Chlorofullerene $C_{60}Cl_6$: a precursor for straightforward preparation of highly water-soluble polycarboxylic fullerene derivatives active against HIV†

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We report for the first time the application of chlorofullerene $C_{60}Cl_6$ as a substrate for straightforward preparation of highly water-soluble fullerene derivatives, promising compounds for investigation of the biological action of fullerenes *in vitro* and *in vivo*. Methyl esters of phenylacetic and benzylmalonic acids were used as reagents in the Friedel–Crafts arylation of $C_{60}Cl_6$ that resulted in the corresponding $C_{60}(Ar)_5Cl$ compounds with 50–60% yields. The following cleavage of ester groups in phenylacetic and benzylmalonic residues was accomplished almost quantitatively to yield the corresponding fullerene-based acids bearing 5 and 10 carboxylic groups, respectively. The relatively-low solubility of these acids in water can be strongly enhanced (up to 150–200 mg ml^{-1}) by their conversion to salts with alkali metal cations. These fullerene salt derivatives showed pronounced anti-HIV action and low toxicity; these two findings point to the necessity for further investigation of the biological properties of the here-reported compounds.

1 Introduction

The list of exciting biological activities of water-soluble fullerene derivatives includes the inhibition of enzymes of human immunodeficiency and hepatitis C viruses,[1,2–3] anticancer action, antiproliferative effects,[4] photodynamic therapy[5–6] and efficient neuroprotective activity.[7] Some fullerene derivatives can be successfully applied as advanced bacteriostatic agents[4,8] and contrast materials in X-ray and magnetic resonance imaging.[9–10] There are numerous published reviews that describe the state of the art in this field.[11,12,13,14]

The development of the biological chemistry of fullerenes is now mostly limited by the availability of novel truly water-soluble fullerene derivatives with specific and well-defined structures. The most detailed investigations were carried out with a family of fullerene-based carboxylic acids represented by two isomers (C_3 and D_3) of malonic acid derivative $C_{60}[C(COOH)_2]_3$ and symmetrical dendro[60]fullerene that have 18 carboxylic groups reaching a solubility of 30 mg ml^{-1} at pH = 7.4 and ~250 mg ml^{-1} at pH = 10. Preparation of these compounds is quite challenging and this, perhaps, hinders their wide application. A fullerene derivative bearing five residues of benzoic acid was synthesized recently from C_{60} in three steps.[15] However, the availability of this compound can be also questioned because the first stage of the synthesis is based on application of organocopper reagents and requires a vigorously-deoxygenated atmosphere, decreased temperatures and HPLC processing for product isolation.

The aim of the present work was to develop a straightforward route for high-yield preparation of water-soluble fullerene-based polycarboxylic acids using cheap and readily-available precursors. Conventional cycloaddition reactions are not efficient enough due to the poor selectivity of multiple additions. Therefore, readily-available chlorofullerene $C_{60}Cl_6$ was considered as a substrate of choice. There are five labile halogen atoms in the molecule of $C_{60}Cl_6$ arranged around one 5-membered ring in the [60]fullerene cage that can be selectively substituted by allyl,[16] methyl,[17] alcoxide[18] and aromatic hydrocarbon residues.[19] Very recently, a substitution of chlorine atoms in $C_{60}Cl_6$ by amine residues was used for preparation of water-soluble compounds.[20] Here we modified the Friedel–Crafts arylation procedure of $C_{60}Cl_6$ reported previously[19] and applied esters of phenylacetic and benzylmalonic acids as reagents to prepare fullerene-based polycarboxylic acids.

2 Results and discussion

2.1 Preparation of $C_{60}Cl_6$

Synthesis and structural characterization of chlorofullerene $C_{60}Cl_6$ was reported in 1993, thus this compound was among the first halides discovered for [60]fullerene.[21] The reaction of C_{60} with ICl in dry benzene within 3 days was the first procedure used for the preparation of $C_{60}Cl_6$.[21] During early studies on the reactivity of $C_{60}Cl_6$, it was shown that a preparation of $C_{60}Cl_6$ in benzene is accompanied by its reaction with the solvent (I_2 is known to participate as a catalyst in such reactions) and the formation of various $C_{60}Ph_xCl_{6-x}$ species that can represent up to 25% of the crude $C_{60}Cl_6$ sample. The isolation and X-ray single-crystal

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structure characterization of pure $C_{60}Me_4PhO_2OH$ derived from $C_{60}PhCl_5$ unambiguously proved this.[17]

These findings showed quite obviously that one should use less reactive solvents for fullerene chlorination to obtain high-purity $C_{60}Cl_6$ samples. The first encouraging results were reported when 1,2-dichlorobenzene (1,2-DCB) was applied as a solvent for the chlorination of C_{60} by ICl and ICl_3.[22] It was shown that the duration of chlorination and reagent ratios are the two main factors that govern the selectivity of the reaction and product composition. In particular, it was shown that pure $C_{60}Cl_6$ can be prepared within 5 minutes if the chlorinating reagent is used in large excess (40 eq. of ICl or 20 eq. of ICl_3).[22]

Some time later, a similar short-time chlorination method was reported, called the "seven minute synthesis of pure $C_{60}Cl_6$".[23] According to the described procedure, C_{60} was treated with 30 eq. of ICl in chlorobenzene within 7 minutes. MALDI TOF mass spectrometry and HPLC analysis showed that the short-time chlorination in chlorobenzene results in an increased purity of $C_{60}Cl_6$ samples in comparison with the reference syntheses in benzene. Unfortunately, this "seven minute" procedure[23] was not compared with the *short-time* chlorination of fullerene in 1,2-DCB reported before.[22]

It was proved here that the short-time chlorination of fullerene in 1,2-DCB can be used for preparation of $C_{60}Cl_6$ and gives as good results as reported recently "seven minute" synthesis. In particular, the reaction of C_{60} with 30 eq. of ICl was conducted in dry 1,2-DCB at a conventional rotary evaporator within 4–8 min at 40 °C under a pressure of ~1 mbar. It was found that a rotary evaporator also gives perfect results with chlorobenzene and, therefore, there is no need for the special equipment designed earlier.[23] The synthesis of $C_{60}Cl_6$ in 1,2-DCB is described in more detail in the Experimental section.

The ^{13}C NMR spectrum confirmed the high purity of the $C_{60}Cl_6$ sample prepared in 1,2-DCB: no signals from any impurities were detected (see the ESI, Fig. S1†). The ^{13}C NMR spectrum of the reference batch of $C_{60}Cl_6$ synthesized in accordance with the "seven minute" procedure was identical. It is notable that $C_{60}Cl_6$ used as a starting material in this work and in our previous studies[20] was synthesized using 1,2-DCB as a solvent according to the method described here and in earlier work.[22]

Notable also is the application of $POCl_3$ for chlorination of C_{60} in the sealed tubes in the absence of any solvent.[24] This method allows preparation of pure $C_{60}Cl_6$ at the 20–30 mg scale, but it seems to be less promising for bulk preparations.

2.2 Synthesis of compounds 2a–b from $C_{60}Cl_6$

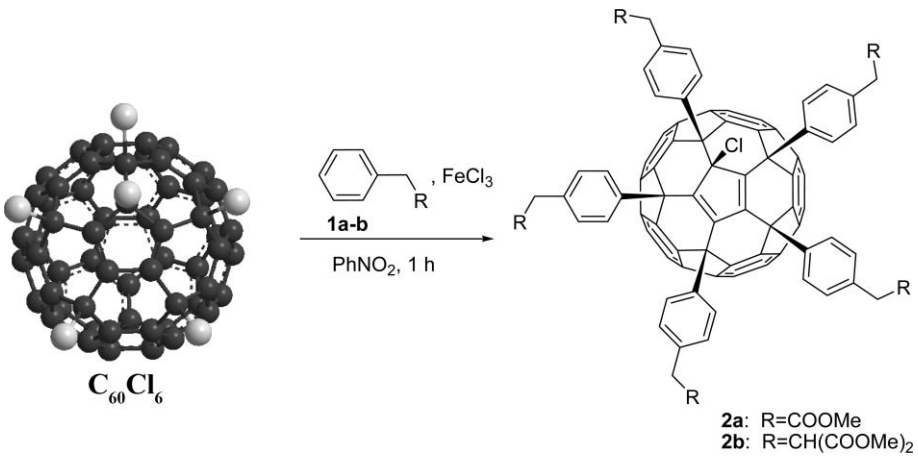
All previously-reported Friedel–Crafts arylation reactions of $C_{60}Cl_6$ were carried out with liquid aromatics such as benzene, toluene, fluorobenzene, anisole, *tert*-butylbenzene, thiophene and phenyltrimethylsilane. A large excess of the reagent served as a solvent in most of these cases.[25] Very active aromatics react with $C_{60}Cl_6$ in dichloromethane at low temperatures (30–40 °C).[25]

Methyl esters of phenylacetic and benzylmalonic acids **1a**–**b** were found to be quite inert towards $C_{60}Cl_6$; the corresponding solvent-free reactions catalyzed by $FeCl_3$ can be initiated only at elevated temperatures (above 90 °C). These syntheses were very reagent-consuming (>10 g of **1a**–**b** to 100 mg of $C_{60}Cl_6$) and did not give high yields of the expected $C_{60}Ar_5Cl$ compounds because of the formation of elimination products such as $C_{60}Ar_4$ and $C_{60}Ar_2$.

To decrease the amount of reagents consumed and improve the selectivity of the reactions, several solvents were examined as potentially-suitable media for these syntheses. It was shown that both chlorobenzene and 1,2-DCB compete with **1a** in the reaction with $C_{60}Cl_6$. A column chromatography separation of the resulting reaction mixture gave multiple badly-resolved fractions that were represented by $C_{60}Ar_x(Ar')_{5-x}Cl$ (Ar′ corresponds to the solvent residue) species was revealed by MALDI TOF mass spectrometry.

Nitrobenzene‡ was the optimal solvent, since it does not undergo a detectable reaction with $C_{60}Cl_6$ in the presence of **1a**–**b** and gives high yields of corresponding products **2a**–**b** (Scheme 1). Compounds **2a**–**b** were eluted as broad bright-orange fractions during chromatographic separation on silica; their high compositional purity was evidenced by MALDI-TOF mass spectrometry, ^1H and ^{13}C NMR spectroscopy (Fig. 1). It is important that these compounds can be isolated without the use of expensive and time-consuming HPLC processing, thus allowing for easy up-scaling of these preparations.

‡ CAUTION: A TOXIC SOLVENT.



Scheme 1

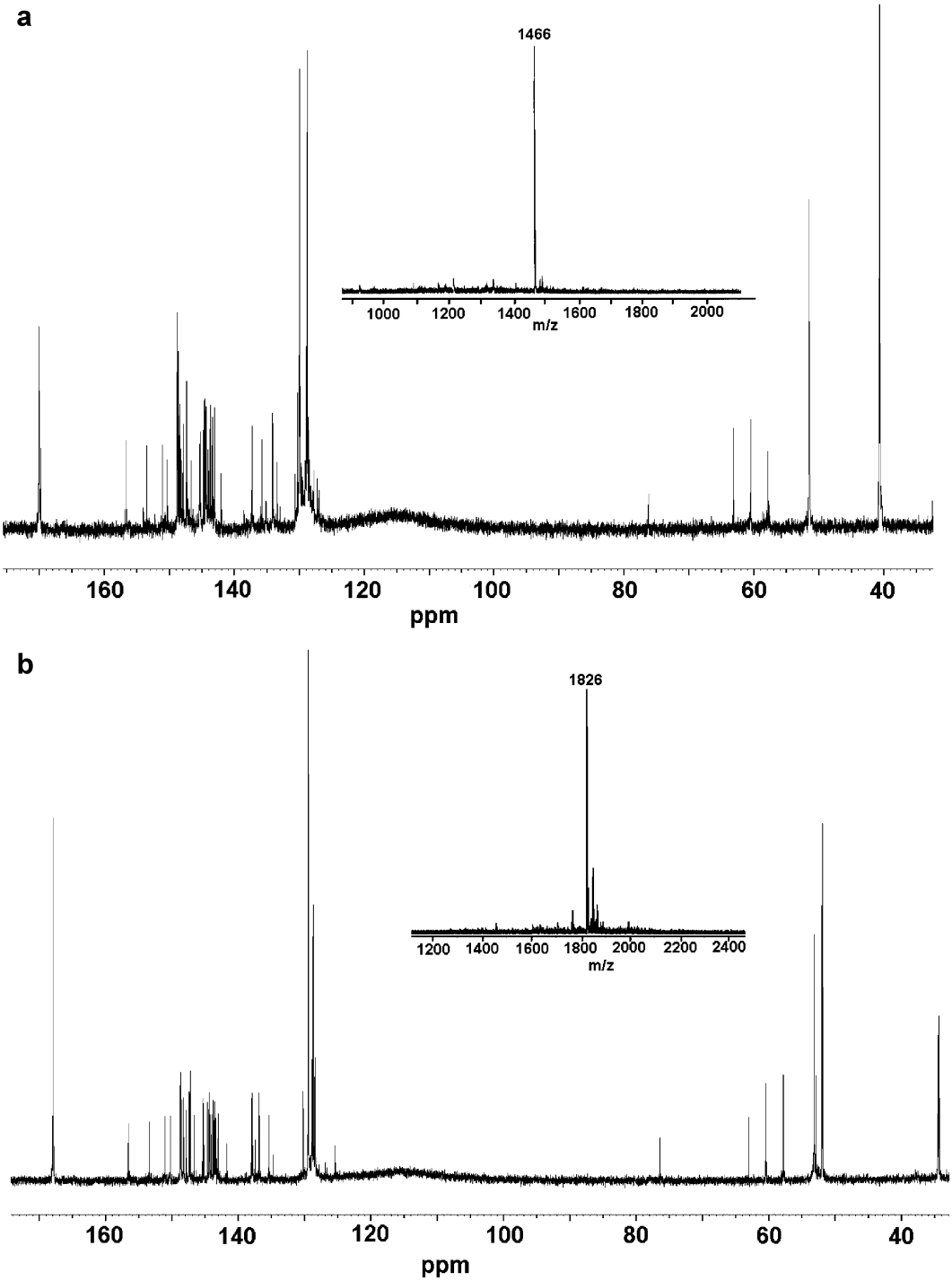


Fig. 1 The ^{13}C NMR spectra of compounds **2a** (a) and **2b** (b) (CS_2-acetone-D6). Insets show corresponding MALDI TOF mass spectra.

The MALDI-TOF mass-spectra of **2a**–**b** exhibited intense peaks corresponding to the fragment ions $[C_{60}Ar_5 \cdot H]^+$ (1466 and 1826 amu for **2a** and **2b**, respectively). The parent molecular ions of $C_{60}Ar_5Cl$ have not been detected due to a facile loss of chlorine atom; similar results were observed previously for fluorophenyl derivatives under EI mass spectrometry conditions.[21] The low-intensity peaks corresponding to the ions $[C_{60}Ar_5OH]^+$ (1842 amu) and $[C_{60}Ar_5OHNa]^+$ (1865 amu) were observed in the mass spectrum of **2b**. $C_{60}Ar_5Cl$ probably undergoes partial hydrolysis to $C_{60}Ar_5OH$ during the sample preparation for the MALDI TOF measurements. The replacement of Cl by OH was documented before for similar compounds.[21]

Nearly 30 independent syntheses were performed where the methyl ester of phenylacetic acid (**1a**) was treated with $C_{60}Cl_6$ in nitrobenzene. The obtained samples of **2a**, as well as small amounts of other isolated products, were analyzed by MALDI TOF MS. It was shown that both $C_{60}(C_6H_4CH_2COOMe)_6$ and $C_{60}(C_6H_4CH_2COOMe)_4$ are formed along with **2a** and can be

isolated with a satisfactory compositional purity (see the ESI, Fig. S2†). However, the amounts of these two compounds were not sufficient for ^{13}C NMR characterization.

Interestingly, Na^+-containing positive ions frequently appeared in the MALDI TOF mass spectra of **2a** and related by-products. For example, the signals of $[C_{60}(C_6H_4CH_2COOMe)_n+Na]^+$ are virtually equal in intensity to the signals of the parent $[C_{60}(C_6H_4CH_2COOMe)_n]^+$ ions (n = 5 and 6; see the ESI, Fig. S2b†). The statistics allowed us to conclude that Na^+ comes from sodium sulfate used in the workup procedure *before* the chromatographic separation (see the Experimental section). No Na^+-containing species were observed in the mass-spectra when sodium sulfate was replaced by $MgSO_4$ and this effect was very reproducible. It seems that **2a** and related products have a strong affinity for Na^+ ions. Indeed, **2a** has 10 oxygen atoms with their lone electron pairs that can be arranged in the right geometry for complex formation with Na^+, like in the case of crown ethers.

The ^{13}C NMR spectra of **2a**–**b** confirmed the high compositional purity of these compounds. Both spectra were similar and corresponded to the C_s symmetrical structures shown in Scheme 1. Particularly, there are 31 fullerene sp^2 carbon signals along with four peaks from sp^3 cage carbons in each spectrum. Three resonances at 57–64 ppm correspond to the fullerene carbons bearing organic addends, while the signal at *ca*. 76 ppm was assigned to the *C*-Cl moiety. Similar chemical shifts of these signals were observed in the ^{13}C NMR spectra of other $C_{60}Ar_5Cl$ derivatives reported previously.[25] The ^1H NMR spectra of **2a**–**b** (not shown) consisted of six doublets with integration intensities 1 : 1 : 2 : 2 : 2 : 2 as it can be expected for their structures.

2.3 Cleavage of ester groups in 2a–b and isolation of polycarboxylic acids 3a–b

The hydrolysis of the ester groups in fullerene derivatives is a quite challenging task. Fullerene cages undergo rapid, irreversible polyhydroxylation in alkaline media,[26] so saponification of ester groups can hardly be applied in this case.

The treatment of fullerene derivatives bearing ester groups with NaH, quenching of the reaction mixture with MeOH and the following acidification was reported to yield the corresponding acids.[7,27] Attempted hydrolysis of ester **2a** according to this procedure gave a mixture of products (instead of the expected acid **3a**), as was revealed by ^1H NMR, chemical analysis and MALDI TOF mass spectrometry (Scheme 2). Identified from the mass spectrum components of this mixture were oxidized fullerenols $C_{60}O_x(OH)_y$ ($x + y$ = 2–8) (that is in line with other studies[28]) and toluene addition products $C_{60}(PhCH_2)_nH_n$, n = 1–6. The peaks corresponding to the fragment ions of the parent ester **2a** (1466 amu, [**2a**-Cl]·H^+) and partially-hydrolyzed compound $[C_{60}(C_6H_4CH_2COOMe)_4(C_6H_4CH_2COOH)\cdot H]^+$ were also quite intensive. Very intensive peaks due to C_{58}^+ and $C_{57}O^+$ ions were also observed, most likely due to the fragmentation of oxidized species $C_{60}O_x(OH)_y$, as it was observed before.[21]

An alternative, very mild hydrolysis procedure utilizing $(CH_3)_3SiI$ as a reagent was reported to give good results with fullerene-based esters.[29] However, **2a** was not hydrolyzed completely, even after 5 days stirring at 50 °C in CCl_4.

Finally, continuous heating of fullerene derivatives bearing ester groups in 1,2-DCB–HCl–CH_3COOH mixtures was reported to produce corresponding acids in good yields.[30] This procedure was also beneficial for our systems and allowed us to accomplish hydrolysis of **2a**–**b** to corresponding acids **3a**–**b** with almost quantitative yields (>85%, Scheme 2). Addition of trifluoroacetic acid into the mixture CH_3COOH–HCl strongly accelerated hydrolysis as it was monitored by TLC. Chlorobenzene was used instead of 1,2-dichlorobenzene since the optimal reaction temperature in our experiments was relatively low (60 °C).

Fullerene-based acid **3a** was initially characterized by high-resolution negative mode ESI-MS (Fig. 2). The ESI mass spectrum of **3a**, similarly to the MALDI TOF mass spectrum of **2a**, did not show the parent Cl-containing molecular ion because of facile dechlorination. However, the presence of chlorine in **3a** was evidenced by chemical analysis data that were in a good agreement with the structure shown in Scheme 2. The mass spectrum of **3a** was represented by intense signals at m/z 1412.20 ([**3a**-Cl+OH]$^-$), 1396.22 ([**3a**-Cl+H]$^-$), 705.60 ([**3a**-Cl+O]$^{2-}$), 697.62 ([**3a**-Cl]$^{2-}$)

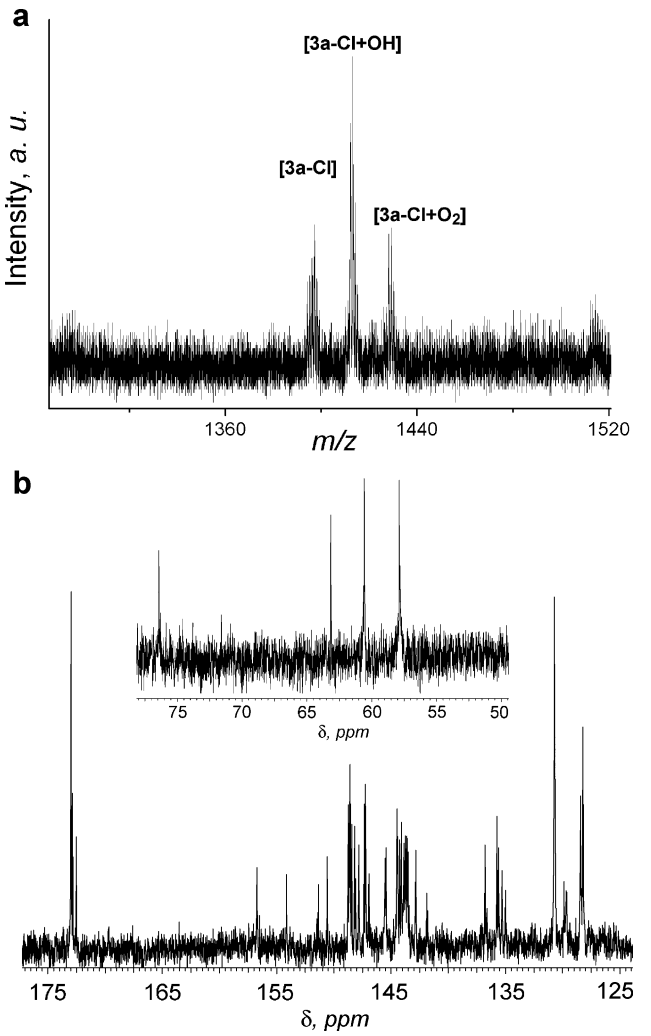
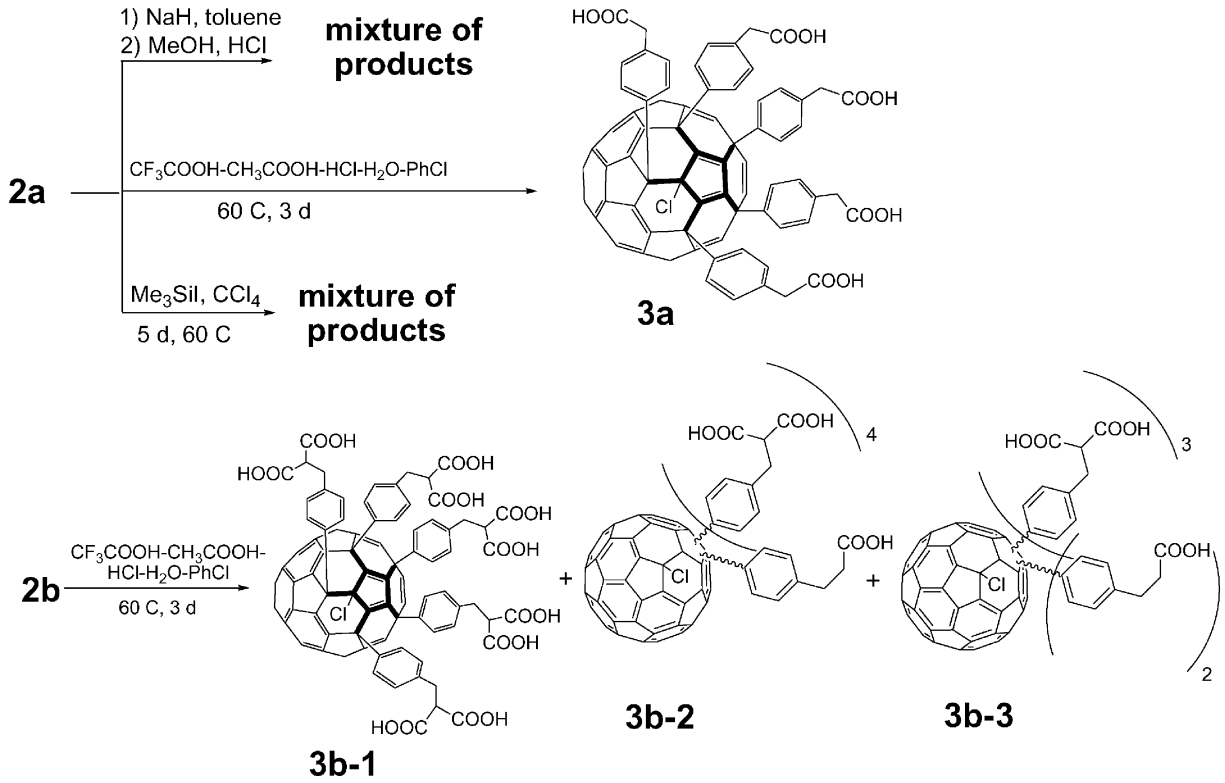


Fig. 2 (a) A part of the ESI mass spectrum which shows the signals of [**3a**–**H**]$^-$ and other detected monoanions. A part of the spectrum where the signals of [**3a**–**2H**]$^{2-}$ and [**3a**–**3H**]$^{3-}$ appeared is not shown. (b) The ^{13}C NMR spectrum of **3a** (DMSO-D6). The inset shows the high-field part of the spectrum. The signals due to the CH_2 groups, presumably overlapped with the DMSO-D6 peak at 38–41 ppm, are not shown.



Scheme 2

amu corresponding to the title compound **3a**. There were also quite pronounced peaks at *m/z* 1530.33 ($[C_{60}(C_6H_4CH_2COOH)_6]^-$), 764.61 ($([C_{60}(C_6H_4CH_2COOH)_6\text{-}H]^{2-}$) and 713.59 ($[\mathbf{3a}\text{-}Cl+O_2]^{2-}$) amu. Since the starting sample of **2a** cannot contain more than 5–8% of impurities (NMR data), the presence of substantial amounts of $C_{60}(C_6H_4CH_2COOH)_6$ or oxidized species as admixtures to **3a** is hardly probable. Perhaps oxygen-containing compounds ([**3a**-Cl+OH], $[\mathbf{3a}\text{-}Cl+O_2]$) were formed from **3a** *via in situ* oxidation under the ESI conditions.

The ^{13}C NMR spectrum of **3a** also supported the high compositional purity of this compound (Fig. 2). The sp^2 part of the spectrum consisted of 44 signals, including three peaks due to the –*C*OOH groups and a number of broadened signals corresponding to five aryl residues. This allows one to make conclusions about the C_s molecular symmetry of **3a**. The absence of signals corresponding to MeO– groups in the high-field part of the spectrum provides evidence of complete hydrolysis of the parent ester **2a**. The signals at 57.88, 60.59 and 63.16 ppm correspond to the fullerene cage sp^3 carbons bearing aryl groups, while the peak at 76.42 was assigned to the carbon bearing chlorine atom. Thus, both the ESI MS and ^{13}C NMR data confirmed that hydrolysis of **2a** to **3a** was accomplished with high selectivity.

In contrast, hydrolysis of **2b** proceeded less smoothly. The ESI MS spectrum (see the ESI, Fig. S3†) of the crude product revealed the presence of several major components: the target compound **3b1** (1720.32 amu) and two other products: **3b2** (1676.33 amu) and **3b3** (1632.29 amu) formed from **3b1** *via* the loss of one or two carboxylic groups, respectively (Scheme 2). Interestingly, the parent molecular ions $[\mathbf{3b1}\text{–}\mathbf{3}]^-$ appeared in the spectrum with comparable intensities to the ions of corresponding dechlorinated species $[(\mathbf{3b1}\text{–}\mathbf{3})\text{-}Cl+H]^-$. The ^{13}C NMR spectrum of **3b** also revealed the presence of at least four components in the sample; there are 3–4 detectable independent sets of signals in the sp^3 region and numerous badly-resolved peaks in the sp^2 part of the spectrum (see the ESI, Fig. S3†).

The formation of **3b2** and **3b3** is a result of decarboxylation under the hydrolysis conditions; this result is very typical for substituted malonic acids. A mixture of acids **3b1**–**3** gives highly water-soluble salts that were also used for biological investigations.

2.4 Solubility of polycarboxylic acids 3a–b and their salts in water

The fullerene acids **3a**–**b** are almost completely insoluble in water. However, they can be easily dissolved in a drop of DMSO and then diluted with a large amount of water (10–100 volumes with respect to the volume of DMSO) without any sign of precipitation. Such a character of solubility was observed for many other "water-soluble" fullerene derivatives and, particularly, for malonic acid derivatives $C_{60}[C(COOH)_2]_3$.[7]

The solubility of **3a**–**b** in water can be strongly enhanced by their conversion to corresponding ionic potassium salts. These salts can be obtained by addition of a concentrated solution of **3a** and **3b1**–**3** (*ca.* 10 mg ml^{-1}) in THF or other hydrophilic solvents (DMSO, 1,4-dioxane, acetone) to aqueous solution containing 2.5 (for **3a**) or 5 (for **3b1**–**3**) equivalents of K_2CO_3. The resulting transparent solutions can be concentrated to dryness on the rotary evaporator to give bright orange solids which are readily soluble in water. The solubility of potassium salts of **3a** and **3b1**–**3** in water was 50–100 mg ml^{-1} at pH < 7.5 that is among the highest values reported for fullerene derivatives.

2.5 Antiviral activity of the synthesized fullerene derivatives

The peculiarity of the molecular structures of **3a**–**b** is in arrangement of all organic addends around one five-membered ring (Fig. 3), while the main part of the fullerene cage remains free and available for interactions with biological targets. Therefore, the synthesized fullerene-based acids can be considered as very interesting entities for biological studies.

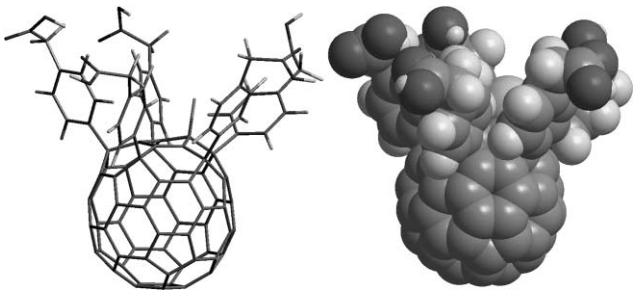


Fig. 3 The optimized 3D molecular structure of compound **3a**.

Here we report the preliminary results of anti-HIV screening of **3a**–**b** in the form of their potassium salts **4a** and **4b**, respectively. All tests were performed in lymphocyte CEM cell cultures infected with HIV-1 or HIV-2.[31] They were based on inhibition of virus-induced cytopathicity (giant cell formation) at day 4–5 post infection by the test compounds. The cytostatic activity (inhibition of cell proliferation) of the compounds was studied in the same cell cultures at day 3 or 4 upon incubation with the test compounds. The results are shown in Table 1, where the anti-HIV data for two recently investigated compounds **5** and **6** are also given for comparison. Note that these two isomeric bispyrrolidinofullerenes are among the best known fullerene-based inhibitors of HIV.[3]

As follows from Table 1, compounds **4a** and **4b** also possess pronounced anti-HIV activity with respect to both virus subtypes. According to the commonly-accepted mechanism of the anti-HIV action of fullerene derivatives, the carbon sphere of C_{60} fits well to the active site of some HIV enzymes (*i.e.* the HIV-protease).[1] Bulky organic groups attached to the fullerene cage can prohibit such interactions between the fullerene derivative and the HIV-encoded enzymes. In the case of our compounds, **4b** is slightly (~2-fold) less active than **4a**, perhaps, because the former has more bulky addends than the latter.

The absolute IC50 values for **4a** are 3–6 times higher than for **5** and **6**. However, **4a** is 6–20 times less cytostatic than **5** and **6**. Thus, **4a** can be considered as a more-selective and, perhaps, more promising fullerene-based anti-HIV agent.

In addition, the synthesis of **4a** is straightforward, the yield is reasonably high and there is no need for using the time-consuming HPLC separation. The solubility of **4a** in water approaches 100 mg ml^{-1}; this is an important prerequisite for easy intravenous administration of this compound. A study of the action of **4a**–**b** against other viruses is currently in progress. No pronounced, if any, activity was observed against a variety of viruses, other than HIV, including herpes simplex virus type 1 (KOS), HSV-2 (G), vaccinia virus, vesicular stomatitis virus, Coxsackie virus B4, respiratory syncytial virus, parainfluenza virus-3, reovirus-1, Sindbis virus and Punta Toro virus. At the same time, **4a** is a good starting point for further design of polycarboxylic derivatives of fullerenes that are more active with respect to HIV inhibition.

3. Conclusions

It was shown that chlorofullerene $C_{60}Cl_6$ is a versatile substrate for the preparation of highly water-soluble fullerene derivatives. Particularly, Friedel–Crafts arylation of $C_{60}Cl_6$ with methyl esters of phenylacetic and benzylmalonic acids was applied in this work for synthesis of two novel polycarboxylic fullerene compounds in good yields. The developed procedures are straightforward and can be used for large-scale preparations.

The synthesized fullerene-based acids possess high solubility in water in the form of alkali metal salts (*ca.* 50–100 mg ml^{-1} at pH < 7.5). Moreover, these compounds exhibited a pronounced anti-HIV activity and low cytotoxity. On the one hand, these findings should stimulate intensive biological studies of the reported fullerene-based polycarboxylic acids. On the other hand, the developed synthetic route allows for the preparation and investigation of a series of other water-soluble compounds that can reveal important structure–biological-activity relationships.

4. Experimental

General remarks

All commercially-available solvents and reagents were purchased from Acros or Aldrich and used as received. Nitrobenzene was vigorously dried after repeated (3–4 times) distillation over phosphorus anhydride. NMR spectra were recorded from solutions in [D_6]acetone (**3a**–**b**) or CS_2–[D_6]acetone solvent mixture (**2a**–**b**), on Bruker Avance (600 MHz-^{1}H) instrument with the solvent residual proton signal or tetramethylsilane (TMS) as a standard. A custom-made high resolution ESI O-TOF MS[32] was used to obtain ESI mass spectra.

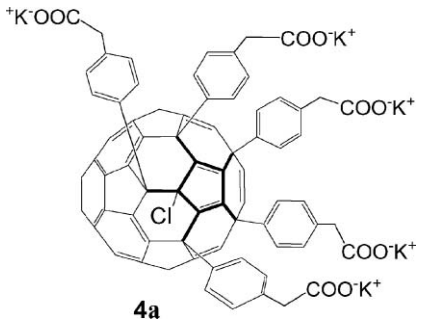
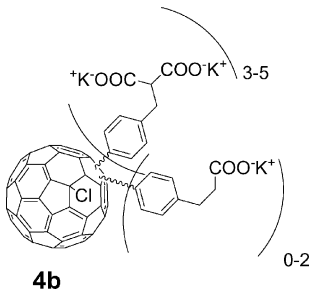
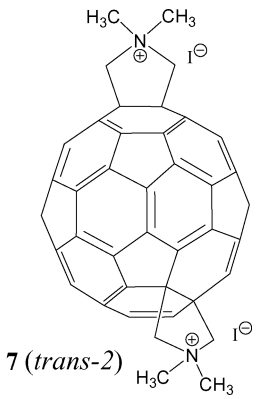
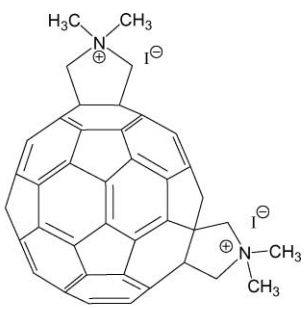
Preparation of $C_{60}Cl_6$

Fullerene C_{60} (120 mg, 0.167 mmol) was dissolved in 1,2-DCB (30 ml) under stirring in air within 2 h in a 250-ml round-bottom flask. Then freshly-prepared solution of ICl (800 mg, 4.92 mmol) in 10 ml of 1,2-DCB was added in one run to the fullerene solution. The resulting reagent mixture was immediately connected to the rotary evaporator, pumped down within 1 minute to *ca*. 1 mm Hg and put into a warm water bath (40 °C). Evaporation of 1,2-DCB usually takes 4–8 minutes. Bright red solid was recrystallized from PhCl, washed with hexane and dried in air. Yield of $C_{60}Cl_6$ was 120 mg (77%). Spectroscopic characteristics of the product (IR, ^{13}C NMR) were identical to reported previously.[21]

Synthesis of 2a–b

Chlorofullerene $C_{60}Cl_6$ (200 mg, 0.214 mmol) was dissolved in 100 ml of vigorously-dried nitrobenzene under stirring at 100 °C within 2 h in argon atmosphere. Then 8 ml of ester **1a**–**b** and several crystals of anhydrous $FeCl_3$ were added to the hot solution of $C_{60}Cl_6$. Resulting mixture was stirred at 100 °C within 1 h, then cooled down to the room temperature and quenched with 10 ml of CF_3COOH. Afterwards, 100 ml CCl_4 was added, resulting solution was washed three times with 150–200 ml of water, dried over

Table 1 The anti-HIV activity of the **6a** and **6b** salts and reference compounds **7** and **8**

Structure	CC50/μM	HIV-1		HIV-2	
		EC50/μM	CC50/EC50	EC50/μM	CC50/EC50
 **4a**	>63	1.2 ± 0.44	>52	4.4 ± 0.9	>14
 **4b**	>48	2.7 ± 0.7	>17	8.5 ± 4.2	>6
 **7** (*trans-2*)	2.9	0.21	14	0.2–1.0	3–14
 **8** (*trans-3*)	9.0	0.35	26	0.7	13

anhydrous Na_2SO_4 or $MgSO_4$, filtered and poured at the top of a silica gel column (silica gel 40–100 μ, 60 Å). Elution with a mixture of MeOH–toluene 0.5–99.5 (v/v) gave a first fraction represented by $C_{60}Ar_4$ and other elimination products. Compounds **2a**–**b** were eluted with MeOH–toluene 0.8–99.2 (v/v) as bright-orange broad fractions. Yields: **2a**: 193 mg, 60%; **3a**: 200 mg, 50%.

Compound 2a. ^{1}H NMR (600 MHz, CS_2-$(CD_3)_2CO$ 10 : 1): δ = 3.40–3.80 (CH_2, O-CH_3, m, 25H), 6.98 (d, 2H), 7.11 (d, 2H), 7.17 (d, 4H), 7.25 (d, 4H), 7.53 (d, 4H), 7.83 (d, 4H) ppm. ^{13}C NMR (150 MHz, CS_2-$(CD_3)_2CO$ 10 : 1): δ = 40.58 (CH_2), 40.65 (CH_2), 40.71 (CH_2), 51.47 (OCH_3), 51.57 (OCH_3), 51.69 (OCH_3), 57.86 (cage), 60.52 (cage), 63.16 (cage), 76.26 (cage), 127.02

(Ar), 127.25 (Ar), 127.81 (Ar), 128.77 (Ar), 128.84 (Ar), 128.96 (Ar), 129.95 (Ar), 129.99 (Ar), 130.06, 130.29, 133.47, 134.12, 134.16, 135.79, 137.28, 142.06, 143.10, 143.43, 143.52, 143.73, 143.76, 144.05, 144.39, 144.48, 144.55, 144.75, 145.29, 145.40, 146.70, 147.32, 147.46, 147.94, 148.23, 148.36, 148.56, 148.76, 148.81, 148.86, 150.40, 151.07, 153.54, 156.72, 169.84(*C*=O), 170.05(*C*=O), 170.10(*C*=O) ppm.

Compound 2b. ^{1}H NMR (600 MHz, CS_2-$(CD_3)_2CO$ 10 : 1): δ = 3.02 (CH_2, d, 2H), 3.16 (CH_2, d, 8H), 3.50–3.70 (C*H*, O-CH_3, m, 35H), 6.91 (d, 2H), 7.03 (d, 2H), 7.11 (d, 4H), 7.16 (d, 4H), 7.44 (d, 4H), 7.75 (d, 4H) ppm. ^{13}C NMR (150 MHz, CS_2-$(CD_3)_2CO$ 10 : 1): δ = 34.37 (*C*H$_2$), 34.47 (*C*H$_2$), 34.56 (*C*H$_2$), 51.90 (O*C*H$_3$), 52.01 (O*C*H$_3$), 52.06 (O*C*H$_3$), 52.97 (C*H*), 53.26 (C*H*), 57.84 (cage), 60.47 (cage), 63.07 (cage), 76.47 (cage), 128.48 (Ar), 128.73 (Ar), 128.87 (Ar), 129.47 (Ar), 130.26 (Ar), 135.41, 136.92, 137.47, 137.91, 138.02, 141.76, 143.08, 143.40, 143.58, 143.67, 143.78, 144.07, 144.37, 144.45, 144.50, 144.72, 145.26, 145.42, 146.71, 147.30, 147.44, 147.91, 148.22, 148.34, 148.54, 148.73, 148.72, 148.74, 148.78, 148.83, 150.32, 151.10, 153.43, 156.62, 167.92(*C*=O), 167.95(*C*=O) ppm.

Synthesis of 3a–b

A portion of **2a** or **b** (100 mg) was dissolved in 30 ml of chlorobenzene and then added to a mixture of 80 ml of CF_3COOH, 70 ml of CH_3COOH and 30 ml of 10 M aqueous HCl in a 1-L round-bottom flask. The resulting mixture was heated at 60 °C within 3 days and then concentrated in vacuum to dryness. Residue was dried in vacuum (*ca.* 10^{-3} mbar) for 4 h to remove remaining traces of acids and water. Yields of **3a**–**b**: 80–85 mg.

Compound 3a. ^{1}H NMR (600 MHz, DMSO-D6): δ = 3.49 (CH_2, d, 2H), 3.63 (CH_2, d, 8H), 7.03 (d, 2H), 7.15 (d, 2H), 7.25 (d, 4H), 7.30 (d, 4H), 7.61 (d, 4H), 7.83 (d, 4H), 12.35 (COO*H*, br. s, 5H) ppm. ^{13}C NMR (150 MHz, DMSO-D6): δ = 57.88 (cage), 60.59 (cage), 63.16 (cage), 76.42 (cage), 128.23 (Ar), 128.44 (Ar), 129.88 (Ar), 130.72 (Ar), 135.01, 135.3, 135.62, 135.76, 136.8, 141.82, 141.88, 142.84, 143.41, 143.53, 143.59, 143.63, 143.71, 143.75, 143.86, 144.1, 144.25, 144.42, 144.47, 145.44, 145.51, 145.55, 146.94, 147.2, 147.24, 147.34, 147.81, 148.12, 148.2, 148.44, 148.59, 148.65, 148.75, 150.58, 151.37, 154.14, 156.73, 172.52 (*C*=O), 172.85 (*C*=O), 172.99 (*C*=O) ppm. ESI MS (THF-Et_3N): 1412.24 ([**3a-Cl+OH**]$^-$), calcd 1412.26 amu. Anal. found/calcd (%): C, 83.61/83.89; H, 2.68/2.46; Cl, 2.74/2.48.

Compounds 3b1–3. ^{1}H NMR (600 MHz, DMSO-D6): δ = 3.30–3.60 (broad m, 15H), 7.04 (br. d, 2H), 7.11 (br. d., 2H), 7.29 (br. d, 4H), 7.49 (br. d, 4H), 7.76 (br. d, 4H) ppm. ESI MS (THF-Et_3N): 1720.32 ([**3b1**]$^-$), calcd 1720.22; 1686.36 ([**3b1**-Cl+H]$^-$), calcd 1686.26; 1676.33 ([**3b2**]$^-$), calcd 1676.23; 1642.27 ([**3b2**-Cl+H]$^-$) calcd 1613.33 amu; 1632.29 ([**3b3**]$^-$), calcd 1632.24 amu; 1598.36 ([**3b3**-Cl+H]$^-$), calcd 1598.28 amu.

Antiviral activity assays

The methodology of the anti-HIV assays was as follows: CEM cells (4.5×10^5 cells per ml) were suspended in fresh culture medium and infected with HIV-1 at 100 $CCID_{50}$ (cell culture infective dose-50) per ml of cell suspension. Then 100 µl of the infected cell suspension were transferred to microplate wells, mixed with 100 µl of the appropriate dilutions of the test compounds, and further incubated at 37 °C. After 4–5 days, giant cell formation was recorded microscopically in the CEM cell cultures. The 50% effective concentration (EC50) corresponded to the compound concentrations required to prevent syncytium formation by 50% in the virus-infected CEM cell cultures.

The antiviral assays other than HIV were based on an inhibition of virus-induced cytopathicity in either HeLa, Vero or HEL cell cultures. Herpes simplex virus type 1 (HSV-1) (KOS), HSV-2 (G), vaccinia virus, vesicular stomatitis virus (VSV) and the thymidine kinase-deficient HSV-1 TK$^-$ (B2005) strain were exposed to HEL cell cultures, parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie B4 virus and Punta Toro virus to Vero cell cultures, respiratory syncytial virus to HeLa cell cultures, and cytomegalovirus (CMV) (AD-169, Davis) and varicella-zoster virus (VSV) (YS, OKA), and the thymidine kinase-deficient VZV (07/1, YS/R) strains to HEL cell cultures. Confluent cell cultures in 96-well microtiter trays were incubated with 100 $CCID_{50}$ of virus, 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After a 1 h virus adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures.

The cytostatic activity assays for the CEM cells were as follows: 6×10^4 cells suspended in growth medium were allowed to proliferate in 200-µl wells of microtiter plates in the presence of five-fold dilutions of the test compounds at 37 °C in a humidified CO_2-controlled atmosphere. After 72 h, the number of cells was counted in a Coulter counter. The IC_{50} value was defined as the compound concentration required to inhibit cell proliferation by 50%.

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