Chemistry of Natural Compounds and Bioorganic Chemistry

Amino acid and peptide derivatives of fullerene

M. E. Vol'pin,† Z. N. Parnes, and V. S. Romanova*

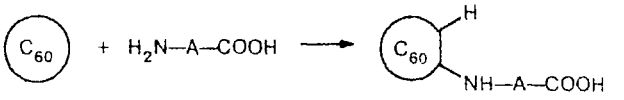
A. N. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, 28 ul. Vavilova, 117813 Moscow, Russian Federation, Fax: +7 (095) 135 5085. E-mail: parnes@ineos.ac.ru

A general method for the synthesis of amino acid and peptide derivatives of fullerene (ADF) was developed, and the physicochemical properties of the compounds obtained were studied. ADF were shown to penetrate into liposomes and to exhibit adjuvant properties and antiviral activity.

Key words: fullerene; amino acid, peptide; solubility in water; diffusion; association; biological activity.

One of the most promising directions in the organic chemistry of fullerene is the synthesis of physiologically active compounds based on it. Organic derivatives of fullerene were first tested as biologically active compounds in 1993, when American[1] and Japanese[2] scientists almost simultaneously demonstrated the biological activity of disubstituted methylenefullerenes and products of cycloaddition to C_{60} synthesized by them. It was shown in these and subsequent works that when these compounds are irradiated in the presence of oxygen, they can cleave DNA chains, display cytotoxicity against tumor cells, and inhibit activity toward various enzymes, including the inhibition of the HIV enzymes. In a short period of time (five years), many studies have been carried out which showed that a lot of organic fullerene derivatives are biologically active.[3] In order to impart biological activity to C_{60} derivatives, amino acid or peptide residues were introduced in their molecules. As a result, a number of fullerene derivatives, in which an amino acid or peptide residue is located in an annelated six-membered ring, were synthesized.[4] Furthermore, such compounds were obtained by esterification of C_{60} phenol derivatives synthesized by the addition of phenyl-substituted carbenes.[1,5] On the other hand, the study of biological properties of fullerene derivatives was hindered by the insolubility of the majority of these compounds in water. They are usually made water-soluble by adding gamma-dextrin or polyvinylpyrrolidone, which results in the formation of inclusion compounds.

We suggested a new method for the addition of amino acid and peptide molecules to fullerene.[6] The reaction with C_{60} involves an acid or peptide amino group, while the carboxyl group remains free.



† Deceased.

Translated from *Izvestiya Akademii Nauk. Seriya Khimicheskaya*, No. 5, pp. 1050—1054, May, 1998.

1066-5285/98/4705-1021 $20.00

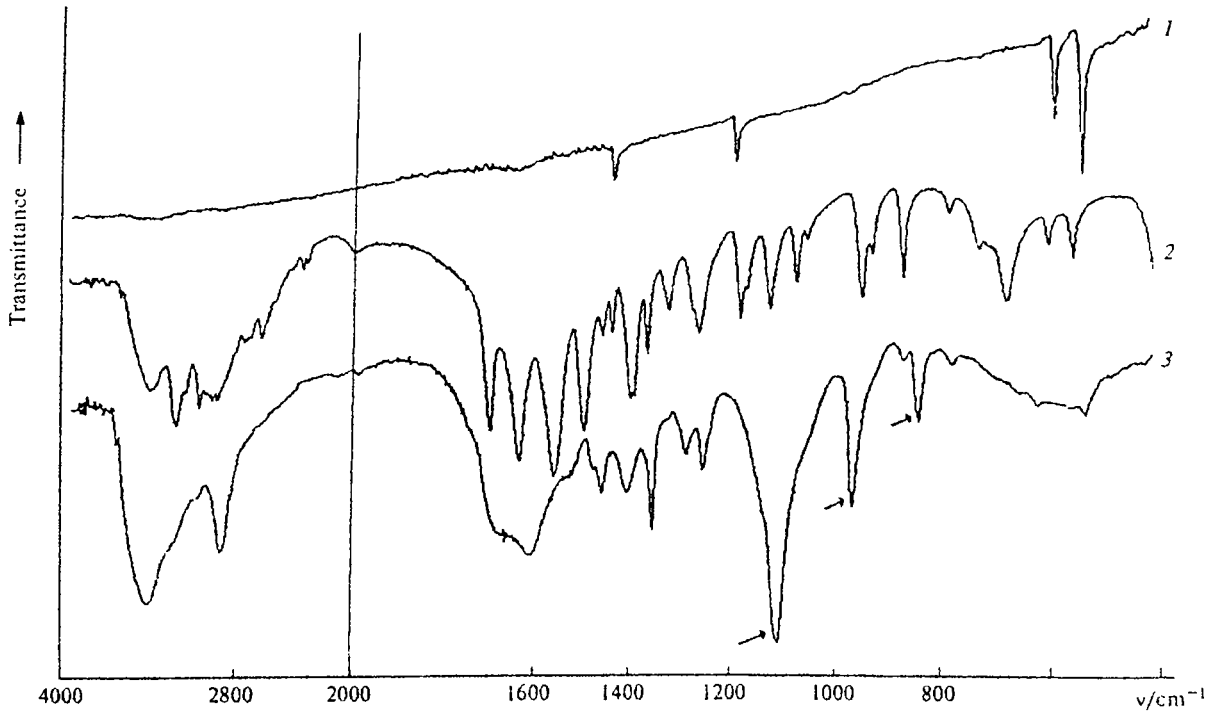


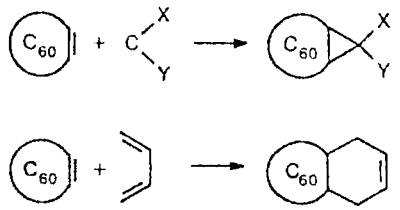
Fig. 1. IR spectra of C_{60} (*1*), glycyl-L-alanine (*2*), and fullereneglycyl-L-alanine (*3*).

Table 1. Results of amino acid analysis of fullerene derivatives

Amino acid or peptide attached to C_{60}	Amount of amino acid in the specimen/μmol		Found / Calculated
	found	calculated	
Gly	4.20	4.40	0.96
Ala	1.36	1.34	1.02
Ser	1.14	1.27	0.91
Pro	1.40	1.44	0.97
$NH_2(CH_2)_5COOH$	9.20	9.60	0.96
p-$NH_2C_6H_4COOH$	1.30	1.46	0.90
GlyGly	1.79	1.80	0.99
Arg	1.30	1.29	1.00
GlyVal	2.40	2.46	1.02
	(1.20+1.20)	(1.23+1.23)	(0.51+0.51)
AlaAla	1.26	1.48	0.92

As a result, fullerene derivatives of the following amino acids and peptides have been obtained in quantitative yield: glycine, L-alanine, L-proline, *p*-aminobenzoic and ω-aminocaproic acids, D,L-serine, glycylglycine, L-alanyl-L-alanine, D,L-alanyl-D,L-alanine, glycyl-L-valine, glycyl-D,L-alanine, L-arginine, and D-arginine. The fullerene derivatives synthesized were purified from excess amino acid by dialysis. The absence of the starting amino acid was determined by a negative reaction with ninhydrin. The homogeneity of the compounds obtained was confirmed by electrophoresis data,[6,7] and their composition was determined by amino acid analysis[8] (Table 1).

The main difference between the structure of the compounds synthesized by us and that of the hitherto known fullerene derivatives is that the amino acid (peptide) adds directly to the double bond of C_{60}. The synthesis of an overwhelming majority of organic fullerene derivatives obtained to date* has been based either on the cyclopropanation of a double bond of C_{60} by a carbene, or on the Diels—Alder reaction resulting in a five- or six-membered cycle.[9a]



The unusual structure of the compounds synthesized is manifested in their physical, chemical, and biological properties. The IR spectra of amino acid and peptide derivatives of C_{60} do not contain fullerene absorption bands[8] (Fig. 1) but contain a characteristic group of three bands: a strong band at 1108 cm^{-1}, a medium intensity band at 960 or 840 cm^{-1}, and a weak band around 1250 cm^{-1}, the positions and comparative intensity of which are almost independent of the structure of

* An exception is Ref. 9b, which reports a direct addition of glycine to fullerene to give a fullerene derivative containing 24 glycine residues. However, no additional data were available about a continuation of this study in the following years. Our attempts to reproduce the synthesis reported in Ref. 9b failed.

the adduct. These bands appear in the spectra of both ionic, acid, and ester forms, as well as of dipeptides. They probably correspond to oscillations of the fullerene molecule fragment in which substitution occurs. One of the bands may correspond to oscillations of the C_{60}—N or C_{60}—H bond.[8]

Unexpectedly for us, the majority of peptide and amino acid fullerene derivatives (ADF) were found to be water-soluble. In order to determine the nature of this phenomenon, the compounds obtained were studied on a scanning electron microscope in secondary electrons. The objects for analysis by scanning electron microscopy (SEM) were prepared according to the technique used in transmission electron microscopy for the study of biological macromolecules[10] but modified somewhat to comply with requirements for SEM specimens. The study showed that aqueous solutions of all the specimens contained large anisodiametric micelle-like particles, the size of which was up to 10 μm. Most of the micelles have oval shape[10] (Fig. 2), and the thickness of the micelles is 2 to 4 times smaller than the length.[11] The particle size depends on the solution concentration. It was found that there is no more than 10% of the solvent inside the micelles.

The ADF which are insoluble in water are soluble in organic media such as DMSO and pyridine. It was found by SEM that ADF also form micelle-like particles, but their structure differs from that of associates in aqueous solutions: they are more dense and spherical[12] (Fig. 3). Furthermore, we were able to study the formation of the structure of associates by scanning tunnel microscopy. In the first step, self-assembling of micelle-like structures occurs. At first, associates of irregularly shaped plates are formed, and then oval-shaped micelle-like structures with loosely packed plates appear. Later, the plates are densely packed, the amount of the solvent and the number of voids in the micelle-like structures decrease, and compact oval particles with smooth surface are formed. Along with structure formation, destruction of micelle-like structures occurs on subsequent storage of solution.[13]

It was shown by the diffusion method that the micelle size depends on the nature of the amino acid (dipeptide), concentration, acidity of the solution, and its ionic strength. In certain cases, association does not occur, and true solutions are formed.[14—17]

An important condition for the application of the compounds obtained in biology and medicine is the possibility of their penetration through cell membranes. We used the luminescent probe method[18,19] to study this propety. Pyrene was added as a fluorescent probe during the formation of bilayer membranes. Pyrene introduced into liposomes is known to be localized in the inside region of a lipid bilayer.[20] The addition of ADF results in fluorescence quenching, which indicates the ability of these compounds to penetrate into the zone

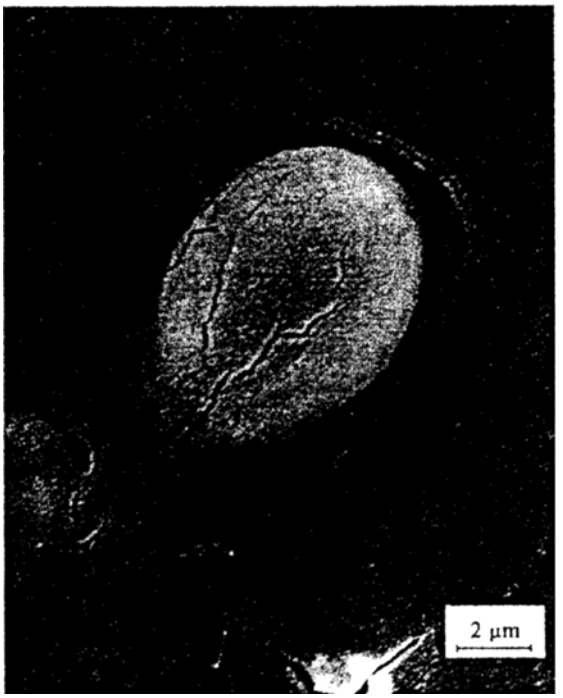


Fig. 2. Electron microscopic image of the associate of alanine fullerene derivative in aqueous solution.

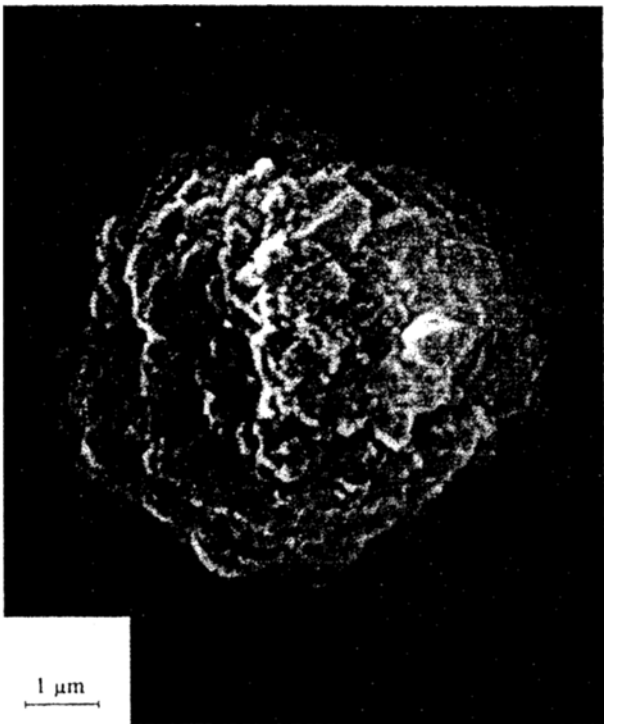
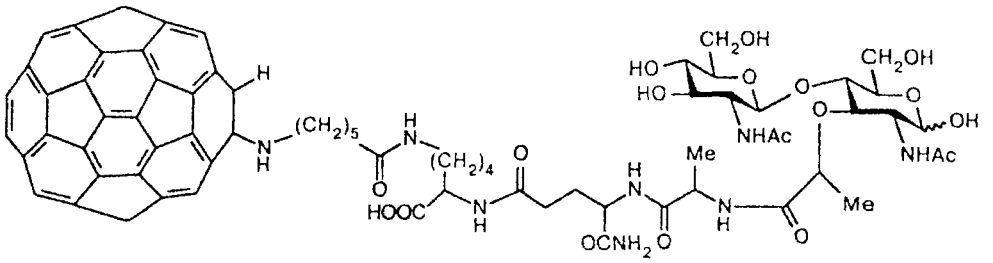


Fig. 3. Electron microscopic image of the associate of *p*-aminobenzoic fullerene derivative in DMSO.



A

where pyrene is localized without changing considerably the structure of the lipid bilayer.

To reveal the ability of the fullerene derivatives synthesized to penetrate through membranes, we used erythrosine B as a triplet probe excited by a nitrogen pulse laser. The kinetics of phosphorescence quenching (the triplet probe method) were recorded on a laser pulse set-up based on a detector of transition processes.[21] Erythrosine is located on the inside and outside surfaces of a bilayer. When the experiment is carried out in an aqueous solution containing a water-soluble cobalt salt, full quenching of phosphorescence of the erythrosine molecules occurs in the aqueous solution and on the outside liposome surfaces. However, Co^{2+} cannot penetrate through the membrane, hence the phosphorescence of erythrosine on the inside membrane surface does not change. It should be noted that if even an insignificant change in the membrane integrity occurs upon exposure to the chemicals studied, the intensity of phosphorescence of internal probes decreases abruptly. The addition of ADF results in efficient quenching in the inside layer. Quenching occurs gradually, *i.e.*, the membrane structure remains unchanged. Thus, we have found that ADF can be localized in the inside regions of an artificial membrane, penetrate into liposomes through a lipid bilayer, and perform active transmembrane transfer of bivalent metal ions.

To date, the greatest success in the study of ADF has been reached in the field of immunology. The commonly used adjuvants for immunization include microorganisms, products of their metabolism, their suspensions in mineral oils (the so-called Freund adjuvant), aluminum hydroxide, insoluble inorganic salts, saponins, and hydrophobic plant extracts. However, immunization using adjuvants in the form of water-oil emulsions and suspensions often leads to undesirable complications, such as local infiltration, ulceration, and allergic reactions.[22] Therefore, the design of new water-soluble synthetic adjuvants is one of the most important problems of biotechnology, biochemistry, and medicine. It was found that although such fullerene derivatives do not possess their own immunogenic activity in tests in mice and rabbits, they display pronounced adjuvant activity. The injection of ADF into rabbits results in an abrupt enhancement (by almost two orders of magnitude) of the formation of antibodies against such a weak antigen as egg albumin.*

The presence of a free carboxyl group in the amino acid and peptide fragments of ADF makes it possible to attach the ADF obtained to other biologically active compounds.

It has been shown that attachment of a well-known adjuvant, a peptide derivative of muramic acid, which is widely used in biology and medicine, to an aminocaproic fullerene derivative results in a new adjuvant (structure **A**), whose activity is more than an order of magnitude higher than that of the original compound.

Thus, a new, highly active adjuvant has been created on the basis of an amino acid fullerene derivative.

The attachment of a 24-membered peptide, which is a fragment of the foot-and-mouth disease virus protein and is active against this disease, to an aminocaproic fullerene derivative gives an antigen, whose immunogenic activity is several orders of magnitude higher than that of the 24-membered peptide.

Preliminary data suggest that certain ADF possess antiviral activity against the cytomegalovirus. Experiments with human erythrocytes showed that even high doses of ADF do not possess hemolytic properties; cells do not agglutinate during incubation, and their morphology does not change. It was found that amino acid fullerene derivatives cause adverse effects on human cells only starting from a very high concentration (300 μg L^{-1}), which is much higher than that used in the experiments (0.3 μg L^{-1}). Thus, the fullerene derivatives in question can be considered virtually nontoxic.

The data presented above demonstrate the prospects of further studies into the physicochemical and biological properties of ADF.

Experimental

IR spectra were recorded on a Bruker IFS-113 Fourier spectrometer. Electron microscopic studies were performed on a Hitachi S-2500 instrument (Japan) at a resolution of 3.5 nm.

Commercial grade amino acids were used.

* Two patent applications have been submitted on the basis of these results: No. 97/105810 (as of April 10, 1997) and No. 97/105811 (as of April 17, 1997).

This study was financially supported by the Russian Foundation for Basic Research (Project No. 95-03-08417), the Russian Foundation for Intellectual Cooperation ("Fullerenes and Atomic Clusters"), and the International Scientific and Technical Center (Program 079).

References

1. R. Sijbesma, G. Srdanov, F. Wudl, J. A. Castoro, C. Wilkins, S. H. Friedman, D. L. DeCamp, and G. L. Kenyon, *J. Am. Chem. Soc.*, 1993, **115**, 6510.
2. H. Tokuyama, S. Yamago, and E. Nakamura, *J. Am. Chem. Soc.*, 1993, **115**, 7918.
3. A. W. Jensen, S. R. Wilson, and D. I. Schuster, *Bioorg. Med. Chem.*, 1996, **4**, 767.
4. Y.-Z. An, J. L. Anderson, and Y. Rubin, *J. Org. Chem.*, 1993, **58**, 4799.
5. M. Prato, A. Bianco, M. Maggini, G. Scorrano, C. Toniolo, and F. Wudl, *J. Org. Chem.*, 1993, **58**, 5578.
6. V. S. Romanova, V. A. Tsyryapkin, Yu. I. Lyakhovetskii, Z. N. Parnes, and M. E. Vol'pin, *Izv. Akad. Nauk, Ser. Khim.*, 1994, 1154 [*Russ. Chem. Bull.*, 1994, **43**, 1090 (Engl. Transl.)].
7. M. E. Vol'pin, V. S. Romanova, and Z. N. Parnes, *Mol. Mat., Sec. C*, 1996, **7**, 53.
8. Z. S. Klemenkova, V. S. Romanova, V. A. Tsyryapkin, V. E. Muradan, Z. N. Parnes, B. V. Lokshin, and M. E. Vol'pin, *Mendeleev Commun.*, 1996, 60.
9. (a) A. Hirsch, *Synthesis*, 1995, 895, (b) L. B. Gan, C. P. Luo, C. H. Huang, G. X. Xu, Z. N. Gu, and X. H. Zhou, *Fullerenes Sci. Technol.*, 1993, **1**, 196.
10. M. E. Vol'pin, E. M. Belavtseva, V. S. Romanova, A. I. Lapshin, L. I. Aref'eva, and Z. N. Parnes, *Mendeleev Commun.*, 1995, 129.
11. E. M. Belavtseva, E. V. Kichenko, V. S. Romanova, Z. N. Parnes, M. E. Vol'pin, *Izv. Akad. Nauk, Ser. Khim.*, 1996, 876 [*Russ. Chem. Bull.*, 1996, **45**, 831 (Engl. Transl.)].
12. E. M. Belavtseva, V. S. Romanova, A. I. Lapshin, E. F. Kuleshova, Z. N. Parnes, and M. E. Vol'pin, *Mendeleev Commun.*, 1996, 171.
13. E. M. Belavtseva, V. S. Romanova, E. F. Kuleshova, Z. N. Parnes, and M. E. Vol'pin, *Biofizika* [*Biophysics*], 1998, in press (in Russian).
14. G. I. Timofeeva, V. S. Romanova, L. A. Lopanova, *Izv. Akad. Nauk, Ser. Khim.*, 1996, 879 [*Russ. Chem. Bull.*, 1996, **45**, 834 (Engl. Transl.)].
15. G. I. Timofeeva, E. F. Kuleshova, and V. S. Romanova, *Mendeleev Commun.*, 1997, 37.
16. G. I. Timofeeva, E. F. Kuleshova, and B. S. Romanova, *Izv. Akad. Nauk, Ser. Khim.*, 1997, 493 [*Russ. Chem. Bull.*, 1997, **46**, 472 (Engl. Transl.)].
17. G. I. Timofeeva, E. F. Kuleshova, and V. S. Romanova, *Mendeleev Commun.*, 1997, 182.
18. R. A. Kotelnikova, A. I. Kotelnikov, G. N. Bogdanov, V. S. Romanova, E. F. Kuleshova, Z. N. Parnes, and M. E. Vol'pin, *FEBS Lett.*, 1996, **389**, 111.
19. G. N. Bogdanov, R. A. Kotelnikova, V. S. Romanova, and Z. N. Parnes, in *Abstrs. of III Intern. Workshop "Fullerenes and Atomic Clusters,"* 1997, St. Petersburg, 47.
20. G. E. Dobretsov, *Fluorestsentnye zondy v issledovanii kletok, membran i lipoproteidov* [*Fluorescent Probes in the Study of Cells, Membranes, and Lipoproteids*], Nauka, Moscow, 1989 (in Russian).
21. R. A. Kotel'nikova, L. V. Tat'yanenko, V. M. Mekler, and A. I. Kotel'nikov, *Mol. Biol.* [*Molecular Biology*], 1982, **16**, 1188 (in Russian).
22. L. Chedid, *Ann. Inst. Pasteur Immunol.*, 1985, **136D**, 283.

Received October 21, 1997;
in revised form November 20, 1997