Synthesis and Anti-HIV Properties of New Water-Soluble Bis-functionalized[60]fullerene Derivatives

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Abstract—A new series of bis-functionalized fullerene C_{60} derivatives bearing two or more solubilizing chains have been evaluated for their activity against HIV-1 and HIV-2 strains. Some of the compounds show activity against HIV-1 type in the low micromolar range. The effect of the positions of the addends on the C_{60} nucleus has been investigated, indicating that only *trans*-2 isomers possess promising activity. The presence of a quaternary pyrrolidinium nitrogen is essential to increase solubility.
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Introduction

In the last few years, many interesting biological properties of fullerene C_{60} and its derivatives have been investigated, such as DNA photocleavage, neuroprotection and apoptosis.[1–4] In 1993, Wudl and co-workers found that the activity of HIV-protease (HIVP) could be inhibited by using a water-soluble C_{60} derivative. In fact, molecular modelling studies showed that the carbon sphere can be allocated inside the catalytic hydrophobic cavity of the enzyme, giving rise to remarkable Van der Waals interactions.[5,6] We also synthesized two compounds bearing two ammonium groups with the aim of better interactions with the aspartate residues.[7] However, the biological tests showed no anti-HIV activity, most probably due to problems of solubility. In fact, the major problem of C_{60} is the complete insolubility in water, which limits its use in pharmacological studies. In the last decade, many efforts have been devoted to solve this problem.[4,8] Recently, some functionalized C_{60} derivatives have been studied as anti-HIV agents with promising results.[9,10] Some of these compounds showed EC_{50} values in the low micromolar range and a total absence of toxicity in Vero and PBM cells.[9] Nevertheless, the low number of derivatives reported in the literature do not allow to establish a structure–activity relationship (SAR) profile of this class of compounds. Starting from these observations, we decided to investigate a new series of fullerene derivatives variously functionalized with the aim to better understand which are the relevant structural features for improving anti-HIV activity. In particular, more than one solubilizing appendage have been attached to C_{60}, generating different isomers (**1–13**). Whereas only one monoadduct is usually generated, the bisaddition of symmetrical addends on the C_{60} sphere can generate up to eight regioisomers. The separation of these many isomers is usually highly painstaking. In addition, not all isomers are produced in the same amount. In this paper, we describe the biological properties of four of the eight isomers, in particular the *trans*- and *equatorial*-adducts, which are produced in higher yields, generally between 5 and 10% of the overall mixture. *cis*-Bisadducts are formed in extremely low yields and have not been isolated in sufficient amounts for biological tests. The nomenclature of the bisadducts was assigned following Hirsch suggestions, considering the relative position between addends (Fig. 1).[11]

All the compounds (Fig. 2) have been purified, fully characterized and tested in order to understand which group and, most importantly, which position confers anti-HIV properties. The detailed experimental procedure for the synthesis of the new compounds will be reported elsewhere.

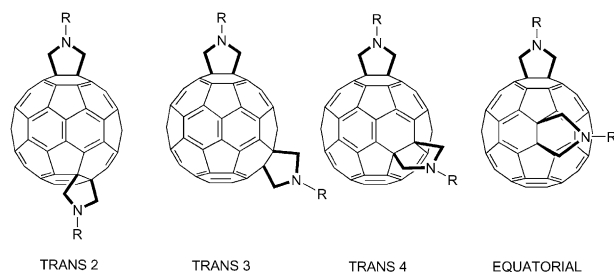*Corresponding authors. Tel.: +39-040-558-7883; fax: +39-040-52572; e-mail: prato@units.it

Figure 1. Four bis-adduct isomers.

As clearly indicated in Table 1, all the compounds (**1**–**13**) were inactive (>4 μM) against HIV-2, but showed different activities against the HIV-1 strain. It is quite evident that the monofunctionalized derivative **1**, and difunctionalized derivatives **2**–**5** and **10**, **11** and **13** do not show any activity while the corresponding quaternary salts at the pyrrolidine nitrogen **6**–**9** proved to be inhibitory at low micromolar concentrations (EC$_{50}$: 0.40–2.60 μM). This experimental observation seems to indicate that electrostatic interactions may be involved in the action of these compounds.

Results and Discussion

Compounds **1**–**13** have been prepared by the well-known cycloaddition of azomethine ylides to C$_{60}$ as briefly summarized in Scheme 1.[1,12,13] While for the achievement of the monoaddition product one equivalent of amino acid was used, two equivalents were necessary to obtain the series of bisadducts.[14] Compounds **10** and **11** were synthesized starting from the monoadduct (**1**), further functionalized by cyclopropanation with a malonic ester derivative.[15]

All isomers have been purified with the help of silica gel chromatography and fully characterized by main spectroscopic techniques. Their purity was checked by HPLC.

All compounds (**1**–**13**) have been tested in lymphocyte CEM cell cultures infected with HIV-1 or HIV-2 as previously described.[16–18] Cytotoxicity has also been evaluated in CEM cell cultures (Table 1).

Table 1. Anti-HIV-1 (III$_B$) and anti-HIV-2 (ROD) activity and cytostatic activity of synthesized compounds in CEM cells cultures (**1**–**13**)

Compd	EC$_{50}$[a] (μM) HIV-1	EC$_{50}$[a] (μM) HIV-2	CC$_{50}$[b] (μM)
1 (monoadduct)	>4	>4	44.3
2 (trans-2)	>4	>4	7.2
3 (trans-3)	>4	>4	7.63
4 (trans-4)	>4	>4	7.4
5 (equatorial)	>4	>4	9.6
6 (trans-2)	0.40 ± 0.0	>4	4.79
7 (trans-3)	0.96 ± 0.39	>4	3.02
8 (trans-4)	2.60 ± 0.88	>4	13.2
9 (equatorial)	1.60 ± 0.0	>4	6.59
10 (trans-2)	>4	>4	ND[c]
11 (equatorial)	>4	>4	ND[c]
12 (trans-2)	2.01 ± 0.0	>4	ND[c]
13 (equatorial)	>4	>4	ND[c]

[a]50% effective concentration or compound concentration required to inhibit HIV-induced cytopathicity by 50%.
[b]50% cytostatic concentration or compound concentration required to inhibit CEM cell proliferation by 50%.
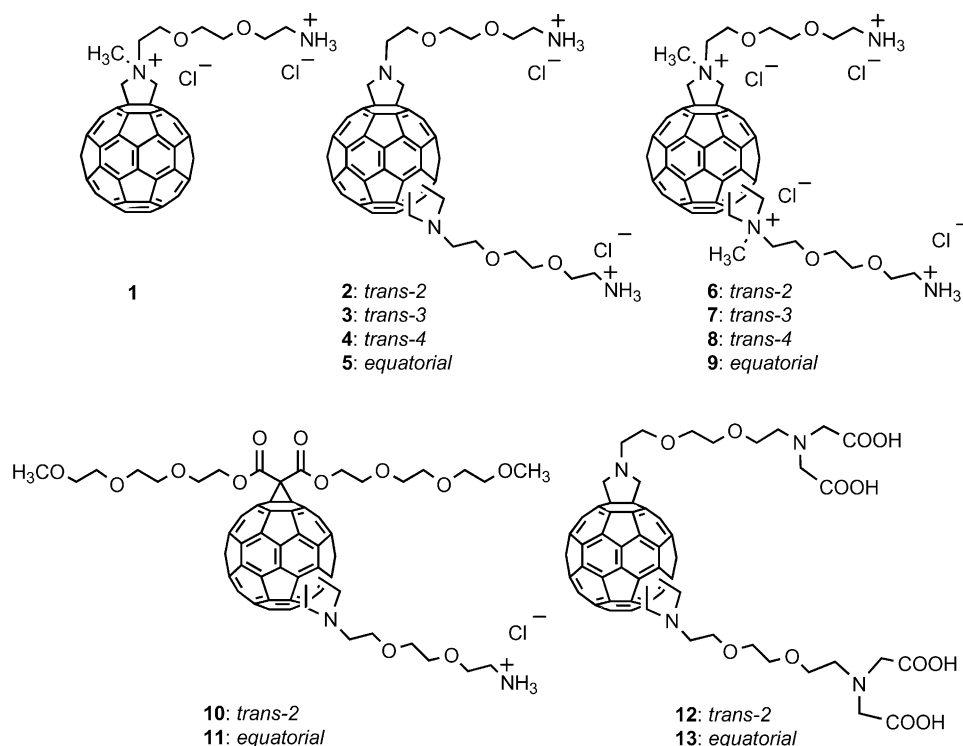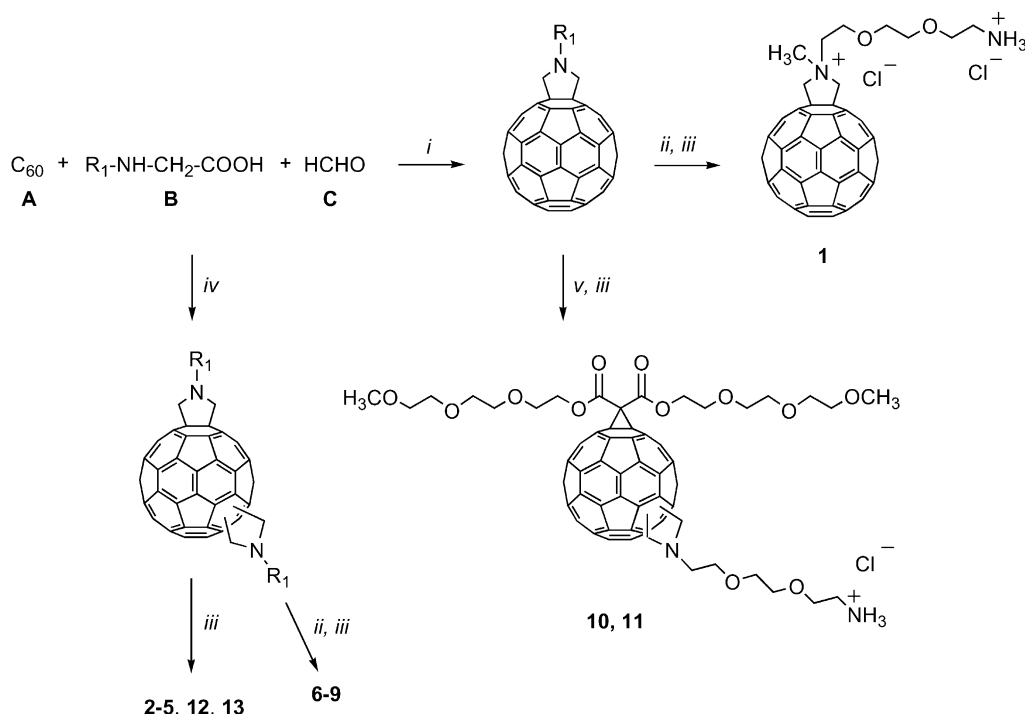[c]ND, not determined.

Figure 2. Structures of synthesized compounds.

Scheme 1. $R_1 = CH_2CH_2OCH_2CH_2OCH_2CH_2NHBoc$ or $CH_2CH_2OCH_2CH_2OCH_2CH_2N(CH_2COOtBu)_2$. Reagent and conditions: (i) molar ratio A:B:C 1:1:3, toluene, Δ; (ii) CH_3I, CH_2Cl_2, 80 °C; (iii) HCl (g), CH_3OH, 0 °C; (iv) mol ratio A:B:C 1:2:5, toluene, Δ; (v) $CH_3O(CH_2CH_2O)_3$-$COCH_2COO(CH_2CH_2O)_3CH_3$, I_2, DBU, toluene, rt.

A careful examination of the biological activities of the most active compounds seems to indicate that the position of the functionalization plays a role in the activity. In fact *trans*-2 isomer (**6**) was more potent (2–6-fold) than the corresponding *trans*-3 (**7**), *trans*-4 (**8**) and *equatorial* (**9**). This observation is confirmed when we consider compounds **12** and **13**. The *trans*-2 tetraacetate derivative **12** showed again a remarkable activity in the micromolar range, while the analogue equatorial **13** results to be inactive. The introduction of malonate moieties (**10–11**) induces a loss of activity. Most of the synthesized compounds showed significant toxicity in human CEM cells (CC_{50}: 4–15 μM) with the exception of monofunctionalized derivative **1** that shows a moderate toxicity (CC_{50}: 44 μM). The toxicity is most probably attributed to the strong amphiphilic character of these derivatives which can lead to cell membrane disruption and subsequent cellular death, as already observed in other series of water soluble C_{60} derivatives.[19] Nevertheless, compound **6** showed a selectivity in the CC_{50}/EC_{50} ratio of about 10, allowing to consider this derivative an excellent starting point for the search of new fullerene derivatives as potential anti-HIV agents.

Conclusions

The study presented herein should increase the knowledge about the SAR of C_{60} derivatives as anti-HIV agents. In particular it seems that positive charges near to the C_{60} backbone could increase potency, while longer solubilizing chain seems to induce cytotoxicity. An extended series of derivatives is under consideration in our laboratories for better understanding these aspects and will be reported in due time.

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