Supporting Information

The development and application of synthetic affinity ligands for the purification of ferritin-based influenza antigens

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1.0. Docking of initial ligand SJ02

The initial ligand design (SJ02) was based on previous work but this proved difficult to synthesise efficiently. Therefore, the design focused on simpler glycine-dervied Ugi products SJ047 and SJ055 which have the amine available as a point of attachment for linking to the support. These were predicted to bind well to the target, and were easy to synthesise in solution.

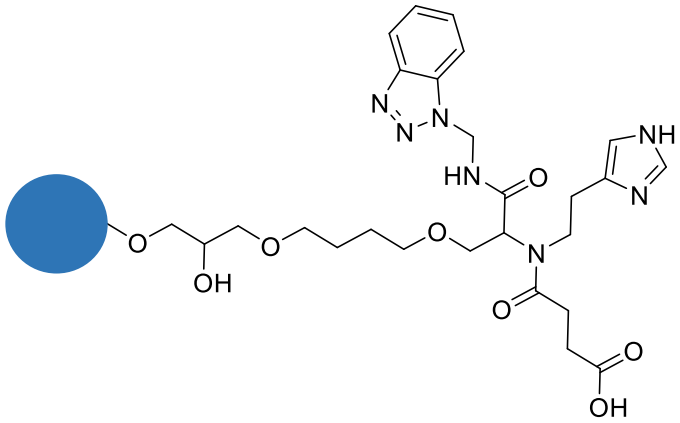


Figure S1. Solid phase ligand SJ02 coupled to 1,4-butanediol diglycidyl ether: 15Å spacer arm.

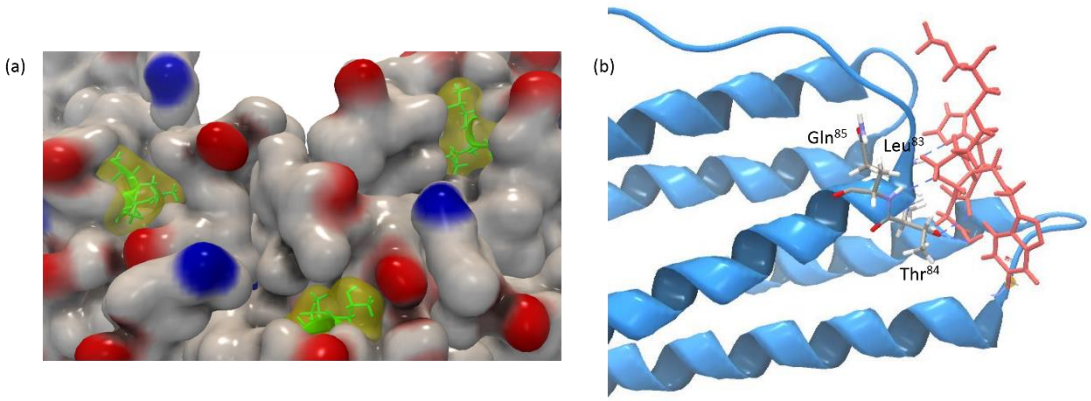
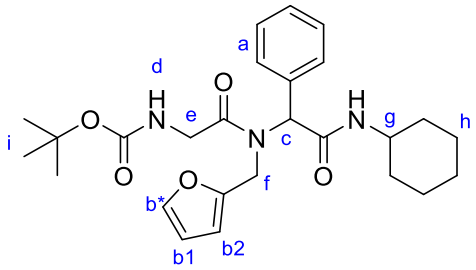


Figure S2. Docking of ligand SJ02 showing the binding interactions between the ligand and the ferritin target site. (a) The magnified binding sites of ferritin (3 subunits). The three residues of the hydrophobic region can be observed (Gln^{85}, Thr^{84} and Leu^{83}) on the surface. b) Highest scoring docking result of ligand SJ02 (orange) interacting with the most accessible hydrophobic binding pocket.Hydrogen bonds are formed between ferritin residues and the ligand SJ02.

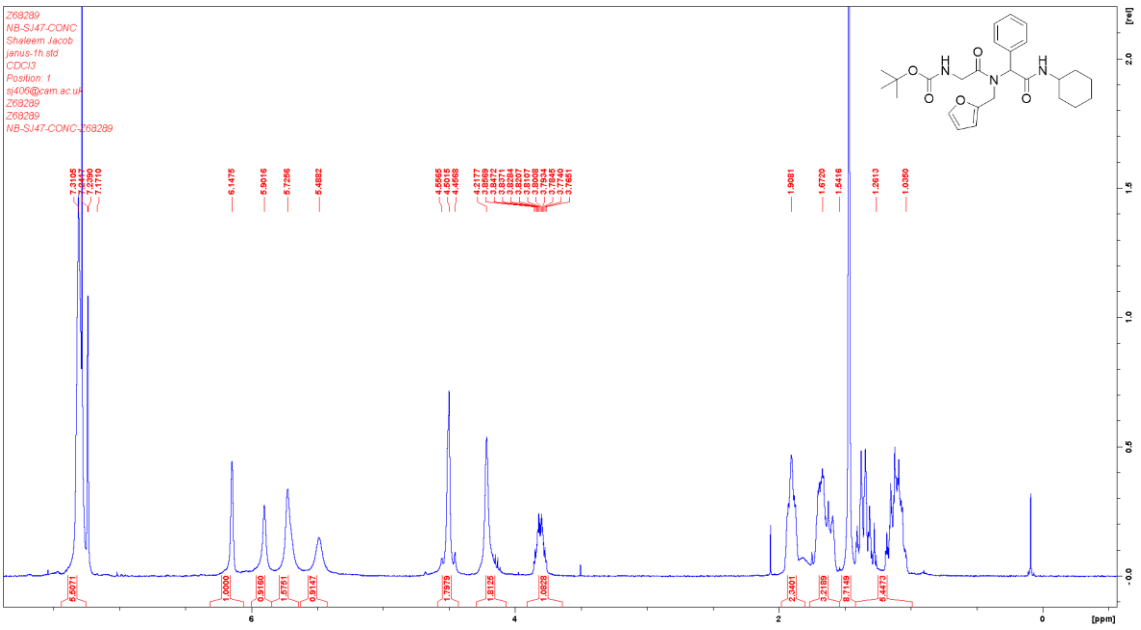
2.0. Characterisation of Boc-SJ047



Boc-SJ047, *tert*-butyl (2-((2-(cyclohexylamino)-2-oxo-1-phenylethyl)(furan-2-ylmethyl)amino)-2-oxoethyl)carbamate

White solid. Yield 95%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31-7.28 (m, 5H, H_a), 7.24 (d,1H, J=1.1 Hz, H_{b*}), 6.15 (s, 1H,H_{b1}), 5.90 (s,1H, H_c), 5.73 (s,1H,H_{b2}), 5.49 (s, 1H, H_d), 4.52 (d,1H,J=17.8 Hz, H_e), 4.50 (d,1H,J=17.8 Hz, H_e), 4.21 (s, 2H, H_f), 3.81 (tq, 1H,J=11.2, 3.8 Hz, H_g), 1.96-1.86 (m, 2H, H_h), 1.73-1.58 (m, 3H, H_h) 1.47 (s, 9H, H_i), 1.42-1.28 (m, 2H, H_h), 1.19-1.05 (m, 3H, H_h). ^{13}C NMR (400 MHz, $CDCl_3$) 155.7, 142.1, 134.4, 129.6, 128.7, 128.6, 110.5, 108.0, 79.6, 64.1, 63.1, 48.7, 42.9, 42.6, 32.8, 28.4, 25. 5, 24.8, 24.7. LRMS (ES+) 470 (M+H). HRMS (ES+) calculated for "$C_{26}H_{36}O_5N_3$" 470.2649 (M+H), found 470.2632.

^{1}H NMR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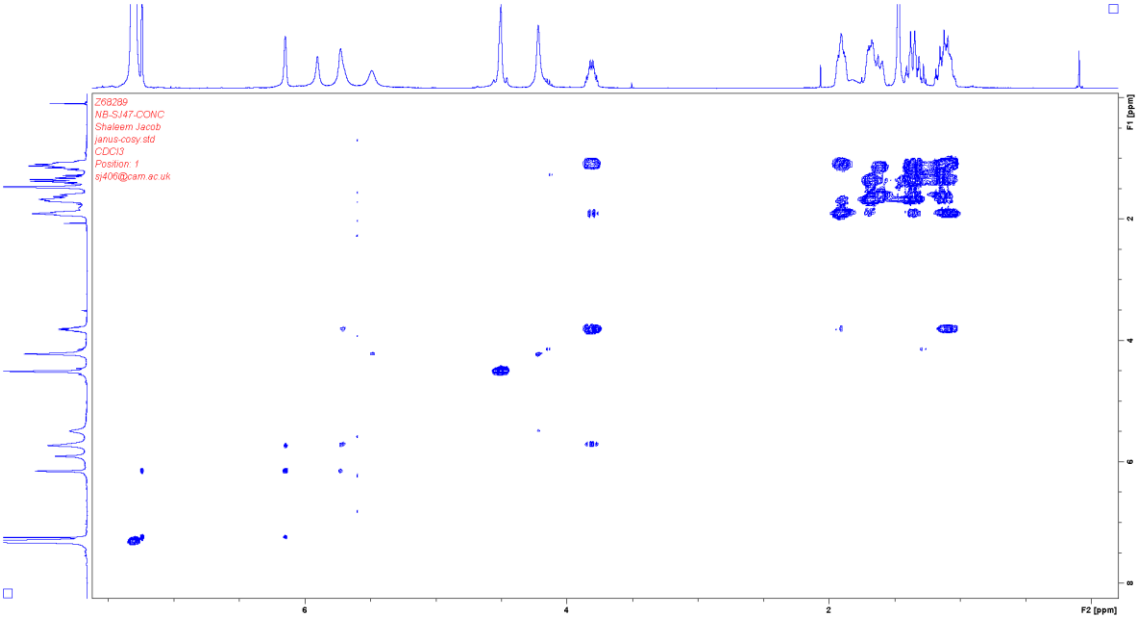
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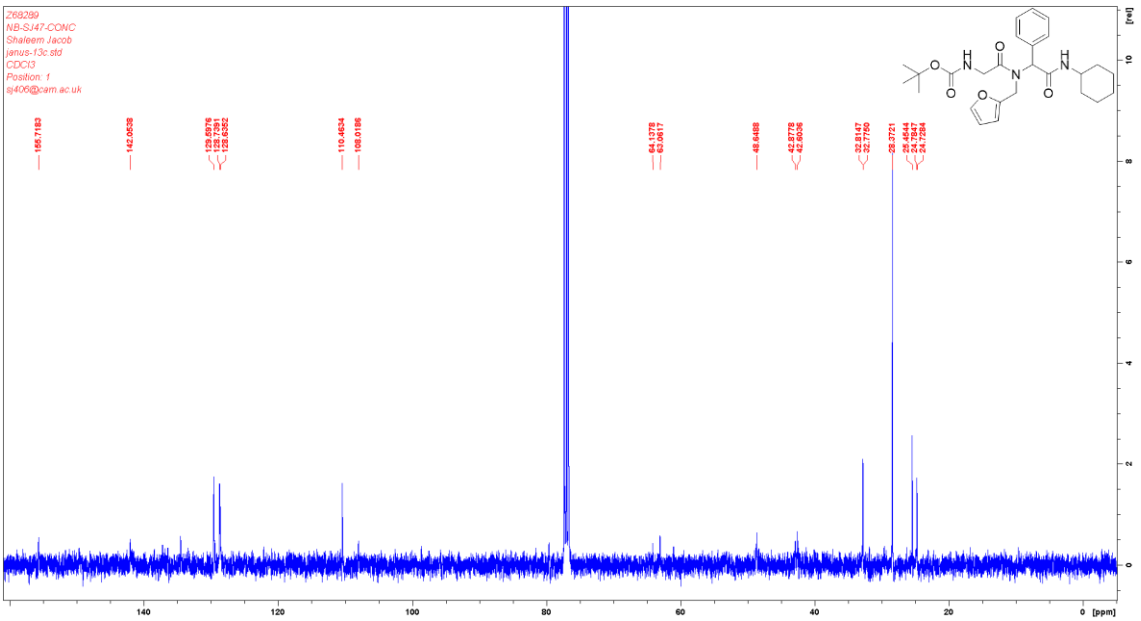
^{13}C NMR



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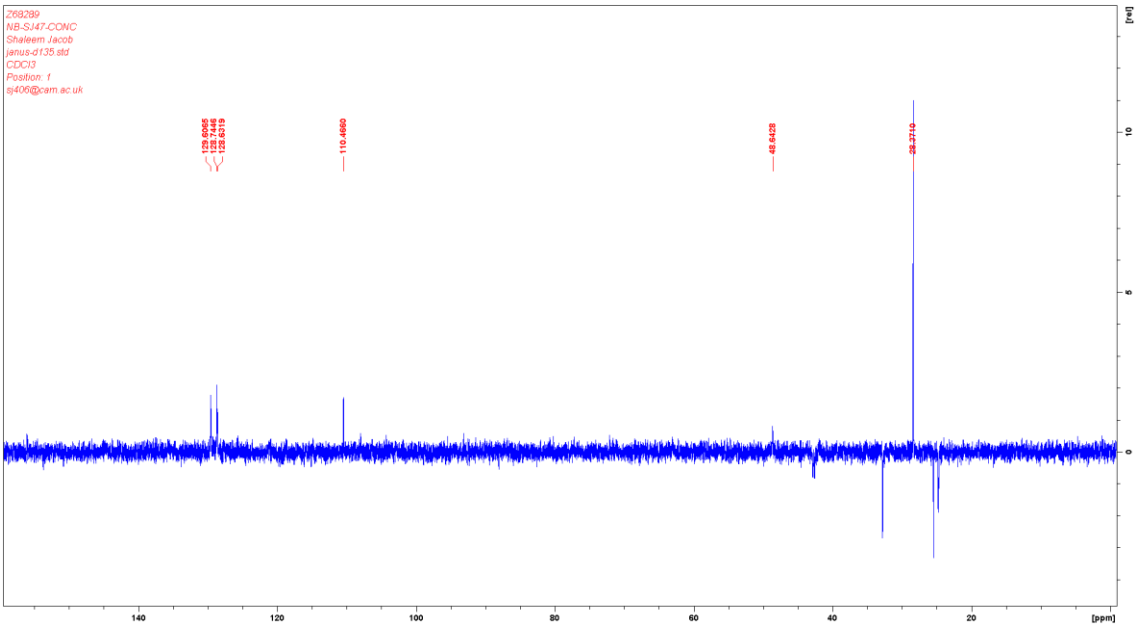

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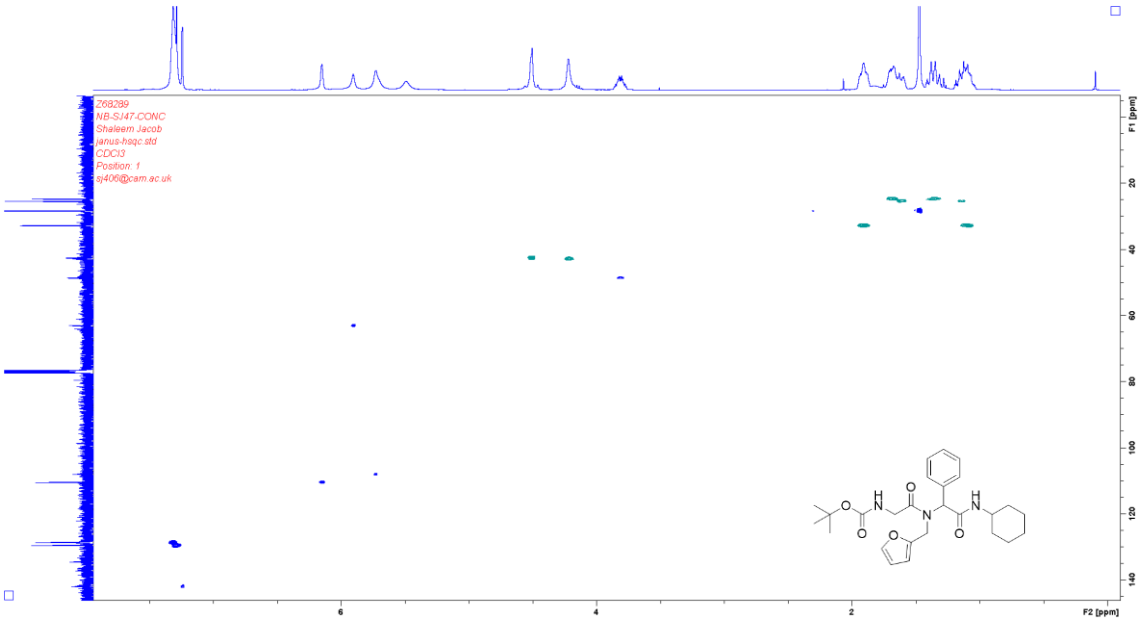
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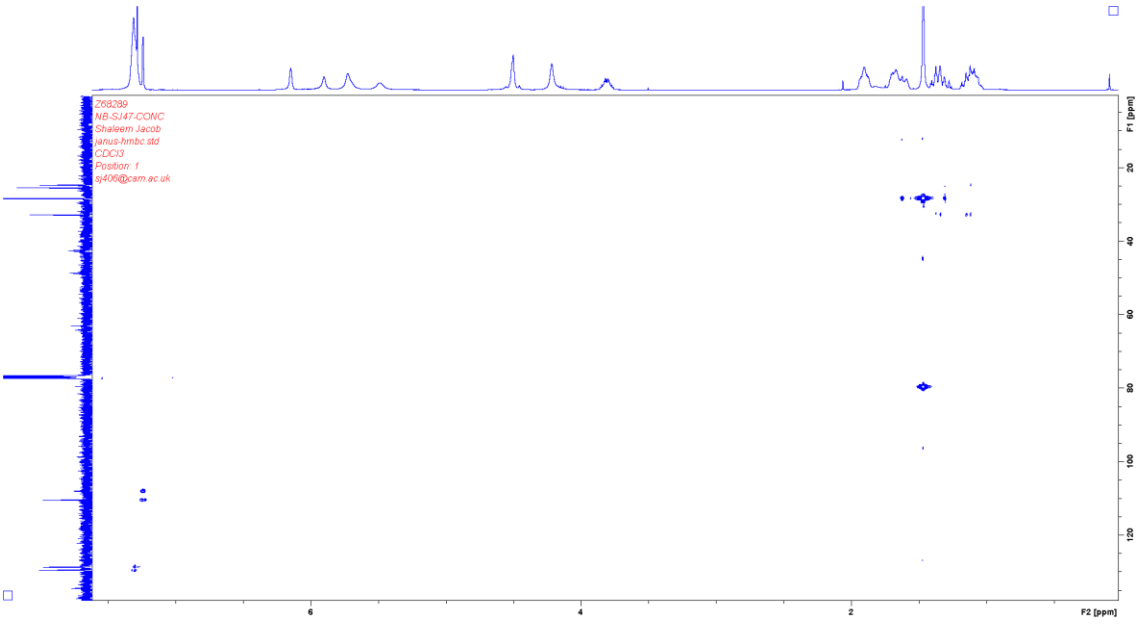
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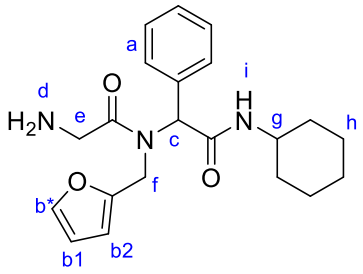
2.1. Characterisation of deprotected ligand SJ047

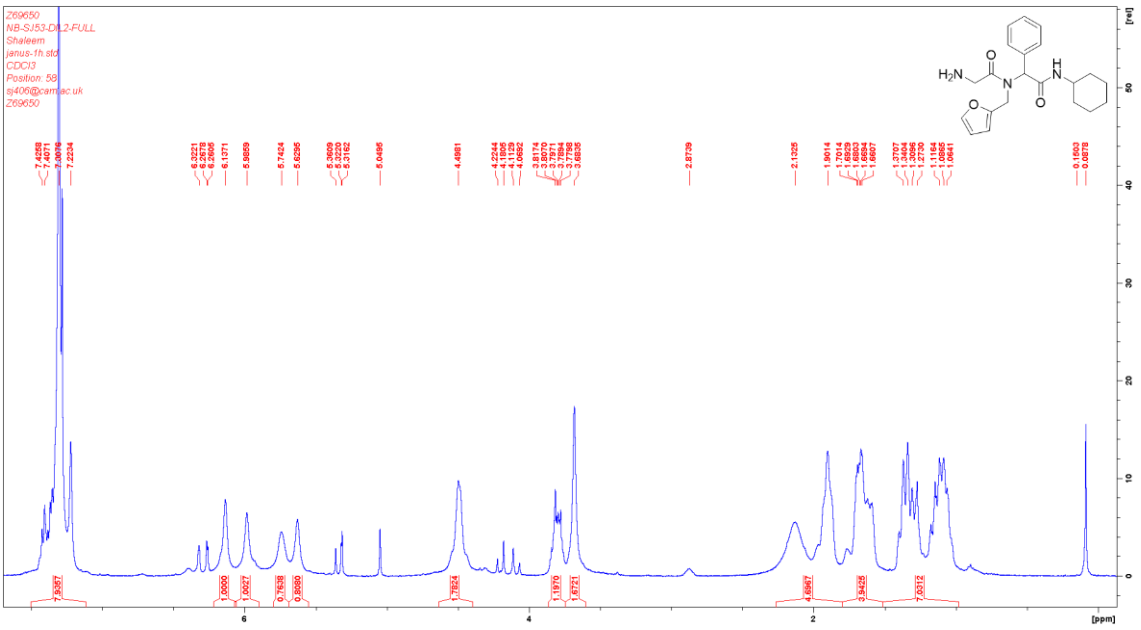
Before immobilisation, ligand SJ047 was deprotected and fully characterised.



Deprotected SJ047, 2-amino-N-(2-(cyclohexylamino)-2-oxo-1-phenylethyl)-N-(furan-2-ylmethyl)acetamide

White solid. Yield 64%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42-7.29 (m, 5H, H_a), 7.22 (s,1H, H_{b*}), 6.14 (s, 1H,H_{b1}), 5.9 (s,1H, H_c), 5.74 (s,1H,H_{b2}), 5.63 (s, 1H, H_i), 4.5 (s, 2H, H_f), 3.82-3.78 (m, 1H, H_g), 3.68 (s,2H, H_e), 2.13 (s, 2H, H_d), 1.90-1.06 (m, 10H, H_h). ^{13}C NMR (400 MHz, $CDCl_3$) 168.5, 161.4, 141.7, 134.9, 129.6, 129.3, 128.7, 128.6, 126.5, 110.5, 108.6, 62.7, 48.6, 43.9, 42.2, 32.8, 25.5, 24.8, 24.7. LRMS (ES+) 370 (M+H). HRMS (ES+) calculated for "$C_{21}H_{28}O_3N_3$" 370.2125 (M+H), found 370.211

^{1}H NMR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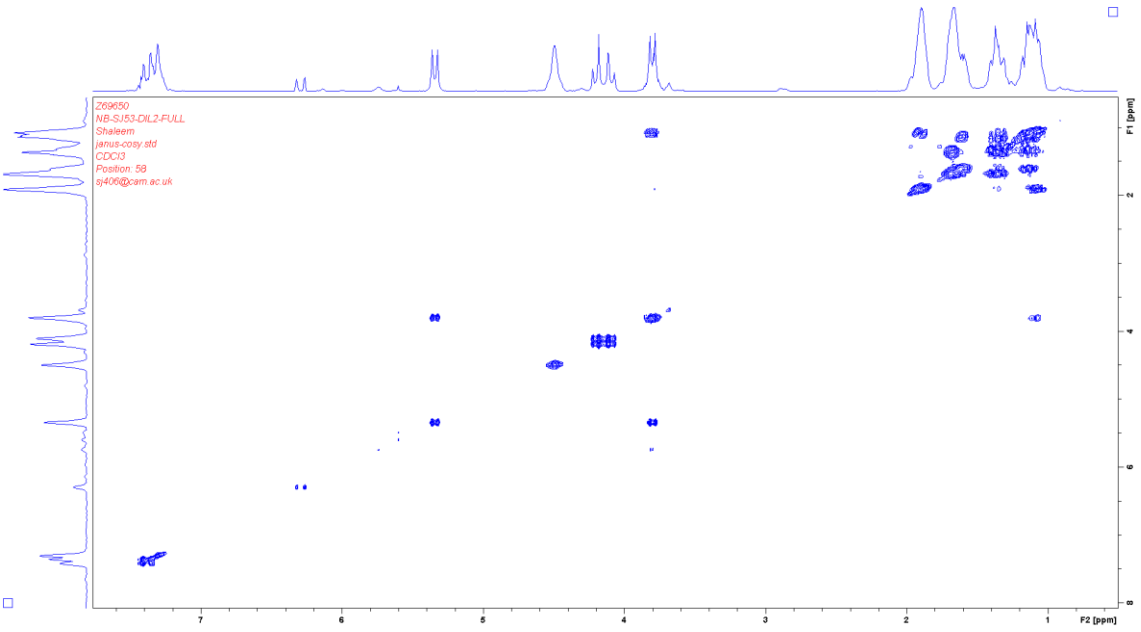
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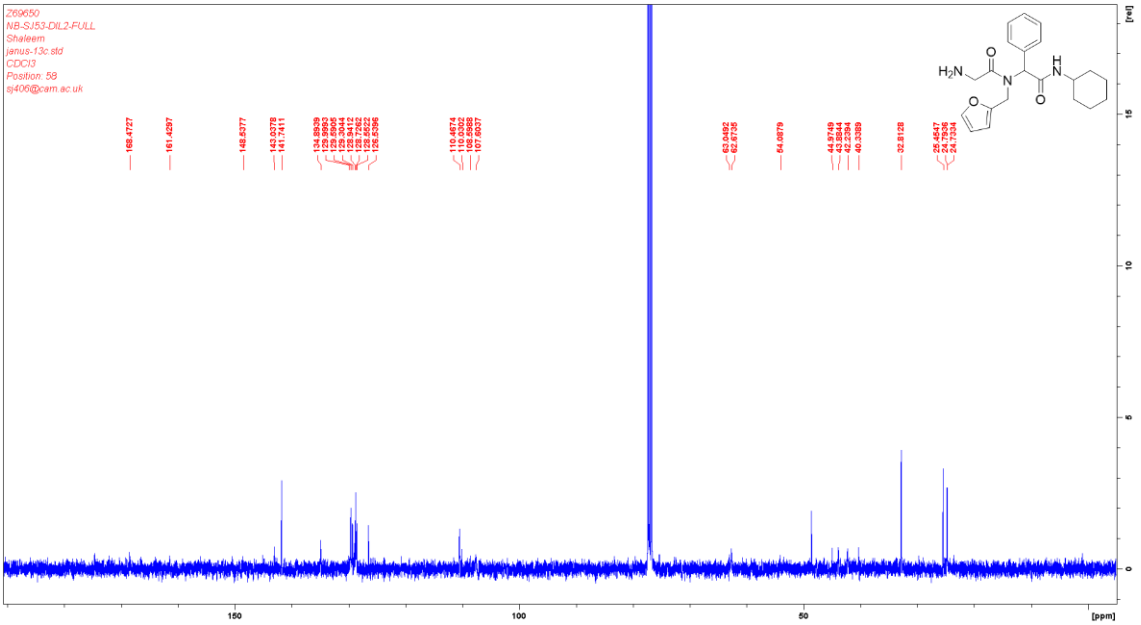
^{13}C NMR



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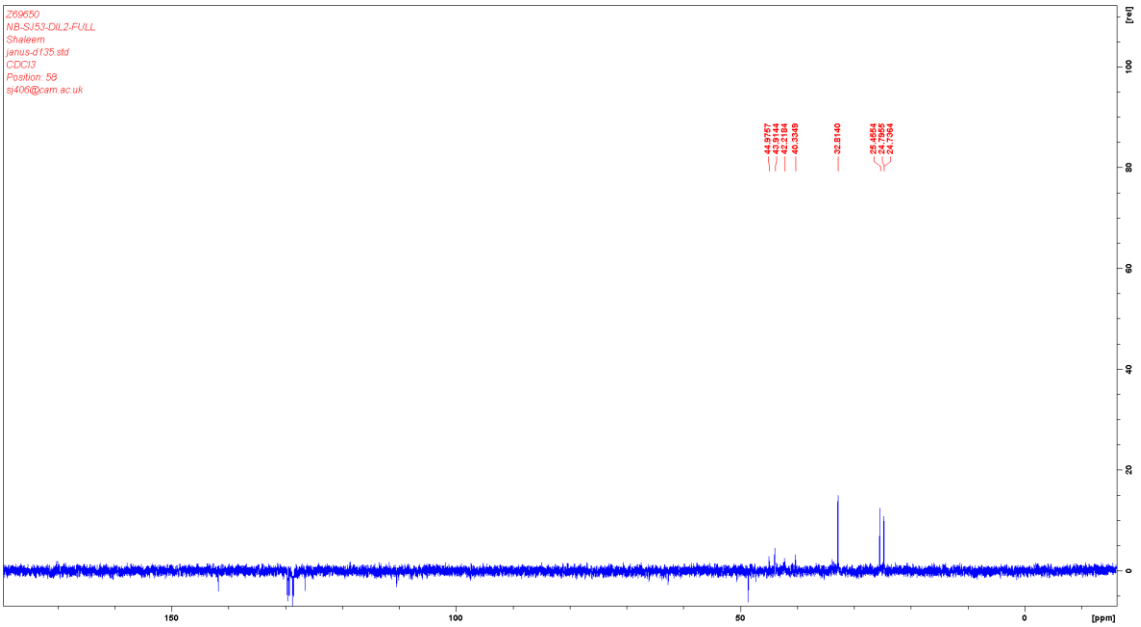

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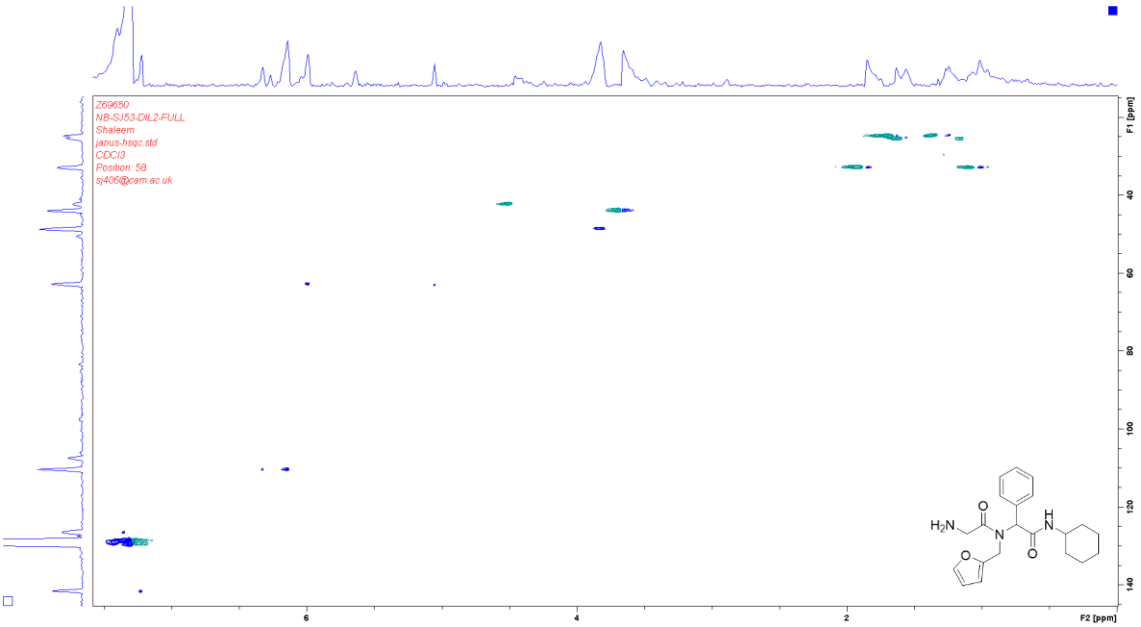
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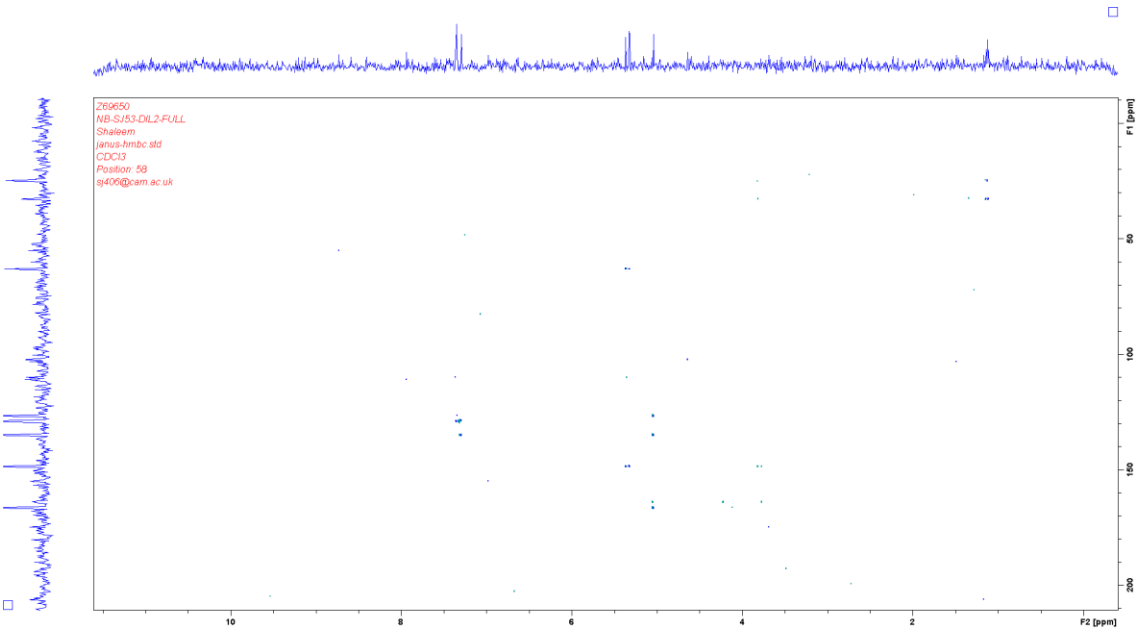
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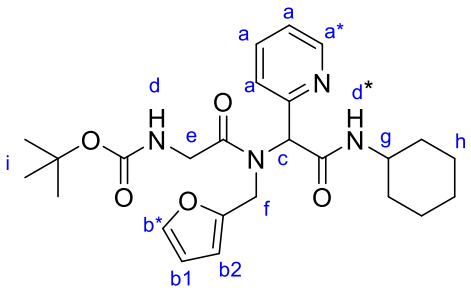
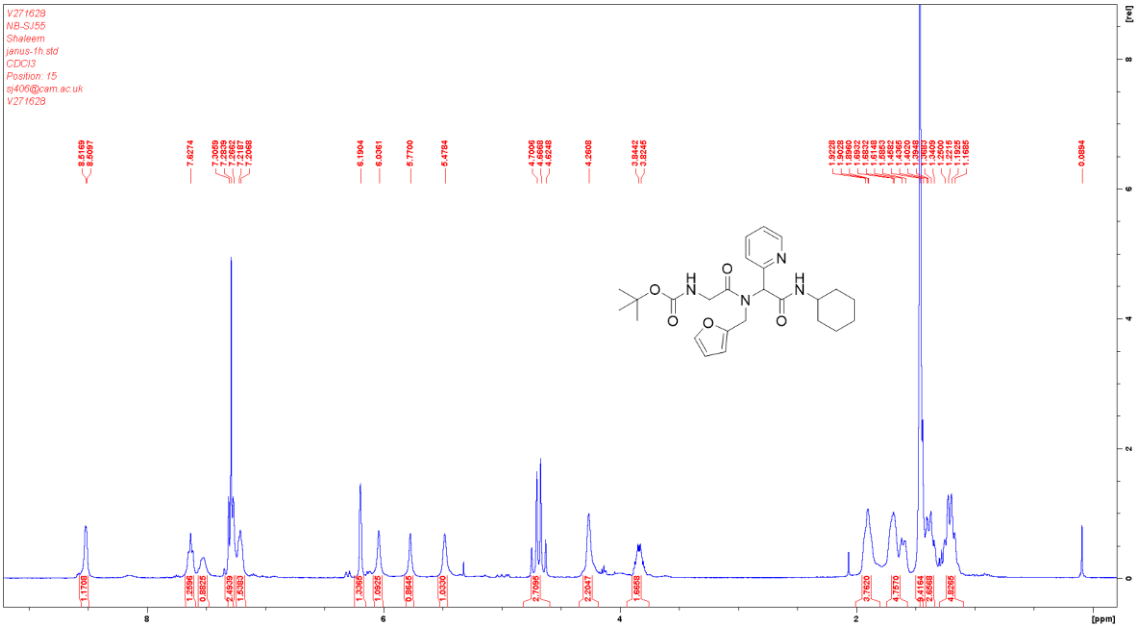
3.0. Characterisation of ligand Boc-SJ055



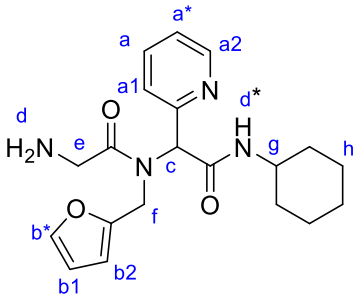
Boc-SJ055, tert-butyl(2-((2-(cyclohexylamino)-2-oxo-1-(pyridin-2-yl)ethyl)(furan-2 ylmethyl)amino)-2-oxoethyl)carbamate.

Yellow oil. Yield 71%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (s, 1H, H_{a*}), 7.64-7.60 (m, 1H, H_a), 7.52 (s, 1H, H_d), 7.30-7.24 (m, 2H, H_a), 7.21-7.19 (m, 1H, H_{b*}), 6.19 (s, 1H,H_{b1}), 6.0 (s,1H, H_c), 5.77 (s,1H,H_{b2}), 5.48 (s, 1H, H_{d*}), 4.71 (d, J=16.9 Hz, 1H, H_e), 4.66 (d, J=16.9 Hz, 1H, H_e)4.26 (s, 2H, H_f), 3.88-3.79 (m, 1H, H_g), 1.95-1.85 (m, 2H, H_h), 1.73-1.57 (m, 3H, H_h), 1.46 (s, 9H, H_i), 1.44-1.33 (m, 2H, H_h), 1.30-1.13 (m, 3H, H_h).

^{1}H NMR



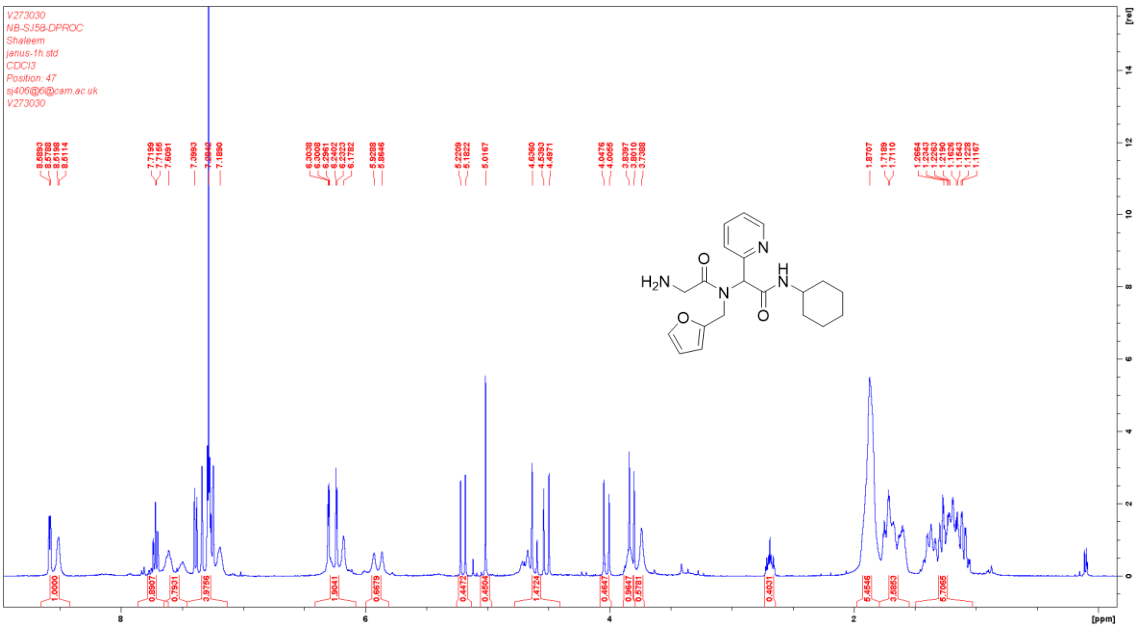
3.1. Characterisation of deprotected ligand SJ055



Deprotected SJ055, 2-amino-N-(2-(cyclohexylamino)-2-oxo-1-(pyridin-2-yl)ethyl)-N-(furan-2-ylmethyl)acetamide

Yellow solid. Yield 62%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58 (d, 1H, J=4.19, H_{a2}) , 7.72 (td, 1H, J=7.7, 1.7 Hz, H_a), 7.6-7.5 (m,1H, H_{a1}), 7.29-7.28 (m,1H,H_{a*}), 7.19 (s, 1H, H_{b*}), 6.3 (dd, J=3.2, 1.7 Hz, 1H, H_{b1}), 6.24 (d, J=3.2 Hz, 1H, H_{b2}), 6.18 (s, 1H, H_{d*}), 5.86 (s,1H, H_c), 5.20 (d, J=15.6 Hz, 1H, H_f), 4.52 (d, J=16.8 Hz, 1H, H_e), 4.03 (d, J=16.8 Hz, 1H, H_e), 3.82 (d, J=15.6 Hz, 1H, H_f), 3.87-3.82 (m, 1H, H_g), 1.87 (s, 2H, H_d), 1.72-1.11 (m, 10H, H_h). ^{13}C NMR (400 MHz, $CDCl_3$) 166.5, 165.5, 154.9, 150.2, 148.8, 148.4, 143.0, 137.1, 123.7, 123.5, 110.5, 110.0, 64.5, 45.6, 40.4, 36.2, 32.8, 32.7, 25.6, 25.1, 24.6. LRMS (ES+) 371 (M+H). HRMS (ES+) calculated for "$C_{20}H_{27}O_3N_4$" 371.2078 (M+H), found 371.2068.

^{1}H NMR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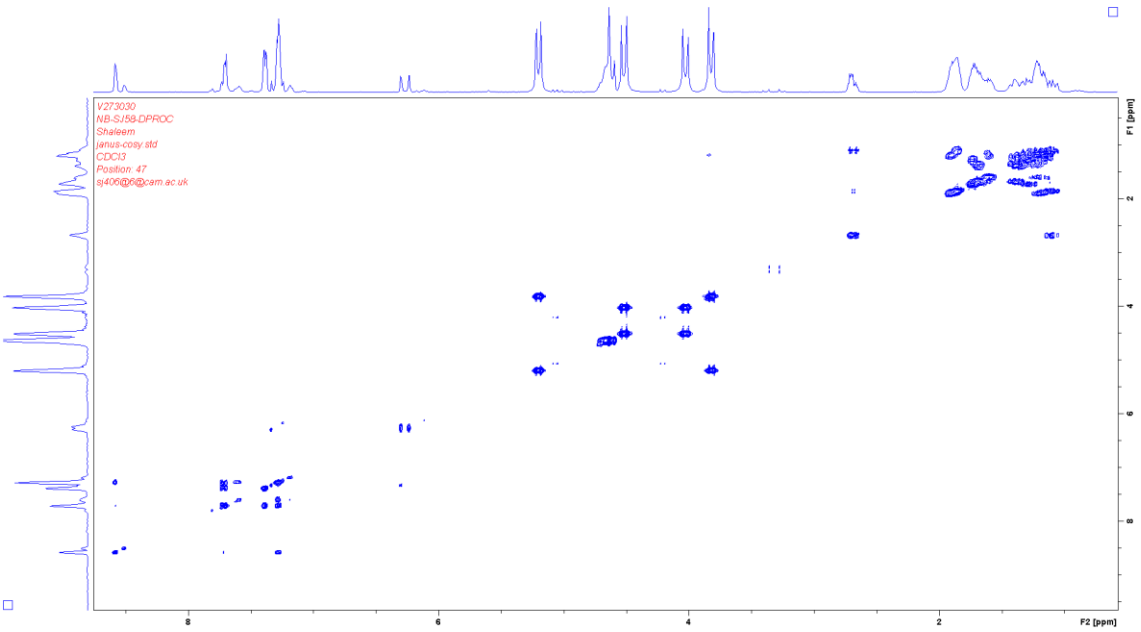
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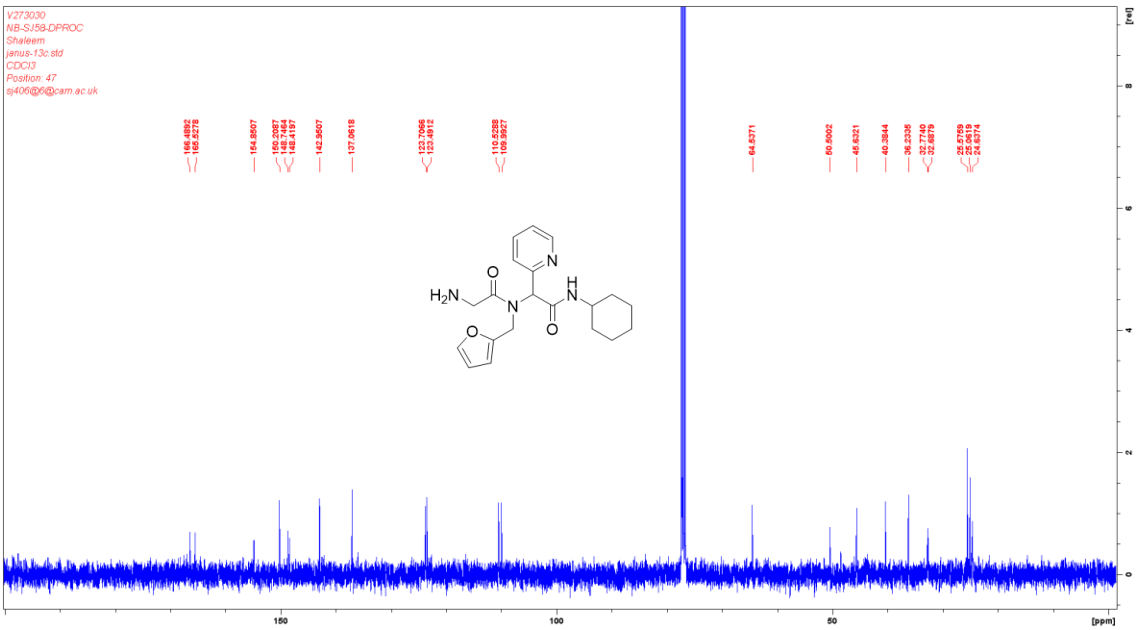

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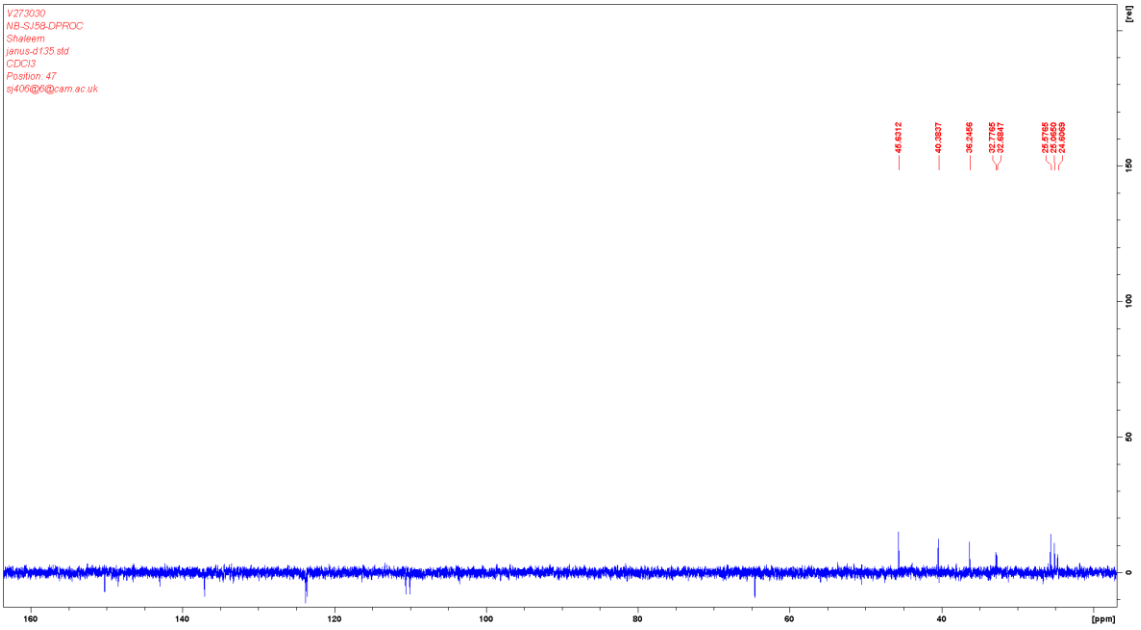

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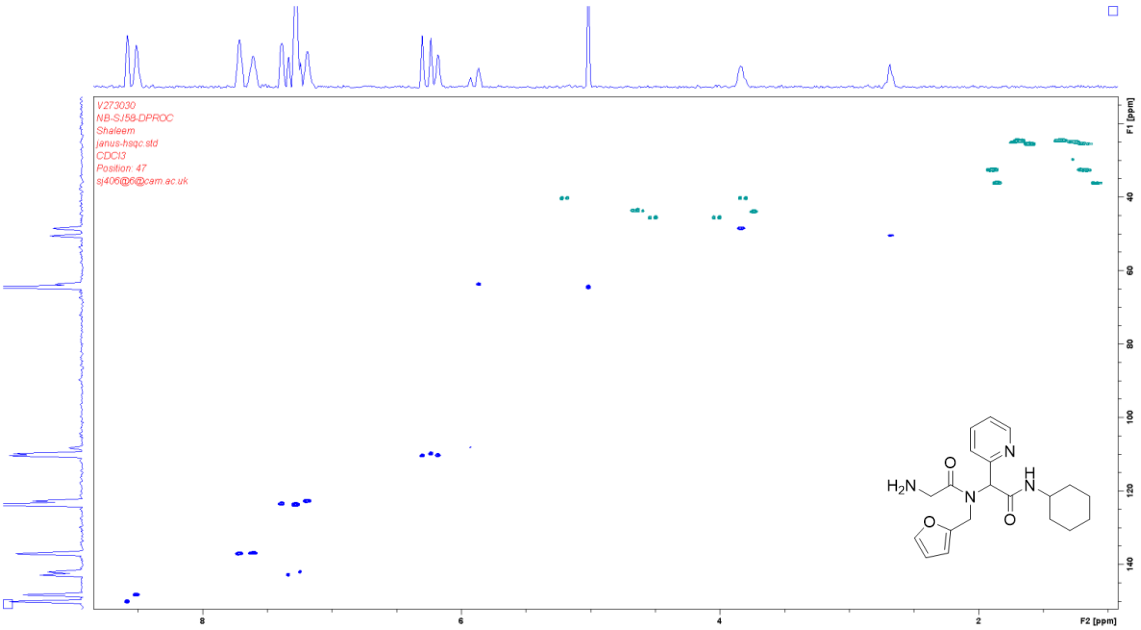
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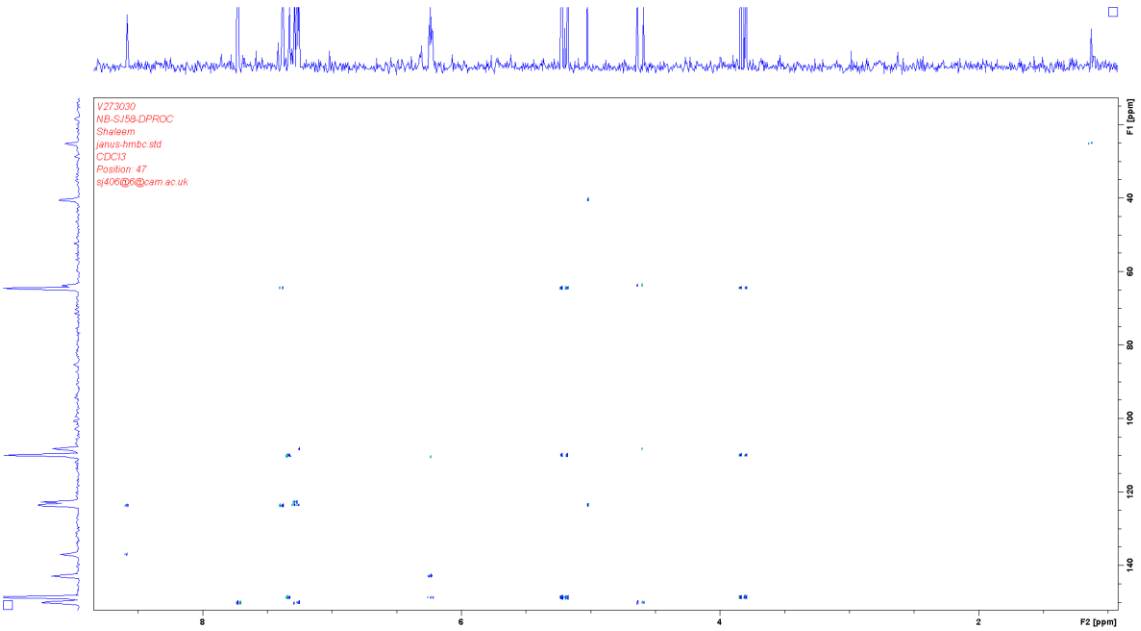
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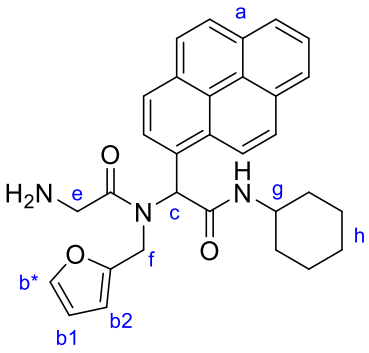
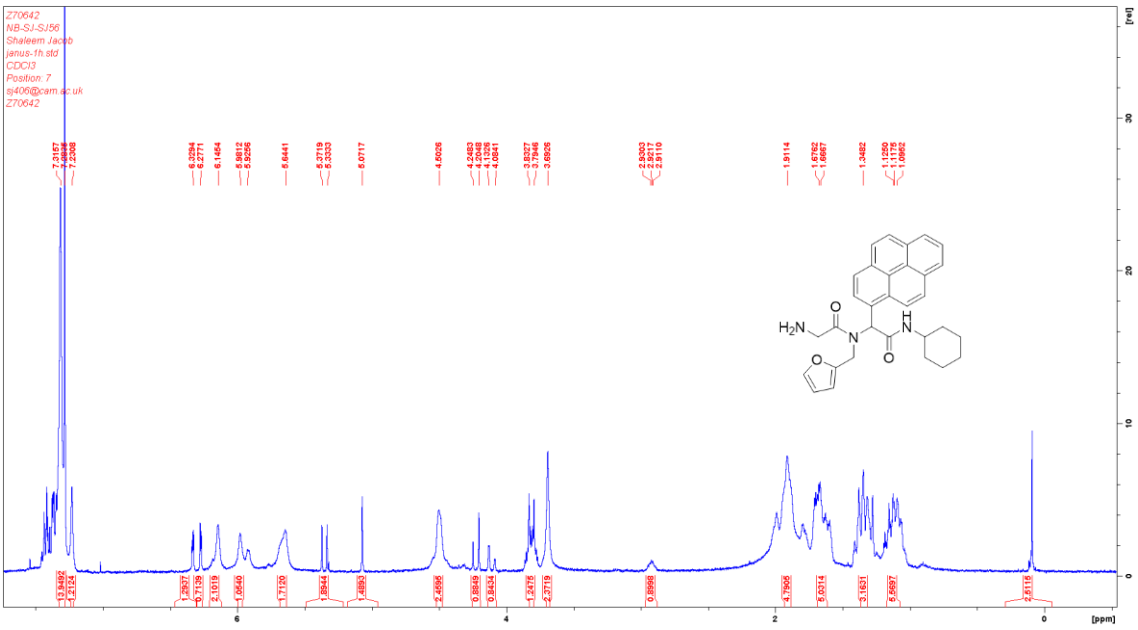


Orange solid. Yield 60%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42-7.28 (m, 9H, H_a), 7.23 (s, 1H, H_{b*}), 6.14 (s, 1H,H_{b1}), 5.98 (s,1H, H_c), 5.64 (s,2H,H_{b2}), 5.64 (s, 2H, H_i), 4.5 (s, 2H, H_f), 3.83-3.79 (m, 1H, H_g), 3.69 (s,2H, H_e), 1.90-1.07 (m, 10H, H_h). LRMS (ES+) 494 (M+H). HRMS (ES+) calculated for "$C_{31}H_{32}O_3N_3$" 494.2438 (M+H), found 494.2424.

^{1}H NMR



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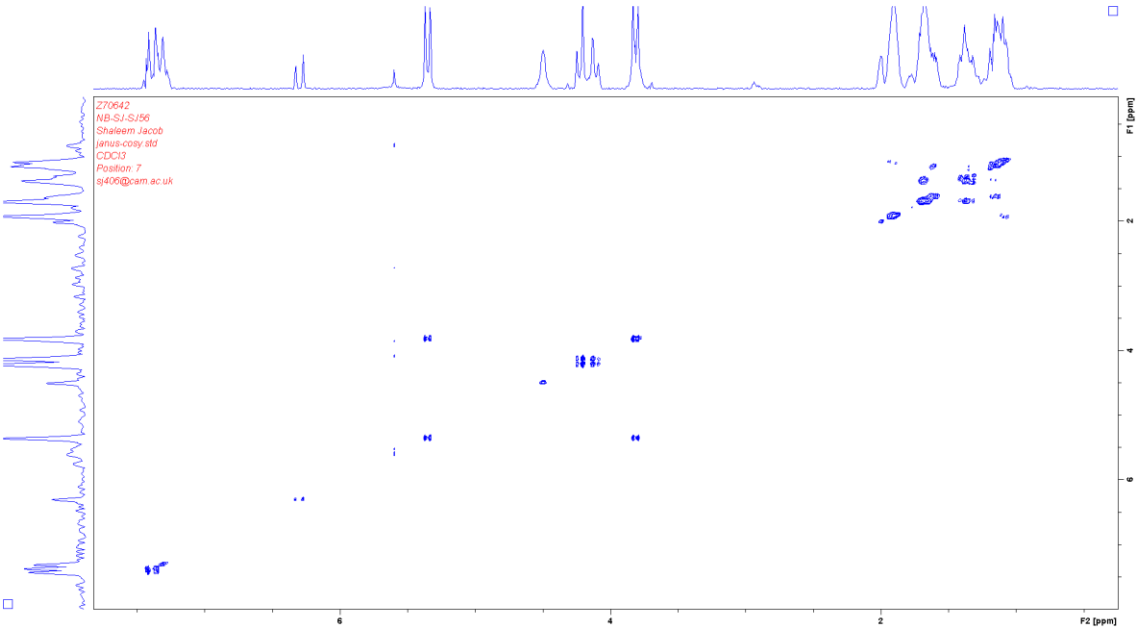
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2

5.0. Control Experiments

First, the unmodified Sepharose CL-4B column was tested with the crude mixture (Figure S3a). It was observed that the recombinant antigen was predominantly in the flow-through and wash fractions. This suggested that even in a salt binding buffer concentration of 20 mM PIPES and 1.6 M ammonium sulphate, the recombinant antigen was not being bound non-specifically to the beads and eluted in the flow-through fractions. In addition, PEGDE 500 was coupled to the Sepharose CL-4B beads and cis-diol functionalised. The epoxide groups of the coupled PEG spacer were converted into cis-diol because the epoxides would interact with the free primary amine groups on the recombinant antigen in a ring-opening process. A similar behaviour was found for the PEG cis-diol functionalised packed column, where, ~73% of the HA (New Caledonia)–ferritin was observed in the flow through and binding fractions (Figure S3b). However, some of the HEK HCP was seen in elution, presumably due to the high salt concentrations encouraging hydrophobic interactions between the PEG and the recombinant antigen along with HEK host cell protein (HCP) impurities. In the molecular modelling, it was suggested that the PEG spacer arm binds to the recombinant antigen; however, in this instance it could be due to the high salt increasing the probability of binding. The PEG cis-diol functionalised packed column, eluted to a purity of ~76% and yield of ~27%. Therefore, these results suggested that at 1.6 M ammonium sulphate concentration the PEG spacer arm binds and elutes HA (New Caledonia)-ferritin and the HCP impurities.

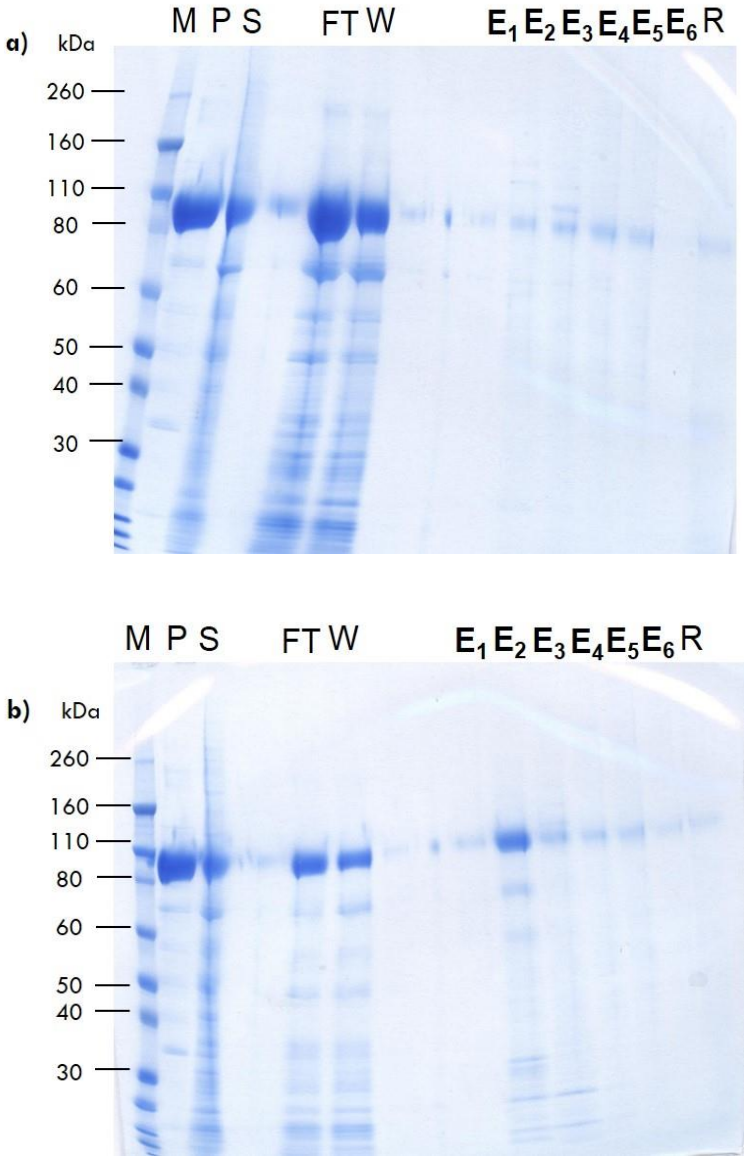
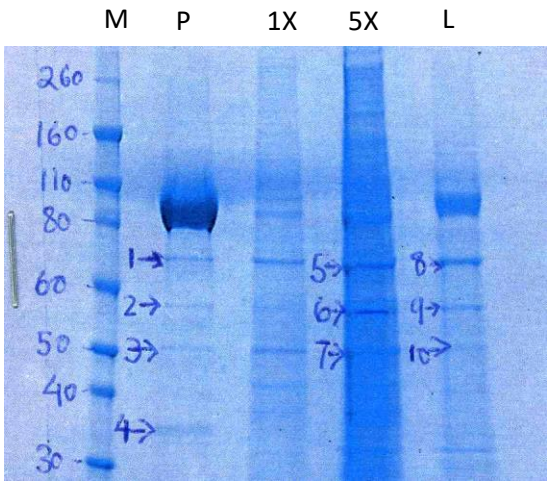


Figure S3. Control experiments of Sepharose beads without immobilised ligands using crude supernatant. Colloidal blue SDS-page of: (a) Clarified HEK expressed HA (New Caledonia)-ferritin supernatant (0.1 mg/mL) loaded onto the ~1 mL unmodified Sepharose CL-4B packed column and ran on the ÄKTA avant. (b) Clarified HEK expressed HA (New Caledonia)-ferritin supernatant (0.1 mg/mL) loaded onto ~1 mL cis-diol PEG coupled Sepharose packed column and ran on the ÄKTA avant.Using densitometry analysis (ImageJ), calculated yield and purity of lane E2 was ~27% and ~76%, respectively. Legend for SDS-PAGE above, M: Marker (Pre-stained 10 kDa ladder), P: Purified HA (New Caledonia)-Ferritin (5 µg), S: Crude HEK HA (NC)-ferritin supernatant (100 µg), FT: Column wash (20 mM PIPES, 1.6 M $(NH_4)_2SO_4$), W: Column wash (20 mM PIPES, 1.6 M $(NH_4)_2SO_4$), Ex: Gradient elution fraction to 20 mM PIPES, R: Regeneration fraction.

6.0. Human embryonic kidney host cell protein (HCP) impurities

Samples were sent for MALDI mass spectrometry analysis of the HEK supernatant proteins (Figure S4). It was evident that all the HCP impurities were derived from the human embryonic kidney cell line and indicating that the supernatant is free from other contaminants (Table 1). The HCP impurities in elution fractions need to be minimised using the functionalised resins to obtain purified recombinant antigens.



Legend:
M: Marker (Pre-stained 10 kDa ladder)
P: Purified HA (New Caledonia)-Ferritin (5 µg)
1X: 1X supernatant of HEK culture
5X 5X supernatant of HEK culture
L: Load ~100µg HA (New Caledonia)-ferritin spiked into HEK supernatant

Figure S4. SDS-PAGE of HCP impurities in the HEK supernatantof purified and spiked HA (New Caledonia)-Ferritin samples.

Table 1. SDS-PAGE protein bands (1-10) analysed using MALDI mass spectrometry.

Band	HEK cell supernatant impurities analysed by MALDI
1	HSP70-2 [Homo sapiens]: Heat shock protein aids with protein folding.
2	Glucose-6-phosphate isomerase isoform 4 [Homo sapiens]: glycolytic enzyme
3	-
4	L-lactate dehydrogenase B chain LDBH [Homo sapiens]: catalysis enzyme
5	HSP70-2 [Homo sapiens]
6	Nucleobindin 1, isoform CRA b [Homo sapiens]: calcium binding protein
7	Chain A, Crystal structure of human enolase 1: glycolytic enzyme
8	HSP70-2 [Homo sapiens]
9	Neuroleukin [Homo sapiens]: neuronal growth factor, Glucose-6-phosphate isomerase isoform 4 [Homo sapiens]

7.0. Capacity studies for HA (NC)-Ferritin from HEK cell supernatants

The Coomasie blue stained SDS-page below shows the capacity of binding the antigen for the immobilised ligands. The HA(NC)-ferritin load and the ammonium sulphate content in the wash buffer was increased as indicated in the figures below.

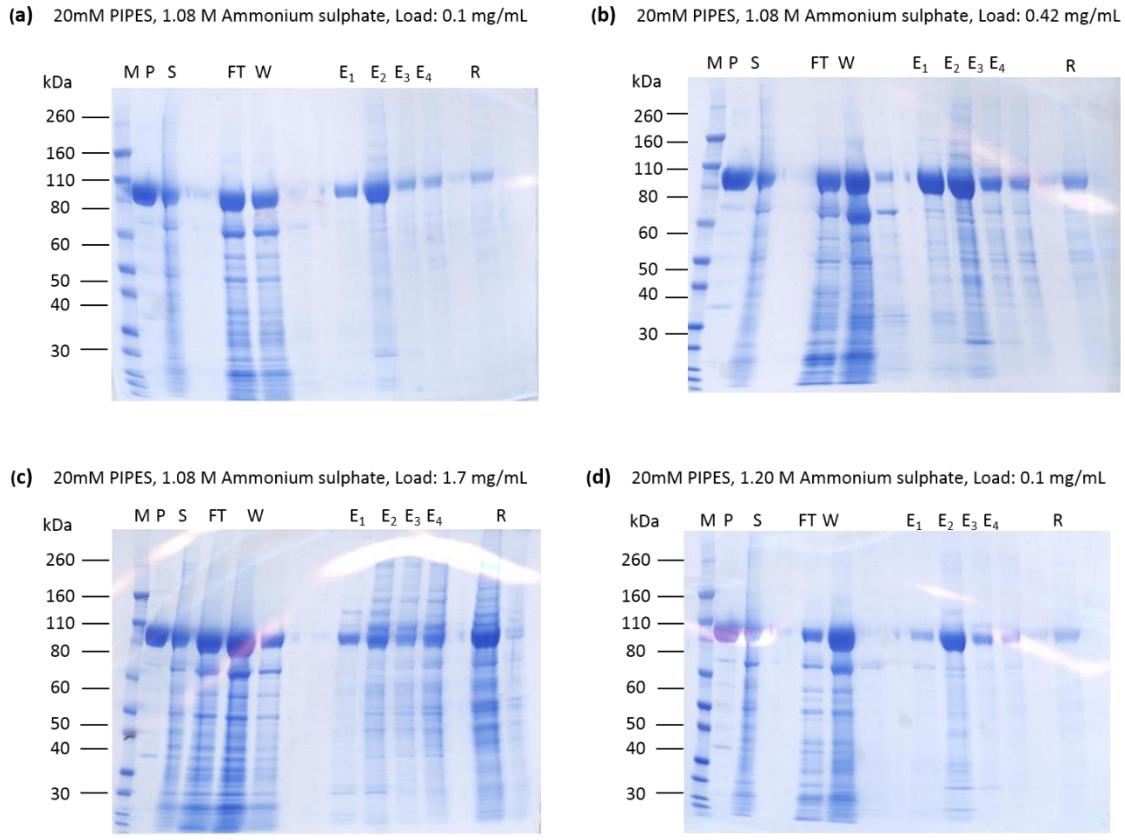


Figure S5. Capacity of SJ047- immobilised resins in increasing concentrations of HA-ferritin load (0.1 – 1.7 mg/mL).(a)-(c) In 20 mM PIPES and 1.08M ammonium sulphate wash buffer, breakthrough of the antigen was observed in the flow through and wash fractions across all loaded concentrations. (d) The wash buffer was changed to 1.2 M ammonium sulphate, breakthrough was till observed in the wash and flow through fractions. However, comparing the salt content of 1.08 M to 1.2 M with the same load of 0.1 mg/mL, more protein was being observed in the elution fractions for 1.2 M concentration. Legend for SDS-PAGE above, M: Marker (Pre-stained 10 kDa ladder), P: Purified HA (New Caledonia)-Ferritin (5 µg), S: Crude HEK HA (NC)-ferritin supernatant load (X mg/mL), FT: Column wash (20 mM PIPES, X M $(NH_4)_2SO_4$), W: Column wash (20 mM PIPES, X M $(NH_4)_2SO_4$), E_X: Gradient elution fraction to 20 mM PIPES, R: Regeneration fraction.

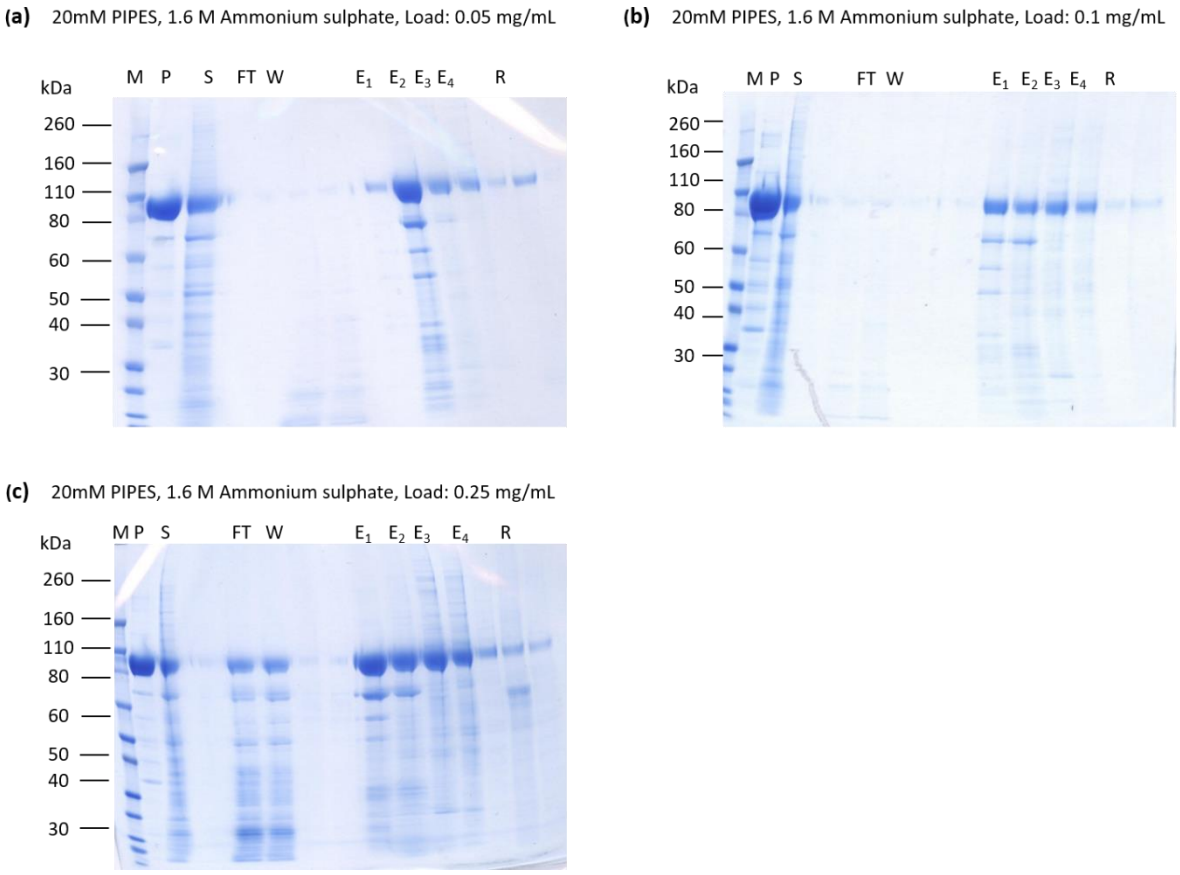


Figure S6. Capacity of SJ047- immobilised resins in increasing the ammonium sulphate concentration to 1.6M.In 20 mM PIPES and 1.6M ammonium sulphate wash buffer, breakthrough was not observed for the (a) 0.05 mg/mL to (b) 0.1 mg/mL. (c) However, as the load increased to 0.25 mg/mL and the column became saturated, breakthrough was seen in the flow through and wash fractions. Legend for SDS-PAGE above, M: Marker (Pre-stained 10 kDa ladder), P: Purified HA (New Caledonia)-Ferritin (5 µg), S: Crude HEK HA (NC)-ferritin supernatant load (X mg/mL), FT: Column wash (20 mM PIPES, X M $(NH_4)_2SO_4$), W: Column wash (20 mM PIPES, X M $(NH_4)_2SO_4$), E_X: Gradient elution fraction to 20 mM PIPES, R: Regeneration fraction.

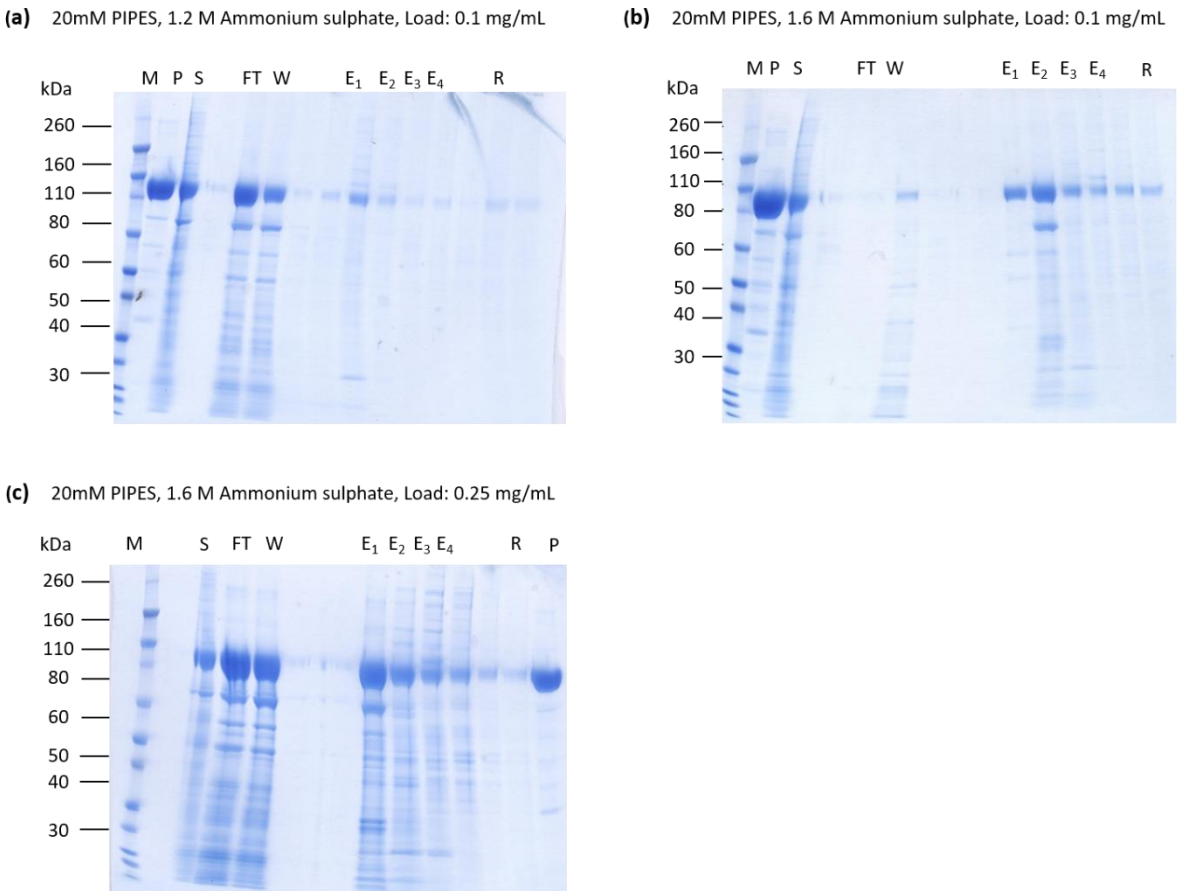


Figure S7. Capacity of SJ055-immobilised resins in increasing the ammonium sulphate concentration from 1.2M to 1.6M (a) In 20 mM PIPES and 1.2M ammonium sulphate wash buffer, breakthrough was observed for the 0.1 mg/mL load. (b) However, as the salt concentration increased to 1.6M and load remained the same to 0.1mg/mL, the salting effect adsorbed the antigen to the hydrophobic ligand and breakthrough was not observed in the wash fractions. (c) However, as the load increased to 0.25 mg/mL and the column became saturated, breakthrough was seen in the flow through and wash fractions. Legend for SDS-PAGE above, M: Marker (Pre-stained 10 kDa ladder), P: Purified HA (New Caledonia)-Ferritin (5 µg), S: Crude HEK HA (NC)-ferritin supernatant load (X mg/mL), FT: Column wash (20 mM PIPES, X M $(NH_4)_2SO_4$), W: Column wash (20 mM PIPES, X M $(NH_4)_2SO_4$), E_X: Gradient elution fraction to 20 mM PIPES, R: Regeneration fraction.

8.0. Optical Density measurement at 350 nm

1 mL samples of 0.1 mg/mL of HA (New Caledonia)- ferritin samples in 20 mM PIPES, 1.6 M ammonium sulphate were agitated and measured to check for recombinant antigen aggregation.

Table 2. Absorbance measurement (at λ=350 nm and 360 nm) of 0.1 mg/mL HA (New Caledonia)- ferritin agitated for 5 hours in 20 mM PIPES and 1.6M Ammonium Sulphate

Time (h)	Absorbance at 350 nm	Absorbance at 360 nm
1	0.0006	0
2	0.0009	0.0008
3	0	0
4	0	0
5	0	0