Synthesis and Biological Properties of Fullerene-Containing Amino Acids and Peptides

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Abstract: Organofullerene derivatives have shown a great potential in a wide variety of biological activities such as DNA photocleavage, HIV-protease inhibition, neuroprotection and apoptosis. Among the plethora of functionalized organofullerenes that have been synthesized, fullerene-based amino acids are particularly appealing for structural studies and biological applications. When the fullerene-framework is incorporated into peptides, its original properties can be substantially modified. In addition, the water-solubility of the fullerene derivatives is enhanced, which makes such molecules amenable to biological studies.

In this review, recent advances in the growing field of medicinal chemistry of fullerene derivatives will be discussed. Emphasis will be given to the synthesis of the biggest unnatural amino acid 3,4-fulleroproline (Fpr) and its derivatives. For example, Fpr derivatives have been found to interact with different hydrolytic enzymes and selectively discriminate between rationally designed peptides. Fullerene-based peptides have been found to substantially activate enzymes involved in the oxidative deamination of biogenic amines. In addition, their membranotropic properties and effects on the structure and permeability of the lipid bilayer of phosphatidylcholine liposomes as well as the transmembrane transport of bivalent metal ions have been studied. Finally, applications in medicinal chemistry of such types of amino acids and peptides will be highlighted.

1. INTRODUCTION

The novel properties exhibited by the fullerenes envisioned innovative uses with diverse applications spanning from materials science to medicine and biotechnology [1-5]. Since fullerenes possess unique geometrical shapes as well as novel photophysical properties and are efficient radical scavengers, a wide variety of biological applications have been considered. Indeed, it has been demostrated that fullerenes display several biological activities such as i) inhibition towards various enzymes including HIV-protease and reverse transcriptase [6,7], ii) cytotoxicity against tumor cells [3] iii) neuroprotection and transfection [8-11] and iv) DNA cleavage under visible light irradiation [12-14]. However, fullerene derivatives cannot be considered as appropriate candidates for photodynamic therapy of solid tumours because they have no absorption in the red spectral region where the tissues present an optimum transmission [15-17]. Some other important biological effects induced by fullerenes include the *in vivo* reaction between C_{60} and vitamin A [18], the penetration of C_{60} inside a variety of cells without any acute toxicities [19] as well as the readily elimination through the kidneys of a highly water soluble fullerene [20]. However, a constant drawback related to fullerenes is their limited solubility in polar organic solvents or aqueous solutions. Fortunately, the chemical functionalization of fullerenes that can be achieved by covalent attachment of appendages on their globular framework has resulted in the preparation of soluble organofullerene compounds.

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In addition, to act as photoactive agents and in order to be selectively delivered to their biological targets, fullerenes must be conjugated with molecules possessing biological affinity to certain nucleic acids, proteins, cell types, organelles, etc. On the other hand, C_{60} itself could facilitate the interactions of certain biologically active molecules with lipophilic membranes of living cells and consequently improve cellular uptake due to its high hydrophobicity [21-23].

Herein, we will review the chemical functionalization of C_{60} that results in organofullerene compounds potentially useful in medicine and biology. Particularly, the methods of preparation and characterization of fulleropyrrolidine methanofullerene and cycloadded fullerene-based amino acids and peptides will be highlighted. Furthermore, results upon their biological and pharmaceutical assays will be also discussed.

2. CHEMICAL FUNCTIONALIZATION OF [60]FULLERENE: SYNTHESIS OF FULLERENE-CONTAINING AMINO ACIDS, PEPTIDES AND PROTEINS

The functionalization of fullerenes has provided access to a broad variety of derivatives that, while retaining the original fullerene properties, exhibit higher solubility and bear functionalities useful for new applications and further modifications.

2.1. Functionalization *via* 1,3-Dipolar Cycloaddition Reaction

One of the most widely used methods for functionalizing C_{60} is the 1,3-dipolar cycloaddition of azomethine ylides

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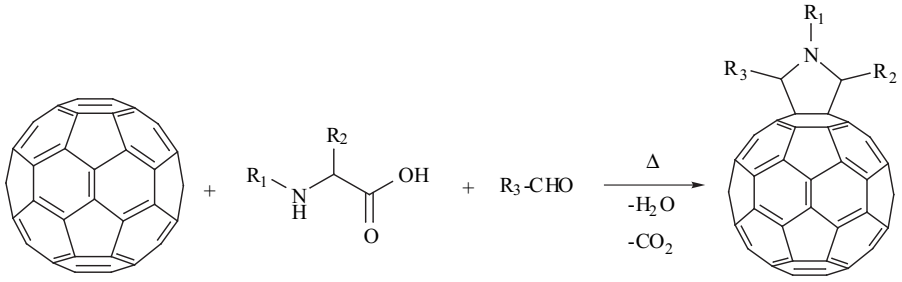


Scheme 1. 1,3-Dipolar cycloaddition of azomethine ylides to C_{60} *via* tautomerization of immonium salts derived from the condensation of α-amino acids with aldehydes.

[24-26]. This method is extremely versatile since both reactants necessary to generate the azomethine ylide intermediates, namely α-amino acid and an aldehyde or ketone, can be chosen with a wide variety of species so that any functional group may be introduced around the fullerene core.

For example, functionalized fullerene derivatives can be prepared by 1,3-dipolar cycloaddition of azomethine ylides to C_{60} *via* decarboxylation of immonium salts obtained from the condensation of α-amino acids with aldehydes, as shown in Scheme 1.

The main advantage of this type of reaction relies on the possibility of incorporating various solubilizing moieties at the nitrogen atom and/or at the C-2 positions of the pyrrolidine ring in one single step. For instance, using triethylene glycol monomethyl ether chain as a hydrophilic group, several fulleropyrrolidine derivatives were prepared that exhibited moderate solubility in a 9:1 water-dimethylsulfoxide solution [27] and were tested for their biological activity [28].

Some representative amino acid derivatives **1**-**3** [29,30] belonging to this new class of molecules are shown in Fig. **1**.

A special attention has been given to the synthesis and properties of fulleroproline (Fpr) the biggest unnatural amino acid [31] featuring a natural α-amino acid proline condensed to a (6,6) ring junction of C_{60} [32]. In addition, di- and tri-peptides have already been prepared by incorporating Fpr at their N- or C- terminal parts [33-35].

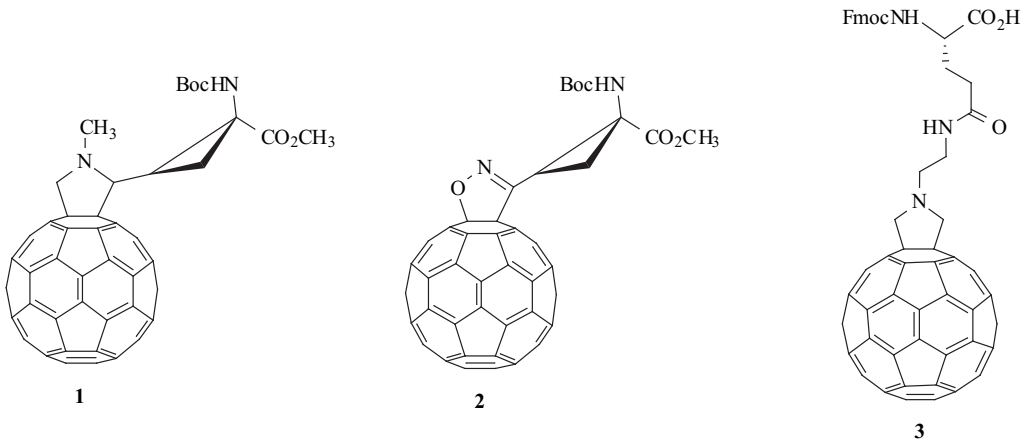
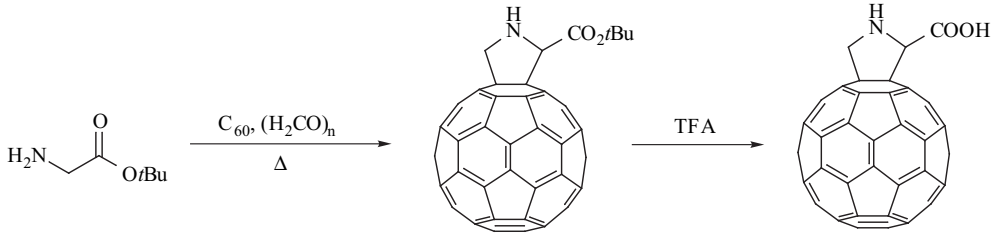


Fig. (1). Examples of fullerene-based amino acids.

Based on described 1,3-dipolar cycloaddition of azomethine ylides, when the amino acid component is replaced by its ester derivative, fulleroprolines are obtained. For example, when glycine *tert*-butyl ester is condensed with *para*formaldehyde in refluxing toluene, followed by isolation of the intermediate product and removal of *tert*-

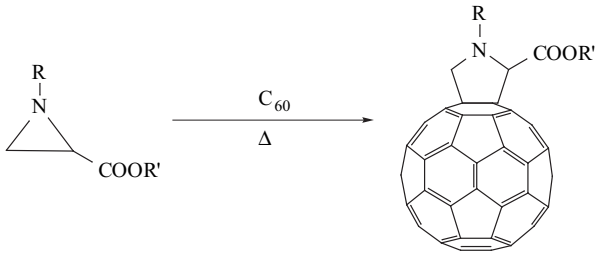


Scheme 2. Synthesis of Fulleroproline (Fpr).

butyl group under acidic conditions, the free fulleroproline (Fpr) is generated (Scheme 2) [24, 35].

This amino acid residue is insoluble in any kind of solvent. However, derivatization of the pyrrolidine nitrogen with either an acylating agent (*i.e.* acetic anhydride) or a protecting group (*i.e.* Fmoc fluorenylmethyloxycarbonyl or Boc, *tert*-butyloxycarbonyl) affords a compound that can be easily characterized and it is amenable to peptide synthesis.

Alternatively, fulleroprolines can be obtained *via* the thermal ring opening of aziridines [36], as shown in Scheme 3. In this case, the fulleroproline results orthogonally protected at the *N*- and *C*-termini.



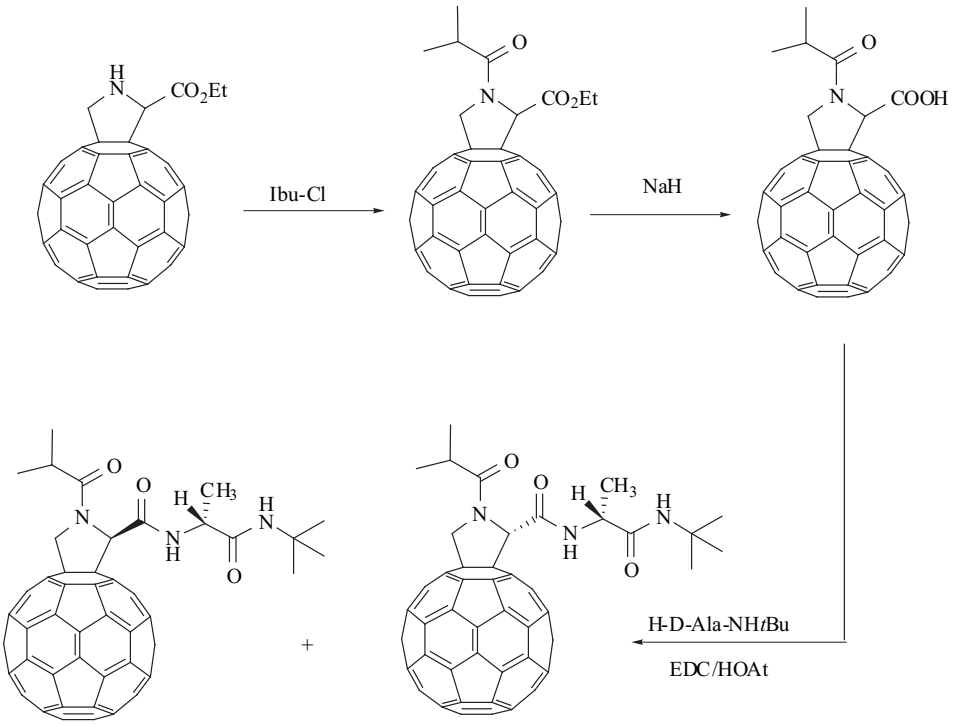
Scheme 3. Thermal ring opening of aziridines leading to orthogonally protected fulleroprolines.

Both methods described above generate a racemic center at the C^{α} atom of the pyrrolidine ring. Therefore, prior to the use of a suitably protected fulleroproline amino acid in peptide synthesis, the resolution of the enantiomeric mixture and the assignment of the configuration of the C^{α} chiral center were mandatory [33, 35]. For this purpose, the fulleroproline racemic mixture was separated by HPLC chiral chromatography and the two isomers characterized by circular dichroism (CD). A sharp absorption band at 428 nm, associated to the characteristic transition around 430 nm, present in the UV-Vis spectra of most monofunctionalized fullerene derivatives, has been used for the configurational assignment of the enantiomers. A positive Cotton effect at 428 nm was associated to the fulleroproline in configuration R, while the S enantiomer gives rise to a strong negative absorption band at the same wavelength. This behaviour has been also found for many other fulleroproline derivatives and enantiomerically pure fulleroproline-containing peptides [34].

A second possibility to solve the racemic fulleroproline derivatives consists in the preparation of diastereoisomeric compounds and their separation using chromatographic methods, including silica column chromatography and semi-preparative RP-HPLC. Similarly, the determination of the chirality of the pyrrolidine C^{α} was done by means of CD measurements and observation of the sign of the Cotton effect at 428 nm [33].

A fulleroproline residue bearing the C_{60} moiety fused to the 3,4-bond of the pyrrolidine ring, can be considered strictly related to its natural proline counterpart. Therefore, it was interesting to evaluate if Fpr presents the same characteristic properties of proline residue: i) the *cis-trans* isomerization equilibrium about the tertiary amide bond, and ii) the propensity to induce the formation of β-turn when inserted within a peptide backbone.

For the study of the *cis-trans* isomerization process in toluene and evaluation of the thermodynamic and kinetic parameters, Ac-Fpr-OMe derivative was prepared and compared with the molecule containing the proline residue [35]. A series of NMR experiments were acquired at increasing temperatures in order to calculate the different kinetic constant rates of the *cis-trans* dynamic process. The entropic and enthalpic activation values were extrapolated and corresponded to -4.4 cal·mol·deg^{-1} and 14.6 Kcal·mol^{-1}



Scheme 4. Synthesis of fulleroproline-containing dipeptides.

for Ac-Fpr-OMe and 6.5 cal·mol·deg^{-1} and 21.2 Kcal·mol^{-1} for Ac-Pro-OMe, respectively. The most remarkable difference is relative to the enthalpic parameter. The significant decrease of the enthalpy for the Fpr derivative is due to the lower availability of the nitrogen amide lone pair for the conjugation with the carbonyl oxygen. The fullerene moiety exerts on the pyrrolidine nitrogen an electron withdrawing effect, thus, reducing the double bond character of the C-N bond.

Proline plays an important role in peptide and protein folding. It is often present in the region where the amino acid sequence forms a turn or a loop. Inside small peptides, proline is able to display typical conformational preferences. In this context, fulleroproline containing di- and tripeptides were prepared as model compounds to evaluate the ability of this new synthetic amino acid to form β-turn structures and to compare its behavior with that of the proline within the cognate oligomers (Scheme 4). Infrared and NMR spectroscopic studies have allowed us to analyze the conformation adopted by the diastereoisomeric Ibu-L-Fpr-D-Ala-NH*t*Bu, Ibu-D-Fpr-D-Ala-NH*t*Bu and Ibu-L-Fpr-D-Ala-L-Ala-OMe (Ibu, isobutyryl; OMe, methoxy) peptides [34]. The heterochiral sequences are able to fold into a β-turn conformation of type II, stabilized by an intramolecular hydrogen bond between the carbonyl oxygen of isobutyryl group and the amide *tert*-butyl NH proton and the amide NH of L-Ala of the dipeptide and tripeptide, respectively. This propensity was also found for the same peptides where the fulleroproline was replaced by the natural residue (Pro). In view of these Fpr features, the design of Fpr-based peptides with predetermined structures and potential biological activity can be conceived.

Other fulleropyrrolidine-based amino acids were introduced within the sequence of several proteins and peptides. For example, a fulleropyrrolidine was functionalized with a maleimido group and bound to azurin **4**, a bacterial protein involved in the electron transfer within the denitrification chain [37]. Recently it also has been developed a protocol for the solid-phase synthesis of fullerene modified peptides using the fulleropyrrolidino-

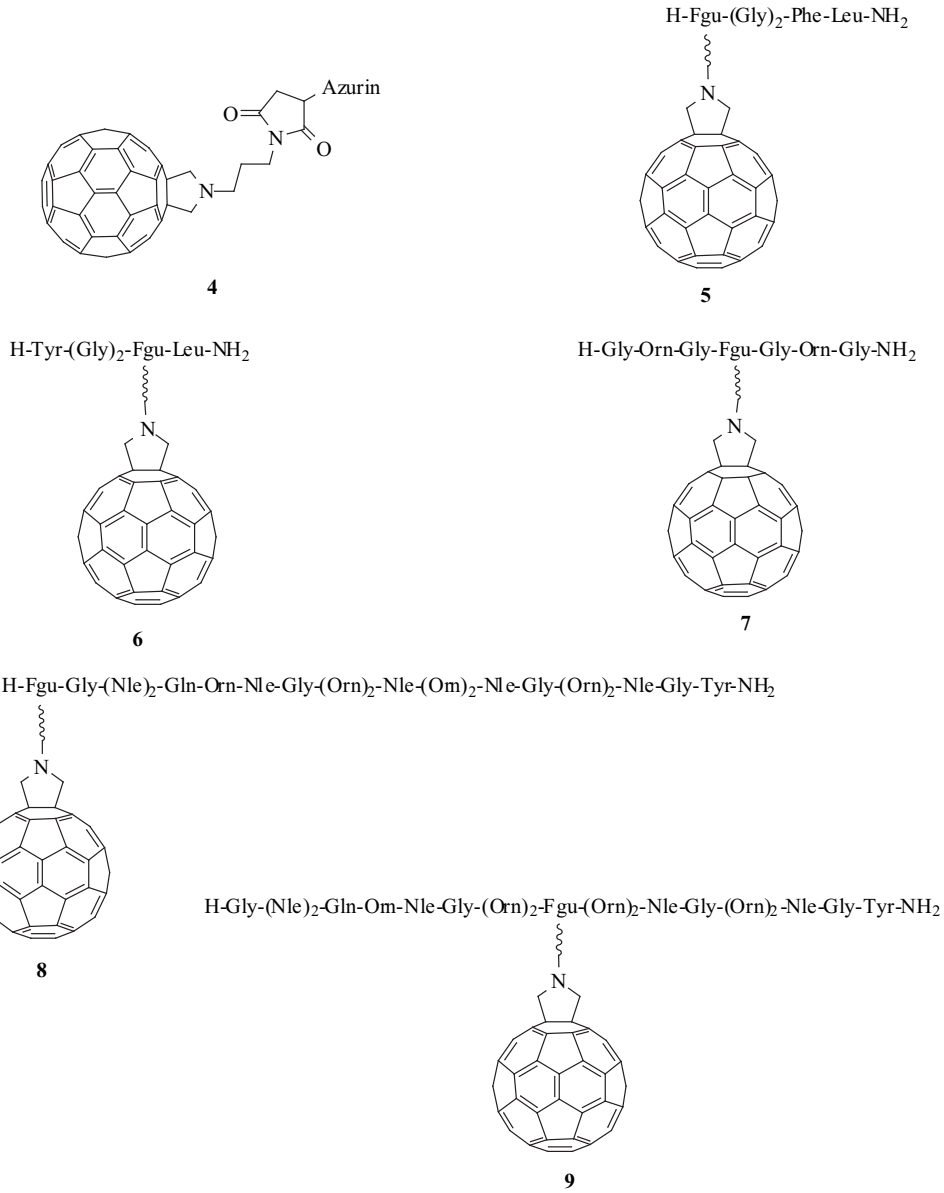
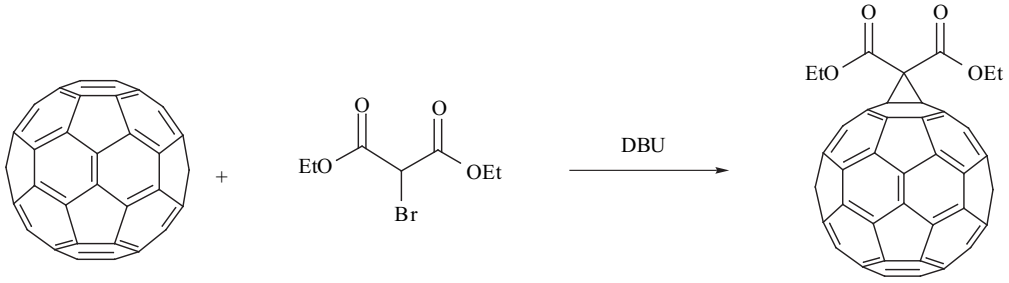


Fig. (2). Examples of fullerene-based peptides and proteins.

glutamic acid (Fgu) **3** (Fig. **1**), which has been inserted in a series of different peptides including Leu-enkephalins analogues **5**, **6** (Fig. **2**) and antimicrobial sequences **7**-**9** (Fig. **2**) [38]. Their structural and biological properties were also studied.

2.2. Functionalization *via* Cyclopropanation Reaction

Another interesting method for the synthesis of fullerene derivatives is the cyclopropanation reaction that has been extensively used for the functionalization of fullerenes. In principle, carbon nucleophiles are generated from α-halo esters and are subsequently added to C_{60} [39]. The addition takes place exclusively to the double bond between two six-membered rings of the fullerene skeleton, thus yielding the methanofullerene. As shown in Scheme 5, addition of diethyl-bromomalonate to C_{60} in the presence of an auxiliary base such as NaH or DBU (1,8-diazabicyclo[5,4,0]undecen-7-ene) leads to methanofullerene possessing two protected carboxylic groups attached to the bridging sp^3 carbon atom.



Scheme 5. Addition of diethyl-bromomalonate to C_{60}.

The novelty of this type of organic functionalization resides on the high number of different methanofullerene compounds that can be reached due to the presence of esters moieties. Either properly functionalized carbon nucleophiles can be conceived, synthesized and ultimately added to C_{60} or additional chemical transformation of the ester groups of the final methanofullerene can be envisaged. As a consequence, these chemical modifications permit to construct organofullerene hybrids endowed of novel biological and/or pharmaceutical properties.

Recently, novel strategies utilizing i) carbanionic precursors to methanofullerenes other than malonates [40] and ii) alternative pathways generating the reactive monohalomalonate intermediate *in situ* [41] have been developed. As a result, the potential of applications of such type of organic functionalization for designing and constructing new organofullerene compounds has been significantly increased [29].

A methanofullerene was also used to synthesize the first fulleropeptide **12** (Fig. **3**) [42]. This revealed the potential use of the C_{60} derivative as a real pharmacophore in medicinal chemistry. An *N*-terminal free pentapeptide with an alternating -Ala-Aib- sequence was covalently attached to the fullerene derivative through a linker based on a benzoic acid moiety. It was demonstrated that after the introduction of the methanofullerene derivative, the peptide still retains its capacity to adopt a typical 3_{10}-helical conformation. Moreover, it was found that when a slightly modified

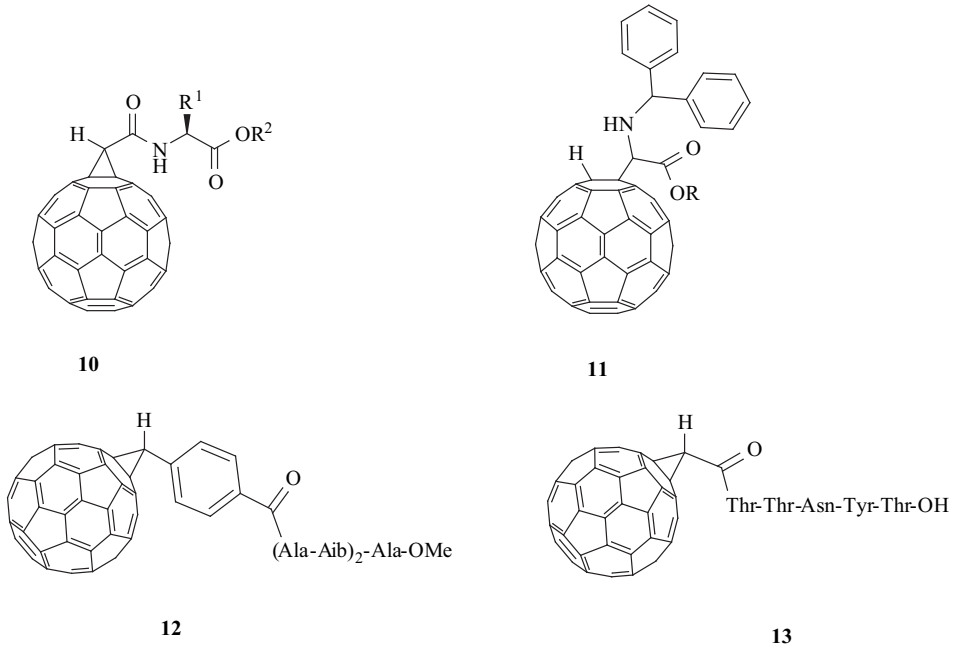


Fig. (3). Examples of fullerene-based amino acids and peptides.

methanofullerene amino acid **10** (Fig. **3**) was linked to pentapeptide T, the derived compound **13** displayed interesting biological activities [43]. In a step forward, more complex systems like proteins and long peptide were chosen for their conjugation to fullerene derivatives.

Finally, 1,2-dihydro[60]fullerylglycine **11** is another compound that can be derived from a modified methanofullerene via a novel cyclopropane ring opening reaction [44].

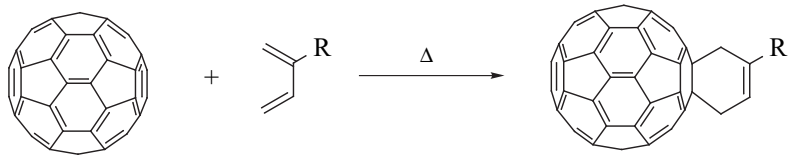
2.3. Functionalization *via* Diels-Alder Reaction and Direct Amination

Among other suitable ways for synthesizing stable and fairly soluble organofullerene compounds, cycloaddition reactions were explored. Fullerenes can function as 2π-electron deficient dienophiles and dipolarophiles thus allowing cycloaddition reactions that give different products depending on the reaction conditions. In this context, [3+2] and [4+2] are the most widely used reactions to form cycloadducts, where the diene always added to the (6,6) double bonds of the fullerene framework (Scheme 6).

Five-membered rings fused to (6,6) junctions are formed upon [3+2] cycloadditions to fullerenes. Several such systems have been prepared with the majority involving heterocyclic structures [45].

In the classical [4+2] Diels-Alder cycloaddition reaction, 1,3 dienes add to fullerenes to form cyclohexene rings fused to (6,6) bonds. Again, this type of cycloaddition offers the great advantage of controlling the degree as well as the site of addition [46]. Some representative amino acid derivatives **14**-**16** [47,48] belonging to this class of molecules are shown in (Fig. **4**).

The reactivity of primary and secondary amines with the fullerene moiety represents another easy method to obtain fullerene-based amino acids. Thus, free Gly, Ala and Ser have been added in equimolar ratio to C_{60} giving water-soluble glycyl-fullerene, alanyl-fullerene and seryl-fullerene **17** respectively [49].



Scheme 6. Diels-Alder reaction of C_{60} with a buta-1,3-dienes bearing a generic functional group.

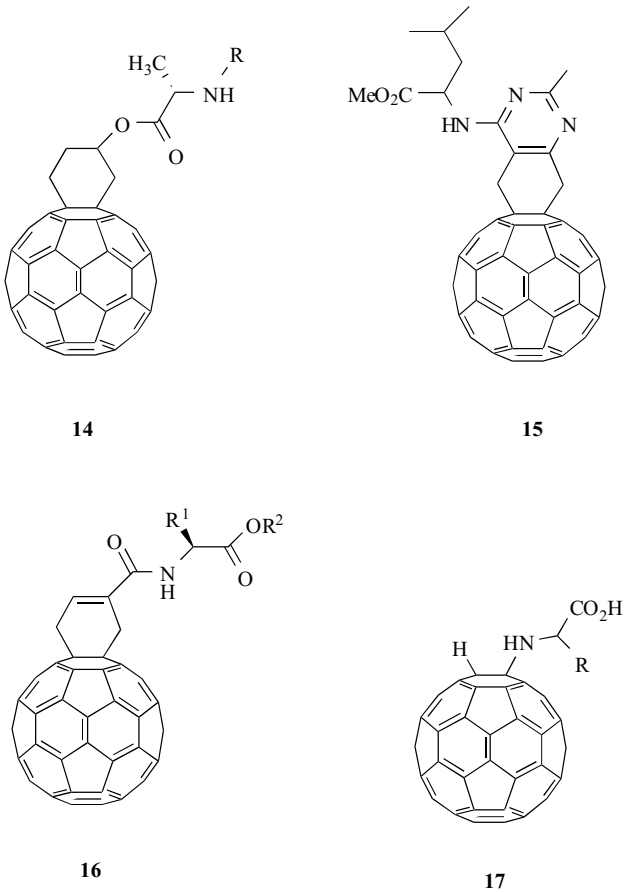


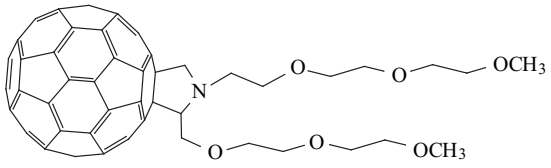
Fig. (4). Examples of fullerene-based amino acids.

3. BIOLOGICAL PROPERTIES

3.1. Antibacterial Properties

During the last decade the resistance of several kinds of bacteria to the strongest antibiotics induced chemists and microbiologists to produce more efficient antibacterial compounds. In this context, fullerene, with its uncommon symmetry and peculiar physico-chemical properties, was properly functionalized and tested against germs [28, 30, 38, 50, 51]. In preliminary tests, water soluble fulleropyrrolidine derivative **18** (Fig. **5**) was found to be active against a variety of microorganisms [28]. Different species of bacteria and fungal strains were killed in a slightly modified agar diffusion test. They included two strains of *Candida albicans* (clinical isolates CA1 and Z11), a fastidious pathogenic eukariote; the strain ATCC 6633 of *Bacillus subtilis*, a spore-forming, Gram positive bacterium; the strain AB1153 of *Escherichia coli*, a Gram negative enteric bacterium; the strain 261/6 of *Mycobacterium avium* clinically isolated, an acid fast emerging pathogen resistant to most antimicrobial drugs. In the latter case, 70% inhibition was observed with a concentration of 26 μg/ml, whereas complete inhibition was achieved with concentrations 10 times higher.

In another interesting work, an unexpected protection effect was observed when young and old mice were treated against the infective Gram negative Coccus *Neisseria Meningitidis*, with the two isomers of *tris*-malonyl carboxyfullerene **19** and **20** (Fig. **5**) possessing C_3 and D_3 molecular symmetry, respectively [51]. The same derivatives were also found active against *E.coli*.



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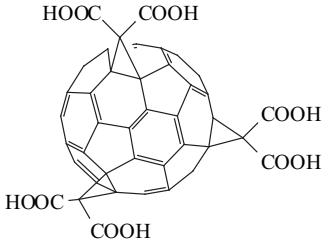

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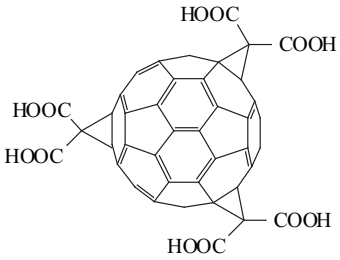

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Fig. (5). Examples of bioactive organofullerene derivatives.

It has been also reported that the natural peptide alamethicin, a well-known Aib-rich (Aib, α-aminoisobutyric acid) 20-mer able to fold into a typical helical structure and endowed of a good antibiotic activity, was coupled to a methanofullerene carboxylic acid activated as succinimmide ester [52]. The peptide was first prepared in solid-phase peptide cleaved from the resin full-protected and finally coupled in solution to the C_{60} derivative. The biological properties of the fulleropeptide cover the conductance modulation in the lipid bilayer by forming aggregates pores. Interestingly, it was found that C_{60}-alamethicine was able to protract the lifetime of the aggregate channel inside synthetic phospholididic micelles when compared to the corresponding natural peptide. This behavior was explained in terms of the high hydrophobicity of fullerene and of affinity of the peptide for the inner part of the bilayer membrane. This preliminary study highlights the potential application of such conjugates as antibacterial agents.

Very recently, the novel Fgu **3** (Fig. **1**) has been synthesized for use in solid phase peptide synthesis (SPPS) [30]. Briefly, a C_{60} derivative containing a free amino group was condensed with Fmoc-Glu-O*t*Bu to give the C_{60} functionalized amino acid **3**. The presence of the orthogonal protection guaranties the employment of such a building block in the SPPS using Fmoc/*t*Bu strategy. The easy and versatile synthesis of **3** opens the way to the general production of such fulleropeptides. Indeed, the SPPS synthesis of a fulleropeptide with an alternate sequence of glycine and ornithine possessing the Fgu at the *N*-terminal part was performed [30]. This peptide displayed an antibacterial activity against Gram positive and Gram negative, with MIC values ranging from 8 μM to 64 μM.

Generally, antimicrobial peptides are often highly cationic, well soluble in physiological media, able to interact strongly with and disrupt the biological membranes of the target microorganisms. The latter task is accomplished by the amphipatic nature of the peptide sequence. The hydrophobic residues are involved in the interaction with the lipid bilayer, while the polar and charged residues remain in contact with the membrane polar head-groups and external aqueous environment. Insertion of the highly hydrophobic C_{60} moiety into an antimicrobial peptide could further modulate its interaction with the membrane. A series of natural and rationaly designed peptides possessed a minimal in the micromolar range necessary to inhibit the growth (MIC) of three reference microorganisms: the Gram-negative bacterium *E. coli*, the Gram-positive bacterium *S. aureus*, and the yeast *C. albicans*. The introduction of the fullero-amino acid **3** at the *N*-terminus of the peptide, showed a remarkable effect on both the antimicrobial and the hemolytic activity [38]. The former activity is considerably reduced for *E. coli* and *C. albicans*, whereas it is increased for *S. aureus*. A similar behavior was observed when the fullerene-based residue lies in the middle of the sequence. A possible explanation for the antimicrobial activity of fullero-peptides is that the C_{60} moiety and/or the increased aggregation resulting from its presence reduce 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, hampering to reach the cytoplasmic membrane. On the contrary, the fullerene appears to promote interaction with the cytoplasmic membrane of the Gram-positive bacterium, as well as the membrane of erythrocytes, leading to increased lysis.

3.2. Membranotropic Properties

The interaction of fullerene derivatives with cellular and subcellular structures was shown in HS69 human fibroblasts and in monkey kidney cells, using compound **21** (Fig. **6**), a slightly water soluble carboxyfullerene [21]. Immunofluorescent microscopy revealed the intracellular distribution of the compound. Moreover, the measure of radioactivity of cells incubated with enriched ^{14}C fullerene derivative, showed a selective localization in proximity of every kind of intracellular membrane like the principal cellular membrane and the membrane of mitochondria [23] and microsomes [53]. The demonstration that the water soluble fullerene derivative used in these studies is able to

cross the cell membrane could be paralleled with the structural analogy between the fullerene cage and that of clathrin-coated vesicles. These structures play a fundamental role during the process of the endocytosis.

In another interesting application, a water soluble amino acid, namely a C_{60} derivative of DL-alanine (compound **17** of (Fig. **4**), with R = CH_3), and a C_{60}-DL-alanine-DL-alanine dipeptide were used to analyze the membranotropic properties of soluble fullerene derivatives [54]. Artificial phosphatidylcholine liposomes were utilized in order to reproduce a simplified model of cell membrane and studied using the luminescence probe technique. Either the fullero-amino acid or the fullero-peptide displayed a strong fluorescence quenching. This feature was used to monitor the membrane affinity of C_{60} derivatives in presence of kindred membrane fluorescent probes. Both compounds were able to penetrate inside the lipid bilayer without changing its structure and inside the liposome without any membrane destruction. Moreover, it was shown that these derivatives were able to perform activated trasmembrane transport of bivalent metal ions.

All these *in vitro* experiments are not completely in agreement with pharmacokinetic results obtained *in vivo* [22]. After intravenous administration of MSAD-C_{60} (**22**) [MSAD, bis-monosuccinimmide-p,p'-bis(2-aminoethyl)di-phenyl] (Fig. **6**), a complete biopharmaceutical study was carried out. A very low value of renal clearance, a high plasma protein binding (>99%) and a steady-state distribution volume, that was three-fold greater than the total body water content, suggested an extensive distribution of the fullerene drug within the tissues. Thus, MSAD-C_{60} possessed higher affinity for the tissues than for the plasma proteins and this was probably due to the its tendency to interact with membranes or in general lipidic structures.

3.3. Enzymatic Interaction

In medicinal chemistry, most of the targets of drugs are enzymes that normally catalyse a very large number of endogenous transformations. Sometimes enzymes are the main cause of the symptoms of certain diseases and for this reason it is necessary to modulate their activity by inhibition or activation.

In addition to photochemical behavior, C_{60} is endowed of unique physico-chemical characteristics including hydrophobicity, electrophilicity, and low reduction potential. The enzyme inhibition activity of fullerene derivatives is probably due to all these properties [2, 12, 55-57]. It has been largely reported the inhibition activity of several kinds of enzymes by numerous organofullerene derivatives, while the same activity exerted by C_{60}-based amino acids or peptides has remained little explored. We might envision that the peptidic structure is able to interact in a more flexible way with the active or allosteric site of an enzyme and thus inhibit or activate it.

Interstingly, an amino acid derivative linked to the fullerene core was demonstrated to be a potent inhibitor of the nitric oxide inducible synthase [58]. Its mechanism of action is explained in terms of interference with the enzymatic electron transfer. It was also reported that *tris*-malonyl-C_{60} derivatives were found excellent free radical scavengers in *in vitro* and *in vivo* experiments [8]. Moreover, compounds **19** and **20** (Fig. **5**) are able to inhibit the endogenous production of nitric oxide synthesized by constitutive and inducible NO synthase [57]. The effect was attributed to interactions of fullerene derivatives with the enzyme and not to their properties as radical sponges [59]. As NOS inhibitors, a significant accumulation of data suggest that there is a very interesting and sensitive structure-function relationship that affects potency, isoform

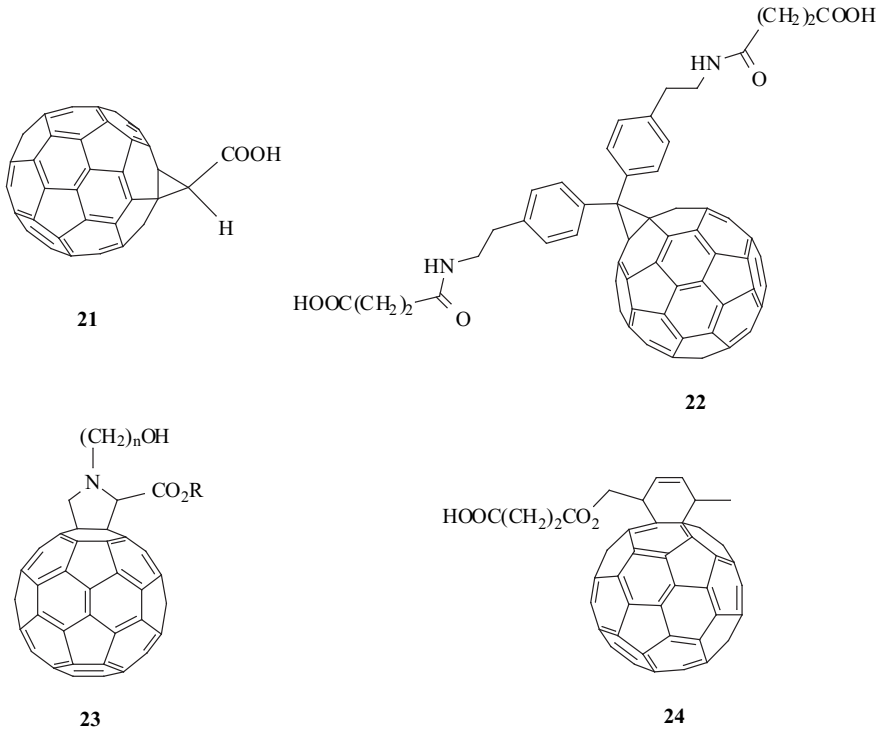

Fig. (6). Examples of bioactive organofullerene derivatives.

selectivity and even the pharmacological mechanism of action of the fullerene derivatives [59]. This points out to a specific interaction of fullerene molecules with the enzyme, and it is almost impossible to understand or explain if the inhibition would be exerted by large aggregates [60].

Fulleroproline **23** (Fig. **6**) can interact as an active substrate with lipase B from *Candida antartica* and with lipoprotein lipase from *Pseudomona Aeruginosa* [62]. Relatively fast reactions and modest enantioselectivities (up to 45% ee) were observed for the enzymatic activity on the chiral centers spatially distant from the carbon sphere. These results seem also to indicate that the C_{60} encumbers the fitting of the substrate either in the acyl- or alcohol binding sites when the reaction center is close to the fullerene spheroid. This may be due to its size, as C_{60} derivatives cannot be easily accommodated inside the lipase active site. However, it is reasonable to assume that C_{60} is able to establish hydrophobic interactions with more superficial regions of the enzyme, while the proline moiety approaches the active site cavity of the lipase and the hydroxy group interacts with the catalytic triad [61].

The active site of the HIV-1 protease (HIVP) is a quasi spherical hydrophobic cavity, with a diameter of about 10 Å. On its surface, two amino acid residues, (Asp^{25} and Asp^{125}) are responsible of the catalysis process. On the basis of molecular modeling [55], it was found that C_{60} can be almost perfectly accommodated inside the HIVP hydrophobic site. If the interactions are sufficiently strong, inhibition of the catalytic activity of the protease is expected. *In vitro* studies, performed using the water soluble fullerene derivative **22** (Fig. **6**), confirmed that inhibition of acutely and chronically affected peripheral blood mononuclear cells (PBMC) indeed occurred with an EC_{50} of 7 μM. On this bases of these results, the first synthesized water soluble fullero peptide **13** (Fig. **3**) was tested *in vitro* as HIV-1 protease inhibitor and an inhibition activity, although weak (IC_{50} of 100 μM) was observed [43].

3.4. Immunological Properties

The technique of vaccination developed over 200 years ago has played a major role in the control and eradication of viral diseases both in man and in animals. Vaccines could be constituted of a whole attenuated microorganism or of an epitopic part of it. At the moment, the immunologists are interested to find the best way to induce the highest B-cell response implicated in the production of precious antibodies. In this context, fullerenes due to their particularly polygonal structure and composition of solely carbon atoms, were considered as potential antigenic compounds.

A series of conjugate fullerene-proteins and fullerene-peptides were prepared in order to immunize animals and to induce the generation of antibodies [62]. Derivative **24** (Fig. **6**) was coupled to bovine thyroglobulin (TG) and to bovine and rabbit serum albumin (BSA and RSA) by activating the carboxylic group with *N*-hydroxysuccinimmide. In both cases the proteins were substituted with about ten molecules of the fullerene derivative per molecule of protein. Two, fullero-peptide conjugates were also prepared by coupling derivative **24** to an L-Lys homotri- and homopentapeptide, respectively.

After the immunization of BALB/c mice *via* intraperitoneal administration, ELISA assays were performed to detect the presence of specific antibodies against fullerene. Therefore, the presence of fullerene did not prevent intracellular processing and subsequent peptide presentation to T cells [62].

Finally, the structure of Fab fragment of the anti-C_{60} monoclonal antibody (IgG) was characterized by X-ray diffraction [63]. It has been found that in the crystal structure it is present a highly hydrophobic fullerene recognition region constituted of Leu, Ile, Tyr, Trp, Pro, Phe and Ala residues. The recognition process involves several parameters such as the hydrophobicity of the fullerene skeleton, its curvature and the possibility to have π-π stacking interactions. Moreover an induced fit mechanism was hypothesized in the fullerene binding process [63]. In conclusion, the production of specific C_{60}-antibodies and therefore their immunogenicity would enhance the use of fullerenes in vaccine delivery and/or in the design of immune modulators and adjuvants.

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