Synthesis, Purification, and Mass Spectrometric Characterization of Stable Isotope-Labeled Amadori-Glycated Phospholipids

Xiaobo He† and Qibin Zhang*†‡

†Center for Translational Biomedical Research, University of North Carolina at Greensboro, North Carolina Research Campus, Kannapolis, North Carolina 28081, United States
‡Department of Chemistry & Biochemistry, University of North Carolina at Greensboro, Greensboro, North Carolina 27412, United States

ABSTRACT: Nonenzymatic glycation of lipids plays an important role in several physiological and pathological processes, such as normal aging and complications of diabetes mellitus. To develop liquid chromatography coupled with mass spectrometric (LC-MS) methods for accurate analysis of Amadori compound-glycated lipids from biological samples, it is essential to obtain isotope-labeled Amadori-lipid standards. Herein, we report optimized methods for the preparation of six stable isotope-labeled Amadori-glycated lipid standards covering four types of lipids, including [13C6]Amadori-phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), -LysoPE, and -LysoPS. Optimal conditions for the synthesis and purification of these four types of Amadori-glycated lipids were detailed in this study. LC-MS and LC-UV analyses showed that destination products were highly purified (＞95%). Accurate mass and MS/MS fragmentation in both positive- and negative-ion modes further validated the identification of these six synthetic [13C6]Amadori-glycated lipid standards. Successful preparation of these highly purified isotope-labeled standards makes it possible to develop targeted LC-MS/MS methods for accurate analysis of Amadori-glycated phospholipids from biological samples.

1. INTRODUCTION

Nonenzymatic glycation, generally known as the Maillard reaction, is triggered by reaction of amino group in biomolecules and carbonyl function of reducing sugars (glucose, fructose, ribose, etc.) to form an unstable Schiff base, followed by the Amadori rearrangement reaction to form a more stable Amadori product. Furthermore, Amadori compounds can undergo complex reactions to form advanced glycation end products (AGEs). The Amadori-modified compounds and AGES are implicated in the pathogenesis of age-related diseases and complications of mellitus.

The Maillard reaction of proteins and peptides has attracted the most attention in glycation and advanced glycation studies. However, aminophospholipids are also important targets of non-enzymatic glycation. For example, the nonenzymatic glycation of membrane lipids can cause peroxidation of proteins and membrane lipids, inactivation of receptors, and other membrane dysfunctions, which are involved in various physiological and pathological processes, such as aging, diabetes, atherogenesis.

In vitro, Amadori products were reported with the ability to generate reactive oxygen species, which can further lead to lipid peroxidation involved in many physiological and pathological processes.

To better understand the roles of Amadori-glycated lipids in physiological and pathological processes, it is essential to develop analytical methods for a comprehensive profiling of Amadori-glycated lipid species in biological samples. To date, reported methods using liquid chromatography coupled with mass spectrometric (LC-MS) technique have focused on Amadori-phosphatidyl ethanolamine (PE) with a few identified molecular species. For lipidomic level investigation on Amadori-glycated lipids, LC-MS/MS methods with high sensitivity should be developed for measuring various types of Amadori lipids. To this end, it is necessary to obtain stable isotope-labeled Amadori-glycated lipid standards with high purity.

Several preparation methods for Amadori-PE were already reported. However, these methods are not well suited for preparing stable-isotope-labeled standards, in that either a complex process of six steps is used or synthesis is carried out in methanol (MeOH) phosphate buffer (PB) medium, which requires a long reaction time (15 days) and a high amount of glucose (500 mM), the latter is prohibitive for isotope-labeled Amadori compound synthesis due to the high cost of [13C6]glucose. A revised method using MeOH medium was also reported for the synthesis of Amadori-PE, which
requires a shorter reaction time (12 h) and less amount of glucose (100 mM). In spite of the methods mentioned above, no data are available about how yields change under various conditions, such as temperature, reaction duration, the ratio of reactants, and reaction medium. Considering the high cost of [13C6]glucose, optimal conditions are required for the preparation of [13C6]Amadori-PE standards. In addition, to our knowledge, preparation of Amadori-phosphatidyl serine (PS), LysoPE, and -LysoPS has not been reported yet. Herein, we present optimized methods for the preparation of [13C6]Amadori-PE, -PS, -LysoPE, and -LysoPS with high purity. The identification of these synthetic [13C6]Amadori-lipid standards was performed by LC-MS/MS analysis in both positive- and negative-ion modes, and distinctive ions were identified for [13C6]Amadori-modified lipids.

2. MATERIALS AND METHODS

2.1. Chemical and Solvent. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE(16:0/18:1(9Z))), 1, 2-dipentadecanoyl-sn-glycero-3-phosphoethanolamine (PE(15:0/15:0)), 1-tridecanoyl-sn-glycero-3-phosphoethanolamine (LysoPE(13:0)), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (PS(16:0/18:1(9Z))), 1,2-dihexadecanoyl-sn-glycero-3-phospho-L-serine (PS(17:0/17:0)), and 1-tridecanoyl-sn-glycero-3-phospho-L-serine (LysoPS(13:0)) were purchased from Avanti Polar Lipids (Abalaster, AL). [U-13C6]-d-glucose (99%) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Butylated hydroxytoluene 2,6-di-tert-butyl-p-cresol (BHT) and all solvents, including MeOH, acetonitrile (ACN), isopropanol (IPA), chloroform (CHCl3), and water (H2O), of LC-MS grade and high performance liquid chromatography (HPLC) grade were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Optimization of Synthetic Conditions. On the basis of the work of Miyazawa and co-workers,15,17,18 we carried out a series of incubations to optimize conditions for the synthesis of [13C6]Amadori-modified lipids. To find which medium (MeOH or MeOH-PB) is better for the synthesis of [13C6]Amadori-lipids and whether BHT could increase the reaction yield, three incubations were performed as follows: (A) 7 μmol PE(16:0/18:1(9Z)) was mixed with 537 μmol [13C6]d-glucose in 5 mL of MeOH-0.1 M PB (1:2, v/v, pH 8) at 37 °C; (B) 7 μmol PE(16:0/18:1(9Z)) was mixed with 107 μmol [13C6]d-glucose in 1 mL of MeOH at 37 °C with and without 0.3 mg of BHT.

To further optimize the reaction temperature and time, 7 μmol PE(16:0/18:1(9Z)) was mixed with 107 μmol [13C6]d-glucose in 1 mL of MeOH and the mixture was stirred at 600 rpm in a Thermomix mixer (Eppendorf, Germany) at 37, 50, or 60 °C. At different time intervals (0–4 days), aliquots of reaction mixtures were collected for analysis. Incubations of [13C6]d-glucose with LysoPE(13:0), PS(16:0/18:1(9Z)), and LysoPS(13:0) were carried out similarly.

Optimization of reactant ratio was performed as follows: 7 μmol PE(16:0/18:1(9Z)) was incubated in 1 mL of MeOH at 60 °C with 54, 107, 161 and 215 μmol of [13C6]d-glucose.

2.3. Purification of Amadori-Glycated Lipids. 2.3.1. Lipid Extraction. For [13C6]Amadori-PE/PS species, the Folch method was performed to remove [13C6]glucose and other water-soluble byproducts. After incubation, the reaction mixture in 1 mL of MeOH was mixed with 2 mL of CHCl3 and 0.6 mL of H2O. The mixture was vortexed for 20 s and then centrifuged at 3000 rpm for 15 min. The bottom layer was transferred out by a glass pipette and then dried with nitrogen.

2.3.2. C18 Solid-Phase Extraction (SPE). Because of the high hydrophilicity of [13C6]Amadori-LysoPE/LysoPS species, the Folch method cannot be used for liquid–liquid extraction. Instead, C18 SPE was employed to separate synthetic products from [13C6]glucose or other water-soluble materials. In brief, incubation mixture was reconstituted in 10% MeOH and then loaded onto an ISOLUTE C18 cartridge (500 mg, Biotage, Sweden) conditioned with the same solvent. After rinsing with an additional 5 mL of 10% MeOH, Amadori lipids were eluted out with 10 mL of MeOH.

2.3.3. Phenylboronic Acid (PBA) SPE. Diol-containing Amadori-glycated lipids can bind to immobilized PBA, which enables isolation of Amadori-glycated lipids from their corresponding lipids. Bond Elut PBA cartridges (500 mg) purchased from Agilent (Palo Alto, CA) were adopted to perform SPE for cleanup. Briefly, PBA cartridges were wetted with 5 mL of MeOH and then conditioned with 3 mL of 150 mM ammonium formate (AF, pH 10), followed by 5 mL of 20% MeOH containing 100 mM AF (pH 8) (loading solvent). The dried resultant lipid extracts were redissolved in MeOH and diluted 10 times with loading solvent before loading onto equilibrated PBA cartridges. The cartridges were rinsed with 5 mL of MeOH containing 0.1% (v/v) ammonium hydroxide, then [13C6]Amadori-lipids were recovered by elution with 5 mL of 90% MeOH containing 1% formic acid. The elution effluent was nitrogen-dried and subjected to the following HPLC separation.

2.3.4. HPLC-UV Separation. Semipreparative separation was performed on a Shimadzu 20A HPLC equipped with LC-8A pumps. The samples were loaded onto a Kromasil C18 column (250 × 10 mm, 10 μm, AkzoNobel, Bohus, Sweden) using four isocratic mobile phases at a flow rate of 3 mL/min for different [13C6]Amadori-lipid species: [13C6]Amadori-PE: 100% MeOH containing 5 mM AF; [13C6]Amadori-PS: 100% MeOH containing 5 mM AF and 0.1% phosphoric acid; [13C6]Amadori-LysoPE: 80% MeOH containing 5 mM AF; and [13C6]-Amadori-LysoPS: 75% MeOH containing 5 mM AF and 0.1% phosphoric acid. The effluent was monitored for UV absorbance at 220 nm, and the injection volume was set at 500 μL.

2.4. LC-MS Analysis of [13C6]Amadori-Lipids. A Vanquish UHPLC system coupled to a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific) was primarily used for the analysis of [13C6]-Amadori-lipids. A core–shell Accucore C30 column (Thermo Fisher Scientific) was used with a column oven temperature of 40 °C and a flow rate of 350 μL/min. Two elution gradients were employed for the analysis of [13C6]Amadori-PE/PS and [13C6]Amadori-LysoPE/LysoPS, respectively, as follows:

Gradient 1: To the method previously published by our laboratory for global lipidomics analysis,20 the mobile phase was composed of solvent A (ACN/H2O, 60:40, v/v) and solvent B (IPA/ACN, 90:10, v/v), both containing 10 mM AF and 0.1% formic acid. The gradient was as follows: −3 to 0 min, 30% B for column equilibration; 0–5 min, 30–43% B; 5–5.1 min, 43–50% B; 5.1–14 min, 50–70% B; 14.1–21 min, 70–99% B; 21–24 min, 99% B; 24–24.1 min, 99–30% B; 24.1–28 min, 30% B for column reequilibration. The total analysis time including column reequilibration was 31 min.

Gradient 2: The mobile phase was composed of solvent A (H2O) and solvent B (MeOH), both containing 5 mM AF and 0.1% formic acid. The gradient was as follows: −3 to 0 min,
isocratic elution with 60% B for the equilibration of the column; 0−3 min, 60% B; 3−13 min, 60−99% B; 13−15 min, 99% B; 15−15.1 min, 99−60% B; 15.1−19 min, 60% B for column reequilibration. The total analysis time including column reequilibration was 22 min.

The TSQ Quantiva mass spectrometer was equipped with a heated electrospray ionization source, with positive- and negative-ion spray voltages set at 3500 and 3000 V, respectively. For both ionization modes, nitrogen was used as the sheath, auxiliary, and sweep gases at flow rates of 20, 7, and 1 (arbitrary units), respectively. Vaporizer and ion-transfer tube temperatures were 400 and 350 °C, respectively. For MS analysis in full-scan mode, the scan range, scan rate, and resolution were m/z 200−1000, 1000 amu/s, and 0.7 (full width at half maximum), respectively. For MS/MS analysis in product ion scan mode, the argon collision gas pressure was 1.5 mTorr. The optimized collision energy was 20 eV for [13C6]Amadori-PE and 16 eV for [13C6]Amadori-PS/LysoPE/LysoPS in positive-ion mode, whereas it is 27 eV for [13C6]Amadori-PE/PS and 22 eV for [13C6]Amadori-LysoPE/LysoPS in negative-ion mode.

For high-resolution mass analysis, a Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) was coupled to a Vanquish UPLC, as described above for TSQ Quantiva. Mass ranges for full-scan MS and mass resolution settings were m/z 100−1000 and 120 000, respectively.

3. RESULTS AND DISCUSSION

3.1. Optimization of Synthetic Conditions. To determine the relative yield of [13C6]Amadori-glycated lipids, LC-MS analysis in full-scan mode was performed to quantify [13C6]-Amadori-lipids synthesized under different conditions. An assumption was made that Amadori-glycated lipids have similar ionization efficiency to their corresponding unmodified lipids, so chromatographic peak areas were used to estimate the relative reaction yield.

\[
\text{relative yield (%) = peak area of Amadori-lipid yielded / peak area of initial lipid reactant × 100%}
\]

Reactions of PE(16:0/18:1(9Z)) with [13C6]D-glucose in MeOH-PB, MeOH, and MeOH with addition of BHT were carried out at 37 °C in parallel. After incubation for 0, 1, 2, 3, and 4 days, aliquots of reaction mixtures were dried and then subjected to Folch extraction. CHCl3 layer was transferred out, dried, reconstituted in MeOH, and then subjected to LC-MS analysis. As shown in Figure 1, reaction yield in complete MeOH medium was 3.2, 4.9, 5.8, and 7.0 times higher than those in MeOH-PB after incubation for 1, 2, 3, and 4 days, respectively.

Figure 1. Reaction yields of [13C6]Amadori-PE(16:0/18:1(9Z)) in the medium of MeOH-PB, MeOH, or MeOH in the presence of BHT.

![Figure 1](image1.png)

![Figure 2](image2.png)

Figure 2. Effect of temperature and time on reaction yields of (A) [13C6]Amadori-PE(16:0/18:1(9Z)), (B) [13C6]Amadori-PS(16:0/18:1(9Z)), (C) [13C6]Amadori-LysoPE(13:0/0:0), and (D) [13C6]Amadori-LysoPS(13:0/0:0).

Table 1. Optimized Synthetic Conditions for [13C6]Amadori-Lipids

|          | medium | temperature (°C) | incubation duration (days) | concentration of lipids (μmol/mL) | concentration of [13C6]D-glucose (μmol/mL) |
|----------|--------|-----------------|---------------------------|-----------------|---------------------------------|
| [13C6]Amadori-PE | MeOH   | 60              | 3                         | 7               | 161                             |
| [13C6]Amadori-PS | MeOH   | 50              | 2                         | 7               | 161                             |
| [13C6]Amadori-LysoPE | MeOH   | 50              | 3                         | 12              | 215                             |
| [13C6]Amadori-LysoPS | MeOH   | 50              | 2                         | 10              | 215                             |

DOI: 10.1021/acsomega.8b01893
ACS Omega 2018, 3, 15725−15733
On the other hand, yields in MeOH were 1.3, 1.4, 1.3, and 1.4 times higher than those in MeOH + BHT after 1, 2, 3, and 4 days, respectively. BHT, a synthetic antioxidant that can effectively inhibit the browning reaction and lipid peroxidation, was expected to prevent the formation of secondary byproducts and thus increase the recovery of destination product; however, our results showed that addition of BHT did not help the production of [13C6]Amadori-PE.

In terms of effects of reaction temperature and time, as shown in Figure 2, yields peaked at 60 °C and 3 days for [13C6]Amadori-PE, 50 °C and 3 days for [13C6]Amadori-LysoPE, and 50 °C and 2 days for [13C6]Amadori-PS/LysoPS.

To optimize the ratio of reactants, incubations of 7 μmol PE(16:0/18:1(9Z)) in 1 mL of MeOH at 60 °C with 54, 107, 161, and 215 μmol [13C6]D-glucose were carried out in parallel, with the corresponding molar ratios of [13C6]glucose over PE(16:0/18:1(9Z)) being 7.7, 15.4, 23.1, and 30.9. After 3 days of incubation, the resulting yields were 19.9, 40.6, 49.9, and 53.0% for the molar ratios of 7.7, 15.4, 23.1, and 30.9, respectively. This result showed that yields increased with increasing molar ratio of [13C6]glucose over PE. However, the increase of yield was not significant when the molar ratio increased from 23.1 to 30.9. As such, molar ratios of [13C6]D-glucose over lipids were optimized to about 23 for the synthesis of all Amadori-glycated lipids. The optimized synthetic conditions for [13C6]-Amadori-PE/PS/LysoPE/LysoPS are listed in Table 1.
extraction, for which the Folch method was employed. Diol-containing glucose has the ability to bind to immobilized PBA, which will likely affect the binding between PBA and diol-containing [13C6]Amadori-lipids. After lipid extraction, PBA SPE was performed to separate [13C6]Amadori-PEs from their corresponding substrates, according to the reversible covalent interaction between PBA and diol-containing [13C6]Amadori-PEs. [13C6]Amadori-PEs were finally purified by semipreparative HPLC.

As for [13C6]Amadori-PS species, however, PBA SPE was not applicable because [13C6]Amadori-PSs could not be retained well on PBA SPE column. A similar poor retention was also observed for [13C6]Amadori-LysoPS. The possible reason is that [13C6]Amadori-PS/LysoPS carries negative charge under alkaline conditions, which results in electrostatic repulsion from immobilized boronate. In contrast to [13C6]Amadori-PE, the separation performance of [13C6]Amadori-PS on C18 column was improved largely by adding acid to mobile phase. Consequently, [13C6]Amadori-PSs were purified on a semipreparative C18 column using isocratic elution with 100% MeOH containing 5 mM AF and 0.1% phosphoric acid, then one more Folch extraction was employed to remove phosphoric acid in eluent from purified compounds.

LC-MS full-scan chromatograms (Figure 4) showed that [13C6]Amadori-PS/LysoPS carries negative charge under alkaline conditions, which results in electrostatic repulsion from immobilized boronate. In contrast to [13C6]Amadori-PE, the separation performance of [13C6]Amadori-PS on C18 column was improved largely by adding acid to mobile phase. Consequently, [13C6]Amadori-PSs were purified on a semipreparative C18 column using isocratic elution with 100% MeOH containing 5 mM AF and 0.1% phosphoric acid, then one more Folch extraction was employed to remove phosphoric acid in eluent from purified compounds.
PS(16:0/18:1(9Z)), and PS(17:0/17:0) were pure (>95%), with purification process recoveries of about 43, 51, 31, and 25%, respectively. LC-UV chromatograms (Supporting Information Figure S1) also validated the high purity of [13C6]Amadori-lipids.

Compared to [13C6]Amadori-PE/PS, [13C6]Amadori-LysoPE/LysoPS are more hydrophilic. Thus, different strategies were employed for purification of [13C6]Amadori-LysoPE/LysoPS. Instead of the Folch method, C18 SPE was performed to remove [13C6]D-glucose for [13C6]Amadori-LysoPE. In terms of [13C6]Amadori-LysoPS, besides its low recovery on PBA SPE, we found it was more unstable than the other species. To make the purification more efficient for [13C6]Amadori-LysoPS, direct purification using HPLC was employed, which utilizes isocratic elution with 75% MeOH containing 5 mM AF and 0.1% phosphoric acid. After HPLC preparation, phosphoric acid in the eluent was removed by C18 SPE. [13C6]Amadori-LysoPS was recovered in MeOH, nitrogen-dried, and kept at −80 °C. LC-MS full-scan chromatograms (Figure S5) showed that [13C6]Amadori-LysoPE(13:0/0:0)/LysoPS(13:0/0:0) were pure (>95%), with purification process recoveries of about 48 and 19%, respectively. LC-UV chromatograms (Supporting Information Figure S2) also validated their high purity.

The mass accuracy of the six purified [13C6]Amadori-lipids was confirmed by high-resolution mass spectrometry. A comparison between measured masses with calculated values exhibited a high mass accuracy (mass errors ranged from −2.6 to 2.4 ppm) (Supporting Information Table S1).

Isotopic purity of these six synthesized compounds was also investigated using high-mass-resolution LC-MS. Extracted ion chromatograms were generated and peak areas were integrated for each of the resolved isotopes related to these compounds.
Table 2. MS/MS Fragmentation Analysis in Negative-Ionization Mode for $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)), -PE(15:0/15:0), -PS(16:0/18:1(9Z)), -PS(17:0/17:0), -LysoPE(13:0/0:0), and -LysoPS(13:0/0:0)

| Product Ions (m/z) and Their Related Structures (Listed in the First Column) after Collision-Induced Dissociation Using a Triple Quadrupole Mass Spectrometer | Proposed product ions |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 884.7 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 820.7 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | 928.7 |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | 940.7 |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | 578.4 |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | 622.4 |

| | $M^{-}\text{H}$ |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 884.7 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 820.7 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | 928.7 |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | 940.7 |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | 578.4 |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | 622.4 |

| | $M^{-}\text{H}-13C_6H_10O_5$ |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 716.6 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 662.5 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | 760.6 |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | 762.7 |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | 410.3 |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | 454.3 |

| | fatty acid-H (sn-1) |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 255.4 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 241.3 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | 255.4 |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | 269.3 |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | 213.3 |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | 213.2 |

| | fatty acid-H (sn-2) |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 281.3 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 241.3 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | 281.4 |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | 269.3 |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | NA |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | NA |

| | LPE-H (sn-1) |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 452.3 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 438.2 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | NA |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | NA |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | 410.2 |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | NA |

| | LPE-H (sn-2) |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 204.2 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 204.2 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | NA |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | NA |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | 204.3 |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | NA |

| | PA-H |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 673.4 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 619.6 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | 673.5 |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | 675.5 |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | 391.3 |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | 391.3 |

| | LPA-H (sn-1) |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 409.5 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 395.6 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | 409.3 |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | 423.4 |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | 367.2 |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | 367.3 |

| | LPA-H (sn-2) |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 435.5 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 395.6 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | 435.4 |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | 423.4 |
| $[^{13}C_6]$Amadori-LysoPE(13:0/0:0) | NA |
| $[^{13}C_6]$Amadori-LysoPS(13:0/0:0) | NA |

| | glycero phosphoethanolamine-H2O-H |
|---|---|
| $[^{13}C_6]$Amadori-PE(16:0/18:1(9Z)) | 196.4 |
| $[^{13}C_6]$Amadori-PE(15:0/15:0) | 196.1 |
| $[^{13}C_6]$Amadori-PS(16:0/18:1(9Z)) | NA |
| $[^{13}C_6]$Amadori-PS(17:0/17:0) | NA |

PE: phosphatidyl ethanolamine; PS: phosphatidyl serine; PA: phosphatidic acid; LPE: LysoPE; LPA: LysoPA; NA: not applicable.
Amadori-lipids unambiguously validated the identification of these novel compounds.

4. CONCLUSIONS

In this study, six stable isotope-labeled [13C6] Amadori-glycated lipids were synthesized, purified, and structurally confirmed using accurate mass measurement and tandem mass spectrometry, covering four types of lipids, including Amadori-PE, -PS, -LysoPE, and -LysoPS. Optimal conditions for the synthesis and purification of these four types of stable isotope-labeled Amadori-glycated lipids were obtained. MS/MS fragmentation in both positive- and negative-ion modes validated the identification of these novel synthetic compounds. The high purity (>95%) of these synthetic compounds was verified by both LC-MS full-scan analysis and LC-UV analysis. As a result, successful preparation of highly purified stable isotope-labeled Amadori-lipid standards makes it possible to develop targeted LC-MS/MS methods for accurate analysis of endogenous Amadori-glycated phospholipids from biological samples.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01893.

HPLC-UV chromatograms, full-scan MS, and MS/MS spectra of synthesized labeled and unlabeled Amadori-PE, -PS, -LysoPE, and -LysoPS compounds (PDF).

Figure 8. MS/MS fragmentation schemes of (A) [13C6] Amadori-PE(16:0/18:1(9Z)) in positive ion mode and (B) negative ion mode, and (C) [13C6] Amadori-PS(16:0/18:1(9Z)) in negative ion mode. The MS/MS spectra corresponding to (A), (B), and (C) are shown in Figures 6A and 7A,C.

[13C6] Amadori-lipids unambiguously validated the identification of these novel compounds.

AUTHOR INFORMATION

Corresponding Author

E-mail: q_zhang2@uncg.edu.

ORCID

Qibin Zhang: 0000-0002-6135-8706

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors gratefully acknowledge funding from the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (R01 DK116731) and the American Heart Association (17CSA33570025) for support of this research.

REFERENCES

(1) Zhang, Q.; Ames, J. M.; Smith, R. D.; Baynes, J. W.; Metz, T. O. A perspective on the Maillard reaction and the analysis of protein glycation by mass spectrometry: probing the pathogenesis of chronic disease. J. proteome Res. 2009, 8, 754–769.

(2) Ledl, F.; Schleicher, E. New Aspects of the Maillard Reaction in Foods and in the Human Body. Angew. Chem., Int. Ed. 1990, 29, 565–594.

(3) Miyazawa, T.; Nakagawa, K.; Shimasaki, S.; Nagai, R. Lipid glycation and protein glycation in diabetes and atherosclerosis. Amino Acids 2012, 42, 1163–1170.
(4) Bucala, R. Lipid and lipoprotein modification by advanced glycosylation end-products: role in atherosclerosis. *Exp. Physiol.* 1997, 82, 327–337.

(5) Bucala, R.; Makita, Z.; Koschinsky, T.; Cerami, A.; Vlassara, H. Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 1993, 90, 6434–6438.

(6) Bucala, R.; Makita, Z.; Vega, G.; Grundy, S.; Koschinsky, T.; Cerami, A.; Vlassara, H. Modification of low density lipoprotein by advanced glycation end products contributes to the dyslipidemia of diabetes and renal insufficiency. *Proc. Natl. Acad. Sci. U.S.A.* 1994, 91, 9441–9445.

(7) Nagashima, T.; Oikawa, S.; Hirayama, Y.; Tokita, Y.; Sekikawa, A.; Ishigaki, Y.; Yamada, R.; Miyazawa, T. Increase of serum phosphatidylcholine hydroperoxide dependent on glycemic control in type 2 diabetic patients. *Diabetes Res. Clin. Pract.* 2002, 56, 19–25.

(8) Kinoshita, M.; Oikawa, S.; Hayasaka, K.; Sekikawa, A.; Nagashima, T.; Toyota, T.; Miyazawa, T. Age-related increases in plasma phosphatidylcholine hydroperoxide concentrations in control subjects and patients with hyperlipidemia. *Clin. Chem.* 2000, 46, 822–828.

(9) Sell, D. R.; Lane, M. A.; Masoro, E. J.; Mock, O. B.; Reiser, K. M.; Fogarty, J. F.; Cutler, R. G.; Ingram, D. K.; Roth, G. S.; Monnier, V. M. Longevity and the genetic determination of collagen glycoxidation kinetics in mammalian senescence. *Proc. Natl. Acad. Sci. U.S.A.* 1996, 93, 485–490.

(10) Sookwong, P.; Nakagawa, K.; Fujita, I.; Shoji, N.; Miyazawa, T. Amadori-glycated phosphatidylethanolamine, a potential marker for hyperglycemia, in streptozotocin-induced diabetic rats. *Lipids* 2011, 46, 943–952.

(11) Oak, J.; Nakagawa, K.; Miyazawa, T. Synthetic preparation of Amadori-glycated phosphatidylethanolamine can trigger lipid peroxidation via free radical reactions. *FEBS Lett.* 2000, 481, 26–30.

(12) Utzmann, C. M.; Lederer, M. O. Identification and quantification of aminophospholipid-linked Maillard compounds in model systems and egg yolk products. *J. Agric. Food Chem.* 2000, 48, 1000–1008.

(13) Nakagawa, K.; Oak, J. H.; Higuchi, O.; Tsuzuki, T.; Oikawa, S.; Otani, H.; Mine, M.; Cai, H.; Miyazawa, T. Ion-trap tandem mass spectrometric analysis of Amadori-glycated phosphatidylethanolamine in human plasma with or without diabetes. *J. Lipid Res.* 2005, 46, 2514–2524.

(14) Kodate, A.; Otoki, Y.; Shinmi, N.; Ito, J.; Kato, S.; Umetsu, N.; Miyazawa, T.; Nakagawa, K. Development of quantitation method for glycated aminophospholipids at the molecular species level in powdered milk and powdered buttermilk. *Sci. Rep.* 2018, 8, No. 8729.

(15) Xenakis, D.; et al. Organic synthesis of Amadori rearrangement products. *Synthesis* 1983, 541–543.

(16) Utzmann, C. M.; Lederer, M. O. Independent synthesis of aminophospholipid-linked maillard products. *Carbohydr. Res.* 2000, 325, 157–168.

(17) Levertir, S.; Shiraishi, M.; Miyazawa, T. Identification of deoxy-D-fructosyl phosphatidylethanolamine as a non-enzyme glycation product of phosphatidylethanolamine and its occurrence in human blood plasma and red blood cells. *Biosci., Biotechnol., Biochem.* 1998, 62, 893–901.

(18) Miyazawa, T.; Oak, J. H.; Nakagawa, K. A convenient method for preparation of high-purity, Amadori-glycated phosphatidylethanolamine and its prooxidant effect. *Ann. N. Y. Acad. Sci.* 2005, 1043, 276–279.

(19) Miyazawa, T.; Kamiyoshihara, R.; Shimizu, N.; Harigae, T.; Otoki, Y.; Ito, J.; Kato, S.; Miyazawa, T.; Nakagawa, K. Amadori-glycated phosphatidylethanolamine enhances the physical stability and selective targeting ability of liposomes. *R. Soc. Open Sci.* 2018, 5, No. 171249.

(20) Navaréz-Rivas, M.; Zhang, Q. Comprehensive untargeted lipidomic analysis using core-shell C30 particle column and high field orbitrap mass spectrometer. *J. Chromatogr. A* 2016, 1440, 123–134.

(21) Lambert, C. R.; Truscott, T. G.; Black, H. S. Reactivity of butylated hydroxytoluene. *Free Radical Biol. Med.* 1996, 21, 395–400.