Supplementary Information

Monitoring Replication Protein A (RPA) dynamics in homologous recombination through site-specific incorporation of non-canonical amino acids

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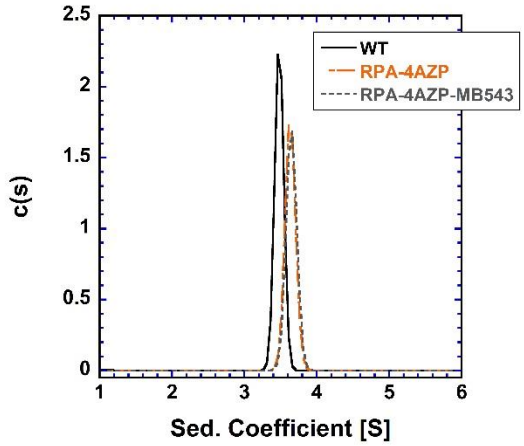
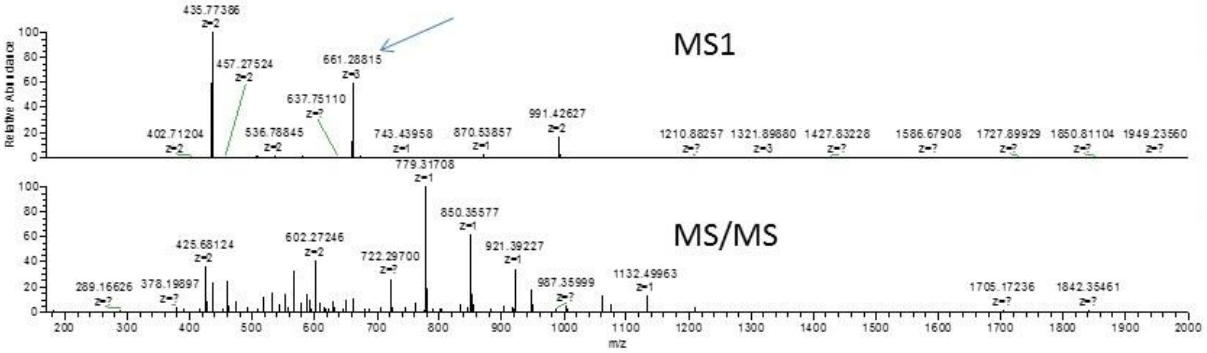
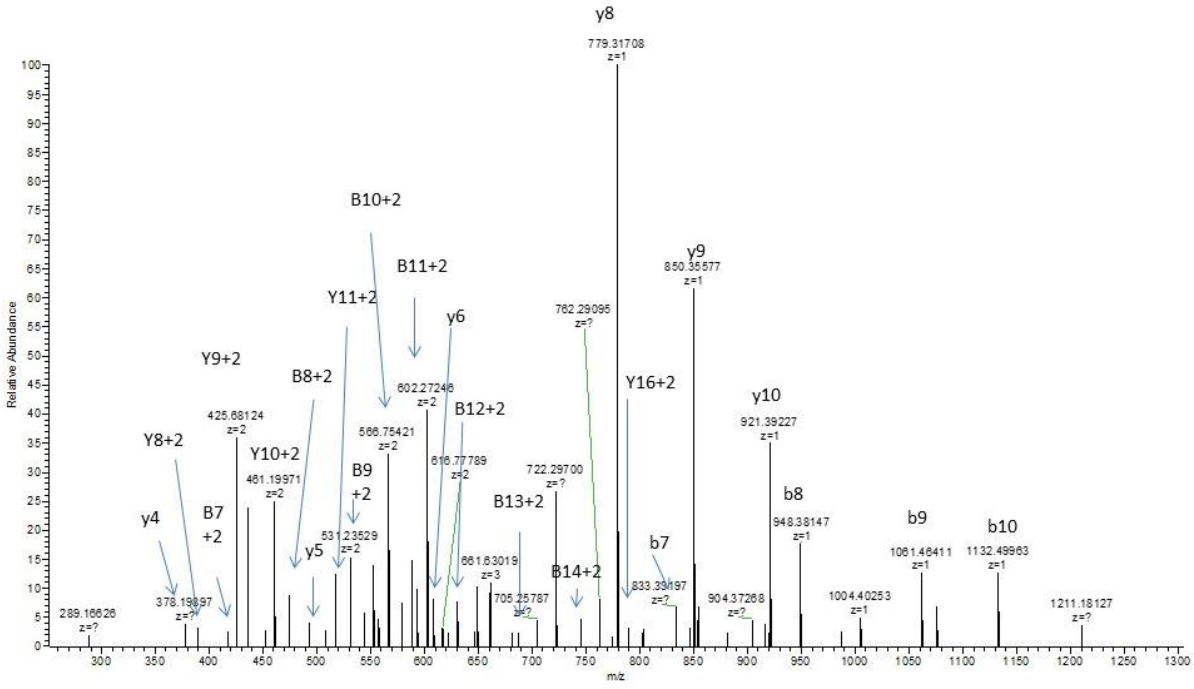


Supplemental Figure 1. Sedimentation velocity analysis of RPA^{f}. Analytical ultracentrifugation analysis of RPA^{WT}, RPA with 4AZP incorporated, and RPA bio-orthogonally labeled with MB543 show the presence of a single heteromeric species with predicted molecular weights – 114 kDa, 116.4 kDa and 117 kDa, respectively. AUC experiments were performed with 3 μM RPA at 25 °C.

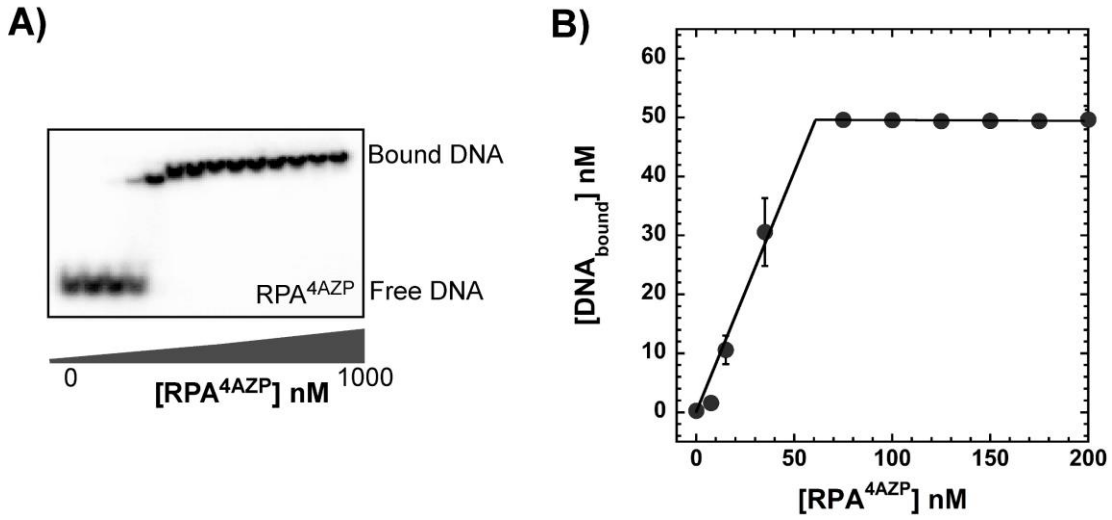
A)



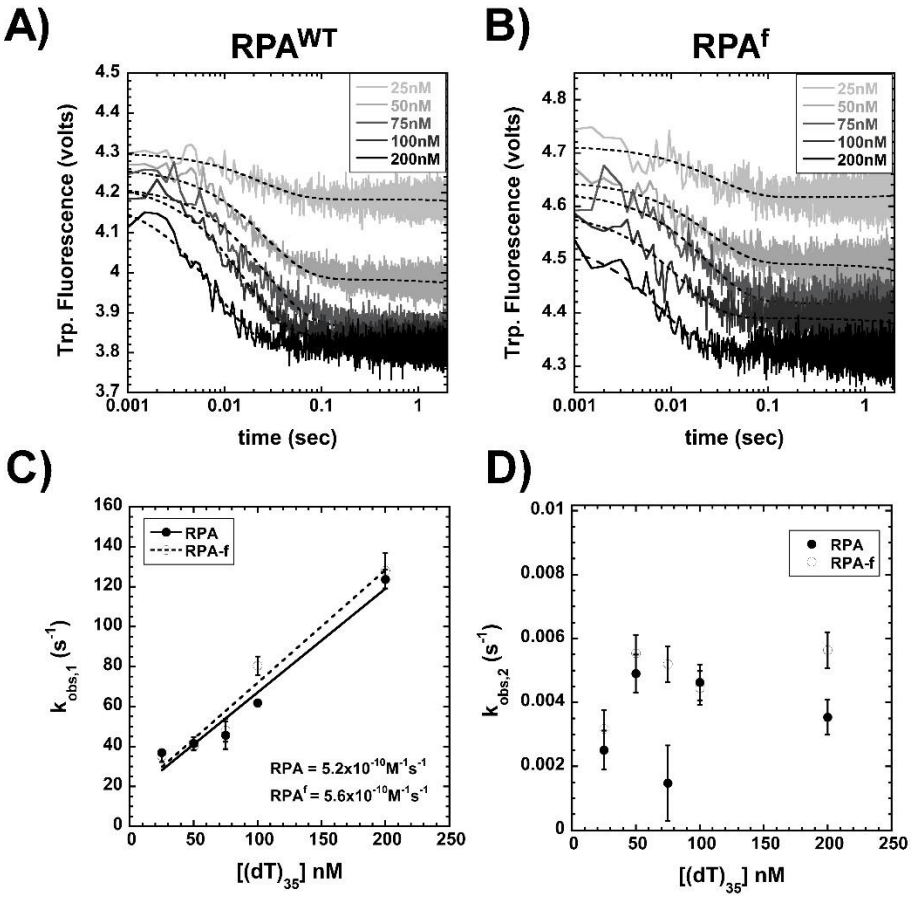
B)



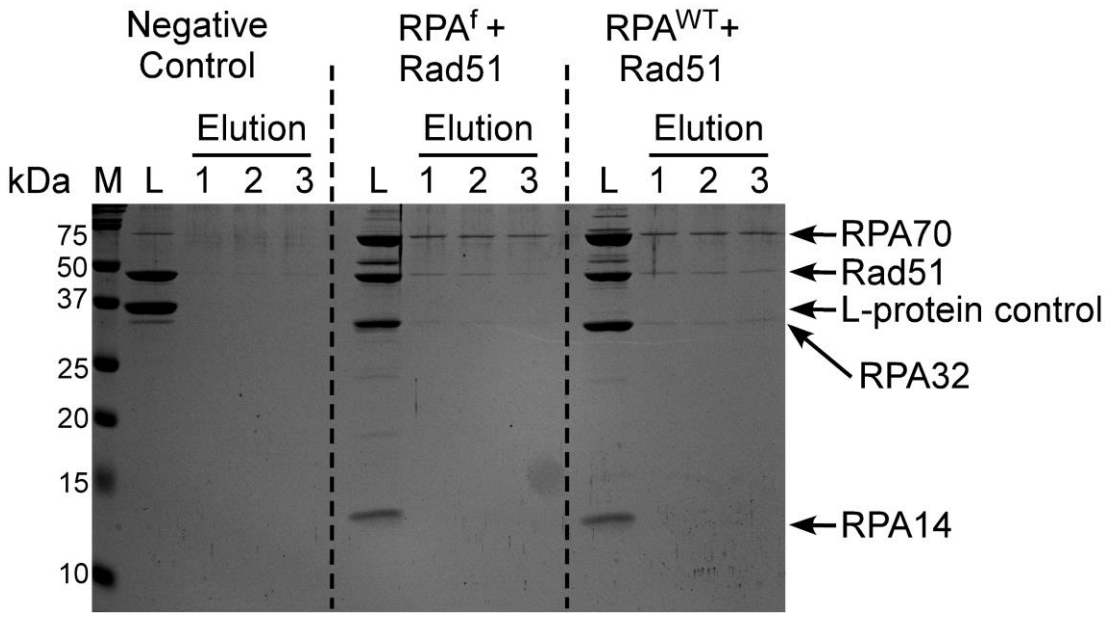
Supplemental Figure 2. Mass spectrometric analysis of RPA^{4AZP}. RPA^{4AZP} was separated on a 12 % SDS-PAGE gel and bands corresponding to RPA32 were excised, extracted, digested with trypsin, and analysed using electrospray orbitrap MS and MS/MS sequencing. **A)** Data from the orbitrap analysis show the presence of peptides carrying 4AZP at position 101. The predicted mass of the peptide with the 4AZP modification [K(4AZP)SEDANDLAAGNDDSSGK] is 661.28. The spectra show the presence of the $(M+3H)^{3+}$ species with mass of 661.28 (denoted by the arrow). **B)** A zoomed version of the MS/MS spectra and analysis of the peptide show excellent coverage and high resolution of the species corresponding to the expected peptide and position of 4AZP.



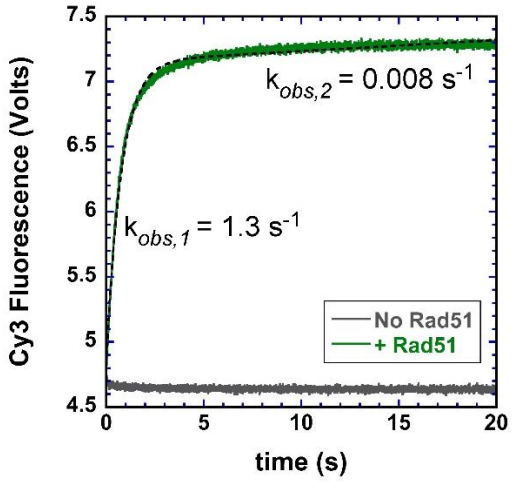
Supplemental Figure 3. Electrophoretic mobility band shift analysis of RPA^{4AZP} DNA interaction. A) 50 nM ^{32}P-end labeled $(dT)_{35}$ was incubated with increasing concentrations of RPA^{4AZP} and the band shift was assessed as described in the methods. **B)** Quantitation of the band shifts in panel A show stoichiometric binding of RPA^{4AZP} to $(dT)_{35}$.



Supplemental Figure 4. Intrinsic tryptophan-based measurement of RPA-$(dT)_{35}$ kinetics. Increasing concentrations of $(dT)_{35}$ were rapidly mixed with 100 nM **A)** RPA^{WT} or **B)** RPA^{f} [post-mixing concentrations], and the change in intrinsic tryptophan fluorescence was measured. Data were fit to a single exponential + linear fit ($\Delta F = A(1 - e^{-k_1 t}) + k_2 t$. Eq.2) and two observed rate constants were obtained. $k_{obs,1}$ and $k_{obs,2}$ describe the exponential and linear phases, respectively; F is change in tryptophan fluorescence and A is the amplitude of signal. **C)** Plot of $k_{obs,1}$ as a function of $[(dT)_{35}]$ yields association constants of 5.2 ± 0.3 x 10^{-10} $M^{-1}s^{-1}$ versus 5.6 ± 0.5 x 10^{-10} $M^{-1}s^{-1}$ for RPA^{WT} and RPA^{f}, respectively. **D)** $k_{obs,2}$ does not change appreciably for either RPA^{WT} or RPA^{f}.



Supplemental Figure 5. RPA-Rad51 interactions. 3 μM of His-RPA^{WT}, His-RPA^{f} or a non-specific protein (L-protein, an unrelated oxidoreductase) were bound to Ni^{2+}-NTA beads in the presence of Rad51 (3 μM), washed and then sequentially eluted with increasing concentrations of imidazole (100, 200 and 400 mM – elutions 1, 2 and 3). Rad51 forms a complex with RPA^{WT} and RPA^{f}, and is found in the elution samples. There is no Rad51 binding to the non-specific control protein. Lanes represent: M = protein marker; L = Total Load; 1, 2, 3 = elutions with increasing imidazole.



Supplemental Figure 6. Kinetics of Rad51 binding to 5'Cy3-$(dT)_{79}$ ssDNA. Stopped flow analysis of Rad51 (1.5 μM) binding to 5'Cy3-$(dT)_{79}$ ssDNA oligonucleotide (40 nM) in reaction buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 5 mM ATP and 6 % v/v glycerol) at 25 °C**.** Cy3 fluorescence was measured by exciting the sample at 515 nm and emission was monitored using a 555 nm long-pass filter. Data were fit to a single-exponential plus linear equation to obtain observed rate constants: $\Delta F = A(1 - e^{-k_1 t}) + k_2 t$. k_1 and k_2 are the two observed rate constants and A is the amplitude of Cy3 fluorescence change. A rapid and slow phase with observed rate constants of 1.3 ± 0.3s^{-1} and 0.008 ± .001 s^{-1} were captured.

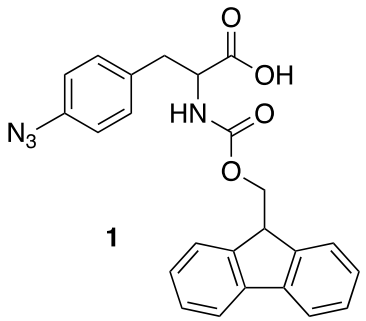
Supplementary Methods

Analytical sedimentation.

Sedimentation velocity experiments were performed using an Optima XL-A analytical ultracentrifuge equipped with an An50Ti rotor (Beckman Coulter, Fullerton, CA) at 25 °C. Experiments were performed with 3 μM RPA, RPA^{4AZP} or RPA^{f} in 30 mM Hepes, pH 7.8, 30 mM KCl, 10 % v/v glycerol and 2 mM TCEP). 380 μl of protein and 392 μl of buffer were loaded into their appropriate sectors of an Epon charcoal-filled two-sector centrepiece and centrifuged at 42000 rpm while both absorbance and interference signal were simultaneously measured. The continuous sedimentation coefficient c(s) was calculated using the program SEDFIT[1] and predicted molecular weights were obtained.

Detailed Procedure for the Synthesis of 4-azidophenylalanine

All reagents and solvents were purchased from commercial vendors and used as received, and water for reactions and workups was deionized and purified by charcoal filtration. Magnetic stirring was used unless otherwise noted. NMR spectra were recorded on Varian 300 MHz or 400 MHz spectrometers as indicated. Proton and carbon chemical shifts are reported in parts per million (ppm; δ) relative to tetramethylsilane (^{1}H δ 0), DMSO (^{1}H δ 2.50, ^{13}C δ 39.5), or MeOH (^{1}H δ 3.31, ^{13}C δ 49.0). NMR data are reported as follows: chemical shifts, multiplicity (obs = obscured, app = apparent, br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, comp = complex overlapping signals); coupling constant(s) in Hz; integration. Unless otherwise indicated, NMR data were collected at 25 °C. NMR purity measurements were performed using dimethylsulfone as internal calibrant according to published procedures.[2] Vacuum filtration was performed using VWR Grade 413 filter paper (11.0 cm in diameter). Tandem liquid chromatography/mass spectrometry (LC-MS) was performed on a Shimadzu LCMS-2020 with autosampler, photodiode array detector, and single-quadrupole MS with ESI and APCI dual ionization, using a Peak Scientific nitrogen generator. Unless otherwise noted, a standard LC-MS method was used to analyze reactions and reaction products: Phenomenex Gemini C18 column (100 x 4.6 mm, 3 μm particle size, 110 A pore size); column temperature 40 °C; 5 μL of sample in MeOH at a nominal concentration of 1 mg/mL was injected, and peaks were eluted with a gradient of 25−95% MeOH/H_2O (both with 0.1% formic acid) over 5 min., then 95% MeOH/H_2O for 2 min. Purity was measured by UV absorbance at 210 or 254 nm. IR spectra were obtained as solids or thin films using a Thermo Scientific Nicolet iS5 spectrometer.



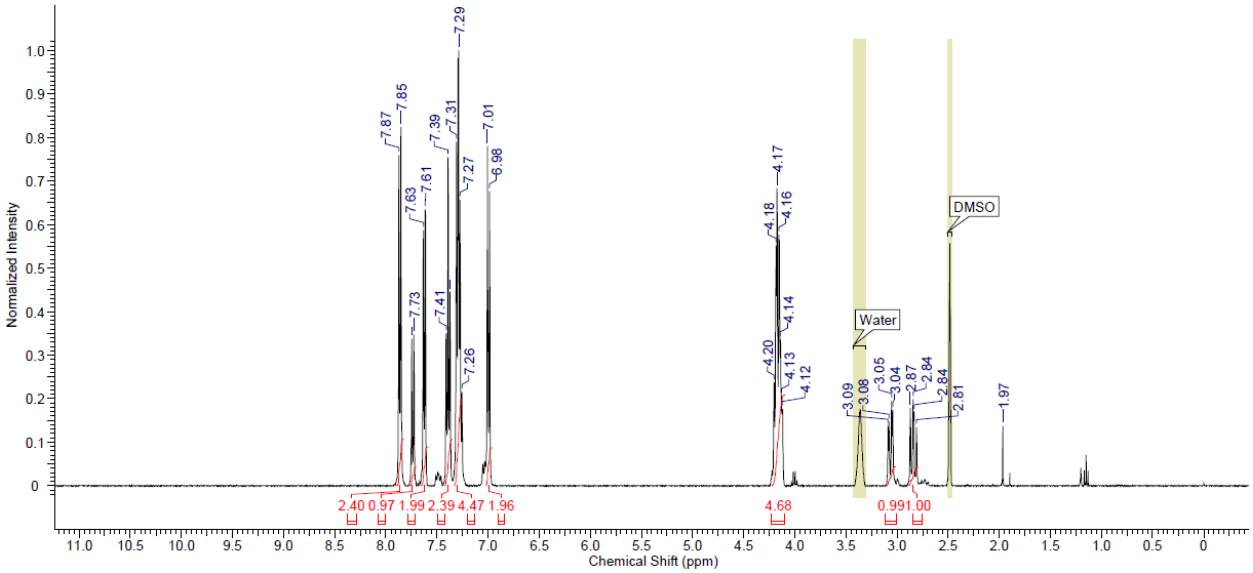
1

3-(4-azidophenyl)-2-{[(9H-fluoren-9-yloxy)carbonyl]amino}propanoic acid (**1**)

To a 4 L Erlenmeyer flask with stir bar were added Fmoc-4-amino-phenylalanine (purchased from Angene International Ltd.) (25.0 g, 62.1 mmol) and water (2.0 L). The flask was placed in an ice bath at 0 °C, and the suspension was stirred while concentrated HCl (37% v/v, 30 mL) and $NaNO_2$ (5.0 g, 73 mmol dissolved in 30 mL of water) were added sequentially over a period of 10 min. Note: This step was performed with magnetic stirring, but we recommend using mechanical stirring for more efficient mixing of the subsequent foamy suspension. The resulting off-white suspension was stirred at 0–5 °C for 4 h, after which time a solution of sodium azide (5.05 g, 77.7 mmol) in water (20 mL) was added dropwise over a period of 20 min. [Caution: Dropwise addition is necessary because of foam production (due to evolution of N_2 gas).] The reaction was warmed to 20 °C and stirred for 90 min. A few drops of the resulting suspension were taken from the reaction via Pasteur pipette, filtered with cotton, and the precipitate was dissolved with LC-MS-grade methanol. LC-MS analysis (see general method) showed consumption of starting material (4.71 min. retention time) and a new distinctive product peak at 7.35 min. The precipitate was filtered with a Buchner funnel and washed with additional water (250 mL), then dried in a vacuum oven at for 8 h (50 °C; 172 Torr), after which time a consistent mass was recorded. 23.2 g (95% yield, 88% pure by NMR) of azide **1** was obtained as a pale yellow solid. The crude material was used without further purification in the subsequent reaction.

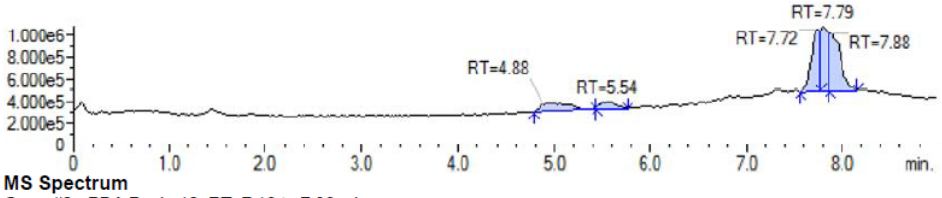
^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.86 (d, *J* = 7.5 Hz, 2 H), 7.74 (d, *J* = 8.6 Hz, 1 H), 7.62 (d, *J* = 7.4 Hz, 2 H), 7.39 (t, *J* = 7.5 Hz, 2 H), 7.29 (dd, *J* = 6.0, 7.8 Hz, 4 H; overlapping with impurity), 7.00 (d, *J* = 8.4 Hz, 2 H), 4.22 - 4.11 (comp, 4 H; overlapping with impurity), 3.07 (dd, *J* = 4.2, 13.8 Hz, 1 H), 2.84 (dd, *J* = 10.8, 13.7 Hz, 1 H). **LC-MS** (ESI^+) calculated for $C_{24}H_{20}N_4O_4$ [M-H] 427.45, found 427.10.

Fmoc-4-AZP-H1



MS Chromatogram

Group#2 Scan(-) EI : TIC



MS Spectrum

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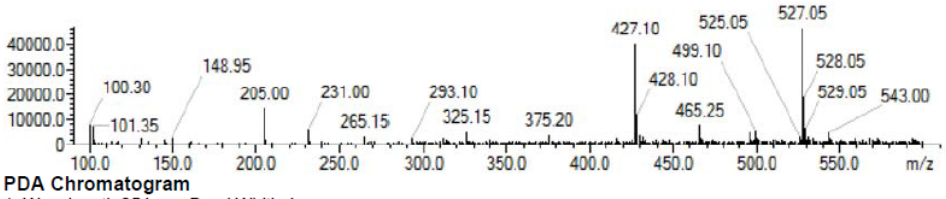
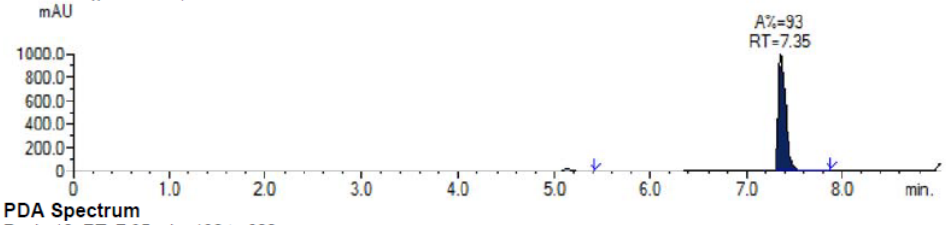


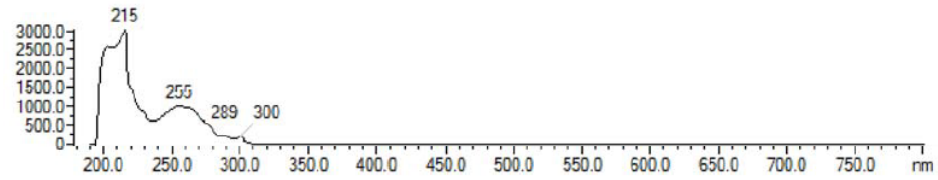
PDA Chromatogram

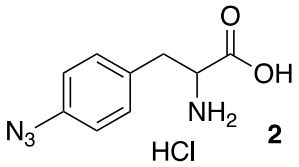
1: Wavelength 254 nm, Band Width 4 nm



PDA Spectrum

Peak: 12, RT: 7.35 min, 190 to 800 nm





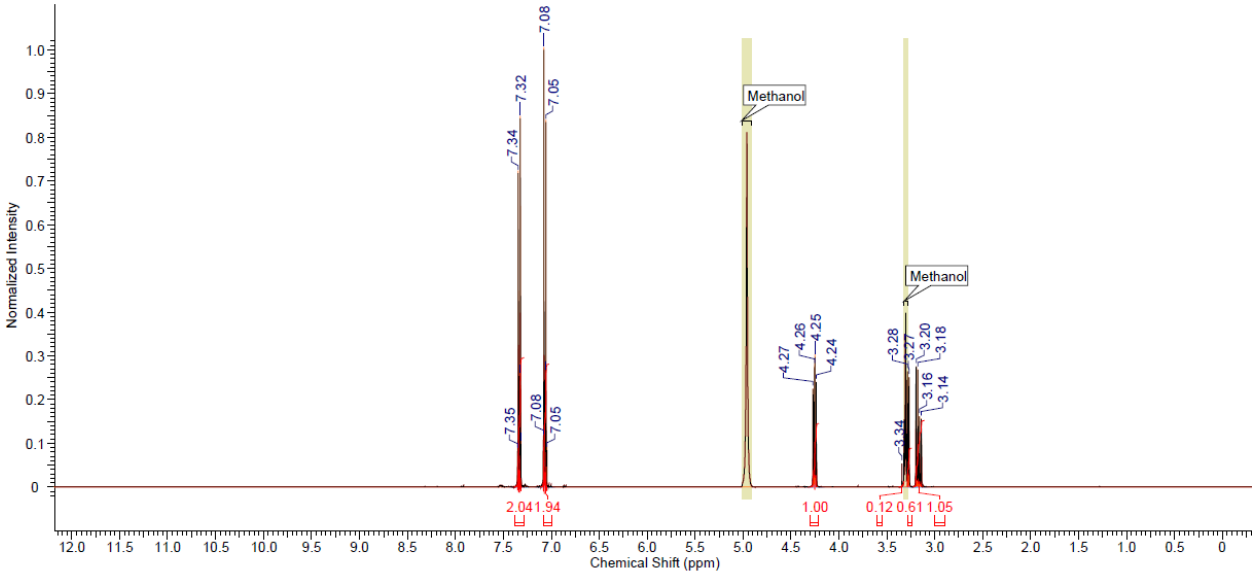
2

2-amino-3-(4-azidophenyl)propanoic acid hydrochloride (**2**)

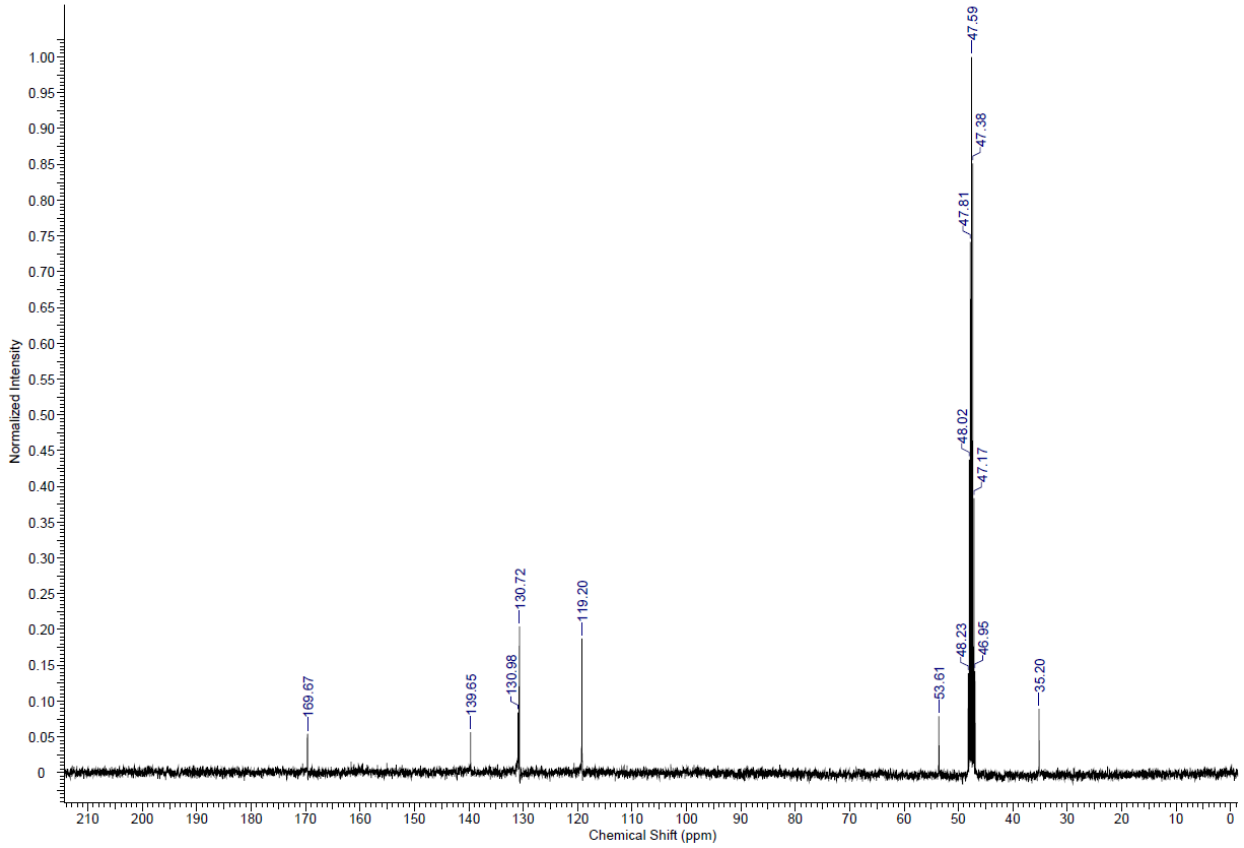
Azide **1** (16.7 g, 39.0 mmol) and dry DCM (400 mL) were added to a 1 L round-bottom flask with stir bar. The stirred reaction was sealed under nitrogen, then piperidine (80 mL, 0.88 mol) was added dropwise over 5 min. by syringe, and the flask was connected with a vent line to an oil bubbler. The reaction was stirred for 90 min. at 20 °C, then a sample was taken via Pasteur pipette (10-12 drops), and extracted with 1 M aq. NaOH in a glass microtube. The aqueous layer was concentrated and dissolved with LC-MS grade methanol; LC-MS analysis showed consumption of starting material and a new product peak at 3.02 min was observed. The reaction was adjusted to pH 11 by addition of 1 M aq. NaOH solution (~75 mL). Water (350 mL) was added to the flask, and the solution was transferred to a separatory funnel. The phases were separated and the alkaline aqueous phase was washed with DCM (3 x 200 mL), then adjusted to pH 7 by addition of 1 N aq. HCl, causing the product to precipitate. The resulting solid was filtered with a Buchner funnel and washed with additional water (150 mL). The obtained solid was transferred to a 250 mL beaker, to which 1 N aq. HCl was added. The crude product was dissolved by heating to 60 °C on a hot plate, then filtered with a Buchner funnel. The filtrate was allowed to cool to 20 °C and then placed in a refrigerator at 5 °C for 12 h to allow recrystallization. The mother liquor was aspirated from the crystals using a Pasteur pipet connected to a vacuum trap, then the crystals were dried under high vacuum (0.6 mm Torr) to give amino acid **2** (7.80 g, 83% yield, >95% pure by NMR)[2] as pale yellow shiny crystals.

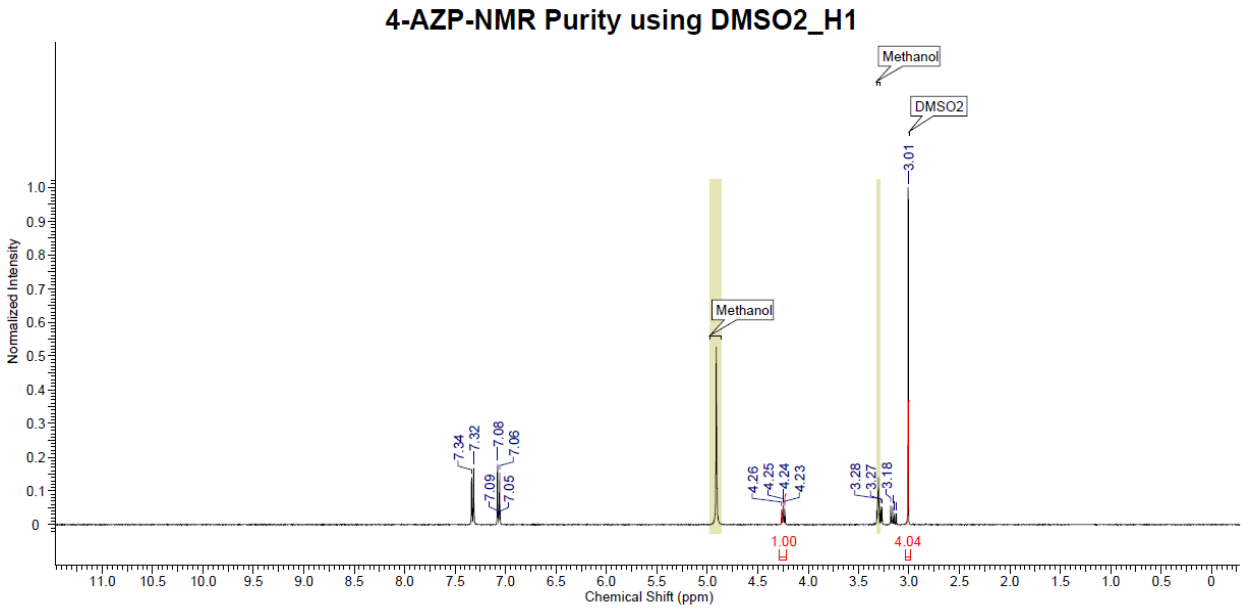
^{1}H NMR (400 MHz, CD_3OD) δ = 7.34 (d, *J* = 8.6 Hz, 2 H), 7.07 (d, *J* = 8.5 Hz, 2 H), 4.26 (dd, *J* = 5.7, 7.5 Hz, 1 H), 3.35 - 3.26 (m, 1 H; overlapping with MeOD peak), 3.17 (dd, *J* = 7.7, 14.4 Hz, 1 H). **^{13}C NMR** (101 MHz, CD_3OD) δ = 169.6, 139.7, 131.0, 130.7, 119.2, 53.6, 35.2. **LC-MS** (ESI^+) calculated for $C_9H_{10}N_4O_2$ [M+H] 207.20, found 206.65. **IR (solid film):** 2861.1, 2098.1, 1731.9, 1484.5, 1197.2, 805.3 cm^{-1}. Amino acid **2** underwent slow degradation above 160 °C and turned to black char at 210 °C.

4-AZP-Crystal_H1



4-AZP-Crystal_C13





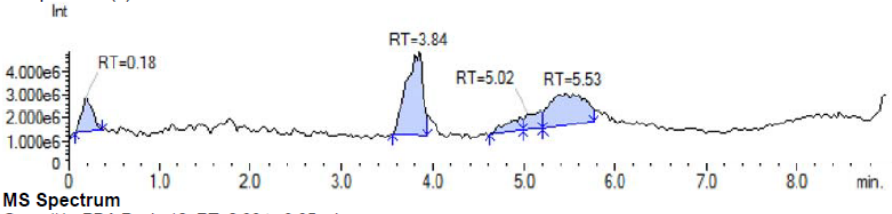
❖ Calculation[1]:

$$P(\%) = \frac{n_{IC}.Int_t\,.MW_t\,.n_{IC}}{n_t.Int_{IC}\,.MW_{IC}\,.m_s}\,.P_{IC}$$

1) m_{IC} = mass of the internal calibrant (IC) $DMSO_2$= 2.4 mg
2) m_S = mass of the 4-AZP crystal = 8.0 mg
3) Int_{IC} = area (integral) of the IC resonance signal being used for quantification = 4.04
4) Int_t = area (integral) of the 4-AZP crystal (*t*) resonance signal being used for quantification = 1
5) n_{IC} = number of protons that give rise to Int_{IC} = 6
6) n_t = number of protons of the 4-AZP crystal that give rise to Int_t = 1
7) MW_{IC} = molecular weight of the $DMSO_2$ = 94.13 g/mol
8) MW_t = molecular weight of the 4-AZP crystal = 206.20 g/mol
9) P_{IC} = purity of the $DMSO_2$, as percent value = >99.0% (Reported from manufacturer TCI)

MS Chromatogram

Group#1 Scan(+) EI : TIC



MS Spectrum

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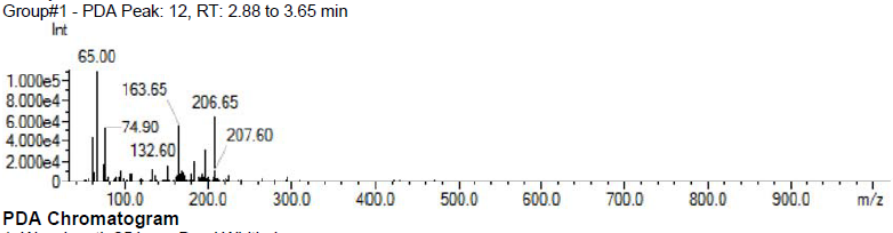
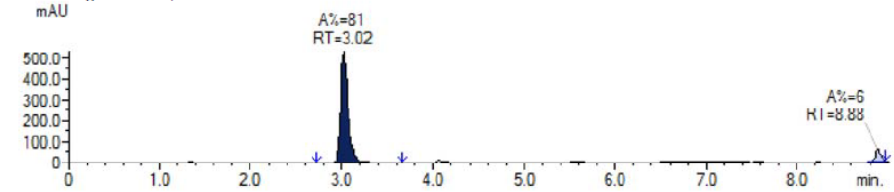


PDA Chromatogram

1: Wavelength 254 nm, Band Width 4 nm



PDA Spectrum

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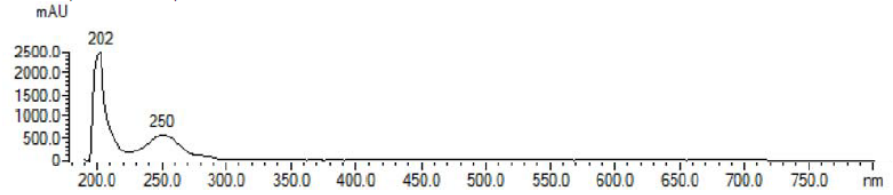

References:

1 Schuck, P. Sedimentation analysis of noninteracting and self-associating solutes using numerical solutions to the Lamm equation. *Biophysical journal* **75**, 1503-1512, doi:10.1016/S0006-3495(98)74069-X (1998).

2 Pauli, G. F. *et al.* Importance of purity evaluation and the potential of quantitative (1)H NMR as a purity assay. *Journal of medicinal chemistry* **57**, 9220-9231, doi:10.1021/jm500734a (2014).