Journal of Peptide Science
J. Peptide Sci. **7**: 208–219 (2001)
DOI: 10.1002/psc.313

REVIEW

Fullerene-based Amino Acids and Peptides

ALBERTO BIANCO[a,*], TATIANA DA ROS[b], MAURIZIO PRATO[b,1] and CLAUDIO TONIOLO[c]

[a] Laboratoire de Chimie Immunologique, IBMC, UPR 9021 CNRS, Strasbourg, France
[b] Dipartimento di Scienze Farmaceutiche, Università di Trieste, Trieste, Italy
[c] Centro di Studio sui Biopolimeri, CNR, Dipartimento di Chimica Organica, Università di Padova, Padova, Italy

Received 2 November 2000
Accepted 4 November 2000

Abstract: Recent advances in the chemistry of fullerene have allowed the synthesis of many classes of novel fullerene derivatives. Among these classes, fullerene-based amino acids and peptides are particularly interesting, both for structural studies and biological applications. In this review, we will discuss our own achievements in this rapidly growing field. In particular, the application of fulleroproline (Fpr) amino acids and peptides to medicinal chemistry and materials science will be highlighted. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: fulleroamino acids; fulleropeptides; fulleroproline

INTRODUCTION

Fullerenes are made exclusively of carbon atoms, in which condensed hexagons and pentagons are arranged in a spherical form similar to a soccer ball [1]. Following their discovery in 1985 [2], it was to be 5 years before a bulky amount of material could be isolated [3]; this allowed the study of the chemical and physical properties of these new molecular forms of carbon [4–10]. Of the several fullerenes detected, the most abundant, C_{60}, has received the most attention in the scientific community. This is because C_{60} molecules display a wide range of interesting features, which include non-linear optical properties, superconductivity, as well as potential use in medicinal chemistry [11–17]. Within the field of biological applications, the neuroprotective, enzymatic, antiapoptotic, antibacterial, DNA photocleavage, nitric oxide synthase inhibition, and chemotactic activities of fullerenes and their derivatives have been studied [15–17]. A most interesting finding was reported early in 1993: based on computer graphics simulations, it was found that the hydrophobic cleft of HIV-1 protease can easily accommodate a C_{60} molecule [18–21]. This theoretical work was followed by the experimental demonstration that a water-soluble C_{60} derivative was able to inhibit the activity of the enzyme in the low μM range.

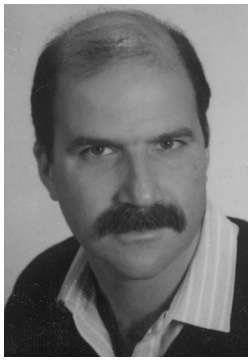
Their broad biological potential has stimulated the synthesis of many different classes of fullerene derivatives. The aim of this review is to analyze the contributions relating to the application of fullerenes, including fulleroamino acids and fulleropeptides, in the field of peptide chemistry. In particular, we will focus our attention on the methods of preparation and characterization of fulleroproline (Fpr)-based amino acids and peptides. Their utility in medicinal chemistry and material science will also be discussed. Finally, we will briefly describe the biological implications of some other interesting fullerene derivatives.

Abbreviations: Ac, acetyl; *t*Bu, *tert*-butyl; Bz, benzoyl; Bzl, benzyl; Et, ethyl; Fpr, fullero-3,4-proline; Ibu, isobutyroyl; Teg, triethylene glycol; TOAC, 2,2,6,6-tetramethylpiperidine 1-oxyl-4-amino-4-carboxylic acid.

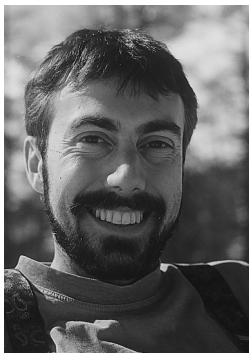
* Correspondence to: Institut de Biologie Moléculaire et Cellulaire, UPR 9021 CNRS, 15, rue René Descartes, 67084 Strasbourg Cedex, France; e-mail: A.Bianco@ibmc.u-strasbg.fr
[1] E-mail: prato@univ.trieste.it

BIOGRAPHIES

Claudio Toniolo obtained his Laurea Degree in Chemistry in 1965 from University of Padova, where he specialized in peptide synthesis. After obtaining the Libera Docenza in Pharmaceutical Chemistry and following a post-doctoral year with M. Goodman (Polytechnic Institute of Brooklyn) working on the conformation of peptides, he returned to the University of Padova, where he was promoted to full professor in 1980. His research interests mainly include synthesis, 3D-structure and application for chemistry, biochemistry and materials science of peptides based on non-coded amino acids.



Maurizio Prato graduated in Chemistry in Padova in 1978, where he joined the Faculty of Science in 1983. He moved to the University of Trieste in 1992, where he is currently Professor of Organic Chemistry. He spent sabbatical terms at Yale University in 1986–1987 and at the University of California, Santa Barbara in 1991–1992. He received the Federchimica Prize in 1995. His current research interests focus mainly on functionalized fullerenes for applications in materials science and medicinal chemistry.

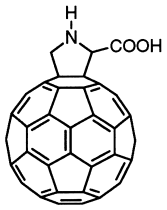


Alberto Bianco received his Laurea degree in Chemistry in 1992 and his PhD in 1995 from the University of Padova, working on fullerene-based amino acids and peptides. As a visiting scientist, he worked at the University of Lausanne (1992), at the University of Tübingen (1996–1997) and at the University of Padova (1997–1998). He is currently at IBMC in Strasbourg, where his research interests focus on the synthesis of pseudopeptides for application in immunotherapy, combinatorial chemistry and HRMAS NMR spectroscopy.

Tatiana Da Ros was born in 1969. She received her Laurea degree in Pharmaceutical Chemistry from the University of Trieste in 1995 working with Professor Marco De Amici (magna cum laude). She then took her PhD in 1999, under the supervision of Professor Maurizio Prato, her dissertation dealing with the biological properties of water-soluble fullerene derivatives. She has a research contract with the University of Trieste and is currently working in the Department of Pharmaceutical Sciences.

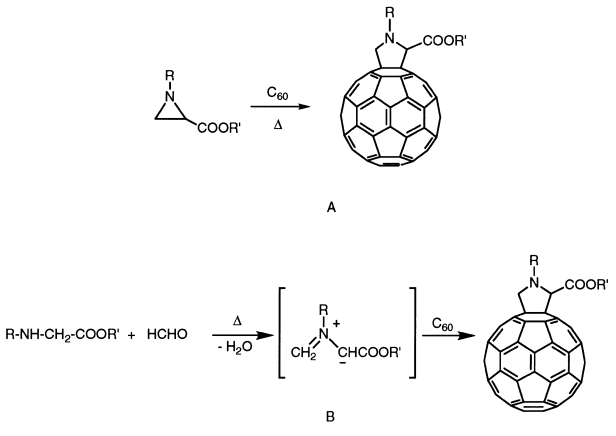
FULLEROPROLINE: SYNTHESIS AND PROPERTIES

Fpr (**1**) is the biggest unnatural amino acid [22] and the first example of an α-amino acid (Pro) condensed to a 6,6-ring junction of the C_{60} sphere (Structure 1) [23].



1

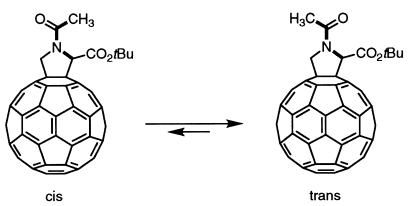
The preparation of Fpr derivatives is based on the 1,3-dipolar cycloaddition of azomethine ylides to C_{60} [24,25]. The reactive intermediates can be generated in different ways: (i) via the thermal ring opening of aziridines (Scheme 1(A)); and (ii) via the tautomerization of immonium salts derived from the condensation of esters of α-amino acids with aldehydes (Scheme 1(B)). One of the major difficulties in the manipulation and characterization of Fpr is its extremely low solubility when obtained as a free amino acid [23]. However, functionalization at the amino and/or carboxylic group leads to species that



Scheme 1 Synthesis of Fpr derivatives.

can be dissolved in the common organic solvents and spectroscopically characterized [23].

Fpr is strictly related to Pro, which is a conformationally restricted amino acid, and plays a central role in the folding of peptides and proteins [26,27]. In this context, we have studied both the *cis–trans* isomerism of Fpr derivatives and the propensity of the Fpr-containing peptides to induce β-turn conformations [28,29]. Ac-Fpr-O*t*Bu and its Pro analogue Ac-L-Pro-O*t*Bu were prepared and analyzed by NMR spectroscopy over a range of temperatures in toluene solution, in order to compare their equilibrium activation parameters for the *cis–trans* interconversion process about the tertiary amide bond (Scheme 2) [28]. These studies gave respective $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values of 21.2 kcal mol^{-1} and 6.5 cal mol^{-1} deg^{-1} for Ac-L-Pro-O*t*Bu and 14.6 kcal mol^{-1} and -4.4 cal mol^{-1} deg^{-1} for Ac-Fpr-O*t*Bu. Interestingly, while the entropic contribution was very small for both molecules, indicating a poor solvent rearrangement during the isomerization, the lower activation enthalpy of the Fpr derivative is related to a lower availability of the lone pair of the amide nitrogen for conjugation with the carbonyl oxygen. As a consequence, a higher rate constant of the *cis–trans* equilibrium at room temperature is displayed by Ac-Fpr-O*t*Bu. The fullerene moiety



Scheme 2 *Cis–trans* equilibrium about the tertiary amide bond of Ac-Fpr-*Ot*Bu.

seems to play an important role in the *cis–trans* interconversion process by withdrawing electron density from the nitrogen atom of the pyrrolidine ring, resulting in a lesser double-bond character of the C–N bond.

Fpr was conceived as a building block for the construction of peptides containing C_{60}. The method for the synthesis of Fpr is devised to only obtain this amino acid as a racemic mixture. Therefore, prior to peptide synthesis, the mixture of enantiomers must be submitted to optical resolution. Fpr in optically pure form can be obtained by separation of the racemic mixture using chiral HPLC (Figure 1) [30].

The CD spectra of the pure isomers exhibited very strong bands, which were attributed to the fullerene chromophore. In particular, in all CD spectra of Fpr derivatives, a sharp band at 428 nm was diagnostic for the attribution of chirality. A positive Cotton effect is indicative of the R configuration, while the negative peak is characteristic of the Fpr S isomer [30]. This conclusion was reached by the resolution of diastereomeric mixtures containing one known chiral center (*cf.* cyclic compounds in Figure 2). By structural correlation, the unknown configuration of the Fpr αCH carbon could be deduced.

Pro residues occur frequently in turns, which are generally defined as the sites where a peptide or a protein backbone reverses its direction [26,27]. These structures are involved in the bioactive sites of several natural peptides. The biological activity is sometimes mediated by the turn conformation. Peptide turns serve as models for turns in proteins, and also to evaluate the role they can play in the protein folding process. For a parallel comparison between the conformational features of Pros and Fprs, a

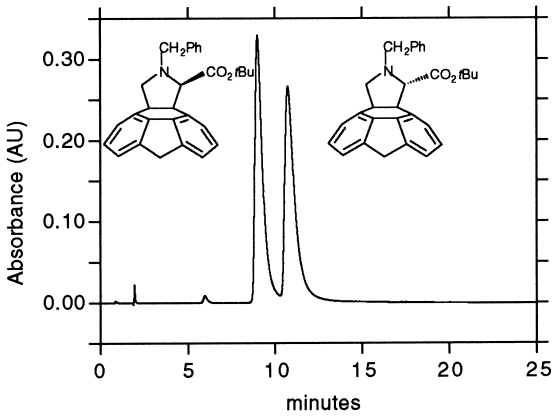


Figure 1 Chiral HPLC trace for the enantiomer resolution of a Fpr derivative.

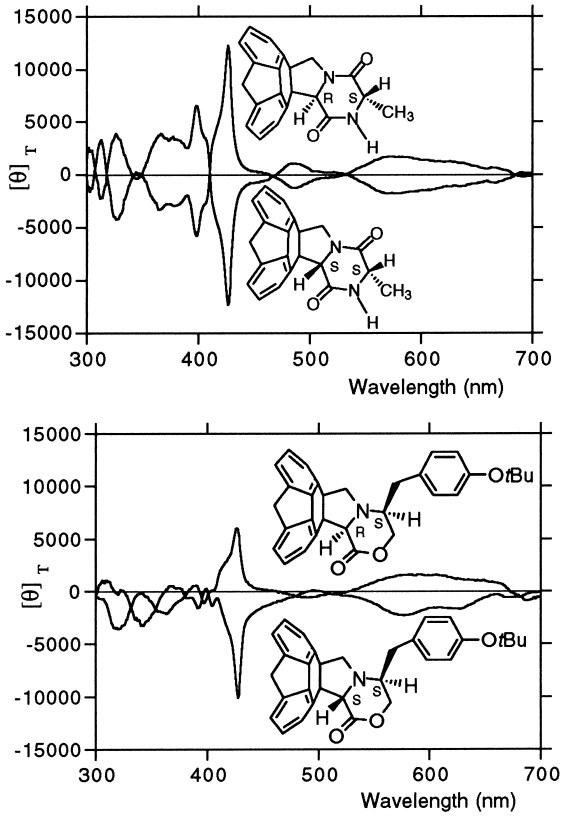
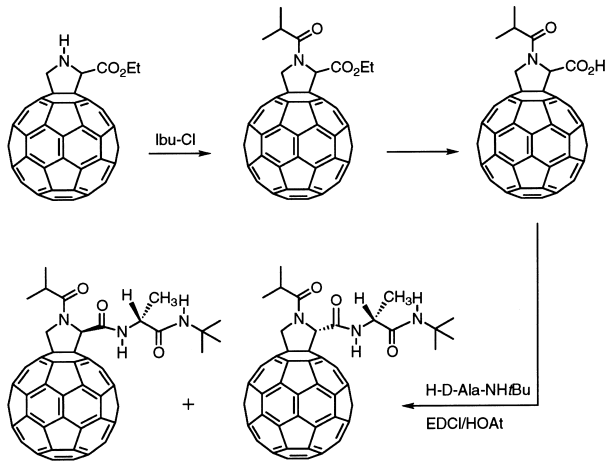


Figure 2 CD spectra of diastereoisomeric cyclic Fpr derivatives.

series of di- and tripeptides containing either the natural or the synthetic amino acid residue were prepared, with the aim to investigate their role as β-turn inducers and to evaluate their ability to form intramolecular H-bonds [29]. The folding structure of the -L-Fpr-D-Ala- sequence was examined in chloroform solutions by FT-IR absorption and ^{1}H-NMR, and compared with the known propensity of the cognate -L-Pro-D-Ala- to adopt a β-turn conformation of type II. The synthesis of diastereomeric isobutyroyl (Ibu)-L,D-Fpr-D-Ala-NH*t*Bu dipeptides was initially accomplished using the method of 1,3-dipolar cycloaddition of azomethine ylides derived from the condensation of H-Gly-OEt with *para*-formaldehyde. Following the separation and chiral assignment of the two diastereoisomers by CD (Scheme 3), it was found that the heterochiral dipeptide sequence is able to fold into a β-turn conformation, stabilized by a $1 \leftarrow 4$ intramolecular H-bond between the *tert*-butylamide N–H and Ibu C=O groups, in agreement with the behavior of the Pro-containing peptide [29].

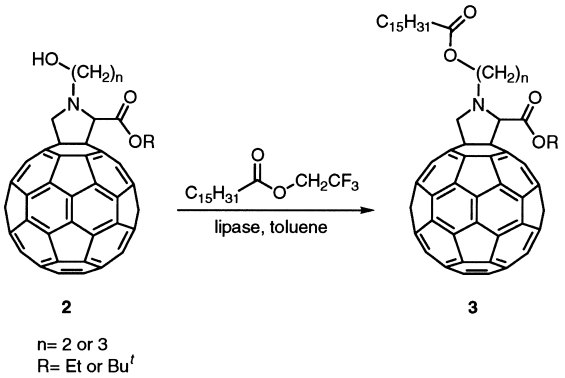
The tripeptide Ibu-L-Fpr-D-Ala-L-Ala-OMe was instead obtained by condensation of a suitable pro-



Scheme 3 Synthesis of diastereomeric (Ibu)-L,D-Fpr-D-Ala-NHtBu dipeptides.

tected aziridine to C_{60} and the subsequent coupling of the Fpr-activated carboxylic function to the *N*-terminal free H-D-Ala-L-Ala-OMe dipeptide. Again, this peptide tends to adopt a β-turn structure, which has been confirmed to be of type II by determination of the characteristic nOe spatial correlation between L-FprαCH and D-Ala NH resonances.

The interactions of Fprs with different hydrolytic enzymes have been reported [31]. Transesterification of Fprs **2** in toluene was successfully achieved using lipase B from *Candida antarctica* (CALB) or lipoprotein lipase from *Pseudomonas* species (LPL), with 2,2,2-trifluoroethyl palmitate as the acylating agent (Scheme 4). Although the experiments were carried out in an organic solvent, the enzymes were able to modify the structure of the fullerene derivatives, thus allowing in some cases the resolution of racemic mixtures and a better understanding of the structural requirements for the interaction with enzymes.



Scheme 4 Enzymatic transesterification of Fpr derivatives.

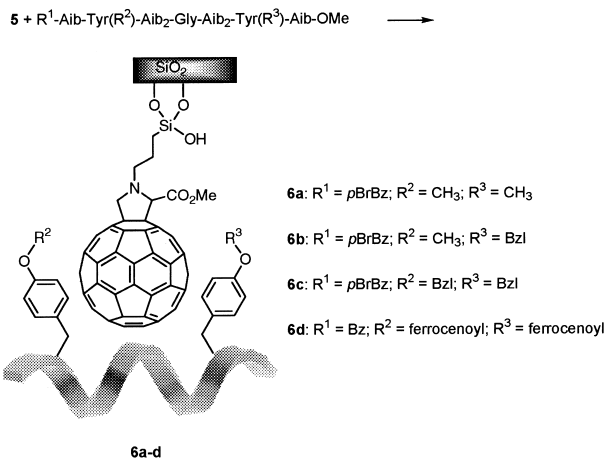
Based on all of the above results, it is feasible to design and prepare peptides containing the conformationally restricted Fpr residue, which may adopt predetermined ordered secondary structures. In addition, all of the fullerene properties can be transferred to its peptides.

FULLEROPROLINE DERIVATIVES: APPLICATIONS IN MATERIALS SCIENCE

An interesting application of Fpr derivatives is related to the preparation of a C_{60}-modified silica gel as a new stationary phase for HPLC column chromatography. A suitable aziridine, functionalized at N with a trialkoxysilane group, was synthesized and allowed to react with C_{60}, affording the silicon-based Fpr **4** (Scheme 5). Grafting of the fullerene derivative **4** to the silica matrix was easily achieved by simple heating in toluene [32].

The new chromatographic material can be used in either organic or aqueous solutions to investigate the binding affinities of potential hosts for the immobilized C_{60} core. In particular, the possibility of using this stationary phase to study the interaction of host–guest systems in aqueous solvents is very advantageous. Calixarenes and cyclodextrins of different size could be selectively separated using the novel fullerene-doped silica gel HPLC column.

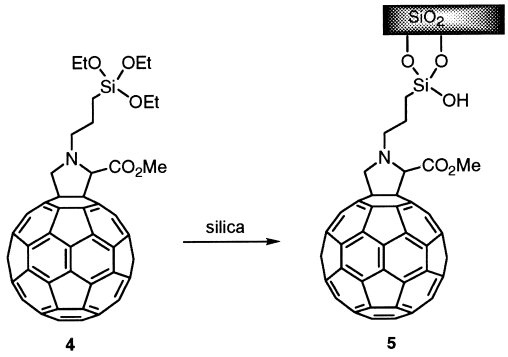
Moreover, selective recognition was demonstrated by analyzing the interaction of the grafted fullerene with a series of rationally designed peptides forming cavities. In particular, Aib-rich nonapeptides (**6a–d**), which fold into a regular 3_{10}-helix and are characterized by two host hydrophobic residues (the side chain substituted Tyr residues) separated by two helical turns, displayed chromatographic retention times that are related to the ability of the host side chains to generate a cleft for the accommodation of a C_{60} molecule (Scheme 6). The retention times were also related to the donor properties of the peptide side chains. It was indeed found that **6c** and **6d** gave the most effective interactions, with the latter being characterized by the strong ferrocene donor.



Scheme 6 Supramolecular complex between a silica grafted Fpr and a series of Aib-rich nonapeptides.

The fact that peptide **6d** possesses a high affinity for the fullerene spheroid was also demonstrated by photophysical measurements. Rapid quenching of the *N*-methylfulleropyrrolidine **7** excited singlet state induced by the nonapeptide Bz-Aib-Tyr(ferrocenoyl) - Aib_2 - Gly - Aib_2 - Tyr(ferrrocenoyl) - Aib - OMe (Figure 3) suggests strong host–guest interactions [33]. The discovery of such specific interactions may open the door to the possibility of studying binding interactions between hydrophobic fullerenes and more complex biological targets, like enzymes and receptors.

The fullerene-C_{60} exhibits a broad absorption spectrum in the ground state, covering almost all of the visible region. Excitation of the ground state



Scheme 5 Functionalization of silica gel using a trialkoxysilane Fpr derivative.

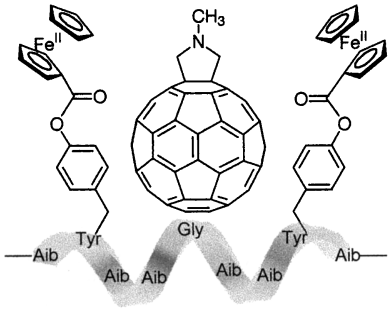


Figure 3 The complex formed by the ferrocene-containing nonapeptide **6d** and *N*-methylfulleropyrrolidine **7**.

generates a short-lived singlet excited state that converts almost quantitatively to a long-lived triplet, which absorbs light very strongly. As a matter of fact, in toluene solution, C_{60} shows a very efficient optical limiting behavior [34,35]. Fpr **4** with a triethoxysilane group (Scheme 5) has been used for the doping of glass films using the sol-gel process (acid-catalyzed polymerization of alkoxysilanes). This methodology allows not only the synthesis of bulk glasses but also that of thin films, which, in principle, can be spread over several different substrates for surface protection. The optical-limiting behavior of these hybrid inorganic–organic materials was studied at different wavelengths and proved to be very efficient, especially at 690 nm.

OTHER FULLEROAMINO ACIDS

Besides Fpr, some other synthetic amino acid derivatives have been synthesized by different groups [36–48]. Representative examples of these compounds (**8**–**15**) are shown in Figure 4. These compounds have been obtained using different addition reactions to [60]fullerene. Compound **8** was derived from a Diels–Alder reaction that generates a hydroxycyclohexanefullerene subsequently esterified with an *N*-protected Ala [36]. Similarly, the same hydroxyfullerene derivative was reacted with the Glu side chain carboxylic function. Another example of esterification is represented by a C_{60} molecule with a fused five-membered ring bearing an alcoholic function, which was allowed to react with the carboxy group of a Phe residue [37]. Diels–Alder cycloadducts of 1,3-butadienes characterized by an electron-withdrawing functionality were also used to prepare the carboxylic precursor of the fulleroamino acid **9**, which is the final product derived from the coupling reaction with a series of α-amino esters [38]. Methanofullerene-substituted amino acids, represented by **10**, were prepared either via the addition of a diazo derivative that affords the methanofullerene carboxylic acid suitable for coupling with different amino acids [39], or by the direct addition to the C_{60} core of the α-amino diazoamides, which are generated by diazoacetylation of primary amino groups in amino acids [40]. Based on the known reactivity of primary and secondary amines with the fullerene moiety [49], free Gly, Ala and Ser have been added in equimolar ratio to C_{60} giving water-soluble glycyl-fullerene, alanyl-fullerene and seryl-fullerene (**11**), respectively [41]. These molecules were able to modify the properties of liposomes and to penetrate artificial membranes [42]. Aggregation of fullerene derivatives, including α-amino ester **12**, in liposome model membranes has also very recently been analyzed using photophysical techniques [43]. Most of the C_{60} amino acid derivatives reported above are not very useful for peptide synthesis because they present either the amino or the carboxylic function suitable for the subsequent reactions. Indeed, these compounds can only be used as terminal amino acids. Finally, the synthesis of 1,2-dihydro[60]fullerylglycine **13**, which is considered an analogue of α-phenylglycine where the phenyl ring is replaced by the fullerene moiety, was previously described [44]. Very recently, Illescas *et al.* [45] reported the synthesis of fulleroamino acid derivatives containing a cyclopropyl ring. They synthesized the diastereomeric *N*-methyl fulleropyrrolidine **14** and the enantiomerically pure isoxazolino[4′,5′:1,2][60]fullerene **15** with retention of the configuration of the starting aldehyde.

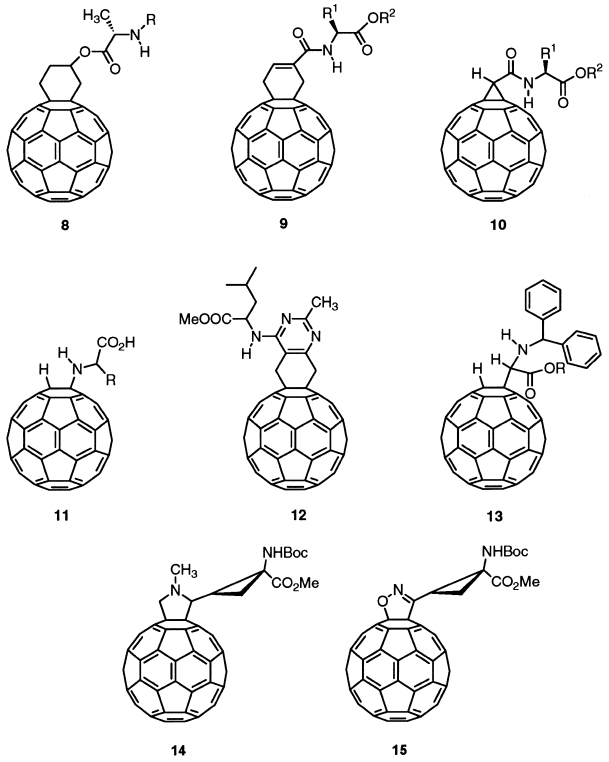


Figure 4 Examples of fulleroamino acids.

FULLEROPEPTIDES AND FULLEROPROTEINS

We have already described some peptide sequences that contain the Fpr residue (see above). Some other

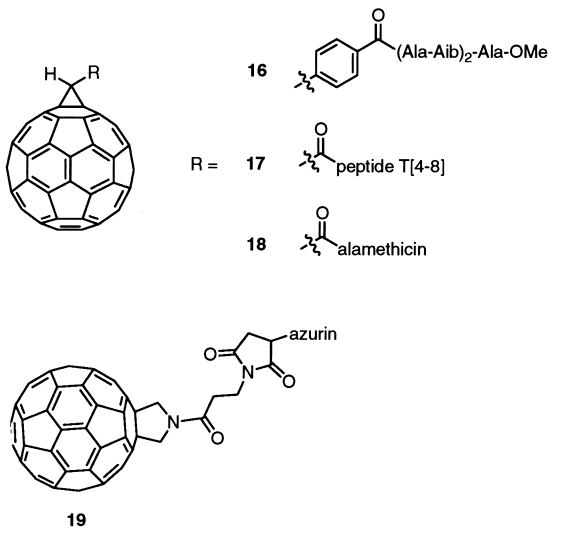


Figure 5 Examples of peptide- and protein-based fullerenes.

fulleropeptides containing differently functionalized C_{60} derivatives have also been prepared (structures **16**–**20**, Figures 5 and 6).

The first peptide based on a fullerene molecule was reported by our group in 1993 [50]. An *N*-terminal free pentapeptide with an alternating -Ala-Aib- sequence was covalently attached to a methanofullerene through a linker based on a benzoic acid moiety (**16**) (Figure 5). This model fulleropeptide was subjected to conformational characterization in order to disclose the propensity of the C_{60}-peptide conjugate to fold into a regular structure. Conventional FT-IR absorption studies in solution and ^{1}H-NMR solvent titration analysis allowed us to show that the peptide is able to adopt an ordered secondary structure (3_{10}-helix) which is stabilized by an array of intramolecular H-bonds. Our work on this model peptide opened up the possibility of extending the synthesis of fulleropeptides to peptides endowed with high biological and pharmacological activities and/or to water-soluble peptides. Accordingly, a methanofullerene carboxylic acid, activated as its acid chloride, was

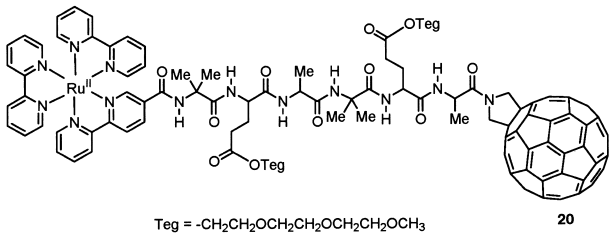


Figure 6 The Ru(bpy$_3$)-peptide-fullerene dyad **20**.

linked to the α-amino group of the hydrophilic *C*-terminal 4–8 sequence of peptide T (**17**) (Figure 5) [51]. This pentapeptide H-Thr-Thr-Asn-Tyr-Thr-OH is known to display a potent human monocyte chemotaxis. The fullerene-peptide T conjugate [4–8] was evaluated for its chemotactic properties, which were shown to be comparable with those of the parent peptide T [4–8]. Moreover, the water-soluble C_{60}-peptide was also able to inhibit, in the μM concentration range, the peptidase activity of HIV-1 protease [18,19].

The formation of C_{60}-derivatized peptides can also be achieved by direct binding of the *N*-terminal free amino function to pristine fullerene. H-Gly$_2$-OMe was reacted with C_{60} via the generation of a nitrene intermediate by addition of bromine to a basic solution, and using phase transfer catalysis to avoid the problem of solubility in aqueous media of the hydrophobic fullerene [52].

Recently, a hexapeptide, constituting a repeated -Aib-Ala-Glu- sequence, was used as a spacer between a C_{60} derivative and a ruthenium complex covalently attached to its *C*- and *N*-termini (compound **20**, Figure 6) [53]. In structure-supporting, chlorinated solvents the peptide-bridged donor–acceptor system undergoes an intramolecular photo-induced electron transfer that causes quenching of the emission associated with the ruthenium metal-to-ligand charge-transfer excited state. The addition of a strong protic solvent leads to deactivation of the electron transfer process. This behavior has been correlated to the protic solvent effect on the conformation of the peptide spacer. Efficient interactions between the acceptor and the donor moieties were referred to the presence of a 3_{10}-helical conformation adopted by the peptide in chlorinated solvents. The unfolding of the peptide-ordered secondary structure by the addition of a protic solvent hampers the onset of a suitable spatial orientation of the chromophores, thereby switching off the photo-induced electron transfer.

A fulleropyrrolidine has been linked to the Asp side chain and the new fulleroamino acid has been subsequently introduced in a pentapeptide of sequence Z-Asp(fulleropyrrolidine)-Aib-Ala-TOAC-Ala-O*t*Bu [54]. This fulleropeptide has been studied by time-resolved electron paramagnetic resonance (EPR) in order to evaluate the sign of the exchange interaction between the TOAC nitroxide free radical and the triplet excited state of the fullerene moiety by means of spin polarization. The value and sign of the electron exchange interaction are important parameters since they determine the polarization pattern, the kinetic behavior, and the magnetic field

effects on the reactivity. The mechanism producing the interaction between the two spin systems of the fullerene–nitroxide conjugate is dependent on the geometry of the system and it occurs through the space.

We can imagine that the next step from small fulleropeptides could be that of linking C_{60} derivatives to more complicated systems such as the proteins. Indeed, a fulleropyrrolidine functionalized with a maleimido group was bound to the thiol function of azurin (**19**) (Figure 5). This redox protein, which binds copper ions and acts as an electron transfer agent in the denitrification chains of several bacteria, has been mutated by the introduction of an exposed Cys residue and reacted to form a C_{60}-based thiol adduct [55]. The electrochemical interaction between fullerene and the protein redox center was then investigated, revealing that there is a direct communication between the two moieties and that no protein denaturation occurs during the electrochemical process.

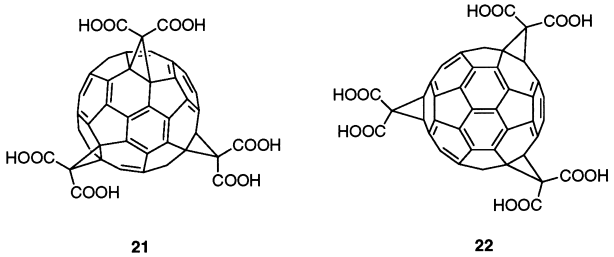
A methanofullerene carboxylic acid, activated as a succinimide ester, was used for the synthesis of C_{60}- and C_{70}-alamethicin peptides **18** (Figure 5) [56]. Alamethicin is an Aib-rich, antibiotic peptide 20 amino acids in length that adopts a helical structure and can induce voltage-dependent conductance in lipid bilayers by forming channel aggregates. Besides the solid-phase synthesis of alamethicin, coupling with the fullerene derivatives was performed in solution. Both conjugates showed channel activities that were different from the natural peptide. In particular, they stabilize channels with distinct pore size. Fullerene-C_{70}-alamethicin forms channel states with a lifetime of seconds.

A series of fullerene derivatives containing a carboxylic acid functionality were conjugated to different proteins, including bovine thyroglobulin, and bovine and rabbit serum albumins, with the aim of studying the possibility of inducing the production of specific antibodies against the carbon atom C_{60} sphere [57]. Immunization of mice with the fullerothyroglobulin generated a population of fullerene-specific IgG antibodies. This demonstrates that the immune system is complete enough to recognize and process molecules such as fullerenes when presented as conjugates with a protein.

MISCELLANEOUS APPLICATIONS

Different fullerene derivatives have been prepared and tested for the evaluation of a series of biological activities and properties that comprise the following: anticancer, antiviral and antibacterial activities; antioxidant and neuroprotective properties; photodynamic therapy; cell signaling and apoptosis; enzyme inhibition; DNA interaction; and genomic applications [15–17]. The tris-malonyl-C_{60} adducts **21** and **22** represented in Figure 7 exhibited very promising biological activity. The two water-soluble isomers were prepared and used as neuroprotective agents [58]. They have been found to be excellent free radical scavengers. Oxygen and/or nitric oxide radicals are associated with many neurodegenerative diseases. The tris-malonyl-C_{60} derivatives display an effective neuroprotective antioxidant activity *in vitro* and *in vivo*. Moreover, the carboxyfullerenes limit the apoptosis of cultured cortical neurons, associated with the presence of amyloid peptide $A\beta_{1-42}$ [58]. They reduce neuronal death resulting from exposure to glutamate receptor agonists, which are known to be cell-death promoters [58]. The free radical nitric oxide is also an important effector molecule in immune and cardiovascular systems. It is produced by three different synthase isoforms that are further responsible of the generation of citrulline. The fullerene adducts **21** and **22** exert some inhibition on the three synthases, with a consequent reduction in the level of nitric oxide production and the rate of citrulline formation [59]. This effect was attributed to interactions of the fullerene molecules with the enzyme and not to their properties as radical sponges. In the same study it was shown that these compounds inhibit the Arg-independent NADPH-oxidase activity of one of the synthase isoforms, without affecting its catalyzed cytochrome c reductive activity.

Another interesting activity of such derivatives is inhibition of bacterial meningitis [60]. This infection is still associated with a high mortality rate in very young and elderly patients, despite the availability of effective antibiotic treatments. It has been found



21 **22**

Figure 7 The C_3 (**21**) and D_3 (**22**) tris-malonyl C_{60} isomers.

that the water-soluble carboxylic acid C_{60} compounds **21** and **22** are able to protect mice from *Escherichia coli*-induced death in a dose-dependent manner. The mice treated with fullerenes had less tumor necrosis factor (TNF)-α and interleukin (IL)-1β production, which are detected in the cerebrospinal fluid of patients infected with bacterial meningitis and in experimental animals, in comparison with the levels of production for the control mice. The C_{60} molecules also inhibit the *E. coli*-induced increases in blood–brain barrier permeability and the inflammatory neutrophil infiltration, which are associated with the production of the proinflammatory mediator cytokines TNF-α and IL-1β. The action of C_{60} derivatives would be exerted when the molecules enter the brain via bloodstream circulation following intraperitoneal injection. The data reported suggest that the carboxyfullerenes could be considered potential agents against bacterial meningitis infection, being more effective than the clinically used dexamethasone. A series of similar fullerene mono- and bis-adducts are also able to suppress *E. coli* growth [61]. This interesting activity allows us to conceive the development of fullerene derivatives against a broader variety of microorganisms and to be used as antimicrobial drugs. Indeed, water-soluble and ionic fullerenes have been synthesized and found to display inhibitory activity against several species of bacteria [62,63]. Very recently, compound **21** was tested *in vitro* against apoptotic neuronal death in rat cerebellar granule cells [64]. Cerebellar granule cells represent one of the best *in vitro* models of neuronal apoptosis, both for the mitochondria and the nucleus, which is strictly related to the generation of reactive oxygen species. The protective role of fullerene compound **21** is likely to be due to the known free radical scavenging characteristics of buckyball molecules. Among the antioxidative action, the tris-malonyl-C_{60} adducts also prevent iron-induced stress in rat brain and the lipid peroxidation induced by superoxide and hydroxyl radicals [65,66].

It has been proven that fullerene and its derivatives sometimes display apparent cytotoxicity, while in other cases they do not seem to damage the organs in which they accumulate following administration [67–69]. To better evaluate the metabolism and excretory properties of fullerene, studies on the pharmacokinetics of water-soluble C_{60}-derivatives were carried out to follow their distribution into the tissues and to understand the mechanism of their clearance [70]. This issue needs to be further investigated, however, since it has been demonstrated that [60]fullerene has, for example, a seriously harmful effect on mouse embryos *in vitro* and *in vivo* following administration to pregnant mice [71].

CONCLUSION AND PERSPECTIVES

Recent advances in the chemistry of fullerene have allowed the synthesis of many classes of novel fullerene derivatives. Among these classes, fullerene-based amino acids and peptides are particularly interesting, both for structural studies and for biological applications. The results achieved to date cannot be considered exhaustive as there is much room for novel derivatives and even for innovative approaches. It is clear that the fullerene spheroid, when incorporated in peptides, modifies substantially the original properties. However, the hydrophobic character and the ability to act as a radical sponge make fullerenes attractive building blocks in biological applications. As our knowledge on the properties of fullerene derivatives in water increases, we may be able to address specific aspects of their complex behavior. For these reasons the synthesis of novel derivatives, and especially peptide- or protein-fullerene conjugates, is to be strongly encouraged.

REFERENCES

1. Kroto HW, Fischer JE, Cox DE. *The Fullerenes*. Pergamon: Oxford, 1993.
2. Kroto HW, Heath JR, O'Brien SC, Curl RF, Smalley RE. C_{60}: buckminsterfullerene. *Nature* 1985; **318**: 162–163.
3. Kratschmer W, Lamb LD, Fostiropoulos K, Huffman DR. Solid C_{60}: a new form of carbon. *Nature* 1990; **347**: 354–358.
4. Hirsch A. *The Chemistry of the Fullerenes*. Thieme: Stuttgart, 1994.
5. Hirsch A (ed.). *Fullerenes and Related Structures*. Top. Curr. Chem., vol. 199. Springer: Berlin, 1999.
6. Smith AB (ed.). *Tetrahedron Symposium-in-Print Number 60. Fuller. Chem.* 1996; **52**: 4925–5262.
7. Diederich F, Thilgen C. Covalent fullerene chemistry. *Science* 1996; **271**: 317–323.
8. Diederich F, Kessinger R. Templated regioselective and stereoselective synthesis in fullerene chemistry. *Acc. Chem. Res.* 1999; **32**: 537–545.
9. Martín N, Sànchez L, Illescas B, Pérez I. C_{60}-based electroactive organofullerenes. *Chem. Rev.* 1998; **98**: 2527–2547.

10. Wudl F. The chemical properties of buckminsterfullerene (C_{60}) and the birth and infancy of fulleroids. *Acc. Chem. Res*. 1992; **25**: 157–161.
11. Haddon RC. Electronic-structure, conductivity, and superconductivity of alkali-metal doped C_{60}. *Acc. Chem. Res*. 1992; **25**: 127–133.
12. Tutt LW, Kost A. Optical limiting performance of C_{60} and C_{70} solutions. *Nature* 1992; **356**: 225–226.
13. Guldi D, Prato M. Excited-state properties of C_{60} fullerene derivatives. *Acc. Chem. Res*. 2000; **33**: 695–703.
14. Prato M. [60]fullerene chemistry for materials science applications. *J. Mater. Chem*. 1997; **7**: 1097–1109.
15. Da Ros T, Prato M. Medicinal chemistry with fullerenes and fullerene derivatives. *Chem. Commun*. 1999; 663–669.
16. Jensen AW, Wilson SR, Schuster DI. Biological applications of fullerenes. A review. *Bioorg. Med. Chem*. 1996; **4**: 767–779.
17. Wilson SR. Biological aspects of fullerenes. In *Fullerenes: Chemistry, Physics, and Technology*, Kadish K, Ruoff R (eds). Wiley: New York, 2000; 437–465.
18. Friedman SH, DeCamp DL, Sijbesma RP, Srdanov G, Wudl F, Kenyon OL. Inhibition of the HIV-1 protease by fullerene derivatives: model building studies and experimental verification. *J. Am. Chem. Soc*. 1993; **115**: 6506–6509.
19. Sijbesma R, Srdanov G, Wudl F, Castoro JA, Wilkins C, Friedman SH, DeCamp DL, Kenyon GL. Synthesis of a fullerene derivative for the inhibition of HIV enzymes. *J. Am. Chem. Soc*. 1993; **115**: 6510–6512.
20. Schinazi RF, Sijbesma RP, Srdanov G, Hill CL, Wudl F. Synthesis and virucidal activity of a water soluble, configurationally stable, derivatized C_{60} fullerene. *Antimicrob. Agents Chemother*. 1993; **37**: 1707–1710.
21. Friedman SH, Ganapathi PS, Rubin Y, Kenyon GL. Optimizing the binding of fullerene inhibitors of the HIV-1 protease through predicted increases in hydrophobic desolvation. *J. Med. Chem*. 1998; **41**: 2424–2429.
22. Holmes AB, Stephenson OR. Organic chemistry. *Chem. Ind*. 1994; 303.
23. Maggini M, Scorrano G, Bianco A, Toniolo C, Sijbesma RP, Wudl F, Prato M. Addition reactions to C_{60} leading to fulleroprolines. *J. Chem. Soc. Chem. Commun*. 1994; 305-306.
24. Maggini M, Scorrano G, Prato M. Addition of azomethine ylides to C_{60}: synthesis, characterization, and functionalization of fullerene pyrrolidines. *J. Am. Chem. Soc*. 1993; **115**: 9798–9799.
25. Prato M, Maggini M. Fulleropyrrolidines: a family of full-fledged fullerene derivatives. *Acc. Chem. Res*. 1998; **31**: 519–526.
26. Yaron A, Naider F. Proline-dependent structural and biological properties of peptides and proteins. *Crit. Rev. Biochem. Mol. Biol*. 1993; **28**: 31–81.
27. Rose G, Gieracsh L, Smith J. Turns in peptides and proteins. *Adv. Protein Chem*. 1985; **37**: 1–109.
28. Bianco A, Lucchini V, Maggini M, Prato M, Scorrano G, Toniolo C. *Trans–cis* amide bond isomerization in fulleroprolines. *J. Peptide Sci*. 1998; **4**: 364–368.
29. Bianco A, Bertolini T, Crisma M, Valle G, Toniolo C, Maggini M, Scorrano G, Prato M. β-Turn induction by C_{60}-based fulleroproline: synthesis and conformational characterization of Fpr/Pro small peptides. *J. Peptide Sci*. 1997; **50**: 159–170.
30. Bianco A, Maggini M, Scorrano G, Toniolo C, Marconi G, Villani C, Prato M. Synthesis, chiroptical properties, and configurational assignment of fulleroproline derivatives and peptides. *J. Am. Chem. Soc*. 1996; **118**: 4072–4080.
31. Schergna S, Da Ros T, Linda P, Ebert C, Gardossi L, Prato M. Enzymatic modification of fullerene derivatives. *Tetrahedron Lett*. 1998; **39**: 7791–7794.
32. Bianco A, Gasparrini F, Maggini M, Misiti D, Polese A, Prato M, Scorrano G, Toniolo C, Villani C. Molecular recognition by a silica-bound fullerene derivative. *J. Am. Chem. Soc*. 1997; **119**: 7550–7554.
33. Guldi D, Maggini M, Scorrano G, Prato M, Bianco A, Toniolo C. Photoinduced electron transfer in supramolecular fullerene/ferrocene systems. *J. Inf. Rec*. 1998; **24**: 33–39.
34. Maggini M, Faveri CD, Scorrano G, Prato M, Brusatin G, Guglielmi M, Meneghetti M, Signorini R, Bozio R. Synthesis and optical limiting behavior of hybrid inorganic–organic materials from sol-gel processing of organofullerenes. *Chem. Eur. J*. 1999; **5**: 2501–2510.
35. Signorini R, Zerbetto M, Meneghetti M, Bozio R, Maggini M, De Faveri C, Prato M, Scorrano G. Optical limiting properties of soluble fullerene derivatives for incorporation in sol-gel materials. *Chem. Commun*. 1996; 1891–1892.
36. An YZ, Anderson JL, Rubin Y. Synthesis of α-amino acid derivatives of C_{60} from 1,9-(4-hydroxycyclohexano)-buckminsterfullerene. *J. Org. Chem*. 1993; **58**: 4799–4801.
37. Yamago S, Tokuyama H, Nakamura E, Prato M, Wudl F. Chemical derivatization of organofullerenes through oxidation, reduction, and C–O and C–C bond-forming reactions. *J. Org. Chem*. 1993; **58**: 4796–4798.
38. Ohno M, Shirakawa Y, Eguchi S. C_{60}-linked carboxylic acid and its derivatives by Diels–Alder cycloaddition of C_{60} with a buta-1,3-diene bearing an electron-withdrawing group. *Synthesis* 1998; 1812–1816.
39. Isaacs L, Diederich F. Structure and chemistry of methanofullerenes: a versatile route into *N*-[(methanofullerene)carbonyl]-substituted amino acids. *Helv. Chim. Acta* 1993; **76**: 2454–2464.
40. Skiebe A, Hirsch A. A facile method for the synthesis of amino acid and amido derivatives of C_{60}. *J. Chem. Soc. Chem. Commun*. 1994; 335–336.

41. Vol'pin ME, Belavtseva EM, Romanova VS, Lapshin M, Aref'eva LI, Parnes ZN. Self-assembling of associates of amino acids and dipeptide derivates of [60]fullerene in aqueous solution: a study by scanning electron microscopy. *Mendeleev Commun.* 1995; 129–131.
42. Kotelnikova RA, Kotelnikov AI, Bogdanov GN, Romanova VS, Kuleshova EF, Parnes ZN, Vol'pin ME. Membranotropic properties of the water soluble amino acid and peptide derivatives of fullerene C_{60}. *FEBS Lett.* 1996; **389**: 111–114.
43. Janot JM, Bienvenüe E, Seta P, Bensasson R, Tomé A, Enes R, Cavaleiro A, Leach S, Camps X, Hirsch A. [60]fullerene and three [60]fullerene derivatives in membrane model environments. *J. Chem. Soc. Perkin Trans. 2*, 2000; 301–306.
44. Burley G, Keller P, Pyne S, Ball G. Synthesis of a 1,2-dihydro[60]fullerylglycine derivative by a novel cyclopropane ring opening of a methano[60]fullerene. *Chem. Commun.* 1998; 2539–2540.
45. Illescas B, Rifé J, Ortuño R, Martín N. Stereoselective synthesis of C_{60}-based cyclopropane amino acids. *J. Org. Chem.* 2000; **65**: 6246–6248.
46. Gan L, Zhou D, Luo C, Tan H, Huang C, Lü M, Pan J, Wu Y. Synthesis of fullerene amino acid derivatives by direct interaction of an amino acid ester with C_{60}. *J. Org. Chem.* 1996; **61**: 1954–1961.
47. Zhang W, Su Y, Gan L, Jiang J, Huang C. Photolysis of C_{60} with cyclic amino acids: preparation of dihydrofullerenes by decarboxylation. *Chem. Lett.* 1997; 1007–1008.
48. Zhou D, Gan L, Luo C, Tan H, Huang C, Liu Z, Wu Z, Zhao X, Xia X, Zhang S, Sun F, Zan ZXY. Langmuir–Blodgett film and nonlinear optical property of C_{60}-glycine ester derivative. *Chem. Phys. Lett.* 1995; **235**: 548–551.
49. Hirsch A, Li Q, Wudl F. Globe-trotting hydrogens on the surface of the fullerene compound $C_{60}H_6(N(CH_2CH_2)_2O)_6$. *Angew. Chem. Int. Ed. Engl.* 1991; **30**: 1309–1310.
50. Prato M, Bianco A, Maggini M, Scorrano G, Toniolo C, Wudl F. Synthesis and characterization of the first fullerene-peptide. *J. Org. Chem.* 1993; **58**: 5578–5580.
51. Toniolo C, Bianco A, Maggini M, Scorrano G, Prato M, Marastoni M, Tomatis R, Spisani S, Palù G, Blair ED. A bioactive fullerene peptide. *J. Med. Chem.* 1994; **37**: 4558–4562.
52. Wang N, Li J, Zhu D, Chan TH. A C_{60}-derivatized dipeptide. *Tetrahedron Lett.* 1995; **36**: 431–434.
53. Polese A, Mondini S, Bianco A, Toniolo C, Scorrano G, Guldi D, Maggini M. Solvent-dependent intramolecular electron transfer in a peptide-linked $[Ru(bpy)_3]^{2+}$-C_{60} dyad. *J. Am. Chem. Soc.* 1999; **121**: 3446–3452.
54. Mazzoni M, Conti F, Corvaja C. The sign of the exchange interaction between excites fullerene and nitroxide free radicals. *Appl. Magn. Reson.* 2000; **18**: 351–361.
55. Kurz A, Halliwell C, Davis J, Hill A, Canters G. A fullerene-modified protein. *Chem. Commun.* 1998; 433–434.
56. Redemann T, Kempter C, Ihlenfeld HG, Lamparth I, Skiebe A, Meder S, Hirsch A, Boheim G, Jung G. Alamethicin–fullerene conjugates adopt preferred channel size in voltage-dependent bilayer experiments. In *Peptides 1996*, Ramage R, Epton R (eds). Mayflower Scientific: Kingswinford, 1998; 747–748.
57. Chen BX, Wilson SR, Das M, Coughlin DJ, Erlanger BF. Antigenicity of fullerenes: antibodies specific for fullerenes and their characteristics. *Proc. Natl. Acad. Sci. USA* 1998; **95**: 10809–10813.
58. Dugan LL, Turetsky DM, Du C, Lobner D, Wheeler M, Almli CR, Shen CKF, Luh TY, Choi DW, Lin TS. Carboxyfullerenes as neuroprotective agents. *Proc. Natl. Acad. Sci. USA* 1997; **94**: 9434–9439.
59. Wolff D, Papoiu A, Mialkowski K, Richardson C, Schuster D, Wilson S. Inhibition of nitric oxide synthase isoforms by tris-malonyl-C_{60}-fullerene adducts. *Arch. Biochem. Biophys.* 2000; **378**: 216–223.
60. Tsao N, Kanakamma P, Luh TY, Chou CK, Lai HY. Inhibition of *Escherichia coli*-induced meningitis by carboxyfullerene. *Antimicrob. Agents Chemother.* 1999; **43**: 2273–2277.
61. Mashino T, Okuda K, Hirota T, Hirobe M, Nagano T, Mochizuchi M. Inhibition of *E. coli* growth by fullerene derivatives and inhibition mechanism. *Bioorg. Med. Chem. Lett.* 1999; **9**: 2959–2962.
62. Da Ros T, Prato M, Novello F, Maggini M, Banfi E. Easy access to water soluble fullerene derivatives via 1,3-dipolar cycloadditions of azomethine ylides to C_{60}. *J. Org. Chem.* 1996; **61**: 9070–9072.
63. Bosi S, Da Ros T, Castellano S, Banfi E, Prato M. Antimycobacterial activity of ionic fullerene derivatives. *Bioorg. Med. Chem. Lett.* 2000; **10**: 1043–1045.
64. Bisaglia M, Natalini B, Pellicciari R, Straface E, Malorni W, Monti D, Franceschi C, Schettini G. C_3-fullerotris-methanodicarboxylic acid protects cerebellar granule cell from apoptosis. *J. Neurochem.* 2000; **74**: 1197–1204.
65. Lin A, Chyi B, Wang S, Yu H, Kanakamma P, Luh TY, Chou C, Ho L. Carboxyfullerene prevents iron-induced oxidative stress in rat brain. *J. Neurochem.* 1999; **72**: 1634–1640.
66. Wang I, Tai L, Lee D, Kanakamma P, Shen CF, Luh TY, Cheng C, Hwang K. C_{60} and water-soluble fullerene derivatives as anti oxidants against radical-initiated lipid peroxidation. *J. Med. Chem.* 1999; **42**: 4614–4620.
67. Tokuyama H, Yamago S, Nakamura E, Shiraki T, Sugiura Y. Photoinduced biochemical activity of fullerene carboxylic acid. *J. Am. Chem. Soc.* 1993; **115**: 7918–7919.
68. Nakamura E, Tokuyama H, Yamago S, Shiraki T, Sugiura Y. Biological activity of water-soluble fullerenes.

Structural dependence of DNA cleavage, cytotoxicity, and enzyme inhibitory activities including HIV-protease inhibition. *Bull. Chem. Soc. Jpn.* 1996; **69**: 2143–2151.

69. Schuster DI, Wilson SR, Schinazi RF. Anti-human immunodeficiency virus activity and cytotoxicity of derivatized buckminsterfullerenes. *Bioorg. Med. Chem. Lett.* 1996; **6**: 1253–1256.
70. Rajagopalan P, Wudl F, Schinazi RF, Boudinot FD. Pharmacokinetics of a water-soluble fullerene in rats. *Antimicrob. Agents Chemother.* 1996; **40**: 2262–2265.
71. Tsuchiya T, Oguri I, Yamakoshi YN, Miyata N. Novel harmful effects of [60]fullerene on mouse embryos *in vitro* and *in vivo*. *FEBS Lett.* 1996; **393**: 139–145.