Supporting Information

Identification and quantification of oxidation products in full-length biotherapeutic antibodies by NMR spectroscopy

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Supplementary Tables

Table. S1. NMR quantification of induced Met(O) in rituximab and adalimumab using ^{1}H-^{13}C HSQC spectra measured at 25°C in 7 M urea-d_4 in D_2O. Both mAbs were incubated with 0.35% H_2O_2 for 30 min at RT.

	Rituximab			Adalimumab		
	Occurrence[a]	Volume (×10^8)	Err %[e]	Occurrence[a]	Volume (×10^8)	Err %[e]
Met(O) Cε/Hε	12×	8.14[b] ± 0.37[d]	4.5	10×	9.0 [b] ± 0.2[f]	2.2
Met Cε/Hε		36.3[b] ± 0.73[d]	2.0		38[b] ± 2[d]	5.3
Ala Cβ/Hβ	80×	297[c] ± 7.9[d]	2.7	78×	363[c] ± 2[d]	0.6
Ile Cδ1/Hδ1	28×	102[c] ± 2.9[d]	2.3	30×	138[c] ± 2[d]	1.4
Ile Cγ2/Hγ2		107[c] ± 5.6[d]	5.3		137[c] ± 5[d]	3.6
Leu Cδ1/Hδ1	90×	337[c] ± 1.9[d]	0.6	104×	500[c] ± 3[d]	0.6
Leu Cδ2/Hδ2		335[c] ± 13[d]	3.9		501[c] ± 9[d]	1.8
Met(O) Cγ/Hγ	12×	5.1[b] ± 6.0[d]	13.0	10×	5.1[c] ± 0.5[d]	9.8
Arg Cδ/Hδ	28×	73[c] ± 1.0[d]	1.4	42×	140[c] ± 3[d]	2.1
Leu Cβ/Hβ	90×	203[c] ± 23[d]	11.4	104×	306[c] ± 16[d]	5.2
Lys Cε/Hε	98×	28[cc] ± 15[d]	0.5	88×	305[c] ± 5[d]	1.6

[a] in the mAb dimer; [b] Gaussian fit for integration; [c] integration over a defined box; [d] maximal deviation between the different integration methods (eqn. 1); [e] error in percentage, determined by dividing the absolute error by the volume; [f] maximal deviation between several integrations of the same integration method.

Table S2. Peptides synthesized by solid phase synthesis, containing methionine sulfoxide (Met(O)), kynurenine (Kyn), 5-hydroxy tryptophan (5HTP) and oxindolylalanine (Oia).

Number	Peptide sequence	M_{found} (M_{calcd}) / Da	t_R /min	Purity / %
1	Ac-$(Gly)_2$-Met(O)-$(Gly)_2$-NH_2	433.31[a,b] (434.16)	6.0[e]	91
2	Ac-$(Gly)_2$-Met-$(Gly)_2$-NH_2	417.09[a,b] (418.16)	16.0[e]	91
3	Ac-$(Gly)_2$-Met(O)-Ala-Gly-NH_2	447.17[a,b] (448.17)	7.2[e]	91
4	Ac-$(Gly)_2$-Met(O)-Pro-Gly-NH_2	473.09[a,b] (474.19)	14.0, 14.4[e]	91
5	Ac-$(Gly)_2$-Kyn-$(Gly)_2$-NH_2	478.03[c,b] (477.20)	17.4[f]	93
6	H-Gly-5HTP-$(Gly)_3$-NH_2	448.18[c,d] (447.19)	14.5[f]	80
7	Ac-$(Gly)_2$-Oia-$(Gly)_2$-NH_2	489.91[c,b] (489.20)	17.2, 17.7[f]	72[g]
8	Ac-$(Gly)_2$-Trp-$(Gly)_2$-NH_2	473.69[c,b] (473.20)	19.7[f]	91

[a] Negative ion mode MALDI-TOF-MS. [b] Matrix: HCCA. [c] Positive ion mode MALDI-TOF-MS. [d] Matrix: DHAP. [e] HPLC gradient: 1% B for 8 min, 1-50% B over 35 min. [f] HPLC gradient: 3% B for 8 min, 3-60% B over 35 min. (A) 0.06% (v/v) TFA in water, and (B) 0.05% (v/v) TFA in ACN. [g] At least 10-15% Kyn in place of Oia was quantified by HPLC and NMR.

Table S3. Chemical shifts of the reference peptides Ac-Gly-Gly-Met(O)-Gly-Gly-NH_2 and Ac-Gly-Gly-Met-Gly-Gly-NH_2 in 7 M urea-d4 in D_2O at two pH values (2.3 and 7.4) and referenced to internal DSS.

	Ac-Gly-Gly-Met(O)-Gly-Gly-NH_2		Ac-Gly-Gly-Met-Gly-Gly-NH_2	
	pH 2.3	pH 7.4	pH 2.3	pH 7.4
	Met(O)	Met(O)	Met	Met
Cα	55.5/55.3[a]	55.5/55.2[a]	55.7	55.6
Cβ	27.1	27.0	32.9	32.9
Cɣ	51.6	51.5	32.3	32.2
Cε	39.5	39.4	17.0	16.9
Hα	4.556/4.568[a]	4.562/4.568[a]	4.524	4.523
Hβ2[b]	2.328	2.326	2.146	2.144
Hβ3[b]	2.178	2.176	2.030	2.026
Hɣ2[b]	2.958	2.954	2.622	2.620
Hɣ3[b]	2.917	2.918	2.541	2.540
Hε	2.717	2.716	2.109	2.108

[a] Two sets of chemical shifts due to the diastereotopic effects of the chiral, tetrahedral sulfoxide group

[b] Not stereochemically assigned, the lower value was tentatively assigned to Hβ3 or Hɣ3.

Table S4. Random coil chemical shifts of tryptophan oxidation products measured at 25°C in 7 M urea-d_4 in D_2O and referenced to internal DSS.

Atom[a]	Oxindolylalanine (Oia)				Kynurenine (Kyn)		5-Hytroxytryptophan (5HTP)		N-Formylkynurenine (NFK)				Hydroxypyrroloindole (HPI)			
	A		B						*Trans*[b]		*Cis*[b]		*Trans*[c]		*Cis*[c]	
	pH 2.3	pH 7.4	pH 2.3	pH 7.4	pH 2.3	pH 7.4	pH 2.3	pH 7.4	pH 2.3	pH 7.4	pH 2.3	pH 7.4	pH 2.3	pH 7.4	pH 2.3	pH 7.4
C	176.2	-	176.3	-	-	-	176.6	176.7	-	-	-	-	-	-	-	-
Cα	53.9	53.9	53.5	53.5	52.5	52.7	57.5	57.5	52.5	52.4	52.5	52.4	63.9	63.9	64.3	64.3
Hα	4.632	4.618	4.418	4.396	5.001	4.964	4.725	4.724	5.002	5.005	5.002	5.005	4.900	4.883	4.517	4.519
Cβ	33.4	33.3	33.1	33.0	43.1	42.8	30.0	29.9	43.8	43.8	43.8	43.8	46.2	46.1	43.0	43.0
Hβ2[d]	2.472	2.490	2.624	2.645	3.688	3.676	3.207	3.207	3.706	3.711	3.706	3.711	2.754	2.752	2.702	2.706
Hβ3[d]	2.438	2.445	2.516	2.512	3.688	3.602	3.244	3.253	3.706	3.711	3.706	3.711	2.619	2.621	2.550	2.558
Cɣ	(45.0)	(46.0)[f]	(46.1)	(46.0)[f]	203.0	203.0	110.9	110.9	204.5	n.d	204.5	n.d	91.0[e]		90.5[e]	
Hɣ	3.746	3.755[f]	3.762	3.771[f]	-	-	-	-	-	-	-	-	-	-	-	-
Cδ1	184.1	n.d.	184.4	184.3	-	-	128.3	128.3	198.7	198.7	167.8	167.8	86.4	86.4	84.8	84.8
Hδ1	-	-	-	-	-	-	7.250	7.250	8.387	8.388	8.888	8.890	5.735	5.734	5.593	5.592
Cδ2	131.5	131.6	130.6	130.6	124.4	120.1	130.3	130.4	n.d		n.d		133.2[e]		132.2[e]	
Cε2	144.2	144.3	144.2	144.3	144.8	153.2	134.0	134.0	138.7[e]		140.2[e]		150.1[e]		151.6[e]	
Cε3	127.0	127.0	127.0	127.0	134.4	134.2	105.3	105.3	133.7	133.7	134.5	134.5	126.0	126.0	(126.9)	(125.7)
Hε3	7.344	7.349	7.344	7.349	7.977	7.818	7.050	7.050	8.010	8.013	8.051	8.054	7.396	7.412	7.347	7.375
Cζ2	113.4	113.3	113.2	113.2	123.7	119.4	115.5	115.5	125.4	125.4	120.6	120.5	115.3	115.2	114.9	114.6
Hζ2	7.021	7.021	6. 993	6.992	7.142	6.783	7.370	7.373	8.246	8.246	7.551	7.555	6.890	6.887	6.845	6.817
Cζ3	125.6	125.7	125.6	125.7	124.8	120.6	151.6	151.8	127.7	127.6	127.7	127.6	124.2	124.2	(123.7)	123.6
Hζ3	7.157	7.161	7.150	7.152	7.167	6.846	-	-	7.364	7.368	7.371	7.375	7.023	7.031	(6.947)	6.971
Cη2	131.2	131.2	131.2	131.2	138.3	138.3	114.5	114.5	137.7	137.7	138.3	138.4	133.4	133.5	(133.1)	(133.1)
Hη2	7.316	7.316	7.316	7.316	7.574	7.403	6.840	6.843	7.679	7.678	7.683	7.683	7.332	7.329	7.299	7.285

[a] Atom nomenclature of parent tryptophan, see also Figures 1 and S12, [b] cis or trans configuration of Hδ1 and Cε2 , [c] cis and trans configuration of the OH group, [d] Not stereochemically assigned, the lower value was tentatively assigned to Hβ3, [e] different conditions: D_2O at pH 2.6, [f] Hɣ exchanges in D_2O, only visible in 7 M urea and 90% H_2O/10% D_2O. Values in brackets are ambiguous.

Table. S5. Chemical shifts of the peptides **4** (Ac-G-G-Met(O)-P-G-NH_2) and **3** (Ac-G-G-Met(O)-A-G-NH_2) measured at 25°C in 7 M urea-d_4 in D_2O and referenced to internal DSS. These peptides were investigated to test the possible change of the reference chemical shifts of Met(O) when coupled to Pro or Ala.

	Ac-Gly-Gly-Met(O)-Pro-Gly-NH_2 (**4**)		Ac-Gly-Gly-Met(O)-Ala-Gly-NH_2 (**3**)	
	pH 2.3	pH 7.4	pH 2.3	pH 7.4
	Met(O) /Met(O)'		Met(O)/ Met(O)'	
Cα	53.5/53.2	53.4/53.1	55.3/55.1	55.3/55.0
Cβ	26.7	26.6	27.2	27.1
Cɣ	51.2	51.2	51.5	51.4
Cε	39.5	39.5	39.5	39.4
Hα	4.818/4.832	4.812/4.830	4.519/4.535	4.520/4.534
Hβ2[a]	2.264	2.261	2.283	2.282
Hβ3[a]	2.139	2.136	2.158	2.157
Hɣ2	2.964	2.949	2.942	2.938
Hɣ3	2.919	2.903	2.910	2.910
Hε	2.719	2.716	2.714	2.713

[a] Not stereochemically assigned, the lower value was tentatively assigned to Hβ3

Table S6. Chemical shifts of methionine sulfone in the peptide Ac-G-G-Met(O_2)-G-G-NH_2, which was generated by treating the peptide Ac-G-G-MetG-G-NH_2 with H_2O_2 (H_2O_2 was removed afterwards in the lyophilization step), measured at 25°C in 7 M urea-d_4 in D_2O and referenced to internal DSS.

Sulfone		
	Chemical shifts (ppm)	
	pH 2.3	pH 7.4
Cα	54.8	54.9
Cβ	26.5	26.5
Cɣ	52.9	53.0
Cε	42.4	42.4
Hα	4.581	4.587
$Hβ_2$[a]	2.405	2.414
$Hβ_3$[a]	2.233	2.242
$Hɣ_2$ + $Hɣ_3$	3.330	3.338
Hε	3.121	3.130

[a] Not stereochemically assigned, the lower value was tentatively assigned to Hβ3

Table S7. Bottom-up MS quantification of methionine sulfoxide in the mAbs rituximab and adalimumab.

Rituximab		Adalimumab	
modified methionine[a]	Relative Oxidation ± SD [%][b]	modified methionine[a]	Relative Oxidation ± SD [%][b]
HC Met20	1.4 ± 0.2	HC Met34	8.0 ± 0.4
HC Met34	4.6 ± 0.5	HC Met83	8.3 ± 0.5
HC Met81	11.5 ± 1.5	HC Met256	54.4 ± 1.7
HC Met256	59.1 ± 2.0	HC Met432	26.9 ± 1.7
HC Met432	29.1 ± 1.9	LC Met4	8.7 ± 1.1
LC Met21	1.9 ± 0.1	Total[c]	21.3 ± 1.2
Total[c]	17.9 ± 1.3		

[a] Position of the modified amino acid. HC= heavy chain, LC= light chain.

[b] Relative quantification of methionine oxidation in %. Relative Abundance = [Peak area modified peptide/(Peak area modified + Peak area non-modified peptide)] x 100. SD = standard deviation of 5 technical replicates.

[c] Average oxidation of all methionine residues. Relative Abundance = Sum of relative abundances of oxidation of individual methionines/number of methionine residues. Added standard deviation = [square root ($SD1^2 + SD2^2 + \ldots SDn^2$)/n].

Figures

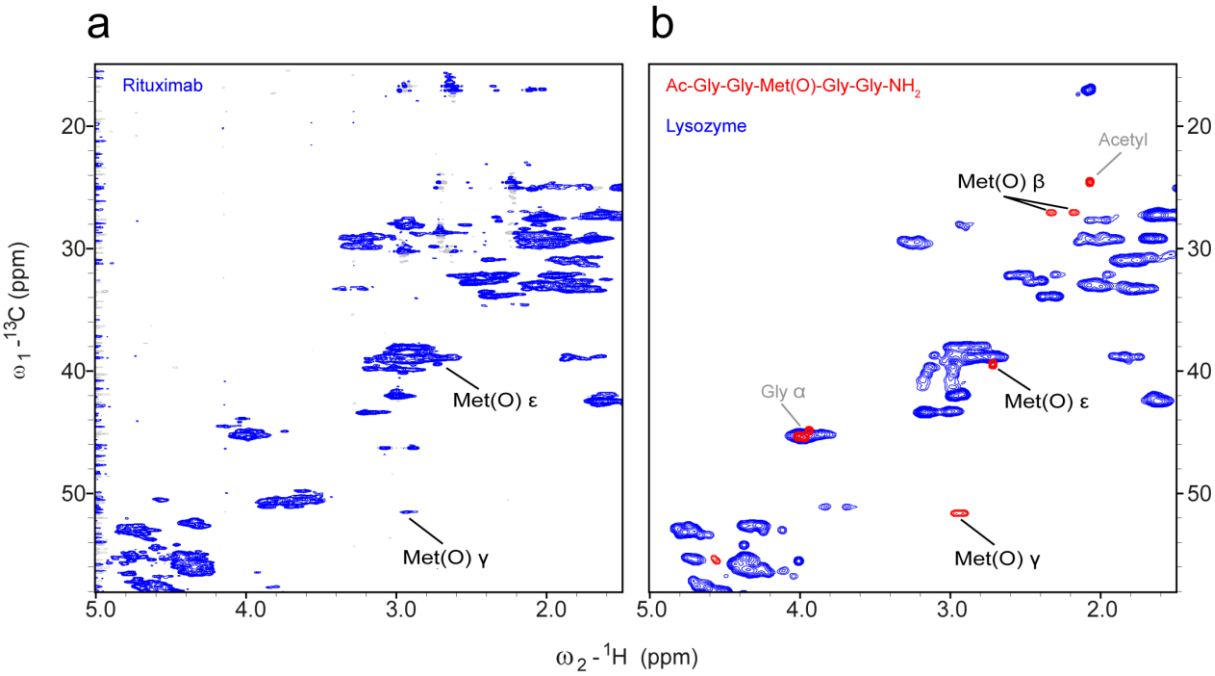


Figure S1 Comparison of the ^{1}H-^{13}C HSQC spectrum of oxidized rituximab (0.35% H_2O_2 for 30 min at RT) with lysozyme and the Met(O)-containing reference peptide measured at 25°C in 7 M urea-d_4 in D_2O at pH 2.3. (a) ^{1}H-^{13}C HSQC spectrum of treated rituximab (512×512 complex points, 104 scans, a recycle delay of 3 s, resulting in a total measurement time of 2 d 14 h) as also shown with a more focused range in Fig. 2c. (b) Overlay of ^{1}H-^{13}C HSQC spectra of the reference peptide Ac-Gly-Gly-Met(O)-Gly-Gly-NH_2 (red) and lysozyme (blue) measured under comparable conditions for comparison.

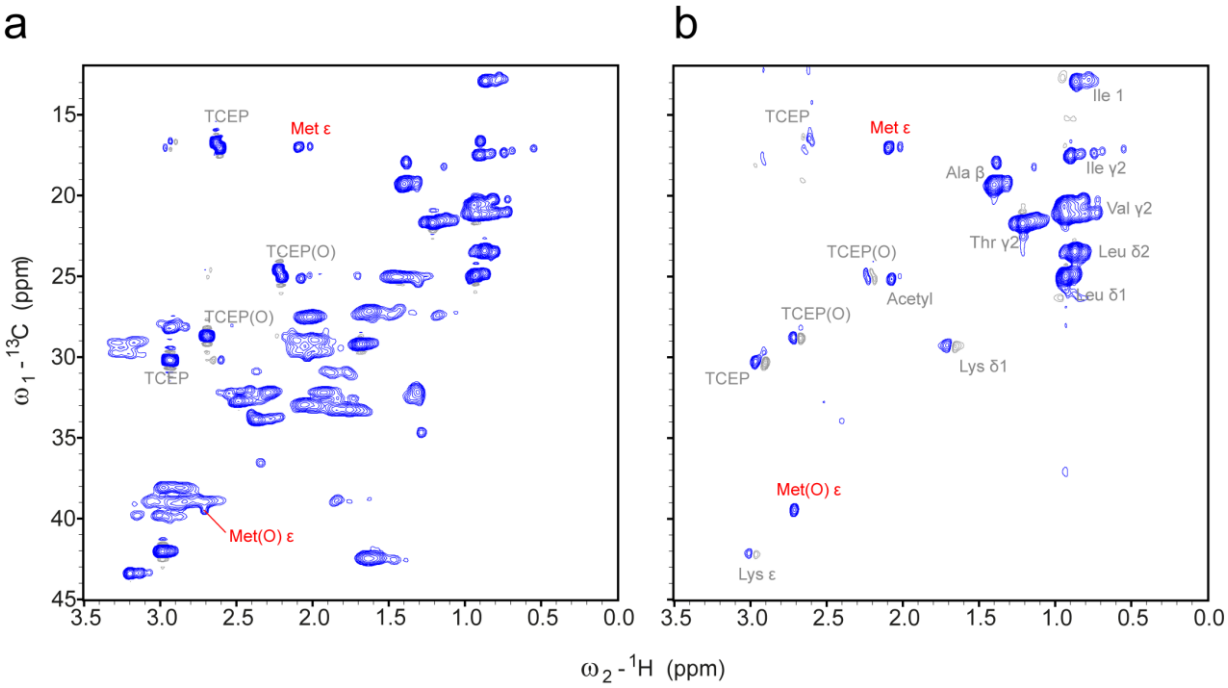


Figure S2. Methyl selective ^{1}H-^{13}C correlation reduces the chemical shifts overlap of the key methyl signal of Met(O) measured at 25°C in 7 M urea-d_4 in D_2O. (a) ^{1}H-^{13}C HSCQ spectrum of rituximab treated with 0.35% H_2O_2 (512×128 complex points, 200 scans, a recycle delay of 2 s, total measurement time of 30 h). (b) ^{1}H-^{13}C HQQC spectrum of the same sample (512×128 complex points, 324 scans, a recycle delay of 1.3 s, total measurement time of 33 h).

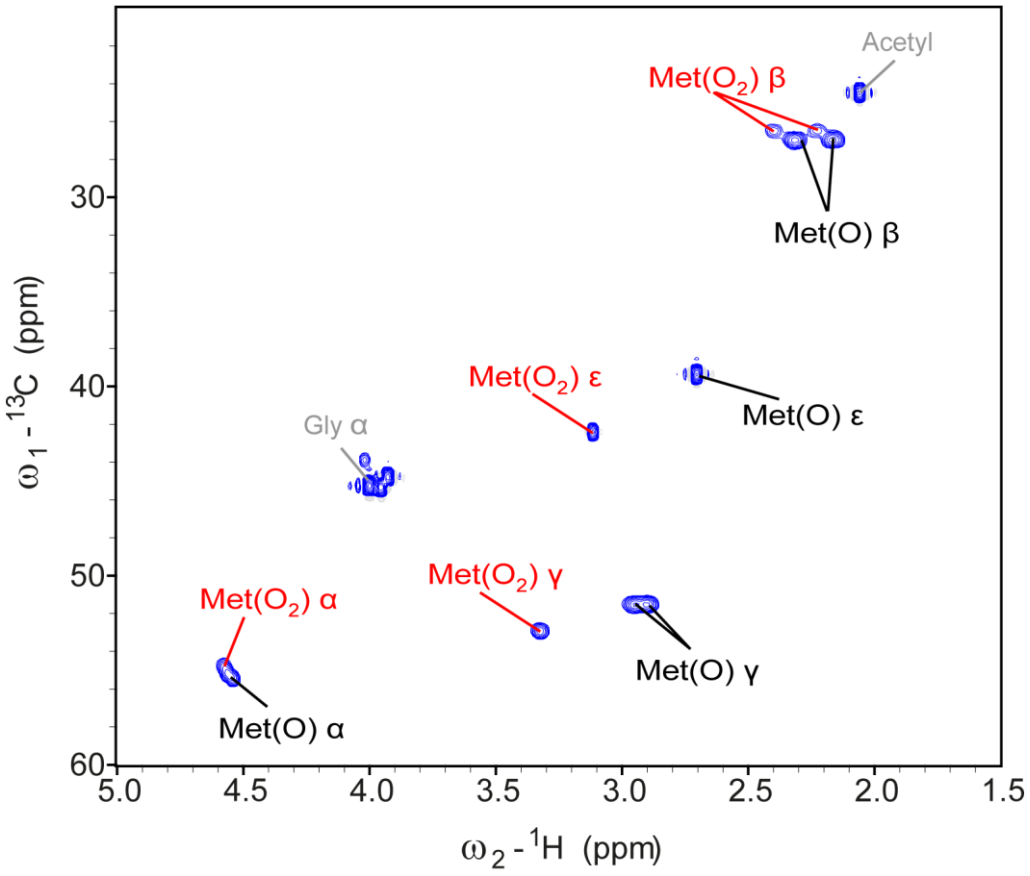


Figure S3. ^{1}H-^{13}C HSQC spectrum of the peptide Ac-G-G-M-G-G-NH_2 treated with H_2O_2 measured at 25°C in 7 M urea-d_4 in D_2O (pH: 2.3) using 512×128 complex points, 110 scans, a recycle delay of 2 s, total measurement time 16.5 h. Methionine was fully oxidized and two species can be detected. One spin-system is related to methionine sulfoxide (Met(O)) (Table S3). All the other unknown cross correlations belong to one spin system, which could be determined by TOCSY spectra (data not shown). The cross correlations at 26.5/2.405, 26.5/2.233 ppm and 52.9/3.330 ppm are CH_2 groups (data not shown). The chemical shifts of this second species match to the carbon and proton shifts for methionine sulfone, reported by Takizawa et al.[1]

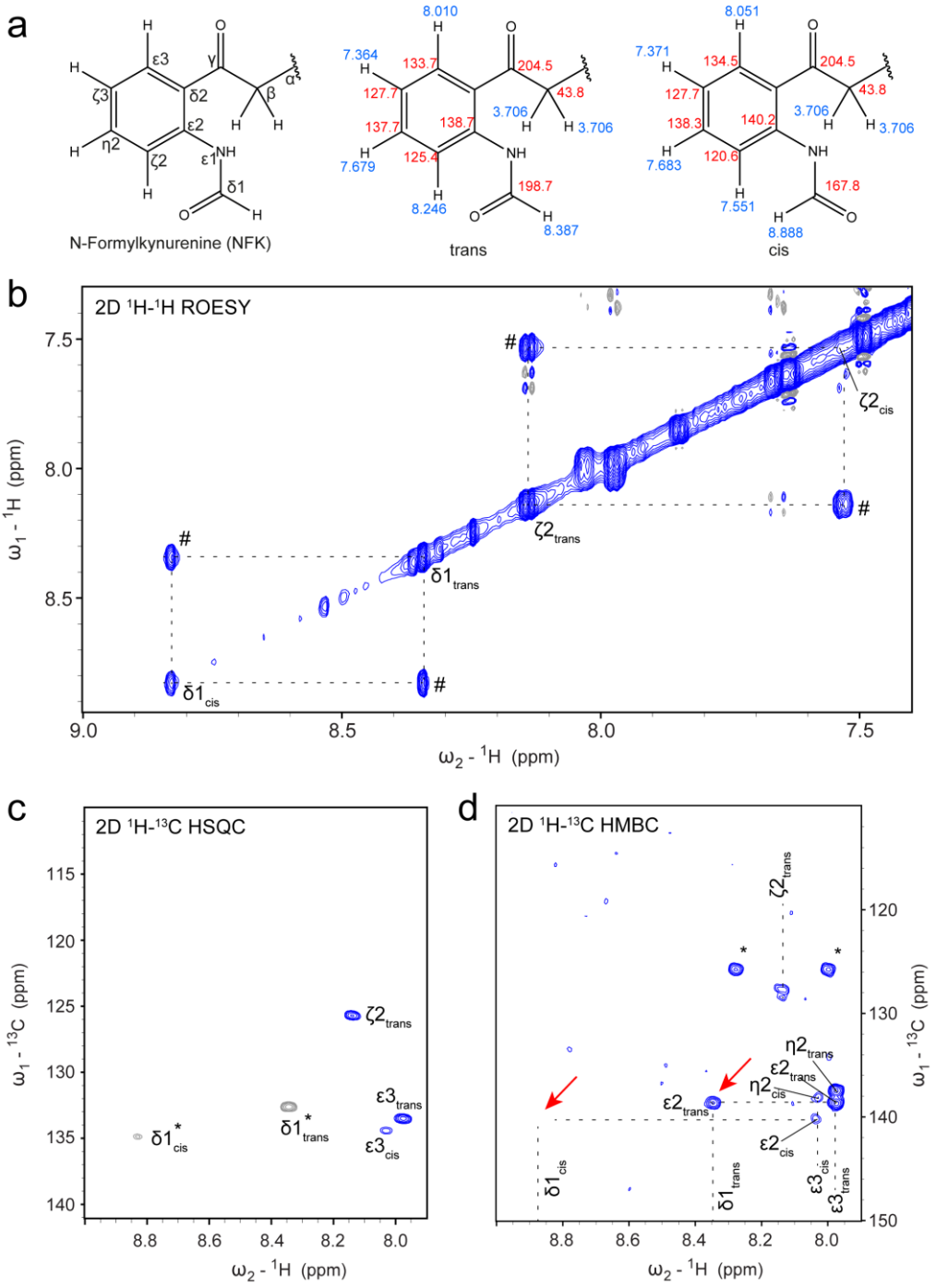


Figure S4. NFK gives rise to two sets of chemical shifts that are in chemical exchange during the NMR experiments measured at 25°C in plain D_2O (pH 2.6). (a) Chemical structure and chemical shifts of NFK. (b) 2D ^{1}H-^{1}H ROESY spectrum showing exchange signals of the two NFK species (cis/trans configuration of the N-aryl-formamide moiety) that have the same sign as the diagonal signals in contrast to NOE cross-peaks that have opposite sign (1024×256 complex points, 32 scans, a recycle delay of 2 s, total measurement time 13 h). (c) 2D ^{1}H-^{13}C HSQC spectrum showing the most prominent differences in the ^{1}H-^{13}C correlation of the two NFK spin systems (512×128 complex points, 60 scans, a recycle delay of 1.2 s, total measurement time 5.5 h). (d) ^{1}H-^{13}C HMBC spectrum reveals that only one species shows strong Hδ1-Cε2 correlation indicative for a *cis* amide bond ($H\delta1_{trans}$, $C\varepsilon2_{trans}$) of the N-aryl-formamide moiety (2048×256 complex points, 64 scans, a recycle delay of 1.5 s, total measurement time of 16 h).

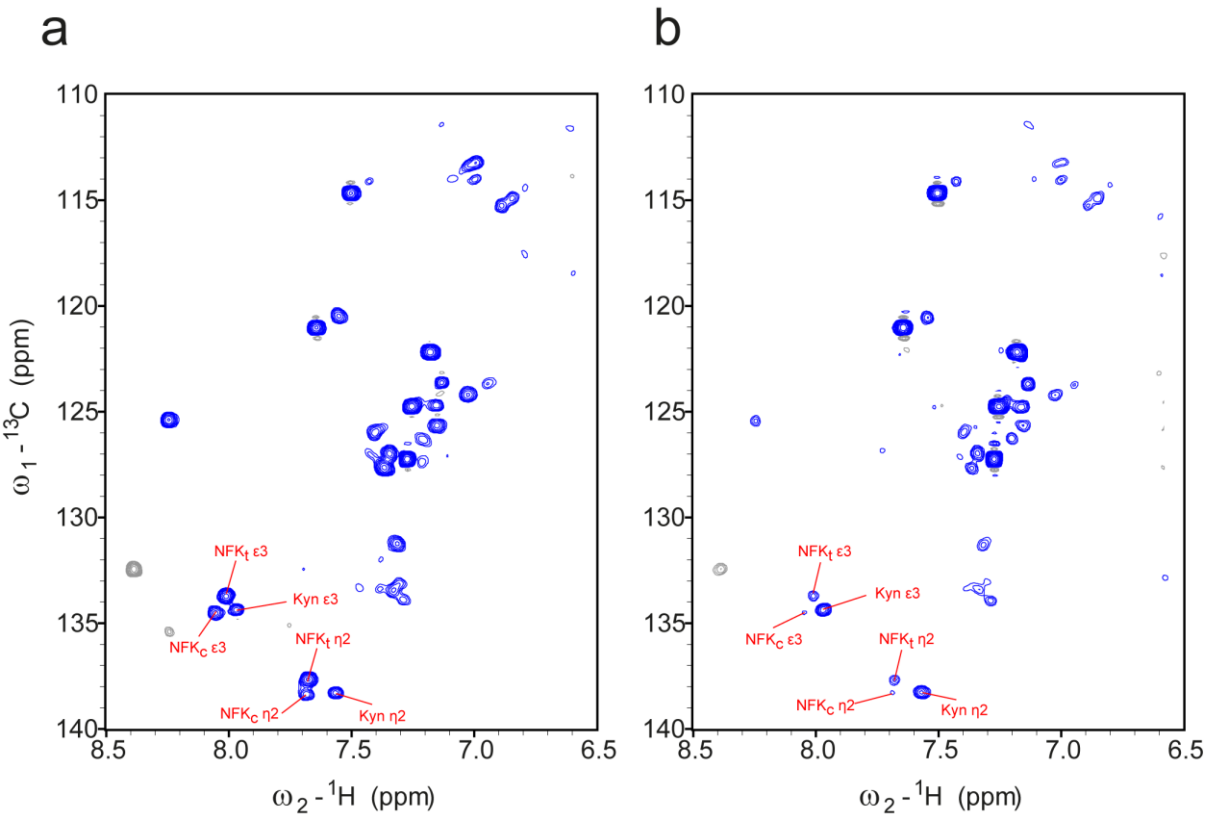


Figure S5. The oxidation product NFK is slowly converted to Kyn as shown in the $^{1}H^{13}C$ HSQC spectra of the peptide Ac-Gly-Gly-Trp-Gly-Gly-NH_2 treated with 1 % H_2O_2 for 5 days measured at 25°C in 7 M urea-d_4 in D_2O at pH 2.3. a) A sample measured shortly after the 5-day H_2O_2 treatment (512×128 complex points, 60 scans, a recycle delay of 1.2 s, total measurement time 5.5 h). b) Measurement of the same 5-day treated sample after 1 day at 298 K and 5 days at 277 K (512×128 complex points, 112 scans, a recycle delay of 1.2 s, total measurement time 10.3 h).

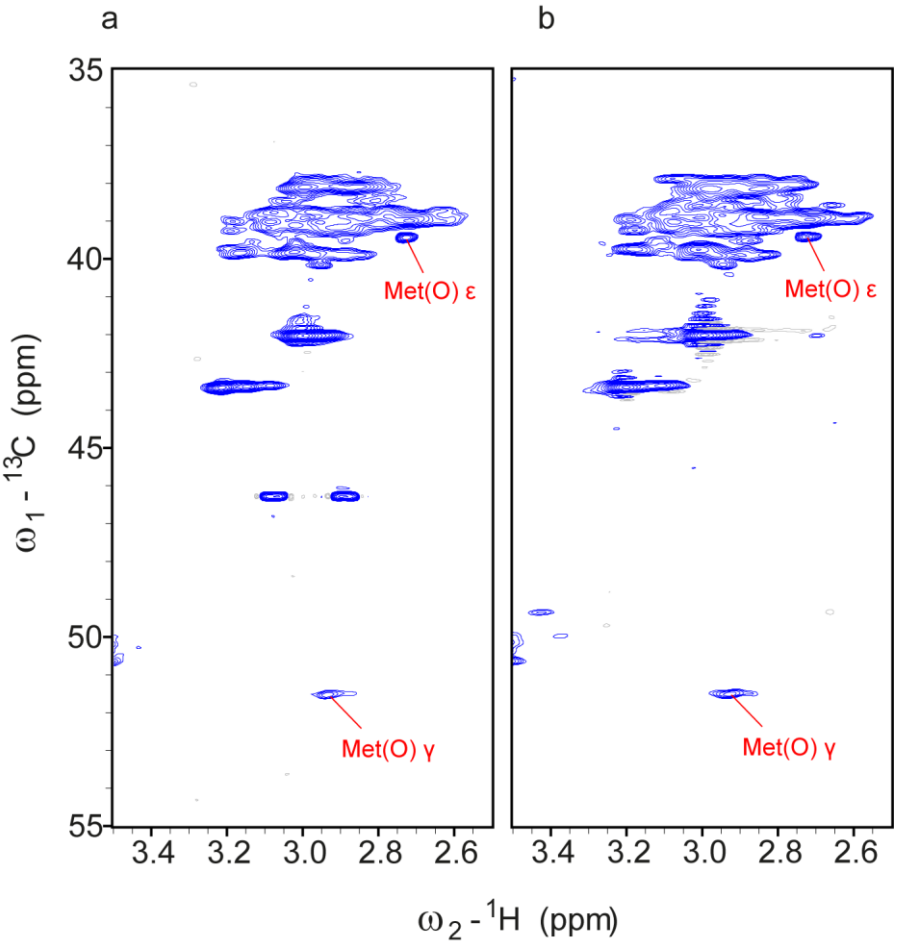


Figure S6. ^{1}H-^{13}C HSQC spectra of stressed (0.35% H_2O_2 for 30 min at RT) rituximab and adalimumab measured at 25°C under denaturing conditions (7 M urea-d_4 in D_2O) at pH 2.3. (a) Spectrum of rituximab measured with 512×512 complex points, 104 scans, a recycle delay of 3 s resulting in a measurement time of 2 d 14 h. (b) Spectrum of adalimumab recorded with 512×512 complex points, 112 scans, a recycle delay of 3 s resulting in a aquisition time of 2 d 19 h.

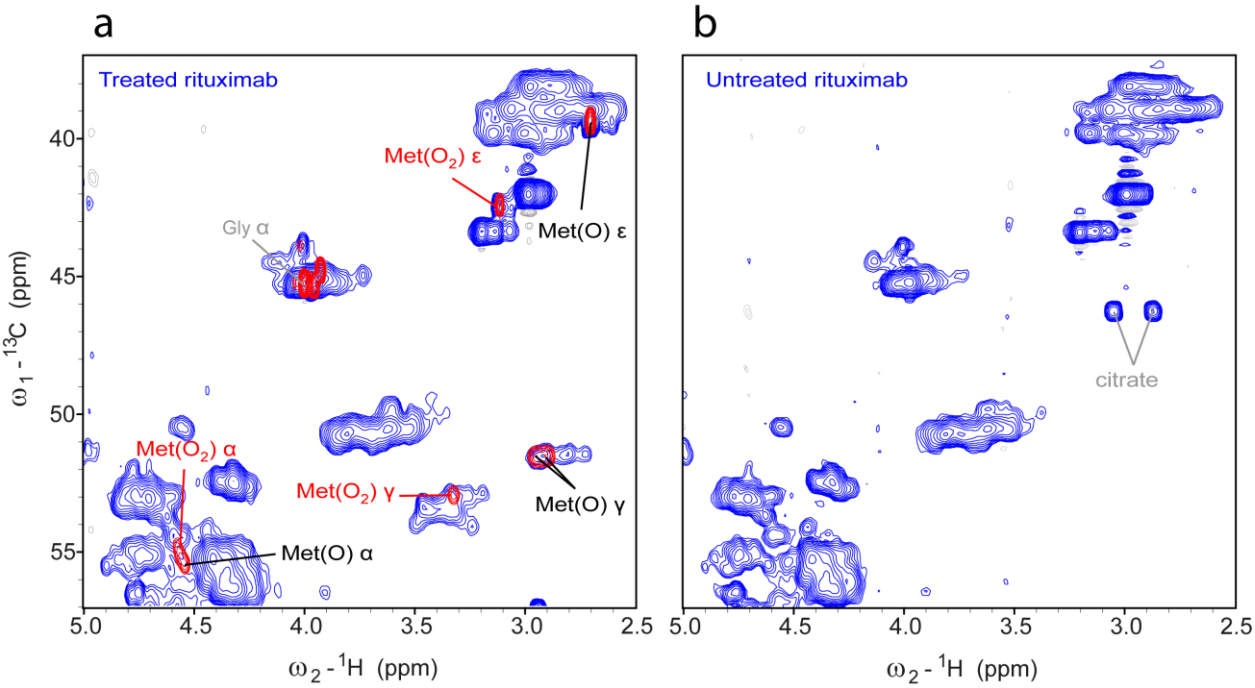


Figure S7. Detection of Met(O2) using a ^{1}H-^{13}C HSQC spectrum of harshly treated rituximab measured at 25°C under denaturing conditions (7 M urea-d_4 in D_2O) at pH 2.3. (a) ^{1}H-^{13}C HSQC (512×128 complex points, 264 scans, a recycle delay of 2 s resulting in a aquisition time of 1 d 15 h) spectrum in blue overlaid with the random coil chemical shifts of Met(O_2) using a H_2O_2 treated reference peptide (Fig. S3) in red. The rituximab spectrum shows unique and isolated cross peaks of Met(O_2). (b) a ^{1}H-^{13}C HSQC (512×128 complex points, 100 scans, a recycle delay of 2 s resulting in a aquisition time of 15 h) spectrum of non-treated denatured rituximab in which the unique Met(O) and Met(O_2) γ cross correlations are missing.

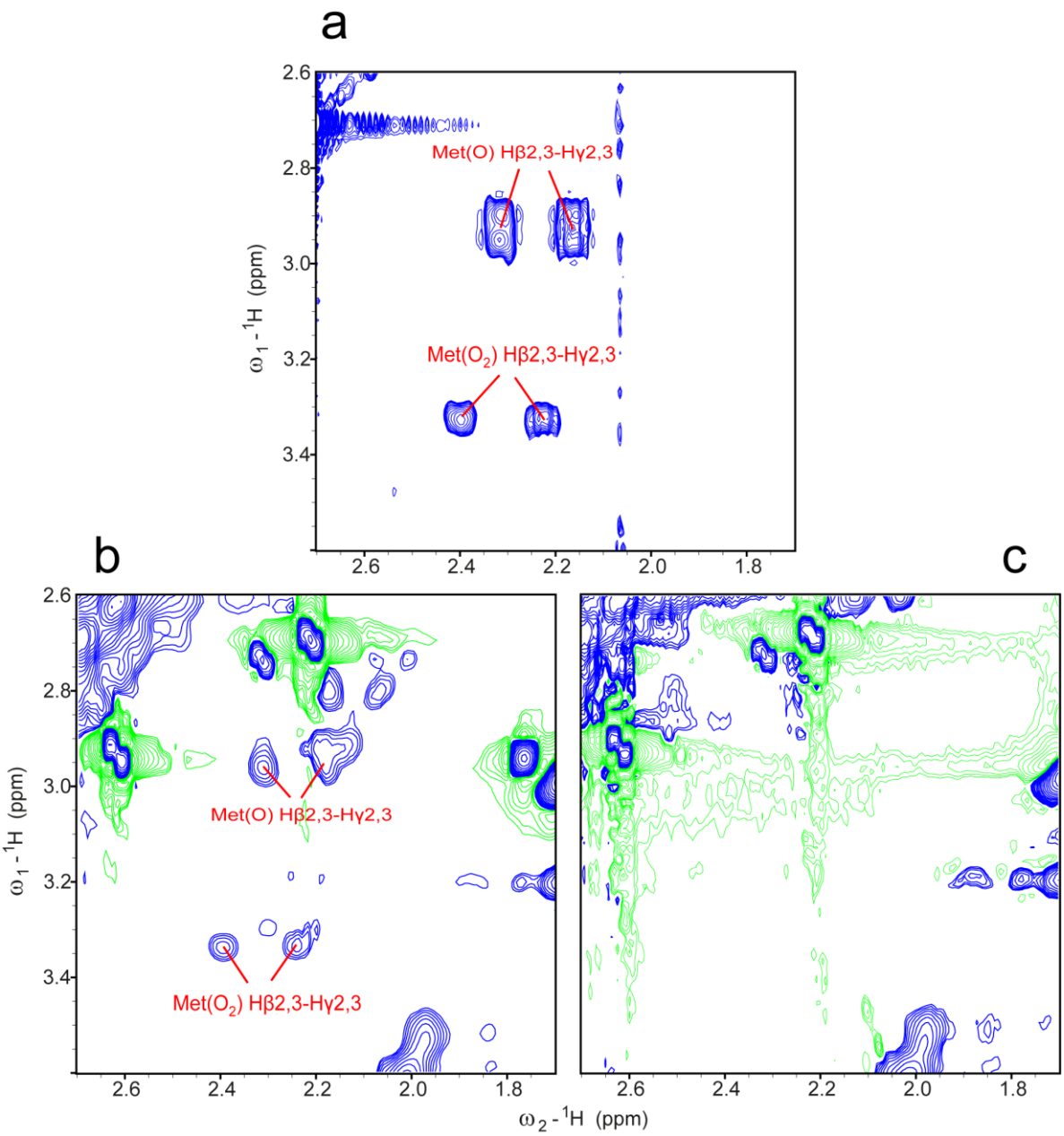


Figure S8. Detection of Met(O) and Met(O_2) using ^{1}H-^{1}H TOCSY spectra measured in 7 M urea-d_4 in D_2O at pH 2.3 and 25°C. (a) H$\beta_{2,3}$ Hγ cross correlations for Met(O) and Met(O_2) in a ^{1}H-^{1}H TOCSY spectrum (13 ms mixing time, 1024×256 complex points, 4 scans, a recycle delay of 1.2 s resulting in a aquisition time of 1 h) of the peptide Ac-G-G-M-G-G-NH_2 which was treated with H_2O_2. These shifts are unique. (b) Identical region of a ^{1}H-^{1}H TOCSY spectrum (12 ms mixing time, 1024×256 complex points, 56 scans, a recycle delay of 2 s resulting in a aquisition time of 17 h) of harshly treated rituximab (incubated for 24h with 1% H_2O_2). (c) Spectrum of untreated rituximab (12 ms mixing time, 1024×256 complex points, 4 scans, a recycle delay of 1.2 s resulting in a aquisition time of 1 h) lacking signals in this region.

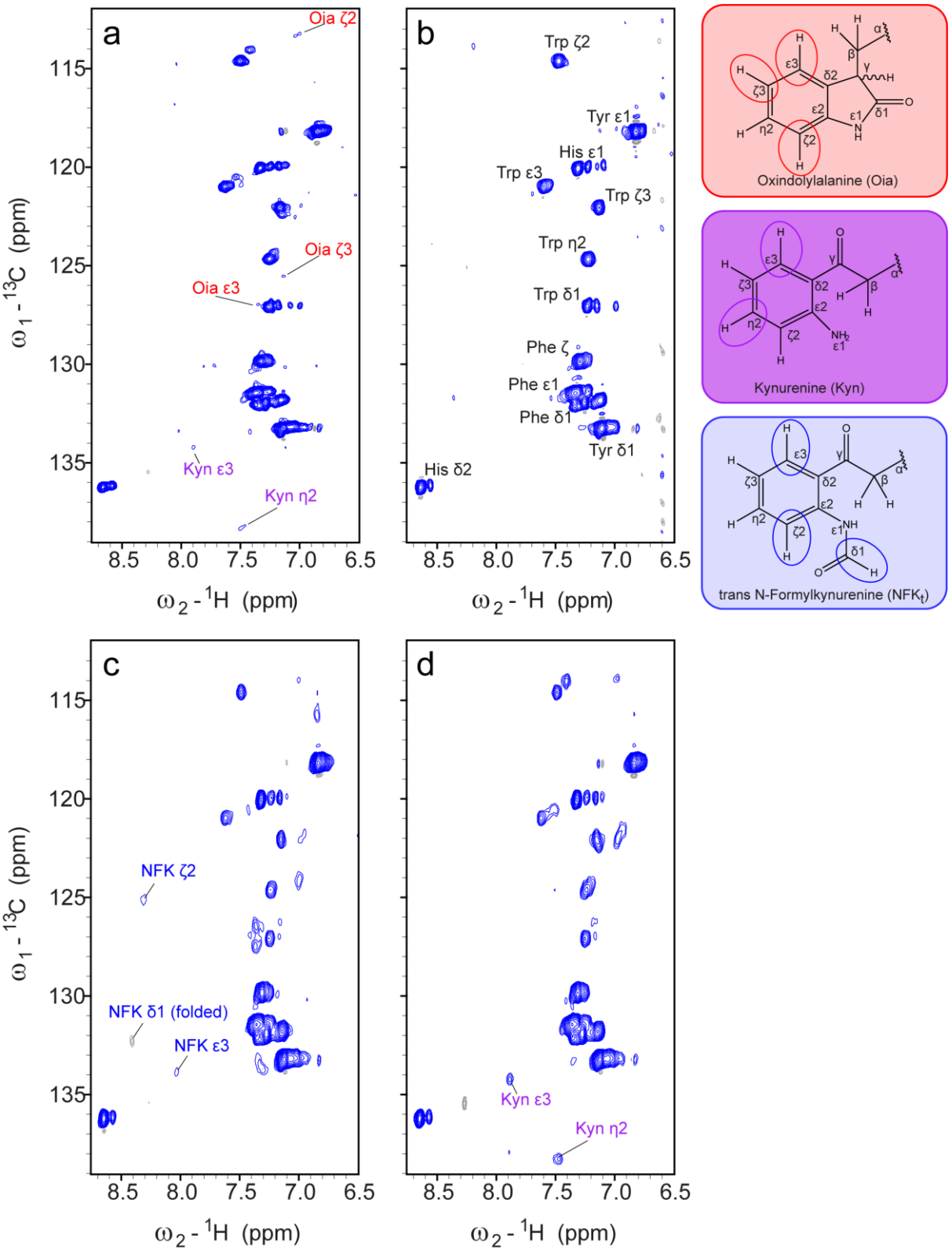


Figure S9. Trp oxidation products observed in rituximab treated with H_2O_2. (a) ^{1}H-^{13}C HSQC spectrum of the aromatic region of rituximab treated with 1 % H_2O_2 for 24 h measured in 7 M urea-d_4 in D_2O at pH 2.3 and 25°C (512×128 complex points, 488 scans, recycle delay of 2 s, total measurement time 72 h). (b) Comparable spectrum of an untreated rituximab sample. (c) and (d) ^{1}H-^{13}C HSQC spectrum of an even harsher oxidized rituximab sample (1% H_2O_2 for 132 h) using 512×128 complex points, 440 scans and a recycle delay of 2 s resulting in a total measurement time of 65 h. The spectrum shown in (d) was measured directly after the spectrum shown in (c).

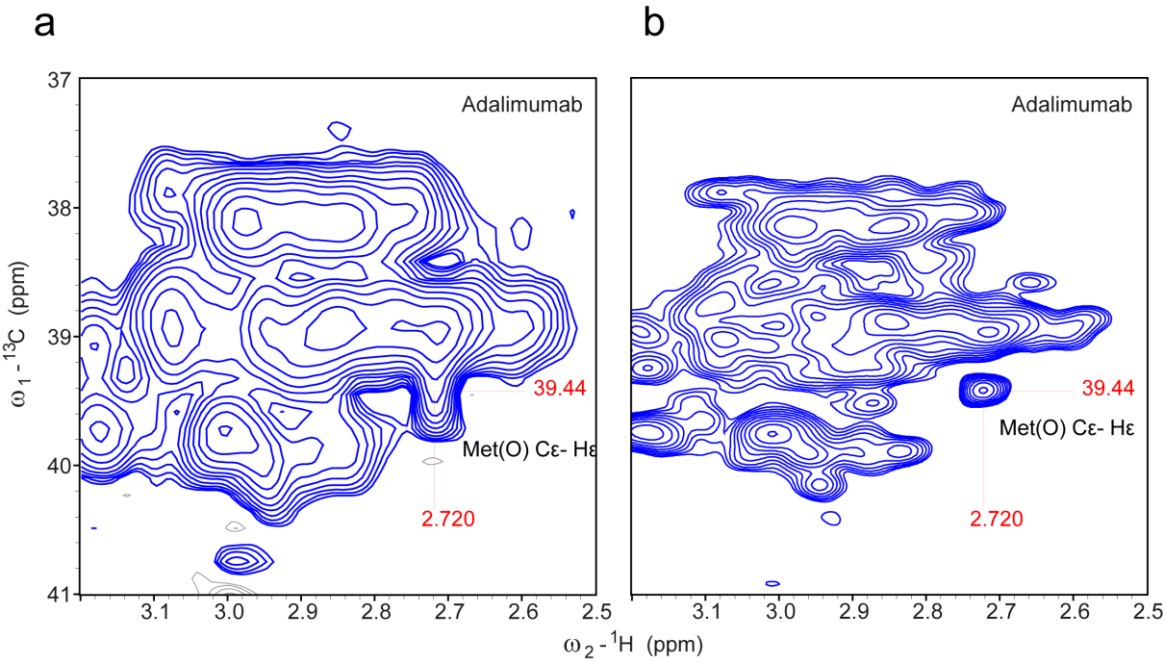


Figure S10. ^{1}H-^{13}C HSQC spectra of adalimumab measured in 7 M urea-d_4 in D_2O at pH 2.3 and 25°C with different resolutions in ω_1. (a) ^{1}H-^{13}C HSQC spectrum measured with 512×128 complex points, 180 scans and a recycle delay of 2 s resulting in a total aquisition time of 27 h). (b) ^{1}H-^{13}C HSQC spectrum of the same sample measured with 512×512 complex points, 112 scans and a recycle delay of 2 s resulting in a total measurement time of 67 h.

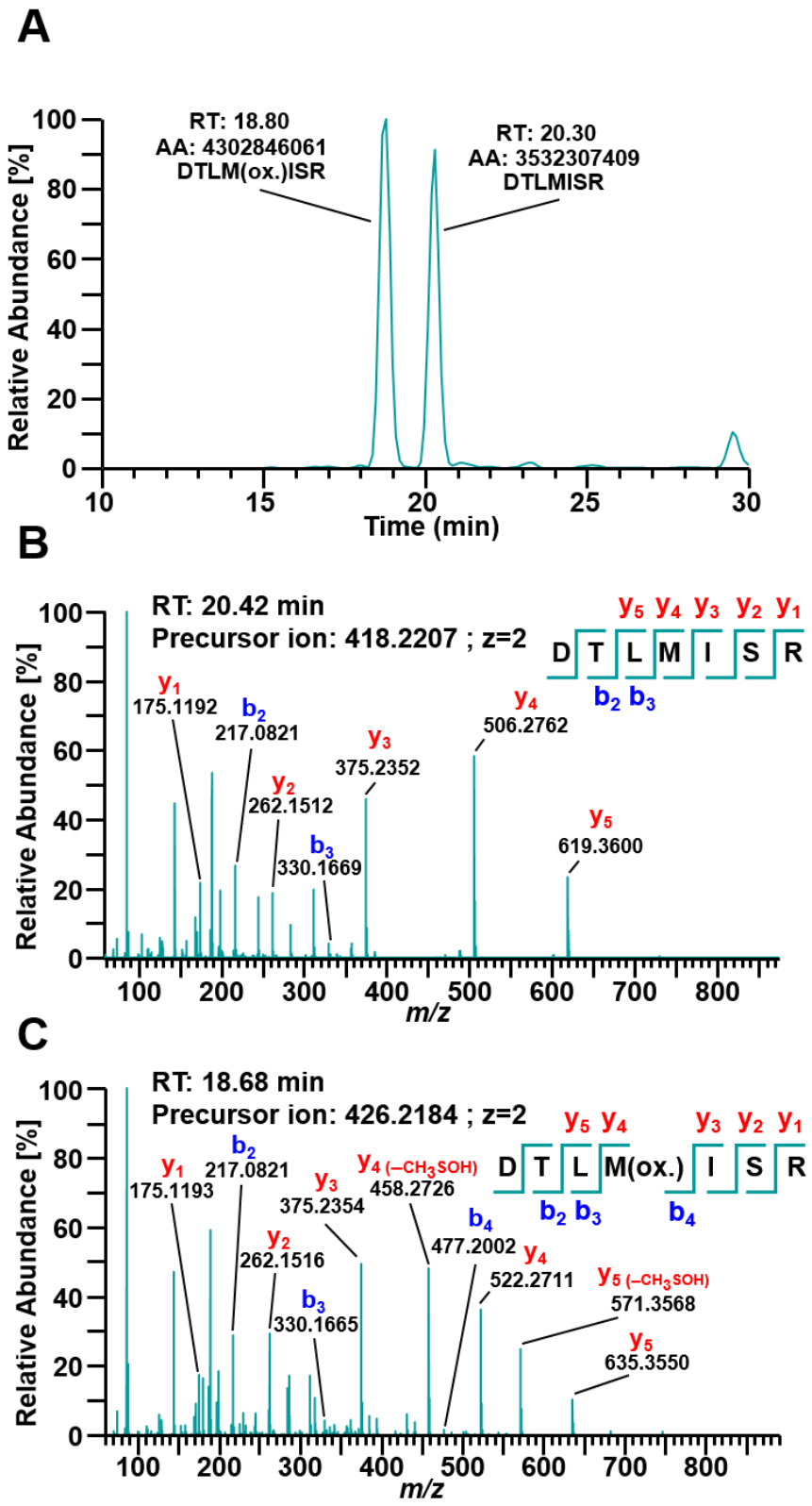
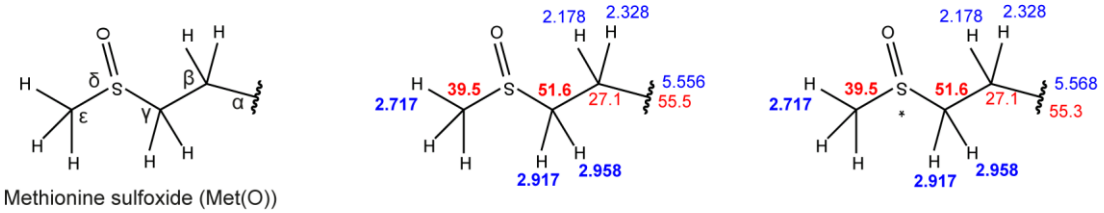


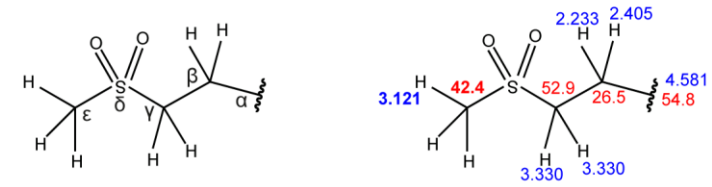
Figure S11. Extracted ion current chromatogram of the heavy chain peptide DTLMISR containing methionine residue Met256 and its oxidized form (A). The peak areas used for relative quantification of oxidation are indicated with AA, RT marks the retention time. Identification of the non-oxidized (B) and the oxidized form (C) of the DTLMISR peptide is based on b- and y-ions detected upon higher-energy fragmentation spectra.

a



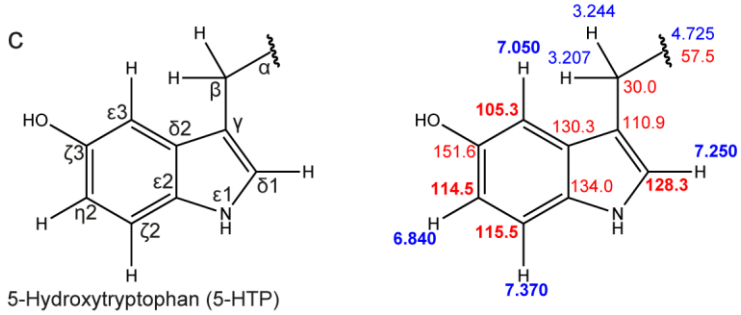
Methionine sulfoxide (Met(O))

b



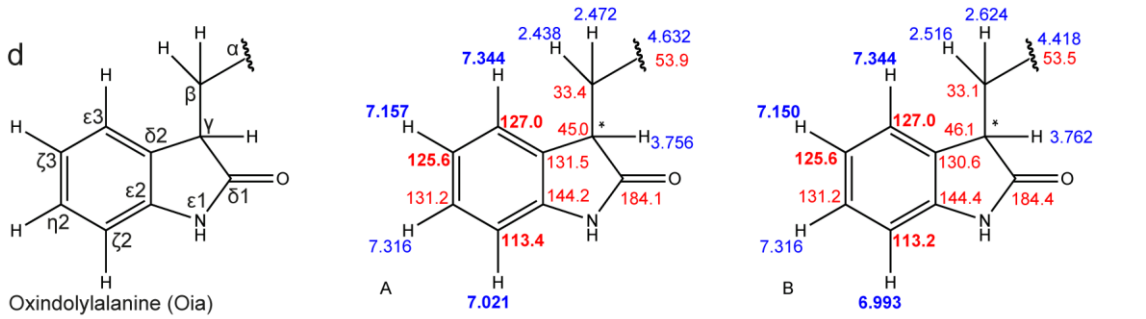
Methionine sulfone (Met(O_2))

c



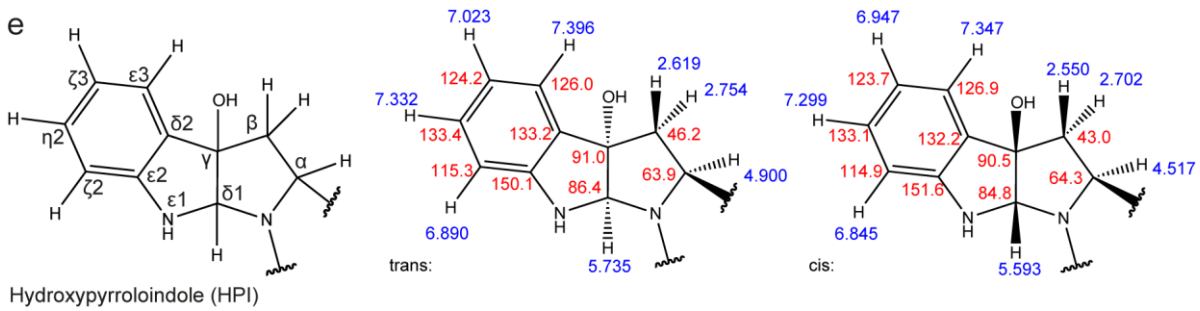
5-Hydroxytryptophan (5-HTP)

d



Oxindolylalanine (Oia)

e



Hydroxypyrroloindole (HPI)

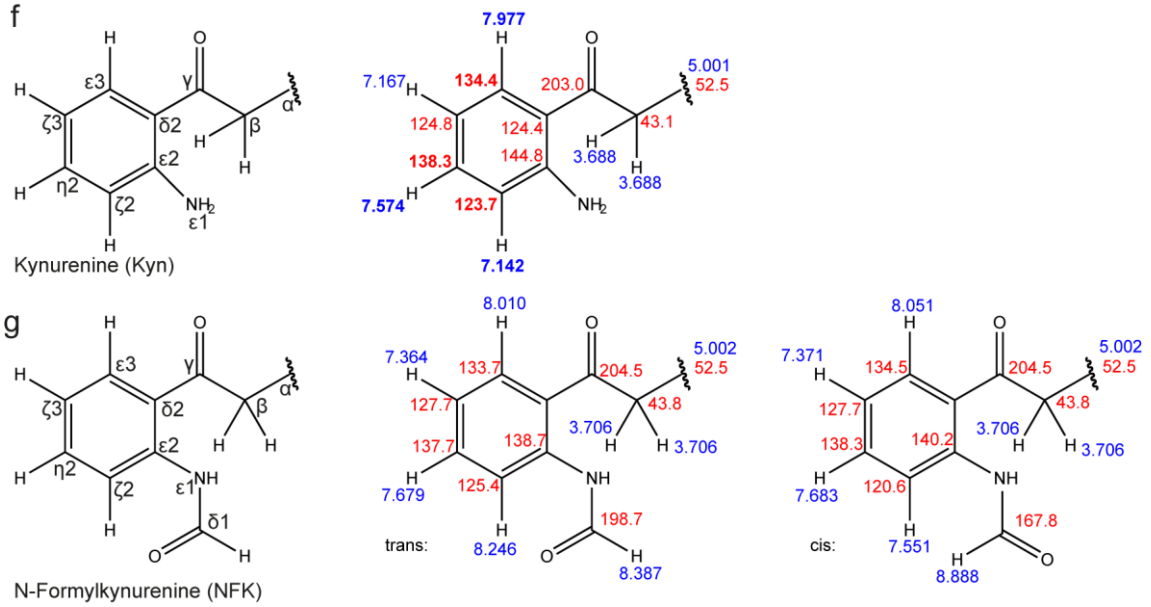


Figure S12. Summary of the random coil chemical shifts of all observed oxidation products indicated by the numbers in red (^{13}C) and blue (^{1}H) referenced to DSS. a) Met(O), b) Met(O_2). b) – f) oxidation products of Trp. Random coil chemical shifts for carbon (red) and hydrogen (blue) in 7 M urea-d_4 in D_2O at pH 2.3 (Tables S3 and S4). An * marks the chiral center, when the stereochemistry is not given. Chemical shifts in bold are suited as references for the detection in proteins, as they do not overlap with the random coil chemical shifts of the natural amino acids.

Supplementary Methods

Chemicals for synthesis

Fmoc-Rink amide MBHA resin, N,N-dimethylformamide (DMF), 1-methyl-2-pyrrolidinone (NMP), dichloromethane (DCM), diethylether (Et_2O), N,N-diisopropylethylamine (DIPEA), and Fmoc-OSu were purchased from Iris Biotech GmbH (Marktredwitz, Germany). N-Methyl-N-(trimethylsilyl)trifluoroacetamide was obtained from Roth (Karlsruhe, Germany). 1-Hydroxybenzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA), and piperidine were obtained from Biosolve (Valkenswaard, The Netherlands). HPLC-grade trifluoroacetic acid (TFA) was from Alfa-Aesar (Karlsruhe, Germany). N-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU) and Fmoc-Met(O)-OH were purchased from Merck (Darmstadt, Germany). HPLC-grade acetonitrile (ACN), triisopropylsilane (TIS), thioanisole (TIA), 1,2-ethanedithiol (EDT), bromotrimethylsilane (TMSBr), piperidine, H-Kyn-OH and H-5HTP-OH were purchased from Sigma Aldrich (Vienna, Austria). All other Fmoc-amino-acids were purchased from Iris Biotech. Fmoc-Oia-OH was prepared starting from H-Trp-OH (Sigma Aldrich (Vienna, Austria)), as reported previously.[2] Briefly, dimethyl sulfoxide (DMSO, 12.5 mmol) was added to 12 M HCl (5 ml) at room temperature (RT). Phenol (1 mmol) was added, followed by a suspension of H-Trp-OH (5 mmol) in 30 mL glacial acetic acid. The mixture was stirred at RT for 5 h. Acetic acid and DMSO were removed under vacuum and the dark-yellow viscous liquid was diluted in 1.5 ml glacial acetic acid and left for precipitation overnight at 4°C. The precipitate was washed with cold diethyl ether and dried under vacuum. The dried product (1 equivalent) was suspended in 7 ml DCM, then N-Methyl-N-(trimethylsilyl)trifluoroacetamide (3 equivalents) was added and refluxed for 1 h. Fmoc-OSu (1 equivalent), suspended in DCM, was added and the reaction mixture was stirred at RT until the reaction was complete. The trimethylsilyl protected carboxyl group was converted into the free acid upon methanol addition. Ether was added and the mixture was extracted three times with 5% potassium carbonate. The pH of the aqueous phase was lowered to 2 with 1 M HCl and the obtained precipitate was recovered by filtration.[3] H-Kyn-OH and H-5HTP-OH were Fmoc protected in the same manner.

Solid phase peptide synthesis

The peptides were synthesized by Fmoc-chemistry using solid-phase peptide synthesis (SPPS) on an automatic peptide synthesizer (Syro I, MultiSynTech GmbH), or manually in the case of the Kyn-, 5HTP- and Oia-containing peptides. For the automatic synthesis, amino-acid double coupling (2x40 min) was performed by using Fmoc-AA-OH/HOBt/HBTU/DIPEA (5:5:4.8:10 equivalents). The same protocol was used for the manual synthesis, except for the double coupling (2x1.5 h) of Fmoc-Oia-OH, Fmoc-Kyn-

OH and Fmoc-5HTP-OH, which was accomplished by using Fmoc-AA-OH/HATU/DIPEA (6:5.8:12 equivalents).[4] Fmoc deprotection was carried out with 25%/12.5% piperidine in DMF/NMP (80:20, v/v) for 3 min/12 min. Acetylation was performed with Ac_2O/DIPEA (10 equivalents each) in DMF for 20 min. The peptides were cleaved from the resin with TFA containing 10% of the scavenger mixture H_2O/TIA/EDT/TIS (1:3:3:3) at room temperature for 1.5 h. The peptides were then precipitated in cold diethylether, recovered by centrifugation at 4 °C, washed three times with cold ether, dried under nitrogen, dissolved in 0.1% aqueous TFA and lyophilized. For the Met-containing peptides, a treatment with TFA containing 2.4% EDT and 1.6% TMSBr for 40 min was carried out after the cleavage from the resin and precipitation from ether, in order to reduce the Met(O) that was eventually formed during the peptide-chain assembly upon air oxidation.

Peptide characterization

Analytical RP-HPLC was performed using a Thermo Scientific™ UltiMate™ 3000 UHPLC system (Germering, Germany) and a Thermo Scientific™ Syncronis C18 column (100 Å, 5 µm, 250 × 4.6 mm) at a flow rate of 1.5 mL/min. The UV detection was set at 220 nm. The elution system was (A) 0.06% (v/v) TFA in water, and (B) 0.05% (v/v) TFA in ACN. The products were dissolved in ACN/H_2O (25:75, v/v) containing 0.1% TFA. Two gradients were used: 3% B for 8 min, 3% to 60% B in 35 min; or 1% B for 8 min, 1% to 50% B in 35 min. Mass spectra were recorded on an Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using α-cyano-4-hydroxy-cinnamic acid (HCCA) or 2,5-dihydroxy-acetophenone (DHAP). All peptides were at least 91% pure (based on the HPLC peak), with the exception of the Oia-containing peptide (72%; at least 10% Kyn in place of Oia was present), and of the 5HTP-containing peptide (80%) (Table S2).

Oxidation and sample preparation of peptides

To induce Trp oxidation in the peptide Ac-Gly-Gly-Trp-Gly-Gly-NH_2, 8 mg peptide were dissolved in 8 ml ddH_2O and incubated with 1% H_2O_2 for 5 days at RT. Afterwards the peptide was lyophilized. To detect additional oxidation products of Met, 1.7 mg of the reference peptide Ac-Gly-Gly-Met-Gly-Gly-NH_2 were incubated with 0.35% H_2O_2 in 10 ml 175 mM ammonium acetate–d_3 for 30 min at RT. Afterwards the peptide was lyophilized, but during the lyophilization over night the sample thawed, so that the oxidation process might have proceeded till the sample turned dry.

For NMR sample preparation of all reference peptides, 1-2 mg were dissolved in 0.5 ml 7 M urea-d_4 98 atom%D (ARMAR Chemicals) in D_2O 100 atom%D (ARMAR Chemicals) and the pH was adjusted to 2.3 or 7.4 with DCl (ARMAR Chemicals) or NaOD (ARMAR Chemicals).

Oxidation and sample preparation of mAbs

For mild oxidation, 2 ml of each of the two biotherapeutics adalimumab (Humira, AbbVie, exp. year: 2016, 10 mg/ml) and rituximab (MabThera, Roche, exp. year: 2013, 10 mg/ml) formulations were used. The formulation buffer was exchanged to 175 mM ammonium acetate buffer (pH 7) using Amicon Ultra-15 Centrifugal Filter Units (Cutoff: 30 kDa, Merck), followed by adjusting the volume to 5 ml. The protein solutions were incubated with 0.35% H_2O_2 (Sigma) for 30 min at RT. For treated and untreated samples, the buffer was exchanged to D_2O using Amicon Ultra-15 Centrifugal Filter Units. Alternatively, buffer exchange to 175 mM deuterated acetate buffer (pH 7) (acetic-d_3 acid-d 99.5 atom%D, ARMAR Chemicals and ammonium hydroxide, Sigma-Aldrich) using Amicon Ultra-15 Centrifugal Filter Units led to comparable results (data not shown). After lyophilization, the proteins were dissolved in 0.5 ml 7 M urea-d_4 98 atom%D (ARMAR Chemicals) solution in D_2O 100 atom%D (ARMAR Chemicals) and the pH was adjusted to 2.3 or 7.4 with DCl (ARMAR Chemicals) or NaOD (ARMAR Chemicals). This resulted in final protein concentrations of approx. 280 µM for both adalimumab and rituximab. For disulfide bond reduction, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma-Aldrich) was added to the samples to a final concentration of 11 mM and samples were incubated for 15 min at 60°C.

To induce a detectable amount of Met(O_2) and Trp oxidation products in a mAb, harsher conditions were required: 2 ml of a rituximab formulation (Mabthera, Roche, exp. year: 2013, 10 mg/ml) were treated with TCEP (11 mM) in 7 M urea (Merck) and heated up to 60°C for 15 min before the oxidation was induced with 1% H_2O_2 for 25 h at RT. For treated and untreated samples the buffer was then changed to ddH_2O with a Spectra/Por dialysis membrane (Cutoff 3.5 kDa), and then the samples were aliquoted and lyophilized. Part of the protein precipitated and the entire suspension was first lyophilized and then further processed as described above.

Details for quantification in 2D NMR spectra

Depending on the dispersion of the signals of interest and their overlap or isolation, different integration methods were suited. For an isolated signal with nearly Gaussian line shape, integration with a Gaussian fit (using Sparky) gave very reproducible integrals. However, for a cluster of many overlapping cross-peaks, integration by Gaussian fit required estimated peak positions and the resulting volume often depended on the number of picked peaks and their initial positions. In contrast, the integral over a box or ellipse (using Sparky) gave reproducible volumes for isolated or a cluster of signals as long as the baseline was flat and centered around zero. However, spectral artefacts like truncation artefacts and small baseline offsets could potentially lead to a systematic error. Since most random coil cross peaks in a 2D ^{1}H-^{13}C HSQC spectrum of a denatured protein consist of a number of overlapping individual signals with very similar chemical shifts, the Gaussian fit integration method

was more sensitive to manually set parameters, whereas the integral over a box or ellipse gave more consistent results and was therefore chosen as the method of choice.

To estimate the error of the volumes, we compared the results between the box integration and Gaussian fit using different numbers of peaks and peak positions, as long as not stated otherwise. With the "slice" option of Sparky, the peak shapes of the signals were compared to the fit of the Gaussian function resulting from the Gaussian integration to judge the quality of the fits. The largest deviation (ΔV) between several trials (eqn. 1) of the volumes (V_i, V_j) from the two integration methods are given in Table S1.

$$\Delta V = |V_i - V_j| \qquad \text{(eqn. 1)}$$

For the overall quantification of Met(O), different approaches were compared to assess the accuracy of the quantification. For the first (a) and third (c) approach the volumes of Met(O) Cγ/Hγ (V_{MetO}) or Met(O) Cε/Hε (V_{MetO}) were compared to the volumes of isolated CH_2 or CH_3 groups of other amino acids (V_X), respectively. To compare V_{Met} and V_x, the values were normalized using the occurrence of an amino acid type in the mAb dimer (n_{Met}, n_X) according to eqn. 2.

$$f_{ox} = \frac{V_{MetO} \cdot n_x}{V_X \cdot n_{Met}} \qquad \text{(eqn. 2)}$$

Concerning the signal of Hγ2 of Ile at approx. 0.91 ppm and Cγ2 at 17.4 ppm we noticed in some spectra a signal nearby at 0.90/16.7 ppm that is not originating from Ile and should therefore be excluded from the integration. The error was calculated using eqn. 3, in which ΔX is the estimated absolute error for the volume of residue X (Table S1).

$$\Delta f_{ox} = \frac{n_x}{n_{Met}} \cdot \frac{V_x \cdot \Delta MetO + V_{MetO} \cdot \Delta X}{{V_X}^2} \qquad \text{(eqn. 3)}$$

The second approach (b) to obtain the fraction of Met(O) (f_{ox}) was calculated as the ratio of the volume of Met(O) Cε/Hε (V_{MetO}) and the sum of the integrals of Met Cε/Hε (V_{Met}) and Met(O) Cε/Hε (V_{MetO}) according to eqn. 4.

$$f_{ox} = \frac{V_{MetO}}{V_{MetO} + V_{Met}} \qquad \text{(eqn. 4)}$$

The error (Δf_{ox}) was calculated according to eqn. 5, in which ΔMetO is the absolute error estimated for the integral of the volume V_{MetO}. Similarly, ΔMet is the absolute error estimated for the non-oxidized volume V_{Met}.

$$\Delta f_{ox} = \frac{V_{Met} \cdot \Delta MetO + V_{MetO} \cdot \Delta Met}{(V_{MetO} + V_{Met})^2} \qquad \text{(eqn. 5)}$$

The fourth, indirect approach (d) was based on the comparison of the volume of the Met Cε/Hε (V_{Met}) signals with the volume of isolated CH_3 groups of other amino acids (V_X), yielding the fraction of non-oxidized methionine. To obtain the Met(O) fraction (f_{ox}) eqn. 6 was applied.

$$f_{ox} = 1 - \frac{V_{Met} \cdot n_x}{V_X \cdot n_{Met}} \qquad \text{(eqn. 6)}$$

The error was calculated according to eqn 7.

$$\Delta f_{ox} = \frac{n_x}{n_{Met}} \cdot \frac{V_x \cdot \Delta Met + V_{Met} \cdot \Delta X}{{V_X}^2} \qquad \text{(eqn. 7)}$$

Quantification of Met(O) by MS

For quantification by MS the same lyophilized adalimumab and rituximab samples as used for the NMR quantification measurements were treated with 20 mmol·L^{-1} iodoacetamide (Sigma-Aldrich, Vienna, Austria) for 30 minutes at 22°C in the dark. Subsequently the samples were rebuffered in 175 mmol·L^{-1} ammonium acetate (Merck, Darmstadt, Germany) using Amicon Ultra-0.5 centrifugal filters with a 3 kDa cut-off (Merck). Subsequently the mAbs were proteolytically digested employing trypsin (Promega, sequencing grade modified trypsin) overnight at 37°C. To suppress additional oxidation during sample preparation, the samples were covered with Argon (SIAD Austria GmbH, St. Pantaleon, Austria). Separation of the obtained peptides was carried out on a Thermo Scientific UltiMate 3000 Rapid Separation system at a flow rate of 80 µL·min^{-1} using a Thermo Scientific™ Hypersil GOLD™ aQ C18 column (100 x 1.0 mm i.d., 1.9 µm particle size, 175 Å pore size), operated at a temperature of 50 °C. Mobile phase A was composed of H_2O (Merck, ultra pure Milli-Q) + 0.1% formic acid (Sigma-Aldrich), mobile phase B of acetonitrile (VWR) + 0.1% formic acid. The gradient applied was: 1.0% B for 2 min, 1.0−5.0% B in 2 min, 5.0−10.0% B in 2 min, 10.0−35.0% B in 30 min, 80% B for 4 min, and 1.0% B for 45 min. The UHPLC system was coupled to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitap™ mass spectrometer equipped with a Thermo Scientific™ Ion Max™ electrospray ionization source with a Thermo Scientific™ heated electrospray ionization (HESI) probe. The instrument settings for analysis were as follows: source heater temperature of 80 °C, spray voltage of 3.5 kV, sheath gas flow of 5 arbitrary units, capillary temperature of 300 °C, S-lens RF level of 60.0. Each scan cycle consisted of a full scan at a scan range of *m/z* 350-2,000 with an AGC target of 1e6, a maximum injection time of 150 ms and a resolution setting of 35,000 at *m/z* 200, followed by ten data-dependent higher-energy collisional dissociation scans at 29% normalized collision energy with an AGC target of 2e5, a maximum injection time of 100 ms and a resolution setting of 17,500 at *m/z* 200. Data analysis

of the acquired mass spectra was conducted using the Thermo Scientific™ BioPharma Finder™ software version 3.0. Identification of modified sites was based on peptide fragmentation, relative quantification of oxidation was based on peak areas of full scan data (% Abundance = [Peak area modified peptide/[Peak area modified + Peak area non-modified peptide]] x 100).

Supplementary References

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