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Multifunctional and Highly Sensitive Precolumn Reagents for Amino Acids in Liquid Chromatography/Tandem Mass Spectrometry

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We have developed novel precolumn derivatization reagent, *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide (TAHS), for sensitive analyses of amino acids using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). TAHS, an activated carbamate, was reacted briefly with the amino group to form a ureide bond under mild condition. The derivatives provided selective cleavage at the binding site between the reagent and the amino acid in the collision cell of the mass spectrometer and produced a characteristic fragment derived from the reagent moiety. Using the precursor ion scan mode of the tandem mass spectrometry, amino acids derivatized with the reagents were simultaneously measured on the chromatogram. Selective cleavage also enabled the straightforward isotope ratio analysis of amino acids by the selected reaction monitoring mode, which was applicable in ^{13}C metabolic flux analysis. TAHS, which contains a cationic quaternary amine, achieved subfemtomole to attomole levels of amino acids detection by measurement in the selected reaction monitoring mode. We also synthesized trideuteriummethyl-substituted TAHS, TAHS-d_3, and demonstrated that the combination of TAHS and TAHS-d_3 is useful in comparing amino acid concentrations between two different samples using a single LC/MS/MS measurement.

Several high-performance liquid chromatography (HPLC) methods to analyze amino acids have been developed over 50 years. In 1958, Moore et al. developed an automatic amino acid analyzer system for the colorimetric detection of amino acids; a purple color was produced after reaction of amino acids with ninhydrin reagent, and a cation-exchange resin was used to separate the amino acids in a stepwise procedure by gradually raising the pH of the citric acid buffer solution.[1] The analyzer is now capable of assaying not only protein hydrolysates but also amino acids in biological fluids, with the progressive improvement of HPLC pumping performance and the development of more effective column resins, although the sensitivity is not very high and the detection limits are a few picomoles.

In addition to the ninhydrin method, a variety of other techniques for converting amino acids to sensitive analyzable fluorescent derivatives have been developed since the late 1970s. The reagents used to produce the derivatives include *o*-phthalaldehyde (OPA),[2] 9-fluorenylmethylchloroformate (FMOC-Cl),[3] 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl-Cl),[4] 7-fluoro-4-nitorobenzo-2oxa-1,3-diazole (NBD-F),[5] and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC).[6] The sensitivity of detection is within the range of subpicomoles to femtomoles.

The mass spectrometer (MS) plays an important role as the detector of HPLC, not only in protein analysis (proteome) but also metabolite analysis (metabolome). One advantage of using an HPLC/mass spectrometer (LC/MS) system is its selectivity, which can distinguish analytes not only by their retention times but also by their *m*/*z* (mass-to-charge ratio) values. HPLC/tandem mass spectrometry (LC/MS/MS) has increased selectivity and sensitivity. Tandem mass spectrometry enables specific detection of an analyte using both the precursor ion and the product ion in a collision-induced dissociation, and decreases the noise level, which improves the limits of detection.

Recently, amino acid analysis by LC/MS has been reported. Piraud et al. separated approximately 80 underivatized amino acids by using ion-paring reversed-phase LC/MS/MS for the diagnosis of inherited disorders of amino acid metabolism.[7] Precolumn derivatization reagents for amino acid analyses using LC/MS or LC/MS/MS were also developed, mainly to achieve greater sensitivity and selectivity. The reagents used include *N*-alkylni-

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cotinic acid, *N*-hydroxysuccinimide ester,[8] and iTRAQ (isobaric tag for relative and absolute quantitation).[9]

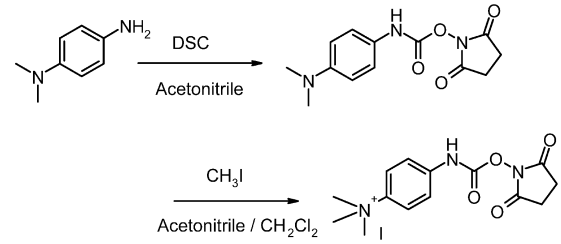
We have recently focused on developing new types of precolumn derivatization reagents of the amino group, for use in LC/MS/MS. One of our most successful reagents, *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide (TAHS), achieved subfemtomole to attomole levels of amino acids detection. Amino acids derivatized with TAHS were selectively cleaved at the binding site between TAHS and the amino acid in the collision cell of the triple-stage quadrupole mass spectrometer. Amino acid analyses, such as simultaneous analysis of compounds with amino groups and biosynthetic pathway studies, including ^{13}C metabolic flux analysis, have been performed.[10,11]

EXPERIMENTAL SECTION

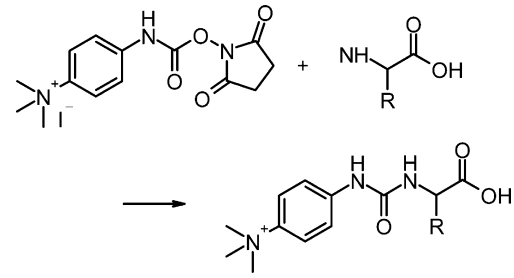
Chemicals. The amino acid standard mixture solution, type H (mixture of L-lysine, L-histidine, L-arginine, L-aspartic acid, L-threonine, L-serine, L-glutamic acid, L-proline, L-glycine, L-alanine, L-cystine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, and L-phenylalanine, with each amino acid at a 2.5 mM concentration) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Tryptophan, L-glutamine, and L-asparagine were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Stable isotope-labeled amino acids, including ^{13}C, ^{15}N uniformly labeled L-glutamine (Gln-IS), ^{15}N uniformly labeled L-arginine (Arg-IS), ^{13}C, ^{15}N uniformly labeled L-histidine (His-IS), ^{13}C, ^{15}N uniformly labeled L-glutamic acid (Glu-IS), ^{13}C, ^{15}N uniformly labeled L-serine (Ser-IS), and ^{13}C, ^{15}N uniformly labeled L-tryptophan (Trp-IS) were obtained from Ajinomoto Co., Inc. (Tokyo, Japan). Glycine-2,2-d_2 (Gly-IS) and proline-d_7 (Pro-IS) were purchased from Cambridge Isotope Laboratories (Andover, U.S.A.). L-Alanine-3,3,3-d_3 (Ala-IS), L-leucine-5,5,5-d_3 (Leu-IS), DL-lysine-4,4,5,5-d_4 (Lys-IS), DL-valine-d_8 (Val-IS), L-phenyl-d_5-alanine (Phe-IS), and L-methionine-d_3 (methyl d_3; Met-IS) were purchased from Isotec (Tokyo, Japan). *N*,*N*-Dimethylamino-*p*-phenylenediamine and *N*,*N*′-dihydroxysuccinimidyl carbonate (DSC) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Iodomethane and iodomethane-d_3 were purchased from Nacalai Tesque (Kyoto, Japan) and Taiyo Nippon Sanso Corporation (Tokyo, Japan), respectively. Acetic acid and acetonitrile were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Deionized water was used after purification through a Milli-Q gradient A10 System (Millipore, Bedford, MA).

Synthesis of *p*-*N*,*N*,*N*-Trimethylammonioanilyl *N*′-Hydroxysuccinimidyl Carbamate Iodide. DSC (1.28 g, 5 mmol) was dissolved in 25 mL of acetonitrile at room temperature. *N*,*N*-Dimethylamino-*p*-phenylenediamine (535 mg, 5 mmol), dissolved in 25 mL of acetonitrile, was added dropwise to the carbonate solution over a period of approximately 2 h. After an additional 22 h of stirring, the reaction mixture was concentrated by rotary evaporation. The residue was resuspended in 5 mL of acetonitrile and then filtered, to obtain *p*-*N*,*N*-dimethylaminoanilyl *N*′-hydroxysuccinimidyl carbamate (597 mg, 48% yield) (Scheme 1).

Scheme 1. Synthesis of TAHS



Scheme 2. Reaction of Amino Acids with TAHS Reagent



^1H NMR, 400 MHz (JEOL α400, Japan) (CD_3CN, ppm, tetramethylsilane as an internal standard) δ7.22 (d, 9.0 Hz, 2 H), δ6.72 (d, 9.0 Hz, 2 H), δ2.88 (s, 6 H), δ2.76 (s, 4 H); MS (micromass Q-Tof-2, Manchester, U.K.), m/z 278.1141 $[M + H]^+$, molecular formula, $C_{13}H_{16}N_3O_4$ (Δ −4.4 ppm).

p-*N*,*N*-Dimethylaminoanilyl *N*′-hydroxysuccinimidyl carbamate (264 mg, 1.1 mmol) was dissolved in 10 mL of acetonitrile/dichloromethane (4:1) at room temperature. Iodomethane (0.4 mL, 8 equiv) was added to the solution, which was then stirred for 23 h at room temperature. After the reaction mixture was filtered, 354 mg of TAHS was obtained (76% yield) (Scheme 1).

^1H NMR, 400 MHz (JEOL α400, Japan) (DMSO-d_6, ppm, tetramethylsilane as an internal standard) δ7.97 (d, 8.4 Hz, 2 H), δ7.62 (d, 8.4 Hz, 2 H), δ3.58 (s, 9 H), δ2.83 (s, 4 H); MS (micromass Q-Tof-2, Manchester, U.K.), m/z 292.1297 $[M]^+$, molecular formula, $C_{14}H_{18}N_3O_4$ (Δ −2.9 ppm).

Synthesis of *p*-*N*,*N*-Dimethyl,*N*-trideuteromethylammonioanilyl *N*′-Hydroxysuccinimidyl Carbamate Iodide (TAHS-d_3). *p*-*N*,*N*-Dimethylaminoanilyl *N*′-hydroxysuccinimidyl carbamate (264 mg, 1.1 mmol) was dissolved in 10 mL of acetonitrile/dichloromethane (4:1) at room temperature. Iodomethane-d_3 (0.4 mL, 8 equiv) was added to the solution, which was then stirred for 50 h at room temperature to yield TAHS-d_3.

Derivatization of the Amino Acids Standard Solution with TAHS. The synthesized TAHS reagent was dissolved in dry acetonitrile to a concentration of 20 mg/mL. The mixed amino acids solution was prepared from the dilution of the commercial amino acid standard mixture solution (type H) and the L-tryptophan, L-glutamine, and L-asparagine solutions. A 10 μL aliquot of the mixed amino acid solution was mixed with 30 μL of 0.2 M sodium borate buffer at pH 8.8, in a sealed 1 mL reaction vial or 1.5 mL polypropylene micro test tube. A 10 μL aliquot of TAHS was added to the above solution, and the mixture was heated at 55 °C for 10 min. A 200 μL aliquot of 0.2% aqueous acetic acid was then added to the reaction mixture before storage in a tightly sealed container at 4 °C until LC/MS/MS analysis (Scheme 2).

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Preparation of Plasma Samples. Blood samples were obtained from Sprague−Dawley (SD) rats (8 week old, male). The samples were collected in tubes containing EDTA as an anticoagulant (NONCLOT-D; Daiichi Pure Chemicals, Tokyo, Japan) and centrifuged for 10 min at 3000 rpm, at 4 °C (model H-103N; Kokusan Centrifuge Ltd., Tokyo, Japan). Plasma samples were collected, pooled, and then stored at −80 °C until analysis.

A 10 μL aliquot of the pooled plasma was added to the 10 μL aliquot of internal standard solution (isotope-labeled amino acids mixture solution) and diluted with 80 μL of Milli-Q water and deproteinized to a final concentration of 50% acetonitrile. The mixture was vortexed and centrifuged for 10 min at 15 000 rpm. A 10 μL aliquot of the deproteinized supernatant, in a sealed 1 mL reaction vial or 1.5 mL polypropylene micro test tube, was mixed with 30 μL of 0.2 M sodium borate buffer at pH 8.8. A 10 μL aliquot of the reagent solution was added to the solution, and the mixture was heated at 55 °C for 10 min. A 200 μL aliquot of 0.2% aqueous acetic acid was added to the reaction mixture. The mixture was diluted 10-fold with 0.2% aqueous acetic acid before storage in a tightly closed container at 4 °C until LC/MS/MS analysis.

***E. coli* Extract Sample.** The *E. coli* extract sample, cultivated with ^{13}C-labeled glucose, was used for isotope ratio analysis of amino acid. The cultivation and the extraction conditions are described in Iwatani et al.[11] A 10 μL aliquot of the TAHS reagent solution was added to the isotope-labeled extract solution; the mixture was then heated at 55 °C for 10 min. A 200 μL aliquot of 0.2% aqueous acetic acid was added to the reaction mixture before storage in a tightly closed container at 4 °C until LC/MS/MS analysis.

Instrumentation. An Agilent 1100 series liquid chromatography system (Agilent Technologies, Waldbrunn, Germany) with a binary pump, degasser, autosampler, column compartment, and UV detector was used.

The system was coupled to a triple-quadrupole mass spectrometer, Applied Biosystems Sciex API 4000 (Applied Biosystems-MDS Sciex, Concord, Canada) equipped with a TurboIonSpray interface. The Applied Biosystems-MDS Sciex Analyst 1.4.2 software was used to control these instruments.

LC/MS/MS of the Precursor Ion Scan by Simultaneous Analysis of TAHS-Tagged Amino Acids. The TAHS-tagged amino acids were injected into a column of Inertsil ODS-3, 3 μm particle size, 100 mm × 2.1 mm i.d. (GL Sciences, Tokyo, Japan). The column temperature was maintained at 40 °C throughout the analysis. Mobile phase A consisted of 0.2% acetic acid in Milli-Q-water (v/v), and mobile phase B consisted of 0.2% acetic acid in acetonitrile. Each mobile phase was filtered through a 0.2 μm membrane (Nalge Nunc International, Rochester, NY) before use. The gradient conditions (B %) were 0−0.10 min = 0%, 0.11−15 min = 5−12% (linear), 15−18 min = 12%, and 18−22 min = 70%. Re-equilibration was performed for 8 min at 100% of mobile phase A. The flow rate was 0.2 mL/min. Mobile phase B was introduced just after the column outlet as the sheath solution, at a flow rate of 50 μL/min. The injection volume was 3 μL.

The TurboIonSpray interface was operated in the positive mode at 5000 V and 700 °C. Other MS parameters, CUR, GS1, GS2, CAD, DP, EP, CE, and CXP, were set to 10, 70, 30, 5, 50, 10, 20, and 14, respectively. The precursor ion monitoring was set to *m/z* 177 amu, which was derived from the TAHS moiety, and the scan range for the precursor ion scan was set to 150−500 amu, with 2.5 s for the scan time.

Table 1. Selected Monitor Ions and Correlation Coefficients and Detection Limits for Each TAHS-Derivatized Amino Acid

amino acid	Q1	Q3	linearity [R^2]	range [nmol/mL]	LOD (S/N = 3) fmol[a]
glycine	252.1	177.1	0.993	0.25−10	0.10
alanine	266.1	177.1	0.991	0.05−10	0.05
serine	282.1	177.1	0.994	0.01−10	0.10
proline	292.1	177.1	0.990	0.01−10	0.05
valine	294.1	177.1	0.995	0.01−25	0.11
threonine	296.1	177.1	0.997	0.01−10	0.07
isoleucine	308.1	177.1	0.997	0.01−25	0.10
leucine	308.1	177.1	0.998	0.01−10	0.10
asparagine	309.1	177.1	0.995	0.01−10	0.09
aspartic acid	310.1	177.1	0.998	0.01−250	0.25
glutamine	323.2	177.1	0.995	0.01−10	0.06
lysine	250.3	177.1	1.000	0.01−50	0.07
glutamic acid	324.1	177.1	0.997	0.025−100	0.15
methionine	326.1	177.1	0.999	0.01−100	0.30
histidine	166.8	177.1	0.998	0.01−10	0.16
phenylalanine	342.2	177.1	0.999	0.01−10	0.05
arginine	176.1	177.1	0.998	0.01−10	0.14
tyrosine	358.2	177.1	0.997	0.01−25	0.06
tryptophan	381.2	177.1	0.993	0.01−25	0.11
cystine	297.8	177.1	0.999	0.025−250	0.34

[a] LOD: limit of detection was calculated from a signal-to-noise ratio of 3 after the Savitzky−Golay smoothing.

LC/MS/MS Selected Reaction Monitoring for Quantitative Analysis of Amino Acid Derivatives. Amino acid derivatives were injected into a column of Capcell Pack MGIII, 3 μm particle size, 100 mm × 2.0 mm i.d. (Shiseido Co., Ltd., Japan). The column temperature was maintained at 40 °C throughout the analysis. Other conditions, including injection volume, gradient conditions, flow rate, and composition of the sheath solution for the analysis of amino acids derivatized with TAHS were the same as those of the precursor ion scan by simultaneous analysis of the TAHS-tagged amino acids.

The TurboIonSpray interface was operated in the positive mode at 5000 V and 700 °C. Other MS parameters for TAHS-tagged amino acids, CUR, GS1, GS2, CAD, DP, EP, CE, and CXP, were set to 10, 30, 30, 5, 50, 10, 20, and 15, respectively. Quantitative analysis of the amino acids was performed by selected reaction monitoring (SRM). The monitored SRM channels for the TAHS-tagged amino acids are summarized in Table 1.

LC/MS/MS for Isotope Ratio Analysis of Amino Acids by TAHS. The HPLC conditions were the same as those of the precursor ion scan by simultaneous analysis of TAHS-tagged amino acids.

The TurboIonSpray interface was operated in the positive mode at 5000 V and 700 °C. Other MS parameters, CUR, GS1, GS2, CAD, DP, EP, CE, and CXP, were set to 15, 50, 30, 5, 71, 10, 29, and 15, respectively. Isotope ratio analysis of amino acids was performed by SRM. The monitored SRM channels are summarized in Table 2.

Comparative Analysis of Amino Acids between Samples Using TAHS and TAHS-d_3. Two aliquots of the same sample, 10 μM amino acid mixture, were derivatized with the TAHS reagent and the TAHS-d_3 reagent, respectively, and then mixed at a 1:1 ratio.

Table 2. Selected Monitor Ions for Isotope Ratio Analysis and Calculated Theoretical Natural Isotope Ratio and Measured Isotope Ratio of TAHS-Derivatized Amino Acids of the Standard Amino Acid Solution and the *E. coli* Extract Sample[a,b]

amino acid	Q1	Q3	calcd [%]	measd [%]	*E. coli* extract [%]	amino acid	Q1	Q3	calcd [%]	measd [%]	*E. coli* extract [%]
Glycine											
M	252.1	177.1	96.9	97.0	65.5	M + 2	254.1	177.1	0.4	0.5	21.6
M + 1	253.1	177.1	2.7	2.6	12.0	M + 3	255.1	177.1	0.0	0.0	0.8
Alanine											
M	266.1	177.1	95.8	95.4	32.0	M + 3	269.1	177.1	0.0	0.0	42.0
M + 1	267.1	177.1	3.7	4.0	20.9	M + 4	270.1	177.1	0.0	0.0	0.3
M + 2	268.1	177.1	0.4	0.6	4.9						
Serine											
M	282.1	177.1	95.6	95.2	35.6	M + 3	285.1	177.1	0.0	0.1	32.0
M + 1	283.1	177.1	3.8	3.9	22.2	M + 4	286.1	177.1	0.0	0.0	0.7
M + 2	284.1	177.1	0.6	0.7	9.6						
Proline											
M	292.1	177.1	93.7	93.0	54.1	M + 3	295.1	177.1	0.0	0.1	11.3
M + 1	293.1	177.1	5.8	6.2	9.3	M + 4	296.1	177.1	0.0	0.0	9.6
M + 2	294.1	177.1	0.5	0.6	9.8	M + 5	297.1	177.1	0.0	0.0	5.5
Valine											
M	294.1	177.1	93.6	92.9	84.1	M + 3	297.1	177.1	0.0	0.0	2.7
M + 1	295.1	177.1	5.8	6.5	7.0	M + 4	298.1	177.1	0.0	0.0	1.3
M + 2	296.1	177.1	0.5	0.6	2.7	M + 5	299.1	177.1	0.0	0.0	2.3
Threonine											
M	296.1	177.1	94.5	93.9	88.7	M + 3	299.1	177.1	0.0	0.0	1.7
M + 1	297.1	177.1	4.8	5.3	6.0	M + 4	300.1	177.1	0.0	0.0	1.5
M + 2	298.1	177.1	0.7	0.8	2.0	M + 5	301.1	177.1	0.0	0.0	0.1
Isoleucine											
M	308.1	177.1	92.6	92.3	87.6	M + 3	311.1	177.1	0.0	0.0	0.4
M + 1	309.1	177.1	6.8	7.0	7.3	M + 4	312.1	177.1	0.0	0.0	0.4
M + 2	310.1	177.1	0.6	0.6	3.9	M + 5	313.1	177.1	0.0	0.0	0.2
Leucine											
M	308.1	177.1	92.6	92.1	90.3	M + 3	311.1	177.1	0.0	0.0	0.2
M + 1	309.1	177.1	6.8	7.2	7.2	M + 4	312.1	177.1	0.0	0.0	0.2
M + 2	310.1	177.1	0.6	0.7	1.7	M + 5	313.1	177.1	0.0	0.0	0.1
Aspartic Acid											
M	310.1	177.1	94.3	94.9	39.3	M + 3	313.1	177.1	0.0	0.1	16.7
M + 1	311.1	177.1	4.8	4.2	15.7	M + 4	314.1	177.1	0.0	0.0	16.8
M + 2	312.1	177.1	0.9	0.8	11.5						
Glutamic Acid											
M	324.1	177.1	93.2	91.6	7.3	M + 3	327.1	177.1	0.1	0.4	30.7
M + 1	325.1	177.1	5.8	6.8	11.6	M + 4	328.1	177.1	0.0	0.1	17.4
M + 2	326.1	177.1	0.9	1.1	19.3	M + 5	329.1	177.1	0.0	0.0	13.7
Methionine											
M	326.1	177.1	89.0	89.1	30.5	M + 3	329.1	177.1	0.3	0.3	16.5
M + 1	327.1	177.1	6.2	6.3	18.0	M + 4	330.1	177.1	0.0	0.1	10.2
M + 2	328.1	177.1	4.5	4.2	14.7	M + 5	331.1	177.1	0.0	0.0	10.1
Phenylalanine											
M	342.1	177.1	89.6	89.3	88.1	M + 5	347.1	177.1	0.0	0.0	0.4
M + 1	343.1	177.1	9.6	9.8	9.0	M + 6	348.1	177.1	0.0	0.0	0.3
M + 2	344.1	177.1	0.8	0.9	1.0	M + 7	349.1	177.1	0.0	0.0	0.3
M + 3	345.1	177.1	0.1	0.0	0.3	M + 8	350.1	177.1	0.0	0.0	0.2
M + 4	346.1	177.1	0.0	0.0	0.3	M + 9	351.1	177.1	0.0	0.0	0.2
Tyrosine											
M	358.2	177.1	89.3	89.1	tr	M + 5	363.2	177.1	0.0	0.0	tr
M + 1	359.2	177.1	9.6	9.8	tr	M + 6	364.2	177.1	0.0	0.0	tr
M + 2	360.2	177.1	1.0	1.1	tr	M + 7	365.2	177.1	0.0	0.0	tr
M + 3	361.2	177.1	0.1	0.1	tr	M + 8	366.2	177.1	0.0	0.0	tr
M + 4	362.2	177.1	0.0	0.0	tr	M + 9	367.2	177.1	0.0	0.0	tr

[a] The theoretical natural isotope ratio of each amino acids was calculated by using MS-Isotope in ProteinProspector (http://prospector.ucsf.edu/) site, University of California, San Francisco. [b] tr: trace.

HPLC conditions were the same as those of the precursor ion scan for simultaneous analysis of the TAHS-tagged amino acids.

The TurboIonSpray interface was operated in the positive mode at 5000 V and 700 °C. The other MS parameters, CUR, GS1, GS2, CAD, DP, EP, CE, and CXP, were set to 10, 50, 30, 5, 50, 10, 25, and 14, respectively. The monitored SRM channel for TAHS-d_3 of the Q3 channel was 180.1, and the Q1 channels for glycine, alanine, serine, proline, valine, threonine, isoleucine, leucine, asparagine, aspartic acid, glutamine, lysine, glutamic acid, methionine, histidine, phenylalanine,

arginine, tyrosine, tryptophan, and cystine were set to 255.1, 269.1, 285.1, 295.1, 297.1, 299.1, 311.1, 311.1, 313.1, 323.1, 253.2, 324.1, 326.1, 168.2, 345.2, 177.7, 361.2, 384.1, and 300.1, respectively.

RESULTS AND DISCUSSION

Design and Synthesis of Precolumn Derivatization Reagents for LC/MS/MS. Precolumn derivatization techniques for amino acid analysis have been developed because they increase detection sensitivity and selectivity of analytes. Mass spectrometry, especially in the tandem mode, has been eagerly developed as a powerful tool in proteome, metabolome, and pharmacokinetics studies due to considerable improvements in its analytical sensitivity and selectivity. In this study, we developed new precolumn derivatization reagents for amino acid analysis by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS).

The derivatization of an amino acid reacted with the reagent was designed to provide characteristic and selective cleavage at the bonding site between the reagent moiety and the amino acid in the collision cell of the triple-stage quadrupole mass spectrometer. Subsequently, each derivative generated the same product ion, which was derived from the reagent moiety. We discovered the formation of a ureide bond (R−NH−(C=O)−NH−R′), in which R is the component of the amino compound and R′ is the reagent moiety, facilitates analysis. To form the ureide bond with the amino groups of the analytes, we selected an activated carbamate-containing reagent (e.g., succinimidyl carbamate), since carbamates are well-known for their rapid and selective reactions with amino groups under mild conditions.

Although the reaction was completed in a minute at room temperature, phenolic hydroxyl group was also reacted with TAHS reagent. Further heating (more than 5 min at 55 °C) was needed to hydrolysis of the phenolic hydroxyl group of tyrosine. And optimized reaction pH for TAHS derivatization was pH 8.0−9.0.

In addition, a cationic group was introduced to the reagent skeleton, to increase the ion efficiency for electrospray ionization. The sensitivity of TAHS derivatives show 7−320 higher than the sensitivity of *p*-*N*,*N*-dimethylaminoanilyl *N*′-hydroxysuccinimidyl carbamate derivatives. Furthermore, a hydrophobic moiety, such as a phenyl, naphthyl, quinoly, or pyridyl group, was combined with the activated carbamate to increase the retention of the derivatives and to facilitate their separation, in comparison with the unreacted amino acids, in reversed-phase chromatography.

To achieve effective ionization, the reagents were designed and have both phenyl group as hydrophobic moiety and cationic group as ionic moiety.

The fragmentation patterns of the derivatives were evaluated by MS/MS product ion scans. The fragment ions from the TAHS adducts were due to regulatory cleavage at the ureide bond of the carbamate moiety and the amino groups and the loss of amino acid moieties from the precursor ions under the optimized positive-ion collision-induced dissociation conditions of $[M + H]^+$. The results were utilized to develop the SRM conditions, which were programmed to include the transitions of all of the protonated molecular ions to the common fragment at *m*/*z* 177, derived from TAHS (Figure 1). All of the derivatives can be monitored in the positive mode, making it easy to add the monitoring

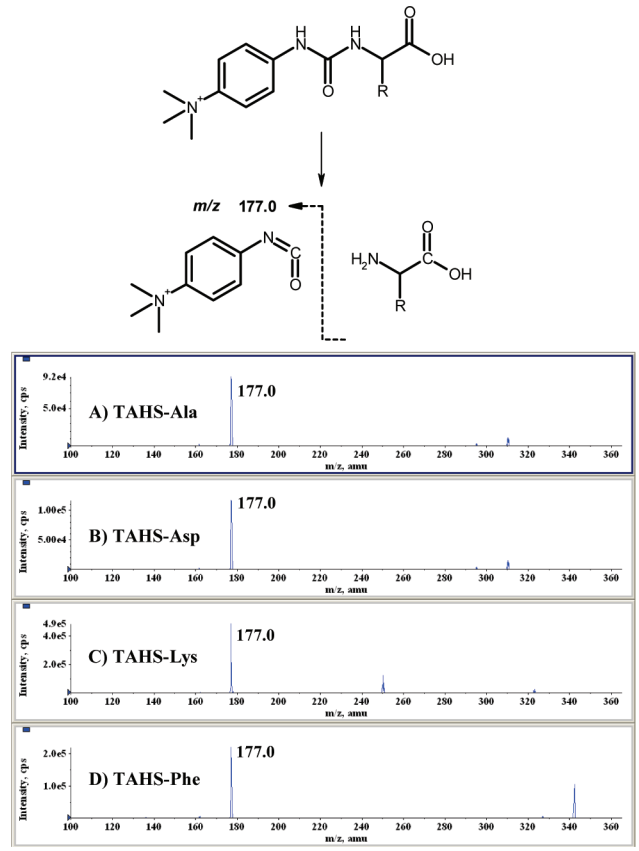

Figure 1. Specific cleavage performed in a tandem mass spectrometer. Weak collision energy produces the unique fragment ion derived from the reagent skeleton. All TAHS derivatives of the amino acids show the same *m*/*z* 177 product ions in the product ion scan mode: (A) TAHS derivative of alanine, (B) TAHS derivative of aspartic acid, (C) TAHS derivative of lysine, and (D) TAHS derivative of phenylalanine.

compounds in tandem mass measurements, without any method optimization for Q3 ions.

The activated carbamates in the reagent can be substituted with isocyanates, which also form a ureide bond with amino groups.

LC/MS/MS Precursor Ion Scan by Simultaneous Analysis of TAHS-Tagged Amino Acids. Figure 2 shows the total ion chromatogram for the precursor ion scan of the amino acid standard mixture solution (type H) derivatized with TAHS; the product ion was set at *m*/*z* 177. All of the amino acids derivatized with TAHS, that is, all of the analytes with amino groups, were selectively and simultaneously monitored by the precursor ion scan. Although the compounds with two amino groups, such as lysine, were bound with 2 mol of TAHS, the product ions were also set at a value of *m*/*z* 177.

The results indicated that this method, combining LC/MS/MS and precolumn derivatization with these reagents, was not only similar to selective amino acid analysis by using the fluorescent reagents for the amino groups but also superior to the method, as it includes additional information on the parent mass number of each peak. Thus, when an unidentified peak is observed on the chromatogram, its structure with amino group(s) may be estimated from its mass number.

Figure 3 shows the total ion chromatograms for the precursor ion scan of deproteinized rat plasma and the TAHS-derivatized

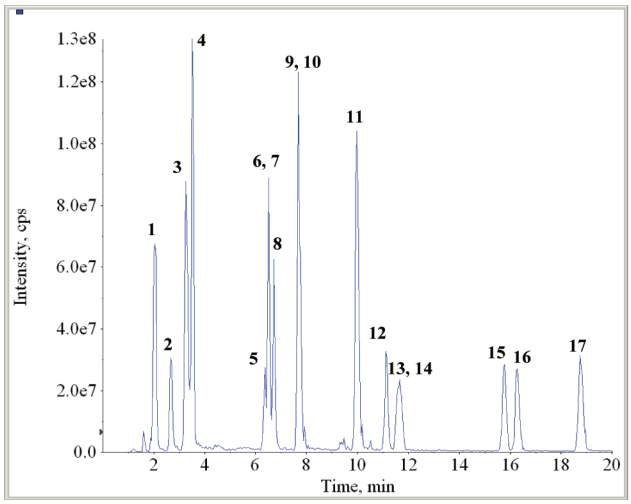

Figure 2. Total ion chromatogram of a 17 amino acids standard mixture derivatized with TAHS reagent. Detection was achieved by a precursor ion scan of the precursor of *m*/*z* 177; the range of *m*/*z* was 150−500. Peak identification: 1, histidine; 2, arginine; 3, serine; 4, glycine; 5, aspartic acid; 6, threonine; 7, glutamic acid; 8, alanine; 9, lysine; 10, proline; 11, cystine; 12, valine; 13, methionine; 14, tyrosine; 15, isoleucine; 16, leucine; 17, phenylalanine.

deproteinized rat plasma. The results show that product ion of *m*/*z* 177 is only present in TAHS-derivatized samples.

LC/MS/MS Selected Reaction Monitoring for Quantitative Analysis of Amino Acid Derivatives. The detection limits, linearity, and quantitative range of TAHS-tagged amino acids were evaluated in the selected ion monitoring mode (Table 1). The 20 different amino acids that make up proteins were used. The detection limits of amino acids derivatized with TAHS, which were calculated from a signal-to-noise ratio of 3 after Savitzky−Golay smoothing using Analyst 1.4 software, were 0.05−0.34 fmol. Ionization of the derivatives with TAHS was very efficient because it has a trimethylammonium group: the sensitivity of the procedure was lower than femtomole levels of amino acids. The aromatic ring moiety of TAHS is also important in increasing the hydrophobicity necessary for efficient electrospray ionization.

To increase the ionization efficiency of the derivatives, we used an exterior pump for the sheath flow. The addition of acidic acetonitrile as the sheath flow increased the sensitivity of analytes, especially those eluted with lower organic solvent compositions. With sheath flow, TAHS derivatives were increased 1−2.2-fold higher intensity. Gln and Asp derivatives increased more than 2-fold with using the sheath flow.

Table 3 shows quantitation of the rat plasma samples. The analysis was performed six times. The relative standard deviations of the precisions were 3−6%. The recovery rates after addition of the 100 μM amino acids mixture to plasma samples were 94−112%. Because of the high sensitivity of this method, samples could be diluted before LC/MS/MS analysis so that the matrix effects were reduced and better precisions and recovery rates were achieved.

We used a Capcell Pack MGIII column in the SRM, although an Inertsil ODS-3 column shows similar separation results for TAHS-tagged amino acids. However, using our method, a Capcell Pack MGIII column shows better signal-to-noise ratio for basic amino acids. TAHS derivative of histidine, arginine, and lysine showed 3.5−7.2-fold higher signal-to-noise ratio. These results might be from the highly end-capped silica gel particles reducing the interaction between the basic part of the TAHS derivative and silanols of the silica gel particles and providing the sharp peaks.

LC/MS/MS for Isotope Ratio Analysis of Amino Acids by TAHS. Selective cleavage at the binding site between the reagent moiety and the amino acid moiety in the derivative facilitated isotope ratio analysis of the analyte itself, without any complex correction calculations. When the selected ion monitoring mode analysis of MS/MS was set at *m*/*z* of 177, derived from the TAHS moiety of the *p*-*N*,*N*,*N*-trimethylammonioanilyl group ($C_{10}H_{13}N_2O$), the product ion distribution reflected the isotope ratio of the amino acids themselves.

Table 2 shows the measured isotope distributions of natural amino acids, after derivatization with TAHS, by selected ion monitoring. A good correlation was observed between the theoretical and observed values. For instance, the theoretical isotope distributions of natural alanine are 95.8% (m (= monoisotopic)), 3.7% (m + 1), and 0.5% (m + 2), whereas the observed isotope distributions were 95.4% (m), 4.0% (m + 1), and 0.6% (m + 2). Correction calculations were needed to obtain the isotope distributions with more than two amino groups, such as lysine.

We also applied this method to the biological sample, *E. coli* extract, which was cultivated with ^{13}C-labeled glucose to measure the non-natural isotope ratio of amino acids. A difference in isotope ratio patterns was observed between labeled and natural amino acids with this method. Increased incorporation of ^{13}C atoms, derived from the ^{13}C-labeled glucose, were observed in amino acids more readily converted for energy metabolism in glycolysis and the citric cycle (TCA cycle). For instance, we observed the isotope ratio (m) for glutamic acid was only 7.3%, suggesting rapid turnover of glutamic acid was performed in the intracellular metabolic dynamics.

Metabolic flux analysis using ^{13}C-labeled substrates is a well-developed method for investigating intracellular metabolite behavior. Recently, several ^{13}C metabolic flux analysis experiments were performed, measuring intracellular amino acids, rather than relying on proteinogenic amino acids, because of their relatively rapid turnover. For example, Kromer et al. utilized the labeling of intracellular amino acids, using gas chromatography/mass spectrometry (GC/MS), to determine the fluxes in the lysine production in different phases of a batch cultivation of *Corynebacterium glutamicum*.[12] Although GC/MS has been one of the most convenient and sensitive tools in measuring isotope distributions for this purpose, to obtain the isotope ratio in the target compound by GC/MS, the observation error derived from the isotope ratio of the reagent itself has to be considered. Our method, involving selective cleavage at the binding site between the reagent moiety and the amino acid in the derivative, allows the isotope ratio of the analyte to be measured, without any complex calculated corrections.

The metabolic flux analysis experiment was very expensive because of the ^{13}C-labeled glucose. The high sensitivity of TAHS will help to reduce costs as the experiments can be performed using low-concentration intracellular amino acids. Iwatani et al. determined the metabolic flux change during the fed-batch cultivation of a lysine-producing strain of *E. coli* using mass

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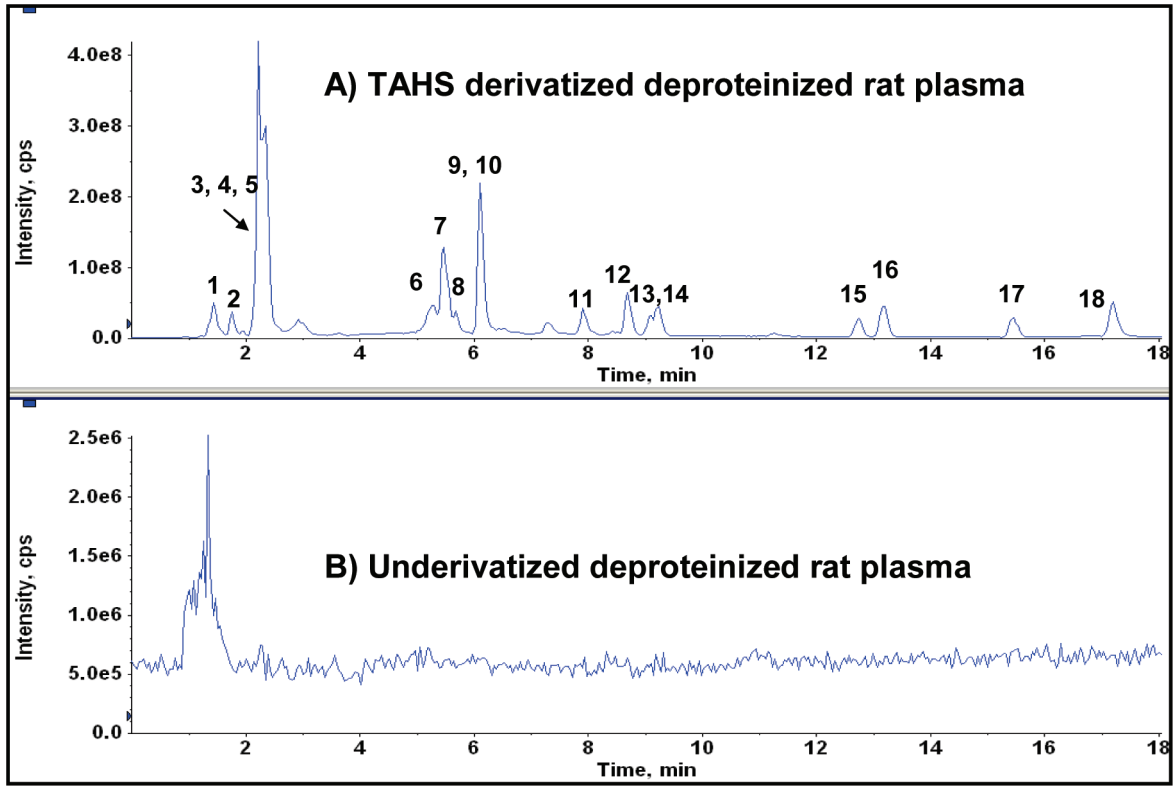

Figure 3. Total ion chromatograms of the precursor ion scan of TAHS-derivatized deproteinized rat plasma (A) and deproteinized rat plasma (B): 1, histidine; 2, arginine; 3, serine; 4, glycine; 5, glutamine; 6, threonine; 7, glutamic acid; 8, alanine; 9, lysine; 10, proline; 11, cystine; 12, valine; 13, methionine; 14, tyrosine; 15, isoleucine; 16, leucine; 17, phenylalanine; 18, tryptophan.

Table 3. Quantitative Data for the Determination of 16 Amino Acids in Rat Plasma Samples ($N = 6$)

	concentration in plasma [μM]	RSD [%]	recovery [%]	internal standard
glycine	102.7	2	103	Gly-IS
alanine	195.6	2	102	Ala-IS
serine	79.6	2	104	Ser-IS
proline	65.5	1	93	Pro-IS
valine	79.0	3	103	Val-IS
threonine	88.8	3	104	Ser-IS
isoleucine	37.7	2	102	Leu-IS
leucine	68.3	3	102	Leu-IS
asparagine	47.8	5	104	Ser-IS
aspartic Acid	5.9	2	104	Gln-IS
lysine	81.9	4	99	Lys-IS
glutamine	134.0	4	97	Gln-IS
glutamic acid	125.8	3	101	Glu-IS
methionine	20.6	2	102	Met-IS
histidine	28.8	9	100	His-IS
phenylalanine	30.2	2	109	Phe-IS
arginine	41.6	6	99	Arg-IS
tyrosine	39.2	3	102	Pro-IS
tryptophan	141.1	2	93	Trp-IS
cystine	0.7	8	107	Lys-IS

distribution data derived from both intracellular free amino acids and proteinogenic amino acids measured using the precolumn LC/MS/MS method.[11]

Comparative Analysis of Amino Acids between TAHS and TAHS-d_3 Samples. In mass spectrometric studies, the reagents labeled with isotopes can be used in quantitative comparisons between samples. For example, isotope-coded affinity tag (ICAT)[13] reagents have been used in proteomics. Yang et al. synthesized isotope-labeled reagents of *N*-alkylnicotinic acid *N*-hydroxysuccinimide ester, for the comparative quantification of amino acids.[8]

In this study, a heavy reagent of TAHS, TAHS-d_3, was synthesized from *p*-*N*,*N*-dimethylaminoanilyl *N*′-hydroxysuccinimidyl carbamate and trideuterized methyl iodide.

Figure 4 shows the sample preparation scheme. One amino acid-containing solution was derivatized with TAHS, and another solution was separately derivatized with TAHS-d_3. Equivalent volumes of each derivatized sample were mixed, and the mixture was analyzed using the selected ion monitoring mode of LC/MS/MS. The detection channels of the product ions were set to the m/z values of TAHS-tagged amino acids and TAHS-d_3-tagged amino acids. The detection channels of the fragment ions were set to an m/z of 177 for the TAHS derivatives and an m/z of 180 for the TAHS-d_3 derivatives. Relative concentrations of the amino acids in the two samples were measured in a single analysis in order to compare the peak intensities derived from the two reagents.

Figure 4 shows the peak area ratio of the TAHS/TAHS-d_3 relative analysis. Each 10 μM amino acid mixture solution was derivatized with the same volume of the TAHS and TAHS-d_3 reagent and analyzed by LC/MS/MS. The mean ratio of six injections for each amino acid except tyrosine was 0.92−1.18. The ratio of the TAHS-tagged tyrosine to TAHS-d_3-tagged tyrosine was 0.77. The reason remains unexplained, and further experiments will be needed to the accuracy of the ratio to obtain optimized methods for this analysis.

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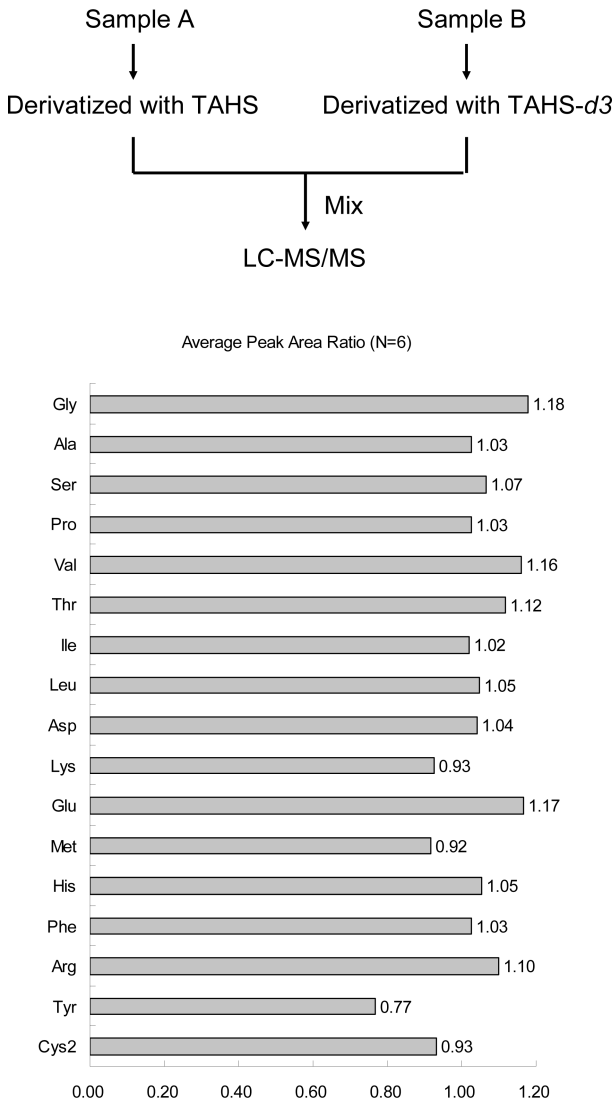

Figure 4. Peak area ratio of selected TAHS derivatives and TAHS-d_3 derivatives (TAHS derivatives/TAHS-d_3 derivatives). Two samples (10 μM amino acid mixture solution) were reacted with each reagent and mixed together before analysis.

When the amino acid standard solution was derivatized with TAHS-d_3 and the actual sample was derivatized with TAHS, we were able to calculate the concentrations of the amino acids in the sample with few ion suppression effects in the analysis. Multiple samples, derivatized with different mass numbers of TAHS reagent, analyzed in the single run will show the same matrix effects.

It would also be easy to compare the concentrations of several samples if other isotope-labeled reagents were used, such as TAHS-d_6 or TAHS-d_9.

We used TAHS-d_3 as the heavy reagent in this study because large isotope effects are known between deuterium and hydrogen, such as retention time shift. It is better to use ^{13}C instead of ^{12}C in the structure of the reagent, as they do not show chromatographic isotope effects, which derive from the interaction between the C−H or C−D bonds and the stationary phase of the reversed-phase HPLC column.[8,14]

CONCLUSIONS

We developed new derivatization reagents for amino acid analysis using LC/ESI-MS/MS. Subfemtomole to attomole levels of amino acid analysis were achieved using the precolumn reagent with cationic groups, TAHS, and selected ion monitoring by LC/MS/MS. This highly sensitive method for amino acid analysis may be utilized to determine the intracellular concentration of amino acids and amino acids present in biological fluids and various organisms where their concentrations are very low. TAHS may be utilized for highly sensitive analyses of proteomes, in combination with its deuterium isomer, TAHS-d_3.

The selective cleavage induced by derivatized reagents was reportedly useful for simultaneous detection during functional group-specific LC/MS/MS analyses.[15,16]

Although other labeling reagents, such as NBD-F or dansyl-Cl,[17,18] can be used in LC/MS/MS analysis to increase sensitivity and selectivity, NBD-F-labeled amino compounds show different fragmentation patterns and are difficult to apply to the precursor ion scan analysis or isotope ratio analysis. With the use of our reagents, the characteristic and selective cleavage of the ureide bond in the derivatives facilitated not only the simultaneous analysis of the compounds with amino groups using LC/MS/MS but also the measurement of the isotope distribution of amino acids. These analyses were performed without complex correction calculations, which was useful in the ^{13}C metabolic flux analysis. Selective cleavage induced by a precolumn reagent may be useful for other functional groups.

Comprehensive analysis approaches using LC/MS or NMR techniques have been widely applicable for the metabolome. Although these methods are useful in discrimination analysis or principal component analysis, there are too many compounds to identify key metabolites. Therefore, the strategy of selective analysis, featuring the functional group, using a combination of the precursor ion scan mode of tandem mass spectrometry with the developed reagent, will be more useful in offering practical knowledge for biochemical studies.

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