Bioorganic & Medicinal Chemistry Letters 26 (2016) 5073–5077

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis of d-labeled and unlabeled ethyl succinic anhydrides and application to quantitative analysis of peptides by isotope differential mass spectrometry

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ARTICLE INFO

Article history:
Received 1 August 2016
Revised 19 August 2016
Accepted 24 August 2016
Available online 24 August 2016

Keywords:
Proteomics
Quantitative analysis
Stable isotope labeling
Amino-group modifiers
Soft ionization mass spectrometry

ABSTRACT

Ethyl succinic anhydride and its d_5-labeled version have been synthesized and applied to quantitative analysis of peptides in combination with MALDI or ESI mass spectrometry. These modifiers react with amino groups in the N-termini and lysine side chains in proteins, and therefore the combination of these modifiers was shown to be a useful tool for quantification of peptides and hence for proteomics research.

Proteomics comprehensively studies a set of proteins expressed under certain external stimuli, and is increasingly becoming an important research area for study of biological samples especially for comparison of protein expression patterns in different physiological conditions. For this purpose, quantitative analysis of proteins and hence peptides derived from enzymatic digestion of proteins, in particular in combination with mass spectrometry, constitutes one of the essential parts of proteomics studies. Many studies for quantitative analysis of proteins by various approaches have therefore been reported. One of the earliest approaches is metabolic labeling.[1] In this approach, cells are cultured in isotope-enriched or in normal media and the relative abundance of specific proteins is quantitatively analyzed from the mass spectra of each species. However, these approaches typically require a long time for cell culturing, and they are not applicable to human proteins. Instead, chemical modifications by covalent tagging of isotope-labeled and unlabeled modifiers on specific functional groups of amino acid residues are expected to be applicable to any protein sample. Therefore, many studies have been developed for quantitative analysis of proteins by covalent labeling of specific amino acid residues in protein samples[2] since the pioneering work by the isotope-coded affinity tag (ICAT) method[3] was reported.

In this context, we have also been developing our methodology with the use of combinations of isotope-labeled and unlabeled small organic compounds that modify specific functional groups of amino acid residues followed by mass spectrometric analysis. The combinations we reported are d_5-labeled and unlabeled *N*-ethyl maleimides,[4–8] $^{13}C_6$ or $^{13}C_7$-labeled and unlabeled iodoacetanilides,[5–10] d_7-labeled and unlabeled *N*-β-naphthyliodoacetamides,[11] and d_5-labeled and unlabeled benzoyloxysuccinimides.[12] We have demonstrated that these modifiers enable quantitative analysis of peptides and proteins, either by electrophoresis or liquid chromatography followed by MALDI or ESI mass spectrometry. However, most of these modifiers react specifically with the sulfhydryl group of the cysteine residues, despite the fact that there are some proteins that do not contain cysteine residues. Although the benzoyloxysuccinimides[12] react with amino groups of N-termini and lysine, they have rather

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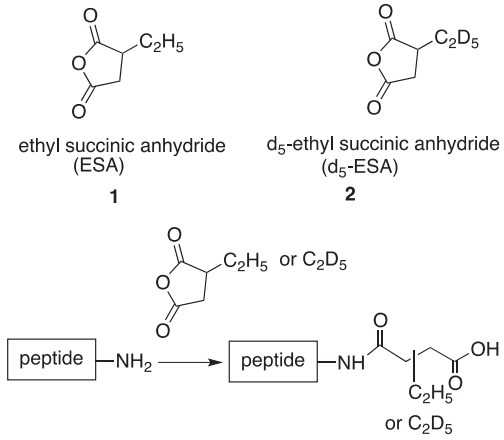
http://dx.doi.org/10.1016/j.bmcl.2016.08.079
0960-894X/

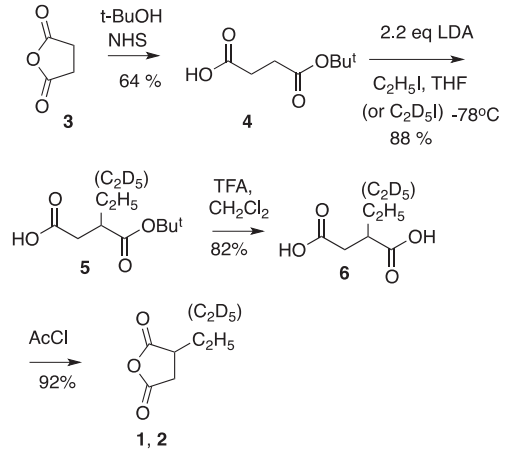
limited solubility in aqueous media, and sometimes undesirable side reactions occur, including reactions with other amino acid residues and methionine oxidations. Therefore, we have been developing additional kinds of modifiers that react with amino groups.

Here we report synthesis of ethyl succinic anhydride (ESA), **1**, and its d_5-labeled version, **2**, and their application to quantitative analysis of peptides in combination with MALDI or ESI mass spectrometry. Succinic anhydride is known to react with amino groups of N-termini of peptides and lysine, and the combination of succinic anhydride and its d_4- or $^{13}C_4$-labeled version has been applied to proteomics research.[13] These combinations introduce 4 Da difference between the isotope-labeled and unlabeled succinic anhydride, however, we had previously found that in our proteomics studies, having at least 5 Da difference facilitates clear separation of the modified peptides.[4] Therefore, we synthesized ethyl-substituted succinic anhydride and its d_5-labeled version, allowing introduction of five deuterium atoms (Scheme 1).



Scheme 1. Ethyl succinic anhydride (ESA), **1**, d_5-ethyl succinic anhydride (d_5-ESA), **2**, and their reactions with an amino group.

Synthesis of ethyl succinic anhydride (ESA), **1**, and its d_5-labeled version, **2**, was performed as in the following scheme (Scheme 2).[14] We initially intended to synthesize them by ethylation of monomethyl succinate prepared by selective monohydrolysis of dimethyl succinate[15] followed by dehydration, but the ethylation yielded greater percentages of the corresponding diacid than the alkylated product, probably because of the small ester functional group. Therefore, we introduced a bulkier and hence more hydrophobic *tert*-butyl group. The preparation of *tert*-butyl succinate, **4**, was performed by the reaction of succinic anhydride, **3**, and t-BuOH in the presence of *N*-hydroxysuccinimide (NHS).[16] This mono *tert*-butyl succinate was ethylated with 2 equiv of LDA and ethyl iodide, followed by the removal of the *tert*-butyl group, and subsequent cyclization to produce ethyl succinic anhydride, **1**, in a high yield. The corresponding d_5-labeled ethyl succinic anhydride, **2**, was obtained by substituting the ethyl iodide with C_2D_5I.



Scheme 2. Synthesis of **1** and **2**.

We tested the applicability of this combination to quantitative analysis of three peptides, substance P, angiotensin, and neurotensin, utilizing both MALDI and ESI mass spectrometry.[17] The amino acid sequences and molecular weights of these peptides are RPKPQQFFGLM-NH_2, DRVYIHPF, and pELYENKPRRPYIL; 1347.8, 1046.6, and 1672.9 Da ($[M+H]^+$), respectively.

The following are ESI MS spectrum charts showing one of the above peptides, angiotensin itself, and angiotensin reacted with d-unlabeled or d_5-labeled ethyl succinic anhydride at pH 8.5 (Fig. 1). As this peptide was identified from the doubly charged ion $[M+2H]^{2+}$, the monoisotopic peak appeared as half of the molecular ion (523.7 Da). Because of the existence of natural isotopes, the ion peaks show the monoisotopic and many isotopic peaks that are several Dalton greater than it. Since this peptide does not contain a lysine residue, the modification of this peptide with ethyl succinic anhydride (ESA) having the molecular weight of 128 Da added 64 Da (=128/2) by the tagging of the terminal amino group at this pH. The d_5-labeled ethyl succinic anhydride added 66.5 Da (=133/2) due to its reactions with this amino group. Although modification of the amino group may be thought to lead to a decrease in ionization efficiencies, we did not observe such a decrease of ionization efficiencies after the modification, even though the ethyl succinic anhydride (ESA) does not contain a nitrogen atom. Essentially the same MS spectra were obtained by MALDI mass spectrometry as a result of the reaction with these modifiers (data not shown).

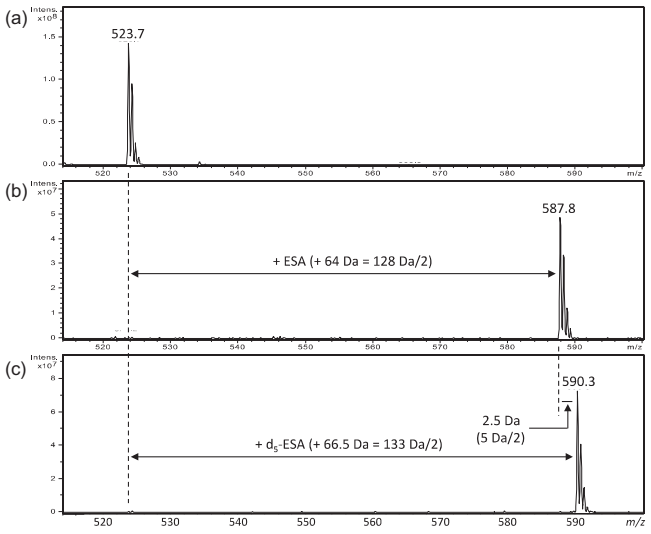


Figure 1. ESI mass spectra of (a) angiotensin, (b) angiotensin-ESA, and (c) angiotensin-d_5-ESA.

We next applied these modifiers to quantitative analysis of the three peptides for assessment of general applicability to quantitative analysis. Solutions of the peptides were prepared at pH 8.5 and were reacted with d-unlabeled or d_5-labeled ethyl