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Supporting Information

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Supporting Information for

A High-Resolution Interaction Map of Three Transcriptional Activation Domains with a Key Coactivator from Photo-Cross-Linking and Multiplexed Mass Spectrometry

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Peptide synthesis

The TADs VP2 DFDLDMLGD(pBpa)DLDMLGC,
Gal4(854-874) CGMFNTTTMDDVYNYL(pBpa)DDEDT
and Gcn4 (105-134) TPMFEYENLEDNSKEWTSL(pBpa)DNDIPVTTDDC
were synthesized using solid phase peptide synthesis in accordance with standard protocols.[1] TADs containing Phe in place of pBpa were synthesized in an analogous manner. FMOC-pBpa was purchased from Chem-Impex International. The pBpa containing peptides were biotin-labeled at the cysteine residue using Biotin-HPDP (*N*-(6-(Biotinamido)hexyl)-3'-(2'-pyridyldithio)-propionamide (Pierce) in 50% DMF, 10% CH_3CN, 0.1 %TFA overnight at 37 °C. Biotin labeling allows enrichment of TAD-Med15 cross-linked peptides following trypsin digestion (described below). Such enrichment is necessary to avoid mass spectrometric ion suppression from smaller non-crosslinked peptides. The peptides without pBpa were labeled using Fluorescein-NHS ester (Pierce) in 10% DMF, 10% CH_3CN, 100 mM $NaHCO_3$ pH 8.6 The products were purified to homogeneity using reversed-phase HPLC on a C18 column with a gradient solvent system (buffer A: 0.1% TFA, buffer B: CH_3CN) and stored at -80 ºC. The identity was verified using electrospray mass spectrometry (LCT Micromass).

Med15(Gal11) bacterial expression

Expression of Med15 fragments fused to the His_6-MBP and/or His_6-mOCR solubility tags was carried out in Rosetta2 (DE3) pLysS *E. coli* cells (Novagen) as previously described.[2] Briefly, cultures (50 mL) from single colonies were grown overnight at 37 °C (300 rpm) in Select APS Super Broth (Difco) supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) before addition to 1L of Select APS Super Broth supplemented with ampicillin (100 μg/mL). After an OD_{600} of 0.3 was reached, the cultures were cooled to 20 °C for 1 h, and expression was induced with IPTG (final concentration 0.1 mM) for ~12 h. The cell pellet was resuspended in Buffer A (100 mM Tris pH 7.5 at 4 °C, 10% glycerol (v/v), 150 mM NaCl, 10 mM β-ME and Roche Complete Protease Inhibitor Cocktail), lysed using sonication, and the His-tagged protein was isolated using Ni NTA-Agarose (Qiagen). The Ni-NTA beads were washed 6 times with Wash buffer (100 mM Tris pH 7.5 at 4 °C, 10% glycerol (v/v), 150 mM NaCl, 10 mM β-ME, 30 mM imidazole). The protein was eluted from the beads at 4 ºC 3 times using Elution buffer (100 mM Tris pH 7.5 at 4 °C, 10% glycerol (v/v), 150 mM NaCl, 10 mM β-ME, 300 mM imidazole). In the case of Med15(1-416), the His_6-mOCR tag was cleaved using TEV protease by incubating with TEV in accordance with manufacturer protocol (Invitrogen). The resulting mixture was placed in dialysis tubing (Pierce) and the buffer exchanged to Storage Buffer (50 mM Tris pH 7.5 at 4 °C, 10% glycerol (v/v), 150 mM NaCl, 10 mM β-ME) overnight at 4 °C. The solution thus obtained (containing Med15(1-416), TEV, His_6-mOCR) was concentrated using a Vivascience 30K centrifugal filter device and then loaded onto a gel filtration column (Superdex 75, GE Healthcare) pre-equilibrated with Storage buffer to remove His_6-mOCR and TEV. Fractions containing Med15(1-416) were pooled and concentrated using a Vivascience

30K centrifugal filter device. The protein concentration was measured using absorbance at 280 nm. The identity and purity of the protein was verified by reducing SDS-PAGE with appropriate molecular weight standards.
Cysteine 306 in Med15 was alkylated to prevent it from cleaving the disulfide linker between the TADs and biotin. For this purpose, Med15(1-416) was treated with 10 mM DTT for 1 h. Subsequently 40 mM iodoacetamide (1M in 100 mM $NaHCO_3$) was added and the protein solution was incubated in the dark for 45 min. Excess iodoacetamide and any reducing agent were removed using a PD-10 column (GE Healthcare) pre-equilibrated with Storage buffer that did not contain any reducing agents.

In vitro cross-linking
For each cross-linking reaction utilized for mass spectrometry analysis, biotinylated TAD was dissolved in minimal DMSO and diluted to a 100 μL volume of 100 mM PBS (pH 7.2) such that the final concentration of DMSO was <10% (final TAD concentration 100-300μM). This solution was mixed with 100 μL Med15 (100-300 μM concentration) and irradiated with a 150W 365 nm UV lamp (BIB-150P, Spectroline) at a distance of 20 cm at 4 °C for 2 h. The irradiated sample was then buffer exchanged with a 1 mL 30K MW cutoff concentrator (Millipore) with 100 mM PBS to remove free TAD (4 x 1mL). This solution was subsequently incubated with 10 μg sequencing grade trypsin (Promega) for 5 h at 37 °C. After digesting the sample, trypsin was inactivated by vortexing and incubating at 65 °C for 10 min. Next the sample was added to 100 μL of neutravidin beads (Pierce) prewashed with 100 mM PBS and incubated for 1 hr at 4 °C with shaking. To remove non-specifically bound tryptic Med15 fragments, the neutravidin beads were washed (6 x 1 mL) with 100 mM PBS (pH 7.2). To elute the biotinylated products the beads were incubated 2x with 100 μL Elution buffer (100 mM PBS, 100 mM DTT) for 1 h at 65 °C. The eluted samples were stored at -80 °C until mass spectrometry analysis was performed. Immediately prior to mass spectrometry analysis, the samples were desalted using a C18 ZIP tips (Millipore) and exchanged to 0.1% formic acid, 70% acetonitrile or 70% methanol solution. A combination of MALDI–TOF/TOF and ESI-FT-ICR mass spectrometry (MS) analysis was performed.

Mass spectrometry analysis
For electrospray ionization (ESI) in both positive and negative mode, samples were analyzed with an actively shielded 7 Tesla quadrupole-Fourier transform ion cyclotron resonance mass spectrometer (APEX-Q, Bruker Daltonics, Billerica, MA). Target analytes in electrospray solution (1:1 CH_3CN:H_2O with 0.1% HCOOH for positive ion mode, and 1:1 CH_3CN: H_2O with 20 mM NH_4HCO_3 for negative ion mode) were directly infused into an ESI source (Apollo II, Bruker Daltonics) at a flow rate of 70 μL/h. A counterflow of hot (240 °C) nitrogen gas was applied to assist desolvation of ESI droplets. Multiply protonated ions generated by ESI were externally accumulated in a hexapole and transferred into an infinity ICR cell[3] through a series of high-voltage optics for m/z analysis. For external collision induced dissociation (CID), precursor ions were mass-selectively accumulated in the hexapole collision cell at collision voltages ranging from - 18-24 V. Argon was used as collision gas. All data were acquired with XMASS software (version 6.1, Bruker Daltonics) in broadband mode from m/z = 200 to 2000 with 512k data points and summed up to 50 scans. Mass spectra were analyzed with MIDAS software.[4] Due to the possibility of cross-linking hindering access to tryptic cleavage sites in the vicinity of the TAD binding sites, up to five trypsin missed cleavages were considered.

For MALDI analysis (performed at the Michigan Proteome Consortium), 5 μL of alpha-cyano-4-hydroxycinnaminic acid (5 mg/mL in 50% acetonitrile, 0.1% TFA, 2mM ammonium citrate) were added to the avidin purified crosslinked tryptic peptides. Samples were taken to dryness and resuspended in 5 uL of 50% acetonitrile/0.1% TFA. 0.5 uL of this solution were hand-spotted onto a 192-well MALDI target and allowed to dry under atmosphere. MALDI mass spectra were acquired on an Applied Biosystems 4800 Proteomics Analyzer (TOF/TOF) in reflector positive ion mode. Peptide masses were acquired up to 8000 Da. MS spectra were obtained with 2000 laser (Nd-YAG laser operating at 355 nm and 200 Hz) shots. At least 3 trypsin autolysis peaks were used for internal calibration. MS/MS spectra were also acquired in positive ion mode. Signals in the MS/MS spectra were accumulated based on 6,000 laser shots, or until up to 5 peptide fragment ions were observed to reach a signal to noise ratio of 100. Atmosphere, at a pressure of around $6x10^{-7}$ torr was used as collision gas with a collision energy of 2 keV.

Fluorescence polarization assays

Apparent dissociation constants for fluorescein labeled TADs and Med15(1-416) were determined using fluorescence polarization as previously described.[1] For each experiment, 50 nM TAD was incubated with varying concentrations of Med15(1-416) in 100 mM PBS pH 7.2 and incubated for 10 min in 384 well low volume plate (Corning) before being detected using a TECAN Genios Pro plate reader and the dissociation constant calculated as previously described.[1]

β-galactosidase assays

Low copy yeast plasmids expressing Gal4(1-100) fused to Gal4(840-881), Gcn4 (105-144) or VP2 under the control of the β-actin promoter were generated using standard molecular biology techniques.[5] Plasmids encoding different fragments of Med15 were generated from Ycplac111 Med15 WT, a low copy plasmid that expresses Med15 under the control of its own promoter.[6] LS41ΔMed15 [JPY9::ZZ41, *Matα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2Δ385 gal4* URA::pZZ41 Med15::TRP] yeast was co-transformed with the plasmids encoding each Gal4(1-100)+TAD fusion and a portion of Med15. The activity of each construct was monitored using β-galactosidase assays as previously described.[6]

References

[1] Z. Wu, G. Belanger, B. B. Brennan, J. K. Lum, A. R. Minter, S. P. Rowe, A. Plachetka, C. Y. Majmudar, A. K. Mapp, *J Am Chem Soc* **2003**, *125*, 12390.

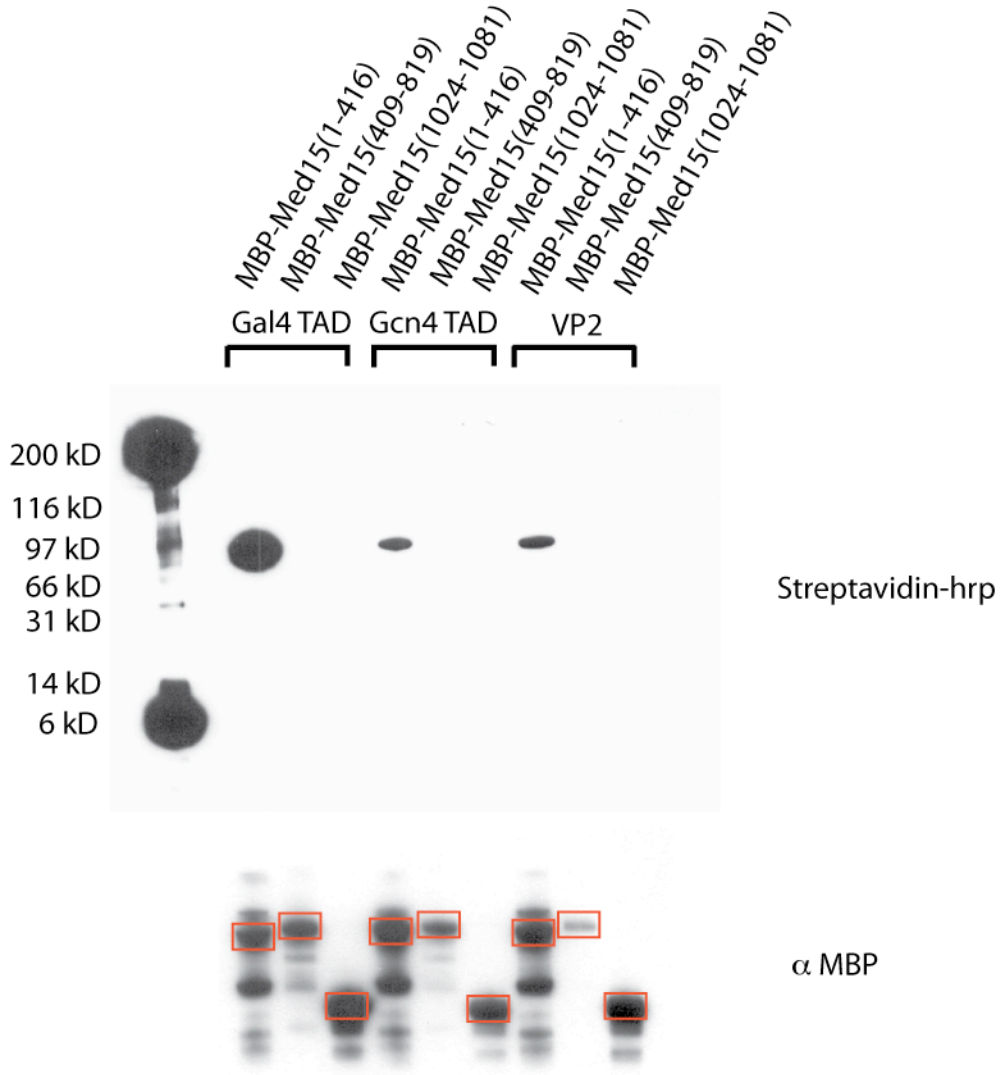
[2] J. DelProposto, C. Y. Majmudar, J. L. Smith, W. C. Brown, *Protein Expr Purif* **2009**, *63*, 40.

[3] P. Caravatti, M. Allemann, *Organic Mass Spectrometry* **1991**, *26*, 514.

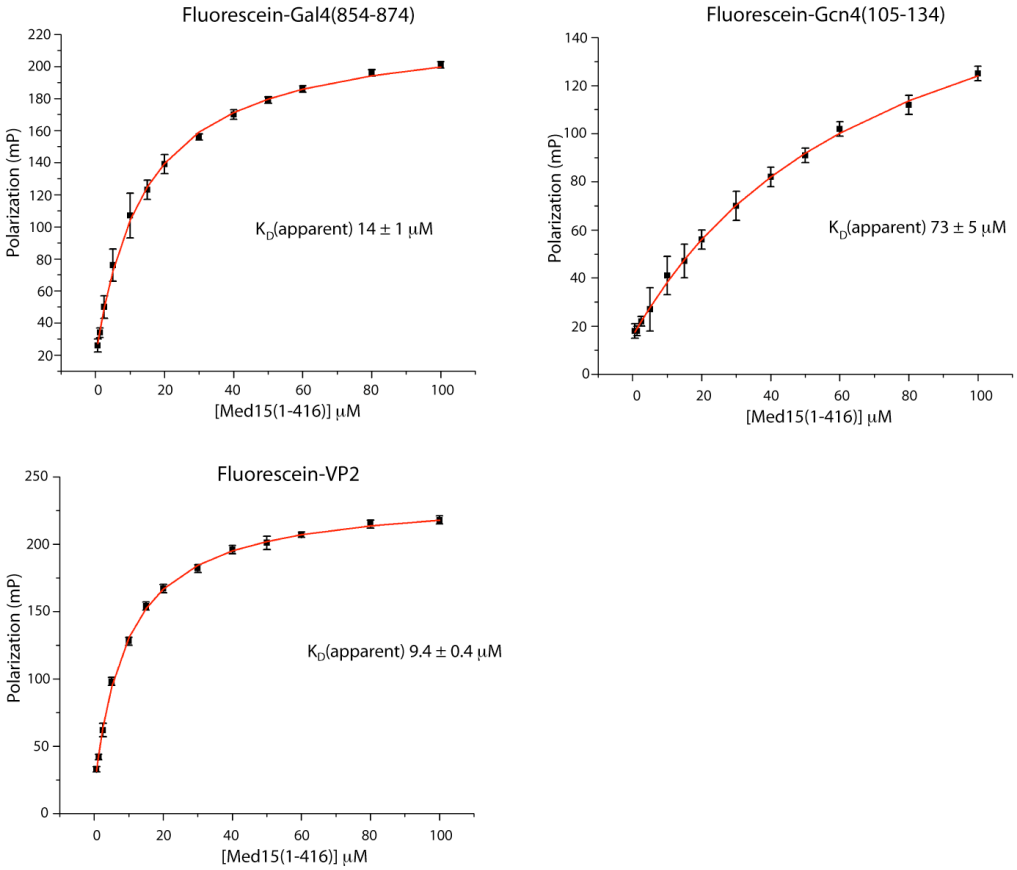
[4] M. W. Senko, J. D. Canterbury, S. H. Guan, A. G. Marshall, *Rapid Communications in Mass Spectrometry* **1996**, *10*, 1839.

[5] J. K. Lum, C. Y. Majmudar, A. Z. Ansari, A. K. Mapp, *ACS Chem Biol* **2006**, *1*, 639.

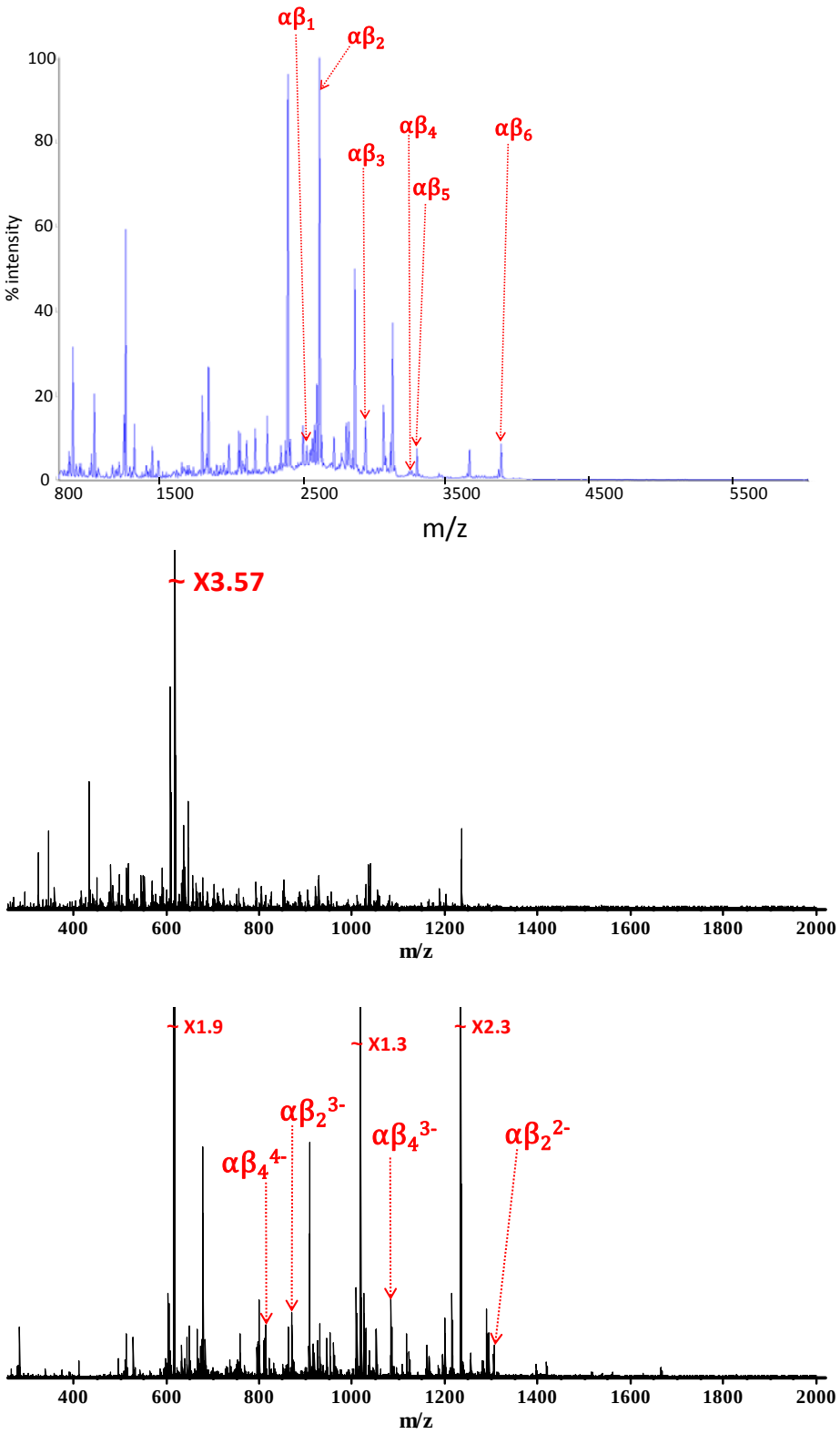
[6] C. Y. Majmudar, J. K. Lum, L. Prasov, A. K. Mapp, *Chem Biol* **2005**, *12*, 313.



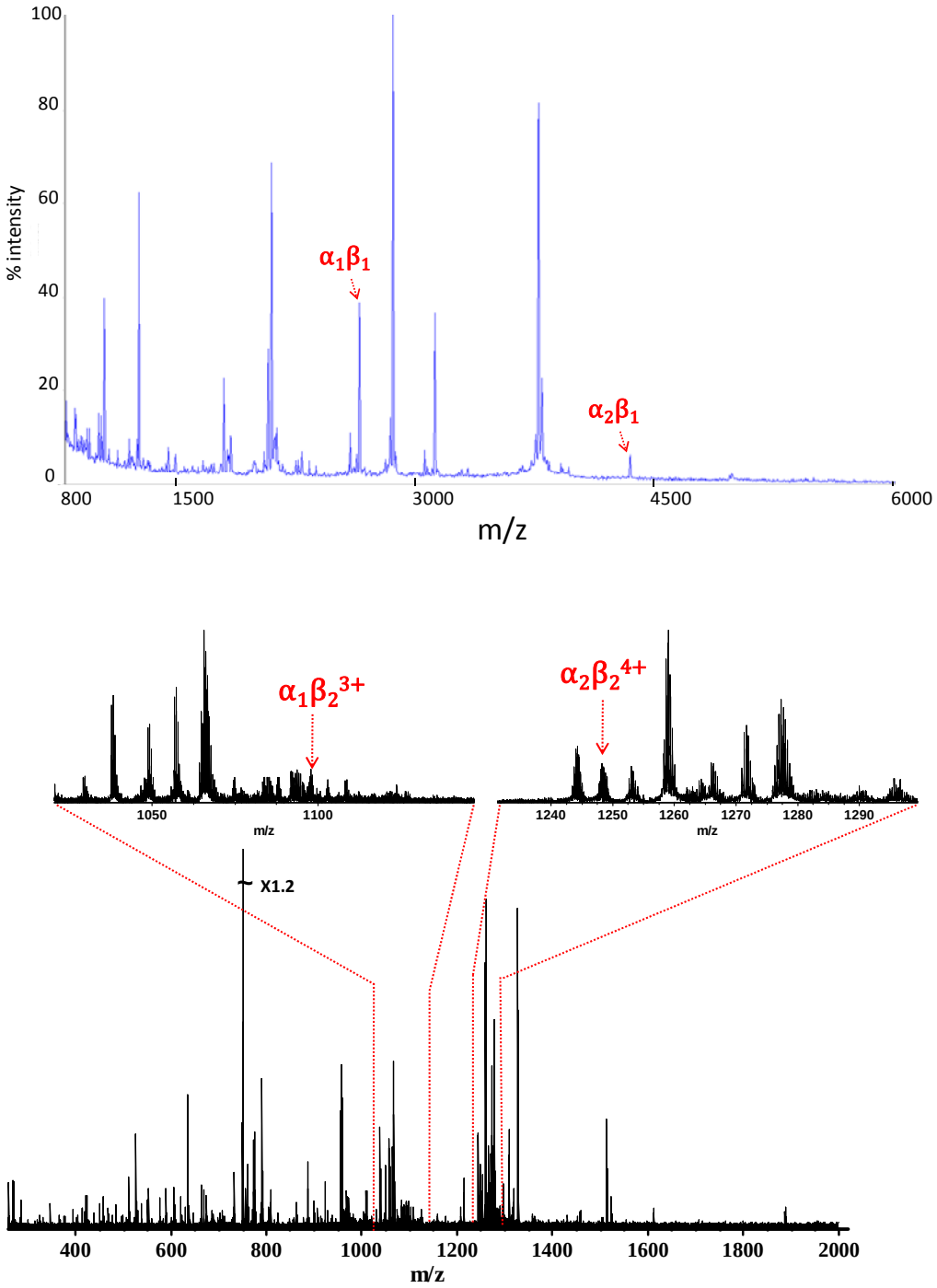
Supporting Figure 1. Localization of TAD binding region on Med15. 10 μM of each biotinylated-pBpa-containing TAD was incubated with 1 μM of a Med15 fragment and irradiated with 365 nm light for 5 min. The samples were analyzed via a western blot using Streptavidin-hrp (top gel) and then subsequently re-probed with an antibody for MBP (bottom gel) to use as a loading control.



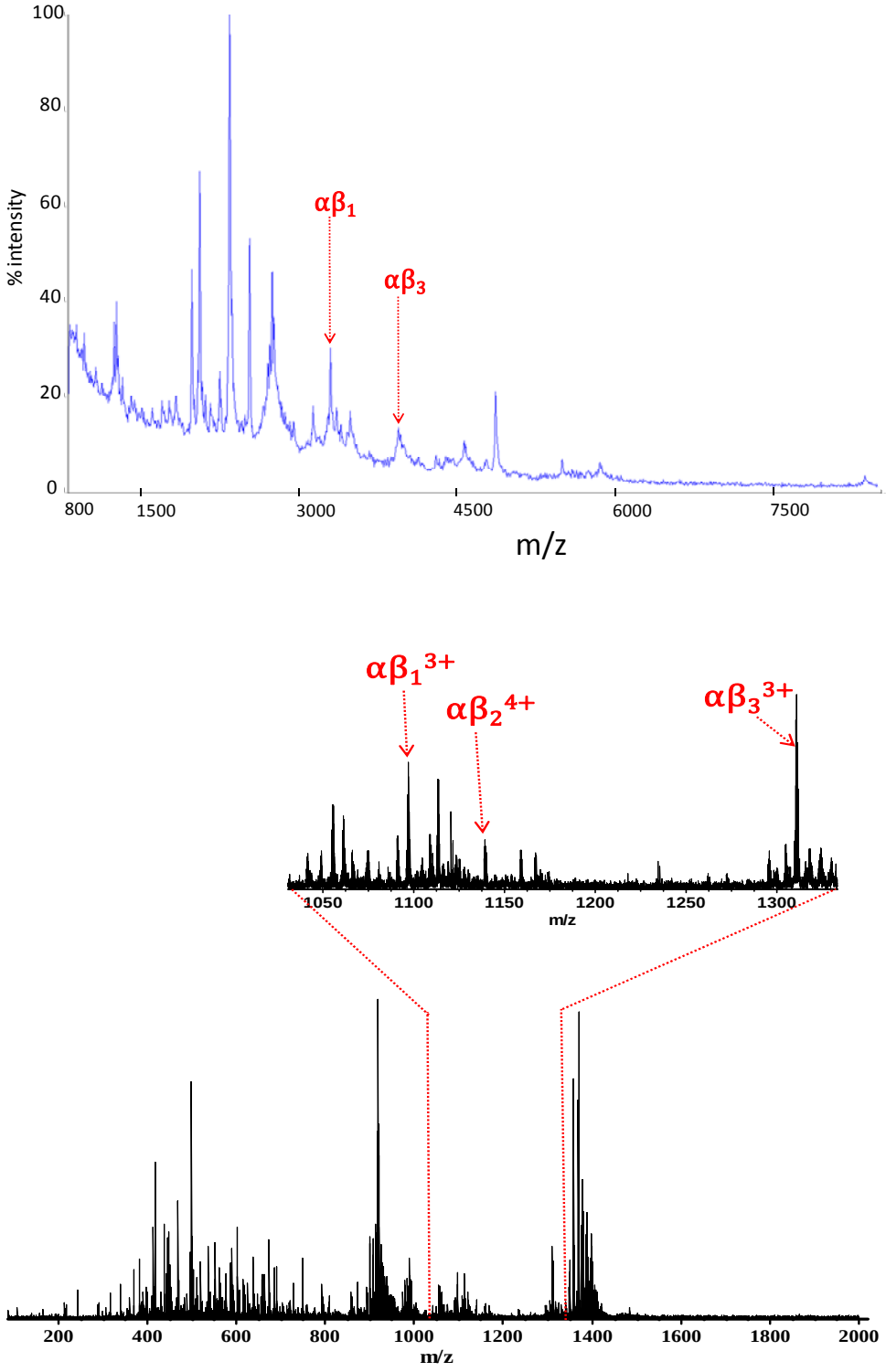
Supporting Figure 2. Dissociation constants for Med15(1-416)-TAD interactions. A constant 50 nM concentration of the fluorescein-labeled variant of each TAD was incubated with varying concentrations of Med15(1-416) for 10 min at rt and the resultant polarization values at each protein concentration obtained on a TECAN Genios Pro plate reader. Each value is the average of three independent experiments with the indicated error (STD).



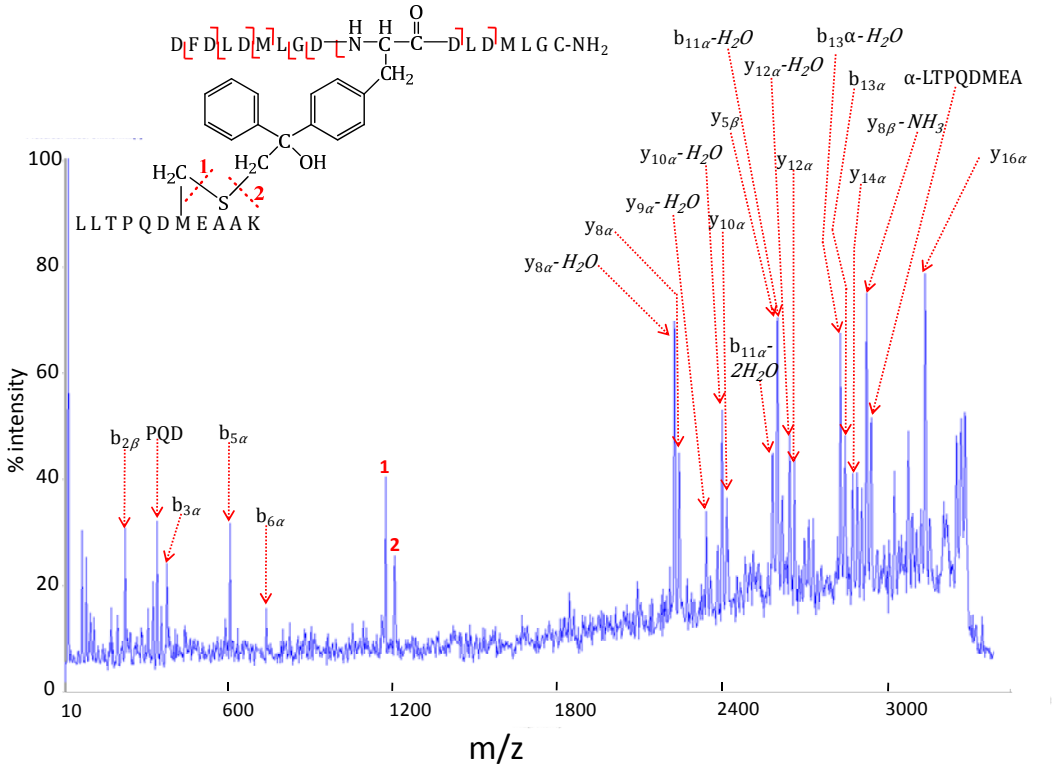
Supporting Figure 3. MALDI-TOF (top) and ESI FT-ICR MS (positive mode, middle; negative mode, bottom) analysis of VP2/Med15 crosslinked products. Following photo-crosslinking, samples were subjected to proteolysis and purification as described in the experimental section. Here, α represents activator VP2, β_1 corresponds to Med15(345-348), β_2 to Med15(74-78), β_3 to Med15(79-85), β_4 to Med15(207-217), β_5 to Med15(222-231), and β_6 to Med15(160-174). Crosslinked products were identified by MALDI-TOF and negative ion mode ESI-FT-ICR MS.



Supporting Figure 4. MALDI-TOF (top) and ESI FT-ICR MS (positive ion mode, bottom) analysis of crosslinked products involving Gcn4 and Med15. Following photo-crosslinking, samples were subjected to proteolysis and purification as described in the experimental section. Here, α_1 represents the activator tryptic peptide Gcn4(119-134); α_2 represents Gcn4(105-134), β_1 corresponds to Med15(74-78), and β_2 to Med15(207-217).



Supporting Figure 5. MALDI-TOF (top) and ESI FT-ICR MS (positive ion mode, bottom) analysis of crosslinked products involving Gal4 and Med15. Following photo-crosslinking, samples were subjected to proteolysis and purification as described in the experimental section. Here, α represents the activator Gal4, β_1 corresponds to Med15 (74-78), β_2 to Med15 (160-174), and β_3 to Med15 (207-217).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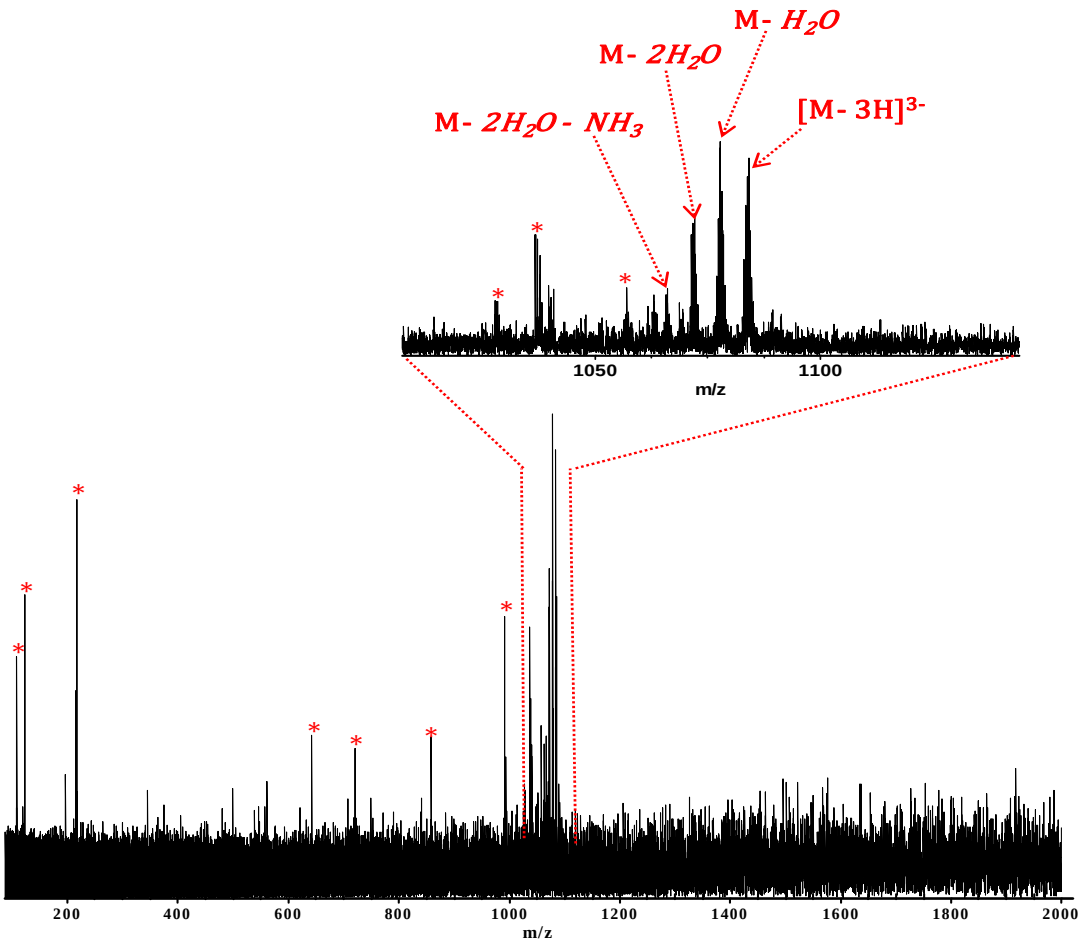
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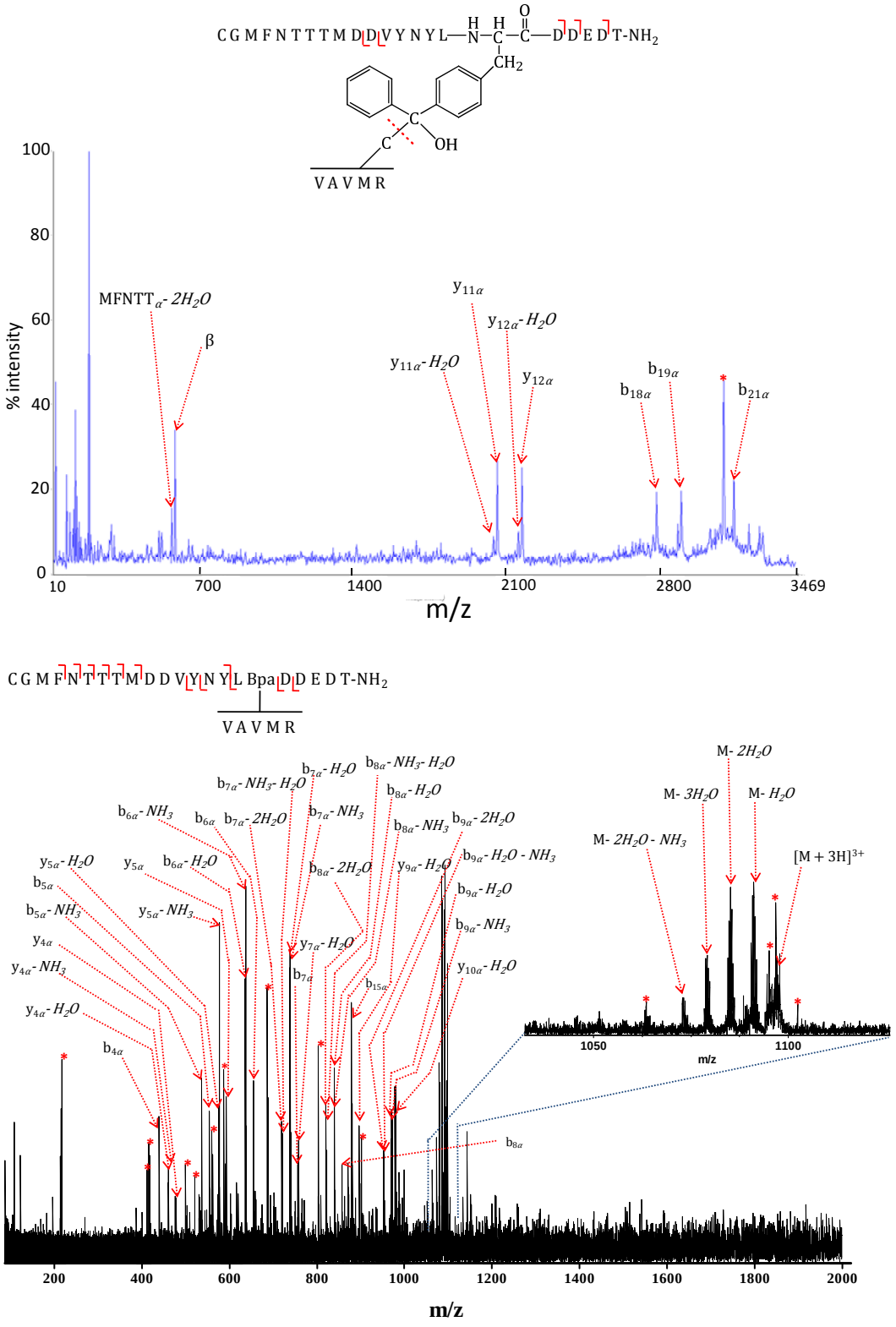
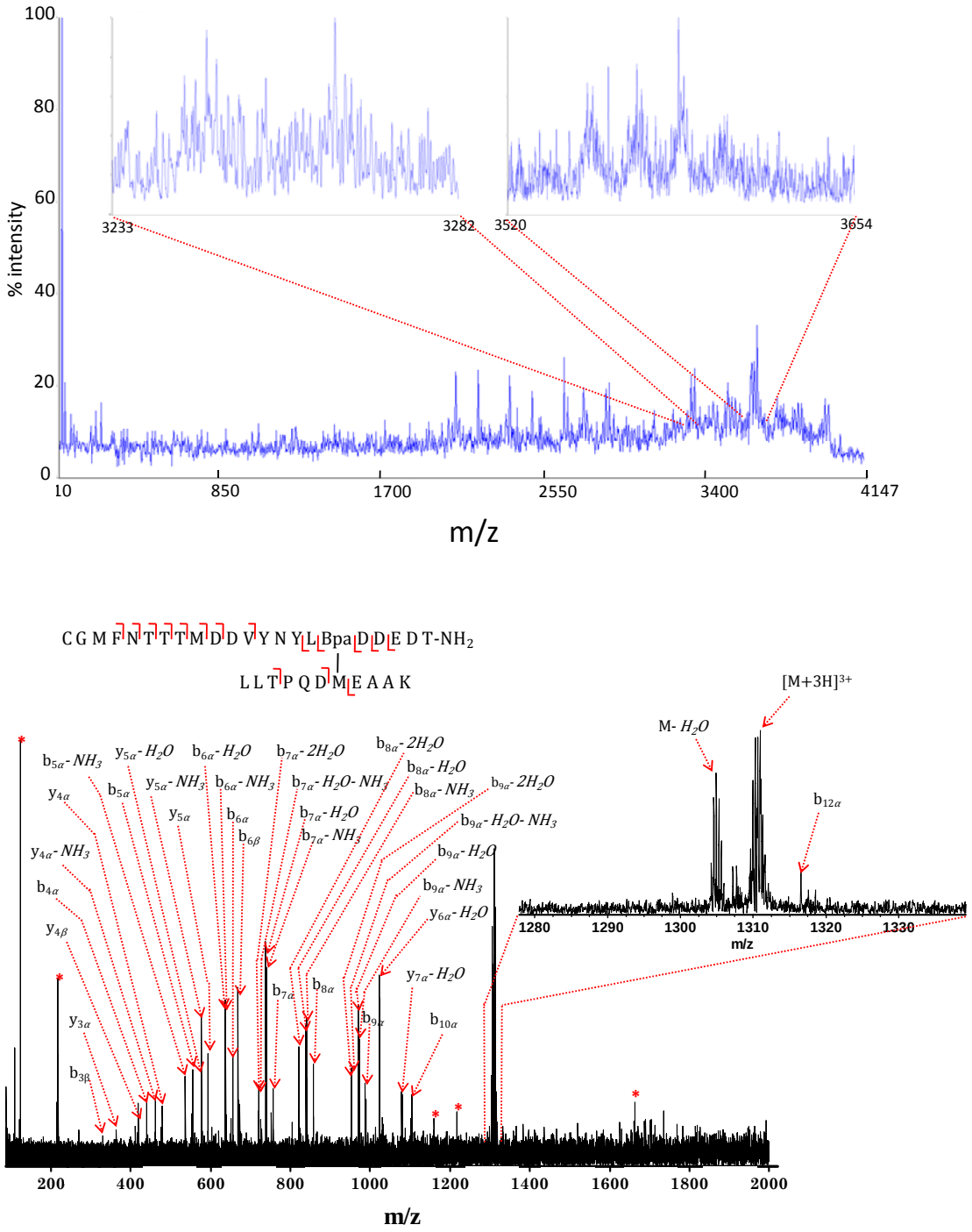
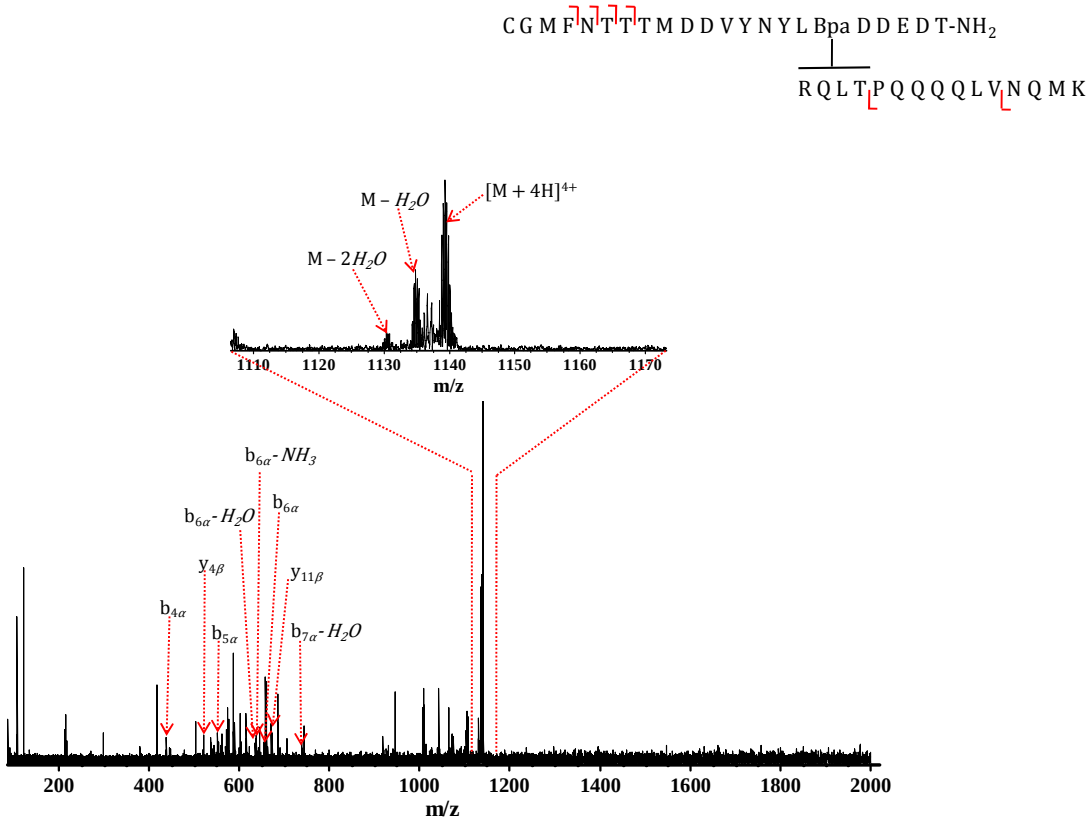
Supporting Figure 6. MALDI-TOF/TOF MS/MS (top) and ESI FT-ICR MS/MS (negative ion mode, bottom) analysis of a potential crosslinked product: VP2:Med15(207-217). Peak 1 and 2 were assigned as C-S bond cleavages, assuming crosslinking occurred at Met^{213} in Med15 (207-217). In MALDI TOF/TOF MS/MS, extensive peptide fragmentation was observed. In negative ion mode ESI FT-ICR MS/MS, only neutral losses of water and ammonia were seen, precluding confident verification of this crosslinked product with the latter technique (* = unidentified peak). Here, α represents VP16 and β corresponds to Med 15 (207-217).



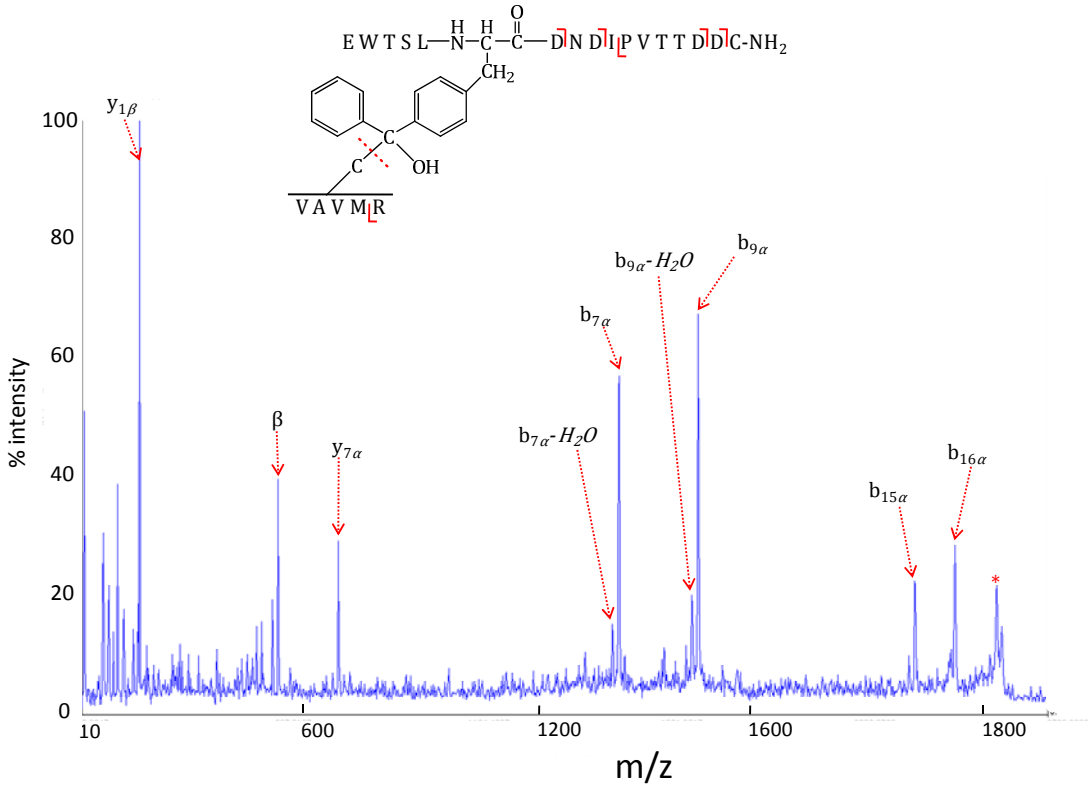
Supporting Figure 7. MALDI TOF/TOF MS/MS (top) and ESI FT-ICR MS/MS (positive ion mode, bottom) analysis of the potential crosslinked product Gal4:Med15(74-78). In MALDI TOF/TOF MS/MS, a limited number of peptide backbone fragments were identified. In addition, cleavage of the C-C bond of the pBpa cross-linker was observed. In positive ion mode ESI FT-ICR MS/MS, significantly more extensive backbone cleavage was observed (* = unidentified peak). Here, α represents Gal4 and β corresponds to Med 15 (74-78).



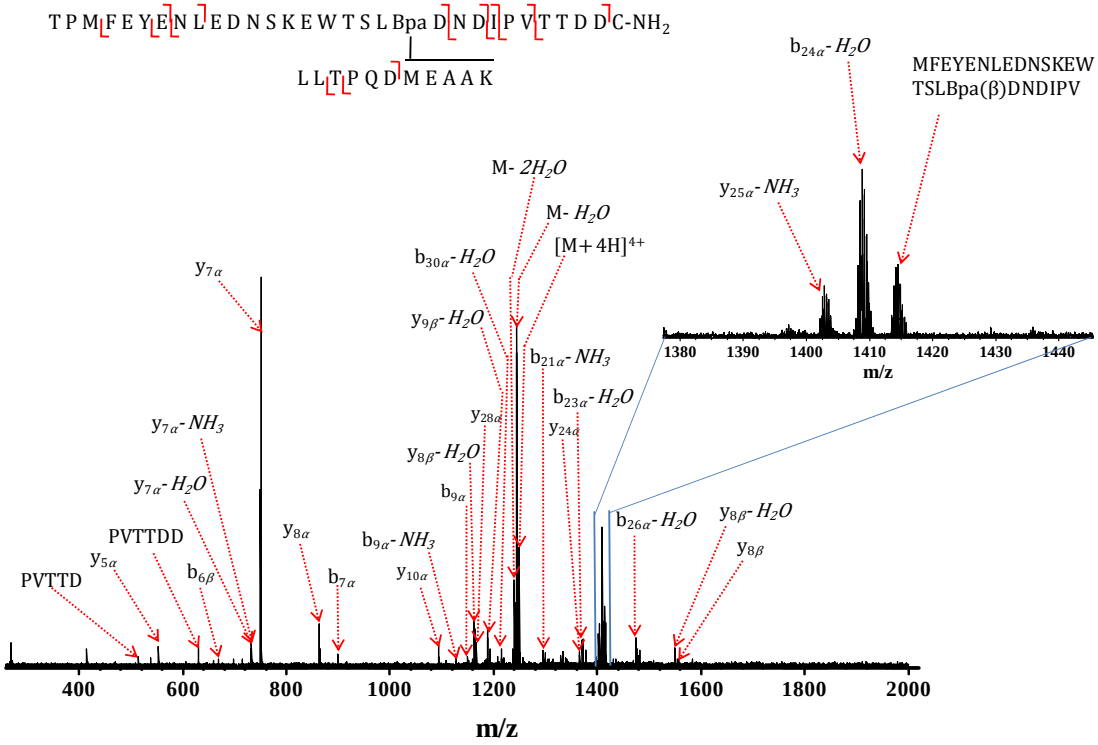
Supporting Figure 8. MALDI TOF-TOF MS/MS (top) and ESI FT-ICR MS/MS (positive ion mode, bottom) analysis of the potential crosslinked product involving Gal4 and Med15(207-217). In MALDI TOF/TOF MS/MS, a limited number of product ion peaks are observed. However, these peaks are ill defined (particularly those in the high m/z area, shown in the insets) and accurate m/z values could not be derived. In positive ion mode ESI FT-ICR MS/MS, a series of backbone cleavages was observed. Based on the fragmentation along the Med15(207-217) peptide backbone, the pBpa crosslinking site can be localized to Met^{213} (* = unidentified peak). Here, α represents Gal4 and β corresponds to Med15(207-217).



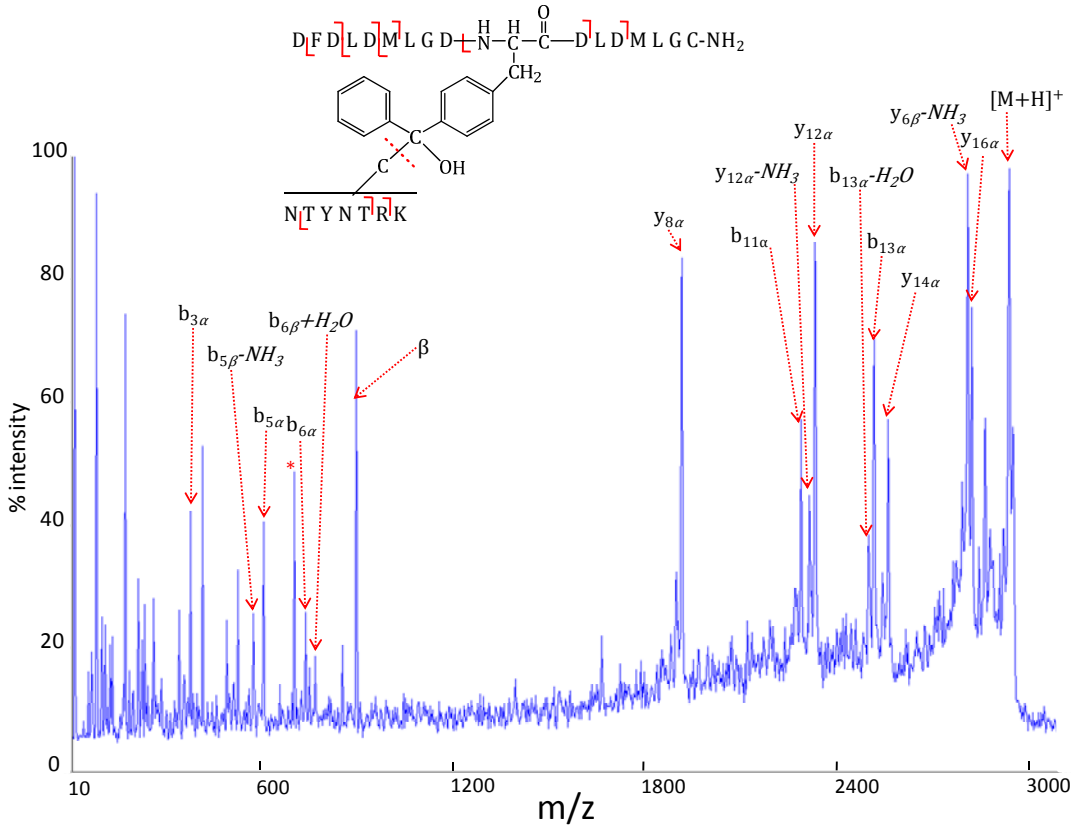
Supporting Figure 9. ESI FT-ICR MS/MS (positive ion mode) analysis of the potential cross-linked product involving Gal4 and Med15(160-174). Several peaks were not identified, perhaps due to imperfect precursor ion isolation. Here, α represents Gal4 and β corresponds to Med 15(160-174).



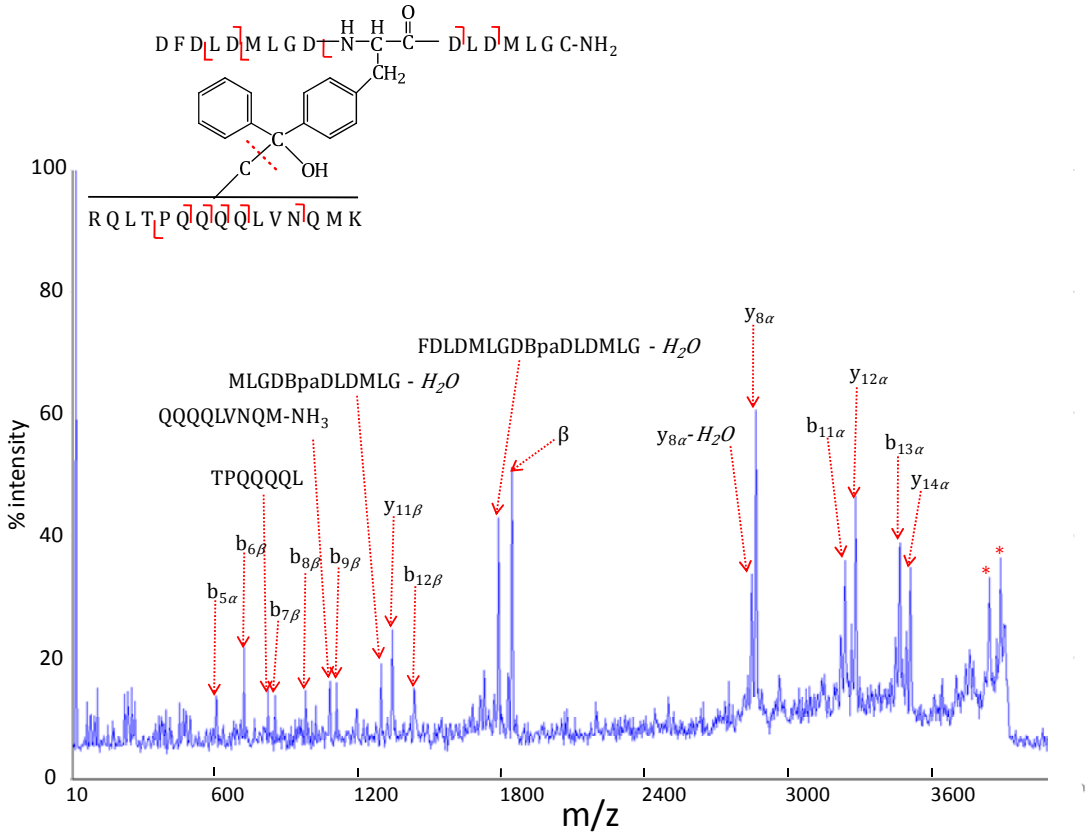
Supporting Figure 10. MALDI TOF-TOF MS/MS analysis of the potential cross-linked product involving Gcn4(119-134) and Med15(74-78). Cleavage of the C-C bond of the pBpa cross-linker was observed. Here, α represents Gcn4(119-134) and β corresponds to Med 15 (74-78) (* = unidentified peak).



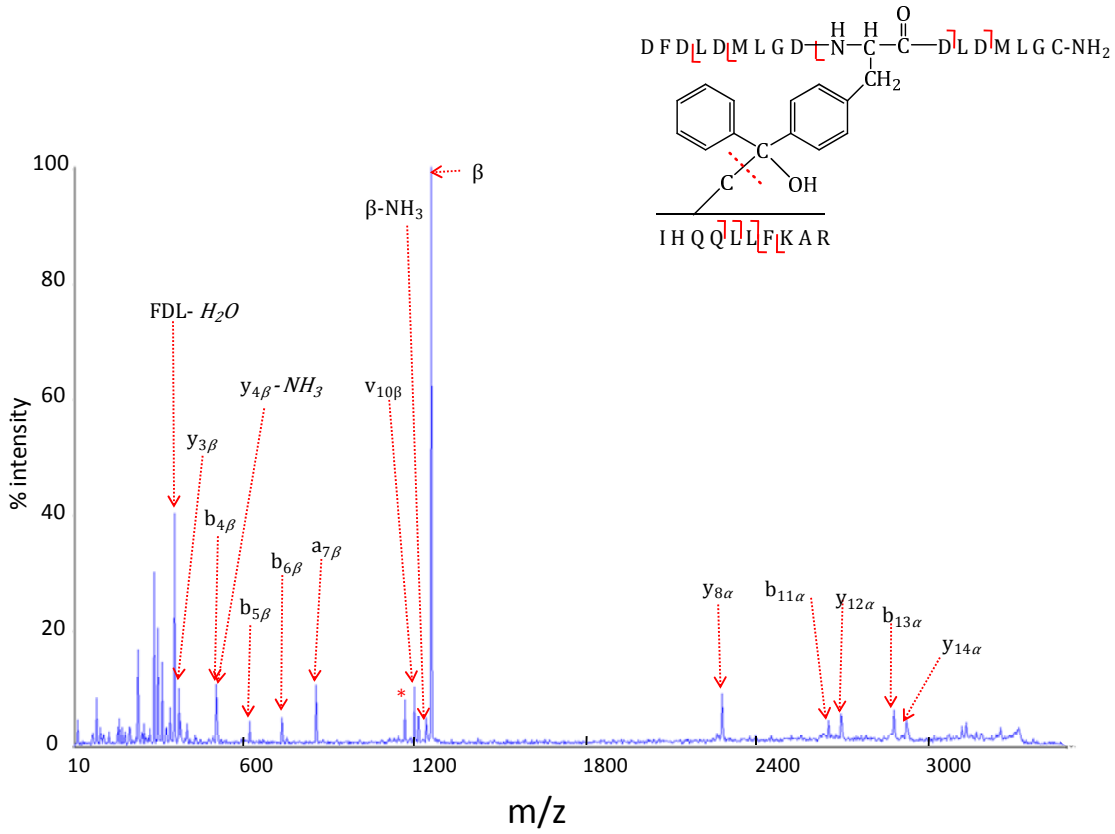
Supporting Figure 11. ESI FT-ICR MS/MS (positive ion mode) analysis of the potential cross-linked product involving Gcn4(105-134) and Med15(207-217). Here, α represents Gcn4(105-134) and β corresponds to Med 15(207-217).



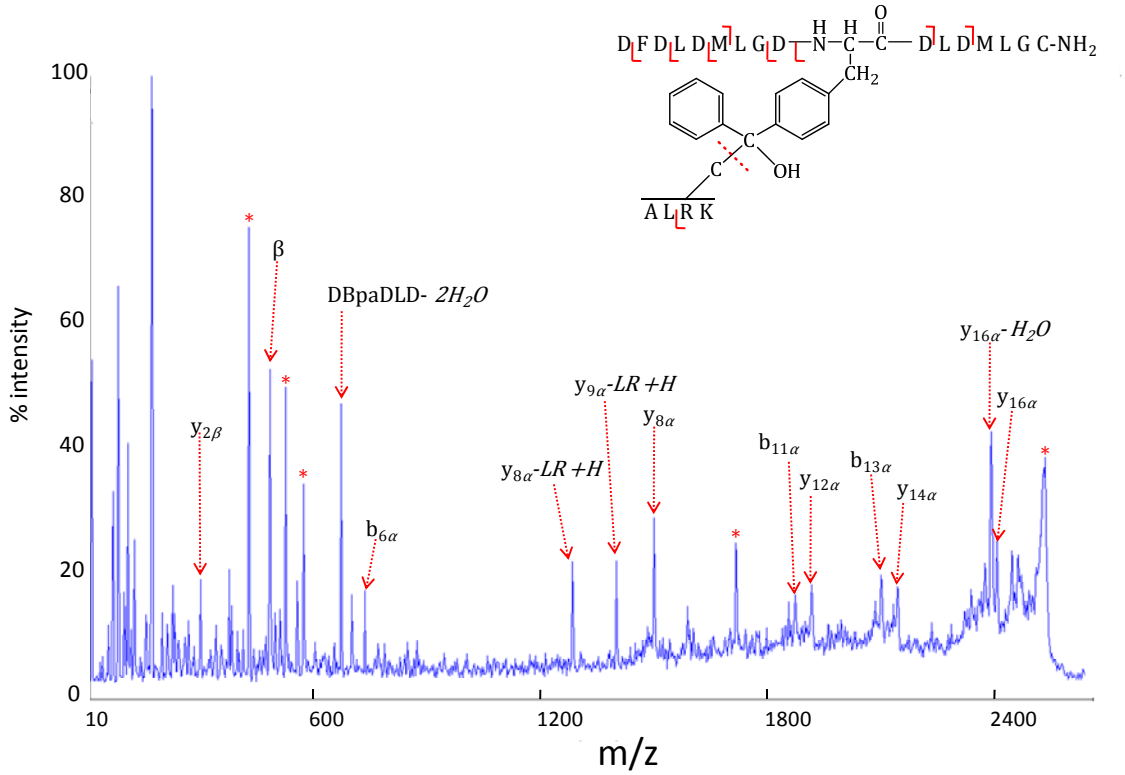
Supporting Figure 12. MALDI TOF-TOF MS/MS analysis of the potential cross-linked product involving VP2 and Med15(79-85). Cleavage of the C-C bond of the pBpa cross-linker was observed. Here, α represents VP2 and β corresponds to Med 15(79-85) (* = unidentified peak).



Supporting Figure 13. MALDI TOF-TOF MS/MS analysis of the potential cross-linked product involving VP2 and Med15(160-174). Cleavage of the C-C bond of the pBpa cross-linker was observed. Here, α represents VP2 and β corresponds to Med 15(160-174) (* = unidentified peak).



Supporting Figure 14. MALDI TOF-TOF MS/MS analysis of the potential cross-linked product involving VP2 and Med15(222-231). Cleavage of the C-C bond of the pBpa cross-linker was observed. Here, α represents VP2 and β corresponds to Med 15(222-231) (* = unidentified peak).



Supporting Figure 15. MALDI TOF-TOF MS/MS analysis of the potential cross-linked product involving VP2 and Med15(345-348). Cleavage of the C-C bond of the pBpa cross-linker was observed. Here, α represents VP2 and β corresponds to Med 15(345-348) (* = unidentified peak).