Efficient isothiocyanate modification of peptides facilitates structural analysis by radical-directed dissociation

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Abstract

Radical-directed dissociation (RDD) is a powerful technique for structural characterization of peptides in mass spectrometry experiments. Prior to analysis, a radical precursor must typically be appended to facilitate generation of a free radical. To explore the use of a radical precursor that can be easily attached in a single step, we modified peptides using a “click” reaction with iodophenylisothiocyanate. Coupling with amine functional groups proceeds with high yields, producing stable iodophenylthiourea modified peptides. Photodissociation yields were recorded at 266 nm and 213 nm for the 2-, 3-, and 4-iodo isomers of the modifier and found to be highest for the 4-iodo isomer in nearly all cases. Fragmentation of the modified peptides following collisional activation revealed favorable losses of the tag, and electronic structure calculations were used to evaluate a potential mechanism involving hydrogen transfer within the thiourea group. Examination of RDD data revealed that 4-iodobenzoic acid, 4-iodophenylthiourea, and 3-iodo-tyrosine yield similar fragmentation patterns for a given peptide, although differences in fragment abundance are noted. Iodophenylisothiocyanate labeling in combination with RDD can be used to differentiate isomeric amino acids within peptides, which should facilitate simplified evaluation of isomers present in complex biological samples.

Introduction

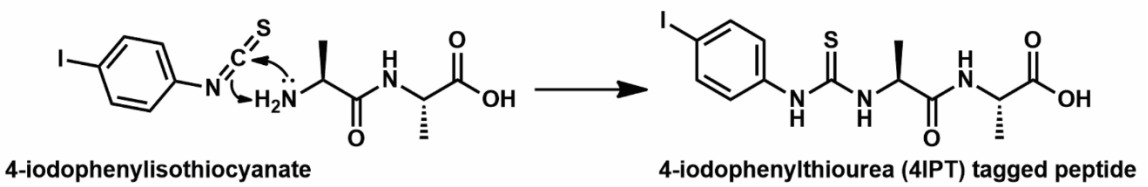
Mass spectrometry (MS) is a powerful technique for the characterization of biomolecules due to its ability to accurately measure the mass of intact analytes and obtain structural information following fragmentation. A variety of fragmentation methods are available, with differing chemical mechanisms creating fragments in each. The most commonly employed fragmentation method is collision-induced dissociation (CID), which involves heating molecules through collisions with a neutral gas.[1] These collisions impart small amounts of energy into the molecule which build up until the lowest energy dissociation pathway is accessible, at which point fragmentation occurs. In peptides, this method produces primarily b- and y-ions via cleavage of the amide bond. Photodissociation can be employed to impart energy into the analyte via absorbed photons. In the longer wavelength region, infrared multiphoton dissociation (IRMPD) heats a peptide slowly and results in a similar spectrum to CID,[2] while ultraviolet photodissociation (UVPD) can be employed at different UV wavelengths to generate diverse fragments by dissociative electronic excitation.[3,4,5,6,7]

As opposed to these methods which operate by imparting energy into even-electron peptides, distinct fragmentation pathways are observed for activation of odd-electron or radical analytes. In electron-transfer dissociation (ETD) or electron-capture dissociation (ECD), a hydrogen abundant radical is initially created by the incoming electron.[8,9,10] Dissociation of this fragile species produces c- and z-ions that are not typically observed in even-electron fragmentation spectra. Radical-directed dissociation (RDD) also generates odd-electron species, but operates through a different mechanism. In RDD, a radical is produced through homolytic cleavage of a labile bond, yielding a hydrogen-deficient molecule that is also susceptible to fragmentation.[11] Photocleavage of a carbon-iodine bond can cleanly produce such radicals with quantitative yields.[12] Suitable carbon-iodine bonds can be incorporated by iodination of tyrosine residues.[13] Alternatively, 4-iodobenzoic acid (4IB) can be appended to the N-terminus or a lysine residue through an NHS-ester reaction.[12,14] In both cases, the iodine atom is coupled directly to a phenyl ring that acts as a chromophore and enhances absorption of incident light, increasing the photodissociation yield. Other methods for radical generation include the use of CID-cleavable tags such as TEMPO[15], nitric oxide[16], and peroxycarbamate[17].

In a hydrogen-deficient peptide, the radical can migrate to other sites by abstracting a hydrogen atom, relocating the hydrogen to the original radical location and generating a radical in the new site. Addition of collisional energy results in unique fragmentation through bond cleavage initiated by the radical to produce a-, x-, and z-ions as well as side-chain losses.[18]

Radical migration takes place between sites which are spatially proximal, a property that is independent of peptide sequence. The mechanism for radical migration lends RDD high structural sensitivity and the ability to identify isomers in lipids,[19] glycans,[20] and peptides[21,22] based on differences in fragmentation intensities among the various forms.

RDD typically requires covalent modification of analytes prior to analysis. Although not commonly employed with mass spectrometry, isothiocyanate functional group tagging of peptides and proteins is commonly employed within biology. Historically, the Edman degradation procedure was ubiquitously used to sequence proteins by capitalizing on the reaction between protein N-terminal amines and isothiocyanates.[23] Isothiocyanates are also naturally occurring in some dietary vegetable species and have been studied for their native reactivity with protein functional groups.[24,25] However, the most common use of isothiocyanates in protein research is the fluorescent labelling of protein amines utilizing labels such as fluorescein isothiocyanate (FITC)[26,27] or rhodamine isothiocyanate.[28] Isothiocyanates are one class of "click" reagents, which react rapidly in a one pot synthesis with high yield.[29] Isothiocyanate reacts with both amines (as illustrated in Scheme 1) and thiols, although the thiol product is less stable and the modification is reversible.[30] Previous uses with mass spectrometry include isothiocyanate reactions employed to attach sulfonic acid groups to peptide digests to aid with gas-phase peptide sequencing.[31,32] Isothiocyanate derivatization has additionally been implemented to enhance separation in liquid chromatography coupled to mass spectrometry.[33]



Scheme 1. Concerted mechanism for the reaction of a free amine with the isothiocyanate functional group to produce a thiourea linkage.

Herein, we characterize isothiocyanate modified peptides for suitability in RDD experiments. Photodissociation yields at 213 nm and 266 nm were measured as a function of iodine position. Fragmentation following radical initiation was characterized, revealing both typical RDD products and fragments unique to the isothiocyanate tag. A mechanism supported by B3LYP/6-31+G(d) calculations that can account for the unique fragments is proposed. The effectiveness of isothiocyanate modified peptides for RDD-based structural characterization and differentiation of isomers is also evaluated.

Experimental Procedures

Materials

4-iodophenylisothiocyanate was purchased from Oakwood Products Inc. 2- and 3-iodophenylisothiocyanate were purchased from Fisher Scientific. All iodine-containing reagents were used without further purification. RRLIEDNEYTARG was purchased from Bachem Inc. RQpsVELHSPQSLPR was purchased from Anaspec Inc. All other peptides were synthesized using an accelerated FMOC-protected solid-phase peptide synthesis protocol[34] using FMOC-protected amino acid residues purchased from Anaspec Inc.

Modification Reaction

Peptides were dissolved in a 1:1 ratio of 50 mM borate buffer pH 8.5:acetonitrile to a concentration of 250 μM in 40 μL. Iodophenyisothiocyanate was dissolved in acetonitrile to a concentration of 50 mM and 0.2, 1, or 2 μL were added to the peptide for a 1:1, 1:5, or 1:10 peptide:isothiocyanate ratio for 30 minutes for the reaction tests in Fig. 1. Subsequent reactions were performed at a 1:10 ratio for maximum yield. Samples were desalted using a Michrom Bioresources MacroTrap peptide trap and re-dissolved in 50:50 H_2O:ACN with 0.1% formic acid to a final concentration of 10 μM for electrospray mass spectrometry.

Mass Spectrometry

Electrospray ionization (ESI) at 4 kV spray voltage, 275°C capillary temperature, and sheath gas flow rate of 5 was used for analysis of peptides in Orbitrap and LTQ mass spectrometers. Other source voltages were optimized for maximum signal. UVPD experiments at 213 nm were conducted in an Orbitrap Velos Pro with an HCD cell modified with a quartz window. Photoactivation was carried out for 50 ms at 1000Hz with 2.5 μJ laser pulses from an FQSS 213-Q4 CryLas laser. UVPD experiments at 266 nm were performed in an LTQ ion trap mass spectrometer modified with a quartz window to allow for photoactivation with an Nd:YAG laser (Continuum, Santa Clara, CA). Photodissociation was achieved by a single-shot excitation with an approximately 4mJ laser pulse.

Electronic Structure Calculations

Theoretical calculations were carried out using density functional theory as implemented in Gaussian09. The B3LYP functional was used with the 6-31+G(d) basis set. Transition states were found using the QST3 approach and verified by calculating vibrational frequencies, for which a single imaginary value was obtained.

Results and Discussion

In this study, model synthetic peptides were incubated with commercially available iodophenylisothiocyanates to modify free amines located at either the N-terminus or a lysine side-chain as shown in Scheme 1. The outcome of this reaction is the attachment of an iodophenyl radical precursor to a peptide via a thiourea linker. The resulting iodophenylthiourea (IPT) tagged-peptide contains a phenyl chromophore attached to a photolabile iodine atom that can be irradiated with 266 nm or 213 nm light to produce homolytic cleavage and generate a site-specific radical. Once generated, the radical can migrate to spatially proximal sites throughout the sequence and cleave bonds to produce structurally-dependent fragments. Reaction yields are shown in Fig. 1a for the incubation of 3-iodophenylisothiocyanate with RRLIEDNEYTARG as a function of stoichiometric excess and temperature. The yields are estimated based on ion counts from all contributing charge states for the modified and unmodified peptides. The additional iodophenyl group in the modified peptides is hydrophobic, which may enhance ionization and lead to slight over-representation of the modified form.[35] The reported yields therefore represent an upper limit, but are valid for comparison of varying conditions. After 30 minutes of incubation at room temperature, modest modification (~9%) is observed even with 10-fold excess isothiocyanate. However, increasing the temperature slightly to 37°C resulted in a significantly higher yield (~96%) for the same 1:10 ratio. Notably, at 37°C even the 1:1 ratio was able to achieve over 70% yield. Isolation and irradiation of the modified peptide is shown in Fig. 1b, where application of 213 nm light produces a site-specific homolytic cleavage of the carbon-iodine bond, generating a radical ion.

To examine the effect of iodine position on subsequent dissociation products, we modified a series of peptides with the 2-, 3-, and 4-iodophenylisothiocyanates. The respective photodissociation yields following activation at 266 nm are shown in Fig. 1c. There is a significant difference observed between the isomers, and the 4-iodo isomer is found to produce the greatest relative yield for all of the examined sequences. The 3-iodo isomer was found to produce a higher or similar yield as the 2-iodo isomer in most cases. The variation in yields between the different sequences is likely due to the presence of additional chromophores found in the aromatic side-chains of tyrosine, phenylalanine, and tryptophan. These chromophores are capable of transferring energy if the peptide structure places them in proximity to the 4IPT tag. The photodissociation yield may also differ based on proximity to charged sites, which can lead to shifts in the absorption profile.[36] Photodissociation yields obtained following 213 nm excitation are shown in Fig. 1d. Smaller differences are observed at 213 nm, but the 4-iodo isomer is generally slightly favored at this wavelength as well.

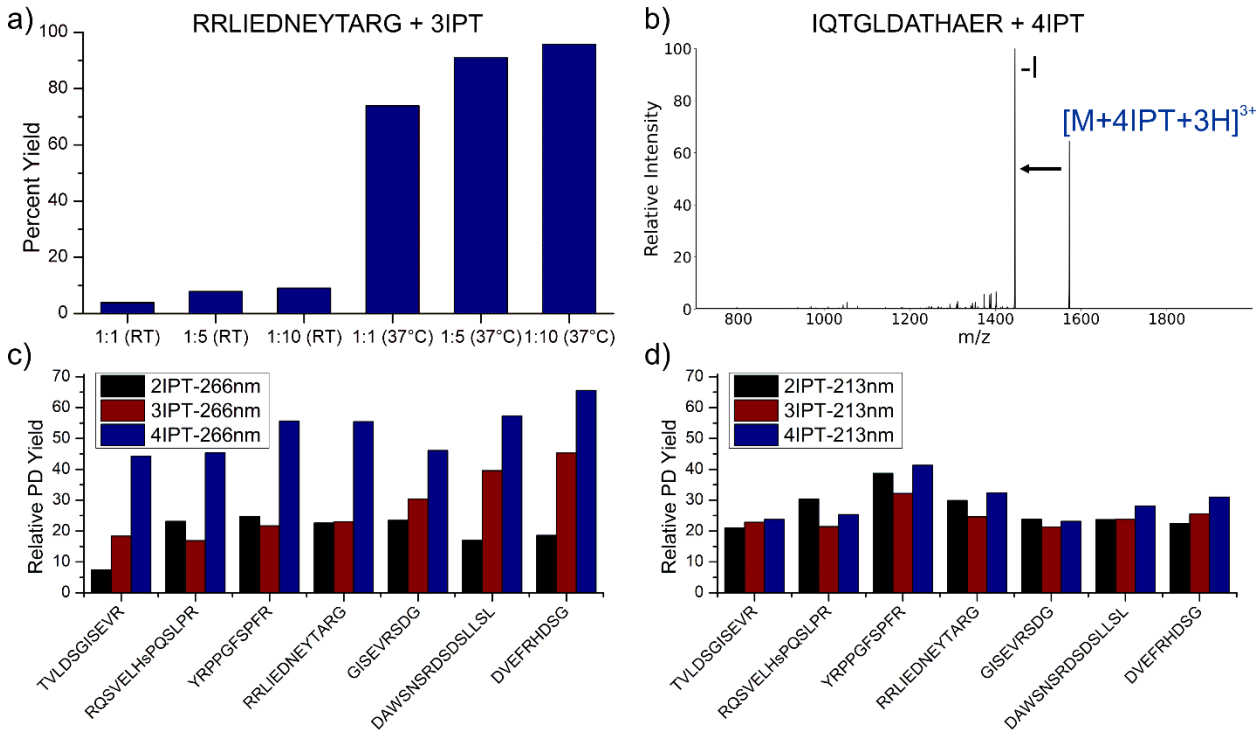


Figure 1. a) Reaction yields based on MS data for modification of the peptide RRLIEDNEYTARG with isothiocyanate. Temperature exerts a greater effect than stoichiometric excess. b) Irradiation with 213 nm light produces site-specific cleavage of the iodine atom to generate a radical peptide. c) Photodissociation yields for a series of peptides irradiated with 266 nm light. d) Photodissociation yields for peptides irradiated with 213 nm light.

Following loss of iodine, collisional activation of the re-isolated radical yields the RDD spectrum, as shown for DVEFRHDSG modified with 4IPT in Fig. 2a. A series of a- and z-ions are observed as well as partial and full side-chain losses which are typical of RDD spectra. Additionally, we observed two unique losses which correspond to partial-tag (-pt) and full-tag (-ft) losses of the 4IPT. RDD of the deprotonated peptide GISEVRSDG is shown in Fig. S1, displaying typical radical fragments in addition to the partial and full tag losses. As opposed to the proton-driven mechanism in CID, the overall peptide charge does not play a central role in radical fragmentation and RDD is relatively unaffected by charge state or polarity.[37] The CID spectrum of the non-radical peptide shown in Fig. 2b also displays abundant loss of the partial- and full-tag fragments, indicating that these losses are primarily a result of vibrational heating. Further isolation and fragmentation of the partial-tag loss is shown in Fig. 2c. In this spectrum, no fragment corresponding to the full-tag loss is observed, indicating that the partial-tag loss is not the first step towards the full-tag loss, rather each is generated independently. CID of the re-

isolated full-tag-loss fragment is shown in the top spectrum of Fig. 2d, compared to fragmentation of the unmodified peptide in the bottom spectrum. The two spectra are nearly identical, suggesting that the full-tag loss leaves behind the original sequence without discernable alteration. Similarly favorable fragmentation has been observed previously in other urea and thiourea containing compounds.[38,39,40]

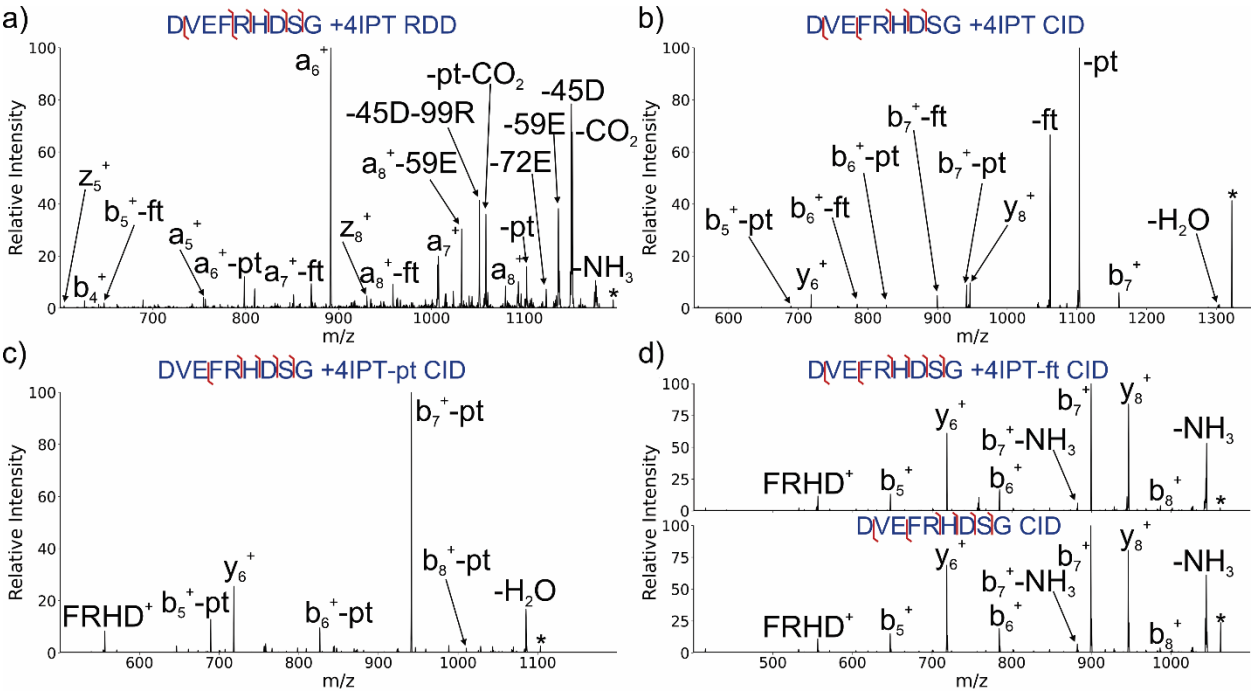
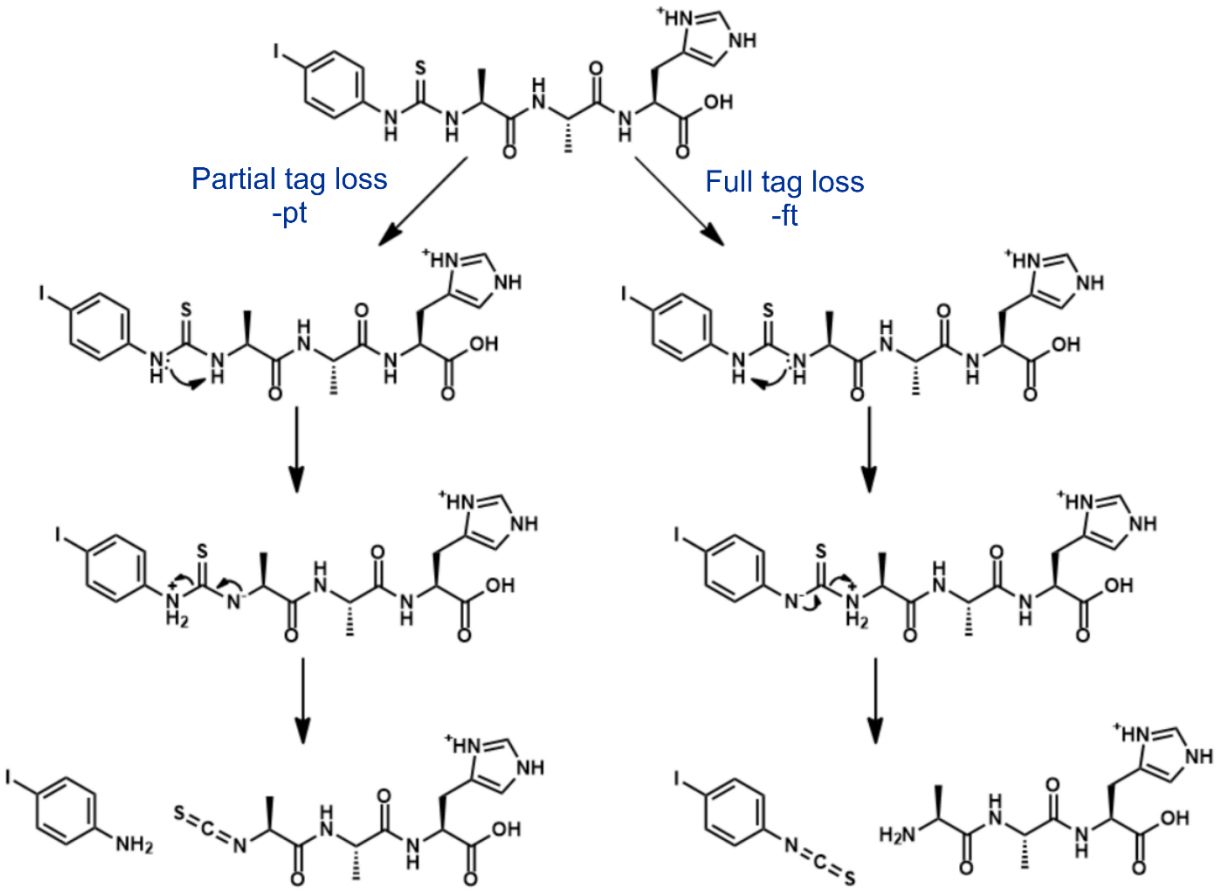


Figure 2. a) RDD fragmentation of DVEFRHDSG [M+4IPT+H]$^+$ produces a-ions and side-chain losses. Unique losses of the partial tag (-pt) and full tag (-ft) are observed in some fragments. b) CID fragmentation of DVEFRHDSG modified with 4IPT produces b/y-ions and partial/full tag loss. c) CID fragmentation of the partial tag loss does not lead to full tag loss indicating different mechanistic origins. d) CID fragmentation of the full tag loss (top) matches CID of the unmodified peptide (bottom).

A proposed mechanism for both unique tag losses is shown in Scheme 2. For the partial-tag loss, the first nitrogen of the thiourea abstracts a hydrogen from the second. This results in dissociation of the bond connecting the first nitrogen with the thiourea carbon, and loss of the iodophenyl group with an amine. The sulfur and carbon from the thiourea group remain on the peptide and form an isothiocyanate group at the N-terminus. In a complementary mechanism for the full tag loss, the second nitrogen deprotonates the first, resulting in loss of the full tag with the two terminal groups swapped. The iodophenyl group in this mechanism is lost as an isothiocyanate, reforming the initial reactant that was used for modification. The peptide ends up

with a terminal amine, regenerating the original peptide structure. As the original structure is reformed, the fragmentation matches the unmodified peptide, as illustrated in Fig. 2d.



Scheme 2. Proposed mechanisms involving hydrogen transfer for the fragmentation pathways of the partial tag and full tag losses observed in the RDD and CID spectra of IPT modified peptides.

Energy minimization and transition state calculations on a model structure (phenyl-thiourea-methyl) were carried out using the B3LYP/6-31+G(d) level of theory. Transition states were confirmed by the presence of a single imaginary frequency that corresponded to transfer of a hydrogen atom, as shown in Fig. 3a. An analogous structure was also optimized for urea, which yielded a similar transition state (see Fig. S2). Structures with two methyl groups were optimized as well (see Fig. S3 and S4). The relative energetics for reactants/transition state/products are shown in Fig. 3b. The transition states for urea and thiourea are structurally quite similar, as are the changes in energy +206 kJ/mol (urea) compared to +181 kJ/mol (thiourea). The final products for each are analogous as well, and the calculations suggest that both pathways are

somewhat endothermic. An alternative proton-driven mechanism has been proposed in model alkyl thiourea and alkyl urea compounds that can also yield dissociation of the same carbon-nitrogen bonds.[39] However these pathways require multiple rearrangements and are guarded by similar transition state barriers that yield more endothermic products.

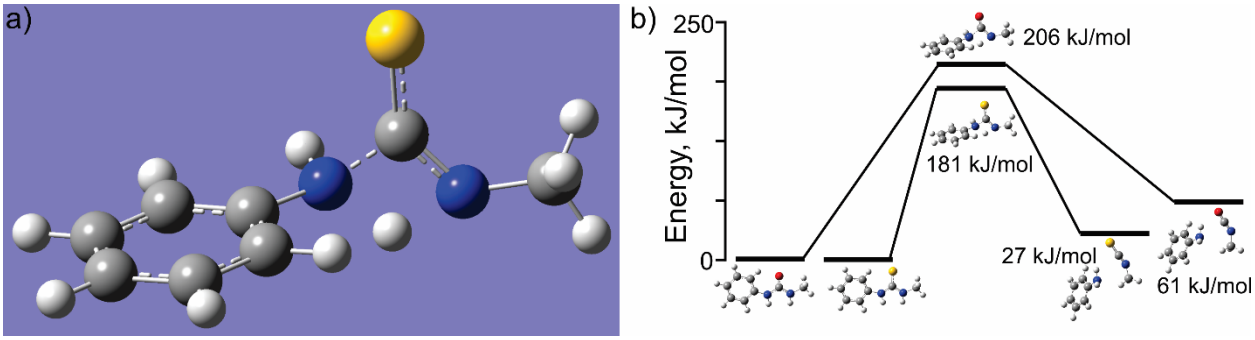


Figure 3. a) Transition state for the proposed mechanism of partial and full tag losses involving the thiourea linker. A hydrogen is transferred between nitrogen atoms, leading to dissociation of one carbon-nitrogen bond. b) Energy diagram for the overall dissociation reaction of the thiourea linker and analogous urea linker. Energies are relative to the corresponding initial reactants.

A comparison of RDD performed with the 4IPT tag versus the previously used 4IB tag and iodo-tyrosine modifications is shown in Fig. 4. In all spectra the common RDD fragments of a-, x-, and z-ions as well as abundant side-chain losses are observed. Additionally, c_9 and z-1 ions are observed in all cases, which are produced by a unique radical mechanism at threonine residues.[18] Some variation in fragment abundance is observed between the tags, most noticeably in the -87R$^+$ and -106Y peaks. Apparent intensity differences are evident in the a_9 and x_{10} fragments as well, which are both reduced in intensity in the 4IPT spectrum. The iodo-tyrosine spectrum is the only one which did not contain the x_{10} ion or the b_6 ion. With the iodo-tyrosine radical precursor, the radical starts in a location remote from the N-terminus, and this change is similarly reflected in the change of fragmentation intensity compared to the N-terminally tagged peptides. These examples illustrate the structural sensitivity of RDD, where differences in the precursor composition, radical starting location, or gas phase structure can influence fragmentation intensities in the resulting spectra. The most notable difference between the spectra is the partial tag loss that yields the most abundant peak in the 4IPT-tag spectrum. In comparison with 4IB, where the tag is attached via an amide group, no analogous loss of the 4IB-tag is observed. This difference may provide further evidence that the mechanism of tag loss is occurring through hydrogen transfer between the nitrogen atoms of the thiourea group,

which cannot occur in an amide group that contains only one nitrogen atom. Each radical precursor has advantages and disadvantages for its application in peptide analysis. 4IB features the highest PD yield, and specifically reacts with amines. However, the procedure to install 4IB requires an extra step for prior activation via an NHS-ester. Iodination of tyrosine is able to utilize a native tyrosine residue in the sequence without prior activation, however this precursor use is limited as the residue is not found in every sequence. Additionally, the use of an oxidizer during the iodination can modify other residues such as methionine, and it is difficult to avoid some addition of multiple iodine atoms that complicate the experiment. IPT requires no prior activation, and additionally contains unique tag losses which can be used to confirm the identity of modified peptides. IPT typically generates lower PD yields than 4IB, however, which reduces the amount of radical ions available for subsequent analysis. The choice of radical precursor can be tailored from these options depending on the needs of the experiment.

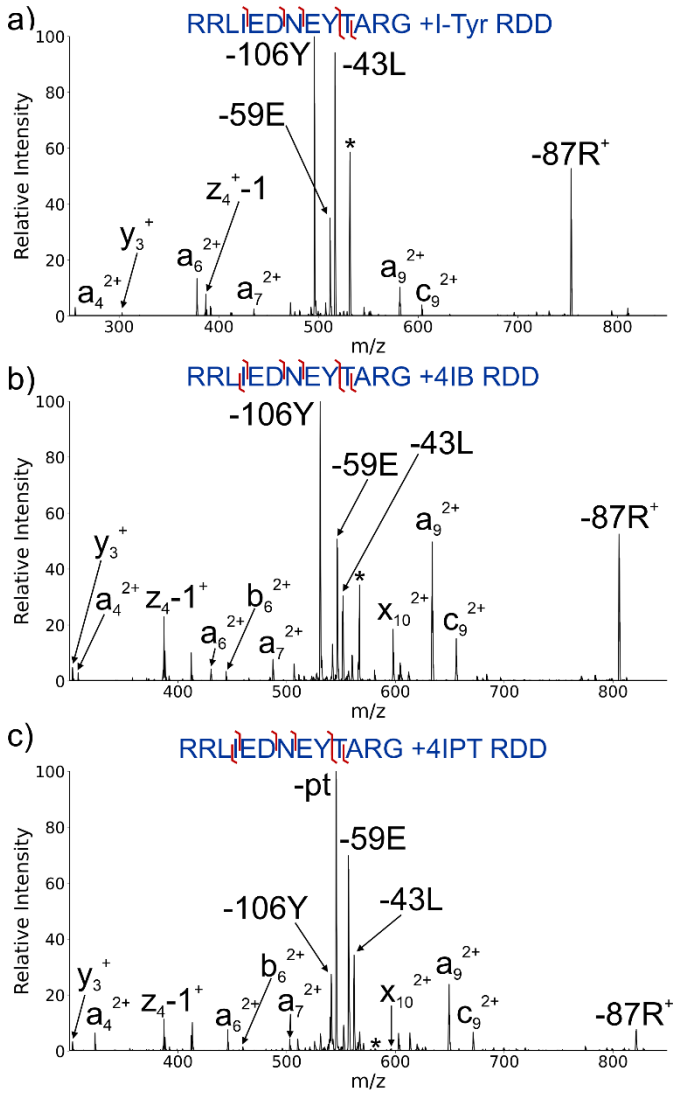


Figure 4. RDD spectra for RRLIEDNEYTARG $[M+3H]^{3+}$ modified by a) iodination of tyrosine to b) 4IB and c) 4IPT. The same major fragments are observed in all spectra with some variations in abundance. Exceptions include b_6 and x_{10} fragments that are not observed iodotyrosine. The 4IPT spectrum) additionally contains a partial tag (-pt) loss that is not observed with 4IB.

One of the most advantageous applications of RDD is structural characterization, due to the sensitivity of radical migration to three-dimensional structure. Radical migration occurs between sites which are nearby in space but may be distant in sequence. Isomerization of residues inherently affects the underlying structure, and these changes further alter the final gas-phase structure of peptide analytes. The variation in radical migration pathway and change in energetic favorability leads to larger changes in the abundance of fragments produced with this technique. As a result, RDD is able to discern isomers more sensitively than other techniques such as CID.

Comparison of RDD spectra for peptide pairs where a single amino acid has been isomerized is shown in Fig. 5. For $[GISEVRSDG+H]^+$ (with either L-Asp or L-isoAsp), the resulting RDD spectra vary most in the abundances of the a_8 and $-CO_2$ losses. To quantitatively assess differences between the spectra, an R_{isomer} score is calculated from the two fragments that change abundance the most between spectra. The ratio of these two fragments in each spectrum are taken and divided to yield a ratio of ratios. A significance threshold R_{isomer} score of 2.4 has been established previously with 4IB.[41] For Fig. 5a, an R_{isomer} score of 6.40 is obtained from the 4IPT RDD spectra for the GISEVRSDG isomers. In Fig. 5b, the sequence HFSPEELK contains the L-Ser and D-Ser epimers, differing in inversion of one chiral bond. Here, the glutamic acid sidechain and partial tag loss fragments change significantly between spectra with a score or 5.16. While these epimers represent smaller modifications to the structure compared to L-isoAsp, they are still discriminated using radical fragmentation. A variety of peptides were tested and the resulting R_{isomer} scores are displayed in Table 1. 4IPT is able to differentiate isomers of Asp, Ser, His, and Glu, demonstrating versatility in RDD isomer analysis. Additional comparisons to 4IB scores are shown for two sequences at the bottom of Table 1. For these particular sequences, 4IPT yields slightly higher scores, but the similar scores indicate that RDD discrimination of isomers will likely be comparable for 4IPT and 4IB.

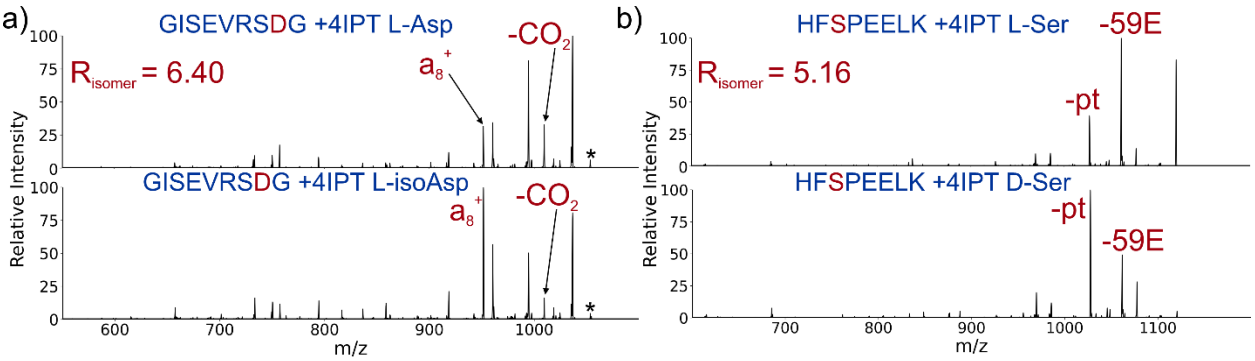


Figure 5. a) RDD examination of GISEVRSDG $[M+4IPT+H]^+$ in the L-Asp form (top) and L-isoAsp form (bottom). b) RDD examination of HFSPEELK $[M+4IPT+H]^+$ epimers. L-Asp (top) and D-Asp (bottom).

Sequence	Isomer 1	Isomer 2	R_{isomer} Score
GISEVRS**D**G	L-Asp8	L-isoAsp8	6.40
WF**D**TGK	L-Asp3	D-Asp3	3.96
DVEFRH**D**SG	L-Asp7	L-isoAsp7	6.16

DAWSNSR**D**SDSLLSL	L-Asp8	L-isoAsp8	4.56
TVLDSGIS**E**VR	L-Glu9	D-isoGlu9	5.15
HF**S**PEELK	L-Ser3	D-Ser3	5.00
IQTGL**D**ATHAER	L-Asp6	D-Asp6	3.30
HFSPEDLTVK	L-His1	D-His1	5.16
IQTGL**D**ATHAER (4IB)	L-Asp6	D-Asp6	2.67
HFSPEDLTVK (4IB)	L-His1	D-His1	3.25

Table 1. R_{isomer} scores obtained from RDD analysis of various peptides isomers. Raw data is included in the supporting information.

Conclusion

We have characterized the modification of peptides with a new radical precursor using a commercially available iodophenylisothiocyanate that easily forms covalent bonds with amines. This modification was found to be well-suited for RDD experiments, producing the typical fragments expected of a radical peptide while maintaining sufficient structural sensitivity to allow for isomer identification. The tag is partially labile and can be lost with sufficient vibrational excitation, producing unique mass losses that could be used to confirm the identity of modified peptides in complex samples. Although isocyanate-reactive reagents have been widely employed in solution-phase studies of proteins, they have found less use in mass spectrometry or proteomics. The ease of modification and stability of the resulting products in both solution and the gas phase suggest that isocyanate coupling may be an excellent candidate for other applications requiring covalent modification of peptides or other biomolecules for mass-spectrometry based experiments.

Supporting Information

Anionic RDD spectrum for DVEFRHDSG (Figure S1), transition state structure for phenyl-urea-methyl (Figure S2), transition state structures for methyl-thiourea-methyl (Figure S3) and methyl-urea-methyl (Figure S4), and mass spectra for the peptide R_{isomer} scores shown in Table 1 (Figure S5-S12).

Acknowledgements

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References

[1] Wells, J.M.; McLuckey, S.A. Collision-Induced Dissociation (CID) of Peptides and Proteins. *Methods Enzymol.* **2005**, *402*, 148-185.
[2] Crowe, M.C.; Brodbelt, J.S. Infrared Multiphoton Dissociation (IRMPD) and Collisionally Activated Dissociation of Peptides in a Quadrupole Ion Trap with Selective IRMPD of Phosphopeptides. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1581-1592.
[3] R. Julian, R. The Mechanism Behind Top-Down UVPD Experiments: Making Sense of Apparent Contradictions. *J. Am. Soc. Mass Spectrom.* **2017**, *28* (9), 1823–1826.
[4] Brodbelt, J.S.; Morrison, L.J.; Santos, I. Ultraviolet Photodissociation Mass Spectrometry for Analysis of Biological Molecules. *Chem. Rev.* **2020**, *120(7)*, 3328-3380.
[5] Girod, M.; Sanader, Z.; Vojkovic, M.; Antoine, R.; MacAleese, L.; Lemoine, J.; Bonacic-Koutecky, V.; Dugourd, P. UV Photodissociation of proline-containing peptide ions: insights from molecular dynamics. *J. Am. Soc. Mass Spectrom.* **2015**, *26(3)*, 432-443.
[6] Zhang, L.; Reilly, J.P. Peptide de Novo Sequencing Using 157 nm Photodissociation in a Tandem Time-of-Flight Mass Spectrometer. *Anal. Chem.* **2010**, *82(3)*, 898-908.
[7] Morgan, J.W.; Hettick, J.M.; Russell, D.H. Peptide sequencing by MALDI 193-nm photodissociation TOF MS. *Methods Enzymol.* **2005**, *402*, 186-209.
[8] Moore, B. N.; Ly, T.; Julian, R. R. Radical Conversion and Migration in Electron Capture Dissociation. *J. Am. Chem. Soc.* **2011**, *133* (18), 6997–7006.
[9] Syka, J.E.P.; Coon, J.J.; Schroeder, M.J.; Shabanowitz, J.; Hunt, D.F. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101(26)*, 9528-9533.
[10] Oh, H.; Breuker, K.; Sze, S.K.; Ge, Y.; Carpenter, B.K.; McLafferty, F.W. Secondary and tertiary structures of gaseous protein ions characterized by electron capture dissociation mass spectrometry and photofragment spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99(25)*, 15863-15868.
[11] Tureček, F.; Julian, R.R. Peptide Radicals and Cation Radicals in the Gas Phase. *Chem. Rev.* **2013**, *113*, 6691-6733.
[12] Ly, T; Zhang, X.; Sun, Q.; Moore, B.; Tao, Y.; Julian, R.R. Rapid, quantitative, and site specific synthesis of biomolecular radicals from a simple photocaged precursor. *Chem. Commun.* **2011**, *47*, 2835-2837
[13] Ly, T.; Julian, R.R. Residue-Specific Radical-Directed Dissociation of Whole Proteins in the Gas Phase. *J. Am. Chem. Soc.* **2008**, *130*, 351-358.
[14] Riggs, D.L.; Gomez, S.V.; Julian, R.R. Sequence and Solution Effects on the Prevalence of D-isomers Produced by Deamidation. *ACS Chem. Biol.* **2017**, *12(11)*, 2875-2882.
[15] Lee, M.; Kang, M.; Moon, B.; Oh, H.B. Gas-phase peptide sequencing by TEMPO-mediated radical generation. *Analyst* **2009**, *134*, 1706-1712.
[16] Hao, G.; Gross, S.S. Electrospray Tandem Mass Spectrometry Analysis of S- and N-Nitrosopeptides: Facile Loss of NO and Radical-Induced Fragmentation. *J. Am. Soc. Mass Spectrom*. **2006**, *17*, 1725-1730.
[17] Masterson, D.S.; Yin, H.; Chacon, A.; Hachey, D.L.; Norris, J.L.; Porter, N.A. Lysine Peroxycarbamates: Free Radical-Promoted Peptide Cleavage. *J. Am. Chem. Soc.* **2004**, *126*, 720-721.
[18] Sun, Q.; Nelson, H.; Ly, T.; Stoltz, B.M.; Julian, R.R. Side Chain Chemistry Mediates Backbone Fragmentation in Hydrogen Deficient Peptide Radicals. *J. Proteome Res.* **2008**, *8(2)*, 958-966.

[19] Pham, H.T.; Ly, T.; Trevitt, A.J.; Mitchell, T.W.; Blanksby, S.J. Differentiation of complex lipid isomers by radical-directed dissociation mass spectrometry. *Anal. Chem.* **2012**, *84(17)*, 7525-7532.
[20] Riggs, D.L.; Hofmann, J.; Hahm, H.S.; Seeberger, P.H.; Pagel, K.; Julian, R.R. Glycan Isomer Identification Using Ultraviolet Photodissociation Initiated Radical Chemistry. *Anal. Chem.* **2018**, *90(19)*, 11581-11588.
[21] Riggs, D.L.; Silzel, J.W.; Lyon, Y.A.; Kang, A.S.; Julian, R.R. Analysis of Glutamine Deamidation: Products, Pathways, and Kinetics. *Anal. Chem.* **2019**, *91(20)*, 13032-13038.
[22] Lyon, Y.A.; Sabbah, G.M.; Julian, R.R. Differences in α-Crystallin isomerization reveal the activity of protein isoaspartyl methyltransferase (PIMT) in the nucleus and cortex of human lenses. *Exp. Eye Res.* **2018**, *171*, 131-141.
[23] Han, K.K.; Belaiche, D.; Moreau, O.; Briand, G. Current Developments in Stepwise Edman Degradation of Peptides and Proteins. *Int. J. Biochem.* **1985**, *17(4)*, 429-445.
[24] Nakamura, T.; Abe-Kanoh, N.; Nakamura, Y. Physiological relevance of covalent protein modification by dietary isothiocyanates. *J. Clin. Biochm. Nutr.* **2018**, *62(1)*, 11-19.
[25] Mi, L.; Pasqua, A.J.D.; Chung, F.-L. Proteins as binding targets of isothiocyanates in cancer prevention. *Carcinogenesis* **2011**, *32(10)*, 1405-1413.
[26] George, G.; Geetha, M.; Appukuttan, P.S. Antigen-Induced Activation of Antibody Measured by Fluorescence Enhancement of FITC Label at Fc. *J. Fluoresc.* **2015**, *25*, 1493-1499.
[27] Kim, B.S.; Oh, J.M.; Kim, K.S.; Seo, K.S.; Cho, J.S.; Khang, G.; Lee, H.B.; Park, K.; Kim, M.S. BSA-FITC-loaded microcapsules for in vivo delivery. *Biomaterials* **2009**, 902-909.
[28] Slevin, M.; Matou-Nasri, S.; Turu, M.; Luque, A.; Rovira, N.; Badimon, L.; Boluda, S.; Potempa, L.; Sanfeliu, C.; De Vera, N.; Krupinski, J. Modified C-Reactive Protein is Expressed by Stroke Neovessels and is a Potent Activator of Angiogenesis In Vitro. *Brain Pathol.* **2009**, *20(1)*, 151-165.
[29] Planas, O.; Gallavardin, T.; Nonell, S. A novel fluoro-chromogenic click reaction for the labelling of proteins and nanoparticles with near IR theranostic agents. *Chem. Commun.* **2015**, *15*, 5586-5589.
[30] Wilderspin, A.F.; Green, N.M. The Reaction of Fluorescein Isothiocyanate with Thiols: A Method for Assay of Isothiocyanates. *Anal. Biochem.* **1983**, *132*, 449-455.
[31] Chen, P.; Nie, S.; Mi, W.; Wang, X.-C.; Liang, S.-P. De novo sequencing of tryptic peptides sulfonated by 4-sulfophenyl isothiocyanate for unambiguous protein identification using post-source decay matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 191-198.
[32] Gargis, S.R.; Heath, H.E.; Heath, L.S.; LeBlanc, P.A.; Simmonds, R.S.; Abbott, B.D.; Timkovich, R.; Sloan, G.L. Use of 4-Sulfophenyl Isothiocyanate Labeling and Mass Spectrometry to Determine the Site of Action of the Streptococcolytic Peptidoglycan Hydrolase Zoocin A. *Appl. Environ. Microbiol.* **2009**, *75(1)*, 72-77.
[33] Santa, T. Isothiocyanates as derivatization reagents for amines in liquid chromatography/electrospray ionization-tandem mass spectrometry. *Biomed. Chromatogr.* **2010**, *24(9)*, 915-918.
[34] Hood, C.A.; Fuentes, G.; Patel, H.; Page, K.; Menakuru, M.; Park, J.H.; Fast conventional Fmoc solid-phase peptide synthesis with HCTU. *J. Pept. Sci.* **2008**, *14(1)*, 97-101.
[35] Shuford, C.M.; Muddiman, D.C. Capitalizing on the hydrophobic bias of electrospray ionization through chemical modification in mass spectrometry-based proteomics. *Expert Rev. Proteomics* **2011**, *8(3)*, 317-323.
[36] Kirk, B.B.; Trevitt, A.J.; Blanksby, S.J.; Tao, Y.; Moore, B.N.; Julian, R.R. Ultraviolet Action Spectroscopy of Iodine Labeled Peptides and Proteins in the Gas Phase. *J. Phys. Chem. A* **2013**, *117*, 1228-1232.

[37] Moore, B.; Sun, Q.; Hsu, J.C.; Lee, A.H.; Yoo, G.C.; Ly, T.; Julian, R.R. Dissociation Chemistry of Hydrogen-Deficient Radical Peptide Anions. *J. Am. Soc. Mass Spectrom.* **2012**, *23*, 460-468.
[38] Iacobucci, C.; Piotrowski, C.; Rehkamp, A.; Ihling, C.H.; Sinz, A. The First MS-Cleavable, Photo-Thiol-Reactive Cross-Linker for Protein Structural Studies. *J. Am. Soc. Mass Spectrom.* **2018**, *30*, 139-148.
[39] Falvo, F.; Fiebig, L.; Dreiocker, F.; Wang, R.; Armentrout, P.B.; Schäfer, M. Fragmentation reactions of thiourea- and urea-compounds examined by tandem MS-, energy-resolved CID experiments, and theory. *Int. J. Mass Spectrom.* **2012**, *330-332*, 124-133.
[40] Müller, M.Q.; Dreiocker, F.; Ihling, C.H.; Schäfer, M.; Sinz, A. Cleavable Cross-Linker for Protein Structure Analysis: Reliable Identification of Cross-Linking Products by Tandem MS. *Anal. Chem.* **2010**, *82*, 6958-6968.
[41] Tao, Y.; Julian, R.R. Identification of Amino Acid Epimerization and Isomerization in Crystallin Proteins by Tandem LC-MS. *Anal. Chem.* **2014**, *86(19)*, 9733-9741.

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