Carboxylic fullerene C$_{60}$ derivatives: efficient microbicides against herpes simplex virus and cytomegalovirus infections *in vitro*

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Some carboxylic fullerene C$_{60}$ derivatives produced by Friedel–Crafts arylation of C$_{60}$Cl$_6$, whose potassium salts are water-soluble, exhibit low cytotoxicity and high antiviral activity against herpes simplex virus and human cytomegalovirus.

Sexually transmitted infections caused by the widespread herpes simplex virus (HSV) and human cytomegalovirus (HCMV) are particularly dangerous in various immunodeficiency states, *e.g.*, pregnancy, organ or tissue post-transplantation period. Vertical transmission of HSV or HCMV often results in abnormal foetal development, diseases of newborns with severe complications and high lethality.[1] In addition, herpesviral infections (HVI) are the most frequent opportunistic infections in HIV patients, being the cause of death in some of them.[2]

There are no effective vaccines against HSV and HCMV. High toxicity of specific antiviral drugs[3] and resistance developing to them during long-course therapy[4,5] limit their use in patients with HVI. Therefore, microbicides (agents that inactivate or destroy microorganisms immediately before coitus, thus preventing sexually

transmitted infections) can be regarded as prospective candidates for anti-HSV and anti-HCMV drugs. A range of compounds (potential microbicides) with different structures and mechanisms of action have been tested.[6–11] However, chemical structures of some of them were not fully identified; others were toxic for human cells and display low antiviral activity. Therefore, further research in that direction is required.

Considerable efforts have been focused on functional derivatives of fullerenes, which cross the plasma membrane,[12] and exert antioxidant effect.[13] Fullerene-based compounds were shown to increase cell resistance to some toxins.[14] Anti-HSV and anti-HCMV activities were demonstrated for some fullerene–amino acid conjugates.[15–18] However, individual and well-characterized water-soluble derivatives remain hardly available and many bio-
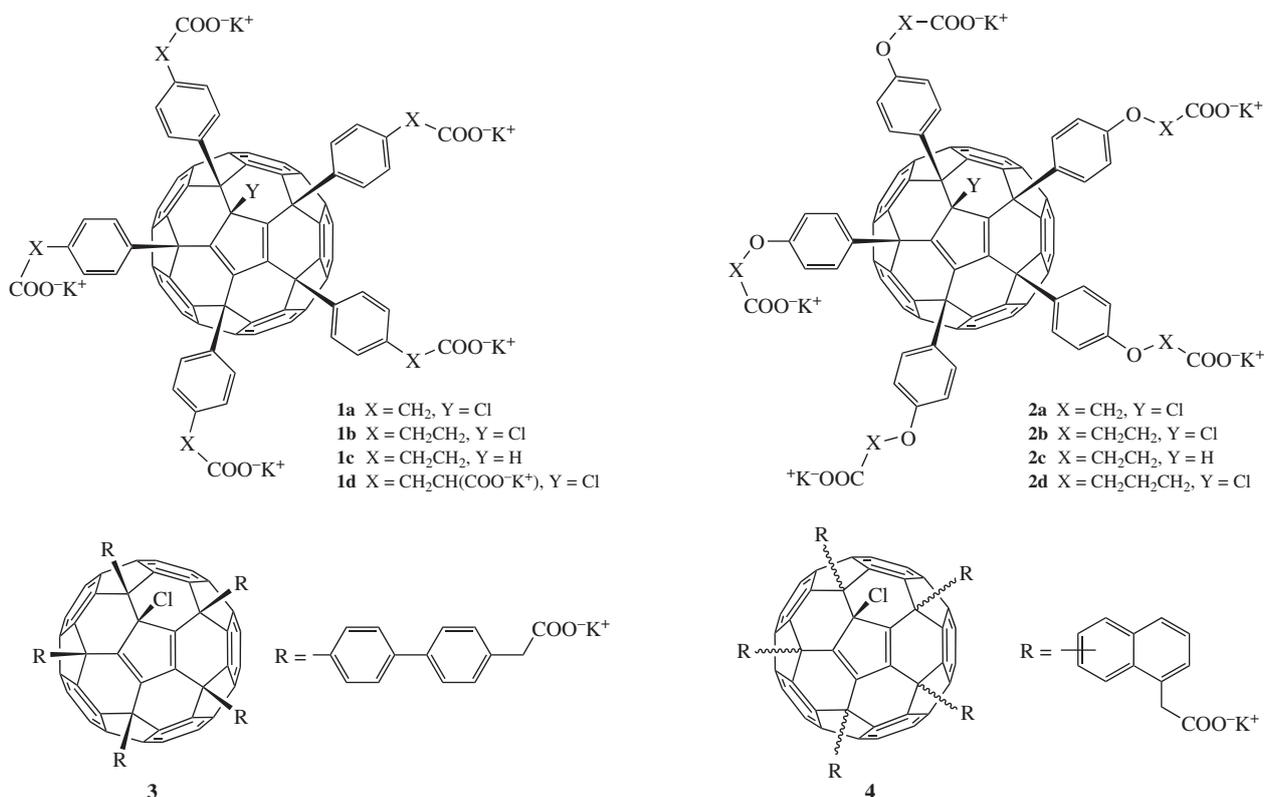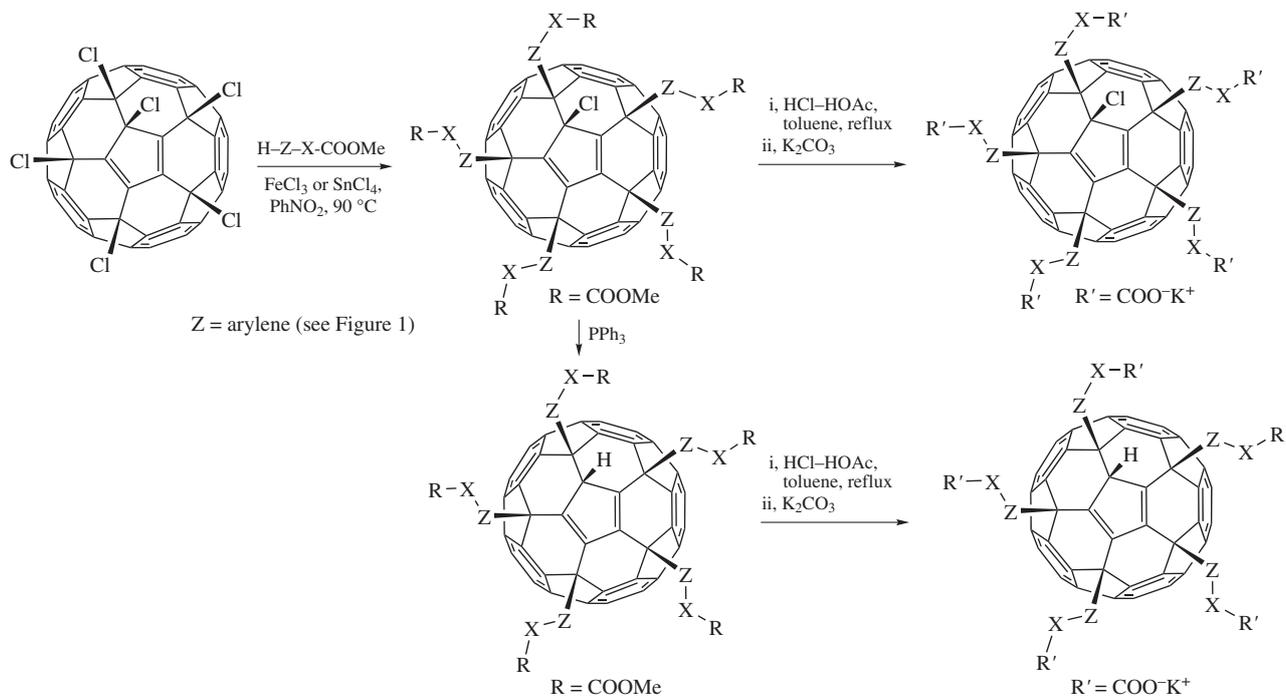

1a X = CH$_2$, Y = Cl
1b X = CH$_2$CH$_2$, Y = Cl
1c X = CH$_2$CH$_2$, Y = H
1d X = CH$_2$CH(COO$^-$K$^+$), Y = Cl

2a X = CH$_2$, Y = Cl
2b X = CH$_2$CH$_2$, Y = Cl
2c X = CH$_2$CH$_2$, Y = H
2d X = CH$_2$CH$_2$CH$_2$, Y = Cl

3

R =

4

R =

Figure 1 Molecular formulas of the investigated water-soluble fullerene C$_{60}$ derivatives.

Scheme 1

logical studies were performed with poorly defined mixtures of fullerene derivatives.

Here we report a systematic study of a big family of individual polycarboxylic fullerene C_{60} derivatives with well defined molecular structures and compositions. Ten water-soluble fullerene derivatives bearing residues of phenylacetic, phenylpropionic, phenylbutyric, phenoxyacetic, phenoxypropionic, phenoxybutyric, 4-biphenylacetic and 1-naphthylacetic acids were appended to the fullerene cage (Figure 1). These compounds have been synthesized following a general synthetic route developed previously[19] and based on the Friedel–Crafts arylation of chlorofullerene $C_{60}Cl_6$ with methyl esters of corresponding arene acid methyl esters followed by hydrolysis of the ester groups (Scheme 1).

The compositions and molecular structures of the synthesized compounds were confirmed by ^1H, ^{13}C and 2D NMR spectroscopy, ESI mass spectrometry, UV-VIS and FT-IR spectroscopy.[†] The potassium salts of the carboxylic fullerene derivatives showed high solubility in water (> 200 mg cm^{-3}) which allows their use in biological research.

We studied cytotoxicity of these compounds in Vero (green monkey kidney epithelial-like cells) and HELF (human embryo lung diploid fibroblasts) culture cells which have the highest sensitivity to HSV and HCMV, respectively. Cell viability was evaluated using dye exclusion assay with Trypan blue. One- and three-day cytotoxicities were determined as concentrations causing death of 50% cells after 24 h and 72 h, respectively. Seven-day cytotoxicity was determined as maximal concentration of the evaluated fullerene derivatives inducing no cytotoxic effects within this period. The concentrations of the fullerene derivatives in the test culture medium were varied from 0.1 to 2500 ug cm^{-3}. Every experiment was repeated three times to ensure reproducibility. The obtained average values are outlined in Table 1.

The obtained results indicate that the carboxylic fullerene C_{60} derivatives have reasonably low one-day cytotoxicity. Vero cells

Table 1 Cytotoxic effects of fullerene C_{60} derivatives in Vero and HELF cell cultures.

Entry	Compound	Cytotoxicity (CC_{50})/ug cm^{-3}					
		One-day[a]		Three-day[b]		Seven-day[c]	
		Vero	HELF	Vero	HELF	Vero	HELF
1	**1a**	977±2	57±2	485±2	50±1	125[d]	1.0
2	**1b**	>2000	80±2	1065±8	40±2	500	6.3±1
3	**1c**	>2000	50±2	1100±18	41±2	500	10.0
4	**1d**	>2000	529±2	1045±17	474±3	1000	10.0
5	**2a**	>2000	60±7	1020±7	45±2	500	6.3±1
6	**2b**	>2000	505±6	1410±6	58±2	1000	—
7	**2c**	>2000	585±2	1400±7	474±26	1000	1.0
8	**2d**	2300±32	1354±5	2000±11	1269±8	1000	10.0
9	**3**	2160±8	>1000	1760±8	670±7	1000	10.0
10	**4**	>2000	662±2	1630±6	486±2	1000	10.0

[a] Concentration causing death of 50% cells, one-day treatment. [b] Concentration causing death of 50% cells, three-day treatment. [c] Non-cytotoxic concentration (100% of cells were viable), seven-day treatment. [d] The concentrations reproducible in three experiments.

displayed higher resistance to the tested compounds compared to the HELF cells. Nine compounds showed very low one-day cytotoxicity (> 2000 ug cm^{-3}) with respect to the Vero cells and only two (**2d** and **3**) fullerene derivatives were low toxic for the HELF cells. Nine fullerene derivatives showed low three-day cytotoxicity in Vero cells with CC_{50} > 1000 ug cm^{-3} (see Table 1). One compound (**1a**) showed CC_{50} = 485 ug cm^{-3}. On the contrary, just a single compound (**2d**) demonstrated very low three-day cytotoxicity (CC_{50} > 1000 ug cm^{-3}) in HELF cell culture. However, four compounds were also low toxic showing CC_{50} in the range of 100–1000 ug cm^{-3}, whereas five other derivatives demonstrated CC_{50} of 10–100 ug cm^{-3}. Seven-day cytotoxicity varied from 1000 to 500 ug cm^{-3} in Vero cells, being < 500 ug cm^{-3} only for one of them (**1a**). At the same time, seven-day cytotoxicity was < 10 ug cm^{-3} for five compounds tested in HELF cell culture.

Different cytotoxicities in Vero and HELF cell lines were reported for other compounds;[21] this effect can be attributed to the tissue-specificity of cultured cells and different chemical

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[†] A detailed description of the experimental procedures and spectral characteristics of the synthesized compounds will be given elsewhere.[20] For synthesis and characteristics of compound **3**, see Online Supplementary Materials.

Table 2 Anti-HSV and anti-CMV activities of fullerene derivatives (an average of three independent experiments).

Entry	Compound	EC$_{50}$^{a}/ug cm^{-3}		CTI^b	
		HSV	CMV	HSV	CMV
1	**1a**	7.4±1	—^{c}	65.5±1.5	
2	**1b**	20±0.5	—^{c}	53±1	—^{c}
3	**1c**	14.8±1.5	0.57±0.1	74±1.5	72±2
4	**1d**	12±2	—^{c}	87±2	—^{c}
5	**2a**	0.52±0.1	—^{c}	1961±7.2	—^{c}
6	**2b**	23±1	3±0.8	61±2.6	19±1.8
7	**2c**	—^{c}	17±1.5	—^{c}	28±1.5
8	**2d**	18±1	10±1	111±8,3	127±9.4
9	**3**	0.98±0.1	—^{c}	1796±10	—^{c}
10	**4**	5.9±1.5	—^{c}	276±5.7	—^{c}

^{a}Concentration inducing 50% inhibition of plaque formation. ^{b}Chemotherapeutic index calculated as CC$_{50}$/EC$_{50}$; CC$_{50}$ – three-day cytotoxicity. ^{c}Antiviral effect was not observed at CC$_{50}$ and lower.

compositions of their plasma membranes. Different cytotoxicities of different fullerene derivatives seem to be related to alternations in the molecular structures of organic addends attached to the fullerene cage. These results are in line with the previous reports indicating that fullerene derivatives produce three-day cytotoxic effects in human fibroblasts in a wide concentration range: CC$_{50}$ = 1500 ug cm^{-3} was obtained for a C$_{60}$-ω-aminocaproic acid-Na conjugate, while C$_{60}$-ω-aminocaproic acid-OH-Na conjugate had CC$_{50}$ = 40 ug cm^{-3}.^{16,22}

Antiviral activities‡ of compounds **1**–**4** are given in Table 2. The obtained results revealed that four compounds have high anti-HSV activity (CTI > 100). Compound **2a** displayed the highest activity with calculated EC$_{50}$ = 0.52 ug cm^{-3} and CTI = = 1961. Compound **3** also inhibited effectively HSV infection (CTI = 1796). Reasonably high anti-HSV activity was revealed for nine fullerene derivatives (see Table 2). Four compounds displayed anti-CMV activity. Compound **1c** inhibited plaque formation at a low concentration of 0.57 ug cm^{-3}, although the CTI did not exceed 72. Compound **2d** was considerably less active (EC$_{50}$ = 10 ug cm^{-3}), however the CTI has approached 127 in this case owing to low cytotoxicity of this compound.

It seems that the antiviral activity of the fullerene derivatives bearing phenoxyalkanoic acid moieties (compounds **2a**–**d**) is superior compared to the characteristics of similar compounds bearing phenylalkanoic ones (compounds **1a**–**d**). Compounds having biphenyl- and naphthylacetic moieties display high anti-HSV activity and do not act against CMV.

In conclusion, we have shown that the water soluble carboxylic fullerene C$_{60}$ derivatives inhibit effectively herpesviral infections in cell cultures. The highest anti-HSV activity was demonstrated by compounds **2a** (CTI = 1961) and **3** (CTI = 1796), whereas **2d** showed potent anti-CMV action (CTI = 127). These compounds seem promising candidates for further investigation and the development of new microbicidal medicines.

‡ Vero and HELF cells were incubated (1 h, 37 °C) with various concentrations of the compounds and then were infected with viruses taken in 10^{-4} PFU/cell concentrations. Virus-specific plaques were revealed and counted three days after HSV-infection and five days after HCMV-infection. Virus-specific plaques were detected by immunocytochemical staining with monoclonal antibodies to HSV1|2 (clone LP1, Abcam, GB) and to HCMV (clone AAC10, DAKO, Denmark). Antiviral activity was assessed by inhibition of the plaque formation in experimental and control groups. Effective concentration (EC$_{50}$) was determined as a concentration of the fullerene derivative inducing 50% inhibition of plaque formation compared to the control series. Chemotherapeutic index (CTI), named also as selectivity index (SI), was calculated as CC$_{50}$/EC$_{50}$.

Online Supplementary Materials
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2012.09.009.

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