Supporting Information

Unambiguous Identification of Pyroglutamate in Full-Length Biopharmaceutical Monoclonal Antibodies by NMR Spectroscopy

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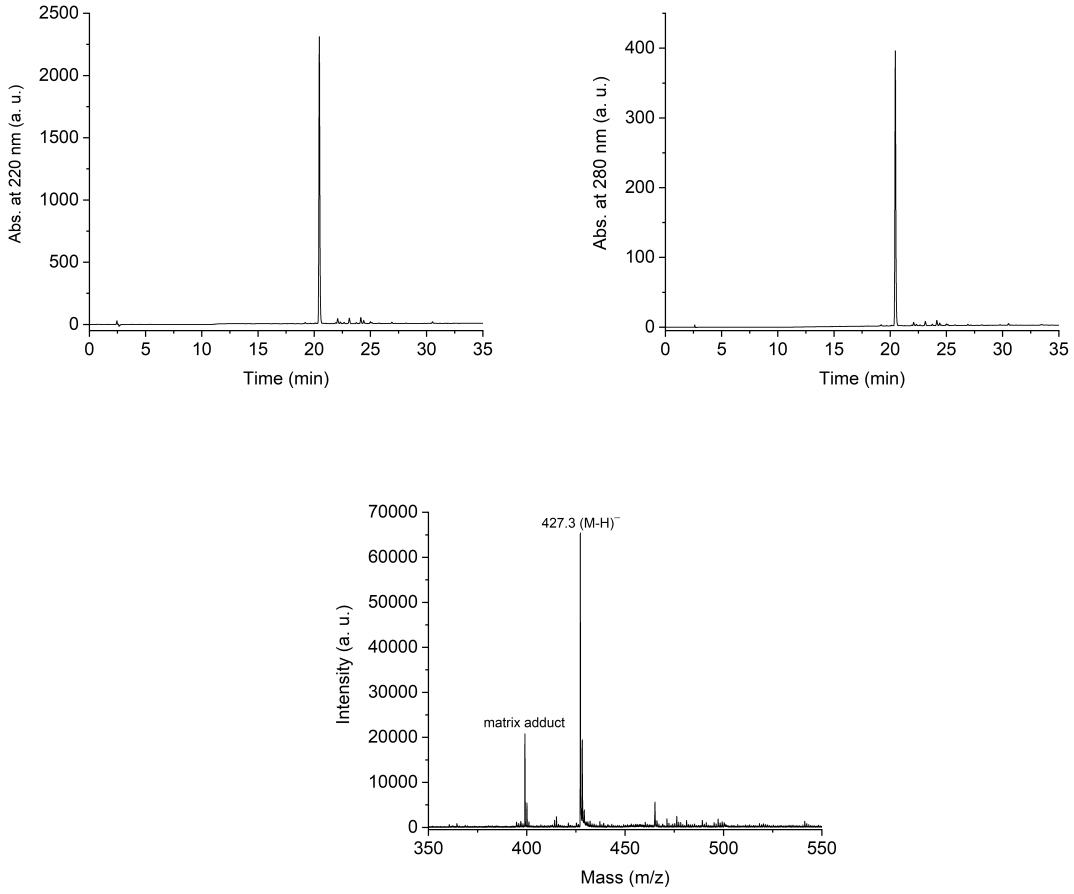

Figure S1: Analytical characterization of the reference peptide used to assess the detection limit (pGlu-G-G-W-NH_2). RP-HPLC profiles recorded at 220 nm (left) and 280 nm (right): area percentage of the peak at 20.4 min: 93 %. (Bottom) MALDI-TOF-MS. The peptide (1 mg/ml) was dissolved in ACN/H_2O (25:75, v/v) containing 0.1% TFA and mixed with a saturated solution of the matrix α-cyano-4-hydroxycinnamic acid in ACN/MeOH (50:50, v/v) (peptide/matrix 2:1 (v/v)). A 5 μl drop was deposited on the stainless steel sample plate and let dry on air. The MS measurement was performed in negative mode: M_{theor} 428.45 Da, $(M-H)^-_{found}$ 427.3 Da (the MS peak at 399.1 Da corresponds to a matrix adduct: $[(C_{10}H_6NO_3^-)\cdot(C_{10}H_6NNaO_3)]$.

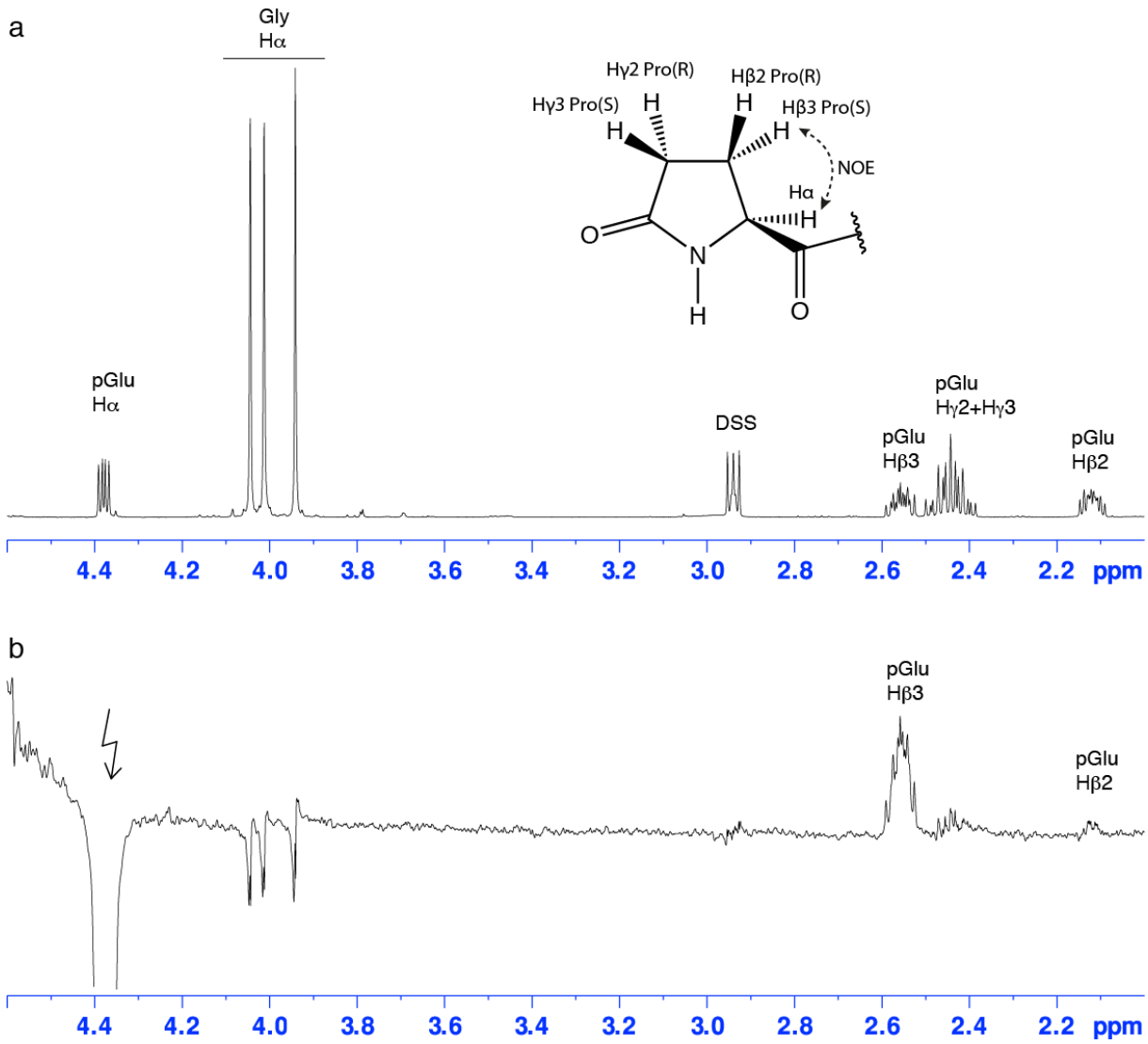

Figure S2: 1D steady-state NOE difference spectrum for the stereo chemical assignment of Hβ2 and Hβ3 of pGlu. The reference peptide pGlu-G-G-G-NH_2 was measured under denaturing conditions at a pH of 7.4. a) Standard 1D ^{1}H spectrum of the reference peptide. b) 1D ^{1}H steady-state NOE difference spectrum at the irradiation frequency of pGlu Hα. The integral of the pGlu Hβ3 signal is much higher than the Hβ2 signal, which indicates that Hα is closer to Hβ3 than to Hβ2.

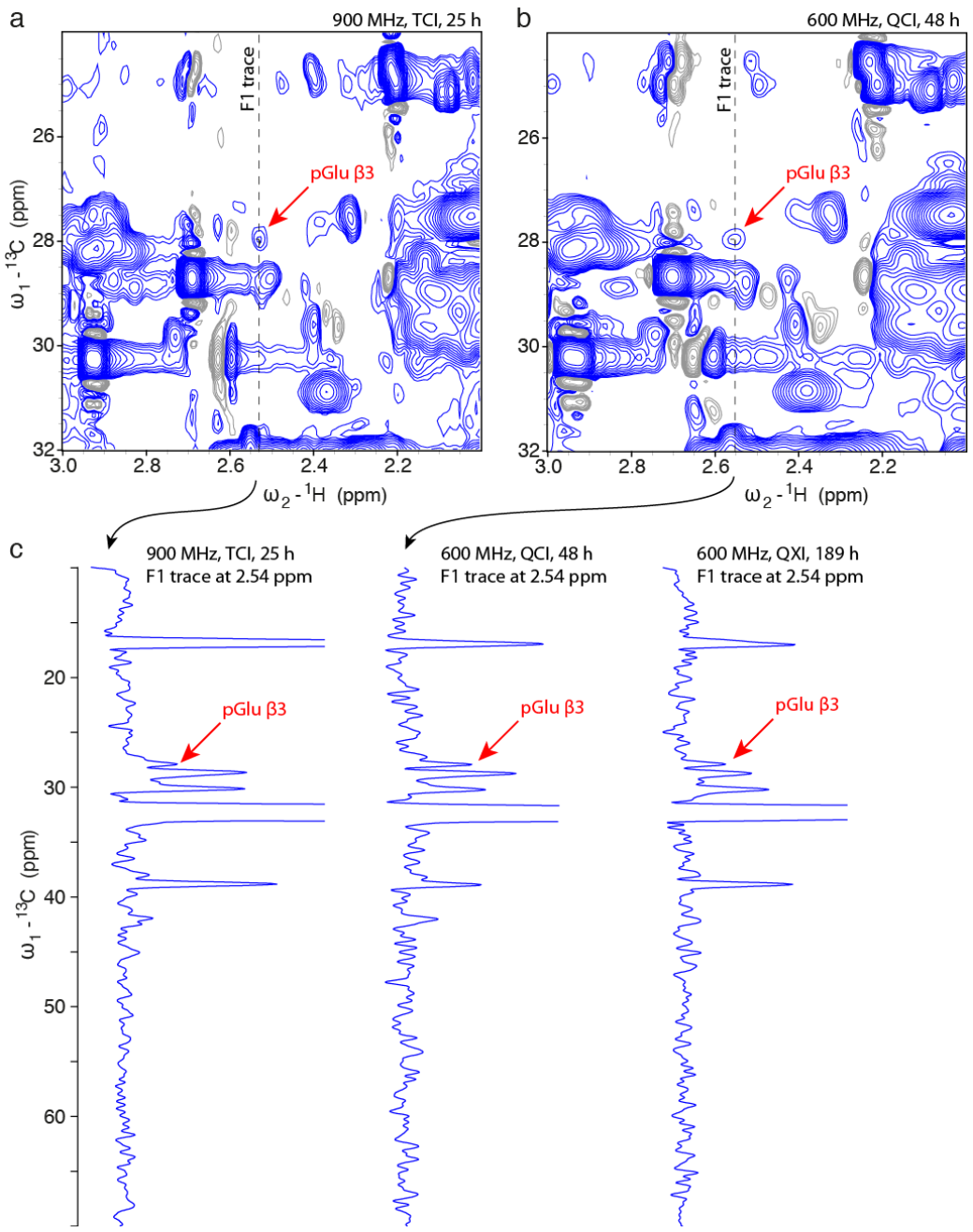

Figure S3: Estimation of the detection limit in ^{1}H-^{13}C HSQC correlation spectra using independent adalimumab samples (220 µM) doped with a peptide containing pGlu (55 µM). a) ^{1}H-^{13}C HSQC spectrum measured at a 900 MHz spectrometer with a cryogenic probe with 120 scans (total measurement time 25 h) using sample 4 (Table S2). Acquisition parameters: d1: 2 s, td: 1024×180 complex points. Positive contour lines are shown in blue and negative ones in gray. b) Comparable spectrum recorded at a 600 MHz spectrometer with a cryogenic probe with 240 scans (total measurement time 48 h) using sample 2 (Table S2). Acquisition parameters: d1: 2 s, td: 512×170 complex points. c) ^{13}C-1D slices at 2.54 ppm of comparable spectra measured at 3 spectrometers using 3 independent samples (Table S2).

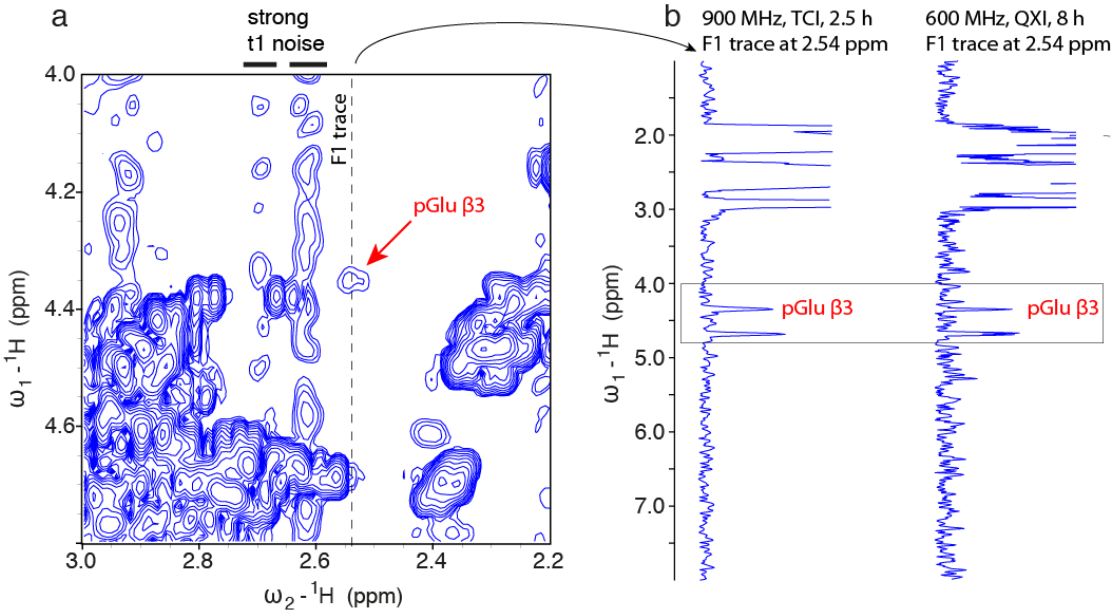

Figure S4: Estimation of the detection limit in ^{1}H-^{1}H COSY spectra using humira samples (220 µM) doped with a peptide containing pGlu (55 µM). a) ^{1}H-^{1}H COSY spectrum measured at a 900 MHz spectrometer with a cryogenic probe with 8 scans (total measurement time 2.5 h) using sample 4 (Table S2). Acquisition parameters: d1: 2 s, td: 1024×256 complex points. b) ^{1}H slices of the indirect dimension at 2.54 ppm of comparable spectra measured at 2 spectrometers using 2 independent samples (Table S2).

Table S1: 2D NMR quantification of pGlu in 3 independent samples of the same batch of rituximab (MabThera). The mAb concentration was 220 μM (volume: 500 μl).

	pGlu residues per mAb	
	reference to Arg Cδ-Hδ	**reference to Lys Cε-Hε**
Measurement 1	4.35	4.34
Measurement 2	4.48	4.53
Measurement 3	3.87	3.85
Arithmetic mean	**4.23**	**4.24**
Standard deviation	**0.32**	**0.35**

Table S2: Estimation of the detection limit at 3 different spectrometer setups measuring independent samples (220 μM humira, 55 μM pGlu peptide). Given are the measurement times and the signal-to-noise ratios of the characteristic signals as calculated by sparky (Hβ3/Hα correlation for ^{1}H-^{1}H COSY and Hβ3-Cβ correlation for ^{1}H-^{13}C HSQC). Some exemplary spectra are displayed in Figures S3 and 4.

		Spectrometer		
Sample	**Experiments**	**600 (QXI RT probe)**	**600 (QCI cryo probe)**	**900 (TCI cryo probe)**
1	^{1}H-^{13}C HSQC	Time: 189 h (S/N: 8)	-	-
2	^{1}H-^{13}C HSQC	-	Time: 48 h (S/N: 10)	-
3	^{1}H-^{13}C HSQC	-	Time: 48 h (S/N: 9)	-
4	^{1}H-^{13}C HSQC	-	-	Time: 25 h (S/N: 12)
1	^{1}H-^{1}H COSY	Time: 8 h (S/N: 7)		
4	^{1}H-^{1}H COSY			Time: 2.5 h (S/N: 7)