# Supporting Information

# Time-Resolved Luminescence Detection of Syk Kinase Activity Through Terbium-Sensitization

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**Optimal luminescence excitation of SAStide and pSAStide $Tb^{3+}$ complexes**

The UV absorbance spectra of the peptide biosensor in its phosphorylated and unphosphorylated form both displayed a single absorbance band. The unphosphorylated form, SAStide, displayed maximum absorption at 275 nm, whereas the maximum absorbance shifted to 266 nm for the phosphorylated form, pSAStide (Figure S1A). These absorbance bands did not shift in the presence of $Tb^{3+}$ (Figure S1B). This shift in the maximum UV absorbance offered an additional advantage for the detection of the phosphorylated form over the unphosphorylated form of the biosensor. Using a monochromator, the excitation source can be filtered to a single wavelength, allowing for specific excitation of a particular chromophore and preferred detection of a particular form of the peptide. The differences in luminescent signal obtained from excitation of each species via either maximum absorption wavelength were characterized. Solutions containing SAStide:$Tb^{3+}$ and pSAStide:$Tb^{3+}$ complexes were excited at 266 nm and 275 nm and the luminescent emission spectra were collected. SAStide:$Tb^{3+}$ excitation with each wavelength resulted in similar emission spectra; however, the area under the spectra decreased by 16% when 266 nm was used as the excitation wavelength compared to 275 nm (Figure S1C). Similarly, when the pSAStide:$Tb^{3+}$ complex was excited with 275 nm compared to 266 nm the emission area under the emission spectra decreased by 32% (Figure S1D).

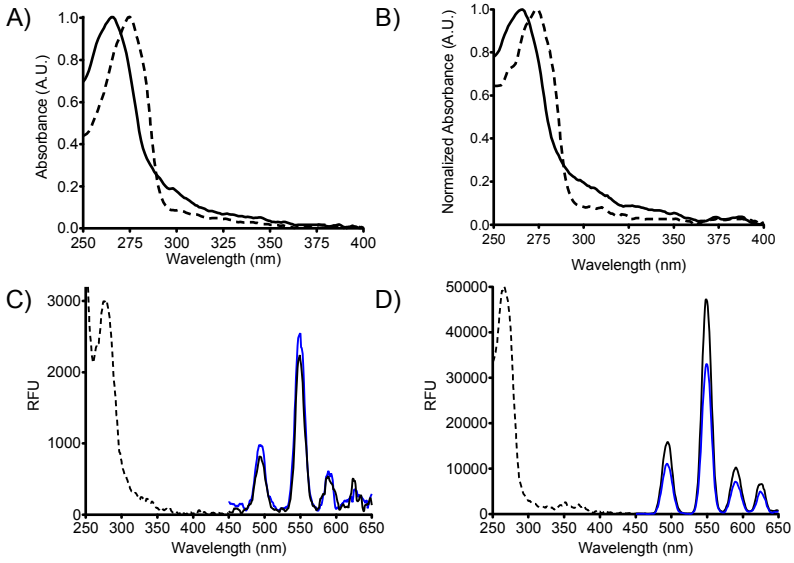


**Figure S1. Absorption and luminescent properties of SAStide and pSAStide. A**) UV-Vis spectra of SAStide (---) and pSAStide (—) without $Tb^{3+}$. **B**) UV-Vis spectra of SAStide (---) and pSAStide (—) in the presence of $Tb^{3+}$. Luminescence excitation (---) and emission spectra with 266 $nm_{Ex}$ (—, black) and at 275 $nm_{Ex}$ (—, blue) for SAStide:$Tb^{3+}$ (**C**) and pSAStide:$Tb^{3+}$ (**D**) complexes. Luminescence emission spectra were collected from 15 µM peptide in the presence of 100 µM $Tb^{3+}$, 10 mM HEPES, 100 mM NaCl with a 50 µs delay and 1 ms collection time. Each spectrum represents that average of three replicates.

**Terbium complexation with SAStide and pSAStide**

Luminescence emission spectra were collected of SAStide or pSAStide in the presence of varying concentrations of terbium. The integrated emission spectral area was normalized to plateau of the first hyperbolic curve. The addition of terbium to both peptides resulted in a hyperbolic increase in terbium-sensitized luminescence characteristic of one site binding (the model for which is further described in the main text). The addition of terbium beyond 50 μM (50 equivalents) to SAStide or pSAStide resulted in increased terbium sensitized luminescence with an inflection and plateau (respectively) at approximately quantized ratios of two- and three- fold greater than the 1:1 complex plateau. Since terbium sensitized luminescence is proportional to the amount of terbium bound in the complex, it is reasonable to interpret that the 2 and 3 fold increase in luminescence corresponds to the formation of the 1:2 and 1:3 pSAStide:$Tb^{3+}$ complexes and the 1:2 SAStide:$Tb^{3+}$ complex. This is further supported by data from electrospray ionization mass spectrometry analysis, which indicates adducts of 1, 2 and 3 $Tb^{3+}$ ions associated with the phosphorylated SAStide peptide (and even also exhibited 1:3 complexation of the unphosphorylated species with $Tb^{3+}$) (Fig. S3).

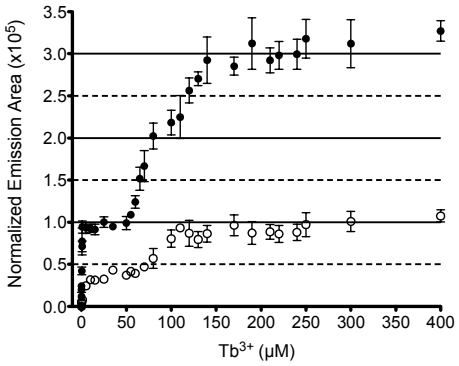


**Figure S2. Terbium binding to SAStide and pSAStide**. Spectra were collected from 1 μM pSAStide (●) or SAStide (○) in the presence of varying concentrations of $Tb^{3+}$ in 10mM HEPES, 100mM NaCl, pH 7.5, $\lambda_{ex}$ = 266 nm for pSAStide and $\lambda_{ex}$ = 275 nm for SAStide, 1000ms collection time, 50 μsec delay time and sensitivity 180. Data represent averages ± SEM of experiment performed in triplicate.

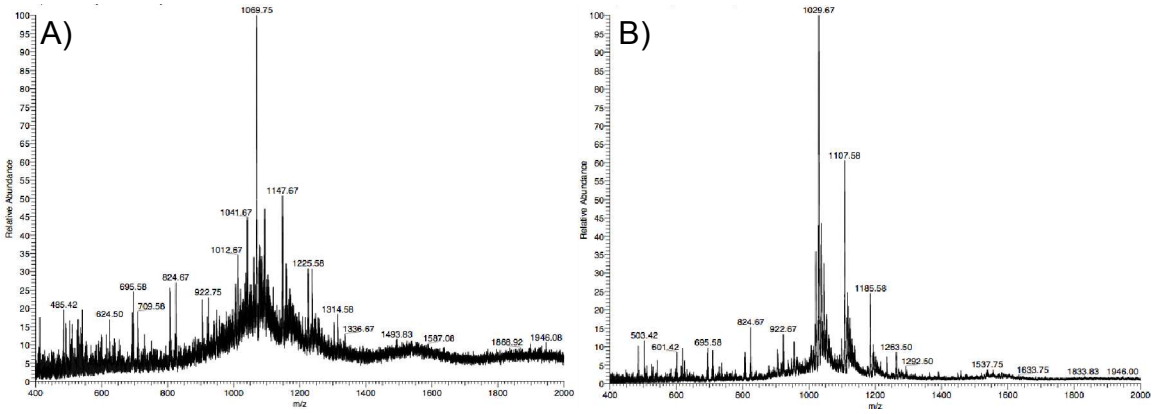


Figure S3. Electrospray ionization mass spectrometric detection of terbium binding to SAStide and pSAStide. Negative mode ESI mass spectra of 15 μM pSAStide (A) or SAStide (B) peptides in the presence of 100 μM $Tb^{3+}$. pSAStide displays the formation of 1:1, 2:1 and 3:1 $Tb^{3+}$:pSAStide and $Tb^{3+}$:SAStide complexes.

**Determination of pSAStide:$Tb^{3+}$ hydration number**

Luminescence emission spectra were collected on a Biotek Synergy4 plate reader at 23°C in black 384-well plates (Greiner Fluortrac 200). Spectra were collected from 450-650 nm after excitation at 266 nm with a delay time starting at 100 μsec, increasing in steps of 100 μsec to 5000 μsec, and a collection time of 1 ms. Samples were prepared with 1 μM pSAStide or SAStide with 1 μM $Tb^{3+}$ in 100 mM NaCl, 10 mM HEPES pH 7.5 with varying percentages of $D_2O$ in 50 μL total volume. The intensity of the emission peak at 549 nm was used as the metric for luminescence lifetime. Data were fitted to a single exponential decay curve using GraphPad Prism and lifetimes in varying percentages of $D_2O$ were determined and plotted against the mole fraction of $H_2O$. (Figure S2) The luminescent decay rate of pSAStide:$Tb^{3+}$ and SAStide:$Tb^{3+}$ in $D_2O$ were interpolated from this plot and applied to the following equation (S1):

$$q = A'(k_{H2O} - k_{D2O} - 0.06) \qquad (S1)$$

where $k_{H2O}$ is the luminescence decay constant in $H_2O$ and $k_{D2O}$ is the luminescence decay constant in $D_2O$ extrapolated from the plot. A' is the lanthanide specific constant, which is 5 for $Tb^{3+}$. The decay rates were 2.02 and 2.44 in $H_2O$ and $D_2O$ respectively for pSAStide:$Tb^{3+}$. These data produced a q value of 0.12 indicating that the hydration number of the pSAStide:$Tb^{3+}$ complex is approximately 0. The SAStide:$Tb^{3+}$ complex displayed luminescence decay rates of 1.88 and 2.92 in $H_2O$ and $D_2O$ respectively. These data in equation S1 gave a q value of 0.66 indicating 1 water molecule in the coordination sphere.

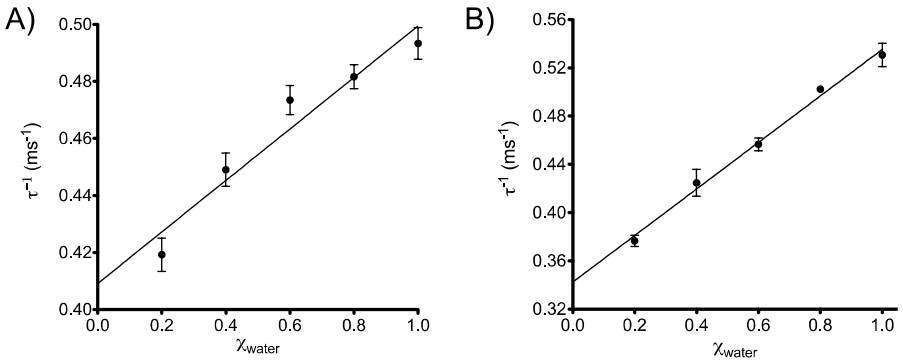


**Figure S4.** Luminescence decay rates with increasing mole fraction of $H_2O$ of pSAStide:$Tb^{3+}$ (**A**) and SAStide:$Tb^{3+}$ (**B**). Extrapolation to a $H_2O$-free solution provides the rate constant in $D_2O$. Data represent the average ± SEM of three individual replicates.

**Determination of pSAStide:$Tb^{3+}$ quantum yield**

The quantum yield of the pSAStide:$Tb^{3+}$ complex was determined using diffusion-enhanced energy transfer between the pSASide:$Tb^{3+}$ complex and FITC. The pSAStide:$Tb^{3+}$ complex (4 μM pSAStide and 10 μM $Tb^{3+}$) was mixed with various concentrations of the fluorescent acceptor, FITC, which was used as a standard to compare excited-state lifetimes and intensities of emission. Luminescence decay spectra were collected with increasing delay times and spectra were normalized to the emission at 495 nm. The area under the emission spectra was integrated. The lifetime of pSAStide:$Tb^{3+}$ was 0.43 ms and decreased to 0.37 ms (15% energy transfer) in the presence of 2 μM FITC, 0.34 ms (21% energy transfer) in 4 μM FITC and 0.28 ms (36% energy transfer) in 8 μM FITC. It was observed that increasing the concentration of FITC decreased the lifetime while increasing the energy transfer (indicated by the increase in the intensity of the FITC emission peak at 520 nm). The quantum yield was determined using this information in the equation below where $Q_a$ is the quantum yield of FITC at pH 7.5, $I_{da}$ is the area under the curve for the luminescence spectrum of the pSAStide:$Tb^{3+}$ complex in the absence of FITC, $I_{ad}$ is the difference in area under the curve for the luminescence spectrum for the pSAStide:$Tb^{3+}$ in the absence vs. the presence of FITC, $\tau_d$ is the excited-state lifetime of the donor (pSAStide:$Tb^{3+}$) luminescence, $\tau_{da}$ is the excited-state lifetime of the donor in the presence of the acceptor (pSAStide:$Tb^{3+}$ in the presence of FITC)

$$Q_{Ln} = Q_a * I_{da} * (\tau_d - \tau_{ad}) / (I_{ad} * \tau_{ad}) \quad \text{(S2)}$$

The quantum yields in the presence of 2 μM, 4 μM and 8 μM FITC were determined to be 0.36, 0.35, and 0.32 respectively for an average quantum yield of 0.34.

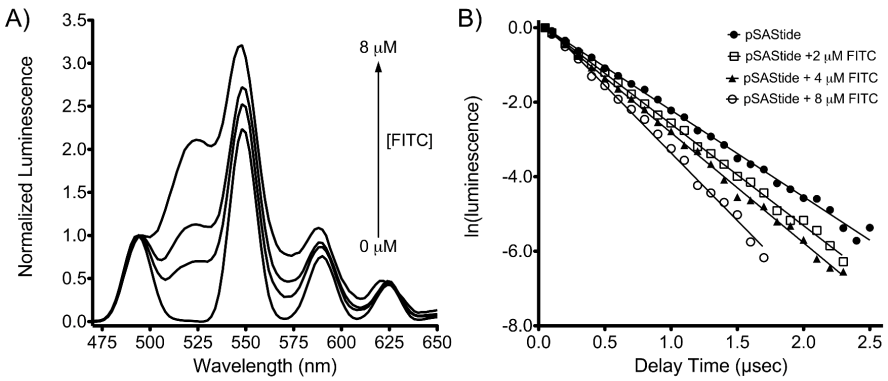


**Figure S5. Determination of pSAStide-$Tb^{3+}$ quantum yield A)** Emission spectra of pSAStide-$Tb^{3+}$ complex in the presence of various concentrations of FITC. **B)** Luminescence decay plots of the pSAStide:$Tb^{3+}$ in the presence of various concentrations of FITC.

**pSAStide:$Tb^{3+}$ signal stability and resistance to interference**

The effect of various buffer components on the luminescence of the pSAStide:$Tb^{3+}$ and SAStide:$Tb^{3+}$ complexes were assessed individually and compared to their luminescence in the absence of interference. Samples were prepared, TR-luminescence spectra were collected in triplicate as described before and the areas under the curves were determined. Most components displayed minimal interference, with the exception of those containing hard bases such as the phosphate groups of ATP, the sulfoxide group of DMSO, and the oxoanion of pervanadate. These components produced increased signal from the unphosphorylated sample and slightly decreased signal from the phosphorylated sample, resulting in the potential for a net, albeit not severe or limiting, decrease in signal to noise (as discussed in the main text).

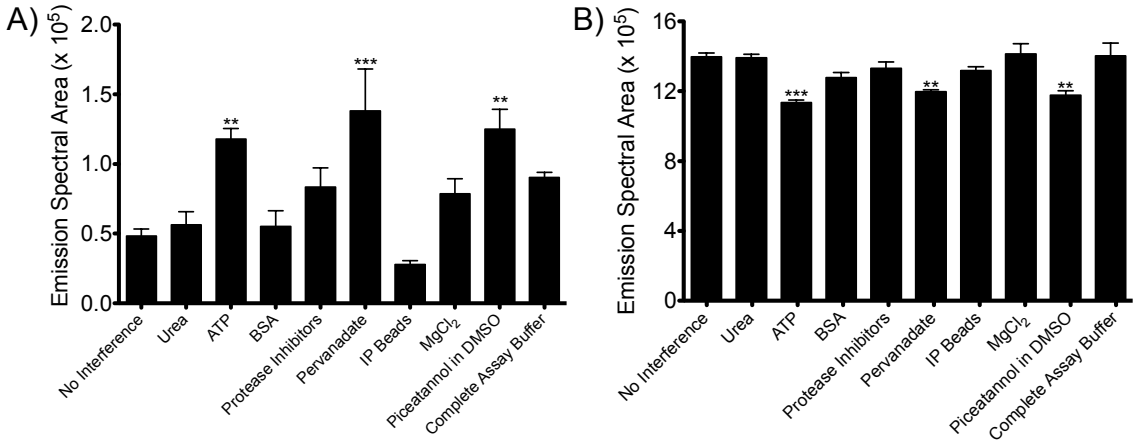


**Figure S6. pSAStide:$Tb^{3+}$ and SAStide:$Tb^{3+}$ luminescence interference from assay components.** TR-luminescence measurements were performed for the negative control SAStide (15 μM) (**A**) or the positive control pSAStide (15 μM) (**B**) in the presence of 100 μM $Tb^{3+}$, 100 mM NaCl, 10 mM HEPES pH 7.5 and the components comprising the *in vitro* kinase assay. 2.4 M Urea, 4 mM $MgCl_2$, 40 μM ATP, 0.4 μM

prevanidate, 0.04 µg/µL aprotinin and leupeptin, 50 ng/µl BSA, 2% DMSO, 100 µM piceannatol. Data were analyzed using repeated measures one-way ANOVA with a Dunnett post-test using the no interference sample as the control for both SAStide and pSAStide. Statistical significance is indicated as follows: *P<0.05, **P<0.01, ***P<0.001.

**Table S1. *In vitro* assay components and change on luminescence signal**

| Component | Concentration | pSAStide:$Tb^{3+}$ Average Area (x $10^5$) | pSAStide:$Tb^{3+}$ Percent Change (± SEM) | SAStide:$Tb^{3+}$ Average Area (x $10^5$) | SAStide:$Tb^{3+}$ Percent Change (± SEM) |
|---|---|---|---|---|---|
| **Urea** | 2.4 M | 13.61 | -2.04 ± 0.80 | 0.56 | +121.29 ± 49.21 |
| **BSA** | 50 ng/µL | 12.62 | -8.46 ± 5.68 | 0.55 | +112.33 ± 18.29 |
| **$MgCl_2$** | 4 mM | 13.96 | +1.01 ± 5.24 | 0.79 | +161.71 ± 11.22 |
| **Protease Inhibitors** | 0.1 µg/µL Aprotinin 0.1 µg/µL Leupeptin | 13.14 | -4.77 ± 2.19 | 0.83 | +171.09 ± 27.23 |
| **ATP** | 40 µM | 11.20 | -18.82 ± 2.47 | 1.18 | +249.78 ± 54.96 |
| **Pervanadate** | 0.4 µM | 11.82 | -14.28 ± 2.77 | 1.38 | +279.91 ± 54.32 |
| **Piceatannol and DMSO** | 100 µM Piceatannol 2% DMSO | 11.62 | -15.81 ± 1.08 | 1.22 | +121.75 ± 23.27 |
| **IP Beads** | 1:25 dilution | 13.01 | -5.72 ± 2.05 | 0.28 | -59.41 ± 18.72 |
| **Assay Buffer** | | 13.84 | -5.62 ± 4.97 | 0.90 | +189.71 ± 24.69 |

A number of buffers are commonly used in biological assays, including phosphate buffers and buffers containing other hard base moieties that could potentially compete for $Tb^{3+}$ binding and interfere with the luminescence of the biosensor. The luminescence of the pSAStide:$Tb^{3+}$ complex was measured in the presence of 4(2-hydroxyethyl)-1-piperzaineethanesulfonic acid (HEPES), 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholine)propanesulfonic acid (MOPS), phosphate and tris(hydroxymethyl)aminomethane (Tris) buffers at various concentrations (1 µM – 70 mM). The luminescence emission spectra of pSAStide:$Tb^{3+}$ were robust for HEPES, MES, MOPS and Tris over all concentrations with only a small decrease in signal at higher concentrations (70 mM). In the presence of the phosphate buffer (from 1 µM to 1 mM) luminescence emission spectra were robust but significantly diminished. At concentrations greater than 1 mM the robustness of the spectra was lost.

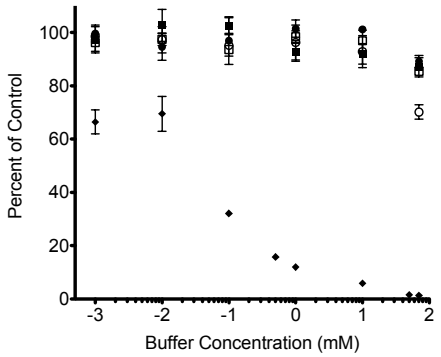

**Figure S7. Compatibility of pSAStide:$Tb^{3+}$ assay with biological buffers.** Luminescent emission spectra of 15 μM pSAStide in the presence of 100 μM of $Tb^{3+}$, 100 mM NaCl and various buffers including HEPES (●), MES (○), MOPS (□), Phosphate (◆) and Tris (▫) at pH 7.5. Spectra were collected in triplicate and the area of each spectrum was calculated and taken as the percent of the no buffer control. Data represent the averages ± SEM.

As demonstrated above phosphate can influence the signal of the pSAStide:$Tb^{3+}$ complex by decreasing the signal and robustness of the emission spectrum (likely because of competition for binding of $Tb^{3+}$). One of the essential cofactors required for phosphorylation is the phosphate source adenosine triphosphate, ATP. The luminescence of the pSAStide:$Tb^{3+}$ complex was measured in presences of various concentrations of ATP. The area under each spectrum was calculated and normalized to the percent of the control in the absence of ATP. The luminescent intensity of the pSAStide:$Tb^{3+}$ decreased in a concentration dependent manner in the presence of ATP with an approximate $K_i$ = 58 μM. These parameters should be taken into account when designing assay and quenching conditions for $Tb^{3+}$-based detection.

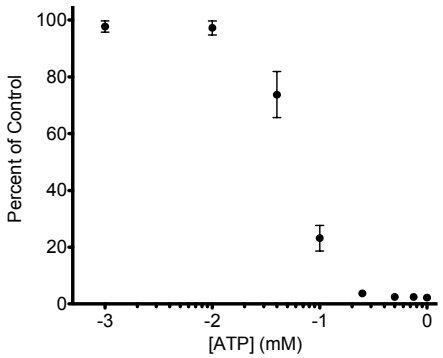


**Figure S8. The influence of ATP on pSAStide:$Tb^{3+}$luminescence.** Luminescence emission spectra of pSAStide (15 μM) in the presence of 100 μM $Tb^{3+}$, 100 mM NaCl, 10 mM HEPES pH 7.5 and various concentrations of ATP ranging from 1 μM to 100 mM. Spectra were collected in triplicate and the area of each spectrum was calculated and taken as the percent of the control (absence of ATP). Data represent the mean ± SEM.

**Validation of SAStide phosphorylation *in vitro***

Phosphorylated SAStide was detected using a chemifluorescent ELISA-based assay in which the quenched reaction mixture was incubated in a 96-well neutravidin coated-plate to allow for affinity capture of the biotinylated substrate. The total amount of peptide in the quenched reaction mixture applied to each well was 37.5 pmol, which ensured that each well was saturated with peptide (15 pmol binding capacity) for analysis. The captured peptide was then incubated an anti-phosphotyrosine primary antibody (4G10) followed by a horseradish peroxidase-conjugated secondary antibody. Chemifluorescent detection was

accomplished by incubating each well with Amplex Red reagent and hydrogen peroxide in phosphate buffer, which gave a fluorescent signal proportional to the amount of horseradish peroxidase-conjugate antibody in each well, and thus reports the degree of phosphotyrosine present. As seen in the with the $Tb^{3+}$ based detection, the ELISA-based assay displayed increasing fluorescent signal over time, demonstrating that SAStide was phosphorylated by Syk *in vitro*.

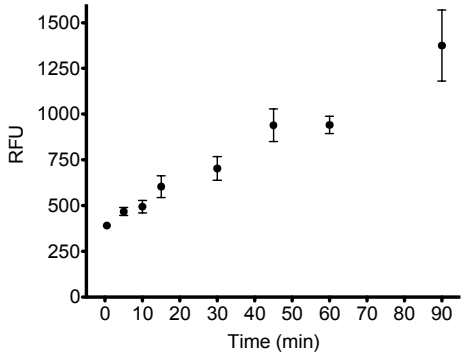


**Figure S9. Validation of *in vitro* phosphorylation of SAStide using ELISA-based chemifluorescence.** The SAStide biosensor was incubated with Syk-EGFP in an in vitro kinase assay as described in the main text. Aliquots were removed at designated time points, quenched with EDTA and the amount of phosphorylated substrate was measured using ELISA-based detection.

**Table S2. HTS assay parameters for controls**

| Percent Phosphorylation | CV (%) | Average (Area $10^5$) | Standard Deviation | Z factor | Signal Window |
|---|---|---|---|---|---|
| **0%** | 4.21 | 0.90 | 0.07 | N/A | N/A |
| **20%** | 9.88 | 3.54 | 0.61 | **0.56** | **4.21** |
| **40%** | 9.91 | 5.04 | 0.86 | **0.61** | **5.06** |
| **60%** | 5.62 | 7.82 | 0.76 | **0.79** | **12.49** |
| **80%** | 8.78 | 11.03 | 1.68 | **0.70** | **7.32** |
| **100%** | 5.25 | 13.83 | 1.26 | **0.82** | **14.64** |

**Table S3. HTS assay parameters for piceatannol dose-response inhibition of Syk**

| Concentration (piceatannol) | CV (%) | Average (% Activity) | Standard Deviation | Z factor | Signal Window |
|---|---|---|---|---|---|
| **10 nM** | 12.33 | 114.22 | 24.39 | **0.60** | **4.73** |
| **100 nM** | 14.39 | 115.48 | 28.78 | **0.53** | **3.62** |
| **1 µM** | 9.86 | 132.41 | 22.61 | **0.68** | **6.73** |
| **10 µM** | 10.59 | 119.12 | 21.85 | **0.65** | **6.01** |
| **50 µM** | 7.94 | 101.65 | 13.99 | **0.73** | **8.92** |
| **100 µM** | 5.90 | 70.30 | 7.19 | **0.78** | **12.64** |
| **500 µM** | 10.63 | 37.53 | 6.91 | **0.58** | **5.04** |
| **1 mM** | 212.93 | 5.33 | 19.67 | -13.15 | -3.01 |
| **10 mM** | 32.59 | 2.74 | 1.54 | N/A | N/A |