Supporting Information

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SI Text

Analytical Size-Exclusion Chromatography. For quaternary structure analysis of WT and mutant mTNF- α , a Superdex 75 10/300 GL gel filtration column (GE Healthcare) was used. Size-exclusion chromatography was performed in PBS buffer at 25°C and using a flow rate of 0.3 ml/min. The column was calibrated with a molecular mass gel-filtration standard from Bio-Rad containing thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44.0 kDa), myoglobin (17.0 kDa), and vitamin B₁₂ (1.35 kDa). Protein elution was followed by measuring the absorption at 280 nm.

NF_KB-Luciferase Reporter Gene Assay. HEK293 cells stably expressing NF_KB-luciferase were used for the reporter gene assay. The stable cells were dissociated with trypsin, resuspended in DMEM containing 10% FBS at 5 × 105 cells per ml, and plated at 20 µl per well in a 384-well white plate (Greiner). After 2-h incubation at 5% CO₂ in a 37°C tissue culture incubator, 20 µl of mTNF- α was added to the cells. The cells were continuously incubated for 24 h. Luciferase activities were measured by the addition of 20 µl of Bright-Glo (Promega), and the plate was read by using a luminescence plate reader.

ELISA. Maxisorp 384-well plates (Nunc) were coated with 30 μ l of 0.5 μ g/ml protein overnight at 4°C. The coated plates were washed with PBS + 0.05% Tween 20 (PBST), blocked with 80 μ l of 1% BSA in PBS, and washed again with PBST. The plates were sequentially incubated with 20 μ l of primary antibody or serum diluted in 1% BSA in PBS, 20 μ l of HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch), and 20 μ l of TMB substrate (KPL) and read at an absorbance of 650 nm. The plates were washed with PBST between incubations.

Purification of WT and Mutant mTNF- α **Under Native Conditions.** All purification steps were performed at 4°C. After thawing the cell pellet for 15 min on ice, the cell paste was resuspended in lysis buffer [50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 10% (vol/vol) glycerol] at 5 ml per gram of wet weight. After addition of Complete Protease Inhibitor Mixture (Roche), 10 ml of cell suspension was treated with 150 μ l of lysozyme (100 mg/ml; MP Biomedicals), 50 μ l of DNase I (5 mg/ml; Roche), 5 μ l of RNase A (100 mg/ml; Sigma–Aldrich), and 125 units of benzonase nuclease (Novagen). The cell suspension was stirred at room temperature for 20 min to allow lysis to occur. The prelysed cells were then flash-frozen in liquid nitrogen and thawed in a 37°C water bath. This freeze–thaw cycle was repeated once. Complete lysis was then achieved by sonication on ice for 2 min. After

centrifugation at 18,000 × g for 20 min, 1 ml of Ni-NTA His-Bind Resin (Novagen) was added to the supernatant and mixed on a rotary shaker for 30 min. The lysate–resin mixture was loaded onto a 5-ml polypropylene column (Qiagen) and washed twice with 20 ml of lysis buffer. Protein was eluted by the addition of 2 ml of elution buffer [50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 250 mM imidazole, 10% (vol/vol) glycerol], concentrated with a 10K molecular mass cut-off Amicon Ultra-15 centrifugal filter device (Millipore), and further purified by a Superdex 75 10/300 GL column (flow rate of 0.3 ml/min) preequilibrated with PBS. All proteins were characterized by MALDI-TOF mass spectrometry, which was performed on a Voyager-DE-STR instrument (Applied Biosystems) with sinapinic acid as a matrix at The Scripps Center for Mass Spectrometry, The Scripps Research Institute (La Jolla, CA).

MS/MS Sequencing of Tryptic Fragment Containing pNO₂Phe. The excised gel slice containing the pNO₂Phe⁸⁶mTNF- α was diced into small pieces and mixed with 100 μ l of 25 mM NH₄HCO₃/50% acetonitrile. After vortexing for 10 min, the supernatant was discarded. This step was repeated twice, before the gel pieces were dried in a Speed Vac for ≈ 20 min. The protein sample was reduced by addition of 25 μ l of 10 mM DTT in 25 mM NH₄HCO₃. The reaction was allowed to proceed at 56°C for 1 h. After removal of the supernatant, the gel pieces were mixed with 25 μ l of 55 mM iodoacetamide. After incubation in the dark for 45 min at room temperature, the gel pieces were subjected to tryptic in-gel digestion. The resultant peptide mixture was purified by C18 ZipTip (Millipore) and subjected to MS/MS fragmentation on a Thermo Finnigan LTO mass spectrometer (Thermo Scientific), which was run in positive-ion mode using the nanospray source at The Scripps Center for Mass Spectrometry.

IgG Preparation. Murine serum was loaded onto a 10-ml Sepharose-conjugated protein G affinity column (GammaBind Plus Sepharose; Amersham Pharmacia Biotech). The column was washed with three column volumes of PBS (pH 7.4). Elution was carried out with two column volumes of 0.1 M acetic acid (pH 3.0). The eluent was then neutralized with 1 M Tris·HCl (pH 9.0) and dialyzed into PBS (pH 7.4).

T Cell Proliferation Assay. CD4⁺ T cells from immunized mice were isolated from lymph nodes by magnetic depletion with MACS beads (Miltenyi Biotec). T cells were then placed into culture with irradiated splenocytes from naïve Bcl-2 mice and increasing amounts of antigen. The cultures were incubated for 48 h and then pulsed with [³H]thymidine overnight. The culture plates were harvested onto filter mats and radioactivity was quantified with a TopCount scintilltion counter (PerkinElmer).



Sample	Retention time (min)	Observed mass (calculated mass of trimer) (kDa)
pNO ₂ Phe ⁸⁶ mTNF- α without His ₆ tag	33.00	55.2 (51.9)
Phe ⁸⁶ mTNF- α without His ₆ tag	33.20	53.8 (51.7)
pNO ₂ Phe ⁴² mTNF- α with His ₆ tag	32.64	58.0 (57.7)
Phe ⁴² mTNF- α with His ₆ tag	32.01	63.3 (57.6)
wt mTNF- α without His ₆ tag	32.97	55.5 (51.8)
pNO ₂ Phe ¹¹ mTNF- α with His ₆ tag	32.55	58.8 (57.6)

Fig. S1. Characterization of the pNO₂Phe⁸⁶mTNF- α mutant. (*A*) Tandem mass spectrum of the octamer fragment FAISXQEK, where X denotes pNO₂Phe, produced from trypsin digestion of pNO₂Phe⁸⁶mTNF- α . The partial sequence of the octamer containing pNO₂Phe can be read from the annotated b (blue) or y (red) ion series. (*B*) The quaternary structures of pNO₂Phe⁸⁶mTNF- α , Phe⁸⁶mTNF- α , pNO₂Phe⁴²mTNF- α , pNO₂Phe¹¹mTNF- α , and WT mTNF- α were determined based on a plot of the logarithm of the molecular mass of the protein standards versus the retention time on a Superdex 75 10/300 GL gel filtration column.



Fig. S2. Mass spectrometric analysis of pNO₂Phe⁸⁶mTNF-α. (A) MALDI-TOF mass spectrometric analysis of pNO₂Phe⁸⁶mTNF-α. (B) MALDI-TOF mass spectrometric analysis of WT mTNF-α.

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Fig. S4. Serum titers against WT mTNF- α and pNO₂Phe⁸⁶mTNF- α for Bcl2 mice immunized with WT mTNF- α or pNO₂Phe⁸⁶mTNF- α . The RIMMS protocol involved eight injections (5 μ g of protein per injection) over a period of 17 days in the presence of CFA for the initial injection and IFA for the remaining seven injections. ELISAs were measured against WT mTNF- α (red and orange bars) or pNO₂Phe⁸⁶mTNF- α (blue and light blue bars). Before measurement, serum samples were diluted either 1:100 or 1:1,000 with 1% BSA in PBS buffer.

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Fig. S5. Immunization with $pNO_2Phe^{86}mTNF-\alpha$ in the absence of adjuvant. Serum titers for Bcl-2 mice immunized with WT mTNF- α (a), or $pNO_2Phe^{86}mTNF-\alpha$ (b) for eight injections (5 μ g of protein per injection) over a period of 17 days in the absence of either CFA or IFA. ELISAs were measured against WT mTNF- α (red bar) or $pNO_2Phe^{86}mTNF-\alpha$ (blue bar). Before measurement, serum samples were diluted 1:1,000 with 1% BSA in PBS buffer.

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Fig. S6. T cell proliferative assay. (A) Proliferation of CD4⁺T cells from Bcl-2 transgenic mice immunized with WT mTNF- α (pink line), pNO₂Phe⁸⁶mTNF- α (orange line), and Phe⁸⁶mTNF- α (green line) and stimulated *in vitro* with serial dilutions of pNO₂Phe⁸⁶mTNF- α . (B) Proliferation of CD4⁺T cells from Bcl-2 transgenic mice immunized with WT mTNF- α (pink line), pNO₂Phe⁸⁶mTNF- α (orange line), and Phe⁸⁶mTNF- α . (B) Proliferation of CD4⁺T cells from Bcl-2 transgenic mice immunized with WT mTNF- α (pink line), pNO₂Phe⁸⁶mTNF- α (orange line), and Phe⁸⁶mTNF- α (green line) and stimulated *in vitro* with serial dilutions of WT mTNF- α .



Fig. S7. Serum titers against WT mTNF- α and Phe⁸⁶ mTNF- α for Bcl2 mice immunized with Phe⁸⁶ mTNF- α in the absence or presence of adjuvant. For mice immunized without adjuvant, the RIMMS protocol involved eight injections (5 μ g of protein per injection) over a period of 17 days. For mice immunized with adjuvant, CFA was used for the first injection and IFA for the remaining seven injections. ELISAs were measured against WT mTNF- α (red and orange bars) or Phe⁸⁶ mTNF- α (blue and light blue bars). Before measurement, serum samples were diluted either 1:100 or 1:1,000 with 1% BSA in PBS buffer.



Fig. S8. Immunogenicity of other surface sites on mTNF- α . (A) Serum titers against WT mTNF- α , pNO₂Phe⁴²mTNF- α , and Phe⁴² mTNF- α for C57BL/6 mice immunized with either pNO₂Phe⁴²mTNF- α or Phe⁴² mTNF- α . (B) Serum titers against WT mTNF- α , PBS, and pNO₂Phe¹¹mTNF- α for C57BL/6 mice immunized with either pNO₂Phe¹¹mTNF- α or WT mTNF- α . The RIMMS protocol involved eight injections (5 μ g of protein per injection) over a period of 17 days in the absence of adjuvant. ELISAs were measured against WT mTNF- α (red bars), pNO₂Phe⁴²mTNF- α /pNO₂Phe⁴²mTNF- α (blue bars), Phe⁴²mTNF- α (yellow bars), or PBS (green bars). Before measurement, serum samples were diluted 1/100 (A) or 1/800 (B) with 1% BSA in PBS buffer.

Table 1. MS/MS sequencing of a tryptic fragment of pNO₂Phe⁸⁶mTNF-a

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yseries ions147.0147.1 $[y_1]^+$ 276.1276.2 $[y_2+hO]^+$ 258.1258.1 $[y_2+hO]^+$ 258.1258.1 $[y_2+hO]^+$ 259.2259.1 $[y_3]^+$ 404.4404.2 $[y_3+hO]^+$ 366.2366.2 $[y_4]^+$ 596.3596.3 $[y_3^+hO]^+$ 665.4665.3 $[y_5+hO]^{2+}$ 333.1333.1 $[y_5+hO]^{2+}$ 333.1333.1 $[y_6]^+$ 796.3766.4 $[y_6]^+$ 796.3766.4 $[y_6]^{2+}$ 398.9398.7 $[y_6+hO]^+$ 778.3778.4 $[y_6+hO]^+$ 778.3778.4 $[y_7+hO]^+$ 867.3867.4 $[y_7+hO]^+$ 849.6849.4beseries ions111419.2 $[b_3]^+$ 32.0332.2 $[b_3]^+$ 32.0332.2 $[b_3]^+$ 35.9306.1 $[b_3]^+$ 611.3611.3 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 35.9306.1 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 35.9306.1 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 850.386.4 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 739.4739.3 $[b_3]^+$ 739.4739.3 $[b_3]^$		Observed mass, Da	Calculated mass, Da
[y1]^{↑}147.0147.1[y2]^{↑}276.1276.2[y2]+h2]^{↑}258.1258.1[y2]+h3]^{↑}259.2259.1[y3]^{↑}404.4404.2[y3]+t2]^{↑}366.2366.2[y4]+t2]^{↑}366.2366.2[y4]^{↑}596.3596.3[y5]^{↑}665.4665.3[y5]+t2]^{↑}665.4666.3[y5]+t2]^{↑}796.3796.4[y6]+t2]^{↑}796.3778.4[y6]+t2]^{↑}796.3778.4[y6]+t2]^{↑}867.3867.4[y6]+t2]^{↑}849.4849.4[y6]^{↑}332.0332.2[y6]^{↑}332.0332.2[y6]^{↑}332.0332.2[b1]^{↑}332.0332.2[b2]^{↑}311.1419.2[b3]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1[b5]^{↑}305.9306.1	y-series ions		
[y] ¹ 276.1276.2[y2-H20] ⁺ 258.1258.1[y2-H20] ⁺ 259.2259.1[y3-H50] ⁺ 362.2366.2[y3+H50] ⁺ 366.3366.2[y4] ⁺ 596.3596.3[y5-H20] ²⁺ 333.1333.1[y5-H20] ²⁺ 388.9388.7[y6-H20] ⁺ 796.3796.4[y6-H20] ⁺ 797.5779.4[y7-H20] ⁺ 867.3867.4[y7-H20] ⁺ 849.6849.4b-series ions11.1411.2[b3] ⁺ 332.0332.2[b3] ⁺ 332.0332.2[b3] ⁺ 332.0332.2[b3] ⁺ 332.0332.2[b3] ⁺ 335.9332.2[b3] ⁺ 335.9332.2[b3] ⁺ 335.9365.1[b3] ⁺ 355.9306.1[b3] ⁺ 355.9305.1[b3] ⁺ 353.2533.3[b6] ⁺ 739.4739.3[b6] ⁺ 739.4739.3[b6] ⁺ 863.3864.4[b7] ⁺ 863.3864.4[b7] ⁺ 863.3864.4[b7] ⁺ 863.3864.4[b7] ⁺ 863.3864.4[b7] ⁺ 865.3864.4[b7] ⁺ 865.3 </td <td>[y₁]⁺</td> <td>147.0</td> <td>147.1</td>	[y ₁] ⁺	147.0	147.1
[y2+h2)]+258.1258.1[y2,NH3]+259.2259.1[y3,I+]404.4404.2[y3,I+C)]+366.2366.2[y4,I+C)]+596.3663.3[y5,I+C)]+663.3663.3[y5,NH3]+666.3666.3[y5,NH3]+666.3666.3[y6,I+C)]+796.3796.4[y6,I+C)]+778.3778.4[y6,I+C)]+779.5779.4[y7]+2,C]+867.3867.4[y7]+2,C]+842.2434.2[y7]+2,C]+332.0332.2[ba]+419.1419.2[ba]+419.1419.2[ba]+2,C]+201.1201.1[ba]+2,C]+201.1201.1[ba]+2,C]+305.9306.1[ba]+2,C]+305.9306.1[ba]+2,C]+739.4739.3[ba]+4,C]+739.4739.3[ba]+4,C]+739.4739.3[ba]+4,C]+739.4739.3[ba]+4,C]+739.4739.3[ba]+4,C]+739.4739.3[ba]+4,C]+739.4739.3[ba]+4,C]+739.4739.3[bb]+4,C]+739.4739.3[bb]+4,C]+741.3721.3[bb]+4,C]+741.3721.3[bb]+4,C]+868.3868.4[bb]+4,C]+868.3868.4[bb]+4,C]+868.3868.4[bb]+4,C]+868.3868.4[bb]+4,C]+868.3868.4[bb]+4,C]+868.3 <t< td=""><td>[y₂]⁺</td><td>276.1</td><td>276.2</td></t<>	[y ₂] ⁺	276.1	276.2
jy-Rhy]+259.2259.1(y_3)+404.4404.2(y_3+Lo)+386.2386.2(y_4)+596.3596.3(y_5)+683.3683.3(y_5+Lo)+665.4665.3(y_5+Lo)2++333.1333.1(y_6)+796.3796.4(y_6)+796.3796.4(y_6)+796.3796.4(y_6)+796.3796.4(y_6)+778.3778.4(y_6+Lo)+778.3778.4(y_6+Lo)+779.4867.3(y_7+Lo)+867.3867.4(y_7+Lo)+867.3867.4(y_7+Lo)+810.4810.4(y_7+Lo)+820.9332.2(y_7+Lo)+111419.2(La)+419.1419.2(La+Lo)+201.1419.2(La+Lo)+401.1401.2(La+Lo)+401.1401.2(La+Lo)+739.4739.3(La+Lo)+739.4739.3(La+Lo)+739.4739.3(La+Lo)+739.4739.3(La+Lo)+739.4739.3(La+Lo)+850.3860.4(La+Lo)+851.1851.4(La+Lo)+850.4860.4	[y ₂ -H ₂ O] ⁺	258.1	258.1
lyg1+404.4404.2lyg-t2Q1+366.2366.2lyg1+596.3596.3lyg1+683.3683.3lyg-t2Q1+333.1333.1lyg-t2Q1+333.1333.1lyg1+666.3666.3lyg1+796.3796.4lyg1+795.3778.4lyg-t4Q01+778.3779.4lyg1+795.5867.4lyg1+2Q1+434.2434.2lyg1+2Q1+434.2434.2lyg1+2Q1+32.0332.2lyg1+2Q1+218.9219.1lbg1+32.0332.2lbg1+2Q1+401.1401.2lbg1+2211.1401.2lbg1+2305.9306.1lbg1+4Q01+401.1401.2lbg1+4Q01+35.9305.1lbg1+479.4593.2lbg1+279.4593.2lbg1+279.4593.2lbg1+279.4593.2lbg1+179.4lbg1+179.4lbg1+279.4lbg1+179.4lbg1+179.4lbg1+179.3lbg1+179.3lbg1+179.4lbg1+179.3lbg1+179.4lbg1+179.4lbg1+179.3lbg1+279.4lbg1+179.4lbg1+279.4lbg1+279.4lbg1+279.4lbg1+179.4lbg1+179.4lbg1+1	[y ₂ -NH ₃] ⁺	259.2	259.1
yg-hz,Ql+386.2386.2yg-l+596.3596.3yg-l+2,Ql+683.3683.3yg-Hz,Ql+665.4665.3yg-Hz,Ql+33.133.1yg-Hz,Ql+796.3796.4yg-Hz,Ql+796.3796.4yg-Hz,Ql+796.3796.4yg-Hz,Ql+796.3796.4yg-Hz,Ql+797.5779.4yg-Hz,Ql+867.3867.4yg-Hz,Ql+849.6849.4yg-Hz,Ql+849.6849.4yg-Hz,Ql+332.0332.2 b_1 +11.1419.2 b_1++739.4419.1 b_1++352.0332.2 b_1++13.3611.3 b_1++13.3611.3 b_1++739.4739.3 b_1++739.4739.3 b_1++739.4739.3 b_1++739.4739.3 b_1++739.4739.3 b_1++739.4739.3 b_1++850.3850.4 b_1++850.3850.4 b_1++850.3850.4 b_1++850.3850.4 b_1++850.3850.4 b_1++851.1851.4 b_1++850.4850.4 b_1++850.4850.4	[y ₃] ⁺	404.4	404.2
[ya] ⁺ 596.3596.3[ys] ⁺ 683.3683.3[ys-H_2O] ⁺ 665.4665.3[ys-H_2O] ²⁺ 333.1333.1[ys-H_2O] ²⁺ 796.3666.3[ya] ⁺ 796.3796.4[ya] ²⁺ 796.3796.4[ya] ²⁺ 796.378.4[ye-H_2O] ¹⁺ 778.3778.4[ye-H_2O] ¹⁺ 779.5779.4[yr] ¹⁺ 867.3867.4[yr] ²⁺ 434.2434.2[yr] ²⁺ 434.2434.2[yr] ²⁺ 18.9219.1[b_2] ¹⁺ 332.0332.2[b_3] ¹⁺ 419.1419.2[b_4+D_2O] ²⁺ 201.1201.1[b_3] ¹⁺ 611.3611.3[b_3] ²⁺ 593.2593.3[b_6] ¹⁺ 739.4739.3[b_6] ¹⁺ 739.4739.3[b_6] ¹⁺ 739.4739.3[b_6] ¹⁺ 739.4739.3[b_7] ¹⁺ 868.3868.4[b_7] ¹⁺ 850.3861.4[b_7] ¹⁺ 851.1851.4[b_7] ¹⁺ 851.1851.4[b_7] ¹⁺ 850.4850.4	[y ₃ -H ₂ O] ⁺	386.2	386.2
[yg]+683.3683.3[yg+LQ]+665.4665.3[yg+LQ]2+333.1333.1[yg-NH3]+666.3666.3[yg]2+796.3796.4[yg]2+796.3796.4[yg+LQ]1+778.3778.4[yg-H4,Q]1+779.5779.4[yr]2+849.6849.4yr_PL2O]+849.6849.4b-series ions111419.2[b_1]+332.0332.2[b_1]+201.1201.1[b_1]2+201.1201.1[b_1]2+35.9306.1[b_1]2+35.9306.1[b_1]2+739.4306.1[b_1]2+859.2306.1[b_1]2+739.4739.3[b_1]2+739.4739.3[b_1]2+739.4739.3[b_1]2+739.4739.3[b_1]2+739.4739.3[b_1]2+850.3868.4[b_7+LQ]1+851.1851.4[b_7]2+850.3868.4[b_7+LQ]1+851.1851.4[b_7]2+850.4850.4	[y ₄] ⁺	596.3	596.3
[y5-H20]+665.4665.3(y5-H20]+333.1333.1[y5-H40]+333.1333.1(y5-H40]+666.3666.3(y6]+796.3796.4(y6]+796.3398.9(y6-H40)+778.3778.4(y6-H410)+779.5779.4(y7)+867.3867.4(y7)+2434.2434.2(y7)+20]+867.3867.4(y7)+20]+849.6849.45-sries ions11419.2[b2]+218.9219.1[b3]+332.0332.2[b4]+419.1419.2[ba]+20]+401.1401.2[ba+H20]+611.3611.3[b3]+20]+593.2593.3[b4]+20]+739.4739.3[b5]+40]+739.4739.3[b5]+40]+850.3868.4[b7-H20]+850.3850.4[b7-H20]+850.3850.4	[y ₅] ⁺	683.3	683.3
İys-H2Ol ²⁺ 333.1 333.1 İys-H2Ol ²⁺ 666.3 666.3 İysl ⁺ 796.3 796.4 İysl ²⁺ 388.9 398.7 İys-H2Ol ⁺ 778.3 778.4 İys-H2Ol ⁺ 779.5 779.4 İyr-H2Ol ⁺ 867.3 867.4 İyr-İ ²⁺ 849.6 849.4 İyr-H2Ol ⁺ 849.6 849.4 İyr-H2Ol ⁺ 218.9 219.1 İyal ⁺ 419.1 419.2 İyal ⁺ 219.1 333.2 İyal ⁺ 419.1 419.2 İyal ⁺ 419.1 419.2 İyal ⁺ 201.1 201.1 İyal ⁺ 305.9 306.1 İyal ⁺ 305.9 306.1 İyal ⁺ 739.3 739.3 İbal ⁺ 739.4 739.3 İbal ⁺ 739.4 739.3 İbal ⁺ 739.4 739.3 İbal ⁺ 721.3 721.3 İbal ⁺ <td>[y₅-H₂O]⁺</td> <td>665.4</td> <td>665.3</td>	[y ₅ -H ₂ O] ⁺	665.4	665.3
[ys-NH3] ⁺ 666.3666.3[ys] ⁺ 796.3796.4[ys] ²⁺ 398.9398.7[ys-H20] ⁺ 778.3778.4[ys-NH3] ⁺ 779.5779.4[yr] ²⁺ 867.3867.4[yr] ²⁺ 434.2434.2[yr-H20] ⁺ 849.6849.4b-series ions11419.2[ba] ⁺ 419.1419.2[ba] ⁺ 401.1401.2[ba] ⁺ 611.3611.3[bs] ²⁺ 305.9306.1[bs] ²⁺ 305.9306.1[bs] ²⁺ 739.4739.3[bs] ¹⁺ 688.3868.4[br-H20] ⁺ 868.3868.4[br-H20] ⁺ 850.3850.4	[y ₅ -H ₂ O] ²⁺	333.1	333.1
[y ₆] ⁺ 796.3 796.4 [y ₆] ² + 398.9 398.7 [y ₆ +l ₂ 0] ⁺ 778.3 778.4 [y ₆ +l ₃] ⁺ 779.5 779.4 [y ₇] ² + 867.3 867.4 [y ₇] ² + 34.2 34.2 [y ₇] ² + 849.6 849.4 b-series ions 849.4 849.4 [b ₃] ⁴ 32.0 32.2 [b ₄] ¹ 419.1 419.2 [b ₄ +l ₂ O] ²⁺ 201.1 201.1 [b ₄ +l ₂ O] ²⁺ 201.1 201.1 [b ₅] ¹ ²⁺ 305.9 306.1 [b ₅] ²⁺ 305.9 306.1 [b ₅] ²⁺ 305.9 306.1 [b ₅] ² 721.3 721.3 [b ₆] ¹ 739.4 739.3 [b ₆] ¹⁺ 868.3 868.4 [b ₇ +l ₂ O] ¹⁺ 868.3 868.4 [b ₇ +l ₂ O] ¹⁺ 850.4 850.4	[y ₅ -NH ₃] ⁺	666.3	666.3
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	[y ₆] ⁺	796.3	796.4
[y ₆ -H ₂ O] ⁺ 778.3 778.4 [y ₆ -H ₂ O] ⁺ 779.5 779.4 [y ₇] ² 867.3 867.4 [y ₇] ² + 849.2 849.2 b-series ions 849.6 849.4 b-series ions 1 1 [b ₂] ⁴ 32.0 332.2 [b ₄] ⁴ 419.1 419.2 [b ₄ -H ₂ O] ⁴ 401.1 401.2 [b ₄ -H ₂ O] ² + 201.1 201.1 [b ₄] ² 35.9 366.1 [b ₅] ⁴ 93.2 593.3 [b ₆] ⁴ 739.4 739.3 [b ₆] ⁴ 739.4 739.3 [b ₆] ⁴ 739.4 739.3 [b ₆] ⁴ 739.4 739.3 [b ₆] ⁴ 739.4 739.3 [b ₆] ⁴ 739.4 739.3 [b ₆] ⁴ 739.4 739.3 [b ₆] ⁴ 739.4 739.3 [b ₆] ⁴ 739.4 850.3 850.4 [b ₇] ⁴ 868.3	[y ₆] ²⁺	398.9	398.7
[y6-NH3]+ 779.5 779.4 [y7]+ 867.3 867.4 [y7]2+ 434.2 434.2 [y7+AQ]+ 89.6 849.4 b-series ions 867.3 867.4 [b_1]+ 218.9 219.1 [b_3]+ 332.0 332.2 [b_4]+ 419.1 419.2 [b_4]+ 419.1 419.2 [b_4+P_Q]+ 401.1 401.2 [b_4+Q_Q]+ 611.3 611.3 [b_5]+2 305.9 306.1 [b_5+H_2Q]+ 593.2 593.3 [b_6]+ 739.4 739.3 [b_6]+2,Q]+ 721.3 721.3 [b_7+A_2Q]+ 868.3 868.4 [b_7+N_3]+ 851.1 851.4 [b_7+A_2Q]+ 868.3 868.4	[y ₆ -H ₂ O] ⁺	778.3	778.4
[y ₇] ⁺ 867.3 867.4 [y ₇] ²⁺ 434.2 434.2 [y ₇ H ₂ O] ⁺ 849.6 849.4 b-series ions 1 1 [b ₂] ⁺ 218.9 219.1 [b ₃] ⁺ 332.0 332.2 [b ₄] ⁺ 419.1 419.2 [b ₄ -H ₂ O] ⁺ 401.1 401.2 [b ₄ -H ₂ O] ²⁺ 201.1 201.1 [b ₅] ²⁺ 611.3 611.3 [b ₅] ²⁺ 593.2 593.3 [b ₆] ⁴ 739.4 739.3 [b ₆ -H ₂ O] ⁺ 868.3 868.4 [b ₇ -NH ₃] ⁺ 850.3 850.4	[y ₆ -NH ₃] ⁺	779.5	779.4
[y ₇] ²⁺ 434.2 434.2 [y ₇ +l ₂ O] ⁺ 849.6 849.4 b-series ions 1 [b ₂] ⁺ 218.9 219.1 [b ₃] ⁺ 332.0 332.2 [b ₄] ⁺ 419.1 419.2 [b ₄ -H ₂ O] ⁺ 401.1 401.2 [b ₄ -H ₂ O] ⁺ 201.1 201.1 [b ₅] ²⁺ 305.9 306.1 [b ₅] ²⁺ 305.9 306.1 [b ₅] ² + 305.9 306.1 [b ₅ -H ₂ O] ⁺ 739.4 739.3 [b ₆] ⁺ 721.3 721.3 [b ₇ -H ₂ O] ⁺ 850.3 850.4 [b ₇ -H ₂ O] ⁺ 851.1 851.4 [b ₇ -H ₂ O] ⁺ 850.3 850.4 [b ₇ -H ₂ O] ⁺ 850.4 850.4	[y ₇] ⁺	867.3	867.4
[y7-H2O] ⁺ 849.6 849.4 b-series ions 219.1 [b_] ⁺ 332.0 332.2 [b_] ⁺ 332.0 332.2 [b_1 ⁺ 419.1 419.2 [b_4-H2O] ⁺ 401.1 401.2 [b_4-H2O] ²⁺ 201.1 201.1 [b_3] ²⁻⁴ 305.9 306.1 [b_3] ²⁺⁴ 305.9 306.1 [b_3-H2O] ⁺ 593.2 593.3 [b_6] ¹⁺ 739.4 739.3 [b_6-H2O] ⁺ 868.3 868.4 [b_7-H2O] ⁺ 850.3 850.4 [b_7-H2O] ⁺ 868.3 868.4 [b_7-H2O] ⁺ 850.4 850.4	[y ₇] ²⁺	434.2	434.2
b-series ions 218.9 219.1 [b_3]+ 332.0 332.2 [b_4]+ 419.1 419.2 [b_4-H_2O]+ 401.1 401.2 [b_4-H_2O]2+ 201.1 201.1 [b_3]2+ 305.9 306.1 [b_5]2+ 305.9 306.1 [b_6]+ 593.2 593.3 [b_6]+ 739.4 739.3 [b_6]+2O]+ 868.3 868.4 [b_7-H_2O]+ 850.3 850.4 [b_7-H_2O]+ 850.3 850.4 [b_7]+2O]+ 868.3 868.4 [b_7]+2O]+ 850.4 850.4	[y ₇ -H ₂ O] ⁺	849.6	849.4
[b_2]+218.9219.1[b_3]+332.0332.2[b_4]+419.1419.2[b_4-H_2O]+401.1401.2[b_4-H_2O]2+201.1201.1[b_5]+611.3611.3[b_5]2+305.9306.1[b_5-H_2O]+593.2593.3[b_6]+739.4739.3[b_7]+868.3868.4[b_7-H_2O]+850.3850.4[b_7]+851.1851.4[b_7]+868.3868.4[b_7]+868.3868.4[b_7]+850.4850.4	b-series ions		
[b3]+332.0332.2[b4]+419.1419.2[b4-H2O]+401.1401.2[b4-H2O]2+201.1201.1[b5]+611.3611.3[b5]2+305.9306.1[b5-H2O]+593.2593.3[b6]+739.4739.3[b6]+721.3721.3[b7]+868.3868.4[b7-H2O]+850.3850.4[b7]+868.3850.4[b7]+868.3868.4[b7]+868.3868.4[b7]+869.3850.4[b7]+869.3868.4[b7]+869.3869.4[b7]+869.3869.4[b7]+869.3869.4[b7]+869.3869.4[b7]+869.4869.4[b7]+869.4869.4[b7]+869.4869.4[b7]+869.4869.4[b7]+869.4869.4[b7]+850.4869.4[b7]+850.4869.4[b7]+850.4869.4[b7]+850.4850.4	[b ₂] ⁺	218.9	219.1
$\begin{array}{llllllllllllllllllllllllllllllllllll$	[b ₃] ⁺	332.0	332.2
	[b ₄] ⁺	419.1	419.2
	[b ₄ -H ₂ O] ⁺	401.1	401.2
$\begin{array}{cccc} [b_{5}]^{+} & 611.3 & 611.3 \\ [b_{5}]^{2+} & 305.9 & 306.1 \\ [b_{5}+h_{2}O]^{+} & 593.2 & 593.3 \\ [b_{6}]^{+} & 739.4 & 739.3 \\ [b_{6}-h_{2}O]^{+} & 721.3 & 721.3 \\ [b_{7}]^{+} & 868.3 & 868.4 \\ [b_{7}-H_{2}O]^{+} & 850.3 & 850.4 \\ [b_{7}-NH_{3}]^{+} & 851.1 & 851.4 \\ [b_{7}]^{+} & 868.3 & 868.4 \\ [b_{7}-H_{2}O]^{+} & 868.3 & 868.4 \\ [b_{7}-H_{2}O]^{+} & 868.3 & 868.4 \\ [b_{7}-H_{2}O]^{+} & 868.3 & 868.4 \\ [b_{7}-H_{2}O]^{+} & 868.3 & 868.4 \\ [b_{7}-H_{2}O]^{+} & 868.3 & 868.4 \\ [b_{7}-H_{2}O]^{+} & 868.3 & 868.4 \\ [b_{7}-H_{2}O]^{+} & 850.4 & 850.4 \\ \end{array}$	[b ₄ -H ₂ O] ²⁺	201.1	201.1
$\begin{array}{llllllllllllllllllllllllllllllllllll$	[b ₅] ⁺	611.3	611.3
$\begin{array}{cccc} [b_5 - H_2 O]^+ & 593.2 & 593.3 \\ [b_6]^+ & 739.4 & 739.3 \\ [b_6 - H_2 O]^+ & 721.3 & 721.3 \\ [b_7]^+ & 868.3 & 868.4 \\ [b_7 - H_2 O]^+ & 850.3 & 850.4 \\ [b_7 - NH_3]^+ & 851.1 & 851.4 \\ [b_7]^+ & 868.3 & 868.4 \\ [b_7]^+ & 868.3 & 868.4 \\ [b_7 - H_2 O]^+ & 868.3 & 868.4 \\ \end{tabular}$	[b ₅] ²⁺	305.9	306.1
$\begin{array}{cccc} [b_6]^+ & 739.4 & 739.3 \\ [b_6-H_2O]^+ & 721.3 & 721.3 \\ [b_7]^+ & 868.3 & 868.4 \\ [b_7-H_2O]^+ & 850.3 & 850.4 \\ [b_7-NH_3]^+ & 851.1 & 851.4 \\ [b_7]^+ & 868.3 & 868.4 \\ [b_7-H_2O]^+ & 850.4 & 850.4 \end{array}$	[b ₅ -H ₂ O] ⁺	593.2	593.3
$\begin{array}{cccc} [b_6-H_2O]^+ & 721.3 & 721.3 \\ [b_7]^+ & 868.3 & 868.4 \\ [b_7-H_2O]^+ & 850.3 & 850.4 \\ [b_7-NH_3]^+ & 851.1 & 851.4 \\ [b_7]^+ & 868.3 & 868.4 \\ [b_7-H_2O]^+ & 850.4 & 850.4 \end{array}$	[b ₆] ⁺	739.4	739.3
[b7]+ 868.3 868.4 [b7-H2O]+ 850.3 850.4 [b7-NH3]+ 851.1 851.4 [b7]+ 868.3 868.4 [b7-H2O]+ 850.4 869.4	[b ₆ -H ₂ O] ⁺	721.3	721.3
[b ₇ -H ₂ O] ⁺ 850.3 850.4 [b ₇ -NH ₃] ⁺ 851.1 851.4 [b ₇] ⁺ 868.3 868.4 [b ₇ -H ₂ O] ⁺ 850.4 850.4	[b ₇] ⁺	868.3	868.4
[b ₇ -NH ₃] ⁺ 851.1 851.4 [b ₇] ⁺ 868.3 868.4 [b ₇ -H ₂ O] ⁺ 850.4 850.4	[b ₇ -H ₂ O] ⁺	850.3	850.4
[b ₇] ⁺ 868.3 868.4 [b ₇ -H ₂ O] ⁺ 850.4 850.4	[b ₇ -NH ₃] ⁺	851.1	851.4
[b ₇ -H ₂ O] ⁺ 850.4 850.4	[b ₇] ⁺	868.3	868.4
	[b ₇ -H ₂ O] ⁺	850.4	850.4

The sequence of the tryptic fragment containing pNO₂Phe is shown in single letter code (X, pNO₂Phe). Observed fragment ions of the y and b series are indicated. Key y and b ions proving the incorporation of pNO₂Phe are represented in red. All masses are reported as monoisotopic masses.