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Synthesis and biological activity of a novel water-soluble methano[60]fullerene tetracarboxylic derivative

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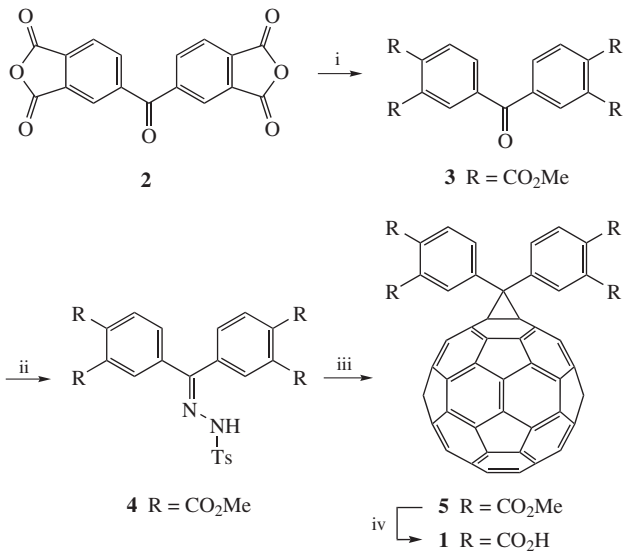
A reaction of 3,3′,4,4′-tetrakis(methoxycarbonyl)benzophenone tosylhydrazone with fullerene C_{60} was used to obtain a novel methano-[60]fullerene derivative bearing four carboxylic groups, whose sodium and potassium salts are water-soluble. The compound possesses antiviral and anticancer activity as well as pronounced antioxidant properties in combination with a low toxicity.

Fullerenes and their functional derivatives possess antiviral, antibacterial and anticancer activity along with efficient antioxidant and radioprotector properties.[1] Some new fullerene derivatives are active against *Mycobacterium tuberculosis*[2] and two viruses of *Herpesviridae* family.[3] They are also promising for drug[4] and gene delivery[5] and in medical diagnostics (*e.g.*, MRI contrast agents).[6] Unmodified fullerenes[7] and some of their derivatives[8] increase lifespan of laboratory animals and improve their cognitive performance which makes the development of fullerene-based pharmaceuticals against age-related disorders promising.

Low solubility of pristine fullerenes in aqueous media leading to non-optimal pharmacokinetics and serious excretion difficulties is a problem to be solved.[9] Incorporation of ionic groups into a molecule is one means to enhance solubility in water.[10–14] Fullerenes modified with protonated or quaternized amine groups often exhibit high toxicity typical of polyamines.[15] On the contrary, fullerene derivatives bearing several carboxyl groups (typically, more than three) show reasonable solubility in water and low toxicity.[16–19]

Previous syntheses of such compounds involved use of large and sophisticated organic addends[18] or multiple additions to the fullerene cage,[19] which can exert undesired influence on stereoelectronic, photophysical and biological properties of a resulting fullerene derivative.

Here we report the synthesis of a novel water-soluble fullerene **1** modified with a reasonably small solubilizing organic addend having four carboxylic groups (Scheme 1).[†] At the first step the



2 **3** R = CO_2Me **4** R = CO_2Me **5** R = CO_2Me **1** R = CO_2H

Scheme 1 *Reagents and conditions*: i, MeOH, $SOCl_2$, Δ, 3 h; ii, $TsNHNH_2$, MeOH, Δ, 2 h; iii, C_{60}, MeONa, Py, 1,2-DCB, 100 °C, 3 h, then Δ, 10 h; iv, MeONa, PhCl–MeOH–H_2O, 70 °C, 15 min, then H_2O/HCl.

† *3,3′,4,4′-Tetrakis(methoxycarbonyl)benzophenone* **3**. Dianhydride **2** (35 g, 0.11 mol) was added to 150 ml of methanol and heated at reflux for 3 h. After cooling, thionyl chloride (105 g, 0.88 mol) was added with intense stirring. The resulting solution was stirred for 3 days at room temperature and then concentrated at the rotary evaporator. The resulting residue was dissolved in 200 ml of diethyl ether, washed with 5% aqueous solution of K_2CO_3 (2×100 ml), dried over calcined $MgSO_4$, filtered and concentrated to give 36.7 g (78%) of compound **3** as a viscous liquid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 3.90 (s, 6H), 3.91 (s, 6H), 7.63 (d, 2H), 7.67 (d, 2H), 7.90 (s, 2H).

Compound **5**. *p*-Toluenesulfonylhydrazine (7.0 g, 37 mmol) was added to a solution of **3** (16.6 g, 37.0 mmol) dissolved in 100 ml of methanol. The mixture was heated at reflux for 2 h and cooled. Methanol was removed under reduced pressure leaving 21.8 g (quantitative) of tosylhydrazone **4** as a cream-coloured resin which was used at the next step without purification. Fullerene C_{60} (1.5 g, 2.13 mmol) was dissolved in 1,2-dichlorobenzene (200 ml) in an argon atmosphere. Hydrazone **4** (2.0 g, 3.43 mmol), MeONa (187 mg, 3.43 mmol) and pyridine (5 ml) were added. The mixture was stirred at 100 °C for 3 h, cooled, filtered, concentrated *in vacuo* to the volume of 50 ml and subjected to the column chromatography on silica (40–60 μm, 70 Å, Acros). Elution with pure toluene produced 210 mg of unreacted C_{60}. The product **5** was eluted with toluene–methanol (99:1 v/v) mixture as a dark red-brown solution giving after concentration 860 mg of a black solid. ^{1}H NMR analysis of the obtained material revealed the presence of *ca.* 20% of unisomerized fulleroid with an open-cage structure as an impurity. To complete the isomerization, product was dissolved again in 50 ml of 1,2-dichlorobenzene in an argon atmosphere, heated at reflux for 10 h and purified by column chromatography as above. The methanofullerene derivative **5** was obtained as a dark solid in 42% yield (840 mg) based on consumed C_{60}. ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 3.96 (s, 6H), 3.98 (s, 6H), 7.87 (d, 2H), 8.35 (d, 2H), 8.49 (s, 2H). ^{13}C NMR, δ: 52.93, 53.02, 55.36, 77.45, 129.64, 131.32, 132.34, 132.51, 133.82, 138.26, 141.08, 141.11, 142.11, 142.16, 143.03, 143.05, 143.07, 143.81, 144.51, 144.79, 144.80, 145.04, 145.25, 145.29, 146.50, 167.16, 167.73. Found (%): C, 87.08; H, 1.78. Calc. for $C_{81}H_{18}O_8$ (%): C, 86.94; H, 1.62.

commercially available 3,3′,4,4′-benzophenonetetracarboxylic dianhydride **2** was converted to tetramethyl ester **3** and then tosylhydrazone **4**. The Hummelen–Wudl reaction[20] of compound **4** with fullerene C_{60} produced methanofullerene **5**.

Our initial attempts to hydrolytically cleave the ester groups in compound **5** under the typical acidic conditions[20,21] were unsuccessful. However, we have found that the ester groups in **5** can be easily hydrolyzed under alkaline conditions using a small excess (1.05 equiv. per one ester group) of methanolic sodium hydroxide solution. The water-soluble target compound **1** was prepared in four steps from easily available precursors with good overall yield of 40% based on consumed C_{60}.

Tetracarboxylic compound **1** in the form of free acid does not show any noticeable solubility in pure water at neutral pH (a behaviour characteristic of vast majority of polycarboxylic fullerene derivatives). However, the corresponding tetrasodium salt (designated below as **1**-Na) and tetrapotassium salt (**1**-K) of **1** exhibited high solubility in water (>100 mg cm^{-3}) giving transparent solutions stable over at least 6 months. High solubility of **1**-Na allowed us to investigate its biological activity in few *in vitro* and *in vivo* systems.

First of all, we studied a cytotoxicity of compound **1**-Na in a range of eukaryotic cell lines (murine leukemia cells L1210/0, human T-lymphocyte cells Molt4/C8 and CEM/0, human cervix carcinoma cells HeLa and human osteosarcoma cells Ost/TK$^-$). At the highest investigated dose of 100 μg cm^{-3} (86 μM) this derivative did not reveal any sign of cytostatic action.

Antiviral activity of **1**-Na was investigated against HIV-1 and HIV-2 in an *in vitro* assay according to procedure described previously.[21] The compound showed comparable activity against both virus strains and was characterized by mean effective inhibitory concentrations IC_{50} of 20±10 (HIV-1) and 29±1 μM (HIV-2). The results suggest that **1**-Na is not a very promising antiviral agent since its activity is *ca*. 10 times lower compared to many other fullerene derivatives.[17,21,22] However, the observed low cytostatic activity of this compound implies that its biological activity might be investigated using various *in vivo* models.

The acute toxicity of **1**-Na was evaluated after intraperitoneal (*i.p.*) injection in mice (male BDF_1 hybrids, weight 20–25 g) with determination of main toxicological parameters: maximum tolerable dose LD_0, median lethal dose LD_{50} and absolute lethal dose LD_{100}. The values were found to be 400, 500 and 700 mg kg^{-1}, respectively, characterizing this compound as a very low-toxic substance.

The anticancer activity of **1**-Na was assessed on murine leukemia P388 tumor-bearing mice. For this purpose, leukemia P388 (10^6 cells) was *i.p.* inoculated into male BDF_1 hybrid mice. After that, compound **1**-Na was given at the dose of 167 mg kg^{-1} ($^1/_3LD_{50}$) as *i.p.* injections on the 1st, 3rd, 4th and 6th days after tumor transplantation. The increase of average lifespan (ILS) was determined to be 46% for the fullerene-treated mice in comparison with the control group. Thus, compound **1**-Na showed a weak, but still evident anticancer action in the mouse tumor model.

Table 1 Antioxidant and antiradical activity of compound **1**-Na compared to butylated hydroxytoluene (BHT).

Compound	MDA concentration (% to control)	Standard deviation (%)	Luminol chemiluminescence (% to control)	Standard deviation (%)
Control	100	12	100	8
1-Na (10^{-5} M)	47	7	82	7
BHT (10^{-5} M)	59	7	81	5

The synthesized fullerene derivative **1** (in the form of sodium salt) exhibited antiviral and anticancer activity. Although, the revealed therapeutic effects are not strong enough to consider practical implementation of this compound, its very low toxicity makes the application of this compound as a biocompatible form of fullerene C_{60} promising, *e.g.*, for antioxidative, neuroprotective or drug delivery applications.

To reveal the performance of **1**-Na as a potential neuroprotective agent, the inhibition of the lipid peroxidation and radical scavenging activity were studied in *ex vivo* models using a well-known antioxidant BHT (3,5-di-*tert*-butyl-4-hydroxytoluene) as a reference compound. Malondialdehyde (MDA) production characterizing lipid peroxidation level was measured spectrophotometrically in the rat brain homogenates after the reaction with thiobarbituric acid.[23] Radical scavenging activity of **1**-Na was determined using the luminol chemiluminescence assay applied to the rat brain homogenates after initiation of the lipid peroxidation with *tert*-butyl hydroperoxide.[24,25] The obtained results are shown in Table 1. Compound **1**-Na at a concentration of 10^{-5} mol dm^{-3} suppressed MDA production down to 47% of its control value in comparison with 59% for BHT applied at the same concentration. Thus, **1**-Na showed somewhat higher antioxidant activity compared to the reference antioxidant. However, the chemiluminescence of luminol was quenched only by 18% which implies that **1**-Na does not perform as an efficient scavenger of active oxygen species formed during the lipid peroxidation. It is even possible that **1**-Na catalyzes peroxide dismutation like some other fullerene derivatives reported previously.[8] Detailed study of this aspect is now underway.

In conclusion, we have synthesized a novel highly water-soluble C_{60} derivative which exhibited promising biological properties such as low toxicity in combination with noticeable antiviral, antitumor and antioxidant activity.

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61,61-Bis(3,4-dicarboxyphenyl)methano[1,9](C$_{60}$-I$_h$)[5,6]fullerene **1**. Compound **5** (300 mg, 0.27 mmol) was dissolved in 150 ml of chlorobenzene under heating at 70 °C. The addition of the solution of 122 mg of MeONa (1.13 mmol, 1.05 equiv.) in 3 ml of MeOH–H_2O (9:1 v/v) to the solution of **5** induced immediate precipitation of some insoluble product and decolouration of the solution. The mixture was heated at 70 °C for further 15 min and cooled. The precipiated solid was collected by centrifugation, dissolved in 10 ml of deionized water and quenched with 1 ml of 5% HCl. The precipitated tetraacid **1** was collected by centrifugation, washed several times with water and dried *in vacuo*. The yield of **1** obtained as a dark brown powder was 275 mg (96%). ^{1}H NMR (DMSO-d_6, 600 MHz) δ: 7.86 (d, 2H), 8.75 (d, 2H), 8.80 (s, 2H). ^{13}C NMR (DMSO-d_6, 150 MHz) δ: 56.34, 65.40, 128.68, 129.37, 132.85, 133.93, 134.32, 137.46, 140.45, 141.36, 142.08, 142.17, 142.85, 142.95, 143.81, 144.24, 144.54, 144.63, 144.77, 145.13, 145.15, 146.50, 148.56, 168.58, 168.94. ESI-MS, *m/z*: 1061.0 [M–H]$^-$.

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