Efficient solid-phase synthesis of fullero-peptides using Merrifield strategy†

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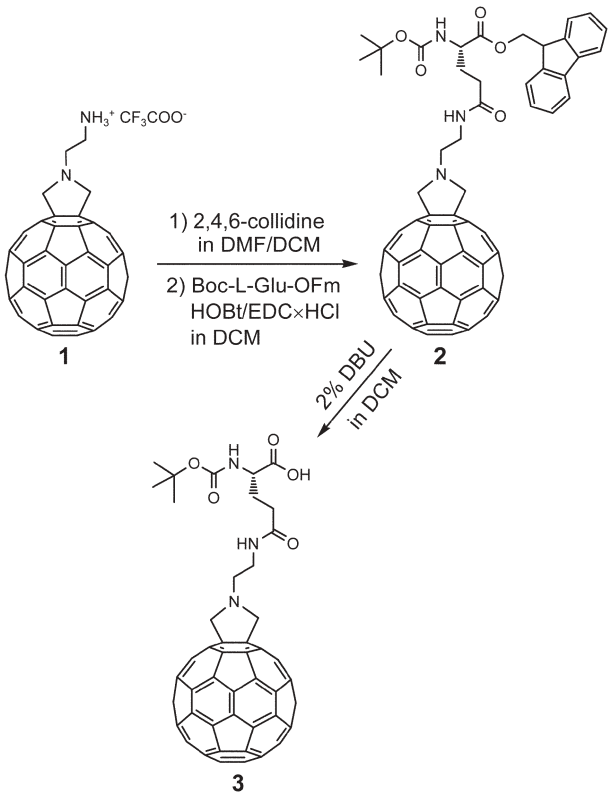
Boc-protected L-fulleropyrrolidino-glutamic acid was readily prepared and employed for the synthesis of fullerene-containing peptides using the solid-phase Boc chemistry developed by Merrifield.

Fullerenes are currently considered as standard building blocks in organic synthesis.[1–3] However, integration of C_{60} and its derivatives into biological systems is to a certain extent limited by their solubility in aqueous solutions.[4] To overcome this problem one approach is based on the design and preparation of suitable amino acids containing the C_{60} moiety and their incorporation into bioactive peptides.[5] The scope is double: 1) the fullerene can be rendered highly soluble in water; and 2) the biological activity of a natural peptide can be modulated by the presence of the fullero-amino acid. In this context, different fullero-amino acids have been prepared and they have been subsequently inserted into different classes of peptides using both solution and solid-phase synthesis.[5–9] Solid-phase synthesis of fullero-peptides is still at the early stage of development. Its feasibility has been recently demonstrated following the preparation of the Fmoc-protected L-fulleropyrrolidino-glutamic acid.[7] The synthesis of fullero-peptides was optimised for the Fmoc/tBu strategy.[8,9] However, this method requires some precautions since the fullerene moiety is particularly sensitive during the deprotection of the Fmoc group under basic conditions.[10]

In this Communication we describe the possibility of applying the alternative methodology invented forty years ago by Merrifield, which implies the use of acid conditions for the cleavage of the amino acid N-protecting groups and of the fullero-peptide from the solid support.[11] For this purpose, Boc-L-fulleropyrrolidino-glutamic acid (Boc-L-Fgu-OH) residue **3** was prepared (Scheme 1). The synthesis of C_{60} derivative **1** was already reported.[7] The first attempt to obtain compound **3** using the commercially available Boc-L-Glu-OtBu was not successful. Indeed, we encountered several difficulties in liberating the *tert*-butyl ester of the fullerene derivative. The selective cleavage of tBu in the presence of Boc described by Marcantoni *et al.* for other types of N- and C-protected amino acids failed due to the limited solubility of the amino acid Boc-L-Fgu-OtBu on the solvents reported in this procedure.[12] We decided to remove simultaneously the N- and C-protecting groups and to subsequently reintroduce the Boc on the amino function. In contrast to the recent results published by Mishino and co-workers for the synthesis of a similar derivative,[5c] the protection of with Boc_2O was ineffective either in a mixture of water/dioxane or in organic solvents. This is probably due to the extremely low solubility of H-L-Fgu-OH.



Scheme 1 Synthesis of Boc-L-fulleropyrrolidino-glutamic acid **3** (L-Fgu).

Therefore, an alternative protection scheme on glutamic acid was chosen. Derivative **1** was coupled to commercially available Boc-L-Glu-OFm, activated in turn with 1-hydroxybenzotriazole (HOBt) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC × HCl), obtaining the fully-protected compound **2** in 82% yield after purification by flash chromatography (Scheme 1).† The carboxylic function was quantitatively liberated by treating **2** with a 2% solution of DBU (1,8-diazabicyclo-[5,4,0]undecen-7-ene) in DCM for 30 seconds under sonication, followed by the immediate precipitation of the amino acid **3** into diethyl ether.

The treatment with the base permitted the isolation of the desired Fgu amino acid as DBU salt, as confirmed by NMR analysis.† The application of this extremely fast cleavage procedure, which also implies a limited excess of DBU, does not produce by-products or multiple additions to the C_{60} spheroid

† Electronic supplementary information (ESI) available: full experimental details. See http://www.rsc.org/suppdata/cc/b5/b504659a/

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although it has been reported that C_{60} could be sensitive to DBU treatment.[1a] Boc-L-Fgu-OH **3** was then used to investigate the solid-phase synthesis of a series of peptides using the Boc/Bzl methodology.[13] We decided to prepare two new analogues of the 64–78 peptide derived from histone H3 corresponding to the sequence KLPFQRLVREIAQDF. The parent peptide 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).[14] In the field of immunology few studies using fullerene derivatives and/or fullero-peptides have been reported until now. A specific antibody against a C_{60} derivative has been elicited by *in vivo* immunisation.[15] It was subsequently isolated and crystallized, thus allowing the C_{60} recognition site to be described at atomic resolution.[16] Sofou *et al.* have recently shown the biological activity of a fullero-peptide against sera from patients attained by SLE and mixed connective tissue disease (MCTD).[5d]

The design of the new 64–78 peptide analogues **4** and **5** containing Fgu was based on the molecular structure of a previous fullero-peptide synthesised in our laboratory.[9] In that fullero-peptide glutamine in position 68 of peptide 64–78 was replaced by a Fgu residue. Concerning the analogues **4** and **5** (Fig. 1), the first fullero-peptide is constituted of a scrambled sequence of the parent peptide where Gln66 is substituted by Fgu, while the second analogue contains Fgu in position 67 instead of phenylalanine.

The solid-phase synthesis of 64–78 Fgu analogue **4** and **5** was performed on PAM resin already derivatised with the first amino acid. The synthesis was carried out on a semi-automated peptide synthesizer for Boc chemistry.[17] Each standard Boc-protected amino acid was coupled twice for 10 min using a 5-fold excess in the presence of Bop (benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate), HOBt and DIEA (diisopropylethylamine) in DMF. Boc was removed with neat TFA in less than 5 min. Fullero-amino acid **3** was coupled in a 3-fold excess using Bop/HOBt activation in the presence of DIEA in a DCM/DMF/NMP mixture. The reaction was accomplished at room temperature over a period of 6 h. Fgu **3** can be used directly as DBU salt. The completeness of the insertion was confirmed by a negative Kaiser test. Although after the insertion of the fullero-amino acid the resin beads turn brown, 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. After the introduction of Fgu **3**, an analytical amount of peptide **4** was cleaved from the resin and analysed by RP-HPLC and mass spectrometry to verify the high purity of the fragment before finishing the synthesis. To cleave the fullero-peptides from the resin it is not possible to use the classical HF cleavage conditions.[18] The reactivity of C_{60} towards fluorides is well known and it has originated in the last years a new class of molecules called fluorofullerenes, used as synthons for different applications.[19] Therefore, the fragment was cleaved using a mixture of TFA/TMSOTf/p-cresol at room temperature for 15 h, followed by direct precipitation into cold diethyl ether.[20,21] Fig. 2 shows the RP-HPLC chromatogram and the UV-Vis spectrum of the crude precursor of fullero-peptide **4**.

The purity of the fragment was considered sufficiently high to achieve the elongation of peptides. Contrary to the synthesis that we optimised with the Fmoc/tBu strategy,[8] it was not necessary to adopt the precautions of inert atmosphere and darkness for the subsequent Boc unmasking and amino acid coupling. Indeed the N-terminus is immediately protonated by TFA and the reactivity of the amino functions with the C_{60} sphere is completely eliminated. This is undoubtedly advantageous if compared to the use of the Fmoc/tBu strategy.

The final products were cleaved from the resin using the same acid cocktail described above. The crude brown fullero-peptides **4** and **5** were precipitated with cold diethyl ether and lyophilised. Although the fullero-peptides strongly interact with the aromatic resins thus remaining embedded during the cleavage step,[8] more than 80% of peptide **4** and **5** was recovered.

The presence of the fullerene moiety increased the hydrophobicity of the peptide analogues. For this reason, RP-HPLC analyses of the crude product were performed on a C_4 column. Fig. 3 shows the relative RP-HPLC chromatogram of **5** where the main peak corresponds to the expected compound, as confirmed by mass spectrometry.† The two analogues were purified using RP-HPLC chromatography on a semi-preparative C_4 column. Pure fullero-peptide **4** and **5** (Fig. 3, inset) were obtained in 6.5 and 12% yield, respectively.

The presence of the fullerene moiety was again assessed by its typical UV-Vis absorption bands from 200 to 700 nm (Fig. 2, inset).[22] Both fullero-peptides are completely soluble in pure water at a concentration between 1 and 5 mg ml^{-1}.

In summary, we have prepared two novel fullero-peptides derived from the nucleosomal H3 protein containing the

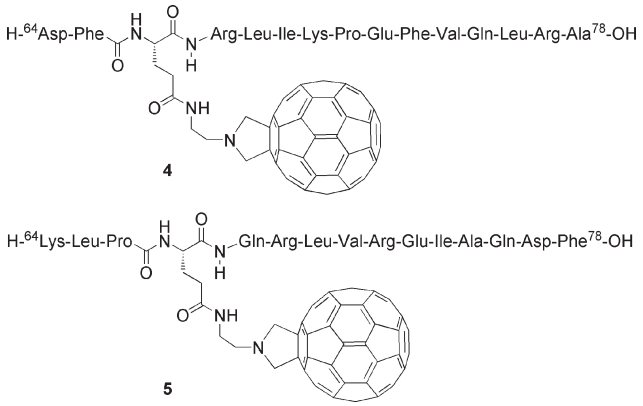

Fig. 1 Amino acid sequences of the analogues of 64–78 peptide derived from the nucleosomal protein H3 containing Fgu.

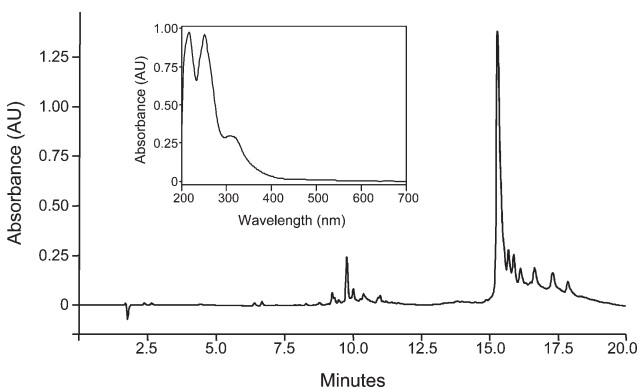

Fig. 2 RP-HPLC chromatogram of crude precursor of fullero-peptide **4** (λ = 214 nm). Inset: UV-Vis spectrum.

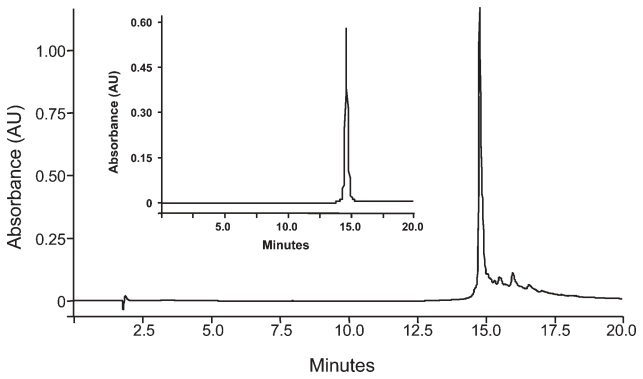

Fig. 3 RP-HPLC chromatogram of crude and purified (inset) fullero-peptide **5** (λ = 254 nm).

L-fulleropyrrolidino-glutamic acid residue using Merrifield based Boc/Bzl solid-phase approach. In this strategy, conditions for the removal of the protecting groups permit working at ambient atmosphere and the direct protonation of the amino function during TFA treatment eliminates almost completely the side-reactions. This methodology can be easily extended to the preparation of other fullero-peptides derived from the modification of biologically important peptides. We are currently studying the interaction of the two Fgu-based analogues with the MHC class II molecules and their ability to modulate the T-cell response.

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