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CCA-2763 *Authors' Review*

Biological Applications of Fullerene Derivatives: A Brief Overview

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Starting soon after the production of fullerenes in 1990, many efforts have been devoted to the application of C_{60} and its derivatives. In fact, [60]fullerene possesses a variety of interesting biological properties, such as HIV-P inhibition, DNA photocleavage, neuroprotection, apoptosis, *etc*. Unfortunately, the low solubility in biological fluids limits the use of these compounds as new pharmacophores for structure-activity relationship studies in medicinal chemistry. This article briefly summarizes recent studies on the functionalization of C_{60} aimed at increasing water solubility as well as the preliminary studies performed on biological targets. In particular, the HIV-P inhibition, DNA photocleavage and antibacterial activity are discussed.

Key words: anti-HIV agent, antibacterial agent, C_{60}, DNA photocleavage, fullerene, fulleropyrrolidine.

INTRODUCTION

In the last few years, fullerene C_{60} (**1**) and its derivatives have started to be investigated due to their promising preliminary biological activities, such as DNA photocleavage, HIV-Protease (HIV-P) inhibition, neuroprotection and apoptosis.[1–3]

Our group has been involved in the synthesis and study of the properties of C_{60} derivatives. In particular, a novel functionalization of C_{60} has been developed *via* cycloaddition of azomethine ylides to fullerene.[4,5] The azome-

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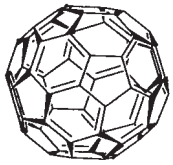

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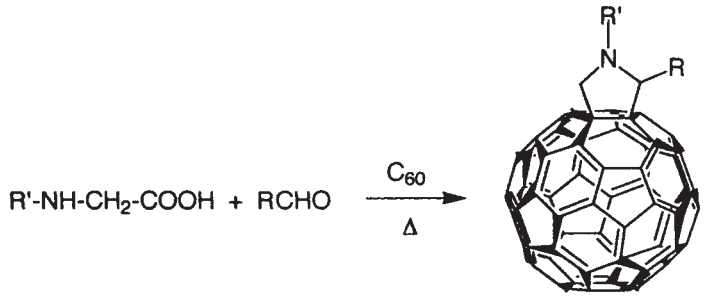
thine ylides are generated *in situ* by condensation of α-amino acids and aldehydes or ketones. In this way, fulleropyrrolidines are obtained with the 5-membered ring fused to a 6,6 bond on the fullerene (Scheme 1).



Scheme 1

This reaction is very useful because it is possible to introduce different substituents on nitrogen and on carbons 2 and 5 using different substituted reagents. This class of fullerene derivatives retain the main properties of the parent molecule, such as the ground state absorptions, which extend throughout the visible region up to 700 nm, and the excited state properties.[6]

In medicinal chemistry, the potential applications of C_{60} derivatives include inhibition of HIV-P,[7] antibacterial activity,[8–10] and photocytotoxicity.[11,12] Being a good radical scavenger, this all-carbon molecule might be also used as an anti-apoptotic and/or anti aging agent.[13–15] All these interesting possibilities of utilizing fullerenes in biology and medicinal chemistry face a significant problem: the natural repulsion of fullerenes to water. Encapsulation of C_{60} in cyclodextrins[16] or in calixarenes[17] or water suspension preparations[18] are useful methodologies for overcoming this limitation, but the most versatile technique is modifying the solubility properties by covalent attachment of water-soluble appendages, such as dendrimers,[19] cyclodextrins[20] or calixarenes.[21,22]

In this review, we briefly summarize the biological profile of some C_{60} derivatives prepared in our laboratory, especially focusing our attention on the synthetic aspects.

INHIBITION OF HIV-P

Earlier experiments performed by Wudl and coworkers demonstrated that there is inhibition of the HIV-P in the presence of C_{60}.[7,23,24] This activity has been supported by molecular modeling studies, which proved that the fullerene can be accommodated inside the hydrophobic cavity present in the enzyme and its location might prevent the interaction between the catalytic portions of the HIV-P and the virus substrates.

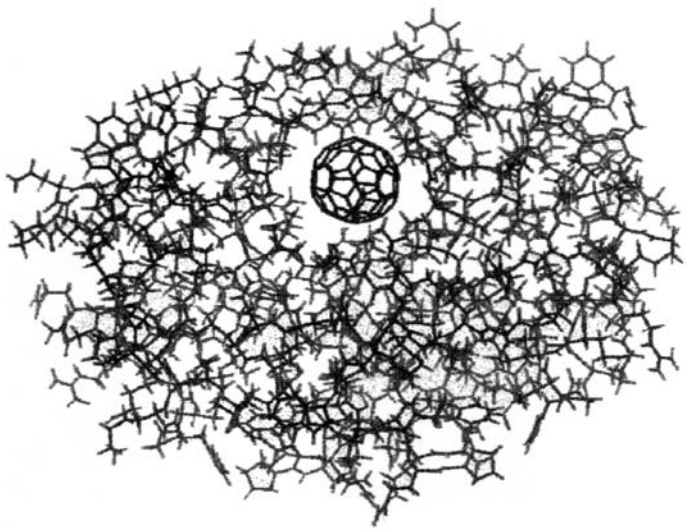


Figure 1. Computer designed accommodation of C_{60} in the HIV protease hydrophobic cavity.

The binding constant found experimentally for a »first generation« inhibitor[7] was not significant in terms of affinity (K_d $10^{-6}/10^{-9}$ M) but represented a starting point for further experiments, which would require a structural optimization of C_{60} derivatives for HIV-P interaction. The catalytic site of HIV-P contains two aspartic residues. A stable interaction with the aspartates could increase the efficiency of the potential inhibitions. On this basis, the same authors proposed an ideal inhibitor **2**, in which two ammonium groups at 5.5 Å distance are directly linked to C_{60} (Figure 2).

Starting from these experimental and theoretical observations, but using a different approach, we synthesized C_{60} derivative **3** in which the distance between the two ammonium residues was 5.1 Å.[25] The synthetic procedure is based on the cycloaddition of *N,N'*-Boc-1,3-diamino-2-propanone (**4**) with sarcosine (**5**) or *N*-(3,6,9-trioxadecyl)glycine (**6**) (Scheme 2). These

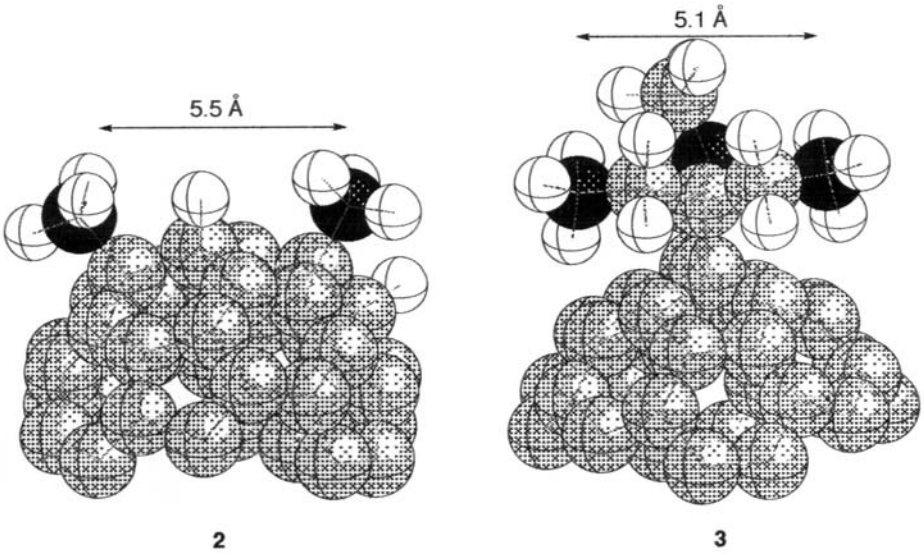
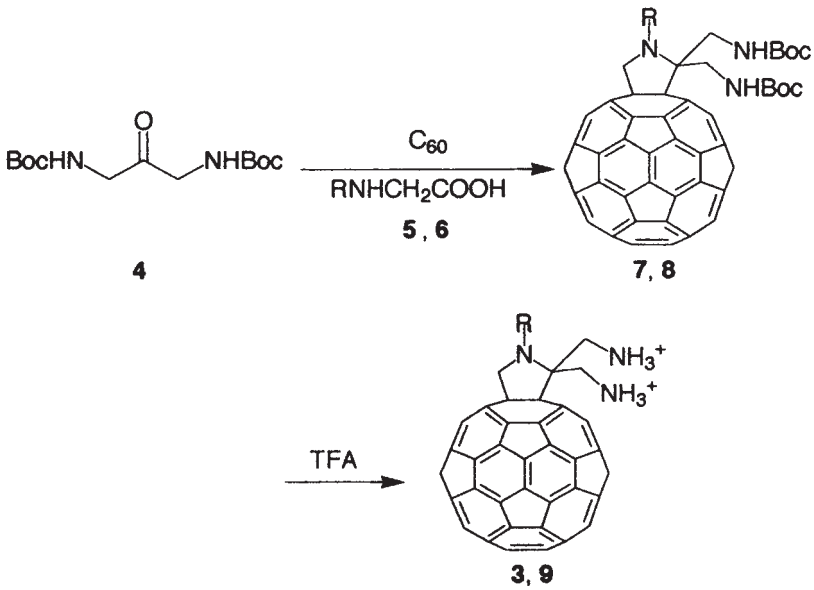


Figure 2. PM3-minimized distances of the two ammonium groups (black) in the ideal inhibitor **2** and in fulleropyrrolidine **3**.



3, **5**, **7**: R= -CH_3
6, **8**, **9**: R= -$CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_3$ (mTEG)

Scheme 2

products (**7** and **8**), after deprotection by TFA, afford the final compounds **3** and **9**, which are still under investigation for their biological profiles.

Molecular modeling studies on derivatives **3** and **9** showed that they could fit very well inside the HIV-P cavity and the electrostatic interactions can take place between the carboxylic residues of aspartates and the ammo-

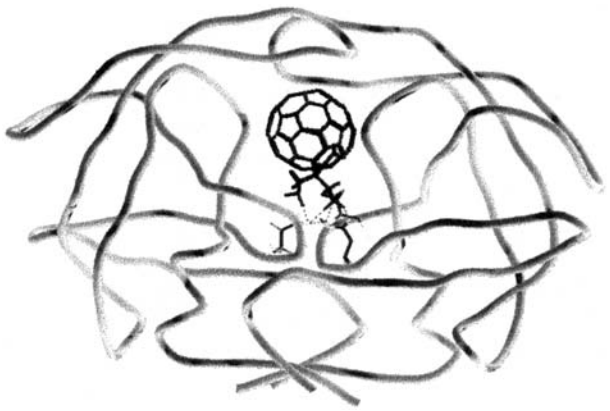


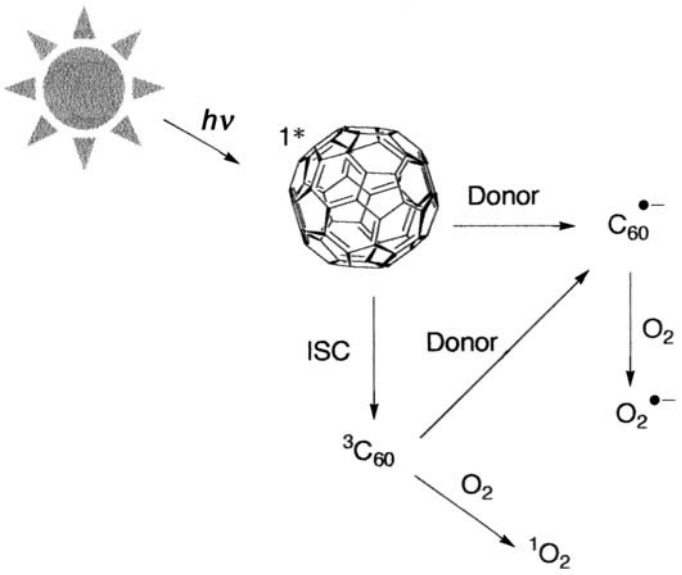
Figure 3. Computer designed complex **3**/HIV-P, showing the interaction between fulleropyrrolidine and Asp 25 and 125.

nium groups. Figure 3 shows the accommodation of the synthesized compound **3** inside the HIV-P cavity. This effect could contribute to stabilizing the complexation of the enzyme by derivative **3**.[25]

DNA-PHOTOCLEAVAGE

Another potential biological application of C_{60} is related to the easy photoexcitation of fullerenes. In fact, from the ground state, the fullerene can be excited to $^1C_{60}$ by photoirradiation. This short-lived species is readily converted to the long-lived $^3C_{60}$ *via* intersystem crossing. In the presence of molecular oxygen, the fullerene can decay from its triplet to the ground state, transferring its energy to O_2, generating $^1O_2$, known to be a highly cytotoxic species. In addition, the high-energy species $^1C_{60}$ and $^3C_{60}$ are excellent acceptors and, in the presence of a donor, can undergo a different process, being easily reduced to $C_{60}^{\cdot -}$ by electron transfer. Again, in the presence of oxygen, the fullerene radical anion can transfer one electron, producing $O_2^{\cdot -}$. The excited fullerene can be reduced in the presence of the guanosine residue present into DNA. Hydrolysis of oxidized guanosines followed by DNA cleavage is a consequence of the electron transfer from G to C_{60}*.[26]

On the other hand, singlet oxygen and superoxide radical anion are well known reactive species towards DNA. The $^1O_2$ in fact modifies G by cycloaddition to the imidazole portion and the resulting modified base is subject to a rapid alkaline hydrolysis of the phosphate bond. Also in this case, the effect is DNA cleavage.



Scheme 3

In this field, many fullerene conjugates with different units possessing biological affinity to nucleic acids or proteins might be particularly interesting. In particular, conjugates between C_{60} and specific agents that interact with nucleic acid, such as acridine,[27] netropsin[11] or complementary oligonucleotides,[26,28] have been synthesized with the aim to understand the mechanism of action of this class of conjugates and to increase both cytotoxicity and sequence selectivity. Many fullerene derivatives linked to an intercalator or a minor groove binder have been reported,[11] however, DNA cleavage occurs at guanine residues without significant sequence selectivity.[29] Only when C_{60} was conjugate to an oligonucleotide, a good selectivity was observed.[28]

In this context, with the aim to obtain higher sequence-selectivity, we undertook to prepare a derivative of C_{60} (**10**) bearing a minor groove binder and an oligonucleotide sequence. The rational design of derivative **10** is based on a reinforced effect due to the simultaneous presence of two different agents able to confer sequence selectivity, such as trimethoxyindole

(TMI) and oligonucleotide. The TMI nucleus is characteristic of a class of natural compounds named duocarmycins (Figure 4), possessing high cytotoxicity (p*M* range, 72 h of incubation for Leukemia Cells L 1210), and high selectivity for AT rich regions of DNA (Figure 5).[30,31]

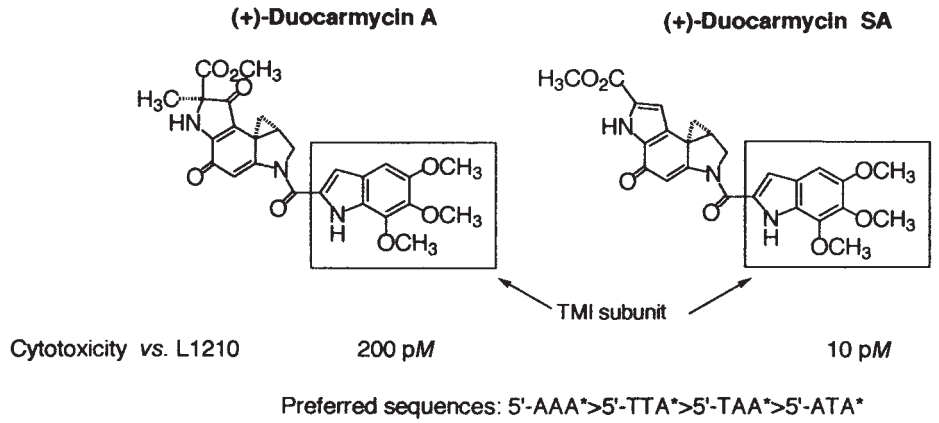


Figure 4. Structures and cytotoxicity values of (+)-Duocarmicin A and (+)-Duocarmicin SA.

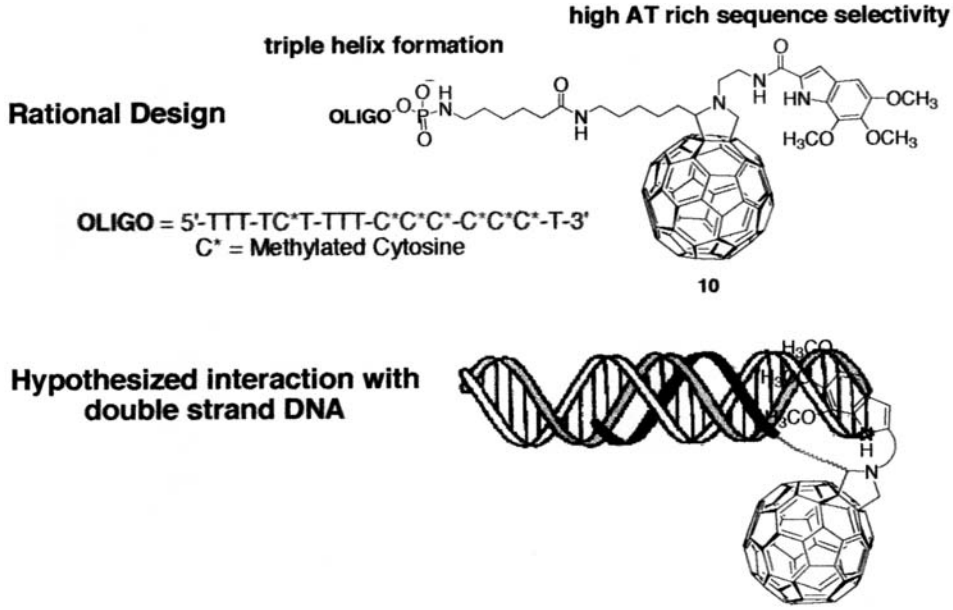
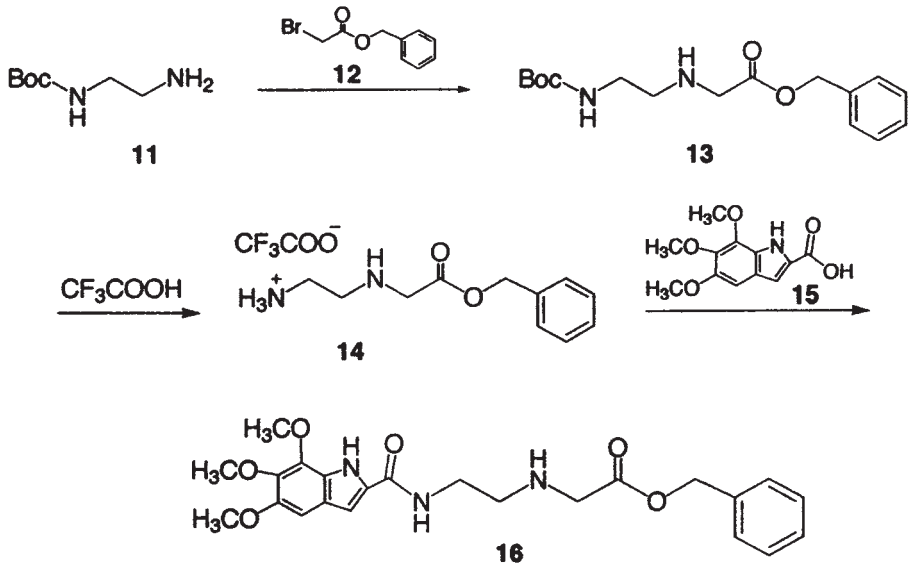


Figure 5. Rational design, possible interaction and triplex helix formation of **10**.

On the other hand, the oligonucleotide chain could increase both the sequence-selectivity and water solubility, the biggest problem of C_{60} derivatives for their biological use.

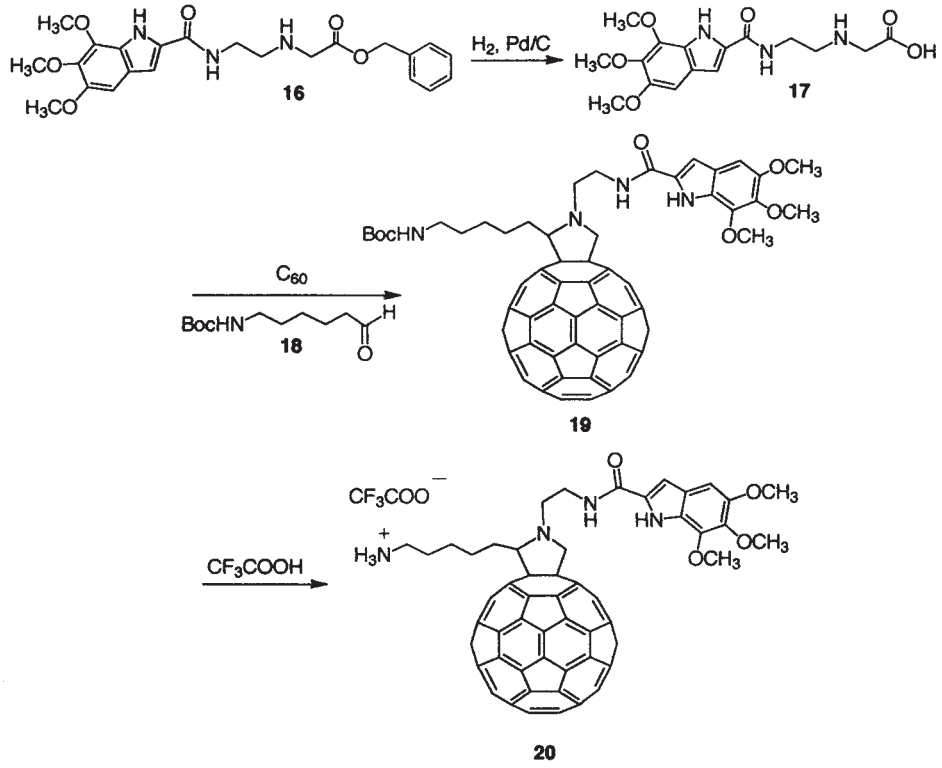
The synthesis of this derivative started from the ethyl diamine *N*-Boc-protected (**11**), which was reacted with benzyl bromoacetate (**12**) to afford compound **13**,[32] which after deprotection of the amino group (**14**), and subsequent condensation with trimethoxy indol 2-carboxilic acid (**15**) in the presence of EDC, gave derivative **16** (Scheme 4).



Scheme 4

The latter (**16**), after catalytic hydrogenation, afforded the corresponding amino acid **17**, which was allowed to react with fullerene (**1**) and the *N*-Boc 6-aminohexanal (**18**) (Scheme 5). Removal of the *N*-Boc protection led to compound **20**, whose amino group could be used for the oligonucleotide coupling reaction.

The designed product **10** was synthesized following a general synthetic strategy for the preparation of oligonucleotide conjugates reported previously[33–35] and summarized in Scheme 6, but this new procedure involved several modifications. Activation of the oligonucleotide terminal phosphate was achieved by the Mukaiyama reagents, triphenylphosphine-dipyridyl-2,2'-disulfide in the presence of DMAP,[36] but the conjugation yield was not satisfactory. Thus, 6-aminocaproic acid (ACA) was utilized as a spacer between the two moieties.

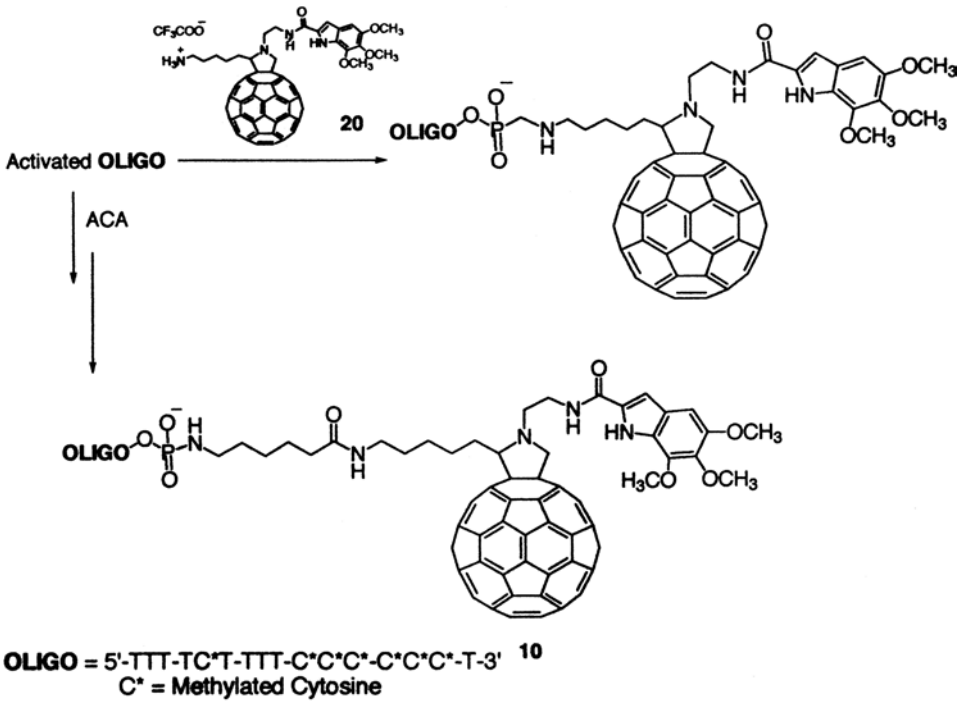


Scheme 5

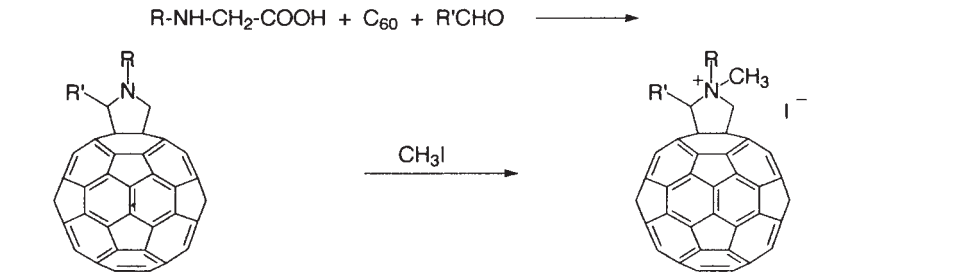
After activation of phosphorylated oligonucleotide (16-mer) at its terminal phosphate and purification,[33] coupling to the ε-amino group of 6-aminocaproic acid in water in the presence of triethylamine was performed, giving the carboxylic acid derivative of oligonucleotide in quantitative yield. The latter was coupled with fullerene derivative **20** in a similar way by activation of carboxylic group with Mukaiyama reagents in organic media, giving the desired conjugate **10**. Purification of **10** was performed by electrophoresis in 1% agarose/0.1% triton X-100 gel using trisacetate buffer, as previously described.[26,28]

ANTIBACTERIAL ACTIVITY

This aspect has been investigated on the hypothesis that C_{60} could produce membrane disruptions by insertion into phospholipidic bilayers. The consequent membrane-disorder could lead to the discharge of metabolites and cell death.



Scheme 6



21: R= $-CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_3$
R'= -H

22: R= $-CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_3$
R'= $-CH_2OCH_2CH_2OCH_2CH_2OCH_3$

23: R= $-CH_3$
R'= $-CH_2OCH_2CH_2OCH_2CH_2OCH_3$

24: R= $-CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_3$
R'= -H

25: R= $-CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_3$
R'= $-CH_2OCH_2CH_2OCH_2CH_2OCH_3$

26: R= $-CH_3$
R'= $-CH_2OCH_2CH_2OCH_2CH_2OCH_3$

Scheme 7

To obtain water-soluble derivatives, we employed different aldehydes (paraformaldehyde or 3,6,9- trioxadecane aldehyde, Scheme 7) to synthesize *N*-mTEG (mTEG = monomethoxytriethylene glycol) substituted fulleropyrrolidines **21**–**23**. The latter were also alkylated with methyl iodide to afford the corresponding ammonium salts **24**–**26**.[8,10]

As shown in Table I and as expected, quaternization ammonium salts **24**–**26** show increased water solubility (DMSO/Water 1/9) compared to neutral compounds (**21**–**23**).

TABLE I

Compound	Solubility/mol dm^{-3}
21	1.5×10^{-5}
22	1.5×10^{-5}
23	3.5×10^{-5}
24	4.3×10^{-5}
25	9.5×10^{-5}
26	1.5×10^{-4}

This allowed us to examine the microbiological profile of these compounds on different microorganisms. The most interesting result was obtained on *Mycobacterium avium* (complete inhibition by compound **23**, 260 μg mL^{-1}) and on *Mycobacterium tubercolosis*. In this case, there is complete growth inhibition by **24**–**26** at a concentration of 50 μg mL^{-1} for **24** and 5 μg mL^{-1} for **25** and **26**. The activity mechanism is under investigation but a plausible explanation could be that the presence of the carbon cage destabilizes the cell wall by intercalation in the hydrophobic part. In any case, the fullerene spheroid must be responsible for the activity, since the same experiments performed using non-fullerenic analogues gave negative results.

Besides, the *in vivo* behavior of compound **26** after *intra*-peritoneal injection at different concentrations (0, 0.5, 1.0 and 1.5 g kg^{-1}) was studied on mice.[37] It was found that the LD_{50} is higher than 1.2 g kg^{-1} but also that this compound is not very well absorbed and produces deposits on different organs, without altering their appearance. Only at very high doses, the livers of the mice show an alteration typical of fibrogenosis, but their microscopic examination, surprisingly, shows normal tissues. It is interesting to note that there is an important deposition in Tyson's glands. This could be explained by the fact that this tissue is rich in ceramides, the same hydrophobic constituents of *Mycobacterium* cell wall.

CONCLUSIONS AND PERSPECTIVES

Although they pose serious manipulation and dosage problems, the preliminary biological investigations of fullerene derivatives have given encouraging results. The hydrophobic spheroid and the radical sponge character of fullerene are responsible for the activity in different fields.

These preliminary findings, along with the low toxicity detected so far in fullerenes, are sufficiently promising to stimulate researchers in chemistry and in biology to unite their efforts and systematically investigate the biological properties of these fascinating molecules.

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SAŽETAK

Biološke primjene derivata fulerena: kratki pregled

Tatiana Da Ros, Giampiero Spalluto i Maurizio Prato

Buckminsterfulleren ima niz zanimljivih bioloških svojstava, kao što su to inhibicija virusa HIV-P, fotocijepanje DNA, apoptoza itd. Nažalost, slaba topljivost u biološkim tekućinama ograničuje primjenu te skupine spojeva u medicinskoj kemiji. Sažeto su prikazani rezultati najnovijih istraživanja funkcionaliziranja C_{60} s ciljem povećanja topljivosti u vodi.