Convenient Synthesis of N^{ε}-(Carboxymethyl)lysine, a Key Advanced Glycation Endproduct Biomarker

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Abstract: Advanced glycation endproducts (AGEs) are formed when sugars react with peptides and proteins without the help of enzymes and by thermal processing of food such as baking and frying. AGEs and especially N^{ε}-(carboxymethyl)lysine (CML) has been used as general key biomarkers for oxidative stress and a number of diseases associated with poor lifestyle. Herein we present the first synthetic pathway to the free zwitter ion of CML via a protected intermediate.

Key words: CML, AGE biomarker, amino acids, amination, natural products

The thermal processing of foods such as baking, broiling, and frying in normal open air accelerates a large number of chemical reactions with a visible, browning or darkening result obvious and well-known to all people. These chemical reactions comprise the Maillard reaction that give rise to Schiff base intermediates which are unstable and subsequently undergo intramolecular Amadori rearrangements, leading to the formation of advanced glycation endproducts (AGEs).[1] These AGEs are typically formed when reducing sugars react with guanidino groups or free amines in the side chain or N-terminus of amino acids, peptides, and proteins. Also amino lipids and nucleic acids can undergo similar reactions resulting in formation of AGEs.[2,3] So far, several AGEs have been isolated and characterized; examples include pentosidine,[4] glyoxal–lysine dimer (GOLD)[5] and N^{ε}-(carboxymethyl)lysine (CML, **1**)[6] as exemplified in Figure 1.

Besides the thermally induced formation of AGEs, they are also generated in vivo, without the help of enzymes.[7] Through many years, human health and nutrition have been two areas showing increasing interest in the fields of AGEs.[8] In medical prognostic research, AGEs and especially CML have been used as a general key biomarker for oxidative stress and a number of diseases associated with poor lifestyle such as atherosclerosis and type 2 diabetes. Moreover, AGEs also serve as an appetite-enhancing agent and thereby encourage overnutrition that leads to obesity and eventually also elevate the intake of more AGEs.[9]

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Some AGE compounds also enhance oxidative stress through the receptor for AGEs, which is found in the endothelium of several organs. The oxidative stress may trigger inflammation and β-cell injury in pancreas, contributing to peripheral insulin resistance and potentially to type 1 or type 2 diabetes.[9] The implications from overload of pro-oxidants such as CML are considerable and call for further preclinical and clinical research. Therefore, new efficient methodologies for the reproducible and robust synthesis of AGEs in high purity and large amounts are needed.

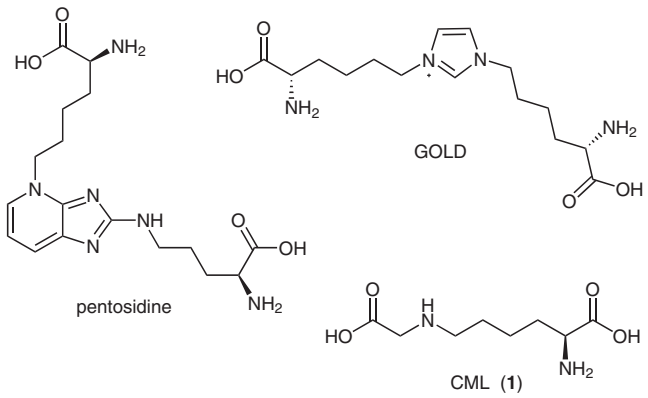


Figure 1 Examples of advanced glycation end products (AGEs)

To date, several syntheses of CML (**1**) have been disclosed, and in all cases the final product was isolated as the corresponding hydrochloric salt.[1,10–15] The synthetic routes either proceed via reductive amination of glyoxylic acid with an N^{α}-protected lysine,[11,15] or via alkylation of an N^{α}-protected lysine with haloacetic acids.[1,10,13,14,16] However, these syntheses suffer from low yields and/or cumbersome purification procedures that generally only allow for the synthesis of CML (**1**) on a milligram scale. The described purification procedures include ion-exchange chromatography and evaporation of large volumes of aqueous HCl. We therefore wished to develop a synthetic pathway to CML (**1**) passing via easy and high-yielding access to protected CML **6**, and allows for facile global deprotection yielding CML (**1**) as the free zwitter ion on a gram scale. Herein we report a new and convenient synthetic pathway to CML (**1**) that passes through benzyl ester- and Cbz-protected CML **6** (Scheme 1) ultimately allowing for the first synthesis of CML (**1**) as the free zwitter ion.

Protected CML **6** was synthesized by reductive amination of aldehyde **5** with appropriately N^{α}-/C-protected lysine **3** as illustrated in Scheme 1. Thus, the required (*Z*)-Lys-OBn (**3**) was prepared from commercially available (*Z*)-Lys(Boc)-OH (**2**), where the acid moiety was first protected as a benzyl ester.[17] Subsequently, the Boc group was removed employing a modified literature procedure[17] using 40% TFA in CH_2Cl_2, maintaining the temperature at 0 °C both during the reaction and the following workup to avoid cleavage of the benzyl ester. Aldehyde **5** was prepared by oxidative cleavage of dibenzyl tartrate **4** using periodic acid as described in literature.[18]

Different reaction conditions for the reductive amination step were screened on a 0.5 mmol scale, including the use of a representative range of reducing agents such as $NaBH_3CN$, $NaBH(OAc)_3$, BH_3·pyridine and BH_3·THF (Table 1). Thus, the initial reductive amination was carried out in ethanol using $NaBH_3CN$ as the reducing agent in the presence of acetic acid (Table 1, entry 1).[19] The amine and the aldehyde (1.0 equiv) were allowed to react for one hour in the presence of acetic acid (0.3 equiv) before $NaBH_3CN$ (1.2 equiv) was added, and the mixture was then allowed to stir for another two hours (Table 1, entry 1).

After workup and flash chromatography, we obtained the desired product **6** in 28% yield. No improvement was observed when increasing the reaction time to four hours (Table 1, entry 2). The obtained 28% yield could be improved to 38% and 44% by increasing the amount of added aldehyde **5** to 2.0 equivalents and 6.0 equivalents, respectively (Table 1, entries 3 and 4). Though not satisfied with these mediocre yields, we still observed no improvement when omitting the addition of acetic acid (Table 1, entry 5) or increasing the amount of $NaBH_3CN$ to 5.0 equivalents (Table 1, entry 6). Attempts to ensure complete imine formation by stirring overnight with molecular sieves (Table 1, entry 7) or by use of microwave irradiation at 150 °C for ten minutes (Table 1, entry 8) likewise proved futile. The latter even led to complete decomposition of the starting materials. Substituting $NaBH_3CN$ with $NaBH(OAc)_3$ resulted in an initial decrease in the yield to 28% (Table 1, entry 9) although an improvement to 35% was observed when using DCE as solvent without the addition of acetic acid (Table 1, entry

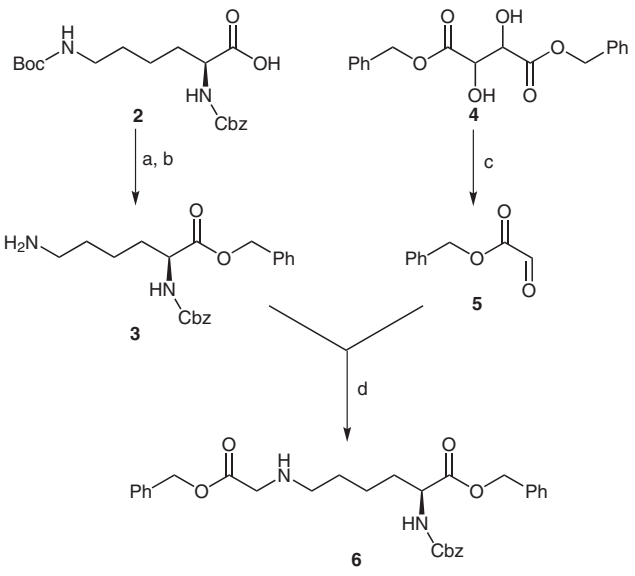
Table 1 Optimization of Reductive Amination

Entry	Imine formation	Aldehyde **5** (equiv)	Reducing agent (equiv)	Solvent	Reaction time	Further conditions	Yield (%)
1	1 h	1.0[b]	$NaBH_3CN$ (1.2)	EtOH	2 h	AcOH (0.3 equiv)	28
2	1 h	1.0[b]	$NaBH_3CN$ (1.2)	EtOH	4 h	AcOH (0.3 equiv)	28
3	1 h	2.0[b]	$NaBH_3CN$ (2.0)	EtOH	2 h	AcOH (0.3 equiv)	38
4	1 h	6.0[a]	$NaBH_3CN$ (2.0)	EtOH	2 h	AcOH (0.3 equiv)	44
5	1 h	2.0[a]	$NaBH_3CN$ (2.4)	EtOH	2 h	–	31
6	1 h	2.0[b]	$NaBH_3CN$ (5.0)	EtOH	2 h	$NaBH_3CN$ added twice, AcOH (0.3 equiv)	38
7	15 h MS	2.0[b]	$NaBH_3CN$ (1.2)	EtOH	2 h	AcOH (0.3 equiv)	29
8	10 min MW, 150 °C	2.0[b]	$NaBH_3CN$ (2.0)	EtOH	2 h	AcOH (0.3 equiv)	0
9	1 h	2.0[a]	$NaBH(OAc)_3$ (1.2)	EtOH	2 h	AcOH (0.3 equiv)	28
10	1 h	2.0[a]	$NaBH(OAc)_3$ (2.0)	DCE	2 h	–	35
11	–	1.0[a]	$NaBH(OAc)_3$ (1.5)	DCE	1.5 h (0.5 h)[c]	–	47
12	–	1.2[a]	$NaBH(OAc)_3$ (1.5)	DCE	1.5 h (0.5 h)[c]	–	63
13	–	1.2[a]	$NaBH(OAc)_3$ (1.5)	DCE	2 h (1 h)[c]	–	55
14	–	1.4[a]	$NaBH(OAc)_3$ (1.5)	DCE	2 h (1 h)[c]	–	47
15	–	1.2[a]	$NaBH_3CN$ (1.5)	EtOH	1.5 h (0.5 h)[c]	AcOH (0.3 equiv)	20
16	–	1.2[a]	BH_3·pyridine (1.5)	CH_2Cl_2–AcOH	1.5 h (0.5 h)[c]	–	trace
17	–	1.2[a]	BH_3·THF (1.5)	CH_2Cl_2–AcOH	1.5 h (0.5 h)[c]	–	trace

[a] Freshly distilled.
[b] In a solution of toluene.
[c] Aldehyde **5** was added slowly to a solution of amine **3** and the reducing agent followed by stirring.

10).[20] In general we had observed that the major byproduct in these reactions was the corresponding dialkylated product. We speculated that the formation of this byproduct could be minimized by adding aldehyde **5** more slowly to a solution of amine **3** and the reducing agent. Thus, by adding aldehyde **5** (1.0 equiv) to a solution of amine **3** and $NaBH(OAc)_3$ (1.5 equiv) over 30 minutes, we were able to increase the yield to 47% while no formation of the dialkylated product was observed (Table 1, entry 11). By increasing the amount of added aldehyde **5** with 0.2 equivalents (Table 1, entry 12), the yield increased to 63%. Further increments in the amount of aldehyde and increase in reaction time both resulted in a decrease in the yield (Table 1, entries 13 and 14). Adaptation of the optimal conditions to the use of $NaBH_3CN$ resulted in a decrease of yield to 20% (Table 1, entry 15). Furthermore, the use of BH_3·pyridine or BH_3·THF as reducing agents only furnished traces of the desired product (Table 1, entries 16 and 17). Using the conditions from entry 12 (Table 1), we then synthesized 3.65 g of protected CML **6** in 62% yield.

With protected CML **6** in hand, we could then conveniently complete the first synthesis of CML (**1**) as the free zwitter ion by a simple hydrogenation step (Scheme 2). The hydrogenation was performed using Pd/C under H_2 with a pressure of five bar and MeOH as solvent.[21] CML (**1**) was isolated by simple filtration and evaporation in 96% yield.



Scheme 1 Synthesis of protected CML **6**. *Reagents and conditions*: (a) BnBr, DIPEA, MeCN, r.t., 90%; (b) TFA–CH_2Cl_2 (2:3), 0 °C, 98%; (c) HIO_4, Et_2O, r.t., 64%; (d) reductive amination (see Table 1 for details).

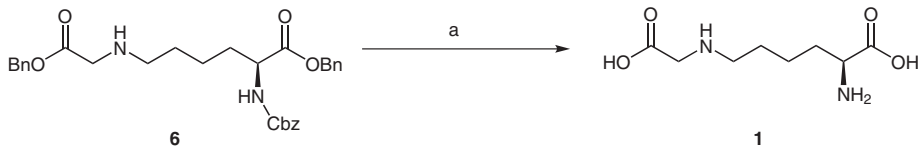
In summary, we have developed a facile, efficient, and convenient method for the synthesis of CML (**1**) on gram scale. The synthesis progresses via benzyl ester- and Cbz-protected CML **6** by a simple hydrogenation to give, for the first time, pure CML as the free zwitter ion in a crystalline state. We expect that the strategy may be modified to enable access to suitably protected CML amino acid building blocks for incorporation of CML in peptide chains[22] and thereby allow for the observation of AGE at work in natural, biological contexts.

(*S*)-Benzyl 6-Amino-2-{[(benzyloxy)carbonyl]amino}hexanoate (3)

To a solution of (*S*)-benzyl 2-{[(benzyloxy)carbonyl]amino}-6-[(*tert*-butoxycarbonyl)amino]hexanoate (5.50 g, 11.7 mmol) in CH_2Cl_2 (63 mL) at 0 °C was added slowly TFA (42 mL). The resulting mixture was stirred at 0 °C for 1.5 h and was then concentrated under reduced pressure at 0 °C. The residue was dissolved in CH_2Cl_2 (250 mL) and was washed with sat. aq Na_2CO_3 (150 mL). The aqueous layer was extracted with CH_2Cl_2 (250 mL), and the combined organic layers were washed with H_2O (250 mL), dried over Na_2SO_4, filtered, concentrated at 0 °C and dried in vacuo, yielding **3** (4.22 g, 98%) as a colorless oil. R_f = 0.40 (EtOAc–MeOH–concd aq NH_3 = 75:20:5). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.40–7.30 (m, 10 H), 5.55–5.40 (m, 1 H), 5.25–5.17 (m, 4 H), 4.48–4.37 (m, 1 H), 2.67–2.53 (m, 2 H), 1.93–1.58 (m, 2 H), 1.47–1.20 (m, 4 H), 1.17–1.05 (s, 2 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.3 (C_q), 155.8 (C_q), 136.2 (C_q), 135.3 (C_q), 128.5, 128.4, 128.4, 128.3, 128.1, 128.0 (10 CH), 67.0 (CH_2), 66.9 (CH_2), 53.8 (CH), 41.7 (CH_2), 33.1 (CH_2), 32.4 (CH_2), 22.3 (CH_2) ppm. NMR spectra were in full accordance with those reported in literature.[17]

(*S*)-Benzyl 6-{[(Benzyloxy)-2-oxoethyl]amino}-2-{[(benzyloxy)carbonyl]amino}hexanoate (6)

To a solution of **3** (4.20 g, 11.4 mmol) in DCE (75 mL) at r.t. was added $NaBH(OAc)_3$ (3.60 g, 17.1 mmol), and then a solution of **5** (2.20 g, 13.7 mmol) in DCE (75 mL) slowly over 30 min. After stirring for 1 h further, H_2O (130 mL) was added, the organic layer was isolated, and the aqueous layer was extracted with CH_2Cl_2 (150 mL). The combined organic layers were dried over Na_2SO_4, filtered, concentrated, and dried in vacuo. Flash column chromatography of the crude product (SiO_2, EtOAc) yielded **6** (3.65 g, 62%) as a colorless oil. R_f = 0.30 (EtOAc). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.40–7.28 (m, 15 H), 5.46–5.32 (m, 1 H), 5.22–5.08 (m, 6 H), 4.45–4.35 (m, 1 H), 3.42 (s, 2 H), 2.58–2.48 (m, 2 H), 1.93–1.60 (m, 2 H), 1.55 (s, 1 H), 1.50–1.40 (m, 2 H), 1.40–1.20 (m, 2 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.4 (C_q), 172.2 (C_q), 155.9 (C_q), 136.3 (C_q), 135.6 (C_q), 135.3 (C_q), 128.6, 128.5, 128.5, 128.4, 128.4, 128.3, 128.2, 128.1 (15CH), 67.1 (CH_2), 66.9 (CH_2), 66.6 (CH_2), 53.9 (CH), 50.6 (CH_2), 48.9 (CH_2), 32.4 (CH_2), 29.3 (CH_2), 22.7 (CH_2) ppm. HRMS (TOF MS ES+): *m/z* calcd for $C_{30}H_{35}N_2O_6$ $[M + H]^+$: 519.2490; found: 519.2488.



Scheme 2 Hydrogenation of protected CML **6** leading to CML (**1**). *Reagents and conditions*: a) Pd/C, H_2 (5 bar), MeOH, 96%.

(2*S*)-2-Amino-6-[(carboxymethyl)amino]hexanoate, (CML) (1)
To a solution of **6** (2.80 g, 5.40 mmol) in MeOH (40 mL) at r.t. was added Pd/C (280 mg, 10 wt%), and the resulting mixture was stirred for 24 h under H_2 (5 bar). The mixture was then filtered through Celite and first washed with MeOH to remove impurities followed by wash with H_2O which dissolved the product. Concentration of the filtrate yielded **1** (1.06 g, 96%) as colorless solid; mp >228 (dec.). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.68 (t, 1 H, *J* = 6.1 Hz), 2.60 (s, 2 H), 3.12–3.00 (m, 2 H), 1.95–1.80 (m, 2 H), 1.80–1.68 (m, 2 H), 1.60–1.40 (m, 2 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 175.7 (C_q), 171.8 (C_q), 54.6 (CH), 49.3 (CH_2), 47.1 (CH_2), 30.4 (CH_2), 25.3 (CH_2), 21.6 (CH_2) ppm. HRMS (TOF MS ES$^+$): *m/z* calcd for $C_8H_{17}N_2O_4$ $[M + H]^+$: 205.1183; found: 205.1188.

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References

(1) Glomb, M. A.; Monnier, V. M. *J. Biol. Chem.* **1995**, *270*, 10017.
(2) Fu, M.-X.; Requena, J. R.; Jenkins, A. J.; Lyons, T. J.; Baynes, J. W.; Thorpe, S. R. *J. Biol. Chem.* **1996**, *271*, 9982.
(3) Zhang, Q.; Ames, J. M.; Smith, R. D.; Baynes, J. W.; Metz, T. O. *J. Proteome Res.* **2009**, *8*, 754.
(4) Sell, D. R.; Monnier, V. M. *J. Biol. Chem.* **1989**, *264*, 21597.
(5) Wells-Knecht, K. J.; Brinkmann, E.; Baynes, J. W. *J. Org. Chem.* **1995**, *60*, 6246.
(6) Ahmed, M. U.; Thorpe, S. R.; Baynes, J. W. *J. Biol. Chem.* **1986**, *261*, 4889.
(7) Uribarri, J.; Cal, W.; Sandu, O.; Peppa, M.; Goldberg, T.; Vlassara, H. *Ann. N. Y. Acad. Sci.* **2005**, *1043*, 461.
(8) Gerrard, J. A. *Trends Food Sci. Technol.* **2006**, *17*, 324.
(9) Vlassara, H.; Striker, G. E. *Nat. Rev. Endocrinol.* **2011**, *7*, 526.
(10) Ahmed, M. U.; Thorpe, S. R.; Baynes, J. W. *J. Biol. Chem.* **1986**, *261*, 4889.
(11) Delatour, T.; Fenaille, F.; Parisod, V.; Arce Vera, F.; Buetler, T. *Amino Acids* **2006**, *30*, 25.
(12) Pashikanti, S.; de Alba, D. R.; Boissonneault, G. A.; Cervantes-Laurean, D. *Free Radical Biol. Med.* **2010**, *48*, 656.
(13) Takanashi, M.; Sakurai, T.; Tsuchiya, S. *Chem. Pharm. Bull.* **1992**, *40*, 705.
(14) Matsutani, H.; Kusumoto, S.; Koizumi, R.; Shiba, T. *Phytochemistry* **1979**, *18*, 661.
(15) Csuk, R.; Stark, S.; Barthel, A.; Kluge, R.; Ströhl, D. *Synthesis* **2009**, 1933.
(16) (a) Grunwald, S.; Krause, R.; Bruch, M.; Henle, T.; Brandsch, M. *Br. J. Nutr.* **2006**, *95*, 1221. (b) Hellwig, M.; Geissler, S.; Matthes, R.; Peto, A.; Silow, C.; Brandsch, M.; Henle, T. *ChemBioChem* **2011**, *12*, 1270.
(17) De León-Rodriguez, L. M.; Kovacs, Z.; Dieckmann, G. R.; Sherry, A. D. *Chem. Eur. J.* **2004**, *10*, 1149.
(18) Bishop, J. E.; O'Connell, J. F.; Rapoport, H. *J. Org. Chem.* **1991**, *56*, 5079.
(19) Messeguer, J.; Masip, I.; Montolio, M.; del Rio, J. A.; Soriano, E.; Messeguer, A. *Tetrahedron* **2010**, *66*, 2444.
(20) Maresca, K. P.; Marquis, J. C.; Hillier, S. M.; Lu, G.; Femia, F. J.; Zimmerman, C. N.; Eckelman, W. C.; Joyal, J. L.; Babich, J. W. *Bioconjugate Chem.* **2010**, *21*, 1032.
(21) Wu, X.; Jiang, Z.; Shen, H.-M.; Lu, Y. *Adv. Synth. Catal.* **2007**, *349*, 812.
(22) Gruber, P.; Hofmann, T. *J. Peptide Res.* **2005**, *66*, 111.

