Supporting Information

for

A polycarboxylic chelating ligand for efficient resin purification of His-tagged proteins expressed in mammalian systems

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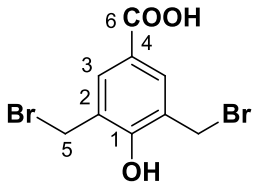
1. Organic Synthesis

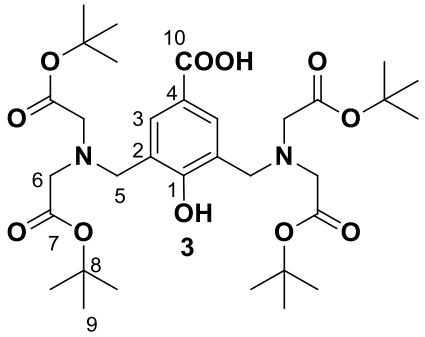
General experimental information

All solvents and reagents were used as commercialized unless otherwise indicated. Thin layer chromatography was performed on silica plates Merck Silica 60 $F_{254,}$. Preparative chromatography was performed on columns manually packed, using silicagel 60 (40-63 μm) and the same elution systems as for TLC. RP-HPLC experiments were carried on a VARIAN Pro Star 210 system, equipped with UV detection, using an analytical C18 VYDAC (300 Å, 4.6 mm × 150 mm, 5 μm,) column, eluting with gradient mixtures of water (containing 0.1% TFA) and acetonitrile. The retention time (R_t) is given in minutes with the gradient in percentage of acetonitrile.Water for RP-HPLC was produced by a Millipore Mili Q Biocel system. Characterization of the compounds was realized by Nuclear Resonance Magnetic (NMR) spectrometry using Bruker Avance III (500 MHz) or Bruker FOURIER (300 MHz) respectively. Chemical shifts (δ) are reported in parts per million (ppm) using the peak values of the residual solvent as an internal reference. The multiplicities are abbreviates as following: s – singlet, d – doublet, t – triplet, dd – doublet of doublets. Melting points of the compounds were determined with open capillary method, using an electrical apparatus STUART SMP3 and are not corrected. The UV spectra in solution state were realized with JASCO V-670. High resolution mass spectrum was recorded on Thermo Scientific (LTQ XL Orbitrap) spectrometer, in positive ion mode, using Electrospray ionisation technique.



2

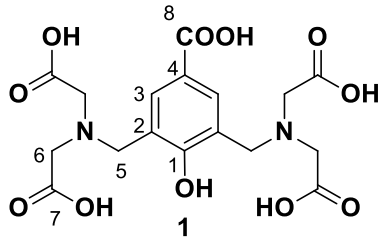
3,5-bis(bromomethyl)-4-hydroxybenzoic acid (2). White solid, *m.p.* = 182-185°C, R_f = 0.55 (silica, ethyl acetate), η= 76%. A mixture of 4-hydroxybenzoic acid (10.87 mmol, 1.5 g) and paraformaldehyde (12.14 eq, 0.13 mol, 3.96 g) was dissolved in 48% aqueous hydrogen bromide (50 mL). The resulted solution was stirred at 60-70°Cfor 6 days. Over this period, the solution colour changed from orange to slightly yellow, then colourless. A white precipitate was formed, filtered and washed with distilled water. The filtrate was further extracted with ethyl acetate (several times). The organic extracts were dried over anhydrous magnesium sulphate and evaporated. The unified solid phases in the form of a white solid did not require any further purification. ^{1}H-NMR (500 MHz, $CDCl_3$): δ= 8.05 (s, 2H, H_{Ar}), 6.20 (s, 1H, O*H*), 4.58 (s, 4H, CH_2) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ= 169.39; 157.91; 133.54; 125.14; 121.94; 28.15 ppm.



3

3,5-bis((bis(2-(tert-butoxy)-2-oxoethyl)amino)methyl)-4-hydroxybenzoic acid (3). White yellowish solid, *m.p.* = 138-141°C, R_f = 0.53 (silica, ethyl acetate: dichloromethane = 1:2),

R_t=12.65 min (RP-C18, 5-95% water (0.1%TFA)/acetonitrile), η= 70%. The brominated compound **2** (0.39 mmol, 0.125 g) was dissolved in acetonitrile (30 mL), di-*tert*-butyl iminodiacetate (2 eq., 0.77 mmol, 0.189 g) and sodium bicarbonate (2 eq., 0.77 mmol, 0.065 g) were added to the solution which was stirred at 80°C overnight. The mixture was left to cool to room temperature, then filtered and washed with ethyl acetate. The filtrate was evaporated, resulting an yellow oil. The residue was purified by column chromatography to yield the pure product in the form of a white yellowish solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ= 7.92 (s, 2H, H_{Ar}), 4.00 (s, 4H, CH_2), 3.46 (s, 8H, CH_2CO_2H), 1.46 (s, 36H, *t*Bu) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$): δ= 170.9; 170.3; 160.9; 132.2; 123.3; 120.1; 81.5; 55.2; 53.3; 28.0 ppm. HRMS (ESI, +, orbitrap) *m/z*: calc. for $C_{33}H_{53}N_2O_{11}$ $[M+H]^+$ 653.3649; found 653.3627.



1

2,2',2'',2'''-(((5-carboxy-2-hydroxy-1,3-phenylene)bis(methylene))bis(azanetriyl)) tetraacetic acid (1) White-off solid, *m.p.* =204-207°C, R_t=3.85 min (RP-C18, 5-95% water(0.1%TFA)/acetonitrile), η= 94%. Compound **3** (0.068 mmol, 44.2 mg) was dissolved in a solution of TFA 25% in dichloromethane (1.6 mL). The mixture was stirred at room temperature until complete conversion of the ester, as followed by RP-HPLC, the solvent was evaporated, the product was washed with diethyl ether, acetonitrile and dried under high vacuum. ^{1}H-NMR (300 MHz, DMSO-d_6): δ= 7.83 (s, 2H, H_{Ar}), 4.11 (s, 4H, CH_2), 3.68 (s, 8H, CH_2CO_2H) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6): δ= 170.8; 166.9; 160.2; 133.0; 121.4; 120.6; 58.6; 53.6 ppm. HRMS (ESI, +, orbitrap) *m/z*: calc. for $C_{17}H_{21}N_2O_{11}$ $[M+H]^+$ 429.1145; found 429.1132.

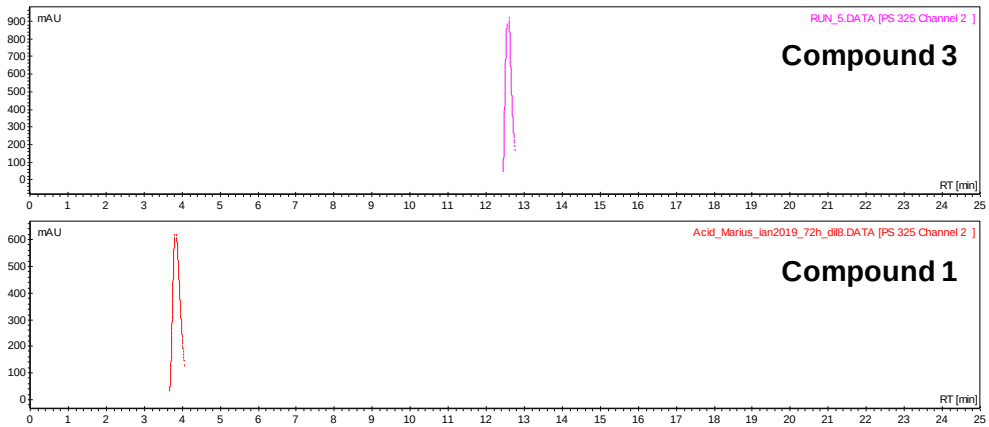


Figure S 1 RP-HPLC chromathograms of compounds **3** and **1**(RP-C18, 5-95% water(0.1%TFA)/acetonitrile)

2. Absorption spectroscopy and determination of the molar absorption coefficient for ligand 1

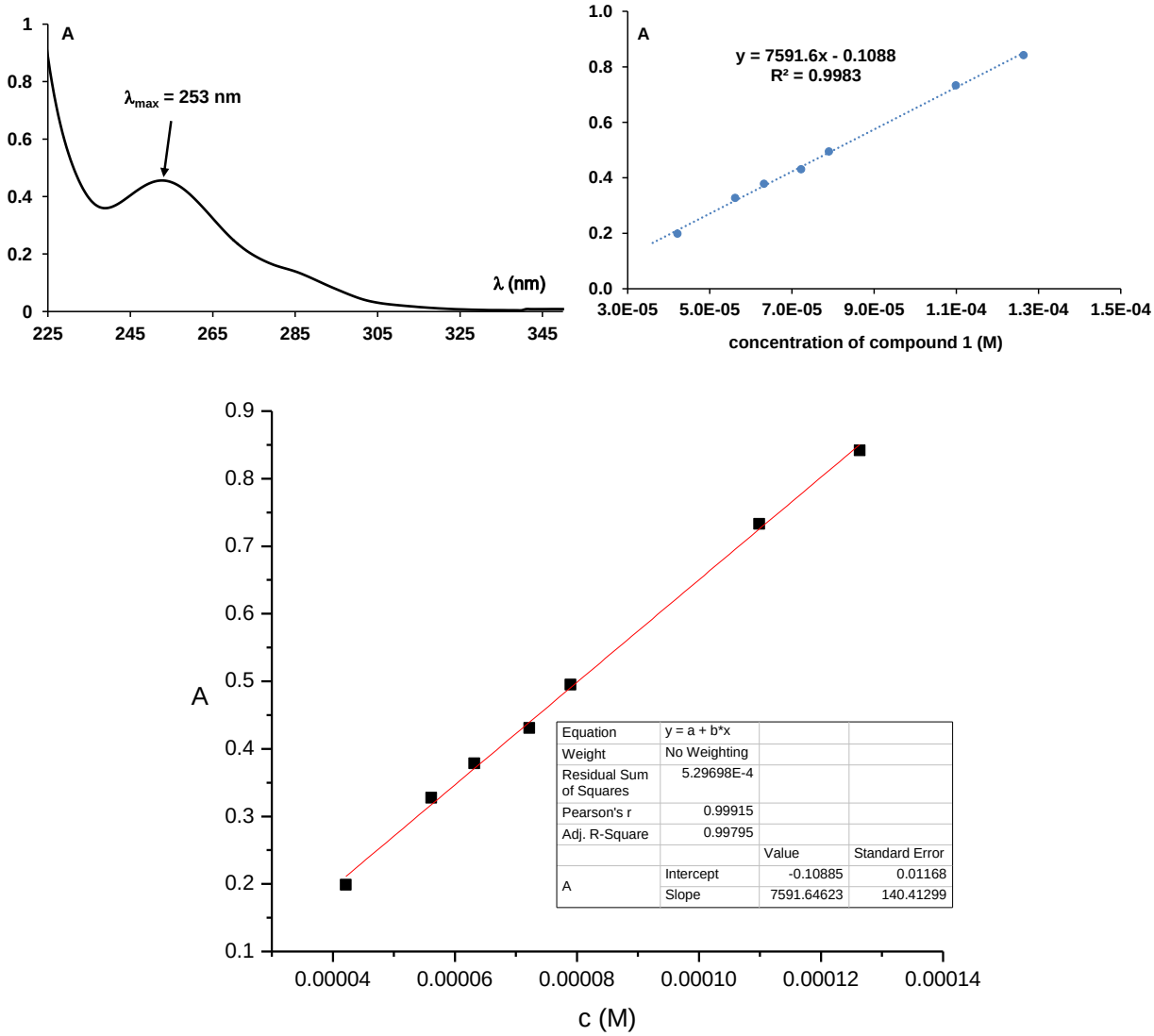


Figure S 2 UV-Vis spectrum of compound **1** recorded in water (20 µM) and molar extinction coefficient determination. Fitting analysis of the data provides the slope which corresponds to the molar extinction coefficient of 7591.6, standard deviation 140.41.

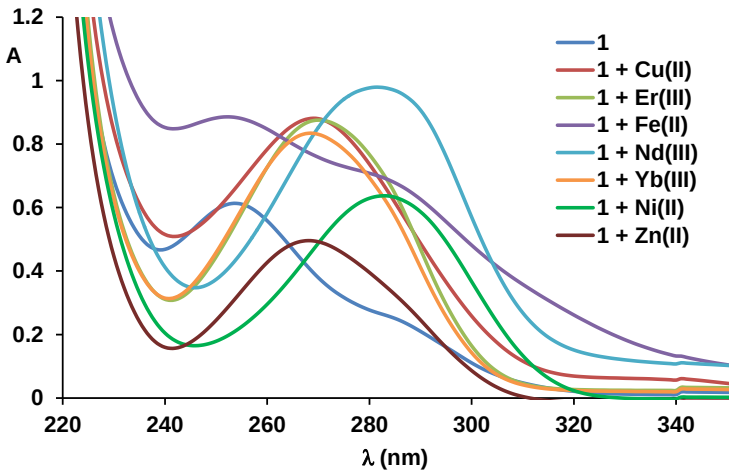
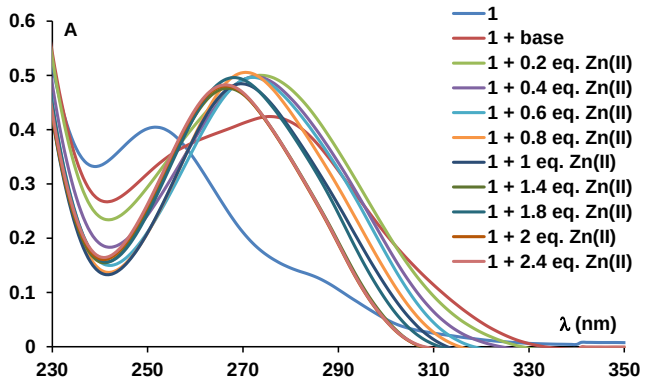


Figure S 3Screening of ligand **1** with different metal ions

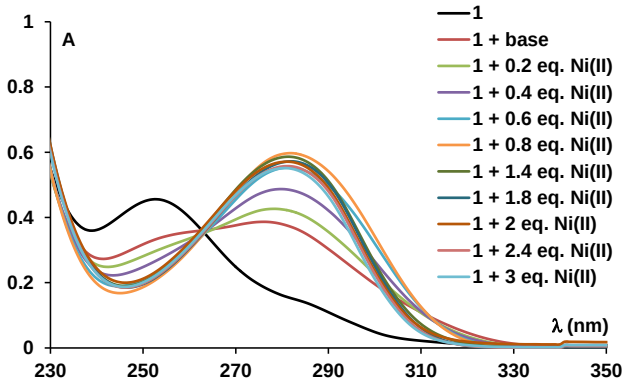
3. UV-Vis absorption experiments for determination of stoichiometry between Zn(II) and ligand 1



conc L (M)	conc Zn (M)	eq	254 nm	266 nm	271 nm	275 nm
5.54E-05	0.00E+00	0	0.348431	0.398246	0.414868	0.42391
5.54E-05	1.11E-05	0.2	0.3412	0.465595	0.494988	0.498947
5.54E-05	2.22E-05	0.4	0.296509	0.461613	0.495143	0.492675
5.54E-05	3.33E-05	0.6	0.270263	0.459626	0.494975	0.488432
5.54E-05	4.44E-05	0.8	0.277454	0.481576	0.505193	0.485666
5.54E-05	5.54E-05	1	0.278561	0.469215	0.48252	0.456828
5.54E-05	6.65E-05	1.2	0.302162	0.470791	0.465704	0.43061
5.54E-05	7.76E-05	1.4	0.323177	0.475571	0.457615	0.414929
5.54E-05	8.87E-05	1.6	0.329436	0.479248	0.457942	0.413649
5.54E-05	9.98E-05	1.8	0.318003	0.491027	0.487614	0.454533
5.54E-05	1.11E-04	2	0.333074	0.480214	0.458129	0.413816
5.54E-05	1.22E-04	2.2	0.325529	0.472355	0.449316	0.404246
5.54E-05	1.33E-04	2.4	0.335752	0.482297	0.459731	0.415203
5.54E-05	1.44E-04	2.6	0.329135	0.477628	0.454901	0.409716
5.54E-05	1.55E-04	2.8	0.323004	0.469548	0.44766	0.40361
5.54E-05	1.66E-04	3	0.32799	0.477083	0.455148	0.410879

Figure S 4: UV-Visdata obtained by titration of ligand **1** with Zn(II)

4. UV-Vis absorption experiments for determination of stoichiometry between Ni(II) and ligand 1



conc L(M)	conc Ni (M)	eq	254	278	282	284
5.99E-05	0	0	0.338728	0.384794	0.370817	0.35885
5.99E-05	1.20E-05	0.2	0.304345	0.426301	0.419316	0.409583
5.99E-05	2.40E-05	0.4	0.276014	0.484986	0.483571	0.475208
5.99E-05	3.60E-05	0.6	0.240406	0.543658	0.552454	0.547622
5.99E-05	4.80E-05	0.8	0.221462	0.584555	0.597072	0.592532
5.99E-05	5.99E-05	1	0.213537	0.614656	0.636331	0.635923
5.99E-05	7.19E-05	1.2	0.221671	0.606855	0.630687	0.63115
5.99E-05	8.39E-05	1.4	0.237151	0.576595	0.585771	0.579512
5.99E-05	9.59E-05	1.6	0.23949	0.565898	0.571474	0.563413
5.99E-05	1.08E-04	1.8	0.228376	0.559448	0.572029	0.567734
5.99E-05	1.20E-04	2	0.24601	0.563801	0.570286	0.562928
5.99E-05	1.32E-04	2.2	0.243764	0.562592	0.569561	0.562856
5.99E-05	1.44E-04	2.4	0.231177	0.549484	0.556758	0.549934
5.99E-05	1.56E-04	2.6	0.226005	0.545072	0.554275	0.547841
5.99E-05	1.68E-04	2.8	0.232708	0.547237	0.553933	0.546615
5.99E-05	1.80E-04	3	0.235202	0.546012	0.549469	0.540505

Figure S 5UV-Vis data obtained by titration of ligand **1** with Ni(II)

5. Derivatization of cross linked beaded agarose support with polycarboxylic compound

2 mL of Thermo Scientific™CarboxyLink™ Coupling Gel (Immobilized Diaminodipropylamine) were added in a peptide synthesis reactor and the aqueous solution was evacuated. The resin was washed in the reactor 6 times with DMF and suspended in 3 mL of DMF. Then compound **3** (2 Eq, 40 μmol, 52 mg), PyBOP reagent (2.2 Eq, 44 μmol, 98 mg) and DIPEA (6 Eq, 120 μmol, 62 mg, 0.1 mL) were dissolved in 1 mL DMF. The resulted solution was added to the resin in the reactor which was left in an active mechanical shaker. The coupling was checked using nynhidrin test (Kaiser test for peptide coupling) and when attachment was complete (different batches required or not addition of ligand excees), the solvent was evacuated and the resin was washed 3 times with DMF and exchanged with water by 6 consecutive washings with MilliQ water. An aqueous solution of $LiOH \cdot H_2O$ (10 Eq, 200 μmol, 10 mg) was added in the reactor which was shaken for 1 h, then an aqueous solution of $Ni(ClO_4)_2 \cdot 6H_2O$ (8 Eq, 160 μmol, 60 mg) was added to the resin and the reactor was again shaken for 24 hours. The resin was thoroughly washed with MilliQ water to afford the modified agarose support.

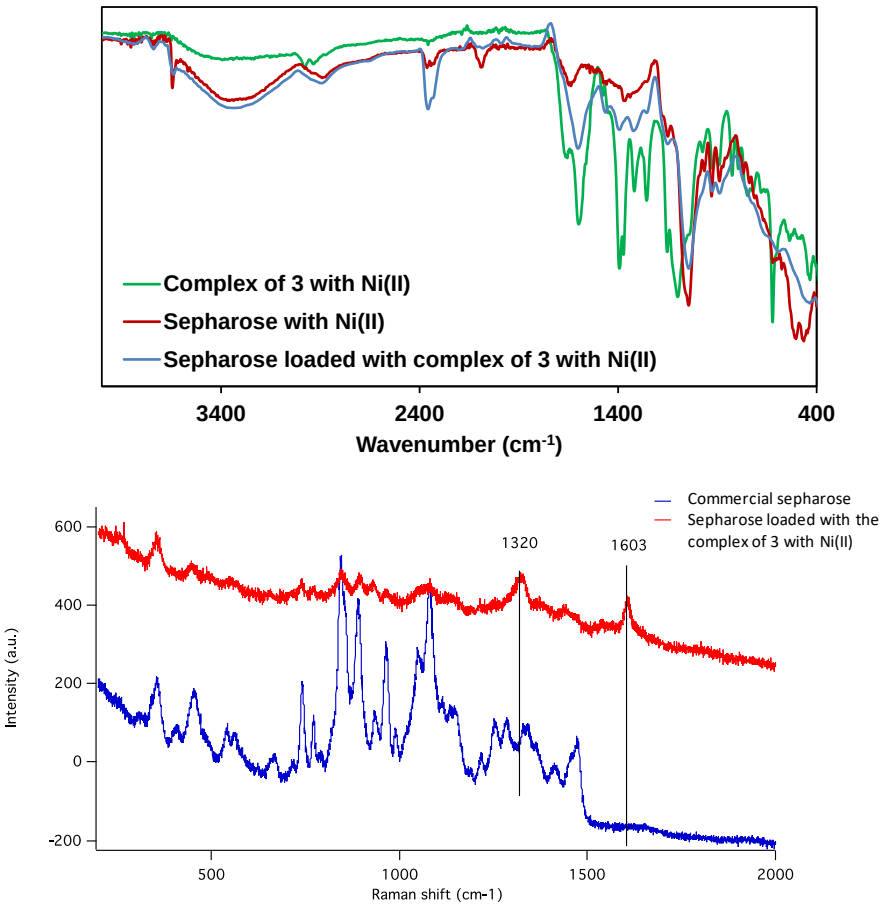


Figure S 6 Top: IR spectra, bottom: RAMAN spectra

6. The affinity test of the modified sepharose support for His-tag derived protein

His-tag functionalized protein used for the retention test was S100B with a molar mass of 13.8 kDa. Purified S100B in 20mM HEPES, 20mM imidazole, 150mM NaCl, 2mM $CaCl_2$,and 10% glycerol at a concentration of 4.18 μg/μL was further used. The control samples for the test comprised of unfunctionalized resin and resin with ligand as negative controls and NiNTA resin as a positive control. Firstly, the resins were equilibrated by 3 washing steps with a His-tag purification binding buffer (20 mM imidazole, 500 mM NaCl, 20 mM HEPES). For each resin, 30 μL were taken froman homogenous suspension of sepharosebeads. Each washing step was followed by centrifugation and removal of the buffer. After the last wash, 500 μL of buffer and 50μg His-S100B (12μL) were added to the resinsand incubated for 1 h at 4ºC in a rotating mixer. Samples were again centrifuged and all of the solvent was collected to determine the quantity of unbound protein. The protein was eluted by incubating the beads with elution buffer (500mM imidazole, 50 mM NaCl, 20 mM HEPES) at 4° C for 15 minutes. After elution,10 μL of loading buffer (62.5 mM Tris-HCl pH 6.8, 2.5 % SDS, 0.002 % Bromophenol Blue, 0.7135 M (5%) β-mercaptoethanol, 10 % glycerol) specifically used for loading protein samples in polyacrylamide gels were added to the elute. After mixing, the samples were heated at 95ºC for 10 minutes to completely denature the protein. Proteins were resolved in a 15% SDS-PAGEat 25 mA and 40mA for 45 minutes. The gel was stained withCoomasie Blue G-250 reagent solution.

7. His tagged protein purification from complex prokaryotic lysates

BL21(DE3) cells were transformed with pHAT2-S100B and the next day a fresh colony was picked and inoculated in LB media for protein induction and expression. At an OD of 0.6, cells were induced with 10mM IPTG for 4 hours. Bacterial cells were pelleted at 5000g, 4°C for 15 minutes and washed with ice cold PBS to be centrifuged again. Pellet was stored until lysis at -80°C.

We incubated 1g pellet of BL21(DE3) *E. coli* cells expressing S100B with 10mL Lysis Buffer for 30 minutes on ice then sonicated for 10 seconds at 70% power for 6 times. The lysate was centrifuged for 30 minutes at 4°40.000 rcf and split into 4 tubes. The 4 supernatants were further incubated with 20uL of NiNTa, S-1-Ni, S-1, and Sepharose beads, respectively, and 700uL Binding Buffer (20mM imidazole, 500mM NaCl, 20mM HEPES) for 1 hour at 4°C with rotation. Samples with resins were centrifuged and the supernatant was removed and kept as the Unbound fraction. The resin was washed with 1mL Buffer 3 times, each time being incubated for 10 minutes at 4°C and centrifuged. Elution was performed in 50μL Elution Buffer (500mM imidazole, 500mM NaCl, 20 mM Tris, pH = 8) at 4°C for 15 minutes. Samples were centrifuged and the supernatant was boiled with Loading Buffer at 95°C to ensure a complete protein denaturation.Protein separation was performed in a 15% gel at 20mA and 40mA for one hour. The gel was stained with Coomassie Blue and distainedover night.

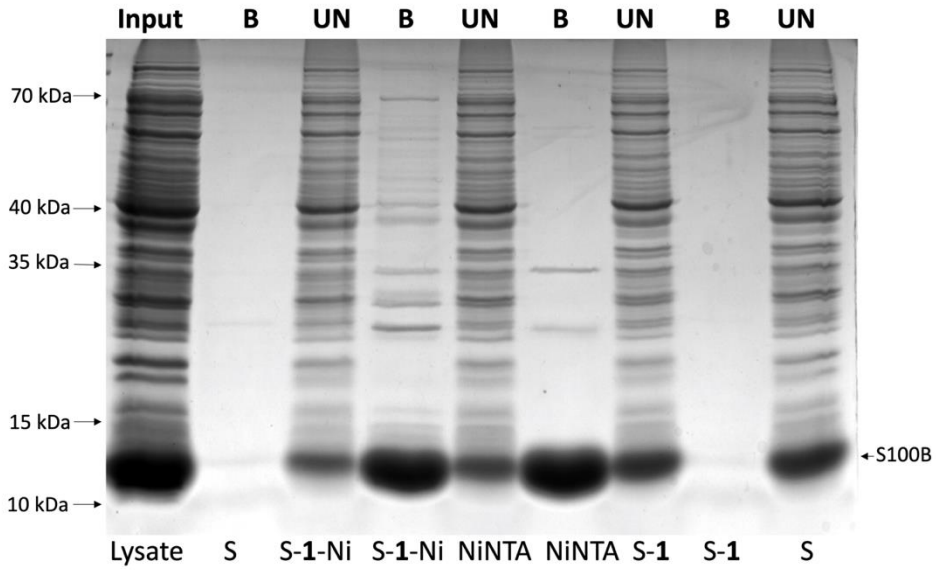


Figure S 7 Purification of recombinant HisS100B from *E. coli* BL21 cell lysate using commercial amino-sepharose (**S**), sepharose resin derivatized with **1 (**S-**1),** commercial Ni-NTA resin (NiNTA), sepharose derivatized with Ni(II) complex of **1** (S-**1**-Ni). Clarified lysate (Input), the eluates (**B**), and 1/30 of the supernatants (**UN**) were separated by SDS-PAGE and visualized with Commassie Blue staining.

To further assess the binding capacity of S-**1**-Ni we decided to quantify the amount of S100B bound to the resins by densitometry. Quantification was performed using Image J (vs.1.53a) based on a purified protein constructed standard curve with ranging concentrations from 24µg/ml to 750ng/ml by serial binary dilutions. Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.We started with 500 µg total loaded protein with an estimated 90 µg HisSB100. The binding capacities of S-1-Ni was calculated 409+/-42 µg HisSB100/ml resin and 412+/-78 µg HisSB100/ml resin of NiNTA. The pull down experiments were performed in triplicates.

8. Soluble His tag protein purification from mammalian cell culture media

To test the specificity of our resin for EK protein expression systems we decided to express a viral protein pcDNA3.1-His-sE2 in HEK293T cells. Cells were sub-cultured and grown to a 70% confluence and transfected using Lipofectamine 3000 in a 6-well plate with pcDNA3.1-His-sE2 and pcDNA3.1 (control). After 24 hours we substituted 1.5mL of complete media (10% FBS) with 1.5mL Optimem media in one of the transfection wells. At 48 hours post transfection, cell media was collected, centrifuged at 10.000 rcf for 5 minutes, and the supernatant was collected. Cells were scraped with PBS and centrifuged for 5 minutes at 400 rcf. The pellet was lysated using PBS Triton100x 1% with protease inhibitors, vortexed, and thawed on ice in between vortexing. The lysate was centrifuged again at 4°C and 14.000 rcf for 20 minutes and the supernatant was frozen.

Clarified media was subjected to affinity chromatography using S-**1**-Ni and NiNTA for both samples containing 2% FBS or 10% FBS as mentioned previously.

Samples of purified protein were separated in a 10% polyacrylamide gel at 20mA and 40mA and then transferred to a nitrocellulose membrane at 300mA for 1 hour. The membrane was further blocked with PBS - Milk 5% for an hour. The primary antibody used was 3/11, which is a specific E2 antibody, at a dilution of 1/2000 and left overnight at 4°C. The membrane was washed with PBS Tween 0.1% 3 times for 40 minutes. Afterwards, an anti rat HRP-linked secondary antibody was added and the membrane was further incubated for an hour. Washing was performed and the membrane was incubated with 2mL of ECL reagent mix for 2 minutes and the film was exposed with the membrane for 10 minutes.

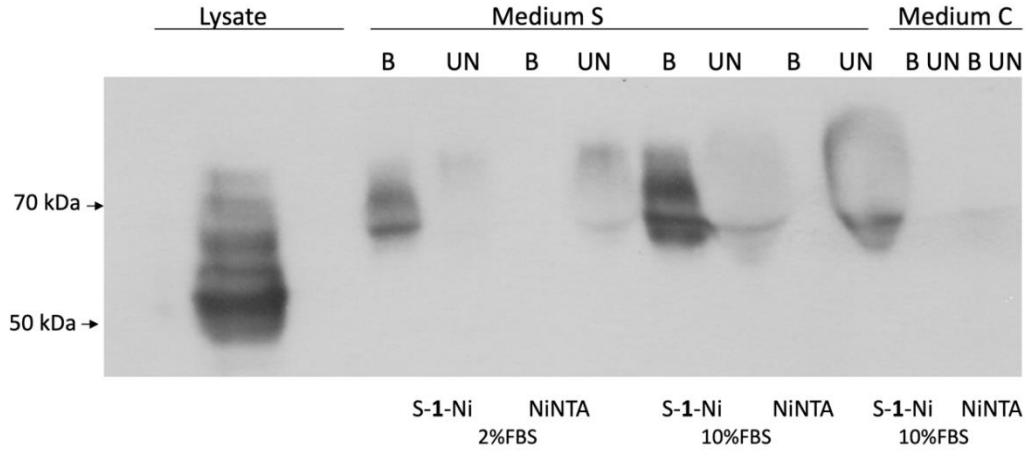


Figure S 8 Purification of recombinant HCV E2s protein. 293T cells were transfected with a plasmid coding for HCV E2 ectodomain (AB154191_E2_C1b, aa 384_656) (S) or pcDNA3.1 empty vector (C). 48h post-transfection, the clarified media was subjected to pulldown using either NiNTA or S-1-Ni resin. Cell lysates, eluates (B), and 1/30 of the supernatants (UN) were separated by SDS-PAGE and HCV E2s was detected by Western Blot with an exposure time of 5 minutes.

To quantify the specific binding capacity of the resins we performed ELISA. A Corning white half-area 96 wells plate was incubated with antigen using 30µL from each pulldown sample (E2 in 2% FBS, E2 in 10% FBS, pcDNA3.1 in 2% FBS, and pcDNA3.1 in 10% FBS purified with Ni-NTA and S-**1**-Ni in triplicate, respectively) overnight and kept at 4ºC. Next day, blocking was performed with 4% milk in PBS 0.05% Tween followed by washing steps using PBS 0.05% Tween. Then, samples were incubated with the primary antibody (3/11, 1/1000) for 1 h, further washed and incubated for another hour with the secondary antibody (goat-anti rat Hrp conjugated). After 3 further washing steps, samples were incubated with 50µL of SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher) and luminescence was immediately assessed using the Mithras LB940 (Berthold Technologies) reader. To estimate the binding capacity of the two resins, we used a standard curve of binary dilutions from 10µg/ml to 125ng/ml of purified HCV E1E2 heterodimer (Clarke et al., 2017, doi: 10.1111/pbi.12743). The binding capacity was estimated after the subtraction of the values obtained for the mock transfected cells.

To further assess the binding capacity of the resin, we performed a Coomassie staining resolving 1/8 of the 10% FBS pcDNA3.1-His-sE2 and pcDNA3.1 eluate.

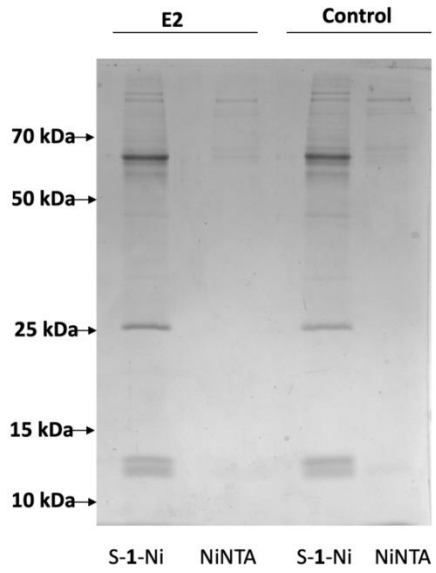


Figure S 9 Mammalian cell media with 10% FBS pulldown using Ni-NTA resin (NiNTA) and sepharose derivatized with Ni(II) complex of **1** (S-**1**-Ni). Eluates (**B**) were separated by SDS-PAGE and visualized with Commassie Blue staining.

9. His tag protein purification with reused resin

To assess the stability of S-**1**-Ni we further used the same resin previously used to purify S100B from BL21(DE3) lysate for another two rounds of pulldown as described previously. After each pulldown, the resins were washed with EtOH 20% 1mL for 3 times and stored in EtOH 20% at 4ºC. Eluted samples were resolved in SDS-PAGE and Coomassie stained as described.

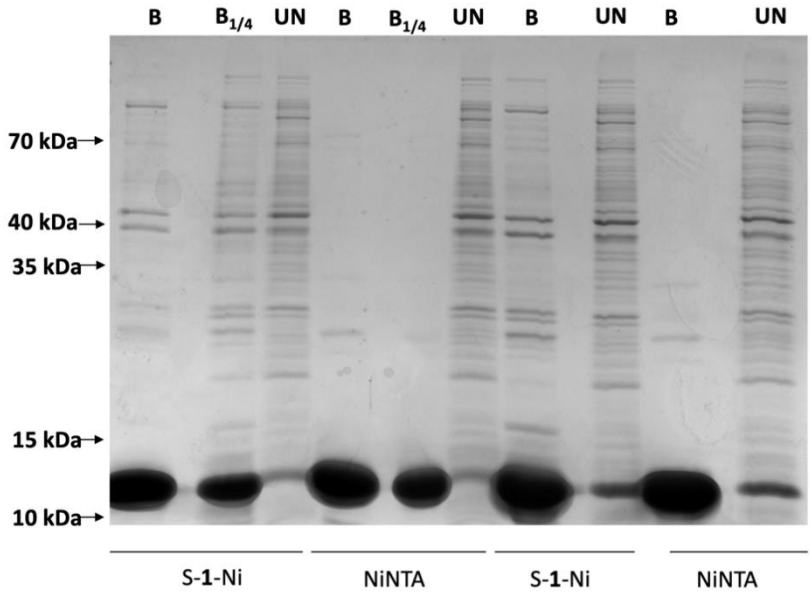


Figure S 10 Purification of recombinant HisS100B from *E. coli* BL21 cell lysate usingcommercial Ni-NTA resin (NiNTA), sepharose derivatized with Ni(II) complex of **1** (S-**1**-Ni). Eluates (**B**), and 1/30 of the supernatants (**UN**) were separated by SDS-PAGE and visualized with Commassie Blue staining. First six lanes represent the eluates of a second use of the resins whereas the last for lanes depict the capacity of the resins at a third use.

9. His-RAGE protein expression and purification

BL21(DE3) cells were transformed with pHAT2-TEV-His-sRAGE and the next day a fresh colony was picked and inoculated in LB media for protein induction and expression. At an OD of 0.6, the culture was moved at 23ºC and induced at an OD = 1 overnight. Bacterial cells were pelleted at 5000g, 4°C for 15 minutes and washed with ice cold PBS to be centrifuged again. Pellet was stored until lysis at -80°C.

We incubated 800mg pellet of BL21 (DE3)*E. coli* cells expressing His-sRAGE with 8mL Lysis Buffer for 30 minutes on ice then sonicated for 10 seconds at 70% power for 6 times. The lysate was centrifuged for 30 minutes at 4°40.000 rcf and pulldown was performed as previously described.

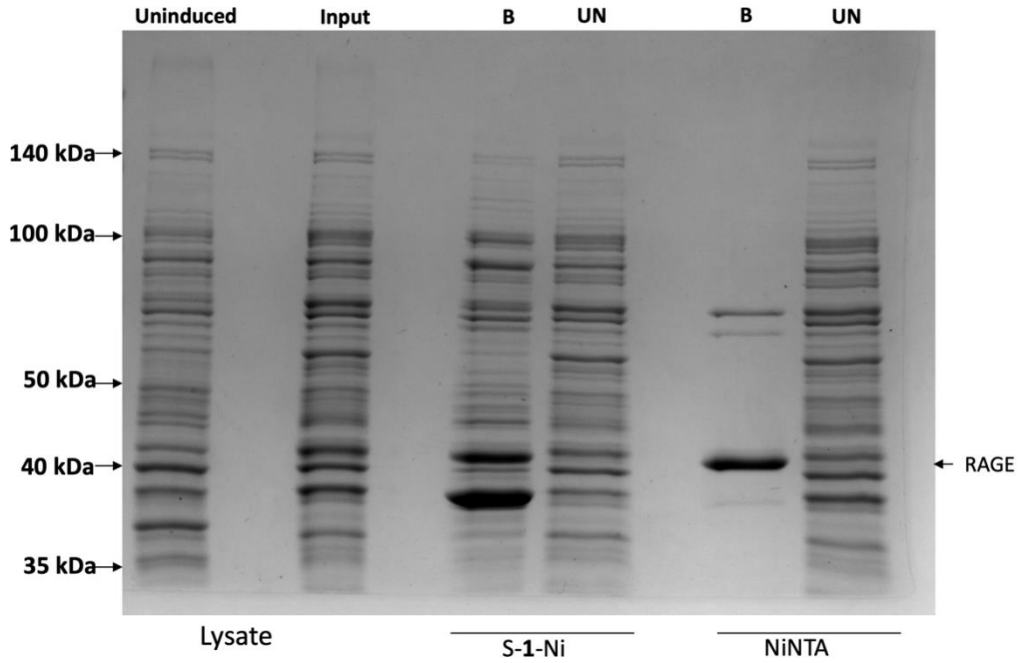
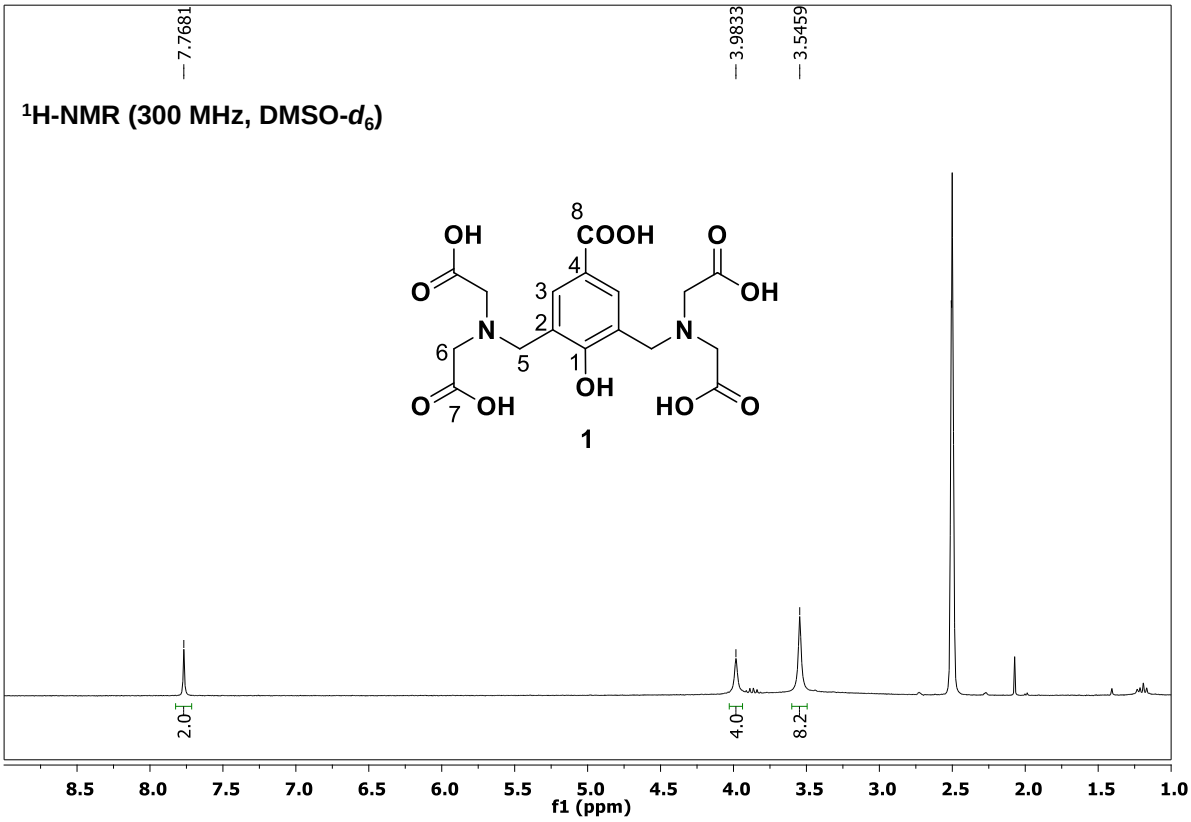
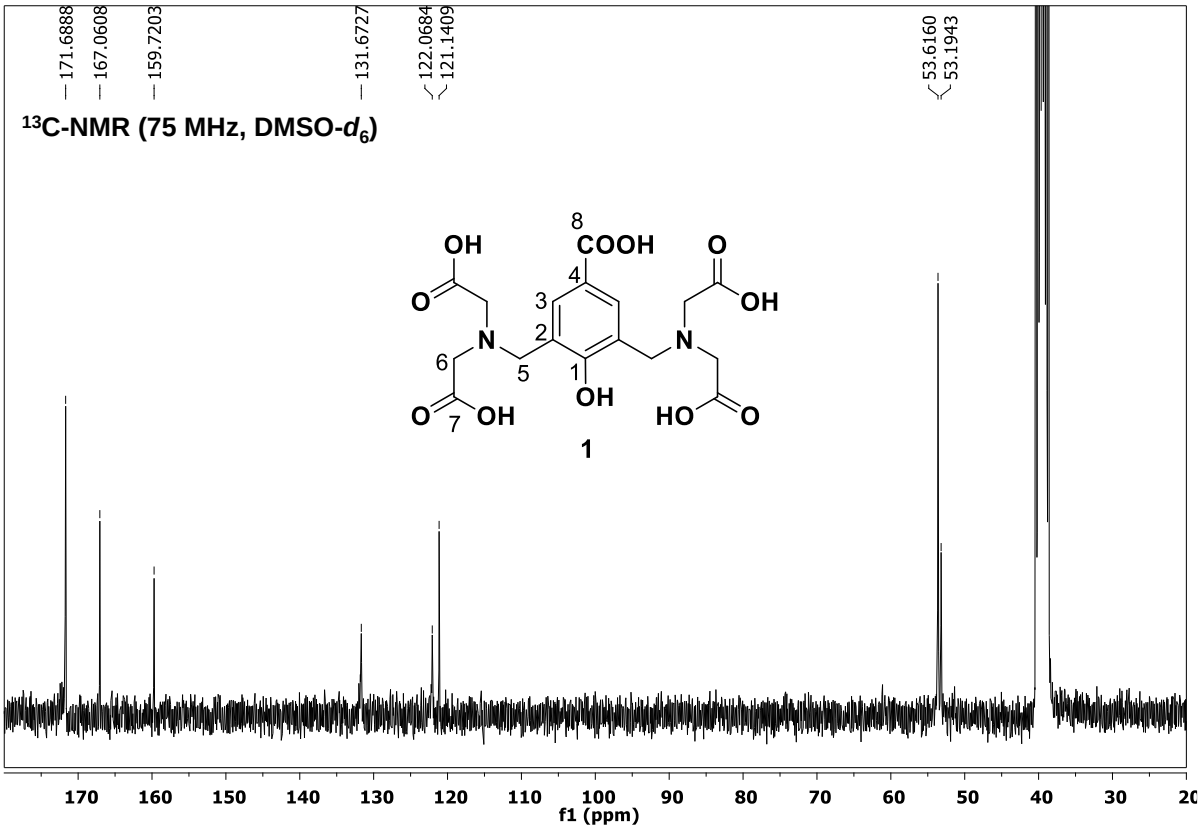
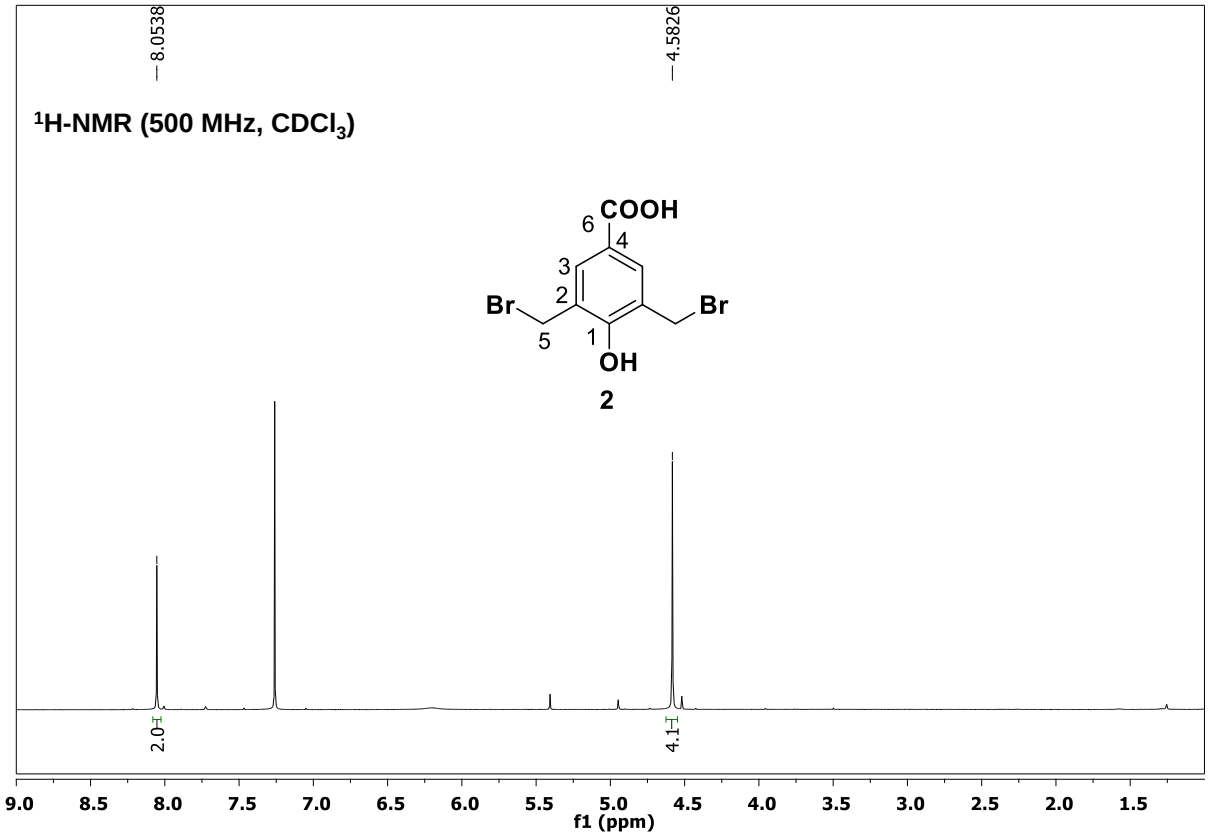


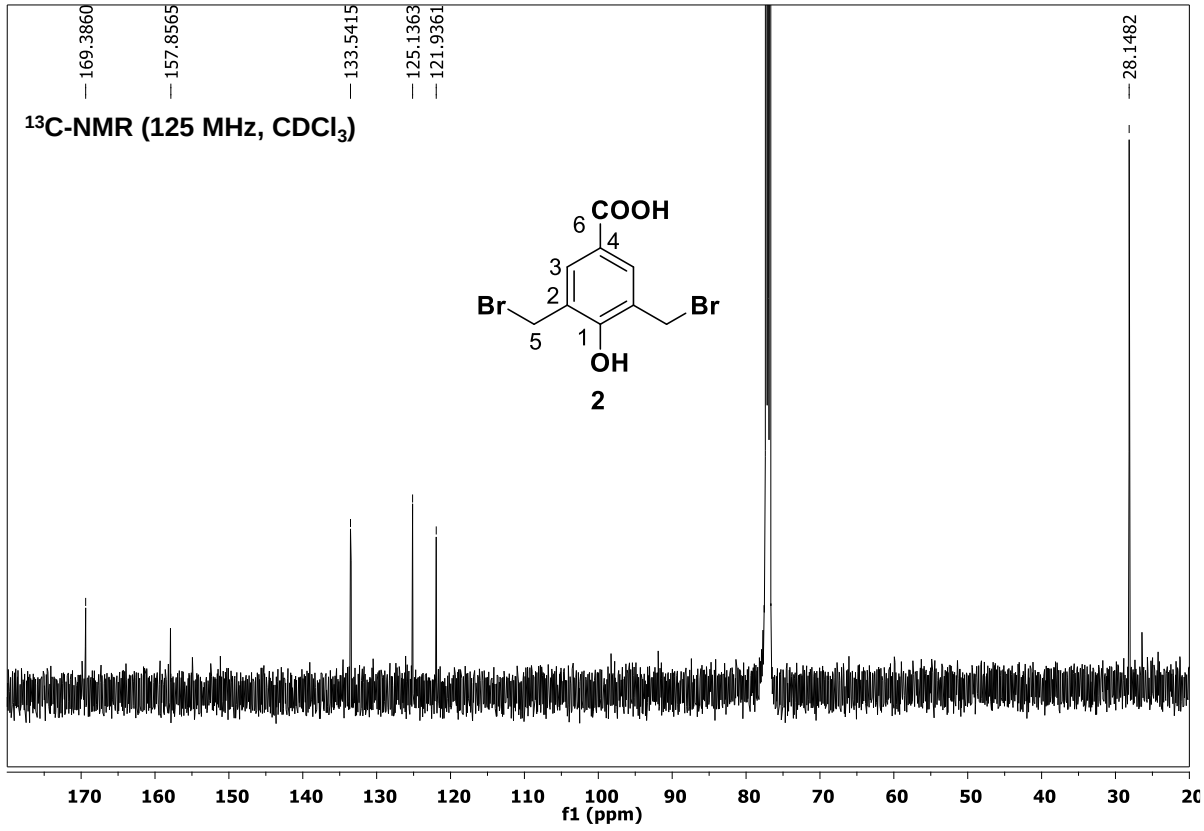
Figure S 11 Purification of recombinant His-sRAGE from *E. coli* BL21 cell lysate usingcommercial Ni-NTA resin (NiNTA), sepharose derivatized with Ni(II) complex of **1** (S-**1**-Ni). Eluates (**B**), and 1/30 of the supernatants (**UN**) were separated by SDS-PAGE and visualized with Commassie Blue staining.

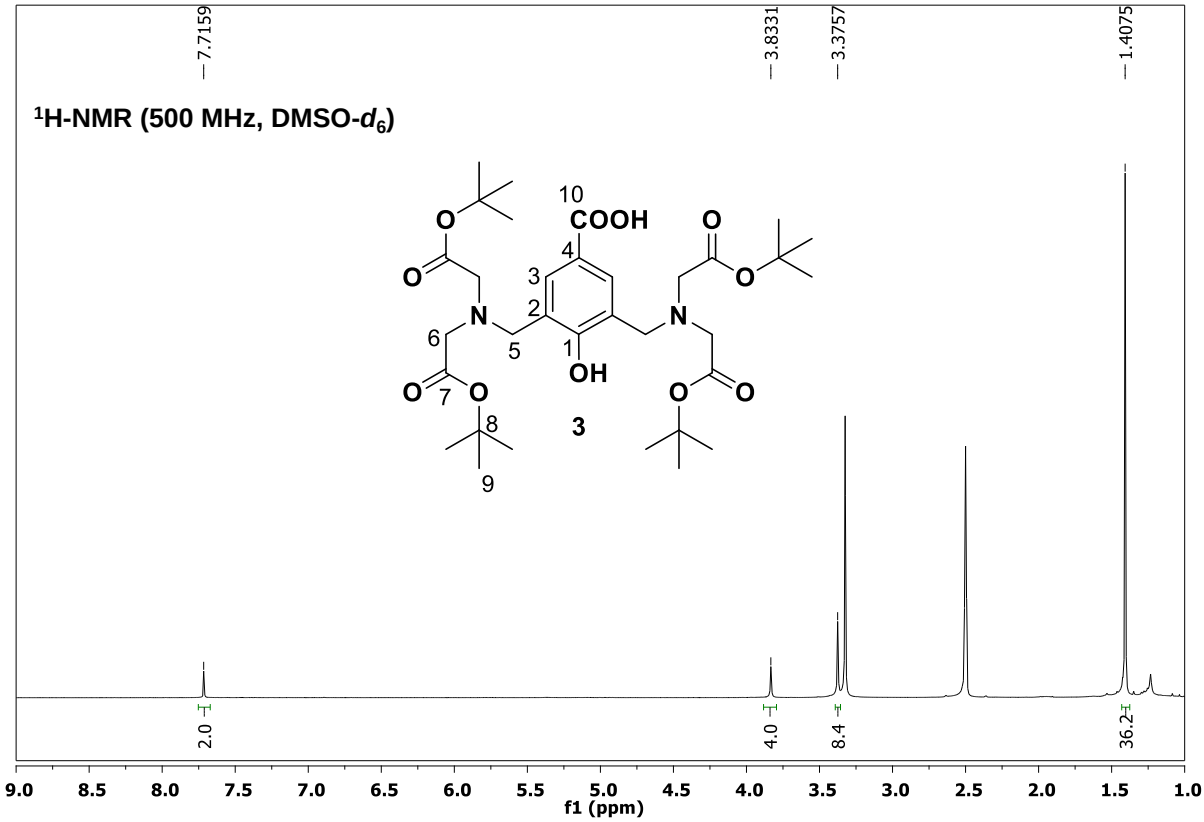
10. NMR spectra

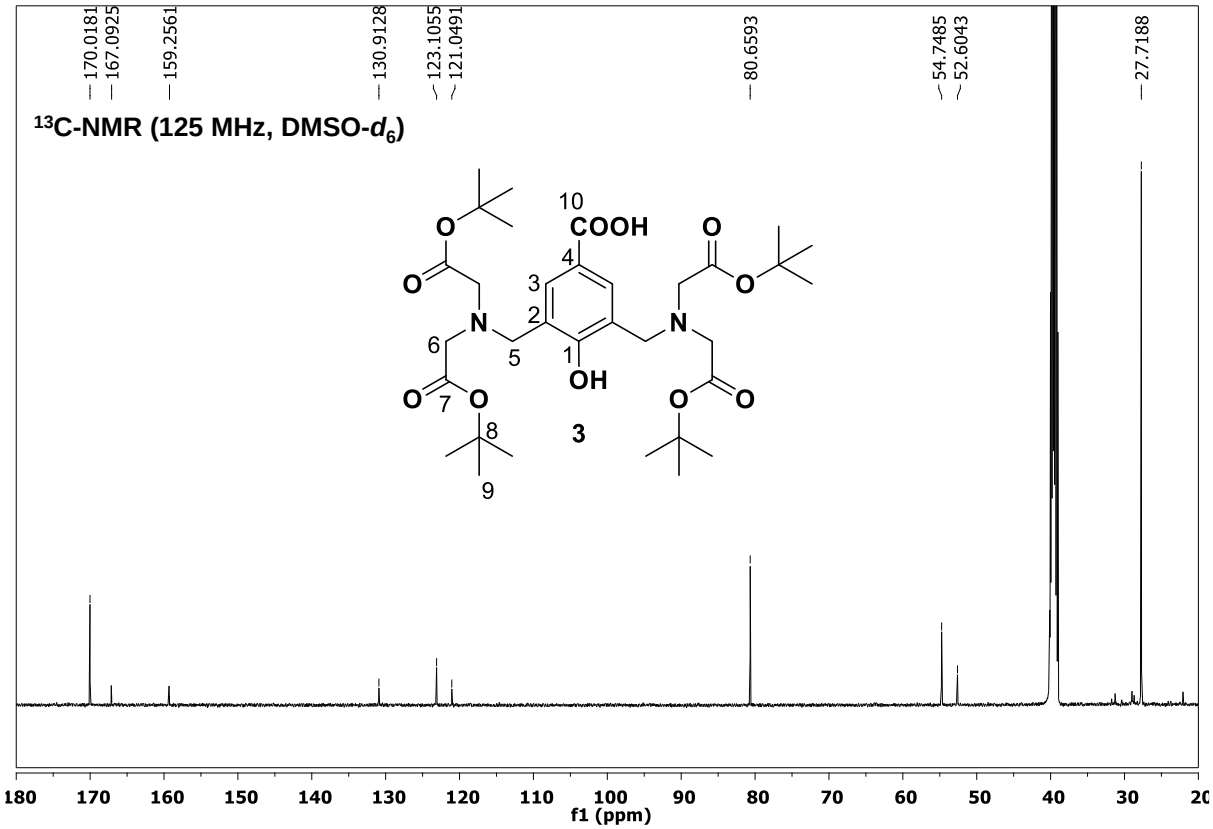





8.0538
4.5826
1H-NMR (500 MHz, CDCl3)
COOH
Br
Br
OH
2
2.0
4.1
f1 (ppm)


169.3860
157.8565
133.5415
125.1363
121.9361
28.1482
13C-NMR (125 MHz, CDCl3)
COOH
Br
Br
OH
2
f1 (ppm)


7.7159
3.8331
3.3757
1.4075
¹H-NMR (500 MHz, DMSO-d6)
COOH
OH
3
2.0
4.0
8.4
36.2
f1 (ppm)


170.0181
167.0925
159.2561
130.9128
123.1055
121.0491
80.6593
54.7485
52.6043
27.7188
¹³C-NMR (125 MHz, DMSO-d6)
COOH
OH
3
f1 (ppm)