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N,N-Dimethyl Leucines as Novel Isobaric Tandem Mass Tags for Quantitative Proteomics and Peptidomics

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Herein, we describe the development and application of a set of novel *N,N*-dimethyl leucine (DiLeu) 4-plex isobaric tandem mass (MS^2) tagging reagents with high quantitation efficacy and greatly reduced cost for neuropeptide and protein analysis. DiLeu reagents serve as attractive alternatives for isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMTs) due to their synthetic simplicity, labeling efficiency, and improved fragmentation efficiency. DiLeu reagent resembles the general structure of a tandem mass tag in that it contains an amine reactive group (triazine ester) targeting the N-terminus and ε-amino group of the lysine side chain of a peptide, a balance group, and a reporter group. A mass shift of 145.1 Da is observed for each incorporated label. Intense a_1 reporter ions at *m/z* 115.1, 116.1, 117.1, and 118.1 are observed for all pooled samples upon MS^2. All labeling reagents are readily synthesized from commercially available chemicals with greatly reduced cost. Labels 117 and 118 can be synthesized in one step and labels 115 and 116 can be synthesized in two steps. Both DiLeu and iTRAQ reagents show comparable protein sequence coverage (~43%) and quantitation accuracy (<15%) for tryptically digested protein samples. Furthermore, enhanced fragmentation of DiLeu labeling reagents offers greater confidence in protein identification and neuropeptide sequencing from complex neuroendocrine tissue extracts from a marine model organism, *Callinectes sapidus*.

It has become increasingly important to determine relative abundance of protein or endogenous peptide expression levels in different biological states using mass spectrometry (MS). Numerous MS-based chemical derivatization quantitation approaches such as mass-difference labeling and isobaric labeling methodologies have been developed and widely used for quantitative proteomics and peptidomics.[1] Mass-difference labeling approaches introduce a mass difference for the same peptide by incorporating a light or heavy isotopic form of the labeling reagent. Light and heavy labeled peptides are combined prior to MS analysis, and quantitation is accomplished by comparing the extracted ion chromatogram peak areas of light and heavy forms of the same peptide. Methods such as isotope-coded affinity tags (ICAT),[2−4] stable isotope labeling with amino acids in cell culture (SILAC),[5] 4-trimethylammoniumbutyryl (TMAB) labels,[6] and reductive formaldehyde dimethylation[7] have been widely used in mass-difference quantitation proteomics. Although being well-established methodologies for quantitative proteomics, this mass-difference labeling has two general limitations. First, only a binary set of samples can be compared due to the use of light and heavy labeling of a peptide. Second, mass-difference labeling increases mass spectral complexity by introducing an extra pair of labeled peptides, thus decreasing the confidence and accuracy of quantitation. The first limitation has been addressed and overcome by several research groups by introducing multiple heavy labeled reagents, rather than just one.[8−10] The second limitation is an inherent drawback of the mass-difference approach, and the spectral complexity is increased with the use of multiple heavy isotope labeling reagents. This problem is solved by the isobaric labeling approach.

Tandem mass tags (TMTs) were the first isobaric labeling reagents used to improve the accuracy for peptide and protein quantitation by simultaneous identification and relative quantitation during MS^2 experiments.[11] Two generations of TMTs were reported (TMT1 and TMT2), and each generation had two isobaric labels. Amine groups (N-terminus and ε-amino group of the lysine side chain) in peptides labeled with TMT1 produce fragments at *m/z* 270 and 273 at 70 V collision energy, whereas TMT2 produces fragments at *m/z* 287 and 290 at 35 V collision

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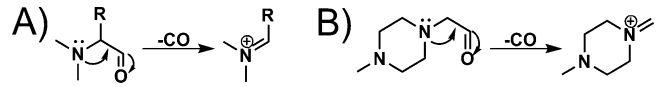
energy. Relative quantitation can be performed by comparing the intensities of these fragments to one another. A 6-plex version of TMTs was reported recently.[12] iTRAQ follows the same principle as TMTs quantitation, but it improves the quantitation further by providing four isobaric labels with signature reporter ions that are one Da apart upon MS^2 fragmentation.[13] iTRAQ allows for the quantitation of proteins present in four different biological states simultaneously. These tags are structurally identical isobaric compounds with different isotopic combinations. Each sample is labeled individually, pooled together, and introduced into the mass spectrometer for quantitative analysis. Since samples are isobarically labeled, the same peptide from four samples produces a single peak in MS mode, but upon MS^2 fragmentation, each labeled sample gives rise to a unique reporter ion (m/z = 114.1, 115.1, 116.1, and 117.1) along with sequence-specific backbone cleavage for identification. Relative quantitation is achieved by correlating the relative abundance of each reporter ion with its originating sample.

Isobaric MS^2 tagging approaches have been successfully used in MS-based quantitative proteomics. However, their application as a routine tool for quantitative MS studies is limited by high cost. The high cost of commercial TMTs and iTRAQ comes from the challenge of synthesizing these compounds; multiple steps involved in synthesis lead to moderate to low yield. A set of 6-plex deuterium-labeled DiART reagents was reported very recently with reduced cost of isobaric labeling. However, seven steps were still required to synthesize these compounds with 30%−40% overall yield.[14] A new type of isobaric MS^2 tags with fewer steps involved in synthesis is desirable to further reduce experimental cost while taking full technical advantages of the isobaric MS^2 tagging approach. Formaldehyde dimethylation represents one of the most affordable approaches among all isotopic chemical derivatization techniques used for MS-based peptide and protein quantitation.[10,15−29] However, isotopic formaldehyde labeling is a mass-difference labeling approach and, thus, lacks the advantages

Scheme 1. Formation of (A) Dimethyl Amino Acid a_1 Ion and (B) iTRAQ Reporter Ion



offered by the isobaric labeling approach. We have used a formaldehyde labeling technique for improved peptide fragmentation, enhanced neuropeptide de novo sequencing, and quantitation.[30−34] A notable feature of this labeling approach is the production of intense immonium a_1 ions when dimethylated neuropeptides undergo MS^2 dissociation.[30,35] The formation of the dimethylated a_1 ion is shown in Scheme 1A.[30,35] The structural similarity of iTRAQ reporter ion (Scheme 1B) and dimethylated amino acid a_1 ion inspired the design of novel dimethyl leucine isobaric MS^2 tags (DiLeu). This manuscript describes the development and evaluation of a set of 4-plex DiLeu reagents for protein quantitation and neuropeptide analysis.

EXPERIMENTAL SECTION

Materials. All isotopic reagents for the synthesis of labels including: leucines (L-leucine and L-leucine-1-^{13}C, ^{15}N), heavy formaldehyde (CD_2O), sodium cyanoborodeuteride ($NaBD_3CN$), ^{18}O water 97% ($H_2{}^{18}O$), and deuterium water (D_2O) were purchased from ISOTEC Inc. (Miamisburg, OH). *N*-Methylmorpholine (NMM) was purchased from TCI America (Tokyo, Japan). Chromasolv water, acetonitrile, and formic acid (FA) for UPLC were purchased from Fluka (Büchs, Switzerland). Neuropeptide standard allatostatin I (AST-I, GDGRLYAFGL-NH_2) and calmodulin dependent protein kinase substrate analog (PLRRTLSVAA-NH_2) were purchased from American Peptide Company (Sunnyvale, CA). Sequencing grade trypsin was purchased from Promega (Madison, WI). ACS grade methanol (MeOH), dichloromethane (CH_2Cl_2), acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF), and 4-(4, 6-dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) were purchased from Fisher Scientific (Pittsburgh, PA). Ethanol was purchased from Pharmco-AAPER (Brookfield, CT). Sodium cyanoborohydride ($NaBH_3CN$), ammonium formate, formaldehyde (CH_2O), tris-(2-carboxyethyl)phosphine (TCEP) (1 M, pH = 8.5), iodoacetamide (IAA), triethylammonium bicarbonate (TEAB), sodium dodecyl sulfate (SDS, ≥98%), α-cyano-4-hydroxy-cinnamic acid (CHCA), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). The 4-plex iTRAQ reagents were provided by Applied Biosystems (Foster City, CA). 2, 5-Dihydroxybenzoic acid (DHB) was obtained from MP Biomedicals, Inc. (Solon, OH).

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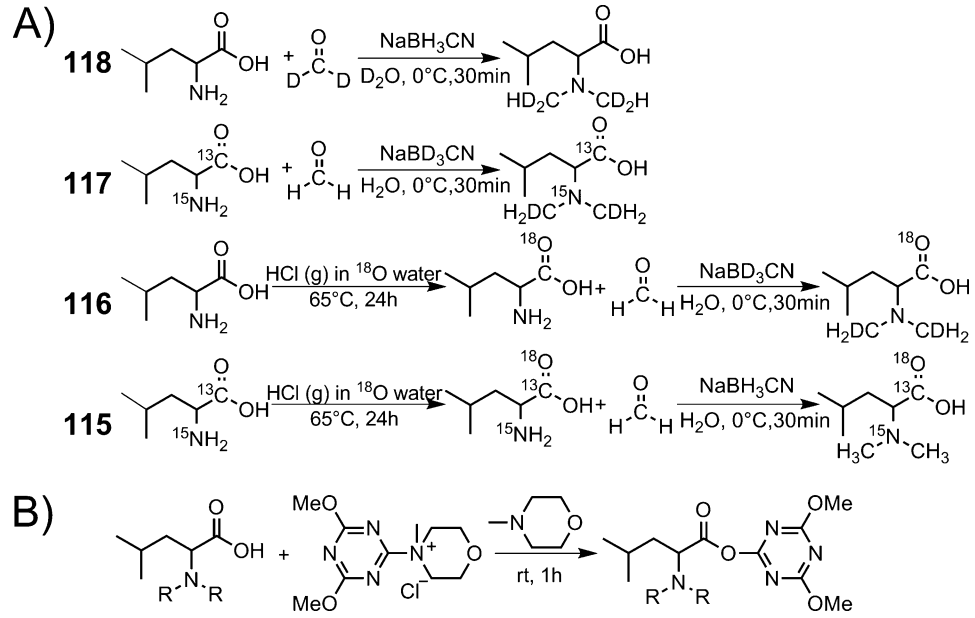
Scheme 2. (A) Synthesis of Isobaric Labels and (B) Activation Using 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride (DMTMM)/*N*-Methylmorpholine (NMM)



Synthesis of *N*,*N*-Dimethylated Leucine (DiLeu). A 120 mg portion of sodium cyanoborohydride ($NaBH_3CN$) was dissolved in 125 μL of H_2O or D_2O. A 100 mg portion of leucine or isotopic leucine was suspended in the mixture, the vial was sealed and mixture was kept in an ice−water bath for 30 min to cool down. Light formaldehyde (CH_2O, 285 μL, 37% w/w) or a heavy formaldehyde (CD_2O, 530 μL, 20% w/w) was then added to the mixture dropwise. The mixture was stirred in an ice−water bath for 30 min. The reaction was monitored by ninhydrin staining on a thin-layer chromatography (TLC) plate. The target molecule was purified by flash column chromatography (MeOH/CH_2Cl_2). Synthetic routes are shown in Scheme 2A.

Caution: Both formaldehyde and sodium cyanoborohydride are very toxic by inhalation, in contact with skin, or if swallowed and may cause cancer and heritable genetic damage. These chemicals and reactions should be handled in a fume hood.

^{18}O Exchange. ^{18}O exchange is required for both the 115 and 116 labels to have the same masses as labels 117 and 118. ^{18}O exchange was carried out according to a procedure previously reported.[36] Briefly, 25 mg of leucine or isotopic leucine was dissolved in 1N HCl $H_2{}^{18}O$ solution (pH 1) and stirred on a hot plate at 65 °C for 24 h, followed by reductive *N*,*N*-dimethylation. Synthetic routes are shown in Scheme 2A.

Synthesis of DiLeu Triazine Ester. One milligram of DiLeu in 50 μL of DMF was combined with 1.86 mg of DMTMM and 0.74 μL of NMM in a 1.6 mL eppendorf vial. Mixing was performed at room temperature for 1 h and stored at −20 °C until needed for future labeling. The general synthetic route is shown in Scheme 2B.

Protein Reduction, Alkylation, and Digestion. BSA (100 μg) aliquots were resuspended in 0.5 M TEAB, pH 8.5, and 2% (w/v) SDS, reduced with 5 mM TCEP for 1 h at 60 °C, and alkylated with 84 mM IAA for 10 min in darkness. After reduction and alkylation, the protein was digested overnight with trypsin at 37 °C at a ratio of 1:10 trypsin to protein. After digestion, samples were pooled and divided into 50 μg aliquots. Aliquots were then dried using a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation, West Palm Beach, FL) and redissolved in 10 μL of TEAB before labeling.

Neuropeptide Extraction. Pericardial organs (POs) of blue crabs were dissected out in chilled physiological saline and then immediately transferred to 20 μL of cold DMF. POs from twenty crabs were divided into four groups (five crabs per group). POs in each group were pooled after dissection and homogenized in a manual glass tissue homogenizer using 200 μL of DMF as the extraction buffer. The homogenate was centrifuged at 13 200 rpm for 10 min at room temperature in an Eppendorf 5415 D microcentrifuge (Brinkmann Instruments Inc., Westbury, NY). The supernatant was transferred to a clean 0.6 mL eppendorf vial and placed on ice. The pellet was then extracted 3 more times with 100 μL aliquots of DMF, and the supernatant from each extraction was combined. The resulting supernatant was concentrated to dryness using SpeedVac and resuspended in 10 μL of TEAB before labeling.

Protein Digest Labeling. In order to compare 4-plex quantitation between DiLeu and iTRAQ, two 4-plex sets of digested protein BSA aliquots (50 μg) were labeled with DiLeu and iTRAQ reagents. DiLeu labeling was performed by transferring 25 μL of labeling solution into the sample vial. iTRAQ labeling was performed by placing 35 μL of ethanol solubilized reagent in the sample vial. Both sample sets were labeled at room temperature for 1 h and quenched for 30 min by adding 100 μL of water. Labeled samples were combined at equal ratios (1:1:1:1) and dried under SpeedVac.

Neuropepide Extract Labeling. The same DiLeu labeling procedure outlined above for protein digest labeling was used to label neuropeptide extracts from blue crab POs.

Strong Cation Exchange Chromatography. Both iTRAQ and DiLeu labeled peptides were fractionated by strong cation exchange (SCX) liquid chromatography using PolySULFOETHYL A 200 mm × 2.1 mm, 5 μm, 300 Å column (PolyLC, Columbia,

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MD). Buffer A was 25 mM ammonium formate, 10% (v/v) acetonitrile, pH 2.9, and buffer B was 25 mM ammonium formate, 500 mM ammonium formate, 10% (v/v) acetonitrile, pH 4.3. The dried labeled samples were resuspended in buffer A and loaded onto the SCX column. After sample loading and washing with buffer A for 3 min, buffer B concentration was increased from 0% to 50% in 20 min and then increased to 100% at a flow rate of 0.2 mL/min. Twelve fractions were collected based on a SCX chromatogram, combined, and then followed by drying under SpeedVac.

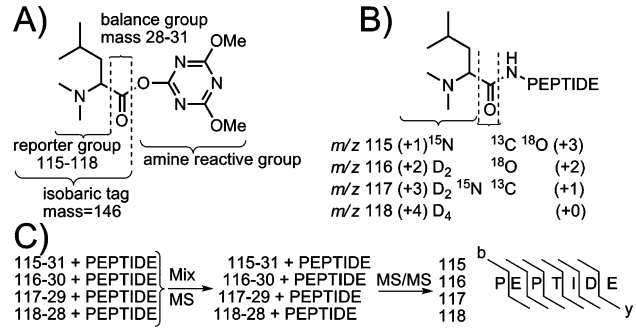
Reversed Phase NanoLC ESI MS/MS. Tryptic peptide and neuropeptide labeled samples were analyzed using a Waters nanoAcquity UPLC system coupled online to a Waters Micromass QTOF mass spectrometer (Waters Corp., Milford, MA). Tryptic peptide samples were dissolved in 0.1% formic $acid_{(aq)}$ (FA), injected, trapped onto a C_{18} trap column for 10 min (Zorbax 300SB-C_{18} Nano trapping column, Agilent Technologies, Santa Clara, CA), and eluted onto a homemade C_{18} column (75 μm × 100 mm, 3 μm, 100 Å) using a linear gradient (300 nL/min) from 5% buffer B (0.1% FA in ACN) to 45% buffer B over 33 min. The linear gradient used for labeled neuropeptide extract solutions was from 5% buffer B to 45% buffer B over 60 min. Buffer A used was 0.1% FA in H_2O. Survey scans were acquired from m/z 400−1800 with up to two precursors selected for MS^2 from m/z 50 to 1800 with 3 min dynamic exclusion. iTRAQ used a collision energy 20% greater than that used for DiLeu.

MALDI FTICR MS. The analysis of DiLeu labeled peptide standard was performed using a Varian/IonSpec Fourier transform mass spectrometer (Lake Forest, CA) equipped with a 7.0 T actively shielded superconducting magnet. A 355 nm Nd:YAG laser was used for ionization/desorption. A saturated solution of 2,5-dihydroxybenzoic acid (DHB, 150 mg/mL of 50: 50 H_2O/MeOH, v/v) was used as the matrix for sample spotting. Spectra were collected in positive ion mode.

MALDI TOF/TOF MS. A model 4800 MALDI TOF/TOF analyzer (Applied Biosystems, Framingham, MA) equipped with a 200 Hz, 355 nm Nd:YAG laser was used. Acquisitions were performed in positive ion reflectron mode. Instrument parameters were set using the 4000 Series Explorer software (Applied Biosystems). Mass spectra were obtained by averaging 900 laser shots covering mass range m/z 500−4000. MS^2 was achieved by 1 kV collision induced activation (CID) using air as collision gas. For sample spotting, equal volumes of a 0.4 μL sample solution and α-cyano-4-hydroxy-cinnamic acid (CHCA) matrix solution in 60% ACN were mixed and allowed to dry prior to analysis.

Data Analysis and Quantitation. DiLeu reagent labeled neuropeptide standards were identified and sequenced using Waters Masslynx peptide sequencing software (PepSeq). BSA tryptic peptide identifications were performed using the X! Tandem search engine (The Global Proteome Machine Organization). The database search of labeled BSA was restricted to tryptic peptides of BSA sequence FASTA file. Carbamidomethyl on cysteine was selected as fixed modification. N-terminal, lysine, tyrosine DiLeu modifications and methionine oxidation were selected as variable; one missed cleavage was allowed, and the precursor error tolerance was set to <100 ppm. Identified labeled peptide spectra were converted to dta files using PLGS 2.1 software (Waters) and quantified by comparing the intensities of the reporter ions using Quant MATLAB scripts.[37] Reporter ions were changed to 115.1, 116.1, 117.1, and 118.1 with a peak detection window of 0.1 Da. Isotope correction factors were applied to correct the peak intensities calculated for each reporter ion to account for the losses to, and gains from, other reporter ions.

Scheme 3. General Structure of Dimethyl Leucine Isobaric Tags Is Shown in (A). Formation of New Peptide Bond at N-Terminus or ε-Amino Group of the Lysine Side Chain and Isotope Combination of Isobaric Tags Is Illustrated in (B). Quantitation of 4-plex Isobarically Labeled Peptide Is Illustrated in (C)



RESULTS AND DISCUSSION

Rationale for the Development of DiLeu Reagents. It was previously shown that the dimethylated a_1 ion can be used as a peptide quantitiation tool due to its high intensity.[7] We also observed that MS^2 of dimethylated neuropeptides with leucine at the N-termini yielded the most intense dimethylated a_1 ions as compared to other N-terminal amino acids. The formation of the iTRAQ reporter ions (as shown in Scheme 1B) shares the same mechanism as the dimethylated amino acid a_1 ion. The difference between these two ions is that the iTRAQ reporter ion is a cyclic immonium ion, and dimethylated a_1 ion is an amino immonium ion. Given that a dimethylated leucine provides an intense a_1 ion which shares the same formation mechanism as an iTRAQ reporter ion and most importantly isotopic leucines with different isotope combinations are commercially available, we propose the design and development of a set of novel DiLeu 4-plex isobaric MS^2 tags. These new tags resemble the structure of the original iTRAQ reagent (reporter group, balance group, amine reactive group) but differ in the use of isotopically dimethylated leucine a_1 ions as signature reporter ions.[34] The structure of our novel isobaric tag, labeling mechanism, and 4-plex quantitation is shown in Scheme 3. The proposed design of the first set of isotopic dimethylated amino acid reagents for isobaric tagging are based on the following considerations: (1) Methods of coupling amino acid to peptides are well established; (2) Intense reporter ion (dimethylated leucine a_1 ion) is generated upon tandem MS fragmentation;[5,28] (3) Isotopic leucines are commercially available which will simplify the preparation of isotopic reporter ions.

Isobaric DiLeu Reagent Synthesis. Labels 117 and 118 (molecular weight = 163) are isobaric after one step synthesis. Labels 115 and 116 (molecular weight = 161) need ^{18}O exchange to incorporate an ^{18}O atom to the carbonyl group to make all

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four labels isobaric. The dimethylation of leucines gave yields from 85−90% after purification by column chromatography. The yield of the ^{18}O exchange experiment was monitored by direct infusion of the labels on Waters Micromass QTOF mass spectrometer. Since after 24 h no unexchanged DiLeu was detected in the direct infusion experiment, we concluded that the ^{18}O exchange rate was quantitative. Because the molar ratio between $H_2{}^{18}O$ to DiLeu is 224:1 (i.e., large excess of $H_2{}^{18}O$ was used), the exchange was pushed to completion despite that the purity of $H_2{}^{18}O$ is 97%. In general, the yield limiting step was the column purification. Because dimethylated leucine was retained strongly on the normal phase flash column, though a large amount of solvent ($MeOH/CH_2Cl_2$) was used to elute the compound off the column, the recovery of DiLeu was not quantitative. Our tags are very stable until activation for labeling. The very first compound we synthesized was two and a half years old and still works great.

Synthesis of DiLeu Amine Reactive Triazine Ester. Due to the high polarity of dimethylated leucine, reaction conditions worked for *tert*-butyloxycarbonyl (Boc) protected leucine *N*-hydroxysuccinimide (NHS) ester activation but did not work on DiLeu NHS ester activation. DMTMM was reported as an in situ condensation reagent for polar substrates with good yield.[38−41] Our experiment showed that DiLeu can be activated by DMTMM/NMM in DMF, and a time course study indicated this reaction reached equilibrium in an hour. Several reactions suggesting a two-step condensation by DMTMM were reported previously.[42−44] Briefly, the acid moiety was first activated with DMTMM/NMM and followed by addition of amines to the reaction mixture. Purification of activated ester was not necessary according to these publications. The activated ester was not purified in our experiments presented here. The quick activation time and absence of purification step in ester preparation provide the advantage of easy access of our labeling reagents. Each label can be freshly activated before use. A preliminary stability test of the activated ester was carried out as follows: The same stock solution was used to label neural tissue extract aliquot a week apart. The freshly made ester showed effective labeling of our sample; a week later, double the amount was needed to achieve acceptable labeling. We suspected that the formed active ester was hydrolyzed over time due to the trace amount of water produced in the DiLeu activation process. After adding $MgSO_4$ to the freshly made labeling solution to dry the labeling solution, the stock labeling solution showed the same labeling efficiency after a week. In general, the activated ester was suitable for labeling for approximately a week when stored in a −20 °C refrigerator with $MgSO_4$.

Peptide Labeling Efficacy. MALDI-FTICR MS detection of labeled neuropeptide allatostatin I (AST-I) showed quantitative labeling within 1 h (Figure 1A,B). Calmodulin dependent protein kinase substrate analog (CaM, m/z 1082.68) was used to test the labeling efficiency of secondary amine. Quantitative conversions of CaM and other two unknown impurities (m/z 1172.66, 1236.65) were detected by MALDI-FTICR MS (Figure 1C,D). Complete labeling of proline residue at the N-terminus of CaM peptide demonstrates high reactivity of triazine ester to secondary amine group. LC-MS/MS analysis of iTRAQ and DiLeu labeled BSA tryptic peptides indicated complete labeling within the same amount of time. LC-MS/MS data was analyzed by a X! Tandem database search using searching parameters described in the Experimental Section. Twenty-three peptides were identified as unique BSA tryptic peptides with a log(e) < −2 cutoff for both iTRAQ and DiLeu labeled samples. Fourteen of these peptides (~61%) were found overlapping (Table 1). Both iTRAQ and DiLeu showed the same sequence coverage at ~43%. The tyrosine side reaction and unlabeled N-termini or lysine side chain were explored. A minimal degree (<3%) of tyrosine derivatization and unlabeled N-termini were found in DiLeu labeled peptides, and reaction with serine or threonine was not observed. In Table 1, the database search results showed that among the labeled tryptic peptides only LCVLHEK was not completely labeled at its N-terminus and the remaining peptides were completely labeled. Equivalent protein sequence coverage and minimal side reactions demonstrate that DiLeu reagents have comparable performance and labeling efficiency to iTRAQ reagents.

In our experiment, the molar ratio of DMTMM to DiLeu is 1.1:1. The remaining DMTMM is very likely to react with other carboxylates of peptides to form the peptide triazine ester in subsequent tagging reactions. However, since the activated DiLeu triazine ester is very reactive to amine group, when activated DiLeu is mixed with peptides, a coupling reaction takes place immediately. Most peptides can be completely labeled by DiLeu within an hour. The side reaction of DMTMM to the carboxylates of labeled peptides can be hydrolyzed by adding a 0.1% FA solution. The trace amount of unlabeled peptides can also be activated by remaining DMTMM and undergo a peptide cyclization reaction. The cyclization reaction was investigated using high concentration peptide standards (10^{-4} M). However, no cyclized peptide was detected in our quantitative experiment.

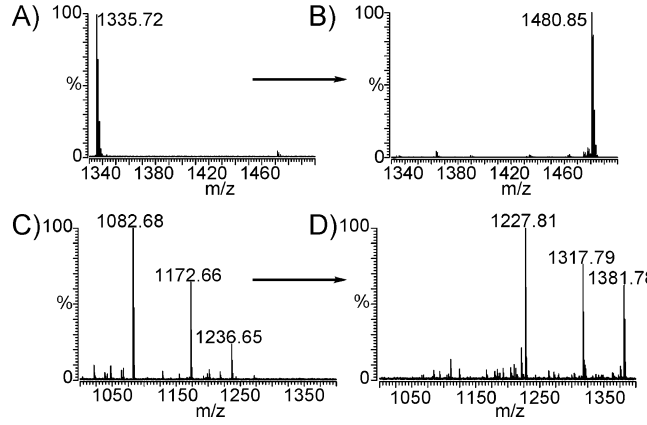

Figure 1. MALDI-FTICR mass spectra of neuropeptide allatostatin (AST)-I (APSGAQRLYGFGL-NH_2) (A) before and (B) after DiLeu labeling and CaM (PLRRTLSVAA-NH_2) (C) before and (D) after DiLeu labeling, 145.1 Da mass shift is produced for each incorporated label.

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Table 1. Identified iTRAQ and DiLeu Labeled BSA Tryptic Peptides by X! Tandem

	Sequence	iTRAQ[a]	DiLeu[a]
1	HLVDEPQNLIK	–4.4	–5.1
2	YICDNQDTISSK[b]	–3.7	–6.3
3	LKPDPNTLCDEFKADEK[b]	–5.2	–6.2
4	LKPDPNTLCDEFK[b]	–4.4	–5.1
5	LVNELTEFAK[b]	–7.0	–6.0
6	MPCTEDYLSLILNR[b]	–6.4	–6.3
7	LVTDLTK[b]	–2.3	–2.3
8	AEFVEVTK	–2.3	–2.1
9	ECCHGDLLECADDR	–4.8	–6.9
10	RPCFSALTPDETYVPK[b]	–3.0	–4.8
11	LGEYGFQNALIVR[b]	–7.5	–5.7
12	QTALVELLK[b]	–4.1	–3.8
13	FYAPELLYYANK[b]	–2.5	–5.6
14	KVPQVSTPTLVEVSR	–4.9	–5.0
15	ECCDKPLLEK	–6.5	n/a
16	KQTALVELLK	–5.3	n/a
17	DDPHACYSTVFDK	–4.7	n/a
18	VHKECCHGDLLECADDR	–4.1	n/a
19	EACFAVEGPK	–4.1	n/a
20	TVMENFVAFVDK	–3.5	n/a
21	EYEATLEECCAK	–3.3	n/a
22	VPQVSTPTLVEVSR	–3	n/a
23	SLHTLFGDELCK	–2.7	n/a
24	CCAADDKEACFAVEGPK	n/a	–10.4
25	TCVADESHAGCEK	n/a	–9.6
26	DAIPENLPPLTADFAEDKDVCK	n/a	–5.6
27	DAFLGSFLYEYSR	n/a	–4.7
28	LCVLHEK	n/a	–2.9
29	GACLLPK	n/a	–2.5
30	QNCDQFEK	n/a	–2.5
31	ATEEQLK	n/a	–2.4
32	NECFLSHK	n/a	–2.1

[a] Note: Log(e) value indicates the confidence of identified peptide; the lower the value, the higher is the confidence for peptide identification. [b] Unfragmented reporter group intensity is higher than the reporter ions labeled by iTRAQ.

Another way to eliminate this problem is to purify the activated ester before labeling by adding 1 mL of CH_2Cl_2 followed by 100 μL of H_2O to the ester activation mixture. After vortexing, the mixture is then separated into two phases; the upper phase is water and DMF, and the lower phase is CH_2Cl_2. Since DMTMM and NMM are water-soluble, removing water and the DMF phase can completely remove the excessive amount of DMTMM and NMM and leave only activated ester in CH_2Cl_2 phase. CH_2Cl_2 can be easily blow dried under nitrogen.

Fragmentation of Labeled Peptides. Besides equivalent labeling efficiency as compared to iTRAQ reagents, DiLeu reagents produced better fragmentation and higher reporter ion intensities than iTRAQ reagents for labeled tryptic peptides, thereby offering improved confidence for peptide identification and more reliable quantitation. There were 14 overlapping labeled peptides between iTRAQ and DiLeu labeling (first 14 peptides in Table 1); 9 out of 14 individual peptides labeled by DiLeu had higher scores than iTRAQ labeled ones. Also, the sum of scores of those 14 peptides were −63 (iTRAQ) and −71.2 (DiLeu), respectively. DiLeu labeling showed ~13% increase of confidence for peptide identification than iTRAQ labeling.

To directly compare the labeled peptide fragmentation and reporter ion intensities by these DiLeu and iTRAQ labeling, de novo sequencing of all those 14 common peptides using PepSeq software was performed. The de novo sequencing results indicated that DiLeu 4-plex labeled peptides showed more intense reporter

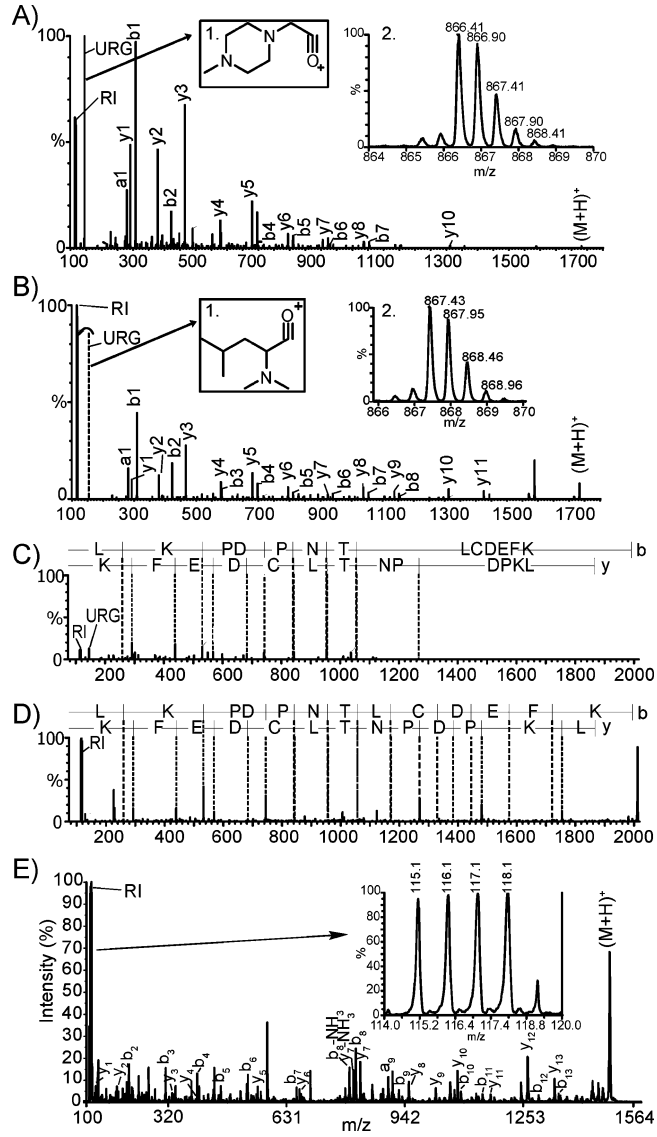

Figure 2. ESI QTOF de novo sequenced MS^2 spectra BSA tryptic peptide (YICDNQDTISSK) labeled by (A) iTRAQ 4-plex and (B) DiLeu 4-plex. General structures of unfragmented reporter group (URG) of iTRAQ (inset 1 in A) and DiLeu (inset 1 in B) are shown. Peptides were isobarically labeled by iTRAQ (inset 2 in A) and DiLeu (inset 2 in B). (C) Low reporter ion (RI) intensities, presence of URG, and incomplete fragments of b and y ions are shown for iTRAQ 4-plex labeled BSA tryptic peptide (LKPDPNTLCDEFK). (D) Intense RI, absence of URG, and a complete set of b and y ions are shown for the same peptide labeled by 4-plex DiLeu. (E) MALDI TOF/TOF MS^2 spectrum of AST-I labeled by DiLeu 4-plex showing quantitation capability in inset.

ions than iTRAQ 4-plex labeled tryptic peptides (Figure 2A,B), resulting from complete fragmentation and production of DiLeu reporter ions. It is worth noting that even after 20% increase of collision energy the peptides labeled by iTRAQ still had an unfragmented reporter group (m/z 145.1, general structure shown in Figure 2A inset 1) whereas no unfragmented DiLeu reporter group (m/z 146.1, general structure shown in Figure 2B inset 1) was found in DiLeu labeled peptide spectra at normal collision energy. Ten out of those fourteen iTRAQ labeled peptides noted in Table 1 had unfragmented reporter groups with intensities higher than the reporter ions indicating less efficient fragmenta-

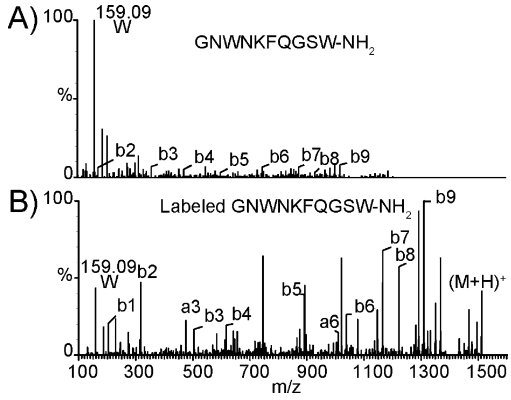

Figure 3. ESI QTOF MS^2 fragmentation (A) before and (B) after DiLeu labeling of B-type allatostatin neuropeptide (GNWNKFQGSW-NH_2).

tion of iTRAQ reporter group (Table 1). In contrast, the DiLeu reporter ions have significantly higher S/N ratios than iTRAQ reporter ions due to complete fragmentation of DiLeu reporter groups. Production of abundant reporter ions is likely to contribute to more robust and reliable quantitation. Improved fragmentation of DiLeu labeled peptide is shown in Figure 2. A complete set of y ions and more balanced fragmentation ions were observed in a DiLeu labeled peptide (Figure 2B,D), whereas fewer y ions were seen for iTRAQ labeled peptides (Figure 2A,C). The fragmentation efficiency of DiLeu labeled neuropeptide allatostatin I (AST-I) was also tested on a MALDI-TOF/TOF MS instrument. The tandem MS spectrum exhibited abundant reporter ions and complete y ion series with typical collisional induced dissociation parameter setting (Figure 2E). Here, abundant reporter ion counts induced by normal collisional energy on the MALDI TOF/TOF platform offer an advantage of reliable quantitation while maintaining sequence-specific fragmentation information using DiLeu labeling reagents, indicating applicability to a wide range of MS platforms.

The enhanced fragmentation has proven to be very helpful in determining the crustacean neuropeptides sequence due to the current lack of database for crustacean model organisms. De novo sequencing is the main tool to interpret neuropeptide MS^2 spectra. DiLeu labeling also improves neuropeptide MS^2 fragmentation. Figure 3 compares the MS^2 spectra of a B-type allatostatin (AST-B) neuropeptide (GNWNKFQGSW-NH_2) before and after DiLeu labeling acquired on an ESI QTOF mass spectrometer. As shown, b ions are substantially enhanced after labeling, which facilitates improved de novo sequencing of neuropeptides. Greater confidence in peptide identification using DiLeu labeling reagents demonstrates that DiLeu provides an attractive alternative reagent for iTRAQ reagent by providing enhanced fragmentation.

Peptide and Protein Quantitation and Reproducibility. Representative aliquots (1:1:1:1) enable a quantitative comparison of BSA tryptic peptides labeled by either iTRAQ or DiLeu reagents. The results are shown in Figure 4A,B with mean values and standard deviations shown in insets. Fourteen overlapping BSA tryptic peptides were selected to calculate peptide ratios. The means and standard deviations of peptide ratios were used to quantify BSA. Furthermore, relative quantitation of the same peptide labeled at ratio 1:5:2:10 by DiLeu was also investigated

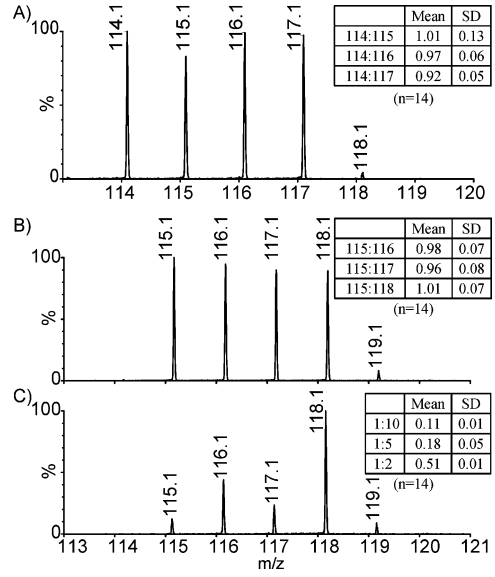

Figure 4. Quantitation of BSA tryptic peptide (YICDNQDTISSK) using (A) iTRAQ and (B) DiLeu. Quantitative dynamic range of the same peptide is illustrated in (C). Means and standard deviations of BSA are shown in insets.

Table 2. Reproducibility of DiLeu Labeled BSA Tryptic Fragments ($N = 14$)

		mean	SD
replicate 1	115:116	0.98	0.07
	115:116	0.96	0.07
	115:116	0.93	0.05
replicate 2	115:117	0.96	0.08
	115:117	0.96	0.08
	115:117	0.98	0.07
replicate 3	115:118	1.01	0.07
	115:118	0.93	0.08
	115:118	0.97	0.06

for linear dynamic range of quantitation (Figure 4C). Quantitation reproducibility was tested in triplicates. Ratios of each quantitation are listed in Table 2. DiLeu shows robust quantitation and excellent reproducibility and has comparable performance as that of commercial iTRAQ reagents (shown in Figure 4 and Table 2). It is worth noting, however, that the average cost of a set of 4-plex DiLeu labels for 100 μg of protein tryptically digested peptide is estimated to be about ten dollars, whereas the same amount of iTRAQ reagents costs more than two hundred dollars. The reduced cost for reagent production coupled with excellent accuracy and reproducibility for quantitation makes the new DiLeu reagents an attractive alternative for protein and peptide quantitation for biological samples.

Crab Neuropeptide Identification and Quantitation. In order to evaluate individual variability in neuropeptide expression, we employed 4-plex DiLeu reagents to label the pericardial organ (PO) extracts from four groups of blue crabs (5 crabs each), *Callinectus sapidus*. A total of 131 MS^2 spectra were acquired in a single LC/MS/MS run and about 95% of the spectra contained DiLeu 4-plex signature reporter ions, indicating that DiLeu reagents can efficiently label neuropeptides present in complex tissue extracts. Six B-type allatostatins (AST-B) and three orcokinins were de novo sequenced in their labeled forms. B-type AST peptides exhibit characteristic sequence motifs of

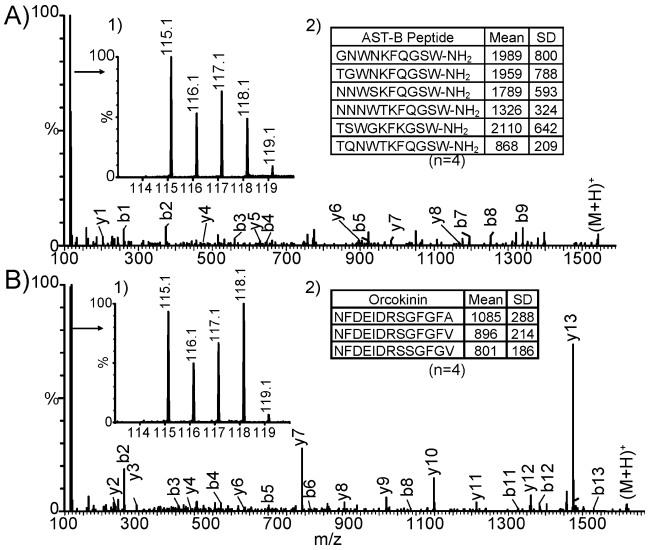

Figure 5. ESI QTOF MS^2 spectra of neuropeptides from the pericardial organ tissue extract of blue crab *C. sapidus*. DiLeu 4-plex labeled (A) B-type allatostatin neuropeptide ($GNWNKFQGSW\text{-}NH_2$) and (B) orcokinin neuropeptide (NFDEIDRSGFGFA). Relative abundance changes for neuropeptide expression levels among different animals are highlighted in inset tables.

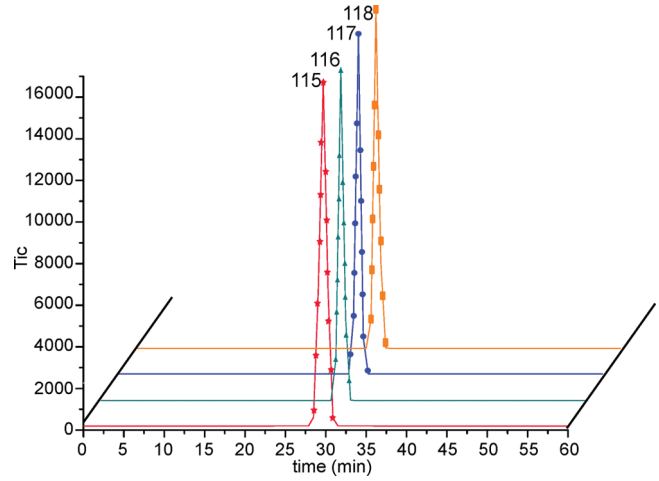

Figure 6. DiLeu 4-plex labeled AST-I peptides show negligible retention time difference (29.65 min) of extracted ion chromatograms.

$W(X)_6$Wamide, where X represents variable amino acid residues. Since the first report of AST-B in crustacean,[45] numerous studies have documented the widespread presence of this novel class of peptides in various decapod crustaceans and have shown the inhibitory effects that these peptides have on the crustacean pyloric neural circuits.[46−49] Here, using the DiLeu labeling reagent reported for the first time, the identification of six AST-B peptides in a commercially important crustacean species, *Callinectus sapidus*, is demonstrated. Figure 5A shows a representative de novo sequencing spectrum from AST-B. Similarly, Figure 5B shows a representative tandem mass spectrum of an orcokinin peptide in *C. sapidus*, another highly conserved neuropeptide family found in numerous crustaceans with myotropic activities.[50−53] Four biological replicates were performed and reporter ion counts were compared, showing a maximum of 2-fold expression differences for several isoforms in both neuropeptide families (Figure 5 insets 1 in A and B). Means and standard deviations (SD) of reporter ion counts were calculated across four samples (Figure 5 insets 2 in A and B). These preliminary results suggest individual animal variability and their potential impact on neuropeptide quantitation at different physiological states.

Deuterium Atom Effect of Labeled Peptides. It is known that deuterium affects the retention time of small- to intermediate-sized peptides in reversed phase chromatography.[54] Obviously, different retention times of these isobarically labeled peptides will affect the accuracy of quantitation if not properly adjusted. However, it was reported that the deuterium effect can be minimized to acceptable values by grouping deuterium atoms around polar functional groups which both minimizes their interaction with the stationary phase and reduces the isotope effect.[6] Using a relatively small number of deuterium atoms in labeling reagents should also help to minimize the isotope effect. In our 4-plex DiLeu tags, the greatest difference of deuterium atoms exists between tags 115 and 118 (4 deuterium atoms located around the more polar dimethylated amine group). The increased polarity of the amine group offsets the small deuterium number difference in our tags. Therefore, peptides labeled with the four DiLeu tags showed negligible differences in retention time on RP chromatography as shown in Figure 6.

CONCLUSIONS

We have successfully developed a novel class of isobaric tandem mass tagging reagents based on isotope-encoded dimethylated amino acid (leucine), DiLeu. Several features of the four-plex DiLeu reagents are summarized below: First, our reagents maintain the isobaric quantitation features present in the iTRAQ reagents, with comparable performance and greatly reduced cost. This makes the DiLeu tandem MS tags a more affordable tool for routine quantitation and methodology development as compared to commercially available reagents. Second, all four reagents can be easily synthesized from commercially available isotope reagents in regular analytical lab settings. Labels 117 and 118 require only one step synthesis and labels 115 and 116 require two steps of synthesis. Third, our methodology offers new

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opportunities to develop an array of natural amino acid-based tagging reagents including mass-difference and isobaric tags. Besides multiplex quantitation, these dimethylated amino acid tags also promote enhanced fragmentation, thereby allowing more confident protein identification from tryptic peptides and de novo sequencing of neuropeptides. Fourth, quantitation data processing can be easily adopted from freely available software. We have demonstrated the utility of these novel DiLeu labeling reagents for protein quantitation and neuropeptide sequencing from complex tissue extracts. Future work will be conducted to extend this methodology to large-scale quantitative proteomics and peptidomics as well as other amino acid-based isobaric tagging strategies for multiplexed quantitation and sequencing.

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