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Synthesis of ^{13}C-Labeled Iodoacetanilide and Application to Quantitative Peptide Analysis by Isotope Differential Mass Spectrometry

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Abstract—^{13}C-Labeled and unlabeled iodoacetanilides have been synthesized for covalent modification of the sulfhydryl groups of cysteine residues in proteins or peptides. A combination of these reagents, coupled with mass spectrometry, is a powerful tool for quantitative analysis of peptides and hence proteins.

Quantitative analysis of proteins is an essential part of proteomics, which studies proteomes, a set of proteins expressed under certain physiological conditions. Examples of conventional methods for quantification include densitometry of the gel and counting radioactivities that are incorporated by metabolic labeling.[1–3] Recently, stable isotope labeling followed by mass spectrometry analysis has been emerging as a powerful technology for more accurate quantification of proteins. This technique is based on the assumption that the isotopically labeled molecule and its parent species behave similarly and their ionization efficiencies are the same.[4] Most common and classical approaches have relied on incorporation of specific isotope atoms such as ^{15}N, ^{18}O, or D into growing cells in the isotope-enriched media or into the digested peptides during the proteolytic cleavage of proteins. However, such methods require a long processing time and are laborious, and may not be applicable to more complex systems such as whole animals. In contrast, chemical modifications by covalent labeling on specific amino acid residues using isotope-labeled reagents followed by mass spectrometry analysis is versatile and convenient, as it can handle any protein extracts. The pioneering work by Aebersold et al. utilized deuterium-labeled isotope-coded affinity tags (ICATs).[5] Their method applies a biotinylated conjugate of iodoacetamide, which is known to be a specific sulfhydryl group modifier in cysteine residues in peptides, and its deuterated derivative. In this method, the biotinyl moiety is used to affinity-purify the resulting peptides that are carrying the modified cysteine residues, making the analysis of whole proteins feasible. If, however, each protein is separated and displayed on a two-dimensional (2-D) gel electrophoresis, the affinity purification of the cysteine-modified peptides will be unnecessary, making the entire protocol significantly simpler compared to the ICAT method. We have developed a prototype of such a method using isotope-labeled alkylmaleimides.[6] The combination of isotope-labeled and unlabeled chemical modification of specific amino acid residues followed by 2-D gel electropheresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) makes the whole process handier and more economical compared to the ICAT method.

Here, we report synthesis of another sulfhydryl-group-specific modifier, $^{13}C_6$-labeled and unlabeled iodoacetanilides (IAA), and the application of this modifier to quantitative analysis of peptides in the context of our proteomics research. We believe that the success of this work signifies success in quantification of tryptic peptides, hence proteins (Scheme 1).

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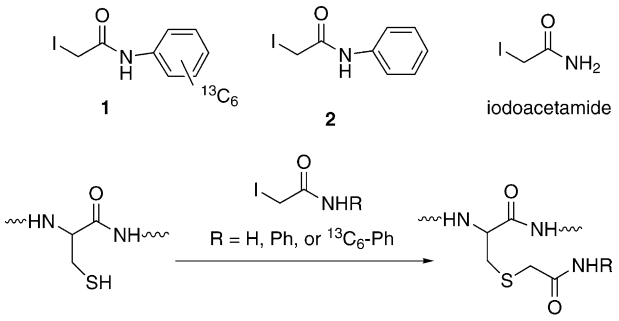
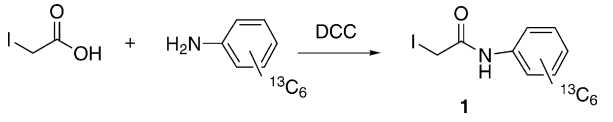


Scheme 1.

Iodoacetanilide possesses the same reaction site toward –SH as does iodoacetamide, the well-known cysteine-specific modifier. These reagents are expected to be more reactive than *N*-alkylmaleimide, they are expected not to interfere with phosphine-based reducing reagents,[7] and the difference in the molecular mass is 6 Da rather than 5 Da; therefore, the combination of $^{13}C_6$-labeled and unlabeled reagents is expected to provide sharper separation than that by *N*-alkylmaleimide modification and hence more accurate quantification.

Synthesis of iodoacetanilide from aniline and iodoacetic acid has been reported.[8] Accordingly, $^{13}C_6$-labeled iodoacetanilide was synthesized from $^{13}C_6$-labeled aniline[9] and iodoacetic acid (Scheme 2).[10]



Scheme 2.

We tested the reactivity of these reagents with three synthetic peptides that possess different amino acid sequences and molecular weights. We chose peptides that have approximately 2000 Da or less, as the molecular weights for most tryptic peptides associated with proteomics are in this range. The three peptides are MAT 31, MAT 13, and MAT 60, and the sequences, monoisotopic masses, and estimated pIs are KEEPPH HEVPESETC, 1746.75 Da, 4.5 for MAT 31, SDTCSSQKTEVSTVSSTQK, 2001.92 Da and 6.2 for MAT 13, and ALVCEQEAR, 1017.49 and 4.4 for MAT 60. Aqueous solutions of these peptides with pH values 9.0 were treated with these labeled or unlabeled iodoacetanilides, and the ion peaks of these solutions were analyzed by MALDI-TOF MS after adding a matrix.

The following charts (Fig. 1) show MAT 31 by itself and MAT 31 reacted with unlabeled iodoacetanilide or labeled iodoacetanilide.[11] The ion peaks show the monoisotopic mass for MAT 31 (1746.77 Da) and a series of isotope peaks that are several Da higher than the monoisotopic peak because of the existence of natural isotopes. The labeled and unlabeled iodoacetanilides completely reacted with this peptide within 10 min. After reacting with the unlabeled or labeled iodoacetanilide, the molecular weight of the IAA-modified or $^{13}C_6$-IAA-modified MAT 31 increased by 133 or 139 Da, respectively, showing that a combination of these modified peptides displays 6 Da difference.

In the next step, in order to examine the applicability of these reagents to quantification of peptides, several aqueous MAT 31 solutions were prepared with pH 8.5 and these solutions were individually treated with labeled or unlabeled iodoacetanilide. Then the differentially labeled MAT 31 solutions were mixed. The ratios of IAA-MAT 31 to $^{13}C_6$-IAA-MAT 31 examined were 9:1, 6:1, 3:1, 1:1, and 1:3.[12] The relative quantities of MAT 31 in each mixed solution were measured from the relative signal intensities for pairs of peptide ions modified with labeled or unlabeled iodoacetanilide.

The following charts show the MALDI MS spectrum for each mixture (Fig. 2). The areas of the monoisotopic peak and the following four isotopic peaks were summed for each modified peptide. The areas of the small 7–10th isotopic peaks of IAA-MAT 31 were subtracted from the monoisotopic peaks and the following high isotopic peaks of $^{13}C_6$-IAA-MAT 31. The relative ratios of MAT 31 in the five sets of the two solutions obtained in these experiments were plotted against their theoretical ratios in the following graph (Fig. 3).

The graph obtained in this way indicates that the observed ratios and the theoretical ratios for ^{13}C-labeled

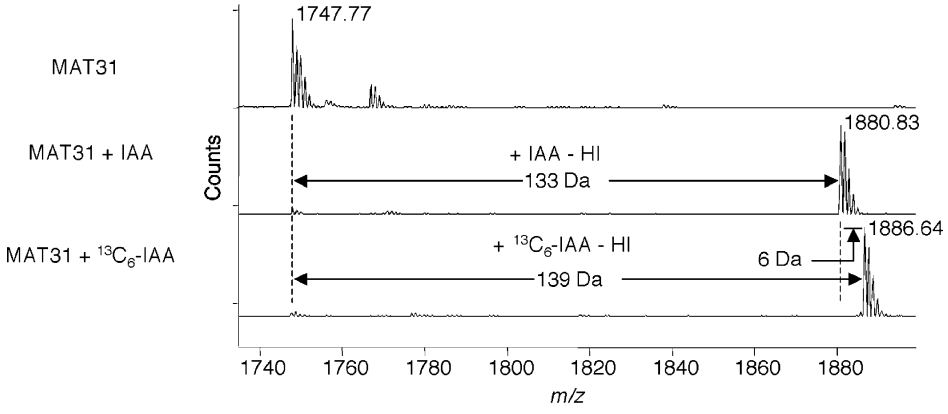


Figure 1. MALDI-TOF MS spectra of MAT 31 andiodoacetanilide-modified MAT 31.

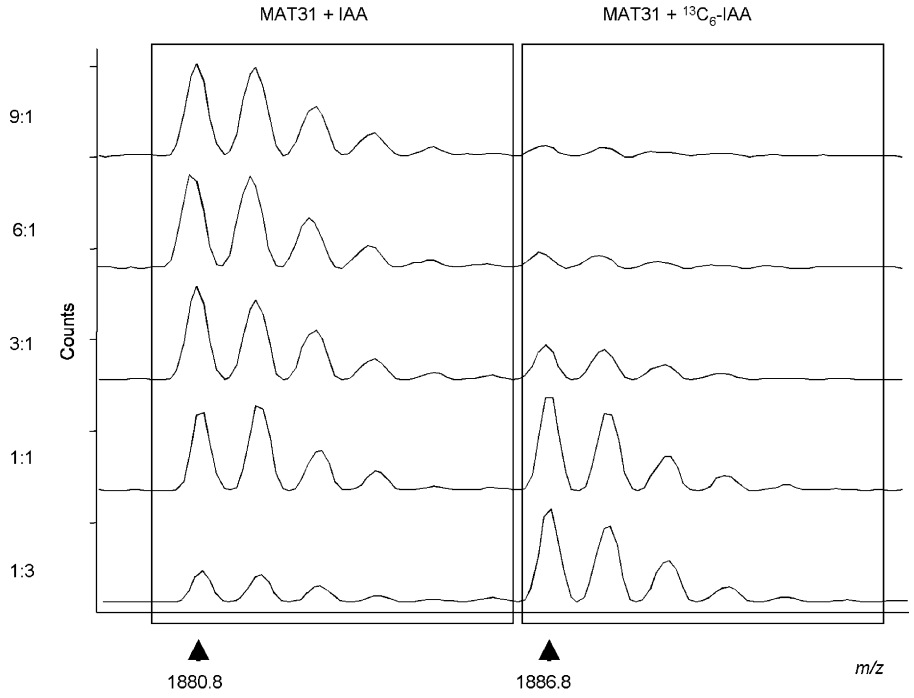


Figure 2. MALDI-TOF MS spectra of IAA-modified/$^{13}C_6$-IAA-modified MAT 31 solutions with varied concentrations.

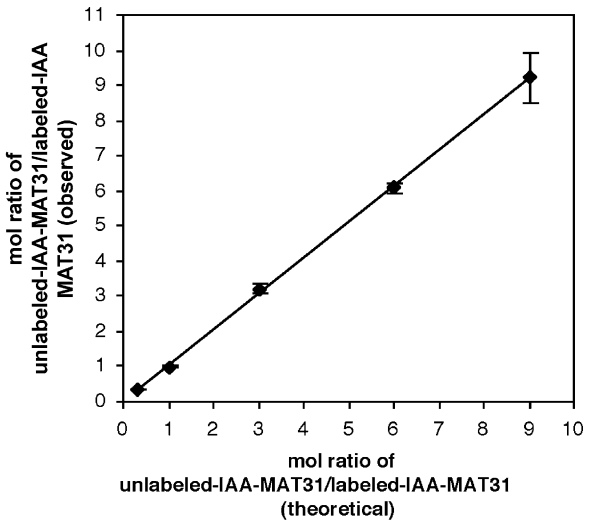


Figure 3. Quantitative analysis of MAT 31.

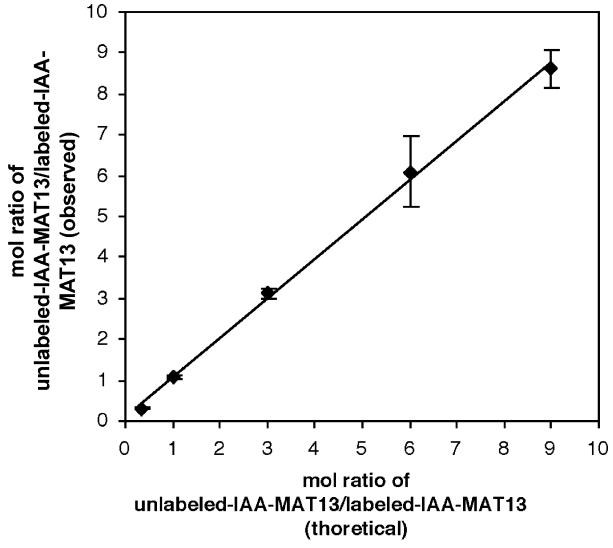


Figure 4. Quantitative analysis of MAT 13.

and unlabeled IAA-modified MAT 31 in the two solutions show excellent correlation ($r^2 = 0.9995$, inclination = 1.0223). Therefore, as had been reported for d-labeled and unlabeled *N*-alkylmaleimide modified peptides earlier,[6a] we conclude that the ionization efficiencies of the ^{13}C-labeled IAA-MAT 31 and those for unlabeled IAA-MAT 31 are the same within the experimental error.

We next applied this method to other peptides, MAT 13 and MAT 60, in order to demonstrate the generalizability of this method. The results are shown in Figures 4 and 5.

As can be seen from these figures, the theoretical and observed relative ratios for ^{13}C-labeled and unlabeled IAA-modified MAT 13 and MAT 60 are also well correlated ($r^2 = 0.9980$ and 0.9995, inclination = 0.9615 and 0.9789 respectively). These results also indicate that the ionization efficiencies of the ^{13}C-labeled and unlabeled IAA-modified MAT 13 and MAT 60 are the same within the experimental error. We safely conclude that the relative molar ratio of the peptide in two different sample solutions can be measured at a high accuracy by this method.

In summary, we developed a new method for quantitative analysis of peptides for the purpose of quantification of proteins. We synthesized $^{13}C_6$-labeled iodoacetanilide and have shown, as a model system for quantification of proteins, that a combination of $^{13}C_6$-labeled and unlabeled iodoacetanilide enables quantitative analysis

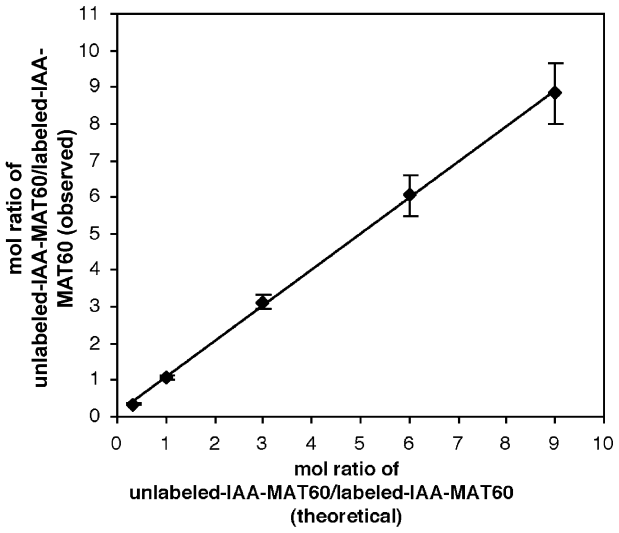


Figure 5. Quantitative analysis of MAT 60.

of a variety of peptides with the molecular weight 1.0–2.0 kDa.

Acknowledgements

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References and Notes

1. Matsumoto, H.; Kurien, B.; Takagi, Y.; Kahn, E. S.; Kinumi, T.; Komori, N.; Yamada, T.; Hayashi, F.; Isono, K.; Pak, W. L.; Jackson, K. W.; Tobin, S. L. *Neuron* **1994**, *12*, 997.
2. Komori, N.; Usukura, J.; Matsumoto, H. *J. Cell Sci.* **1992**, *102*, 191.
3. Gygi, S. P.; Rochon, Y.; Franza, B. R.; Aebersold, R. *Mol. Cell. Biol.* **1999**, *19*, 1720.
4. (a) For example Oda, Y.; Huang, K.; Cross, F. R.; Cowburn, D.; Chait, B. T. *Proc. Natl. Acad. Sci, U.S.A.* **1999**, *96*, 6591. (b) Veenstra, T. V.; Martinovic, S.; Anderson, G.; Pasa-Tolic, L.; Smith, R. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 78. (c) Pasa-Tolic, L.; Jensen, P. K.; Anderson, G. A.; Lipton, M. S.; Peden, K. K.; Martinovic, S.; Tolic, N.; Bruce, J. E.; Smith, R. D. *J. Am. Chem. Soc.* **1999**, *121*, 7949. (d) Mirgorodskaya, O. A.; Kozmin, Y. P.; Titov, M. I.; Körner, R.; Sönksen, C. P.; Roepstorff, P. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1226. (e) Chen, X.; Smith, L. M.; Bradbury, E. M. *Anal. Chem.* **2000**, *72*, 1134. (f) Geng, M.; Ji, J.; Regnier, F. E. *J. Chromatgr. A* **2000**, *870*, 295. (g) Wang, S.; Regnier, F. E. *J. Chromatgr. A* **2001**, *924*, 345. (h) Sechi, S. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1416. (i) Smith, R. D.; Anderson, G. A.; Lipton, M. S.; Pasa-Tolic, L.; Shen, Y.; Conrads, T. P.; Veenstra, T. D.; Udseth, H. R. *Proteomics* **2002**, *2*, 513. (j) Zhang, R.; Sioma, C. S.; Wang, S.; Regnier, F. E. *Anal. Chem.* **2001**, *73*, 5142. (k) Yao, X.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenseleau, C. *Anal. Chem.* **2001**, *73*, 2836. (l) Gu, S.; Pan, S.; Bradbury, E. M.; Chen, X. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 1.
5. Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994.
6. (a) Niwayama, S.; Kurono, S.; Matsumoto, H. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2257. (b) Kurono, S.; Niwayama, S.; Matsumoro, H. In American Society for Mass Spectrometry Conference, Mass Spectrometry and Allied Topics, Orlando, FL, 2002. (c) Kurono, S.; Niwayama, S.; Matsumoro, H., 91st Annual Technical Meeting of the Oklahoma Academy of Science, Oklahoma City, OK, 2002.
7. Getz, E. B.; Xiao, M.; Chakrabarty, T.; Cooke, R.; Selvin, P. R. *Anal. Biochem.* **1999**, *273*, 73.
8. Benesch, R.; Edalji, R.; Benesch, R. E. *J. Biochem. Biophys. Methods* **1979**, *1*, 129.
9. The $^{13}C_6$-labeled aniline was purchased from Aldrich, Milwaukee, WI, USA.
10. The procedure for synthesis of **1** is as follows. Iodoacetic acid (470 mg, 2.53 mmol) was dissolved in ethyl acetate and the mixture was cooled to 0 °C. To this solution, $^{13}C_6$-labeled aniline (250 mg, 2.53 mmol) was added, and subsequently a solution of dicyclohexylcarbodiimide (520 mg, 2.53 mmol) was added slowly with stirring. A white precipitate formed immediately after addition of the dicyclohexylcarbodiimide. The mixture was stirred at 0 °C for 30 min and at rt 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 (638 mg, 95% yield). The product was recrystallized from $CHCl_3$ (a yellowish solid, mp 140–141 °C). ^{1}H NMR (300MHz, acetone-d_6) δ 9.51 (1H, br. s), 7.9–6.7 (5H, m), 3.88 (2H, s), ^{13}C NMR (75 MHz, acetone-d_6) 167.0, 140.0, 129.6, 124.5, 119.9, 0.71, HRMS *m*/*z* calcd for $^{12}C_2{}^{13}C_6H_8INO$ $(M+H)^+$: 267.9933, found: 267.9933.
11. The MAT31 aqueous solution (0.6 mM, 2 μL), 2 μL of 50 mM TRIZMA® Pre-set crystals pH 9.0 (Sigma Chemical Co., St Louis, MO, USA) and 2 μL of 10 mM IAA or $^{13}C_6$-IAA DMSO solution were mixed to approximately pH 8.5. This mixture was incubated for 10 min at room temperature. Then, 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (prepared by dissolving 10 mg of CHCA in 1 mL of 50% acetonitrile/0.1% trifluoroacetic acid) was added to 1 μL of the sample solution, and 1 μL of this mixture was analyzed by MALDI-TOF MS after evaporation of the solvent in vacuo. The MALDI spectra were obtained from a Voyager Elite BioSpectrometry Research Station, equipped with a delayed extraction option (Applied Biosystems, Foster City, CA, USA) operated at accelerating voltage, 20 kV; grid voltage, 74–75%; guide wire voltage, 0.05%; pulse delay time, 100–225 ns; vertical scale, 3000 mV; and vertical offset, 3%. A pulsed nitrogen laser operating at 337 nm was used as a desorption/ionization source. Mass spectrometry was performed in a reflector with positive ion detection. The ion signal was recorded using a 500 MHz transient digitizer. The data were analyzed using GRAMS/386 (Galactic Industries Corp., Salem, NH, USA).
12. The aqueous solution of MAT 31 (0.6 mM) was used for quantitative analysis. The solution was incubated with an excess amount of unlabeled or $^{13}C_6$-labeled IAA DMSO solution for 10 min at room temperature, and the two solutions were mixed with peptide molar ratios of 9:1, 6:1, 3:1, 1:1 or 1:3, and were subjected to MALDI-TOF MS analysis as described in ref 11. Five data points were collected for analysis of each ratio, and these values were averaged to be plotted on the graph. Aqueous solutions that have the same concentration (0.6 mM) were used for quantitative analysis of two other peptides, MAT 13 and 30, as well.