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A new procedure for the synthesis of peptide-derived Amadori products on a solid support☆

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Abstract—A new and straightforward solid-phase synthesis of a series of site-specific Amadori-modified peptides is described. The method involves reductive alkylation of the ε-amino groups of lysine with 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose in the presence of sodium cyanoborohydride on a solid support.

The non-enzymatic reaction of glucose with amino groups of proteins yields Amadori products. These compounds are key intermediates in the formation of advanced glycation end products (AGEs) involved in aging, diabetes, and other pathological processes.[1] Peptide-derived Amadori products are the subject of interest in clinical chemistry as markers of diabetes mellitus. Recent reports suggest a significant influence of the glycation process on the function of relatively short-lived hormones and regulatory peptides.[2] The dynamic progress in the studies of the biochemical consequences of the Mailard reaction requires the development of a fast, convenient, and general method of glycated peptide synthesis. To the best of our knowledge, the procedures reported in the literature concerning Amadori-modified peptides are based on the 'in solution' approach only, which makes them tedious and time consuming.

Our previous investigations on the solid-phase synthesis of peptide-derived Amadori products were based on the direct reaction of deprotected ε-amino groups of lysine with a saturated solution of glucose in DMF at 60–70 °C.[3] A similar approach was reported recently by Frolov et al.[4] These authors developed a site-specific method of synthesizing Amadori-modified peptides on a solid support. However, this method has several drawbacks as it requires a relatively high temperature (70 or 110 °C) and long reaction time, while the yield is relatively low (30–35%). Moreover, according to our experience, prolonged incubation of peptides in DMF at high temperature leads to the loss of the Fmoc protection of the N-terminal amino group and the Boc protection of the ε-amino groups of the lysine side chain.

Here we report the solid-phase synthesis of peptide-derived Amadori products by direct alkylation of the ε-amino groups of the lysine residue with 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose[5] **1** (Fig. 1) in the presence of sodium cyanoborohydride. This reagent was utilized previously in the solution-phase synthesis of glycated peptides.[6] The isopropylidene group in derivative **1** provides permanent protection of the hydroxyl groups during peptide synthesis and can be removed relatively easily during cleavage of the peptide from the resin. This makes **1** a very convenient reagent for the solid-phase synthesis of glycated peptides. Preliminary experiments were conducted on the model peptide Lys-Ala-Ala-Phe. After assembling the peptide on Wang resin by the standard Fmoc procedure, the Mtt group protecting the ε-amino group in the side chain of lysine was removed using 1% TFA in

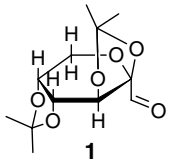

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Figure 1. Structural formula of 2,3:4,5-di-*O*-isopropylidene-β-D-arabino-hexos-2-ulo-2,6-pyranose.

Keywords: Solid-phase peptide synthesis; Amadori rearrangement; Glycation.

☆ Preliminary results were presented during the 18th Polish Peptide Symposium, Wrocław, Poland, 2005 (http://www.18pps.uni.wroc.pl/Abstracts/Kapczynska.doc).

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DCM. The reductive alkylation was performed at room temperature. Various concentrations of **1** were tested to optimize the reaction conditions. The progress of the reaction was monitored by ESI-MS. Although ESI-MS analysis is not quantitative, it provides a reasonable approximation of the glycation level.[7] We found that the best conversion rate was achieved using a twofold excess of **1** and a fivefold excess of sodium cyanoborohydride.[8] Under these conditions, more than 80% of the ε-amino groups of Lys were converted to the glycated form. The unconverted peptide could be removed during HPLC purification.

To test whether this procedure was suitable for site-specific synthesis of peptide-derived Amadori products, we synthesized a fragment of bovine serum albumin (BSA): Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys-Glu and its two possible derivatives glycated on specific ε-amino groups in the side chains of Lys7 and Lys9 (isomeric peptides: Gln-Asp-Thr-Ile-Ser-Ser-Lys(Glc)-Leu-Lys-Glu and Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys(Glc)-Glu, respectively) on solid support. An example of the synthesis is presented in Figure 2. The peptides were assembled on solid support using the standard Fmoc synthetic procedure. The first amino acid attached to the Wang resin was Glu(O*t*Bu). The Fmoc-protecting group was removed in the presence of 25% piperidine in DMF and consecutive amino acid residues were coupled using TBTU in DMF. The side chain of the Lys residue for glycation was protected with the Mtt group and the other Lys side chain with a Boc group. The side-chain protecting groups for other Fmoc-amino acids were *tert*-butyl for Thr, Asp and Ser, and Trt for Gln. After deprotection of the ε-amino group in the side chain of lysine, the resin was incubated with **1** and sodium cyanoborohydride for 2 h at room temperature. The obtained conjugate was treated with $(Boc)_2O$ to protect the secondary amino group formed during Lys modification, and the synthesis of the peptide was continued by the standard Fmoc method. Finally, the reaction product was cleaved from the resin using TFA containing 5% water and 5% of TIS (triisopropyl silane) for 24 h at room temperature. The obtained peptides were precipitated with cold diethyl ether. The yields and HPLC purities of the crude peptides are given in Table 1. The glycated peptides were purified by preparative HPLC. The homogenity of the peptides was tested by HPLC and ESI-MS (Table 2). The structures of the glycated peptides were confirmed by tandem mass spectrometry using a Q-TOF hybrid mass spectrometer. The observed neutral losses of 18, 36, and 54 mass units could be explained by elimination of one, two, and three water molecules, respectively, while the neutral loss of 84 mass units corresponds to the elimination of three water molecules and one molecule of formaldehyde. The resulting pyrylium and furylium (or immonium tautomer) ions are characteristic for the pyranose moiety.[9] This fragmentation pattern is characteristic for Amadori-modified peptides.[4,10] Furthermore, MS/MS confirmed unambiguously, which Lys residue underwent the glycation. The peptide Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys(Glc)-Glu showed an abundant peak (b8, see Supplementary data) at *m*/*z* 873.5 resulting from elimination of the Lys(Glc)-Glu fragment. On the other hand, the peptide Gln-Asp-Thr-Ile-Ser-Ser-Lys(Glc)-Leu-Lys-Glu produced a peak at *m*/*z* 981.5 resulting

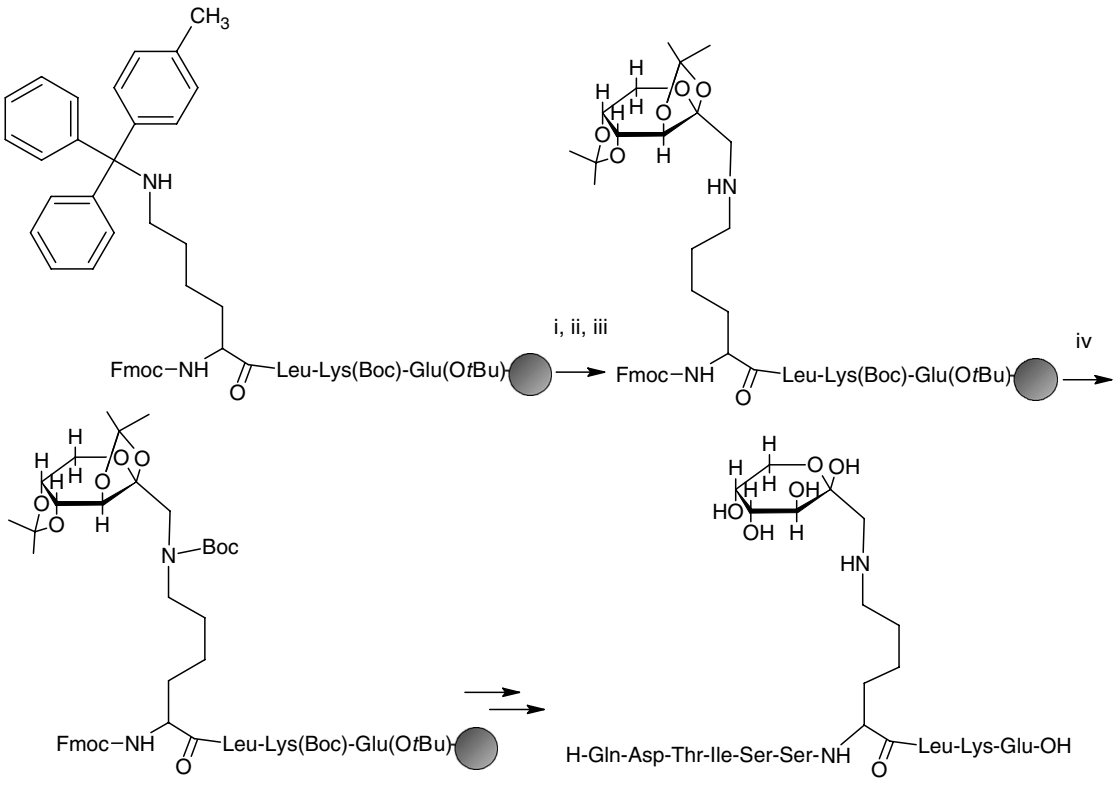

Figure 2. Reagents and conditions: (i) 1% TFA in DCM, 10 min, rt, 7×; (ii) 2 M DIEA in DMF, 10 min, rt; (iii) **1** (2 equiv), $NaBH_3CN$ (5 equiv) in 1% AcOH in DMF, 2 h, rt; (iv) Boc_2O (20 equiv), DIEA (4 equiv) in DMF, 12 h, rt.

Table 1. Characterization of crude peptide-derived Amadori products

PEPTIDE	t_R[a] (min)	Crude yield (%)	HPLC[b] purity (%)
H-Lys(Glc)-Ala-Ala-Phe-OH	14.21	77	80
H-Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys(Glc)-Glu-OH	14.60	93	56
H-Gln-Asp-Thr-Ile-Ser-Ser-Lys(Glc)-Leu-Lys-Glu-OH	14.69	74	58
H-Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys-Glu-OH	15.54	65	62

[a] C18 column Vydac (250 × 4.6 mm): gradient 0–80% B in A in 60 min, A: water + 0.1% TFA, B: acetonitrile + 0.1% TFA.
[b] Purity based on the integral of the crude product absorption at λ 220 nm on RP-HPLC.

Table 2. Analytical data for purified glycated peptides

Glycated peptide	t_R[a] (min)	HPLC[b] purity (%)	Formula/predominant ion	*m/z* Calcd/found[c]	MS/MS analysis[d]
H-Lys(Glc)-Ala-Ala-Phe-OH	14.17	99	$C_{27}H_{43}N_5O_{10}$ $[MH]^+$	598.3088/598.3091	580.3 (–H_2O); 562.3 (–2H_2O); 544.3 (–3H_2O); 514.2 (–3H_2O and –CH_2O); 436.2 (y4-glucose) 308.1 (y3)[e]
H-Gln-Asp-Thr-Ile-Ser-Ser-Lys-Leu-Lys(Glc)-Glu-OH	14.60	97	$C_{48}H_{85}N_{13}O_{19}$ $[MH_2]^{2+}$	655.8385/655.8351	1292.7 (–H_2O); 1274.7 (–2H_2O); 1256.7 (–3H_2O); 1226.7 (–3H_2O and –CH_2O); 873.5 (b8)
H-Gln-Asp-Thr-Ile-Ser-Ser-Lys(Glc)-Leu-Lys-Glu-OH	14.69	97	$C_{48}H_{85}N_{13}O_{19}$ $[MH_2]^{2+}$	655.8385/655.8361	1292.7 (–H_2O); 1274.7 (–2H_2O); 1256.7 (–3H_2O); 1226.7 (–3H_2O and –CH_2O); 981.5 (b8-3H_2O)

[a] C18 column Vydac (250 × 4.6 mm): gradient 0–80% B in A in 60 min, A: water + 0.1% TFA, B: acetonitrile + 0.1% TFA.
[b] Purity based on the integral of the crude product absorption at λ 220 nm on RP-HPLC.
[c] ESI-MS spectrum, MicrOTOFQ (Bruker Daltonics).
[d] Selected fragmentation ions. Full MS/MS spectra of glycated peptides are given as Supplementary data.
[e] Nomenclature for peptide fragment ions according to Roepstrof and Fohlmann.[11]

from elimination of a Lys-Glu fragment and three molecules of water. The interpreted MS/MS spectra of glycated peptides are given as Supplementary data. Moreover, the molecular formulas of glycoconjugates obtained were confirmed by high resolution mass spectrometry.

Further experiments showed that the reaction at the N-terminal α-amino group did not occur even when a significantly enhanced excess of the reagents (20 × **1** and 45 × $NaBH_3CN$) and longer reaction times (20 h) were applied.

In summary, we have developed an efficient and simple procedure for the solid-phase synthesis of site-selectively glycated peptides. Our method is compatible with the Fmoc strategy of solid-phase peptide synthesis. It does not require high temperature and gives good yields and relatively pure products (Table 1). To our knowledge, this is the first method allowing the routine synthesis of peptide-derived Amadori products.

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Supplementary data

MS/MS spectra of glycated peptides are available as Supplementary data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.12.022.

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8. Procedure for solid phase glycation: after deprotection (1% TFA in DCM; 10 min, rt, 7×) of the ε-amino group of lysine, the resin (470 mg) was suspended in 17 ml of a 1% solution of acetic acid in DMF. Then a twofold molar excess of **1** in 1 ml of DMF and a fivefold molar excess of sodium cyanoborohydride (with respect to loading of the resin) in 1 ml of DMF were added and the resin was agitated for 2 h at rt. The final reaction product was cleaved from the resin using TFA containing 5% water and 5% of TIS for 24 h at rt.
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