Expedient Synthesis of Peptides Containing \( \text{N}^\varepsilon \)-Carboxymethyllysine

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Supplementary Information
Materials and Methods

All solvents and reagents were used as supplied from commercial sources. Analytical thin-layer chromatography was performed using Kieselgel F254 0.2 mm (Merck) silica plates with visualization by ultraviolet irradiation (254 nm) followed by staining with ninhydrin. Flash chromatography was performed using Kieselgel S63-100 μm (Riedel-de-Hahn) silica gel. All solvent compositions reported are on a volume/volume (v/v) basis. $^1$H-NMR spectra were recorded on a 400 MHz Bruker spectrometer and are reported in parts per million (ppm) on the δ scale relative to $d_6$-dimethyl sulfoxide (DMSO). $^{13}$C-NMR spectra were recorded on a 100 MHz Bruker spectrometer and are reported in parts ppm on the δ scale relative to $d_6$-DMSO. The multiplicities of $^1$H signals are designated by the following abbreviations: $s$ = singlet, $d$ = doublet, $t$ = triplet, $m$ = multiplet, $dd$ = doublet of doublets. Mass spectra were obtained by electrospray ionization in positive ion mode.

Synthesis of $N^\alpha$-Fmoc-$N^\varepsilon$-Ns-L-Lysine 6

To an ice-cooled solution of Fmoc-Lys-OH (1.0 g, 2.82 mmol, 1 equiv) in 1 M aqueous $K_2CO_3$ (7 ml) and 1,4-dioxane (10 ml) was added NsCl (0.74 g, 3.36 mmol, 1.2 equiv) as a solution in dioxane (5 ml). The reaction was allowed to warm to rt and stirred for 2 h. The reaction mixture was partitioned between ethyl acetate (25 ml) and $H_2O$ (25 ml) and the layers separated. The aqueous layer was extracted with ethyl acetate (2 x 20 ml) and the combined extracts washed with brine, dried over $\text{MgSO}_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (100% ethyl acetate) afforded the title compound as a white solid (1.35 g, 90% yield). Spectroscopic data were in good agreement with those previously reported.$^1$ $^1$H NMR (400 MHz, $d_6$-DMSO) δ 8.08 (t, $J = 5.4$ Hz, 1H), 7.99-7.93 (m, 2H), 7.90-7.81 (m, 4H), 7.72-7.70 (m, 2H), 7.60-7.57 (m, 1H), 7.42-7.29 (m, 4H), 4.28-4.26 (m, 2H), 4.23-4.19 (m, 1H), 3.90-3.82 (m, 1H), 2.87 (dd, $J = 12.8$, 6.8 Hz, 2H), 1.66-1.46 (m, 2H), 1.45-1.20 (m, 4H). $^{13}$C NMR (100 MHz, $d_6$-DMSO) δ 174.0, 156.2, 147.8, 143.8,
Peptide Synthesis

Automated SPPS

Standard SPPS was performed via the Fmoc strategy on aminomethyl resin equipped with a Rink Amide linker using a Biotage Alstra peptide synthesiser (Uppsala, Sweden) on 0.1 mmol scale. The Fmoc group was deprotected with 20% piperidine in \textit{N,N}-dimethylformamide (DMF) for 2 + 3 minutes at 60 °C. The coupling step was performed with 5 equiv of Fmoc-protected amino acid in DMF (0.2 M), 4.5 equiv of \( O\)-(benzotriazol-1-yl)-\textit{N,N,N',N'}-tetramethyluronium hexafluorophosphate (HBTU) in DMF (0.45 M) and 10 equivalents of \textit{N,N}-diisopropylethylamine (DIPEA) for 5 min at 75 °C. The final Fmoc-group was removed and the free amine was acetylated by \textit{Ac}_2\text{O} in the presence of DIPEA at room temperature for 10 min.

Alkylation with tert-butyl bromoacetate

Resin-bound peptide 10 or 12 (0.1 mmol, 1 equiv) was swollen in dichloromethane (30 min), then in DMF (10 min), and drained. A solution of tert-butyl bromoacetate (70 μl, 0.5 mmol, 5 equiv) with DIPEA (175 μl, 1 mmol, 10 equiv) in DMF (5 ml) was added in one portion and the reaction mixture shaken overnight at rt to afford peptides 11 or 13 respectively, which were drained and washed with DMF.

Ns Deprotection

Resin-bound peptide 8 or 11 (0.1 mmol, 1 equiv) was swollen in dichloromethane (30 min), then in DMF (10 min) and filtered. A solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (90 μl, 0.6
mmol, 6 equiv) and 2-mercaptoethanol (42 μl, 0.6 mmol, 6 equiv) in DMF (5 ml) was added in one portion and the mixture shaken for 15 min, drained, and washed with DMF.

**Peptide Purification**

Following completion of syntheses, the peptides were released from resin with concomitant removal of the remaining side-chain protecting groups by treatment with trifluoroacetic acid (TFA)/triisopropyl silane (TIS)/H₂O (38:1:1, 5 ml) at rt for 2 h. The crude peptides were precipitated with cold diethyl ether, isolated by centrifugation, washed in cold diethyl ether, dissolved in 1:1 acetonitrile/H₂O containing 0.1% TFA and lyophilized. The peptides were analysed for purity by liquid chromatography mass spectrometry (LCMS) (Agilent 1120 compact LC system equipped with Agilent 6120 Quadrupole MS and a UV detector at 214 nm) using a Zorbax C3 column (3.5 μ; 3 x 150 mm; Agilent) at 0.3 ml/min using a linear gradient. The solvent system used was A (0.1% formic acid in H₂O) and B (0.1% formic acid in acetonitrile). Purification of crude peptides was performed by semipreparative high-performance liquid chromatography (HPLC) (Dionex Ultimate 3000 equipped with a 4 channel UV detector) at 210, 230, 254, and 280 nm using a Gemini C18 column (10 μ; 250 x 10 mm; Phenomenex) at 5 ml/min using a shallow linear gradient. The solvent system used was A (0.1% TFA in H₂O) and B (0.1% TFA in acetonitrile). The resulting purified peptides were analysed by the LCMS system used for crude peptide analysis. The purities were extrapolated from integrating the peaks corresponding to peptide 1 (13.1 min) and to peptide 2 (12.7 min) (Table 1).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MS [M+H]^+</th>
<th>Purity (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>1512.9 (calcd. 1512.7)</td>
<td>98</td>
</tr>
<tr>
<td>2 (Approach A)</td>
<td>1571.6 (calcd. 1571.7)</td>
<td>96</td>
</tr>
<tr>
<td>2 (Approach B)</td>
<td>1571.6 (calcd. 1571.7)</td>
<td>98</td>
</tr>
<tr>
<td>2 (Approach C)</td>
<td>1571.6 (calcd. 1571.7)</td>
<td>97</td>
</tr>
</tbody>
</table>

Table 1 Mass Spectrum Data and HPLC purities for synthetic peptides
**Trypsin Digest**

Bovine trypsin (0.3 mg, type XI, 9090 units/mg, Sigma) was dissolved in H₂O (1 ml) and 3.3 μl (9 units) of this solution diluted to 1 ml using Tris buffer (pH 8.0) and incubated at 37 °C for 30 min. Substrate peptide (0.21 μmol) was added in one portion and 50 μl aliquots removed every minute, quenched with 1 M HCl (50 μl) and analysed by analytical reverse phase-HPLC (Dionex Ultimate 3000 equipped with a 4 channel UV detector) at 210 nm using a Luna C18(2) column (3μ; 150 x 3 mm; Phenomenex) at 0.3 ml/min using linear gradient. The concentrations were extrapolated from integrating the peaks corresponding to peptide 1 (14.9 min) and to peptide 2 (15.0 min).
Fig SI-1 HPLC profile of crude 2 with MS at 12.8 min made by the building block approach (A), by alkylation post-synthesis (B), and by alkylation mid-synthesis in the presence of Fmoc-group (C).