

Supplemental Information for

**Macrocyclic peptides inhibit the interaction of p53 with  
HDM2 and HDMX by accommodating a functional  $\alpha$ -helix  
motif**

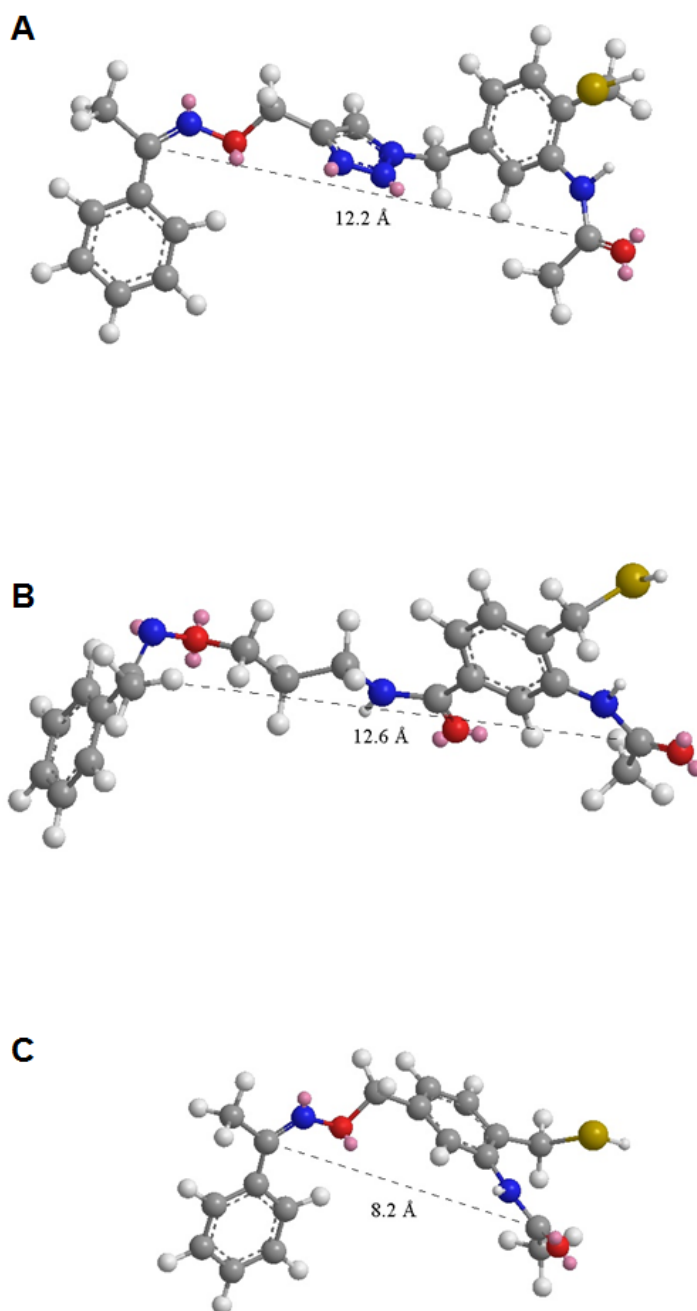
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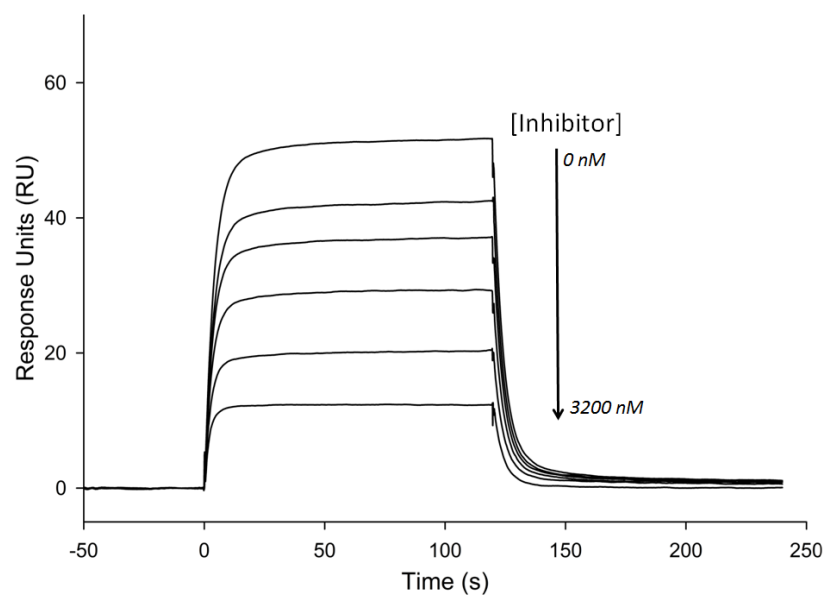
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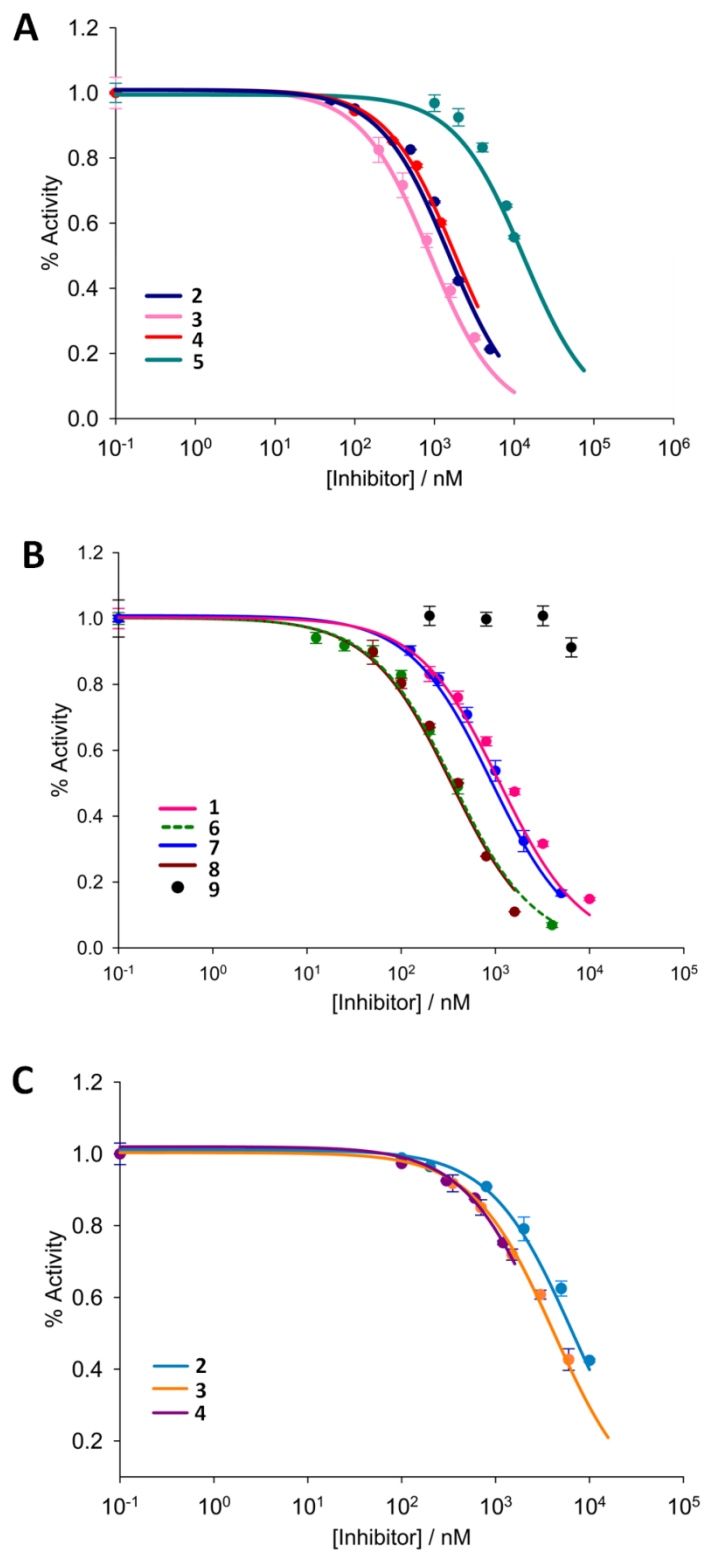
**Figure S1.** Energy minimized models of the organic linkers SP6 (A), SP8 (B), and SP4 (C) illustrating the near-maximal spanning distance between the two ligation points within the target peptide. The side-chain attachment site is mimicked by an acetophenone moiety, whereas the C-terminal attachment site is mimicked by an acetyl moiety. The distance between the carbon atoms of the oxime and amide groups is indicated.



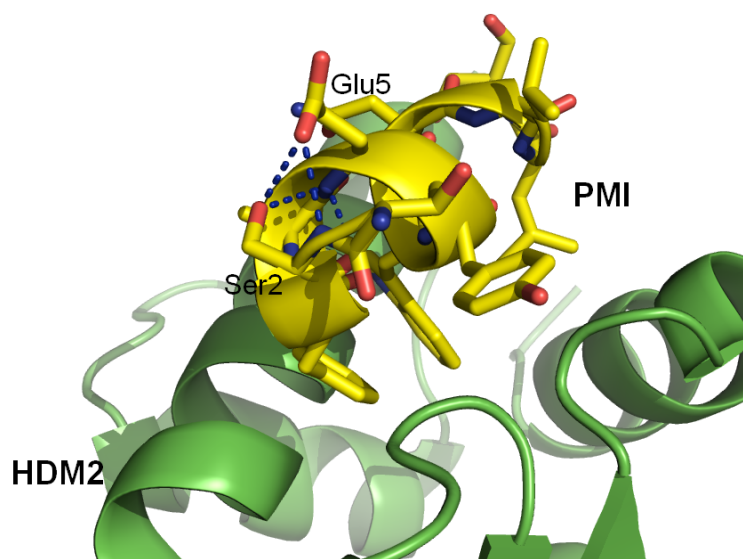
**Figure S2.** Biacore sensogram response curves of HDM2 binding to immobilized p53 peptide in the presence of varying concentrations of peptide **1** (p53<sub>15-29</sub>).



**Figure S3.** Concentration dependent inhibition of HDM2:p53<sub>15-29</sub> and HDMX:p53<sub>15-29</sub> complex formation by MOrPHs and linear peptide controls. A) Inhibition of HDM2 with peptides **2**, **3**, **4**, and **5**; B) inhibition of HDMX with peptides **1**, **6**, **7**, **8**, and **9**; C) inhibition of HDMX with peptides **2**, **3**, and **4**.



**Figure S4.** H-bond network between Glu5 side-chain carboxylic group and Ser2 amide NH and side-chain hydroxy group in the PMI peptide bound to HDM2 (pdb 3EQS).<sup>1</sup>



## EXPERIMENTAL PROCEDURES

**Cloning, Expression, and Purification of HDM2 and HDMX proteins.** Genes encoding for the p53-binding domain of human HDM2 (residues 1-109) and human HDMX (residues 1-109) were cloned into a pET22 vector (Novagen). The template for PCR amplification of the HDM2 gene was plasmid pGEX-4T MDM2 WT (AddGene # 16237).<sup>2</sup> A template for PCR amplification of the HDMX gene was kindly provided by Prof. Mike Dyer in the Division of Developmental Biology at the St. Jude Children's Research Hospital (Memphis, TN). In the final plasmid constructs (pET22-HDM2-YFP-His and pET22-HDMX-YFP-His), the HDM2/X protein was C-terminally fused to Yellow Fluorescent Protein (YFP) containing a C-terminal His tag. Fusion to the YFP protein was found to improve the solubility and stability of the protein constructs. To isolate the HDM2-YFP and HDMX-YFP fusion proteins, pET22-HDM2-YFP-His and pET22-HDM2-YFP-His plasmids were each transformed into BL21(DE3) cells followed by plating and overnight growth in LB medium containing ampicillin (50 mg L<sup>-1</sup>). The overnight cultures were used to inoculate a 500 mL LB culture (ampicillin at 50 mg L<sup>-1</sup>), which was induced with 0.5 mM IPTG at OD<sub>600</sub> ~ 0.6, and incubated for 16 hours at 27 °C. Cells were harvested by centrifugation and lysed by sonication. The clarified lysate was loaded onto a Ni-NTA affinity column and the protein was eluted with 50 mM Tris, 150 mM NaCl, 300 mM imidazole (pH 7.4). After buffer exchange with potassium phosphate 50 mM, NaCl 150 mM buffer (pH 7.5), aliquots of the protein solutions were stored at -80°C. Protein concentration was determined using the extinction coefficient at 280 nm ( $\epsilon_{280}$ ) calculated based on the protein primary sequence. The identity of the isolated protein was confirmed by MALDI-TOF and SDS-PAGE.

### **Cloning, Expression, and Purification of PMI-2 and PMI-3 Containing Biosynthetic Precursors.**

The protein precursors containing the target peptide sequence PMI-3 (GTSFA(pAcF)YWNLLA) and PMI-2 (G(pAcF)SFAEYWNLLA) followed by *Mxe* GyrA(N198A) intein and a C-terminal His tag were prepared as follows. First, genes that encode for these peptide sequences (an amber stop codon, TAG, is used for incorporation of pAcF) fused to the GyrA gene were generated by PCR using the pET22b-based plasmid pMG-G8T<sup>3</sup> as template, forward primers PMI\_for1 5'-GCGATTGGAACCTGCTGGCGTGCATCACGG-GAGATGCACTAGT-3' and PMI\_for2 5'-CTAGACATAT-GGGCTAGAGCTTCGCGGAATATTGGAACCTGCTGGCGTGCAT-3', and the reverse primer T7\_terminator 5'-GCTAGTTATTGCTCAGCGGTGGC-3'. The resulting PCR products (~ 0.75 Kbp) were cloned into pET22 plasmid (Novagen) using *Nde* I and *Xho* I restriction enzymes, to produce the plasmids pPMI-2-GyrA and pPMI-3-GyrA. In these constructs, the gene encoding for the biosynthetic precursor protein is under the control of an IPTG-inducible T7 promoter. The precursor proteins were expressed by co-transforming pPMI-2-GyrA (or pPMI-3-GyrA) and a pEVOL-based vector

encoding for a reported *Mj* tRNA<sub>CUA</sub> / aminoacyl-tRNA synthetase pair<sup>4</sup> for amber stop codon suppression with *para*-acetylphenylalanine (pAcF), into BL21(DE3) *E. coli* cells. Protein expression was carried out as described.<sup>3</sup> After expression, the proteins were purified Ni-affinity chromatography as described above. The identity of the isolated protein was confirmed by MALDI-TOF and SDS-PAGE. MS analysis indicated complete cleavage of the initial methionine in the purified proteins.

**Synthesis and Purification of PMI-based MOrPHs.** The PMI-2 and PMI-3-based MOrPHs were prepared by large scale macrocyclization reactions between precursor protein PMI-2-GyrA (or PMI-3-GyrA) and the appropriate synthetic precursor (SP6, SP8, or SP4). In addition to providing the desired compounds, this approach was chosen (over solid-phase peptide synthesis) to demonstrate the scalability of our catalyst-free chemobiosynthetic method for MOrPH synthesis.<sup>3</sup> In a typical reaction, the protein (200  $\mu$ M in potassium phosphate 50 mM, NaCl 150 mM buffer (pH 7.5)) was mixed with 10 mM synthetic precursor and 10 mM TCEP (total volume: 6 mL). After 30 hrs, the pH of the reaction mixture was adjusted to 8.5 and incubated with iodoacetamide (15 mM) for 1 hour to cap the free thiol group. The reaction was centrifuged at 4000 x g for 2 minutes, after which the supernatant (*a*) was separated from the pellet. The pellet was resuspended in 20% acetonitrile/H<sub>2</sub>O by vortexing for several minutes to dissolve the MOrPH product, then centrifuged at 4000 x g for 2 minutes to provide supernatant (*b*). The supernatants (*a* and *b*) were combined and applied to a solid-phase extraction C<sub>18</sub> column pre-washed with 10 column volumes (CV) MeOH, 10 CV acetonitrile, and 10 CV water. The macrocyclic product was eluted using a gradient of acetonitrile in water from 10% to 80%. The eluted MOrPH was further purified by HPLC using a GraceSmart RP C18 column (250 x 4.6mm, 5  $\mu$ m) maintained at 25°C, a flow rate of 0.9 mL/min, a binary mobile phase system consisting of A: water + 0.1% TFA and B: acetonitrile + 0.1% TFA, and a linear gradient from 10% to 90% solvent B (12 min). The identity of the isolated MOrPH was confirmed by LC-MS and MS/MS. Masses for all MOrPH products are listed in Table S1.

**Table S1.** Calculated and observed masses of MOrPH ligands

Peptide	Mass Calc. [M+H] <sup>+</sup>	Mass Obs. [M+H] <sup>+</sup>
3	1718.5	1719.1
4	1708.9	1708.4
5	1637.9	1637.5
7	1746.4	1746.2
8	1736.9	1737.3
9	1608.8*	1608.5*

\* denotes free thiol (no acetamide alkylation)

**Inhibition Assays.** The surface plasmon resonance (SPR)-based inhibition assays were performed using a BIAcore T100 instrument. A HDM2/X binding surface was first generated by immobilizing ~500 RU of a biotinylated p53 peptide (biotin-SGSG-p53<sub>15-29</sub>) on a streptavidin-coated biosensor chip (SA chip, GE Healthcare). Running buffer and sample buffer contained 10 mM HEPES buffer, pH 7.4 with 150 mM NaCl, 3 mM EDTA and 0.05% v/v Tween 20. For the inhibition studies, increasing concentrations of inhibitor were added to a fixed concentration (150 nM) of purified HDM2-YFP or HDMX-YFP and the mixture was injected over the functionalized surface. With increasing concentrations of the inhibitor, binding of HDM2 (or HDMX) to the surface is inhibited, leading to a decrease in biosensor response. HDM2/HDMX plus inhibitor samples were injected at a rate of 30  $\mu$ L/min over a 2 minute interval (Figure S4) by a 2 minute dissociation period and a 10-second regeneration step using 10 mM HCl. The chip was allowed to re-equilibrate for 1 minute between runs in running buffer. Specific binding curves for each concentration of inhibitor were obtained by subtracting the response in the reference surface from the response in the p53-coated surface. Response values were calculated by averaging the last 10 s of each sample injection. The data was analyzed with SigmaPlot 12.5 software and the sigmoidal plots fitted to the Hill equation for a one site competitive binding to derive IC<sub>50</sub> values (Figure S2). Reported IC<sub>50</sub> average values and standard deviations were derived from at least two independent experiments.

**Circular Dichroism Studies.** CD spectra were recorded with a JASCO J-710 CD spectropolarimeter using a 0.1 cm path length cuvette at room temperature. The purified peptides were dissolved in 5 mM potassium phosphate buffer (pH 7.0) containing 40% trifluoroethanol to a final concentration of 20-50  $\mu$ M. Spectra were averaged over 2 scans recorded from 195 to 250 nm wavelength range with a speed of 10 nm/min, a response time of 1.0 s, and a resolution of 0.5 nm. The bandwidth was set to 2.0 nm and the sensitivity of the spectrometer set to 100 mdeg. The mean residue ellipticity was plotted vs. wavelength and the helical content of each peptide derived based on the following formula:  $[\theta]_{222}/[40000 \times (n - 4)/n]$  where n = number of peptide bonds.<sup>5</sup>

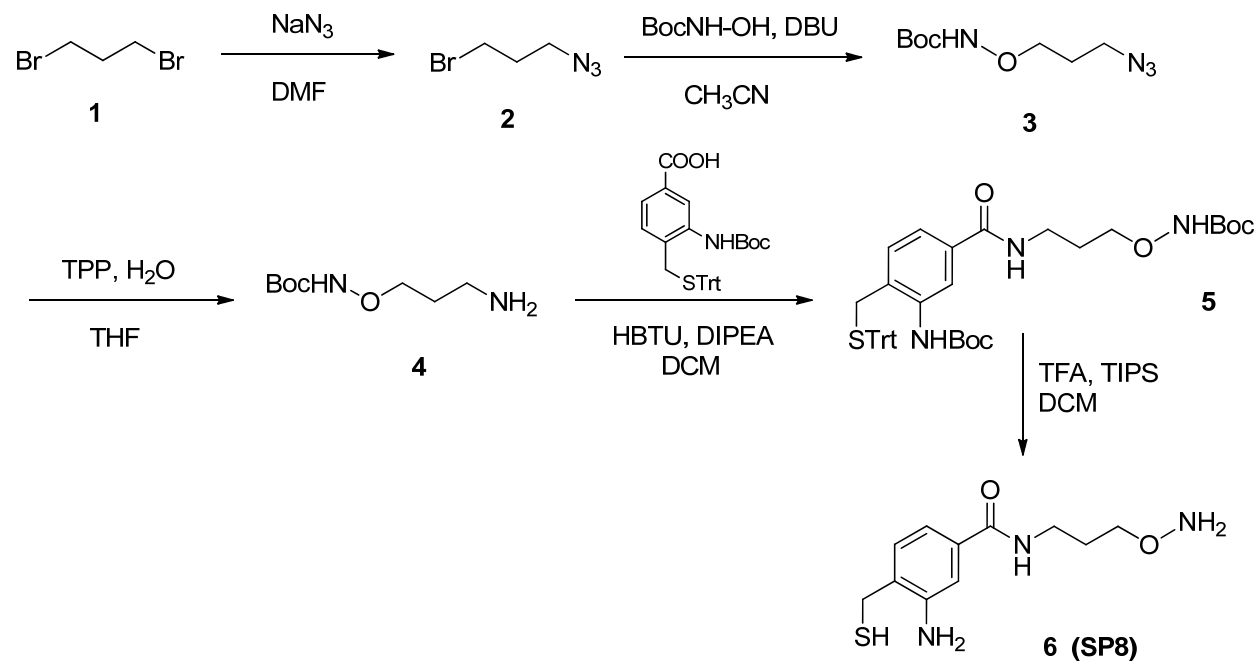
**Analysis of Proteolytic Resistance.** Each peptide (10  $\mu$ M) was dissolved in 50 mM potassium phosphate buffer (pH 7.5) containing 150 mM NaCl and 10% DMSO. Chymotrypsin (Sigma-Aldrich) was added to a final concentration of 1.0  $\mu$ g / mL and incubated at room temperature. At each time point, a 50  $\mu$ L aliquot of the mixture was removed, quenched by TFA addition (5  $\mu$ L) followed by HPLC analysis. Peptide cleavage was monitored based on the decrease of the peak area corresponding to the integer peptide. Experiments were performed at least in duplicate. HPLC analyses were carried out using a GraceSmart RP C18 column (250 x 4.6mm, 5  $\mu$ m) maintained at 25°C, a flow rate of 0.9 mL/min, a binary mobile phase system consisting of A: water + 0.1% TFA and B: acetonitrile + 0.1% TFA, and a linear gradient from 10% to 90% solvent B in 12 min.



## SYNTHETIC PROCEDURES

**Synthesis of SP6 and SP4.** Synthetic precursors SP6 and SP4 were synthesized as described previously.<sup>3</sup>

### Synthesis of SP8.



**1-azido-3-bromopropane (2):** A solution of 1,3-dibromopropane (**1**) (4.0 g, 19.8 mmol) in dry DMF (30 mL) was placed under argon. Sodium azide (1.16 g, 17.8 mmol) was added and the reaction stirred at 50 °C for 16 hours. The reaction was cooled to room temperature and extracted from ice-cold water with ethyl acetate (3 x 20 mL). The combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* to yield a yellow oil. The crude product was purified by silica gel flash chromatography (100% hexanes) to yield **2** (1.62 g, 50%) as a clear oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 2.06-2.12 (m, 2 H), 3.47-3.51 (m, 4 H).

***tert*-butyl (3-azidopropoxy)carbamate (3):** 1-azido-3-bromopropane (**2**) (0.73 g, 4.45 mmol) was dissolved in acetonitrile (14 mL). DBU (1.33 mL, 8.91 mmol) and *N*-Boc-hydroxylamine (0.889 g, 6.68 mmol) were added to the solution, which was placed under argon and connected to a reflux condenser. The reaction was heated to 50 °C. After 20 h, water (35 mL) was added and the product was extracted with ethyl acetate (3 x 20 mL). The combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo*. The crude product was then purified by silica gel flash chromatography with hexanes : ethyl acetate (95:5) to afford **3** as a clear oil (0.8 g, 83%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 1.48 (s, 9 H), 1.88-1.91 (m, 2 H), 3.45 (t, 2 H, J = 6.6), 3.93 (t, 2 H, J = 6.0), 7.11 (br, 1 H).

**tert-butyl (3-aminopropoxy)carbamate (4):** Triphenylphosphine (1.16 g, 4.44 mmol) was added to a solution of *t*-butyl (3-azidopropoxy)carbamate (**3**) (0.8 g, 3.70 mmol) in THF (17 mL) and water (0.3 mL). The reaction mixture was stirred at room temperature for 12 hours. THF was then removed under reduced pressure and the crude product was purified by silica gel flash chromatography with dichloromethane : methanol (80:20) to yield **4** as a yellow oil (0.51 g, 71%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 1.47 (s, 9 H), 1.77-1.81 (m, 2 H), 2.88 (t, 2 H, J = 6.5), 3.95 (t, 2 H, J = 5.5). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 28.23, 30.50, 39.02, 74.89, 81.69, 157.1. MS (ESI) calcd for C<sub>8</sub>H<sub>18</sub>N<sub>2</sub>O<sub>3</sub> [M+H]<sup>+</sup> *m/z*: 191.24; found: 191.55.

**tert-butyl(5-((3-(((tert-butoxycarbonyl)amino)oxy)propyl)carbamoyl)-2-((tritylthio)methyl)-phenyl)carbamate (5):** 3-((tert-butoxycarbonyl)amino)-4-((tritylthio)methyl)benzoic acid (0.677 g, 1.31 mmol) was dissolved in dichloromethane (15 mL) and the solution was added with *tert*-butyl-3-aminopropoxycarbamate (**4**) (0.25 g, 1.31 mmol), HBTU (0.745 g, 1.96 mmol), and DIPEA (0.55 mL, 3.15 mmol) under argon. The reaction mixture was stirred at room temperature for 3 hours, after which it was diluted with water and extracted with dichloromethane (3 x 40 mL). The organic layers were concentrated under reduced pressure to afford a crude residue, which was then purified by silica gel flash chromatography using hexanes : ethyl acetate (70:30) to yield **5** (0.658 g, 72%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 1.45 (s, 9 H), 1.52 (s, 9 H), 1.85-1.90 (m, 2 H), 3.18 (s, 2 H), 3.56-3.61 (m, 2 H), 3.98 (t, 2 H, J = 5.6), 7.14 (d, 1 H, J = 8.0), 7.22-7.26 (m, 3 H), 7.30-7.34 (m, 6 H), 7.48-7.52 (m, 7 H), 8.18 (s, 1 H).

**3-amino-N-(3-(aminooxy)propyl)-4-(mercaptomethyl)benzamide (6):** *tert*-butyl (5-((3-(((tert-butoxycarbonyl)amino)oxy)propyl)carbamoyl)-2-((tritylthio)methyl)phenyl)carbamate (**5**) (0.48 g, 0.69 mmol) was dissolved in dichloromethane (7.5 mL) under argon at 0 °C. Triisopropylsilane (0.36 mL, 1.75 mmol) was added, followed by TFA (1.6 mL, dropwise). The reaction was stirred for 30 minutes at 0 °C. Volatiles were then removed *in vacuo* and the yellow residue placed under high vacuum over night. The product was triturated with ice-cold hexanes and dried *in vacuo* to yield SP8 as a solid (0.18 g, quant.). <sup>1</sup>H NMR (500 MHz, d<sub>4</sub>-MeOD): δ 1.95-2.01 (m, 2 H), 3.48 (t, J = 6.8, 2 H), 3.72 (s, 2 H), 4.11 (t, 2 H, J = 6.0), 7.09 (d, J = 8.0, 1 H), 7.13 (d, J = 8.0, 1 H), 7.23 (s, 1 H). <sup>13</sup>C NMR (125 MHz, d<sub>4</sub>-MeOD): δ 29.14, 37.34, 40.14, 74.16, 116.3, 116.6, 117.8, 126.5, 132.7, 135.9, 170.5. MS (ESI) calcd for C<sub>11</sub>H<sub>17</sub>N<sub>3</sub>O<sub>2</sub>S [M+H]<sup>+</sup> *m/z*: 256.34; found: 255.92.

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