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SUPPORTING INFORMATION

Potent pan-group quorum sensing inhibitors in *Staphylococcus aureus* revealed by N-terminal tailoring of peptidomimetics

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Contents.

Materials.

Fmoc-protected amino acids and hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) were purchased from Chem-Impex international. All carboxylic acid derivatives used for capping were purchased from Sigma-Aldrich and TCI. Diisopropylethylamine (DIPEA) and piperidine were purchased from VWR international. Dichloromethane (DCM), dimethyl formamide (DMF), 4-nitrophenyl chloroformate, triisopropylsilane (TIPS), trifluoracetic acid (TFA), acetonitrile, guanidinium chloride, sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), diethyl ether, dimethyl sulfoxide (DMSO), and chloramphenicol (for bacterial culture) were purchased from Sigma-Aldrich. Dawson Dbz resin was purchased from Novabiochem. Triton X-100 was purchased from Thermo Scientific. DCM was distilled and dried over 3 Å molecular sieves overnight prior to use. Brian-heart infusion (BHI) powder used for bacterial culture was purchased from Research Products International and dissolved in 18 MΩ water (prepared using a Sartorius Arium Pro water purifier), and this solution was autoclaved prior to use. Phosphate buffered saline (PBS, pH 8) buffer was prepared from purified water and contained 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. Rabbit red blood cells (10% in saline) were purchased from LAMPIRE. Costar 96-well black polystyrene microplates for bacterial assays were purchased from Fisher Scientific.

Instrumentation.

Purification of linear and cyclic peptidomimetics was performed via semi-preparative reverse-phase liquid chromatography (RP-HPLC) on a Shimadzu instrument composed of C18 Kromasil Eternity column (10 mm × 250 mm), SCL-10Avp controller, DGU-14A degasser, FCV-10Alvp solvent mixer, LC-10ATbp pump, CTO-10ASvp oven, SPD-M10Avp detector, and FRC-10A collector. Purity of the peptidomimetics was analyzed via RP-HPLC using an analytical C18 Kromasil Eternity column (4.6 mm × 250 mm). The masses of the peptidomimetic products were determined by mass spectrometry (MS) using a Thermo Scientific Q Exactive Plus ESI-Q-IT mass spectrometer. Absorbance (at 600 nm; i.e., optical density (OD) readings of bacterial cultures, and at 405 nm for hemolysis assays) and fluorescence measurements (excitation at 500 nm and emission at 540 nm; for bacterial reporter assays) were obtained on Biotek Synergy2 plate reader running Gen5 software (version 1.05). Fluorescence excitation and emission spectra for coumarin-derivatized peptidomimetics were obtained using an ISS PC1 photon counting spectrofluorimeter. Samples were measured in Hellma fluorescence cuvettes.

Peptidomimetic synthesis methods.

Linear and macrocyclic peptidomimetics were synthesized using our previously reported solid-phase and solution-phase methods.[1-3] In brief, 42.8 mg of Fmoc Dawson AM resin was used for each peptidomimetic. The resin was swelled in 2 mL of DCM before the first amino acid coupling reaction. After washing with 2 mL DCM and 2 mL DMF (3x each), the resin was deprotected by treatment with 2 mL 20% piperidine (in DMF) for 10 min (2x) while shaking on a shaker platform at room temperature. Meanwhile, 3 eq. of Fmoc protected amino acid, 3 eq. of HATU, and 6 eq. of DIPEA were dissolved in 1 mL DMF, and this reaction mixture was allowed to activate for 2.5 min at room temperature. The deprotected resin was then washed with 2 mL DMF (3x), and the activated mixture was added to the drained resin. The reaction mixture was shaken for 30 min on a shaker platform at room temperature, after which the Fmoc group was cleaved as described above. This procedure (HATU coupling/Fmoc cleavage) was repeated iteratively to generate each peptidomimetic sequence; 8-(Fmoc-amino)-3,6-dioxaoctanoic acid was incorporated into this workflow to install the n7 oxo linker. Each solid-phase synthesis was

terminated with a final coupling of carboxylic acid derivative using the standard HATU method. The resin was then washed with DMF (3x) followed by DCM (3x).

To initiate cleavage of the linear peptidomimetic from the resin, 5 eq. of 4-nitrophenyl chloroformate was dissolved in 2 mL DCM and added to the resin. The reaction mixture was shaken for 30 min at room temperature, followed by washing with DCM and DMF (3x each). Next, 2 mL of 1:4 DIPEA:DMF was added to the resin, and the mixture was shaken for 10 min at room temperature; the reagents were drained from the resin and the same DIPEA:DMF treatment was performed two additional times. Thereafter, the resin was washed with DMF, DCM, and diethyl ether (3x each) and lyophilized overnight.

The final peptidomimetic cleavage step was performed by adding 1.8 mL TFA, 0.1 mL DCM, 0.05 mL TIPS, and 0.05 mL water to the resin and shaking the mixture for 2 h at room temperature. The resin was drained, and the eluent was isolated and incubated with 10 mL diethyl ether at - 20 °C for 1 h to precipitate the cleaved linear peptidomimetic. The peptidomimetic was isolated by centrifuging the mixture at 3500 rpm and removing the liquid layer. The isolated peptidomimetic was dissolved in 1:1 water:acetonitrile and lyophilized overnight. The peptidomimetic was redissolved in 5 mL of 25% acetonitrile in water and purified via RP-HPLC using a 40% → 45% or 45% → 55% acetonitrile gradient; fractions containing the peptidomimetic were combined and lyophilized.

To effect macrocyclization, peptidomimetic was dissolved in 4 mL of cyclization buffer containing 80% 6M guanidinium chloride in 0.1 M Na_2HPO_4 and 20% acetonitrile (pH 6.8). The macrocyclization reaction was allowed to proceed for 2 h with shaking at 50 °C, and the product was immediately purified using RP-HPLC with a 10% → 95% acetonitrile gradient and lyophilized. The identities of the macrocyclic products were confirmed by ESI-MS (listed in Table S1) and their purities were determined using analytical RP-HPLC (traces included at the end of this document). Starting with 42.8 mg of resin, 0.5-1 mg of purified macrocyclic peptidomimetic product was typically obtained (~3-7% yield). For testing in bacterial reporter assays, all peptidomimetics were dissolved in DMSO to generate 1 mM stock solutions.

Table S1. ESI-MS data and HPLC % purity for the peptidomimetics in this study. m/z represents [M+H$^+$] unless specified.

compound	calculated m/z [Da]	observed m/z [Da]	purity [%]
Bn(2F)-n7OFF	679.2596	679.2600	98.6
Bn(3F)-n7OFF	701.2416[a]	701.2414[a]	97.7
Bn(4F)-n7OF	679.2596	670.2600	>99
Bn(2Cl)-n7OFF	679.2301	679.2301	96.6
Bn(3Cl)-n7OFF	695.2301	695.2306	>99
Bn(4Cl)-n7OFF	695.2301	695.2309	>99
Bn(2Br)-n7OFF	739.1796	739.1813	98.2
Bn(3Br)-n7OFF	761.1615[a]	761.1629[a]	97.5
Bn(4Br)-n7OFF	739.1796	739.1782	>99
Bn(2I)-n7OFF	809.1476[a]	809.1470[a]	>99
Bn(3I)-n7OFF	809.1476[a]	809.1469[a]	>99
Bn(4I)-n7OFF	809.1476[a]	809.1472[a]	97.6
Bn(3CF_3)-n7OFF	751.2384[a]	751.2384[a]	96.6
Bn(4CF_3)-n7OFF	751.2384[a]	751.2386[a]	>99
Bn(2NO_2)-n7OFF	728.2361[a]	728.2358[a]	95.6
Bn(3NO_2)-n7OFF	728.2361[a]	728.2353[a]	99.0
Bn(3Me)-n7OFF	675.2847	675.2847	98.0
Bn(4Me)-n7OFF	675.2847	675.2847	>99
Bn(3OMe)-n7OFF	691.2796	691.2803	>99
Bn(4OMe)-n7OFF	691.2796	691.2802	>99
Bn(3SMe)-n7OFF	707.2568	707.2572	>99
Bn(4SMe)-n7OFF	707.2568	707.2570	>99
Bn(4Ph)-n7OFF	737.3000	737.3006	99.0
Bn(3$CF_3$4F)-n7OFF	747.2470	747.2470	99.0
Bn(3NH_2)-n7OFF	676.2800	676.2802	>99
Bn(4NH_2)-n7OFF	676.2800	676.2799	>99
PhPr-n7OFF	675.2847	675.2843	98.7
PhBu-n7OFF	689.3004	689.300	98.0
PhPr(2F)-n7OFF	693.2753	693.2754	95.1
PhPr(3F)-n7OFF	693.2753	693.2751	98.8
PhPr(4F)-n7OFF	715.2572[a]	715.2574[a]	96.2
PhPr(2Cl)-n7OFF	709.2467	709.2454	95.0
PhPr(3Cl)-n7OFF	731.2277[a]	731.2277[a]	95.5
PhPr(4Cl)-n7OFF	731.2277[a]	731.2278[a]	95.3
PhPr(2Br)-n7OFF	753.1952	753.1956	97.0
PhPr(3Br)-n7OFF	775.1772[a]	775.1769[a]	98.0
PhPr(4Br)-n7OFF	775.1772[a]	775.1764[a]	95.4

PhPr(2I)-n7OFF	801.1814	801.1813	97.6
PhPr(3I)-n7OFF	823.1633[a]	823.1636[a]	97.5
PhPr(4I)-n7OFF	801.1814	801.1820	>99
PhPr(3Br)-Bnc3	785.1980[b]	785.1984[b]	95.1
Nap-n7OFF	711.2847	711.2849	>99
Coumarin-n7OFF	745.2538	745.2536	97.7
Coumarin-Bnc3	755.2757	755.2757	96.1

[a][M+Na]$^+$. [b][M+K]$^+$.

Bacterial reporter strains.

All peptides were evaluated for *agr* inhibitory activity using the reporter strains AH1677, AH430, AH1747, and AH1872 for *S. aureus* groups-I, -II, -III, and -IV, respectively. These strains each contain the pDB59 plasmid with the yellow fluorescence protein (YFP_{10B}) gene under control of the P3 promoter.[4] In these strains, reductions or increases in fluorescence (relative to background fluorescence levels) can be interpreted as inhibition or activation of the *agr* system, respectively.

Fluorescence *agr* reporter assay protocol.

The reporter assay protocol is based on our previously reported method.[3] Briefly, the bacterial strain was grown at 37 °C with shaking at 220 rpm overnight in BHI medium with 10 μg/mL chloramphenicol. The peptides to be tested were serial diluted 1:3 in DMSO, and 2-μL aliquots of each of these serial dilutions were added to 96-well black plates. The overnight bacterial culture was diluted 1:50 with BHI medium, and 198-μL aliquots of culture were added to each well. The plate was incubated at 37 °C with shaking at 1000 rpm for 24 h. No compound displayed any appreciable effect on cell growth over the time course of these assays (see Figure S5A below for representative data).

The OD_{600} and fluorescence of each well was measured using a plate reader, and the data was normalized to vehicle (100%) and media (0%) controls. Dose response activity curves were generated from these normalized values using GraphPad Prism software (v. 9) and are shown in Figures S1–S4. The IC_{50} values and 95% confidence intervals were determined by Prism using a non-linear regression fit ([compound] vs. percent activity, 4-parameters) and listed in Table 1 (in the main text) and Table S2.

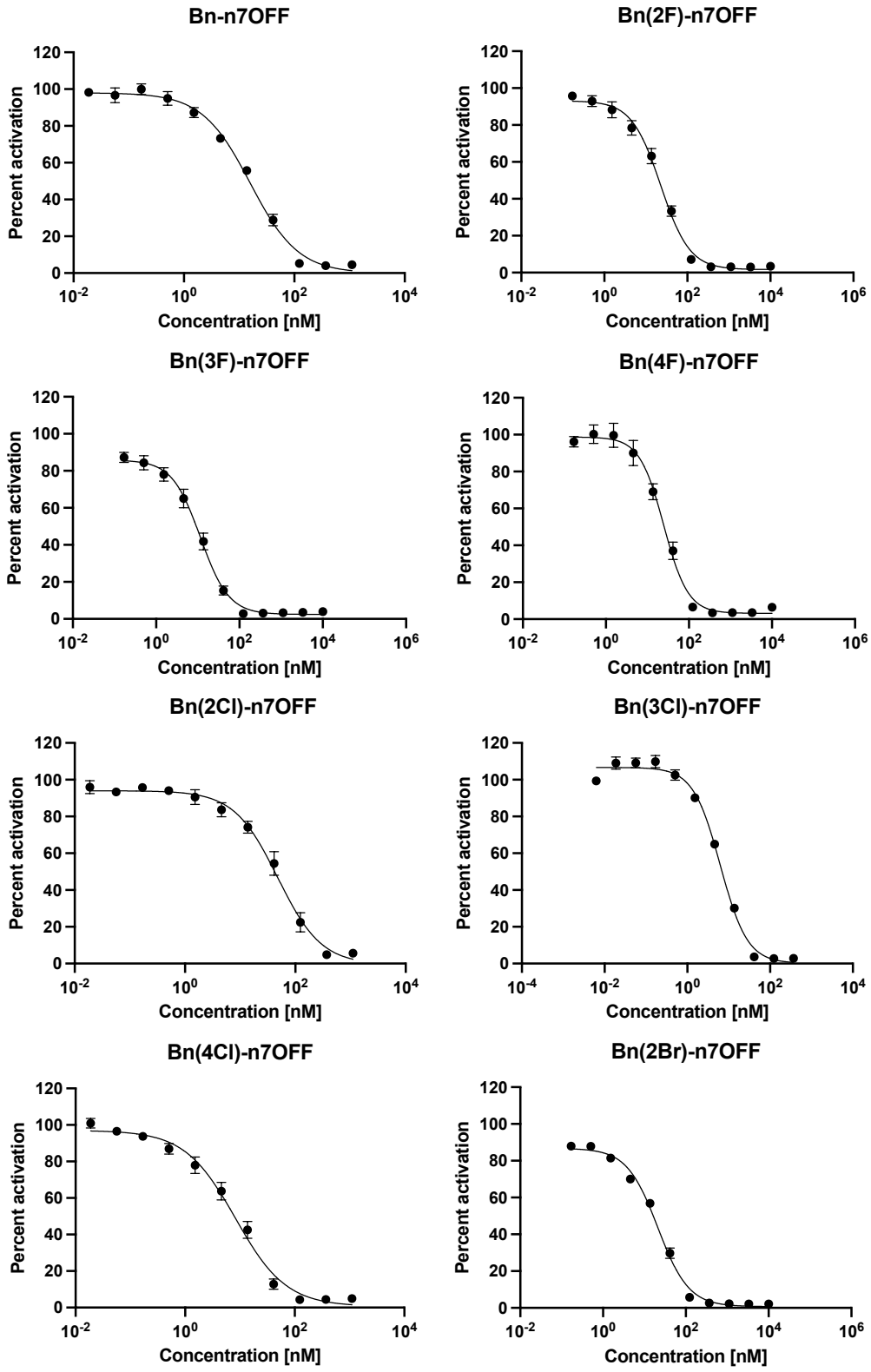


Figure S1. Dose response *agr* activity curves in group-I *S. aureus* (AH1677). Compound names are indicated at the top of each plot. All experiments performed in three technical replicates on three separate days. The values on the curves are the average of the replicates; error bars represent SEM.

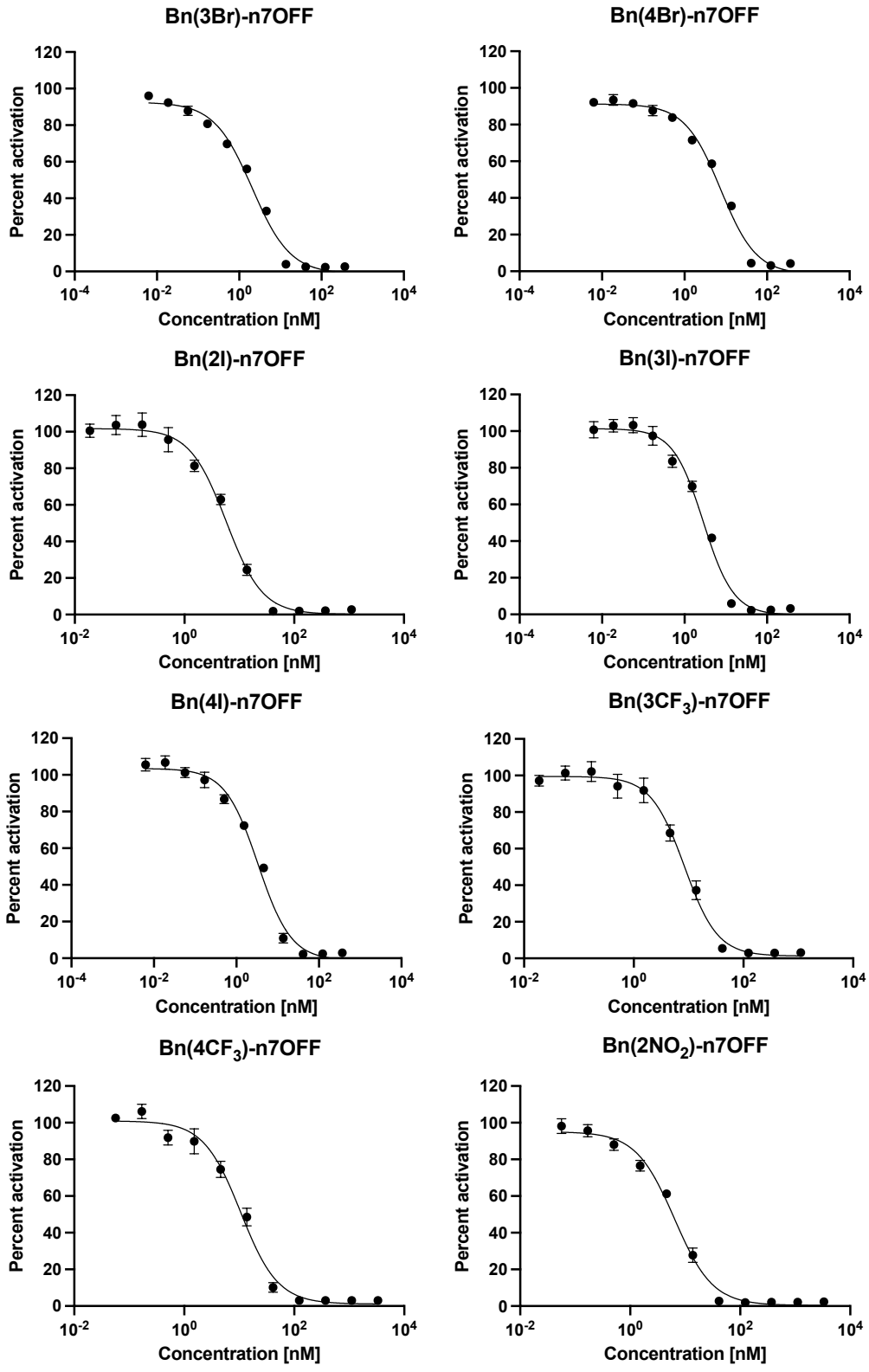
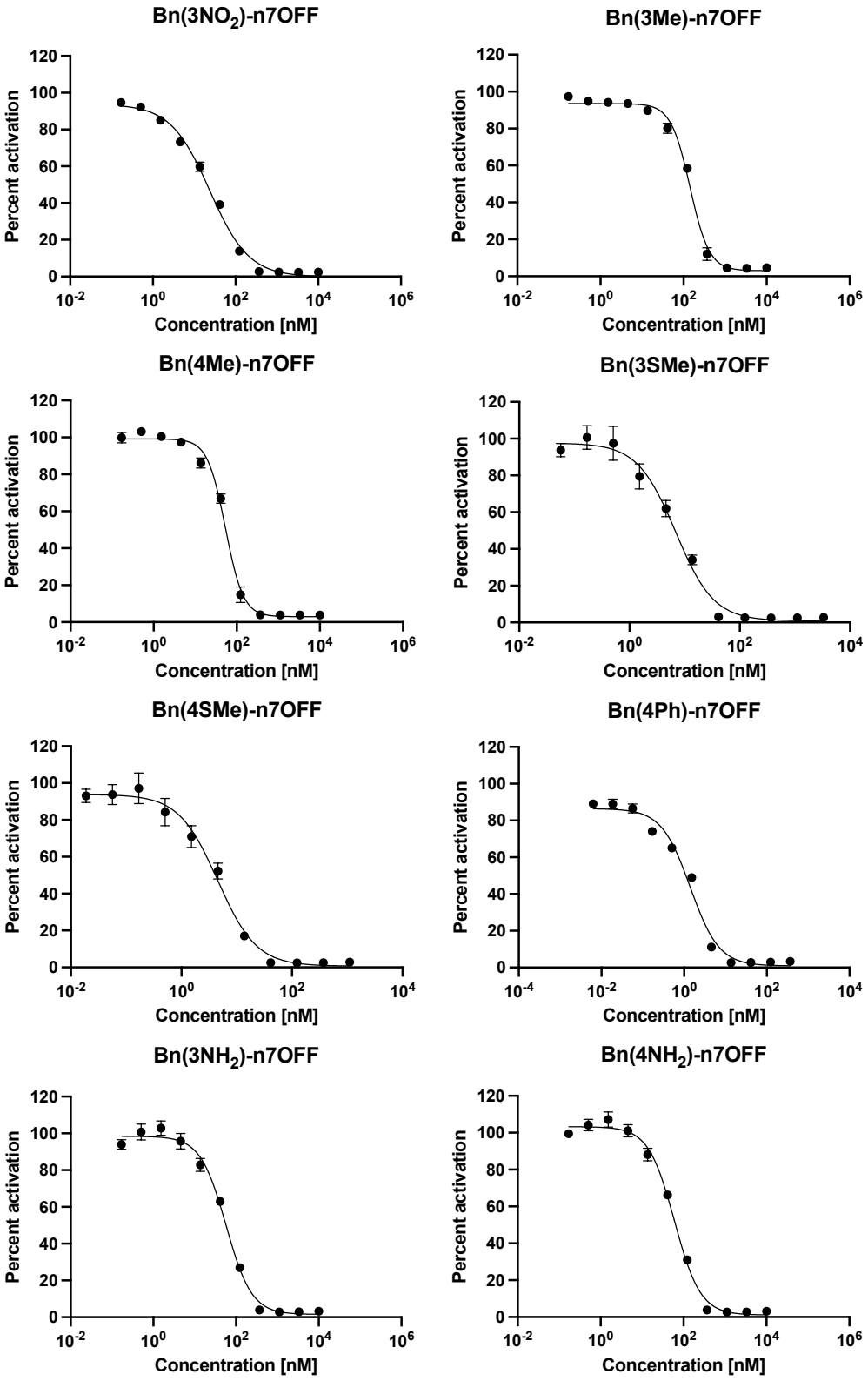


Figure S1. *(continued)* See legend above.



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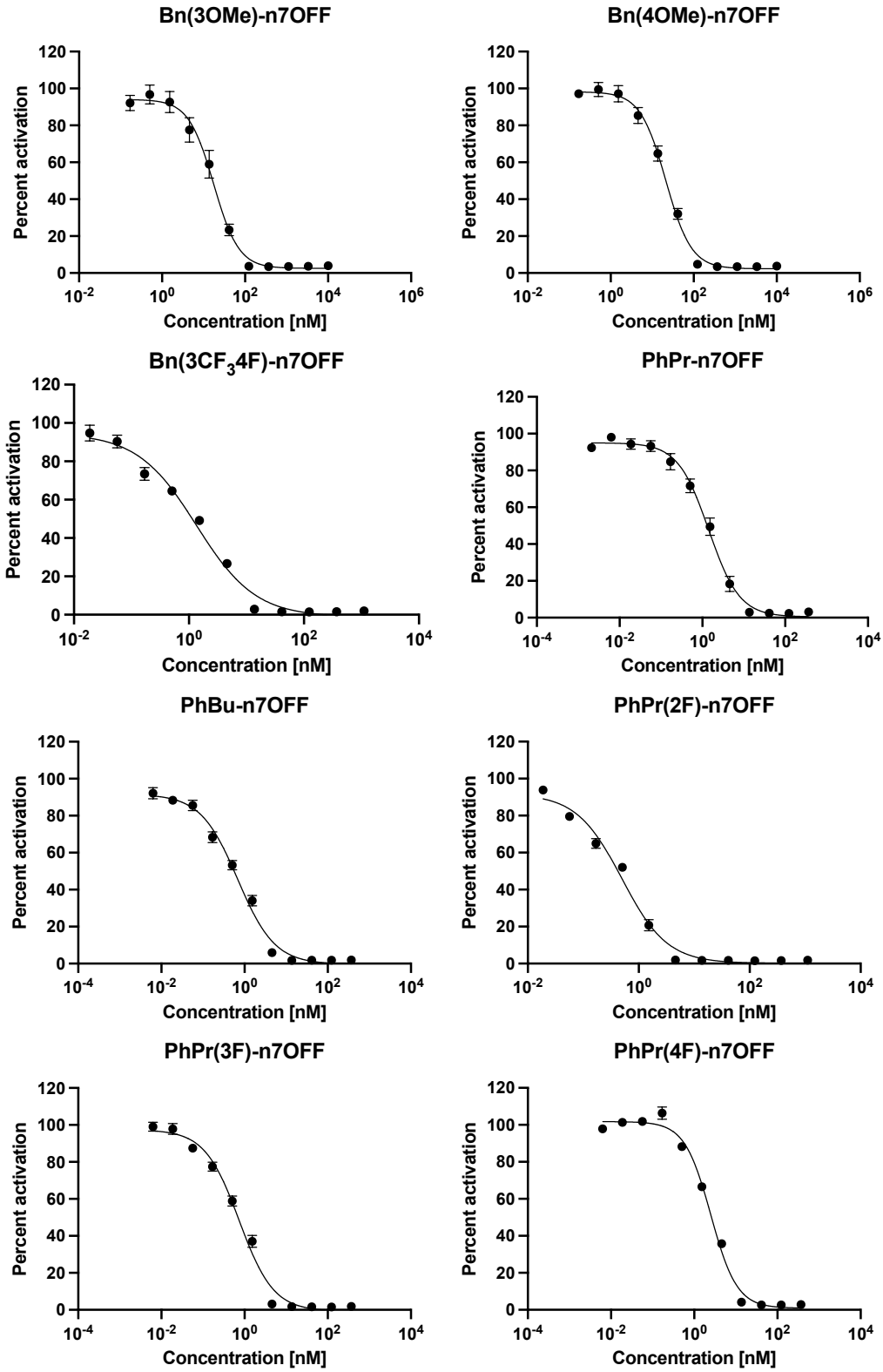

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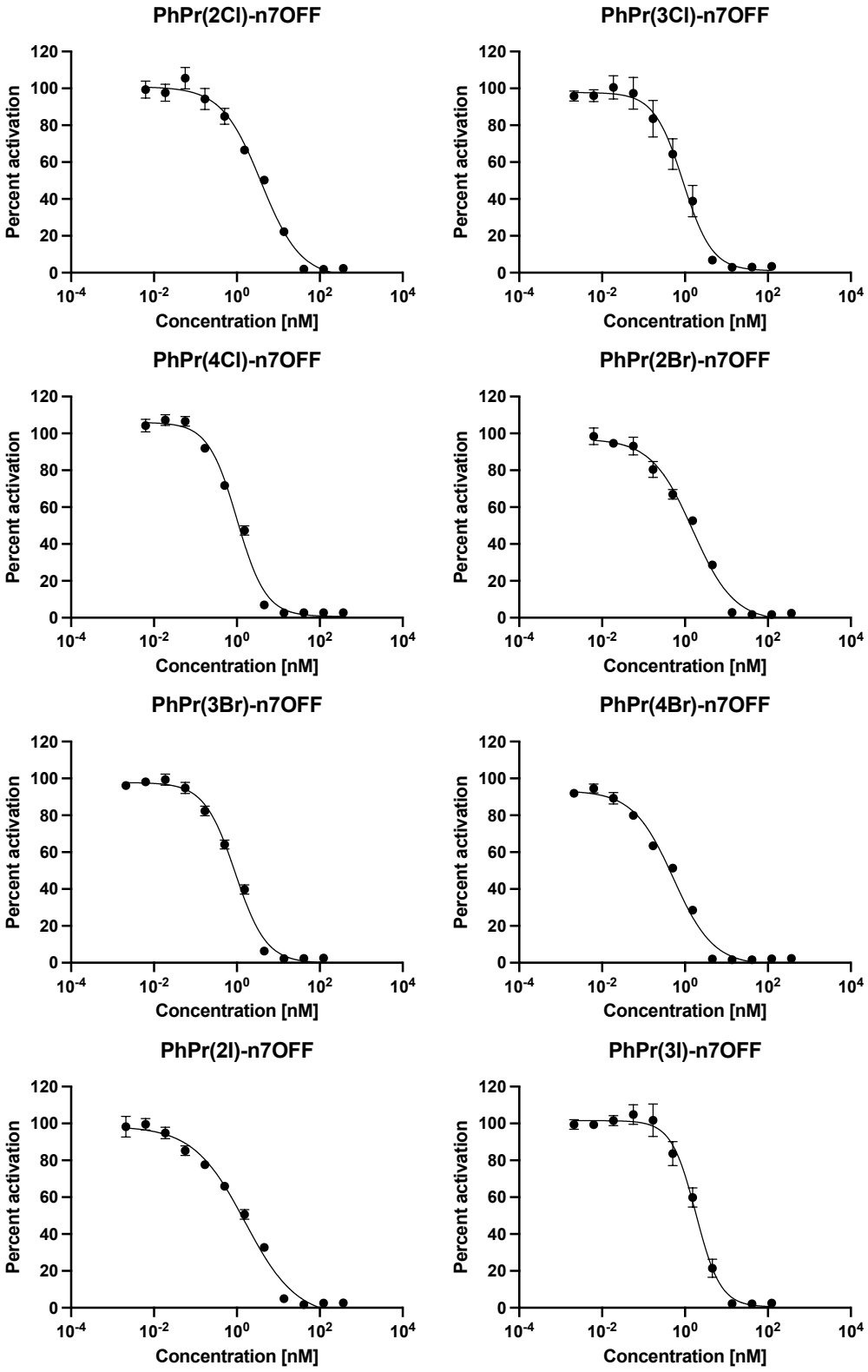

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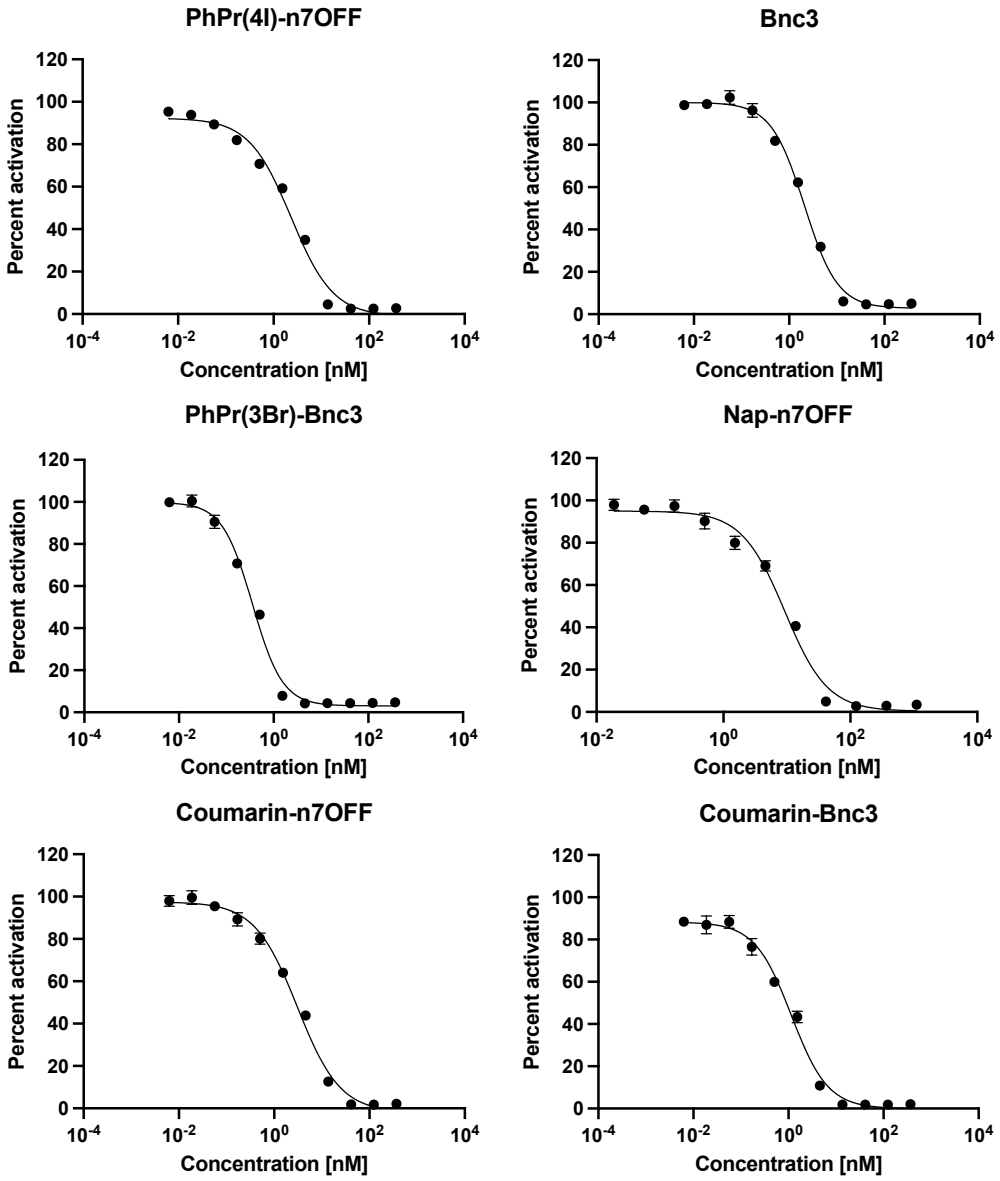

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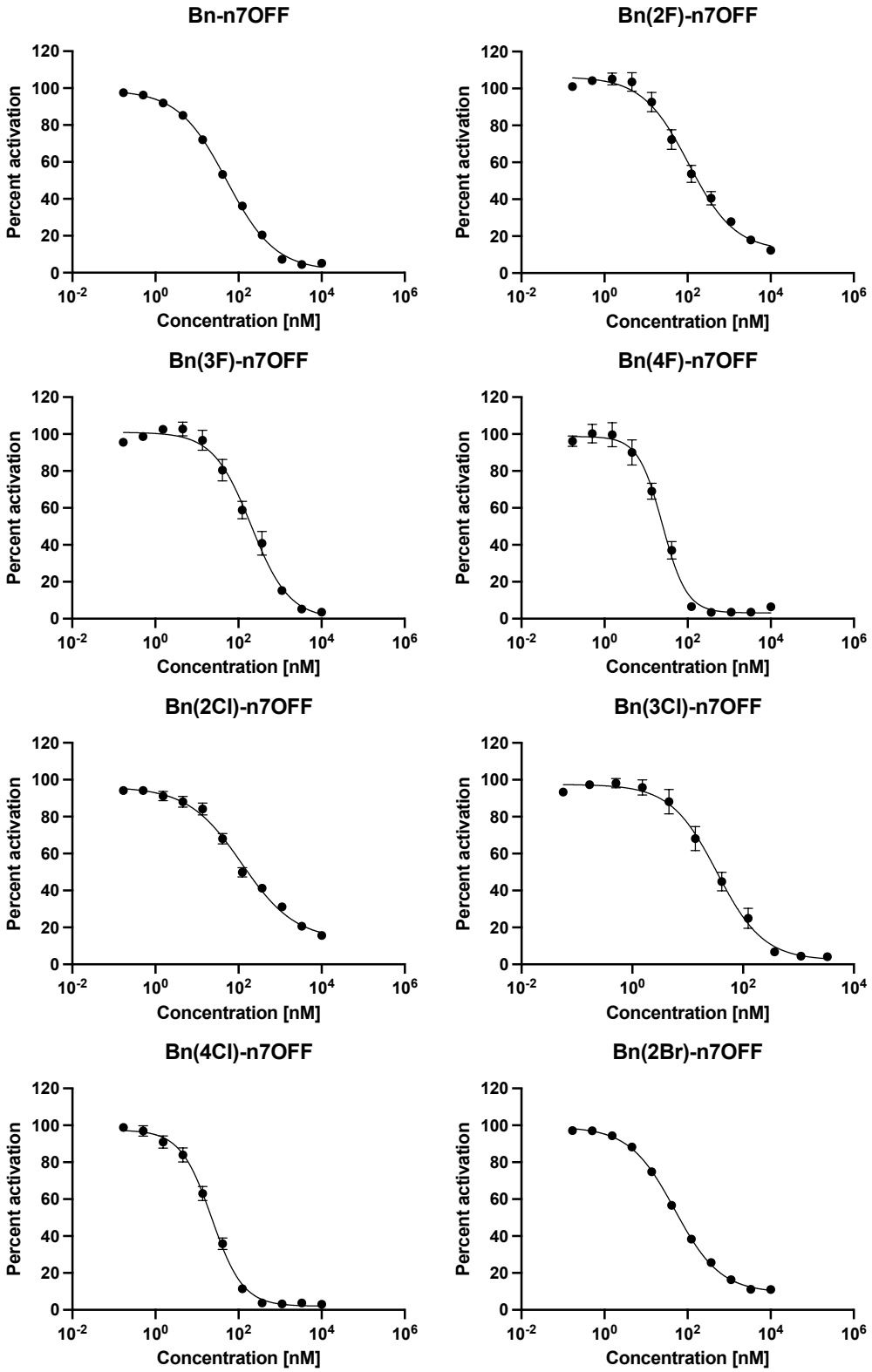
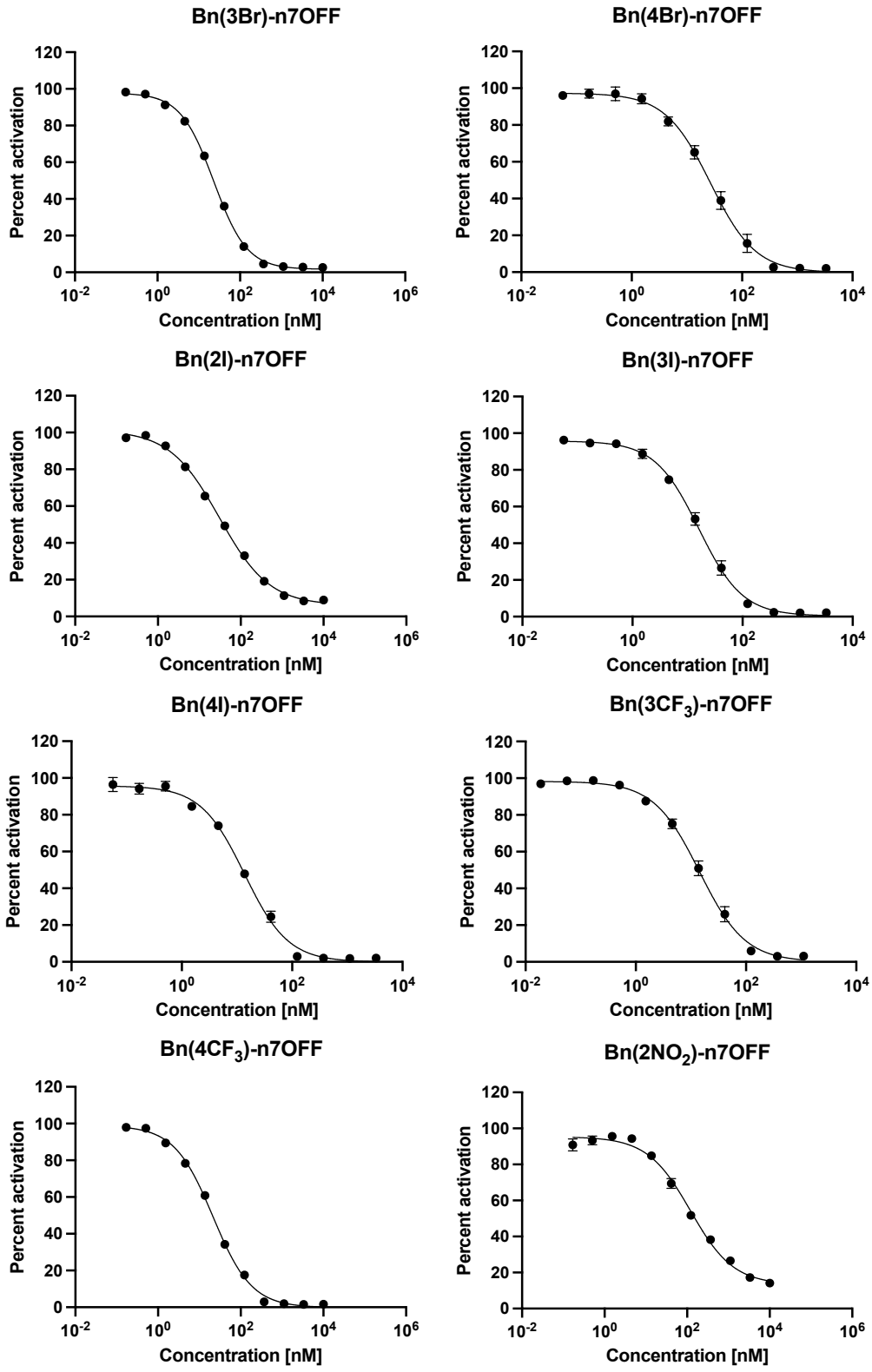


Figure S2. Dose response *agr* activity curves in group-II *S. aureus* (AH430). Compound names are indicated at the top of each plot. All experiments performed in three technical replicates on three separate days. The values on the curves are the average of the replicates; error bars represent SEM.



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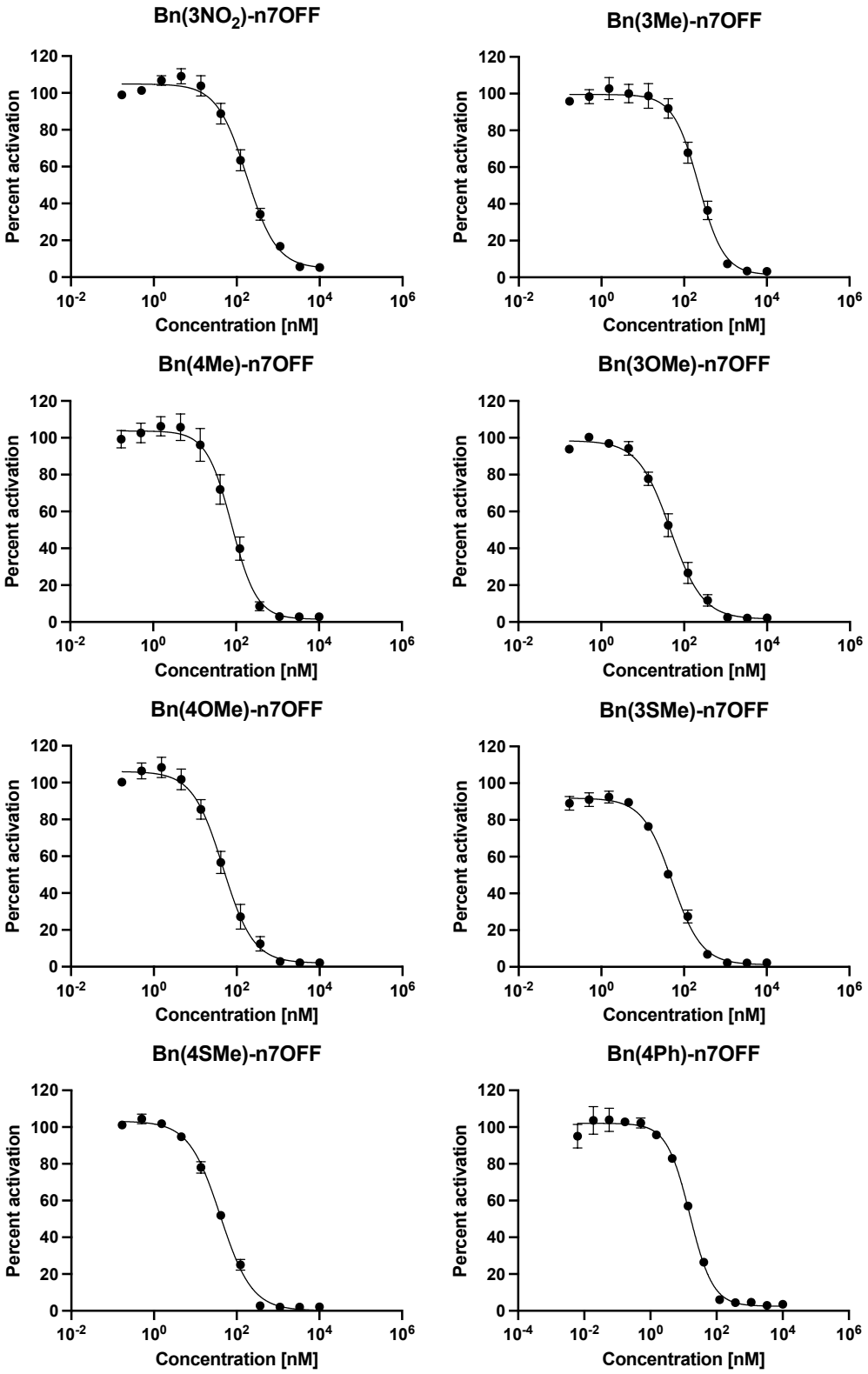

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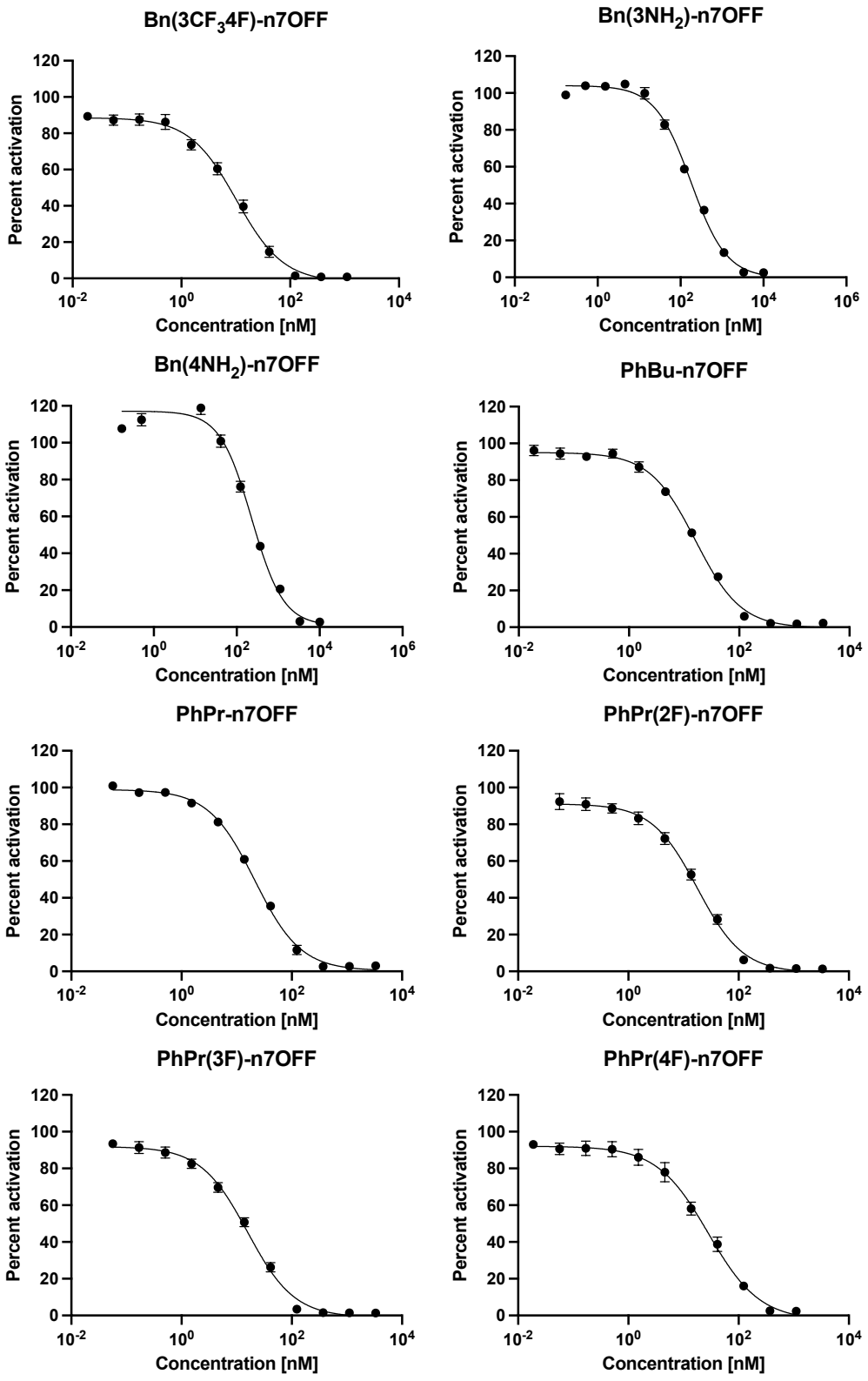

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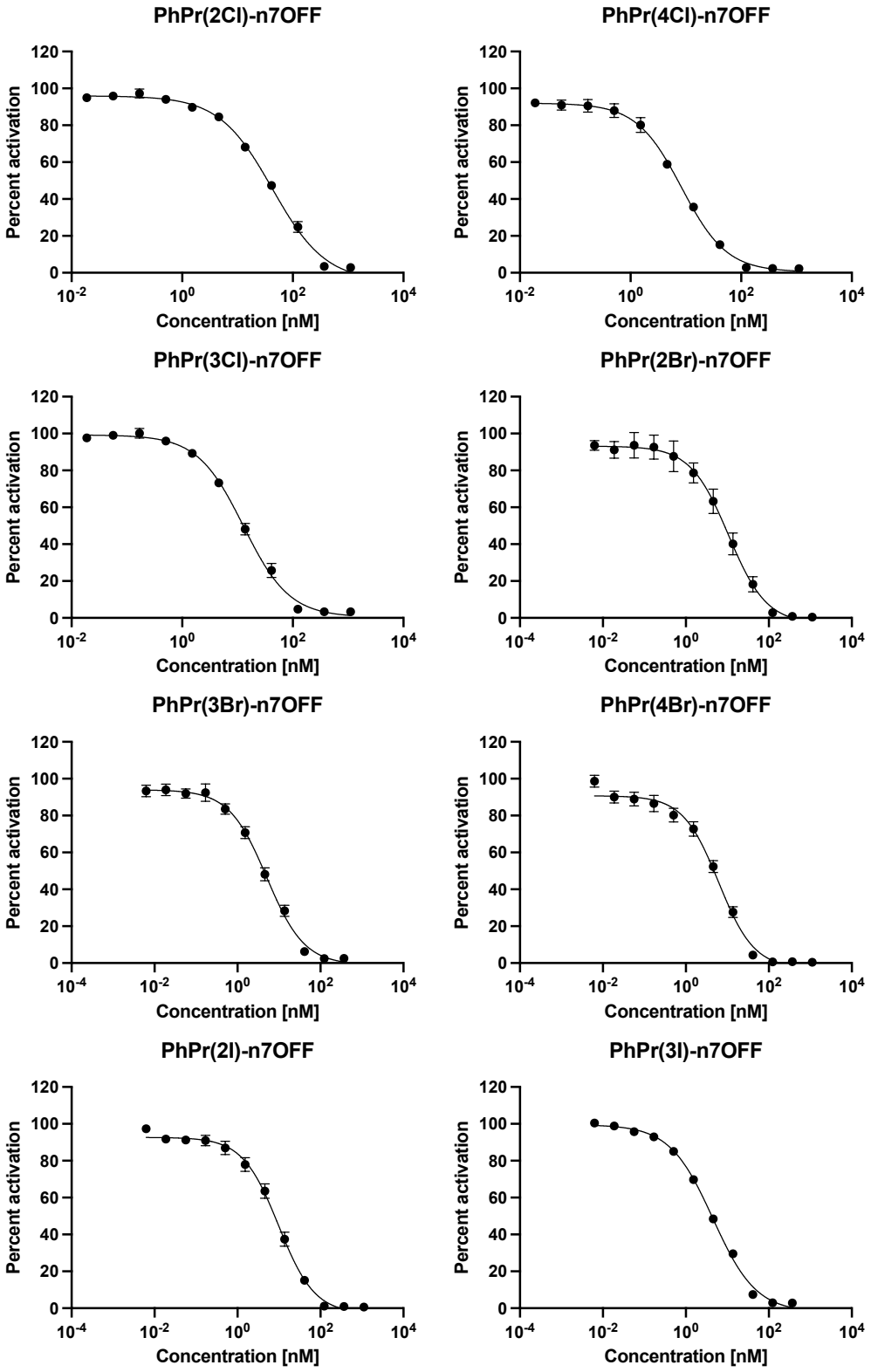

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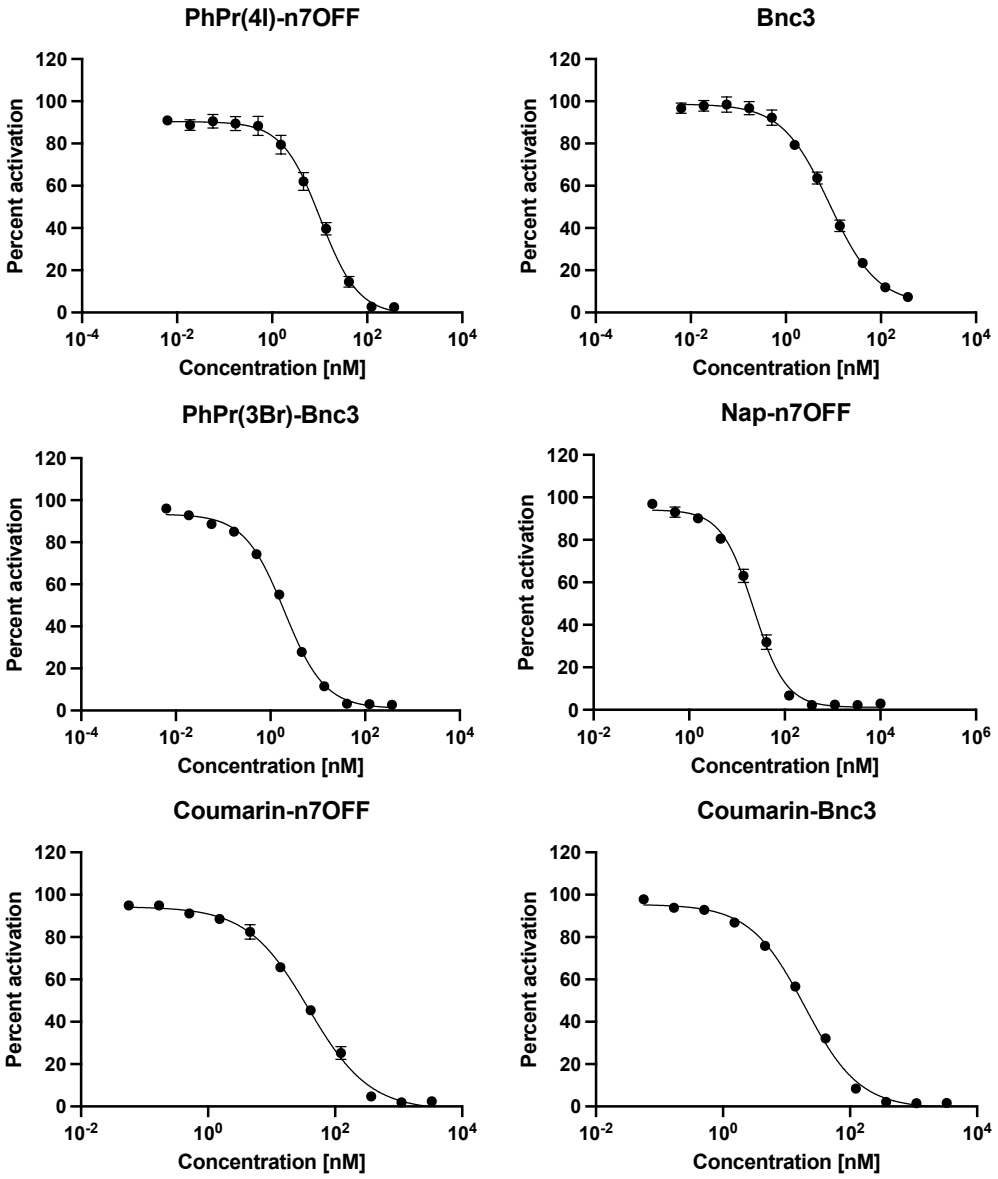

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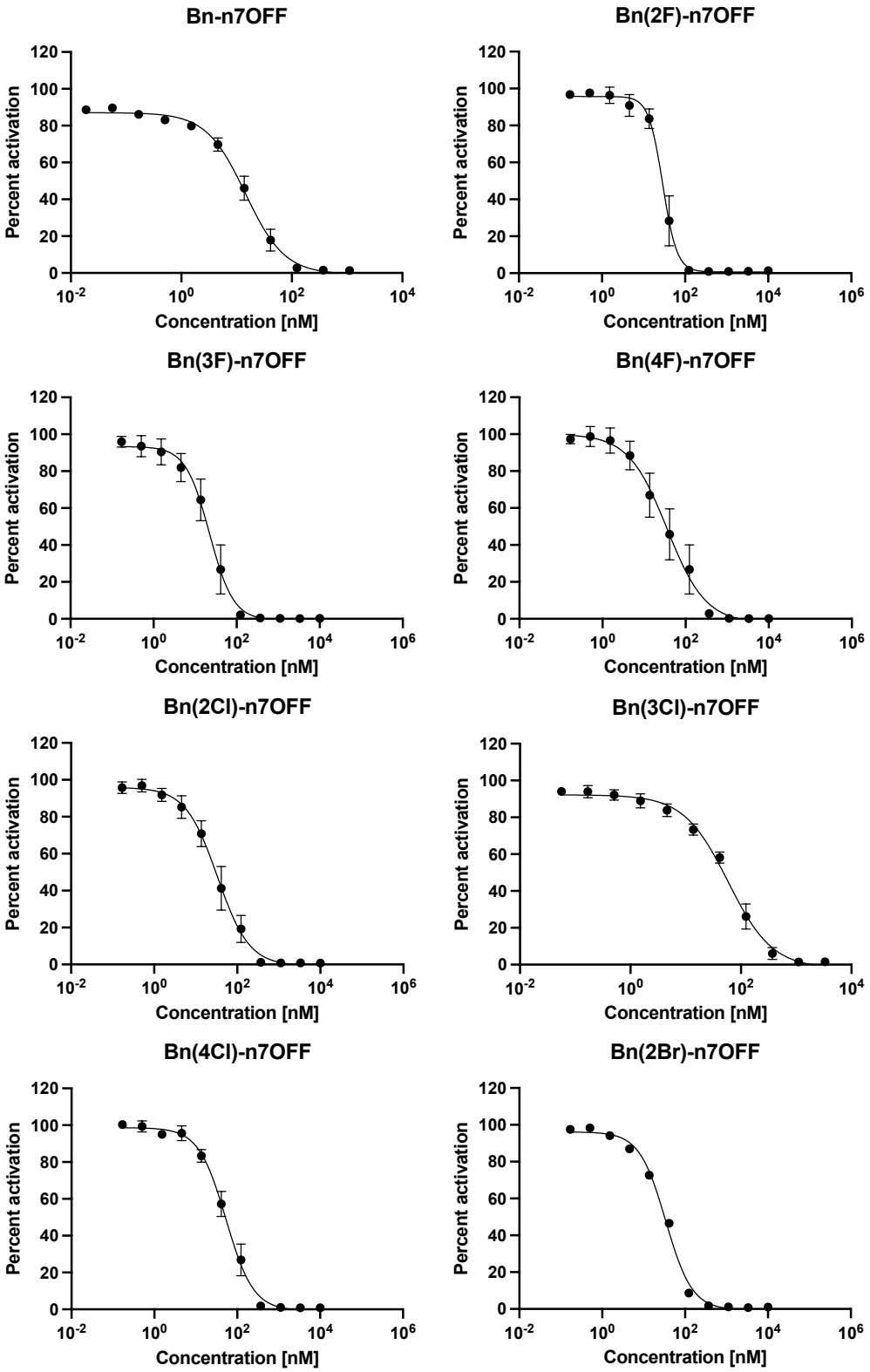
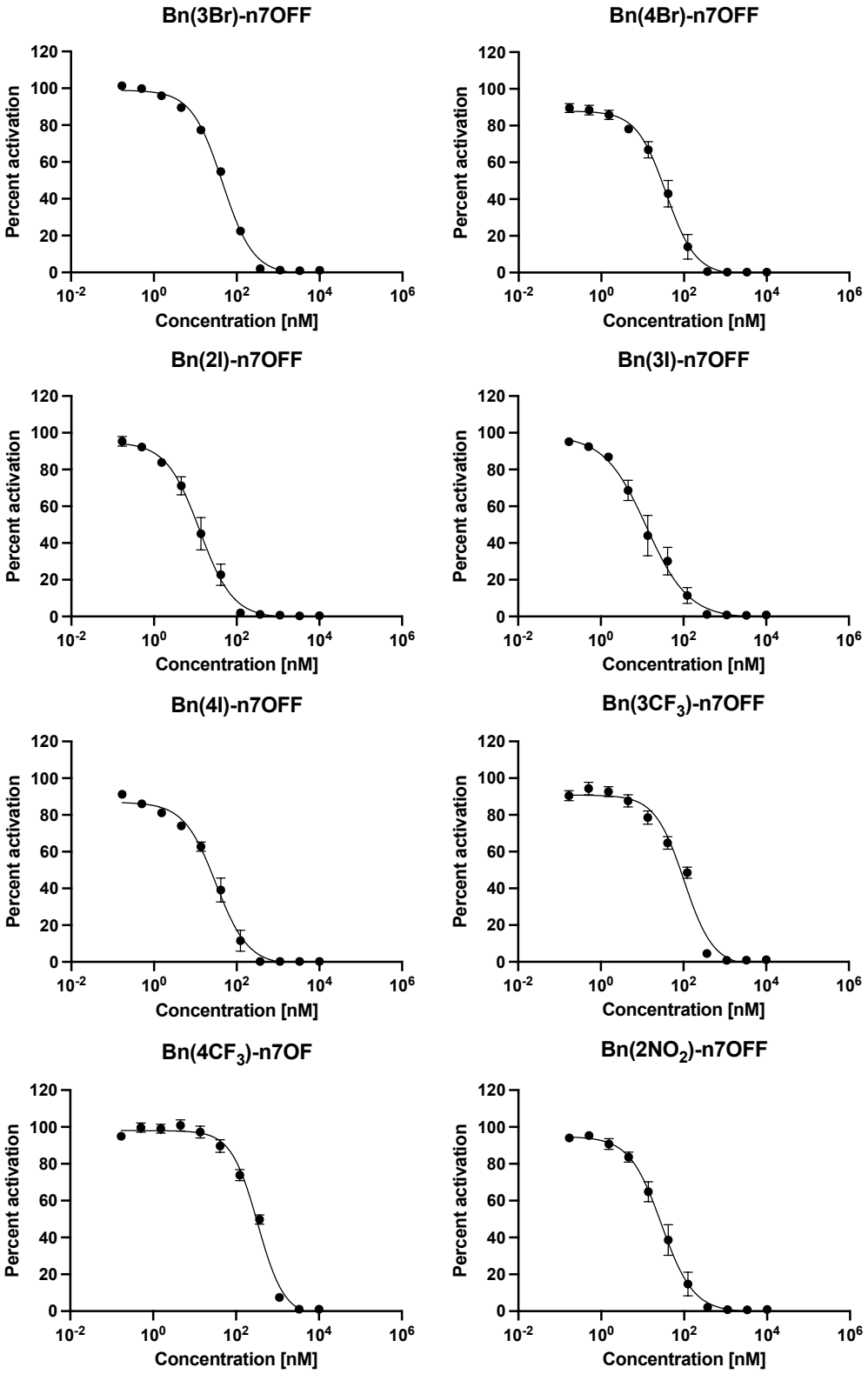


Figure S3. Dose response *agr* activity curves in group-III *S. aureus* (AH1747). Compound names are indicated at the top of each plot. All experiments performed in three technical replicates on three separate days. The values on the curves are the average of the replicates; error bars represent SEM.



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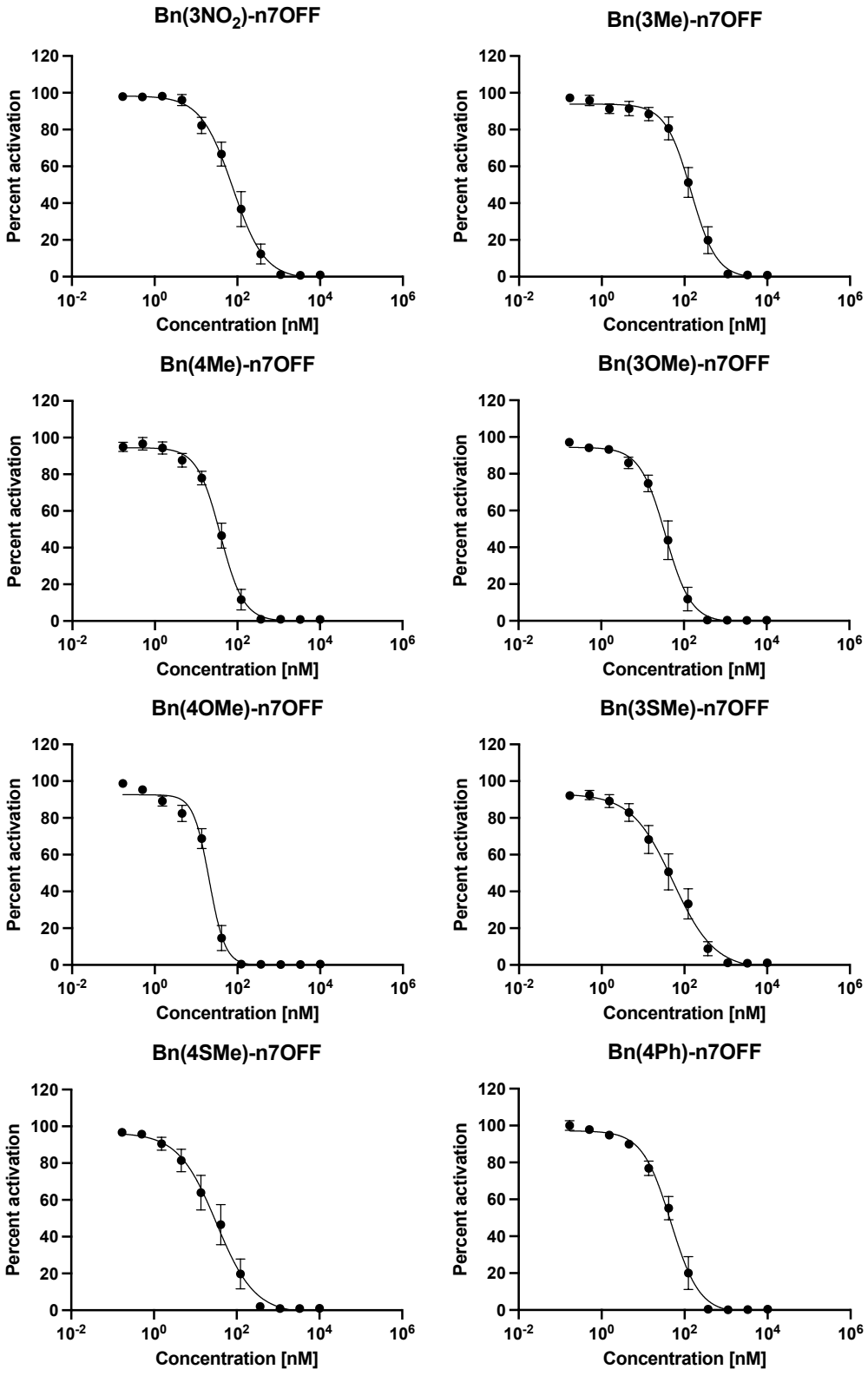

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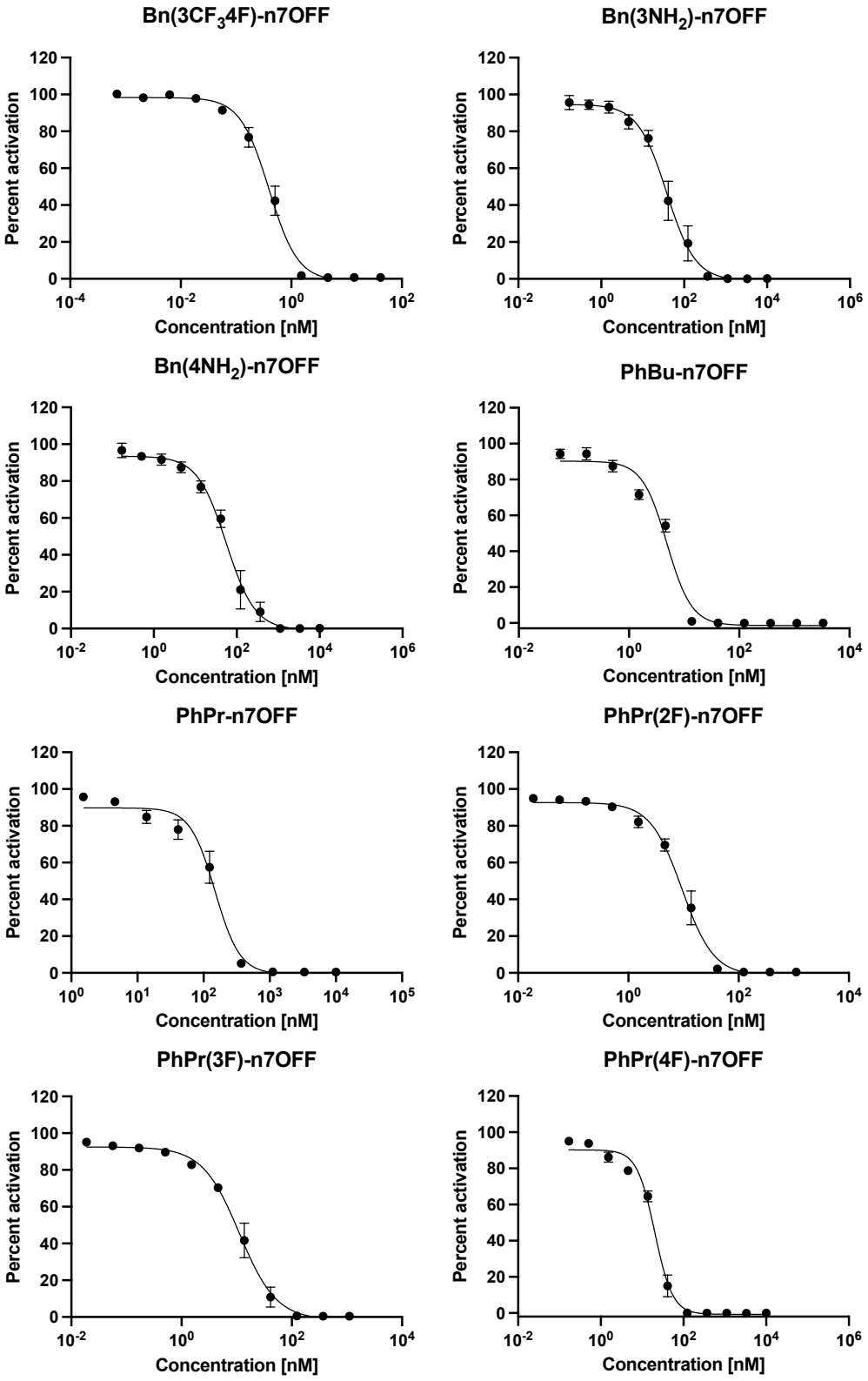

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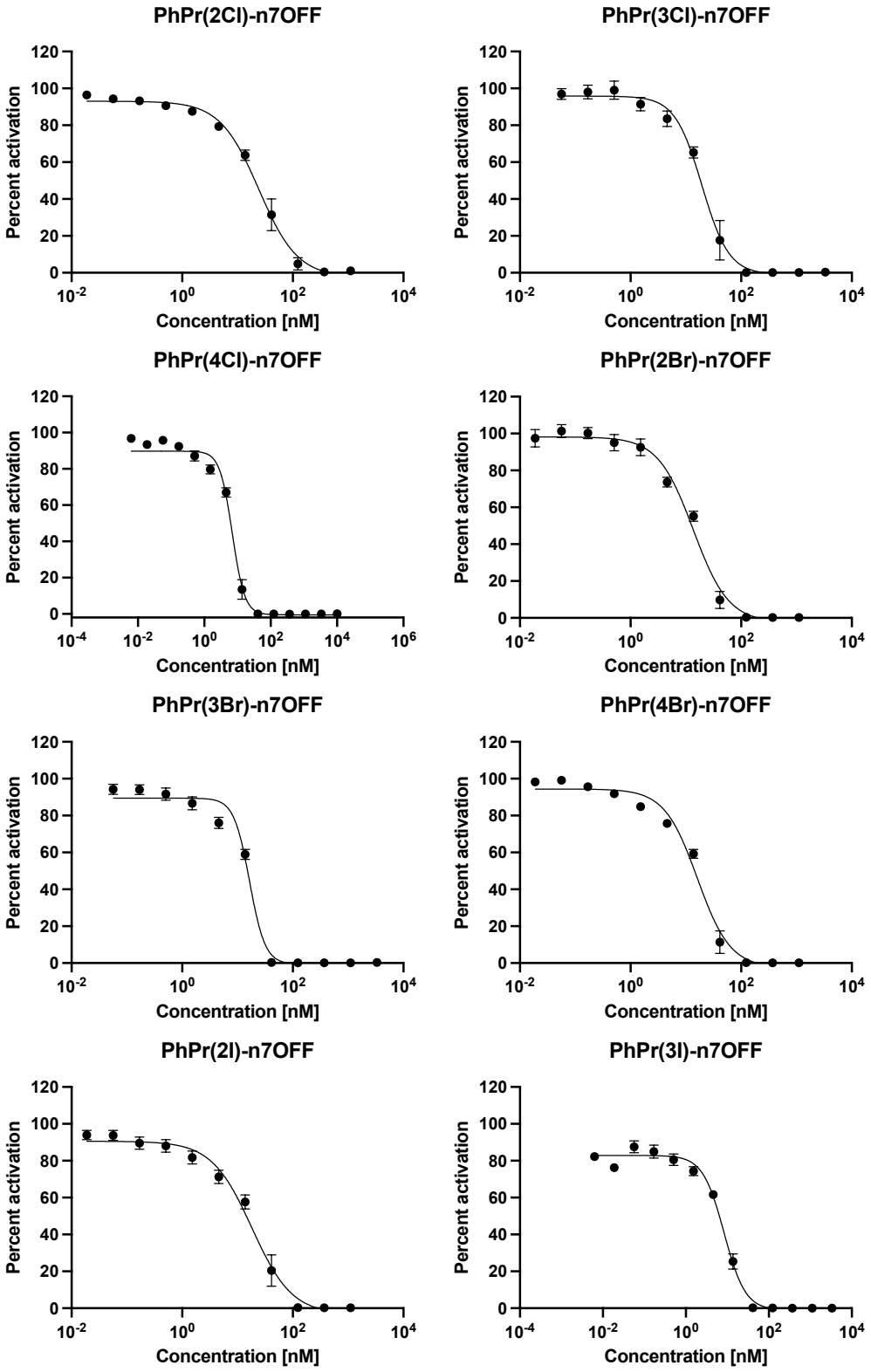

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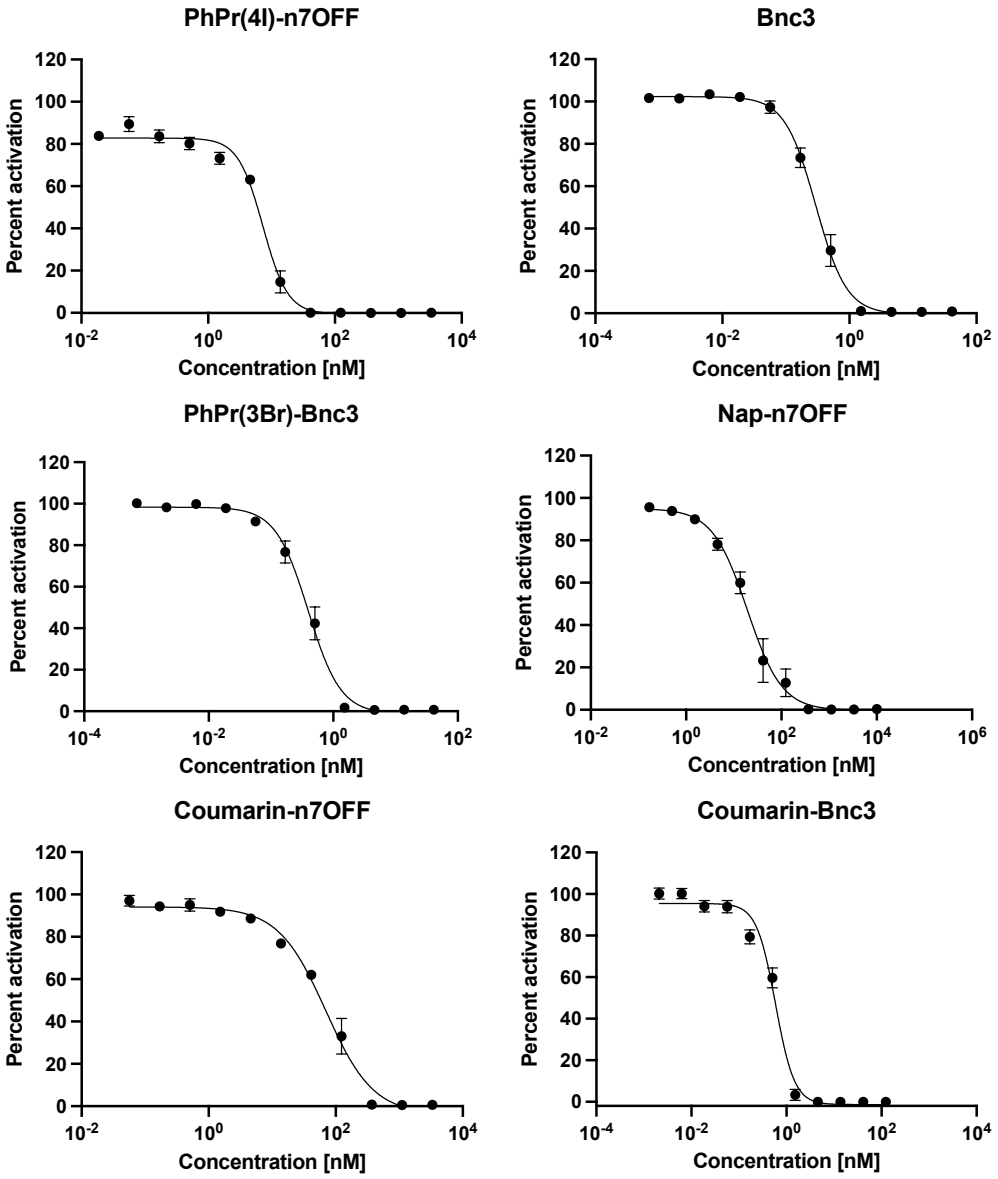

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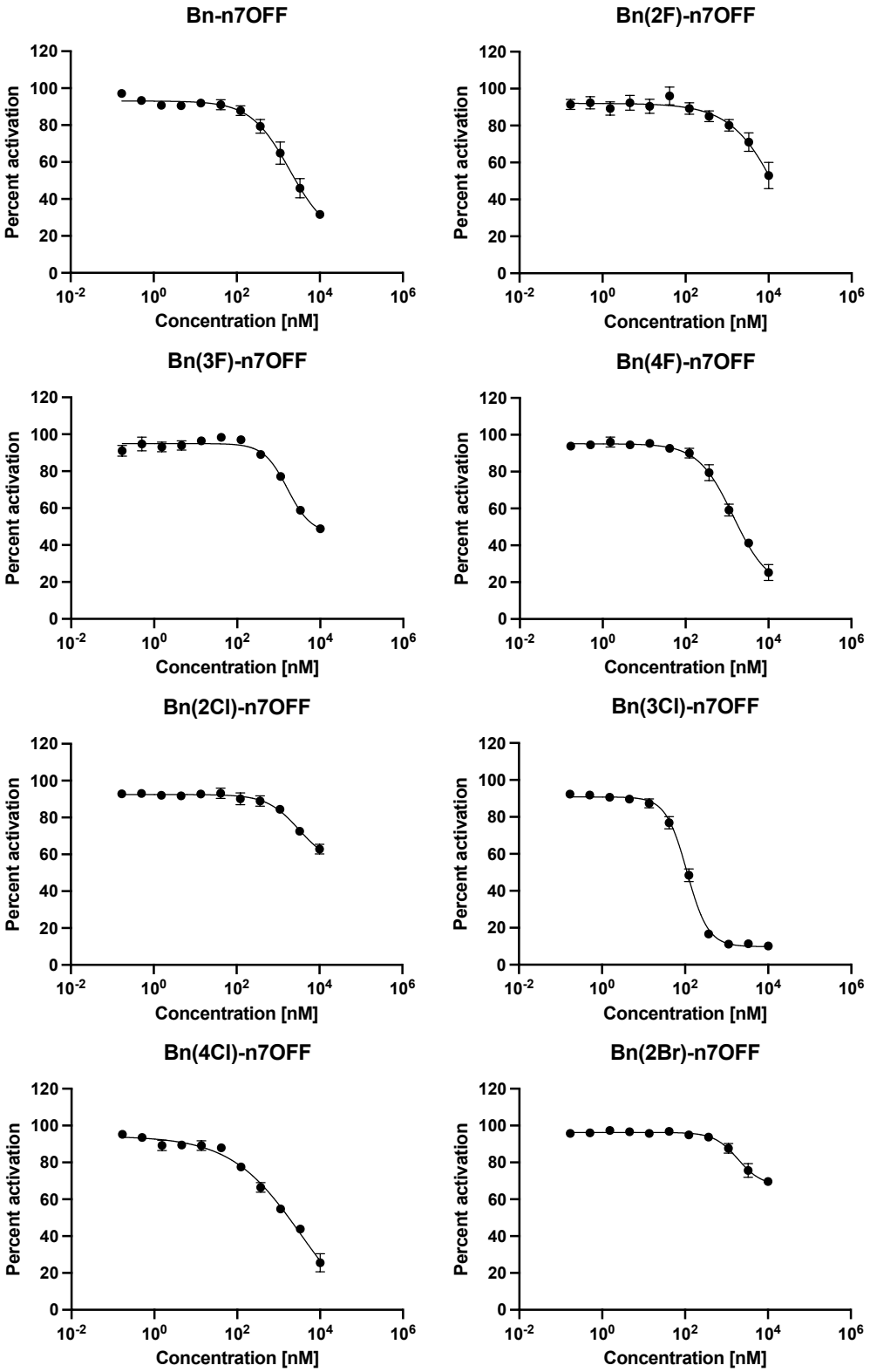
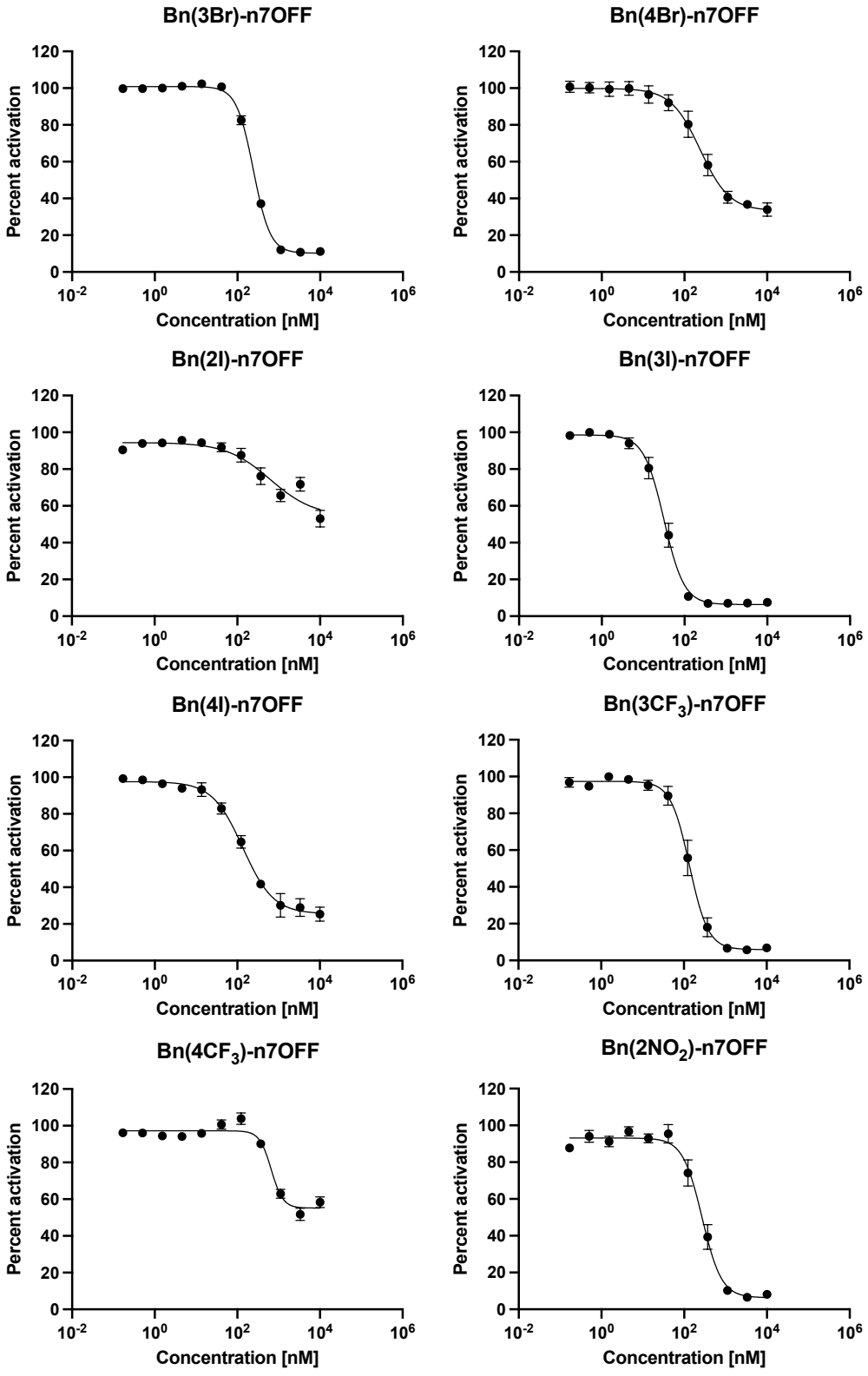


Figure S4. Dose response *agr* activity curves in group-IV *S. aureus* (AH1872). Compound names are indicated at the top of each plot. All experiments performed in three technical replicates on three separate days. The values on the curves are the average of the replicates; error bars represent SEM.



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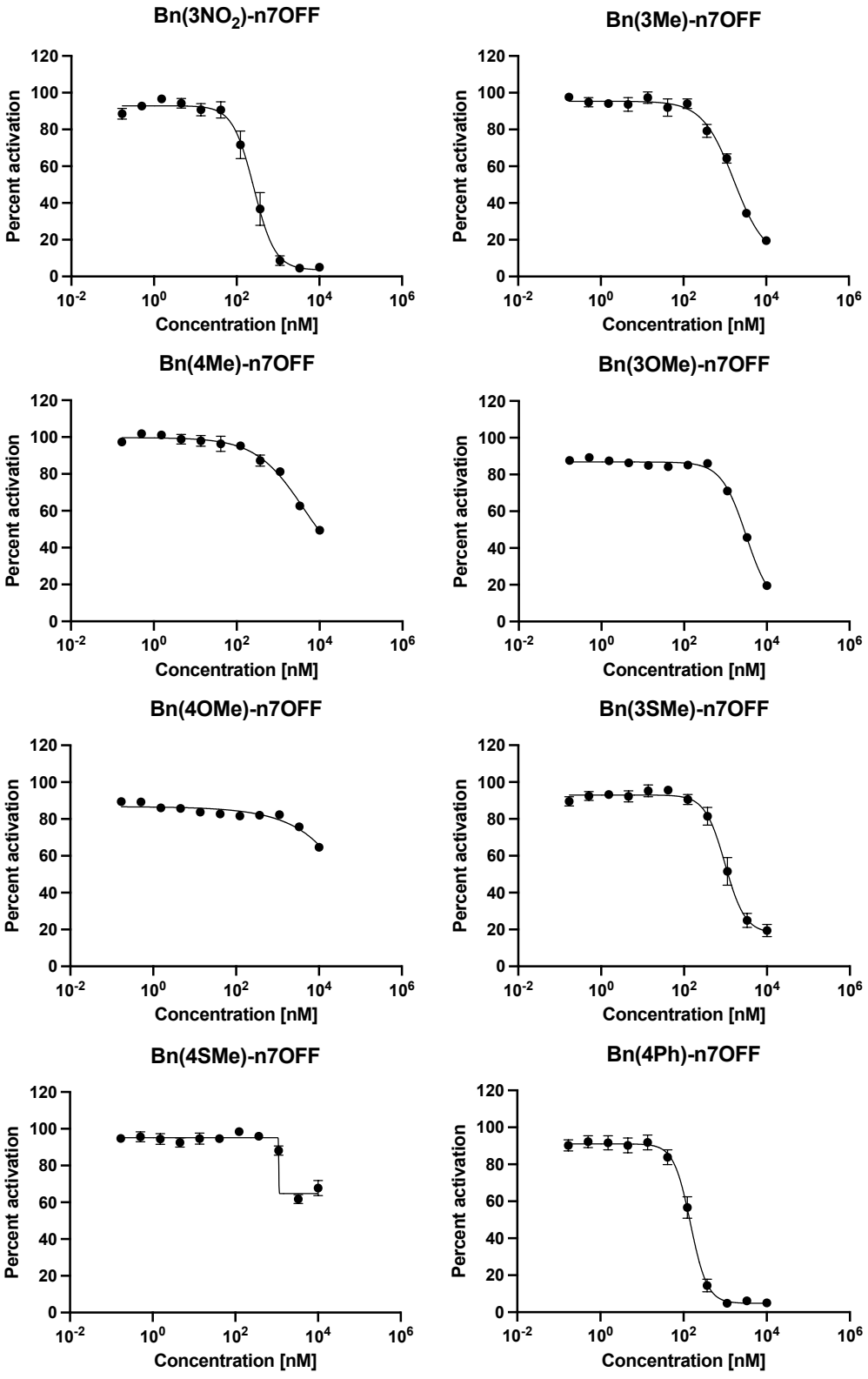

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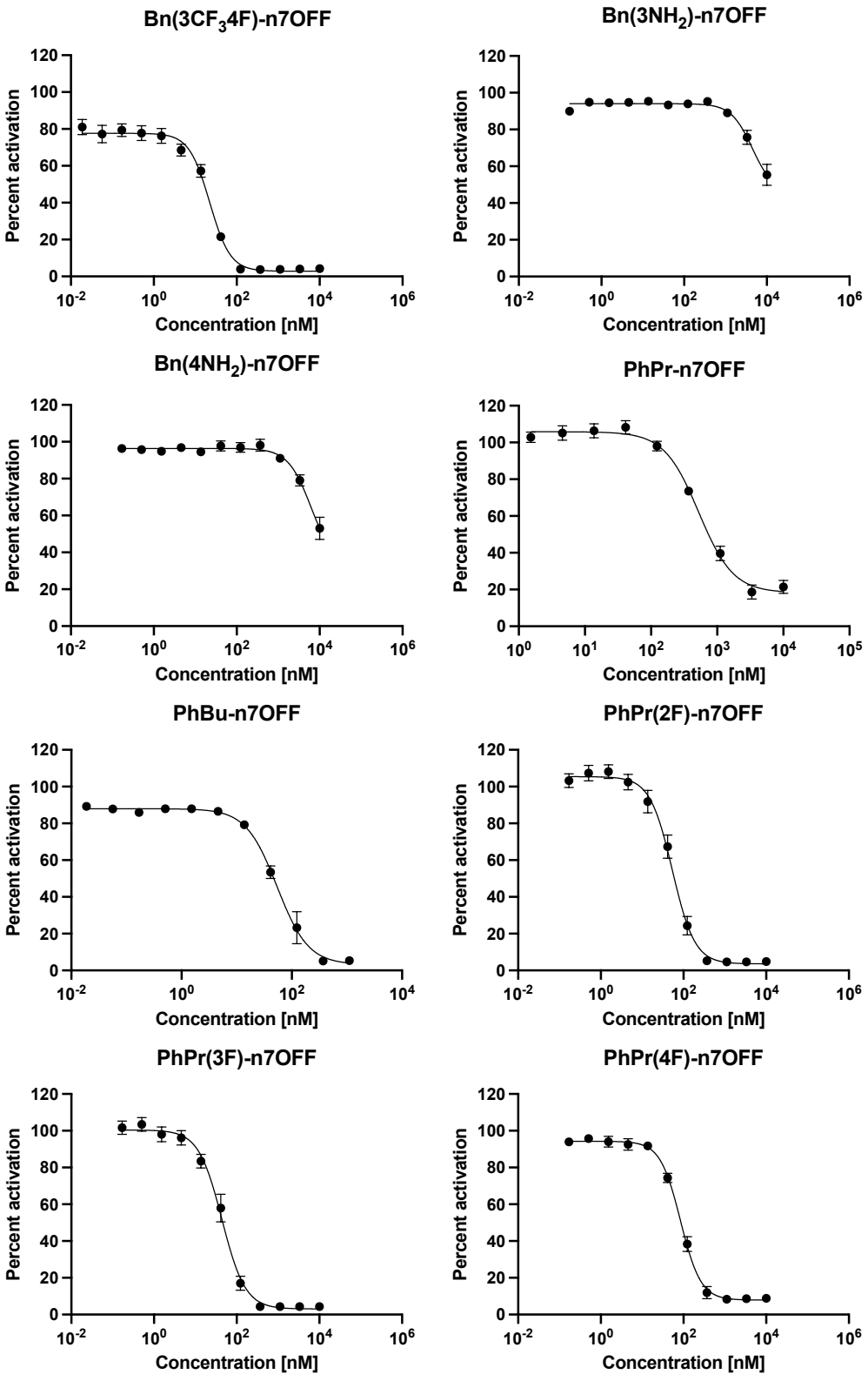

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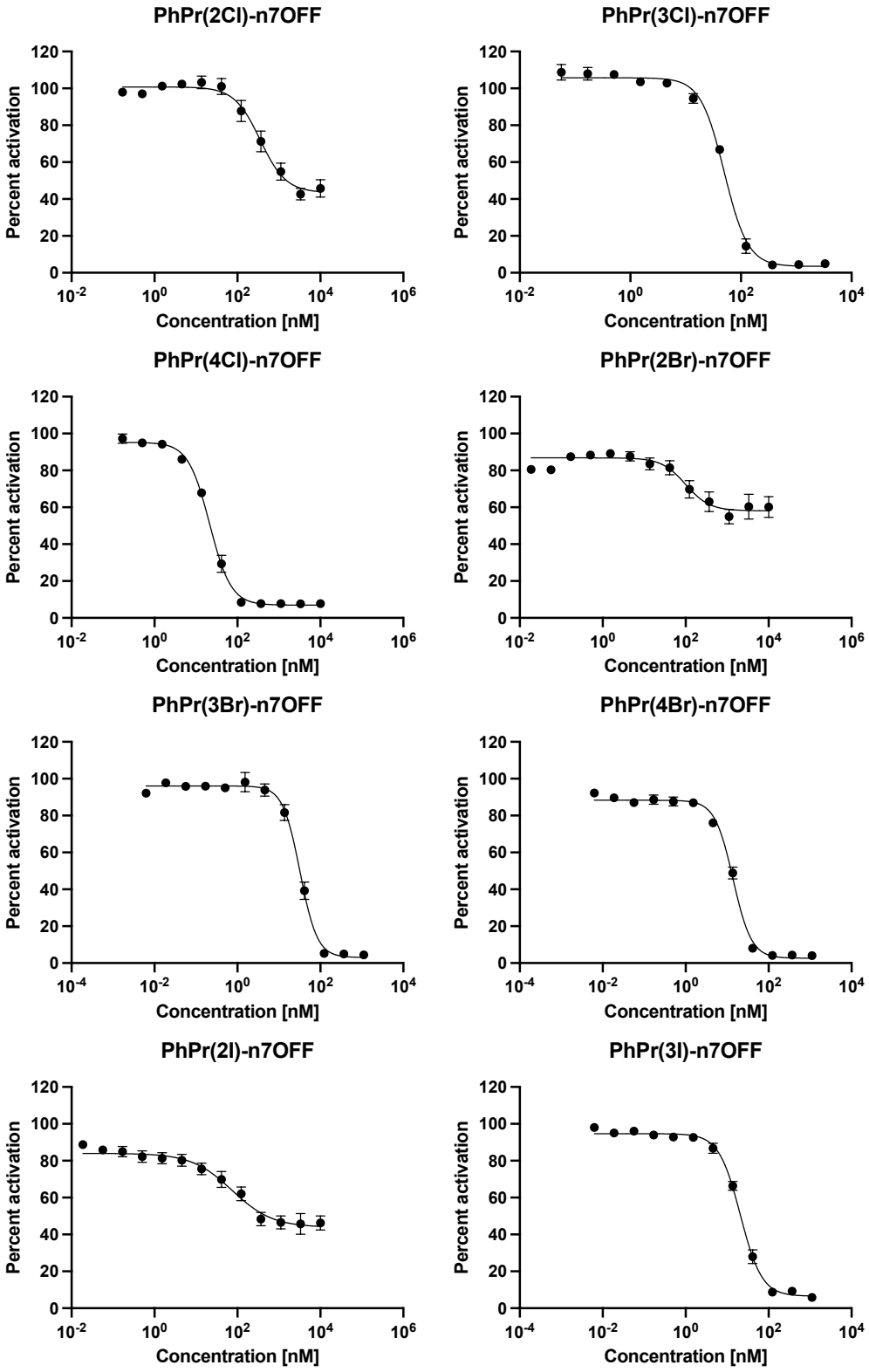

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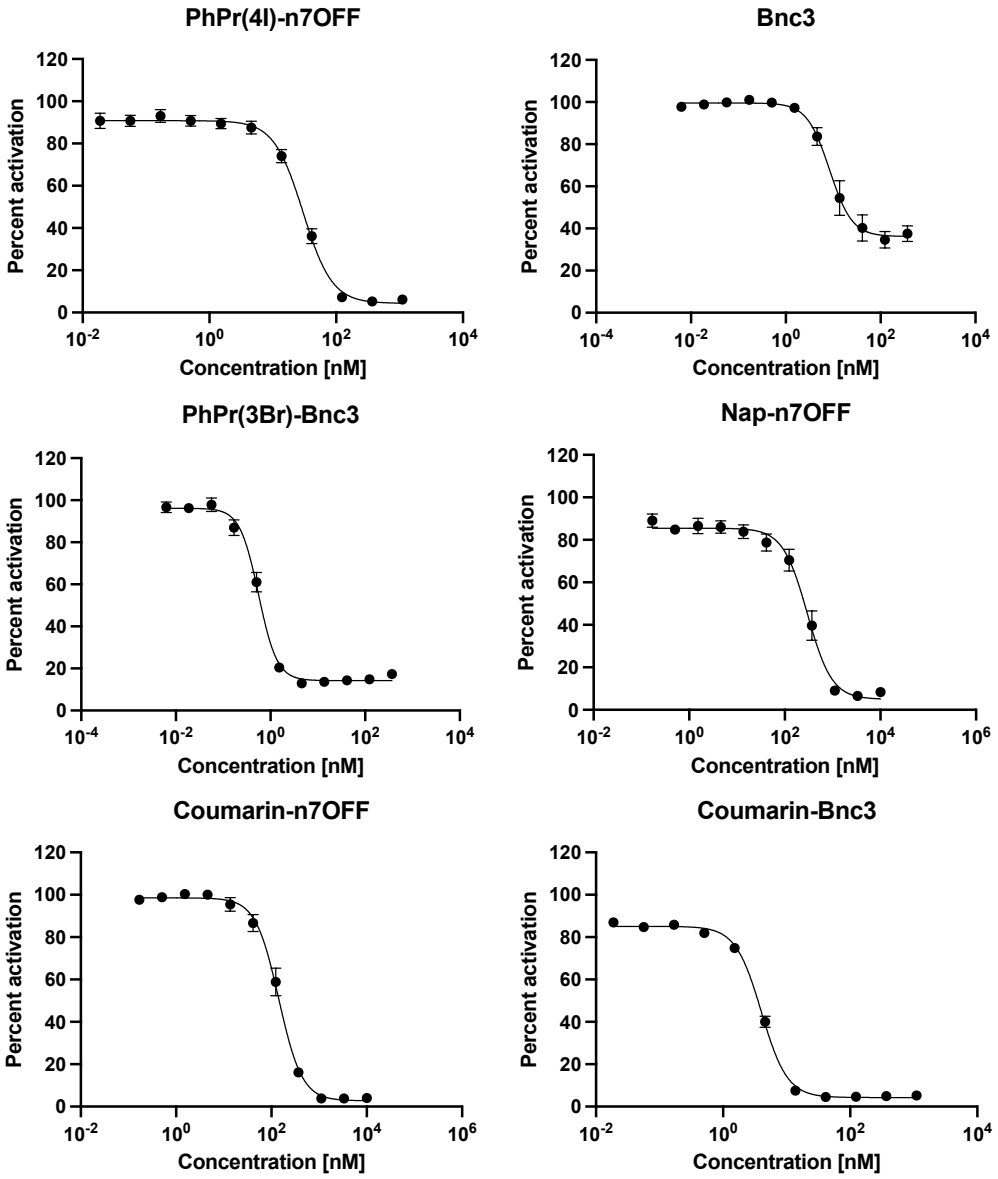


Figure S4. *(continued)* See legend above.

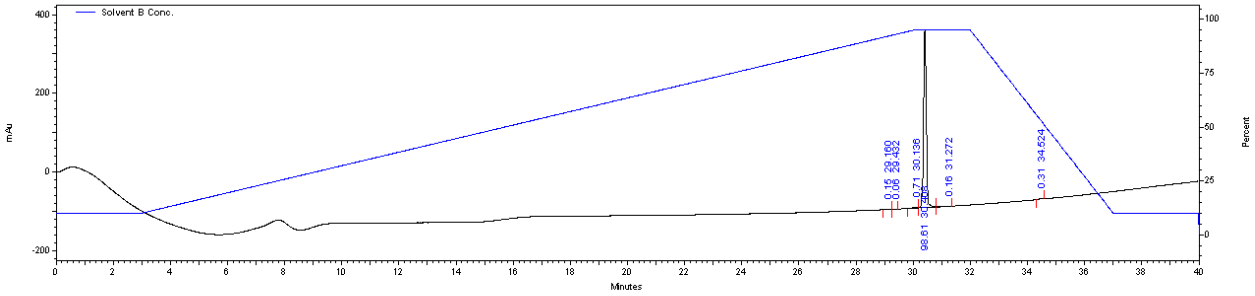
Table S2. Additional IC_{50} values for Bn-n7OFF and Bnc3 analogues in the group I–IV *S. aureus agr* reporter strains. All units in nM. 95% confidence intervals indicated in parentheses.

compound	group-I	group-II	group-III	group-IV
Bn(2F)-n7OFF	22.7 (19.0-27.0)	106 (72.9-165)	29.0 (23.7-34.9)	>26600[a]
Bn(3F)-n7OFF	11.5 (9.34-14.0)	202 (149-283)	22.2 (15.4-30.9)	1620 (1190-2490)[a]
Bn(4F)-n7OFF	24.7 (20.0-30.4)	24.7 (19.9-30.4)	35.7 (19.6-63.7)	1410 (937-3030)[a]
Bn(2Br)-n7OFF	21.0 (18.6-23.7)	49.2 (43.1-56.3)	34.1 (31.7-36.8)	>1900[a]
Bn(4Br)-n7OFF	7.48 (6.26-8.95)	26.6 (21.7-32.7)	36.9 (28.9-46.6)	240 (164-367)
Bn(2I)-n7OFF	5.75 (4.63-7.07)	30.6 (25.1-37.1)	12.6 (9.52-16.5)	>624[a]
Bn(4I)-n7OFF	3.36 (2.81-4.00)	14.0 (11.8-15.9)	31.5 (25.2-40.0)	136 (102-184)[a]
Bn(4CF_3)-n7OFF	10.7 (8.59-13.1)	21.3 (19.1-23.8)	332 (285-387)	661 (515-826)[a]
Bn(3NO_2)-n7OFF	23.7 (20.5-27.2)	174 (138-222)	77.1 (58.7-101)	260 (206-327)
Bn(3Me)-n7OFF	143 (129-158)	224 (175-287)	143 (112-183)	1650 (1180-2950)[a]
Bn(3OMe)-n7OFF	17.5 (13.6-22.1)	46.6 (37.6-58.0)	35.8 (28.2-45.0)	>3150[a]
Bn(4OMe)-n7OFF	21.2 (18.0-24.9)	46.4 (36.0-60.2)	20.8 (18.1-23.8)	No activity
Bn(3SMe)-n7OFF	6.88 (5.16-9.02)	51.4 (43.8-60.3)	53.0 (34.1-82.0)	977 (768-1290)[a]
Bn(3NH_2)-n7OFF	57.3 (49.2-66.5)	177 (153-207)	37.7 (28.0-50.7)	>4490[a]
Bn(4NH_2)-n7OFF	60.4 (52.8-66.9)	229 (192-276)	55.8 (42.6-72.6)	>6330[a]
PhPr(4F)-n7OFF	2.50 (2.25-2.79)	28.2 (21.0-39.3)	20.2 (17.8-22.8)	84.6 (74.2-96.2)[a]
PhPr(2Cl)-n7OFF	3.84 (3.90-5.00)	42.6 (35.5-52.4)	24.2 (19.8-29.8)	363 (239-541)[a]
PhPr(2Br)-n7OFF	1.53 (1.20-1.95)	10.7 (6.95-17.3)	13.6 (11.2-16.4)	No activity
PhPr(2I)-n7OFF	1.57 (1.20-2.05)	9.78 (7.94-12.2)	18.2 (14.1-23.3)	No activity
PhPr(4I)-n7OFF	2.32 (1.99-2.71)	10.3 (8.08-13.2)	7.29 (6.44-8.27)	30.0 (26.3-34.0)

[a] Compound unable to fully inhibit *agr* over concentrations tested.

Analytical HPLC traces.

Bn(2F)-n7OFF



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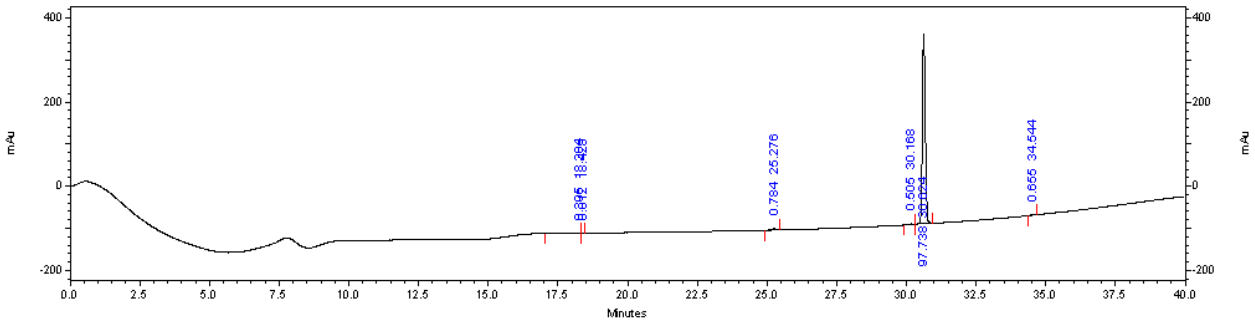

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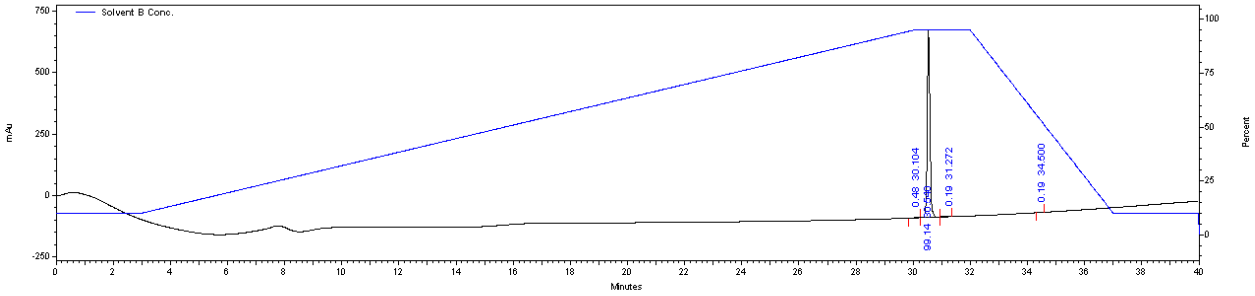

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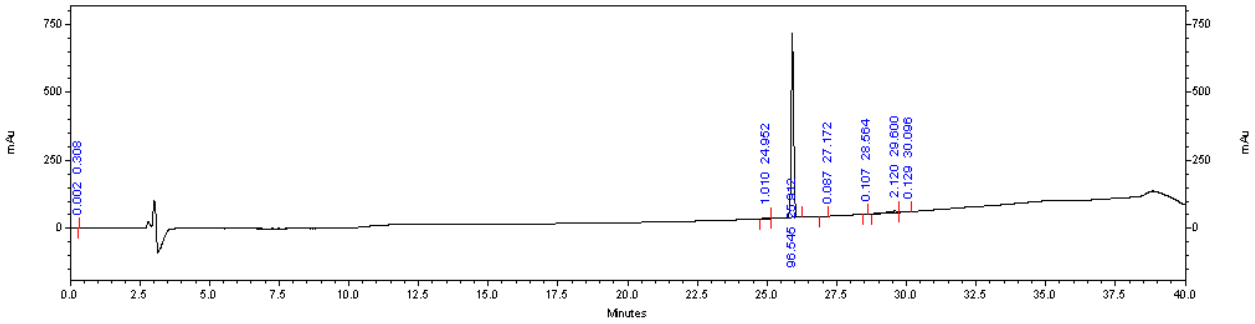

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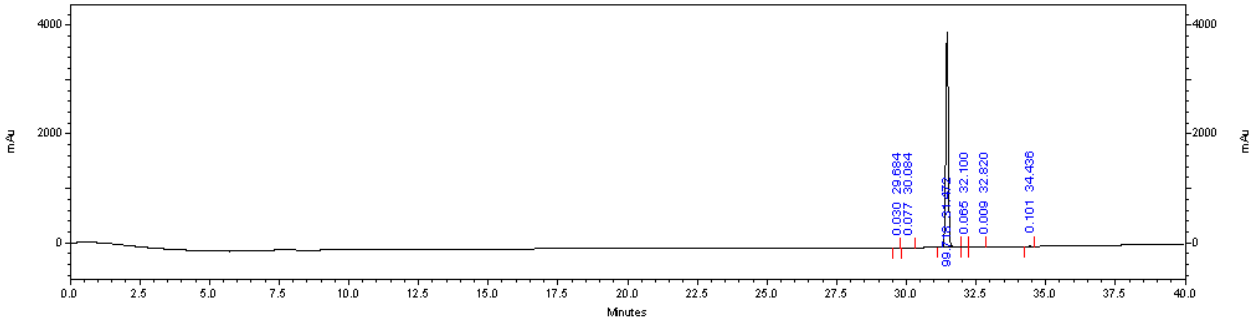

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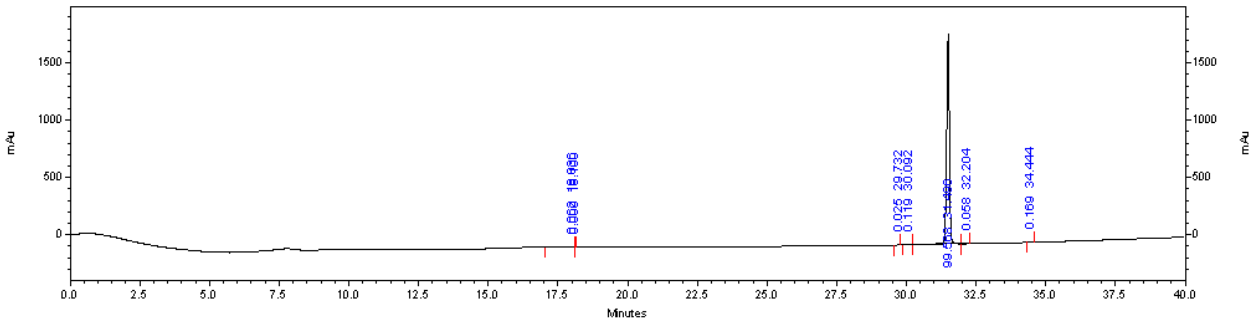

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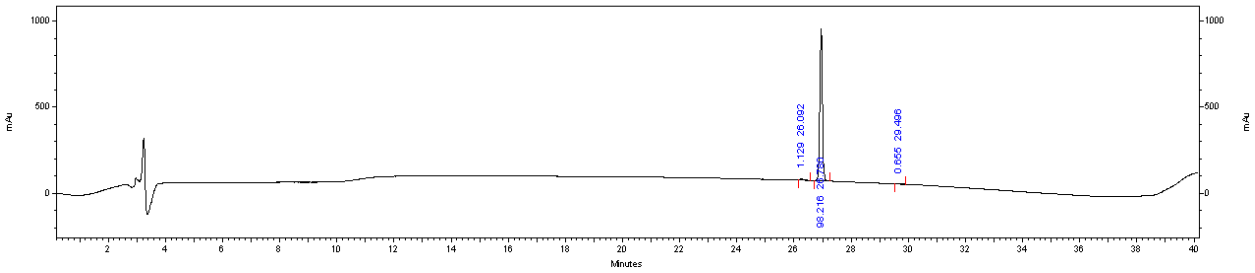

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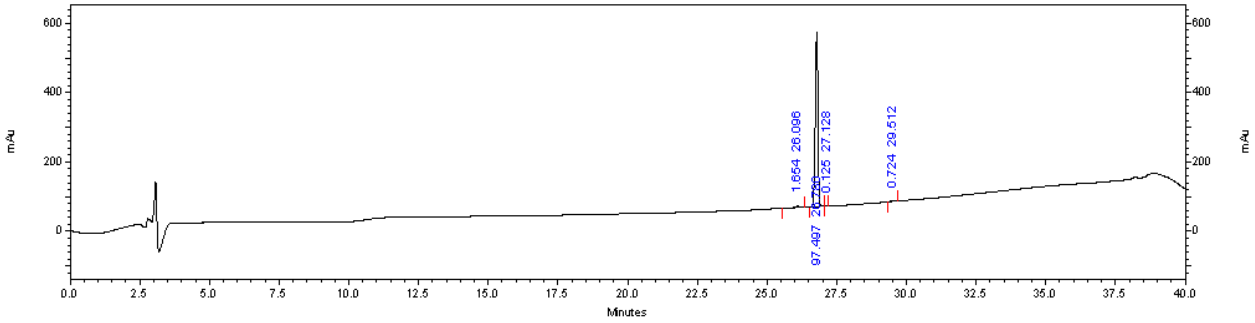

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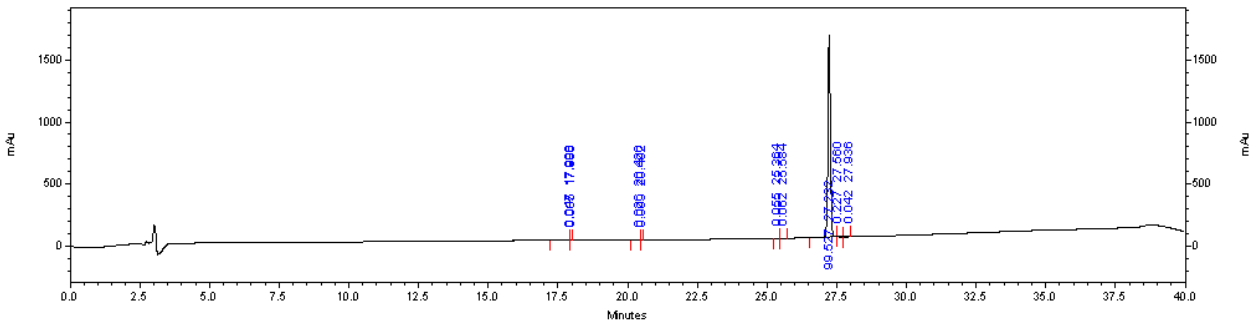

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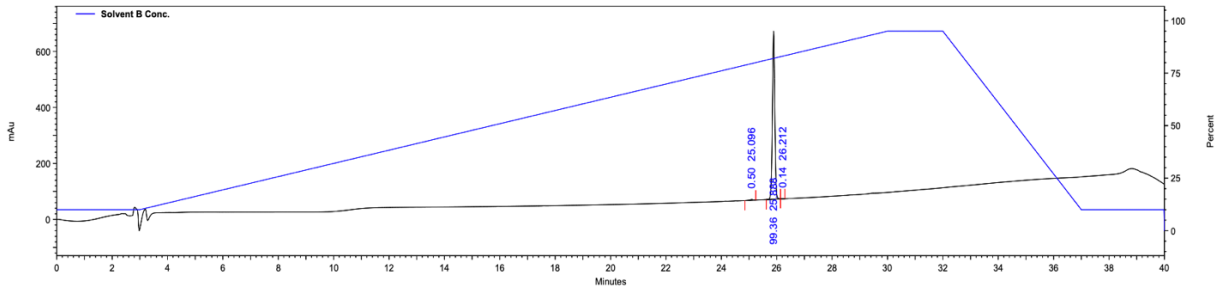

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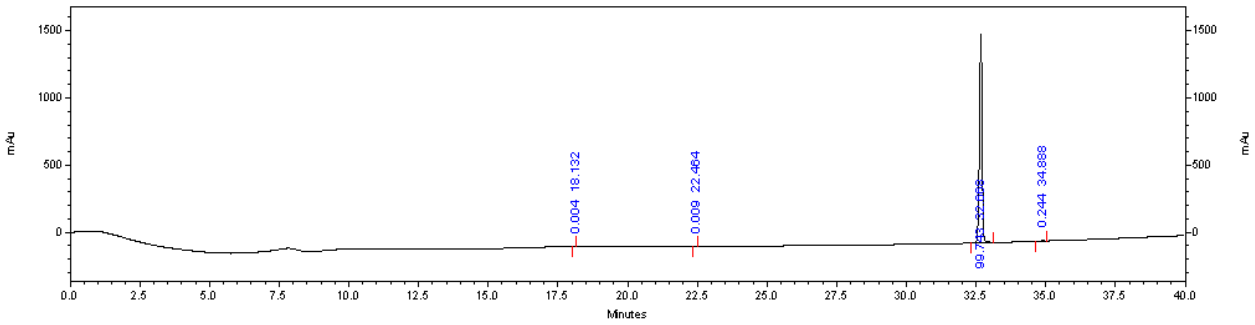

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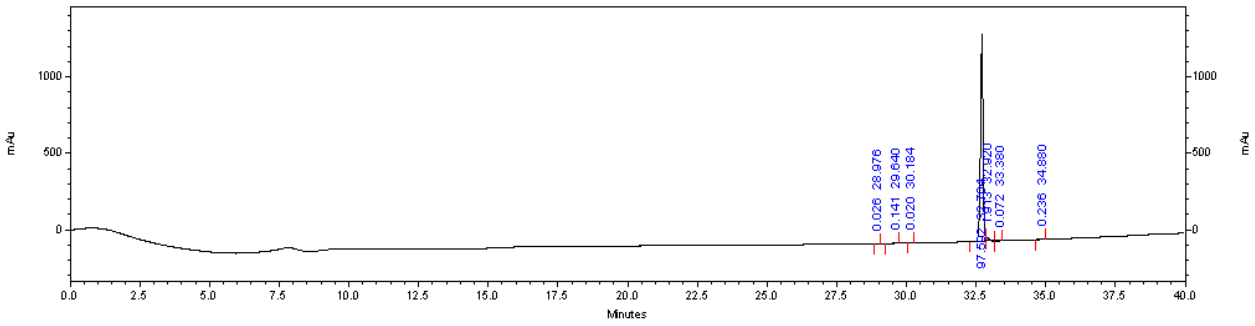

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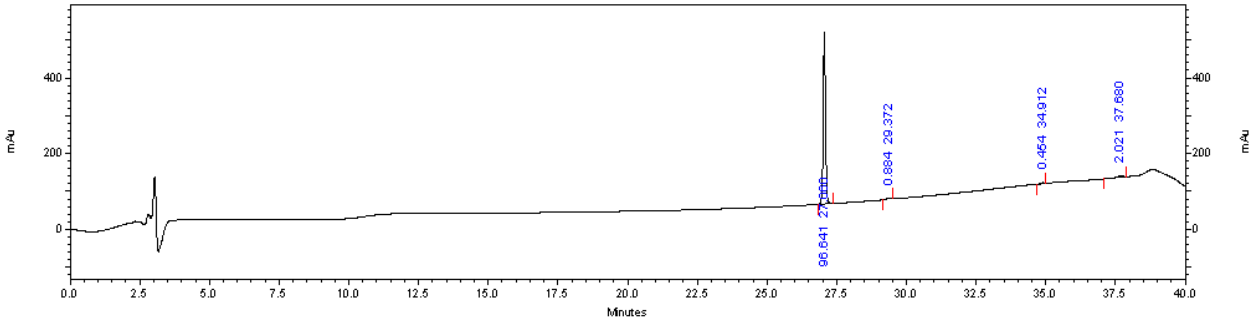

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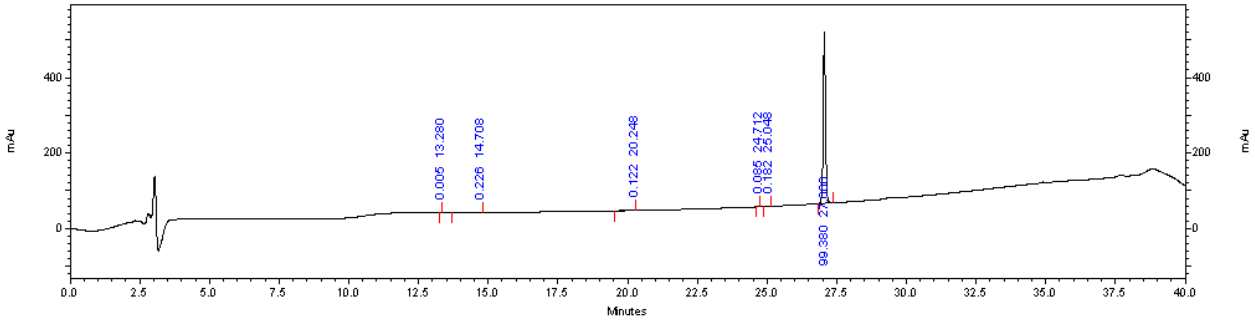

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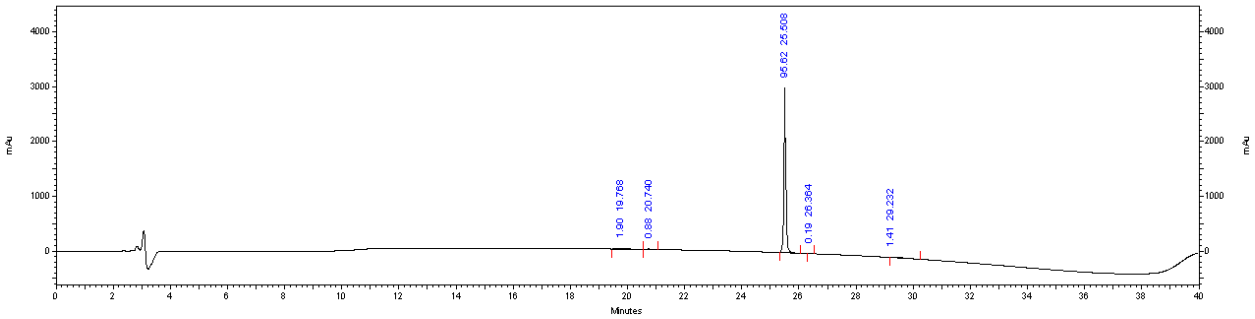

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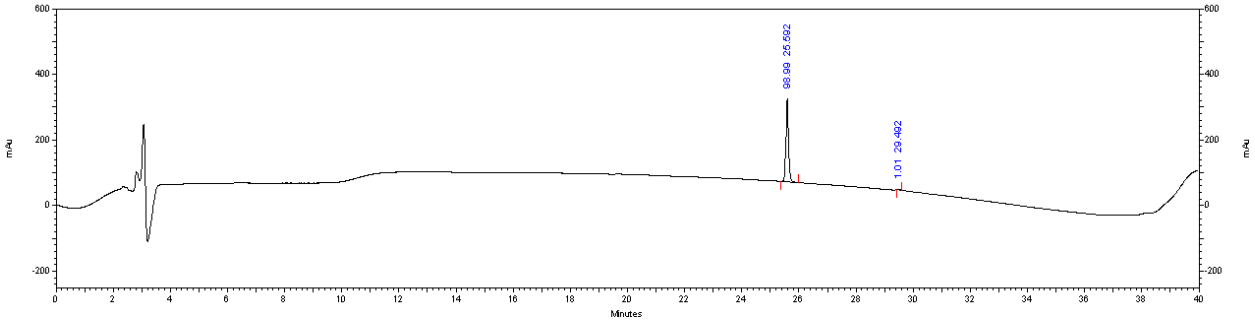

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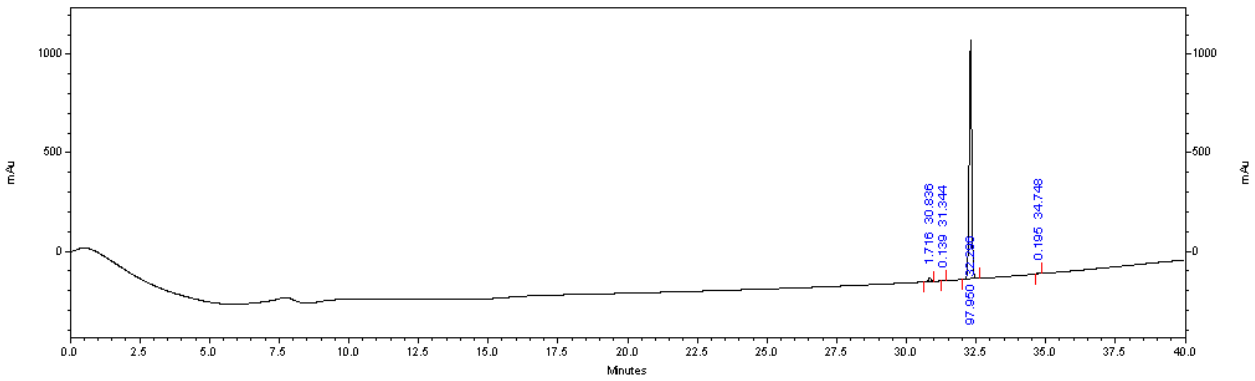

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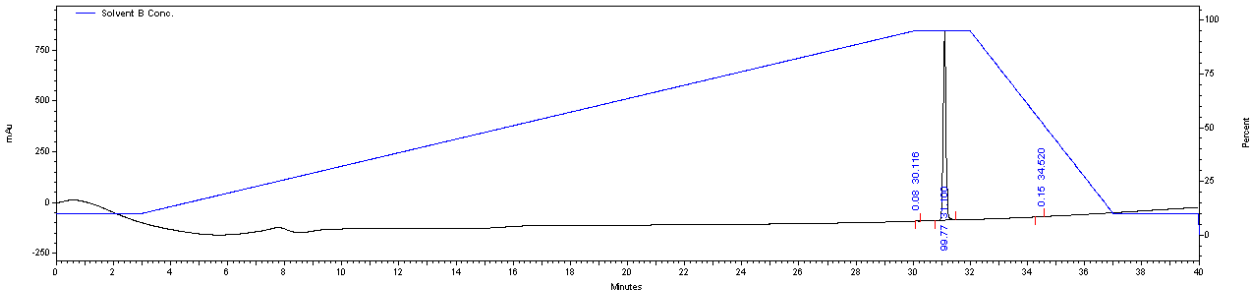

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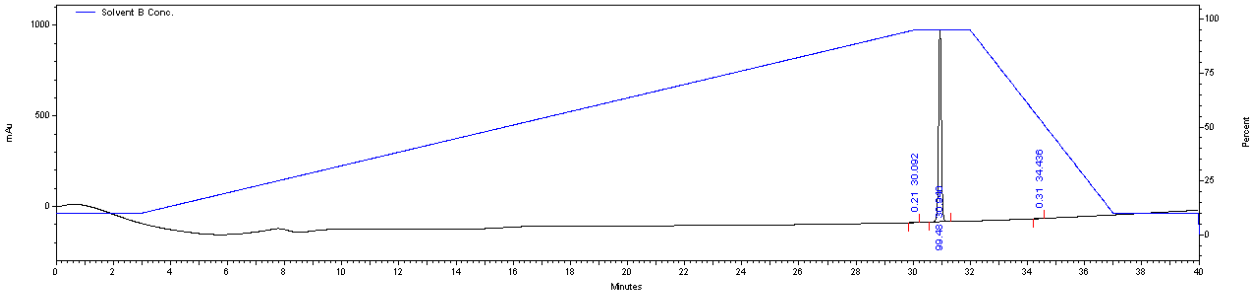

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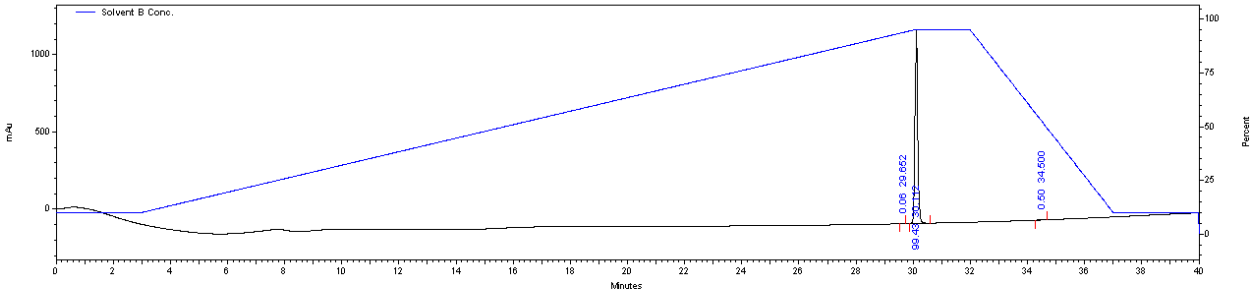

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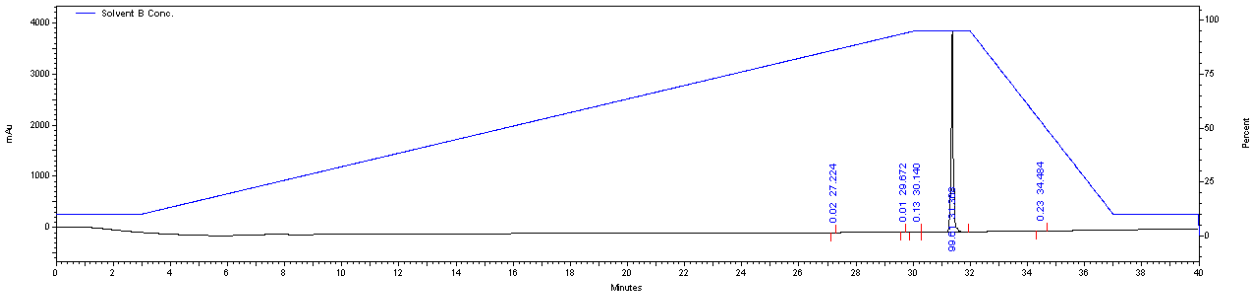

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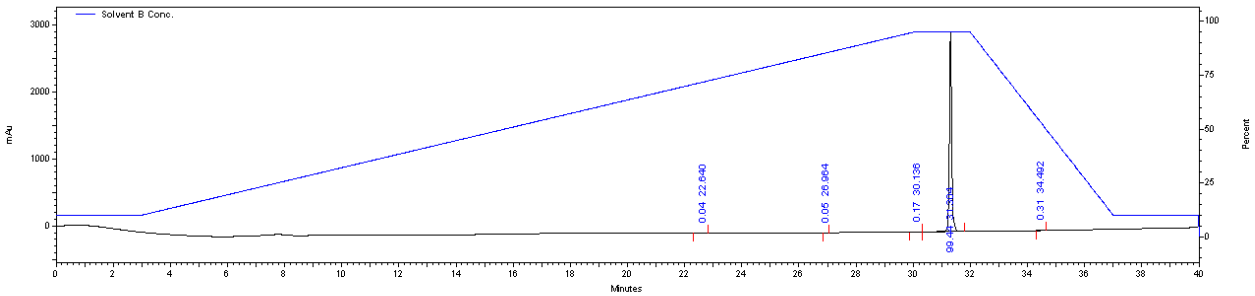

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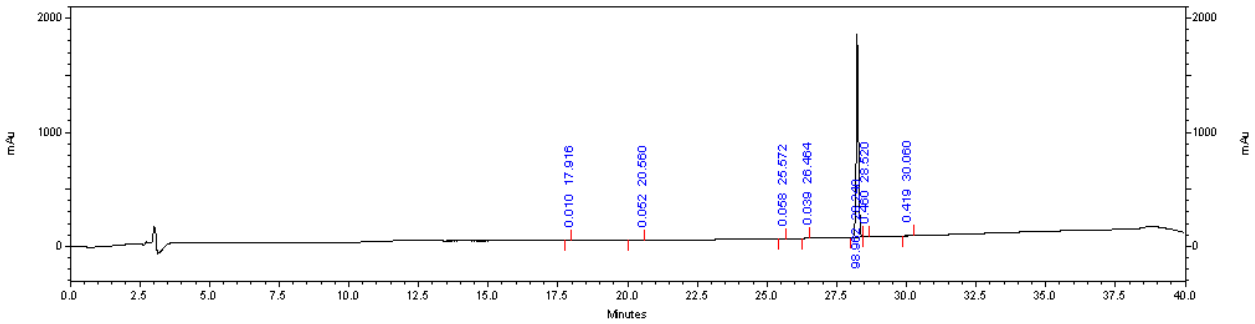
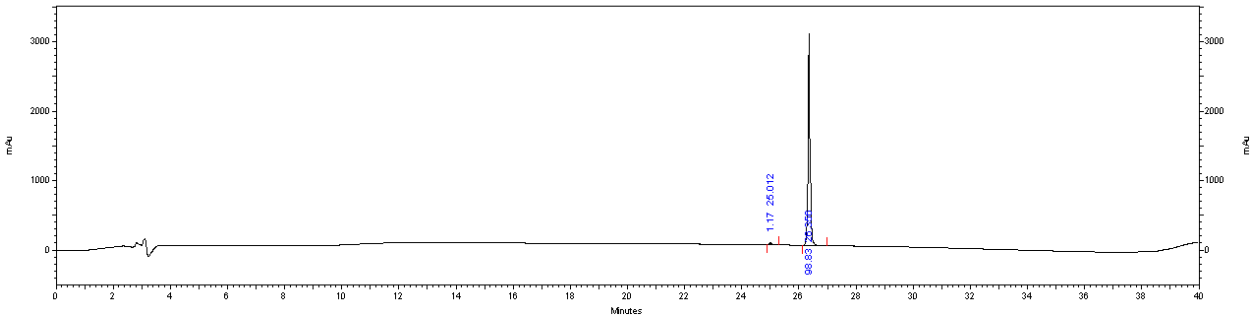
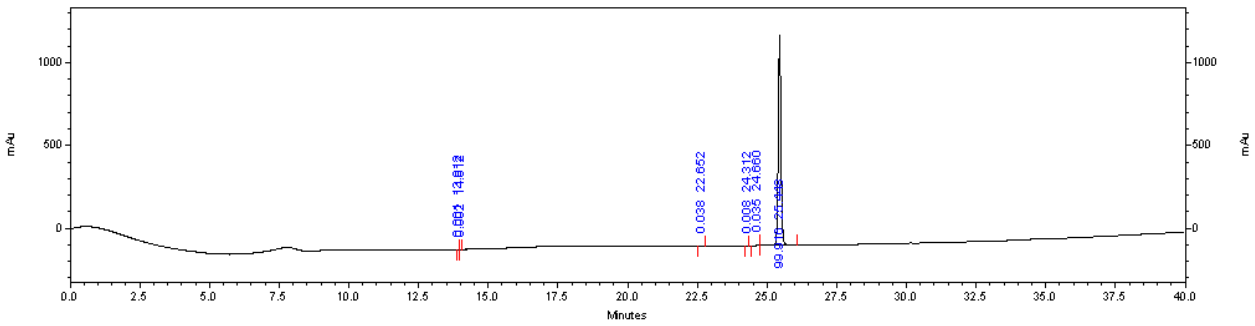


Bn(3CF$_3$4F)-n7OFF



Bn($3NH_2$)-n7OFF



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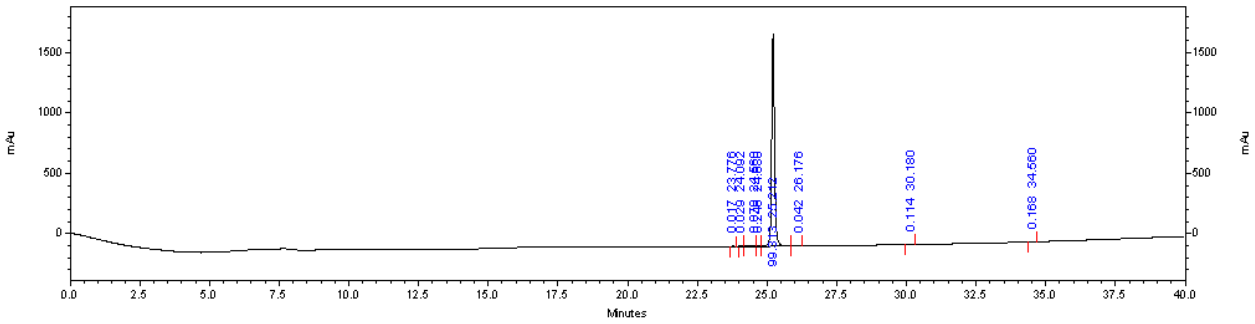
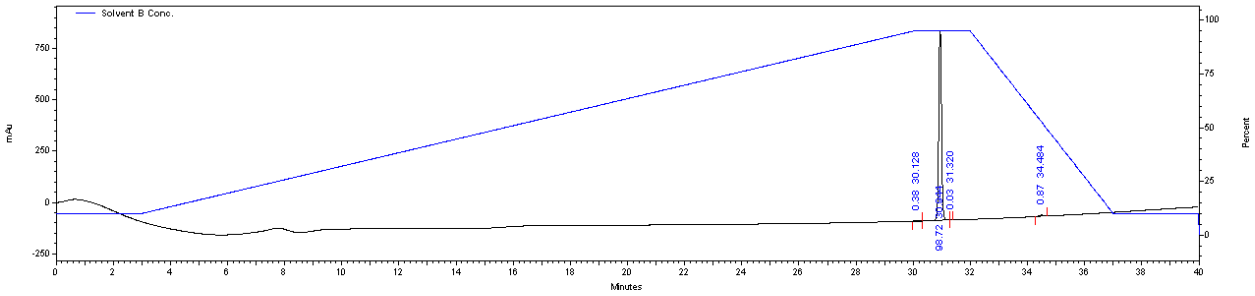
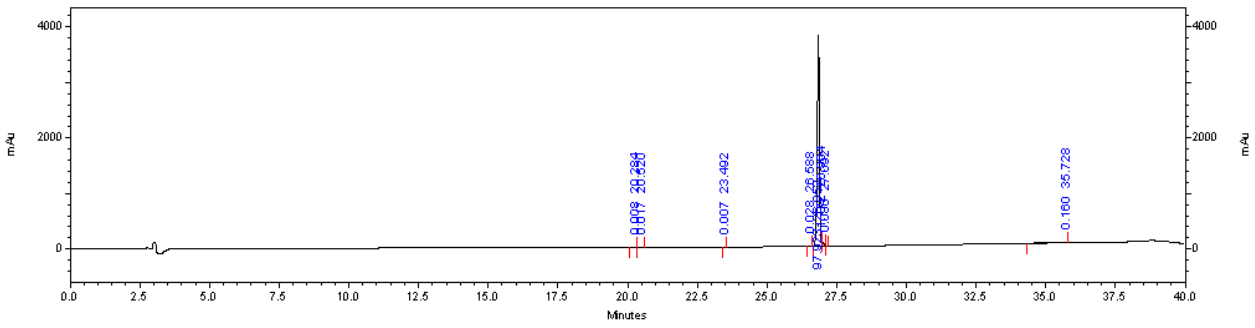


PhPr-n7OFF



PhBu-n7OFF



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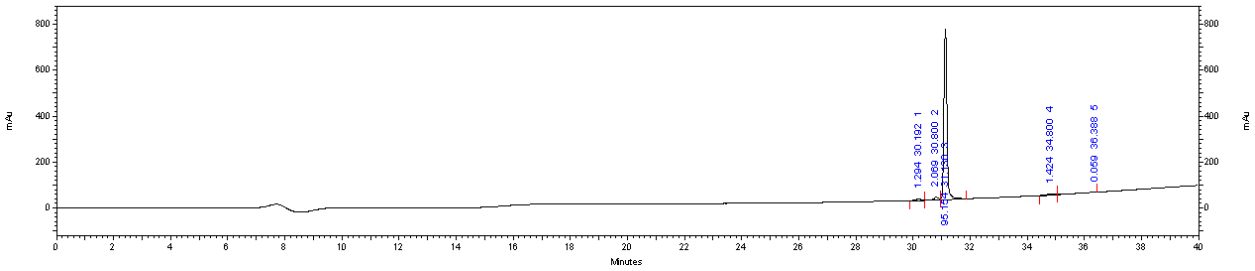

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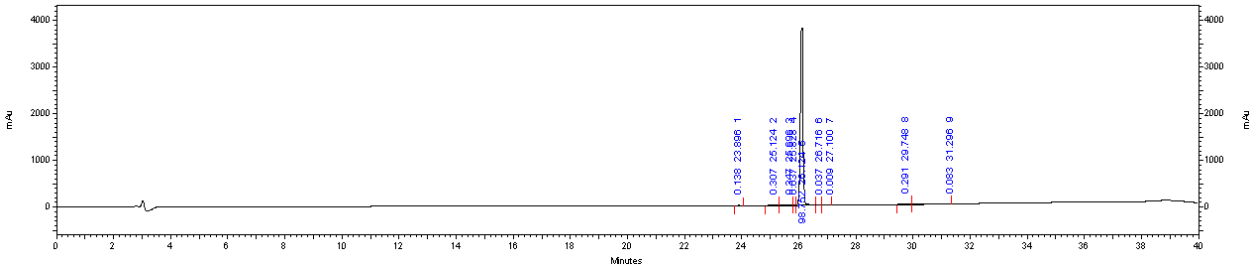

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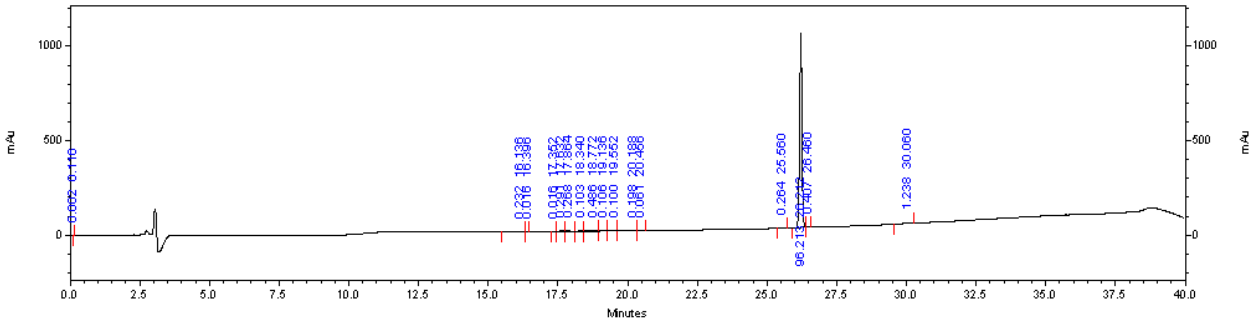

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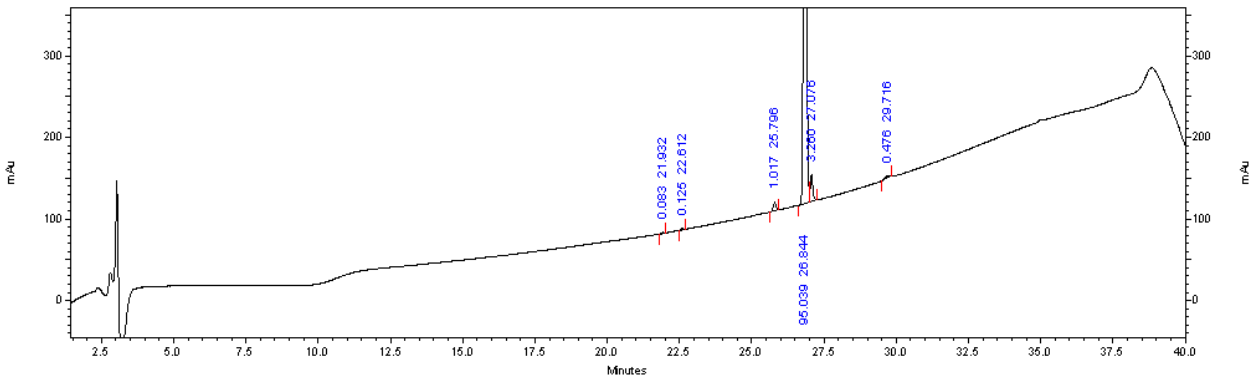

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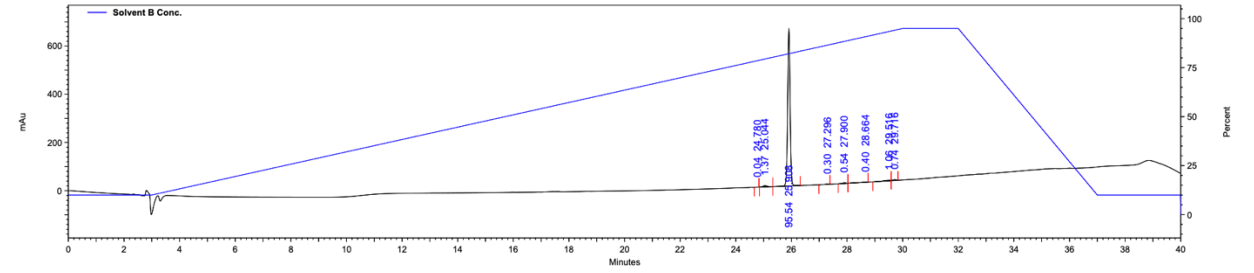

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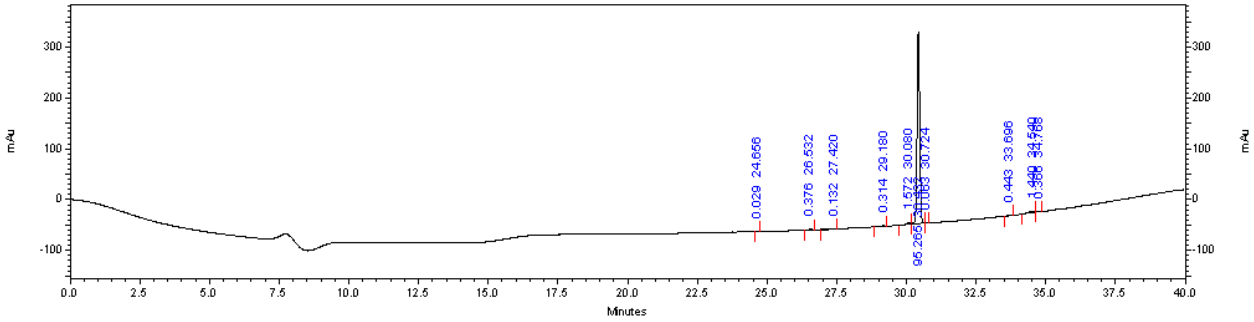
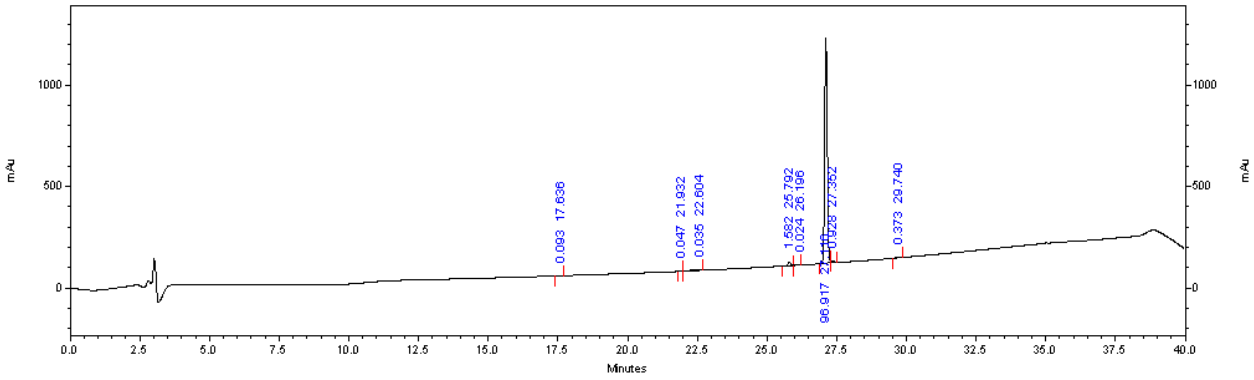


PhPr(2Br)-n7OFF



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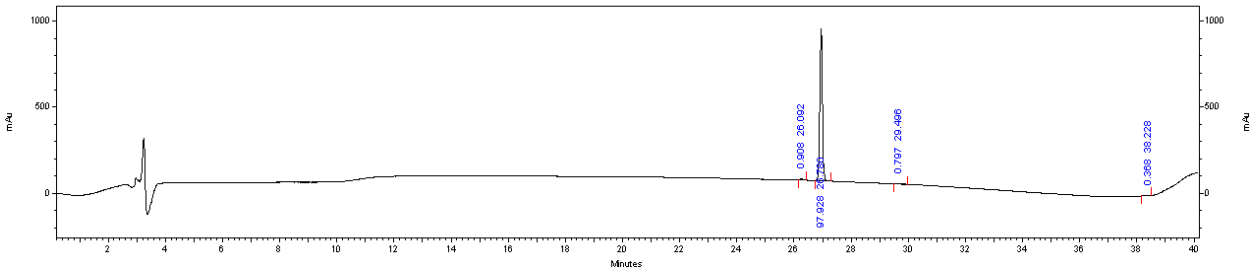

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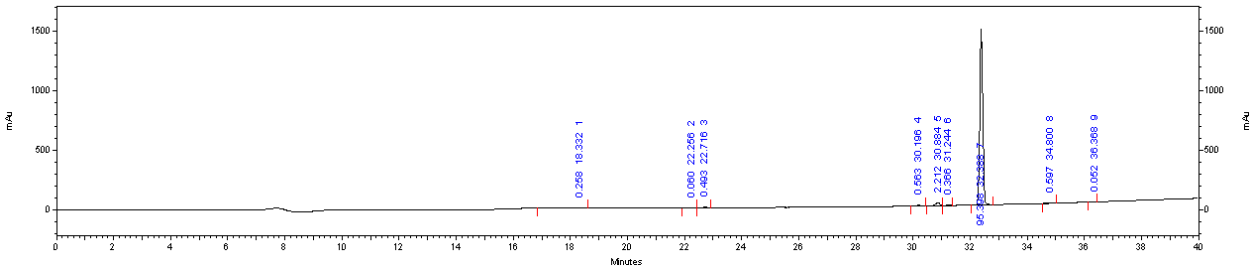

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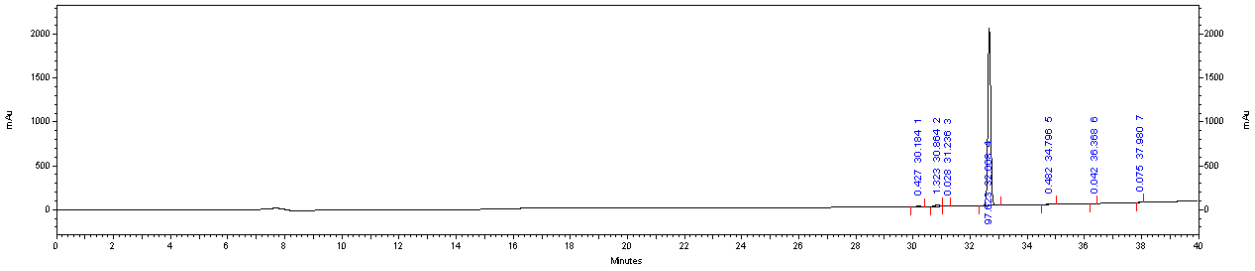

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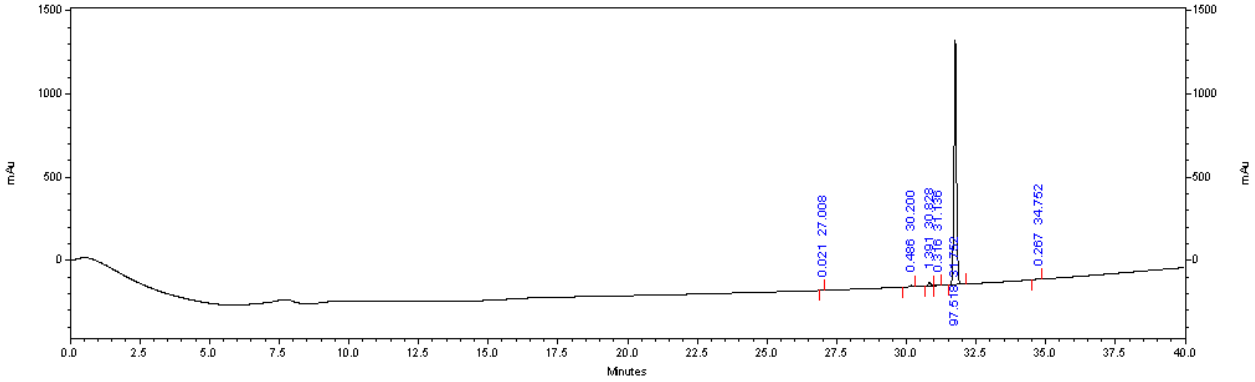

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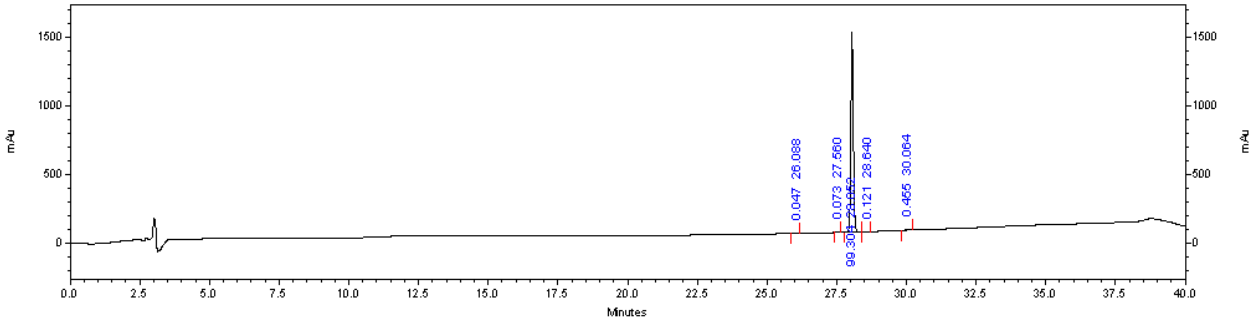
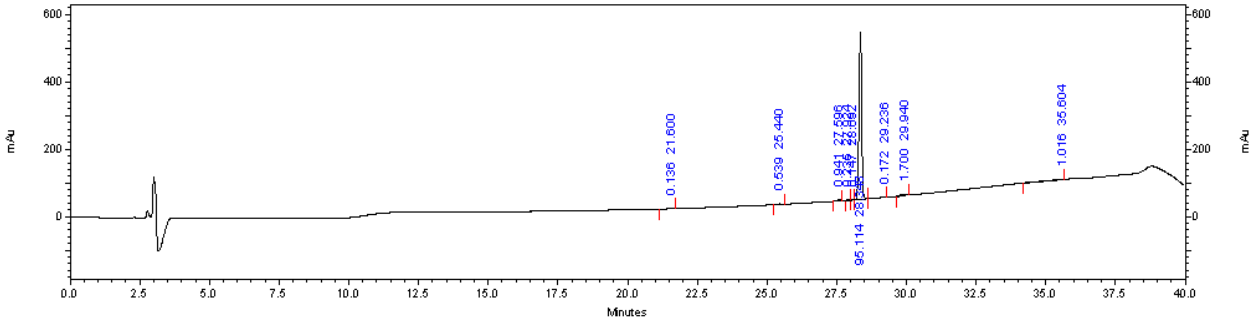
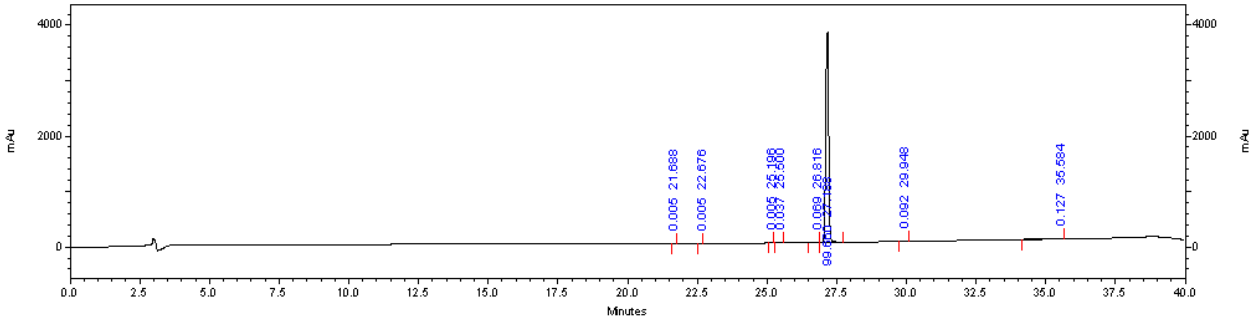


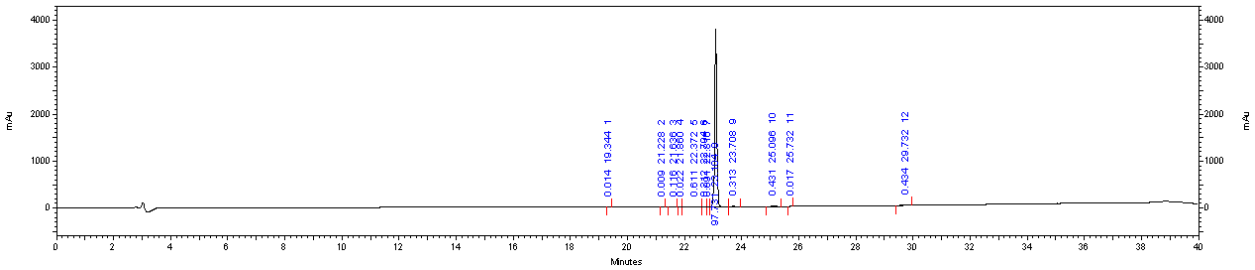
PhPr(3Br)-Bnc3



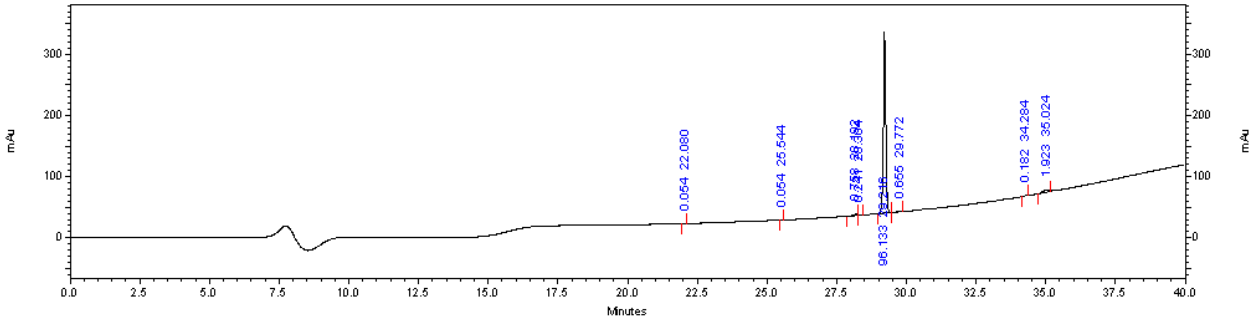
Nap-n7OFF



Coumarin-n7OFF



Coumarin-Bnc3



Hemolysis assay protocol.

To gauge the gross toxicity of the lead peptidomimetic PhPr(3Br)-Bnc3 in mammalian cells, we performed hemolysis assays using suspensions of rabbit blood cells according to our previously reported method.[5] The level of cell lysis in the presence of compound can be correlated with its overall toxicity. A 1-mL aliquot of 10% rabbit red blood cells (in saline) was pelleted by centrifugation at 500 g. The pellet was resuspended in an equal amount of PBS. The resulting suspension of cells was aliquoted (in 25-μL volumes) into Eppendorf tubes and diluted with 470 μL of PBS. PhPr(3Br)-Bnc3 was serial diluted with DMSO, and 5 μL portions of serial diluted compound, DMSO (vehicle, negative control), or Triton X-100 (positive control) were added to tubes. The mixtures were incubated overnight (16 h) at 37° C. Next, the mixtures were centrifuged at 500 g, and a 200-μL volume of supernatant from each tube was transferred to wells in a 96-well clear microtiter plate. The absorbance of each well was measured at 405 nm using a Biotek Synergy2 plate reader running Gen5 software (version 1.05). Data was processed using GraphPad Prism software (v. 9).

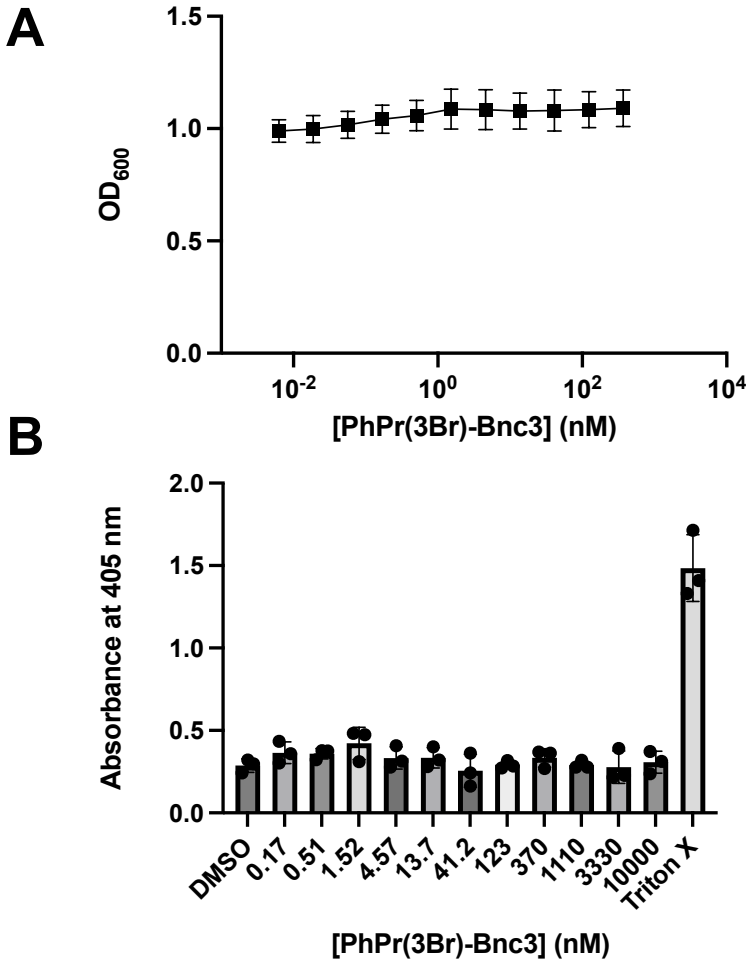


Figure S5. Effects of PhPr(3Br)-Bnc3 on bacterial cell growth and mammalian cells. (**A**) Normalized OD_{600} of *S. aureus* cells treated with PhPr(3Br)-Bnc3 over a range of concentrations and cultured for 24 h at 37 °C. PhPr(3Br)-Bnc3 does not affect *S. aureus* growth over the concentrations tested. OD_{600} normalized to the DMSO vehicle control. Plot shows the average of three replicates; error bars represent SEM. (**B**) Absorbance (at 405 nm) of supernatants of rabbit red blood cells treated with PhPr(3Br)-Bnc3 over a range of concentrations. DMSO vehicle = negative control; the surfactant Triton X-100 = positive control. PhPr(3Br)-Bnc3 does not have any lytic activity over the concentrations tested. Bar plot shows average of three replicates; error bars represent SD.

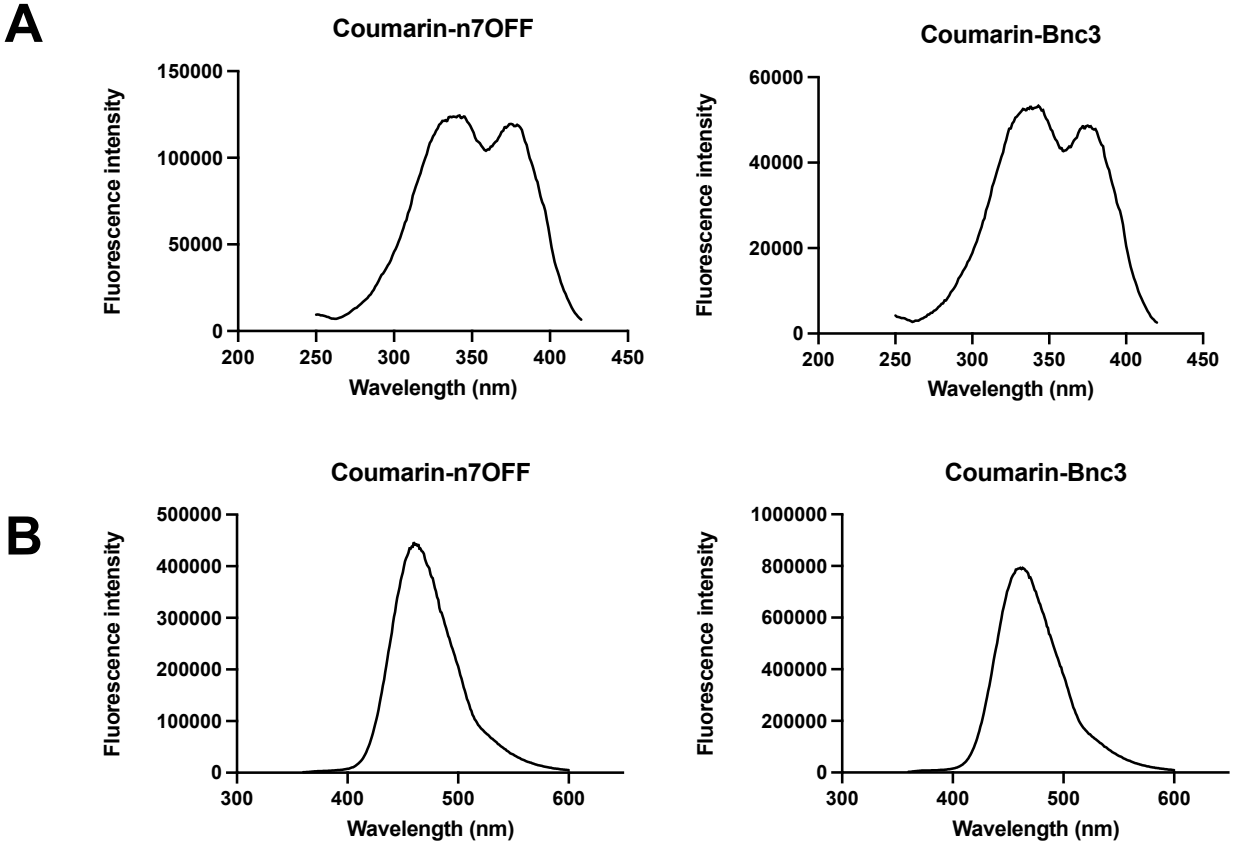


Figure S6. Fluorescence (**A**) excitation (with emission at 460 nm) and (**B**) emission (with excitation at 330 nm) spectra for coumarin-n7OFF and coumarin-Bnc3. Samples were diluted to 100 nM in PBS buffer (0.01% DMSO). Excitation was measured from 250 nm to 460 nm in 1 nm increments. Emission was measured from 360 nm to 600 nm in 1 nm increments. Each plot is a single replicate.

References.

1. W. Chan and P. White, *Fmoc solid phase peptide synthesis: a practical approach*, OUP Oxford, 1999.

2. J. B. Blanco-Canosa and P. E. Dawson, *Angew. Chem., Int. Ed. Engl.*, 2008, **47**, 6851-6855.

3. J. K. Vasquez and H. E. Blackwell, *ACS Infect. Dis.*, 2019, **5**, 484-492.

4. C. L. Malone, B. R. Boles, K. J. Lauderdale, M. Thoendel, J. S. Kavanaugh and A. R. Horswill, *J. Microbiol. Methods*, 2009, **77**, 251-260.

5. Y. Tal-Gan, D. M. Stacy, M. K. Foegen, D. W. Koenig and H. E. Blackwell, *J. Am. Chem. Soc.*, 2013, **135**, 7869-7882.