Supporting Information for:

Cysteinylated Protein as Reactive Disulfide: an Alternative Route to Affinity Labeling.

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Materials:

All reagents used were of the highest purity available commercially, used without further purification if not stated otherwise. Pure water (18 MΩ-cm) was used in all instances. In addition, all glassware and plasticware was soaked overnight in 3M HCl and thoroughly washed with pure water.

Reagents: (S)-2-(4-aminobenzyl)-DOTA was purchased from Macrocyclics (Dallas, TX). $^{90}YCl_3$ was purchased from Perkin Elmer (Wellesley, MA).

High Performance Liquid Chromatography:

Analytical and preparative reversed phase HPLC was performed on an Altima 5 µm, 4.6×250 mm C18 column and an Altima 5 µm, 22×250 mm C18 column (Alltech Associates, Inc., Deerfield, IL). Reversed-phase HPLC: solvent A, 0.1 % CF_3COOH in H_2O ; solvent B, 0.075% CF_3COOH in CH_3CN; 5-30% B, 0-35 min; 30-80% B, 35-45 min; 100-5% B, 45-50 min, 4 mL/min.

Liquid Chromatography/Mass Spectrometry: An Atlantis 3 µm, 2.1×100 mm C18 column (Waters, Milford, MA) was used for HPLC separation of peptide samples for mass spectrometric analysis. HPLC gradient: solvent A, H_2O with 0.2% formic acid; solvent B, CH_3CN with 0.175% HCOOH, 0–55 min, 3–55% B, linear solvent B, 250 µL/min. All mass spectrometry was performed on a ThermoFinnigan LCQ Deca electrospray ion-trap instrument (Waltham, MA).

Nano-LC-MS/MS: A UPLC NanoAcquitity (Waters, 100µm ID, 100mm length) C18 column was used for ECAT. HPLC gradient: solvent A, H_2O with 0.1% formic acid; solvent B, CH_3CN with 0.1% formic acid, 0-30min, 2-80% B, linear solvent, 250nL/min. MS and MS/MS were both performed on a Thermo-Electron LTQ-FT electrospray instrument.

Proton NMR: ^1H NMR was performed on a Varian Mercury 300 MHz NMR (Palo Alto, CA) with either D_2O or $CDCl_3$. The proton shift was assigned at 4.79 ppm for HDO and 7.26 ppm for $CHCl_3$. All chemical shift predictions prior to actual experiment were conducted using ChemDraw Ultra software (CambridgeSoft Corp., Cambridge, MA).

Cobalt-binding Assay: Assays were carried out as described *(1)*. Briefly, equal amounts of 29.3 mM $[^{57}Co]Co^{+2}$ solution was added to a titration series of chelate solution diluted in 0.5 M ammonium acetate, pH 6.0, mixed and then incubated for 1 hour at 37 °C. Aliquots were applied to plastic backed silica gel TLC plates (0.2-mm-thick 60 F254, Merck) which were run using a 10% (w/v) sodium acetate /methanol (1:1 v/v) solution as the eluent. In this system, unchelated metal stays at origin while chelated metal migrates to an R_f of 0.5 to 0.7. Quantitative analysis was done by cutting developed TLC plates and performing chelated and non-chelated activity γ-counts on a LKB/Wallac 1282 CompuGamma CS (Pharmacia, New York, NY) counter.

Metallation of Chelates:

10 µL of 2 mM solutions of **1, 2, 3** and Nitrobenzyl-DOTA were incubated with ^{90}Y to obtain metallated complexes. Briefly, 0.24 nM ^{90}Y was incubated at 37 °C with 2 mM **1** or **2** for 1.5h, in 0.5 M ammonium acetate buffer (pH =6). Afterwards, excess cold yttrium was added and the resulting solution incubated for an additional 1.5h at 37 °C. DTPA was then added to scavenge free metal. TLC analysis *(1)* was performed to confirm complete complexation of ^{90}Y.

Synthesis of Sulfhydryl-DOTA reagents:

A synthetic route first characterized by Weiss et al in 1947 was used to add a mercaptoacetyl group to (S)-2-(4-aminobenzyl)-DOTA to yield **1** *(2-4)*. In addition, (S)-2-(4-aminobenzyl)-DOTA was allowed to react with 2-iminothiolane to form (S)-2-(4-(4-mercapto-butyrimidoyl)-aminobenzyl)-DOTA (chelate-**2)**.

Thiocyanatoacetic acid (**a1**): 20 mL of H_2O was added to a 200 mL flask along with 38.6 g (0.28 mol) of bromoacetic acid. The solution was neutralized with sodium carbonate and 5.0 g (0.28 mol) of NaSCN in 10 % aqueous solution was added. After several hours, the product was collected and recrystallized from hot ethanol, yielding white crystals upon cooling. This was dried under reduced pressure to yield 34.5 grams of final product. TLC analysis showed a single product migrating at $R_f = 0.72$, with bromoacetic acid migrating at $R_f = 0.6$ using a solvent mixture of MeOH/Ethyl acetate (1:3). ^1H NMR of final product in D_2O showed a singlet at 3.8ppm, relative to 4.0ppm for bromoacetate.

(S)-2-(4-(2-carbamoylsulfanyl-acetylamino)-benzyl)-DOTA (**a2**)*:* 100 mg (1.60×10^{-1} mmol) of aminobenzyl-DOTA was dissolved in 30 mL of 18 MΩ-cm H_2O in a 25mL roundbottom flask. A small amount of 3M HCl was added to bring the compound into solution. The sodium thiocyanatoacetate from step 1 (0.1 mol) was added and the resulting solution was acidified to pH 4 with 3M HCl. The pH was adjusted with dilute NaOH as needed. This solution was stirred gently for several minutes, after which a brown precipitate formed. After several hours, the solid was filtered and dried. ESI-MS *m/z* calcd (MH+): 626.7. Found: 627.4 (Figure s1).

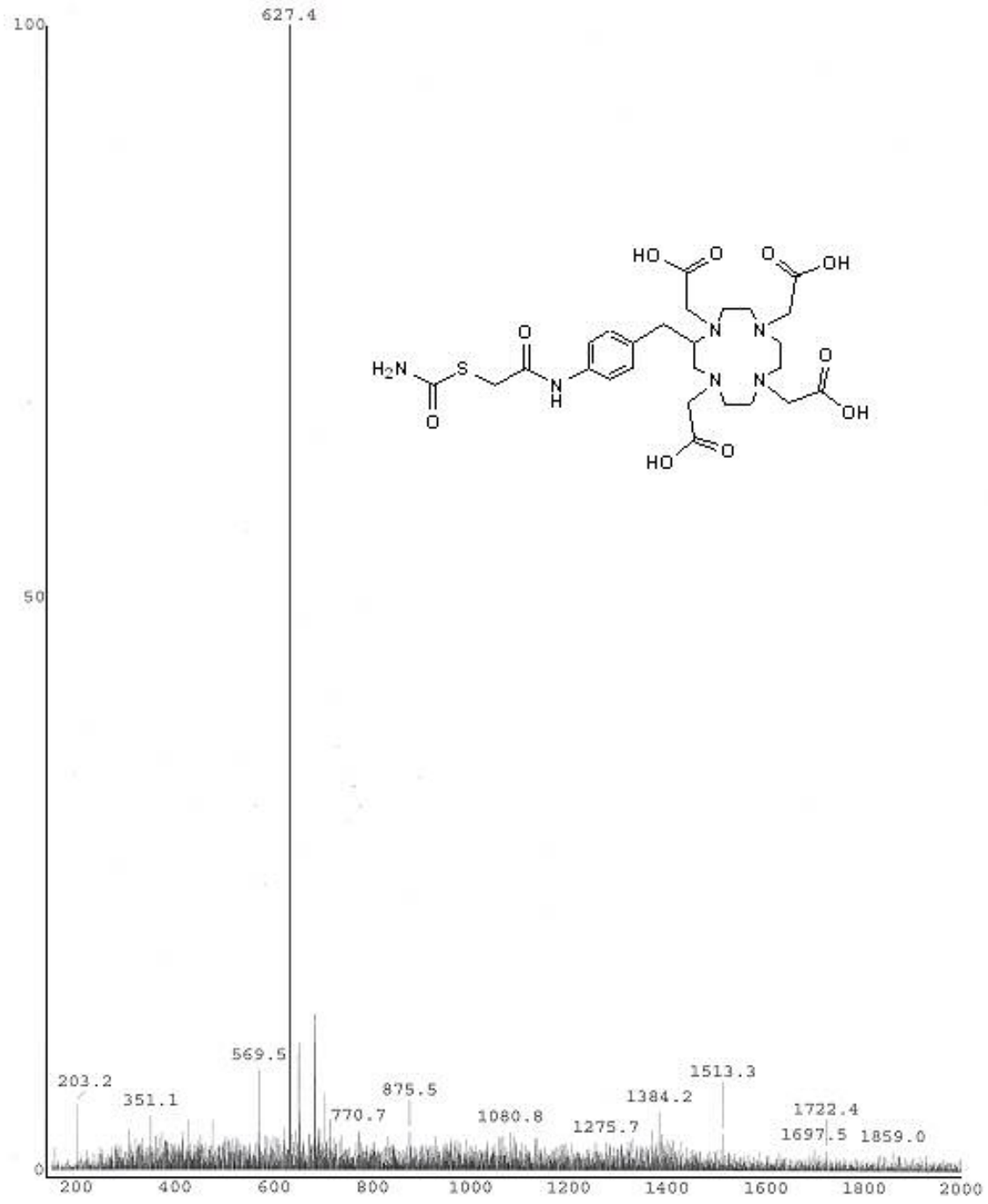


Figure s1: MS of (S)-2-(4-(2-carbamoylsulfanyl-acetylamino)-benzyl)-DOTA (**a2**).

Formation of *(S)-2-(4-(2-mercapto-acetylamino)-benzyl)-DOTA* (Chelate-**1**): The crystals of **a2** were dissolved in a minimal amount of 7M NH_3(aq), and the resulting yellowish

solution was refluxed for 20 min. The solution was immediately put on ice and adjusted to pH 4 with 6 M HCl. Crystals appeared spontaneously and the resulting mixture was kept overnight at -4 ^{0}C. The product was purified by reversed-phase HPLC: solvent A, 0.1 % CF_3COOH in H_2O ; solvent B, 0.075% CF_3COOH in CH_3CN ; 5-30% B, 0-35 min; 30-80% B, 35-45 min; 100-5% B, 45-50 min; product peak, 21 min. MS and NMR spectra are provided in Figures s2 and s3. LC/MS was performed; product eluted at 9.3 min; ESI-MS *m/z* calcd (MH+): 583.30. Found: 583.70.

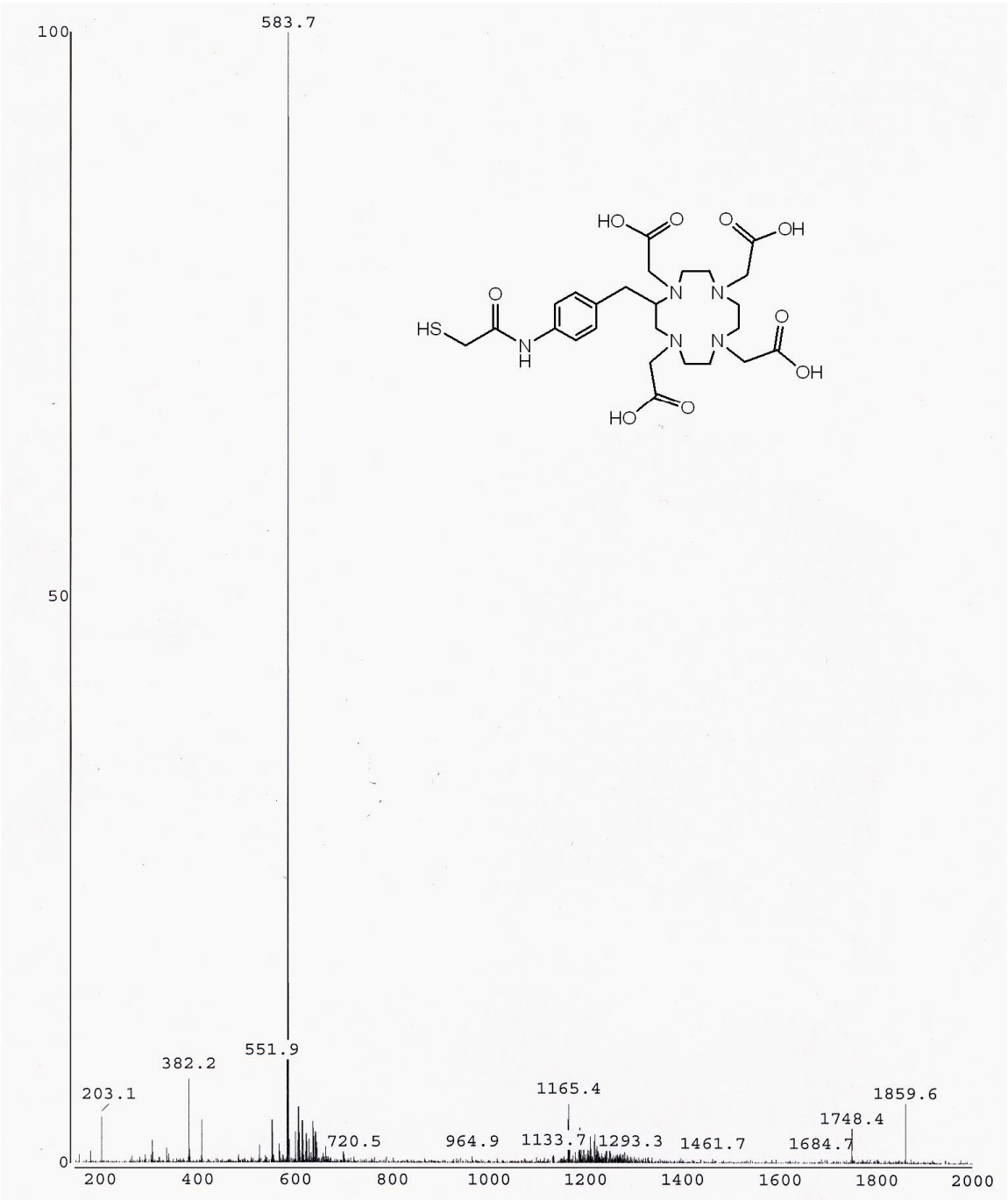


Figure s2: MS of 2-(S)-(4-(2-mercapto-acetylamino)-benzyl)-DOTA (Chelate-**1**) after HPLC purification.

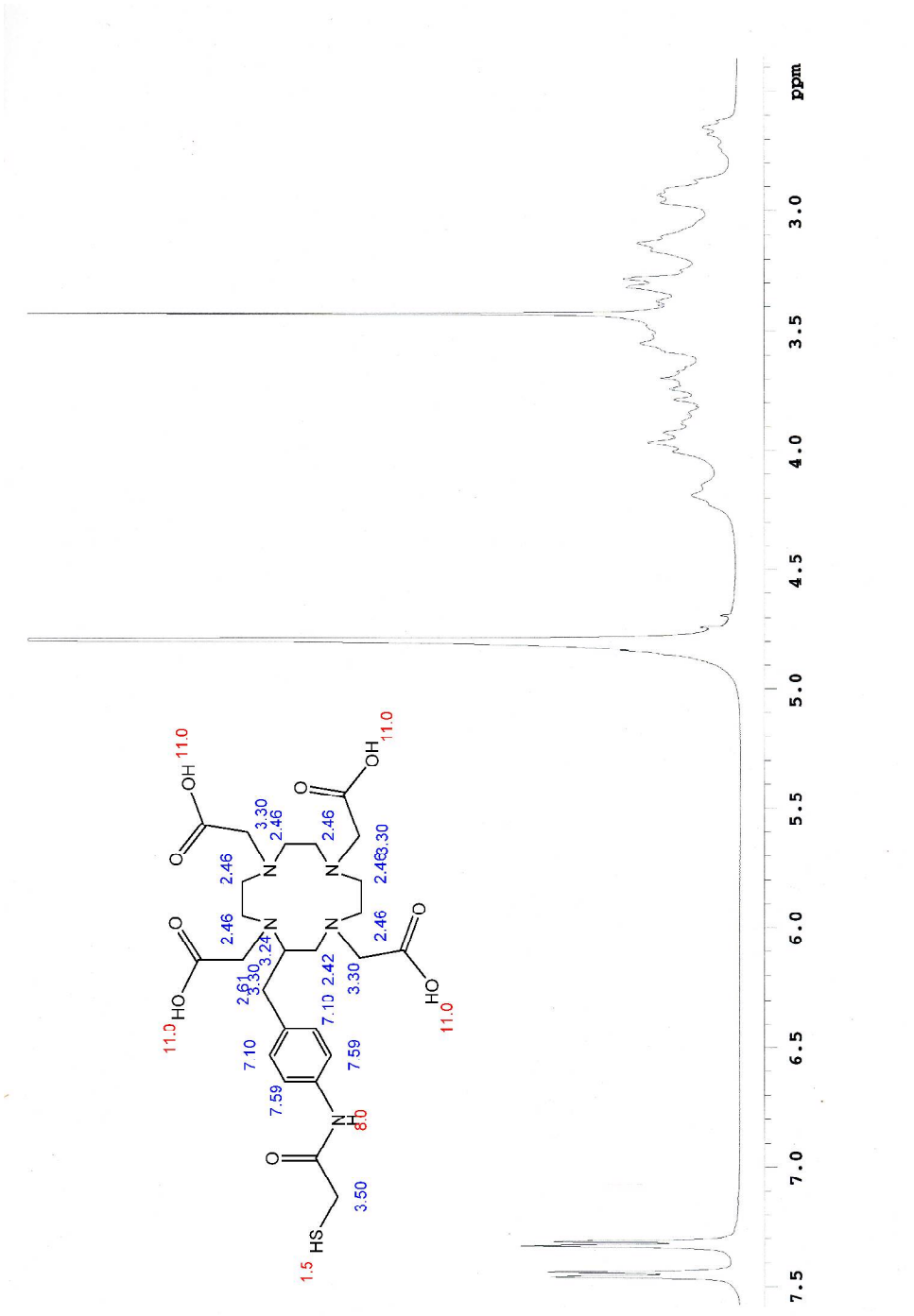


Figure s3: NMR of (S)-2-(4-(2-mercapto-acetylamino)-benzyl)-DOTA(Chelate-**1**) after HPLC purification.

Preparation of *(S)-2-(4-(4-mercapto-butyrimidoyl)-aminobenzyl)-DOTA* (Chelate-**2**). 50 mg (9.8×10^{-2} mmol) of (S)-2-(4-aminobenzyl)-DOTA was dissolved in 10 mL H_2O in a 50mL roundbottom flask. 50 mg (4.9×10^{-1} mmol) of dry 2-iminothiolane (2IT) was immediately added to the solution and allowed to stir overnight at ambient temperature under argon atmosphere. The resulting yellowish solution was lyophilized and characterized by LC/MS. The peak of interest eluted at 15.02 minutes (Figure s4) with ESI-MS *m/z* calcd (MH+): 612.09. Found: 612.20 (Figure s5). NMR of the purified product is shown in Figure s6. TLC analysis showed a single product migrating at R_f = 0.72, with (S)-2-(4-aminobenzyl)-DOTA migrating at R_f = 0.6 using MeOH/0.1M Et_3NHOAc(aq), pH 8.0 (3:1).

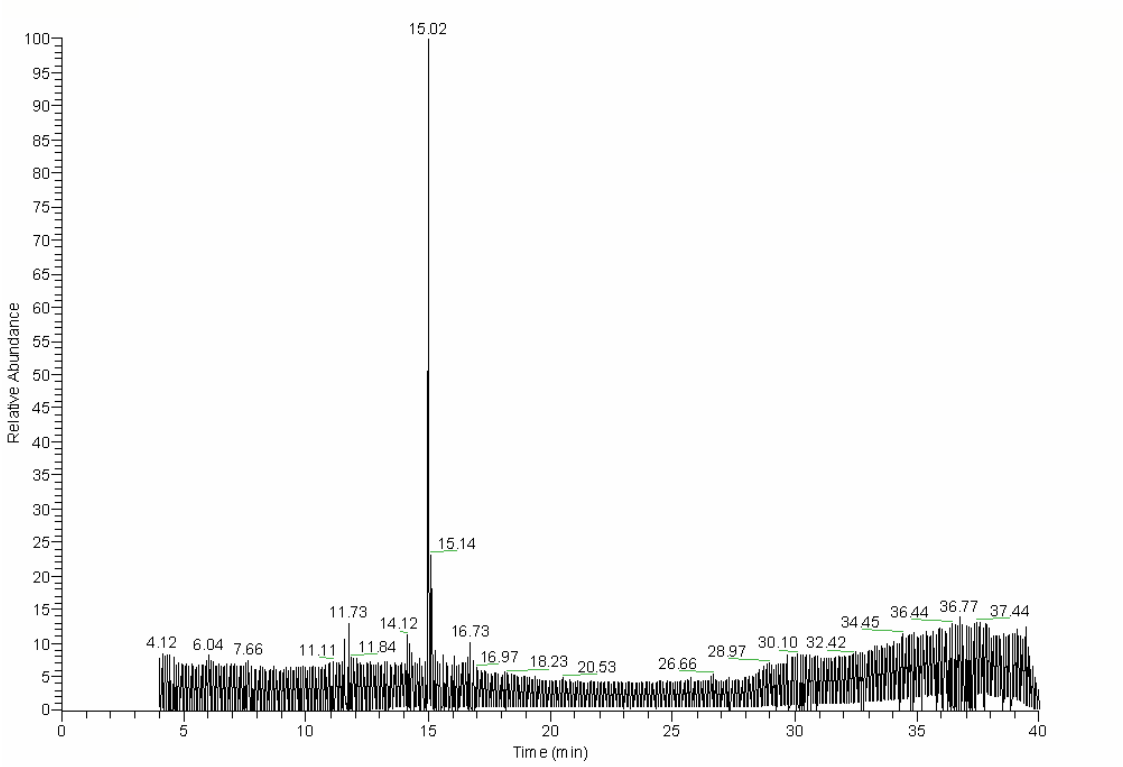


Figure s4: Elution of **2** at 15.02 minutes, monitoring m/z 612.2.

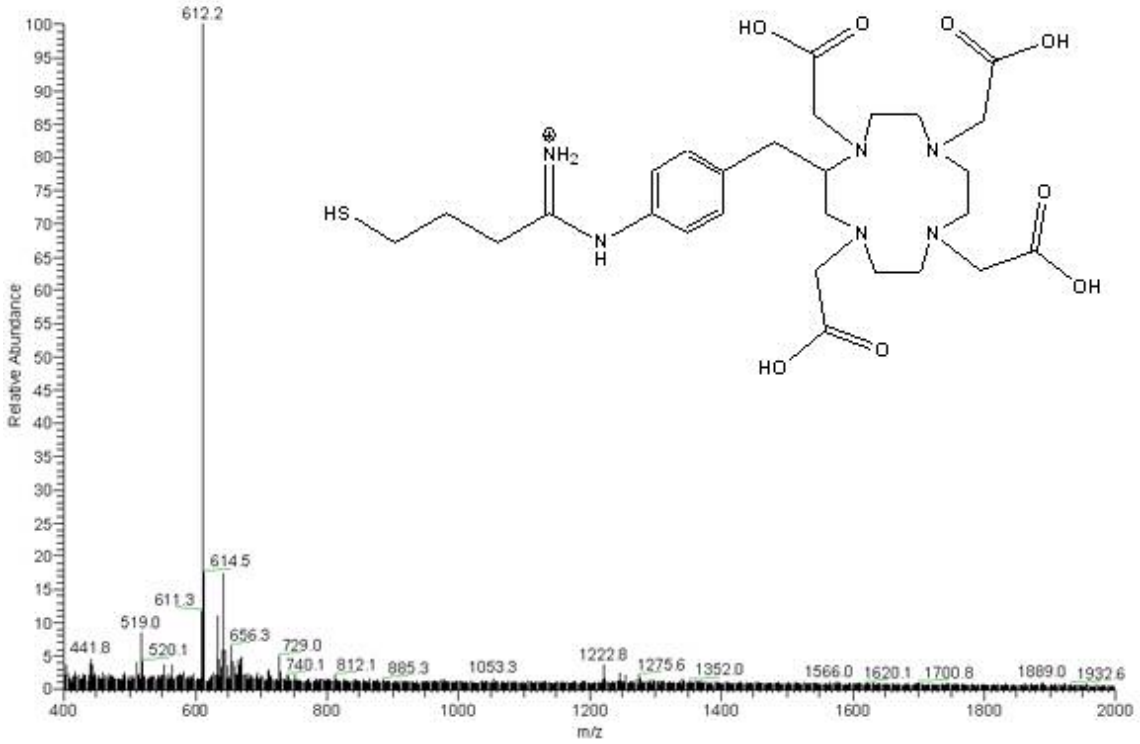


Figure s5: Elution of **2** at 15.02 minutes with the expected mass of 612.2.

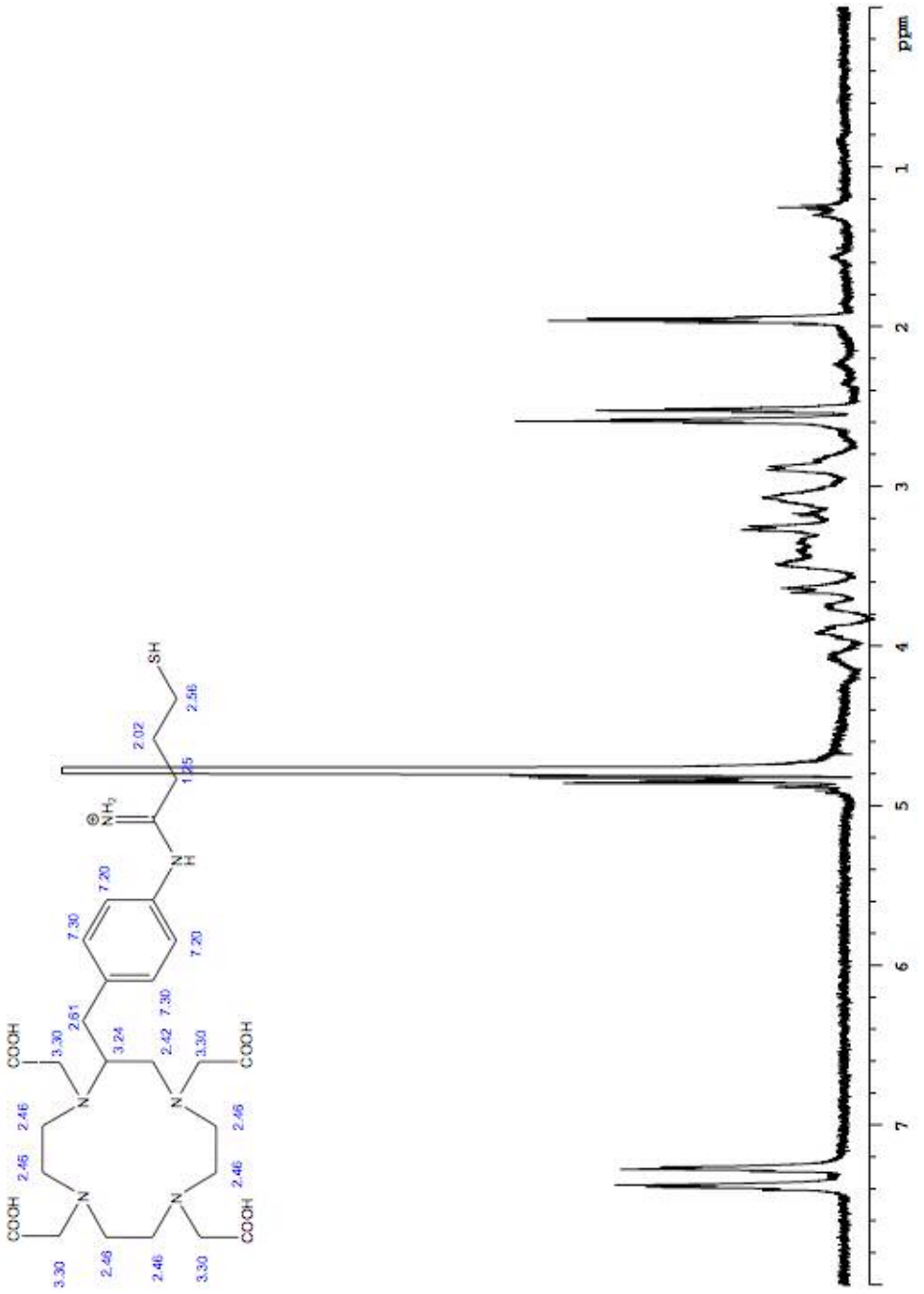


Figure s6: NMR of (S)-2-(4-(4-mercapto-butryimidoyl)-aminobenzyl)-DOTA (Chelate-**2**) after HPLC purification.

Analysis of bond formation of chelate-1 and chelate-2 to antibody 2D12.5 G54C mutants:

All reactions were carried out in metal free eppendorf tubes, and PBS buffer was used throughout unless otherwise noted. Solutions of **1** and **2** were incubated with $^{90}Y^{3+}$ to obtain complexes and characterized as described above. Either solution was used without further purification in the following experiment: 19 µL aliquots of 1.05 µM 2D12.5 G54C Fab in PBS buffer (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) were added to 24 eppendorf tubes. Twelve of these tubes were assigned to ^{90}Y-**1**, and twelve were assigned to ^{90}Y-**2**. At appropriate times, 1 µL of the appropriate ^{90}Y complex was added to each tube, bringing the final Fab concentration to 1 µM and the final chelate concentration to 10 µM, incubated at 37 ^{0}C for the following times in min: 160, 128, 96, 64, 32, 16, 8, 4, 2, 1. After incubation the reaction was stopped by addition of non-reducing SAB and heating at 95ºC for 5 min. Samples were flash frozen in liquid N_2 and stored at -80ºC before SDS-PAGE analysis. The time 0 negative control was addition of SAB to 19 µL of 2D12.5 G54C and heating at 95ºC before addition of 1 µL of ^{90}Y chelate. Each of the time points for ^{90}Y-**1** and ^{90}Y-**2** were analyzed on SDS-PAGE followed by quantitation using a phosphor screen. Results are shown in Figure 2 of the paper.

Analysis of the percentage of covalent bond formation between the cysteinylated 2D12.5 G54C Fab protein and chelate-1 and chelate-3 before and after mild reduction:

All reactions were carried out in metal free eppendorf tubes, and PBS buffer was used throughout unless otherwise noted. Solutions of **1** and **3** were incubated with $^{90}Y^{3+}$ to

obtain complexes and characterized as described above. Either solution was used without further purification in the following experiment: 24 µL aliquots of 2D12.5 G54C Fab before and after mild reduction in PBS buffer (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) was added to 9 eppendorf tubes. 24 µL aliquots of 2D12.5 mAb were made by the same conditions as a negative control. Three of these tubes were assigned to ^{90}Y-**1**, three were assigned to ^{90}Y-**3**, and three were assigned to ^{90}Y-Nitrobenzyl-DOTA (NBD). The protein and chelate final concentration was 5 µM and 50 µM respectively. These were incubated for 30 min at 37ºC before being added to a bio-spin 6 column (Bio-Rad) to remove any unbound chelate. The proteins were then denatured before being run on a SDS-PAGE gel. Covalently bound chelate migrates with the protein, while chelate that was not covalently bound to the protein migrated with the dye front. The gels were imaged by phosphor screen before being analyzed by volume analysis to determine the percentage of covalently bound chelate. The gel images are shown in figures s7-9.

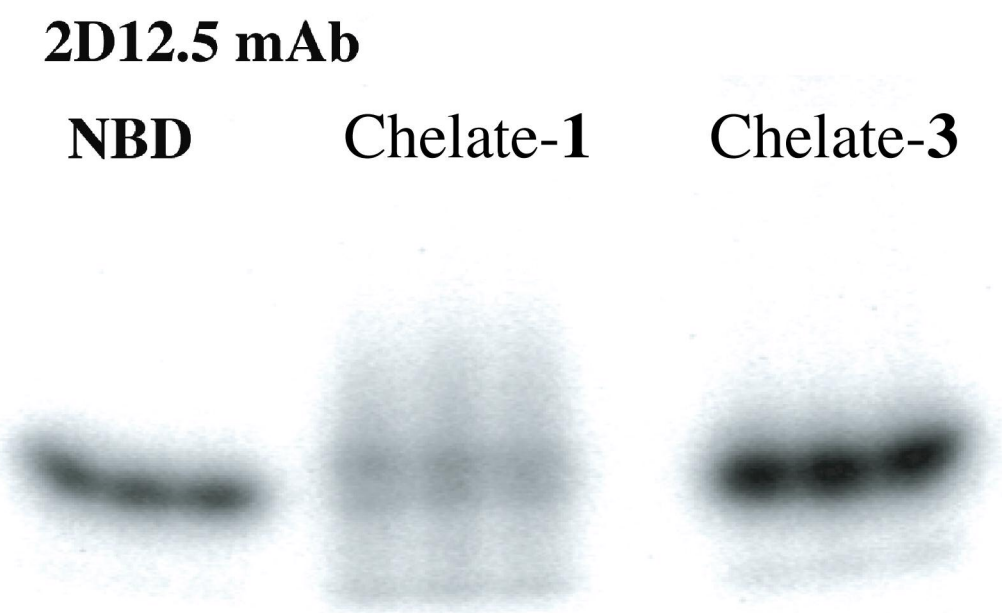


Figure s7 Phosphorimage of a denaturing SDS-PAGE gel of parental 2D12.5 mAb following incubation with Y-90 chelates. None of the chelates covalently bound, so all

move with the dye front. NBD (p-nitro-benzyl-DOTA); chelate-**1**; chelate-**3**.

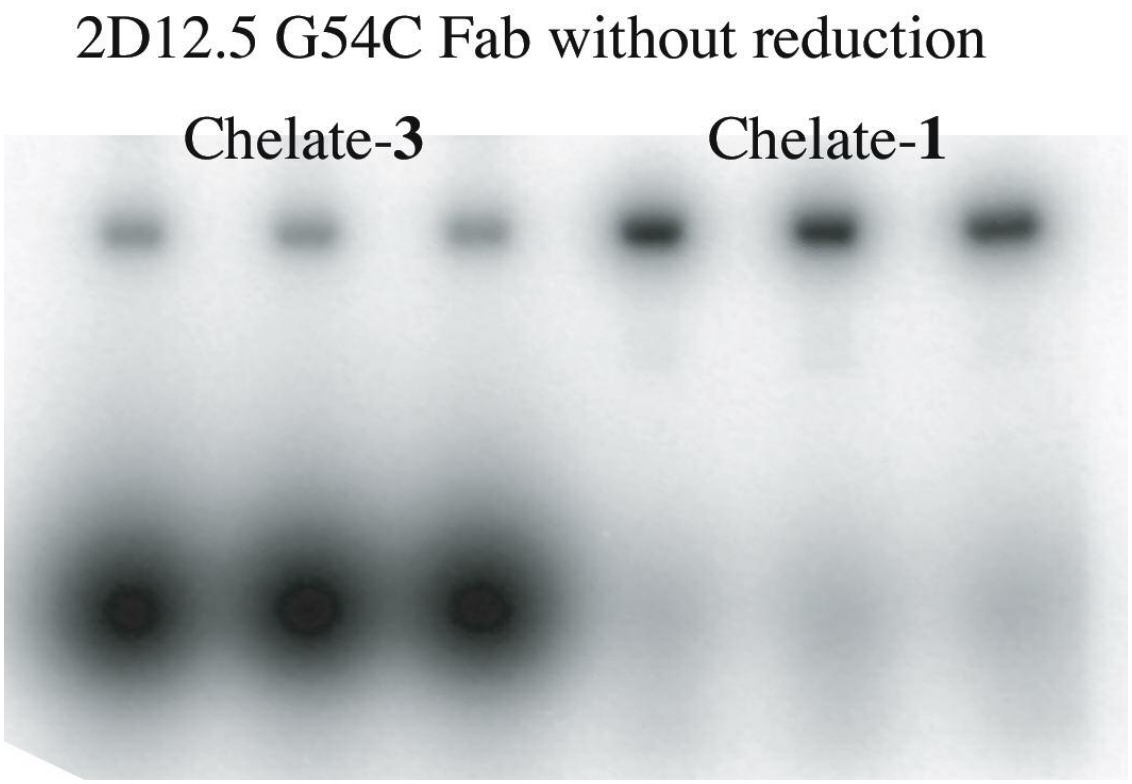


Figure s8. Phosphorimage of a denaturing SDS-PAGE gel of 2D12.5 G54C Fab (without prior treatment with DTT) following incubation with Y-90 chelates **1** and **3**. The bands at the top are the chelates migrating with the protein (those that are covalently bound). The bands at the bottom are the free chelates that migrate with the dye front (not covalently bound to the protein). Volume analysis showed that 7.6 ± 0.1 % of chelate-**3** and 83 ± 4 % of chelate-**1** was covalently bound to the protein.

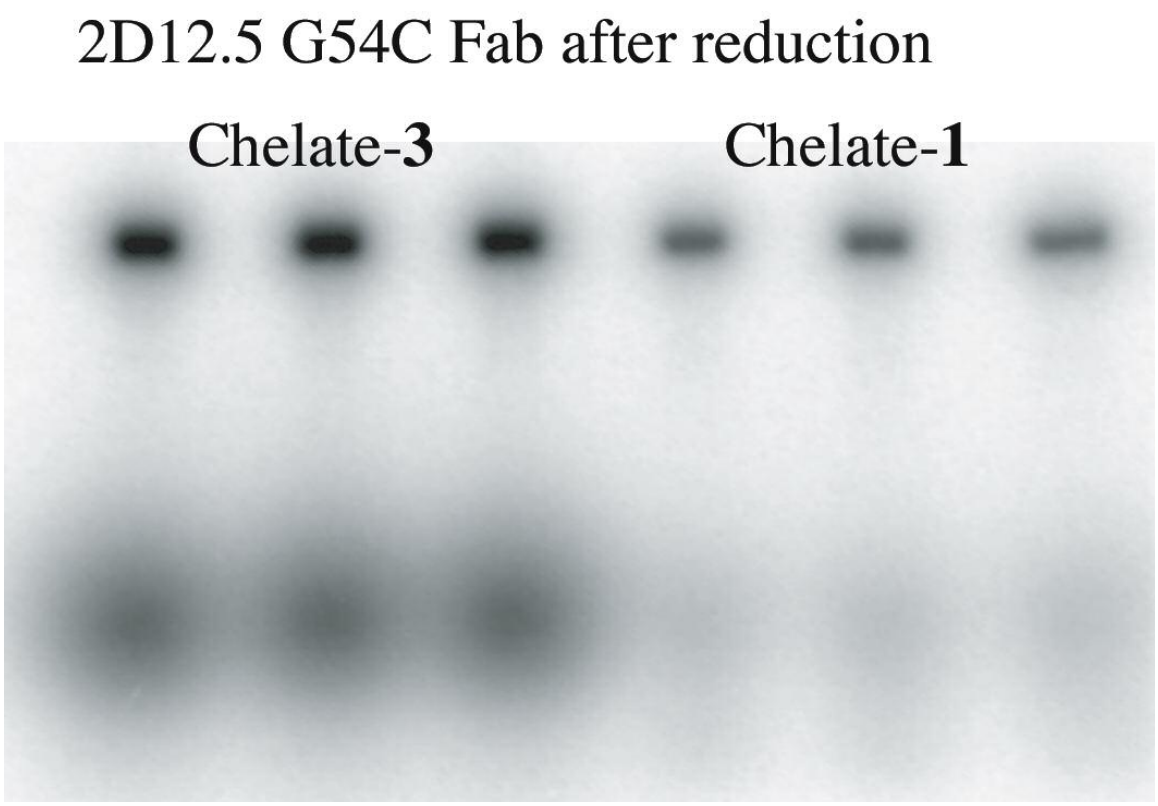


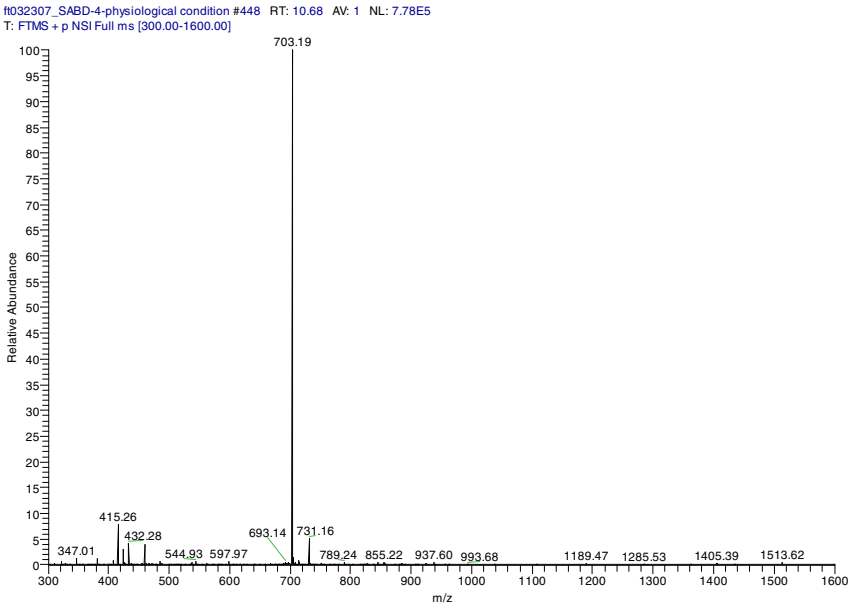
Figure s9. Phosphorimage of a denaturing SDS-PAGE gel of 2D12.5 G54C Fab that had been prepared by treatment with DTT before incubation with Y-90 chelates. Volume analysis showed that 47 ± 1 % of chelate-**3** and 77 ± 5 % of chelate-**1** was covalently bound to the protein. The reason that chelate-**3** was able to have such a high efficiency of covalent binding to the protein is that after metallation 29% of the chelate is in the disulfide form, allowing it to bind to G54C in the free sulfhydryl form.

Determination of the specificity of binding of chelate-1 with the 2D12.5 G54C Fab:

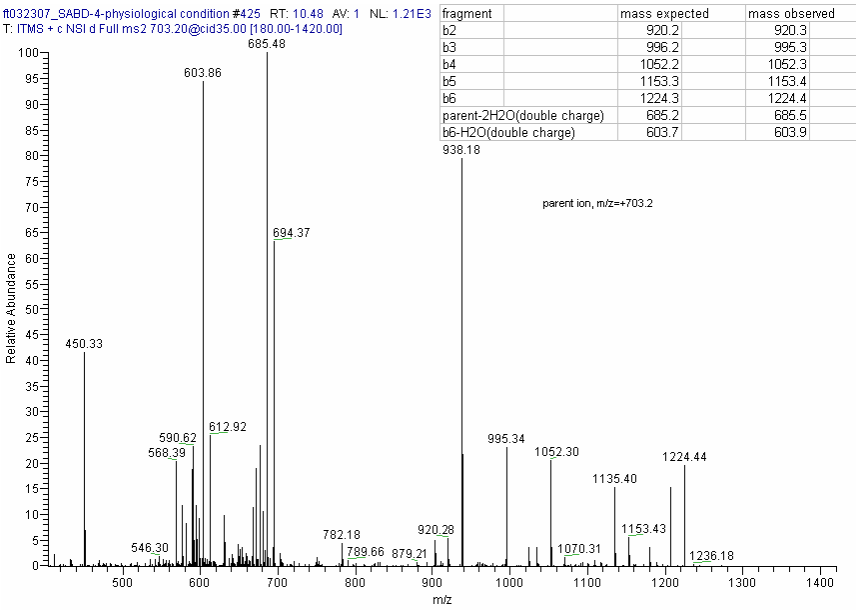
1 µl of 1.4mM **1**-Tm (14 µM final concentration) was added to 100 µl of 20µM un-deblocked Fab-G54C in sodium phosphate buffer (pH7.4) supplemented with 47µM cystine, 9µM cysteine, 0.05 µM oxidized glutathione and 2 µM of reduced glutathione (to mimic the redox equilibrium of human serum). The eppendorf tubes were flushed with argon and incubated at 37 °C for 2 h. Unconjugated **1**-Tm was removed using a bio-spin-6 column (Bio-Rad). The **1**-tagged Fab was buffer exchanged to 5M urea supplemented with 125mM Tris-HCl, pH8, which was then incubated at 55 ºC for 45 min to denature the protein. The solution was cooled down to room temperature before addition of 4µl of chymotrypsin (25µg/20µl) was added before being incubated overnight at RT. Then PMSF was added to 1mM final concentration before the **1**-Tm tagged peptide was purified with 2D12.5 mAb column according to previous published protocol [2, 6]. **1**-Tm tagged peptide was eluted with 0.4%TFA in 50% acetonitrile. Solvent was removed by speed-vac. The sample was analyzed by LC-MS/MS to observe the **1**-Tm tagged peptide. The results are shown in figure s10.

Figure s10. FTMS and MS/MS of **1**-Tm tagged peptide

A



B



fragment	mass expected	mass observed
b2	920.2	920.3
b3	996.2	995.3
b4	1052.2	1052.3
b5	1153.3	1153.4
b6	1224.3	1224.4
parent-2H2O(double charge)	685.2	685.5
b6-H2O(double charge)	603.7	603.9

C

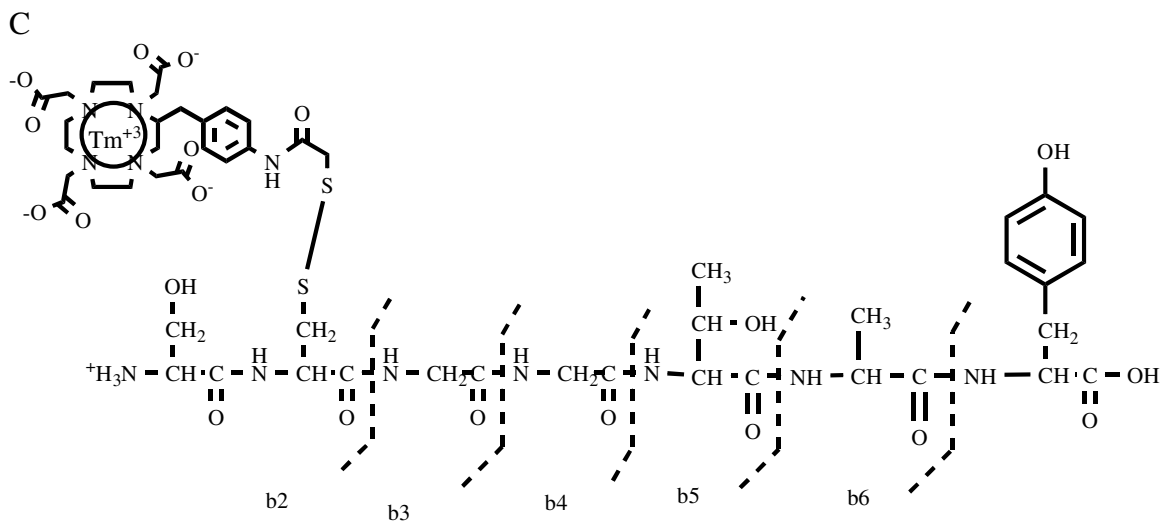


Figure s10. The Fab G54C that was treated with **1**-Tm before chymotrypsin digestion followed by a 2D12.5 mAb column purification before analysis with (A) LC/FTMS and (B) MS^2 of **1**-Tm. (C) MS^2 analysis confirmed that the sequence of the peptide tagged by **1**-Tm matches with Fab G54C mutation site fragment sequence SCGGTAY, as expected.

Amino acid analysis of the Fab-G54C blocking residue

1mg/ml of non-reduced Fab-G54C was dialyzed against water and concentrated to 2mg/ml by centrifuge ultrafilter. The filtrate of non-reduced Fab-G54C was saved as a negative control. DTT and Mops were added to 1ml of both Fab-G54C and filtrate of non-reduced Fab-G54C to final concentrations of 4mM and 2.5mM. The solution was incubated at RT overnight. Protein was separated from blocking residue by centrifuge ultrafilter. The filtrate of the reduced Fab-G54C was saved (sample). The volume of filtrate of the reduced Fab-G54C and the filtrate of non-reduced Fab-G54C (with both DTT and Mops in it) were reduced to about 100µl by speed-vacuum. Samples were snap frozen and kept at -80 °C. Preparation for amino-acid analysis by performic acid (PO)

oxidation and HCl hydrolysis: 30µl of the above sample was removed and put in a hydrolysis tube; 1ml fresh PO solution (made by adding 9ml of formic acid to 1ml of 30% hydrogen peroxide and incubating at 0 °C for 1h) was added and the mixture allowed to stand overnight, after which it was dried. Cysteine was oxidized to cysteic acid during this process *(7, 8, 9)*. Liquid phase hydrolysis (200µl 6N HCl/0.1% phenol at 100 °C for 24 h) was performed, and the sample dried. Samples were taken up in 75 µl of 50 mM citrate buffer pH 2.2 with 150 mM sodium chloride with 100 nmol/mL of AE-Cys as internal standard. The samples were then vortex mixed, and centrifuged. 50 µl was loaded to L-8800 Hitachi analyzer with "post-column" ninhydrin reaction detection system (figure s11).

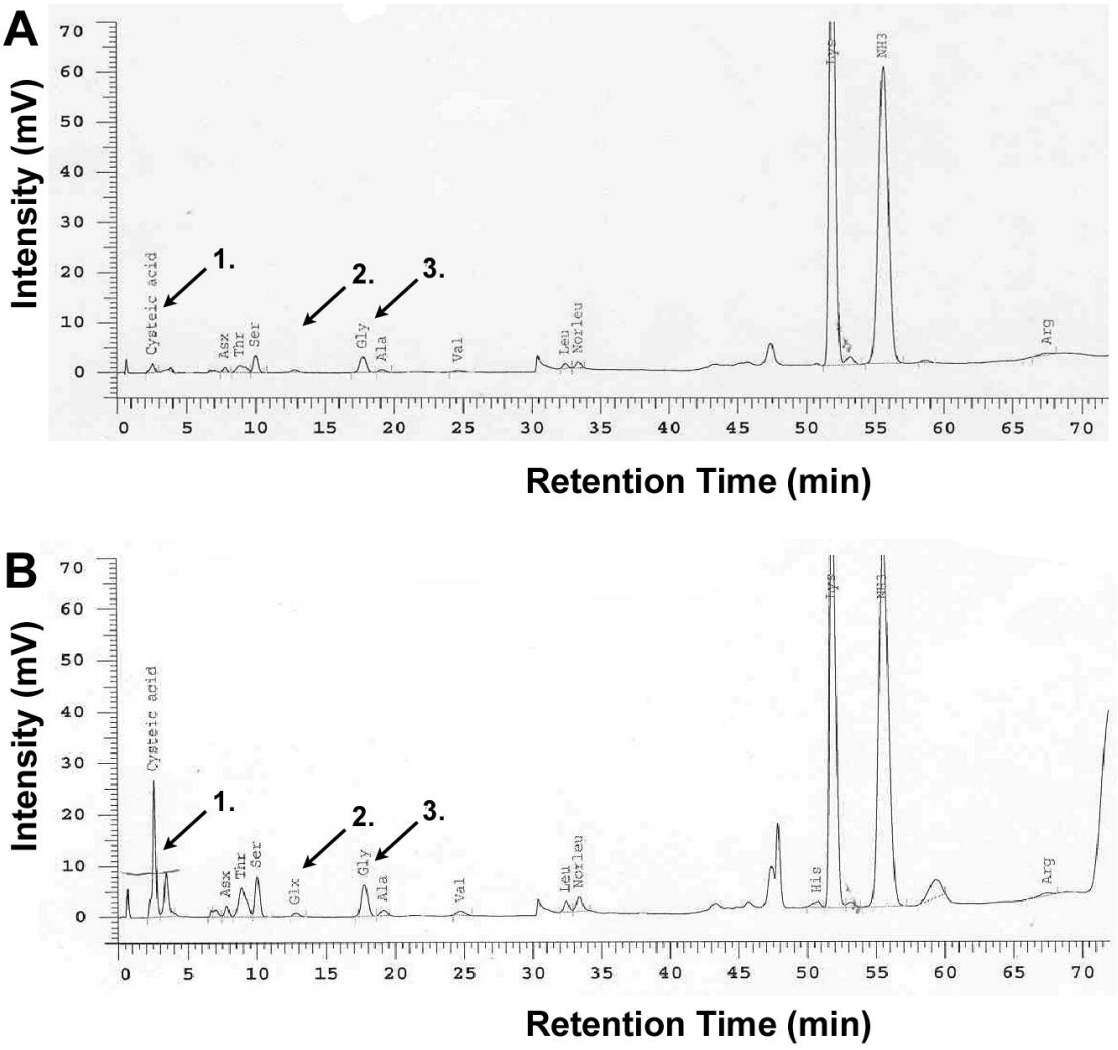


Figure s11: Amino acid analysis of Fab-G54C blocking group. A: amino acid analysis of the filtrate of non-reduced Fab-G54C. B: amino acid analysis of the filtrate of reduced Fab-G54C. There was a significant amount of cysteic acid (arrow 1) present in B: 0.60 nmol, compared with 0.058 nmol in A. The absence of similar intensities for glycine (arrow 3) and glutamic acid (arrow 2) showed that the blocking residue is not glutathione.

Determination of the stability of the disulfide bond between chelate-2 and the 2D12.5 G54C Fab:

1 μM 2D12.5 G54C Fab in PBS was incubated for 1.5 hour at 37 ºC with 10 μM Y-**2**. Afterwards 5 μL of varying concentrations of β-mercaptoethanol (BME) was added to 75 μL of chelate-**2**-2D12.5 Fab solution with the final concentration for the BME being the following: 0, 3.2 μM, 10 μM, 32 μM, 0.1 mM, 0.32 mM, 1 mM, 3.2 mM, 10 mM, 32 mM, and 100 mM. All samples were then incubated for 1.5 h at 37 ºC. Afterwards excess BME and **2** were removed by a Bio-spin 6 desalting column (Bio-Rad) according to the manufacturer's protocols. The recovered samples were immediately denatured by addition of SAB before being analyzed by denaturing (non-reducing) gel electrophoresis. The samples were analyzed by a sypro-ruby total protein stain (Bio-Rad) or a western blot with the primary antibody being a biotin-labeled 2D12.5 mAb and the secondary conjugate being streptavidin-HRP. The western blot was visualized by a west dura chemiluminescent substrate (Pierce). The gel and blot are shown in Figure 3 of the paper. Quantitation of the intensity of the bands on the sypro gel and western blot was performed and plotted vs. the concentration of BME (figure s12). The reduced Fab and non-reduced Fab values were obtained from the quantitation of the 25kD and 50kD band respectively in the total protein stained gel. The DOTA values were from the sum of these bands in the western. The most intense band on the gel or western was set as 100% with 0% being set from the intensity in a blank lane. The maximum for the reduced Fab and the minimum for the non-reduced Fab for the regression analysis were set from the analysis of the other sample set. The minimum for the DOTA sample set was obtained from the analysis of a 0.5 M DTT treated lane. The calculated EC50 of the nonlinear regression analysis is 0.01 M (95% confidence limits 0.008 to 0.02) for the reduced and

non-reduced Fab, while the estimated EC50 of the DOTA is 10× larger at ≈0.1 M (95% confidence limits 0.08 to 0.2). The difference between the two is significant, indicating that the disulfide between Y-**2** and the Fab is stronger than the interchain disulfide.

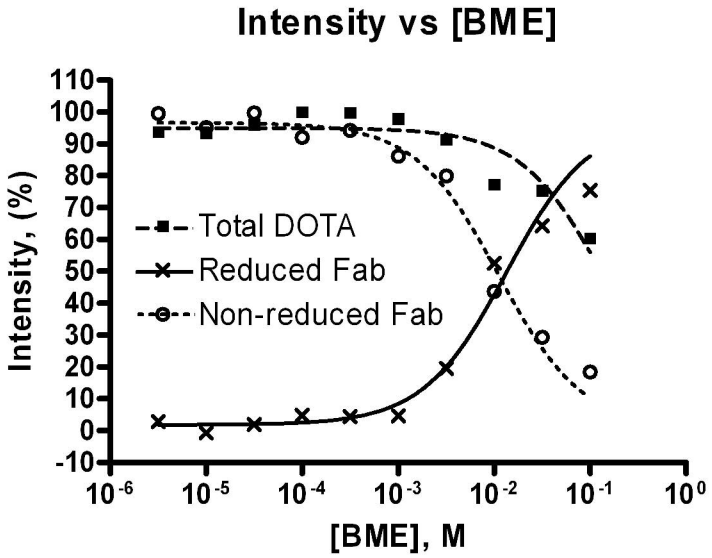


Figure s12. Graph showing the percent intensity vs. the concentration of BME. The percent intensity was obtained by analyzing the intensity of the bands on the non-reducing sypro total protein stain PAGE gel and anti-DOTA western blot shown in Figure 3 of the publication. Nonlinear regression was performed using the PRISM software package.

References for Supporting Information:

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