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Synthesis of D-labeled naphthyliodoacetamide and application to quantitative peptide analysis by isotope differential mass spectrometry

Satomi Niwayama,[a,*] Sadamu Kurono,[b] Hanjoung Cho[a] and Hiroyuki Matsumoto[c]

[a]*Department of Chemistry and Biochemistry, Texas Tech University, Box 41061, Lubbock, TX 79409-1061, USA*
[b]*IBERICA HOLDINGS Co., Ltd, Kurume University Translational Research Center, Kurume, Fukuoka 830-0011, Japan*
[c]*Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA*

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Abstract—D-labeled and -unlabeled *N*-β-naphthyliodoacetamides have been synthesized for specific modification of the sulfhydryl groups of cysteine residues in proteins or peptides, and have been applied to quantitative analysis of several peptides. A combination of these reagents, coupled with mass spectrometry, is anticipated to serve as a useful tool for quantitative analysis of peptides and hence proteins.

Proteomics studies proteomes, which are sets of proteins expressed by a cell, tissue, or organism under a specific set of conditions, and it is rapidly becoming an important research area in the postgenome era. Proteomics is originally defined as the complete characterization of the protein complement of a cell, including post-translational modifications, and as a part of such goals, a great deal of effort has been directed to developing methods to quantitatively measure changes in relative abundance of proteins expressed under two different physiological conditions. In this sense, it is essential to develop efficient methodologies for quantitative analysis of proteins within complex mixtures expressed under certain physiological conditions.

Some classical examples that are still common for certain purposes of quantitative analysis of proteins include two-dimensional densitometry of the gel,[1] metabolic radioisotope labeling,[2] or metabolic stable-isotope labeling,[3] or stable-isotope labeling during proteolytic digestion of proteins.[4] Recently, more sophisticated methods using stable-isotope tagging by chemical reactions followed by mass spectrometry analysis have been emerging as a more versatile technology for quantification of proteins.[5] Some of the earliest work applies deuterium-labeled isotope-coded affinity tags (ICAT), a biotinylated conjugate of a sulfhydryl group modifier and its deuterated derivative, which allow purification of peptides by LC and subsequent quantitative analysis by MS/MS.[6] Other researchers have also invented similar LC-based methods using different modifiers.[7] However, several fundamental problems in these methods have been reported, perhaps due to the use of large organic molecules and a large amount of aqueous media. These include fragmentation of the tags during the CID conditions and primary isotope effects causing differential elution during liquid chromatography.[5a,b] These problems suggest the need to eliminate the LC and the use of large hydrophobic organic molecules. Earlier, we reported such methods using small organic molecule modifiers, isotope-labeled and -unlabeled alkylmaleimides[8] and iodoacetanilides.[9] We found that the combination of isotope-labeled and -unlabeled chemical modification of cysteine residues followed by 2-D gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) makes quantification of peptides and proteins feasible. This method also allows identification of proteins prior to quantitative analysis through peptide mass fingerprinting without

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Keywords: Proteomics; Quantitative analysis; Peptides; Stable isotope-labeling.

* Corresponding author. Tel.: +1 806 742 3118; fax: +1 806 742 1289; e-mail: satomi.niwayama@ttu.edu

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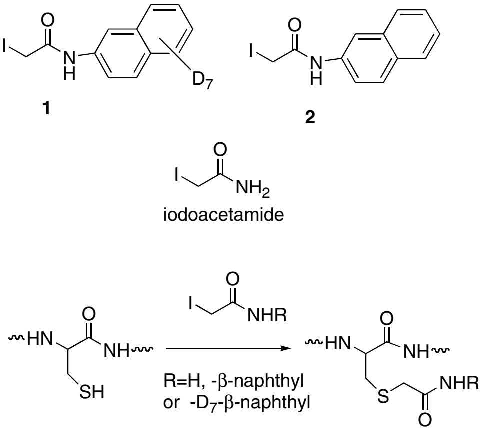
the use of MS/MS unlike other shotgun-LC-based methods, making our method more economical and accessible to diverse researchers.

Here we report synthesis of another set of sulfhydryl group-specific modifiers that are small organic molecules, D_7-labeled and -unlabeled *N*-β-naphthyliodoacetamides (NBN), **1** and **2**, and the application of these modifiers to quantitative analysis of peptides. As has been demonstrated in our earlier work using alkylmaleimides and iodoacetanilides, we believe that the success of this work signifies success in quantification of tryptic peptides, hence proteins (Scheme 1).

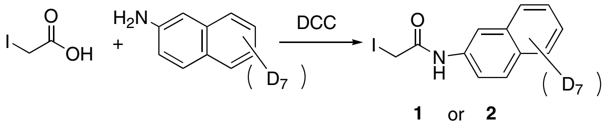
N-β-naphthyliodoacetamide possesses the reaction site toward the sulfhydryl group, –SH, that is the same as iodoacetamide, which is a well-known sulfhydryl group modifier. The combination of D_7-labeled and -unlabeled *N*-β-naphthyliodoacetamides (NBN) exhibits 7Da difference, which is greater than in our earlier studies of $^{13}C_6$-labeled and -unlabeled iodoacetanilide as well as D_5-labeled *N*-ethylmaleimide and -unlabeled *N*-ethylmaleimide, and therefore is anticipated to be more effective.

Synthesis of D_7-labeled and -unlabeled *N*-β-naphthyliodoacetamides (NBN), **1** and **2**, was carried out from iodoacetic acid and D_7-labeled or -unlabeled β-aminonaphthalene, as reported in our earlier synthesis of $^{13}C_6$-labeled iodoacetanilide (Scheme 2).[10]

The applicability of these reagents was tested for three synthetic peptides, PEP 31, PEP 13, and PEP 60, in which the amino acid sequences, molecular weight, and p*I* values are KEEPPHHEVPESETC, 1746.75 Da, 4.5, SDTCSSQKTEVSTVSSTQK, 2001.92 Da, 6.2, and ALVCEQEAR, 1017.49, and 4.4, respectively. These peptides are the same as those reported in the studies of iodoacetanilide and $^{13}C_6$-iodoacetanilides.[9,11]



Scheme 1.



Scheme 2.

Various aqueous solutions of these peptides with pH 9.0 were reacted with D_7-labeled or -unlabeled *N*-β-naphthyliodoacetamides (NBN), and the relative molar ratios were quantitatively analyzed by MALDI-TOF MS after a matrix was added.

The following charts show peptide PEP 31 itself and PEP 31 reacted with -unlabeled iodoacetanilide or D_7-labeled *N*-β-naphthyliodoacetamides.[12] The ion peaks show the monoisotopic mass for PEP 31 (1746.75 Da) and a series of isotope peaks that are several daltons greater than the monoisotopic peak due to the existence of natural isotopes. The excess amount of D_7-labeled and -unlabeled *N*-β-naphthyliodoacetamides completely reacted with this peptide within 30 min. By reacting with the unlabeled or labeled *N*-β-naphthyliodoacetamides (NBN), the molecular weight of the NBN-modified or D_7-NBN-modified PEP 31 increased by 183 or 190 Da, respectively, indicating that a combination of these modified peptides shows 7 Da difference (Fig. 1).

In order to examine general applicability of these reagents to quantitative analysis of peptides, several aqueous PEP 31 solutions with pH 8.5 were prepared, and these solutions were individually treated with D_7-labeled or -unlabeled *N*-β-naphthyliodoacetamides. Then the differentially labeled PEP 31 solutions were mixed in various ratios. The ratios of NBN-PEP 31 to D_7-NBN-PEP 31 examined were 6:1, 3:1, 1:1, and 1:3. The relative quantities of PEP 31 in each mixed solution were measured from the relative signal intensities for pairs of peptide ions modified with D_7-labeled or -unlabeled *N*-β-naphthyliodoacetamides.[13]

Figure 2 shows the MALDI MS spectrum for each set of the PEP 31 mixtures above. After measuring the height of the most abundant peak among the monoisotopic

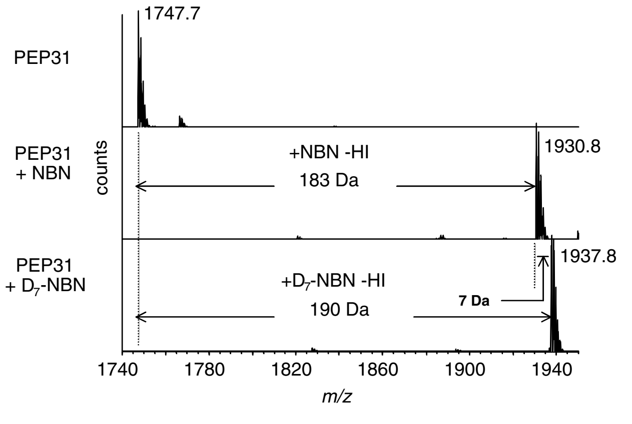


Figure 1. MALDI spectra of PEP 31 and *N*-β-naphthyliodoacetamide-modified PEP 31.

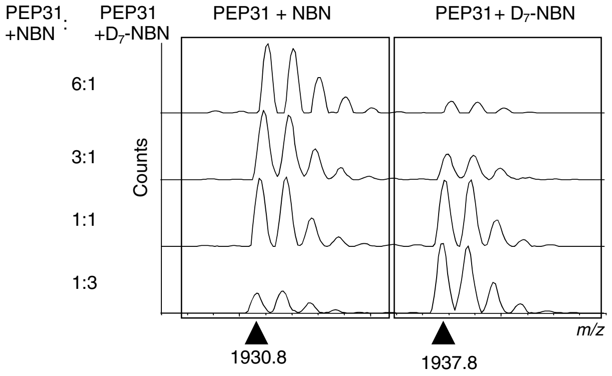


Figure 2. MALDI spectra of the sets of *N*-β-naphthyliodoacetamide-modified PEP 31 with varied concentrations.

and the following few isotopic peaks for each modified peptide, the relative ratios of the modified peptide were calculated. The relative ratios of modified PEP 31 thus obtained were plotted against their theoretical ratios as in the following graph (Fig. 3).[13]

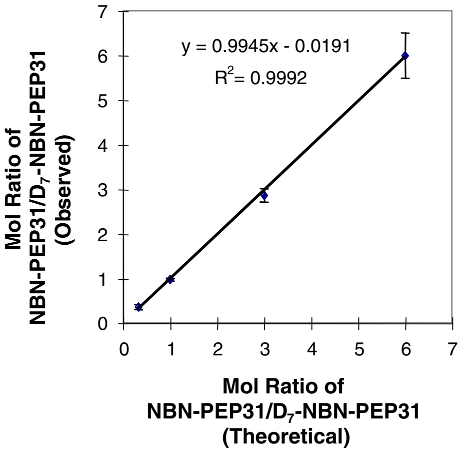


Figure 3. Quantitative analysis of PEP 31.

As can be seen in the graph, the observed relative ratios and the theoretical ratios for D_7-labeled and -unlabeled NBN-modified PEP 31 in the two solutions have an excellent correlation. From these data, it is concluded that ionization efficiencies of the D-labeled and those for -unlabeled NBN-PEP 31 are identical within the experimental errors, as we had reported for other stable isotope-labeled and -unlabeled small molecule modifiers.

In order to assess the generality of this method, we further applied this method for quantitative analysis to two other peptides, PEP 13 and 60. The following two graphs were obtained in the same manner as described above (Fig. 4).

From these figures, it is also concluded that the ionization efficiencies of the D_7-labeled and -unlabeled NBN-modified PEP 13 and 60 are the same within the experimental errors, and hence molar ratios of the peptides in two sample solutions can be measured at a high accuracy according to this method. While primary isotope effects have been reported for a combination of D-labeled and -unlabeled compounds in other quantification methods, we observed no isotope effect during the quantitative analysis of proteins/peptides in this study as well as in the methods we previously reported.

In summary, we demonstrated that a combination of D_7-labeled and -unlabeled *N*-β-naphthyliodoacetamides (NBN) enables quantitative analysis of various peptides with molecular weight 1000–2000 Da. Since these sizes of peptides are typically used in proteomics, this method is anticipated to be a useful tool for proteomic research.

Acknowledgments

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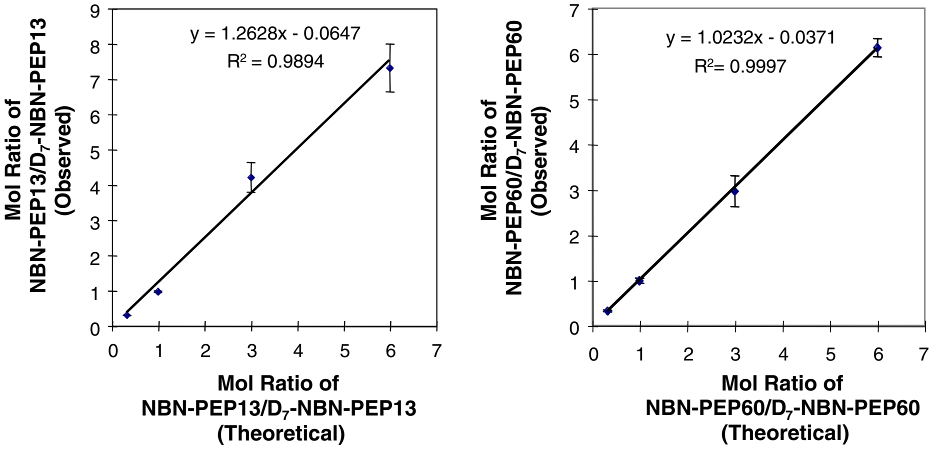

Figure 4. Quantitative analysis of PEP 13 and 60.

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10. The procedure for synthesis of *N*-β-naphthyliodoacetamides, **1**, is as follows. The D_7-labeled β-aminonaphthalene was purchased from Aldrich, Milwaukee, WI, USA. Iodoacetic acid (136 mg, 0.73 mmol) was dissolved in ethyl acetate and the mixture was cooled to 0 °C. To this solution, D_7-labeled β-aminonaphthalene (100 mg, 0.67 mmol) was added, and subsequently dicyclohexylcarbodiimide (137 mg, 0.67 mmol) dissolved in ethyl acetate (2 mL) was added dropwise with stirring. A white precipitate formed immediately after addition of the dicyclohexylcarbodiimide. The mixture was stirred at 0 °C for 30 min and at room temperature for an hour. The dicyclohexylurea was removed by Celite filtration and the filtrate was evaporated to dryness and purified by silica gel column chromatography (CH_2Cl_2-$CHCl_3$ = 1/1 then CH_2Cl_2) to afford the product (210 mg, 99% yield). The product was recrystallized from $CHCl_3$. Compound **1**: white solid, mp 181–182 °C. ^1H NMR (300 MHz, acetone-d_6) δ 9.75 (1H, br s), 3.96 (2H, s), HRMS *m/z* calcd for $C_{12}H_3D_7NOI$ $[M+H]^+$: 319.0324. Found: 319.0321. The corresponding D-unlabeled *N*-β-naphthyliodoacetamide, **2**, was synthesized in the same manner from β-aminonaphthalene. Compound **2**: white solid, mp 181–182 °C (lit. 180–182 °C)[14] ^1H NMR (300 MHz, acetone-d_6) δ 9.75 (1H, br s), 7.6–7.1 (7H, m) 3.90 (2H, s), HRMS *m/z* calcd for $C_{12}H_{10}NOI$ $[M+H]^+$: 310.9807. Found: 310.9807.
11. PEP 13 and 60 were purchased from Sigma–Aldrich, and PEP 31 was prepared at the Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center. The sequences of amino acids for PEP 13, 31, and PEP 60 are identical to those of the peptides we reported earlier (Ref. 9) and were referred to as MAT 13, 31, and 60, respectively.
12. The PEP 31 aqueous solution in 40 mM Tris buffer at a concentration of 50 μM was reduced with tributylphosphine (5 mM as the final concentration). To this solution 5 mM NBN or D_7-NBN DMSO solution was mixed at a concentration of 480 μM at around pH 9. This mixture was incubated for 30 min at 37 °C, treated with ZipTip$^{\circledR}_{\mu-C18}$ (Millipore) and mixed with α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix solution (prepared by dissolving 10 mg of CHCA in 1 mL of 50% acetonitrile/0.1% trifluoroacetic acid), and then analyzed by an AXIMA-CFR Plus (Shimadzu-Kratos) MALDI-TOF-MS in a reflector and the positive ion mode.
13. The two solutions of PEP 31 reacted with an excess amount of NBN or D_7-NBN as described in reference 12 were mixed with peptide molar ratios of 6:1, 3:1, 1:1, or 1:3 (NBN-PEP31:D_7-NBN-PEP31). These peptide mixtures were treated with ZipTip$^{\circledR}_{\mu-C18}$ (Millipore), and subjected to MALDI-TOF MS analysis as described in reference 12. Five mass spectra were collected for quantitative analysis of each ratio, and the heights of the most abundant peaks of NBN or D_7-NBN-modified peptides were used for the calculation of the relative ratios of NBN or D_7-NBN-modified peptides in the four sets of 6:1, 3:1, 1:1, or 1:3. The five observed relative ratios were averaged to be plotted on the graph with standard deviations. Quantitative analysis of two other peptides, PEP 13 and PEP 60, was carried out in the same manner.
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