Supporting information for:

Use of electrochemical oxidation and model peptides to study nucleophilic biological targets of reactive metabolites: The case of rimonabant

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Rimonabant was electrochemically oxidized at 900 mV and incubated with either leucine-enkephalinamide or angiotensin II for 4 hours at 37 ºC. The final concentrations were 18 μM and 9.1 μM for the peptides and rimonabant oxidation products, respectively, in 50% acetonotrile and potassium phosphate buffer (pH 7.4, 38 mM). There was an approximate 2-fold molar excess of peptide to oxidized rimonabant. The incubations were diluted after incubation 1:1 with water and analyzed by LC-MS.

Incubation of EC oxidized rimonabant with either leucine-enkephalinamide or angiotensin II resulted in peptide adducts with a mass increment of 64 Da (PX). No other peptide modifications could be detected in the peptide incubations (Figure S1). Extraction of modified leucine-enkephalineamide (*m/z* 619.3) revealed three peaks. The retention time for the major adduct (PX) was 6.7 min, while the minor peaks eluted after 7.7 and 12.1 minutes. All peaks were unique for the sample corresponding to peptide incubated with oxidized rimonabant and absent in the controls (Figure S1). Fragmentation analysis of all three peaks at *m/z* 619.3 unambiguously identified the adducted peptide and assigned the modification site to the N-terminus. Inspection of the full scan spectra from intact and not fragmented peptides showed that *m/z* 619.3 was an in-source fragment of both ions at *m/z* 646.3 and *m/z* 1079.4 (Figure S2). Thus, the mass differences were +27.0 and +460.1 Da suggesting further adduction of cyanide and M7 or M7' to the adducted peptide (PX) on the N-terminus, respectively. Acetonitrile, used as a solvent in these incubations, is known to contain trace amount of cyanide. In order to confirm the +27 Da mass increment, cyanide was added to the incubations. The cyanide addition totally consumed *m/z* 619.3 in favor for *m/z* 646.3 corresponding to the modified peptide +27 Da (Figure S3). The observation of an ion at *m/z* corresponding to M7 and/or M7' having their typical chlorine isotopic pattern in the spectra of *m/z* 1079.4 confirmed further adduction of M7 or M7' to the

adducted peptide (PX). Similar results were found for angiotensin II, where the 6.4 and 9.1 min peaks correspond to further adduction with cyanide or M7 and/or M7', respectively (Figure S1E). No peptide modifications by other oxidation products could be detected in peptide incubations based on the LC/MS analysis.

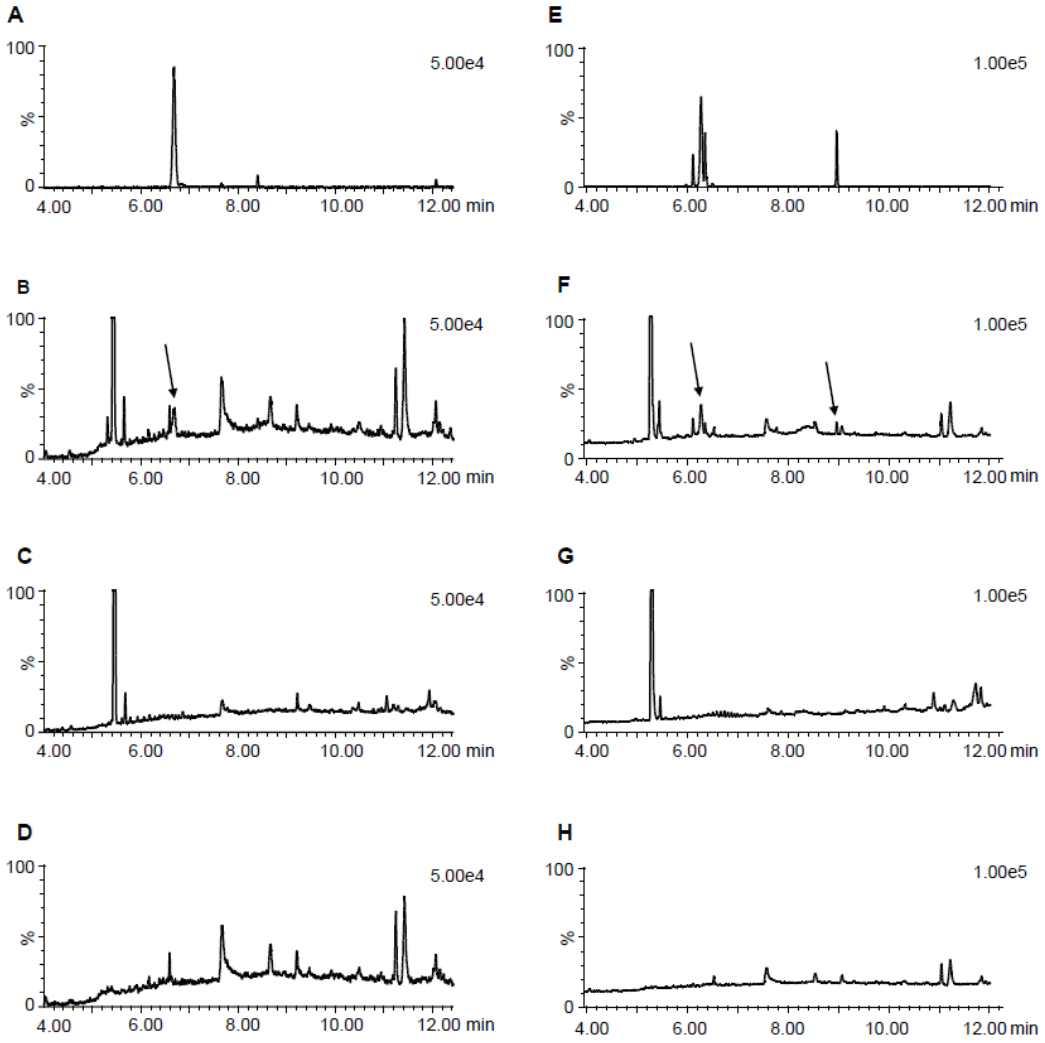


Figure S1. Representative chromatograms of leucine-enkephalinamide, angiotensin II and control incubations (A) extracted ion chromatogram of adducted leucine-enkephalinamide ($[M+H]^+$, *m/z* 619.3) in peptide sample incubated with rimonabant oxidized at 900 mV (B) Total ion current (TIC) of leucine-enkephalinamide incubated with rimonabant oxidized at 900 mV (C) TIC of leucine-enkephalinamide incubated in absence of rimonabant (D) TIC of oxidized

rimonabant incubated with buffer (E) extracted ion chromatogram of adducted angiotensin II ($[M+2H]^{2+}$, *m/z* 555.8) in peptide sample incubated with rimonabant oxidized at 900 mV (F) TIC of angiotensin II incubated with rimonabant oxidized at 900 mV (G) TIC of angiotensin II incubated in absence of rimonabant (H) TIC of oxidized rimonabant incubated with buffer.

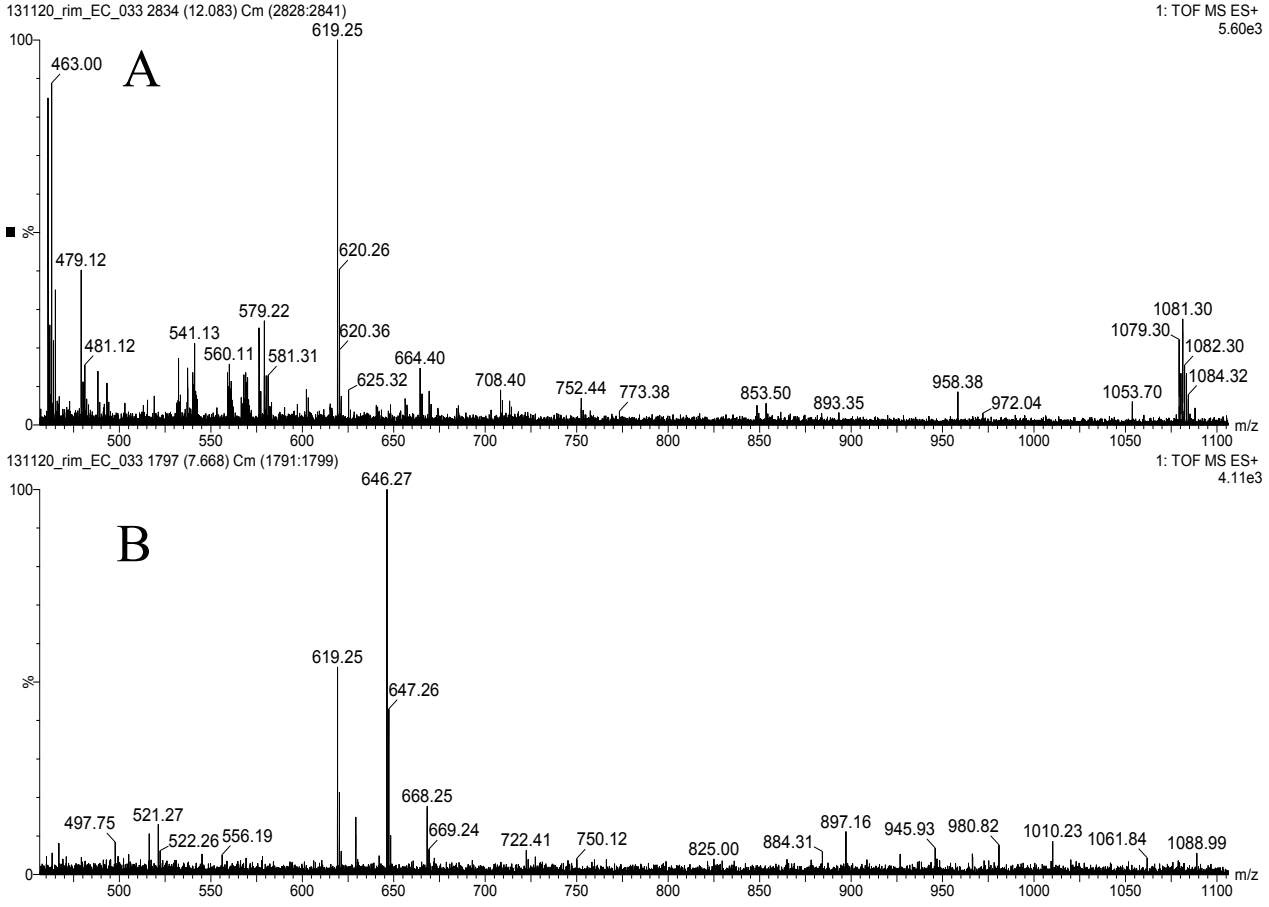


Figure S2. MS spectra for the adducted leucine-enkephalineamide at (A) retention time 12.1 min where PX is further adducted with M7 and/or M7' giving a mass increment of 460 Da to *m/z* 1079.3. The ions at *m/z* 461.0 and 619.3 correspond to M7 and/or M7' and PX, respectively. (B) retention time 7.7 min where PX is further adducted with cyanide giving a mass increment of +27 Da to *m/z* 646.3

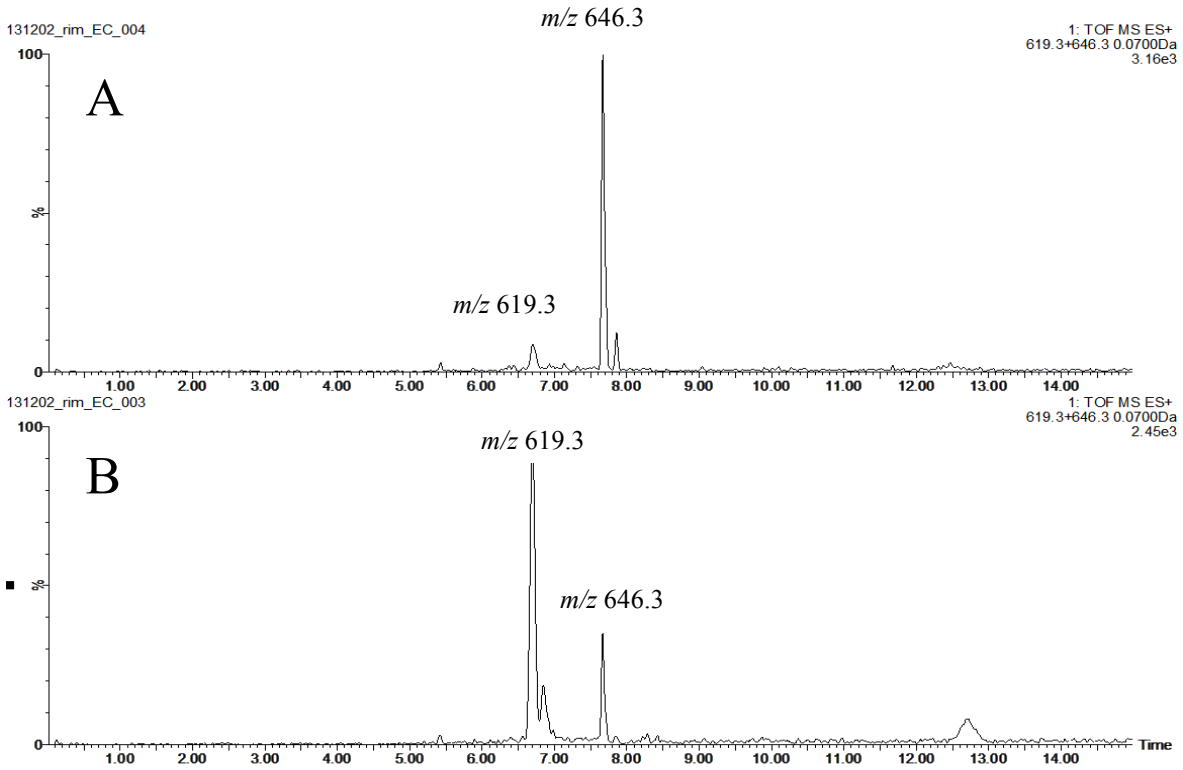


Figure S3. Extracted chromatogram of the adducted leucine-enkephalineamide (PX, *m/z* 619.3) and PX further adducted with cyanide (+27 Da, *m/z* 646.3) (A) addition of cyanide to the incubation of peptide and oxidized rimonabant (B) incubation of peptide and oxidized rimonabant

Fragmentation analysis of angiotensin II and its corresponding adducted peptide (PX) confirmed the mass increment of 64 Da for the b-and a-ions extending from the N-terminus of the adducted peptide (Figure S4).

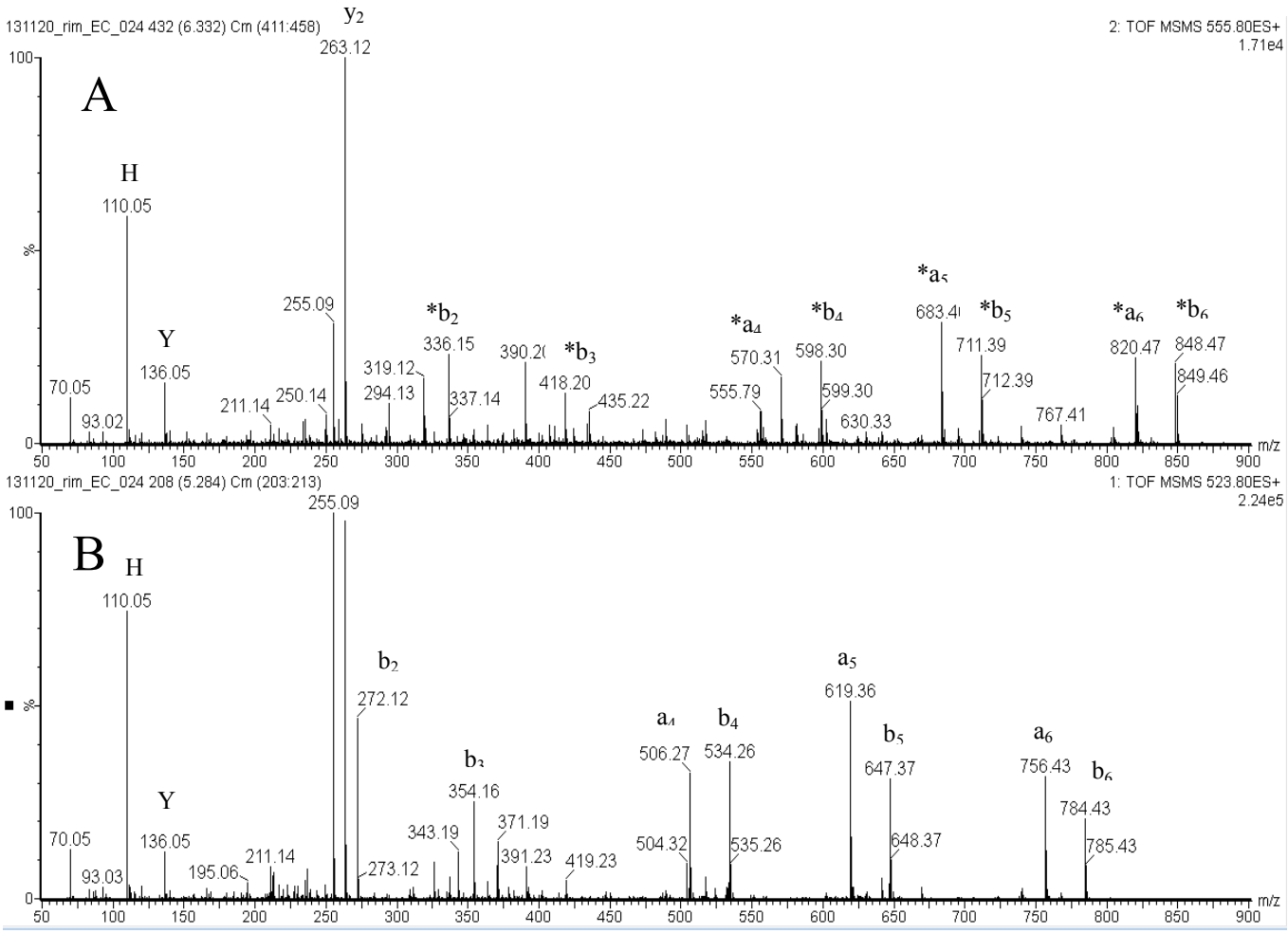


Figure S4. Product ion spectra of angiotensin II corresponding to the sequence NRVYIHPF (A) adducted peptide (B) unadducted peptide *indicates a modified ion with a mass increament of +64 Da

Fragmentation of adducted leucine-enkephalinamide incubated with either [^{14}C]-labeled rimonabant or rimonabant oxidized at 900 mV revealed a shift in the isotope distribution for all product ions containing the N-terminus after incubation with [^{14}C]-labeled rimonabant (Figure S5). The shift in isotope distribution is in agreement with the [^{14}C]-labeling on the pipiridine ring.

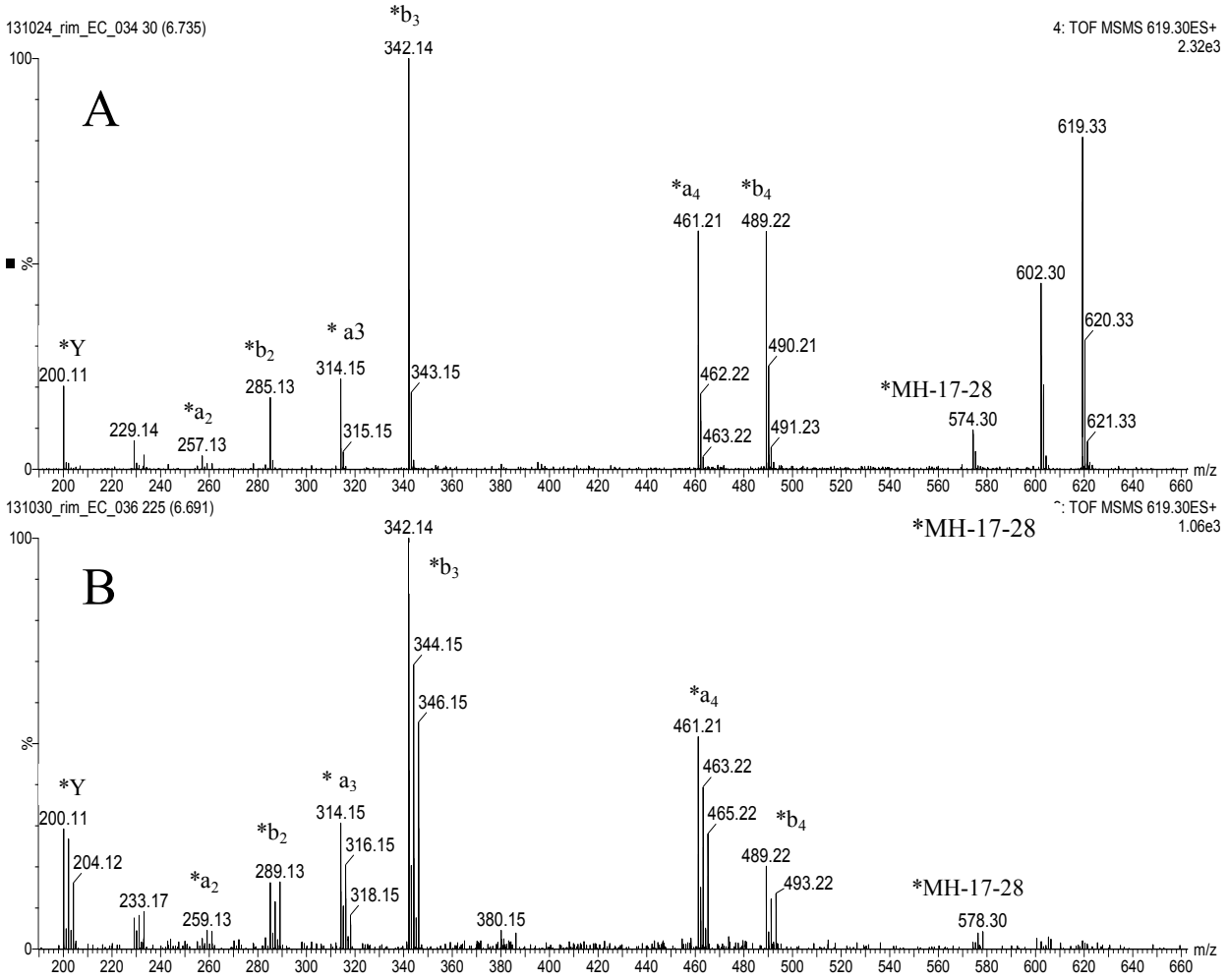


Figure S5. Product ion spectra containing the N-terminal tyrosine (Y) at *m/z* 200.1 from fragmentation of adducted leucine-enkephalinamide (A) incubated with unlabeled rimonabant (B) incubated with [^{14}C]-labeled rimonabant * indicates ions found in both spectra with a shift in the isotopic pattern for the [^{14}C]-labeled sample

Leucin enkephalinamide and angiotensin II were incubated separately with pentanedial. The final concentrations were 18 μM for the peptides and 0.1, 0.4 0.9 and 1.8 μM for pentanedial in 50% acetonitrile and potassium phosphate buffer (pH 7.4, 38 mM). The incubations were diluted after incubation 1:1 with water and analyzed by LC-MS. The same peptide modification (PX) as in the EC experiments was observed for both peptides at all pentanedial concentrations and there were

increasing levels of adducted peptides with increasing concentration of pentanedial (Figure S6). The additional adduction by the adduct (PX) of the cyanide impurity in the acetonitrile was also observed (*vide supra*). The adducted peptides were unambiguously identified by fragmentation. Their product ion spectra were identical to those of the adducted peptides from the samples of peptides incubated with rimonabant oxidation products (Figure S7).

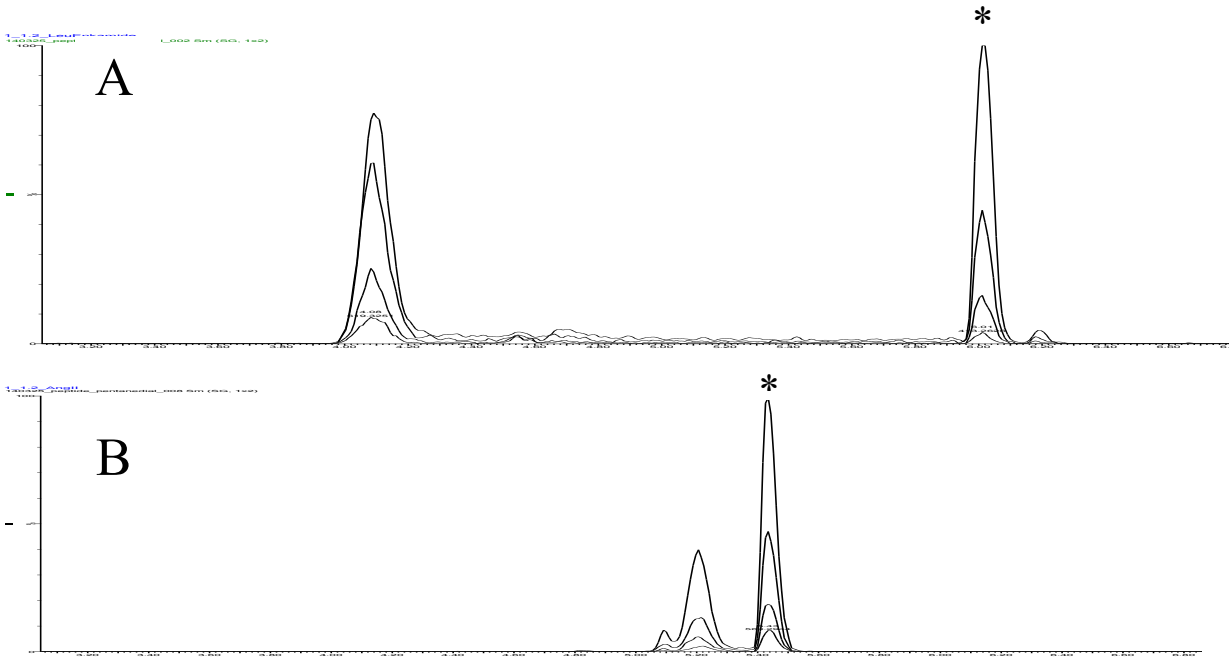


Figure S6. Extracted ion chromatogram of adducted model peptides (PX) after pentanedial incubation at 0.1, 0.4, 0.9 and 1.8 µM (A) leucine-enkephalineamide (*m/z* 619.3) (B) angiotensin II (*m/z* 555.8) *indicates further adduction by PX of +27 Da corresponding to the cyanide impurity in the acetonitrile

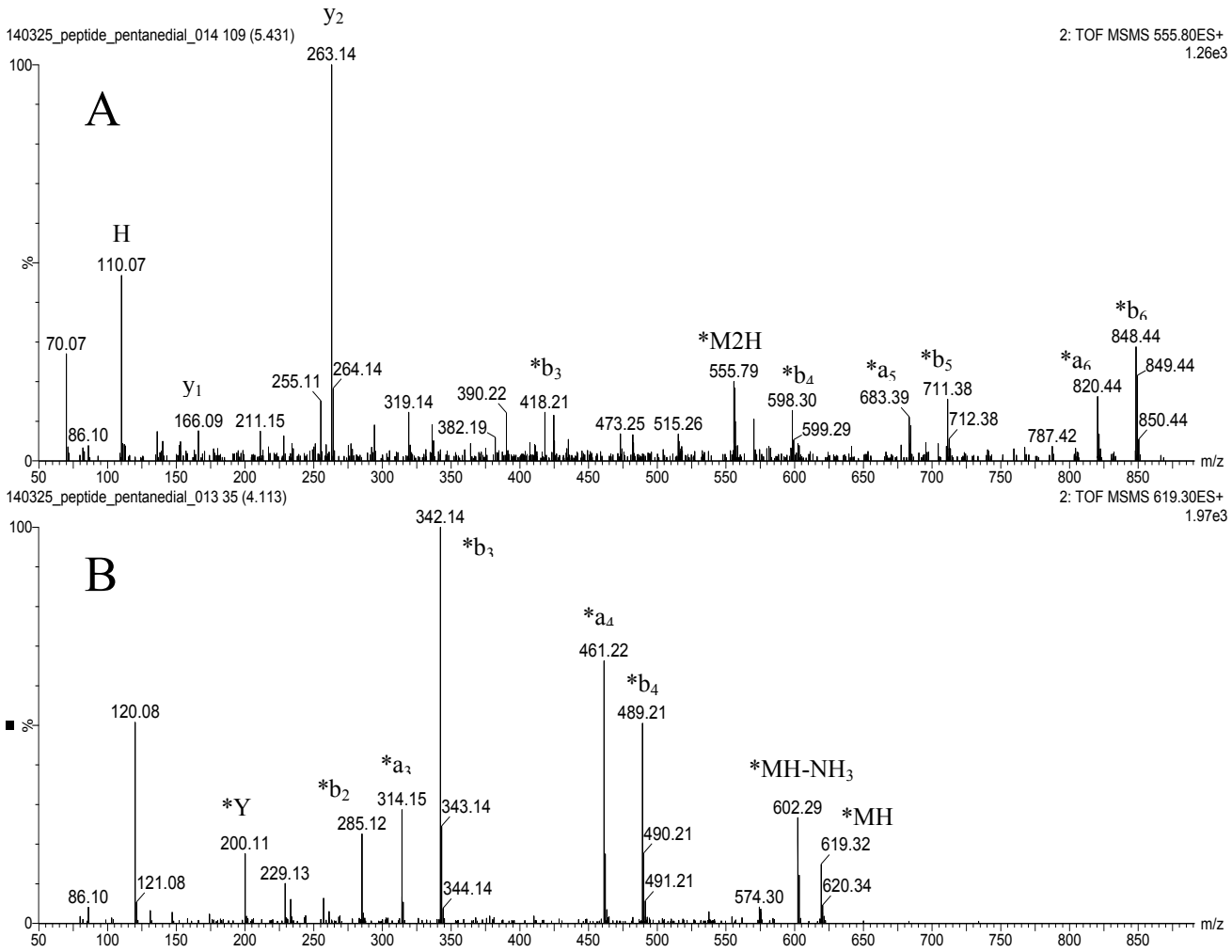
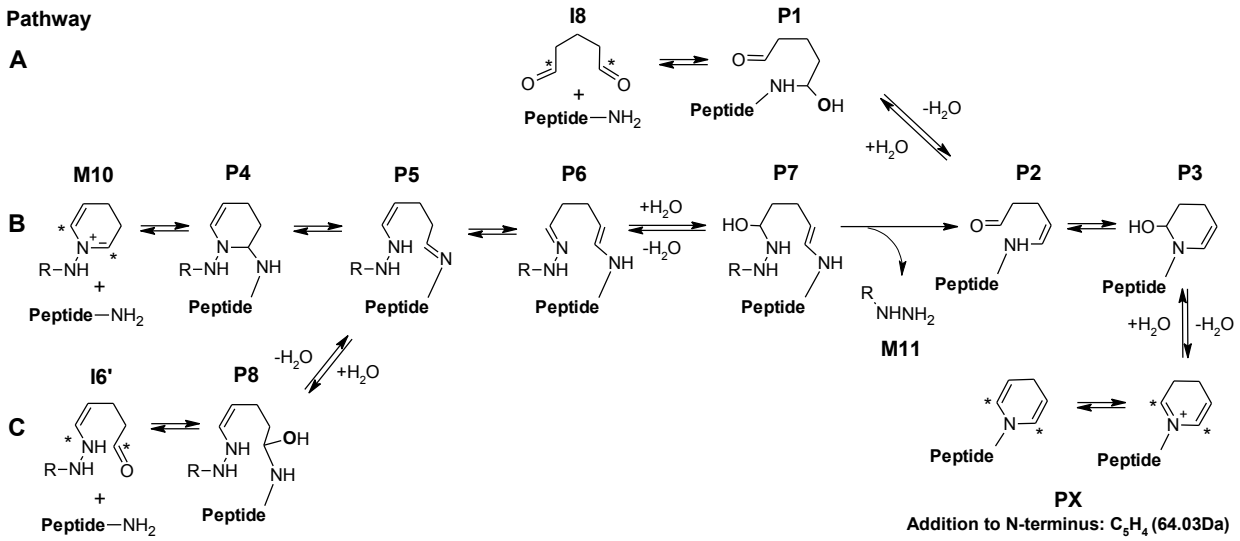


Figure S7. Product ion spectra of adducted model peptides (PX) after pentanedial incubation at 0.4 μM (A) angiotensin II (B) leucine-enkephalineamide *indicates a modified ion with a mass increament of +64 Da

Addition of the peptide N-terminal NH_2 to pentenedial (I8) would give the carbinolamine intermediate P1 followed by dehydration of P1 to the aldehyde intermediate P2. Subsequent addition of the secondary N-terminal nitrogen to the aldehyde would close the ring and give the carbinolamine intermediate P3 that, finally, would eliminate a molecule of water to form the stable peptide adduct PX (Scheme S1, Pathway A). An alternative route to initiate the formation of PX is addition of the peptide N-terminal NH_2 to the imine of the 3,4-dihydropyridinium (M10) that would give the α-carbon modified tetrahydropiperidine P4. P4 is expected to be in equilibrium with the ring-opened intermediate P5. Two possible enamine/imine equilibriums exist for P5 and one possible conformation is P6 that, in an aqueous environment, would be in equilibrium with the hydrated intermediate P7. The carbinolamine motif of P7 is expected to be unstable and degrade to the hydrazide product M11 and the aldehyde intermediate P2 from which PX can be formed as described above (Scheme S1, Pathway B). A third plausible route to initiate the formation of PX is addition of the peptide N-terminal NH_2 to one of the aldehyde intermediates I6, I6' or I7 in Scheme 2. This concept is illustrated in Scheme S1 for the aldehyde intermediate I6´. Dehydration of the carbinolamine intermediate P8 would then give the ring-opened imine intermediate P5 from which PX can be formed as described above (Scheme S1, Pathway C).



Scheme S1. Potential mechanisms for the formation of PX. * indicates the positions of the [^{14}C]-labels in the reactive species and the final PX adduct.