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A Novel [60]Fullerene Amino Acid for Use in Solid-Phase Peptide Synthesis

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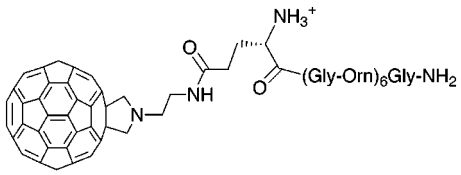
ABSTRACT



A fullerene derivative containing a free amino group has been condensed with *N*-Fmoc-L-glutamic acid α-*tert*-butyl ester to give a C_{60}-functionalized amino acid. The carboxylic end of this amino acid has been deprotected in acidic conditions, and the resulting acid has been used for solid-phase peptide synthesis. The final peptide, cleaved from the resin, was very soluble in water solutions and showed antimicrobial activity against two representative bacteria.

Considerable effort in fullerene chemistry has been directed to establishing this novel form of carbon as a standard building block in organic synthesis.[1−3] This task has proven not to be trivial, since C_{60}, the most popular representative of the fullerene family, is reactive under a variety of conditions, causing many standard reactions required for further derivatization to be inapplicable. For instance, C_{60} reacts readily with nucleophiles and also reacts under hydrogenation conditions, which represents a major obstacle for its use in chemical processes such as peptide synthesis. This notwithstanding, the exceptionally hydrophobic nature and spheroidal shape of C_{60} make it a very interesting pharmacophore in biologically active molecules, favoring hydrophobic interactions with receptors or membranes.[4−6] Thus, incorporation of the C_{60} moiety into some biologically active peptides might be desirable. Because of the above-mentioned problems, however, only a few examples of fullerene-modified peptides are known, mostly prepared under conditions of standard solution chemistry.[5,7−9] In particular, no examples have been reported to date of the potentially more facile solid-phase synthesis of fulleropeptides.

Here, we report the synthesis of a protected, fullerene-functionalized amino acid and its use in solid-phase synthesis for the preparation of a water-soluble peptide. This compound, which combines the lipophilic properties of C_{60} with the water solubility and the electrostatic attraction to membranes conferred by the small basic peptide, was found to acquire remarkable antimicrobial properties.

The synthesis of α-amino acid **1** was performed according to Schemes 1 and 2. 1,3-Dipolar cycloaddition of the azomethine ylide generated by condensation of the known

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(1) Hirsch, A. *The Chemistry of the Fullerenes*; Thieme: Stuttgart, 1994.
(2) *Fullerenes and Related Structures*; Hirsch, A., Ed.; Springer: Berlin, 1999; Vol. 199.
(3) *The Chemistry of Fullerenes*; Taylor, R., Ed.; World Scientific: Singapore, 1995.
(4) Jensen, A. W.; Wilson, S. R.; Schuster, D. I. *Bioorg. Med. Chem.* **1996**, *4*, 767−779.
(5) Da Ros, T.; Prato, M. *Chem. Commun.* **1999**, 663−669.
(6) Wilson, S. R. In *The Fullerene Handbook*; Kadish, K., Ruoff, R., Eds.; Wiley: New York, 2000; pp 437−465.
(7) Prato, M.; Bianco, A.; Maggini, M.; Scorrano, G.; Toniolo, C.; Wudl, F. *J. Org. Chem.* **1993**, *58*, 5578−5580.
(8) Toniolo, C.; Bianco, A.; Maggini, M.; Scorrano, G.; Prato, M.; Marastoni, M.; Tomatis, R.; Spisani, S.; Palù, G.; Blair, E. D. *J. Med. Chem.* **1994**, *37*, 4558−4562.
(9) Bianco, A.; Da Ros, T.; Prato, M.; Toniolo, C. *J. Pept. Sci.* **2001**, *7*, 208−219.

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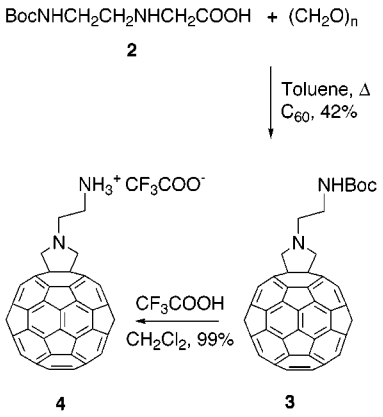
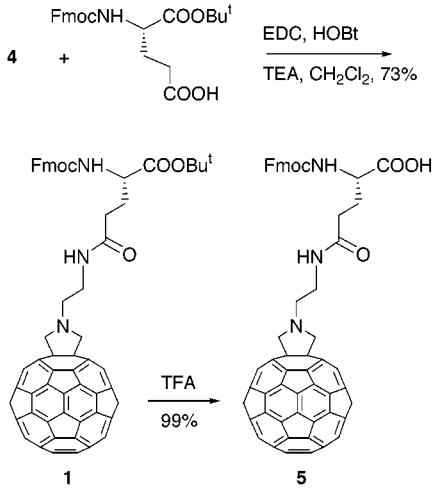
N-substituted amino acid **2**[10] with formaldehyde to C_{60} led to good yields of Boc-protected aminoalkyl fulleropyrrolidine **3** (Scheme 1).[11,12]

Scheme 1



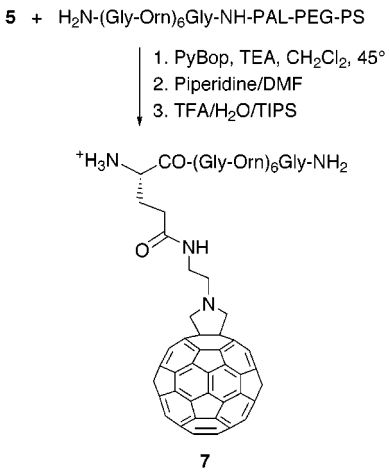
Deprotection of **3** was achieved with excess trifluoroacetic acid. The resulting ammonium salt **4** was suspended in dichloromethane and condensed with *N*-Fmoc-L-glutamic acid α-*tert*-butyl ester in the presence of EDC, HOBt, and triethylamine (TEA), to give the protected fulleroamino acid **1** (Scheme 2). This compound has the amino and the

Scheme 2



carboxylic acid functionalities protected with groups that are removable under orthogonal conditions, providing a suitable building block for solid-phase peptide synthesis. The acid function was liberated in the presence of excess TFA, leading to **5** (Scheme 2). We have designed a fulleropeptide in which **5** is conjugated with the highly cationic peptide H-(Gly-Orn)$_6$-Gly-NH$_2$ (**6**) to give a water-soluble fulleropeptide (**7**, Scheme 3). The key step in this process, namely the

Scheme 3



condensation of **5** with **6**, was performed through solid-phase synthesis with fully protected **6** attached to the resin. This avoided any problem related to the different solubility properties of the two partners. Peptide **7** is likely to interact with anionic bacterial membranes but lacks the amphipathic conformation required for antimicrobial activity per se. In this case, the peptide should only act as carrier, while any biological activity should derive from the fullerene moiety. A possible structure of this new peptide was computer-generated as shown in Figure 1. No attempt was made to minimize the structure, its only purpose being to indicate the relative size of the hydrophobic fullerene with respect to the hydrophilic peptide moiety.

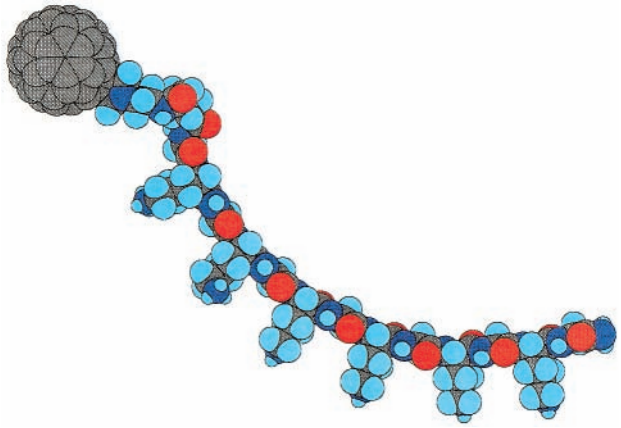


Figure 1. Computer-generated structure of peptide **7** in extended conformation, using Spartam program running on a Silicon Graphics workstation.

(10) Muller, D.; Zeltser, I.; Bitan, G.; Gilon, C. *J. Org. Chem.* **1997**, *62*, 411−416.

(11) Maggini, M.; Scorrano, G.; Prato, M. *J. Am. Chem. Soc.* **1993**, *115*, 9798−9799.

(12) Prato, M.; Maggini, M. *Acc. Chem. Res.* **1998**, *31*, 519−526.

Solid-phase synthesis of **6** was carried out using standard conditions, on a PAL-PEG-PS resin (loading 0.15 mmol/g), using a PE Pioneer automated synthesizer.

For each coupling, a 6-fold excess was used of amino acid, TBTU, and DIPEA in 1:1:1.7 ratio, respectively. Fmoc deprotection was performed using 20% piperidine in DMF at each cycle. UV−vis analysis of the FMOC cleavage byproduct dibenzofulvene indicated very high yields (>90%) for each step. After a final Fmoc deprotection, part of the resin-supported peptide was cleaved for the production of a reference peptide, which was purified by reverse phase HPLC. The correct structure was confirmed by Electrospray Mass Spectrometry (ES-MS, calculated MW = 1101.3 Da, measured MW = 1102.0). The rest of the resin was used for the coupling with fullerene-functionalized glutamate (**5**). To spare material, the excess amount of fullerene amino acid used was limited to three times that of the reactant. In this case, PyBop was preferred as the condensing agent, since it has a longer lifetime under these reaction conditions, and the reaction was carried out at 45 °C.

The Fmoc group from the N-terminal, fullerene-functionalized glutamic acid residue was then removed, using piperidine (20% in DMF, yield >80% based on dibenzofulvene analysis). Cleavage from the resin was achieved by employing TFA/H_2O/TIPS 95:2.5:2.5, for 2 h at room temperature. Peptide **7** was then precipitated and washed several times with *tert*-butyl methyl ether to eliminate any trace of TFA.

Peptide **7** proved to be very soluble in water, and the crude product was found to be of acceptable purity. It was further purified by reverse phase HPLC using a Waters Symmetry C18 column, with 45% CH_3CN and 55% H_2O. Under these isocratic conditions, the elution time of **7** was 16.9 min. The UV−vis absorption spectrum, measured with a diode array detector during elution, indicated a low level of aggregation.[13]

The mass spectrum (ES-MS, H_2O) of **7**, reported in Figure 2, shows the presence of minor $(M + H)^+$ and $(M + 2H)^{2+}$ peaks and major peaks attributed to $(M + 3H)^{3+}$ and $(M + 4H)^{4+}$ and confirms the correct structure of the fulleropeptide (calcd M_w = 2019.1; measd MW = 2019.2).

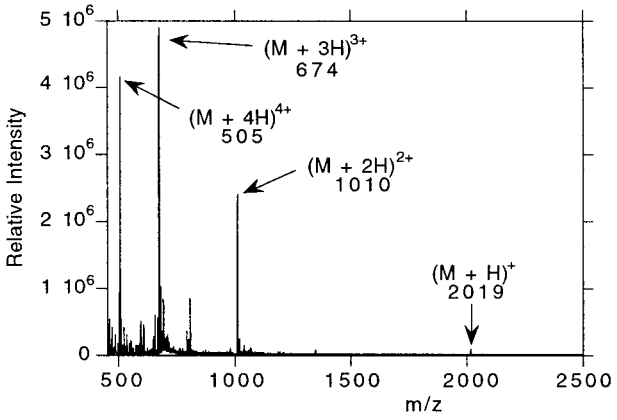


Figure 2. Electrospray mass spectrum of peptide **7** (H_2O).

The activity of this peptide as an antimicrobial agent was tested on two representative microorganisms, *S. aureus* 710A and *E. coli* ML-35, respectively, a Gram-positive and a Gram-negative bacterium, and compared to that of the unmodified peptide **6**. Minimum inhibitory concentrations (MIC) were determined by the microdilution susceptibility method, as described previously.[14] Briefly, serial 1:1 dilutions of each peptide in 50 mL of Mueller−Hinton (MH) broth were made in 96-well microtiter plates and 50 μL of 3×10^5 CFU/mL of microorganism suspensions were added to each well. Plates were incubated at 37 °C overnight and visually inspected to determine the MIC, corresponding to the first well not to exhibit growth.

As shown in Table 1, the nonfullerene peptide **6** was inactive on both microorganisms. Fullero-amino acid **5** is very insoluble, even if deprotected from the FMOC group, so that biological assays did not give meaningful results. Although **6** is highly cationic (seven positive charges), it lacks the hydrophobicity and amphipathicity required for activity in cationic antimicrobial peptides.[15] However, the attachment of the fullerene moiety to this highly hydrophilic carrier renders the fullero-peptide **7** quite active against the Gram-positive bacterium and somewhat less active against the Gram-negative species.

Table 1. Antimicrobial Activity of Peptides **6** and **7**, Reported as MIC (Minimum Inhibitory Concentrations). Results Are the Mean of at Least Four Independent Evaluations Run in Duplicate (na = nonactive)

peptide	**6**	**7**
S. aureus	na	8 μM
E. coli	na	64 μM

An explanation could be that the basic peptide guides the fullerene moiety down to the cytoplasmic membrane with which it can interact, leading to cellular inactivation. In this respect, the outer membrane of the Gram-negative bacterium appears to be a more efficient barrier to the peptide than the thick peptidoglycan layer of the Gram-positive microorganism. This might be due to interaction with the outer membrane lipopolysaccharide in the former case or to aggregation at this level due to locally high peptide concentrations.

These results are novel and promising, particularly in view of the fact that the fullerene-modified, Fmoc-protected amino acid **1** can, to all effects, be utilized as any other amino acid in peptide synthesis and inserted anywhere in the sequence. Its use is now being considered at intermediate positions of peptides analogous to **7**. Furthermore, we plan to include **1** in peptides that already have a potent activity per se, such as amphipathic α-helical antimicrobial peptides,[14,15] so as to modulate their hydrophobicity in a potentially useful manner.

(13) Guldi, D. M.; Prato, M. *Acc. Chem. Res.* **2000**, *33*, 695−703.
(14) Tossi, A.; Tarantino, C.; Romeo, D. *Eur. J. Biochem.* **1997**, *250*, 549−558.
(15) Tossi, A.; Sandri, L.; Giangaspero, A. *Biopolymers* **2000**, *55*, 1−30.

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Supporting Information Available: Full experimental data pertaining to the preparation of the new compounds **1**, **5**, **6**, and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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