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Click Chemistry Facilitates Formation of Reporter Ions and Simplified Synthesis of Amine-Reactive Multiplexed Isobaric Tags for Protein Quantification

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Abstract

We report the development of novel reagents for cell-level protein quantification, referred to as Caltech Isobaric Tags (CITs), which offer several advantages in comparison with other isobaric tags (*e.g.*, iTRAQ and TMT). Click chemistry, copper-catalyzed azide-alkyne cycloaddition (CuAAC), is applied to generate a gas-phase cleavable linker suitable for the formation of reporter ions. Upon collisional activation, the 1,2,3-triazole ring constructed by CuAAC participates in a nucleophilic displacement reaction forming a six-membered ring and releasing a stable cationic reporter ion. To investigate its utility in peptide mass spectrometry, the energetics of the observed fragmentation pathway are examined by density functional theory. When this functional group is covalently attached to a target peptide, it is found that the nucleophilic displacement occurs in competition with formation of b- and y-type backbone fragment ions regardless of the amino acid side-chains present in the parent bioconjugate, confirming that calculated reaction energetics of reporter ion formation are similar to those of backbone fragmentations. Based on these results, we apply this selective fragmentation pathway for the development of CIT reagents. For demonstration purposes, duplex CIT reagent is prepared using a single isotope-coded precursor, allyl-d_5-bromide, with reporter ions appearing at *m/z* 164 and 169. Isotope-coded allyl azides for the construction of the reporter ion group can be prepared from halogenated alkyl groups which are also employed for the mass balance group via N-alkylation, reducing the cost and effort for

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Supporting Information: Complete list of authors for ref 23; details of the synthesis and characterization of compounds by ESI-MS and NMR; peptide labeling of CIT reagents; Cul1 pull-down from HEK 293 cells; mass spectrometry data processing; instrument conditions for MALDI TOF, ESI-LTQ ion trap mass spectrometers for direct infusion, and nanoLC-LTQ-Orbitrap experiments; details of the computational methods; and tables of calculated cartesian coordinates and energies of model systems. This material is available free of charge via the Internet at http://pubs.acs.org.

synthesis of isobaric pairs. Owing to their modular designs, an unlimited number of isobaric combinations of CIT reagents are, in principle, possible. The reporter ion mass can be easily tuned to avoid overlapping with common peptide MS/MS fragments as well as the low mass cut-off problems inherent in ion trap mass spectrometers. The applicability of the CIT reagent is tested with several model systems involving protein mixtures and cellular systems.

Introduction

Recent achievements in mass spectrometry (MS)-based proteomics have provided essential methodologies for a deeper understanding of protein expressions in cells.[1,2] MS-based proteomics allows high-throughput identification and quantification of proteins of interest. Currently, state-of-the-art liquid chromatography (LC)-MS instruments can analyze the whole-cell yeast lysate within a day, identifying several thousand distinct proteins.[3,4] Quantitative approaches in MS-based proteomics aim to investigate the relative and absolute expression levels of proteins in cells.[5,6] By employing those approaches, various biological processes can be monitored by tracking changes in protein expression.[7]

The simplest method for quantitative mass spectrometric measurement is the label-free analysis. After successive runs of samples of interest under the same instrument conditions, protein abundances are determined by either integrating ion chromatograms or spectral counting of peptide MS signals.[8] Yet, current label-free quantification approaches require highly consistent analyses, which are mostly hampered by fluctuations in ionization efficiencies and difficulties in processing discrete MS data.[8,9]

In another label-free approach (which is also often performed with internal standards and is thus not label-free), selected reaction monitoring (SRM), and its extension plural multiple reaction monitoring (MRM)[10], examines the transitions (*i.e.*, one or more targeted fragment ions from the precursor ions) by scanning specific mass regions using triple quadruple mass spectrometers. These permit highly sensitive identification and concomitant quantification of peptides.[11–13] SRM, however, requires pre-knowledge of fragmentation behaviors of analytes, for which the dissociation pathways must be determined through tedious assays prior to actual SRM analyses. In addition, the high cost for the preparation of required synthetic peptides may limit its wide application in shotgun proteomics involving complex mixtures. SRM also suffers from problems associated with fluctuations in ionization efficiencies and chromatographic reproducibility.

To address problems associated with label-free quantification, stable isotopes are incorporated into samples to be used as internal (or mutual) standards.[14] A conceptual breakthrough for protein quantification was achieved by Aebersold and coworkers by introduction of isotope-coded tags that can be used to selectively label peptide digests.[15] In this approach, cysteine-containing peptides from different sources are tagged by light or heavy isotope-coded affinity tag (ICAT) reagents, and enriched from the complex mixture using an attached biotin affinity tag. Because these tagged peptides share the same physico-chemical properties, they are not differentiated by ionization and chromatography steps. Therefore, a simple comparison of MS signal intensities between light and heavy isotope-coded peptides directly yields the relative protein expression levels.

Another quantification approach takes advantage of *in vivo* metabolic incorporation of isotope labels.[16–18] Among various metabolic labeling methods, SILAC (Stable Isotope Labeling with Amino acids in Cell culture)[19] has been popular due to its simplicity in the incorporation of stable isotopes for mammalian cells. Two cell populations are grown with identical culture media except for stable isotope-labeled amino acids (*e.g.*, ^{13}C and/or ^{15}N labeled lysine and/or arginine). The resulting heavy isotope-coded cell populations behave

identically with their light isotope-coded controls. After applying a perturbation to one of the light or heavy cell populations, the two samples are combined for MS analysis. Direct comparison of the peptide signals from the light- and heavy-labeled cell populations yields the relative protein expression levels. Unlike ICAT, which quantifies only cysteine-containing peptides, metabolic labeling is capable of quantifying the global proteome, because all tryptic peptides are labeled with isotope-coded lysine and/or arginine.[20]

The LC-MS ion signals from isotope-coded peptides in both ICAT and metabolic labeling methodologies are divided into two signals for each labeled peptide, causing an increase in the complexity of MS scans and a reduction in the sensitivity of subsequent sequence analysis by tandem mass spectrometry (MS/MS). As an alternative chemical-labeling method, isobaric tags such as Tandem Mass Tag (TMT)[21,22] and isobaric Tags for Relative and Absolute Quantification (iTRAQ)[23,24] were developed. An isobaric tag is composed of three parts: the reporter ion group, the mass balance group, and the reactive group.[25] A clever feature in the design of these approaches is that the combined mass of the reporter ion group and the mass balance group is isobaric despite each part having different masses. When the identical peptides from different biological experiments are labeled by isobaric tags, they possess the same masses, appearing as a single peak. Mass differentiated reporter ions are produced and detected in MS/MS scans, in which their relative intensities reflect the initial amounts of each peptide from the original sources. All backbone fragment ions are also isobaric, allowing simultaneous peptide sequencing and quantification. One advantage of isobaric tags over ICAT- and metabolic labeling-based methodologies, especially when quantifying more than two system states, is that the combined signals from multiple biological samples reduce the complexity of the MS scans and increase the sensitivity for the following MS/MS analyses. Preparing each part of the tag with various isotopomers in principle allows for the facile construction of multiplexed reagents capable of quantifying multiple samples in a single MS analysis. The primary amines that are usually the target functional groups for isobaric tag labeling exist in virtually all peptides (*i.e.*, the N-terminal amine and ε-amine of lysine side-chain), enabling researchers to investigate the whole proteome. Isobaric tags have been employed for quantification of tissue samples as well as samples from human, where metabolic labeling methodology is not an option.[26]

In spite of the improvements achieved by isobaric tags, their applications have been somewhat constrained by the cost of commercially available reagents.[27] In addition, the number of the current multiplexed isobaric tags (*e.g.*, iTRAQ) is limited to a maximum of eight[24] due to their inherent designs and difficulties in the synthesis of various isotope-coded functional groups. The low mass cut-off in resonance type ion trap mass spectrometers, one of the most popular proteomics platforms, also hinders the simultaneous monitoring of reporter ions and peptide sequence ions.

The design of new isobaric tags is hindered by the fact that very few low-energy fragmentation pathways suitable for production of the reporter ions are known in peptide MS/MS. One of the widely known fragmentation pathways, the preferential cleavage of the aspartic acid-proline peptide bond via a salt-bridged intermediate[28,29], was applied in the first TMT report (Scheme 1, I).[21] In iTRAQ, the formation of the reporter ions proceeds through the facile *N*-methylpiperazine-acetyl bond—mediated cleavage process (Scheme 1, II). Most isobaric tags that have been proposed to provide cheaper synthetic routes are still based on similar tertiary amine-branched methylene-amide bonds.[30–32] Designing suitable gas-phase fragmentation pathways is thus a critical task to facilitate the development of novel isobaric tags and other mass spectrometric analyses. Yet, to the our best knowledge, it has not been reported that one can intentionally design small molecular tags using bio-orthogonal chemistry whose peptide conjugates leads to highly selective gas-phase reactions, generating a reporter ion.

Here, we report a novel isobaric reagent (Scheme 2), referred to as Caltech Isobaric Tag (CIT), that is based on a newly found gas-phase cleavable linker constructed by copper-catalyzed azide-alkyne cycloaddition (CuAAC) or simply click chemistry[33–35], affording a significant advance in cost effective MS-based protein quantification methodology.

CIT has several advantages over current isobaric labeling reagents. Most importantly, CIT is easy to synthesize and the possible number of multiplexed isobaric tags, in theory, is unlimited. Energetics for the reporter ion and peptide sequence ion formations are balanced, guaranteeing simultaneous quantification and sequencing of target peptides. CIT is inspired by the observation of a highly selective gas-phase fragmentation triggered by a nucleophilic displacement of the N3 nitrogen in the 1,2,3-triazole ring (Scheme 3) produced by CuAAC. Previously, the 1,2,3-triazole ring is known to be inert in the peptide gas-phase fragmentation.[36–38] We show that by designing proper linkers it is possible to induce highly selective gas-phase reactions to be useful in peptide tandem mass spectrometry. The design of a duplex CIT reagent is described, and the applicability of the reagent is validated in model systems using various mass spectrometers.

Methods

The synthetic route for generating CIT reagents is depicted in Figure 1. Briefly, the isotope-coded isobaric tags were prepared via sequential *N*-alkylations of L-alanine methyl ester by 6-hexynyl iodide and allyl-d_0 or d_5 bromides, CuAAC of mass balanced allyl-d_0 or d_5 azides, and deprotection/activation of methyl ester to *N*-hydroxysuccinimidyl ester. The resulting CIT reagents were conjugated to peptides and analyzed by various mass spectrometers. The detailed synthetic scheme and experimental methodologies can be found in Supporting Information.

Results and Discussion

Rationale of CIT Design

At the inception of this study, a key goal was to find an appropriate gas phase fragmentation pathway for the formation of the reporter ions. At that time, we were interested in the application of bio-orthogonal CuAAC reactions to MS-based proteomics studies. Observation of a highly selective gas-phase fragmentation triggered by a nucleophilic displacement of the N3 nitrogen in the 1,2,3-triazole ring competitive with the formation of b- and y-type ions in collision-induced dissociation (CID) of covalently labeled peptides inspired us to create novel isobaric tags (Scheme 3). In multiply protonated CIT-labeled peptides, the tertiary amine in the CIT reagent would be protonated due to its higher proton affinity than most backbone amides and amino acid side-chains. A nucleophilic displacement of the N3 of the 1,2,3-triazole ring at the alpha-carbon position of the protonated *N,N*-alkylated alanine residue in the CIT reagent releases a stable quaternary ammonium reporter ion, forming a six-membered ring.

The energetics of reporter ion formation is investigated by density functional theory calculations (Figure 2). If this process is significantly favored compared to backbone fragmentation, less sequence information would be acquired by having fewer and weaker intensity b- and y-type ions in the MS/MS spectrum. It is desirable that activation parameters associated with reporter ion formation are balanced with those of backbone fragmentation. This ensures that accurate protein quantification is achieved while not reducing sequencing efficacy using MS/MS. In our calculation model, the formation of the reporter ion is simulated by the *N*-protonated *N,N*-dimethyl-4-(1-methyl-1*H*-1,2,3-triazol-4-yl)butan-1-amine. At the B3LYP/6-311++G(d,p) level of theory, the reaction barrier and enthalpy at 1 atm and 298.15 K are determined as 33.7 and 13.3 kcal/mol, respectively

(Figure 2). The usual reaction barrier for amide bond cleavage in protonated peptides ranges from 25 to 40 kcal/mol.[39] Therefore, it is anticipated that most backbone cleavages will occur competitively with formation of the reporter ion. In addition to the formation of reporter ions, this feature also validates the usefulness of the observed fragmentation pathway for other applications involving mass spectrometry such as the controlled release of affinity tags, used in the separation of targeted peptides, by collisional activation.

Deriving advantage from the described gas-phase cleavage reaction to form reporter ions, we proceeded to design novel isobaric tags. Figure 3 depicts the structure of CIT, the construction of the theoretical N-plex reagents and the schematic drawing of their application to peptide conjugates. CIT is composed of three parts: the reporter ion group, the mass balance group, and the amine reactive group found in other commercially available isobaric tags.

The major improvement of CIT that distinguishes it from other isobaric tags is the *modularization* of the isotope-coded residues, both for the mass balance group and the reporter ion group. Any functional groups (R) that do not contain other reactive or interfering functionalities can be implemented into the mass balance group by inserting a good leaving group such as bromine, iodine, or tosylate. Via a simple S_N2 reaction in the mild conditions employing DMF/NaN_3, the isotope-coded reporter ion group can be easily prepared from an activated R group that is used for the mass balance group (Figure 3). Each isobaric pair of R_m-X (X: leaving group) and R_n-N_3 forms a building block for an isobaric tag with a certain reporter ion mass (R_n + 123 Da). By preparing a set of the N different isotope-coded R−X, it is possible to construct the N-plex isobaric reagents. This modularity of CIT significantly reduces the effort and cost of synthesis. In addition, the mass of the reporter ion is *tunable*; this property enables us to bypass the mass cut-off problem in ion trap mass spectrometers and target open windows of m/z values normally found in peptide MS/MS (*e.g.*, sequence ions, immonium ions or internal fragments).

This general experimental and synthetic methodology is applied to the creation of a prototype duplex CIT reagent using allyl bromide-d_0 and d_5 as the isotope-coded starting materials. Duplex CIT reagents were synthesized, with stable isotopes having the reporter ions at *m/z* 164 and 169, respectively. We adopt the NHS group for facile amine-reactive coupling to peptides as in other commercially available isobaric tags. NHS has been popular in bioconjugation due to the compatibility with most biological buffer solutions. Most importantly, its target functional groups (N-termini of peptides and the ε-amine of lysine) are ubiquitous among tryptic peptides. The size of the overall modification by this duplex CIT reagent is 279 Da, which is not much larger than most of the commercially available isobaric tags (iTRAQ 4-plex, 144 Da; TMT 6-plex, 224 Da; iTRAQ 8-plex, 304 Da).

MS/MS of CIT-labeled Peptides

The light and heavy duplex CIT reagents were used to label the model tryptic peptide, HSDAVFTDNYTR. Figure 4 depicts matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS spectra of CIT-labeled model peptides. The masses of CIT-labeled peptides are 279 Da larger than the original peptides as expected (Figure 4). The labeling yields of light and heavy CIT reagents are both ~99% estimated by the peak height comparison between unmodified and CIT-labeled peaks in the MALDI TOF MS spectra (Figure 4). The exact masses of light- and heavy-labeled peptides are identical, appearing as one peak in all mass spectrometric analyses.

Pulsed-Q dissociation (PQD) in the linear ion trap of an LTQ-Orbitrap Classic mass spectrometer was used to fragment protonated CIT-labeled peptide ions produced by electrospray ionization (ESI). PQD of the 1:1 mixture of the heavy and light CIT-labeled

peptides in the ion trap generates abundant reporter ions at *m/z* 164.1 and 169.1 as well as sequence ions, confirming N-terminal labeling (Figure 5a and b). PQD of the triply protonated precursor ion yields abundant backbone fragment ions along with reporter ions, but less prominent backbone fragments are observed for the doubly protonated precursor ion. These results are presumably caused by sequestering of mobile protons at the CIT and arginine residues, increasing the reaction barrier for backbone cleavage. The H/L ratio of the CIT reporter ion is determined to be 0.8 in both +2 and +3 charge states (Figures 5a and b), which we attribute to an initial experimental mixing error. Note that for the PQD experiment, light- and heavy-labeled peptides are prepared and purified separately by C18 ziptips, and later mixed for linear response testing with various mixing ratios to reduce the effort for desalting each mixed sample. In the actual quantification experiment, the sample mixing step is prior to the C18 purification to avoid dissimilar sample loss. This systematic error appears as the y-axis intercepts in the linear fitting lines in Figure 6.

For comparison of reporter ion yields to the CIT reagent, the iTRAQ-113 reagent having no isotope substitution was conjugated to the same model peptide and the resulting peptides were subject to PQD (Figures 5c and d). The overall sequence coverage of the iTRAQ-labeled peptide by PQD is very similar to that of the CIT-labeled peptides. Yet, the relative reporter ion intensities in the PQD spectra of the doubly and triply protonated iTRAQ-labeled peptide ions are lower than those of the CIT-labeled peptides (Figures 5c and d). This result indicates that the process for reporter ion formation of CIT is slightly favored over that of iTRAQ, enabling more reliable quantification with the CIT reporter ions.

To test the dynamic range of the CIT reagent, we monitored reporter ion formation with various ratios of light- and heavy-labeled peptides using PQD in an LTQ-Orbitrap Classic mass spectrometer. The PQD spectra of doubly and triply charged CIT-labeled peptides were recorded in profile and centroid modes, and the intensities or areas of the reporter ions were used to plot the linear dependency on the initial mixing ratio (Figure 6, log2-log2 plot). All of the methods for data processing show a good correlation ($R^2 \sim 0.99$) between the initial mixing ratio of light and heavy CIT-labeled peptides. The overall linearity (slopes = ~1.0) and quality of fitting ($R^2 = \sim 0.99$) indicate a very good linear response of the CIT reagents from 1/9 to 9 H/L mixing ratios. This demonstrates roughly a two-orders-of-magnitude dynamic range with the performance of the CIT reagents for relative quantification.

Chromatographic Separation

The tailing of deuterated counterparts in LC elution profiles has been reported previously and may affect the accuracy of quantification.[30,40,41] To address this possibility, the retention times in nanoLC were measured by injection of the 1:1 mixture of the heavy and light CIT-labeled model peptides. For monitoring HCD signals of the reporter ions, the MS/MS analysis was performed repeatedly by turning off the data-dependent acquisition mode. Figure 7 depicts nanoLC chromatograms of the CIT-labeled peptides (Figure 7a) and their HCD scans (Figures 7b and c) filtered by *m/z* 164 (red) and 169 (blue) mass ranges to monitor the elution profiles of the light and heavy CIT-labeled peptides. As seen in Figures 7b and c, ion current profiles for both reporter ions appear identical with no apparent *differential* tailing effect. The mean H/L ratio of CIT-labeled model peptides eluted at 17 min is determined as 0.97 ± 0.05. This result indicates that both heavy and light CIT-labeled peptides have the same chromatographic properties, validating the suitability of the CIT reagents for protein quantification using LC-MS platforms.

Protein Labeling

The applicability of the CIT reagent is tested with model systems involving protein mixtures. Protein digests prepared from the mixture of bovine serum albumin, ovalbumin, α- and β-caseins, and lysozyme were used for CIT labeling with the initial H/L mixing ratio of 1:1. The nanoLC-LTQ-Orbitrap analyses using PQD (Orbitrap classic) and higher-energy collisional dissociation (HCD, Orbitrap Velos) generally reproduce the initial mixing ratio, in which hundreds of peptides tagged by CIT reagents are quantified (Table 1). Importantly, both PQD and HCD resulted in very similar heavy to light ratios although the confidence intervals of the medians at 95% confidence level reported in PQD are relatively large (approximately 40% of the observed medians). Judging from the same phenomenon observed independently in the previous report using PQD for the quantification of iTRAQ-labeled peptides,[42] we believe that the relatively large confidence intervals results, in large part, from the poor performance of PQD. Compared to PQD, HCD in a LTQ-Orbitrap Velos mass spectrometer systematically yielded smaller confidence intervals (approximately 20% of the observed medians) with more peptide identification, showing its superior performance in both detection and quantification of CIT-labeled peptides.

Next, the CIT reagent is applied to quantify biologically relevant samples. Cul1 is a ubiquitin ligase that forms a large protein complex with dozens of known binding partners.[43] This protein complex was purified from HEK 293 cells, and quantified using CIT after tryptic digest. To facilitate purification of the Cul1 complex, we constructed a stable cell line that expresses tandem-tagged Cul1 upon tetracycline treatment.[44] Trypsin digests of Cul1 protein complexes affinity-purified from the HEK 293 cell line were split with the ratio of 1:1 for labeling with heavy or light CIT reagents. The labeled samples were combined, and the resulting mixture was analyzed by HCD/CID in a LTQ-Orbitrap XL mass spectrometer. Table 2 lists identified proteins with the H/L ratios determined by Mascot. The calculated medians are close to 1 for identified proteins with multiple quantified peptide hits, indicating that CIT is suitable for quantification of complex biological samples.

In quantifying more complex mixtures such as whole cell lysates, other isobaric tags have often yielded less reliable quantification results by coincidently isolating impurities for MS/MS analyses due to the high sample complexity.[45] Our CIT reagents may also suffer from interferences by having overlaps among precursor ions in complex mixtures, resulting in poor quantification results. By applying the gas-phase purification via proton transfer reaction methodology developed by Coon and coworkers[46] or the MS3 analysis by Gygi and coworkers,[47] one can avoid these interferences in complex mixture analysis using our isobaric tags.

Lastly, the applicability of the CIT reagent for the quantification of relative protein expression levels *in vivo* is investigated. In this study, different amounts of Cul1 were expressed in stable HEK 293 cells by treating two populations of cells with either 0.5 μg/mL of tetracycline for 1 h or 2.0 μg/mL for 4 h. These two samples were subject to Western blot analysis. As shown in Figure 8, the level of Cul1 expression differs by ratios of 5.3 (Lane 2/Lane1), and 5.2 (Lane 4/Lane 3), respectively. For MS analyses, Cul1 was purified from the two differentially expressed samples and digested by Lys-C/trypsin. After CIT labeling, the resulting peptides were analyzed by HCD/CID in an LTQ-Orbitrap XL mass spectrometer.[42,48] The median for the H/L ratio of 12 Cul1 tryptic peptides is 5.6, which agrees well with the ratio determined by Western blot experiments. These results demonstrate that the CIT-based quantification is an accurate, reliable methodology for the determination of protein abundance involving complex *in vivo* samples.

Conclusion

A novel isobaric tag is developed for protein quantification, referred to as Caltech Isobaric Tag (CIT), with excellent demonstrated performance in a range of typical proteomics investigations employing model systems. The design of the CIT reagents is based on a novel gas-phase fragmentation pathway reported here for the first time. In this pathway, a nucleophilic displacement of the N3 of the 1,2,3-triazole ring releases a stable reporter ion resulting from formation of a six-membered ring. It should be noted that DFT-calculated reaction energetics of reporter ion formation are similar to those of backbone fragmentations in collisional activation, permitting the effective quantification and sequencing simultaneously.

In the preparation of CIT reagents, the mass of the reporter ion can be easily tuned by varying azide groups in the preparation of the 1,2,3-triazole ring via Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC), better known as click reaction. The number of the possible isobaric tags is determined by the number of isotope-tagged azide groups. These azides can be prepared from halogenated alkyl groups, which are also used for the alkylation of the linker amino acids, reducing both the cost of reagents and effort required for the synthesis of isobaric tags. This modular feature expands the possible number of combinations of CIT reagents. The properties of CIT reagents can be tuned by using larger isotope-coded halogenated alkyls that yield higher *m/z* reporter ions and these avoid the low mass cut-off problems normally associated with ion trap mass spectrometers. Mixtures of light and heavy CIT-labeled model peptides showed good linear correlations with a two-orders-of-magnitude dynamic range. Observed ratios of the light and heavy CIT-labeled protein digests from the mixture of bovine serum albumin, ovalbumin, α- and β-caseins, and lysozyme also exhibited good agreement with the initial mixing ratio. Lastly, we have demonstrated the applicability of CIT reagents in quantifying complex biological samples using affinity-purified Cul1 ubiquitin ligase complexes from HEK 293 cells.

The key aspects of CIT reagents are realized by having a gas-phase cleavable linker constructed using click chemistry, which has activation parameters comparable to those for peptide backbone fragmentation in MS/MS. We are pursuing other applications that use this well-defined reporter ion formation process when the new chemical reagents are coupled to biomolecules of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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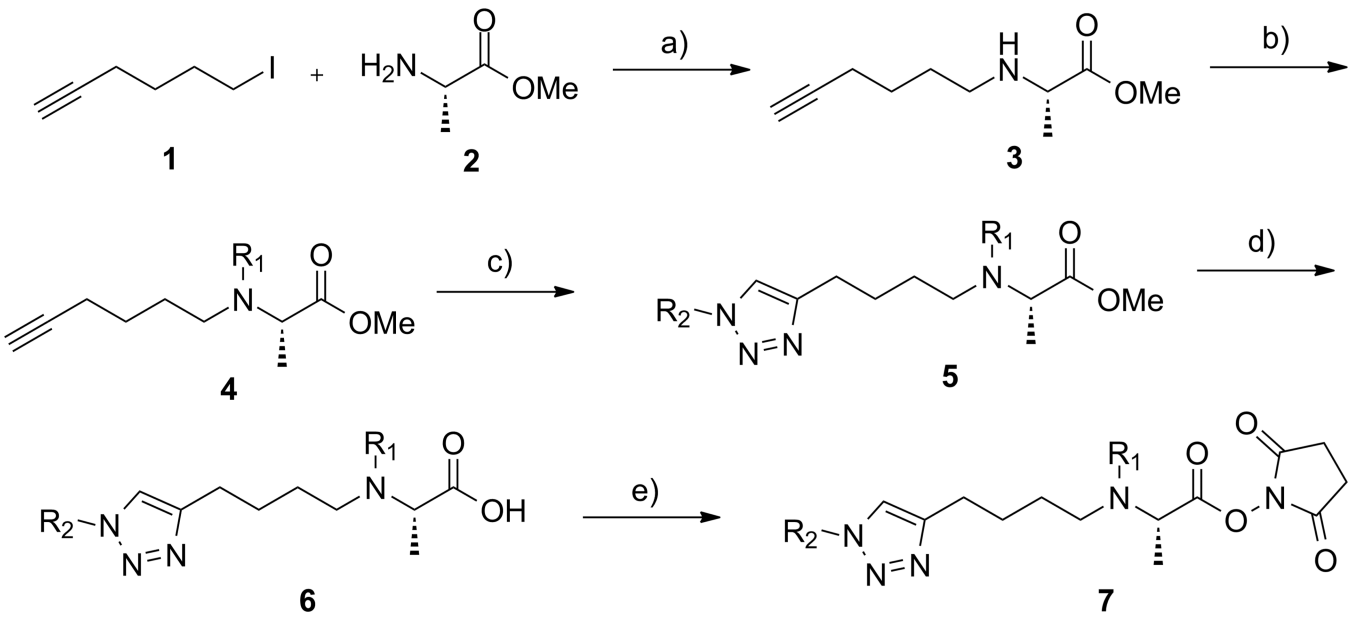


Figure 1. Synthesis of CIT

Synthesis of CIT reagents) THF, K_2CO_3, TEAI, reflux 18 h, 56%. b) THF, K_2CO_3, TEABr, reflux 18 h, R_1 = Allyl-d_0-bromide (**4a**), 56%, R_1 = Ally-d_5-bromide (**4b**), 67%. c) 0.4 eq Na ascorbate, 0.1 eq CuSO4, 0.01 eq TBTA, DMSO/H_2O, RT 4 h, R_2 = Allyl-d_5-azide (**5a**), 72% (heavy tag), R_2 = Allyl-d_0-azide (**5b**), 69% (light tag). d) 2M KOH, THF, RT overnight, *quantitative* (heavy tag, **6a**), 97% (light tag, **6b**). e) TFA-NHS, DMF, RT overnight, 24% (heavy tag, **7a**), 23% (light tag, **7b**). THF = tetrahydrofuran, TEAI = tetraethylammonium iodide, TEABr = tetraethylammonium bromide, TBTA = tris[(1-*t*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, DMSO = dimethyl sulfoxide, TFA-NHS = trifluoroacetic *N*-hydroxysuccinimide ester, DMF = *N,N*-dimethyl formamide.

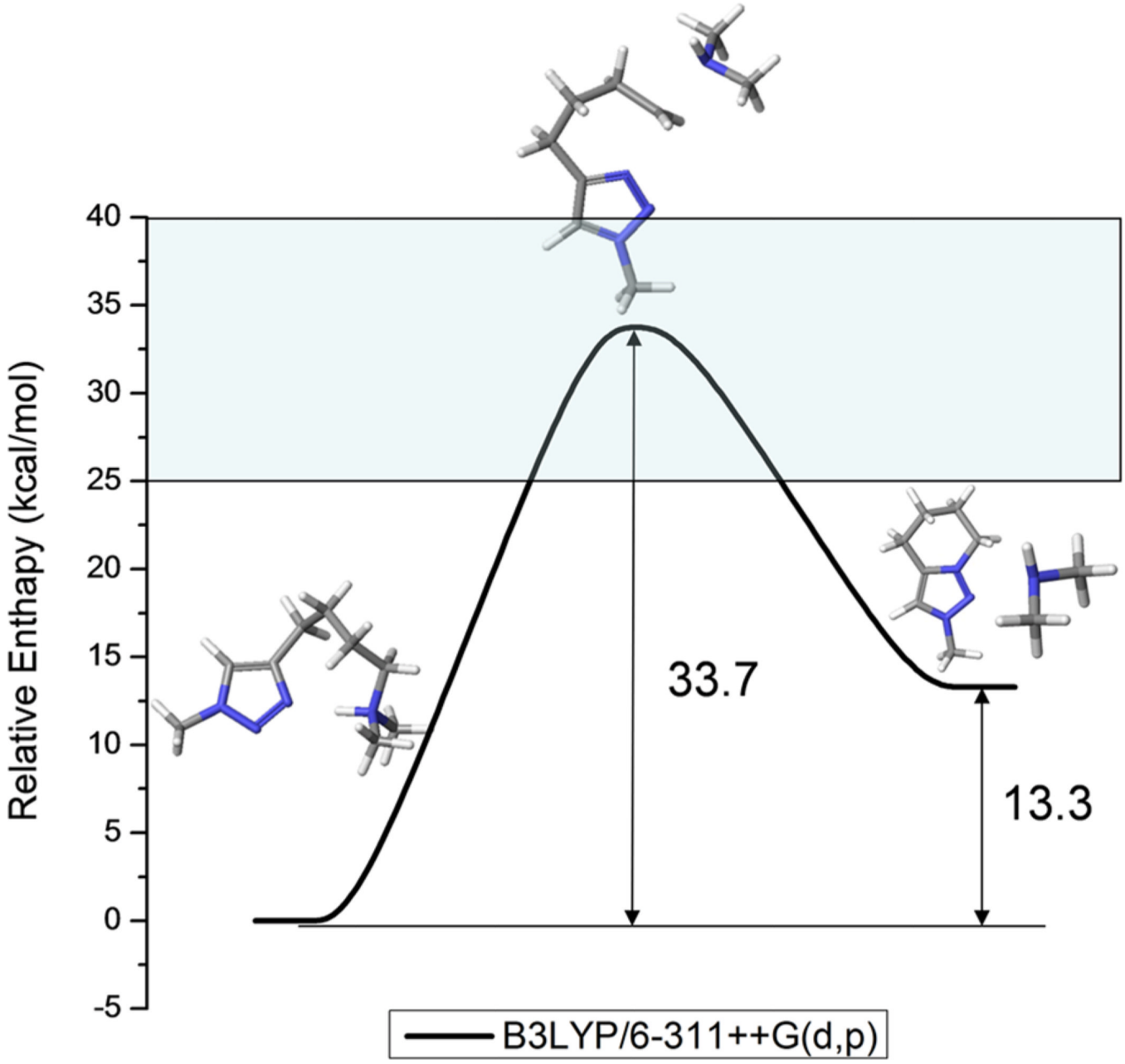


Figure 2. Energetics of Reporter Ion Formation

Energetics of reporter ion formation. The model system, *N,N*-dimethyl-4-(1-methyl-1*H*-1,2,3-triazol-4-yl)butan-1-amine was chosen for calculation. Geometry optimization and thermochemical calculations were performed at the B3LYP/6-311++G(d,p) level of theory. The shaded area indicates the range of enthalpies of activation for amide cleavage of protonated peptides to form b- and y-type ions via collisional activation.

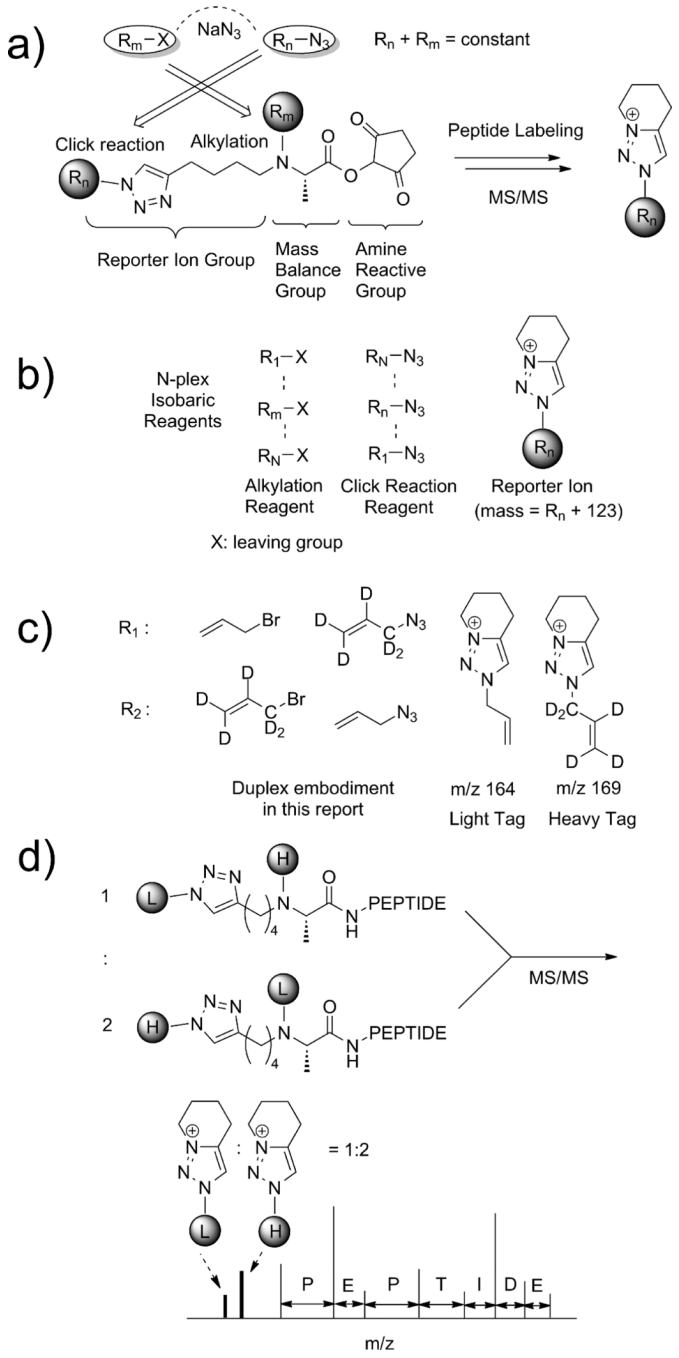


Figure 3. Design and structure of CIT

Design and structure of CIT reagents) The components of N-plex CIT reagents: the reporter ion group, the mass balance group, and the amine reactive group. b) Each reporter ion group and mass balance group can be prepared from a series of isotope-coded iodinated R_n groups. CIT labeled peptides are fragmented by various ion activation methods (*e.g.,* PQD, and Higher energy Collisional Dissociation (HCD)), yielding the reporter ions whose masses are R_n + 123 Da. c) the duplex embodiment of the CIT reagents in this report by using allyl bromide-d_0 and d_5. Note that the reporter ion is formed regardless of the structure of the attached R_n or R_m groups. d) schematic drawing for the quantification and sequencing of 1:2 light and heavy CIT-labeled peptides by MS/MS.

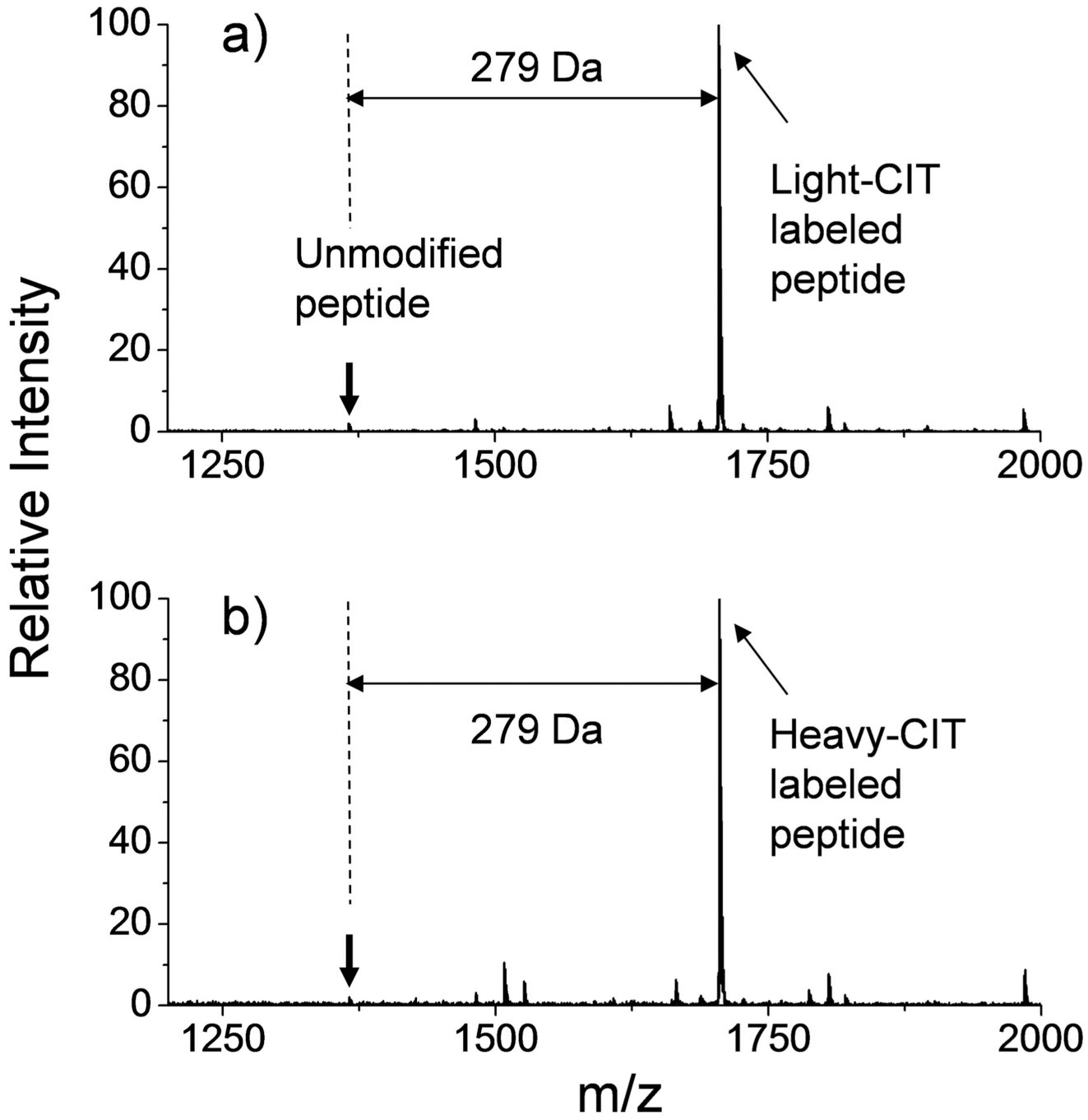


Figure 4. MALDI TOF MS Spectra of CIT-labeled Peptides

MALDI TOF MS spectra of a) light and b) heavy CIT reagent labeling of the model peptide, VIP(1–12), HSDAVFTDNYTR. The labeling reaction was performed for 2 h and quenched using 0.1 M hydroxylamine. The conversion yield was approximately ~99%. Quenching by 0.1 M hydroxylamine reverses unwanted by-products, which contain CIT reagent conjugation on tyrosine residues. Some of the impurities were observed but their contributions were appropriately considered for the calculation of H/L ratios.

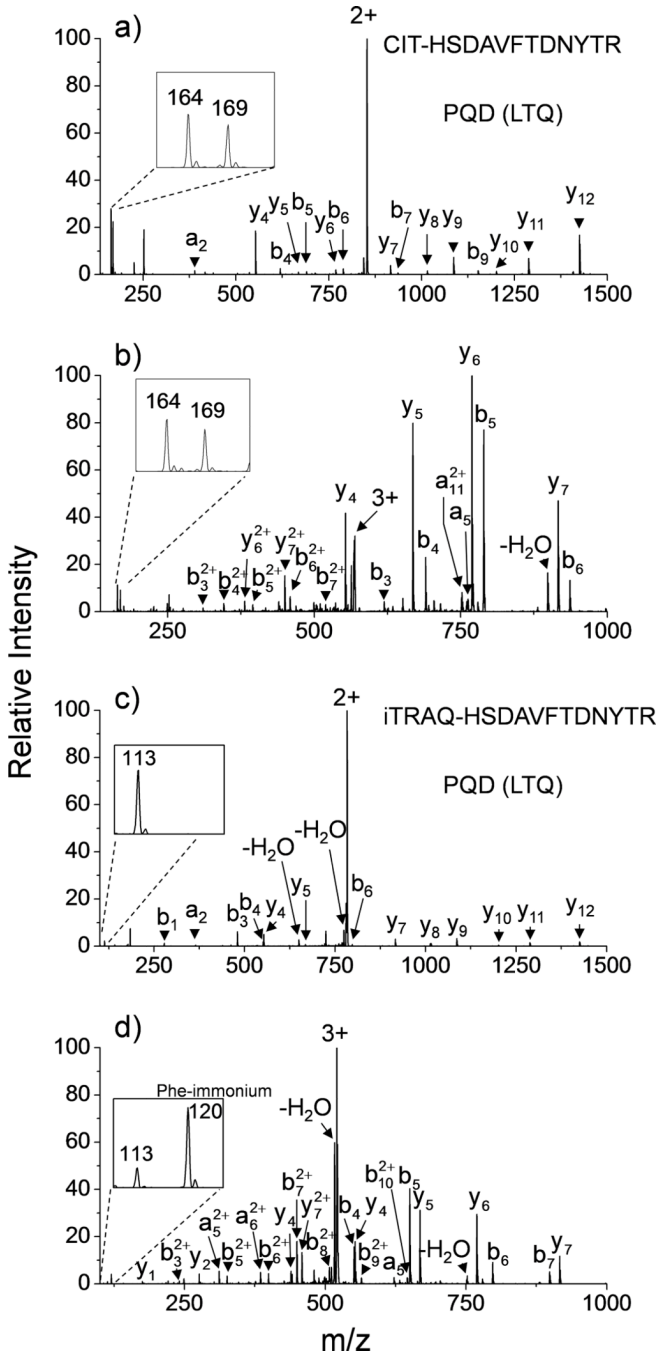


Figure 5. PQD of CIT-labeled Peptides

PQD of doubly and triply protonated CIT- and iTRAQ-113-labeled model peptide ions, HSDAVFTDNYTR with 1:1 = light:heavy ratio. Both a) and b) show abundant reporter ions at *m/z* 164 and 169, respectively, whereas the intensities of the reporter ions at *m/z* 113 in c) and d) are relatively small, indicating the favored energetics of the fragmentation pathway used for the CIT reporter ions. PQD of the triply protonated peptide ions in b) and d) also yields abundant sequence ions. For PQD of the doubly protonated peptide ions, the sequestered protons at the CIT residue and arginine side-chain increase the barrier for amide bond cleavage, yielding less backbone fragments. Experimentally observed reporter ion ratios (H/L) were 0.80 for 2+ and 0.81 for 3+, compared to the expected ratio of 1.0. These

deviations likely result from initial experimental mixing errors (see the main text for the details).

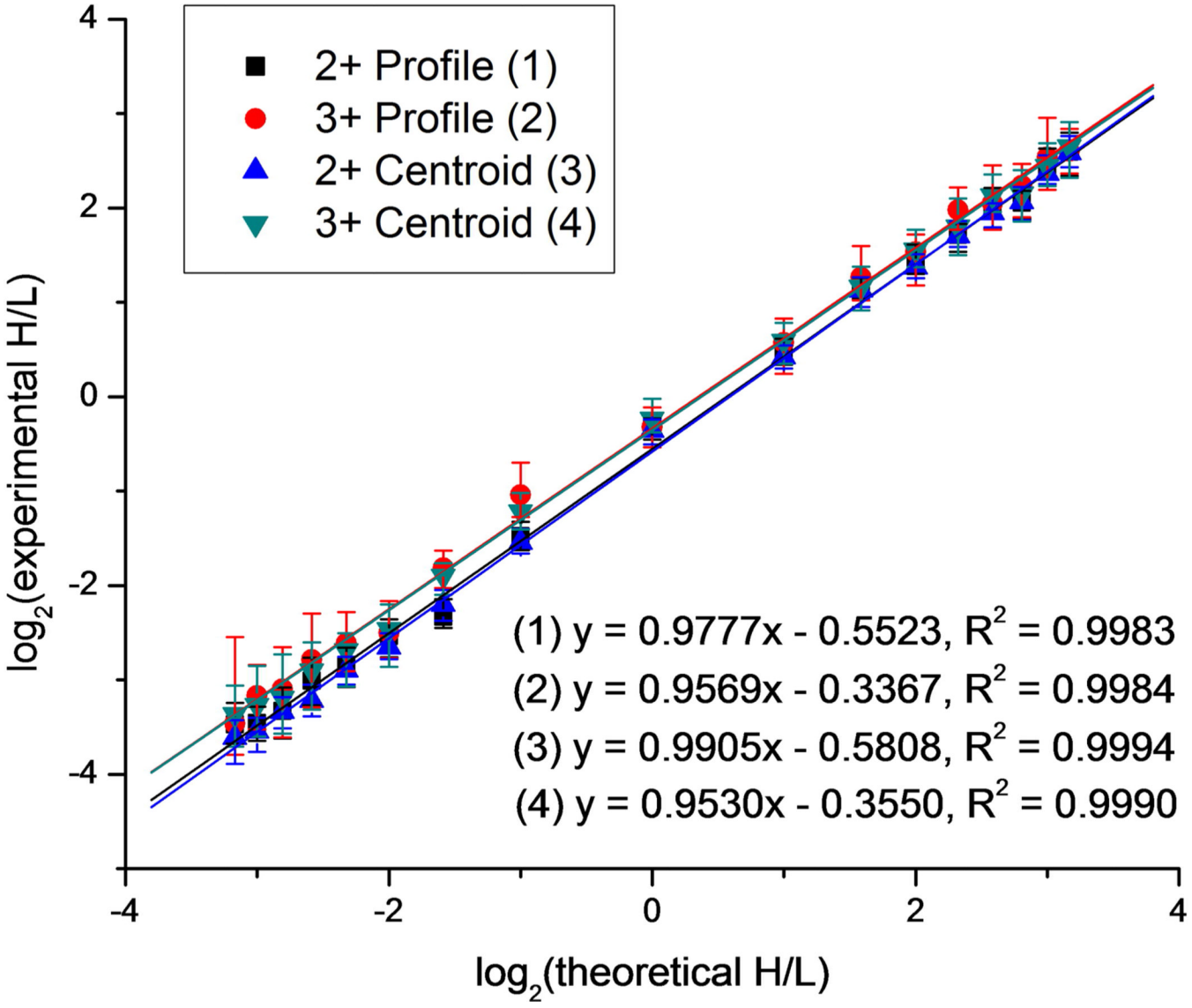


Figure 6. Linearity Test of CIT Reporter Ions

The linear fitting trend lines obtained by calculating the log2 of summations of a) the integration of all areas of isotopes in each reporter ions (*m/z* 164, 165, and 166 for the light tag; *m/z* 168, 169, 170, and 171 for the heavy tag), b) the integration of only *m/z* 164 and 169 peak areas, c) peak heights at *m/z* 164, 165, and 166 for the light tag, and at *m/z* 168, 169, 170, and 171 for the heavy tag, and d) peak heights only at *m/z* 164 and 169 for y-axis and the log2 of intended initial mixing ratios for x-axis. Relatively large (~0.4–0.5) y-axis intercepts in all figures are originated from systematic sources such as initial experimental mixing errors (see the main text for the details). Therefore, the overall linearity (slopes = ~1.0) and quality of fitting (R^2 = ~0.99) are not affected.

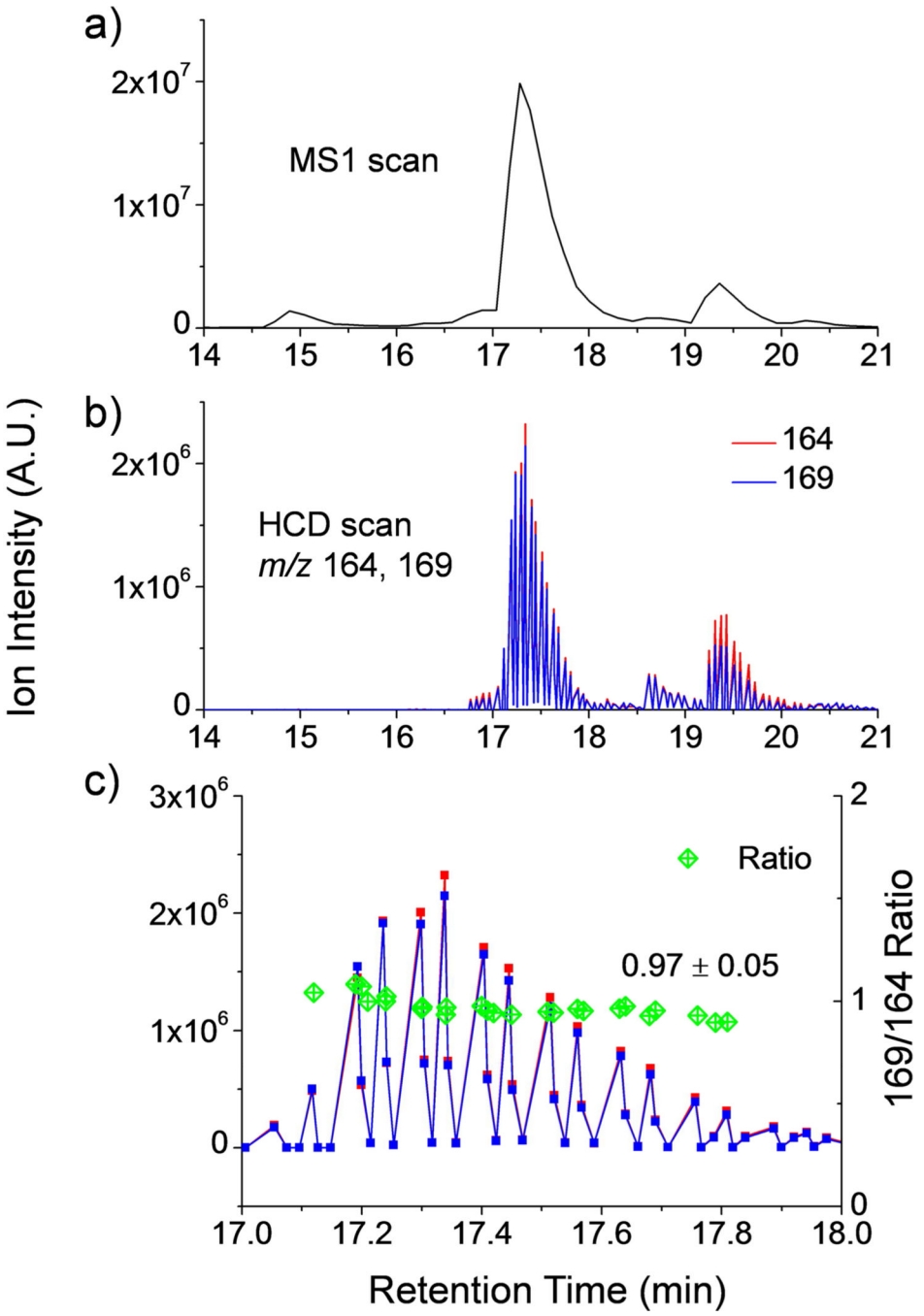


Figure 7. Chromatographic Identity of Light and Heavy CIT-labeled Peptides

The nanoLC chromatograms of a) MS1 scan, b) *m/z* 164 (red) and 169 (blue) reporter ions observed in HCD scans generated by the 1:1 mixture of light and heavy CIT labeled model peptides, HSDAVFTDNYTR and c) a close look of the reporter ion counts and 169/164 ratios around 17.3 min. The base peaks in all chromatograms are related to CIT-labeled model peptides. Note that the red and blue lines are very similar, indicating almost identical chromatographic behavior of light and heavy CIT-labeled peptides (0.97 ± 0.05). The peak at 14.9 min in a) is a non-labeled model peptide. The peak appearing around 19.4 min in a) is from CIT-labeled peptide fragments, AVFTDNYTR. Because this fragment peptide existed in each peptide stock independently, their initial mixing ratios were different from

the original intact peptide, yielding dissimilar 169/164 ratios in (b). The small peak at 18.6 min is associated with doubly CIT-labeled peptides including tyrosine and N-terminal modifications.

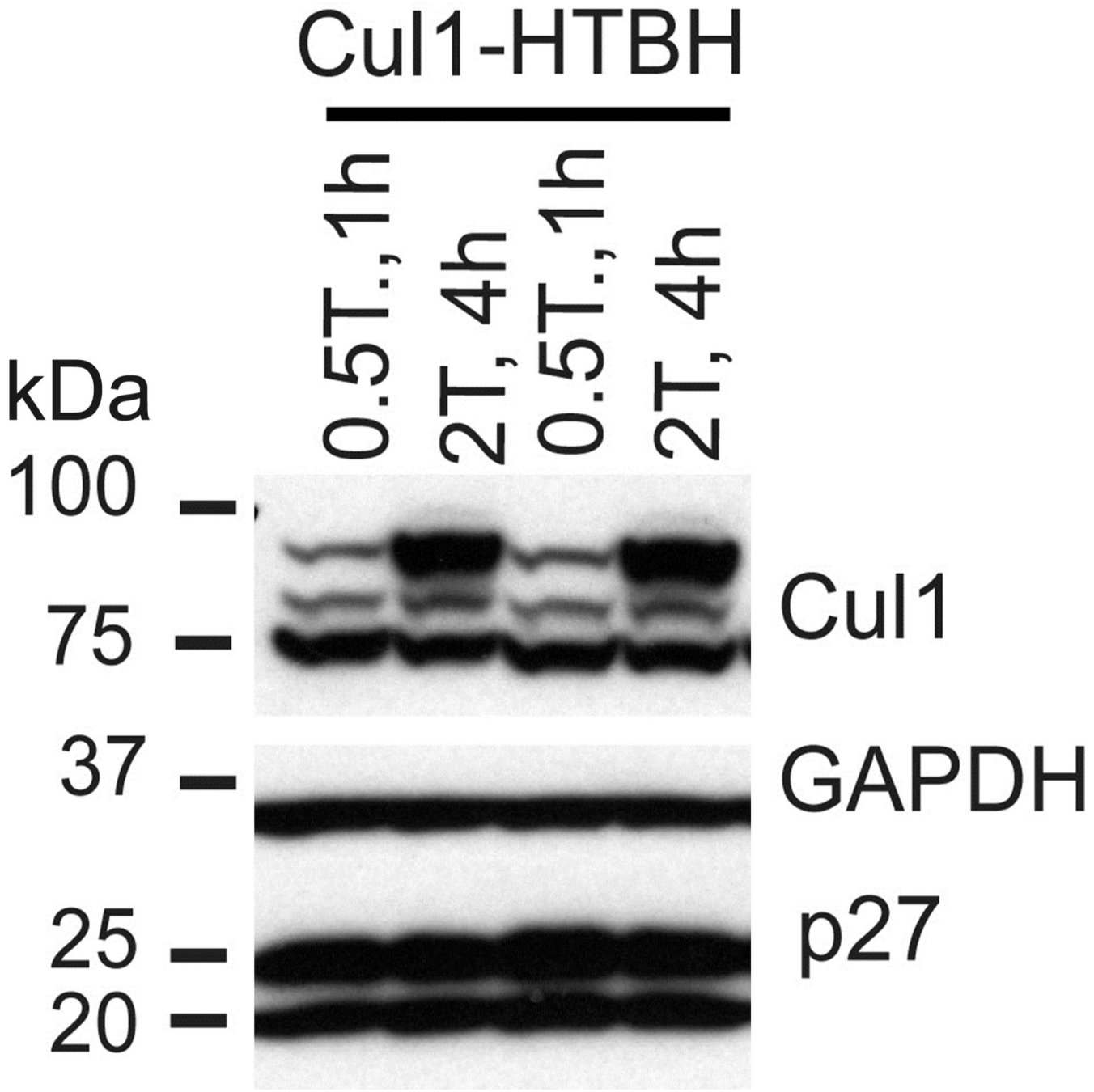
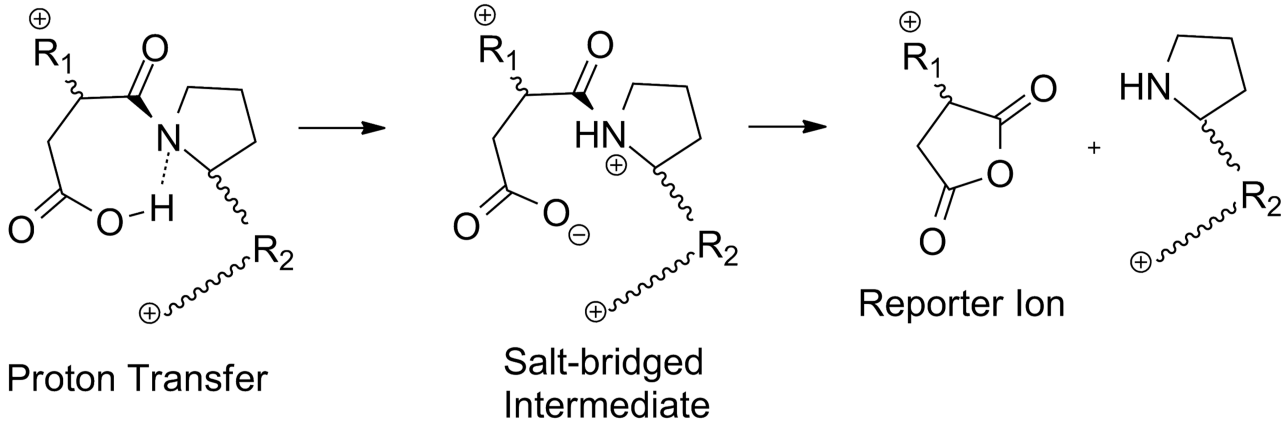


Figure 8. Western Blot Analysis of Cross-linked Cul1

Western blot analysis of differentially expressed HTBH (His_6, tobacco etch virus protease site, *in vivo* biotinylation sequence and another His_6) tagged Cul1 from HEK 293 cells. The amounts of GAPDH and p27 were analyzed as reference proteins. Differential induction was performed by adding 0.5 or 2.0 μg/mL of tetracycline to the growth medium for 1 h or 4 h, respectively (Lane 1 and 2). Lane 3 and 4 are the replicates of Lane 1 and 2, respectively. The relative Cul1 expression level was 1:5.3 for Lane 1 and Lane 2, and 1:5.2 for Lane 3 and Lane 4. HCD/CID of CIT-labeled Cul1 digests yielded median H/L ratio of 5.6 using 12 peptides. The geometric standard deviation was 2.2 and the confidence interval with 95% confidence was (5.3, 8.9).

I



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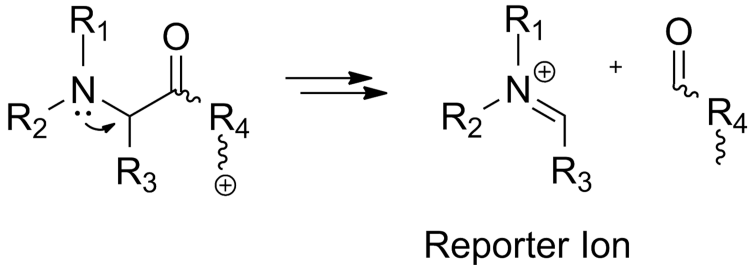
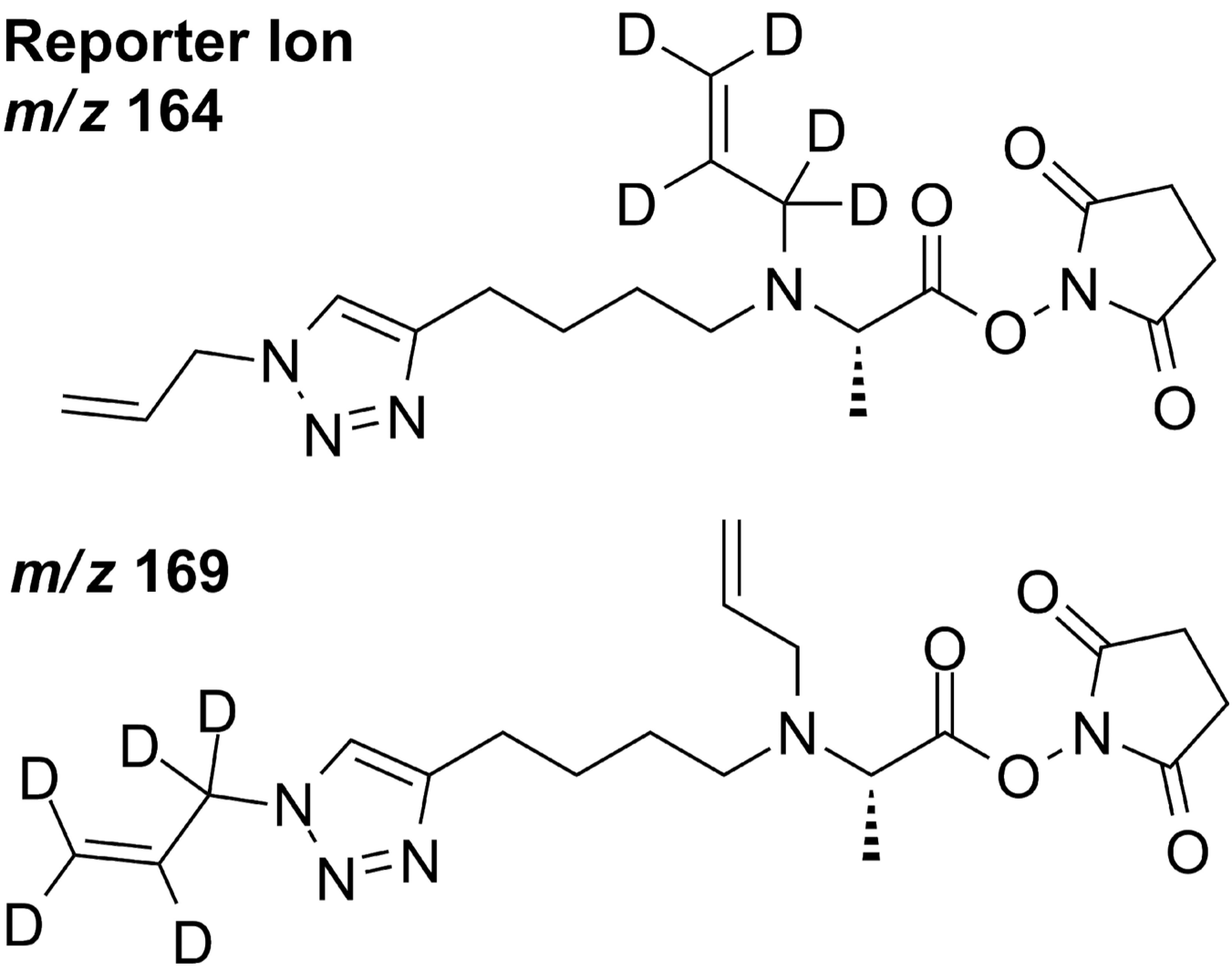
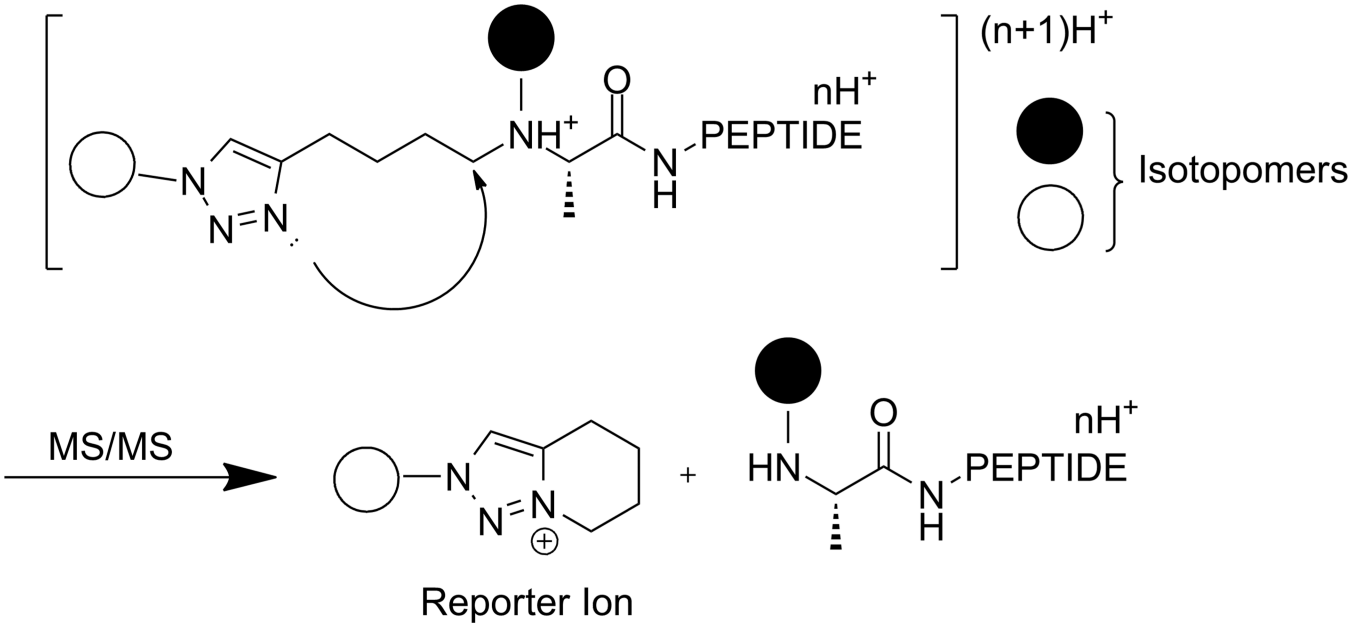


Scheme 1.



Scheme 2.



Scheme 3.

Table 1

Quantification Results of CIT-labeled Protein Mixture

Protein	H/L Ratio		# of Quantified Peptide Hits		Confidence Interval (95%)	
	PQD	HCD	PQD	HCD	PQD	HCD
BSA	0.95	0.95	45	119	(0.80, 1.19)	(0.88, 1.03)
Ovalbumin	0.87	0.82	44	48	(0.73, 1.10)	(0.65, 0.86)
Lysozyme	1.05	1.04	4	27	(0.69, 1.19)	(0.96, 1.26)
Alpha-S1-casein	0.89	0.91	27	25	(0.69, 1.19)	(0.70, 1.15)
Beta-casein	1.06	1.06	7	17	(0.59, 1.93)	(0.97, 1.29)

Table 2

Quantification Results of CIT-labeled Cul1 Complex

Protein	H/L Ratio	# of Quantified Peptide Hits	Confidence Interval (95%)
ACTB	1.02	13	(0.87, 1.20)
CUL1	0.94	11	(0.62, 1.14)
POTEE	1.00	11	(0.83, 1.18)
CAND1	1.00	11	(0.61, 1.37)
ACTN4	0.89	9	(0.63, 1.20)
ACTN1	0.87	6	(0.60, 1.09)
FLNA	1.07	5	(0.93, 1.17)
ACTA1	1.06	3	(1.02, 1.32)
SPTAN1	0.92	2	N/A
UBB	0.78	1	N/A
CORO1C	0.80	1	N/A
SKP1	1.59	1	N/A
MYL6B	1.02	1	N/A
COPS8	0.96	1	N/A
COPS6	0.50	1	N/A
COPS2	0.62	1	N/A