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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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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.

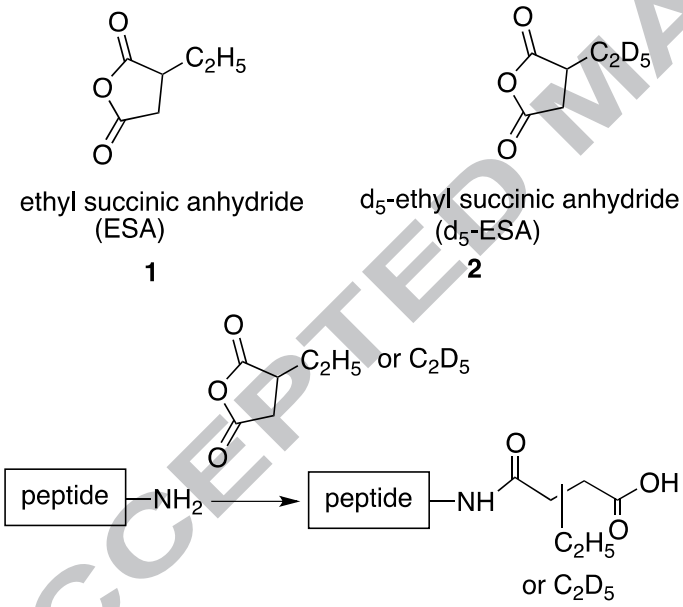
Keywords: Proteomics, Quantitative analysis, Stable isotope labeling, Amino-group modifiers, Soft ionization mass spectrometry

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,[4,5,6,7,8] $^{13}C_6$ or $^{13}C_7$-labeled and unlabeled iodoacetanilides,[5,6,7,8,9,10] 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 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.

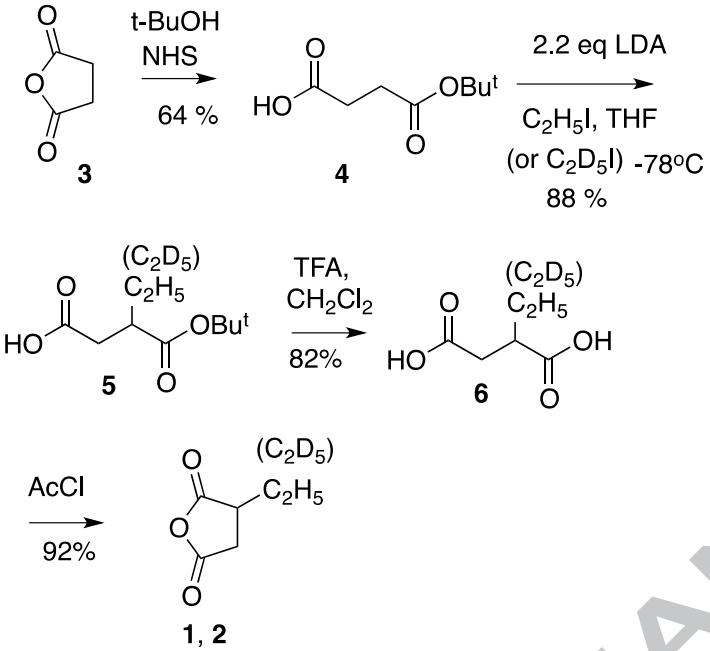
Here we report synthesis of ethyl succinic anhydride (ESA), **1**, and its d_5-labeled version, **2**, and their application to quantitative analysis of peptides in combination with MALDI or ESI mass spectrometry. Succinic anhydride is known to react with amino groups of *N*-termini of peptides and lysine, 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 equivalents 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 anhydide, **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 (Figure 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).

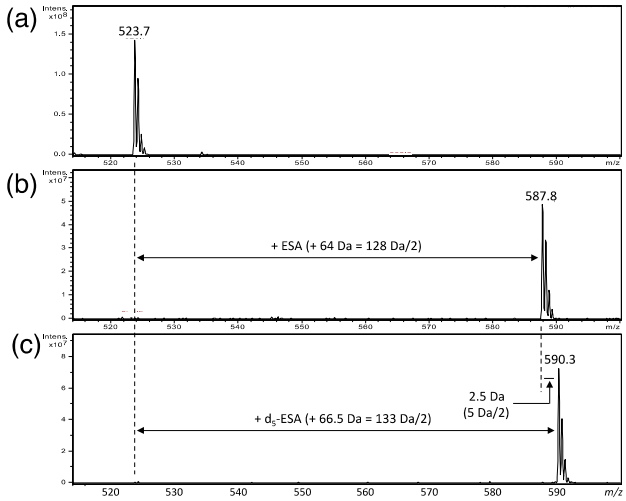
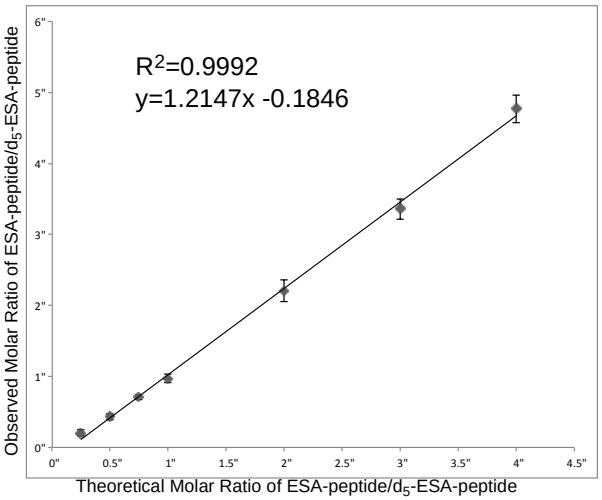
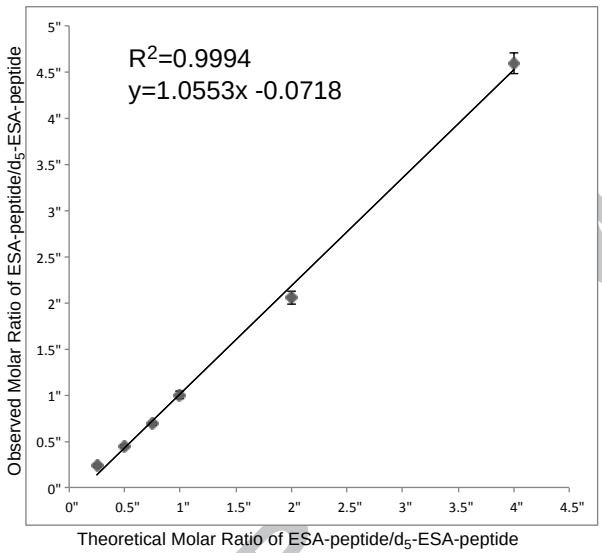


Figure 1. ESI mass spectra of (a) angiotensin, (b) angiotensin-ESA, and (c) angiotensin-d_5-ESA

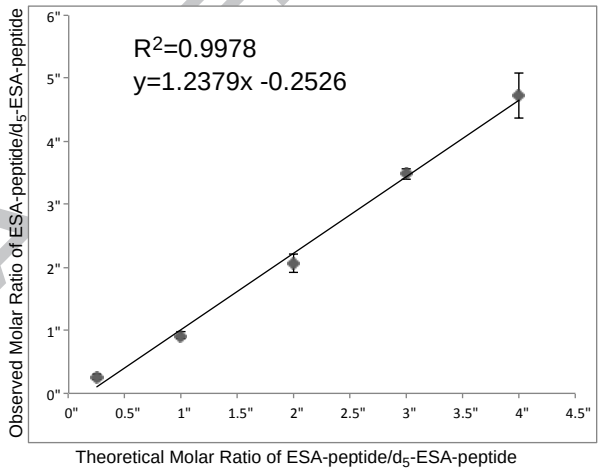
We next applied these modifiers to quantitative analysis of the three peptides for assessment of general applicability to quantitative analysis. Solutions of the peptides were prepared at pH 8.5 and were reacted with d-unlabeled or d_5-labeled ethyl succinic anhydride (ESA) solution at room temperature. The ESA-reacted peptide solution and d_5-ESA-reacted peptide solution were mixed at several molar ratios, such as 0.25, 0.5, 0.75, 1, 2, 3, and 4 (d_0/d_5), and the relative ratios of the peptides in each mixed solution were measured from the relative intensities of the monoisotopic peaks of the ESA-modified and d_5-ESA-modified peptides by MALDI TOF mass spectrometer. In this way, the observed relative ratios of ESA-modified and d_5-modified peptides were plotted against the theoretical ratios for all three peptides. Figure 2 shows the results obtained by MALDI mass spectrometry.[18]



(a) quantitative analysis of substance P



(b) quantitative analysis of angiotensin



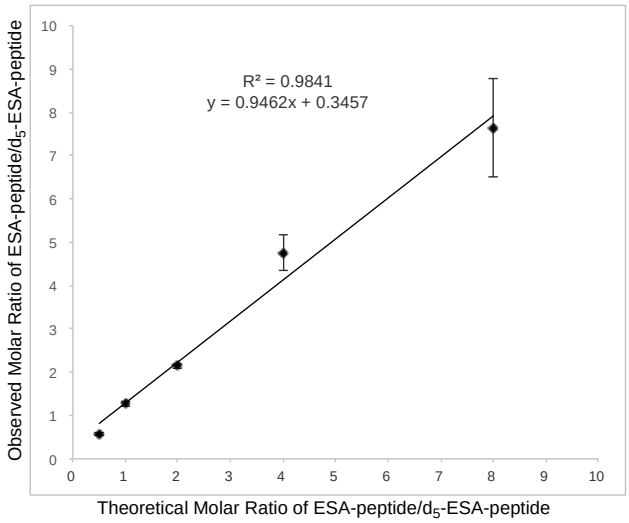
(c) quantitative analysis of neurotensin

Figure 2. Quantitative analysis of 3 peptides with MALDI

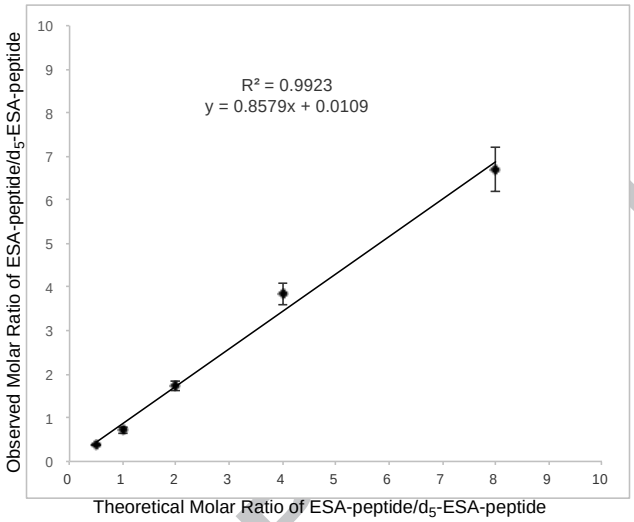
As shown in the graphs, a good correlation between the theoretical ratios and observed ratios was found for all the peptides. Two peptides, substance P and neurotensin, contain one and two lysine residues respectively, in which the amino group also reacts with

these ESA or d_5-ESA, but significant differences among these three peptides were not observed. Therefore, we conclude that ionization efficiencies of the ESA-modified peptides and those of the d_5-ESA-modified peptides are the same within experimental errors, and relative quantitative analysis of two peptide solutions is possible with the combination of ESA and d_5-ESA.

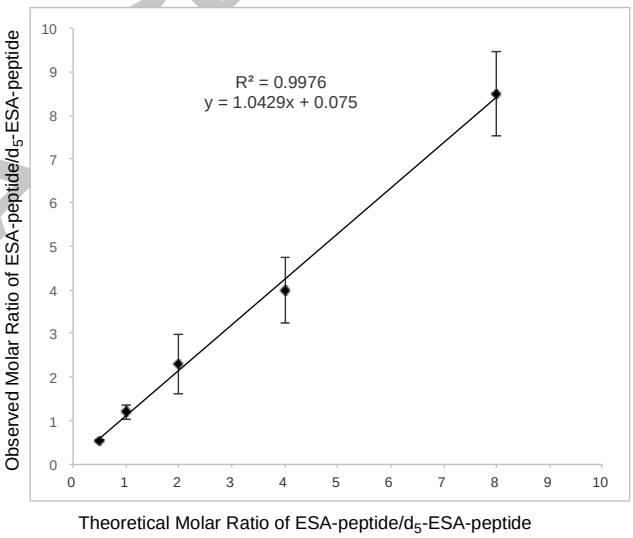
However, at higher ratios of ESA-peptide/d_5-ESA-peptide, occasionally the observed molar ratios for some of these peptides were notably higher than the theoretical molar ratios, perhaps indicating "the suppression effect" known in the MALDI-ionization process due to high concentration of salts.[19] Therefore, we compared the quantitative analysis with ESI mass spectrometry.[20] The following are the results of quantitative analysis of the same peptides with ESI mass spectrometry (Figure 3).



(a) quantitative analysis of substance P



(b) quantitative analysis of angiotensin



(c) quantitative analysis of neurotensin

Figure 3. Quantitative analysis of 3 peptides with ESI

As can be seen from the graphs, the observed ratios and theoretical ratios show excellent correlation within experimental errors. Although the errors tend to become larger with high ratios as in the previously reported quantitative analysis in our shotgun approach, the correlation is still reasonably good, because the R^2 is close to 1 in quantitative analysis of all the peptides. This somewhat better correlation may be attributed to the homogeneous nature of the analyte solutions in measuring ESI mass spectra and to reduced "ion suppression effect" in ESI compared to MALDI because of the non-existence of excessive salts in the analytes.

In summary, we synthesized d_5-labeled and unlabeled ethyl succinic anhydrides (ESAs), which specifically react with the amino group of lysine residues and *N*-termini of peptides, and demonstrated that the combination of these modifiers and mass spectrometry enables quantitative analysis of peptides with high accuracy. The reactions of these modifiers with the amino group requires a longer time than those of cysteine modifiers, sometimes necessitating overnight because of the reduced nucleophilicities of the amino group compared to the sulfhydryl group, but quantitative analysis is possible with either MALDI or ESI mass spectrometry, which are commonly applied soft-ionization mass spectrometry. Although a side reaction of succinic anhydride with the hydroxyl group of tyrosine residue is sometimes reported,[21] no such side reaction was observed in our experiments. Therefore, the addition of hydroxylamine for removal of the undesirable side reaction was unnecessary for modification with these d_5-labeled and unlabeled ethyl succinic anhydrides (ESA), unlike the benzoyloxysuccinimides, which induced some side reactions as we reported previously.[12] The solubility of ESA and d_5-ESA also appear to have improved over benzoyloxysuccinimides compared to the benzoyloxysuccinimides, as the reaction mixtures were more homogeneous, allowing a reasonable quantification with ESI mass spectrometry. In addition, while isotope effects are commonly observed during quantitative analysis with the use of d-labeled modifiers, as in other modifiers we reported for our shotgun approach,[4,5,6,7,9,10,11] we did not observe an isotope effect in this quantitative analysis either with MALDI or ESI. These modifiers can also be synthesized from readily available reagents relatively inexpensively. Therefore, it is anticipated that the combination of d_5-labeled and unlabeled ethyl succinic anhydrides (ESA) is a useful tool for quantification of peptides for proteomics research without requiring existence of specific amino acid residues.

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References and Notes

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14. Synthetic procedures for ESA is as follows:

Synthesis of 4

Succinic anhydride, **3** (5.0 g, 49.96 mmol) was suspended in 30 mL of toluene under nitrogen atmosphere. *N*-Hydroxysuccinimide (1.72 g, 14.98 mmol), 4-(dimethylamino)pyridine (0.610 g, 4.9 mmol), dry *tert*-butyl alcohol (15 mL, 150 mmol), and Et_3N (2.10 mL, 14.98 mmol) were added to the solution. Dissolution occurred upon refluxing it for one day. After cooling, ethyl acetate (20 mL) was added, and the organic layer was washed three times with 10 % citric acid and once with brine, and dried over Na_2SO_4, filtered, and evaporated to afford 5.40 g (64% yield) of brown solid. Recrystallization with ether gave white crystals of **4**. The structure was confirmed by ^{1}H NMR. mp: 49-50 °C (lit.[22] 49-51 °C) ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.61 (t, *J* = 6.7 Hz, 2H), 2.56–2.49 (m, 2H), 1.43 (s, 9H)); ^{13}C NMR (125 MHz, $CDCl_3$) 178.7, 171.6, 81.2, 30.2, 29.3, 28.2.

Synthesis of 5

A THF (10 mL) solution of LDA (6.3 mL, 12.64 mmol) was stirred at -78 °C for 15 minutes, and a solution of *tert*-butylsuccinate, **4**, (1.0 g. 5.74 mmol) in THF (5 mL) was added slowly. The reaction mixture was warmed to 0 °C, stirred for 2h, cooled again to -78 °C. To this mixture was added ethyl iodide (0.64 mL, 8.04 mmol) dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 24h. The reaction mixture was quenched with H_2O (3 mL) and concentrated. To this residue was added ethyl acetate (30mL) and cold 1 M HCl (20 mL), and extracted with ethyl acetate. The organic layer was dried (Na_2SO_4), filtered, concentrated, and purified by column chromatography (25% ethyl acetate in hexanes) to afford pure product **5** (1.02 g, 88%) as an oil. The structure was confirmed by ^{1}H NMR spectrum as follows.

^{1}H NMR (500 MHz, $CDCl_3$) δ 2.74–2.58 (m, 2H), 2.47–2.34 (m, 1H), 1.72–1.50 (m, 2H), 1.42 (s, 9H), 0.92 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.6, 174.1, 80.9, 43.4, 35.7, 28.1, 25.2, 11.4.

Synthesis of 6

Trifluoroacetic acid (12.34 mL, 160.25 mmol) was added to a solution of 2-ethyl-*tert*-butylsuccinate (1.029 g, 5.09 mmol) in dry CH_2Cl_2 (40 mL). The reaction mixture was stirred at r.t. for 24 h, and was then concentrated and purified by column chromatography (50% ethyl acetate in hexane) to produce diacid **6** (0.609 g, 82 %) as white crystals. The structure was confirmed by the melting point and ^{1}H NMR. mp: 96-97 °C (lit.[16] 96-97 °C) ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.15 (brs, 2H), 2.48–2.34 (m, 2H), 2.28 (dd, *J* = 16.5, 4.7 Hz, 1H), 1.59–1.36 (m, 2H), 0.81 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ = 176.0, 173.4, 42.1, 35.2, 24.5, 11.3.

Synthesis of ethyl succinic anhydride, 1

A mixture of 2-ethyl succinic acid, **6**, (0.100 g, 0.684 mmol) and acetyl chloride (0.29 mL, 4.21 mmol) was refluxed for 3 h. The solution was concentrated to give the desired anhydride **1** (0.080 g, 92 %) as an oil. The structure of ethyl succinic anhydride, **1,** was confirmed by spectral data as follows.

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.24–2.94 (m, 2H), 2.83–2.55 (m, 1H), 2.09–1.78 (m, 1H), 1.80–1.60 (m, 1H), 0.92 (t, *J* = 7.6 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.8, 170.4, 42.0, 33.7, 24.3, 11.1.; HRMS *m/z* calcd for $C_6H_8O_3$ $(M+H)^+$: 129.0551, found: 129.0554.

The corresponding d_5-ethyl succinic anhydride, **2**, was synthesized by substituting C_2H_5I with C_2D_5I in the above procedure. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.16–2.99 (m,

2H), 2.74–2.60 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.5, 170.2, 41.7, 33.5, 23.23, 9.87; HRMS *m/z* calcd for $C_6H_3D_5O_3$ $(M+H)^+$: 134.0865, found: 134.0870.

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16. Bergmeier, S. C.; Ismail, K. A. *Synthesis*, **2000**, *10*, 1369.

17. These peptides were purchased from Sigma-Aldrich and Akaal Organics.

18. The typical procedure with a MALDI mass spectrometer is as follows: The stock solution of each peptide was prepared at the concentration of 0.19 mM in triethylammonium bicarbonate (TEAB) buffer (pH=8.5, 50 mM). The stock solution of ethyl succinic anhydride (ESA) and that of d_5-ESA were prepared at the concentration of 20 mM in the solution containing 60% acetonitrile and 40% TEAB buffer (pH=8.5, 50 mM). The reaction started by mixing 2 μL of the peptide stock solution with 2 μL of ESA or d_5-ESA stock solution, which was left at room temperature to an elevated temperature for one hour to overnight. In order to stop the reaction, 2 μL of lysine solution (20 mM in TEAB buffer) was added to the mixture and left at room temperature for an additional half hour. For the quantitative analysis, the ESA-peptide solution and the d_5-ESA-peptide solution were mixed together at the molar ratios of 0.25, 0.5, 0.75, 1, 2, 3, and 4 (ESA-peptide/d_5-ESA-peptide), and subjected to MALDI MS analysis by MALDI TOF/TOF 4800 plus™ (Applied Biosystems). The sample at each ratio was spotted 5 times to overcome the problem of sample inhomogeniety in MALDI analysis.
The MS spectra were acquired automatically in the positive mode as previously reported.[12] Five data points were collected for each ratio, and the S/N values of the monoisotopic peaks of ESA or d_5-ESA-modified peptides were used for the calculation of the relative ratios. The averages of these data were plotted on the graph.

19. Knochenmuss, R. and Zenobi, R. *Chem. Rev.* **2003**, *103*. 441.

20. The typical procedure with an ESI mass spectrometer is as follows: Each peptide was dissolved in 50 mM ammonium bicarbonate at around pH 9 for modification with ESA (light, 1) or d_5-ESA (heavy, 2). Each peptide solution was divided into the designated ratios (8:1, 4:1, 2:1, 1:1, and 1:2 for light/heavy modification) and then incubated with an excess amount of 100 mM ESA (**1**) or d_5-ESA (**2**) acetonitrile solution overnight at 60 ℃Next, the peptide solutions were mixed in the designated ratios above as sample solutions. Subsequently, 10% trifluoroacetic acid corresponding to 5% of the solution was added to make the pH acidic. The amount of 5 μL of sample solution was injected into a nano LC (UltiMate 3000, Thermo Scientific Dionex, San Jose, CA, USA) with a L-column 2 C18 (150 x 0.075 mm id analytical column, containing 3 μm particles; Chemicals Evaluation and Research Institute (CERI), Kitakatsushika, Saitama, Japan), equipped with a C18 guard column (5 x 0.3 mm id, containing 5 μm particles; CERI). The measurement time and the flow rate were 45 min and 300 nL/min, respectively; mobile phases A and B consisted of 0.1% formic acid (pH 2.0) and 0.1% formic acid in acetonitrile, respectively. Sample separation was accomplished with the following linear gradient: 2-8% B over 1 min; 8-20% B over 19 min; 20-35% B over 5 min; 35-95% B over 0.01 min; held at 95% B over 5 min; 95-2% B over 0.01 min; and held at 2% B until the end of the measurement. The LC was connected directly to a nanoelectrospray ionization ion

trap (nano-ESI-IT)-MS (HCTultra, Bruker Daltonik GmbH, Bremen, Germany) equipped with a nano-ESI ion source. A PicoTip emitter (10 μm id, New Objective, Woburn, MA, USA) was used to generate nano-ES(Electrospray). The heated nitrogen gas temperature, gas flow rate, and spray voltage were 160 °C, 4.0 L/min, and 1.2 kV, respectively. The LC/MS measurements collected from *m/z* 300 to 1500 were obtained three times for each sample. The LC/MS/MS measurements were conducted for data-dependent ion trap MS/MS acquisition of the four most abundant precursor ions, and an active exclusion time of 30 s was used to differentiate these precursor ions from previously analyzed ions. For the quantitative analysis of the peptides, the relative ratio of the EIC peak areas corresponding to the monoisotopic ions of a peptide modified with ESA (**1**) or d_5-ESA (**2**) was calculated and taken as the average of three measurements.

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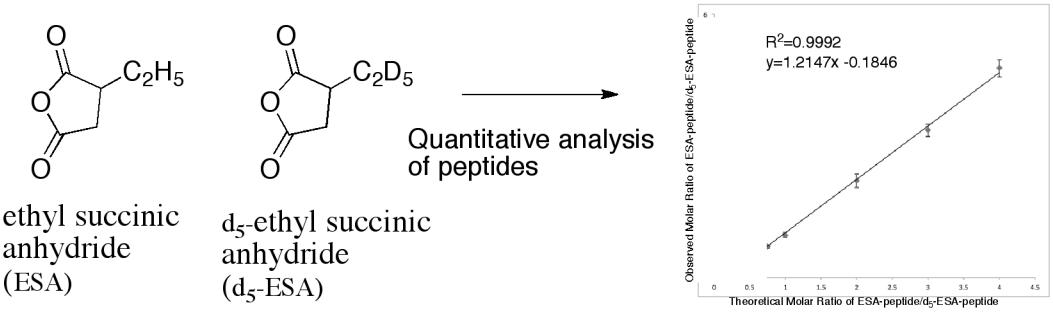
22. Guzzo, P. R.; Miller, M. J. *J. Org. Chem*. **1994**, *50,* 4862.

Graphical abstract



ethyl succinic anhydride (ESA)

d_5-ethyl succinic anhydride (d_5-ESA)