Supporting Information for:

Identifying Modulators of Protein-Protein Interactions Using Photonic Crystal Biosensors

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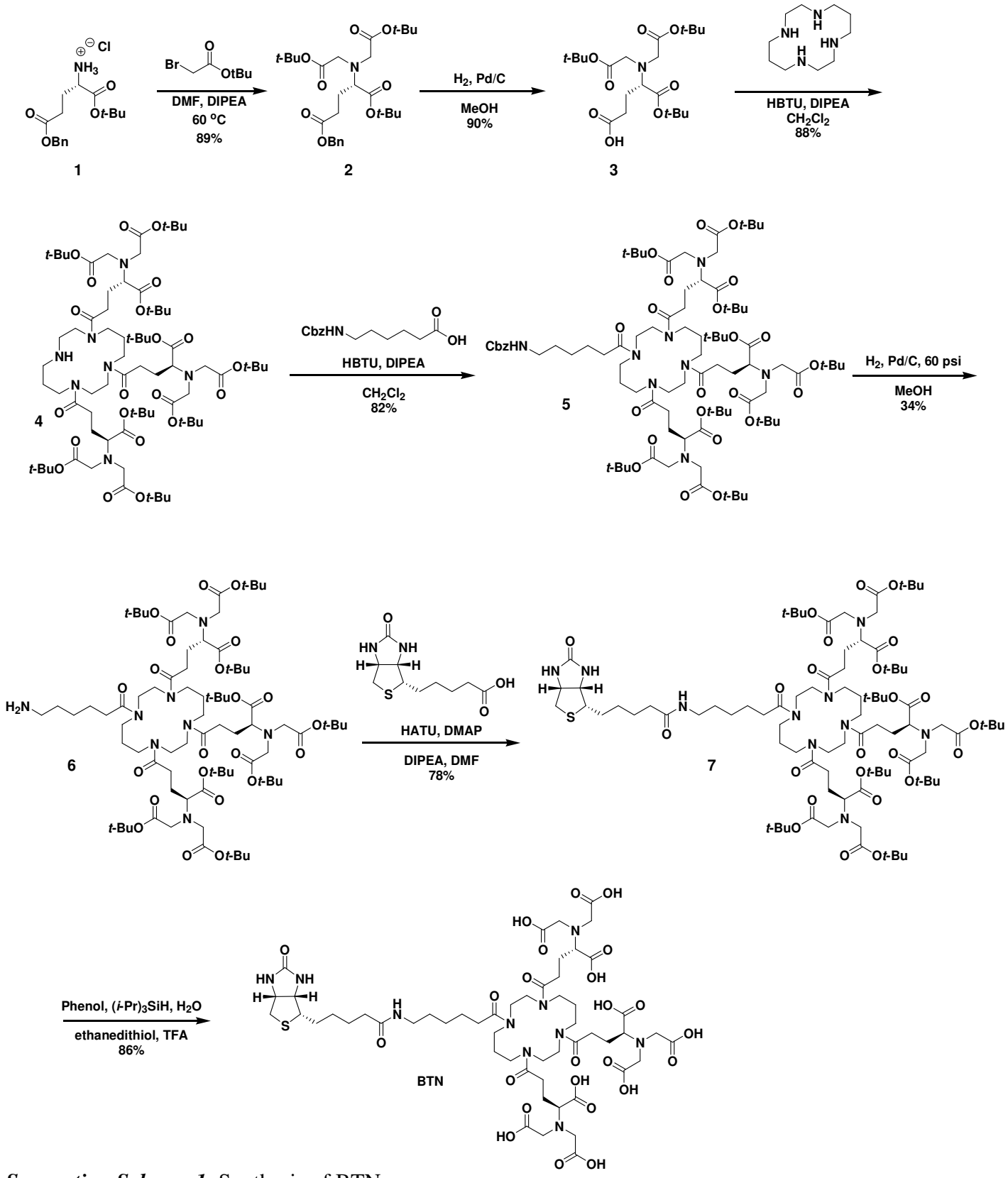
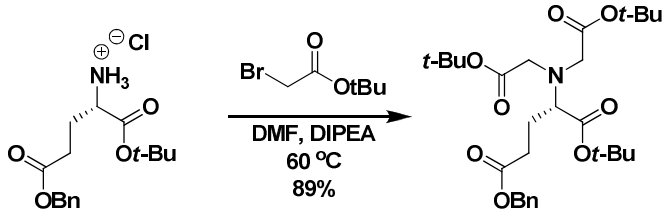
Materials: All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Biosensor plates (384-SA1) were purchased from SRU Biosystems (Woburn, MA).

Instrumentation: Flash column chromatography was performed using 230-400 mesh silica gel. ^{1}H-NMR spectra were recorded on Varian Unity 500 (500 MHz) spectrometers in $CDCl_3$ unless otherwise noted. ^{13}C-NMR spectra were recorded on Varian Unity 500 (125 MHz) spectrometers in $CDCl_3$ unless otherwise noted. Mass spectra were obtained in the University of Illinois Mass Spectroscopy Center and are reported in the form m/z.



Supporting Scheme 1: Synthesis of BTN.

Synthesis of Compound 2:

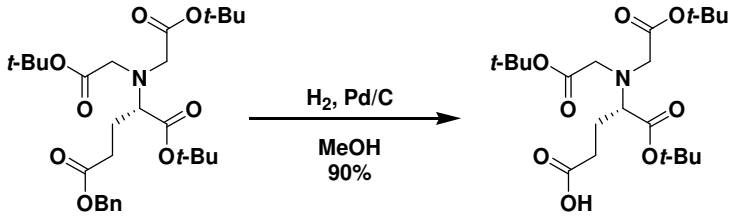


1 **2**

To a stirred solution of **1** (3.10 g, 9.41 mmol, 1.0 eq., ChemImpex) in DMF (40 mL) was added *t*-butyl bromoacetate (5.6 mL, 37.64 mmol, 4.0 eq.), and DIPEA (8.20 mL, 47.05 mmol, 5.0 eq.). The reaction mixture was then stirred for 13 hrs at 60°C. The volatiles were removed by evaporation then EtOAc (50 mL) was added and the slurry was filtered. The filtrate was concentrated and purified by silica gel column chromatography (EtOAc:hexanes=1:10) affording **2** (4.36 g, 8.36 mmol, 89%) as a red oil.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.36-7.29 (m, 5H), 5.11 (s, 2H), 3.44 (s, 4H), 3.38 (dd, 1H, *J* = 10.3, 5.7 Hz), 2.73-2.57 (m, 2H), 2.05-1.88 (m, 2H), 1.46 (s, 9H), 1.43 (s, 18H).
The spectral data match with the reported data from Konig et. al *Chemistry--A European Journal* **2008**, *14*, 2536-2541.

Synthesis of Compound 3:

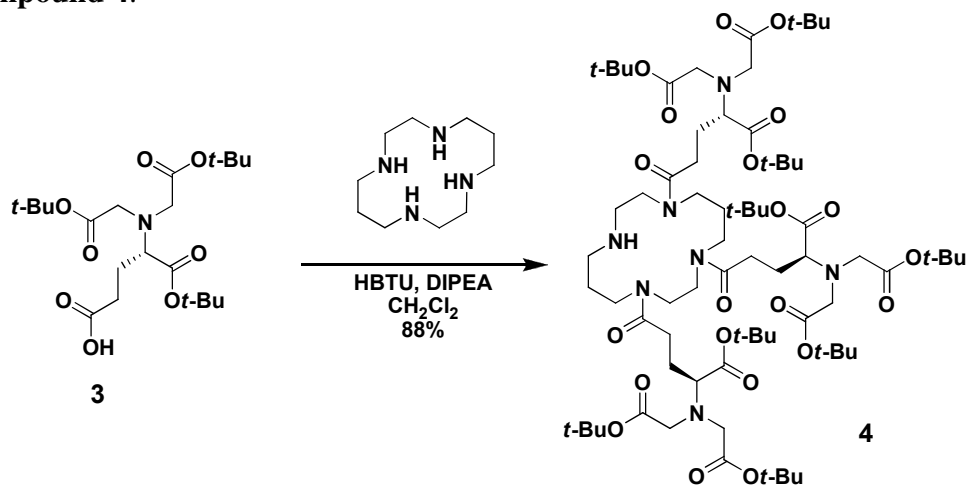


2 **3**

Compound **2** (3.07 g, 5.89 mmol) was dissolved in MeOH (50 mL), Pd/C (100 mg) was added to the solution, and the flask was fitted with a balloon charged with H_2. The reaction mixture was vigorously stirred for 4 hrs. After celite filtration with diethyl ether, concentration of the filtrate afforded **3** (2.29 g, 5.30 mmol, 90%) as a red oil.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.45 (s, 4H), 3.38 (dd, 1H, *J* = 10.5, 5.0 Hz), 2.73-2.57 (m, 2H), 2.04-1.86 (m, 2H), 1.47 (s, 9H), 1.44 (s, 18H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 177.6, 171.7, 170.8, 81.8, 81.4, 64.8, 54.2, 31.1, 28.4, 28.3, 28.2, 25.5.
The spectral data match with the reported data from Konig et. al *Chemistry--A European Journal* **2008**, *14*, 2536-2541.

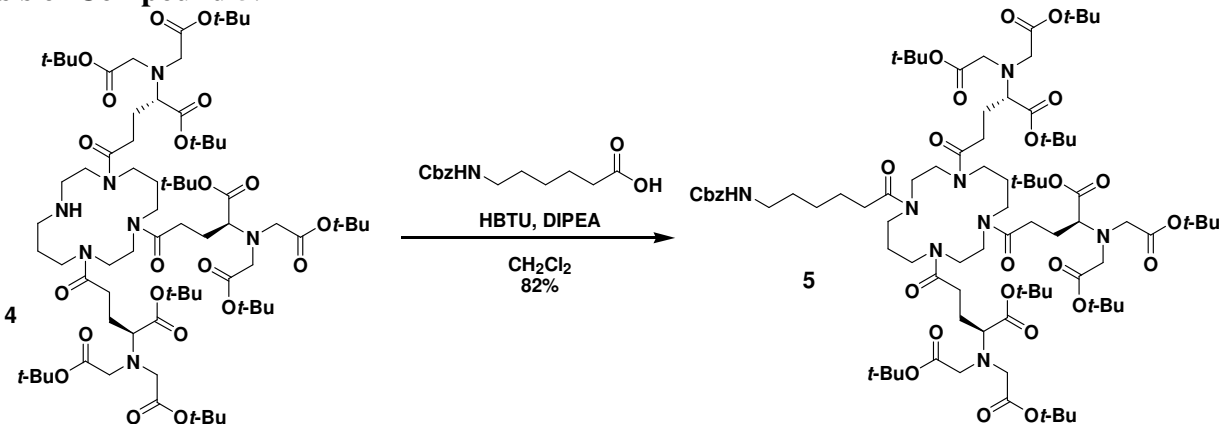
Synthesis of Compound 4:



To a stirred solution of **3** (2.42 g, 5.60 mmol, 2.90 eq.) in CH_2Cl_2 was added amine (387 mg, 1.93 mmol, 1.0 eq.), HBTU (2.70 g, 7.72 mmol, 4.0 eq.), and DIPEA (1.68 mL, 9.65 mmol, 5.0 eq.) sequentially then stirred for 14 hrs at room temperature. The volatiles were removed by evaporation then CH_2Cl_2 (100 mL) was added. The organic phase was washed with H_2O (10 mL, 3 times), brine (10 mL), then dried over Na_2SO_4, and concentrated *in vacuo*. Purification by silica gel column chromatography (CH_2Cl_2:MeOH=10:1) afforded the tris-NTA (compound **4**, 2.47 g, 1.71 mmol, 88%) as a red oil.

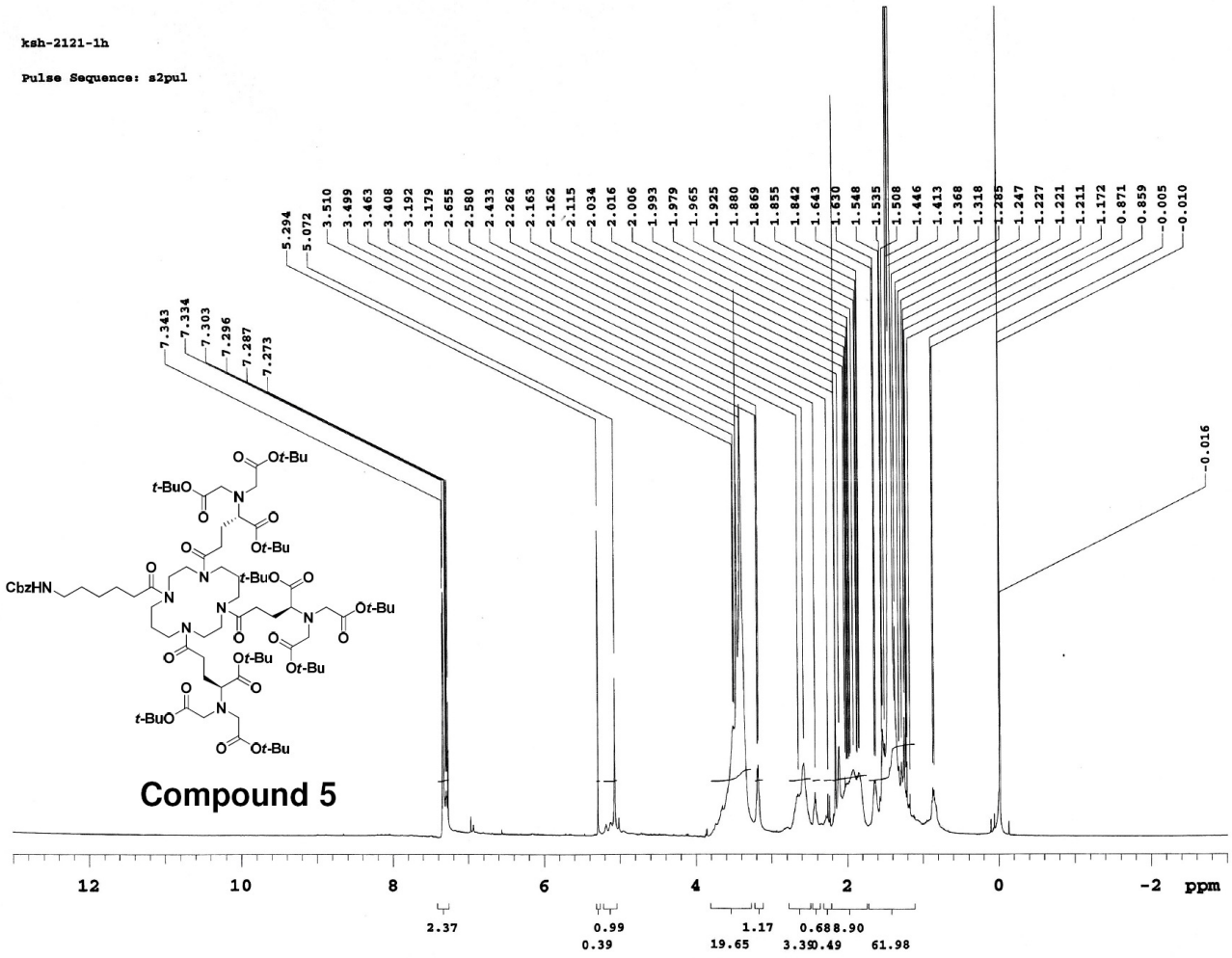
^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.52-3.39 (m, 27H), 2.82 (bs, 2H), 2.63-2.53 (m, 8H), 2.03-1.77 (m, 11H), 1.43 (s, 27H), 1.40 (s, 54H); MS (ESI$^+$, $C_{73}H_{130}N_7O_{21}$) Calculated mass: 1439.9 Observed mass: 1441.0 $[MH]^+$
The spectral data match with the reported data from Lata et. al *J. Am. Chem. Soc.* **2008**, *29*, 10205-15.

Synthesis of Compound 5:



To a stirred solution of compound **4** (164 mg, 0.114 mmol, 1.0 eq.) in CH_2Cl_2 (10 mL) was added Cbz-amino caproic acid (36 mg, 0.137 mmol, 1.2 eq.), HBTU (52 mg, 0.137 mmo, 1.2 eq.), DIPEA (39 µL, 0.228 mmol, 2.0 eq.), then stirred for 24 hrs. The reaction mixture was washed with water, then brine, then dried over $MgSO_4$ and concentrated *in vacuo*. Purification by silica gel chromatography (CH_2Cl_2:MeOH=15:1) afforded compound **5** (157 mg, 0.093 mmol, 82%) as a colorless oil.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.31-7.27 (m, 6H), 5.04 (s, 2H), 3.62-3.38 (m, 31H), 3.16 (bd, 2H, J = 6.5 Hz), 2.90-2.29 (m, 8H), 2.01-1.78 (m, 16H), 1.69-1.39 (m, 81H); MS (ESI$^+$, $C_{87}H_{146}N_8O_{24}$) Calculated mass: 1688.1 Observed mass: 1689.0 $[MH]^+$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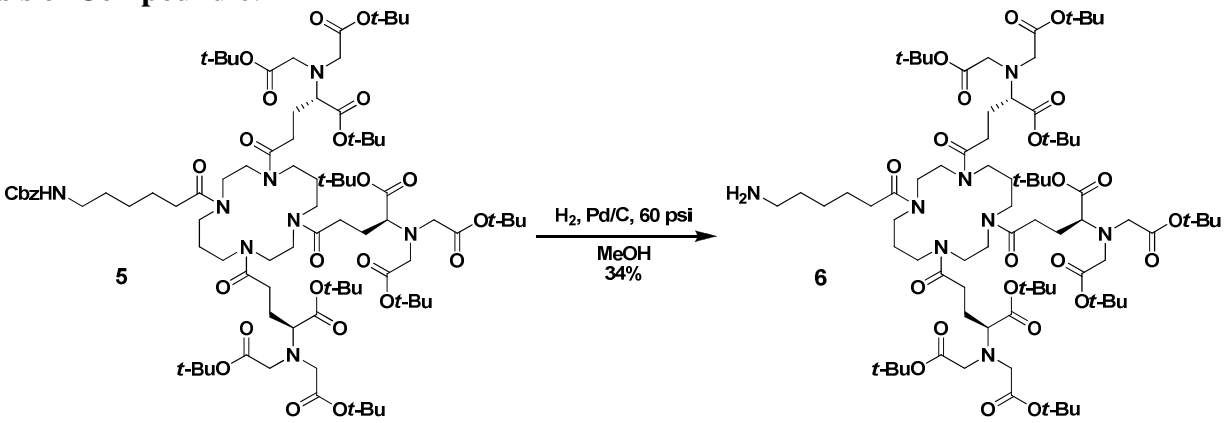
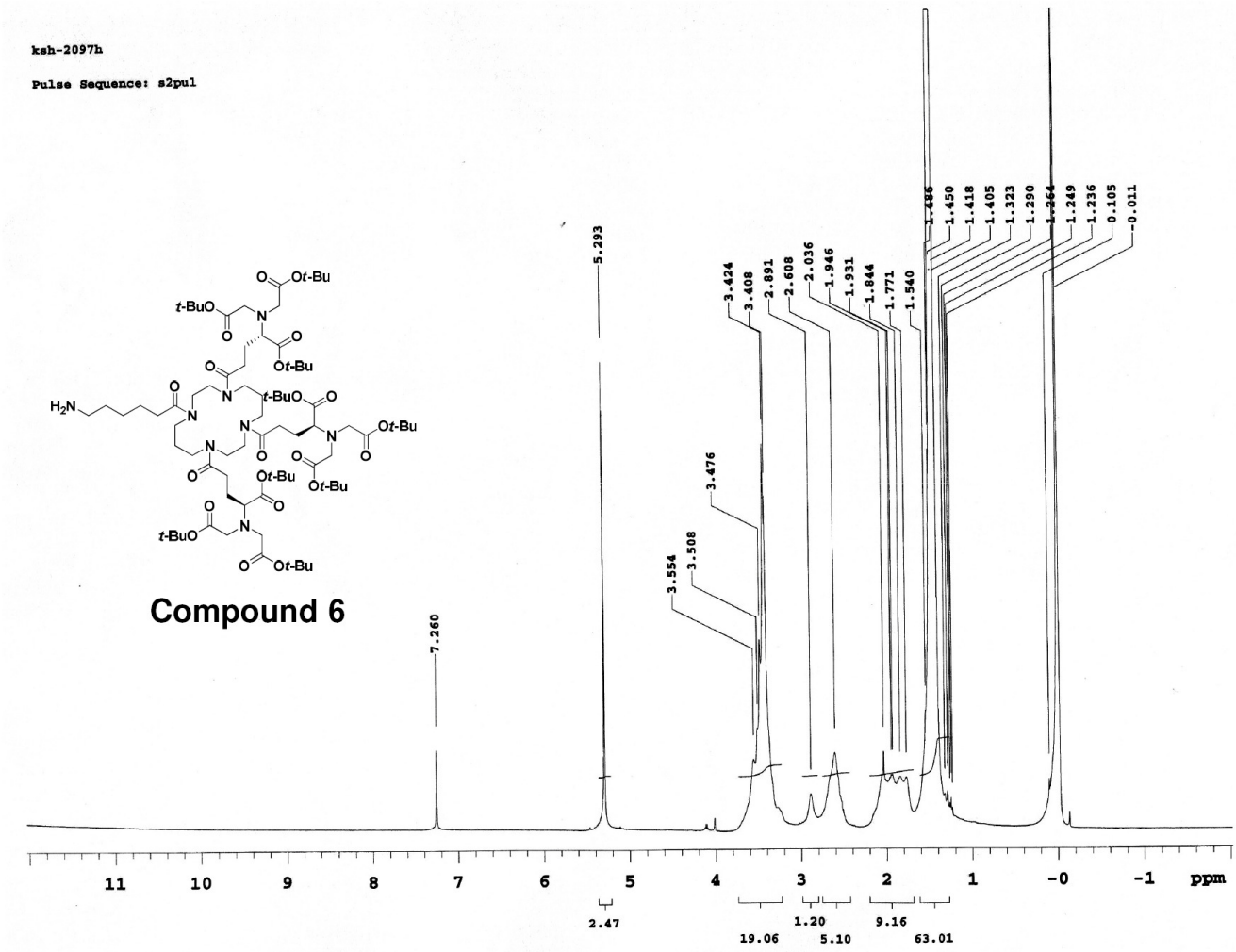
Synthesis of Compound 6:

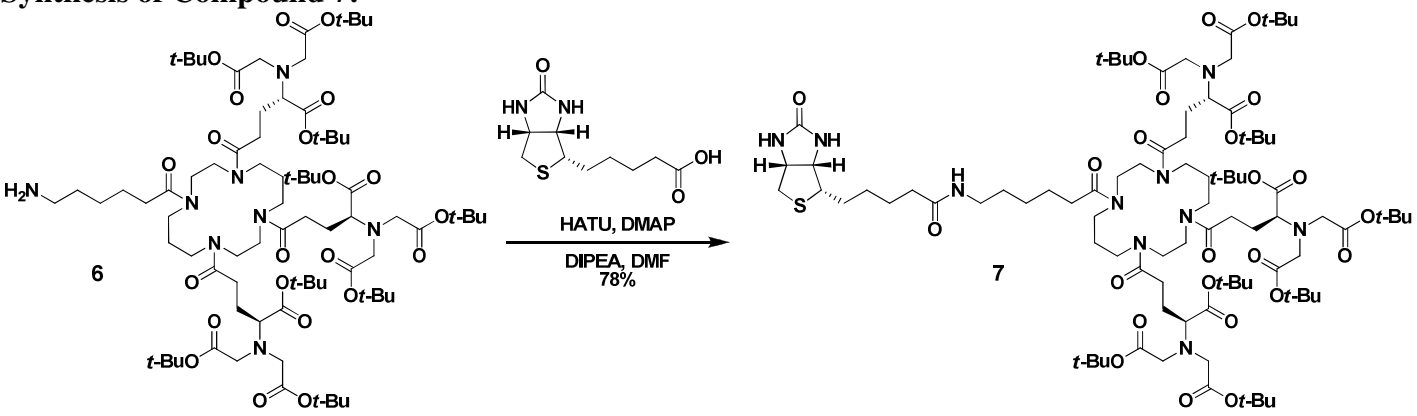


Compound **5** (320 mg, 0.190 mmol) was dissolved in MeOH (10 mL) and Pd/C (30 mg) was added to the solution and the flask was fitted with a balloon filled with H_2. The reaction mixture was vigorously stirred for 12 hrs. After celite filtration with diethyl ether, silica gel column chromatography (CH_2Cl_2:MeOH=10:1) afforded compound **6** (100 mg, 0.064 mmol, 34%) as a colorless oil.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.55-3.37 (m, 29H), 2.60-2.59 (m, 6H), 2.05-1.25 (m, 105H); MS (ESI^+, $C_{79}H_{140}N_8O_{22}$) Calculated mass: 1554.0 Observed mass: 1554.7 $[MH]^+$

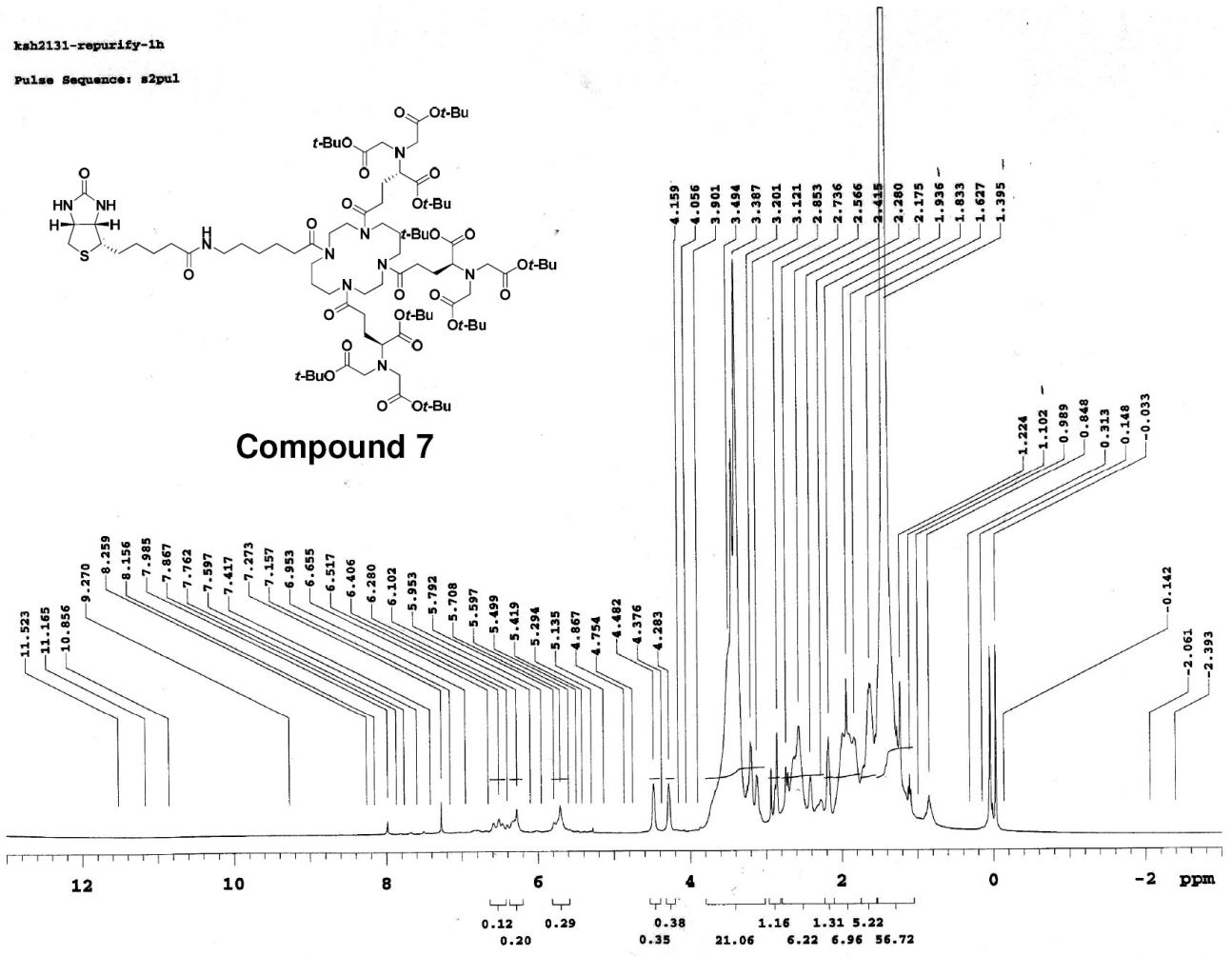


Synthesis of Compound 7:

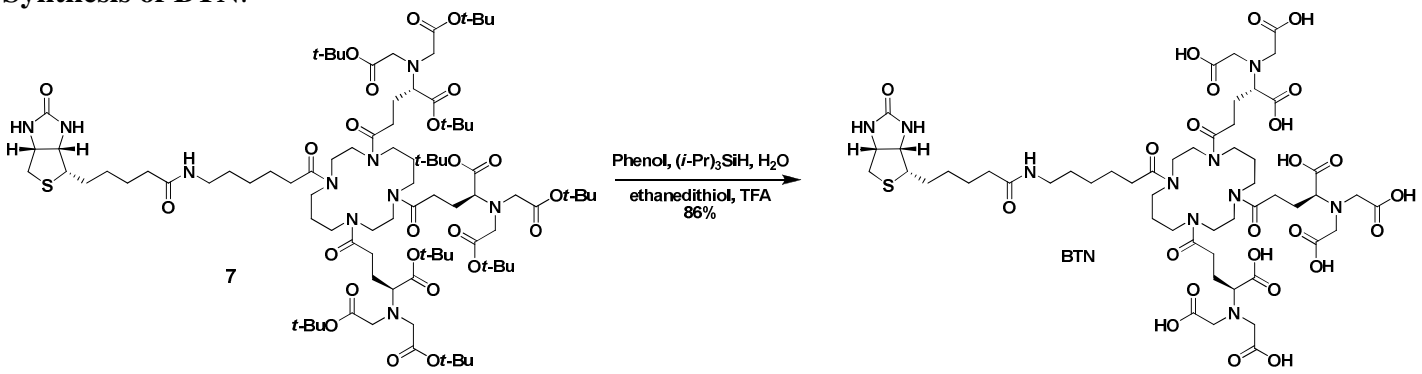


To a stirred solution of D-biotin (23 mg, 96.5 μmol, 1.5 eq.) in DMF (3 mL) was added HATU (37 mg, 96.5 μmol, 1.5 eq.), DMAP (5 mg, cat. amount), DIPEA (25 mg, 193.2 μmol, 2.0 eq.). This mixture was stirred for 20 minutes. The reaction mixture was added to the solution of compound **6** (100 mg, 64.4 μmol) in DMF (1 mL), then stirred for 36 hrs. The volatiles were removed and the mixture was added to water (10 mL), then extracted with EtOAc (50 mL, 3 times). The combined organic layers were washed with water, brine, dried over $MgSO_4$, and concentrated *in vacuo*. Silica gel column chromatography (CH_2Cl_2:MeOH=15:1) afforded compound **7** (90 mg, 51.0 μmol, 78%) as a colorless oil.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 6.55-6.27 (m, 2H), 5.70 (s, 1H), 4.48 (s, 1H), 4.28 (s, 1H), 3.49-3.12 (m, 32H), 2.85 (bs, 2H), 2.73-2.41 (m, 10H), 2.28 (s, 2H), 1.93-1.10 (m, 103H); MS (ESI$^+$, $C_{89}H_{154}N_{10}O_{24}S$) Calculated mass: 1780.2 Observed mass: 1780.9 $[MH]^+$

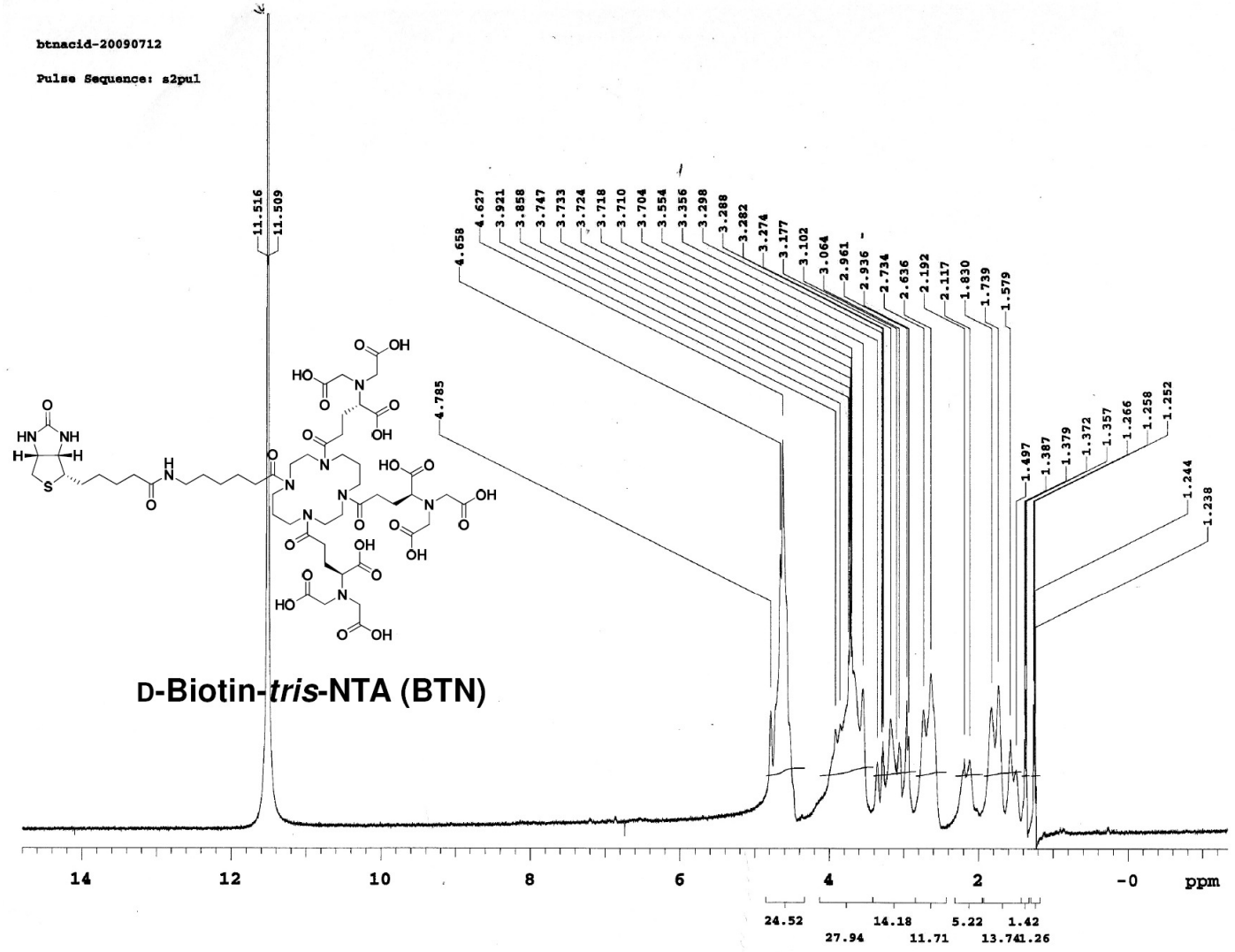


Synthesis of BTN:



To a stirred solution of compound **7** (86 mg, 48.3 μmol) in TFA (0.3 mL) was added a solution of phenol (100 mg), (i-Pr)$_3$SiH (100 μl), ethanedithiol (100 μl), H_2O (100 μl) in TFA (0.5 mL). The reaction mixture was stirred for 5 hrs at room temperature. The volatiles were evaporated *in vacuo* and then re-dissolved in TFA (0.3 mL). The product was precipitated with cold diethyl ether then washed with cold diethyl ether (5 mL, 3 times). The product was dried to afford **BTN** (53 mg, 41.5 μmol, 86%) as a white solid.

^{1}H-NMR (500 MHz, CF3COOD) δ 4.78-4.62 (m, 17H), 3.92-3.55 (m, 19H), 3.35-2.93 (m, 10H), 2.73-2.63 (m, 8H), 2.19-2.11 (m, 4H), 1.83-1.57 (m, 10H), 1.36 (t, 1H, J = 7.5 Hz), 1.25 (t, 1H, J = 7.5 Hz); MS (ESI$^+$, C53H82N10O24S) Calculated mass: 1275.3 Observed mass: 1276.1 $[MH]^+$



Recombinant Protein Expression:

His6-FKBP12. The cDNA encoding human FKBP12 was provided in a pGEX vector by Prof. Jie Chen (University of Illinois at Urbana-Champaign). Primers containing flanking *Eco*RI and *Bam*HI sites were designed, and after PCR amplification and digestion, the FKBP12 cDNA was cloned in to pET28a (Novagen). Correct insertion of FKBP12 was confirmed by DNA sequencing. The pET28a-FKBP12 vector was transformed into Rosetta 2 *E. coli* (Invitrogen) and protein expression was induced via the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 1 mM at 37ºC for 4 h. Bacteria were harvested by centrifugation and stored at -20ºC.

GST-FRB. The cDNA encoding the FRB domain of human mTOR was provided in a pGEX vector by Prof. Jie Chen. The correct cDNA sequence was confirmed by DNA sequencing. The pGEX-FRB vector was transformed into Rosetta 2 *E. coli* and protein expression was induced via the addition of IPTG to 0.3 mM at 25ºC for 6 h. Bacteria were harvested by centrifugation and stored at -20ºC.

His6-caspase-3/7. The cDNA encoding human caspase-3 or caspase-7 was provided by Dr. Guy Salvesen (The Burnham Institute) in a pET23b vector. The correct cDNA sequence was confirmed by DNA sequencing. The pET23b-caspase-3/7 vector was transformed into Rosetta 2 *E. coli* and protein expression was induced via the addition of IPTG to 1 mM at 37ºC for 4 h. Bacteria were harvested by centrifugation and stored at -20ºC.

His6-caspase-9. The cDNA encoding human caspase-9 was provided by Dr. Qian Tin (Cornell University) in a pET28a vector. The correct cDNA sequence was confirmed by DNA sequencing. The pET28a-caspase-9 vector was transformed into Rosetta 2 *E. coli* and protein expression was induced via the addition of IPTG to 1 mM at 37ºC for 4 h. Bacteria were harvested by centrifugation and stored at -20ºC.

GST-XIAP. The cDNA encoding human XIAP was provided by Dr. Colin Duckett (University of Michigan) in a pGEX vector. The correct cDNA sequence was confirmed by DNA sequencing. The pGEX-XIAP vector was transformed into BL21(DE3) *E. coli* and protein expression was induced via the addition of IPTG to 1 mM at 37 ºC for 4 h. Bacteria were harvested by centrifugation and stored at -20ºC.

Protein Purification:

His6-tagged proteins. All pellets stored at -20ºC were thawed at 25ºC in 10-15 mL of NTA binding buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole pH 8.0). Bacteria were lysed by sonication, centrifuged at 35,000 x *g*, and incubated with 1 mL Ni-NTA Agarose (Qiagen) slurry for 1 h at 4ºC. Resin was washed with an additional 30 mL of NTA binding buffer, then washed with 30 mL of wash buffer (50 mM Tris, 300 mM NaCl, 20 mM imidazole pH 8.0), followed by elution with 10 mL of elution buffer (50 mM Tris, 300 mM NaCl, 500 mM imidazole pH 8.0). All proteins were dialyzed overnight into Assay Buffer (10 mM HEPES, 150 mM NaCl, 20 µM EDTA, 0.005% Tween 20 pH 7.4) and concentrated using an Amicon 3K MWCO spin concentrator. Protein concentration was determined by BCA assay (Pierce).

GST-tagged proteins. All pellets stored at -20ºC were thawed at 25ºC in 10-15 mL of binding buffer (PBS + 1 mM DTT). Bacteria were lysed by sonication, centrifuged at 35,000 x *g*, and incubated with 1 mL glutathione Sepharose 4B (GE Biosciences) slurry for 1 h at 4 ºC. Resin was washed with an additional 30 mL of PBS + 1 mM DTT, followed by elution with 10 mL of elution buffer (PBS + 20 mM glutathione). All proteins were dialyzed overnight into Assay Buffer (10 mM HEPES, 150 mM NaCl, 20 µM EDTA, 0.005% Tween 20 pH 7.4) and concentrated using an Amicon 3K MWCO spin concentrator. Protein concentration was determined by BCA assay (Pierce).

Photonic Crystal (PC) Biosensor Assays and Data Analysis:

SRU 384-well SA1 plates were allowed to warm to 25 ºC, and were washed 5 times with Assay Buffer (AB) to remove glycerol. Each well was equilibrated with 40 µL of AB; this volume was kept consistent through subsequent steps. Equilibration reads were performed on a SRU Profiler BIND® Reader. After signal stabilization, the wells were aspirated of AB, and 10 µM of BTN in AB was added to each well and allowed to incubate at 4ºC overnight. Sensor plates were allowed to warm to 25 ºC, and wells were read to confirm the PWV shift due to BTN, then washed 3 times with AB, and read again; at this point there was a consistent 0.2-0.3 nm of shift due to BTN remaining bound to the surface (Fig. S1). BTN-functionalized PC biosensors were charged with Charging Buffer (10 mM HEPES, 150 mM NaCl, 500 µM $NiCl_2$, 20 µM EDTA, 0.005% Tween 20 pH 7.4) read, washed 3 times with AB, and then read again prior to His6-tagged protein addition. The dose- and Ni^{2+}-dependence of His6-tagged protein binding is shown in Figure S2. Further detail of experiments performed for each protein—protein interaction is described below. After the final wash of each experiment, Stripping Buffer (10 mM HEPES, 150 mM NaCl, 350 mM EDTA, 0.005% Tween 20 pH 7.4) was added and the plate was incubated overnight at 4 ºC. After the overnight incubation, wells were washed 5 times with AB, and could be recharged for reuse (Figure S6, His6-FKBP12—GST-FRB section).

An advantage of the software used for data analysis using PC biosensor technology is the ability to reference each well to any previous step in the experiment. For example, in Figure 2 each well has been referenced to the assay buffer wash following overnight incubation with 10 µM BTN. This allows the visualization of the bulk shift observed upon the addition of 500 µM $NiCl_2$ in the charging buffer.

His6-caspase-3/7/9—GST-XIAP: His6-caspase-9 or His6-caspase-7 (100 µg/mL) was incubated with charged BTN-functionalized PC biosensors for 10 min, washed 3 times with AB, and then incubated with 0, 50, or 100 µg/mL GST-XIAP for 10 min and finally washed 3 times with AB. GST-XIAP was also shown to bind His6-caspase-3 in analogous experiments (Figure S3). GST-XIAP binding to His6-caspase-9 or His6-caspase-7 was inhibited by a 30 min preincubation with SM-164. SM-164 was also able to disrupt preformed GST-XIAP—His6-caspase-9 complexes (Figure S4).

His6-FKBP12—GST-FRB: His6-FKBP12 (100 µg/mL) was incubated with charged BTN-functionalized PC biosensors for 15 min, washed 3 times with AB, and then incubated with 100 µg/mL GST-FRB for 15 min and finally washed 3 times with AB. No increase in PWV was observed in the absence of rapamycin. Rapamycin was added to wells containing His6-FKBP12 at concentrations of 0, 0.1, 0.5, and 1 µM 5 min prior to GST-FRB addition, which greatly enhanced the PWV shift observed due to GST-FRB binding.

To demonstrate the HTS capability of the PC biosensor assay, a whole 384-well plate was coated with 10 µM BTN, charged, and bound with 100 µg/mL His6-FKBP12 except for wells A1 and B1. A library plate from an in-house library containing 10 mM small molecules in DMSO was diluted to 200 µM in AB, and rapamycin was added to well G16 at a concentration of 4 µM. GST-FRB was incubated with the diluted library plate for 30 min so that the final concentration of GST-FRB was 200 µg/mL, library compounds were 100 µM, and rapamycin was 2 µM. Finally, GST-FRB + cmpds was added to the His6-FKBP12 bound plate so that the final concentration of GST-FRB was 100 µg/mL, library compounds were 50 µM, and rapamycin was 1 µM. This "needle-in-a-haystack" assay was repeated in an analogous fashion for SM-164 and the GST-XIAP—caspase-9 interaction (Figure S5). GST-XIAP (0.1 mg/mL) was incubated with 375 library compounds (80 µM) or SM-164 (10 µM) for 30 min at 25 ºC. This mixture was applied to wells previously coated with 0.1 mg/mL His6-caspase-9, and washed after a 15 min incubation.

To demonstrate the reusability of the BTN-functionalized PC biosensor, His6-FKBP12 was repeatedly bound, stripped, and rebound. As shown in Figure S6, charged BTN-functionalized PC biosensors were incubated with 100 µg/mL His6-FKBP12, washed, and then incubated with Stripping Buffer. In experiments

1-3, Stripping Buffer was used repeatedly until no further decrease in PWV was observed, while experiments 4 and 5 were performed on successive days following overnight incubation with Stripping Buffer.

Supporting Figures

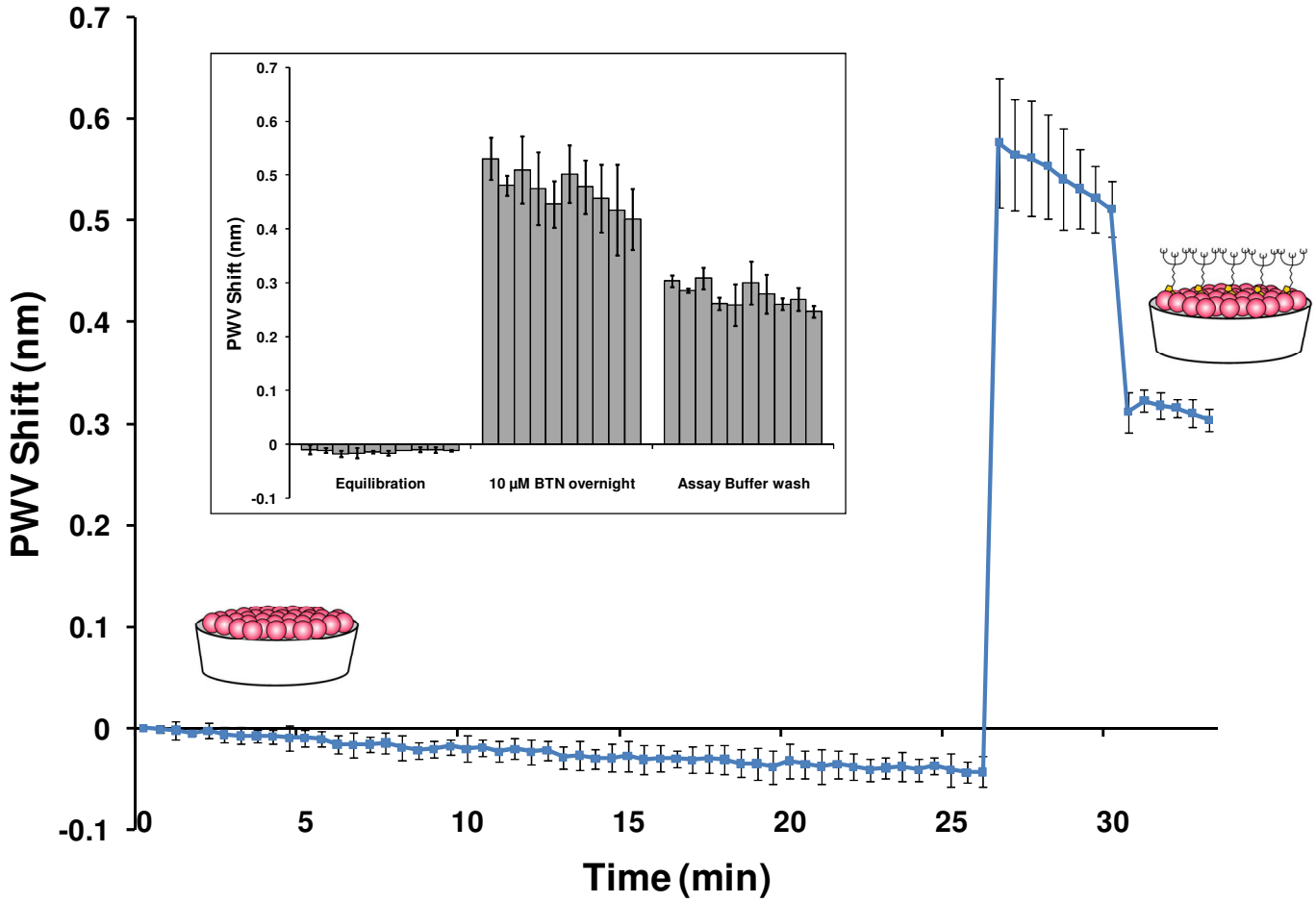


Figure S1: Overnight incubation of BTN with SA-functionalized PC biosensors produces a 0.2-0.3 nm shift. BTN (10 µM) in AB was added to SRU SA1 plates and allowed to incubate at 4 ºC overnight, and the PWV shift was recorded before and after a wash step with AB. Time course analysis displays the PWV shift due to equilibration (0-26 min), overnight incubation with BTN (26-30 min), and after washing with Assay Buffer (30-33 min). 10 replicates (in duplicate) are displayed in the inset. All error bars represent the range (n = 2).

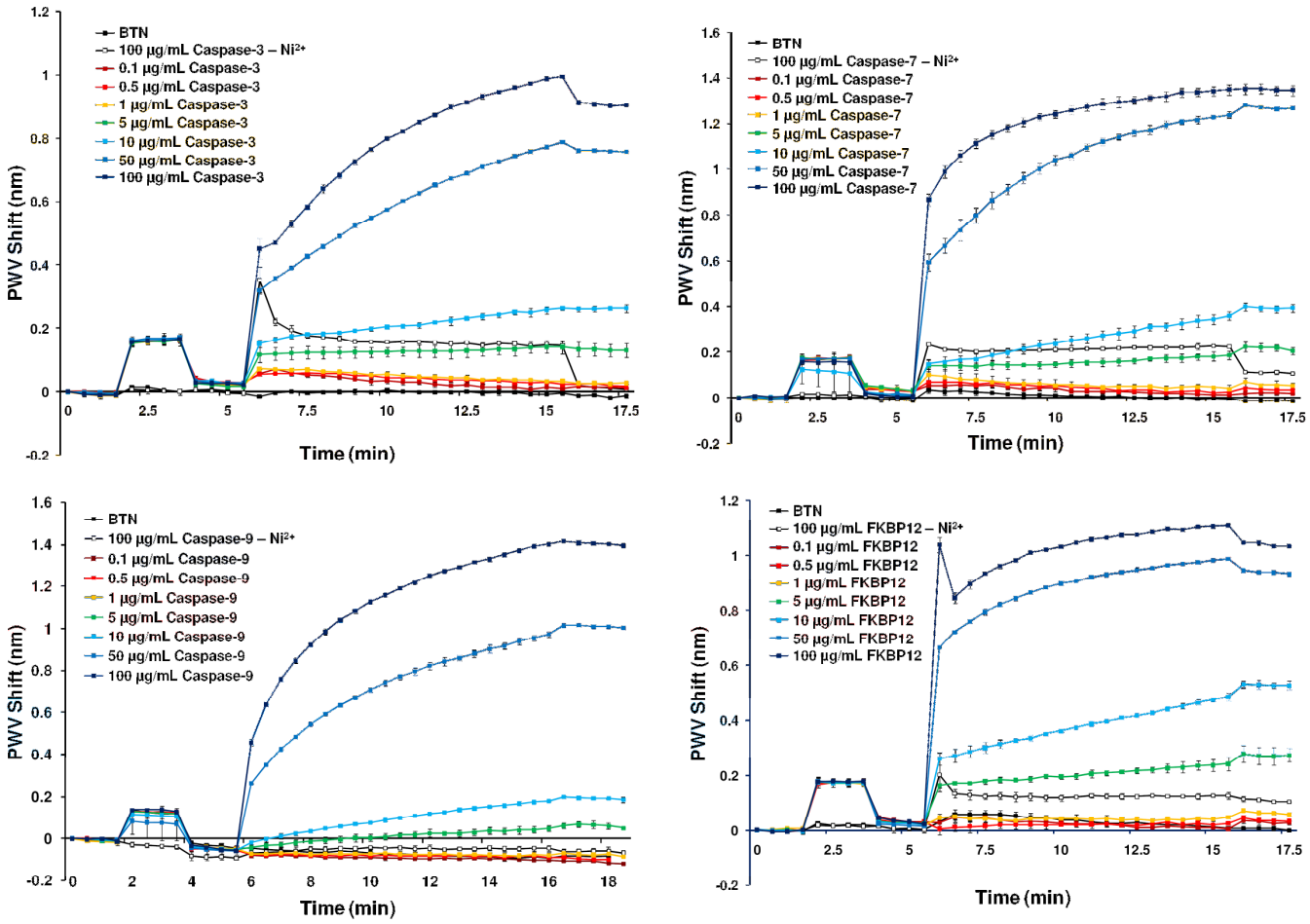


Figure S2: Dose-dependent binding of His6-tagged proteins (Caspase-3, Caspase-7, Caspase-9, and FKBP12) to BTN-functionalized PC biosensors. Varying concentrations of His6-tagged proteins were added to BTN functionalized PC biosensors, and the PWV Shift was recorded for 10 min. All error bars represent the range (n = 2).

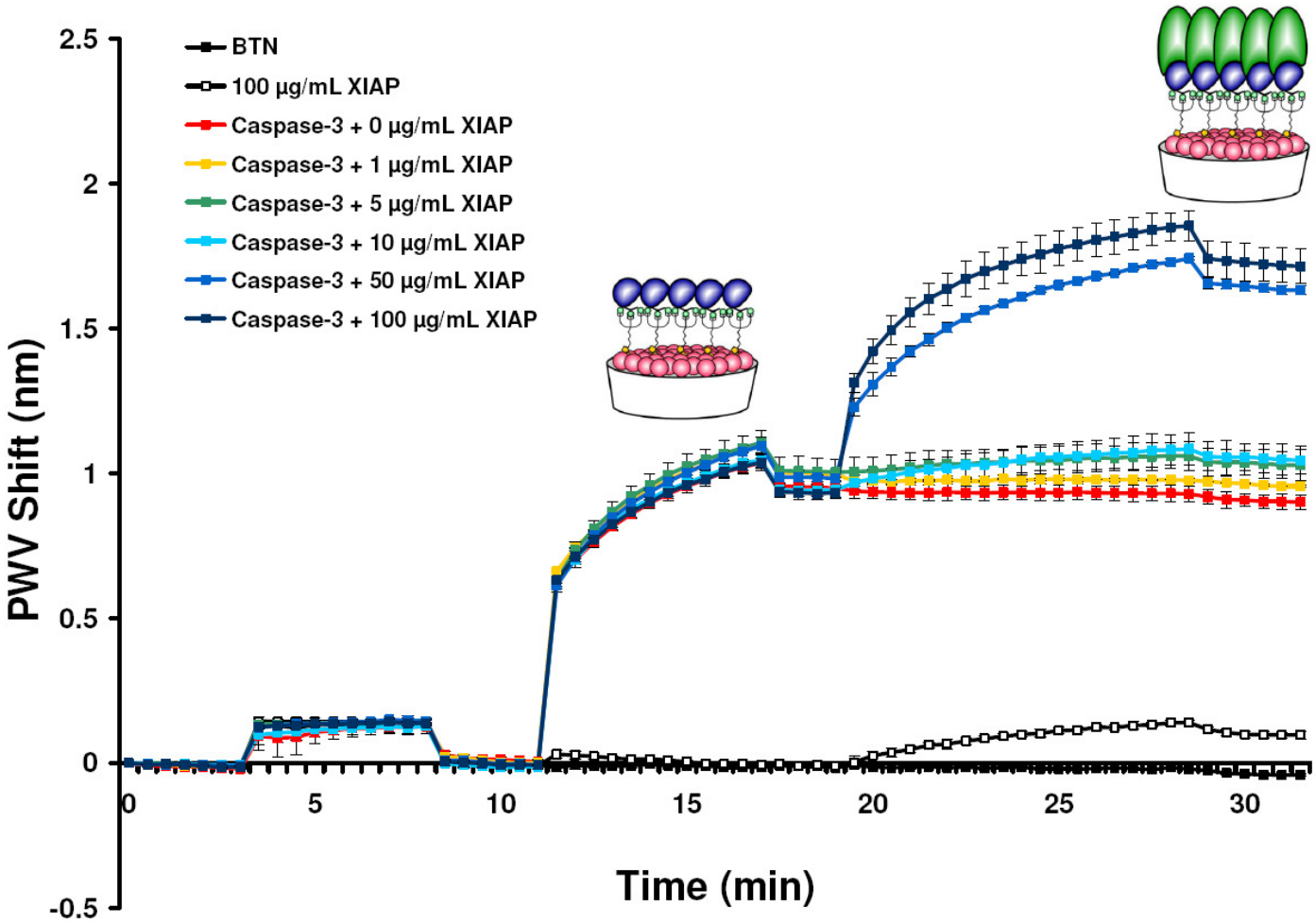


Figure S3: Dose-dependent binding of GST-XIAP to immobilized His6-caspase-3. His6-caspase-3 (100 µg/mL) was immobilized on $NiCl_2$ charged BTN-functionalized PC biosensors, then washed with Assay Buffer. Increasing PWV shifts are seen when increasing concentrations of GST-XIAP are added to the His6-caspase-3 containing wells. All error bars represent the range (n = 2).

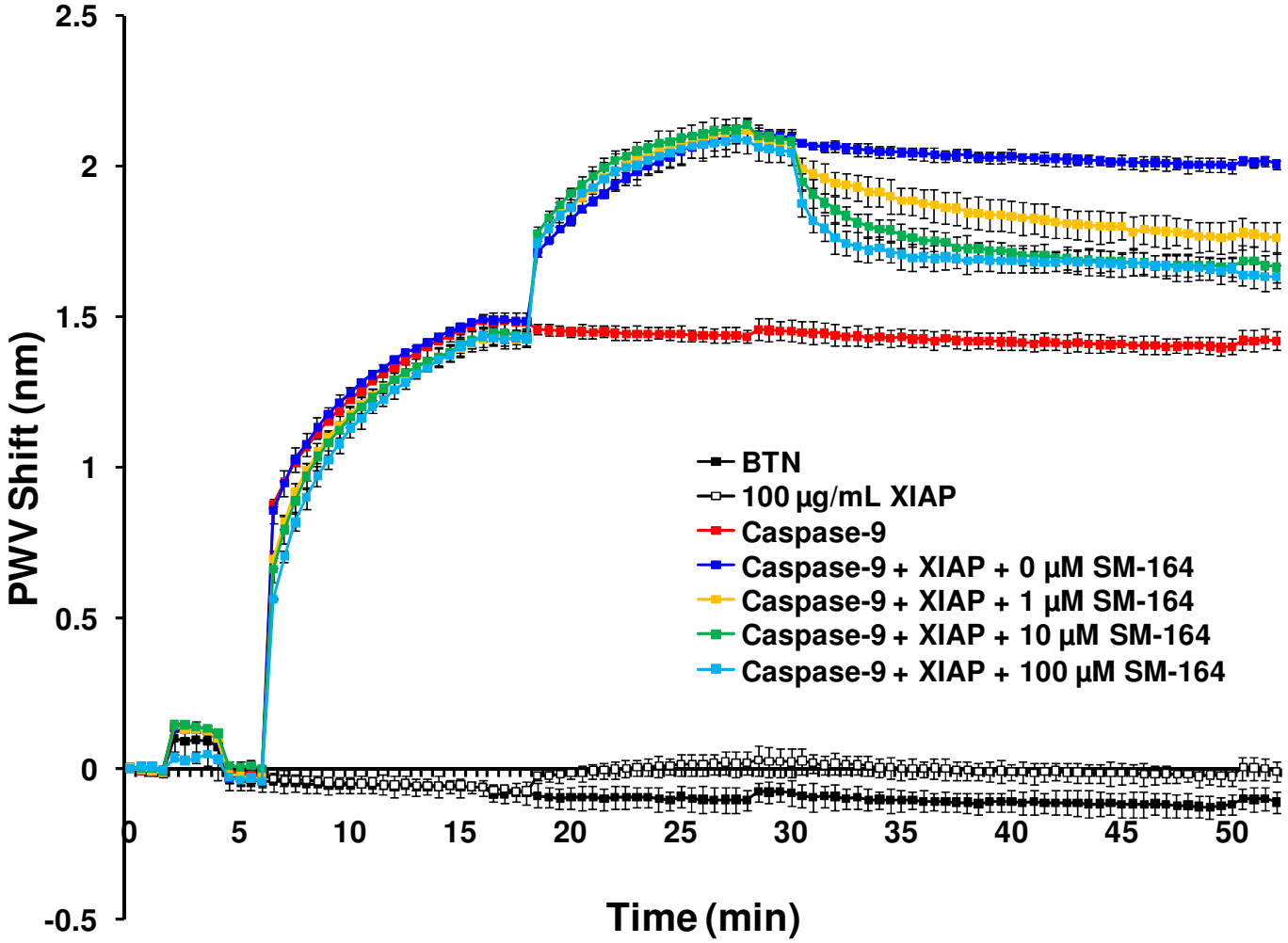


Figure S4: Dose dependent disruption of a preformed caspase-9—XIAP complex by SM-164. GST-XIAP was allowed 10 minutes to bind to immobilized His6-caspase-9, washed, and then incubated with increasing concentrations of SM-164. All error bars represent the range (n = 2).

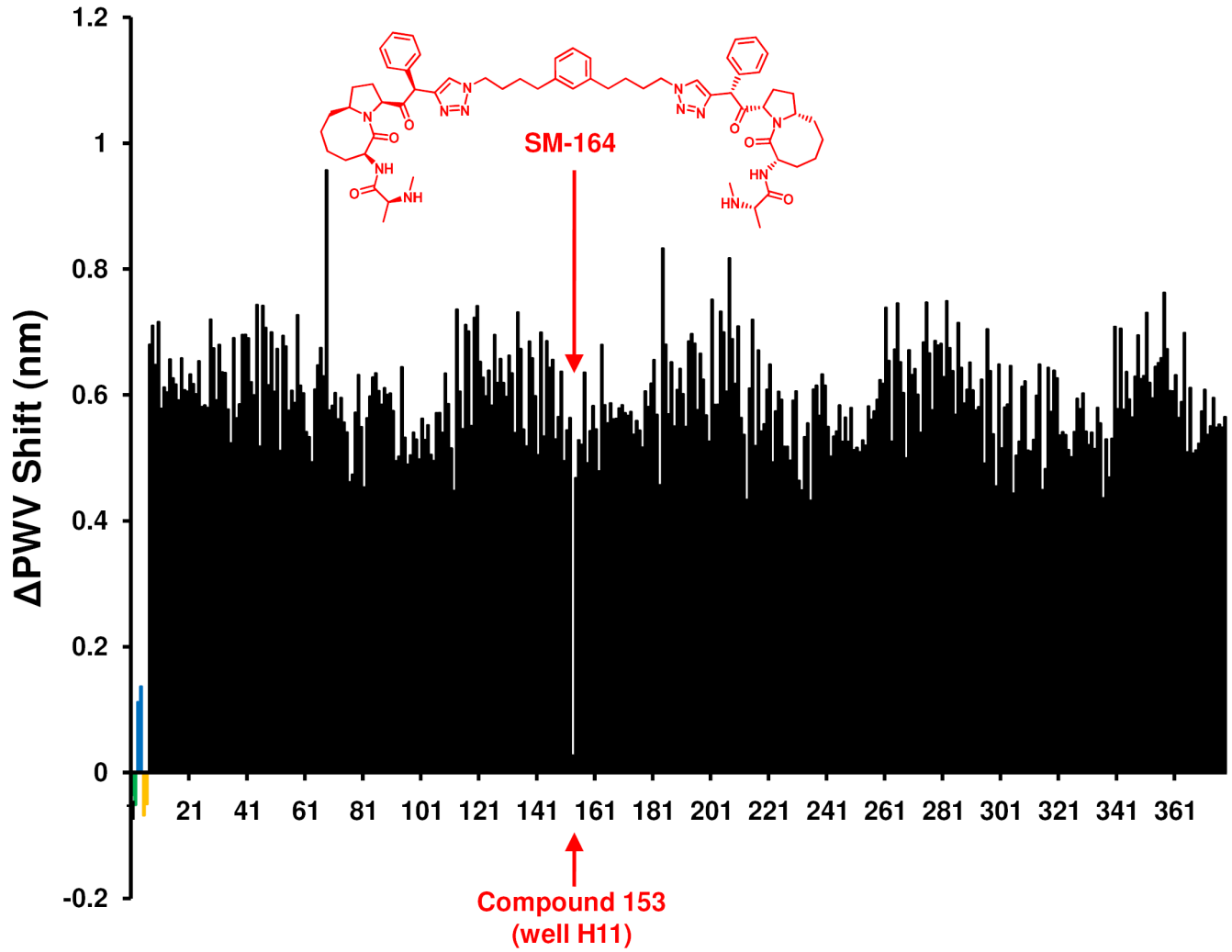


Figure S5: SM-164 is detectable in a "needle-in-a-haystack" mini-HTS. All wells of a 384-well BTN-coupled PC biosensor plate were washed, charged, and washed as described above. GST-XIAP (0.1 mg/mL) was incubated for 30 min at 25 °C with a library plate of 375 compounds (each at 80 μM), with SM-164 (10 μM) added to well H11. During this time, 0.1 mg/mL His6-caspase-9 was added to the charged BTN-coupled PC biosensor plate in all wells except 4 control wells, and the plate was incubated for 15 min at 25 °C. After a wash step, the GST-XIAP/compound mixture was added to all wells except 4 control wells, and allowed to incubate for 15 min at 25 °C. SM-164 shows robust inhibition of the GST-XIAP—His6-caspase-9 interaction in this assay. ΔPWV Shift (nm) represents the difference in signal observed after the addition of the GST-XIAP/compound mixture. Green bars: BTN only, no protein added; blue bars: 0.1 mg/mL GST-XIAP only, no His6-caspase-9 added; orange bars: 0.1 mg/mL His6-caspase-9 only, no GST-XIAP added.

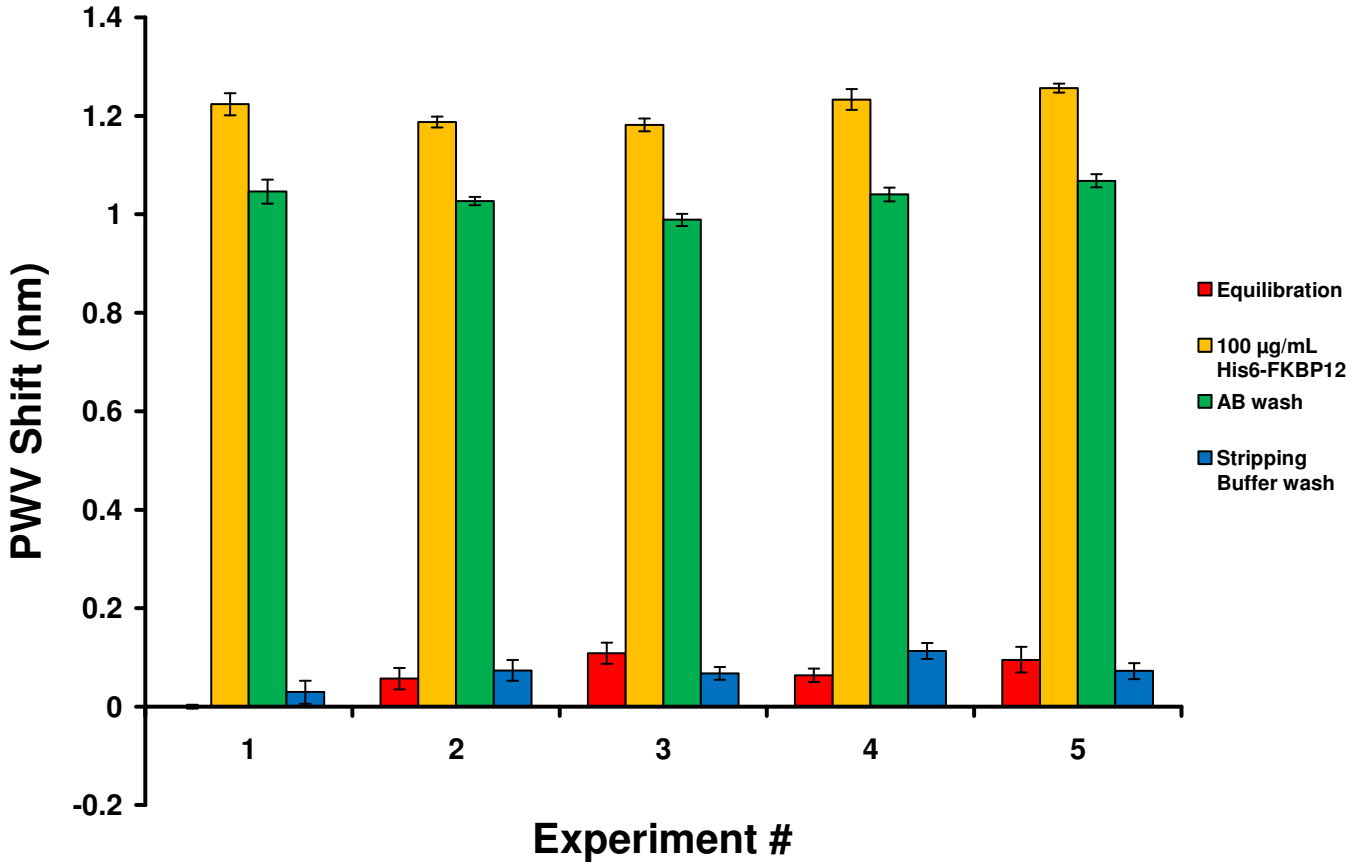


Figure S6: BTN-coupled PC biosensors are reusable at least 5 times. His6-FKBP12 was bound to charged BTN-functionalized PC biosensors, washed with Assay Buffer (AB), and then with Stripping Buffer. For experiments 1-2, Stripping Buffer was repeatedly added to each well until it was determined no further decrease in PWV could be obtained. In experiments 3-5, Stripping Buffer was added, and the plate was incubated overnight at 4 ºC, with experiments 4 and 5 performed on subsequent days after experiments 1-3. All error bars represent the range (n = 2).

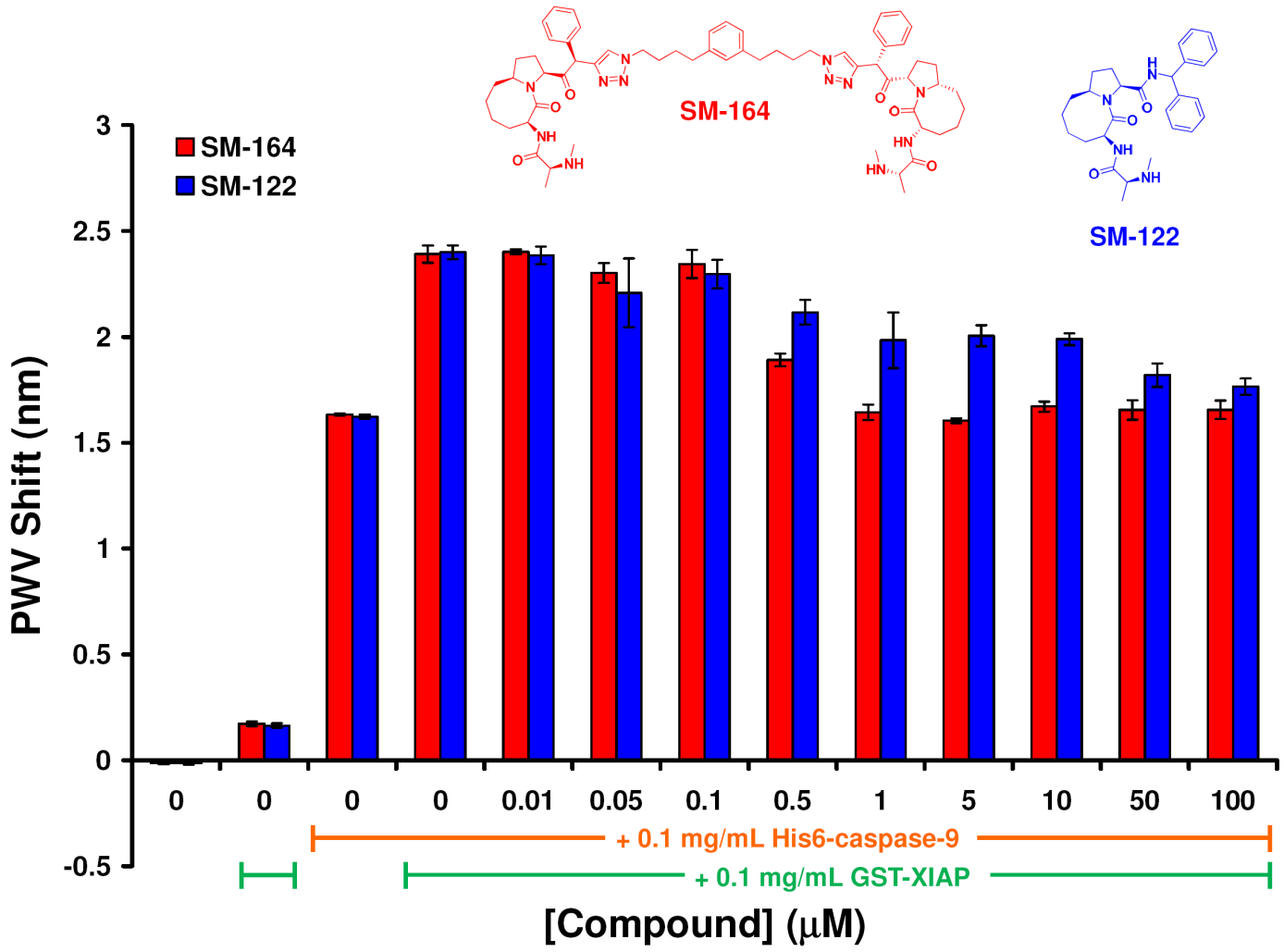


Figure S7: SM-164 is a more potent inhibitor of the GST-XIAP—His6-caspase-9 interaction than SM-122. This assay was performed exactly the same as Figure 2b, where GST-XIAP was preincubated with either SM-164 or SM-122 for 30 min at 25 °C, prior to addition to a His6-caspase-9 coated sensor. SM-164 inhibits the GST-XIAP interaction with an IC_{50} = 0.39 μM, while SM-122 has an IC_{50} = 2.72 μM. IC_{50} values were calculated and averaged over two separate experiments; shown here is a representative graph. IC_{50} values were calculated using logarithmic dose-response curve fit analysis software (TableCurve). All error bars represent the standard deviation from the mean (n = 3).