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Short communication

Chromatographic separation and evaluation of the lipophilicity by reversed-phase high-performance liquid chromatography of fullerene-C_{60} derivatives

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Abstract

Two water-soluble regioisomers of *tris*-dicarboxymethanofullerene-C_{60} with D_3 (**3**) or C_3 (**4**) symmetry have been shown to possess interesting neuroprotective properties, among which the free radical scavenging activity is particularly relevant. Here we report a faster preparative scale separation of the two trisadducts along with analytical RP-HPLC data of **3** and **4** in order to provide additional information for the evaluation of their membrane permeability.

Keywords: Lipophilicity; Regioisomer separation; Fullerenes

1. Introduction

Fullerene-C_{60} is a reactive molecule that can be functionalized by means of a large number of reactions. Representative examples include cycloadditions, addition of nucleophiles, carbenes, nitrenes, halogens and radicals [1]. One of the main goals of the functionalization of C_{60} is the production of water-soluble, biologically interesting derivatives endowed with enhanced chemical and physical properties compared to fullerene itself while retaining its main electronic properties. Recently [2] two regioisomers of *tris*-dicarboxymethanofullerene-C_{60} with D_3 [*trans-3,trans-3,trans-3*-$C_{63}(CO_2H)_6$, **3**] or C_3 [*e,e,e*-$C_{63}(CO_2H)_6$, **4**] symmetry have been shown to possess interesting neuroprotective properties. Indeed, both **3** and **4** are effective free radical scavengers, able to block intense, rapidly triggered *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity in cortical neuronal cultures, with **4** more potent and effective than **3**. The difference in biological activity was estimated by EPR spectroscopy measurements and has been attributed to a difference in the dipole moment and the relative ability to intercalate into lipid bilayers.

With the aim of better defining the physico-chemical properties of **3** and **4** and to devise an additional evaluation of the hydrophobic–hydrophilic balance useful for membrane permeability studies, we have synthesized them and analyzed both regioisomers by RP-HPLC. Unlike the flask-shaking method for determining the logarithm of 1-octanol–water partition coefficient (log *P*) as an index of the hydrophobic character of a bioactive compound, the RP-HPLC technique carried out on a large variety of stationary phases is now widely accepted as a lipophilicity measurement [3]. Indeed, the chromatographic method offers some advantages: the measurement is dynamic, and lower sample purity and smaller sample size are required. We herein report

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preliminary results of the application of *n*-alkylsilyl bonded phases for the evaluation of the hydrophobicity of the two carboxyfullerene derivatives **3** and **4**.

2. Experimental

2.1. Chemistry

While the reaction scheme for the synthesis of **3** and **4** was the same as previously described [2,4], a faster purification step of the regioisomeric trisadducts was obtained by combining flash and medium pressure chromatography in order to shorten the long and tedious procedure required by gravity chromatography (Fig. 1). Accordingly, a solution of fullerene-C_{60} and dry diethyl bromomalonate in toluene was magnetically stirred for 15 h in an argon atmosphere and at room temperature in the presence of dry 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The reaction mixture was then evaporated and the brownish residue submitted to flash chromatography by means of the Biotage Flash 40 system (Biotage

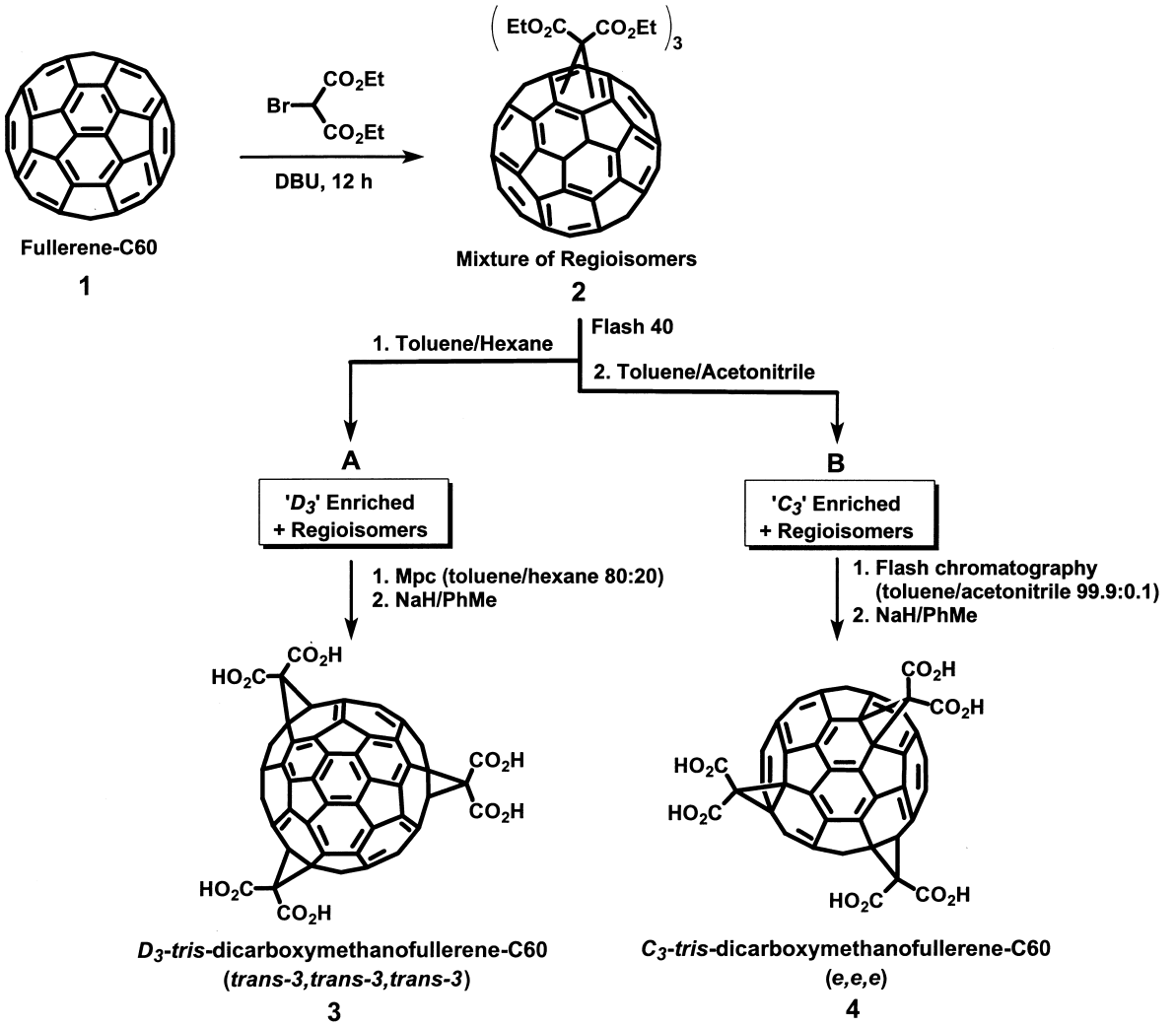

Fig. 1. Synthesis and separation of D_3- and C_3-*tris*-dicarboxymethanofullerene-C_{60} regioisomers (**3** and **4**).

UK, Flash 40M, KP-SIL cartridges). Elution with toluene–hexane (8:2) to toluene afforded a fraction (A) enriched in the D_3-adduct. Following elution with toluene–acetonitrile (99.5:0.5) gave a fraction (B) enriched in the C_3-adduct. Fraction A was then submitted to medium pressure chromatography and elution with toluene–hexane (8:2) to afford pure (84.2%) D_3-adduct in 19.5% yield. Fraction B was further purified by flash chromatography using toluene–acetonitrile (99.9:0.1) thus obtaining pure (90.1%) C_3-adduct in 15.5% yield. Both the tris-adducts showed analytical and spectroscopic data (UV, ^{13}C-NMR) identical with those already reported [5]. Alkaline hydrolysis of the C_3-adduct was performed with sodium hydride in toluene as previously reported [2,4] with a work-up procedure slightly modified. Accordingly, after stirring for 30 min at room temperature, the reaction mixture was cooled (0°C) and methanol was added. Stirring was continued for 45 min at room temperature, the red-orange precipitate was collected by centrifugation and washed with toluene and hexane. An equimolar amount of 4 M sulfuric acid was then added to the residue dissolved in water and the resulting solution was concentrated in vacuo and re-dissolved in methanol. Filtration and evaporation of the solvent afforded the pure acid **4** in 70% yield [4]. An analogous sequence was applied to the D_3-adduct, thus obtaining the free acid **3** in a 37% yield.[1]

2.2. HPLC analysis

2.2.1. Instrumentation

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-Workstation Class LC-10 equipped with a CBM-10A system controller, two LC-10AD high-pressure binary gradient delivery systems, a SPD-10A variable-wavelength UV–Vis detector and a Rheodyne 7725i injector (Rheodyne, Cotati, CA, USA) with a 20 μl stainless-steel loop.

[1] ^{13}C-NMR (Bruker DRX-400) for **3**: (^2H_2O) δ 170.28, 149.24, 148.46, 147.74, 147.15, 145.60, 143.06, 142.44, 140.63, 139.75, 76.80, 63.02; **4**: (^2H_2O) δ 170.92, 149.86, 148.41, 146.65, 146.26, 146.15, 146.02, 145.77, 145.08, 144.94, 143.57, 143.42, 143.14, 141.97, 140.82, 140.14, 140.04, 77.79, 76.95, 66.64.

2.2.2. Materials

All HPLC solvents were purchased from Merck (Darmstadt, Germany). Water was purified using a Millipore Milli-Q system (Bedford, MA, USA). 25 mM Phosphate buffers of pH 5.5, 8.0 and 9.8 were prepared starting from Na_2HPO_4 and NaH_2PO_4 and filtered with Durapore (Millipore) membrane filters HV of 0.45 μm. The acidic aqueous phase was prepared by adding 0.1% (v/v) of trifluoroacetic acid. The HPLC columns used were: Supelcosil LC-Si (Supelco, Bellefonte, PA, USA) 250×4.6 mm, 5 μm, 100 Å; Jupiter C_{18} (Phenomenex, Torrance, CA, USA) 150×4.6 mm, 5 μm, 300 Å, coupled with a 30×4.6 mm guard column; LiChrospher 100 RP8 (Merck) 250×4.0 mm, 5 μm, 100 Å; LiChrospher 100 RP18 (Merck) 250×4.0 mm, 5 μm, 100 Å, coupled with a 4.0×4.0 mm guard column.

2.2.3. Methods

All the measurements were performed at room temperature. The analytical control of the reaction mixtures of trisadducts was assessed by the Supelcosil LC-Si column with detection at 220 and 280 nm. The mobile phases were made up of toluene containing 1% (v/v) of hexane (A) or 2% (v/v) of ethyl acetate (B). The gradient program was as follows: 0% B for 10 min at 0.9 ml min^{-1}, 0–100% B in 45 min scaling down to 0.7 ml min^{-1}, 100% B for 35 min at 0.7 ml min^{-1}, 100–0% B in 5 min at 0.9 ml min^{-1} and 10 min re-equilibration. The purity of the isolated regioisomeric trisadducts was then detected isocratically on the same column with the B mobile phase at 0.7 ml min^{-1}. RP-HPLC of the final acids was best accomplished with the acidic methanol–water (90:10) mobile phase, detected at 220 and 280 nm, and isocratically eluted at 1 ml min^{-1}.

3. Results and discussion

Different mobile phase compositions were tested on three *n*-alkylsilyl bonded stationary phases. Many attempts were initially made with the LiChrospher 100 RP18 column by using different proportions of methanol to buffer as the mobile phase; none of them gave useful results as evidenced by the presence in the chromatogram of several peaks showing various

Table 1
Retention times (t_R) and capacity factor (k') values[a]

	t_R (min)			k'		
	A	B	C	A	B	C
3	2.55	2.28	2.55	0.70	0.20	0.34
4	4.82	4.63	6.93	2.21	1.44	2.65

[a] A, Jupiter C-18; B, LiChrospher RP-8; C, LiChrospher RP-18.

and complex degrees of deprotonation. Better results were obtained with the same column by employing methanol–water (90:10, v/v) with the aqueous phase containing 0.1% (v/v) of trifluoroacetic acid. Under these conditions, the two regioisomers showed the retention times (t_R) and the capacity factor (k') values reported in Table 1. While the peak of **3** showed at least two unresolved impurities, the peak of **4** was well separated from the small impurity peaks eluting after it. Nevertheless, unlike the buffered mobile phases, the presence of a small amount of trifluoroacetic acid in the eluting mixture enables its use for RP-HPLC preparative scale separations. The three stationary phases behaved similarly: the LiChrospher 100 RP18 column provided better capacity factor values together with a better separation index (7.79) while the RP-8 column afforded more symmetrical peaks. The regioisomer **4** showed higher hydrophobic interactions with the bonded silica surface than that observed for the regioisomer **3**, thus revealing a very different retention which correlates well with the membrane interactions already reported [2]. This behavior can be tentatively rationalized on the basis of some of their physicochemical properties. The simple inspection of the three-dimensional structure of **3** and **4** already reveals that **3** is endowed with an isotropic distribution of the carboxy groups on the surface of the spherically shaped C_{60} molecule, whereas these groups are concentrated in one of the two hemispheres in **4**, thus affecting the polarity of the two compounds. We

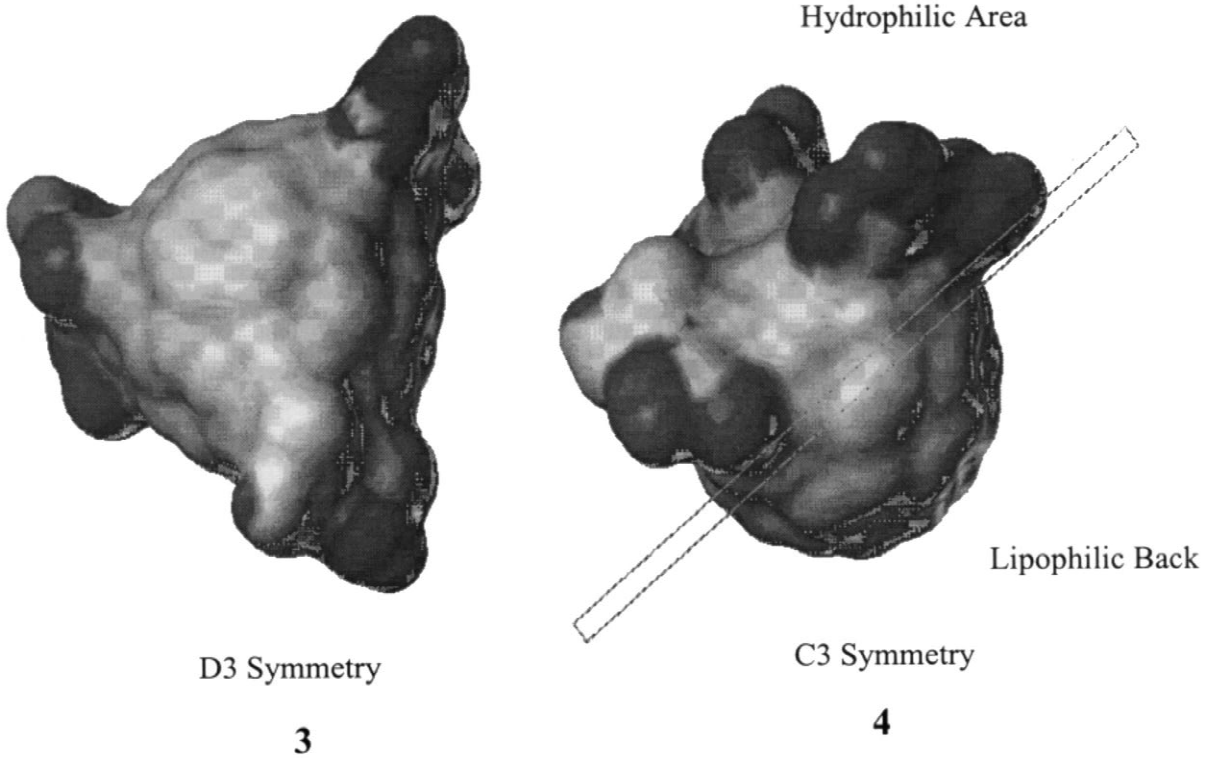

Fig. 2. Solvent accessible surfaces of D_3 (**3**) and C_3 (**4**) isomers. The hydrophilic portions are colored in dark gray while the hydrophobic ones are colored in white.

have performed a dipole moment calculation on the fully protonated form of the two regioisomers, based on a Gasteiger-Hükel scheme of point charges, and the results confirm that **4** is much more polar (18.7 D) than **3** (2.9 D). On the other hand, the same pattern of distribution of substituents on the surface of the fullerene molecule is responsible for the extent of the so-called hydrophobic and hydrophilic surfaces, which can be the major determinants for the interaction with the stationary phase. While in **3** the hydrophobic surface is broken by the high hydrophilic component constituted by the carboxylic moieties, in **4** a 'hydrophobic back' of the molecule can be recognized, clearly distinct from the small hydrophilic area (Fig. 2). This latter feature, that can be seen as a sort of amphiphilicity, is responsible for the higher retention time of **4** with respect to **3**. In conclusion, our results are in agreement with the previously reported findings [2] and confirm that **4** is endowed with a higher lipophilic character compared with **3**, as evidenced by the stronger hydrophobic interactions with the *n*-alkylsilyl bonded stationary phases.

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