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ORIGINAL ARTICLE

Synthesis of stable isotope-labelled monolysyl advanced glycation endproducts

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Abstract Advanced Glycation Endproducts (AGEs) are modified amino acids that form on proteins and are known to be implicated in the pathogenesis of diabetes and related diseases. Ready access to synthetic stable isotope-labelled AGEs allows for quantitative mass spectrometry studies to be undertaken, providing key insights into the roles AGEs play in the progression of such diseases. However, the majority of current syntheses of these compounds suffer from poor yields and lengthy procedures and are not suitable for the purposes required here. Here, we report robust syntheses of stable isotope-labelled monolysyl AGEs, N^{ε}-(carboxymethyl)lysine, N^{ε}-(carboxyethyl)lysine and pyrraline, that provide straightforward access to these compounds for quantitative amino acid analysis. This work will facilitate future investigations with these compounds and lead to a better understanding of the roles they play in diabetes and related diseases.

Keywords Stable isotope labels · Synthesis · Advanced glycation endproducts · Internal standards · Diabetes · Glycation

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Introduction

The Advanced Glycation Endproducts (AGEs) are a large and structurally diverse group of compounds that result from post-translational non-enzymatic modification of proteins with reducing sugars (Henle 2005; Rabbini and Thornalley 2012; Ledl and Schleicher 1990). These modifications usually occur at lysine and arginine residues and are most problematic on long-lived proteins, such as collagens and lens crystallins (Verzijl et al. 2000a; b). The formation and accumulation of AGEs in the body is thought to play a significant role in the pathogenesis of many debilitating diseases including diabetes (Ahmed et al. 2007; Stitt 2001; Peppa and Vlasarra 2005), atherosclerosis (Basta et al. 2004) and age-related neurodegenerative diseases, such as Alzheimer's disease (Münch et al. 2012).

As part of an ongoing research program investigating the roles of AGEs in the pathogenesis of diabetes, we required readily available access to stable isotope labelled AGE standards for quantitative amino acid analysis using mass spectrometry. Several other groups have also required access to these types of compounds in the course of their studies and have synthesized them according to known literature procedures (Thornalley et al. 2003; Teerlink et al. 2004). However, upon consulting the literature, we found that the majority of these routes suffered from either difficult and lengthy procedures and/or low yields and did not represent attractive routes. Consequently, there is a need for the development of improved protocols/procedures for the preparation of AGEs in their free amino acid forms. Recently, we reported the synthesis of the three monolysyl AGEs, N^{ε}-(carboxymethyl)lysine **1** (CML), N^{ε}-(carboxyethyl)lysine **2** (CEL) and pyrraline **3** as both free amino acids and Fmoc-protected analogues suitable for incorporation into peptide synthesis (Woods et al. 2012). In this

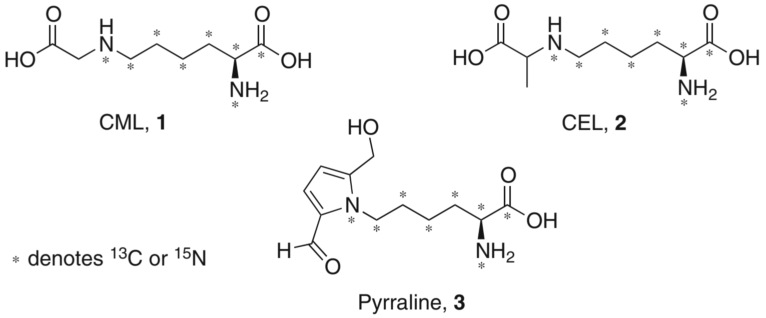


Fig. 1 The stable isotope-labelled monolysyl AGEs synthesized

communication, we report efficient and straightforward syntheses of the stable isotope labelled forms of these three important AGEs (Fig. 1), which are suitable as internal standards for mass spectrometry studies.

Experimental

General

[U-13C6, U-15N2]-L-lysine.2HCl was purchased from Cambridge Isotope Laboratories. Unless stated, all solvents and reagents were used as supplied from commercial sources. All moisture sensitive reactions were performed in an inert, dry atmosphere of nitrogen in oven-dried glassware. Analytical thin-layer chromatography (TLC) was performed using Kieselgel F254 0.2 mm (Merck) silica plates with visualisation by ultraviolet irradiation (254 nm) followed by staining with either potassium permanganate or ninhydrin. Flash chromatography was performed using Kieselgel S 63–100 μm (Riedel-de-Hahn) silica gel. The solvent compositions reported for all chromatographic separations are on a volume/volume (v/v) basis. Infrared (IR) spectra were recorded on a PerkinElmer Spectrum 100 FT-IR Spectrometer. Unless stated, ^{1}H-NMR spectra were recorded on 400 MHz Bruker spectrometer and are reported in parts per million (ppm) on the δ scale relative to $CDCl_3$ (δ 7.26) or TMS (0.00) and ^{13}C-NMR spectra were recorded on a 100 MHz Bruker spectrometer and are reported in ppm on the δ scale relative to $CDCl_3$ (δ 77.16). The multiplicities of ^{1}H signals are designated by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublets, dt = doublet of triplets and dm = doublet of multiplets. All coupling constants J = are reported in hertz. Mass spectra were obtained by electrospray ionisation in positive ion mode.

Procedures for amino acid synthesis

9BBN-lysine-5: ammonium hydroxide solution (28–30 %, 21.0 mL) was cooled in an ice bath then added to a flask containing [U-$^{13}C_6$, 99 %, U-$^{15}N_2$, 99 %]-L-lysine dihydrochloride **4** (500 mg, 0.22 mmol, 1.0 eq.) and the reaction was allowed to warm to room temperature and stirred for 30 min. The reaction mixture was concentrated under reduced pressure then dried under high vacuum affording the free base as a white solid. This solid was added to a refluxing solution of 9-BBN dimer (322 mg, 1.32 mmol, 0.6 eq) in MeOH (8 mL, HPLC grade) and the reaction mixture refluxed for 1.5 h (the reaction mixture becomes homogeneous after 30 min) before cooling to room temperature. The MeOH was removed under reduced pressure affording a gummy solid that was triturated in hot hexanes (2 × 5 mL) then hot diethylether (2 × 5 mL), collected by filtration and dried under high vacuum affording the title compound as a white solid (895 mg). TLC $R_f = 0.18$ (2:18:80 NH_4OH/MeOH/DCM). HRMS $[M + H]^+$ 275.2385, $^{12}C_8^{13}C_6H_{28}B^{15}N_2O_2$ requires M^+ 275.2380. This compound was used without further purification.

9BBN-Ns-lysine-6: to a cold (0 °C) solution of **5** (100 mg, 0.25 mmol, 1.0 eq., calculated from lysine used in previous step) in 1 M K_2CO_3 aq. (1 mL) and dioxane (1 mL) was added NsCl (81 mg, 0.37 mmol, 1.5 eq.) as a solution in dioxane (0.5 mL). The reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was partitioned between ethyl acetate (2 mL) and H_2O (2 mL) and the layers separated. The aqueous layer was extracted with ethyl acetate (2 × 2 mL) and the combined organics washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (1:1 ethyl acetate/hexanes then ethyl acetate) afforded the title compound as a pale yellow solid (100 mg, 0.22 mmol, 87 %). TLC R_f = 0.32 (8:2 ethyl acetate/hexanes). $[\alpha]_D^{22} = -10.7\,°$ (c = 0.169, MeOH). MP = sinters and decomposes. IR (ATR) υ 3,419–3,011, 2,850, 1,660, 1,595, 1,537, 1,324, 1,249, 1,218, 1,161, 1,126, 1,057, 1,004, 962, 853, 781, 733, 654 cm^{-1}. ^{1}H NMR (400 MHz, MeOH-d4) δ 8.08 (1H, m), 7.87–7.77 (3H, m), 6.35 (0.22H, dm, J_{NH} = 74 Hz, NH), 5.62 (0.73H, dm, J_{NH} = 72 Hz, NH), 3.60 (1H, dm, J_{CH} = 141 Hz), 3.07 (2H, dm, J_{CH} = 139 Hz), 2.07 (0.5H, br), 1.25–1.95 (17.5H, m), 0.55 (2H, br). ^{13}C NMR (100 MHz, MeOH-d4) δ 177.3 (C = O, d,

$J_{CC} = 53$ Hz), 149.6 (quat), 135.0 (CH), 134.8 (quat), 133.6 (CH), 131.5 (CH), 125.9 (CH), 56.2 (CH, *m*), 43.9 (CH_2, dt, $J_{CC} = 36$ Hz, $J_{NC} = 5$ Hz), 32.63 (CH_2), 32.58 (CH_2), 32.3 (CH_2), 32.2 (CH_2), 31.5 (CH_2, tt, $J_{CC} = 36$ Hz, $J_{NC} = 5$ Hz), 30.4 (CH_2, td, $J_{CC} = 36$ Hz, $J_{NC} = 4$ Hz), 25.6 (CH_2), 25.3 (BCH), 25.2 (CH_2) 24.1 (BCH), 24.0 (CH_2, *t*, $J_{CC} = 35$ Hz). HRMS $[M + Na]^+$ 482.1966, ${}^{12}C^{13}_{14}C_6H_{30}BO_6S^{14}N^{15}N_2Na$ requires M^+ 482.1983.

9BBN-Et-CML-7a: to a cold (0 °C) suspension of **6** (112 mg, 0.24 mmol, 1 eq.) and K_2CO_3 (135 mg, 0.98 mmol, 4 eq.) in dry DMF (0.6 mL) was added ethyl-2-bromoacetate (54 μL, 0.49 mmol, 2 eq.) as a solution in DMF (0.2 mL). The reaction was allowed to warm to room temperature and stirred at room temperature for 18 h. The reaction mixture was cooled to 0 °C and thiophenol (130 μL, 1.22 mmol, 5 eq.) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for a further 2 h (complete by TLC). The reaction mixture was partitioned between ethyl acetate (2 mL) and H_2O (3 mL) and the layers separated. The aqueous layer was extracted with ethyl acetate (3 × 3 mL) and the combined organics washed with H_2O (5 × 1 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (5:95 MeOH/DCM) afforded the title compound as a colourless oil (68 mg, 0.19 mmol, 77 %). TLC $R_f = 0.38$ (1:9 MeOH/DCM). $[\alpha]^{22}_D = -15.6$ ° ($c = 0.192$, MeOH). MP = sinters and decomposes. IR (ATR) υ 3,700–2,500, 2,922, 2,845, 1,734 (C = O), 1,660, (C = O), 1,607, 1,455, 1,214, 1,115, 1,003, 964, 920, 843 cm^{-1}. ${}^1H$ NMR (400 MHz, $CDCl_3$) δ 5.60 (1H, dm, $J_{NH} = 73$ Hz), 4.90 (1H, dm, $J_{NH} = 72$ Hz), 4.17 (2H, q, J = 7.2 Hz), 3.79 (1H, dm, $J_{CH} = 141$ Hz), 3.40 (2H, *d*, $J = 4.7$ Hz), 2.68 (1H, dm, $J_{CH} = 134$ Hz), 2.61 (1H, dm, $J_{CH} = 134$ Hz), 2.20 (0.5H, *m*), 2.00 (1H, *m*), 1.34–1.98 (17.5H, *m*), 1.28 (3H, *t*, $J = 7.1$ Hz), 0.56 (2H, br). ${}^{13}C$ NMR (100 MHz, $CDCl_3$) δ 174.4 (C = O, *d*, $J_{CC} = 53$ Hz, 172.8 (C = O), 61.2, 55.4 (m), 50.9, 48.4 (*d*, $J_{CC} = 37$ Hz), 31.9, 31.6, 31.5, 31.3, 29.8 (*t*, $J_{CC} = 35$ Hz), 28.3 (*t*, $J_{CC} = 35$ Hz), 24.5, 24.1, 23.3 (BCH), 23.2 (BCH), 22.6 (*t*, $J_{CC} = 35$ Hz), 14.3. HRMS (ESI^+) found $[M + H]^+$ 361.2748, ${}^{12}C^{13}_{12}C_6H_{34}BO^{15}_4N_2$ requires M^+ 361.2750.

9BBN-Et-CEL-7b: to a mixture of **6** (100 mg, 0.22 mmol, 1 eq.) and K_2CO_3 (180 mg, 1.31 mmol, 6 eq.) in dry DMF (0.6 mL) was added ethyl-2-bromopropionate (85 μL, 0.65 mmol, 3 eq.) as a solution in DMF (0.2 mL). The reaction was allowed to warm to room temperature and stirred at room temperature for 18 h. The reaction mixture was cooled to 0 °C and thiophenol (130 μL, 1.22 mmol, 5 eq.) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for a further 2.5 h (complete by TLC). The reaction mixture was partitioned between ethyl acetate (2 mL) and H_2O (3 mL) and the layers separated. The aqueous layer was extracted with ethyl acetate (3 × 3 mL) and the combined organics washed with H_2O (5 × 1 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (1:99 then 5:95 MeOH/DCM) afforded the title compound as a colourless oil (52 mg, 0.14 mmol, 64 %). TLC $R_f = 0.35$ (1:9 MeOH/DCM). $[\alpha]^{23}_D = -19.4°$ ($c = 0.25$, $CHCl_3$). MP = Sinters and decomposes. IR (ATR) υ 3,600–2,500, 2,920, 2,843, 1,719 (C = O), 1,659 (C = O), 1,452, 1,357, 1,251, 1,215, 1,144, 1,066, 1,003, 962, 835 cm^{-1}. ${}^1H$ NMR (400 MHz, $CDCl_3$) δ 5.56 (1H, dm, $J_{NH} = 73$ Hz), 4.57 (1H, *m*), 4.17 (2H, *m*), 3.78 (1H, dm, $J_{CH} = 143$ Hz), 3.34 (1H, *m*), 2.62 (1H, dm, $J_{CH} = 135$ Hz), 2.57 (1H, dm, $J_{CH} = 135$ Hz), 2.11–2.33 (2H, *m*), 1.20–2.08 (24H, *m*), 0.56 (2H, br). ${}^{13}C$ NMR (100 MHz, $CDCl_3$) δ 176.1, 176.0, 174.3 (C = O, *d*, $J = 53$ Hz), 61.19, 61.15, 56.9, 56.8, 55.5 (*m*), 46.9 (dt, $J_1 = 36$ Hz, $J_2 = 4$ Hz), 30.7 (*t*, $J = 35$ Hz), 30.0 (*t*, $J = 35$ Hz), 28.8 (*m*), 24.5, 24.0, 23.2 (*t*, $J = 35$ Hz), 22.6 (*t*, $J = 35$ Hz), 19.1, 14.4. HRMS (ESI^+) found $[M + H]^+$ 375.2904, ${}^{12}C^{13}_{13}C_6H_{36}BO^{15}_4N_2Na$ requires M^+ 375.2905.

CML bis-hydrochloride-8a (method 1): to a flask containing **7a** (35 mg, 0.97×10^{-4} mol, 1 eq.) was added 6 M HCl aq. (1 mL) and the mixture was stirred at reflux for 1 h. After cooling to room temperature, the mixture was extracted with ethyl acetate (1 × 2 mL) and the layers separated. The aqueous layer was loaded directly onto a C-18 reverse phase plug and eluted with 1 % $MeCN/H_2O$. Fractions of 1 mL were collected and were visualised by ninhydrin stain. The fractions that stained positive were combined and lyophilised affording the title compound as a white solid (28 mg, quant.).

CML bis-hydrochloride-8a (method 2): a solution of **5** (100 mg, 0.25 mmol, 1 eq., based on lysine used in previous step) and glyoxylic acid hydrate (140 mg, 1.5 mmol, 6 eq.) in water (1 mL) was heated at 40 °C for 22 h after which time almost no starting material remained by TLC analysis. The reaction mixture was cooled to room temperature and extracted with ethyl acetate (3 × 2 mL), the combined organic layers were washed with saturated brine (1 × 2 mL), dried over $MgSO_4$, filtered and concentrated to a pale yellow oil (76 mg). $R_f = 0.32$ (1:19:80 AcOH/MeOH/DCM). 1 M aq. HCl (1.5 mL) was added to this intermediate (insoluble) and the reaction mixture was refluxed for 2 h after which time a pale yellow homogeneous solution was observed and the intermediate had been consumed (TLC). The reaction mixture was cooled to room temperature and partitioned between DCM (2 mL) and H_2O (2 mL) and the layers separated, the DCM layer was washed with H_2O (1 × 1 mL) and the combined aqueous layers lyophilised affording the crude material as a pale brown solid. The crude material was dissolved in H_2O (1 mL) and passed down a short C-18 reverse phase column eluting with 1 % MeCN/H_2O and 1 mL fractions collected. The fractions that stained positive by ninhydrin reagent were combined and lyophilised affording the title compound as an amorphous white solid (46 mg, 0.16 mmol, 66 %). This compound is extremely hygroscopic. $[\alpha]_D^{23} = +10.8°$ ($c = 0.166$, H_2O). IR (ATR) υ 3,660–2,220, 1,743 (C = O), 1,697 (C = O), 1,582, 1,496, 1,412, 1,196, 812 cm^{-1}. ^{1}H NMR (400 MHz, D_2O) δ 4.06 (1H, dm, J_{CH} = 147 Hz), 3.93 (2H, *d*, *J* = 2 Hz), 3.11 (2H, dm, J_{CH} = 144 Hz), 2.13 (1H, *m*), 1.50–2.0 (4H, *m*), 1.35 (1H, *m*). ^{13}C NMR (100 MHz, D_2O) δ 171.9 (*d*, *J* = 59 Hz), 169.0, 52.5 (m), 47.2, 47.0 (dt, J_1 = 35 Hz, J_2 = 4 Hz), 29.1 (td, J_1 = 34 Hz, J_2 = 5 Hz), 24.8 (td, J_1 = 35 Hz, J_2 = 4 Hz), 21.4 (*t*, J_1 = 34 Hz). HRMS (ESI$^+$) found $[M + H]^+$ 213.1330, $^{12}C_2{}^{13}C_6H_{17}{}^{15}N_2O_4$ requires M^+ 213.1325.

CEL bis-hydrochloride-8b: to a flask containing **7b** (52 mg, 0.14 mmol, 1 eq.) was added 6 M HCl aq. (1 mL) and the mixture was stirred at reflux for 1 h. After cooling to room temperature, the mixture was extracted with ethyl acetate (1 × 3 mL) and the layers separated. The aqueous layer was loaded directly onto a C-18 reverse phase plug and eluted with 1 % MeCN/H_2O. Fractions of 2 mL were collected and were visualised by ninhydrin stain. The fractions that stained positive were combined and lyophilised affording the title compound as an amorphous white solid (40 mg, 0.13 mmol, 96 %). This compound is very hygroscopic. $[\alpha]_D^{23} = +8.5°$ ($c = 0.188$, H_2O). IR (ATR) υ 3,670–2,174, 1,740 (C = O), 1,701 (C = O), 1,577, 1,451, 1,388, 1,191, 1,107, 798, 732 cm^{-1}. ^{1}H NMR (400 MHz, D_2O) δ 4.02 (1H, dm, *J* = 146 Hz), 3.96 (1H, qd, J_1 = 7 Hz, J_2 = 2 Hz), 3.08 (2H, dm, J_1 = 144 Hz), 1.46–1.99 (4H, *m*), 1.53 (3H, dd, J_1 = 7 Hz, J_2 = 3 Hz), 1.35 (1H, *m*). ^{13}C NMR (100 MHz, D_2O) δ 172.1 (*d*, *J* = 59 Hz), 171.9, 55.7, 52.6 (*m*), 45.4 (dt, J_1 = 35 Hz, J_2 = 4 Hz), 29.2 (td, J_1 = 34 Hz, J_2 = 5 Hz), 25.2 (td, J_1 = 35 Hz, J_2 = 4 Hz), 21.4 (*t*, J_1 = 34 Hz), 14.1. HRMS (ESI$^+$) found $[M + H]^+$ 227.1485, $^{12}C_3{}^{13}C_6H_{19}{}^{15}N_2O_4$ requires M^+ 227.1481.

Fmoc-TBS-pyrraline-12: to a solution of Fmoc-Lys TFA salt (140 mg, 0.29 mmol, 1.0 eq.) in THF (5 mL) was added DIPEA (51 μL, 0.29 mmol, 1.0 eq.) and the reaction stirred at room temperature for 3 h. A white precipitate starts forming immediately. After 3 h, the reaction mixture was concentrated to dryness and then dissolved in a THF/H_2O mixture (1:1, 1 mL), the enone **11** was then added (68 mg, 0.29 mmol, 1 eq.) and the reaction was heated at 40 °C for 18 h. The reaction mixture was diluted with H_2O (2 mL) and extracted with ethyl acetate (3 × 2 mL). The combined organics were washed with H_2O (2 mL) then saturated brine (2 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (1. 25, 2. 50, 3. 75 % ethyl acetate/hexanes with 0.25 % AcOH) afforded recovered enone starting material (24 mg, 35 %) and the title compound as an amorphous off-white solid (85 mg, 1.42×10^{-4} mol, 54 %). All the lysine starting material was consumed in the reaction. TLC R_f = 0.39 (10 % MeOH/DCM, UV and ninhydrin). MP = sinters and decomposes above 40 °C. $[\alpha]_D^{24} = +15.0°$ ($c = 0.240$, DCM). IR (ATR) υ 3,500–2,400, 2,930, 2,857, 1,692, 1,656, 1,451, 1,251, 1,062, 836, 776, 759, 738 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.42 (1H, *d*, *J* = 4 Hz, CHO), 7.75 (2H, *d*, *J* = 7 Hz), 7.60 (2H, *t*, *J* = 7 Hz), 7.39 (2H, *t*, *J* = 7 Hz), 7.30 (2H, *t*, *J* = 7 Hz), 6.87 (1H, *t*, *J* = 4 Hz), 6.15 (1H, *t*, *J* = 4 Hz), 5.49 (1H, dd, J_1 = 91 Hz, J_2 = 8 Hz, NH), 4.66 (2H, *d*, *J* = 2 Hz), 4.05–4.65 (5H, *m*), 4.22 (1H, *t*, *J* = 7 Hz), 1.15–2.22 (6H, *m*), 0.988 (9H, *s*), 0.06 (6H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.6, 175.8 (*d*, *J* = 59 Hz), 156.3 (*d*, *J* = 24 Hz), 144.0, 143.9, 142.5 (*d*, *J* = 10 Hz), 141.5, 132.1 (*d*, *J* = 10 Hz), 127.9, 127.2, 125.3 (2 × CH), 120.1, 110.5, 67.3, 57.4, 53.8 (*m*), 47.3, 45.6 (*m*), 31.9 (*t*, *J* = 26 Hz), 30.6 (*t*, *J* = 26 Hz), 25.9, 22.6 (*t*, *J* = 26 Hz), 18.4, −5.2. HRMS (ESI$^+$) found $[M + Na]^+$ 621.2836, $^{12}C_{27}{}^{13}C_6H_{42}{}^{15}N_2O_6SiNa$ requires M^+ 621.2846.

Fmoc-pyrraline-13: a solution of **12** (31 mg, 0.52×10^{-4} mol, 1 eq.) in a 1 % conc. HCl solution in THF/H_2O (9:1, 1 mL) was stirred at room temperature for 8 h. The reaction was diluted with H_2O (2 mL) and extracted with ethyl acetate (3 × 2 mL). The combined organics were washed with saturated brine (2 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (1. 5, 2. 10 % MeOH/DCM with 0.25 % AcOH) afforded the title compound as an amorphous white solid (24 mg, 0.50×10^{-4} mol, 96 %). TLC R_f = 0.13 (10 % MeOH/DCM). MP = sinters and decomposes above 40 °C. $[\alpha]_D^{23} = +3.5°$ ($c = 0.40$, $CHCl_3$). IR (ATR) υ 3,650–2,326, 2,931, 1,688, 1,645, 1,501, 1,449, 1,325, 1,191, 1,023, 760, 739 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.40 (1H, *d*, *J* = 4 Hz, CHO), 7.74 (2H, *d*, *J* = 7 Hz), 7.56 (2H, *t*, *J* = 7 Hz), 7.38 (2H, *t*, *J* = 7 Hz), 7.29 (2H, *t*, *J* = 7 Hz), 6.83 (1H, *t*, *J* = 4 Hz), 6.17 (1H, *t*, *J* = 4 Hz), 5.63 (1H, dd, J_1 = 92 Hz, J_2 = 8 Hz, NH), 4.00–4.78 (6H, *m*), 4.37 (1H, *d*, *J* = 7 Hz), 4.18 (1H, *t*, *J* = 7 Hz), 1.10–2.17 (6H, *m*). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.8, 175.5 (*d*, *J* = 59 Hz), 156.7 (*d*, *J* = 24 Hz), 144.0, 143.8, 141.9 (*d*, *J* = 13 Hz), 141.4, 132.3 (*d*, *J* = 13 Hz), 127.9, 127.3, 125.2 (2 × CH), 120.1, 110.9, 67.3, 56.3, 53.4 (*m*), 47.3, 45.6 (*m*), 31.9 (*t*, *J* = 34 Hz), 30.4 (*t*, *J* = 34 Hz), 22.3 (*t*, *J* = 34 Hz). HRMS (ESI$^+$) found $[M + Na]^+$ 507.1985, $^{12}C_{21}{}^{13}C_6H_{28}{}^{15}N_2O_6Na$ requires M^+ 507.1982.

Pyrraline-14: a solution of **13** (53 mg, 1.09×10^{-4} mol, 1 eq.) in 5 % piperidine/DCM (1.5 mL) was stirred at room temperature for 1.5 h. The reaction mixture was concentrated under reduced pressure then under high vacuum (1 h). Purification by silica flash chromatography (1. 9:2:1, 2. 9:2:2 MeCN/EtOH/H_2O) afforded the title compound as a pale yellow solid (23 mg, 0.88×10^{-4} mol, 80 %). TLC $R_f = 0.25$ (9:2:2 MeCN/EtOH/H_2O). $[\alpha]_D^{22} = +3.5°$ ($c = 0.40$, H_2O). IR (ATR) υ 3,600–2,400, 1,656, 1,541, 1,392, 1,343, 1,153, 1,000, 779, 722 cm^{-1}. ^{1}H NMR (400 MHz, D_2O) δ 9.35 (1H, *d*, $J = 4$ Hz, CHO), 7.15 (2H, *d*, $t = 4$ Hz), 6.39 (1H, *t*, $J = 4$ Hz), 4.70 (2H, *d*, $J = 2$ Hz), 4.31 (2H, dm, $J_1 = 140$ Hz), 3.73 (1H, dm, $J_1 = 145$ Hz), 1.88 (2H, bd, $J = 130$ Hz), 1.76 (2H, bd, $J = 130$ Hz), 1.49 (2H, bd, $J = 130$ Hz). ^{13}C NMR (100 MHz, D_2O) δ 181.4, 174.7 (*d*, $J = 54$ Hz), 143.4 (*d*, $J = 14$ Hz), 131.7 (*d*, $J = 14$ Hz), 126.3, 111.0, 54.8, 54.7 (ddt, $J_1 = 54$ Hz, $J_2 = 34$ Hz, $J_3 = 5$ Hz), 44.9 (*m*), 30.3 (td, $J_1 = 34$ Hz, $J_2 = 4$ Hz), 30.1 (td, $J_1 = 34$ Hz, $J_2 = 4$ Hz), 21.6 (*t*, $J = 34$ Hz). HRMS (ESI^+) found $[\mathrm{M} + \mathrm{Na}]^+$ 285.1304, ${}^{12}\mathrm{C}_6{}^{13}\mathrm{C}_6\mathrm{H}_{18}{}^{15}\mathrm{N}_2\mathrm{O}_4\mathrm{Na}$ requires M^+ 285.1301.

Results and discussion

The first consideration when planning our synthesis was that the desired compounds have a sufficient mass difference from their non-labelled analogues to easily distinguish the peaks in the mass spectrum. Therefore, the synthesis of the three isotopically labelled monolysyl AGEs commenced with [U-$^{13}C_6$, 99 %, U-$^{15}N_2$, 99 %]-L-lysine dihydrochloride **4**, providing a mass difference of 8 amu from the non-labelled parent analogue.

For the synthesis of stable isotope labelled CML **1** and CEL **2** it was decided to employ the 9-BBN group to concomitantly protect the alpha amino and carboxylic acid groups in lysine according to the method of Dent (Dent et al. 2002). This approach had the advantage of selectively protecting the alpha-NH_2 group in the presence of the epsilon-NH_2, whilst reducing the number of protection/deprotection steps required in the syntheses. More importantly, we envisaged that when used with other acid labile protecting groups, the BBN group would enable a final global deprotection step to access the desired free amino acids, while providing a tractable organic compound up to this final step. In addition, it was decided to use the nosyl (Ns) group alkylation methodology as a reliable and efficient method to introduce the required side chain functionality (Kan and Fukuyama 2004).

Our synthesis (Scheme 1) started with treatment of [U-$^{13}C_6$, 99 %, U-$^{15}N_2$, 99 %]-L-lysine dihydrochloride **4** with 9-BBN dimer in MeOH affording BBN protected lysine **5**. With **5** in hand, the desired nosyl group was cleanly introduced using standard conditions and furnished BBN-Ns-lysine **6** in an 89 % yield over two steps from **4**. At this stage, a one-pot alkylation/deprotection reaction was developed. Hence, a solution of **6** and K_2CO_3 in DMF was treated with either ethyl bromoacetate or ethyl bromopropionate and stirred for 24 h after which time thiophenol was added and the reaction was stirred for an additional 2 h affording BBN-OEt-CML **7a** and BBN-OEt-CEL **7b** in 77 and 64 % yields, respectively. As envisaged, use of the 9-BBN and ethyl ester protecting groups enabled the final global deprotection step to be carried out by simply refluxing **7a** or **7b** in 6 M aq HCl for 1 h. After cooling to room temperature, the reaction mixture was partitioned between H_2O and ethyl acetate to remove the organic by-products (BBN) and the aqueous layer was freeze-dried. The crude material was purified using a short reverse phase C-18 column eluting with 0.5 % MeCN/H_2O to remove any remaining organic by-products. Finally, the dihydrochloride salts of CML **8a** and CEL **8b** were isolated by lyophilization in 99 and 98 % yields and overall yields of 68 and 56 %, respectively. It is noteworthy that there are no difficult or lengthy isolation or purification steps required in these syntheses.

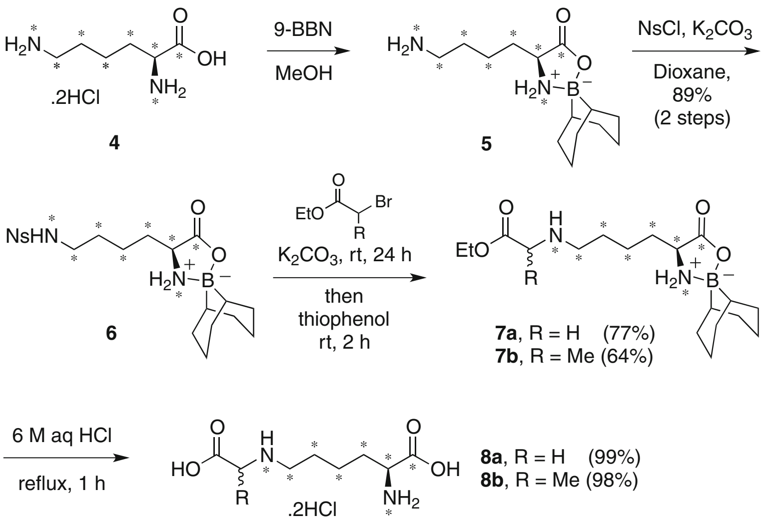
A second, more direct synthesis of CML using BBN protected lysine **5** was also developed (Scheme 2) and employed an approach similar to that reported by Kilhberg et al. (1983). The BBN group was introduced as described above and the BBN protected lysine **5** was then treated with an excess of glyoxylic acid in H_2O at 40 °C for 22 h. After cooling to room temperature, the mixture was partitioned with ethyl acetate and the organic layer concentrated to dryness affording the formylated intermediate **9**, which was then refluxed in 1 M aq HCl to cleave the formyl and BBN groups. The only purification required in this route is a simple filtration after the final step using a short C-18 column as described above, which furnished the desired CML dihydrochloride **8a** in an overall yield of 66 % from **4**.

Unfortunately, attempts to synthesize CEL by replacing glyoxylic acid with pyruvic acid were unsuccessful.

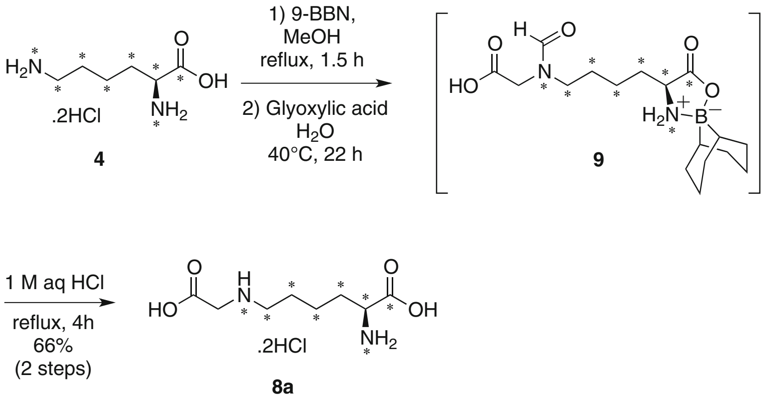
Our strategy for the synthesis of pyrraline (Scheme 3) relied on the condensation of dihydropyranone **11** with the side chain amine of a lysine derivative. We had hoped to utilise BBN-protected lysine **5** as the starting material for each AGE synthesis, however, despite several attempts the condensation between **5** and **11** proved unsuccessful. Therefore, we resorted to the use of Fmoc-lysine and the required [U-$^{13}C_6$, 99 %, U-$^{15}N_2$, 99 %]-L-Fmoc-lysine trifluoroacetate **10** was prepared from [U-$^{13}C_6$, 99 %, U-$^{15}N_2$, 99 %]-L-lysine dihydrochloride **4** according to a literature procedure (Wiejak et al. 2001).

Thus, the TFA lysine derivative **10** was treated with diisopropylethylamine and the resulting free base reacted

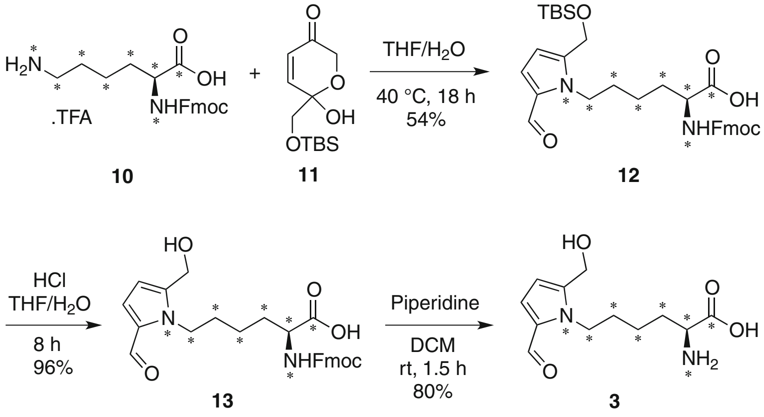
Scheme 1 Synthesis of the dihydrochloride salts of CML (**8a**) and CEL (**8b**)



Scheme 2 More direct synthesis of CML hydrochloride (**8a**) using glyoxylic acid



Scheme 3 Synthesis of pyrraline (**3**)



with dihydropyranone **11** (Geng et al. 2012) affording the desired protected pyrraline **12** in a 54 % yield. It is worth noting that this compound is a suitable building block for incorporation into peptides using automated solid phase peptide synthesis. With **12** in hand, removal of the TBS group was easily achieved by treatment with 1 M aq HCl in THF (1:9 v:v) furnishing **13** in excellent yield (96 %). Finally, the Fmoc group was removed under standard conditions affording the desired free amino acid of pyrraline **3** in a good 80 % yield. As with CML and CEL, there are no difficult isolation or purification steps required in this route.

Conclusion

In summary, we have developed robust and straightforward syntheses of ^{13}C/^{15}N stable isotope-labelled monolysyl AGEs CML **1**, CEL **2** and pyrraline **3** in overall yields of 68, 56 and 41 %, respectively. These routes are noteworthy for their practicality, requiring no difficult or lengthy isolation or purification steps. The final compounds are suitable as standards for quantitative mass spectrometry studies and will facilitate investigations into the role that these AGEs play in the pathogenesis of diabetes and related diseases. These studies are important steps towards the development of new and improved therapeutic strategies to combat these debilitating diseases.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Ahmed N, Thornalley PJ (2007) Advanced glycation endproducts: what is their relevance to diabetic complications. Diabetes Obes Metab 9:233–245

Basta G, Schmidt AM, De Caterina R (2004) Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc Res 63:582–592

Dent WH III, Erickson R, Fields SC, Parker MH, Tromiczak EG (2002) 9-BBN: an amino acid protecting group for functionalization of amino acid side chains in organic solvents. Org Lett 4:1249–1251

Geng HM, Chen JLY, Furkert DP, Jiang S, Brimble MA (2012) A convergent synthesis of the 2-formylpyrrole spiroketal natural product Acortatarin A. Synlett 23:855–858

Henle T (2005) Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. Amino Acids 29:313–322

Kan T, Fukuyama T (2004) Ns strategies: a highly versatile synthetic method for amines. Chem Commun 4:353–359

Kihlberg J, Bergman R, Wickberg B (1983) Synthesis of strombine: a new method for monocarboxymethylation of primary amines. Acta Chem Scand B 37:911–916

Ledl F, Schleicher E (1990) The Maillard reaction in food and in the human body—new results in chemistry, biochemistry and medicine. Angew Chem Int Ed 102:597–626

Münch G, Westcott B, Menini T, Gugliucci A (2012) Advanced glycation endproducts and their pathogenic roles in neurological disorders. Amino Acids 42:1221–1236

Peppa M, Vlasarra H (2005) Advanced glycation end products and diabetic complications: a general overview. Hormones 4:28–32

Rabbani N, Thornalley PJ (2012) Glycation research in amino acids: a place to call home. Amino Acids 42:1087–1096

Stitt AW (2001) Advanced glycation: an important pathological event in diabetic and age related ocular disease. Br J Ophthalmol 85:746

Teerlink T, Barto R, ten Brink HJ, Schalwijk CG (2004) Measurement of N”-(carboxymethyl)lysine and N”-(carboxyethyl)lysine in human plasma protein by stable-isotope-dilution tandem mass spectrometry. Clin Chem 50:1222–1228

Thornalley PJ, Battah S, Ahmed N, Karachalias N, Agalou S, Babaei-Jadidi R, Dawnay A (2003) Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 375:581–592

Verzijl N, DeGroot J, Oldehinkel E, Bank RA, Thorpe SR, Baynes JW, Bayliss MT, Bijlsma JWJ, Lafeber FPJG, TeKoppele JM (2000a) Age-related accumulation of Maillard reaction products in human articular cartilage collagen. Biochem J 350:381

Verzijl N, DeGroot J, Thorpe SR, Bank RA, Shaw JN, Lyons TJ, Bijlsma JWJ, Lafeber FPJG, Baynes JW, TeKoppele JM (2000b) Effect of collagen turnover on the accumulation of advanced glycation end products. J Biol Chem 275:39027–39031

Wiejak S, Masiukiewicz E, Rzeszotarska B (2001) Improved scalable synthesis of mono- and bis-urethane derivatives of ornithine. Chem Pharm Bull 49:1189–1191

Woods TM, Kamalov M, Harris PWR, Cooper GJS, Brimble MA (2012) Synthesis of monolysyl advanced glycation endproducts and their incorporation into collagen model peptides. Org Lett 14:5740–5743