Published in final edited form as:

J Org Chem. 2009 October 2; 74(19): 7309–7314. doi:10.1021/jo901369k.

Synthesis of Red-Shifted 8-Hydroxyquinoline Derivatives Using Click Chemistry and Their Incorporation into Phosphorylation Chemosensors

Juan A. González-Vera[†], Elvedin Luković[†], and Barbara Imperiali^{†,‡,*}

[†] Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139

[‡] Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Abstract

Protein phosphorylation is a ubiquitous post-translational modification and protein kinases, the enzymes that catalyze the phosphoryl transfer, are involved in nearly every aspect of normal, as well as aberrant, cell function. Here we describe the synthesis of novel, red-shifted 8-hydroxyquinoline-based fluorophores and their incorporation into peptidyl kinase activity reporters. Replacement of the sulfonamide group of the sulfonamido-oxine (**1**, Sox) chromophore, which has been previously used in kinase sensing, by a 1,4-substituted-triazole moiety, via click chemistry, resulted in a significant bathochromic shift in the fluorescence excitation (15 nm) and emission (40 nm) maxima for the Mg^{2+} chelate. Furthermore, when a click derivative was incorporated into a chemosensor for MK2, the kinase accepted the new substrate as efficiently as the previously reported Sox-based sensor. Taken together, these results extend the utility range of kinase sensors that are based on chelation-enhanced fluorescence (CHEF).

Introduction

The ubiquitous process of protein phosphorylation is central to signal transduction and regulation in all living organisms. By catalyzing transfer of the γ -phosphoryl group of ATP to

imper@mit.edu.

Supporting Information Available: Experimental procedures, characterization of chromophores and peptides, fluorescence spectra, and enzyme assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

the side chains of serine, threonine, and/or tyrosine, protein kinases play an important role in regulating many aspects of cellular function in eukaryotes, including proliferation, cell cycle, metabolism, transcription, and apoptosis.¹ Since many protein kinases are associated with a wide variety of diseases, from cancer to inflammation,^{2–6} they have also emerged as attractive targets for drug discovery. Thus, tools that allow for facile monitoring of kinase activity are in great demand in both pharmaceutical and academic settings. Recently, our laboratory reported versatile peptide sensors for the continuous fluorescence-based assays of Ser/Thr and Tyr kinase activities that utilized the sulfonamido-oxine (Sox) chromophore, which was introduced into peptides either as the Sox^{7,8} or C-Sox⁹ residue (Figure 1). The Sox-containing peptide substrates show low fluorescence, but upon phosphorylation the chromophore binds to Mg²⁺ with greater avidity and undergoes chelation-enhanced fluorescence (CHEF). Such probes have been successfully used to monitor various kinases both with recombinant enzymes and in crude cell lysates.¹⁰ To expand the scope of these sensors in order to visualize kinase activities in living cells, the photophysical properties of the chemosensor should be improved by modulating the excitation and the emission wavelengths of the quinoline reporter to longer, lower energy wavelengths. The use of lower energy to irradiate such fluorophores would minimize photodamage to biological systems, and would mitigate problems with high background signals that commonly complicate *in cellulo* studies. Finally, the addition of another distinctly colored chromophore could allow simultaneous visualization of activities among multiple kinases.

Toward that goal, herein we report studies on the chemical modification of the 8-hydroxyquinoline (Oxn) moiety in order to develop it as a building block for the assembly of new phosphorylation sensors. In particular, shifts in fluorescence excitation (15 nm) and emission wavelengths (40 nm) are reported through the substitution of *p*-bromophenyl triazole into the 5 position of Oxn (Clk). The Clk fluorophore was then incorporated into peptidyl kinase substrates through the cysteine residue side chain (C-Clk). Moreover, the C-Clk-based chemosensor for MK2 was shown to be an efficient reporter of MK2 activity when compared to the existing C-Sox-based probe.

Results and Discussion

Synthesis and Screening of Oxn Derivatives

In the first phase of studies, a selection of substituted hydroxyquinoline derivatives related to the original sulfonamide derivative (**1**), but bearing different acceptor groups at position 5 (**2–7**) were prepared using previously described methods (see the Supporting Information). These analogs were screened for fluorescence in the presence of excess Mg²⁺ and under buffered aqueous conditions.

Table 1 lists key spectroscopic data for compounds **1–7**. Notably, the excitation and emission wavelengths of the magnesium-bound complexes are similar to those of the original Sox derivative (**1**). However, the quantum yields, measured by standard methods,¹¹ are poorer. Indeed, only the aldehyde-substituted chromophore (**2**) showed an improved quantum yield. Nonetheless, this derivative was not appropriate for further studies because it exhibited a shorter λ_{em} , and the aldehyde group can be easily oxidized in biological systems. In light of these findings, we focused on 8-hydroxyquinoline derivatives with substitution at position 5 that extended the conjugation of the quinoline ring system. In the extended aromatic systems the π - π^* gap would be reduced,¹² thereby resulting in longer excitation and emission wavelength maxima. Since the triazole ring is a versatile and readily installed linkage that has been used to extend the conjugation of diverse aromatic systems,^{13,14} we envisioned that installation of this moiety into the Oxn core could potentially provide red-shifted derivatives. The azide substitution at position 5 of the hydroxyquinoline would afford a non-fluorescent

intermediate that could be readily subject to 1,3-dipolar cycloaddition with a variety of terminal alkynes using the Cu(I)-catalyzed Huisgen reaction.¹⁵

For this purpose azide **8** was prepared using previously reported methods (Scheme 1).¹⁶ As expected, **8** showed no fluorescence due to the quenching effect of the electron-rich azido group.¹³ In the presence of catalytic Cu(I) and ascorbic acid **8** reacts readily at room temperature with 1-ethynylcyclohexene (**9a**) in DMF/4-methylpiperidine (8:2) to afford the cycloaddition product **10a** in excellent yield. In the presence of 10 mM MgCl₂ quinoline **10a** has a λ_{ex} of 371 nm and a λ_{em} of 522 nm due to the elimination of azide quenching after formation of the triazole ring. Despite the poor quantum yield of **10a** ($\Phi = 0.033$), this initial result in the shift of the emission wavelength encouraged us to screen additional triazole-substituted quinolines by investigating the spectroscopic properties of products from the 1,3-dipolar cycloaddition reaction of **8** with 21 additional alkynes (**9b-v**).

The cycloaddition reactions were generally complete in 12 h at room temperature and were monitored by TLC and mass spectrometry. The formation of the fluorescent triazole compounds could be easily established upon exposure to a hand-held UV lamp. The fluorophores were then qualitatively compared in a 96-well plate format on a transilluminator ($\lambda_{\text{ex, max}} = 365$ nm) to identify promising compounds (Figure 2) and were further subjected to quantitative analysis in a fluorescence plate reader (Table 2 and the Supporting Information). The excitation and emission wavelengths of the magnesium-bound triazole products (**10b-v**, $\lambda_{\text{ex}} = 360\text{--}375$ nm, $\lambda_{\text{em}} = 510\text{--}530$ nm) were improved compared to those for Sox (**1**, $\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 485$ nm, $\Phi = 0.342$, $\epsilon_{355} = 8,247$ cm⁻¹ M⁻¹). Based on the preliminary screening, selected targets were then synthesized in larger quantities and the quantum yields of the corresponding hydroxyquinoline derivatives were determined (Table 2). Compared to **10a** ($\Phi = 0.033$) a 3.5-fold improvement in quantum yield was obtained in the case of the bromide **10u** ($\lambda_{\text{ex}} = 375$ nm, $\lambda_{\text{em}} = 525$ nm, $\Phi = 0.111$, $\epsilon_{355} = 7,905$ cm⁻¹ M⁻¹). In view of the fluorescent properties of this derivative, we chose to use it as a chelation-sensitive fluorophore to prepare peptide-based probes for the MAPK-activated protein kinase-2 (MK2),¹⁷ and sarcoma kinase (Src),^{18–20} used as models of Ser/Thr and Tyr kinases, respectively. For the purposes of comparison, the analogous fluorescent peptides containing the original Sox chromophore were also prepared as previously described.⁹

Synthesis and Evaluation of Clk-based Peptidyl Kinase Substrates

The synthesis of the probes, outlined in Scheme 2, was performed using a strategy similar to that used for the preparation of the Sox-based recognition-domain focused (RDF) chemosensors.⁹ Diazotization of 5-amino-2-methylquinolin-8-ol (**12**, prepared from 8-hydroxyquinaldine using literature methods)²¹ followed by treatment of the diazonium salt with NaN₃ gave the corresponding azide **13** (66% yield). Protection of the phenolic hydroxyl group as a *tert*-butyldiphenylsilyl ether produced **14** (98 %), which was then brominated under free radical conditions to afford the bromide **15** (30%). To avoid dibromination, the reaction was stopped after 20 min, thereby providing a mixture of the desired product (**15**) and the starting material (**14**), which could not be separated using standard chromatographic methods and was used in the next step without purification. Fmoc-based solid phase peptide synthesis (SPPS) was utilized to assemble the intact peptide that included an appropriately placed cysteine residue protected with monomethoxytrityl (Mmt), which is a hyper acid-labile protecting group (Scheme 2). After selective on-resin sulfhydryl deprotection, the free thiol was alkylated with **15**. Then a 1,3-dipolar cycloaddition reaction with 1-bromo-4-ethynylbenzene in the presence of catalytic Cu(I) gave the corresponding triazole-substituted peptide. Standard TFA cleavage from the resin and concomitant removal of all side-chain protecting groups revealed the desired chemosensor with excellent conversion to the final product (> 95%).

Table 3 shows the substrate sequences of the Sox- and click (Clk)-based RDF probes for MK2 and Src kinases, as well as the fluorescence increases that were observed with the corresponding phosphopeptides. The difference in fluorescence was determined by comparison of the fluorescence intensity at the maximum emission wavelength (485 nm for Sox and 525 nm for Clk) of phosphorylated and unphosphorylated peptides in the presence of Mg^{2+} . The fluorescence increases of the click peptides (2- to 2.5-fold) are significant changes that are useful in enzymatic assays (*vide infra*). More specifically, the click-based RDF peptides exhibited larger fluorescence increases than the Sox-based RDF peptides in the case of Src (entries 3 and 4) while this trend is reversed in the case of MK2 (entries 1 and 2).

As an example, Figure 3a shows a comparison between the fluorescence spectrum of the synthetically-obtained phosphopeptide MK2(Sox) [**P1**] and that from the analogous MK2(Clk) [**P2**]. In the case of the Src sensors the result was the same (see the Supporting Information). Neither species was fluorescent at pH 7.0 (50 mM HEPES, 150 mM NaCl) in the absence of the metal ion. Upon addition of Mg^{2+} the fluorescence spectra of Mg^{2+} -bound **P1** and Mg^{2+} -bound **P2** exhibited emission maxima of 485 and 525 nm, respectively, indicating a red-shift of 40 nm. In contrast, in the corresponding excitation spectra the Mg^{2+} -bound **P1** and **P2** reached maxima at 364 and 368 nm, respectively representing a slightly decreased bathochromic shift relative to that observed with the parent chromophores. The change in absorption maxima (and thus the excitation spectra) is most likely due to the difference in coordination spheres. For the chromophore this would be a 1:1 or 2:1 chelate with Mg^{2+} ; in contrast, phosphopeptides bearing the chromophore would exclusively form 1:1 complex, engaging the quinoline and phosphate groups with Mg^{2+} .

Evaluation of Clk-based Substrates in Enzymatic Assays

Having validated the utility of the new fluorophore through photophysical characterization, we also evaluated its efficacy in kinase reactions. Following established protocols,⁹ Sox- and Clk-based substrates (entries 1 and 2, respectively, in Table 3) were subjected to MK2 under identical conditions and then the overall turnover of each substrate was compared. As shown in Figure 3b, MK2 phosphorylated the Clk-substrate just as efficiently as the Sox-substrate indicating that the size of Clk chromophore does not adversely influence reaction kinetics. Due to previous work, which showed that Sox-based substrates had at least comparable, if not better kinetics than parent peptides, we believe that the Clk-based reporters will also follow the same trend for Src and other kinases. However, kinase substrate kinetics are highly empirical and, thus, new substrates will have to be experimentally evaluated, and can be improved using high-throughput, mass spectrometry-based methods.²²

Conclusions

In conclusion, this report presents the synthesis, peptide incorporation and characterization of new phosphorylation chemosensors that contain 1,4-triazole-substituted 8-hydroxyquinolines and exhibit improved fluorescence properties compared to Sox. This modification results in significant red-shifts in the excitation (15 nm) and emission maxima (40 nm) of the chromophore when complexed to Mg^{2+} . Although, the Clk fluorophore exhibits a somewhat decreased excitation shifts when incorporated into a peptide to monitor kinase activity, the chromophore does not inhibit the ability of MK2 to recognize and efficiently phosphorylate the Clk-bearing probe. Together, these results effectively expand the potential capabilities of the hydroxyquinoline-based kinase sensing strategy.

Experimental Section

Synthesis of the Triazolyl Derivatives **10a-v**

5-Azido-8-hydroxy-2-methylquinoline (**13**, 50 mg, 0.26 mmol) and the corresponding alkyne (**9a-v**) (0.26 mmol) were suspended in a 8:2 mixture of DMF/4-methylpiperidine (2 mL). Ascorbic acid (7.1 mg, 0.04 mmol) and copper iodide (2.5 mg, 0.01 mmol) were added suspended in a 8:2 mixture of dimethylformamide/4-methylpiperidine (1 mL) and the heterogeneous mixture was stirred vigorously overnight in the dark at room temperature. TLC analysis indicated complete consumption of the reactants in 12 h. The mixture was dissolved in ethyl acetate (40 mL), was washed with H₂O (5 mL), brine (5 mL), dried over Na₂SO₄, and evaporated to yield the corresponding triazolyl derivatives **10a-v**.

Peptide Synthesis

All peptides were synthesized using the standard Fmoc-based amino acid protection chemistry. Peptides were synthesized on Fmoc-PAL-PEG-PS resin (Applied Biosystems, 0.19 mmol/g) using on resin alkylation. The resin was swelled in CH₂Cl₂ (5 min.) and then DMF (5 min) prior to synthesis. All the amino acids were coupled according to the following procedure: Fmoc deprotection (20% 4-methylpiperidine in DMF, 3 × 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; CH₂Cl₂, 5 x). The coupling was repeated if necessary as determined by the TNBS test. At the end of the synthesis, the Fmoc group was removed with 20% 4-methylpiperidine in DMF (3 × 5 min.) and the resin was rinsed with DMF (5 x). The resin-attached free amines were capped by exposure to Ac₂O (20 equiv.) and pyridine (20 equiv.) in DMF for 30 min. The resin was rinsed with DMF (5 x), CH₂Cl₂ (5 x) and subjected to 20% 4-methylpiperidine in DMF (3 × 5 min.). The resin was finally washed with DMF, CH₂Cl₂, MeOH (5 x each) and dried under vacuum.

On-resin Alkylation of Peptides with **15**

Resin-attached peptides (50 mg, 0.0095 mmol, 1 equiv.) incorporating Cys(Mmt) were swelled in CH₂Cl₂, then DMF. The Mmt protecting group was removed from the resin-bound peptide by bubbling N₂ through a solution of 1% TFA, 5% TIS in CH₂Cl₂ (4 × 20 min). The resin was washed with CH₂Cl₂ (5 x) and DMF (5 x). Anhydrous DMF (200 µL) was added to the resin followed by freshly distilled tetramethylguanidine (5.96 µL, 0.0475 mmol, 5 equiv.). The mixture was incubated for 2–3 min. **15** (17 mg, 0.0285 mmol, 3 equiv.) was dissolved in anhydrous DMF (150 µL) and added to the resin. After ca. 12 hours of reaction time, the excess reagents were drained and the resin washed with DMF, CH₂Cl₂, MeOH, CH₂Cl₂ (5 x).

On-resin Click Chemistry of Peptides with **9u**

Resin-attached peptides (50 mg, 0.0095 mmol, 1 equiv.) incorporating 5-Azido-8-Hydroxyquinoline were swelled in CH₂Cl₂, then DMF (5 min). A mixture of 1-bromo-4-ethynylbenzene (**9u**; 34.4 mg, 0.19 mmol), ascorbic acid (0.75 mg, 0.0043 mmol) and copper iodide (0.27 mg, 0.0014 mmol) was added to the resin suspended in a 8:2 mixture of DMF/4-methylpiperidine (1.5 mL). After ca. 12 hours of reaction time, the excess reagents were drained and the resin washed with DMF, CH₂Cl₂, MeOH, CH₂Cl₂ (5x). The resin cleavage and protecting group removal was achieved by exposing the resin-bound peptides to TFA/H₂O/TIS (95:2.5:2.5% v/v). The resulting solution was concentrated under a stream of N₂ and precipitated by addition of cold Et₂O. The pellet was triturated with cold Et₂O, redissolved in water, filtered and lyophilized. The peptides were purified by preparative reverse-phase HPLC using UV detection at 228 nm (amide bond absorption) and 316 nm (8-hydroxyquinoline absorption). Only fractions showing a single peak of correct mass by analytical HPLC were used in further experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the NIH Cell Migration Consortium (GM064346) and the A. M. Escudero Foundation postdoctoral fellowship for J.A.G-V. We also thank the Department of Chemistry Instrumentation Facility (NIH-1S10RR013886-01).

References

1. Hunter T. Cell 2000;100:113–127. [PubMed: 10647936]
2. Adams JA. Chem Rev 2001;101:2271–2290. [PubMed: 11749373]
3. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. Science 2002;298:1912–1934. [PubMed: 12471243]
4. Skorski T. Nat Rev Cancer 2002;2:351–360. [PubMed: 12044011]
5. Johnson LN, Lewis RJ. Chem Rev 2001;101:2209–2242. [PubMed: 11749371]
6. Yarden Y, Sliwkowski MX. Nat Rev Mol Cell Biol 2001;2:127–137. [PubMed: 11252954]
7. Shults MD, Imperiali B. J Am Chem Soc 2003;125:14248–14249. [PubMed: 14624552]
8. Shults MD, Carrico-Moniz D, Imperiali B. Anal Biochem 2006;352:198–207. [PubMed: 16600168]
9. Lukovic E, Gonzalez-Vera JA, Imperiali B. J Am Chem Soc 2008;130:12821–12827. [PubMed: 18759402]
10. Shults MD, Janes KA, Lauffenburger DA, Imperiali B. Nat Methods 2005;2:277–283. [PubMed: 15782220]
11. Montes VA, Pohl R, Shinar J, Anzenbacher P Jr. Chem Eur J 2006;12:4523–4535.
12. Ghedini M, La Deda M, Aiello L, Grisolia A. Inorg Chim Acta 2004;357:33–40.
13. Sivakumar K, Xie F, Cash BM, Long S, Barnhill HN, Wang Q. Org Lett 2004;6:4603–4606. [PubMed: 15548086]
14. Sawa M, Hsu TL, Itoh T, Sugiyama M, Hanson SR, Vogt PK, Wong CH. Proc Natl Acad Sci USA 2006;103:12371–12376. [PubMed: 16895981]
15. Kolb HC, Finn MG, Sharpless KB. Angew Chem Int Ed 2001;40:2004–2021.
16. Dunkin IR, ElAyeb AA, Gallivan SL, Lynch MA. J Chem Soc, Perkin Trans 2;1997:1419–1427.
17. Roux PP, Blenis J. Microbiol Mol Biol Rev 2004;68:320–44. [PubMed: 15187187]
18. Martin GS. Nat Rev Mol Cell Biol 2001;2:467–475. [PubMed: 11389470]
19. Frame MC. Biochim Biophys Acta 2002;1602:114–130. [PubMed: 12020799]
20. Schlessinger J. Cell 2000;100:293–296. [PubMed: 10676810]
21. Gershon H, McNeil MW. J Heterocycl Chem 1972;9:659–667.
22. Gonzalez-Vera JA, Lukovic E, Imperiali B. Bioorg Med Chem Lett 2009;19:1258–1260. [PubMed: 19157873]

Figure 1.
Derivatives of 8-hydroxyquinoline-based amino acids used to report kinase activity through CHEF.

Figure 2.

Qualitative comparison of emission wavelengths of **10a-v**. The colors shown here do not represent the true fluorescent wavelengths (shown in the Supporting Information). The fluorophores (10 μ M) were illuminated ($\lambda_{\text{ex}} = 365$ nm) in 50 mM HEPES (pH 7.4), 15% DMSO, 150 mM NaCl, 10 mM MgCl_2 at 25 $^{\circ}\text{C}$.

Figure 3.

Spectral characterization and enzymatic evaluation of Clk-based substrates. (a) Fluorescence excitation and emission spectra of **P1** (--) and **P2** (—) with Mg^{2+} . Samples were prepared in 50 mM HEPES (pH 7.4) and 150 mM NaCl. Spectra were acquired at 25 °C and were baseline-corrected using a sample of the buffer solution. (b) Percentage of turnover of the Clk-based (■, entry 2) or Sox-based (□, entry 1) substrate with MK2 after 10 min. Assays were performed in 20 mM HEPES (pH 7.4), 10 mM MgCl_2 , 0.1 mM EGTA, 0.01% Brij 35, 0.1 mg/mL BSA, 1 mM DTT, 1 mM ATP, 5 μM substrate and 10 ng MK2 at 30 °C. Plotted values indicate the mean $\pm$ s.e.m. for triplicate measurements.

Scheme 1.
1,3-Dipolar Cycloaddition Reactions of **8** with **9a-v**

Scheme 2.
Synthesis of the RDF Chemosensors

Table 1
Relevant Spectroscopic Data for Oxn Derivatives that Form Fluorescent Complexes with Mg^{2+}

Complex	R ¹	R ²	λ_{ex} (nm)	λ_{em} (nm)	$\Phi^{a,b}$
1^c	SO ₂ N(CH ₃) ₂	CH ₃	360	485	0.342
2	CHO	CH ₃	373	460	0.597
3	CN	CH ₃	362	485	0.276
4	COCH ₃	CH ₃	370	465	0.168
5	COCH ₂ Cl	CH ₃	373	460	0.034
6	CO ₂ H	CH ₃	362	508	0.004
7	CO-Ph	H	375	460	0.002

^a Spectra were acquired in 150 mM NaCl, 50 mM HEPES (pH 7.4), 25 °C with 10 μ M **1–7** and 10 mM MgCl₂.

^b Excitation of all species is provided at λ_{max} (355–425 nm). Quantum yields were calculated with reference to a quinine sulphate standard (in 0.05 M H₂SO₄).

^c Extinction coefficient: $\epsilon_{355} = 8,247 \text{ cm}^{-1} \text{ M}^{-1}$.

Table 2

Fluorescence Data for Magnesium Chelates of Selected Triazole-Substituted Oxn Derivatives

Complex	λ_{ex} (nm)	λ_{em} (nm)	$\Phi^{a,b}$
10a	371	522	0.033
10b	370	525	0.041
10c	367	523	0.043
10d	365	525	0.071
10i	375	525	0.067
10u^c	375	525	0.111

^a Spectra were acquired in 150 mM NaCl, 50 mM HEPES (pH 7.4), 25 °C with 10 μM **10** and 10 mM MgCl_2 .

^b Excitation of all species is provided at λ_{max} (355–425 nm). Quantum yields were calculated with reference to a quinine sulphate standard (in 0.05 M H_2SO_4).

^c Extinction coefficient: $\epsilon_{355} = 7905 \text{ cm}^{-1} \text{ M}^{-1}$.

Table 3
Substrate Sequences of the RDF Chemosensors and Their Fluorescence Increases

Entry	Name	Target kinase	Location of the chromophore ^a	Peptide Sequence ^b	Fold Fluorescence Increase ^c
1	P1	MK2	C	Ac-AHL <u>QRQLS</u> *I-C(Sox)-HH-CONH ₂	4.4 ± 0.2
2	P2	MK2	C	Ac-AHL <u>QRQLS</u> *I-C(Clk)-HH-CONH ₂	2.1 ± 0.3
3	P3	Src	N	Ac-AEE-C(Sox)-IY* <u>GEFEAKKKK</u> -CONH ₂	2.0 ± 0.1
4	P4	Src	N	Ac-AEE-C(Clk)-IY* <u>GEFEAKKKK</u> -CONH ₂	2.4 ± 0.2

^a Location determined in reference to the chromophore. C denotes C-terminus and N stands for N-terminus.

^b Asterisk (*) denotes the residue that is phosphorylated. In cases where it has been determined, residues important in kinase recognition are underlined.

^c Measured in triplicate as a quotient of fluorescence intensity at 485 nm (for Sox peptides) or 525 nm (for Clk peptides) of phosphopeptide and substrate in 20 mM HEPES (pH 7.4), 10 mM MgCl₂, and 10 μM peptide (for Sox peptides λ_{ex} = 360 nm; for Clk peptides λ_{ex} = 375 nm).