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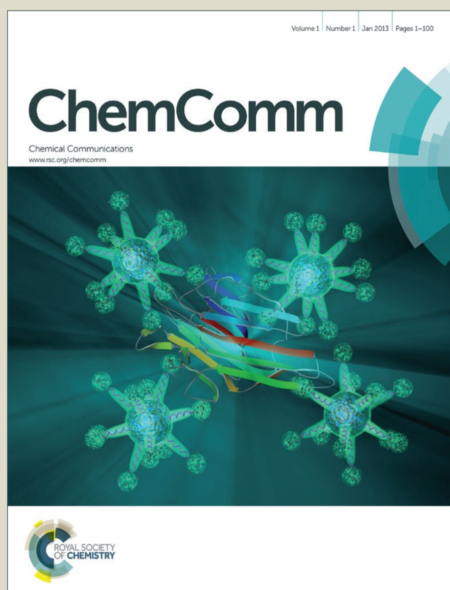

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ARTICLE TYPE

On resin synthesis and cross-linking of collagen peptides containing the advanced glycation end-product pyrraline via Maillard condensation

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Glycation and its products cause a host of pathological conditions but their exact roles are yet to be determined. Pyrraline, a key product of glycation, and a novel pyrraline-derived cross-link have been incorporated into collagenous peptides via Maillard condensations performed on resin-bound peptide sequences.

Advanced glycation end-products (AGEs) have attracted considerable scientific interest in a wide range of disciplines.[1] AGEs are known to accumulate in a number of tissues *in vivo* and AGE levels have been correlated with the severity of age- and diabetes-related conditions such as retinopathy and nephropathy.[2] Despite the apparent role of AGEs as pathogens or biomarkers of other important pathological processes, the exact chemical properties of AGEs and of glycated proteins have not been fully characterised. In depth understanding of the properties of AGEs will enable development of novel detection and prevention strategies. Therefore new methods have to be developed in order to study the exact effect of AGEs on the biochemistry of the host proteins.

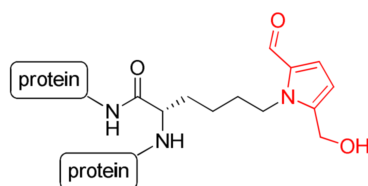

Fig. 1 Protein-bound pyrraline

As part of an ongoing research program, we have developed techniques for site-specific incorporation of particular AGEs (carboxymethyl lysine, carboxyethyl lysine, glyoxal and methylglyoxal lysine dimers) into peptide sequences.[3,4] The synthetic incorporation of AGEs is a promising avenue for investigating the precise impact of individual AGEs on the host systems. In particular, our model studies have demonstrated the dramatic impact AGEs have on the proteolytic digestibility of collagenous peptides and their ability to prevent triple helix formation.[3,4] Importantly, incorporation of AGEs into peptides has also resulted in enhanced copper binding properties of the host peptides with direct implications for the pathogenic properties of AGEs in diabetes and diabetic complications.[5]

Pyrraline (Fig. 1) is an important member of the AGE family. It derives from the glycation of lysine by carbohydrates and carbohydrate degradation products. Pyrraline has been shown to form in small amounts on protein side-chains in living tissues,[6] and in larger quantities during food preparation,[7] where pyrraline can be allergenic.[8] Urinary levels of pyrraline spike with the onset of type 2 diabetes,[9] therefore pyrraline has been used as a biomarker of diabetic organ damage.[10] However, the precise levels of pyrraline and the effects of its formation on the host systems have not yet been determined.

Previously our group has incorporated pyrraline into a peptide sequence via synthesis of the pyrraline modified amino acid building block followed by its introduction into peptide sequences via solid phase peptide synthesis (SPPS).[11] However, this approach is inefficient due to the lengthy preparation of the suitably protected pyrraline building block and the large quantities required for its incorporation into the peptide sequence.

Herein we report an expedient and novel solid phase method for incorporating pyrraline into peptide sequences. In this approach pyrraline is generated directly by reacting an unprotected lysine side chain on the resin-bound peptide in a Maillard-type condensation. This solid phase approach significantly facilitates access to peptides containing pyrraline. Equipped with this technique, we have incorporated pyrraline into two different collagen peptides and investigated the impact this AGE has on a number of physiologically relevant properties of the host peptide.

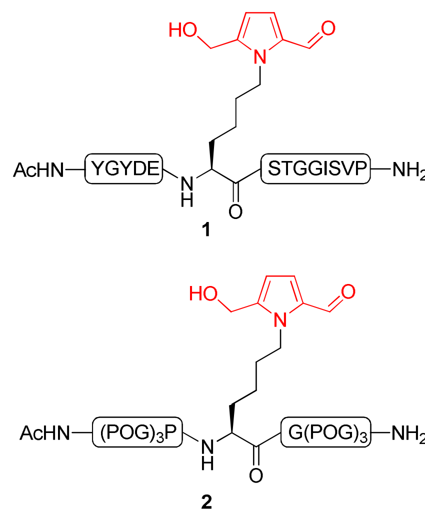

Fig. 2 Structures of peptides **1** and **2**, where O denotes *4R*-hydroxyproline

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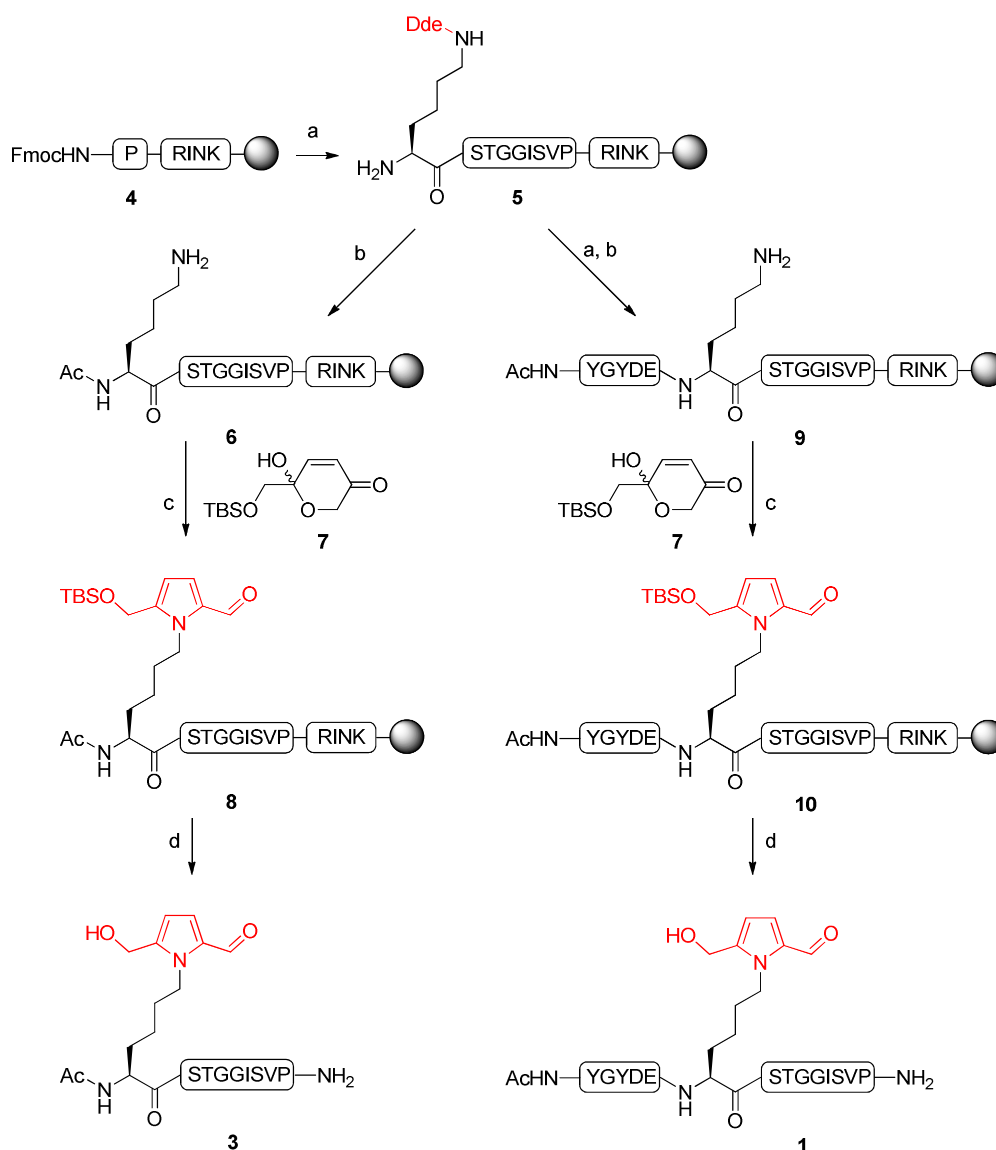
Importantly, a novel pyrraline-derived peptide cross-link was characterised while optimising the solid phase synthesis of pyrraline-containing peptides. It is proposed that this new cross-link is formed via amination of the aldehyde on pyrraline by a lysyl amine of a neighbouring peptide sequence.

Collagen is the most abundant protein in mammals.[12] The terminal telopeptide regions of collagen are common sites for proteolysis *in vivo*, and therefore these regions are important in tissue recycling and regeneration.[13] The telopeptide regions have also been shown to be common sites for glycation.[14] In order to suitably mimic the telopeptides, a sequence from the *N*-terminal telopeptide region of human type I α1 collagen was chosen for incorporation of pyrraline (peptide **1**, Fig. 2). Another region of collagen that is important for protein stability is the triple helical region. Collagen model peptides (CMPs) have been developed by others to mimic this structural motif[15] hence we aimed to incorporate pyrraline into a CMP (peptide **2**, Fig. 2).

Firstly, to establish solid phase methodology for incorporation of pyrraline into peptide sequences, we investigated the synthesis of the pyrraline-containing peptide **3** (Scheme 1), which is a version of the telopeptide **1** truncated at the pyrraline residue. Resin-bound Fmoc-proline **4** was employed in the synthesis of the suitably protected peptide **5** using HATU/DIPEA for coupling of the amino acids and 20% piperidine in DMF for Fmoc removal. The terminal lysine in peptide **5** was incorporated with a Dde-protected side-chain to allow for optimal removal by treatment with hydrazine. However, since the Fmoc group is not stable to treatment by hydrazine, the terminal lysyl Fmoc group was removed and the N-terminus was acetylated with Ac_2O. The lysyl side-chain Dde group was subsequently deprotected with 2% hydrazine in DMF to afford peptide **6**. With resin-bound peptide **6** in hand, attention next focused on conversion of the side-chain amine of the terminal lysine into the pyrraline heterocycle. Optimisation of the Maillard-type condensation between peptide **6** and dihydropyranone **7** was carried out at a 5 μmol scale.



Scheme 1 Synthesis of pyrraline-containing peptides **1** and **3**. *Reagents and conditions:* (a) Fmoc-SPPS *i.* 20% piperidine in DMF, *ii.* Fmoc-AA-OH, HATU, DIPEA in DMF; iii. steps *i.* and *ii.* repeated until the desired sequence is built (b) i. Ac_2O, DIPEA in DMF, ii. 2% hydrazine in DMF, 2 x 3 min; (c) **7** (3 equiv.), 20% piperidine in DMF, overnight; (d) TFA/H_2O, rt, 2 h.

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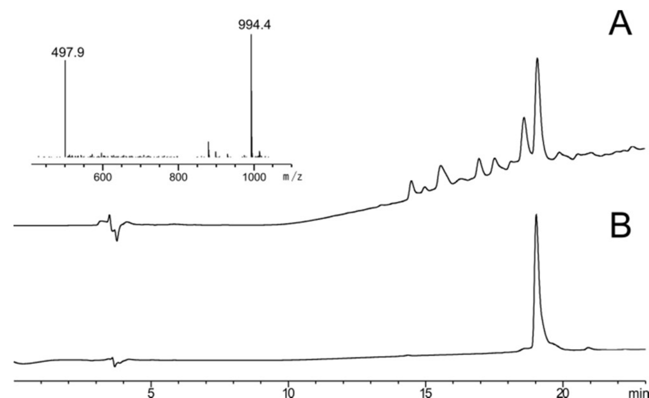

Fig. 3 LCMS chromatograms of crude (A) and purified (B) peptide **3**.

The Maillard-type condensation to afford the desired peptide **8** was optimised by varying: a) the solid phase matrix and b) the organic base used to carry out the reaction (Table SI-1). The optimal reaction conditions required the use of 20% piperidine solution in DMF in conjunction with high loading polystyrene resin (0.92 mmol/g), which resulted in 36% yield of peptide **3**. The crude and purified LC-MS profiles of peptide **3** obtained using these conditions are depicted in Fig. 3. Such site-specific incorporation of pyrraline into a peptide sequence is convenient primarily because it is accomplished on the resin bound peptide sequence and does not require lengthy workup and purification procedures generally associated with pyrraline synthesis.[16]

Having established the conditions for on resin incorporation of pyrraline into peptide **3** we were now in a position to embark on the synthesis of the full length telopeptide **1** (Scheme 1). Resin-bound peptide **5** underwent further Fmoc-SPPS, *N*-terminal acetylation and lysyl side chain deprotection to afford the intermediate peptide **9**, which was subjected to side-chain Maillard-type condensation with **7** by treatment with 20% piperidine in DMF for 16 h. Subsequent treatment of the resulting resin-bound and pyrraline-modified peptide **10** by the pyrraline-specific cleavage cocktail (5% H_2O in TFA) and HPLC purification afforded the desired pyrraline peptide **1** in 33% yield (>95% purity, $[M+H]^+$ calc. 1622.7, obs. 1622.7).

Both the native telopeptide Ac-YGYDEKSTGGISVP-NH_2 **11** and the AGE-modified telopeptide **1** were subjected to a bovine trypsin digest. Under the experimental conditions, trypsin digested 90% of the native peptide in 10 minutes, whereas the pyrraline-modified peptide **1** remained unchanged (Fig. SI-1). This result demonstrates the effect the presence of the pyrraline system has on host protein proteolysis, specifically by trypsin as representative of the proteases present in the digestive tract.

In an analogous fashion to telopeptide **1**, the CMP **2** (Fig. 2) was prepared via solid phase peptide synthesis. On-resin condensation with dihydropyranone **7** followed by resin cleavage afforded the peptide **2**, which was recovered by HPLC following cleavage (21% yield, >95% purity, $[M+2H]^{2+}$ calc. 1027.5, obs. 1027.4).

The distinguishing feature of CMPs is their capacity to form triple helices in solution. This property renders CMPs as suitable model peptides to investigate the structural and functional properties of native collagenous triple helices. Collagenous triple helices have a signature circular dichroism (CD) profile that consists of a maximum at 225 nm and a minimum near 200 nm. A thermal unfolding experiment monitors the spectral maximum at 225 nm as the temperature is increased which, in the presence of a triple helix, shows a cooperative transition. The CD profiles of **2** and unmodified peptide Ac-$(POG)_3$PKG$(POG)_3$–NH_2 **12**, where O denotes *4R*-hydroxyproline, were analysed in the range of 190-260 nm at 20 °C. The CD spectrum of **12** displayed a maximum at 222 nm and a minimum at 198 nm suggesting that the peptide formed a triple helix (Fig. SI-2A). The CD profile of **2** also displayed a maximum at 222 nm and a minimum at 198 nm, suggesting that the pyrraline-containing peptide also formed stable triple helices. When thermal melting experiments were performed from 5 to 50 °C on samples at 0.5 mM, both **2** and **12** displayed cooperative transitions (Fig. SI-2B). Major transitions occurred in the first derivative curves at 23 °C for **2** and 29 °C for **11**. This indicates that pyrraline formation does not substantially hinder formation of the collagenous triple helix.

Importantly, during optimisation studies on resin-bound peptide **3** we observed a notable by-product **13** (Fig. 4). This product formed in the highest yield when the condensation was carried out using a lower loading (0.37 mmol/g) Tentagel resin and 20% piperidine in DMF. This Maillard cyclisation was repeated at a higher scale (0.02 mmol) and a sample of **13** was subsequently isolated by HPLC (Fig. 4C).

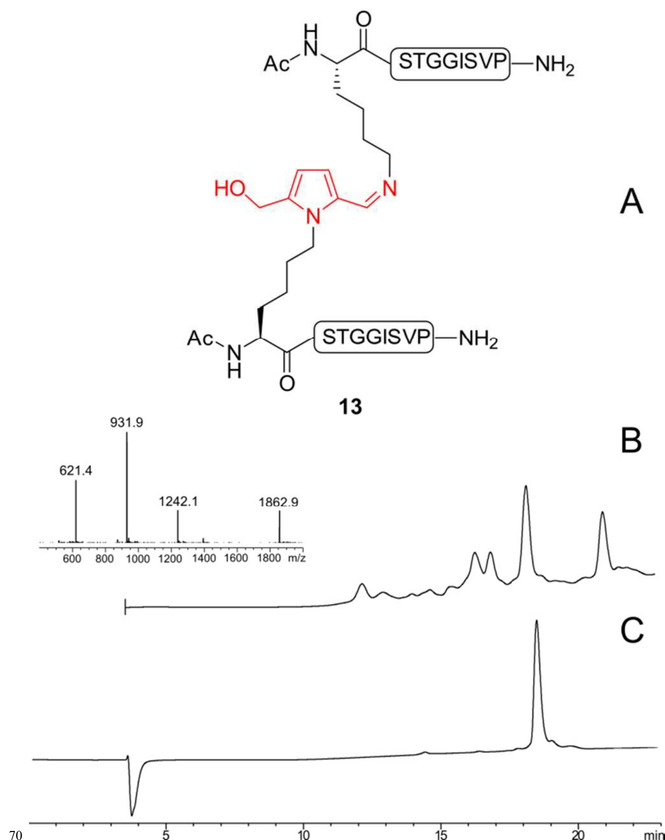

Fig. 4 Structure (A) and LCMS chromatograms of crude (B), and of purified (C) peptide **13**.

Analysis of the mass spectrum of purified sample revealed four charged states, three of which were in accordance with the proposed dimeric peptide structure depicted in Fig. 4, where a cross-link at the *N*-terminus occurred via Schiff base formation

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between the pyrraline aldehyde and a side-chain lysyl amine of an adjacent peptide sequence. The calculated *m/z* for $[M+H]^+$ species of **13** is 1863.1, for $[M+2H]^{2+}$ is 932.0, and for $[M+3H]^{3+}$ is 621.7, which correspond well to the recorded ESI-MS spectrum of **13** (Fig. 4B). Furthermore, the mass spectrum shows evidence of cluster formation, at *m/z* 1242.1 which closely correlates with the $[2M+3H]^{3+}$ species for peptide **13** with calculated *m/z* of 1242.4.

Evidence for similar dimerization has been reported in our previous work focused on the solution phase synthesis of 2-formylpyrrole-containing natural products.[17] Similar conjugates have also been reported by Nissl *et al.*, who showed that certain 2-formylpyrrole-containing Maillard reaction products can bind to free amines in proteins.[18] Reactive aldehydes are known to form Schiff bases *in vivo* and this reaction is commonly believed to be the first step in the process of glycation between sugars and reactive protein side-chains.[1] Since pyrraline is known to form in living tissues,[6] cross-linking in a manner similar to that in **13** may take place *in vivo*, in which case dimer **13** could represent a new cross-linking member of the AGE family. Covalent dimerisation of peptides and proteins is a common feature of AGEs[1] and it appears to be particularly relevant to the pathogenesis of Alzheimer's disease.[19]

The stability of the dimeric peptide **13** was probed in aqueous solutions at different pH values. The peptide showed remarkable stability in aqueous solutions at pH < 7. However an increase to pH 8 quickly hydrolysed the putative Schiff base **13** affording the respective pyrraline-peptide **6** and peptide Ac-KSTGGISVP-NH_2 (Fig. SI-8). The reversible nature of the cross-link in **13** can represent a "post-it"-type glue for peptide and protein duplexes analogous to recently reported reversible cross-linking of DNA.[20] Such reversible conjugation of peptides is valuable for the design and production of delivery systems that can release cargo in response to an outside stimulus.[20]

Conclusions

In summary, a convenient and economical method for site-specific introduction of pyrraline into peptides attached to a solid support has been developed. The synthetic strategy uses standard Fmoc SPPS conditions, does not require preparation of the respective building block, and gives access to pyrraline-containing peptides using a single solid phase Maillard-type condensation. The method developed herein enabled ready access to collagen telopeptides containing pyrraline thus facilitating a study of their relative stability to proteolytic digestion. These studies conclusively demonstrated the significant impact that incorporation of pyrraline has on the overall stability of the peptides. Additionally, synthetic CMPs that contained pyrraline were prepared and were shown to maintain the triple helical superstructure. Importantly, a novel reversible type of AGE-peptide cross-link has been characterised and further work on the practical application of this reversible cross-linking for the preparation of conjugated functional systems is currently underway.

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Notes and references

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