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Synthesis of Monolysyl Advanced Glycation Endproducts and Their Incorporation into Collagen Model Peptides

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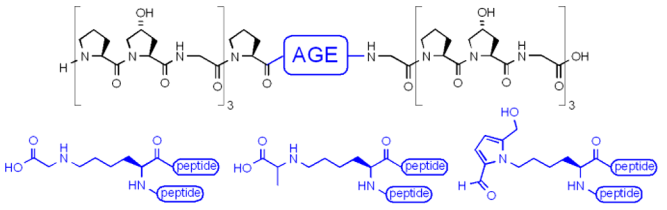
ABSTRACT



The synthesis of advanced glycation endproducts (AGEs), CML, CEL, and pyrraline and their incorporation into collagen model peptides is reported. AGEs are modified amino acids that form on proteins such as collagen and are thought to play a significant role in the pathogenesis of many diseases, particularly diabetes. The synthesis and incorporation of these compounds into synthetic peptides is a key step in developing model systems with which to investigate AGE-modified proteins.

Advanced glycation endproducts (AGEs) are a group of compounds that are formed by nonenzymatic reactions between reducing sugars and amino acid side-chain residues in proteins. These post-translational modifications mainly involve reactions at lysine and arginine residues and give rise to a large number of structurally diverse compounds ranging from simple alkylations to more complex heterocycles and cross-linked structures.[1] 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[2] and age-related neurodegenerative diseases, such as Alzheimer's disease.[3] AGE formation and accumulation is most problematic on long-lived proteins such as collagen and lens-crystallins,[4] and these are the most relevant proteins to study when investigating AGEs. However, the precise molecular mechanisms by which AGEs influence the progression of these diseases are not clear, and there remains a need to develop new chemical tools to investigate them. A key step to achieve this is the site-specific incorporation of AGEs into synthetic peptide sequences using solid phase peptide synthesis (SPPS). This will enable access to synthetic AGE-modified peptides that will facilitate the development of suitable model systems to undertake future detailed molecular studies. The initial step required to address these fundamental questions is the development of

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practical and efficient synthetic strategies for the preparation of suitably protected AGE building blocks for incorporation into SPPS.

Here we report the synthesis of the three monolysyl AGEs, N^{ϵ}-(carboxymethyl)lysine (**1**) (CML), N^{ϵ}-(carboxyethyl)-lysine (**2**) (CEL), and pyrraline (**3**) (Figure 1) as both Fmoc-protected building blocks suitable for SPPS and as free amino acids. Furthermore, the AGE building blocks were readily incorporated into 21 amino acid residue collagen model peptide (CMP) sequences thus establishing their utility in SPPS. The CMPs were subsequently shown to form triple helices in solution demonstrating their potential as model systems for investigating AGE-modified collagens.

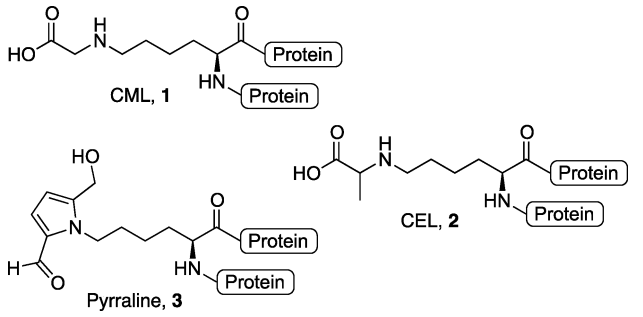
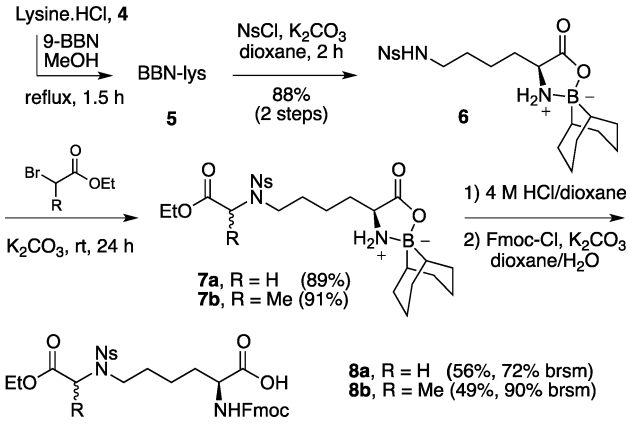


Figure 1. Three monolysyl AGEs, CML, CEL, and pyrraline.

Although the synthesis of the free amino acid forms of CML,[5] CEL,[5f,6] and pyrraline[7] are well documented, the majority of these approaches suffer from lengthy and difficult procedures and/or poor yields. Furthermore, the synthesis of CML and CEL as building blocks suitable for incorporation into SPPS has only been reported once,[8] while the synthesis of a suitably protected pyrraline building block has not been reported. Importantly, the existing syntheses of the CML and CEL Fmoc building blocks required starting materials that are neither commercially available nor easy to prepare, and yields for their preparation were not reported. Therefore, our principal aim was to develop practical syntheses of suitably protected Fmoc building blocks of CML, CEL and pyrraline and demonstrate their use in automated microwave SPPS. The routes were also designed to provide ready access to the free amino acid forms of these important AGEs.

Our strategy to prepare CML and CEL hinged on the use of the nosyl (Ns) group alkylation methodology to alkylate the ε-NH_2 group of a lysine derivative.[9] Additionally, it was expected that the Ns group would also serve as an orthogonal protecting group in SPPS. To facilitate the selective nosylation of the ε-NH_2, the 9-BBN group was used to concomitantly protect the α-NH_2 and carboxylic acid group in lysine.[10] We envisaged that when used with other acid labile protecting groups the BBN group would enable a final global deprotection step to access the free amino acids while providing a tractable organic compound prior to this. The synthesis started with lysine hydrochloride **4**, which was treated with 9-BBN dimer affording BBN-lysine **5**. The Ns group was then introduced affording BBN-Ns-Lys **6** in 88% yield over two steps. Alkylation of **6** with ethyl bromoacetate or ethyl bromopropionate afforded compounds **7a** (89%) and **7b** (91%), respectively. Selective deprotection of the BBN-moiety was achieved by treatment of **7a** and **7b** with 4 M HCl/dioxane. Nonaqueous conditions were essential to avoid concomitant hydrolysis of the ethyl ester. After removal of the HCl/dioxane under reduced pressure, the crude products were treated with Fmoc-Cl and K_2CO_3 in dioxane, affording the desired Fmoc building blocks **8a** and **8b** ready for incorporation into SPPS (Scheme 1).

Scheme 1. Synthesis of Fmoc-Protected CML and CEL Building Blocks **8a** and **8b**



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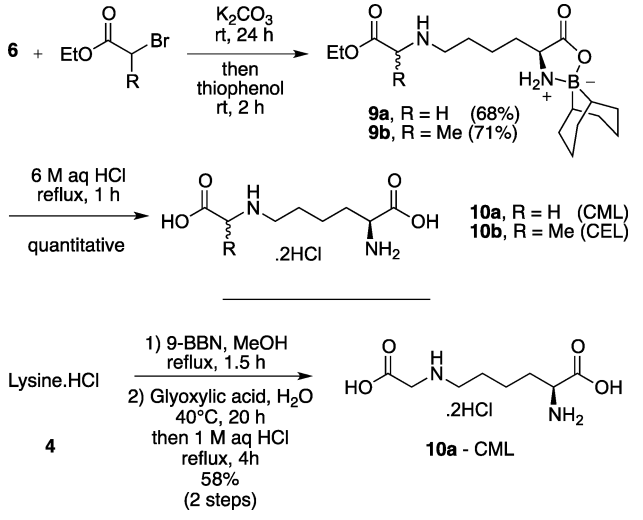
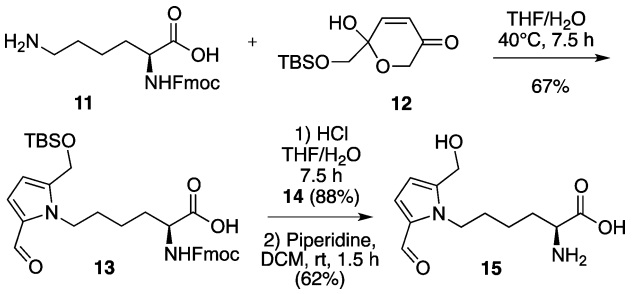
This strategy also enabled ready access to the free amino acid forms of CML and CEL. Thus, starting from 9-BBN-Ns-lysine **6**, a one-pot alkylation/Ns deprotection with either ethyl bromoacetate or ethyl bromopropionate furnished BBN-OEt-CML **9a** (68%) and BBN-OEt-CEL **9b** (71%), respectively. Use of the 9-BBN and ethyl ester protecting groups enabled a final global deprotection to be carried out by treatment of **9a** and **9b** with 6 M aq HCl. Purification of the final compounds using a short reverse-phase C-18 column eluting with 0.5% MeCN/H_2O removed the organic byproducts (BBN). Finally, CML **10a** and CEL **10b**, were isolated by lyophilization as their *bis*-hydrochloride salts in quantitative yields (Scheme 2). BBN-Lys precursor **5** also provided the starting material for a more direct synthesis of CML using an approach similar to that described by Kihlberg et al.[5m] Whereas Kihlberg used either formic or trifluoroacetic acid as solvent we were able to use H_2O, simplifying both the procedure and purification. Hence, 9-BBN-Lys **5** and glyoxylic acid were heated at 40 °C in H_2O for 24 h. The resulting formylated intermediate (not shown) was then heated at reflux in 1 M aq HCl. Purification using a C-18 column afforded CML bis-hydrochloride salt **10a** in 58% yield over two steps (Scheme 2).

Scheme 2. Synthesis of the Free Amino Acids of CML and CEL

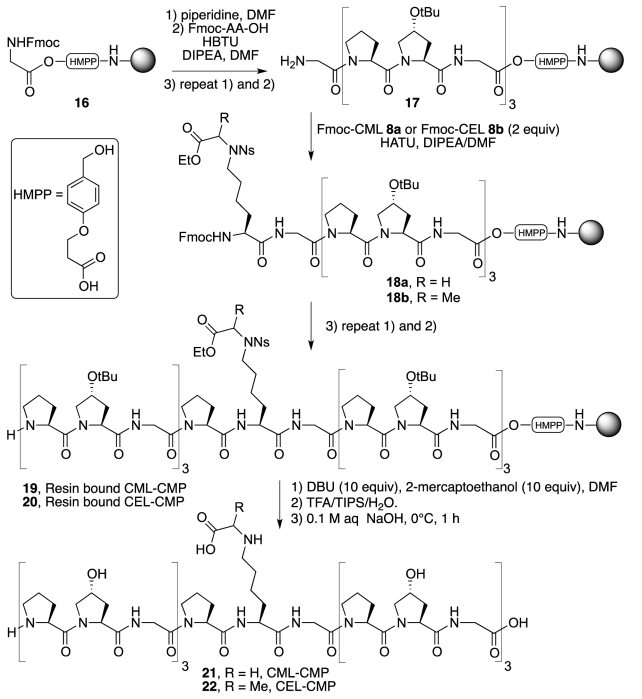


Our strategy for pyrraline relied on the condensation of a protected lysine derivative with TBS protected dihydropyranone **12** (Scheme 3). A similar condensation using dihydropyranone **12** has previously been employed within our group for the synthesis of acortatarin A.[11] Hence, a suspension of Fmoc-lysine **11** and dihydropyranone **12** in THF/H_2O (1:1) was heated at 40 °C for 7.5 h, furnishing the desired Fmoc protected pyrraline derivative **13** in 67% yield. Deprotection of the TBS ether in **13** by treatment with dilute HCl afforded **14** in 88% yield and final deprotection of the

Scheme 3. Synthesis of Pyrraline Derivatives



Scheme 4. Synthesis of CMPs Containing CML and CEL



Fmoc group with 5% piperidine/DCM afforded the free amino acid form of pyrraline **15** in 62% yield (Scheme 3).

CMPs are synthetic peptides based on the repeat unit Pro-Hyp-Gly that are able to form triple helices in solution, thereby mimicking the tertiary structure of collagen. They have been used extensively as model systems to study the structure and stability of collagen.[12] The synthesis of homotrimeric CMPs containing CML, CEL and pyrraline will establish the general utility of our AGE building blocks in SPPS while also demonstrating that AGE-modified CMPs are viable and accessible chemical tools for studying

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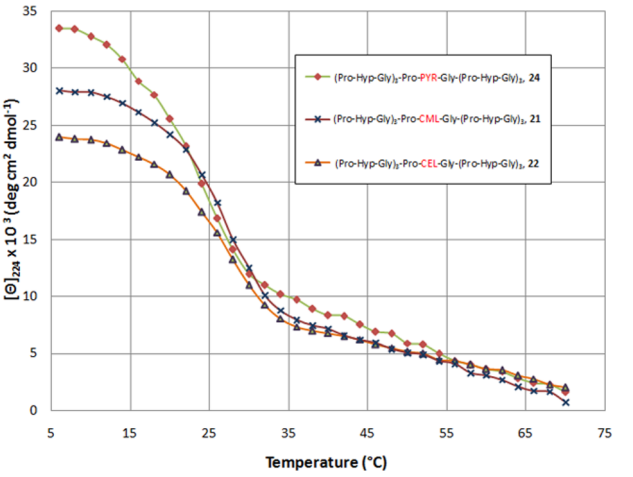

Figure 2. CD thermal transition curves for CMPs **21**, **22**, and **24** measured in 50 mM aqueous AcOH (0.5 mM peptide).

AGE-modified collagens. Surprisingly, given their biological relevance, there are only two examples in the literature of synthetic AGE building blocks being incorporated site specifically into synthetic peptides.[8,13]

Our aim was to synthesize CMPs comprising seven repeat units of (Gly-Hyp-Pro) where the central Hyp residue (position 11) was replaced with our synthetic AGE building blocks. Thus, aminomethyl polystyrene resin was coupled to a preloaded Fmoc-Gly-hydroxymethylphenoxypropionic acid (HMPP) linker affording resin **16**. Standard couplings were then carried out by SPPS furnishing resin **17**. The CML and CEL building blocks were coupled manually using 2 equiv of building block and HATU affording resin-bound peptides **18a** and **18b**. Standard couplings then gave the desired resin-bound peptides **19** and **20** (Scheme 4). Pleasingly, the Ns group was readily deprotected on resin by treatment with 2-mercaptoethanol and DBU without complication. Cleavage from the resin using a TFA/TIPS/H_2O cocktail and subsequent hydrolysis of the ethyl ester by treatment with aqueous NaOH afforded CML−CMP **21** (19% yield and >95% purity) and CEL−CMP **22** (30% yield and >95% purity).

Using the same protocol, pyrraline building block **13** was also incorporated into a CMP affording the desired resin bound peptide **23**. In this case, when cleaving the peptide from the resin using TFA/TIPS/H_2O the mass of the major product was found to be 16 mass units less than the mass of the desired peptide. This was attributed to a TIPS facilitated reduction of the benzylic like alcohol following removal of the TBS group. This problem was easily circumvented by removing TIPS from the resin cleavage mixture, which afforded the desired pyrraline−CMP **24** in 27% yield and >95% purity (see the Supporting Information).

A key feature of CMPs is their propensity to form triple helices in solution, a phenomenon observed by CD spectroscopy by the appearance of a small maximum around 225 nm and a large minimum around 200 nm. The formation and relative stabilities of the triple helices can be verified by thermal transition curves that show the denaturation of the triple helices.[14] The CD spectra for the three AGE-modified CMPs all exhibited a small positive and large negative peak at the expected wavelengths (see the Supporting Information). In addition, each AGE−CMP showed a clear thermal transition (T_m, **21** = 26 °C, **22** = 25 °C, **24** = 23 °C) upon heating the solutions from 5 to 70 °C (Figure 2), thus demonstrating their potential as useful model systems to study AGE-modified collagens.

In summary, we have developed efficient syntheses of the Fmoc-protected and free amino acid forms of the three monolysyl AGEs: CML, CEL, and pyrraline. Moreover, we have successfully incorporated each Fmoc-protected AGE building block site-specifically into a CMP highlighting the utility of these building blocks in SPPS. Importantly, the AGE-modified CMPs were shown to form triple helices in solution, thus establishing their potential as model systems for studying AGE-modified collagens. The synthesis of the free amino acids, Fmoc building blocks, and AGE-modified peptides will facilitate future investigations into the roles these AGEs play in the pathogenesis of diabetes and other relevant diseases thereby enabling the development of improved therapeutic strategies. The synthesis of the CMPs reported herein provides the impetus for the synthesis of further AGE-modified CMPs and related peptides, thereby facilitating the development of suitable model systems to probe the chemical, physical and mechanical properties of AGE modified collagens and related proteins.

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Supporting Information Available. Experimental procedures and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.