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RESEARCH ARTICLE

Peptide Dimethylation: Fragmentation Control via Distancing the Dimethylamino Group

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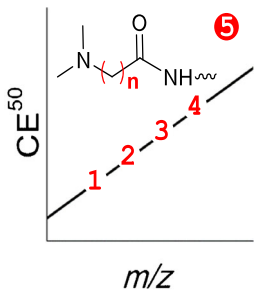

Abstract. Direct reductive methylation of peptides is a common method for quantitative proteomics. It is an active derivatization technique; with participation of the dimethylamino group, the derivatized peptides preferentially release intense a^1 ions. The advantageous generation of a_1 ions for quantitative proteomic profiling, however, is not desirable for targeted proteomic quantitation using multiple reaction monitoring mass spectrometry; this mass spectrometric method prefers the derivatizing group to stay with the intact peptide ions and multiple fragments as passive mass tags. This work investigated collisional fragmentation of peptides whose amine groups were derivatized with five linear ω-dimethylamino acids, from 2-(dimethylamino)-acetic acid to 6-(dimethylamino)-hexanoic acid. Tandem mass spectra of the derivatized tryptic peptides revealed different preferential breakdown pathways. Together with energy resolved mass spectrometry, it was found that shutting down the active participation of the terminal dimethylamino group in fragmentation of derivatized peptides is possible. However, it took a separation of five methylene groups between the terminal dimethylamino group and the amide formed upon peptide derivatization. For the first time, the gas-phase fragmentation of peptides derivatized with linear ω-dimethylamino acids of systematically increasing alkyl chain lengths is reported.

Key words: Peptide fragmentation, Collisional dissociation mechanism, Energy-resolved mass spectrometry, Peptide derivatization, Active derivatization, Passive derivatization, Peptide dimethylation, Dimethylamino peptides, Quantitative proteomics, Multiple reaction monitoring mass spectrometry

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Introduction

The use of peptide derivatization is a staple of proteomic quantitation [1]. Derivatization-based mass spectrometry (MS) quantitation is characterized by its sample-throughput capabilities allowing multiple samples to be analyzed simultaneously. Different applications are dependent upon fragmentation control of the derivatized peptide. In applications of isobaric mass tagging reagents [1, 2], the derivatized peptides are isobaric, but after fragmentation produce differentiable reporter ions in tandem MS (MS/MS) spectra. Peptide derivatization with these types of reagents are Type I active derivatizations (Scheme 1), classified by the strong signals directly observed from the fragmented derivatization group [3]. One significant pitfall of these reagents is the limited dynamic range, which is caused by all the derivatized peptides sharing common reporter ions; therefore, peptides with similar masses and elution time produce elevated background for the peptides in quantitation [4, 5].

Reductive methylation has been used as both mass-difference tagging for MS quantitation [6–8] and, recently, isobaric mass tagging for MS/MS quantitation [9–11]. Dimethylation is a relatively simple reaction only requiring a reducing agent and formaldehyde. This reaction is expeditious without any substantial formation of side products. When stable isotope labels are used in the reducing agent and/or formaldehyde, peptides can be introduced with a designed number of stable isotope labels for MS-based quantitative measurements. This method has been broadly applicable because of its easy implementation [12].

The molecular basis for MS/MS quantitation of peptides with direct dimethylation is the facile collisional cleavage of the first N-terminal amino acid residues. Strong signals for derivatized a_1 ions (stable quaternary amine ions) are characteristic in MS/MS spectra of the derivatized peptides [13–15]. Dimethylation of peptides is considered a Type II active

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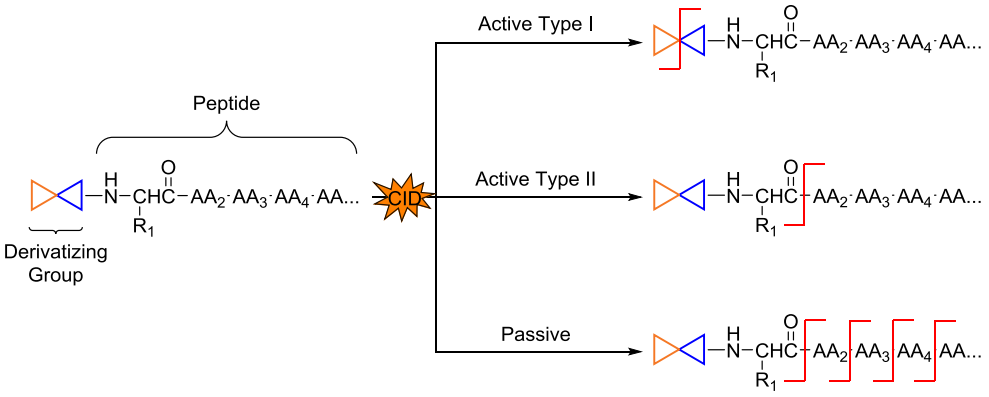


Scheme 1. Categorization of peptidyl chemical derivatizations

derivatization, where the derivatizing group promotes the cleavage of the first amide bond of peptides (Scheme 1). Collisional fragmentation of directly dimethylated peptides has been studied in detail [14, 15]. When the methyl groups carry differential stable isotope labels, the derivatized peptides can, in principle, be quantified based on the correspondingly labeled a_1 ions. This quantitation using MS/MS measurement could be advantageous. These a_1 ions are peptide-specific and, thus, lessen the interference from co-eluting peptides experienced for peptides derivatized with isobaric mass tagging reagents. In order to observe such interference, derivatized peptides would need to have similar elution times and masses as well as the same first N-terminal amino acid residue; a rare occurrence. However, authentic quadrupole sampling of dimethylated peptide ions with different numbers of isotope labels as precursors can be problematic. Efforts have been made to develop an isobaric mass tagging capability using peptide dimethylation. One method derivatizes peptides at both N- and C-termini, but with complementary numbers of stable isotope labels. In this method, triplex isobaric peptide termini labeling with dimethylation, peptide precursors are isobaric and fragment ions carry differentiable stable isotope labels and thus the ion intensity of the fragments can be used for quantitation [9].

In line with recent advancements in the utilization of ultra-high resolving MS for proteomics, differences in mass defects of carbon, hydrogen, oxygen, and nitrogen isotopes have been exploited [10, 11, 16–19]. The scope of isobaric mass tagging is, thus, being expanded, opening exciting new opportunities in proteome quantitation. In principle, all of the currently practiced, derivatization-based quantitative proteomic methods can be adapted to better utilize contemporary mass spectrometers. Two recent reports use peptide dimethylation. Peptides in comparison carry derivatizing groups with different isotopologue labels (i.e., two atoms of ^{13}C versus ^{2}H). Therefore, during the low-resolution selection of precursor ions, the differentially-labeled peptides are co-selected authentically. However, when MS/MS are recorded with ultra-high resolving power, fragment ions can be baseline-separated for quantitation. Both works use Lys-C for protein digestion so that each resulting peptide carries two derivatizing groups. It is interesting to note that there are more derivatized y ions than b ions available to be used for quantitation [10, 11]; in other words the ε-dimethylamino group on the lysine side chain at the peptide C-terminus is more stable than the α-dimethylamino group at the N-terminus.

Multiple reaction monitoring (MRM) MS is at the forefront of targeted, quantitative MS because of its high selectivity, sensitivity, and method robustness [20]. Stable isotope labeled references are commonly used to assemble methods for stable isotope dilution (SID) MRM MS. The generation of quantitation reference peptides can be achieved by derivatizing peptides with stable isotope labeled chemicals for mass-difference tagging [21, 22]. Although the quantitation utility of the derivatized reference peptides can be comparable to those produced by metabolic labeling techniques [23], these reagents have yet to be broadly adopted. The limited adoption of the commercial reagents for MRM MS exemplifies a common problem for using active derivatizations of peptides for MRM-based MS quantitation: the derivatizing groups are not efficiently held as intact mass tags for the precursor ions of the derivatized peptides or for their fragments.

MRM-based methods require the detection of strong signals for both precursor ions and fragment ions to obtain low quantitation limits. Furthermore, multiple distinguishable fragment ions are preferred to secure the method's specificity. If the derivatizing group is cleaved from the fragment ions, these fragments are no longer distinguishable among peptides with differential derivatizations. Therefore, MRM-based quantitation can benefit from passive derivatization of peptides (Scheme 1) [3]: during MRM analysis, derivatized peptides preserve the derivatizing groups on the intact peptide precursor ions in the ionization region of the instrument and keep these derivatizing groups on multiple fragment ions during fragmentation of the derivatized peptides in the collision cell. We hypothesize that if the dimethylamino group on derivatized peptides is disengaged from the adjacent amide bond, a passive derivatization will be achieved for MRM-based peptide analysis. This derivatization will keep the increased gas-phase basicity of dimethylamino peptides from the tertiary amine of the dimethylamino group and, accordingly, the enhanced MS signals [24], but not activate particular peptide bonds in a biased manner. Herein, we present a systematic study of how

diversely a dimethylamino group participates in collisional fragmentation of peptides with the increased distance from the first N-terminal amino acid residue. In addition, the change of role for the dimethylamino group from an active participant in fragmentation to a passive mass tagging one is reported.

Experimental

Chemicals

2-Aminoacetic acid (>99%), 3-aminopropanoic acid (99%), 4-aminobutanoic acid (>99%), 5-aminopentanoic acid (>97%), 6-aminohexanoic acid (>99%), methanol (>99.9%), diethyl ether (>99%), glacial acetic acid (>99.7%), *N,N*-diisopropylethylamine (99.5%, DIEA), *N*-hydroxysuccinimide (98%, NHS), dimethyl sulfoxide (>99.7%, DMSO), anhydrous *N,N*-dimethylformamide (>99.8%, DMF), 4-(4,6-dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium chloride (≥96.0%, DMTMM), *N*- (3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (≥98.0%, EDC), and anhydrous dichloromethane (>99.5%, DCM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (88% and 99.5%), trifluoroacetic acid (97%, TFA), acetonitrile (99.9%, ACN), and formaldehyde (37%) were purchased from Fisher (Hanover Park, IL, USA). Concentrated hydrogen chloride acid (36.5%–38%) was purchased from J. T. Baker (Center Valley, PA, USA). *N,N*′-diisopropylcarbodiimide (99%, DIC) was purchased from Alfa Aesar (Ward Hill, MA, USA). The ultrapure water was obtained from a Direct-Q 3 UV water purification system (EMD Millipore, Billerica, MA, USA). The peptides LSLVPDSEQGEAILPR (95%), LSEPAELTDAVK (99%), YGGFLR (98%), and SVILLGR (99%) were purchased from Peptide 2.0 (Chantilly, VA, USA). The peptide NSILTETLHR (95%) was purchased from Anaspec (Fremont, CA, USA). The isotopic peptide SVIL[L-$^{13}C_6{}^{15}N$]GR (99%) was purchased from Thermo Fisher (Rockford, IL, USA).

Dimethylamino Acid Synthesis

Each amino acid (8 mmol) was dissolved in formic acid (5 mL, 88%) and formaldehyde (1 mL). The synthesis of 3-(dimethylamino)-propanoic acid (denoted as dim-3), 4-(dimethylamino)-butanoic acid (denoted as dim-4), 5-(dimethylamino)-pentanoic acid (denoted as dim-5), and 6-(dimethylamino)-hexanoic acid (denoted as dim-6) was carried out in a microwave reactor (CEM Discover-S; Matthews, NC, USA), with the following conditions: 100 W, 110°C, 20 to 120 min. 2-(dimethylamino)-acetic acid (denoted as dim-2) was conventionally refluxed at 100°C for 1 h. Concentrated HCl (1 mL) was added after heating. The solvent was dried in vacuo. After drying, a white precipitate was obtained and washed with glacial acetic acid (5 mL) three times. The dimethylamino acid was then recrystallized with methanol and diethyl ether. After purification, the reagents were characterized with nuclear magnetic resonance (NMR) spectroscopy (Avance III 400 MHz; Bruker, Billerica, MA, USA) and high-resolution MS (AccuTOF DART; JEOL, Peabody, MA, USA). Detailed syntheses and characterizations can be found in Supplementary Information (SI) 1–5.

Peptide Derivatization

The dimethylamino acid (1.0 equivalent), NHS (1.2 equivalents), and DIC (1.0 equivalent) were first dissolved in DMSO, and then diluted with anhydrous DCM. The solution was incubated overnight at room temperature. A solution containing five peptides, NSILTETLHR, LSLVPDSEQGEAILPR, LSEPAELTDAVK, YGGFLR, and SVILLGR dissolved in 10% DIEA/DMF, was added to the activated dimethylamino acid. The solution was incubated overnight at room temperature. The coupling was then quenched with 20% formic acid/H_2O on ice. The solution was first dried with a speed-vac (Savant SC100; Thermo Fisher), then lyophilized (Labconco FreeZone Plus, Kansas City, MO, USA). After drying, the sample was desalted via an empty spin column (Thermo Fisher) packed with hydrophilic-lipophilic-balanced (HLB, Oasis Waters, Milford, MA, USA) reversed-phase sorbent. The sample was then dried and reconstituted with FA/H_2O for liquid chromatography (LC)-MS analysis (Shimadzu HPLC pumps/controller, Kyoto, KYT, Japan and HTC PAL autosampler, Carrboro, NC, USA).

LC-MS and LC-MS/MS Studies

A 10 cm, 1.0 mm i.d. Hypersil Gold HPLC column (Thermo Fisher Scientific) with, 175 Å, 3 μm C18 resin was used. Solvent A was composed of 98.8% H_2O, 1.0% ACN, and 0.2% FA and solvent B was 98.8% ACN, 1.0% H_2O, and 0.2% FA. The LC gradient was 5% to 35% solvent B for 45 min. The column temperature was 60°C. The hybrid mass spectrometer used was a QSTAR Elite (AB SCIEX, Framingham, MA, USA). Positive electrospray ionization (ESI) was used, with parameters of 5.5 kV for the spray voltage and source temperature of 300°C. The initial collision energies (CE) for the doubly- and triply charged peptides were obtained from Equations 1 and 2, respectively [25].

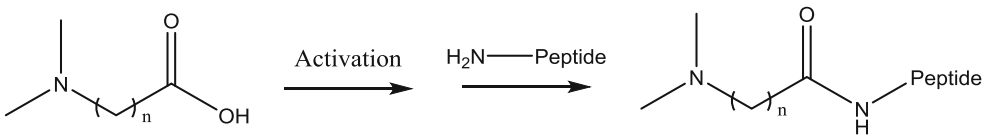
$$CE = (0.057 \times m/z) - 4.265 \quad (1)$$

$$CE = (0.031 \times m/z) + 7.082 \quad (2)$$

These equations were empirically adjusted for the non-derivatized peptides (Supplementary Table S1), and for dim-2-peptides (Supplementary Table S2). The new equations from the dim-2-peptides were applied to the dim-3- through dim-6-peptides.

Energy Resolved (ER)-MS Studies

An AB SCIEX 4000 QTRAP triple quadruple mass spectrometer with direct syringe infusion of the sample solutions was used for the ER-MS studies. The ESI was in positive mode with



n = 1 for dim-2, 2 for dim-3, 3 for dim-4, 4 for dim-5, and 5 for dim-6

Scheme 2. General reaction for derivatizing peptidyl amines with dimethylated amino acids

parameters of 5.5 kV for the ion spray and a source temperature of 200°C. Each peptide (500 fmol/μL) was individually infused with a flow rate at 5 μL/min. The DP was optimized before the collision studies were started. The CE was ramped from 5 to 40 V in 0.5 V/s increments. The resolution was set to unit for Q1 and high for Q3. The dwell time was 200 ms. Three replicates were taken for each peptide measured (non-derivatized YGGFLR through dim-6-YGGFLR).

Results and Discussion

Microwave-Assisted Synthesis of Dimethylamino Acids

The established Eschweiler-Clarke reaction [26, 27] was used to dimethylate the primary amines of five amino acids: 2-aminoacetic acid, 3-aminopropanoic acid, 4-aminobutanoic acid, 5-aminopentanoic acid, and 6-aminohexanoic acid. They have increasing numbers of methylene groups, one through five (dim-2 through dim-6, respectively), distancing the amino group from the carboxylic acid. Full conversion of 3-aminopropanoic acid to dim-3 required 8 h of conventional refluxing. Other amino acids were even less reactive towards amine dimethylation. In an attempt to expedite the dimethylation of 3-aminopropanoic acid to 6-aminohexanoic acid, microwave irradiation was utilized. All of the initial five substrates were converted to the corresponding products within 20 to 120 min of irradiation (SI 1–5).

Preparation of Dimethylamino Peptides

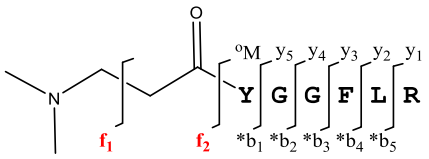
Five synthetic peptides were used as models to investigate collisional fragmentation mechanisms of dimethylated peptides: NSILTETLHR, LSLVPDSEQGEAILPR, LSEPAELTDAVK, YGGFLR, and SVILLGR. They possess a wide variety of properties (Supplementary Table S3). Peptide LSEPAELTDAVK was chosen as a model peptide for lysine-containing peptides, which have two primary amines. Tandem mass spectra for these non-derivatized peptides are shown in Supplementary Figures S1a–f.

Peptidyl amino groups on the model peptides were derivatized with dimethylamino acids whose carboxylic groups were activated in situ (Scheme 2). The activation of carboxylic groups was mediated by DIC and NHS. Solvent played a dominating role in this peptide derivatization. The dimethylamino acids were soluble in aqueous solution. However, derivatization of the peptides using water-soluble mediation reagents, including EDC/NHS and DMTMM, resulted in low and varying yields for the derivatization products. A mixed solvent of DMSO and DCM served well for activating the dimethylamino acids using DIC/NHS. This solvent system was miscible with the mixture of DIEA and DMF, which was used as the solvent for preparing peptide solutions for derivatization.

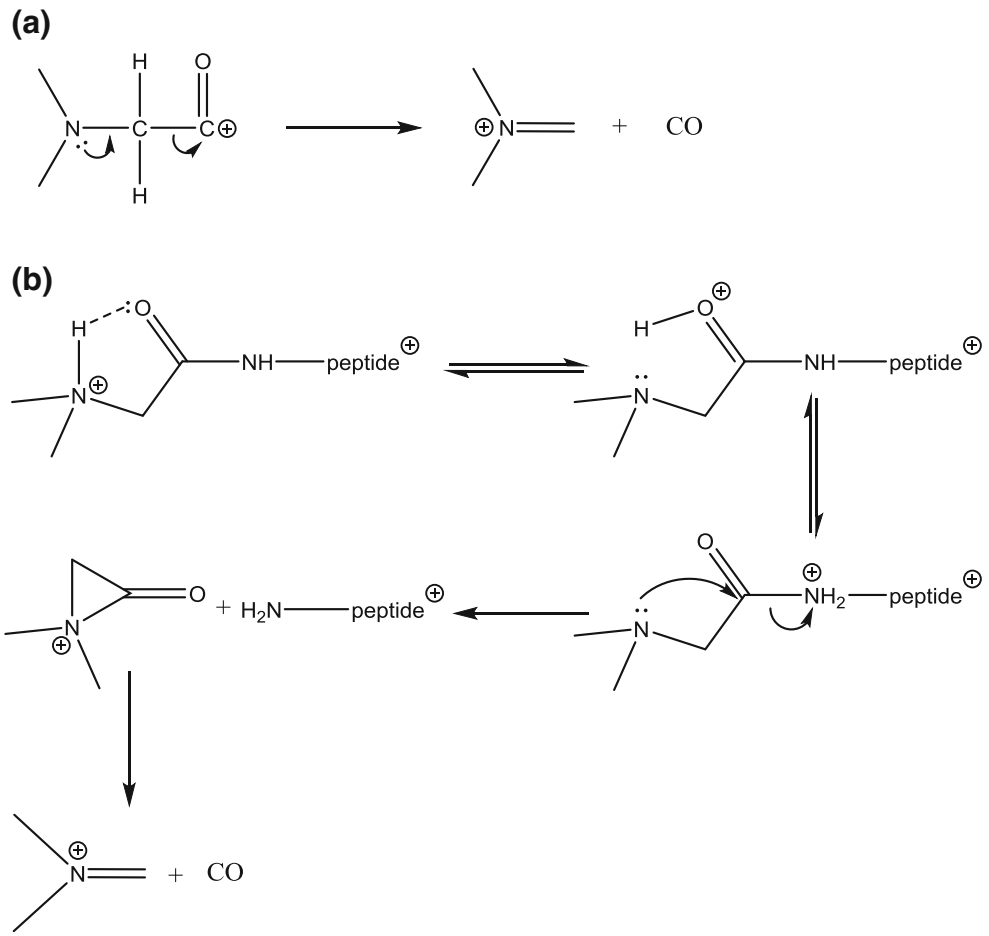
MS Signal Enhancement of Dimethylamino Peptides

The percentage of chemical conversion (PCC) and signal yield for mass spectrometry (SYMS) were quantitatively measured for peptides dim-2-SVILLGR through dim-6-SVILLGR. After quenching the derivatization reaction, an equal amount of isotopically labeled SVIL[L-$^{13}C_6{}^{15}N$]GR was added as the quantitation reference, although GSVIL[L-$^{13}C_6{}^{15}N$]GR would be a closer reference. The MS signal for the residual unreacted SVILLGR, compared with that for the isotopic quantitation reference, allowed for calculation of the PCC value for each derivatization. The average PCC for all five derivatizations of SVILLGR was 85% (Supplementary Equation S1). Further optimization of derivatization reactions was not performed in this work because these reactions were used to generate sufficient amounts of dimethylated peptides for mechanistic investigations not to be directly applied for proteome quantitation. The SYMS value for a derivatization was calculated based on the MS signal of the derivatized peptide (e.g., dim-2-SVILLGR) and that of the isotopic quantitation reference. The average SYMS for all five derivatized peptides was 170% (Supplemental Equation S2). This average SYMS was further corrected for the derivatization completeness (or average PCC), giving an adjusted SYMS of 200% (Supplementary Equation S3), for the addition of dimethylamino acids to the N-terminus of SVILLGR.

The enhanced MS signal for the derivatized peptides can be attributed to the increased gas-phase basicity, compared with the underivatized SVILLGR. These derivatizations convert a primary N-terminal amino group to a tertiary amine, which is more basic and results in more favorable protonation in the gas phase. Signal enhancement of 130% to 240% was also reported



Scheme 3. Nomenclature of fragment ions as represented by dim-3-YGGFLR



Scheme 4. Mechanism of f_1 production from dim-2 derivatized peptides

for peptides with dimethylated lysine side chains, compared with their non-dimethylated counterparts [24]. The increased basicity of peptides carrying the dimethylamino group is likely also attributing to the charge-state shift from a mixture of singly- and doubly charged ions for SVILLGR and YGGFLR to the mostly doubly charged for their derivatized counterparts, and predominantly doubly charged LSLVPDSEQGEAILPR to a doubly- and triply charged mixture (Supplementary Figure S7a–c). The coalescence to doubly charged SVILLGR

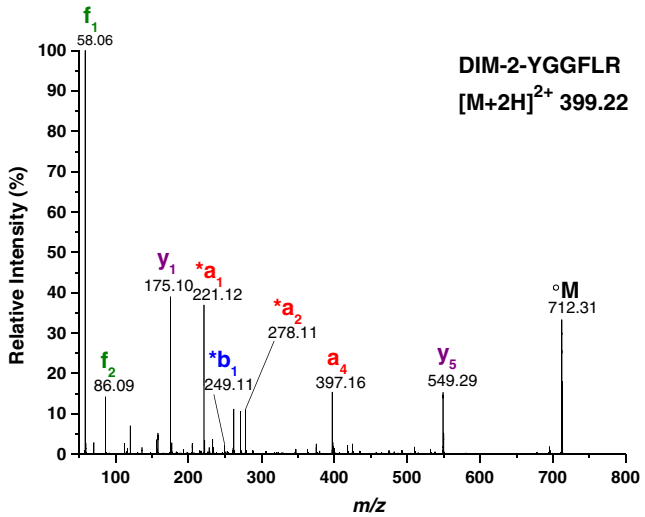


Figure 1. MS/MS spectrum of dim-2-YGGFLR

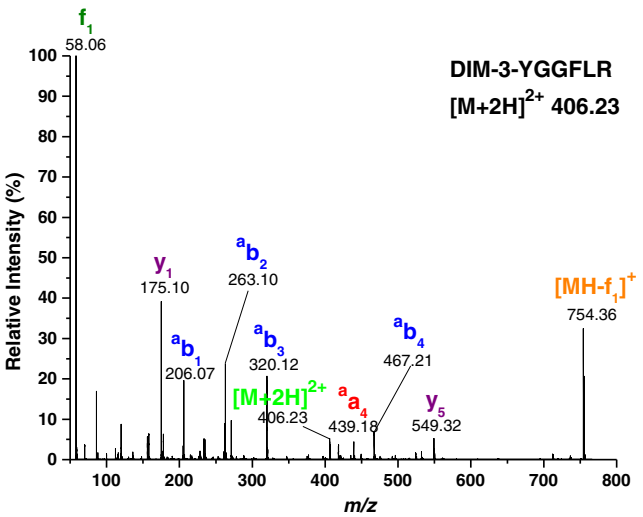
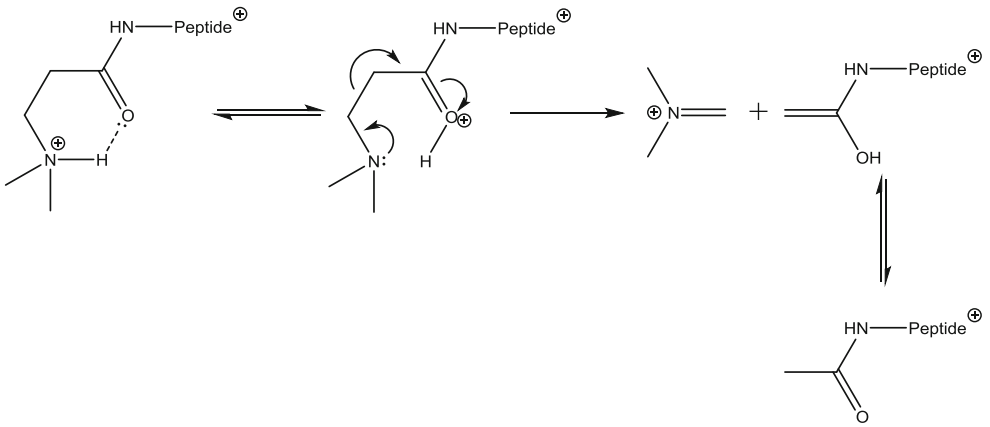


Figure 2. MS/MS spectrum of dim-3-YGGFLR



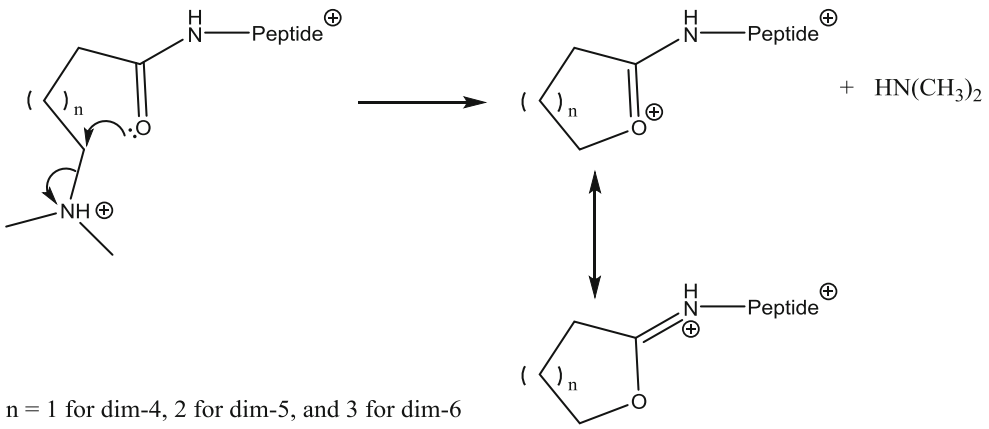
Scheme 5. Mechanism of f_1 production from dim-3 derivatized peptides

from the singly- and doubly charged mixture could also attribute to the increase in SYMS.

Fragmentation Dependence of Dimethylamino Peptides on Alkyl Chain Length

Tandem MS was performed on a QqTOF mass spectrometer for all of the derivatized peptides. The derivatized YGGFLR peptides are used as the exemplary peptides for discussion, with all others in the SI (Supplementary Figures S1–S6). The CE was empirically adjusted using Equations 1 and 2 to ensure proper fragment ion production. The dim-2-peptides were used for the empirical adjustment, and the CE was increased by 1.1 to 1.5 times, compared with the suggested values from the equations (Supplementary Table S3). The CE was adjusted until an approximate <10% precursor ion was observed after collision-induced dissociation (CID). Equations 1 and 2 were then corrected accordingly for other dimethylamino peptides. As shown in Scheme 3, fragment ions are denoted with typical nomenclature except for the following: (1) b and a ions carrying the derivatizing group are denoted as *b and *a respectively, (2) b and a ions carrying a cyclization product of the derivatizing group are denoted as ^{c}b and ^{c}a respectively, (3) ions from the bond cleavage between the α and β carbon from the dimethylamino group are denoted as f_1, (4) b and a ions carrying an acetyl group (the complementary products from the α-β cleavage) are denoted as ^{a}b and ^{a}a, respectively, and (5) the ions from the amide bond cleavage between the derivatizing group and the original N-terminus of the peptide are denoted as f_2, and the corresponding production of the singly charged ion as the original peptide is denoted as ^{o}M. A general trend of the increased coverage of b and derivatized b ions was observed for the derivatized peptides compared with their underivatized counterparts (Supplementary Figures S1–S6) [6].

Dim-2 derivatization results in peptides with the same chemical structure as peptides with direct reductive dimethylation; collisional fragmentation mechanism for the directly methylated peptides has been studied in detail (Scheme 4a) [14, 15]. For instance, dim-2-YGGFLR (MS/MS spectrum shown in Figure 1) is the same as dimethylated GYGGFLR. Dimethylated peptides are known to produce enhanced signals for $*a_1$ ions [13–15]. In the nomenclature system used in this paper, the f_1 ion was observed for dim-2-YGGFLR (or $*a_1$ for dimethylated GYGGFLR) at *m/z* 58.06 (Figure 1; Scheme 4). Generation of f_2 ions (or $*b_1$ for dimethylated GYGGFLR) at *m/z* 86.20 can be accounted for by the oxozolium mechanism [28] or the aziridin-2-one pathway (Scheme 4b), which further produces the f_1 ion [29].



Scheme 6. Imido lactone formation at the N-terminus of dim-4-, dim-5-, and dim-6-YGGFLR

Generation of f_2 ions suggests the favorable protonation of the dimethylamino group and a facile intramolecular proton transfer to activate the adjacent amide group in the derivatized peptides (Scheme 4b). In other words, there is an active participation of the derivatizing group in the peptide fragmentation.

Dim-3 derivatization extends the distance by one additional methylene group between the dimethylamino group and the first amide group on the derivatized peptides, which is formed upon the attachment of a dimethylamino propionic acid. Two intense ions at *m/z* 58.06 (f_1) and *m/z* 754.36 $[MH\text{-}f_1]^+$ were observed for dim-3-YGGFLR, together with significant signals for acetyl b ions ($^a b_1$, $^a b_2$, $^a b_3$, and $^a b_4$), shown in Figure 2. The sum of *m/z* 58.06 and *m/z* 754.36 made up the mass balance for dim-3-YGGFLR, suggesting the existence of a labile bond. Generation of these two ions can be readily explained by the McLafferty-type rearrangement (Scheme 5) [30]. It should be noted that although the ion structure for f_1 ions for dim-2-peptides and dim-3-peptides are the same, mechanisms for the ion generation are different (Schemes 4 and 5). In general, the f_1 ion was observed for all dim-3 peptides (Figure 2 and Supplementary Figure S3). On the other hand, not all of the corresponding $[MH\text{-}f_1]^+$ ions were observed (Supplementary Figure S3), which could be due to further breakage of the primary $[MH\text{-}f_1]^+$ fragment ions under experimental conditions. According to the categorization of peptide derivatization shown in Scheme 1, the dim-3 reaction would be classified as a Type I active derivatization, where there is preferential cleavage within the derivatizing group.

A common, neutral loss of dimethylamine was observed for doubly charged ions of dim-4-, dim-5-, and dim-6-YGGFLR, producing corresponding doubly charged fragments (Scheme 6). Dim-4 derivatization produced a very strong fragment ion at *m/z* 390.75 (cyc^{2+}, Figure 3). Dim-5-YGGFLR gave a doubly charged ion at *m/z* 397.73 (cyc^{2+}) as the most intense fragment (Figure 4). These two ions are proposed as YGGFLR carrying an imido lactone at the N-terminus (Scheme 6). A complement of b and a ions carrying the imido

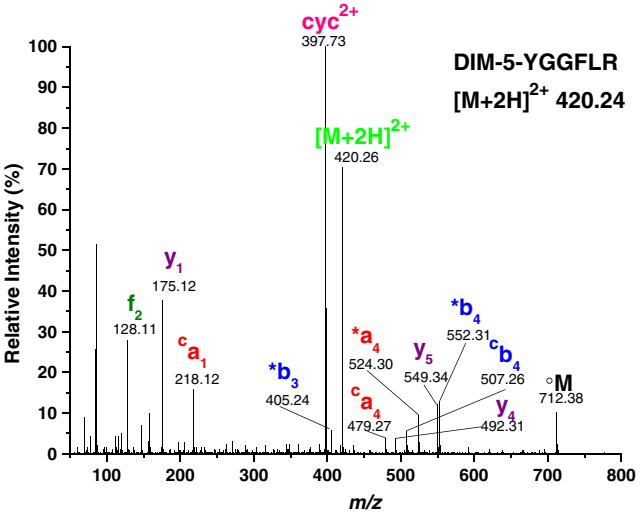


Figure 4. MS/MS spectrum of dim-5-YGGFLR

lactone (i.e., ^{c}b and ^{c}a ions) were observed for dim-4- and dim-5-YGGFLR. Formation of these ions can be explained by nucleophilic substitution of the amide oxygen to the α-methylene carbon of the dimethylamino group (Scheme 6). Similar mechanisms were reported in a fragmentation study of lysylglycine [31] and used for explaining the charge mobilization of peptides derivatized with a quaternary amine [32] and loss of dimethylamine from N^{ε}-dimethyllysine [33]. Dim-6-YGGFLR produced a similar cyclization product at *m/z* 404.72 (cyc^{2+}, Figure 5), but at a much reduced intensity and without observable complementary ^{c}a and ^{c}b ions. The intensity differences could be related to the stability of imido lactone rings.

Doubly charged dim-6-YGGFLR, compared with dim-4- and dim-5-YGGFLR, produced full series of *a and *b ions carrying the derivatization group, together with y ions (Figures 3, 4, and 5). This suggests that in the dim-6-peptide the preferential cleavage via the mechanism in Scheme 5

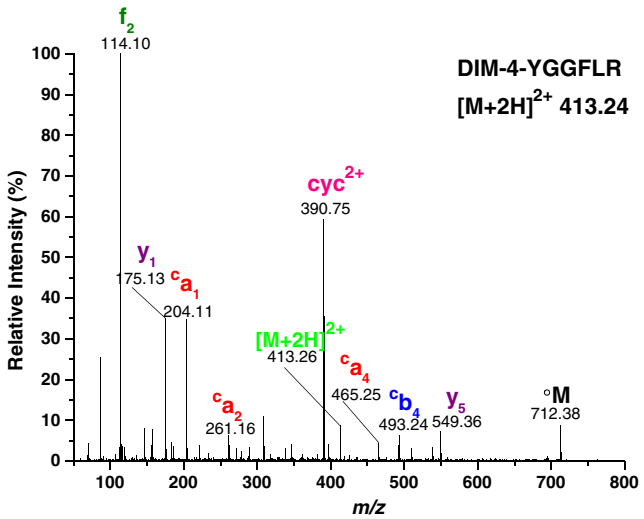


Figure 3. MS/MS spectrum of dim-4-YGGFLR

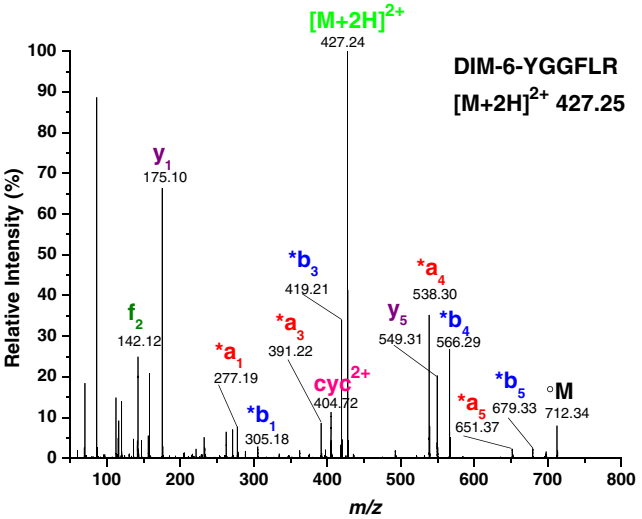
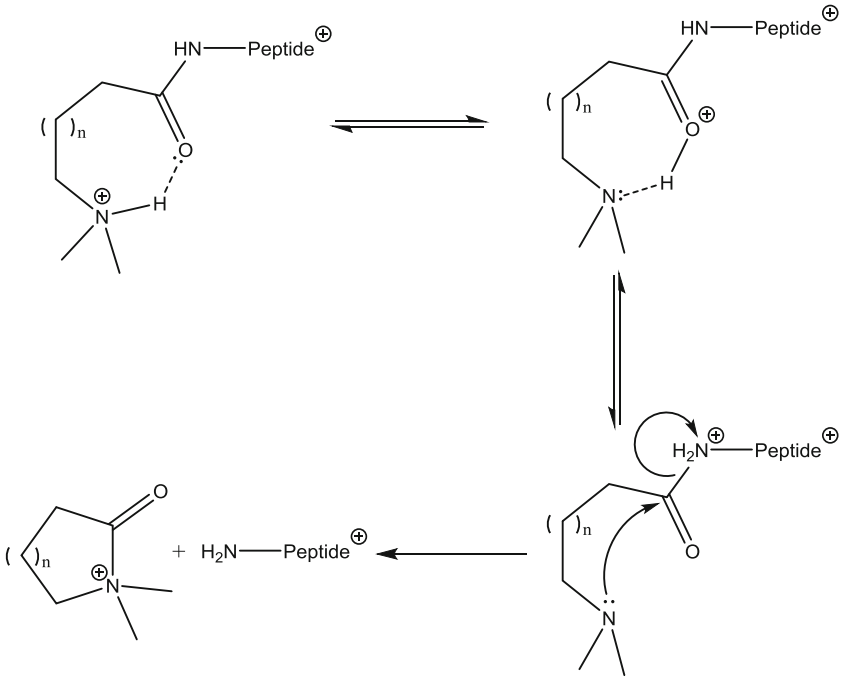


Figure 5. MS/MS spectrum of dim-6-YGGFLR



Scheme 7. *N,N*-dimethyl lactam ion loss from dim-4-, dim-5-, and dim-6-YGGFLR

becomes much less competitive. In other words, the dim-6 derivatization is passive (Scheme 1). Similar results were also observed for other dim-6 derivatized peptides (Supplementary Figure S6). It is also interesting to note that the intensity for the residual doubly charged precursor was high (Figure 5), using the collision energy obtained from the same calculation as those for other derivatized peptides (Supplementary Table S2), dim-2-YGGFLR through dim-5-YGGFLR.

Double derivatization of LSEPAELTDAVK was obtained at both the N-terminus and the lysine side chain. For example, dim-4-LSEPAELTDAVK (Supplementary Figure S4d) produced fragments that corresponded to the cyclization occurring at both sites. The first cyclization product (*m/z* 727.45) was 80% relative intensity and the second (*m/z* 704.90) was 30% relative intensity. In comparison, one cyclization product (*m/z* 741.43) was observed for dim-5-LSEPAELTDAVK at 15% relative intensity (Supplementary Figure S5e), which reports the bond cleavage at the N-terminus.

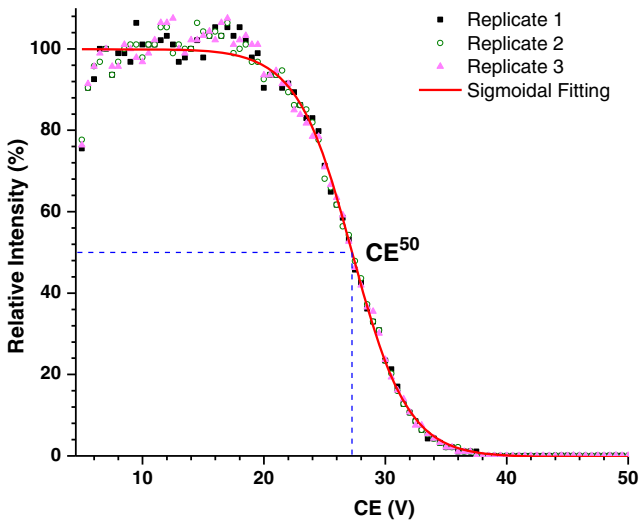


Figure 6. Survival curve of dim-6-YGGFLR. The precursor was selected in Q1 and Q3 with the CE being ramped from 5 to 35 V in 0.5 V/s increments. Three replicates were obtained for each YGGFLR peptide, and the data was normalized to the maximum intensity. After sigmoidal dose-response fitting the CE^{50} was obtained

Another Preferential Cleavage Pathway of Dim-4-, Dim-5-, and Dim-6-Peptides

Two ions related to cleavage of the derivatizing group from the doubly charged dim-4-YGGFLR precursor were observed; one dominantly at *m/z* 114.10 (f_2) and the other strong ion at *m/z* 712.38 (^oM, Figure 3). Dim-5-YGGFLR and dim-6-YGGFLR, cleaving at the same amide bond, also produced ion pairs at *m/z* 128.15 and 712.38 and at *m/z* 142.12 and 712.34, respectively. However, the relative ion intensities for the corresponding f_2 ions, although strong, were not dominantly high for dim-5- and dim-6-YGGFLR (Figures 4 and 5). The favorable cleavage of the amide bond can be accounted for by the mechanism proposed in Scheme 7. The intensity differences among these ions are on the same order of the facility for intramolecular proton transfer, requiring the formation of a pseudo ring of 7, 8, or 9 atoms, with the increasing entropy penalty. Although dim-2- and dim-3-peptides have a more facile intramolecular proton

transfer through a pseudo 5- or 6-membered ring (Schemes 4b and 5), the nucleophilic attack of the deprotonated dimethylamine to the carbon of protonated amide would experience high ring constraints; the generation of similar f_2 ions would require the formation of a ring with three or four atoms following the mechanism in Scheme 7. Protonated aziridin-2-ones, however, are viable intermediates for a_1 ions of peptides upon collisional dissociation (Scheme 4b) [29].

ER-MS to Categorize Peptides into Active and Passive Derivatization Groups

ER-MS is an excellent tool to evaluate the energy requirements for the generation of fragment ions and elucidation of fragmentation patterns and mechanisms [28]. ER-MS was performed for YGGFLR and dim-2- through dim-6-YGGFLR. The CE profiles, in the laboratory frame, for the breakdown of the derivatized peptides were recorded on a triple quadrupole instrument. After normalization to the initial precursor ion intensity, triplicate measurements were combined as a single data set for sigmoidal dose-response fitting to produce the survival curve (Figure 6), giving a CE value at 50% (CE^{50}) of residual precursor ions. Fitting plots for all of the six YGGLFR peptides are shown in Supplementary Figure S8 and the formulae and the goodness of fitting are reported in Supplementary Table S4. CE^{50} values for these peptides were plotted against *m/z* values of doubly charged precursor ions (Figure 7). Dim-2-, dim-3-, dim-4-, and dim-5-YGGFLR correlated linearly with a slope of 0.153 and adjusted R^2 of 0.9994. Together with the fact that different pathways operate to preferentially produce f_1 and/or f_2 ions and their complementary fragment ions (Schemes 4, 5, 6, and 7), this linear correlation suggests that these different mechanisms result in minimal differential effects on CE required for the breakage of doubly charged precursor ions.

The underivatized YGGFLR showed positive deviation of 1.81 V from the linear correlation (Figure 7). This deviation signifies the peptide as a mechanistic indicator, reporting that there is no special bond in the peptide, which is particularly labile to gas-phase collision. The same is true for the positive deviation of 2.59 V for dim-6-YGGFLR (Figure 7). Although dim-4- to dim-6-YGGFLR share a common fragmentation pathway releasing the derivatizing groups as *N,N*-dimethyl lactams (Scheme 7), the increased distance between the dimethylamino group and the amide bond formed upon the peptide derivatization leads to a discrete increase in the stability of intact precursors from dim-5- to dim-6-YGGFLR, shown as the positive deviation (Figure 7). This agrees with the observation that the ε-dimethylamino group on the lysine side chain is a passive mass tag (Supplementary Figure S2e), stable to collisional fragmentation in the gas phase [10, 11].

Two Routes for Selective Activation of Dimethylamino Peptides

There are two general mechanisms for a derivatizing group actively participating in preferential fragmentations around the N-termini of derivatized peptides. Both start from the preferential protonation of the dimethylamino group. This proton then either (1) transfers to the adjacent amide group acting as

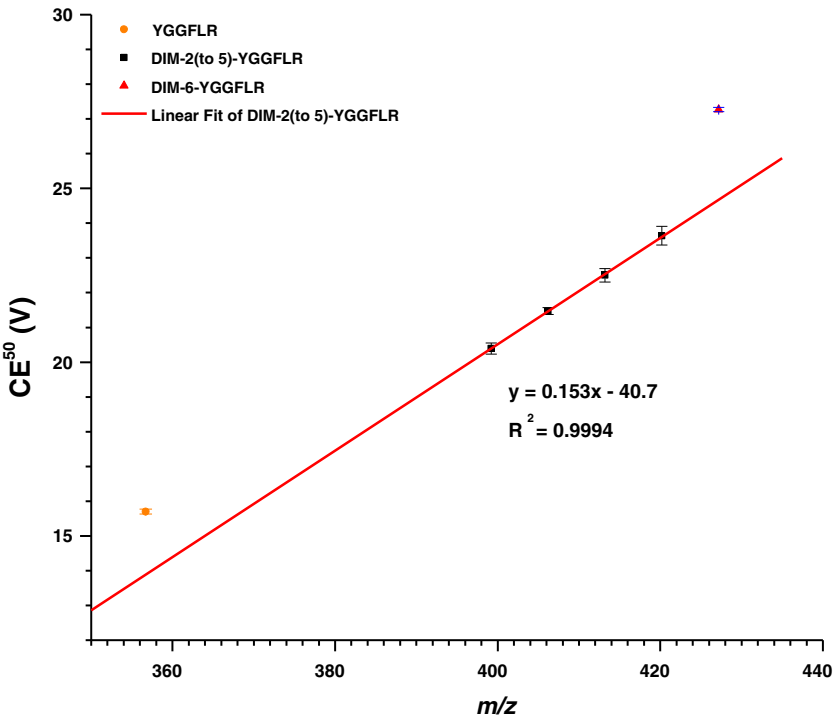


Figure 7. CE^{50} and *m/z* correlation for dimethylamino YGGFLR peptides

an acid catalyst to facilitate fragmentation reactions (the mobile-proton regime) as in Schemes 4b, 5, and 7, or (2) directly polarizes the α-methylene carbon of the protonated dimethylamino group for the nucleophilic substitution by the adjacent amide oxygen (the charge-directed regime) as in Scheme 6. Proton migration from basic amino acids, including histidine, lysine, and arginine, has been shown to promote preferential neighboring cleavages [34–36]. The first general mechanism also operates in the preferential fragmentation of peptides derivatized with isothiocyanates [37, 38]. The importance of the intramolecular proton transfer is evident in this study. When the intramolecular proton transfer becomes less favorable for dim-6-YGGFLR, the preferential activation of the adjacent amide bond diminishes and so do the subsequent fragmentation products. In comparison, proton transfer from non-methylated ε-amine at the peptide N-terminus faces competing paths because of the complex intramolecular solvation of the protonated amine group [39]; therefore activation of the first amide group via acid catalysis confronts similar competitions.

For the second general mechanism, although activation of the α-methylene carbon of the protonated dimethylamino group stays the same more or less, the direct nucleophilic substitution by the amide oxygen becomes less entropically favorable with the distancing of the protonated dimethylamino group from the neighboring amide. Succinctly, a quantized change happens from dim-5-YGGFLR to dim-6-YGGFLR or from active derivatization to passive derivatization (Scheme 1).

Conclusion

Reagents for quantitative MS are essential tools in proteomic technologies. Full utilization of contemporary MS advancement requires a clear appreciation of chemical principles governing the gas-phase fragmentation of derivatized peptides [40]. In certain applications, *active* cleavage of the derivatizing group is preferred to produce quantitative reporter ions and simple spectra of sequence ions for concurrent peptide identification; common examples are proteomic peptides derivatized with tandem mass tagging reagents. In other applications such as MRM MS, it is advantageous to have the derivatizing group associated with multiple fragment ions. This requires a derivatizing group staying *passive* as a ubiquitous mass tag during fragmentation processes of derivatized peptides [3]. Understanding the underlying principles of gas-phase chemistry of derivatized peptides, as revealed by the investigational reagents in this study, can guide rational design of novel reagents for quantitative MS.

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