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Building blocks for the synthesis of post-translationally modified glycated peptides and proteins

Stefano Carganico,[a,b] Paolo Rovero,[a,c] Jose A. Halperin,[d,e] Anna Maria Papini[a,b] and Michael Chorev[d,e]*

Growing interest in synthetic peptides carrying post-traslational modifications, in general, and the Amadori modification in particular, raises the need for specific building blocks that can be used in stepwise peptide synthesis. Herein, we report the synthesis of N^{α}-Fmoc-Lys-OH derivatives containing N^{ε}-1-deoxyfructopyranosyl moiety. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

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Glycation of proteins through non-enzymatic reactions between glucose or other reducing sugars and reactive amino groups represents one of the more abundant processes involved in post-translational modification of proteins [1]. Spontaneous and reversible condensation of a reducing sugar and a free amino group of a protein forms an aldimine also known as the Schiff base that undergoes a rearrangement into the more stable ketoamine known also as the Amadori product [2]. In the case of glucose, the initially formed Schiff base rearranges into the more stable 1-deoxyfructopyranosyl moiety. Subsequent dehydration, condensation, fragmentation, oxidation, and cyclization reactions lead to the irreversible formation of advanced glycation end products (AGEs). This process leads to inactivation of proteins and is involved in pathologies such as senile cataract [3], arteriosclerosis [4], vascular complications of diabetes [5], dysfunction of skin collagen [6], and neurodegenerative diseases such as Alzheimer's disease [7,8] and Parkinson disease [9].

Growing evidence suggests that glycation occurs preferentially at specific glycation motifs characterized by acidic amino acids, mainly glutamate and lysine residues that catalyze the glycation of nearby lysines [10,11]. Proximity to histidine either in the primary or in the secondary structure was also suggested to promote glycation of adjacent lysines [12,13]. Recent interest to fully characterize the glycation products and to use them as biomarkers and antigens for diagnosis and prognosis of disease, monitoring its progress and evaluation of the efficiency of therapy generated the need for glycated peptides representing the glycation motifs specifically modified by the 1-deoxyfructopyranosyl. Today, syntheses of site-specific Amadori-modified peptides are carried out on partially protected synthetic peptides in which only the lysyl residues designated for glycation are exposed while the rest are protected [14–17]. This approach involves orthogonal protection strategies and suffers from low yields and elaborated purification schemes.

Stepwise assembly of site-specific Amadori-modified peptides requires N^{α}-protected-N^{ε}-glycated-Lys building blocks and represents a fully controlled and effective synthetic strategy. Herein, we report the synthesis, purification, and characterization of N^{α}-Fmoc, N^{ε}-Boc, N^{ε}-(1-deoxyfructopyranosyl)lysine building blocks needed for Fmoc-based solid phase synthesis of Amadori-modified peptides.

This study offers a controlled side-specific introduction of N^{ε}-Amadori-modified Lys residue into synthetic peptides during a stepwise assembly either in solution or in solid phase methodologies. This strategy will overcome three major problems associated with the modification of already assembled peptides: (i) lack of site-specificity in the introduction of the modification, (ii) need for elaborate orthogonal protection scheme in an effort to achieve site-specificity, and (iii) extremely low yields and complicated reaction mixtures due to side reactions following the direct thermal glycation. Adapting the conditions for generating Amadori peptides by direct thermal glycation in the presence of excess D-glucose [14] to the direct glycation of N^{α}-Fmoc-lysine led to the synthesis of N^{α}-Fmoc-Lys[N^{ε}-1-deoxyfructopyranosyl)]-OH (**1a**) in 67% yield (Scheme 1, pathway A). Preliminary attempt to use

* *Correspondence to: Michael Chorev, Laboratory for Translational Research, Harvard Medical School, One Kendall Square, Building 600, Cambridge, MA 02139, USA. E-mail: michael_chorev@hms.harvard.edu*

a *Laboratory of Peptide and Protein Chemistry and Biology, Polo Scientifico e Tecnologico, University of Firenze, Sesto Fiorentino I-50019, Italy*

b *Dipartimento di Chimica Organica, Polo Scientifico e Tecnologico, University of Firenze, Via della Lastruccia 13, Sesto Fiorentino I-50019, Italy*

c *Dipartimento di Scienze Farmaceutiche, University of Firenze, Via Ugo Schiff 3, Polo Scientifico e Tecnologico, Sesto Fiorentino I-50019, Italy*

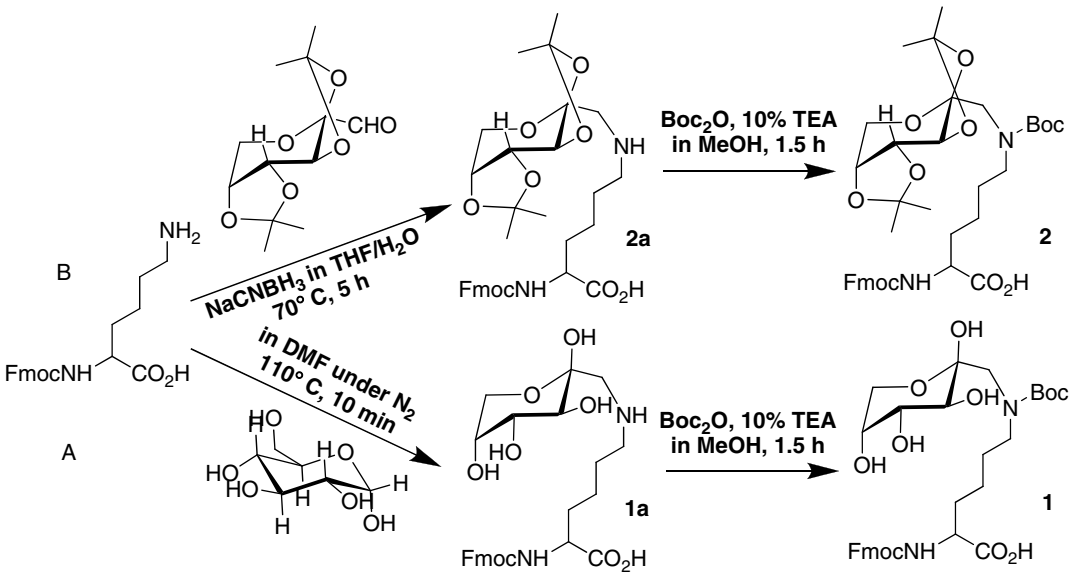
d *Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA*

e *Laboratory for Translational Research, Harvard Medical School, One Kendall Square, Building 600, Cambridge, MA 02139, USA*

1a as a building block in stepwise assembly of peptides suggested that further protection of the ε-amino by the orthogonal Boc group may eliminate some of the observed side products. To this end Boc protection of **1a** yielded the pure N^{α}-Fmoc-Lys[N^{ε}-1-deoxyfructopyranosyl, N^{ε}-Boc)]-OH (**1**) in 45% yield.

Anticipating that quantitative incorporation of **1** may require highly activated N^{ε}-Amadori-modified building block and extended reaction times we sought the synthesis of an exhaustively protected building block in which in addition to the primary and secondary amino function we also protected the hydroxyls on the carbohydrate moiety. To this end, reductive alkylation of the N^{α}-Fmoc-Lys-OH by 2,3:4,5-di-*O*-isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose [18–20] in the presence of $NaCNBH_3$ led to the formation of **2a** in 22% (Scheme 1, pathway B). The pure fully protected N^{α}-Fmoc-Lys[N^{ε}-(2,3:4,5-di-*O*-isopropylidene-1-deoxyfructopyranosyl, N^{ε}-Boc)]-OH (**2**) was obtained in 67% yield. Figure 1 depicts the analytical RP-HPLC tracings obtained for the purified building blocks **1** and **2**, and their precursors **1a** and **2a**.

In contrast from what was previously reported for short post-synthetically glycated peptides, in which the incorporated sugar moiety presents several tautomeric forms in equilibrium [21,22], the sugar moiety of N^{ε}-Amadori-containing N^{α}-Fmoc-Lys-OH derivatives **1** and **1a** displays a single β-pyranose tautomer (NMR



Scheme 1. Synthesis of N$^{\alpha}$-Fmoc-Lys[N$^{\varepsilon}$-1-deoxyfructopyranosyl, N$^{\varepsilon}$-Boc)]-OH **(1)** and N$^{\alpha}$-Fmoc-Lys[N$^{\varepsilon}$-(2,3:4,5-di-O-isopropylidene-1-deoxyfructopyranosyl, N$^{\varepsilon}$-Boc)]-OH **(2)**.

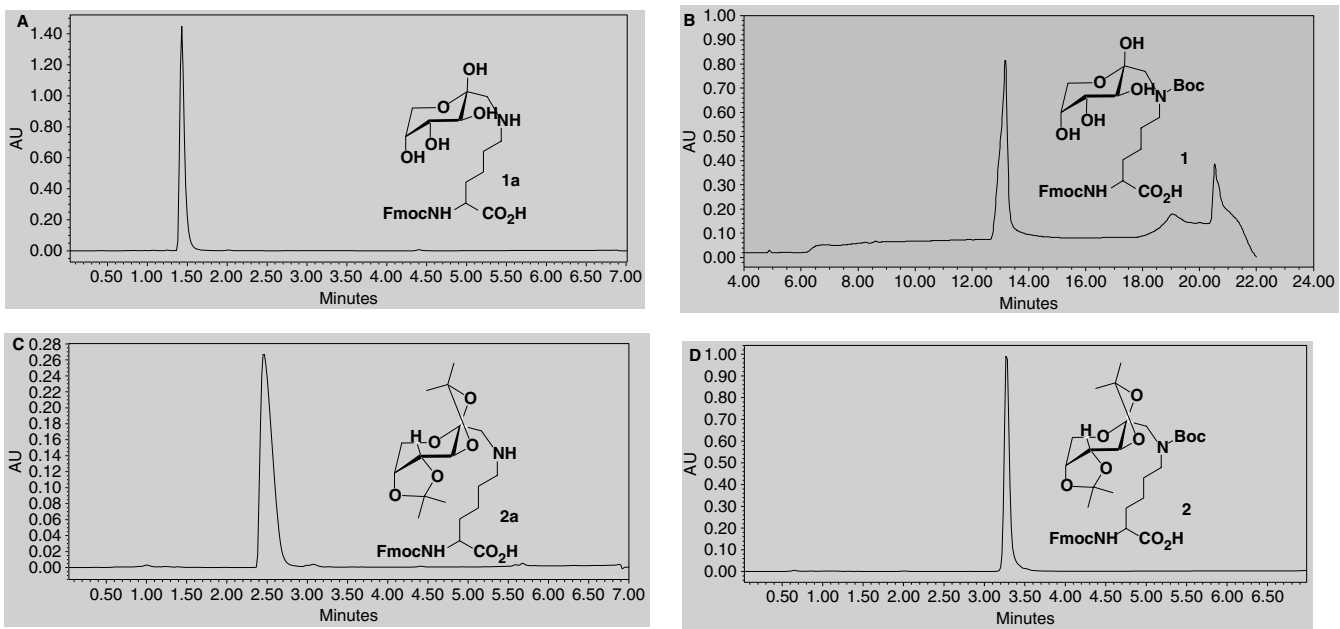


Figure 1. (A) Analytical HPLC of purified **1a**, gradient 40–80% B in A over 3 min, R_t = 1.45 min, 254 nm; **(B)** Analytical HPLC of purified **1** gradient 40–70% B in A over 15 min, R_t = 13.3 min, 254 nm; **(C)** Analytical HPLC of pure **2a**, gradient 40–80% B in A over 3 min, R_t = 2.5 min, 254 nm; **(D)** Analytical HPLC of pure **2**, gradient 70–100% B in A over 3 min, R_t = 3.42 min, 254 nm.

data). The β-pyranose form, which is the predominant tautomer of glucose in solution, is also the only detectable tautomer in **1** and **1a**. We speculate that in the presence of peptide, as in the case of post-synthetic glycation, the tautomerization of the sugar moiety is kinetically driven allowing the less stable tautomers such as α-pyranose, α- and β-furanose, the linear keto and linear form to be detected.

In conclusion, we have developed N^{ε}-Amadori-containing N^{α}-Fmoc-Lys-OH derivatives as new building blocks for the synthesis of site-specific Amadori-modified peptides. Contrary to previously published post-synthetically Amadori-modified peptides, these building blocks will enable efficient and site-specific incorporation of a major post-translational modification into bioactive and antigenic peptides by stepwise assembly.

Unless otherwise noted, all the materials and solvents were obtained from commercial suppliers and used without further purification. Protected amino acids were purchased from Novabiochem. Analytical HPLC was performed on a Waters 2695 Alliance equipped with a photodiode-array detector and a Waters XBridge C18 column (5 µm/4.6 × 100 mm), operating at a flow rate of 1 ml/min. LC-ESI-MS was performed on either a Waters 2695 Alliance coupled with a Waters Micromass ZQ spectrophotometer and equipped with a UV–VIS detector and a Waters Symmetry C18 column (3.5 µm/2.1 × 100 mm), operating at flow rate 0.5 ml/min or on a Thermo Finnigan Surveyor chromatograph coupled with a LCQ Advantage spectrophotometer and equipped with a photodiode-array detector and a Waters XTerra C18 column (3.5 µm/3 × 100 mm), operating at a flow rate of 1 ml/min. Semi-preparative HPLC was performed on a BioRad BioLogic DuoFlow chromatograph equipped with a UV–VIS detector and a Waters XBridge Prep C18 column (5 µm/10 × 250 mm) operating at a flow rate of 3 ml/min. A Biotage SP System Flash Chromatograph (RP-FC) (equipped with a UV–VIS detector) was used for the purification of the building blocks and of their precursors. The solvent system used in the analytical and semi-preparative reversed-phase HPLC and RP-FC, unless otherwise noted, was eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in MeCN. NMR spectra were measured on a Varian I 500 MHz spectrometer.

N^{α}-Fmoc-Lys[N^{ε}-(1-Deoxyfructopyranosyl)]-OH (1A)

N^{α}-Fmoc-Lys-OH (1 g, 2.71 mmol, 1 eq) and D-glucose (1.22 g, 6.77 mmol, 2.5 eq) were suspended in 40 ml of anhydrous DMF under N_2 atmosphere [18]. The reaction mixture was then warmed at 110 °C for about 10 min: Fmoc-Lys-OH dissolved and before the colour of the reaction mixture changed from yellow to brown (index of diglycation), the solution was immediately cooled in an ice bath. DMF was evaporated under vacuum and the crude residue was purified by RP-FC using a gradient 15–40% B in A over 20 min, elution occurred at ~25% B. Homogeneous fractions were then evaporated and the water solution lyophilized yielding pure **1a** as a white, highly hygroscopic solid (390 mg, 67%). For additional information, please see section *S1* in the Supporting Information available online.

LC-ESI-MS (*m/z*): $[M + H]^+$ calculated for $C_{27}H_{34}N_2O_9$ 531.23; found, 531.38. M.P. = 115 °C (caramelization and decomposition were observed). $[\alpha]^{26}_D = -20.70$ (c = 1.015, H_2O). Elemental analysis calculated for $C_{27}H_{34}N_2O_9 \cdot TFA \cdot H_2O$: C, 49.86; H, 5.92; N, 4.01; found: C, 49.88; H, 6.26; N, 4.10.

^{1}H NMR (D_2O, 400 MHz): δ 7.2 (broad s, 4H, fluorenyl 4-H, 5-H, 1-H and 8-H), 6.9 (broad s, 4H, fluorenyl 3-H, 6-H, 2-H and 7-H), 4.1–3.9 (m, 2H, CH_2, Fmoc), 3.85 (broad s, CH Fmoc), 3.8 (broad s, CH_α, Lys), 3.8–3.7 (m, CHOHx3, 1-deoxyfructopyranosyl), 3.6 (t, 2H, J = 10.0 Hz, 1-deoxyfructopyranosyl), 3.1–2.9 (m, CH_2NH, 1-deoxyfructopyranosyl), 2.8–2.6 (m, $CH_{2\varepsilon}$, Lys), 1.6–1.2 (m, 4H, $CH_{2\beta,\delta}$), 1.1–0.8 (m, $CH_{2\gamma}$, Lys).

^{13}C NMR ($CDCl_3$, 50 MHz): δ 175.8 (C, COOH), 162.7 (q, J = 35.4 Hz, C, COOH, TFA), 157.1 (C, CONH, Fmoc), 116.3 (q, J = 291.0 Hz, CF_3, TFA), 143.2 (CH_{Ar} x2, Fmoc), 140.6 (CH_{Ar} x2, Fmoc), 127.5 (CH_{Ar} x2, Fmoc), 126.9 (CH_{Ar} x2, Fmoc), 124.7 (CH_{Ar} x2, Fmoc), 119.7(CH_{Ar} x2, Fmoc), 95.3 (C_q, C1 1-deoxyfructopyranosyl), 69.5, 69.3, 68.9 (CHOH x3, 1-deoxyfructopyranosyl), 69.4 (CH_2, 1-deoxyfructopyranosyl), 63.9 (CH_2, Fmoc), 53.9, 52.8 (CH_2NH 1-deoxyfructopyranosyl, C_α Lys) 48.0 ($CH_{2\varepsilon}$, Lys), 46.7 (CH, Fmoc), 30.5 ($CH_{2\beta}$, Lys), 24.8 ($CH_{2\gamma}$, Lys), 22.2 ($CH_{2\delta}$, Lys).

N^{α}-Fmoc-Lys[N^{ε}-(1-Deoxyfructopyranosyl), N^{ε}-Boc)]-OH (1)

A solution of Boc_2O (514 mg, 2.35 mmol, 2.5 eq) in 5 ml of MeOH was added, in N_2 atmosphere, under stirring at 0 °C, to a solution of **1a** in MeOH (500 mg, 0.94 mmol, 1 eq, 5 ml). The reaction was left under stirring at room temperature for 1.5 h and then the solvent was evaporated and the residue purified by RP-FC with a linear gradient of 30–55% B in A over 20 min. Elution occurred at ~50% B. Homogeneous fractions were then evaporated and the water solution yielded pure **1** as a white hygroscopic solid (270 mg, 45%). For additional information please see section *S2* in the Supporting Information available online.

LC-ESI-MS (*m/z*): $[M + Na]^+$ calculated for $C_{32}H_{42}N_2O_{11}$ 653.28; found, 653.26. M.P. = 105–106 °C. $[\alpha]^{26}_D = -16.18$ (c = 0.94, MeOH). Elemental analysis calculated for $C_{32}H_{42}N_2O_{11} \cdot 5H_2O$: C, 53.32; H, 7.27; N, 3.88; found: C, 53.03; H, 7.09; N, 4.09.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.8–7.6 (m, 2H, fluorenyl 4-H, 5-H), 7.6–7.4 (m, 2H, fluorenyl 1-H and 8-H), 7.4–7.2 (m, 4H, fluorenyl 3-H, 6-H, 2-H and 7-H), 4.5–4.3 (m, 2H, CH_2, Fmoc), 4.3–4.1 (m, 2H, CH Fmoc and CH_α Lys), 4.1–3.8 (m, CHOHx3, 1-deoxyfructopyranosyl), 3.8–3.5 (m, 1-deoxyfructopyranosyl), 3.5–2.9 (m, 4H, CH_2NH 1-deoxyfructopyranosyl and $CH_{2\varepsilon}$ Lys), 2.0–1.4 (m, 4H, $CH_{2\beta,\delta}$), 1.4 (s, CH_3x3, Boc), 1.4–1.0 (m, $CH_{2\gamma}$, Lys).

^{13}C NMR ($CDCl_3$, 50 MHz): δ 174.9 (C, COOH), 158.5 (C, CONH, Boc), 156.0 (C, CONH, Fmoc), 143.5 (CH_{Ar} x2, Fmoc), 141.1 (CH_{Ar} x2, Fmoc), 127.6 (CH_{Ar} x2, Fmoc), 127.0 (CH_{Ar} x2, Fmoc), 125.0 (CH_{Ar} x2, Fmoc), 119.9 (CH_{Ar} x2, Fmoc), 98.7 (C_q, C1 1-deoxyfructopyranosyl), 81.4 (C_q, Boc), 70.6, 70.1, 69.5 (CHOH x3, 1-deoxyfructopyranosyl), 67.2 (CH_2, 1-deoxyfructopyranosyl), 63.3 (CH_2, Fmoc) 54.6, 53.6 (CH_2NH 1-deoxyfructopyranosyl, C_α Lys) 50.0 ($CH_{2\varepsilon}$, Lys), 47.2 (CH, Fmoc), 31.9 ($CH_{2\beta}$, Lys), 28.5 (CH_3x3, Boc), 27.8 ($CH_{2\delta}$, Lys), 22.2 ($CH_{2\gamma}$, Lys).

N^{α}-Fmoc-Lys[N^{ε}-(2,3:4,5-Di-*O*-Isopropylidene-1-Deoxyfructopyranosyl)]-OH (2A)

A fresh solution of $NaCNBH_3$ (600 mg, 9.5 mmol, 2.5 eq) in 5 ml H_2O/THF (1 : 1, v/v) was added to a stirred solution of N^{α}-Fmoc-Lys-OH (1.4 g, 3.8 mmol, 1 eq) and 2,3:4,5-di-*O*-Isopropylidene-aldehydo-β-D-arabino-hexos-2-ulo-2,6-pyranose [18–20] (2.45 g, 9.5 mmol, 2.5 eq) in 15 ml H_2O/THF (1 : 1, v/v) under N_2 at 50 °C. After 4 h, the solvent was removed under reduced pressure and

the crude product was purified by RP-FC, eluted with the linear gradient 30–50% B in A over 20 min (eluted at ~37% B). The homogeneous fractions were pooled, evaporated and lyophilized yielding the pure **2a** as a white solid (500 mg, 22%). For additional information, please see section *S3* in the Supporting Information available online.

LC-ESI-MS (*m/z*): $[M + H]^+$ calculated for $C_{33}H_{42}N_2O_9$ 611.29; found, 611.17. M.P. = 92–93 °C. $[\alpha]^{26}_D = -12.04$ (c = 0.89, MeOH). Elemental analysis calculated for $C_{33}H_{42}N_2O_9 \cdot TFA \cdot 2H_2O$: C, 55.26; H, 6.23; N, 3.68; found: C, 55.35; H, 6.15; N, 3.39.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.74 (d, 2H, J = 7.4 Hz, fluorenyl 4-H and 5-H), 7.59 (d, 2H, J = 7.4 Hz, fluorenyl 1-H and 8-H), 7.37 (t, 2H, J = 7.4 Hz, fluorenyl 3-H and 6-H), 7.28 (t, 2H, J = 7.4 Hz, fluorenyl 2-H and 7-H), 5.85 (broad d, NH_α), 4.65–455 (m, CH Fmoc), 4.4–4.3 (3H, CH_2, Fmoc and CH_α Lys), 4.25–4.15 (m, CHOHx3, 1-deoxyfructopyranosyl), 3.8–3.7 (m, CH_2 1-deoxyfructopyranosyl), 3.35–3.30 (m, CH_2NH 1-deoxyfructopyranosyl), 3.30–3.20 (m, $CH_{2\varepsilon}$ Lys), 1.95–1.65 (m, 4H, $CH_{2\beta,\delta}$) 1.5–1.45 (m, $CH_{2\gamma}$, Lys) 1.47, 1.41, 1.33, 1.28 (s, CH_3x4, isopropylidene).

^{13}C NMR ($CDCl_3$, 50 MHz): δ 174.5 (C, COOH), 161.9 (q, J = 38.2 Hz, C, COOH, TFA), 156.2 (C, CONH, Fmoc), 116.5 (q, J = 290.9 Hz, CF_3, TFA), 143.6 (CH_{Ar} x2, Fmoc), 141.1 (CH_{Ar} x2, Fmoc), 127.6 (CH_{Ar} x2, Fmoc), 127.0 (CH_{Ar} x2, Fmoc), 125.1 (CH_{Ar} x2, Fmoc), 119.8 (CH_{Ar} x2, Fmoc), 110.0 (C_q, isopropylidene), 109.3 (C_q, isopropylidene), 99.56 (C_q, C1 1-deoxyfructopyranosyl), 72.2, 70.3, 69.6 (CHOH x3, 1-deoxyfructopyranosyl), 67.1 (CH_2, 1-deoxyfructopyranosyl), 61.58 (CH_2, Fmoc) 54.3, 53.7 (CH_2NH 1-deoxyfructopyranosyl, C_α Lys) 48.7 ($CH_{2\varepsilon}$, Lys), 47.2 (CH, Fmoc), 31.7 ($CH_{2\beta}$, Lys), 26.1, 25.9, 24.6, 24.1 (CH_3x4, isopropylidene), 25.5 ($CH_{2\gamma}$, Lys), 22.0 ($CH_{2\delta}$, Lys).

N^α-Fmoc-Lys[N^ε-(2,3:4,5-Di-*O*-Isopropylidene-1-Deoxyfructopyranosyl), N^ε-Boc]-OH (2)

A stirred solution of **2a** (500 mg 0.81 mmol, 1 eq) in methanol (5 ml) at 0 °C was treated with Boc_2O (445 mg, 2.07 mmol, 2.5 eq). The reaction was left stirring at room temperature for 1.5 h and then the solvent was evaporated and the residue purified by RP-FC employing a linear gradient of 50–100% B in A over 20 min (the product **2** eluted at ~70% B). Acidification of the concentrate, obtained after evaporation of acetonitrile from the pooled fractions, to pH 3 with acetic acid, resulted in a white precipitate that was filtered off, washed with water and dried under vacuum. The pure **2** was obtained as a white solid (390 mg, 67%). For additional information, please see section *S4* in the Supporting Information available online.

LC-ESI-MS (*m/z*): $[M + H]^+$ calculated for $C_{38}H_{50}N_2O_{11}$ 711.34; found, 711.43. M.P. = 97–98 °C. $[\alpha]^{26}_D = -16.97$ (c = 1.03, MeOH). Elemental analysis calculated for $C_{38}H_{50}N_2O_{11} \cdot H_2O$: C, 62.62; H, 7.19; N, 3.84; found C, 62.56; H, 7.29; N, 3.84.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.75 (d, 2H, J = 7.4 Hz, fluorenyl 4-H and 5-H), 7.59 (d, 2H, J = 7.4 Hz, fluorenyl 1-H and 8-H), 7.38 (t, 2H, J = 7.4 Hz, fluorenyl 3-H and 6-H), 7.30 (t, 2H, J = 7.4 Hz, fluorenyl 2-H and 7-H), 5.45 (broad d, NH_α), 4.6–4.55 (m, CH Fmoc), 4.50–4.45 (m, CH_α Lys), 4.45–4-30 (m, CHORx3, 1-deoxyfructopyranosyl), 4.25–4.15 (m, CH_2 Fmoc) 3.75–3.60 (m, CH_2 1-deoxyfructopyranosyl), 3.35–3.25 (m, CH_2NH 1-deoxyfructopyranosyl), 3.35–2.25 (m, $CH_{2\varepsilon}$ Lys), 2.00–1.70, 1.55–1.45, 1.35–1.25 (m, 6H, $CH_{2\beta,\delta,\gamma}$, Lys) 1.44 (s, CH_3x3, Boc),1.50, 1.47, 1.34, 1.31 (s, CH_3x4, isopropylidene).

^{13}C NMR ($CDCl_3$, 50 MHz): δ 175.5 (C, COOH), 159.1 (C, CONH, Boc), 156.1 (C, CONH, Fmoc), 143.7 (CH_{Ar} x2, Fmoc), 141.1 (CH_{Ar} x2, Fmoc), 127.6 (CH_{Ar} x2, Fmoc), 127.0 (CH_{Ar} x2, Fmoc), 125.0 (CH_{Ar} x2, Fmoc), 119.8 (CH_{Ar} x2, Fmoc), 108.8 (C_q, isopropylidene), 108.0 (C_q, isopropylidene), 104.5 (C_q, C1 1-deoxyfructopyranosyl), 80.8 (C_q, Boc), 71.4, 70.7, 70.5 (CHOH x3, 1-deoxyfructopyranosyl), 67.2 (CH_2, 1-deoxyfructopyranosyl), 61.3 (CH_2, Fmoc), 53.9, 51.4 (CH_2NH 1-deoxyfructopyranosyl, C_α Lys) 48.5 ($CH_{2\varepsilon}$, Lys), 47.3 (CH, Fmoc), 32.5 ($CH_{2\beta}$, Lys), 28.6 (CH_3x3, Boc), 27.4 ($CH_{2\delta}$, Lys), 26.4, 26.1, 25.1, 24.1 (CH_3x4, isopropylidene), 22.7 ($CH_{2\gamma}$, Lys).

Supporting information

Supporting information may be found in the online version of this article.

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