Supporting Information

A Rapid Method for Generation of Selective Sox-based Chemosensors of Ser/Thr Kinases Using Combinatorial Peptide Libraries

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I. General methods

Unless otherwise noted, all solvents and reagents were obtained commercially and used without further purification. N^{α}-Fmoc-protected amino acids[§] were purchased from Novabiochem. Whenever anhydrous and/or degassed CH_2Cl_2 was necessary it was distilled from calcium hydride and degassed by bubbling argon for at least 20 min. Peptides were purified *via* preparative reverse-phase HPLC employing a gradient of solvents A (H_2O with 0.1% v/v TFA) and B (CH_3CN with 0.1% v/v TFA). Compounds were characterized by ^1H and ^{13}C NMR and mass spectroscopy. Peptide purity was determined by analytical reverse-phase HPLC.

II. Instrumentation

HPLC: HPLC was carried out on Waters Prep LC 4000 System or Waters Delta 600 System equipped with Waters 2487 dual wavelength absorbance detectors. Columns used: C_{18} analytical (flow rate = 1 mL/min), Beckman Ultrasphere ODS, 5 μm, 150 x 4.6 mm; C_{18} preparatory (flow rate = 15 mL/min), YMC-Pack Pro, 5 μm, 250 x 20 mm.
MALDI-TOF MS: PerSeptive Biosystems Voyager MALDI-TOF instrument.
UV-Vis Spectrophotometer: Shimadzu UV-2401PC.
Fluorometer: Fluoromax 3 from Jobin Yvon. Cuvette: Starna Cells (16.100F-Q-10) 100 μL sub-micro cuvette, 1 cm path length.

III. Peptide synthesis

III.1. Peptide library synthesis

Solid-phase peptide synthesis was performed manually by using Fmoc chemistry on Fmoc-PAL-PEG-PS resin (0.19 mmolg^{-1}). The resin was swelled in CH_2Cl_2 (5 min) and then DMF (5 min) prior to synthesis. Fixed positions were introduced by treating the amino acid (3 equiv/equiv resin) with benzatriazole-1-yloxy-tris-pyrrolidino-phosphonium hexafluorophosphate (3 equiv/equiv resin) and diisopropylethylamine (8 equiv/equiv resin) in dimethylformamide (DMF) for one hour at room

[§] Abbreviations: ATP: adenosine triphosphate, Bn: benzyl, tBu: *t*-butyl, Boc: *t*-butoxycarbonyl, BSA: bovine serum albumin, DIEA: diisopropylethylamine, DMF: *N,N*-dimethylformamide, DMSO: dimethyl sulfoxide, DTT: dithiothreitol, EDT: 1,2-ethanedithiol, EDTA: ethylenediaminetetraacetic acid, EGTA: glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, HEPES: 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, HOAt: 7-aza-1-hydroxybenzotriazole, HOBt: 1-hydroxybenzotriazole, HPLC: high performance liquid chromatography, MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry, NMR: nuclear magentic resonance, Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, PKA: protein kinase A, PyBOP: Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, Sox-Br: 2-bromomethyl-8-*t*-butyldiphenylsilyloxy-5-(*N,N*-dimethyl)sulfonamidoquinoline, SPPS: solid-phase peptide synthesis, TFA: trifluoroacetic acid, TIS: triisopropylsilane, UV: Ultra-Violet.

temperature. To install the randomized position, 10 equivalents of an isokinetic mixture of Fmoc-amino acids (Fmoc-amino acid, mole%: Fmoc-Ala-OH, 7.1; Fmoc-Arg(Pbf)-OH, 13.6; Fmoc-Asp(O-*t*-Bu)-OH, 7.3; Fmoc-Gly-OH, 6.1; Fmoc-His(Boc)-OH, 7.3; Fmoc-Lys(Boc)-OH, 13.0; Fmoc-Phe-OH, 5.2; Fmoc-Trp(Boc)-OH, 8.0; Fmoc-Tyr(O-*t*-Bu)-OH, 8.6; Fmoc-Val-OH, 23.7) were preactivated with DICI (diisopropylcarbodiimide, 10 equivalents), and HOBt(1-hydroxybenzotriazole, 10 equivalents) in DMF (3 ml). The coupling was repeated after introducing the randomized position. Standard deprotection conditions were employed (20% piperidine in DMF for 10 minutes t room temperature). At the end of the synthesis the Fmoc group was removed with 20% 4-methylpiperidine in DMF, the resin was rinsed with DMF and exposed to Ac_2O (20 equivlents) and pyridine (20 equivalents) for 30 min. The resin was finally washed with DMF, CH_2Cl_2, MeOH (5 x each) and dried under vacuum. Side chains were deprotected by treatment with 94% trifluoroacetic acid (TFA), 2.5% H_2O, 2.5% triisopropylsilane and 1% of 1,2-ethanedithiol, for 3 h at room temperature.

III.2. Synthesis of Sox-peptides

Selected peptides from the screening were synthesized using the standard Fmoc-based amino acid protection chemistry on Fmoc-PAL-PEG-PS resin (Applied Biosystems, 0.19 mmol/g) using Fmoc-C(Sox[TBDPS])-OH building block. The resin was swelled in CH_2Cl_2 (5 min.) and then DMF (5 min) prior to synthesis. All the amino acids except for Fmoc-C(Sox[TBDPS])-OH were coupled according to the following procedure: Fmoc deprotection (20% 4-methylpiperidine in DMF, 3 x 5 min), rinsing step (DMF, 5 x), coupling step (amino acid/PyBOP/HOBt/DIEA, 6:6:6:6, 0.15 M in DMF, 30-45 min), rinsing step (DMF, 5 x; CH_2Cl_2, 5 x). Fmoc-C(Sox[TBDPS])-OH was coupled in the following manner: amino acid/PyAOP/HOAt/DIEA, 2:2:2:5, 0.15 M in DMF, 2-12 hr. The coupling was repeated if necessary (amino acid/PyAOP/HOAt/DIEA, 1:1:1:3, 0.15 M in DMF, 2-12 hr) as determined by the TNBS test for free amines. It is important to wash the resin rigorously (DMF followed by CH_2Cl_2) to remove excess amino acids before performing any tests for free amines. This is particularly necessary after coupling of Fmoc-C(Sox[TBDPS])-OH due to its deep red color, which does not affect its coupling efficiency. At the end of the synthesis, the Fmoc group was removed with 20% 4-methylpiperidine in DMF (3 x 5 min.) and the resin was rinsed with DMF (5 x). The resin-attached free amines were capped by exposure to Ac_2O (20 equiv.) and pyridine (20 equiv.) in DMF for 30 min. The resin was rinsed with DMF (5 x), CH_2Cl_2 (5 x) and subjected to 20% 4-methylpiperidine in DMF (3 x 5 min.) to remove any Sox aryl esters that might have formed during acetylation. The resin was finally washed with DMF, CH_2Cl_2, MeOH (5 x each) and dried under vacuum. Side chains were deprotected by treatment with 94% trifluoroacetic acid (TFA), 2.5% H_2O, 2.5% triisopropylsilane and 1% of 1,2-ethanedithiol, for 3 h at room temperature.

Peptide Sequence	*Mol. Formula*	*HPLC Ret. Time (min)*[a]	*$[M+xH]^{x+}$ Calc.*	*$[M+H]^+$ found*[b]
Ac-ALRRASL-CSox-AA-$CONH_2$	$C_{56}H_{92}N_{18}O_{16}S_2$	25.8	1336.6	1337.7
Ac-ALRRApSL-CSox-AA-$CONH_2$	$C_{56}H_{93}N_{18}O_{19}PS_2$	24.6	1416.6	1417.8
Ac-ALRRFSL-CSox-AA-$CONH_2$	$C_{62}H_{97}N_{19}O_{15}S_2$	23.4	1412.7	1412.2
Ac-ALRRFpSL-CSox-AA-$CONH_2$	$C_{62}H_{98}N_{19}O_{18}PS_2$	23.9	1492.6	1492.5
Ac-ALRRFSL-CSox-GA-$CONH_2$	$C_{61}H_{95}N_{19}O_{15}S_2$	24.4	1398.6	1398.8
Ac-ALRRFpSL-CSox-GA-$CONH_2$	$C_{61}H_{96}N_{19}O_{18}PS_2$	23.7	1478.6	1478.9
Ac-AGRRYSL-CSox-DAA-$CONH_2$	$C_{58}H_{89}N_{19}O_{16}S_2$	26.7	1372.6	1374.1
Ac-AGRRYpSL-CSox-DAA-$CONH_2$	$C_{58}H_{90}N_{19}O_{19}PS_2$	24.8	1452.5	1453.2

[a]Retention times reported and HPLC conditions were used for the analytical runs. For these results a reverse-phase C_{18} column was used with the flow rate of 1 ml/min (anal.). Solvent A: H_2O with 0.1 % v/v TFA; solvent B: CH_3CN with 0.1 % v/v TFA. The run was started at 5 % B (over 5 min.). increase to 15 % B (over 1 min) and followed by a linear gradient to 45 % B (over 30 min). [b]For all peptides a reverse-phase analytical C_{18} column was used with the flow rate of 1 ml/min. The run was started at 5 % B (over 5 min.) and increased linearly to 95 % B (over 30 min) and was kept at 95 % B (5 min.).

IV. Phosphorylation of the peptide libraries

AurA: 200 μM of the corresponding peptide library were added to a solution of 25 mM Tris, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 0.01 % Triton x100, 1mM ATP, 2.5 mM DTT, 0.5 mM EGTA and 200 ng AurA (Biosource, diluted with 20 mM Tris, 0.05 mM Triton x100, 2 mM DTT, 0.1 mg/mL BSA, 10% glycerol) in a total volume of 100 μL.
PKA: 200 μM of the corresponding peptide library were added to a solution of 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1mM ATP, 1 mM DTT, 0.1 mM EGTA and 100 ng PKA catalytic subunit (catalytic subunit, Calbiochem, diluted with 50 mM Tris–HCl [pH 7.5], 10 mM $MgCl_2$, 1mM DTT, and 0.15 mg/ml BSA) in a total volume of 100 μL.
Five incubation reactions for each peptide library that were stopped at five different times (10 min, 30 min, 1 h, 2 h and 24 h) starting the derivatization reaction.

V. Chemical derivatization reaction

400 μL of a saturated solution of $Ba(OH)_2$ and 20 μL of MEP (Toronto Research) were added to the corresponding phosphorylation reactions. The reaction was carried out for 2h at 37 ºC and the solution was acidified with 20 μL of formic acid, desalted and concentrated using Zip-Tip$_{C18}$ and analyzed by MALDI. The samples were dissolved in 100 μL of 0.1% TFA in water and one alicuot (5 μL) of each sample was taken to be desalted using a Zip-Tip$_{C18}$ and analyzed by MALDI.

VI. Stock solutions

Due to the affinity of the phosphorylated peptides for selected transition metal ions (including Zn^{2+}), by analogy with a previously reported peptide,[3] only reagents of the highest purity and lowest metal content were used to avoid the need to remove metal ion impurities after preparation.

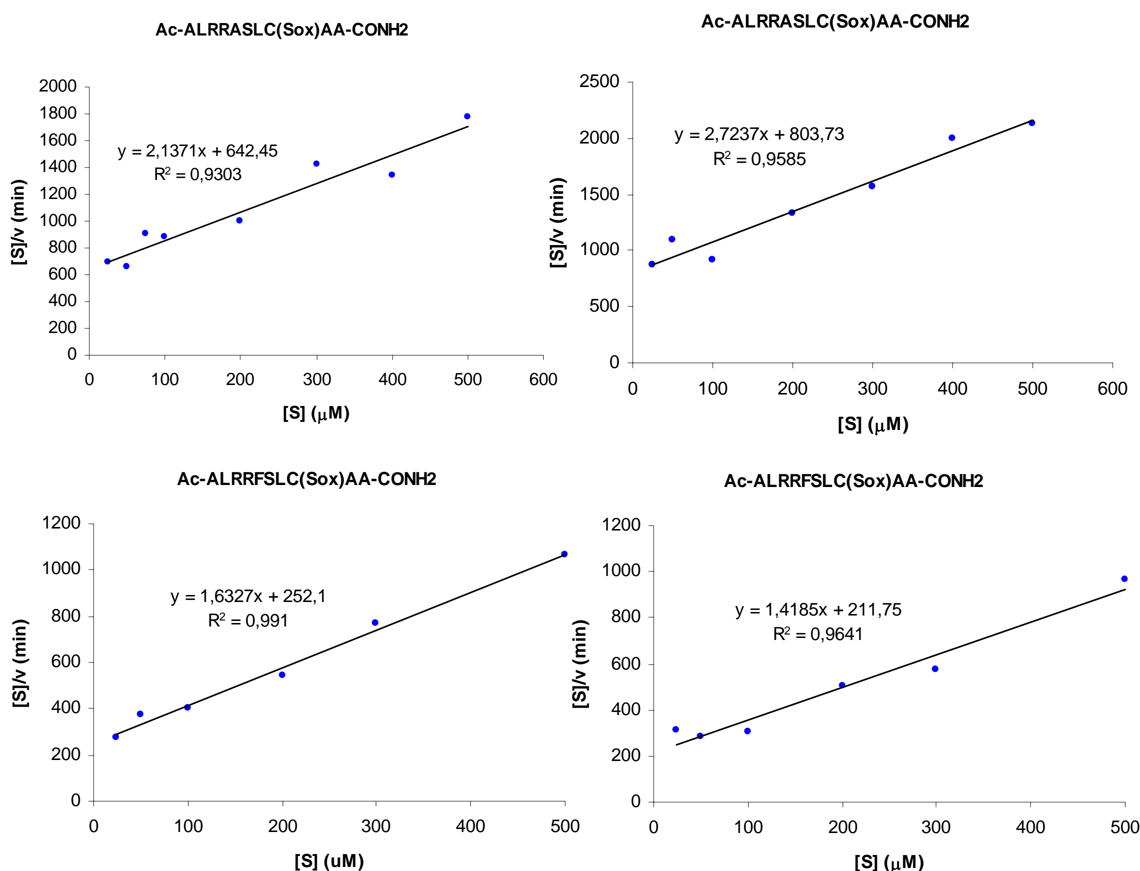
1. Stock solutions of the peptides were prepared in doubly deionized water and concentrations were determined by UV-Vis (based on the determined extinction coefficient of the fluorophore unit, 5-(*N*,*N*-dimethylsulfonamido)-8-hydroxy-2-methylquinoline, ε_{355} = 8247 M^{-1} cm^{-1} at 355 nm in 0.1 M NaOH with 1 mM Na_2EDTA).[3] An average of the values from three separate solutions, each prepared using a different volume of the stock solution, was read on UV-Vis spectrophotometer. Purified peptide stock solutions could be stored at 4 °C for at least 6 months or -20 ^oC for longer periods.
2. A magnesium chloride stock solution of 2.66 M and a calcium chloride stock solution of 354 mM were prepared using Alfa Aesar's Puratronic grade salts. Most commercially available salts contain Zn^{2+} as significant impurities and should not be used due to the high affinity of the phosphorylated peptides for Zn^{2+}. The Mg^{2+} and Ca^{2+} concentrations were determined by titration with a standardized solution of EDTA (Aldrich) in the presence of an Eriochrome Black T (Aldrich) as described previously.[3]
3. 500 mM HEPES (SigmaUltra) was prepared and adjusted to pH 7.4 with NaOH (99.998+%, Aldrich) solution.
4. 10 mM DTT (Biotechnology grade, Mallinckrodt) was prepared in degassed ultrapu re water and stored in aliquots at -80 ^oC.
5. 20 mg/mL BSA (Heat Shock Fraction V, Roche) was prepared in ultrapure water, filtered through a 0.45 micron syringe filter to remove particulates and stored at 4 ^oC.
6. 0.1% Brij-35 P (Fluka) solution was prepared by dissolving an appropriate amount in doubly deionized water and was stored at room temperature.
7. 100 mM ATP (Disodium salt, Low Metals Grade, Calbiochem) was prepared in ultrapure water. The solution was stored in aliquots at -80 ^oC.
8. 500 mM EGTA (SigmaUltra) was prepared in 2 M NaOH and stored at 4 ^oC.
9. Enzyme Dilution Buffer (EDB) was prepared by combining appropriate volumes of above solutions to give the final concentrations of: 20 mM HEPES (pH 7.4), 1 mM DTT, 10 mM $MgCl_2$, 0.3 mM $CaCl_2$,

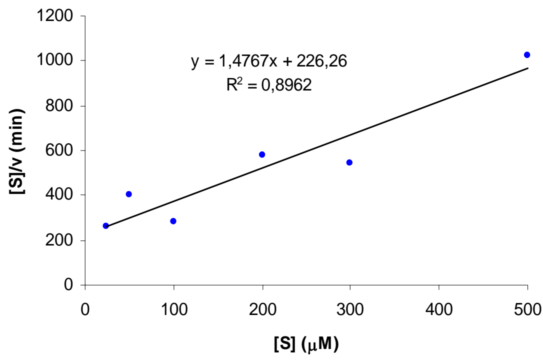
10 mg/mL BSA, 0.01% Brij-35 P.

VII. Fluorescence experiments

Recombinant enzyme was added to begin each reaction. All of the assays to obtain kinetic parameters were performed in the fluorometer (λ_{ex} = 410 nm, λ_{em} = 485 nm). The reactions were carried out using a fluorescence microcuvette (120 μL per reaction) containing varying chemosensor concentrations (generally 0.2–5 K_M) at 30 °C. The Kinetic constants from fluorescence data where determined according to the procedures previously described in our group.[3]

Hanes-Plots




Ac-ALRRFSLC(Sox)AA-CONH2
y = 1,4767x + 226,26
R² = 0,8962
[S]/v (min)
[S] (μM)
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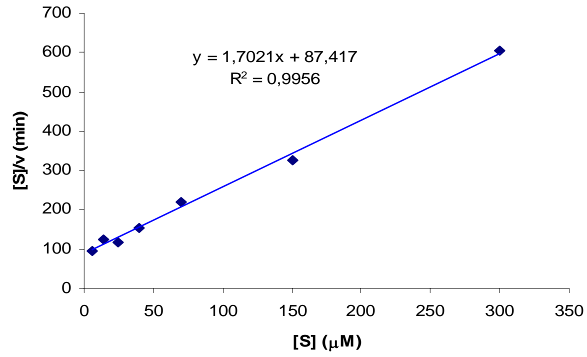
200
400
600
800
1000
1200
0
100
200
300
400
500


Ac-ALRRFSLC(Sox)GA-CONH2
y = 1,7021x + 87,417
R² = 0,9956
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[S] (μM)
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100
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200
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300
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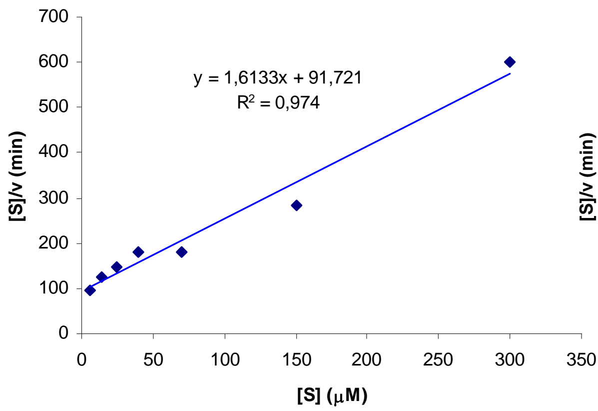
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R² = 0,974
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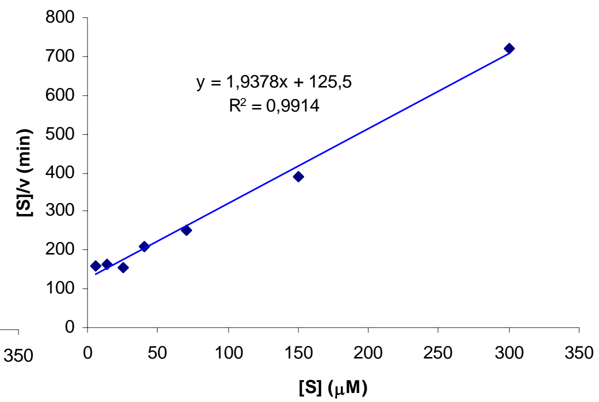
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y = 1,9378x + 125,5
R² = 0,9914
[S]/v (min)
[S] (μM)
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600
700
800
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100
150
200
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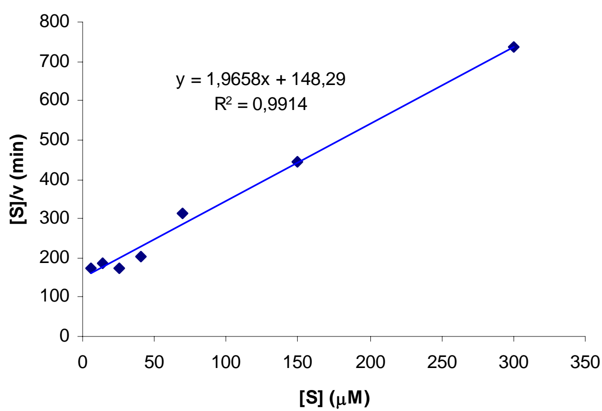
300
350


Ac-AGRRYSLC(Sox)DAA-CONH2
y = 1,9658x + 148,29
R² = 0,9914
[S]/v (min)
[S] (μM)
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200
300
400
500
600
700
800
0
50
100
150
200
250
300
350