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New non-ionic, highly water-soluble derivatives of C_{60} designed for biological compatibility

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Abstract—The most water-soluble (>240 mg C_{60} mL^{-1}) fullerene derivatives to date, the new malonodiserinolamide fullerenes $C_{60}[C(COSer)_2]_n$ ($n=4$, 5, 6, Ser = 2-amino-1,3-propanediol), have been synthesized and characterized. The compounds were designed to provide maximum water solubility and biological compatibility. The facile synthesis is based on Bingel additions to C_{60} and should be applicable to the solubilization of any prospective fullerene-based drug.

Organically-functionalized derivatives of C_{60} are currently being investigated for applications in the fields of biology and medicine.[1] In general, prospective diagnostic/therapeutic agents will be required to have sufficient biological availability, and thus, adequate water solubility. C_{60} itself may be coerced to form a colloidal suspension in water,[2] but it is intrinsically insoluble.[3] Therefore, finding simple, well-characterized methods to solubilize fullerene materials in biological environments is the subject of great current interest.[4–8]

To date, the methods proposed to water solubilize fullerenes have taken one of four approaches: (1) chemical/electrochemical reduction of C_{60} or its derivatives to a water-soluble carbon-based anion (e.g. C_{60}^{n-} or $Ph_5C_{60}^-$)[2b,7] (Method 1); (2) complexation of C_{60} in a host–guest fashion within hosts such as the cyclodextrins[4a,4b] and calixarenes,[4c] effectively shielding the hydrophobic fullerene from the polar aqueous environment[9] (Method 2); (3) covalent modification of C_{60} with one or more organic functionalities that contain(s) one or more ionic group(s)[5] (Method 3), and finally (4) covalent modification of C_{60} with one or more organic functionalities that contain(s) strongly hydrophilic group(s) that are non-ionic[6] (Method 4).

Using Method 1, aqueous solutions of C_{60}^{n-} ($n=1$, 2) with concentrations of $\sim$1 mg mL^{-1} may be obtained, which are reasonably stable in the absence of oxygen.[2b] Similarly, the carbon-based anion $Ph_5C_{60}^-$ is soluble in water, with a maximum solubility of $\sim$3.8 mg C_{60} mL^{-1}.[7] For medicinal applications, however, the oxygen sensitivity and expected biological instability of such carbon-based anions, coupled with relatively low water solubility, makes them unattractive for use in vivo.

C_{60}-containing host–guest complexes (Method 2) are also limited in medicinal applicability due to very low aqueous solubilities ($\sim$0.07 mg C_{60} mL^{-1}).[4b] Other potential problems with this approach include an effective shielding by the host of a fullerene compound from biological availability or undesired release and precipitation of C_{60} in vivo which, in mammals, leads to accumulation in the liver.[10]

Most literature examples of water-soluble fullerenes involve functionalization of the fullerene with ionic organic groups (Method 3).[5] The trifluoroacetic acid salt of an octahedrally-functionalized (T_h) derivative of C_{60} containing 12 primary amine groups was found to have a solubility of 89 mg C_{60} mL^{-1} in water, the highest value reported to date.[5b] A monofunctionalized dendrimeric C_{60} derivative with 18 carboxylic acid groups produced a solubility of 65 mg C_{60} mL^{-1} at pH 10 but only 8.7 mg C_{60} mL^{-1} at pH 7.[5a] Although this approach imparts excellent water solubility, the solubility is pH dependent with only marginal solubility at biological pH of 7.4. In addition, the hyperosmolality caused by the multiple ionic groups (and their counterions) of these types of fullerene compounds is undesirable for medical applications such as magnetic

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resonance imaging (MRI) and X-ray radiography, where a contrast-enhancing agent is administered intravenously in very high concentration.[11]

Finally, the covalent modification of C_{60} with strongly water-solubilizing, non-ionic groups (Method 4) in theory circumvents the above noted problems of hyperosmolality, chemical instability, limited water solubility, and pH dependence of solubility. To date, however, derivatives of this type have met with only limited success. Examples are a cyclodextrin–fullerene conjugate made by nucleophilic addition to C_{60} of an amine-modified cyclodextrin,[6b] a polyethylene glycol–C_{60} conjugate,[6c] a *N*-(3,6,9-trioxadecyl)-glycine functionalized fulleropyrrolidene,[6a] and the fullerenols.[12] In all these cases, the compounds were either ill-defined or only slightly soluble in water.

In this work, we report the design, synthesis, and characterization of new Type 4 water-soluble fullerene derivatives with exceptionally high water solubility at biological pH. These new malonodiserinolamide fullerenes (Fig. 1) are non-ionic and are based on the biologically-stable serinolamide structures used to water solubilize commercially available X-ray contrast agents such as iohexol[13] and iopamidol,[14] which are also designed to be non-ionic and non-toxic. In addition, we have previously used serinolamides to water-solubilize Mn^{III} metalloporphyrins for use as MRI contrast agents.[15]

In a typical synthesis of **3** (Fig. 1), two equivalents of serinol (2-amino-1,3-propanediol) were condensed with diethylmalonate at elevated temperature, with the loss of ethanol, to give the corresponding malonodiamide.[16] Esterification of the alcohol groups in pyridine/Ac_2O (76% overall yield for two steps starting from serinol) was followed by electrophilic bromination (79% yield) at the central acidic carbon of the malonodiamide with Br_2 in EtOAc/NEt_3. The bromomalonodiamide, **1**, was then appended to C_{60} via a Bingel type reaction[17] in the presence of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), in toluene or toluene/acetone to produce the desired compound **2**.[18] The stoichiometry was varied depending on the degree of addition desired. Deprotection of **2** was then effected to yield **3** in essentially quantitative yield with K_2CO_3 in CH_3OH/H_2O, followed by removal of potassium (as confirmed by energy dispersive X-ray spectroscopy) with 50W-X2 cation exchange resin (H^+ form). Anhydrous samples of **3**, confirmed by thermogravimetric analysis (TGA), were obtained after drying over P_2O_5 at $\sim$100°C under vacuum.

Initial efforts focused on the synthesis of the $n=1$ adduct, which was isolated in 44% yield as a reddish-brown solid after column chromatography on silica (acetone/toluene). The ^{13}C spectrum (Fig. 2) of the malonodiamide derivative verified a structure completely analogous to comparable structures containing malonodiester addends. That is, the malonodiamide added exclusively across one of the 6–6 double bonds of C_{60} forming a cyclopropane ring.[17] Deprotection of the $n=1$ adduct yielded the expected tetrahydroxy product which was found to be completely insoluble in water.

Efforts then turned to the multiadduct products. Using a 10-fold excess of **1** relative to C_{60}, a cherry red solution of mixed products was obtained. After flash chromatography on silica (acetone/toluene), the red solid (69% yield) was analyzed by MALDI-TOF MS and determined to consist of $\sim$15% tetra-, $\sim$80% penta-, and $\sim$5% hexa-functionalized product (Fig. 3). With some difficulty, a sample consisting of only an isomer mixture of pure $n=5$ product could be obtained by multiple passes on silica, as confirmed by HPLC and MS. However, the bulk solubility and partition coefficient (K_{OW}) measurements reported below were performed on the deprotected mixture.

The water solubility of **3** ($n=4$–6) was determined using UV–vis spectrophotometry at pH 7. In a 1 mm quartz cell containing 400 μL of water, the material was dissolved in 5.0 mg increments and spectra taken sequentially. The material continued to dissolve and the spectrum continued to increase linearly ($R^2=0.995$) in intensity even after 255 mg of **3** had been added. At this point, further addition was discontinued because of solution viscosity. The degree of water solubilization observed corresponds minimally to a remarkable ***240 mg C_{60} mL^{-1}***! Additionally, no visible change in the solution was noted in the pH 2–12 range. This result

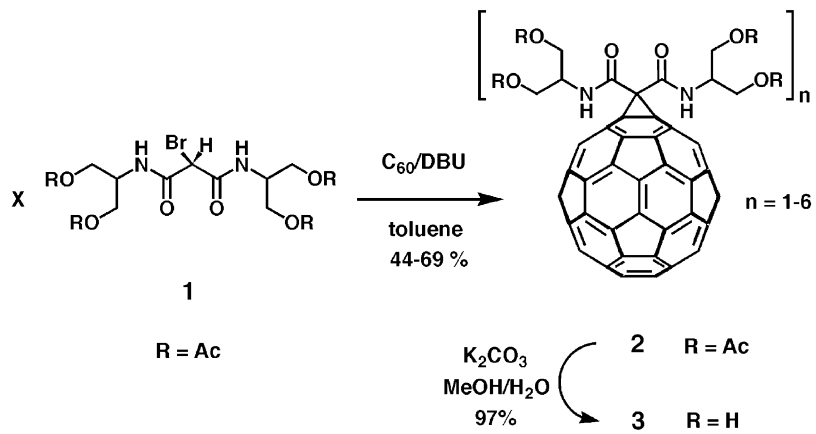

Figure 1. Synthesis of the malonodiserinolamide fullerenes. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, Ac = acetate.

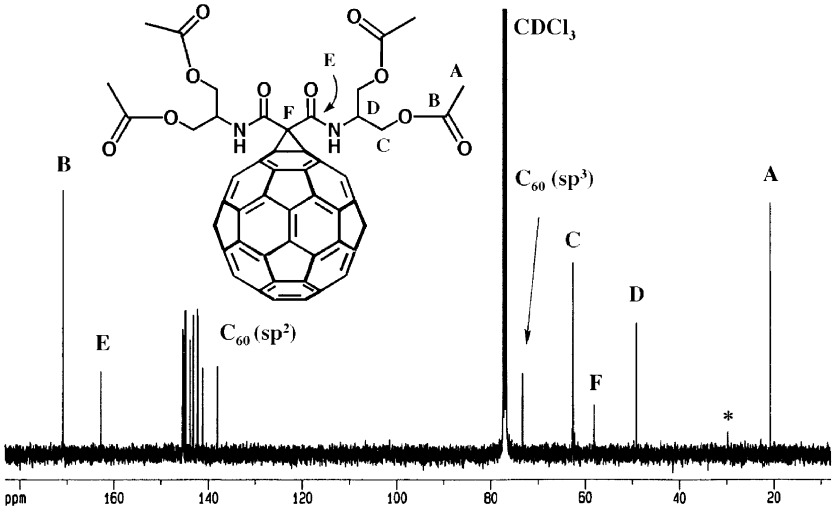

Figure 2. ^{13}C NMR of **2** ($n=1$). 400 MHz, $CDCl_3$, * = solvent impurity.

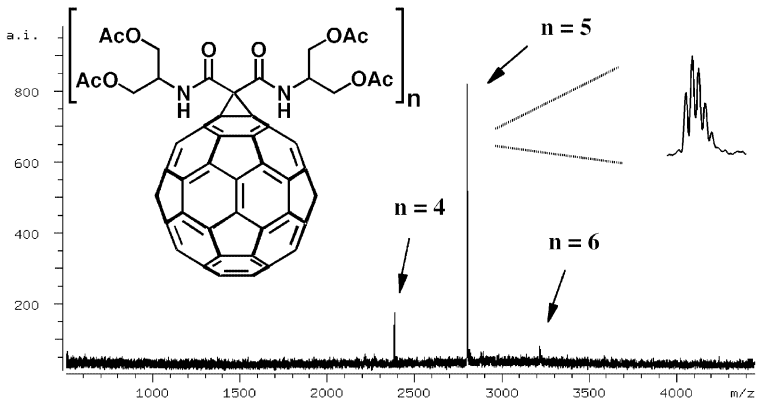

Figure 3. MALDI-TOF mass spectrum of **2** ($n=4$, 5, 6) as a product mixture. CS_2/S, $CHCl_3$, negative ion mode.

suggests no practical solubility limitations to the development of fullerene-based diagnostic/therapeutic agents using malonodiserinolamide addends, even in conjunction with other active drug components appended to the same fullerene molecule.

The *n*-octanol/water partition coefficient of **3** ($n=4$–6) was determined at 25°C by the method of Leo.[19] Accordingly, a 2.0 mg mL^{-1} solution of **3** in *n*-octanol-saturated water was shaken for 5 min with an equal volume of *n*-octanol, followed by centrifugation. The UV–vis spectrum of the aqueous layer was unchanged from that of the starting solution, and the organic phase remained colorless. Thus, for **3**, $K_{OW} \approx 0$ which predicts that the material will have negligible hydrophobic interactions in vivo. This value compares well with the very small K_{OW} values ($<10^{-3}$)[13] of currently-used X-ray contrast agents which, in vivo, are restricted to the extracellular space and are rapidly and completely cleared from the body. Compound **3**, with its high degree of derivatization ($n=4$–6), is therefore also well suited to extracellular applications. However, lesser degrees of functionalization ($n=2$–3) may allow a tailoring of K_{OW} for intracellular uses, such as drug delivery, while still providing adequate water solubility.

In conclusion, a new synthetic methodology for water solubilizing fullerene materials has been developed. The resulting fullerene derivatives are exceedingly soluble in water without a significant pH dependence. This straightforward approach for water-solubilizing fullerene-based materials leads to well-defined, biologically-compatible products and should be generally applicable to empty fullerenes, as well as to endohedral metallofullerenes, $M@C_n$, with M being any one of several medically-interesting metal ions.[1,20] Water-solubilized metallofullerene materials are presently under investigation.

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18. All new compounds gave satisfactory spectral data. For **2** ($n=1$): reddish brown powder; TLC R_f 0.26 in 9:1 toluene:MeOH; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.10 (s, 12H, CH_3), 4.34–4.41 (m, 8H, CH_2), 4.68–4.72 (m, 2H, C*H*), 7.37 (br d, $J=6.4$ Hz, 2H, N*H*); ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm) 170.95 (ester *C*=O), 162.90 (amide *C*=O), 145.57, 145.44, 145.49, 145.40, 145.06, 144.99, 144.80, 144.01, 143.41, 143.32, 143.24, 142.40, 142.29, 141.30, 138.21 (C_{60} sp^2 C), 73.45 (C_{60} sp^3 C), 62.72 (*C*H$_2$), 58.28 (bridgehead), 49.30 (*C*H), 20.96; FT-IR (KBr) ν (cm^{-1}) 1745 (s, ester C=O), 1685 (m, amide C=O), 1228 (s, asym. -OCOCH$_3$), 526 (m); HPLC (Cosmosil 5 PBB column), retention time 3.43 min in toluene:MeOH (40:1), detection at 290 nm; UV (40:1 toluene:MeOH) λ_{max} 285 nm; MALDI-TOF MS calcd for $C_{77}H_{24}O_{10}N_2$ (M^+) 1136, found 1136. Anal. ($C_{77}H_{24}O_{10}N_2$ acetone (C_3H_6O)) calcd: C, 80.4; H, 2.53; N, 2.34, found: C, 79.79; H, 2.41; N, 2.45.
19. The *n*-octanol/water partition coefficient (K_{OW}) of compounds has proven to be useful for the determination of biological structure–activity relationships. It is defined as $K_{OW}=c_o c_w^{-1}$, where c_o and c_w are the equilibrium concentrations of an analyte in *n*-octanol and water, respectively. The partition coefficient has been used to predict the rate of biological uptake,[18b] the degree of biomagnification,[18b] and the degree of binding of solutes to serum albumin and other blood proteins.[18a] (a) Leo, A.; Hansch, C.; Elkins, D. *Chem. Rev.* **1971**, *71*, 525–554; (b) Chiou, C. T.; Freed, V. H.; Schmedding, D. W.; Kohnert, R. L. *Environ. Sci. Technol.* **1977**, *11*, 475–478.
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