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# Solid-phase synthesis and characterization of a novel fullerene-peptide derived from histone H3

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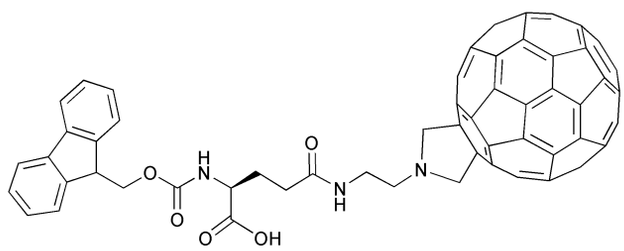
**A peptide analogue from a histone H3 protein containing the L-fulleropyrrolidino-glutamic acid has been prepared by a solid-phase approach and has been fully characterized. By molecular modelling it was verified that this peptide derivative is able to retain a binding capacity to the MHC (major histocompatibility complex) molecule similar to that of the cognate epitope.**

The applications of fullerenes in medicinal chemistry are in continuous expansion, since functionalised, soluble fullerene derivatives are now accessible.[1–4] These molecules can be further manipulated and integrated into biological systems, where they display a wide range of activity including neuroprotection, DNA photocleavage, enzyme inhibition, antiapoptosis.[5–9] Among the different classes of compounds, fullerene-based amino acids and peptides are particularly interesting.[5,6] Most of the compounds belonging to this family of molecules have been prepared using solution methods. For example 3,4-fullero-proline (Fpr) was considered the biggest unnatural amino acid and was inserted into model peptide sequences for the evaluation of its conformational properties.[10–13] Since the preparation of Fpr affords a racemic mixture,[13] a new, homochiral fullerene-based amino acid has been recently conceived and synthesised.[14] The molecular structure of the novel Fmoc-*N*-protected L-fulleropyrrolidino-glutamic acid **1** (L–Fgu) is shown in Fig. 1. This residue was inserted first at the *N*-terminus of an antimicrobial peptide,[14] opening the possibility of exploring new strategies for the insertion of Fgu also within a peptide sequence. A protocol of Fgu coupling, Fmoc deprotection and resin cleavage was optimised to obtain highly pure fullero-peptides.[15] This protocol was applied to the synthesis of antimicrobial and Leu-enkephalin peptide analogues.[15]

The use of automated solid-phase peptide synthesis (SPPS) allows a rapid increase in of the number of fullero-peptides available for biological applications. Herein, we present the solid-phase synthesis of a novel fullero-peptide which represents an analogue of the 64–78 peptide derived from histone H3. In particular, we have replaced residue Gln68 by Fgu (Fig. 2). The parent 64–78 peptide **2** has been identified in our laboratory as a dominant T-cell epitope that may play an important role in the development of the immune response in systemic lupus erythematosus (SLE).[16] Its modification by the introduction of non-coded amino acids could allow one to obtain new ligands able to restore self-tolerance in the autoimmune response. In the field of immunology only a limited number of reports on the use of fullerenes have appeared until now. A specific antibody against a $C_{60}$ derivative has been elicited by *in vivo* immunization.[17] It was subsequently isolated and crystallized thus enabling a description of the $C_{60}$ recognition site at atomic resolution.[18]



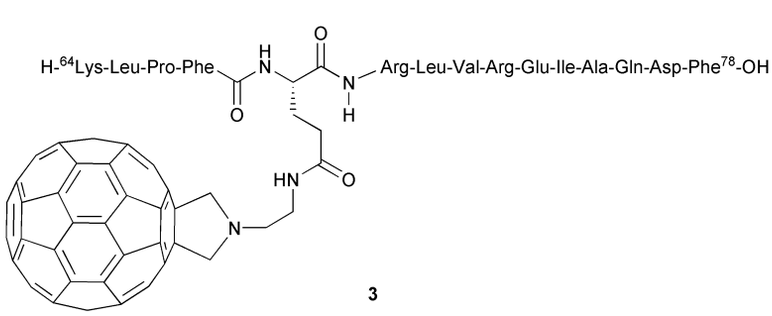
**1**

**Fig. 1** Molecular structure of the Fmoc-L-fulleropyrrolidino-glutamic acid (L–Fgu).

Using SYFPEITHI,† a database for MHC ligands and peptide motifs, we have initially verified that the glutamine in position 5 of the peptide sequence is not a key residue for binding to the human leukocyte antigen-group DR (HLA-DR) molecules.[19,20] Indeed, the program has predicted that the residues in positions 1, 4, 6 and 9 are the main anchors for this type of major histocompatibility complex (MHC) class II molecule.

H-$^{64}$Lys-Leu-Pro-Phe-Gln-Arg-Leu-Val-Arg-Glu-Ile-Ala-Gln-Asp-Phe$^{78}$-OH

**2**



**3**

**Fig. 2** Amino acid sequences of the parent 64–78 peptide derived from the nucleosomal protein H3 and the analogue in which residue Gln68 has been replaced by Fgu.

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In a recent article, it has been shown that the native 64–78 epitope strongly binds to HLA DRB1*1101 subtype.[16] The crystal structure of this MHC allele is not available. On the basis of the crystal structure of DRB1*0101 (pdb access number 1AQD) containing an antigenic peptide,[21] we have replaced the residues of the MHC α and β chains of the binding groove with those of the HLA DRB1*1101 sequence, using a pairwise BLAST for sequence alignment.[22] Using the Biopolymer and Discover modules of Accelrys (San Diego, CA) to build a new complex, we have subsequently replaced the original peptide present in the complex by our 64–78 epitope **2**, pointing the anchor residues P1, P4, P6 and P9, inside the groove. These key residues, as determined by SYFPEITHI, correspond to Lys64, Phe67, Arg69 and Arg72, respectively. The entire complex was minimized, *in vacuo*, by fixing the MHC binding pocket trace in the presence of the peptide. It has to be pointed out that the last four *C*-terminal amino acids were truncated because they are completely outside the MHC cavity and therefore not important for the stabilization of the complex. We first applied the steepest descent process until a RMS (root mean square) of 0.1 Kcal $mol^{-1}$ $Å^{-1}$, followed by the conjugate gradient algorithm fixing the MHC structure and enabling the peptide to relax inside the binding pocket, until a RMS of 0.001 Kcal $mol^{-1}$ $Å^{-1}$ was obtained. The same process was repeated by inserting the Fgu residue at position 68 of the native epitope. Fig. 3 shows that the molecular structures of both the natural and the modified peptide contained in the cavity are nicely superimposed and that all the hydrogen bonds between the peptide backbone and the binding pocket were retained as in the B1*0101 structure. It can be observed also that the anchors P1, P6 and P9 are deeply buried into the MHC cleft, thus stabilising the entire structure. We have calculated that the total non-covalent energy (van der Waals and electric interactions) corresponds to −161.3 and −138.3 Kcal $mol^{-1}$, for peptides **2** and **3**, respectively. The replacement of the glutamine by the fullero-pyrrolidino-glutamic acid does not perturb the conformation of the peptide, suggesting that it should maintain a high capacity to bind to the MHC molecule.

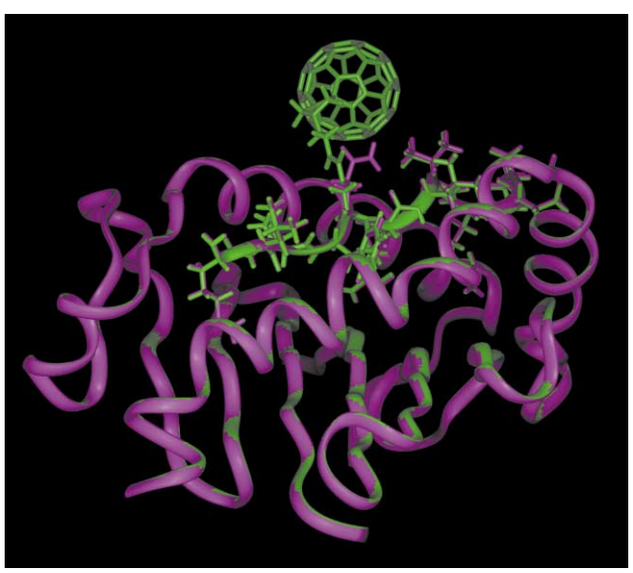

**Fig. 3** Superposition of the molecular structures of the MHC complexes containing the native epitope **2** and the fullero-peptide **3**.

The natural peptide H3(64–78) **2** and its Fgu analogue **3** were synthesized on a Wang resin (0.42 mol $g^{-1}$) using the Fmoc–*t*Bu strategy on a 15 μmol scale.[23,24] In the case of the natural epitope **2** the synthesis was fully automated.[25] Each standard Fmoc-protected amino acid was coupled in a 5-fold excess in the presence of Bop (benzotriazole-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate), HOBt (1-hydroxybenzotriazole) and DIEA (diisopropylethylamine) in DMF. Fmoc deprotection was performed twice using a 20% piperidine solution in DMF for 10 minutes. Concerning the Fgu-based peptide **3**, we used the same automated procedure as for **2** until Arg69. Before the introduction of the precious Fgu **1** residue, a small amount of decapeptide 69–78 was cleaved from the resin and analyzed by RP-HPLC and mass spectrometry to verify the high purity of the fragment. Then, the fullero-amino acid, replacing the glutamine residue at position 68, was inserted following a manual procedure. We reduced the excess of Fgu residue **1** to 4-fold excess by activating with Bop–HOBt–DIEA in DCM–DMF–NMP and by coupling at room temperature to the free amino function for 4 h. The completeness of the coupling was confirmed by a negative Kaiser test. This test becomes somewhat ambiguous after the insertion of the fullero-amino acid, since the colour of the resin beads turns brown. Nonetheless, the free amino function on the resin can be revealed by the release of blue colour into the test solution, which instead remains pale yellow after a successful coupling. The Fmoc-protecting group was then removed with 1 ml of a solution of 2% of DBU (1,8-diazabicyclo[5,4,0]undecen-7-ene) in DMF under argon and in the dark. This cleavage was repeated three times for 2 minutes. The same procedure was applied to all the deprotection steps of the residues following Fgu. The final product was removed from the resin using 5 ml of a 88 : 5 : 5 : 2 TFA–TIS–DTT–$H_2O$ mixture (TFA, trifluoroacetic acid; TIS, triisopropylsilane; DTT, dithiothreitol) for 2.5 h. The cleavage was repeated twice. The crude brown peptide **3** was precipitated with cold diethyl ether and after lyophilization 30 mg of crude material were recovered. The presence of the fullerene moiety increased the hydrophobicity of the peptide analogue. For this reason, the RP-HPLC analysis of the crude product was performed on a $C_4$ column monitoring at 254 nm. Fig. 4 shows the relative HPLC chromatogram where the main peak corresponds to the expected compound, as confirmed by mass analysis.

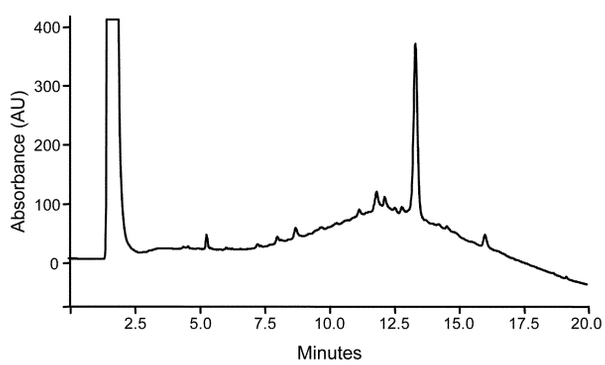


**Fig. 4** RP-HPLC chromatogram of the crude fullero-peptide **3**.

Fgu analogue **3** was purified using RP-HPLC chromatography on a semipreparative $C_4$ column and a gradient of 30% acetonitrile in water (0.1% TFA) to 100% acetonitrile (0.08% TFA) in 20 minutes. After six HPLC runs, 2.9 mg (7% overall yield) of pure fullero-peptide **3** was isolated (Fig. 5).

The presence of the fullerene moiety was confirmed by its typical UV-Visible absorption bands from 200 to 700 nm (Fig. 4, inset).[26] The identity of the fullero-peptide **3** was finally assessed by Maldi-Tof mass spectrometry and amino acid analysis.

Preliminary tests have evidenced that the fullero-peptide **3** is not completely soluble in pure water between 0.5–1 mM, the typical concentration range for measuring a biological activity. However, its solubility can be increased by adding 5 to 10% of DMSO, an amount which is still compatible with physiological conditions.

As we recently published the optimised protocol for the synthesis of fullerene-containing peptides endowed with promising

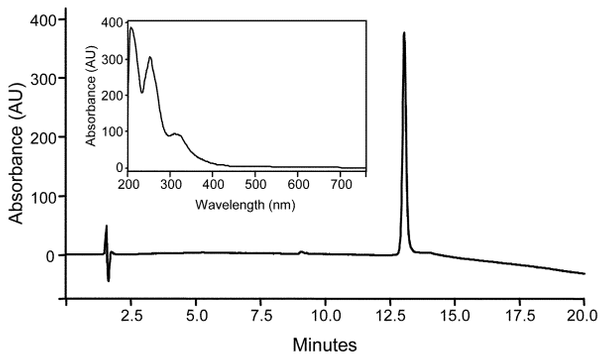


**Fig. 5** RP-HPLC chromatogram of the purified fullero-peptide **3**. Inset: UV-Vis spectrum of **3**.

biological activity, we confirmed in this report the reliability of our method. The critical step is certainly the removal of the base labile Fmoc-protecting group. Since the fullerenes are sensitive to the treatment with bases and nucleophiles,[27–29] the latter step must be carried out carefully.[15] Nevertheless, the complete SPPS procedure is prone to be fully automated thus speeding up the production of new fullero-peptides for biological tests.

In summary, we have prepared a novel optically homogenous fullero-peptide derived from the nucleosomal H3 protein containing the L-fulleropyrrolidino-glutamic acid residue. Molecular modelling suggests that this peptide analogue conserves the same binding capacity to the MHC molecule as the parent peptide. This successful synthesis is certainly encouraging toward the design and preparation of other biologically relevant fullero-peptides. We are currently studying the interaction of the histone H3 Fgu-based analogue with the MHC class II molecules and verifying if it is able to stimulate or block T-cell responses.

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## Notes and references

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