Supporting Information

Identification of Small-Molecule Antagonists of the *Pseudomonas aeruginosa* Transcriptional Regulator PqsR: Biophysically Guided Hit Discovery and Optimization

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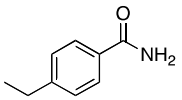
METHODS

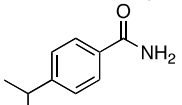
Chemical and Analytical Methods. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 instrument. Chemical shifts are given in parts per million (ppm), and tetramethylsilane (TMS) was used as internal standard for spectra obtained in $CDCl_3$, MeOH-d_4 and DMSO-d_6. Coupling constants (*J*) are given in hertz. Mass spectrometry (LC/MS) was performed on a MSQ® electro spray mass spectrometer (Thermo Fisher). The system was operated by the standard software Xcalibur®. A RP C18 NUCLEODUR® 100-5 (125 × 3 mm) column (Macherey-Nagel GmbH) was used as stationary phase with water/acetonitrile mixtures as eluents. All solvents were HPLC grade. Reagents were used as obtained from commercial suppliers without further purification. The reaction progress was determined by thin-layer chromatography (TLC) analyses on silica gel 60, F_{254} (Merck). Visualization was accomplished with UV light. Melting points were measured using melting point apparatus SMP3 (Stuart Scientific). The apparatus is uncorrected.

General procedure for the synthesis of amides.[1]



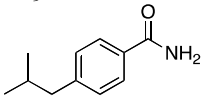
7

Synthesis of 4-ethylbenzamide[2] **(7).** A solution of 4-ethylbenzoic acid (746 mg, 500 mmol), thionyl chloride (1 mL) and *N*,*N*-dimethylformamide (3 drops) was heated at 80 °C for 1 h. The reaction mixture was evaporated under reduced pressure. The residue was redissolved in toluene (10 mL). At 0 °C the mixture was added to concentrated ammonia (20 mL). After stirring for 30 minutes the precipitate was collected by suction filtration, washed with water and *n*-hexane, and dried under reduced pressure at 50 °C. Recrystallization from ethyl acetate afforded compound **7** (0.41 g, 55%). Mp: 164-165 °C. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.25 (t, *J* = 7.6 Hz, 3H), 2.69 (q, *J* = 7.6 Hz, 2H), 6.12 (bs, 2H), 7.26 (d, *J* = 8.2 Hz, 2H), 7.74 (d, *J* = 8.5 Hz, 2H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 15.2, 28.8, 127.4, 128.1, 130.8, 148.7, 169.5. LC/MS: m/z = 150.06 $(M + H)^+$, 191.13 $(M + H + CH_3CN)^+$, t_R = 8.77 min, 98.6% pure (UV).



8

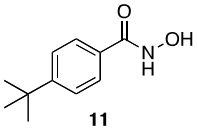
Synthesis of 4-isopropylbenzamide[3] **(8).** Compound **8** was prepared from 4-isopropylbenzoic acid according to the general procedure (0.24 g, 49%). Mp: 150.5-151.5 °C. ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 1.20 (d, *J* = 6.9 Hz, 6H), 2.92 (septet, *J* = 6.9 Hz, 1H), 7.26 (bs, 1H), 7.30 (d, *J* = 8.2 Hz, 2H), 7.80 (d, *J* = 8.2 Hz, 2H), 7.89 (bs, 1H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 23.6, 33.3, 126.1, 127.6, 131.9, 151.7, 167.8. LC/MS: m/z = 164.11 $(M + H)^+$, 205.09 $(M + H + CH_3CN)^+$, t_R = 8.54 min, >99% pure (UV).



10

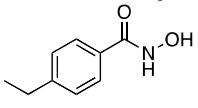
Synthesis of 4-isobutylbenzamide[4] **(10).** Compound **10** was prepared from 4-isobutylbenzoic acid according to the general procedure. The final product was crystallized from ethyl acetate (0.45 g, 51%). Mp: 154-155 °C. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 0.89 (d, *J* = 6.6 Hz, 6H), 1.89 (nonet, *J* = 6.8 Hz, 1H), 2.52 (d, *J* = 7.2 Hz, 2H), 6.00 (bs, 2H), 7.21 (d, *J* = 8.5 Hz, 2H), 7.73 (d, *J* = 8.1 Hz, 2H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 15.2, 28.8, 127.4, 128.1, 130.8, 148.7, 169.5. LC/MS: m/z = 178.18 $(M + H)^+$, 219.05 $(M + H + CH_3CN)^+$, t_R = 10.72 min, 97.3% pure (UV).

General procedure for the synthesis of hydroxamic acids.[5]



11

Synthesis of 4-(*tert*-butyl)-*N*-hydroxybenzamide (11). *O*-(Tetrahydro-2-pyranyl)hydroxylamine (352 mg, 3.00 mmol) and sodium carbonate (159 mg, 1.50 mmol) were dissolved in a water-chloroform mixture (1:1, 10 mL). 4-*tert*-Butylbenzoyl chloride (590 mg, 3.00 mmol) was added and the solution was stirred for 30 minutes at room temperature. Both layers were separated and the water layer was extracted with chloroform. The combined organic layers were evaporated under reduced pressure leaving a foam. The solid was dissolved in methanol (20 mL). 0.1 N aqueous HCl (20 mL) was added and the solution was stirred overnight at room temperature. The solvent was removed under reduced pressure leaving compound **11** as a white solid (573 mg, 99%). Mp: 135.3-136.8 °C (lit. [6] 141-144 °C). ^{1}H-NMR (500 MHz, MeOH-d_4): δ = 1.28 (s, 9H), 7.45 (d, *J* = 8.5 Hz, 2H), 7.69 (d, *J* = 8.5 Hz, 2H), 11.14 (bs, 1H). ^{13}C-NMR (125 MHz, MeOH-d_4): δ = 31.6, 35.8, 126.5, 128.1, 131.3, 156.4, 169.8. LC/MS: 235.04 $(M + H + CH_3CN)^+$, t_R = 8.32 min, 96.0% pure (UV).



12

Synthesis of 4-ethyl-*N*-hydroxybenzamide (12). Compound **12** was prepared from 4-ethylbenzoyl chloride according to the general procedure (258 mg, 52%). Mp: 103.0-104.8 °C (lit. [7] 106.5-107 °C). ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.17 (t, *J* = 7.6 Hz, 3H), 2.61 (q, *J* = 7.6 Hz, 2H), 7.17 (d, *J* = 7.9 Hz, 2H), 7.66 (d, *J* = 7.9 Hz, 2H), 8.56 (bs, 1H), 10.66 (bs, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 15.1, 28.6, 126.9, 127.8, 128.7, 148.1, 165.7. LC/MS: m/z = 165.94 $(M + H)^+$, 207.03 $(M + H + CH_3CN)^+$, 330.81 $(2M + H)^+$ t_R = 6.68 min, 95.0% pure (UV).

Reporter Gene Assay in *E. coli*. The ability of the compounds to either stimulate or antagonize the PqsR-dependent transcription was performed as previously described using a β-galactosidase reporter gene assay [8] in *E. coli* expressing PqsR with some modifications to enable a higher throughput [9]. PQS and PqsR ligands were diluted in ethyl acetate, added to the wells of a 96 deep well plate and the solvent was evaporated. Overnight cultures of *E. coli* DH5α cells containing the plasmid pEAL08-2 which encodes PqsR under the control of the *tac* promoter and the β-galactosidase reporter gene *lacZ* controlled by the *pqsA* promoter, were diluted 1:100 in Luria-Bertani medium with ampicillin (50 µg ml^{-1}). The culture was incubated at 37 °C with shaking until it reached an OD_{600} of 0.2. For the determination of agonistic activities, 1 ml aliquots were supplemented with either PQS (50 nM) or the test compound (100 µM). Ethyl acetate was used as control. Antagonistic effects of the compounds (100 µM) were evaluated in the presence of 50 nM PQS. The β-galactosidase activity was determined after a 2.5 h incubation period at 37 °C with shaking (150 rpm). OD_{600}, OD_{420} and OD_{550} were measured and the activity is expressed as ratio of the ethyl acetate control relative to the cultures that received either PQS, a test compound or both.

Reporter Gene Assay in *P. aeruginosa* PA14. The ability of the compounds to either stimulate or antagonize the PqsR-dependent transcription was performed as previously described using a β-galactosidase reporter gene assay [8] in *PA 14ΔpqsA*[9]. PQS and PqsR ligands were diluted in ethyl acetate, added to the wells of a 96 deep well plate and the solvent was evaporated. Overnight cultures of *PA14* cells containing the plasmid pEAL08-2 which encodes PqsR under the control of the *tac* promoter and the β-galactosidase reporter gene *lacZ* controlled by the *pqsA* promoter, were diluted 1:100 in Luria-Bertani medium with carbenicillin (250 µg ml^{-1}). The culture was incubated at 37 °C with shaking until it reached an OD_{600} of 0.2. For the determination of agonistic activities, 1 ml aliquots were supplemented with either PQS (50 nM) or the test compound (100 µM). Ethyl acetate was used as control. Antagonistic effects of the compounds (100 µM) were evaluated in the presence of 50 nM PQS. The β-galactosidase activity was determined after a 2.5 h incubation period at 37 °C with shaking (150 rpm). After the incubation period the aliquots were shaked at least for 1 min (1000rpm). OD_{600}, OD_{420} and OD_{550} were measured and the activity is expressed as ratio of the ethyl acetate control relative to the cultures that received either PQS, a test compound or both.

Pyocyanin assay. Pyocyanin produced by *P. aeruginosa* PA14 was determined using the method of Essar et al.[10] with some modifications: Cultures were inoculated with a starting OD_{600} = 0.02 in 24 well plates (Greiner, Cellstar) containing 1.5 ml PPGAS medium per well. PPGAS medium (20 mM NH_4Cl, 20 mM KCl, 1.6 mM $MgSO_4$, 120 mM Tris-HCl, pH 7.2, 0.5% (w/v) glucose, 1% (w/v) Bacto™ Tryptone)[11] was found to enhance pyocyanin production. DMSO as a control or DMSO solutions of inhibitors were added to the cultures to a final DMSO concentration of 1%. Plates were incubated at 37 °C, 200 rpm and a humidity of 75% for 16 hrs. For pyocyanin determination, 900 µl of each culture were extracted with 900 µl of chloroform. The solution was centrifuged (14.000 rpm, 15 min) to separate the phases. 800 µl of the organic phase were mixed with 250 µl of 0.2 M HCl. After centrifugation (14.000 rpm, 15 min), 80 µl of the aqueous phase was transferred to 96 well plates (Nunc U96 MicroWell™) for measurement of OD_{520} using FLUOstar Omega (BMG Labtech). For each sample, cultivation and extraction were performed in triplicates.

SUPPLEMENTARY FIGURES

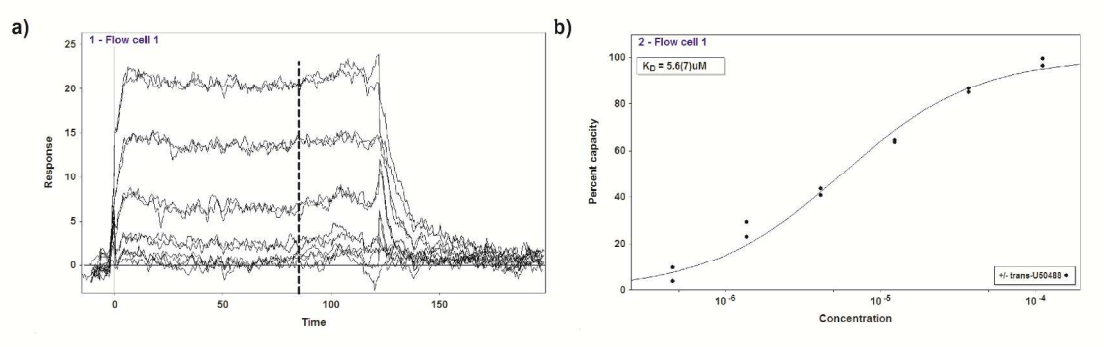


Figure S1. Binding affinity for (±)-*trans*-U50488 **a)** Overlay of sensorgrams for confirmed hit binding to H_6SUMO-PqsR^{C87} measured in duplicates at 12°C; double referenced. **b)** Fit of the duplicate (±)-*trans*-U50488 equilibrium response data from the H_6SUMO-PqsR^{C87} surface to a 1:1 interaction.

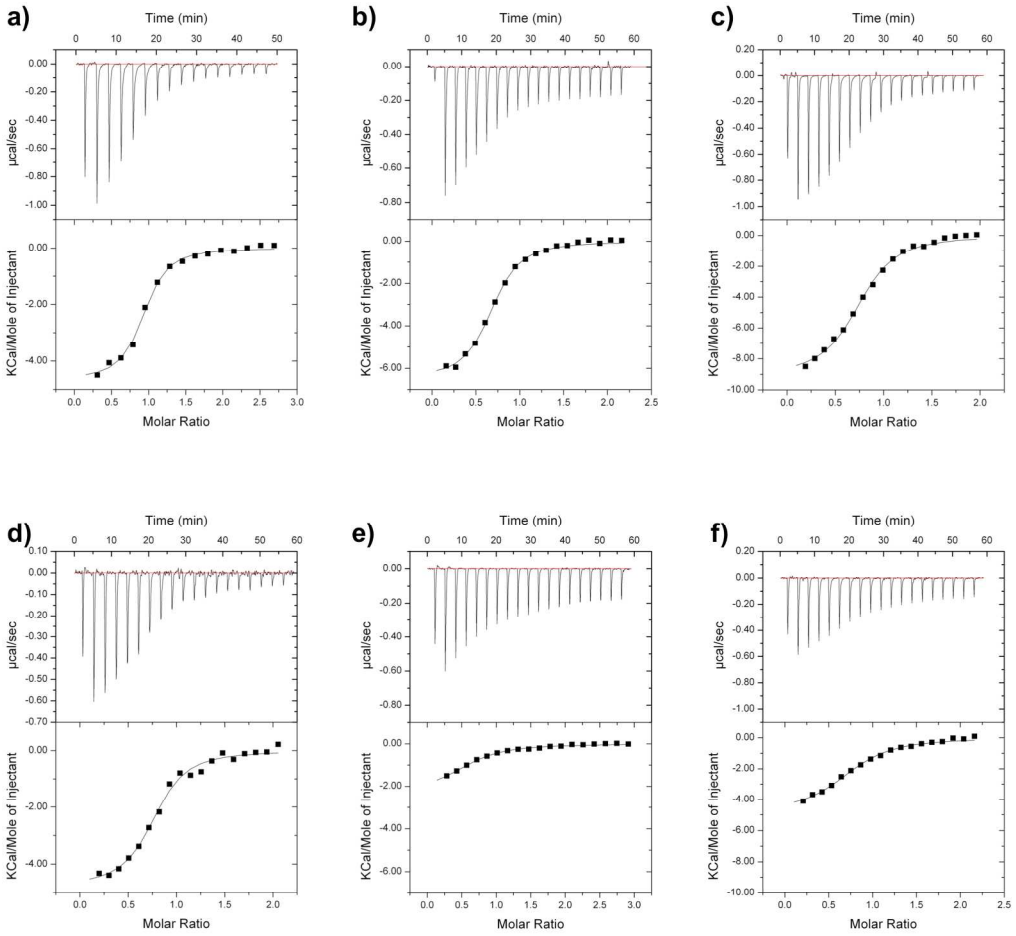


Figure S2. Representative ITC titrations. **a)** ITC titration of **1** (1.4 mM) against H_6SUMO-PqsR^{C87} (111 µM). **b)** ITC titration of **8** (900 µM) against H_6SUMO-PqsR^{C87} (83 µM). **c)** ITC titration of **11** (900 µM) against H_6SUMO-PqsR^{C87} (94 µM). **d)** ITC titration of **1** (1 mM) against H_6SUMO-Q194APqsR^{C87} (100 µM). **e)** ITC titration of **8** (2 mM) against H_6SUMO-Q194APqsR^{C87} (141 µM). **f)** ITC titration of **11** (1 mM) against H_6SUMO-Q194APqsR^{C87} (95 µM). The recorded change in heat is shown in units of µcal sec^{-1} as a function of time for successive injections of the ligand (upper row). Integrated heats (black squares) plotted against the molar ratio of the binding reaction. The continuous line represents the results of the non-linear least squares fitting of the data to a binding model (lower row).

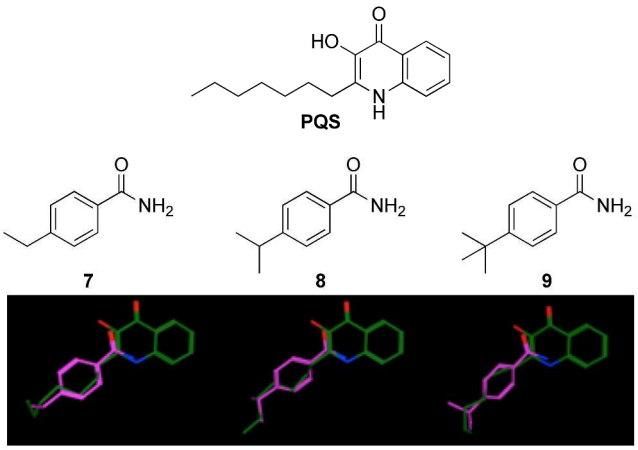


Figure S3. Flexible alignment of **7**, **8**, and **9** (magenta) with the natural ligand PQS (green). Alignment was done using the flexible alignment utility of MOE2010.10 (Chemical Computing Group Inc.).

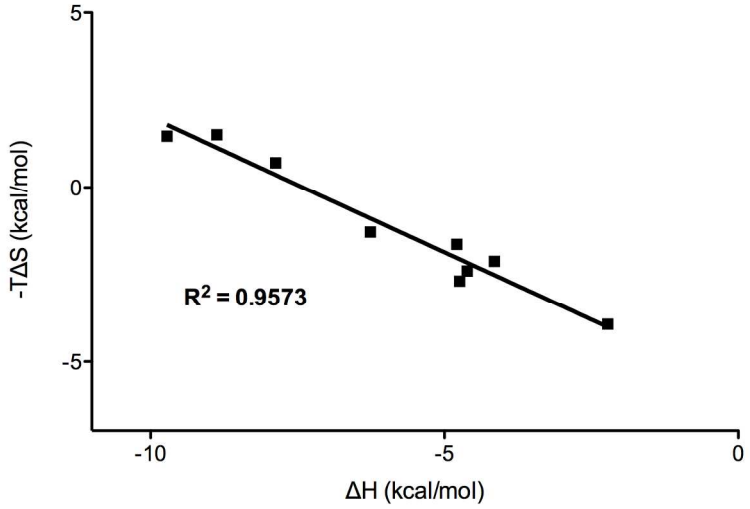


Figure S4. Plot of enthalpy (Δ*H*) vs entropy (-*T*Δ*S*). Δ*H* and –*T*Δ*S* are highly correlated thereby clearly evidencing an enthalpy-entropy compensation effect in the protein-ligand complexes of the current study.

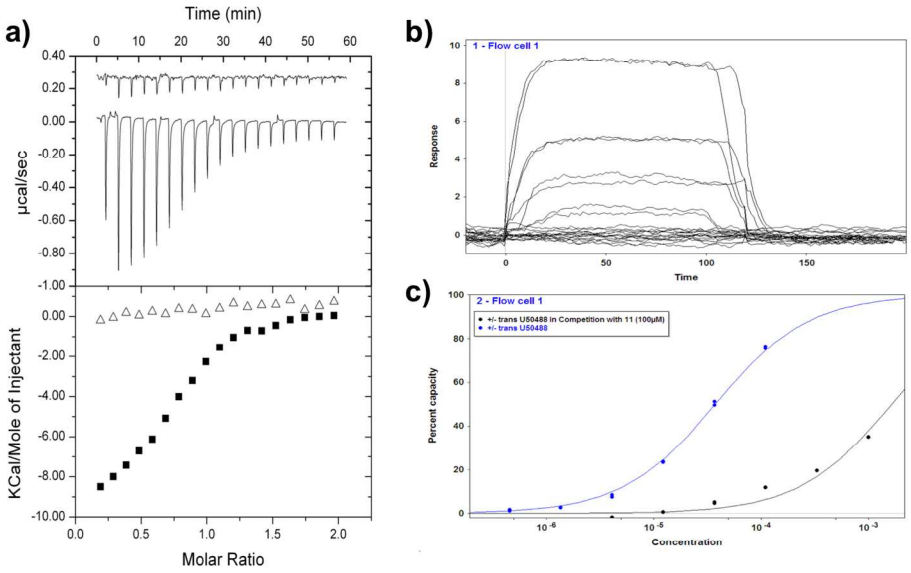


Figure S5. Competitive binding of ligands to PqsRC^{87} as measured by ITC and SPR. **a)** Raw ITC data (top) and integrated normalized data (bottom) for titrations of 94 µM H_6SUMO-PqsR^{C87} with 900 µM **11** (□) in the absence of **1** and in the presence of 1 mM compound **1** (Δ). **b)** Overlay of sensorgrams for compound **1** in competition with **11** to H_6SUMO-PqsR^{C87} measured in duplicates at 12°C; double referenced and DMSO calibrated. **c)** Blue line: Fit of the duplicate (±)-*trans*-U50488 equilibrium response data from the H_6SUMO-PqsR^{C87} surface to a 1:1 interaction; black line: Fit of the duplicate (±)-*trans*-U50488 equilibrium response data from the H_6SUMO-PqsR^{C87} surface to a 1:1 interaction in the presence of 100µM of compound **11**.

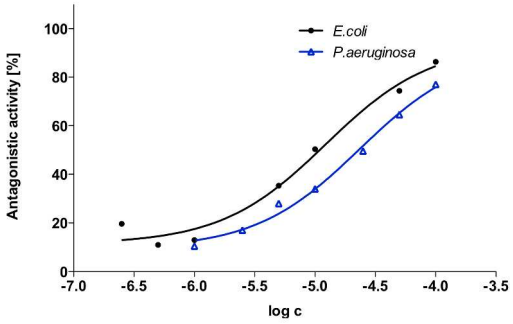


Figure S6. Determination of IC_{50} values in *E.coli* and *P. aeruginosa*. IC_{50} values were determined in the reporter gene assays. Compound **11** was tested at seven concentrations in competition with 50 nM PQS. IC_{50} value in *E.coli*: 12.5 µM; mean value of three experiments. IC_{50} value in *P. aeruginosa*: 23.6 µM; mean value of two experiments with n = 4. The log (inhibitor) vs. response model (Prism 5.0) was applied for nonlinear regression and determination of IC_{50}-values; the Hill slope was constrained equal to 1.0.

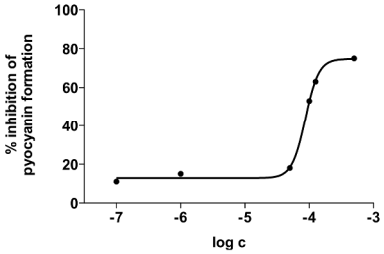


Figure S7. Effect of **11** on the pyocyanin production in *P. aeruginosa* PA14. For the determination of the IC_{50} value the pyocyanin levels in *P. aeruginosa* PA14 were spectrophotometrically determined at A_{520} nm in the presence of compound **11** at six concentrations. IC_{50} value in: 87.3 µM; mean value of three experiments. The log (inhibitor) vs. response-variable slope model (Prism 5.0) was applied for nonlinear regression and determination of the IC_{50}-value.

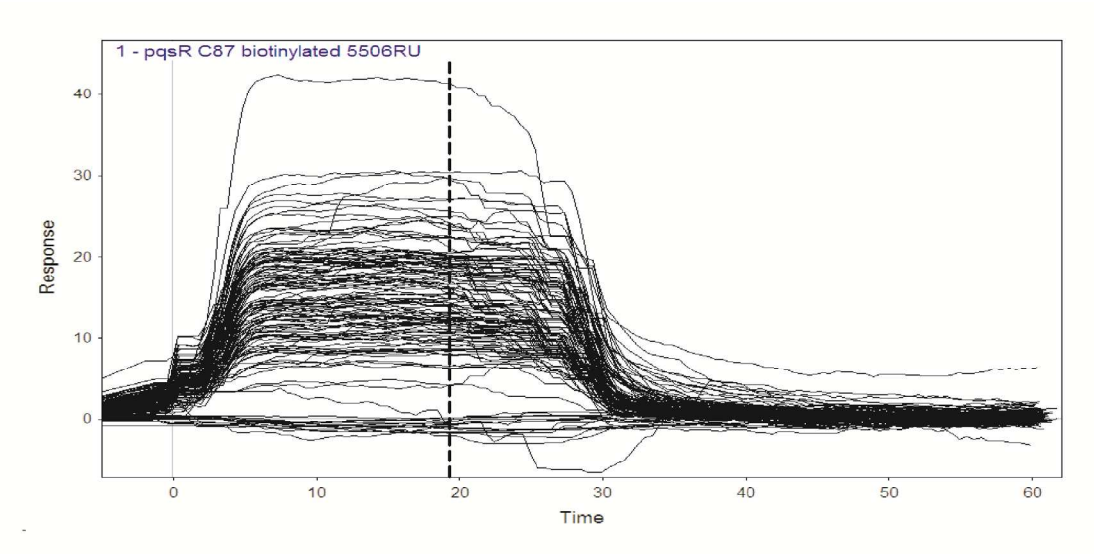


Figure S8. Overlay of 110 sensorgrams for tested compounds against biotinylated H_6SUMO-PqsR$^{C87'}$. The data read-out line is indicated by the dashed line.

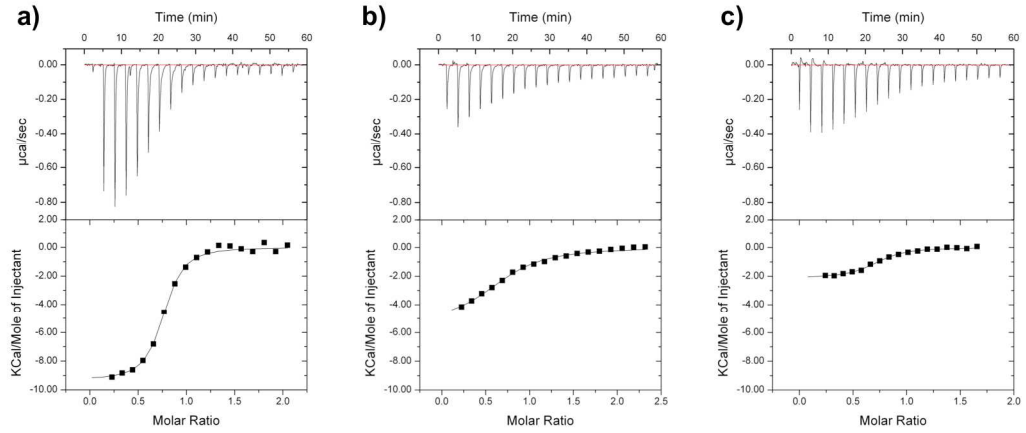


Figure S9. Representative ITC titrations. **a)** ITC titration of **9** (700 µM) against H_6SUMO-PqsR^{C87} (67 µM). **b)** ITC titration of **9** (700 µM) against H_6SUMO-Q194APqsR^{C87} (66 µM). **c)** ITC titration of **9** (1 mM) against H_6SUMO-F221APqsR^{C87} (124 µM). The recorded change in heat is shown in units of µcal sec^{-1} as a function of time for successive injections of the ligand (upper row). Integrated heats (black squares) plotted against the molar ratio of the binding reaction. The continuous line represents the results of the non-linear least squares fitting of the data to a binding model (lower row).

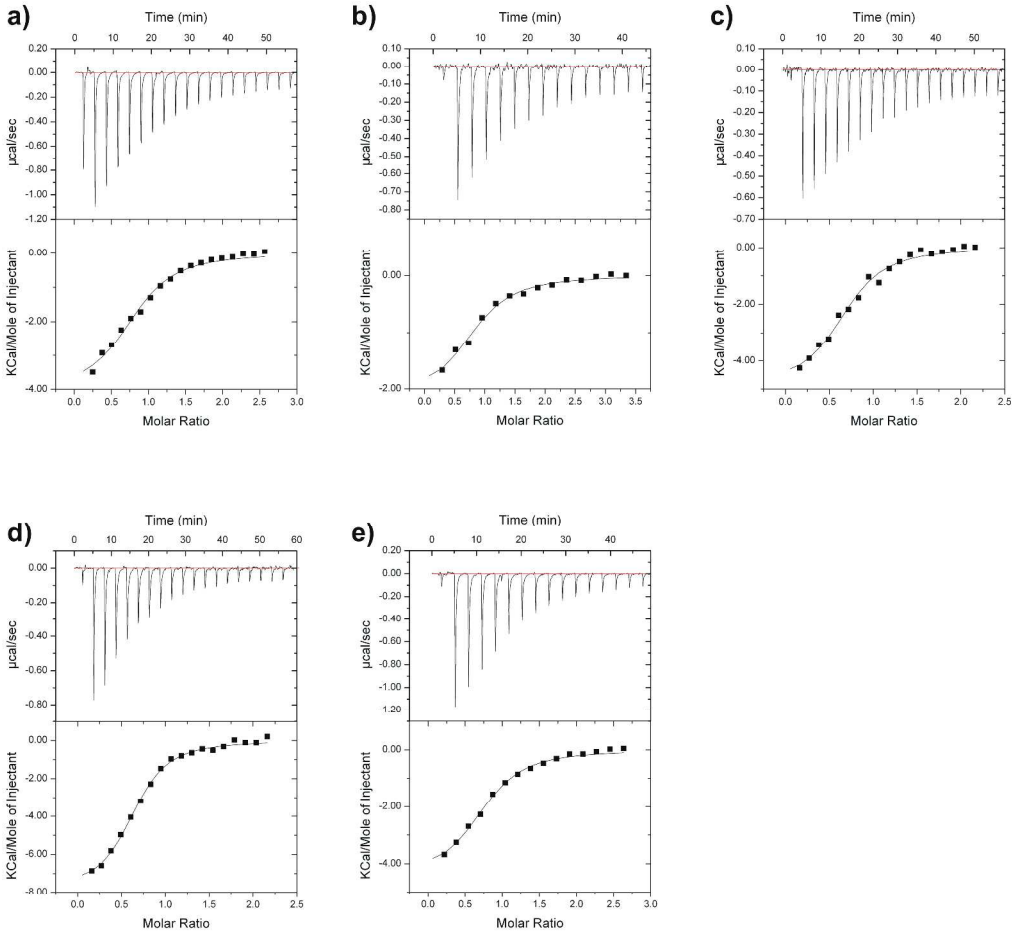


Figure S10. Representative ITC titrations. **a)** ITC titration of **4** (2.5 mM) against H_6SUMO-PqsR^{C87} (200 µM). **b)** ITC titration of **6** (2.5 mM) against H_6SUMO-Q194APqsR^{C87} (157 µM). **c)** ITC titration of **7** (900 µM) against H_6SUMO-PqsR^{C87} (83 µM). **d)** ITC titration of **10** (900 µM) against H_6SUMO-Q194APqsR^{C87} (83 µM). **e)** ITC titration of **12** (2 mM) against H_6SUMO-Q194APqsR^{C87} (158 µM). The recorded change in heat is shown in units of µcal sec^{-1} as a function of time for successive injections of the ligand (upper row). Integrated heats (black squares) plotted against the molar ratio of the binding reaction. The continuous line represents the results of the non-linear least squares fitting of the data to a binding model (lower row).

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