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# Solid-Phase Synthesis of Fullerene-peptides

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***Abstract:*** The solid-phase synthesis of peptides (SPPS) containing [60]fullerene-functionalized amino acids is reported. A new amino acid, fulleropyrrolidino-glutamic acid (Fgu), is used for the SPPS of a series of analogues of different length based on the natural Leu[5]-Enkephalin and on cationic antimicrobial peptides. These fullero-peptides were prepared on different solid supports to analyze the influence of the resin on the synthesis. Optimized protocols for the coupling and deprotection procedures were determined allowing the synthesis of highly pure peptides in sufficient quantities for evaluation of biological activities. In particular, to avoid side reactions of the fullerene moiety with bases and nucleophiles, the removal of the protecting groups was performed under inert conditions (nitrogen or argon in the dark). We have encountered serious problems with the recovery of the crude compounds, especially when Fgu was inserted in the proximity of the resin core as fullero-peptides tend to remain embedded inside the resin. Eventually, all of the fullero-peptides were easily purified, and the cationic peptides were tested for their antimicrobial activities. They displayed a specific activity against the Gram-positive bacterium *S. aureus* and also lysed erythrocytes. The availability of a fullero-amino acid easily useable in the SPPS of fullero-peptides may thus open the way to the synthesis of new types of biologically active oligomers.

## Introduction

The recent progress in the chemistry of fullerenes has allowed the use of this allotropic form of carbon as a novel building block in organic synthesis.[1] Of the several fullerenes that have been characterized, the most abundant, $C_{60}$, has received the highest attention, as $C_{60}$-based molecules display a wide range of interesting features, which include nonlinear optical properties and superconductivity.[2] The hydrophobic nature of $C_{60}$ and its unique shape also render this molecule very interesting for its potential use in medicinal chemistry.[3] Indeed, a series of [60]-fullerene derivatives displays a wide spectrum of biological properties, including neuroprotective, enzymatic, antiapoptotic, antibacterial, DNA photocleaving, nitric oxide synthase inhibiting, and chemotactic activities.[3] Recently, it has also been demonstrated that $C_{60}$-protein conjugates are able to induce the production of specific antibodies.[4,5] Among the different classes of derivatives, fullerene-based amino acids and peptides are particularly interesting, both for structural studies and for biological applications.[3a] For example, $C_{60}$-based 3,4-fullero-proline (Fpr), which is the fullerene homologue of the natural proline residue, has been inserted into small peptides for studying its propensity to induce $\beta$-turn conformations and to influence the cis−trans equilibrium around the tertiary amide

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bond.[3a,6] Fulleroproline amino acid derivatives are also shown to interact with different hydrolytic enzymes in model transesterification reactions, and to form supramolecular complexes with, and selectively discriminate between, different size calix-[*n*]arenes, cyclodextrins, and other rationally designed peptides forming cavities.[7]

The synthesis of the first fullero-peptide was reported in 1993.[8] A methanofullerene was linked to the N-terminal part of a pentapeptide with an alternating -Aib-Ala- sequence, and this model fullero-peptide was able to adopt a $3_{10}$ helical structure. This work opened up the possibility to extend the preparation of fullero-peptides endowed with biological and pharmacological activities characterized by enhanced solubility in aqueous solutions.[9] However, all of the reported [60]fullerene-containing peptides have been prepared by solution methods, while the solid-phase synthesis of peptides containing fullero-amino acids has not been properly explored nor developed. This is in part due to synthetic problems related to the use of [60]-fullerene-based amino acids, but also to the sensitivity to base and nucleophile treatments of the carbon cage.[1a,10] A novel $C_{60}$-functionalized amino acid, a derivative of glutamic acid, has been recently prepared for use in solid-phase peptide synthesis.[11] This amino acid was coupled on the resin at the N-terminus of a water-soluble peptide, and the resulting oligomer displayed antimicrobial activity. This exciting result stimulated us to further explore the solid-phase synthesis of fullero-peptides and to develop suitable protocols for the introduction of this fulleropyrrolidino-glutamic acid residue (abbreviated hereafter as Fgu) within peptide sequences. In this work, we describe the possibility of inserting the Fgu residue at different positions of antimicrobial peptides of various lengths and into small model opioid peptides.[12] The optimization of the synthetic protocol is described in detail, together with the difficulties and problems encountered during the solid-phase coupling and deprotection steps. The role of the solid support and the position of Fgu along the peptide backbone have been analyzed and correlated to the synthesis yield and quality. The effect of the fullerene substituent on the biological activities of the antimicrobial analogues will also be briefly discussed. Indeed, some simple [60]fullerene derivatives already display interesting antibacterial properties against a broad range of microorganisms,[13] but a wider application of fullerenes in biology is heavily limited by their poor solubility in aqueous media. The idea was to explore if association of the fullerene characteristics with those of highly soluble, membrane active antimicrobial peptides of natural or synthetic origin could lead to a new class of oligomers potentially useful as lead compounds for the development of novel antiinfective agents.

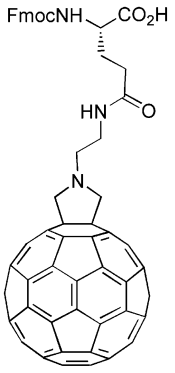
***Table 1.*** Leu[5]-Enkephalin and Antimicrobial Sequences of Peptides and Analogues Containing Fgu **1**

| entry | peptide sequences[a] | resin[b] |
|---|---|---|
| 2 | H-Tyr-$(Gly)_2$-Phe-Leu-$NH_2$ | PAL-PEG-PS |
| 3 | H-Tyr-$(Gly)_2$-Phe-Leu-OH | NovaSyn-HMP |
| 4 | H-Tyr-$(Gly)_2$-**Fgu**-Leu-$NH_2$ | PAL-PEG-PS |
| 5 | H-Tyr-$(Gly)_2$-**Fgu**-Leu-OH | NovaSyn-HMP<br>POEPOP-HMP |
| 6 | H-**Fgu**-$(Gly)_2$-Phe-Leu-$NH_2$ | PAL-PEG-PS<br>POEPOP-Rink |
| 7 | H-Gly-$(Nle)_2$-Gln-Orn-Nle-Gly-$(Orn)_2$-Nle-$(Orn)_2$-Nle-Gly-$(Orn)_2$-Nle-Gly-Tyr-$NH_2$ | PAL-PEG-PS |
| 8 | H-**Fgu**-Gly-$(Nle)_2$-Gln-Orn-Nle-Gly-$(Orn)_2$-Nle-$(Orn)_2$-Nle-Gly-$(Orn)_2$-Nle-Gly-Tyr-$NH_2$ | PAL-PEG-PS |
| 9 | H-Gly-$(Nle)_2$-Gln-Orn-Nle-Gly-$(Orn)_2$-**Fgu**-$(Orn)_2$-Nle-Gly-$(Orn)_2$-Nle-Gly-Tyr-$NH_2$ | PAL-PEG-PS |
| 10 | H-Gly-Orn-Gly-**Fgu**-Gly-Orn-Gly-$NH_2$ | PAL-PEG-PS<br>POEPOP-Rink |

[a] The fullero-amino acid (Fgu) is written in bold to highlight its position. [b] Type of resin used for the solid-phase synthesis of each peptide.

## Results and Discussion

**Design of the Fullero-peptides.** With the purpose of systematically studying the solid-phase synthesis of peptides containing the novel Fmoc-protected fulleropyrrolidino-glutamic acid (Fgu) **1**,[11] we have decided to consider two classes of peptides (Table 1).




**1**

The first class of peptides (**4**−**6**) consists of analogues of the small natural Leu[5]-Enkephalin **2** and **3**, often used as model peptides.[14] Fgu **1**, in this case, has been used to replace the hydrophobic residues Tyr[1] and Phe[4]. These fullero-peptides were also prepared using different resins to analyze the role of the solid support on the yield and the efficiency of the synthesis

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(Table 1). The second series of derivatives includes cationic peptides (**8**−**10**) of different length, where the Fgu **1** residue has been introduced either at the N-terminus or in the middle of the sequence to test the flexibility of synthetic protocol and to evaluate the influence of the $C_{60}$-based residue position on the biological activity.[13] Fullero-peptides **8** and **9** are based on the previously characterized antimicrobial peptide **7**,[12a,b] whereas **10** is based on a simple hexapeptide devoid of intrinsic antimicrobial activity.[11]

**Synthesis of Leu$^5$-Enkephalin Peptide Analogues.** Our work has initially focused on the role of the resin on peptide yields while using Fgu **1** in SPPS. The small peptide analogues (**4**−**6**) of Leu$^5$-Enkephalin (Table 1) in which the aromatic residues Tyr$^1$ and Phe$^4$ have been replaced by Fgu **1** have been prepared manually on Tentagel-like and cross-linked PEG resins (Table 1) to evaluate the influence of the different type of solid support on the quality of the synthesis. The functionalization of the resins containing hydroxyl groups was carried out employing Fmoc-Leu-OH in the presence of MSNT and methyl imidazole as activating agents.[15] The other amino acids were coupled via activation with BOP, HOBt, and DIAE. Fmoc deprotection was performed using 20% piperidine in DMF until the introduction of Fgu **1**, and then with a solution of 5% DBU in DMF without using inert conditions (vide infra). 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, because the color of the resin beads turns brown. Nonetheless, the free amino group on the resin can be revealed by the release of blue color into the test solution, which instead remains pale yellow after a successful amino acid coupling. The final peptide analogues were obtained with a free C-terminal carboxylic function or as C-terminal amides, after cleavage from the different resins using a mixture of TFA/TIS/$H_2O$. While the synthesis of the reference peptides **2** and **3** proceeded properly, those of the analogues containing the fullero-amino acid Fgu **1** were less successful. In the case of fullero-peptides **4** and **5** with Fgu **1** in position 4, a very low yield of crude product (less than 25%) was recovered, although the spectrophotometrical analysis following each Fmoc cleavage indicated a complete coupling/deprotection according to the resin loading. Furthermore, the expected compound was not detected by MALDI-Tof analysis. We reasoned that the nature of resin could have played a role in determining the synthetic yield of these fullero-peptides, where the Fgu **1** residue is inserted close to the polystyrene-based polymeric matrix. The high affinity of fullerene derivatives for aromatic molecules is in fact well known.[16] A further indication of interaction was that after the insertion of Fgu **1**, we observed a dramatic decrease in the swelling properties of the peptide-resin conjugate. Again, we speculated that following cleavage of the peptide from the resin, part of the product could remain embedded within the aromatic core of the solid support. The intense brown color of the resin after the final acid treatment was in fact indicative of a certain difficulty in the recovery of the product, and persisted even after further treatment with fresh TFA solution, and extensive washings of the resin with different solvents, while heating or sonicating the resin beads.

To analyze in more detail the influence of the resin on the synthetic yields, fullero-peptide **5** was also prepared on POE-POP-HMP resin,[15] whose polymeric structure consists of cross-linked polyoxyethylene−polyoxypropylene and is devoid of aromatic substituents. However, the recovery of the crude compound was again difficult, and only 29% of the theoretical amount was obtained, while the expected compound was not detected in the mass spectrum. Finally, we attempted to evaluate the influence of the position of Fgu **1** with respect to the resin linker, by introducing the fullero-amino acid at the N-terminus of Leu$^5$-Enkephalin, thus replacing Tyr$^1$ residue. The synthesis of fullero-peptide **6** on PAL-PEG-PS resin led to a yield of crude product comparable to that of fullero-peptide **4**, where Fgu is at the C-terminal part of the sequence, but when the POEPOP-Rink resin was used instead, an increase in the recovery of crude product to about 48% was observed. Furthermore, the fullero-peptide **6** was detected by mass spectrometry both as the N-terminal Fmoc protected analogue and, after final deprotection, as the free peptide. According to the MALDI-Tof spectrum, Fmoc-**Fgu**-Gly-Gly-Phe-Leu-$NH_2$ was obtained in high purity, while the deprotected fullero-peptide **6** presented a more complex spectrum. Indeed, the mass spectrum showed a series of multiple adducts of higher mass consistent with the addition of a DBU molecule and of an unidentified species, probably because the Fmoc cleavage was performed under noninert conditions (vide infra). Furthermore, it was impossible to separate these derivatives from the correct fullero-peptide using analytical HPLC column chromatography.

Apparently, even by using a resin without an aromatic core it is difficult to obtain a quantitative recovery of the fullero-peptide. This is also consistent with the reduced swelling properties of the peptide-resin conjugate before the acid treatment, probably due to a certain degree of aggregation which decreased the mobility of the linked molecule.

**Synthesis of Antimicrobial Peptide Analogues.** Peptides **7**−**10** (Table 1) were synthesized using an automated synthesizer on PAL-PEG-PS resin. Each standard amino acid was coupled in the presence of TBTU and DIEA in DMF, and Fmoc deprotection was performed using a 20% piperidine solution in DMF. The introduction of Fgu **1** at the N-terminus of peptide **8**, in the middle of peptide **9**, replacing an apolar norleucine at position 8 of the peptide, and in the middle of peptide **10** was carried out overnight at 50 °C using PyBOP/HOBt as activating agents, as this mixture is more suitable for lengthy couplings. For fullero-peptides **9** and **10**, the coupling of the standard amino acids after the introduction of the Fgu residue was optimized by employing the HATU/HOAt activator couple. At the end of the synthesis, the peptides were removed from the solid support by treating with reagent K (TFA/ETD/thioanisole/$H_2O$/phenol).[17] Following precipitation, the crude compounds were analyzed by electrospray mass spectrometry (Figure 1A and B). A number of species were present, characterized by higher masses relative to the expected molecular ion, although the main byproducts were multiionizable molecules, which behaved like the expected product. In the case of fullero-peptide **9**, with the fullero-amino acid in the center of the sequence, the mass of the expected

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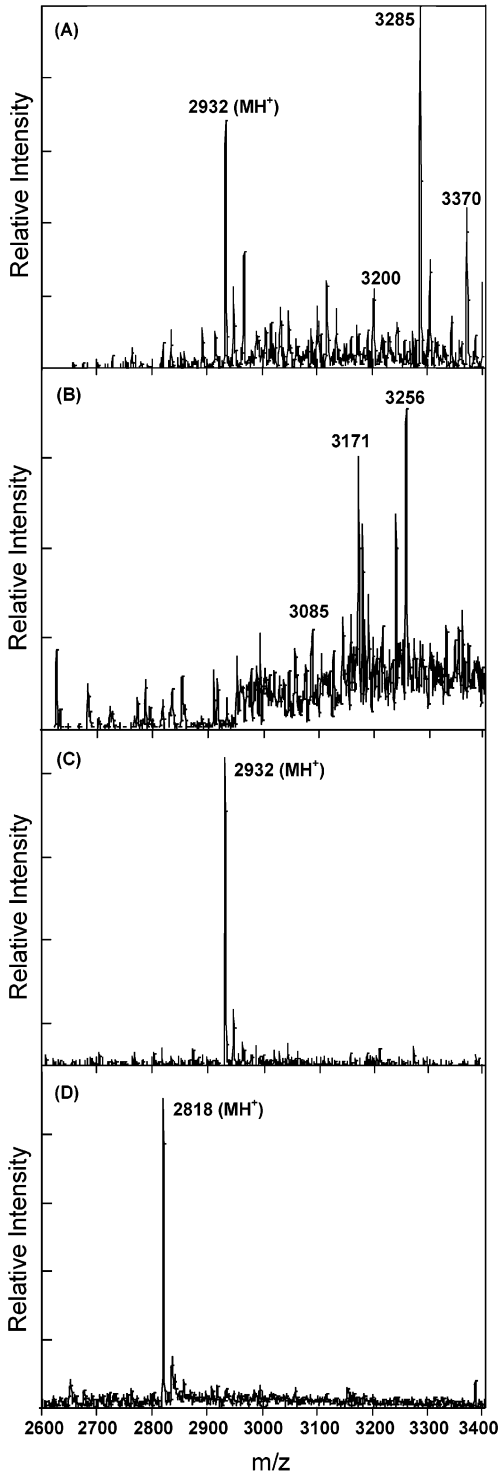
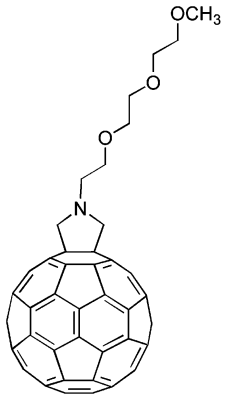



***Figure 1.*** ESI-MS spectra of crude fullero-peptide **8** (A) and **9** (B), cleaved from the resin, after removal of N-terminal Fmoc-protecting group using 20% piperidine in DMF, and ESI-MS spectra of crude fullero-peptide **8** (C) and purified fullero-peptide **9** (D) using a solution of 5% DBU in DMF for 2 min under inert conditions.

compound was not detected (Figure 1B). A careful analysis of the spectra allowed us to assign all of the various species to multiple adducts of piperidine to the $C_{60}$ core. We also observed species with a mass difference very close in weight to the dibenzofulvene-piperidine adduct, and thus likely due to the addition of benzofulvene anion formed during the Fmoc cleavage. From this analysis, we realized that the successive treatments with piperidine required in SPPS generate polyadducts to the fullerene moiety. This effect is relatively less relevant when the fullero-amino acid **1** is inserted at the N-terminus of the peptide (Figure 1A), while it considerably hampers the synthesis of the expected compound when the Fgu residue is inserted in the middle of the sequence (Figure 1B). Indeed, fullerene derivatives are electron-poor species, sensitive to treatment with bases and nucleophiles.[1d,10] Similar results were also obtained during the synthesis of peptide **10**, again with the fullero-amino acid inserted in the middle part, albeit of a shorter peptide.

The highly water-soluble fullero-peptide **8** was easily purified by reverse phase HPLC, while it was instead impossible to isolate fullero-peptides **9** and **10**. In light of the above results, we had to reconsider our synthetic strategy for the preparation of these analogues in which Fgu **1** is coupled in the middle of a peptide chain.

**Optimization of the Fmoc Cleavage Conditions.** The addition of piperidine and morpholine to $C_{60}$ has been already described and results in a 1,4-addition reaction in the presence of oxygen, leading to the generation of bis- and tetra-adducts.[1d,10] To further analyze the difficulties associated with an extensive treatment of fullerene derivatives with piperidine in DMF, we have studied the behavior of the model fulleropyrrolidine triethylene glycol monomethyl ether **11**, during treatment with different Fmoc cleavage conditions. This derivative in fact presents a good solubility in dimethylformamide, is resistant to photodegradation, and has already been fully characterized.[13a,18]




**11**

First, we carried out a series of experiments in which compound **11** was treated under four different reaction conditions: (i) 20% piperidine in DMF (standard Fmoc cleavage for SPPS); (ii) 20% piperidine in DMF in the dark; (iii) 20% piperidine under argon; and (iv) 20% piperidine in DMF under argon in the dark. For each condition, aliquots of solution were taken at different times, the fullerene derivative precipitated and washed prior to mass characterization. In Figure 2, the ESI-MS spectra of the compound **11** before and after piperidine treatment for 20 min using condition i are compared, showing the presence of multiple fullerene-piperidine adducts, and similar mass spectra were observed for conditions ii, iii, and iv (results not shown). These experiments confirm the sensibility of the

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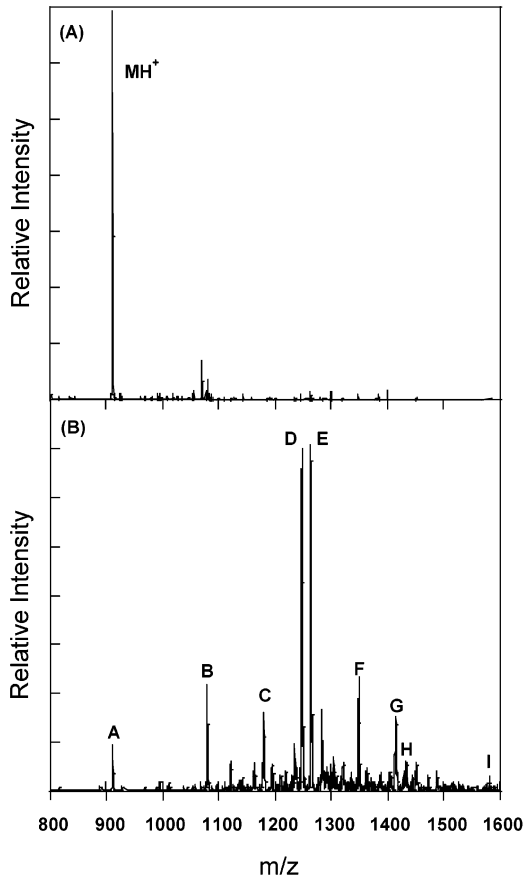


***Figure 2.*** ESI-MS spectra of compound **11** before treatment with piperidine (A) and after treatment with piperidine for 20 min (B). The different polyadducts correspond to the following: (A) starting material **11**; (B) **11** plus two piperidines; (C) **11** plus three piperidines plus one oxygen; (D) **11** plus four piperidines; (E) **11** plus four piperidines plus one oxygen; (F) **11** plus five piperidines plus one oxygen; (G) **11** plus six piperidines; (H) **11** plus six piperidines plus two oxygens; (I) **11** plus eight piperidines.

$C_{60}$ core to the addition of secondary amines under the cleavage conditions normally used in SPPS. Furthermore, the extent of polyadduct formation tends to increase with treatment duration, so that the formation of side product is expected to increase with repeated piperidine treatments.

We therefore treated compound **11** with 5% DBU in DMF, another commonly used reagent for Fmoc removal in SPPS,[19] under the same four different conditions described above. A series of fractions was collected, and, after precipitation, the products were again analyzed by mass spectrometry. In all cases, a mass signal corresponding to a monoadduct of DBU was clearly detected after 10 min of treatment, which tended to decrease until its complete disappearance after 30 min of treatment. This experimental observation can be explained by the generation of a zwitterionic complex $C_{60}$-DBU, which is dissociable to the $[C_{60}]^{\bullet-}$ and $DBU^{\bullet+}$ free radicals.[1d] Furthermore, we found that the starting material **11** was not degraded by side reactions. On the basis of these results, we decided to substitute the piperidine solution with 5% DBU in DMF, under inert conditions (nitrogen or argon in the dark), for the cleavage of the Fmoc protecting group of all residues following insertion

(19) (a) Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161−214. (b) Jung, G.; Beck-Sickinger, A. G. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 367−383. (c) Wade, J. D.; Bedford, J.; Sheppard, R. C.; Tregear, G. W. *Pept. Res.* **1991**, *4*, 194−199.

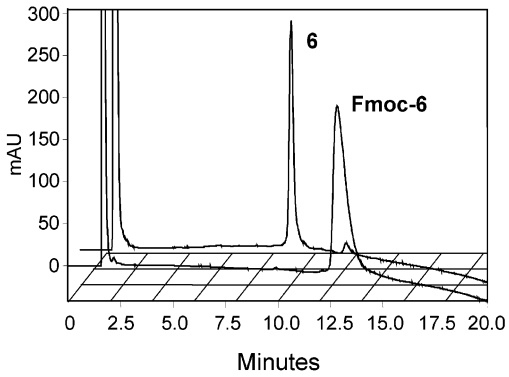


***Figure 3.*** RP-HPLC chromatograms of the fullero-peptide **6** and its Fmoc-protected precursor using a $C_4$ column and a gradient of 30−100% of acetonitrile along 20 min.

of Fgu, in the SPPS of fullero-peptides. The Fmoc cleavage time was fixed to 2 min, repeated twice. A comparison of the mass spectrum corresponding to the crude fullero-peptide **8**, synthesized using this new Fmoc cleavage protocol (Figure 1C), with that of the peptide from the original synthesis clearly shows the synthetic improvement.

**Improved Synthesis of Fullero-peptides 6, 9, and 10.** The synthesis of fullero-peptide **6** was therefore repeated using the optimized Fmoc removal conditions. The compound was cleaved from the resin both as the Fmoc-protected analogue or after Fmoc deprotection. Treatment with the TFA cocktail was performed twice for 3 and 24 h, respectively, to improve recovery. A certain amount of fullero-peptide was in fact recovered by precipitation also after the longer treatment. Figure 3 shows the HPLC chromatograms of both the Fmoc-protected and the free peptides deriving from the short treatment, as identified by MALDI-Tof. The presence of the $C_{60}$ moiety on these peptides was further verified by detection of the characteristic UV−visible absorption bands from 200 to 700 nm.

The synthesis of antimicrobial fullero-peptide **9**, with the Fgu replacing norleucine in position 8, was repeated on the PAL-PEG-PS resin using an automated synthesizer working under nitrogen flow during the Fmoc cleavage with a solution of 5% of DBU.[20] The recovery of the peptide required three successive treatments with TFA for 4, 24, and 72 h, respectively, allowing one to obtain 46% of crude material. The analytical HPLC showed the expected compound together with a significant amount of unidentified byproducts. However, it was possible to isolate fullero-peptide **9** with greater than 90% purity. The identity of the compound was confirmed by MALDI-Tof and ESI-MS analysis (Figure 1D). The synthesis of fullero-peptide **10**, with the Fgu **1** inserted in the middle of an alternating -Gly-Orn- sequence, was instead repeated on POEPOP-Rink resin, using the same synthetic strategy as that described for compound **6**. The complete fullero-peptide was cleaved from the resin with a 49% yield of product. The electrospray mass spectrum of the crude peptide confirmed the presence of the target structure with only a small percentage of a DBU adduct that was easily eliminated leading to pure fullero-peptide **10**.

**Influence of the Resin Type and of the Position of Fgu 1 along the Peptide Chain on the Synthesis.** Considering the yield of crude peptide after cleavage, we could discern that the main limiting factor for a good quantitative outcome to the

(20) Neimark, J.; Briand, J. P. *Pept. Res.* **1993**, *6*, 219−228.

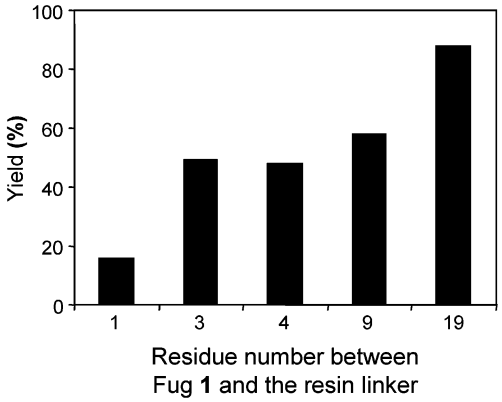


***Figure 4.*** Histogram of the recovered yield of crude peptide in the cleavage step as a function of the distance of the fullerene-based residue from the resin. The *x*-axis indicates the number of amino acids between Fgu **1** and the linker.

***Table 2.*** Biological Activity of Antimicrobial Fullero-peptides

| | antimicrobial activity (MIC[a], $\mu$M) | | | % hemolysis[b] | |
|---|---|---|---|---|---|
| peptide | *E. coli* ML-35 | *S. Aureus* 710A | *C. albicans* (c.i.) | 10 $\mu$M peptide | 100 $\mu$M peptide |
| 7 | 1 | 16 | 4 | 5% | 15% |
| 8 | 16−32 | 8 | >64 | 85% | >90% |
| 9 | 32 | 8−16 | >64 | n.d. | n.d. |
| 10 | >64 | 16 | >64 | 20% | n.d. |

[a] Results are the mean of at least three independent evaluations performed in duplicate. [b] Results are the mean of two independent evaluations performed in duplicate.

reaction is the distance between the position of Fgu insertion and the polymer matrix. A qualitative trend of the synthetic efficiency is reported in Figure 4, where it is clear that an increased separation from the solid support results in both an increased yield and the release of the fullero-peptide from the polymer core. The overall length of the peptide chain also seems to play a role, as the recovery of the short peptides is difficult even when the Fgu residue is inserted at the N-terminus. On the other hand, the nature of the resin seems to play a less fundamental role in the final recovery of fullero-peptides. Part of the compound appears to remain trapped inside the polymeric matrix also when it is devoid of an aromatic structure, as is the case for PEOPOP. In this respect, the proximity of the fulleropyrrolidino-glutamic acid to the resin core seems to play a critical role as its presence influences the swelling properties of the solid support. As a consequence, longer TFA cleavage times are required for this type of peptides with respect to classical solid-phase peptide synthesis protocols, and the optimization of cleavage conditions needs to be further studied.

**Antimicrobial Activity.** Antimicrobial peptides are often highly cationic, and therefore very soluble, and yet able to interact strongly with, and disrupt, the biological membranes of the target microorganism. They do this by virtue of their amphipathic nature, so that hydrophobic residues are relegated to a sector which interacts with the lipid bilayer, while polar and charged residues remain in contact with the membrane polar headgroups and external aqueous environment.[12a] Insertion of the highly hydrophobic $C_{60}$ moiety could therefore further modulate the interactions with the membrane. As fullerene derivatives already show an intrinsic antimicrobial activity,[13] it was conceivable that a synergistic effect might result. Table 2 shows the minimal concentration that inhibits the growth (MIC)

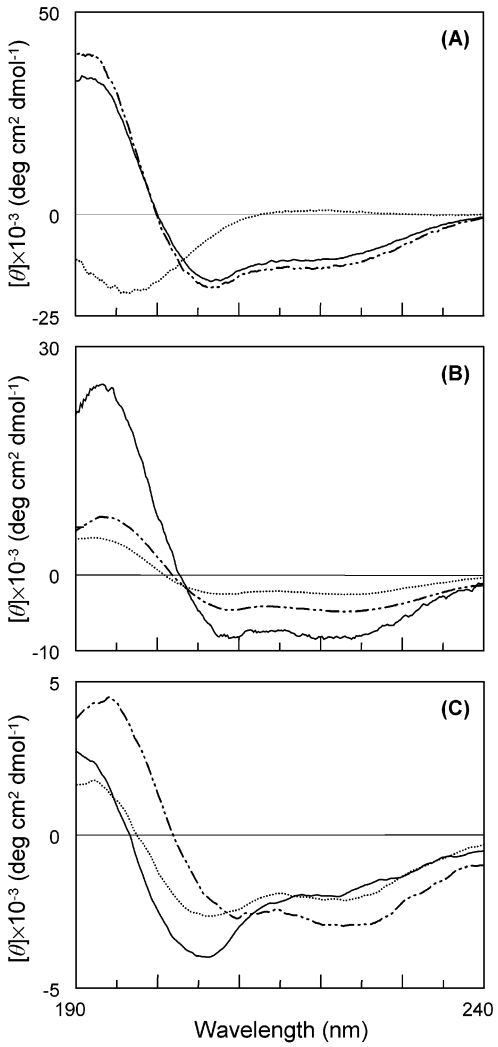


***Figure 5.*** CD spectra of peptide **7** (A) and the corresponding fullero-peptides **8** (B) and **9** (C) at $4 \times 10^{-5}$ M concentration, in aqueous 5 mM phosphate buffer (pH 7) (···) or in the presence of 50% TFE (−··−) or 10 mM SDS (−) in phosphate buffer.

of three reference microorganisms: the Gram-negative bacterium *E. coli*, the Gram-positive bacterium *S. aureus*, and the yeast *C. albicans*. Peptide **7**, also previously called P19(8), had already been extensively characterized[12a] and shows quite a high activity toward the Gram-negative bacterium and yeast but has a lower activity toward the Gram-positive microorganism. It was also found to have a relatively low cytotoxic activity[12b] as indicated by the low hemolytic activity (Table 2). The nonamphipathic peptides based on an alternating $(Gly\text{-}Orn)_n$ sequence, where $n = 2$, 4, or 6, were instead found to be devoid of antimicrobial or hemolytic activity.[11]

Introduction of Fgu **1** at the N-terminus of **7**, leading to the fullero-peptide **8**, has a remarkable effect on both the antimicrobial and the hemolytic activity. The former activity is considerably reduced for *E. coli* and *C. albicans*, whereas it is increased for *S. aureus*. A similar behavior was observed for **9**, where the fullerene-based residue lies in the middle of the sequence. The intrinsic antimicrobial activity of the $C_{60}$ moiety was confirmed with fullero-peptide **10**, in which it was inserted in the middle of a $(Gly\text{-}Orn\text{-}Gly)_2$ peptide. Also in this case, the highest activity was observed with the Gram-positive bacterium. The presence of $C_{60}$ also appeared to greatly increase

the ability of the peptides to lyse red blood cells. This was particularly evident for peptide **7**, which due to its structural characteristics[12b] has a low hemolytic activity, whereas it is quite high for the fullero-peptide **8**. The peptide and fullerene moieties seem to collaborate in this respect; as for the smaller fullero-peptide **10**, the hemolytic activity was lower. This may be in part due to a greater tendency of this peptide to aggregate, and in fact it precipitates at higher concentrations, which prevented the determination of hemolysis at 100 $\mu$M. For the fullero-peptide **8**, the presence of the $C_{60}$ moiety may also promote aggregation, which could have important implications for its biological activity.

Circular dichroism (CD) analysis of peptide **7** (Figure 5A) indicates that this derivative is unstructured in aqueous solution and assumes a partly helical structure (~40% helical content) in the presence of the helix-promoting solvent trifluoroethanol, or sodiumdodecyl sulfate micelles, a simple model for biological membranes. This behavior is typical of amphipathic α-helical antimicrobial peptides.[12b] The shape of the CD spectra for fullero-peptides **8** and **9** instead indicates a significant degree of helix formation already in aqueous solution (Figure 5B and C), although the intensity of the band at 222 nm is low with respect to that shown by peptide **7**, which could be an indication that the peptides aggregate to form helical bundles. Unlike peptide **7**, the spectra for both fullero-peptides **8** and **9** change more markedly in the presence of SDS micelles than of TFE (Figure 5B and C). The shape of the spectrum for fullero-peptide **9**, in particular, changes in a manner consistent with the presence of an extended conformation. In both cases, a strong interaction with the lipid-like environment is indicated.

A possible explanation for the antimicrobial behavior of fullero-peptides **8** and **9** could be that the $C_{60}$ moiety and/or the increased aggregation that results from its presence reduces the capacity of the peptide to penetrate the LPS-rich outer membrane of the Gram-negative bacterium, or the outer surface of the yeast cell, and thus to reach the cytoplasmic membrane. On the contrary, it appears to promote interaction with the cytoplasmic membrane of the Gram-positive bacterium, as well as the membrane of erythrocytes, leading to increased lysis. Work is in progress to better elucidate membrane permeabilization in all of these contexts.

## Conclusion

We have successfully performed the solid-phase synthesis of a series of fullero-peptides containing the new Fgu amino acid **1** and based on Leu[5]-Enkephalin and on cationic antimicrobial peptides. A suitable protocol of coupling and deprotection was optimized to avoid side reactions due to the reactivity of the fullerene moiety against bases and nucleophiles. All types of resins have shown a strong tendency to retain the fullerene-based peptides, a phenomenon that created serious problems during the removal step. In this respect, the position of the Fgu residue inside the peptide sequence appears to be critical. The recovery of the material decreases dramatically as the fullerene approaches the solid support. The fullero-amino acid also influences the swelling properties of the resin probably due to a certain degree of aggregation which decreases the mobility of the linked molecule. However, the recovered fullero-peptides could be easily purified and tested for their biological activity. They displayed a high and specific activity against Gram-positive bacteria and therefore become interesting lead molecules for the discovery of new antiinfective agents. Moreover, SPPS of [60]fullerene-based peptides can now be extended to other classes of biologically relevant peptides.

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**Supporting Information Available:** Experimental section including abbreviations, instrumentation, analytical characterization, biological assays, and experimental details for the synthesis of peptides **2**, **3**, **6**−**10** and for the treatment of fullerene derivative **11** with bases (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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