Phosphorylation of BlaR1 in Manifestation of Antibiotic Resistance in Methicillin-Resistant *Staphylococcus aureus* and its Abrogation by Small Molecules

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Syntheses of compounds 2 to 9

General information. Reagents for chemical synthesis were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.) or Alfa Aesar (Ward Hill, MA, U.S.A.). ^{1}H and ^{13}C NMR spectra were acquired on a Varian DirectDrive 600 or a Varian INOVA-500 NMR spectrometer. High-resolution mass spectra were acquired on a Bruker microTOF/Q2 mass spectrometer (Bruker Daltonik, Bremen, Germany) by electrospray ionization. Thin-layer chromatography was done on EMD Millipore (Billerica, MA, U.S.A.) 0.25 mm silica gel 60 F_{254} plates. Column chromatography was done either manually using silica gel 60, 230-400 mesh (40-63 μm particle

size) purchased from Sigma-Aldrich Chemical Co., or on a Teledyne Combiflash Rf 200i automated chromatography system (Teledyne Isco, Lincoln, NE, U.S.A.) using disposable silica gel columns. The known compounds, 4-(((*tert*-butyldimethylsilyl)oxy)methyl)pyridine (**2**), 4-fluoro-*N*-methoxy-*N*-methylbenzamide **(3**), 2-(((*tert*-butyldimethylsilyl)oxy)-1-(4-fluorophenyl)-2-(pyridin-4-yl)ethan-1-one (**4**), and 1-methoxymethyl-2,4,5-tribromoimidazole (**5**) were synthesized according to literature procedures.[1-3]

1-Methoxymethyl-2-(4-*iso*-butylphenyl)-4,5-dibromoimidazole (6). Compound **5** (3.66 g, 10.2 mmol) and 4-*iso*-butylphenylboronic acid (1.89 g, 10.6 mmol) were dissolved in toluene/MeOH (5:1, 105.0 mL), and an aqueous solution of K_2CO_3 (2.0 M, 11.5 mL) was added. The mixture was degassed with argon for 20 minutes while stirring, followed by the addition of $Pd(PPh_3)_4$ (1.23 g, 1.1 mmol). The mixture was stirred at reflux for 18 h. It was cooled to room temperature and diluted with water (40 mL) and EtOAc (20 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layer was washed with brine (20 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo*, and the crude product was purified by flash chromatography (silica, 100% hexanes to 9:1 hexanes/EtOAc) to give a pale-yellow viscous oil (3.83 g, 86%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.91 (d, 6H, J = 6.8 Hz, $CH_2CH(CH_3)_2$)), 1.89 (nonet, 1H, J = 6.8 Hz, $CH_2CH(CH_3)_2$), 2.52 (d, 2H, J = 6.8 Hz, $CH_2CH(CH_3)_2$), 3.44 (s, 3H, OCH_3), 5.27 (s, 2H, NCH_2O), 7.23 (d, 2H, J = 8.2 Hz, ArH), 7.65 (d, 2H, J = 8.2 Hz, ArH); ^{13}C NMR (150 MHz, $CDCl_3$) δ 22.5, 30.4, 45.4, 56.8, 76.4, 105.1, 118.0, 126.8, 128.8, 129.7, 144.1, 150.0; HRMS (ESI): calcd for $C_{15}H_{19}Br_2N_2O$ 400.9859, found 400.9895 $[MH]^+$.

1-Methoxymethyl-2-(4-*iso*-butylphenyl)-4-bromo-5-(tributylstannyl)imidazole (7). *n*-Butyl lithium (1.6 M in hexanes, 6.1 mL, 9.8 mmol) was added dropwise to a solution of **6** (3.76 g, 9.4 mmol) in THF (46.0 mL) at -78 ^{o}C, and the mixture was stirred at this temperature for 15 min. Tri-*n*-butyltin chloride (2.8 mL, 10.3 mmol) was then added dropwise, and the reaction mixture was stirred at -78 ^{o}C for 30 min, before being poured into saturated $NaHCO_3$ (40 mL). The aqueous layer was extracted with EtOAc (3 x 20 mL), and the combined organic layer was dried over anhydrous Na_2SO_4. The suspension was filtered, and the solvent in the filtrate was evaporated to dryness *in vacuo*. The residue was purified by column chromatography (silica,

100% hexanes to 95:5 hexanes/EtOAc) to give the product as a pale-orange viscous oil (3.92 g, 68%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.90-0.92 (m, 15H, 5 x CH_3), 1.20-1.23 (m, 6H, 3 x CH_2), 1.36 (sextet, 6H, *J* = 7.3 Hz, 3 x CH_2), 1.54-1.59 (m, 6H, 3 x CH_2), 1.89 (nonet, 1H, *J* = 6.8 Hz, $CH_2CH(CH_3)_2$), 2.51 (d, 2H, *J* = 6.8 Hz, $CH_2CH(CH_3)_2$), 3.09 (s, 3H, OCH_3), 5.14 (s, 2H, NCH_2O), 7.21 (d, 2H, *J* = 8.2 Hz, ArH), 7.47 (d, 2H, *J* = 8.2 Hz, ArH); ^{13}C NMR (150 MHz, $CDCl_3$) δ 11.0, 13.9, 22.5, 27.5, 29.1, 30.4, 45.4, 53.3, 77.6, 120.3, 127.3, 129.2, 129.5, 130.5, 143.2, 152.6.; HRMS (ESI): calcd for $C_{27}H_{46}BrN_2OSn$ 613.1804, found 613.1839 $[MH]^+$.

1-Methoxymethyl-2-(4-*iso*-butylphenyl)-4-bromo-5-(4-pyridyl)imidazole (8). Stannane **7** (1.61 g, 2.6 mmol), 4-iodopyridine (0.60 g, 2.9 mmol), and $Pd(PPh_3)_4$ (0.61 g, 0.53 mmol) were dissolved in DMF (26.5 mL), and argon was bubbled through the mixture for 20 min. It was then heated at 110 ^{o}C for 45 h, at which point it was cooled to room temperature, poured into water (30 mL), and extracted with EtOAc (3 x 15 mL). The combined organic layer was washed with brine (15 mL) and dried over anhydrous Na_2SO_4. The suspension was filtered, and the filtrate was evaporated to dryness *in vacuo*. Purification by column chromatography (100% hexanes to 1:1 hexanes/EtOAc) gave the title compound as a sticky solid (0.86 g, 82%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.92 (d, 6H, *J* = 6.8 Hz, $CH_2CH(CH_3)_2$), 1.91 (nonet, 1H, *J* = 6.8 Hz, $CH_2CH(CH_3)_2$), 2.54 (d, 2H, *J* = 6.8 Hz, $CH_2CH(CH_3)_2$), 3.32 (s, 3H, OCH_3), 4.99 (s, 2H, NCH_2O), 7.27 (d, 2H, *J* = 8.2 Hz, ArH), 7.62 (d, 2H, *J* = 5.4 Hz, ArH), 7.71 (d, 2H, *J* = 8.2 Hz, ArH), 7.75 (d, 2H, *J* = 5.4 Hz, ArH); ^{13}C NMR (150 MHz, $CDCl_3$) δ 22.5, 30.4, 45.4, 55.4, 75.8, 117.1, 124.0, 126.4, 128.9, 129.8, 132.3, 136.5, 144.2, 150.4, 150.9; HRMS (ESI): calcd for $C_{23}H_{23}BrN_3O$ 400.1019, found 400.1046 $[MH]^+$.

1-Methoxymethyl-2-(4-*iso*-butylphenyl)-4-bromo-5-(4-fluorophenyl)imidazole (9). Stannane **7** (1.70 g, 2.8 mmol) and 4-fluoroiodobenzene (0.32 mL, 2.8 mmol) were dissolved in DMF (73.0 mL), and argon was bubbled through the mixture for 20 min. Tris(dibenzylideneacetone)dipalladium(0)-chloroform adduct ($Pd_2(dba)_3{\cdot}CHCl_3$, 0.45 g, 0.44 mmol), $AsPh_3$ (0.68 g, 2.2 mmol), and CuI (1.36 g, 7.1 mmol) were then added, and the mixture was stirred at room temperature for 2 h. It was then poured into water (200 mL), and the solution was extracted with EtOAc (3 x 75 mL). The combined organic layer was washed with water (2 x 50 mL) and brine (50 mL), then dried over anhydrous Na_2SO_4. After filtration and removal of the

solvent from the filtrate *in vacuo*, the crude product was purified by column chromatography (silica, 100% hexanes to 9:1 hexanes/EtOAc) and recrystallization from EtOAc/hexanes to give off-white crystals. (0.87 g, 75%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.92 (d, 6H, J = 6.8 Hz, $CH_2CH(\mathit{CH_3})_2$), 1.91 (nonet, 1H, J = 6.8 Hz, $CH_2\mathit{CH}(CH_3)_2$), 2.53 (d, 2H, J = 6.8 Hz, $\mathit{CH_2}CH(CH_3)_2$), 3.25 (s, 3H, OCH_3), 4.97 (s, 2H, NCH_2O), 7.19 (t, 2H, J = 8.7 Hz, ArH), 7.25 (d, 2H, J = 8.4 Hz, ArH), 7.59 (dd, 2H, J = 8.7, 5.3 Hz, ArH), 7.70 (d, 2H, J = 8.4 Hz, ArH); ^{13}C NMR (150 MHz, $CDCl_3$) δ 22.5, 30.4, 45.4, 55.6, 75.6, 115.6, 116.0 (d, $^{2}J_{CF}$ = 22.4 Hz), 124.7 (d, $^{4}J_{CF}$ = 3.4 Hz), 126.9, 128.8, 129.7, 130.7, 132.4 (d, $^{3}J_{CF}$ = 7.9 Hz), 143.7, 149.5, 163.1 (d, $^{1}J_{CF}$ = 249.1 Hz); HRMS (ESI): calcd for $C_{21}H_{23}BrFN_2O$ 417.0972, found 417.0981 $[MH]^+$.

Experimental procedures

Bacterial strains. *Staphylococcus aureus* strain MRSA252 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.); *S. aureus* strains NRS70, NRS123, and NRS128 were acquired from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA, Chantilly, VA, U.S.A.).

Minimal-inhibitory concentration (MIC) determination. Determination of MICs was done by the microdilution method cation-adjusted in Mueller-Hinton II Broth (CAMHB II, BBL) in accordance with the protocols of CLSI.[4] A final bacterial inoculum of 5 x 10^5 CFU/mL was used, and the results were recorded after incubation for 16-20 h at 37 ^{o}C.

Identification of phosphorylated domain of BlaR1. As the two fragments of proteolyzed BlaR1 are roughly the same size, it is difficult to determine the site of phosphorylation without separation. To accomplish this, we immunoprecipitated whole-cell extracts of NRS128 grown in the absence and presence of CBAP using an antibody raised against the sensor domain of BlaR1 ($BlaR^S$) immobilized on Protein A-agarose. $BlaR^S$-Agarose resin for immunoprecipitation of *S. aureus* extracts was prepared as previously described[5] by crosslinking $BlaR^S$ antibody to Protein A Agarose beads (Thermo Scientific) with dimethylpimelimidate (DMP) in sodium phosphate buffer (pH 7.4) with 150 mM NaCl. Crosslinked resin was stored in PBS at 4 °C. Whole-cell extracts were first cleared of Protein A and other immunoglobulin-binding proteins (as described above), then were incubated with $BlaR^S$-Agarose resin overnight at 4 °C with end-over-end

rotation. Unbound protein was removed after centrifugation. Bound proteins (containing $BlaR^S$, including full-length BlaR1) were eluted from the resin using Laemmli sample buffer. Unbound and bound fractions were loaded onto an 11% SDS-PAGE gel and subjected to western blot analysis with phosphotyrosine, phosphoserine, and $BlaR^S$ antibodies (Fig. S2). Probing with the $BlaR^S$ antibody revealed the C-terminal BlaR1 fragment that contains the sensor domain only in the bound fraction (band at ~30 kDa; red arrow), while it revealed the full-length BlaR1 both in the bound and unbound fractions (band at ~60 kDa; green arrows). The presence of the full-length BlaR1 in the unbound fraction is not unexpected since its interaction with lipids (it has four transmembrane helixes) makes its affinity-purification less efficient. Probing with the phosphotyrosine antibody also revealed a band at ~60 kDa both in the unbound and bound fractions. The correlation of the intensity of the full-length BlaR1 bands in the unbound and bound protein fractions of the membranes probed with the $BlaR^S$ antibody, with the intensity of the bands at ~60 kDa in the unbound and bound protein fractions of the membranes probed with the phosphotyrosine antibody indicates that the phosphorylated protein is BlaR1. Probing with the phosphotyrosine antibody revealed a ~30 kDa fragment only in the unbound fraction. This indicated that the site of tyrosine phosphorylation is located in the N-terminal fragment of BlaR1 (residues 1-283), which contains the cytoplasmic protease domain. This observation makes good sense, as ATP, the source of phosphate, is found only in the cytoplasm. A repeat of these experiments with anti-phosphoserine antibody indicates that the N-terminal half is also phosphorylated at a serine (Fig. S2c). Incidentally, the protease domain contains eleven serine and ten tyrosine residues, any of which could be the sites of phosphorylation. We cannot predict with any confidence the sites of phosphorylation in bacteria, as the predictive power is not good.

Cloning, expression, and purification of *S. aureus* Stk1 protein kinase. The *stk1* gene for Stk1 protein kinase (SA1063 in *S. aureus* NRS70) is conserved in all known genomic sequences for *S. aureus*.[6-8] We PCR-amplified the DNA fragment corresponding to the entire coding sequences of *stk1* from *S. aureus* strain NRS70 chromosomal DNA with primers STK1fw (5'-CCCCCCCATATG ATAGGTAAAATAATAAATGAACGATAT-3') and STK1rev (5'-CCCCCCCTCGAG TTAAATATCATCATAGCTGACTTCTTTTTC-3'). The amplified DNA fragments were then digested with NdeI and XhoI restriction enzymes and cloned into pET28a. After verification of the inserts by DNA sequencing on both strands, the resulting plasmid

(termed pETstk1) was introduced into *E. coli* strain BL21(DE3) for protein expression. For purification of Stk1, 5 mL of an overnight culture of BL21(DE3) harboring pETstk1 was inoculated into 500 mL fresh LB medium and grown at 37 °C until the OD_{600} reached 0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to the culture to a final concentration of 0.5 mM and the culture was shaken at 15 °C overnight. The culture was then centrifuged at 5000 rpm at 4 °C for 15 min. The pellet was resuspended in 20 mL of lysis buffer (25 mM HEPES, 500 mM NaCl, 10 mM imidazole, pH 7.4). After sonification, the lysate was centrifuged at 18,000 *g* at 4 °C for 45 min. The resulting supernatant containing the His-tagged Stk1 was loaded onto a 5-mL Hitrap Chelating column (GE Healthcare), followed by elution with a linear gradient of imidazole (0-500 mM) in lysis buffer. The fractions containing Stk1 were pooled, concentrated and the buffer was exchanged to 25 mM HEPES, pH 7.4. The resulting sample was then subjected to Q anion-exchange chromatography and eluted with a linear gradient of NaCl (0-1 M). Protein purity was ascertained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S5).

In vitro **phosphorylation assay.** The Stk1 protein kinase was assayed for its autophosphorylation (on serine and threonine residues) and for phosphorylation of myelin basic protein (MBP), a commercially available, nonspecific substrate of Ser/Thr protein kinases. The assay was used for assessment of inhibition of the protein by the synthetic kinase inhibitors **10-12**. The reactions were carried out in 20 µL of 25 mM Tris, pH 7.4, 1 mM dithiothreitol and 10 mM $MgCl_2$ and contained 1 µg purified Stk1 and varying concentrations of compounds **10-12.** Reactions to monitor the effect of the compound on phosphorylation of MBP by Stk1 also included 4 µg MBP. All reactions were initiated by the addition of 4 µCi [γ-^{32}P]-ATP (20 µM final). The assay mixture was incubated at room temperature for 20 min and it was stopped by the addition of 5x SDS-PAGE sample buffer. After boiling for 5 minutes, the mixtures were subjected to SDS-PAGE. The gel was then exposed to storage phosphor screen overnight and the screen was scanned with an Amersham Storm 840. Band intensities were quantified using GelQuant software. The band intensities in the presence of compounds **10-12** were divided by the intensities in the absence of inhibitors to obtain the relative band intensities. Relative band intensities of the Stk1 or MBP bands were plotted against the concentration of compounds **10-12** (µM) (Fig. S6) and GraphPad Prism 5 was used to calculate the IC_{50} values by non-linear

regression, using the equation $Y=IC_{50}/[IC_{50}+X]$ as previously described,[9] with R^2 values ranging from 0.87 to 0.91.

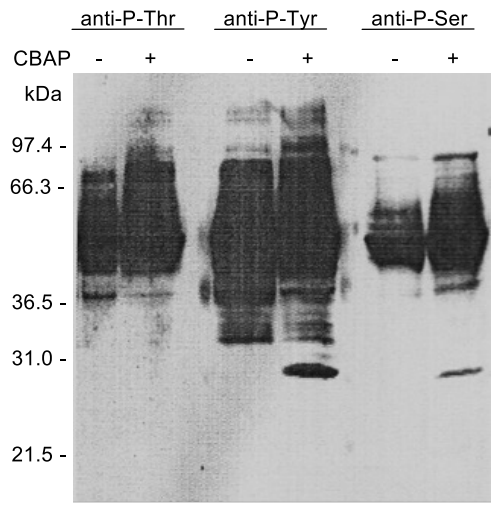

Figure S1. Western-blot analysis of NRS128 whole-cell extracts grown in the absence and presence of 10 μg/mL CBAP using antibodies against phosphothreonine, phosphotyrosine, and phosphoserine. The ~30 kDa bands seen with anti-phosphotyrosine and anti-phosphoserine correspond to fragmented BlaR1. No such band was detected using anti-phosphothreonine antibody. The bands between 36.5 kDa and 97.4 kDa are attributed to the ubiquitous Protein A and other immunoglobulin-binding proteins, which was cleared from the extract in subsequent experiments.

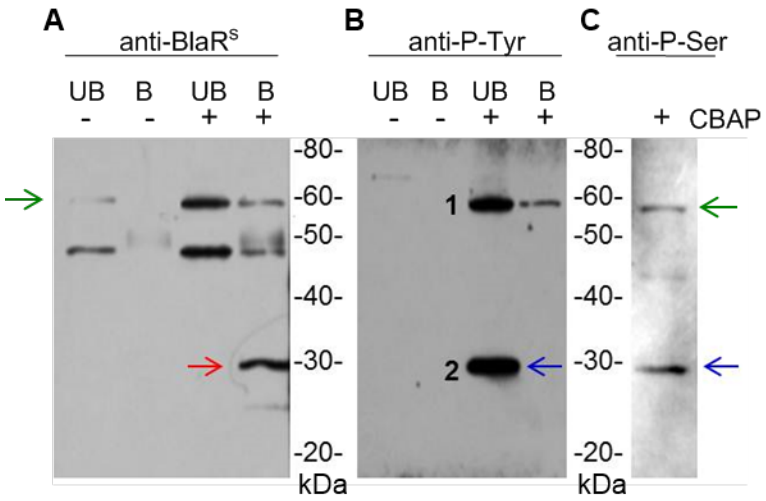

Figure S2. Western-blot analysis of CBAP-induced (+) and non-induced (-) extracts of NRS128 after immunoprecipitation with anti-BlaR^S-agarose. Nitrocellulose membrane containing unbound ("UB") and eluted bound ("B") fractions were probed with (**A**) anti-BlaR^S or (**B**) anti-P-Tyr. The red arrow indicates the C-terminal fragment of BlaR1, seen only in the bound fraction. The blue arrows indicate the unbound N-terminal fragment containing the phosphorylated cytoplasmic domain. The green arrows indicate the full-length BlaR1, which is phosphorylated and partially recovered in the bound fraction; most of the full-length protein remains in the unbound fraction, most likely due to its interaction with lipids. Bands identified by anti-BlaR^S between 40-50 kDa are undefined proteolytic fragments of BlaR1.[9] (**C**) Western-blot analysis of CBAP-induced whole-cell extract using antibody against phosphoserine (anti-P-Ser) is shown. Both the full-length and N-terminal fragment of BlaR1 (blue arrow) were detected.

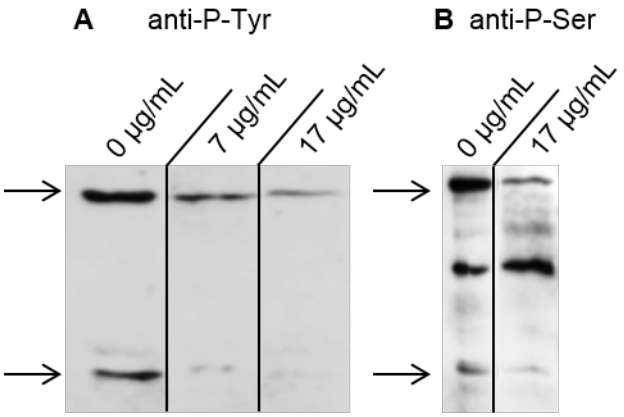

Figure S3. BlaR1 phosphorylation in the absence and presence of 7 or 17 µg/mL of compound **1**. Whole-cell extracts of CBAP-treated NRS128 in the absence or the presence of compound **1** were cleared of IgG-binding proteins by incubation with IgG Sepharose and analyzed by western blot using antibodies against (**A**) phosphotyrosine and (**B**) phosphoserine.

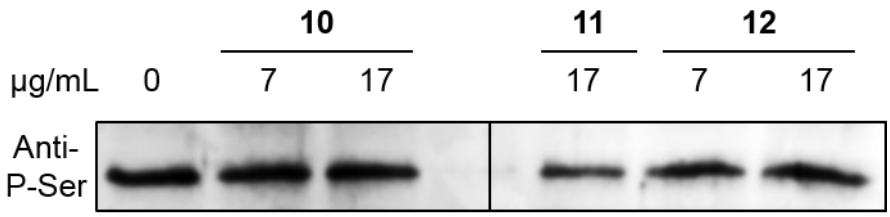

Figure S4. The effect of compounds **10**, **11**, or **12** (0, 7 or 17 µg/mL) on serine phosphorylation of BlaR1 fragment. NRS70 whole-cell extracts were cleared of Protein A and analyzed by Western Blot using antibody against phosphoserine.

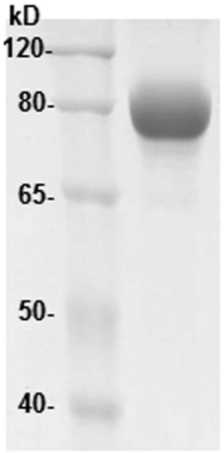

Figure S5. SDS-PAGE of purified Stk1 from *S. aureus* strain NRS70.

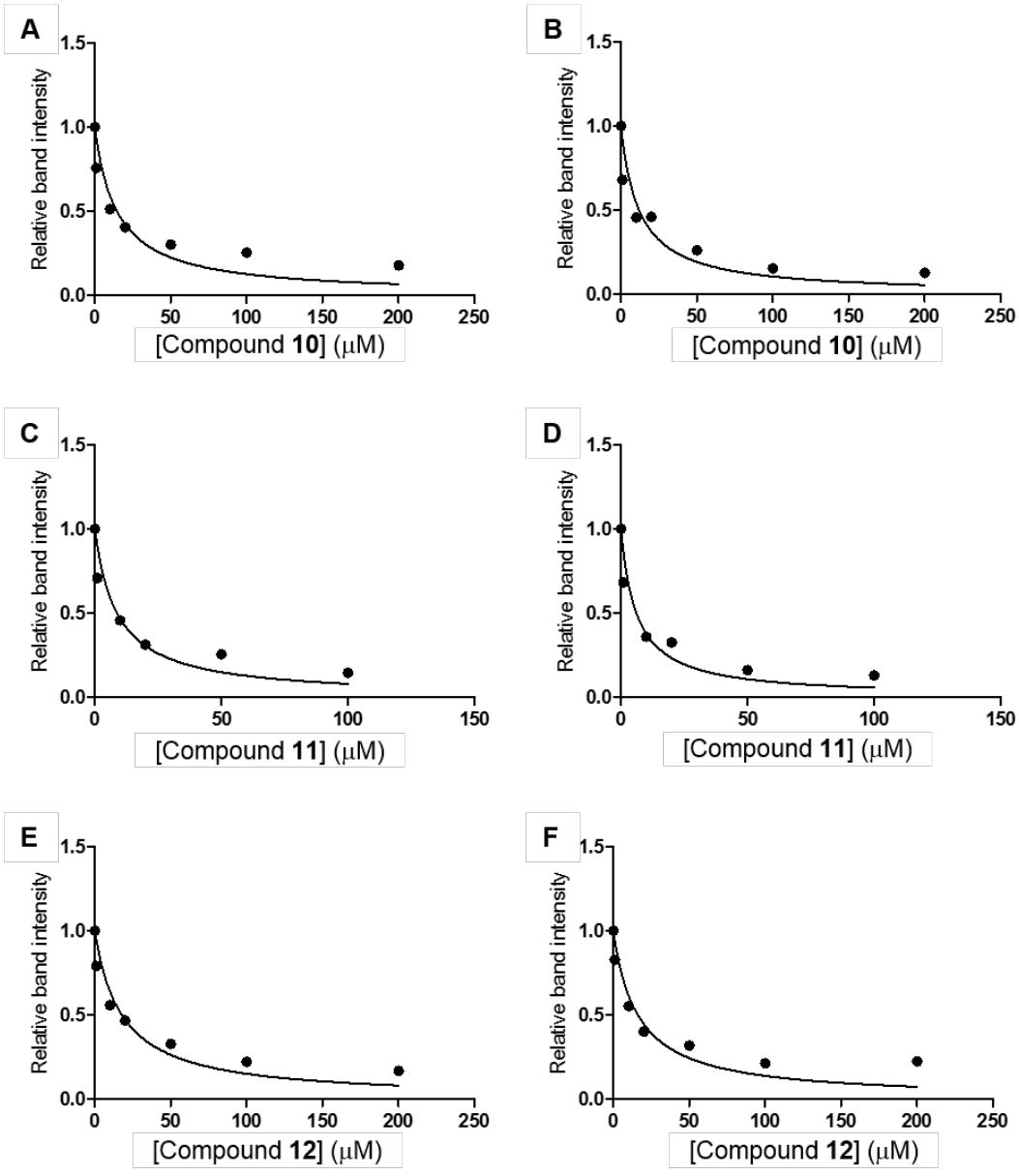

Figure S6. Inhibition of autophosphorylation of purified Stk1 or myelin basic protein (MBP) by compounds **10**-**12**. Purified Stk1 or myelin basic protein (MBP) was radiolabelled by [γ-^{32}P]-ATP (20 μM) in the presence of increasing concentrations of synthetic inhibitors **10-12**. Inhibition of Stk1 autophosphorylation by **10-12**, giving IC_{50} values of 3.1 ± 0.8 μg/mL (9 ± 2 μM), 5 ± 1 μg/mL (15 ± 4 μM), and 6 ± 1 μg/mL (18 ± 4 μM), respectively (panels **A**, **C**, and **E**). Inhibition of MBP phosphorylation by compounds **10-12**, giving IC_{50} values of 2.1 ± 0.6 μg/mL (6 ± 2 μM), 4 ± 1 μg/mL (12 ± 4 μM), and 6 ± 1 μg/mL (16 ± 4 μM), respectively (panels **B**, **D**, and **F**).

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