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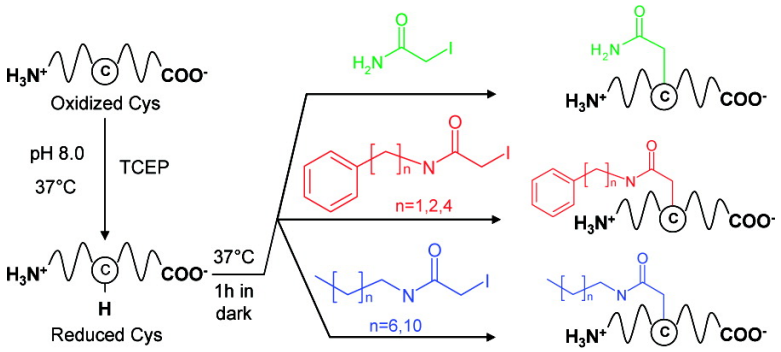

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Synthesis, Characterization, and Application of Iodoacetamide Derivatives Utilized for the ALiPHAT Strategy

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Since the completion of the human genome project and with subsequent advances in databases, mass spectrometry has emerged as a leading technology in proteomics.[1−3] To analyze proteomic samples, cysteines are normally reduced and alkylated to eliminate the protein's tertiary structure.[4−6] The alkylation step is utilized in many chemical tagging strategies because the reaction is simple and efficient.[5] Hydrophobic tagging of large biomolecules (>500 Da) was first described by Null et al.[7] One tagging strategy is the ALiPHAT strategy (augmented limits of detection for peptides with hydrophobic alkyl tags),[8] which is a method previously developed by Frahm et al. in which electrospray response of peptides was increased via a cysteine specific hydrophobic tag. In the previous study, peptides modified with one hydrophobic tag, 2-iodo-*N*-octylacetamide, were demonstrated to have improved limits of detection relative to peptides alkylated with iodoacetamide. Here in, we report the synthesis, characterization, and application of four new hydrophobic tags as well as the application of a previously developed tag for the ALiPHAT strategy.[8]

The ALiPHAT application of these hydrophobic tags was completed on peptide E-76 (Table 1, peptide 1), whose amino acid sequence is shown in Figure 1, and two additional peptides utilizing liquid chromatography Fourier-transform ion cyclotron resonance mass spectrometry (LC-FT-ICR-MS). The E-76 peptide was chosen because of its biological significance as a potent inhibitor of coagulation factor VIIIa[9] and because its amino acid sequence contained cysteines. Iodoacetamide was the standard alkylating agent to which the other tags were compared. The reaction of iodoacetamide with cysteine groups results in a carboxyamidomethyl (CAM) modification. The peptides modified with the hydrophobic tags were combined with their CAM-modified counterpart such that identical molar amounts of each were injected on-column. Scheme 1 shows general reduction of protein and alkylation reactions of the hydrophobic tags used in these experiments.

The ratio of peak areas from the extracted ion chromatograms (XICs) describes both the chromatographic and electrospray ionization consequences of addition of the hydrophobic tag. This ratio was utilized to quantify the results described herein.[7]

E-76 peptide alkylated with 2-iodo-*N*-(4-phenylbutyl)acetamide (Scheme 1, $n = 4$) was observed to elute 6.05 min after the CAM-modified peptide. This represents an increase in mobile phase B by 8.5%, which demonstrates that this modified peptide is indeed more hydrophobic than the CAM modified peptide. The overlaid XICs of this and the CAM modified peptides are shown in Figure 1. The ratio of the area of these peaks demonstrates an improvement of 429 for the peptide modified with 2-iodo-*N*-(4-phenylbutyl)-acetamide versus the standard CAM-modified peptide. This hydrophobic tag provided the greatest improvement over the CAM modification for the E-76 peptide.

Also shown in Figure 1 are mass spectra; one representing the CAM-modified peptide and one representing the peptide modified with 2-iodo-*N*-(4-phenylbutyl)acetamide which have theoretical *m*/*z*'s of 1164.0202 and 1296.1140, respectively, in the 2+ charge state. MS/MS data is shown for this modified peptide which demonstrates that the tag itself does not fragment. This is important

Table 1. Investigated Peptide Sequences

Peptide #	Sequence
Peptide 1 (E-76)	Ac-ALCDDPRVDRWYCQFVEG-NH_2
Peptide 2	SCSLPQTSGLQKPES-NH_2
Peptide 3	CYFQNCPRG-NH_2

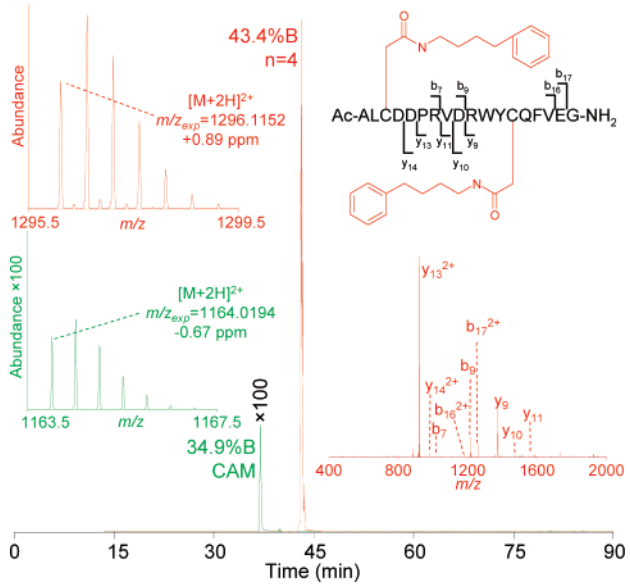
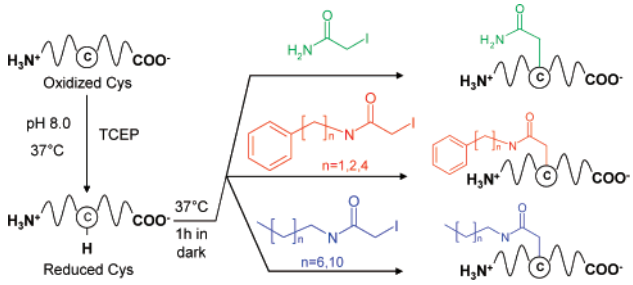


Figure 1. The overlaid XICs of the CAM (green) and $n = 4$ (red) modified peptide are shown as well as their mass spectra. Denoted is the charge state, theoretical *m*/*z*, and observed mass measurement accuracy. The sequence of the E-76 peptide as well as a labeled MS/MS spectrum demonstrating the $n = 4$ tag does not fragment under conditions of CID nor hinder MS/MS experiments are also shown.

Scheme 1. Reduction and Alkylation with Hydrophobic Tags



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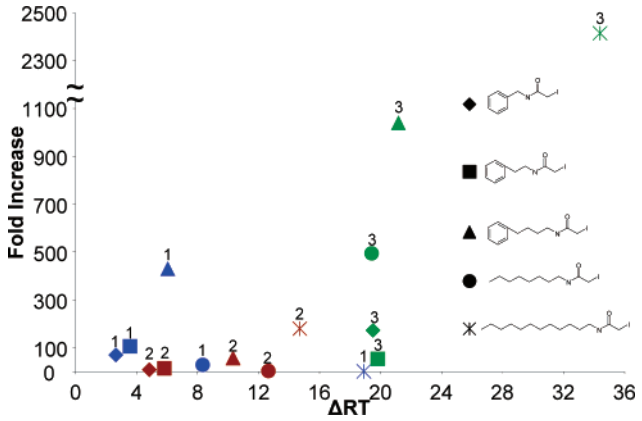

Figure 2. ESI response fold improvement vs change in retention time in relation to the CAM-modified peptide. Tags are represented by point shape and peptides represented by numbers corresponding to Table 1.

to note because if the tag were to fragment under conditions of collision induced dissociation (CID), it would make the interpretation of MS/MS data much more difficult. Tandem MS data were analyzed for all peptides with each of the tags discussed herein. None of the tags were observed to fragment. The fragmentation pattern remained the same between peptides modified with iodoacetamide and the new hydrophobic tags, which demonstrates that the new tags do not adversely affect the CID mechanism for fragmentation.

E-76 peptide alkylated with 2-iodo-*N*-benzylacetamide (Scheme 1, $n = 1$) improved the chromatography and ESI response. Figure 2 shows the improvement of 69. Modification of the peptide with this tag resulted in a shift in retention time by 2.85 min. This shift in retention time is a result of the peptide being more hydrophobic which causes it to elute in a concentration of mobile phase B increased by 3.4% when compared to the CAM-modified peptide. The improvement in chromatography is visible by a narrower peak which results from the more hydrophobic modified peptide being able to be captured more efficiently at the head of the column which favors sample concentration prior to elution, as compared to the CAM-modified peptide. This phenomenon is also observed with the other tags examined in this study.

2-Iodo-*N*-(phenethyl)acetamide reacts with cysteines to create a modification observed in Scheme 1 with $n = 2$. This modified E-76 peptide resulted in a slightly more hydrophobic peptide than when modified with 2-iodo-*N*-benzylacetamide. This increased hydrophobicity results in an increase in retention time of 3.57 min over the CAM modified peptide. This difference in retention time corresponded to the elution of this tagged peptide in mobile phase with an increase of 4.5% B over the CAM-modified peptide. The ratio of the peak areas demonstrated an improvement of 105 for the modified peptide, shown in Figure 2.

As previously shown, alkylation with 2-iodo-*N*-octylacetamide (Scheme 1, $n = 6$) creates an octylcarboxyamidomethyl (OCAM) modification to cysteines in a peptide or protein sequence.[8] The addition of the OCAM tag to the cysteines of E-76 improved both the chromatography and electrospray response of the peptide which is evident by the increase in peak area by 27 times versus the CAM-modified peptide, shown in Figure 2. The difference in retention time was 8.33 min which corresponds to an increase of 13.4% B for the OCAM peptide to elute. The increase in hydrophobicity is evident by the increased retention time; however, the OCAM tag does not improve the ESI response as well as the tags with the phenyl group.

The final tag examined was 2-iodo-*N*-dodecylacetamide (Scheme 1, $n = 10$), which generates a dodecylcarboxyamidomethyl (DCAM) modification to cysteines. The DCAM-modified E-76 peptide led to an increase in retention time of 16.90 min versus the CAM-modified peptide. This difference in retention time represents an increase in mobile phase B by 25.9%. DCAM modification of the E-76 peptide results in the most hydrophobic of any of the tagged peptides observed in this study. The peak ratio of the XIC between the DCAM- and CAM-modified peptide yielded a result of 0.6.

Figure 2 demonstrates the relationship between the differences in retention time between the different peptides (Table 1) with the iodoacetamide derived tags and their CAM-modified counterpart. The modifications with the phenyl terminal group yielded better improvements when compared to the tags with only alkyl chains for the E-76 peptide. 2-Iodo-*N*-dodecylacetamide performed the best for peptides 2 and 3 and provided for electrospray response improvements of 179 and 2441, respectively.

These hydrophobic tags have been applied to three peptides whose electrospray response improvements are summarized in Figure 2. The data, presented herein, clearly show alkylation of the E-76 peptide, with the hydrophobic tags synthesized and characterized in this study, can improve ESI response >400 fold as well as provide for improved chromatographic behavior. Peptides 2 and 3 showed improvement in electrospray response for all hydrophobic tags in comparison to their CAM-modified counterpart. Furthermore, peptide 3 was able to achieve an improvement of >2000-fold improvement over its CAM-modified counterpart! These improvements in electrospray response come at essentially no experimental cost since the alkylation step is facile and carried out in nearly all bottom-up proteomic analyses. These benefits will be able to aid in the investigation of cysteine-containing peptides and proteins that have low electrospray response or concentration.

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Supporting Information Available: Synthesis and characterization data for the hydrophobic tags. This material is available free of charge via the Internet at http://pubs.acs.org.

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