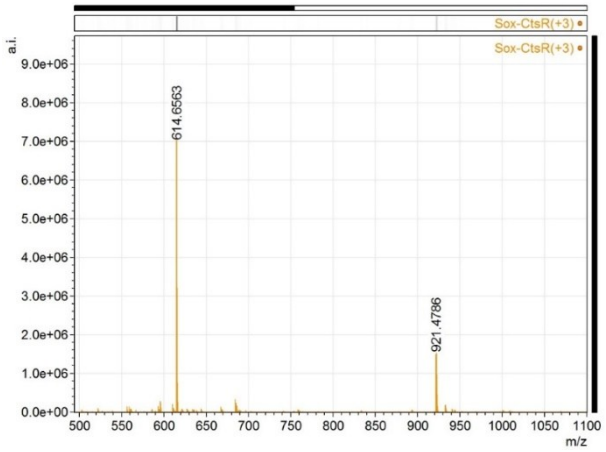
Sox-CtsR(+3): Ac-KIVQSKRGGC(Sox)GYIK-NH_2

HPLC



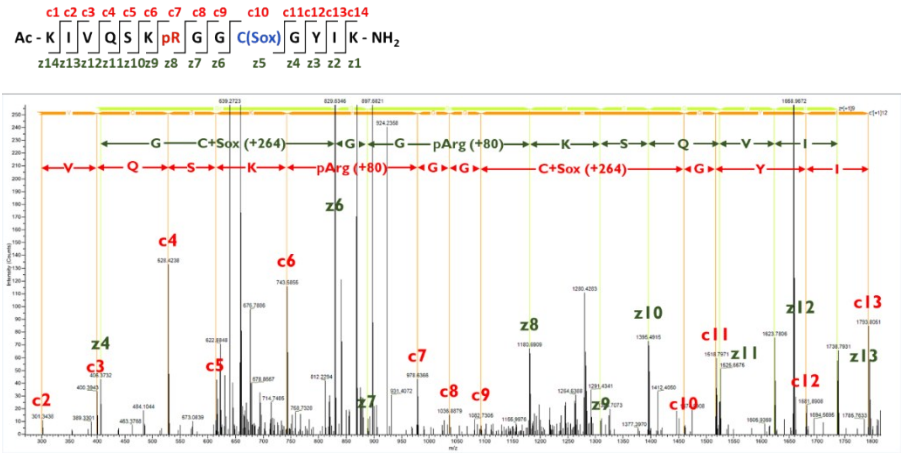
Peak #	RetTime [min]	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.783	0.0656	809.05225	190.27736	98.8952
2	14.796	0.0977	9.03851	1.32335	1.1048

ESI-MS (HR-MS): a. Calculated $[M+2H]^{+2}$: 921.4793 m/z, Observed $[M+2H]^{+2}$: 921.4786 m/z / b. Calculated $[M+3H]^{+3}$: 614.6553 m/z, Observed $[M+3H]^{+3}$: 614.6563 m/z



Sox-CtsR(+3)P: Ac-KIVQSKpRGGC(Sox)GYIK-NH_2

LC-MS/MS (ETD)



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