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Research article

A novel crosslinked type of advanced glycation end-product derived from lactaldehyde

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ABSTRACT

Glycation of amino or guanidino groups of proteins with glucose and glucose-derived reactive aldehydes, such as α-hydroxyaldehydes, leads to accumulation of advanced glycation end-products (AGEs) in the body, resulting in diabetic complications and age-related pathology. Although molecular structures of glycolaldehyde- and glyceraldehyde-derived AGEs have been described in previous studies, little is known about lactaldehyde-derived AGEs of α-hydroxyaldehydes. Here, we report a novel crosslinked type of AGE, named as lactaldehyde-derived lysine dimer (LAK2), which is produced due to non-enzymatic glycation of N^{α}-acetyl-*L*-lysine with lactaldehyde under physiological conditions. We have identified the molecular structure of LAK2 by extensive mass spectrometry and nuclear magnetic resonance analyses. Furthermore, we propose a reaction pathway to produce LAK2, in which it is formed through an intermediate common with the recently reported lactaldehyde-derived pyridinium-type lysine adduct (LAPL). Since lactaldehyde is known to be produced from L-threonine in a myeloperoxidase (MPO)-mediated reaction at sites of inflammation, LAK2 has the potential to be an oxidative stress marker of MPO-mediated reactions induced in inflammation.

1. Introduction

Advanced glycation end-products (AGE) are non-enzymatic chemical modifications of proteins typically formed between glucose and glucose-derived reactive aldehydes and protein amino or guanidino groups, and are known to accumulate *in vivo* under hyperglycemic conditions. Of the sugar-derived reactive aldehydes, α-hydroxyaldehydes such as glycolaldehyde and glyceraldehyde have a unique reactivity toward amines involving Amadori rearrangement that facilitates AGE formation. They are also reported to be associated with various diseases such as diabetic complications [1, 2]. For example, glycolaldehyde has been demonstrated to produce the AGE, GA-pyridine, *in vivo*, as well as carboxymethyl lysine [3]. There is growing interest in the pathogenic roles of AGEs, as well as their use as therapeutic targets and diagnostic markers [4, 5]. Interestingly, glycolaldehyde is also produced from L-serine through the myeloperoxidase (MPO)-hydrogen peroxide system, another important pathway to generate AGE precursor aldehydes from amino acids. Overexpression of MPO and MPO-mediated AGE formation has been described to play a critical role in tissue damage at sites of inflammation [6, 7].

Lactaldehyde, a member of the α-hydroxyaldehyde family, is reportedly produced from L-threonine by the MPO-hydrogen peroxide system [6]. This led us to examine a similar reaction of the ε-amino group of lysine with lactaldehyde to explore the possibility that lactaldehyde-derived AGEs could be biomarkers for oxidative stress related to inflammation, and might be another AGE that accumulates under abnormal metabolic conditions. Additionally, lactaldehyde is produced during the detoxification of methylglyoxal, which has been shown in glyoxalase 1 knock-out Schwann cells [8]. No reports had been published on lactaldehyde-derived AGEs until a recent study by Fujimoto et al. [9] that identified a 3-hydroxypyridinium-type new AGE named as lactaldehyde-derived pyridinium-type lysine adduct (LAPL), in the Maillard reaction between N^{α}-acetyl-*L*-lysine (Ac-Lys) and lactaldehyde.

In this report, we have described our results regarding a novel crosslinked type of AGE, lactaldehyde-derived lysine dimer (LAK2), that we have identified as one of the major products in the Maillard reaction of Ac-Lys with lactaldehyde under physiological conditions at pH 7.4 and 37 °C.

2. Material and methods

2.1. Maillard reaction of Ac-Lys with lactaldehyde

N^{α}-acetyl-*L*-lysine (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in 0.5 M phosphate buffer (pH 7.4) at a concentration of 0.5 M

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and mixed with an equal volume of DL-lactaldehyde solution (1 M, Sigma-Aldrich, St. Louis, MO, USA). The resulting solution containing 0.5 M of both Ac-Lys and lactaldehyde was incubated at 37 °C for one week.

2.2. Mass-based purification of LAPL and LAK2

LAPL and LAK2 were analyzed and purified from the reaction mixture containing Ac-Lys and lactaldehyde using an Agilent 1260 Infinity II Preparative Liquid Chromatography/Mass Selective Detector (LC/MSD) system (Agilent Technologies, Santa Clara, CA, USA). Detailed instrumentation and chromatographic conditions are summarized in Tables S1 to S3. Collected fractions were evaporated *in vacuo* and lyophilized to give the product as formate salt.

2.3. Accurate mass measurement and collision-induced dissociation of LAK2

LAK2 was dissolved in 0.1% formic acid in water at a final concentration of 5 ppm. The chemical composition of LAK2 was then analyzed using an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/Mass Spectrometry (MS) system (Agilent Technologies) equipped with a dual electrospray ionization (ESI) source in a positive ion mode. Detailed instrumentation, chromatographic conditions, and MS parameters are summarized in Tables S4 to S6. Obtained data were analyzed with the MS-FINDER software [10].

2.4. Structural determination of LAK2

LAK2 (25 mg as formate) was dissolved in 0.6 mL of deuterium oxide containing 0.05% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP-d_4, Sigma-Aldrich). Nuclear magnetic resonance (NMR) measurements were performed using an AVANCE III HD 500 MHz NMR spectrometer equipped with a QCI CryoProbe (Bruker, Billerica, MA, USA) at 298 K. The ^{1}H- and ^{13}C-NMR chemical shifts were recorded relative to the internal reference TSP-d_4. The molecular structure of LAK2 was determined based on the NMR spectra of one-dimensional (1D)-^{1}H, 1D-^{13}C, ^{1}H–^{1}H COSY, NOESY, ^{1}H–^{13}C edited-HSQC, ^{1}H–^{13}C HMBC, 1,1-ADEQUATE, and ^{1}H–^{15}N HMBC. Instrument operation, data processing, and data analysis were performed using the TopSpin software 3.6 (Bruker).

3. Results and discussion

3.1. LC/MS profile of the Maillard reaction of Ac-Lys with lactaldehyde

After incubating Ac-Lys with an equimolar amount of lactaldehyde under physiological conditions for one week, the reaction mixture was subjected to a semi-preparative LC/MS system with mass-based fraction collection. We first observed an intense peak at a retention time (R_t) of 3.5 min when detected at its absorption maximum of 290 nm (Figure 1A and 1B, dashed line). The MS spectrum of this peak showed a positive ion with mass-to-charge (*m/z*) of 281.1 (Figure 1C, left panel). We then performed mass-based fraction collection of this peak to elucidate the molecular structure by NMR analyses (Figures S1 to S3). The NMR spectra revealed a structure that corresponded to LAPL, which was recently identified by Fujimoto et al. under similar reaction conditions [9]. Using a diode array detector at 254 nm, another peak was observed at an R_t of 5.8 min (Figure 1A), of which the ultraviolet (UV)-visible spectrum demonstrated absorption maxima at longer wavelengths of 232, 268, and 348 nm when compared to LAPL. This suggested the existence of an additional molecule bearing an aromatic structure (Figure 1B, solid line). The single quadrupole MS of LAK2 showed a positive ion at *m/z* 451.3, which suggested that LAK2 was probably a crosslinked dimer derivative of lysine (Figure 1C, right panel). As the peak of LAK2 seemed to be separable by LC/MS, we decided to isolate this peak by mass-based fraction collection for subsequent structural analysis.

3.2. MS fragmentation analysis of LAK2

To determine the molecular structure, isolated LAK2 (as formate) was subjected to accurate mass measurement and product ion scan using a Q-TOF LC/MS instrument. Figure 2A shows the exact mass of LAK2 as an ion to be *m/z* 451.2555 in the positive ion mode, which is estimated to be a $C_{22}H_{35}N_4O_6^+$ positive ion (calculated mass 451.2557). Additionally, the tandem MS (MS/MS) spectrum showed several fragment peaks at *m/z* 84.0806, 109.0754, 280.1652, 320.2335, and 409.2434 (Figure 2B),

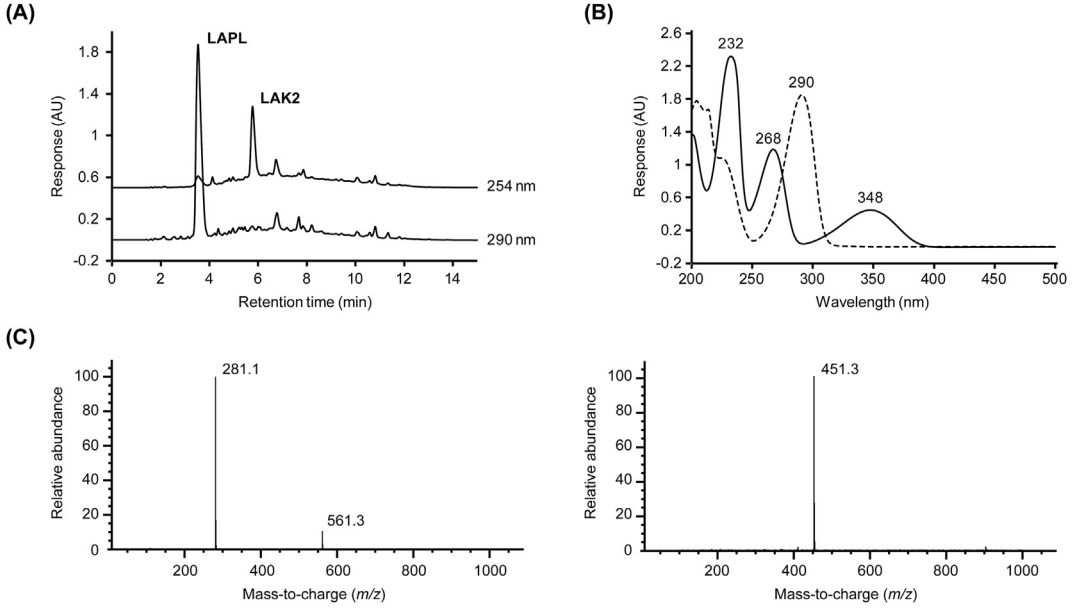


Figure 1. Semi-preparative LC/MS analysis of the Maillard reaction mixture of Ac-Lys with lactaldehyde. (A) UV chromatograms of the reaction mixture observed at 254 nm and 290 nm. (B) UV-visible wavelength absorption spectra of LAPL (dashed line) and LAK2 (solid line). (C) MS spectra of LAPL (left panel) and LAK2 (right panel).

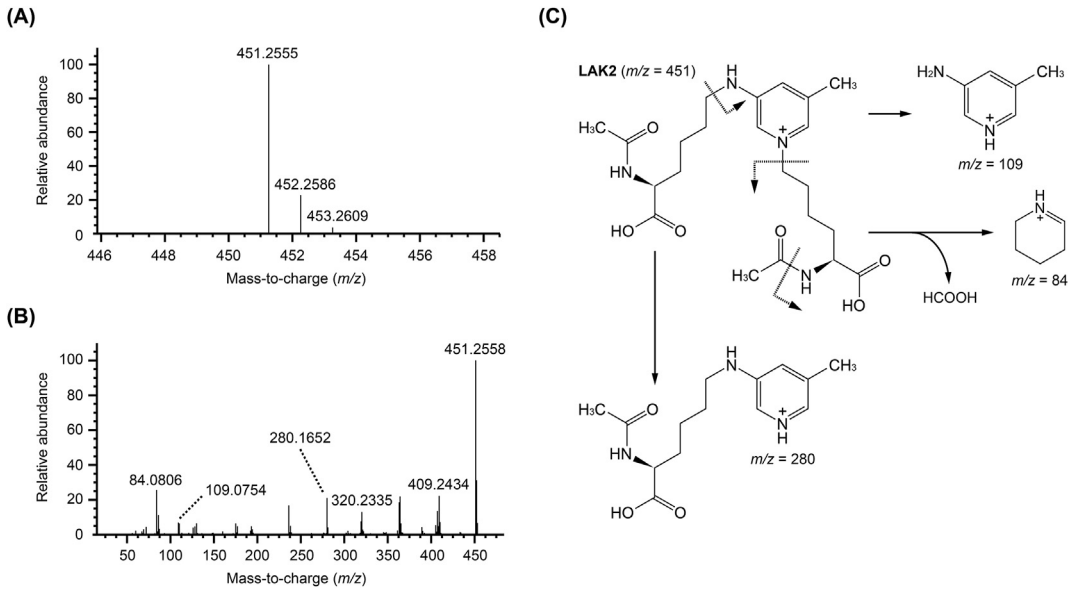


Figure 2. Structural analysis of purified LAK2 by Q-TOF LC/MS. (A) An accurate mass spectrum of LAK2. A monoisotopic ion peak at *m/z* 451.2555 $[M]^+$ calculated as $C_{22}H_{35}N_4O_6^+$ (*m/z* 451.2557). (B) A tandem MS spectrum of LAK2 of the peak at *m/z* 451.2555. The collision energy in MS/MS experiments of LAK2 was 30 eV in positive ion mode. (C) Predicted fragmentation scheme of LAK2 on MS/MS analysis.

which we could assign to the fragments from LAK2 using the MS-FINDER software [10]. Experimental *m/z* 84.0806 was estimated as $C_5H_{10}N$ (calculated *m/z* 84.0813), 109.0754 as $C_6H_9N_2$ (109.0766), 280.1652 as $C_{14}H_{22}N_3O_3$ (280.1661), 320.2335 as $C_{18}H_{30}N_3O_2$ (320.2338), and 409.2434 as $C_{20}H_{33}N_4O_5$ (409.2451). As shown in Figure 2C, the mass of *m/z* 84.0806 possibly due to 2,3,4,5-tetrahydropyridinium ion of deacetylated Ac-Lys was also previously described as a specific MS/MS fragment ion of glycated lysine products observed by LC-ESI-MS/MS with collision-induced dissociation [11]. From the molecular formula of $C_{22}H_{35}N_4O_6^+$ as analyzed above, LAK2 is thought to be comprised of two lysine moieties (C_{16}) and two lactaldehyde-derived 3-carbon units (C_6).

3.3. Molecular structure of LAK2

After isolating LAK2, we identified the molecular structure utilizing several NMR techniques, including ^1H, ^{13}C, DEPT, COSY, NOESY, ^1H–^{13}C edited-HSQC, ^1H–^{13}C/^{15}N HMBC, and 1,1-ADEQUATE (Figure 3 and S4). The 1D ^1H- and ^{13}C-NMR spectra obtained supported the structure of LAK2, as shown in Figures S5 and S6, respectively. All ^1H as well as ^{13}C chemical shifts were distinguishably identified, except for the methylene protons of two lysine moieties that appeared in multiplet. 2D NMR experiments were particularly helpful in assigning the aromatic protons of the ring that crosslinked the two lysine moieties. As shown in Figure S7, the ^1H–^{15}N HMBC data supported the 3-amino-5-methylpyridinium ring of LAK2 with coupling constants consistent with the structure. All these NMR data indicated LAK2 to be 1-((*S*)-12-acetamido-12-carboxypentyl)-3-((*S*)-(12′-acetamido-12′-carboxypentyl)amino)-5-methylpyridin-1-ium (Figure 3). In support of the NMR data, the MS/MS fragmentation pattern corroborated the structure with the following fragments: *m/z* 109.0754 (3-amino-5-methylpyridinium) and *m/z* 280.1652 ((*S*)-3-((12′-acetamido-12′-carboxypentyl)amino)-5-methylpyridinium) (Figure 2B and 2C).

We have identified in this study a novel lactaldehyde-derived AGE, LAK2, in which two molecules of lysine are crosslinked *via* a 5-methylpyridinium ring structure derived from two molecules of lactaldehyde. To support the novel chemical structure, we hypothesized a theoretical reaction pathway generating LAK2 according to reaction mechanisms previously established in AGE generation. Scheme 1 illustrates our proposed pathway to produce both LAK2 and LAPL. Briefly, Schiff base formation of Ac-Lys with lactaldehyde followed by Amadori

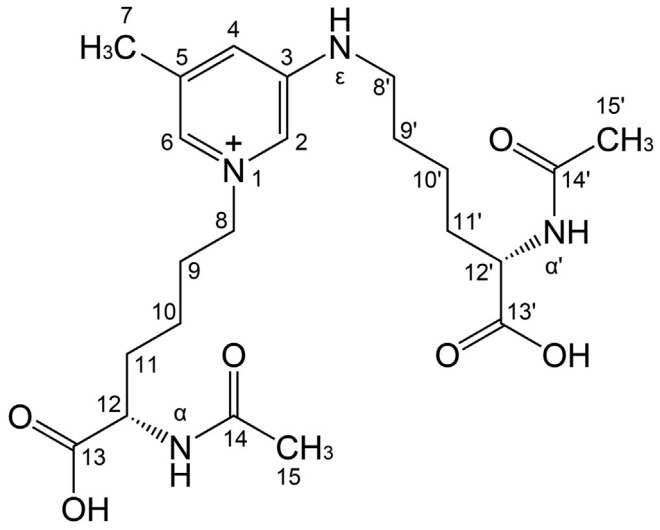
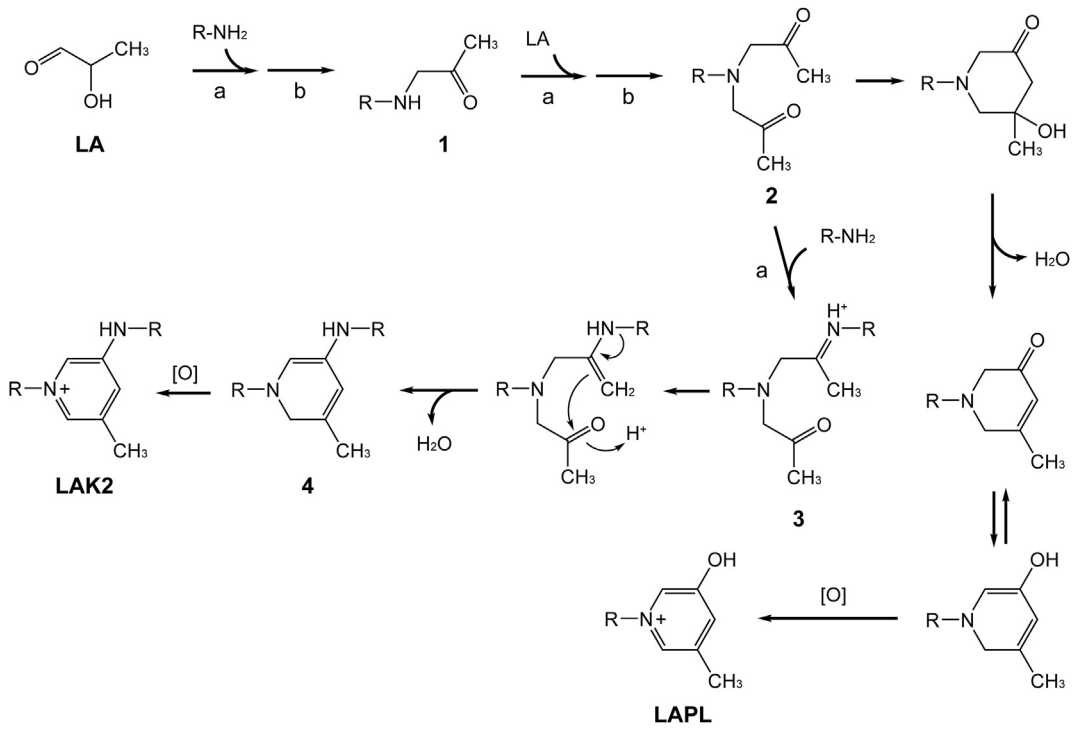


Figure 3. LAK2 structure determined by several NMR spectra. NMR assignment of LAK2 is summarized in the supplementary information.

rearrangement gives the Amadori product 1, which further reacts in similar steps with lactaldehyde to yield intermediate 2, a common precursor both to LAK2 and LAPL. The key step in this pathway is that further Schiff base formation of 2 with Ac-Lys should take place to give 3, which tends to cyclize and is subsequently dehydrated to yield 4. Finally, dihydropyridine 4 is spontaneously oxidized to yield LAK2, where aromatization possibly drives the two-electron oxidation in the presence of dissolved oxygen. Similar to LAK2, LAPL reported by Fujimoto et al. [9] was identified in the same Maillard reaction of Ac-Lys with lactaldehyde under similar conditions with the same substrates at lower concentrations, which favored the intramolecular cyclization of intermediate 2 rather than the intermolecular Schiff base formation with Ac-Lys (Scheme 1). The reason that the same intermediate 2 produced LAK2 in this study instead of LAPL might be attributed to Ac-Lys and lactaldehyde being used at a much higher equimolar concentration (0.5 M) than those employed by Fujimoto et al. (0.02–0.04 M) [9].



Scheme 1. A proposed pathway to produce LAK2 and LAPL in the Maillard reaction between lactaldehyde (LA) and N^{α}-protected-L-lysine (R–NH_2). Reaction conditions: a, Schiff base formation; b, Amadori rearrangement; [O], oxidation.

This study demonstrated the production of LAK2 and LAPL, both of which have a common 5-methylpyridinium structure, in the Maillard reaction of lactaldehyde with Ac-Lys. We consider that the major difference between LAK2 and LAPL is whether they are a crosslinked type of AGE or not. The difference induces a shift in UV absorption as observed with LAK2 compared to LAPL (Figure 1B). In addition, we also think that intra- and/or intermolecular crosslinking of a protein (modified by LAK2 in this case) might lead to destabilization of the protein followed by insolubilization *in vivo*. A previous paper has summarized that crosslinked type of other AGEs including pentosidine, vesperlysine, lysine-lysine pyridinium (K2P) and glucosepane accumulate with aging in the human lens, leading to protein insolubilization [12]. Therefore, measurement of LAK2 in physiological samples might be valuable to estimate a long-term accumulation of AGEs induced by inflammation, since lactaldehyde is one of the major reactive aldehydes produced from amino acids by MPO system at sites of inflammation [6].

Interestingly, Fujimoto et al. [9] have reported LAPL formation in a reaction of Ac-Lys with threonine and hypochlorous acid in addition to that of Ac-Lys with lactaldehyde, although the experimental conditions were not detailed and the data not shown. The reaction of threonine with hypochlorous acid has been reported to produce lactaldehyde *via* chloramine formation [7]. The formation of both LAK2 and LAPL was confirmed in the same reaction of Ac-Lys with lactaldehyde under the conditions described above. We therefore consider that LAK2 can be also produced in the reaction of Ac-Lys with threonine and hypochlorous acid. Further research will be conducted to prove LAK2 formation under *in vitro* conditions as above, as well as to detect LAK2 *in vivo* to elucidate cellular functions of LAK2 and to explore the importance of LAK2 measurements.

We also compared LAK2 and LAPL in their yields of formation in the Maillard reaction of Ac-Lys with lactaldehyde under physiological condition, by isolating each product through mass-based peak collection. In the Maillard reaction followed by purification, which was carried out two times independently, LAK2 was obtained in 5.2 % and 4.5 % yield, respectively (based on Ac-Lys). Similarly, in the same runs, we obtained LAPL in 11.8 % and 11.4 % yield, respectively (based on Ac-Lys). Although we did not monitor the product formation in a time-dependent manner, we estimated that the rate of LAK2 formation to be 2.4-fold lower than that of LAPL based on these reaction yields. This suggests that intermediate 2 tends to undergo intramolecular cyclization rather than intermolecular Schiff base formation with Ac-Lys (Scheme 1).

Analogous crosslinked types of AGEs derived from α-hydroxyaldehydes were described to be only obtained from the Maillard reaction of N^{α}-protected-lysine with glyceraldehyde under similar conditions [13]. Using extensive MS analyses, several products called triosidines that involve two lysine moieties tethered by a 3-aminomethyl-5-hydroxypyridinium ring structure were reported, similar to LAK2. It is noteworthy that, despite being in the same family of α-hydroxyaldehydes, lactaldehyde, which is identical to glyceraldehyde except for the β-hydroxy group, gives a much less complicated Maillard reaction profile compared to glyceraldehyde as observed in LC chromatograms with UV detection (Figure 1A).

In conclusion, we have identified a novel lactaldehyde-derived AGE in the Maillard reaction between Ac-Lys and lactaldehyde under physiological conditions, in which two lysine molecules are crosslinked *via* a pyridinium ring. Little attention has been paid thus far to lactaldehyde-derived AGEs, but we surmise that lactaldehyde and lactaldehyde-derived AGEs are potentially important, since lactaldehyde is one of the major reactive aldehydes produced from amino acids by the MPO system at sites of inflammation [6]. LAK2 has not been detected *in vivo*, and will require future studies. We are focusing our current research efforts toward finding more applications for LAK2 as an AGE produced during inflammation.

Declarations

Author contribution statement

Tomoaki Shigeta: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kazumi Sasamoto, Tetsuro Yamamoto: Analyzed and interpreted the data; Wrote the paper.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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