Bioorganic & Medicinal Chemistry Letters 21 (2011) 4629–4632

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of d-labeled and unlabeled benzoyloxysuccinimides and application to quantitative analysis of peptides and a protein by isotope differential mass spectrometry

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ARTICLE INFO

Article history:
Received 10 February 2011
Revised 21 May 2011
Accepted 23 May 2011
Available online 30 May 2011

Keywords:
Amino group modifier
Stable isotope-labeling
Proteomics
Mass spectrometry

ABSTRACT

Benzoyloxysuccinimide and its d_5-labeled version, which react with amino groups in the N-termini and lysine side chains in proteins, were synthesized and applied to quantitative analysis of peptides and a commercially available protein in combination with a MALDI mass spectrometer.

Proteomics is becoming an important research area in the study of proteomes, a set of proteins expressed under specific physiological conditions, for example, the examination of comprehensive protein expression patterns expressed under different external stimuli. In particular, quantitative analysis of relative amounts of expressed proteins and identification of the proteins in combination with mass spectrometry are central in proteomics studies. Many research groups have reported methodologies for quantitative analysis of proteins or peptides in this context, in particular, in the incorporation of stable isotopes. These methods include metabolic labeling,[1] enzyme labeling,[2] and chemical modifications.[3] Among these methods, chemical modification is expected to be applicable to any protein samples, including mammalian proteins. Most of these methods of chemical modification rely on liquid chromatography (LC) for separation and purification of the proteins and on subsequent mass spectrometry analysis for identification and quantitative analysis of the peptides, exemplified by the ICAT method and its derivatives.

However, due to the problems associated with LC separation, such as isotope effects, at our laboratory we have been developing a practical methodology that makes use of electrophoresis rather than LC, as well as small organic molecules that specifically react with certain amino acid residues and their stable isotope-labeled versions. Small organic molecules are expected to have greater solubility in sample solutions. Furthermore, we have thus far not observed isotope effects in our methods. We have reported four kinds of modifiers and their d- or ^{13}C-labeled versions that specifically react with the sulfhydryl group of cysteine residues, and have demonstrated that all these reagents can successfully measure the relative amounts of peptides or proteins in combination with electrophoresis and soft ionization mass spectrometry such as MALDI or ESI.[4–9] However, these methods rely on the existence of cysteine residues. Since some proteins do not contain cystein residues, there is a need to develop a method for noncysteine-containing proteins. Here we report synthesis of a new modifier that reacts with the amino group and its d_5-labeled version and their application to quantitative analysis of peptides and a protein in combination with MALDI TOF MS.

N-hydroxysucciniminyl esters are among the most commonly used compounds for modification of amino groups in peptides (Scheme 1). Münchbach et al. also reported 1-(nicotinoyloxy) succinimide and its deuterated versions for quantitative analysis of proteins in combination with electrophoresis,[10] and later other groups also reported derivatives of (nicotinoyloxy)succinimide.[11] Although these nicotinoylating reagents are also small organic molecules, the difference between the isotope-labeled and unlabeled versions are 4 Da or less. In our case, we can introduce a 5 Da difference, which potentially allows more accurate quantitative analysis. In addition, benzoic acid and deuterated benzoic acid are far less expensive than nicotinic acid and deuterated nicotinic acid, thus making our reagents more accessible.

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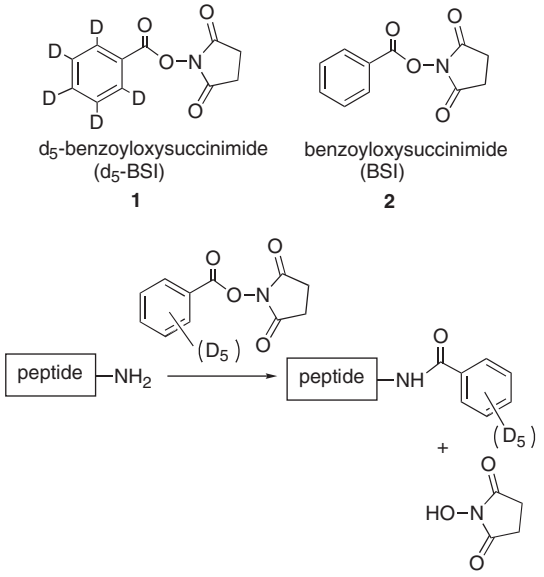
doi:10.1016/j.bmcl.2011.05.090



Scheme 1. D_5-benzoyloxysuccinimide, benzoyloxysuccinimide, and their reactions with an amino group.

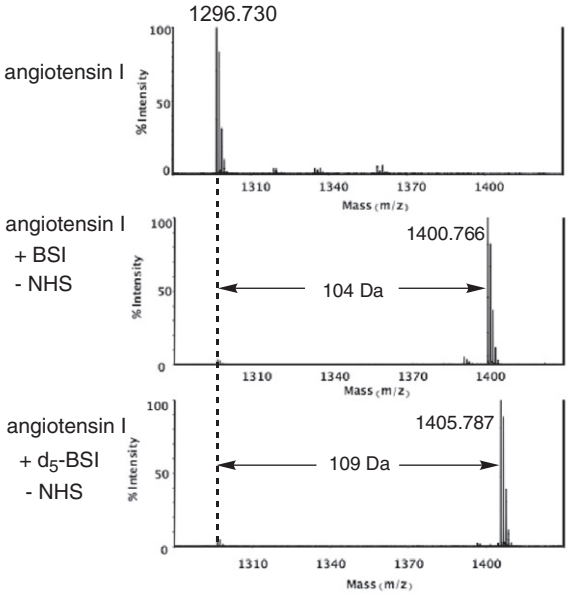
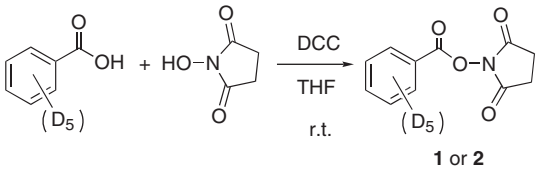


Figure 1. MALDI MS spectra of angiotensin I and benzoyloxysuccinimide(BSI)-modified angiotensin I.

D_5-labeled benzoyloxysuccinimide (BSI), **1**, and unlabeled benzoyloxysuccinimide (BSI), **2**, were synthesized by a coupling reaction of benzoic acid and *N*-hydroxysuccinimide (NHS) by modification of a known procedure (Scheme 2).[12,13]



Scheme 2. Synthesis of d_5-benzoyloxysuccinimide and benzoyloxysuccinimide.

In order to test the applicability of the combination of these reagents to quantitative analysis of peptides, solutions of two model peptides, angiotensin I (human) and ACTH 18-39, were prepared, and the amino modifiers, **1** and **2**, were reacted with these peptides. The amino acid sequences and molecular weights of these peptides are DRVYIHPFHL, 1296.7 Da, and RPVKVYPNGAEDESAEAFPLEF, 2465.6 Da, respectively.[14] Various aqueous solutions of these model peptides with pH 8.5 were reacted with d_5-labeled or unlabeled reagents and the relative molar ratios were quantitatively analyzed by MALDI TOF MS.

The following charts show a peptide, angiotensin I itself, and angiotensin I reacted with the d-unlabeled or d_5-labeled modifier (Fig. 1). Due to the natural isotopes, the ion peaks show the monoisotopic mass as well as a series of isotopic peaks several Daltons greater than it. As this peptide does not contain a lysine residue, this reaction increased 104 Da as a result of the modification of the terminal amino group, although the reagents can react with both the N-terminal amino group and the ε-amino group of the lysine at this pH. The d_5-labeled version added 109 Da. Although reactions with other amino acid residues such as tyrosin are commonly reported for other *N*-hydroxysuccinimide, these esters were hydrolyzed by addition of hydroxylamine. In addition, reactions with these reagents did not diminish the ionization efficiencies of the peptide, even though the benzoyl group from these modifiers does not contain a nitrogen atom.

We next examined the general applicability of these reagents to quantitative analysis of peptides.[15] Several aqueous solutions of the peptide, angiotensin I, were prepared with a pH of 8.5, and these aqueous solutions were treated with d_5-labeled or unlabeled benzoyloxysuccinimide, **1** or **2**, and an aqueous hydroxylamine solution was added to this mixture. The differentially labeled angiotensin I solutions were then mixed in the ratio of 0.5, 1, 2, 4, 6, and 9 (d_0/d_5). The relative quantities of this peptide in each mixed solution were measured from the relative intensities of the signals corresponding to monoisotopic peaks for the peptide ions modified with d_5-labeled or unlabeled benzoyloxysuccinimide. The relative ratios of BSI-modified and d_5-BSI-modified peptides observed in this way were plotted against the theoretical ratios as in the graph. An excellent correlation between the theoretical ratios and the observed ratios is shown here (Fig. 2a).

In order to further examine the general applicability of these reagents, we applied this method to quantitative analysis of another peptide, ACTH 18–39, in the same way, and plotted the observed relative ratios against the theoretical relative ratios. This peptide has one lysine residue which has an ε-amino group, and therefore the reactions with these modifiers increased 208 and 218 Da, respectively, but the results were similar to those of angiotensin I. As can be seen in the following graphs, there is excellent correlation between the theoretical ratios and the observed ratios (Fig. 2b).

Next we applied the combination of these reagents to quantitative analysis of a commercially available protein, bovine serum albumin (BSA), after the cysteine residues were blocked with a cysteine modifier, iodoacetamide.[16] Several solutions with differing amounts of BSA were prepared and loaded in each well of 1D electrophoresis. The proteins were excised and in-gel digested, and the extracted peptide solutions were modified with the d_5-labeled or unlabeled modifier. Six peptides were identified from the mixtures in this way. The d_5-labeled and unlabeled peptides were mixed in various ratios and quantitatively analyzed in the same manner as the peptides above, and the averages of the observed ratios were plotted against theoretical ratios. As can be seen from the graph below, good correlation between the theoretical ratios and observed ratios was obtained in accordance with the results obtained from the two model peptides (Fig. 3).

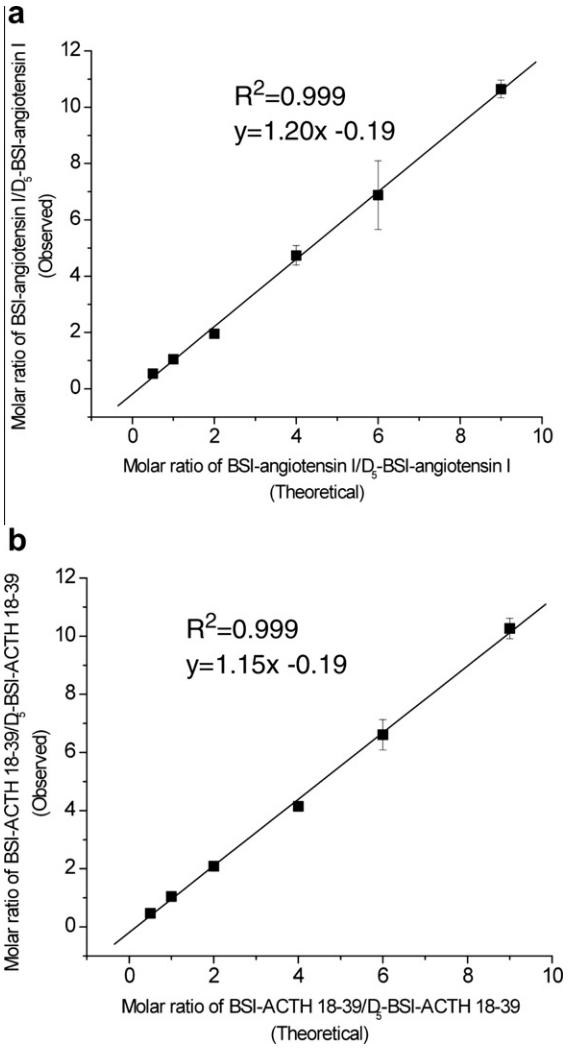


Figure 2. Quantitative analysis of two model peptides. (a) Quantitative analysis of angiotensin I. (b) Quantitative analysis of ACTH 18-39.

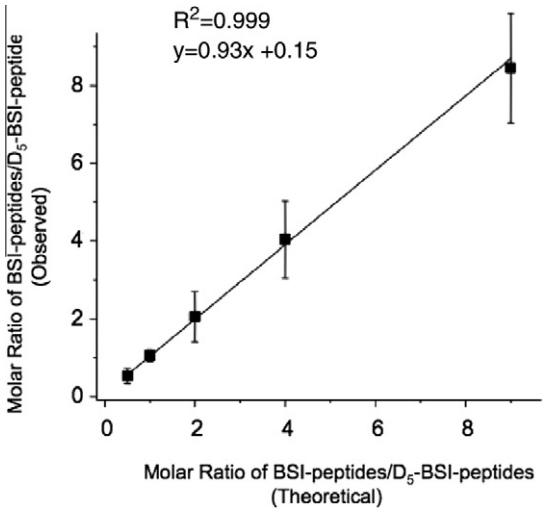


Figure 3. Quantitative analysis of bovine serum albumin (BSA).

In summary, we synthesized benzoyloxysuccinimide and its deuterated version, which react with amino groups in peptides, and demonstrated that the combination of these reagents enables quantitative analysis of peptides and proteins with high accuracy. As with other reagents we reported earlier that specifically modify cysteine residues, we did not observe any isotope effects. Although a decrease in the ionization efficiencies as a result of blocking the amino groups may be anticipated, we did not observe a decrease in ionization efficiencies in the model peptides or the commercial protein reported here. While addition of hydroxylamine for removal of excessive reactions with hydroxyl groups is required for these modifiers, they do not require the existence of particular amino acid residues and are thus expected to be applicable to quantitative analysis of a wider variety of proteins. These modifiers are prepared relatively inexpensively from readily available sources. We are developing additional kinds of amino-group modifiers and the results will be reported in due course.

Acknowledgments

This work is supported by the Texas Tech University-Texas Tech University Health Sciences Center Joint Initiative Grant. We also thank the Center for Biotechnology and Genomics, Texas Tech University for allowing us to use the research facilities.

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13. Synthesis of the benzoyloxysuccinimide (BSI), **2**, is as follows: benzoic acid (0.353 g, 2.89 mmol) was dissolved in 50 mL of dry THF, and *N*-

hydroxysuccinimide (0.40 g, 3.47 mmol) and DCC (0.776 g, 3.76 mmol) were added. The mixture was stirred at room temperature for 2 days. The soluble portion was separated from the viscous residue and the solvent was removed under reduced pressure. The residue was dissolved in $CHCl_3$ (50 mL) and washed with brine (30 mL). After the $CHCl_3$ layer was separated and dried over anhydrous Na_2SO_4, the solvent was removed by evaporation to dryness. The crude product was purified by column chromatography with 40% ethyl acetate in hexane to afford a white solid (0.586 g, 93%). The white solid was recrystallized from 2-propanol.
mp 132–133 °C (lit. 134–136 °C).[17] ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.88 (4H, s), 7.49–8.13 (4H, m), Anal. Calcd for $C_{11}H_9NO_4$: C, 60.29; H, 4.14; N, 6.39. Found: C, 60.52; H,4.00; N, 6.40. MS (MALDI TOF) *m/z* calcd for $C_{11}H_9NO_4Na$ $(M+Na)^+$ 242.0, found: 242.0.
The corresponding d_5-labeled version, **1**, was synthesized in the same manner from d_5-benzoic acid (purchased from Cambridge Isotope Laboratories, Inc.) in 91% yield. mp 132–133 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.88 (4H, s), MS (MALDI TOF) *m/z* calcd for $C_{11}H_4D_5NO_4Na$ $(M+Na)^+$ 247.1, found: 247.1.

14. These peptides were purchased from Sigma–Aldrich.
15. *The typical procedure is as follows:* the stock solutions of the two peptides were prepared at a concentration of 0.19 mM in triethylammonium bicarbonate (TEAB) buffer (50 mM, pH 8.5). The stock solutions of the d_5-labeled or unlabeled BSI were prepared at a concentration of 20 mM in 50% acetonitrile/water solution. The reaction was started by mixing 2 μL of the peptide stock solution with 2 μL of BSI or d_5-BSI stock solution, and left at room temperature for 1 h. Another 1 μL of BSI was added to this mixture and left for one additional hour to ensure completion of the reaction. To this mixture was added 3 μL of hydroxylamine (1.4 M, pH 10–12) and left for 10 min to hydrolyze additional esters potentially formed on other amino acids. The sample mixture was analyzed by MALDI mass spectrometer every 30 min in order to examine the completion of the reaction. The BSI-peptide and d_5-BSI-peptide solutions prepared in this way were mixed in the molar ratios of BSI/d_5-BSI = 0.5, 1, 2, 4, 6, and 9. These mixtures were diluted with 50% acetonitrile, 0.1% TFA and subjected to MALDI MS analysis by MALDI TOF/TOF 4800 plus™ (Applied Biosystems). The MS spectra were acquired automatically in the positive mode and a total of 1000 shots were accumulated per spectrum. The mass range was selected between 600 and 4000 *m/z*. Five data points were collected for each ratio, and the *S/N* values of the monoisotopic peaks of BSI or d_5-BSI-modified peptides were used for calculation of the relative ratios. The averages of these data were plotted on the graph.
16. The procedure for quantitative analysis of bovine serum albumin (BSA) is as follows: the stock solutions of **1** and **2** were prepared at a concentration of 100 mM in 75% acetonitrile/water. The stock solutions of BSA were prepared at a concentration of 1.25 mg/mL in a sample buffer (triethylammonium bicarbonate (TEAB), 50 mM, pH 8.5). This stock solution (50 μL) was mixed with 20 μL of DTT (10 mM) solution, heated to 100 °C, and cooled to room temperature. To this mixture was added 20 μL of iodoacetamide (IA) solution (250 mM). The mixture was left for two additional hours in darkness for completion of the alkylation by IA, and was then subjected to 1D electrophoresis. Different amounts (1.5, 3, 6, 12, and 27 pmol) of this BSA solution were loaded in each well, and the purification of the protein in this 1D electrophoresis was performed for 1 h at 120 V. The gel was stained with Coomassie staining solution, and the protein bands were excised. After the decoloration of the excised bands, protein digestion was performed overnight by addition of 50 ng of Trypsin in TEAB buffer (50 mM, pH 8.5). After extraction of the peptides, the samples were dried and mixed with 20 μL of TEAB buffer. The samples prepared this way (1.5, 3, 6, 12, and 27 pmol) were modified with 10 μL of d-unlabeled benzoyloxysuccinimide, **2**, and another 3 pmol sample was separately modified with d_5-benzoyloxysuccinimide, **1**, and 10 μL of acetonitrile was added to each sample in order to avoid potential precipitation. After 1 h, an additional 5 μL of the solution of **1** or **2** was added, and the mixture was left for two additional hours at room temperature. For removal of the esters derived from esterification reactions, 10 μL of *N*-hydroxylamine (1.4 M, pH 11–12) was added to each mixture. The samples were dried, re-suspended in 0.5% TFA and 5% acetonitrile for purification with the use of PepClean C-18 Spin Colums, again dried, and dissolved in 20 μL of 50% acetonitrile and 0.1% TFA for further analysis by MALDI TOF. For quantitative analysis, 2 μL of each sample solution modified with **2** was mixed with 2 μL of the sample solution modified with **1**, and 0.5 μL of this mixture was spotted on the MALDI-plate followed by mixing with 0.5 μL of a matrix solution consisting of 5 mg/mL of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA).
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