Improved Performance of Positive-ion mode Free Radical-Initiated Peptide Sequencing with para-TEMPO-Bz

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ABSTRACT: Free radical-initiated peptide sequencing (FRIPS) is a tandem mass spectrometry (MS/MS) technique that generates sequence informative ions via collisionally-initiated radical chemistry. Collision activation homolytically cleaves an installed radical precursor, initiates radical formation, extensive hydrogen atom transfer, and peptide backbone dissociation. While the FRIPS technique shows great promise, when applied to multiply charged derivatized peptide ions, a series of high abundance mass losses are observed which syphon ion abundance from radicaly generated sequence ions. This loss of ion abundance reduces the sequence coverage generated by FRIPS fragmentation. In this work, we hypothesized that these mass losses were assisted by the ortho-orientation of the radical precursor undergoing facile conversion into five- or six-membered intermediates or products and that when combined with the lower bond dissociation energy of the para-precuror, conjugated peptides would not undergo this chemistry. To test this assertion, we synthesized para-TEMPO-Bz, conjugated it to these peptides, and collisionally activated each. And indeed, we see dramatic attenuation of these undesired collisional processes and the significant increase in radical precursor ion abundance. The increase in ion abundance leads to a significant increase in the sequence coverage generated. These results demonstrate that p-TEMPO-Bz significantly improves the performance of positive-ion mode FRIPS and may be a compelling alternative to the currently utilized ortho-TEMPO-Bz-based FRIPS.

Free radical initiated peptide sequencing (FRIPS) is a radical-driven fragmentation technique that has been extensively applied to peptide ions.1–5 Its major advantages over the predominantly utilized electron-driven technology (electrospray dissociation, ETD) is its applicability to all peptides regardless of charge state, high duty cycle, and that it can be implemented on any mass spectrometer capable of collisional activation.1,3,12,13,4–11 These attributes stem from how the radical is initiated in each technique. In ETD, the radical is transferred to a peptide ion most frequently from a chemical ionization-derived radical anion of fluoranthene.14 This transfer neutralizes a charge prior to stimulating N→C bond dissociation and thus, cannot be applied to singly protonated peptide ions. In FRIPS, the radical is introduced through the installation of a radical precursor into the peptide.1,3,6 Collisional activation of this derivatized peptide promotes the homolytic cleavage of the radical precursor, radical initiation, and the dissociation of the peptide backbone.

Robust implementation of FRIPS requires efficient conjugation of the radical precursor with the peptide and radical initiation through the selective homolytic dissociation of the radical precursor. Collisional activation, however, is not selective and even-electron processes that are instigated with lower or comparative amounts of energy, will be in direct competition with radical initiation.1,10,15–17 These competitive processes syphon ion abundance from sequence informative ions, decreasing signal-to-noise ratios and sequence coverage. Mobile proton-driven processes are often the lowest energy even-electron processes and have been avoided through the study of anions, sequestration of protons via the guanidination of lysine residues, development of a new FRIPS precursors, or focus on ions with fewer protons than arginine residues.1,2,10,16–21 Recently, a para-substituted FRIPS precursor was shown to more efficiently couple to peptides and better instigate single-step peptide sequencing due to removal of a carbonyl decreasing the stability of the generated radical but its influence on the minimization of this undesired charge-directed processes has not been studied.22 To be employed in more complex systems and proteomics workflows, the efficiency of sequence ion generation must be improved for multiply charged ions which are frequently generated in proteomics workflows.

In this work, we examine several proton-driven neutral and cationic losses and identify that these processes are highly abundant when the most utilized FRIPS tag, ortho-TEMPO-Bz is employed. We further demonstrate that these undesired mass losses are significantly reduced or even eliminated when the para-substituted arene is utilized. Attenuation of these competing processes increases efficiency of radical generation, product ion abundance, and sequence coverage.

Experimental Section

Materials and Reagents

2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO), substance P (RPKQPQFFGLM), 4,4′-dimonyl-2,2′-dipyridyl, N-hydroxysuccinimide, methyl(4-bromomethyl) benzoate were purchased from Sigma Aldrich (St. Louis, MO). Water, MS-grade methanol, acetonitrile, 2-(bromomethyl)benzoic acid, tolune, dichloromethane, tetrahydrofuran, copper (II) trifluoromethanesulfonate, thionyl chloride, N, N-dicyclohexylcarbodiimide, triethylamine (TEA), and formic acid were acquired from Fisher Scientific (Waltham, MA). ACTH (1–14) (SYSMEHRWGPV), angiotensin I (DRVYIHPFHL), melittin (GIGAVLKVLTGLPALISW1KRKRQQ-NH2), and...
amyloid β-peptide (10-20) (YEVHHQKLVFF) were purchased from Bachem (Bubendorf, Switzerland).

**Syntheses of o-TEMPO-Bz-NHS and p-TEMPO-Bz-NHS**

Detailed synthetic procedures for o-TEMPO-Bz-NHS and p-TEMPO-Bz-NHS can be found in the supporting information.

**Conjugation of FRIPS Tag (o- and p-TEMPO-Bz-NHS)**

Working solutions of o-TEMPO-Bz-NHS and p-TEMPO-Bz-NHS were made in acetonitrile. The peptides were buffered with 0.1 M triethylamine and the reaction was initiated by the addition of 30 µL of the o- and p- TEMPO-Bz-NHS stock solution, respectively. The conjugation reaction with each peptide was performed with a 30-fold excess of label for 2 hours at 37°C. The total volume of the reaction was 50 µL. After completion, the reaction solution was evaporated using a ThermoSavant ISS110 SpeedVac system. Each labeled peptide was redissolved in 50 µL water. For all experiments these purified stock solutions were diluted into 50/50 methanol/water to a working concentration of 1 µM.

**Mass Spectrometry**

All FRIPS-based MS/MS analyses were completed using a Thermo Scientific Orbitrap Fusion (Waltham, MA) mass spectrometer. For all experiments, the Orbitrap was used as the mass analyzer, the needle voltage was set to 4400 V, and the sample solutions were directly infused into the instrument at a rate of 2 µL/min. The ion transfer tube temperature was operated at 275°C. The automatic gain control (AGC) was set to 2.0 x 10⁶. The ion source temperature was 37°C. The total volume of the reaction was 50 µL. After completion, the reaction solution was evaporated using a ThermoSavant ISS110 SpeedVac system. Each labeled peptide was redissolved in 50 µL water. For all experiments these purified stock solutions were diluted into 50/50 methanol/water to a working concentration of 1 µM.

Data from the instrument was exported, plotted on Origin Pro 2016 and peaks were manually assigned with a 10 ppm cutoff. Hydrogen atoms were explicitly tracked using the nomenclature described by the Zubarev group. Calculated m/z values for peptide ions and their product ions were acquired from Protein Prospector (http://prospector.ucsf.edu/prospector/mshome.html).

**BDE Calculation**

The bond dissociation energy of ortho- and para-TEMPO benzoic acid was calculated using the: A machine-Learning derived Fast Accurate Bond dissociation Enthalpy Tool (https://bde.ml.nrel.gov/). The SMILES strings CC1(C)C(C)C(C)C(C)C(C)(C)N1OCc1ccccc1C(=O)O and CC1(C)C(C)C(C)C(C)C(C)(C)N1OCc1ccccc1C(=O)O were utilized for the ortho and para isomers, respectively.

**Results and Discussion**

FRIPS is typically implemented in an MS³ workflow (Scheme 1). The objective of the first activation step is to maximize homolytic cleavage, neutral loss of the TEMPO moiety (156.14 Da), and production of the radically initiated species. The formation of the benzyl radical is frequently the lowest energy dissociation pathway available to the peptide ion and thus, resonance CID is typically employed to both promote radical initiation and minimize any subsequent activation of this newly generated off-resonance species. The ensuing activation of the radical initiated ion promotes hydrogen atom transfer and backbone dissociation. While this is often performed with resonance CID to precisely control the precursor ion’s internal energy, minimize competition with even-electron dissociation pathways, and ensure that radical processes dominate, implementation of the auto-MS/MS functionality and this precise control will not be possible. Thus, in this work, beam-type CID will be employed as the second activation step in all MS³ experiments and collisional and radical product ions will simultaneously be generated.

Scheme 1. Illustration of free radical initiated peptide sequencing workflow.

MS² of o-TEMPO-Bz and p-tempo-Bz-conjugated peptide ions

When ACTH 1-14 (SYSMEHRWGPVG), β-amyloid 10-20 (YEVHHQKLVFF), melittin (GIGAVLKVLTGLPAlISWIKRKQO), angiotensin I (DRVYIHPFHL), and substance P (RPKQQFFGLM) are derivatized with o-TEMPO-Bz-NHS and ionized via electrospray ionization, the protonated cations of each conjugated peptide are observed (indicated by the “R” subscript). When these precursor ions are subjected to gentle collisional activation with resonance CID, a prominent neutral loss of 156.14 Da (indicated with the “r” subscript) is generated in all spectra indicating that all peptides are in-part undergoing radical initiation (Scheme 1, Figures S1, 1A, 2, S3, 1B, and Table S1).
Collision energies were chosen to maximize the generation of the (M + 2H)^+ ion.

We hypothesize that use of a para-TEMPO-Bz reagent will reduce the abundance of these undesired products for two major reasons. 1) para-TEMPO-Bz will restrict the formation of the hypothesized ring structures and decrease the efficiency at which these undesired products are formed (Scheme 2D), and 2) radical initiation of para-TEMPO-Bz requires 1.5 kcal mol^(-1) less energy^{24,25} (see methods) than the ortho precursor and thus, may better compete with these low-energy heterolytic processes.

Figure 1. CID spectra of α-(top) and p-TEMPO-Bz-conjugated (bottom) for (A) doubly protonated β-amylloid (10-20) and (B) substance P 2+ cations. The colored region is magnified to the right of each spectrum and highlights the heterolytic loss of tempo.

In addition to the desired radical product, collisional activation of α-TEMPO-Bz derivatized ACTH 1-14, β-amylloid 10-20, and substance P generates an undesired series of highly abundant neutral and cationic losses (Figures S1, 1A, 1B, and Table S1). The neutral loss of 157.15 Da is highly abundant in all three spectra and represents the heterolytic elimination of TEMPO. A similar product was initially documented by Ihling et al. and was shown to be proton dependent. In addition to this neutral loss, we observe charge reduction and the mass loss of 140.14, 158.15, and 274.18 Da from these peptide ions (Figures 1A, 1B, S1, and Table S1). Unlike these peptides, conjugated melittin demonstrates minor losses of 140.14 and 158.15 Da and angiotensin I displays no neutral or cationic losses (Figures S2 and S3). The lack of these losses in angiotensin I may be due to the N-terminal aspartic acid interacting with both the conjugated N-terminus and the initiated radical. The proximity of the aspartic acid side chain to the radical is supported by the highly abundant neutral loss of CO₂ and COOH upon MS² activation of the ortho-conjugated peptide (Figure 3C). Future work is needed to better understand how the identity of nearby residues influence product ion generation in FRIPS.

To explain the observed loss in their manuscript, Ihling et al. proposed a mechanism that resulted in the formation of a carbocation. Their proposed structure, however, results in a carbocation with an electron withdrawing group in the ortho position which is known to be destabilizing. The high abundance of this neutral loss observed by them and us, however, indicate that this loss is a product of a highly favorable process. Thus, we propose that the efficiency of this and the 140.14 Da loss may be better explained by the facile formation of five-, or six-membered ring intermediates or products (Scheme 2A and B).

Scheme 2. (A-C) Proposed Mechanisms for abundant mass losses from α-TEMPO-Bz-conjugated peptide ions. (D) Dissociation of p-TEMPO-Bz-conjugated peptides.

To test this hypothesis, we synthesized the para isomer, conjugated it with ACTH 1-14, β-amylloid 10-20, melittin, angiotensin I, and substance P, and subjected each to collisional activation (Figures S1, 1A, 1B, S2, S3, and Table S1). Interestingly, homolytic cleavage of these para-conjugated peptides required a ≈14% decrease in collisional energy. This may be due to a decrease in the energy necessary to initiate homolytic cleavage, as the ortho and para isomers were calculated^{24,25} to require 44.5 and a 43.0 kcal/mol, respectively. As with the ortho isomer, the major product generated from this collisional activation is the loss of 156.14, again representative of robust radical initiation (Figure 1A and B). Unlike α-TEMPO-Bz, however, no other high abundant neutral or cationic losses are observed (Figures 1A and B). Low abundance losses of 140.14 Da are observed in some para-conjugated peptides (Figure 1B and Table S1) and indicate that protonation of the radical precursor still occurs in para-conjugated peptides although to a lesser extent. The absence of the 157.15 Da loss in para-conjugated peptides suggests that the carbocation product is not a highly efficient process and much of this product formation in ortho-conjugated peptides is potentially due to this proposed alternative mechanism (Scheme 2B). To quantitatively assess the influence...
that para-TEMPO-Bz has on radical initiation efficiency, we divided the ion abundance of each radically initiated species by the sum of all significant species present in each MS^3 spectrum. On average, collisional activation of para-TEMPO-Bz conjugated peptides results in approximately 23% more radical initiation than ortho-TEMPO-Bz conjugated species (Figure 2). While the elimination of the undesired heterogeneous processes is the basis of the majority of these gains, in angiotensin I the primary benefit is more efficient dissociation of the precursor ion (Figure S3). While conjugation of melittin with p-TEMPO-Bz results in the elimination of the cationic losses of 140.14 and 158.15 Da, no significant change in the relative abundance of the radical initiated species is observed (Figure 2 and S2).

**Figure 2. Fraction ion abundance of radically initiated species following CID activation of o- and p-TEMPO-Bz-conjugated peptides.**

MS^3 of o- and p-tempo-Bz-conjugated peptide ions

To examine if the increased abundance of the radically initiated species results in tangible benefits in sequence coverage, we isolated, collisionally activated, and sequenced the radical initiated peak of each conjugated peptide. MS^3 activation of the radically initiated ortho-and para-TEMPO-Bz conjugated ACTH 1-14 and β-amyloid 10-20 results in a robust series of product ions (Figure 3A, 3B, and Tables S2, S3, S4, and S5). The increased energetics of beam-type CID does promote significant of b- and y-type product ion formation, but a series of c- and z-type ions are also generated, indicating progression through radical-driven mechanisms. In both ACTH 1-14 and β-amyloid 10-20, the para-isomer promoted the dissociation of a bond between every amino acid and generated 100% sequence coverage (Figure 3A and B). This is significantly greater than the sequence coverage generated by the ortho-isomer which generated 69 and 80 % sequence coverage for ACTH 1-14 and β-amyloid 10-20, respectively (Figure 4). In addition to the reduced sequence coverage, the isolation of ortho-TEMPO-Bz conjugated ACTH 1-14, substance P, and β-amyloid 10-20 is complicated by the proximity of the undesired even-electron loss of 157.15 Da (Figure S1, 1B, and 1A). This even-electron species is more robust to collisional activation and thus, when MS^3 is applied, the benzyl radical species is depleted semi-selectively (Figures S4, S5, 3A and 3B). The prominence of this surviving even-electron species and the relatively low abundance (frequently less than 20%) of any generated product ions further complicates and reduces the confidence of any potential bioinformatic analyses.

**Figure 3. MS^3 mass spectra for both ortho- and para-Bz-conjugated ACTH 1-14 (A), β-amyloid 10-20 (B), and angiotensin I (C).**

Unlike ACTH 1-14 and β-amyloid 10-20, when doubly charged angiotensin I and substance P are subjected to MS^3 activation, few c- and z-type ions are generated (Figures 3C, S4, and Tables S6, S7, S8, and S9), and the spectrum is dominated by b- and y-type ions. This is different behavior than that observed when resonance CID is utilized (Figure S6, S7, and Tables S10, S11, S12, and S13). Interestingly, despite the dominate mechanism of fragmentation being heterolytic the para-conjugated substance P still demonstrated increased sequence coverage (Figure 4).

In the absence of guanidination, NHS-based derivatization could lead to labeling of either the N-terminus or any existing lysine residue. To ensure that the dissociation patterns demonstrated by the ortho- and para-precursors were not a result of differing labeling preferences, we utilized the robust product ion generation upon MS^3 analyses to assign the location of each precursor conjugation site. These product ions enabled the assignment of both the ortho and para precursor to the N-terminus and lysine 7 in angiotensin I and β-amyloid 10-20, respectively.
In substance P, both FRIPS isomers could be localized to either the N-terminus or lysine 3, but no product ions were observed to differentiate between these two sites. Unlike the other three peptides, when the para-TEMPO-Bz conjugated ACTH 1-14 spectrum is interpreted, conjugation at the N-terminus and lysine 11 are indicated. The lysine 11 isomer appears to be more abundant based on the relative abundance of the \( e_9, e_3', a_9, \) and \( a_9' \) product ions (Figure 3A). Product ion abundance is significantly reduced in the spectrum of ortho-conjugated ACTH 1-14 and thus, only the presence of an underivatized \( x_{12} \) ion indicates N-terminal conjugation. Overall, these results suggest that the differences in product ion generation are unlikely to be related to differences in conjugation site.

Interestingly, both the ortho- and para-radical precursor required approximately the same amount of energy (26%) to initiate peptide fragmentation in MS3. This is different behavior than that demonstrated in the recent work by Lee et al. where they demonstrated that activation of their new p-TEMPO-Bz-Sc required lower amounts of energy for MS3. A22 This difference in behavior supports the author’s contention that removal of the carbonyl group from their new precursor decreases the stability of the generated radical.

**Figure 4.** Sequence coverage for each indicated o- and p-TEMPO-Bz-conjugated peptides.

**Free radical initiated peptide sequencing of melittin**

As the size of the polypeptides increase, CID is typically funneled towards a handful of labile peptide bonds significantly limiting sequence coverage.28 To examine the applicability of this new FRIPS precursor to peptides of this size, we examined melittin, a 26 amino acid peptide. When melittin is sequenced with CID, few product ions are generated in the C-terminal region leading to limited sequence coverage.29 When FRIPS is employed and triply charge ions of both the o- and p-TEMPO-Bz conjugated melittin are activated, a-, c-, z-, and w-type product ions are generated indicating robust radical-driven dissociation. We also observe the abundant loss of amino acid residue side chains which is also indicative of radical-driven dissociation.9 The sequence coverages generated by both radical precursors are significantly higher than that generated by typical CID-based analyses (Figure 5 and Tables S14 and S15). A28 Similarly to the previously examined peptides, the para-orientation generates significantly higher sequence coverage than the ortho-precursor dissociating 23 of the 25 (92%) inter-residue bonds (Figure 4). As with the other peptides, MS3 analyses identify similar conjugation profiles between the ortho and para radical precursors with Lys23 and N-terminal modifications being assignable for both reagents (Tables S14 and S15). The performance of FRIPS towards this larger peptide makes this reagent a potential promising option to middle-down workflows that frequently generate poor sequence coverage.

**Figure 5.** (A) HCD MS3 o- and p-TEMPO-Bz-conjugated Melittin 3+ cations (B) Fragment ion type generated.

**Conclusion**

In this work, we demonstrate that peptide cations conjugated with the ortho-substituent pattern of TEMPO-Bz undergo significant neutral and charged losses when collisionally activated. These mass losses shuttle ion abundance away from sequence informative product ions leading to decreased ion abundance and reduced sequence coverage. In contrast, para-TEMPO-Bz-conjugated peptide ions demonstrate drastically lower abundance of these losses and on average generate significantly greater sequence coverage. Lastly, we employed both the para- and ortho-substituent patterns of FRIPS to dissociate melittin. Both tags promote robust fragmentation of this large peptide ion but again the para-isomer generates superior sequence coverage. The improved sequence coverage and product ion abundance generated by this para-isomer make it a compelling alternative to the typically utilized FRIPS precursors.

**ASSOCIATED CONTENT**

**Supporting Information**

Synthetic schemes and procedures, additional mass spectra, and mass tables can be found in the supporting information. Raw mass spectra can be found under the accession code MSV00091064 in the MassIVE data repository (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp). The Supporting Information is available free of charge on the ACS Publications website.

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